SISSA
Scuola Internazionale Superiore di Studi Avanzati

Joint PhD Programme in Molecular Biology

Alterations of the secretory pathway induced by a mutant p53/miR-30d axis

PhD candidate

Marco Fantuz

Advisor

Prof. Giannino del Sal

Co-advisor

Valeria Capaci, PhD

Academic year 2019-2020

I. Index

II. List of abbreviations

5'-CAGE	5-cap analysis of gene expression
acetyl-CoA	acetyl-coenzyme A
AKT	v-Akt murine thymoma viral oncogene homolog
ALDH	aldehyde dehydrogenase
AMPK	AMP-activated protein kinase
ATF	activating transcription factor
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia and Rad3-related protein
AP2A1	component of the adaptor-protein complex 2 subunit A1
ARID5b	AT-rich interaction domain protein 5b
ARL4C	ADP-ribosylation factor-like 4C
BCL	B-cell lymphoma protein
BFA	brefeldin A
BID	BH3 interacting-domain death agonist protein
BiP	binding immunoglobulin protein
CAF	cancer-associated fibroblast
CDKN	cyclin-dependent kinase inhibitor
CDIP	cell death-inducing p53 target
ChIP	chromatin immunoprecipitation
CHOP	C/EBP transcription factor homologous protein
CLEM	correlative light electron microscopy
CM	conditioned medium
CoIP	co-immunoprecipitation
COP	coat complex subunit
CREB3	c-AMP responsive element-binding 3
DAG	diacylglycerol
DDB2	damage-specific DNA-binding protein 2
DGKZ	diacylglycerol-kinase zeta
dy-30d	decoy construct for miR-30d
ECM	extracellular matrix

EDEM	ER degradation-enhancing α-mannosidase-like proteins
eIF	eukaryotic initiation factor
EMT	epithelial-to-mesenchymal transition
ENTPD5	ectonucleoside triphosphate diphosphohydrolase 5
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum-associated degradation
ERBB2	v-Erb avian erythroblastic leukemia viral oncoprotein homolog 2
ERK	extracellular signal-regulated kinase
$FADH_2$	reduced flavin adenine dinucleotide
FBS	fetal bovine serum
FDR	false discovery rate
FSH	follicle-stimulating hormone
GA	Golgi apparatus
GADD	growth arrest and DNA damage-inducible protein
GALNT7	oolypeptide N-acetylgalactosaminyltransferase 7
GBP2	interferon-induced guanylate-binding protein 2
GFP	green fluorescent protein
GLUT	glucose transporter
GM-130	Golgi matrix protein 130
GOCC	Gene Ontology: cellular compartment
GPI	glycosylphosphatidylinositol
GSEA	gene set enrichment analysis
GST	glutathione S-transferase
HLA	human leukocyte antigen
HIF	hypoxia-inducible factor
HS	horse serum
Hsp	heat-shock protein
IRE1α	inositol-requiring enzyme 1α
IQGC	IQGAP-related protein
ITSN1	intersectin 1
JNK	c-JUN NH2-terminal kinase
kbp	kilobase pairs

LC-MS/MS	liquid chromatography-coupled mass spectrometry
lnc-30d	long non-coding RNA containing miR-30d
HDAC	histone deacetylase
HPF	high-power field
MAPK	mitogen-activated protein kinase
MDH1	nucleocytoplasmic malate dehydrogenase 1
MDM	mouse double-minute homolog
mRNA	messenger RNA
MSH2	MutS homolog 2
MSI2	Musashi RNA-binding protein 2
mTOR	mammalian target of rapamycin
MUTYH	MutY DNA glycosylase
miR	microRNA
miRNA	microRNA
mutp53	mutant p53
NADH	reduced nicotinamide adenine dinucleotide
NRF2	nuclear factor erythroid 2-related factor 2
NQO1	NAD(P)H:quinone oxidoreductase 1
OGG1	8-oxoguanine DNA glycosylase 1
PDI	prolyl-disulfide isomerase
PD-L1	programmed-death ligand 1
PERK	PKR-like endoplasmic reticulum kinase
PGC	PPARG coactivator
PGM	phosphoglycerate mutase
PIG	p53-induced gene
PML	promyelocytic leukemia protein
PNUTS	phosphatase 1 nuclear targeting subunit
PPP3CB	protein phosphate 3 catalytic subunit beta
PTEN	phosphatase and tensin homolog
PUMA	p53-upregulated modulator of apoptosis
qRT-PCR	quantitative real-time PCR
RAS	rat sarcoma viral proto-oncogene

RB1	retinoblastoma-1
RIDD	regulated IRE1α-dependent decay
RHO	RAS-homolog family member
RNAi	RNA intereference
ROCK	RHO-associated coiled-coil-containing protein kinase
ROS	reactive oxygen species
S1P	site-1 protease
S2P	site-2 protease
SCO2	synthesis of cytochrome c oxidase protein 2
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SESN	sestrin
SHARP	enhancer-of-split and hairy-related protein
shRNA	short hairpin RNA
siRNA	small interfering RNA
SOD	superoxide dismutase
SREBP	sterol regulatory element-binding transcription factor
ssGFP	signal sequence-harboring secreted GFP
TCA cycle	tricarboxylic acid cycle
TFE3	transcription factor binding to IGHM enhancer 3
Tg	thapsigargin
TIGAR	TP53-induced glycolysis regulatory phosphatase
Tm	tunicamycin
TSS	transcription start site
TXN	thioredoxin
UGT	UDP-glucuronosyltransferase
UPR	unfolded protein response
UTR	untranslated region
VEGF	vascular endothelial growth factor
VHL	von-Hippen Lindau tumor suppressor protein
VPS26B	vacuolar protein sorting-associated protein 26B
XBP1	X-box binding protein 1

1. Abstract

Cancer is, nowadays, among the most prevalent and deadly diseases worldwide. This term describes a group of pathologies characterized by an abnormal growth of a mass of cells harboring mutations in their DNA, resulting in uncontrolled growth, evasion from the cell control checkpoint mechanisms and spreading throughout the body. In recent years, the investigation of the interplay between cancer cells and the tumor micro-environment has gained a central spot in the comprehension of the neoplastic development and outgrowth. At a cellular level, the main hub regulating the communication with the surrounding tissues is the secretory pathway, which is deputed to the movement of proteins and lipids between the ER, the Golgi apparatus, and, through the secretory vesicles, to the extracellular space. Alterations in the functions of the secretory pathway could have an important role in helping the development and progression of the malignancies, fostering metastasis, invasion, altered secretion patterns and cytoskeletal remodeling.These phenotypes, among the others, have been linked by a vast amount of data to the mutated forms of p53, derived by missense point mutations in the TP53 gene which lose the oncosuppressive function of the wild-type form, and acquire, in many cases, novel pro-oncogenic features.

mutp53s exert their pro-neoplastic functions through a plethora of interactors, both coding and non-coding. In our laboratory, we identified miR-30d as a new target gene of mutp53, which appears to be regulated through the interaction of this protein with the hypoxia-inducible factor HIF1α. Preliminary evidence show that miR-30d-regulated genes are enriched for factors involved in the unfolded protein response activation and protein secretion, suggesting an effect on the structure and functions of the secretory pathway.

Our results show that miR-30d expression is able to blunt the activation of the UPR following drug-induced ER stress and concomitantly to induce major alterations in the secretory pathway organelles, mainly represented by a strong vesiculo-tubulation of the Golgi apparatus.

Moreover, miR-30d, through the modulation of its direct and indirect targets, as its upstream regulators mutant p53 and HIF1α, is able to strongly promote the secretion of proteins by cancer and normal cells.

Taken all together, the findings reported in this thesis suggest a role for this newly described mutant p53/HIF1α/miR-30d axis in the regulation of the structures and functions of the secretory pathway, and particularly on the Golgi apparatus.

2. Introduction

2.1. Cancer: a global burden

Cancer accounts for the 17.08% of the yearly deaths worldwide, establishing itself as the second leading mortality cause after cardiovascular pathologies[1], matching to a striking number of 8.93 million people who die from neoplastic diseases every year.

The determinant of these malignancies is mainly the accumulation of diverse mutations in the DNA of the cells, that in normal conditions leads to the activation of suppressive mechanism promoting senescence and programmed cell death, which both effectively act as a barrier towards neoplastic transformation; however, when the number of mutations builds up in the cells, they acquire the ability to evade the normal regulatory feedbacks, becoming able to divide and to grow without control [2,3]. Moreover, their consequent rapid replications, together with the failure of important checkpoint regulatory mechanisms, promote the genetic instability of these cells, helping them to progress towards a neoplastic state [4,5].

Despite their heterogeneity, most of cancers share common altered features, which are considered hallmarks of tumor development and progression, giving them the capability to evade the organism's surveillance systems and allowing for their disordered and uncontrolled growth. Tumor cells become self-sufficient in growth signals and insensitive to growth inhibitors, evading the normal growth suppressive mechanisms, thus acquiring a limitless replicative potential. Additionally, they gain new and abnormal capabilities, including the possibility to promote angiogenesis, helping to sustain the needs of the growing neoplastic mass, but also to spread to adjacent tissues, invading them, and to colonize distant loci in the organism, forming metastases[6] (*Fig. 1)*. Interestingly, in recent years it has also been proposed that the stress stimuli and the deregulation of the consequent homeostatic pathways could be tightly linked to the acquisition of pro-aggressive cancer phenotypes[7] (*Fig. 1*).

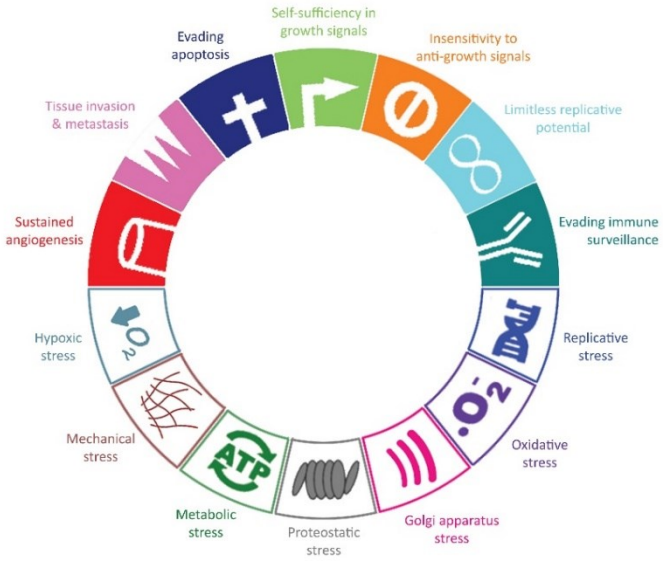

Fig. 1 – The hallmarks of cancer and their interplay with stress pathways.
In the figure (adapted from[236]) are reported the main features of cancer transformation, the so-called hallmarks of cancer (top half, colored background), and the different stress stimuli that can act on cancer cells

2.2. The hallmarks of cancer development and progression

As stated, cancer cells, during their evolution, acquire common features which promote the transformation of phenotypically normal cells into malignant ones, favoring the progression of neoplastic cells through the exploitation of the healthy host tissue[7]. In fact, it must be considered that tumors are not only insular masses of proliferating cancer cells, but instead they are composed of different cell types, all participating in heterotypic interactions with one another. The surrounding normal cells, together with the cues acting from the stroma towards the neoplasm, form the tumor microenvironment, which has been described as an active contributor in tumorigenesis and cancer progression[6].

2.2.1. Selective growth and proliferative advantage

Cancer cells display limitless replicative capacity thanks to self-sufficiency in growth signaling and decreased sensitivity to anti-mitogenic stimuli. These features, together with the resistance to apoptosis and the aberrant regulation of the replicative stress signaling, give to cancer cells their selective growth properties and a strong proliferative advantage compared to the surrounding host tissue.

Cancer cells can acquire the capability to sustain proliferation by promoting growth factor production in order to sustain autocrine signaling or paracrine stimulation from the tumor-associated stroma, but also favoring increased levels or alterations in mitogenic receptor proteins on the cell surface; contemporaneously, they induce a downregulation of anti-proliferative pathways and cell cycle checkpoints[7]. Even in the presence of aberrant growth signaling, nonetheless, the tight regulation of the cell cycle is able, in normal cells, to keep cell divisions in check. In cancer, however, the disruption of the cell cycle checkpoints and its consequent deregulation is essential for the neoplastic growth.

Normally, cells which fail to completely replicate their genomic information in the S phase activate the DNA damage checkpoints, mainly regulated by the activation of the ATM/ATR kinases and of the downstream p53 pathway, leading to transient cell cycle arrest, and, eventually, if the stress cannot be resolved, to cell senescence and apoptosis[8]. If, however, such cells proceed through mitotic division, the under-replicated genomic regions can be converted in gaps and breaks in the genomic sequence and, moreover, if these regions are not correctly recognized and processed, their presence can lead to genomic instability[9].

Lastly, cancer cells often harbor oncogenes, both mutated, such as RAS^{V12} forms, or overexpressed due to genomic amplification, such as MYC, which promote and force the cell cycle progression even in the presence of incorrectly or partially replicated DNA, therefore prompting the cells to develop and accumulate new genetic mutations[10].

2.2.2. Metabolic rewiring in cancer cells

In order to face the needs of their uncontrolled growth, tumors promote an enhanced metabolism for rapid acquisition and availability of energy, of anabolic building substrates and of reducing equivalents to counteract the metabolic side-effects of oncogene activation and of other cellular stresses; moreover, cancer cells also enhance, if possible, the availability of nutrients and oxygen in the surrounding regions of the growing neoplastic mass [11–13].

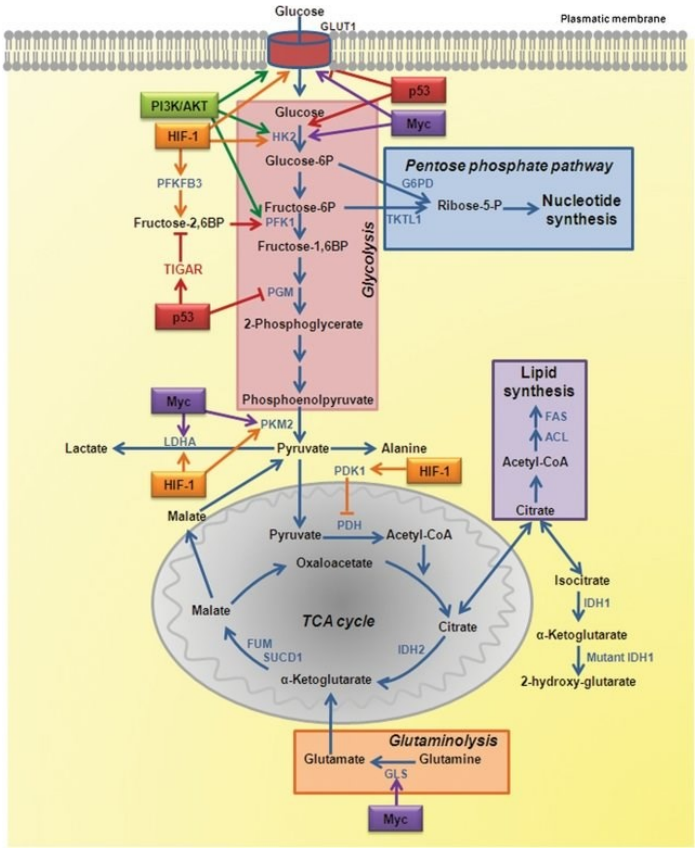

Fig. 2 – Metabolic reprogramming of cancer cells
Exemplified view of the remodeling of the main metabolic pathways in cancer cells and their regulation by oncogenes and tumor suppressor genes[237].

The metabolic rewiring in cancer cells appear to be an universal phenomenon in malignancies, independently from the mutational pattern own to the specific neoplasm[14], and its best known feature is the Warburg effect[15], a metabolic switch characterized by an enhanced glucose consumption and glycolytic metabolism. This response, normally, is peculiar of cells that undergo hypoxia, but, when hijacked by cancer cells, produces intermediate glycolytic metabolites that supply subsidiary processes, including the pentose-phosphate pathway, lipogenesis and one-carbon metabolism[16] (*Fig.2)*. Accordingly, the TCA cycle is not used anymore as a supply chain for the mitochondrial respiration, but mainly as a source of intermediates (e.g. oxaloacetate, citrate) that will be used as building blocks in many anabolic pathways[16].

Moreover, it has to be taken into account that, in addition to the glycolysis' pyruvate production, several other molecules can be used as input for the TCA and thus employed to yield energy. For

example, beta-oxidation of lipid moieties provides both acetyl-CoA, which can enter the TCA cycle, and NADH and $FADH_2$, that can be used for ATP production in the electron transport chain[17]; moreover, glutaminolysis, which is the ensemble of processes derived from glutamine metabolism, can provide anaplerotic fluxes of α-ketoglutarate and citrate that enter the Krebs' cycle [18] (*Fig.2*).

2.2.3. Tumor micro-environment physical properties modulate cancer progression

Tumors, while growing as a mass of altered cells in a context in which the tissue is not organized to accommodate it, are subjected to physical and mechanical constraints, which have been described in the recent years as a key microenvironmental cue in cancer development.

A central part of this assumption is that mechanical inputs, which include the tumor expansion leading to physical compression of the neoplastic mass and of its surrounding tissues, increased extracellular matrix (ECM) stiffness and increased interstitial pressure, together with the alteration of the pathways deputed to sensing these stresses, can lead to modifications in the cell and tissue tension both in the malignant ones and in the surrounding stroma. These alterations leads to the release, concentration and activation of several growth factors in an aberrant way, and to the promotion of intracellular responses that, through coordinated actomyosin contraction and signaling pathways activation, can ultimately lead to enhanced tumor cell growth, survival and invasion[19].

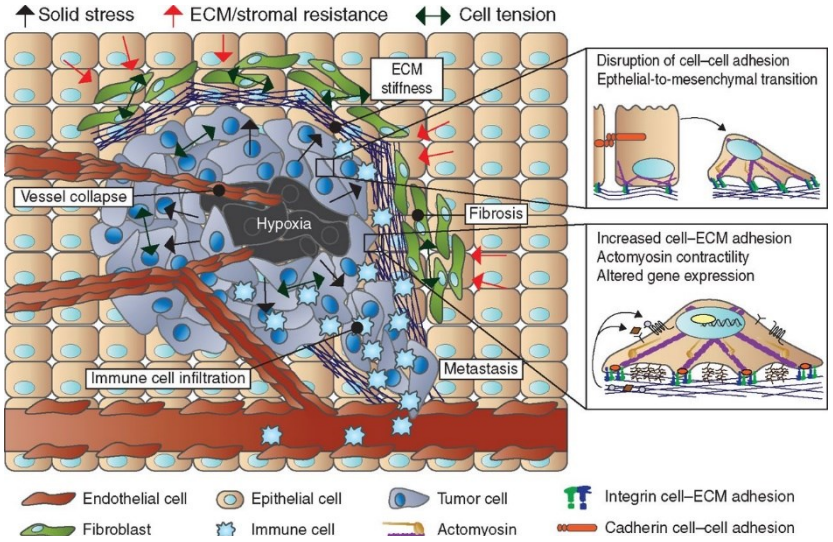

Fig.3 – Mechanical stress in cancer
Exemplified view of the mechanical forces acting on the tumor mass and their consequences in signaling and aberrant cell modifications (adapted from [238]).

Additionally, the rapid expansion of the tumor mass can lead to the compression of the tumor interior and to the simultaneous distention of the surrounding stromal tissues, in a process termed solid stress. This results in an increased ECM tension and remodeling, with deposition and crosslinking of collagens, metalloprotease-mediated alterations and general disruption of the normal tissue architecture surrounding the growing cancerous mass[20] (*Fig. 3*). Moreover, tumor-

associated fibroblast proliferation, promoted by the sustained juxtacrine and paracrine signaling derived from the cancer cells, can also contribute to the solid stress displayed in tumors, ultimately promoting cancer growth[20]. Lastly, solid stress-generated compression leads to partial clamping of blood vessels in the tumor mass, promoting hypoxic stress and consequent metabolic reprogramming[21] (*Fig.3)*.

2.2.4. Evasion from the immune response

Other than the physical properties of the microenvironment, the cellular composition of the stroma surrounding the neoplastic mass can also modulate its development and growth. In particular, when the immune cells that infiltrate both the tumor and the stroma try to eliminate transformed cells without being successful, consequently lead to the development, in the tumor region, of a chronic inflammatory microenvironment. This condition, opposing to its original aim, helps reducing the anti-tumoral immune response and favors the escape of the malignancy from immune elimination[22].

Moreover, both cancer cells and other non-immune components of the tumor microenvironment, such as cancer-associated fibroblasts, can produce immunosuppressive components, including growth factors[23], cytokines[24] and chemokines[25], which have been demonstrated to be closely involved in tumor immune escape. Additionally, cancer cells are also able to downregulate the levels and the presentation on the cell surface of key molecules that mediate immune-related functions, such as the HLA-I and HLA-II complexes, therefore reducing the recognition both by cytotoxic T-cells and the presentation to the immune system of cancer-related neoantigens[26]. Lastly, cancer cells often gain the expression of novel immunosuppressive factors, including the HLA-G and PD-L1[27] proteins, which directly downregulate the function and the activation of immune cells towards the malignancy[28].

2.2.5. Oxygen need and sustained angiogenesis

As cancer grows, its nutrient requirements will eventually exceed the capacity of the vascular bed, and, although many neoplasms are able to adapt by promoting neoangiogenesis, eventually their core will become hypoxic and in need of nutrients. Since cancer is an intensively proliferating and expanding tissue, the demand for oxygen is vastly overcoming the oxygen supply, and the distance between each cell and the closest existing blood vessel increases, hampering oxygen diffusion and further increasing the hypoxic conditions[29].

Enduring changes in blood flow and low oxygen availability, in fact, result in chronic hypoxia, a feature typical especially of larger tumors, with broad impacts on the cellular functions. Long exposure to hypoxia is associated to high frequency of DNA breaks, both due to increased ROS generation and to the hampering of the DNA repair systems, potentially leading to increased mutation rate and genomic instability[30].

Hypoxia induces a plethora of complex intracellular signaling pathways, involved in cell proliferation, survival, apoptosis, metabolism, migration and inflammatory response (*Fig.4*). Nevertheless, cellular adaptation to hypoxia is primarily mediated by the activation of the hypoxia-inducible factors (HIFs), a family of three transcription factors, HIF1α, HIF2α and HIF3α, which protein levels are induced and stabilized upon hypoxic stimuli. In oxygenated cells, in fact, HIFs subunits are hydroxylated by two classes of oxygen-sensing enzymes, prolyl-hydroxylases (PHDs) and the asparagine hydroxylase FIH-1; this hydroxylation leads to recognition of the HIFs by the VHL protein, which promotes their ubiquitination and consequent degradation. When the oxygen levels, instead, are low, the oxygen-sensing enzymes lose their activity, and therefore HIFs degradation is halted; these factors are then stabilized and translocate to the nucleus, where they dimerize with the constitutively expressed HIFβ subunits, leading to the promotion of an adaptive transcriptional program, bringing the cells to cope with hypoxia[29]. Indeed, it is estimated that 50–60% of solid tumors contain areas of hypoxic and/or anoxic tissue that develop as a result of an imbalance between oxygen supply and consumption in proliferating neoplasms[31].

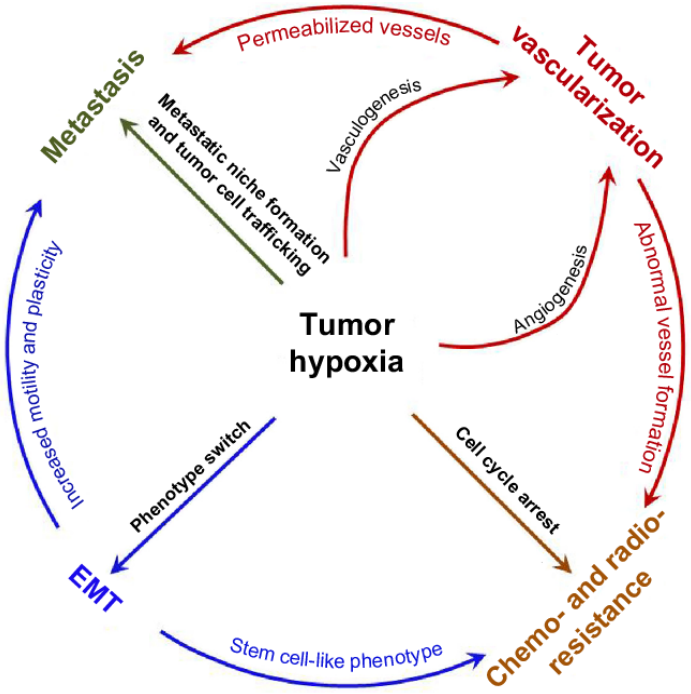

Fig. 4 – Consequences of hypoxia in tumors
Overview of the effects of the hypoxic stress in the neoplastic disease, which acts as a driving force in tumor growth and progression, modulating on a plethora of different cancer-related phenotypes[53].

Therefore, both the hypoxic microenvironment in which the tumor grows and the aberrant activation of HIFs stabilizing pathways lead to a pathological hypoxic response in cancer cells, which facilitates neoplastic development and spreading. In fact, hyperactivation of HIFs leads

firstly to an increase in blood vessel formation, mainly endorsed through the induction of the vascular endothelial growth factors (VEGFs), but also by the promotion of the expression of matrix-remodeling enzymes and growth factors to support and recruit all the accessory components needed for blood vessel formation[32]. However, in tumors, neovessels are often abnormal and leaky, plenty insufficient to support the growing tumor tissue in terms of oxygen and nutrients. Therefore, neoangiogenesis can further promote metabolic and hypoxic stresses in the growing tumor mass, closing a vicious circle that boost neoplastic progression[33] (*Fig. 4)*. Concordantly, the ECM-remodeling capabilities of cells which undergo hypoxic stress can promote the invasive and migratory behavior of cancer cells through the induction of epithelial-to-mesenchymal transition (EMT) and through direct alteration of the surrounding extracellular matrix[34,35] (*Fig. 4*).

Another consequence of the altered vascularization and oxygen levels in cancer is the increased generation of reactive oxygen species (ROS): while, in normal cells, low concentrations of these molecules are required for signal transduction before their elimination, in cancer cells, instead, due to the accelerated metabolism and high proliferation rate, ROS levels are highly increased due to their augmented production[36]. This accumulation leads to an imbalance between ROS generation and elimination, which must be counteracted by cancer cells by elevated antioxidant defense mechanisms in order to survive[37].

Cancer cells employ, to this aim, a plethora of difference homeostatic stress-response mechanisms, therefore hijacking the beneficial effects of high ROS signaling, including promotion of angiogenesis and of cell survival, without being damaged by the increased oxidative stress condition (*Fig. 5*): firstly, neoplastic cells promote glycolysis and downregulate mitochondrial function, therefore decreasing the rate of production of ROS at its primary source[38]; moreover, it has been reported that several oncogenes are able to upregulate and stabilize the NRF2 transcription factor, which comprises among its target antioxidant detoxification enzymes, such as heme oxygenase

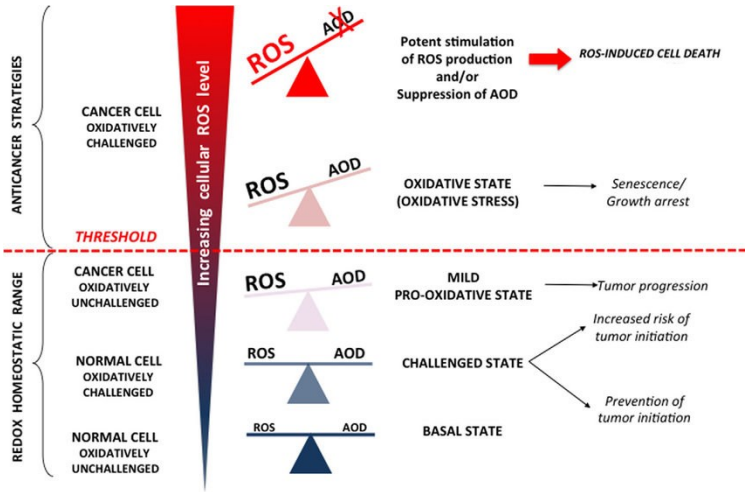

Fig. 5 – Redox balance in cancer cells and its consequences
Schematic representation of altered balance of ROS and antioxidant molecules (AOD), which can have both pro-tumoral and anticancer effects[239].

(HMOX1), NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs)[39,40]. Moreover, aside from the regulation by NRF2 on the enzyme catalyzing its synthesis, the anabolic pathways leading to the production of glutathione are modified and altered in many tumor types, including glucose, serine, glycine and glutamine metabolism, all providing anaplerotic fluxes leading to increased glutathione levels[41–43].

2.2.6. Aberrant stress responses along the secretory pathway

The reduced nutrient and oxygen levels present in most tumors have, among their effects, the result of leading to impaired ATP generation, ROS accumulation and metabolic imbalance, and therefore cause a reduction of the protein folding capabilities of the endoplasmic reticulum (ER). The accumulation of misfolded proteins in the ER leads the cells to undergo proteostatic stress, an unbalance in the protein folding, secretion and degradation pathways, promoting the activation of complex and intertwined stress response mechanisms[44].

In normal conditions, proteins are brought in the ER co-translationally, where they begin folding from a linear aminoacidic chain into more stable conformations characterized by lower free energy. In the organelle's lumen, a unique set of modifications is imposed onto the nascent proteins, including the removal of signal sequences, disulfide bond formation, N-glycosylation and glycosylphosphatidylinositol (GPI) addition. These changes have not only effects on the functionality of the proteins but also serve as a checkpoint mechanism which ensures that only correctly folded ones exit the ER[45].

Nevertheless, a central role in protein folding in the ER is represented by the activity of the chaperone BiP. Mechanistically, in normal cells the nascent polypeptides chains which enter the ER are bound by several folding-assisting enzymes, and among them, one prominent place is taken by the Hsp70 chaperone BiP; this protein recognizes in an ATP-dependent manner exposed hydrophobic patches on a broad spectrum of protein chains, helping their folding, preventing premature aggregation, and keeping incorrectly folded proteins to exit from the ER[46]. If unfolded proteins, however, continue to accumulate, the cells activate a stress-response pathway termed unfolded protein response (UPR), a tripartite pathway that leads first to adaptation of the cell to the proteostatic stress by enhancing protein folding and globally attenuating translation, in order to reduce the burden of the misfolded intermediates in the ER lumen; eventually, if the cell cannot resolve the stress, the UPR is able to promote apoptosis[47].

Notably, BiP binds the intraluminal domains of the three receptors which promote the UPR process, the inositol-requiring enzyme 1α (IRE1α), the PKR-like endoplasmic reticulum kinase (PERK) and the activating transcription factor 6 (ATF6), keeping them in an inactive state. However, when misfolded proteins accumulate in the ER lumen, BiP is sequestered by the unfolded intermediates, detaching from the receptors and leading to their activation[48].

The IRE1α pathway is activated by the dissociation of BiP from the luminal portion of the receptor, leading to its homo-oligomerization followed by autophosphorylation. IRE1α activation enables its endoribonuclease activity, leading to the cleavage of a 26-nucleotide intron from the XBP1 mRNA, thus allowing the production of sXBP1, a potent transcription factor promoting the expression of adaptation proteins that include chaperones and ERAD components[49]; moreover, IRE1α is also a serine/threonine kinase which, upon UPR triggering, promotes the phosphorylation of several substrates, among which the c-JUN NH2-terminal kinase JNK occupies a central spot; its activation, in fact, promotes the function of the Bcl2-like protein BIM, which induces cytochrome c mitochondrial release and caspase activation, ultimately leading the cell to apoptosis[50]. Additionally, IRE1α is able to exert its RNAse activity not only on the XBP1 mRNA, but also cleaving mRNA encoding for ER-targeted proteins in a process called regulated IRE1α-dependent decay (RIDD), helping to reduce the influx of proteins in the ER and therefore the burden to be carried by the folding machinery[51].

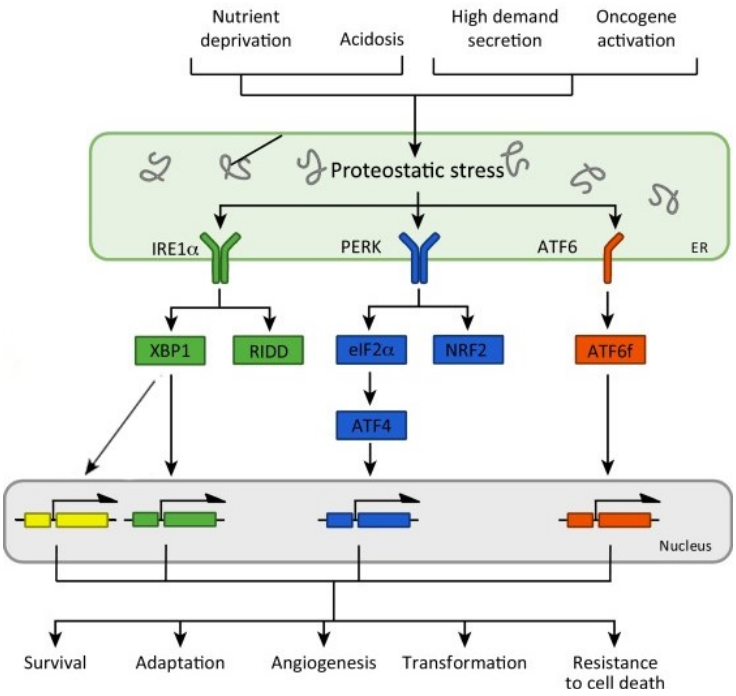

Fig. 6 – Proteostatic stress in cancer
Exemplified overview of the main stimuli able to promote proteostatic stress in cancer and of the outcomes of the downstream UPR activation in regulating tumor-related phenotypes (adapted from[240]).

Similar consequences are caused by BiP detachment from the intraluminal portion of PERK, which leads to its activation by dimerization and consequent autophosphorylation. PERK activity then induces phosphorylation of the eukaryotic initiation factor 2α (eiF2α), leading to attenuation of global protein translation, thus reduced ER folding load and promoting ERAD activity. Interestingly, decreased protein translation is not universal, because transcripts harboring internal ribosome

entry sites (IRES) in their 5'UTR bypass the phospho-eiF2α-induced translation block[46]. Among them we find the activating transcription factor 4 (ATF4), which drives the expression of pro-survival effectors under UPR conditions, including amino acid transporters and producers, redox reactions regulators and protein secretion promoters[52]. However, ATF4 induces also the expression of the C/EBP transcription factor homologous protein CHOP, which activity promotes the repression of the BCL2 anti-apoptotic protein, ultimately leading to cell death[53].

Conversely, upon BiP detachment from ATF6, this protein is translocated from the ER to the Golgi apparatus, where it is cleaved by two proteases, S1P and S2P, in order to produce a fragment, termed ATF6f, which translocates in the nucleus promoting a pro-survival transcriptional program. ATF6f, in fact, upregulates the expression of chaperones, including BiP, of protein disulfide isomerases and of ERAD components[54]. At the same time, ATF6-induced gene expression program promotes BCL2 activity, exerting a potent cytoprotective role[55].

Moreover, UPR pathways can exert a plethora of other roles in promoting cancer cell oncogenic phenotypes, including promotion of neoangiogenesis[44] and EMT-driven cell invasion[56] (*Fig. 6*).

While the UPR and the protein folding machinery in the ER controls the correct arrangement and consequent functionality of proteins, a relevant part in the control of the proteostatic stress is deputed to the ERAD degradation pathway, which contributes to lighten the burden of accumulated misfolded intermediates in the ER. Proteins that fail to comply to the ER quality control are in fact recognized by the ER degradation-enhancing alpha-mannosidase-like proteins, or EDEMs, which extract misfolded intermediates from the folding cycles and then associate with a disulfide isomerase protein and with BiP, leading to the unfolding of the partially folded structure and consequent retrotranslocation in the cytosol through the SEC61 complex in a way that has still to be fully characterized. At this point, unfolded proteins are ubiquitinated by several E3-ubiquitin ligases and targeted to the proteasome for their degradation[57].

Additionally, in a similar fashion to what happens in the ER, where a reduction in the function of the organelle triggers a complex spectrum of stress responses, the decrease of the function of the Golgi apparatus (GA) leads to the activation of a number of homeostatic responses, collectively named Golgi stress; this process is triggered when the production of secretory and membrane proteins increases and overwhelms the capacity of the GA, in order to answer to the fact that these proteins cannot be modified or transported properly due to insufficiency of the Golgi function[58]. While the stress responses in the ER have been extensively characterized, not much is known about the homeostatic pathways that regulate GA stress, and only in recent years some of

them have been described; however, the biggest part of this process has still to be unveiled and elucidated.

The TFE3 pathway is one of the Golgi stress processes which has been described, and is deputed to induce a general augmentation of the function of the Golgi apparatus; in normal cells, this protein is phosphorylated and retained in the cytosol, while upon Golgi stress, is dephosphorylated, translocates into the nucleus and activates the transcription of Golgi structural proteins, N-glycosylation enzymes and of vesicular transport components[59]. Interestingly, the activation of ER stress does not activate TFE3, identifying these two processes as independent one from the other[60].

As for the UPR, GA stress response appears to be in control of a fine balance between apoptosis and cell survival. Regarding cell death, in fact, in recent years the transcription factor CREB3 has gained novel importance in relationship to GA stress: in normal conditions, this protein is retained on the ER membrane, while upon Golgi stress is transported to the GA, where it is cleaved to produce a fragment which translocates to the nucleus to induce the transcription of the ARF4 and DR4 genes, ultimately inducing apoptosis[61] . On the other hand, there have been reports that the ER chaperone HSP47 could be in charge of promoting cell survival upon Golgi stress, but the sensors, transcription factors and transducers of this pathway are still unknown[62].

Even if the pathways controlling the response to Golgi apparatus stress are still poorly characterized, the importance of the Golgi apparatus in tumor biology has been rising in the recent years, since alterations in glycosylation[63,64], protein secretion[65,66] and in the Golgi apparatus structure[67,68] have been extensively linked to neoplastic features, and therefore this organelle is acquiring a central spot in the biology of cancer cells and as a feasible target in the development of novel anti-neoplastic drugs.

2.2.7. Invasion and metastasis

Aside from the regulation subtending primary tumor development and growth, a key point in malignancies aggressiveness is the capacity of cancer cells to invade local tissues and eventually to spread to distal sites, forming metastases.

The process of invasion and metastasis has been described as a multistep sequence of discrete processes, often termed the invasion-metastasis cascade. This representation shows a succession of changes in the morphology and functions of the neoplastic cells, starting with local invasion of the surrounding stroma, intravasation by cancer cells into nearby blood and lymphatic vessels,

transit of cancer cells through the lymphatic and blood circulation systems, followed by extravasation of cancer cells from the lumen of vessels to transit into the parenchyma of distant tissues, leading to the formation of small nodules of cancer cells (micrometastases), and eventually to colonization of the distant tissue, which is the process of growth of micrometastatic lesions into macroscopic tumors[6]. However, even if the schematic description of these processes appear to be starting from the established primary tumor and moving further along time, in recent years, it has been proven that metastatic dissemination is a precocious process, often developing in parallel to the primary cancer mass, posing novel hurdles to metastasis targeting and elimination[69].

2.3. The p53 protein and its mutants: master regulators of cancer hallmarks

As stated, cancer arises and develops in a multistep process, with an accumulation of genetic mutations and epigenetic changes which lead normal cells to progressively evolve towards a neoplastic state[70,71]. DNA mutations, in cancer, can take place in three main types of genes, called proto-oncogenes, tumor suppressors and caretaker genes. Proto-oncogenes are genes implied in processes like cell growth and survival, cell cycle progression and metabolic control; mutations that activate such genes, like KRAS or ERBB2, lead to the deregulation of different pathways, promoting the neoplastic progression. Moreover, other proto-oncogenes, like pro-metastatic and pro-invasive factors, give the neoplastic cell new and atypical features implied in its aggressive phenotypes[72]. On the other side, tumor suppressors, like RB1 or PTEN, are genes that keep in control processes like cell proliferation, apoptosis, senescence, maintenance of cell polarity and of epithelial features, anoikis and contact inhibition; these genes, if inactivated, can favor the neoplastic development[72]. Lastly, caretaker genes encode for a group of genes whose protein products control DNA repair and maintain genome integrity. Mutations in these genes or functional inactivation of their protein products lead to an increased rate of DNA mutations and genomic instability[73].

Among the other factors involved in cancer development and progression, a central spot is taken by the p53 protein. This factor is a potent tumor suppressor which, upon different kind of cellular stresses, including DNA damage, hyperproliferation, hypoxia, oxidative stress and nutrient starvation, is able to trigger transient cell cycle arrest, cell senescence and apoptosis[74–76].

Notably, almost 50% of all the malignancies carry a p53 mutation, and of those cancer that do not harbor mutated p53, a large proportion of them have inactivated p53 function by another altered mechanism[77].

Taken together, these evidences strongly support the central relevance of p53 in cancer, and prompt to the investigation of its roles in the regulation of the hallmark features of malignancies.

2.3.1. Wild-type p53

The human TP53 gene encodes for a DNA-binding protein that acts primarily as a tetrameric transcription factor; this protein, termed p53, belongs to a family of similar ones, including p63 and p73, which possess relevant tumor suppressive activities (*Fig. 7*). Interestingly, the TP53 gene appears to be a very complex genomic locus, giving rise to 13 different transcripts derived both from alternative splicing and alternative translation initiation; the most represented isoform, p53α, has been extensively characterized, while the role of the other isoforms is still unclear, but in recent years several works have shown that they might have pivotal roles in the regulation of physiological and pathological processes[78–80].

In unstressed cells, p53 is maintained at very low levels through the constant ubiquitin-proteasome-dependent degradation induced by the MDM2 E3-ubiquitin ligase and its co-factor MDMX. Upon induction of stress, post-translational modifications of both p53 and MDM2 block their interaction, leading to p53 stabilization, accumulation and induction of transcriptional activity[76].

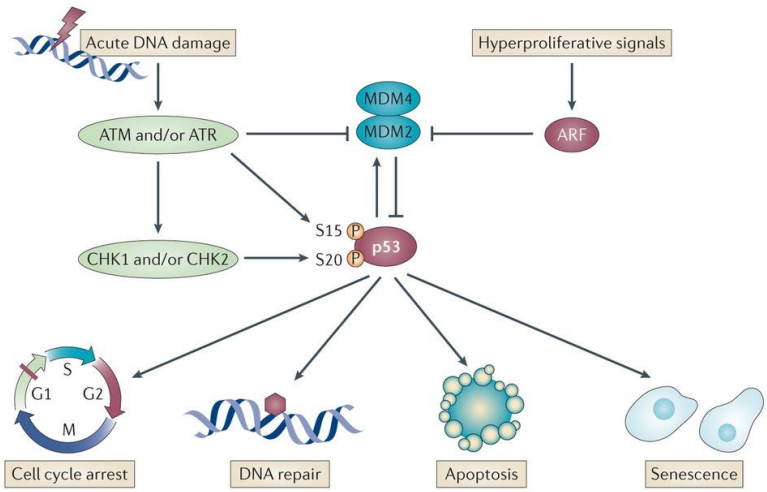

Fig. 7 – Wild-type p53 is a potent oncosuppressive factor
Schematic view of p53 regulation and its downstream functions[241].

p53 and its pathway control the G1/S and G2/M cell cycle checkpoint in order to arrest cell cycle progression and therefore prevent the propagation of DNA damage while the cells attempt to repair it (*Fig. 7)*. p53 can induce G1 cell cycle arrest through transcriptional induction of the cyclin-dependent kinase inhibitor CDKN1A/p21[81], and G2 arrest by promoting the levels of GADD45[82] and SFN[83]. Beyond triggering cell cycle arrest, p53 stimulates the functionality of various DNA repair mechanisms[76].

When, however, the damage is too relevant and cannot be repaired by p53, this protein can promote senescence or apoptotic cell death. Regarding the first, stabilized p53 is able to transcriptionally increase potent senescence inductors, such as p21, which induces G1 cell cycle arrest, and PML, which recruits the p16 and pRb proteins, modulating the expression of their downstream targets, leading eventually to senescence[84]. Concerning the latter, instead, p53 is able to induce the expression of pro-apoptotic genes, including BAX, BID, PUMA and NOXA, while repressing anti-apoptotic genes[76]; moreover, it can promote apoptosis in a transcription-independent fashion, both by directly inducing permeabilization of the mitochondrial membrane by forming complexes with the Bcl-XL and Bcl-2 proteins, resulting in cytochrome c release[85], or by favoring transfer of calcium from the ER to the mitochondria, altering the morphology of this organelle and leading to cell death[86].

2.3.2. p53 interplay with the cancer hallmarks

Given its functions, is appears evident that efficient control of p53 is essential for cell growth. An increasing amount of evidences is showing, in fact, that many different stress signals can lead to p53 activation; interestingly, different stresses seem to utilize different pathways to promote activation of p53[87].

The most well-known inducer of p53 activation is DNA damage, which can be originated both by external insults and by DNA replicative stress. Injuries to the DNA lead to activation of the ATM and ATR kinases, which results in the phosphorylation of p53, MDM2 and MDMX[88]; these post-translational modifications both disrupt the interaction between p53 and MDM2, leading to decreased ubiquitination of the first and consequently to its stabilization, and promote the degradation of MDM2 and MDMX, further releasing p53 from its inhibition[89]. In addition to stabilizing p53, DNA damage-induced kinases may also play a role in the activation of p53, in part by promoting acetylation of the C-terminus of the protein[87]. In turn, p53 is able to promote the activity of DNA repair machineries, both at a transcriptional level, by inducing genes involved in nucleotide excision repair (DDB2, XPC), base excision repair (OGG1, MUTYH), mismatch repair (MSH2), non-homologous end joining and homologous recombination (RAD51), as well as directly interacting and modulating *in trans* the activity of several members of these pathways, therefore promoting the homeostatic stress response to DNA damage, including the one induced by replicative stress[90].

Reduced nutrient and energy amounts have also been shown to strongly impact on p53 function: in fact, low levels of the formers result in a failure to stimulate the AKT/mTOR pathway and in a consequent AMPK activation; both mechanism can lead to stimulation of p53: AKT, in fact, activates by phosphorylation MDM2, and therefore its reduced activity acts a stabilizing agent for p53[91], while activation of AMPK leads to the increased transcription of the TP53 gene and promotion of p53 activity through its direct phosphorylation[92]. Additionally, the nucleocytoplasmic malate dehydrogenase MDH1 has been shown to directly bind and activate p53 upon glucose starvation[93]. Concordantly, the balance between ATP and ADP, a direct readout of the cell's energetic state, can regulate p53 function: ADP, in fact, promotes its binding to the DNA, while ATP inhibits this interaction[94]. In turn, several studies have shown that p53 has a role in the regulation of both glycolysis and oxidative phosphorylation: by slowing the former, in fact, this protein can inhibit the Warburg effect, which is characteristic of most of malignancies. p53 can inhibit the expression of the glucose transporters GLUT1 and GLUT4[95] and of the phosphoglycerate mutase PGM[96], while increasing the expression of TIGAR[97], resulting in an impediment at various steps of the glycolytic pathway[98]. On the other hand, p53 matches the restraint imposed on the glycolytic rate with an induction on mitochondrial oxidative phosphorylation, exerted by transcription of several key players of this pathway, including subunit I of the cytochrome c oxidase[99], SCO2[100] and p52R2[101]. Additionally, p53 is able to inhibit mTOR signaling, promoting autophagy[102], while endorsing AMPK-dependent fatty acid β-oxidation[103], providing further levels of complexities to p53 contribution in the cell survival upon metabolic stress.

Similarly, decreased oxygen availability promotes the activation of p53 by inducing stabilization of the protein via the induction of a DNA-damage response in the absence of detectable DNA damage[104]. Under hypoxia-induced replication stress, p53 is indeed phosphorylated mainly by ATR and, to a lesser extent, by ATM[104,105]. In addition, p53 stabilization is helped by the HIF1α-dependent suppression of MDM2 activity, through a mechanism that is still not well understood[106]. Moreover, it has been postulated that increased p53 stability upon hypoxia could also be helped through the induction of hypoxia-regulated genes: in fact, the hypoxia-induced PNUTS protein could increase p53 activity via inhibition of protein-phosphatase-1, which targets p53[107], as well as the activity of the VHL protein, which is a HIF1α transcriptional target, could enhance p53 translation in a pathway which involves the RNA-binding protein HuR[108]. In most cases, the increased p53 activity in hypoxic cells is able to promote apoptosis, and the presence of HIF1α appears essential for this activity[109]. Interestingly, however, it appears that known p53 pro-

apoptotic gene targets, including PUMA and BAX, are not induced under hypoxic condition[110]. In fact, it has been proposed that p53 might not be able to induce its target genes because of the lack of recruitment of the CBP and p300 co-activators, which are sequestered by HIF1α in order to induce its target genes[111]. Indeed, the main activity of p53 under hypoxic conditions has been reported to be *trans*-repression of apoptosis inhibitors, even if the genes involved in this regulation have not been defined yet[112].

Notably, mitochondrial generation of reactive oxygen species upon hypoxia could also be one of the factors contributing to p53 induction[113]. Indeed, aside from low oxygen conditions, ROS increased levels have been shown to promote p53-activating phosphorylation by p38α MAPK[114], ATM[115] and ERK[116]; however, if the effect of ROS amount on these kinases is direct or mediated by ROS-induced DNA damage, is still not clearly known. Oxidative stress-induced p53 stabilization can have different outcomes, ranging from cell cycle arrest to apoptosis, but the knowledge on how ROS can regulate p53 target genes selection is still in very preliminary status[117]. On the other hand, a broad amount of data have helped to clarify p53 function in regulating the cellular ROS levels; in fact, physiological levels of p53 are able to maintain redox homeostasis by inducing antioxidant genes, including SESN1, GPX1 and AIF[118], and metabolic genes like TIGAR, SCO2 and PGM, which can control the metabolic flux that lead to generation of ROS[117]. Additionally, stabilization of p53 by different cellular stresses is able to promote instead pro-oxidant genes (e.g. BAX, PUMA, NQO1) and suppress antioxidant ones (e.g. SOD, PIG12, ALDH4), therefore promoting oxidative stress, mitochondria permeabilization and consequent p53-mediated apoptosis[117].

Lastly, the interplay between proteostatic stress regulation and p53 function is still not clear: in fact, contrasting evidences have been reported, showing both that ER stress can promote p53 nuclear localization and activity[119] and that, instead, it could promote cytoplasmic localization of the protein and destabilization[120]. However, interestingly, a possible explanation for these opposite findings could rely in the fact that proteostatic stress could promote the transcription of specific p53 isoforms, resulting in transient G2 cell cycle arrest and sensitization to DNA damage[121], leading to the hypothesis that ER stress regulation on p53 levels could involve different layers of complexity, and still needs to be further addressed. Nevertheless, p53 activation has been shown to repress the IRE1α/XBP1 pathway[122] and to induce the CDIP1 pro-apoptotic ER stress mediator[123]. These data suggest that p53 could exert a part of its oncosuppressive function by hindering ER function and promoting proteostatic stress-induced cell death[124].

2.3.3. mutant p53: from oncosuppressor to oncogene

The primary outcome of TP53 mutations is the loss of the wild-type form of the p53 proteins and, consequently, of its oncosuppressive functions. These alterations are extremely prevalent in human cancers, with a mutation rate for the TP53 gene that spans around the 35-40 % of the total cancer patients; moreover, p53 is often inactivated or downregulated by other mechanisms in tumor cells[125].

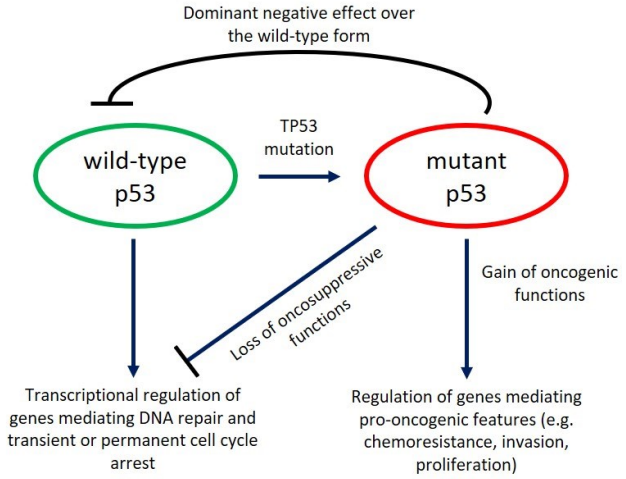

Fig. 8 – mutant p53 hijacks the functions of the wild-type form
Schematic view of the alterations in the function of the p53 protein upon missense mutations.

However, differently from most other tumor suppressors genes in human cancers, the majority of TP53 mutations are missense and produce single aminoacidic changes in loci situated mainly in the DNA-binding domain of the protein. p53 missense mutants (mutp53s) not only lose the normal oncosuppressive functions of the protein, being uncapable of activating canonical p53 target genes, but also acquire dominant negative effects over the wild-type form[126]. Moreover, mutp53s gains novel pro-oncogenic functions, becoming able to reshape the tumor cells' transcriptome and proteome through the interplay with a plethora of intracellular effectors, both coding and non-coding[127] (*Fig.8*).

Interestingly, mutp53s become stabilized and activated in response to various stress conditions, similarly to the wild-type counterpart, and, of note, several evidences are showing how mutp53s can help cancer cells to cope with the challenging condition that are originated during tumor initiation, outgrowth and spreading[128].

2.3.4. mutp53s regulates mechanisms involved in cancer arisal and development

Similarly to the wild-type form of the protein, mutp53s are constantly subjected to the degradation promoted by MDM2 protein function; however, in cancer cells, the interaction of these mutants with the HSP90 chaperones, which increased levels are promoted by the accumulation of altered and misfolded proteins typical of cancer cells, is able to specifically inhibit MDM2-mediated mutp53 degradation[129]. Additionally, several stress stimuli that frequently target cancer cells and stabilize the wild-type p53 are also able to promote accumulation and activation

of the mutant proteins, therefore favoring their oncogenic functions[126], which include induction of proliferation, chemoresistance, invasion and metastasis[126] (*Fig. 9*).

Exemplifying, the genomic instability and the rapid growth of cancer cells trigger constitutive replicative stress, promoting continuous activation of the DNA-damage response machineries; in particular, the derived aberrant activation of the ATM kinase is able to drive stabilization of mutp53, thus inducing its oncogenic functions[130]. Additionally, these altered mutant p53 proteins can become important regulators of cancer cell metabolism: mutp53, accordingly, have been reported to sustain the glucose intake and the Warburg effect by the RHOA-ROCK-dependent membrane translocation of the GLUT1 glucose transporter[131]. In addition, the altered protein can induce aerobic glycolysis by direct inhibition of the AMPK kinase[132] and can induce, through its interaction with the SREBP factors[133], the production of fatty acids and cholesterol and the activity of the mevalonate pathway[134]. Interestingly, it has been reported that mutp53 associates and fosters PGC1α function, enhancing oxidative phosphorylation in cancer cells[135]. These apparently contrasting evidences in reality hint to the fact that mutp53 is probably able to promote a metabolic plasticity state in tumors, facilitating their adaptation to the constantly shifting needs of the growing, invading and distally spreading neoplastic disease[126]. A fascinating interplay among stress pathways regulated by mutp53 is provided by the fact that, as the hosting lab recently demonstrated, induction of the mevalonate pathway by mutp53/SREBPs interaction is able to promote the geranyl-geranylation of RHOA[136]. The modified form of the protein is then able to exert its normal functions, transducing extracellular mechanical stimuli through the actin cytoskeleton: interestingly, by investigating the pathways protecting mutant p53 from degradation, our laboratory discovered that HDAC6/HSP90-dependent mutant p53 accumulation is sustained by RHOA and actin-dependent transduction of mechanical inputs, such as the stiffness of the extracellular environment[137].

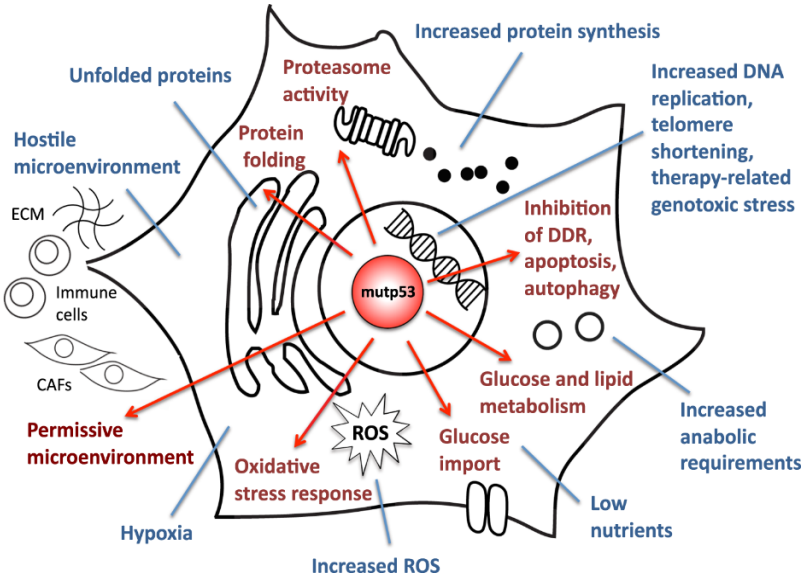

Fig. 9 – Interplays between mutant p53 and cellular stress pathways
Schematic representation of the regulation exerted by mutant p53 on several homeostatic stress response pathways and its relevance in the cancer cell[179].

Moreover, the activity of mutant p53 can be promoted also by hypoxic conditions: in fact, while not changing the total levels of the p53 protein, it has been reported that HIF1α, binding to mutp53, is able to promote its the localization on chromatin, regulating the transcription of several target genes, promoting the aggressiveness of lung cancer cells[138]. Closing a positive feedback loop, mutp53 appears to stimulate HIF1α stabilization inducing its dissociation from MDM2 in hypoxic conditions[139] and antagonizing the SHARP1 gene, a factor which promotes ubiquitin-mediated degradation of HIF1α[140]. On the opposite side of the spectrum, our group recently demonstrated that mutp53, through the binding to NRF2, contributes to a selective modulation of its transcriptional program, promoting a pro-survival oxidative stress response, repressing genes with a cytotoxic effect in cancer cells, such as HMOX1, and favoring the expression of pro-survival mediators, including the TXN gene; this regulation allows cancer cells to cope with the high levels of oxidative stress typical of cancer transformation and growth[141].

Additionally, proteasome hyperactivation by mutp53 also impinges on the regulation of the proteostatic stress, which is typical of cancer cells due to the accumulation of mutated and misfolded proteins in the ER. In fact, increased proteasomal activity could cooperate in relieving the burden of unfolded intermediates, therefore reducing ER stress. Recently, we demonstrated also that mutant p53 is able to shift the balance of pro- and anti- apoptotic signals downstream of the UPR activation by dampening the function of the IRE1α and PERK pathways and promoting the activation of the ATF6 pro-survival mediator, thus contributing to cancer cells' survival in condition of imbalanced protein homeostasis[142].

Taken together, these evidences put mutant p53 in the center of a complex regulatory network in which several stress stimuli can act on the protein, shifting its activation, and mutp53 in turn is able to regulate several homeostatic stress response pathways in order to promote cancer cell growth, survival and aggressiveness exerting its pro-oncogenic gain of function.

3. Preliminary data

Work performed in the hosting laboratory has been focused on understanding the role of missense gain-of-function mutant forms of p53 in cancer, and on the action of microRNAs as effectors of the oncogenic properties of mutp53s. To identify novel microRNAs regulated by mutant p53, the expression levels of a panel of known onco-miRNAs previously reported to be amplified or overexpressed in solid tumors[143] was evaluated by qRT-PCR in MDA-MB-231, a breast cancer cell line harboring an endogenous mutant $p53^{R280K}$ form, where this factor was silenced using a specific siRNA. This analysis highlighted miR-30d as one of the top hits regulated by mutp53 (*Fig.10A*). microRNA-30d is an intergenic miRNA located on the chromosome 8q24, which levels has already been reported as frequently increased in multiple types of human solid tumors[144]. Moreover, miR-30d mediates several processes related to cancer development and progression in different types of malignancies, including migration[145], invasion[145–147], EMT[145], autophagy[148], angiogenesis[149] and metastasis[146,147].

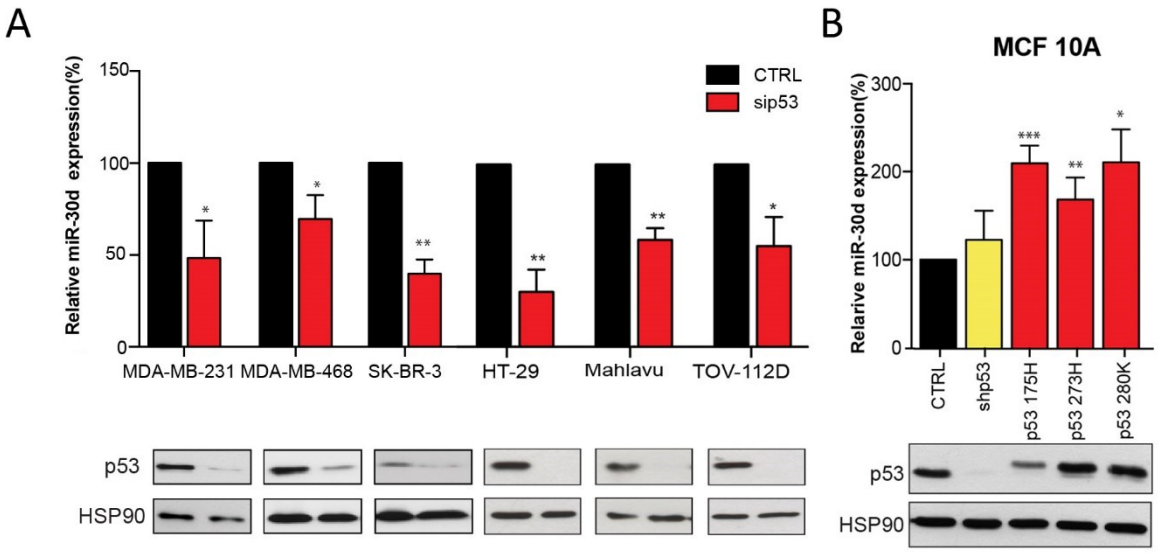

Fig. 10– mutp53 regulates miR-30d expression levels
A. Top: expression levels of miR-30d were analyzed by qRT-PCR, normalized to U6B RNA expression, upon silencing of mut-p53 in the indicated human cancer cell lines; *bottom:* Western blot for p53 is shown as silencing control, Hsp90 levels as normalizer. *B.* Endogenous wild-type p53 was stably silenced in MCF-10A mammary epithelial cells (shp53); exogenous shRNA resistant forms of the indicated p53 mutants were introduced by viral transduction. miR-30d expression was then evaluated as in *A*. All histograms represent the average ± SEM of three independent experiments. *=p<0.05, **=p<0.01, ***=p<0.001. All Western blots are representative of n=3 biological replicates.

We confirmed mutant p53-dependent miR-30d regulation in other cancer cell lines endogenously expressing different forms of the mutant protein (*Fig. 10A*), including SK-BR-3 and TOV112-D ($mutp53^{R175H}$), Mahlavu ($mutp53^{R249S}$), MDA-MB-468 and HT-29 ($mutp53^{R273H}$). Moreover, we reported that silencing of the endogenous wild-type p53 protein in MCF-10A cells, a normal breast epithelial immortalized cell line, had no effect of miR-30d levels, whereas ectopic reintroduction of

mutp53^{R175}, mutp53^{R273H} or mutp53^{R280K} was able to upregulate the expression of the microRNA (*Fig. 10B).*

Preliminary experiments have also suggested that the mutp53/miR-30d axis promotes aggressive cancer phenotypes, such as migration, invasion, EMT and epithelial cell polarity disruption (*data not shown*).

Interestingly, evidences reported in literature[150] have shown that miR-30d expression is controlled, under hypoxic conditions, by the hypoxia-inducible factor HIF1α through its binding to a genomic site located approximatively 27 kbp upstream of the MIR30D gene.

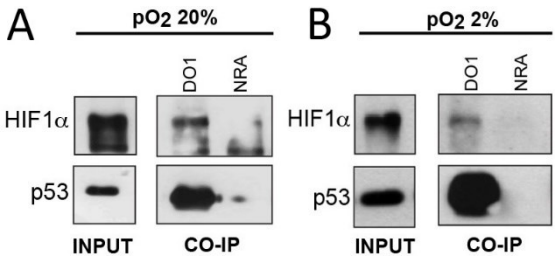

Fig. 11 – mutant binds HIF1α both in hypoxic and normoxic conditions
Co-immunoprecipitation experiments showing mutant p53 binding to HIF1α in normoxic (A) and under hypoxic (pO_2 2% for 24h) (B) conditions. DO1: p53 specific antibody; NRA: Non-Related Antibody

Moreover, by performing co-immunoprecipitation experiments of mutp53 and HIF1α proteins from lysates of MDA-MB-231 cells, grown under different oxygen pressure, we demonstrated that mutp53 physically interacts with HIF1α both in hypoxic and in normoxic/hyperoxic conditions, putatively regulating its activities. (*Fig. 11)*

Given the importance of both these factors in human malignancies, these data prompted us to investigate the existence and the functions of this newly identified mutp53/HIF1α/miR-30d molecular axis.

4. Aim of the thesis

Cancer cells modulate their surrounding microenvironment in order to favor the growth of the neoplastic mass, and, in turn, receive and respond to a plethora of stimuli coming from the stromal components of the surrounding healthy tissue.

The homeostatic responses that become activated downstream of these microenvironmental cues are modulated by a vast number of intracellular signaling factors, among which p53 and its mutated forms gain a central role. Their activities are exerted through a broad spectrum of effectors, both coding and non-coding.

In the laboratory in which this thesis was developed, microRNA-30d, a known onco-miRNA, reported to be under the transcriptional control of the hypoxia-inducible factor HIF1α, has been identified as a novel mutant p53 target, able to mediate several of its known oncogenic functions such as the induction of cell invasion, EMT and promotion of cell migration.

Given these evidences, the aims of this thesis are:

1. to investigate the existence of a mutant p53/HIF1α molecular axis, and eventually to characterize its action on the induction of miR-30d levels;
2. to elucidate the functions of miR-30d in promoting novel pro-oncogenic functions of mutant p53;
3. to investigate the molecular targets of miR-30d which downregulation could be responsible for its functions downstream of mutant p53 regulation.

The results of this research will shed a light on how mutant p53, putatively cooperating with HIF1α in normoxic conditions, and thus hijacking its canonic functions, could regulate the gain of novel pro-oncogenic phenotypes through its non-coding effector microRNA-30d.

5. Results

5.1. mutant p53 induces miR-30d through HIF1α

Having shown that miR-30d levels are modulated by mutant p53, I wanted to characterize the mechanism underlying this regulation. Notably, the current model describing the transcriptional regulation activity by mutp53 indicates that this protein is not able to recognize specific sequences on DNA as its wild-type counterpart, but instead establishes protein-protein complexes with several DNA-binding transcription factors[151]. Therefore, to evaluate the possibility that mutant p53 could be interacting with a specific factor on miR-30d regulatory regions, I inspected the MIR30D genomic locus and noticed the presence of a putative HIF1a bind site around 27kbp upstream of gene encoding for miR-30d, as previously reported in literature[152].

To better characterize this hypothetic regulatory region, I employed the RIKEN FANTOM5 Phase1 and Phase2 data, which contain a database of 5' CAGE reads aligned to the reference genome; using these data is possible to map the 5' ends of mRNAs, and therefore their hypothetical transcriptional start site (TSS); accordingly, in the putative promoter region of miR-30d I reported two 5'-CAGE peaks, supporting the notion that this locus could represent the TSS of this non-coding RNA (*Fig. 12A).*

In the same direction, this region contained also a binding site for the RNA polymerase II, as reported in a published ChIP-seq experiment[153], and high levels of H3K4me3, low levels of H3K4me1, together with an increase of the H3K27Ac mark, representing the epigenetic configuration of an active promoter[154] (*Fig. 12A).* Taken together, these data support the notion that this genomic region could be the putative promoter of miR-30d, and therefore responsible for its transcriptional regulation.

Although the putative miR-30d promoter region lays 27 kbp upstream of the genomic sequence of the MIR30D gene, CHIP-seq data reported in literature identified that hypoxia-inducible factors HIF1α and HIF2α, under hypoxic conditions, are able to bind within the same region to regulate the levels of miR-30d,[150], suggesting that HIF1α could be actually regulating an uncharacterized host transcript harboring miR-30d in its sequence.

In order to confirm the existence of this hypothetic host transcript, I took advantage of the expressed sequence tags (EST) mapping[155] present in the UCSC Genome Browser suite[156], which shows the alignment to the reference genome of single-read sequences, typically about 500 bases in length, that usually represent fragments of transcribed genes.

Indeed, I was able to report the presence of a ~43 kbp locus, termed LOC102723694, encoding for multiple isoforms of an uncharacterized long non-coding RNA, which I will hereby name lnc-30d; moreover, these isoforms displayed a putative start site close to the region of binding for HIF1α as reported by previous works in literature[150,152], and could contain miR-30d in one of their introns (*Fig. 12A).*

In order to evaluate whether mutp53 could be associated to this genomic region, I took advantage of the data derived from a ChIP-seq of mutant p53 performed in MDA-MB-231 available in previously published work from the hosting laboratory[157]. Since mutant p53 is unable to directly bind the DNA, the identified ChIP-seq peaks indicate putative genomic loci in which mutp53 associates with specific DNA-binding transcription factors. Concordantly, this analysis revealed the presence of ChIP-seq peaks for mutp53 which overlap the HIF1α putative binding region, suggesting that these two factors could bind the putative promoter region of lnc-30d (*Fig. 12A).*

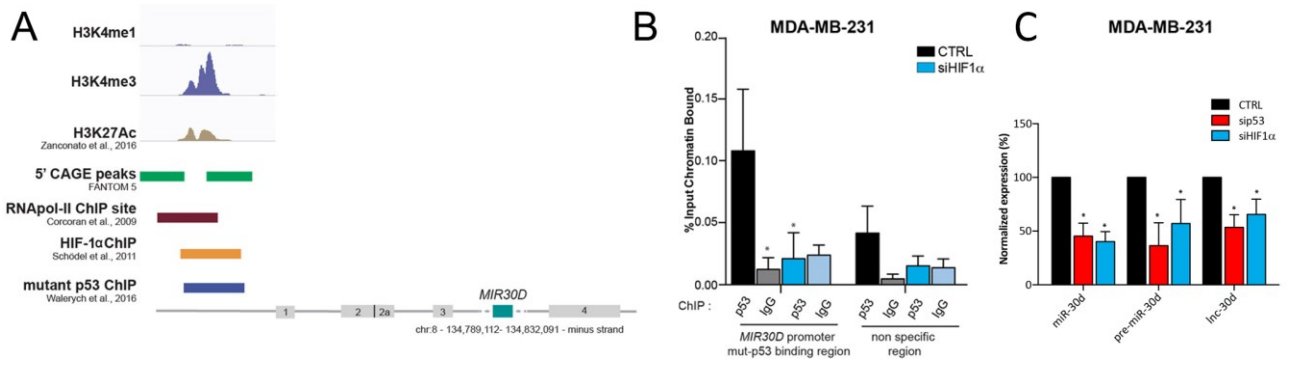

Fig. 12 – mutant p53 cooperates with HIF1α to transcribe lnc-30d and increase miR-30d levels

A. Schematic representation of the lnc-30d/miR-30d locus, comprising the features annotated on the putative promoter. *B.* MDA-MB-231 cells were subjected to chromatin immunoprecipitation (ChIP) analysis with anti-p53 FL-393 antibody (p53) or rabbit nonspecific IgG as control. Relative DNA binding of p53 to lnc-30d/miR-30d putative promoter region was calculated as a fraction of the input chromatin. *=p<0.05 – unpaired Student's T-test. *C.* Bar graph showing quantification of qRT-PCR experiments (n=3) analyzing the levels of lnc-30d, pre-miR-30d and mature miR-30d after silencing by RNAi of either mutant p53 or HIF1α in MDA-MB-231 cells; the bars are shown as average ± SEM, *=p<0.05, **=p<0.01, ***=p<0.0001, unpaired Student's T-test; the levels of miR-30d and pre-miR-30d are normalized on U6B expression, while the ones of lnc-30d on H3 expression levels.

To formally prove this hypothesis, I assessed whether the binding of mutp53 on the lnc-30d/miR-30d promoter could be dependent on HIF1α. To this aim, I performed chromatin immunoprecipitation experiments in MDA-MB-231 cells cultured in normoxic conditions and, as shown (*Fig. 12B*), I demonstrated that mutant p53 strongly binds to lnc-30d/miR-30d putative promoter, in the same genomic region as described by the reported ChIP-seq experiments, but not to an unrelated nearby region. Interestingly, this binding was reduced upon HIF1α silencing by RNAi, confirming that mutant p53 depends on the presence of the hypoxia-inducible factor in order to attach to the putative promoter region of the long non-coding RNA.

Lastly, silencing of either mutp53 or HIF1α by RNAi in the same experimental conditions was able to strongly downregulate the levels of lnc-30d, of the precursor form pre-miR-30d and of the mature miR-30d, supporting the notion these two factors act at a transcriptional level to regulate microRNA-30 (*Fig. 12C*).

Taken together, these evidences point to the fact that mutp53s, by binding to HIF1α both in hypoxic and in normoxic/hyperoxic conditions, are able to attach to the putative promoter of lnc-30d, which acts as an host transcript for miR-30d harboring it into one of its introns, thus regulating the levels of both the long non-coding RNA and of the microRNA.

5.2. Evaluation of the functional relevance of the mutant p53/HIF1α/miR-30d molecular axis

5.2.1. Analysis of miR-30d effects in cancer cells

Having demonstrated that the expression of miR-30d is regulated by mutant p53 in association to HIF1α, I investigated the functional effects of the upregulation of microRNA-30d in cancer cells.

To this aim, I employed a dataset derived from a DNA microarray transcriptomic analysis generated in the hosting laboratory, performed in MDA-MB-231 cells stably expressing a decoy construct for miR-30d (dy-30d), which dampens the levels and the functions of the endogenous microRNA. This vector was constructed similarly to what has been previously described in literature[158], and it encodes for a GFP protein fused with a synthetic 3'UTR containing two miR-30d-responsive elements, and is thus able to act as a molecular sponge, binding and sequestering the mature miRNA in cells in which it is expressed.

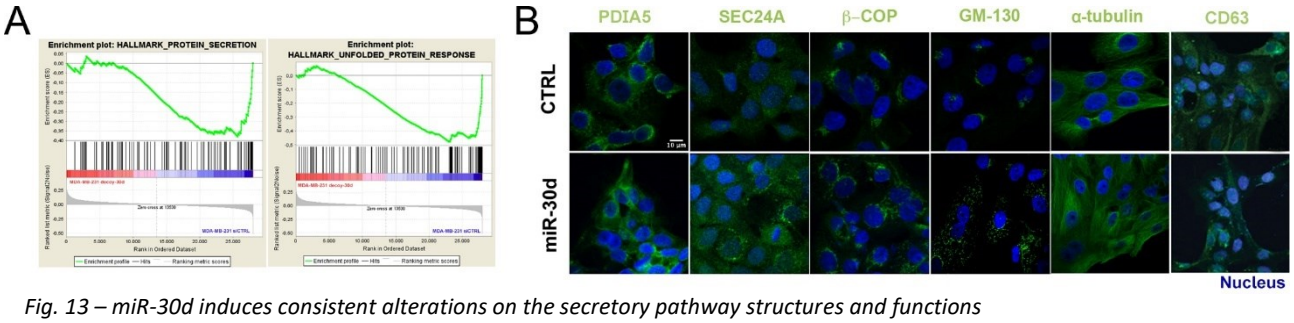

Fig. 13 – miR-30d induces consistent alterations on the secretory pathway structures and functions

A. Enrichment plots for the protein secretion and unfolded protein response clusters derived from GSEA analysis of the transcriptomic data from MDA-MB-231 transduced with the decoy-30d construct compared to the control condition (n=3). *B.* Immunofluorescent staining in MCF-10A cells with miR-30d overexpression of the ER (anti-PDIA5), COPII vesicles (anti-SEC24A), COPI vesicles (anti-β-COP), Golgi apparatus (anti-GM-130), microtubules (anti-α-tubulin) and endosomes/multivesicular bodies (anti-CD63).

Gene Set Enrichment Analysis (GSEA)[159] performed on these transcriptomic data showed a strong enrichment for two gene clusters, one related to the protein secretion process and one to the unfolded protein response activation (*Fig. 13A*). These pathways are two of the main determinants

of the regulation of the secretory proteostasis, an ensemble of processes which oversees the modulation of the homeostatic balance of proteins along the secretory pathway.

In order to elucidate whether miR-30d could mediate alterations in the secretory pathway organelles, I analyzed the morphology of the endoplasmic reticulum (ER), the COPII and COPI transport vesicles, the Golgi apparatus (GA), the microtubules and the endosomal/multivesicular body compartments by immunofluorescence. As shown (*Fig. 13B*), miR-30d overexpression in MCF-10A cells was able to produce an enlargement of the ER, analyzed *via* PDIA5 staining, an increase in the number of both anterograde and retrograde transport vesicles, shown respectively by SEC24A and β-COP staining, an important disorganization of the GA, evaluated by GM-130 staining, and a mild stabilization in the microtubules, stained for alpha-tubulin. These evidences point to a relevant miR-30d-induced general structural alteration of the secretory pathway; however, the strongest alterations in miR-30d-overexpressing cells concerned the morphology of the GA, with a loss of the normal perinuclear ribbon-like structure of the organelle and its conversion in multiple mini-stacks dispersed within the cytoplasm, a morphology hereafter defined as vesiculation.

5.2.2. mutant p53 and HIF1α induce structural alterations of the secretory pathway via miR-30d

Since miR-30d expression promotes structural alterations of the secretory pathway organelles, particularly altering the morphology of the GA, I wanted to evaluate whether the upstream regulators of miR-30d, mutp53 and HIF1α, could lead to the same modifications.

To this aim, I focused on the Golgi apparatus, as it showed the most altered phenotype, represented by enhanced vesiculation; therefore, I employed MCF-10A cells engineered to express three different missense mutant p53 variants: in these cells, the endogenous expression of wild-type p53 was stably depleted by a specific shRNA and three silencing-resistant mutant forms of p53, namely mut $p53^{R175H}$, mutp-53^{R273H} and mut $p53^{R280K}$, were ectopically expressed. As shown (*Fig. 14A*), all three of the mutant p53 proteins strongly promote the vesiculation of the GA, while the silencing of the wild-type forms does not produce any significant effect. To evaluate if this mutp53 effect was dependent on miR-30d presence, I co-transduced, in these same conditions, the dy-30d construct; strikingly, co-expression of the decoy-30d vector was able to rescue the normal organelle structure, proving that mutant p53 forms are able to induce alterations in the Golgi apparatus structure through the action of miR-30d (*Fig. 14A*). Similarly, upon treatment of MCF-10A cells with cobalt chloride, a known hypoxia-mimicking drug able to

stabilize the levels of HIF1α[160], a similar level of vesiculation of the GA was observed (*Fig. 14A*). Under this conditions, concomitant expression of the decoy construct for miR-30d partially restored the GA morphology to the control conditions (*Fig. 14B*).

These evidences demonstrate that both mutp53 and HIF1α promote alterations of the Golgi apparatus morphology *via* their effector miR-30d

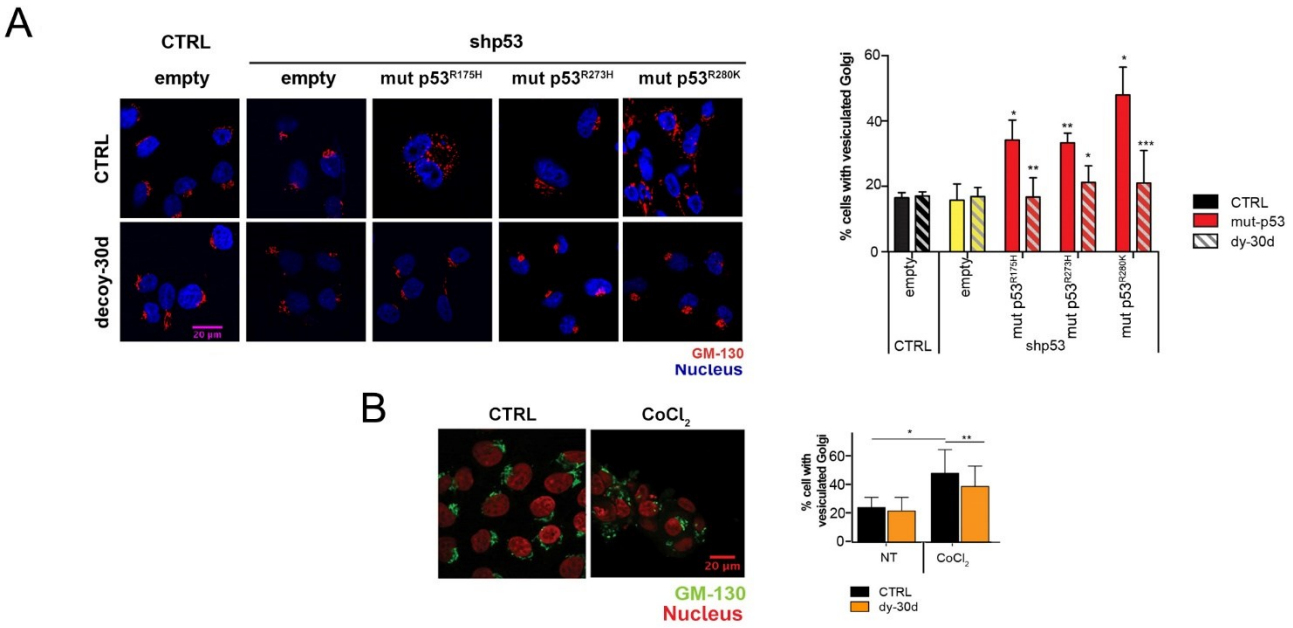

Fig. 14 – mutant p53 and HIF1α induce alterations of the Golgi apparatus via miR-30d

A. Immunofluorescence analysis of the Golgi apparatus in MCF-10A cells upon silencing of endogenous wild-type p53 and ectopic overexpression of the indicated different forms of mutant p53 in conjunction with the decoy for miR-30d. Bar graph is showing the quantification of cells with vesiculated GA as average ± SEM ; n=30 HPF, *=p<0.05, **=p<0.01, ***=p<0.001, unpaired Student's T-test. *B.* Immunofluorescence analysis of MCF-10A cells upon $CoCl_2$ treatment and concomitant transduction with dy-30d, analyzed as in *A*.

5.2.3. Ultrastructural characterization of the Golgi apparatus alterations

To characterize the reported alteration of the GA, Correlative Light Electron Microscopy (CLEM) experiments[161] were performed in collaboration with dr. Alexandre Mironov and dr. Galina Beznoussenko from the IFOM Institute in Milan. This technique consists in the consequent imaging of the same sample through super-resolution immunofluorescence and transmission electron microscopy (TEM). The obtained TEM image is then used to provide a 3D reconstruction of the selected structures, which in this case are individual Golgi apparatuses immunostained with fluorescent anti-GM130 antibody. As shown (*Fig. 15*), overexpression of miR-30d in MCF-10A cells induces a strong modification of the Golgi apparatus structures, which can be described as a vesiculo-tubulation, evidentiating an increase both in the tubular connections between Golgi

cisternae and in the COPI vesicles, which have been previously described as indicators of upregulated secretory rate in cells[162,163].

Moreover, strikingly, while the silencing of wild-type p53 in the same cells did not produce any significant alteration in the Golgi apparatus, as I previously shown by immunofluorescence staining, I confirmed that ectopic overexpression of mutp53^{R280K} recapitulates the reported alterations induced by miR-30d; concordantly, expression of the decoy-30d construct in presence of mutant p53 rescues the structure of the GA back to the normal condition (*Fig. 15*).

These ultrastructural analyses demonstrate that mutant p53, via miR-30d, induces a structural alteration of the GA, described as an enhanced tubulo-vesiculation of the organelle.

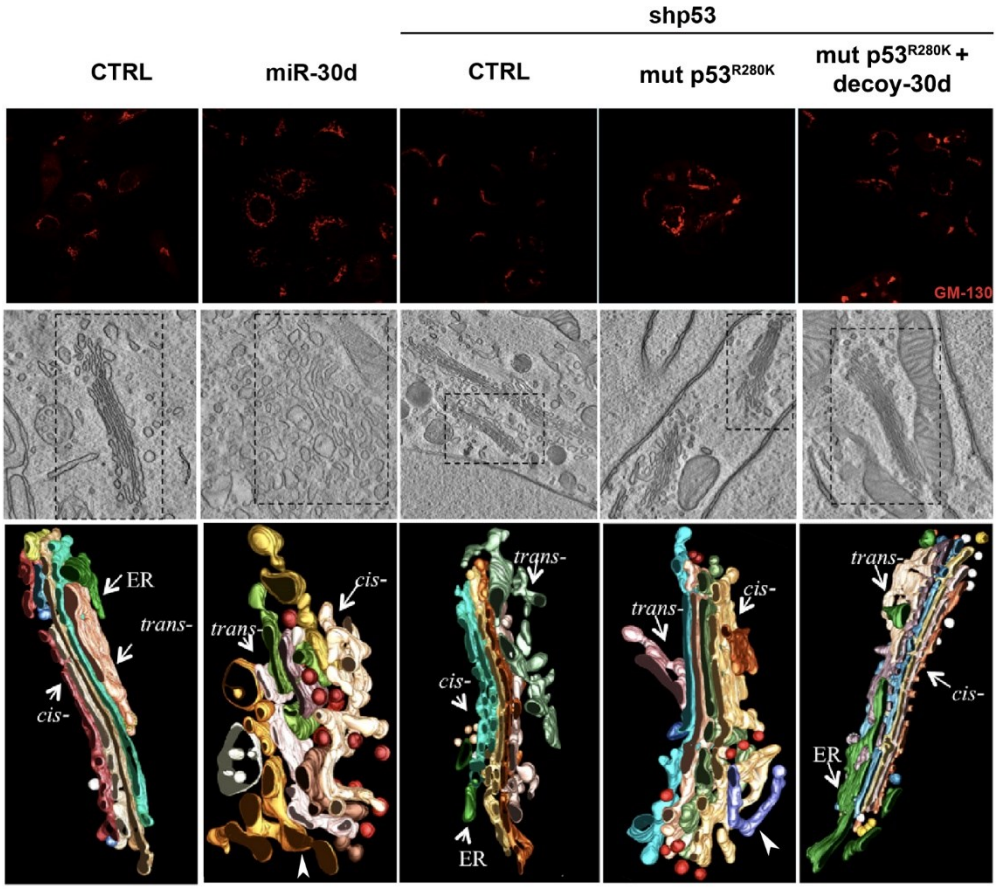

Fig. 15. Ultrastructural analysis of mutant p53/miR-30d-induced alteration of the Golgi apparatus.

CLEM analyses of the Golgi apparatus in MCF-10A cells with overexpression of miR-30d or silencing of wild-type p53 and ectopic introduction of mutp53^{R280K} concomitantly with decoy-miR-30d. *Top:* confocal super-resolution microscopy analysis of Golgi structure stained for GM-130 antigen; *middle:* electron tomography of the above samples; *bottom:* three-dimensional model of the Golgi stacks highlighted in the middle panels, showing the Golgi cisternae. Arrowheads indicate vesicular-tubular clusters in cells overexpressing miR-30d or mutp53^{R280K}.

5.3. Functional outcomes of the mutant p53/HIF1α/miR-30d-induced alterations of the secretory pathway

The results of the gene ontology clustering from the transcriptomic data derived from dy-30d-expressing MDA-MB-231 cells (*Fig. 13A)* revealed an enrichment for differentially expressed genes involved in the regulation of the unfolded protein response and of protein secretion. These processes, coupled with alterations of proteasomal protein degradation, already shown to be regulated by mutant p53[141], are in charge of regulating the secretory proteostasis. This term refers to an ensemble of pathways subtending the homeostatic regulation of protein levels throughout the secretory process, which could have major impact in miR-30d-regulated cancer-associated phenotypes.

5.3.1. miR-30d expression controls the activation of the Unfolded Protein Response

The evidence that the enlargement of the ER observed by immunofluorescence analysis of miR-30d-overexpressing MCF-10A cells (*Fig. 13B*) has already been correlated with UPR activity[164], and, coupled to the data obtained from transcriptomic analysis of MDA-MB-231 cells transduced with the dy-30d construct (*Fig. 13A*), which showed an enrichment cluster of differentially regulated genes correlated with the Unfolded Protein Response (UPR), led me to hypothesize that miR-30d levels could regulate the UPR activation upon ER stress induction.

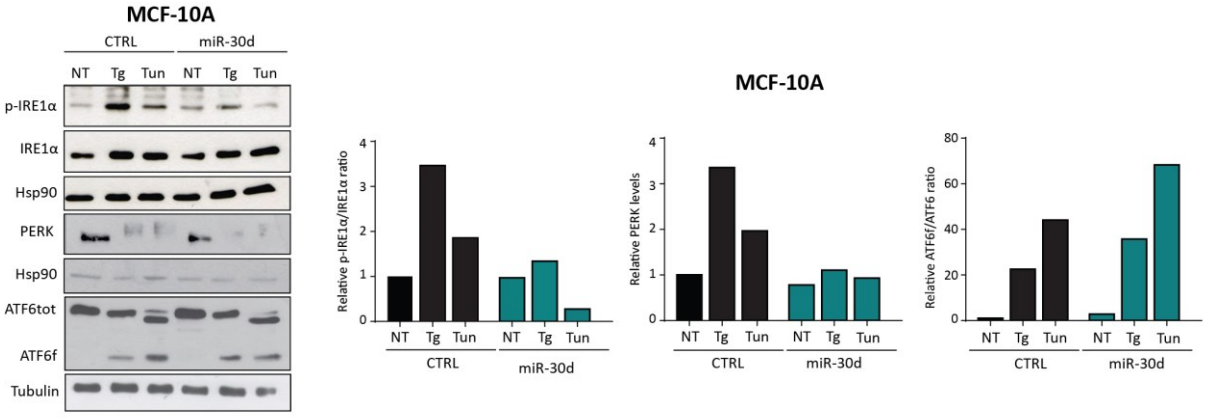

Fig. 16. miR-30d dampens the activation of the IRE1α/PERK branches of the UPR upon ER stress induction, while promoting the activation of ATF6

Western blot analyses (n=3) showing the levels of UPR activation marks (p-IRE1α/IRE1α ratio, PERK phosphorylation, ATF6 f/ATF6total ratio) upon ER stress induction by treatment with thapsigargin (Tg) or tunicamycin (Tun) in miR-30d-overexpressing MCF-10A cells. Hsp90 and tubulin (TUB) are shown as loading controls. Bar graphs are showing the quantification of the depicted Western blot band, accounting for the according normalizers.

To this aim, I performed Western blot analyses checking the ratio of phospho-IRE1α/IRE1α proteins, phospho-PERK and ATF6f/total ATF6 levels, as markers of UPR activation, in miR-30d-overexpressing MCF-10A cells upon induction of ER stress through treatment with thapsigargin or tunicamycin. As shown (*Fig. 16*), both in basal conditions and particularly upon ER stress induction, miR-30d presence was able to strongly downregulate the amount of phosphorylated IRE1α and PERK, while at the same time promoting the increase of the cleaved active ATF6 nuclear fragment. Since IRE1α and PERK pathways represent the pro-apoptotic branches of the UPR homeostatic response, while ATF6, on the contrary, promotes cell survival, these data point to a novel role for miR-30d, which could be sustaining cancer cells viability upon ER stress induction, a condition typical of proliferating malignant cells.

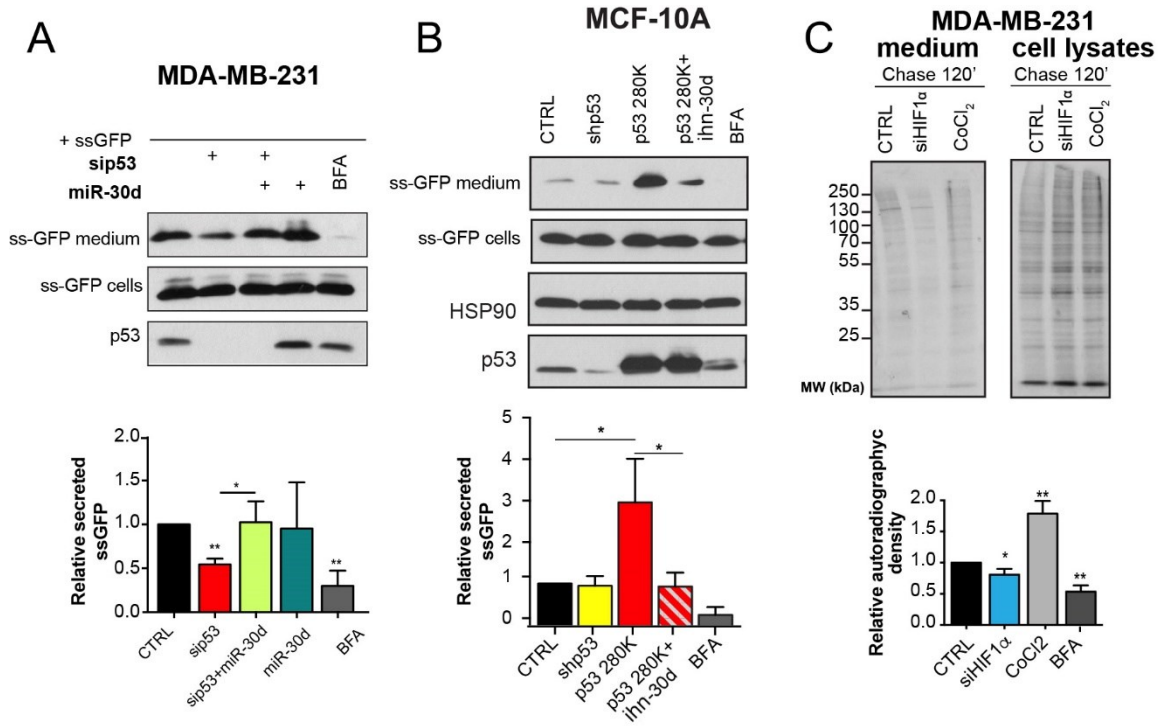

Fig. 17. Protein secretion is increased by the activity of the HIF1α/mutant p53/miR-30d axis

A. Western blot analyses of secreted ss-GFP reporter in MDA-MB-231 silenced for mutant p53 and with ectopic reintroduction of miR-30d and in *B.* MCF-10A cells silenced for the endogenous wild-type p53, with ectopic overexpression of mutant p53 concomitantly with transfection with the inhibitor of miR-30d. *C.* Autoradiography of secreted and intracellular radiolabeled protein from MDA-MB-231 cells either silenced for HIF1α or treated with cobalt chloride. All images are representative pictures of experiments conducted in triplicate; intracellular GFP (*ss-GFP cells*) is shown as normalizer of the secreted ss-GFP medium secreted reporter. p53 is shown as silencing control, Hsp90 as loading control. Intracellular amount of radiolabeled proteins is shown in the cell lysates autoradiography as normalizer for the amount of secreted proteins. Bar graphs are showing the average ± SEM of the normalized secreted proteins amount. *=p<0.05, **=p<0.01, unpaired Student's T-test. BFA: brefeldin A treatment, used as a negative control of cell secretion.

5.3.2. The HIF1α/mutant p53/miR-30d axis promotes protein secretion

Functional annotation of MDA-MB-231 dy-30d transcriptomic data (*Fig. 13A*) revealed an enrichment for a cluster of differentially regulated genes implied in protein secretion, a process which has been shown by several reports[162,165] to be closely related to the GA morphology.

Starting from the evidence that both mutant p53 and miR-30d induce, as shown (*Fig. 15)* a consistent vesiculo-tubulation of the Golgi apparatus, which has been previously described as an indicator of increased secretory rate in cells[162,163], I wanted to evaluate the impact of the mutant p53/miR-30d molecular axis on the cell secretion. To this aim, I generated, similarly to what has been previously described in literature[166], a reporter vector expressing a secreted form of the GFP protein, termed ssGFP, by cloning the signal sequence for the import in the ER derived from the rat FSHβ hormone at the N-terminus of GFP.

As shown (*Fig. 17A*), silencing of mutp53^{R280K} in MDA-MB-231 cells caused a reduction of the amount of secreted reporter, while the ectopic reintroduction of miR-30d rescued it back to the control level. Conversely, when mutp53 was ectopically overexpressed in MCF-10A cells, the amount of secreted GFP resulted increased, while inhibition of miR-30d in the same cells caused a significant reduction of the reporter secretion. (*Fig. 17B*).

To evaluate whether also if HIF1α stabilization was able to produce similar outcomes, I performed pulse-chase experiments with metabolic S^{35} protein radiolabeling. As shown (*Fig. 17C*), silencing endogenous HIF1α protein by RNAi in MDA-MB-231 decreased the levels of protein secretion, while treatment with cobalt chloride, conversely, increased the amount of radiolabeled proteins present in the culture medium.

Altogether, these results suggest that the mutp53/HIF1/miR-30d axis is able to stimulate protein secretion in cancer cells.

5.3.3. mutant p53 induces an altered secretome through miR-30d

In order to characterize which was the spectrum of modifications of the cell secretome induced by mutant p53 through miR-30d upregulation, a quantitative LC-MS/MS analyses of the secreted media from MDA-MB-231 cells either silenced for mutant p53^{R280K} or with the ectopic reintroduction of miR-30d in the same conditions has been performed in collaboration with Jacek Wisniewski from the Max Planck Institute in Munich.

To reduce intracellular contaminant proteins, and to retrieve only the specifically secreted ones, the data were filtered through an *in silico* pipeline previously described in literature[167]. Briefly, the

full list of proteins retrieved in the mass spectrometry experiments was used as input for the SignalP[168] online tool, which predicts the presence of signal peptides and the location of their cleavage sites in proteins. The remaining negative hits were screened for annotation of the keyword *signal* in the UniProtKB database[169], which indicates as well the presence of an annotated signal sequence in the protein. From these tools, 448 proteins resulted as containing the signal sequence for the entry in the ER, which is typical of classically secreted proteins. The remaining proteins were annotated via the GO cellular compartments terms[170], and the ones which were included in the GO:0005576 – extracellular region term, that includes proteins present in the space external to the outermost structure of a cell, were enlisted as 555 non-classically secreted proteins of type I.

From the left out proteins, I removed the ones enlisted in the GO:0005622 – intracellular gene ontology term, which includes the matter contained in, but not including, the plasma membrane, comprising the nucleus and the cytoplasm, and subjected the remaining ones to the SecretomeP web tool[171], which produces *ab initio* predictions of non-classical i.e. not signal peptide triggered protein secretion. The method queries a large number of other feature prediction servers to obtain information on various post-translational and localization aspects of the protein, which are integrated into the final secretion prediction. The resulting 33 positive hits were grouped as non-classically secreted proteins of type II. Altogether, in the mass spectrometry data, I reported a total of 1036 secreted proteins *(Fig. 18A*).

At this point, I performed differential expression analysis of the secreted proteins, in order to evaluate if mutant p53 and miR-30d had a relevant impact on their amount, and, as shown (*Fig. 18B*), mutant p53 significantly modulated the protein secretome of MDA-MB-231 cells, with both up- and down-regulation of a broad number of proteins; moreover, accordingly to the previous results, overexpression of miR-30d strongly reverted the effects of mutant p53 silencing. Additionally, I compared the analyzed secretome data with a previously published mutant p53-dependent transcriptome and proteome obtained in the same cell line in previous work produced by the hosting laboratory[157], revealing that only about the 30% (247 out of 815) of the proteins differentially secreted in relation to mutant p53 presence were also regulated at the transcript or protein levels upon mutant p53 knockdown (*Fig. 18C*). Even strongly, when comparing the secretome with the previously described miR-30d-depedent MDA-MB-231 transcriptome, only the 11% (107 proteins out of 988) of hits were common among the two analyses. *(Fig. 18D*). These results indicate that the effect of the depicted molecular axis on protein secretion is probably only

partly due to changes in the expression or stability of the secreted proteins and support previously described evidences that, instead, the mutp53/mir-30d axis might directly impinge on the secretory machinery function.

Interestingly, gene ontology clustering for GO biological processes terms using the DAVID bioinformatic platform[172,173] (*Fig.18E*) shows an enrichment of differentially secreted factors related to cell adhesion, extracellular matrix organization, angiogenesis, positive regulation of cell proliferation and repression of apoptosis, which are all functions that could have relevant impact on cancer cell development and aggressiveness, suggesting a putative role for mutant p53/miR-30d-dependent secretome in regulating oncogenic phenotypes in cancer cells.

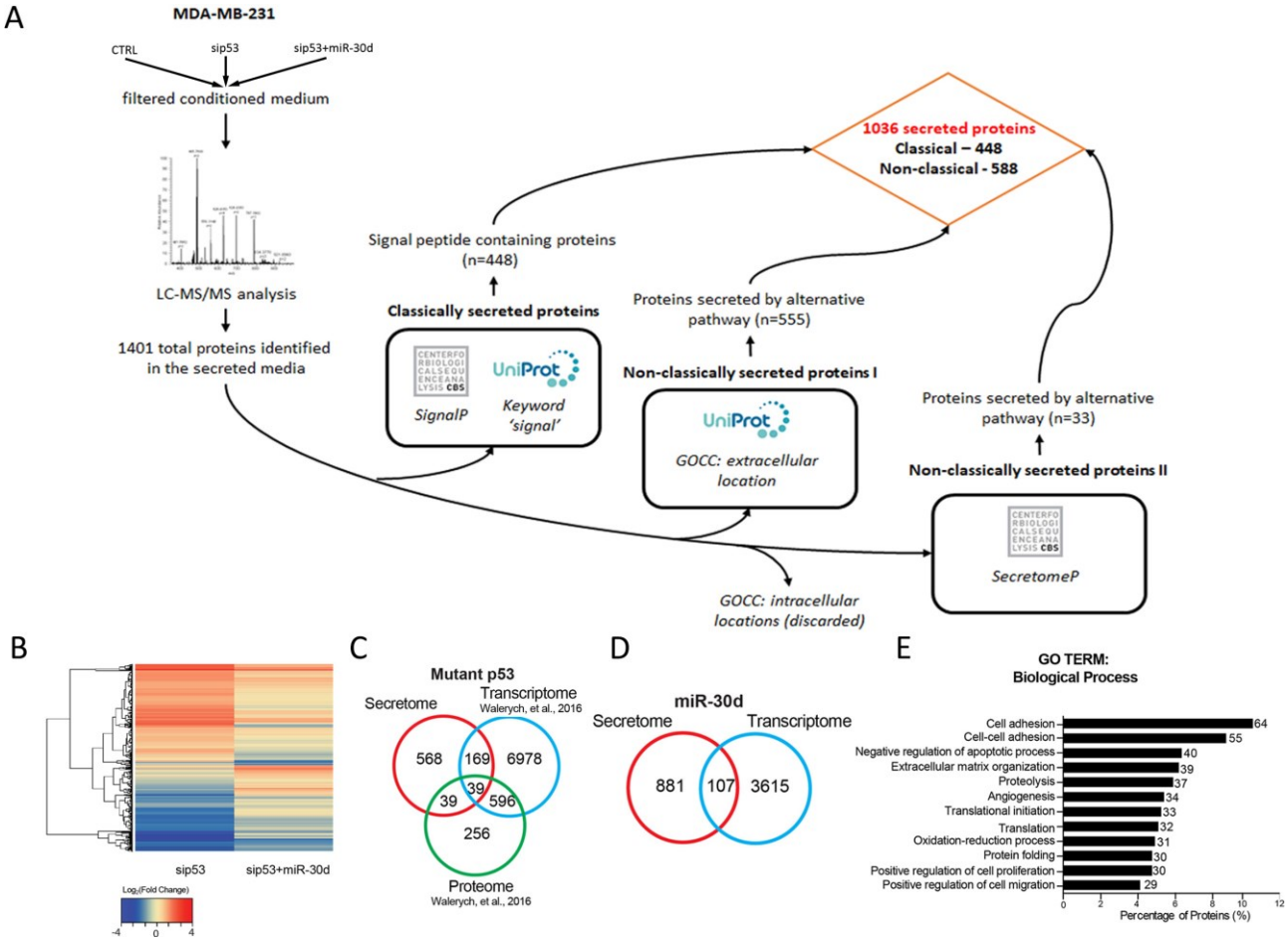

Fig. 18. Protein secretion is increased by the activity of the mutant p53/HIF1α/miR-30d molecular axis

A. Schematic representation of the workflow resulting in the selected secreted proteins. *B.* Heatmap with hierarchical dendrogram clustering of filtered genes differentially secreted upon silencing of mutant p53 in MDA-MB-231 cells and with ectopic reintroduction of miR-30d. *C.* Venn diagram showing the overlap between mutant p53-dependent secretome, transcriptome and proteome from MDA-MB-231 cells. *D.* Venn diagram showing the overlap between miR-30d-dependent secretome and transcriptome in MDA-MB-231 cells. *E.* Gene ontology analysis for GO biological process terms using the mutant p53/miR-30d-dependent secretome as input list. Bars are showing gene cluster coverage, while numbers are the absolute protein number included in the enrichment cluster from the input secretome. All terms were significant ($p<0.05$) upon Fisher exact test with Benjamini-Hochberg correction.

5.4. The mutant p53/HIF1α/miR-30d axis exerts its effects on the secretory pathway through the downregulation of specific miR-30d targets

5.4.1. The mutant p53/HIF1α/miR-30d axis downregulates DGKZ and VPS26B

The effect of miR-30d in controlling the vesiculo-tubulation of the Golgi apparatus, together with the consequent increase in secretion, could depend on its post-transcriptional regulation on direct and indirect targets. To identify direct targets of miR-30d, based on the evidence that this microRNA is under the transcriptional control of mutp53 and HIF1α, I intersected the lists of regulated genes from mutant p53 silencing in a transcriptome published by the hosting laboratory[157], from the silencing of HIF1α in a MDA-MB-231 transcriptome present in literature[140]

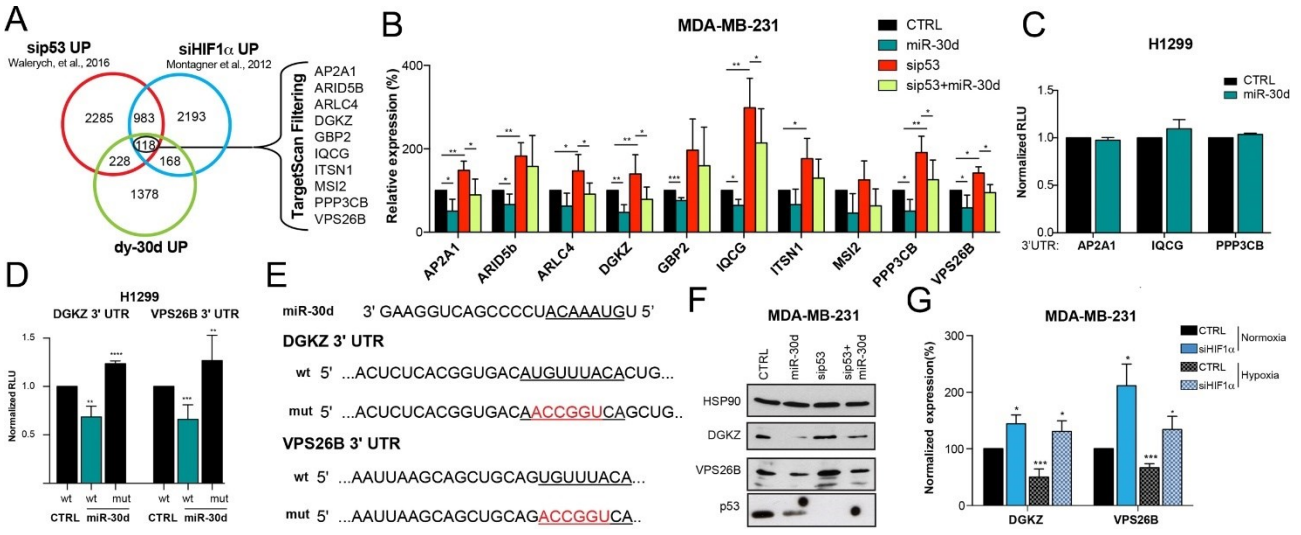

Fig. 19. DGKZ and VPS26B are bona fide targets of the mutant p53/HIF1α/miR-30d molecular axis

A. Venn diagram showing the overlap among genes regulated by mutant p53, HIF1α and miR-30d in the related transcriptomes, and successive filtering using the list of Targetscan predicted miR-30d targets. *B.* qRT-PCR experiments showing the expression levels of the putative miR-30d targets upon silencing of mutant p53 in MDA-MB-231 and ectopic reintroduction of miR-30d. Bars are showing the average ± SEM of three experiments normalized on the H3 expression levels; *=p<0.05, **=p<0.01, unpaired Student's T-test. *C-D.* Bar graph showing the relative luciferase units of the reporter vectors for the predicted miR-30d targets normalized on the firefly luciferase levels in H1299 cells overexpressing miR-30d. Bars are showing average ± SEM of three experiments. *E.* Schematic representation of miR-30d and its pairing with the wild-type and mutated sequence of the miRNA-responsive elements in the 3'UTR of DGKZ and VPS26B. *F.* Western blot showing the levels of DGKZ and VPS26B upon silencing of mutant p53 and concomitant expression of miR-30d in MDA-MB-231 cells. The image is representative of three experiments; p53 levels are shown as silencing control, Hsp90 levels as loading control. *G.* qRT-PCR experiments showing the expression levels of DGKZ and VPS26B upon silencing of HIF1α either in normoxic or hypoxic condition, normalized to the H3 expression levels. Bars are showing the average ± SEM of three experiments; *=p<0.05, ***=p<0.001, unpaired Student's T-test.

and in the decoy-30d-dependent transcriptome described in the previous paragraphs, reporting a list of 118 genes which are commonly regulated by the three members of the mutant p53/HIF1α/miR-30d molecular axis. Furthermore, by filtering this list with the miR-30d targets

predicted by the bioinformatic tool TargetScan[174], I annotated a list of ten miR-30d putative targets (*Fig. 19A*) which I proceeded to validate by qRT-PCR experiments in MDA-MB-231 silenced for mutant p53 and with ectopic reintroduction of miR-30d. As shown (*Fig. 19B*), among the ten selected genes, only five of them, namely AP2A1, DGKZ, IQCG, PPP3CB and VPS26B, showed increased levels upon mutant p53 silencing and rescue to control levels upon miR-30d reintroduction, and therefore I decided to proceed in the validation only with these five positive hits.

To provide formal demonstration of miR-30d action on these putative targets, I cloned the 3'UTR of these genes downstream of the *Renilla reniformis* luciferase gene in a dual-luciferase reporter pSI-CHECK2 vector, and transfected these constructs in H1299 cells, a non-small cell lung cancer cell null for p53 gene, together with miR-30d. As depicted (*Fig. 19C*), regarding AP2A1, IQCG and PPP3CB, miR-30d expression was not able to downregulate the expression of the reporter for these genes, while DGKZ and VPS26B were strongly diminished upon miR-30d transfection. Furthermore, mutating the miRNA-30d-responsive element (*Fig. 19D*) in the 3'UTR of these genes completely abolished miR-30d-induced downregulation (*Fig. 19E*), confirming DGKZ and VPS26B as *bona fide* direct miR-30d targets.

Concordantly, silencing of mutant p53 in MDA-MB-231 cells was able to upregulate the expression levels of these targets (*Fig. 19F*), whereas ectopic reintroduction of miR-30d brought back the levels of the proteins to the control ones. Analogously, while induction of hypoxia was able to downregulate the mRNA levels of both targets, contemporaneous silencing of HIF1α partially rescued their normal expression levels *(Fig. 19G*).

Summarizing, DGKZ and VPS26B are strong candidates as directs targets of the depicted mutant p53/HIF1α/miR-30d molecular axis, and these data prompted to further investigation of their role as putative mediators of the effects of these factors on the secretory pathway.

5.4.2. DGKZ and VPS26B downregulation mimics miR-30d effects on the secretory pathway structure and function

After validating DGKZ and VPS26B as probable targets of the described molecular axis, I wanted to evaluate their relevance and connection to the protein secretion process.

Giving the putative relation between these targets and the protein secretion pathway, they appeared as plausible mediators of miR-30d functions, and therefore I moved on to investigate if they could be mediating the depicted effects on the Golgi apparatus and on protein secretion.

To this aim, I performed immunofluorescence staining of the GA with anti-GM-130 antibody in MCF-10A cells silenced for either of the two targets, reporting a strong vesiculation of the Golgi apparatus very similar to the one described for miR-30d overexpression in the previous paragraphs (*Fig. 20A*).

Additionally, silencing of DGKZ or VPS26B, either alone or in combination, resulted in a strong downregulation of the secretion of the ss-GFP reporter in MCF-10A cells (*Fig. 20B*).

Together, these evidences confirm that downregulation of DGKZ and VPS26B is able to produce similar effects compared to miR-30d overexpression on the structure of the Golgi apparatus and on protein secretion in MCF-10A cells, and suggest that these two factors could play a role in the alterations of the morphology and functions of the secretory pathway induced by the mutant p53/HIF1α/miR-30d molecular axis.

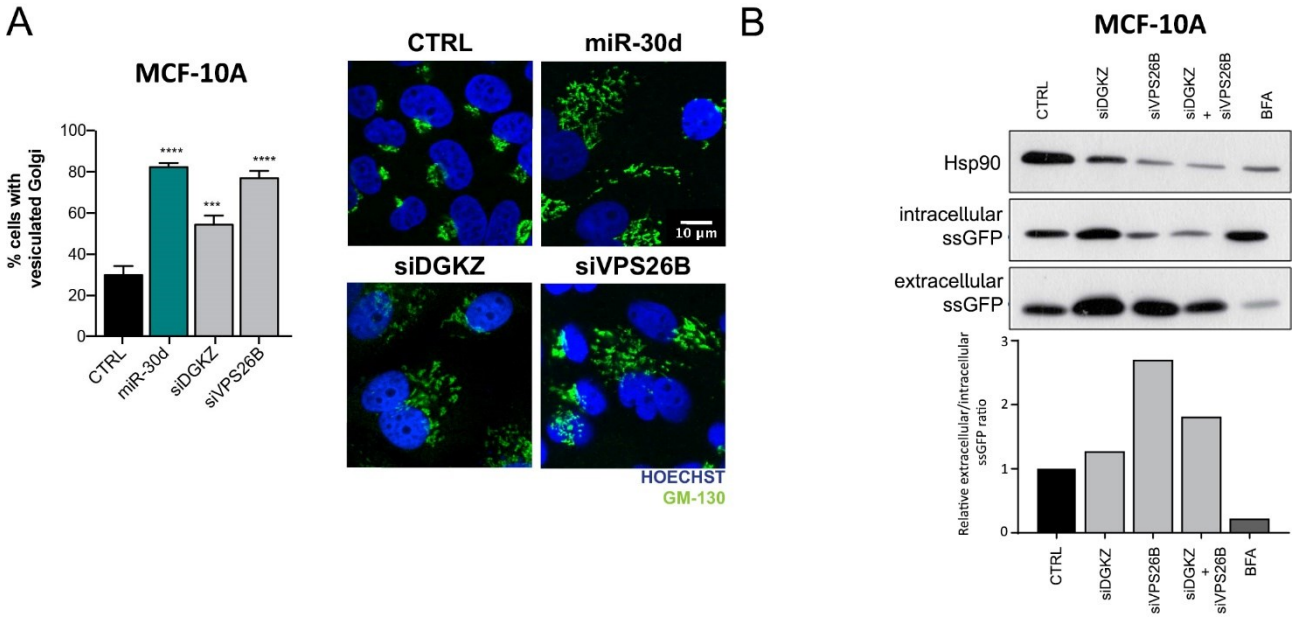

Fig. 20. Inhibition of DGKZ and VPS26B mediates the effect of the mutant p53/HIF1α/miR-30d molecular axis on the secretory pathway

A. Immunofluorescence analysis of the Golgi apparatus (anti-GM130 staining) in MCF-10A cells silenced for either DGKZ or VPS26B, or with miR-30d overexpression. Bar graph is showing the percentage of cells with vesiculated Golgi as average ± SEM of 30 HPF; ***=p<0.001, ****=p<0.0001, unpaired Student's T-test. *B.* Western blot of secreted and intracellular ssGFP reporter levels in MCF-10A cells silenced for DGKZ and VPS26B, either alone or in combination. Hsp90 is shown as loading control, BFA: brefeldin A treatment, used as negative control of cell secretion. Bar graph is showing the quantification of the ratio for the ssGFP levels in the depicted Western blot experiment, accounting for the according normalizer.

6. Discussion

The crosstalk between the cancer cells and the tumor microenvironment, comprising both its cellular and non-cellular components, is crucial for tumor growth, progression and metastasis. In fact, tumor cells actively reprogram the components of the related stroma, including activation of the cancer-associated fibroblasts (CAFs)[175], increased formation of blood vessels[176], recruitment of infiltrating immune cells[177] and increased ECM deposition and remodeling[178]. In this complex ecosystem, the interplay with the tumor microenvironment is exerted by the cancer cells both out of direct contact with the stromal cells, acting *via* membrane factors, and by indirect influence, through the release of secreted factors. Therefore, understanding the regulation of the secretory pathway and the consequent impact on the cell secretome appears pivotal for unveiling the communication network established within the tumor microenvironment.

In this thesis, I demonstrated that missense mutant forms of p53 induce structural and functional alterations of the morphology of the secretory pathway organelles *via* transcriptional induction of miR-30d, leading to enhanced secretory trafficking and release of a promalignant secretome that, in turn, might impact on the tumor microenvironment.

microRNA-30d is a member of the miR-30 family, which in humans comprises six members, namely miR-30a, -30b, -30c1, -30c2, -30d and -30e, sharing a common seed sequence, but harboring different regulatory flanking elements, allowing them to target different genes.

Regarding their function in cancer, miR-30 family members have been shown to display both oncosuppressive and oncogenic roles. In fact, miR-30a has been reported to inhibit the growth of renal cell carcinoma[179], medulloblastoma[180] and cervical cancer cells[181], while miR-30e is able to dampen the proliferation of breast cancer cells[182]. Similarly, miR-30c1 and miR-30c2 block the proliferation of osteosarcoma[183] and glioblastoma cells[184]. Conversely, contrasting evidences have been reported for miR-30b, showing both oncosuppressive[185] and oncogenic[186] roles in different cellular contexts.

Nevertheless, miR-30d gene has been reported to be frequently amplified and overexpressed in different types of tumors[187], and its expression has been clearly associated with aggressive neoplastic features in a broad range of contexts, including breast cancer, in which it promotes invasion and migration of cancer cells[145], and prostate cancer, where it favors neoangiogenesis and tumor growth[149]. Moreover, miR-30d has been shown to be secreted by various cell types, and to mediate different non cell-autonomous phenotypes both in physiological[188,189] and in pathological processes[190–192]. Nevertheless, evidences regarding the cell-autonomous role of miR-

30d in controlling the communication with the tumor microenvironment are still lacking in literature.

The results reported in this thesis suggest that mutp53 regulates miR-30d expression at a transcriptional level, since both pre- and pri-miR-30d appear to be regulated. Thus, given the fact that mutant p53s are not able to bind directly the DNA as their wild-type counterpart, the identification of an interactor that could act as a transcriptional regulator for miR-30d expression appeared pivotal. As reported, mutant p53 is able to interact with HIF1α, both in normoxic and hypoxic conditions, and its binding to lnc-30d/miR-30d promoter is dependent on HIF1α presence. Of note, the hypoxia-inducible factors HIF1α and HIF2α are able to promote the transcription of miR-30d also in non-mutant p53 hypoxic contexts[150], pointing to a putative role for the downstream effects of miR-30d in the regulation of the hypoxic stress responses. Therefore, microRNA-30d, downstream of the oncogenic action of mutant p53 exerted through the interaction with HIF1α, regulates the secretory pathway morphology and functions, putatively promoting cancer aggressive phenotypes.

In physiological contexts, increased secretion is linked to structural adaptations of the trafficking hubs, including enlargement of the ER[193] and tubulo-vesiculation of the Golgi apparatus[163]; therefore, it is conceivable that oncogenes may hijack these programs in order to enhance pro-malignant secretion in cancer cells. Accordingly, expansion of the Golgi network has been observed in cancer cell lines with high tumorigenic potential[194,195], and upregulation of an ER-Golgi trafficking signature has been shown to correlate with breast cancer metastasis[196].

Nevertheless, while it is widely accepted that alterations in the unfolded protein response activation[197–199] and in the Golgi apparatus morphology and secretory function are linked to cancer progression[67,200,201], little is known about the role of mutant p53 in the direct modulation of the secretory proteostasis. In fact, a compelling amount of works point to a central role of missense mutant forms of p53 in regulating the crosstalk between cancer cells and the surrounding stroma, but until now these phenotypes have been shown to be exerted through transcriptional regulation of genes encoding for secreted proteins[202–204].

Recent evidences, published in literature by our group[142] and by others[205], showed that missense p53 mutants are able to regulate the unfolded protein response, both by promoting the levels of the UDPase ENTPD5, which favors the folding of N-glycosilated proteins, and by shifting the balance between the UPR branches, dampening the activity of IRE1α and PERK, simultaneously promoting ATF6 activation, ultimately resulting in increased cancer cell survival.

Interestingly, preliminary evidences reported in this thesis show that miR-30d is able to produce a similar modulation of the UPR, dampening the pro-apoptotic IRE1α and PERK response and promoting the activation of ATF6 mediated prosurvival pathway. Altogether, these data suggest a novel way for mutant p53 to promote cell survival under ER stress conditions, which are typical of many solid cancers during development and outgrowth.

Interestingly, miR-30d, together with miR-30b, has been reported to target and downregulate the Golgi-resident GalNAc transferase enzyme GALNT7 in melanoma, indirectly promoting invasion and metastasis of cancer cells[146], and to downregulate the levels of BiP/GRP78, one of the key regulators of the ER unfolded protein response, in prostate cancer cells[206]; these data support our evidences that identify miR-30d as a regulator of the secretory pathway homeostasis.

Additionally, I discovered that the mutant p53/miR-30d axis is able to perturb the structure of the Golgi apparatus, promoting the formation of tubular continuities across the Golgi cisternae, resulting in rapid diffusion of soluble cargos and increased protein secretion.

Indeed, the expression of microRNA-30d is linked to major structural alterations of the secretory pathway components, resulting in an enlargement of the endoplasmic reticulum, an increase in number of both the COP-I and COP-II vesicles, a stabilization of the microtubules and a vesiculo-tubulation of the Golgi apparatus. Moreover, these morphologic alterations are linked to functional modifications of the related processes, comprising an aberrant UPR activation and an enhancement of general cell protein secretion.

Indeed, HIF1α activity has been widely linked to unfolded protein response modulation[207,208] and to the secretory pathway trafficking[209,210], further corroborating the notion that the mutant p53/HIF1α/miR-30d axis could play a central role in the regulation of the secretory proteostasis in cancer cells. Notably, activated HIF1α can induce miR-30d even in the absence of mutant p53, implying that hypoxia and other stimuli able to promote the levels of this factor might modify the secretory proteostasis *via* miR-30d induction even in non-mutant p53 contexts.

Interestingly, the data presented in this thesis show that the mutant p53/miR-30d axis fosters general protein secretion from cancer cells. In fact, when comparing the MDA-MB-231 cell secretome regulated by this axis to the transcriptomics and proteomics analysis previously published from our laboratory[211], we found that the majority of the secreted proteins did not appear to be regulated nor at the transcript nor at the intracellular protein levels, suggesting that the main action of the depicted molecular axis is exerted through a direct regulation on the protein secretion machinery.

Mechanistically, I demonstrated that miR-30d affects the Golgi structure via direct downregulation of two of its targets, DGKZ and VPS26B.

DGKZ is a kinase that acts as a negative regulator of diacylglycerol (DAG) in cell membranes; DAG impacts on the secretory proteostasis at multiple levels, promoting Golgi apparatus tubulo-vesiculation[212,213] and facilitating vesicular transport and secretion[214–216]; of note, a previously published high-throughput RNAi screening reported a fragmentation of the Golgi apparatus upon knockdown of the DGKZ expression[217], corroborating the evidences presented in this thesis work.

VPS26B, instead, is a member of the retromer complex, which is deputed to the backtrafficking of *trans*-plasmamembrane receptors to the endosomal compartment and to the retrograde transport from the endosomes to the trans-Golgi network[218], putatively remodeling endosomal membranes generating tubulo-vesicular structures[219], possibly cooperating in the modulation of the Golgi apparatus morphology.

In addition to these direct targets, looking at the miR-30d dependent transcriptomic profile we found that miR-30d enhances the expression of key components of the ER-Golgi vesicular trafficking machinery and reduced the expression of negative regulators of ER-Golgi trafficking and kinesin-mediated retrograde transport. Moreover, miR-30d induced the expression of genes involved in ER-related transport and folding and reduced the expression of genes involved in the ER stress program.

Therefore, the selected direct miR-30d targets, together with the indirect ones, point to a consistent regulation of the secretory pathway structures and functions by the described molecular axis.

Nevertheless, the functional relevance of the enhanced secretion and whether the mut-p53/miR-30d secretome impacts on the properties and activities of the tumor microenvironment needs to be better clarified. Interestingly, gene ontology analysis of the differentially secreted factors reported in the mutant p53/miR-30d-dependent secretome, unveiled that most of the described proteins are related to the extracellular matrix composition and remodeling, suggesting that the final outcome of these regulations could be tightly linked to cancer cell invasion, migration and metastatic spreading.

This work therefore unveils a novel mechanism by which cancer cell could regulate the secretory proteostasis and offers several targets for potential therapeutic intervention.

Indeed, the available strategies include the interference with mutant p53 gain of function, either through inhibition of its stabilization, *via* statin[137] or HSP90 inhibitors[220] treatment, or through

other mutant p53 inhibitors, such as PRIMA-1Met/APR-246[221]. Additionally, molecules downregulating HIF1α levels, such as paclitaxel or methoxyestradiol[222,223], could have a relevant use in the blockade of this axis. Moreover, the direct inhibition of miR-30d by the use of antisense oligonucleotides or miRNA decoys, together with the protection of the mRNAs of the selected targets *via* modified interfering oligonucleotides, could constitute a further step in effective therapeutical strategies.

Lastly, the functional outcome of miR-30d action could constitute an ensemble of druggable processes, through the use of molecules modulating the outcome of the UPR response[224], *via* the use of secretion inhibitors[66] and by the development of Golgi apparatus recompacting drugs, which development is still in the early phases[225].

7. Materials and methods

7.1. Cell cultures

7.1.1. Cell lines

MDA-MB-231, HEK-293T and HEK-293GP cells were cultured in DMEM medium (Lonza) supplemented with 10% FBS (Fetal Bovine Serum, Lonza). H1299 cells were cultured in RPMI (Lonza) medium supplemented with 10% FBS. MCF-10A cells were maintained in DMEM/Ham's F12 medium (Lonza) in a 1:1 ratio, supplemented with 5% HS (Horse Serum, Lonza), insulin (10 µg/ml, Sigma-Aldrich), hydrocortisone (0.5 µg/ml, Sigma-Aldrich) and Epidermal Growth Factor (EGF, 20 ng/ml, Peprotech). All media were supplemented with penicillin and streptomycin (100 IU/mL each, Lonza). All the cells described were maintained in a 37°C, 5% CO2 incubator.
Hypoxic conditions were obtained treating cells with 150µM CoCl2 (Sigma-Aldrich) for 24h.

7.1.2. Stably transduced cell lines

MDA-MB-231 cells with stable inhibition of miR-30d were obtained by lentiviral transduction with TWEEN-3'UTR-EGFP empty, that was used as a negative control, and with the decoy-miR-30d construct. The TWEEN-3'UTR-EGFP empty was kindly provided by R. De Maria, and the decoy-miR-30d was cloned as previously described[158].

MCF-10A cells silenced for endogenous wild-type p53 and overexpressing the mutp53^{R175H}, mutp53^{R273H} and mutp53^{R280K} forms were obtained by retroviral transduction with pRS-shp53 and the matching pRS-empty control vector, kindly provided by R. Agami, and either pMSCV-empty, pMSCV-mutp53^{R175H}, pMSCV-mutp53^{R273H} or pMSCV-mut p53^{R280K} vector, obtained by site-directed mutagenesis from the pMSCV-wild-type-p53 vector.

MCF-10A cells cells silenced for endogenous wild-type p53 and overexpressing the mutp53^{R175H}, mutp53^{R273H} and mutp53^{R280K} forms, together with the TWEEN-3'UTR-EGFP or decoy-30d vector, were obtained from the previously described cells, via lentiviral transduction of the inhibitory construct for miR-30d and its relative control vector as described for MDA-MB-231.

MCF-10A cells overexpressing miR-30d were obtained by retroviral transduction with pRS-Blast and miR-Vec-30d, kindly provided by R. Agami.

Infected cell populations were selected using puromycin (Sigma-Aldrich) and/or blasticidin (InvivoGen), 2µg/mL each, for at least one week.

7.1.3. Transfection

Cells were transfected when the culture reached 50-80% confluence level. For DNA transfections, the appropriate amount of DNA, depending on the total surface of the culture vessel, was used together with Lipofectamine 2000 or LTX transfection reagents, following manufacturer's instructions; for siRNA/miRNA mimic/miRNA inhibitor transfections, cells were transfected with 40nM siRNA oligonucleotides (Eurofins Genomics), 3nM miRNA mimic (Ambion) or 20nM miRNA inhibitor (Dharmacon) together with Lipofectamine RNAiMax (Invitrogen) following manufacturer's instructions; as a negative control, the Qiagen AllStars Negative Control siRNA was used. The sequences of these oligonucleotides are the following:

Oligonucleotide	***Sequence***	***Manufacturer***
control siRNA	Unknown	Qiagen AllStars Negative Control siRNA (Qiagen)
control miRNA inhibitor	miRIDIAN inhibitor - Unknown	Dharmacon
miR-30d inhibitor	miRIDIAN inhibitor - Unknown	Dharmacon
miR-30d mimic	Unknown	Ambion
sip53	GACUCCAGUGGUAAUCUAC	Eurofins MWG
siHIF1α	AGGAAGAACUAUGAACAUA	Eurofins MWG
siDGKZ	GAGGAACGACUUCUGUAAG	Eurofins MWG
siVPS26B	GAAGUUCUCUGUGCGCUAU	Eurofins MWG

For retrovirus production, low-confluence HEK-293GP packaging cells were transfected using calcium phosphate with the appropriate plasmids in combination with the pMD2.G packaging vector. For lentivirus production, low-confluence HEK-293T packaging cells were transfected using calcium phosphate with the appropriate plasmids in combination with the pMD2.G and ps-PAX2 packaging vectors. After 48–72 h the virus-containing medium was collected and filtered with 0.45 µM syringe filter to remove cellular debris and was added to the target cells.

7.2. Protein analyses

7.2.1. Protein extraction

Total cell extracts were lysed with Lysis Buffer (50mM Tris-HCl pH 7.5, 300mM NaCl, NP-40 1%, EDTA 1mM, all from Sigma-Aldrich) supplemented with PMSF 1 mM (Sigma-Aldrich), NaF 5mM (Sigma-Aldrich), Na_3VO_4 1mM (Sigma-Aldrich), 10 µg/ml CLAP (Sigma-Aldrich). Cells were then

centrifuged at 10000 rpm for 10 minutes at 4°C. Protein concentration was determined with Bio-Rad Protein Assay Reagent (Bio-Rad). Samples obtained were denatured in Laemmli Sample Buffer 2x or 6x. For the recovery of secreted proteins, 1 ml of CM was precipitated by addition of 100% Trichloroacetic acid (TCA, Sigma-Aldrich) to a final concentration of 10% and incubation at 4°C overnight. The following day, the samples were centrifuged at 13200 rpm for 15 min at 4°C. Pellets were resuspended in 0.4 ml 100% chilled acetone by vortexing, and protein precipitates were recovered by centrifugation at 13200 rpm for 10 min at 4°C and air-dried at room temperature for approximately 30 min. Finally, pellets were dissolved in Laemmli Sample Buffer 2x. All the samples were denatured by heating at 95°C for 5 min. For brefeldin-A (BFA) treatment, 2.5 μg/ml of BFA was added to the medium during all phases of the experiments

7.2.2. Western blot

Lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham). Blocking was performed in Blotto-tween (PBS, 0.2% Tween-20, not fat dry milk 5%, all from Sigma-Aldrich) or with TBST-milk (0.2% Tween-20, Tris/HCl 25mM pH 7.5, plus 5% not fat dry milk, all from Sigma-Aldrich) depending on the antibody. Anti-mouse and anti-rabbit HRPO-conjugated (Sigma-Aldrich) were used as secondary antibodies. Membranes were analyzed by chemiluminescence using ECL™ Western Blotting Reagents (Amersham) or LiteAblot Extend Long Lasting Chemiluminescent Substrate (EuroClone). The following primary antibody were used:

Target	***Antibody***
α-tubulin	T5168 (Sigma-Aldrich)
ATF6	ab122897 (Abcam)
β-actin	a9718 (Sigma-Aldrich)
DGKZ	HPA051336 (Sigma-Aldrich)
GFP	Home-made produced rabbit polyclonal
HIF1α (D2U3T)	#14179 (Cell Signaling Technologies)
Hsp90 (HSP90 α/β, F-8)	sc-13119 (Santa Cruz)
IRE1α	#3294 (Cell Signaling Technologies)
PERK	#3192 (Cell Signaling Technologies)
phospho-IRE1α (S724)	ab48187 (Abcam)
p53 (DO-1)	sc-129 (Santa Cruz)

p53 (FL-393)	sc-6243 (Santa Cruz)

Anti-mouse and anti-rabbit HRPO-conjugated (Sigma-Aldrich) antibodies were used as secondary antibodies for Western blot. Bands were quantified by densitometry of autoradiographic films using FIJI software[226].

7.2.3. Metabolic protein labeling

Cells were cultured in DMEM without L-methionine and L-cysteine for 3 h and then pulsed with 25μCi/ml of ^{35}S methionine and cysteine (EasyTag™ EXPRESS35S Protein Labeling Mix Perkin Elmer) for 1 h. The chase was performed in DMEM containing 0.25M L-methionine and 0.25M L-cysteine. For brefeldin-A (BFA) treatment, 2.5 μg/ml of BFA was added to the medium during all phases of the experiments. Medium and cell lysates were collected as described below and analyzed by SDS-PAGE followed by autoradiography. Lanes were quantified calculating the densitometry of autoradiographic film by the FIJI software[226], and normalized to the intracellular counterpart.

7.2.4. Secretome analysis

CM was collected as above, treated with 2% SDS and 0.05 M DTT in 0.1 M Tris-HCl and boiled for 5 min. Total protein concentration in the lysates and the peptide contents in the digests (see below) were assayed using a tryptophan fluorescence based WF-assay in microtiter plate format[227]. The lysates were processed using the MED FASP method with modifications as described[228]. Briefly, proteins were first cleaved overnight by endoproteinase LysC, and subsequently digested with trypsin (enzyme to protein ratio 1:50) for 2 h. Aliquots containing 5 μg total peptide were concentrated to a volume of ~5 μL and stored at -20°C. Analysis of peptide mixtures were performed using a QExactive HF mass spectrometer (Thermo-Fisher). Aliquots containing 2 □g total peptide were chromatographed on a 50 cm column with 75 μm inner diameter packed C18 material (Dr. Maisch GmbH). Peptide separation was carried out at 300 nL/min for 45 min using a two-step acetonitrile gradient 5-40% over the first 35 min and 40-95% for the following 10 min. The temperature of the column oven was 55□C. The mass spectrometer operated in data-dependent mode with survey scans acquired at a resolution of 50 000 at m/z 400 (transient time 256 ms). Top 15 most abundant isotope patterns with charge ≥ +2 from the survey scan (300-1650 m/z) were selected with an isolation window of 1.6 m/z and fragmented by HCD with normalized collision energies of 25. The maximum ion injection times for the survey scan and the MS/MS

scans were 20 and 60 ms, respectively. The ion target value for MS1 and MS2 scan modes was set to 3×106 and 105, respectively. The dynamic exclusion was 25 s and 10 ppm. Spectra were searched using MaxQuant software.

7.2.5. Imaging

Immunofluorescence staining was performed as previously described[137]. Briefly, cells were fixed in 4% paraformaldehyde for 10 min, washed in PBS, permeabilized with Triton 0.1% for 10 min and blocked in FBS 3% in PBS for 30 min. Antigen recognition was performed by incubating primary antibody for 1 h at 37°C and with secondary antibody for 30 min at 37°C (goat anti-mouse, and goat anti-rabbit Alexa Fluor 488, 568, 647, Life Technologies). Nuclei were counterstained with Hoechst 33342 (Life Technologies).

Golgi morphology was analyzed on ~300 cells for each condition/experiment. To quantify the numbers of Golgi stacks, cells were immunostained with an anti-GM130 antibody and imaged by LSM510 Meta (Zeiss) confocal microscope. CLEM analysis was performed as described[161,229]. The following primary antibodies were used:

Target	***Antibody***
α-tubulin	T5168 (Sigma-Aldrich)
β-COP	CSB-PA005783LA01HU (Cusabio)
CD-63 (H-193)	sc-15363 (Santa Cruz)
GM-130	610822 (BD)
PDIA5 (RL-90)	ab2792 (Abcam)
SEC24A	CSB-PA020950GA01HU (Cusabio)

7.3. Nucleic acids analyses

7.3.1. Plasmids

Plasmids pRS-shp53 and pRS vectors were kindly provided by R. Agami. miR-Vec constructs were part of the miR-Lib, provided in collaboration by R. Agami[230].

pLPC-ssGFP was obtained fusing the rat FSHb signal peptide upstream of the eGFP gene in the pLPC construct, as previously described[166].

psiCHECK2 3'UTR reporter constructs were obtained by cloning each 3'UTR into the psiCHECK2 (Promega) plasmid, downstream of the *Renilla reniformis* luciferase gene, between NotI and XhoI restriction sites. The psiCHECK2 vector also expresses *Photinus pyralis* luciferase, which is used to

normalize for the efficiency of plasmid transfection. The 3'UTRs sequences of AP2A1, DGKZ, IQCG, PPP3CB were obtained from Ensembl[231] and UCSC[155] databases and amplified from MDA-MB-231 genomic DNA with AccuPrime™ Taq DNA Polymerase High Fidelity (Invitrogen), following manufacturer's instructions. psiCHECK2-VPS26B-3'UTR was purchased from GenScript. In the psiCHECK2-DGKZ-3'UTR and psiCHECK2-VPS26B-3'UTR reporters the miR-30d putative binding sites were mutated by using Quick change II XL Site-Directed Mutagenesis kit (Stratagene), following manufacturer's instructions.

The lentiviral vector pTWEEN-3'UTR-EGFP empty was kindly provided by R. De Maria, and the miR-30d decoy was cloned as described[158]. p53 overexpressing constructs pMSCV-empty, pMSCV-mut $p53^{R175H,}$ pMSCV-mut $p53^{R273H}$ and pMSCV-mut $p53^{R280K}$ were previously described[141].

The primers used for the production of these vectors are the following:

Gene target	***Sequence***	***Direction***
PPP3CB 3'UTR	CATCATGACGTCCCCACTACTTCCCAGG	FW
PPP3CB 3'UTR	CATCATCATATGTGCAATTATCACTAATATTTTTCTTATTGT	RV
DGKZ 3'UTR	CATCATGACGTCACGAGCGCCTTCC	FW
DGKZ 3'UTR	CATCATCATATGAAAGAAAAAAAAATCCACTTTACTGAG	RV
AP2A1 3'UTR	CATCATGACGTCCCCTGGACTCTGCC	FW
AP2A1 3'UTR	CATCATCATATGTAGCGTCTCTCTGTTTATTCG	RV
IQCG 3'UTR	CATCATGACGTCCCAAGTTCCTTGTGTTCTG	FW
IQCG 3'UTR	CATCATCATATGATGGTTTACAGCTTTCGTTTTAT	RV
DGKZ 3'UTR mut	TTTACTGAGTCACACCCAGCTGACCGGTTGTCACCGTGAG AGTCCCGCCC	FW
DGKZ 3'UTR mut	GGGCGGGACTCTCACGGTGACAACCGGTCAGCTGGGTGT GACTCAGTAAA	RV
VPS26B 3'UTR mut	GCCATTGAAAAGATGACACATTAAGAAACATGACCGGTCT GCAGCTGCTTAATTACAAGTTGCACTGCTT	FW
VPS26B 3'UTR mut	AAGCAGTGCAACTTGTAATTAAGCAGCTGCAGACCGGTCA TGTTTCTTAATGTGTCATCTTTTCAATGGC	RV
Annealing of the rat FSHbeta ss	AGCTTATGATGAAGTCGATCCAGCTTTGCATCCTACTCTGG TGCTTGAGAGCAGTCTGCTGCCAT	UP
Annealing of the	GATCCATGGCAGCAGACTGCTCTCAAGCACCAGAGTAGGA	DW

rat FSHbeta ss	TGCAAAGCTGGATCGACTTCATCAT	
eGFP	CATCATGGATCCGTGAGCAAGGGCGAG	FW
eGFP	CATCATGAATTCTACTTGTACAGCTCGTCCA	RV
decoy-30d	TCGAGCTTCCAGTCGGGGATGTTTACAAGAGAACTTAGAG AACTTCTTCCAGTCGGGGATGTTTACAT	UP
decoy-30d	TAGATGTAAACATCCCCGACTGGAAGAAGTTCTCTAAGTT CTCTTGTAAACATCCCCGACTGGAAGC	DW

7.3.2. Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed as previously described[141]. Chromatin was immunoprecipitated with the p53 FL-393 (sc-6243, Santa Cruz Biotechnology) antibody. IgGs purified from rabbit serum were used as negative control (Santa Cruz Biotechnology). Co-immunoprecipitated DNA was analyzed by real-time PCR. Promoter occupancy was calculated as percent of input chromatin immunoprecipitated using the 2-ΔCt method. The primers used are the following:

Gene target	***Sequence***	***Direction***
MIR30D ChIP Binding	TATCTTGACACTTGAAGGCCCC	FW
MIR30D ChIP Binding	GCCTTGGCTGCCTGTGATA	RV
Non-specific region	CAACCAAAGCCCATGTCCTC	FW
Non-specific region	AGGCACGCTACAGGGCTTC	RV

7.3.3. RNA extraction and qRT-PCR

Cells were harvested in Qiazol lysis reagent (Qiagen) for total RNA extraction, and contaminant DNA was removed by DNAse treatment. qRT-PCR analyses were carried out on cDNAs retrotranscribed with Quantitect reverse transcription kit (Qiagen), and analyzed genes were amplified using SsoAdvancedTMSYBR® Green Master Mix (Biorad) on a CFX96™ Real-Time PCR System (Biorad). For miRNAs and the housekeeping control genes RNU6B and SNORD25 small nuclear RNA, 0.5 µg of total RNA were retrotranscribed and amplified with miScript PCR System (Qiagen) following manufacturer's instructions. The data were analyzed with the Biorad CFX Manager software. Experiments were performed at least three times, and each sample is the average of a technical duplicate. The quantification is based on the 2-ΔΔCt method using the proper housekeeping gene levels as normalization reference.

The primers used are the following:

qPCR primers miRNAs		
Gene target	***Accession number***	***miScript Primer Assay Catalog Number (Qiagen)***
SNORD25	NR_002565.1	MS00014007
RNU6B	NR_004394.1	MS00014000
hsa-miR-30d	MI0000255	MS00009387
hsa-pre-miR-30d	MI0000255	MP00001848
qPCR primers mRNAs		
Gene target	***Primer sequence***	***Direction***
AP2A1	TCATCTCCGACATCCGGAAC	FW
AP2A1	TCCAAGGCTTTGTCTCCTTTGA	REV
ARID5B	CACCTTTGACCACCCGACTC	FW
ARID5B	ATTTCACCTTGGCAACGGCT	REV
ARL4C	GAAACGCAGGAAGTCCCTCA	FW
ARL4C	TTGGTTCGCTCTTTGTTCGC	REV
DGKZ	AGCAGTACTGTGTAGCCAGGAT	FW
DGKZ	CACGGAAGGACGGCTTACAG	REV
GBP2	CTGCACAGGGACAGTGAGAG	FW
GBP2	AGTCATCTCGCCTTGCTTCC	REV
IQCG	CGAACTCACTGAGCTGGAAGT	FW
IQCG	AGTCTTCCAGGCTGTCTTCTTC	REV
ITSN1	GGTCCACTGCAGAAAAAGGTC	FW
ITSN1	GGGTTCTCCAGTTTGGCTTTC	REV
lnc-30d		FW
lnc-30d		REV
MSI2	AGCAAGAGGATCAGGCTCCA	FW
MSI2	GCCGTTGCAATCAAAGGTCC	REV
PPP3CB	CTCTGTTCTCAGGGAGGAGAGT	FW
PPP3CB	TCAGCCTCAATAGCCTCAACTG	REV

VPS26B	TTGGGATTGAGGACTGTCTGC	FW
VPS26B	TTCTCATGGTACACGTTGGGG	REV
Actin	CGCCGCCAGCTCACCATG	FW
Actin	CACGATGGAGGGGAAGACGG	REV
H3	GAAGAAACCTCATCGTTACAGGCCTGGT	FW
H3	CTGCAAAGCACCAATAGCTGCACTCTGGAA	REV

7.3.4. Luciferase reporter assays

H1299 cells were seeded in 60 mm dishes and transfected with 2 µg of psiCHECK2 3'UTR reporter vectors. After 24 h the cells were splitted in 2 wells of a 24-well plate, and transfected either with 3nM of miR-30d-mimic or with miR-Negative Control. 6h after transfection, medium was changed and 18h later luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega) on a Promega luminometer. Relative Luciferase Units (RLU) were calculated by normalizing the luciferase units measured for the *Renilla reniformis* luciferase on the luciferase units of the *Photinus phyralis* luciferase in each sample.

7.4. Omic data generation and analyses

7.4.1. Microarray data generation and analysis

For microarray analysis of genes regulated by miR-30d, total RNA (2 µg) was isolated from MDA-MB-231 cells expressing miR-30d decoy or control vector. For each experimental condition, three biological replicates were prepared and processed in parallel. RNA concentration, quality and purity were determined using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc.). Synthesis of cDNA and biotinylated cRNA (from 500 ng total RNA) was performed using the Illumina TotalPrep RNA Amplification Kit (Ambion), according to the manufacturer's protocol. Quality assessment and quantification of total RNA and cRNAs were performed with Agilent RNA kits on a Bioanalyzer 2100 System (Agilent). Hybridization of cRNAs (750 ng) was carried out using Illumina Human 48K gene chips (Human HT-12 v4 Expression BeadChip). Array chip washing was performed in High Temp Wash Buffer (Illumina) at 55°C for 10 min, followed by staining using streptavidin-Cy3 dyes (Amersham Biosciences). Hybridized arrays were stained and scanned in a BeadStation 500 System (Illumina). GenomeStudio Data Analysis Software's Gene Expression Module (GSGX) Version 1.9 was used and cubic spline normalization was applied to the data. The average signal was used for performing the analysis ("AVG_Signal") using limma in the

Bioconductor suite[232]. Differentially expressed genes were identified using Significance Analysis of Microarray algorithm coded in the samr R package[233], estimating the percentage of false-positive predictions (FDR).

7.4.2. Gene ontology analysis of the secreted proteins

For the secretome analysis, the differentially secreted proteins were identified performing t-test analysis of calculated LFQ-values. Then, starting the list of 1036 putative secreted proteins, was filtered including differentially secreted proteins with a Benjamini-Hochberg adjusted p-value ≤0.05 in cells depleted of mutant p53 compared to control cells. These proteins were further filtered comparing the obtained list of mut-p53 regulated proteins with the proteins whose basal secretion is restored by miR-30d reintroduction. The heatmap was produced with and ggplot2 package for R, from the Bioconductor suite[234].

7.4.3. Selection of microRNA-30d putative targets

RNA sequencing data from MDA-MB-231 cells with siRNA of mut-p53 were obtained from the GEO dataset GSE682481[141], from where we selected differentially expressed genes as those with a reported Benjamini-Hochberg adjusted p-value ≤0.05. Microarray data from MDA-MB-231 cells with shRNA depletion of HIF1α were obtained from the GEO dataset GSE339502[140], we selected as differentially expressed genes the ones with a False Discovery Rate (FDR) ≤0.05. Microarray data from MDA-MB-231 cells with functional inhibition of miR-30d function were obtained (see above), and we selected as differentially expressed genes the ones with a Benjamini-Hochberg adjusted p-value ≤0.05. Intersecting the lists of upregulated genes using Venny[235], 112 genes that were upregulated by mutant p53 depletion, HIF1α depletion, or miR-30d functional inhibition were obtained. This list was then intersected with the 1569 genes that are predicted as miR-30d targets by TargetScan[174] obtaining the 10 genes shown.

8. Bibliography

1. Dicker, D. *et al.* Global, regional, and national age-sex-specific mortality and life expectancy, 1950-2017: A systematic analysis for the Global Burden of Disease Study 2017. *Lancet* **392**, 1684–1735 (2018).
2. Nordling, C. O. A new theory on cancer-inducing mechanism. *Br. J. Cancer* **7**, 68–72 (1953).
3. Knudson, a G. Mutation and cancer: statistical study of retinoblastoma. *Proc. Natl. Acad. Sci. U. S. A.* **68**, 820–823 (1971).
4. Nowell, P. C. The clonal evolution of tumor cell populations. *Science* **194**, 23–28 (1976).
5. Mardis, E. R. Insights from Large-Scale Cancer Genome Sequencing. *Annu. Rev. Cancer Biol.* **2**, 429–444 (2018).
6. Hanahan, D. & Weinberg, R. a. Hallmarks of cancer: the next generation. *Cell* **144**, 646–74 (2011).
7. Fouad, Y. A. & Aanei, C. Revisiting the hallmarks of cancer. *American Journal of Cancer Research* (2017).
8. Cadwell, C. & Zambetti, G. P. The effects of wild-type p53 tumor suppressor activity and mutant p53 gain-of-function on cell growth. *Gene* (2001). doi:10.1016/S0378-1119(01)00696-5
9. Hickson, I. D. & Bhowmick, R. The 'enemies within': Regions of the genome that are inherently difficult to replicate. *F1000Research* (2017). doi:10.12688/f1000research.11024.1
10. Kitao, H. *et al.* DNA replication stress and cancer chemotherapy. *Cancer Science* (2018). doi:10.1111/cas.13455
11. Dong, W., Keibler, M. A. & Stephanopoulos, G. Review of metabolic pathways activated in cancer cells as determined through isotopic labeling and network analysis. *Metab. Eng.* **43**, 113–124 (2017).
12. Heiden, M. G. Vander, Cantley, L. C. & Thompson, C. B. Understanding the warburg effect: The metabolic requirements of cell proliferation. *Science* (2009). doi:10.1126/science.1160809
13. Boroughs, L. K. & DeBerardinis, R. J. Metabolic pathways promoting cancer cell survival and growth. *Nat. Cell Biol.* **17**, 351–359 (2015).
14. Smith, B. *et al.* Addiction to Coupling of the Warburg Effect with Glutamine Catabolism in Cancer Cells. *Cell Rep.* **17**, 821–836 (2016).
15. Warburg, O. On the origin of cancer cells. *Science (80-.).* (1956). doi:10.1126/science.123.3191.309
16. Liberti, M. V & Locasale, J. W. The Warburg Effect: How Does it Benefit Cancer Cells? *Trends Biochem. Sci.* **41**, 211–218 (2016).
17. Carracedo, A., Cantley, L. C. & Pandolfi, P. P. Cancer metabolism: Fatty acid oxidation in the limelight. *Nat. Rev. Cancer* (2013). doi:10.1038/nrc3483
18. Yang, L., Venneti, S. & Nagrath, D. Glutaminolysis: A Hallmark of Cancer Metabolism. *Annu. Rev. Biomed. Eng.* **19**, 163–194 (2017).
19. Provenzano, P. P. & Keely, P. J. Mechanical signaling through the cytoskeleton regulates cell proliferation by coordinated focal adhesion and Rho GTPase signaling. *J. Cell Sci.* (2011). doi:10.1242/jcs.067009
20. Levayer, R. Solid stress, competition for space and cancer: The opposing roles of mechanical cell competition in tumour initiation and growth. *Semin. Cancer Biol.* 0–1 (2019). doi:10.1016/j.semcancer.2019.05.004
21. Padera, T. P. *et al.* Cancer cells compress intratumour vessels. *Nature* (2004). doi:10.1038/427695a
22. Clevers, H. At the crossroads of inflammation and cancer. *Cell* (2004).

doi:10.1016/j.cell.2004.09.005
23. Eftekhari, R. *et al.* Study of the tumor microenvironment during breast cancer progression. *Cancer Cell Int.* (2017). doi:10.1186/s12935-017-0492-9
24. Setrerrahmane, S. & Xu, H. Tumor-related interleukins: Old validated targets for new anti-cancer drug development. *Molecular Cancer* (2017). doi:10.1186/s12943-017-0721-9
25. Gál, P. *et al.* How Signaling Molecules Regulate Tumor Microenvironment: Parallels to Wound Repair. *Molecules* (2017). doi:10.3390/molecules22111818
26. Rodríguez, J. A. HLA-mediated tumor escape mechanisms that may impair immunotherapy clinical outcomes via T-cell activation (Review). *Oncology Letters* (2017). doi:10.3892/ol.2017.6784
27. Glodde, N. & Hölzel, M. RAS and PD-L1: A Masters' Liaison in Cancer Immune Evasion. *Immunity* (2017). doi:10.1016/j.immuni.2017.12.001
28. Nicolini, A., Ferrari, P., Rossi, G. & Carpi, A. Tumour growth and immune evasion as targets for a new strategy in advanced cancer. *Endocr. Relat. Cancer* (2018). doi:10.1530/ERC-18-0142
29. Muz, B., de la Puente, P., Azab, F. & Azab, A. K. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. *Hypoxia* 83 (2015). doi:10.2147/hp.s93413
30. Luoto, K. R., Kumareswaran, R. & Bristow, R. G. Tumor hypoxia as a driving force in genetic instability. *Genome Integrity* (2013). doi:10.1186/2041-9414-4-5
31. Vaupel, P. & Mayer, A. Hypoxia in cancer: Significance and impact on clinical outcome. *Cancer and Metastasis Reviews* (2007). doi:10.1007/s10555-007-9055-1
32. Carmeliet, P. Angiogenesis in life, disease and medicine. *Nature* (2005). doi:10.1038/nature04478
33. Carmeliet, P. & Jain, R. K. Molecular mechanisms and clinical applications of angiogenesis. *Nature* (2011). doi:10.1038/nature10144
34. Azab, A. K. *et al.* Hypoxia promotes dissemination of multiple myeloma through acquisition of epithelial to mesenchymal transition-like features. *Blood* (2012). doi:10.1182/blood-2011-09-380410
35. Thiery, J. P. & Sleeman, J. P. Complex networks orchestrate epithelial-mesenchymal transitions. *Nature Reviews Molecular Cell Biology* (2006). doi:10.1038/nrm1835
36. Trachootham, D., Alexandre, J. & Huang, P. Targeting cancer cells by ROS-mediated mechanisms: A radical therapeutic approach? *Nat. Rev. Drug Discov.* **8**, 579–591 (2009).
37. Gorrini, C., Harris, I. S. & Mak, T. W. Modulation of oxidative stress as an anticancer strategy. *Nat. Rev. Drug Discov.* **12**, 931–947 (2013).
38. Gogvadze, V., Orrenius, S. & Zhivotovsky, B. Mitochondria in cancer cells: what is so special about them? *Trends in Cell Biology* (2008). doi:10.1016/j.tcb.2008.01.006
39. Denicola, G. M. *et al.* Oncogene-induced Nrf2 transcription promotes ROS detoxification and tumorigenesis. *Nature* (2011). doi:10.1038/nature10189
40. Li, W. & Kong, A. N. Molecular mechanisms of Nrf2-mediated antioxidant response. *Molecular Carcinogenesis* (2009). doi:10.1002/mc.20465
41. Possemato, R. *et al.* Functional genomics reveal that the serine synthesis pathway is essential in breast cancer. *Nature* (2011). doi:10.1038/nature10350
42. Locasale, J. W. *et al.* Phosphoglycerate dehydrogenase diverts glycolytic flux and contributes to oncogenesis. *Nat. Genet.* (2011). doi:10.1038/ng.890
43. Jain, M. *et al.* Metabolite profiling identifies a key role for glycine in rapid cancer cell proliferation. *Science (80-.).* (2012). doi:10.1126/science.1218595
44. Wang, Y. *et al.* The Unfolded Protein Response Induces the Angiogenic Switch in Human Tumor Cells through the PERK/ATF4 Pathway. *Cancer Res.* **72**, 5396–5406 (2012).

45. Anelli, T. & Sitia, R. Protein quality control in the early secretory pathway. *EMBO J.* **27**, 315–327 (2008).
46. Schröder, M. & Kaufman, R. J. THE MAMMALIAN UNFOLDED PROTEIN RESPONSE. *Annu. Rev. Biochem.* (2005). doi:10.1146/annurev.biochem.73.011303.074134
47. Gong, J. *et al.* Molecular signal networks and regulating mechanisms of the unfolded protein response. *Journal of Zhejiang University: Science B* (2017). doi:10.1631/jzus.B1600043
48. Chakrabarti, A., Chen, A. W. & Varner, J. D. A review of the mammalian unfolded protein response. *Biotechnology and Bioengineering* (2011). doi:10.1002/bit.23282
49. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T. & Mori, K. XBP1 mRNA Is Induced by ATF6 and Spliced by IRE1 in Response to ER Stress to Produce a Highly Active Transcription Factor. *Cell* **107**, 881–891 (2015).
50. Urano, F. *et al.* Coupling of Stress in the ER to Activation of JNK Protein Kinases by Transmembrane Protein Kinase IRE1. *Sci.* **287**, 664–666 (2000).
51. Maurel, M., Chevet, E., Tavernier, J. & Gerlo, S. Getting RIDD of RNA: IRE1 in cell fate regulation. *Trends in Biochemical Sciences* (2014). doi:10.1016/j.tibs.2014.02.008
52. Harding, H. P. *et al.* An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol. Cell* (2003). doi:10.1016/S1097-2765(03)00105-9
53. McCullough, K. D., Martindale, J. L., Klotz, L.-O., Aw, T.-Y. & Holbrook, N. J. Gadd153 Sensitizes Cells to Endoplasmic Reticulum Stress by Down-Regulating Bcl2 and Perturbing the Cellular Redox State. *Mol. Cell. Biol.* (2001). doi:10.1128/mcb.21.4.1249-1259.2001
54. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T. & Mori, K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* (2001). doi:10.1016/S0092-8674(01)00611-0
55. Wang, H. G. *et al.* Ca2+-Induced Apoptosis Through Calcineurin Dephosphorylation of BAD. *Science (80-.).* (1999). doi:10.1126/science.284.5412.339
56. Tanjore, H. *et al.* Alveolar epithelial cells undergo epithelial-to-mesenchymal transition in response to endoplasmic reticulum stress. *J. Biol. Chem.* (2011). doi:10.1074/jbc.M110.181164
57. Meusser, B., Hirsch, C., Jarosch, E. & Sommer, T. ERAD: The long road to destruction. *Nat. Cell Biol.* **7**, 766–772 (2005).
58. Sasaki, K. & Yoshida, H. Organelle autoregulation - Stress responses in the ER, Golgi, mitochondria and lysosome. *Journal of Biochemistry* (2015). doi:10.1093/jb/mvv010
59. Taniguchi, M. *et al.* TFE3 is a BHLH-ZIP-type transcription factor that regulates the Mammalian Golgi stress response. *Cell Struct. Funct.* (2015). doi:10.1247/csf.14015
60. Sasaki, K. & Yoshida, H. Golgi stress response and organelle zones. *FEBS Lett.* (2019). doi:10.1002/1873-3468.13554
61. Reiling, J. H. *et al.* A Creb3–arf4 Signalling Pathway Mediates the Response to Golgi Stress and Susceptibility to Pathogens. *Nat. Cell Biol.* (2013). doi:10.1038/ncb2865
62. Miyata, S., Mizuno, T., Koyama, Y., Katayama, T. & Tohyama, M. The Endoplasmic Reticulum-Resident Chaperone Heat Shock Protein 47 Protects the Golgi Apparatus from the Effects of O-Glycosylation Inhibition. *PLoS One* (2013). doi:10.1371/journal.pone.0069732
63. Burchell, J. M., Beatson, R., Graham, R., Taylor-Papadimitriou, J. & Tajadura-Ortega, V. O-linked mucin-type glycosylation in breast cancer. *Biochemical Society Transactions* (2018). doi:10.1042/BST20170483
64. Padler-Karavani, V. Aiming at the sweet side of cancer: Aberrant glycosylation as possible target for personalized-medicine. *Cancer Letters* (2014). doi:10.1016/j.canlet.2013.10.005
65. Karagiannis, G. S., Pavlou, M. P. & Diamandis, E. P. Cancer secretomics reveal pathophysiological pathways in cancer molecular oncology. *Mol. Oncol.* **4**, 496–510 (2010).

66. Dejeans, N. *et al.* Addicted to secrete - novel concepts and targets in cancer therapy. *Trends in Molecular Medicine* (2014). doi:10.1016/j.molmed.2013.12.003
67. Petrosyan, A. Onco-Golgi: Is Fragmentation a Gate to Cancer Progression? *Biochem. Mol. Biol. J.* (2015). doi:10.21767/2471-8084.100006
68. Wortzel, I., Koifman, G., Rotter, V., Seger, R. & Porat, Z. High Throughput Analysis of Golgi Structure by Imaging Flow Cytometry. *Sci. Rep.* **7**, 788 (2017).
69. Klein, C. A. Parallel progression of primary tumours and metastases. *Nature Reviews Cancer* (2009). doi:10.1038/nrc2627
70. Vogelstein, B. & Kinzler, K. W. The multistep nature of cancer. *Trends in Genetics* (1993). doi:10.1016/0168-9525(93)90209-Z
71. Suvà, M. L., Riggi, N. & Bernstein, B. E. Epigenetic reprogramming in cancer. *Science* **339**, 1567–70 (2013).
72. Croce, C. M. Oncogenes and cancer. *New England Journal of Medicine* (2008). doi:10.1056/NEJMra072367
73. Levitt, N. C. & Hickson, I. D. Caretaker tumour suppressor genes that defend genome integrity. *Trends in Molecular Medicine* (2002). doi:10.1016/S1471-4914(02)02298-0
74. Giaccia, A. J. & Kastan, M. B. The complexity of p53 modulation: Emerging patterns from divergent signals. *Genes and Development* (1998). doi:10.1101/gad.12.19.2973
75. Hu, W., Feng, Z. & Levine, A. J. The Regulation of Multiple p53 Stress Responses is Mediated through MDM2. *Genes and Cancer* (2012). doi:10.1177/1947601912454734
76. Bieging, K. T. Unravelling mechanisms of p53-mediated tumour suppression - Bieging et al 2014. *Nat. Rev. Cancer* **14**, 359–370 (2014).
77. Bykov, V. J. N. & Wiman, K. G. Novel cancer therapy by reactivation of the p53 apoptosis pathway. *Annals of Medicine* (2003). doi:10.1080/07853890310017152
78. Khoury, M. P. & Bourdon, J. The Isoforms of the p53 Protein The Isoforms of the p53 Protein. *Spring* 1–11 (2010). doi:10.1101/cshperspect.a000927
79. Joruiz, S. M. & Bourdon, J. C. P53 isoforms: Key regulators of the cell fate decision. *Cold Spring Harb. Perspect. Med.* **6**, (2016).
80. Bourdon, J. C. p53 and its isoforms in cancer. *British Journal of Cancer* (2007). doi:10.1038/sj.bjc.6603886
81. el-Deiry, W. S. *et al.* WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res.* (1994).
82. Fornace, A. J., Wolff, A. C., Chen, C. Y. & Kastan, M. B. The p53-dependent G1 Cell Cycle Checkpoint Pathway and Ataxia-Telangiectasia. *Cancer Res.* (1994).
83. Hermeking, H. *et al.* 14-3-3σ is a p53-regulated inhibitor of G2/M progression. *Mol. Cell* (1997). doi:10.1016/S1097-2765(00)80002-7
84. Qian, Y. & Chen, X. Senescence regulation by the p53 protein family. *Methods Mol. Biol.* (2013). doi:10.1007/978-1-62703-239-1_3
85. Mihara, M. *et al.* p53 has a direct apoptogenic role at the mitochondria. *Mol. Cell* (2003). doi:10.1016/S1097-2765(03)00050-9
86. Giorgi, C. *et al.* P53 at the endoplasmic reticulum regulates apoptosis in a Ca 2+ -dependent manner. *Proc. Natl. Acad. Sci. U. S. A.* (2015). doi:10.1073/pnas.1410723112
87. Horn, H. F. & Vousden, K. H. Coping with stress: Multiple ways to activate p53. *Oncogene* **26**, 1306–1316 (2007).
88. Maréchal, A. & Zou, L. DNA damage sensing by the ATM and ATR kinases. *Cold Spring Harb. Perspect. Biol.* (2013). doi:10.1101/cshperspect.a012716
89. Moll, U. M. & Petrenko, O. The MDM2-p53 Interaction. *Molecular Cancer Research* (2003).
90. Williams, A. B. & Schumacher, B. p53 in the DNA-damage-repair process. *Cold Spring Harb.*

Perspect. Med. **6**, 1–15 (2016).
91. Mayo, L. D. & Donner, D. B. The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network. *Trends in Biochemical Sciences* (2002). doi:10.1016/S0968-0004(02)02166-7
92. Okoshi, R. *et al.* Activation of AMP-activated protein kinase induces p53-dependent apoptotic cell death in response to energetic stress. *J. Biol. Chem.* (2008). doi:10.1074/jbc.M705232200
93. Lee, S. M., Kim, J. H., Cho, E. J. & Youn, H. D. A nucleocytoplasmic malate dehydrogenase regulates p53 transcriptional activity in response to metabolic stress. *Cell Death Differ.* (2009). doi:10.1038/cdd.2009.5
94. Okorokov, A. L. & Milner, J. An ATP/ADP-Dependent Molecular Switch Regulates the Stability of p53-DNA Complexes. *Mol. Cell. Biol.* (1999). doi:10.1128/mcb.19.11.7501
95. Schwartzenberg-Bar-Yoseph, F., Armoni, M. & Karnieli, E. The Tumor Suppressor p53 Down-Regulates Glucose Transporters GLUT1 and GLUT4 Gene Expression. *Cancer Res.* (2004). doi:10.1158/0008-5472.CAN-03-0846
96. Kondoh, H. *et al.* Glycolytic enzymes can modulate cellular life span. *Cancer Res.* (2005).
97. Bensaad, K. *et al.* TIGAR, a p53-Inducible Regulator of Glycolysis and Apoptosis. *Cell* (2006). doi:10.1016/j.cell.2006.05.036
98. Vousden, K. H. & Ryan, K. M. P53 and metabolism. *Nat. Rev. Cancer* **9**, 691–700 (2009).
99. Okamura, S. *et al.* Identification of seven genes regulated by wild-type p53 in a colon cancer cell line carrying a well-controlled wild-type p53 expression system. *Oncol. Res.* (1999).
100. Matoba, S. *et al.* p53 regulates mitochondrial respiration. *Science (80-.).* (2006). doi:10.1126/science.1126863
101. Bourdon, A. *et al.* Mutation of RRM2B, encoding p53-controlled ribonucleotide reductase (p53R2), causes severe mitochondrial DNA depletion. *Nat. Genet.* (2007). doi:10.1038/ng2040
102. Levine, A. J., Feng, Z., Mak, T. W., You, H. & Jin, S. Coordination and communication between the p53 and IGF-1-AKT-TOR signal transduction pathways. *Genes and Development* (2006). doi:10.1101/gad.1363206
103. Buzzai, M. *et al.* Systemic treatment with the antidiabetic drug metformin selectively impairs p53-deficient tumor cell growth. *Cancer Res.* (2007). doi:10.1158/0008-5472.CAN-06-4447
104. Hammond, E. M., Denko, N. C., Dorie, M. J., Abraham, R. T. & Giaccia, A. J. Hypoxia Links ATR and p53 through Replication Arrest. *Mol. Cell. Biol.* (2002). doi:10.1128/mcb.22.6.1834-1843.2002
105. Hammond, E. M., Dorie, M. J. & Giaccia, A. J. ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. *J. Biol. Chem.* (2003). doi:10.1074/jbc.M212360200
106. Chen, D., Li, M., Luo, J. & Gu, W. Direct interactions between HIF-1α and Mdm2 modulate p53 function. *J. Biol. Chem.* (2003). doi:10.1074/jbc.C200694200
107. Lee, S. J. *et al.* Protein phosphatase 1 nuclear targeting subunit is a hypoxia inducible gene: Its role in post-translational modification of p53 and MDM2. *Cell Death Differ.* (2007). doi:10.1038/sj.cdd.4402111
108. Galban, S. *et al.* Influence of the RNA-Binding Protein HuR in pVHL-Regulated p53 Expression in Renal Carcinoma Cells. *Mol. Cell. Biol.* (2003). doi:10.1128/mcb.23.20.7083-7095.2003
109. Hammond, E. M. & Giaccia, A. J. The role of p53 in hypoxia-induced apoptosis. *Biochemical and Biophysical Research Communications* (2005). doi:10.1016/j.bbrc.2005.03.154
110. Fei, P. *et al.* Bnip3L is induced by p53 under hypoxia, and its knockdown promotes tumor growth. *Cancer Cell* (2004). doi:10.1016/j.ccr.2004.10.012
111. Schmid, T., Zhou, J., Köhl, R. & Brüne, B. p300 relieves p53-evoked transcriptional repression of hypoxia-inducible factor-1 (HIF-1). *Biochem. J.* (2004). doi:10.1042/BJ20031299
112. Hammond, E. M. *et al.* Genome-Wide Analysis of p53 under Hypoxic Conditions. *Mol. Cell.*

Biol. (2006). doi:10.1128/mcb.26.9.3492-3504.2006
113. Chandel, N. S., Vander Heiden, M. G., Thompson, C. B. & Schumacker, P. T. Redox regulation of p53 during hypoxia. *Oncogene* (2000). doi:10.1038/sj.onc.1203727
114. Bragado, P., Armesilla, A., Silva, A. & Porras, A. Apoptosis by cisplatin requires p53 mediated p38α MAPK activation through ROS generation. *Apoptosis* (2007). doi:10.1007/s10495-007-0082-8
115. Kurz, E. U. & Lees-Miller, S. P. DNA damage-induced activation of ATM and ATM-dependent signaling pathways. *DNA Repair* (2004). doi:10.1016/j.dnarep.2004.03.029
116. Persons, D. L., Yazlovitskaya, E. M. & Pelling, J. C. Effect of extracellular signal-regulated kinase on p53 accumulation in response to cisplatin. *J. Biol. Chem.* (2000). doi:10.1074/jbc.M004267200
117. Liu, B., Chen, Y. & St. Clair, D. K. ROS and p53: A versatile partnership. *Free Radical Biology and Medicine* (2008). doi:10.1016/j.freeradbiomed.2008.01.011
118. Sablina, A. A. *et al.* The antioxidant function of the p53 tumor suppressor. *Nat. Med.* (2005). doi:10.1038/nm1320
119. Lin, W. C. *et al.* Endoplasmic reticulum stress stimulates p53 expression through NF-κB activation. *PLoS One* (2012). doi:10.1371/journal.pone.0039120
120. Qu, L. K. *et al.* Endoplasmic reticulum stress induces p53 cytoplasmic localization and prevents p53-dependent apoptosis by a pathway involving glycogen synthase kinase-3β. *Genes Dev.* (2004). doi:10.1101/gad.1165804
121. Mlynarczyk, C. & Fåhraeus, R. Endoplasmic reticulum stress sensitizes cells to DNA damage-induced apoptosis through p53-dependent suppression of p21 CDKN1A. *Nat. Commun.* (2014). doi:10.1038/ncomms6067
122. Namba, T. *et al.* Loss of p53 enhances the function of the endoplasmic reticulum through activation of the IRE1α/XBP1 pathway. *Oncotarget* (2015). doi:10.18632/oncotarget.4598
123. Namba, T. *et al.* CDIP1-BAP31 Complex Transduces Apoptotic Signals from Endoplasmic Reticulum to Mitochondria under Endoplasmic Reticulum Stress. *Cell Rep.* (2013). doi:10.1016/j.celrep.2013.09.020
124. Byun, S., Namba, T. & Lee, S. W. Losing p53 loosens up ER-stress. *Aging* (2015). doi:10.18632/aging.100847
125. Bailey, M. H. *et al.* Comprehensive Characterization of Cancer Driver Genes and Mutations. *Cell* (2018). doi:10.1016/j.cell.2018.02.060
126. Mantovani, F., Collavin, L. & Del Sal, G. Mutant p53 as a guardian of the cancer cell. *Cell Death and Differentiation* (2019). doi:10.1038/s41418-018-0246-9
127. Kim, M. P. & Lozano, G. Mutant p53 partners in crime. *Cell Death Differ.* 1–8 (2017). doi:10.1038/cdd.2017.185
128. Senft, D. & Ronai, Z. A. Adaptive Stress Responses During Tumor Metastasis and Dormancy. *Trends in Cancer* **2**, 429–442 (2016).
129. Schulz-Heddergott, R. & Moll, U. M. Gain-of-function (GOF) mutant p53 as actionable therapeutic target. *Cancers* (2018). doi:10.3390/cancers10060188
130. Frum, R. A. *et al.* Constitutive activation of DNA damage checkpoint signaling contributes to mutant p53 accumulation via modulation of p53 Ubiquitination. *Mol. Cancer Res.* (2016). doi:10.1158/1541-7786.MCR-15-0363
131. Zhang, C. *et al.* Tumour-associated mutant p53 drives the Warburg effect. *Nat. Commun.* (2013). doi:10.1038/ncomms3935
132. Zhou, G. *et al.* Gain-of-Function Mutant p53 Promotes Cell Growth and Cancer Cell Metabolism via Inhibition of AMPK Activation. *Mol. Cell* (2014). doi:10.1016/j.molcel.2014.04.024

133. Freed-Pastor, W. A. *et al.* Mutant p53 disrupts mammary tissue architecture via the mevalonate pathway. *Cell* (2012). doi:10.1016/j.cell.2011.12.017
134. Parrales, A., Thoenen, E. & Iwakuma, T. The interplay between mutant p53 and the mevalonate pathway. *Cell Death and Differentiation* (2018). doi:10.1038/s41418-017-0026-y
135. Basu, S. *et al.* Mutant p53 controls tumor metabolism and metastasis by regulating PGC-1α. *Genes Dev.* (2018). doi:10.1101/gad.309062.117
136. Sorrentino, G. *et al.* Metabolic control of YAP and TAZ by the mevalonate pathway. *Nat. Cell Biol.* **16**, 357–366 (2014).
137. Ingallina, E. *et al.* Mechanical cues control mutant p53 stability through a mevalonate-RhoA axis. *Nat. Cell Biol.* (2018). doi:10.1038/s41556-017-0009-8
138. Amelio, I. *et al.* p53 mutants cooperate with HIF-1 in transcriptional regulation of extracellular matrix components to promote tumor progression. *Proc. Natl. Acad. Sci.* **115**, E10869–E10878 (2018).
139. Kamat, C. D. *et al.* Mutant p53 facilitates pro-angiogenic, hyperproliferative phenotype in response to chronic relative hypoxia. *Cancer Lett.* (2007). doi:10.1016/j.canlet.2006.08.017
140. Montagner, M. *et al.* SHARP1 suppresses breast cancer metastasis by promoting degradation of hypoxia-inducible factors. *Nature* **487**, 380–384 (2012).
141. Walerych, D. *et al.* Proteasome machinery is instrumental in a common gain-of-function program of the p53 missense mutants in cancer. *Nat. Cell Biol.* **18**, 897–909 (2016).
142. Sicari, D. *et al.* Mutant p53 improves cancer cells' resistance to endoplasmic reticulum stress by sustaining activation of the UPR regulator ATF6. *Oncogene* 1–13 (2019). doi:10.1038/s41388-019-0878-3
143. Volinia, S. *et al.* A microRNA expression signature of human solid tumors defines cancer gene targets. (2005).
144. Li, N. *et al.* A combined array-based comparative genomic hybridization and functional library screening approach identifies mir-30d as an oncomir in cancer. *Cancer Res.* **72**, 154–64 (2012).
145. Han, M. *et al.* microRNA-30d mediated breast cancer invasion, migration, and EMT by targeting KLF11 and activating STAT3 pathway. *J. Cell. Biochem.* (2018). doi:10.1002/jcb.26767
146. Gaziel-Sovran, A. *et al.* miR-30b/30d regulation of GalNAc transferases enhances invasion and immunosuppression during metastasis. *Cancer Cell* **20**, 104–18 (2011).
147. Yao, J. *et al.* MicroRNA-30d promotes tumor invasion and metastasis by targeting Galphai2 in hepatocellular carcinoma. *Hepatology* **51**, 846–56 (2010).
148. Yang, X. *et al.* mir-30d Regulates multiple genes in the autophagy pathway and impairs autophagy process in human cancer cells. *Biochem. Biophys. Res. Commun.* **431**, 617–22 (2013).
149. Lin, Z. *et al.* MicroRNA-30d promotes angiogenesis and tumor growth via MYPT1 / c-JUN / VEGFA pathway and predicts aggressive outcome in prostate cancer. 1–14 (2017). doi:10.1186/s12943-017-0615-x
150. Camps, C. *et al.* Integrated analysis of microRNA and mRNA expression and association with HIF binding reveals the complexity of microRNA expression regulation under hypoxia. *Mol. Cancer* **13**, 28 (2014).
151. Kim, E. & Deppert, W. Interactions of mutant p53 with DNA: Guilt by association. *Oncogene* (2007). doi:10.1038/sj.onc.1210312
152. Schödel, J. *et al.* High-resolution genome-wide mapping of HIF-binding sites by ChIP-seq. *Blood* **117**, (2011).
153. Corcoran, D. L. *et al.* Features of mammalian microRNA promoters emerge from polymerase II chromatin immunoprecipitation data. *PLoS One* **4**, (2009).
154. Zanconato, F. *et al.* Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers

drives oncogenic growth. *Nat. Cell Biol.* **17**, 1218–1227 (2015).
155. Haeussler, M. *et al.* The UCSC Genome Browser database: 2019 update. *Nucleic Acids Res.* (2019). doi:10.1093/nar/gky1095
156. Kent, W. *et al.* UCSC Genome Browser. *Genome Research* **12**, 996–1006 (2002).
157. Walerych, D. *et al.* Proteasome machinery is instrumental in a common gain-of-function program of the p53 missense mutants in cancer. *Nat. Cell Biol.* **18**, 897–909 (2016).
158. Bonci, D. *et al.* The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities. *Nat. Med.* **14**, 1271–7 (2008).
159. Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 15545–50 (2005).
160. Muñoz-Sánchez, J. & Chánez-Cárdenas, M. E. The use of cobalt chloride as a chemical hypoxia model. *Journal of Applied Toxicology* (2019). doi:10.1002/jat.3749
161. Mironov, A. A. & Beznoussenko, G. V. Correlative light-electron microscopy: A potent tool for the imaging of rare or unique cellular and tissue events and structures. in *Methods in Enzymology* (2012). doi:10.1016/B978-0-12-391857-4.00010-0
162. Beznoussenko, G. V. *et al.* Transport of soluble proteins through the Golgi occurs by diffusion via continuities across cisternae. *Elife* (2014). doi:10.7554/eLife.02009
163. Trucco, A. *et al.* Secretory traffic triggers the formation of tubular continuities across Golgi sub-compartments. *Nat. Cell Biol.* (2004). doi:10.1038/ncb1180
164. Oslowski, C. M. & Urano, F. Measuring ER stress and the unfolded protein response using mammalian tissue culture system. in *Methods in Enzymology* (2011). doi:10.1016/B978-0-12-385114-7.00004-0
165. Chia, J. *et al.* RNAi screening reveals a large signaling network controlling the Golgi apparatus in human cells. *Mol. Syst. Biol.* **8**, 629 (2012).
166. Roh, J. Y. *et al.* Modification of enhanced green fluorescent protein for secretion out of cells. *Biotechnol. Bioprocess Eng.* **18**, 1135–1141 (2013).
167. Deshmukh, A. S., Cox, J., Jensen, L. J., Meissner, F. & Mann, M. Secretome analysis of lipid-induced insulin resistance in skeletal muscle cells by a combined experimental and bioinformatics workflow. *J. Proteome Res.* **14**, 4885–4895 (2015).
168. Petersen, T. N., Brunak, S., von Heijne, G. & Nielsen, H. SignalP 4.0: discriminating signal peptides from transmembrane regions. *Nat. Methods* **8**, 785–6 (2011).
169. Bateman, A. UniProt: A worldwide hub of protein knowledge. *Nucleic Acids Res.* (2019). doi:10.1093/nar/gky1049
170. Carbon, S. *et al.* AmiGO: Online access to ontology and annotation data. *Bioinformatics* (2009). doi:10.1093/bioinformatics/btn615
171. Bendtsen, J. D., Jensen, L. J., Blom, N., Von Heijne, G. & Brunak, S. Feature-based prediction of non-classical and leaderless protein secretion. *Protein Eng. Des. Sel.* **17**, 349–356 (2004).
172. Huang, D. W., Lempicki, R. A. & Sherman, B. T. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**, 44–57 (2009).
173. Huang, D. W., Sherman, B. T. & Lempicki, R. a. Bioinformatics enrichment tools: Paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* **37**, 1–13 (2009).
174. Agarwal, V., Bell, G. W., Nam, J. W. & Bartel, D. P. Predicting effective microRNA target sites in mammalian mRNAs. *Elife* **4**, (2015).
175. Liu, T., Zhou, L., Li, D., Andl, T. & Zhang, Y. Cancer-associated fibroblasts build and secure the tumor microenvironment. *Frontiers in Cell and Developmental Biology* (2019). doi:10.3389/fcell.2019.00060
176. Farnsworth, R. H., Lackmann, M., Achen, M. G. & Stacker, S. A. Vascular remodeling in cancer.

Oncogene (2014). doi:10.1038/onc.2013.304
177. Gonzalez, H., Hagerling, C. & Werb, Z. Roles of the immune system in cancer: From tumor initiation to metastatic progression. *Genes and Development* (2018). doi:10.1101/GAD.314617.118
178. Laplane, L., Duluc, D., Larmonier, N., Pradeu, T. & Bikfalvi, A. The Multiple Layers of the Tumor Environment. *Trends in Cancer* **4**, 802–809 (2018).
179. Wang, C. *et al.* MicroRNA-30a-5p Inhibits the Growth of Renal Cell Carcinoma by Modulating GRP78 Expression. *Cell. Physiol. Biochem.* 2405–2419 (2017). doi:10.1159/000484394
180. Singh, S. V. *et al.* Restoration of miR-30a expression inhibits growth, tumorigenicity of medulloblastoma cells accompanied by autophagy inhibition. *Biochem. Biophys. Res. Commun.* **491**, 946–952 (2017).
181. Zhao, J., Li, B., Shu, C., Ma, Y. & Gong, Y. Downregulation of miR-30a is associated with proliferation and invasion via targeting MEF2D in cervical cancer. *Oncol. Lett.* 7437–7442 (2017). doi:10.3892/ol.2017.7114
182. Liu, M. M. *et al.* MiR-30e inhibits tumor growth and chemoresistance via targeting IRS1 in Breast Cancer. *Sci. Rep.* **7**, 1–10 (2017).
183. Zhang, X. D. *et al.* Biological function of microRNA-30c/SOX9 in pediatric osteosarcoma cell growth and metastasis. *Eur. Rev. Med. Pharmacol. Sci.* (2018). doi:10.26355/eurrev-201801-14102
184. Liu, S., Li, X. & Zhuang, S. miR-30c impedes glioblastoma cell proliferation and migration by targeting SOX9. *Oncol. Res.* (2019). doi:10.3727/096504018X15193506006164
185. Sun, X. *et al.* MicroRNA-30b Suppresses Epithelial-Mesenchymal Transition and Metastasis of Hepatoma Cells. *J. Cell. Physiol.* (2017). doi:10.1002/jcp.25466
186. Jin, L. *et al.* Identification of miR-30b as an oncogene in renal cell carcinoma. *Mol. Med. Rep.* (2017). doi:10.3892/mmr.2017.6197
187. Li, N. *et al.* A combined array-based comparative genomic hybridization and functional library screening approach identifies mir-30d as an oncomir in cancer. *Cancer Res.* (2012). doi:10.1158/0008-5472.CAN-11-2484
188. Vilella, F. *et al.* Hsa-miR-30d, secreted by the human endometrium, is taken up by the pre-implantation embryo and might modify its transcriptome. *Development* **142**, 3210–3221 (2015).
189. Balaguer, N. *et al.* MicroRNA-30d deficiency during preconception affects endometrial receptivity by decreasing implantation rates and impairing fetal growth. *Am. J. Obstet. Gynecol.* (2019). doi:10.1016/j.ajog.2019.02.047
190. Zhu, B. *et al.* Serum miR-30d as a novel biomarker for multiple myeloma and its antitumor role in U266 cells through the targeting of the MTDH/PI3K/Akt signaling pathway. *Int. J. Oncol.* **53**, 2131–2144 (2018).
191. Xie, L. *et al.* Cell-free miRNAs may indicate diagnosis and docetaxel sensitivity of tumor cells in malignant effusions. *BMC Cancer* **10**, 591 (2010).
192. Fleming, N. H. *et al.* Serum-based miRNAs in the prediction and detection of recurrence in melanoma patients. *Cancer* **121**, 51–59 (2015).
193. Plate, L. & Wiseman, R. L. Regulating Secretory Proteostasis through the Unfolded Protein Response: From Function to Therapy. *Trends Cell Biol.* **xx**, 1–16 (2017).
194. Wortzel, I., Koifman, G., Rotter, V., Seger, R. & Porat, Z. High Throughput Analysis of Golgi Structure by Imaging Flow Cytometry. *Sci. Rep.* (2017). doi:10.1038/s41598-017-00909-y
195. Halberg, N. *et al.* PITPNC1 Recruits RAB1B to the Golgi Network to Drive Malignant Secretion. *Cancer Cell* **29**, 339–353 (2016).
196. Howley, B. V., Link, L. A., Grelet, S., El-Sabban, M. & Howe, P. H. A CREB3-regulated ER-Golgi

trafficking signature promotes metastatic progression in breast cancer. *Oncogene* **37**, 1308–1325 (2018).
197. Wang, S. & Kaufman, R. J. The impact of the unfolded protein response on human disease. *J. Cell Biol.* **197**, 857–867 (2012).
198. Vandewynckel, Y. P. *et al.* The paradox of the unfolded protein response in cancer. *Anticancer Research* (2013).
199. Ma, Y. & Hendershot, L. M. The role of the unfolded protein response in tumour development: Friend or foe? *Nature Reviews Cancer* (2004). doi:10.1038/nrc1505
200. Kellokumpu, S., Sormunen, R. & Kellokumpu, I. Abnormal glycosylation and altered Golgi structure in colorectal cancer: Dependence on intra-Golgi pH. *FEBS Lett.* **516**, 217–224 (2002).
201. Farber-Katz, S. E. *et al.* DNA damage triggers golgi dispersal via DNA-PK and GOLPH3. *Cell* **156**, 413–427 (2014).
202. Cordani, M. *et al.* Mutant p53 proteins alter cancer cell secretome and tumour microenvironment: Involvement in cancer invasion and metastasis. *Cancer Letters* (2016). doi:10.1016/j.canlet.2016.03.046
203. Shakya, R. *et al.* Mutant p53 upregulates alpha-1 antitrypsin expression and promotes invasion in lung cancer. *Oncogene* **36**, 4469–4480 (2017).
204. Stein, Y., Aloni-Grinstein, R. & Rotter, V. Mutant p53 – a potential player in shaping the tumor–stroma crosstalk. *J. Mol. Cell Biol.* (2019). doi:10.1093/jmcb/mjz071
205. Vogiatzi, F. *et al.* Mutant p53 promotes tumor progression and metastasis by the endoplasmic reticulum. 1–10 (2016). doi:10.1073/pnas.1612711114
206. Su, S.-F. *et al.* miR-30d, miR-181a and miR-199a-5p cooperatively suppress the endoplasmic reticulum chaperone and signaling regulator GRP78 in cancer. *Oncogene* **32**, 4694–4701 (2013).
207. Feldman, D. E., Chauhan, V. & Koong, A. C. The unfolded protein response: A novel component of the hypoxic stress response in tumors. *Molecular Cancer Research* (2005). doi:10.1158/1541-7786.MCR-05-0221
208. Chipurupalli, S., Kannan, E., Tergaonkar, V., D'andrea, R. & Robinson, N. Hypoxia induced ER stress response as an adaptive mechanism in cancer. *International Journal of Molecular Sciences* (2019). doi:10.3390/ijms20030749
209. Bensellam, M. *et al.* Hypoxia reduces ER-to-Golgi protein trafficking and increases cell death by inhibiting the adaptive unfolded protein response in mouse beta cells. *Diabetologia* (2016). doi:10.1007/s00125-016-3947-y
210. Mennerich, D., Kellokumpu, S. & Kietzmann, T. Hypoxia and reactive oxygen species as modulators of endoplasmic reticulum and golgi homeostasis. *Antioxidants and Redox Signaling* (2019). doi:10.1089/ars.2018.7523
211. Walerych, D. *et al.* Proteasome machinery is instrumental in a common gain-of-function program of the p53 missense mutants in cancer. **18**, (2016).
212. Asp, L. *et al.* Early stages of Golgi vesicle and tubule formation require diacylglycerol. *Mol. Biol. Cell* **20**, 780–90 (2009).
213. Fernández-Ulibarri, I. *et al.* Diacylglycerol is required for the formation of COPI vesicles in the Golgi-to-ER transport pathway. *Mol. Biol. Cell* **18**, 3250–63 (2007).
214. Roy, A., Ye, J., Deng, F. & Wang, Q. J. Protein kinase D signaling in cancer: A friend or foe? *Biochim. Biophys. Acta - Rev. Cancer* **1868**, 283–294 (2017).
215. Wang, Y., Mousley, C. J., Lete, M. G. & Bankaitis, V. A. An equal opportunity collaboration between lipid metabolism and proteins in the control of membrane trafficking in the trans-Golgi and endosomal systems. *Curr. Opin. Cell Biol.* **59**, 58–72 (2019).
216. McMahon, H. T. & Boucrot, E. Molecular mechanism and physiological functions of clathrin-

mediated endocytosis. *Nature Reviews Molecular Cell Biology* (2011). doi:10.1038/nrm3151
217. Chia, J. *et al.* RNAi screening reveals a large signaling network controlling the Golgi apparatus in human cells. *Mol. Syst. Biol.* **8**, 629 (2012).
218. Klinger, S. C., Siupka, P. & Nielsen, M. S. Retromer-mediated trafficking of transmembrane receptors and transporters. *Membranes* (2015). doi:10.3390/membranes5030288
219. Wang, J. *et al.* Endosomal receptor trafficking: Retromer and beyond. *Traffic* **19**, 578–590 (2018).
220. Wiech, M. *et al.* Molecular Mechanism of Mutant p53 Stabilization: The Role of HSP70 and MDM2. *PLoS One* (2012). doi:10.1371/journal.pone.0051426
221. Lehmann, S. *et al.* Targeting p53 in vivo: A first-in-human study with p53-targeting compound APR-246 in refractory hematologic malignancies and prostate cancer. *J. Clin. Oncol.* **30**, 3633–3639 (2012).
222. Tan, C. *et al.* Identification of a novel small-molecule inhibitor of the hypoxia-inducible factor 1 pathway. *Cancer Res.* (2005).
223. Mabjeesh, N. J. *et al.* 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. *Cancer Cell* (2003). doi:10.1016/S1535-6108(03)00077-1
224. Hetz, C., Chevet, E. & Harding, H. P. Targeting the unfolded protein response in disease. *Nature Reviews Drug Discovery* (2013). doi:10.1038/nrd3976
225. Zappa, F., Failli, M. & De Matteis, M. A. The Golgi complex in disease and therapy. *Current Opinion in Cell Biology* (2018). doi:10.1016/j.ceb.2018.03.005
226. Schindelin, J. *et al.* Fiji: An open-source platform for biological-image analysis. *Nature Methods* (2012). doi:10.1038/nmeth.2019
227. Wiśniewski, J. R. & Gaugaz, F. Z. Fast and sensitive total protein and peptide assays for proteomic analysis. *Anal. Chem.* (2015). doi:10.1021/ac504689z
228. Wiśniewski, J. R., Vildhede, A., Norén, A. & Artursson, P. In-depth quantitative analysis and comparison of the human hepatocyte and hepatoma cell line HepG2 proteomes. *J. Proteomics* (2016). doi:10.1016/j.jprot.2016.01.016
229. Beznoussenko, G. V. & Mironov, A. A. Correlative video-light–electron microscopy of mobile organelles. *Methods Mol. Biol.* (2015). doi:10.1007/978-1-4939-2309-0_23
230. Voorhoeve, P. M. & Agami, R. The tumor-suppressive functions of the human INK4A locus. *Cancer Cell* **4**, 311–319 (2003).
231. Zerbino, D. R. *et al.* Ensembl 2018. *Nucleic Acids Res.* (2018). doi:10.1093/nar/gkx1098
232. Smyth, G. K. Limma: linear models for microarray data BT - Bioinformatics and Computational Biology Solutions Using R and Bioconductor. *Bioinforma. Comput. Biol. Solut. Using R Bioconductor* (2005). doi:citeulike-article-id:5722720
233. Tusher, V. G., Tibshirani, R. & Chu, G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. U. S. A.* (2001). doi:10.1073/pnas.091062498
234. Ginestet, C. ggplot2: Elegant Graphics for Data Analysis. *J. R. Stat. Soc. Ser. A (Statistics Soc.* (2011). doi:10.1111/j.1467-985x.2010.00676_9.x
235. Oliveros, J. C. VENNY. An interactive tool for comparing lists with Venn Diagrams. *BioinfoGP of CNB-CSIC* http://bioinfogp.cnnb.csic.es/tools/venny/index.ht (2007).
236. Luo, J., Solimini, N. L. & Elledge, S. J. Principles of Cancer Therapy: Oncogene and Non-oncogene Addiction. *Cell* **136**, 823–837 (2009).
237. Marie, S. K. N. & Shinjo, S. M. O. Metabolism and brain cancer. *Clinics* (2011). doi:10.1590/S1807-59322011001300005
238. Northey, J. J., Przybyla, L. & Weaver, V. M. Tissue force programs cell fate and tumor aggression. *Cancer Discovery* (2017). doi:10.1158/2159-8290.CD-16-0733
239. Vučetić, M., Cormerais, Y., Parks, S. K. & Pouysségur, J. The central role of amino acids in

cancer redox homeostasis: Vulnerability points of the cancer redox code. *Frontiers in Oncology* (2017). doi:10.3389/fonc.2017.00319
240. Urra, H., Dufey, E., Avril, T., Chevet, E. & Hetz, C. Endoplasmic Reticulum Stress and the Hallmarks of Cancer. *TRENDS in CANCER* **xx**, 1–11 (2016).
241. Bieging, K. T., Mello, S. S. & Attardi, L. D. Unravelling mechanisms of p53-mediated tumour suppression. *Nature Reviews Cancer* (2014). doi:10.1038/nrc3711