



Inter-institutional Interdepartmental Program of Postgraduate Studies
“Molecular and Cellular Biology and Biotechnology”



Laboratory of Biology
Faculty of Medicine
Department of Health Sciences
University of Ioannina

The interplay between the JAML-CXADR axis and YAP-1 in the pathogenesis of Basal Cell Carcinoma (BCC)

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Master's Thesis
Ioannina, 2026

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Disclaimer: No generative Artificial Intelligence (AI) has been used for any part of the writing process of the current thesis, nor for the creation of its figures, and I strictly forbid the use of my thesis as input for the training of any Large Language Model (LLM).

Acknowledgements

This Master's thesis was carried out at the Laboratory of Biology of the School of Medicine, Department of Health Sciences, University of Ioannina, between April 2025 and September 2026, under the supervision of **Assistant Professor Achilleas Floudas**, to whom I want to express my most sincere gratitude. Working alongside Assistant Professor Floudas, sharing his knowledge and expertise, has been of invaluable importance for my scientific, but also my personal growth. His lab has been an area of constant scientific curiosity, exchanging of ideas, and inspiration, which, alongside his treasured feedback, unwavering guidance, patience and support, have helped shape my identity as an emerging researcher, and have made me feel extremely lucky for having the opportunity to collaborate with and learn from him. I am forever indebted to him for believing and trusting in me even in times of self-doubt, and I look forward to working under his precious mentorship during my upcoming PhD journey.

I would like to extend my heartfelt thanks to **Professor Evangelos Kolettas** and **Associate Professor Evangeli Lampri**, for the excellent collaboration, valuable resources and insight, which were essential for the realization of my thesis, as well as to the other two members of my thesis committee, **Professor Efstathios Frilingos**, and **Dr. Dimitrios Liakopoulos** for their valuable time and constructive feedback.

My earnest appreciation extends to **Dr. Sofia Bellou** (Senior Laboratory Teaching Staff, University of Ioannina) for her vital contribution to the confocal microscopy experiments and her valuable help with the scratch assay set-up. I would also like to express my profound gratitude to **PhD candidate Roozbeh Sanaei**, whose work has laid the foundation for my own project, for the valuable training during my first months in the lab and his continuing guidance thereafter. I would also like to thank **PhD candidate Stefanos Tsavdaridis** for the insightful conversations and productive feedback on the bioinformatic analysis, including his help with the initial steps of the spatial transcriptomic data examination.

I extend my appreciation to all the other lab members with who I had the opportunity to work with these past few months, fellow MD and MSc student Charis Roumbas, and undergraduate students Grigoris Dimas, Stavroula Harinou and Giorgos Margetis, as well as to the entire staff of the Laboratory of General Biology. A special thank you is reserved for **Dr. Maria Karageorgou**, currently Senior Laboratory Teaching Staff in the Laboratory of Biology, who I was lucky enough to also have as my Biology teacher in middle school, and who I deeply admire both as an educator and as a person.

I would also like to acknowledge **Alexandra Elbakyan** for her valuable contribution towards an open access scientific world, without which a big part of my thesis' introduction couldn't have been completed.

I want to genuinely thank my friends for their continuous encouragement and for bearing with me in all the times that I complained during this academic journey. And last but not least, I want to express my deepest gratitude and love to my parents, Olga and Dimitris, for always supporting and encouraging me in everything I pursue – without them I wouldn't have achieved anything of what I have so far.

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Abbreviations

%MT: percentage of mitochondrial DNA

2D: two-dimensional

3D: three-dimensional

5-FU: 5-Flourouracil

6-4PP: Pyrimidine-Pyrimidone (6-4) photoproduct

AAD: Allergic Airway Disease

ABCA12: ATP Binding Cassette subfamily A member 12

aBCC: advanced Basal Cell Carcinoma

ACOT6: Acyl-CoA Thioesterase 6

ACT1: Act 1 adaptor protein

ACTA2: Actin Alpha 2, smooth muscle

ACTRT1: Actin Related Protein T1

ACYP2: Acylphosphatase 2

AD: Atopic Dermatitis

Ad5FK: Adenovirus serotype 5 Fiber Knob protein

ADAM: A Disintegrin And Metalloproteinase domain

ADCC: Antibody-Dependent Cellular Cytotoxicity

ADGRA2: Adhesion G protein-coupled Receptor A2

AE: Adverse Event

AFDN: Afadin

AHR: Aryl Hydrocarbon Receptor

ALA: 5-Aminolaevulinic Acid

ALA-PDT: 5-Aminolaevulinic Acid Photodynamic Therapy

ALDH1A1: Aldehyde Dehydrogenase 1 family member A1

AMOT: Angiomotin

AMOTL: Angiomotin-Like

AMP: Adenosine Monophosphate

AMPK: AMP-activated protein Kinase

ANKRD1: Ankyrin Repeat Domain 1

ANS: Adjacent Normal Skin

ANXA2: Annexin A2

AP-1(X): clathrin Adaptor Protein complex 1(X)

AP-1: Activator Protein-1

APC: Adenomatous Polyposis Coli

APC: Antigen-Presenting Cell

APL: Acute Promyelocytic Leukemia

APMAP: Adipocyte Plasma Membrane Associated Protein

APOE: Apolipoprotein E

APS: Ammonium Persulfate

AQP5: Aquaporin 5

AR: Androgen Receptor

AREG: Amphiregulin

ARID1A: AT-Rich Interaction Domain 1A

As: Arsenic

ASNS: Asparagine Synthetase (glutamine-hydrolyzing)

ASPM: Assembly factor for Spindle Microtubules

ASS1: Argininosuccinate Synthase 1

ATAC-seq: Assay for Transposase-Accessible Chromatin with high-throughput sequencing

ATF: Activating Transcription Factor

ATP1B3: ATPase Na⁺/K⁺ Transporting subunit Beta 3

ATR: Ataxia Telangiectasia and Rad3-related

ATRA: All-Trans Retinoic Acid

AURK: Aurora Kinase

BACE1: Beta-site Amyloid precursor protein Cleaving Enzyme 1; also known as β -secretase 1

BARD1: BRCA1 Associated RING Domain 1

BATF: Basic leucine zipper ATF-like Transcription Factor

Bax: BCL2-Associated X protein

BCA: Bicinchoninic Acid

BCC: Basal Cell Carcinoma

BCL: B-Cell Lymphoma

Bcl-xL: B-cell lymphoma-extra large

BDCA: Blood Dendritic Cell Antigen

BID: BH3 Interacting domain Death agonist

BioID: proximity-dependent biotin identification

BirA: Biotin—(acetyl-CoA-carboxylase) ligase

BIRC5: Baculoviral IAP Repeat Containing 5

BLIMP1: B Lymphocyte-Induced Maturation Protein 1; also known as PRDM1: PR/SET Domain 1

BNC2: Basonuclin zinc finger protein 2

BRCA(X): Breast Cancer type (X) susceptibility protein

BSA: Bovine Serum Albumin

BSC: Basosquamous Carcinoma

BST: Basosquamous Transition

BTLA: B and T Lymphocyte Associated; also known as CD272

C&E: Curettage and Electrodesiccation

C2-type: Constant 2 type

CADM1: Cell Adhesion Molecule 1

CALML5: Calmodulin Like 5

CAPN1: Calpain 1

CASP: Caspase

CAV: Caveolin

CBP: CREB Binding Protein

CCL: CC motif chemokine Ligand

CCN: Cellular Communication Network factor

CCN: Cyclin

CCR: CC motif chemokine Receptor

CD: Cluster of Differentiation

CD300LG: CD300 Molecule Like family member G

CD40L: CD40 Ligand

CDC(X): Cell Division Cycle (X)

CDC: Complement-Dependent Cytotoxicity

cDC: conventional Dendritic Cell

CDH(X): Cadherin (X)

CDK: Cyclin Dependent Kinase

cDNA: complementary DNA

CDSN: Corneodesmosin

CEA: Carcinoembryonic Antigen

CEBPB: CCAAT Enhancer Binding Protein Beta

CEL: Carboxyl Ester Lipase

CENP: Centromere Protein

CEP20: Centrosomal Protein 20; also known as FOPNL: FOP-related Protein of 20 kDa

CFLAR: CASP8 and FADD Like Apoptosis Regulator

cGAS-STING: cyclic GMP-AMP Synthase and Stimulator of Interferon Genes

CHAC1: ChaC glutathione specific gamma-glutamylcyclotransferase 1

CHCHD10: Coiled-Coil-Helix-Coiled-Coil-Helix Domain containing 10

CHEK1: Checkpoint Kinase 1

CHEK2: Checkpoint Kinase 2

CHS: Contact Hypersensitivity

CIDEA: Cell death Inducing DFFA like Effector A

CK: Casein Kinase

CKAP2: Cytoskeleton Associated Protein 2

CLDN: Claudin

CLEC(XY): C-Type Lectin domain family (X) member (Y)

CLIP1: CAP-Gly domain containing Linker Protein 1

CLMP: Cocksackie- and adenovirus receptor-Like Membrane Protein; also known as CXADR
Like cell adhesion molecule

CLSTN3: Calsyntenin 3

CNA: Copy Number Alteration

CNFN: Cornifelin

CNTRL: Centriolin

CNV: Copy Number Variation

CO₂: carbon dioxide

COL(X)A(Y): Collagen type (X) Alpha (Y) chain

COX-2: Cyclooxygenase-2

CPD: Cyclobutane Pyrimidine Dimers

CR: Complete Response

CRABP2: Cellular Retinoic Acid Binding Protein 2

CRC: Colorectal Cancer

CRNKL1: Crooked Neck pre-mRNA splicing factor 1

CRNN: Cornulin

CSC: Cancer Stem Cell

cSCC: cutaneous Squamous Cell Carcinoma

CSF: Colony Stimulating Factor

CSPG4: Chondroitin Sulfate Proteoglycan 4

CTL: Cytotoxic Lymphocyte

CTLA-4: Cytotoxic T-Lymphocyte Associated protein 4

CTNN: Catenin

CTSB: Cathepsin B

CuSO₄: Copper sulfate

CVB3: Coxsackievirus B3

CXADR: Coxsackie and Adenovirus Receptor

CXCL: CXC motif chemokine Ligand

CXCR: CXC motif chemokine Receptor

CYR61: Cysteine-Rich angiogenic inducer 61

D: Domain

DAB: 3,3'-diaminobenzidine

DAPI: 4',6-diamidino-2-phenylindole

DC: Dendritic Cell

DCD: Dermicidin

ddH₂O: double distilled water

DDIT3: DNA Damage Inducible Transcript 3

DEG: Differentially Expressed Gene

DETC: Dendritic Epidermal T Cell

DKO: Double Knockout

DLL1: Delta Like canonical Notch Ligand 1

DMSO: Dimethyl Sulfoxide

DNFB: 2,4-dinitro-1-flourobenzene

DPBS: Dulbecco's Phosphate-Buffered Saline

DPH3: Diphthamide biosynthesis 3

DSC: Desmocollin

DSG: Desmoglein

dTMP: Deoxythymidine Monophosphate

dUMP: Deoxyuridine Monophosphate

DVL: Dishevelled

E2F: E2F Transcription Factor

EADO: European Association of Dermato-Oncology

EC: Endometrial Cancer

ECD: Extracellular Domain

ECL: Enhanced Chemiluminescence

ECM: Extracellular Matrix

ECT: Electrochemotherapy

EGF: Epidermal Growth Factor

EGFLAM: EGF Like, Fibronectin Type III and Laminin G domains

EGFR: Epidermal Growth Factor Receptor

EGR1: Early Growth Response 1

ELISA: Enzyme-Linked Immunosorbent Assay

ELOVL: ELOVL fatty acid elongase

EM: Electron Microscopy

EMA: Epithelial Membrane Antigen

EMA: European Medicines Agency

EMP2: Epithelial Membrane Protein 2

EMT: Epithelial to Mesenchymal Transition

ENTPD1: Ectonucleoside Triphosphate Diphosphohydrolase 1

EOMES: Eomesodermin

EPAS1: Endothelial PAS domain protein 1

EPCAM: Epithelial Cell Adhesion Molecule

ERBB2: Erb-B2 receptor tyrosine kinase 2

ERCC1: Excision Repair Cross-Complementation group 1

ERK: Extracellular signal-Regulated Kinase

ESCC: Esophageal Squamous Cell Carcinoma

EXPH5: Exophilin 5

F11R: F11 receptor; also known as JAM1: Junctional Adhesion Molecule 1; also known as JAM-A: Junctional Adhesion Molecule A

FABP7: Fatty Acid Binding Protein 7

FACS: Fluorescence-Activated Cell Sorting

FAK: Focal Adhesion Kinase; also known as PTK2: Protein Tyrosine Kinase 2

FAM(XY): Family with sequence similarity (X) member (Y)

FasL: Fas Ligand

FBS: Fetal Bovine Serum

FC: Flow Cytometry

FCER1A: Fc Epsilon Receptor 1a; also known as High affinity immunoglobulin Epsilon Receptor subunit Alpha

FCGR1, FCGR1A: Fc Gamma Receptor 1a; also known as CD64a

FCGR3A: Fc Gamma Receptor 3a; also known as CD16a

FDA: Food and Drug Administration

FEN1: Flap structure-specific Endonuclease 1

FFPE: Formalin-Fixed Paraffin-Embedded

FGF: Fibroblast Growth Factor

FGFR3: Fibroblast Growth Factor Receptor 3

FIZZ1: Found In Inflammatory Zone 1

FKBP5: FKBP Prolyl isomerase 5

FLG: Filaggrin

FN1: Fibronectin 1

FOPNL: FOP-related Protein of 20 kDa; also known as CEP20: Centrosomal Protein 20

FOXM1: Forkhead box M1

FOXO1: Forkhead box O1

FOXP3: Forkhead box P3

FRMD4A: FERM Domain containing 4A

FRZB: Frizzled related protein

FST: Follistatin

FXIIIa: Factor XIIIa

FZD: Frizzled class receptor

GATA3: GATA binding protein 3

GC: Gastric Carcinoma

G-CSF: Granulocyte Colony Stimulating Factor; also known as CSF3: Colony Stimulating Factor 3

GEO: Gene Expression Omnibus

GJ: Gap Junction protein

GLI: Glioma-associated oncogene homolog

GM-CSF: Granulocyte/Macrophage Colony Stimulating Factor; also known as CSF2: Colony Stimulating Factor 2

gp130: Glycoprotein 130

GPCR: G-Protein Coupled Receptor

GREM1: Gremlin 1, DAN Family BMP antagonist

GSK3β: Glycogen Synthase Kinase 3 beta

GTP: Guanosine Triphosphate

GZMB: Granzyme B

H&E: Hematoxylin and Eosin

H₂O₂: Hydrogen peroxide

HAVCR2: Hepatitis A Virus Cellular Receptor 2; also known as TIM3: T-cell Immunoglobulin and Mucin domain-containing protein 3

HC: Healthy Control

HCC: Hepatocellular Carcinoma

HDM: House Dust Mite

HES1: Hes family BHLH transcription factor 1

HF: Hair Follicle

HFG: Hepatocyte Growth Factor

Hh: Hedgehog

HHI: Hedgehog pathway Inhibitor

HIBADH: 3-Hydroxyisobutyrate Dehydrogenase

HIF: Hypoxia-Inducible Factor

HIV: Human Immunodeficiency Virus

HLA: Human Leucocyte Antigen

HMGA2: High Mobility Group AT-hook 2

HNSCC: Head and Neck Squamous Cell Carcinoma

HR: Hazard Ratio

HRAS: Harvey Rat Sarcoma viral oncogene homolog

HRNR: Hornerin

HRP: Horseradish Peroxidase

ICAM-1: Intercellular Adhesion Molecule 1

ICD: Intracellular Domain

ICD-O: International Classification of Diseases for Oncology

ICI: Immune Checkpoint Inhibitor

ICOS: Inducible T cell Costimulator

ID1: Inhibitor Of DNA Binding 1

IF: immunofluorescence

IFE: Interfollicular Epidermis

IFN: Interferon

IFNGR: Interferon gamma receptor

Ig: Immunoglobulin

IGF1R: Insulin like Growth Factor 1 Receptor

IGF2BP1: Insulin like Growth Factor 2 mRNA Binding Protein 1

IgSF: Immunoglobulin Superfamily

IHC: immunohistochemistry

IL(X)R: Interleukin (X) Receptor

IL: Interleukin

IL-2R γ c: Interleukin 2 Receptor gamma common

IL3RA: Interleukin 3 Receptor subunit Alpha; also known as CD123

ILC3: type 3 Innate Lymphoid Cell

ILR2A: Interleukin-2 Receptor subunit alpha; also known as CD25

IMPA2: Inositol Monophosphatase 2

IMQ: Imiquimod

iNOS: inducible Nitric Oxide Synthase; also known as NOS2: Nitric Oxide Synthase 2

IRF: Interferon Regulatory Factor

IRS: Inner Root Sheath

ITG: Integrin subunit

IVL: Involucrin

JAG2: Jagged canonical Notch ligand 2

JAGN1: Jagunal vesicle mediated transporter 1

JAK: Janus Kinase

JAM: Junctional Adhesion Molecule

JAML: Junctional Adhesion Molecule Like

JNK: c-Jun N-terminal Kinase

JUP: Junction Plakoglobin

K: Keratin (protein)

KC: Keratinocyte

KCa: Keratinocyte Cancer

KCNN4: Potassium Calcium-Activated Channel Subfamily N Member 4

KDR: Kinase insert Domain Receptor; also known as VEGFR2: Vascular Endothelial Growth Factor Receptor 2

KEGG: Kyoto Encyclopedia of Genes and Genomes

KGF-1: Keratinocyte Growth Factor 1

Ki-67: Antigen Kiel 67

KIBRA: Kidney And Brain Protein

KIF22: Kinesin Family member 22

KLK: Kallikrein related peptidase

KLRK1: Killer Cell Lectin Like Receptor K1; also known as CD314

KO: Knockout

KPRP: Keratinocyte Proline Rich Protein

KRAS: Kirsten Rat Sarcoma viral oncogene homolog

KRT: Keratin (gene)

KRTAP: Keratin Associated Protein

KRTDAP: Keratinocyte Differentiation Associated Protein

laBCC: locally advanced Basal Cell Carcinoma

LAG3: Lymphocyte Activating 3; also known as CD223

LATS: Large Tumor Suppressor kinase

LC: Langerhans Cell

LEKTI: Lympho-Epithelial Kazal-Type-related Inhibitor; also known as SPINK5: Serine Peptidase Inhibitor Kazal type 5

LGALS: Galectin

LGR: Leucine rich repeat containing G protein-coupled receptor

LHX2: Lim Homeobox 2

LNK2: Ligand of Numb-protein X 2

LOF: Loss Of Function

LOH: Loss Of Heterozygosity

LOR: Loricrin cornified envelope precursor protein

LPCAT3: Lysophosphatidylcholine Acyltransferase 3

LRIG1: Leucine Rich repeats and Immunoglobulin like domains 1

LUAD: Lung Adenocarcinoma

LUSC: Lung Squamous Carcinoma

Mac-1: Macrophage-1 antigen

MAFB: MAF BZIP transcription factor B

MAGI-1: Membrane-Associated Guanylate kinase 1

MAL: Methyl Aminolevulinate

MAL-PDT: Methyl Aminolevulinate Photodynamic Therapy

MAP4K: MAP kinase kinase kinase kinase

MAPK: Mitogen-Activated Protein Kinase

MATN4: Matrilin 4

Mb: Megabase

mBCC: metastatic Basal Cell Carcinoma

MC1R: Melanocortin 1 Receptor

MCCC: Methylcrotonyl-CoA Carboxylase subunit

MCM: Minichromosome Maintenance complex component

MCP-1: Macrophage Chemotactic Protein-1

M-CSF: Macrophage Colony Stimulating Factor; also known as CSF1: Colony Stimulating Factor 1

MDSC: Myeloid-Derived Suppressor Cell

MERLIN: Moesin-Ezrin-Radixin Like

MET: Mesenchymal to Epithelial Transition

MET: N-methyl-N'-nitro-N-nitrosoguanidine; MET proto-oncogene, receptor tyrosine kinase; also known as HGFR: Hepatocyte Growth Factor Receptor

MGST1: Microsomal Glutathione S-Transferase 1

MHC-(X): Major Histocompatibility Complex class (X)

MIP: Macrophage Inflammatory Protein

MKI67: Marker of proliferation Ki-67

MLANA: Melan-A

MLH1: MutL Homolog 1

MMP: Matrix Metalloproteinase; also known as Matrix Metalloproteinase

MMR: Mortality Rate Ratio

MMS: Mohs's Micrographic Surgery

M-MuLV: Moloney Murine Leukemia Virus

MNN: Mutual Nearest Neighbor

MOB: Mps One Binder

moDC: monocyte-derived Dendritic Cell

MOSPD1: Motile Sperm Domain containing 1

MRTF: Myocardin-Related Transcription Factor

MS4A1: Membrane Spanning 4-domains A1; also known as CD20

MSH: MutS Homolog

MSLN: Mesothelin

MST: Mammalian STE20-like

MST: Minimum Spanning Tree

mTOR: mechanistic Target Of Rapamycin kinase

MUCL1: Mucin Like 1

MUPP-1: Multi-PDZ domain Protein-1

MXRA5: Matrix Remodeling Associated 5

MYCN: Neuroblastoma MYC oncogene

NBN: Nibrin

NCAM1: Neural Cell Adhesion Molecule 1; also known as CD56

NDR: Nuclear Dbf2-Related kinase

NE: Neutrophil Elastase

NEBL: Nebulette

NECTIN: Nectin cell adhesion molecule

NES: Nestin

NF2: Neurofibromin 2

NFAT: Nuclear Factor of Activated T cells

NFATC1: Nuclear Factor of Activated T Cells 1

NF- κ B: Nuclear Factor kappa B

NHEK: Normal Human Epidermal Keratinocyte

NHK: Normal Human Keratinocyte

NICD: Notch1 Intracellular Domain

NK: Natural Killer cell

NKT: Natural Killer T cell

NLK: Nemo-Like Kinase

NLRP3: NLR family Pyrin domain containing 3

NMSC: Non-Melanoma Skin Cancer

NNAT: Neuronatin

NRAS: Neuroblastoma RAS viral oncogene homolog

NRP1: Neuropilin 1; also known as BDCA4: Blood Dendritic Cell Antigen 4; also known as CD304

NS: Normal Skin

NSCLC: Non-Small Cell Lung Carcinoma

NT5E: 5'-Nucleotidase Ecto; also known as CD73

NUPR1: Nuclear Protein 1

O₂: oxygen

OCLN: Occludin

OCT: Optical Coherence Tomography

ORR: Objective Response Rate

ORS: Outer Root Sheath

OS: Overall Survival

OTR: Organ Transplant Recipient

OVA: Ovalbumin

OVOL: Ovo Like zinc finger

p-: phosphorylated

p53: tumor protein P53 (protein)

p63: tumor protein 673 (protein)

p73: tumor protein p73 (protein)

PALB2: Partner And Localizer of BRCA2

pBCC: primary Basal Cell Carcinoma

PBMC: Peripheral Blood Mononuclear Cell

PC: Principal Component

PCNA: Proliferating Cell Nuclear Antigen

PCR: Polymerase Chain Reaction

PD-1: Programmed cell Death 1

PDAC: Pancreatic Ductal Adenocarcinoma

pDC: plasmacytoid Dendritic Cell

PDGF: Platelet-Derived Growth Factor

PDK1: Phosphoinositide-Dependent Kinase 1

PDL: Programmed cell Death 1 Ligand

PDT: Photodynamic Therapy

PDZ: Post-synaptic density protein 95 (PSD-95), Discs large tumor suppressor (Dlg1) and Zonula occludens 1 (ZO-1)

PECAM1: Platelet and Endothelial Cell Adhesion Molecule 1; also known as CD31

PEX: Peroxisomal biogenesis factor

PHGDH: Phosphoglycerate Dehydrogenase

PHLDA1: Pleckstrin Homology-Like Domain, family A, member 1

PHLPP2: PH domain and Leucine rich repeat Protein Phosphatase 2

PI3K: Phosphatidylinositol 3-Kinase; also known as Phosphoinositide 3-Kinase

PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-Kinase Catalytic subunit Alpha

PKA: Protein Kinase A

PKCδ: Protein Kinase C delta

PKP: Plakophilin

PLA2G2A: Phospholipase A2 Group IIA

PLA2R1: Phospholipase A2 Receptor 1

PLCB4: Phospholipase C Beta 4

PLET1: Placenta Expressed Transcript 1

PLIN2: Perilipin 2

PLK1: Polo-Like Kinase 1

PLXND1: Plexin D1

PMA: Phorbol 12-Myristate 13-Acetate

PMEL: Premelanosome protein

PMN: Polymorphonuclear leucocyte

PMPCB: Peptidase, Mitochondrial Processing subunit Beta

POLA1: DNA Polymerase Alpha 1, catalytic subunit

PP2Ac (or **PPP2CA**): Protein Phosphatase 2 Catalytic subunit Alpha

PPP2R5C: Protein Phosphatase 2 Regulatory subunit B'Gamma

PPP6C: Protein Phosphatase 6 Catalytic subunit

PRDM1: PR/SET Domain 1; also known as BLIMP1: B Lymphocyte-Induced Maturation Protein 1

PRIM2: DNA Primase subunit 2

PROM2: Prominin 2

PRR: Proline Rich

PSAT1: Phosphoserine Aminotransferase 1

PSPH: Phosphoserine Phosphatase

PTCH: Patched

PTEN: Phosphatase and Tensin homolog

pTh5: pathogenic type 5 T helper cell

PTH1H: Parathyroid Hormone Like Hormone

PTM: Post-Translational Modification

PTPN(X): Protein Tyrosine Phosphatase Non-receptor type (X)

PTPR(X): Protein Tyrosine Phosphatase Receptor type (X)

PVDF: Polyvinylidene Difluoride

PYGB: Glycogen Phosphorylase B

QoL: Quality of Life

RA: Rheumatoid Arthritis

RAC1: Ras-related C3 botulinum toxin substrate 1

Rap1: Ras-Associated Protein 1

RB1: Retinoblastoma protein 1

rBCC: recurrent Basal Cell Carcinoma

RBL2: RB transcriptional corepressor Like 2

RBM47: RNA Binding Motif protein 47

RCC: Renal Cell Carcinoma

rcf: relative centrifugal force

RCM: Reflectance Confocal Microscopy

resBCC: resistant Basal Cell Carcinoma

RET: Rearranged during Transfection

RETNLB: Resistin Like Beta

RFC(X): Replication Factor C subunit (X)

RhoA: Ras homolog family member A

RIP: Regulated Intramembrane Proteolysis

RIPA: Radioimmunoprecipitation Assay

RNF5: Ring Finger protein 5

ROCK: Rho-associated protein kinase

RORC: RAR related Orphan Receptor C (gene)

RORγ: RAR-related Orphan Receptor gamma (protein)

ROS: Reactive Oxygen Species

RP: Replication Protein

rpm: revolutions per minute

RPMI: Roswell Park Memorial Institute

RPTN: Repetin

RRM: Ribonucleotide Reductase catalytic subunit M

RS: Relative Survival

RSR: Relative Survival Rate

RT: Room Temperature

RTK: Receptor Tyrosine Kinase

RT-qPCR: Reverse Transcription quantitative real-time Polymerase Chain Reaction

RUNX: Runt-related transcription factor

S6K: ribosomal protein S6 Kinase

SAMM50: SAMM50 Sorting and Assembly Machinery component

SAV1: Salvador homologue 1

SBSN: Suprabasin

SC: Stem Cell

SCC: Squamous Cell Carcinoma

SCGB(XYZ): Secretoglobin family (XY) member (Z)

SCLC: Small-Cell Lung Carcinoma

scRNAseq: single-cell RNA sequencing

SDC1: Syndecan 1; also known as CD138

SDS: Sodium Dodecyl Sulfate

SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SE: Surgical Excision

SERPINB1: Serpin family B member 1

SETD2: SET Domain containing 2

SFRP: Secreted Frizzled Related Protein

SHH: Sonic Hedgehog

SIAH2: Siah E3 ubiquitin protein ligase 2

siRNA: small interfering RNA

SIRPA: Signal Regulatory Protein Alpha; also known as CD172a

SIRT1: Sirtuin 1

sJAML: soluble Junctional Adhesion Molecule Like

SKP2: S-phase Kinase-associated Protein 2

SLC(X)A(Y): Solute Carrier family (X) member (Y)

SLURP1: Secreted LY6/PLAUR domain containing 1

SMC: Structural Maintenance of Chromosomes

SMO: Smoothed

SMOi: Smoothed inhibitor

SMPD2: Sphingomyelin Phosphodiesterase 2

SMR: Standardized Mortality Ratio

SND1: Staphylococcal Nuclease and Tudor Domain containing 1

SNV: Single Nucleotide Variant

SOCS1: Suppressor Of Cytokine Signaling 1

SOX: SRY-box transcription factor

SPINK5: Serine Peptidase Inhibitor Kazal type 5; also known as LEKTI: Lympho-Epithelial Kazal-Type-related Inhibitor

SPRR: Small Proline Rich Protein

Src: Sarcoma

SRF: Serum-Response Factor

SRM: Significantly Recurrent Mutation

ST: Spatial Transcriptomics

STAG1: Sister chromatid cohesion 3 homolog A

STAG2: Sister Chromatid Cohesion 3 Homolog B

STAT: Signal Transducer And Activator of Transcription

STK19: Serine/Threonine-protein Kinase 19; also known as WHR1: Winged Helix Repair factor 1

STRIPAK: Striatin-Interacting Phosphatase and Kinase

SUFU: Suppressor of Fused

SV40: Simian Virus 40

SWI/SNF: SWItch/Sucrose Non-Fermentable

TAC: Transit-Amplifying Cell

TAD: Transactivation Domain

TAGLN: Transgelin

TAK1: TGF-beta Activated Kinase 1; also known as MAP3K7: Mitogen-Activated Protein Kinase Kinase Kinase 7

TAM: Tumor-Associated Macrophage

T-bet: T-Box Expressed In T cells

TBS-T: Tris-Buffered Saline with Tween[®] 20 detergent

TBX: T-Box transcription factor

Tc(X): type (X) cytotoxic T cell

TCF3: Transcription Factor 3

TCGA: The Cancer Genome Atlas

TCHH: Trichohyaline

TCHHL1: Trichohyaline Like 1

Tcrd: T cell receptor delta locus

TEAD: TEA Domain transcription factor

TEAE: Treatment-Emergent Adverse Event

TEM: Transendothelial Migration

TEMED: Tetramethylethylenediamine

TEpM: Transepithelial Migration

TERT: Telomerase Reverse Transcriptase

TF: Transcription Factor

Tfh: T follicular helper cell

Tfr: T follicular regulatory cell

TGF: Transforming Growth Factor

TGFR2: Transforming Growth Factor Beta Receptor 2

TGM3: Transglutaminase 3

Th(X): type (X) T helper cell

THBD: Thrombomodulin; also known as BDCA3: Blood Dendritic Cell Antigen 3; also known as CD141

THY1: Thy-1 cell surface antigen; also known as CD90

TID: TEAD-interaction domain

TIGIT: T cell immunoreceptor with Ig and ITIM domains

TIL: Tumor-Infiltrating Lymphocyte

TIM3: T-cell Immunoglobulin and Mucin-domain containing-3; also known as HAVCR2: Hepatitis A Virus Cellular Receptor 2;

TINAGL1: Tubulointerstitial Nephritis Antigen Like 1

TJP: Tight Junction protein

TLR: Toll-Like Receptor

TM4SF1: Transmembrane 4 L Six Family member 1

TMB: Tumor Mutational Burden

TME: Tumor Microenvironment

TMED4: Transmembrane P24 trafficking protein 4

TMEM97: Transmembrane protein 97

TNC: Tenascin C

TNFR: Tumor Necrosis Factor Receptor

TNFRSF(X): TNF Receptor Superfamily member (X)

TOP2A: DNA Topoisomerase II Alpha

TOX: Thymocyte selection-associated high mobility group box

TP53: Tumor Protein P53 (gene)

TP63: Tumor Protein P63 (gene)

TP73: Tumor Protein P73 (gene)

TRDC: T cell Receptor Delta Constant

Treg: regulatory T cell

TREM2: Triggering Receptor Expressed on Myeloid cells 2

TRGC: T cell Receptor Gamma Constant

TRM: Tissue-resident memory

TRPV4: Transient Receptor Potential cation channel subfamily V member 4

TRUB2: TruB pseudouridine synthase family member 2

TUNEL: Terminal deoxynucleotidyl transferase dUTP Nick-End Labeling

T-VEC: Talimogene laherparepvec

TXNIP: Thioredoxin Interacting Protein

TYMS: Thymidylate Synthetase

TYR: Tyrosinase

UBE2C: Ubiquitin conjugating Enzyme E2 C

UK: United Kingdom

UM: Uveal Melanoma

UMAP: Uniform Manifold Approximation and Projection

USA: United States of America

USP3: Ubiquitin Specific Peptidase 3

USP9X: Ubiquitin Specific Peptidase 9 X-linked

UV: Ultraviolet

UVR: Ultraviolet Radiation

VANGL2: VANGL planar cell polarity protein 2

VCL: Vinculin

VEGF: Vascular Endothelial Growth Factor

VEGFR2: Vascular Endothelial Growth Factor Receptor 2; also known as KDR: Kinase insert Domain Receptor

VGLL4: Vestigial Like family member 4

V-type: Variable type

VWF: Von Willebrand Factor

WB: Western Blot

WFDC: WAP Four-Disulfide Core domain

WHO: World Health Organization

WHR1: Winged Helix Repair factor 1; also known as STK19: Serine/Threonine-protein Kinase 19

WIF1: Wnt Inhibitory Factor 1

WT: wild-type

WWTR1: WW domain containing Transcription Regulator 1; also known as TAZ

XCR1: X-C Motif Chemokine Receptor 1

XP(X): Xeroderma Pigmentosum group (X)-complementing protein

XP: Xeroderma Pigmentosum

YAP: Yes-Associated Protein

ZEB1: Zinc finger E-box binding homeobox 1

ZNF750: Zinc Finger protein 750

ZO: Zonula Occludens

α -SMA: alpha-Smooth Muscle Actin

β cDC: β -catenin Destruction Complex

βTr-CP: beta-transducin repeat containing E3 ubiquitin protein ligase

γδ T cell: gamma delta T cell

Abstract

Basal cell carcinoma (BCC) comprises the most common type of skin cancer, and the most common malignancy overall in white people. Despite its very low fatality, morbidity is significant, and for the difficult-to-treat cases, recurrence and/or resistance to therapy is not uncommon. Epithelial cell-expressed CXADR and its cognate ligand, immune cell-expressed JAML, have both been implicated in tumorigenesis, in a context-dependent manner, while CXADR has recently been proposed to potentially interact, in human bronchial epithelial cells, with YAP-1, which is known for promoting BCC development. The aim of the current study was to investigate the role of the potential interplay between the JAML-CXADR axis and YAP-1 in the pathogenesis of BCC. To this end, publicly available scRNAseq (8 BCC and 14 HC skin biopsies) and spatial transcriptomic (ST) (8 BCC, 22 HC) data were analyzed, followed by immunofluorescence (IF) examination of HaCaT cells, to investigate the co-localization of CXADR and YAP-1, while BCC biopsies were immunohistochemically (IHC) evaluated for CXADR and YAP-1 expression. Scratch assay on HaCaT cells, as well as Western Blot (WB) analysis, under T helper (Th)2 (IL-4, IL-5, IL-13) or recombinant JAML cell culture conditions, was also performed. Results revealed a distinct T cell infiltrating profile for BCC compared to HC skin. Importantly, CXADR and YAP-1 co-localize at areas of intercellular contact between KCs. While in ST and IHC analysis, CXADR and YAP-1 were uniformly expressed within the BCC aggregates, with *JAML*-positive T cells preferentially located at the periphery rather than within the tumor. Altogether, the present study highlights a novel potential interaction between CXADR and YAP-1 that could drive the malignant phenotype of BCC KCs, and shape the tumor-associated immune responses in the BCC tumor microenvironment (TME).

Περίληψη

Το βασικοκυτταρικό καρκίνωμα (BCC) αποτελεί το συχνότερο τύπο καρκίνου του δέρματος, και την πιο συχνή κακοήθεια γενικά στους λευκούς πληθυσμούς. Παρά την πολύ χαμηλή θνητότητα, χαρακτηρίζεται από σημαντική νοσηρότητα, και για τις περιπτώσεις αυτού που είναι «δύσκολες στο να θεραπευτούν» (difficult-to-treat), οι υποτροπές ή/και η αντίσταση στην αγωγή δεν αποτελούν σπάνιο φαινόμενο. Ο επιθηλιακά εκφραζόμενος υποδοχέας CXADR, και ο ενδογενής προσδέτης του, JAML, των ανοσοκυττάρων, έχουν συσχετιστεί αμφότεροι με τη διαδικασία της καρκινογένεσης, με τρόπο που εξαρτάται από τις εκάστοτε συνθήκες, ενώ ο CXADR έχει πρόσφατα προταθεί να αλληλεπιδρά πιθανά με την πρωτεΐνη YAP-1, η οποία είναι γνωστό πως προάγει την ανάπτυξη του BCC. Ο στόχος της παρούσας μελέτης ήταν να διερευνήσει το ρόλο της πιθανής αλληλεπίδρασης μεταξύ του άξονα JAML-CXADR και της YAP-1 στην παθογένεση του BCC. Για το σκοπό αυτό, έγινε ανάλυση δημοσίων διαθέσιμων scRNAseq (8 βιοψίες BCC και 14 βιοψίες υγιούς δέρματος-HC), και spatial transcriptomic (ST) (8 BCC, 22 HC) δεδομένων, ακολουθούμενη από μελέτη ανοσοφθορισμού σε κύτταρα HaCaT για διερεύνηση της συνεντόπισης CXADR και YAP-1, ενώ έγινε επίσης ανοσοϊστοχημική (IHC) χρώση βιοψιών BCC προκειμένου να εκτιμηθεί η έκφραση των δύο πρωτεϊνών. Πραγματοποιήθηκε επιπλέον scratch assay σε κύτταρα HaCaT, καθώς και ανάλυση ανοσοτύπωσης κατά Western (Western Blot) στα εκχυλίσματα αυτών, σε συνθήκες καλλιέργειάς τους που προσομοιάζουν την παρουσία Th2 βοηθητικών κυττάρων (IL-4, IL-5, IL-13) ή παρουσία ανασυνδυασμένου JAML. Τα αποτελέσματα ανέδειξαν ένα διακριτό προφίλ T λεμφοκυτταρικής διήθησης στα BCC σε σχέση με το υγιές δέρμα. Σημαντική ήταν ειπλέον η ανάλυση της συνεντόπισης CXADR και YAP-1 σε περιοχές διακυτταρικής επαφής μεταξύ των KCs. Παράλληλα, από την ανάλυση των ST δεδομένων και την IHC, αναδείχθηκε μια ομοιογενής κατανομή της έκφρασης CXADR και YAP-1 εντός των εστιών του BCC, με τα T λεμφοκύτταρα που εκφράζουν JAML να εντοπίζονται κατά προτίμηση στην περιφέρεια παρά στο εσωτερικό του όγκου. Συνολικά, η παρούσα μελέτη αναδεικνύει μια νέα πιθανή αλληλεπίδραση μεταξύ CXADR και YAP-1, που μπορεί να ωθεί τον κακοήγη φαινότυπο των BCC KCs, και να διαμορφώνει τις ανοσολογικές αποκρίσεις που σχετίζονται με τον όγκο εντός του μικροπεριβάλλοντος του BCC.

1. Introduction

1.1 Basal Cell Carcinoma

Basal cell carcinoma (BCC) is the most common type of skin cancer, accounting for approximately 80% of all skin cancer cases in the USA ¹, and comprises the most common cancer in white humans overall ². BCC falls under the umbrella of non-melanoma skin cancer (NMSC), along with the other major keratinocyte (KC) malignancy, cutaneous squamous cell carcinoma (cSCC), representing, approximately 70% of all NMSCs ¹.

1.1.1 Epidemiology of BCC

The exact prevalence and incidence rate of BCC are challenging to estimate, due to the lack of systematic reporting of BCC cases in national cancer registries and cancer surveillance programs in most countries ³⁻⁵. This discrepancy has been attributed mainly to the high incidence and low fatality and mortality of BCC ⁵ that render its reporting both demanding and of secondary importance compared to other cancer types, the lack of histopathological confirmation for various BCC cases, especially in the setting of metachronous BCCs of the same patient ^{5,6}, as well as the absence of distinct reporting of BCCs and SCCs, which are often studied as one, under the term “NMSC”, in the literature ⁷. Keeping this variability in mind, studies have reported a BCC prevalence of 525 per 100000 persons in the USA ⁸, and have estimated an incidence of around 2 million new BCC cases in the USA annually ³. The number of BCC cases globally in 2019 was 4 million ⁹, while adjusted incidence rate has been estimated at approximately 196 and 257 BCC cases per 100000 person-years worldwide and in Europe, respectively ¹⁰. The highest incidence rate of BCC is reported in Australia, where more than 1000 new BCC cases per 100000 persons are diagnosed annually ¹¹⁻¹³.

White-skinned individuals are far more likely to develop BCC compared to people of color ^{3,14-17}, although estimations for their lifetime risk vary between different studies. Previous calculations in white people from the USA highlight an average lifetime risk of approximately 30% (23-28% for women, 33-39% for men) ^{6,18}, while more recent studies on the Danish and Spanish populations report an average BCC lifetime risk of 21.2% (19.7% for women, 22.4% for men) ¹⁹, and of 5.8% (5.02% for women and 7% for men) ¹⁰, respectively. Another recent study reports an average lifetime risk for all NMSCs of 19.7% (17.5% for women, 22.3% for men) in the English population ²⁰, whereas the cumulative risk for the development of NMSC before the age of 70 in Australian people is around 58% for women and 69% for men ²¹.

Furthermore, several studies have highlighted an overall increase in incidence rates of BCC in white populations in recent years ^{11,22-24}. Indicatively, age-adjusted incidence rates of BCC increased by approximately 100% for women and 145% for men between 1986/1988 and

2006²⁴. A 5.5% annual increase in BCC incidence rates in males in Europe between a similar time period¹¹, and an increase of 4% annually in both men and women between 1979-1980 and 1993-1994²³ have been reported. Importantly, projections estimate that BCC incidence rates will increase by more than 140% by 2050²⁵.

In addition, the risk for BCC increases with age^{3,26–29}, with most BCC cases arising after 40 years, and with a mean age of patients at diagnosis at approximately 66 years old²⁷. Moreover, BCC is generally more common in males than females^{3,30–32} – however, in individuals under 40 years old, the male/female ratio tends to be reversed, due to a significant increase in the incidence of the disease in young women, as has been observed in the Netherlands^{22,33}, Denmark³⁴, the UK²⁰ and USA³⁵. This has been speculated to be in part attributable to the increased use of artificial tanning methods (sunbeds) by young women and/or to more frequent medical visits in regard to skin-associated cosmetic practices^{5,36}.

1.1.2 Risk Factors for BCC

As has already been established, age^{3,26–29}, male sex^{3,30–32}, and light skin type^{3,17}, specifically Fitzpatrick type I and type II skin^{37,38} constitute risk factors for the development of BCC. Additionally, it has been shown that individuals with red/blonde hair and/or light-colored eyes are also more susceptible to the development of the disease^{17,39,40}.

However, the most important – environmental – risk factor for BCC is UV radiation (UVR)^{2,17}, and especially UVB exposure⁴¹ – in fact, this explains the spatiotemporal distribution of BCCs; most of these tumors arise in sun-exposed areas of the skin, including the face, head, scalp and neck^{17,42}, and their incidence is higher (more than double) in locations close to the Equator²⁸. Interestingly, it appears that it isn't cumulative UVR exposure which associates with an increased risk for BCC, but rather intermittent, intense sun exposure, especially during childhood and adolescence^{2,43–45} – accompanied by events of sunburns during childhood that exert the same effect^{43,46}. It is also not surprising that indoor tanning has been found to significantly increase the risk of BCC, even more so when performed in early life^{41,47}. Accordingly, it has been observed that a high number (>150) of psoralen plus UVA radiation (PUVA) treatments in patients with psoriasis leads to increased risk of BCC^{48,49}, as does a high number (>300) of UVB treatments^{49,50}.

Aside from the non-ionizing solar UV exposure, ionizing radiation also increases the risk for BCC development^{17,51–53}. Such type of exposure can be either iatrogenic (e.g. radiotherapy for various cancer types, total body irradiation preceding haemopoietic stem cell transplantation), occupational (e.g. medical workers exposed to radiation) or environmental (e.g. atomic bomb survivors)^{51–53}.

Both UV and ionizing radiation contribute to the development of BCC due to their mutagenic potential, which, in the case of UVR can be exerted primarily through the formation of photoproducts, such as cyclobutane pyrimidine dimers (CPD) and pyrimidine-pyrimidone (6-4) photoproducts (6-4PP), but also DNA strand breaks or crosslinks, or via the production of reactive oxygen species (ROS) ^{41,54–56}, while in the case of ionizing radiation mainly through DNA breaks or ROS production ⁵⁷. If DNA repair mechanisms fail, there is probable development of mutations in proto-oncogenes and/or tumor suppressor genes, which can lead to aberrant cell proliferation, dysregulation of cell death and/or differentiation, and ultimately to BCC formation ⁵⁵. However, another important mechanism that contributes to the tumorigenicity of UV (and ionizing) radiation is its immunosuppressive effect, that can be exerted either locally or systemically. As Hart & Noval thoroughly examine in their review ⁵⁸, this process involves KC-derived production of a variety of immunological factors, including cytokines and chemokines such as interleukin (IL)-10, IL-6, transforming growth factor (TGF)- α , and CC motif chemokine ligand (CCL)20, as a result of UV-induced changes in DNA, lipids or other chromophores of the KCs. Moreover, UVR-activated vitamin D also facilitates immunosuppression, for instance through reduction of dendritic cell (DC) activity and induction of regulatory T cells (Tregs) ^{59,60}, while CPD formation in cutaneous antigen-presenting cells (APCs) has been found to be associated with their impaired antigen presenting ability following UV irradiation ⁶¹. UVR can also mediate the migration of (immature or UV-damaged) Langerhans cells (LCs) to draining lymph nodes, where they facilitate the induction of Tregs and natural killer T cells (NKTs) ^{62,63}, as well as the nodal migration of mast cells, which secrete IL-10 and enable the production of regulatory B cells, further supporting the immunomodulatory effect of UVR ^{58,64,65}. Additionally, studies from murine models propose a potential effect of UVR on myeloid progenitors in the bone marrow, ultimately leading to long-lasting systemic immunosuppression due to disrupted DC function ^{66,67}.

Another factor that has been associated with increased risk for BCC is arsenic (As) exposure ^{17,68,69}, which may occur mainly through ingestion of contaminated drinking water or food ⁷⁰. Although the exact mechanisms have not been fully elucidated, ROS production, DNA damage, impaired DNA repair and immune dysfunction have all been implicated in the tumorigenic function of As in skin cancers ⁷¹. Interestingly, recent studies also suggest that As can interfere with the Hippo/YAP signaling pathway ^{72,73}, which constitutes an important regulator of cell proliferation, differentiation and apoptosis, and has extensively been associated with tumorigenesis ^{74–96}, including that of BCC ^{97–101}.

Unsurprisingly, immunosuppression increases the risk for BCC development as well. Organ transplant recipients (OTRs) display a 3- to 5- fold increased risk for malignancies compared to the general population, with NMSCs being among the most common ones ^{102–104}.

Although the risk for cSCC is much more prominently increased (65-fold or higher), placing it as the number one neoplasm in OTRs ^{103,104}, the risk for BCC is also significantly increased, 10-fold compared to that of the rest of the population ^{105–107}. This also marks an inverted ratio of cSCC:BCC observed in OTRs compared to the general population ¹⁰⁸. This discrepancy in the relative risks for cSCC and BCC in OTRs is mainly attributed to the increased susceptibility of these individuals to virus-associated malignancies, as is the case for cSCC, but not for BCC ^{104,108}. While post-transplant cSCCs appear to be more aggressive compared to those of the general population, this is not the case for post-transplant BCCs, which display a similar behavior to those of non-OTRs ^{105,109,110}. Although to a lesser degree compared to OTRs, patients with HIV also display an increased risk for the development of BCC, which has been estimated at approximately 2 times higher than that of the general population ^{105,111,112}, and also maintain a similar cSCC:BCC ratio to the general population ^{112,113}. Non-transplant patients receiving immunosuppressive agents for the treatment of diseases such as rheumatoid arthritis (RA), as well as patients with malignancy-induced immunosuppression, can also be at increased risk for the development of skin cancer ¹⁰⁴ – Scurtu and colleagues have specifically corroborated this observation for BCC, reporting that a significantly higher percentage of BCCs was detected in patients with comorbidities compared to individuals without, and of those, the significant majority (>80%) had immunosuppressive comorbidities, including diabetes mellitus, history of solid or hematogenous cancer in the last 5 years, chronic kidney disease, chronic infections, or were receiving antirheumatic immunosuppressive therapies ¹¹⁴.

One of the most important predictors for the development of BCC is personal and/or family history of NMSC ^{17,115,116}. Meta-analyses have shown that patients with a previous KC cancer (KCa) are at least 10 times more likely to develop new BCCs (or cSCCs) compared to the general population ^{115,116}. A recent study, however, highlights that the risk for subsequent new KCa is substantially higher after a non-first KCa diagnosis compared to that after a first lifetime KCa diagnosis (with this applying to both the risk for new BCCs after BCC, and the risk for new cSCCs after cSCC) ¹¹⁷. Also, it appears that familial history of skin cancer is associated with increased risk for early-onset BCC ^{118,119}.

1.1.2.1 Genetic Predisposition for BCC

A series of hereditary disorders are also associated with increased risk for BCC, including Xeroderma Pigmentosum, Gorlin-Goltz Syndrome (or Basal Cell Naevus Syndrome or Nevroid Basal Cell Carcinoma Syndrome), Bazex-Dupre-Christol Syndrome, and Rombo Syndrome. Briefly, (i) Xeroderma Pigmentosum (XP) is an autosomal recessive genodermatosis that results from mutations in the nucleotide excision repair system (*XPA*, *XPB*, *XPC*, *XPD*, *XPE*, *XPF*, *XPG*, *XPV*, *ERCC1* genes), which is essential for UVR-induced

DNA damage repair, thus this condition associates with increased photosensitivity and an increased risk for (early-onset) cutaneous malignancies, including BCCs ¹²⁰. It has been estimated that the risk for NMSC in patients with XP is 10000-fold that of the general population ¹²¹. (ii) Gorlin-Goltz Syndrome is an autosomal dominant familial syndrome, that is characterized by skin manifestations, including multiple early-onset BCCs – mean age of appearance of around 20 years ¹²² – along with skeletal abnormalities (such as odontogenic keratocysts), ocular lesions, and neurological manifestations, including the risk for medulloblastomas ^{123–127}. Gorlin-Goltz Syndrome is caused, in the majority of cases, by germline mutations in the tumor suppressor *PTCH1* gene (located on chromosome 9q) ^{128–133} of the Hedgehog signaling pathway – a pathway that is abnormally upregulated in most BCC cases ^{134–136}. More recent studies have also associated the condition with mutations in the tumor suppressor *SUFU* (chromosome 10q) ^{137,138} and *PTCH2* (chromosome 1p) ^{139–141} genes. (iii) Bazex-Dupre-Christol syndrome is a rare X-linked condition characterized by the triad of BCC, follicular atrophoderma and hypotrichosis, and associated with mutations of the *ACTRT1* gene (encoding for the actin related protein T1) ^{142,143}, while (iv) Rombo syndrome is an extremely rare autosomal dominant disorder also associated with BCCs which reportedly arise around the age of 35 ^{144,145} – its genetic background, however, is still under investigation.

Furthermore, variants of the melanocortin 1 receptor (*MC1R*) gene, which encodes for a G-protein coupled receptor (GPCR) known for its contribution to skin and hair pigmentation determination ^{146,147}, have also been associated with increased susceptibility to the development of BCC (and skin cancer in general), independently of skin color ^{148–152}. This has been hypothesized to be potentially attributable to non-pigmentary effects exerted by MC1R when expressed in non-melanocytic cell populations, including immune cells, such as macrophages ^{148,153}.

A summary of the individual (non-environmental) and environmental risk factors associated with the development of BCC is shown in **Table 1**.

1.1.3 BCC Pathogenesis and Molecular Genetics

As for most malignancies, development of BCC is the result of an interplay between environmental factors and genetic predisposition (see Risk Factors for BCC). Genomic analyses, immunohistochemistry (IHC) and immunofluorescence (IF) examinations of human BCCs, along with murine model experiments, have allowed us to gain further insight into the molecular drivers of this malignancy. Overall, BCC is characterized by a very high tumor mutational burden (TMB), especially in its sporadic form, for which more than 65 mutations per megabase (Mb) of coding DNA have been reported ^{154–156}, making it one of, if not the most, mutated cancers overall ^{157,158}. Familial (Gorlin-Goltz syndrome) cases of BCC display a lower,

Risk factor	Ref.
Individual/Non-environmental	
Increased age	3,26–29,159
Male sex	3,33,159
Light skin phototype (Fitzpatrick scale I/II)	3,37,38
Natural red/blonde hair color	39,40
Light-colored eyes (green/blue)	39,40
Personal and/or familial history of NMSC	115,116,118,119
Immunosuppression – including organ transplant recipients (OTRs)	102–107,111,112,114
Hereditary disorders: Xeroderma Pigmentosum, Gorlin-Goltz Syndrome, Bazex-Dupre-Christol Syndrome, Rombo Syndrome	120–127,142,144,145
<i>MC1R</i> gene variants	148–152
Environmental	
UV (mainly UVB) radiation – especially episodes of intermittent intense sun exposure; indoor tanning	2,41,43–49
Ionizing radiation	51–53
Arsenic (As) exposure	68,69

Table 1: Individual and environmental risk factors associated with the development of basal cell carcinoma (BCC). NMSC = Non-Melanoma Skin Cancer, MC1R = Melanocortin 1 Receptor, UV = Ultraviolet

but still very high TMB, at 21–33 mutations/Mb^{154,156}. In concordance with UVR exposure being the most important risk factor for its development, BCC is characterized by C>T and CC>TT transitions^{154–156,160} – in fact, 90% of BCC's single nucleotide variants (SNVs) have been reported to be C>T changes – which constitute a UVR-induced mutational signature, as they arise at sites of pyrimidine dimers, such as those of the UV-induced CPD and 6,4-PP photoproducts^{161–163}.

From a mechanistic point of view, it has been established that the Hedgehog (Hh) signaling pathway plays a crucial role in the pathogenesis of BCC^{134–136}. As is true for several other developmental pathways in vertebrates, Hh signaling is dependent on the primary cilium, a microtubule-based cell membrane protrusion, which is coordinately assembled and disassembled throughout the cell cycle^{164–166}. Physiologically, in the absence of the Hh protein (the Sonic human homologue, SHH, is the most well studied and the most relevant to BCC), the transmembrane receptors Patched1 (PTCH1) and Patched2 (PTCH2) inhibit the

translocation of the transmembrane protein Smoothed (SMO) to the ciliary membrane; this results in the formation of a repressive form of the glioma-associated oncogene homolog GLI3 transcription factor (TF), and enables the association of the Suppressor of Fused (SUFU) negative regulator of hedgehog signaling protein with GLI2 and GLI3, which suppresses their activity, thus overall inhibiting the expression of nuclear target genes ^{165,167}. Contrarily, in the presence of the Hh ligand, its binding to the PTCH1/PTCH2 receptor releases inhibition on SMO, enabling its accumulation at the primary cilium, which ultimately results in the generation of activating forms of the GLI2/3 TFs, thus facilitating the expression of target genes in the nucleus ^{165,167}. These include, among others, both the *GLI1* and *PTCH1* genes, as well as genes associated with cell cycle and related pathways, such as *MYCN*, *CCND1*, *CCND2*, *BCL2*, *JAG2*, *GREM1*, *PRDM1* (*BLIMP1*) and *CFLAR* ¹⁶⁸. Nevertheless, it is worth noting that Hh signaling can be mediated in a SMO- and/or GLI- independent manner as well, for which the term “non-canonical” Hh signaling is being used ^{169,170}. Either directly or indirectly through its interactions with other pathways, Hh signaling overall promotes cellular proliferation and survival and supports a stem cell (SC) phenotype ¹⁶⁸.

Mutated forms or loss of the PTCH1/2 protein fail to exert an inhibitory effect on SMO, thus mimicking the presence of the Hh ligands ¹⁷¹. Similarly, activating mutations in *SMO* lead to a constitutive activation of downstream target genes, whereas inactivating mutations of *SUFU*, and amplifications of *GLI1/GLI2* genes also result in Hh target gene overexpression ¹⁷¹. It is therefore unsurprising that hyperactivation of the Hh pathway has been associated with carcinogenesis, including that of BCC ¹⁶⁶. In fact, the inappropriate activation of the Hh signaling cascade has been characterized as the most important driver of BCC ^{136,172}, with even early studies ^{130,173} supporting that it is required for its pathogenesis. Nevertheless, recent data from murine models suggest that although necessary, it is not sufficient for the progression of the disease ¹⁷⁴.

Our current knowledge about the implication of the Hh pathway in BCC pathogenesis stems from early linkage studies in families with Gorlin-Goltz Syndrome, which suggested the existence of a putative tumor suppressor gene in chromosome 9q22.3–q31, an area where microsatellite polymorphisms and allelic losses had been described in patients with the disorder ^{128,130}. It was later revealed that the affected loci harbor the *PTCH1* gene, encoding for the PTCH1 protein ^{131,132}. Alterations at this genomic region were identified in sporadic BCCs as well ^{173,175}, ultimately placing loss of heterozygosity (LOH) on chromosome 9q22 (found in 55%-69% of sporadic BCCs ^{130,154,176}) and somatic point mutations of the *PTCH1* gene (found in more than 70% of BCCs) as the most frequent genetic alterations in sporadic BCC tumors ^{130,154,176–183}. *PTCH1* mutations have also been reported to significantly correlate with the superficial rather than the nodular subtype of BCC ¹⁷⁷ (see Clinicopathological

characteristics & Diagnosis of BCC). In lower frequencies, mutations of the *SMO* gene have also been described in patients with BCC ¹⁸¹, and have particularly been associated with resistance (either intrinsic or acquired) to Hh pathway inhibitors-HHIs (SMO inhibitors; SMOi) ^{154,156,180,184} – present in 43% of resistant BCCs compared to 20% of treatment-naïve sporadic BCCs ¹⁵⁴. Similarly, mutations of *GLI1/GLI2* have been identified in BCCs ¹⁸⁰, and amplification of these genes ¹⁸⁵ has also been implicated in resistant and recurrent cases ^{154,156}. Inactivating mutations or chromosomal losses of the *SUFU* gene have additionally been reported in around 8% of BCCs ^{154,156,181} and appear to contribute to, although probably not be sufficient for, HHI resistance development ^{154,156}.

Accordingly, it has been shown that mice with heterozygous deletion of *Ptch1* develop BCC-like lesions following UV or ionizing irradiation ^{186–188}, while in basal layer cell-specific *Ptch1* KO mice BCC tumors also arise, irrespective of irradiation ^{188,189}. Similarly, expression of a constitutively active SMO mutant ^{97,99,190–192}, overexpression of *GLI1* ¹³⁵ or *GLI2* ¹⁹³, or *Sufu* deletion ¹⁹⁴ result in a similar BCC-like phenotype in murine models.

Following the hyperactivation of the Hh signaling pathway, the next most important genetic event implicated in BCC carcinogenesis is the inactivation of the *TP53* gene ^{154,155,160}, a well-known tumor suppressor involved, among others, in cell cycle arrest and induction of apoptosis, and commonly mutated in many human cancers ¹⁹⁵. Approximately half of BCCs (from 40% up to more than 60%) harbor *TP53* mutations ^{154,155,177,181,196,197}, the majority (60%–72%) of which are UVR-associated C>T and CC>TT nucleotide changes ^{181,197,198} – in contrast to a lower proportion of the *PTCH1* mutations being characteristic of UVR exposure (33–40%) ^{181,198}. Copy number alterations (CNAs) of the *TP53* gene locus (17p13.1) are not as common as those of the *PTCH1* locus (9q22.32) in BCCs, and studies have revealed BCC cases where only one *TP53* allele was inactivated, an observation which is interesting given that *TP53* usually requires biallelic inactivation in order to contribute to carcinogenesis ^{155,183}.

Aside from Hh, other pathways that have been implicated in the pathogenesis of BCC include Wnt, Notch, and Hippo/YAP – all important regulators of critical cellular processes such as proliferation, apoptosis and differentiation ^{199–201}. Expression, including nuclear, of β -catenin, the downstream effector of canonical Wnt signaling, is frequently identified in human BCCs, and has been suggested to be positively associated with tumor aggressiveness ^{202–204}. Expression of the transcriptional co-repressor transcription factor 3 (TCF-3), which has been reported to act as a negative regulator of β -catenin degradation ²⁰⁵, has been found upregulated in BCCs ²⁰⁶. Furthermore, an interaction between the Hh and Wnt pathways has been recognized and suggested to promote BCC development ^{207,208}. Indicatively, it has been shown that *SUFU* can suppress ²⁰⁹, whereas *GLI1* can promote ²¹⁰ β -catenin signaling, while

the Wnt inhibitory factor 1 (WIF1) can antagonize the activity of both Wnt and Shh proteins ²¹¹. Although, to my knowledge, point mutations and CNAs of Wnt-associated genes have not been identified in BCCs, hypermethylation of the gene promoters of adenomatosis polyposis coli (*APC*) and secreted frizzled related protein 5 (*SFRP5*) has been found in BCC tumors compared to normal skin (NS) ²¹². In addition, based on murine model experiments, Wnt signaling has been suggested to be involved in the development of resistance against HHIs ²¹³.

The Notch signaling pathway, on the other hand, appears to be downregulated in BCCs compared to healthy skin ^{214,215}, and its stimulation has been shown to promote BCC tumor cell apoptosis ²¹⁴. Mutations of the *NOTCH1* and *NOTCH2* genes have been identified in approximately 29% and 26% of BCCs, respectively ^{154,155,177,180}, although, intriguingly, *NOTCH2* mutations appear to be absent from metastatic cases ²¹⁶. *NOTCH1* mutations have been reported to be significantly higher in superficial compared to nodular BCCs, as well as in BCCs arising in the trunk rather than those arising in head/neck and extremities ¹⁷⁷. Mouse model experiments have implicated Notch signaling in the response of BCCs to HHIs as well ²¹⁷.

Hippo/YAP signaling, dependently or independently of its interaction with the Hh pathway, has also been involved in BCC development (see Role of YAP/TAZ in BCC). On a molecular level, at least four of the Hippo/YAP pathway genes have been found mutated in BCC tumors, namely: (i) large tumor suppressor kinase (*LATS1*) (mutations in 8% of BCCs) and (ii) *LATS2* (12%) ^{154,218} – which encode for tumor suppressor kinases that prevent the nuclear translocation of the Yes-associated protein (YAP)-1 transcriptional co-activator ^{219,220}, (iii) protein tyrosine phosphatase non-receptor type 14 (*PTPN14*) (23%) ^{154,218} – encoding for a non-receptor phosphatase that mediates the nuclear-to-cytoplasmic translocation of YAP-1 ^{221,222}, thus acting as a putative tumor suppressor in BCC, and (iv) protein phosphatase 6 catalytic subunit (*PPP6C*) (15%) ^{154,216} – that encodes for a phosphatase potentially involved in the activation of *LATS1/2* ^{223–225}. Moreover, in a small cohort of patients with metastatic BCC (mBCC) (n = 8) one case with a *YAP1* mutation (S127F) was identified, along with three cases/patients (five lesions in total) with missense *TEAD1/2/4* mutations within the YAP/TAZ binding locus ²¹⁶. Interestingly, although Bonilla and colleagues had also observed *TEAD* gene mutations in their series of BCCs, these were all silent ¹⁵⁴.

Additional genes that can be mutated in BCCs include: *MYCN* (30% of BCCs) ^{154,180,216}, *ARID1A* (26%) ^{154,180} – which is also indirectly related to the Hippo/YAP pathway ²²⁶ – *NRAS*, *HRAS* and *KRAS*, although much less commonly ^{154,227–229}, *STK19* (also known as *WHR1*), *PIK3CA* ^{154,216}, *RAC1*, *ERBB2*, *CASP8*, *RB1* ¹⁵⁴, *PTPRD*, *FLG* and *FLG2* (the latter two

encoding for filaggrin and filaggrin 2, respectively, two proteins important for maintenance of skin homeostasis and barrier function)¹⁸⁰, *STAT5B*, *CRNKL1* and *NEBL*¹⁵⁵. Non-coding mutations in the promoter regions of *TERT* and *DPH3* are also common (present in more than 40-50% and more than 19-42% of BCCs, respectively)^{177,179,180,230–232}.

It is also worth noting that infiltrative BCCs do not appear to have a distinct somatic mutational profile compared to non-infiltrative ones, although they do display significant differences at the transcriptomic level, including the differential expression of *IGF2BP1*, matrix metalloproteinase (*MMP*)7 and fibronectin 1 (*FN1*)¹⁶⁰. Metastatic BCCs (mBCCs), nevertheless, tend to harbor unique mutational features, including deleterious mutations of the *STAG1*, *PPP2R5C* and *CNTRL* genes²¹⁶, which are implicated in cell cycle^{233–235}, as well as mutations in hepatocyte growth factor (*HGF*) and the *MET* (also known as hepatocyte growth factor receptor) and *RET* proto-oncogenes, which have been found mutated significantly more frequently in mBCCs compared to either low- or high- risk BCCs from the cohort by Bonilla and colleagues²¹⁶. Moreover, hematogenous BCC metastases, in comparison to lymphatic/regional ones, have been shown to display exclusive mutational signatures, including mutations in *MATN4*, *PECAM1*, *EGFLAM*, *ADGRA2*, *PLXND1* and *USP9X*²¹⁶, genes which are involved in processes like cell adhesion, migration and/or invasion^{236–241}, as well as in *PTPN21*²¹⁶, which similarly to *PTPN14*, can mediate the nuclear to cytoplasmic shuttle of YAP-1²⁴².

Lastly, aside from the CNAs observed at the *PTCH1* (9q22.32) and *TP53* (17p13.1) loci, additional somatic CNAs in BCCs may involve many of the aforementioned genes^{154,155,180}, while interestingly, recently Nawrocka and colleagues also reported a recurring amplification of the chr9p24.1 locus which harbors the programmed cell death 1 ligand (*PDL*)1 (also known as *CD274*), *PDL2* (also known as *CD273*) and Janus kinase 2 (*JAK2*) genes¹⁸⁰, which had only been previously identified in a case of mBCC with excellent response to anti-programmed cell death 1 (PD-1) immunotherapy²⁴³.

A summary of the genes most commonly affected by point mutations and/or CNAs in BCC tumors is shown in **Table 2**.

1.1.4 BCC and Other Malignancies

Aside from the hereditary syndromic BCC cases, such as those of Gorlin-Goltz syndrome, that are commonly accompanied by increased risk for other malignancies, like medulloblastomas^{123–127}, it has interestingly been observed that non-Gorlin-Goltz syndrome patients who develop BCCs at a high frequency (defined as at least 6 BCC tumors within a 10-year period), are significantly more prone to the development of any other malignancy in

Gene (locus)	Ref.
<i>PTCH1</i> (9q22.32)	130,154,176–183
<i>PTCH2</i> (1p34.1)	136,141,155
<i>SMO</i> (7q32.1)	181
<i>GLI1</i> (12q13.3)	154,156,180,185
<i>GLI2</i> (2q14.2)	154,156,180,185
<i>SUFU</i> (10q24.32)	154,156,181
<i>TP53</i> (17p13.1)	154,155,177,181,196,197
<i>STK19</i> (<i>WHR1</i>) (6p21.33)	154
<i>LAST1</i> (6q25.1)	154,218
<i>LATS2</i> (13q12.11)	154,218
<i>PTPN14</i> (1q32.3-q41)	154,218
<i>PPP6C</i> (9q33.3)	154
<i>MYCN</i> (2p24.3)	154,180
<i>NOTCH1</i> (9q34.3)	154,155,177,180
<i>NOTCH2</i> (1p12)	154,155,180
<i>ARID1A</i> (1p36.11)	154,180
<i>TERT</i> promoter (5p15.33)	177,179,180,230–232
<i>DPH3</i> promoter (3p25.1)	177,179,180,230–232

Table 2: Genes commonly affected by point mutations and/or copy number alterations in BCC.

general compared to the control population, including prostate, ovarian, breast cancer, as well as hematological malignancies ²⁴⁴. This increased susceptibility may be, at least partly, attributable to the significantly higher presence of pathogenic mutations in several DNA repair-associated genes (*BRCA1*, *BRCA2*, *BARD1*, *PALB2*, *MLH1*, *MSH2*, *MHS6*, *CHEK2*, and *NBN*) in patients with high-frequency BCCs compared to the control population ²⁴⁴. Even more intriguingly, there is evidence in the literature suggesting a significant positive correlation between BCC and the development of subsequent non-cutaneous malignancies irrespective of BCC multiplicity and/or frequency ^{245,246}. For instance, an increased risk has been observed for lip ^{247–249}, oropharyngeal and/or laryngeal, salivary gland carcinomas ^{247,248}, connective tissue malignancies ²⁴⁸, Non-Hodgkin ^{247,249} and/or Hodgkin ²⁴⁸ lymphomas, and potentially prostate ²⁵⁰, breast and lung ²⁵¹ cancer as well – although there is substantial controversy between the results of the various studies, with some even proposing a reduced risk for specific

cancer subtypes, such as colorectal carcinoma (CRC), in patients with BCC compared to the general population ²⁵². Overall, these observations propose an interesting opportunity for potentially identifying individuals at higher risk for more aggressive malignancies, although, it is important to keep in mind that their interpretation in regard to potential underlying confounding factors should be carried out with caution, and that their translation to a clinical setting would be a demanding task, given the extremely high frequency of BCC in the general population.

1.1.5 Cellular Origin of BCC

The cellular origin of BCC has been controversial and still remains under investigation. The initial hypothesis proposed that BCCs originate from the basal layer of the interfollicular epidermis (IFE) ²⁵³, but was soon questioned by many researchers ^{254–259}, who observed varying degrees of differentiation of several BCCs towards the appendages of the skin – sebaceous glands, apocrine glands, or hair follicles (HFs) – thus, supporting a tumor origin from these structures or from the undifferentiated cells of the primary epithelial germ (the embryonic ectodermal layer that gives rise to the aforementioned structures) ^{256,260}; this was also supported by electron microscopy studies ²⁶¹. However, IHC studies continued to contradict the appendage-derived origin of BCC, mainly due to the absence of connection of tumor cells to HFs or glands, and still favored their IFE derivation ²⁶².

Nevertheless, overall, the body of evidence which accumulated recently points toward a hair follicular origin of BCC, especially from the outer root sheath (ORS) and the bulge SCs. For context, the ORS is the outermost layer of the HF and is considered to be continuous with the basal layer of the IFE, while the bulge comprises a specific area of the ORS, between the insertion point of the arrector pili muscle and the opening of the sebaceous gland ^{263,264}, which is a known reservoir of epithelial SCs, responsible for the regeneration of HFs, as well as of sebaceous glands, and the IFE ^{265–273} – for the latter, however, only in the setting of epithelial wounding and not under homeostatic conditions ^{274–276}. The cytokeratin (keratin; K) profile of BCC, as determined by both IHC and Western blotting (WB), has been found almost identical to that of the ORS (K 5, 6, 14, 16 and 17), and distinct from that of the IFE (K 1, 5, 10 and 14) ^{277,278}. It has also been revealed that many BCCs, as well as trichoepitheliomas and other follicular tumors express K15 ²⁷⁹, which is considered a bulge SC-specific marker ^{268,269}. Additionally, the central role of the Hh pathway in both BCC formation (as previously discussed) and HF development ^{280–282} further supports the hypothesis. It has further been shown that the expression of the SRY-box transcription factor 9 (SOX9), necessary for the hair differentiation process, is mainly restricted to the ORS and HF bulge SCs (and is absent in the IFE), and appears to be regulated by upstream signaling of the Hh pathway, while also having been characterized as a marker for human BCCs and other adnexal-associated neoplasms ^{283–286}.

The expression pattern of SOX9, as well as that of β -catenin²⁸⁴ and epithelial cell adhesion molecule (EpCAM)²⁸⁷ in human BCCs closely resembles that in HF during embryonic development, thus supporting a connection between BCC and the HF. This is further established by the proposed regulatory effect of the canonical Wnt/ β -catenin pathway, which is essential for HF development and SC differentiation^{288,289}, on the Hh signaling pathway in the epidermis, including in an oncogenic context^{208,290,291}. Accordingly, distribution of Notch1 intracellular domain (NICD) in murine BCC tumors is reminiscent of that in anagen HF¹⁷⁴. Moreover, another study in mice has demonstrated that conditional *Ptch1* deletion results in BCC-like tumor formation from HF SC populations, but not efficiently from IFE SCs²⁹². And similarly, X-ray-induced BCC development in *Ptch1*+/- mice is restricted to K15+ HF bulge SCs²⁹³.

Nonetheless, there are relatively recent studies that still oppose the HF origin of BCC. For example, it has been shown that expression of a constitutively active SMO mutant (*SmoM2*) in different murine epidermal compartments, results in the development of BCCs from long-term progenitor cells of the IFE and upper infundibulum, but not from HF bulge SCs and their transient amplifying progenitors²⁹⁴. Moreover, interestingly, conditional knockout (KO) of the *Tp53* gene, aside from increasing the susceptibility of bulge SCs to tumor formation, also resulted in BCC development from the IFE – an observation partly attributed to induction of increased SMO expression by the loss of p53²⁹³.

Overall, it seems very likely that BCCs originate from SCs – however, whether those are of follicular origin or/and of interfollicular epidermal origin still remains to be elucidated.

1.1.6 Clinicopathological Characteristics & Diagnosis of BCC

1.1.6.1 Localization of BCC

The majority (almost 90%) of BCCs are located in sun-exposed areas of the skin, specifically, and in descending order of frequency, on the nose, orbital region, cheeks, scalp, forehead, the auricular region, upper and lower lip, chin, neck, and less frequently on the trunk and extremities^{42,295,296}. However, different subtypes display different patterns of localization: for example, nodular and morpheaform BCCs have a tendency to develop on the face and head, while superficial BCCs show a preference for the sun-protected areas of the trunk, and extremities, especially in men^{296–299}.

1.1.6.2 Subtypes of BCC and their Clinicopathological Characteristics

1.1.6.2.1 Nodular and Superficial BCC

Many different subtypes of BCC have been characterized, differing both in their clinical presentation and histopathological features, as well as in their biological behavior, with nodular being the most common one (>50% and even up to almost 80% of BCCs depending on the study) ^{159,295,296,298,300,301}. Nodular BCC usually presents as a round, shiny, pearly papule or nodule, with telangiectasias, that can often bleed, and may also develop ulceration ^{2,159,299,302}. Microscopically, it is typically characterized by well-defined nests or islands of malignant basaloid cells within the papillary and/or reticular dermis, which display peripheral nuclear palisading, while the stroma can be mucoid/myxoid, and a cleft retraction artifact between the tumor mass and the surrounding stroma is often present ^{301–304}. Apoptotic cells within the tumor mass, as well as amyloid depositions within the surrounding stroma can also be observed ³⁰⁵.

The second most common subtype is superficial BCC, accounting for approximately 10-30% of the cases ^{298,300,305}. This BCC occurs in significantly younger individuals ^{297,298,306}, and many studies also report a higher frequency in women ^{17,298,306}. Superficial BCC usually presents macroscopically as a well-defined thin erythematous plaque with or without scaling and possible central clearing, and microscopically as multiple foci of malignant basaloid cells with peripheral palisading, that are attached to the epidermis and can extend into the papillary dermis; myxoid stroma and clefting artifact can also be present, while another common feature is the presence of a band-like lymphoid infiltrate ^{159,301–303,307}. Both nodular and superficial BCCs are classified as indolent-growth subtypes ^{2,305}.

1.1.6.2.2 Morpheaform, Infiltrative, Micronodular BCC, and Basosquamous Carcinoma

On the other hand, more aggressive, but rarer subtypes include morpheaform (or morpheic or sclerosing), infiltrative, micronodular BCC, and basosquamous carcinoma (BSC) ². Briefly, the morpheaform subtype presents as a white or yellowy, waxy, firm sclerotic plaque without tendency for ulceration or bleeding, that clinically mimics morphea ^{2,159,301,302,308} (a form of localized scleroderma ³⁰⁹), and corresponds microscopically to very thin strands of neoplastic cells extending into a sclerotic collagenous stroma, deep within the dermis or even the subcutaneous tissue, usually lacking retraction artifact as well as palisading of the nuclei ^{2,159,301,302,308}.

Infiltrative BCCs are also characterized by neoplastic cells forming strands, but additionally nests or islands of variable sizes and forms, with an irregular configuration of the peripheral cells and thus irregular contours of the tumor masses, that extend deep into the dermis or even subcutaneously, while the stroma might be relatively collagenous rather than myxoid – this invasive growth pattern is clinically reflected in the lesion's poorly-defined

margins, and similarly to the morpheaform ones, infiltrative BCCs usually manifest as white or yellowish plaques ^{159,301,302,304,310}. As is easily evident, there are many similarities between the latter two types, and the International Classification of Diseases for Oncology (ICD-O) ³¹¹ actually considers morpheic BCC as a subtype of infiltrating sclerosing BCC.

Micronodular BCC shares common features with the nodular subtype, but, as its name suggests, displays tumor islands of smaller size (<0.15 mm in diameter), with round, well-demarcated contours, but less prominent peripheral palisading, and with a more dispersed pattern into the dermis, which can be more collagenous compared to the nodular type, and thus not display prominent retraction artifact; deeper infiltration into the tissues is more common compared to nodular BCC, which also explains its more aggressive behavior ^{301,302,305,312}. Clinically it usually presents as an erythematous macule or a papule-like or plaque-like lesion with ill-defined borders, and depending on the extent of stroma sclerosis, may be softer or more indurated ^{159,301}. Its invasive pattern and higher risk of deeper infiltration and recurrence, which is similar to that of sclerosing and infiltrative BCC has prompted a recent call for the unified classification of all these three subtypes as one, under the name “infiltrative BCCs” ³¹³.

Lastly, BSC (or by others metatypical BCC) comprises a considerably debated subtype of BCC, with some previous studies classifying within this category cases of BCC/SCC collision tumors ^{314,315} – meaning the co-existence of BCC and SCC with distinct borders ³¹⁶ – and/or cases of keratinizing BCC with squamous metaplasia ³¹⁷. However, in recent years there seems to be a consensus that restricts the term specifically to tumors with both BCC and SCC elements (the latter highlighted, among others, by eosinophilic cellular cytoplasm and intercellular bridges), that are characterized, by a transitional zone between the two, thus suggesting a differentiation process of the basaloid to squamoid cells ^{159,318–324}. Clinically, BSC does not display any characteristic features that help distinguish it from a typical BCC; it usually presents as a persistent nodule which ultimately converts into an ulcerative lesion ³²⁵. Dermatoscopic evaluation could raise suspicion of a BSC if at least one criterion for BCC and one for SCC are recognized, however diagnosis is confirmed with histological examination ³²⁵. Not unexpectedly, these tumors display higher aggressiveness, as is evident by their higher rates of recurrence and metastasis, resembling the biological behavior of cSCC ^{319,326–328}.

1.1.6.2.3 Differentiated BCCs: BCC with sarcomatoid differentiation, BCCs with adnexal differentiation, and Fibroepithelial BCC

Historically, BCCs have been divided into undifferentiated and differentiated subclasses – the former including the subtypes described above, and the latter including BCCs which display features of differentiation towards specific cell types or structures, particularly: BCC

with sarcomatoid differentiation, BCCs with adnexal differentiation, and fibroepithelial BCC^{302,305,329}.

BCC with sarcomatoid differentiation comprises a very rare biphasic tumor with a BCC element accompanied by a malignant stroma which exhibits features of differentiation towards osteo-, chondro-, leiomyo-, rhabdomyo- or/and undifferentiated pleiomorphic sarcoma^{305,330–335}. The existing literature suggests that the sarcomatoid stromal cells result through a dedifferentiation process of the malignant epithelial cells, as the two have been found to share common genetic alterations³³⁶, including *PTCH1* mutations³³². Nonetheless, it has also been hypothesized that the epithelial and stromal malignant components may originate from a common undifferentiated progenitor cell³³⁷. Although varied outcomes in terms of prognosis have been reported from distinct patient cohorts^{331–336}, owing partly to the overall very low frequency of this subtype, the World Health Organization (WHO) classifies BCC with sarcomatoid differentiation as of high risk for recurrence³⁰⁵.

BCC with adnexal differentiation is an “umbrella term” for BCCs which display features of differentiation towards HF components, apocrine sweat glands or sebaceous glands³⁰⁵. Aside from the histopathological findings of “typical” BCCs, a series of distinct histological features are utilized as diagnostic criteria for the adnexal differentiation of these tumors³³⁸. For instance, BCCs with matrical (hair matrix) differentiation^{339–341} are characterized by the presence of the so-called “shadow cells”^{305,338} (cornified cells with eosinophilic cytoplasm and a ghost-like contour of their nucleus, which are commonly present in pilomatrixomas³⁴²), while infundibulocystic BCCs display, among the anastomosing strands and cords of basaloid cells, small keratin-filled cystic structures outlined by epithelial cells with squamoid features^{302,305,338,343,344}. Interestingly, multiple infundibulocystic BCCs have been associated with Gorlin-Goltz syndrome, and particularly with specific *SUFU* mutations^{345,346}. Moreover, BCCs with sebaceous differentiation are characterized by the presence of mature sebocytes with foamy cytoplasm^{305,338,347,348}, whereas BCCs with apocrine differentiation are recognized by the characteristic decapitation secretion pattern of their cells^{305,338,349,350}. BCCs with ductal differentiation can be of either eccrine, apocrine or sebaceous type^{305,338,351}. Overall, a thorough microscopic examination with the use of an appropriate panel of IHC markers is required for the differential diagnosis between the several BCCs with adnexal differentiation patterns and the respective true adnexal neoplasms which they can mimic, and which can display more aggressive biological behavior than BCCs³⁰⁵. Compared to the “conventional” BCC subtypes, the presence of adnexal differentiation does not seem to carry any prognostic significance³⁰⁵.

Lastly, fibroepithelial BCC, also known as fibroepithelioma of Pinkus (FeP) is a controversial entity, in the sense that some argue for its classification as a benign adnexal tumor (trichoblastoma) rather than a BCC ^{305,352–354}. It most commonly arises on the trunk, and usually presents as a flesh-like or pink nodule ^{302,305,355–357}, while on histological examination, thin anastomosing strands of basaloid cells extending from the epidermis within an abundant fibrotic/fibroblastic stroma are identified; peripheral palisading of cell nuclei and retraction artifact – typical of BCC – can also be recognized ^{302,305,355–359}. FeP is considered an indolent tumor with an overall excellent prognosis ^{305,352,355}.

1.1.6.2.4 Mixed-subtype BCCs

It is important to note, that mixed subtypes of BCC are not uncommon – they can account for up to 40% of all cases according to some studies ^{301,360} – and most commonly include combinations of a non-aggressive and an aggressive subtype, such as nodular/micronodular ³⁰¹, nodular/infiltrative or nodular/morpheaform – in which case their biological behavior seems to be determined by the aggressive-growth component ^{301,360} – but can also include combinations of two non-aggressive subtypes, e.g. nodular/superficial, or two aggressive subtypes, e.g. micronodular/infiltrative BCCs ^{360,361}.

1.1.6.2.5 Pigmented BCC

Pigmented BCC is a variant of the tumor characterized by increased pigmentation both macro- and micro-scopically, that can otherwise be of either nodular, superficial, micronodular or any other “classic” subtype ^{2,362}. It is more frequent in individuals with darker skin tones, specifically those of African, Hispanic and Asian descent, compared to light-skinned people ^{363–370}. Macroscopically, these lesions can mimic melanomas ^{371–373}, and even in dermatoscopic evaluation they can present with features suggestive of melanocytic malignancies (in significantly higher rates compared to non-pigmented BCCs) ³⁷⁴ – however, they lack a pigmented network which is characteristic of melanocytic lesions ³⁷⁵. Diagnosis can be confirmed only through pathological examination, in which the classic findings of the respective BCC subtype are recognized, along with non-malignant melanocytes within the tumor ^{363,376–378}, as well as transferred (phagocytosed) melanosomes inside the epithelial tumor cells ^{261,363,376,379,380}, and/or phagocytosed melanin inside peritumoral stromal histiocytes (melanophages) ^{363,378,381}. Interestingly, BCC pigmentation has been observed to positively associate with increased peritumoral inflammation ³⁸², while evidence from Asian populations additionally suggests a positive correlation between pigmentation and less aggressive behavior of BCC ³⁸³; this hypothesis is accepted by the WHO classification of skin tumors as well ³⁰⁵.

Overall, many different clinicopathological subtypes of the tumor have been described in the literature, with sometimes varying, however, definitions, leading to some confusion regarding the classification of BCC and occasionally confounding the comparison of results from different studies. A summary of the characteristics of the main BCC subtypes, as those are adopted by WHO ³⁰⁵, is shown in **Table 3**.

BCC subtype	Clinical presentation	Histopathological characteristics	Biological behavior – Risk of recurrence	Ref.
Nodular BCC	Round, shiny, pearly papule or nodule with telangiectasias Bleeding is common Ulceration may be present Most commonly on the head and neck region	Well-defined nests or islands of malignant basaloid cells within the papillary and/or reticular dermis Peripheral nuclear palisading Mucoid/myxoid stroma Cleft retraction artifact between tumor mass and surrounding stroma	Indolent – low	2,159,299,301–305
Superficial BCC	Well-defined thin erythematous plaque with or without scaling and/or central clearing More common in younger individuals and women	Multiple foci of malignant basaloid cells attached to the epidermis, potentially extending into the papillary dermis Peripheral nuclear palisading With or without myxoid stroma and cleft retraction artifact Band-like lymphoid infiltrate	Indolent – low	17,159,297,298,301–303,305–307
Morpheaform BCC	White or yellowy, waxy, firm sclerotic plaque No tendency for ulceration or bleeding	Thin strands of malignant cells extending into a sclerotic collagenous stroma, deep within the dermis and/or subcutaneously Cleft retraction artifact and peripheral nuclear palisading are usually absent	More aggressive – high	2,154,301,302,305,308
Infiltrative BCC	White or yellowish plaque with ill-defined borders	Strands, nests or islands of neoplastic cells, of variable sizes and forms, with irregular	More aggressive – high	159,301,302,304,305,310

	More common on the upper trunk, head and neck regions	peripheral cell configuration, extending deep into the dermis and/or subcutaneously Relatively collagenous stroma		
Micronodular BCC	Erythematous macule, papule- or plaque- like lesion with ill-defined borders Softer or more indurated Most commonly on the head and neck region	Small-size (<0.15 mm in diameter) tumor islands with round, well-demarcated contours, but less prominent peripheral palisading, more dispersed arrangement into the dermis (satellite pattern), and/or deeper infiltration Stroma can be more collagenous compared to nodular BCC Not prominent cleft retraction artifact	More aggressive – high	301,302,305,312
Basosquamous carcinoma (BSC)	No specific features discerning it from typical BCC Usually, persistent nodule that transforms into an ulcerative lesion	Elements of both BCC and SCC (SCC: eosinophilic cytoplasm, intercellular bridges), characterized by a transitional zone between the two	More aggressive – high	159,305,318–323,325–328
BCC with sarcomatoid differentiation	Usually large at diagnosis (>2 cm in diameter) May present as an exophytic nodular lesion with or without ulceration	Malignant epithelial component with BCC features and malignant stromal component with spindle cell morphology, giant cell morphology, or characteristics of differentiation towards other sarcoma types	More aggressive – high	305,330–335
BCC with adnexal differentiation	No specific clinical feature Some preferentially arise in the periocular region	Features of differentiation towards HFs (e.g. shadow cells, small keratin-filled cysts outlined with squamous cells, trichohyaline granules etc.), apocrine sweat glands	Depends on the BCC features, not affected by the adnexal differentiation pattern	302,305,338–341,343,344,347–351

		(decapitation secretion), sebaceous glands (vacuolated cells with foamy cytoplasm), or glandular ducts (tubular structures of cuboidal cells)		
Fibroepithelial BCC (Fibroepithelioma of Pinkus)	Flesh- or pink- colored nodule Usually on the trunk	Thin anastomosing basaloid strands of reticular pattern starting from the epidermis and extending within a fibrotic/fibroblastic stroma With or without peripheral palisading and retraction artifact	Indolent – low	302,305,352,355 –359
Pigmented BCC	Pigmented lesion with features of any BCC subtype Can mimic melanoma	Features of any BCC subtype, along with non-malignant melanocytes, transferred melanosomes inside tumor cells and/or phagocytosed melanin inside peritumoral stromal histiocytes (melanophages)	Potential inverse correlation between pigmentation and tumor aggressiveness	261,305,325,363 ,376–381,383

Table 3: Summary of clinicopathological characteristics and biological behavior of BCC subtypes. SCC = Squamous Cell Carcinoma, HF = Hair Follicle

1.1.6.3 Diagnosis of BCC

Overall, diagnosis of BCC is initially based on clinical presentation, which, as discussed, can vary substantially depending on the subtype. The lesions are usually asymptomatic, but patients may report discomfort, itching, crusting, a history of recurrent bleeding, or (rarely) pain in the area ^{384,385}. Dermatoscopy is necessary for guiding the differential diagnosis; according to recent, systematic reviews ^{386–388} dermatoscopic features suggestive of BCCs include arborizing vessels and short fine telangiectasias, erosions and ulcerations, shiny white strands and/or blotches, MAY globules (standing for Multiple Aggregated Yellow-white globules, which have also been associated with more aggressive BCC subtypes), blue ovoid nests, while yellow structures should not rule out the BCC diagnosis (although they are more commonly present in other pathologies), and of equal importance is the absence of features that characterize other neoplasms, such as the absence of a pigment network for pigmented BCCs. Other non-invasive imaging techniques include reflectance confocal microscopy (RCM) and optical coherence tomography (OCT) that, if available, can

be used in cases of difficult-to-recognize BCCs ³²⁹. However, definite diagnosis can be confirmed only through histological examination.

Aside from the standard microscopic evaluation with hematoxylin and eosin (H&E) staining which aims to reveal histological features of the different subtypes, IHC analysis can be performed in ambiguous cases in order to aid differential diagnosis. For example, when the possibility of SCC needs to be ruled out, staining against EpCAM (using the anti-EpCAM antibody, clone BerEP4), and against the epithelial membrane antigen (EMA) (using the namesake antibody, clone E29) has been traditionally regarded as standard of care, as these two proteins are considered markers for BCC and SCC, respectively ^{287,389–395}. It is worth noting, however, that, as some studies suggest, the sensitivity and specificity of using BerEP4 for the differential diagnosis between BCC and SCC may not be 100%, as 1 out of 31 BCCs was not stained positive with the antibody in one study ³⁹⁶, while a small percentage of SCCs was focally stained positive in another ³⁹⁷. Furthermore, BerEP4 cannot distinguish between BCC and trichoepitheliomas, trichoblastomas or Merkel cell carcinomas (all of which are usually stained positive), while even cases of sebaceous adenomas can display EpCAM expression in IHC ^{397,398}. This is the reason why, when the need for differential diagnosis arises, IHC panels of more than one antibodies are used; aside from BerEP4 and EMA, these can include antibodies against various keratins, such as K20, K19, K17, K15 and K14, antibodies against androgen receptors (AR), carcinoembryonic antigen (CEA), CD10, Ki-67, proliferating cell nuclear antigen (PCNA), pleckstrin homology-like domain, family A, member 1 (PHLDA1), B-cell lymphoma 2 (Bcl-2) (useful only for differential diagnosis from tumors without follicular differentiation) – and this list is not exhaustive ^{398–409}.

Once the diagnosis of BCC has been established, however, staining with the BerEP4 antibody may be useful in identifying malignant cells that can be missed in H&E slides in difficult BCC cases, such as those with intense inflammation ³⁹³ – although this has been disputed by Katz et al. ⁴¹⁰, those with sclerotic stroma, or those with perineural invasion, and may also help determine the clearance of resection margins on tumor specimens more accurately compared to a simple H&E examination ³⁹³. Lastly, a recent study has shown that loss of EpCAM (defined as a staining intensity of tumor borders or tumor islands infiltrating the dermis that is less than 50% of that of the rest of the tumor), especially along the tumor invasive front, is significantly associated with infiltrative BCC cases compared to nodular ones, with potential implications for the management of high-risk BCCs ³⁹⁰.

1.1.7 Management of BCC

The management of BCC generally depends on the staging of the disease, its subtype and localization (which, along with other factors, also determine the risk of recurrence), as well as patient characteristics, such as age, fitness status and personal wishes ³²⁹.

1.1.7.1 Definition of advanced BCC & Classification into “easy-to-treat” and “difficult-to-treat”

Although very rare, advanced BCC (aBCC) includes cases of locally advanced BCC (laBCC) and cases of mBCC. An established definition of laBCC does not exist, however it generally refers to cases of the disease that have remained long (for years) without intervention and/or have failed to respond or relapsed multiple times after treatment, and/or are characterized by extensive local tissue destruction, and thus, are not candidates for surgical or radiotherapeutic treatment ^{329,411–415}. According to the study of Goldenberg and colleagues, less than 1% (0.8%) of BCCs are locally advanced ⁴¹². Even rarer is the development of nodal and/or distal metastases, with traditionally reported rates of mBCC ranging between 0.028% and 0.55%, and resulting mainly from more aggressive BCC subtypes – however, the lack of systematic staging procedures in the past, and the only recent introduction of registries for aBCC ^{416,417} could have led to an underestimation of these numbers ³²⁹.

The European Association of Dermato-Oncology (EADO) has recently proposed a new classification system for the disease to help guide treatment, according to which BCCs are divided into common, “easy-to-treat” BCCs (accounting for >90% of the cases), and the less frequent “difficult-to-treat” BCCs ³²⁹. According to Peris and colleagues, the latter category includes: (i) common BCCs that are difficult to treat because of reasons related to the tumor (e.g. poorly defined margins, location that requires technical expertise, etc.) and/or to the patient (e.g. comorbidities, refusal of a treatment modality etc.), (ii) BCCs that are difficult to treat because of multiple lesions, (iii) large and/or destructive BCCs either outside or within critical areas, and (iv) giant and/or deeply invasive BCCs with extracutaneous spread ³²⁹. All the rest BCC cases are considered as “easy-to-treat” ³²⁹. Based on this classification they have also introduced a new staging system for the disease, the prognostic value of which, however, has not been validated ³²⁹.

1.1.7.2 Classification of BCCs according to risk of recurrence & Risk factors for recurrence

Regarding the risk of recurrence, BCCs are categorized into low-risk and high-risk groups. According to the latest guidelines by EADO ³²⁹, as high-risk BCCs are considered (a) all the difficult-to-treat cases (essentially because they may not enable the implementation of

the ideal treatment modality or the ideal safety margins in the case of surgical excision), as well as some seemingly easy-to-treat cases that are located in areas of high risk for recurrence, specifically the H area of the face (which includes eyelids, periorbital, periauricular regions, temples, ears, central face, lips, and nose ⁴¹⁸), (b) those with more aggressive histopathological characteristics (infiltrative, morpheaform, micronodular, BCC with sarcomatoid differentiation, BSC, and/or perineural/perivascular/muscular/bone invasion) ^{419–424}, as well as (c) BCCs in immunocompromised patients ⁴²⁵.

History of recurrence of a specific lesion also increases the risk for its future recurrence. Several studies have revealed, compared to primary BCCs (pBCCs), increased recurrence rates for recurrent BCCs (rBCCs) (BCCs that have relapsed after initial treatment, which for most studies was surgical excision, either standard or micrographically controlled, while for some it was curettage and electrodesiccation) ^{419,421,426–428,428–433}, with some of the highest numbers reported being a 2-year recurrence rate of 46.2% for rBCCs vs. 9.2% for pBCCs of the periocular region ⁴¹⁹. However, some have argued that this is an overestimation and no statistically significant difference exists between recurrence rates of pBCC and rBCC ⁴³⁴. As expected, incomplete excision also increases significantly the risk of recurrence ^{420–422,435} — nevertheless, the risk of incomplete excision itself can be influenced by other factors, such as an aggressive BCC subtype ^{420,422,435} or a “difficult” location, such as the postauricular and nasogenian folds ⁴³⁵. Several studies have also highlighted a positive correlation between tumor size and risk of recurrence; various research teams have reported that a tumor size greater than 2 cm significantly increases the risk for recurrence ^{425,436,437}, while the group of Yildizdal and colleagues has also uncovered a significant positive correlation between both tumor diameter and tumor depth and the BCC recurrence rate, reporting a mean tumor width of 15.6 mm in patients experiencing recurrence vs. 10.6 mm in those not, and a mean tumor thickness of 5 mm vs. 2.9 mm, respectively ⁴²⁴. This association may be attributed to the reduced ability of achieving negative margins during the excision of larger lesions; Bozan and colleagues revealed a significant correlation between a lesion size greater than 2 cm and increased risk for positive margins, and suggested that an even lower cut-off of 9 mm for lesion diameter could serve as a valuable predictive factor for margin positivity ^{426,438}. Others, however, have not corroborated a correlation between tumor size and residual tumor at resection margins ⁴³⁹, implying that tumor size may act as an independent risk factor for recurrence. Furthermore, one of the most important factors that influences recurrence rates is the treatment modality, as will be elaborated shortly after.

From a molecular point of view, several IHC markers have been associated with the biological behavior of BCC tumors. For instance, it has been suggested that a higher epidermal growth factor receptor (EGFR) expression and/or a lower AR expression may be correlated

with more aggressive BCC subtypes (infiltrative, micronodular) ⁴⁴⁰, while for EGFR in particular significantly higher IHC levels have been observed in recurrent compared to non-recurrent BCCs as well ⁴⁴¹. High p53 ^{442,443} and/or, interestingly, low Bcl-2 ^{442,444} IHC positivity have been correlated with high-risk/aggressive compared to low-risk/non-aggressive BCC tumors. Not unexpectedly, Ki-67 levels have been associated with more aggressive BCC subtypes, higher disease staging ⁴⁴⁵ and/or increased risk of recurrence ⁴⁴⁶, while stromal and/or epithelial α -SMA expression has been shown to positively correlate with an increased BCC recurrence risk ⁴⁴⁷. Nonetheless, the existing evidence is still controversial enough to not allow for their establishment as treatment-guiding parameters.

Overall, it is evident that the histopathological subtype and/or molecular profile, the localization, tumor size, previous recurrence, treatment approach, and complete or incomplete resection are all factors that can influence the recurrence risk for BCC ^{17,329,419–428,428–433,435–437,440–448} – however, whether some of these are independent risk factors, or are affected by the rest is not totally clear.

1.1.7.3 Surgical management of BCC

Although delving into the various therapeutic strategies for BCC is outside the scope of this study, a brief outline of the therapeutic options and their major indications will be provided, as those are formulated based on the latest guidelines by the EADO ³²⁹.

Most BCCs can be treated with surgery, either a classic 2D surgical excision (SE) with safety margins, or a 3D micrographically controlled stepwise surgical procedure, the most common of which is called Mohs's micrographic surgery (MMS) ³²⁹. The standard 2D procedure, with safety margins of 3-4 mm, is the recommended modality for primary low-risk BCC cases, such as nodular BCCs outside the H area of the face, and superficial BCCs either in the trunk and extremities or on the face and scalp ³²⁹. Bigger safety margins (>5 mm) should be used for high-risk BCCs, including nodular BCCs on the H zone, BCCs with poorly defined margins, recurrent BCCs, multiple BCCs, aggressive subtypes (as previously defined), or in the case of suspicion of subclinical extension, such as perineural invasion ³²⁹. However, according to the guidelines, in most cases of high-risk tumors, if available, micrographically controlled surgery should be offered as first line of treatment, as it has been shown to achieve lower recurrence outcomes, especially in the setting of rBCCs ³²⁹. Two prospective clinical trials from the Netherlands have demonstrated significantly higher recurrence rates for rBCCs following traditional 2D SE compared to micrographically controlled excision – in the first ⁴⁴⁹, 12.1% and 2.4% of patients with rBCCs experienced recurrence after treatment with SE and MMS, respectively (statistically significant), while for patients with pBCCs the respective numbers were 4.1% and 2.5% (although not statistically significant). In the second study ⁴⁵⁰,

the 10-year cumulative probability of recurrence for rBCCs was estimated at 3.9% and 13.5% for MMS and SE, respectively (statistically significant), while for primary, high risk BCCs it was 4.4% after MMS and 12.2% after SE (although not statistically significant).

In case of incomplete excision, lesions are re-excised ^{329,451}. Moreover, although surgery is not the first line of treatment for aBCC cases due to difficulty in achieving excision with clear margins and/or an acceptable cosmetic and functional result, it can be offered in patients with aBCC in the setting of palliative treatment, or following tumor size reduction by a systematic treatment modality ³²⁹.

1.1.7.4 Non-surgical management of BCC

1.1.7.4.1 Destructive treatments for BCC

In patients with primary, low-risk, superficial and nodular BCCs that are not suitable for surgery (e.g. due to poor general status) or refuse surgery, the option of destructive treatments, topical treatments and photodynamic therapy (PDT) exists ³²⁹. Regarding destructive treatments, these include curettage with or without electrodesiccation (diathermy), cryosurgery (cryotherapy), and laser ablation. With the exception of curettage (for which margin assessment is also not possible nonetheless), none of these approaches allows for histopathological examination due to destruction of the tissue, which constitutes one of their limitations ⁴⁵².

Nevertheless, curettage plus electrodesiccation (C&E) has shown comparable results to standard SE when it comes to primary low-risk BCCs ⁴⁵³, with reported recurrence rates varying between 3% and 20% ^{429,430,454}. The efficacy of C&E has further been corroborated in a systematic review and meta-analysis, demonstrating that the recurrence rate after C&E (6.9% [CI, 0.9% to 36.6%]) is comparable not only to that after SE (3.8% [95% CI, 1.5% to 9.5%]), but also after MMS (3.8% [CI, 0.7% to 18.2%]) ⁴⁵⁵. However, it is important to keep in mind that the lack of a common manner of rate calculation in the different studies may have affected the observed result.

Several studies have also assessed the outcomes of cryotherapy and laser ablation therapy, compared to other treatment modalities and/or compared to one another ^{456–459}. Both seem to achieve a significantly lower level of remission and worse cosmetic outcome compared to surgery, however no significant difference is evident between them ^{456–459} – although this may also depend on the specific type of laser used in each study. According to the previously mentioned meta-analysis ⁴⁵⁵, the estimated recurrence rate for cryotherapy was 22.3% [CI, 10.2% to 42.0%].

1.1.7.4.2 Photodynamic therapy (PDT) for BCC

Photodynamic therapy with methyl aminolevulinate (MAL) (MAL-PDT) has demonstrated comparable efficacy to SE for small superficial BCCs in terms of initial clearance (92% for PDT vs. 99% for surgery)⁴⁶⁰, but not for nodular BCCs⁴⁶¹. The same outcome for nodular BCC was also observed for PDT with 5-aminolaevulinic acid (ALA) (ALA-PDT) compared to surgery⁴⁶². Recurrence rates reported by the meta-analysis of Drucker and colleagues are 18.8% [CI, 10.1% to 32.5%] for MAL-PDT and 16.6% [CI, 7.5% to 32.8%] for ALA-PDT⁴⁵⁵.

1.1.7.4.3 Topical pharmacological treatments for BCC

Topical treatments mainly include imiquimod (IMQ) 5% and 5-fluorouracil (5-FU) 5%. IMQ is an immunomodulatory compound that principally exerts its action through agonistic binding to toll-like receptor (TLR)-7 and TLR-8, ultimately leading to NF- κ B-mediated expression and production of several pro-inflammatory cytokines, including IFN- α , TNF- α , IL-1, IL-6, IL-8, IL-10, IL-12, granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), macrophage inflammatory protein (MIP) and macrophage chemotactic protein-1 (MCP-1) by immune cells and/or stromal cells, including KCs^{463–468}. IMQ has also been shown to facilitate the recruitment of plasmacytoid DCs (pDCs) in the skin, including in the setting of BCC, along with myeloid DCs^{469,470}, and contribute to their activation and expression of several IFN- α -associated genes⁴⁷¹, while it has also been suggested to facilitate the migration of LCs to lymph nodes^{469,472}. Furthermore, it has been shown that IMQ induces the accumulation of an immune cell infiltrate in BCC tumors following its topical application, potentially mediated by the observed increase in intercellular adhesion molecule (ICAM)-1 expression on endothelial cells⁴⁷³. The immune cell infiltrate consists of both CD4+ and CD8+ lymphocytes with enhanced effector function as reflected by their increased production of IFN- γ , perforin and granzyme following IMQ treatment^{382,473,474}. The recruitment of other immune cell types has also been reported, including CD68+ monocytes/macrophages⁴⁷³, CD20+ B cells⁴⁶⁹, CD56+/CD94+ NK cells, and CD15+/HLA-DR- neutrophils⁴⁷⁰. Importantly, Bcl-2 expression on tumor cells was found to be reduced after IMQ treatment, implying a pro-apoptotic effect of the drug, although the expression levels of Bax, Fas/FasL and p53 were unaffected^{469,473}. Surprisingly, it been observed that even in the absence of the active compound IMQ, the vehicle cream itself is capable of inducing an inflammatory skin response, which has been attributed to the isostearic acid component of the Aldara® cream⁴⁷⁵.

In the setting of BCC, IMQ can be offered in the form of topical 5% cream as a treatment option in immunocompetent patients with (mainly small) primary superficial or nodular BCC

tumors, who refuse surgery or for whom surgery is contraindicated ³²⁹. Five-percent (5%) IMQ cream has been shown to be superior to both MAL-PDT and 5-FU cream for the treatment of primary superficial BCCs ⁴⁷⁶, and although it is inferior to surgery for the treatment of both superficial and nodular BCCs ^{477,478}, its clinical efficacy is still sufficient to make it an acceptable solution in these cases ^{478–484}.

Several combinational therapeutic strategies involving IMQ have also been evaluated, with the most promising ones including: (i) curettage (without electrodesiccation) followed by topical IMQ treatment ^{485–490}, (ii) curettage and electrodesiccation (C&E) followed by topical IMQ treatment ⁴⁹¹ – both approaches demonstrated overall appealing results in terms of efficacy and cosmetic outcomes, mainly in the case of nodular and superficial BCCs, (iii) cryosurgery performed during the course of a 5-week regimen of daily topical IMQ application (immunocryosurgery), which has proven a very promising treatment option for patients with non-superficial BCCs, with an efficacy comparable to SE for tumors up to 2 cm in diameter ^{492–496}, and (iv) IMQ pretreatment for MMS in facial nodular BCCs, which was shown to significantly reduce tumor size before MMS, surgical defect size, as well as reconstruction time ⁴⁹⁷.

Topical 5% 5-FU, an antimetabolite drug that inhibits the thymidylate synthase which is responsible for the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTTP) ⁴⁹⁸, can also be considered for the treatment of superficial BCCs in immunocompetent patients when surgery is not an option ³²⁹, although it has been demonstrated to be inferior to the 5% IMQ cream ⁴⁷⁶.

1.1.7.4.4 Radiotherapy for BCC

Radiotherapy is an additional alternative option for patients that are not candidates for surgery or for whom a surgical approach would not be considered best due to reasons either related to the patient, or to the tumor (e.g. for large facial BCCs and/or BCCs where curative surgery would lead to severe disfigurement) ³²⁹. According to Drucker and colleagues, external-beam radiation (the most common modality of radiotherapy for BCC) has an estimated recurrence rate of 3.5% [CI, 0.7% to 16.8%], making it comparable to that of surgical procedures ⁴⁵⁵.

1.1.7.4.5 Systematic pharmacological treatments for BCC

1.1.7.4.5.1 Hedgehog pathway inhibitors (HHIs)

Systematic pharmacological treatment includes, first of all, HHIs, which are offered to patients with laBCCs that are not suitable for surgery or radiotherapy, or have experienced disease recurrence after surgery or radiotherapy, and to patients with mBCCs ³²⁹. Both vismodegib and sonidegib are SMOi, exerting their action through binding to subparts of the

transmembrane binding-pocket of the SMO receptor^{499–501}, therefore inhibiting downstream activation of the Hh pathway. It has been shown, in a series of matched biopsies from 4 patients with laBCC, before and 4 weeks after HHI treatment, that HHIs lead to a significant reduction in Ki-67, SOX9, EpCAM and Bcl-2 expression, as assessed by IHC⁵⁰². Additionally, a significant increase was observed in the IHC expression levels of MHC-I molecules by the malignant cells following HHI administration, with a proposed potential mechanism for this effect implicating the suppressor of cytokine signaling 1 (SOCS1) protein⁵⁰³, which is known for its suppressive function on IFN- γ and downstream JAK/STAT1 cell signaling^{504,505}, including in KCs⁵⁰⁶. IFN- γ /STAT1 signaling mediates (either directly or indirectly) the expression of various proteins, including ICAM-1^{507,508}, HLA-DR⁵⁰⁸, and MHC-I^{509–512}. Laner-Plamberger and colleagues⁵⁰³ identified *SOCS1* as a direct gene target of the GLI TFs, and thus proposed that activation of the Hh pathway can lead to suppression of IFN- γ signaling and its downstream effects. They further showed by IHC that human BCCs display a higher expression of SOCS1 compared to healthy KCs, and in combination with studies that have highlighted the lower expression of IFN- γ target genes in these tumors^{513–517}, their proposed positive regulatory mechanism of GLI TFs on SOCS1 expression could, at least partly, explain how the hyperactivation of the Hh pathway in BCCs leads to reduced expression of MHC-I molecules, and how blockade of this pathway with HHIs results in upregulation of MHC-I expression. Of interest to this, YAP-1 has also been proposed as a positive regulator of SOCS1 expression, at least in astrocytes⁵¹⁸.

Furthermore, the IHC examination of the matched BCC biopsies pre- and 4 weeks post-HHI treatment⁵⁰² also revealed a significant increase in the numbers of CD8+ T cells intratumorally, CD4+ T cells both intra- and peri-tumorally, as well as in MHC-II-expressing mononuclear cells, and in CD68+ macrophages both within the tumor and at its periphery. Intriguingly, HHI treatment also significantly increased Forkhead box P3 (FOXP3)+ Tregs both intratumorally and peritumorally⁵⁰².

The suggested immunological effect of HHIs may further be supported by other studies. Such is a case report of lichenoid dermatitis developing within the successfully treated lesional area of a patient with a giant BCC receiving oral vismodegib, followed by minimal surgery⁵¹⁹. Although increased apoptosis could partly contribute to the observed local immune cell infiltration, the existing evidence regarding HHIs appears to be somewhat controversial, in contrast to the established pro-apoptotic effects of IMQ^{520,521}, with some researchers supporting that, at least for vismodegib, induction of apoptosis is not a mechanism of action^{522,523}, whereas other studies support a pro-apoptotic effect of vismodegib in several human and murine tumor models^{524–527}.

Still, an immunological effect of HHIs would not be surprising, given that many studies have revealed involvement of the Hh signaling pathway in both central and peripheral T cell immunological processes, including TCR signaling and T cell activation, which are suppressed by Hh activity^{528–530}. Furthermore, it has been shown that T cell-specific constitutive inhibition of GLI2-mediated transcription in murine models of allergic airway disease (AAD) significantly reduces the recruitment of CD4⁺ and Th2 T cells in both lungs and lymph nodes⁵³¹, whereas in murine models of atopic dermatitis (AD) it leads to an exacerbation of skin inflammation, reflected in increased gene expression of *IL4* and *IL13*, and increased protein expression of IFN- γ , TNF- α and IL-6⁵³². Systemic administration of a SMOi in another murine model of AAD also resulted in decreased *IL4* and *IL13* expression in the lung, as well as reduced infiltration of CD4⁺ T cells (in both lung and lymph nodes), mast cells, eosinophils and basophils (in lungs), and reduced IgE levels in the serum⁵³³ – however, these observed systematic outcomes of SMO inhibition may not represent primary, but rather secondary effects due to the local immunomodulation⁵³⁴. Overall, the existing evidence suggests that HHIs do exert modulatory functions on local (and possibly on systemic) immune responses – however, these appear to be tissue/organ-specific, and further research is needed in order to elucidate the specific immunological role of these drugs in the setting of BCC.

Irrespective of their mechanisms of action, the clinical efficacy of vismodegib and sonidegib in aBCC treatment has been established mainly through two multicenter phase II clinical studies, namely the ERIVANCE^{535–537} and the BOLT^{538–540} trial, respectively, which led to the approval by both the FDA (Food and Drug Administration) and the EMA (European Medicines Agency), of their use in the treatment of laBCCs and mBCCs. Despite the appealing efficacy, however, HHI treatment is often discontinued due to patient intolerance to the drugs' adverse events (AEs). At the long-term evaluation in the vismodegib trial, most patients had discontinued treatment, with 21.2% of them due to treatment-emergent adverse events (TEAE), while all patients had experienced at least one TEAE; AEs of at least grade 3 were reported in almost 60% of the patients⁵³⁷. Similarly, for sonidegib, almost all patients experienced TEAEs, with grade 3–4 TEAEs being reported in 32% and 43.3% of the patients receiving the 200 mg and 800 mg dose, respectively, and these led to discontinuation of the drug in approximately 14% of the patients in both dosing populations⁵³⁸. These results are also corroborated by a recent meta-analysis⁵⁴¹.

Aside from the cases of intrinsic resistance to SMOi, reflected in part in the percentage of non-responders, an important factor that further limits their clinical use is the emergence of acquired resistance during treatment, leading to disease recurrence after an initial phase of response^{542,543}. It is also important to note that a big proportion of patients that do display sustained response to SMOi treatment, end up experiencing tumor recurrence after treatment

cessation, with an approximate 50-60% patients with laBCC that achieved complete response (CR) with vismodegib being reported to develop recurrence after its discontinuation ^{544,545}.

1.1.7.4.5.2 Anti-PD-1 immunotherapy

For patients with aBCC in whom HHIs have failed, are not tolerated or are contraindicated, a recently approved immunotherapeutic agent called cemiplimab, can be offered as monotherapy ³²⁹. Cemiplimab is an immune checkpoint inhibitor (ICI), specifically a human recombinant IgG4 monoclonal antibody that targets the PD-1 protein (also known as CD279). Briefly, PD-1 is expressed on activated T cells, B cells, macrophages, natural killer (NK) cells and DCs, and acts as a co-inhibitory molecule, through binding to its ligands, PD-L1 and PD-L2 ^{546,547}. Although the PD-1/PD-L1 axis plays an important role in homeostasis by modulating immune responses and maintaining self-tolerance ⁵⁴⁸, in the setting of malignancy it comprises a mechanism that can be “highjacked” by cancer cells in order for them to promote “immune escape”, through high expression of PD-L1 (or PD-L2) on their surface, leading to suppression of anti-tumor immunity, especially that exerted by tumor-infiltrating lymphocytes (TILs) and tumor-associated macrophages (TAMs) ^{546,549}. Thus, blocking the interaction between PD-1 and PD-L1 with the use of monoclonal antibodies has become a promising target of immunotherapy in the last few years, with established implementation of their use in the treatment of various malignancies ^{546,550–553}.

One of the main characteristics of BCC that made the use of an ICI appealing for its treatment is its very high mutational burden, as it has been suggested by many studies that a high TMB is associated with better response to immunotherapeutic agents and better overall outcomes in patients with several types of solid tumors, especially melanoma and non-small cell lung carcinoma (NSCLC) ^{554–562}. The proposed underlying mechanism supports that a higher TMB favors the expression of cancer-specific antigenic epitopes (neoantigens), which can be recognized and targeted by tumor-specific CD8+ T cells, and thus, preventing the exhaustion of said T cells through the use of ICIs can enhance anti-tumor immune responses and lead to better clinical outcomes ^{559–561,563}. Other studies, however, support the hypothesis that the observed discrepancy in response to ICIs based on TMB may be confounded by factors such as tumor subtype, and the clinical utility of the TMB status in guiding ICI treatment has repeatedly been questioned ^{564–566}. Nonetheless, the significantly higher rates of BCC in immunocompromised patients, especially in OTRs ^{104–107,111,114}, highlight the importance of adaptive and/or innate immunity in BCC pathogenesis, and thus further support the notion of ICI use in BCC treatment.

It has been shown that higher expression of PD-L1, especially in tumor-infiltrating immune cells, is associated with better response to anti-PD-1 and anti-PD-L1 monoclonal

antibodies in various cancers, including melanoma and NSCLC ^{567,568}. Regarding BCC, PD-L1 expression has been detected both in tumor cells and in associated immune cells, including TILs and macrophages ^{569–572}. Intratumoral PD-L1 expression has been found significantly enriched in low-risk compared to high-risk tumors, with nodular BCCs exhibiting a significantly higher level of PD-L1 both intra- and peri- tumorally compared to superficial and morpheaform BCC ⁵⁷¹. Approximately 90% and 95% of the BCCs (including nodular, superficial and infiltrative) in another series (n = 138, from 62 patients) were IHC positive for PD-L1 in tumor cells and in TILs, respectively, and moreover, the IHC intensity of PD-L1 in both cell types was significantly higher in treated compared to treatment-naïve BCCs ⁵⁶⁹. In a cohort of 40 locally aggressive or recurrent, primary BCCs from the same number of patients, 22% of them displayed IHC positivity for PD-L1 in tumor cells, and 82% of them in TILs and associated macrophages, while PD-1 positivity was identified for lymphocytes in all samples, including the ones (40%) with sparse T cell infiltration ⁵⁷². All cases (33/33) which exhibited PD-L1 positivity on malignant cells or macrophages were associated with the presence of PD-1+ TILs in close proximity to those cells ⁵⁷². Moreover, several case reports of patients with aBCCs who received anti-PD-1 antibodies (including nivolumab and pembrolizumab) following failure of HHI treatment, also revealed some encouraging results ^{243,573–576}, even in the case of no PD-L1 expression by the tumor cells ⁵⁷⁷, and paved the way for the evaluation of such treatments in clinical studies.

Despite the overall promising background for PD-1/PD-L1 blockade in BCC treatment, cemiplimab still remains far from the ideal therapeutic intervention. The pivotal phase II multicenter clinical trial on 84 patients with laBCC and 54 patients with mBCC that were treated with 350 mg of cemiplimab intravenously every 3 weeks, revealed an independently-assessed objective response rate (ORR) of approximately 32% and 22% in the laBCC and mBCC cohorts, respectively, with CR being identified in 7% of the laBCC patients and 4% of the mBCC ones ^{578–580}. Among responders, more than 80% and more than 55% in the laBCC and mBCC groups respectively, sustained response at 12 months, and approximately 50% at 24 months in both cohorts. Nevertheless, almost half of the laBCC patients experienced grade 3-4 TEAEs ^{579,580}. Safety profile was similar in the mBCC group analysis as well ⁵⁷⁸. Overall, almost 18% of laBCC patients and 7% of mBCC patients discontinued cemiplimab due to TEAEs ^{578–580}. Importantly, 4 patients (4.8%) in the laBCC cohort and 2 patients (4%) in the mBCC cohort died as a result of a serious AE, although these were not deemed treatment-related, but were rather attributed to co-existing medical conditions ^{579,580}.

1.1.7.4.6 Electrochemotherapy and Chemotherapy for BCC

Electrochemotherapy (ECT) is a relatively new approach in the treatment of cutaneous malignancies, in which the intracellular delivery to tumor cells of cytotoxic chemotherapeutic

agents, such as bleomycin or cisplatin, is mediated through the topical application, via electrodes, of short-term high-voltage electric pulses, which enable the permeabilization of their cellular membranes ^{581,582}. ECT in the setting of BCC can be considered for patients that are not suitable for either surgery or radiotherapy, and it can also be offered to patients with recurrent or (locally) advanced BCCs ³²⁹, as the response and recurrence rates that have been observed suggest even non-inferiority compared to the gold-standard SE ^{583–585}.

Conventional chemotherapy, on the other hand, may be considered, either as monotherapy or in combination with radiotherapy, in patients with aBCC (and especially mBCC) that do not respond to or display disease progression after treatment with HHIs or anti-PD-1 antibodies ³²⁹.

1.1.7.4.7 Treatments for BCC under investigation

Research is still ongoing in the field of BCC treatment, especially for the aBCC cases, for which a sustainable therapeutic approach is lacking. Several trials either recently completed or still in progress have been evaluating systematic treatments (either as monotherapy or in combinations), including, for example, second-generation HHIs patidegib ^{586,587}, taladegib ⁵⁸⁸, and silmitasertib (NCT03897036), that aim to circumvent the resistance currently observed with vismodegib and sonidegib. Nivolumab (another anti-PD-1 monoclonal antibody) monotherapy is being investigated in treatment-naïve and patients previously treated with HHIs, and combination of nivolumab and relatlimab [an anti-lymphocyte-activation gene 3 (LAG-3) monoclonal antibody] or nivolumab and ipilimumab [an anti-cytotoxic T-lymphocyte associated protein 4 (CTLA-4) monoclonal antibody] is evaluated in patients with anti-PD-1-refractory aBCC (NCT03521830) ⁵⁸⁹. A combination of cemiplimab (anti-PD-1) and pulsed administration of sonidegib (SMOi) in patients with aBCC is also being explored (NCT04679480) ⁵⁹⁰, while a recently completed study investigated the intralesional administration of cemiplimab (anti-PD-1) in patients with either BCC or cSCC (NCT03889912).

In the setting of neoadjuvant therapy, a combination of relatlimab (anti-LAG-3) and nivolumab (anti-PD-1) versus nivolumab only is being assessed in patients with resectable high-risk BCCs prior to surgery (NCT06624475) ⁵⁹¹, while a recent study also investigated the intralesional oncolytic virotherapy with Talimogene laherparepvec (T-VEC) in patients with difficult-to-resect BCC, with promising results ⁵⁹². Neoadjuvant/adjuvant pembrolizumab (anti-PD-1) along with standard SE is also being explored lately in patients with resectable laBCC of the head and neck with some promising, although still preliminary, results ^{593,594}.

1.1.8 Prognosis of BCC

Overall, the prognosis of BCC is exceptionally good, with reported 5-year and 10-year relative survival rates (RSR) being close to 100% ^{595,596}. Accordingly, mortality has been found similar to that of the control population in various studies [hazard ratio (HR): 0.96 (95% CI 0.77-1.19) ⁵⁹⁷, summary relative mortality: 0.92 (95% CI 0.83-1.02) ⁵⁹⁸, 10-year mortality rate ratio (MMR): 0.91 (95% CI 0.89-0.92) ⁵⁹⁹, standardized mortality ratio (SMR): 0.97 (95% CI 0.96-0.98) ⁶⁰⁰]. Tumor localization seems to exert only a small effect on survival, with observed 5-year RSR ranging from 99.8% for BCCs on the lips to 105.0% for BCCs on overlapping body sites while age appears to either not affect survival ^{596,599} or be positively associated with better 5-year and 10-year relative survival (RS) outcomes ⁵⁹⁵. There is also evidence supporting that comorbidities, including immunodeficiency, do not influence survival in patients with BCC ^{109,599}. For patients with mBCC specifically, 5-year survival rate is estimated at 89.3% for those with nodal metastases and at 61.0% for those with distal metastases, with the former group achieving complete remission in 50% (11/22) of the cases, while the latter only in 3% of the cases (1/31) in a recent study ⁶⁰¹.

Altogether, it is evident that for the vast majority of patients, BCC will not be a life-threatening condition, and most of them will be successfully treated with the standard of care SE – however, it is essential to note that the overall disease burden is far from negligible, with increasing trends for it observed in the last two decades, and projections estimating that it will rise even further by 2050, especially in the 55+ age group ^{4,9,25,602–605}. Although the percentage of patients with BCC that are affected by locally advanced or metastatic disease is very low, owing to the tumor's high prevalence and incidence rate this actually corresponds to a considerable absolute number of patients each year that develop aggressive forms of the disease and are likely to suffer from disease recurrence, and/or not respond to therapy, and/or experience severe TEAEs that can result in treatment discontinuation, or in rare cases even die due to the disease. Additionally, the cosmetic damage that BCC can cause (either directly or indirectly due to the necessary therapeutic interventions), particularly in advanced cases, and in combination with its frequent localization on facial areas, can also negatively impact the quality of life (QoL) of the patients ^{606,607}, especially in elderly ones ⁶⁰⁸. Moreover, the BCC-associated direct and indirect costs present a significant burden for healthcare systems, with an estimated \$715 million spent in USA in 2013 for all BCC cases ⁶⁰⁹. It is, therefore, of paramount importance that new/better therapeutic options for patients with aBCC become available, and one potential field that could hold promise towards this goal is the immunological environment of BCC, which still remains to be fully elucidated.

1.1.9 Immunological Profile of BCC

1.1.9.1 Overview of immune cell types of interest

1.1.9.1.1 *CD8+ T cells*

CD8+ T cells have been widely known for their cytotoxic functions against both infected and malignant cells ⁶¹⁰. They are activated following recognition of antigenic peptides presented on MHC-I molecules on the surface of either APCs or target cells themselves, by their TCR along with CD8 (signal 1), followed by the interaction between co-stimulatory molecules, such as CD28 on T cells and CD80 or CD86 on APCs (signal 2) ^{611–613}. Activation of CD8+ T cells leads to the formation of pores on target cells, primarily mediated by the glycoprotein perforin, and to the intracellular transfer of granules containing granzymes (A, B, K), cathepsin C and/or granulysin, all of which are responsible for cytotoxic effector functions, via both apoptotic and non-apoptotic pathways ^{613–615}. Surface expression of Fas ligand (FasL) on CD8+ T cells further enhances their cell-killing properties, through binding to the Fas receptor on target cells, ultimately resulting in activation of their extrinsic apoptotic pathway ^{613,616}. CD8+ T cells can additionally produce IFN- γ , TNF- α and/or IL-2, which exert non-contact-dependent effects on nearby malignant cells, as well as contribute to modulation of immune cells ^{617–619}.

IFN- γ , also produced by T helper (Th)1 CD4+ T cells, NK and NKT cells, binds to the IFN γ receptor (IFNGR), consisting of the IFNGR1 and IFNGR2 chains, and acts mainly through downstream JAK1/2-STAT1 signaling ⁶²⁰. IFN- γ has been shown to induce tumor cell apoptosis ^{621–623}, inhibit cancer cell proliferation ^{624,625}, and enable a state of tumor dormancy/senescence ^{626,627}. It may additionally facilitate the autophagy of malignant cells ⁶²⁸, and favor the upregulation of MHC-I molecules on the surface of tumor cells ^{509–512}. Furthermore, IFN- γ induces the recruitment of various immune cells in the TME, including cytotoxic lymphocytes (CTLs) ⁶²⁹ and NK cells ⁶³⁰, promotes the CD4+ Th1 ^{631,632} and the M1 macrophage ⁶³² polarization processes, can destabilize the pro-tumorigenic Treg identity ⁶³³, and might also play a role in the maturation and activity of CTLs ⁶³⁴. Nevertheless, there is substantial evidence for various tumor-promoting effects of IFN- γ as well. For instance, IFN- γ has been observed to promote a stemness state of malignant cells ^{635,636}, as well as upregulate the expression of the PD-L1 immune checkpoint molecule on tumor cells ^{637,638}. It has been also proposed to enhance apoptosis of activated T lymphocytes ^{632,639} and downregulate the formation of memory CD8+ T cells ^{632,640}, increase the migration of Tregs in the TME through a CCL5/CC motif chemokine receptor (CCR)5-dependent mechanism ^{632,641}, as well as inhibit the cytotoxicity of NK cells ^{632,642}.

Overall, the density of CD8⁺ T cell infiltration has been associated with favorable tumor characteristics and/or better clinical outcomes in various cancer types, including CRC ^{643–645}, GC ^{643,646}, gallbladder ⁶⁴⁷, breast ⁶⁴⁸, ovarian cancer ⁶⁴⁹, and melanoma ^{650–653}, whereas the frequency of *memory* (including tumor tissue-resident memory, TRM) CD8⁺ T cells has been correlated with better response to immunotherapy in a series of malignancies ⁶⁵⁴, such as melanoma ⁶⁵⁵ and NSCLC ⁶⁵⁶.

It is important to underline, however, that the aforementioned cytotoxic profile corresponds to the “typical” CD8⁺ T cells that belong to the Tc1 subtype, the polarization towards which is primarily mediated by the T-bet (encoded by the *TBX21* gene), Eomesodermin (EOMES) and signal transducer and activator of transcription (STAT)4 TFs, following IL-12 stimulation of naïve CD8⁺ T cells ^{610,657}. In reality, however, additional CD8 categories have been identified, displaying reduced cytotoxic activity and being characterized by TF and cytokine profiles reminiscent of specific CD4⁺ T cell subtypes. For instance, Tc2 CD8⁺ cells are regulated by GATA3 and produce type 2 cytokines such as IL-4, IL-5 and IL-13 (similarly to CD4⁺ Th2 cells), whereas Tc17 cells express RORγt (*RORC*) and STAT3 and produce IL-17 (similarly to CD4⁺ Th17 cells) ^{610,657}.

1.1.9.1.2 CD4⁺ T cells

Along with CD8⁺, CD4⁺ T cells are also crucial players of cellular adaptive immunity, and are responsible for a variety of functions, including the activation of CD8⁺ T cells, B cells, cells of innate immunity (DCs, NKs, macrophages) and even non-immune cells, as well as the regulation/containment of the responses mediated by them ⁶⁵⁸. Naïve CD4⁺ T cells are activated following recognition, through their TCR along with CD4, of antigenic peptides in complex with MHC-II molecules on the surface of the APCs (signal 1), followed by interaction between co-stimulatory molecules (e.g. CD28 on T cells and CD80/CD86 on APCs) (signal 2) ^{611,612,659,660}. Subsequently, naïve CD4⁺ T cells acquire unique characteristics according to the differentiation program that is triggered in each case, which depends on several factors, such as the cytokine profile of their microenvironment (signal 3) ^{611,612,661} and/or the strength of antigenic stimulation ⁶⁶². CD4⁺ subpopulations include, but are not limited to, Th1, Th2, Th9, Th17, Th22, pathogenic (p)Th5 cells, follicular helper T (Tfh) cells, and Treg cells ^{658,663} – although it has now been established that CD4⁺ T cells can be characterized by plasticity and polyfunctionality, meaning that they can either “transform” from one “classic” subtype to another (plasticity), or simultaneously produce cytokines of different subtypes (polyfunctionality) ^{658,663–667}.

1.1.9.1.2.1 T helper 1 (Th1) cells

Th1 cells have an important role in the immune responses against intracellular pathogens, including bacteria and viruses^{668–672}, but have also been implicated as essential effectors in anti-tumor immunity^{672,673}. Th1 cells are characterized by the production of IFN- γ ⁶⁷⁴, and the main TF responsible for their differentiation is T-bet (*TBX21*)⁶⁷⁵; its induction is, however, mediated by two other important TFs, STAT4 and STAT1^{676,677}, which are activated following IL-12^{678–680} and IFN- γ stimulation^{681,682} of naïve CD4⁺ cells, respectively. T-bet (in cooperation with additional TFs) facilitates the expression of the Th1 signature *IFNG* gene^{675,683,684}, in so creating a positive feedback loop for Th1 polarization, and suppresses the *IL4* gene⁶⁸³ and GATA3 protein⁶⁸⁵, as well as the *RORC* gene⁶⁸⁶, thus, shifting naïve CD4⁺ T cells away from the Th2 and Th17 phenotypes, respectively⁶⁷⁵. Similarly to their CD8⁺ Tc1 counterparts, Th1 cells can also produce the pleiotropic TNF- α and IL-2 cytokines, which along IFN- γ , have been shown to contribute to the anti-tumor properties attributed to Th1 cells^{673,687–689}, which are mediated by their influence either on malignant cells or other immune cells⁶⁹⁰. The latter include DCs, which upregulate their MHC-II expression and/or their cytotoxic function^{691,692}, macrophages, which are polarized towards an inflammatory, anti-tumor M1 phenotype⁶³², and CTLs, which are also activated to exert their cytotoxic function⁶⁹³ under Th1 influence. However, as with CD8⁺ T cells, chronic exposure of Th1 cells to these cytokines within the TME may result in tumor-favoring effects⁶⁹⁴. Expectedly, the presence of Th1 cells has generally been associated with better clinical outcomes for patients with several malignancies^{695–698}.

1.1.9.1.2.2 T helper 2 (Th2) cells

Th2 cells are primarily known for their role in immunity against extracellular pathogens, such as helminths^{699–701}, as well as their involvement in allergic diseases, such as asthma and AD^{702–704}, through their ability to recruit and activate mast cells, basophils and eosinophils, as well as stimulate the production of antigen-specific IgE antibodies by B cells^{705,706}. Th2 polarization is dependent on IL-4 and IL-2 stimulation of naïve CD4⁺ T cells^{707–710}. In the presence of TCR signaling, IL-4-induced activation of STAT6^{711–713} is crucial for the sustained expression of^{714–716} the master regulator TF, GATA3^{710,717,718}, which in turn mediates the transcription of target genes^{717,719}, including *IL4*, *IL5* and *IL13*^{720–728}, encoding for the namesake signature cytokines of the Th2 phenotype. Additionally, IL-2-mediated activation/stabilization of STAT5 is necessary, in synergy with GATA3, for the expression of the *IL4* gene⁷²⁹, through binding to distinct sites of its promoter. Importantly, GATA3 is also responsible for suppression of the Th1 polarization program⁷¹⁸, although the underlying mechanism is quite controversial, including potential negative effects on IL-12 receptor (IL-12R β 2) expression⁷¹⁵, on IFN- γ production^{708,715,730}, or on STAT4⁷³⁰ in T cells.

In the context of malignancy, the Th2 environment has traditionally been considered as pro-tumorigenic ⁷³¹. For instance, it has been observed that IL-4, signaling through its type II receptor (which consists of one IL-4R α and one IL-13R α 1 chain) on cancer cells, can mediate their enhanced proliferation as well as reduced apoptosis ^{732–736}. Importantly, aside from immune, tumor cells can also be sources of IL-4 within the TME, thus acting in an autocrine manner ^{732–735,737}; and the same has specifically been shown for cancer SCs (CSCs) as well in CRC models ^{738,739}. IL-4 has also been implicated (directly or indirectly) in promoting the metastatic potential of malignant cells, in several tumor models ^{740–743}. In immune cells, IL-4 exerts its effect through either its type I receptor (consisting of one IL-4R α and one IL-2R γ chain, and mainly expressed on T cells, NKs, mast cells and basophils) or its type III receptor (consisting of all three IL-4R α , IL-13R α 1 and IL-2R γ chains, and mainly expressed in B cells and monocytes) ⁷⁴⁴. Importantly, IL-4/IL-4R signaling is an established mediator of macrophage polarization towards the immunosuppressive M2 phenotype ^{745–747}, while it has also been proposed to assist the immunomodulatory activity of Tregs ⁷⁴⁸, and restrict the migration of the antigen-presenting LCs (at least in vitro) ⁷⁴⁹. In humans, the expression of IL-4R α has been found increased in several tumors, with an observed inverse correlation between it and prognosis ^{750–753}. Similarly, IL-13 signaling, in part mediated via its shared with IL-4 type II receptor ⁷⁵⁴ and/or via its type III receptor (consisting of the IL-13R α 2, IL-13R α 1 and IL-4R α chains) ^{755–761} has also been associated with tumor-promoting effects in the setting of several human malignancies ^{755,756,759–774}. Regarding IL-5, not much is known about its role within the tumor context, although the majority of existing evidence points toward a pro-tumorigenic effect exerted from it as well ^{775–777}.

Overall, the proportion of Th2 cells in the TME has mostly been associated with worse clinical outcomes in the setting of several malignancies, including PDAC and HCC ^{778–780}. Nonetheless, there is substantial evidence suggesting a potential tumor-suppressing function of the Th2 lineage as well ^{781–798}, with a recent study in patients with CRC demonstrating that Th2 density, similarly to Th1 density, positively correlates with improved survival – although the ratio of Th1/Th2 infiltration did not display any association with prognosis ⁷⁹⁹.

1.1.9.1.2.3 *T helper 17 (Th17) cells*

Th17 cells comprise an important subset of helper T cells, characterized by the secretion of IL-17A and IL-17F (although IL-21 and IL-22 can also be produced), and are mainly involved in immunity against extracellular pathogens ⁸⁰⁰, as well as in autoimmune and inflammatory disorders, including psoriasis and RA ^{801,802}. Various functions of Th17 cells have, nonetheless, been characterized in the setting of anti-tumor immunity as well ^{663,693,803–807}. IL-21, IL-1 β , IL-6, IL-23 and, although quite controversially, TGF- β have been recognized as important cytokines in the induction and stability of the Th17 differentiation program from naïve

CD4⁺ T cells^{808–813}, while ROR γ t^{809,813–817} and STAT3^{817–822} have been identified as crucial TFs for the Th17 lineage, being responsible for the expression of the *IL17A* and *IL17F* genes^{816,823–825}. STAT3 is furthermore implicated in the expression of the *RORC* gene itself, as well as in the suppression of *TBX21* and *FOXP3* gene transcription, thus inhibiting the differentiation process towards the closely related, but immunomodulatory, Treg phenotype⁸²². IL-17-producing cells, including, aside from CD4⁺ Th17 cells, CD8⁺ T cells (Tc17) and innate immune cells, such as $\gamma\delta$ T cells ($\gamma\delta$ T17), NKT cells and type 3 innate lymphoid cells (ILC3), have been identified within the TME of various malignancies^{826–829}.

The influence of Th17 cells on anti-tumor immune responses and/or prognosis appears to be context-dependent. On one hand, a positive correlation between the TME density of Th17 cells (and IFN γ -producing T cells) has been reported in ovarian carcinomas, whereas an inverse correlation was observed between Th17 (CD4⁺ IL-17⁺) and Treg (CD4⁺ FOXP3⁺) populations⁸³⁰. Evidence from several in vitro and vivo studies has proposed that IL-17, in synergy with IFN- γ production by Th17 cells, can indirectly induce the chemoattraction of Th1 cells, CD8⁺ T cells and/or NK cells through stimulation of the production of CXCL9 and CXCL10 by malignant cells^{830–832}. Accordingly, a significant positive correlation between the density of IL-17A⁺ cells and CD1a⁺ DCs in the TME has been observed in human ESCC tumors, with the accumulation of the latter population associating further with better overall survival (OS)⁸³¹. In addition, IL-17 has been shown to facilitate the recruitment of neutrophils in the TME^{833,834}, potentially by upregulating CXCL2 and CXCL3 on tumor cells, and furthermore enhance their cytotoxic properties, in so contributing to reduction in tumor size in murine ESCC models⁸³³.

On the other hand, tumor-promoting functions of Th17 cells have been described as well. Indicatively, it has been shown by both in vitro and in vivo analyses, that IL-17 can promote angiogenesis, via inducing the secretion of several pro-angiogenic factors (including VEGF) by both tumor cells and fibroblasts^{835–839}, and favor tumor growth in general^{836,840,841}, in part by stimulating the production of IL-6 and activation of downstream STAT3 signaling^{840,841}. Moreover, Th17 cells have been shown to be able to express functional CD39 (ENTPD1) and CD73 (NT5E)⁸⁴², two ectonucleotidases responsible for the production of the immunosuppressive adenosine^{843–846}, and thus repress the effector functions of both CD8⁺ and CD4⁺ T cells⁸⁴². In addition, IL-17 may induce the tumor-promoting M2-like phenotype of macrophages, in a NF- κ B-dependent manner⁸⁴⁷. Although existing evidence is controversial⁸⁴⁸, it has been suggested that IL-17 may favor the recruitment and activity of myeloid-derived suppressor cells (MDSCs) in the TME, in so promoting tumor growth in mouse models^{849,850}. Importantly, the close association between the Th17 and Treg phenotypes, and the observed

plasticity of both populations can further complicate their functional role within the TME and render the significance of their infiltration in tumors context-dependent^{663,803–807}.

1.1.9.1.2.4 *T helper 22 (Th22) cells*

Th22 cells are closely related to, but still discrete from Th2 and Th17 cells^{851,852}, and are mainly involved in inflammatory diseases, including cutaneous ones, like psoriasis⁸⁵¹. Th22 cells are characterized by the production of IL-22, without production of IL-17, IL-4 or IFN- γ , although they may also secrete TNF- α and IL-13^{853,854}. Plasticity of Th22 cells towards Th1 and Th2 cells in vitro has also been observed⁸⁵². Differentiation of naïve CD4⁺ T cells towards the Th22 phenotype is orchestrated by aryl hydrocarbon receptor (AhR) and ROR γ t activation^{852,855} and T-bet downregulation⁸⁵², following stimulation by IL-21, IL-6 and/or TNF- α ^{854–856}. The STAT3 TF is also implicated in promoting IL-22 expression via its co-operative interaction with AhR on the *IL22* gene promoter⁸⁵⁵. Of relevance to cutaneous immunity, dermal DCs, and even more so LCs, have been recognized as important inducers of the Th22 phenotype in naïve CD4⁺ T cells⁸⁵⁷.

The main effector of Th22 cells' functions, IL-22, binds to the IL-22 receptor (IL-22R) and primarily activates the downstream STAT3 signaling cascade in target cells^{858–861}. In the context of malignancy, the effects of IL-22 have mainly been described as pro-tumorigenic⁸⁵⁴. For instance, it has been shown that IL-22 can enhance the proliferation and inhibit the apoptosis of CRC cells in vitro, as well as promote CRC tumor growth and/or metastasis in murine models in vivo, in a STAT3-dependent manner^{862,863}. Similar observations have been made for NSCLC⁸⁶⁴, as well as for triple negative breast cancer, in the setting of which IL-22 has additionally been implicated in development of chemotherapy resistance⁸⁶⁵. Th22 cells have been identified as the main source of IL-22 in liver metastases, where they appear to contribute to angiogenesis induction within the tumor foci⁸⁶⁶. Upregulation of inducible nitric-oxide synthase (iNOS) expression in cancer cells may serve as an additional tumor-promoting mechanism of IL-22^{867,868}. Unsurprisingly, in clinical cohorts of patients with GC, higher tumor densities of Th22 cells and IL-22-producing CD4⁺ T cells have been observed to be significantly correlated with advanced disease staging and/or worse prognosis^{869,870}. Although TNF- α production by Th22 cells has mainly been reported within infectious and/or inflammatory contexts^{871,872}, their ability to produce this predominantly tumor-suppressing cytokine may imply Th22 cells' capacity to exert anti-tumor functions as well, under specific circumstances⁶⁹³.

1.1.9.1.2.5 *T follicular helper (Tfh) cells*

Tfh cells are a specialized CD4⁺ T cell subpopulation with the ability to closely interact with B cells and promote humoral immunity. In particular, Tfh cells are necessary for the

activation and differentiation of B cells, including class-switch recombination and somatic hypermutation of immunoglobulins, which take place in the germinal centers of secondary lymphoid organs ^{873,874}. Proper function of Tfh cells is essential for homeostasis, with their dysregulation having been associated with the development of autoimmune conditions ^{873–875}. The differentiation of naïve CD4⁺ T cells towards the Tfh phenotype is a complex process which is not yet fully understood ⁸⁷⁶; nevertheless, it appears to be triggered by IL-6 and IL-21, in the absence of TGF- β , and is dependent on the master regulator TF Bcl-6, the activity of which is antagonized by Blimp-1 ^{877–881}. Among the functions of the Bcl-6 transcriptional repressor, is its binding to the *Tbx21* and *Rorc* promoters, leading to repression of the Th1 and Th17 differentiation programs, respectively ⁸⁸¹. Tfh cells are characterized by high surface expression of the CXCR5 receptor, which facilitates their response to B cell-derived signals and thus homing to B lymphoid follicles, a process which is further facilitated by downregulation of surface CCR7 ^{881–883}. Within germinal centers, Tfh cells also express a series of co-stimulatory and co-inhibitory molecules, such as CD40 ligand (CD40L) and inducible T cell costimulator (ICOS), and PD-1, respectively ^{874,882,883}.

All Tfh cells are characterized by high and consistent secretion of the IL-21 cytokine, which is crucial for their B cell helper function, while it additionally creates a positive feedback loop for Tfh identity maintenance ^{873,877,884,885}. Nevertheless, several discrete subpopulations of Tfh cells have been described, according to the rest of their cytokine profile and associated TFs, with the ability to drive distinct antibody class switching and mediate immune responses that are reminiscent of other Th subtypes ^{873,875}. These specifically include: (i) Tfh1 cells, which, aside from IL-21, produce IFN- γ and/or IL-10, (ii) Tfh2 cells, which produce the Th2-associated cytokines IL-4, IL-5 and IL-3, as well as (iii) Tfh17 cells, which secrete IL-17 and/or IL-22 ^{873,875}. Moreover, T follicular regulatory (Tfr) cells share properties of both Tfh and Treg (see below) cells, and thus express the Treg master regulator TF FOXP3 (aside from Bcl-6), express the surface Treg-associated molecules CD25 and CTLA-4 (aside from CXCR5), as well as secrete IL-10, TGF- β and/or granzyme B – in so, being capable of suppressing the function of both B cells and Tfh cells ^{875,886,887}.

1.1.9.1.2.6 Regulatory T (Treg) cells

Tregs comprise a, classically CD4⁺ CD8⁻ (although both CD4⁻ CD8⁺, and CD4⁻ CD8⁻ / double negative populations have also been described) ^{888–893}, T cell subpopulation, characterized by immunomodulatory activity ^{894,895}. In healthy human adults, Tregs are considered to constitute around 5% of CD4⁺ cells in peripheral blood ^{896–899}, and approximately 8-20% of CD4⁺ cells in the skin ^{897,899}. The FOXP3 TF has been long considered as the master regulator of Treg identity, mediating, among others, the constitutive high expression of the following two surface proteins ^{900,901}: (i) CD25 – also known as the α chain of the (high affinity)

IL-2 receptor complex (ILR2 α), which is involved in the suppression of both effector and helper T cell subsets' survival, proliferation and activity by enabling IL-2 depletion within the microenvironment^{902,903}, while it is also crucial for the overall homeostasis and phenotypic profile of Tregs, through downstream intracellular signaling^{902,904–908}, and (ii) CTLA-4, a known immune checkpoint receptor, which facilitates the depletion (e.g. through trans-endocytosis or trogocytosis) of the co-stimulatory CD80/CD86 molecules on the surface of APCs following the formation of immune synapse with Tregs^{909,910}, a process which can additionally favor the upregulation of PD-L1 on APCs, thus further inhibiting the function of PD-1-expressing effector T cells⁹¹⁰. FOXP3 is additionally responsible for inhibiting the production of the pro-inflammatory IL-2 and IFN- γ cytokines by Tregs^{901,911–915}, whereas secretion of IL-10, TGF- β and IL-35, all of which can exert anti-inflammatory effects^{916–918}, comprises another hallmark of Treg identity⁹¹².

Tregs can inhibit the activation or induce the exhaustion of cytotoxic CD8+ T cells⁹¹⁹, or interfere with the function of CD4+ helper T cells, DCs, NKs and even B cells⁹²⁰, and are thus crucial for maintaining peripheral self-tolerance, with their dysregulation having been implicated in several autoimmune disorders^{920–923}. Unsurprisingly, Treg activity also plays a vital role in anti-tumor immunity, in the context of which Treg presence usually favors tumor growth, owing to their immunomodulatory profile, and has thus been associated with more aggressive subtypes and/or worse prognosis in various malignancies, including ovarian⁹²⁴, breast^{925,926}, gastric⁹²⁷, esophageal⁹²⁸, colorectal⁹²⁹, pancreatic⁹³⁰, lung⁹³¹, as well as cSCC⁹³², and melanoma^{933,934}. Interestingly, it has been observed that tumor-infiltrating Tregs can display unique characteristics, such as up-regulation of surface PD-1, PD-L1, PD-L2, IL-1 receptor 2 (IL1R2), and CCR8, as well as increased expression of IL-10, potentially mediated through the influence of various aspects of the TME, such as hypoxia or metabolic dysregulation, which appear to ultimately stabilize and even enhance their immunosuppressive activity^{926,935–938}.

It is important to note, however, that the significance of Tregs' presence within the TME regarding anti-tumor immunity and overall prognosis is not so straightforward, but may rather depend on the context, and, thus under certain circumstances can be associated with better prognosis^{644,646,939–942}. In line with the plasticity that characterizes CD4+ T cell subpopulations, and its implications in human disease^{658,663,664,666,667}, Tregs are capable, under certain circumstances, of converting into cells with a pro-inflammatory phenotype. For instance, it has been shown that, in the setting of autoimmune arthritis, FOXP3+ CD4+ T cells can, under the influence of synovial fibroblast-derived IL-6, lose FOXP3 expression and transdifferentiate into IL-17-producing Th17 cells, which possess an enhanced pathogenic potential, while IL-17 FOXP3 double positive T cells have been identified in the synovium of patients with active RA,

potentially representing an intermediate phenotype during the Treg to Th17 conversion process ⁹⁴³.

Overall, Treg functional identity cannot solely be defined by the expression of FOXP3 ⁹⁴⁴, and accordingly, for the rest of T cell subtypes their characterization cannot usually be determined by one sole factor. Additionally, the activation or exhaustion status of each cell population comprises a very important aspect when evaluating their functional impact within the TME, however its assessment usually lacks in existing studies; whereas T cell plasticity can further hinder our understanding of the potential effects of a specific T cell subtype within a certain microenvironment.

1.1.9.1.3 Macrophages

Macrophages are part of the mononuclear phagocyte system, along with circulating monocytes and DCs, and thus constitute important mediators of innate immune responses ⁹⁴⁵. Moreover, both macrophages and DCs are considered “professional” APCs, driving the activation and proliferation of T cells ⁹⁴⁶. Macrophages have traditionally been categorized into M1, which, regarding malignancy, are associated with tumor-suppressing functions, and M2, which correlate with tumor-promoting outcomes, including their contribution to angiogenesis and immunomodulatory properties ⁹⁴⁵. Nonetheless, nowadays several distinct M2 subtypes are recognized, including M2a, M2b, M2c and M2d, each one with unique properties and markers (surface, secreted, and TFs), which remain outside the scope of this study ⁹⁴⁵. In short, though, M1 macrophages display, among others, high expression of MHC-II, CD80, CD86, TLR-2, TLR-4 and iNOS, and secrete pro-inflammatory cytokines, such as IL-1 β , IL-8, IL-12 and TGF- α , whereas M2 macrophages are characterized by the surface expression of CD163, CD204, CD206, CD209 and FIZZ1 (encoded by the resistin like beta; RETNLB gene), along with reduced expression of MHC-II molecules, and produce several anti-inflammatory mediators, including IL-10 ⁹⁴⁵.

1.1.9.1.4 Dendritic Cells (DCs)

DCs are professional APCs, functioning as vital bridging cells between innate and adaptive immunity, and essentially all of them express CD11c and HLA-DR on their surface ⁹⁴⁷. Nonetheless, different DC subtypes have been described according to their progenitor cell, the TFs driving their differentiation, their pool of secreted factors, and their associated functions ^{947–950}. These include the following: (i) conventional DCs (cDCs), which are further divided into cDC1 and cDC2. Human cDC1 cells are dependent on the BATF TF, are characterized by surface expression of XCR1, CLEC9A, BTLA, CADM1 and CD141 (THBD, BDCA3), and are mainly responsible for the cross-presentation of antigens to CD8⁺ T cells via MHC-I molecules ^{947–950}. cDC2 cells, the most common DC subtype, comprise an overall heterogenous

subpopulation, which, in humans, is driven by several TFs, most importantly IRF4. They can express a diverse pattern of surface markers, depending on their localization, but are generally characterized by CD1c (BDCA1), CD11b, CD2 and CD172a (SIRP α), and are implicated in the activation of naïve CD4⁺ T cells via antigen presentation on MHC-II molecules ^{947–950}. (ii) Plasmacytoid DCs (pDCs) lack the dendritic shape which characterizes the other subsets in their mature, steady state, and instead display a plasma cell-like morphology. They also express lymphocytic markers, such as CD45RA, as well as IL3RA (CD123), CLEC4C (CD303, BDCA2), and NRP1 (CD304, BDCA4) in humans, while they display decreased CD11c and MHC-II levels, and are primarily involved in antiviral immune responses, producing pro-inflammatory cytokines, such as type I and type III IFNs, TNF- α and IL-6 ^{947–950}. (iii) Monocyte-derived DCs (moDCs), as their name suggests, differentiate from monocytes, at sites where the latter have been recruited under inflammatory conditions, and expectedly, display markers of both cDCs (MHC-II, CD11c, CD11b) and monocytes (CD64, FCGR1). They also produce several pro-inflammatory factors, including TNF- α and iNOS ^{947–950}. (iv) Langerhans cells (LCs), residing in the epidermis, have traditionally been considered as a specialized DC subtype, however, according to recent studies they are now classified as a special subset of macrophages (see below). Importantly, DCs are also categorized into mature and immature populations, with the latter exhibiting a more rounded morphology, lower levels of surface CD80, CD86, CD83, and MHC-II expression, and a lower cytokine secreting activity ⁹⁵¹. Unsurprisingly, DCs exert important roles in orchestrating anti-tumor immune responses, with their presence and functionality in different cancer settings though still being under investigation ^{948,952}.

1.1.9.1.5 Langerhans Cells (LCs)

Langerhans cells (LCs) had long been considered as a specific subtype of DCs resident to the epidermis, owing to their migratory and antigen-presenting properties. However, more recent studies have proposed the reclassification of LCs as a specialized macrophage subset, based on their embryological origin ^{953–955}. LCs are characterized by the expression of CD1a, CD207 (also known as langerin), E-cadherin, and MHC-II ^{956,957}, their high ATPase activity, expressing a plasma membrane-bound form of the enzyme (ecto-ATPase) ^{958,959}, as well as the cytoplasmic presence of rod-shaped organelles (specific to LCs) called Birbeck granules, the formation of which is mediated by langerin and whose function still remains incompletely understood ^{960–963}. LCs, through their capacity for nodal migration and antigen presentation to T cell populations there, as well as their proposed in situ functions in the epidermis (for example, interaction with Tregs and/or resident memory T cells) ⁹⁶⁴ are essential for skin homeostasis, and may, accordingly, be implicated in aspects of the anti-tumor immunity against both melanoma and NMSCs ^{965–967}.

1.1.9.2 Aspects of the Immune Microenvironment of BCC

Several studies have intended to shed light into the immune microenvironment of BCC, and although many of its aspects still remain poorly understood or controversial, the relative presence or absence of the various immune cell types, as has been identified so far, implies the existence of both immunosuppressive mechanisms and host-derived anti-tumor immunity influencing BCC pathogenesis. The majority of existing evidence has highlighted that the immune microenvironment of BCC consists of a predominantly lymphocytic, and especially T cell infiltrate^{514,968–975}, which tends to localize at the periphery of the tumors^{968,971,976–980}, and the extent of which has been suggested to be inversely correlated with tumor aggressiveness^{571,971,974,975,981}. Several studies support the prevalence of CD4+ over CD8+ T cells within the BCC infiltrate^{973,977}, with different CD4+ subtypes having been identified, including Th1, Th2, Th17 and Treg cells^{382,977–979,982}. However, their relative proportion within the BCC TME, in comparison to healthy skin, still remains disputable, probably in part due to the distinct approaches implemented in different studies for their examination, as well as differences in BCC subtypes (including in regard to staging, aggressiveness or location) and inconsistencies in localization of the skin used as the healthy control (facial or from trunk and limbs) in each study.

Based on various IHC, IF and/or reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) analyses, a significant enrichment in the proportion of CD4+ FOXP3+ Treg cells has been identified in BCCs compared to peri-tumoral and/or distant healthy skin, with an observed accumulation of Tregs predominantly at the periphery of the tumor^{977–979,983}. In accordance, mRNA levels of *CCL17*, *CCL18*, *CCL20* and *CCL22* – chemokines that are associated with Treg recruitment – have been found significantly increased in BCC compared to NS^{978,979}. A recent IHC study, however, highlights that despite the significant discrepancy in absolute numbers of CD3+ FOXP3+ cells between tumor center, tumor periphery and adjacent normal skin (ANS) (with tumor periphery exhibiting the highest Treg cell count), the proportion of FOXP3+ cells among all CD3+ cells does not display significant differences between these areas⁹⁸⁴. Nonetheless, the ratio between CD8+ IFN- γ + T cells and CD4+ FOXP3+ T cells is significantly downregulated in BCC compared to healthy skin⁹⁷⁷, thus supporting the proposed immunosuppressive aspect of the BCC TME. In contrast, a bioinformatic study has reported a significantly decreased Treg gene set enrichment score in BCC compared to NS⁹⁸⁵; however, this observation on its own is not sufficient to challenge the in vitro findings.

In addition to Tregs, significantly increased levels (either mRNA and/or protein) of Th2-related cytokines (IL-4, IL-5, IL-13) have been observed in BCC compared to NS^{978,986,987};

contrastingly, though, a recent flow cytometry (FC)-based study has highlighted a significant decrease of IL-4-producing CD4+ T cells in BCC tissues compared to NS ⁹⁷⁷.

The mRNA levels of Th1-associated cytokines IL-12 (*IL12*), TNF- α (*TNF*) and IFN- γ (*IFNG*) have been found significantly enriched in BCC compared to healthy skin, although no significant differences were reported between BCC and peritumoral skin ^{978,979}. Nevertheless, in two recent studies, protein expression of IFN- γ was significantly upregulated in BCC vs. NS by IHC ³⁸², and IFN- γ -producing CD4+ T cells were verified as significantly enriched in BCC vs. NS by FC ⁹⁷⁷. Moreover, it has been suggested that IFN- γ protein expression is significantly higher in superficial compared to nodular BCCs ³⁸², while the mRNA levels of *IFNG* have been observed to be significantly upregulated in actively regressing BCCs compared to non-regressing BCCs ⁹⁸⁸ – this could potentially imply the involvement of Th1 cells in the BCC regression process.

Cells positive for IL-17 and/or IL-22 – two cytokines that have been associated with tumor-promoting functions in the setting of BCC ⁹⁸² – or the related IL-23 cytokine, have been identified in BCC biopsies, especially at tumor periphery ^{382,982,989}. IL-17-producing T cells have been found to be significantly enriched in BCCs compared to NS ⁹⁷⁷, as well as compared to patient-matched PBMCs ⁹⁸².

BCCs have also been reported to be depleted of $\gamma\delta$ T cells compared to healthy skin ⁹⁷⁷.

CD8+ T cells are implicated in the BCC TME as well, as part of the host anti-tumor immune response. Similarly to the CD4+ FOXP3+ (Treg) populations, CD8+ T cells mainly localize at the periphery of BCCs rather than within tumor nests, contributing to the peritumoral inflammatory niche ^{977,978,983,990}. CD8 IHC expression, especially stromal one, is significantly increased in BCC samples compared to healthy skin ^{571,977}, although there is evidence that the frequency of CD8+ among all CD3+ T cells does not differ significantly between BCCs and NS ⁹⁷⁷. Nevertheless, the proportion of IFN- γ -producing CD8+ T cells is significantly increased in BCC samples compared to healthy skin ⁹⁷⁷, potentially suggesting an increased cytotoxic activity exerted by CD8+ T cells in the setting of BCC. Interestingly, BCC-infiltrating CD8+ T cells are among the immune cells staining positive for IL-17 expression ³⁸², highlighting the presence of a population with suggested enhanced anti-tumor activity ⁹⁹¹ in the BCC TME. In accordance with their anti-tumor functions, the intratumoral levels of CD8+ T cells have been positively associated with lower risk BCC subtypes compared to high risk ones, although, interestingly, the same has been proposed to hold true for FOXP3+ cells as well ⁵⁷¹. No significant difference has been observed in the proportion of CD8+ T cells between actively regressing BCC, BCC with previous regression and non-regressing BCC ⁹⁹². Overall, the

degree to which CD8+ T cells are involved in the BCC TME, and the potential association of their involvement with several clinical aspects needs to be investigated further.

Regarding B cell infiltration, the majority of IHC studies support a very low number of B cells within the BCC immune infiltrate^{968,970,971,973}, although the presence of plasma cells has been suggested to be quite prominent in BCCs⁹⁷⁵. Interestingly the proportion of plasma cells in the peritumoral infiltrate has been found to be significantly and positively correlated with high risk BCC subtypes compared to low risk ones⁹⁷⁴. As with CD8+ T cells, no difference has been reported for the frequency of B cells depending on the BCC regression status⁹⁹².

Most IHC studies report only a scarce NK infiltration in BCC tissues^{968,971,973}, which can however, reach up to approximately 10% of the total infiltrating immune cells, as measured by FC analysis in a recent series of BCCs⁹⁸². One in silico study has proposed that NKs are enriched in BCC tumors compared to healthy skin⁹⁸⁵, while another hasn't reported any significant differences in the frequency of NKT cells between BCC and NS samples⁹⁹³.

Aside from lymphocytes, several non-lymphocytic populations have been identified in BCC. Mast cells are accumulated in BCC tumors, especially in their periphery^{968,971,994–998}, with the degree of their infiltration being inversely correlated with that of T cells⁹⁷¹. Moreover, mast cell density has been positively associated with higher risk subtypes of BCC^{971,996,998}. Unsurprisingly, BCC-infiltrating mast cells have been shown to produce tumor-promoting and angiogenic factors, such as VEGF, IL-8 and CCL5, with their degree of infiltration correlating with the extent of tumor vascularization⁹⁶⁸. Nonetheless, BCC-associated mast cells may also potentially exert tumor-suppressing effects through TNF- α production⁹⁹⁵.

Macrophages are present in BCC tumors, although to a much lower extent compared to T cells^{973,974}; but seemingly in an increased fashion compared to healthy skin⁹⁹³. Macrophages (CD68+) preferentially localize at the peritumoral BCC stroma⁹⁹⁷, as well as in the tumor invasive front^{983,990}, and have been found to be positively correlated with the tumor infiltration depth and the microvessel density. In fact, it has been hypothesized that the interaction between TAMs of an M2-like phenotype and malignant BCC cells, drives the pathogenic properties of the latter, including their increased invasive capacity and angiogenic potential, in a cyclooxygenase-2 (COX-2)-dependent manner^{999,1000}. Moreover, it has been proposed that triggering receptor expressed on myeloid cells 2 (TREM2)-expressing TAMs, aside from their involvement in effector CD8+ T cell exhaustion and resistance to anti-PD-1 treatment^{1001,1002}, in the setting of BCC in particular may also produce oncostatin M and activate downstream JAK-STAT3 signaling in BCC tumor cells, in so promoting their proliferation¹⁰⁰³. In accordance with these observations, there is evidence supporting the predominance of M2 compared to M1 macrophages in BCCs⁹⁷⁸. However, no significant

association has been observed for either total, M1 or M2 macrophages and the risk of recurrence or the tumor size of BCCs ¹⁰⁰⁴. Nonetheless, higher macrophage infiltration and lower T cell infiltration has been associated with advanced compared to non-advanced BCCs, as well as HHI-resistant compared to HHI-sensitive BCCs ⁹⁹³.

DCs comprise another immune cell subset predominantly located at the peritumoral area of BCCs rather than within tumor aggregates ^{1005,1006}. However, their presence (both of total DCs and pDCs in particular) in BCCs is significantly reduced compared to healthy skin ⁹⁸⁵. Moreover, their functionality appears to be impaired, based on their reduced antigen presenting capacity, evident in T cell proliferation assays, and mediated by the reduced CD80/CD86 expression that they display compared to DCs from skin with allergic and/or inflammatory conditions, matched healthy skin or matched peripheral blood-derived DCs ¹⁰⁰⁵. Despite observing a similar degree of cDC infiltration in the immediate peritumoral dermis/juxtatumoral dermis and the adjacent non-lesional papillary dermis of BCC biopsies, Kaporis and colleagues, importantly, highlighted a predominantly immature phenotype of BCC-associated cDC ⁹⁷⁸. On the contrary though, a recent bioinformatic analysis has challenged this notion, reporting a significantly lower score for immature DC in BCC samples compared to NS samples ⁹⁹³. In addition, no significant difference has been observed for the stromal density of factor XIIIa (FXIIIa)-positive dermal DCs between BCC types in terms of aggressiveness (sclerosing, morpheaform, micronodular BCCs vs. nodular, superficial BCCs) ¹⁰⁰⁶.

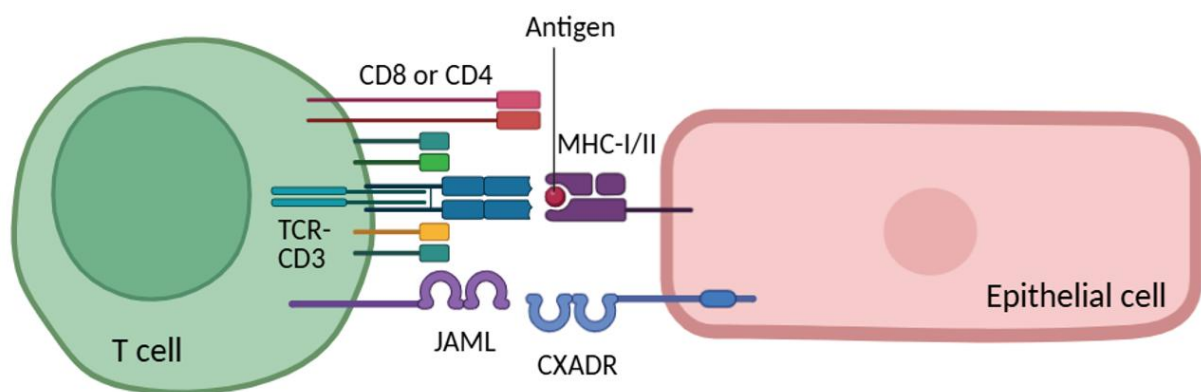
Unsurprisingly, along DCs and macrophages, LCs have also been implicated in the pathogenesis of BCC, with the majority of existing evidence supporting a decreased accumulation in tumor samples compared to matched healthy skin ^{976,978,1007–1010}, and with a preferential distribution at the periphery of tumor aggregates rather than within them ^{978,1007–1009}, or within the supratumoral epidermis ⁹⁷⁶. Importantly, even when no significant differences in LC density were noted, the morphology of BCC-associated LCs, especially of supratumoral and not peritumoral ones ^{1007,1011}, was altered, characterized mainly by a more round-shaped cellular body, and fewer, thinner and less branching dendrites stemming from it, compared to LCs from healthy skin ^{976,1007,1008,1012}. Although UVR exposure has been associated with such LC perturbations, along with LC migration to lymph nodes ^{1013–1017}, that could, at least partly, explain the altered density and morphological phenotype of LCs in BCC, the identification of discrepancies even between BCC samples and *sun-exposed* healthy skin ¹⁰⁰⁸, implies the existence of additional mechanisms that contribute to this observation. Moreover, given that previous analyses have associated UVR exposure with disruption of normal LC function and a shift towards a tolerogenic profile through downregulation of CD80/CD86 ¹⁰¹⁷, one can hypothesize that the normal antigen-presenting activity of LCs may be dysregulated in BCC

and potentially contribute to its development. In support of this, a relatively recent study in the *Ptch*^{flox/flox} *ERT2*^{+/-} murine BCC model has shown that liposomal clodronate-mediated depletion of macrophages, LCs and dermal MHC-II+ cells in the tumor area of BCC results in enhanced tumor growth, thus underlining the potential significance of macrophage, LC and dermal DC involvement in induction of the antitumor immunity against BCC ¹⁰¹⁸.

Moreover, it is worth underlining that BCC tumor cells and/or stromal cells play an important role in modulating the immune microenvironment of BCC, either through the expression of immune checkpoint molecules like PD-L1 ^{569–572} and HLA-G ^{980,1019}, through the downregulation of antigen-presenting MHC-I molecules ^{514,1020–1022}, and/or through the secretion of cytokines that lead to the recruitment of Tregs or other immune cells which ultimately support a pro-tumorigenic state, including CCL20 and CCL22 ^{1023,1024}.

1.2 The JAML-CXADR Axis

Coxsackie and Adenovirus Receptor (CXADR) as well as its cognate ligand, Junctional Adhesion Molecule-Like (JAML) (**Introductory Figure 1**), are both type I transmembrane glycoproteins, belonging to the junctional adhesion molecule (JAM) group, “canonical” members of which are considered the previously identified JAM-A, JAM-B and JAM-C molecules¹⁰²⁵. JAMs belong to the CTX subfamily within the umbrella of the immunoglobulin superfamily (IgSF) and are structurally characterized by two extracellular immunoglobulin (Ig) domains, one single-pass transmembrane region, and a cytoplasmic tail, which traditionally includes a PDZ-binding motif, that enables their interaction with PDZ domain-possessing scaffolding proteins^{1025–1027}. The distinct JAM proteins display diverse cell expression patterns (mainly across epithelial, endothelial and leucocyte populations), and constitute essential molecules for the assembly of intercellular connections, predominantly tight junctions, as well as adherens junctions, while also being able to interact with integrins^{1025,1028–1032}. Thus, they are implicated in a variety of physiological processes and functions, including cell polarity, formation and integrity of the epithelial barrier, blood-brain barrier and blood-testis barrier, as well as in cell migration, especially the transendothelial migration (TEM) of immune cells^{1029,1033–1047}.



Introductory Figure 1: Interaction between CXADR-expressing epithelial cells and JAML-expressing T cells. CD = Cluster of Differentiation, TCR = T cell Receptor, MHC = Major Histocompatibility Complex, CXADR = Coxsackie and Adenovirus Receptor, JAML = Junctional Adhesion Molecule Like

1.2.1 Junctional Adhesion Molecule-Like (JAML)

1.2.1.1 Structure and Cellular expression pattern of JAML

JAML was initially identified in 2003 as a novel gene upregulated in human (cell line and primary) acute promyelocytic leukemia (APL) cells following treatment with all-trans retinoic acid (ATRA)¹⁰⁴⁸ – known to induce differentiation of APL blasts towards the

granulocytic phenotype ^{1049,1050} – and was mapped to the q23 locus of chromosome 11 ¹⁰⁴⁸. Characterization of the *JAML* cDNA sequence and predicted protein structure highlighted its homology with previously known JAM molecules, while the JAML protein cellular localization was described as confined to cell membrane areas involved in intercellular interactions ¹⁰⁴⁸.

The JAML protein has a total length of 394 amino acids, and differs structurally from the other JAM molecules due to its longer extracellular Ig domains ^{1037,1048}, both of which are, parenthetically, of the V (variable) type ¹⁰⁵¹, and like the rest of non-canonical JAMs (including CXADR) ¹⁰⁵² possesses a longer cytoplasmic tail compared to canonical JAMs ^{1037,1048,1053,1054}, which, however, does not contain a conventional PDZ-binding motif ¹⁰³⁷. Nevertheless, JAML possesses a dimerization motif similar to that of the canonical JAM molecules ¹⁰⁴⁸, and early on it was proposed that JAML dimerization in cis is required for its heterophilic interactions with CXADR in trans ¹⁰⁵³. In subsequent studies, a dimeric form of the JAML-CXADR complex was identified by crystallography and, when in solution, a markedly increased PI3K assembly to the intercellular domain of JAML was observed following its activation by *dimeric* forms of CXADR or a stimulatory antibody, compared to when activated by their monomeric form – thus, suggesting that dimerization of JAML is implicated in downstream signal transduction ¹⁰⁵¹. Nonetheless, when in an immobilized setting, dimeric and monomeric forms of the JAML-stimulating antibody did not result in any significant difference regarding JAML-mediated outcomes ¹⁰⁵⁵.

In contrast to most other JAMs that are commonly found on the surface of epithelial and/or endothelial cells, JAML is preferentially, although not exclusively, expressed in mature hematopoietic cells, including neutrophils, monocytes and T lymphocytes, especially under homeostatic conditions ^{1037,1048,1053,1056}. Both CD4⁺ and CD8⁺, including memory, T cell populations, express JAML ¹⁰⁵³; while, in addition, JAML expression has been observed in T cell subpopulations that are characterized by surface expression of molecules associated with T cell homing to the skin (cutaneous leucocyte antigen; CLA, CCR4) or to the gut (integrin $\alpha 4\beta 7$) ¹⁰⁵³.

1.2.1.2 JAML and CXADR interaction

Although it was initially suggested that the interaction between JAML and CXADR is mediated through the membrane-proximal (D2) domain of the former and the membrane-distal (D1) domain of the latter ¹⁰⁴⁷, it was later established by crystallography that it is the membrane-distal (D1) domain of JAML that is responsible for binding to CXADR ^{1051,1057}. Nevertheless, the membrane-proximal (D2) domain of JAML appears to facilitate an increase in the affinity of JAML D1 and CXADR D1 domain binding ^{1051,1057}. The interacting area between the membrane-distal epitopes of both molecules is characterized by numerous hydrophilic bonds

and the presence of highly evolutionarily conserved charged amino acids, implying their significance for the JAML-CXADR interaction ¹⁰⁵⁵. The association of JAML and CXADR through their D1 domains displays a relatively low binding affinity (order of magnitude: micromolar – μM) and relatively fast kinetics with quick dissociation of the majority of molecules ¹⁰⁵⁵.

1.2.1.3 JAML as a T cell co-stimulatory molecule

Even though JAML was first described as a surface molecule predominantly of neutrophils and monocytes, responsible for facilitating their TEM ^{1047,1048,1058}, focus soon shifted to JAML's role in T cell populations, especially since it was established that JAML acts as a co-stimulatory molecule for (cutaneous) $\gamma\delta$ T cells ¹⁰⁵⁷. $\gamma\delta$ T cells are a T cell subset characterized by the expression of TCRs consisting of the γ and δ chains instead of the “conventional” α and β ones ^{1059,1060}, which grants them the ability for MHC-independent antigen recognition ¹⁰⁶¹; thus, they are often considered as a “bridging” population between innate and adaptive immunity ¹⁰⁶². Although they comprise a rare T cell subset in peripheral blood and lymphoid tissues of humans (0.5-5% of total T cells), they can be found in considerable proportions in epithelial tissues of mice (even more than 50% of total T cells), while in humans they constitute approximately 20% of T cells in the skin ^{1063–1067}. $\gamma\delta$ T cells are involved in both homeostatic and pathologic states, including carcinogenesis ^{1068–1070}. In this context, they have been associated with mostly anti-tumorigenic properties, as T cell receptor delta locus (*Tcrd*) KO (*Tcrd*^{-/-}) mice exhibit increased susceptibility to tumorigenesis ¹⁰⁷¹, whereas in clinical cohorts of patients with several different malignancies a positive correlation between $\gamma\delta$ T cell infiltration and overall prognosis has been identified ^{1072–1076}.

In line with its co-stimulatory function in $\gamma\delta$ T cells, JAML shares a YMxMxPxxP intracellular motif with the CD28 co-stimulatory molecule ^{1051,1057}. Similarly to CD28 ¹⁰⁷⁷, the YMxM sequence of JAML has been proven to serve as a binding site for PI3K following ligation to CXADR, thus activating downstream Akt signaling ^{1051,1057}. Aside from PI3K/Akt activation, the concomitant $\gamma\delta$ TCR and JAML stimulation additionally results in upregulation of the activity of the JNK kinase (of the MAPK pathway), to a degree bigger compared to stimulation of either molecule alone, ultimately leading to $\gamma\delta$ T cell proliferation and production of pro-inflammatory cytokines, including TNF- α ¹⁰⁵⁷.

Contrasting initial reports which claimed that JAML does not act as a co-stimulatory molecule in $\alpha\beta$ T cells ¹⁰⁵⁷, two recent studies ^{1078,1079} have highlighted, by in vitro and ex vivo experiments, JAML's potential as a co-stimulatory receptor in both murine and human $\alpha\beta$ TCR-expressing populations, including CD4+, but most prominently CD8+ T cells. Specifically, ex-vivo restimulation with anti-CD3, and one of the anti-JAML (HL4E10), anti-CD28 or IgG control

antibodies, of previously isolated naïve and subsequently activated and expanded splenic CD8⁺ and $\gamma\delta$ T cells from wild-type (WT) mice, corroborated the previously described co-stimulatory role of JAML in $\gamma\delta$ T cells ¹⁰⁵⁷, but also further highlighted its potential function as a co-stimulatory molecule in CD8⁺ T cells, as reflected by the significant upregulation in CD69 expression as well as TNF- α and IFN- γ production ¹⁰⁷⁹. Similarly, in vitro co-stimulation of human CD4⁺ or CD8⁺, as well as murine CD8⁺ T cells, with an anti-CD3 antibody and either recombinant CXADR (human) or a stimulating anti-JAML antibody (mice) resulted in upregulation of the surface expression of early activation marker proteins, such as CD69, PD-1, CD25 (IL2RA), and 4-1BB (TNFRSF9, CD137), and/or in increased T cell proliferation rate ¹⁰⁷⁸. Accordingly, it has been demonstrated that CD8⁺ TRM cells (identified as CD45⁺ CD3⁺ CD8⁺ CD69⁺ CD103⁺ by FC) sorted from tumor samples of patients with NSCLC, when stimulated in vitro with anti-CD3 and recombinant CXADR, exhibited a significantly upregulated production of IFN- γ and TNF- α , compared to when stimulated with either factor alone, and interestingly, the level of the two cytokines detected following CXADR co-stimulation was comparable to that observed after CD28 co-stimulation, thus indicating that JAML on CD8⁺ TRM cells can serve as a robust co-stimulatory molecule ¹⁰⁸⁰. Moreover, IF analysis highlighted the spatial proximity between JAML⁺ CD8⁺ CD108⁺ TRM cells and CXADR⁺ epithelial cells, both within malignant and healthy lung tissue, supporting their interaction in vivo ¹⁰⁸⁰. Importantly, in a B16F10 melanoma mouse model, significant reduction in tumor volume was achieved by intratumoral administration of recombinant CXADR in *Itgae*^{f/f} mice, but this was abolished in mice with CD8-specific conditional KO of *Itgae* (*Itgae* KO), thus suggesting that the interplay between CXADR and JAML enhances the anti-tumor properties of CD8⁺ TRM cells within the TME ¹⁰⁸⁰.

What's more, Eschweiler and colleagues have observed that TCR stimulation leads to JAML upregulation in human and murine $\alpha\beta$ (especially CD8⁺) T cells, and by analyzing Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq) data from human CD8⁺ and CD4⁺ T cells, as well as other immune cell types, they have proposed a mechanism through which TCR activation promotes JAML expression in a T cell-specific manner, potentially through stimulation of the nuclear factor of activated T cells (NFAT) TF, which subsequently triggers the *CD3D* gene promoter, and in so, through long-range cis-regulatory interactions with the JAML promoter, ends up upregulating the expression of the *JAML* gene as well ¹⁰⁷⁸.

1.2.1.4 Soluble JAML

Interestingly, JAML does not solely exist and act as a transmembrane protein, but in a soluble form (sJAML) as well ¹⁰⁸¹. Weber and colleagues initially observed that polymorphonuclear leucocytes (PMNs) transmigrating through either an epithelial monolayer

in vitro or the colonic epithelium in vivo, downregulated their JAML expression; an observation that was potentially associated with their adhesion to the epithelial cells, and/or their activation. Follow-up analyses proposed that the underlying mechanism comprises shedding of the extracellular domain (ECD) of JAML, most probably mediated through its cleavage by a zinc-dependent metalloprotease. Importantly, this soluble JAML ectodomain was capable of binding to an epithelial cell line in vitro, impeding restoration of barrier integrity and/or function ¹⁰⁸¹. Interestingly, sJAML interaction with the epithelial CXADR in vitro did not affect epithelial cell migration during wound closure, but rather inhibited epithelial cell proliferation. Similar outcomes were also observed in vitro by utilizing soluble adenovirus serotype 5 fiber knob protein (Ad5FK) (which binds to the CXADR D1 domain) instead of sJAML, and were then further validated in in vivo murine models of colonic mucosal injury ¹⁰⁸¹. Overall, it is proposed that JAML shedding from the surface of PMNs during (colonic) epithelial transmigration (following injury and/or infection) can result in disruption of CXADR interactions and/or signaling at the site of tight junctions, including suppression of epithelial cell proliferation, thus interfering with the wound healing process ¹⁰⁸¹.

1.2.1.5 Role of JAML in Wound healing and Skin inflammation

Through a different mechanism, the interaction between JAML and CXADR in the *skin* has also been implicated in wound healing ¹⁰⁵⁷. In murine models, upregulation of CXADR in adjacent to the wound KCs has been observed, while dendritic epidermal T cells (DETCs), a specialized $\gamma\delta$ T cell subset in murine skin, co-stimulated by CXADR in vitro, increase their production of keratinocyte growth factor 1 (KGF-1) ^{1057,1082–1084}.

Furthermore, JAML is involved in cutaneous inflammatory reactions, as demonstrated in the contact hypersensitivity (CHS) murine model, which is representative of allergic contact dermatitis in humans ¹⁰⁸⁵. *Jaml* KO mice displayed a significantly reduced inflammatory response following sensitization with the 2,4-dinitro-1-fluorobenzene (DNFB) contact allergen compared to WT mice – an observation that is probably attributable to the significant reduction in epidermal CD8⁺ and CD4⁺ T cells in *Jaml* KO mice compared to the WT ¹⁰⁸⁵. Furthermore, similar outcomes were observed for the elicitation phase of CHS, when *Jaml* KO and *Jaml* WT mice were re-challenged with DNFB days after initial sensitization ¹⁰⁸⁵. Adoptive transfer experiments further supported the significance of JAML during the inflammatory response after exposure to contact allergens; transfer of T cells from *Jaml* KO mice resulted in significantly reduced ear thickness, lower levels of CD8⁺ TRM cells, and downregulated expression of IFN- γ , TNF- α and IL-1 β , compared to mice that received WT T cells, following DNFB (re-)challenge ¹⁰⁸⁵. Moreover, significant upregulation of CXADR expression was observed in murine KC cell line cells following stimulation with several contact allergens, while significant increase in KC

CXADR expression was further observed in vivo in murine models following treatment with DNFB, both during the sensitization and the elicitation phase ¹⁰⁸⁵.

1.2.1.6 Role of JAML in Malignancies

Of particular interest to the present study is the role of JAML in the context of malignancies and anti-tumor treatment. Importantly, JAML expression has been identified both in immune cells and malignant cells within the TME of different cancers, with the former mainly associating with tumor-suppressing outcomes, whereas the latter with tumor-promoting ones (see below).

Several studies have highlighted the importance of JAML expression on CD8+ and $\gamma\delta$ T cell populations in mediating tumor-suppressing outcomes, with a recent focus on CD8+ TRM cells ^{1078,1080}. For instance, *Jaml* KO murine melanoma (B16F10/B16F10-OVA) models demonstrate significantly bigger tumor burden compared to WT mice, whereas treatment with stimulatory anti-JAML antibody significantly improves tumor control; however this anti-tumor effect is lost in *Trcd*-KO or CD8+ T cell-depleted mice, implying that both $\gamma\delta$ and CD8+ T cells are required for the tumor-suppressing properties of JAML ^{1078,1079,1086}. Moreover, the anti-tumor effect of anti-JAML stimulating antibodies appears to be dependent on malignant cell expression levels of CXADR, with only tumors of low CXADR expression seemingly benefitting from anti-JAML immunotherapy ^{1078,1079}. Importantly, combinational treatment of anti-JAML and anti-PD-1 monoclonal antibodies benefits both CXADR-high and CXADR-low tumors (to a significantly higher degree compared to either monotherapy alone) ^{1078,1079,1086}, potentially due to the proposed TCR-mediated positive regulation of JAML expression, which is restored following inhibition of PD-1 signaling ^{1078,1087}. Furthermore, in human patient cohorts of several cancer types, including melanoma, HNSCC, NSCLC, CRC and HCC, JAML expression has been identified preferentially on CD8+ TRM cells, and directly or indirectly associated with improved patient outcomes ^{1079,1080,1088,1089}, while also potentially serving as a positive predictive factor for response to immunotherapy in patients with HCC ¹⁰⁹⁰.

On the other hand, JAML can also be expressed on malignant (epithelial) cells, where it usually mediates tumor-promoting functions. For example, in several in vitro experiments in human GC, CRC and LUAD cell lines, which were further validated in murine models in vivo, JAML overexpression resulted in significantly increased proliferative, migratory and invasive cell capacity, whereas JAML silencing significantly suppressed these cancer cell properties ^{1091–1094}. The proposed mechanisms implicated here involve a positive regulatory effect of JAML on p38 (MAPK), PI3K/Akt/mTOR and/or Wnt/ β -catenin signaling pathways in tumor cells ^{1091–1094}. In accordance, JAML expression levels in tumor tissue have been positively correlated with worse clinicopathological characteristics and/or reduced OS in various human

malignancies, including GC, CRC and LUAD ^{1091–1093}. Intriguingly, it has also been observed, in both human and murine CRC samples, that JAML tumor tissue expression is inversely correlated with CD8+ T cell infiltration, presumably due to JAML's ability to regulate the secretion of several cytokines and chemokines by the tumor cells ¹⁰⁹².

A recent body of evidence suggests that JAML expression in human EC or LUAD cells exerts the opposite effects, in particular suppressing proliferation, migration and EMT of malignant cells, and modulating the immune microenvironment in a tumor-suppressing fashion, potentially through activation of the cyclic GMP-AMP synthase and stimulator of interferon genes (cGAS-STING) pathway ^{1088,1089}, which constitutes an important signaling cascade of the innate immune system, implicated in the recognition of foreign and/or damaged cytosolic DNA, and mediating the production of pro-inflammatory cytokines, such as TNF- α , IFN- γ , and IL-1 β , through NF- κ B activation ^{1095,1096}. In addition, it is also proposed that EC cell-expressed JAML can favor the shift of macrophage populations toward the pro-inflammatory M1 phenotype and away from the tumor-promoting M2 phenotype ¹⁰⁸⁹, whereas CRC cell-expressed JAML may enhance the radiosensitivity of malignant cells, via downregulation of the Ataxia telangiectasia and Rad3-related (ATR) and checkpoint kinase 1 (CHEK1) pathway ¹⁰⁹⁷; however, further research is certainly required in this area.

Lastly, it has recently been proposed that JAML expressed on endothelial cells can support tumor growth, by favoring tumor angiogenesis, through activation of the Focal Adhesion Kinase (FAK)/Src/ERK/Akt and the VEGFA/VEGF Receptor 2 (VEGFR2; also known as Kinase insert domain receptor; KDR) pathways ¹⁰⁹⁸. Indeed, endothelial cell-specific *Jaml* KO results in significantly reduced CRC tumor growth and angiogenesis, as well as in normalization of tumor vessels in vivo ¹⁰⁹⁸, thus highlighting a potential novel role of JAML as an anti-angiogenic target, aside from immunotherapy target. Interestingly, endothelial cell-specific silencing of JAML also resulted in a significantly increased accumulation of both CD8+ and CD4+ T cell populations within tumor tissue ¹⁰⁹⁸, mirroring the findings of Fang and colleagues regarding tumor cell-derived JAML influencing T cell chemoattraction ¹⁰⁹².

Altogether, the role of JAML within the tumor context appears to be cell-dependent as well as context-dependent, with both tumor-suppressing and tumor-promoting functions having been demonstrated in the literature in in vitro, in vivo, and human cohort examinations, and several signaling pathways having been identified as downstream targets of its activation. A summary of the most important tumor-suppressing and tumor-promoting functions that have been described for JAML is provided in **Table 4**. The discrepancies observed for the role of JAML in anti-tumor immunity for certain malignancies, such as CRC and LUAD, could also be partly attributable to the different methods (in silico, in vitro, in vivo) that were utilized and/or to

the different disease models that were generated in each study. To the best of my knowledge, no evidence exists for the role of JAML in cutaneous non-melanocytic malignancies (including BCC), and further research is required in order to elucidate the significance of this molecule, through its interaction with its CXADR counter-receptor, to the development and progression of human cancers.

Observation/Mechanism	Tumor type	Study type	Ref.
<i>Suspected tumor-suppressive effects of JAML</i>			
Significantly lower JAML expression in malignant compared to non-malignant tissue and/or significant inverse correlation between JAML expression and worse clinicopathological characteristics	Lung (inc. LUAD and LUSC), EC, breast, CRC, HNSCC, melanoma, bladder, RCC (chromophobe)	Clinical cohort, in silico, in vitro	1079,1086, 1088,1089
JAML expression is significantly increased in CD8+ TRM cells, which correlate with significantly better survival, compared to CD8+ non-TRM cells	HNSCC, NSCLC	Clinical cohort, ex vivo	1078,1080
JAML expression significantly correlates with CD69 and Ki-67 expression in $\gamma\delta$ T cells	Melanoma (B16F10-C57BL/6J mice)	In vivo, ex vivo	1079
JAML overexpression significantly enhances, while JAML knockdown significantly suppresses the proliferation and cytotoxicity of CD8+ T cells	Irrespective of type	In vitro	1086
JAML expression characterizes a less exhaustive CD8+ T cell state	Melanoma (B16F10-C57BL/6J mice), HCC (Hepa1-6-C57BL/6J mice)	In vivo, ex vivo, in vitro, in silico	1079,1090, 1099
<i>JAML</i> overexpression in tumor cells significantly inhibits, while <i>JAML</i> silencing significantly enhances, their proliferation and/or invasion and migration	EC (Ishikawa, HEC-1-A), LUAD (A549, H1437)	In vitro	1088,1089
<i>JAML</i> overexpression, and <i>JAML</i> knockdown in tumor cells promote an M1-like and an M2-like macrophage phenotype, respectively – potentially via cGAS-STING pathway-dependent cytokine secretion modulation	EC (Ishikawa)	In vitro	1088,1089
<i>Jaml</i> KO significantly enhances tumor growth <i>in vivo</i>	Melanoma (B16F10-C57BL/6J mice)	In vivo	1079,1080

<i>JAML</i> overexpression in tumor cells may enhance their radiosensitivity – potentially via ATR/CHEK1 pathway inhibition	CRC (LOVO, DLD-1, LOVO- or DLD-1-Balb/c-nu mice)	In vitro, in vivo, ex vivo	1097
Suspected tumor-promoting effects of JAML			
Significantly higher JAML expression in malignant compared to non-malignant tissue and/or significant positive correlation between JAML expression and worse clinicopathological characteristics	LUAD, CRC, GC, HCC, RCC (clear cell, papillary)	Clinical cohort, ex vivo	1089,1091–1093
<i>JAML</i> knockdown in tumor cells significantly reduces, while <i>JAML</i> overexpression significantly increases cell proliferation, migration and/or invasion – potentially in a p38- or PI3K/Akt/mTOR- dependent manner	CRC (HCT116, LOVO, DLD-1, SW480), GC (AGS, HGC-27, MKN-28), LUAD (A549, H1299, PC9, H1975)	In vitro	1091,1092
<i>JAML</i> overexpression in tumor cells promotes EMT characteristics – potentially via a Wnt/ β -catenin- and/or PI3K/Akt/mTOR- dependent mechanism	LUAD (A549, H1299, PC9, H1975)	In vitro	1093,1094
<i>JAML</i> silencing in tumor cells significantly reduces, while <i>JAML</i> overexpression significantly enhances, their tumorigenic capacity <i>in vivo</i> – potentially in a PI3K/Akt/mTOR- and/or Wnt/ β -catenin- dependent manner	CRC (LOVO-BALB/c nude mice, MC38-C57BL/6J mice), LUAD (A549- or PC9-BALB/c mice)	In vivo	1092,1093
<i>Jaml</i> KO in endothelial cells significantly reduces tumor growth and angiogenesis <i>in vivo</i> – potentially via suppression of the FAK/Src/ERK/Akt and/or VEGFA/VEGFR2 pathways	CRC (MC38-C57BL/6J mice)	In vivo, in vitro	1098

Table 4: Tumor-promoting and tumor-suppressing functions of JAML, including proposed underlying mechanisms. LUAD = Lung Adenocarcinoma, LUSC = Lung Squamous Carcinoma, EC = Endometrial Cancer, CRC = Colorectal Carcinoma, HNSCC = Head and Neck Squamous Cell Carcinoma, RCC = Renal Cell Carcinoma, NSCLC = Non-Small Cell Lung Carcinoma, PD-1 = Programmed cell Death 1, Eomes = Eomesodermin, TIM-3 = T-cell Immunoglobulin and Mucin-domain containing-3, HCC = Hepatocellular Carcinoma, cGAS-STING = cyclic GMP-AMP Synthase and Stimulator of Interferon Genes, ATR = Ataxia Telangiectasia and Rad3-related, CHEK1 = Checkpoint Kinase 1, GC = Gastric Cancer, PI3K = Phosphatidylinositol 3-Kinase, mTOR = mechanistic Target Of Rapamycin kinase, FAK = Focal Adhesion Kinase, ERK = Extracellular signal-Regulated Kinase, VEGFA = Vascular Endothelial Growth Factor A, VEGFR2 = Vascular Endothelial Growth Factor Receptor 2

1.2.2 CXADR

1.2.2.1 Structure and Interacting partners of CXADR

CXADR was identified in 1997 as a receptor of high affinity for group B (B1-B6) Coxsackie viruses and serotype 2 and 5 Adenoviruses ^{1052,1100,1101}, later expanded to various A, C, D, E and F subgroup Adenoviruses ¹¹⁰², while in 2005 it was additionally recognized as the epithelial cell-expressed receptor of PMN-derived JAML, responsible for facilitating PMN migration across tight junctions ¹¹⁰³; overall highlighting its role as both a viral entry receptor and a JAM participating in intercellular contacts ¹¹⁰⁴. The murine *Cxadr* gene is located on chromosome 16 ¹¹⁰⁵, whereas the human one (*CXADR*) on chromosome 21 (21q21.1 locus) (GeneCards) ¹¹⁰⁶. It consists of eight exons in humans, and eight or nine exons in mice ¹¹⁰⁷, and undergoes alternative splicing, ultimately being able to give rise to at least three membrane isoforms (mCAR1, mCAR2, mCAR3) in mice, and at least three membrane (hCAR1, hCAR2, hCAR6), as well as three soluble (hCAR4/7, hCAR3/7, hCAR2/7) isoforms in humans ^{1105,1108–1113}.

CXADR is most commonly encountered in its transmembrane form, which resides inside lipid rafts of the plasma membrane ^{1114–1116}, and consists of two extracellular Ig domains – a membrane-distal one (D1), which is of V-type, and a membrane-proximal one (D2), which is of C2 (constant 2)-type ^{1051,1117} – a single-pass transmembrane region, and a cytoplasmic tail ¹⁰⁵², which, similarly to most JAMs, but unlike JAML, possesses a PDZ-binding motif ^{1037,1052,1118,1119}. The membrane CXADR isoforms differ only in their C-terminus; the canonical hCAR1 protein, also denoted as CAR^{Ex7} since it results from transcription of the seven first exons of the *CXADR* gene, consists of 365 amino acids and possesses a class I PDZ-binding motif (X-S/T-X-Φ*, specifically GSIV); isoform 6 (hCAR6), also referred to as CAR^{Ex8} since it results from alternative splicing within exon 7 and contains exon 8 of the *CXADR* gene, consists of 352 amino acids, differing in total in the last 26 residues compared to hCAR1, but similarly contains a class I PDZ-binding motif (ITVV); whereas, hCAR2 lacks the last 20 amino acids of hCAR1, and contains five altered residues in its carboxyterminal end (differing in total in the last 26 residues compared to hCAR1), thus ending in a class II PDZ-binding motif (X-Φ-X-Φ*, specifically KYPY) (where X corresponds to any amino acid, Φ to a hydrophobic amino acid: V, I, L, F, W, Y, or M, and * to COOH terminus) ^{1052,1118–1121}. These PDZ-binding sequences enable the interaction of CXADR with PDZ domain-containing proteins, including, but not limited to, zonula occludens (ZO)-1, membrane-associated guanylate kinase 1 (MAGI-1), multi-PDZ domain protein-1 (MUPP-1), and ligand of Numb-protein X 2 (LNK2), contributing to the essential structural and functional role that CXADR exerts at tight and/or adherens junctions ^{1054,1118,1122–1124}. Aside from its indirect binding to F-actin through these scaffolding

proteins, CXADR has also been shown to directly interact with both actin ¹¹²⁵ and microtubules ¹¹²⁶, as well as act as a negative regulator of Rho-associated protein kinases (ROCKs) ¹¹²⁷, further highlighting its involvement in cytoskeletal dynamics and signaling regulation.

In this direction, of great significance are also the homophilic interactions of CXADR in trans ¹¹²². CXADR can form homophilic interactions both in trans and in cis, primarily mediated by the D1 ECDs; however, it has been proposed that the in trans homophilic interactions may be further reinforced by reciprocal D1-D2 domain binding, which is favored by conformational changes resulting from the initial engagement of D1 domains of opposing molecules ^{1117,1122,1128,1129}. Furthermore, CXADR is involved in several in trans heterophilic interactions with membrane proteins, including JAML ^{1051,1057,1103} and JAM-C ¹¹³⁰, but also extracellular matrix (ECM) proteins, such as fibronectin, laminin-1, agrin and tenascin-R ¹¹²⁹. As already described, CXADR-JAML binding is mediated by their membrane-distal (D1) domains, with JAML D2 domain amplifying the affinity of this interaction ^{1051,1057}, while dimeric forms of CXADR are usually required for complete JAML activation ^{1051,1055}. The binding site for JAML on CXADR overlaps with the one for adenoviruses ¹⁰⁵¹, and it has been demonstrated that the Ad5FK interferes with the JAML-CXADR interaction ^{1051,1057}.

1.2.2.2 Post-translational modifications (PTMs) of CXADR

The CXADR molecule can undergo several post-translational modifications (PTMs). First of all, each of the ECDs (D1, D2) contains a glycosylation site (Asn 106 and Asn 201, respectively); although their modification does not significantly affect CXADR trafficking to the cell membrane, nor its localization at tight junctions, they have been shown to influence homophilic interactions of CXADR, and in so cell adhesion, as at least one of them needs to be glycosylated for cells to form clusters in vitro, whereas the glycosylation of the D2 domain affects the binding and infection potential of adenoviruses ^{1129,1131}. Regarding its intracellular domain (ICD), CXADR possesses two palmitoylation sites (Cys 259, Cys 260), which, when modified, are responsible for its proper and stable localization to the plasma membrane, without affecting adenoviral infection dynamics ¹¹³². In addition, phosphorylation can take place at specific ICD residues, including Thr 290 and Ser 293 ^{1054,1133}; ICD phosphorylation of CXADR is mediated by protein kinase C delta (PKCδ) and/or PI3K ^{1133,1134}, and has been implicated in destabilization of CXADR at the epithelial cell membrane ^{1135,1136}, regulation of endocytosis-dependent E-cadherin trafficking at intercellular junctions ¹¹³³, and thus also in transepithelial migration (TEpM) of leucocytes under inflammatory conditions ¹¹³⁴. Lastly, a number of CXADR Lys residues can be ubiquitinated, in so facilitating its proteasomal degradation ¹¹³⁷.

1.2.2.3 Signaling pathways activated downstream of CXADR

Several signaling cascades have been reported to act downstream of CXADR, highlighting its importance as a signaling molecule. First of all, the levels of phosphorylated p44/42 (MAPK) protein have been found significantly upregulated in CXADR-overexpressing cells in vitro, while it has also been proposed that this CXADR-mediated stimulation of p44/42 may contribute to β 1 and β 3 integrin activation, thus enhancing cellular adhesion to ECM, among other effects ¹¹²⁸. Moreover, following stimulation of A549 (human LUAD cell line) cells with the Ad5FK or complete fiber protein, activation of ERK1/2 ¹¹³⁸ and JNK kinases of the MAPK pathway, as well as NF- κ B nuclear translocation, have been observed ¹¹³⁹. Moreover, β -catenin has been recognized as one of the intracellular partners of CXADR ¹¹⁴⁰, and its activity appears to be regulated by the receptor. Sertoli cell-specific deletion of *Cxadr* in murine models results in a significant increase in the levels of non-phosphorylated β -catenin (favoring its activated state), as well as in a significant decrease in the ratio of cell division cycle 42 (Cdc42)-GTP (active) to total Cdc42, implying that CXADR expression in this cellular context normally suppresses β -catenin signaling and promotes Cdc42 activation ¹¹⁴¹, which can further regulate both the cytoskeleton organization and gene expression ^{1142,1143}. Contrarily, though, when *Cxadr* was overexpressed in cardiomyocytes in vivo, it associated with nuclear translocation of β -catenin, and upregulated expression of β -catenin target genes, including *c-Myc* ¹¹⁴⁴ – overall, this could imply a cell type-specific role of CXADR in β -catenin signaling regulation. In addition, the CXADR-overexpressing murine cardiomyocytes displayed significantly higher levels of p-Akt ¹¹⁴⁴, but, intriguingly, the same outcome was observed in murine breast cancer cell line cells when *Cxadr* was knocked down in vitro ¹¹⁴⁵. Nevertheless, these results suggest that CXADR is also involved in Akt signaling regulation, probably through its effects on the PTEN and PHLPP2 phosphatases ¹¹⁴⁵, and it is suggested that through its influence on Akt-induced GSK3 β phosphorylation CXADR ultimately regulates β -catenin activity ^{1135,1144}. Furthermore, as demonstrated in human bronchial epithelial cells (16HBE), CXADR depletion also results in a significant upregulation of the p-SMAD2/3 TFs, potentially via GSK3 β activity modulation as well ¹¹³⁵.

1.2.2.4 Membrane dynamics of CXADR

Membrane dynamics of CXADR are quite complex and fall outside the scope of this study. However, it is worth mentioning that, under certain stimuli, including viral infection ¹¹¹⁶ and ubiquitination ¹¹³⁷, CXADR can be endocytosed from lipid rafts ¹¹¹⁶, in a seemingly clathrin-independent manner ^{1116,1146}, despite the established interaction of its ICD with the clathrin adaptor proteins AP-1A and AP-1B, which regulates its basolateral or apical membrane sorting (see below) ^{1147,1148}. Both dynamin and actin are implicated in the CXADR internalization

process ¹¹¹⁶. Moreover, it has been demonstrated that CXADR can follow the endolysosomal pathway, contributing to its membrane fate determination ¹¹¹⁶.

Importantly, in polarized epithelial cells, including human airway epithelial ones, CXADR is mainly confined to the basolateral membrane rather than the apical ¹¹⁴⁹. Interestingly, though, it has been proposed that different membrane isoforms display distinct membrane localization patterns, with CAR^{Ex7} (hCAR1) preferentially localizing at the basolateral membrane of polarized epithelial cells, while CAR^{Ex8} (isoform 6, hCAR6) is preferentially located at the apical membrane ^{1150,1151}. Since the membrane isoforms differ only in their cytoplasmic tail, this finding by Excoffon and colleagues essentially corroborates previous observations that it is various regions within the ICD of CXADR that are responsible for its specific basolateral membrane sorting ^{1149,1152,1153}. This process is mediated by the interaction of a specific tyrosine-based YxxΦ motif of the CXADR ICD with AP-1A and AP-1B proteins ^{1147,1148}.

1.2.2.5 Soluble CXADR isoforms

In contrast to the membrane ones, all three soluble isoforms of CXADR lack the exon encoding for the transmembrane region of the protein, and contain segments of the ECD, along with an altered ICD, compared to the canonical membrane molecule; nevertheless, they maintain their ability to interact with the ECD of the full-length CXADR ¹¹⁵⁴. Furthermore, hCAR4/7 has been shown to bind to Coxsackievirus B3 (CVB3), and, interestingly, all three soluble isoforms (hCAR4/7, hCAR3/7, hCAR2/7) demonstrate an inhibitory effect on CVB3 infection of target cells in vitro ¹¹⁵⁴. Importantly, their expression has also been positively associated with worse clinical outcomes in the setting of ovarian carcinoma, potentially due to their interference with the membrane CXADR-mediated intercellular junctions ¹¹⁵⁵. Moreover, it has been suggested that these soluble CXADR molecules may function as an impeding mechanism for adenoviral target gene therapy efficacy ¹¹⁵⁶.

1.2.2.6 Regulated intramembrane proteolysis (RIP) of CXADR

Interestingly, CXADR has also been demonstrated to undergo regulated intramembrane proteolysis (RIP) ^{1157–1159}, a process of intramembrane cleavage of the ICD of a specific transmembrane protein, following its ectodomain shedding ¹¹⁶⁰. Specifically, it has been observed that CXADR exhibits both constitutive and inducible shedding of its ECD, mediated by A Disintegrin And Metalloproteinase domain 10 (ADAM10) ¹¹⁵⁹ or other proteinases, including beta-site amyloid precursor protein cleaving enzyme 1 (BACE1), also known as β-secretase 1 ¹¹⁶¹, and neutrophil elastase (NE) ¹¹⁵⁸. For the CAR^{Ex8} isoform in particular, ADAM17 has been implicated in its ECD cleavage ¹¹⁵⁷. Succeeding ECD shedding, CXADR can be cleaved intracellularly by the γ-secretase complex, in so releasing a

cytoplasmic fragment, which can subsequently translocate to the nucleus¹¹⁵⁹. Overall, CXADR ECD shedding can act as a mechanism of adenoviral infection inhibition¹¹⁵⁷ and intercellular junction regulation, while RIP could further potentially implicate nuclear-translocated CXADR in gene expression modulation.

1.2.2.7 Cellular and developmental expression pattern of CXADR & its role in non-malignant conditions

The expression of CXADR is tissue- and developmental stage- dependent. CXADR can be detected in several organs, including, but not limited to, skin (epithelial cells, lymphatic endothelial cells)^{1162–1164}, lungs (airway and pulmonary epithelial cells)^{1109,1162,1163}, heart (cardiomyocytes)^{1109,1162,1163}, brain (including cortical neurons, the pituitary, and olfactory system)^{1109,1129,1162,1163,1165–1170}, liver (hepatocytes & bile duct cells)^{1109,1162,1163}, pancreas (islet, acinar and duct cells)^{1109,1162,1163}, stomach¹¹⁶³, esophagus^{1162,1163}, colon^{1086,1163}, kidneys (glomerular epithelium, proximal and distal convoluted tubule cells)^{1162,1163}, and testes (Sertoli and germ cells)¹¹⁶³, where its expression is finely tuned according to the developmental phase. Overall, CXADR expression is abundant during embryonic development and early postnatal periods, but is gradually downregulated and/or restricted to specific cellular areas and/or cell types in adult subjects^{1129,1167,1168,1171}, including, of interest to this study, the tight and adherens junctions between epithelial cells.

CXADR has been established as an essential mediator of normal heart development, as its deletion (either non-specific or cardiac-specific) in mice results in embryonic lethality between days E11.5 and E13.5 due to cardiac malformation, which is, among others, attributable to the observed misorganization of myofibrils, dysregulated intercellular junctions (at the intercalated discs) and altered signal transduction, as well as hyperplasia of cardiomyocytes of *Cxadr* KO mice^{1172–1175}. Conditional *Cxadr* KO in adult mice has been shown to cause complete atrioventricular block (as far as the cardiovascular system is concerned)¹¹⁷⁶, but it additionally results in intestinal dilation (however without affecting intestinal epithelial barrier integrity), exocrine pancreas atrophy, as well as dysregulated thymopoiesis, with increased total number of thymocytes compared to control mice¹¹⁷⁷; thus, highlighting the importance of CXADR in the development of several other organs in vivo. Accordingly, CXADR exerts an important regulatory role in the developing brain^{1165,1166,1168,1170}; central nervous system (CNS)-specific deletion of *Cxadr* has been demonstrated to impact adult neurogenesis and synaptic signaling, especially in the hippocampal region, in so affecting mouse behavior¹¹⁷⁰.

In accordance with its central role in the homeostasis of several tissues and organs, CXADR has been implicated in a plethora of pathological conditions, both malignant and non-

malignant ones. Regarding the latter, aside from all the aforementioned effects of CXADR deletion or overexpression in adult mouse models ^{1170,1176,1177}, CXADR has been implicated in a wide range of cardiological diseases, including several types of cardiomyopathy and myocarditis ^{1171,1175,1178–1186}, while it also exhibits an emerging role in the pathogenesis of neurodegenerative diseases, such as Alzheimer's, with human hippocampal tissues of patients with early-stage disease displaying significantly reduced CXADR expression compared to controls, and similar results being observed in murine models as well ^{1169,1170}. These conditions, however, remain outside the scope of this study.

1.2.2.8 CXADR and Hypoxia

In regard to tumorigenesis, given that hypoxia constitutes a central aspect of the TME ¹¹⁸⁷, a small number of in vitro experiments have examined the effects of hypoxia on CXADR expression, providing, however, inconclusive results. On one hand, downregulation in the expression of the CXADR gene has been observed under hypoxic conditions in human GC (AGS), CRC (SW480), and prostate (PC3) cancer cell lines, which has been, at least partly, attributed to the negative regulatory effect of the hypoxia-inducible factor-1 α (HIF-1 α) on the CXADR gene promoter ¹¹⁸⁸. Nonetheless, an earlier study had supported that in two different human CRC cell lines (HT-29, DLD-1) no significant change in CXADR expression was induced by hypoxia ¹¹⁸⁹.

1.2.2.9 Role of CXADR in Malignancies

Despite the wide collection of existing studies, the function of CXADR within the tumor context still remains elusive, and appears to be dependent on the tumor type and/or stage and the cellular and subcellular localization of CXADR. Additionally, the contradictory nature of some of the results reported in distinct studies may be further influenced by the organism studied (human or mouse) in each case, the use of primary cells or cell lines, as well as the accurate simulation of human cell in vivo conditions.

1.2.2.9.1 CXADR expression pattern in human malignancies

First of all, studies that have evaluated the protein and/or mRNA expression levels of CXADR in human tumor specimens compared to non-malignant tissue samples provide an indicative interpretation for the role of CXADR in the tumorigenesis of distinct tumor types ^{1080,1086,1163,1190–1193}. Among the most well-studied cancer types in regard to CXADR expression are lung carcinomas, especially NSCLC, in the setting of which there is congruence that CXADR probably acts as a tumor promoting factor, as malignant samples express significantly higher levels of the protein compared to non-malignant tissue samples ^{1080,1163,1190–1192}. A similar, pro-tumorigenic role of CXADR, based on its expression level in clinical samples, has been hypothesized for brain tumors, including gliomas ^{1163,1191,1192}, for PDAC ¹¹⁹⁴, for breast

cancer ¹¹⁹⁵ as well as for several gynecological malignancies, including ovarian carcinomas ^{1155,1163}. On the contrary, among others, in HNSCC ¹¹⁹⁶, as well as in gastrointestinal malignancies such as CRC ^{1086,1163,1193} and GC ¹¹⁹⁷, CXADR expression is downregulated compared to healthy tissue, suggesting that CXADR may function as a tumor-suppressor in this context. Regarding bladder and prostate cancer, observations from existing studies are divergent ^{1163,1191,1192,1198,1199}.

Furthermore, several associations between CXADR expression levels and clinicopathological parameters of the various malignancies have been observed. For instance, in accordance with the proposed pro-tumorigenic role of CXADR in lung cancer, its expression levels in LUSC have been observed to positively correlate with higher histological grading ¹¹⁹⁰. Similarly, in HNSCC ¹²⁰⁰, breast cancer ¹¹⁹⁵ and PDAC ¹¹⁹⁴ it has been proposed that higher CXADR expression associates with worse pathological characteristics and/or poorer prognosis. Interestingly, in ovarian carcinomas, such a correlation was identified for the *soluble*, rather than the membrane isoforms of CXADR ¹¹⁵⁵. On the contrary, in cohorts of patients with CRC ^{1086,1163} or GC ¹¹⁹⁷, where CXADR has been suspected to exert a tumor suppressor function, progressive loss of CXADR has been associated with disease progression, more advanced staging and/or worse survival. Moreover, an analogous trend has been reported in three independent studies examining bladder cancer, where CXADR downregulation was significantly correlated with more invasive disease stages and/or decreased tumor differentiation (higher grading) ^{1198,1199,1201}; this, in fact, could support the otherwise debatable notion that CXADR functions as a tumor-suppressing factor in bladder cancer after all. Nonetheless, in a study by Reeh and colleagues ¹¹⁶³ many of the aforementioned associations have been questioned as non-statistically significant.

To date, only limited evidence exists for the role of CXADR in BCC, or non-melanocytic malignancies in general. Reeh and colleagues have observed that IHC expression of CXADR is significantly higher in BCC biopsies compared to non-malignant skin samples ¹¹⁶³, implying a potential tumor-promoting function of CXADR in BCC, with no significant correlation identified between CXADR levels and disease staging or grading ¹¹⁶³.

It is important to note, however, that it is not just the expression level of CXADR, but also its subcellular localization and/or cellular distribution that influences its observed tumor-associated functions. Overall, CXADR is predominantly identified in the epithelial populations of both malignant and non-malignant tissues ^{1190,1193,1197}, but with a general tendency to shift or expand cytoplasmically, on top of its plasma membrane localization, in tumor cells ^{1193,1195,1197}, or in specific tumor subtype cells ¹²⁰².

1.2.2.9.2 Role of CXADR in tumor cell growth, proliferation and apoptosis regulation

Aside from the clinical cohort studies, several in vitro and vivo analyses have attempted to untangle and further characterize the role of CXADR in tumor development and progression. First of all, CXADR has been implicated in the regulation of cancer cell growth and proliferation, as well as cancer cell viability through modulation of apoptosis. Consistent with the patient sample observations, in vitro silencing of *CXADR* in human NSCLC cell lines (NCI-H1703, NCI-H157, A549, H1975) significantly downregulates their proliferation, as well as their colony formation ability in soft agar and/or culture plates, and importantly, significantly inhibits their tumorigenic potential in vivo, when transplanted into nude mice ^{1203–1205}, with tumors resulting from the *CXADR*-KO cells being characterized by significantly reduced size and weight, as well as significantly lower Ki-67 IHC expression, compared to those originating from control cells ¹²⁰³. Similar in vitro observations have also been made for human SCC (HSC-2, HSC-3, Ca9-22) and PDAC (PK-8, PANC-1) cell lines ¹¹²⁷. Moreover, in murine breast cancer in vivo, and in human cervical squamous carcinoma (CaSki), cervical adenocarcinoma (HeLa), and ovarian adenocarcinoma (A2780) cell lines in vitro, CXADR has been proposed to promote an anti-apoptotic phenotype of tumor cells, with its expression correlating with an increase of Bcl-2 and/or Bcl-xL anti-apoptotic protein levels ¹²⁰⁶. On the contrary, *CXADR* downregulation in human GC (AGS, MKN28, MKN45) ¹¹⁹⁷ and CRC (DLD1, HCT116, SW480, SW620, HT29) ¹¹⁹³ cell lines results in significantly enhanced proliferation; whereas, *CXADR* overexpression inhibits their proliferative and/or enhances their apoptotic capacity in vitro ^{1086,1193}, reflected additionally in significantly increased levels of caspase-3 and cleaved caspase-3 and significantly reduced levels of Bcl-2 ¹⁰⁸⁶.

From a mechanistic point of view, CXADR has been shown to participate in the regulation of postmitotic daughter cell separation, favoring more stable connections and faster daughter cell spreading ¹²⁰³; whereas it has also been proposed that an epidermal growth factor (EGF)/EGFR signaling-dependent mechanism can promote the PKC δ -mediated phosphorylation of the CXADR cytoplasmic tail, and the subsequent formation of a CXADR-kinesin family member 22 (KIF22) protein complex, which facilitates the re-localization of CXADR within intercellular junctions, and ultimately promotes the cancer cell proliferation rate ¹²⁰³. Moreover, it has been shown that CXADR, through its ICD, can interact with and inactivate the Rho-associated ROCK-I and ROCK-II kinases, and in so suppress the RhoA-ROCK-mediated apoptotic pathway ¹¹²⁷. The importance of the CXADR ICD phosphorylation has been further validated in murine lung cancer cells, where, although both WT CXADR (WTCAR)- and unphosphorylatable Thr290Ala and Ser293Ala CXADR mutant (AACAR)- overexpressing tumor cells could significantly enhance tumorigenicity in vivo compared to control cells, the tumors originating from the AACAR-tumor cells were significantly smaller than those resulting

from the WTCAR-tumor cells ¹²⁰⁷. Furthermore, studies in human bladder cancer (T24, RT4, 253J) ¹²⁰¹ and glioma (U-118 MG) ^{1115,1208} cell lines have suggested that the ECD (specifically the D2, but not the D1 domain), as well as the transmembrane region of CXADR are also necessary, in addition to the ICD, for the tumor-suppressive effects that CXADR appears to exert in these tumor types in vitro and in mouse models in vivo ^{1115,1201,1208} – in contrast to what has been postulated for gliomas based on the clinical sample examinations ^{1163,1191,1192}. However, partly in disagreement with this proposed role of the D2 rather than the D1 CXADR domain, it has been suggested that the perturbed proliferation and viability observed in CXADR-silenced tumor cells does not depend on the loss of cell-ECM contacts (mainly mediated by the D2 domain), but rather from loss of the intercellular contacts (mainly mediated by the D1 domain) ¹¹²⁷. Nonetheless, it is generally accepted that, CXADR participates in cellular interactions with ECM proteins, such as collagen I and Matrigel in vitro, a phenomenon which is supported by the reversal of the homophilic in trans CXADR interactions, and additionally regulates the formation of focal adhesions, by binding, via its phosphorylated ICD, to activated $\beta 1$ integrins, as well as Src, FAK and paxillin, most probably in a Ras-associated protein 1 (Rap1) signaling pathway-mediated manner ¹²⁰⁷. However, in the setting of murine melanoma, an inverse correlation between the expression of CXADR and that of $\alpha 4$, αv , $\beta 1$ and $\beta 3$ integrins has been reported ¹²⁰⁹. Interestingly, Owczarek and colleagues have also observed that CXADR exhibits a mechanoresponsive behavior, with p-CXADR levels of murine lung cancer cells significantly increasing in substrates of elevated stiffness in vitro, compared to when cultured in lower stiffness conditions; this observation is further supported by the preferential distribution of CXADR, including p-CXADR, at the periphery of murine lung tumor tissue, at the junction between malignant mass and adjacent non-malignant ECM ¹²⁰⁷.

1.2.2.9.3 Role of CXADR in tumor cell migration and invasion regulation

In accordance with the proposed involvement of CXADR in cell-ECM interactions, it has also been shown that CXADR can regulate the migratory and invasive phenotype of malignant cells. *CXADR* silencing in human NSCLC cell lines significantly impairs their adhesive and invasive potential in vitro, while *CXADR* overexpression significantly enhances both of these characteristics ¹¹⁹⁰. Contrarily, in human GC cell line cells, *CXADR* knockdown promotes their migration, invasion and proliferation, whereas *CXADR* overexpression downregulates all three properties in vitro ¹¹⁹⁷; similarly, in human CRC cell lines *CXADR* inhibition enhances their invasive potential in vitro as well as their metastatic potential in vivo ¹¹⁹³. Intriguingly, it has been proposed that CXADR may play a prominent role in the *initial* stages of malignant cell engraftment, as only the co-administration with tumor cells, and not the subsequent administration, of a CXADR D1-blocking monoclonal antibody could achieve a reduction in tumor volume in transplant mouse models of NSCLC ^{1204,1205}. Interestingly,

however, in murine melanoma (B16) and CRC (CT26) cells, *transient* CXADR upregulation results in a significant *reduction* of their metastatic potential in vivo, and/or a significantly reduced accumulation in mouse lung parenchyma within the first 48 hours after i.v. administration of tumor cells ¹²⁰⁹, thus corroborating, on one hand, the participation of CXADR in the early phase of the metastatic process, but contradicting, on the other hand, CXADR's proposed role and ultimate outcome observed in murine lung cancer ^{1204,1205}. Furthermore, the overexpression of *CXADR* significantly inhibits the invasiveness of glioma cell line (U87-MG) spheroids in vitro ¹¹¹⁵, consistent with the previous non-clinical observations for the role of CXADR in human brain tumors ^{1115,1208} – an effect which has been shown to be dependent on the ICD of CXADR ¹¹¹⁵. Mechanistically, it has been proposed that, among others, the cytoplasmic tail of CXADR is responsible for interaction with the microtubules, and this could serve as a tumor cell migration-promoting mechanism, including in gliomas ¹¹²⁶.

1.2.2.9.4 Role of CXADR in EMT and CSC state regulation

Furthermore, CXADR also appears to be involved in the regulation of EMT and a potential CSC phenotype in various malignancies. On one hand, it has been observed that human NSCLC cells with epithelioid morphology (NCI-H292, Calu-3) are characterized by low CXADR expression, whereas NSCLC cells with a mesenchymal-like phenotype (NCI-H1703, NCI-H157) display high CXADR levels; furthermore, a positive association between the expression of CXADR and vimentin, Snail, Zeb and Twist, whereas a negative association between CXADR and E-cadherin, occludin, α -catenin and γ -catenin levels, has been observed ¹²⁰⁵, implying that CXADR expression drives a more mesenchymal-like tumor cellular profile. In addition, CXADR has been shown to characterize a CSC-like state of chemotherapy- or radiotherapy- resistant human NSCLC cells ¹²¹⁰. On the other hand, in murine breast carcinomas, CXADR has been suggested to function as a critical regulator of a tight junction-associated signalosome, which consists of CXADR, MAGI-1, PTEN and PHLPP2, and is thus capable, via a positive effect on the two phosphatases, of inhibiting the Akt pathway activity, and ultimately, the Akt-mediated EMT process in vivo ¹¹⁴⁵. This proposed mechanism has been further validated in human breast (MCF-7, T47D) and human NSCLC (A549) cells in vitro, where CXADR overexpression was shown to increase the levels of MAGI-1, PHLPP2 and PTEN, inhibit the activity of Akt, promote a more epithelial-like cellular phenotype, and even partially reverse the effects of TGF- β 1-induced EMT ¹¹⁴⁵. Nonetheless, in another recent study, it has been suggested that CXADR, irrespective of its ICD phosphorylation state, does not participate in the EMT process, as neither protein levels nor localization of E-cadherin, β -catenin, and vimentin were significantly affected by either overexpression or knockdown of CXADR in murine lung cancer cells ¹²⁰⁷.

1.2.2.9.5 Role of CXADR in tumor immune microenvironment regulation

In addition to the aforementioned potential mechanisms through which CXADR expression can either promote or inhibit cancer growth and metastasis by directly affecting the dynamics and kinetics of tumor cells themselves, it has recently become clear that CXADR can also participate in the regulation of the immune TME. Intriguingly, Niu and colleagues have implicated CXADR in an epigenetically regulated mechanism of TME remodeling towards an immunosuppressive phenotype in human pancreatic cancer – specifically, they have demonstrated that loss of the SET domain containing 2 (SETD2) histone lysine methyltransferase in pancreatic cancer cells results in suppression of CXADR gene expression, which in turn mediates the activation of the PI3K/Akt pathway, and through it, the enhanced expression of CXCL1 and GM-CSF, ultimately facilitating chemoattraction, but also reprogramming of neutrophils towards a tumor-promoting phenotype ¹²¹¹. Aside from this proposed indirect effect on the immune TME, it is also reasonable to hypothesize that either tumor cell- or endothelial cell- expressed CXADR can directly modulate the TME immune responses via its interaction with immune cell-expressed JAML – and gradual loss of CXADR during tumor progression has been suggested to serve as an immune evasion mechanism ^{1078,1079,1201}. Interestingly, it has also been speculated that, in the case of PDAC, CXADR may contribute to tumorigenesis via its viral entry receptor function as well ¹¹⁹⁴. In particular, CVB3 infection, mediated by the presence of CXADR, and detected in 50% of clinical PDAC samples, has been implicated in mouse models of an oncogenic KRAS mutational background as a contributor to tumor-promoting chronic inflammation and loss of cellular senescence of pre-malignant lesions ¹¹⁹⁴.

A summary of the suggested tumor-promoting and tumor-suppressing functions of CXADR is depicted in **Table 5**.

Observation/Mechanism	Cancer type	Study type	Ref.
Proposed tumor-promoting effects of CXADR			
Significantly higher CXADR expression in malignant compared to non-malignant tissue and/or positive correlation between CXADR expression levels and worse clinicopathological characteristics	Lung (inc. NSCLC), brain (inc. glioma, neuroblastoma), PDAC, breast, EC, cervical, ovarian, HNSCC (inc. laryngeal), bladder, prostate, thyroid, BCC	Clinical cohort, ex vivo	1080,1155,1163, 1190–1192,1194,1195, 1200

CXADR silencing significantly inhibits the proliferation and/or colony formation capacity of tumor cells	NSCLC (NCI-H1703, NCI-H157, A549, H1975), SCC (HSC-2, HSC-3, Ca9-22), PDAC (PK-8, PANC-1)	In vitro	1203–1205
CXADR silencing significantly inhibits the <i>in vivo</i> tumorigenic potential of cancer cells (significantly reduced tumor size, weight and Ki-67 expression, compared to control cells)	NSCLC (H1975-BALB/c nude mice)	In vivo	1203
CXADR expression correlates with apoptosis inhibition and/or increased Bcl-2 and/or Bcl-xL expression	Cervical squamous carcinoma (CaSki), cervical adenocarcinoma (HeLa), ovarian adenocarcinoma (A2780)	In vitro	1206
CXADR silencing significantly reduces, while CXADR overexpression significantly enhances tumor cell adhesion and invasion	NSCLC (A549)	In vitro	1190
CXADR expression correlates with increased invasiveness of tumor cells <i>in vivo</i>	Breast (mouse mammary epithelial cell lines-BALB/cMed mice)	In vivo, ex vivo	1206
CXADR expression is associated with mesenchymal-like cell characteristics, increased expression of vimentin, Snail, Zeb and Twist, and reduced expression of E-cadherin, occludin, α -catenin and γ -catenin	NSCLC (NCI-H292, Calu-3, NCI-H1703, NCI-H157)	In vitro	1205
CXADR expression characterizes CSC-like treatment-resistant tumor cells	NSCLC (A549, H460, H460-nu/nu nude mice)	In vitro, in vivo	1210
CXADR-dependent CVB3 infection can establish a pro-tumorigenic chronic inflammatory environment and reverse pre-malignant cell senescence	PDAC	Clinical cohort, ex vivo, in vitro	1194

Proposed tumor-suppressing effects of CXADR			
Significantly lower CXADR expression in malignant compared to non-malignant tissue and/or correlation between loss of CXADR expression and worse clinicopathological characteristics	HNSCC, CRC, GC, bladder, prostate, RCC subtypes	Clinical cohort, ex vivo	1086,1163,1193, 1197–1199,1201
CXADR silencing significantly increases, while CXADR overexpression significantly suppresses tumor cell proliferation	CRC (DLD1, HCT116, SW480, SW620), GC (AGS, MKN45)	In vitro	1193,1197
CXADR overexpression significantly increases tumor cell apoptosis, upregulates caspase-3 and cleaved caspase-3, and downregulates Bcl-2 levels	CRC (HT29, SW620)	In vitro	1086
CXADR overexpression significantly inhibits the <i>in vivo</i> tumorigenic potential of cancer cells (significantly reduced tumor size, compared to control cells)	CRC (SW480)	In vivo	1193
CXADR silencing significantly induces, while CXADR overexpression significantly inhibits, tumor cell migration and/or invasion	GC (AGS, MKN45, MKN28), CRC (DLD1, HCT116), glioma (U87-MG)	In vitro	1115,1193,1197
CXADR knockdown significantly promotes the <i>in vivo</i> metastatic potential of tumor cells	CRC (SW480- athymic SCID mice)	In vivo	1193
Loss of CXADR promotes EMT, while CXADR overexpression promotes MET – potentially via a PHLPP2/PTEN-Akt-dependent mechanism	Breast (EpRas, EpXT, and MCF-7, T47D), NSCLC (A549), C57BL/6J mice	In vitro, in vivo	1145
Suppression of CXADR expression facilitates the recruitment and reprogramming of neutrophils towards a pro-tumorigenic state, in a PI3K/Akt-dependent manner	PDAC (KPC1199, KPC mice)	In vitro, in vivo	1211

Table 5: Tumor-promoting and tumor-suppressing functions of CXADR, including potential underlying mechanisms. NSCLC = Non-Small Cell Lung Carcinoma, PDAC = Pancreatic Ductal Adenocarcinoma, HNSCC = Head and Neck Squamous Cell Carcinoma, BCC = Basal Cell Carcinoma, SCC = Squamous Cell Carcinoma, CRC = Colorectal Carcinoma, GC = Gastric Cancer, RCC = Renal Cell Carcinoma, CSC = Cancer Stem Cell, EMT = Epithelial to Mesenchymal Transition, MET = Mesenchymal to Epithelial Transition

1.2.2.9.6 CXADR and anti-tumor treatments

It is worth underlining that CXADR can serve as a mediator and/or target for anti-cancer treatments ¹¹³⁶. First of all, expression of CXADR in tumor tissues can function as a determinant of adenoviral cancer therapy efficacy, with CXADR-low tumor cells being more resistant to adenoviral transfection compared to CXADR-high ones ^{1212–1217}. Several earlier studies had sought to modify CXADR expression on malignant cells ^{1218–1220}, or modify the adenoviral particle so that its internalization is mediated by a CXADR-independent mechanism ^{1221–1223}, aiming in increased gene transfer. More importantly, though, a recent study has successfully attempted to circumvent the reduced transfection efficiency in breast cancer of low CXADR expression through liposomal encapsulation of the adenoviral particles ¹²⁰².

Furthermore, in the last few years, an effort to develop therapeutic anti-CXADR monoclonal antibodies has also been carried out ^{1191,1192}. After the initial design of a murine anti-hCXADR monoclonal antibody that showed promising results in murine models of human prostate cancer, CRC and pancreatic cancer ¹¹⁹¹, Sakamoto and colleagues developed a chimeric (mouse-human) anti-hCXADR monoclonal antibody, which exhibited tumor-suppressing properties in vivo, in murine models of human prostate cancer and human SCLC ¹¹⁹². In vitro assessment of the antibody uncovered its potential to mediate both antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) ¹¹⁹². Importantly, CXADR may not only serve as a target for regulating the tumor-associated properties of malignant cells, but as an “immune checkpoint” molecule as well, through its ability to influence the immune TME via its interaction with JAML ^{1051,1054,1057,1078,1079,1103,1134}.

Although still preliminary, these findings hold promise for the development of novel targeted therapeutics against CXADR for personalized anti-cancer treatment – nonetheless, determining the appropriate administration time, given the context/stage-dependent function of CXADR, and attaining an acceptable safety profile, given its wide tissue expression, will be two major challenges in this area.

A summary of the potential therapeutic applications for the CXADR and JAML molecules in the field of oncology is shown in **Table 6**.

1.2.2.10 CXADR and Inflammation

Taking into consideration the long-characterized role of CXADR in viral infections, the established interaction of CXADR with the immune cell-expressed JAML, the homophilic in trans CXADR interactions that regulate barrier integrity, as well as the diverse upstream signals and mediators that regulate CXADR expression, it is not surprising that the inflammatory responses can both be modulated by and modulate CXADR. Interestingly, it has been

observed that CXADR expression in both neuronal ¹¹⁷⁰ and non-neuronal (germ, endothelial, normal bronchial epithelial, and to a lesser extent, malignant pulmonary epithelial) cells ^{1137,1224} is downregulated by pro-inflammatory cytokines, particularly IFN- γ and TNF- α . This has, expectedly, been associated with reduced adenoviral gene transfer in target cells in vitro ¹²²⁴, and more importantly, it has been implicated in the pathogenesis of neurodegenerative disorders, such as Alzheimer's Disease, in vivo, where, during the initial disease stages, systemic inflammation mediates the loss of CXADR in hippocampal neurons, thus disrupting normal synaptic signaling ¹¹⁷⁰. Moreover, in patients with inflammatory dilated cardiomyopathy, elevated serum IL-6 levels have been found to be significantly and positively correlated with the membrane expression levels of CXADR in cardiomyocytes, whereas among patients with CXADR-positive platelets, serum IL-6 and TNF- α levels are significantly upregulated compared to patients with CXADR-negative platelets ¹¹⁷⁹. Intriguingly, it has been observed that the pro-inflammatory IL-8 cytokine facilitates an increase in the expression of the apically-localized CAR^{Ex8} isoform in human airway epithelial cells [through activation of the Akt/ribosomal protein S6 kinase (S6K) pathway and inhibition of GSK3 β], without affecting the levels of the predominant, basolateral CAR^{Ex7} isoform ¹²²⁵. This was further suggested to function as an underlying mechanism for the increased neutrophil adhesion that is observed in the apical surface of epithelial cells following IL-8 treatment, which is in turn able of enhancing adenoviral entry ¹²²⁵.

A recent study by Ortiz-Zapater and colleagues ¹¹³⁵ identified CXADR as a key regulator of lung inflammation and tissue remodeling induced by aeroallergens like house dust mites (HDM). Eliminating *Cxadr* specifically in lung epithelial cells (Ep-*Cxadr* KO) significantly reduced immune cell infiltration, particularly of neutrophils and $\gamma\delta$ T cells, in pulmonary tissue, following HDM exposure ¹¹³⁵. This protective effect was further characterized by a marked decrease in the production of Th2 cytokines and pro-inflammatory factors, including IL-4, IL-13, IL-33, IL-1 β , CCL20, IL-17, and CXCL1. In vitro experiments with human bronchial epithelial (16HBE) cells confirmed these findings, as CXADR-silenced-cell-conditioned medium resulted in reduced migration of myeloid leukemia cells. While CXADR silencing did not affect total protein levels of E-cadherin or ZO-1, it did disrupt their localization, impairing epithelial barrier function and decreasing cellular binding to collagen I and Matrigel. Mechanistically, it was proposed that HDM challenge initiates a PKC δ -induced phosphorylation of CXADR ICD, that downregulates junctional CXADR localization ¹¹³⁵. The study indicates that targeting CXADR could be a potential therapeutic strategy to mitigate allergic airway inflammation and tissue remodeling.

Potential therapeutic application	Ref.
CXADR as a receptor for adenoviral gene transfer or oncolytic virus therapy	1213,1217
CXADR as a tumor cell target for anti-CXADR monoclonal antibodies	1191,1192
JAML as a target for stimulatory monoclonal antibodies in CD8+ and/or $\gamma\delta$ T cells, for tumors with high CXADR expression	1078,1079
JAML as a target for stimulatory monoclonal antibodies in CD8+ and/or $\gamma\delta$ T cells, in combination with anti-PD-1/anti-PD-L1 monoclonal antibodies, for tumors with either low or high CXADR expression	1078,1079,1087,1090

Table 6: Potential applications of CXADR and JAML in anti-tumor treatments. PD-1 = Programmed cell Death 1, PD-L1 = Programmed cell Death 1 Ligand 1

1.2.2.11 Novel proposed interaction between CXADR and YAP-1

Importantly, a novel interaction between CXADR and YAP-1, the nuclear effector protein of the Hippo/YAP-1 signaling pathway, was proposed ¹¹³⁵. Ortiz-Zapater and colleagues performed proximity-dependent biotin identification (BioID) in CXADR-silenced 16HBE cells re-expressing CXADR-BirA (biotin ligase), and among the more than 700 CXADR interacting partners that were recognized, they also identified YAP-1 ¹¹³⁵, without, however, exploring the potential implications of this interaction.

Overall, along with all the previously discussed effects and underlying mechanisms regarding CXADR's cognate ligand, JAML, within the tumor context, it becomes evident that the JAML-CXADR axis is an important aspect that can contribute to regulation of tumor development and progression, both in terms of influencing malignant cell dynamics, and in terms of modulating the anti-tumor immune response. However, in the setting of BCC and other non-melanocytic cutaneous malignancies a lot remains to be elucidated. In this direction, one potentially promising element that deserves to be further explored is the recently proposed interaction between CXADR and the tumor-associated YAP-1 protein.

1.3 The Hippo/YAP Signaling Pathway

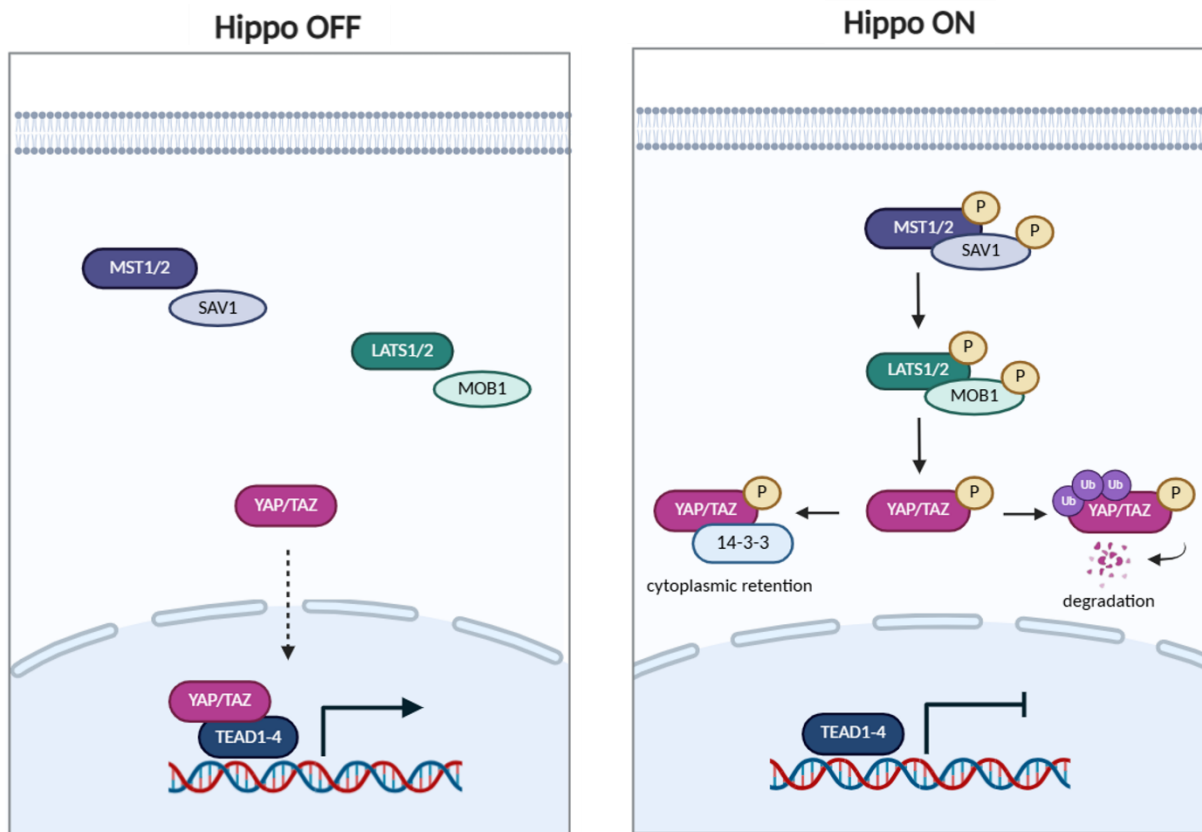
1.3.1 Main Components & Brief Overview of the Hippo/YAP Cascade

The Hippo/YAP signaling pathway was initially described in *Drosophila melanogaster* in the late 1990s - early 2000s, as an important signaling cascade for regulating tissue/organ growth^{1226–1232}. Evolutionarily conserved, the *mammalian* Hippo pathway consists primarily of the serine/threonine Mammalian STE20-like (MST) kinase 1/2, the serine/threonine kinase LATS 1/2, their respective binding partners, Salvador homologue 1 (SAV1) and MOB kinase activator 1A/B (MOB1A/B), as well as YAP-1, its paralog WW domain containing transcription regulator 1 (WWTR1 or TAZ), and the TEA domain transcription factor (TEAD) 1-4¹²³³. SAV1 binding to MST1/2 can enhance the kinase activity of the latter¹²³⁴, while MOB1A/B can both facilitate the interaction between MST1/2 and LATS1/2, as well as promote the kinase activity of its partner (LATS1/2) through conformational changes¹²³⁵.

Briefly, when the Hippo pathway is inactive, unphosphorylated YAP-1/TAZ are able to translocate from the cytoplasm to the nucleus, bind to the TEAD1-4 TFs, and by functioning as transcriptional co-activators, mediate the expression of target genes (**Introductory Figure 2**), which are implicated in the regulation of critical cellular processes, including proliferation, differentiation, apoptosis, and SC self-renewal – *typically*, although not invariably, target gene activation is associated with enhanced cellular proliferation and growth and inhibited differentiation^{1236–1240}. Interestingly, although there is substantial overlap (and thus redundancy) between YAP and TAZ activity, YAP is overall a more potent regulator of several cellular functions compared to TAZ¹²⁴¹. On the other hand, when the Hippo pathway becomes activated by any of the several potential upstream signals (see below), MST1/2 kinase is phosphorylated and activated, and in turn phosphorylates and activates the LATS1/2 kinase, which ends up phosphorylating the YAP-1/TAZ proteins, ultimately resulting either in their cytoplasmic retention by 14-3-3 chaperone proteins, or in their ubiquitination and proteasomal degradation – either way preventing the transcription of target genes in the nucleus^{219,220,1233} (**Introductory Figure 2**), especially since TEAD TFs display very low transcriptional activation potential on their own^{1242,1243}. Moreover, the transcriptional co-activating activity of YAP-1/TAZ is generally antagonized by the nuclear presence of Vestigial like family member 4 (VGLL4) proteins, which share common binding sites with YAP-1/TAZ on TEADs¹²⁴⁴; although a recent report suggests that VGLL4 might contribute to the recruitment of YAP-1 to TEAD4¹²⁴⁵.

In addition, it is worth noting that, although TEADs are the most common and well characterized TFs with which YAP-1/TAZ interact, a series of additional TFs have been reported to associate with YAP/TAZ in an either TEAD- dependent or independent manner^{1246,1247}, in so promoting the expression of their own target genes. Indicatively, these TFs

include activator protein (AP)-1^{1240,1248}, STAT3¹²⁴⁸, Runt-related transcription factor (RUNX)2 or 3¹²⁴⁹, Smad2/3-4 complexes¹²⁵⁰, Myc¹²⁵¹, the EMT-associated Snail family transcriptional repressor (SNAI)1 (or SNAI1)^{1252,1253}, SNAI2 (or SLUG)^{1252,1253}, and zinc finger E-box binding homeobox 1 (ZEB1)¹²⁵⁴, as well as p73^{1255,1256}, p63^{1257,1258} and mutant forms of p53¹²⁵⁹. Of interest to this study, several of the aforementioned interactions have been implicated in the regulation of skin homeostasis¹²⁴⁷.



Introductory Figure 2: The main components of the Hippo/YAP signaling cascade. MST = Mammalian STE20-like, SAV1 = Salvador homologue 1, LATS = Large Tumor Suppressor kinase, MOB1 = MOB kinase activator 1, YAP = Yes-Associated Protein, TEAD = TEA Domain transcription factor, P = Phosphorylation, Ub = Ubiquitination

1.3.2 Main Gene Targets of YAP/TAZ

Gene targets of YAP/TAZ constitute a lengthy list, among which cellular communication network factor (*CCN*)1 – also known as cysteine-rich angiogenic inducer 61 (*CYR61*), and *CCN2* – also known as connective tissue growth factor (*CTGF*) comprise the most well characterized ones^{1233,1260–1263}. This list further encompasses ankyrin repeat domain (*ANKRD*)1¹²⁶⁴, amphiregulin (*AREG*)¹²⁶⁵, fibroblast growth factors (*FGFs*), *VEGFC*, several cell-cell or cell-ECM junction components, including cadherins, such as E- (*CDH1*) and N- (*CDH2*) cadherin, integrin (*ITG*)A11, *ITGB2*, and tight junction protein (*TJP*)1 and *TJP2* (also known as ZO-1 and ZO-2, respectively), numerous genes involved in cell cycle regulation,

including genes encoding for cyclins and cyclin-dependent kinases, genes involved in transcription and translation, including activating transcription factor 3 (*ATF3*) and tumor protein (*TP*)63 (p63), apoptosis regulation genes, such as BH3 interacting domain death agonist (*BID*), the *MYC* and *MYCN* oncogenes, as well as *IL17R*^{219,1260,1266,1267}. Among the TF genes that are upregulated by YAP/TAZ, members of the AP-1 TF, including *FOS* (encoding for c-Fos) are recognized – in fact, it has been shown that YAP/TAZ-TEAD complexes bind directly to the promoter of *FOS* and induce its expression, in so contributing to cell proliferation and growth¹²⁶⁸. Reversely, YAP/TAZ expression also appears to be dependent on AP-1 activity¹²⁶⁸, with AP-1 binding sites having been identified in the cis-regulatory elements of the *YAP1* gene (GeneHancer)¹²⁶⁹. Additionally, a co-operative interaction of YAP/TAZ-TEAD and AP-1 takes place at the enhancer regions of genes responsible for cell proliferation, and can contribute to skin tumorigenesis¹²⁴⁰. Intriguingly, despite the transcriptional co-activator function that characterizes YAP in general, a number of genes have been observed to be downregulated following its activation; these include, for example, bone morphogenetic protein 2 (*BMP2*), *CXCL1*, *IL1A*, *IL1B*, *IL6*, *ITGA5*, and *JAM2*²¹⁹. Of relevance to BCC, *Ptch1* has also been recognized among the genes regulated by YAP¹²⁷⁰, although, as holds true for several of the aforementioned genes, it is not clear whether this regulation is direct or indirect. Moreover, YAP/TAZ can either directly or indirectly induce the activity of LATS1, and especially LATS2 kinase, thus contributing to the establishment of a negative feedback regulatory loop^{1271–1273}.

1.3.3 Protein Structure of YAP/TAZ

The human *YAP1* gene is located on chromosome 11q22.1^{1274,1275} and encodes for several alternate isoforms of the protein, most common of which are YAP1-2γ (commonly referred to as YAP1 or YAP-1) and YAP1-2α (also known as YAP2 or YAP-2)^{1276,1277}. Canonical YAP-1 protein consists of 504 amino acids, and possesses (i) a proline-rich region in its N-terminus, (ii) two coiled-coil regions, the first of which represents the TEAD-interaction domain (TID), (iii) one WW domain (two in the case of YAP-2), which mediates its interactions with a series of other TFs, such as RUNX, Smad, p63 or p73, (iv) an SH3-binding domain, responsible for interacting with SH3 domain-containing proteins, such as tyrosine-protein kinase Yes and non-receptor tyrosine kinase Src, (v) an unstructured transactivation domain (TAD) in its C-terminus, rich in serine, threonine and acidic residues, as well as (vi) a PDZ-binding motif (LTWL) at its C-tail, which enables, among others, the interaction of YAP-1 with ZO-2^{1260,1278–1280}. The human *WWTR1* gene is mapped to the 3q25.1 locus^{1281–1283} and encodes for TAZ, the canonical form of which consists of 400 residues and displays an overall similar domain layout to YAP-1, lacking, however, the proline-rich region and the SH3-binding domain^{80,1278,1283}.

1.3.4 Regulatory Mechanisms of Hippo/YAP Pathway Activity

1.3.4.1 Post-translational modifications (PTMs) of YAP/TAZ

The YAP/TAZ proteins can undergo several PTMs, which contribute to the regulation of their localization and function. Among the most well characterized ones is the phosphorylation that takes place in Ser127 of YAP-1 (or Ser89 of TAZ) and is responsible for its recognition by 14-3-3 proteins, thus defining its cytoplasmic localization ^{1284,1285}. Phosphorylation in this residue can be carried out by LATS1/2, but also by other kinases, including Akt and nuclear dbf2-related (NDR) kinases ^{1/2} ^{219,1286}. Competitively, phosphorylation of the neighboring Ser128 residue of YAP-1 by Nemo-like kinase (NLK) or cyclin-dependent kinase (CDK) ⁷ restricts the S127 phosphorylation and thus favors the nuclear localization of YAP-1 ^{1287,1288}. Moreover, phosphorylation of Ser94 in YAP-1 (or Ser51 in TAZ), mediated by the energy stress-induced AMP-activated protein kinase (AMPK) inhibits its interaction with the TEAD TFs ^{1263,1289}. In addition, YAP-1 can be phosphorylated in Tyr357 by the c-Abl kinase, as a response to DNA damage, favoring its binding to the p73 (TP73) protein, and in so promoting the transcriptional activity of the latter on pro-apoptotic genes ^{1256,1290,1291}. JNK kinases can also modify several residues of YAP-1, including Ser138, Ser317 and Thr362, following UV-induced stress in KCs; however, these phosphorylations have been implicated in YAP-1 binding to the anti-apoptotic p63 (TP63) protein, favoring its stabilization ¹²⁵⁷. Nevertheless, genotoxic stress-associated JNK- or p38 (MAPK14)- mediated poly-phosphorylation of YAP has also been implicated in its activation and the exertion of anti-apoptotic outcomes, independently of p73 ¹²⁹². Furthermore, a specific type of glycosylation (O-GlcNAcylation) can take place at Ser109 or Thr241 of YAP-1 (but not in TAZ) in an AMPK-independent manner, and inhibit its interaction with LATS1/2, thus downregulating the Ser127 phosphorylation and favoring the nuclear translocation of YAP-1 ^{1293,1294}.

YAP-1 can also be ubiquitinated in several Lys residues, with either proteolytic or non-proteolytic outcomes. On one hand, LATS1/2-phosphorylated Ser381 of YAP-1 is essential for the casein kinase (CK)1δ/ε-mediated phosphorylation of Ser384, which in turn is responsible for the beta-transducin repeat containing E3 ubiquitin protein ligase (βTrCP)-induced ubiquitination of YAP-1, which results in its proteasomal degradation ¹²⁹⁵. On the other hand, YAP-1 can be poly-ubiquitinated in a Lys68-linked, Hippo pathway-independent manner, by the S-phase kinase-associated protein 2 (SKP2) E3 ligase complex, which does not result in its proteasomal degradation, but instead favors its nuclear translocation and binding to TEAD1-4, thus enhancing their transcriptional output ^{1296,1297}.

YAP-1 has further been reported to become acetylated in Lys residues of its C-terminal by CREB binding protein (CBP) and p300, following its translocation to the nucleus (as a result

of its reduced phosphorylation), in response to alkylating agents¹²⁹⁸. Acetylation has also been observed in upstream Lys residues inside the TID of YAP, which when deacetylated by sirtuin 1 (*SIRT1*), enhance YAP's interaction with TEAD TFs^{1298–1300}. Overall, this PTM can influence YAP's stability and/or binding capacity to TEADs, thus modulating nuclear target gene expression^{1298–1301}. Lastly, mono-methylation of two distinct YAP-1 residues, Lys494 and Lys342, can promote its cytoplasmic retention or block its nuclear export, respectively, thus influencing target gene transcription^{1302,1303}.

1.3.4.2 Regulatory proteins interacting with Hippo/YAP pathway components & Other cues influencing its activity

Numerous upstream signals have been described as regulators of the Hippo pathway, and numerous binding partners of YAP-1 or other Hippo components have been identified. Among others, cell polarity, intercellular contacts, mechanical cues, including ECM stiffness, cell density and cell shape/geometry, stress signals such as hypoxia, energy, heat, osmotic or shear stress, as well as several soluble factors exerting their action through their respective receptors, have all been described to act upstream of and influence the activity of Hippo pathway proteins, as have extensively been reviewed by Fu and colleagues¹²³³.

First of all, aside from MST1/2, LATS1/2 can also become activated by members of the MAP kinase kinase kinase kinase (MAP4K) family, underlining the redundancy of MST and MAP4K activity in suppressing YAP/TAZ^{1304,1305}, whereas the striatin-interacting phosphatase and kinase (STRIPAK) complex, via its phosphatase component, can inhibit both the MST1/2 and the MAP4Ks, thus ultimately enabling the nuclear translocation of YAP/TAZ^{1306–1308}. Autophosphorylation of MST in the residues that become recognized by STRIPAK has in fact been shown to act as a self-regulating homeostatic mechanism¹³⁰⁸. Mammalian tumor suppressor neurofibromin 2 (NF2), and its *Drosophila* ortholog moesin-ezrin-radixin like (Merlin) protein, the localization of which at the plasma membrane is influenced by cell polarity and other mechanical cues^{1309,1310}, have been shown to facilitate, in conjunction with WWC1 or kidney and brain protein (KIBRA), respectively, the recruitment of MST/Hippo kinase (directly), as well as of LATS/Wts kinase (either directly or indirectly) to the cellular membrane, in so favoring the phosphorylation and activation of the latter, and ultimately resulting in suppression of YAP-1/Yki activity^{1311–1315}. Interestingly, NF2/Merlin has also been implicated in regulating the nuclear/cytoplasmic shuttle of YAP/TAZ in a Hippo pathway-independent manner, by directly interacting with them and preventing their nuclear translocation, following its dissociation from adherens junctions subsequent to contraction of the circumferential actin belt of epithelial cells¹³¹⁶.

As expected, several additional intercellular contact components are involved in Hippo pathway regulation – for instance, PTPN14, another adherens junction-associated protein ¹³¹⁷, the expression of which becomes upregulated under high cell density conditions, has been shown to directly interact with YAP-1, in a Ser127 phosphorylation-independent manner, dephosphorylate its Tyr357 residue, and mediate its nuclear to cytoplasmic translocation, thus inhibiting YAP target gene expression ^{221,222}. In addition, angiomin (AMOT) and angiomin-like (AMOTL)1 and AMOTL2 proteins, which associate with cadherins ¹³¹⁸, have also been reported to interact with YAP-1 (independently of Ser127 phosphorylation) and facilitate its cytoplasmic sequestration ^{1319,1320}. Moreover, it has been observed that the AMOT family members can serve as scaffolding proteins for LATS1/2, the SAV1-MST1/2 complex, and YAP-1, as well as enable the autophosphorylation of LATS1/2 ¹³²¹; while they can also interact with the NF2/Merlin protein, reverse its auto-inhibition, and thus favor its association with LATS1/2 ¹³²² – ultimately facilitating the inactivation of YAP-1.

Furthermore, it has been demonstrated that the adherens junction component α -catenin can indirectly interact with (Ser127 phosphorylated) YAP-1 through 14-3-3 proteins, and in so prevent its dephosphorylation and nuclear translocation in KCs, with loss of α -catenin and subsequent constitutive nuclear localization of YAP having been associated with the pathogenesis of cSCCs ^{1323,1324}. Moreover, the ZO-2, tight junction-associated protein, has been implicated in YAP localization regulation, by interacting with either YAP-1 and/or LATS1 ^{1325,1326}; formation of the ZO-2/YAP-1/LATS1 complex at tight junctions promotes YAP-1 phosphorylation by LATS1, thus supporting the inactivation of its transcriptional activity ¹³²⁶.

It is essential to underline that the actin cytoskeleton also participates in a critical fashion in orchestrating the regulation of YAP/TAZ activity in response to intercellular contact-mediated signals, via either Hippo- dependent or independent mechanisms ¹³²⁷.

As expected, all the aforementioned interactions between Hippo/YAP components and intercellular junction elements can function as regulators of contact inhibition-modulated cell proliferation, placing cell confluency as an essential factor influencing YAP activity; simply put, the formation of cell-cell interactions when cells exist in a relatively high density favors the cytoplasmic retention of the YAP protein, either by its direct binding to protein components of the junctions, or indirectly, by promoting its LATS-mediated phosphorylation, which results in its sequestration by 14-3-3 proteins, thus ultimately suppressing YAP nuclear activity that normally favors cell proliferation ^{221,222,1233,1311–1315,1323,1326,1327}.

Cell-ECM contacts and associated mechanical cues can also affect the activity of the Hippo/YAP pathway. Specifically, it has been demonstrated that low ECM stiffness results in Hippo activation and YAP activity suppression, whereas high ECM stiffness inhibits the Hippo

pathway, and thus promotes YAP nuclear accumulation and transcriptional activity^{1233,1327,1328}. Among the ECM proteins that have been observed to transduce mechanical signals to the Hippo/YAP network is agrin, which indirectly facilitates the inhibition of Merlin/NF2 and LATS1, and thus the activation of YAP¹³²⁹, as well as fibronectin¹³³⁰ and laminin β 1¹³³¹, both of which have also been implicated in negative Hippo regulation (and in so in YAP activation). Conversely, tenascin-C has been reported to activate the Hippo pathway, resulting in suppression of YAP/TAZ activity^{1332,1333}. Interestingly, agrin, fibronectin and laminin, along with another tenascin family member, tenascin-R, have been recognized as binding partners of CXADR¹¹²⁹.

Moreover, it is worth noting, that within the nucleus, under low ECM stiffness conditions, YAP/TAZ can interact with the SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin remodeling complex, specifically with its AT-rich interactive domain-containing protein 1A (ARID1A) component, in so preventing their interaction with the TEAD TFs²²⁶. When ECM stiffness is high, on the other hand, polymerized nuclear F-actin associates with the ARID1A/SWI/SNF complex, releasing YAP/TAZ and enabling their binding to TEADs and ultimately, target gene expression²²⁶.

Cell morphology is another aspect that has been observed to influence YAP activity, primarily through actin cytoskeleton dynamics^{1334,1335}. And similarly, shear stress has also been shown to result in YAP activation, via an actin regulatory mechanism^{1336,1337}. Other forms of cell stress can also modulate the Hippo/YAP pathway, including, for instance, energy stress – under such conditions, AMPK activation, on top of phosphorylating Ser94 of YAP-1, which inhibits its interaction with TEADs^{1263,1289}, can also mediate the phosphorylation of a specific residue of the AMOTL1 protein, which contributes to its stabilization and thus supports its inhibitory effect on YAP signaling¹³³⁸.

1.3.4.3 Hippo/YAP and Hypoxia

In relevance to oncogenesis, a reciprocal regulatory mechanism has also been described between Hippo/YAP signaling and hypoxia, with hypoxic conditions and HIF1 overall promoting YAP activity^{1339–1343}. For instance, it has been shown that hypoxia favors the destabilization of LATS1 by enhancing the Siah E3 ubiquitin protein ligase 2 (SIAH2) function¹³⁴¹, while the HIF1 TF has been reported to suppress LATS1 expression, and lead to increased YAP-1 activity¹³⁴⁰. Additionally, HIF1 has been reported to induce the expression of a specific GPCR (GPCR5A) in human CRC, which, via a potentially Rho-dependent pathway, results in YAP activation, and ultimately tumor cell survival¹³³⁹. Moreover, YAP may form a complex with HIF1 α , increasing its stability, and leading to accumulation of HIF1 α ^{1341,1344}.

1.3.4.4 Interactions between Hippo/YAP and other signaling pathways

The Hippo/YAP pathway displays interactions with several other signaling networks. One of its most well characterized crosstalks is that with the Wnt pathway, with Hippo being able to downregulate the activity of Wnt/ β -catenin signaling^{1345–1348}. The proposed underlying mechanisms involve: (i) binding of phosphorylated cytoplasmic YAP/TAZ to the Dishevelled (DVL) protein, in so restricting DVL phosphorylation, which is normally necessary for inhibition of the β -catenin destruction complex (β cDC), ultimately affecting β -catenin stability¹³⁴⁸, (ii) incorporation of cytoplasmic YAP/TAZ to the β cDC, when Wnt is inactive, which facilitates the recruitment of the β -TrCP E3 ligase, thus enhancing β -catenin degradation; conversely, when Wnt signaling is activated, YAP/TAZ are released from the β cDC, enabling both the stability and nuclear translocation of β -catenin, as well as their own nuclear localization¹³⁴⁵, and/or (iii) direct interaction of phosphorylated YAP/TAZ with β -catenin, which results in restriction of its nuclear translocation¹³⁴⁷. Additionally, YAP activity in epidermal KCs has been shown to promote paracrine canonical Wnt signaling, through Wnt16 secretion, in so enhancing KC proliferation¹³⁴⁹. Conversely, active Wnt signaling (dependent or independently of β -catenin) has been shown to promote the expression and/or nuclear activity of YAP^{1350–1352}. Overall, it is evident that Hippo pathway activation results in suppression of both β -catenin and YAP/TAZ target gene expression, whereas Wnt pathway activation upregulates both β -catenin and YAP/TAZ transcriptional output. Moreover, it has been observed that YAP and β -catenin can cooperatively regulate the expression of specific target genes, such as *SOX2* and *SNAI2* (*SLUG*)¹³⁴⁶.

In contrast to the reciprocal positive regulation between YAP/TAZ and Wnt/ β -catenin signaling, YAP/TAZ downregulate the activity of the Notch pathway^{101,1353} – the other major developmental pathway involved in stemness and differentiation regulation¹³⁵⁴ – to control cell fate in the epidermis^{101,1353}. Interestingly, though, a positive feedback network has been described between Notch signaling and YAP/TAZ in murine models of HCC, which, nevertheless, was abrogated by Wnt/ β -catenin stimulation, in so inhibiting its pro-tumorigenic effects¹³⁵⁵.

Another well studied interplay is that between the Hippo/YAP and TGF- β pathways, which has been implicated in the regulation of the SC phenotype and ECM remodeling^{1250,1356–1358}. YAP/TAZ have been shown to bind to Smad2/3-4 heterodimeric complexes, following TGF- β stimulation, and facilitate their nuclear localization¹²⁵⁰, as well as cooperatively regulate the expression of target genes^{1356,1358}. Downregulation of YAP/TAZ has also been associated with induction of the TGF- β /Smad3-suppressive protein Smad7, via an AP-1-dependent mechanism¹³⁵⁹. TGF- β 1, on the other hand, has been observed to induce the expression of TAZ in a Smad3-independent, and rather p38 (MAPK14)- and MRTF- dependent manner¹³⁶⁰.

Of note, co-operativity between the YAP/TAZ-TEAD and AP-1 factors has been described, and has been implicated in YAP target gene activation regulating cell cycle and driving oncogenesis¹²⁴⁰. Regulatory interactions between YAP/TAZ and NF-κB signaling have also been described, although their exact nature is not yet completely understood^{1361–1363}.

Lastly, an additional Hippo/YAP signaling interplay, which is of primary interest to the particular study, is the one with the Hh pathway, the most common driver of BCC pathogenesis^{130,136,172,173}. Briefly, it has been described, in both epidermal¹⁹⁰ and non-epidermal^{1270,1364–1366} cells, the existence of a reciprocal positive regulatory network between Hh and YAP activity, which contributes to homeostasis regulation, but also to the development of several malignancies^{1364,1365}, including BCCs^{190,192} and the related medulloblastomas¹³⁶⁵. Nevertheless, reports of a negative effect of YAP expression on Hh activity (in human embryonic kidney cells) also exist¹³⁶⁷.

1.3.4.5 Regulation of YAP signaling by Cytokines

It has also been observed that several cytokines can modulate the activity of YAP. For instance, pro-inflammatory factors TNF-α and IL-1β can promote the proteasomal degradation of YAP, either by an MST-LATS-dependent mechanism, or through activation of the TGF-beta activated kinase 1 (TAK1 or MAP3K7)¹³⁶¹. Contrarily, IL-17A can promote the transcriptional activity of YAP; IL-17RA stimulation by IL-17 is followed by recruitment of the Act 1 adaptor protein (ACT1), which in turn facilitates the sequestration of MST1, in so inhibiting its interaction with LATS, and thus enabling YAP nuclear translocation – in fact, this mechanism has been proposed to be implicated in the pathogenesis of psoriasis, via promotion of YAP-mediated KC proliferation¹³⁶⁸. IL-6 has also been shown to activate YAP in a gp130-Src-mediated manner^{1369,1370}.

Overall, the regulatory interactions between the Hippo/YAP pathway components and their upstream or downstream binding partners, despite still not fully elucidated, are extensive, and it is impossible to recapitulate their entirety within the extent of this study. For a more complete overview of the underlying mechanisms of YAP regulation, the review works by Han¹²⁷⁸, Fu and colleagues¹²³³, as well as by Kwon et al.¹²⁶⁰ provide a very good starting point.

1.3.5 Implications of the Hippo/YAP Pathway in Tumorigenesis

Before delving into its role in skin homeostasis and BCC pathogenesis in particular, which is the focus of the present study, it is worth noting that the Hippo/YAP pathway is one of the most common signaling cascades dysregulated in human malignancies^{75,80,93}, although mutations and CNAs of the genes encoding for its components are not so frequently present in tumors¹³⁷¹. In general, within the tumor context, YAP/TAZ are considered to act as

oncoproteins, as in most instances the downstream target gene activation has been associated with promotion of (tumor) cell growth, proliferation ^{76,77,79,81,83,91,92}, invasion and metastasis ^{79,85,92,96,1372}, metabolic reprogramming ^{82,88,89,95,1373,1374}, and angiogenesis ⁸⁷, as well as inhibition of apoptosis ^{77,90,1375} and of differentiation ^{77,86}, in so further supporting a CSC phenotype and enhancing the EMT process ^{74,78,84,92,94}. Accordingly, the MST1/2 and LATS1/2 kinases are generally considered as suppressors of tumor progression and metastasis ^{1376–1378}. Evidence supporting this notion has been provided by studies on the homeostasis of several tissues and organs and their respective tumors, including, but not limited to, HCC ^{1315,1379,1380}, GC ^{85,1381,1382}, CRC ^{1376,1377,1383,1384}, PDAC ^{81,83,86,92,94,1369,1385–1387}, lung cancer ^{91,1375,1388–1391}, breast cancer ^{78,1378}, ovarian cancer ^{1392,1393}, RCC ^{76,1394}, bladder cancer ⁷⁹, SCCs – both cutaneous and non-cutaneous ones ^{97,1240,1395,1396}, cutaneous ^{96,1372} and uveal (UM) ¹³⁹⁷ melanoma, as well as several nervous system tumors, including glioblastomas ^{77,1398,1399}, ependymomas ¹⁴⁰⁰, and schwannomas ¹⁴⁰¹.

Interestingly, YAP/TAZ signaling has also been implicated in tumor progression via promotion of an immune escape mechanism. Firstly, it has been demonstrated that, through TEAD binding and activation, YAP can upregulate the expression of PD-L1 in malignant cells ^{1402–1405}. In addition, it can facilitate the recruitment of MDSCs and/or M2 macrophages in the TME by inducing the secretion of specific cytokines by tumor cells, including IL-6, TNF- α , GM-CSF [colony stimulating factor (CSF)2], G-CSF (CSF3), macrophage colony stimulating factor (M-CSF or CSF1), CXCL2 and CCL2 ^{1406,1407}. Furthermore, YAP signaling in immune cells (instead of tumor cells) has been suggested to be involved in promoting the M2 macrophage polarization ^{1408,1409}, supporting the immunomodulatory Treg phenotype ^{1410,1411}, as well as inhibiting the differentiation and effector function of CD8+ and/or CD4+ populations ^{1412,1413}. In accordance with these and additional observations ^{1414,1415}, YAP signaling has mainly been implicated in non-response to and/or development of resistance against immunotherapeutic agents, including anti-PD-1 monoclonal antibodies ^{1416,1417}, as well as against targeted therapies ¹⁴⁰⁴, chemotherapeutic drugs ^{94,1418–1420}, and radiotherapy ¹⁴²¹.

On the other hand, Hippo-mediated *downregulation* of YAP has been reported to *promote* chemoresistance in the setting of PDAC ¹⁴²². Accordingly, there has been a series of studies, including in CRC ^{1423–1425}, breast cancer ^{1426–1428}, LUSC ¹⁴²⁹, several neuroendocrine tumors ¹⁴³⁰ and hematological malignancies ¹⁴³¹, where the nuclear activity of YAP/TAZ has been proposed to hinder the tumorigenic process. The proposed tumor-suppressing functions of YAP/TAZ may be independent of TEAD activation, and may instead be mediated by other YAP/TAZ-interacting TFs, such as RUNX3 ¹²⁴⁹, or via other binding partners of YAP/TAZ that can regulate transcriptional output, such as annexin A2 (ANXA2) ⁸⁵.

Pearson and colleagues ¹⁴³⁰ have in fact classified several human malignancies into YAP^{on} (pro-tumorigenic role of YAP) and YAP^{off} (tumor-suppressing role of YAP) categories, identifying disparate underlying pathogenetic mechanisms. Briefly, they propose that YAP^{on} tumors are associated with increased expression of cell cycle-promoting genes, whereas YAP^{off} tumors (including many of neural or neuroendocrine origin), are characterized by downregulation of ITGAV, ITGB5 and other adhesion molecules, via a unique/alternate enhancer activity compared to that in YAP^{on} tumors ¹⁴³⁰. Accordingly, they outline distinct vulnerabilities between the two tumor classes, that can potentially be utilized as therapeutic targets ¹⁴³⁰.

1.3.6 Role of YAP/TAZ in Skin Development and KC Proliferation & Differentiation

In accordance with its central role in organ development and function, the Hippo/YAP signaling pathway exerts a crucial regulatory effect in skin differentiation and homeostasis. First of all, the expression level and the subcellular localization of YAP/TAZ is dynamically altered during skin development: in murine subjects, during the early embryonic stages, YAP/TAZ is highly expressed in the early epidermal monolayer and is predominantly localized within the nucleus ^{101,1432}. As the epidermis begins its stratification process, YAP/TAZ is mainly restricted to the basal layer, which essentially contains the proliferating cells, in which it remains predominantly nuclear ^{101,1432}. Postnatally, the expression and nuclear localization of YAP/TAZ are markedly downregulated ¹⁰¹. However, still, the basal layer retains a higher expression, with either nuclear or cytoplasmic subcellular distribution, compared to the overlaying, more differentiated strata, which display a lower and more diffuse YAP/TAZ expression pattern, outside of the nucleus ^{1323,1432,1433}. Accordingly, during HF formation and maturation, YAP/TAZ is identified in the nucleus of ORS cells and transit-amplifying cells (TACs) of the HF matrix, whereas it localizes cytoplasmically in the more differentiated and terminally differentiated cells of the IRS and hair shaft ^{101,1432}. In postnatal mice, YAP expression is predominantly present in the cytoplasm of HF bulge cells (which correspond to the HF SCs), and to a lower degree is also detected in the cytoplasm of ORS cells ¹⁴³³. Importantly, a similar pattern for YAP/TAZ subcellular localization is detected in adult human skin as well ^{1432,1434}. Overall, it has been established that nuclear YAP/TAZ activity is predominantly restricted to the epidermal stem/progenitor cell compartment, where it plays an important role in promoting cell proliferation and inhibiting differentiation ^{101,1323,1432,1434}.

Accordingly, it has been shown that in vitro overexpression of YAP in primary normal human keratinocytes (NHKs) leads to a significant increase in their proliferative and colony-forming capacity, in so resulting in their immortalization, as well as to a blockade of the normal clonal evolution process, “entrapping” KCs primarily in the SC-associated holoclone phenotype ¹⁴³⁴. As expected, this is accompanied by a disruption of the normal differentiation process,

with observed upregulation of progenitor-associated markers, such as p63, and downregulation of differentiation-associated markers, such as involucrin and serine peptidase inhibitor kazal type 5 (SPINK5 or lympho-epithelial kazal-type-related inhibitor; LEKTI) ¹⁴³⁴. Nevertheless, YAP overexpression is not capable of inducing EMT characteristics in the primary NHKs in vitro ¹⁴³⁴.

Similarly, basal (K14) KC-specific induction of increased nuclear YAP activity in transgenic mice with a mutated YAP isoform (S127A) which cannot be phosphorylated on the critical Ser127 residue, results, among others, in increased IFE thickness, characterized by a significantly higher cell density and a markedly altered cell morphology at the basal layer, along with a thinner stratum corneum, compared to the WT littermate, as well as in evagination of the HFs, which further lack the IRS and hair shaft populations ^{101,1323}. In addition, these mice either became sick or displayed lethality shortly after birth ^{101,1323}. Further analyses confirmed that YAP overexpression results in increased proliferative and reduced differentiation capacity of epidermal stem and progenitors cells, with the IHC positivity for proliferation markers, such as Ki-76, and additional basal layer/SC markers, like K14, K5 and p63, being partly expanded in suprabasal layers, whereas the expression of differentiation markers, such as K10, involucrin and loricrin was reduced, compared to the healthy epidermis ^{101,1323}. Accordingly, the expression of protein markers associated with late-stage HF differentiation, including K6, K40, and trichohyaline, was downregulated or delayed ¹⁰¹. In addition, in vitro assays utilizing the transgenic-mice-derived KCs corroborated their enhanced proliferation and restricted differentiation following YAP overexpression, but further highlighted YAP's inhibitory effect on cellular apoptosis as well ¹⁰¹. Importantly, engraftment of newborn YAP-overexpressing transgenic murine skin into nude mice led to the development of cutaneous masses resembling human cSCCs, which gradually progressed into invading tumors displaying an EMT profile ¹³²³.

On the other hand, basal (K14) KC-specific YAP *downregulation*, resulted in the exact opposite outcomes, restricting the proliferation and favoring the differentiation, with or without increasing the apoptosis of basal KCs in vitro ^{101,1323}; and more importantly, producing an in vivo mouse phenotype with thinner, fragile skin, devoid of its barrier function, and complete epidermal loss in distal limb regions, which was characterized by embryonic or perinatal lethality ¹³²³. Histological examination highlighted the hypoplastic basal layer of the *Yap*-silenced skin, as well as the altered basal layer cell morphology, which resembled that of upstream differentiated cells (more flattened appearance) ¹³²³. Double YAP and TAZ conditional KO in the basal (K5) KC layer leads to a similar, although not equally severe phenotype, when induced in neonate or adult mice, overall impacting hair growth in the former, and causing patch-like hair loss in the latter, accompanied by a reduction in the proliferation rate of basal KCs ¹⁴³².

Moreover, based on a series of experiments in transgenic mice that express a basal cell (K5)-specific YAP mutant which is resistant to MST-LATS phosphorylation but also lacks the TAD of the C-terminus (YAP2-5SA-ΔC)¹²⁸⁵, Beverdam and colleagues proposed that the C-terminal domain of the YAP protein plays an important role in regulating the shift between the proliferative stem/progenitor to the suprabasal differentiated epidermal cell phenotype¹⁴³³.

From a mechanistic point of view, YAP-mediated stimulation of KC proliferation overall appears to be dependent on its interaction with its “classic” nuclear binding partners, the TEAD TFs¹³²³, as well as on the expression of the bona fide YAP target gene, *Ccn1* (*Cyr61*)¹⁰¹. However, upstream of YAP, the “typical” regulator, Hippo pathway, may not be the one that modulates YAP-dependent cellular processes in the epidermis, as neither *Mst1/2* nor *Lats1/2* knockdown in KCs (in vitro or in vivo) seem to significantly impact YAP nuclear activity and/or its phenotypic outcomes¹³²³. Instead, via a series of in vitro and in vivo experiments, it has been proposed that YAP activity in epidermal cells is moderated, at least in part, through its interaction with α-catenin, which, as already discussed, is indirect, and facilitated by the Ser127-phosphorylated YAP’s binding to 14-3-3¹³²³.

Recruitment of YAP to the adherens junctions appears to be mediated by α-catenin (and not β-catenin, E- or P- cadherin), and as expected, negatively regulates its transcriptional activity – however, the proposed underlying mechanism may involve the prevention of YAP Ser127 dephosphorylation by the PP2Ac phosphatase owing to YAP’s association with α-catenin¹³²³. In conjunction with previous studies which have revealed that downregulation of α-catenin results in epidermal hyperproliferation and development of cSCCs^{1435,1436} – a phenotype resembling that of YAP-overexpressing transgenic mice – it is overall speculated that α-catenin plays a central role in inhibiting the pro-proliferative, oncogenic, nuclear activity of YAP in the skin¹³²³.

Furthermore, in a series of in vitro (and in vivo) experiments on murine and human skin KCs, it has been demonstrated that focal adhesion signaling, via ITGB1, FAK and Src, as well as PI3K signaling, presumably downstream of the EGFR, play an essential role in mediating the nuclear localization of YAP in the skin epithelium, both under normal and inflammatory conditions – thus, implying that the association of the basal layer KCs with the basement membrane (via focal adhesion formation) is imperative for their nuclear YAP expression¹⁴³². In accordance with this, the phenotype of skin-specific *Itgb1*-KO mice^{1437,1438} closely resembles that of the skin-specific double YAP-TAZ KO mice described earlier¹⁴³².

Overall, Elbediwy and colleagues have proposed a regulatory mechanism for the localization of YAP, and the concomitant proliferation or differentiation of epithelial cells, in squamous compared to columnal epithelia¹⁴³². Briefly, as already described, in squamous

epithelia, such as that of the skin, focal adhesion-dependent retention of cellular contact with the basement membrane ECM facilitates the nuclear translocation of YAP, and thus the maintenance of a transcriptional program that favors proliferation and restricts differentiation¹⁴³². As suprabasal cells begin to lose contact with the basement membrane, YAP shifts to the cytoplasm and enables promotion of differentiation and downregulation of proliferation. In contrast, in columnar epithelia, such as those of the airways, colon or endometrium, even differentiated cells retain their contact to the basement membrane, however, YAP is cytoplasmic¹⁴³². This is achieved via the negative regulatory effect of apical domain proteins that are expressed in mature columnar epithelial cells. The presence of an apical domain results in the cytoplasmic sequestration of YAP – whereas, loss of the apical domain during carcinogenesis of these columnar epithelia allows the nuclear translocation of YAP and the exertion of its pro-tumorigenic effects¹⁴³².

Importantly, the research team of Coulombe has recently highlighted the significance of the ratio between K14 (keratin-marker mainly of the basal epidermis layer cells) and K15 (generally considered a HF bulge SC marker) in regulating the subcellular localization and activity of YAP in KCs – a role dependent on specific residues of theirs or lack thereof^{1439,1440}. Briefly, they propose that, owing to certain amino acids and/or motifs, K14 indirectly mediates the cytoplasmic sequestration of YAP, whereas K15, lacking the same sequences, is unable to do so, and thus facilitates its nuclear translocation, ultimately contributing to sustaining the progenitor cell phenotype¹⁴⁴⁰. This functional discrepancy between the two keratins may also explain their distribution pattern within the epidermis, with K15 staining being recognized almost exclusively in the basal layer of the IFE, whereas, gradually decreasing K14 staining can be identified within suprabasal layers as well¹⁴⁴⁰.

Aside from its direct effects in regulating epidermal cell proliferation and differentiation, YAP can also control these cellular processes in the skin, via modulation of other signaling pathways. For instance, it has been shown that YAP activation in the IFE can enhance the nuclear activity of β -catenin, and ultimately the proliferation of neighboring KCs, in a paracrine, canonical Wnt/ β -catenin signaling cascade-dependent manner, as a result of increased secretion of the Wnt16 ligand by the YAP-stimulated KCs^{1349,1441}.

The regulatory effect of YAP/TAZ signaling on epidermal cell fate determination and overall skin homeostasis regulation, as well as their upstream regulatory signals and interacting signaling pathways in the epidermis have been recently thoroughly reviewed by Pankratova and colleagues¹²⁴⁷.

1.3.7 Role of YAP/TAZ in Skin Homeostasis and Cutaneous Immunity

Of clinical significance, YAP/TAZ are required for the wound healing process, both in general, and in the skin particularly, through their contribution to cell proliferation and migration induction, and apoptosis prevention ^{1247,1442}. Following skin wounding, epidermal upregulation of both YAP and TAZ is observed, especially in the basal layer cells, with a predominantly nuclear localization ^{1432,1443}. Increased nuclear YAP/TAZ is also detected in the dermis, potentially corresponding to dermal fibroblasts ¹⁴⁴³. Basal cell (K5)-specific double conditional YAP-TAZ KO mice display a significantly impaired/delayed wound healing process, accompanied by a significantly reduced number of proliferating (Ki-67+) cells at the wound area, compared to WT ¹⁴³². Interestingly, though, single KO of either protein (YAP, TAZ) alone in the epidermis does not seem to affect skin wound healing ¹⁴³². However, treatment of mouse skin wounds with either YAP- or TAZ- siRNAs, which silence the respective proteins in both epidermal and dermal cells locally, did result in significantly reduced wound healing capacity compared to the control siRNA-treated wounds ¹⁴⁴³, overall potentially suggesting a more prominent role of *dermal*-derived YAP/TAZ in regulating wound closure.

The Hippo/YAP pathway contributes, through both immune and non-immune cell-mediated mechanisms to the regulation of immune responses. Indicatively, it has been demonstrated that YAP in macrophages can enable the activation of the NLR family pyrin domain containing 3 (NLRP3) inflammasome ¹⁴⁴⁴, which is an essential component of the innate immune response ¹⁴⁴⁵, along with downstream IL-1 β and IL-18 production ¹⁴⁴⁴. Moreover, YAP can modulate macrophage polarization – although contradicting findings have been reported in the literature ¹⁴⁴⁶, the most predominant hypothesis is that YAP promotes the anti-inflammatory M2 phenotype ^{1408,1409}. In addition, YAP has been shown to support the immunomodulatory properties of Tregs ^{1410,1411}, favor the Th17 differentiation of CD4+ T cells ¹⁴⁴⁷, as well as interfere with the differentiation and effector function of CD8+ and/or CD4+ populations ^{1412,1413}. Non-immune-cell-secreted cytokines downstream of YAP signaling, especially in the setting of cancer, can also contribute to the recruitment of MDSCs and/or M2 macrophages ^{1406,1407}.

As a “bridging” observation between YAP’s role in regulating the cutaneous immune microenvironment and the wound healing process, Gao and colleagues, based on their findings in a murine model of skin wound healing, have recently proposed that inhibition of autophagy, which promotes the wound healing process, does so, at least in part, by increasing MST expression, in so inhibiting YAP activity, and thus, leading to an increase in the production of IL-33 (which was separately shown to favor wound closure) and IL-10, as well as in a decrease in the levels of TNF- α , IL-1 β and IL-6 ¹⁴⁴⁸.

Unsurprisingly, YAP/TAZ signaling (especially fibroblast-derived) has been implicated in the skin aging process ^{1449,1450}, as well as in the pathogenesis of several cutaneous conditions, including inflammatory and hyperproliferative diseases, such as psoriasis ^{1368,1451} and AD ¹⁴⁵², in both melanoma ^{96,1453} and NMSCs ^{97,1323,1396,1454}, as well as in diseases characterized by KC hypoproliferation and disruption of the skin barrier, like epidermolysis bullosa ¹⁴⁵⁵ – as have recently been reviewed by Cai and colleagues ¹⁴⁴². An overview of the various YAP-associated signaling cascades that participate in skin homeostasis and pathology are further provided by Jia and colleagues ¹⁴⁵⁶.

1.3.8 Role of YAP/TAZ in BCC

In contrast to CXADR and JAML, for which only limited or no evidence at all exists regarding their role in BCC tumorigenesis, the Hippo/YAP pathway has been shown to be involved in its initiation and progression. First of all, genetic analysis of 293 BCCs from 236 patients has revealed the presence of mutations in several components or regulators of the Hippo/YAP cascade ¹⁵⁴. In particular: (i) *PTPN14* mutations were identified in 23% of the samples, with 61% of them being truncating mutations ¹⁵⁴. In accordance with the protein's function in mediating the nuclear to cytoplasmic shuttle of YAP ^{221,222}, BCC tumors with LOF *PTPN14* mutations (n = 8) displayed IHC YAP staining within the nucleus, in contrast to WT *PTPN14* BCCs (n = 3) which exhibited a predominantly cytoplasmic YAP staining ¹⁵⁴. (ii) *LATS1* and *LATS2* mutations were identified in 16% and 12% of the BCCs, respectively ¹⁵⁴. Twenty-four (24) % of the *LATS1* mutations were truncating, with Arg995Cys being recognized as a significantly recurrent mutation (SRM), and in silico analysis further predicting the potential structural and functional importance of this residue ¹⁵⁴. *LATS2* mutations were mainly accumulated in its C-terminal end, of which, 10% were impacting Pro1004, a residue, which based on computational approaches, was suspected to exert a role in the protein's functionality. (iii) Mutations in the *PPP6C* gene were also present in 15% of BCCs ¹⁵⁴. Among other functions, PPP6C has been shown to interact with MOB1, and potentially regulate the activation of LATS by MST ^{223–225}. *PTPN14* and *LATS1* mutations have further been validated in a more recent cohort of patients with BCC ²¹⁸. *PTPN14* mutations were more common in *SMO*-driven tumors compared to *PTCH1*-driven ones ¹⁵⁴. In addition, *PPP6C* and *PTPN14* mutations (along with those of *MYCN*) were associated with BCCs of increased recurrence risk (morpheaform and metatypical; BSC), compared to low-recurrence-risk BCCs (nodular and superficial), whereas vismodegib-resistant tumors exhibited enrichment for YAP-1 and N-Myc target genes compared to treatment-naïve ones ¹⁵⁴. Also, in a case report of a 61 year old Japanese patient with Gorlin-Goltz syndrome, genetic analysis of her BCCs revealed a biallelic disruption of the *LATS1* gene (a nonsense mutation in the position encoding for Gln315, and a loss of the WT allele on chromosomal locus 6q25.1) only in the infiltrative, and not in the

superficial tumors ¹⁴⁵⁷. Interestingly, a recent study has further proposed the potential implication of Hippo/YAP signaling in the metastatic process of BCC, as it identified missense mutations of the YAP/TAZ-binding domain of the *TEAD1/2/4* TFs in metastatic cases, that were lacking in non-metastatic ones, as well as the presence of mutations in *PTPN21* ²¹⁶ (another negative regulator of YAP-1, similarly to *PTPN14* ²⁴²) exclusively in the hematogenously spread compared to the regionally spread BCCs ²¹⁶.

IHC examination of human BCC specimens in several independent studies has highlighted an increased YAP expression (reportedly ten-fold), especially within the nucleus, of malignant cells, compared to those of the healthy epidermis ^{97–101}. This holds true for all the different BCC subtypes examined (including nodular, superficial and infiltrative), with no significant differences having been reported between them ⁹⁷. YAP expression was mainly present within the BCC *epidermal* compartment, and only minimal in the surrounding stroma ¹⁰⁰. TAZ expression has also been observed in most infiltrative, and in areas of nodular and superficial BCCs ⁹⁷. This increased nuclear localization of YAP/TAZ in BCC cells is not unexpected given their established role in promoting epidermal cell proliferation, inhibiting differentiation, and potentially regulating EMT ^{101,1323,1432,1434}. In their cohort of patients with mBCC, Vergara and colleagues, observed increased nuclear and decreased cytoplasmic YAP/TAZ IHC positivity in the *PTPN21*-mutated hematogenous lung metastatic foci compared to the non-*PTPN21*-mutated local nodal metastases of BCCs, whereas the YAP/TAZ-binding domain *TEAD*-mutated lymph node metastases displayed the lowest nuclear YAP/TAZ and the highest cytoplasmic p-YAP intensity compared to both non-*TEAD*-mutated nodal metastases and pulmonary metastases, further supporting a potential role of Hippo/YAP signaling in BCC metastasis ²¹⁶.

On the other hand, no significant difference has been observed between recurrent (n = 8) and non-recurrent (n = 38) BCCs for IHC YAP expression, in terms of both staining intensity and percentage of positive cells ¹⁴⁵⁸. However, YAP-regulated *CCN2* (*CTGF*) IHC expression (intensity and percentage) was significantly lower in recurrent compared to non-recurrent tumors ¹⁴⁵⁸. In addition, YAP target gene *CCN1* (*CYR61*) and *CCN2* (*CTGF*) expression has been found significantly upregulated in BCC aggregates compared to the healthy epidermis ¹⁰⁰, with stromal cells adjacent to the tumor borders displaying increased expression of *CCN2* (*CTGF*) targets, such as fibronectin, type I collagen, and α -smooth muscle actin, accompanied by increased stiffness, compared to the normal dermis ¹⁰⁰. Along with a series of in vitro functional experiments in primary NHKs, Quan and colleagues ultimately proposed that *CCN1* (*CYR61*), but not *CCN2* (*CTGF*), is required for the proliferation and survival of malignant KCs, whereas *CCN2* (*CTGF*) produced by those at BCC borders contributes to the ECM remodeling

observed in the peritumoral stroma ¹⁰⁰ – which in turn may further modulate the activity of YAP signaling ^{1233,1327}.

Regarding studies performed in mice, several different BCC murine models have been described in the literature, with two of the most common ones being: (i) one which results from basal epidermal cell-specific *Ptch1* conditional KO (*K14CreER/Ptch1*^{-/-} mice), and (ii) one which results from basal epidermal cell-specific activation of the expression of a constitutively active mutant of the Smo2 protein (*K14-CreER/Rosa-SmoM2* mice). Debaugnies and colleagues have demonstrated nuclear IHC YAP positivity in BCC tumors from both of these models, as well as an enrichment in YAP gene signatures in the *K14-CreER/Rosa-SmoM2* (referred to as *SmoM2*) BCCs, compared to the normal IFE, including enrichment in bona fide YAP target genes *Ccn1* (*Cyr61*) and *Ccn2* (*Ctgf*) ⁹⁷. Double YAP and TAZ conditional KO performed at the same time point as the induction of *SmoM2* expression in the *K14-CreER/Rosa-SmoM2* mice (resulting in so-called *SmoM2* YAP-TAZ DKO mice) led to *no* BCC development in the course of the 8 weeks post-tamoxifen treatment, in contrast to the control *SmoM2* population ⁹⁷. Interestingly, only a small population of *SmoM2*-expressing epidermal cells were identified in the *SmoM2* YAP-TAZ DKO mice, associated nonetheless with the presence of rare dysplastic lesions, which were revealed to additionally exhibit YAP/TAZ expression, indicative of incomplete KO of these proteins – thus, overall, suggesting that YAP/TAZ activity is required for the initiation of *SmoM2*-dependent BCCs ⁹⁷. Moreover, by performing a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay in their epidermis 3.5 days following tamoxifen administration, they observed a higher number of apoptotic cells in *SmoM2* YAP-TAZ DKO mice compared to *SmoM2* mice, proposing that YAP/TAZ activity is also necessary for the survival of *SmoM2*-expressing epidermal cells ⁹⁷. Overall, the work of Debaugnies and colleagues provides an important piece of evidence for the role of YAP/TAZ signaling in BCC initiation (and similarly for SCC initiation as well, although this remains outside the scope of the particular study) ⁹⁷.

Accordingly, in another hallmark study in the field, Maglic and colleagues verified the expression of *Yap* and *Taz* (*Wwrt1*) within the BCCs of *K14-CreER/Rosa-SmoM2* mice by in situ RNA hybridization, pointing out, however, that, in contrast to *Yap*, which mainly localizes in the epidermal compartment, and especially at the periphery of tumor aggregates, *Taz* (*Wwrt1*) is mainly expressed in the dermal cell populations ⁹⁹. Moreover, in another series of experiments, they identified the presence of a subpopulation of cells with YAP transcriptional activity (reflected in *Ccn1/Cyr61* promoter activity) within the uniformly-*SmoM2*-expressing tumor cells ⁹⁹.

Overall, the existing data from both human and murine BCC examinations supports a tumor-promoting function of YAP (and to a lesser degree of TAZ) in the setting of BCC, consistent with the increased nuclear YAP levels identified in malignant KCs of clinical BCC specimens, compared to those of the normal human epidermis^{97–101}. In addition, there is, interestingly, substantial evidence supporting the notion that YAP and Hh (the main BCC driver^{130,136,172,173}) signaling positively regulate one another within the context of BCC carcinogenesis^{1270,1364–1366,1441}, with the YAP-mediated upregulation of Hh activity potentially being TEAD-independent¹⁹². Moreover, it has been proposed that suppression of the upstream Gα_s-protein kinase A (PKA) axis may contribute to the activation of both YAP and GLI TFs, in so promoting BCC growth⁹⁸. Co-operativity between YAP-TEAD and JNK-c-Jun signaling has also been suggested to facilitate the development of BCC⁹⁹. Lastly, aside from the ones on Hh activity, additional TEAD-independent effects of YAP/TAZ have been suggested for the NF-κB, STAT3 and IFN-γ pathways, in so potentially modulating the BCC immune TME¹⁹².

1.3.9 Pharmacological Targeting of the Hippo/YAP Pathway

Several pharmacological agents that target the Hippo/YAP/TAZ/TEAD signaling cascade are currently under investigation (at pre-clinical or clinical level) for the treatment of either malignant or non-malignant human diseases. Given the *usually* pro-proliferative and growth-promoting function of nuclear YAP/TAZ, drugs which inhibit YAP/TAZ activity can potentially be used in antineoplastic treatment of the so-called “YAP^{on}” malignancies, whereas drugs which enhance YAP/TAZ activity may potentially be utilized in the treatment of diseases requiring stimulation of the regenerative process and/or wound healing, including traumatic brain injury, diabetes melitus, liver pathologies and heart failure, or in malignancies where YAP exerts a tumor-suppressing role^{75,1233,1459}. The first pharmacological category includes: (i) agonists of MST1/2 kinases, (ii) inhibitors of YAP (either of its expression or activation), including CA3 and YAP antisense oligonucleotides (ION537), and (iii) inhibitors of YAP/TAZ-TEAD interaction, such as the photosensitizing agent verteporfin, and VGLL4-mimetic peptides^{75,80,1233,1460}. The second pharmacological category includes: (i) antagonists of MST1/2 kinases, (ii) inhibitors of LATS1/2 kinases, (iii) agonists of TAZ, and (iv) agonists of YAP/TAZ-TEAD interaction. Several *indirect* regulators of the respective molecules are also being explored, including PI3K-AKT/PDK1 inhibitors, AP-1 inhibitors, Src inhibitors, and, intriguingly, statins, which can prevent the nuclear localization of YAP via their modulation of the mevalonate pathway^{75,80,1233,1460}.

A couple of the aforementioned compounds have specifically been investigated in the field of NMSCs, especially in cSCC models, including dasatinib – an Src inhibitor¹⁴⁶¹, and BEZ-235 – a dual PI3K/mTOR inhibitor¹⁴⁶¹. To my knowledge, as of now, no direct or indirect Hippo/YAP regulators have specifically been investigated for BCC treatment; however, taking

into consideration the role and interactions of the Hippo/YAP pathway in driving its pathogenesis, YAP and/or YAP-TEAD inhibitors, as well as AP-1 inhibitors, either alone or in combination with the established HHIs, could theoretically hold promise of a therapeutic potential. Nonetheless, it is important to note, that, given the significance of Hippo/YAP in orchestrating a series of critical cellular processes, it is unsurprising that the drugs targeting its activity can be accompanied by several adverse effects^{75,1233}. Therefore, shedding light into additional aspects of this pathway's regulatory interactions with proteins/molecules in a more tumor cell-specific manner, could enable the development of novel therapeutics with more targeted outcomes – in that direction, elucidating the interplay between CXADR and YAP-1 in the setting of BCC could potentially be of great importance for the management of difficult-to-treat BCC cases.

2. Aim-Importance of the Study

The present study aims to elucidate the potential contribution of the interplay between the JAML-CXADR axis and YAP-1 in the pathogenesis of BCC, the most common cancer overall. Understanding the immune and non-immune mechanisms that drive BCC pathogenesis could potentially lead to novel therapeutic strategies for the management of difficult-to-treat BCCs. Importantly, tumor-promoting molecular mechanisms are shared between BCC and other malignancies, therefore, knowledge of these pathways may lead to better patient stratification and refined treatment options for other skin malignancies. This study specifically aims to:

- i. characterize the influence of the JAML-CXADR axis on the immune cell infiltrate of BCC.
- ii. investigate the effect of the potential CXADR-YAP-1 interaction on the immune-stromal cell interactions in BCC.

In order to address these questions, a series of *in silico* and *in vitro* approaches were employed. Publicly available scRNAseq and ST data of BCC and HC skin biopsies were analyzed. The analysis included characterization of the T lymphocytic infiltrate and its *JAML* expression, of the malignant KC populations, as well as the spatial proximity and/or potential interaction between *JAML*-expressing T cells and *CXADR*-expressing KCs, in BCCs compared to HC skin. Immunofluorescence (IF) was performed to investigate the potential co-localization of CXADR and YAP-1 in human KCs, while IHC examination of human BCC biopsies was carried out in order to explore the level, distribution and potential correlation between CXADR and YAP-1 expression in clinical BCC specimens, as well as the potential association with other clinicopathological parameters. Lastly, scratch assays and Western blot analysis were performed in order to examine the effect of Th2 cytokines or recombinant JAML on the migration of human KCs and on their protein expression levels of CXADR and YAP-1, respectively.

3. Materials and Methods

3.1 Single-cell RNA Sequencing Data Analysis

3.1.1 Data Availability

Two publicly available scRNAseq datasets were initially included in the study. The first one, reported by Yao and colleagues ¹⁴⁶², is available on the Gene Expression Omnibus (GEO) under the accession number GSE141526, and consists of four (4) BCC samples and two (2) ANS samples. The second one, reported by Ganier and colleagues ¹⁴⁶³, is available on ArrayExpress under the accession number E-MTAB-13085, and consists of eight (8) BCC samples and fourteen (14) HC skin samples. Due to the presence of withstanding batch effects despite the performed integration of the two datasets, analysis was ultimately focused solely on the second one (E-MTAB-13085) – thus, all subsequent parameters and workflows described apply to the samples of E-MTAB-13085 (BCC, n = 8; HC, n = 14). Clinical information and number of cells for each sample of the E-MTAB-13085 dataset are displayed on **Table 7**. The skin tone of all patients and healthy donors was of Fitzpatrick scale I/II ¹⁴⁶³.

3.1.2 Quality Control (QC)

Quality control was performed as follows: first, samples with more than 20000 cells loaded on the Chromium chip were excluded (BCC_CHEEK1, BCC_EAR1) to limit the possibility of multiplets ¹⁴⁶⁴. For each of the remaining samples (BCC, n = 6; HC, n = 14), single-cell reads were loaded in R (version 4.5.1) utilizing the Seurat package (version 5.0.0), and elimination of empty droplets was performed using the emptyDrops function of the DropletUtils package (version 1.28.1) ^{1465,1466}. Briefly, emptyDrops examines how distinct the transcript profile of each droplet (each barcode) is compared to that of the ambient RNA pool, in order to distinguish between droplets containing cells with low number of transcripts and droplets devoid of cells which may just contain ambient RNA ^{1465,1466}.

Next, a Seurat object was created for each sample, filtering out features (genes) detected in less than 3 cells. An upper cut-off for mitochondrial DNA percentage (%MT) was set at 20% to exclude apoptotic and/or stressed cells. Lower and upper cut-offs for unique feature counts were set at 350 and 6000, respectively, in order to exclude ambient RNA, apoptotic blebs and cell fragments, and multiplets, respectively. Normalization of the feature expression data for each cell by total expression (NormalizeData), identification of the most variable features (FindVariableFeatures), and scaling of all features (ScaleData) was followed by principal component analysis (PCA) for linear dimensionality reduction (RunPCA) and Uniform Manifold Approximation and Projection (UMAP) for non-linear dimensionality reduction (RunUMAP) under Seurat package (version 5.0.0), before performing exclusion of

<i>Sample name</i>	<i>Condition</i>	<i>Age (years)</i>	<i>Sex</i>	<i>Face/Body</i>	<i>Location</i>	<i>N of cells loaded</i>	<i>N of cells after QC</i>
BCC_NOSE1	BCC	91	Male	Face	Nose	6888	5725
BCC_EAR1	BCC	70	Male	Face	Ear	23915	N/A
BCC_EAR2	BCC	69	Male	Face	Ear	12054	10976
BCC_FOREHEAD1	BCC	73	Male	Face	Forehead	18255	15906
BCC_TEMPLE1	BCC	51	Male	Face	Temple	13513	11349
BCC_TEMPLE2	BCC	59	Male	Face	Temple	9116	7971
BCC_CHEEK1	BCC	39	Male	Face	Cheek	32153	N/A
BCC_CHEEK2	BCC	85	Male	Face	Cheek	10672	9141
FACE_NOSE1	HC	56	Male	Face	Nose	15752	13144
FACE_EAR1	HC	77	Male	Face	Ear	11432	10441
FACE_EAR2	HC	86	Male	Face	Ear	10123	7507
FACE_FOREHEAD1	HC	85	Male	Face	Forehead	15516	13574
FACE_FOREHEAD2	HC	70	Male	Face	Forehead	9154	6798
FACE_FOREHEAD3	HC	80	Male	Face	Forehead	15415	14049
FACE_FOREHEAD4	HC	90	Male	Face	Forehead	12440	11095
FACE_FOREHEAD5	HC	73	Male	Face	Forehead	8388	7707
FACE_TEMPLE1	HC	59	Male	Face	Temple	13602	11909
FACE_TEMPLE2	HC	78	Male	Face	Temple	5801	4796
FACE_CHEEK1	HC	85	Male	Face	Cheek	10763	9456
FACE_CHEEK2	HC	62	Male	Face	Cheek	4522	2875
FACE_CHEEK3b	HC	81	Female	Face	Cheek	8920	7571
FACE_CHEEK4	HC	76	Male	Face	Cheek	14640	13324

Table 7: Demographic and clinical information, and number of cells of each sample from the E-MTAB-13085 dataset. BCC = Basal Cell Carcinoma, HC = Healthy Control, QC = Quality Control, N/A = Not Applicable. *Adapted from Ganier et al., 2024.*

doublets/multiplets using the DoubletFinder package (version 2.0.6)¹⁴⁶⁷. In brief, DoubletFinder creates artificial doublets from the existing scRNAseq data, performs PCA on the merged artificial and real data, and identifies the proportion of artificial k nearest neighbors (pANN) for each droplet¹⁴⁶⁷. pANN values are filtered according to the expected number of doublets, which depends on the number of cells loaded on the chip. Essentially, droplets that cluster most closely to artificial doublets in the principal component (PC) space are identified as doublets and are excluded from downstream analysis¹⁴⁶⁷.

3.1.3 Integration of samples & Cell clustering

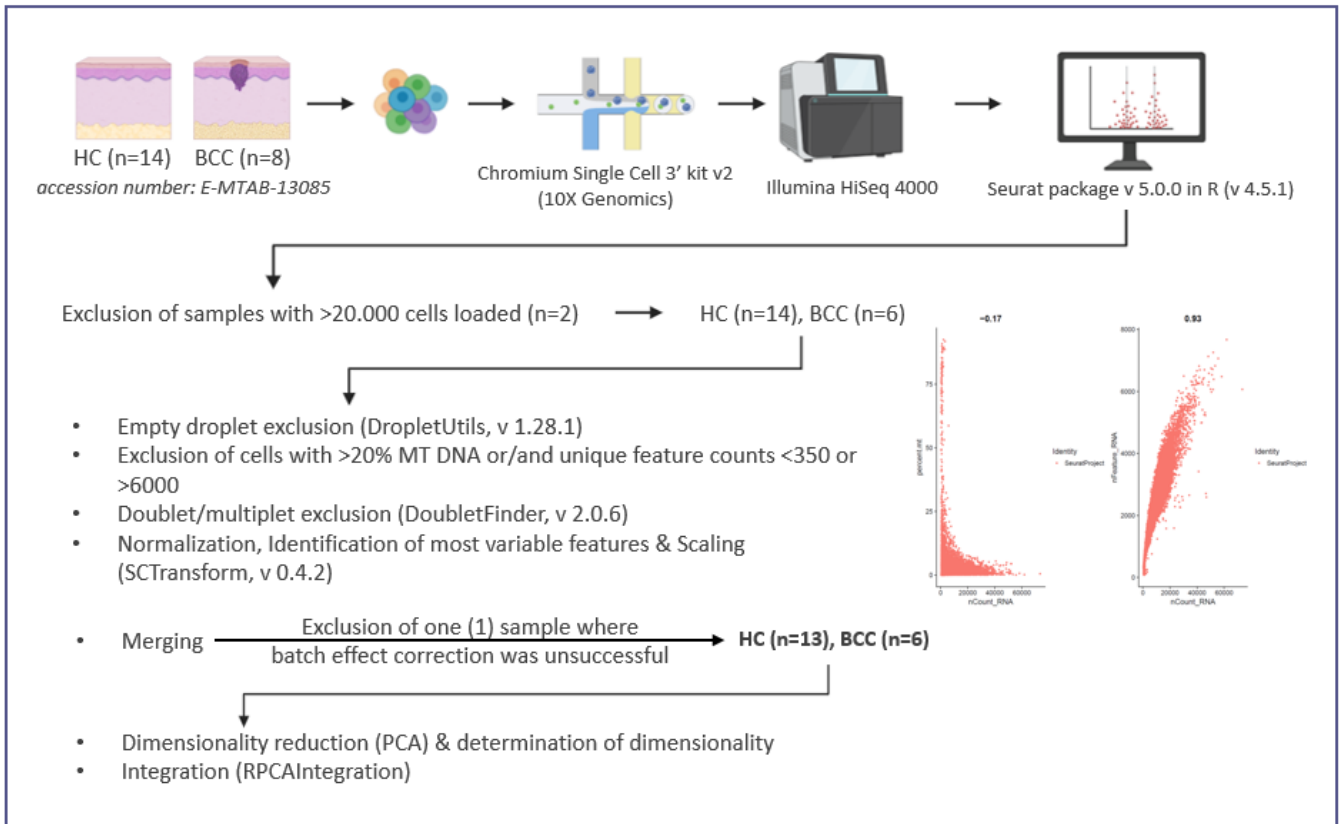
Following the exclusion of multiplets, normalization of count data, identification of the top 3000 most variable features and scaling of said features was performed, with the SCTransform function (using v2 regularization, `vst.flavor="v2"`) of the `sctransform` package (version 0.4.2) ¹⁴⁶⁸. The SCTransformed samples were then merged into one common Seurat object. At this point one additional (HC) sample (FACE_EAR2) was excluded from downstream analysis, due to the presence of prominent batch effects which were not corrected during the subsequent integration step. For the rest of the samples (BCC, $n = 6$; HC, $n = 13$), which comprised a total of 187000 cells, linear dimensionality reduction was performed (RunPCA) utilizing the scaled most variable features of the merged object, followed by integration of the samples (each one represented by a Seurat object), using the `IntegrateLayers` function of the Seurat package, with `RPCAIntegration` as the selected method. A summary of this workflow is depicted in **Methods Figure 1**.

After determining the dimensionality of the dataset (first 30 PCs) based on the `ElbowPlot`, the `FindNeighbors` and `FindClusters` functions from Seurat package were implemented for the identification of cell clusters. A resolution of 0.2 was used, leading to a total of eighteen (18) clusters, which were visualized on a UMAP plot (`RunUMAP`). The `FindAllMarkers` function of Seurat package, with the default Wilcoxon test, was utilized to identify the DEGs for each cell cluster compared to the rest – DEGs were filtered so that only positive ones, detected in a minimum of 25% (0.25) of either of the two compared clusters, and with a minimum log-scale fold change of 0.25, were returned. Cell cluster annotations were carried out based on the DEG profile of each cluster, in conjunction with prior knowledge from the literature for gene markers representative of the expected cell types.

3.1.4 T cell Subclustering

For the higher resolution analysis of T lymphocytes, the four clusters originally annotated as T cells 1, T cells/Innate-like T cells, T cells 2, and Proliferating T cells were subsetted from the object, were merged into a new Seurat object, followed by PCA analysis (`RunPCA`) utilizing the scaled most variable features of the merged object, and ultimately, integration of the discrete samples was performed with the `IntegrateLayers` function and `RPCAIntegration` as the selected method. Clustering of the cells was performed similarly to before, using the `FindNeighbors` function on the first 25 PCs (based on the `ElbowPlot`), and the `FindClusters` function with a selected resolution of 0.4. A total of twelve (12) T cell clusters were generated, and were visualized in a UMAP plot with `RunUMAP`. `FindAllMarkers` was run in order to determine the DEGs of each T cell cluster compared to the rest – these were again

utilized, together with existing knowledge based on the literature, for the annotation of the T cell clusters.



Methods Figure 1: Summary of the workflow followed for the quality control and integration of the scRNAseq data. MT = Mitochondrial, PCA = Principal Component Analysis, RPCA = Reciprocal Principal Component Analysis

3.1.5 KC Subclustering

Similarly to what was followed for the T cell populations, for the higher resolution analysis of the KCs, the cluster originally annotated as Keratinocytes was subsetted from the object, and PCA analysis (RunPCA) with the scaled most variable features of this new object was carried out. Integration of the discrete samples was performed with the IntegrateLayers function and RPCAIntegration as the chosen method. At this step, one (1) HC sample (FACE_CHEEK2) was excluded due to its low number of cells, which was interfering with the integration process. Clustering of the cells was performed by using the FindNeighbors function on the first 25 PCs (based on the ElbowPlot), followed by the FindClusters function with a resolution of 0.2, which led to the identification of nine (9) KC clusters in total, that were visualized in a UMAP plot using RunUMAP. Annotation of the KC clusters was performed on the basis of the DEGs of each cluster compared to the rest, as these were identified by the FindAllMarkers function, along with prior knowledge from the existing literature.

3.1.6 Pathway Enrichment Analysis

Pathway enrichment analysis was performed with the pathfindR package (version 2.6.0)¹⁴⁶⁹. Briefly, based on the DEGs between the clusters or conditions of interest, pathfindR searches for active subnetworks, i.e. groups of interconnected genes within a protein-protein interaction network that are mainly composed of significantly altered genes, and then, based on the genes of filtered active subnetworks identifies the significantly enriched terms (gene sets, pathways), as reported from a reference pathway database¹⁴⁶⁹. Specifically in our data, for each T cell cluster examined, FindMarkers was run to identify the cluster's DEGs compared to the rest of T cell clusters. DEGs were filtered, keeping those expressed in at least 25% (0.25) of the clusters, with a log₂ fold-change greater than 0.5 (either positive or negative), and an adjusted p-value lower than 0.05. run_pathfindR was then called using the gene sets from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, with a minimum and maximum number of genes for each term set at 5 and 500, respectively, and with an adjusted p-value threshold for the enrichment results of 0.01. For pathway enrichment analysis performed on the BCC-derived compared to the HC-derived cells of each T cell cluster, the same workflow was followed, with the only difference being that DEGs were identified between the BCC- and HC- derived cells of each distinct T cell cluster. The same workflow was also implemented for pathway enrichment analysis in the KC clusters.

3.1.7 Copy Number Variation (CNV) Analysis

Copy Number Variation (CNV) analysis of the KCs was performed with the InferCNV algorithm^{1470,1471}. In a glance, based on the scRNAseq data, InferCNV assesses the expression intensity for genes across chromosomal positions and compares it to the average expression intensity or to that of a reference group of cells that are considered normal – in that way it provides an estimation for the presence of genomic gains or losses in the chromosomal areas of the (tumor) cells of interest compared to normal cells (if defined) or to the “average” cell (if no normal cells are determined). InferCNV requires three files as input for creating the Infercnv object (CreateInfercnvObject). With regard to the particular study these were: the matrix containing the raw feature counts for each cell of the KC object (as that was formed following the processing described above) (raw counts matrix), a file with the cluster annotation of each cell, and a file with the chromosomal gene positions. No defined group of cells was used as reference. Infercnv was run on the infercnv object, utilizing a cut-off of 0.1 for the minimum average read counts per gene among the reference cells.

3.1.8 Generation of Gene Modules for KC Clusters

Gene lists were manually curated for different epidermal cell-associated states (stratum basale, stratum granulosum, stratum spinosum, stratum corneum, hair follicle-HF, eccrine

sweat gland, sebaceous gland, proliferating cells, stem cells-SC), based on the existing literature. Two distinct SC gene modules were generated – one containing a series of well-characterized epidermal SC-related genes (*stem_score_a*), and one containing a list of genes identified as DEGs between holoclones and meroclones or paraclones of human epidermal cells in a recent study ¹⁴⁷² (*stem_score_b*). The genes included in each module are demonstrated in **Table 8**. The AddModuleScore function of Seurat was utilized to calculate the average expression level of each module of genes in each of the BCC-derived KC clusters on a single cell level, subtracted by the aggregate expression of a control gene set (n = 100, default). The gene module scores were visualized by Violin plot.

Name of module	Genes included	Ref.
stem_score_a	<i>ITGA6, ITGB4, ITGA2, ITGB1, ITGA3, CD34, CD46, CD200, TP63, SOX9, TCF3, LHX2, NFATC1, TBX1, LGR5, LGR6, LRIG1, SHH, GLI1, COL17A1, PLET1, THY1, DLL1, CSPG4, FRMD4A, KIT, KRT5, KRT14, KRT15, KRT18, KRT19, KRT23, FST, NES, TNC, YAP1, FZD2, FZD3, FZD7, AXIN2</i>	1463,1473–1481
stem_score_b	<i>CASP14, ASS1, ASNS, LGALS7, LGALS7B, LGALS1, IMPA2, TXNIP, CHAC1, SLC7A11, CEL, VEGFA, CD68, THBD, SLC3A2, MSLN, PLA2R1, EMP2, WNT4, ADAM17, SFRP1, FGFR3, EGFR, SLC1A4, BACE1, HNF4A, NUPR1, HIF1A, EPAS1, TP53, TP63, KRT15, DLL1, ATF4, FOS, FOXO1, EGR1, DDIT3, SOX7, ALDH1A1, GJA1, GJB2, PHGDH, PSPH, PSAT1, WEE1, RBL2, VANGL2, EXPH5, USP3, FOPNL (CEP20), ID1, MXRA5</i>	1472
proliferation_score	<i>MKI67, ASPM, PTHLH, HMGA2, CENPE, CENPF, CENPA, TOP2A, E2F1, E2F3, MYC, CCNA2, CCNB1, CCNB2, CCND1, TBX22, FOXM1, PCNA, TYMS, MCM2, MCM3, MCM4, MCM5, MCM6, MCM7, CDK1, CDK2, CDC20, UBE2C, AURKA, AURKB, PLK1, BIRC5, FEN1, RRM1, RRM2, RFC2, RFC3, RFC4, RFC5, PRIM2, POLA1, RPA1, RPA2, RPA3, SMC1A, SMC3, STAG2</i>	1477,1482
basal_score	<i>KRT5, KRT14, TP63, EGFR, IGF1R, TGFBR2, DLL1, ITGA6, ITGB4, ITGB1, ITGA3, TP73, CDH3, CDH1, CTNNB1, CTNNA1, CTNND1, JUP, VCL, NECTIN1, NECTIN2, NECTIN3, NECTIN4, AFDN, OVOL2, CAV1, CAV2, CKAP2, CD46</i>	1463,1473–1475,1477,1479–1481,1483–1486, proteinatlas.org

spinous_score	<i>KRT1, KRT10, KRT2, HES1, NOTCH1, NOTCH3, CEBPB, MAFB, DSG1, DSG2, DSG3, DSG4, DSC1, DSC2, DSC3, JUP, PKP1, PKP2, PKP3, PKP4, KRTDAP, SBSN, CASP14, SPRR1A, SPRR1B, SPRR2A, SPRR2B, SPRR2C, SPRR2D, SPRR2E, SPRR2F, SPRR2G, SPRR3, OVOL1, CTSB</i>	1463,1473– 1475,1477,1479– 1481,1483–1488, proteinatlas.org
granular_score	<i>KRT1, KRT10, KRT2, FLG, FLG2, LOR, IVL, TCHH, CRNN, HRNR, CDSN, CNFN, CLDN1, CLDN2, CLDN3, CLDN4, CLDN5, CLDN6, CLDN7, CLDN8, CLDN9, CLDN10, CLDN11, CLDN12, CLDN14, CLDN15, CLDN16, CLDN17, CLDN18, CLDN19, CLDN20, CLDN22, CLDN23, CLDN24, CLDN25, OCLN, TJP1, TJP2, TJP3, F11R, JAM2, JAM3, CEBPB, KPRP, TGM3, ZNF750, SPINK5, CALML5, DSG1, DSG2, DSG3, DSG4, DSC1, DSC2, DSC3, JUP, PKP1, PKP2, PKP3, PKP4, SLURP1, SPRR1A, SPRR1B, SPRR2A, SPRR2B, SPRR2C, SPRR2D, SPRR2E, SPRR2F, SPRR2G, SPRR3, OVOL1, SMPD2, PLA2G2A, ABCA12, CDSN, KLK5, KLK7, KLK14, RAB11A, CLIP1</i>	1463,1473– 1475,1477,1479– 1481,1483–1491, proteinatlas.org
corneal_score	<i>KRT3, KRT12, FLG, FLG2, LOR, CNFN, CRNN, HRNR, CDSN, KLK5</i>	1473– 1475,1480,1481,148 3,1484,1486, proteinatlas.org
HF_score	<i>MGST1, S100A2, S100A3, S100P, ATP1B3, GJB2, GJB6, SFRP1, FRZB, TM4SF1, CHCHD10, CRABP2, WFDC3, MUCL1, SOX9, SFRP1, TCHH, TCHHL1, FLG, KRT5, KRT6B, KRT9, KRT14, KRT16, KRT17, KRT19, KRT25, KRT26, KRT27, KRT28, KRT31, KRT33A, KRT33B, KRT34, KRT35, KRT40, KRT71, KRT72, KRT73, KRT74, KRT75, KRT79, KRT81, KRT82, KRT83, KRT84, KRT85, KRT86, KRTAP11-1, KRTAP16-1, KRTAP2-1, KRTAP2-2, KRTAP2-3, KRTAP2-4, KRTAP3-2, KRTAP3-3, KRTAP24-1, PRR9, RPTN, TYR, KLK5</i>	1530,1542,1544, 1550–1553, proteinatlas.org
eccrine_score	<i>FAM180B, FAM160A1 (FHIP1A), PLA2R1, CAPN1, KCNN4, PLCB4, PROM2, CLDN7, COL26A1, COL17A1, SCGB2A2, SCGB1D2, CD300LG, ACOT6, PRR4, TRPV4, AQP5, CTNNA2, DCD, KRT17, KRT19, KRT77</i>	1530,1542,1550, 1551,1553, proteinatlas.org

sebaceous_score	<i>SDC1, MGST1, APOE, KRT7, KRT17, FABP7, ELOVL5, ELOVL3, PLIN2, CIDEA, CLMP, ACYP2, APMAP, CLSTN3, FKBP5, HIBADH, JAGN1, LPCAT3, MCCC1, MCCC2, MOSPD1, PEX11A, PEX16, PMPCB, PYGB, RBM47, RNF5, SAMM50, SERPINB1, SLC1A3, SND1, TMED4, TMEM97, TRUB2, NNAT, IL1R2, TINAGL1, WFDC2</i>	1530,1542,1550, 1551,1553,1559, 1560, proteomics.org
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Table 8: List of genes included in each of the epidermal cell-associated state module scores.

3.1.9 Trajectory Analysis

Trajectory analysis of BCC-derived KCs was performed at the cluster level using the TSCAN (pseudo-Time reconstruction in Single-Cell RNA-seq ANalysis) package (version 1.46.0) ^{1494,1495}. TSCAN aims to construct a pseudo-temporal path/ordering of the cells of interest according to the progressive change of their transcriptomic profile. Ordering of the cells is performed with the generation of a Minimum Spanning Tree (MST), which connects the center of the clusters to which cells have been assigned; each cell is projected onto the MST and in so the pseudo-time is acquired. MST was generated (createClusterMST) using the distances between mutual nearest neighbor (MNN) pairs between the clusters. The MST was visualized on top of the KC clusters in a UMAP plot with reportEdges and PlotUMAP. mapCellsToEdges was used to move each cell onto the closest MST edge. The starting point of the trajectory (“root node”) was selected based on the stem_score_a gene module score (see above), together with the entropy value of each cluster, as that was calculated with perCellEntropy. Entropy reflects the diversity of each cluster’s transcriptomic profile, with higher values being suggestive of less differentiated cell clusters ^{1496,1497}. Then, a pseudotime ordering of the cells was obtained, using the orderCells function, by calculating the distance, along the MST, between the “root node” and the new position to which each cell has been mapped with mapCellsToEdges.

3.1.10 Receptor-Ligand Interaction Analysis

Cell-cell interactions between KCs and T cells were analyzed with the nichenet package (version 2.2.1) ¹⁴⁹⁸. In short, nichenet incorporates information for the expression profile of cells of interest from the scRNAseq data with pre-existing information on gene regulatory networks, in order to identify potential interactions between distinct cell populations. The DEGs between the conditions of interest in the “receiver” population are used to predict, which ligands from the “sender” population may have influenced their differential expression, and their cognate receptors in the “receiver” population are uncovered ¹⁴⁹⁸.

More specifically, nichenet utilizes predetermined receptor-ligand networks deposited in Zenodo ¹⁴⁹⁹, including: (i) a ligand-target prior model that describes the potential of a specific ligand regulating the expression of a target gene, (ii) a ligand-receptor network with data on potential ligand-receptor bindings, and (iii) a weighted ligand-receptor network with weights that represent the potential of a ligand binding to a receptor. One KC cluster (KC_0 or KC_3) was assigned as the “receiver” population, while all twelve T cell clusters were assigned the role of the “sender” populations. The list of potential ligands was determined based on the ligands from the receptor-ligand network that are expressed in at least 25% of the cells of the sender (T cell) clusters derived from the condition of interest (BCC or HC) in each case and whose cognate receptors are expressed in the receiver population. The gene set of interest was determined as the list of DEGs between the two conditions (BCC vs. HC, or HC vs. BCC, respectively), filtering, in each case, for those expressed in at least 10% (0.10) of the cells belonging to each condition, with a positive $\log_2$ fold-change of at least 0.25 and an adjusted p-value of at least 0.05. As background genes, the list of all genes in the receiver population that are expressed in at least 25% of its cells was used. Ligand activity analysis was performed with the `predict_ligand_activities` function. The potential ligands were ranked based on the presence of their target genes (as per the ligand-target matrix) in the gene set of interest, compared to the background gene set. Active target genes comprise the genes in the gene set of interest that, based on the ligand-target prior model, display the highest regulatory potential for each of the top-ranked ligands; in other words, they are the top n (here n = 200) targets of a given ligand. These are identified using the `get_weighted_ligand_target_links` function. For better visualization of the prioritized ligands and their active target genes in a heatmap it is recommended that a quantile cut-off is set for the scores in the ligand-target prior model, so that score values below that are set to 0. The cut-off used here was 0.33. In the heatmap, the ligands that pass the score filtering are ordered according to their ranking. The receptors of the receiver cells (KC_0 or KC_3) that display the highest interaction potential with the top-ranked ligands of sender cells (T cells from BCC or HC) can be identified by the `get_weighted_ligand_receptor_links` function, based on the weighted ligand-receptor network. The inferred receptor-ligand interactions are visualized in a heatmap, where both the receptors and the ligands are ordered by hierarchical clustering (essentially meaning that molecules of similar interaction profiles are visualized next to each other).

3.2 Spatial Transcriptomic Data Analysis

3.2.1 Data Availability

A publicly available dataset of ST data, reported by Ganier and colleagues ¹⁴⁶³, and available on ArrayExpress under the accession number E-MTAB-13084, was analyzed. The dataset consists of eight (8) BCC samples and twenty-two (22) HC skin samples. Analysis was performed using primarily the Seurat package (version 5.0.0) in R (version 4.5.1). Clinical information and number of spots for each sample of the E-MTAB-13084 dataset are displayed on **Table 9**. The skin tone of all patients and healthy donors was of Fitzpatrick scale I/II ¹⁴⁶³.

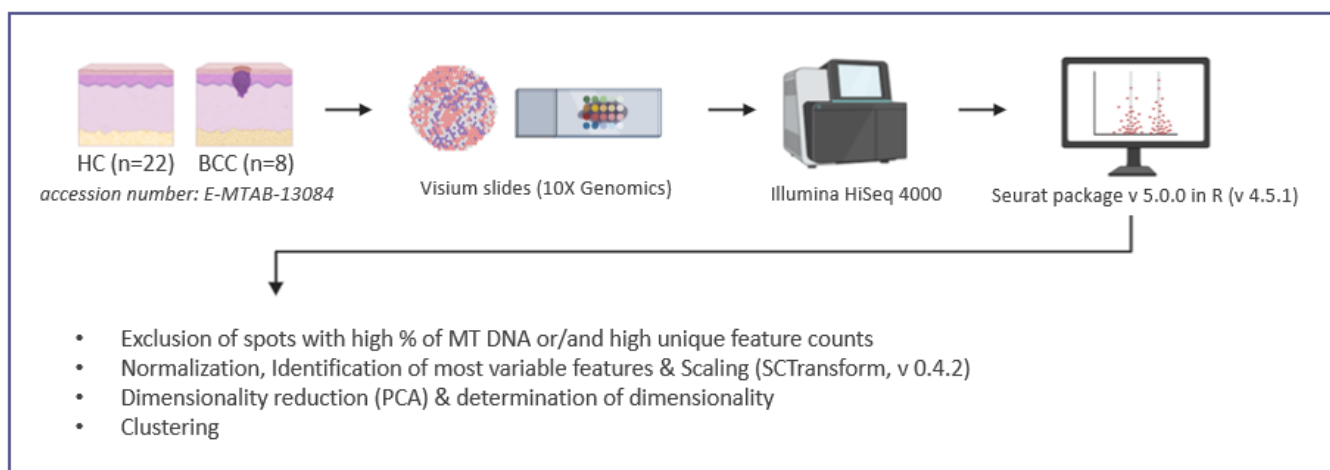
<i>Sample name</i>	<i>Condition</i>	<i>Age (years)</i>	<i>Sex</i>	<i>Face/ Body</i>	<i>Location</i>	<i>N of spots before QC</i>	<i>N of spots after QC</i>
BCC_FACE_NOSE1	BCC	91	Male	Face	Nose	1200	1146
BCC_FACE_NOSE2	BCC	72	Female	Face	Nose	1334	1309
BCC_FACE_NOSE3	BCC	82	Female	Face	Nose	952	916
BCC_FACE_EAR1	BCC	90	Male	Face	Ear	1148	1127
BCC_FACE_FOREHEAD1	BCC	73	Male	Face	Forehead	1043	1007
BCC_FACE_FOREHEAD2	BCC	64	Female	Face	Forehead	932	919
BCC_FACE_CHEEK1	BCC	39	Male	Face	Cheek	813	785
BCC_FACE_CHEEK2	BCC	85	Male	Face	Cheek	1870	1811
FACE_NOSE1a	HC	77	Male	Face	Nose	1552	1499
FACE_NOSE1b	HC	77	Male	Face	Nose	1642	1607
FACE_FOREHEAD1a	HC	55	Male	Face	Forehead	1163	967
FACE_FOREHEAD1b	HC	55	Male	Face	Forehead	1987	920
FACE_FOREHEAD2	HC	73	Male	Face	Forehead	1987	1925
FACE_TEMPLE1a	HC	68	Male	Face	Temple	986	957
FACE_TEMPLE1b	HC	68	Male	Face	Temple	927	853
FACE_TEMPLE2a	HC	33	Female	Face	Temple	1547	1471
FACE_TEMPLE2b	HC	33	Female	Face	Temple	1494	1454
FACE_CHEEK1	HC	39	Male	Face	Cheek	2036	1982
FACE_CHEEK2	HC	76	Male	Face	Cheek	1383	1360
FACE_GLABELLA1	HC	68	Male	Face	Glabella	1577	1540
FACE_SCALP1	HC	68	Male	Face	Scalp	1344	1328
BODY_BACK1a	HC	60	Male	Body	Back	2418	1408
BODY_BACK1b	HC	60	Male	Body	Back	2546	2429

BODY_ABDOMEN1b	HC	47	Male	Body	Abdomen	1755	1482
BODY_INGUINAL1a	HC	55	Male	Body	Inguinal	2097	2071
BODY_INGUINAL1b	HC	55	Male	Body	Inguinal	2247	2204
BODY_INGUINAL2	HC	63	Male	Body	Inguinal	1958	1918
BODY_PUBIS1	HC	52	Male	Body	Pubis	1632	1607
BODY_THIGH1b	HC	53	Male	Body	Thigh	1826	1751
BODY_BREAST1	HC	42	Female	Body	Breast	2097	2071

Table 9: Demographic and clinical information, and number of spots of each sample from the E-MTAB-13084 dataset. BCC = Basal Cell Carcinoma, HC = Healthy Control, QC = Quality Control. *Adapted from Ganier et al., 2024.*

3.2.2 Quality Control (QC) & Downstream Analysis

Quality control included exclusion of spots with high %MT or/and high number of unique feature counts; an upper cut-off for the total number of counts was also used. Unique parameters were set for each separate ST object, according to their characteristics. Cut-off used for the %MT varied between 5 and 15 for the BCC samples, and 5 and 16 for the HC ones. The lower cut-off for unique features was set to 200 in all samples, while the upper cut-off varied between 8000 and 11000 for BCC samples and 6500 and 10000 for HC samples. Upper threshold for total counts ranged between 40000 and 80000 for BCC samples, and between 25000 and 70000 for HC ones. SCTransform, from the sctransform package (version 0.4.2), was performed on each separate object for normalization of count data, identification of the top 3000 most variable features, and scaling of those features, followed by linear dimensionality reduction with RunPCA, clustering with FindNeighbors and FindClusters, and non-linear dimensionality reduction with RunUMAP. The dimensionality of the dataset was determined individually for each sample according to the ElbowPlot, and so was the resolution used for the clustering. Spatially variable genes were identified with the FindSpatiallyVariableFeatures function. A summary of this workflow is depicted in **Methods Figure 2**.



Methods Figure 2: Summary of the workflow followed for the quality control of the Spatial Transcriptomic data. MT = Mitochondrial, PCA = Principal Component Analysis

3.3 In Vitro Experiments

3.3.1 Human Primary Cells, Human Cell Lines, and Cell culture

3.3.1.1 Human primary T cells

Human primary naïve CD4⁺ T cells were isolated from healthy donor (n = 5) peripheral blood samples obtained from the blood transfusion department of St James's University Hospital, Dublin, Ireland, as described below (see Naïve CD4⁺ T cell Stimulation and Polarization & Flow Cytometry). Ethical approval was obtained by the Ethics Committee of the St James's University Hospital.

3.3.1.2 Human KC cell lines

HaCaT (human epidermal cell line) and modified HaCaT cell lines were kindly provided by *Professor Evangelos Kolettas* (Department of Biology, Faculty of Medicine, University of Ioannina). Specifically, three distinct cell lines were utilized: (i) WT HaCaT cells, (ii) a c-Fos-overexpressing HaCaT cell line (referred to as CFOS), and (iii) a HaCaT cell line carrying the plasmid vector of CFOS cells without the *FOS* gene (referred to as XIT) and serving as the negative control of the CFOS cells. Briefly, as previously described in Markopoulou, Kontargiris et al. (2009) ¹⁵⁰⁰, the CFOS HaCaT cells carry the pZipNeoSV(X)-c-Fos (PM43.1) plasmid, which results from the cloning of the almost full-length human *FOS* cDNA into the pZipNeoSV(X) retroviral vector, downstream of its Moloney murine leukemia virus (M-MuLV) promoter. The pZipNeoSV(X) vector contains two cleavage sites for distinct restriction endonucleases (Bam HI and Xho I) in the place of the retroviral gag, pol, env sequences, as

well as a neomycin phosphotransferase resistance gene (NeoR)¹⁵⁰¹. The human *FOS* cDNA is inserted within the Bam HI site of pZipNeoSV(X), while the Xho I site harbors the pBR322 and SV40 sequences, which are required for the propagation and replication of the vector DNA in host cells, as well as a transposon Tn5 DNA sequence. Phoenix cells were transfected with the pZipNeoSV(X)-c-Fos (PM43.1) plasmid, and supernatants containing the generated viral particles were collected and used for infection of HaCaT cells¹⁵⁰⁰. Transfected HaCaT cells were selected using G418, resistance against which is provided in mammalian cells by the transposon Tn5 DNA sequence of the retroviral vector. The same sequence is also responsible for resistance against kanamycin in *E. coli*.

3.3.1.3 Cell culture of human KC cell line cells

HaCaT cells were cultured in complete medium (RPMI 1640 with L-glutamine) supplemented with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin. Cells were incubated in 37°C, under humidified (90% humidity), 5% CO₂ and atmospheric (20.8%) O₂ concentration conditions, in a CellXpert® C170i cell culture incubator (Eppendorf).

Cell passage was carried out as follows (reported amounts apply to cells cultured in 100 mm x 20 mm cell culture dishes): Medium was aspirated from the dish, and cells were washed with 5 ml of Dulbecco's phosphate-buffered saline (DPBS). One (1) ml of Accutase® was added, and cells were incubated for approximately 15 minutes. Accutase® was neutralized by the addition of 10 ml DPBS, and the cell suspension was collected in a 15 ml falcon tube. Collected cells were centrifuged at 400 relative centrifugal force (rcf) for 5 minutes. After the supernatant was discarded, the cell pellet was resuspended in complete medium (RPMI 1640 with L-glutamine) supplemented with 10% FBS and 2% penicillin-streptomycin, and cells were re-seeded in new plates and put back in the incubator. When required, cells were frozen in 10% dimethyl sulfoxide (DMSO) in FBS.

3.3.2 Human Biopsies

Archived formalin-fixed, paraffin-embedded (FFPE) tissue sections of lesional skin biopsies from patients with BCC (n = 4) were used for IHC examination, in collaboration with *Associate Professor in Pathology, Evangelia Lampri* (Faculty of Medicine, University of Ioannina). Ethical approval was obtained by the Ethics Committee of the University Hospital of Ioannina. Demographic and clinicopathological characteristics of the patients with BCC and their tumors are shown in **Table 10**.

Sample	Sex	Age (years)	Localization	Max. diameter (cm)	Subtype
bcc1	Male	78	Forehead	2.5	Infiltrative
bcc2	Male	82	Cheek	1.8	Nodular
bcc3	Female	77	Nose	1.3	Mixed; nodular & infiltrative
bcc4	Male	65	Back	2.4	Mixed; nodular & infiltrative

Table 10: Demographic and clinicopathological characteristics of the patients with BCC and their tumors.

3.3.3 Naïve CD4+ T cell Stimulation and Polarization & Flow Cytometry (FC)

Isolation, polarization and FC analysis of human primary naïve CD4+ T cells was performed by *PhD candidate Roozbeh Sanaei* (Dublin City University, Dublin, Ireland).

3.3.3.1 *Reagents used*

The reagents used for the isolation, polarization and FC analysis of human primary naïve CD4+ T cells are listed in **Table 11**.

Reagent	Source	Catalogue Number
Antibodies		
ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator	STEMCELL Technologies	# 10990
Purified anti-human IL-4 antibody	Biolegend	# 500701
Purified anti-human IFN-γ antibody	Biolegend	# 506501
Purified anti-human IL-17A antibody	Biolegend	# 512702
PE/Cyanine7 anti-human CD3 antibody	Biolegend	# 344815
Brilliant Violet 605™ anti-human CD4 antibody	Biolegend	# 344645
Brilliant Violet 650™ anti-human CD8 antibody	Biolegend	# 344729
BD OptiBuild™ BV510 mouse anti-human JAML (AMICA)	BD Biosciences	# 749804
Brilliant Violet 421™ anti-human IFN-γ antibody	Biolegend	# 506537
PE anti-human IL-4 antibody	Biolegend	# 500808
Cytokines		
Recombinant human TGF-β1 (carrier-free)	Biolegend	# 781802
Recombinant human IFN-γ (carrier-free)	Biolegend	# 570204

Recombinant human IL-2 (carrier-free)	Biolegend	# 589104
Recombinant human IL-4 (carrier-free)	Biolegend	# 574004
Cell Stimulation		
eBioscience™ Cell Stimulation Cocktail (500X)	Invitrogen	# 00-4970-93
eBioscience™ Brefeldin A Solution (1000X)	Invitrogen	# 00-4506-51
eBioscience™ Monensin Solution (1000X)	Invitrogen	# 00-4505-51
Solutions & Buffers		
RPMI 1640 with L-glutamine	Biosera	# LM-R1640/500
Fetal Bovine Serum (FBS) Europe	Biosera	# FB-1200/500
Penicillin-Streptomycin Solution 100X	Biowest	# L0022-100
Dulbecco's PBS (1x) without Ca & Mg, without Phenol Red	Capricorn Scientific	# PBS-1A
Human TruStain FcX™ (Fc Receptor Blocking Solution)	Biolegend	# 422301
Powders		
Sodium azide ReagentPlus®	Sigma-Aldrich	# S2002
Paraformaldehyde, 95%, powder	Sigma-Aldrich	# 158127
Kits		
MojoSort™ Human CD4 Naïve T Cell Isolation Kit	Biolegend	# 480041
Human IL-4 DuoSet ELISA	R&D Systems	# DY204
Human IFN-gamma DuoSet ELISA	R&D Systems	# DY285B
Zombie NIR™ Fixable Viability Kit	Biolegend	# 423105
eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set	Invitrogen	# 00-5523-00

Table 11: Reagents used for the isolation, stimulation, polarization and FC analysis of human primary naïve CD4+ T cells.

3.3.3.2 Isolation, stimulation and polarization of human primary peripheral naïve CD4+ T cells

PBMCs were isolated from healthy donor buffy coats and lethally irradiated buffy coats. Naïve CD4+ T cells were then isolated by utilizing the MojoSort™ Human CD4 Naïve T Cell Isolation Kit (Biolegend, 480041), based on manufacturer's instructions. The isolated CD4+ T

cells were cultured with complete medium (RPMI 1640 with L-glutamine) supplemented with 10% FBS and 2% penicillin-streptomycin in flat bottom 96-well plates, at a concentration of 4×10^6 cells/ml, in the presence of 5×10^5 lethally irradiated PBMCs/ml, at 37°C, under humidified (90% humidity), 5% CO₂ and atmospheric (20.8%) O₂ concentration conditions. Cells were stimulated with anti-CD3/anti-CD2/anti-CD28 beads (STEMCELL Technologies, 10990) as per manufacturer's instructions. For the polarization of naïve CD4⁺ T cells towards Th1, the cultures were supplemented with purified anti-human IL-4 antibody (10 µg/ml, Biolegend, 500701), recombinant human TGF-β (10 µg/ml, Biolegend, 781802), IFN-γ (20 µg/ml, Biolegend, 570204) and IL-2 (20 µg/ml, Biolegend, 589104), while for the polarization of naïve CD4⁺ T cells towards Th2, cultures were supplemented with purified anti-human IFN-γ (10 µg/ml, Biolegend, 506501) and anti-human IL-17 (2 µg/ml, Biolegend, 512702) antibodies, and recombinant human TGF-β (10 µg/ml, Biolegend, 781802), IL-4 (20 µg/ml, Biolegend, 574004) and IL-2 (20 µg/ml, Biolegend, 589104). Cells were cultured for 13 days, with medium changes every 3 days.

Polarization was assessed on day 13 by ELISA, utilizing the human IL-4 (R&D Systems, DY204) and human IFN-γ (R&D Systems, DY285B) DuoSet ELISA kits, as per manufacturer's instructions, for IFN-γ and IL-4 detection, as well as by FC analysis (see below).

3.3.3.3 Flow Cytometric analysis

FC analysis was performed to assess the efficiency of Th1 and Th2 polarization, as well as examine the expression of JAML in the stimulated Th1 and Th2 polarized cells compared to the non-polarized.

T cells were transferred to wells of a round bottom 96-well plate (1×10^6 cells/well), were washed once with DPBS (200 µl/well), and were incubated with (50 µl/well of) diluted 1:250 in DPBS LIVE/DEAD stain (Zombie NIR™ Fixable Viability Kit, Biolegend, 423105) for 30 minutes at 4°C. Cells were then washed once with DPBS (200 µl/well) by being spun at 400 rcf for 3 minutes, were resuspended in (20 µl/well of) Fc receptor blocking solution (Human TruStain FcX™, Biolegend, 422301), as per manufacturer's instructions, and were incubated with the Fc block for 10 minutes at 4°C. Without removing the Fc block, the antibody master mix was added to the cells (30 µl/well), and cells were incubated in it for 30 minutes at 4°C. The antibodies used here were the following, in dilutions according to the manufacturer's instructions: PE/Cyanine7 anti-human CD3 (Biolegend, 344815), BV605 anti-human CD4 (Biolegend, 344645), BV650 anti-human CD8 (Biolegend, 344729) and BV510 anti-human JAML (BD Biosciences, 749804). Then, cells were washed with FACS buffer (2% FBS in

DPBS, supplemented with 0.005% sodium azide); 200 µl of FACS buffer were added per well and cells were spun at 400 rcf for 3 minutes.

For the Th1 and Th2 polarization characterization, intracellular staining for IFN-γ and IL-4 (respectively) was performed. First, T cells were incubated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (eBioscience™ Cell Stimulation Cocktail, Invitrogen, 00-4970-93), as per manufacturer's instructions, for 1h at 37°C, 5% CO₂, atmospheric (20.8%) O₂ and 90% humidity conditions, in order to induce cytokine production. Then, brefeldin A (eBioscience™ Brefeldin A Solution, Invitrogen, 00-4506-51) and monensin (eBioscience™ Monensin Solution, Invitrogen, 00-4505-51) were added to the cells, according to the manufacturer's instructions, and cells were incubated for 5 h at the same conditions, in order to inhibit cytokine secretion. Next, following the LIVE/DEAD staining and Fc receptor blocking, performed as described above, for the intracellular staining, T cells were resuspended, after the FACS wash step, in fixation-permeabilization buffer (eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set, Invitrogen, 00-5523-00) for 30 min at 4°C. Then, cells were washed once with permeabilization buffer (eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set, Invitrogen, 00-5523-00), and were resuspended in 30 µl/sample of the antibody master mix. The antibodies were diluted in permeabilization buffer, according to the manufacturer's instructions. The antibodies used for the intracellular staining were: BV421 anti-human IFN-γ (Biolegend, 506537) and PE anti-human IL-4 (Biolegend, 500808). T cells were incubated with the antibodies for 30 minutes at 4°C, and were then washed once with permeabilization buffer, followed by one wash with FACS buffer.

After the FACS buffer washing step, irrespective of intracellular staining or not, cells were fixed with 1% paraformaldehyde (Sigma-Aldrich, 158127) solution by adding 50 µl of it per well and incubating at 4°C for 10-30 minutes, followed by a final wash step with FACS buffer. Cells were ultimately analyzed on the CytoFLEX LX Flow Cytometer (Beckman Coulter), located at the University College Dublin (UCD), Dublin, Ireland.

3.3.4 Scratch Assay

3.3.4.1 *Reagents used*

The reagents used for scratch assays performed on HaCaT cells are listed in **Table 12**.

Reagent	Source	Catalogue Number
<i>Cytokines and other recombinant proteins</i>		
Recombinant human IL-4	Biolegend	# 574004
Recombinant human IL-5	Biolegend	# 791304
Recombinant human IL-13	Biolegend	# 571104
Recombinant mouse JAML	Biolegned	# 776806
<i>Solutions & Buffers</i>		
RPMI 1640 with L-glutamine	Biosera	# LM-R1640/500
Fetal Bovine Serum (FBS) Europe	Biosera	# FB-1200/500
Penicillin-Streptomycin Solution 100X	Biowest	# L0022-100
Dulbecco's PBS (1x) without Ca & Mg, without Phenol Red	Capricorn Scientific	# PBS-1A

Table 12: Reagents used for the scratch assay performed on HaCaT cells.

3.3.4.2 Scratch assay procedure

Scratch assay was performed on HaCaT cells; all three HaCaT cell lines (WT, CFOS, XIT) were utilized. Cells were seeded in 24-well plates (seeding density: 90000 cells/well for the WT and XIT cells, 100000 cells/well for the CFOS cells) and were grown to confluency. Once they had formed a confluent monolayer, cells underwent serum starvation for 24 hours (culture with serum-low medium: RPMI supplemented with 1% FBS plus 2% penicillin-streptomycin) in order to arrest cell proliferation. Next, two perpendicular scratches were introduced to each monolayer manually, using a 200 µl pipette tip. Wells were washed twice with DPBS to remove detached scratched cells from the well. Finally, the treatments of interest were introduced to the cells in serum-low medium; HaCaTs of all three lines were treated with either 20 ng/ml of recombinant human IL-4 (Biolegend, 574004), 20 ng/ml of recombinant human IL-5 (Biolegend, 791304), 20 ng/ml of recombinant human IL-13 (Biolegend, 571104), a combination of IL-4 (20 ng/ml), IL-5 (20 ng/ml) and IL-13 (20 ng/ml), or 1.35 µg/ml of recombinant mouse JAML (Biolegned, 776806). Cells treated with serum-low medium were used as the negative control. Wounds were photographed directly after their introduction (t = 0), and at indicated time points (4h, 20h, 24h) after the cells were placed back in the incubator at 37°C, 5% CO₂, atmospheric (20.8%) O₂ and 90% humidity.

3.3.4.3 Image acquisition & Image analysis

Photos were taken under phase-contrast microscopy, with the Motic AE2000 microscope, using the 4x objective lens and the Motic Images Plus 3.0 software. To account for the small, yet existing, differences of the initial wound area between the different wells (which were expected given that the wound is generated manually), when calculating the migration rate of the cells under the different conditions, the wound area of each well at each time point was first normalized to the average of the initial ($t = 0$) wound area from all WT controls (from all three experiments). Then, the wound area of each time point of interest was normalized to the initial ($t = 0$) wound area of the respective condition, and multiplied by 100. The difference between 100% and this number corresponded to the percentage of the initial wound area covered at a given time point for each condition. The measurement of wound area (in pixels²) was performed using the Wound_healing_size_tool (updated) on ImageJ/Fiji.

3.3.5 Western Blot Analysis

3.3.5.1 Reagents used

The reagents used for the WB experiments are listed in **Table 13**.

Reagent	Source	Catalogue Number
Cytokines and other recombinant proteins		
Recombinant human IL-4	Biolegend	# 574004
Recombinant human IL-5	Biolegend	# 791304
Recombinant human IL-13	Biolegend	# 571104
Recombinant mouse JAML	Biolegend	# 776806
Antibodies		
CAR rabbit polyclonal antibody	Invitrogen	# PA5-110995
YAP1 mouse monoclonal antibody	Proteintech	# 66900-1-Ig
Alpha-tubulin rabbit polyclonal antibody	Cell Signaling Technology	# 2144
HRP-conjugated purified goat anti-rabbit IgG antibody	Nepenthe	# NA0010201M1
HRP-conjugated goat anti-mouse IgG antibody	ABclonal	# AS003
Solutions & Buffers		

Radioimmunoprecipitation assay (RIPA) protein extraction buffer 5x	Bioworld	# 41820002-4
2X Odorless Protein Loading Buffer, (reducing)	NEPENTHE	# NB053146020
Acrylamide/Bis-acrylamide, 30% solution	Sigma-Aldrich	# A3699
Hydrochloric acid	Honeywell-Fluka	# 30721-2.5L
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich	# T9281-25ML
FastGene prestained protein marker, 7-band protein ladder (15 - 130 kDa)	NIPPON Genetics EUROPE	# MWP01
Methanol	Fisher Scientific	# M-4000-17
Tween® 20	Sigma-Aldrich	# P9416
NeoPRO Femto Western ECL Substrate	Neo Biotech	# NB-59-0001-250ML
2-Mercaptoethanol (β-Mercaptoethanol)	Sigma-Aldrich	# M6250
Powders		
Sodium dodecyl sulfate (SDS), BioReagent, suitable for electrophoresis	Sigma-Aldrich	# L3771-100G
Ammonium persulfate (APS)	Sigma-Aldrich	# A3678-25G
Trizma® base	Sigma-Aldrich	# T-1503
Glycine	Sigma-Aldrich	# G-8898
Sodium Chloride (NaCl)	Sigma-Aldrich	# 31434
Sodium azide ReagentPlus®	Sigma-Aldrich	# S2002
Enzymes		
Proteinase K	Transgen biotech	# ER101
Kits		
Pierce™ bicinchoninic acid (BCA) protein assay kit	ThermoFisher Scientific	# 23227

Table 13: Reagents used for HaCaT cell lysate preparation, total protein quantification and Western Blot analysis.

3.3.5.2 Lysate preparation

HaCaT cells (WT, CFOS, XIT) were seeded into 6-well plates and were incubated for 24 hours at 37°C, 5% CO₂, atmospheric (20.8%) O₂ and 90% humidity. Then, cells were treated with the following factors at the indicated final concentrations: recombinant human IL-4 (20

ng/ml, Biolegend, 574004), recombinant human IL-5 (20 ng/ml, Biolegend, 791304), recombinant human IL-13 (20 ng/ml, Biolegend, 571104), a combination of IL-4 (20 ng/ml), IL-5 (20 ng/ml) and IL-13 (20 ng/ml), or recombinant mouse JAML (1.35 µg/ml, Biolegend, 776806). Untreated cells were used as negative control. Cells were incubated with the treated medium, under the same conditions, until they reached a confluency of at least 70%.

Next, supernatants were collected into 1.5 ml Eppendorf tubes and stored in the freezer (-20°C). Cells were washed with DPBS, and were lysed with diluted 1:5 with double distilled water (ddH₂O) Radioimmunoprecipitation assay (RIPA) protein extraction buffer 5x (Tris-HCL 250mM, NaCL 750mM, NP-40 5%, sodium deoxycholate 2.5%, SDS 0.5%) (Bioworld, 41820002-4) supplemented with proteinase K (1:1000, Transgen biotech, ER101) for 30 minutes while being kept in ice. Lysates were then collected into 1.5 ml Eppendorf tubes and were centrifuged at 10000 revolutions per minute (rpm) for 5 minutes at 4°C. Supernatants were collected and transferred to new Eppendorf tubes, and were stored in the freezer (-20°C), while the cell debris pellet was discarded.

3.3.5.3 Total protein quantification

Before running the gel electrophoresis, total protein quantification of each cell lysate was performed using the Pierce™ bicinchoninic acid (BCA) protein assay kit (ThermoFisher Scientific, 23227), as per manufacturer's instructions. Briefly, the BCA assay is a colorimetric biochemical method for the detection and quantification of total protein in a solution. It relies on the reduction of Cu²⁺ ions to Cu¹⁺ in the presence of peptide bonds, under alkaline conditions (potassium tartrate), and the subsequent formation of a complex between two BCA molecules and one Cu¹⁺ cation – this complex is characterized by its purple color and displays strong absorbance at 562 nm, the intensity of which increases in a linear fashion with increasing protein concentrations ^{1502,1503}.

In a 96-well plate, seven serial dilutions (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64) of bovine serum albumin (BSA) with a stock concentration of 2 mg/ml were performed, using a 1:10 diluted solution of the one used for cell lysis (essentially, RIPA protein extraction buffer 0.1x, plus proteinase K 1:10000). These represent the “standards”. Additionally, two wells received only the 1:10 diluted lysis solution, serving as the “blanks”. Next, the samples were loaded in neighboring wells: cell lysates were thawed, and a small amount of each required for the assay (approximately 5 µl) was diluted 1:10 with ddH₂O (final volume 50 µl) before being loaded into the respective well. In all wells the BCA reagents A and B were added in a 50:1 ratio, respectively, and cells were placed in the incubator at 37°C for 30 minutes (as higher temperatures promote the formation of the reaction product). Finally, absorption at 562 nm for each well was measured using the BioTek Synergy HTX multi-mode microplate reader

(Agilent), and total protein concentration for each sample was automatically calculated, according to the standard curve generated from the wells of known BSA concentration ("standards"), taking into consideration the "background" optical density of the cell lysis buffer ("blanks").

3.3.5.4 Sample preparation and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Based on the calculated total protein concentration, the appropriate amount of each lysate was mixed at a 1:1 ratio with a 2x reducing odorless protein loading buffer (NEPENTHE, NB053146020). Protein samples were denatured at 100°C in a dry bath for 5 minutes.

Hand-cast 10% acrylamide resolving gels, along with 4% acrylamide stacking gels were prepared in-house. The following ingredients were used for preparation of: (i) the resolving gel: ddH₂O, 30% w/v acrylamide/bis-acrylamide solution, 1.5 M Tris-HCl (pH 8.8), 10% w/v SDS solution, 10% w/v ammonium persulfate (APS) solution, tetramethylethylenediamine (TEMED), and (ii) the stacking gel: ddH₂O, 30% acrylamide/bis-acrylamide solution, 1.0 M Tris-HCl (pH 6.8), 10% w/v SDS solution, 10% w/v APS solution, TEMED. Approximately 20 µl of each sample were loaded into the wells of the gel. For part of the experimental repetitions, a protein ladder was also used [FastGene prestained protein marker, 7-band protein ladder (15 - 130 kDa), NIPPON Genetics EUROPE, MWP01]; 5 µl of the protein ladder were loaded in a separate well. Electrophoresis was performed with constant voltage settings; for two gels: 100 V, until the dye passes the bottom of the stacking gel, and 150 V onwards until the dye reaches the bottom of the resolving gel. Running time ranged between 40 and 90 minutes. The running buffer used for SDS-PAGE consisted of 24.9 mM Trizma® base, 250.4 mM glycine and 0,1% w/v SDS.

3.3.5.5 Protein transfer

Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (pore size 0.2 µm, SERVA, 42515.01), activated with methanol (Fisher Scientific, M-4000-17), using a wet transfer protocol, with constant current settings; for two gels: 250 mA, and a transfer time of 1 hour. The transfer buffer consisted of 48 mM Trizma® base, 39 mM glycine, 0.05% w/v SDS, and 20% v/v methanol.

3.3.5.6 Immunodetection

Membranes were incubated with 5% w/v milk powder in Tris-buffered saline (TBS; 20 mM Trizma® base, 136.9 mM NaCl, pH 7.6) with 0.1% Tween® 20 detergent (TBS-T) for 1 hour at RT while on a rocker or overnight at 4°C, for the blocking step. Incubation with the primary antibody was performed either overnight at 4°C on a rocker (in the first case), or for at

least 2 hours at RT on a rocker (in the second case). The following primary antibodies with the indicated dilutions were used: CAR (CXADR) rabbit polyclonal antibody (1:2500, Invitrogen, PA5-110995), YAP1 mouse monoclonal antibody (1:2500, Proteintech, 66900-1-Ig), and α -tubulin rabbit polyclonal antibody (1:1000, Cell Signaling Technology, 2144). All primary antibodies were diluted in 0.5% w/v milk powder plus 0.05% sodium azide in TBS-T, and were kept in -20°C for up to 10 uses. Following incubation with the primary antibodies, membranes were washed three times (5 minutes each) with TBS-T, while on the rocker at RT, and were then incubated with the secondary antibodies for at least 1 hour on the rocker at RT. The secondary antibodies used and their respective dilutions are as follows: Horseradish peroxidase (HRP)-conjugated purified goat anti-rabbit IgG antibody (1:3000, Nepenthe, NA0010201M1) and HRP-conjugated goat anti-mouse IgG (H+L) antibody (1:3000, ABclonal, AS003). Secondary antibodies were diluted in 0.5% w/v milk powder in TBS-T. Since no sodium azide was used because of its interference with HRP, secondary antibodies were not stored for re-use. Following incubation with the secondary antibodies, membranes were washed at least three times (5 minutes each) with TBS-T, before being developed.

3.3.5.7 Image acquisition & Image analysis

To activate HRP on the secondary antibodies for image acquisition, 1 ml of the NeoPRO Femto solutions A and B (Neo Biotech, NB-59-0001-250ML) in a 1:1 ratio, containing an enhanced chemiluminescence (ECL) substrate, was added to each membrane, and chemiluminescence signal detection was carried out with the Molecular Imager® ChemiDoc™ XRS System (Bio-Rad).

Image analysis was performed with the use of ImageJ/Fiji. Pixel density for each band minus that of its background was measured with the according ImageJ/Fiji tools, and was normalized to the loading control protein (α -tubulin).

3.3.5.8 Stripping and Reprobing of WB membranes

Some of the WB membranes underwent stripping and reprobing. In order to release the antibodies from the protein, the PVDF membrane was first washed three times (5 minutes each) with TBS-T, while gently agitated on the rocker at RT, and was then incubated for 15 minutes, under the same conditions, with the stripping buffer, which consists of 62.5 mM 1.0 Tris-HCl (pH 6.8), 2% w/v SDS and 0.8% v/v β -mercaptoethanol and is heated to 55°C prior to use. The membrane was then washed at least five times (5 minutes each) with TBS-T, and was blocked with 5% w/v milk powder in TBS-T for 1 hour at the rocker at RT, or overnight at 4°C. Stripped membranes were either immediately reprobed with a new primary antibody (derived from a different species than that of the previous primary used on the same membrane) followed by incubation with the appropriate secondary antibody, or were stored at

-20°C until the time to reprobe them. Membranes were stripped and reprobed a maximum of two times each.

3.3.6 Immunofluorescence (IF)

3.3.6.1 Reagents used

The reagents used for the immunofluorescence staining of HaCaT cells for CXADR and YAP-1 are listed in **Table 14**.

Reagent	Source	Catalogue Number
Antibodies		
CAR rabbit polyclonal antibody	Invitrogen	# PA5-110995
YAP1 mouse monoclonal antibody	Proteintech	# 66900-1-Ig
Alexa Fluor™ 488 donkey anti-rabbit IgG (H+L)	Invitrogen	# A21206
Alexa Fluor™ 568 donkey anti-mouse IgG (H+L)	Invitrogen	# A10037
Solutions & Buffers		
Dulbecco's PBS (1x) without Ca & Mg, without Phenol Red	Capricorn Scientific	# PBS-1A
Formaldehyde solution 37 wt. % in H ₂ O	Sigma-Aldrich	# 252549
Triton X-100	Atom Scientific	# GPS9031-E
Fetal Bovine Serum (FBS) Europe	Biosera	# FB-1200/500
DAPI Fluoromount-G®	SouthernBiotech	# 0100-20

Table 14: Reagents used for the fixation, permeabilization and immunofluorescence staining of HaCaT cells.

3.3.6.2 Immunofluorescence staining

WT HaCaT cells were seeded into 8-well chambered coverslips with an ibiTreat treated-surface (Ibidi µ-Slide 8 Well high, Ibidi, 80806) at two different densities: 20000 cells/well and 10000 cells/well. Cells were incubated at 37°C, 5% CO₂, atmospheric (20.8%) O₂ and 90% humidity for 24 hours. Next, the medium was aspirated from each well, and cells were washed twice with DPBS. Cells were fixed with 10% formaldehyde (Sigma Aldrich, 252549) diluted in DPBS for 10 minutes, and then washed twice with DPBS. Cell permeabilization was achieved by adding a 0.5% Triton X-100 (Atom Scientific, GPS9031-E) in DPBS solution for 10 minutes;

cells were again washed twice with DPBS. The blocking step was performed by the addition of 5% FBS plus 0.2% Triton X-100 diluted in DPBS for 1 hour at RT. Cells were incubated overnight at 4°C with the following primary antibodies at the indicated dilutions: CAR (CXADR) rabbit polyclonal antibody (1:250, Invitrogen, PA5-110995), YAP1 mouse monoclonal antibody (1:400, Proteintech, 66900-1-Ig), or a combination of the two. All antibody dilutions (including those of the secondary antibodies; see below) were performed in a solution of 1.25% FBS plus 0.05% Triton X-100 in DPBS (referred to hereafter as antibody diluent). The next day, cells were washed thrice with antibody diluent, and incubated for 1 hour at RT with the following secondary antibodies: Alexa Fluor™ 488 donkey anti-rabbit IgG (H + L) (1:1000, Invitrogen, A21206) for the anti-CXADR primary, and/or Alexa Fluor™ 568 donkey anti-mouse IgG (H + L) (1:1000, Invitrogen, A10037) for the anti-YAP1 primary, succeeded by five washes with antibody diluent. Ultimately, DAPI (4',6-diamidino-2-phenylindole) Fluoromount-G® (SouthernBiotech, 0100-20) was added to each well and was left to air dry for at least five minutes before microscopic examination. Cells in one well (referred to as blank) did not receive any antibodies (either primary or secondary); this served for the calculation of potential autofluorescence of the cells, whereas cells of another well were incubated only with the secondary antibodies, in order to measure any potential background signal from non-specific binding of the secondary antibodies.

3.3.6.3 Image acquisition & Image analysis

Image acquisition for the IF was carried out in collaboration with *Dr. Sofia Bellou* (Senior Laboratory Teaching Staff, University of Ioannina). Images were acquired using a Nikon AX laser scanning confocal microscopy system equipped with a Nikon Spatial Array Confocal (NSPARC) detector and controlled via NIS-Elements imaging software, located at the Department of Biological Applications and Technology, University of Ioannina. Fluorescence excitation was achieved using 405 nm, 488 nm, and 564 nm laser lines. Samples were imaged using either a CFI Plan Apochromat 40x oil immersion objective (NA 1.25) or a CFI Plan Apochromat 100x oil immersion objective (NA 1.40) lens. For standard confocal imaging, a resonant scanner was utilized. Where specified, super-resolution and enhanced signal-to-noise imaging were performed by capturing spatial information through the 25-element NSPARC single-photon counting detector array.

3.3.7 Hematoxylin & Eosin (H&E) Staining and Immunohistochemistry (IHC)

The H&E staining and IHC procedures were carried out in collaboration with *Associate Professor Evangelia Lampri* (Pathology, Faculty of Medicine, University of Ioannina).

3.3.7.1 Reagents used

The reagents used in the H&E staining and the IHC staining of human BCC biopsies are listed in **Table 15**.

Reagent	Source	Catalogue Number
Antibodies		
Monoclonal mouse anti-human epithelial antigen, clone Ber-EP4	Dako	# M0804
CAR (CXADR) rabbit polyclonal antibody	Invitrogen	# PA5-110995
YAP1 mouse monoclonal antibody	Proteintech	# 66900-1-Ig
Solutions & Buffers		
Mayer's Hematoxylin solution	Sigma-Aldrich	# MHS1
Antibody diluent	Roche	# 05261899001
Kits		
Dako H&E Reagent Package, Large	Agilent	# CS10130-2
OptiView DAB IHC Detection Kit	Roche	# 06396500001

Table 15: Reagents used for the Hematoxylin & Eosin and the immunohistochemical staining of human BCC biopsies.

3.3.7.2 Formalin Fixation and Paraffin Embedding of BCC tissues

H&E and IHC staining were performed on FFPE tissue sections. For the FFPE process, specimens were initially fixed in neutral buffered formalin (37% aqueous formaldehyde solution) for 24h at RT, utilizing approximately a 10:1 formalin to tissue volume ratio. Specimens were then sectioned according to guidelines, were sequentially dehydrated in a step-wise manner in ethanol solution of increasing concentrations ranging from 50% to 100% (v/v), cleared with xylene, and finally embedded in paraffin wax.

3.3.7.3 Hematoxylin & Eosin (H&E) staining

For the H&E staining, serial tissue sections of approximately 3-4 μm in thickness were cut on the transverse plane of the FFPE blocks using a microtome, and were mounted on glass slides, utilizing the DakoCytomation Autostainer Instrument (DakoCytomation, Kyoto, Japan). Tissue sections were deparaffinized in xylene (three changes, 5 minutes each), and were rehydrated through graded ethanol solutions; first 100% ethanol (two changes, 2 minutes

each), followed by 95% ethanol (one change, 2 minutes), and 70% ethanol (one change, 2 minutes), and were subsequently rinsed in running tap water for 2 minutes. Next, tissue sections were submerged in hematoxylin solution (Dako Hematoxylin, Dako H&E Reagent Package, Agilent, CS10130-2) for 4 minutes, and were rinsed thoroughly in running tap water. They were briefly differentiated in acid alcohol and rinsed in running tap water to remove excess dye, and were then blued in appropriate bluing reagent (Dako Bluing Buffer, Dako H&E Reagent Package, Agilent, CS10130-2) for 45 seconds (for conversion of hematoxylin color from red to blue), followed by thorough rinsing in running tap water. Sections were then counterstained with eosin by being submerged in eosin Y solution (Dako Eosin, Dako H&E Reagent Package, Agilent, CS10130-2) for approximately 90 seconds, and were briefly rinsed in 95% ethanol solution. Next, slides were dehydrated by sequential immersion in 95% ethanol (one change, 1 minute) and 100% ethanol (two changes, 1.5 minute each), were cleared in xylene (two changes, 2.5 minutes each), and were ultimately mounted using a permanent mounting medium (Dako Mounting Medium, CS10130-2) and a glass coverslip.

3.3.7.4 Immunohistochemical staining

For the IHC staining, serial tissue sections of approximately 3-4 μm in thickness were cut on the transverse plane of the FFPE blocks using a microtome, and were mounted on positively charged glass slides. The IHC procedure was performed utilizing the Ventana BenchMark ULTRA system (Roche Diagnostics, North America). Tissue sections were dewaxed in xylene, and rehydrated in ethanol solutions of gradually decreasing concentration ranging from 100% to 50% (v/v). Endogenous peroxidase activity was blocked by incubation of tissue sections in 3% hydrogen peroxide (H_2O_2) solution (OptiView peroxidase inhibitor, OptiView DAB IHC Detection Kit, Roche, 06396500001) for 5 minutes, as HRP-conjugated tertiary antibodies would be used. Heat-induced antigen retrieval was performed by autoclave treatment of sections for 30 minutes in Dako target retrieval solution, citrate pH 6 (Agilent). Tissue sections were then incubated for 32 minutes at 36°C with the following primary antibodies at the indicated dilutions: monoclonal mouse anti-human epithelial antigen (EpCAM) clone Ber-EP4 (1:100, Dako, M0804), CAR (CXADR) rabbit polyclonal antibody (1:300, Invitrogen, PA5-110995) and YAP1 mouse monoclonal antibody (1:1000, Proteintech, 66900-1-Ig); all primary antibodies were diluted in commercially available antibody diluent (Roche, 05261899001). For signal detection tissue sections were first incubated with a cocktail containing HQ-labeled secondary goat anti-mouse and HQ-labeled secondary goat anti-rabbit antibodies (OptiView HQ Universal Linker, OptiView DAB IHC Detection Kit, Roche, 06396500001), followed by incubation with mouse monoclonal HRP-conjugated anti-HQ tertiary antibody (OptiView HRP Multimer, OptiView DAB IHC Detection Kit, Roche, 06396500001) for 30 minutes, as per manufacturer's instructions. Subsequently, the 3,3'-

diaminobenzidine (DAB) chromogen solution (0.2% w/v, OptiView DAB, OptiView DAB IHC Detection Kit, Roche, 06396500001), which is an HRP substrate, along with H₂O₂ solution (0.04%, OptiView H₂O₂, OptiView DAB IHC Detection Kit, Roche, 06396500001) and copper sulfate (CuSO₄) solution (5 g/L, OptiView Copper, OptiView DAB IHC Detection Kit, Roche, 06396500001) were added, ultimately resulting in the formation of a brown reaction product at the sites of antigen expression. Tissue sections were then counterstained with Mayer's hematoxylin solution (Sigma-Aldrich, MHS1), rinsed in running tap water, dehydrated in gradually increasing alcohol concentrations, cleared in xylene, and permanently mounted with a coverslip. Hematoxylin serves as a nuclear counterstain in IHC, by staining the nuclei blue-purple. Appropriate positive and negative controls were included in each staining run according to the Pathology laboratory's established QC procedures.

3.3.7.5 Image acquisition

Photographs of the sections were taken under an Olympus BX43 microscope equipped with an Olympus SC30 digital camera (Olympus Optical, Tokyo, Japan). Total magnification (objective lens x 10x eyepiece lens) is indicated in the respective figure legends.

3.4 Statistical Analysis

Statistical analysis was carried out using GraphPad Prism 10 (version 10.6.1, Dotmatics). The statistical test applied in each experiment is indicated in the respective figure legend. Briefly, Mann-Whitney test was used for comparative analysis of all variables in the scRNAseq data between BCC and HC skin biopsies (cell cluster frequency, T cell cluster frequency, pooled *JAML* expression, *JAML* expression in each distinct T cell cluster, *CXADR-FOS* co-expression). Two-way ANOVA and student's t-test were used for comparative analysis of the percentage of initial wound area covered at given time points between conditions of interest (IL-4, IL-5, IL-13, combination of all three, or recombinant *JAML*) and the negative control in scratch assays. P-values reported are as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

4. Results

4.1 The cellular landscape of BCC

Initially, two different publicly available datasets with scRNAseq data from lesional biopsies of patients with BCC and biopsies from healthy human skin were included in the study. The first one (accession number: GSE141526) consists of 4 BCC and 2 ANS samples, while the second (accession number: E-MTAB-13085) consists of 8 BCC and 14 HC samples. An extensive QC was performed for samples of both datasets. A detailed description for the E-MTAB-13085 samples is provided in Materials and Methods; QC for the GSE141526 samples was carried out in a similar manner. Following QC, a total of 51969 cells from GSE141526 and 194607 cells from E-MTAB-13085 were merged, and sample integration was performed (HarmonyIntegration). Cells were clustered into 18 different cell populations, which were annotated based on prior knowledge of cell type-specific markers. The 4 clusters annotated as KCs, and the 5 clusters annotated as T cells, were separately subjected to a higher resolution analysis, which revealed 9 distinct KC subpopulations and 11 distinct T cell subpopulations, respectively. However, it was observed that several T cell clusters exhibited signs of “contamination” with *KRT*-expressing cells. Therefore, we implemented an in-house developed stepwise process of filtering T cell clusters for *KRT*⁺ cells and KC clusters for *PTPRC*⁺ cells. Despite this approach, it was still not possible to eliminate cross-contamination between these two populations. Ultimately, it was uncovered that the cause of this phenomenon was the existence of batch effect at the dataset level in one of the initial clusters which was annotated as T cells. It is possible that the observed batch effect, which was not addressable with bioinformatic methods, is due to cellular overloading of the microfluidic cartridge. Nevertheless, given that the observed batch effect could not be corrected, it was decided to re-perform the analysis on only one of the datasets. Since all HC samples were derived from the E-MTAB-13085 dataset, which further contained a higher number of BCC samples compared to GSE141526, E-MTAB-13085 was chosen for this purpose.

Following exclusion of the GSE141526 samples, the already quality-controlled samples from the E-MTAB-13085 dataset (BCC; n = 8, HC; n = 13 – one HC sample was also excluded due to persisting batch effects), were re-integrated, and the resulting 187100 cells were clustered into 18 populations (**Figure 1 A**). Cluster annotation was performed manually based on prior knowledge of cell type-specific markers, according to an in-house curated list of genes (**Figure 1 A and B**) These comprised one cluster of fibroblasts (*COL1A1/2*, *THY1*, *FN1*), one of smooth muscle cells (*ACTA2*, *TAGLN*), one of KCs (*KRT14*, *KRT5*), one of melanocytes (*PMEL*, *MLANA*), two clusters of endothelial cells (*VWF*, *PECAM1*, *CD34*), several immune cell populations (*PTPRC*), in particular four T cell clusters (including one with innate-like

characteristics and one of proliferating T cells) (*CD3D/E/G*, *CD4*, *CD8A/B*; *NCAM1*, *FCGR3A*; *MKI67*), one cluster of B cells/plasma cells [*MS4A1* (*CD20*), and *CD19*, or *SDC1*], two DC clusters (*XCR1*, *CLEC9A*, *CLEC4E*; *IRF4*, *CD1C*), one mixed macrophage/DC cluster (*CD14*, *CD68*, *CD1C*), one cluster of proliferating macrophages (*CD68*, *MKI67*), and one population of mast cells (*KIT*, *FCER1A*), as well as one cluster of undifferentiated cells (several non-specific genes) (**Figure 1 A**). Differential cell cluster distribution was observed between the BCC and HC skin biopsies (**Figure 1 C**). Further analysis on the pooled populations of each distinct cell type highlighted the statistically significant enrichment in melanocytes (* $p \leq 0.05$) and B cells/plasma cells (* $p \leq 0.05$), and decrease in fibroblasts (* $p \leq 0.05$) in BCC compared to HC skin biopsies (**Figure 1 D**).

4.2 JAML-enriched T cell populations in BCC and HC skin biopsies

T cells play a central role within the BCC TME^{514,571,968–980,982–984,990,992,993,1504}; hence, a higher resolution analysis of the T cell populations was performed. The four T cell clusters (T cells 1, T cells/Innate-like T cells, T cells 2, and Proliferating T cells; total cell number $n = 65616$) were subsetted, re-integrated and re-clustered into 12 distinct groups, in particular: Th2/Th22 cells (*CD4*, *IL13*, *GATA3*, *STAT5A/B*, *IL22*, *AHR*), Th17 cells (*CD4*, *IL17A/F*, *RORC*), Tfh cells (*CD4*, *IL21*, *BCL6*), Tregs (*CD4*, *FOXP3*, *IL2RA*, *CTLA4*), three clusters of CD8+ T cells (*CD8A/B*), including one with innate-like characteristics (*TRGC1/2*, *TRDC*, *KLRK1*), a cluster of early activated T cells (high *CD69* and very low *CD4* and *CD8A/B* expression), one of NKs/ $\gamma\delta$ T cells (*NCAM1*, *TRGC1*, *TRDC*), one of proliferating T cells (*CD4*, *CD8A/B*, *MKI67*), as well as one population of uncategorized T cells (which did not exhibit a transcriptomic profile representative of any one T cell subtype) and lastly, a small cluster of mixed-origin cells (**Figure 2 A and B**). The frequency of cells within these clusters was distinct between the BCC and HC samples (**Figure 2 C**). Regarding the *CD4*+ populations, BCC samples were enriched in Tregs (* $p \leq 0.05$), but intriguingly, displayed a significantly reduced frequency of Th2/Th22 cells (** $p \leq 0.01$) and a significantly higher frequency of Tfh cells (***) $p \leq 0.001$), compared to HCs (**Figure 2 D**). Th17 cells did not differ significantly between the two groups (**Figure 2 D**). Additionally, BCCs exhibited a significant enrichment in Innate-like CD8+ T cells (* $p \leq 0.05$) and Proliferating T cells (* $p \leq 0.05$), and a significant downregulation of Early activated T cells (** $p \leq 0.01$) and Uncategorized T cells (* $p \leq 0.05$), compared to HCs (**Figure 2 E**). For the remaining T cells clusters no statistically significant difference was observed between BCCs and HCs (**Figure 2 E**).

JAML was identified as a DEG in Th2/Th22 cells (significantly downregulated in BCC), Tfh cells (significantly enriched in BCC), Uncategorized T cells (significantly downregulated in BCC), and Th17 cells, compared to all other skin T cell clusters (**Figure 2 F**). However, the total T cell *JAML* expression did not differ between BCC and HC (**Figure 2 G**), and neither did

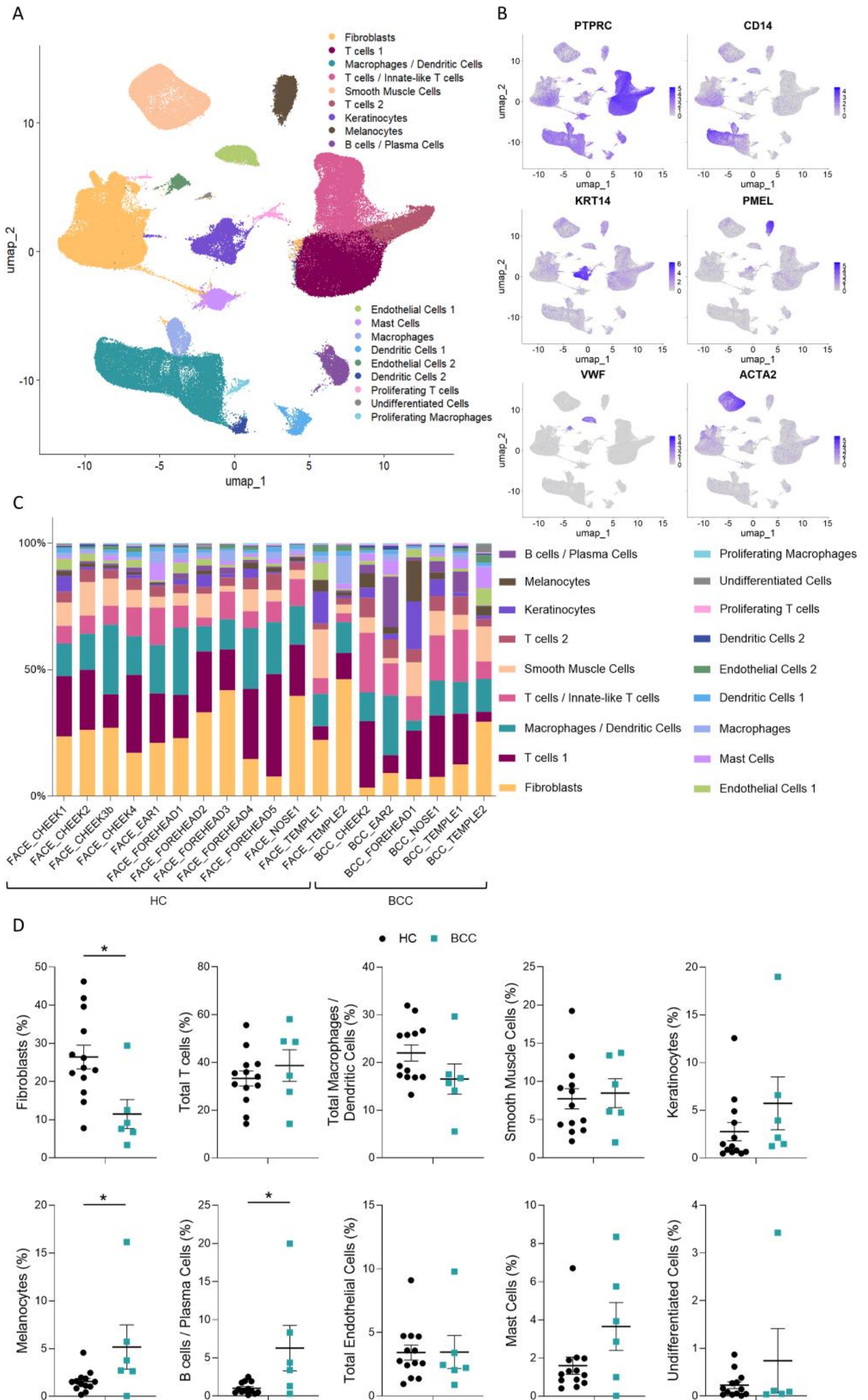


Figure 1: Differential frequency of cell populations between BCC and HC skin biopsies. **A.** Uniform Manifold Approximation and Projection (UMAP) plot depicting all clusters identified from clustering of 187100 cells from six BCC and thirteen HC samples. A total of eighteen clusters were identified. **B.** Selection of feature plots for representative markers used for cluster annotation. **C.** Bar chart depicting the per sample distribution of cells among the clusters, between BCC and HC samples. **D.** Relative percentage of each cell cluster among all cell clusters in BCC compared to HC samples. Each symbol represents a distinct biological sample, bars indicate mean $\pm$ standard error of the mean (SEM). PTPRC = Protein Tyrosine Phosphatase Receptor Type C, CD14 = Cluster of Differentiation 14, KRT14 = Keratin 14, PMEL = Premelanosome Protein, VWF = Von Willebrand Factor, ACTA2 = Actin Alpha 2, smooth muscle. Mann-Whitney test was used for comparative analysis (* p-value ≤ 0.05).

the *JAML* expression of each separate T cell cluster when compared between BCC and HC samples (**Figure 2 H**).

4.3 Pathway enrichment analysis of T cell clusters

Pathway enrichment analysis was performed in the JAML-enriched T cell populations of interest (Th2/Th22, Th17, and Tfh) in comparison to the rest of the T cell clusters, and the top 5 relevant most enriched pathways for each one are depicted in **Figure 3 A**. Some pathways, such as the Th17 cell differentiation pathway, are shared between them. Accordingly, pathway enrichment analysis was performed within each one of the aforementioned clusters for the BCC-derived T cells compared to the HC-derived ones (**Figure 3 B**). The PD-L1 expression and PD-1 checkpoint pathway in cancer was identified among the top 5 relevant most enriched pathways in both BCC Th17 and BCC Tfh populations, compared to their HC counterparts, while the MAPK signaling pathway was identified as the top enriched pathway in both the BCC Th2/Th22 and BCC Tfh cells compared to HC. The upregulated and downregulated genes of each of the top 5 pathways reported for the BCC vs. HC Th2/Th22 and Tfh cells are shown (**Figure 3 C**). The complete lists of the enriched pathways and their associated genes are shown in **Supplementary Tables 1-6**.

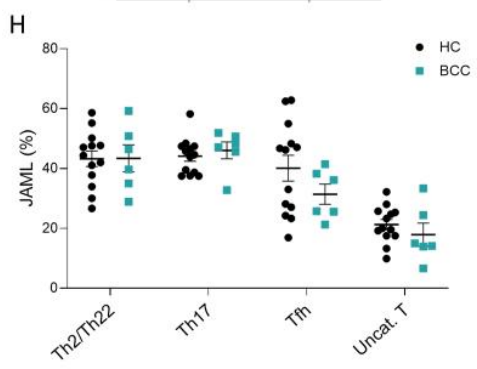
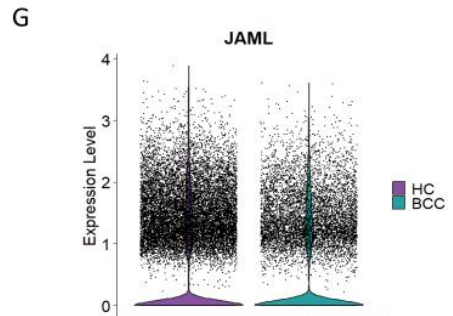
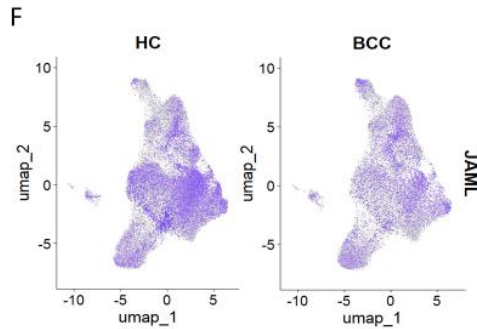
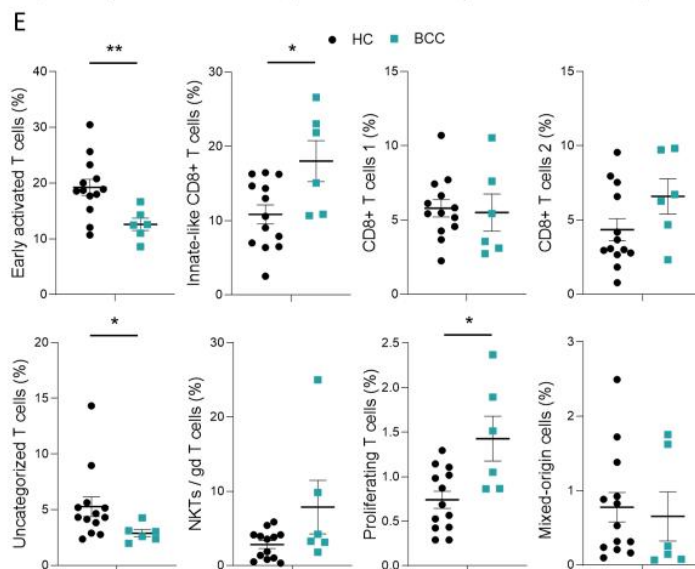
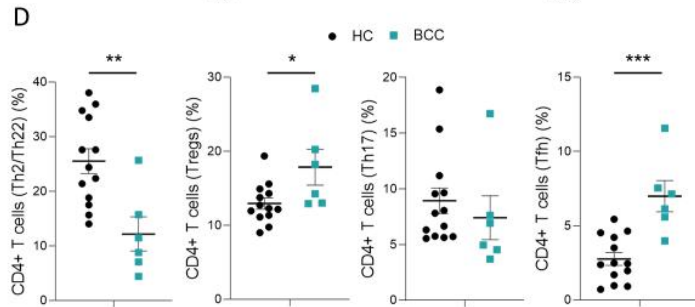
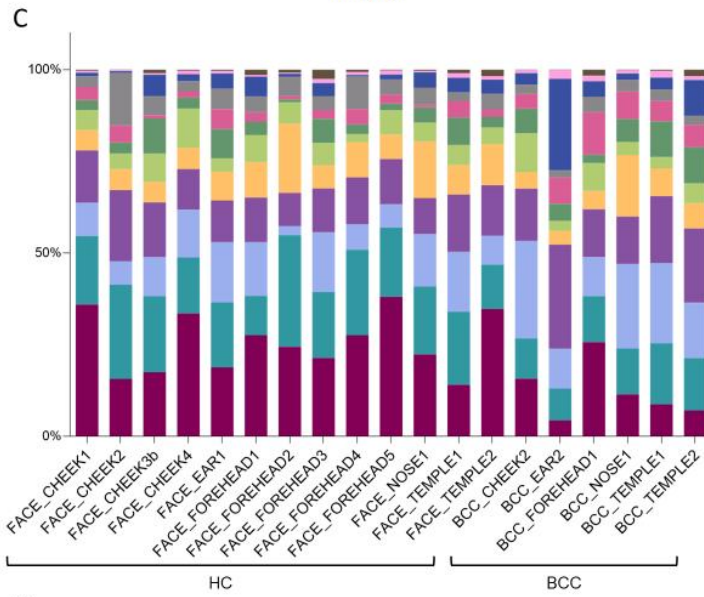
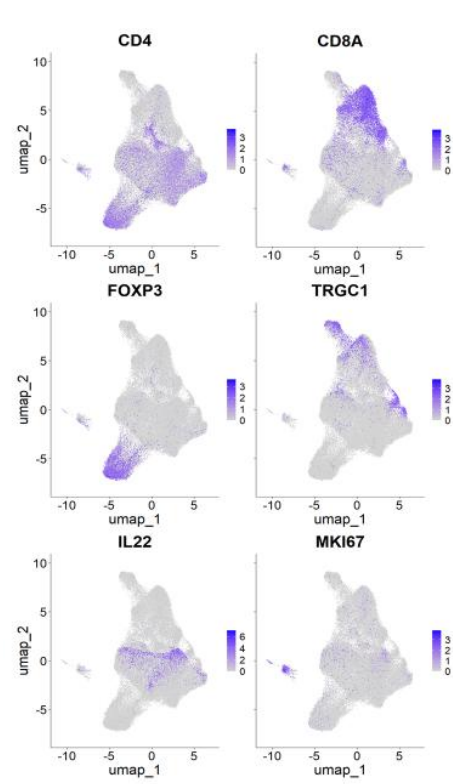
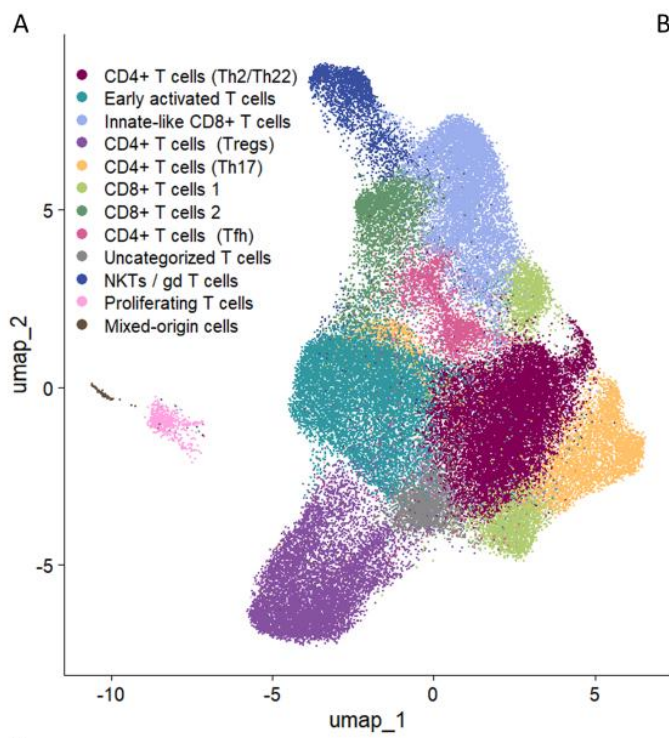


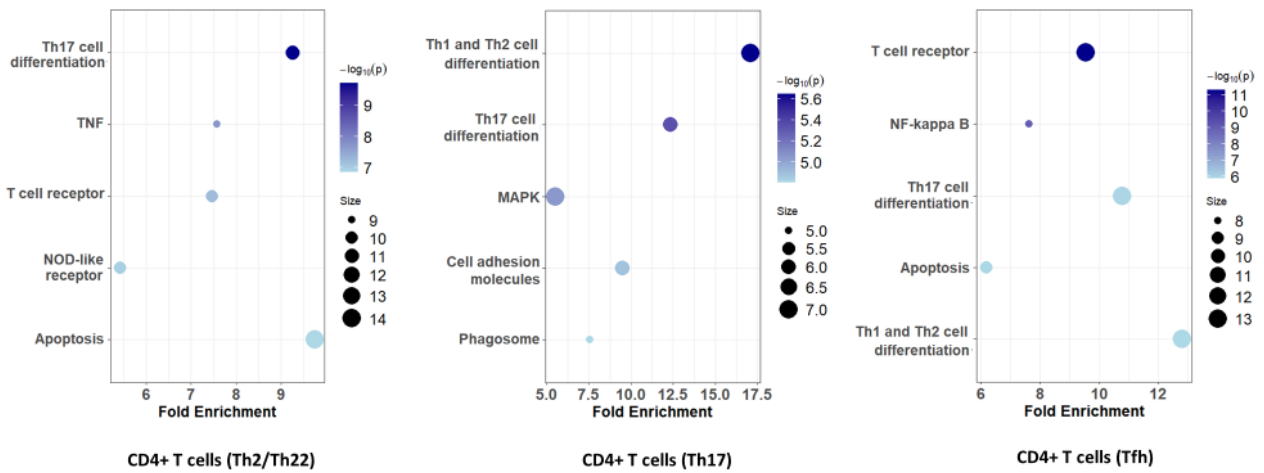
Figure 2: Identification of *JAML*-expressing T cells in BCC and HC skin biopsies. **A.** UMAP plot depicting all T cell clusters identified following re-integration and re-clustering of cells originally annotated as T cells 1, T cells/Innate-like T cells, T cells 2, and Proliferating T cells (n = 65616). A total of twelve clusters were identified. **B.** Selection of feature plots for representative markers used for cluster annotation. **C.** Bar chart depicting the per sample distribution of T cells among the T cell clusters, between BCC and HC samples. **D.** Relative percentage of each CD4+ T cell cluster among all T cell clusters in BCC compared to HC samples. **E.** Relative percentage of each of the non-CD4+ T cell clusters among all T cell clusters in BCC compared to HC samples. **F.** Feature plot depicting the level and distribution of *JAML* expression in T cell clusters in BCC compared to HC samples. **G.** Violin plot depicting the log-normalized pooled expression of *JAML* in all T cell clusters in BCC compared to HC samples. **H.** Relative percentage of *JAML*-expressing T cells among all T cells for the indicated T cell clusters in BCC compared to HC samples. Each symbol represents a distinct biological sample, bars indicate mean $\pm$ SEM. Mann-Whitney test was used for comparative analysis (* p-value $\leq$ 0.05, ** p-value $\leq$ 0.01, *** p-value $\leq$ 0.001). Uncat. T = Uncategorized T cells

4.4 *JAML* is upregulated under Th2 but not under Th1 polarization conditions

In order to further elucidate the role of *JAML* in CD4+ T cell populations, human primary naïve CD4+ T cells were isolated from the peripheral blood of healthy individuals (n = 5) via negative selection, and were stimulated (anti-CD3, anti-CD28, anti-CD2 beads) in vitro, under either Th1 polarization (anti-IL-4, TGF- β , IFN- γ , IL-2) or Th2 polarization (anti-IFN- γ , anti-IL-17, TGF- β , IL-4, IL-2) conditions, in the presence of lethally irradiated alloreactive APCs (**Figure 4 A**). Interestingly, FC analysis revealed an increase in the *JAML* levels in T cells stimulated under Th2 polarization conditions compared to the control non-polarized stimulated T cells (**Figure 4 B**). Contrarily, T cells stimulated under conditions promoting Th1 polarization displayed a relative downregulation of *JAML* expression, compared to the control (**Figure 4 B**).

A

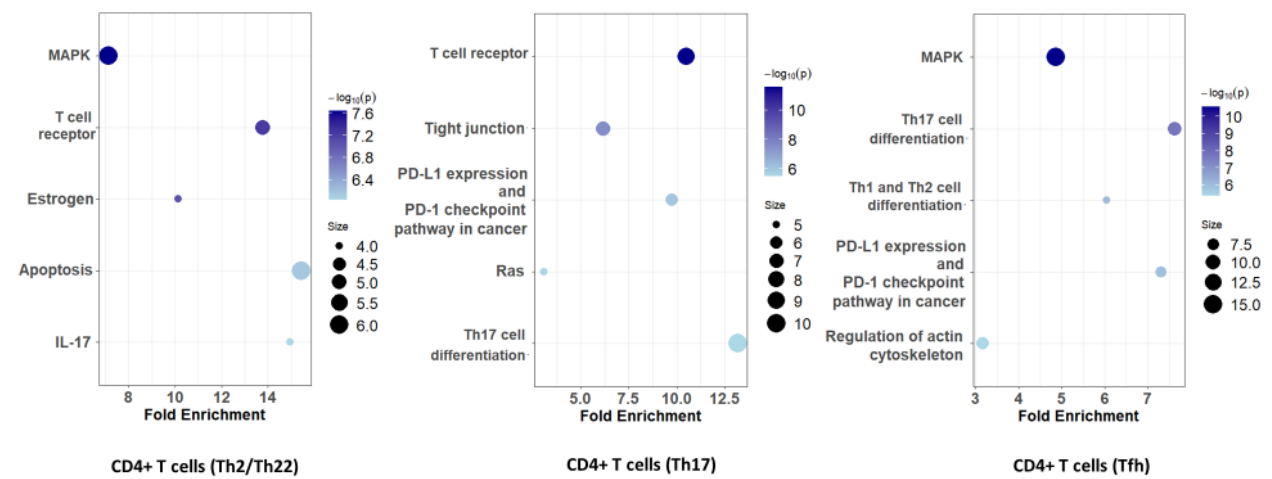
signaling pathway



B

signaling pathway

BCC vs. HC



C

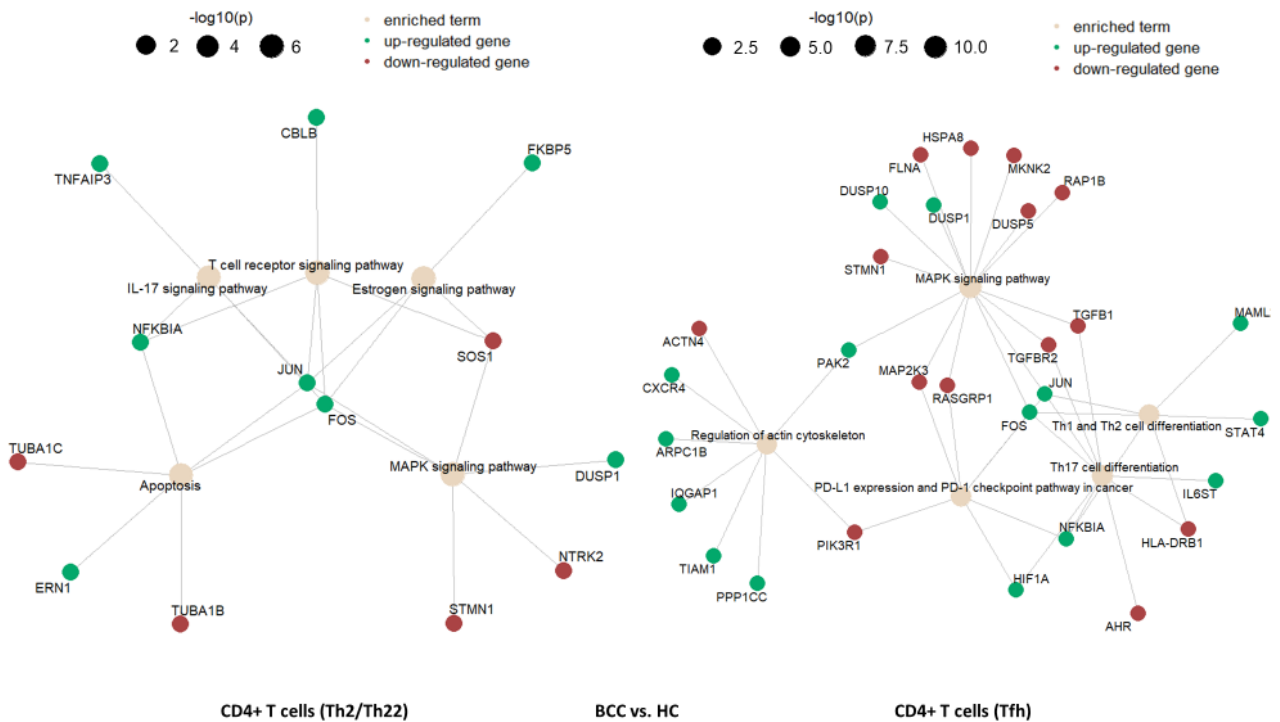
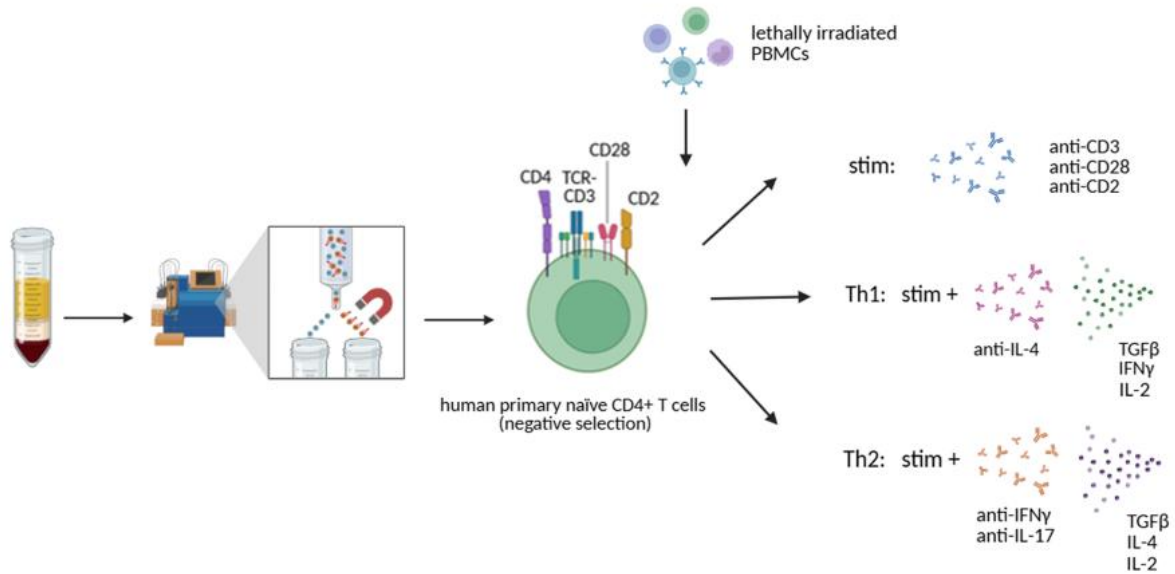


Figure 3: Pathway enrichment analysis in T cell clusters marked by high *JAML* expression compared to the rest. **A.** Dot plots for KEGG pathway enrichment analysis depicting the top 5 relevant enriched pathways in Th2/Th22 (left), Th17 (center) and Tfh (right) cells compared to the rest of the T cell clusters. **B.** Dot plots for KEGG pathway enrichment analysis depicting the top 5 relevant enriched pathways in BCC-derived Th2/Th22 (left), Th17 (center) and Tfh (right) cells compared to the HC-derived ones. Dot size is indicative of the number of enriched genes from each pathway. Color gradient indicates p-value. **C.** Term-gene graphs depicting common upregulated or downregulated genes for the indicated pathways for Th2/Th22 (left) and Tfh (right) cells. Dot size indicates p-value of the enriched pathway.

A



B

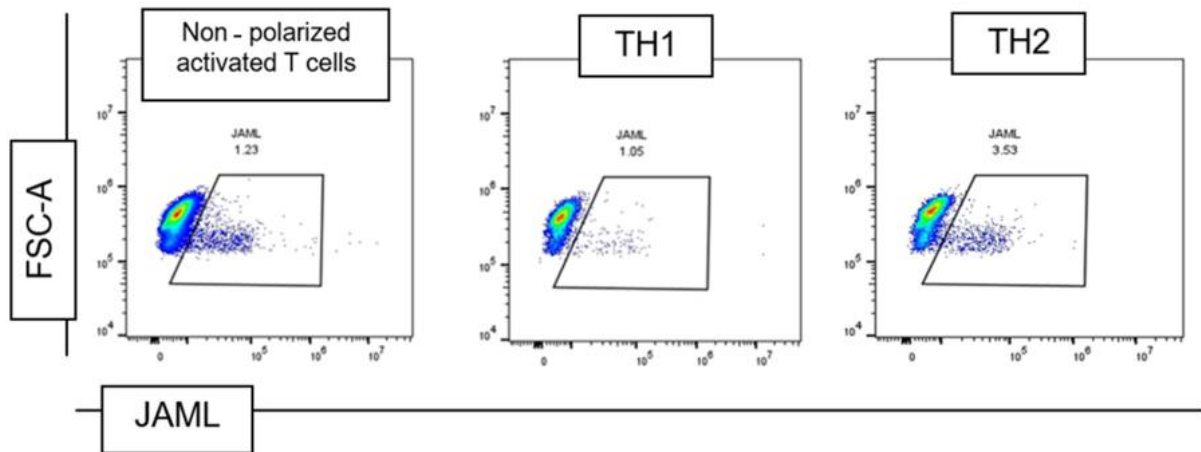


Figure 4: JAML is upregulated under Th2- but not under Th1- polarization conditions. A. Schematic of the workflow followed for the polarization of human primary naïve CD4⁺ T cells, isolated from healthy donor (n = 5) peripheral blood, and cultured under Th1- or Th2- promoting conditions (created with bioRender). **B.** Flow cytometry density dot plot for control (non-polarized), Th1-polarized, and Th2-polarized activated T cells. FSC-A = Forward Scatter-Area. *Unpublished data of the Floudas lab, from experiments performed by Roozbeh Sanaei (PhD candidate – Dublin City University, Erasmus student – University of Ioannina).*

4.5 Identification of malignant KCs and interrogation of CXADR and YAP-1 expression

To further interrogate the role of the JAML-CXADR axis in the BCC context, a higher resolution examination of the KCs was carried out, in a manner similar to that described for the T cell populations. Re-clustering of the 7974 cells originally annotated as Keratinocytes

revealed a total of 9 distinct subpopulations (**Figure 5 A**). Among these, clusters KC_0 and KC_3 were characterized as enriched in malignant KCs, on the basis of their observed increased expression of a series of cancer- and more specifically BCC- associated genes, especially *EPCAM*, *KRT17*, and basoon zinc finger protein 2 (*BNC2*) (**Figure 5 B and C**). *EpCAM* is a widely used marker for BCC differential diagnosis^{287,389,391–395,407}. *K17* is normally absent from the healthy IFE, but is induced under inflammatory and malignant skin conditions; its expression is identified in several cancers (including non-cutaneous ones), and appears to be associated with the proliferation and EMT of tumor cells^{1481,1505–1511}. *K17* can also aid the differential diagnosis of BCC in clinical practice^{1512,1513}. *BNC2* encodes for a TF that was recently associated with ECM remodeling¹⁵¹⁴, and which has been implicated in the pathogenesis of BCC^{1515–1518}; BCC specimens have been reported to display increased *BNC2* expression compared to healthy skin^{1515,1518}, in accordance with its proposed regulation by the GLI TFs¹⁵¹⁵. Expression of these markers was also detected in cluster KC_6, which comprises a population of proliferating KCs, according to its increased expression of proliferation-associated genes, such as *MKI67* (**Supplementary Figure 1**), and is thus, anticipated to include malignant cells as well. *KRT17* expression was also identified, to a lower degree, in additional clusters (**Figure 5 B and C**), potentially corresponding either to pilosebaceous unit-derived cells or non-malignant stressed/activated KCs^{1481,1507,1509}.

Moreover, CNV analysis was performed, utilizing the InferCNV algorithm, which revealed losses in chromosome 9 for clusters KC_0 and KC_3, potentially corresponding to *PTCH1* losses (9q22)¹⁵⁴, as well as gains in chromosome 6 of the same clusters, which have previously been detected in BCC¹⁵⁴ and other malignancies¹⁵¹⁹ (**Supplementary Figure 2**). This observation further supported the notion that clusters KC_0 and KC_3 were the ones enriched in malignant KCs.

Following the identification of the malignant cell-enriched KC clusters, a significantly higher (** $p \leq 0.01$) co-expression percentage of *CXADR* and *FOS* was identified in the BCC KCs vs. HC KCs (**Supplementary Figure 3**). Interestingly, in earlier studies, it has been reported that normal human adult skin exhibits an overall high *FOS* expression, both at the transcriptional and protein level¹⁵²⁰, and that BCC cells display lower *FOS* levels compared to the basal layer cells of the healthy epidermis¹⁵²¹. Moreover, c-Fos (encoded by the *FOS* gene) expression has been found higher in invasive compared to non-invasive forms of BCC^{1522,1523}, and has recently also been implicated in basal to squamous cell carcinoma transition (basosquamous transition; BST)^{1524,1525}, which is expected to associate with increased aggressiveness of the disease^{597,1526}.

We reported herein that type 2 polarization of CD4+ T cells results in increased JAML expression (**Figure 4**). Therefore, the expression of CXADR and YAP-1 in KCs may be influenced by conditions mimicking the presence of Th2 cells and the presence of recombinant JAML. Th2 cells have traditionally been considered to exert immunomodulatory functions within the TME and promote tumor growth⁶⁹³, and particularly for BCC, increased levels of IL-4 and/or IL-5 have previously been observed in comparison to healthy skin^{978,986,987}. In order to test this feed-forward loop, we cultured WT, CFOS and XIT HaCaT cells with either recombinant IL-4, IL-5, IL-13, a combination of the three, or recombinant JAML, and assessed the protein levels of CXADR and YAP-1 by WB analysis. Interestingly, although preliminary, our data indicate a potential positive influence of IL-5 on the expression of both CXADR and YAP-1 in WT HaCaT cells, but not in CFOS HaCaT cells (**Supplementary Figure 4**). As expected, protein levels in the XIT group were resembling those of the WT cells (data not shown).

Furthermore, scratch assay was performed on WT, CFOS and XIT HaCaT cells treated with recombinant IL-4, IL-5, IL-3, a combination of all three cytokines, or recombinant JAML. HaCaT cells treated with serum-low (1% FBS) medium were used as negative control in each case. As an estimate of their migratory capacity, the percentage of the initial wound area covered at predetermined time points (0, 4, 20, 24 hours) was calculated as described in Materials and Methods, and results are depicted in **Supplementary Figure 5**. No significant difference was observed for the percentage of initial wound area covered at any time point between cells treated under the different conditions compared to the control (**Supplementary Figure 5**).

4.6 Pathway enrichment analysis of KC clusters

In order to further characterize the transcriptomic profile of the malignant KCs, a pathway enrichment analysis was performed in the malignant KC-enriched clusters (KC_0, KC_3) compared to the rest of the KC clusters (**Figure 6 A**). Among the top 5 relevant most enriched pathways in both clusters in comparison to the rest, the Chemical carcinogenesis – reactive oxygen species pathway was identified. Oxidative phosphorylation was also enriched in KC_0, as was PI3K-Akt signaling, MAPK signaling, and the p53 signaling pathways in KC_3, compared to the other KC clusters. The upregulated or downregulated genes of each of the top 5 pathways, in clusters KC_0 and KC_3 compared to the rest of the KC clusters, is presented in **Figure 6 B**. Of note, in both of the malignant KC-enriched KC_0 and KC_3 clusters, AP-1 family members – in particular *JUN* and *FOS* in KC_0, and *JUND* and *FOS* in KC_3 – were found upregulated compared to the rest of the KC clusters (**Figure 6 B**). The

complete lists of the enriched pathways and their associated genes are shown in **Supplementary Tables 7 and 8**.

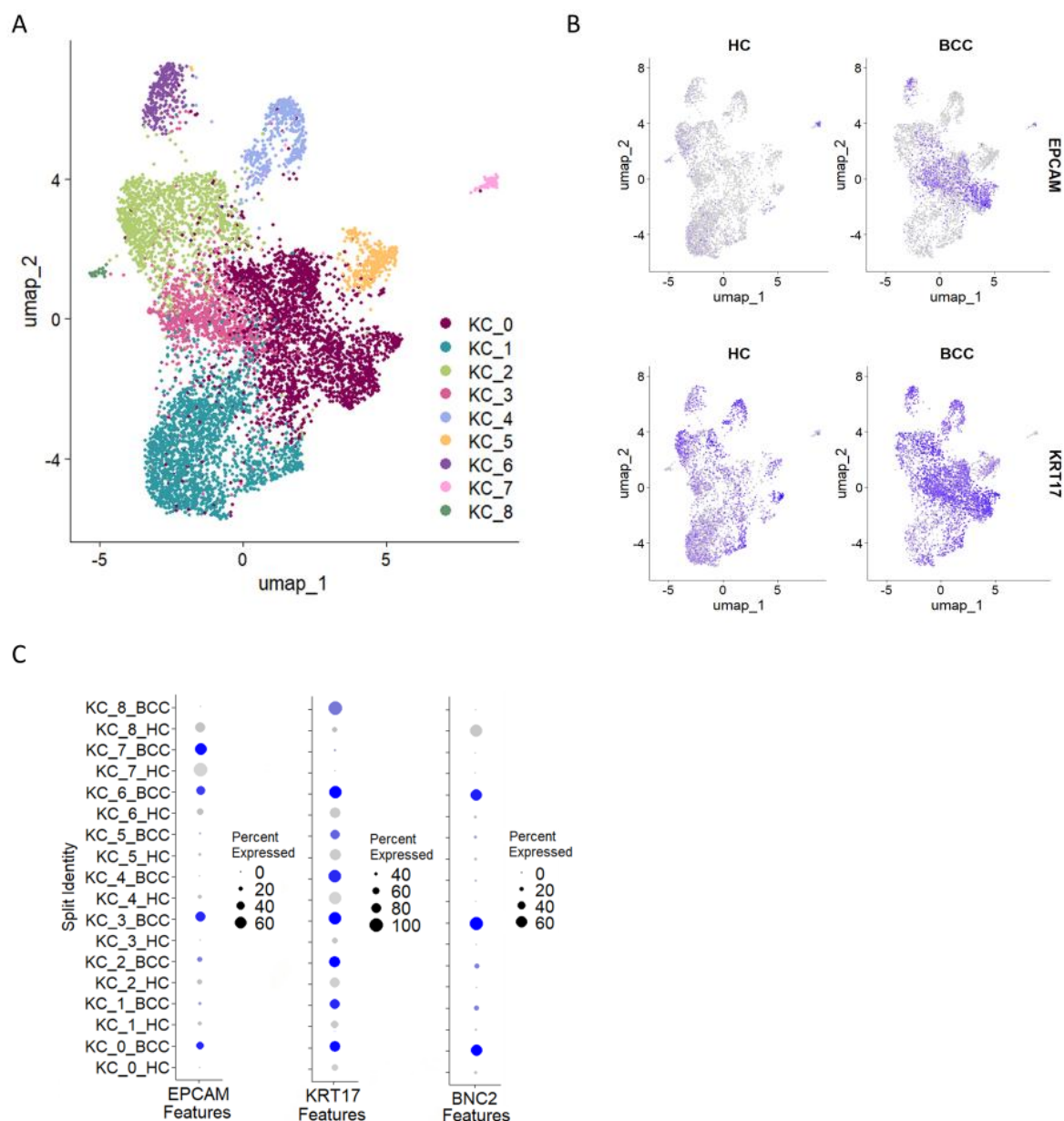
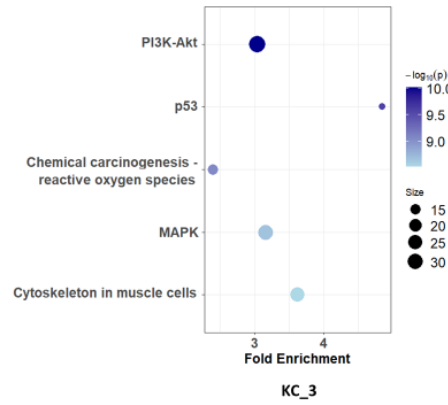
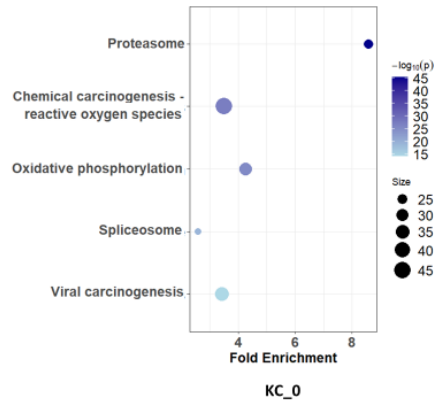


Figure 5: Identification of malignant cell-enriched keratinocyte (KC) clusters. **A.** UMAP plot depicting all KC clusters identified following re-integration and re-clustering of cells originally annotated as Keratinocytes (n = 7974). A total of nine clusters were identified. **B.** Feature plots depicting the level and distribution of expression of malignancy-associated genes *EPCAM* and *KRT17* in KCs in BCC compared to HC samples. **C.** Dot plots depicting the percentage of cells per KC cluster expressing malignancy-associated genes *EPCAM*, *KRT17* and *BNC2*, in BCC compared to HC samples.

A

signaling pathway



B

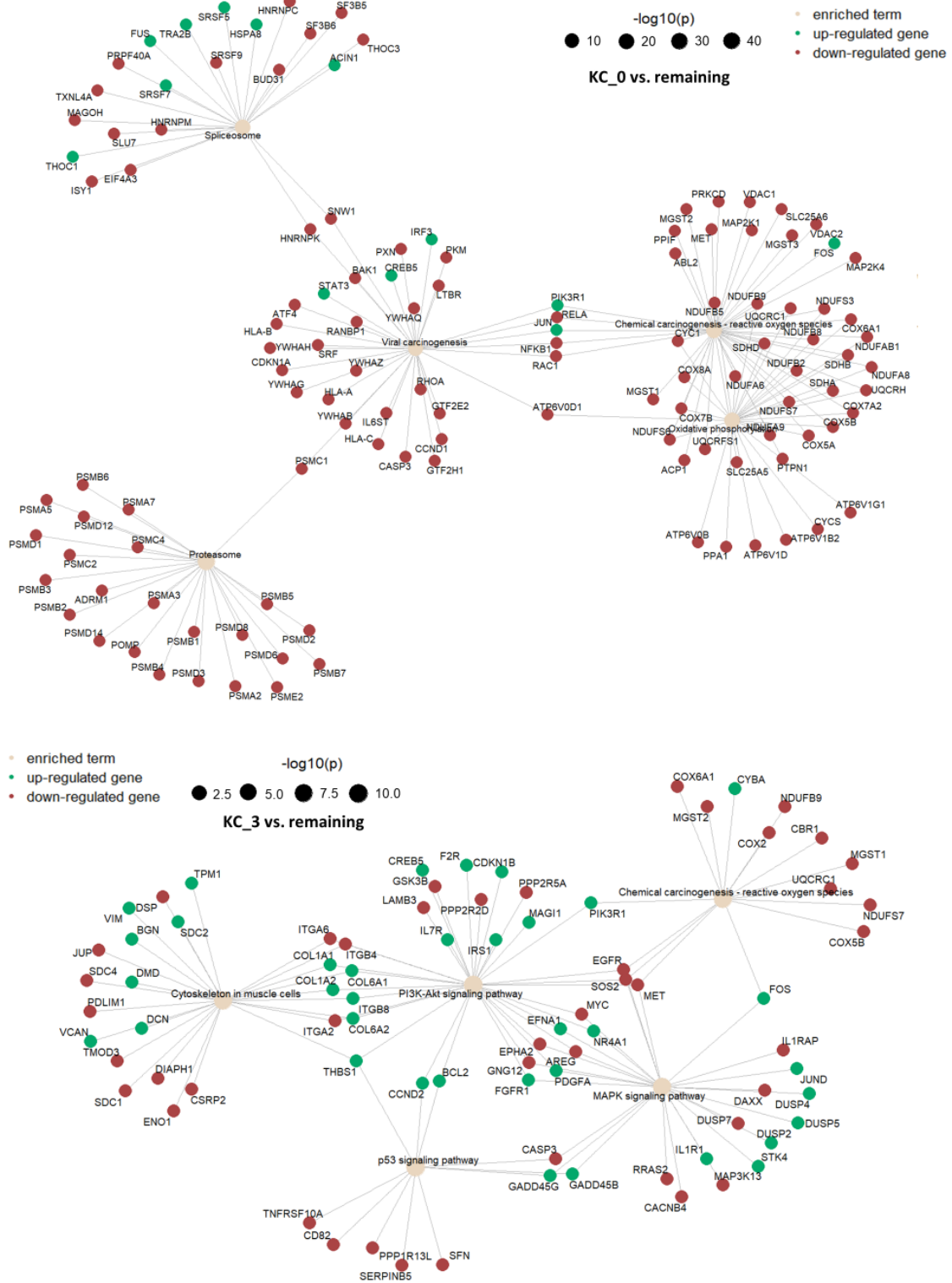


Figure 6: Pathway enrichment analysis in malignant-cell-enriched compared to the rest of the KC clusters. A. Dot plots for KEGG pathway enrichment analysis depicting the top 5 relevant enriched pathways in KC_0 (left) and KC_3 (right) compared to the rest of the KC clusters. Dot size is indicative of the number of enriched genes from each pathway. Color gradient indicates p-value. **B.** Term-gene graphs depicting common upregulated or downregulated genes for the indicated pathways for KC_0 (top) and KC_3 (bottom). Dot size indicates p-value of the enriched pathway.

4.7 Trajectory analysis of BCC-derived KC reveals progression from stem cell-like cluster to malignant KC-enriched clusters

Next, we sought to investigate the cellular point of origin of the malignant KC clusters. A series of modules for the distinct epithelial subpopulation phenotypes that are expected to be identified among the KC clusters was generated. A module is defined as a set of genes, the expression of which is coordinately modified within a given context ¹⁵²⁷. In particular, modules were constructed for all four epidermal layers (stratum basale; basal_score, stratum granulosum; granular_score, stratum spinosum; spinous_score, and stratum corneum; corneal_score), for the HF-associated cells (follicular_score), for the eccrine sweat gland (eccrine_score) and the sebaceous gland (sebaceous_score) cells, as well as for proliferating cells (proliferation_score). In addition, two discrete SC modules were created: (i) one comprising a set of well-characterized epidermal SC-associated markers, including, but not limited to *ITGA6*, *ITGB4*, *ITGA2*, *ITGB1*, *ITGA3*, *CD34*, *CD200*, *SOX9*, *TP63*, *COL17A1*, *LGR5*, *KRT5*, *KRT14*, *KRT15*, *KRT18*, *KRT19*, and *KRT23* ^{1463,1473–1481} (stem_score_a), and (ii) one consisting of genes differentially expressed between holoclones and meroclones or paraclones in cultured human epidermal epithelial cells ¹⁴⁷² (stem_score_b). The score (expression level) of each module (except for the corneal_score which was very low in all clusters) for the BCC KC populations is depicted in **Figure 7 A**. Cluster KC_8 was excluded as it contained only one cell. Based on the module scores, we verified the proliferative identity of KC_6 cells, we confirmed that KC_2 comprises a population of suprabasal KCs, and additionally, we characterized KC_4 as HF cells, KC_7 as eccrine sweat gland cells, and KC_5 as sebaceous gland cells (**Figure 7 A**). According to these annotations, clusters KC_5, KC_6 and KC_7 were excluded from the subsequent trajectory analysis; KC_5 and KC_7 are not expected to be biologically related to the malignant KCs, while KC_6 constitutes a non-uniform population of KCs which cluster together on the basis of their proliferative profile.

Construction of the trajectory was performed at a cluster level utilizing the TSCAN algorithm (**Figure 7 B**). Minimum spanning tree (MST) generation did not reveal any unrelated clusters (“outgroups”) (**Figure 7 C**). Based on stem_score_a, and in conjunction with the entropy calculated for each cluster (**Supplementary Figure 6**), KC_1 was chosen as the most

likely starting point of the trajectory. Noted is the direct progression from the stem-like KC_1 cells to the malignant KC_3 and then KC_0 cells, which further corroborates the suggested SC origin of BCC ^{283–286,292}.

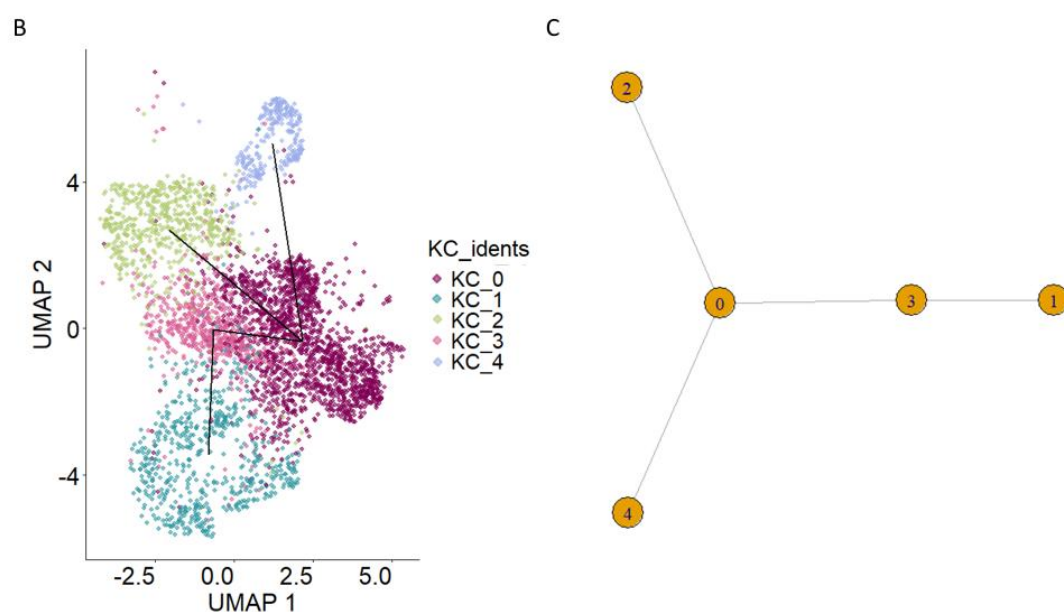
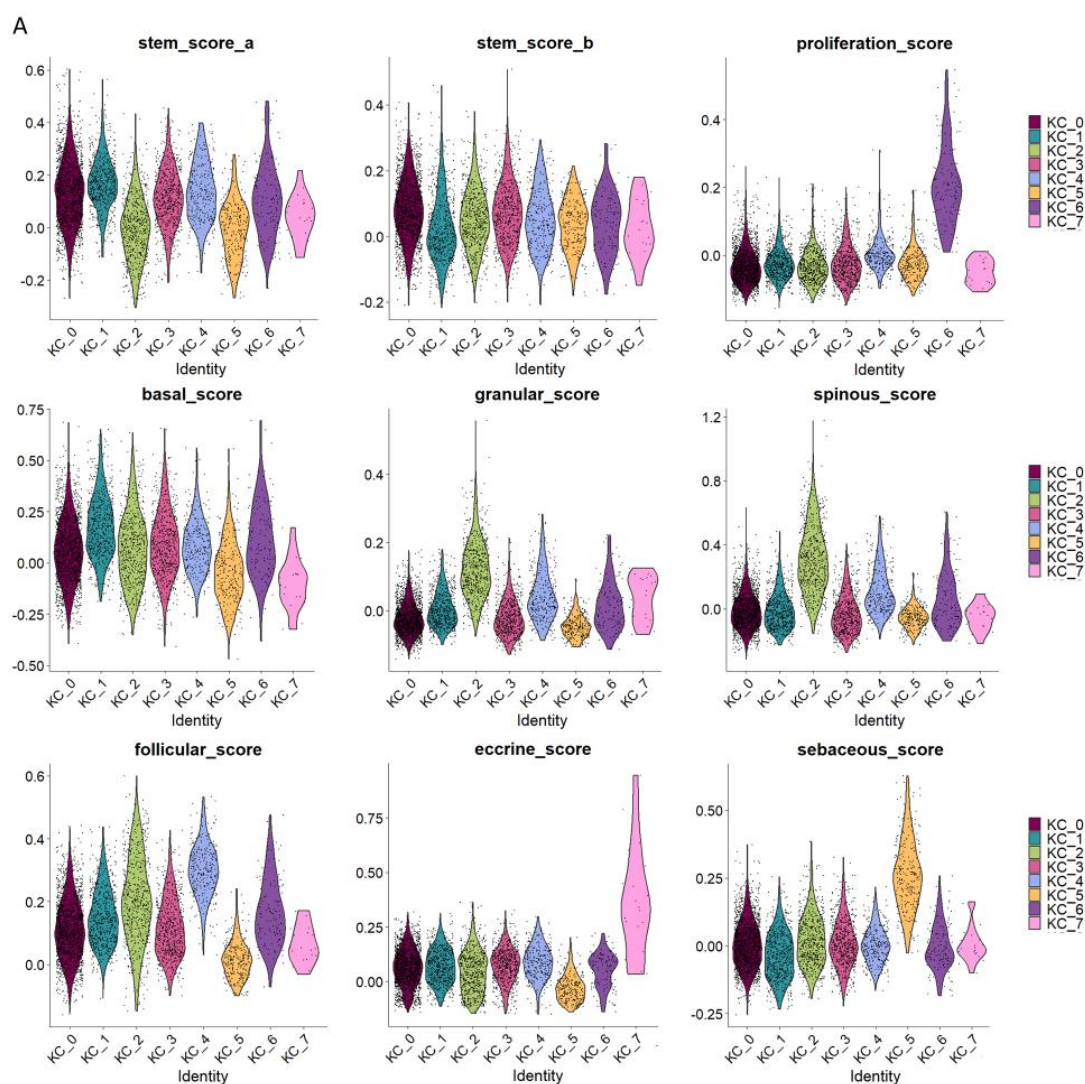


Figure 7: Trajectory analysis of BCC-derived keratinocytes reveals progression from stem cell-like cluster to malignant KC-enriched clusters. **A.** Violin plots depicting the expression score of gene modules manually curated for stem cell, proliferation, basal layer, granular layer, spinous layer, hair follicle, eccrine sweat gland and sebaceous gland cell genes, in the BCC-derived KC clusters. **B.** UMAP plot depicting the KC clusters included in the construction of the trajectory, where each point represents a cell and is colored according to its cluster assignment, with the cluster-based minimum spanning tree (MST) obtained overlaid on top. Cluster KC_1 was assigned as the starting point of the trajectory. **C.** Cluster-based MST, joining the centroids of KC clusters that were included in the construction of the trajectory.

4.8 Receptor-ligand interaction analysis between malignant cell-enriched KC clusters and T cell populations

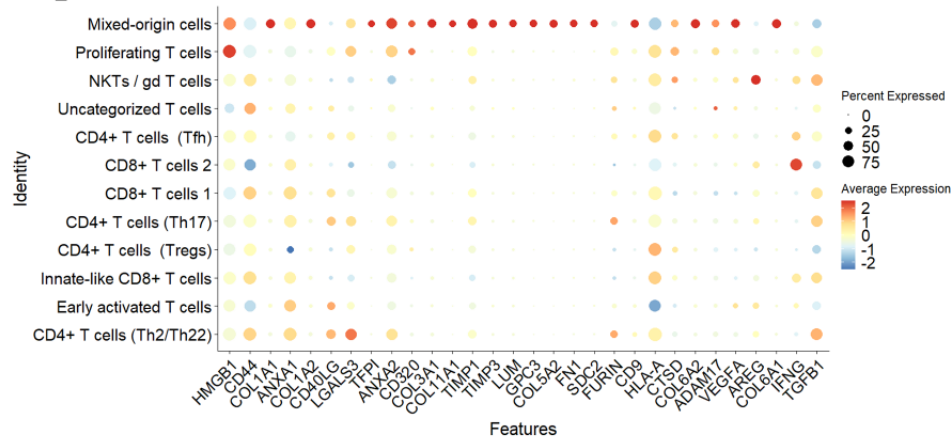
In order to gain a better understanding of the intercellular interactions within the BCC TME, a receptor-ligand interaction analysis was performed (NicheNet), focusing particularly on the interactions between KC populations of interest (KC_0, KC_3; which were individually set as the “receiver”) and the T lymphocytic populations (which were chosen as “senders”). Analysis was performed separately for the BCC- and the HC- derived KC clusters, utilizing the set of upregulated DEGs between BCC and HC or HC and BCC, respectively, as the gene set of interest. The top 30 T cell-derived ligands that potentially drive the transcriptomic profile of BCC- and HC- associated KC_0 and KC_3 cells, and their T cell cluster of origin are depicted in **Figure 8 A and B**. The active target genes of KC_0 and KC_3, the differential expression of which has been predicted to be regulated by the prioritized T cell ligands in HC and BCC are demonstrated in **Supplementary Figure 7** and **Supplementary Figure 8**, whereas the receptors of the top-ranked T cell ligands that are expressed in KC_0 or KC_3 in BCC and HC are demonstrated in **Supplementary Figure 9** and **Supplementary Figure 10**. Interestingly, *JAML* was identified among the top 30 ligands for the HC-derived KC_3 cells, but not for the BCC-derived KC_3 cells; specifically, *JAML* ranked 24th out of 82 in the top ligands for HC KC_3 (top 29%), and 33rd out of 75 in the top ligands for BCC KC_3 (top 44%) (**Figure 8A and B**). Unsurprisingly, *JAML* expression primarily originated from Th2/Th22, Th17 and Tfh cells, and to a lesser extent from Proliferating and Uncategorized T cells (**Figure 8B**).

4.9 CXADR and YAP-1 co-localize at areas of intercellular membrane contact between human KCs

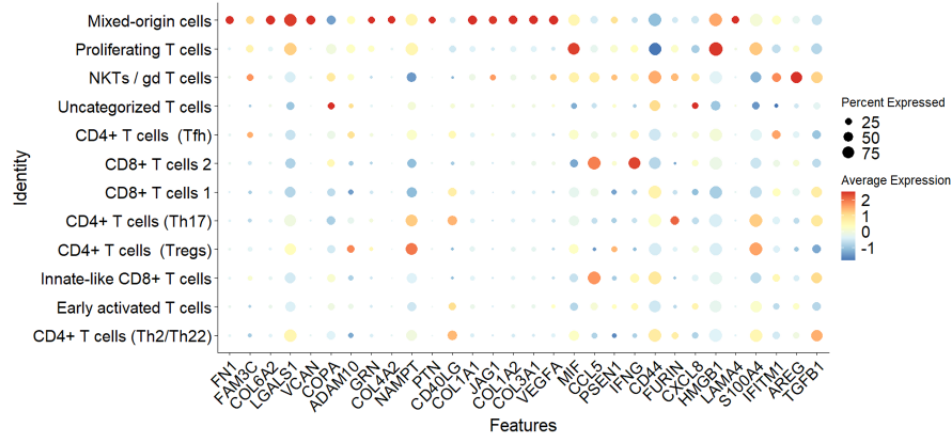
In order to further investigate the proposed potential interaction between CXADR and YAP-1¹¹³⁵, we performed an IF examination of WT HaCaT cells, using antibodies against CXADR and YAP-1, followed by super resolution confocal microscopy. CXADR (green) was diffusely present within the cytoplasm of KCs, and also accumulated at the regions of

A

HC-KC_0

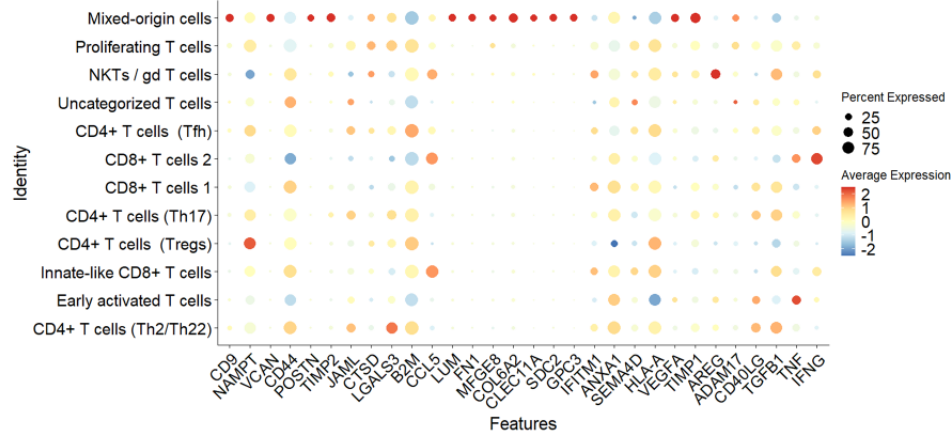


BCC-KC_0



B

HC-KC_3



BCC-KC_3

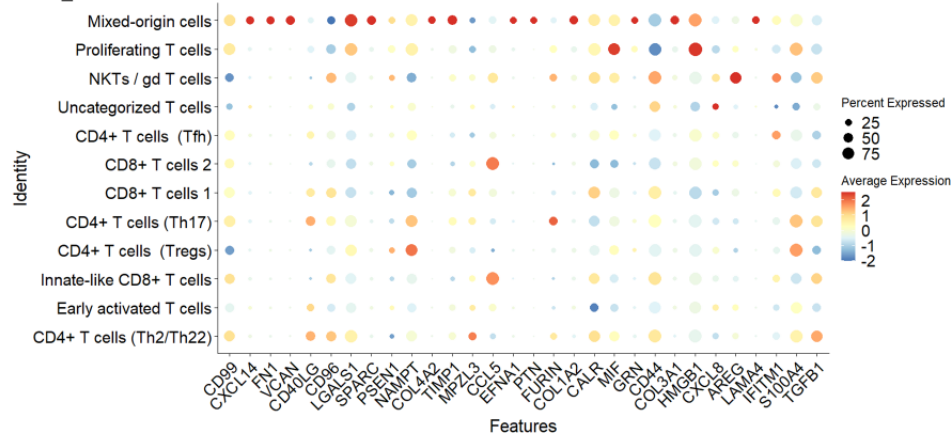


Figure 8: T cell-derived JAML is included in the top ligands of HC-associated KC_3 receptors. Dot plots depicting the expression of the top 30 ranked T cell-derived ligands for BCC compared to HC keratinocyte cluster KC_0 (A) and KC_3 (B) receptors. Dot size indicates percentage of positive cells in the corresponding cluster. Color gradient indicates level of expression

intercellular contacts (**Figure 9**). YAP-1 (red) localized both within the nucleus and the cytoplasm of KCs (**Figure 9**), and this distribution appeared to be influenced by the presence or absence of cell-cell interactions and the degree of cell confluency – an observation which is in accordance with the existing literature ^{1233,1317,1323–1327}. Interestingly, co-localization of YAP-1 with CXADR was identified at the intercellular contact regions, as evidenced by the presence of yellow fluorescence at these foci (**Figure 9**). The observed co-localization of CXADR and YAP-1 is indicative of their interaction, although it cannot be determined solely from this experiment whether this interaction is direct or indirect.

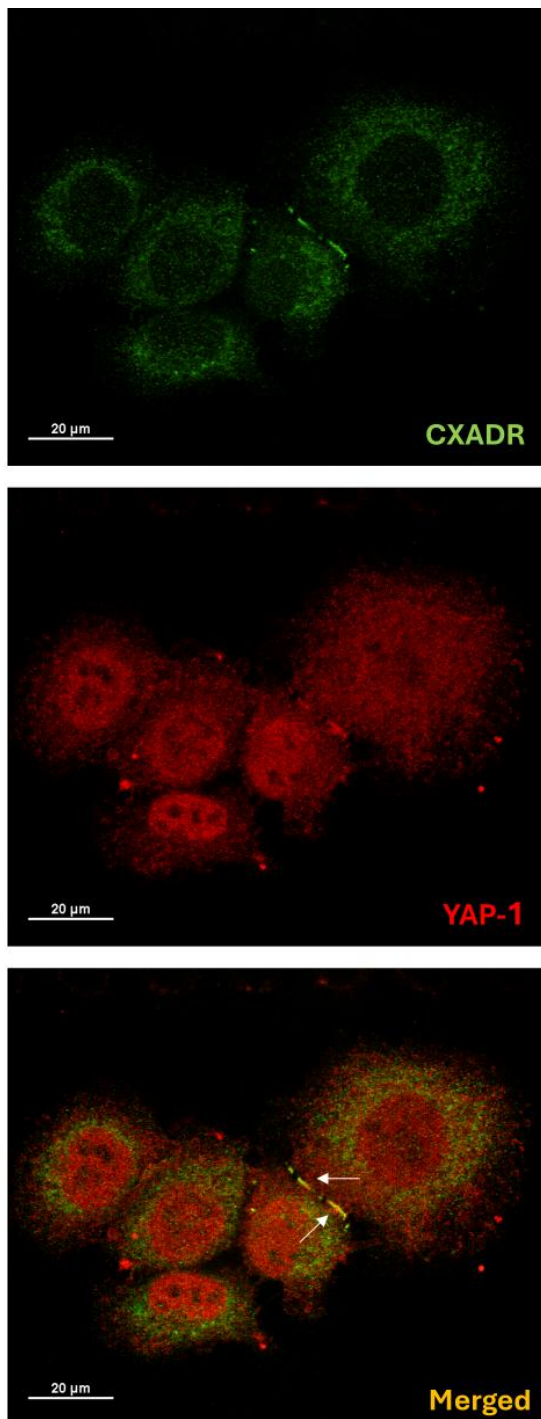


Figure 9: CXADR and YAP-1 co-localize at areas of membrane contact between human keratinocytes. Super resolution confocal microscopy images (100x) obtained following immunofluorescence (IF) staining of WT HaCaT cells for CXADR (green) and YAP-1 (red) (top and middle). Concomitant positive IF staining of the cells for both proteins was evaluated (bottom). Arrows indicate regions of CXADR and YAP-1 co-localization (yellow). Images depicted are representative of three (n = 3) individual experiments.

4.10 *CXADR* and *YAP1* display a homogenous spatial pattern of expression within BCCs

Given that the TME can vary significantly on a spatial level, a subsequent analysis of ST data was performed, originating from a publicly available dataset (accession number: E-MTAB-13084) that consists of BCC (n = 8) and HC (n = 22) samples. Five of these (BCC; n = 3, HC; n = 2) were matched to the scRNAseq samples (E-MTAB-13085). Spatial feature plots of a representative sample are shown in **Figure 10**. The BCC region was identified based on its high expression of *EPCAM*, *KRT17* and *BNC2* (**Figure 10 A**). Importantly, it was uncovered that *CXADR* and *YAP1* display a homogenous expression pattern within BCC areas (**Figure 10 B**), while JAML-expressing T cells accumulate at the periphery of the tumor (**Figure 11**).

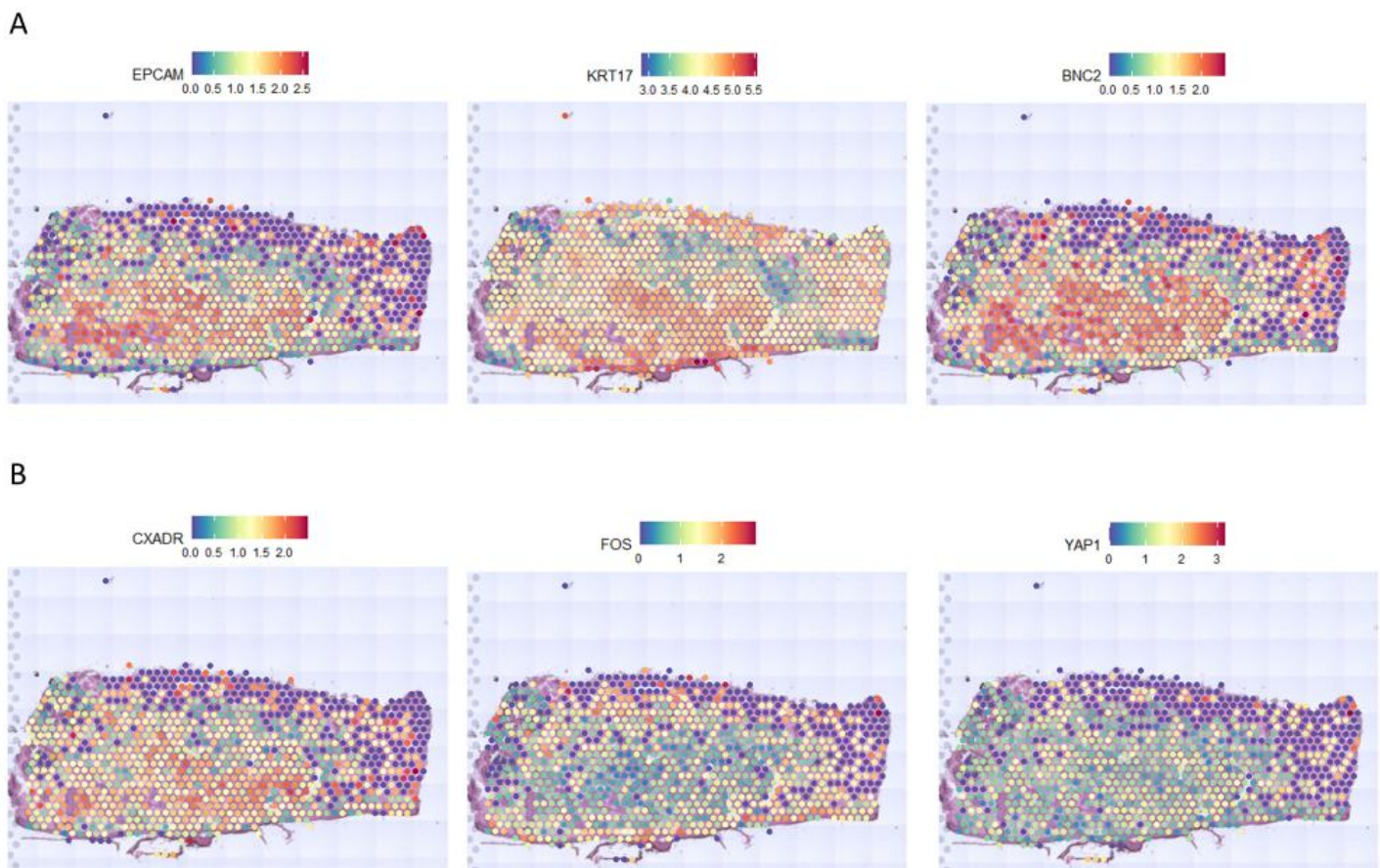


Figure 10: *CXADR* and *YAP1* display a homogenous pattern of expression within BCC aggregates. Spatial feature plots depicting the level and distribution of expression of malignancy-related genes (*EPCAM*, *KRT17*, *BNC2*) (**A**), and *CXADR*, *YAP1* and *FOS* (**B**) in a representative BCC sample following spatial transcriptomic analysis. BNC2 = Basoon zinc finger protein 2.

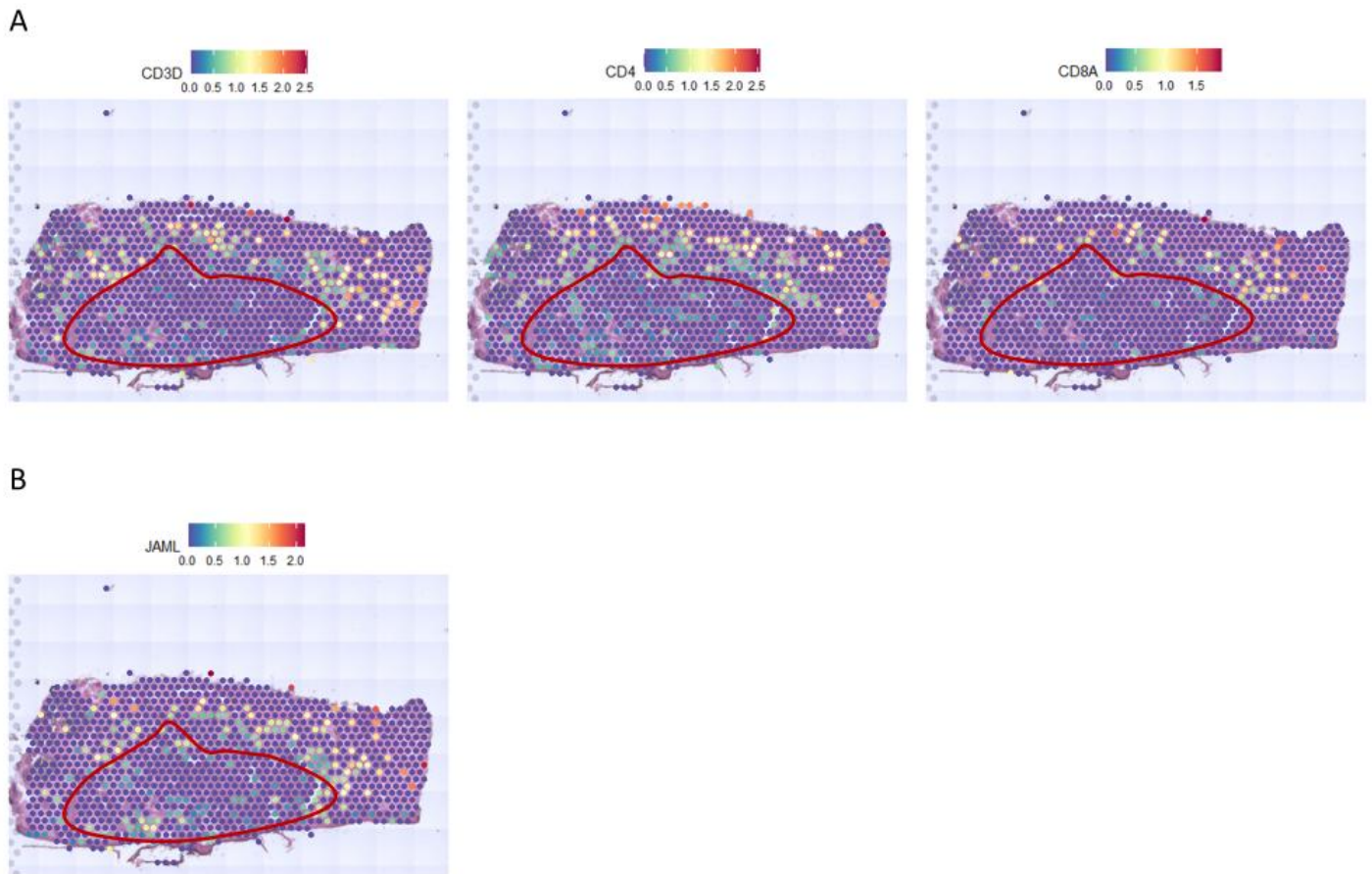


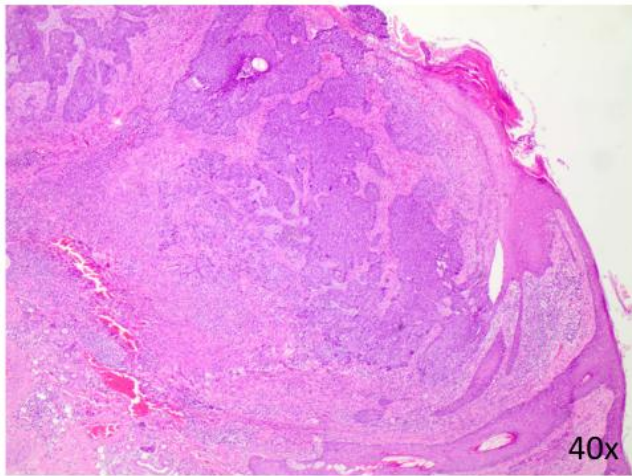
Figure 11: *JAML*-expressing T cells localize preferentially at BCC periphery. Spatial feature plots depicting the level and distribution of expression of T cell-related genes (*CD3D*, *CD4*, *CD8A*) **(A)** and *JAML* **(B)** in a representative BCC sample following spatial transcriptomic analysis. The red outline marks the area of the tumor based on the expression of *EPCAM*.

4.11 CXADR and YAP-1 display a similar spatial pattern of expression in human BCC biopsies

In order to investigate the relevance of the ST observations in clinical specimens, an IHC examination of lesional biopsies from patients with BCC ($n = 4$) was performed (**Figure 12**). The expression of CXADR and that of YAP-1, at the protein level, exhibited a similar pattern within the BCC tumor islands (**Figure 12 C**). This was evident both within nodular BCC foci (characterized by their nodular morphology, peripheral palisading of nuclei, and the presence of a cleft retraction artifact) as well as invasive BCC strands (characterized by their infiltrating, “finger-like” projection within the stroma morphology, and lack of nuclei palisading and cleft retraction artifact) (**Figure 12 C**), with no marked difference between them. A tendency for more prominent CXADR and/or YAP-1 expression at the periphery of the tumor islands (compared to their center) was observed, although this requires further validation (**Figure 12 C**). In malignant cells comprising the tumor masses, CXADR expression was found

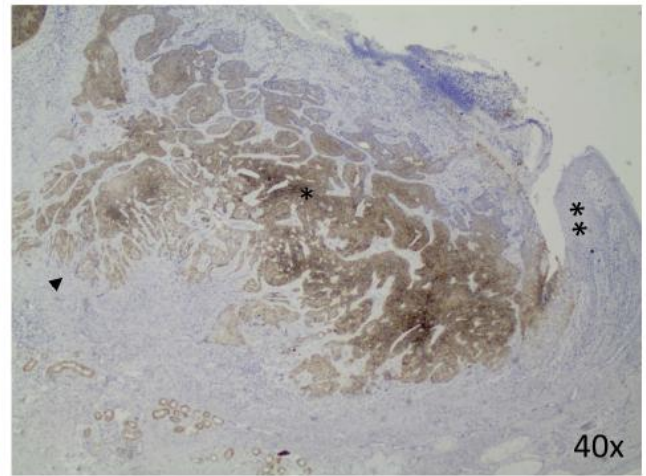
to be mainly membranous (plasma membrane), whereas YAP-1 expression was distributed both in the cytoplasm and in the nucleus (**Figure 12 D**). The intensity of CXADR IHC positivity displayed a degree of variation between the malignant KCs (**Figure 12 D**). Importantly, though, the staining intensity for both proteins was markedly higher in BCC tumor cells, compared to the epithelial cells of the adjacent normal epidermis and those of the HFs (**Figure 12 C**). YAP-1 IHC positivity was also identified within the stroma, although to a much lower degree compared to the epithelial compartment, most probably corresponding to dermal fibroblasts (**Figure 12 C and D**).

A



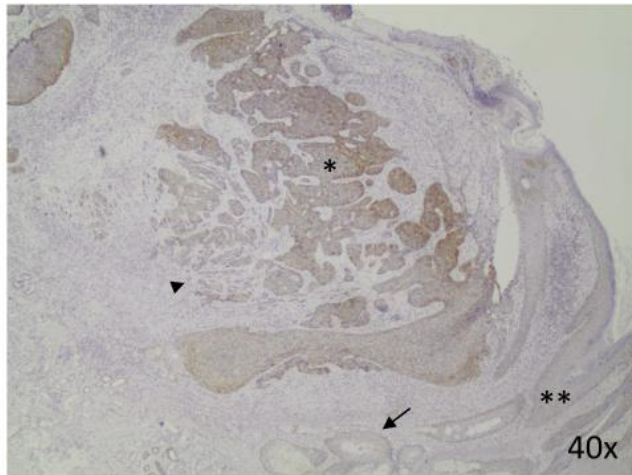
B

EPCAM

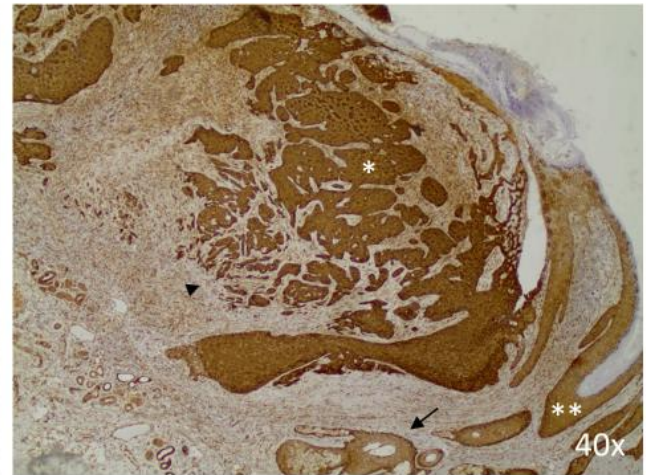


C

CXADR

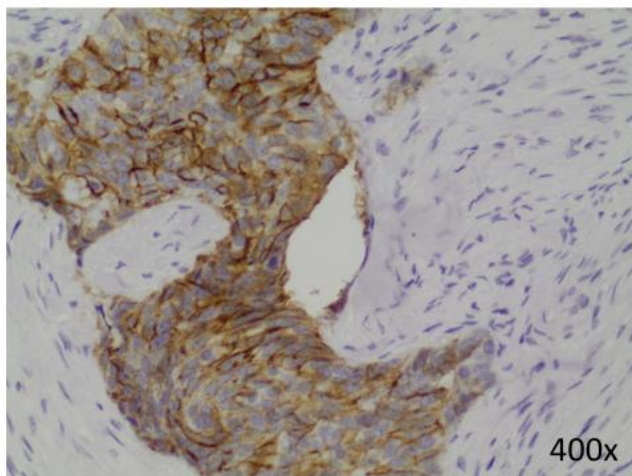


YAP-1



D

CXADR



YAP-1

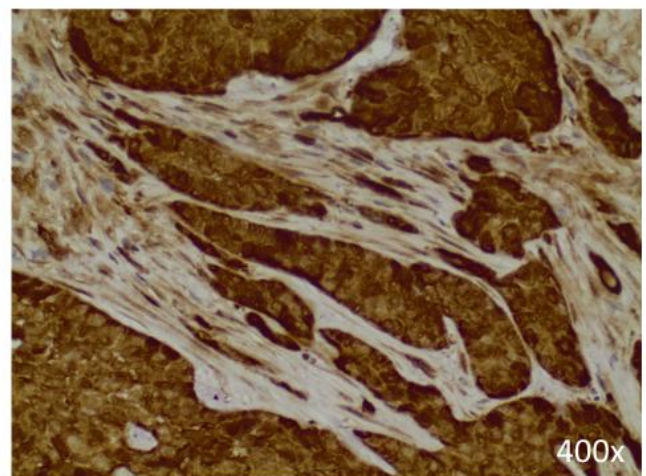


Figure 12: BCC biopsies display a similar pattern of CXADR and of YAP-1 expression, which is markedly higher than that of normal epidermis and hair follicles. A. Low-magnification (40x total magnification) image from Hematoxylin & Eosin (H&E) staining of a representative BCC biopsy of mixed subtype (nodular and infiltrative). **B and C.** Low-magnification (40x total magnification) images from the immunohistochemical (IHC) examination of the same BCC biopsy for EPCAM (**B**), CXADR and YAP-1 (**C**). **B.** IHC positivity for EPCAM is identified within nodular (single asterisk) and invasive (arrow head) tumors aggregates, with a lower expression level observed in the latter compared to the former. EPCAM expression is absent from the adjacent healthy epidermis (double asterisk). **C.** IHC positivity for both CXADR and YAP-1 is recognized within tumor aggregates, both nodular ones (single asterisk) and invasive ones (arrow head), with a similar distribution. Their level of expression is higher than that of epithelial cells in the adjacent healthy epidermis (double asterisk) and HFs (arrow). **D.** Higher magnification (400x total magnification) images of IHC evaluation of the same BCC biopsy for CXADR and YAP-1. IHC positivity for CXADR is identified mainly at the plasma membrane, and to a lesser degree within the cytoplasm, while IHC positivity for YAP-1 is distributed both within the nucleus and the cytoplasm of the malignant epithelial cells. The intensity of CXADR staining exhibits some variability between the malignant epithelial cells. Images depicted are representative of four (n = 4) individual BCC biopsies.

5. Discussion

The present study provides novel insight into the pathogenetic mechanisms underlying BCC, with a focus on the potential role of the JAML-CXADR axis in orchestrating immune (especially T cell)-stromal cell communications, and highlights the potential interaction between CXADR and YAP-1 in driving the malignant phenotype of skin epithelial cells. First of all, analysis of scRNAseq data from human BCC (n = 6) and HC skin (n = 13) biopsies highlighted a disparity in the T cell infiltrate between BCCs and NS – in particular, the relative percentage of Treg cells was found significantly increased in BCCs compared to HCs (**Figure 2 D**), in so supporting previously reported findings regarding the central role of Tregs in the BCC TME^{977–979,983,1504}. This observation is not unexpected given the established immunomodulatory properties of Tregs and the observed association between their infiltration density and worse clinicopathological outcomes in several different malignancies^{693,924–931}, including cSCC⁹³² and melanoma^{933,934}.

Interestingly, a significant downregulation in the relative percentage of Th2/Th22 cells in patient with BCC compared to HC biopsies was identified (**Figure 2 D**). Previous studies in the field have underlined a Th2 cell-associated cytokine profile (IL-4, IL-5, IL-13) dominance in the BCC TME^{978,986,987}, which has traditionally been associated with tumor-promoting effects^{693,732–736,740–743,745–749,755,760,762–769,773,775–780,1528}. Similarly, Th22 cells, through the production of IL-22, have also been implicated in mediating mainly pro-tumorigenic outcomes^{693,854,862–870}, including in the setting of BCC⁹⁸². The observed decrease in Th2/Th22 cells in BCCs vs. HC may be attributable to the following: (i) It may reflect an aspect of the anti-tumor immune response, “compensating” for the presence of other tumor-supporting stimuli, including the increased Treg infiltration. (ii) It is possible that this stems from the method/approach used for the characterization of Th2/Th22-associated responses. In particular, in the studies by Kaporis et al.⁹⁷⁸, Elamin et al.⁹⁸⁶, and Yamamura et al.⁹⁸⁷, identification of IL-4, IL-5 and/or IL-13 happens at the cytokine mRNA and/or protein level, however, no marker is utilized to verify the presence of the related Th2 cells themselves. Given that these cytokines, especially IL-4, may be secreted by additional immune and non-immune cell types, including tumor cells^{732–735,737–739}, the findings of the aforementioned studies may not necessarily reflect an increased Th2 cell infiltration in the BCC TME. Contrarily, in our study, the characterization of the Th2/Th22 cluster was made according to additional markers (aside from *IL4*, *IL5*, *IL13* and *IL22*) including Th2- and Th22- associated TFs, such as *GATA3* & *STAT5A/B*, and *AHR* & *RORC*, respectively. (iii) Furthermore, it is essential to note that, although not as common, context-dependent tumor-suppressive functions have also been reported for the Th2- and Th22- associated cytokines in the literature^{693,781–798,1529,1530}; thus, the reduced Th2/Th22 infiltration observed in our study may reflect a tumor-promoting aspect of the BCC TME.

Another intriguing observation from our analysis is the significant enrichment of BCC samples in Tfh cells, compared to healthy skin (**Figure 2 D**). Tfh cells are mostly associated with anti-tumor functions^{874,884,1531–1540}, owing, first of all, to their critical contribution in the organization and maturation of germinal centers, and in so, in the proliferation, activation, and high-affinity antibody production by B cells^{873,874,1541,1542}. Several additional tumor-suppressing outcomes have been described for their signature cytokine, IL-21, mediated through its effect either on immune^{1542–1546}, tumor¹⁵⁴⁷, or stromal¹⁵⁴⁸ cells within the TME. (i) Similarly to what was proposed for the observed reduced Th2/Th22 infiltration in BCC, one potential explanation for the increased Tfh presence in BCC would be that it represents part of the anti-tumor immune responses, aiming to counterbalance the pro-tumorigenic effects of the TME. (ii) However, there is also compelling evidence for the pleiotropic nature of IL-21, with some studies supporting tumor-promoting outcomes exerted by it, even in the context of solid tumors^{1549,1550}. (iii) Moreover, among the Tfh cluster, Tfh2 subtype and/or Tfr cells may be present, which are associated with immunomodulatory functions^{873,875,886,887}, and could thus promote the BCC carcinogenic process. Indeed, part of the Tfh cluster cells in our data display *GATA3* and *STAT5A/B* expression (indicative of Tfh2), although no Tfr-associated *FOXP3* expression is identified. (iv) More importantly though, when assessed for the expression of several exhaustion-related markers¹⁵⁵¹ (*LAG3*, *PDCD1*, *CTLA4*, *HAVCR2*, *TIGIT*, *TOX*), the BCC-associated Tfh cells displayed a significantly higher expression of *TOX* (***) p-value ≤ 0.001) and *CTLA4* (* p-value ≤ 0.05) compared to HC-associated ones (data not shown), thus suggesting that their increased abundance in the BCC TME may not actually translate to anti-tumor effects, but rather be accompanied by a state of increased Tfh cellular exhaustion, compared to healthy skin.

Importantly, both the Th2/Th22 (downregulated in BCC) and Tfh (upregulated in BCC) clusters, along with Th17 cells (which did not display any significant difference in their frequency between BCC and HC) were enriched in *JAML* expression, and shared some common enriched molecular pathways, such as “Th17 cell differentiation” and “TCR signaling pathway”. In addition, our ST analysis highlighted the preferential accumulation of *JAML*-expressing T cells at the periphery of BCC tumors rather than within them (**Figure 11**). To my knowledge, no previous study has examined the distribution of *JAML*+ T cells in regard to the tumor mass. Although in vitro examinations are required to corroborate our in silico observations, this proposed preferential localization of *JAML*+ T cells at BCC periphery is in accordance with the observed tendency for CXADR IHC expression to be relatively higher at the outer edge of the BCC tumor islands compared to their center (**Figure 12 C**). Future in vitro studies are necessary in order to explore additional potential underlying mechanisms and

the potential clinicopathological significance of this unique localization pattern of JAML and CXADR expression.

From the perspective of tumor epithelial cells, our IHC staining of clinical BCC samples indicated a markedly higher expression of both CXADR and YAP-1 in BCC cells compared to adjacent healthy KCs of the IFE and HFs, corroborating previous observations^{97–101,1163}. Signaling by the YAP-1 transcriptional co-activator has been positively implicated in BCC development by several studies on human and/or murine BCCs^{97–101,190,192}, while very limited evidence supports a potential BCC-promoting effect of CXADR as well¹¹⁶³. Nonetheless, to my knowledge, no study until now has examined the potential interplay between these two proteins in the pathogenesis of BCC.

Furthermore, our IHC examination highlighted a similar pattern of distribution for the expression of both CXADR and YAP-1 within the BCC tumor islands (**Figure 12 C**). Although our ST examination had demonstrated an overall homogenous pattern of *CXADR* and *YAP1* expression within BCC aggregates, our IHC observations suggest a potential tendency for the expression of both proteins to be relatively higher at the peripheral area of the tumor masses (**Figure 12 C**). This is partly in accordance with the findings of Maglic and colleagues⁹⁹, who have reported a relatively increased peripheral expression of YAP-1 at the mRNA level in murine BCC and at the protein level in human BCC tumors. Interestingly, a recent IHC study in human BCC biopsies has underlined that Ki-67 and p53 expression is also more prominent in the peripheral palisading BCC tumor regions¹⁵⁵². Whether there is indeed a significant upregulation in CXADR and YAP-1 expression at the tumor edges, and whether/how this correlates with the proliferative/mutational status of the BCC cells remains to be elucidated in future studies.

In addition, the mainly membranous IHC positivity for CXADR in tumor cells (**Figure 12 D**) is in accordance with the protein's predominant transmembrane localization at the plasma membrane^{1114,1116,1136}, and comes to add to the scarce existing evidence regarding the expression profile of CXADR in human BCC tumors¹¹⁶³. On the other hand, YAP-1 was identified in the cytoplasm, but also the nucleus of malignant epithelial cells, as was expected based on its pro-tumorigenic role in BCC, and in accordance with previous observations^{97–101}.

Although our sample size is relatively small ($n = 4$), the identification of the aforementioned observations in both nodular and infiltrative BCC areas in our series supports the findings by Debaugnies and colleagues, who have reported that no significant differences in YAP-1 expression between distinct BCC subtypes (including superficial, nodular and infiltrative) exist⁹⁷, and, moreover, provides preliminary evidence for the absence of marked differences in CXADR expression as well between BCC subtypes.

Further interrogation of our scRNAseq data, and especially execution of a cell-cell interaction analysis between the KC clusters and the T cell populations, suggested that Th2/Th22-, Tfh- and/or Th17- expressed JAML, may be, at least partly, responsible for driving the transcriptomic profile of specific HC KC clusters via its engagement to CXADR. This observation could potentially further implicate the downregulated JAML-CXADR axis in the pathogenesis of BCC.

To further characterize the potential role of CXADR and/or YAP-1 in this observed interaction in the setting of cutaneous carcinogenesis, we sought to investigate the effect of classically-considered as tumor-promoting Th2 cytokines (IL-4, IL-5, IL-13) and recombinant JAML on the protein expression level of CXADR and YAP-1 in human epithelial cell line (HaCaT) cells. Our preliminary WB data indicate that recombinant JAML may not influence the expression of either CXADR or YAP-1. However, IL-5 may possibly upregulate both CXADR and YAP-1 expression in WT HaCaT cells. One potential mechanism underlying this observation entails the activation of the MAPK pathway downstream of the IL-5R^{1553–1555}. MAPK stimulation results in the activation of the AP-1 TF^{1556–1558}, binding sites for which have been recognized in the enhancer/promoter region of both *CXADR* and *YAP1* genes (GeneHancer)¹²⁶⁹. Moreover, both the *CXADR* and *YAP1* cis-regulatory elements contain binding sites for the p300 protein (GeneHancer)¹²⁶⁹ which is also known to be regulated by the MAPK pathway^{1559,1560}. On the contrary, IL-4 and IL-3, which share a common receptor, primarily stimulate STAT6, and do not usually activate the MAPK pathway^{754,1561,1562}, so it would make sense for them to not alter CXADR or YAP-1 expression, as no STAT6-CXADR or STAT6-YAP1 regulatory mechanism has been established.

Moreover, neither recombinant Th2 cytokines nor recombinant JAML exerted any significant effect on the migratory capacity of HaCaT cells. This is partly in accordance with a previous study by Weber and colleagues, in which they demonstrated that sJAML interacting with intestinal epithelial cell-expressed CXADR, despite interfering with the wound healing process in vitro, does so by inhibiting the proliferative capacity of cells, and not by suppressing their migration¹⁰⁸¹. In contrast to our experimental procedure (see Materials and Methods), in the study by Weber and colleagues^{1081,1563}, no serum starvation step is described prior to the introduction of the wound and sJAML treatment – this could explain the discrepancy between our results and theirs, as the 24h serum starvation step implemented in our case has potentially eliminated any possible effects of cell proliferation in wound closure. Performing a proliferation assay in our epidermal cell line under recombinant JAML treatment would help clarify this hypothesis. Nonetheless, it cannot be ruled out that the different origin of the cell lines and the use of fractioned (sJAML) vs. full-length recombinant JAML may have contributed to the observed discrepancy in cell behavior in the two studies. Furthermore, another interesting

future approach would be to examine whether *immobilized* recombinant JAML (instead of one in solution) exerts any different influence on the migration of KCs, or in the localization pattern of YAP-1 in KCs, in vitro. We hypothesize that ligand-mediated CXADR dimerization in cis may be necessary for downstream signaling activation (including any potential effects exerted through its suspected interaction with YAP-1), as for other JAM family members, including JAML^{1051,1053,1055} and JAM-A^{1564–1566}, their dimeric in cis form has been shown to modulate the molecule's functionality and ultimately cell behavior. Utilizing cell culture plates pre-coated with bound recombinant JAML would favor the formation of CXADR dimers (in contrast to unbound JAML molecules), and it would also facilitate the generation of shear stress force via CXADR as KCs move along the JAML-coated surface, which we hypothesize may be another factor implicated in the activation of the CXADR receptor.

Regarding Th2-related cytokines, although several studies have assessed the influence of IL-4 on KCs in vitro^{1567–1571}, only limited evidence exists for its effect on KC migration in particular, with reported results being ambiguous. For instance, one study on primary human foreskin KCs identified a significant negative effect of IL-4 on their migratory capacity in vitro¹⁵⁷², whereas another study on primary normal human epidermal KCs (NHEK) demonstrated no significant alteration in their migration under IL-4 stimulation¹⁵⁷³, similarly to our observations. Nonetheless, IL-4 has been suggested to negatively impact epidermal wound healing in vivo¹⁵⁷³ – but additional, complex, and not solely KC-dependent mechanisms may be involved in this case. To my knowledge, the individual effect of IL-5 or IL-13 on human epidermal KC migration has not been studied. Further research is required in order to shed light into the potential role of the Th2-associated cytokines, that have consistently been reported to be present in the BCC TME^{978,986,987}, in its pathogenesis.

Due to the effect of Th2 polarization on T cell JAML expression, the expression of CXADR in BCC KCs was investigated and was found to be co-expressed to a significantly higher degree with *FOS* in BCC-derived KCs compared to HC-derived ones. Interestingly, *FOS* expression on its own has previously been reported to be lower in human BCC cells compared to basal layer cells of the normal epidermis¹⁵²¹. In addition, the protein encoded by the *FOS* gene (c-Fos) has been found increased in invasive compared to non-invasive BCCs^{1522,1523}, and has been implicated in basal to squamous cell carcinoma transition (BST)^{1524,1525}, a process which is anticipated to correlate with increased tumor aggressiveness^{597,1526}. c-Fos has mainly been associated with the differentiation process, rather the proliferation of KCs^{1574–1578}, while it has, intriguingly, been proposed to promote the cutaneous chemoattraction of CD4⁺ T cells, including IL-22-producing ones, via the induction of MMP10 and S100A15 secretion by the malignant epithelial cells¹⁵⁷⁶. In addition, although preliminary, our WB data suggest that the potential positive effect of IL-5 on CXADR and YAP-1 protein levels observed

in WT HaCaT cells is abrogated when *FOS* is overexpressed (CFOS HaCaT cells), and moreover, that CXADR expression is relatively downregulated in CFOS cells (under all conditions) compared to WT cells. Taking all of the above into consideration, we hypothesize that c-Fos may negatively regulate the expression of CXADR, while in the context of BCC tumorigenesis, the upregulation of CXADR expression via an unrelated (still undetermined mechanism) may result in the “compensatory” increase of *FOS* expression as well. Nevertheless, the significance of the increased *FOS* and CXADR co-expression in BCCs compared to NS needs to be further explored in future studies.

Ortiz-Zapater and colleagues had recently proposed a potential novel interaction between CXADR and YAP-1 in human bronchial epithelial cell line (16HBE) cells, as they reported YAP-1 among the binding partners of CXADR in a BioID assay ¹¹³⁵. Importantly, our series of IF experiments corroborates this notion, and in fact further expands it in the setting of human epidermal epithelial cell line (HaCaT) cells, as co-localization of CXADR and YAP-1 is revealed at sites of intercellular membrane contact between them – indicative of their interaction (**Figure 9**). Nevertheless, neither of these two observations can determine whether the proposed interaction between CXADR and YAP-1 is direct or indirect. CXADR comprises a known and important component of tight and adherens junctions, including in epithelial cells ^{1107,1118,1122,1140}, while several regulatory proteins acting upstream of YAP-1 exhibit established interactions with adherens (and/or tight) junction components. For instance, both the MST and LATS kinases can be recruited to the cell membrane through their interaction with the adherens junction-associated NF2 protein ^{1311–1315}. More importantly, PTPN14 ^{221,222}, as well as AMOT family members ^{1319,1320}, which are known to associate with adherens junction proteins ^{1317,1318}, can interact with YAP-1, while α -catenin has also been shown to indirectly bind to YAP-1 via 14-3-3 proteins ^{1323,1324}. It would, therefore, not be surprising, at least from a functional point of view, if CXADR were found to interact with MST or LATS, and in so indirectly recruit YAP-1 to the intercellular junction areas. In addition, another probable underlying mechanism would be the indirect interaction between CXADR and YAP-1 via their mutual binding to a PDZ-containing scaffolding protein, especially ZO (zonula occludens), since both CXADR ^{1037,1052,1118,1119} and YAP-1 ^{1260,1278} possess a C-terminal PDZ-binding motif, and are known to interact with ZO-1 ¹¹²² and ZO-2 ^{1325,1326}, respectively. Moreover, CXADR has been found to co-localize with α -catenin (as well as modulate its expression), despite not directly interacting with it ¹⁵⁷⁹ – α -catenin, nonetheless, known to indirectly associate with YAP-1 ^{1323,1324}, was identified as a binding partner of ZO-1 ^{1323,1324}, which comprises an established interactor of CXADR ¹¹²². Thus, one can hypothesize that a CXADR/ZO-1/ α -catenin/14-3-3/YAP-1 protein complex may also explain the observed CXADR-YAP-1 co-localization. Taking into account the above, along with the structural and/or functional association of YAP-1 with caveola and

clathrin-coated pits ¹⁵⁸⁰ – specialized plasma membrane regions that might also be related to CXADR ^{1114–1116,1135,1147,1148}, we aim to focus our future studies on elucidating the structural and functional aspects of the CXADR-YAP-1 interaction. Indicatively, an interesting approach would be to interrogate whether the targeted inhibition of ZO-1 and/or ZO-2 expression in primary KCs from HC and patient with BCC skin biopsies abolishes the co-localization pattern of CXADR and YAP-1. In addition, the implementation of computational approaches ¹⁵⁸¹ could provide us with evidence on whether CXADR and YAP-1 are capable of directly interacting with one another, and in so guide the design of our future in vitro experiments.

Limitations of the study & Future directions

First of all, despite the robustness of our scRNAseq analysis, owing to the high number of cells (n = 187100) included, this method, by definition, allows us to gain insight only on the transcriptomic profile of the cells, with no assessment of their proteomic and/or functional status. For the absence of spatial information in scRNAseq, we compensated with the inclusion of ST data; however, ST analysis also exhibits inherent limitations, including its non-single-cell resolution, as more than one cells (up to 10) are included in each spot. Overall, as holds true for all in silico observations, it is imperative for our scRNAseq and ST findings to be validated in vitro and/or in vivo.

Another important consideration, regarding our receptor-ligand interaction analysis, is that the uncovered JAML-CXADR interaction may, at least partly, mask the effect of the in trans homophilic CXADR-CXADR interactions on the transcriptomic profile of the KCs. The homophilic CXADR interaction is not included in the ligand-receptor network from Zenodo that is used as reference in nichenet; hence, when we examined the autologous interactions between KC_0 or KC_3 and the rest of the KC clusters in BCC and HC, CXADR did not show up as a potential ligand. It is probable, nevertheless, that at least part of the transcriptomic differences between HC- and BCC- derived KC_3 cells that have been ascribed to JAML's binding to CXADR may be attributable to CXADR's binding to the CXADR of neighboring KCs.

Regarding the in vitro experiments performed in this study, it should be noted that in certain instances sample sizes were small, but are currently being expanded. Furthermore, the HaCaT cell line (utilized for WB, scratch assay and IF), although widely used in the field of cutaneous research, is not the ideal in vitro model. HaCaT cells comprise a spontaneously immortalized, aneuploid cell line derived from human adult epidermis, which resembles non-malignant basal KCs and retains the ability for proliferation and differentiation ¹⁵⁸². However, as holds true for all cell lines, HaCaT cells may have potentially underwent unknown modifications in the course of their passages since their establishment in 1988, and overall display several disparities in their expression and behavioral profile compared to primary KCs

^{1583–1585}. Especially in terms of their response to Th cell-associated cytokines (including IL-4), it has been shown that HaCaT cells differ substantially from human primary KCs ¹⁵⁸⁶. Thus, in relevance to our study, although they can provide useful preliminary information for the biological processes/interactions of interest, the degree to which this is actually representative of what takes place in the setting of BCC malignant epithelial cells in vivo is debatable.

For this reason, we are currently focusing on examining the expression level and pattern, as well as the potential co-localization of CXADR and YAP-1 in primary KCs isolated from patient with BCC biopsies in comparison to primary KCs from HC skin. Moreover, expanding our series of BCC biopsies for IHC examination will help us further investigate the existence of any potential associations between the degree and/or distribution of CXADR and YAP-1 (co-)expression and clinicopathological features of higher tumor aggressiveness.

Another interesting approach would be to generate a basal cell-specific CXADR KO BCC model by crossing *Cxadr*-floxed with K14-Cre mice in the background of SmoM2 (K14-CreER/Rosa-SmoM2) mice, one of the most widely used BCC murine models ^{97,99,190,192}, and investigate how several pro-tumorigenic stimuli (including, for example, the Th2-associated cytokines) can affect barrier integrity and immune cell infiltration.

The overall aim of the present, and for future studies to follow, is to help elucidate the potential aspect of BCC pathogenesis involving an interplay between the JAML-CXADR axis and YAP-1, as this could have an important impact on current therapeutic options for difficult-to-treat BCCs.

6. References

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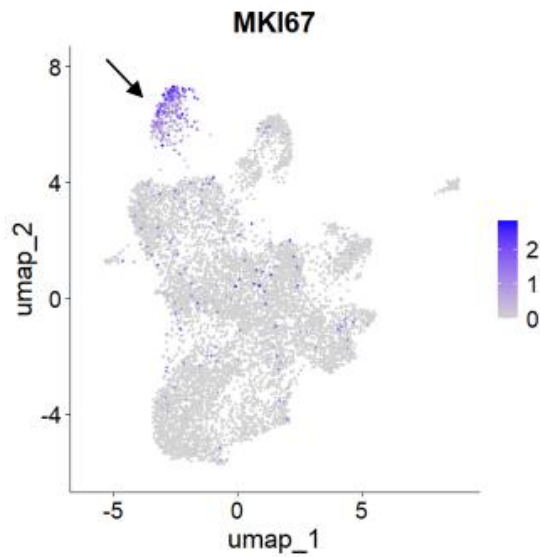
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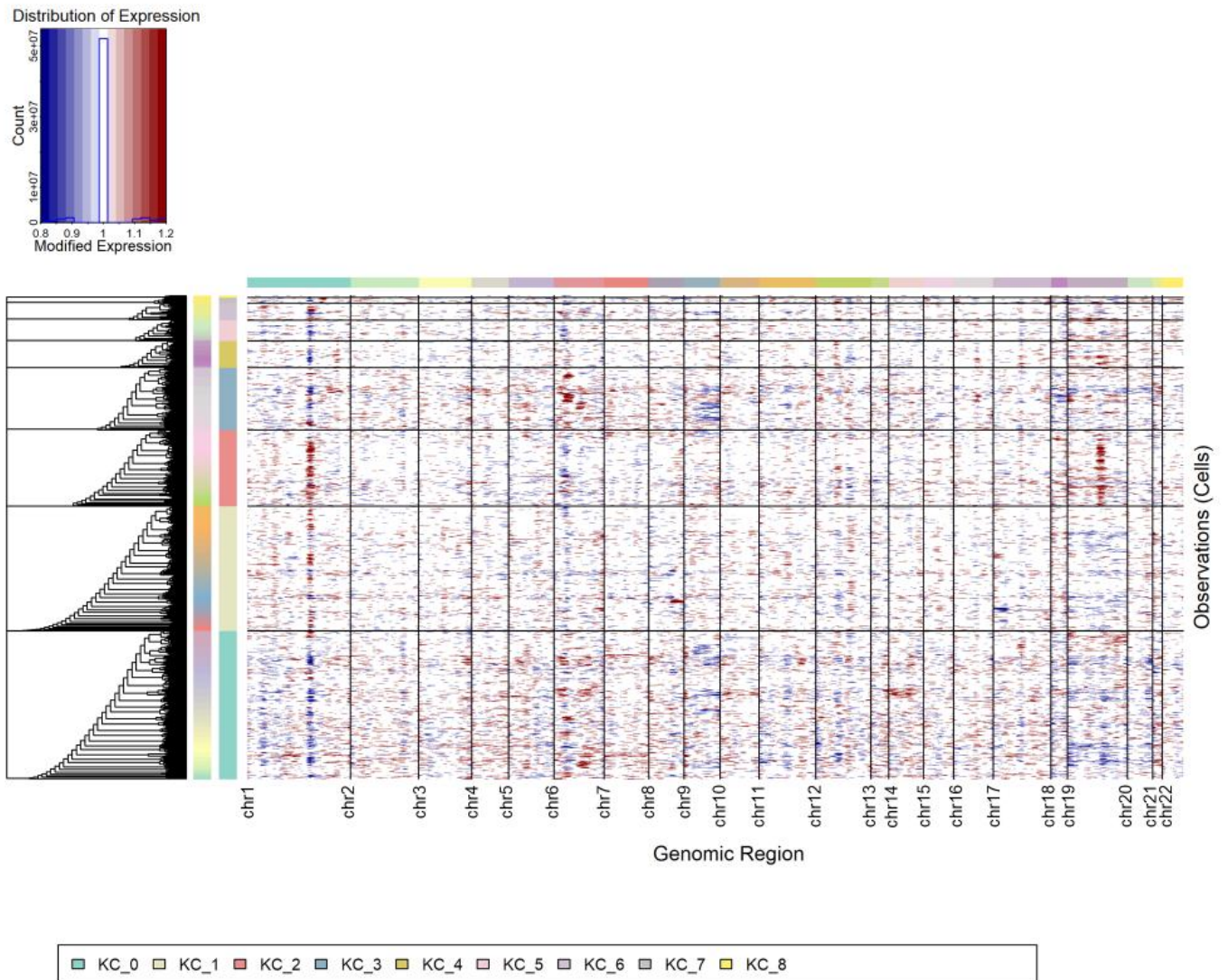
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7. Supplementary Figures

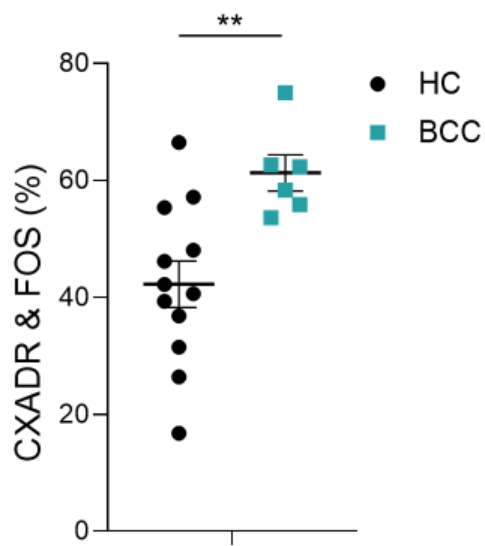


Supplementary Figure 1: Cluster KC_6 contains proliferating keratinocytes. Feature plot showing the level and distribution of expression of representative proliferation-associated gene *MKI67* in KC clusters. The arrow denotes cluster KC_6. *MKI67* = Marker Of Proliferation Ki-67.

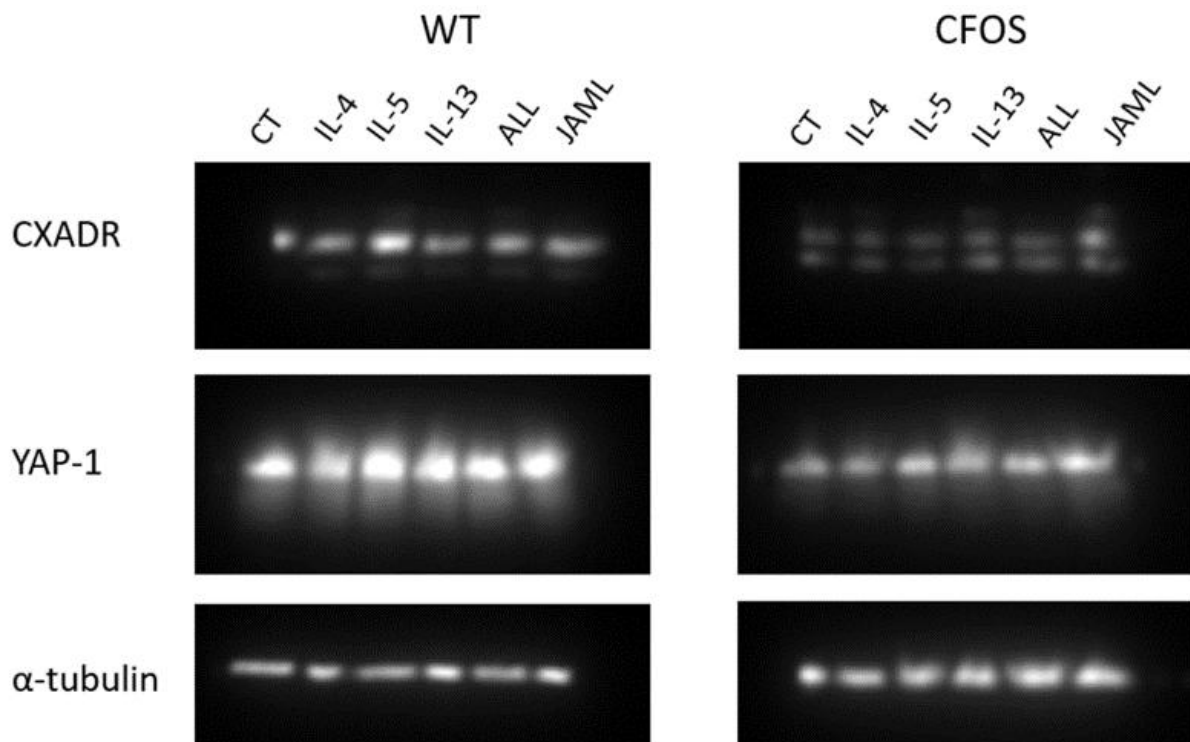
inferCNV



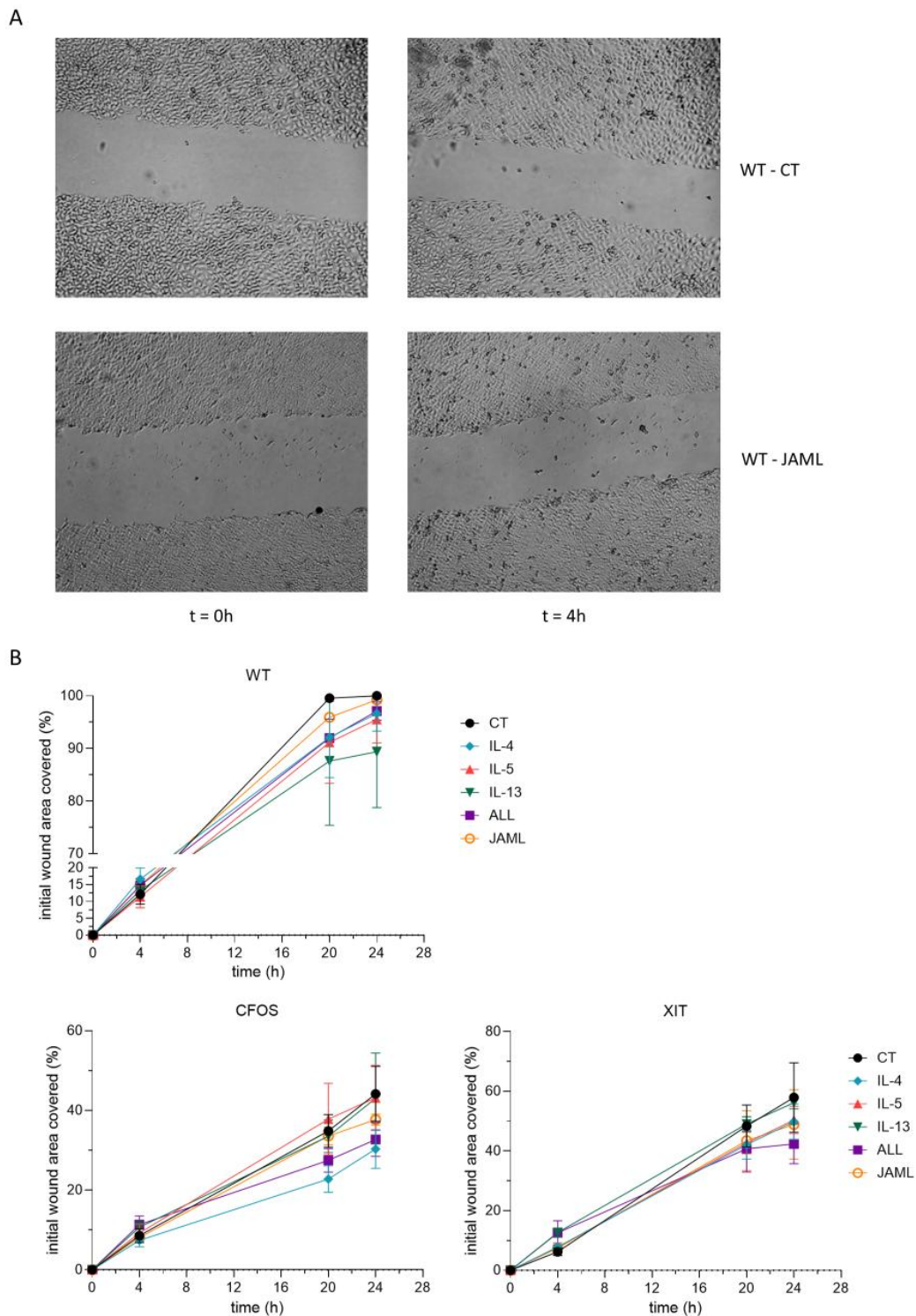
Supplementary Figure 2: Keratinocyte clusters KC_0 and KC_3 display BCC-associated copy number variations (CNVs). InferCNV plot displaying the CNVs inferred for the KC clusters based on the single-cell RNA sequencing data. Red color indicates inferred copy gain, while blue color indicates inferred copy loss, at the respective chromosomal area. chr = chromosome.



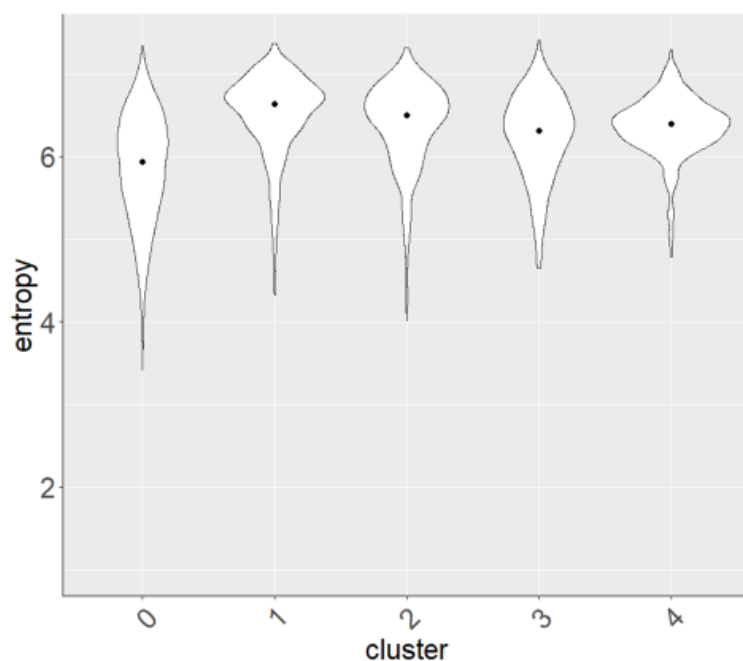
Supplementary Figure 3: BCC-derived keratinocytes display a significantly higher percentage of *CXADR* and *FOS* co-expression compared to HC-derived keratinocytes. Relative percentage of KCs co-expressing *CXADR* and *FOS* among all KCs, in BCC compared to HC samples. Each symbol represents a distinct biological sample, bars indicate mean $\pm$ SEM. Mann-Whitney test was used for comparative analysis (** p-value ≤ 0.01).



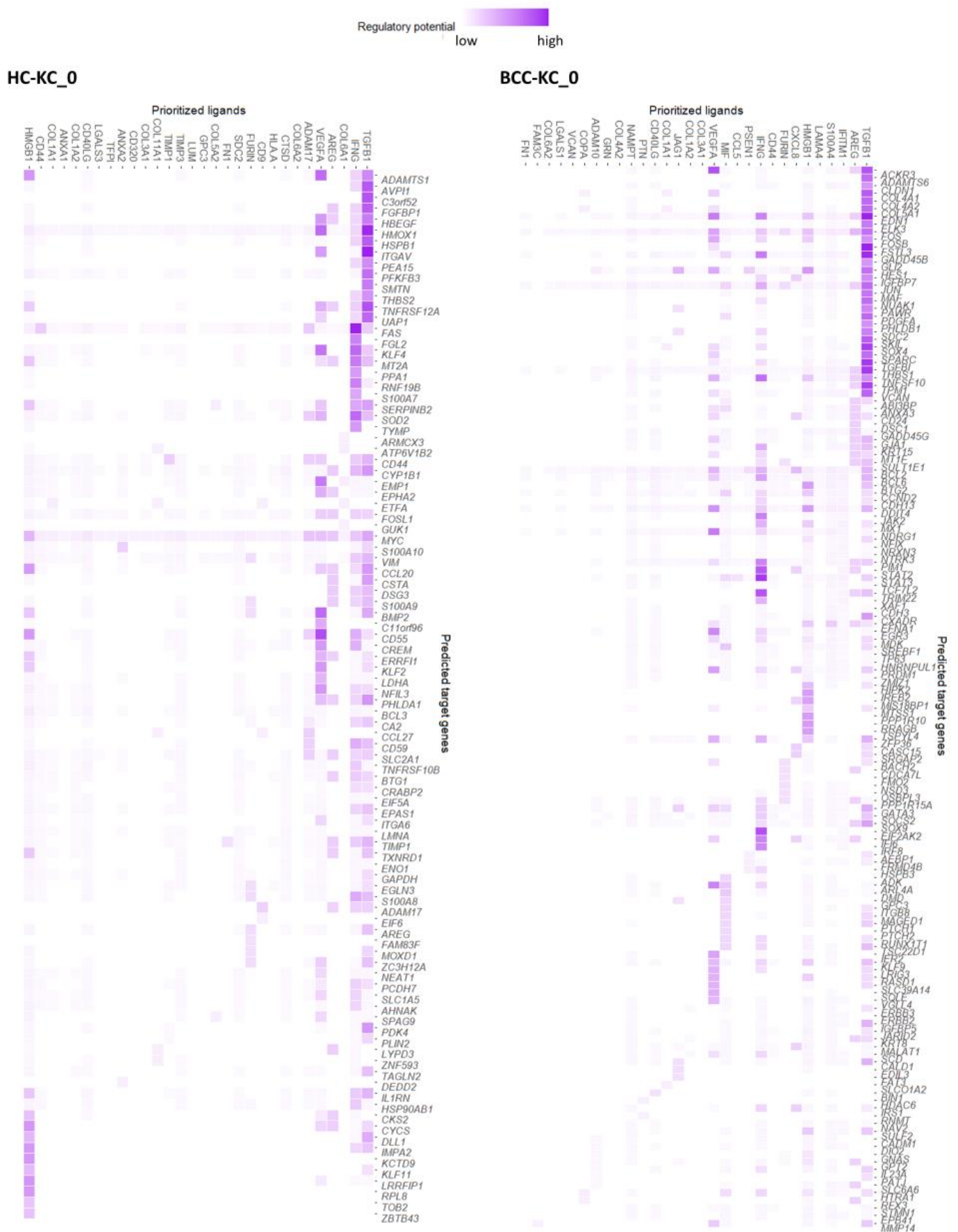
Supplementary Figure 4: IL-5 may upregulate the protein expression of CXADR and YAP-1 in human keratinocytes. Western Blot analysis images depicting the protein expression of CXADR, YAP-1 and α -tubulin in WT and CFOS HaCaT cells cultured with either recombinant IL-4, recombinant IL-5, recombinant IL-13, a combination of all three (ALL), or recombinant JAML. Cells treated with medium were used as controls, (n = 1).



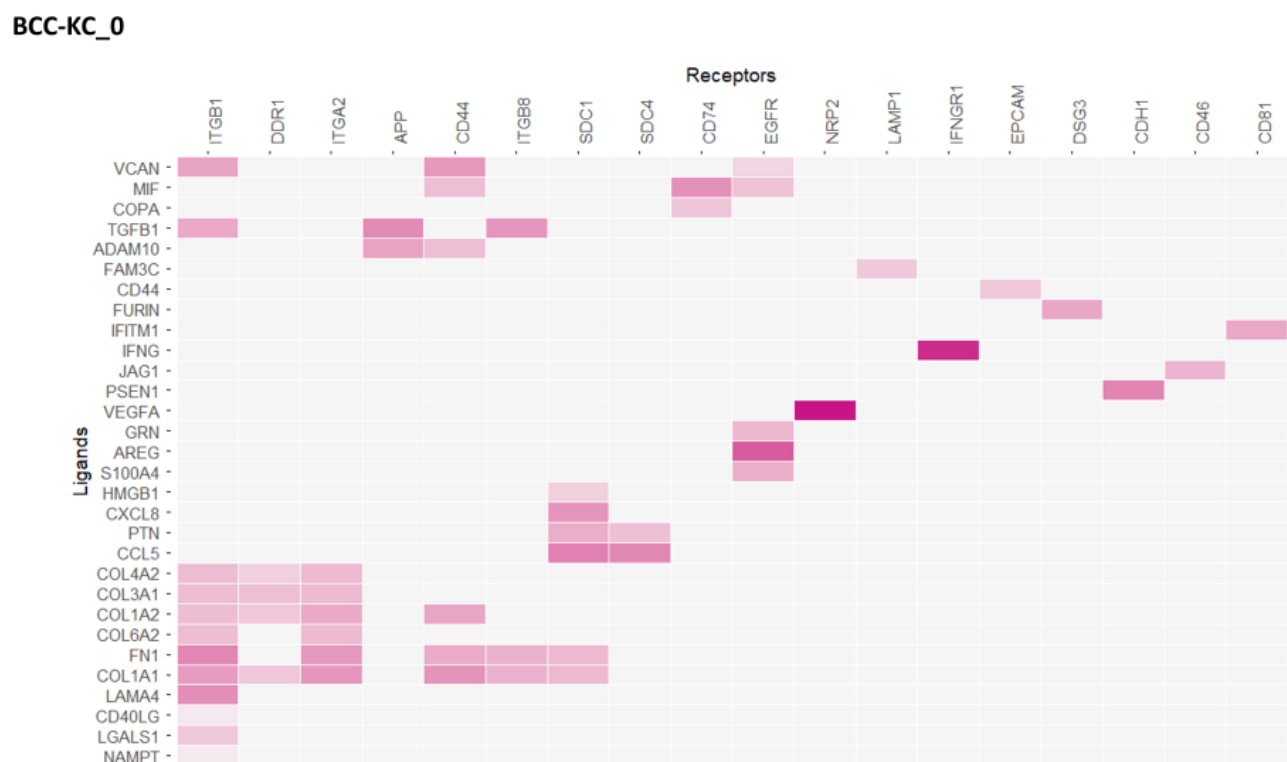
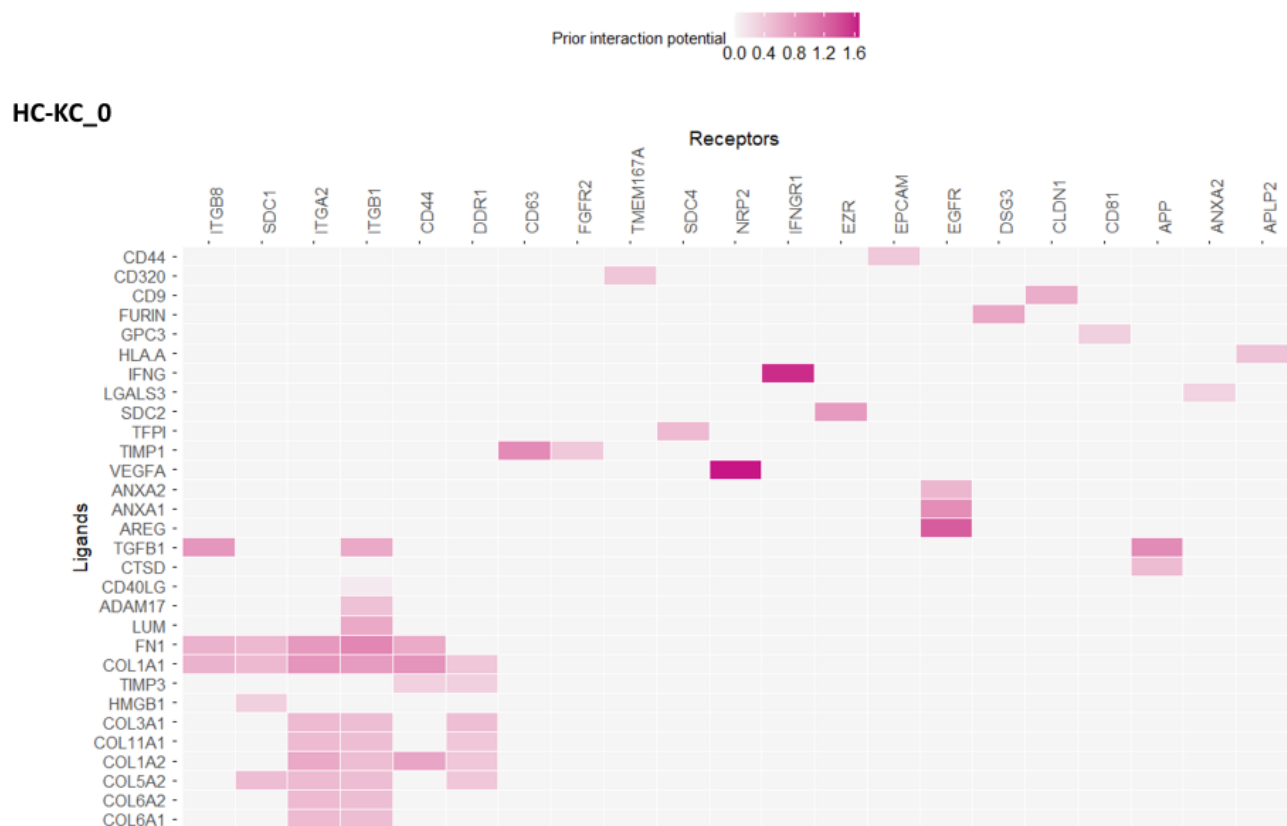
Supplementary Figure 5: Scratch assay of HaCaT cells following treatment with recombinant Th2 cytokines or recombinant JAML. **A.** Representative phase-contrast microscopy images (4x) of WT HaCaT cells treated with serum-low (1% FBS) medium (CT) or recombinant JAML (JAML) immediately after introduction of the scratch (t = 0h) and four hours later (t = 4h). **B.** Graph depicting the normalized percentage of the initial wound area covered at given time points in WT, CFOS and XIT HaCaT cells treated with either recombinant IL-4, recombinant IL-5, recombinant IL-13, a combination of all three (ALL), or recombinant JAML. WT, CFOS and XIT HaCaT cells, respectively, treated with serum-low (1% FBS) medium were used as negative controls (CT). Each symbol represents the mean percentage from three (n = 3) independent experiments, error bars indicate SEM. Two-way ANOVA and Student's t-test were used for comparative analysis.



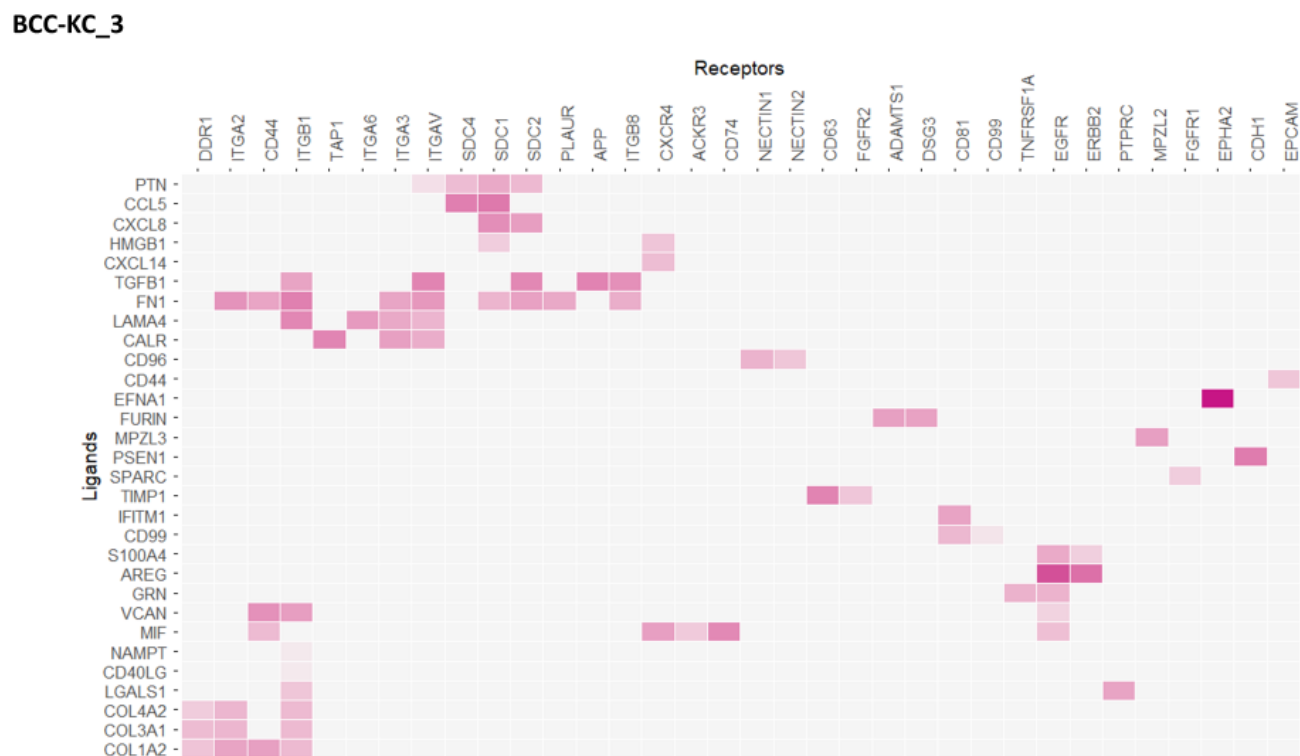
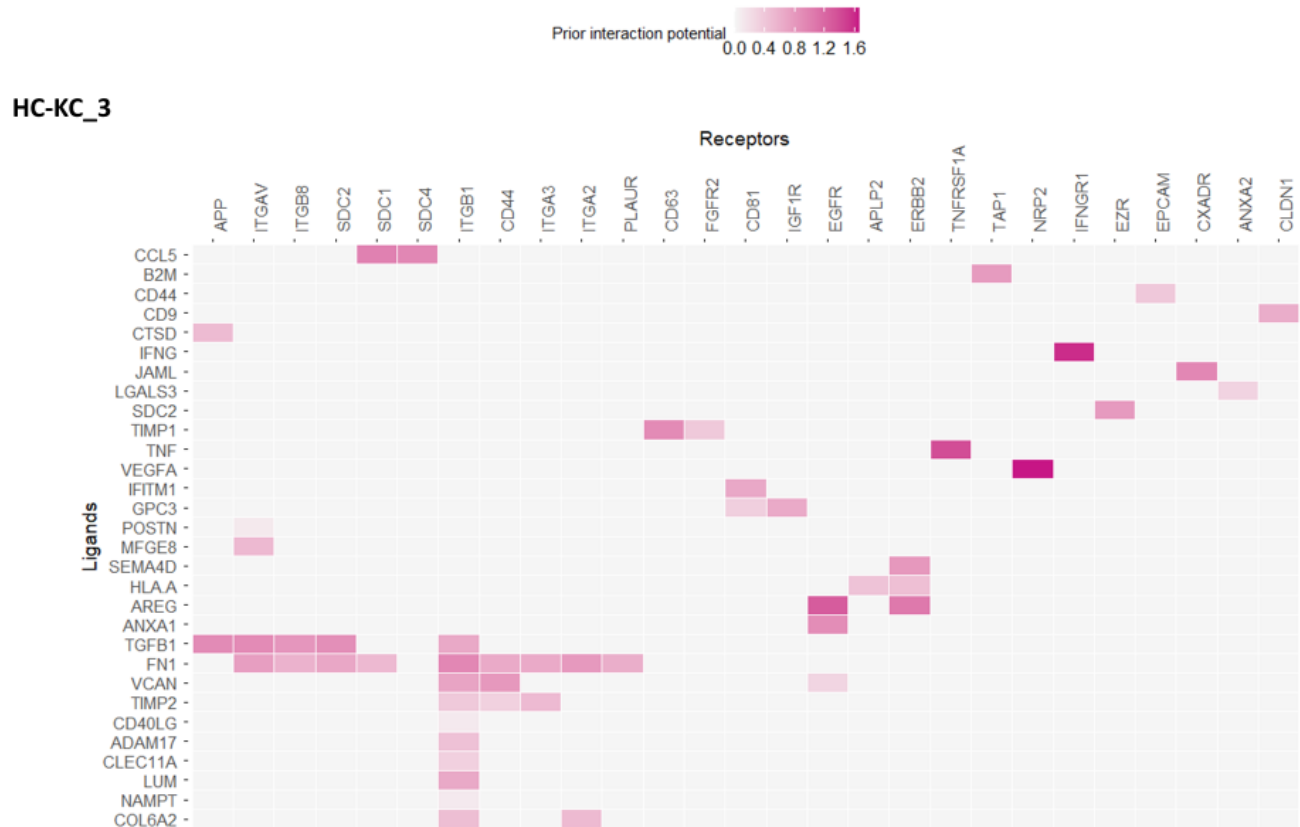
Supplementary Figure 6: Entropy calculation for the BCC-derived keratinocyte clusters in the trajectory. Violin plots displaying the distribution of the per-cell entropy for the BCC-associated KC clusters included in the trajectory analysis. The dot in each violin plot represents the median entropy of the cluster.



Supplementary Figure 7: Heatmaps depicting the T cell (sender)-derived prioritized ligands with the active target genes that they have been predicted to regulate in HC-associated (left) compared to BCC-associated (right) KCs from cluster KC_0. Prioritized ligands (top-ranked ligands with a regulatory score of more than 0.33 for their active target genes) are ordered according to their ranking.



Supplementary Figure 9: Heatmaps depicting the KC (receiver)-derived receptors corresponding to the top-ranked T cell ligands for HC-associated (top) compared to BCC-associated (bottom) KCs from cluster KC_0. Both ligands and receptors are ordered by hierarchical clustering.



Supplementary Figure 10: Heatmaps depicting the KC (receiver)-derived receptors corresponding to the top-ranked T cell ligands for HC-associated (top) compared to BCC-associated (bottom) KCs from cluster KC_3. Both ligands and receptors are ordered by hierarchal clustering.