UNIVERSIDAD DE MURCIA

ESCUELA INTERNACIONAL DE DOCTORADO

Impact of Inflammation on Melanoma Development and Aggressiveness

Impacto de la Inflamación en el Desarrollo y Agresividad del Melanoma

Dna. Elena Gómez Abenza

2019

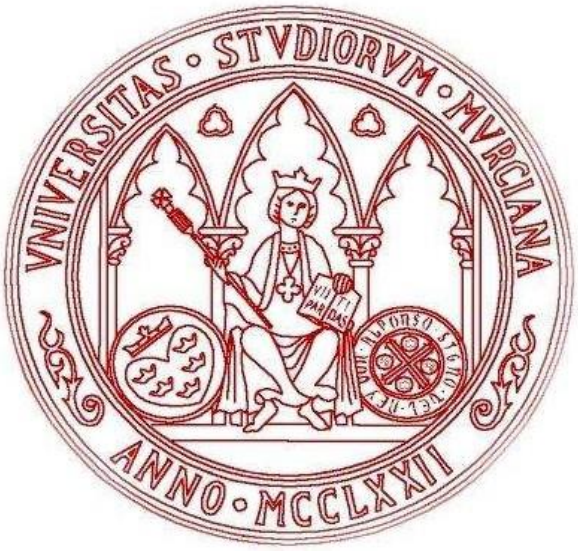

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Table of Content

List of Figures

Introduction

Materials and Methods

Results

List of Tables

Introduction

Materials and Methods

Abbreviations

Ab	Antibody
ACTH	Adrenocorticotropic hormone
AIM	Absent in melanoma
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AP-1	Activator protein 1
AP-3	Clathrin adaptor protein complex-3
APC	Adenomatous polyposis coli
ARID2	AT-rich interaction domain 2
ASP	Agonist stimulating protein
ATP	Adenosine triphosphate
BCL2	B-cell lymphoma 2
bFGF	Basic fibroblast growth factor
BLOC	Biogenesis of lysosome-related organelle complex
BMP	Bone morphogenetic protein
BRAF	Serine/threonine-protein kinase B-RAF
C+	Positive control
cAMP	cyclic AMP
CCL2	chemokine (C-C motif) ligand 2
CDK2	Cyclin-dependent kinase 2
CDKN2A	Cyclin-dependent kinase inhibitor 2A / p16INK4a
cDNA	Complementary DNA
C-KIT	KIT proto-oncogene receptor tyrosine kinase

COX	Cyclooxygenase
CRE	cAMP response elements
CREB	cAMP response element-binding
CSD	Chronically sun damaged melanoma
CSFs	Colony stimulating factors
C-terminal	Carboxi-terminal
CTL	Cytotoxic T cells
CXCR2	Chemokine (C-X-C Motif) receptor 2
DCT	DOPAchrometautomerase
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DN	Dominant negative
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DOPA	Dihydroxyphenylalanine
dpf	Days post-fertilization
DRG	Dorsal root ganglia
DsRed	Red fluorescent protein from *Discosoma* sp.
DUOX	Dual oxidase
E-cadherin	Epithelial cadherin or Cadherin-1
EDNRB	Endothelin receptor type B
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced GFP

ENA	European nucleotide archive
EP1/EP3/FP	Prostanoid receptors
ERB	Family of proteins contains four receptor tyrosine kinases, structurally related to the epidermal growth factor receptor (EGFR)
ERK	Extracellular Receptor Kinase
ET-1	Endothelin 1
ETBR	Endothelin B receptor
EU	European Union
F	Forward primer
FACS	Fluorescence-activated cell sorting
FGFR1/2	fibroblast growth factor receptor 1/2
Fli	Friend leukemia integration
GFP	Green fluorescent protein
Gln	Glutamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GM-CSFR	GM-CSF receptor
GMP	Guanosine monophosphate
GPCR	G-protein coupled receptor
GTP	Guanosine-5'-triphosphate
GTPase	Hydrolase enzyme that can bind and hydrolyze GTP
h	Hours
H.	Hamilton

HAI1	Hepatocyte growth factor activator inhibitor 1 (also known as SPINT1)
HEK293	Human Embryonic Kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	Hepatocyte growth factor
HGFA	Hepatocyte growth factor activator (also known as HGFAC)
HIF1a	Hypoxia-inducible factor 1a
HK	Head kidney
hpf	Hours post-fertilization
hpi	Hours post-injection
IACUC	Institutional animal care and use committee
ID	Identification
Ig	Immunoglobulin
IGFBP-rP1	Insulin-like growth factor binding protein-related protein-1 (also known as IGFBP7)
IL	Interleukin
INF	Interferon
ISDCI	International Society of Developmental and Comparative Immunology
JNK	c-Jun N-terminal kinase
LB	Luria Bertani Broth
Ly	Lymphocytes
Lyz	Lysozyme C
mAbs	Monoclonal antibodies

MAPK	Mitogen-activated protein (MAP) kinase
MC1R	Melanocortin 1 receptor
MCH	Melanin-concentrating hormone
MEK	MAPK/ERK Kinase
MELAN-A	Melanoma antigen recognized by T cells 1 or MART-1
MET	Member of the receptor tyrosine kinases family (RTKs)
MHC	Major histocompatibility complex
MITF	Microphthalmia-associated transcription factor
MITF-M	Melanocyte-specific MITF isoform
MMP	Matrix metalloproteinase
MO	Morpholino
Mpeg1	Macrophage expressed protein 1
Mpx	Myeloperoxidase
mRNA	Messenger RNA
MSA	Migration staging area
MSC	Melanocyte stem cell
MSH	melanocyte-stimulating hormone
MT-SP1	Matriptase, membrane-type serineprotease 1 or Matriptase 1
MΦ	Macrophages
N	Notochord
NADPH	Nicotinamide adenine dinucleotide phosphate
NC	Neural crest
N-cadherin	Neural cadherin (NCAD) or Cadherin-2 (CDH2)

NCCs	Neural crest cells
NF1	Neurofibromin 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
NGFR	NGF receptor
NO	Nitric oxide
NOTCH	NOTCH homolog 1
NRAS	Neuroblastoma RAS viral oncogenehomolog
ns	Not significant
NT	Neural tube
N-terminal	Amino-terminal
ºC	Celsius degrees
OCA	Oculocutaneous albinism
p	p-value
PAMPs	Pathogen-associated molecular patterns
PAR2	Protease activated receptor 2
PAX3	Paired box gene 3
PCR	Polymerase chain reaction
PG	Prostaglandin
PhD	Philosophiae doctor
PIs	Principal investigators
PK	Protein kinase
PLC	Phospholipase C

PMEL17	Premelanosome protein 17/ GP1000
PN	Peripheral nerves
POMC	proopiomelanocortin
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative PCR
R	Reverse primer
RAB27A	Ras-like GTPase 27 A (also known as PMEL17)
RAF	Rapidly accelerated fibrosarcoma protein kinase
RAS	Small G protein GTPase
RNA	Ribonucleic acid
RNIs	Reactive nitrogen intermediates
ROS	Reactive oxygen species
rps11	Ribosomal protein S11
RT	Reverse transcription
RTK	Receptor tyrosine kinase
S	Somites
S.E.M	Standard error of the mean
SCF	Stem cell factor
SCPs	Schwann cell precursors
SEER	The Surveillance, Epidemiology, and End Results, an authoritative source for cancer statistics in the United States
SKCM	skin cancer melanoma
SLUG	Zinc finger protein SNAI2 or a zinc finger transcriptional repressor

SOX10	(SRY-related HMG-box) transcription factor 10
SPINT1	Serine protease inhibitor, kunitz-type, 1 (also known as HAI1)
St14	Suppression of tumorigenicity 14
STAT3	Signal transducer and activator of transcription 3
Std	Standard control
Tbx2	T-box transcription factor 2
TERT	Telomerase reverse transcriptase
Tg	Transgenic
TGF	Transforming growth factor
Th	Helper T cell
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TNFA	Tumor necrosis factor A
TNFR	TNF receptor
TNFR1	TNF receptor 1 also known as TNFRSF1A or P55
TNFR2	TNF receptor 2 also known as TNFRSF1B or P75
Tp53	Tumor protein p53
TYR	Tyrosinase
TYRP1	Tyrosinase-related protein 1
TYRP2	Tyrosinase-related protein-2 (also known as DCT)
uPA	Urokinase plasminogen activator
USA	United States of America
UV	Ultraviolet

UVR	UV Radiation
WNT	Wingless-related integration site
wt/vol	Weight/Volume
YAP	Yes-associated protein 1
ZDM	Zebrafish Disease Models
ZFNs	Zinc finger nucleases
ZIRC	Zebrafish international resource center

Summary

Skin cutaneous melanoma (SKCM) originates from melanocytes, neural-crest derived pigment-producing cells located in the epidermis, where their major function is to protect keratinocytes from UV-induced DNA damage. The malignant transformation of melanocytes generates this fatal form of skin cancer with a complex multigenicetiology that becomes extremely difficult to treat once it has metastasized. For that reason SKCM is the most lethal form of skin cancer and while incidence rates are declining for most cancers, they have been steadily rising for SKCM.

The relationship between inflammation process and cancer is yet considered ambiguous. Indeed, inflammation, especially chronic inflammation, may exert protumorigenic effects but on the other hand tumor cells spreading may be counteracted by inflammatory cells. Besides, immunosuppression is known to increase the cancer risk.

The zebrafish, *Danio rerio*, has emerged as a new experimental organism to model cancer, thanks to the conservation of several molecular and cellular pathways in involved in tumorigenesis. Also, it offers other important advantages as a tumor model organism. Zebrafish embryos are characterized by small size, external development and transparency that allow melanocyte tracking during all stages of their development. The early developmental processes of melanocyte transformation and the methods for their early detection are important for disease eradication.

In this study, *in silico* analysis of the occurrence and relevance of TNFA, TNFR, and SPINT1 genetic alterations of the SKCM TGCA cohort also analyzed. It was evidenced that genetic alterations in TNFA and TNFR2 genes occurred in 5 and 6 % of melanoma patients, respectively, and are associated with bad prognosis. In the case of SPINT1, we found a high prevalence of genetic alterations in SKCM patients (10%) and their association with altered tumor immune microenvironment with a concomitant poor patient survival.

Functional experiments in zebrafish using morpholino gene-mediated inactivation of TNFR1 and TNFR2, and TNFR2 overexpression in transformed melanocytes using the Gal4-UAS system, showed that inflammation through the TNFA/TNFR2 signaling axis accelerates the onset of melanoma, enhancing oncogenic transformation and melanoma progression, pointed to TNFR2 signaling in stromal cells as responsible for the increased proliferation of transformed cells. In addition, the impact of inflammation in promoting melanoma cells proliferation *in vivo* was evaluated by using a spontaneous model of melanoma in zebrafish expressing the oncogenic human $HRAS^{G12V}$ driven by the melanocyte cell-specific promoter *kita*. Furthermore, the

relevance of chronic skin inflammation in early transformation, progression and metastatic invasion of SKCM was studied using the Spint1a-deficient zebrafish model.SPINT1 is a type II transmembrane serine protease inhibitor that has been shown to be involved in the development of several types of cancer, such as squamous cell carcinoma and colorectal cancer. In addition, the Spinta-deficient zebrafish line exhibited chronic skin inflammation and areas of epidermal hyperproliferation due to the overactivation of Matriptase1, a major target of Spint1.The results reveal that Spint1a deficiency facilitates oncogenic transformation, regulates the tumor immune microenvironment crosstalk, accelerates the onset of SKCM and promotes metastatic invasion. Notably, Spint1a deficiency is required at both cell autonomous and non-autonomous levels to enhance invasiveness of SKCM.

These results reveal that inflammation may promote tumor cell aggresiveness, identifying a key role of TNFA/TNFR2 and SPINT1 signaling in the cross-talk between oncogenically transformed cells and the tumor microenvironment, and point to TNFA/TNFR2 and SPINT1as novel therapeutic targets for SKCM.

Introduction

1. The Skin

The skin, the largest organ of the body, represents an effective barrier between the organism and the environment. It prevents the organism of different external factors as well as, pathogens invasion, chemical and physical assaults and the unregulated loss of water and solutes helping in the organism homeostasis (Proksch, Brandner et al. 2008). Beyond these essential features, other functions of the skin are the regulation of body temperature and fat storage. In human adults, skin surface is around $2m^2$ and its color, thickness and texture depends on the corporal zone.

The skin is an epithelium formed by three principal layers in which each one has a specific function (**Figure 1**).

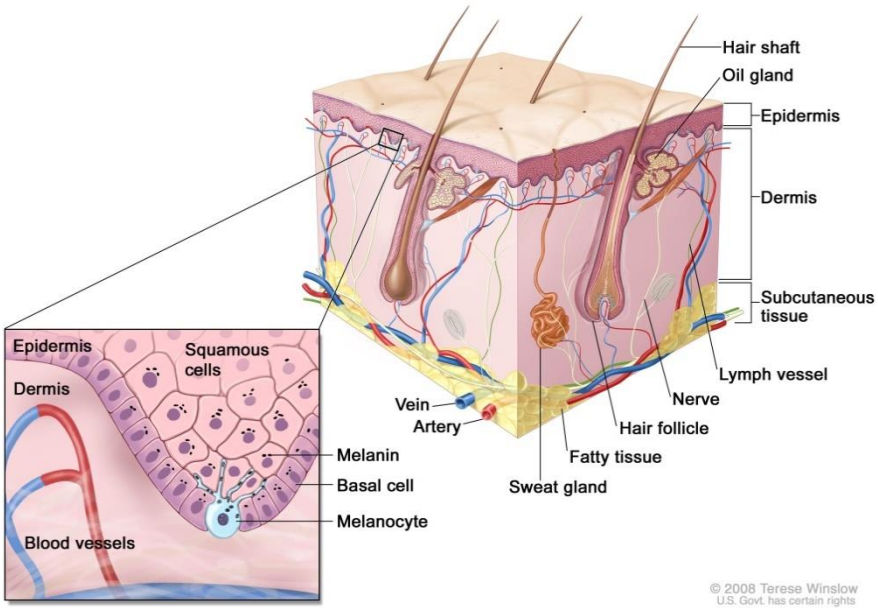

Figure 1: The skin and its layers: epidermis, dermis, and subcutaneous tissue. Melanocytes are located at the deepest part of the epidermis in the basal cells stratum (https://visualsonline.cancer.gov/details.cfm?imageid=7279).

The outermost layer is the **epidermis**. It is a keratinized epithelium composed by 5 different cell types: the keratinocytes which are the most abundant and they synthesize keratins, the Langerhans and the dendritic cells which are responsible of the epidermal immune system response, the Merkel cells that belongs to the peripheral nervous system and the melanocytes located in the basal epidermis. The main function of melanocytes is to provide melanin pigment to their neighboring keratinocytes (Shain and Bastian 2016).

The middle layer of the skin is the **dermis**. Principally, it is constituted by fibroblasts, which synthetize the extracellular matrix components, such as collagen and

elastin fibers, giving elasticity and mechanical resistance to the dermis complex. The dermis also includes Ruffini corpuscles from the nervous system and blood vessels responsible of supplying nutrients and removing wastes.

The **subcutaneous layer** or **hypodermis** is the deeper layer of the skin. It is constituted by connective tissue, blood vessels, nerve endings and adipocytes that help to preserve the body temperature and cushion from external lesions.

In direct contact with the skin there are other structures like nails, hair and moreover fatty and sweat glands. Differences in skin and hair color are genetically determined and are mainly due to variation in the amount, type, and packaging of melanin polymers produced by melanocytes and secreted into keratinocytes (Rees 2003).

1.1. The pigmentary system: melanocytes, melanosomes and melanin

The vertebrate skin shows a wide variety in terms of its pigmentation index. This interspecies variation gives rise to differences in the coloration, patterning and function of the pigmentation. Since the skin is almost constantly exposed to high levels of ultraviolet light (UV), the main function of pigmentation is protects against this potential mutagen (White and Zon 2008). The principal pigment responsible of protectionagainst UV-induced damages to the skin is the melanin.

Melanocytes are pigment-producing cells in the skin of humans and other vertebrates located in the basal layer of the epidermis, close to keratinocytes (**Figure 1**). This type of dendritic cells are neural crest-derived cells that arise during embryonic development, colonize the skin, eye and to a lesser degree, a broad range of other tissues thoughtout the body (Uong and Zon 2010, Mort, Jackson et al. 2015, Shain and Bastian 2016). The neural crest arise during gastrulation stage, in the area between the neural and non-neural ectoderm (Erickson and Reedy 1998). Further, neural crest cells delaminate from the dorsal-most aspect of the neural tube by a process of epithelial-to-mesenchymal transition (Mort, Jackson et al. 2015). These neural crest cells are highly migratory and they contribute to create many specialized structures and tissues in the developing embryo through processes of migration, proliferation and differentiation (Mayor and Theveneau 2013). As shown in **Figure 2**, early migrating neural crest cells in the trunk region, move through the dorso-ventral pathway reaching the space between the somites and the neural tube, giving rise,

among others, to neurons of the dorsal root ganglia. Before migrating along the dorso-lateral pathway, melanoblast precursors stall in the migration staging area, and then move between the somites and the ectoderm to ultimately give rise to primary wave melanocytes while a secondary wave of melanocytes arises from the Schwann cell precursors associated with the peripheral nerves. Melanocytes are found near the nerve endings. Beyond melanocytes, neural crest cells give rise to a number of cell types (**Figure 3**), including osteocytes, chondrocytes and sensory neurons (White and Zon 2008).

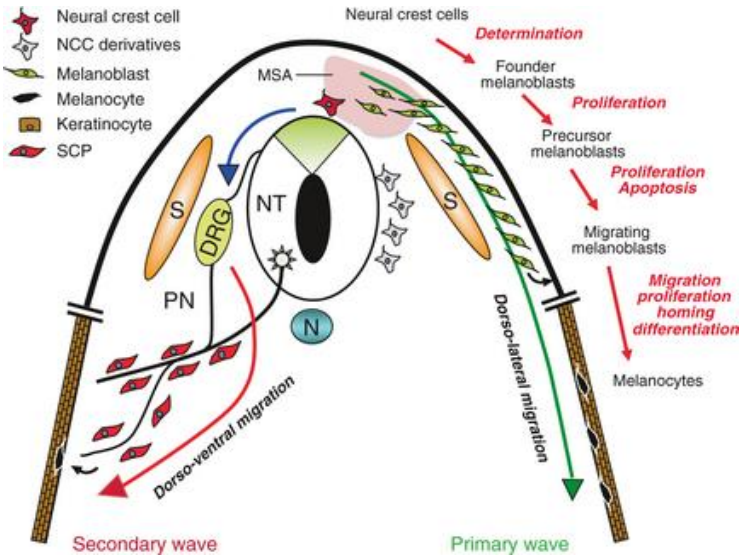

Figure 2: Melanocyte specification from neural crest cells (NCCs). (Bonaventure, Domingues et al. 2013).

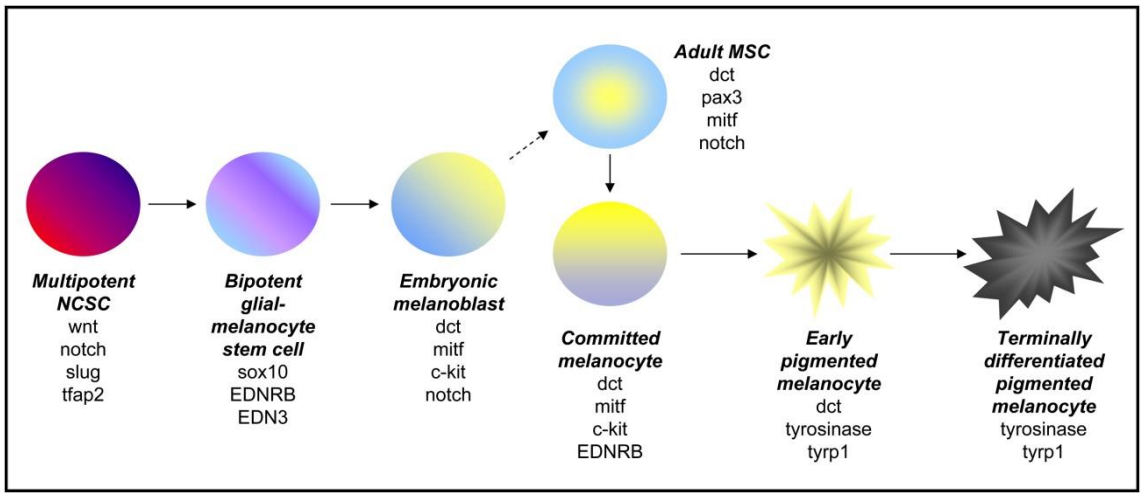

Figure 3: Different cell populations derived from neural crest (NC) cells and the transduction signaling pathways involved. (Sauka-Spengler and Bronner-Fraser 2008).

As shown in **Figure 4**, early neural crest specification is dependent upon interactions between wingless-related integration site (WNT), NOTCH, and bone morphogenetic protein (BMP) signaling. SLUG (a zinc finger protein SNAI2 or a zinc finger transcriptional repressor) transcription marks an early specified neural crest cell and is expressed during the early migration phase of melanocyte precursors. Gradual lineage restriction toward the melanoblast fate is dependent upon microphthalmia-associated transcription factor (MITF), endothelin receptor type B (EDNRB), and KIT proto-oncogene receptor tyrosine kinase (C-KIT) signaling. The embryonic melanoblast and adult melanocyte stem cell (MSC) share some overlapping molecular markers, particularly DOPAchrometautomerase (DCT). It is unclear if there are some adult MSCs which arise independently of the embryonic melanoblast (White and Zon 2008). Terminally, differentiated melanocytes can be identified by the expression of specific molecular markers such as tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1), DCT or tyrosinase-related protein-2 (TYRP2), premelanosome protein 17 (PMEL17/GP1000), MELAN-A or melanoma antigen recognized by T cells 1 or MART-1 and MITF (D'Mello, Finlay et al. 2016).

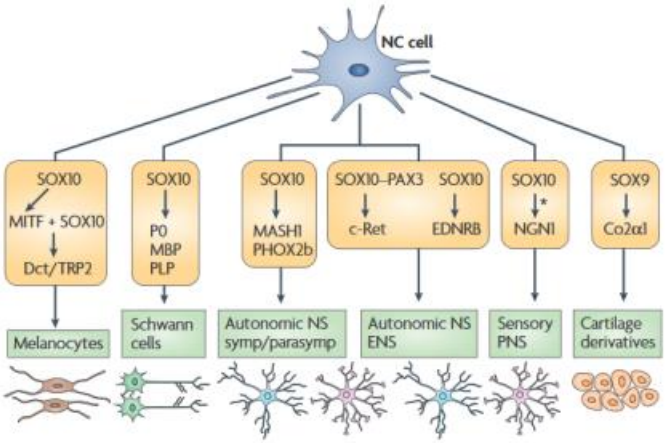

Figure 4: Interactions between various molecular pathways involved in the specification from NCCs into pigmented melanocytes. (White and Zon 2008).

There are approximately 1,500 melanocytes per mm^2 of human epidermis, corresponding to nearly 3 billion cutaneous melanocytes in the skin of an average human (Kanitakis 2002). The so-called "epidermal melanin unit" represents the anatomical relationship between keratinocytes and melanocytes and it has been estimated that each melanocyte is in contact with 36-40 keratinocytes in the basal and suprabasal layers (**Figure 5**) (Costin and Hearing 2007, D'Mello, Finlay et al. 2016). Its density is variable, depending on the anatomical location, but it is very similar between

the individuals of different races. The neural crest-derived melanoblasts are initially multipotent but gradually become lineage-restricted in terms of developmental potential, depending on their anatomical location (White and Zon 2008). The skin color depends on melanocyte activity more than cell number and it is determined by a combination of genetics and environmental factors. An excessive melanocyte proliferation produce freckles, nevus, lentigus and as the last resort melanoma (melanocytic malignization). In contrast there are others phenotypic manifestations due to the absence of melanocytes, like vitilligo (depigmented areas of the skin caused by autoimmune responses or by toxics substances that affect melanocyte development) (Rashighi and Harris 2017). Melanocytes are also found in other tissues of the body such as the central nervous and cardiovascular system, the uvea of the eye, cochlea and even adipose tissue (D'Mello, Finlay et al. 2016).

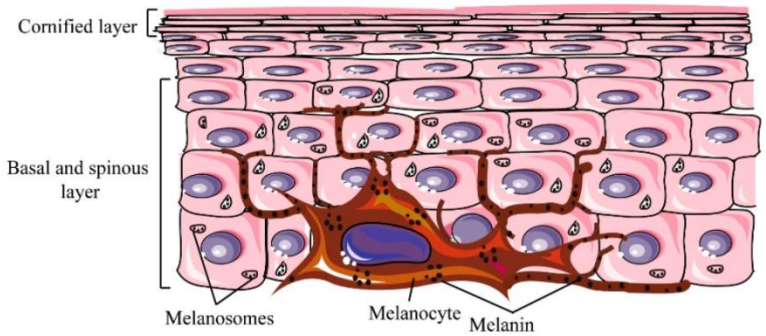

Figure 5: Association of keratinocytes and melanocytes.(D'Mello, Finlay et al. 2016).

Melanosomes are organelles within the melanocytes in which melanin pigments are synthesized and stored (Bonaventure, Domingues et al. 2013) before being transferred through dendrites to surrounding keratinocytes, where they play the critical photoprotection role (Costin and Hearing 2007). Therefore, the skin microenvironment is able to adapted to external stimuli, thanks the crosstalk between keratinocytes and melanocytes (Wang, Fukunaga-Kalabis et al. 2016).

The biogenesis of these electrodense organelles require a number of specific enzymatic and structural proteins to mature and become competent in order to produce melanin (Bonaventure, Domingues et al. 2013). Among them, the enzymes TYR and DCT/TYRP2 affect both the quantity and quality of melanin, while PMEL17 and MART-1 represent the main structural proteins. On the other hand, clathrin adaptor protein complex-3 (AP-3), biogenesis of lysosome-related organelle complex (BLOC)1 and oculocutaneous albinism (OCA)2 have important roles in sorting and

trafficking melanosomes(Sitaram and Marks 2012, Bonaventure, Domingues et al. 2013).

Melanosome development is constituted by four stages, identified by morphological analysis of skin melanocytes by classical electron microscopy (Wasmeier, Hume et al. 2008) (**Figure 6**). In particular, these maturation stages (I–IV) are characterized by peculiar structure and the quantity, quality and arrangement of the melanin produced (Costin and Hearing 2007). Premelanosomes (Stage I) are a round, small vesicles with an amorphous matrix. Melanosomes at Stage II present an organized, structured fibrillar matrix but even if tyrosinase is expressed, synthesis of pigment has not begun yet. Indeed, melanin production starts at Stage III, where pigment is deposited on protein fibrils whileat the last Stage IV pigment fills the whole melanosome. At this later stage, tyrosinase activity is not detectable in melanosomes which are further transported to surrounding keratinocytes through a cytoskeletal-dependent mechanism system (Costin and Hearing 2007, Cichorek, Wachulska et al. 2013).

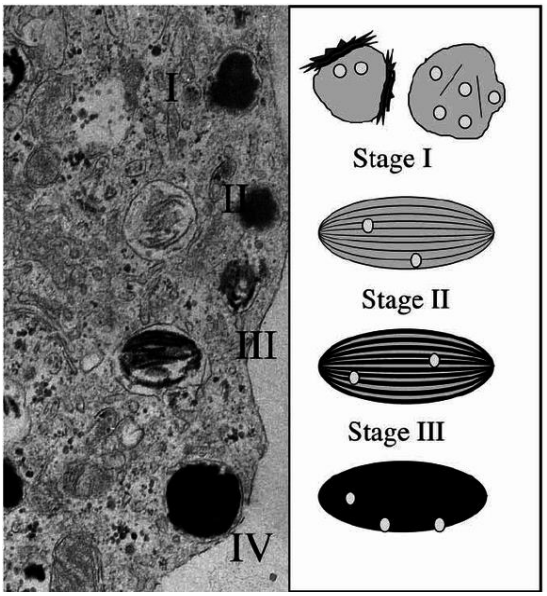

Figure 6: The melanosome and it four stages of development represented by an electron micrograph. Adapted from (Raposo and Marks 2002).

Melanin is the principal component of the melanocyte and represents one of the most potent free radical scavengers in humans. Indeed, its role is to counteract reactive oxygen species (ROS) helping to prevent DNA damage in skin cells (White and Zon 2008). Indeed, melanin has numerous properties which are beneficial to the body: UV light absorption and scattering, free radical scavenging, coupled oxidation-reduction reactions and ion storage (Cichorek, Wachulska et al. 2013).

The term melanin denotes pigments of diverse structure and origin mainly derived by the oxidation and polymerization of tyrosine in animals or phenolic compounds in lower organisms (d'Ischia, Wakamatsu et al. 2015). The biosynthesis of this complex macromolecule is controlled by an enzymatic pathway referred as "the Raper-Mason pathway" (**Figure 7**), leading to eumelanin and pheomelanin production (d'Ischia, Wakamatsu et al. 2015).

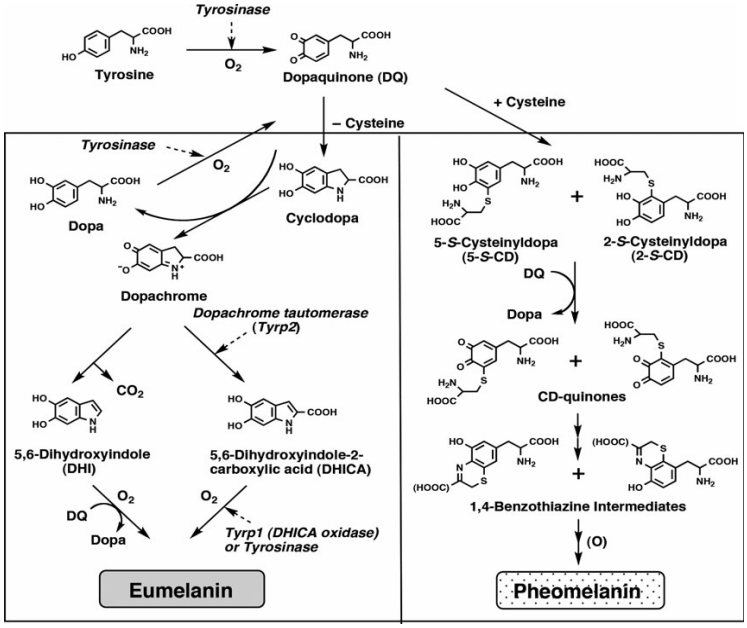

Figure 7: The Raper-Mason pathway.

The enzyme tyrosinase catalyzes the first step of melanin synthesis, converting tyrosine to dihydroxyphenylalanine (DOPA). Individuals affected by albinism carry mutations in tyrosinase gene. In mammals and birds, melanin is present in two forms: eumelanin, which is black or dark brown, and phaeomelanin, which is red or yellow. The biosynthetic pathways for the synthesis of this two pigments diverge downstream of DOPA, and the choice of pathway is determined by the signalling activity of the melanocortin 1 receptor MC1R (Mort, Jackson et al. 2015). While eumelanin synthesis requires the enzymatic activities of TYR, TYRP1 and DCT/TYRP2,only TYR (and the amino acid cysteine) is necessary for the production of pheomelanin(d'Ischia, Wakamatsu et al. 2015). Eumelanins are the black-brown subgroup of insoluble melanin pigments derived at least in part from the oxidative polymerization of L-DOPA via 5,6-dihydroxyindole intermediates. Pheomelanins are yellow-to-reddish brown subgroup of melanin pigments derived from the oxidation of cysteinyldopa precursors via benzothiazine and benzothiazole intermediates (d'Ischia, Wakamatsu et al. 2015). Eumelanin has better photoprotective properties when compared to pheomelanin along

with higher resistance to degradation and ability to ROS neutralization. As a consequence, the risk of skin cancer is increased of 30-40 times in individuals with lighter skin than in those with darker one (Cichorek, Wachulska et al. 2013).

1.2. Molecular pathways involved in melanogenesis

There is a basal or constitutive skin pigmentation that exists naturally in absence of external factors genetically determined by the individual race. Although, there are some relevant physiological mechanisms that stimulate melanogenesis through inducing proliferation and differentiation of melanocytes, and it is based on the cross-talk between this cell type and keratinocytes and dermal fibroblasts. On the other hand, also distant organs like the pituitary gland may be involved in this process, as well as environmental factors, such as UV radiation (UVR) (Cichorek, Wachulska et al. 2013). In particular, undifferentiated basal keratinocytes control melanocyte growth and cell surface receptor expression through a direct cell-cell contact (Achmatowicz, Thiel et al. 2008). In addition, keratinocytes are able to control melanocyte growth and activity through a system of paracrine signals including growth factors, cell adhesion molecules (Cichorek, Wachulska et al. 2013) and hormones which are able to control melanocyte proliferation, melanogenesis and the formation of melanocytic dendrites. These signaling pathways take part in the complex epidermal network responsible of the maintenance of skin homeostasis. **Figure 8** includes an overview of the factors secreted by keratinocytes that activate the signal transduction pathways in melanocytes.

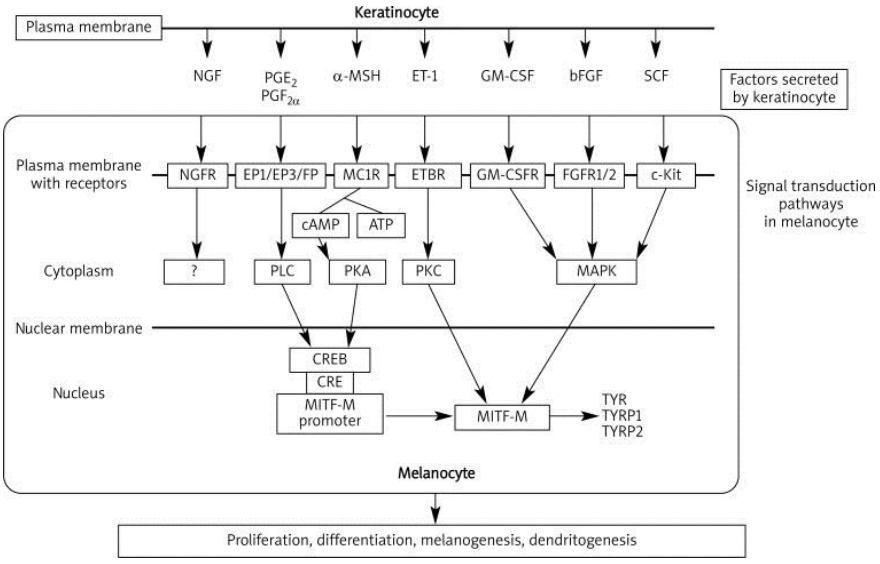

Figure 8: Graphical presentation of the basic elements in keratinocytes-melanocytes cooperation.(Cichorek, Wachulska et al. 2013).

Melanocytes act as a very active element in the epidermal melanin unit because they release a number of signal molecules targeting also skin immunological system cells in addition to keratinocytes. The substances secreted by active melanocytes are proinflammatory cytokines (interleukin (IL) IL-1α, IL-2, IL-3, IL-6, IL-10 and and tumor necrosis factor-alpha (TNFA), chemokines (IL-8, chemokine (C-C motif) ligand 2 (CCL2)), transforming growth factor (TGF-β), catecholamines, eicosanoids, serotonin, α-melanocyte-stimulating hormone (also known as proopiomelanocortin, gene symbol POMC and hereafter referred to as α-MSH) and nitric oxide (NO) (Cichorek, Wachulska et al. 2013). On the other hand, other mediators secreted by melanocytes also act as autocrine factors, e.g. IL-1, IL-6 and TNFA, inhibiting melanogenesis while the level of melanin synthesis is elevated due to the influence of eicosanoids and α-MSH (Cichorek, Wachulska et al. 2013).

Factors driving melanocyte function, whether intrinsic or extrinsic, are either genetically or post-transcriptionally regulated by the melanogenesis-related genes. These regulatory mechanisms not only influence melanocyte development and differentiation but also melanin production (D'Mello, Finlay et al. 2016).

Melanin production is initiated and regulated by a number of signaling systems and transcription factors including the receptor KIT, its ligand Stem Cell Factor (SCF, secreted by dermal fibroblasts), as well as mitogens like hepatocyte growth factor (HGF), endothelins, α-MSH, adrenocorticotropic hormone (ACTH) and MITF. In particular, the tissue specific bHLH-Zip transcription factor MITF is a evolutionarily conserved 'master regulator' of melanocyte function (**Figure 9**) since it is essential for melanocyte development because it influences proliferation, dendrite formation, melanin synthesis and induces the expression of antiapoptotic BCL2 (B-cell lymphoma 2) gene (Cichorek, Wachulska et al. 2013).

Nevertheless, genetic, biochemical and pharmacological evidences have established that MC1R signaling (**Figure 9**) is the main factor dictating melanogenesis (D'Mello, Finlay et al. 2016). This receptor (ligands: α-MSH, ACTH and agonist stimulating protein [ASP]) and others like KIT (ligand: SCF) activate a number of signaling pathways including protein kinase C (PKC), cyclic AMP (cAMP), MAPK/ERK Kinase (MEK) (ERK Extracellular Receptor Kinase) and WNT which regulate the activity of the MITF. Production of melanogenic enzymes is driven by this transcription factor, leading to the synthesis of eumelanin and pheomelanin within melanosomes of melanocytes.

These signaling pathways are active during melanocyte proliferation, including mitogen-activated protein kinase (MAPK), α-MSH/cAMP/PKA (PKA protein kinase A), Endothelin/PKC, signalingpathways. On the contrary, TGF-β, interferon-β (INF-β), IL-1, IL-6 and TNFA signaling pathways cause the opposite effect, arresting melanocyte growth (Cichorek, Wachulska et al. 2013).

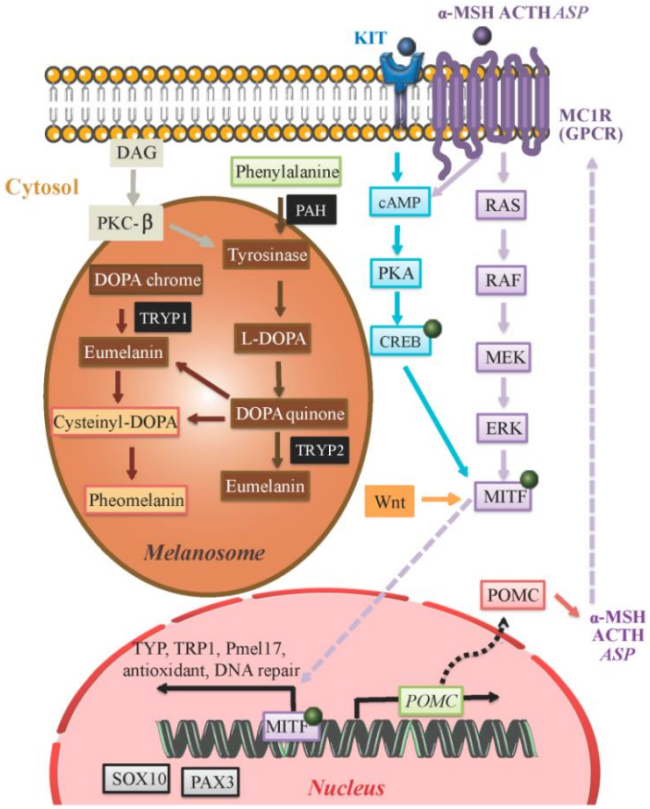

Figure 9: Eumelanin and pheomelanin synthesis by specific enzymes inside the melanosomes of melanocytes (black). Production These enzymes production is driven by the MITF transcription factor whose activity is regulated by a number of signaling pathways including PKC (brown), cAMP (blue), MEK (purple), and WNT (orange). These signaling pathways are activated upstream by receptors such as KIT (ligand: SCF) and MC1R (ligands: α-MSH, ACTH and ASP). The MITF transcription factor drives the expression of a number of genes including (SRY-related HMG-box) transcription factor 10*(SOX10)* and paired box gene 3 *(PAX3)* (D'Mello, Finlay et al. 2016).

1.3. MAP-Kinase pathway: a crucial player in melanocytes proliferation and differentiation

Factors secreted by keratinocytes under basal conditions or in response to UV, stimulate a broad spectrum of intracellular signaling in melanocytes. However, the activation of the MAPK/ERK pathway is the major downstream event resulting from the activity of extracellular stimuli: once triggered, the MAPK/ERK pathway orchestrates

the balance between melanocyte differentiation and proliferation (Wellbrock and Arozarena 2016).

The MAPK/ERK signaling cascade is constituted by a sequential phosphorylation of a number of kinases, including a small G protein (RAS) and three protein kinases (RAF [rapidly accelerated fibrosarcoma], MEK, ERK), and its activation determines a rapid alteration of cellular behavior (Chin 2003).

In normal melanocytes the ERK/MAPK pathway is governed by G-protein coupled receptor (GPCR) (i.e., MC1R), induced by cAMP signaling, and receptor tyrosine kinase (RTK) signaling (**Figure 10**) (Wellbrock and Arozarena 2016). After membrane receptor activation, the GTPase RAS is then recruited to the plasma membrane, leading to its activation. Indeed, RAS proteins are monomeric membrane-associated GTPases, which communicate binary ON/OFF messages to downstream effector proteins by changing between an active GTP bound and inactive GDP bound state (Knight and Irving 2014). RAS binds to and promotes dimer formation of kinases belonging to RAF family, a process crucial for their activation and downstream signal transduction (Gibney, Messina et al. 2013). The activation of this pathway culminates in the regulation of gene transcription in the nucleus by the last pathway component, the extracellular signal-regulated kinase ERK which phosphorylates several cellular substrates (Knight and Irving 2014). For example, the main ERK target is the transcription factor MITF, which regulates the expression of genes controlling differentiation (e.g., TYR) proliferation (e.g., cyclin-dependent kinase 2 [CDK2]) and survival (e.g.,BCL2, BCL2A1) of melanocytes (Wellbrock and Arozarena 2016).

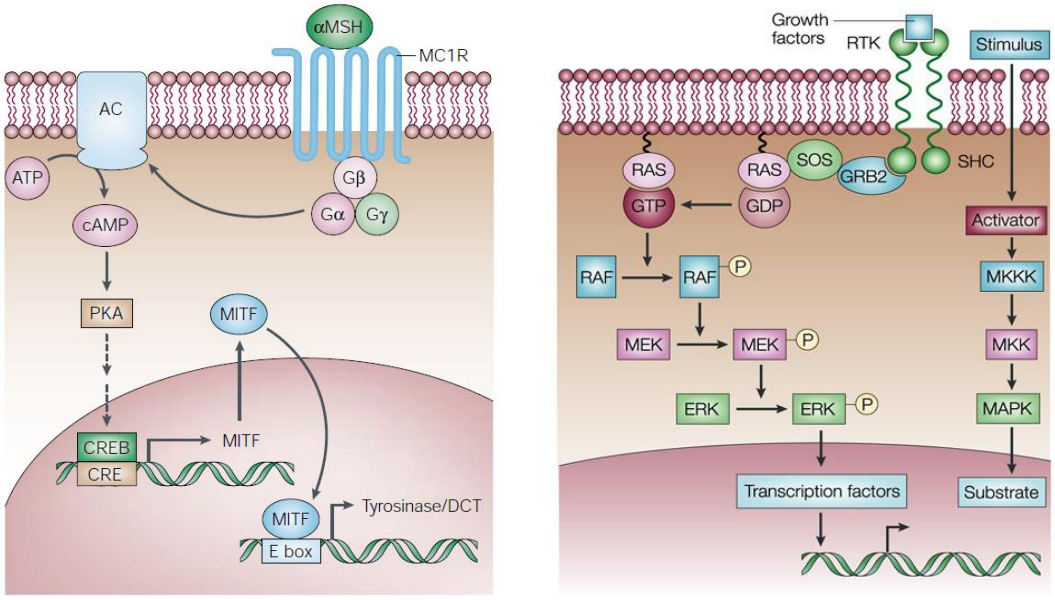

Figure 10: A simplified MAPK signalling module with the RAS–RAF–MEK–ERK pathway. Adapted from (Chin 2003).

1.4. Environmental activator of melanogenesis: UVR

The recurrent interaction of skin with sunlight is an intrinsic constituent of human life, exhibiting both beneficial and detrimental effects. Beyond influencing human skin shades, sunlight stimulates vitamin D synthesis and also influences circadian rhythms (Bogh, Schmedes et al. 2010, Hart, Gorman et al. 2011, Janich, Toufighi et al. 2013).

Solar radiation that reaches the earth's surface predominantly consists of UV (280–380 nm), visible (380–770 nm) and near-infrared (770–1,000 nm) spectra. Depending on the wavelength, the UVR that get on to our skin consist of UVB (280–315 nm) and UVA (315–380 nm). The UVR represents between 5-10% (Yamaguchi, Brenner et al. 2007), although is the major risk factor for melanoma and other skin cancers (Natarajan, Ganju et al. 2014). The skin responds to UVR exposure by developing two defensive barriers: thickening of the stratum corneum and the elaboration of a melanin filter in cells of the epidermis (Costin and Hearing 2007) even if melanin synthesis is an energy-intensive pro-oxidative process (Natarajan, Ganju et al. 2014).

UVA radiation is able to penetrate deeply in the skin reaching the level where melanocytes are: this stimulus activates melanin production but the resultant tan appears to be transient (Yamaguchi, Brenner et al. 2007). On the other hand, UVB radiation is the responsible of the melanogenesis induction as a consequence of sun exposure, the cause of the sunburned skin, and it is able to induce carcinogenesis after repeated and prolonged exposures. The contribution of UVA wavelengths to both UV-induced carcinogenesis and UV-mediated regulation of the immune system is controversial. While some studies demonstrated a suppression of immune response induced by UVA radiation , in others UVA radiation modulated the regulatory effects of UVB radiation (Hart, Gorman et al. 2011).

Along with tumorigenic activity, UVR are also associated to other inflammatory disorders disrupting keratinocytes in the skin epithelia. Indeed, human skin also contains endogenous photosensitizers, whose level can be altered in certain diseases, which leads to phototoxic stress (Natarajan, Ganju et al. 2014).

The synthesis of melanin, in particular black eumelanin, is stimulated primarily by UV irradiation which generates DNA photoproducts and leads to the release of various autocrine and paracrine factors previously mentioned (Wasmeier, Hume et al. 2008). In particular, α-MSH activates the G-protein couple receptor MC1R in the

plasma membrane of skin melanocytes, which results in cAMP-dependent signaling and the stimulation of the expression of MITF (d'Ischia, Wakamatsu et al. 2015). As already mentioned, the activity of MITF enhances the expression of target genes that are involved in melanocyte survival (CDK2,cyclin-dependent kinase inhibitor 2A (CDKN2A)/$p16^{INK4a}$, T-box transcription factor 2 [Tbx2] and p21/CDKN1A), motility (MET, a member of the receptor tyrosine kinases family [RTKs]), differentiation and apoptosis (BCL2 and hypoxia-inducible factor 1a [HIF1a]), and in melanin synthesis and melanosome differentiation (TYRP1, DCT, MALAN-A, absent in melanoma (AIM)1 and PMEL17) and transport (RAB27A [Ras-like GTPase 27 A])(Wasmeier, Hume et al. 2008).

1.5. UV light-mediated ROS production and photoaging

As already mentioned, UVR activates a complex cascade of biochemical reactions in human skin. Inflammation and the production of ROS are two key causes of photodamage. Prolonged exposure to UV irradiation lead to depletion of cellular antioxidants and associated to a reduction of the efficiency of antioxidant enzyme systems, culminating in DNA damage. Accumulation of thymidine dimers activates the neuroendocrine system, causing an increased release of proinflammatory mediators. In addition to enhanced ROS levels, inflammation causes oxidative damage to cellular proteins, lipids and carbohydrates, and these molecules accumulate in the dermal and epidermal compartments, contributing to the etiology of photoaging (Natarajan, Ganju et al. 2014).

Moreover, skin contains endogenous photosensitizers that induce formation of ROS (Denat, Kadekaro et al. 2014). When activated to an excited state, photosensitizers interact with molecular oxygen through direct transfer of energy-generating singlet oxygen, $^1O_2$ (type I photosensitization mechanism). This process can also produce oxygen free radicals $O_2\cdot^-$ by transferring an electron, which can then result in formation of H_2O_2 (type II photosensitizing mechanism). In addition, NO· could be generated through UV light–induced decomposition of endogenous nitrite anion, which results in reactive nitrogen species and photoisomerization of urocanic acid to the *cis* isomer is known to induce immunosuppression (Natarajan, Ganju et al. 2014).

2. The Melanoma: general characteristics, classification, incidence and relevance in the population

Cutaneous melanoma originates from melanocytes, whose major function is to protect keratinocytes from UV-induced DNA damage, as previously described (Wellbrock and Arozarena 2016). The malignant transformation of melanocytes generates this fatal form of skin cancer with a complex multigenic etiology that becomes extremely difficult to treat once it has metastasized.

The incidence of melanoma is increasing at an alarming rate. It produces more than 40.000 dead per year and the 81% of the cases occurs in developed countries (https://www.aecc.es/SobreElCancer/CancerPorLocalizacion/melanoma/Paginas/incidencia.aspx). Nowadays, melanoma is the deadliest form of skin cancer (75% of deaths related to skin cancer) and it is common in the Western world. Its global incidence is 15–25 per 100,000 individuals (Schadendorf and Hauschild 2014). While incidence rates are declining for most cancers, they have been steadily rising for melanoma worldwide (van Rooijen, Fazio et al. 2017).

Melanoma has undergone a rapid increase since the 1950 due to aesthetic and leisure reasons among the population. Indeed, the incidence of melanoma and epithelial skin cancers rise up in the last decades, now reaching epidemic levels, mainly due to the change of leisure-time habits, which include a prolonged exposure to sun and, as consequence, to UV (Schadendorf, Fisher et al. 2015). Unfortunately, melanoma is one of the most recurrent types of cancer and its genetic heterogeneity has led in recent years to join forces to determine melanoma causes and develop effective therapies.

Clinically, cutaneous melanoma occurs most commonly in individuals who are between the ages of 40 years and 60 years, but it can affect those in adolescence and in late life (≥80 years). The median age at diagnosis is 57 years, which is almost one decade before most solid tumors (Schadendorf, Fisher et al. 2015). It is more common in men than women and among individuals of fair complexion and those who have been exposed to natural or artificial sunlight (such as tanning beds) over long periods of time (https://seer.cancer.gov/statfacts/html/melan.html). For that, cutaneous melanoma is one of the most common cancers in young adults aged 20–29 years in the United States of America (USA). Nevertheless, in Europe is more frequent in females(https://www.aecc.es/SobreElCancer/CancerPorLocalizacion/melanoma/Paginas/incidencia.aspx,http://eco.iarc.fr/eucan/Country.aspx?ISOCountryCd=724)and

curiously, Spain has one of Europe's lowest melanoma incidence and mortality rates (https://ecis.jrc.ec.europa.eu/explorer.php?$0-0$1-All$4-1,2$3-27$6-0,14$5-2008,2008$7-7$2-All$CEstByCountry$X0_8-3$X0_19-AE28E$X0_20-No$CEstRelative$X1_8-3$X1_9-AE28$X1_19-AE28E$CEstByCountryTable$X2_19-AE28E). Caucasian are more at risk of getting skin cancer than any other racial/ethnic group. The number of new cases of melanoma of the skin was 22.3 per 100,000 men and women per year based on 2010-2014 cases. In 2017, it was estimated 87,110 new cases of melanoma of the skin and 9,730 people deaths of this disease (https://seer.cancer.gov/statfacts/html/melan.html).

Melanoma pathogenesis is driven by both genetics and environmental risk factors. Its incidence is influenced by skin pigmentation, sun exposure history and geographical location. Moreover, a number of heritable factors contribute to a patient's overall melanoma risk, including response to UV, nevus number, and pigmentation characteristics, such as eye and hair color. Approximately 5-10% of melanoma cases are familial, yet the majority of familial cases lack identifiable germ-line mutations in known susceptibility genes. Furthermore, immunosuppression plays an important role in driving melanoma. Organ transplant patients present a significant increased risk of all skin cancer types, including melanoma (1.6 to 2.5 times higher than in general population). Nevertheless, immunosuppressed patients are at a tremendously higher risk for non-melanoma skin cancers (65 to 250 times higher for squamous cell cancer and a 10-fold increase for basal cell cancer) (Hawkes, Truong et al. 2016).

Early detection is fundamental, since localized, early stage SKCM can be surgically excised with little chance of recurrence with a 98.2% of patient survival rate after 5-year survival as reported by The Surveillance, Epidemiology, and End Results (SEER) (https://seer.cancer.gov/statfacts/html/melan.html). Metastatic melanoma, however, is still an often fatal disease with a 5-year survival rate of 15-20% (van Rooijen, Fazio et al. 2017).

Melanocytes at these diverse sites can give rise to phenotypically diverse types of melanoma (Shain and Bastian 2016). Melanoma is often found on the trunk (the area from the shoulders to the hips) or the head and neck in men while in women, melanoma mostly arises on the arms and legs. When melanoma occurs in the eye, it is called intraocular or uveal melanoma (https://www.cancer.gov/types/skin/patient/melanoma-treatment-pdq#section/all). These cutaneous melanomas can be broadly categorized by their origins from skin that is chronically sun damaged (CSD) or not (non-CSD) (**Figure 11**).

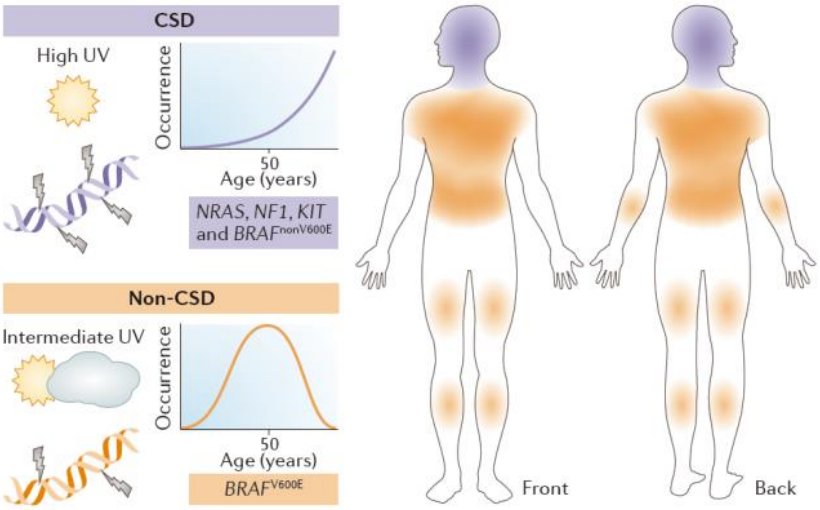

Figure 11: Different subtypes of melanoma: Chronically sun damaged (CSD) and non-CSD (Shain and Bastian 2016).

CSD-melanomas have higher mutation burdens and late age of onset, and occupy anatomical sites with the highest levels of sun exposure, such as the head and neck areas. Non-CSD melanomas present earlier in life are often associated with nevi, have comparatively lower mutation burdens and occupy anatomical sites with intermediate levels of sun exposure. Each subtype of melanoma is characterized by distinct mutations, such as $BRAF^{V600E}$ (serine/threonine-protein kinase B-Raf).in non-CSD melanoma and NRAS (Neuroblastoma RAS viral oncogene homolog), neurofibromin 1 (NF1), KIT and non$BRAF^{V600E}$ mutations in CSD melanoma (Shain and Bastian 2016).

Usually, skin melanomas show two distinct phases of local invasion (http://atlasgeneticsoncology.org/Tumors/SkinMelanomID5416.html) (**Figure 12**):

- The **radial-growth phase**, during which tumor cells acquire the ability to proliferate intraepidermally. The malignant cells tend to stay within the tissue of origin, the epidermis, in an 'in-situ' phase for a prolonged period (months to decades) (Schadendorf, Fisher et al. 2015). At first, superficial spreading melanoma grows horizontally on the skin surface (http://www.dermnetnz.org/topics/superficial-spreading-melanoma/). These types of melanomas are usually associated with a good prognosis because it can be removed by surgical excision (Michailidou, Jones et al. 2009, Leilabadi, Chen et al. 2014).
- The **vertical-growth phase**, which is characterized by tumor invasion of the dermis in form of an expansive nodule. This phase implies a bad prognosis because this infiltration into the dermis creates the possibility for the neoplastic cells to reach

distant skin areas, disseminating along the body through the lymphatic or blood vessels. Beyond lymph node metastases (Massague and Obenauf 2016), metastatic melanomas may finally metastasize to lungs, liver, central nervous system and other organs (Schadendorf, Fisher et al. 2015). At this stage, few therapeutic options are available, and melanoma frequently relapses and becomes incurable (Fernandez Del Ama, Jones et al. 2016).

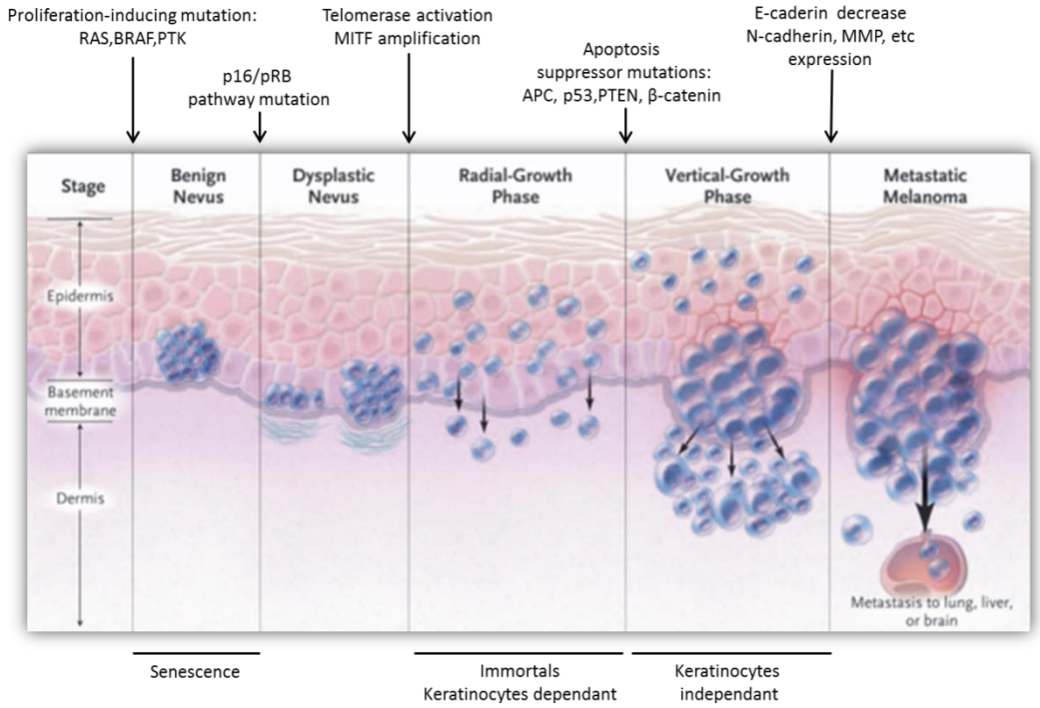

Figure 12: Stages and events in melanocytes malignant transformation.
Adapted from (Miller and Mihm 2006).

Classically, melanomas fall into four basic categories. Three of them begin *in situ*, since they are only present in the upper layers of the skin, and sometimes they become invasive; the fourth category is invasive from the beginning. Invasive melanomas are more serious, as they have penetrated deeper into the skin and may have spread to other areas of the body (Ossio, Roldan-Marin et al. 2017). In 1969 and 1970, physicians Wallace H. Clark, Jr. and Alexander Breslow independently proposed a method to identify melanoma stage, based on the evaluation of tumor invasiveness and thickness in strict relation to prognosis. In particular, melanoma microstaging (T-stage) using the Clark Classification was based on the anatomic level of local invasion, whereas the Breslow Classification was based on the vertical thickness of the invasion in millimeters (**Table 1**). In accordance with the recommendations of the American Joint Commission on Cancer (AJCC), T microstaging now is determined exclusively by the Breslow classification (Leilabadi, Chen et al. 2014).

Nowadays, there are other well-defined clinical of histopathological variants. All of them are represented in **Table 2** (Schadendorf, Fisher et al. 2015).

Clark Level	Histological Tumor Characteristics	Breslow's Thickness
Level I	Confined to the epidermis; *"in situ"* melanoma	<0.76 mm
Level II	Invasion of the papillary dermis	0.76-1.50 mm
Level III	Filling of the papillary dermis but not extending to the reticular dermis	1.51-2.25 mm
Level IV	Invasion of the reticular dermis	2.26-3.0 mm
Level V	Invasion of the deep, subcutaneous tissue	>3mm

Table 1: Melanoma stage using the Clark (based on the anatomic level of local invasion) **and the Breslow classification** (based on the vertical thickness of the invasion in millimeters). Adapted from (Leilabadi, Chen et al. 2014).

Major histopathological subtypes	
Name	*Description*
Superficial spreading melanoma	Characterized by a radial or horizontal growth phase with melanocytes arranged in nests or solitary units displayed in a pagetoid pattern.
Nodular melanoma	Usually occurs exclusively in the vertical growth phase (that is, no melanoma *in situ* or melanoma *in situ* confined to no more than three adjacent rete ridges beyond the margins of the tumour nodule).
Lentigo maligna melanoma	It has cells that are characteristically singly dispersed along the dermal–epidermal junction and skin appendages; signs of chronic UV radiation are prominent.
Acral lentiginous melanoma	It has cells that are present as single units along the dermal–epidermal junction and as confluent foci; this type of melanoma most commonly arises at acral sites but occasionally occurs at mucosal sites.
Other well-defined clinical or histopathological variants	
Name	*Description*
Naevoid melanoma	It shows histopathological features of a banal naevus (that is, 'small-cell melanoma').
Spitzoid melanoma	It has histopathological features of a Spitz naevus.
Desmoplastic melanoma	It displays unique histopathological features, including 'spindle-shaped' melanoma cells that morphologically resemble fibroblasts found in scar tissue.
Ocular melanoma	It arises within the uvea of the eye.
Mucosal melanoma	It originates at a mucosal site (for example, mouth, nasopharynx, larynx, conjunctiva, vagina or anus).
Acral melanoma	It forms on the palms of hands, soles of feet, and nails; the majority of acral melanomas, but not all, are of the acral lentiginous histopathological subtype
Amelanotic melanoma	It lacks clinically evident pigment and often appears pink in colour; any of the major histopathologic subtypes or variants can be amelanotic.

Table 2: Histopathological melanoma variants. Adapted from (Schadendorf, Fisher et al. 2015).

2.1. Melanocyte malignant transformation

A complex interplay of exogenous and endogenous events are responsible of melanocytes transformation into primary and then metastatic melanoma (Schadendorf, Fisher et al. 2015). Indeed, the process of transformation involves both genetics and functional changes detailed in **Figure 12**.

The classical melanoma progression models imply an evolution from nevus, then to dysplastic nevus, to melanoma *in situ* and finally, invasive melanoma. However, the situation is more complex as there are multiple melanoma types, which can be linked to different precursor lesions (**Figure12**, **13** and **Table II**) (Shain and Bastian 2016). More than 50% of the tumors originate from normal skin rather than from dysplastic nevi, suggesting that SKCM not only appears to be due to the transformation of mature melanocytes (Hoerter, Bradley et al. 2012). In this way, the identification of melanoma initiating cells is really important to devising methods for early detection and eradication of melanoma (Santoriello, Gennaro et al. 2010, Kaufman, Mosimann et al. 2016). Melanoma stem cell populations have been characterized and associated with tumor progression, immunoevasion, drug resistance and metastasis (Nguyen, Couts et al. 2015). The **Figure 13** shows an example of the phenotypic heterogeneity of malignant melanoma.

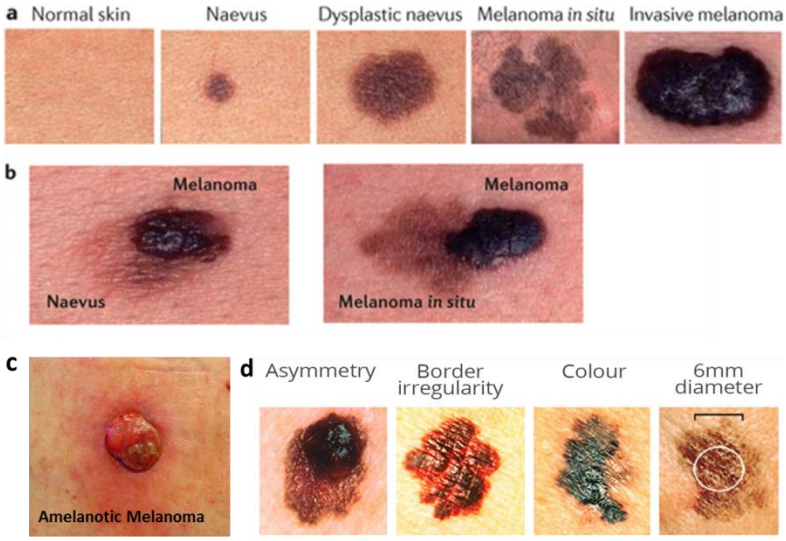

Figure 13: **An example of the phenotypic heterogeneity of malignant melanoma. a)** The different stages of the melanocytes transformations from the normal skin to invasive melanoma. **b)** An example from combined neoplasm, melanomas rarely pass through every histopathological stage. **c)** Amelanotic melanoma. **d)** An example of A (Asymmetry), B (Border), C (Color), D (Diameter) rule for clinical melanoma detection. Adapted from (Shain and Bastian 2016), (http://nchsbands.info/new/amelanotic-nodular-melanoma.html) and (http://www.dermapixel.com/2012/06/melanoma-el-lobo-feroz-de-la.html).

Multiple pathogenic mutations have been clearly associated to melanoma progression. During the very first steps of melanocyte transformation, the most recurrent somatic mutations, both CSD in and non-CSD melanomas arise in key-genes usually governing proliferation (BRAF, NRAS and Tyrosine kinase (PTK)), cell growth and metabolism (Phosphatase and tensin homolog (PTEN) and KIT), cell identity (AT-rich interaction domain 2 (ARID2)), resistance to apoptosis (Tumor protein p53 (TP53)), cell cycle control (cyclin dependent kinase inhibitor 2A (CDKN2A) which encodes $p16^{INK4A}$ and $p14^{ARF}$), and replicative lifespan (telomerase reverse transcriptase (TERT)).

As already mentioned, the RAS GTPases have a central role linking between activated growth factor receptors and downstream signal transduction pathways during melanogenesis. For this reason, the three RAS isoforms (HRAS, KRAS and NRAS) collectively represent the most commonly mutated oncogene in human malignancies and distribute across a variety of cancers (Sullivan and Flaherty 2013). Given the high-frequency BRAF mutations in melanocytic neoplasms, activation of the RAS–RAF–ERK signaling pathway seems to be yet another near obligate event in melanoma development (Chin 2003). Indeed, the relevance of the MAPK-pathway for melanoma is reflected in the overall rate of mutations leading to deregulation of the pathway. These include not only the ~50% of BRAF mutations, but also >25% NRAS mutations and ~14% of melanomas with mutations in the RAS suppressor NF1(Cancer Genome Atlas 2015). The most important genes involved in malignant transformation of melanocytes are sum up in the **Table 3**.

Pathway	Gene	Mutation	Subtype*	Progression phase‡	Role
MAPK	*BRAF*	V600E	Non-CSD	Naevi	Initiation
	BRAF	V600K, K601E and G469A, among other clustered nonV600E alterations	CSD	Intermediate and MIS lesions	Initiation
	NRAS	Q61R and Q61K, among other less common alterations affecting codon 61 or 12	CSD	Intermediate and MIS lesions	Initiation
	NF1	Disabling mutations occurring throughout the gene and deletions	CSD	MIS	Initiation
Telomerase	*TERT*	Promoter mutations affecting hg19 coordinates 1,295,228 or 1,295,250, among less common, nearby mutations	CSD and non-CSD	Intermediate and MIS lesions	Progression
RB	*CDKN2A*	Deletions and disabling mutations occurring throughout the coding region	CSD and non-CSD	Invasive melanoma	Progression
Chromatin remodelling	*ARID1A*, *ARID1B* and/or *ARID2*	Disabling mutations occurring throughout the protein	CSD and non-CSD	Invasive melanoma	Progression
PI3K	*PTEN*	Disabling mutations occurring throughout the protein and deletions	Non-CSD	Thicker invasive melanomas	Advanced progression
p53	*TP53*	Disabling mutations occurring throughout the protein	CSD	Thicker invasive melanomas	Advanced progression

ARID, AT-rich interaction domain; *CDKN2A*,cyclin-dependent kinase inhibitor 2A; CSD, chronically sun damaged; MIS, melanoma *in situ*; *NF1*, neurofibromin 1; *TERT*, telomerase reverse transcriptase. *Subtype refers to the melanoma subtype(s) predominantly associated with the mutation. ‡Progression phase refers to the earliest progression phase at which the mutation typically occurs.

Table 3: Common mutations during melanoma progression. Adapted from (Shain and Bastian 2016).

3. Inflammation

Inflammation is triggered in response to tissue injury, including during host defense against pathogens. It is indeed triggered in a condition of massive cell death or microbial/viral infection to accelerate regeneration of injured tissues and to eliminate pathogens (Pesic and Greten 2016). Several molecular signals may set off and maintain the host response towards the tissue healing (Coussens and Werb 2002). Suboptimal inflammation can be deleterious for tissues, while anomalous activation of inflammatory process in involved in different pathologies including fibrosis, metaplasia and cancer (Pesic and Greten 2016). This signal-mediated response can be produce by different stimuli, including physical agents (e.g. UVR), mechanical injuries, chemical agents (tar products, arsenic, immunomodulatory drugs, toxins), biological agents (bacteria, viruses, fungi, parasites), and it can be associated to immunologic disorders (hypersensitivity reactions, autoimmunity, immunodeficiency states) (Maru, Gandhi et al. 2014).

The immune system includes different cells and molecular mediators that sustain the inflammation process and are responsible of coordinated response against non-host molecules. In fact, after pathogen or foreign material recognition, the immune response is the main player of that mechanism able to finally remove it (Male, 2006). We can distinguish two types of immune response (**Figure 14**): the innate and adaptive, which is acquired, specific, long-lasting and requires the recognition of specific "non-self" antigens (Pancer and Cooper 2006). Adaptive immunity is highly specific for a particular antigen and in the case of a repeated encounter with the same antigen results to be more effective. Therefore, specificity and memory are the two key features of the adaptive immune response (Male, 2006).

Innate immune response represents the first line of defense against infection until the specific response is triggered and it comprises physical barriers, like skin, phagocytic cells, such as macrophages, neutrophils or dendritic cells, eosinophils, natural killer cells and finally various blood molecules (complement and acute phase proteins) (Zen and Parkos 2003), (Mollen, Anand et al. 2006).

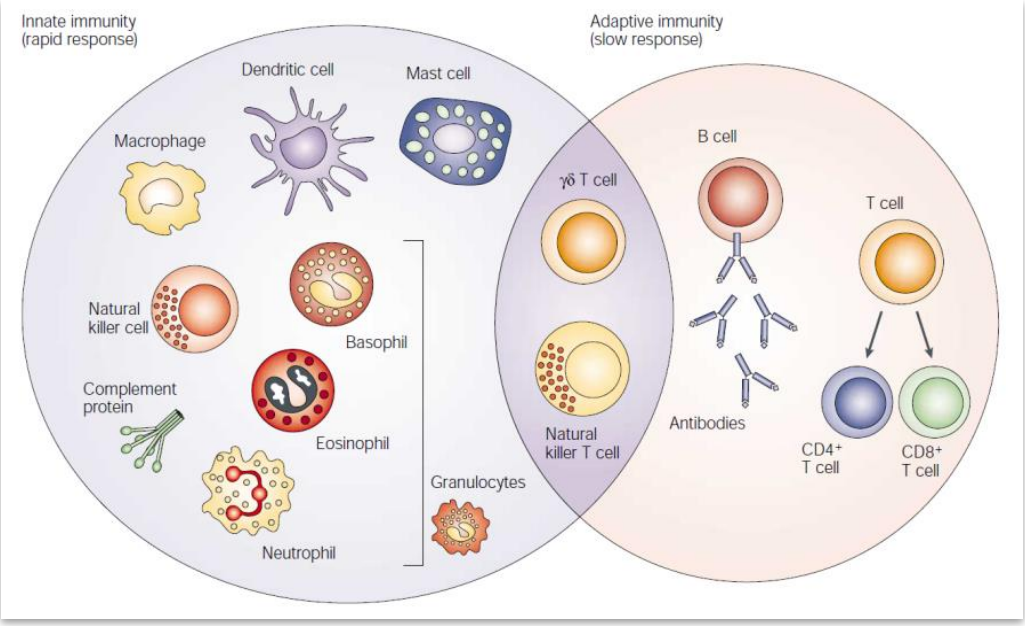

Figure 14: The innate and adaptive immune response. Adapted from (Dranoff 2004).

Lymphocytes (Ly) and secreted antibodies are the main players of adaptive immune response, which are present exclusively in vertebrates (Pancer and Cooper 2006). The Ly differentiate from hematopoietic stem cells in the bone marrow(Janeway and Medzhitov 2002) and specifically recognize individual pathogens. Two main categories of Ly can be distinguished: T Ly (T cells) and B Ly (B cells). B cells recognize and oppose extracellular pathogens and their products through secretion of antibodies (Ab). These molecules are able to bind a foreign molecule, called antigen, with high specificity (Sproul, Cheng et al. 2000). Besides, T cells elicit different functions during inflammatory process. T helper Ly (Th) extert regulatory functions and are involved in the development and production of Ab by B cells as well as in sustain the activity of phagocytic cells, for a complete elimination of previously phagocytized pathogens. These cells are not characterized by cytotoxic activity,indeed they are not able to directly eliminate infected cells or the same pathogens. On the other hand cytotoxic T cells (CTL) may recognize and destroy - cells infected by virus or other pathogens, damaged or dysfunctional cells,including tumoral ones (Harty, Tvinnereim et al. 2000).

The entire immune system represents a very complex interacting network and its activity is the result of the coordinationion between the innate and adaptive immune systems, including processes such as cell-cell interactions in relation to antigen presentation or the secretion of soluble molecules, such as cytokines or chemokines.

These systems of cellular communication are not necessarily exclusive and most importantly the cross-talk between the innate and adaptive systems may be bidirectional (Getz, 2005). In this process, there is considerable interaction between Ly and phagocytes (Male, 2006). For example, some phagocytes capture and degrade antigens and then present them to T cells through attaching the antigens to the surface to major histocompatibility complex receptor (MHC) (Holtmeier and Kabelitz 2005). In response to antigen presentation, the Ly secrete soluble factors (cytokines) which activate phagocytes to destroy the pathogens that they have phagocytized before. So in the early stages of infection, the innate response predominates but Ly subsequently begin to generate the adaptive response.

Different characteristics distinguished acute inflammation from chronic one (see **Table 5**). Acute inflammation is a rapid, self-limiting process, but may develop to a chronic status, which could be responsible of the onset of various diseases, including cardiovascular diseases, cancer, diabetes, arthritis, Alzheimer's disease, pulmonary diseases, and autoimmune diseases (Zhao, Tang et al. 2017).

Characteristics	Acute inflammation	Chronic inflammation
Duration	Short	Relatively long
Pattern	Stereotyped	Varied
Predominant cell	Neutrophils, leukocytes	Lymphocytes, macrophages, plasma cells, giant cells, fibroblasts
Tissue destruction	Mild to moderate	Marked
Fibrosis	Absent	Present
Inflammatory reaction	Exudative	Productive

Table 5: Characteristics of Inflammation. Adapted from (Maru, Gandhi et al. 2014).

3.1. Chronic inflammation

Acute inflammation is self-limiting, since the release of anti-inflammatory cytokines closely follows the production of pro-inflammatory cytokines. However, chronic inflammation seems to be due to the persistence of the initiating factors or a failure of mechanisms required for resolving the inflammatory response (Coussens and Werb 2002).

If the condition causing acute inflammation is not resolved, the inflammation may evolve to a longer term chronic phase. In addition, some pathology tends to directly determine chronic rather than acute inflammation. Many of the features of acute inflammation are persistent once inflammation becomes chronic, including increased blood flow and enhanced capillary permeability. Accumulation of white blood cells is still present, even if the composition of the cells changes.

After the unleashing of the pro-inflammatory program, the predominant cellular specie is the short-lived neutrophils, which quickly enter the infected or damaged tissue. Further, macrophages and lymphocytes quickly begin to be recruited. All these cells share the sequence by which they bind to cell adhesion molecules and pass through the endothelium

The long-lasting macrophages ingest pathogens and other material at the site of the inflammation and indigestible material may remain included in subcellular vesicles for long periods. Moreover, macrophages secrete inflammatory paracrine growth factors, as well as several other proteins.

The activity of macrophages in resolving inflammation may be sustained by Ly especially for dealing with difficult pathogens. But this process may be observed in the context of autoimmune diseases, in which activated macrophages are often the major responsible for tissue damage. In addition, B cells secreting Ab can be present in the site of inflammation, locally integrating the amount of Ab deriving from blood circulation.

3.2. Inflammation and cancer

Today, it is known that inflammation can play a key role in cancer, from initiation of the transformed phenotype to metastatic spread. Nevertheless, inflammation and cancer have a profound yet ambiguous relationship. Inflammation (especially chronic inflammation) has protumorigenic effects, but inflammatory cells also mediate an immune response against the tumor and immunosuppression is known to increase the risk for certain tumors (Shalapour and Karin 2015). Already in 1863, Virchow hypothesized that the origin of cancer was at sites of chronic inflammation, in part based on his hypothesis that some classes of irritants, together with the tissue injury and ensuing inflammation they cause, enhance cell proliferation. He noticed a similarity between the chronic inflammatory processes associated with wound healing and tumor development since both processes are characterized by a significant leukocyte infiltration. Today, the causal relationship between inflammation, innate

immunity and cancer is more widely accepted but, most of the molecular and cellular mechanisms mediating this relationship remain unresolved. Furthermore, tumor cells may usurp key mechanisms by which inflammation interfaces with cancers, to further sustain malignant cells colonization of the host (Balkwill and Mantovani 2001, Maru, Gandhi et al. 2014).

Nowadays, skin cancers are also attributed to chronically injured or non-healing wounds and scars or ulcers that occur at sites of previous burns, sinuses, trauma, osteomyelitis, prolonged heat and chronic friction (**Figure 15**). The incidence of malignancy in scar tissues is 0.1–2.5 % and it is estimated that underlying infections and inflammatory responses are linked to 15–20% of all deaths from cancer worldwide (Maru, Gandhi et al. 2014). Furthermore, chronic inflammation contributes to about 20% of all human cancers (Tang and Wang 2016); it is known that exist a wide range of inflammatory diseases able to induce or produce cancerous malignancies. It is the case of psoriasis patients that have a high probability of developing skin cancer or melanoma (Beyaert, Beaugerie et al. 2013), or other several clinical conditions such as discoid lupus erythematosus, dystrophic epidermolysis bullosa and chronic wound sites. All of them are associated with cutaneous inflammation and appear to predispose the individual to increased susceptibility for skin cancer (Coussens and Werb 2002, Beyaert, Beaugerie et al. 2013).

In chronic inflammatory diseases, several cytokines recruit activated immune and inflammatory cells to the site of lesions, thereby amplifying and perpetuating the inflammatory state. These activated cells produce many other mediators of inflammation. An inflammatory niche, containing stromal fibroblasts, endothelial cells, infiltrated lymphocytes and secreted cytokines, chemokines and growth factors is not only essential for the normal wound healing process but also contributes significantly to the development and progression of cancer (Liu, Lin et al. 2015). Later on, Peyton Rous was the first to recognize that cancers develop from "subthreshold neoplastic states" caused by viral or chemical carcinogens that induce somatic changes (Rous and Kidd 1941). Over the past decades, our understanding of the inflammatory environment of malignant tissues has dramatically increased and has been substantiated by data showing a somewhat altered malignancy-related nature of the inflammatory reaction.

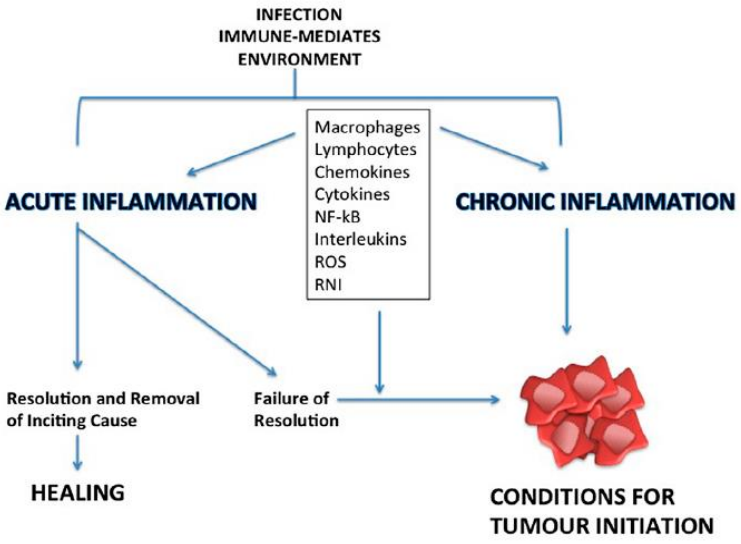

Figure 15: A switch from acute to chronic inflammation during inflammation and tumor initiation. Adapted from (Raposo, Beirao et al. 2015)

As it has been commented previously, if in the normal inflammation the production of anti-inflammatory cytokines follows the pro-inflammatory cytokines closely, however in the chronic inflammation the mechanisms required for resolving this response fail, why does the inflammatory response to tumors persist? Chronic inflammation is characterized by the dominating presence of macrophages in the injured tissue (Tang and Wang 2016). These cells are powerful defensive agents of the body, but the toxins they release (including ROS) are injurious to the organism's own tissues as well as to invading agents. Consequently, chronic inflammation is almost always accompanied by tissue destruction. Under persistent infection or injury, chronic inflammation drives the transformation of cancer-originating cells by producing ROS and reactive nitrogen intermediates (RNI) that are capable of inducing DNA damage and genomic instability (**Figures 15 and 16**). These molecules also inactivate mismatch repair functions, supporting tumor initiation. In a positive feedback loop, DNA damage can also lead to inflammation, supporting tumor progression (Raposo, Beirao et al. 2015). In addition to the defense functions, inflammatory cells are also an important source of growth factors and cytokines such as IL-1 and TNFA that are necessary for cell recruitment, activation, and proliferation (Coussens and Werb 2002, Maru, Gandhi et al. 2014). Besides tumor promotion and progression, the inflammatory microenvironment provides the basis for epithelial-mesenchymal transition (EMT), invasion and metastasis (**Figure 16**). Pro-inflammatory cytokines trigger signaling cascades that activate key transcription factors directly or indirectly (activator protein 1

(AP-1), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), Signal transducer and activator of transcription 3 (STAT3), yes-associated protein 1 (YAP) or NOTCH) that control cell-cycle, cell death, dedifferentiation, stemness, motility and migration. All these processes are involved in normal tissue regeneration and repair but in diseases caused by chronic, unresolved inflammation such as cancer, have gone astray (Shalapour and Karin 2015, Pesic and Greten 2016, Tang and Wang 2016).

On the other hand, UVR also serves as a link between skin cancer and inflammation, as its exposure alters immunological functions in the skin. For example, exposure to UV light results in the upregulation of cyclooxygenase-2 (COX-2) protein in keratinocytes and increased production of prostaglandin E_2 (PGE_2), which leads to cutaneous tissue inflammation. UV exposure also adversely affects skin immune system by suppressing the function of antigen-presenting cells, inducing the expression of immune-suppressive cytokines and modulating contact and delayed-type hypersensitivity reactions. The suppression on adaptive immunity by UVR has been proposed to contribute to the evasion of skin cancer cells from immune surveillance. UVR therefore promotes skin carcinogenesis through both direct action on skin cells and indirect modulating effect on local microenvironment that is shaped by the process of chronic inflammation and immune response (Tang and Wang 2016).

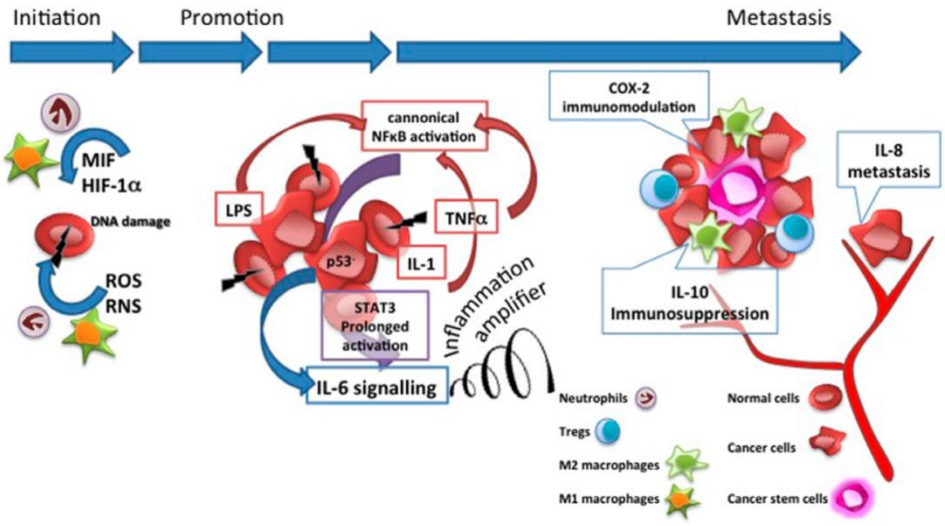

Figure 16: Summary of the cells and actors within the tumor microenvironment. The role of inflammatory and stromal cells interaction with cancer cells in tumor progression, angiogenesis and metastasis. Adapted from (Raposo, Beirao et al. 2015).

Summing up, tumor infiltrating myeloid and lymphoid cells can either promote or inhibit cancer development, depending on the nature of the immune-cancer interaction. Through the production of cytokines, chemokines and extracellular

enzymes, tumor infiltrating immune cells may serve as tumor promoter by supporting tumor cell proliferation and inhibiting programmed cell death. On the other hand, innate and adaptive immune cells recognize tumor-specific antigens and molecular patterns and actively destroy transformed cells. Deregulation of the profile and level of any of cytokines/chemokines that persists at sites of inflammation result in the development of cancer and many other pathologies. The disease process may results from interplay of genetic and microenvironmental factors (**Figure 16**) (Raposo, Beirao et al. 2015, Shalapour and Karin 2015, Pesic and Greten 2016, Tang and Wang 2016). For this reason, inflammation and the tumor-promoting inflammatory environment is now considered a dominant feature and a hallmark of cancer (Hanahan and Weinberg 2011).

4. The Zebrafish

4.1. Description, distribution, taxonomy, ecology and reproduction

Zebrafish (*Danio rerio*) is a shoaling cyprinid fish and its small size is around 60 mm (**Figure 17**). The exact distribution of zebrafish has not been clarified but its origin is the Indian subcontinent, from tropical fresh waters around the Ganges and Brahmaputra river basins in north-eastern India, Bangladesh, and Nepal. Common natural habitat is shallow and slow-flowing water especially in the areas dedicated to rice cultivation (Spence, Gerlach et al. 2008). Zebrafish owes its name for characteristic stripes running along the body and the fins.

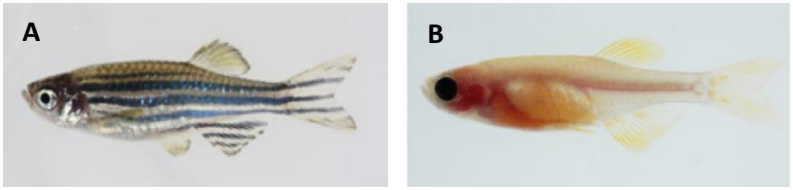

Figure 17: A) adult zebrafish. B)Casper zebrafish.

Taxonomically, the zebrafish is a derived member of the genus *Danio*, of the family Cyprinidae, order Cypriniformes. For many years it was referred to*Brachydanio rerio* in scientific literature*,* until its reassignment to the genus *Danio* (Mayden, Tang et al. 2007) .

Zebrafish are omnivorous, feeding primarily on zooplankton and insects, as well as phytoplankton, filamentous algae and vascular plant material, spores and invertebrate eggs, fish scales and arachnids (Spence, Gerlach et al. 2008).

Zebrafish are promiscuous and breed seasonally during monsoon season, from April to August although spawning has also been evidenced outside wet season, indicating that food availability may influence the seasonal breeding. Photoperiod is a determinant for mating, as spawning begins immediately at first light during breeding season and last more or less an hour. Courtship starts when about 3 - 7 males chase females, guiding them to a spawning site by nudging her and/or swimming around her in a tight circle or figure eight. Spawning sites are typically bare substrate that tends to be well vegetated. In captivity, gravel spawning sites are preferred while silt-bottomed habitats are preferred by wild zebrafish. Once arrived to an adapted spawning site, the male aligns his genital pore with the female one and starts to tremble. That determines sperm release as well as the release of 5 to 20 eggs from the female. This event is repeating for about an hour. The initiation of courtship behavior in the male depends on the presence of female pheromones, while ovulation requires female exposure to male gonadal pheromones (Spence, Jordan et al. 2006).

Zebrafish do not prepare a nest, laying non-adhesive eggs in groups. Hatching moment strongly depends on water temperature chorion thickness and embryo activity but in general, most embryos leave their chorion between 48 and 72 hours after fertilization. . Once outside, the approximately 3 mm-long zebrafish are able to swim, feed, and exhibit active avoidance behaviors within 72 hours from fertilization (Engeszer, Ryan et al. 2004, Engeszer, Barbiano et al. 2007, Engeszer, Patterson et al. 2007).

4.2. The zebrafish as a vertebrate research model

For many decades, zebrafish has been both a very popular aquarium fish and an important research model in several fields of biology (notably, toxicology and developmental biology). Since it was first used in a scientific laboratory 30 years ago, its popularity in biomedical research has significantly increased due to their unquestionable advantages respect other vertebrate models. The development of zebrafish as a model organism for modern biological investigation began with the pioneering work of George Streisinger and colleagues at the University of Oregon (Streisinger, Walker et al. 1981, Dooley and Zon 2000, Briggs 2002).

The use and importance of zebrafish in biological research has exploded and diversified to the point that these fish are extremely important vertebrate models in an extraordinary array of research fields (Vascotto, Beckham et al. 1997), due to multiple advantages:

- Small size. Low maintenance cost and small space needed.
- Robust fish. High resistance to pathogens.
- High fecundity and large production of embryos (around 200 eggs/female/week) makes phenotype-based forward genetics doable.
- Short generation time (for a vertebrate). Typically 3 to 4 months, making it suitable for selection experiments.
- Zebrafish eggs are large relative to other fish (0.7 mm in diameter at fertilization time), optically transparent and externally developed following fertilization, making them easily accessible to embryonic manipulation and imaging.
- Transparency of zebrafish embryos, together with the large availability of transgenic lines, let *in vivo* tracking of cells.
- Rapid development, which is very similar to the embryogenesis in higher vertebrates including humans, the precursors develop to all major organs within 36 hours, and larvae display food seeking and active avoidance behavior within five days after fertilization (2 to 3 days after hatching).
- As a vertebrate, zebrafish has special value as a model of human disease and for the screening of therapeutic drugs (Chakraborty, Hsu et al. 2009, Bootorabi, Manouchehri et al. 2017) and is often more tractable for genetic and embryological manipulation and cost effective than other vertebrate models such as mice (Trede, Langenau et al. 2004).
- The zebrafish genome has been completely sequenced, making it an even more valuable research organism.
- The zebrafish genome contains 26,206 protein-coding genes, and shares 71.4% similarity with the human genome, including 82% of human disease-related genes having at least one zebrafish orthologous (Bootorabi, Manouchehri et al. 2017, van Rooijen, Fazio et al. 2017).
- It is relatively easy to knockdown specific genes by using morpholinos and to overexpress proteins by injecting mRNA or plasmids.
- Use of reverse genetics approaches using zinc finger nucleases (ZFNs) (Meng, Noyes et al. 2008) and a transposon strategy (Kawakami, 2004) and the more

recent CRISPR/Cas9 gene editing strategy for generating transgenic and mutant zebrafish lines, which help in characterizing novel roles of genes during larval development and in adult zebrafish.

- Existence of a centralized online resource for the zebrafish research community (http://zfin.org), making easier the work with this model. Indeed zebrafish-based reagent sharing is quite simple given the easy transferring of zebrafish embryos between different labs.

All these advantages have led to the increased interest of scientists using zebrafish as an animal model research in the last years (**Figure 18**). In the past, it was a major vertebrate model especially in the developmental and genetic research (Hill, Teraoka et al. 2005), whereas now, the zebrafish gains also growing importance in other fields. Nowadays, it has been proposed as an excellent model for the study of the immune system (Renshaw and Trede 2012), hematopoiesis (Martin, Moriyama et al. 2011), vascular development (Isogai, Hitomi et al. 2009, Gore, Monzo et al. 2012, Quaife, Watson et al. 2012), neurogenesis (Schmidt *et al.,* 2013). In addition, the zebrafish has been established as one of the most important model organisms for biomedical research (Tavares and Santos Lopes 2013), including cancer research (Stern and Zon 2003, Mione and Trede 2010, Bootorabi, Manouchehri et al. 2017), among others. This model is particularly suitable for live cell imaging and high-throughput drug screening in a large-scale fashion (Bootorabi, Manouchehri et al. 2017). Some researchers have even used zebrafish to investigate the genetic basis of vertebrate behavior (Dooley and Zon 2000, Miklósi and Andrew 2006, Spence, Gerlach et al. 2008, Norton and Bally-Cuif 2010)and development (Bruneel and Witten 2015). Moreover, zebrafish has become a popular model in pharmacological studies and drug screening (Langenheinrich, 2003).

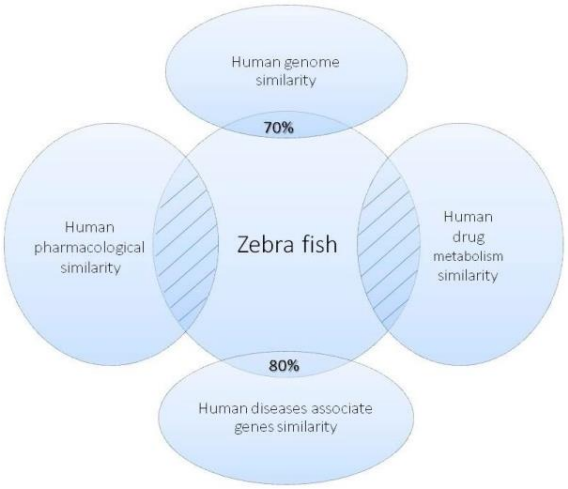

Figure 18: Zebrafish as a relevant model for human disease and cancer therapy. Adapted from (Bootorabi, Manouchehri et al. 2017).

4.3. Immune system of teleost fish

In teleost fish, the immune system shows similar characteristics to those of birds and mammals, displaying cellular and humoral responses that have the features of specificity and memory (Van Muiswinkel, 1995), although there are some important differences (Uribe *et al.*, 2011). Teleost is the first animal group that have well-structured and differentiated innate and adaptive immune systems. Its innate response comprises physical barriers (epithelium and mucosa), cellular effectors (phagocytic cells and nonspecific cytotoxic cells) and humoral factors (complement and other acute phase proteins), whereas adaptive response comprises a cellular (Ly) and humoral (Ab) components (Uribe *et al.*, 2011). However, despite their similarities with other vertebrate immune systems, there are clear differences as fishes depend more heavily on innate defense mechanisms, mainly at low temperature conditions (the fish are poikilothermic) since the adaptive immune response is dependent on the temperature (Cuchens and Clem 1977, Abruzzini, Ingram et al. 1982, Clem, Faulmann et al. 1984, Clem, Sizemore et al. 1985). In contrast to higher vertebrates, fish are free-living organisms from early embryonic stages of life and depend on their innate immune system to survive (Rombout, Huttenhuis et al. 2005).

The organs and tissues of the immune system in teleosts have been classified, as in mammals, in primary and secondary organs (Zapata *et al.,* 1996). Fish lack bone marrow; its function is taken by the kidney, a primary organ that is the largest site of hematopoiesis in adults. Kidney consists of two parts: the anterior or cephalic (head kidney, HK), with mainly hematopoietic function, and subsequent or posterior, basically with excretory function. Regarding to secondary lymphoid organ, the spleen is the most important but presents few Ly, although may increase in number by administration of an antigen. The spleen in zebrafish remains a small organ that contains large amounts of erythroblasts at 30 dpf. At three months, when lymphoblasts are evident in the spleen, emerging ellipsoids are involved in the capture of antigen. A similar developmental pattern has been described for other teleost spleen, such as that of the Atlantic salmon, grouper and catfish (dos Santos, Romano et al. 2000, Petrie-Hanson and Ainsworth 2000).

4.4. The zebrafish as a model of chronic inflammation

A. Tumor necrosis factor A (TNFA)

TNFA is a multifunctional cytokine that mediates key roles in acute and chronic inflammation, antitumor responses, and infection. It is produced by activated macrophages, CD4+ lymphocytes, natural killer (NK) cells and neurons (Faustman and Davis 2013). TNFA binds TNF receptor 1 (TNFR1, also known as TNFRSF1A or P55) and TNFR2 (also known as TNFRSF1B or P75) for stimulation of two opposing signaling events (Shalaby, Sundan et al. 1990) (**Figure 19**). In general, TNFR1 signaling results in the trigger of a cascade that can result in apoptosis, depending upon the cell type, the state of activation of the cell, and the cell cycle. In contrast, a TNFR2 signal induces cell survival pathways that can result in cell proliferation (Aggarwal 2003).

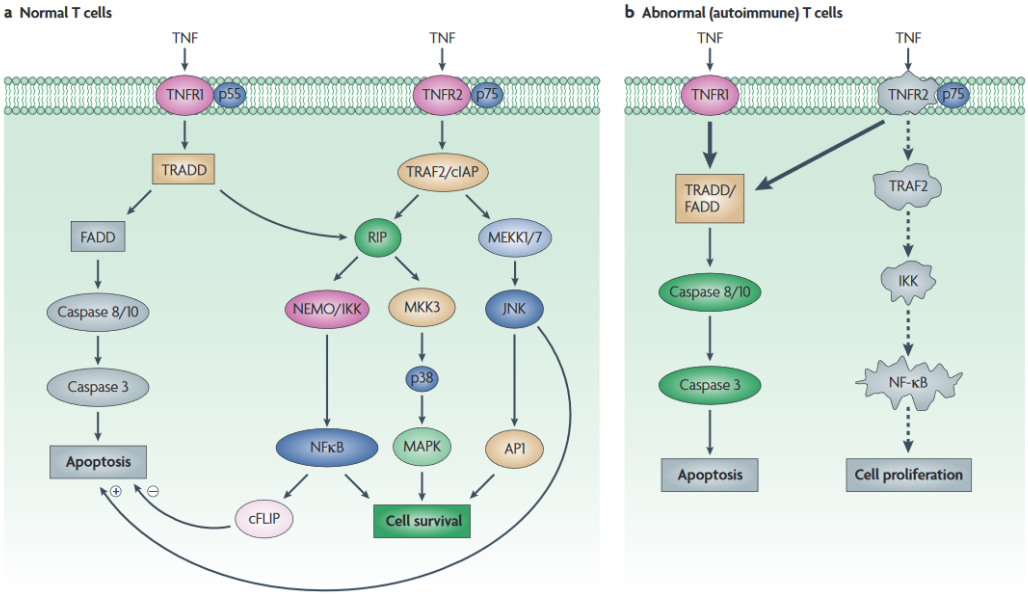

Figure 19: TNF signaling though TNFR1 and TNFR2 in normal T cells compared to abnormal (autoimmune) T cells. Adapted from (Faustman and Davis 2013).

TNF is a pleiotropic cytokine with abundant roles in the organism. Due to the fact that the TNFA plays key roles in the immune system, its unbalances is being widely recognized different diseases. There were some studies where associate the TNFA overexpression with several chronic inflammatory diseases: psoriasis, lichen planus, rheumatoid arthritis and inflammatory bowel disease (IBD). In addition, the inhibition of TNFA activities in these diseases has been remarkably successful (Palladino, Bahjat et al. 2003, Faustman and Davis 2013, Candel, de Oliveira et al. 2014). However, there are more and more studies showing that TNFA is a central

inflammatory mediator of carcinogenesis. Through binding to specific receptors, TNFA can induce more than five pathways that end up with inflammation, apoptosis, proliferation, invasion, angiogenesis, metastasis, or morphogenesis (Maru, Gandhi et al. 2014). It has also been demonstrated that patients with Chronic Inflammatory Diseases (psoriasis, IBD, rheumatoid arthritis) treated with anti-TNFA therapy may develop skin cancer (both melanoma and carcinoma) (Raaschou, Simard et al. 2013, Smith, Sanchez-Laorden et al. 2014). Since TNFR2 expression in melanoma has been associated to good prognosis, nowadays current studies tried to develop higher efficiency therapies based on the antitumor activity of TNFA, proposing TNFR2 as a novel target for cancer immunotherapy (Torrey, Butterworth et al. 2017, Vanamee and Faustman 2017).

TNFA and TNFRs are conserved in all vertebrates. In zebrafish, TNFA is a pro-inflammatory cytokine and TNFRs signaling plays an important role in the homeostasis of endothelial cells (Candel, de Oliveira et al. 2014). Recently, it was found that depletion of TNFA or its receptor TNFR2 caused skin inflammation and hyperproliferation of keratinocytes through the activation of a dual oxidase (DUOX)1/H_2O_2/NF-kB positive feedback inflammatory loop. Specifically, TNFA or TNFR2 deficiency results in neutrophil mobilization to the skin, triggers the expression of genes encoding pro-inflammatory mediators in keratinocytes, inducing NF-kB activation in the skin which promotes H_2O_2 production (Candel, de Oliveira et al. 2014). Given these premises, a relevant model of skin inflammation may be established in zebrafish, to study the implication of TNFA/TNFR2 axis in the oncogenic transformation.

B. Serine peptidase inhibitor, Kunitz type 1 (SPINT1)

Serine proteases regulate diverse cellular features, including in development, tumorigenesis and wound healing (**Figure 20**) (Kataoka, Itoh et al. 2002). They exert the proteolytic activity on a protein precursor, converting it into a functional form or by the inactivation/degradation of a substrate. Strict control of protease activity and expression is thus crucial for proper tissue ontogeny and homeostasis, and deregulated activity of a number of proteases is associated with human disease states (Carney, von der Hardt et al. 2007, Chen, Wu et al. 2011).Serine peptidase inhibitor, Kunitz type 1 (SPINT1) also known as Hepatocyte growth factor activator inhibitor 1 (HAI1) is a membrane-bound serine protease inhibitor that plays a crucial regulation of the

proteolytic activity of Matriptase 1 (also known as Matriptase, membrane-type serineprotease 1 (MT-SP1) and Suppressor tumorigenicity 14 (St14)) (Lin, Anders et al. 1999, Benaud, Dickson et al. 2001, Tseng, Chou et al. 2008), which is a type II transmembrane serine protease, first identified in human breast cancer cells (Lin, Anders et al. 1999). While SPINT1 is almost invariably co-expressed with Matriptase 1 (**Figure 21**) (Oberst, Anders et al. 2001), it plays a dual role in the regulation of Matriptase, as a conventional protease inhibitor and as a factor required for zymogen activation of Matriptase. As a consequence, activation of Matriptase is immediately followed by SPINT1-mediated inhibition, being the activated Matriptase sequestered into SPINT1 complexes (Tseng, Chou et al. 2008).

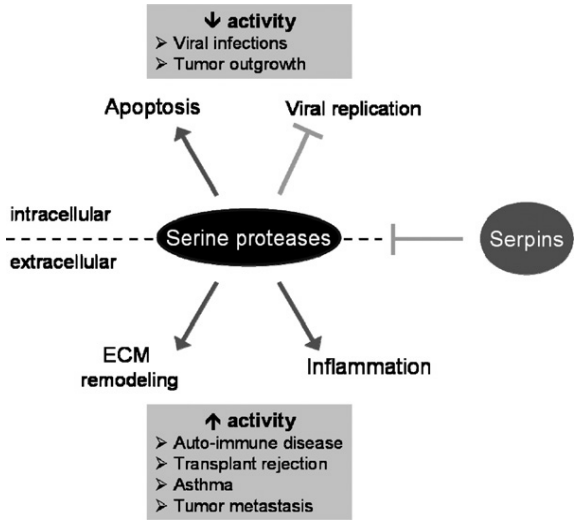

Figure 20: Schematic representation of serine proteases intracellular and extracellular functions. Adapted from (Heutinck, ten Berge et al. 2010).

The inhibitory activity of SPINT1 is conferred via its extracellular Kunitz domains, which bind directly to the protease sites (**Figure 21**) (Kirchhofer, Peek et al. 2003).SPINT1, was first described as an inhibitor of the circulating serine protease Hepatocyte growth factor activator (HGFA, also known as HGFAC) (Shimomura, Denda et al. 1997). Matriptase-SPINT1 complexes were detected in the conditioned media of cultured breast cancer cells besides in immortal mammary epithelial cells (Tseng, Chou et al. 2008). However, Matriptase is expressed in a broad range of epithelia playing critical roles in the establishment and maintenance of epithelial integrity (Mildner, Bauer et al. 2015). Furthermore, is also found on mast cells, peripheral blood leukocytes, such as monocytes and macrophages, and B cells, implicating that the protease might also participate in immune response (Kilpatrick, Harris et al. 2006, Heutinck, ten Berge et al. 2010, Zhao, Yuan et al. 2013). In contrast, SPINT1are not

expressed in monocytes and macrophages, suggesting that these leukocytes possess alternative, SPINT1-independent mechanisms regulating the zymogen activation and protease inhibition of Matriptase(Tseng, Chou et al. 2008). It has been shown that Matriptase is able to activate other proteins and zymogens, such as urokinase plasminogen activator (uPA), protease activated receptor 2 (PAR2), MMP3, insulin-like growth factor binding protein-related protein-1 (IGFBP-rP1, also known as IGFBP7), etc. (Carney, von der Hardt et al. 2007).

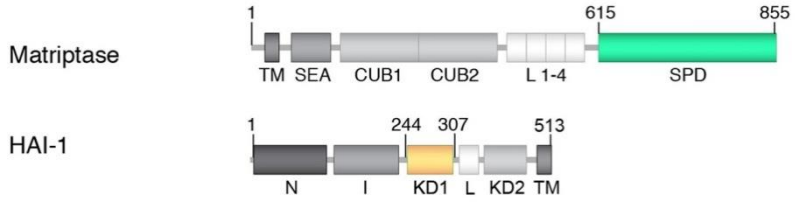

Figure 21: Schematic representations of the structures of matriptase and HAI-1 (SPINT1). Matriptase contains a transmembrane domain (TM), a sea urchin sperm protein, enterokinase, agrin domain (SEA), two complement C1r/C1s, urchin embryonic growth factor, bone morphogenic protein 1 domains (CUB), four low-density lipoprotein receptor class Adomains (L1– 4), and a serine protease domain (SPD). HAI-1 consists of an N-terminal domain (N), an internal domain (I), two Kunitz domains (KD1 and KD2), an LDLRA (L) and a C-terminal transmembrane domain (TM). The structure of HAI-1 KD1 (yellow) bound to the catalytic domain of matriptase (green). Adapted from (Zhao, Yuan et al. 2013).

The close functional relationship between Matriptase and SPINT1 was also observed in the skin of zebrafish. The first zebrafish chronic inflammation mutant was identified by Mathias et al. (Mathias, Dodd et al. 2007). They identified a mutant line with an insertion in the *spint1a* gene that showed accumulation of neutrophils in the fin. The mutant embryos exhibited inflammation in areas of epidermal hyperproliferation that was rescued by knock-down *matriptase 1*, suggesting a novel role for SPINT1-Matriptase 1 pathway in regulating inflammation (Carney, von der Hardt et al. 2007, Mathias, Dodd et al. 2007).

On the other hand, it was demonstrated that the functional linkage between Matriptase and SPINT1 also has important implications for the development of cancer. Matriptase activity, which is only partially opposed by endogenous SPINT1, enhances spontaneous tumorigenesis in the skin of keratin-5-matriptase transgenic mice exposed to carcinogenic compunds. Matriptase induced malignant transformation and its strong prooncogenic potential can, however, be counteracted by increasing epidermal SPINT1 expression (List, Szabo et al. 2005). Indeed, other substrates of matriptase, pro-uPA and pro-HGF, have both been implicated in cancer invasion and metastasis.

Furthermore, high expression levels of matriptase have been found in various cancer forms such as breast, cervix, ovaries, prostate, esophagus, and liver cancers (Kawaguchi, Yamamoto et al. 2016, Tervonen, Belitskin et al. 2016). In some cases, the malignant transformation results fromthe loss of balance between the protease and its cognate inhibitor SPINT1(List, Szabo et al. 2005), suggesting that Matriptase requires very strict regulation by its inhibitor and serves as a potential anti-cancer therapeutic target (Mathias, Dodd et al. 2007).

During embryonic and larval stages, the zebrafish epidermis is bilayered, consisting of a basal and an outer layer of keratinocytes. As in mammals, basal keratinocytes are attached to the basement membrane via hemi-desmosomes and to each other via desmosomes. Zebrafish *spint1a*, its paralogue*spint1b*(Kirchhofer, Peek et al. 2003) and *matriptase1a* are expressed in the developing basal layer of the epidermis (Carney, von der Hardt et al. 2007), as in the human skin (Chen, Wu et al. 2011). Via live imaging and marker analysis, it has been reported both epithelial and inflammatory skin phenotypes caused by loss of Spint1a activity, providing a direct genetic evidence for an essential role of Spint1a to maintain epithelial integrity of the epidermis during development in zebrafish (Carney, von der Hardt et al. 2007).

Given the strong correlation between alterations of Spint1a-Matriptase1 levels with the progression of skin tumor, the *spint1a*, mutant zebrafish may represent an attractive model of chronic inflammation to study the role of SPINT1-Matriptase1 in epithelial hyperproliferation and skin inflammation during the melanoma development.

4.5. The zebrafish as a model of cancer

To better understand the mechanisms underlying tumor initiation and progression, including in the field of melanoma research, zebrafish can be used as an excellent tool, through the use of xenograft technique and transgenic models of spontaneous formation of tumors of different hystotypes (Bootorabi, Manouchehri et al. 2017). Zebrafish models carrying mutations in tumor suppressor genes have been generated, along with transgenic zebrafish lines expressing oncogenes driven by tissue-specific or ubiquitous promoters. Interestingly, expression of human oncogenes is able to transform zebrafish cells demonstrating a conservation of the molecular mechanisms of carcinogenesis between the two organisms (Mione and Trede 2010). Another advantage of using zebrafish model in cancer research is the availability of fluorescent reporters, to monitor tumor initiation and progression, including xenotrasplant experiments. The injection of fluorescent-labeled cancer cells into

zebrafish larvae enables the study of cancer angiogenesis and tumor cell spread (Bootorabi, Manouchehri et al. 2017), taking advantage of the lacking of the adult immune system in zebrafish larvae, which is completely functional only at 28 days of development. Since adult zebrafish are no longer transparent, in order to visualize melanoma cells by fluorescent gene expression, a transparent adult fish, named Casper, has been created by White et al. (White, Sessa et al. 2008). These Casper and nacre zebrafish (**Figure 17**) lack melanocyte development and are extensively used as a recipient for xenotransplantation of cancer cells.

4.5.1 The zebrafish as a tool to study Melanoma

Zebrafish are valuable to study melanocyte biology and to model human melanoma, as underlying molecular mechanisms share a high degree of conservation between species (Mort, Jackson et al. 2015). Similar to mice and humans, zebrafish melanocytes originate from the highly migratory NC lineage during embryonic development, and differentiate into specialized melanin-producing pigment cells that protect the skin from the damaging effects of UV light (Zeng, Richardson et al. 2009). The melanocyte location is the same in humans than in zebrafish. Compared to humans, zebrafish have two additional pigment cells: the yellow xanthophore, and the reflective/silver iridophore, that together with black melanocytes form an intricate pigmentation pattern that plays an important role during adaptive responses to its environment (Kelsh 2004, Logan, Burn et al. 2006). As the same as in humans, MC1R/MSH/cAMP is the signaling pathway in charge of the dynamic dispersion of melanosomes (Richardson, Lundegaard et al. 2008). However, fish melanocytes only produce eumelanin and the melanin granules are retained by cells. MC1R enzymatic activity in fish is associated to a movement of the melanin granules out along microtubules, which results in a darkening of the fish as camouflage. The reverse movement of melanin into the center of the fish melanocyte is produced via a different signalling system involving the melanin-concentrating hormone (MCH) receptor (Mort, Jackson et al. 2015).

The terminal differentiation of melanocytes from neural crest-derived precursors critically depends on expression of the tyrosine kinase receptor *KIT* and the transcription factor *MITF* (**Figure22**). The zebrafish *KIT* orthologous *kita* is expressed in developing melanocyte progenitors and plays a role in migration and survival of embryonic melanocytes (van Rooijen, Fazio et al. 2017).

Zebrafish have two *mitf* genes (*mitfa/b*). *mitfa* is essential for melanocyte development. Patton et al. demonstrates that the abrogation of *mitfa* activity in melanoma led to rapid tumor regression and critical thresholds of MITF lead to dramatically different melanoma outcomes (Zeng, Johnson et al. 2015). Curiously, in zebrafish, there are distinct embryonic and adult pigmentation patterns. The melanoblasts that form both these patterns originate from a SOX10-positive NC-derived progenitor. While the embryonic pattern is formed by melanocytes that develop directly from this progenitor via a MITF+ melanoblast, the melanoblasts that form the adult pattern are derived from a melanocyte stem cell population that resides at the DRG and is specified by an ERB- and KIT-dependent pathway in the embryo (Mort, Jackson et al. 2015).

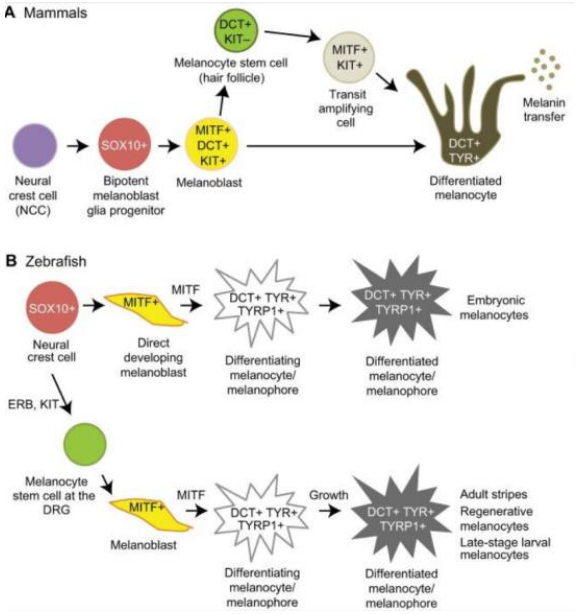

Figure 22: Melanocyte differentiation variances between mammals and zebrafish (Mort, Jackson et al. 2015).

However, it has not been reported that wild zebrafish populations are able to develop melanoma spontaneously like other fish species (eg. *Xiphophorus* or the coral *Plectropomusleopardus*) (Akke, Liu et al. 1998), while a powerful UV-independent melanoma models has been generated in zebrafish through expressing human oncogenes under the control of melanocyte-specific promoters. These models faithfully recapitulate human disease and can be used to study the molecular mechanisms involved in human melanoma development and progression using zebrafish.

4.5.2 Zebrafish models of melanoma: the *kita*:RAS model

As previously mentioned, activating mutations in either BRAF (~52%) or NRAS (~28%) oncogenes are present in nearly all human cutaneous melanomas (Cancer Genome Atlas, N, 2015), leading to aberrant activation of the MAPK-signaling pathway (Cancer Genome Atlas, N, 2015). For that, most zebrafish melanoma models have been developed with these oncogenes (van Rooijen, Fazio et al. 2017).

The zebrafish melanoma model used for this thesis is the *Et(kita:GalTA4,UAS:mCherry)hzm1; Tg(UAS:eGFP-HRAS-G12V)io6*. It is a p53-independent spontaneous melanoma model that expresses oncogenic $HRAS^{GV12}$ under the *kita* promoter and develops melanoma by 1–3 months of age without the need of coadjuvating mutations in tumor suppressors like p53 (Santoriello, Gennaro et al. 2010).

The model is based on the combinatorial GAL4-UAS system, employing a driver line containing the transactivator GAL4 under the *kita* promoter, and a responder line expressing human $HRAS^{GV12}$. The result is an increased hyperproliferation of embryonic melanocytes at 3 days post-fertilization. Indeed, HRAS oncogene expression promotes cell growth and maintains melanocytes in a proliferative state, whereas normally melanocytes are postmitotic by 3 dpf. By 2–4 weeks, transformed melanocytes accumulated in the tail stalk, and transgenic *kita*-GFP-$HRAS^{GV12}$ formed melanoma by 4 weeks of age (Santoriello, Gennaro et al. 2010). In this stable zebrafish melanoma line, not all of these lesions progress to a malignant transformation; approximately only 20% of 1–3 month old fish tumors show an invasive phenotype. Most of these adult tumors are hyper-pigmented, with a very low percentage of them (1–2%) being hypo-pigmented.

This model allow the identification of melanoma initiating cells thanks the constitutive expression of eGFP present in the UAS:$HRAS^{GV12}$ transgene (Santoriello, Gennaro et al. 2010). In this way, zebrafish offer a unique model through which the *in vivo* visualization of initiating cancer will allow to address other important aspects in melanoma initiation, for instance how the microenvironment or the immune system may participate in this process.

4.5.3Zebrafish as a model of metastasis

Metastasis is a multistep process, resulting in cancer cells leaving their site of origin and migrating through the body to colonize distant niches. Proliferation in these landing sites, such as the lungs, liver, bones, and brain in melanoma, results in organ failure and eventually death (Akke, Liu et al. 1998). Genetic, epigenetic, and post-translational events, as well as interaction with the local microenvironment and the immune system, affect melanoma cell progression (Damsky, Theodosakis et al. 2014, Massague and Obenauf 2016).

Due to the lack of adequate models, in the recent last years, zebrafish has emerged as a new tool to study metastasis devoting particular attention to the interaction between melanoma cells and their tumor microenvironment. As already mentioned, the transparency of larvae and the availability of Casper mutant fish allow the long-term evaluation of cancer cells and the environmental response to cancer cells, providing a unique angle to evaluate host responses (Bootorabi, Manouchehri et al. 2017). The injection of fluorescent-labelled melanoma cells into zebrafish either larvae or adults as a host,both from human tumors or endogenous oncogene-transformed cells, provides the opportunity to study cancer angiogenesis, tumor cell spread immune responses the early phases of melanoma progression and the phenotype switching toward metastatic behaviour metastasis evaluation (Dee, Nagaraju et al. 2016).

Therefore, the development of zebrafish models surely will contribute to bridge the gap between *in vitro* cell culture and *in vivo* mammalian models for a rapid pre-clinical drug development.

Objectives

1. Study the relevance of genetic alterations of SPINT1 and TNFA/TNFR2 in the cross talk between inflammation and skin cancer melanoma (SKCM) and in the prognosis of SKCM.

2. Study the relevance of the inflammation driven by Spint1a and Tnfr2 in early oncogenic transformation in zebrafish larval model.

3. Develop zebrafish larval and adult models to study the role played by Spint1a driven inflammation in SKCM progression and aggressiveness.

4. Study the role played by inflammation driven by Spint1a in zebrafish models of SKCM.

Materials and Methods

1. Animals

The experiments complied with the Guidelines of the European Union Council (Directive 2010/63/EU) and the Spanish RD 53/2013. Experiments and procedures were performed as approved by the Consejería de Agua, Agricultura, Ganadería y Pesca de la CARM (authorization number #A13180602).

Wild-type zebrafish (*Danio rerio* H. Cypriniformes, Cyprinidae) were obtained from the Zebrafish International Resource Center (ZIRC, Oregon, USA) and mated, staged, raised and processed as described in the zebrafish handbook (Westerfield, 2000). Zebrafish fertilized eggs were obtained from natural spawning of *wild-type* and transgenic fish held at our facilities following standard husbandry practices. Animals were maintained in a 12 h light/dark cycle at 28°C.

Transgenic zebrafish *Et(kita:GalTA4,UAS:mCherry)*^{hzm1} was crossed with *Tg(UAS:eGFP-H-RAS_G12V)*^{io6} line (Santoriello, Gennaro et al. 2010) (both provided by Prof. M. Mione) to express oncogenic human HRAS^{G12V} driven by the melanocyte cell-specific promoter *kita*. Moreover, the *Tg*(*4xUAS*:tnfrsf1b, *myl7*:EGFP)^{ums1} (referred to as UAS:tnfr2 throughout the thesis) and *Tg(mpx:EGFP)*^{i114}(provided by Prof. SA Renshaw) expressing GFP under the neutrophil-specific promoter of myeloperoxidase were also used.

The zebrafish line *spint1a* (also known as *hai1*) (Mathias, Dodd et al. 2007) carries a hypomorphic*spint1a* mutant allele because an insertion (*hi2217*) within the promoter of the zebrafish *spint1a* gene. This line was used to promote the skin inflammation model because of the embryos exhibited inflammation in areas of epidermal hyperproliferation, showing a phenotype that resembles the common human skin disease psoriasis. This line was provided by Prof. A. Huttenlocher.

The transparent *roy*$^{a9/a9}$; *nacre*$^{w2/w2}$(Casper) in which pigment cell production is inhibited (White, Sessa et al. 2008) was used for xenotransplantation in larvae and transplantation in 4-8 month old adult of at least 1 g of weight and 3 cm in length.

2. Anesthesia

Zebrafish larvae were anesthetized by a solution of 0.16 mg/ml tricaine (MS-222) (Sigma, St. Louis, MI) in embryo medium.

Adult zebrafish were anesthetized by a duel anesthetic protocol to minimize over-exposure to tricaine, in long-term studies (up to 40 min). At the beginning of the procedure, the anesthesia was induced by MS-222-only solution that consisted of 4 ml

of MS-222 (Sigma-Aldrich) stock 4 g/l in a light-protected bottle into 100 ml of fish water. Following, fish were transferred to a MS-222/isoflurane solution constituted by 2 ml of the MS-222 stock 4 g/l and 100 µl of diluted isoflurane into 100 ml of fish water. The diluted isoflurane was stored at 4°C in a light-protected bottle and composed of undiluted forane (Fatro) and ethanol (Merck) in a 1:9 ratio (Dang, Henderson et al. 2016).

3. Morpholinos and Chemical Treatments

Specific MOs (Gene Tools) were resuspended in nuclease-free water to reach the concentration of 2 mM for Standard-MO and *tnfr1*-MO and 1 mM of *tnfr2*-MO (**Table 1**).

Gene	Ensembl ID	Target	Sequence (5'→3')
tnfr1	ENSDARG00000018569	e6/i6	CTGCATTGTGACTTACTTATCGCAC
tnfr2	ENSDARG00000070165	i1/e2	GGAATCTGTGAACACAAAGGGACAA

Table 1: morpholinos used in this study. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html). ENA, European Nucleotide Archive.

MOs were mixed in microinjection buffer (0.5X Tango buffer and 0.05% phenol red solution) and 0.5–1 nl per embryo (200 pg/egg) were microinjected into the yolk sac of one-cell-stage embryos using a microinjector (Narishige). The same amounts of MOs were used in all experimental groups. The efficiency of the MOs was checked previously (Espin, Roca et al. 2013).

To inhibit melanogenesis, zebrafish larvae were exposed to 0.3% phenylthiourea (PTU, Sigma).

4. Human SKCM dataset analysis

Normalized gene expression, patient survival data, genetic alterations, neutrophil/macrophage infiltration and data for correlation studies were downloaded from SKCM repository of The Cancer Genome Atlas (TCGA, Provisional) from cBioPortal database (https://www.cbioportal.org/), as well as data to perform the GO enrichment analysis of biological process. Transcript levels of *TNFA* and *TNFR2* and *SPINT1* in human samples from normal skin, benign nevus and malignant melanoma was

collected from Gene Expression Omnibus (GEO) (GDS1375 dataset and 207113_at and 203508_at probe for *TNFA* and *TNFR2, respectively,* while for *SPINT1*,GDS1375 dataset and 202826_at probe were used). Gene expression plots, regression curves for correlation studies and GO enrichment analysis of biological process were obtained using GraphPad Prism 5.03 (GraphPad Software).

5. *In vivo* larval allotransplantion assays

kita:RAS melanomas were disaggregated, then labelled with 1,1'-di-octa-decyl-3,3,3',3'-tetra-methyl-indo-carbo-cya-nine perchlorate (DiI, ThermoFisher) and finally resuspended in a buffer containing 5% FBS in PBS. Between 25 to 50 cells/embryo were then injected in the yolk sac of 60-90Casper or *spint1* mutants zebrafish larvae of 48 hours post-fertilization (hpf) and after 5 days at 28°C, larvae were scored manually in blind samplesfor zebrafish melanoma cells dissemination by fluorescence microscopy (Marques, Weiss et al. 2009).

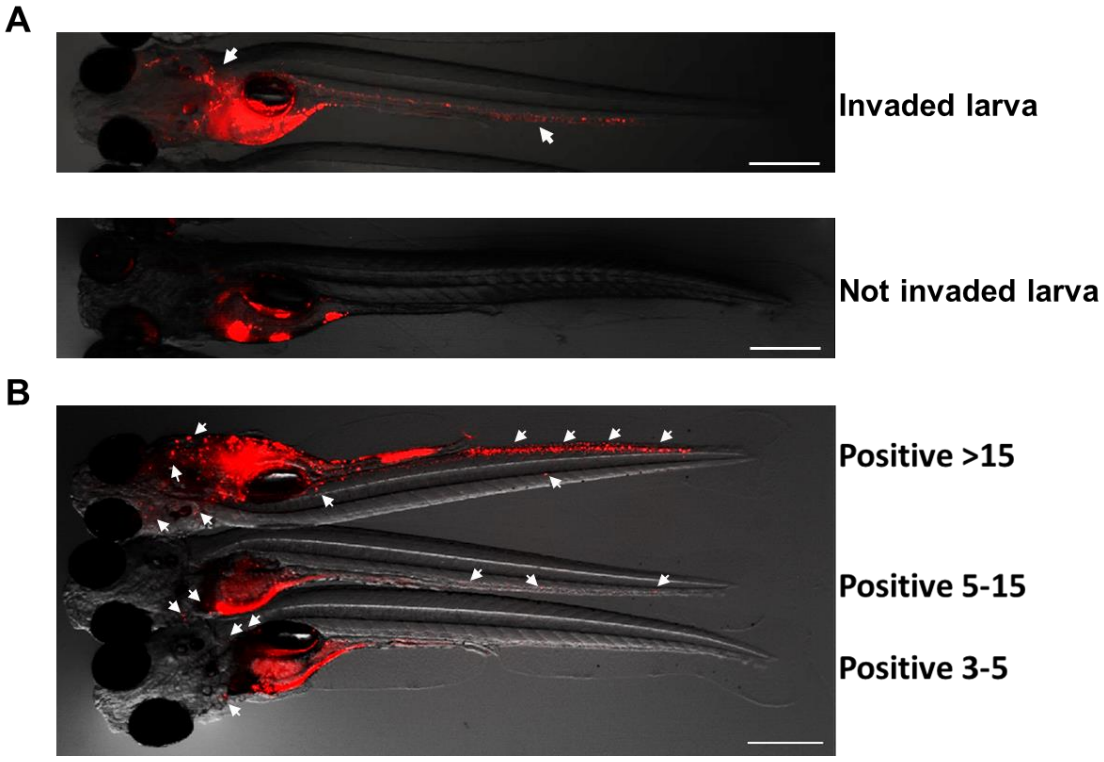

Figure 1: A) Representative images of invaded larvae or not (at 5 dpi) by zebrafish melanoma cells which are showing in red by the red fluorescent dye DiI. **B)** Images of the 3 invasion phenotypes showing the number of positive foci per larvae is represented.Scale bars: 500 µm.

Melanoma cell invasion score was calculated as the percentage of zebrafish melanoma cell-invaded larvae over the total number of larvae analyzed taking into account also the number of tumor foci per larvae. Three tumor foci were established to detrermine a larva as positive for invasion (**Figure 1A**). Furthermore, larvae positive for invasion were also distinguished in three groups considering the number of positive foci per larvae: 3-5 foci per larvae, 5-15 foci per larvae and >15 foci per larvae (**Figure 1B**).

6. Live imaging of zebrafish larvae

Images were captured using an epifluorescence LEICA MZ16FA stereomicroscope equipped with green and red fluorescent filters at 28.5 °C. All images were acquired with the integrated camera on the stereomicroscope. Subsequently, those images were used to count the number of $HRAS^{G12V}$ positive cells in 3 dpf larvae and for the *in vivo* larvae invasion assay in the 5 dpi larvae. Samples were mounted in 1% (w/v) low-melting-point agarose and a confocal microscope was used to imaging 3dpf larvae to count neutrophils (*Tg(mpx:EGFP)*^{i114}) and $HRAS^{G12V}$ positive cells contact in 3 dpf larvae in four ROIs per larvae. The cell contact ratio was obtained as a mean of the four ROIs per larvae in 3-4 larvae per treatment.

7. *In vivo* melanoma incidence analysis

For melanoma formation curve, zebrafish were housed in groups of ≤30 and survivors were counted from 28 dpf to 120 dpf. During that period of time, fish were monitored weekly by visual inspection for exophytic tumor growth and scored starting at the first appearance of raised lesions. For each curve at least three repeats were performed.

8. Adult allotransplantion assays

8.1. Biopsy of zebrafish melanoma tumors

Primary melanoma tumors were excised from adult zebrafish once they had reached between 3-5 mm in diameter. Some individuals were euthanized according the European Union Council and IUAC protocol and others were monitored and maintained still alive, after the tumor biopsy, in fresh fish water treated with conditioners to reduce fish stress and heal damaged tissue and wounds (STRESS COAT, API), as well as to protect of bacterial (MELAFIX, API) and fungal infections (PIMAFIX, API).

8.2. Tumor cells disaggregation

The tumor was excised with scalpel and razor blade, placed in 2 ml of dissection media, composed by DMEM/F12 (Life Technologies), 100U/mlpenicillin-streptomycin (Life Technologies), 0.075 mg/ml of Liberase (Roche). After manually disaggregation with a clean razor blade and incubation at room temperature for 30 min, 5 ml of wash media, composed by DMEM/F12 (Life Technologies), penicillin-streptomycin (Life Technologies), and 15% heat-inactivated FBS (Life Technologies), was added to the tumor slurry and manually disaggregated one last time. Next, the tumor cells suspensions were passed through a 40 µm filter (BD) into a clean 50 ml tube. An additional 5 ml of wash media was added to the initial tumor slurry that was filtered again. This procedure was repeated twice. Cell numbers were calculated with a hemocytometer and the tubes of resuspended cells were centrifuged at 800 g for 5 min at 4ºC. The pellet of tumor cells were resuspended in the appropriate volume of PBS containing 5% FBS and kept on ice prior to transplantation (Dang, Henderson et al. 2016).

8.3. Cell Sorting

The resulting cell suspension from zebrafish melanoma tumors (as previously described) was passed through a 40 mm cell strainer and propidium iodide (PI) was used as a vital dye to exclude dead cells. The Cell Sorting was performed on a "Cell Sorter" SONY SH800Z in which eGFP positive cells were sorted from the negative ones of the same cell tumor suspension.

8.4. Adult zebrafish immunosuppression

Adult zebrafish used as transplant recipients were immunosuppressed to prevent rejection of the donor material. Thus, the recipients were anesthetized, as previously described, and treated with 30 Gy of split dose sub-lethal X-irradiation (YXLON SMART 200E, 200 kV, 4.5 mA) two days before the transplantation. Then the immunosuppressed fish were maintained in fresh fish water treated with conditioners preventing infections onset and the consequent recipient deaths.

8.5. Subcutaneous injection

Once the adult zebrafish were anesthetized using the 2-step anesthetic protocol detailed above, injections were performed using a 10 µl beveled, 26S-guaged Hamilton

syringe (Hamilton). Anesthetized fish (10-20 per tumor) were placed dorsal side up on a damp sponge and stabilized with one hand. Using the other hand, the needle was positioned midline and ahead to the dorsal fin. 30,000, 100,000, 300,000 and 500,000 cells resuspended in PBS were injected into the dorsal subcutaneous cavity. The syringe was washed in 70% ethanol and rinsed with PBS between uses.

Following transplantation, recipient's post-transplant care was needed to prevent fish infection as previously mentioned. Thus, fishes were placed into a recovery tank of fresh fish water and kept off-flow with daily water changes for 7 days. Large and pigmented tumors engrafted and were observed to expand by 10 days post-transplantation.

8.6. Imaging and tumor measurements in adult zebrafish

Photographs of all experimental subjects from adult transplantation assays were obtained at 1, 2, 3 and 4 weeks post injection (wpi) of the experimental timeline. Zebrafish were anesthetized, placed in a dish of fish water, and photographed using a mounted camera (Nikon D3100 with a Nikon AF-S Micro Lens). The pigmented tumor size was represented by the number of pigmented pixels (Adobe Photoshop CS5).

9. Generation and maintenance of zebrafish melanoma cell lines

ZfMRH (+/-) and ZfMRH (-/-), referred as zebrafish cell lines Wt or mutant for *spint1a*, were cultured from a primary zebrafish melanoma tumor that, as described above, overexpresses oncogenic human $HRAS^{G12V}$ in a pattern driven by the melanocyte cell-specific promoter *kita*, along with eGFP. The cells were maintained in DMEM (Life Technologies) supplemented with 20% FBS (Life Technologies), 1% Glutamax (Life Technologies), 1% penicillin-streptomycin (Life Technologies) and kept in a sterile 28°C incubator. The cells were washed with sterile PBS (Life Technologies) and trypsinized when confluent. Cell numbers were quantified using a hemocytometer.

10. Analysis of gene expression

Once zebrafish tumors reached between 3-5 mm of diameter, they were excised and total RNA was extracted with TRIzol reagent (Invitrogen), following the manufacturer's instructions, and then treated with DNase I, amplification grade (1

U/µg RNA; Invitrogen). SuperScript III RNase H^- Reverse Transcriptase (Invitrogen) was used to synthesize first-strand cDNA with oligo$(dT)_{18}$ primer from 1 µg of total RNA at 50°C for 50 min. Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems) using SYBR Green PCR Core Reagents (Applied Biosystems).

Gene	ENA ID	Name	Sequence (5'→3')
rps11	NM_213377	F1	GGCGTCAACGTGTCAGAGTA
		R1	GCCTCTTCTCAAAACGGTTG
sox10	NM_131875.1	F	CCTCACGCTACAGGTCAGAG
		R	CGAAGTCGATGTGCGGTTTC
mitfa	NM_001362262.1	F	CGACTGGTCAGTTCTTGCAC
		R	AGGTGGGTCTGAACCTGGTA
tyr	NM_131013.3	F	TGTATTCATGAACGGCTCCA
		R	GATGAAGGGCACCATGAAGT
dct	NM_131555.2	F	TGGACAGTAAACCCTGGGGA
		R	CCGGCAAAGTTTCCAAAGCA
cdh1	NM_131820.1	F	TGGCAAAAGACTAGGCAAAGTGAC
		R	AAACACCTTGTGGCCCTCAT
slug	NM_001008581.1	F1	AGTCCAACAGTGTTTATTTCTCCA
		R1	GCAGGTTGCTGGTAGTCCAT
mmp9	NM_213123.1	F1	GCTGCTCATGAGTTTGGACA
		R1	AGGGCCAGTTCTAGGTCCAT
il1b	NM_212844.2	F5	GGCTGTGTGTTTGGGAATCT
		R5	TGATAAACCAACCGGGACA
lyz	NM_139180.1	F	TGGCAGTGGTGTTTTTGTGT
		R	TCAAATCCATCAAGCCCTTC
mpx	NM_212779	F1	AGGGCGTGACCATGCTATAC
		R1	AGGCTCAGCAACACCTCCTA
mpeg1	NM_212737.1	F	ACAGCAAAACACCCATCTGGCGA
		R	TGCGGCACAATCGCAGTCCA
b2m	NM_001159768.1	F	AACCAAACACCCTGATCTGC
		R	CAACGCTCTTTGTGAGGTGA
mxb	NM_001128672.1	F	AATGGTGATCCGCTATCTGC
		R	TCTGGCGGCTCAGTAAGTTT
pkz	NM_001040376.2	F1	GGAGCACCGTACAGGACATT
		R1	CTCGGGCTTTATTTGCTCTG

Table 2: Primers used in this study for RT-qPCR. The gene symbols followed the Zebrafish Nomenclature Guidelines (http://zfin.org/zf_info/nomen.html). ENA, European Nucleotide Archive (http://www.ebi.ac.uk/ena/).

Reaction mixtures were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and finally 15 s at 95°C, 1 min 60°C and 15 s at 95°C. For

each mRNA, gene expression was normalized to the ribosomal protein S11 (rps11) content in each sample Pfaffl method (Pfaffl 2001). The primers used are shown in Table 2. In all cases, each PCR was performed with triplicate samples and repeated at least in two independent samples.

11. Statistical analysis

Data are shown as mean ± SEM and they were analysed by analysis of variance (ANOVA) and a Tukey multiple range test to determine differences between groups. The survival curves were analysed using the log-rank (Mantel-Cox) test. All the experiments were performed at least three times, unless otherwise indicated. The sample size for each treatment is indicated in the graph or in the figure legend or in corresponding section in M&M. Statistical significance was defined as $p < 0.05$.

Results

1. Genetic alterations in the TNFA / TNFR2 axis are associated with bad prognosis of melanoma patients

Inflammation has emerged as a major factor promoting cancer development (Ganesan, Johansson et al. 2013, Zelenay, van der Veen et al. 2015). Tumor-promoting inflammation is characterized by the presence of neutrophils, macrophages, dendritic cells and T-lymphocytes that support cancer progression (Mantovani, Allavena et al. 2008, Ganesan, Johansson et al. 2013). These cells secrets mediators such us cytokines, chemokines and grow factors that could promote cancer cell growth.

TNFA is a multifunctional cytokine that mediates key roles in acute and chronic inflammation, antitumor responses and infection. Its overexpression has been associated with several chronic inflammatory diseases, such as psoriasis, lichen planus, rheumatoid arthritis and inflammatory bowel disease (IBD) (Candel, de Oliveira et al. 2014). However, patients treated with anti-TNFA therapy may develop skin cancer (both melanoma and carcinoma) (Palladino, Bahjat et al. 2003). Moreover, high TNFR2 expression in melanoma has been associated with good prognosis and recently it has been proposed as a novel target for cancer immunotherapy (Vanamee and Faustman 2017). Because of that, *TNFA* and *TNFR2* genes of SKCM human samples of the TCGA cohort were analyzed *in silico*. **Figure 1A** shows a schematic representation of genetic alterations in *TNFA* and *TNFR2* compared with those of other frequent and relevant oncogenic genes in melanoma. Thereby, *TNFA* and *TNFR2* genetic alteration occurred in 5 and 6 % melanoma patients, respectively, at the same extent of *HRAS* oncogene. In the case of *TNFR2*, mutations and upregulation are the most frequent alterations found. In addition, the survival curve (**Figure 1B**) of patients with high vs. low expression of *TNFR2* and *TNFA* of melanoma was analyzed. It revealed that the low expression of those genes significantly correlated with poor prognosis. Furthermore, genetic expression of *TNFA* and *TNFR2* in human samples from normal skin, benign nevus and malignant melanoma from GEO database is represented in **Figure 1C**. The expression of *TNFR2* gene appeared significantly lower in melanoma human samples than in normal skin. In the case of *TNFA* gene, however, no significant changes were observed.

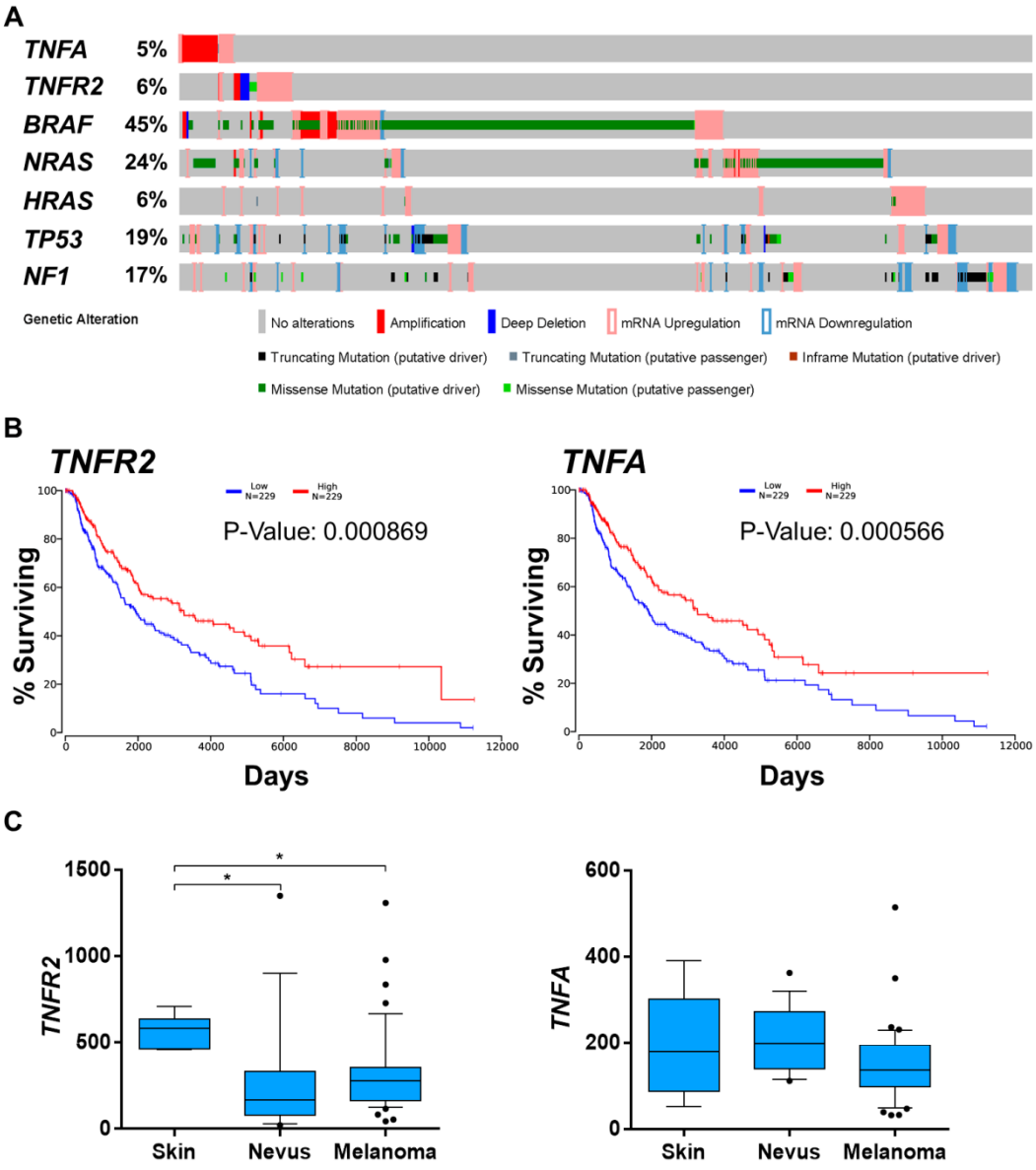

Figure1: TNFA/TNFR2 axis genetic alterations are associated with poor prognosis of SKCM patients. A) Percentage of genetic alterations (color code indicated on the graph) for *TNFR2* and *TNFA* and other major oncogenic genes in SKCM human samples of the TGCA cohort. **B)** Survival curves of SKCM patients (TCGA cohort) with high (red) vs. low (blue) expression of *TNFR2* and *TNFA* from Oncolnc database. Kaplan–Meier Gehan-Breslow-Wilcoxon (Median representation) and nonparametric Log-rank Test. **C)** Genetic expression of *TNFA* and *TNFR2* in human samples from normal skin, benign nevus and malignant SKCM from GEO (GDS1375 dataset and 207113_at and 203508_at probes, number of samples: Skin n=7, Nevus n=18, Melanoma n=45) *p< 0.05 ANOVA and Dunnett's Multiple Comparison Test.

2. TNFA signaling is associated with the establishment and progression of melanoma

In order to elucidate how TNFA/TNFR2 axis affects oncogenic transformation *in vivo*, Tnfr expression was modulated in a zebrafish model using two approaches: (I) morpholino-mediated gene inactivation of *Tnfr1* and *Tnfr2* and (II) *Tnfr2* overexpression in transformed melanocytes using the combinatorial Gal4-UAS system. To generate the genetic depletion of both receptors (**Figure 2A**), morpholinos previously tested in our lab (**Table 1**) were injected in *tg(kita:GalTA4,UAS:mCherry)*^{hzm1}*;(UAS:eGFP-HRAS-G12V)*^{io6} transgenic embryos.

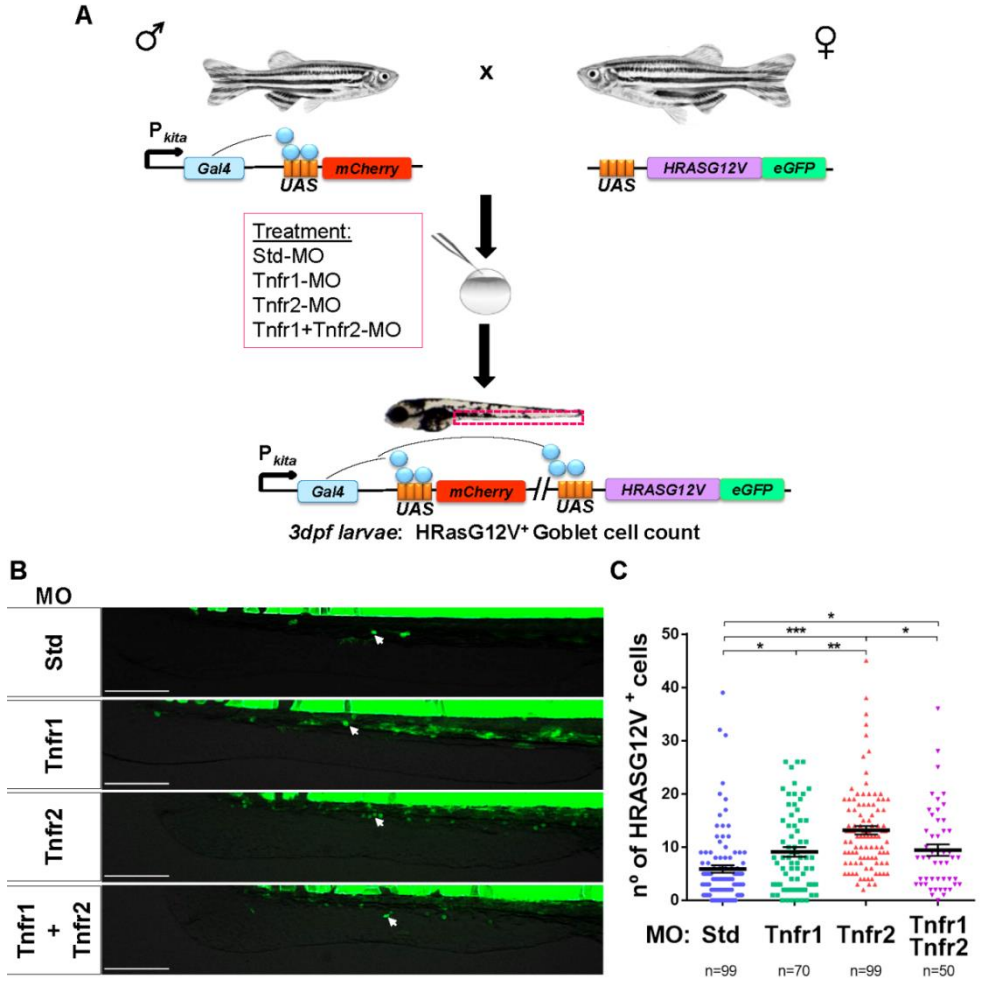

Figure 2: **Oncogenically transformed cells abundance modulation through TNF receptors**. **A)** Schematic diagram of the generation of melanoma driver line*kita:Gal4;UAS:eGFP-H-RAS_G12V*and injection strategy used to transiently inhibit the expression of Tnfr1, Tnfr2, or Tnfr1+Tnfr2 in melanocytes. Zebrafish one-cell *kita:Gal4;UAS:eGFP-H-RAS_G12V* embryos were injected with standard control (Std), Tnfr1, Tnfr2 or Tnfr1+Tnfr2 morpholinos (MO). **B)** Representative images, bright field and green fluorescent channel, of the indicated region of morphants at 3 dpf showing the HRASG12V^{+} cell abundance differences. Scale bars: 250 µm. **C)** Quantification of the number of HRASG12V^{+} cell in control, Tnfr1-, Tnfr2- and Tnfr1+Tnfr2-deficient larvae. The mean ± S.E.M. for each group is shown. *p<0.05; **p<0.01; ***p<0.001 according to ANOVA and Tukey's Multiple Comparison post-Test.

While the genetic inhibition of Tnfr1 slightly increased the abundance of oncogenically transformed cells (HRASG12V^{+} goblet cells) in larvae at 3 dpf, *Tnfr2* deficiency robustly increased the number of oncogenically transformed cells (**Figures 2B** and **2C**). Curiously, genetic inhibition of both receptors had a similar effect as depletion of Tnfr1 alone (**Figures 2B** and **2C**).

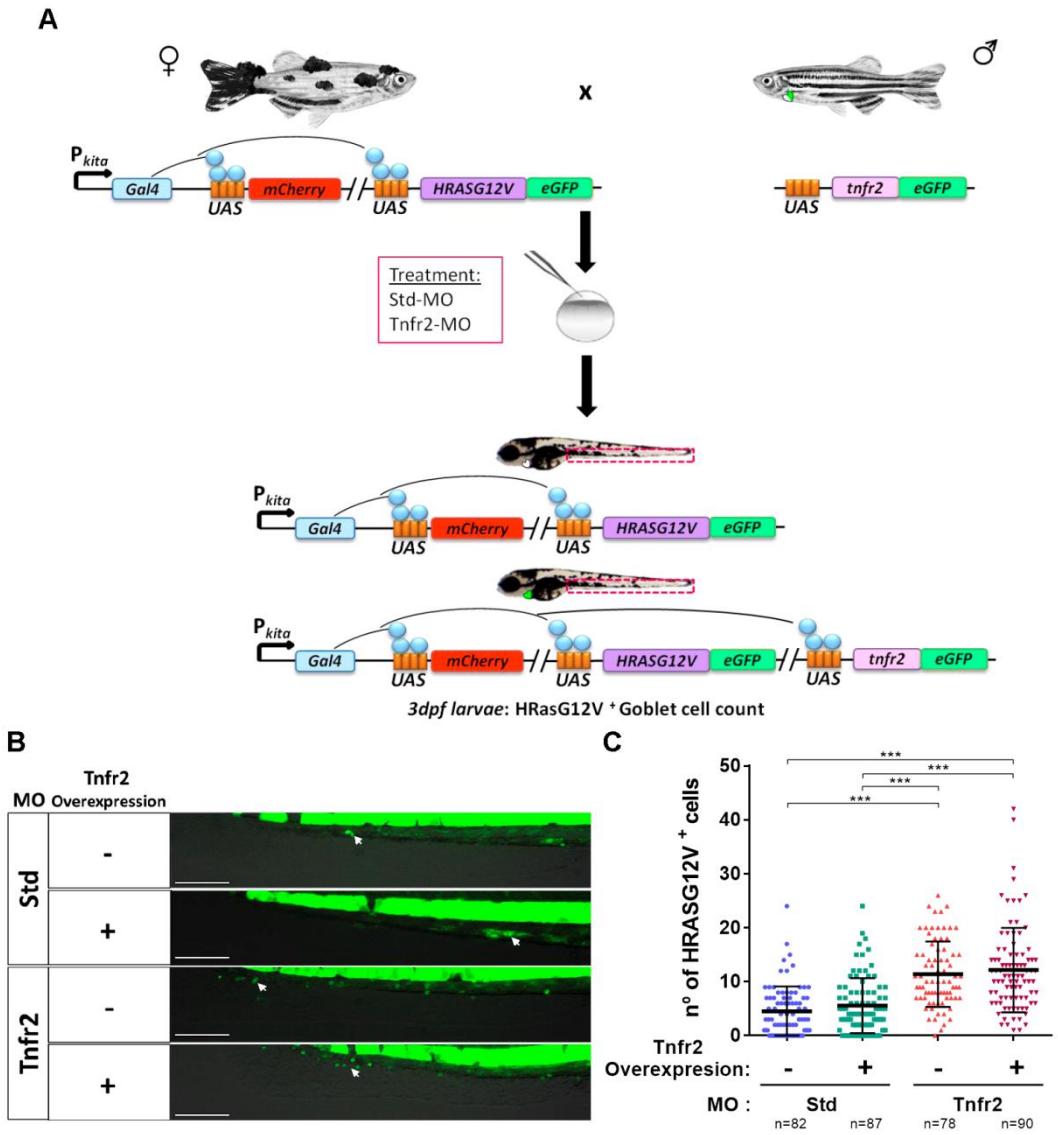

Figure 3: **Tnfr2 expression rescue assay for HRASG12V^{+} cells proliferation**. **A)** Schematic diagram of the generation of melanoma *driver line kita:Gal4*;HRasG12V and injection strategy used to transiently inhibit Tnfr1, Tnfr2 or Tnfr1+Tnfr2 in melanocytes. Zebrafish *kita:Gal4;UAS:eGFP-H-RAS_G12V* one-cell embryos were injected with standard control (Std), Tnfr1, Tnfr2, or Tnfr1+Tnfr2 morpholinos (MO). **B)** Representative images, bright field and green channels, of the indicated region of morphants at 3 dpf showing the HRASG12V^{+} cell differences. Scale bars: 250 µm. **C)** Quantification of the number of HRASG12V^{+} cell in control, Tnfr1-, Tnfr2- and Tnfr1+Tnfr2-deficient larvae. The mean ± S.E.M. for each group is shown. *p<0.05; **p<0.01; ***p<0.001 according to ANOVA and Tukey's Multiple Comparison post-Test.

On one hand, rescue of Tnfr2 expression in transformed melanocytes by using the transgenic line *(UAS:tnfr2)*^{ums1} failed to reverse the increased number of HRASG12V^{+} cells (**Figure 3B** and **C**), indicating that Tnfr2 signaling in stromal cells was responsible for the increased proliferation of oncogenically transformed cells.

It has been reported that while most neutrophils are located in the caudal hematopoietic tissue (CHT) in wild type zebrafish larvae by 72 hpf, their Tnfr2-deficient counterparts showed strong infiltration of neutrophils into the skin (Candel, de Oliveira et al. 2014). In order to elucidate if neutrophils were involved in the effect of Tnfr2 deficiency on HRASG12V^{+} cell abundance, a new approach was designed *in vivo* (**Figure 4**). In particular the double transgenic line *tg(kita:GalTA4,UAS:mCherry)*^{hzm1};*(mpx:*eGFP*),* previously developed in the hosting laboratoty, was cross with the *tg(UAS:TNFR2)*^{ums1} line to visualize neutrophils by analyzing green fluorescence in transgenic larvae to evaluate a possible contact with oncogenic transformed cells.

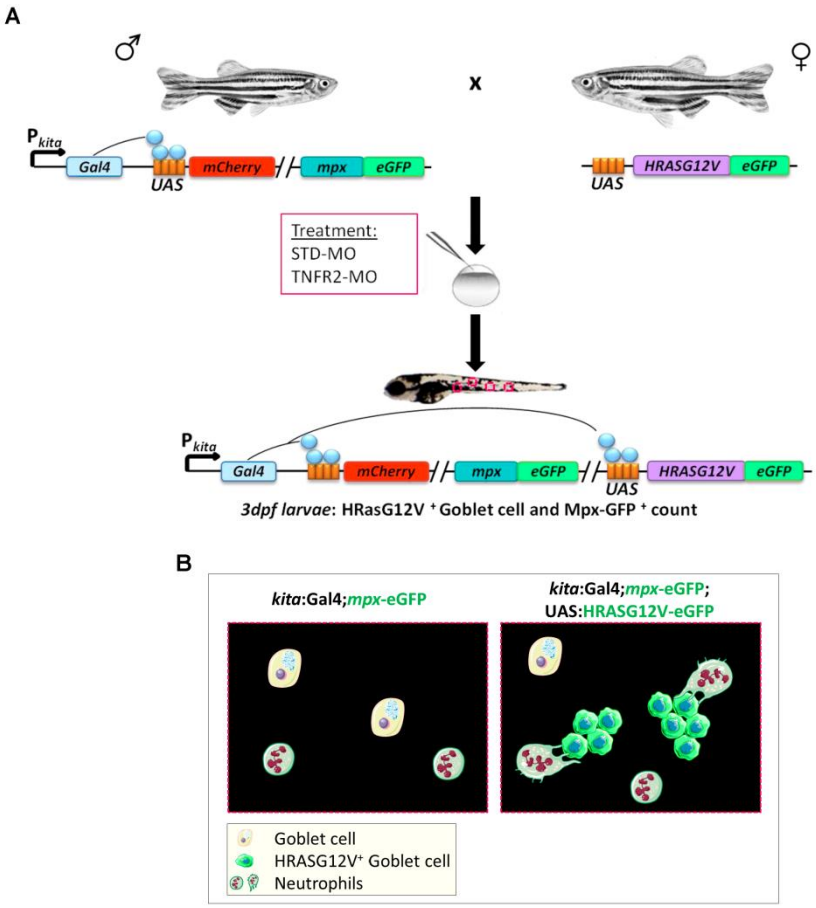

Figure 4: **Generation of an *in vivo* zebrafish model to study the cross-talk between oncogenically transformed cells and neutrophils from the tumor microenvironment**. **A)** Schematic diagram of the generation of melanoma driverline *kita:Gal4*;HRASG12V which shows neutrophils in green (*mpx*:eGFP) and injection strategy used to transiently inhibit Tnfr2 in 3dpf larvae. **B)** Schematic diagram of the expected results of wild type and Tnfr2-deficient larvae.

The cell contact ratio was quantified as the mean number of contacts between HRASG12V^{+} cells and neutrophils (**Figure 5A**). **Figure 5B** shows that neutrophils from Tnfr2-deficient larvae interacted more with transformed cells than their control counterparts. Furthermore, while the number of neutrophils was the same in both groups, the number of HRASG12V^{+} cells was significantly higher in Tnr2-deficient larvae (**Figure 5C**).

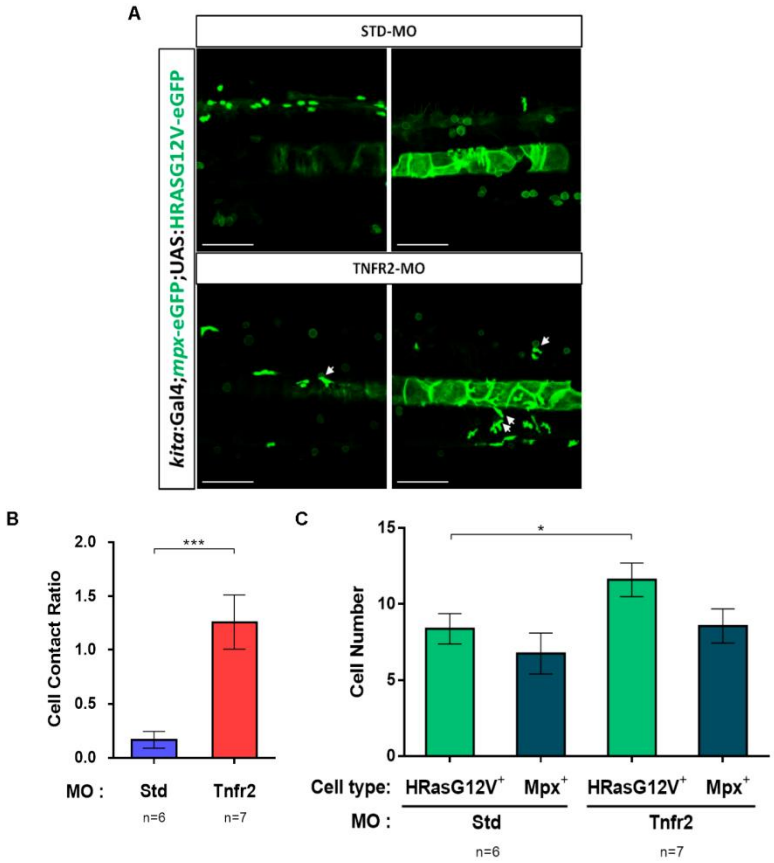

Figure 5: **Tnfr2-deficient larvae neutrophils interaction with HRASG12V^{+}cells**. **A)** Representative images of 2 different ROIs of Tnfr2 morphants and controls at 3 dpf showing the differences between HRASG12V^{+} cell and neutrophils, both characterized by green fluorescence. Scale bars: 250 µm. **B)** Quantification of the cell contact ratio in control and Tnfr2 deficient larvae. Zebrafish one-cell *kita:Gal4;UAS:eGFP-H-RAS_G12V;mpx:gfp* embryos were injected with standard control (Std) and Tnfr2 morpholinos (MO). The cell contact ratio was quantified as the mean number of contacts between HRASG12V^{+} cells and neutrophils assigning 1 for the contact and 0 for no contact. 4 random ROIs were evaluated per larvae. The media was obtained analyzing from 3 to 5 larvae for each morphant group, performing 3 experiments. The mean ± S.E.M. for each group is shown. *p<0.05; ***p<0.001 according to ANOVA and Tukey's Multiple Comparison post-Test.

This data suggest that skin inflammation promoted by Tnfr2 deficiency may facilitate oncogenic transformation and/or tumor cell proliferation, identifying a key role of Tnfr2 signaling in the cross-talk between oncogenically transformed cells and the tumor microenvironment *in vivo*.

3. *SPINT1* genetic alterations are associated with poor prognosis of SKCM patients

To study the impact of SPINT1 in promoting SKCM progression and aggressiveness, an *in silico* analysis of human SKCM samples of the TCGA cohort was performed. This analysis disclosed that genetic alterations occurred in 10% of melanoma patients; a relevant percentage comparing with major melanoma driven oncogenes and tumor suppressors (**Figure 6A**).

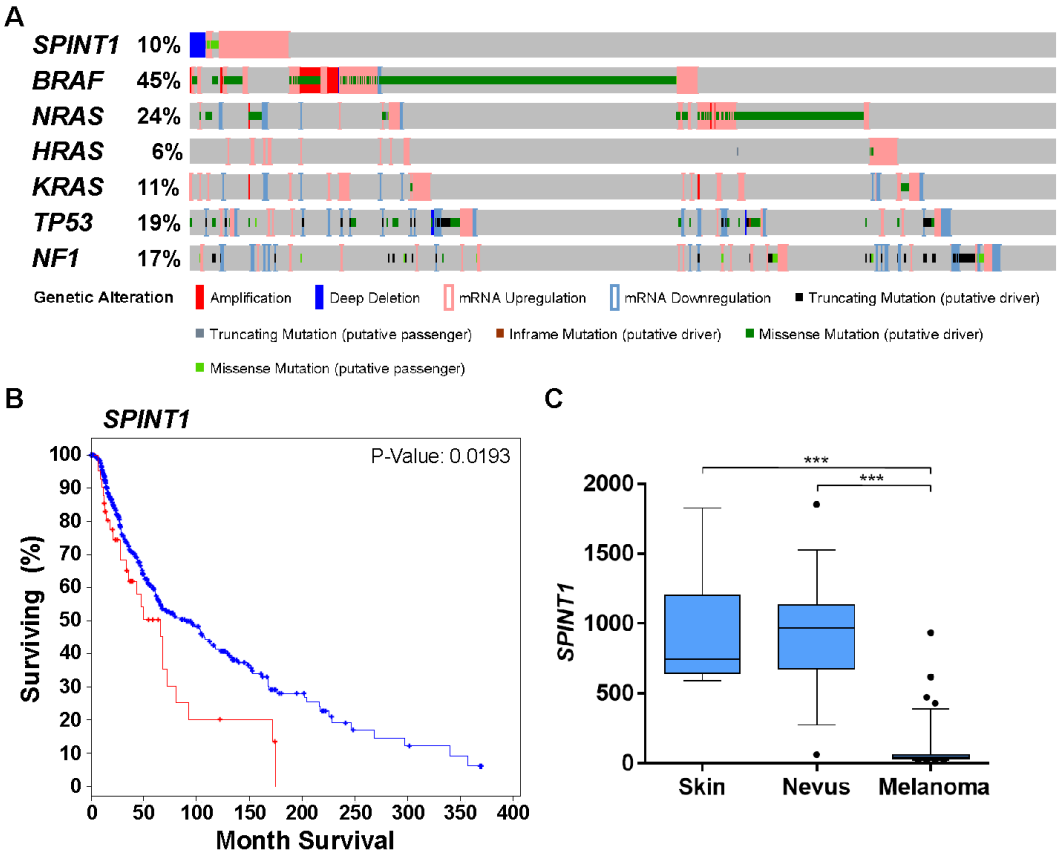

Figure 6: SPINT1 genetic alterations are associated with poor prognosis of SKCM patients.A) Percentage of genetic alterations in oncogenic genes and *SPINT1* in SCKM patients of the TCGA cohort (n=479). **B)**Survival curve of patients with genetic alteration (increased mRNA level, missense mutations and deep deletions, red line, 10% prevalence, n=46) vs. wild type *SPINT1* (blue line, n=433) of SCKM of the TCGA cohort. Kaplan–Meier Gehan-Breslow-Wilcoxon and nonparametric Log-rank Test. Hazard ratio= 1.625; 95% CI of ratio= 1.099-3.033. **C)**Genetic expression of *SPINT1* in human samples from normal skin, nevus and malignant melanoma from GEO dataset GDS1375 and 202826_at probe (number of samples: Skin n=7, Nevus n=18, Melanoma n=45). ***p< 0.0001 according to ANOVA and Tukey's Multiple Comparison Test.

Among these genetic alterations, an increased mRNA level was the most prevalent alteration (7%), while 1.9% missense mutations of unknown significance and 1.9% deep deletions were also observed. Furthermore, a survival curve of patients with altered vs. wild type *SPINT1* of SKCM was analyzed (**Figure 6B**) revealing that the presence of genetic alteration in this gene, significantly correlated with poor patient prognosis and *SPINT1* expression was significantly inhibited human SKCM comparing with nevus or normal skin (**Figure 6C**). In addition, the analysis of patient survival with increased SPINT1 transcript levels also revealed their poor prognosis but, unfortunately, the analysis of deep deletions and missense mutations separately gave no statistical significance because the number of patients for each condition was very low (data not shown).The inhibition of *SPINT1*expression during melanoma progression, together with its correlation with a poor prognosis, suggests an important role in promoting melanoma aggressiveness.

We next performed a GO enrichment analysis of biological process (**Figure 7**)analyzingthe differentially expressed (DE) genes in SKCM samples of the TCGA cohort with missensemutation or copy number alteration of *SPINT1*. The results showed that regulation of immune system, inflammatory response, cell cycle, cell adhesion, and extracellular matrix organization represent key pathways significantly affected in human SKCM samples with genetic alteration in *SPINT1*. Collectively, these results suggest that both high and low levels of SPINT1 result in an unbalanced crosstalk between tumor cells and their microenvironment promoting higher aggressiveness.

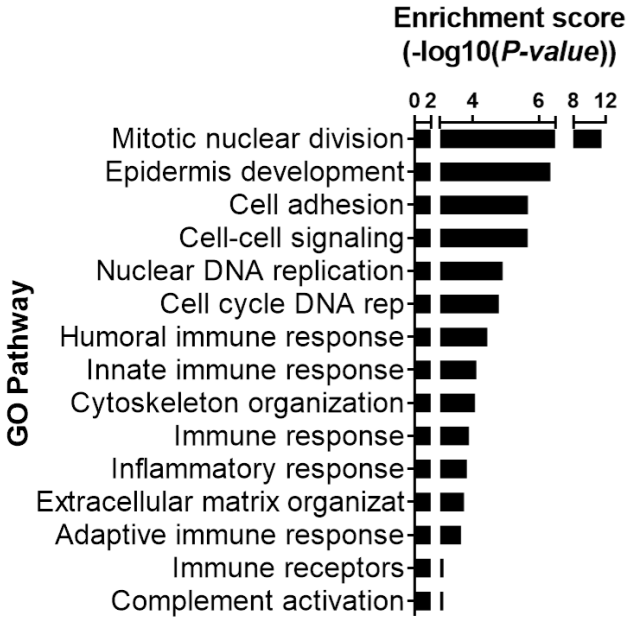

Figure 7:Enrichment analysis of GO biological process. Representation of the most significant GO biological process altered when *SPINT1* is affected by missense mutations or copy-number alterations. Analysis Type: PANTHER Overrepresentation Test (Released 05/12/2017), Test Type: FISHER.

4. *SPINT1* regulates inflammatory cell infiltration in human skin cutaneous melanoma

The tumor microenvironment contains diverse leukocyte populations, including neutrophils, eosinophils, dendritic cells, macrophages, mast cells and lymphocytes (Coussens and Werb 2002). It is known that tumor-associated macrophages (TAM),are able to interact with tumor cells to promote cancer progression(Zaidi, Davis et al. 2011, Colegio, Chu et al. 2014, Gordon, Maute et al. 2017). Melanomas release molecules that can recruit macrophages to melanoma sites (Wang, Yang et al. 2017). This reason, together with the crucial role reveled for *SPINT1* in melanoma aggressiveness and activation of the immune response, previously described, led us to study inflammatory cells infiltration in human SCKM samples. As shown in **Figure 8**, the number of TAM in human SCKM samples correlated with the mRNA levels of *SPINT1* in metastatic melanoma. However, the number of tumor-associated neutrophils (TAN) was independent of SPINT1 levels in both primary and metastatic melanoma. These data further confirmed the role of SPINT1 in the regulation of the crosstalk between tumor cells and inflammatory cells in human SKCM.

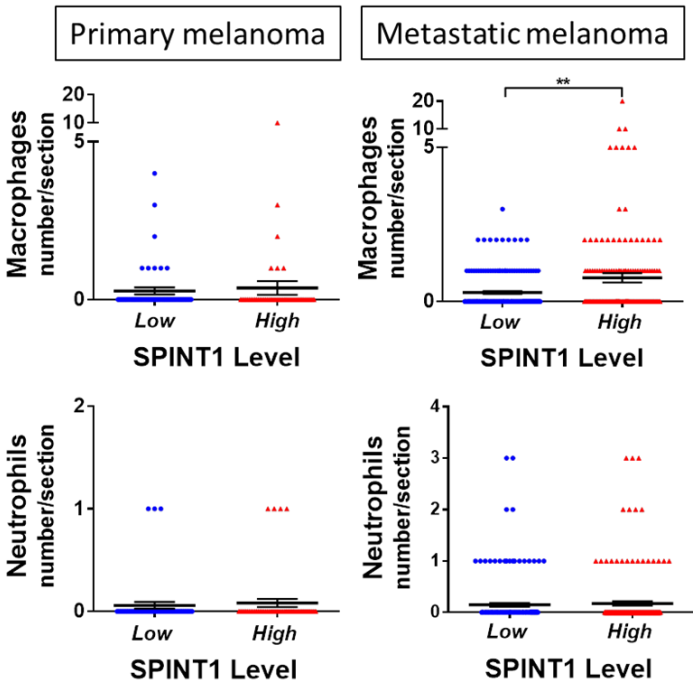

Figure 8: **Inflammatory cells infiltration in human melanoma samples**. Number of infiltrated macrophages and neutrophils in human SKCM samples of the TCGA cohort (n=479) analyzed by histology. The number of infiltrated cells in SKCM samples with low (blue) or high (red) *SPINT1* mRNA levels according to the median. The mean ± S.E.M. for each group is shown. *p<0.05; **p<0.01; t- Test.

5. The expression of SPINT1, positively correlates with both inflammation and macrophage markers in human SCKM biopsies

In order to further understand the role of SPINT1 in SKCM, the RNA Seq database of the large TCGA cohort of SCKM was analyzed. *SOX10*, *TYR*, *DCT* have been shown to be important in melanocyte development (Ronnstrand and Phung 2013, Ordonez 2014) and in particular *SOX10* is a recognized biomarker for the diagnosis of SKCM (Ronnstrand and Phung 2013). It was found that *SPINT1* positively correlated with those of *SOX10* and TYR, while a negative correlation was found between the expression of *SPINT1* and *DCT* (**Figure 9A**). Furthermore, the EMT markers *ZEB* and *TWIST* were analyzed. Both are transcription factors that plays an important role in embryonic development and ZEB protein likely plays a role in transcriptional repression of interleukin-2 (Wang, Lee et al. 2009, Omilusik, Best et al. 2015). The expression of *ZEB1*, *ZEB2* and *TWIST1*, but not *TWIST2*, negatively correlated with that of *SPINT1* (**Figure 9B**) in SKCM biopsies. Collectively, these data suggest that *SPINT1* regulates melanoma cell differentiation and aggressiveness.

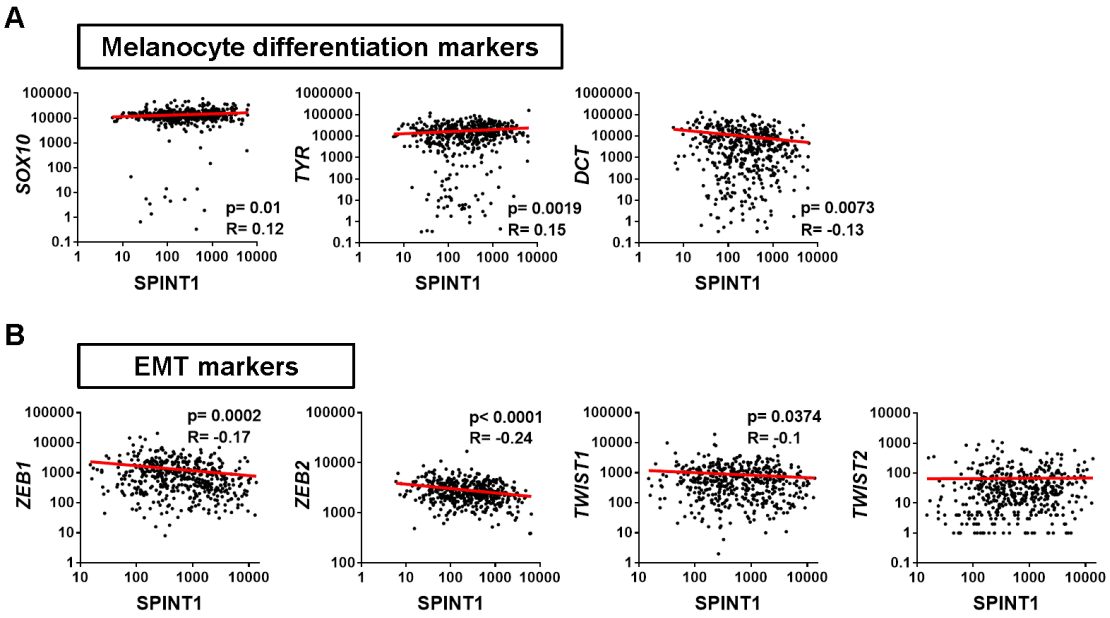

Figure 9: ***SPINT1* expression correlates with aggressiveness marker expression in human SKCM biopsies. A)**Correlation of *SPINT1* gene expression with those of the melanocyte differentiation markers *SOX10, TYR* and *DCT* and **B)**the EMT markers *ZEB1, ZEB2, TWIST1* and *TWIST2* in human SKCM biopsies of the TCGA cohort (n=479). The statistical significance of the correlation was determined using Pearson's correlation coefficient. A linear regression-fitting curve in red is also shown.

SKCM cells release several cytokines and chemokines that recruit and polarize macrophages (Wang, Yang et al. 2017). Therefore, several inflammation markers were analyzed and only the expression of the genes encoding the receptor of the pro-inflammatory cytokine TNFα (TNFR1) and, the receptor of the pro-inflammatory chemokine interleukin 8 (CXCR2), positively correlated with *SPINT1* levels (**Figure 10A**). Notably, the macrophage marker *MFAP4* also positively correlated with *SPINT1* expression (**Figure 10B**). However, the M2 polarization marker *CD163* and several interferon-stimulated genes (ISGs) were all unaffected by *SPINT1* levels (**Figure 10B-C**). These results further suggest that SPINT1 may regulate macrophages infiltration.

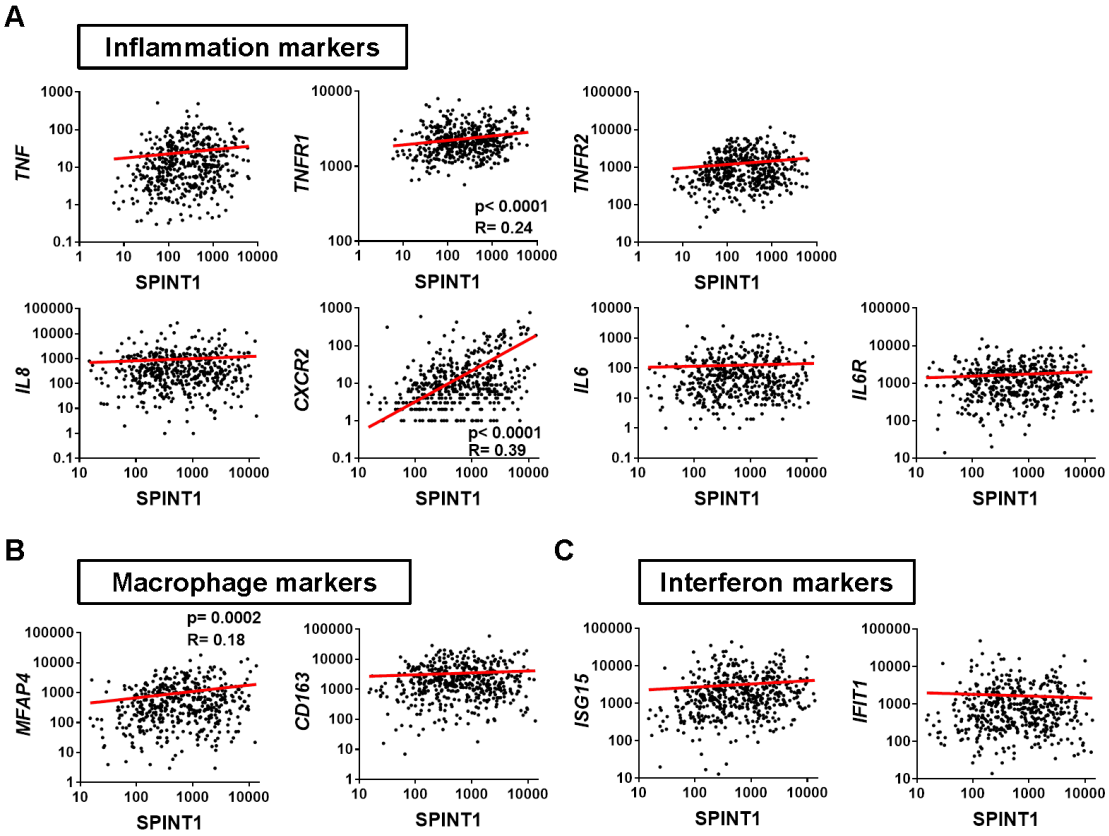

Figure 10: The expression of *SPINT1* inflammation and macrophage markers positively correlates in human SKCM biopsies. A)Correlation of *SPINT1* gene expression with the inflammation markers *TNFA*, *TNFR1*, *TNFR2*, *IL8(CXCL8)*, *CXCR2*, *IL6*, *IL6R* in human melanoma biopsies. **B)** Correlation of *SPINT1* gene expression with the macrophage markers *MFAP4,* and *CD163* in human SKCM biopsies. **C)** Correlation of *SPINT1* gene expression with the interferon markers *ISG15* and *IFIT1* in human SKCM biopsies of the TCGA cohort (n=479). The statistical significance of the correlation was determined using Pearson's correlation coefficient. A linear regression-fitting curve in red is also shown.

6. Melanoma development in a zebrafish model of chronic skin inflammation

Given the strong correlation between alterations of *SPINT1* levels with the progression of SKCM and the crosstalk with the tumor immune microenvironment, we generated a zebrafish line, which spontaneously develop SKCM in a chronic skin inflamed condition. To accomplish this, we outcrossed the zebrafish line *tg*(*kita:Gal4;HRAS-G12V*)(Santoriello, Gennaro et al. 2010), which expresses the human oncogene *HRAS-G12V* in melanocytes by using the specific melanocyte promoter *kita* and the UAS:Gal4 system (Abe, Suster et al. 2011) for the spontaneous SKCM generation (Santoriello, Gennaro et al. 2010), with the zebrafish mutant line *spint1a*$^{hi2217Tg/hi2217Tg}$ *(Mathias, Dodd et al. 2007)*, which presents chronic skin inflammation.

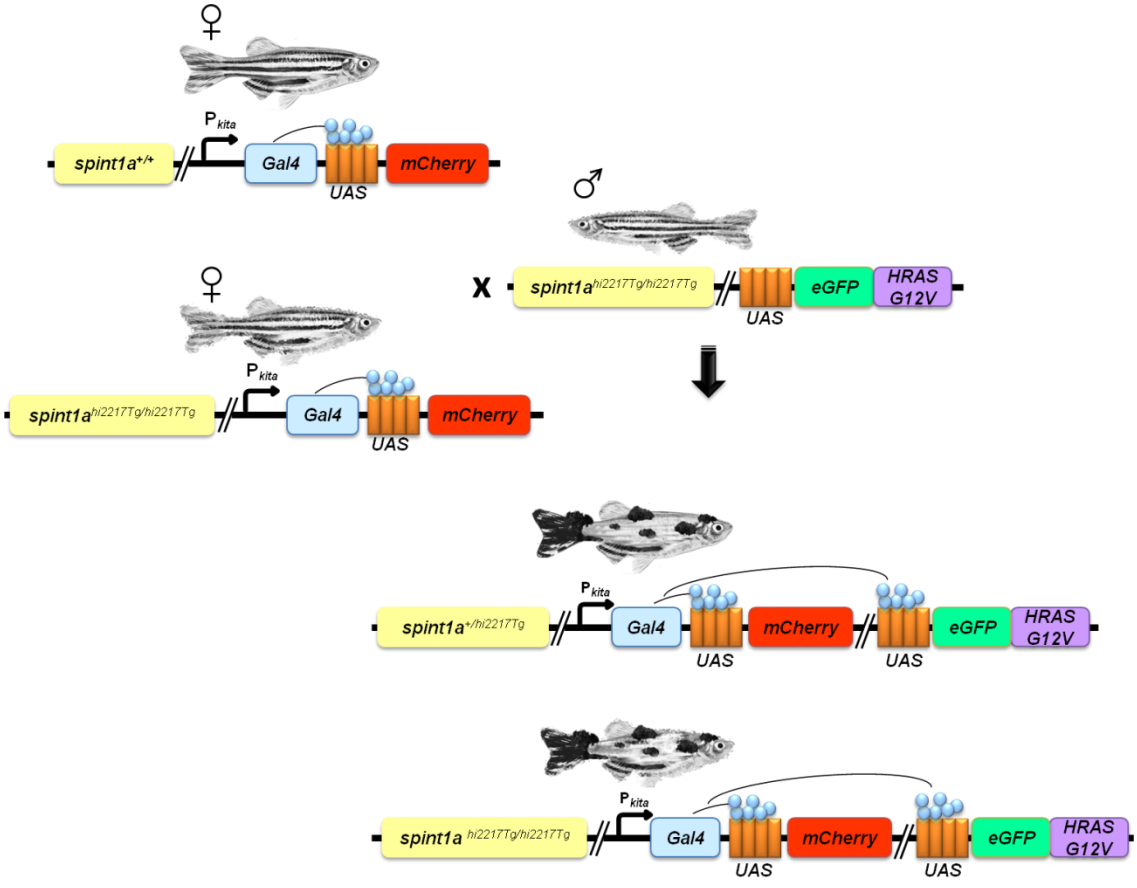

Figure 11: **Schematic diagram of the generation of melanoma-prone zebrafish line with chronic skin inflammation induced by Spint1a deficiency.** Transgenic zebrafish *Et(kita:GalTA4,UAS:mCherry)*^{hzm1} was outcrossed with *spint1a*$^{hi2217Tg/hi2217Tg}$ to get the *spint1a*$^{+/hi2217Tg}$ and *spint1a*$^{hi2217Tg/hi2217Tg}$. Then, both were crossed with *Tg(UAS:eGFP-H-RAS_G12V)*^{io6} lineto develop melanomas spontaneously in a non-inflamed microenvironment, used as a control group, and *spint1a*$^{hi2217Tg/hi2217Tg}$; *kita:Gal4*;HRAS-*G12V* fish to develop melanomas in a chronic skin inflammation microenvironment.

In that way, we got the *spint1a*$^{+/hi2217Tg}$; *kita:Gal4*;HRAS-*G12V* fish that develop melanomas in a non-inflamed microenvironment and *spint1a*$^{hi2217Tg/hi2217Tg}$; *kita:Gal4*;HRAS-*G12V* fish that produce melanomas in a chronic skin inflamed microenvironment (**Figure 11**).

7. Chronic skin inflammation increases oncogenic transformation

As previously discussed, inflammation can play a key role in cancer, from initiation of the transformed phenotype to metastatic spread. Therefore, we first studied whether chronic skin inflammation induced by spint1a deficiency is able to enhance early oncogenic transformation by using the *kita:Gal4;HRAS-G12V*. Thus, we quantified by fluorescence microscopy the number of early oncogenically transformed goblet cells, which also expressed GPF under the control of *kita* promoter as well as melanocytes (Feng, Santoriello et al. 2010), in *spint1a*-deficient larvae and their wild type siblings **(Figure12A)**. The results showed that *spint1a* deficiency resulted in increased number of HRAS-G12V^{+} cells **(Figure12B)**, suggesting that chronic inflammation increases oncogenic transformation.

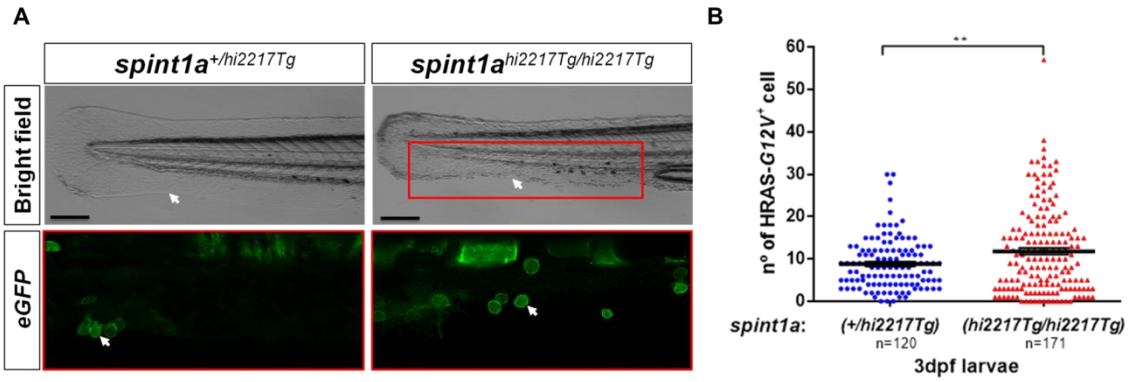

Figure 12: Number of early oncogenically transformed HRAS-G12V^{+}cells in larvae with chronic skin inflammation. A) Representative images showing HRAS-G12V^{+} cells (white arrows) in the boxed area, and Spint1a-deficient (*spint1a*$^{hi2217Tg/hi2217Tg}$) larvae and control (*spint1a*$^{+/hi2217Tg}$) siblings at 3 dpf. Note the morphological alterations observed in the inflamed skin of the mutants (white arrows). Scale bar 250µm. **B)** Quantification of the number of HRAS-G12V^{+} cells in the indicated area (red box in panel A) from larvae of both groups. Each point on the scatter plot represents one larva and the mean ± SEM is shown. ** $p<0.05$ according to an unpaired Student *t* test with Welch's correction.

8. Inflammation accelerates the onset of melanoma

To determine if the enhanced Spint1a deficiency-driven oncogenic transformation was also able to promote SKCM aggressiveness, SKCM development in *spint1a*$^{hi2217Tg/hi2217Tg}$ fish were compared with wild type (*spint1a*$^{+/hi2217Tg}$) from the end of metamorphosis stage (between 28-30 dpf) to 120 dpf (adult stage) (**Figure 13**). **Figure 13A** shows an example of an adult individual of each genotype. The ocular inspection revealed that fish of both genotypes used to develop large nodular melanomas in the tail area but, in the case of *spint1a* deficiency the tumors were more extended. **Figure 13B** exhibit a detail of that nodular melanoma in the tail area developed in both, wild type and *spint1a* mutant where the phenotypic tumor differences are shown.

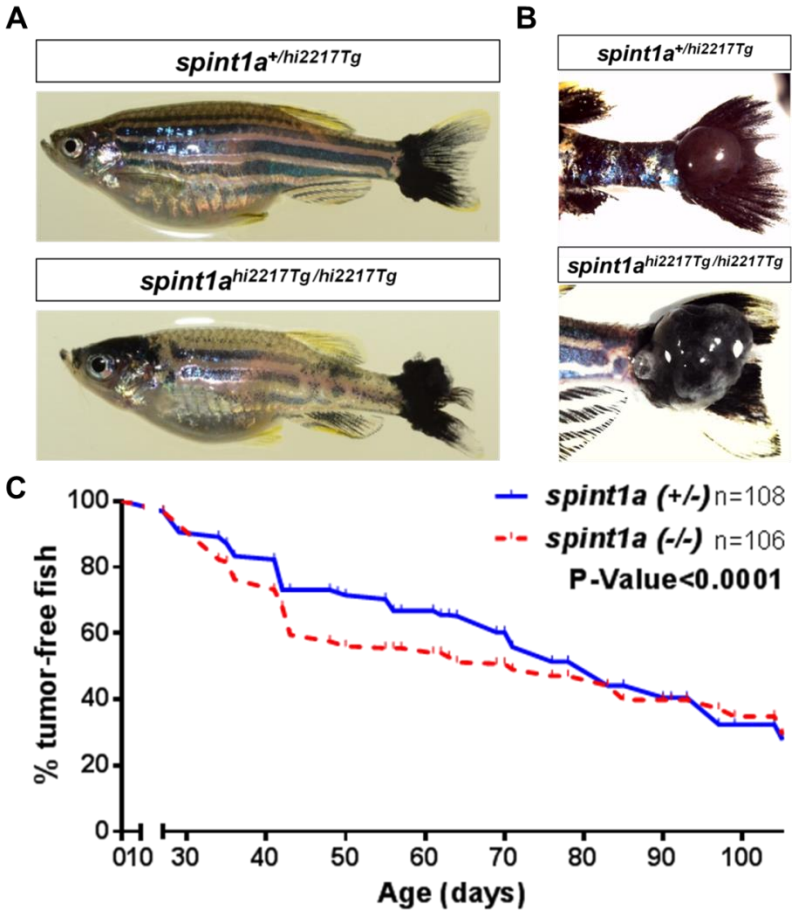

Figure 13: Impact of Spint1a deficiency on SKCM appearance in zebrafish. Representative images of SKCM developed in *spint1a*$^{hi2217Tg/hi2217Tg}$ and *spint1a*$^{+/hi2217Tg}$*kita:Gal4;HRAS-G12V* of whole adult fish, **A)** and of nodular tail tumors, **B)**. **C)** Kaplan-Meier curve showing the percentage of SKCM-free fish in control (blue) and Spint1a-deficient adult fish(red). $p < 0.0001$ according to a Log rank Mantel-Cox test; Hazard ratio= 0.7962; 95% CI of ratio= 0.6834-0.9056.

The monitoring of tumor appearance in both zebrafish lines resulted in a Kaplan-Meier curve which shows a significant increased incidence of melanoma in the spint1a-deficent fish. In this spint1a-deficent fish, SKCM were developed in more than 50% of cases at 50-60 dpf compared with the wild type siblings which reached only 30% at this age (**Figure 13C**). These data suggest that Spint1a deficiency increases oncogenic transformation and accelerates SKCM onset *in vivo*.

9. Spint1a deficiency is required at cell autonomous and non-autonomous levels to enhance SKCM cell dissemination in a zebrafish larval allotrasplantation model

To assess the in vivo role of Spint1a deficiency in SKCM invasiveness, SKCM tumors from *spint1a*$^{hi2217Tg/hi2217Tg}$; *kita:Gal4;HRAS-G12V* and *spint1a*$^{+/hi2217Tg}$; *kita:Gal4;HRAS-G12V* were disaggregated, stained and transplanted into the yolk sac of 2 dpfCasper zebrafish larvae (**Figure 14**).

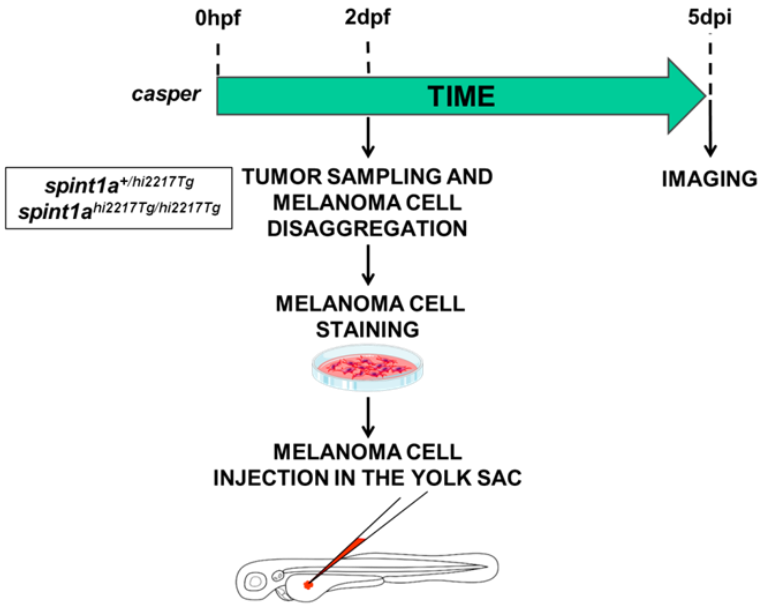

Figure 14: Experimental design of wild type and *Spint1a*-deficient SKCM allotransplants in wild type larvae. Nodular SKCMs from *spint1a*$^{hi2217Tg/hi2217Tg}$; *kita:Gal4;HRAS-G12V* and *spint1a*$^{+/hi2217Tg}$; *kita:Gal4;HRAS-G12V*zebrafish were sampled, disaggregated, stained and injected into the yolk sac of 2 dpf Casper larvae. Imaging to analyse the SKCM invasion was performed at 5 dpi as indicated in the Materials and Methods section.

The results showed that Spint1adeficiency inSKCM cells enhanced the dissemination of SKCM, assayed as the percentage of invaded larvae and the number of foci per larva, compared to wild type SKCM cells-injected larvae (**Figure 15**).

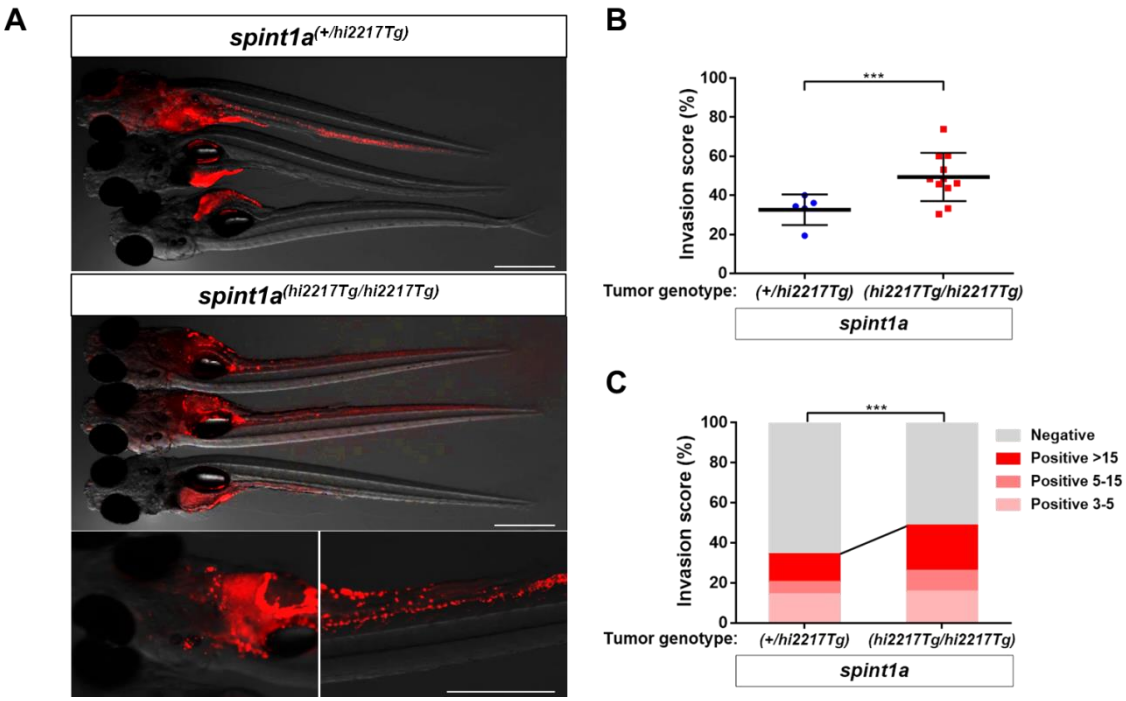

Figure 15: Cell autonomous Spint1a deficiency enhances dissemination of SKCM cells in a larval zebrafish allotrasplant model. A) Representative images (overlay of bright field and red channels) of *spint1a*$^{hi2217Tg/hi2217Tg}$ and *spint1a*$^{+/hi2217Tg}$SKCM cells invasion in Casper zebrafish larvae at 5 dpi. Magnification bars: 500 µm. **B)** Percentage of invaded larvae for both tumor genotypes. Each dot represents the percentage of invaded larvae (n=75-100) injected with a single tumor sample and the mean ± SEM is also shown. ***p< 0.0001 according to unpaired Student t test. **C)** Number of tumor foci per larva. ***p< 0.0001 according to Chi-square Tests.

We next examined whether Spint1a deficiency in the stromal, i.e. in a non-autonomous manner, also promoted SKCM aggressiveness. Therefore, Spint1a wild typeSKCMs, which developed in absence of a chronic skin inflammation, was transplanted into the yolk sac of Spint1a-deficient larvae and their wild type siblings (**Figure 16**). It was found that Spint1adeficiency in tumor microenvironment also promoted a significantly higher dissemination of SKCM, assayed as the percentage of invaded larvae and the number of foci per larva, compared to control tumor microenvironments (**Figure 17**).

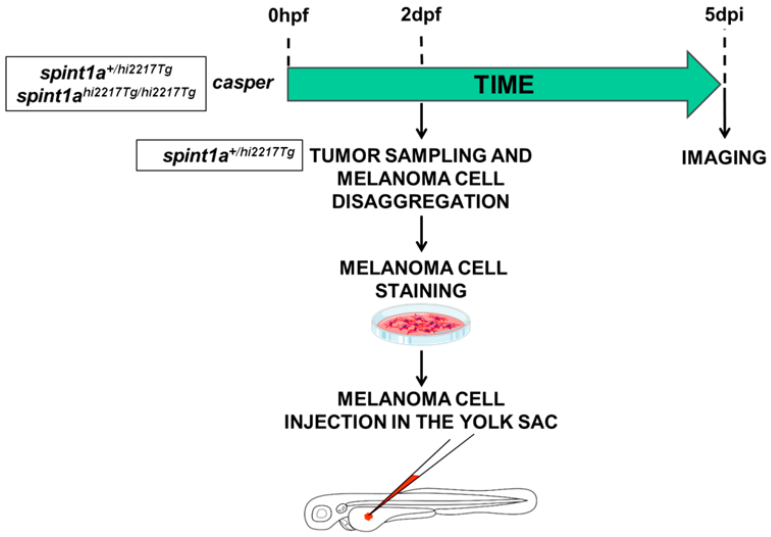

Figure 16: **Experimental design of SKCM allotransplants in wild type and Spint1a-deficient zebrafish larvae.** Nodular SKCMs from *spint1a*$^{+/hi2217Tg}$; *kita:Gal4;HRAS-G12V*zebrafish were sampled, disaggregated, stained and injected into the yolk sac of 2 dpf *spint1a*$^{+/hi2217Tg}$ and *spint1a* $^{hi2217Tg\ /hi2217Tg}$ Casper larvae. Imaging to analyze SKCM invasion was performed at 5 dpi as indicated in the Materials and Methods

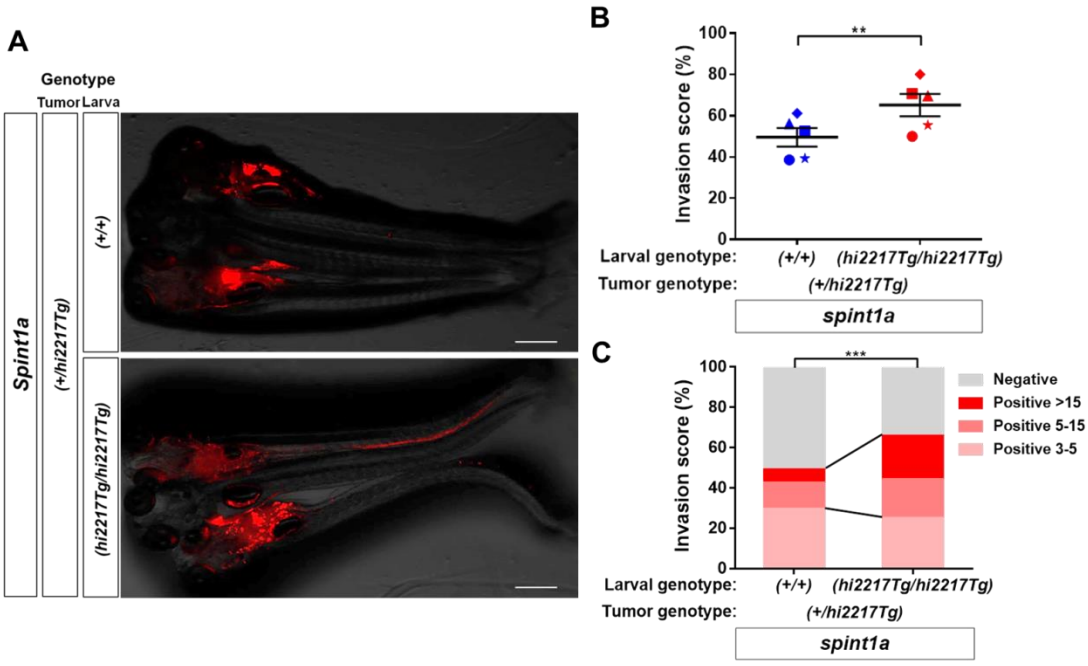

Figure 17: Spint1a deficiency in stromal cells enhances cell dissemination of SKCM in a larval zebrafish allotrasplant model.A) Representative images (overlay of bright field and red channels) of *spint1a* $^{+/hi2217Tg}$ SKCM cells invasion in *spint1a*$^{hi2217Tg/hi2217Tg}$ and *spint1a*$^{+/hi2217Tg}$larvae at 5 dpi. Bars: 500 µm. **B)** Percentage of invaded larvae of both genotypes. Each dot represents the percentage of invaded larvae (n=75-100)injected with the same tumor sample (n=5 SKCM tumors)and the mean ± SEM is also shown.**p<0.01according to unpaired Student t test.**C)**Number of tumor foci per larva. ***p< 0.0001 according to Chi-square Tests.

To further confirm a role of Spint1a in both SKCM and tumor microenvironment cells, we next sorted tumor ($eGFP^+$) and stromal ($eGFP^-$) cells from tumor samples obtained from both genotypes and the cellular types were then mixed in equal proportions in the 4 possible combinations (**Figure 18**). It was found that all tumor samples were constituted by ~90% of GFP-positive tumor cells and ~10% of stromal cells (data not shown).

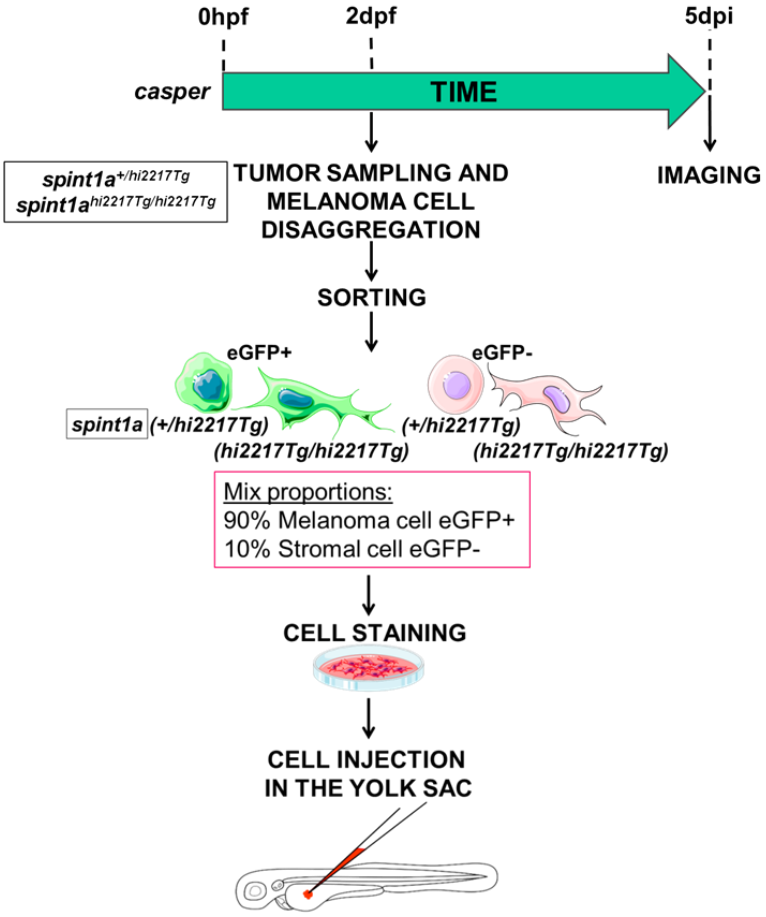

Figure 18: Allotransplant experimental design where combinations of *Spint1a*-deficient tumor and stromal cells from SKCMs were mixed with wild type tumor and stromal cells. SKCMs from *spint1a*$^{hi2217Tg/hi2217Tg}$ and *spint1a*$^{+/hi2217Tg}$zebrafish were sampled, disaggregated and the resulting cell suspensions sorted in tumor ($eGFP^+$) and stromal ($eGFP^-$). The 4 possible cell combinations were obtained maintaining the original *in vivo* ratio between tumor and stromal cells, stained and injected in the yolk sac of 2 dpf Casper larvae. Imaging to analyze SKCM invasion was performed at 5 dpi as indicated in the Materials and Methods section.

As previously observed, Spint1a-deficient tumor cells showed an increase of invasiveness when compared to wild type ones. Notably, not only Spint1a-deficiency in tumor cells but also Spint1a-deficiency in stromal cells was able to increase significantly SKCM cell invasion, but is the combination of both Spint1a-deficent cells which enhance the agressiveness (**Figure 19**).

Collectively, these results suggest that Spint1a deficiency enhances SKCM invasion by both cell autonomous and non-autonomous mechanisms.

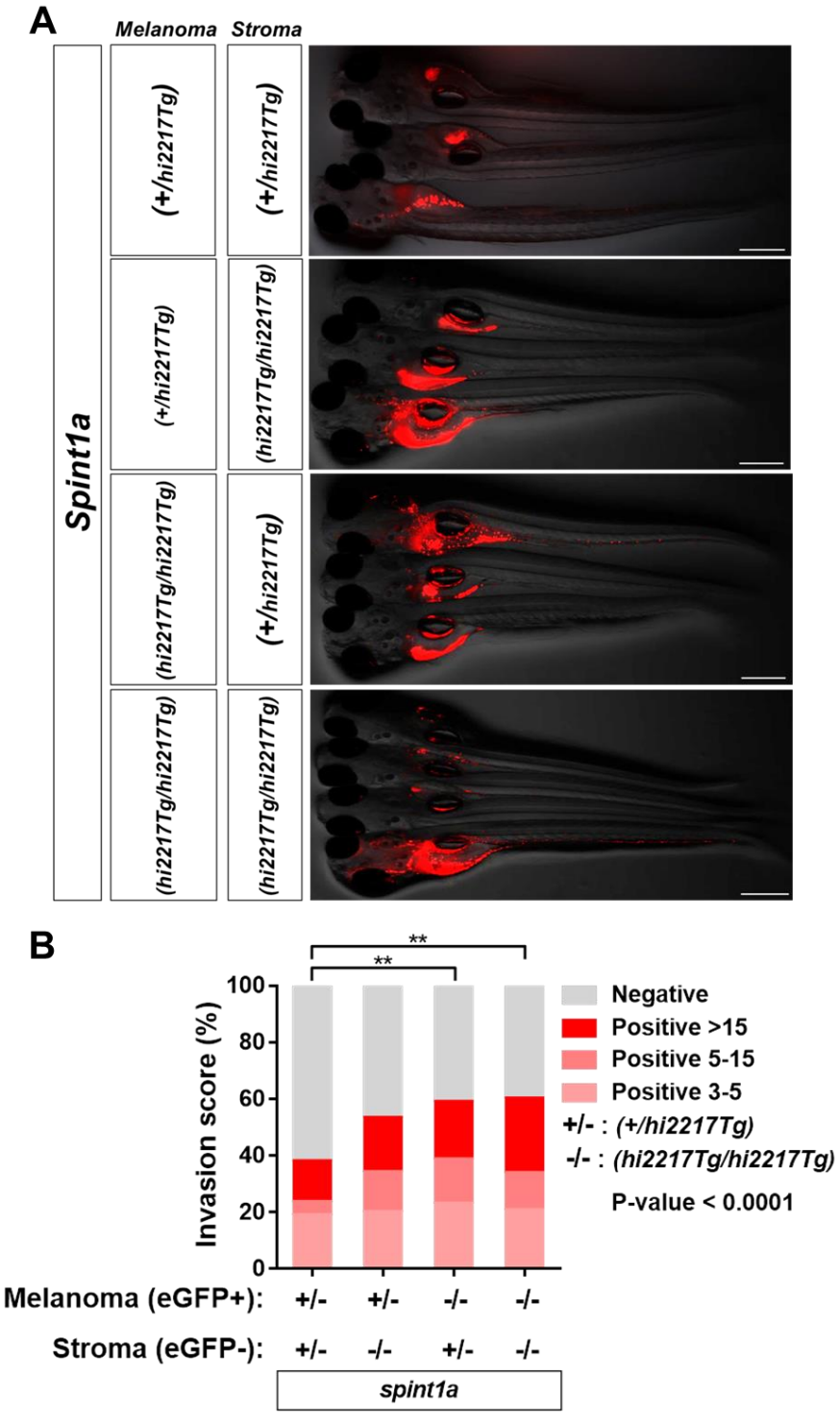

Figure 19: Spint1a deficiency in tumor cells enhances SKCM dissemination in zebrafish larval model independently from Spint1a status of stromal cells. A) Representative images (bright field and red fluorescence channel) of the invasion of the 4 possible cell combination between *spint1a*$^{hi2217Tg/hi2217Tg}$ and *spint1a*$^{+/hi2217Tg}$ tumor and stromal cells in wild type recipient larvae at 5 dpi. Magnification bars: 500 µm. **B)** Number of tumor foci per larva (n=5 SKCM tumors). **p< 0.01 according to Chi-square Tests.

10. Spint1a-deficient SKCMs showed enhanced aggressiveness in adult zebrafish allotransplantation model

The results obtained in allotransplantation assay in larvae prompted us to analyze the role of Spint1a in SKCM aggressiveness in adult Casper zebrafish to directly visualize tumor cell proliferation and dissemination *in vivo* over time. *spint1a*$^{hi2217Tg/hi2217Tg}$ and *spint1a*$^{+/hi2217Tg}$ SKCMs were sampled, disaggregated and subcutaneously injected (300,000 cells) in the dorsal sinus of adult Casper recipients previously irradiated with 30 Gy (**Figure 20**).

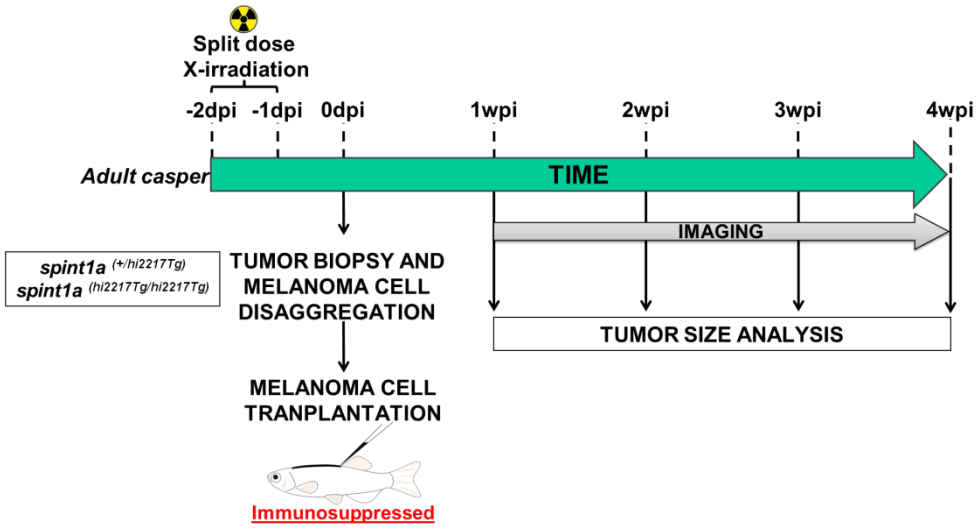

Figure 20: Experimental workflow of adult allotransplantation assays. SKCMs from *spint1a*$^{hi2217Tg/hi2217Tg}$ and *spint1a*$^{+/hi2217Tg}$ zebrafish were sampled, disaggregated and subcutaneously injected (300,000 cells) in pre-irradiated adult Casper zebrafish. Images to analyze tumor size were taken weekly during the following 4 weeks after transplantation and analyzed as indicated in the Materials and Methods section.

Tumor engraftment was visible as early as 7 days post-transplantation in both genotypes. While 90% engraftment was obtained with wild type SKCM cells, Spint1a-deficient cells showed a significant enhancement of tumor engraftment rate, around 95% (**Figure 21A)**. In addition, adult zebrafish recipient transplanted with Spint1a-deficient SKCMs developed tumors with a significant higher growth rate than those injected with wild type SKCMs **(Figure 21B)**. Notably, Spint1a-deficient SKCMs cells were able to invade the entire dorsal area, part of ventral cavity and the dorsal fin **(Figure 21B)**.

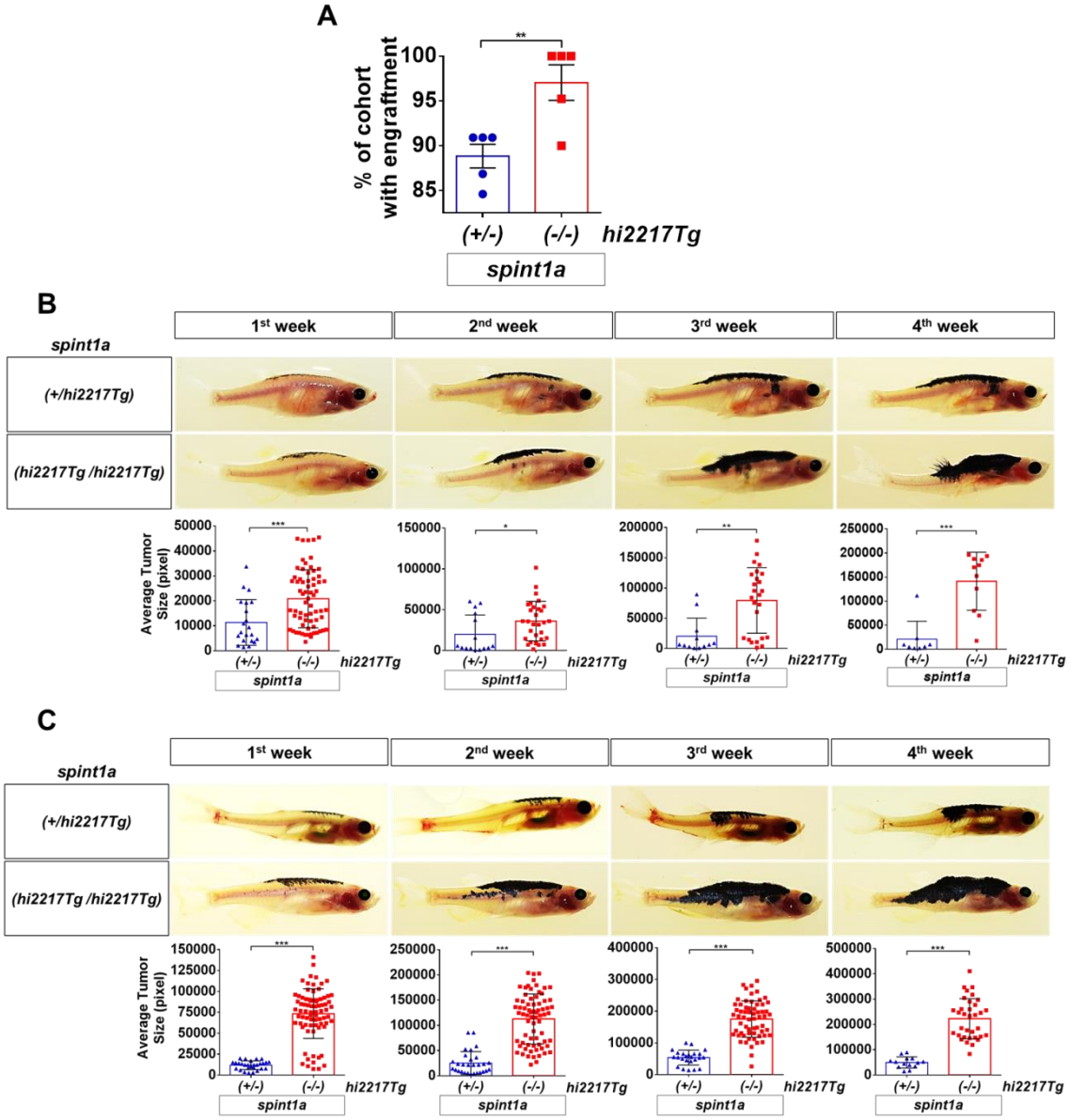

Figure 21: Spint1a-deficiency enhanced SKCMs aggressiveness in adult zebrafish allotransplantation assays. **A)** Percentage of engraftment for both control and Spint1a-deficient tumors. Each dot represents a single SKCM tumor and the mean ± SEM is also shown (n=5). **B)** Growth rate after subcutaneous injection of 300.000 *spint1a*$^{hi2217Tg/hi2217Tg}$ and *spint1a*$^{+/hi2217Tg}$ –derived SKCM cells in pre-irradiated adult Casper zebrafish. **C)** *In vivo* tumor growth rate after subcutaneous injection of 500.000 *spint1a*$^{hi2217Tg/hi2217Tg}$ and *spint1a*$^{+/hi2217Tg}$–derived SKCM cells from primary transplants described in **B)**in pre-irradiated adult Casper zebrafish. **B-C)**Fish was analyzed for average tumor size (pixels) weekly. The top row shows representative images for both genotypes and the bottom row shows the quantification of the average tumor size (pixels) from 1 to 4 weeks post-transplant. Each dot corresponds to a recipient-transplanted fish and the mean ± SEM is shown. *p < 0.05, **p<0.01, ***p<0.001 according to unpaired Student *t* test.

We next performed a secondary transplant following the same work-flow but injecting an increased number of cells (500,000 cells per recipient fish), that ensured a 100 % of engraftment for both genotypes (data not shown). From the first week after injection, Spint1a-deficient SKCMs tumor size was significantly larger than their control counterparts (**Figure 21C**). In addition, the recipients injected with Spint1a-deficient SKCM cells developed larger tumors spanning the entire dorsal area and even exceed the notochord line and grew vertically, a clear aggressiveness signature of SKCM.

To further investigate the aggressiveness potential of Spint1a-deficient SKCMs, a serial dilution assay was performed following the work-flow previously described in **Figure 20**.Cells from both Spint1a-deficient and wild type SKCMs were serially diluted and 3 different cell amounts (30,000 cells, 100,000 cells and 300,000 cells) were transplanted into recipients as described above. Notably, while 30,000 and 100,000 Spint1a-deficient SKCM cells were able to engraft and the tumor grew over the time, wild type SKCMs hardly grew (**Figures 22A** and **22B**). However, injection of 300,000Spint1a-deficient SKCM cells resulted in large tumor spanning the entire dorsal area and invading part of the ventral cavity (**Figure 22C**), confirming previous results. Collectively, all these results confirm that Spint1a deficiency enhances SKCM aggressiveness.

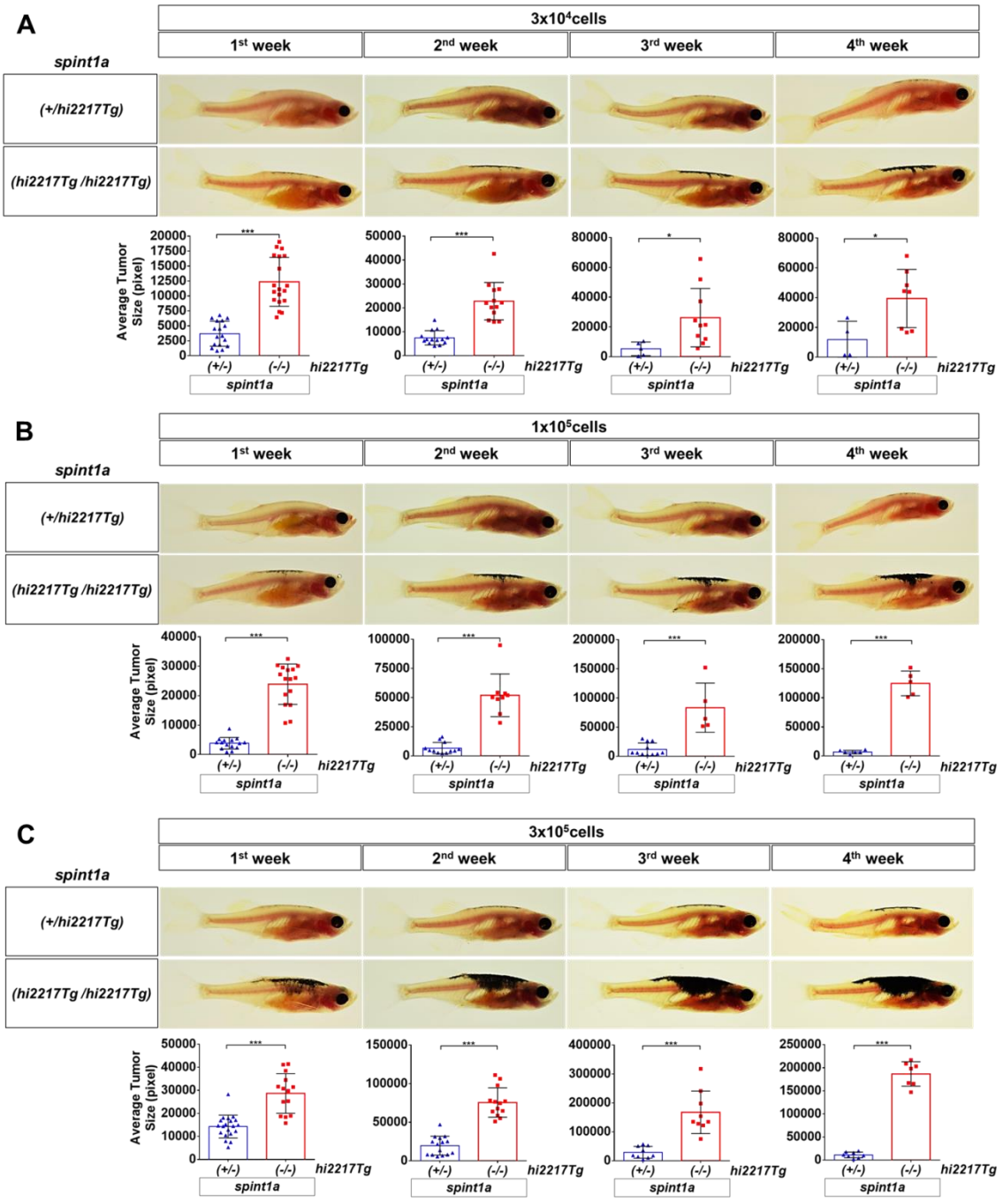

Figure 22: Limiting dilution cell transplantation assay of Spint1a-deficient SKCM cells in Casper adult zebrafish. Control and Spint1a deficient SKCMs were disaggregated and 30,000 **(A)**, 100,000 **(B)** or 300,000 cells **(C)** were injected subcutaneously in pre-irradiated (30 Gy, split dose in two days prior injection) adult Casper zebrafish. Fish were analyzed for average tumor size (pixels) from 1 to 4 weeks post-transplant. Representative images and quantification of the average tumor size are shown. Each dot corresponds to a recipient-transplanted fish and the mean ± SEM is also shown (n=2 SKCM tumors). *p < 0.05, ***p<0.001 according to unpaired Student *t* test.

11. Spint1a deficiency promotes SKCM dedifferentiation

To understand the mechanisms involved in the Spint1a-mediated aggressiveness of SKCM, the expression of genes encoding important biomarkers was analyzed by RT-qPCR. The mRNA levels of the differentiation markers *sox10*, *tyr*, *dct* and *mitfa* were lower in Spint1a-deficient SKCMs than in their wild type counterparts (**Figure 23A**). Furthermore, we extended our analysis to EMT markers and data revealed that while the transcript levels of *mmp9 and slug* were similar in Spint1a-deficient and wild type SKCM, *cdh1* levels were significantly decreased in Spint1a-deficient compared to wild type SKCM, (**Figure 23B**).

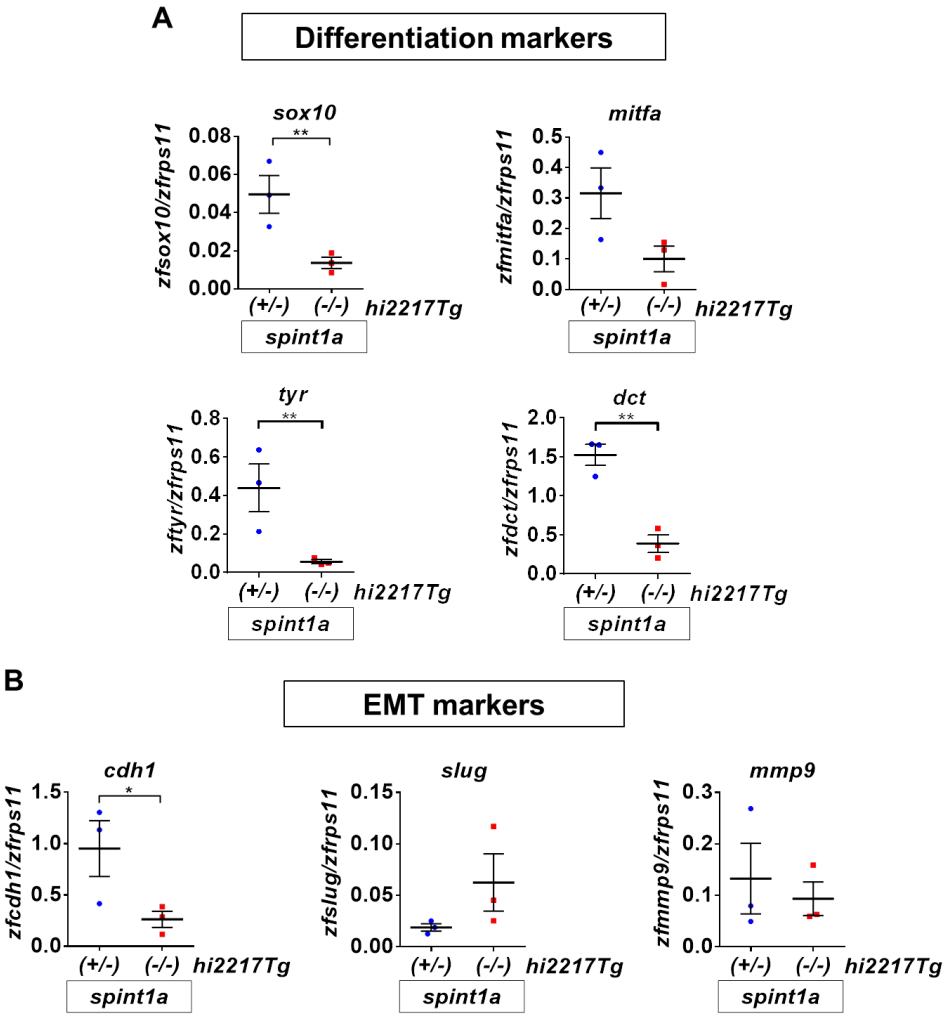

Figure 23: Expression analysis of differentiation melanocyte and EMT markers in zebrafish SKCM. The mRNA levels of the genes encoding the differentiation melanocyte markers Sox10, Mitfa, Tyr, Dct **(A)** and the EMT markers Cdh1, Slug and Mmp9 **(B)** were analyzed by RT-qPCR in *spint1a*$^{hi2217Tg/hi2217Tg}$ and *spint1a*$^{+/hi2217Tg}$ SKCMs . *p < 0.05, **p<0.01 according to one-tailed Student *t* test.

12. Spint1a deficiency promotes inflammation in SKCMs

Next, we analyzed genes encoding key inflammatory molecules and immune cell markers, including the pro-inflammatory cytokine Il1b, the neutrophil markers Lyz and Mpx, the macrophage marker Mpeg1 (**Figure 24A**) and the IFN-stimulated genes (ISGs) B2m, Mxb and Pkz (**Figure 24B**), in Spint1a-deficient and wild type SKCMs. Curiously, it was found that while *il1b*, *lyz* and *mpx* mRNA levels were not affected by Spint1a deficiency, those of *mpeg1* were elevated in Spint1a-deficient SKCMs (**Figure 24A**). Furthermore, the ISGs *b2m*,*mxb* and*pkz*genes showed enhanced mRNA levels in Spint1a-deficient SKCMs (**Figure 24B**). These results point out to macrophages and IFN as key players of the aggressiveness of Spint1a–deficient SKCMs.

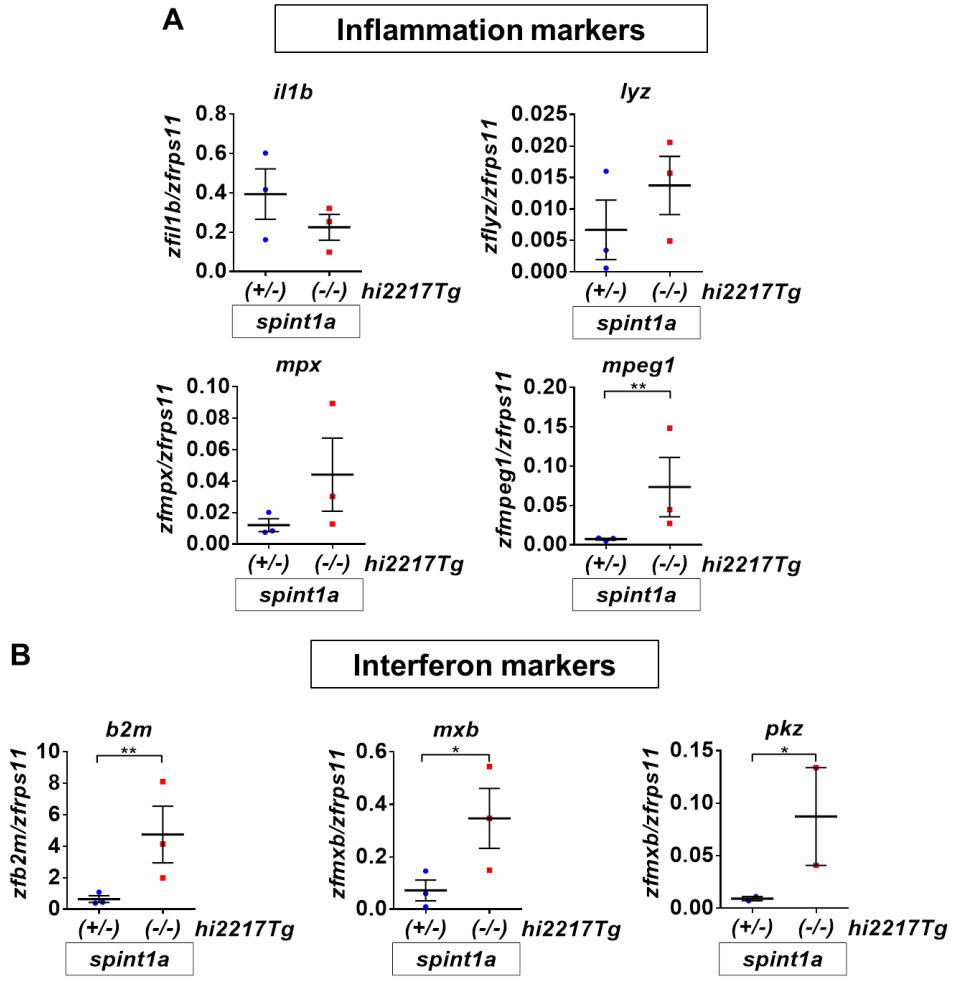

Figure 24: Expression analysis of inflammation and immune cell markers in zebrafish SKCM. The mRNA levels of the genes encoding the inflammation marker Il1b, the neutrophil markers Lyz and Mpx, the macrophage markers Mpeg1 **(A)** and the ISGsB2m, Mxb and Pkz **(B)** were analyzed by RT-qPCR in *spint1a*$^{hi2217Tg/hi2217Tg}$ and *spint1a*$^{+/hi2217Tg}$ SKCMs. *p < 0.05, **p<0.01 according to one-tailed Student *t* test.

13. Creation of new tool for drug screening: generation of a stable zebrafish spint1a-deficient melanoma cell line

Once we characterized the enhanced aggressiveness of spint1a-deficient zebrafish melanoma tumors, we generated a stable zebrafish melanoma cell lines to plan large-scale drug screening.

ZfMRH (-/-) and ZfMRH (+/-) cell lines were generated from spint1a-deficentand control nodular tumors obtained from melanoma-prone zebrafish lines *spint1a*$^{hi2217Tg/hi2217Tg}$; *kita:Gal4*;HRAS-*G12V* and *spint1a*$^{+/hi2217Tg}$; *kita:Gal4*;HRAS-*G12V*, respectively.

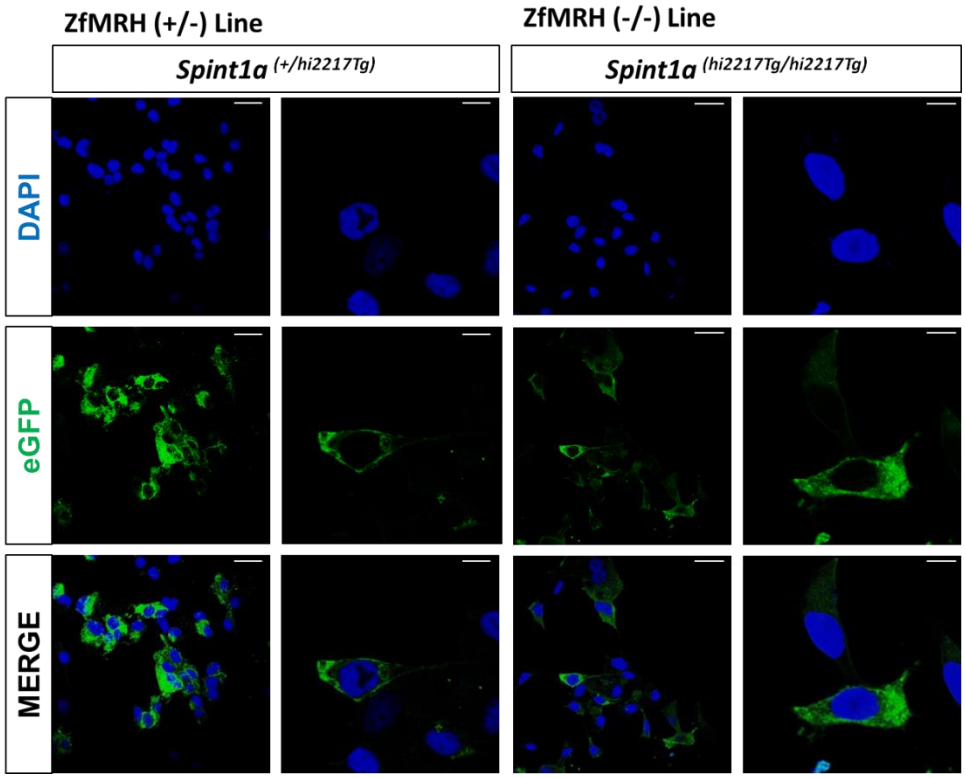

Figure 25: Primary zebrafish SKCM cells immortalization. Representative images of control ZfMRH (+/-) and spint1-deficient ZfMRH (-/-) cell lines in a monolayer visualized under a fluorescence microscope. Both lines are positive for green fluorescence since they express a chimeric protein including eGFP along with the human oncogene HRAS-G12V. Nuclei are evidenced by DAPI staining. Magnification bar: 5 µm.

For cell lines generation, tumors were cleanly dissected and disaggregated as previously described, cells were resuspended in complete zebrafish media and then plated in a single well of a 48-well plate that be been previously coated with fibronectin. The fibronectin coating was needed until they were passaged 10-20 times,

point at which cells were cultured in normal tissue culture plates without fibronectin. As previously indicated in *in vivo* experiments, when cultured *in vitro* both cell lines still express eGFP fused to the human oncogene HRAS-G12V which is exactly localized in the membranes of the melanoma cells (**Figure 25**).

Discussion

The SKCM incidence and mortality is increasing at a worrying rate. Although it is true that this incidence and mortality widely differ by country, more than 232.100 (1.7%) of all newly diagnosed primary malignant cancers are SKCM cases. Annually, it is supposing more than 55.500 deaths that correspond to 0.7% of all cancer deaths (Schadendorf, van Akkooi et al. 2018). Due to this incidence, SKCM is reaching epidemic levels. Furthermore, SKCM is considering the deadliest form of skin cancer since once SKCM has spread, rapidly becomes life-threatening.

In addition, SKCM is characterized by a great genetic heterogeneity that makes difficult to identify a successful treatment. Indeed, for more than 40 years, few therapeutic options were available. Surgically excision of localized lesion has been the more effective treatment even if it can only be applied in cases of SKCM at early stage (Schadendorf, van Akkooi et al. 2018). Because of that, the early detection and primary care become essential to prevent the most recurrent type of cancer.

Thanks to the efforts aimed to characterize the molecular bases of the crosstalk between tumor cells and tumor microenvironment, nowadays is known that inflammatory responses can play decisive roles during the different stages of tumor progression; from tumor initiation to malignant transformation and metastatization (Grivennikov, Greten et al. 2010) but the relationships between inflammation and cancer are still ambiguous (Shalapour and Karin 2015). Recently, inflammation has been recognized by the broad cancer research community as a hallmark of cancer (Taniguchi and Karin 2018). Even though infections and inflammatory responses are estimated to be linked to 15–20% of all deaths from cancer worldwide (Maru, Gandhi et al. 2014), immunosuppression is known to increase the risk for certain tumors (Shalapour and Karin 2015).

On the whole, these evidences have led to join forces to determine the role of inflammation and immune system in malignant SKCM to develop effective therapies for this type of cancer (Carreau and Pavlick 2019). Indeed, some new approaches based on ligand-receptor interactions between cancer cells and host immune cells within the tumor microenvironment became potential therapeutic strategies in cancer therapy. Chimeric antigen receptor (CAR) T cells form part of a broad wave of immunotherapies that are optimistic in early phase cancer clinical trial (Kalaitsidou, Kueberuwa et al. 2015). This treatment consists in the expression of synthetic receptors that redirect polyclonal T cells to surface antigens for the tumor elimination. Recently, Vemurafenib, Ipilimumab, and checkpoint inhibitors like anti-Programmed Cell Death-1(PD-1) and Ligand (PD-L1) antibodies have demonstrated expectation in the treatment of

metastatic SKCM (Burgeiro, Mollinedo et al. 2013, Lang, Peveling-Oberhag et al. 2018, Constantinidou, Alifieris et al. 2019). Ipilimumab is a humanized IgG1 monoclonal antibody that is able to produce immune-modulation through cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4) blockade. It generates favorable antitumor immune system responses reducing the tolerance to tumor-associated antigens. Nevertheless, Vemurafenib is a novel oral small-molecule kinase inhibitor with high selectivity and efficacy toward specific mutated oncogenic BRAF-signalling mediator (Lang, Peveling-Oberhag et al. 2018). In the case of the PD-1/PD-1L inhibitors, they repress pathways involved in adaptive immune suppression producing the immune checkpoint blockade (Constantinidou, Alifieris et al. 2019). Furthermore, combination regimens have demonstrated higher efficiency over monotherapy treatments, especially for targeted agents since each therapeutic combination possesses different advantages but, as opposed, also different side effect profiles (Luther, Swami et al. 2019).

Despite of all these promising advances, the number of durable responses using these new immunotherapies was limited (Enewold, Sharon et al. 2017) and the high toxicity of combination regimens should be taken into account (Kalaitsidou, Kueberuwa et al. 2015). This emphasizes the importance of developing novel treatment strategies able to overcome drug resistance (Zaretsky, Garcia-Diaz et al. 2016). But it is true that, immunotherapy is considered nowadays the most favorable cancer therapy for the next future because CTLA-4 antibodies and anti- PD-1/PD-1L are much better tolerated and greatly improves the overall survival and progression-free survival for many patients (Carreau and Pavlick 2019). These approaches together with the high SKCM heterogeneity open new opportunities for personalized-based therapies to counteract SKCM aggressiveness minimizing the harmful side effects.

In this context, the aim of this study was the evaluation of the chronic inflammation impact in promoting SKCM. Tumor-promoting inflammation is characterized by the presence of neutrophils, macrophages, dendritic cells and T-lymphocytes that support cancer progression secreting mediators such as cytokines, chemokines and grow factors that could promote cancer growth and progression (Tang and Wang 2016). TNFA plays key role in acute and chronic inflammation, antitumor responses and inflammatory diseases (Candel, de Oliveira et al. 2014). Patients affected by chronic inflammatory diseases, such as psoriasis, treated present an higher risk to develop skin cancer after anti-TNFA therapy (Palladino, Bahjat et al. 2003) and high TNFR2 expression in melanoma has been associated with good prognosis so that

it has been proposed as a novel target for cancer immunotherapy (Vanamee and Faustman 2017). Indeed TNFA / TNFR2 axis was analyzed *in silico* demonstrating that genetic alterations in *TNFA* and *TNFR2* genes occur in 5 and 6% of melanoma patients, respectively. These results may have important clinical impact since these alterations are at the same extent of *HRAS* oncogene (6% in the same SKCM patients) that plays a main role in human cancer development because it is involved in regulating cell division in response to growth factor stimulation (Knight and Irving 2014, Simanshu, Nissley et al. 2017, Stephens, Yi et al. 2017). Even though the main TNFA and TNR2 gene alterations are amplification and mRNA upregulation, survival analysis of SKCM patients revealed that low expression of these genes in human samples correlate with poor prognosis.

The transparency of the zebrafish embryos and the rapid development, together with the large availability of transgenic lines, make zebrafish as a perfect preclinical model to study SKCM in early stages. To characterize the role of TNFA / TNFR2 axis in melanoma initiation and progression, we took advantage of a zebrafish spontaneous SKCM model in which we modulated the expression of TNFR2 receptor.

We found that the *in vivo* skin inflammation driven by *Tnfr2* loss of function (Candel, de Oliveira et al. 2014), robustly increased the number of oncogenically transformed cells, while the same did not occur when the other TNFA receptor, Tnfr1, was genetically inhibited. The rescue of Tnfr2 expression in transformed melanocytes failed to reverse the increase of oncogenically transformed cells, suggesting that tumor microenvironment would play an extrinsic role through TNFA/TNFR2 signaling in melanoma establishment. Indeed, when the cross-talk between oncogenically transformed cells and neutrophils was analyzed *in vivo*, we found that the interaction between neutrophils with transformed cells was increased by TNFR2 overexpression and this process could be responsible for the consequent proliferation of these transformed cells. Nevertheless, neutrophil infiltration has been found in many types of solid tumors making TANs relevant in malignant disease but, in spite of neutrophils may be potent antitumor effector cells, increasing clinical evidences show that TANs presence strongly correlates with poor prognosis (Ding, Zhang et al. 2018). This is because the tumor microenvironment is able to control TAN recruitment helping, then, for the tumor progression and metastasis (Fridlender, Albelda et al. 2015, Sionov, Fridlender et al. 2015, Uribe-Querol and Rosales 2015).

To study in depth the impact of inflammation on melanoma onset and progression, we established a novel spontaneous SKCM zebrafish line characterized by Spint1a-driven skin chronic inflammation. We found that Spint1a deficiency increased oncogenic transformation, as observed in Tnfr2-deficient larvae, also enhancing tumor incidence when the individual were analyzed during near 3 months. Furthermore we found that, Spinta is required at both cell autonomous and non-autonomous levels to enhance cell dissemination of SKCM by promoting tumor dedifferentiation and altered immune surveillance. These results may have important clinical impact, since genetic alterations of SPINT1 were found in 10% of SKCM patients and correlated with altered cell cycle, differentiation and innate and adaptive immune signaling pathways and, more importantly, with a poor prognosis. In addition, a positive correlation of both inflammation and macrophage marker with SPINT1 levels was found in tumor samples, fitting the increased number of TAM in SKCM with high SPINT1 levels. However, a negative correlation of SPINT1 and EMT marker levels was observed, suggesting that SPINT1 levels critically regulate tumor and immune cell crosstalk. Therefore, both high and low levels of SPINT1 result in an unbalanced cross talk between tumor cells and their microenvironment promoting higher SKCM aggressiveness.

Another interesting observation is that SPINT1 transcript levels positively correlated with macrophage infiltration, but not neutrophil one, in SKCM tumor samples. Similarly, our gene expression studies also suggest that Spint1a regulates macrophage infiltration in the zebrafish SKCM model. Curiously, activated neutrophils in a condition of repeated wounding, have been shown to interact with pre-neoplastic cells promoting their proliferation through the release of prostaglandin E2 and more importantly, SKCM ulceration correlates with increased neutrophil infiltration and tumor cell proliferation, which are both associated with poor prognosis (Antonio, Bonnelykke-Behrndtz et al. 2015). Although we found a robust positive correlation between the transcript levels of *SPINT1* and *CXCR2*, which encodes a major IL-8 receptor involved in SKCM neutrophil infiltration(Jablonska, Wu et al. 2014), CXCR2 has also been shown to promote tumor-induced angiogenesis and increased proliferation through activation of CXCL8/CXCR2 intrinsic pathway (Gabellini, Trisciuoglio et al. 2009, Singh, Sadanandam et al. 2009, Gabellini, Gomez-Abenza et al. 2018). The increase of CXCR2 expression could be due to an increase of neutrophil infiltration but the contribution of other stromal cells cannot be excluded since CXCR2 is also expressed by macrophages, as well as endothelial cells and fibroblasts, cells may sustain melanoma dissemination

through IL8/CXCR2 pathway. Therefore, SPINT1/CXCR2 axis may regulate SKCM aggressiveness by neutrophil-independent pathways.

The zebrafish model developed in this study uncovered a role for Spint1a in facilitating oncogenic transformation which probably accelerates the SKCM onset. Curiously, Spint1a deficiency seems to have a prominent role in oncogenic transformation accelerating SKCM onset *in vivo*. However, the strong oncogenic activity of HRAS-G12V, which is even able to induce melanoma without the need of coadjuvating mutations in tumor suppressors, results in a similar tumor burden in wild type and Spint1a-deficient fish at later stages. It would be interesting, therefore, to analyze in future studies the impact of Spint1a in SKCM models driven by other major oncogenes, such as Braf, Kras, Nras and Nf1. Although SPINT1 is a serine protease inhibitor with several targets, including ST14 and HGFA, deregulation of the SPINT1/ST14 axis leads to spontaneous squamous cell carcinoma in mice (List, Szabo et al. 2005) and keratinocyte hyperproliferation in zebrafish (Carney, von der Hardt et al. 2007, Mathias, Dodd et al. 2007) preceded by skin inflammation in both models. In addition, intestine-specific Spint1 deletion in mice induces the activation of the master inflammation transcription factor NF-κB and accelerates intestinal tumor formation (Kawaguchi, Yamamoto et al. 2016). Strikingly, pharmacological inhibition of NF-κB activation reduces the formation of intestinal tumors in Spint1-deficient ApcMin/+ mice (Kawaguchi, Yamamoto et al. 2016), unequivocally demonstrating that Spint1-driven inflammation promotes tumorigenesis.

The SKCM allotransplant assays in larvae revealed for the first time that Spint1a deficiency in both tumor and stromal cells increases SKCM invasiveness. In addition, Spint1a deficiency in both cell types does not show to enhance invasiveness compared to the condition in which Spint1a is deficient in each single cell type. We confirmed this result by the injection of wild-type Spint1-a SKCM cells in Spint1a-deficient larvae demonstrated that Spint1a tumor microenvironment is sufficient to enhance SKCM invasion. Unfortunately, when we conducted SKCM allotransplant experiment trying to define the contribution of tumor stromal cell in this process, we found that Spint1a deficiency in this cellular compartment is not sufficient to significantly enhance SKCM invasiveness, even there is a tendency. In addition, wild type Spint1a tumor microenvironment fails to compensate its loss in tumor cells, since Spint1a-deficient tumor cells show enhanced invasiveness in wild type larvae and adult recipients, and vice versa. This result, apparently in contrast with SKCM allotransplant experiment in Spint1a-deficient larvae, enforces our hypothesis that the global chronic skin

inflammatory environment may play a role in enhancing SKCM invasion, and the contribution of infiltrated Spint1a deficient cells, including fibroblasts, endothelial and immune cells is not sufficient to sustain SKCM dissemination. This is an interesting observation, since SPINT1 is a membrane-bound protein that may, therefore, inhibit their targets in both tumor cell autonomous and non-autonomous manners. Importantly, transplantation experiments of serial diluted SKCM cells revealed the crucial cell-autonomous role of Spint1a in inhibiting tumor aggressiveness. Similarly, our result underlines the crucial autocrine role of Spint1a in inhibiting tumor aggressiveness as demonstrated in human pancreatic orthotropic xenograft models (Ye, Kawaguchi et al. 2014).

We observed that genetic alterations in *SPINT1* transcript levels in SKCM patient samples negatively correlate with EMT markers and indeed Spint1a-deficient zebrafish SKCM showed reduced *cdh1* mRNA levels. EMT phenotype switching has been clearly shown to be involved in acquisition of metastatic properties in the vertical growth phase of SKCM (Bennett 2008) and loss of E-cadherin, with gain of N-cadherin and osteonectin, was associated with SKCM metastasis (Alonso, Tracey et al. 2007). Importantly, the presence of aberrant E-cadherin expression in primary and metastatic SKCM is associated with a poor overall patient survival (Yan, Holderness et al. 2016). Therefore, our results suggest that SPINT1 loss may facilitate metastatic invasion of human SKCM through EMT phenotype switching, confirming the possible impact of Spint1a deficiency on cell-autonomous invasive pathways. Moreover, in the analysis of the differentiation markers (*sox10*, *tyr*, *dct* and *mitfa*) in zebrafish tumor samples, their reduction was evidenced in Spint1a-deficient SKCMs comparing with their wild type counterparts, a result that is in line with the enhanced tumor aggressiveness induced by Spint1a deficiency.

Furthermore, the increased expression of macrophages marker (*mpeg1*) induced by Spint1a deficiency in zebrafish SKCM is in line with human tumor samples analysis as well as the lack of significant modulation of neutrophil markers (*lyz* and *mpx*) by Spint1a altered expression in both zebrafish and human samples. In addition, since*il1b* level is not changed; others chemokine could be involved in establishing an inflammatory status within the Spint-1a deficient tumors.

In summary, we have developed two preclinical models to study the role of chronic inflammation in promoting SKCM. Both, the transient inhibition of TNFR by morpholino and germ-line SPINT1 deficiency facilitate the early transformation and progression of SKCM. This model has revealed that the TNFA / TNFR2 axis has a key

role in the cross-talk between oncogenically transformed cells and the tumor microenvironment *in vivo*, and that Spint1a deficiency facilitates oncogenic transformation, regulates the tumor/immune microenvironment crosstalk and is associated to SKCM aggressiveness. In addition, Spint1a deficiency in either tumor or microenvironment compartment increases SKCM aggressiveness. The high prevalence of *TNFA, TNFR2* and *SPINT1* genetic alterations in SKCM patients and their association with a poor prognosis, suggest the relevance of clinical intervention on these signaling pathways to develop effective treatments that could open new opportunities for personalized-based therapies to counteract melanoma aggressiveness in the precision medicine.

Conclusions

The lead conclusions derived from the obtained results of this work are:

1. Genetic alterations in *TNFA* and *TNFR2* genes occurred in 5 and 6 % melanoma patients, respectively, and are associated with bad prognosis.

2. Inflammation through TNFA/TNFR2 signaling axis accelerates the onset of melanoma, enhancing oncogenic transformation and melanoma progression.

3. While overexpression of TNFR2 has no effects on oncogenic transformation, TNFR2 signaling in stromal cells is responsible for the increased proliferation of transformed cells. Even though the two conditions are characterized by the same number of neutrophils, Tnfr2-deficient larvae evidence increased interaction with transformed cells when compared to their control counterparts.

4. *SPINT1 genetic* alterations occur in 10% melanoma patients and are associated with poor prognosis. *SPINT1* expression was significantly inhibited in human SKCM comparing to nevus or normal skin. In addition, *SPINT1* expression correlates with altered cell cycle, differentiation and innate and adaptive immune signaling pathways.

5. *SPINT1* transcript levels in human melanoma samples positively correlated with macrophage infiltration, but not neutrophil, in SKCM tumor samples.

6. Chronic skin inflammation driven by Spint1a deficiency enhances SKCM aggressiveness in preclinical zebrafish models, increasing oncogenic transformation and accelerating the onset of oncogenic *HRAS*-driven SKCM.

7. Allotransplant assays in both larvae and adult zebrafish showed a key role of chronic inflammation, triggered by Spint1a deficiency, in the dissemination of SKCM cells.

8. Spint1a deficiency in both melanoma and stromal tumor microenvironment contributes to enhanced melanoma aggressiveness.

9. SKCM clinical data and zebrafish models indicate that Spint1a loss facilitate EMT phenotype switching in SKCM.

10. SPINT1 plays a crucial role in the regulation of the crosstalk between tumor cells and inflammatory cells in SKCM, opening new opportunities for clinical intervention and personalized-based therapies to counteract SKCM aggressiveness.

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Resumen en Castellano

La piel, el mayor órgano del cuerpo, actúa como una barrera defensiva entre el organismo y el ambiente (Proksch, Brandner et al. 2008). Se trata de un epitelio compuesto por 3 capas: epidermis, dermis e hipodermis o tejido subcutáneo. Cada una de estas capas está formada por diferentes tipos celulares como queratinocitos, y componentes de la matriz extracelular, colágeno, elastina además de vasos sanguíneos, adipocitos y nervios.

En la capa basal de la epidermis se encuentran los Melanocitos que son un tipo de células dendríticas productoras de un pigmento llamado melanina, derivadas de la cresta neural (Sauka-Spengler and Bronner-Fraser 2008, White and Zon 2008, Bonaventure, Domingues et al. 2013). Su principal función es proteger a los queratinocitos del daño oxidativo del ADN inducido por los rayos UV (Mort, Jackson et al. 2015, Shain and Bastian 2016). En el proceso de diferenciación de los melanocitos están implicadas varias vías de transducción de señales, pero es la vía MAP-Kinasa la más esencial en el proceso de proliferación y diferenciación de los melanocitos. Los melanocitos diferenciados pueden ser identificados por marcadores moleculares específicos como son el factor de transcripción asociado a microphthalmia (MITF), tyrosinasa (TYR), dopacromotautomerasa (DCT), entre los más destacados.

La melanina, sintetizada por la vía Raper-Mason (d'Ischia, Wakamatsu et al. 2015) y almacenada en los melanosomas, es uno de los depuradores más potentes de radicales libres en humanos. Principalmente se encarga de la absorción y dispersión de luz UV, eliminación de radicales libres, de reacciones de oxidación-reducción acopladas y del almacenamiento de iones (Costin and Hearing 2007, Bonaventure, Domingues et al. 2013).

La cantidad de melanina y de melanocitos presente en la piel es variable dependiendo de la ubicación anatómica, pero es muy similar entre los individuos de la misma raza. Esto es debido a que existe un pigmentación basal genéticamente determinada por la raza del individuo e independientemente de factores externos.

Además del factor genético, existen factores externos que pueden influir en la pigmentación de la piel. El principal factor externo que induce la melanogenesis es la radiación ultravioleta (UVR). Si bien es cierto que la exposición a la radiación solar ofrece efectos beneficiosos para el organismo como la estimulación de la síntesis de la vitamina D (entre otros), la sobreexposición a este tipo de radiación conduce al agotamiento de los antioxidantes celulares generando daño en el ADN por la acumulación de dímeros de timidina provocando una mayor liberación de mediadores proinflamatorios (Natarajan, Ganju et al. 2014). Esta transformación maligna de los

melanocitos origina la aparición del melanoma cutáneo (SCKM), un tipo de cáncer de piel con una etiología multigénica compleja que se vuelve extremadamente difícil de tratar una vez que ha hecho metástasis. Por esa razón, el SKCM es la forma más letal de cáncer de piel y, aunque las tasas de incidencia están disminuyendo para la mayoría de los cánceres, el SKCM sigue aumentando de manera constante. Hoy en día el 75% de las muertes por cáncer de piel son debidas al melanoma. La incidencia de esta enfermedad se ha visto incrementada desde 1950 debido a los cambios producidos entre la población, tanto por razones estéticas como de ocio, que incluyen un exceso de exposición prolongada a UVR. El SKCM suele ser diagnosticado en individuos comprendidos entre 40 y 60 años y es mucho más común en hombres que en mujeres (https://seer.cancer.gov/statfacts/html/melan.html).

Los melanocitos, dependiendo del lugar anatómico donde se encuentren, pueden dar lugar a melanomas fenotípicamente distintos. Así, por ejemplo, podemos encontrar melanoma uveal, cuando el melanoma se produce en el ojo (https://www.cancer.gov/types/skin/patient/melanoma-treatment-pdq#section/all). En el caso de los melanomas cutáneos, se pueden clasificar en términos generales atendiendo a su origen: los originados en la piel con daño solar crónico (CSD) o los que no (non-CSD) (Shain and Bastian 2016).

Generalmente, SCKM muestra dos fases distintas de invasión local: la fase de crecimiento radial y la fase de crecimiento vertical (http://atlasgeneticsoncology.org/Tumors/SkinMelanomID5416.html). Fue en los años 1969 y 1970 donde los médicos Clark y Breslow propusieron, de manera independiente, un método estandarizado de identificación del estado del SKCM. En estos métodos se evalúa la invasión del melanoma a otros tejidos y su grosor, para así relacionarlo, de manera estricta, con el pronóstico de la enfermedad. Hoy en día solamente se usa método de clasificación de Breslow (Leilabadi, Chen et al. 2014) y también se han definido otras variantes clínicas e histopatológicas del SKCM (Schadendorf, Fisher et al. 2015).

Fenotípicamente, el melanoma se presenta con una gran heterogeneidad pero clásicamente, los modelos de progresión implican el paso desde nevus a nevus displástico y de ahí a melanoma *in situ*, para finalmente acabar en un melanoma invasivo. Se han asociado múltiples mutaciones patogénicas claramente asociadas al establecimiento y progresión del melanoma desde los estadios más tempranos de la transformación maligna del melanoma. Las Ras GTPsas tienen un papel central en la unión entre receptores de factores de crecimiento activados y vías de transducción de

señales posteriores durante la melanogénesis. Es por esta razón, por la que las tres isoformas del oncogén RAS (HRAS, KRAS and NRAS) representan el oncogén más mutado junto con la activación de la ruta de señalización RAS–RAF–ERK (Chin 2003, Sullivan and Flaherty 2013). Además de más del 25% de mutaciones NRAS, también se incluyen mutaciones en BRAF (~ 50%) y mutaciones en el supresor RAS NF1 (~ 14%).

Por otra parte, la inflamación es un proceso en el que el sistema inmune induce diferentes células y mediadores moleculares en respuesta a una lesión tisular, infección patógena o muerte celular masiva para generar una regeneración tisular (Coussens and Werb 2002). La activación anómala de estos procesos están envueltos en diferentes patologías incluidas fibrosis, metaplasia y cáncer (Pesic and Greten 2016). Se pueden distinguir dos tipos de respuesta inmune: la innata y la adaptativa. La innata representa la primera línea de defensa del organismo siendo los principales actores neutrófilos y macrófagos (Mollen, Anand et al. 2006) y la adquirida, es específica, duradera y requiere el reconocimiento de antígenos específicos "no propios" a través de anticuerpos (Pancer and Cooper 2006). La inflamación puede ser aguda o crónica. La inflamación aguda es un proceso rápido y autolimitado pero, en ocasiones, puede convertirse en un estado crónico, que podría generar la aparición de distintos tipos de enfermedades como: cardiovasculares, diabetes, artritis, Alzheimer, enfermedades pulmonares, enfermedades autoinmunes y cáncer (Zhao, Tang et al. 2017).

Hoy en día se sabe que la inflamación puede jugar un papel esencial en el proceso del cáncer, desde su transformación inicial hasta su diseminación metastásica, pero esta relación es ambigua. La inflamación (especialmente la inflamación crónica) tiene efectos protumogénicos, pero las células inflamatorias también median una respuesta inmune contra el tumor. Además, se sabe que la inmunosupresión aumenta el riesgo de producir cáncer así como su promoción y progresión, y el microambiente inflamatorio proporciona la base para la transición epitelial-mesénquima (EMT), invasión y metástasis.

El pez cebra, *Danio rerio*, se ha convertido en un organismo experimental para modelar numerosas enfermedades entre ellas el cáncer. Esto es gracias a la conservación de varias vías moleculares y celulares involucradas en sus procesos. Se sabe también, que su sistema inmune muestra características similares a las de las aves y los mamíferos. Así en este estudio, se ha usado el pez cebra como modelo de inflamación crónica a través de la vía de señalización del TNFA. TNFA es una citoquina proinflamatoria que media en importantes procesos de inflamación aguda y crónica

pero también en infecciones y respuestas antitumorales. Específicamente, se ha visto que la deficiencia de TNFA o TNFR2 resulta en la movilización de neutrófilos a la piel produciendo mediadores proinflamatorios en los queratinocitos que induce la activación de NF-kB en la piel generando H_2O_2 (Candel, de Oliveira et al. 2014). Por tanto, se puede establecer un modelo relevante de inflamación en piel, en el pez cebra, para estudiar la implicación del eje TNFA / TNFR2 en la transformación oncogénica.

Por otro lado, el inhibidor de la Serin Proteasa, Tipo kunitz, 1 (SPINT1), también conocido como Inhibidor del Activador del factor de crecimiento de Hepatocitos 1 (HAI1), es un inhibidor de la serin proteasa transmembrana de tipo II que desempeña un papel crucial en la regulación de la actividad proteolítica tanto del supresor de tumorigenicidad 14 (ST14), también conocida como Matriptase-1 (Lin, Anders et al. 1999, Benaud, Dickson et al. 2001, Tseng, Chou et al. 2008), como del activador del factor de crecimiento de hepatocitos (HGFA) (Shimomura, Denda et al. 1997). El enlace funcional entre ST14 y SPINT1 tiene implicaciones importantes para el desarrollo del cáncer (List, Szabo et al. 2005). Además, también se ha demostrado que la expresión de ST14 está aumentada en varios tipos de cáncer humano como el cáncer de mama, de cuello uterino, ovario, próstata, esófago e hígado (List 2009).

La estrecha relación funcional entre ST14 y SPINT1 también se observó en un modelo de pez cebra de inflamación de la piel, portando una mutación hipomórfica de spint1a. De hecho, la hiperproliferación epidérmica y la infiltración de neutrófilos observados en las larvas de pez cebra mutantes son rescatadas por la desactivación del gen *st14a*, lo que sugiere un nuevo papel para el eje SPINT1-ST14 en la regulación de la inflamación (Carney, von der Hardt et al. 2007, Mathias, Dodd et al. 2007).

Además, el pez cebra también ofrece otras ventajas importantes como organismo modelo para el estudio del melanoma: embriones de pequeño tamaño, alta fecundidad, desarrollo externo y transparencia (entre otras) que permiten el seguimiento de melanocitos durante todas las etapas de su desarrollo.

Los procesos de desarrollo temprano de la transformación de melanocitos y los métodos para su detección temprana son importantes para la erradicación del melanoma, por tanto, el pez cebra es una excelente herramienta para el estudio de esta enfermedad y sus procesos mediante el uso de la técnica de xenoinjerto y modelos transgénicos de formación espontánea de tumores de diferentes histotipos (Bootorabi, Manouchehri et al. 2017).

Por tanto los objetivos que se plantean en esta tesis doctoral son:

1. El estudio de la relevancia de las alteraciones genéticas de SPINT1 y del eje TNFA/TNFR2 en la relación entre la inflamación y el melanoma así como ello influye en pronóstico de la enfermedad.
2. Estudio de la relevancia de la inflamación producida por Spint1a y Tnfr2 en la transformación oncogénica temprana en el modelo de larva de pez cebra.
3. Desarrollo de modelos de pez cebra adulto y larva para el estudio del papel desempeñado por la inflamación producida por Spint1a en la progresión y agresividad del melanoma.
4. Estudio del papel desempeñado por la inflamación producida por Spint1a en modelos de SKCM de pez cebra.

Para llevar a cabo el estudio de la implicación del eje TNFA/TNFR2 en el desarrollo y agresividad del SKCM, se hizo un análisis *in silico* de muestras humanas de la base de datos TCGA demostrando que TNFA y TNFR2 presentaban alteraciones genéticas en estos pacientes. Estas alteraciones se encontraban al mismo nivel que prensentaba el oncogén HRAS. Además se observó que los pacientes que presentaban una baja expresión en esos genes, correlacionaban con un peor pronóstico de la enfermedad.

Para entender como el eje TNFA/TNFR2 afectaba a la transformación oncogénica *in vivo* se moduló la expresión de Tnfr en el modelo de pez cebra utilizando dos enfoques: la inactivación génica mediada por morfolino de Tnfr1 y Tnfr2 y la sobreexpresión de Tnfr2 en melanocitos transformados usando el sistema combinatorio Gal4-UAS. Esto demostró que la deficiencia de Tnfr2, aumentaba el número de células oncogénicamente transformadas. Además el rescate de la expresión de Tnfr2 en los melanocitos transfomados no revirtió este aumento de células HRASG12V^{+}.

Para dilucidar si los neutrófilos eran los responsables de este incremento de células oncogénicamente transformadas, se llevó a cabo un experimento en el que se midió los contactos producidos entre los neutrófilos y las células oncogénicamente transoformadas, demostrando que los neutrófilos deficientes en Tnfr2 interactionaban más con las células transformadas que los controles, aun habiendo el mismo número de neutrófilos en ambos casos. Esto sugiere que la inflamación promovida por la deficiencia en Tnfr2 puede facilitar la transformación oncogénica asociando la señalización de la vía TNFA con el establecimiento y la progresión del melanoma.

Para estudiar el impacto de SPINT1 en la progresión y agresividad de SKCM, se hizo un análisis *in silico* de muestras humanas de la base de datos TCGA que reveló que las alteraciones genéticas en este gen ocurren en el 10% de los pacientes con melanoma. De manera significativa, estas alteraciones genéticas de SPINT1 se correlacionaron significativamente con el mal pronóstico del paciente SKCM y la expresión de SPINT1 se mostró inhibida en SKCM humano en comparación con nevus y piel normal. A través de un análisis de enriquecimiento GO se observó que los procesos biológicos afectados por estas alteraciones eran los implicados con la regulación del sistema inmune, la respuesta inflamatoria, el ciclo celular, la adhesión celular y la organización de la matriz extracelular. Además, el número de TAM en muestras de SKCM humano se mostró correlacionado con los niveles de expresión de SPINT1 en SKCM metastásico. Esto sugiere que tanto los niveles altos y bajos de SPINT1 provocan desbalance entre las células tumorales y su microambiente promoviendo una mayor agresividad.

Para comprender mejor el papel de SPINT1 en SKCM se analizó la expresión de genes implicados en el desarrollo de melanocitos, en la transición epitelio-mesénquima (EMT) y en varios marcadores de inflamación. Los resultados obtenidos sugieren que SPINT1 regula la diferenciación y agresividad de SKCM, y la infiltración de macrófagos en biopsias SKCM humanas.

Dada la fuerte correlación entre las alteraciones de los niveles de SPINT1 con la progresión del SKCM y con la comunicación entre el microambiente inmunológico y el tumor, cruzamos la línea de pez cebra kita:Gal4; HRAS-G12V, que expresa el oncogén humano HRAS-G12V en melanocitos y desarrolla melanomas espontáneamente (Santoriello, Gennaro et al. 2010), con la línea mutante de pez cebra *spint1a*$^{hi2217Tg/hi2217Tg}$ (Mathias, Dodd et al. 2007), que presenta inflamación crónica de la piel. Después de la cuantificación de las células oncogénicamente transformadas en estadios tempranos del desarrollo, *spint1a* mostró un aumento en el número de las mismas (células HRAS-G12V^{+}). Para averiguar si este aumento era capaz de promover la agresividad de SKCM, se comparó el desarrollo de SKCM en individuos con inflamación crónica y sin ella hasta los 120 dpf. Los resultados mostraron un aumento significativo de la incidencia de melanoma en los peces con deficiencia de Spint1a. Esto sugiere que la deficiencia de Spint1a aumenta la transformación oncogénica temprana y acelera la aparición de SCKM *in vivo.*

Para testar cómo afecta la deficiencia de Spint1a *in vivo* en la invasión del SKCM, se llevaron a cabo alotransplantes de células procedentes de tumores

desarrollados en peces con y sin inflamación crónica, mostrando que la deficiencia en Spint1a en células de SKCM aumentaba su invasión. No sucedió lo mismo en el caso de la deficiencia de Spint1a en las células del estroma tumoral. En este caso, se encontró que la deficiencia de Spint1a en el microambiente tumoral también promovía la diseminación en el modelo de alotransplante en larvas. Para confirmar el papel de Spint1a tanto en las células de melanoma como en las del microambiente tumoral, se llevó a cabo un ensayo de alotransplante en larvas inyectando en este caso las diferentes combinaciones de ambos tipos celulares. Estos resultados sugirieron que la deficiencia en Spint1a aumenta la invasión de SKCM tanto por mecanismos autónomos como no autónomos.

Tras varios ensayos de alotransplante llevados a cabo en peces adultos previamente inmunodeprimidos, se demostró que la deficiencia de Spint1a en células SKCM también aumentaba la agresividad.

Para comprender los mecanismos implicados en la agresividad de SKCM mediada por Spint1a se analizó la expresión de genes que codifican biomarcadores importantes relacionados con la diferenciación, EMT, inflamación e interferón demostrando que la deficiencia de Spint1a promueve la diferenciación y la inflamación de SKCM señalando a los macrófagos y al interferón como importantes en el papel de la agresividad producida por la deficiencia de Spint1a en SKCM.

Por último se llevó a cabo la generación de una línea celular estable de melanoma de pez cebra deficiente en spint1a con el objetivo de ser usada como herramienta para el chequeo masivo de drogas para posibles futuros tratamientos.

Así, las principales conclusiones derivadas de los resultados obtenidos en este trabajo son:

1. Las alteraciones genéticas en *TNFA* y *TNFR2* tienen lugar en un 5 y 6 % de los pacientes de melanoma, respectivamente, y estas alteraciones están asociadas con mal pronóstico.
2. La inflamación producida a través del eje de señalización TNFA/TNFR2 acelera la aparición del melanoma, mejorando la transformación oncogénica y la progresión del melanoma.
3. Mientras que la sobreexpresión de TNFR2 no presenta efectos en la transformación oncogénica, la señalización vía TNFR2 en células del estroma es responsable del incremento de la proliferación de células transformadas. Aun cuando las dos condiciones están caracterizadas por

presentar el mismo número de neutrófilos, las larvas deficientes en Tnfr2 muestran un aumento de la interacción con las células transformadas cuando fueron comparadas con sus controles.

4. Las alteraciones genéticas en *SPINT1* están presentes en el 10% de pacientes con melanoma y además están asociadas con un pronóstico desfavorable. La expresión de *SPINT1* se mostró significativamente inhibida en SKCM humano en comparación con nevus o piel normal. Además dicha expresión se correlaciona con una alteración del ciclo celular, diferenciación y vías de señalización del sistema inmune innato y adaptativo.
5. Los niveles de transcripción de *SPINT1* en muestras de melanoma humano se correlacionaron positivamente con la infiltración de macrófagos, pero no de neutrófilos, en muestras de tumores SKCM.
6. La inflamación crónica de la piel impulsada por la deficiencia de Spint1a aumenta la agresividad de SKCM en los modelos preclínicos de pez cebra, incrementando así la transformación oncogénica y acelerando la aparición de SKCM impulsado por el oncogén HRAS.
7. Los ensayos de alotransplante en larvas y peces cebra adultos mostraron un papel clave de la inflamación crónica, desencadenada por la deficiencia de Spint1a, en la diseminación de las células SKCM.
8. La deficiencia de Spint1a tanto en el melanoma como en el estroma del microambiente tumoral contribuye a aumentar la agresividad del melanoma.
9. Tanto los datos clínicos como los datos obtenidos con el modelo del pez cebra indican que la pérdida de Spint1a facilita el cambio al fenotipo de EMT en SKCM.
10. SPINT1 juega un papel crucial en la regulación de la comunicación entre las células tumorales y las células inflamatorias en SKCM, abriendo nuevas oportunidades para la intervención clínica permitiendo la creación de nuevas terapias personalizadas que ayuden a contrarrestar la agresividad del melanoma.

ANEXXE I: Participation in national and international conferences

1. García-Moreno D, Bernabé M, **Gómez-Abenza E**, Tyrkalska SD, Cayuela ML, Mulero V.*Generation of zebrafish mutants using talen and crispr-cas technologies: a valuable tool to study hematopoiesis, inflammation and cancer.* Zebrafish disease models 6 (Zdm6),Haematopoiesis, Inflammation, Immunity, Infection, Cancer and Vascular Biology Workshop, Murcia (Spain), 14-17 July 2013. Poster.

2. **Gómez-Abenza E**; Gabellini C.; García-Moreno D; Mione M; Mulero V. *Impact of inflammation in a spontaneous model of melanoma in zebrafish.* 23rd Biennial Congress of the European Association for Cancer Research (EACR23). From Basic Research to Personalized Cancer Treatment, Munich (Germany), 5-8 July 2014. Poster.

3. Gabellini C; **Gómez-Abenza E**; De Oliveira S; Del Bufalo D; Mulero V. *Bcl-xL protein overexpression enhances tumor progression of human melanoma cells in zebrafish xenograft model: involvement of interleukin 8*. Biennial Congress of the European Association for Cancer Research (EACR23). From Basic Research to Personalized Cancer Treatment, Munich (Germany), 5-8 July 2014. Poster.

4. **Gómez-Abenza E**; Gabellini C.; Mione M; Cayuela ML; Mulero V. *Chronic skin inflammation enhances tumorigenesis in a spontaneous model of melanoma in zebrafish*.Cell Symposia: Cancer, Inflammation and Immunity, Sitges (Spain). 14-16 July 2015. Poster.

5. Gabellini C; **Gómez-Abenza E**; De Oliveira S; Del Bufalo D; Mulero V. *Bcl-xL protein overexpression enhances tumor progression of human melanoma cells in zebrafish xenograft model: Involvement of interleukin 8*.Cell Symposia: Cancer, Inflammation and Immunity, Sitges (Spain). 14-16 July 2015. Poster.

6. García-Moreno D; Bernabé M; Alcaraz-Pérez F; **Gómez-Abenza E**; Gabellini C; Martínez-Morcillo FJ; Adatto I; Zon LI; Cayuela ML; Mulero V.*In vivo genome editing technologies in zebrafish to study immunity and inflammation*. The 13rd ISDCI Congress, Murcia (Spain). 28 June 2015. Poster.

7. Ibáñez-Molero S; **Gómez-Abenza E**; Mulero V. *Obtención de modelos de animales para estudiar el impacto del estrés oxidativo en el cáncer de piel*. IV Congreso de InvestigaciónBiomédica (CIB 2016), Valencia (Spain). 10-12 February 2016. Oral presentation.

8. Ibáñez-Molero S; **Gómez-Abenza E**; Mulero V. *Modelling the impact of oxidative stress and inflammation in melanoma using the zebrafish*. Jornadas de Inicio a la Investigación en Biología, Murcia (Spain). 2-23 June 2016. Oral presentation.

9. **Gómez-Abenza E**; Gabellini C.; Mione M; Cayuela ML; Mulero V. *A spontaneous model of melanoma in zebrafish as a tool to evaluate the impact of inflammation in tumor development*. II Jornadas Doctorales de la Universidad de Murcia, Murcia (Spain). 2 June 2016. Poster.

10. Ibáñez-Molero S; **Gómez-Abenza E**;Martínez-Morcillo FJ; García-Moreno D; Mulero V. *Modelling the impact of oxidative stress and inflammation in melanoma using thezebrafish*. I Jornadas científicas del IMIB- Arrixaca, Murcia (Spain). 21 November 2016. Poster.

11. **Gómez-Abenza E**; Gabellini C.; Mione M; Zon LI; Cayuela ML; Mulero V. *Chronic skin inflammation promotes melanoma aggressiveness in zebrafish*. IJornadas científicas del IMIB - Arrixaca, Murcia (Spain). 21 November 2016. Oral presentation.

12. **Gómez-Abenza E**;Ibáñez-Molero S;Gabellini C.; Mione M; Zon LI; Cayuela ML; Mulero V. *Inflammation promotes melanoma aggressiveness in zebrafish*. Cell Symposium: Hallmarks of Cancer, Ghent (Belgium). 11-13 December 2016. Poster.

13. Ibáñez-Molero S; **Gómez-Abenza E**; Martínez-Morcillo FJ; García-Moreno D; Mulero V. *Identification of new therapeutical targets for melanoma using the zebrafish*. Cell Symposium: Hallmarks of Cancer, Ghent (Belgium). 11-13 December 2016. Poster.

14. **Gómez-Abenza E**; Ibáñez-Molero S;Gabellini C.; Meseguer J; García-Moreno D; Pérez-Oliva AB; Cayuela ML;Mulero V. *Modelling the crosstalk between inflammation and cancer using the zebrafish*. Congreso Nacional de Biotecnología (Biotec 2017), Murcia (Spain). 18-21 June 2017. Poster.

15. Rodríguez-Ruiz L; Ibañez-Molero S; **Gómez-Abenza E**; Martínez-Balsalobre E; Fernández-Lajarín M; Naranjo E; García-Moreno D; Cayuela ML; Pérez-Oliva AB; Mulero V.*Modelling the role of melanoma secretoma in the education of the pre-metastatic nicheusing the zebrafish*. European Association forCancer Research (EACR) Conference Series: Defence is the Best Attack. Immuno-Oncology Breakthroughs, Barcelona (Spain). 9-11 October 2017. Poster.

16. Ibañez-Molero S; **Gómez-Abenza E**; Rodríguez-Ruiz L;Fernández-Lajarín M; Martínez-Balsalobre E; Naranjo E; García-Moreno D; Cayuela ML; Pérez-Oliva AB; Mulero V. *Deciphering the crucial role of oxidative stress and inflammation in cancer: Silencing ofDuox1 enhances melanoma aggressiveness*. European Association for Cancer Research (EACR) Conference Series: Defence is the Best Attack. Immuno-Oncology Breakthroughs, Barcelona (Spain). 9-11 October 2017. Poster.

17. **Gómez-Abenza E**; Gabellini C.; Ibáñez-Molero S; Mione M; Zon LI; Cayuela ML; Mulero V. *Chronic skin inflammation increases melanoma aggressiveness in zebrafish*. 1stAsociación Española de Investigación sobre el Cáncer (ASEICA) Educational Symposium, Madrid (Spain). 14-15 November 2017. Poster.

18. **Gómez-Abenza E**; Gabellini C.; Ibáñez-Molero S; Mione M; Zon LI; Cayuela ML; Mulero V.*Impact of chronic skin inflammation in melanoma aggressiveness in zebrafish*. II Jornadas científicas del IMIB – Arrixaca, Murcia (Spain). 27 November 2017. Poster.

19. **Gómez-Abenza E**; Gabellini C.; Ibáñez-Molero S; Mione M; Zon LI; Cayuela ML; Mulero V. *Chronic inflamed microenvironment as a key player to enhance melanoma aggressiveness*. 16th ASEICA International Congress, Valencia (Spain). 6-8 November 2018. Poster.

20. **<u>Gómez-Abenza E</u>**; Gabellini C.; Ibáñez-Molero S; Mione M; Zon LI; Cayuela ML; Mulero V. *Effect of inflamed microenvironment in melanoma: friend or foe*. III Jornadas científicas del IMIB- Arrixaca, Murcia (Spain). 19-20 November 2018. Oral presentation.

ANEXXE II: Short stays at international research centers

1. *Host institution:* **Institute of Toxicology and Genetics.**
Karlsruhe Institute of Technology (KIT).

Country: **Karlsrhue, Germany.**

Responsible person in the Host: **Dr. Maria Caterina Mione**

Stay period: **5th April 2014 – 2nd August 2014**

2. *Host institution:* **Division of Hematology/Oncology,**
Department of Medicine,
Children's Hospital Boston.
Harvard Medical School, Harvard University.

Country: **Boston, United States of America.**

Responsible person in the host: **Dr. Leonard I. Zon, M.D.**

Stay period: **5th April 2016 – 27th July 2016.**

ANEXXE III: Publications derived from the thesis

1. **Gómez-Abenza,E.**, Ibáñez-Molero,S., García Moreno, D., Fuentes, I., Zon, L.I., Mione, M. C., Cayuela, M. L., Gabellini, C., Mulero,V. (2019). Zebrafish modeling reveals that SPINT1 regulates the aggressiveness of skin cutaneous melanoma and its crosstalk with tumor immune microenvironment. *Journal of Experimental & Clinical Cancer Research*: CR, 38(1), 405. doi:10.1186/s13046-019-1389-3

ANEXXE IV: Other publications related to the thesis

1. Gabellini, C., **Gómez-Abenza, E.**, Ibáñez-Molero, S., Tupone, M.G., Pérez-Oliva, A.B., de Oliveira, S., Del Bufalo, D. and Mulero, V. (2018), Interleukin 8 mediates bcl-xL-induced enhancement of human melanoma cell dissemination and angiogenesis in a zebrafish xenograft model. *Int. J. Cancer*, 142: 584-596. doi:10.1002/ijc.31075.

2. García-Moreno, D., Tyrkalska, S.D., Valera-Pérez, A.,**Gómez-Abenza, E.**, Pérez-Oliva, A.B., Mulero, V. (2019), The zebrafish: A research model to understand the evolution of vertebrate immunity. *Fish Shellfish Immunol*, 90:215-222. doi: 10.1016/j.fsi.2019.04.067.

ANEXXE V: Tutor of undergraduate students

1. *Student:* **Sofía Ibáñez Molero.**

Project title: The zebrafish as a model of biomedical research.
Type of project: TFG*.
Defense date: 2016.
Centre: Universidad de Murcia.
Grade: 9.8/10 (Excellent).

2. *Student:* **Isabel Sánchez Lozoya.**

Project title: The zebrafish as a model of biomedical research.
Type of project: TFG*.
Defense date: 2016.
Centre: Universidad de Murcia.
*Grade:*8.7/10 (B).

*TFG: Final Grade Thesis