# UNIVERSIDAD AUTÓNOMA DE MADRID

Facultad de Medicina

Departamento de Bioquímica

UAM
UNIVERSIDAD AUTÓNOMA
DE MADRID

## Doctoral Thesis

## Mechanistic characterization of p62 as a driver of melanoma metastasis

## PANAGIOTIS KARRAS

Madrid, 2017

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Madrid, 2017

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CERTIFIES:

That the study **"Mechanistic characterization of p62 as a driver of melanoma metastasis"** developed by **Panagiotis Karras** meets the necessary requirements to obtain the **PhD Degree** and, to this purpose, will be presented at the Universidad Autónoma de Madrid. This work has been carried out under my direction and hereby I authorize its defense to a specific PhD Committee assembled for this purpose.

I hereby issue this certification in Madrid on May 22nd 2017.
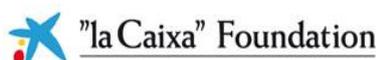
María S. Soengas

PhD, Thesis Director

The work in this doctoral thesis was carried out in the Melanoma Group at the Spanish National Cancer Research Centre (CNIO) from November 2011 to June 2017 under the supervision of María S. Soengas.

*Dedicated to my father*

*Αφιερωμένη στον πατέρα μου*

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# CONTENTS

# I.ABBREVIATIONS

**4-HT**        4-hydroxytamoxifen

**ActD**        Actinomycin D

**AJCC**        American Joint Committee on Cancer Melanoma Staging

**ALM**         Acral Lentigious Melanoma

**AMBRA1**      Autophagy And Beclin 1 Regulator 1

**aPKC**        atypical protein kinase C

**ASH**         Alcoholic  SteatoHepatitis

**ATCC**        American Type Culture Collection

**ATG5**        Autophagy protein 5

**ATGs**        AuTophaGy-related genes

**BAP1**        BRCA1 associated protein 1

**BECN1**       Beclin 1

**BRAF**        v-raf murine sarcoma viral oncogene homolog B

**BRCA1**       BReast CAncer 1

**BSA**          Bovine serum albumin

**CAFs**        Cancer-Associated Fibroblasts

**CCLE**        Cancer Cell Line Encyclopedia

**CCND1**       CyCliN D1

**CDK1**        Cyclin-Dependent Kinase 1

**CDK4**        Cyclin-Dependent Kinase 4

**CDKN2A**      Cyclin Dependent Kinase Inhibitor 2A

**cDNA**        complementary DNA

**CNIO**        Centro Nacional de Investigaciones Oncológicas

**CNS**         Central Nervous System

**CREB**        cAMP Response Element-Binding protein

**CSCs**        Cancer Stem Cells

**DAPI**         4,6-diamidino-2-phenylindole

**DCT**         DopaChrome Tautomerase

**DFCP1**       Double FYVE-Containing Protein 1

**DMEM**        Dulbecco's Modified Eagle's Medium

**ECAR**        Extra-Cellular Acidification Rate

**eCLIP**       enhanced UV CrossLinking and Immunoprecipitation

**EDTA**        Ethylenediaminetetra-acetic acid

**EMT**         Epithelial to Mesenchymal Transition

**ESCRT**       Endosomal Sorting Complex Required for Transport

**FBS**         Fetal Bovine Serum

| | |
|---|---|
| **FDA** | Food and Drug Administration |
| **FDR** | False Discovery Ratio |
| **FERMT2** | Fermitin family homolog 2 |
| **GABARAP** | GABA(A) Receptor-Associated Protein |
| **GEMM** | Genetically Engineered Mouse Model |
| **GNAQ** | guanine nucleotide binding protein α q polypeptide |
| **GO** | Gene Ontology |
| **GSEA** | Gene Set Enrichment Analysis |
| **HCC** | hepatocellular carcinoma |
| **HDACis** | Histone DeACetylase inhibitors |
| **HES1** | Hairy/Enhancer of Split |
| **HKGF** | Human Keratinocyte Growth Factors |
| **HRAS** | v-Ha-ras harvey rat sarcoma viral oncogene homolog |
| **HRP** | HorseRadish Reroxidase |
| **IGF2BP** | Insulin-like Growth Factor 2 mRNA-Binding Proteins |
| **IgG** | Immunoglobulin G |
| **IHC** | ImmunoHistoChemistry |
| **IPA** | Ingenuity Pathway Analysis |
| **iTRAQ** | isobaric Tag for Relative and Absolute Quantitation |
| **JNK** | c-Jun N-terminal kinases |
| **KEAP1** | Kelch Like ECH Associated Protein 1 |
| **KIR** | Keap1-Interacting Region |
| **KO** | knock out |
| **LC3** | Microtubule-associated proteins 1A/1B light chain 3B |
| **LC-MS/MS** | Liquid Chromatography tandem-Mass Spectrometry |
| **LIR** | LC3-Interacting Region |
| **LMM** | Lentigo Malignant Melanoma |
| **MAPK** | Mitogen-Activated Protein Kinase |
| **MC1R** | melanocortin 1 receptor |
| **MEFs** | Mouse Embryonic Fibroblasts |
| **MITF** | microphthalmia-associated transcription factor |
| **MM** | Mucosal Melanoma |
| **mRNA** | messenger RNA |
| **mTOR** | mechanistic Target Of Rapamycin |
| **mTORC1** | mechanistic Target Of Rapamycin Complex 1 |
| **MVB** | MultiVesicular Body |

**MYC**          V-Myc Avian Myelocytomatosis Viral Oncogene Homolog

**NASH**         Non Alcoholic  SteatoHepatitis

**NBR1**         Neighbor of BRCA1 gene 1

**NDP52**        Nuclear Dot Protein 52

**NF1**          NeuroFibromin 1

**NF-κB**        Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1

**NM**           Nodular Melanoma

**NQO1**         NAD(P)H Quinone dehydrogenase 1

**NRAS**         Neuroblastoma RAS viral oncogene homolog

**NRF2**         NFE2-Related Factor 2

**OCR**          Oxygen Consumption Rate

**OIS**          Oncogene-Induced Senescence

**PanIN**        Pancreatic Intraepithelial Neoplasia

**PAX3**         Paired box gene 3

**PB1**          Phox1 and Bem1p

**PCR**           Polymerase Chain Reaction

**PE**           PhosphatidylEthanolamine

**PI(3)K**       Phosphatidylinositol-4,5-bisphosphate 3-Kinase

**PKA**          protein kinase A

**PtdIns3P**     Phosphatidylinositol-3-Phosphate

**PTEN**         Phosphatase and TEnsiN homologue

**PyMT**         Polyoma Middle T antigen

**qPCR**         quantitative Polymerase Chain Reaction

**RB1**          RetinoBlastoma 1

**RBP**          RNA Binding Protein

**RGP**          Radial-Growth Phase

**RIP**          RNA ImmunoPrecipitation

**ROS**          Reactive Oxygen Species

**RT- qPCR**     Reverse transcription- quantitative polymerase chain reaction

**RT-PCR**       Real time  Polymerase Chain Reaction

**RXR**          Retinoid X Receptor

**S6K1**         Ribosomal protein S6 Kinase beta-1

**SDS-PAGE**     Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

**SEM**          Standard error of estimate of mean

**SF3B1**        Splicing Factor 3B subunit 1

**sgRNA**        short guide RNA

| | |
|---|---|
| **shRNA** | short haipin RNA |
| **siRNA** | small (or short) interfering RNA |
| **SMG** | Significantly Mutated Genes |
| **SOX10** | SRY-box-containing gene 10 |
| **SQSTM1** | Sequestosome-1 |
| **SRY** | Sex-determining Region Y |
| **SSM** | Superficial Spreading Melanoma |
| **STRING** | Search Tool for the Retrieval of Interacting Genes/Proteins |
| **SWI/SNF** | SWItch/Sucrose Non-Fermentable |
| **TB** | TRAF6 Binding |
| **TCGA** | The Cancer Genome Atlas |
| **TMA** | Tissue MicroArray |
| **TNM** | Tumor Node Metastasis |
| **TP53** | Tumor protein 53 |
| **TRAF6** | TNF Receptor-Associated Factor 6 |
| **TYRP1** | tyrosinase-related protein-1 |
| **UBA** | UBiquitin-Associated |
| **ULK1** | Unc-51-Like Kinase 1 |
| **UM** | Uveal Melanoma |
| **UTR** | UnTranslated Region |
| **UV** | UltraViolet |
| **UVRAG** | UV irradiation Resistance-Associated Gene |
| **VDR** | Vitamin D Receptor |
| **VGP** | Vertical-Growth Phase |
| **Vps34** | Vacuolar protein sorting-associated protein 34 |
| **WHO** | World Health Organisation |
| **αMSH** | α-melanocyte stimulating hormone |

# II.SUMMARY/RESUMEN

**Summary**

Cutaneous melanoma is the most aggressive form of skin cancer. The aggressive nature of this disease is manifested by the fact that lesions of barely 2 mm in depth already bear a high potential for metastasis. High throughput analyses have identified recurrent alterations in oncogenic pathways (i.e involving BRAF, NRAS, PI3K>AKT, NF1 or NOTCH, among others) that have paved the way for the development of molecularly-targeted therapies. However, melanoma is also a prime example of how a complex (epi)genome can activate compensatory protective responses (also against recently-approved immune checkpoint blockers). A rather unexplored strategy to search for putative tumor drivers is to focus on lineage-specific oncogenic dependencies rather on common cancer hallmarks conserved with other malignancies. In melanoma, these lineage-specific oncogenes have been traditionally linked to melanosome and pigmentation pathways. However, our group has recently identified new signalling cascades distinctively enriched in melanoma cells. These include specific RNA binding proteins (CPEB4, UNR), as well as over 30 endolysosomal factors. Analysis of autophagy, another lyososmal-associated process also revealed selective heterozygous loses in ATG5 as a feature favouring poor patient prognosis.

p62/SQSTM1 (sequestosome 1) was an attractive candidate to pursue for its known links in autophagy and as a signaling hub for multiple protein-protein complexes. Importantly, although p62 has been found accumulated in a broad spectrum of tumor types, there is no clear consensus on whether it acts as a tumor promoting or tumor suppressive agent, particularly in the context of metastasis.

Here we present a comprehensive analysis of p62 expression and functional requirement in melanoma cells, clinical biopsies and newly generated genetically engineered mouse models. This strategy was used to assess key pending questions in this field such as: *(i)* what is the relative impact of p62 copy number in the stroma vs benign and malignant melanocytic lesions, particularly in the context of metastasis; *(ii)* does p62 share autophagy and non-autophagy roles with other tumor types, or *(iii)* does it act in a tumor-specific manner?, and if so, *(iv)* what are the underlying mechanisms. To this end, particular attention was dedicated to genome-wide analyses of the p62 interactome and its global impact on the transcriptome and proteome of melanoma cells. Computational analyses, studies in GEMM and proteomic analyses were also performed in comparison to ATG5. In addition, targeted gene depletion was exploited to assess best known functions of p62 as a signaling hub. Together, this integrated approach revealed a distinct impact of p62 in the control of melanoma-enriched gene networks. Finding an unexpected role of p62 opposing mRNA decay of *FERMT2* and other pro-metastatic factors, our data ultimately revealed a new function of p62 as a hub of RNA binding proteins. Together, the results of this PhD thesis offer a platform for tumor-type specific functions of p62 in cancer.

## Resumen

El melanoma cutáneo es la forma más agresiva y letal de cáncer de piel, tanto es así, que lesiones de menos de 2 mm de grosor ya poseen una gran capacidad metastásica. A través del desarrollo de múltiples análisis a gran escala se ha se han podido identificar una serie de alteraciones recurrentes en diversas rutas de señalización oncogénicas (BRAF, NRAS, PI3K >AKT, NF1 y/o NOTCH entre otros), lo que ha facilitado el desarrollo de terapias genéticamente dirigidas. A pesar de ello, el melanoma es uno de los mejores ejemplos de cómo un (epi) genoma tan complejo y con tal número de alteraciones, puede resultar en la activación de mecanismos compensatorios de adaptación y protección frente a los tratamientos vigentes. Una estrategia aún poco explorada en la identificación de nuevas dianas pro-oncogénicas es el estudio restringido de las dependencias oncogénicas asociadas a linaje, en lugar de perseguir alteraciones comunes a otros tipos de tumores. En melanoma estos oncogenes específicos de linaje se han asociado clásicamente a rutas de pigmentación y producción de melanosomas. Sin embargo nuestro grupo ha identificado nuevas rutas de señalización selectivamente enriquecidas en melanoma. Entre sus componentes se han encontrado proteínas de unión a RNA (CPEB4, UNR) y más de 30 proteínas endolisosomales. Por otro lado, estudios previos de nuestro grupo sobre autofagia, un proceso asociado a los lisosomas, muestran que la pérdida selectiva de una copia del gen que codifica para la proteína ATG5 se encuentra asociada a un pronóstico desfavorable en pacientes. Por todo lo anteriormente mencionado, p62/SQSTM1 (sequestosoma 1) se presenta como un candidato atractivo en melanoma, tanto por su relevante función en autofagia, como por funcionar como plataforma de señalización en numerosos tipos tumorales. Sin embargo, aunque se ha observado que p62 se encuentra sobre-expresado en múltiples tipos de cáncer, aún no existe un consenso sobre su papel como promotor o supresor tumoral, particularmente en metástasis. Por todo ello, esta tesis se planteó con los siguientes objetivos: (i) determinar del impacto en el número de copias de p62, tanto en el estroma tumoral como en lesiones melanocíticas (benignas y malignas), y particularmente en melanoma metastásico; (ii) definir si las funciones de p62, en autofagia y otros procesos celulares, son específicos en melanoma o comunes a otros tumores (iii), y por último, (iv) caracterizar el mecanismo de acción específico en melanoma. Para responder a estas cuestiones se ha realizado un exhaustivo análisis de la función de p62 en líneas celulares de melanoma, biopsias clínicas y modelos animales modificados genéticamente. A su vez, se ha dedicado especial énfasis al interactoma de p62 y al impacto que esta proteína tiene en el transcriptoma y proteoma celular. En paralelo, se ha analizado mediante estudios de proteómica y computacionales el papel de p62 y ATG5 en animales modificados genéticamente para ambos genes. Y por último, mediante ensayos de pérdida de función, se ha caracterizado la función de p62 como plataforma de señalización.

Integrando las aproximaciones anteriormente descritas, se ha descubierto que p62 tiene un papel protector en la estabilidad del mRNA de FERMT2 y otros factores pro-metastásicos, por lo que se describe por primera vez la función de p62 como plataforma de señalización a través de proteínas de unión a RNA (RBPs). En conjunto, estos resultados revelan nuevas funciones de p62 y determinan su especificidad en melanoma.

# III.INTRODUCTION

## 3.1    Melanocyte: the cell of origin of melanoma

Melanocytic neoplasms range from benign lesions (melanocytic nevi), to malignant ones, termed melanomas. All originate from melanocytes, which are neural crest-derived cells[1]. Although melanocytes are located in mucosas, leptomeninges and uveal sites, these cells reside primarily in the skin[2-5]. Consequently, most melanomas are of cutaneous origin, accounting for ~90% of deaths from skin cancer[6] due to the high aggressive capacity to metastasize[7-10].
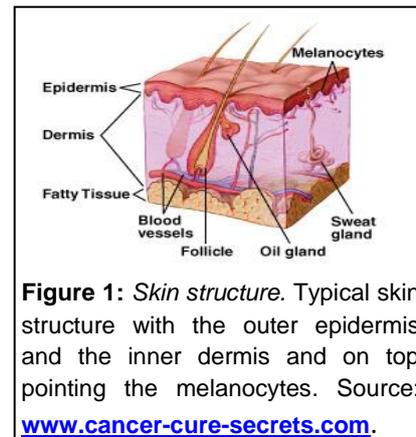


**Figure 1:** *Skin structure.* Typical skin structure with the outer epidermis and the inner dermis and on top pointing the melanocytes. Source: **www.cancer-cure-secrets.com**.

The melanocyte lineage is derived from the neural crest, which has its origins in the neural tube. Following its formation, neural crest cells delaminate from the dorsal-most aspect of the neural tube by a process of epithelial-to-mesenchymal transition. These cells are highly migratory and form many specialised structures in the developing embryo by migration, proliferation and differentiation[11]. **Melanoblasts**, the melanocytes precursors, migrate during embryonic development mostly to the basal layer of the skin (**Figure 1**), where melanocyte:keratinocyte number ratio is typically of 1:10[12,13]. Keratinocytes, together with surrounding cells like dermal fibroblasts sustain melanocyte function and survival in response to environmental signals such as UV radiation[14]. There are approximately 1,500 melanocytes per square millimetre of human epidermis, corresponding to nearly 3 billion cutaneous melanocytes in the skin of an average human[15]. Melanocytes represent a minority cell population within the basal epidermis, and divide infrequently, less than twice a year[16] and mainly provide melanin pigment to their neighbouring keratinocytes[17]. Melanin is a complex macromolecule that scatters and absorbs UV radiation, and epidermal keratinocytes use it to protect their nucleus from UV radiation-induced DNA damage[18]. The pigments are synthesized within specialized organelles called **melanosomes**. Mechanistically, the proliferation and pigment production of melanocytes is stimulated by UV radiation-induced DNA damage to keratinocytes, which subsequently secrete α-melanocyte stimulating hormone (αMSH) in a p53-dependent manner[19]. αMSH binds to the melanocortin 1 receptor (MC1R), which is expressed on melanocytes activates the cAMP/protein kinase A (PKA)/CREB signaling pathway and enhances the level of the microphthalmia-associated transcription factor (MITF). MITF promotes the synthesis of melanin, which melanocytes deliver to keratinocytes[20]. A reduction in its activity is coupled to reduce melanin synthesis and pigmentation[21]. MITF also regulates melanosome biogenesis[22,23] and trafficking[24].

In addition to MITF, during embryogenesis, the survival and migration of melanocytes rely on other signaling pathways such as Wingless signaling (Wnt)/$\beta$-catenin, the endothelin B

receptor and its ligand endothelin-3, the receptor tyrosine kinase KIT and its ligand KIT-ligand/SCF (stem cell factor), NOTCH[25,26], paired box gene 3 (PAX3), SRY-box10 (SOX10), hairy/enhancer of split (HES1)[27-31]. Outside the skin, melanocytes are present in considerable numbers in the uveal tract of the eye and at lower densities in other tissues, such as the meninges and the anogenital tract. However, the melanocytic neoplasms that originate from these melanocytes are distinctly different in their histopathological and genetic status from cutaneous melanomas[5,32-34].

The treatment landscape for advanced-stage, unresectable or metastatic melanoma has shifted dramatically over a short period of time. From a devastating disease with poor survival (~9 months) the discovery of FDA approved targeted agents and immunotherapeutic antibodies (**Table S1**, appendix) has led to a tremendous improvement of the response rates and the increase in overall survival with the simultaneous reduction of the adverse effects[35].

## 3.2 Classification of melanocytic lesions

### 3.2.1 Bening melanocytic nevi

Melanocytic nevi are benign proliferations of melanocytes with theoretically a low likelihood of progressing to melanoma. Nevertheless, their high prevalence makes them contributors to a considerable portion of melanomas[36-38]. **Congenital nevi** are presented at birth or shortly after, while **acquired nevi** tend to appear with more than six months of age and are accumulated during life time[32]. Acquired nevi can exhibit a *lentiginous growth pattern* or a *congenital growth pattern*. The latter category does not necessarily imply that the nevi was present at birth as in bona fide congenital nevi, but indicates that it has a similar growth pattern. Lentiginous and congenital pattern nevi can be stratified as **i.  junctional**, in which melanocytes appear at the dermo-epidermal junction; **ii. dermal,** depending on whether melanocytes appear on the dermis; and **iii. compound**, when melanocytes appear in both places, respectively[39-41]. **Spitz nevi** are another type of melanocytic neoplasm characterized by a predominance of large polygonal (epithelioid) or spindled melanocytes with enlarged nuclei and often multinucleated cells[42,43]. **Blue nevi** are characterized by the proliferation of dendritic, spindled, ovoid, or epithelioid melanocytes in tissues without any significant epithelial involvement. While the majority involve the skin, they can be encountered in many other organs[39,44]. A long-standing debate and controversial category of benign lesions is termed as **intermediate melanocytic neoplasm**. This grey zone of neoplasms contains lesions with overlapping benign and malignant histopathological features. The overall mutation burden is higher than those of unequivocally benign lesions. This category also contains the contentious category of lesions known as **dysplastic nevi**[45-

[47]. There is clear evidence that individuals with multiple dysplastic nevi are at increased risk of developing melanoma over their lifetime[48,49].

The mutational status of oncogenes in benign nevi such as **NRAS** in congenital nevi[50], **HRAS** in spitz nevi[43], and later **BRAF** in acquired nevi[51], **GNAQ** or **GNA11** in blue nevi[34,52] came as a surprise as many of them are common in melanomas. After acquiring an initiating mutation, a melanocyte will undergo limited proliferation to form a nevus, before entering a state that has been described as "senescence-like"[53,54]. However, several observations indicate that at least some melanocytes within a nevus retain the ability to proliferate[55,56].

### 3.2.2 Malignant melanoma

*Classification by anatomical site*

Melanoma has been classified in several ways taking into account various variables such as histopathological type, vertical growth, whether it has spread to nearby lymph nodes or any other organ, among other factors. The traditional clinical and pathological classification scheme for melanoma includes four subtypes based on the pioneering work of Wallace Clark, more than forty years ago[60], and has been included to the current World Health Organisation (**WHO**) classification[61]. The 4 categories are briefly described below with representative images and the most common mutated genes depicted in **Figure 2[57-59]**.



| | Image | Histology | Common Mutated Genes |
|---|---|---|---|
| SSM | | | **BRAF 59-78%** NRAS 3-22% |
| NM | | | **BRAF 43-68%** NRAS 12-31% |
| ALM | | | BRAF 12-23% NRAS 8-15% **KIT 9-36%** |
| LMM | | | **BRAF 40-60%** NRAS 15-29% KIT 16-28% |
| UM | | | **GNAQ 46-50%** KIT 1-76% |
| MM | | | BRAF 3-11% NRAS 5-13% **KIT 15-39%** |
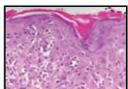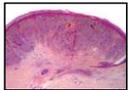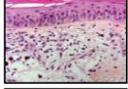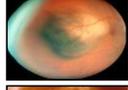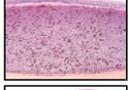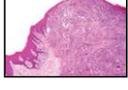
**Figure 2:** *Traditional melanoma subtypes and their most frequently mutated oncogenes.* Source: Refs.[57-59].

I. **Superficial Spreading Melanoma (SSM)** is the most common subtype, comprising approximately 75% of all malignant melanomas. A quarter of this type of melanoma are originate from with a pre-existing nevi, while most develop de novo[62]. **SSM** is usually flat with an intra-epidermal component and is linked to episodes of severe sunburn[63-65].

II. **Nodular Melanoma (NM)** represents 15 to 30% of all melanomas. They progress to invasiveness more quickly than any other type of melanoma. Although it is an aggressive lesion, the prognosis for a patient with a NM is the same as for any other subtype[65,66].

III. **Acral Lentigious Melanoma (ALM)** is the least common variant, representing 2% to 8% of all melanomas in white people and 29% to 72% in dark skin population. ALM commonly develops in palmar regions, plantar regions, and occasionally subungual surfaces[65,67]

IV. **Lentigo Malignant Melanoma (LMM)** accounts for 4% to 15% of melanomas. LMM represents *in situ* melanoma that 5% to 8% of the cases may progresses to invasive lentigo malignant melanoma. Transformation is slow and the lesion can be present for 10 to 50 years before undergoing vertical growth[68,69].

Furthermore, other rare forms of melanoma such as **uveal melanoma** (UM) and **mucosal melanoma** (MM) belong to the category of **non-cutaneous melanoma**. UM, account for approximately 5% of all melanomas in the US with a 10-year survival rates at 50% and is among the most lethal presentations of melanoma[70,71]. MM, account for 1% of all melanomas and primarily involves the mucosa of the anogenital region, nasal cavity, and paranasal sinuses, but can occur in all mucosal sites[72].

*Classification by clinicopathological criteria (Clark model-linear evolution)*

Cancer progression has been conceptualized as a multistep process whereby normal cells accumulate genetic alterations that enable tumor growth and metastatic dissemination[73]. Classically, the different steps of melanoma progression are characterized by different histologic lesions according to the Clark model[8,74,75]. Melanoma is thought to arise in one of two ways: (1) with no visible precursor lesion or (2) in association with a benign melanocytic proliferation (nevus or mole). Five distinct stages have been proposed in the evolution of melanoma on the basis of such histological criteria (**Figure 3**):

**I. Benign nevi.** This first step refers to the proliferation of structurally normal melanocytes leading to the benign nevi. The absence of progression is probably due to **Oncogene-Induced Senescence (OIS)**, in which growth that is stimulated by the activation of oncogenic pathways is limited[76]. Hotspot mutations in ***BRAF*** (up-to 82% of acquired nevi[51]) or ***NRAS*** genes (up-to 81% of congenital nevi[77]) lead to the MAPK pathway activation which triggers a stress response leading to permanent G1 arrest[78,79]. However, these features cannot be used as markers of benign melanocytic tumors since malignant melanoma cells may also display similar phenotypic and molecular signatures[80-84]. It has been reported that senescence-like phenotypes can be induced genetically in metastatic melanoma cells suggesting that induction of senescence could be a novel therapeutic approach to treat melanoma[85-87].

**II. Dysplastic nevi.** They may arise from pre-existing benign nevi or as new lesions. Genetically, in 25-40% of cases of familial melanoma, a genetic defect inactivates **CDKN2A**, a single gene that encodes two tumor suppressor proteins, p16[INK4A] and p19[ARF88-90]; in 25-50% of non-familial melanoma, a different tumor-suppressor gene, phosphatase and tensin homologue (**PTEN**), is inactivated by mutation[91-93]. **CDKN2A** or **PTEN** mutations are not

enough to the development of melanoma, but it is unclear precisely when such mutations occur[8].

**III. Radial Growth Phase (RGP). RGP** consist in the intraepidermal proliferation of melanoma cells, which can be confined to the epidermis forming an ***in situ* melanoma** or penetrate the papillary dermis growing as small nests[94]. Multiple pathogenic alterations are accumulated over a considerable period of time, as reflected by their relatively late age of onset and slow development. This is more notable for neurofibromin 1 (**NF1**) mutant neoplasms, which affect older patients[95-98]. Melanoma *in situ* lesions are more common on chronically sun-exposed anatomical sites and tend to have a high mutation[39,95,99].

**IV. Vertical Growth Phase (VGP).** The invasiveness begins once the melanoma cells leave the epithelium of the epidermis and enter the subjacent mesenchymal tissue[100]. In the **VGP** melanoma cells inherit driver mutations that activate the **MAPK** pathway, as well as **TERT** and members of the **SWI/SNF** chromatin-remodelling complex[45]. A small fraction of melanomas appear suddenly as nodular growths indicating that either accumulating mutations were in rapid succession, or perhaps more likely, they come directly from melanocytes that already harboured the secondary and/or the tertiary genetic alterations[39,45]. There seems to be no further substantial increase in the burden of point mutations from **RGP** to **VGP**[45,101,102]. In the molecular level, secretion of soluble factors and extracellular matrix proteins enable melanoma cells to communicate with their microenvironment cells[103-106].

**V. Metastatic Melanoma**. Once melanoma cells have disseminated beyond the local site they are termed as **metastatic**, which generally appear first in the lymph nodes of the draining area of the primary tumour. Apart from lymph nodes; brain, liver, bone and intestine are the sites that are most frequently colonized by metastatic melanoma cells[107]. Sentinel lymph node resection does not experience an extension of life expectancy in patients[108,109]. Moreover, circulating tumour cells are commonly found in patients who present with only regional metastasis, or even no metastases at all[110-112]. Metastatic melanoma tends to have the highest proliferative index of all stages of melanoma. Mutations that confer resistance to targeted therapies can also lead to simultaneous emergence of resistant clones at many separate body sites[113]. The last decade, studies showed that melanoma cells secrete signals **(exovesicles)** from the very early stages of progression; and these signals "**educate**" the organs and prepare the so-called "**pre-metastatic niche**" to be metastasized for the colonization of melanoma cells to come[114-117].

*Classification by clinicopathological criteria (AJCC-non linear evolution)*

For years, Clark classification has been known to have prognostic significance, and has served as a criterion in several melanoma staging systems. However, only 20–30% of melanomas arise in association with a nevus precursor[119,120]. Several investigators have demonstrated that the level of invasion is less reproducible among pathologists, and is less accurate in providing prognostic information compared to tumor thickness[121,122]. As there are many routes to primary tumor formation, there are likely also many routes to the establishment of clinically meaningful metastatic disease (**Figure 3**). This prompted
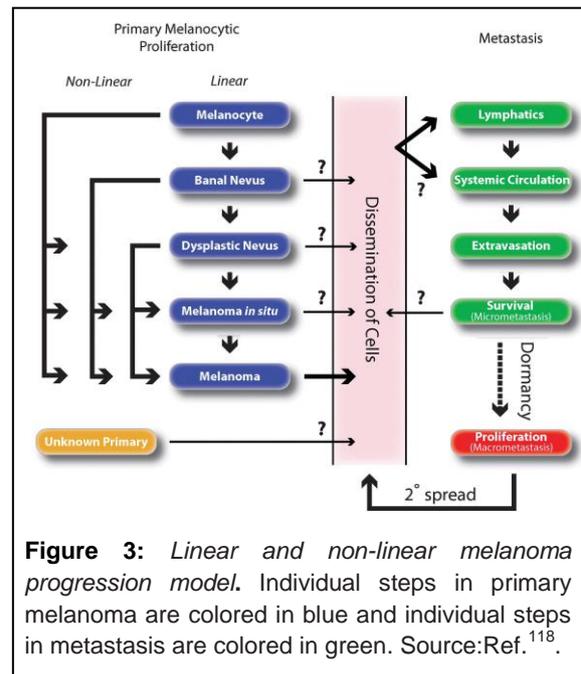


**Figure 3:** *Linear and non-linear melanoma progression model.* Individual steps in primary melanoma are colored in blue and individual steps in metastasis are colored in green. Source:Ref.[118].

the definition of a revised model for melanoma progression which also theorizes melanoma as developing *de novo*. In the new staging system, Clark's level has far less importance. The **TNM** (Tumor, Node, Metastasis) classification and stage grouping of melanoma patients is based on clinical and histopathological prognostic factors, endorsed by AJCC database (American Joint Committee on Cancer Melanoma Staging)[100]. The **T** category is based on the thickness (or depth) of the primary melanoma and is further subcategorized to include the presence or absence of ulceration and mitotic rate- two very important prognostic features of a primary melanoma. The **N** category is determined by the presence and extent of regional lymph node involvement, and is further subcategorized according to number of involved lymph nodes and whether the metastases were macroscopic or microscopic (palpable or determined by sentinel-node evaluation, respectively). In-transit and satellite metastases are also included in the N staging. The **M** category is determined by the presence or absence of distant metastases (including skin), by site of disease, and serum LDH level[100]. The new revised AJCC (8th) classification includes molecular markers and newly acquired insights into the molecular underpinnings of cancer. This edition features 12 entirely new staging systems and a refined emphasis on a personalized-medicine approach[123].

*Classification by genetic alterations*

Due to melanoma complexity, integration of molecular data with relevant clinical and histopathological information are likely to be an important interim step before research

advances to a level permitting a mechanism-based classification. Detailed genetic studies using massively parallel sequencing have clarified the prevalence of driver oncogenes and have identified unique genetically-defined subpopulations of melanoma. Melanoma exhibits the highest rate of genetic alterations among cancer type[124] and whole-genome sequencing studies have confirmed that 80% of those changes are related with UV mutagenesis[125]. However, the above (and additional) studies have either focused on single high-throughput platforms of large sample sets or multi-platform analyses of fewer samples[126,127]. The Cancer Genome Atlas (**TCGA**) is the largest systematic multi-platform characterization of 333 cutaneous melanomas at the DNA, RNA, and protein levels to create a catalogue of somatic alterations and describe their potential biological and clinical significance (**Figure 4**)[95].



**Figure 4:** *Landscape of driver mutations in melanoma.* Total number of mutations, age at melanoma accession, and mutation subtypes are indicated for each sample. Color-coded matrix of individual mutations (specific BRAF and NRAS mutations indicated) (middle), type of melanoma specimen (primary or metastasis), and mutation spectra for all samples (bottom) are indicated. Source:Ref.[95].

From this study a framework for genomic classification into four subtypes based on the pattern of the most prevalent significantly mutated genes (**SMGs**) was established: i. **BRAF mutant**, ii. **RAS mutant**, iii. **NF1 mutant**, iv. and **Triple-WT (wild-type)**. The largest genomic subtype was defined by the presence of **BRAF hot-spot mutations**. Of the 318, 52% (n=166) harboured **BRAF somatic mutations**. Of those, 145 targeted the well documented V600 amino acid residue: **V600E** (n=124), **V600K** (n=18), and **V600R** (n=3). The second major subtype is defined by the presence of **RAS hot-spot mutations**, including known amino acid changes with functional consequences, in all three RAS family members (N-, K- and H-RAS). Overall, 28% had **NRAS** somatic mutations. The third most frequently observed SMG in the MAPK pathway was **NF1**, which was mutated in 14% of samples. More than half of its mutations were predicted to be loss-of-function (LoF) events

(as an alternative way to activate the canonical MAPK pathway). The **Triple-WT subtype** (n=46) was defined as a heterogeneous subgroup characterized by a lack of hot-spot BRAF, N/H/KRAS, or NF1 mutations[95]. The above **SMGs,** and additional that were found mutated in lower fraction samples, are components of the **MAPK** and the **PI(3)K** (Phosphatidylinositol-4,5-bisphosphate 3-Kinase) pathways and were also confirmed to be activated.

## 3.4   "Classical" versus "Lineage-Specific" oncogenes in melanoma

As shown in **Figure 4** and in **Table S2** (Appendix) the majority of melanoma oncogenes signal through the **MAPK** and/or **PI3K** pathways. Other examples of initiating oncogenic events are the amplification of the cell cycle regulators CCND1 or CDK4[128]. These oncogenic mutations are accompanied by alterations in tumor suppressor genes such as CDKN2A[129,130], PTEN[128,129], TP53[131] and RB1[132], among others. These factors suffer activating genetic aberrations which are frequently shared among different tumor types and have been termed **"classical oncogenes"**[133].

Besides these aberrantly activated/deactivated genes which are shared among different types of cancer, there seems to be a **lineage-specific element** to certain pathways that are able to transform melanocytes and that contribute to the robust metastatic potential of melanoma. The best characterized lineage specific gene in melanoma is **MITF**[134]. MITF has been defined as the master regulator of melanocyte development, differentiation and pigmentation[135]. Amplification of MITF has been identified in approximately 15% of melanomas[134]. Loss-of-function mutations of MITF result in the complete absence of the melanocytic lineage[136]. Loss of MITF expression is lethal to most melanoma cell lines *in vitro* (by inducing cell cycle arrest[136] or DNA damage[137]). Intriguingly, MITF depleted melanoma cells are also highly invasive[138]. These contradictions have led to a proposed **rheostat model** where low MITF is a hallmark of stem-like cells (invasive pheonotype), whereas MITF-positive cells either proliferate or differentiate depending on the expression levels or post-translational modifications. As MITF levels and activity are strongly influenced by the microenvironment, the effect of MITF expression on melanoma cell identity is dynamic[139-141]. Importantly, our group recently identified the endolysosomal GTPase RAB7 as an MITF-independent mechanism of lineage-regulation in melanoma[142].

## 3.5   Non-Oncogene Addiction in melanoma

Cancer cells are addicted to **"classical"** or to **"Lineage-Specific"** oncogenes in order to sustain hyper-activated signaling pathways and progress. However, there are many genes

that are unable to directly promote tumor formation but whose reduction in activity can become rate-limiting to the pathway. By this rationale, cancer cells are addicted to both oncogenes and non-oncogenes. To describe this addiction of cancer cells to the functions of non-oncogenes, this phenomenon was termed as **"non-oncogene addiction"**[143,144]. One cellular process in melanoma proposed to be mediated by oncogenic and non-oncogenic mediators is **autophagy**.

### 3.5.1 Autophagy

Autophagy is the major intracellular degradation process by which cytoplasmic materials are delivered to and degraded in the lysosome. However, the purpose of autophagy is not the simple elimination of materials; but instead, serves as a dynamic recycling system that produces new building blocks and energy for cellular renovation and homeostasis. The term was introduced in 1966 by de Duve, derived from the Greek words



**Figure 5:** *Main steps of (macro)autophagy.* Schematic representation of key regulatory proteins involved in the formation and maturation of autophagosomes. Shown also are pharmacological inhibitors and small interfering RNAs (red blocking arrows). *Source:* Ref.[145].

"*auto*" and "*phagy*" meaning "*self*" and "*eating*", to describe the occurrence of distinct intracellular, membranous vesicles that contained degraded cytoplasmic material[146].

Autophagy is classified as **microautophagy**, **chaperone-mediated autophagy** and **macroautophagy[147]**. In microautophagy, the lysosome itself engulfs small components of the cytoplasm by inward invagination of the lysosomal membrane. Membrane dynamics during microautophagy may be quite similar to that of endosomal sorting complex required for transport (ESCRT)-dependent multivesicular body (MVB) formation, which occurs in the late endosome[148]. The chaperone-mediated autophagy does not involve membrane reorganization; instead, proteins directly translocate across the lysosomal membrane during chaperone-mediated autophagy[149]. Macroautophagy (herein will be referred as ''**autophagy**'') is thought to be the major type of autophagy, and it has been studied most extensively compared to other categories. Although autophagy was initially considered a non selective cellular process, the specific catabolism of cellular organelles like mitochondria,

peroxisomes, endoplasmic reticulum, and ribosomes has been discovered and is termed "**mitophagy**"[150,151], "**pexophagy**"[152,153], "**ERphagy/reticulophagy**"[154], and "**ribophagy**"[155], respectively. In general, the role of autophagy to maintain cellular homeostasis requires the versatility to recognize a diverse range of substrates and stimuli[156]. Autophagy is orchestrated by a number of highly conserved AuTophaGy-related genes (**ATGs**), which were originally identified in yeast, with many of these genes having orthologues in mammalian cells[157,158]. The main steps and the core components of this process are highlighted in **Figure 5** and in **Table S3** in the Appendix. Briefly, double-membrane-bound **autophagosomes** develop in a multi-step process starting by the engulfment of cytoplasmic material by the **phagophore**[159]. This process involves the de-repression of the mTOR Ser/Thr kinase which inhibits autophagy by phosphorylating the Atg13 (*step 1*). This leads to the dissociation of Atg13 from a protein complex that contains Atg1 kinase (the fly orthologue of ULK1) and Atg17. When mTOR is inhibited, re-association of Atg13 with Atg1 induces autophagy[160,161]. Among the initial steps of vesicle nucleation is the activation of mammalian Vps34 (a class III PI3K) to generate phosphatidylinositol-3-phosphate (PtdIns3P) (*step 2*). Activation of Vps34 depends on the formation of a complex in which beclin-1 (Becn1; the mammalian orthologue of Atg6), UVRAG (UV irradiation resistance-associated tumour suppressor gene) and a myristylated kinase (Vps15, or p150 in humans) participate. Two ubiquitin-like conjugation systems are part of the vesicle elongation process (*step 3*). One pathway involves the covalent conjugation of Atg12 to Atg5, with the help of the E1‑like enzyme Atg7 and the E2‑like enzyme Atg10. The second involves the conjugation of phosphatidylethanolamine (PE) to LC3/Atg8 (LC3 is one of the mammalian homologues of Atg8). Lipid conjugation leads to the conversion of the soluble form of LC3 (named **LC3-I**) to the autophagic vesicle-associated form (**LC3-II**)[162,163]. LC3-II is used as a marker of autophagy because its lipidation and specific recruitment to autophagosomes provides a shift from diffuse to punctate staining of the protein[164]. The mechanism of retrieval in which the Atg9 complex participates is poorly studied (*step 4*). Autophagosomes undergo maturation by fusion with lysosomes to create **autolysosomes** (*steps 5 and 6*). In the autolysosomes, the inner membrane and the cargo are degraded by lysosomal enzymes that act optimally within this acidic compartment[145,165-167]. In the phagosome formation, autophagy related proteins participate in the orchestrated fusion of Golgi-, endosome- and plasma-membrane-derived membranes[168-171].

### 3.5.2 Autophagy: A "double-edged" sword in cancer

Autophagy plays a key role in the maintenance of cellular homeostasis. In healthy cells, such a homeostatic activity constitutes a robust barrier against malignant transformation. In

cancer, autophagy can be **tumor-suppressive**[172,173] or **tumor promoting**[174,175] in different contexts[176]. Large-scale analyses from **TCGA** indicate that the core autophagy machinery largely escapes genomic mutation in human cancers[177]. Autophagy impacts cellular metabolism, the proteome, and organelle numbers and quality, which alter cell functions in diverse ways. Additionally, autophagy can promote a cross-talk between the tumor and the stroma, which can support tumor growth. Thus, the role of autophagy is determined by nutrient availability, microenvironment stress, and the presence of an immune system[178].

*Autophagy as a tumor suppressor*

Autophagy was initially thought to be a tumor-suppression mechanism. This concept derived from early reports that the essential autophagy gene **ATG6/BECN1** (part of the class III PI3K complex) was monoallelically lost in 40% to 75% in various cancer types. *Becn1*$^{+/-}$ mice (*Becn1*$^{-/-}$ are not viable) spontaneously develop various malignancies, including lymphomas as well as lung and liver carcinomas[179-183]. However, in human tumors, to assess the possibility that **BECN1** is acting as a tumour



**Figure 6:** *Tumor suppressive roles of autophagy in malignant transformation.* Several mechanisms have been proposed such as: the preservation of genetic/genomic stability; the disposal of endogenous sources of potentially mutagenic reactive oxygen species (ROS); the maintenance of normal bioenergetic functions; the degradation of oncogenic proteins; the optimal activation of oncogene-induced senescence (OIS) and oncogene-induced cell death (OICD); the maintenance of a normal stem cell compartment. Source: Ref.[176].

suppressor is complicated because of the close proximity of the **BECN1** gene and the potent tumor suppressor **BRCA1**[184]. Mutations in BRCA1 are known drivers in human breast and ovarian cancers. This makes difficult to rule out the possibility that in human tumors, it is loss of BRCA1 rather than BECN1 that is important. Indeed, TCGA data shows there is no significant loss of BECN1 independent of BRCA1 loss[185].

The mosaic deletion of *Atg5* in mice or *Atg7* in mouse livers produces only benign hepatomas, suggesting that complete and specific autophagy deficiency promotes liver

tumor initiation but restricts progression to malignant disease[186]. Similarly, loss of *Atg5* or *Atg7* in the mouse pancreas promotes benign pancreatic intraepithelial neoplasia (**PanIN**) formation but prevents progression to malignant disease[187,188]. Autophagy loss in the liver and pancreas causes oxidative stress, chronic tissue damage, and inflammation, all factors that are well known to promote tumor initiation[189]. This chronic state of tissue damage, cell death, and inflammation promotes compensatory proliferation, producing cells vulnerable to mutation and oncogenic transformation. *Atg7* deletion increased oxidative stress and enhanced tumor growth at early stages, but promoted abnormal mitochondria accumulation, proliferation defects, a decrease in tumor burden, and increased survival of animals in more advanced stages of tumorigenesis[190]. Similarly, deletion of *Atg7* in mice expressing an activating mutation of B-Raf (Braf$^{+/V600E}$) promotes early tumor development in the lung but also inhibits the progression to a more malignant phenotype and increases mouse survival[191]. An additional autophagy-promoting factor that has tumor suppressor functions is **Atg4C,** where its deletion in mice leads to the development of fibrosarcomas[192]. Finally, it has been proposed that autophagy contributes to oncogene induced cell death or oncogene-induced senescence, two oncosuppressive mechanisms. The activation of various oncogenes imposes a significant stress on healthy cells, a situation that is normally aborted through the execution of a cell death program[193,194], or cell senescence[195].

The above studies, and other, have shown that autophagy deficiency causes oxidative stress, mitochondrial defects, activation of the DNA damage response, genome instability and other features that are highlighted in **Figure 6**. It remains unclear if these premalignant tumors in autophagy-deficient mice are in a "**benign**" state that requires additional genetic alterations to promote malignant transformation (e.g. p53 mutation), or if autophagy inhibition can only promote premalignant/benign tumor formation but somehow protects cells from advancing to malignant cancers[189,196-199].

*Autophagy as a tumor promoter*

In established tumor cells, autophagy is hijacked to serve as a cell survival mechanism that plays a vital role in facilitating tumor cell growth. Several potential mechanisms in cancer have been suggested and are depicted in **Figure 7**. The notion that autophagy represents a mechanism that promotes tumor growth is based on the need of tumor cells to adapt to an environment that is hypoxic, as well as growth factor and nutrient deprived. Furthermore, tumor cells generally have high proliferation rates, which translate into higher bioenergetic and biosynthetic needs compared to non-transformed cells. These requirements can be fulfilled by increasing autophagy as a mechanism that permits obtaining both ATP and metabolic intermediates[190]. Importantly, for tumor cells in which the oncogene **Ras** is activated, high levels of basal autophagy and dependence on this mechanism for survival

are observed. For these reasons, autophagy is thought to promote tumor cell survival by increasing stress tolerance and providing a pathway that permits obtaining the nutrients necessary to meet the enhanced energetic requirements of these cells[187,200]. A first indication that autophagy may promote cancer came from the observations that autophagy is up-regulated in hypoxic tumor regions, suppresses tumor-induced inflammation, and promotes tumor cell survival[201].



**Figure 7:** *Tumor-supporting functions of autophagy in malignant transformation.* Such tumor supporting functions reflects the ability of autophagy to: improve the resistance of cancer cells to endogenous conditions that normally provoke cell death, render transformed cells less sensitive to therapy-induced cell death, sustain the survival of cancer cells that enter a state of dormancy or senescence in response to therapy and ensure the maintenance of the cancer stem cell compartment. Source: Ref.[176].

In GEMM for lung cancer, sporadic activation of an oncogenic allele of Kras (**Kras^G12D**) results in tumor initiation and gradual progression to adenomas and adenocarcinomas upon acquisition of TP53 mutations. Deletion of *Atg7* dramatically alters the progression of these tumors, producing the accumulation of defective mitochondria, accelerated induction of p53, and proliferative arrest. *ATG7* loss promotes the formation of oncocytomas instead of adenomas and carcinomas, proliferation is suppressed, and tumor burden is reduced. Surprisingly, mice with Atg7$^{+/+}$ tumors die from cancer, whereas mice with Atg7$^{-/-}$ tumors die from inflammatory pneumonia, demonstrating that Atg7-deficient lung tumors trigger an immune response[202].

In pancreatic adenocarcinoma, KRAS^G12D-driven cells enter a state of dormancy in response to oncogene ablation and exhibit activated autophagy to efficiently counteract metabolic stress. Interestingly, these cells (characterized as cancer stem cells) have the ability to efficiently form tumors *in vivo,*and this relies on autophagy[203-205].

**FIP200** (key component of autophagosome) deficiency in mammary cancer suppresses tumor growth and prolongs mouse survivalvia the disruption of autophagy machinery[206,207]. FIP200 loss in mammary tumors activates an immune response and T-cell depletion partly restores the growth of FIP200-deficient tumors. This suggests that targeting FIP200 or other

components of the autophagy initiation complex may suppress breast cancer by activating innate and adaptive anti-tumor immune responses[208].

In glioblastoma it is demonstrated that autophagy is crucial for both glioma initiation and sustained growth in a Kras-driven mouse model where shRNAs against Ulk1, Atg7, or Atg13 promote senescence and extended mouse survival[209].

Epithelial to Mesenchymal Transition (**EMT**) is one of the leading biological processes of metastasis during cancer[210,211]. Several works show that EMT requires autophagy to support viability of potentially metastatic cancer cells[174,212-214].

Autophagy is an evolutionarily conserved mechanism and its role in cancer depends on many factors like tissue type, tumor stage, and the type of oncogenic mutation involved[215].

### 3.5.3 Autophagy in melanoma

Melanomas have a high level of basal autophagosomes suggestive of increased autophagy. Melanoma patients with with higher levels of autophagosomes have decreased survival[216]. BRAF targeted therapy results in a transient response in some patients with BRAF mutant melanoma, only to relapse shortly thereafter. In a GEMM of melanoma ($Braf^{V600E}/Pten^{-/-}$), *Atg7* deficiency prevents melanoma initiation and proliferation and prolongs survival. Although the mechanism is not firmly established, Atg7 prevents oxidative stress, apoptosis, fibrosis, and, notably, senescence, a known barrier to melanoma growth and progression[217]. Moreover, autophagy in Braf-resistant melanoma cells either by *ATG5* knockdown or pharmaceutically was not sufficient in restoring sensitivity to Vemurafenib (Braf$^{V600E}$ inhibitor) treatment; however, combinational treatment increased melanoma cell death[218].

On the other hand, two autophagy markers, ATG5 and LC3, were found significantly decreased in patients with early-stage cutaneous melanoma as compared with those with benign nevi[219]. Importantly, studies in our lab using GEMM, *in vitro* studies and data from TCGA database revealed that specific heterozygous loss of *ATG5* favors melanoma metastasis and it's a poor indicator of survival (**Figure 8**). Of note, the tumors in the *ATG5* heterozygous mice were more resistant to targeted therapy with a Braf$^{V600E}$ inhibitor (Dabrafenib)[220]. Although mechanistically was not characterized how the specific loss triggers metastasis, it is speculated that the expression levels of autophagy proteins should be fine tuned in order to regulate downstream factors that favor invasion.

Whether other proteins of autophagy machinery behave in the same manner is poorly characterized.

**Figure 8:** *ATG5 heterozygous loss favors melanoma metastasis.* **A.** Graphical representation of the percentage of cases with the indicated alterations in the *Atg5* gene in specimens with clinical annotations in the TCGA melanoma database. Amp and del stand for amplifications and deletions, respectively. **B.** Overall survival for melanoma patients with diploid, partial losses or deep deletions of the ATG5 locus (TCGA database). **C.** Higher metastatic potential of Atg5$^{+/\Delta}$ melanomas determined by macroscopic examinations of lungs. Source: Ref.[220]

## 3.6 Cargo receptors in autophagy

As was mentioned above, the turnover of damaged organelles, removal of protein aggregates, and elimination of intracellular pathogens, are highly selective and tightly regulated processes that require selective sequestration of the specific cargo into autophagosomal membranes[221,222]. An autophagy receptor is defined by its ability to bridge cargo and autophagosomal membrane, leading to the engulfment of cargo by the autophagic vesicle. Selectivity is achieved through autophagy receptors. The interaction of cargo receptors with **LC3/GABARAP** relies on the formation of an intermolecular β-sheet to which the cargo receptor contributes a single strand, the so-called LC3-interacting region (**LIR**). LIR motifs in general interact with autophagy modifier proteins of the



**Figure 9:** *Cargo receptors.* Proteins with the interacting domains involved in selective autophagy and their domain organization. Source: Ref.[221]

LC3/GABARAP family[223]. In mammalian cells, more than two dozen autophagy receptors were identified by the yeast two-hybrid system and proteomic approaches[224]. There is an additional class of proteins **autophagy adaptors** that also bind to members of the LC3/GABARAP family (**Figure 9**). However, they do not facilitate engulfment and
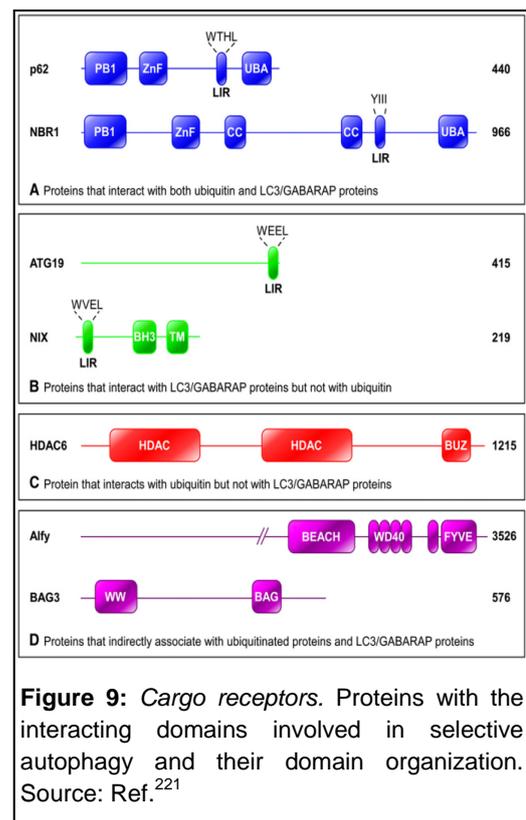
subsequent degradation of cargo, but serve as anchor points for the autophagy machinery and regulate initiation, conjugation, transport and fusion of autophagosomes[225].

One of the most characterized cargo receptors in the higher organisms is **p62** also known as **Sequestosome-1 (SQSTM1)**. Unexpectedly, p62 localizes to the phagophore even in the absence of LC3 interaction[226]. Self-oligomerization of p62 rather than binding to LC3 is critical for the translocation of p62 to the autophagosomal membrane[227].

## 3.7   p62 as an autophagy adaptor and a signaling hub

**p62** was first identified as a 62 kDa protein which binds lymphocyte-specific protein tyrosine kinase (Lck), a Src family member[228]. Later, p62 was also reported to be a binding partner of atypical protein kinase C (aPKC)[229,230]. Since its initial discovery, p62 through the well-defined structural elements has been characterized to act as an **autophagy adaptor** and also as a **signaling hub** due to its ability to interact with key signaling proteins.

### 3.7.1 Structure of p62

p62 is ubiquitously expressed in various cell types, mainly in the cytoplasm, and is also present in the nucleus, autophagosomes, and lysosomes[231-233]. It contains multiple major domains (**Figure 9**) such as the  **PB1** (Phox1 and Bem1p regions, aa 20–102), the **ZZ** (ZZ-type zinc finger, aa 122-166), the **TB** (TRAF6 binding, aa 225-250) and the **KIR** (Keap1-interacting region, aa 346–359) that confer the ability to interact with key components involved in essential signaling pathways[234,235]. Two other domains, **UBA** (Ubiquitin-associated domain, aa 391–436) and the **LIR** (LC3-interacting region, aa 337-343) domains, cause p62 to function as an adaptor between autophagy and ubiquitinated proteins[236].

The **UBA** domain of p62 can bind both K48-linked and K63-linked Ub chains[237-239]. Exposure to proteasome inhibitors or expression of polyglutamine-expanded huntingtin, UBA is phosphorylated in Ser407 by ULK1 kinase, which is required during the early steps of autophagosome biogenesis. This phosphorylation destabilizes the UBA dimer interface. Subsequently, Ser403 of the UBA domain is phosphorylated by casein kinase 2 or TANK-binding kinase 1 to increase the binding affinity of p62 for ubiquitin chains[240]. The UBA is frequently mutated in Paget's disease. This pathology is characterized by an increase in the number and the activity of osteoclasts, leading to bone architecture abnormalities[241,242].

The **LIR** domain, via an 11 amino-acid portion located in the 332-343 region, is responsible for the association of p62 with all isoforms of the LC3 protein and consequently the targeting in the autophagic machinery[243]. There are a series of salt bridges and hydrogen bonds between p62 and LC3. The hydrophobic (Trp340–Leu343) and acidic cluster

(Asp337–Asp339) of p62 are very important for LC3 binding, and these motifs are conserved across species. Disruption of the interaction is sufficient to impair the degradation of p62, resulting in the formation of ubiquitin- and p62-positive inclusions[244].

The **KIR** domain interacts with Keap1, a Cullin3-ubiquitin E3 ligase complex adaptor protein. In turn, Keap1-promoted polyubiquitinylation and subsequent proteasomal degradation of the transcription factor Nrf2 are inhibited. As a consequence, the expression of cytoprotective, antioxidant Nrf2 target genes is increased[199,245]. Moreover, the p62 gene itself is a target for Nrf2; thus, the appropriate oxidative stress response is supported by a positive feedback regulation between p62 and Nrf2[246].Autophagy has a strong impact on Nrf2 activation, since p62 not only disrupts Keap1-Nrf2 interaction but also removes Keap1 from the cytosol via selective autophagy[247].

The **ZZ** domain is related to NF-kB signaling and glucose regulation. This domain could associate with RIPK1. Recently, some studies reveal that the ZZ domain of SQSTM1 may links autophagy to N-end rule pathway[248,249].

The **TB** domain of p62 interacts with TRAF6, an E3 ubiquitin ligase that regulates mTORC1 activity by K63-ubiquitination in a p62-interaction dependent manner. Through this TB domain, p62 could function as a crucial mediator for mTORC1 activity regulation[233].

The **PB1** domain was characterized initially by its ability to bind atypical protein kinase C (PKC ζ and ι)[229,250]. Heterodimerization of p62 with atypical PKC is crucial for the regulation of the NFκB pathway. Indeed, following binding of the RANK ligand to its cognate receptor RANK in osteoclasts, the association of p62 with either atypical PKC (or with TRAF6 via TB domain) contributes to the activation of IKK (Ik-B Kinase) and to the nuclear translocation of NFκB. p62 binds and inhibits ERK1, which is crucial for the negative regulation of adipogenesis and obesity and the ensuing inflammation[251]. The PB1 domain plays a role in p62 self and hetero-oligomerization with other PB1 domain containing proteins, such as NBR1[252]. Finally, PB1-mediated p62 self-oligomerization is essential for its localization to the autophagosome formation site, a process that is independent of LC3 localization[227].



**Figure 10:** *Domain structure of p62 and its interacting partners.* There are six main domains in the p62 protein, necessary for its interaction with the autophagic machinery and with signaling pathways. The N-terminal **PB1** domain is involved in the self-oligomerization of p62 or in heterodimerization. The **ZZ** domain and the **TB** interact with the RIP and TRAF6 proteins, respectively, to regulate the NF-κB pathway. Through **LIR** and the **UBA**, p62 links the autophagic machinery to ubiquitinylated protein substrates to promote the selective degradation. Finally, **KIR** binds Keap1 leading to stabilization and nuclear translocation of the transcription factor Nrf2.

### 3.7.2 Transcriptional regulation of p62

One of the main modes of transcriptional regulation of p62 is dependent on the **NRF2** transcription factor[245]. NRF2 binds to the antioxidant-responsive element (ARE motif) located in the p62 promoter to promote the expression of *p62 mRNA*. In the absence of oxidative stress, NRF2 is maintained as an inactive protein in the cytoplasm through its interaction with the KEAP1. In addition to its specific regulation by NRF2, p62 can interact directly with KEAP1 via its KIR domain and is able to dissociate NRF2 from KEAP1, thus promoting the activation and nuclear translocation of NRF2[199,253]. Additionally, the constitutive activity of the **Ras/MEK/ERK1/2**[3,254] and the **JNK/c-Jun**[255,256] pathways regulates p62 at transcriptional level in different systems. Finally, other stimuli including phorbol 12-myristate 13- acetate (PMA), calcium and IL-3, drastically increase the expression of p62 mRNA in a very short time[257].

### 3.7.3 p62 in cancer progression and metastasis

As mentioned above, p62 expression is elevated in response to a variety of cellular stresses, especially those that result in impaired autophagic flux. p62 is overexpressed in many human cancers. Studies have shown that p62 is overexpressed in hepatocellular carcinoma (HCC)[189,258-263], intrahepatic cholangiocarcinoma[264], pancreatic cancer[265], lung cancer[262,266,267], oral, head and neck cancer[268,269], esophageal cancer[270], gastric cancer[265,271], colon cancer[262,272], breast cancer[273-278], prostate cancer[278-284], melanoma[285], endometrial cancer[286], ovarian cancer[287], glioblastoma multiforme[288] and kidney cancer[289], as well as chronic liver diseases, such as alcoholic and non-alcoholic steatohepatitis (ASH, NASH) that increase HCC risk[259,290-293]. In the **Table S4** (Appendix) are listed the main functions of p62 in different pathological diseases and mainly in different types of cancer.

*p62 autophagy dependent roles in cancer*

p62 has been identified as one of the many autophagy receptors and may promote autophagic degradation of cargos through at least three mechanisms. First, p62 is directly associated with early autophagosome formation by recruiting Atg proteins to the autophagosome formation site through its PB1 domain independent of its LIR[227]. Second, p62 binds to ubiquitinated proteins to form protein aggregates via its UBA domain. Third, p62 also directly interacts with LC3 via its LIR motif (**Figure 10**)[236,243]. Along with its PB1 partner, NBR1 (Neighbor of BRCA1 gene 1), it has been proposed to regulate the packing and delivery of polyubiquitinated, misfolded, aggregated proteins and dysfunctional organelles for their clearance through autophagy in mammalian cells and in the fruit fly Drosophila[243,294,295].

Defects in autophagy promote a failure of protein and organelle quality control in cells, which leads to p62 accumulation, resulting in perturbation of gene expression, increased genome damage and tumorigenesis[189]. Importantly, in autophagy deficient and apoptosis-incompetent tumor cells, metabolic stress leads to the accumulation of p62, elevated expression of endoplasmic reticulum (ER) chaperones, damaged mitochondria, and reactive oxygen species (ROS). This increase in p62 levels is critical for tumorigenesis, as overexpression of p62 in this cell model leads to increased tumor volume in mouse xenograft experiments[189,296].

A relevant *in vivo* study described that complete inhibition of autophagy in hepatocytes; induced by deletion of the essential autophagy genes, *Atg5* or *Atg7*; causes spontaneous liver injury, hepatomegaly, and appearance of liver tumors[186]. These tumors, however, are benign adenomas[297]. Liver adenomas in liver-specific *Atg7*^{-/-} mice show strong accumulation of p62 and subsequent NRF2 activation due to autophagy inhibition, and both tumor growth and liver injury are markedly suppressed by p62 loss[186,298]. Liver injury is alleviated by NRF2 ablation and exacerbated by Keap1 deletion in liver-specific *Atg7*^{-/-} mice[199]. This indicates that p62 may also be a central element in a quality-control mechanism for the disposal of toxic aggregates.

p62 has been proposed as a critical regulator of the EMT factor, Twist1. Insufficient autophagy causes accumulation of p62 and promotes cell proliferation and migration through stabilization of the oncogenic transcription factor Twist1[299].

In established breast tumors **FIP200**-mediated autophagy is required for maintaining tumor growth *in vivo* and the accumulated p62 upon autophagy blockage stimulated the growth of autophagy-deficient tumors. These results revealed a rather synergistic mechanism of p62 signaling and autophagy in the promotion of cancer growth[207]. Of note, recent studies found that p62 is not only required for the proper elimination of ubiquitinated proteins but also participates in the autophagic clearance of non-ubiquitinated substrates[300].

Several studies highlight that although p62 usually recruits ubiquitinated cargos, loss of p62 has little effect on selective autophagy. This lack of phenotypic impact might be explained by the presence of other autophagy receptors including **NBR1**, **NDP52** (Nuclear Dot Protein 52 kDa) and **Optineurin**[236,301,302].

*p62 as a signaling hub in cancer*

Unlike other autophagy adaptors, with the exception of NBR1, p62 is also a central hub due to its ability to interact with key signaling proteins through well-defined structural elements. Therefore, it is possible that autophagy only intervenes as a facilitator or an inhibitor of the prooncogenic action of p62[296,303].

In ductal pancreatic adenocarcinoma, the constitutive activation of Ras activates the NF-κB pathway to trigger increased p62 synthesis, and this newly synthesized p62, in turn, interacts with **TRAF6** to amplify Ras signaling in a positive feedback loop[304]. The same observations arise from studies on lung cancer in which Ras activation stimulates the TRAF6-p62-NF-κB complex that is necessary to inhibit deleterious ROS production[253]. In glioblastoma multiforme, p62 is strongly overexpressed harboring a mesenchymal oncogenic signature and this overexpression is concomitant with **MAPK** activation[288].

**mTORC1** is sensitive to inhibition by rapamycin and senses multiple cellular and environmental cues including nutrient availability, energy levels, protein misfoldingand growth signals. p62 can promote mTORC1 activation by directly interacting with **Raptor**, a key component of mTORC1. In response to stimulation with amino acids, deletion of p62 results in reduced mTORC1 activity. Conversely, overexpression of p62 enhanced mTORC1 activation. Moreover, p62 serves as a docking site and is required for mTORC1 localization to the lysosomal membrane in response to amino acid stimulation. Therefore, **p62-mTORC1** signaling may also establish another line of feedback regulating p62 levels.[282].

The positive regulation of p62 and c-Myc was also addressed in breast cancer stem cells by delaying the degradation of *MYC mRNA* through the repression of let-7a and let-7b, thus promoting *MYC mRNA* stabilization at the post-transcriptional level[277].

Interestingly, chronic p62 accumulation occurs in human pancreatitis and most liver degenerative diseases[305]. High p62 levels promote the activation of NRF2 that results in the transcriptional activation of the p62 gene making a feed-forward loop that promotes cancer initiation[199,245,306]. Although p62 inactivation in liver parenchymal cells abrogates the expression of the NRF2-dependent anti-oxidant response, it also results in the disappearance of hepatocytes that accumulate ROS, which presumably serve as HCC-initiating cells. The loss of these cells is most likely due to inactivation of the NRF2-mediated protective response as well as inhibition of **mTORC1** activation and **c-Myc** expression (**Figure 11**). These studies demonstrate that p62 accumulation in a chronically damaged liver is one of the most important factors that lead to HCC development. The proof of principle that p62 is an oncogenic protein is that its overexpression *in vivo* is sufficient to induce HCC. This effect of p62 is independent of autophagy because overexpression of a p62 variant lacking the UBA domain, and therefore stripped of its autophagy adaptor function, was also sufficient to drive HCC[260,307]. In contrast, a recent study revealed that p62 is downregulated in stellate cells (another type of cells in the liver) acting as a suppressor of HCC development. More specifically, it was shown that p62 is a negative regulator of liver inflammation and fibrosis through its ability to promote Vitamin D Receptor signaling (i.e. **VDR:RXR** heterodimerization) in HSCs, whose activation supports HCC[308]. The high

complexity of the liver clearly demonstrates that p62 may exert different levels of expression and opposing roles in cell populations even in the same cancer type.

Tumor-suppressive functions of p62 were also found during mitosis. During the early mitotic phase p62 is phosphorylated by **CDK1** allowing the cells to properly progress through mitosis. Cancer cells expressing a non phosphorylatable p62 mutant display higher tumorigenic properties *in vivo* and *in vitro*[309].

Apart from cancer cells, many tumors display reduced levels of p62 in their stroma, especially in cancer-associated fibroblasts (CAFs) (**Figure 11**). p62-deficient stromal fibroblasts exhibit increased production of IL-6, which is critical for **TGF-β** synthesis that converts CAFs into activated myofibroblasts that promote tumor progression. This downregulation of p62 in CAFs is needed for acquiring their tumor-promoting function. Decreased **mTORC1** activity due to the downregulation of p62 results in reduced **c-Myc** levels and the concomitant increase in **ROS** levels under conditions of autophagy competence. Interestingly, the increased oxidative stress in these stromal cells translated into higher levels of IL-6 that promoted TGF-β synthesis, being both required for CAF activation in p62-deficient prostate cancer stroma[278].



**Figure 10:** *p62 key functions in cancer.* Role of p62 in the tumor epithelium during cancer initiation (left) and p62 negative expression in the tumor stroma in metabolic reprogramming and inflammation (right). Source: Ref.[307]
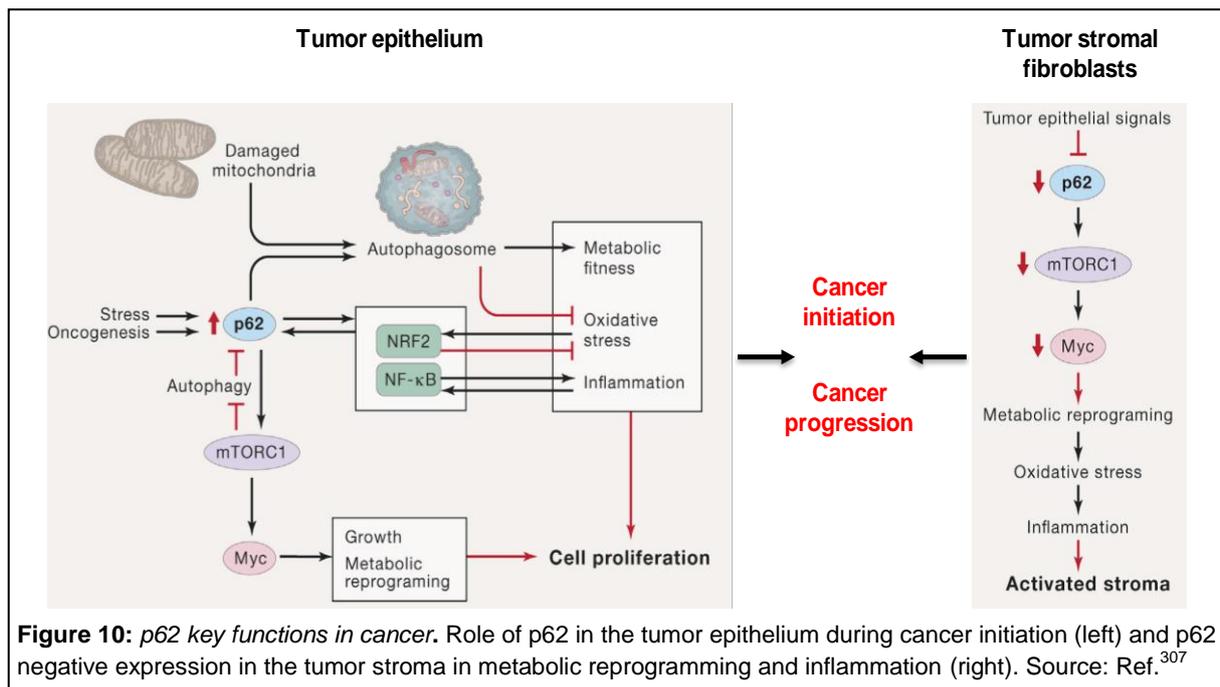
The above results can explain why genetic inactivation of p62 at an organism level is associated with increased tumor progression[278], whereas the selective inactivation of p62 in cancer epithelial cells restrains cancer initiation[260,307].

# IV.OBJECTIVES

This PhD thesis stemmed from our interest in the identification of melanoma drivers, with a particular interest in lineage-specific or lineage-enriched metastatic pathways that may represent new prognostic factors and putative targets for drug development.

The adaptor protein p62/SQSTM1 (sequestosome 1) was selected for its previously reported functions at the crossroads of autophagy[189,300] and lysosomal-associated pathways, both with melanoma-selective features. Moreover, p62 can also function as a signaling hub[260,282], although there is no clear consensus on whether it acts as a tumor promoting[253,260] or tumor suppressive agent[278,308]. For example, while p62 is an indisputable autolysosome cargo[186,227,236], other roles as a hub for multiple protein-protein complexes can be highly cell type dependent (mTOR, KEAP1-NRF2 pathway, MYC metabolism)[260,282,299,308]. These pleiotropic functions of p62 illustrate the need for integrated expression and functional studies in physiologically-relevant systems.

The main goal of this PhD thesis was to dissect the expression and functional contribution of p62 to melanoma progression. To this end, we integrated a comprehensive analysis in melanoma cell lines, tissue specimens and newly-generated mouse models. In addition an integrated –omic strategy (transcriptome/proteome/interactome studies) was performed.

Therefore, the main goal was pursued in the following **specific objectives**:

1. ***THE CHARACTERIZATION OF THE PATHOPHYSIOLOGICAL ROLES OF P62*** *IN VIVO BY GENERATING MELANOMA MOUSE MODELS HARBOURING ALLELIC LOSS FOR P62.*

2. *THE **FUNCTIONAL** CHARACTERIZATION OF P62 IN MELANOMA BY AN -OMIC COMPREHENSIVE ANALYSES.*

3. *THE DISSECTION OF THE **MOLECULAR MECHANISMS** OF P62 IN MELANOMA.*

# V.MATERIALS AND METHODS

## 5.1  Cell Culture

The human melanoma cell lines SK-Mel-5, SK-Mel-19, SK-Mel-28, SK-Mel-103, UACC-62, WM-115, WM-164, WM-209 and LU-1205, were selected for their genetic background, recapitulating the most frequent genetic alterations in this disease (see **Table 1**). B16-F10 (ATCC) mouse melanoma cells were used for subcutaneous injections in mice. All these cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza, cat. No. BE12-604F/U1) supplemented with 10% fetal bovine serum (FBS, Lonza, cat. No. DE14-801F) and 100 µg/mL Penicillin/Streptomycin (Invitrogen, cat. No. 15070-063). Primary human melanocytes, keratinocytes and fibroblasts were isolated as described before[310] from neonatal foreskins obtained from the Hospital Niño Jesús, Madrid, Spain. Melanocytes were cultured in Medium 254 (Invitrogen, cat. No. M-254CF-500) supplemented with 1% melanocyte growth factors (HMGS, Invitrogen, cat. No. S-002-5), 0.2 mM $CaCl_2$ and 100 µg/mL Penicillin/Streptomycin; keratinocytes were cultured in Epilife Medium (Invitrogen, cat. no. M-Epi-500) supplemented with 1% human keratinocyte growth factors (HKGS, Invitrogen, cat. No. S-001-5), 0.2 mM $CaCl_2$ and 100 µg/mL Penicillin/Streptomycin and fibroblasts were cultured in DMEM, supplemented with 10% FBS, and 100 µg/mL Penicillin/Streptomycin.

**Table 1:** Human melanoma cell lines used in this PhD thesis study.

| CELL LINE | STAGE | BRAF | NRAS | PTEN* | p62* |
|-----------|-------|------|------|-------|------|
| **SK-Mel-5** | LN met | V600E | WT | + | ++ |
| **SK-Mel-19** | Primary Melanoma | V600E | WT | + | ++ |
| **SK-Mel-28** | LN met | V600E | WT | + | +++ |
| **SK-Mel-103** | Primary Melanoma | WT | Q61R | + | + |
| **UACC-62** | Primary Melanoma | V600E | WT | - | +++ |
| **WM-115** | Primary Melanoma | V600E | ND | - | ++ |
| **WM-164** | LN met | V600E | WT | + | ++ |
| **WM-209** | met | WT | G12A | ND | + |
| **LU-1205** | Lung met | V600E | WT | - | ++ |

**\***: protein levels; **WT**: wild type; **ND**: not determined

## 5.2  Protein Immunobloting

Cells were harvested and total cell lysates were obtained using 1x **Laemmli buffer** (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol and 5% β-mercaptoethanol) and boiled at 95°C for 5 minutes or **RIPA buffer** [50mM Tris-HCl pH 8, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.5 % Sodium Deoxycholate, 0.1% SDS, 1mM EDTA pH 7.4 and supplemented

protease (Roche Diagnostics, cat. No. 11836170001) and phosphatase inhibitors (Santa Cruz, cat, No. sc-45045). Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories) according to manufacturer's protocol. Protein immunoblots were performed by standard SDS-PAGE electrophoresis in polyacrylamide gels and subsequently transferred to Immobilon-P membranes (Millipore, cat. No. IPVH00010) using Mini Trans-Blot Cell system (Bio-Rad Laboratories). Transfer was performed at 100V during 1 hour at 4°C. Membranes were blocked with 5% milk in tris-buffered saline with 0.05% Tween-20 (TBS-T) for 1 hour at RT. Primary antibodies (**Table 2**) were diluted in 5% milk or 5% BSA in TBS-T and incubated overnight at 4°C or 1 hour at RT. Secondary antibodies were diluted in 5% milk TBS-T or in PBS and incubated for 1 hour at RT (**Table 3**).

**Table 2:** Primary Antibodies used in this PhD thesis.

| Primary antibody | Company | Cat. Number | Isotype |
|---|---|---|---|
| Atg5 | Cell Signaling Technology | 2630 | Rabbit |
| Atg7 | Cell Signaling Technology | 2631 | Rabbit |
| Beta-Actin (AC-15) | Sigma-Aldrich | A5441 | Mouse |
| GAPDH | Monoclonal core unit (CNIO) | - | Mouse |
| Histone 3 | Abcam | ab1791 | Rabbit |
| HMGA2 | Cell Signaling Technology | 8179 | Rabbit |
| IgG | Sigma-Aldrich | I5006 | Rabbit |
| IGF2BP1 /IGF2BP1 | MBL  Ribonomics | RN007P | Rabbit |
| KEAP1 | Proteintech Group | 10503-2-AP | Rabbit |
| Kindlin-2/FERMT2 | Millipore | MAB2617 | Mouse |
| Kindlin-2/FERMT2 | Abcam | ab74030 | Rabbit |
| LC3B | Cell Signaling Technology | 2775 | Rabbit |
| Myc | Millipore | 06-340 | Rabbit |
| NBR1 | Abnova | H00004077-M01 | Mouse |
| NQO1 | Abcam | ab28947 | Mouse |
| p62 | BD Biosciences | 610832 | Mouse |
| p62 | Abcam | ab101266 | Rabbit |
| p62 | Novus Biologicals | NBP1-49956 | Rabbit |
| p65 | Santa Cruz Biotechnology, Inc. | sc-372 | Rabbit |
| phospho-NF-kB p65 | Cell Signaling Technology | 3037 | Rabbit |
| phospho-S6 (Ser240/244) | Cell Signaling Technology | 2215 | Rabbit |
| S6 (5G10) | Cell Signaling Technology | 2217 | Rabbit |
| Tubulin | Sigma-Aldrich | T 9026 | Mouse |

**Table 3:** Secondary Antibodies used in this PhD thesis.

| Secondary antibody | Company | Cat. Number | Isotype |
|---|---|---|---|
| TrueBlot Rabbit IgG-HRP | Rockland antibodies | 18-8816-31 | mouse |
| TrueBlot Mouse IgG-HRP | Rockland antibodies | 18-8817-33 | rat monoclonal |

| Secondary antibody | Company | Cat. Number | Isotype |
|---|---|---|---|
| **Rabbit IgG-HRP** | GE Healthcare | NA934 | donkey |
| **Mouse IgG-HRP** | GE Healthcare | NA9310 | donkey |

## 5.3  RNA extraction, PCR and qPCR

Total RNA was extracted and purified from cell pellets using RNeasy Mini-Kit (QIAGEN, cat. No. 74104) following the manufacturer's instructions. RNA concentration was determined by NanoDrop Spectrophotometer ND-100 (NanoDrop Biotechnologies). 1-2µg total RNA was reverse-transcribed into cDNA using the high capacity cDNA reverse transcriptase kit (Applied Biosystems, cat. No. 4368814), according to manufacturer´s protocol. 20ng of the total cDNA were subjected to real-time quantitative polymerase chain reaction (RT-qPCR - 60°C annealing temperature) using Power SYBR® Green PCR Master Mix (Applied Biosystems, cat. No. 4367659). Assays were run in triplicates on the 7900HT Fast Real-Time PCR system (Applied Biosystems) or QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). The primer sequences and annealing temperatures listed on **Table 4**. GAPDH and β-actin were used as loading control to normalize mRNA expression.

**Table 4:** Primers used in this PhD thesis.

| Gene | Forward Primer (5' – 3') | Reverse Primer (5' – 3') | °C |
|---|---|---|---|
| **CD2AP** | GGTGGCTGGAAGGAGAACTA | GTTTGATGGGCAAACTGTCA | 60 |
| **EHMT2** | GGAGGAAGCTGAACTCAGGAGG | GACTGAAGTCATCACCCACCAC | 60 |
| **FERMT2** | GATGCTAAGCTTCAGTTCACC | GGGTGTCTGATATTAAAAGTCTTAC | 60 |
| **FLOT1** | CCCATCTCAGTCACTGGCATT | CCGCCAACAT CTCCTTGTTC | 60 |
| **GAPDH** | GAAGGCTGGGGCTCATTTGC | CCAGGGGTGCTAAGCAGTTG | 60 |
| **HMGA2** | GAAGCAGCAGCAAGAACCAAC | GCTTCTGCTTTCTTTTGAGCTG | 60 |
| **NCEH1** | TGGACAACAGTTTACTCAAGA | GATCATTGCCTGCACAAAGT | 60 |
| **OGFOD1** | CCCTGGACCTGTACAGCATT | GACCATGAAACCAGCCACTT | 60 |
| **p62** | TAGCGCTACCGGTCGCCA | CCGGAATTTCGAGCGGCCAA | 60 |
| **TOP2A** | ACAAGACATCAAAGTGAAGTAAAGCC | GCAGACTCAAAACACAGACAAAGC | 60 |
| **β-actin** | GGCACCACACCTTCTACAATG | GTGGTGGTGAAGCTGTAGCC | 60 |

## 5.4  Cloning of genes into lentiviral constructs

The primers that were used in the cloning of p62 and FERMT2 are indicated in the **Table 5**. A Kozak sequence was added in the forward sequence. For p62 cloning amplified fragments and empty FG12-CMV-GFP-Puro vector were digested by NheI (NEB Inc, cat. no. R0131S) and XhoI (NEB Inc, cat. No. R0146S). For FERMT2 cloning amplified fragments

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and empty pLV-CMV-SV40-Puro vector were digested by XhoI (NEB Inc, cat. No. R0146S) and NheI (NEB Inc, cat. no. R0131S).The products were digested at $37^{o}C$ for 3 hours in the presence of NEBuffer Cutsmart (NEB Inc, cat. No. B7204S) and BSA (NEB Inc, cat. No. B9001). Digested PCR products were purified by PCR purification kit following manufacturer instructions (QIAquick PCR Purification Kit, cat. No. 28104). Ligation was performed (3:1, insert:vector molar ratio) with T4 DNA Ligase (NEB Inc, cat. No. M0202). For bacterial transformation Stbl3 cells were used (Thermo Scientific, cat. No. C737303). Plasmid isolation was performed following manufacturer instructions (QIAGEN-tip 500, cat. No. 10063). Insertion was checked by DNA sequencing.

**Table 5:** Primers for cloning used in this PhD thesis.

| Gene | Forward Primer (5' – 3') | Reverse Primer (5' – 3') | °C |
|------|--------------------------|--------------------------|-----|
| p62 | TTAACGCTAGCGGAGCCATGGCGTCGCTCACCGTG | GTTAACTCGAGTCACAACGGCGGGGGGATGC | 62 |
| FERMT2 | TTAACCTCGAGGGAGCCATGGCTCTGGACGGGATAA | GTTAAGCTAGCTCACACCCAACCACTGGTAAGTT | 59 |

## 5.5 Gene Silencing and ectopic expression via lentiviral transduction of shRNAs

Lentiviral supernatants were generated using HEK 293FT cells transfected via standard protocols of calcium phosphate precipitation: 500μL of HBS solution (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4.2H_2O$, 12 mM dextrose and 50 mM HEPES), 250 nM of $CaCl_2$, 4 μg of vector plasmid, and 4 μg of each of the lentiviral packaging constructs (pRSV-REV, pMDLg/pRRE and the VSV-G expression plasmid pHCMVG). Media was changed 8 hours after transfection and the viral supernatants were collected 24 hours later. Cells were infected with the viral supernatants and 4 μg/mL of Polybreen (Sigma-Aldrich). Infected cells were selected with 1 μg/mL of Puromycin (Sigma-Aldrich).

For genetic inactivation of p62, FERMT2 and NBR1, melanoma cells were infected with short hairpin RNAs purchased from Sigma-Aldrich (**Table 6**).

For ectopic expression of p62 and FERMT2, melanoma cells were stably infected with the lentiviral constructs, pLV-CMV-p62-mcherry-Puro and pLV-CMV-FERMT2-SV40-Puro in parallel with the corresponding empty vectors.

**Table 6:** short haiprin RNAs used in this PhD thesis

| Gene | TRC number | Sequence | region |
|------|------------|----------|--------|
| shcon | SHC001 | CAACAAGATGAAGAGCACCAA | - |
| p62 | TRCN0000007237 | CCGGCCTCTGGGCATTGAAGTTGATCTCGAGATCAACTTCAATGCCCAGAGGTTTTT | CDS |
| FERMT2 | TRCN0000128058 | CCGGGCGGACAGTTCTTACAACTTACTCGAGTAAGTTGTAAGAACTGTCCGCTTTTTTG | CDS |
| FERMT2 | TRCN0000128511 | CCGGCCGAAGAACTTTCTCTCTTAACTCGAGTTAAGAGAGAAAGTTCTTCGGTTTTTTG | CDS |
| NBR1 | TRCN0000123159 | CCGGGCTTCATAGTTATTTGGCATTCTCGAGAATGCCAAATAACTATGAAGCTTTTTG | 3-UTR |
| NBR1 | TRCN0000123160 | CCGGGCAGCATTTGTGGATGAGAATCTCGAGATTCTCATCCACAAATGCTGCTTTTTG | CDS |

## 5.6 siRNA-mediated transient genetic inactivation of p62

Cells were transfected with specific short interfering RNA (siRNA) molecules using Lipofectamine 2000 Transfection Reagent (Invitrogen, cat. No. 11668027) according to manufacturer´s protocol. Specifically, for genetic silencing of p62 pool of siRNAs (Dharmacon, cat. No. L-010230-00-0005) was used at a final concentration of 100nM. Expression analyses were performed at 72h post-transfection, by protein immunbloting and/or RT-PCR.

## 5.7 p62 gene targeting in cells using CRISPR/Cas9.

Gene-specific sgRNA oligos were cloned into the lentiCRISPR_v2 expression vector (Addgene, cat. No. 52961), which bicistronically expresses sgRNA and Cas9 nuclease. The sgRNA sequence was determined by the CRISPR Design Tool (http://crispr.mit.edu/) targeting the first exon of all p62 isoforms. The designed sgRNAs were **sgp62_1**:CACCGCACCGTGAAGGCCTACCTTC and **sgp62_2**: CACCGCGCTACACAAGTCGTAGTCT. Lentiviruses were produced in 293FT cells and infections were performed as previously described[311]. Gene silencing efficiency was determined by immunoblotting.

## 5.8 Clonogenic capacity of cells (Colony Formation)

Low confluency colony formation assays were performed by seeding $1x10^3$ (SK-Mel-103) or $5x10^3$ (SK-Mel-28, UACC-62) cells per well onto 6-well plates. Cells were allowed to grow for 10-14 days, followed by fixation with cold methanol for 10 minutes. Colonies were stained

with 0.4g/L crystal violet (Sigma). The number of colonies was quantified from micrographs of the plates using the ImageJ software.

## 5.9 Matrigel invasion assays

The invasive activity of melanoma cells was determined by matrigel transwell invasion assays using Boyden chambers (0.8 µm BD BioCoat™ Matrigel™ Invasion Chambers; from BD Biosciences, cat. No. 354480), according to the manufacturer's guidelines. Briefly, $10^5$ cells were serum-starved overnight and were seeded in serum-free DMEM onto the upper chamber. DMEM containing 10% FBS was placed in the lower chamber. After incubation for the indicated time intervals, invading and non-invading cells were first fixed with 4% paraformaldehyde and then stained with DAPI. Single cells were visualized by confocal detection of DAPI-stained nuclei through the 40x objective of a TCS-SP5-WLL (AOBS-UV) spectral microscope (Leica Mycrosystems, Wetzlar, Germany). The transwell membrane was also visualized by laser reflection. LAS AF Matrix screening Software was used for an automated high-throughput acquisition across the total width of the matrigel membrane in 9 different fields per experimental condition. IMARIS 6.3 Software was used to quantify the % of invading cells (normalized to the total cell number per field). Data are presented as means ± SEMs of three independent experiments performed in triplicates.

## 5.10 Drug treatments in vitro

To assess the autophagic flux in melanoma, cells were treated with autophagy activator Rapamycin at 25nM (Selleckchem, cat. No S1039) and autophagome-lysosome inhibitor Chloroquine used at 20µM (Sigma Aldrich, cat. No. C6628) at indicated times. Cells were fixed or collected for further Immunofluorescence and Immunoblotting assays.

## 5.11 RNA stability assay (Actinomycin D treatment)

p62 genetic silenced cells and cells with non-targeting shRNA were seeded on day 4 after infection (250000 cells/6cm plate). Cells were treated with Actinomycin D (Sigma Aldrich, cat. No. A9415) at 5 µg/ml concentration. After treatment, cells were washed with ice cold PBS and collected via scraping at indicated time points (0, 1, 2, 4 and 6 hours). RNA extraction, quantification, reverse transcription and qPCR were performed as described above.

## 5.12 Isobaric Tag for Relative and Absolute Quantitation (ITRAQ)

Melanoma cell pellets from two melanoma cell lines (SK-Mel-103 and UACC62) were extracted 3 days upon p62 or ATG5 genetic inactivation and proteins were subjected to proteome analysis by iTRAQ.

### (I) Sample Preparation

Cell pellets were washed 3 times with cold PBS containing protease inhibitors (Mini Complete, Roche) and then resuspended in 500 μL of ice-cold RIPA lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM EGTA, 50 mM Tris, pH 8.0 plus protease inhibitors) and 0.1% Benzonase Nuclease (Novagen, Darmstadt, Germany). Samples were vortexed, sonicated and clarified by centrifugation at 4 °C and 16,100 × g for 15 min. The supernatants containing the protein fraction were collected and cleaned up by acetone precipitation with 6 volumes of ice-cold acetone. Pellets were dissolved in 7M urea 2M tiourea. The protein concentration of the samples was determined according to the Bradford assay using BSA as standard (Protein Assay Kit, Bio-Rad, Hercules, CA).

### (II)    Protein Digestion and labeling with ITRAQ reagents

Samples were digested using the filter aided sample preparation (FASP) method as described above. Briefly, 100 ug of each sample dissolved in 7M urea 2M tiourea was loaded on the filter, reduced with 10 mM DTT 1 h at 37ºC and alkylated using 50 mM iodoacetamide for 20 min in the dark. The excess of reduction and alkylation reagents was washed. The proteins were digested overnight using endoproteinase Lys-C from Acromobacter lyticus M497-1 (Wako Pure Chemical Industries, Osaka, Japan) with 1:50 enzyme to protein ratio. Finally, trypsin (Promega, Madison, WI) was added and samples were subjected to a second digestion for 6 h. Each tryptic digest was labeled according to the manufacturer's instructions (AB Sciex, Framingham, MA) with one isobaric amine-reactive tag as follows: Tag114; Tag116; Tag115; Tag117. After one hour incubation, labeled samples were pooled, and evaporated to dryness in a vacuum centrifuge. The iTRAQ sample was cleaned up using a Sep-Pak C18 cartridge for SPE (Waters Corp., Milford, MA)[312]. Eluted peptides were vacuum-dried and reconstituted in OFFGEL solution (5% glycerol, 1% ampholytes pH 3-10) prior to electrofocusing.

### (III)   OFFGEL Fractionation

For pI-based peptide separation, we used the 3100 OFFGEL Fractionator system (Agilent Technologies, Böblingen, Germany) with a 24-well set-up. The IPG gel strips of 24 cm-long (GE Healthcare, München, Germany) with a 3–10 linear pH range were rehydrated for 15 min with the Peptide IPG Strip Rehydratation Solution according to the protocol of the manufacturer. Subsequently, 150 µL of sample was loaded in each well. Electrofocusing of the peptides was performed at 20°C and 50 µA until the 50 kVh level was reached. After focusing, the 24 peptide fractions were withdrawn and the wells rinsed with 100 µL of a solution of 0.1%TFA. Rinsing solutions were pooled with their corresponding peptide fraction. All fractions were evaporated by centrifugation under vacuum. Solid phase extraction and salt removal was performed with home–made columns based on Stage Tips with C8 Empore Disks (3M, Minneapolis, MN)[313] filled with Poros Oligo R3 resin (Life Technologies). Eluates were evaporated to dryness and maintained at 4°C. Just prior nano-LC, the fractions were resuspended in H2O with 0.1% (v/v) formic acid (FA).

### (IV)   Peptide analysis by nanoLC-MS/MS

Digested samples were separated by on-line reversed-phase nanoscale capillary LC and analyzed by electrospray MS/MS. The experiments were performed on an Eksigent nano LC system (Eksigent) coupled to an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark). Peptides were resuspended in 0.1% FA and loaded from a cooled nanoLC AS-2 autosampler (Eksigent). In order to pre-concentrate and desalt the samples before switching the pre-column in line with the separation column, 5 µL from each sample was loaded onto a reversed-phase ReproSil Pur C18-Aq 5 µm 0.3 x 10 mm trapping cartridge (SGE Analytical, Australia), and washed for 15 min at 2.5 µL/min with loading buffer (0.1% FA). The peptides were eluted from a RP ReproSil Pur C18-AQ 3 µm 200 x 0.075 mm (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) by application of a binary gradient consisting of 2% ACN in 0.1% FA (buffer A) and 100% ACN in 0.1%FA (buffer B), with a flow rate of 300 nL/min. Peptides were separated using the following gradient: 0-5 min 2% B, 5-150 min 40% B and 150-165 min 98% B. The column was operated at a constant temperature of 30ºC.  The LTQ Orbitrap Velos was operated in positive ionization mode. The MS survey scan was performed in the FT analyzer scanning a window between 250 and 1750 m/z. The resolution was set to 60 000 FWHM at m/z 400. The m/z values triggering MS/MS with a repeat count of 1 were put on an exclusion list for 60 s. The minimum MS signal for triggering MS/MS was set to 1000 counts. In all cases, one microscan was recorded. The lock mass option was enabled for both MS and MS/MS mode and the

polydimethylcyclosiloxane ions (PDMS, protonated (Si(CH3)2O))6; m/z 445.120025) were used for internal recalibration of the mass spectra[314]. For the HCD, up to the 15 most abundant isotope patterns with charge ≥2 from the survey scan were selected with an isolation window of 2 m/z fragmented in the C-trap collision cell. Normalized collision energy was set to 42%, the Q value to 0.25 and an activation time to 0.10 ms. Waveform filter was activated. The resulting fragments were detected in the Orbitrap system with a resolution of 7500 FWHM at m/z 400. The maximum ion injection times for the survey scan and the MS/MS scans were 500 ms and 250 ms respectively and the ion target values were set to 1E6 and 2E4, respectively for each scan mode.

### (V)   Data analysis

The raw files were processed using the Proteome Discoverer 1.3.0.339 software suite (Thermo Scientific). The fragmentation spectra were searched against the concatenated SwissProt Human database (release date: March 21, 2012; 20329 entries) using MASCOT[315] as the search engine (v 2.2) with the precursor and fragment mass tolerances set to 10 ppm and 0.075 Da, respectively, and with up to two missed cleavages. Lysine and peptide N-termini labeling with iTRAQ-4plex reagent as well as carbamidomethylation of cysteine were considered as fixed modifications, while oxidation of methionine was chosen as variable modification for database searching. Minimal peptide length was set to 6 amino acids and Mascot ion score > 20. Peptide identification false discovery rate (FDR) was less than 1%. In case that identified peptides were shared by two or more proteins (homologs or isoforms), they were reported by Proteome Discoverer as one protein group. The results were then exported into Excel for manual data interpretation. Although relative quantification and some statistical data were provided by the Proteome Discoverer software, an additional 1.3-fold change cutoff for all iTRAQ ratios (ratio <0.77 or >1.3) was selected to classify proteins as up- or down-regulated[316-318]. Proteins with iTRAQ ratios below the low range (0.77) were considered to be under-expressed, while those above the high range (1.3) were considered overexpressed. Protein classification (biological process and protein class) was performed by Ingenuity Pathway Analysis (IPA) software, using the entire list of identified proteins as the reference data set to analyze the regulated proteins.

## 5.13 Co-Immunoprecipitation assay followed by Mass Spectrometry

### 5.13.1   Subcellular Fractionation followed by Co-Immunoprecitation assay

Cells at 80% confluency were washed twice with ice cold PBS and collected by scraping with PBS. Cells were lysed first on ice for 5min in cytoplasmic extraction buffer (Tris/HCl

pH=8 50mM, EDTA pH=8 2mM, NP-40 0.1%, Glycerol 10%). Samples were centrifuged at 3000 rpm for 5min. Pellets were washed once with cytoplasmic extract buffer then resuspended in high-salt NP-40 buffer (50 mM Tris pH=8, 150 mM NaCl, 5mM EDTA, 1% nonionic detergent NP40). The buffers were supplemented with protease (Applied Biosystems, cat. No. 88266) and RNase inhibitors (Applied Biosystems, cat. No. N8080119). Extracts were incubated for 20 minutes at 4°C under rotation and sonicated in a Bioruptor Standard (Diagenode) for 10 minutes at medium amplitude. The extracts were centrifuged at maximum speed and the supernatant was collected.  After preclearing by protein A Dynabeads (Invitrogen, cat No. 10001D) for 30 minutes at 4°C, protein concentrations were determined and 2mg of total protein extract per condition was subjected to immunoprecipitation  using p62 antibody (Abcam, ab101266) or rabbit IgG (Sigma-Aldrich, I5006) as a negative control coupled to protein A Dynabeads  for 3h at 4°C. Immunoprecipitates were washed extensively, 4-6 times with the corresponding lysis bufffers. For Mass Spectrometry Analysis two sequential elutions with 100µl of urea 8M each step (Tris-HCl 0,1M + protease inhibitor) at 37°C for 15 min under high agitation. The two elution products were combined for Mass Spectrometry injection. For validation of protein-protein interactions independent co-IP experiments were performed and the immunoprecipitates were eluted in NP-40 buffer mixed with loading dye, boiled at 95°C for 5 minutes and supernatant were collected for immunobloting assays.

## 5.13.2  Mass Spectrometry

### (I)  Sample Preparation

Eluted proteins were subjected to label free proteome analysis. Samples were digested by means of the standard FASP protocol[319]. Briefly, samples were eluted with UT buffer (8M urea in 100 Mm Tris-HCl, pH=8.01) with continuous mixing at 37°C and transfer to a filter unit. Proteins were then reduced with 10 mM DTT, alkylated using 50 mM iodoacetamide for 20 min in the dark and the excess of reagents was washed out with UA twice. Proteins were digested with endoproteinase Lys-C (Wako) during 6 hours in a wet chamber (1:50 enzyme to substrate ratio). Finally, samples were diluted in 50 mM ammonium bicarbonate to reduce the urea concentration to 1M and subsequently digested with Trypsin Gold (Promega) overnight at 37 °C. Resulting peptides were further desalted and concentrated using homemade reversed phase micro-columns filled with Poros Oligo R3 beads (Life Technologies). The samples were dried using the Speed-Vac and dissolved in 30 µL of 0.1% formic acid (FA).

## (II)   LC-MS/MS analysis

Desalted peptides were separated by reversed-phase chromatography using a nanoLC Ultra system (Eksigent), directly coupled with a LTQ-Orbitrap Velos instrument (Thermo Fisher Scientific) via nanoelectrospray source (ProxeonBiosystem). Peptides were loaded onto the column (Dr. Maisch, Reprosil-Pur C18 GmbH 3 μm, 200x0.075 mm), with a previous trapping column step (Prot Trap Column 0.3 x 10 mm, ReproSil C18-AQ, 5 μm, 120Å, SGE), during 10 min with a flow rate of 2.5 μl/min of loading buffer (0.1% FA). Elution from the column was made with a 90 min linear gradient (buffer A: 2% ACN, 0.1%FA; buffer B: 100% ACN, 0.1%FA) at 300 nL/min. The peptides were directly electrosprayed into the mass spectrometer using a PicoTip emitter (360/20 OD/ID μm tip ID 10 μm, New Objective) a 1.4 kV spray voltage with a heated capillary temperature of 325°C and S-Lens of 60%. Mass spectra were acquired in a data-dependent manner, with an automatic switch between MS and MS/MS scans using a top 20 method with a threshold signal of 800 counts. MS spectra were acquired with a resolution of 60000 (FWHM) at 400 m/z in the Orbitrap, scanning a mass range between 350 and 1500 *m/z*. Peptide fragmentation was performed using collision induced dissociation (CID/CAD) and fragment ions were detected in the linear ion trap. The normalized collision energy was set to 35%, the Q value to 0.25 and the activation time to 10 ms. The maximum ion injection times for the survey scan and the MS/MS scans were 500 ms and 100 ms respectively and the ion target values were set to 1E6 and 5000, respectively for each scan mode. Samples were run in duplicates.

## (III)   Data analysis

Raw files were analyzed by Proteome Discoverer (version 1.4.1.14) against a forward-reverse concatenated human database (UniProtKB/Swiss-Prot, 88354 sequences, 26/03/2013 release). Oxidation of methionines was set as variable modification whereas carbamidomethylation of cysteines was considered as fixed modification in the SequestHT search engine. Minimal peptide length was set to 6 amino acids, a maximum of two missed-cleavages were allowed. Peptides were filtered at 1% FDR using Percolator. In case that identified peptides were shared by two or more proteins (homologs or isoforms), they were reported by Proteome Discoverer as one protein group. As stated above, each sample was run in triplicate. Label-free analysis was performed with Proteome Discoverer using the spectral count values determined by the software. Further analysis was done using Excel and MultiExperiment Viewer software (version 4.8.1). The changes in protein abundance between the different samples were measured via a direct comparison of the spectral count values. Protein classification (molecular function, biological process and protein class) was performed by STRING[320] software. Raw files from the eluted samples were also analyzed by

MaxQuant[321] (version 1.4.1.2) (UniProtKB/Swiss-Prot human database, canonical and isoform sequences, 39748 sequences, 01/22/2014 release), including phosphorylation on serine, threonine and tyrosine residues as variable modification. The rest of search parameters were set as above. For protein assessment, at least two unique peptides provided by Andromeda search engine[322] with a FDR = 1% were required. Results at peptide label were exported to excel for further analysis.

## 5.14  RNA Immunoprecipitation (RIP) followed by qPCR

p62 infected cells or control cells (non targeting shRNA) at 80% confluence were fixed in 1% formaldehyde for 10 minutes at room temperature (RT), and fixation was stopped by adding 1M Glycine for 5 minutes at room temperature. After washing with ice cold PBS, cells were collected by scraping and lysed with in NT2 buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1mM MgCl2, 0.5% Nonidet P-40 and 1 mM EDTA) supplemented with protease and RNase inhibitors. For the solubilization of crosslinked complexes, lysates were sonicated for 10 minutes at medium intensity. The lysates were centrifuged at maximum speed and the supernatant was collected. After preclearing by protein A Dynabeads for 30 minutes at 4°C, samples were immunoprecipitated using IGF2BP1 antibody (MBL Ribonomics, cat. No. RN007P) or rabbit IgG (Sigma, cat. No. I5006) as a negative control coupled to protein A Dynabeads for 3h at 4°C. Immunoprecipitates were washed extensively, 4-6 times and resuspended in NT2 buffer containing 50µg proteinase K (Roche Applied Science, cat. No. 03115836001), 1% SDS, 200mM NaCl and 10mM EDTA. Samples were split into two (1:4 for protein, 3:4 for RNA extraction). The protein fraction was mixed with loading dye, boiled at 95°C for 5 minutes and supernatant was collected. RNA elution was done by consecutive incubations at 55°C for 30 minutes and at 65°C for 45 minutes. Supernatants were collected and digested with DNase I for 10 minutes at RT. RNA was extracted with the TRI Reagent (Sigma, cat No. T9424) following manufacturer´s protocol. The total amount of RNA immunoprecipitated and 1µg of RNA extracted from inputs were retrotranscribed using cDNA reverse transcriptase Superscript III (ThermoScientific, cat No. 18080044) and qPCR were performed as described before. Fold binding enrichment of target mRNAs in the immunoprecipitated fraction was calculated after normalization with the gene expression from the inputs or with the negative controls. All the experiments were performed in three biological replicates. The primers used for RIP-qPCR are listed in **Table 7**.

## 5.15 Immunofluorescence in fixed cells

Cells were cultured on cover slips and were fixed with 4% paraformaldehyde in PBS at room temperature or with ice cold methanol for 20 min. Cells were then washed twice with 0.1M glycine in PBS for 10min each, permeabilized with 0.2% Triton X-100 in PBS for 5 min, washed twice with PBS and incubated with 1% BSA in PBS at room temperature for 30 min. Fixed cells were incubated with primary antibody diluted in blocking buffer (1% BSA in PBS) at room temperature for 1 h. Cells were then washed three times with PBS and incubated with Invitrogen´s Alexa-conjugated secondary antibodies at room temperature for 1h. Cells were washed 3 times with PBS and counterstaining of nuclei with 4,6-diamidino-2-phenylindole (DAPI) and mounting of the cover slips were done with Prolong Gold (Invitrogen, cat. No. P36930) 20 minutes before imaging. Negative controls were obtained by omitting the primary antibody. The following primary antibodies were used: p62 (Novus Biosciences, cat No. 610832); IGF2BP1 (Cell Signaling Technology, cat. No. 8482); LC3B (Cell Signaling Technology, cat. No. 2775). Alexa Fluor 555 anti-rabbit IgG (Invitrogen, cat. No. A-31572) and Alexa Fluor 488 anti-mouse IgG (Invitrogen, cat. No. A-21202) were used as secondary antibodies.

## 5.16 Tissue Immunostaining

### (I) Immunofluorescence

Paraffin-embedded tumor samples were stained for p62 (Novus Biologicals, cat. No. NBP1-49956), LC3B (Cell Signaling Technology, cat. No. 2775d), phospho-p65 (Cell Signaling Technology, cat. No. 3037). After deparaffinization and antigen retrieval (10 mmol/L sodium citrate buffer at pH 6) the samples were incubated overnight with primary antibodies at 4 °C in a humidified chamber. Mouse IgG (M.O.M.) Blocking Reagent (Vector Laboratories; MKB-2213); and Image-iT FX signal enhancer (Invitrogen; cat. No. I36933) were used before the primary antibody incubation according to manufacturer's protocols for mouse saples. Then rinsed and incubated with fluorescent secondary antibodies for 1 hour at room temperature. For detection, Alexa Fluor 555 anti-rabbit IgG (Invitrogen, cat. No. A-31572) and Alexa Fluor 488 anti-mouse IgG (Invitrogen, cat. No. A-21202) were used. Nuclei were counterstained with DAPI and mounted with Prolong Gold (Invitrogen, cat. No. P36930) 20 minutes before imaging. The fluorescence emission was acquired using a confocal TCSSP5-WLL (AOBS-UV) spectral microscope (Leica Mycrosystems, Wetzlar, Germany).

## (II)   Immunohistochemistry in human and mouse biopsies

### a. Tissue microarrays (TMAs) and immunohistochemistry (human samples)

For p62, melanocytic tumors including dermal nevi (n=31), dysplastic nevi (n=29), primary radial growth phase (RGP) malignant melanoma (n=16), primary vertical growth phase (VGP) melanoma (n=105), cutaneous metastasis (n=30), lymph node (n=28) and visceral (n=22) melanoma metastases were used for immunohistochemistry (IHC) and TMAs construction as previously described[323]. FERMT2 staining was performed using anti-FERMT2 antibody, clone 3A3 (Merck Millipore). For FERMT2, melanocytic tumors including dermal nevi (n=34), primary vertical growth phase (VGP) melanoma (n=82), cutaneous (n=33), lymph node (n=28) and visceral (n=22) melanoma metastases were used. p62-specific staining was scored blinded and considered positive for p62 taking into account both the percentage of positive cells and the intensity of its expression. It was blinded scored as follows: negative (no expression), low (weak intensity or <50% of the cells with strong intensity of expression) and high (≥50% of the cells with strong intensity of expression). For FERMT2 the scoring system was as follows: negative (no expression), low (weak intensity or <50% of the cells with strong intensity of expression) and high (≥50% of the cells with strong intensity of expression).

IHC was performed on 4-µm-thick sections of formalin-fixed, paraffin-embedded samples. p62 (anti-p62 Ick ligand BD Biosystem) and FERMT2 (anti-Kindlin-2 antibody, clone 3A3 Merck Millipore) staining were performed on a Bond™ Automated System (Leica Microsystems)[323]. The IHCs were performed in the Dermatology and Pathology Department at the Hospital 12 de Octubre Medical School and the scoring was blinded by the Drs. Erica Riveiro-Falkenback, Pablo Ortiz-Romero and Jose L Rodríguez-Peralto.

### b. Immunohistochemistry (mouse samples)

Tissue samples were fixed in 10% neutral buffered formalin (4% formaldehyde in solution), paraffin-embedded and cut at 3 µm, mounted in superfrost® plus slides and dried overnight. For different staining methods slides were deparaffinized in xylene and and re-hydrated through a series of graded ethanol until water. Consecutive sections were stained with hematoxylin and eosin (H&E) and for immunohistochemistry an automated immunostaining platform was used (Ventana Discovery XT, Roche). Antigen retrieval was first performed with high or low pH buffer,  depending on the primary antibody (CC1m, Roche or RiboCC, Roche); endogenous peroxidase was blocked (peroxide hydrogen at 3%) and slides were then incubated with the appropriate primary antibody as detailed:  rabbit monoclonal anti-ATG5 (EPR1755 (2); 1:600; Abcam ab108327), rabbit polyclonal anti-p62 (SQSTM1; 1:150; Novus Biologicals NBP1-49956), rabbit polyclonal anti-S100  (1:500;

Dako, Z0311), and goat polyclonal anti-Sox10 (1/300; Santa Cruz sc-17342. After the primary antibody, for Sox10, slides were incubated with the corresponding secondary antibodies (rabbit anti goat biotinylated, Dako) and visualization systems when needed (OmniRabbit, Ventana, Roche) conjugated with horseradish peroxidase. The rest of the antibodies were incubated with UltraMap Rabbit (Ventana, Roche) conjugated with alkaline phosphatase. Immunohistochemical reaction was developed using 3,30-diaminobenzidine tetrahydrochloride (DAB) (Chromomap DAB, Ventana, Roche) and Naphtol with Fast Red (FR) (Chromomap RED, Ventana, Roche) as a chromogen . Nuclei were counter stained with Carazzi's hematoxylin. Finally, the slides was dehydrated, cleared and mounted with a permanent mounting medium for microscopic evaluation.

## 5.17 Mitochondrial Respiration

$5\times10^3$ cells of p62 deficient SK-Mel-103 and UACC-62 cells with the corresponding shcon cells were plated per well in XF 96-well microplates and incubated for 24 hr at 37 °C in 5% $CO_2$. Respiration was measured under basal conditions, and in response to Oligomycin (ATP synthase inhibitor; 0.5 µM; Sigma Aldrich, cat. no. 75351) and the electron transport chain accelerator ionophore FCCP (Trifluorocarbonylcyanide Phenylhydrazone; 0.5 µM; Quimigen S.L., cat. no. SC-203578). Finally, respiration was stopped by adding the electron transport chain inhibitors Rotenone and Antimycin A (1 µM each; Sigma Aldrich, cat. no. R8875 and A8674, respectively). Oxygen consumption rate (OCR) and extra-cellular acidification rate (ECAR) were measured with a XF96 Extracellular Flux Analyzer (Seahorse Biosciences) as per the manufacturer's recommended protocol. OCR and ECAR values were normalized to cell number. Four technical replicates were used per condition in three independent experiments.

## 5.18 Mice breedings and activation of Tyr::CreER transgene

The following strains were used in this study: Tyr::CreERT2[/1Lru 324], p62-deficient mice[325], Atg5[tm1Myok 326], Braf[tm1Mmcm] (Braf[CA/CA])[327], Pten[tm2Mak 328]. For simplicity these lines are herein referred as to Tyr:CreERT2, p62KO, Atg5[flox/flox], Braf[CA/CA], and Pten[flox/flox], respectively. For the analysis of melanomas, breedings were set to generate Tyr:CreERT2; Braf[CA/CA] Pten[flox/flox] background with different p62 or/and Atg5 copy number (herein referred to as Atg5^{+/+}, Atg5^{-/+}, Atg5^{-/-} or p62^{+/+}, p62^{-/+}, p62^{-/-}, respectively).

Topical administration was performed by applying 2µl (2mg/ml) of (Z)-4-Hydroxytamoxifen (Sigma Aldrich, cat. No.H7904) in ethanol on the skin back at postnatal days 2, 3 and 4. For systemic administration, 8 week-old mice were induced by 100 ml intraperitoneal injection of

tamoxifen (Sigma, T5648; stock 8 mg/ml) for 3 consecutive days. Tumor growth was measured every 5 days blinded to the experimental conditions at the indicated time intervals after post-mortem depilation. Tumor volume was estimated using a calliper and calculated as $V = a \times b^2 \times \pi/6$, where "a" stand for the bigger and "b" for the smaller diameter of the tumor. Studies for tumor growth and survival were performed. Mice were sacrificed 8 weeks upon 4-hydroxytamoxifen administration or when total tumor volume was exceed $2cm^3$. Necropsy was performed collecting tumor samples and mouse tissues for histopathological and protein detection studies.

## 5.19 Subcutaneous inoculation of B16-F10 cells into p62 deficient mice

$5 \times 10^5$ B16-F10 (ATCC), resuspended in PBS, mouse melanoma cells were inoculated subcutaneously in the back of 8 week old mice expressing different copies of p62. Development of subcutaneous tumor was monitored every week, blinded to the experimental conditions at the indicated time intervals, and measured by caliper, as described above. The mice were sacrificed 3 weeks after injection and tumors were collected for histopathological and protein detection studies. All experiments with mice met the Animal Welfare guidelines and were performed in accordance with protocols approved by the Institutional Ethics Committee of CNIO.

## 5.20 Genotyping

Genotyping of every strain was obtained by DNA extraction from the tails of each mouse after the weaning. Briefly, mouse tails were digested overnight with DNA digestion buffer (50mM Tris-HCl with ph 8, 100mM EDTA with pH 8, 100mM NACl, 1% SDS and 20mg/ml proteinase K (Roche, cat. No. 03115852001) at $55^{\circ}$C. Then, saturated NaCl was added and after full speed centrifugation the supernatant was collected equal volume of ice cold isopropanol was added. Precipitated DNA was washed with 70% ethanol and then resuspended in TE buffer (10mM Tris, ph 8 and 1mM EDTA). PCRs were performed for target gene amplification. The samples were loaded into agarose gels. Primers that were used for genotyping are indicated in the **Table 7**.

**Table 7:** Primers for genotyping used in this PhD thesis.

| Gene | Forward Primer (5' – 3') | Reverse Primer (5' – 3') | °C |
|---|---|---|---|
| p62-WT | CTTACGGGTCCTTTTCCCAAC | TCCTCCTTGCCCAGAAGATAG | 57 |
| p62-KO | CTGCATGTCTTCTCCCATGAC | TAGATACCTAGGTGAGCTCTG | 57 |
| Pten | CTCCTCTACTCCATTCTTCCC | ACTCCCACCAATGAACAAAC | 57 |
| Cre | GGCTGGACCAATGTAAATATTG | CATCATCGAAGCTTCACTG | 55 |

| Gene | Forward Primer (5' – 3') | Reverse Primer (5' – 3') | °C |
|---|---|---|---|
| m-Actin | TCATCAGGTAGTCAGTGAGGTCGC | CACCACACCTTCTACAATGAGCTG | 55 |
| Braf | TGAGTATTTTTGTGGCAACTGC | CTCTGCTGGGAAAGCGGC | 55 |
| Atg5-WT | ACAACGTCGAGCACAGCTGCGCAAGG | GAATATGAAGGCACACCCCTGAAATG | 60 |
| Atg5-KO | CAGGGAATGGTGTCTCCCAC | GTACTGCATAATGGTTTAACTCTTGC | 60 |

## 5.21 GO terms, Networks, Heatmaps and Venn Diagrams

GSEA was applied to previously reported data sets from the Cancer Cell Line Encyclopedia evaluating gene lists as described before[329]. Gene sets showing FDR<0.25 after Kolmogorov-Smirnoff testing were considered enriched between classes under comparison. Significantly enriched (p≤0.05) GO biological processes and functions for differentially expressed proteins upon p62 genetic inactivation and p62 interactome were identified by using Ingenuity Pathway Analysis (IPA) software and String database[330]. Heatmap and correlation graphs for RNA and protein levels were created by Perseus v1.5.1.6. Venn diagrams were created by using online tools Venny v2.0[331] and jvenn[332].

## 5.22 Statistical Analyses

Unless otherwise indicated, statistical analyses were performed using Graphpad Prism software with unpaired two-tailed t-test. p62 and FERMT2 *in vivo* correlation was analyzed by Chi-square test. For microarray hybridization and gene expression analyses, gene sets showing FDR<0.25 after Kolmogorov-Smirnoff testing were considered enriched between classes under comparison. For survival analysis, clinical data and immunohistochemical scoring were performed blind, and data were compiled only after all analyses were completed. p62 and FERMT2 IHC stainings were quantified by two independent investigators. The distribution of the patients according to p62 and FERMT2 expression was analyzed by Chi-square test (sex, ulceration and clinical staging) and Student´s t test (age and Breslow). Disease Free Survival (DFS) curves were estimated with Kaplan-Meier product-limit method and survival curves were compared using logrank test. DFS was defined as the time interval between diagnosis (made by histopathological study of primary melanoma) and the development of the first metastasis. It was considered censored for patients who did not present metastasis at last follow-up. The hazard ratio was calculated using Cox regression and adjusted with univariate and multivariate models adjusting by Breslow, Clinical Stage and T3 category.

# VI.RESULTS

## 6.1 Expression and prognostic studies support pro-metastatic roles of p62 in human melanomas

Databases such as The Cancer Genome Atlas (TCGA) are being consolidated as a versatile platform to screen for oncogenic drivers and tumor suppressors across large cohorts of clinically-annotated biopsies[333]. For example, TCGA served as the starting point of selective dose-dependent downregulation of ATG5 and multiple lysosomal-associated factors we have reported in aggressive melanomas[142,220].

Therefore, we interrogated the TCGA for genomic alterations and expression changes that could guide in subsequent functional studies of p62 in this
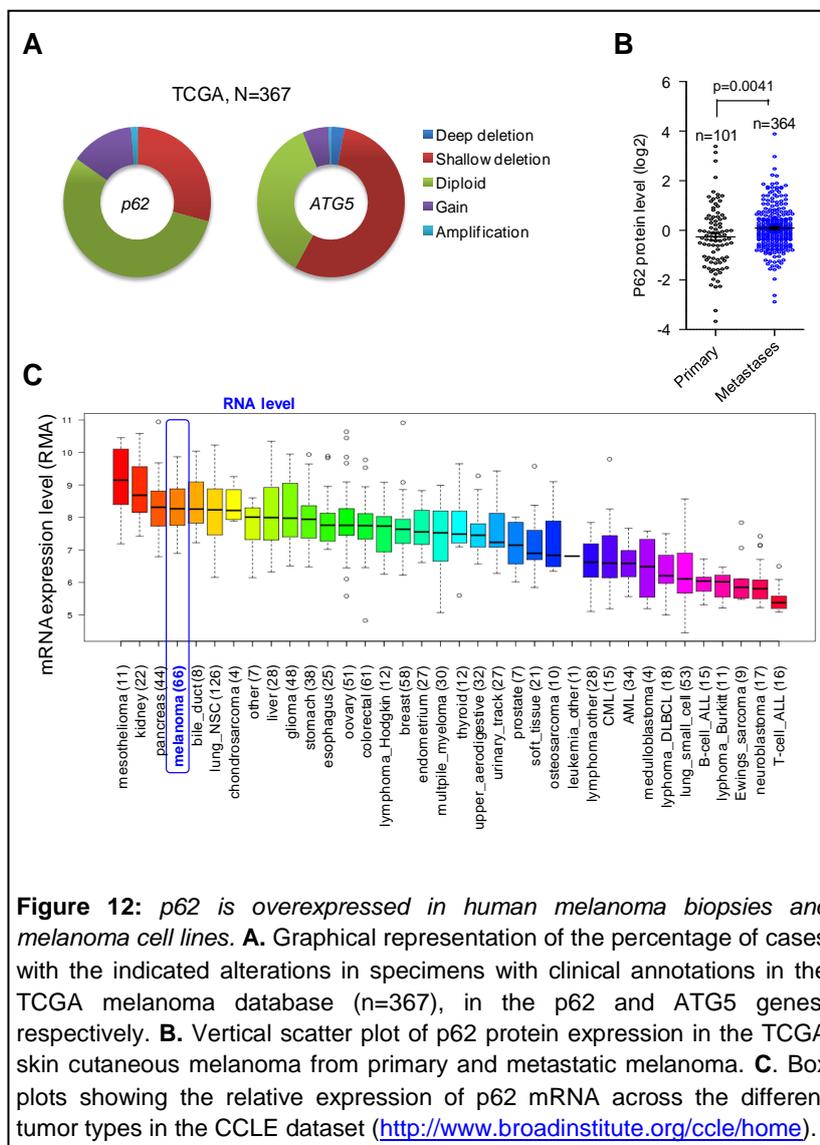


**Figure 12:** *p62 is overexpressed in human melanoma biopsies and melanoma cell lines.* **A.** Graphical representation of the percentage of cases with the indicated alterations in specimens with clinical annotations in the TCGA melanoma database (n=367), in the p62 and ATG5 genes, respectively. **B.** Vertical scatter plot of p62 protein expression in the TCGA skin cutaneous melanoma from primary and metastatic melanoma. **C**. Box plots showing the relative expression of p62 mRNA across the different tumor types in the CCLE dataset (http://www.broadinstitute.org/ccle/home).

disease. Interestingly, allelic losses of the p62 locus were notably less frequent than for ATG5 in the TCGA melanomas (<28% vs 56%; **Figure 12A**) indicating distinct roles of these two proteins in melanoma. Additionally, TCGA revealed a significant upregulation of p62 protein expression at metastatic sites (skin in transit, regional lymph nodes and distal visceral organs), when compared to primary melanomas (see number of specimens analyzed and the corresponding *p*-values in **Figure 12B**). Of note, the relatively high expression of p62 in melanoma was supported by Cancer Cell Line Encyclopedia database (**Figure 12C**; CCLE, encompassing over 700 cell lines of 25 different cancer types).

The above data were independently validated by immunohistochemical staining of p62 in high density tissue microarrays (TMAs; n=213 samples) covering a broad spectrum of melanocytic lesions at different stages of progression (provided by E. Riveiro-Falkenbach)[334]. These included common and dysplastic nevi, primary melanomas at radial and vertical growth phase of expansion (RGP and VGP, respectively), as well as metastases from skin, lymph node and visceral sites. All acquired human dermal nevi analyzed were found with a largely undetectable p62 staining (see representative examples in **Figure 13A** and quantification in **Figure 13B**). These data therefore, support the concept that this protein is not involved in suppressor mechanisms that maintain common nevi in a benign state[285]. Interestingly, a retrospective 5-year follow-up study in an independent cohort of 101 clinically-annotated melanoma specimens showed that patients with positive p62 expression presented an increased risk for the development of metastasis with respect to negative cases (hazard ratio 2.96 and p=0.022; **Figure 13C**).



**Figure 13:** *p62 is progressively increased in human melanoma biopsies.* **A.** Representative histological images of p62 immunohistochemistry in tissue microarrays containing the indicated human benign and malignant melanocytic tumors. **B.** p62 quantification for 213 melanocytic tumors. The staining was classified into negative, low and high p62-expressing tumors (p≤0.0001). **C.** Kaplan–Meier plot of Disease Free Survival (DFS) curves subgrouped by p62 expression (p62 negative and p62 positive with the indicated number of cases in each group). Analysis time represents years of follow-up. Data provided by E. Riveiro-Falkenbach, ref.[334].

Altogether, these data suggest that p62 may be involved not only in cutaneous melanomas[285], but also, at advanced and metastatic stages of this disease.

## 6.2 Tumor-, but not stromal-driven p62 favors melanoma progression in GEMM

p62 has been found overexpressed in multiple neoplasias[189,258-263,265-267]. However, high p62 levels are not an unequivocal indicator of functional requirement, as pro- and antitumoral roles of this protein have been reported depending on the system analyzed. In particular, genetically-engineered mouse models (GEMM) reported in the context of cancer for prostate and hepatocellular carcinomas[260,282] have revealed distinct roles of p62 in tumor and stromal cells; in the latter with suppressive function[278,308]. Therefore, we generated new GEMM to dissect the contribution of p62 to melanoma progression. To mimic benign nevi, p62-deficient mice[325] were crossed into the *Tyr::CreERT2;Braf^{V600E}* strain, where constitutively active Braf (Braf^{V660E}) is induced by means of a tamoxifen-inducible Cre-recombinase restricted to the melanocytic compartment[327]. In turn, crosses into *Tyr::CreERT2;Braf^{V600E};Pten^{-/-}* animals were used to assess the impact of p62 in the context of the characteristic BRAF^{V600E} and PTEN loss of human cutaneous melanomas[327]. Pigmented lesions were optically and histopathologically monitored at different time points after topical application of 4-hydroxytamoxifen (4-HT).

In the normal (uninduced) skin, histological analyses revealed p62 mostly confined to the hair bulge expressing low levels (**Figure 14A**, pink staining). Consistent with dysplastic human nevi shown in **Figure 13A**, hyperplastic lesions in *Tyr::CreERT2;Braf^{V600E}* animals (herein referred to as *Braf^{V600E}* for simplicity) showed a modest induction of p62 staining (pink signal in pigmented-brown-melanocytic cells in middle panels of **Figure 14A**).



**Figure 14:** *p62 is highly induced in Braf^{V600E};Pten^{-/-} mouse model.* **A.** Histological images (p62 staining upper and H&E lower) upon topical administration of 4-HT on the back skin of *Braf^{V600E}* and *Braf^{V600E};Pten^{-/-}* mice. **B.** Histological images for p62 (upper) and S100 (lower) of different tumor sizes developed on the back skin of *Braf^{V600E};Pten^{-/-}* mice upon topical administration of 4-HT.

In contrast, *Braf^V600E;Pten^-/-* melanomas showed a marked increased p62 staining in histological sections (**Figure 14A**, right panel). Interestingly, p62 accumulation was particularly evident as melanomas progressed and became invasive (**Figure 14B**).

To determine the contribution of p62 in benign nevi, mice with monoallelic and biallelic loss of p62 were crossed into *Tyr::CreERT2;Braf^V600E* strain. Nevus formation was followed by visual inspection after 4-HT administration (**Figure 15A**). As summarized in **Figures 15B-C** p62 was largely dispensable for the generation of benign melanocytic lesions, as neither their onset, number nor progression were significantly affected by heterozygous or homozygous loss of this gene (see the corresponding quantifications in **Figure 15D**). In this Tyr::CreERT2;Braf^V600E background, melanomas were not developed, independently on the presence or absence of p62.



**Figure 15:** *p62 is dispensable for nevi formation in Braf^V600E mice.* **A.** Graphical illustration of post-natal administration of 4-HT on the back skin of newborn *Braf^V600E* mice. **B.** Representative images acquired 8 months after topical administration of 4-HT on the back of inducible *Braf^V600E* mice in different p62 genotypes. **C.** Histological images of H&E and p62 stainings from (**A**). **D.** Distribution of lesions after topical administration of 4-HT on the back of inducible *Braf^V600E* mice in different p62 genotypes. Light blue corresponds to number of lesions range from 0-50, blue from 50-100 and dark blue >100.

We then questioned the effect of allelic loss of *p62* in melanoma, with particular emphasis in the transition from radial to vertical/metastatic growth (e.g. under similar settings that have revealed protumorigenic roles of *Atg5* heterozygous loss[220]). This was performed by topical administration of 4-HT on the back of the skin of *Braf^V600E;Pten^-/-* strains with different allelic status of *p62* (**Figure 16A**). In these animals, heterozygous *p62* deletion led to a visibly compromised melanoma development, which was further inhibited in p62^-/- background (see representative macrographs of **Figure 16B**). The relevance of *p62* in the *Braf^V600E;Pten^-/-* melanomas was confirmed histologically (IHC or IF) by staining for the melanocytic markers S100 or TRP2 (**Figures 16C-D**, respectively). Quantitative analyses of tumor volume

emphasized deleterious effects of mono and bi-allelic deletions of *p62* in the *Braf^V600E^;Pten^-/-^* background (**Figures 16E-F**) ; *p*≤0.0001).



**Figure 16:** *p62 loss abrogates tumor growth and diminishes the metastatic capacity of melanoma cells in Braf^V600E^;Pten^-/-^ mice.* **A.** Graphical illustration of post-natal administration of 4-HT on the back skin of newborn *Braf^V600E^;Pten^-/-^* mice mice. **B.** Representative images acquired 6 weeks after topical administration of 4-HT on the back of inducible *Braf^V600E^;Pten^-/-^* mice in different p62 genotypes. **C.** *Upper panel*, representative images of p62 immunofluorescence (red) in the 3 different genotypes for p62. *Lower panel,* histology slides of back skin showing representative examples of p62 and S100 expression in the 3 different genotypes of p62. **D.** Representative images of double-immunofluorescence for p62 (red) and TRP2 (green) of inducible *Braf^V600E^;Pten^-/-^* mice in the 3 different genotypes of p62. **E.** Tumor outgrowth of *Braf^V600E^;Pten^-/-^* mice in different p62 genotypes shown at 40 days after 4-HT topical administration (mm$^3$). Results are mean ± SEM. **F.** Tumor outgrowth follow-up of *Braf^V600E^;Pten^-/-^* mice in different p62 genotypes shown at mm$^3$. Results are mean ± SEM.

*p62* loss not only reduced tumor growth of cutaneous melanomas (**Figures 16E-F**), but also led to a markedly decreased metastatic efficiency to proximal (inguinal) and distal (axilar) lymph nodes (**Figures 17A-B**). To address the relevance of *p62* in the metastatic potential a to distal organs (i.e. lungs), *Braf^V600E^;Pten^-/-^* adult mice were induced systemically

by 4-HT (intraperitoneal injections, see Materials and methods, 5.18). The enhanced lung (micro)metastases that were observed in p62$^{+/+}$ background were diminished in mice with bi-allelic deletion of *p62,* four weeks upon induction (**Figures 17C-D**). Consequently, follow-up studies revealed a dose-dependent impact of p62 loss on the survival of melanoma-bearing *Braf$^{V600E}$;Pten$^{-/-}$* mice (**Figure 17E**). The results above support a key role of melanoma-expressed p62 in tumor progression and metastasis.

To rule out possible effects on the stroma[278], a separate set of *p62$^{+/+}$*, *p62$^{+/-}$* and *p62$^{+/-}$* mice were used as hosts for implantation of syngenic B16 melanoma cells. Interestingly, the allelic status of p62 in this system had no significant impact on these B16-tumors (**Figures 17F**).



**Figure 17:** *p62 loss inhibits melanoma metastasis and prolongs the lifespan of Braf$^{V600E}$;Pten$^{-/-}$ mice.* **A.** Histology stainings in lymph nodes for p62 and S100 of *Braf$^{V600E}$;Pten$^{-/-}$* mice in different genotypes of p62. **B.** Metastases count in lymph nodes 8 weeks after 4-HT administration on the back of the skin of *Braf$^{V600E}$;Pten$^{-/-}$;p62$^{(+/+)}$* (n=9), *Braf$^{V600E}$;Pten$^{-/-}$;p62$^{(-/+)}$* (n=7) and *Braf$^{V600E}$;Pten$^{-/-}$;p62$^{(-/-)}$* (n=4) mice, respectively. **C.** H&E staining (highlighting pigmented-brown-melanocytic cells) of isolated lungs from *Braf$^{V600E}$;Pten$^{-/-}$;p62$^{(+/+)}$* and *Braf$^{V600E}$;Pten$^{-/-}$;p62$^{(-/-)}$* mice, 4 weeks upon intraperitoneal injection with 4-HT. **D.** Quantification of pigmented-brown-melanocytic cells of (**C.**). Lung metastases were quantified as area per lung section. In a section, single cells and aggregated cells (3 and 5) were counted in every area and were considered as (micro)metastases. Results are mean ± SEM. **E.** Kaplan–Meier survival curve of the 3 different groups: *Braf$^{V600E}$;Pten$^{-/-}$;p62$^{(+/+)}$* with mean time to death of 45 days (n=6), *Braf$^{V600E}$;Pten$^{-/-}$;p62$^{(-/+)}$* 66 days (n=12) and *Braf$^{V600E}$;Pten$^{-/-}$;p62$^{(-/-)}$* 88 days (n=18). **F.** Tumor growth (mm$^3$) upon subcutaneous implantation of B16-F10 cells in *C57BL/6J* mice expressing different alleles of p62 (n=6 for p62$^{+/+}$, n=5 for p62$^{-/+}$ and n=6 for p62$^{-/-}$). Results are mean ± SEM.

Together, these data: (i) provide physiological relevance of dose-dependent roles of p62 in melanoma progression; and importantly, (ii) separate melanomas from other tumor types where p62 has a more dominant suppressive role in the stromal compartments.

## 6.3   p62 controls key proliferative and metastatic signatures in melanoma cells

As indicated above, the roles of p62 in cancer cells are highly dependent on the tumor type. Ultimately, these can range from proper entry and exit through mitosis[309,335] to dispensable effects on cell cycle, but a key role in the control of cell viability[253].

Previous studies in our laboratory have demonstrated the functional role of p62 in human melanoma cells by means of lentiviral-driven transduction of short hairpin interfering RNAs (shRNAs)[334]. In short, sustained p62 depletion (**Figure 18A**) resulted in phenotypic changes visualized by cell flattening and reduced growth capacity in human melanoma cell lines (**Figure 18B**). This effect is independent of the main genetic alterations in melanoma such as *BRAF* and *NRAS* mutations. p62 shRNA effectively reduced p62 protein expression in all the cell lines tested (**Figure   18A**), with   the concomitant   inhibition   of   their proliferative capacity in cell monolayers (see quantifications in **Figure 18C**).



**Figure 18:** *p62 loss inhibits melanoma proliferation in vitro.* **A.** Immunoblot analysis for p62 upon p62 knockdown in different melanoma cell lines. **B.** Bright field images of different melanoma cell lines upon p62 knockdown. **C.** Bar graph representing the percentage of cells upon p62 knockdown relative to control cells. Results are mean ± SEM. (**A**) and (**B**) were provided by E Riveiro-Falkenback ref.[334].

The inhibited proliferative capacity was found translated into a marked decreased tumorigenic growth in mouse xenograft models[334]. Moreover, p62 depleted cells revealed that the compromised proliferation was associated to reduced entry in S-phase[334]. Finally, transcriptomic profiles at early time points after p62 depletion in SK-Mel-103 and UACC-62,
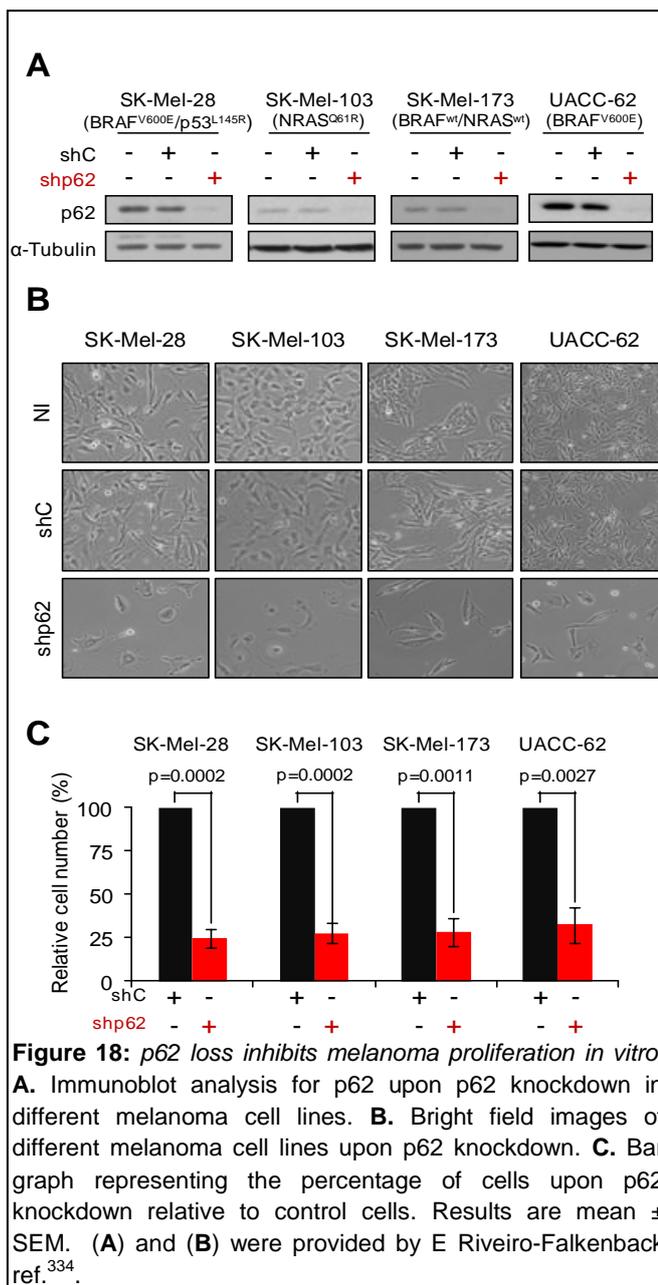
as representative examples of NRAS and BRAF-mutated melanomas, identified cellular pathways (**Table S5**, appendix) related to vesicular trafficking (upregulated) and to cell cycle (downregulated)[334]. These results provide therefore, a mechanistic explanation for the association between p62 and proliferation that we had found in aggressive human melanomas and the inhibited tumor development in genetically engineered melanoma mouse models.

Functions of p62 in metastasis, as indicated above, are less understood. To study this process in human melanoma cells, matrigel-based assays were used upon p62 depletion in different metastatic cell lines. As shown in **Figures 19A**, melanoma cells showed a decreased invasive capacity upon p62 depletion compared with the control cells. Together, these results reinforce the concept of p62 as modulator of poor prognosis in melanoma patients and the aggressive and metastatic potential of the newly generated p62-GEMM. By text mining and guided searches via the Molecular Signatures Database (MSigDB) from GSEA platform we identified 3 comprehensive transcriptomic molecular signatures to be selectively upregulated in metastatic melanomas[336-338]. Interestingly, these melanoma-metastasis signatures were significantly downregulated in the transcriptome of p62-depleted SK-Mel-103 and UACC-62 cells (**Figures 19B-C**).

Therefore, these data support a coordinated effect of p62 on multiple signaling cascades, with new insight on signatures specifically linked to metastasis.



**Figure 19:** *p62 loss decreases invasive capacity of melanoma cells.* **A.** Invasiveness of indicated melanoma cell lines upon p62 knockdown, evaluated by matrigel invasion assay. Results are mean ± SEM. (lower part, immublots for p62 knockdown). **B.** Molecular signatures of metastatic melanoma downregulated in the transcriptome of p62 depleted cells obtained from GSEA. **C.** Representative enrichment plot obtained from (**B**).

## 6.4 p62-deficient melanoma cells support new protumorigenic activities of this protein

Next, we set to study the molecular basis underlying the dependency of melanoma cells on p62. To this end, we tested main known roles of this protein (e.g., modulation of autophagy, mTOR, NF-κB, MYC signaling, oxidative stress and mitochondrial function) that have been described in other systems[253,260,282,339,340]. It is likely that these functions may be differentially regulated under endogenous or exogenous stress inducers (i.e. nutrient deprivation or therapeutic agents). However, here we selected to study basal conditions, where p62 was found essential to maintain malignant features of melanoma cells as described above. First, to define whether p62 is a substrate of autophagy, histological stainings were performed in generated autophagy-deficient melanomas, using GEMM models we have reported before for conditional depletion of *Atg5* (*TyrCre::ERT2*;*Braf*$^{V600E}$;*Pten*$^{-/-}$;*Atg5*$^{fl/fl}$)[220]. As shown in **Figure 20A**, *Atg5* deficiency lead to the accumulation of p62 foci, reminiscent of autophagosome defects in these cells. Accumulation of p62 was also observed in human melanoma cells (UACC-62) blocking lysosomal degradation with chloroquine (**Figure 20B**). Interestingly, in p62 depleted cells, activation (Rapamycin) or inhibition (Chloroquine) of autophagy did not affect the levels of the autophagosomal marker LC3 (**Figure 20C**). However, only a fraction of p62 foci colocalized with the LC3 in CQ-treated cells (**Figure 20B**) suggesting the participation of p62 in additional complexes. In fact, no obvious differences were found in the focal staining of LC3, and in the amount of the autophagosome-lipidated LC3-II form when p62 was depleted *in vivo* -murine *Braf*$^{V600E}$;*Pten*$^{-/-}$ melanomas (see immunomicrographs in **Figure 20D**; and representative immunoblots for LC3-II and ATG5 in **Figure 20E**). Similarly, neither p62 overexpression nor p62 depletion in human cultured melanoma cells significantly altered basal LC3-II and ATG5 levels (**Figure 20F**).

To further characterize the p62 dependency in autophagy, newly generated GEMM *TyrCre::ERT2*;*Braf*$^{V600E}$;*Pten*$^{-/-}$;*Atg5*$^{fl/fl}$;*p62KO* were induced by topical administration of 4-HT and were followed for 8 weeks. From the **Figure 20G** (results in double compound mice) it was observed that the inhibitory effect of p62 allelic loss in the development of *Braf*$^{V600E}$;*Pten*$^{-/-}$ melanomas was not affected by the genomic status of *Atg5*.

All together, these data conclusively demonstrate that p62 is a target of autophagy in melanoma, but not an essential driver of the process.
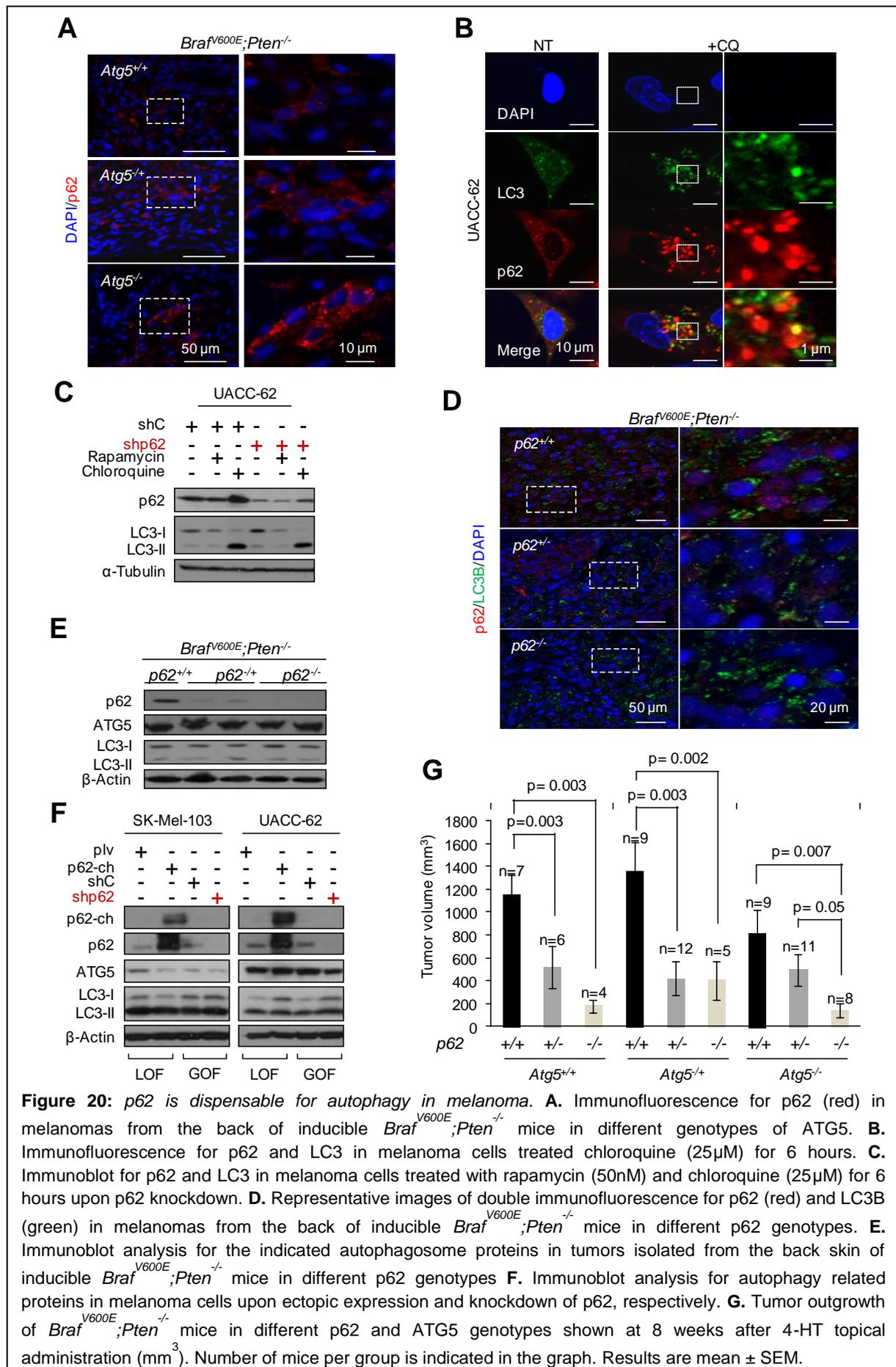
**Figure 20:** *p62 is dispensable for autophagy in melanoma.* **A.** Immunofluorescence for p62 (red) in melanomas from the back of inducible *Braf*[V600E]*;Pten*^{-/-} mice in different genotypes of ATG5. **B.** Immunofluorescence for p62 and LC3 in melanoma cells treated chloroquine (25μM) for 6 hours. **C.** Immunoblot for p62 and LC3 in melanoma cells treated with rapamycin (50nM) and chloroquine (25μM) for 6 hours upon p62 knockdown. **D.** Representative images of double immunofluorescence for p62 (red) and LC3B (green) in melanomas from the back of inducible *Braf*[V600E]*;Pten*^{-/-} mice in different p62 genotypes. **E.** Immunoblot analysis for the indicated autophagosome proteins in tumors isolated from the back skin of inducible *Braf*[V600E]*;Pten*^{-/-} mice in different p62 genotypes **F.** Immunoblot analysis for autophagy related proteins in melanoma cells upon ectopic expression and knockdown of p62, respectively. **G.** Tumor outgrowth of *Braf*[V600E]*;Pten*^{-/-} mice in different p62 and ATG5 genotypes shown at 8 weeks after 4-HT topical administration (mm$^3$). Number of mice per group is indicated in the graph. Results are mean ± SEM.

We then tested the mTOR pathway using three approaches: (i) mining TCGA for a correlation between the effector *S6K1* and *p62 mRNA* in clinical biopsies as described before[282] and performing (ii) gain and loss of function studies in two independent melanoma cell lines (i.e. overexpressing and depleting p62). Results from patient specimens revealed no correlation of *S6K1* with *p62* expression (**Figure 21A**). Consistent with these negative results, neither upregulation nor depletion of p62 had detectable effects on the phosphorylation of S6, another classical reporter of mTOR function (**Figure 21B**). Similar studies failed to reveal significant correlations between *p62* and the cytoprotector *NRF2* and its downstream target *NQO1* (**Figures 21A-B**). This was also the case for MYC, an oncogene that may be regulated by p62 under oxidative stress (**Figures 21A-B**). Regarding NF-κB, p62-IKKβ levels were not correlated in TCGA, and no changes in the phosphorylation of p65 were observed upon p62 loss (**Figure 21C**). Finally, the mitochondrial function of melanoma cells was not impaired by p62 loss as defined by monitoring oxygen consumption rate (OCR) in two melanoma cell lines (**Figure 21D**, *left panels*). This study revealed also that the ratio of oxidative phosphorylation and the glycolysis was not altered suggesting that the mitochondrial respiration of p62 depleted cells was functional (**Figure 21D**, *right panels*). Altogether, these results separate melanomas from other tumor types, suggesting yet additional roles of p62 in malignancy.

**Figure 21:** *p62 described functions in other systems are not altered in melanoma.* **A.** Correlation of *p62* with its downstream targets (*S6K1 and MYC*) and direct targets (*KEAP1* and *IKKβ*) in skin cutaneous melanoma obtained from TCGA database (n=472). **B.** Immunoblot analysis for known p62 targets (mTOR pathway, KEAP1/NRF2 pathway and MYC) upon ectopic expression and knockdown of p62, respectively, in melanoma cells. **C.** Immunoblot analysis for phospho-p65 and total levels of p65 upon ectopic expression and knockdown of p62, respectively**. D.** *Left panels* indicate the mitochondrial respiration/function of melanoma cells upon p62 knockdown determined by oxygen consumption rate (OCR) utilizing the Seahorse XF96 Extracellular Flux Analyzer (Seahorse Bioscience). OCR was measured as pmol/min/cells ($5 \times 10^{3}$) in four technical replicates at basal conditions and after the administration of the indicated compounds (n=3). *Right panels* indicate the ratio of basal oxygen consumption rate (OCR, indicative of mitochondrial oxidative phosphorylation) with basal extracellular acidification rate (ECAR, representing glycolysis) ratio upon p62 knockdown of melanoma cell lines. Results are mean ± SEM.

## 6.5 Quantitative proteomics (iTRAQ) for the identification of downstream effectors of p62

While high throughput proteomics have been reported to identify interactor networks in autophagy pathways[341]no similar studies have been described for p62 in cancer cells. Therefore, multiplexed isobaric Tags for Relative and Absolute Quantification (**iTRAQ**) and subsequent LC-MS/MS (liquid chromatography tandem-mass spectrometry) were performed for parallel comparisons of control and p62-shRNA melanoma cells (SK-Mel-103 and UACC-62, in triplicates). In addition, proteomic analyses were run in both cell lines for ATG5 depletion as a reference for autophagy-associated changes in gene expression. Filters and cut off parameters for the identification of significantly deregulated proteins followed standards in the field[317] (as indicated in the Materials and Methods, 5.12). Interestingly, the fraction of significantly downregulated proteins after depletion of p62 and ATG5 was surprisingly low in both, SK-Mel-103 and UACC-62 cells (6.4 and 6.6% affected proteins for p62 shRNA cells, respectively; and 3.2% and 2.5% for ATG5 siRNA cells; respectively), as summarized in scatter plots of **Figures 22A-B** (see legends for the specific number of affected proteins per cell line and **Tables S6-9** in the appendix). Therefore, we concluded that although p62 and ATG5 (autophagy) may have pleiotropic roles; these ultimately affect a rather restricted sets of proteins. However, the p62- and ATG5-associated iTRAQ changes were unexpectedly divergent (i.e. proteins upregulated upon p62 depletion, were downregulated for the ATG5 counterparts; see venn diagram and the specific list of affected proteins in **Figure 22C**). Furthermore, we found the p62-iTRAQ data in melanoma displaying also a minor overlap with proteins annotated in the *Human Autophagy Database* (**Figure 22D**). These results confirm p62 acting primarily beyond autophagy in melanoma cells.

| Protein | p62_iTRAQ | ATG5_iTRAQ |
|---|---|---|
| SERPINB2 | 1.484952175 | -0.474747479 |
| KRT1 | 1.439174513 | 0.922574263 |
| TMEM164 | 1.381958928 | -0.397199857 |
| TSPAN4 | 1.35452902 | -0.507565454 |
| PARL | 1.326542541 | 0.508665138 |
| SACS | 0.761199879 | -0.478049981 |
| NGLY1 | 0.759356178 | 0.510889607 |
| TMEM33 | 0.758818952 | -0.462960063 |
| SLC16A1 | 0.752437106 | -0.428489879 |
| C1orf85 | 0.724264495 | 0.583104884 |
| UHRF1BP1L | 0.721366105 | -0.607848119 |
| MLIP | 0.71655045 | -0.454295611 |
| CLTCL1 | 0.678814473 | 0.455462387 |
| TRIM22 | 0.667316393 | -0.405152263 |
| RYR2 | 0.65968485 | 0.488247693 |
| SAMD9 | 0.628492426 | -0.398033055 |
| CTNNAL1 | 0.625218139 | -1.129654013 |
| ANKRD52 | 0.603294864 | 0.418153388 |
| CKS2 | 0.591871596 | 0.573811881 |
| VAT1L | 0.575096137 | 0.533874511 |
| NDUFAF6 | 0.563371888 | -1.274292598 |
| FTSJD1 | 0.560018151 | -0.718975305 |
| TMEM102 | 0.559956597 | -0.675430977 |
| DDX58 | 0.519019059 | -0.474102225 |
| UBE2L6 | 0.518886773 | -0.395496677 |
| ISG15 | 0.486100484 | -0.379416501 |
| IFIT3 | 0.266864374 | -0.524574522 |
| IFIT1 | 0.216701472 | -0.451545366 |
| IFIT2 | 0.118177484 | -0.650754357 |

**Autophagy Related Proteins**

| Protein | Fold Change (log2) |
|---|---|
| EGFR | 1.231501368 |
| ITPR1 | 0.798460888 |
| PIK3C3 | 0.793997948 |
| ATG9A | 0.523409777 |
| GABARAPL2 | 0.501340669 |
| ARNT | 0.455867002 |
| CHMP2B | 0.427351755 |
| WDR45L | 0.313113749 |
| ITGB1 | -0.389314911 |
| ITGA6 | -0.481327993 |
| EIF2AK2 | -0.663620739 |
| EEF2K | -0.742812005 |
| CASP3 | -0.8317655 |
| SQSTM1 | -1.815009589 |

**Figure 22:** *p62 proteomic analysis (iTRAQ) identifies downstream targets in melanoma.* **A.** Scatter plots of expression levels of identified proteins from two melanoma cell lines (*left panel*, SK-Mel-103 and *right panel*, UACC-62) upon p62 shRNA transduction estimated with respect to shRNA controls, obtained from iTRAQ LC-MS/MS analysis. Number of up- and down-regulated proteins (red and green, respectively) is highlighted. **B.** Scatter plots of expression levels of identified proteins from two melanoma cell lines (*left panel*, SK-Mel-103 and *right panel*, UACC-62) upon ATG5 silencing by siRNA pool estimated with respect to siRNA controls, obtained from iTRAQ LC-MS/MS analysis. Number of up- and down- regulated proteins (red and green, respectively) is highlighted. **C.** *Left panel*, venn diagram depicting the low overlap of p62 and ATG5 differentially regulated proteins from iTRAQ analysis. *Right panel*, table of common deregulated proteins indicating the fold change. **D.** *Left panel*, venn diagram showing low overlap of p62 differentially regulated proteins from iTRAQ analysis and autophagy related proteins obtained from (*Human Autophagy Database*). *Right panel*, table of common detected proteins indicating the fold change.

Ingenuity Pathway Analysis (**IPA**) was then performed on iTRAQ-p62 data, aggregating proteomic data from SK-Mel-103 and UACC-62 cells. For this study, we focused on downregulated factors after p62 depletion (adjusted p-values<0.05), under the hypothesis that those may reflect tumor drivers controlled by p62. Enriched signaling cascades found in this IPA analysis corresponded to (i) Cell Morphology, (ii) Cellular Assembly and Organization, (iii) Cellular Development, (iv) Cellular Function and Maintenance, and (v) Cellular Growth and Proliferation (**Figure 23A**). These 5 functional categories shared 30 proteins (**Figure 23A**), which were then ranked using two criteria: (i) novelty, namely, no reported links to melanoma, and (ii) annotated roles in invasion and metastasis -as these are the least understood functions of p62. To further narrow down these candidates to those with a higher likelihood of physiologically relevance, we mined the TCGA database for differential expression in metastatic vs primary melanomas.

**A**



Cellular Assembly and Organization **59**

Cellular Development **93**

Cell Morphology **61**

Cellular Growth and Proliferation **124**

Cellular Function and Maintenance **56**

**B**

| gene | Expression (log2 value) | Melanoma | Invasion | TCGA p-values | |
|---|---|---|---|---|---|
| **FERMT2** | **-0.690** | **-** | **+** | **0.0001** | Up |
| NEFL | -0.400 | - | + | 0.8910 | - |
| LAMA2 | -1.150 | - | - | 0.3439 | - |
| UBA6 | -0.410 | - | - | **<0.0001** | Up |
| SPTBN1 | -0.390 | - | - | **0.0002** | Up |
| GALNS | -0.390 | - | - | 0.4927 | |
| DPYSL5 | -0.200 | + | - | **0.0399** | Up |
| RYR2 | -0.096 | + | - | 0.4129 | - |
| MAGI2 | 0.152 | + | + | 0.0799 | - |
| CASP3 | -0.488 | + | + | **<0.0001** | Up |
| NGFR | 0.016 | + | + | 0.4567 | - |
| SERPINE1 | -0.155 | + | + | 0.1419 | |
| MAP2 | 0.032 | + | + | 0.8115 | - |
| L1CAM | 0.012 | + | + | 0.7922 | - |
| WASF2 | -0.086 | + | + | 0.1420 | - |
| FMR1 | -0.285 | + | + | **0.0212** | Up |
| SCARB1 | 0.135 | + | + | 0.2558 | - |
| PLAT | 0.152 | + | + | 0.9268 | - |
| SNCA | 0.326 | + | + | **0.0002** | Down |
| MECP2 | 0.158 | + | + | 0.6808 | - |
| ITGA6 | -0.005 | + | + | 0.9438 | - |
| ARID1A | -0.637 | + | + | **<0.0001** | Up |
| CAV1 | 0.596 | + | + | **0.0292** | Down |
| THBS1 | -0.208 | + | + | 0.0736 | - |
| GSN | 0.034 | + | + | 0.0568 | - |
| EPS8 | -0.276 | + | + | **0.0074** | Up |
| SRC | 0.435 | + | + | 0.3508 | - |
| PLD1 | -0.045 | + | + | 0.8459 | - |
| ITGB1 | -0.462 | + | + | **0.0021** | Up |
| S100B | 0.258 | + | + | - | Up |

**C**



**Figure 23:** *Proteome analyses and data mining identify FERMT2 as a downstream target of p62 in melanoma.* **A.** Molecular and cellular functions of downregulated proteins upon p62 knockdown in two melanoma cell lines (SK-Mel-103 and UACC-62). **B.** Table list of common downregulated proteins implicated in molecular and cellular functions upon p62 knockdown that are mined to be novel in melanoma, with described roles in invasion and enriched in metastatic melanoma compared with primary (TCGA, skin cutaneous melanoma). Bold indicates the significantly enriched in metastatic melanoma versus primary melanoma. **C.** Vertical scatter plot of FERMT2 expression in TCGA (skin cutaneous melanoma) in primary and metastatic melanoma.

RNA sequencing-based information from TCGA database was mined for our candidates in n=101 primary melanomas and in n=364 metastatic cases (see p-values in **Figure 23B**). From this analysis and the applied selection criteria, we identified 6 proteins (FERMT2, NEFL, LAMA2, UBA6, SPTBN1, GALNS) not previously annotated in melanoma and no direct links with p62. Two of the identified proteins (FERMT2, NEFL) have been linked with roles in metastasis (**Figures 23B**). However, *FERMT2* was the putative target that was significantly enriched in metastatic biopsies compared with primary in TCGA database and downregulated in both melanoma cell lines (**Figures 23C**). FERMT2 (Fermitin Family Member 2/KINDLIN-2) is a scaffold protein involved in cell adhesion and migration[342], with well-reported links to metastasis in different cancer types, although regulatory mechanisms are not well understood[343-345]. Together, these results pointed to FERMT2 as a key driver of malignant features of melanoma cells, acting downstream of p62.

## 6.6   FERMT2 is a novel risk factor for poor prognosis in melanoma acting downstream of p62

Before proceeding to functional analyses, histological analyses were performed to confirm a melanoma-associated accumulation of FERMT2 at the protein level. These were performed on TMAs containing melanocytic benign and malignant lesions at different stages of the disease (see **Table S10** in the appendix for detail on the specimens analyzed, n=199 in total). Interestingly, and as the case for p62, FERMT2 was undetectable (or very low) in the dermal nevi studied. Vertically invading melanomas (VGPs) and metastases (particularly at the skin) were found highly positive by immunohistochemistry (**Figures 24A-B**). In an independent validation cohort of 82 VGPs with annotated 10-year follow up, high FERMT2 expression showed a significant correlation to poor disease free survival (**Figure 24C**, **Tables S10-12** in the appendix; double analyses for p62 and FERMT2 expression are discussed below).



**Figure 24:** *FERMT2 is a novel risk factor for poor prognosis in melanoma acting downstream of p62.* **A.** Representative histological images of FERMT2 immunohistochemistry in tissue microarrays containing the indicated human benign and malignant melanocytic tumors. **B.** FERMT2 quantification for 199 melanocytic tumors. The staining was classified into negative, low and high FERMT2-expressing tumors. **C.** Kaplan–Meier plot of Disease Free Survival (DFS) curves subgrouped by FERMT2 expression (FERMT2 low in blue and FERMT2 highin red with the indicated number of cases in each group). Analysis time represents years of follow-up.

To demonstrate the relevance of FERMT2 as a downstream effector of p62, loss and gain of function studies were performed. Specifically, p62 was depleted (shRNA or siRNA pool) or

overexpressed by means of lentiviral transfer. The effect on FERMT2 protein expression by these strategies was consistent: downregulation upon p62 depletion, and increase through ectopic p62 expression (**Figures 25A-C**). To independently validate FERMT2 as bona fide target of p62, two short guide RNAs (sgp62_1 and sgp62_2) were generated for CRISPR/Cas9-mediated gene depletion. As shown in **Figure 25D**, sgRNAs effectively eliminated p62 expression and consequently the levels of FERMT2 (versus scrambled sgRNA). *In vivo*, FERMT2 levels were decreased in *Braf*$^{V600E}$*;Pten*$^{-/-}$ GEMM melanomas as a function of p62 allelic loss (see the differential staining in *p62*$^{+/+}$ with respect to *p62*$^{+/-}$ and *p62*$^{-/-}$ in **Figure 25E**). Similar results were found for xenografts of human melanoma cells (SK-Mel-103) expressing p62 shRNA and implanted in mice compared to the corresponding control (**Figure 25F**).



**Figure 25:** *p62 regulates FERMT2 in vitro and in vivo in melanoma.* **A.** Immunoblot analysis for p62 and FERMT2 upon p62 knockdown in the indicated melanoma cell lines. **B.** Immunoblot analysis for FERMT2 upon p62 knockdown by siRNA pool in melanoma cells. **C.** Immunoblot analysis for p62 and FERMT2 upon ectopic expression of p62 in melanoma cells. **D.** Immunoblot analysis for the indicated proteins in melanoma cells transduced with lentiviruses expressing the indicated sgRNAs for p62 inacrtivation. **E.** Histology slides of back skin of *Braf*$^{V600E}$*;Pten*$^{-/-}$ mice showing representative examples of FERMT2 expression in the 3 different genotypes of p62. **E.** Representative immunohistological images for p62 and FERMT2 upon knockdown of p62 in mice xenografts.

Next, we determined whether depletion of FERMT2 phenocopied p62 loss. Two independent shRNAs were identified to provide a sustained and efficient downregulation of FERMT2 (see immunoblots in **Figure 26A** for three melanoma cell lines). FERMT2-deficient melanoma cells showed a markedly reduced proliferative capacity in long term colony formation assays (**Figure 26B-C**). Moreover, depletion of FERMT2 (as the case for p62)

resulted in a significant reduction in invasive potential of otherwise aggressive melanoma cells (**Figure 26D**).

To determine if FERMT2 levels are regulated by p62 through degradation (main function of p62), melanoma cells were treated with inhibitors of the proteasome (Bortezomib) and lysosome (Chloroquine). While p62 was accumulated (especially in CQ-treated cells), FERMT2 levels were not affected. Additionally, another main cargo receptor protein, NBR1, was depleted in melanoma cells. NBR1 exhibits similar domain architecture to p62 and it has been reported that these two proteins cooperate in autophagy[294]. NBR1 depletion did not affect the expression levels of FERMT2 (**Figure 26F**), strengthening the concept that p62 acts on FERMT2 expression in a manner beyond autophagy.



**Figure 26:** *FERMT2 loss leads to inhibition of proliferation and invasion in melanoma.* **A.** Immunoblot analysis for FERMT2 upon FERMT2 knockdown in different melanoma cell lines using two different shRNAs. **B.** Crystal violet staining of colonies formed in the indicated cell populations upon FERMT2 genetic inactivation by shRNAs **C.** Relative quantification of crystal violet staining of colonies formed in the indicated cell lines upon FERMT2 genetic inactivation. Results are mean ± SEM **D.** Invasiveness of SK-Mel-103 upon p62 and FERMT2 knockdown evaluated by matrigel invasion assay. Results are mean ± SEM. **E.** Representative immunoblot analysis of p62 and FERMT2 upon proteasomal (Bortezomib) and lysosomal (Chloroquine) degradation in melanoma cell lines. **G.** Representative immunoblot analysis for the indicated proteins upon NBR1 knockdown in melanoma cells.

Importantly, to demonstrate that FERMT2 is indeed a physiologically-relevant p62 target, rescue experiments were performed. To this end, melanoma cells were infected with lentiviruses expressing ectopic FERMT2, or an empty vector control. Each of these subpopulations was then depleted for p62 (**Figure 27A**). As shown in **Figures 27B-D**, this ectopic FERMT2 expression diminished the effect of p62 depletion in the proliferative and invasive features of melanoma cells. Therefore, while p62 may certainly impinge on multiple signaling cascades (**Figure 23A**), main roles in proliferation and invasion largely depend on FERMT2.

The corollary of the data above is that FERMT2 and p62 expression should be correlated in patient biopsies. To this end, we constructed a TMA containing 111 melanocytic lesions, and stainings for p62 and FERMT2 were performed in consecutive sections. As illustrated in **Figure 27E**, 20% of p62 negative melanomas still expressed high FERMT2, but overall, Chi-squared test indicated a positive correlation between the two proteins (p=0.001; see representative examples in **Figure 27F**).

Altogether, these results identify FERMT2 as a physiologically-relevant novel indicator of poor prognosis in melanoma, acting downstream of p62.



**Figure 27:** *p62 and FERMT2 positively correlate in vitro and in human melanoma biopsies.* **A.** Immunoblot analysis of FERMT2 ectopically expressed in melanoma cells followed by p62 knockdown. **B.** Crystal violet staining of colonies formed in FERMT2 ectopically expressed cells upon p62 knockdown. **C.** Relative quantification of (**B**). Results are mean ± SEM. **D.** Invasive capacity of FERMT2 ectopically expressed SK-Mel-103 cells followed by p62 knockdown, evaluated by matrigel invasion assay. Results are mean ± SEM. **E.** p62 and FERMT2 quantification showing the positive correlation between p62 and FERMT2 expression in tissue microarrays containing 111 melanocytic lesions. **F.** Representative histological images of p62 and FERMT2 negative and positive tissues from (**E**).

## 6.7 The p62 interactome identifies RNA binding proteins as novel binding partners in melanoma

Next, we investigated the mechanism by which p62 regulates FERMT2. As p62 has been reported to act as a hub to define the localization and stability of other proteins, we questioned whether this was the case for FERMT2. However, no interaction between these two proteins was found by immunoprecipitation assays (data not shown). Interestingly, quantitative RT-PCR revealed a 50% reduction *FERMT2* RNA in p62 depleted melanoma cells (**Figure 28A**). Therefore, we set to study roles of p62 in RNA regulation. As p62 does not have RNA binding domains[277], we hypothesized that the regulation of FERMT2 could be indirect, perhaps via transcription factors or RNA binding proteins.



**Figure 28:** *p62 interactome in melanoma cells.* **A.** qPCR for *p62* and *FERMT2* upon p62 knockdown in melanoma cells. Results are mean ± SEM. **B.** Immunoblot analysis for the indicated proteins upon cellular fractionation of isolated cytoplasm and nucleus subjected to immunoprecipitation against p62. **C**. Cellular localization of the identified p62 interactors. **D.** *Left panel*, venn diagram highlighting the low overlap of common p62 interactors identified in melanoma cells compared with other systems suggesting cell type specificity (data obtained from Biogrid database). *Right panel*, common detected interactors of p62 with other systems.

No genome-wide proteomic information is available for p62 interactors in cancer which we could use as a guideline. However, mining the *Biological General Repository for Interaction Datasets* (*BioGRID*)[346] 256 unique p62 interactors were identified (extracted from low and high throughput analyses in various systems). A global inspection of these known p62 interactors did not reveal reported regulators of *FERMT2*. Therefore, affinity purification followed by LC-MS/MS were performed to dissect interactors of endogenous p62 in melanoma cells. More specifically, these analyses were performed on UACC-62 cells using a highly efficient p62-immunoprecipitating antibody upon subcellular fractionation of the cytoplasm and the nucleus (**Figure 28B**; see Materials and methods, 5.13). The eluted samples were analyzed with MaxQuant bioinformatic tools, using Andromeda as a peptide

search engine for probabilistic scoring[321,322]. Using an FDR = 1%, this strategy identified 153 proteins as putative interactors of p62 in melanoma cells (**Table S13**, appendix). 54.5% of p62 interactors were found to localize in the cytoplasm; 18.8% in the nucleus; and 26.6% in both compartments (**Figure 28C**) supporting the dynamic role of p62 mainly in the cytoplasm, but also as a shuttling protein from the cytoplasm to the nucleus[232,308]. Surprisingly, the overlap between p62-melanoma interactome and the p62-BioGRID data was rather low, with only 12 proteins in common (**Figure 28D**). These results therefore, suggest a melanoma-specific nature of the p62 bound proteins. This hypothesis was indeed confirmed by GSEA using as a reference the Cancer Cell Line Encyclopedia (CCLE), which contains information from 752 cell lines, 54 of which are from melanomas (see heatmap plots of this comparative analysis in **Figures 29A-B**; FDR=0.0047).

STRING[330] (Search Tool for the Retrieval of Interacting Genes/Proteins) was then used to explore the molecular functions of the p62-affinity precipitated proteins in melanoma cells. Surprisingly, the most enriched GO terms found were related to a particular enrichment in RNA binding processes (see highly significant FDR values in **Figure 28C**). Therefore, these results support the attractive scenario of p62 having new cellular roles as a hub for RNA binding proteins (RBPs).



**A.**

MELANOMA

p62 Interactors

FDR= 0.0047

1. Lung
2. Kidney
3. Haematopoietic and Lymphoid Tissue
4. Stomach
5. Central Nervous System
6. Endometrium
7. Pancreas
8. Breast
9. Bone
10. Ovary
11. Salivary Gland
12. Soft Tissue
13. Urinary Tract
14. Thyroid
15. Upper Aerodigestive Tract
16. Liver
17. Large Intestine
18. Autonomic Ganglia
19. Oesophagus
20. Prostate
21. Biliary Tract
22. Small Intestine
23. Pleura

**B.**

p62 interactors

NES= 1.97
FDR= 0.005

SKIN          REST OF TUMORS

**C.**

### Molecular Function (GO)

| pathway description | count in gene set | FDR |
|---|---|---|
| RNA binding | 44 | 1.94E-12 |
| poly(A) RNA binding | 37 | 1.68E-11 |
| nucleotide binding | 41 | 2.04E-05 |
| nucleoside phosphate binding | 41 | 2.04E-05 |
| small molecule binding | 43 | 3.41E-05 |
| carbohydrate derivative binding | 39 | 3.41E-05 |
| anion binding | 42 | 0.00013 |
| purine nucleotide binding | 33 | 0.00032 |
| purine ribonucleotide binding | 32 | 0.000416 |
| purine ribonucleoside triphosphate binding | 32 | 0.000416 |

**Figure 29:** *p62 interactors are enriched in melanoma cell lines among other cancer cell lines.* **A.** Heatmap summarizing a comparative analysis of p62 interactors (in vertical) across 24 tumor types of the CCLE (n=54 and n=752 cell lines from melanoma and other cancers, respectively). Note the differential expression profile of melanoma vs the rest of tumors. **B.** Enrichment plot of p62 interactors from (**A**). **C.** Gene ontology terms of molecular functions (obtained from String database) of p62 interactors (p≤ 0.05).

The data above open new avenues of research in the p62 and RBP fields that could be explored in future studies. Here, we focused on the possibility of a "p62-RBP axis" as a modulator of *FERMT2*. From the interacome analyses of **Figures 28-29**, we found 44 RBPs as putative p62 interactors. It was not practical to assess in detail each of these proteins. Therefore, candidates for functional characterization were selected in two steps: first, the 44 p62-interactors were screened through the CLIPdb 2:POSTAR[347] database for binding to *FERMT2 mRNA.* Specifically, CLIPdb 2:POSTAR was selected as it represents the largest collection of experimentally probed and computationally predicted RBP binding sites in the human and mouse transcriptomes[347]. Secondly, for a more guided analyses, text mining was performed on the 44 p62-precipitated RBPs for functions related with cancer cell proliferation and invasion. As summarized in **Figures 30A**, this approach yielded 2 RBPs (IGF2BP1 and IGF2BP3) that fitted the three criteria: (i) p62 interaction, (ii) binding to *FERMT2 mRNA* in other settings, (iii) and functions that could account for roles of p62 in tumor progression and metastasis.

IGF2BP1 and IGF2BP3 are homologues of the insulin-like growth factor 2 (IGF2) messenger RNA (mRNA)-binding proteins[348,349]. Both are highly conserved oncofetal RBPs that regulate RNA processing at several levels, including localization, translation, and stability[349]. Importantly, IGF2BP1 and IGF2BP3 have been previously shown to be upregulated during melanoma progression, with key roles in tumor cell invasion[350,351], although with no links to p62. The overexpression of IGF2BP1 and IGF2BP3 was validated by immunoblot analysis in freshly isolated normal skin cells and in a panel of melanoma cells (**Figure 30B)**. Using double immunofluorescence analyses we found a visible colocalization of p62 with IGF2BP1 but not with IGF2BP3 (**Figures 30C** and data not shown). Therefore, we centered on IGF2BP1 for further characterization. This selection was further reinforced by finding that large scale analyses of IGF2BP1 binding sites in human pluripotent stem cells included *FERMT2* as a direct target[352].

**Figure 30:** *p62 and IGF2BP1 colocalize in melanoma cells.* **A.** *Left panel*, pie chart indicating the enriched binding of RBPs with p62 in melanoma cells. *Right panel*, table of RBPs that interact with p62. Purple boxes indicates the RBPs reported to bind mRNA *FERMT2*, orange the RBPs implicated to proliferation and grey the RBPs related with invasion. **B.** Immunoblot for the indicated proteins in freshly isolated skin cells and melanoma cell lines. **C.** Representative images of double-immunofluorescence for p62 (red) and IGF2BP1 (green) in melanoma cells.

From the results above, the hypothesis we set to demonstrate is that p62 by binding IGF2BP1 may favor the ability of this RBP to bind and stabilize *FERMT2* mRNA. First, p62-IGF2BP1 interaction was confirmed in different melanoma cell lines by reciprocal co-immunoprecipitations assays (see immunoblots in **Figures 31A**). Secondly, data obtained from enhanced UV crosslinking and immunoprecipitation fort IGF2BP1[352] to analyze the binding to *FERMT2* mRNA. From the data analyzed it was observed that IGF2BP1 binds to the 3'UTR of FERMT2 (**Figure 31B**). Independent RNA Immunoprecipitation (RIP) with anti-IGFBP1 antibodies followed by qPCR proved that IGF2BP1 indeed binds *FERMT2* mRNA (immunoblot for IGF2BP1 immunoprecipitation in **Figure 31C**; see 25-fold difference in binding with respect to IgG control in **Figure 31D**). Thirdly, RIP-qPCR was performed in melanoma cells depleted for p62, finding a reduced IGF2BP1 binding to *FERMT2* by 50% (**Figures 31E**).

**Figure 31:** *p62 regulates the stability of FERMT2 mRNA in melanoma via the interaction with IGF2BP1.* **A.** Melanoma cell lysates were subjected to co-immunoprecipitation against p62, IGF2BP1 and rabbit IgG (negative control). Indicated proteins were detected by immunoblot analysis. Data are representative Immunoblot of three independent assays. *Left panel* indicates the cell line SK-Mel-103 and right panel the cell line UACC-62. **B.** Read density tracks show read density for IGF2BP1 (red), RBFOX2 (as a non related RBP), and paired SMInputs (black) across the 3-UTR of FERMT2 (blue). **C.** Immuprecipitation against IGF2BP1 in melanoma cells followed by immunoblot for IGF2BP1. **D.** RNA Immunoprecipitation (RIP) followed by qPCR analysis for *FERMT2* in melanoma cells (n=3). Results are mean ± SEM. **E.** RIP followed by qPCR analysis for *FERMT2* in melanoma cells upon p62 knockdown in melanoma cells (n=3). Results are mean ± SEM. **F.** mRNA levels of *FERMT2* prior and after actinomycin D treatment upon p62 knockdown in melanoma cells. Results are mean ± SEM.

Finally, *FERMT2* mRNA half life was assessed in the presence or absence of p62 (through actinomycin D treatments to prevent new transcription). Melanoma cells depleted for p62 exhibited a significant acceleration of *FERMT2* mRNA decay (**Figures 31F**, *p*= 0.006).

In conclusion, this comprehensive -omic approach (trascriptomic, proteomic and interactomic analyses) revealed a p62>IGF2BP1>FERMT2 axis embedded within a newly-identified function of p62 as binding partner for RBPs.

## 6.8  p62-IGF2BP1 interaction controls the mRNA stability of multiple pro-metastatic factors

IGF2BP family members are highly pleiotropic RBPs[348,349,353]. Therefore, we considered unlikely that FERMT2 would be the only pro-metastatic factor regulated by the p62-IGF2BP1 interaction. In particular, we questioned whether indeed IGF2BP1 could contribute to a broader impact of p62-related functions. The transcriptomic and proteomic analyses of p62 showed mild positive correlation in the detected genes (**Figure 32A**) and we therefore focused on genes positively controlled by p62 where changes at RNA levels had a significant im  pact on protein expression.



**Figure 32:** *p62 regulates pro-metastatic factors via IGF2BP1 in melanoma.* **A.** Scatter plots for commonly differentially expressed genes at RNA and protein level in SK-Mel-103 (left panel) and UACC-62 (right panel) obtained from iTRAQ LC-MS/MS and transcriptomic arrays upon p62 knockdown. **B.** Venn diagram of downregulated genes upon p62 knockdown depicting a high fraction (28%) of them as targets of IGF2BP1. **C.** Heatmap of downregulated genes (at least in one cell line) obtained from transcriptomic and proteomic analysis upon p62 knockdown that are targets of IGF2BP1, mined for roles in melanoma and metastasis (red indicates upregulation; green, downregulation; black, no changes; grey, no detected). **D.** Venn diagram depicting the low overlap of IGF2BP1 targets regulated by p62 compared with ATG5 downregulated proteins.

This gene set, found to include 146 genes, was obtained by merging aggregated transcriptomic and proteomic data in SK-Mel-103 and UACC-62 (**Figure 32B**). These 146 p62-regulated genes were crossed with IGF2BP1-binding targets described by **eCLIP** (enhanced UV CrossLinking and Immunoprecipitation) in other cell types (human stem cells)[352]. This approach revealed a highly significant set of p62-modulated genes (40/146; i.e. 27%) with the potential to be under the control of IGF2BP1 in melanoma (**Figure 32C**). Of note, these identified genes were not overlapping in ATG5-regulated genes from iTRAQ analysis gene list (**Figure 32D**) suggesting that these factors are not regulated by p62 via the autophagy machinery. These results therefore, further support a key role of this RBP in melanoma progression. For validation, representative genes considered attractive for not being linked to melanoma, but reported with pro-metastatic functions were selected (**Figure 32C**). We thus chose different gene categories: (i) *EHMT2* and *TOP2A* as examples for transcriptional regulators[354,355]; (ii) modulators of vesicular trafficking (*FLOT1* and *CD2AP),* that favored progression in different types of cancer[356-358]; (iii) and pro-metastatic genes with roles in metabolism (*NCEH1* and *OGFOD1)*[359,360]. Intriguingly, the selected factors neither exhibit high mutational status nor copy number alterations (deletions or amplifications) in human melanoma biopsies (**Figure 33A**; data extracted from TCGA, n=470 specimens). However, the levels of expression are increased in metastatic compared with primary melanoma biopsies (**Figure 33B**).



**Figure 33:** *p62-IGF2BP1 targets are upregulated in metastatic melanoma.* **A.** Frequency (%) of melanoma patients with mutations and/or copy number alterations (deletions or amplifications) in the indicated genes. Data were retrieved from the melanoma TCGA database (n=470 specimens) using cBioPortal. *BRAF and NRAS* are included as references for classical melanoma-associated oncogenes. **B.** Gene expression of primary versus metastastic human melanoma patients for the indicated genes obtained from TCGA database.

As IGF2BP1 was found to bind the 3' UTR of these factors in other systems[352] we set to determine whether these candidates were also IGF2BP1 targets in melanoma. RIP followed by qPCR demonstrated that this was the case (see the differential binding of the IGF2BP1 antibody with respect to IgG control in **Figure 34A**). Moreover, this binding was significantly decreased in p62 deficient cells (**Figure 34B**), ultimately resulting in reduced mRNA expression of all the candidates studied (**Figure 34C**).



**Figure 34:** *p62 regulates the stability of pro-metastatic factors via IGF2BP1.* **A.** RNA immunoprecipitation followed by qPCR analysis for the selected targets in melanoma cells (n=3). **B.** RNA immunoprecipitation followed by qPCR analysis for the selected targets in melanoma cells upon p62 knockdown by shRNA in melanoma cells (n=3). **C.** Expression levels of indicated targets by qPCR analysis upon p62 knockdown by shRNA (n=5). **D.** mRNA levels of pro-metastatic targets prior and after actinomycin D treatment upon p62 knockdown by shRNA in melanoma cells (n=3).
Results are mean ± SEM.

To determine whether this inhibited mRNA levels were a consequence of accelerated degradation, cells were treated with actinomycin D to blunt new mRNA synthesis and thus define the half-life of these transcripts. Results showing the requirement of p62 to sustain the stability of all these factors (*EHMT2, TOP2A*, *FLOT1*, *CD2AP, NCEH1* and *OGFOD1*) *are* summarized in **Figure 34D**.

Collectively, these results strongly illustrate that p62 regulates the stability of a broad range of pro-metastatic factors, and described the RNA binding factor IGF2BP1 as a key mediator in this newly identified function.

# VII.DISCUSSION

Melanomas are prime examples of aggressive tumors that proceed with a plethora of changes in the transcriptome and proteome[95,361-364]. Characterization and identification of oncogenes provides new features and traits in melanoma progression, especially at the metastatic stages of the disease. However, the oncogenic factors rarely act via single targets. This complexity is particularly notorious for proteins such as Sequestosome 1/p62, which function by pleiotropic protein-protein interactions.

In this PhD thesis we performed a global analysis of p62 expression and mechanism of action in melanoma cells, clinical biopsies and newly-generated mouse models. The selection of p62 as a putative new modulator of selective gene sets enriched in melanoma was prompted by previously described roles of this protein in lysosomal-associated functions, primarily autophagy[186,298], which we had found with a distinct lineage-selective regulation in this disease[220]. Therefore, we questioned whether p62 (like the autophagy effector ATG5) may be tuned down in invasive or metastatic stages of melanoma progression. In parallel, we also set to define to which extent p62 was required for autophagosome formation. These studies were combined with a comprehensive characterization of the p62-modulated transcriptome, proteome and interactome. Briefly, it was found that (i) the *p62* locus does not suffer frequent heterozygous loses we had reported for *ATG5*[220]; moreover, p62 protein was found overexpressed in TCGA-metastatic melanomas, a feature we confirmed by (ii) histological analyses in independent patient cohorts (in collaboration with Hospital 12 de Octubre), where positive expression was associated with poor prognosis in melanoma patients[334]. (iii) The new GEMM confirmed that p62 is required for the progression and the metastasis of malignant melanoma, and more interestingly the loss of p62 prolongs the survival of the mice. (iv) Genetic silencing of p62 abrogated the proliferation and invasion capabilities of cultured melanoma cells. (v) At molecular level, it was found that known roles of p62 in the modulation of mTOR, NF-κB, KEAP1-NRF2, MYC and mitochondrial respiration were not affected. (vi) More interestingly, it was found that p62 exhibits distinct roles and does not impair the autophagic machinery. (viii) Additionally, from a comprehensive –omics analyses in melanoma cells we identified metastatic signatures significantly downregulated upon p62 depletion. The downstream effector of p62, FERMT2 was identified and proposed as a novel risk factor for poor prognosis in melanoma. (ix) Surprisingly, a large set of RBPs was identified to interact with p62. Among them, IGF2BP1 (via the interaction with p62) was found to regulate the stability of the pro-metastatic factor FERMT2 (and others). Together, these results provide new insights in the role of p62 in melanoma and ultimately separate this disease from other pathologies, where this protein is also overexpressed.

The **Figure 34** illustrates the findings of this PhD thesis highlighting the main steps that were followed to characterize and identify the new role of p62 in melanoma progression and metastasis.



**Figure 35:** *Graphical representation of steps followed to characterize and identify the pleiotropic roles of p62 in melanoma progression and metastasis.*
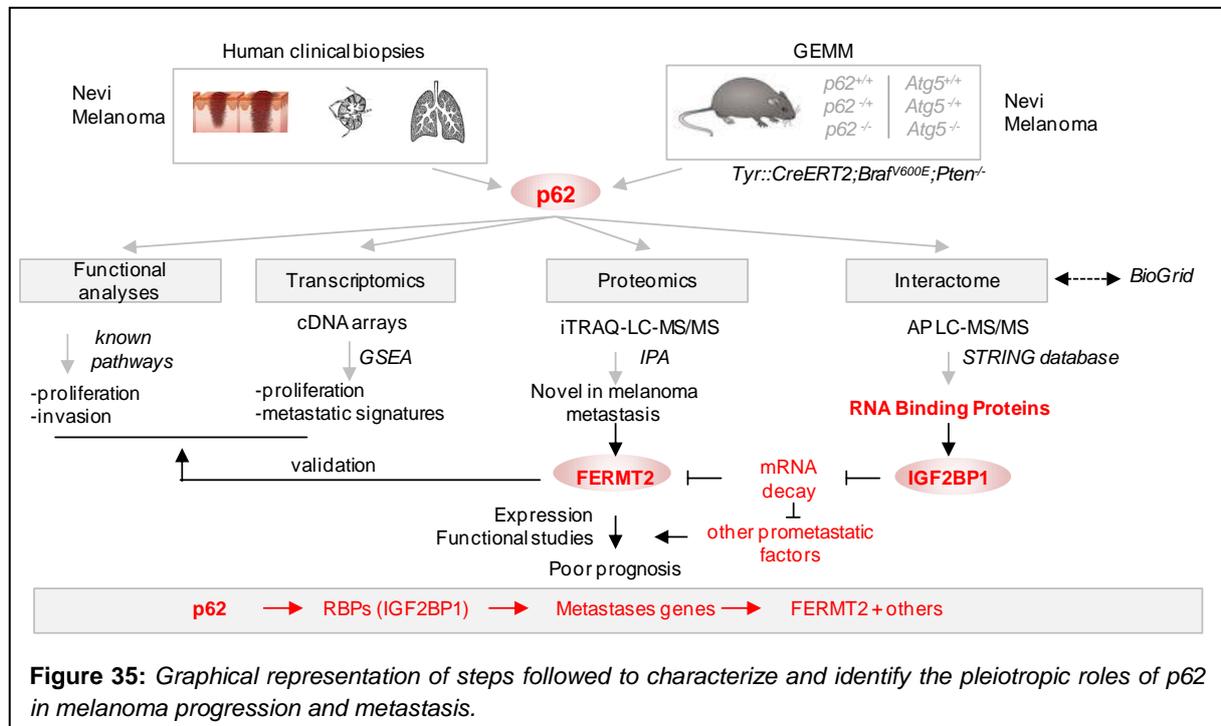
## 7.1 Expression and prognostic studies of p62 in human melanomas

Publicly available databases are being consolidated as a versatile platform to screen for oncogenic drivers and tumor suppressors across large cohorts of clinically-annotated biopsies (**TCGA**)[333] and cell lines (**CCLE**)[329]. Of high importance, the TCGA platform has performed a systematic multi-platform characterization of cutaneous melanomas (~300 biopsies) at the DNA, RNA, and protein levels to create a catalogue of somatic alterations and describe their potential biological and clinical significance. These analyses established a genomic/transcriptomic framework of classification that has potential implications for prognosis and therapy[95]. Moreover, it provides evidence for exploring how additional molecular alterations may explain observed biological and clinical differences among the subtypes. It also provides signposts for identification of druggable targets and predictive biomarkers, as well as potentially useful guidance for decisions about therapy. p62 overexpression has been addressed in various cancers types (see introduction and **Table S4** in the appendix) but still its expression pattern during tumor progression and metastasis is not well characterized. Intriguingly, there is no clear consensus on whether it acts as a tumor promoting[253,260] or tumor suppressive agent[278,308] (i.e. liver cancer). In melanoma, a

recent study characterized the expression of p62 in primary tumors[285]; however the contribution of the protein at later stages was not addressed.

In this PhD thesis, mining TCGA database we found a significant upregulation of p62 protein expression at metastatic sites (skin in transit, regional lymph nodes and distal visceral organs), when compared to primary melanomas. The relatively high expression of p62 in melanoma was supported by CCLE (encompassing over 700 cell lines of 25 different cancer types). These findings were independently validated by immunohistochemical studies for p62 in high density tissue microarrays covering a broad spectrum of melanocytic lesions at different stages of progression[334]. The significant upregulation of the protein at the later stages of the disease raise the possibility that during metastasis p62 levels are tune up to maintain the tumorigenic features of the cells. Furthermore, from a retrospective 5 year-follow up analysis of p62 expression in clinically-annotated primary melanomas it was demonstrated that p62 can be an independent prognostic indicator of patient outcome[334].

Collectively, results obtained from databases and independent validation support the oncogenic roles of p62 in melanoma, especially in metastasis.

## 7.2 Pathophysiological roles of p62 *in vivo*

Genetically-modified mouse models (GEMM) have been instrumental in defining cell-type dependent roles of p62 in cancer development. For example, in GEMM of hepatocellular carcinoma (HCC), p62 was found to act as a sufficient and necessary oncogene in stressed cancer initiating cells (hepatocytes)[260], but as a tumor suppressor in hepatic stellate cells[308]. Prostate cancer models have revealed also tumor suppressive functions of p62 exerted by rewiring the metabolism of stromal fibroblasts[278]. In this PhD thesis two GEMM were generated to dissect the contribution of p62 to melanoma progression. For the benign nevi, the *Tyr::CreERT2;Braf^V600E* strain was crossed with *p62* mice (*p62^+/+*, *p62^-/+*, *p62^-/-*). From these studies it was observed that the allelic loss of p62 (monoallelic and biallelic) was dispensable for the generation of benign melanocytic lesions. Moreover, melanomas were not developed in the presence or absence of p62. These findings (i) recapitulate our clinical data where the different types of nevi exhibit negative or very low levels of p62. (ii) Moreover,support that p62 is dispensable for the transformation of the benign nevi to melanomas in the context of these individual oncogenic changes *in vivo*. However, when *p62* mice (*p62^+/+*, *p62^-/+*, *p62^-/-*) were crossed to *Tyr::CreERT2;Braf^V600E;Pten^-/-* strain, to recapitulate melanoma progression, the effect was different. Allelic loss of *p62* in these mice led to a significantly (i) compromised melanoma development, (ii) nodular and lung metastasis were diminished (iii) and the survival was extended. (iv) Interestingly, we observed that the p62-deficient microenvironment does not override the requirement for p62

in the melanoma tumors. Indeed, the contribution of stroma cells (where p62 levels are low) did not result in increased melanomagenesis when B16 melanoma cells were implanted in *p62 KO* mice. This is different as in the case of prostate cancer where the p62 negative expression of stromal fibroblasts led to epithelial cell proliferation[278]. These findings support the cell type specific roles of p62 in the contribution of tumor progression and also shed light to devising therapeutic strategies depending on the cancer type.

Autophagy has been shown to play complex roles in the regulation of cancer development and progression where p62 functions as a cargo receptor[236]. We and other, have shown that GEMM with defective autophagy lead to the accumulation of p62[186,217,220,236]. An important finding of this study was that p62 has distinct roles from autophagy in melanoma. Using GEMM and functional studies *in vitro* we found that p62 is not essential for autophagosome formation and the autophagic machinery. Classical autophagy markers did not change *in vitro* and *in vivo* upon p62 loss. Interestingly, *Atg5* defective melanoma mice (*TyrCre::ERT2*;*Braf*$^{V600E}$;*Pten*$^{-/-}$;*Atg5*$^{fl/fl}$) with accumulated p62 aggregates display no synergistic effect in tumor progression when the mice were crossed with *p62 KO* mice. Thus p62 may be essential (clearance of ubiquitinated proteins) but it is not necessary for autophagy machinery during tumor progression. These results are supported from the fact that other cargo receptors including NBR1 (neighbor of BRCA1 gene 1) and NDP52 (nuclear dot protein 52), OPTN (optineurin), and TAX1BP1 (Tax1-binding protein 1)[365,366] can compensate the loss of p62 and act in the degradation process of autophagy. Of note, ablation of p62 in hepatocytes or pancreatic acinar cells does not result in any obvious autophagy defects[260,302], suggesting that its function is redundant with that of other cargo receptors. Finally, the findings from proteomic studies strengthen our data that p62 acts beyond autophagy.

## 7.3   p62 known functions are not altered in melanoma

The data obtained from human biopsies and the GEMM reinforced the concept of p62 as modulator of aggressive and metastatic potential. These data were supported by the significantly decreased melanoma metastatic signatures (obtained from GSEA) from the transcriptomic arrays in p62 depleted cells. Considering the pleiotropic effects of p62 in different cancer types our studies failed to characterize an active role of the protein to any of the known pathways described.

One well described role of p62 is the hyperactivation of mTOR pathway by tethering proteins to endo/lysosomes (e.g. Raptor and RAGs; TRAF6; MEKK3)[282,283,339] in prostate cancer. However, (i) through data mining in TCGA (ii) and functional studies *in vitro* it was found no dependency with *p62* expression. These data were supported from the

transcriptomic and proteomic studies where no enrichment of mTOR pathway was observed. Accordingly, the interactome analysis failed to identify mTOR related proteins as interactors of p62. The findings that p62 is not involved in mTOR activation, first support the hypothesis that p62 acts in a cell type specific manner. Secondly, it raises the possibility that this pathway can be regulated by other lysosomal genes where melanoma exploits a lineage-specific wiring of the endolysosomal pathway[142]. Thirdly, these studies were performed at basal conditions, where p62 was found essential to maintain malignant features of melanoma cells. It would be of high interest to explore the possible role of p62 under situations of cellular stress and nutrient availability where mTOR has been found to be hyperactivated by p62[282,339].

In the same manner, studies for KEAP1-NRF2 pathway (antioxidant response) failed to show positive correlation with *p62*. Keap1 binds NRF2 and promotes its degradation in a p62-dependent manner[247,367], whereas Keap1 itself is degraded through autophagy on p62 binding[27]. In cancer, NRF2 is stabilized by p62 via degradation of Keap1 resulting in the induction of antioxidant response elements[199,306]. Although p62 was found to interact with KEAP1 in the interactome arrays, our studies revealed no changes of both proteins (and downstream targets, such as NQO1) in melanoma. Various studies have shown that KEAP1-NRF2 pathway plays important role in cancer progression[260,261,368,369] through p62; however there are studies supporting that this pathway can be regulated in a p62 independent manner[370,371]. Thus, this suggests that in melanoma this pathway can be regulated independently of p62 or under stress conditions (ROS-inducers or chemotherapeutic agents) that need to be addressed in future studies.

NF-κB is considered to be a central player in linking inflammation and cancer[372] where previous studies have shown that p62 induces its activation in different diseases[207,253,304,373,374]. Following the same strategy, NF-κB downstream targets (phospho-p65, IKKβ) were not correlated with p62 in melanoma cells. Moreover, NF-κB players were not found to interact with p62 in the interactome arrays.

p62 positively regulates MYC oncogene via (i) mTORC1[260], (ii) KEAP1-NRF2[260] or by repressing the expression of the microRNAs let-7a/b[277]. In our study, neither correlation was observed (TCGA, *in vitro*) nor the stability of MYC was affected in p62 depleted cells (data not shown). A possible explanation comes from the fact that the above pathways were not affected by p62, consequently MYC levels remained stable.

Finally, the mitochondrial bioenergetics that p62 alters in other systems[340] remain stable in p62 depleted cells raising the possibility that other mitophagy related proteins (Parkin[375], Optineurin[376]) act in a compensatory way in the absence of p62 in melanoma.

Overall, the above data indicated that the basal levels of p62 that are sufficient to maintain tumorigenesis in melanoma are not altering described pathways in other systems supporting the hypothesis of cell type specific roles of this protein.

## 7.4  Novel roles of p62 in melanoma progression and metastasis

Our initial studies to assess mechanistically how p62 maintains tumorigenesis and promotes metastasis indicated that this protein with the pleiotropic effects does not alter known pathways described in other systems. In the light of the above results, we decided to proceed to an unbiased transcriptomic, proteomic and interactome analyses in melanoma cells. Gene Set Enrichment Analyses (GSEA) in p62 shRNA-associated transcriptomic profiles identified a large variety of pro-tumorigenic factors in melanoma cells that included expected modulators of cell cycle and mitosis[309,334]. However, these studies also revealed mRNA-regulated factors not previously assigned to p62. Importantly, these included the gene signatures we and others had reported to be enriched in metastatic melanomas, as well as a large set of pro-metastatic factors with no reported links to this disease.

The proteomic analyses provides a useful platform for downstream targets that p62 regulates in melanoma. From this study the first observation was that the percentage of the affected proteins by p62 depletion was rather low (6.5% for SK-Mel-103 and 7% for UACC-62) indicating that p62 ultimately affects a rather restricted sets of proteins. Ingenuity Pathway Analysis (IPA) in p62 depleted cells identified molecular and cellular functions altered that are essential for the oncogenic features of the protein. Setting selection criteria to the identified proteins and combining data with RNA sequencing-based information from TCGA revealed that 24 of these proteins have been linked to melanoma with roles in invasion and metastasis; 9 of these proteins were found enriched in metastatic biopsies (**Figure 23**).  However these proteins have not previously linked with p62 indicating it as an upstream regulator in melanoma. It will be interesting in future studies to characterize the connection of p62 with these proteins and shed light to new pathways that the protein is implicated.

Of interest, 6 proteins were identified with no previous link to melanoma (FERMT2, NEFL, LAMA2, UBA6, SPTBN1, GALNS) and two of them were found with positive roles in metastasis in other systems (FERMT2[377,378], NEFL[379,380]). FERMT2 was more significantly enriched in metastatic melanoma (TCGA) and was found to be downregulated in both melanoma cell lines. FERMT2 is itself a signaling hub, best known for its roles in the binding and recycling of integrins[381,382], and the activation of multiple signaling cascades involved in cell adhesion, cell motility and invasion[342,383]. To date, FERMT2 regulation has been reported primarily by post-transcriptional mechanisms, involving phosphorylation[384,385] and

integrin-dependent conformational changes[386,387]. We demonstrated the positive regulation of p62-FERMT2 (i) performing functional studies, (ii) using GEMM and (iii) a large cohort of human melanocytic lesions. Our studies identified FERMT2 as a physiologically-relevant novel indicator of poor prognosis in melanoma, acting downstream of p62. Although, the oncogenic potential of FERMT2 has been reported in other systems[344,388-390], this is the first study that shows the clinical relevance of the protein in melanoma. In this context, the data suggested that these two proteins may have a clinical relevance in melanoma and can be used as potential biomarkers.

## 7.5   p62 interactors in the regulation of mRNA stability

A relevant finding that stemmed from this PhD thesis was the identification of the interactors of p62 in melanoma which they were found to be distinct from other systems (BIOGRID) and they were specifically enriched in melanoma among other cancer cell lines (GSEA using as a reference the Cancer Cell Line Encyclopedia). This result comes in agreement with the above data that p62 is acting in a cell type specific manner controlling different signaling pathways. It is tempting to speculate that p62 adapts its activity depending on the cell type and the corresponding signals of the upstream regulators in order to act as a scaffold.

The most unanticipated finding of this study was the interaction of p62 with an increased number of RNA Binding Proteins (RBPs). The p62 interactors that corresponded to RBPs were almost 30% suggesting a new function of p62 as a hub for RBPs. Although, further studies need to be performed to address if the high number of RBPs are protein-protein interactions or p62 interacts with complexes of RBPs, these data imply possible roles of p62 in processes mediated through RBPs such as post-transcriptional gene regulation and RNA processing.

A fundamental role of RBPs (among others) is the regulation of mRNA stability[391,392]. The mRNAs are packaged into ribonucleoprotein particles (mRNPs). Thus, we investigated if any of the precipitated RBPs can directly regulate the stability of *FERMT2*. Our analyses indicated IGF2BP1 as the intermediate player acting in the p62-FERMT2 axis. Moreover, previous studies have shown the IGF2BP1 pro-metastatic roles in melanoma[393,394]. p62 via the interaction with IGF2BP1 stabilizes the mRNA of *FERMT2* resulting in protein translation. p62-mediated regulation linked to the inhibition of mRNA decay, offers therefore an additional strategy to sustain FERMT2 levels under situations of high demand for dynamic focal adhesions during invasive processes. Loss of p62 (i) prevents IGF2BP1 binding to *FERMT2*, (ii) destabilization and (iii) ultimately decreased invasive capacity of melanoma cells.

IGF2BP1 acts by preventing miRNA-mediated silencing, especially for the let-7 family[395-399]. It is possible that IGF2BP1 by counteracting miRNAs protects the degradation of *FERMT2* mRNA. Hence, through this mechanism FERMT2 can favor the invasiveness of melanoma cells. This is an attractive scenario that needs to be investigated and more specifically it would be important to identify the miRNAs that can target *FERMT2* and also to characterize how FERMT2 promotes invasion. Finally, it was observed that the p62-IGF2BP1 axis in mRNA decay is broader, as other pro-metastatic factors were regulated under similar settings as *FERMT2*. This implies that FERMT2 and other pro-metastatic factors can positively promote invasion under the control of p62-IGF2BP1 axis (**Figure 36**).

Collectively, this PhD thesis underscores the relevance of p62 in melanoma progression and metastasis by unraveling a new mechanism of action, where p62 via interaction with RBPs can modulate the stability of pro-metastatic factors.



**Figure 36:** *Proposed model of p62 in melanoma progression and metastasis.* p62 via interaction with IGF2BP1 favors the stability of pro-metastatic factors promoting melanoma invasion. Loss of p62 prevents the binding of IGF2BP1 in the mRNA of pro-metastatic factors resulting in their degradation and consequently to inhibition of invasion.
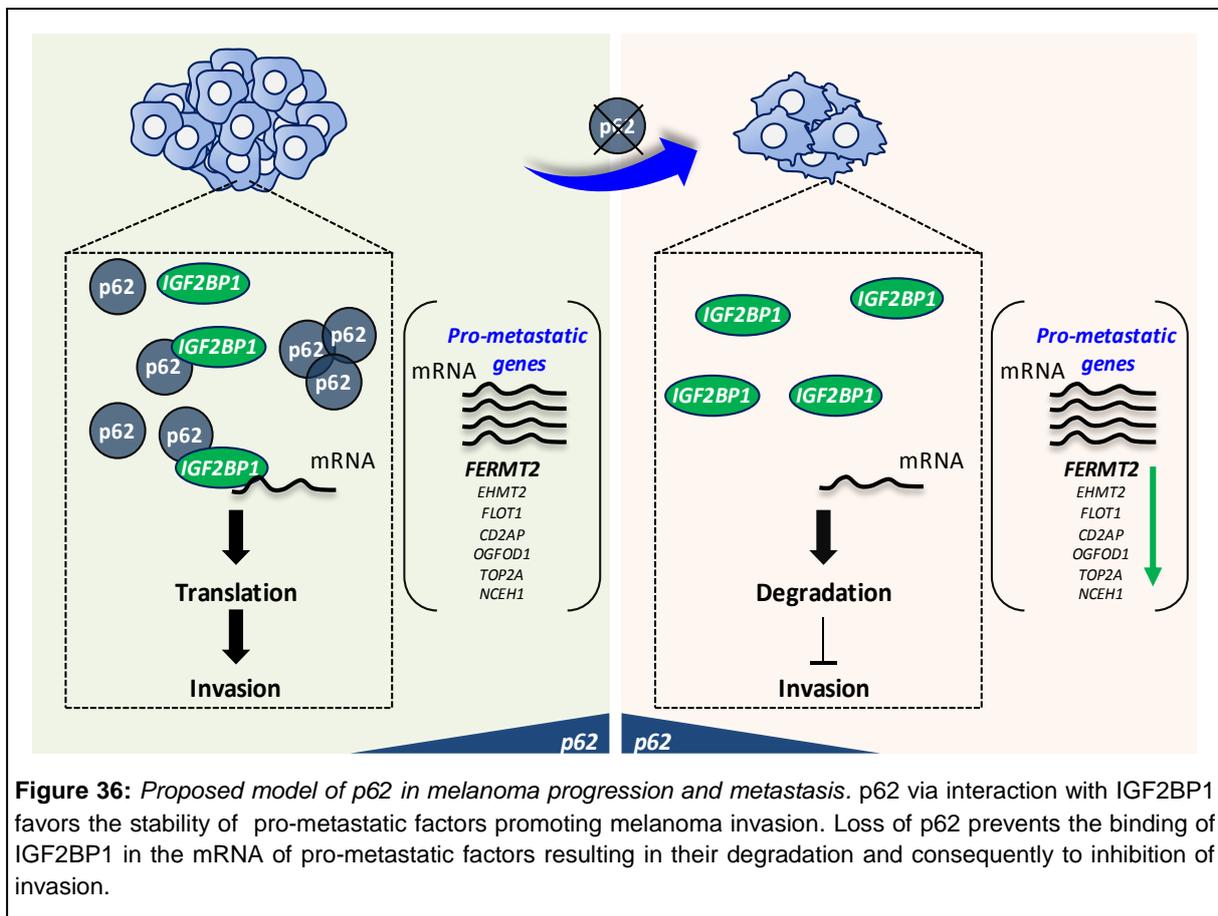
# VIII.CONCLUSIONS/ CONCLUSIONES

## Conclusions

In the light of the results presented in this PhD thesis we conclude the following:

1. Analyses of publicly available databases (TCGA and the CCLE) demonstrate that p62 is not a subject of heterozygous copy number loss in melanoma (as the case for ATG5). Instead, p62 was found to be overexpressed in human melanomas, especially at metastatic stages of the disease, a feature validated by histopathological analyses.

2. New Genetically Engineered Mouse Models (GEMM) revealed that p62 is dispensable for benign nevi, but it ıs required for the   progression and the metastastic potential of malignant melanoma. These results distinguish melanoma from other tumor types where p62 exerts inhibitory roles via the stroma.

3. Targeted depletion of p62 compromises the proliferation and invasion capabilities of cultured melanoma cells, independently on the status of main melanoma-associated drivers (BRAF, NRAS, PTEN or p53).

4. Known roles of p62 in the modulation of mTOR, NF-κB, KEAP1-NRF2, MYC and mitochondrial respiration were not affected by targeted depletion or overexpression of p62 in melanoma cells (also different from other cancer types).

5. *In vitro* studies and newly generated GEMM revealed that p62 loss or ectopic expression does not affect autophagosome formation.

6. Transcriptome analyses and subsequent computational studies by GSEA identified melanoma-associated metastatic signatures significantly downregulated upon p62 downregulation.

7. A comprehensive transcriptomic/proteomic/interactomic approach identified downstream effectors of p62 implicated in metastasis. In particular, the scaffold protein FERMT2 was demonstrated to be a novel risk factor for poor prognosis in melanoma. Accordingly, p62 and FERMT2 were positively correlated in human melanoma biopsies.

8. Interactome analysis identified a large set of RNA Binding Proteins (RBPs) as new p62 binding partners. In particular, IGF2BP1 was validated as a p62-interacting factor that controls the mRNA half-life of *FERMT2*.

9. p62 was found to oppose mRNA decay of other pro-metastatic factors (i.e. EHMT2, CD2AP, TOP2A, FLOT1, OGFOD1, and NCEH1) through the interaction with IGF2BP1.

Collectively, the data presented in this PhD thesis underscore a novel mechanism of p62 in the stability of pro-metastatic factors via the interaction with RBPs.

## Conclusiones

A la luz de los resultados presentados en esta tesis doctoral, se presentan las siguientes conclusiones:

1.  Un detallado análisis mediante bases de datos públicas (TCGA y CCLE) muestra que p62, a diferencia de ATG5, no está sujeto a una pérdida parcial del número de copias en melanoma. Por el contrario, se encuentra sobre-expresado especialmente en estadíos metastásicos. Este dato se ha podido validar a través de estudios histopatológicos. .

2. Los experimentos con nuevos modelos de ratones modificados genéticamente revelan que p62 es prescindible en la formación de lesiones melanocíticas benignas (nevi), y sin embargo, es requerido en la iniciación y progresión del melanoma. Esto diferencia al melanoma de otros tumores, en los que p62 presenta un efecto inhibidor a través del estroma.

3. La depleción de p62 *in vitro* compromete la capacidad proliferativa e invasiva de las células de melanoma en cultivo, con independencia del estado mutacional en los principales oncogenes como BRAF, NRAS, PTEN o p53.

4. Las funciones ya definidas de p62 en la modulación de factores como mTOR, NF-κB, KEAP1-NRF2, y MYC; o de procesos como la respiración mitocondrial, no se ven afectadas tras la supresión o expresión ectópica en células de melanoma. Esto remarca nuevamente el papel diferenciador de p62 en melanoma.

5. Tanto el silenciamiento como la sobreexpresión de p62 (*in vitro o in vivo*) carecen de efecto en la formación de autofagosomas.

6. Mediante análisis transcripcionales y estudios computacionales GSEA, se han podido identificar factores característicos asociados a la metástasis de melanoma cuya expresión disminuye al deplecionar p62.

7. Mediante un análisis global de la transcriptómica, proteómica e interactoma se han podido identificar proteínas efectoras de p62 implicadas en metástasis. En particular, hemos identificado la proteína de FERMT2 como un nuevo factor de riesgo y prognóstico desfavorable en pacientes de melanoma. Además, análisis histopatológicos de biopsias humanas confirman una correlación entre los niveles de p62 y FERMT2 .

8. El análisis del interactoma de p62 permitió identificar un grupo de proteínas de unión a RNA (RBPs) como nuevos factores de interacción. Entre ellos, se ha validado cómo

IGF2BP1 actua como mediador en la regulación de la vida media del mRNA de FERMT2 por p62.

9. Además, la interacción de p62 con IGF2BP1 regula la estabilidad del mRNA de otros factores prometastáticos (EHMT2, CD2AP, TOP2A, FLOT1, OGFOD1, y NCEH1).

Todos los datos presentados en esta tesis confirman una nueva función de p62 como modulador  de los niveles de factores pro-mestastásicos a través de la interacción con RBPs.

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# X.APPENDIX

## 10.1 Supplementary tables and figures

**Table S1:** Systemic therapies approved since 2011 for advanced-stage melanoma.Source:Ref.[35]

| | Agent | Mechanism | FDA-approved indications |
|---|---|---|---|
| **Targeted therapies** | Vemurafenib | BRAF inhibitor | As monotherapy and in combination with cobimetinib for BRAF$^{V600E/K}$-mutant disease |
| | Dabrafenib | BRAF inhibitor | As monotherapy and in combination with trametinib for BRAF$^{V600E/K}$-mutant disease |
| | Trametinib | MEK inhibitor | As monotherapy and in combination with dabrafenib for BRAF$^{V600E/K}$-mutant disease |
| | Cobimetinib | MEK inhibitor | In combination with vemurafenib for BRAF$^{V600E/K}$-mutant melanoma |
| **Immunotherapies** | Ipilimumab | Anti-CTLA-4 antibody | As monotherapy and in combination with nivolumab |
| | Pembrolizumab | Anti-PD-1 antibody | As monotherapy |
| | Nivolumab | Anti-PD-1 antibody | In combination with ipilimumab, or as monotherapy |
| **Oncolytic viral therapy** | Talimogene laharparepvec | Modified oncolytic herpes virus | Local treatment of unresectable cutaneous, subcutaneous, and nodal lesions in patients with recurrent melanoma after surgery |

**Table S2:** Prominent mechanisms of pathway alterations in BRAF, RAS, NF1 and Triple Wild-Type (WT) subtypes with potential predictive genetic alterations indicated ([1, 2, 3, 4, 5, 6]). Source:Ref.[95]

| Mutation Subtypes | BRAF | RAS | NF1 | Triple Wild-Type |
|---|---|---|---|---|
| [1]**MAPK pathway** | [1]BRAFV600,K601 | [1](N/H/K)RASG12, G13,Q61 | [1]NF1 LoF mut;(BRAF non-hot-spot mut) | [1]KITC OSMIC mut/amp, PDGFRa amp,KDR (VEGFR2)amp;rare COSMIC GNA11 mut, GNAQ mut |
| [2]**Cell-cycle pathway** | CDKN2Amut/del/h-meth (60%);[2](CDK4 COSMIC mut) | CDKN2A mut/del/h-meth (70%);CCND1 amp (10%),[2](CDK4 COSMIC mut) | CDKN2A mut/del/h-meth (70%);RB1 mut (10%) | CDKN2A mut/del/h-meth (40%);CCND1 amp (10%),[2]CDK4 amp (15%) |
| [3]**DNA damage response and cell death pathways** | TP53mut(10%); [3](note:TP53wild-type in 90% of BRAF subtype) | TP53mut(20%) | TP53 mut(30%) | [3]MDM2 amp(15%); [3]BCL2 upregulation |
| [4]**PI3K/Akt pathway** | [4]PTEN mut/del(20%); [4](rareAKT1/3 and PIK3CA COSMIC mut) | [4]AKT3 overexpression (40%);[4](rare AKT1/3 and PIK3CA COSMIC mut) | [4]AKT3 overexpression (30%) | [4]AKT3 overexpression (20%) |
| [5]**Epigenetics** | [5]IDH1mut,[5](rare EZH2 COSMIC mut);[5]ARID2 mut (15%) | [5]IDH1mut,[5](rare EZH2 COSMIC mut);[5]ARID2 mut(15%) | [5]IDH1mut,[5](EZH2 mut); [5]ARID2mut(30%) | [5]IDH1 mut,[5](rare EZH2 COSMIC mut) |
| **Telomerase pathway** | Promoter mut (75%) | Promotermut(70%) | Promoter mut(85%) | Promoter mut(<10%); TERT amp(15%) |
| **Other pathways** | PD-L1amp, MITF amp, PPP6C mut(10%) | PPP6C mut(15%) | | |
| [6]**High immune infiltration (pathology)** | ~30% | ~25% | ~25% | ~40% |

**Table S3:** Functions of AuTophaGy (**ATG**) related proteins during autophagosome formation. Source:Ref.[400]

| Protein | Yeast | High eukaryotes | Function |
|---|---|---|---|
| Atg1/ULKs | + | + | Protein kinase involved in the induction of autophagy and possibly in PAS/phagophore biogenesis |
| Atg2 | + | + | Interacts with Atg18/WIPI4; possibly involved in PAS/phagophore biogenesis |
| Atg3 | + | + | E2-like enzyme for the ubiquitin-like conjugation system that catalyses Atg8/LC3's lipidation involved in phagophore expansion |
| Atg4 | + | + | Cysteine protease processing and delipidating Atg8/LC3, thus involved in phagophore expansion |
| Atg5 | + | + | Covalently linked to Atg12, generating the Atg12–Atg5 conjugate involved in phagophore expansion |
| Atg6/beclin 1 | + | + | Component of various PI(3)K complexes, one of which is involved in induction of autophagy and PAS/phagophore biogenesis |
| Atg7 | + | + | E1-like enzyme for the two ubiquitin-like conjugation systems, thus involved in phagophore expansion |
| Atg8/LC3s | + | + | Ubiquitin-like protein involved in phagophore expansion |
| Atg9 | + | + | Transmembrane protein involved in the induction of autophagy and possibly in PAS/phagophore biogenesis |
| Atg10 | + | + | E2-like enzyme for the ubiquitin-like conjugation system that mediates the formation of the Atg12–Atg5 conjugate involved in phagophore expansion |
| Atg12 | + | + | Ubiquitin-like protein involved in phagophore expansion |
| Atg13 | + | + | Binding partner and regulator of Atg1/ULKs, thus involved in the induction of autophagy and possibly PAS/phagophore biogenesis |
| Atg14 | + | + | Component of the PI(3)K complex I involved in induction of autophagy and possibly PAS/phagophore biogenesis |
| Atg16 | + | + | Associates with Atg12–Atg5 to form a large complex, which acts as an E3 ligase to direct LC3 lipidation on autophagosomal membranes, and thus involved in phagophore expansion |
| Atg17/FIP200 | + | + | Binding partner and regulator of Atg1/ULKs, thus involved in the induction of autophagy and possibly PAS/phagophore biogenesis |
| Atg18/WIPIs | + | + | PtsIns3P-binding proteins possibly involved in PAS/phagophore biogenesis |
| Atg23 | + | - | Binding partner and regulator of Atg9, thus involved in the induction of autophagy and possibly in PAS/phagophore biogenesis |
| Atg27 | + | - | Binding partner and regulator of Atg9, thus involved in the induction of autophagy and possibly in PAS/phagophore biogenesis |
| Atg29 | + | - | Binding partner and regulator of Atg1, thus involved in the induction of autophagy and possibly in PAS/phagophore biogenesis |
| Atg31 | + | - | Binding partner and regulator of Atg1, thus involved in the induction of autophagy and possibly in PAS/phagophore biogenesis |
| Atg101 | - | + | Binding partner and regulator of ULKs, thus involved in the induction of autophagy and possibly in PAS/phagophore biogenesis |
| Ambra1 | - | + | Regulator of the PI(3)K and Atg1/ULK complexes, and thus involved in the induction of autophagy |
| DFCP1 | - | + | PtdIns3P-binding proteins concentrating at the omegasome, possibly involved in the induction of autophagy |
| VMP1 | - | + | Transmembrane protein regulating autophagy induction |
| Vps15/p150 | + | + | Kinase regulating Vps34/hVps34 activity; component of various PI(3)K complexes, one of which is involved in the induction of autophagy and PAS/phagophore biogenesis |
| Vps34/PtdIns3PKC3 | + | + | Component of various PI(3)K complexes, one of which is involved in the induction of autophagy and PAS/phagophore biogenesis |

**Table S4:** p62 main described functions in disease and in different cancer types.

| system | Domain | interaction/ Regulation | cancer/ disease | Functions | reference |
|---|---|---|---|---|---|
| HeLa, CD4+ , Jurkat T | PB1 | p56[lck] | - | T cell activation | [228] |
| COS | ZZ | PKC-ζ | - | scaffold | [229] |
| HeLa/293 | PB1 | λ/ι/ζ PKC | - | endolysosomal trafficking | [230] |
| mouse brain | TB | CYLD | - | turnover of Lys63-polyubiquitinated proteins | [238] |
| patients | UBA | - | Paget's disease | mutations in PDB patients | [241] |
| HeLa/MEFs | LIR | LC3 | - | degradation of polyubiquitinated proteins | [243] |
| HeLa/MEFs/HEK293 | KIR | KEAP1 | - | activation of NRF2 target genes | [245] |
| Atg7::Keap1-Alb double-mutant mice into p62- and Nrf2-null backgrounds | KIR | KEAP1 | - | oxidative stress | [247] |

| system | Domain | interaction/ Regulation | cancer/ disease | Functions | reference |
|---|---|---|---|---|---|
| HeLa, PC3 and HEK293. ATG5 and p62 KO cells | ZZ | R-BiP | - | ER-residing proteins degradation | [248] |
| BMDMs from p62KO mice | PB1/TB | aPKCs, TRAF6 | - | osteoclastogenesis | [250] |
| p62 KO mice | - | ERK | - | obesity | [251] |
| HeLa/p62$^{-/-}$, or Atg5$^{-/-}$ MEFs. p62$^{-/-}$ mice, and Atg7F/F:Mx1:p62$^{-/-}$ mice | PB1 | NBR1 | - | Autophagosomal Degradation of Ubiquitinated Substrates | [252] |
| HeLa and HEK293/p62 KO MEFs | PB1 | STAT5A_ΔE18 | - | autophagic clearance of a non-ubiquitylated substrate | [300] |
| BMK/TRIM21 KO mice | PRYSPRY | TRIM2 | - | redox homeostasis | [401] |
| Hela | - | PML bodies/ataxin/p olyUb | prostate cancer | Recruitment of Nuclear Polyubiquitinated Proteins to PML bodies (Nucleocytoplasmic Shuttling) | [232] |
| iBMK ATG5 KO and Beclin1 KO cell lines | - | defective autophagy | hepatocellular carcinomas | oxidative stress/ DNA damage/ NF-κB/ autophagy | [189] |
| Tissue sections of patients with HCC | - | - | hepatocellular carcinomas | associated with HCC | [258] |
| Tissue sections of patients with HCC | - | - | hepatocellular carcinomas | associated with HCC | [259] |
| liver-specific ATG7-deficient mice | KIR | KEAP1 | hepatocellular carcinomas | persistent activation of Nrf2 | [263] |
| Tissue sections of patients with HCC | | - | hepatocellular carcinomas | In Japanese HCC, NRF2 activation is associated with phosphorylation of p62, but not with KEAP1 status | [402] |
| HCC cell lines/Atg7 and NRf2 liver deficient mice. Human biopsies | KIR | KEAP1 | hepatocellular carcinomas | Phosphorylation of p62 and metabolism | [261] |
| Tissue sections of patients | - | - | cholangiocarcin oma with chronic liver | Hyaline Inclusions | [264] |
| Tissue sections of patients | - | - | Gastrointestinal Carcinomas | p62 and ubiquitin are highly expressed in gastric, colonic, and pancreatic carcinomas | [265] |
| Tissue sections of patients | - | - | lung adenocarcinom a | RF2 and p62 are independent prognostic factors for NSCLC | [266] |
| Tissue sections of patients | - | - | lung adenocarcinom a | LC3 and p62 are differentially expressed in NSCLC | [267] |
| cell lines/tissue samples | - | - | Squamous Cell Carcinoma of Head and Neck | resistance to PI3Ki | [268] |
| cell lines/tissue samples | - | - | oral squamous cell carcinomas | p62 accumulation and resistance to radiation or chemotherapy | [269] |
| cell lines/tissue samples | - | - | Esophageal adenocarcinom as | Tumors with low LC3B and p62 expression show the most aggressive behavior | [270] |
| Tissue sections of patients | - | - | gastric cacner | Autophagy determined by LC3, Beclin 1, and p62 significantly correlated with lymph node metastasis, vessel invasion, and hepatic metastasis | [271] |
| Tissue sections of patients | - | - | colon cancer | p62 is overexpressed in 85% of the samples | [272] |
| cell lines/Tissue sections of patients | - | - | breast cancer | PDEF stimulates the p62 promoter | [273] |
| Tissue sections of patients | - | - | breast cancer (TNB) | p62 in TNBC is associated with a higher risk of distant metastases | [274] |
| Tissue sections of patients | - | - | breast cancer | p62 expression correlated with grade and distant metastasis and EGFR, HER2, HER3 and HER4 | [275] |
| cell lines/Tissue sections of patients | - | - | prostate cancer | high-grade prostatic intraepithelial neoplasia | [279] |
| Tissue sections of patients | - | - | prostate cancer | p62 expression was related with LC3A and B reactivity and also with extraprostatic invasion | [280] |
| Tissue sections of patients | - | - | melanoma | p62 expression was related with melanoma progression | [285] |
| cell lines/Tissue sections of patients | - | - | endometrial cancer | High expression of cytoplasmic p62 correlates with endometrial cancer | [286] |
| Tissue sections of patients | - | - | ovarian cancer | p62 expression epithelial ovarian cancer and platinum resistance during chemotherapy | [287] |
| A498, A704, Caki-2, HK-2, RCC4, 769-P, 786-O,UMRC-2, UMRC-6, and UOK101 | - | NRF2, NF-kB and mTOR | kidney cancer | selection pressure to amplify 5q in ccRCC is driven, at least partly, by p62 | [289] |
| Tissue sections of patients | - | - | hepatocellular carcinomas | p62 in Mallory bodies (MBs) and intracellular hyaline bodies (IHBs) | [291] |
| Tissue sections of patients | - | - | non-alcoholic fatty liver disease | suppression of autophagic proteolysis by hepatic steatosis is involved in the pathogenesis of NAFLD | [292] |
| Tissue sections of patients/mouse models | | increased ER stress | non-alcoholic fatty liver disease | autophagic flux is impaired in NAFLD human mouse NAFLD due to elevated ER stress leading to apoptosis | [293] |
| Human GSC | - | - | glioblastoma | DRAM1 and p62 regulate cell motility and invasion | [288] |

| system | Domain | interaction/ Regulation | cancer/ disease | Functions | reference |
|---|---|---|---|---|---|
| | | | multiforme | in GSCs | |
| Pca | - | - | prostate cancer | IL-1β can drive Pca progression in an inflammatory microenvironment through AR repression and p62 induction to promote the development and survival of androgen independent Pca | [281] |
| Tissue sections of patients/mice xenografts | - | raptor | prostate cancer | critical mediator for mTOR activation | [282] |
| Tissue sections of patients/mouse models | LIR/UBA | TRB3 | HCC, colon cancer and lung cancer | TRB3 interacts with p62, which causes p62 deposition and suppresses autophagic/proteasomal degradation | [262] |
| mouse prostate epithelial cells floxed or Cre-deleted Map3k7 | - | - | prostate cancer | p62 Regulates the Switch between Apoptotic and Necroptotic Cell Death | [284] |
| cell lines/p62 KO, PTEN$^{+/-}$, and TRAMP$^{+}$ | - | mTORC1/c-Myc | prostate and breast cancer | p62 levels were reduced in the stroma of several tumors and that its loss in the tumor microenvironment or stromal fibroblasts resulted in increased tumorigenesis of epithelial prostate cancer cells | [260] |
| HEK 293T/Liver-specific TRAF6 KO mice | TB | TRAF6 | - | TRAF6, through its interaction with p62 and activation of mTORC1, modulates autophagy and is an important mediator in cancer cell proliferation | [339] |
| cell lines and PTEN$^{+/-}$ and PTEN$^{fl/fl}$-PBcre mice | PB1 | MEKK3 | prostate cancer | p62 is phosphorylated via a cascade that includes MEK3/6 and p38δ and is driven by the PB1-containing kinase MEKK3 | [283] |
| cell lines/Ikkα$^{Δpan}$ | - | - | pancreatitis | p62 accumulation through IKKα and ATG12L2 | [305] |
| Atg5,Atg3,Atg7,Atg9,Atg12,Atg14,p62 KO MEFs, HaCaT,HEK-293T,A431,A375. | UBA | Twist1 | MEFs/melanoma cells | p62 promotes loss of Ecadherin, cell proliferation and migration in vitro, and tumor growth and metastasis in vivo through stabilizing Twist1 (EMT modulation) | [299] |
| Mel624,A375,A431,p62 KO MEFs | - | - | squamous cell carcinoma cells/melanoma cells | CQ induced NF-kB activation and the expression of its target genes HIF-1a, IL-8, Bcl-2 and Bcl-XL through the accumulation of autophagosomes, p62, and JNK signaling. | [403] |
| COS-7 and HeLa cells | - | - | - | Mammalian cells use a common pathway involving ubiquitin and p62 for targeting diverse types of substrates for autophagy | [295] |
| *Atg7$^{F/F}$*:Mx1:GFP-LC3 mice*Atg7$^{F/F}$*:Mx1:*p62$^{-/-}$*, *Atg7$^{F/F}$*:Mx1:*p62$^{-/-}$*:GFP-LC3, and *Atg7$^{F/F}$*:Nes:*p62$^{-/-}$* mice | LIR | LC3 | liver injury and neurodegeneration | role of homeostatic level of p62, which is regulated by autophagy, in controlling intracellular inclusion body formation | [298] |
| FIP200f/f;MMTV-PyMT and p62 KO mice/HEK293, MEFs, and MDA-MB-231 cells | - | - | mammary tumor cells | p62 synergizes with autophagy for tumor growth in vivo | [207] |
| Flp-in Neuro2a/Atg5-KO mice and MEF cells | - | - | mefs | phosphorylation of p62 at S403 regulates autophagic clearance of ubiquitinated proteins and protein aggregates that are poorly degraded by proteasomes | [240] |
| Rpt2f/f;Alb-Cre mice/ p62 KO MEFs | KIR | KEAP1 | hepatocellular carcinomas | p62 phosphorylation induces expression of cytoprotective Nrf2 targets through interaction with KEAP1 | [404] |
| PDAC mouse models based on KRAS$^{G12D}$ | - | - | Pancreatic Ductal Adenocarcinoma | Dual feed forward loops of IL-1α/p62 through which IKK2/β/NF-κB is activated by Kras$^{G12D}$ in PDAC | [304] |
| p62KO, CCSP-rtTA, and KRASG12D mice/ p62KO MEFs | - | - | lung adenocarcinoma | p62 is induced by Ras, activates IKK through TRAF6 polyubiquitination, is overexpressed in human tumors, and is necessary for survival of human lung adenocarcinoma cells | [253] |
| *Atg7$^{F/F}$*:Mx1:*Nrf2$^{-/-}$* /Keap$^{F/F}$:Atg7$^{F/F}$:Alb mice | KIR | KEAP1 | MEFs | p62 accumulation results in hyperactivation of Nrf2 and delineates unexpected roles of selective autophagy in controlling the transcription of cellular defence enzyme genes | [199] |
| HEK293/A549/p62 KO MEFs | - | - | MEFs K-Ras$^{V12}$ | lack of p62 phosphorylation by cdk1 leads to enhanced cell proliferation and tumorigenesis in Ras-transformed cells. | [309] |
| DA-MB-231, BT-549, MCF-7 and SKBR-3 | - | - | breast cancer stem cells | p62 promotes MYC mRNA stabilization at the post-transcriptional level in breast cancer cells | [277] |
| p62 KO mice/ hepatic stellate cells | several domains | VDR:RXRα dimer | | Loss of p62 in hepatic stellate cells results in increased fibrosis, inflammation, and HCC | [308] |

**Table S5:** Common enriched pathways from GSEA ( using KEGG and Reactome) upon p62 knockdown in two melanoma cell lines (SK-Mel-103 and UACC-62) and two different time points (day 3 and day 6).

| CELLULAR PATHWAYS | SK-Mel-103 | | UACC-62 | | |
| --- | --- | --- | --- | --- | --- |
| | day 3 | day 6 | day 3 | day 6 | |
| | FDR-q value | FDR-q value | FDR-q value | FDR-q value | |
| REACTOME__EGFR DOWNREGULATION | 0.0049 | 0.0408 | 0.0438 | 0.0749 | trafficking |
| REACTOME__MEMBRANE TRAFFICKING | 0.0565 | 0.0605 | 0.2141 | 0.0870 | |
| KEGG_ENDOCYTOSIS | 0.0124 | 0.0817 | 0.0608 | 0.0633 | |
| REACTOME__CELL CYCLE, MITOTIC | <0.0001 | <0.0001 | 0.0706 | <0.0001 | Cell cycle |
| REACTOME__G2_M CHECKPOINTS | <0.0001 | <0.0001 | 0.0639 | <0.0001 | |
| REACTOME__G1_S TRANSITION | <0.0001 | <0.0001 | 0.0685 | <0.0001 | |
| REACTOME__CELL CYCLE CHECKPOINTS | <0.0001 | <0.0001 | 0.0567 | <0.0001 | |
| REACTOME__E2F MEDIATED REGULATION OF DNA REPLICATION | <0.0001 | <0.0001 | 0.0704 | <0.0001 | |
| REACTOME__M_G1 TRANSITION | 0.0003 | <0.0001 | 0.0621 | 0.0001 | |
| REACTOME__CYCLIN E ASSOCIATED EVENTS DURING G1_S TRANSITION | 0.0134 | 0.0009 | 0.0842 | 0.0492 | |
| REACTOME__DNA REPAIR | 0.0036 | 0.0040 | 0.2150 | 0.0012 | DNA damage |
| REACTOME__ACTIVATION OF ATR IN RESPONSE TO REPLICATION STRESS | <0.0001 | <0.0001 | 0.0603 | <0.0001 | |
| KEGG_DNA REPLICATION | <0.0001 | 0.0044 | 0.1642 | <0.0001 | DNA replication |
| REACTOME__ACTIVATION OF THE PRE-REPLICATIVE COMPLEX | <0.0001 | <0.0001 | 0.0962 | <0.0001 | |
| REACTOME__DNA REPLICATION | <0.0001 | <0.0001 | 0.0691 | <0.0001 | |
| REACTOME__DNA REPLICATION PRE-INITIATION | <0.0001 | <0.0001 | 0.0626 | <0.0001 | |
| REACTOME__ASSEMBLY OF THE PRE-REPLICATIVE COMPLEX | 0.0004 | <0.0001 | 0.0649 | 0.0002 | |
| REACTOME__CHOLESTEROL BIOSYNTHESIS | 0.0029 | <0.0001 | 0.0475 | 0.0029 | Metabolism |
| REACTOME__METABOLISM OF CARBOHYDRATES | 0.0118 | 0.0039 | 0.0768 | 0.0017 | |
| REACTOME__ORNITHINE METABOLISM | 0.0331 | 0.0084 | 0.0495 | 0.1479 | |
| REACTOME__GLUCONEOGENESIS | 0.1432 | 0.0139 | 0.1369 | 0.0109 | |
| REACTOME__CITRIC ACID CYCLE (TCA CYCLE) | 0.1627 | 0.0093 | 0.1833 | 0.0064 | |
| KEGG_STEROID BIOSYNTHESIS | 0.0123 | 0.0511 | 0.1142 | 0.0653 | |
| KEGG_PENTOSE PHOSPHATE PATHWAY | 0.0227 | 0.0589 | 0.1087 | 0.0115 | |
| KEGG_CARBON METABOLISM | 0.0481 | 0.0456 | 0.0877 | 0.0063 | |
| KEGG_BIOSYNTHESIS OF AMINO ACIDS | 0.0830 | 0.0653 | 0.1419 | 0.0320 | |
| KEGG_CITRATE CYCLE (TCA CYCLE) | 0.2296 | 0.0408 | 0.1633 | 0.0012 | |
| KEGG_BIOSYNTHESIS OF UNSATURATED FATTY ACIDS | 0.2362 | 0.0679 | 0.2280 | 0.0854 | |

*Red indicates upregulation and green downregulation, respectively. Intensity of colours indicates the degree of significance (*dark: FDR < 0.01; light: 0.01 < FDR < 0.25*)

**Table S6:** Upregulated proteins detected by iTRAQ analysis upon p62 knockdown in melanoma cell lines.

| SK-Mel-103 | | | | | |
| --- | --- | --- | --- | --- | --- |
| UPREGULATED PROTEINS | | | | | |
| Accession | Gene name | log$_2$FC (shp62/shcon) | Accession | Gene name | log$_2$FC (shp62/shcon) |
| P52926 | HMGA2 | 2.380 | Q9Y277 | VDAC3 | 1.383 |
| Q99856 | ARID3A | 1.907 | Q5U3C3 | TMEM164 | 1.382 |
| Q8WUH1 | CHURC1 | 1.831 | P55786 | NPPS | 1.381 |
| P00414 | MT-CO3 | 1.810 | Q9BWS9 | CHID1 | 1.380 |
| A6NKG5 | RTL1 | 1.771 | O15156 | ZBTB7B | 1.380 |
| Q9HCG8 | CWC22 | 1.745 | Q9C0H2 | TTYH3 | 1.377 |
| Q14643 | ITPR1 | 1.739 | P09486 | SPARC | 1.376 |
| P15954 | COX7C | 1.704 | P27540 | ARNT | 1.372 |
| P33947 | KDELR2 | 1.700 | Q5BJD5 | TMEM41B | 1.370 |
| Q9NPH3 | IL1RAP | 1.668 | Q5NDL2 | EOGT | 1.369 |
| O75909 | CCNK | 1.657 | Q9NRX4 | PHPT1 | 1.368 |
| O00622 | CYR61 | 1.627 | Q8N5L8 | RPP25L | 1.364 |
| P43355 | MAGEA1 | 1.624 | Q9NW08 | POLR3B | 1.364 |
| Q9NUD5 | ZCCHC3 | 1.616 | Q8WWI5 | SLC44A1 | 1.363 |
| P09669 | COX6C | 1.612 | P48060 | GLIPR1 | 1.361 |

| SK-Mel-103 | | | | | |
|---|---|---|---|---|---|
| **UPREGULATED PROTEINS** | | | | | |
| **Accession** | **Gene name** | **log$_2$FC (shp62/shcon)** | **Accession** | **Gene name** | **log$_2$FC (shp62/shcon)** |
| O75152 | ZC3H11A | 1.610 | Q86TP1 | PRUNE | 1.359 |
| P52823 | STC1 | 1.604 | P35555 | FBN1 | 1.359 |
| Q14978 | NOLC1 | 1.596 | Q9Y6B6 | SAR1B | 1.359 |
| P11047 | LAMC1 | 1.583 | Q9BSV6 | TSEN34 | 1.355 |
| Q92521 | PIGB | 1.576 | O14817 | TSPAN4 | 1.355 |
| Q96DC9 | OTUB2 | 1.573 | Q9UPT9 | USP22 | 1.354 |
| O14686 | MLL2 | 1.567 | P15923 | TCF3 | 1.354 |
| O60331 | PIP5K1C | 1.567 | Q5T9L3 | WLS | 1.349 |
| Q9NWT6 | HIF1AN | 1.557 | Q17RY0 | CB4 | 1.349 |
| Q13542 | EIF4EBP2 | 1.527 | O14802 | POLR3A | 1.349 |
| P98153 | DGCR2 | 1.513 | P49356 | FNTB | 1.348 |
| P24311 | COX7B | 1.512 | O75340 | PDCD6 | 1.347 |
| Q9H7F0 | ATP13A3 | 1.511 | O94887 | FARP2 | 1.347 |
| P35908 | KRT2 | 1.511 | P43251 | BTD | 1.347 |
| Q9UI42 | CPA4 | 1.503 | O00592 | PODXL | 1.347 |
| Q15047 | SETDB1 | 1.501 | Q8NDZ6 | TMEM161B | 1.346 |
| P05120 | SERPINB2 | 1.485 | Q3YEC7 | RABL6 | 1.345 |
| Q9UBU8 | MORF4L1 | 1.469 | Q00534 | CDK6 | 1.345 |
| P19021 | PAM | 1.465 | O75143 | ATG13 | 1.344 |
| O95470 | SGPL1 | 1.464 | A6NLU0 | RFPL4A | 1.342 |
| Q9BPZ3 | PAIP2 | 1.450 | P51161 | FABP6 | 1.340 |
| Q9UPY3 | DICER1 | 1.449 | Q7LBR1 | CHMP1B | 1.338 |
| O00507 | USP9Y | 1.447 | P32121 | ARRB2 | 1.338 |
| P04264 | KRT1 | 1.439 | P05496 | ATP5G1 | 1.337 |
| Q7Z3C6 | ATG9A | 1.437 | P10606 | COX5B | 1.331 |
| Q5XKK7 | FAM219B | 1.431 | Q9Y2K7 | KDM2A | 1.331 |
| Q13277 | STX3 | 1.430 | P01583 | IL1A | 1.330 |
| O75794 | CDC123 | 1.430 | Q9Y2V2 | CARHSP1 | 1.328 |
| Q8WU17 | RNF139 | 1.428 | Q9UMX5 | NENF | 1.328 |
| Q9BW60 | ELOVL1 | 1.427 | Q9NUM3 | SLC39A9 | 1.327 |
| O95182 | NDUFA7 | 1.425 | Q9H300 | PARL | 1.327 |
| Q9BY49 | CR | 1.421 | O95857 | TSPAN13 | 1.323 |
| O14672 | ADAM10 | 1.420 | P20933 | AGA | 1.323 |
| P00403 | MT-CO2 | 1.418 | O96011 | X11B | 1.321 |
| P60520 | GABARAPL2 | 1.416 | P17612 | PRKACA | 1.321 |
| O95168 | NDUFB4 | 1.411 | O14880 | MGST3 | 1.319 |
| O95835 | LATS1 | 1.405 | Q14696 | MESDC2 | 1.316 |
| P15407 | FOSL1 | 1.405 | Q3SXM5 | HSDL1 | 1.315 |
| Q14980 | NUMA1 | 1.402 | Q9H3K6 | BOLA2 | 1.313 |
| A5PLL7 | TMEM189 | 1.398 | Q8N954 | CCDC75 | 1.310 |
| Q9ULU4 | ZMYND8 | 1.394 | P11171 | EPB41 | 1.309 |
| Q99985 | SEMA3C | 1.393 | Q9H5X1 | FAM96A | 1.306 |
| Q5TCZ1 | SH3PXD2A | 1.392 | Q92833 | JARID2 | 1.306 |
| Q8IVP5 | FUNDC1 | 1.391 | Q01432 | AMPD3 | 1.304 |
| O43731 | KDELR3 | 1.388 | Q9NRR4 | DROSHA | 1.303 |
| Q5HYI8 | RABL3 | 1.384 | P13645 | KRT10 | 1.301 |
| P20674 | COX5A | 1.384 | Q96AQ6 | PBXIP1 | 1.300 |

| UACC-62 | | | | | |
|---|---|---|---|---|---|
| **UPREGULATED PROTEINS** | | | | | |
| **Accession** | **Gene name** | **log$_2$FC (shp62/shcon)** | **Accession** | **Gene name** | **log$_2$FC (shp62/shcon)** |
| O60315 | ZEB2 | 4.375 | Q9NYB0 | TERF2IP | 1.425 |
| P30508 | HLA-C | 3.020 | Q6NXE6 | ARMC6 | 1.424 |
| Q9NUM3 | SLC39A9 | 2.902 | Q9UKN8 | GTF3C4 | 1.422 |
| Q6N063 | OGFOD2 | 2.708 | Q9NVV4 | MTPAP | 1.422 |
| O14646 | CHD1 | 2.699 | Q86XA9 | HEATR5A | 1.420 |
| P35908 | KRT2 | 2.662 | Q2TAL8 | QRICH1 | 1.408 |
| Q9H9Q2 | COPS7B | 2.635 | P19022 | CDH2 | 1.407 |
| P00533 | EGFR | 2.348 | O14734 | ACOT8 | 1.407 |
| P61244 | MAX | 2.327 | Q6PKG0 | LARP1 | 1.406 |
| Q7Z5J4 | RAI1 | 2.222 | Q69YN2 | CWF19L1 | 1.406 |
| P04264 | KRT1 | 2.186 | Q7Z3J2 | C16orf62 | 1.403 |
| Q9UII2 | ATPIF1 | 2.163 | Q15004 | PAF | 1.403 |
| P14136 | GFAP | 2.147 | P05026 | ATP1B1 | 1.400 |
| P11908 | PRPS2 | 1.943 | O14521 | SDHD | 1.397 |
| Q7Z569 | BRAP | 1.917 | Q14738 | PPP2R5D | 1.385 |

| SK-Mel-103 | | | | | |
|---|---|---|---|---|---|
| **UPREGULATED PROTEINS** | | | | | |
| **Accession** | **Gene name** | **log₂FC (shp62/shcon)** | **Accession** | **Gene name** | **log₂FC (shp62/shcon)** |
| Q13131 | PRKAA1 | 1.889 | Q16890 | TPD52L1 | 1.383 |
| Q9Y3D8 | TAF9 | 1.813 | O15259 | NPHP1 | 1.381 |
| Q7Z4Q2 | HEATR3 | 1.801 | P23497 | SP100 | 1.379 |
| Q8NEB9 | PIK3C3 | 1.734 | Q9C0C9 | UBE2O | 1.373 |
| Q9BRS2 | RIOK1 | 1.723 | Q96ME7 | ZNF512 | 1.372 |
| P52926 | HMGA2 | 1.700 | Q8WVJ2 | NUDCD2 | 1.371 |
| P13645 | KRT10 | 1.698 | Q9UNX4 | WDR3 | 1.366 |
| Q9H5K3 | SGK196 | 1.687 | P40937 | RFC5 | 1.366 |
| Q9BTZ2 | DHRS4 | 1.663 | Q96QU8 | XPO6 | 1.366 |
| P08651 | NFIC | 1.656 | Q92922 | SMARCC1 | 1.359 |
| P53667 | LIMK1 | 1.637 | O75030 | MITF | 1.359 |
| Q9NR12 | PDLIM7 | 1.618 | Q9Y3A6 | TMED5 | 1.358 |
| Q9UKJ3 | GPATCH8 | 1.617 | P35227 | PCGF2 | 1.358 |
| Q12933 | TRAF2 | 1.614 | Q5T9L3 | WLS | 1.358 |
| O00141 | SGK1 | 1.607 | Q9NZ32 | ACTR10 | 1.349 |
| O43264 | ZW10 | 1.597 | Q00653 | NFKB2 | 1.348 |
| O75506 | HSBP1 | 1.596 | P49356 | FNTB | 1.348 |
| Q6DKK2 | TTC19 | 1.596 | Q9Y294 | ASF1A | 1.348 |
| Q8NEZ5 | FBXO22 | 1.593 | Q15003 | NCAPH | 1.347 |
| Q9H074 | PAIP1 | 1.590 | Q9NRF9 | POLE3 | 1.345 |
| Q12986 | NFX1 | 1.580 | Q15334 | LLGL1 | 1.345 |
| P31323 | PRKAR2B | 1.562 | Q9BT09 | CNPY3 | 1.345 |
| Q9HCG8 | CWC22 | 1.558 | Q9UQN3 | CHMP2B | 1.345 |
| Q9UJY4 | GGA2 | 1.556 | Q5NDL2 | EOGT | 1.342 |
| Q9BWE0 | REPIN1 | 1.550 | Q04721 | NOTCH2 | 1.342 |
| Q6ZT12 | UBR3 | 1.537 | O75122 | CLASP2 | 1.341 |
| P57076 | C21orf59 | 1.535 | Q9NR50 | EIF2B3 | 1.338 |
| Q14653 | IRF3 | 1.519 | P61421 | ATP6V0D1 | 1.337 |
| Q9NPH3 | IL1RAP | 1.513 | Q8TAE8 | GADD45GIP1 | 1.335 |
| P07992 | ERCC1 | 1.512 | O00139 | KIF2A | 1.332 |
| Q86WA6 | BPHL | 1.506 | O14545 | TRAFD1 | 1.329 |
| Q9NPH0 | ACP6 | 1.498 | Q9BUL8 | PDCD10 | 1.328 |
| Q7Z4S6 | KIF21A | 1.489 | P15927 | RPA2 | 1.328 |
| O75439 | PMPCB | 1.484 | P19387 | POLR2C | 1.325 |
| Q9Y3T9 | NOC2L | 1.484 | Q15796 | SMAD2 | 1.322 |
| Q9Y383 | LUC7L2 | 1.482 | Q86UU1 | PHLDB1 | 1.321 |
| Q6BCY4 | CYB5R2 | 1.481 | P05121 | SERPINE1 | 1.319 |
| P49366 | DHPS | 1.481 | Q8TBQ9 | TMEM167A | 1.319 |
| Q15843 | NEDD8 | 1.473 | Q6ZNE5 | ATG14 | 1.318 |
| Q5MNZ6 | WDR45L | 1.471 | Q96GC9 | VMP1 | 1.317 |
| Q6WCQ1 | MPRIP | 1.469 | P62714 | PPP2CB | 1.312 |
| Q96AX1 | VPS33A | 1.466 | Q9ULV0 | MYO5B | 1.309 |
| Q96GQ7 | DDX27 | 1.464 | Q6ZSR9 | 1 SV | 1.309 |
| P51116 | FXR2 | 1.464 | Q92878 | RAD50 | 1.305 |
| O00499 | BIN1 | 1.464 | Q9UKM9 | RALY | 1.305 |
| Q8WW12 | PCNP | 1.455 | Q9P253 | VPS18 | 1.304 |
| P14923 | JUP | 1.455 | Q8N128 | FAM177A1 | 1.303 |
| P78362 | SRPK2 | 1.452 | Q9UHQ4 | BCAP29 | 1.303 |
| O14976 | GAK | 1.452 | O43765 | SGTA | 1.303 |
| O14524 | TMEM194A | 1.445 | O75152 | ZC3H11A | 1.302 |

**Table S7:** Downregulated proteins detected by iTRAQ analysis upon p62 knockdown in melanoma cell lines.

| SK-Mel-103 | | | | | |
|---|---|---|---|---|---|
| **DOWNREGULATED PROTEINS** | | | | | |
| **Accession** | **Gene name** | **log₂FC (shp62/shcon)** | **Accession** | **Gene name** | **log₂FC (shp62/shcon)** |
| Q9P2R3 | ANKFY1 | 0.767 | Q13287 | NMI | 0.693 |
| Q9BQE5 | APOL2 | 0.767 | Q9UPN7 | PPP6R1 | 0.689 |
| Q6ZTR7 | FAM92B | 0.766 | Q9HC78 | ZBTB20 | 0.687 |
| O43741 | PRKAB2 | 0.766 | Q00587 | CDC42EP1 | 0.687 |
| Q7Z7A4 | PXK | 0.766 | Q9Y3Z3 | SAMHD1 | 0.686 |
| Q8N8A2 | ANKRD44 | 0.766 | Q8IWA0 | WDR75 | 0.685 |
| P43121 | MCAM | 0.766 | Q14592 | ZNF460 | 0.683 |
| O94919 | ENDOD1 | 0.766 | Q06787 | FMR1 | 0.683 |
| Q96RT1 | ERBB2IP | 0.765 | Q9UIQ6 | LNP | 0.682 |
| Q15050 | RRS1 | 0.764 | Q96KB5 | PBK | 0.682 |

| SK-Mel-103 | | | | | |
|---|---|---|---|---|---|
| **DOWNREGULATED PROTEINS** | | | | | |
| **Accession** | **Gene name** | **log₂FC (shp62/shcon)** | **Accession** | **Gene name** | **log₂FC (shp62/shcon)** |
| Q31612 | HLA-B | 0.764 | Q9Y2H2 | INPP5F | 0.682 |
| Q86VI3 | IQGAP3 | 0.763 | Q9NUQ6 | SPATS2L | 0.681 |
| Q14534 | SQLE | 0.763 | Q5SSJ5 | HP1BP3 | 0.680 |
| Q15345 | LRRC41 | 0.762 | P23381 | WARS | 0.680 |
| Q9Y2U8 | LEMD3 | 0.761 | P16298 | PPP3CB | 0.679 |
| Q01082 | SPTBN1 | 0.761 | P06280 | GLA | 0.677 |
| Q9BV79 | MECR | 0.761 | O14519 | CDK2AP1 | 0.677 |
| Q13393 | PLD1 | 0.760 | O95864 | FADS2 | 0.673 |
| Q8N6L1 | KRTCAP2 | 0.760 | P51788 | CLCN2 | 0.673 |
| Q96IV0 | NGLY1 | 0.759 | Q6ICB0 | DESI1 | 0.672 |
| Q63HN8 | RNF213 | 0.759 | P49247 | RPIA | 0.669 |
| Q9BRJ6 | C7orf50 | 0.759 | Q8IYM9 | TRIM22 | 0.667 |
| Q99598 | TSNAX | 0.759 | O00562 | PITPNM1 | 0.665 |
| Q9BXS9 | SLC26A6 | 0.759 | Q6PIJ6 | FBXO38 | 0.661 |
| P12931 | SRC | 0.759 | Q92736 | RYR2 | 0.660 |
| P07196 | NEFL | 0.759 | P0CW20 | LIMS3L | 0.658 |
| A3KMH1 | VWA8 | 0.758 | Q9Y653 | GPR56 | 0.658 |
| Q8TCS8 | PNPT1 | 0.758 | P80217 | IFI35 | 0.653 |
| Q8IWS0 | PHF6 | 0.758 | Q53GL7 | PARP10 | 0.653 |
| Q96EU7 | C1GALT1C1 | 0.758 | Q9Y5Q0 | FADS3 | 0.652 |
| Q9UKL6 | PCTP | 0.757 | O95249 | GOSR1 | 0.651 |
| Q15025 | TNIP1 | 0.757 | Q14202 | ZMYM3 | 0.651 |
| Q12929 | EPS8 | 0.756 | O15554 | KCNN4 | 0.650 |
| Q12765 | SCRN1 | 0.756 | O43493 | TGOLN2 | 0.649 |
| Q8N543 | OGFOD1 | 0.756 | O60307 | MAST3 | 0.648 |
| O00442 | RTCA | 0.756 | Q5D862 | FLG2 | 0.647 |
| Q14693 | LPIN1 | 0.756 | Q8NHU6 | TDRD7 | 0.645 |
| Q92743 | HTRA1 | 0.755 | Q9NYZ1 | FAM18B1 | 0.643 |
| P06396 | GSN | 0.755 | Q13438 | OS9 | 0.643 |
| P43360 | MAGEA6 | 0.755 | P30281 | CCND3 | 0.642 |
| Q9ULT8 | HECTD1 | 0.755 | Q9Y5A7 | NUB1 | 0.640 |
| P46976 | GYG1 | 0.754 | P40306 | PSMB10 | 0.640 |
| P07996 | THBS1 | 0.754 | Q9NUG6 | PDRG1 | 0.640 |
| Q16666 | IFI16 | 0.754 | P19474 | TRIM21 | 0.638 |
| Q8TEL6 | TRPC4AP | 0.753 | P36954 | POLR2I | 0.634 |
| P53985 | SLC16A1 | 0.752 | Q9H5Z1 | DHX35 | 0.634 |
| O15234 | CASC3 | 0.751 | Q15417 | CNN3 | 0.632 |
| Q5T3I0 | GPATCH4 | 0.751 | P19525 | EIF2AK2 | 0.631 |
| A0AVT1 | UBA6 | 0.751 | Q16222 | UAP1 | 0.630 |
| Q68D91 | MBLAC2 | 0.751 | P32004 | L1CAM | 0.629 |
| Q05193 | DNM1 | 0.750 | Q5K651 | SAMD9 | 0.628 |
| Q99538 | LGMN | 0.749 | P11137 | MAP2 | 0.628 |
| Q9UBV2 | SEL1L | 0.749 | Q9Y3R5 | DOY2 | 0.628 |
| P51884 | LUM | 0.748 | Q9UBT7 | CTNNAL1 | 0.625 |
| O00767 | SCD | 0.747 | Q9Y6K5 | OAS3 | 0.625 |
| Q9BRP1 | PDCD2L | 0.746 | Q9BRQ6 | CHCHD6 | 0.622 |
| Q9H7E9 | C8orf33 | 0.746 | Q96AC1 | FERMT2 | 0.618 |
| Q03135 | CAV1 | 0.744 | Q92624 | APPBP2 | 0.617 |
| P60602 | ROMO1 | 0.743 | P05121 | SERPINE1 | 0.613 |
| P30460 | HLA-B | 0.740 | O75955 | FLOT1 | 0.610 |
| Q9BSU1 | C16orf70 | 0.739 | Q9Y2Z2 | MTO1 | 0.607 |
| Q9BTE7 | DCUN1D5 | 0.738 | Q9C0D9 | EPT1 | 0.604 |
| Q8TB03 | CXorf38 | 0.737 | Q8IXQ6 | PARP9 | 0.604 |
| Q8NBM8 | PCYOX1L | 0.737 | Q460N5 | PARP14 | 0.601 |
| Q9H814 | PHAX | 0.737 | O00418 | EEF2K | 0.598 |
| Q9NWS0 | PIH1D1 | 0.735 | O75886 | STAM2 | 0.596 |
| O43592 | XPOT | 0.735 | Q9NQ25 | SLAMF7 | 0.594 |
| P69905 | HBA1 | 0.734 | Q9NY97 | B3GNT2 | 0.589 |
| Q9NZR1 | TMOD2 | 0.733 | Q96EN8 | MOCOS | 0.588 |
| A1X283 | SH3PXD2B | 0.733 | P06899 | HIST1H2BJ | 0.587 |
| Q9H6X4 | TMEM134 | 0.733 | Q8TDB6 | DTX3L | 0.582 |
| O00472 | ELL2 | 0.732 | P08138 | NGFR | 0.582 |
| Q8NC69 | KCTD6 | 0.732 | Q15800 | MSMO1 | 0.578 |
| Q15631 | TSN | 0.730 | Q99541 | PLIN2 | 0.578 |
| Q9Y5K6 | CD2AP | 0.729 | O60814 | HIST1H2BK | 0.576 |
| P09455 | RBP1 | 0.729 | Q9HCJ6 | VAT1L | 0.575 |
| P28838 | LAP3 | 0.729 | P30508 | HLA-C | 0.569 |
| Q96S19 | C16orf13 | 0.728 | Q14699 | RFTN1 | 0.562 |

| SK-Mel-103 | | | | | |
|---|---|---|---|---|---|
| **DOWNREGULATED PROTEINS** | | | | | |
| **Accession** | **Gene name** | **log₂FC (shp62/shcon)** | **Accession** | **Gene name** | **log₂FC (shp62/shcon)** |
| Q96EP0 | RNF31 | 0.728 | P42574 | CASP3 | 0.562 |
| P38159 | RBMX | 0.728 | Q8IYT2 | FTSJD1 | 0.560 |
| Q96D46 | NMD3 | 0.728 | Q8N9M5 | TMEM102 | 0.560 |
| Q9H6R7 | C2orf44 | 0.725 | P48723 | HSPA13 | 0.559 |
| Q8WWB7 | C1orf85 | 0.724 | Q6PIU2 | NCEH1 | 0.551 |
| Q96BN2 | TADA1 | 0.723 | Q9BYK8 | PRIC285 | 0.541 |
| A0JNW5 | UHRF1BP1L | 0.721 | Q9UMR5 | PPT2 | 0.541 |
| O14763 | TNFRSF10B | 0.721 | Q9Y2V0 | C15orf41 | 0.535 |
| Q49A26 | GLYR1 | 0.719 | Q9NUL5 | C19orf66 | 0.531 |
| Q7L5N7 | LPCAT2 | 0.719 | Q13501 | SQSTM1 | 0.525 |
| Q9P0R6 | GSKIP | 0.719 | O94856 | NFASC | 0.521 |
| Q6P996 | PDXDC1 | 0.719 | O95786 | DDX58 | 0.519 |
| P55265 | ADAR | 0.717 | O14933 | UBE2L6 | 0.519 |
| P23229 | ITGA6 | 0.716 | P61960 | UFM1 | 0.506 |
| Q03518 | TAP1 | 0.716 | Q5QJ74 | TBCEL | 0.501 |
| Q9UDT6 | CLIP2 | 0.716 | P42224 | STAT1 | 0.492 |
| Q13938 | CAPS | 0.714 | P05161 | ISG15 | 0.486 |
| Q8N2F6 | ARMC10 | 0.713 | P41226 | UBA7 | 0.485 |
| O95365 | ZBTB7A | 0.713 | Q9BPU6 | DPYSL5 | 0.481 |
| Q96JQ2 | CLMN | 0.711 | P52630 | STAT2 | 0.474 |
| O00635 | TRIM38 | 0.710 | P35610 | SOAT1 | 0.474 |
| Q9H330 | TMEM245 | 0.710 | Q7Z417 | NUFIP2 | 0.463 |
| Q15785 | TOMM34 | 0.708 | Q9P2E3 | ZNFX1 | 0.460 |
| Q2VPB7 | AP5B1 | 0.708 | P32455 | GBP1 | 0.456 |
| Q08AF3 | SLFN5 | 0.706 | Q13325 | IFIT5 | 0.446 |
| Q9H0P0 | NT5C3 | 0.705 | P62805 | HIST1H4A | 0.424 |
| Q96JG6 | CCDC132 | 0.704 | Q8TCB0 | IFI44 | 0.383 |
| Q9Y3C0 | CCDC53 | 0.704 | Q8N4J0 | C9orf41 | 0.382 |
| Q96MU7 | YTHDC1 | 0.702 | P61587 | RND3 | 0.374 |
| P00750 | PLAT | 0.701 | O15162 | PLSCR1 | 0.318 |
| P61769 | B2M | 0.700 | O14879 | IFIT3 | 0.267 |
| O95210 | STBD1 | 0.700 | Q9NQC7 | CYLD | 0.258 |
| Q9BZQ8 | FAM129A | 0.698 | P09914 | IFIT1 | 0.217 |
| Q9BTE3 | MCMBP | 0.697 | Q86UL8 | MAGI2 | 0.210 |
| P16190 | HLA-A | 0.696 | P09913 | IFIT2 | 0.118 |
| P09884 | POLA1 | 0.694 | | | |

| UACC-62 | | | | | |
|---|---|---|---|---|---|
| **DOWNREGULATED PROTEINS** | | | | | |
| **Accession** | **Gene name** | **log₂FC (shp62/shcon)** | **Accession** | **Gene name** | **log₂FC (shp62/shcon)** |
| Q86U38 | NOP9 | 0.768 | P82914 | MRPS15 | 0.714 |
| P53634 | CTSC | 0.767 | Q7Z417 | NUFIP2 | 0.712 |
| P04271 | S100B | 0.767 | O43592 | XPOT | 0.712 |
| P33897 | ABCD1 | 0.767 | Q9Y653 | GPR56 | 0.710 |
| P01011 | SERPINA3 | 0.765 | O43719 | HTATSF1 | 0.709 |
| O15541 | RNF113A | 0.764 | P27338 | MAOB | 0.709 |
| P61604 | HS1 | 0.764 | P37840 | SNCA | 0.703 |
| Q8NHU6 | TDRD7 | 0.764 | P16403 | HIST1H1C | 0.703 |
| Q9BX68 | HINT2 | 0.764 | Q9UBD5 | ORC3 | 0.701 |
| P05556 | ITGB1 | 0.763 | Q15050 | RRS1 | 0.700 |
| P34059 | GALNS | 0.763 | P20338 | RAB4A | 0.699 |
| Q9UBB9 | TFIP11 | 0.762 | Q96RF0 | SNX18 | 0.699 |
| Q8N511 | TMEM199 | 0.762 | Q8WTV0 | SCARB1 | 0.697 |
| Q9NZJ4 | SACS | 0.761 | Q9Y250 | LZTS1 | 0.691 |
| O95070 | YIF1A | 0.761 | O95249 | GOSR1 | 0.691 |
| Q9Y5U9 | IER3IP1 | 0.761 | O00762 | UBE2C | 0.689 |
| Q969X5 | ERGIC1 | 0.759 | Q9H2J7 | SLC6A15 | 0.686 |
| P57088 | TMEM33 | 0.759 | Q03154 | ACY1 | 0.683 |
| P41240 | CSK | 0.757 | Q9BW72 | HIGD2A | 0.683 |
| O95149 | SNUPN | 0.757 | Q6PIU2 | NCEH1 | 0.683 |
| Q9UJW0 | DCTN4 | 0.757 | Q96PU8 | QKI | 0.681 |
| Q8WUA2 | PPIL4 | 0.756 | Q9NVR2 | INTS10 | 0.681 |
| Q9NW82 | WDR70 | 0.756 | P53675 | CLTCL1 | 0.679 |
| Q8WW59 | SPRYD4 | 0.755 | P46976 | GYG1 | 0.677 |
| Q7Z333 | SETX | 0.755 | Q9Y6W5 | WASF2 | 0.675 |
| Q96D46 | NMD3 | 0.754 | P23229 | ITGA6 | 0.672 |
| Q96J42 | TXNDC15 | 0.754 | Q99538 | LGMN | 0.670 |

| UACC-62 | | | | | |
|---|---|---|---|---|---|
| **DOWNREGULATED PROTEINS** | | | | | |
| **Accession** | **Gene name** | **log₂FC (shp62/shcon)** | **Accession** | **Gene name** | **log₂FC (shp62/shcon)** |
| Q9Y606 | PUS1 | 0.754 | Q14146 | URB2 | 0.663 |
| Q15005 | SPCS2 | 0.753 | Q6P996 | PDXDC1 | 0.663 |
| Q969E4 | TCEAL3 | 0.753 | O60443 | DFNA5 | 0.661 |
| O14773 | TPP1 | 0.753 | Q9Y5B9 | SUPT16H | 0.655 |
| Q86WB0 | ZC3HC1 | 0.752 | Q9BV36 | MLPH | 0.654 |
| Q6RW13 | AGTRAP | 0.751 | Q6P2C8 | MED27 | 0.650 |
| P61513 | RPL37A | 0.750 | Q9H4A3 | WNK1 | 0.649 |
| Q15785 | TOMM34 | 0.749 | Q9UBM4 | OPTC | 0.644 |
| Q7Z739 | YTHDF3 | 0.749 | Q9C0D9 | EPT1 | 0.641 |
| O43847 | NRD1 | 0.747 | Q9NRL2 | BAZ1A | 0.641 |
| Q92743 | HTRA1 | 0.747 | Q9NVN8 | GNL3L | 0.640 |
| Q9BY43 | CHMP4A | 0.747 | Q16222 | UAP1 | 0.636 |
| Q15631 | TSN | 0.746 | Q6ICB0 | DESI1 | 0.631 |
| Q86VU5 | COMTD1 | 0.746 | P55795 | HNRNPH2 | 0.630 |
| Q6L8Q7 | PDE12 | 0.745 | P49247 | RPIA | 0.630 |
| Q9H6W3 | NO66 | 0.743 | Q9H0W9 | C11orf54 | 0.629 |
| Q8N131 | TMEM123 | 0.742 | Q9H814 | PHAX | 0.627 |
| Q9Y3A2 | UTP11L | 0.742 | Q8WU79 | SMAP2 | 0.623 |
| P15586 | GNS | 0.741 | P49711 | CTCF | 0.623 |
| O43776 | NARS | 0.740 | Q96AC1 | FERMT2 | 0.616 |
| P62341 | SELT | 0.740 | P11388 | TOP2A | 0.615 |
| Q8N6V9 | TEX9 | 0.739 | Q96E52 | OMA1 | 0.610 |
| O14497 | ARID1A | 0.737 | Q8NB46 | ANKRD52 | 0.603 |
| Q12765 | SCRN1 | 0.736 | P27694 | RPA1 | 0.594 |
| Q9P2Q2 | FRMD4A | 0.735 | P33552 | CKS2 | 0.592 |
| P43360 | MAGEA6 | 0.729 | Q9BUP3 | HTATIP2 | 0.588 |
| Q9P1U0 | ZNRD1 | 0.727 | Q9UDW1 | UQCR10 | 0.585 |
| Q96SZ5 | ADO | 0.726 | Q96KQ7 | EHMT2 | 0.583 |
| Q96GQ5 | C16orf58 | 0.726 | P51692 | STAT5B | 0.583 |
| Q9BRX2 | LO | 0.726 | Q5TAQ9 | DCAF8 | 0.567 |
| P53367 | ARFIP1 | 0.726 | Q330K2 | NDUFAF6 | 0.563 |
| Q5J8M3 | EMC4 | 0.725 | Q9UNN8 | PROCR | 0.555 |
| O60518 | RANBP6 | 0.725 | Q9Y5Q0 | FADS3 | 0.527 |
| Q99598 | TSNAX | 0.724 | Q14739 | LBR | 0.519 |
| Q9BU61 | NDUFAF3 | 0.723 | P42574 | CASP3 | 0.498 |
| O60671 | RAD1 | 0.723 | Q13510 | ASAH1 | 0.492 |
| Q9H8Y8 | GORASP2 | 0.723 | Q8N2G6 | ZCCHC24 | 0.476 |
| P61163 | ACTR1A | 0.722 | P48426 | PIP4K2A | 0.470 |
| Q86YV9 | HPS6 | 0.720 | Q8TDX7 | NEK7 | 0.459 |
| O43172 | PRPF4 | 0.719 | P48723 | HSPA13 | 0.456 |
| P02794 | FTH1 | 0.718 | Q8N4J0 | C9orf41 | 0.451 |
| Q9Y6Y0 | IVNS1ABP | 0.717 | P24043 | LAMA2 | 0.450 |
| P82675 | MRPS5 | 0.717 | Q6GQQ9 | OTUD7B | 0.424 |
| Q5VWP3 | MLIP | 0.717 | P50443 | SLC26A2 | 0.397 |
| P51608 | MECP2 | 0.714 | Q13501 | SQSTM1 | 0.284 |

**Table S8:** Upregulated proteins detected by iTRAQ analysis upon ATG5 knockdown in melanoma cell lines.

| SK-Mel-103 | | | | | |
|---|---|---|---|---|---|
| **UPREGULATED PROTEINS** | | | | | |
| **Accession** | **Gene name** | **log₂FC (siATG5/shcon)** | **Accession** | **Gene name** | **log₂FC (siATG5/shcon)** |
| Q92581-3 | SLC9A6 | 3.006 | H3BPR7 | TYRO3 | 1.410 |
| Q8TD22 | SFXN5 | 2.028 | A6NM62 | LRRC53 | 1.407 |
| G3V0H6 | NARG2 | 2.003 | B4DGV4 | RYR2 | 1.403 |
| Q6ZTA4-2 | TRIM67 | 1.996 | Q8NI37 | PPTC7 | 1.397 |
| Q9UBN7-2 | HDAC6 | 1.901 | O15504 | NUPL2 | 1.390 |
| A8MZ59 | LEUTX | 1.859 | O00429-4 | DNM1L | 1.382 |
| Q9BV19 | C1orf50 | 1.745 | B4E3L6 | D2HGDH | 1.381 |
| Q92871 | PMM1 | 1.693 | Q9NUH8-2 | TMEM14B | 1.374 |
| P35527 | KRT9 | 1.689 | P53675-2 | CLTCL1 | 1.371 |
| Q8WXG9-3 | GPR98 | 1.651 | C9JZR9 | SLC30A6 | 1.370 |
| Q8NBP0-2 | TTC13 | 1.641 | P02533 | KRT14 | 1.367 |
| F8WEM9 | ZNF655 | 1.592 | Q9Y248 | GINS2 | 1.364 |
| D6RJA4 | AMOTL2 | 1.586 | P60660 | MYL6 | 1.364 |
| Q9NZ71-5 | RTEL1 | 1.584 | E9PPF2 | TYK2 | 1.363 |
| F8W036 | 2 | 1.583 | P20749 | BCL3 | 1.361 |

| SK-Mel-103 | | | | | |
|---|---|---|---|---|---|
| **UPREGULATED PROTEINS** | | | | | |
| Accession | Gene name | log$_2$FC (siATG5/shcon) | Accession | Gene name | log$_2$FC (siATG5/shcon) |
| Q9H2H9 | SLC38A1 | 1.570 | Q9Y3Y2-4 | CHTOP | 1.361 |
| Q68DH5 | LMBRD2 | 1.541 | Q9BYC5-3 | FUT8 | 1.354 |
| Q9BWW5 | SSDP4 | 1.528 | P10412 | HIST1H1E | 1.350 |
| C9J9V6 | NME6 | 1.501 | H3BTL1 | MAP1LC3B | 1.348 |
| Q8WWB7-2 | C1orf85 | 1.498 | Q96BR1-2 | SGK3 | 1.347 |
| Q9NS91 | RAD18 | 1.493 | Q9NVN3-3 | RIC8B | 1.342 |
| Q16384 | SSX1 | 1.491 | E7ET89 | DTX2 | 1.341 |
| P33552 | CKS2 | 1.488 | Q8NB46 | ANKRD52 | 1.336 |
| O15069 | NACAD | 1.488 | B4DJJ3 | TAPT1 | 1.334 |
| D3DWC4 | TMEM23 | 1.488 | Q05D32-2 | CTDSPL2 | 1.331 |
| Q71DI3 | HIST2H3A | 1.487 | O43854-2 | EDIL3 | 1.331 |
| A6NDU8 | C5orf51 | 1.480 | Q9NSY1 | BMP2K | 1.324 |
| Q7L0J3-2 | SV2A | 1.474 | Q9BZM4 | ULBP3 | 1.324 |
| Q86SX6 | GLRX5 | 1.460 | Q9Y233 | PDE10A | 1.322 |
| A8MUN2 | APOB | 1.439 | Q9NZD2 | GLTP | 1.321 |
| P07585 | DCN | 1.438 | Q96HY7 | DHTKD1 | 1.316 |
| Q9NQG6 | SMCR7L | 1.433 | E9PP15 | LRP8 | 1.312 |
| C9JEZ4 | CDC42EP3 | 1.433 | O60725 | ICMT | 1.311 |
| C9J5B3 | KCTD20 | 1.432 | Q9NP73-2 | ALG13 | 1.311 |
| Q8IWT0 | ZBTB8OS | 1.427 | H3BVI5 | RHOT2 | 1.311 |
| Q96IV0-3 | NGLY1 | 1.425 | Q9NR31 | SAR1A | 1.308 |
| H7C0U0 | PARL | 1.423 | Q12913 | PTPRJ | 1.306 |
| P12259 | F5 | 1.419 | Q9BV23 | ABHD6 | 1.304 |
| Q96S97 | MYADM | 1.414 | Q96DX7 | TRIM44 | 1.302 |
| Q86UT6-2 | NLRX1 | 1.411 | P62745 | RHOB | 1.300 |

| UACC-62 | | | | | |
|---|---|---|---|---|---|
| **UPREGULATED PROTEINS** | | | | | |
| Accession | Gene name | log$_2$FC (siATG5/shcon) | Accession | Gene name | log$_2$FC (siATG5/shcon) |
| P35527 | KRT9 | 5.091 | P51397 | DAP | 1.403 |
| Q9UQL6 | HDAC5 | 2.742 | P51512 | MMP16 | 1.402 |
| P02533 | KRT14 | 2.333 | P08582-2 | MFI2 | 1.398 |
| P14859-3 | POU2F1 | 2.283 | F8VUA5 | RAB5B | 1.397 |
| Q15366-6 | PCBP2 | 2.001 | H0YL93 | FMN1 | 1.392 |
| Q95IE3 | HLA-DRB1 | 1.922 | E9PPF2 | TYK2 | 1.391 |
| P59542 | TAS2R19 | 1.915 | P16402 | HIST1H1D | 1.385 |
| P04264 | KRT1 | 1.895 | A5A3E0 | POTEF | 1.380 |
| Q4G0P3-2 | HYDIN | 1.838 | Q9HCE3 | ZNF532 | 1.375 |
| B1AZU4 | HLA-F | 1.815 | Q08AT0 | SGCZ | 1.372 |
| E7ENQ1 | MAP4K4 | 1.805 | Q9BWW5 | SSDP4 | 1.370 |
| Q4VC05 | BCL7A | 1.794 | Q12769-3 | NUP160 | 1.368 |
| O14893-2 | GEMIN2 | 1.787 | Q9NQS3 | PVRL3 | 1.367 |
| Q76I76 | SSH2 | 1.744 | A8MXF6 | RABL2B | 1.360 |
| Q9BQQ3 | GORASP1 | 1.690 | Q9NRF2-2 | SH2B1 | 1.359 |
| Q13015 | MLLT11 | 1.655 | H7C1Q1 | 2 | 1.357 |
| Q9Y248 | GINS2 | 1.650 | P17535 | JUND | 1.354 |
| Q9UPN3 | MACF1 | 1.631 | P25440 | BRD2 | 1.341 |
| F8WEM9 | ZNF655 | 1.614 | P17066 | HSPA6 | 1.339 |
| K7EJS5 | MFSD12 | 1.564 | Q9UMN6 | WBP7 | 1.337 |
| P61601 | NCALD | 1.549 | E9PDF6 | MYO1B | 1.336 |
| Q6T4R5-4 | NHS | 1.547 | Q9NTU7 | CBLN4 | 1.333 |
| O00329 | PIK3CD | 1.542 | Q7L7V1-2 | DHX32 | 1.332 |
| P13284 | IFI30 | 1.536 | Q8TD30 | GPT2 | 1.329 |
| D6RGY8 | YIPF3 | 1.524 | H0Y853 | WDR52 | 1.327 |
| H7C4F8 | CD86 | 1.518 | Q9BVS4-2 | RIOK2 | 1.325 |
| C4AMC7 | WASH3P | 1.492 | O43854-2 | EDIL3 | 1.324 |
| O76061 | STC2 | 1.468 | Q6NTF9 | RHBDD2 | 1.322 |
| M0QX48 | EPHX3 | 1.464 | E9PSI1 | TM9SF1 | 1.313 |
| Q9Y2S2-2 | CRYL1 | 1.451 | P12074 | COX6A1 | 1.312 |
| Q9HCJ6 | VAT1L | 1.448 | Q9NVH2-4 | INTS7 | 1.311 |
| C9J9D7 | SIAH2 | 1.445 | O60218 | AKR1B10 | 1.308 |
| Q2MD36 | MBD4 | 1.442 | P17028 | ZNF24 | 1.307 |
| P58107 | EPPK1 | 1.437 | Q7Z7G8-2 | VPS13B | 1.303 |
| G3V217 | CCDC63 | 1.405 | Q9UGU5 | HMGXB4 | 1.302 |

**Table S9:** Downregulated proteins detected by iTRAQ analysis upon ATG5 knockdown in melanoma cell lines.

| SK-Mel-103 | | | | | |
|---|---|---|---|---|---|
| **DOWNREGULATED PROTEINS** | | | | | |
| Accession | Gene name | log₂FC (siATG5/shcon) | Accession | Gene name | log₂FC (siATG5/shcon) |
| Q5JTQ6 | CTNNAL1 | 0.457 | E9PSH3 | TSPAN4 | 0.703 |
| B4E0J7 | RRN3 | 0.466 | O43194 | GPR39 | 0.714 |
| C9J473 | MPV17 | 0.485 | Q8TBN0-2 | RAB3IL1 | 0.714 |
| E5RFF3 | TRPS1 | 0.525 | O14556 | GAPDHS | 0.717 |
| E7ER97 | ASMTL | 0.537 | Q96AC1 | FERMT2 | 0.717 |
| Q9H1Y0-2 | ATG5 | 0.554 | Q9NZJ4 | SACS | 0.718 |
| Q13495-4 | MAMLD1 | 0.565 | P05120 | SERPINB2 | 0.720 |
| B7ZKP8 | UBXN2A | 0.568 | Q14676-3 | MDC1 | 0.720 |
| O94817 | ATG12 | 0.577 | Q16739 | UGCG | 0.722 |
| E7ENQ1 | MAP4K4 | 0.579 | H0Y9R9 | IDUA | 0.723 |
| Q9H469 | FBXL15 | 0.594 | E7EMN6 | PPP1R2 | 0.728 |
| Q8N3X1 | FNBP4 | 0.607 | P15104 | GLUL | 0.728 |
| Q8IYT2 | FTSJD1 | 0.608 | Q9P0S9 | TMEM14C | 0.729 |
| Q8IYQ7 | THNSL1 | 0.609 | D3DR31 | IFIT1 | 0.731 |
| Q58FG1 | HSP90AA4P | 0.623 | E5RG63 | PSEN2 | 0.735 |
| Q15173-2 | PPP2R5B | 0.628 | P12532 | CKMT1A | 0.736 |
| Q8TCZ2-3 | CD99L2 | 0.632 | Q9Y2I1 | NISCH | 0.737 |
| Q8NHU2-4 | C20orf26 | 0.635 | Q15366-6 | PCBP2 | 0.740 |
| P09913 | IFIT2 | 0.637 | Q5SW96 | LDLRAP1 | 0.740 |
| Q99675 | CGRRF1 | 0.639 | Q5T0D9 | TPRG1L | 0.743 |
| O14578-3 | CIT | 0.640 | P53985 | SLC16A1 | 0.743 |
| P12074 | COX6A1 | 0.641 | C9J0J0 | CCDC126 | 0.748 |
| P38432 | COIL | 0.641 | Q9H7E2-2 | TDRD3 | 0.749 |
| A8MX49 | PPM1F | 0.641 | B3KRF7 | ZKSCAN1 | 0.749 |
| O95785 | WIZ | 0.642 | P58511 | SMIM11 | 0.749 |
| I3L196 | ARL16 | 0.644 | D6RC69 | CEP120 | 0.749 |
| Q01518-2 | CAP1 | 0.645 | Q9BYT5 | KRTAP2-2 | 0.750 |
| E9PCV0 | GUSB | 0.649 | O75600 | GCAT | 0.750 |
| J3KN27 | FBRSL1 | 0.650 | H7C2M2 | POLM | 0.750 |
| B4E2D5 | SUPT20H | 0.652 | P25445-6 | FAS | 0.750 |
| Q9UI10-3 | EIF2B4 | 0.653 | Q9UPS8-2 | ANKRD26 | 0.750 |
| P58107 | EPPK1 | 0.653 | Q9UNW1 | MINPP1 | 0.751 |
| Q9UJU3-2 | ZNF112 | 0.654 | B8ZZR0 | CLK1 | 0.753 |
| F5GYA1 | CAPRIN2 | 0.658 | P02790 | HPX | 0.754 |
| Q96SI9-2 | STRBP | 0.661 | K7EIN1 | WBP2 | 0.754 |
| K7ERE1 | 4 | 0.662 | Q8IYM9-2 | TRIM22 | 0.755 |
| Q9P1Y5 | CAMSAP3 | 0.667 | Q3MIP1 | ITPRIPL2 | 0.755 |
| Q58FF3 | HSP90B2P | 0.669 | Q6UWH4-3 | FAM198B | 0.755 |
| H3BRL3 | UBFD1 | 0.670 | Q02156 | PRKCE | 0.758 |
| Q08345 | DDR1 | 0.672 | O60502 | MGEA5 | 0.759 |
| Q676U5-3 | ATG16L1 | 0.675 | C9JKF1 | SAMD9 | 0.759 |
| F5H4R4 | DCP1B | 0.677 | G3V1U5 | GOLT1B | 0.759 |
| Q8WWQ0 | PHIP | 0.679 | C9JMQ9 | KLHL24 | 0.759 |
| Q8N1W1-4 | ARHGEF28 | 0.679 | B4DN24 | FANCL | 0.760 |
| Q8NDV7-2 | TNRC6A | 0.679 | Q6GYQ0-4 | RALGAPA1 | 0.760 |
| K7EJS5 | MFSD12 | 0.680 | Q12959-4 | DLG1 | 0.760 |
| P43356 | MAGEA2 | 0.681 | O14933 | UBE2L6 | 0.760 |
| G3V1V8 | MYL2 | 0.681 | Q9NQL2 | RRAGD | 0.760 |
| H7BXR5 | SNX29 | 0.681 | Q9Y2F5 | KIAA0947 | 0.762 |
| Q8IVT1 | PGPEP1 | 0.685 | Q9NR16-2 | CD163L1 | 0.764 |
| Q9Y6V0-2 | PCLO | 0.688 | Q9H2G4 | TSPYL2 | 0.764 |
| Q5EBL4-2 | RILPL1 | 0.688 | P82909 | MRPS36 | 0.764 |
| P54646 | PRKAA2 | 0.694 | Q15434 | RBMS2 | 0.764 |
| Q15651 | HMGN3 | 0.694 | Q9Y3Q8 | TSC22D4 | 0.765 |
| Q9Y6R7 | FCGBP | 0.696 | Q6P1X5 | TAF2 | 0.766 |
| Q9Y289 | SLC5A6 | 0.700 | Q96EH3 | MALSU1 | 0.769 |
| Q96DX8 | RTP4 | 0.702 | | | |

| UACC-62 | | | | | |
|---|---|---|---|---|---|
| **DOWNREGULATED PROTEINS** | | | | | |
| Accession | Gene name | log₂FC (siATG5/shcon) | Accession | Gene name | log₂FC (siATG5/shcon) |
| Q330K2-2 | NDUFAF6 | 0.413 | Q9Y3R5-2 | DOPEY2 | 0.715 |
| E9PBG7 | CAMK2D | 0.415 | Q9NQN1 | OR2S2 | 0.715 |
| Q0VDD8-3 | DNAH14 | 0.433 | Q9NQA3 | WASH6P | 0.718 |
| C9J9V6 | NME6 | 0.475 | Q8N1W1-4 | ARHGEF28 | 0.719 |

| UACC-62 | | | | | |
|---|---|---|---|---|---|
| **DOWNREGULATED PROTEINS** | | | | | |
| **Accession** | **Gene name** | **log$_2$FC (siATG5/shcon)** | **Accession** | **Gene name** | **log$_2$FC (siATG5/shcon)** |
| O94817 | ATG12 | 0.496 | F5H5W6 | DDX58 | 0.720 |
| Q8TD22 | SFXN5 | 0.497 | Q9H4E5-2 | RHOJ | 0.721 |
| Q13495-4 | MAMLD1 | 0.532 | Q5XG87 | PAPD7 | 0.724 |
| Q9UGJ0-2 | PRKAG2 | 0.535 | O14578-3 | CIT | 0.724 |
| K7EQ93 | UNK | 0.540 | A8MU44 | HOOK1 | 0.725 |
| K7ES52 | TK1 | 0.596 | P57088 | TMEM33 | 0.725 |
| Q5T0D9 | TPRG1L | 0.599 | P07108 | DBI | 0.727 |
| Q9ULP9-2 | TBC1D24 | 0.615 | Q9Y5N5-2 | N6AMT1 | 0.728 |
| Q9H1Y0-2 | ATG5 | 0.615 | Q14676-3 | MDC1 | 0.729 |
| Q676U5-3 | ATG16L1 | 0.616 | D6RD42 | MLIP | 0.730 |
| B3KU92 | LOC90826 | 0.617 | P78358-2 | CTAG1A | 0.731 |
| Q8N9M5 | TMEM102 | 0.626 | Q08345 | DDR1 | 0.731 |
| Q9Y289 | SLC5A6 | 0.627 | Q9UBX3 | SLC25A10 | 0.731 |
| K7EIN1 | WBP2 | 0.631 | Q9NY84-3 | VNN3 | 0.732 |
| P07602-2 | PSAP | 0.639 | O43194 | GPR39 | 0.737 |
| Q6N069 | NAA16 | 0.640 | Q8NHU2-4 | C20orf26 | 0.739 |
| Q13424 | SNTA1 | 0.648 | Q6N075 | MFSD5 | 0.740 |
| Q6EEV4-2 | POLR2M | 0.654 | Q8IYQ7 | THNSL1 | 0.741 |
| A0JNW5 | UHRF1BP1L | 0.656 | Q9H334-2 | FOXP1 | 0.743 |
| Q8IYT2 | FTSJD1 | 0.658 | Q07020 | RPL18 | 0.743 |
| H0Y7G7 | HLA-DPB1 | 0.660 | O75487 | GPC4 | 0.745 |
| Q13433 | SLC39A6 | 0.664 | Q9P086 | MED11 | 0.746 |
| Q86V85 | GPR180 | 0.670 | Q9UIL1-3 | SCOC | 0.748 |
| Q9NPG3-2 | UBN1 | 0.679 | A6NN40 | SHROOM1 | 0.750 |
| Q96SI9-2 | STRBP | 0.685 | H0YLN8 | TRPM7 | 0.750 |
| Q92994 | BRF1 | 0.686 | F5H050 | DYNC1I1 | 0.752 |
| K7ENR6 | PSMG2 | 0.689 | Q96T88 | UHRF1 | 0.754 |
| Q9BZG9-3 | LYNX1 | 0.689 | B4DWK3 | TFAP2C | 0.758 |
| Q9NQ34 | TMEM9B | 0.692 | K7ENE2 | GID4 | 0.758 |
| O14879 | IFIT3 | 0.695 | F5H5D4 | PAAF1 | 0.758 |
| Q8N2Y8 | RUSC2 | 0.696 | B1AHG3 | PRR5 | 0.759 |
| Q69YL0 | 4 | 0.698 | Q9H617 | TMEM164 | 0.759 |
| Q9ULX9-2 | MAFF | 0.700 | J3KST3 | GLTPD1 | 0.760 |
| H3BRL3 | UBFD1 | 0.701 | F8W036 | 2 | 0.760 |
| Q9H0F6-2 | SHARPIN | 0.703 | E7EMN6 | PPP1R2 | 0.761 |
| B7ZKP8 | UBXN2A | 0.704 | D3DWC4 | TMEM23 | 0.762 |
| Q99808 | SLC29A1 | 0.708 | Q86X10-2 | RALGAPB | 0.763 |
| E9PB91 | TIMM23 | 0.709 | B4DXS1 | NMT2 | 0.763 |
| Q8NDV7-2 | TNRC6A | 0.709 | C9JSE3 | FANCG | 0.764 |
| Q6PID8-2 | KLHDC10 | 0.710 | K7EK07 | H3F3B | 0.764 |
| J3KPS2 | FAM83H | 0.710 | K7ERE1 | 4 | 0.767 |
| Q96HN2-3 | AHCYL2 | 0.712 | Q9Y385 | UBE2J1 | 0.768 |
| E9PCV0 | GUSB | 0.713 | P05161 | ISG15 | 0.769 |
| F6RY50 | SIPA1 | 0.713 | H3BPT0 | CMC2 | 0.769 |
| P53794 | SLC5A3 | 0.714 | | | |

**Table S10.** Quantification of FERMT2 immunostaining in tissue microarrays of melanocytic tumors.

| **Melanocytic tumors** | **FERMT2 negative n (%)** | **FERMT2 low n (%)** | **FERMT2 high n (%)** | **Total n** |
|---|---|---|---|---|
| Dermal nevi | 28 (82.4%) | 5 (14.7%) | 1 (2.9%) | 34 |
| VGP melanomas | 20 (24.4%) | 24 (29.3%) | 31 (37.8%) | 82 |
| Cutaneous metastases | 9 (27.3%) | 8 (24.2%) | 16 (48.5%) | 33 |
| Lymph node metastases | 7 (25.0%) | 14 (50.0%) | 7 (25.0%) | 28 |
| Visceral metastases | 4 (18.2%) | 13 (59.1%) | 5 (22.7%) | 22 |
| **Total** | 68 (34.2%) | 64 (32.2%) | 60 (30.2%) | 199 |

**Table S11.** Clinical and histological data of melanoma cases included in the study of FERMT2 immunostaining (total n=82).

| Age (years) | 25–89 (mean 58.88) |
|---|---|
| **Gender** | **n (%)** |
| Female | 43 (52.44%) |
| Male | 39 (47.56%) |
| **Region of primary melanoma** | **n (%)** |
| Trunk | 24 (29.27%) |
| Limbs | 27 (32.93 %) |
| Hands and feet | 16 (19.51%) |
| Head and neck | 12 (14.63%) |
| NA | 3 (3.66%) |
| **Histological type of melanoma** | **n (%)** |
| Superficial spreading | 40 (48.78%) |
| Nodular | 17 (20.73%) |
| Acral | 19 (23.17%) |
| Lentigo maligna | 4 (4.88%) |
| NA | 2 (2.44%) |
| **Breslow thickness (mm)** | 0.4–19 (mean 3.68) |
| **Clark level** | **n (%)** |
| I | 0 (0%) |
| II | 3 (3.66%) |
| III | 32 (39.02%) |
| IV | 33 (40.24%) |
| V | 10 (12.20%) |
| NA | 4 (4.88%) |
| **Ulceration status** | **n (%)** |
| Not ulcerated | 44 (53.66%) |
| Ulcerated | 37 (45.12%) |
| NA | 1 (1.22%) |

(Abbreviation; NA=not available)

**Table S12.** Univariate and multivariate survival analyses using the Cox proportional hazards model in patients with melanoma according to FERMT2 immunostaining.

| Characteristics | Univariate analysis | | | Multivariate analysis | | |
|---|---|---|---|---|---|---|
| **Disease free survival** | **HR** | **95% CI** | **p value** | **HR** | **95% CI** | **p-value** |
| FERMT2 high expression | 1.945 | 1.107-3.417 | 0.021* | 1.845 | 1.040-3.272 | 0.036* |
| Breslow | - | - | - | 1.085 | 0.992-1.187 | 0.073 |
| **Distant metastasis free survival** | **HR** | **95% CI** | **p value** | **HR** | **95% CI** | **p-value** |
| FERMT2 high expression | 2.541 | 1.260-5.126 | 0.009* | 2.357 | 1.163-4.774 | 0.017* |
| Breslow | - | - | - | 1.102 | 0.981-1.238 | 0.100 |
| **Overall survival** | **HR** | **95% CI** | **p value** | **HR** | **95% CI** | **p-value** |
| FERMT2 high expression | 1.882 | 0.986-3.593 | 0.055 | 1.716 | 0.895-3.288 | 0.104 |
| Breslow | - | - | - | 1.157 | 1.043-1.283 | 0.006* |

(HR, hazard ratio. CI, confidence interval. *Significantly different)

**Table S13:** p62 interactors with the corresponding number of peptides identified by co-IP followed by Mass spectrometry in melanoma cell lines.

| Gene Name | p62_CytoREP_01 | p62_CytoREP_02 | p62_CytoREP_03 | p62_NucREP_01 | p62_NucREP_02 | p62_NucREP_03 |
|---|---|---|---|---|---|---|
| SQSTM1 | 175 | 176 | 169 | 45 | 52 | 46 |
| DARS2 | 175 | 171 | 157 | 63 | 58 | 63 |
| UTRN | 84 | 80 | 79 | | | |
| ZC3H4 | 76 | 65 | 67 | 19 | 18 | 20 |
| AZI1 | 73 | 70 | 67 | | | |
| RBM12B | 62 | 58 | 59 | 53 | 50 | 54 |
| ATAD3A | 62 | 58 | 57 | 42 | 40 | 38 |
| ARHGEF18 | 57 | 60 | 48 | | | |
| BCKDK | 52 | 49 | 45 | 4 | 3 | 5 |
| TUBB | 50 | 55 | 50 | 8 | 6 | 4 |
| GBAS | 45 | 48 | 45 | 1 | 2 | 2 |
| TUBB4B | 43 | 42 | 42 | 6 | 4 | 3 |
| RAPGEF2 | 42 | 41 | 39 | | | |
| TAOK1 | 41 | 45 | 41 | | | |
| NIPSNAP1 | 40 | 35 | 39 | | | |
| FN1 | 39 | 47 | 42 | 12 | 11 | 10 |
| TUBB2A | 38 | 41 | 39 | 6 | 5 | 4 |
| CEP350 | 34 | 29 | 29 | | | |
| AHNAK | 33 | 36 | 34 | 18 | 21 | 22 |
| ATAD3B | 32 | 31 | 31 | 22 | 22 | 19 |
| PCNT | 30 | 23 | 29 | | | |
| TUBA1B | 30 | 34 | 30 | 4 | 5 | 3 |
| HADHB | 26 | 28 | 26 | 3 | 4 | 3 |
| SOD3 | 25 | 26 | 24 | 1 | | |
| TUBA1C | 25 | 31 | 29 | 4 | 5 | 3 |
| CDK5RAP2 | 24 | 21 | 30 | | | |
| SLC25A4 | 24 | 28 | 25 | 57 | 59 | 57 |
| GOLGB1 | 22 | 19 | 20 | | | |
| ROBO1 | 22 | 26 | 27 | | | |
| MYO1B | 22 | 24 | 27 | 23 | 26 | 25 |
| WDR82 | 20 | 22 | 19 | 10 | 9 | 12 |
| SMG1 | 18 | 12 | 15 | | | |
| RAI14 | 18 | 11 | 17 | 1 | | 1 |
| TNC | 16 | 19 | 19 | | | 1 |
| GAPVD1 | 15 | 12 | 14 | | | |
| PRRC2A | 15 | 12 | 14 | | | |
| OFD1 | 15 | 18 | 12 | | | |
| SLC25A3 | 15 | 17 | 15 | 30 | 29 | 29 |
| TJP1 | 14 | 16 | 11 | | 1 | 1 |
| RRBP1 | 14 | 20 | 15 | 24 | 18 | 20 |
| HNRNPUL1 | 14 | 10 | 11 | 12 | 14 | 15 |
| MIB1 | 14 | 14 | 16 | | | |
| MYO5A | 13 | 14 | 12 | | | |
| PRKCI | 12 | 12 | 11 | | | |
| MTDH | 12 | 15 | 12 | 18 | 15 | 15 |
| FGFR1OP | 12 | 10 | 12 | | | |
| CD44 | 12 | 10 | 9 | | | |
| AIFM1 | 12 | 13 | 12 | 14 | 16 | 16 |
| PIBF1 | 12 | 11 | 12 | | | |
| POLRMT | 11 | 7 | 10 | | | |
| SPATA20 | 11 | 11 | 10 | | | |
| PGAM5 | 11 | 9 | 12 | 6 | 6 | 6 |
| CEP290 | 11 | 10 | 12 | | | |
| CCDC77 | 11 | 14 | 15 | | | |
| SNTB1 | 10 | 10 | 10 | | | |
| GLG1 | 10 | 8 | 10 | | | |
| CHCHD3 | 10 | 8 | 9 | 2 | 3 | 3 |
| ATP5B | 10 | 11 | 7 | 22 | 16 | 16 |
| PARD3 | 9 | 7 | 9 | | | |
| MOGS | 9 | 10 | 8 | 7 | 7 | 6 |
| EPDR1 | 9 | 9 | 10 | | | |
| CNST | 9 | 9 | 9 | | | |
| IQGAP1 | 8 | 9 | 7 | 1 | | 1 |
| MAGI1 | 8 | 8 | 6 | | | |
| CTHRC1 | 8 | 8 | 7 | | | |
| ATP5A1 | 8 | 8 | 8 | 26 | 24 | 22 |
| SNTB2 | 7 | 7 | 6 | | | |

| Gene Name | p62_CytoREP_01 | p62_CytoREP_02 | p62_CytoREP_03 | p62_NucREP_01 | p62_NucREP_02 | p62_NucREP_03 |
|---|---|---|---|---|---|---|
| IGF2BP3 | 7 | 4 | 7 | | | |
| LLGL1 | 7 | 6 | 6 | | | |
| MPRIP | 7 | 7 | 5 | 1 | 1 | 2 |
| OSBPL8 | 7 | 6 | 7 | | | |
| FRYL | 7 | 6 | 8 | | | |
| IQSEC1 | 6 | 3 | 3 | | | |
| TPX2 | 6 | 4 | 3 | 7 | 8 | 7 |
| CKAP4 | 6 | 6 | 6 | 20 | 13 | 11 |
| RPS16 | 6 | 5 | 5 | 10 | 10 | 9 |
| RPS24 | 6 | 6 | 5 | 7 | 5 | 5 |
| GDF15 | 6 | 5 | 6 | 1 | 2 | 1 |
| EIF5B | 5 | 3 | 5 | 1 | 1 | 1 |
| SERPINE2 | 5 | 5 | 6 | | | |
| RPS5 | 5 | 6 | 5 | 12 | 11 | 11 |
| EPRS | 5 | 4 | 5 | | | |
| CT45A2 | 5 | 6 | 5 | 11 | 12 | 13 |
| RPS15 | 5 | 3 | 3 | 5 | 5 | 6 |
| ACTR1A | 5 | 4 | 6 | | | |
| CCDC61 | 5 | 5 | 3 | | | |
| SLC25A11 | 5 | 4 | 5 | 17 | 21 | 19 |
| ITIH5 | 5 | 6 | 5 | | | |
| SLC25A1 | 5 | 6 | 6 | 16 | 15 | 15 |
| KEAP1 | 4 | 4 | 4 | | | |
| SMG8 | 4 | 4 | 4 | | | |
| PRPF38A | 4 | 2 | 4 | 1 | 2 | |
| CEP170 | 4 | 4 | 4 | | | |
| PAFAH1B1 | 4 | 2 | 3 | | | |
| PPP2R1A | 4 | 4 | 4 | 1 | | |
| MRPS28 | 4 | 2 | 4 | | | |
| CNP | 4 | 5 | 5 | 12 | 10 | 15 |
| GNB1 | 4 | 5 | 3 | | | |
| CD59 | 4 | 4 | 5 | | | |
| SMU1 | 3 | 1 | 1 | 13 | 12 | 11 |
| TAOK2 | 3 | 3 | 2 | | | |
| AP2B1 | 3 | 4 | 2 | 2 | 1 | |
| MRPS22 | 3 | 3 | 4 | | | |
| ERAL1 | 3 | 3 | 3 | | | |
| MRPS26 | 3 | 4 | 2 | | | |
| SRPR | 3 | 4 | 3 | 3 | 3 | 2 |
| MRPS18B | 3 | 4 | 3 | | | |
| UQCRH | 3 | 2 | 2 | 3 | 4 | 4 |
| HSD17B12 | 3 | 2 | 2 | | | |
| CEP250 | 3 | 4 | 3 | | | |
| GNAI2 | 3 | 7 | 3 | | | |
| NBR1 | 2 | 6 | 3 | | | |
| DYNC1LI2 | 2 | 3 | 3 | | | |
| IGF2BP1 | 2 | 2 | 4 | | | |
| DAP3 | 2 | 2 | 3 | | | |
| MRPS34 | 2 | 2 | 3 | | | |
| SRPK2 | 2 | 4 | 5 | | | |
| DYNC1LI1 | 2 | 2 | 4 | | | |
| MRPS9 | 2 | 3 | 3 | | | |
| HS2ST1 | 2 | 2 | 2 | | | |
| BICD1 | 2 | 2 | 2 | | | |
| FARP1 | 2 | 2 | 3 | | | |
| MTX2 | 2 | 3 | 3 | | 1 | |
| GOLGA2 | 2 | 2 | 4 | | | |
| SNAP23 | 2 | 3 | 3 | | | |
| TBC1D31 | 2 | 4 | 4 | | | |
| IK | 1 | | | 7 | 6 | 4 |
| SRP14 | 1 | 2 | 1 | 2 | 2 | 2 |
| CETN2 | 1 | 2 | 2 | 2 | 2 | 2 |
| EIF4B | | | | 2 | 3 | 2 |
| NOL8 | | | | 6 | 10 | 8 |
| NOL9 | | | | 6 | 8 | 4 |
| CHAMP1 | | | | 5 | 3 | 3 |
| PWP1 | | | | 4 | 4 | 3 |
| SMPD4 | | | | 4 | 2 | 4 |
| AP2A2 | | | | 3 | 3 | 3 |
| UTP23 | | | | 3 | 4 | 2 |

| Gene Name | p62_CytoREP_01 | p62_CytoREP_02 | p62_CytoREP_03 | p62_NucREP_01 | p62_NucREP_02 | p62_NucREP_03 |
|---|---|---|---|---|---|---|
| RFC4 | | | | 2 | 2 | 4 |
| NFIX | | | | 2 | 2 | 3 |
| DHX33 | | | | 2 | 3 | 2 |
| SCO1 | | | | 3 | 2 | 3 |
| ZNF384 | | | | 2 | 2 | 2 |
| ATP5H | | | | 2 | 2 | 2 |
| SMARCD1 | | | | 2 | 2 | 2 |
| CAPZA2 | | | | 2 | 2 | 2 |
| SLC25A10 | | | | 2 | 2 | 2 |
| S100A9 | | | | 4 | 4 | 4 |
| SSR3 | | | | 2 | 2 | 2 |
| CSTF3 | | | | 2 | 2 | 2 |
| PTRH2 | | | | 4 | 4 | 2 |
| FKBP11 | | | | 2 | 2 | 2 |
| ACSL3 | | | | 4 | 3 | 4 |
| PNKP | | | | 4 | 4 | 3 |

| Gene Name | p62_CytoREP_01 | p62_CytoREP_02 | p62_CytoREP_03 | p62_NucREP_01 | p62_NucREP_02 | p62_NucREP_03 |
|---|---|---|---|---|---|---|

## 10.2 Publications

✓ **p62 sustains a protumorigenic gene signature in melanoma opposing mRNA-dependent decay** (manuscript in preparation)

**P. Karras**, E. Riveiro-Falkenbach, E. Cañón, T. G. Calvo, R. Martinez, E. Pérez-Guijarro, C. Tejedo, M. Cifdaloz, P. Ximenez-Embun, J. Muñoz, D. Megías, P. Ortiz-Romero, JL. Rodríguez-Peralto and María S. Soengas.

✓ **Cross-tumor differences in the landscape of RNA binding proteins identify a protumorigenic regulon controlled by CELF1 in melanoma** (under revision)

M. Cifdaloz, L. Osterloh, O. Graña, E. Riverio-Falkenbach, J. Muñoz, T. G. Calvo, **P. Karras**, D. Olmeda, B. Miñana. G. Gómez-López, E. Perez-Guijarro, E. Cañón, E. Eyras, P L. Ortiz-Romero, J L. Rodríguez-Peralto, J. Valcárcel, and MS. Soengas

✓ **Lineage-specific control of melanoma cell proliferation by the cytoplasmic polydenylation factor CPEB4.** **Nature Communications,** 2016

E. Pérez-Guijarro, **P. Karras**, M. Cifdaloz, R. Martínez-Herranz, E. Cañón, O. Graña, C. Horcajada, D. Alonso Curbelo, T. G. Calvo, G. Gomez, N. Bellora, E. Riveiro-Falkenbach, P. Ortiz-Romero, JL. Rodríguez-Peralto, L. Maestre, G. Roncador, JC. Agustín Asensio, C. Goding, D. Megías, E. Eyras, R. Méndez and María S. Soengas.

✓ **Metastatic risk and resistance to BRAF inhibitors in melanoma defined by selective allelic loss of ATG5.** **Autophagy,** 2016

M. García-Fernández, **P. Karras**, A. Checinska, E. Cañón, G. Gómez-López, T. Guadalupe Calvo, G. Gomez-Lopez, M. Cifdaloz, A. Colmenar, L. Espinosa, D. Olmeda and M. Soengas.

✓ **Vascular endothelial cadherin downregulation as a feature of endothelial transdifferentiation in monocrotaline- induced pulmonary hypertension.** **A.J.P.-Lung,** 2016

Nikitopoulou I, Orfanos SE, Kotanidou A, Maltabe V, Manitsopoulos N, **Karras P**, Kouklis P, Armaganidis A, Maniatis NA.

✓ **RAB7 counteracts PI3K-driven macropinocytosis activated at early stages of melanoma development**. **Oncotarget,** 2015

Alonso-Curbelo D, Osterloh L, Cañón E, Calvo TG, Martínez-Herranz R, **Karras P**, Martínez S, Riveiro-Falkenbach E, Romero PO, Rodríguez-Peralto JL, Pastor J, Soengas MS

✓ **RAB7 controls melanoma progression by exploiting a lineage-specific wiring of the endolysosomal pathway**. **Cancer Cell,** 2014

D. Alonso-Curbelo, E. Riveiro-Falkenbach, E. Perez-Guijarro, M. Cifdaloz, **P. Karras**, L. Osterloh, D. Megías, E. Cañón, TG. Calvo, D. Olmeda, G. Gómez-Lopez, O. Graña, VJ. Sánchez-Arévalo, DG. Pisano, H-W. Wang, P. Ortiz-Romero, D. Tormo, K. Hoeck, JL. Rodríguez-Peralto, JA. Joyce, MS. Soengas.

## 10.3 Presentations

### Oral

✓ **Hallmarks of Cancer**

**Title:** *Fine tuning of pro-metastatic factors by RNA complexes controlled by p62/Sequestosome-1 in melanoma* Ghent, Belgium, December 2016

✓ **CNIO Lab day**

**Title:** *p62/SQSTM-1, more than a scavenger in melanoma progression and metastasis* Madrid, Spain, December 2015

**Posters**

✓ **13th International Congress of the Society for Melanoma Research**     Boston, Massachusetts

P. Karras, E. Riveiro-Falkenbach, D. Megias, J. Muñoz, P.Ortiz-Romero, JL. Rodríguez-Peralto and Maria S. Soengas.

*Fine tuning of pro-metastatic factors by RNA complexes controlled by p62/Sequestosome-1 in melanoma*                                                          06-09/11/2016

✓ **12th International Congress of the Society for Melanoma Research**   San Francisco, California

P. Karras, E. Riveiro-Falkenbach, D. Megias, J. Muñoz, P.Ortiz-Romero, JL. Rodríguez-Peralto and M.S. Soengas.

*Global control of the melanoma transcriptome by the p62/Sequestosome-1 autophagy modulator.*

8-21/11/2015

✓ **6th European Melanoma Workshop**                    Las Palmas, Canary islands,Spain

P. Karras, E. Riveiro Falkenbach, J. Munoz, P. Embun, I. Ruppen, O. Grana; P. Ortiz Romero; JL. Rodríguez-Peralto, M. Soengas.

*p62/Sequestosome-1, more than a scavenger protein in melanoma progression.*      27-29/06/2014

✓ **10th International Meeting of the Society for Melanoma Research**    Philadelphia, Pennsylvania

P.Karras, E. Riveiro-Falkenbach, P. Ximénez-Embún, I.l Ruppen, P. Ortiz-Romero, JL. Rodríguez-Peralto and M.S. Soengas.

*p62/Sequestosome-1, more than a scavenger protein in melanoma progression.*      17-20/11/2013

## 10.4 Fellowships/Awards

✓ Travel Award, Hallmarks of Cancer                           Ghent, Belgium, December 2016
✓ Best poster at CNIO Lab day                            Madrid, Spain, December 2013
✓ Best poster at 10th International Meeting of the Society for Melanoma Research
                                                  Philadelphia, November 2013
✓ La Caixa"/CNIO International PhD Programme Scholarship          Nov. 2011 – Nov. 2015

## 10.5 Certificates

✓ **Course of Flow Cytometry**       Spanish National Cancer Research Center (CNIO) ,  2013

✓ **Course of Laboratory Animal Sciences for Researchers: Categories B and C**
   Spanish National Cancer Research Center (CNIO) and Animalaria, Formación y Gestion S.L.,
                                                       Madrid, Spain, 2013
✓ **Course on "Functional Analysis of gene Expression Experiments"**
   Spanish National Cancer Research Center (CNIO). Madrid, Spain.                    2012