UNIVERSITÀ DEL PIEMONTE ORIENTALE

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Thesis:

Transglutaminase 2 regulates the susceptibility of metastatic melanoma cells to ferroptotic death mediated by the epithelial-to-mesenchymal transition

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1. SUMMARY

Melanoma is a cancer derived from the malignant transformation of melanocytes. Its incidence has consistently increased in the last 50 years, and it is characterized by aggressiveness and resistance to standard therapy. The more frequent mutations involve the MAPK signaling pathway, particularly BRAF/NRAS. The current treatment of metastatic melanoma includes the selective inhibition of BRAF and its downstream partner MEK, and the targeting of the immune checkpoints PD-1 and CTLA-4. Nonetheless, melanoma is often intrinsically resistant or rapidly develops acquired resistance to those therapies, thus raising the urgency to develop alternative strategies and therapies to contrast metastatic spreading. In response to certain anticancer agents, such as erastin, cancer cells may undergo a type of iron-dependent cell death, named ferroptosis, induced by the inhibition of cysteine uptake by the X_c^- system, located on the cell membrane. This causes a reduction of intracellular glutathione, with consequent inhibition of the enzyme glutathione peroxidase 4 (GPX4) and an increase in the levels of lipid peroxides, which are the main executioners of this form of cell death. We demonstrated that metastatic melanoma cells are heterogeneously resistant to ferroptosis, evidencing a potential correlation with a mesenchymal phenotype. Therefore, we focused on the study of the relationship between the epithelial-mesenchymal transition (EMT), UPR, and resistance to ferroptosis.

Our results indicate that metastatic melanoma cells characterized by enhanced EMT-LP (EMT-Like Profile) index are more sensitive to the induction of ferroptosis, compared to those with a lower index. Furthermore, we found that it is possible to sensitize resistant cells to ferroptosis by pushing forward the EMT-LP index, by TGF-β. Finally, we found that transglutaminase 2 (TG2) might have a key role in this signaling, through ER stress induction.

2. INTRODUCTION

2.1. Melanoma

Human cutaneous melanoma is the leading cause of skin cancer death, with the highest incidence in Western countries. In 2022 skin melanoma represented ~20% of all skin cancer cases worldwide, while it caused almost half of all the deaths in the same period[1]. Melanoma incidence in the US increased by 270% from 1973 to 2002 and continues to rise, mainly affecting fair-skinned populations[2].

Melanoma arises from the malignant transformation of melanocytes that proliferate abnormally due to the accumulation of mutations[3]. Melanocytes are specialized cells that produce melanin pigment and originate from the neural crest, a transient embryonic structure deriving from the neuroectoderm[4]. Although melanocytes predominantly reside the epidermis and the hair follicles[5,6], they are also found in the eyes, inner ear, central nervous system, oral and sinonasal mucosa, and anogenital tracts[7,8].

Based on the morphologic aspects of the early growth phase and the anatomical site of the primary lesion, cutaneous melanoma has been originally classified into four main types: superficial spreading melanoma (SSM), lentigo malignant melanoma (LMM), nodular melanoma (NM), and acral-lentiginous melanoma (ALM)[9]. Currently, the World Health Organization's classification of melanoma, also including mucosa and uveal melanoma, distinguishes nine subtypes, based on their epidemiology, histologic morphology, anatomical location, the evidence of exposure to chronic sun damage (CSD), and genomic characteristics[10]. Nevertheless, certain tumors don't completely fit into just one of these broad categories but share similarities with others. Depending on the histopathological and clinical subsets, the melanocytic lesions present a unique oncogenic signature of driver mutations[11]; common nevi, e.g., have the highest rate of mutation in BRAF, while acral lesions and sun-induced damage frequently involve mutations in KIT[12,13].

Among all the malignancies, melanoma is the one with the highest mutation rate, followed by lung cancers[14]. This is attributed to exposure to UV radiations; nevertheless, in areas not associated with skin exposure to UVs, it might reflect other biological factors, such as the different origin from the neural crest. Over 40 genes harbor driver mutations in melanoma progression and development[15]. In cutaneous melanoma, these driver mutations have been classified into four major classes: (1) BRAF (>60%) associated with non-CSD, (2) NRAS (28%), and (3) NF1 (14%), both associated with CSD, and (4) triple-wild type (15%), without mutations in BRAF, NRAS, or NF1, more frequent in CSD melanomas[14–18]. Usually, in triple

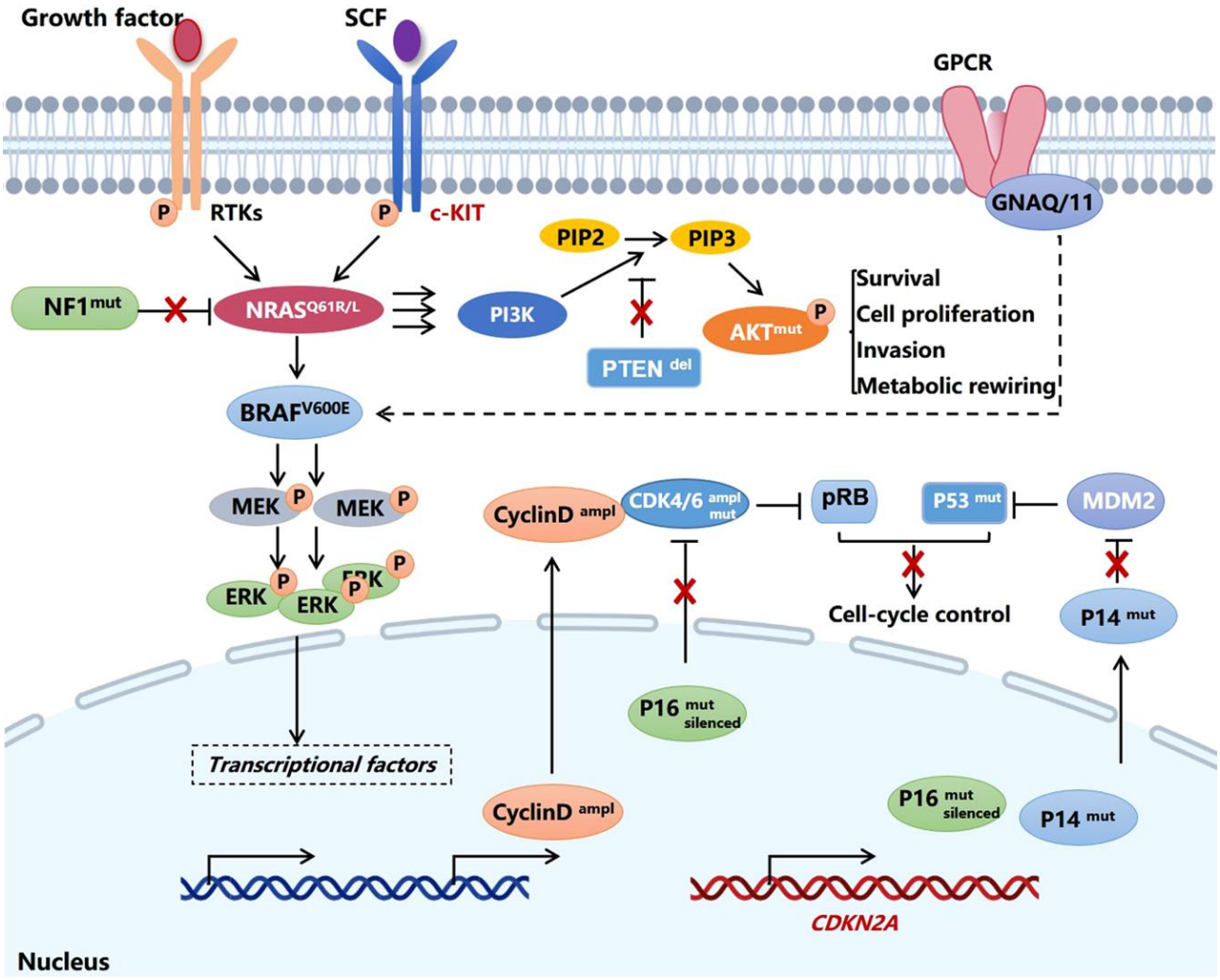

Figure 1. Mutated driver genes and downstream signal pathways in melanoma. Ampl. amplification, CDK cyclin-dependent kinase, Del deletion, GPCR G protein-coupled receptor, Mut mutation, P (in a pink circle) phosphate, $p14^{ARF}$ and $p16^{INK4A}$ splice variant encoded by CDKN2A gene, PIP2 phosphatidylinositol-(4,5)-bisphosphate, PIP3 phosphatidylinositol-(3,4,5)-trisphosphate, PTEN phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase, and dual-specificity protein phosphatase, RB retinoblastoma-associated protein, RTK receptor tyrosine kinase, SCF stem cell factor[155].

wild-type melanomas, the mutated genes are GNA11, GNAQ, SF3B1, and KIT[16–18]. BRAF is a serine/threonine protein kinase that activates the MAPK/ERK downstream pathway; V600E is the BRAF characteristic mutation, which results in constitutive activation, that represents 80-90% of all BRAF mutations in melanoma[19,20]. On the other hand, NRAS belongs to the family of G-regulatory proteins, in which Q61K and Q61R mutations result in constitutive activation which, in turn, stimulates BRAF, thus causing dysregulated cell proliferation[21]. Rarely, mutations in BRAF and NRAS arise simultaneously in a tumor[22]. NF1 is a tumor suppressor that harbors inactivating mutations. Indeed, wild-type NF1 negatively regulates RAS by converting RAS-GTP into inactive RAS-GDP, thus NF1 inactivation leads to constitutive activation of MAPK and PI3K pathways[23]. Amplifications or activating mutations in telomerase reverse transcriptase (TERT) exist in over 25% of melanomas. The

loss of the tumor suppressor phosphatase and tensin homolog (PTEN), a key regulator of the PI3K pathway, is found in 14% of melanomas[24]. Mutations in PTEN often co-occur with BRAF mutations but not with NRAS[23]. Finally, TP53-inactivating mutations are present in about 19% of melanomas[25].

The primary treatment for localized melanoma is the surgical removal of the tumor and the surrounding healthy tissue, and sentinel lymph node biopsy is conducted in patients with tumors greater than 0,8 mm thick[26]. Before 2010, the prognosis for metastatic melanoma was considerably poor, due to the inefficiency of traditional chemotherapy. Therefore, early detection of melanoma was vital for improving patients' prognosis[27]. Nowadays, patients with advanced BRAF-mutant melanoma receive targeted therapy with a combination of BRAF (vemurafenib, dabrafenib, encorafenib) and MEK (trametinib, binimetinib, cobimetinib) inhibitors. Despite the high initial response rates, with some degree of tumor regression and rapid symptom improvement in almost all patients[28–31], ~50% of them develop acquired resistance within 1 year, and 80% of them in 5 years[32,33].

Due to the high rate of mutation[14], melanomas produce a wide array of highly immunogenic tumor antigens. Hence, the most effective treatments for metastatic melanoma up to now are the immune checkpoint inhibitors. They are suitable for patients with both BRAF-mutant and wild-type melanoma. Checkpoint immunotherapy has less initial activity than targeted therapy; nevertheless, it potentially controls the disease over time and probably cures it in many patients, though it may have severe immune-related adverse events[34–36]. The most studied immune checkpoint inhibitors target PD-1, which normally regulates the immune system by inducing the apoptosis of maturing T-cells that recognize self-antigens in the lymph nodes and prevents apoptosis of regulatory T-cells[37]. Other exploited immune checkpoints are PD-L1/2, which are commonly overexpressed in cancers, including melanoma, allowing tumor cells to evade the immune response[37,38], and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which is constitutively expressed on T-reg cells. CTLA-4 competes with CD28 on T-cells to bind the B7-1/2 receptor on APCs, thus repressing the immune response[39]. The developed antibodies targeting PD-1, PD-L1/2, and CTLA-4 effectively block the binding to the respective ligands and the corresponding signal that causes tolerance, maintaining the immune response active[38–40]. Three immune checkpoint inhibitors have been approved for metastatic melanoma treatment: ipilimumab, an anti-CTLA-4 antibody, nivolumab and pembrolizumab, anti-PD1 antibodies[38]. Several studies have been conducted to determine the best combination of the available antibodies[34,35,41–45], resulting in the ipilimumab plus nivolumab combination having the

highest response rate (58%) and the most durable survival (5-year progression-free survival 52%), albeit at the cost of more severe and frequent toxicity (grade 3 or 4 adverse events 55%)[46].

2.2. Ferroptosis

In 2012, Dixon and Stockwell found a novel form of cell death they named ferroptosis by connecting the dots from previous studies performed in Stockwell's and Conrad's labs[47]. Ferroptosis is regulated, iron-dependent, and driven by lipid peroxidation, and is distinct from other forms of cell death, like apoptosis, necroptosis, or pyroptosis[47]. Its discovery arose from the identification, in 2003 and 2008, of two small molecules named erastin (for eradicator of RAS-transformed cells)[48] and RAS-selective-lethal-3 (RSL3), respectively[49], both inducing a non-apoptotic cell death dependent on the accumulation of oxidative stress and cellular iron[47].

For ferroptosis to occur, polyunsaturated fatty acids (PUFAs) must undergo peroxidation. This reaction involves the replacement of a hydrogen atom attached to a carbon atom with a peroxyl group (O-O), which is due to PUFAs' weak C-H bonds between adjacent C=C double bonds[50]. As demonstrated, free fatty acids are not drivers of ferroptosis, but rather PUFAs oxidized tails must be incorporated into membrane phospholipids (PLs), particularly phosphatidylethanolamine (PEs)[51]. The first pro-ferroptotic genes that have been identified encode for the enzymes acyl-coenzyme A synthetase long-chain family member 4 (ACSL4) and lysophosphatidylcholine acyltransferase 3 (LPCAT3), both involved in the incorporation of PUFAs in membrane lipids[52–54]. The peroxidation of membrane PUFA-PLs is due to the labile iron pool (LIP) regulated by transferrin receptor 1 (TfR1) and the degradation of ferritin via ferritinophagy, both promoting the Fenton reaction[55–57], and by iron-dependent enzymes like lipoxygenases (LOXs), that promote the lipid peroxidation of their substrates[58].

The first two classes of ferroptosis-inducing small molecules, erastin and RSL3, act on glutathione peroxidase 4 (GPX4) function, blocking its reduction of peroxidized lipids. RSL3 directly inhibits GPX4, while erastin inhibits the uptake of cystine (the cysteine oxidized form) through the system x_c^- cystine/glutamate antiporter, which provides the cysteine needed for the glutathione (GSH) synthesis, which regenerates reduced GPX4[47]. Although GPX4 is the main suppressor of ferroptosis, other mechanisms independent from it exist, acting through CoQ_{10}, tetrahydrobiopterin (BH_4), and ESCRT-III. The first is regenerated in its reduced form

by ferroptosis suppressor protein 1 (FSP1) thanks to NADPH[59] and in mitochondria by dihydroorotate dehydrogenase (DHODH)[60], while the second is regenerated by GTP cyclohydrolase 1 (GHC1)[61]. On the other hand, the efflux of calcium ions by the ferroptotic pores activates ESCRT-III, which mediates plasma membrane repair and thus delays ferroptotic cell death[62].

Three main ferroptosis mechanisms of resistance have been identified over the last decade, involving the transsulfuration pathway, the mechanistic target of the rapamycin (mTOR), and the master regulator of antioxidant response NRF2. The transsulfuration pathway promotes ferroptosis resistance by producing cysteine from methionine, overcoming the inhibition of the system x_c^-[63]. The mTOR pathway stimulates an increased GPX4 protein synthesis and sterol response element binding protein (SREBP)-mediated lipogenesis[64]. The antioxidant

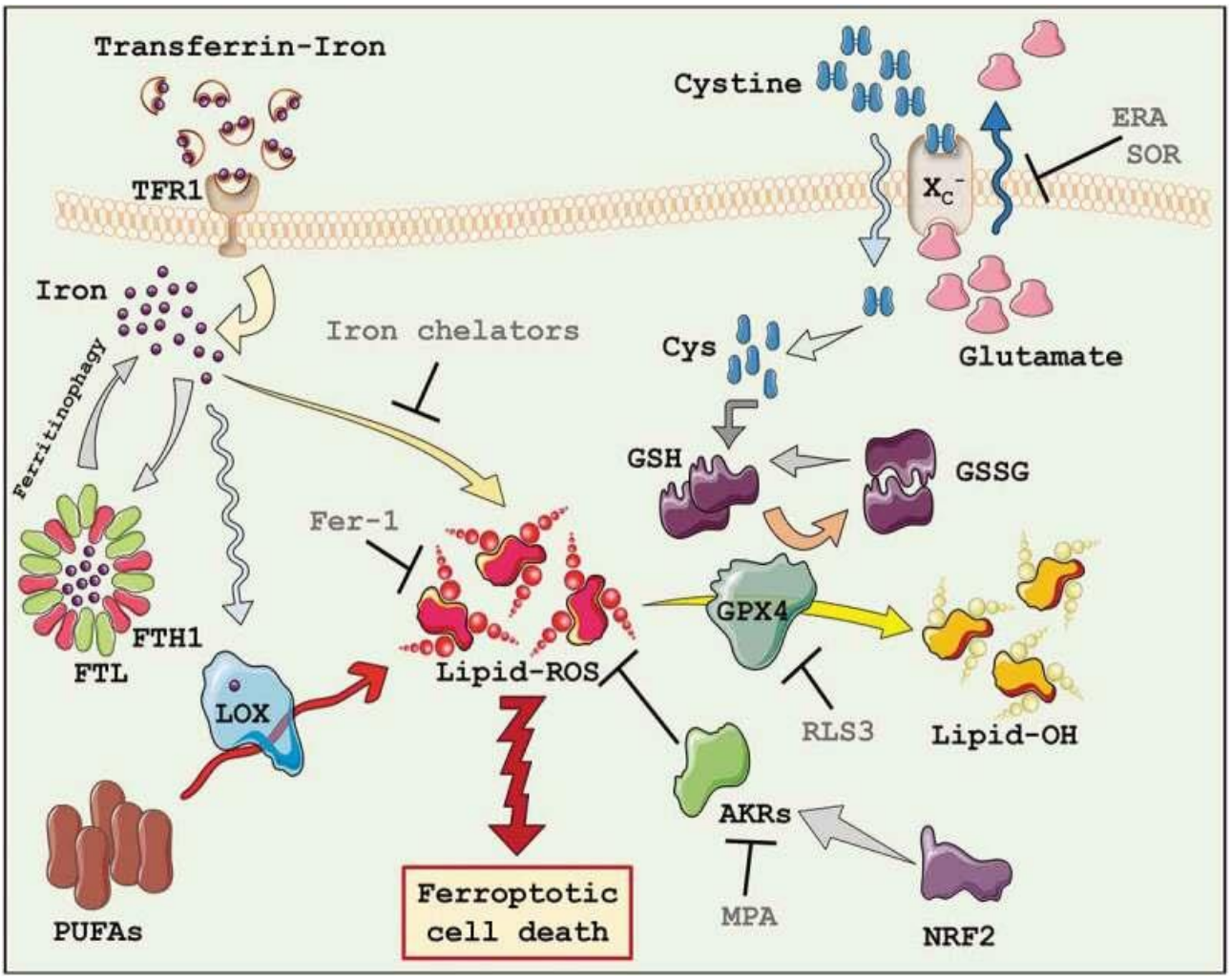

Figure 2. Ferroptosis pathway. The key players of the ferroptotic cell death process have been highlighted. AKRs = Aldo-keto reductases; Cys = cysteine; FTH1 = Ferritin heavy chain 1; FTL = Ferritin light chain; GSH = Glutathione; GSSG = Oxidized Glutathione; GPX4 = Glutathione peroxidase 4; LOX = Lipoxygenases; PUFAs = Polyunsaturated fatty acids; TFR1 = Transferrin receptor 1; ERA = Erastin; Fer-1 = Ferrostatin-1; MPA = Medroxyprogesterone; RLS3 = Ras-selective lethal small molecule 3; SOR = Sorafenib[156].

regulator NRF2 drives the resistance to ferroptosis through transcriptional response, varying depending on cell and tissue context[65]. Particularly, our laboratory demonstrated that in metastatic melanoma NRF2 regulates the expression of members of the aldo-keto reductase family 1 subfamily C (AKR1C1-3), thus reducing the lipid peroxides and inhibiting ferroptosis execution[66].

Different organelles, such as mitochondria, lysosomes, Golgi, and endoplasmic reticulum (ER) have been identified as ferroptosis initiators or amplifiers, or both[67–70], and inhibitors of ferroptosis accumulate in them[71]. Nevertheless, these compounds do not influence the ability of lysosomes, Golgi, and mitochondria to induce ferroptosis, suggesting that ER is the most critical site of lipid peroxidation and that inhibiting the process should be sufficient to suppress ferroptosis.

Ferroptosis occurs in both physiological and pathological processes. For example, it has been demonstrated that 4-hydroxy-2-nonenal (HNE), a biomarker of ferroptotic cells and an end product of lipid peroxidation, is involved in erythrocytes differentiation and aging of different organs in rats[72]. Ferroptosis is reported to be implicated in different neurogenerative diseases, among which Alzheimer's disease, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis[73], in infectious diseases, sometimes promoting and others suppressing the infection[74,75], and also in autoimmune diseases[76,77]. Regarding cancer, activated $CD8^+$ T cells induce ferroptosis in tumor cells through interferon-gamma (IFNγ) secretion, causing the downregulation of solute carrier family 7 member 11 (SLC7A11), one of the two genes encoding for the system x_c^-, and the upregulation of ACSL4[78,79]. Invasive melanoma cells are reported to be protected from ferroptosis by the lymphatic system environment, conferring an increased spread of metastasis, while metastasis of tumor cells undergoes ferroptosis in the blood[80]. Increased susceptibility to ferroptosis is associated with aggressive cancers that have undergone EMT, the ones that develop acquired resistance to targeted agents that induce apoptosis, and the ones with amplification of BRAF[81–83]. Finally, ferroptosis inducers may be useful if combined with radiotherapy and immunotherapy, indeed radiation can induce ferroptosis, and ferroptosis may sensitize tumors to radiation with the aid of immunotherapy[84,85].

2.3. Unfolded Protein Response

Membrane and secretory protein production is essential to maintain the survival of cells. The secretory pathway, comprising ER and Golgi, must adjust the molecular network involved in protein biogenesis to adapt and cope with the cellular demands and the surrounding environment. The network includes chaperons, foldases, glycosylation enzymes, and molecules involved in protein quality control[86]. In physiological conditions, some newly synthesized proteins are misfolded and are translocated to the ER-associated degradation (ERAD) system. When the folding demand exceeds the ER capacity, the proteins accumulate in the ER lumen, causing ER stress. To overcome this state and return to proteostasis, an adaptive cellular stress response named UPR is triggered[87]. During cancer development, the UPR is exacerbated because tumor cells require an acute demand for protein synthesis due to oncogene expression and are exposed to extreme extracellular conditions, such as hypoxia and low nutrient availability[88].

Three ER-resident transmembrane proteins, acting as ER stress sensors, are responsible for the UPR activation: the inositol-requiring enzyme 1 alpha (IRE1α), the protein kinase RNA-like ER kinase (PERK), and the activating transcription factor 6 (ATF6)[89]. All three sensors are regulated by the ER-resident chaperone GRP78/BiP, which is bound in basal conditions to the luminal domains of the ER stress sensors, repressing them[90]. Upon accumulation of misfolded proteins in the ER lumen, GRP78 is released, activating the three proteins and triggering their pathway to reprogram cells to cope with stress, or to die if the stress is not resolved[91].

IRE1 has two enzymatically distinct domains in the cytoplasmatic region, one with a serine/threonine activity and the other with an endoribonuclease (RNase) activity. Once GRP78 is released, IRE1 dimerization/oligomerization leads to a trans-autophosphorylation that activates the RNase domain[88]. This domain can excise 26 nucleotides of a short intronic region of the X-box binding protein 1 (XBP1) mRNA, catalyzing the unconventional splicing of XBP1 and generating a novel open-reading frame. The spliced-XBP1 is a transcription factor for protein folding, secretion, ERAD, and lipid synthesis genes[88]. If ER stress is not resolved, the IRE1 RNase domain catalyzes the degradation of ER-localized mRNA, rRNA, and microRNAs, through regulated IRE1-dependent decay of RNA (RIDD), participating in the reduction of the global mRNA translation[87]. On the other hand, the kinase domain interacts with the adaptor protein TRAF2, triggering a phosphorylation cascade resulting in JNK and NFκB activation[92,93]. If the ER stress is persistent, the IRE1

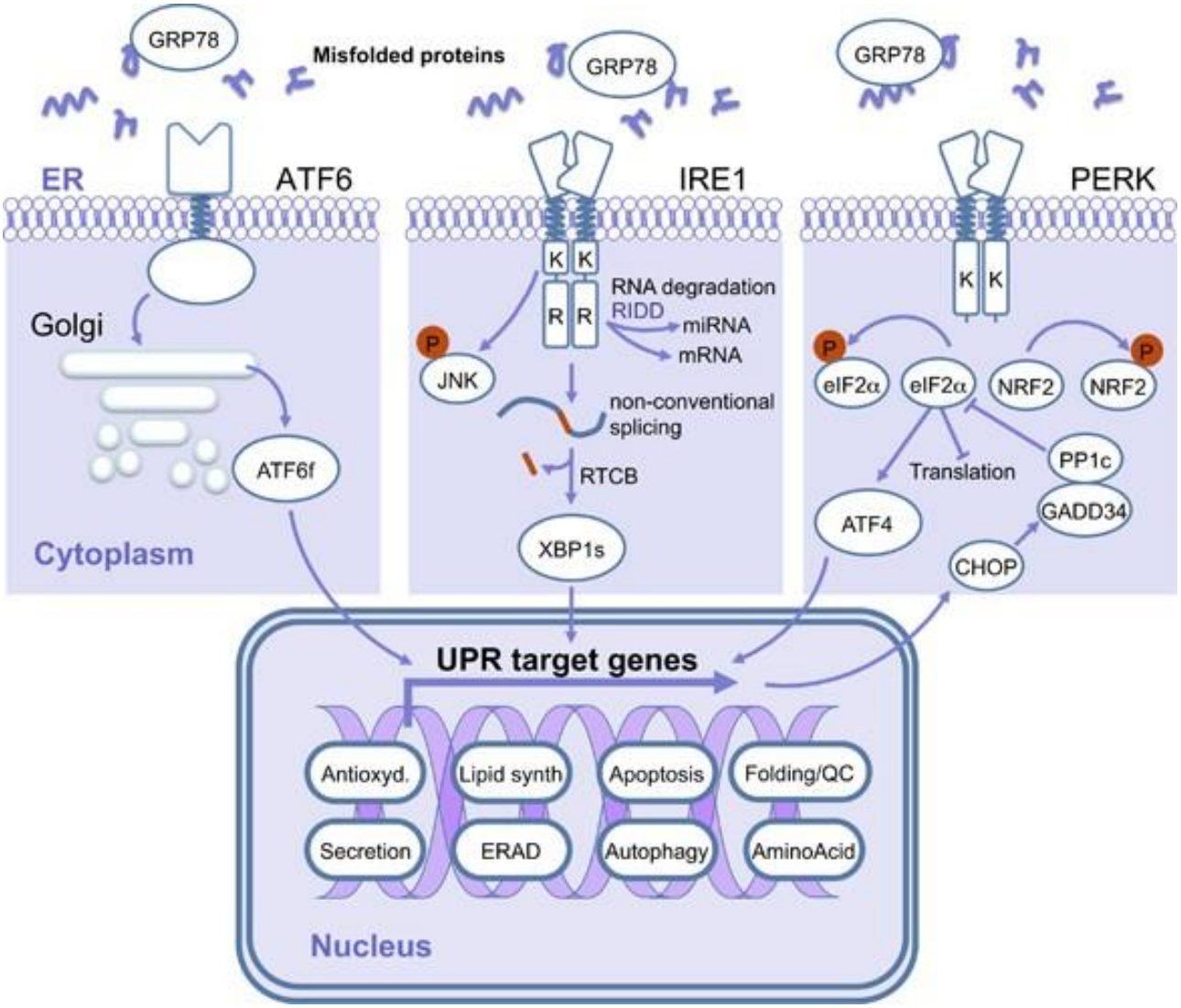

Figure 3. The UPR sensors and their downstream partners. During ER stress, GRP78 is released from IRE1α, PERK, and ATF6 sensors allowing their dimerization/oligomerization or export to the Golgi apparatus PERK activation leads to phosphorylation of NRF2 and eIF2α. Phosphorylation of eIF2α induces the attenuation of global translation and stimulates that of AFT4. ATF4 and NRF2 induce the expression of genes involved in antioxidant response, protein folding, amino-acid metabolism, autophagy and apoptosis. The negative feedback loop activated downstream of PERK dephosphorylates eIF2α to restore translation. IRE1α activation leads to JNK phosphorylation, regulated RIDD activity and XBP1 splicing that induces expression of genes involved protein folding, secretion, ERAD and lipid synthesis. Activation of ATF6 leads to its export in the Golgi apparatus where its cytosolic domain is released to translocate to the nucleus and activate the transcription of genes involved in protein folding and ERAD. Antioxid, antioxidant response; Lipid synth, lipid synthesis; QC, quality control[157].

activity leads to apoptosis through the non-specific cleavage of mRNAs through terminal RIDD.

During ER stress, PERK trans-autophosphorylates itself and thus phosphorylates the eukaryotic translation initiation factor 2 alpha (eIF2α) and the transcription factor NRF2[88]. Phosphorylation of eIF2α reduces the folding demand of the ER by attenuating the global cellular translation[94] and favors the translation of the transcription factor ATF4[95]. Phosphorylated NRF2 dissociates from KEAP1 and translocates into the nucleus[96]. ATF4

and NRF2 induce the expression of genes involved in protein folding, antioxidant response (NQO1), autophagy, and apoptosis (CHOP)[88,89]. The translation is restored by a negative feedback loop regulated by CHOP, resulting in eIF2α dephosphorylation catalyzed by GADD34/PP1c complex[97]. If the proteostasis is not re-established, sustained ATF4/CHOP activation causes apoptosis by inducing pro-apoptotic genes of the BCL2 family, such as BIM and PUMA[98,99].

ATF6 is an unstable protein existing in two isoforms (α and β) that homo- and heterodimerize[100]. Of the two, ATF6α is a powerful transcription factor[101]. Upon GRP78 dissociation, ATF6 is stabilized by the protein disulfide isomerase PDIA5, promoting its translocation to the Golgi apparatus[102]. There, ATF6 is cleaved and thus activated by the S1P and S2P proteases[103]. This causes the release of ATF6f, a membrane-free transcription factor, which translocates to the nucleus and mediates the expression of protein folding and ERAD genes[88].

UPR activation has been found in several types of primary and metastatic tumors, including melanoma[104]. Indeed, GRP78 is expressed at high levels in melanoma cell lines and its expression on melanoma tissue sections increases with melanoma progression[105]. Nonetheless, ER stress induction in melanocytic cells may represent a barrier to melanoma initiation[106]. Interestingly, UPR response is also observed in tumor-associated cells such as endothelial cells, infiltrating lymphocytes, and macrophages to better support tumor growth by promoting angiogenesis and releasing growth factors[107]. Tumor invasion is facilitated by the change in secreted matrix metalloproteases, like MMP2 and MMP9, mediated by ATF4 and XBP1[108,109]. Chronic UPR induction mediated by IRE1 and PERK, leads to NF-κB production, resulting in an EMT phenotype[110]. Finally, UPR activation is also known to protect many cancer types against apoptosis induced by chemotherapeutic drugs, due to the activation of the XBP1 pathway in melanoma[111].

2.4. Epithelial-to-mesenchymal transition

Epithelial-to-mesenchymal transition (EMT) is an essential embryonic process, which provides motility properties and drives the reversible programming of polarized epithelial cells into mesenchymal cells[112]. Microenvironmental signals regulate this cellular plasticity process, and a specific set of transcriptional factors drives it, commonly referred to as EMT-inducing transcription factors (EMT-TFs), predominantly represented by the SNAIL, ZEB,

and TWIST protein families[113]. EMT-TFs are latent in most adult tissues, while they are transiently activated during embryogenesis, e.g., neural crest cells delaminate from the dorsal neural tube through EMT, allowing them to migrate as individual cells[112]. In addition to fibroblasts, other differentiated cell types express EMT-TFs, including melanocytes, endothelial cells, neurons, and immune cells, where they play major roles in cell differentiation and tissue homeostasis[114].

The aberrant reactivation of EMT occurs in pathological conditions, like organ fibrosis and tumorigenesis, particularly in carcinomas[115]. The aberrant expression of EMT-TFs is known to favor dissemination, metastasis, malignant transformation, and tumor initiation[116,117]. This oncogenic potential arises from their ability to foster the escape from oncogene-induced senescence and apoptosis, by providing stem-like properties through the interference with p53 and pRB1 pathways[118]. EMT is not a simple commitment from a fully epithelial tissue to a fully mesenchymal one, rather it encompasses a wide spectrum of states that sustains environment-dependent cell plasticity thanks to reversible transitions both at the primary and distant sites[119].

Melanoma cells may undergo a re-differentiation at the metastatic site, reminiscent of the mesenchymal-to-epithelial transition (MET) process in carcinomas[120], in which ectopic ZEB2 expression facilitates the outgrowth of dormant disseminated melanoma cells, forming successful metastasis[121]. Alternatively, the collective migration of clusters of proliferative and invasive cells may underlie metastatic colonization[122].

In melanoma cells, the transforming growth factor β (TGF-β) is largely produced, inducing EMT, and it is one of the promoters of the ZEB2/ZEB1 switch[123]. TGF-β is a pleiotropic cytokine and has three isoforms encoded by different genes in mammalians that are TGF-β1, TGF-β2, and TGF-β3[124]. It is a potent inhibitor of epithelial cell proliferation; as such it acts as a tumor suppressor during the early stages of carcinogenesis by inducing cell cycle inhibitors like p21 and p15[125]. However, at later stages of carcinogenesis, TGF-β exerts tumor-promoting activities, due to tumor escape of its inhibitory effects[126].

TGF-β signals by dimerizing and then binding to the type II receptor (TβRII). This leads to the incorporation of the type I receptor (TβRI), and the formation of a large complex involving a ligand dimer and four receptor molecules. Afterward, TβRI phosphorylates SMAD2 and SMAD3, which associate with SMAD4, and they translocate to the nucleus as a heterotrimer, where it acts as a transcription factor[127]. Activated TGF-β receptors may signal via SMAD-independent pathways that involve PI3K, ERK, JNK, and MAPK, possibly leading to a pro-oncogenic response of cells to TGF-β[124]. As a paracrine factor, it promotes the remodeling

of the microenvironment to support tumor growth and facilitate metastasis, through the induction of angiogenesis and the regulation of extracellular matrix deposition and degradation[128].

The term EMT *in stricto sensu* cannot be formally used with non-epithelial-derived cancers, nevertheless, mesenchymal transition processes occur also in other cancers, which are, as melanocytes, derived from neural crest cells, like glioblastoma[129]. Particularly, melanomas harbor a high intra-tumoral heterogeneity that does not rely on the cancer stem cells (CSCs) hypothesis, but rather a sort of phenotype switching reminiscent of EMT, and for this reason, called EMT-like profile (EMT-LP)[130,131].

Two members of the EMT-TFs, SNAIL2, and ZEB2, are fundamental for differentiating melanoblasts into melanocytes and regulating their homeostasis. While SNAIL2 and ZEB2 expression is maintained in adult melanocytes, TWIST1 and ZEB1 are not detected[132]. The expression switch from ZEB2/SNAIL2 to ZEB1/TWIST1 is associated with the dedifferentiation of melanocytes, leading to melanomagenesis and the switch represents a poor prognostic factor for melanoma patients[133]. The alteration of the switch is sufficient to impair BRAF-dependent melanocyte transformation, thus ZEB1, TWIST1, or both promote not only invasive features but also display intrinsic oncogenic functions[132], while ZEB2 knock-out is not sufficient to initiate melanoma and it even impairs its formation[121]. High levels of ZEB1/TWIST1 cause a decrease in the expression of E-Cadherin and an increase in Vimentin, MMP2/9, and N-Cadherin[132].

Finally, approximately 40% of BRAFi/MEKi-resistant melanoma do not harbor a known genetic mutation, this has been attributed to phenotypic adaptations caused by cellular reprogramming, such as EMT[134]. Indeed, it has been demonstrated that elevated levels of ZEB1 expression are associated with an innate resistance to MAPKi and that TWIST1 may complement ZEB1 when not activated, even though ZEB1 is the main driver of resistance[135].

2.5. Transglutaminase 2 activity during ferroptotic cell death

Transglutaminase 2 (TG2), also referred to as tissue transglutaminase (tTG), is an acyltransferase belonging to a family of eight isoenzymes (TG1-7 and coagulation factor XIII) that catalyzes the formation of covalent bonds between glutamine or lysine residues on peptides or proteins[136]. TG2 is ubiquitously expressed in tissues and is reported to be localized in every cellular district, being in the cytosol, nucleus (translocated via an importin-

α3-related pathway), mitochondria, ER, and extracellular environment (via unconventional PX27 receptor activation, endosomal and plasma vesicles)[136]. Besides the transamidase activity, TG2 works as a GTPase, isopeptidase, deamidase, adaptor/scaffold protein, DNA nuclease, kinase, and protein disulfide isomerase[137]. GTP and calcium ions are allosteric mediators of transamidase activity. In physiological conditions, TG2 is inactive, because of high intracellular GTP availability, but disturbances in calcium homeostasis readily activate it[138]. The transcription of TG2 is upregulated by stressor stimuli, such as inflammatory cytokines (particularly TGF-β, TNF-α, and IL-6), ER stress, hypoxia, ROS, tissue injury, and UV radiation[138]. Because of this, TG2 participates in biological mechanisms including cell growth, differentiation, cell death, autophagy, inflammation, macrophage phagocytosis, tissue repair, ECM assembly, and remodeling[139].

Interestingly, metastasizing and chemoresistant tumors show a significant increase in TG2 biosynthesis than primary malignancies, suggesting that TG2 participates in the progression rather than in the development of cancers[140]. Moreover, our laboratory has recently found the potential involvement of TG2 in the modulation of ferroptosis. Indeed, cells lacking TG2 show an innate resistance to this type of cellular death[141]. Nevertheless, its role in ferroptotic execution remains enigmatic and elusive, requiring further investigations. TG2 was found to affect the therapy-resistant phenotype of different cancer types, including melanoma, where it impairs the melanoma sensitivity to ionizing radiation and chemotherapeutics (such as alkylating cytostatic agents, cisplatin, and dacarbazine)[140].

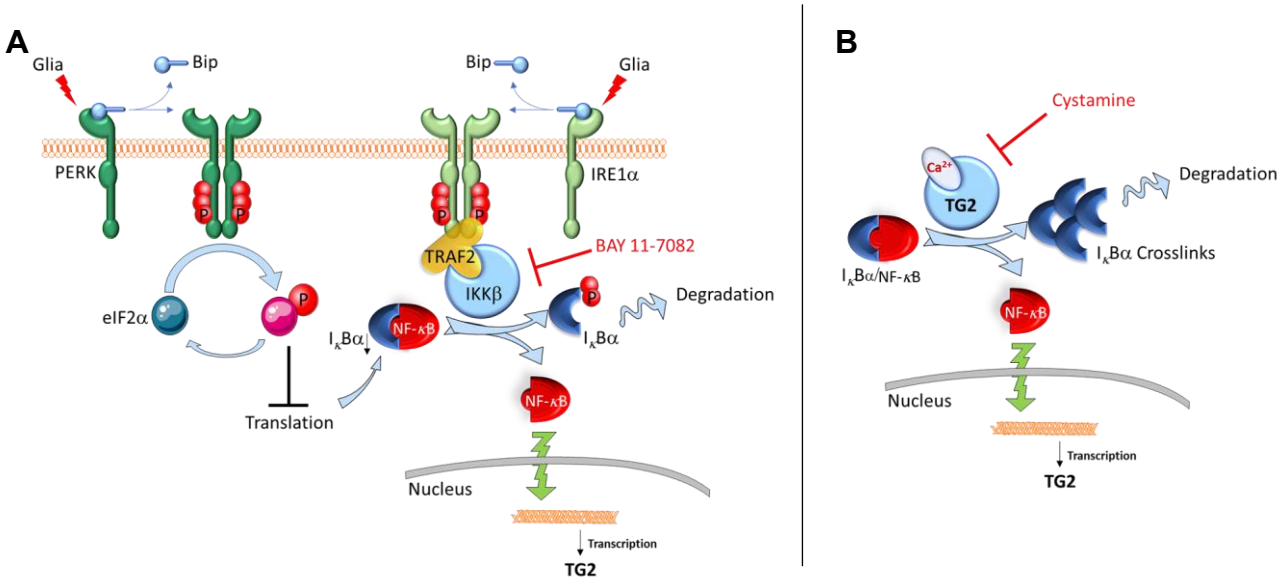

Figure 4. NF-κB canonical and non-canonical pathways. A) In the canonical pathway PERK phosphorylates eIF2α, thus blocking translation of the NF-κB inhibitor IκBα, and IRE1 activates IKKβ through the adapter protein TRAF2, causing the phosphorylation and consequent degradation of IκBα. **B**) In the non-canonical pathway, free calcium ions stimulate TG2 activity, which crosslinks and leads to degradation IκBα. Both pathways result in the transcription of NF-κB target genes, which include TG2.

Furthermore, it has been reported that TG2 induces EMT via the activation of NF-κB, a transcriptional factor essential for the function of the innate and humoral immune response[142,143]. NF-κB can be activated by two signaling pathways: the canonical and the non-canonical. The canonical pathway signals via the activation of PERK, which phosphorylates eIF2α and thus blocks the translation of the NF-κB inhibitor IκBα, and of IRE1α, which activates IKKβ through the adapter protein TRAF2; then, IKKβ phosphorylates IκBα, causing its degradation, hence NF-κB translocates to the nucleus and leads the transcription of its targets[144]. On the other hand, the non-canonical pathway involves the activation of TG2 by calcium ions released by the ER following stress; afterward, IκBα is degraded due to its crosslinking mediated by TG2, therefore NF-κB conducts its function in the nucleus[145]. Interestingly, one of the transcriptional targets of NF-κB is TG2 itself, thus creating a positive feedback loop that favors EMT during cancer progression[146].

3. AIM OF THE THESIS

The steadily rising incidence of metastatic melanoma worldwide has made it a significant public health concern. New therapeutic approaches, such as targeted therapy and immunotherapy, have greatly improved the quality of life and overall survival of the affected population in the last 15 years. However, the cancer's inherent aggressiveness and propensity to relapse partly undermine the progress made, thus new treatment strategies are required to be developed.

The induction of ferroptotic cell death has been suggested as a new potential therapeutic approach for the treatment of metastatic melanoma. In this thesis, the molecular pathways that lead to cancer progression of melanoma and the role carried out by TG2 in the regulation of the EMT (-LP) and of the resistance to ferroptosis have been investigated.

4. EXPERIMENTAL PROCEDURES

4.1. Cell lines and treatments

Human melanoma cell lines used were CHL-1 ($BRAF^{wt}$) and A375 ($BRAF^{V600E}$) were cultured in Dulbecco's (DMEM, EuroClone), supplemented with 10% fetal bovine serum (FBS, EuroClone), 2 mM L-Glutamine (EuroClone), and 1% penicillin/streptomycin (EuroClone) at 37 °C under 5% CO_2. Cells were treated with Erastin 10 μM (Sigma-Aldrich), TGF-β1 5 ng/ml (PEPROTech), Tunicamycin 50 ng/ml (Sigma-Aldrich), 4-phenylbutyric acid 5 μM (4PBA, SantaCruz). Mycoplasma testing was routinely performed monthly using the EZ-PCR Mycoplasma Detection Kit (Sartorius).

4.2. Animal studies

Female 6- to 8-week-old C57BL/6 (Charles River Laboratories, USA) (n=8 for each group) mice were bred under pathogen-free conditions in the animal facility of the University of Eastern Piedmont and treated in accordance with the University Ethical Committee and European guidelines (IACUC No 1240/2020-PR). The mice were injected subcutaneously with B16 and B16-F10 ($1x10^5$ in 100 μL/mouse) and the tumor growth was monitored daily. Ten days after tumor induction, the mice were randomized into two groups: (1) CTRL group treated via i.p. with DMSO solvent (used as a vehicle) and (2) imidazole ketone erastin (IKE, 20mg/kg, dissolved in 5% DMSO/ 95% Hank's Balanced Salt Solution pH 4) group treated via i.p., an erastin analog, for two weeks. At this time point, the mice were sacrificed, and tumor growth was measured.

4.3. Cell transfection

An empty vector was used as negative control and a vector encoding for TG2 (pcDNA4/HisMax) was used for ectopically expressing TG2 in our cells. Briefly, $25×10^4$ cells/well were transfected with 1,5 μg of DNA in 6 well plates using JetPRIME (Polyplus) for 8 h, as recommended by the supplier. 24 h after transfection, cells were trypsinized, plated at $25×10^4$ cells/well in six wells for RNA analysis and at $12×10^4$ cells/well in twelve wells for viability analysis, and then treated as indicated.

Human lentiviral vector (shRNA pLKO-TG2) and non-targeting scramble (shRNA pLKO-GFP, used as negative control) were obtained from Sigma-Aldrich. A total of $25x10^4$

cells/well were transfected with 1,5 µg of DNA in 6-well plates using JetPrime (Polyplus) for 8h, as recommended by the supplier. 24h after transfection, cells were trypsinized, plated at 25x10^4 cells/well in 6-well plates for RNA analysis and 12×10^4 cells/well in 12-well plates for cell viability analysis, and then, treated as indicated. RNA was checked by qPCR analysis.

4.4. qPCR analysis

Total RNA was isolated using TripleXtractor reagent (Grisp) and ExcelRT Reverse Transcriptase (Smobio) to produce cDNA using 2 µg of total RNA, following the manufacturer's recommendations. Quantitative PCR (qPCR) reactions were performed by using the Excel-Taq FAST qPCR SybrGreen (Smobio) and a CFX96 thermocycler (Bio-Rad). The melting curve protocol was used to check for probe specificity. Primer sets for all amplicons were designed using the online IDT PrimerQuest Tool software (IDT; https://eu.idtdna.com/Primerquest/Home/Index). Results were normalized using human and murine L34 mRNA levels as internal control, and the comparative Ct method (ΔΔCt) was used for relative quantification of gene expression.
The sequence of human primers was as follows:

HUMAN PRIMERS	***FORWARD***	***REVERSE***
L34	GTCCCGAACCCCTGGTAATAGA	GGCCCTGCTGACATGTTTCTT
XBP1S	AGAGAAAACTCATGGCCTTGTAGTTG	CATTCCCCTTGGCTTCCG
ATF4	CCCGGAGAAGGCATCCTC	GTGGCCAAGCACTTCAAACC
ATF6	TTTGCTGTCTCAGCCTACTGTGGT	TCCATTCACTGGGCTATTCGCTGA
SNAIL1	GATGAGGACAGTGGGAAAG	CCAAGGAAGAGACTGAAGTAG
ZEB1	GTGGCGGTAGATGGTAATG	ACCTTGTTGTATGGGTGAAG
TWIST1	CAGGTACATCGACTTCCTCTA	CATCCTCCAGACCGAGAA
VIM	CCAGCTAACCAACGACAAA	TCCTCTCTCTGAAGCATCTC
TG2	CAGCTACAATGGGATCTTGG	GTAAGGCAGTCACGGTATTT

4.5. Cell viability

Fluorescein diacetate (FDA)/7AAD staining was used to identify and measure the percentage of live/dead cells. Briefly, the cells were plated and treated as indicated, then, recovered at the indicated time point, trypsinized, washed by PBS, and pelleted at 1'800g, at room temperature. Finally, the pellet was incubated for 10 min with PBS containing FDA (7 pg/ml) and 7AAD (50 ng/ml), and 10'000 events were acquired by flow cytometry. The percentage of FDA positive and 7AAD negative cells was measured and indicated as 'Cell Viability (%)'.

4.6. Statistical analysis

All experiments were performed at least in triplicate and statistical analyses were performed using GraphPad Prism 8. The student's *t-test* was used to determine statistical significance. A *p*-value equal to or less than 0.05 was considered significant.

5. RESULTS

5.1. Sensitivity of metastatic melanoma cell lines to the induction of ferroptotic cell death.

A previous study from our laboratory has shown a different sensitivity of human metastatic melanoma cell lines to erastin-induced ferroptosis[66] (Fig. 5.1A). We expanded this analysis using two murine melanoma metastatic cell lines B16 and B16-F10. To this aim, the two cell lines were treated with the ferroptosis inducer erastin (ERA 0,5 μM and 1 μM), and cell viability was measured after 16 hours by flow cytometry. Data shown in Fig. 5.1B indicate that the two cell lines have a different sensitivity to the induction of ferroptosis, and the B16 are more resistant to ferroptosis execution than the B16-F10, which are more sensitive, in a dose-dependent manner. Finally, B16 and B16-F10 murine cell lines were subcutaneously injected in C57BL/6 mice, letting the tumors grow, and treating the mice with imidazole ketone erastin (IKE), an erastin analog. Our results demonstrated a greater reduction of tumor volume in mice injected with B16-F10 cells and treated with IKE, compared to the reduction that occurred in the ones injected with B16 cells in the same experimental condition (Fig. 5.1C).

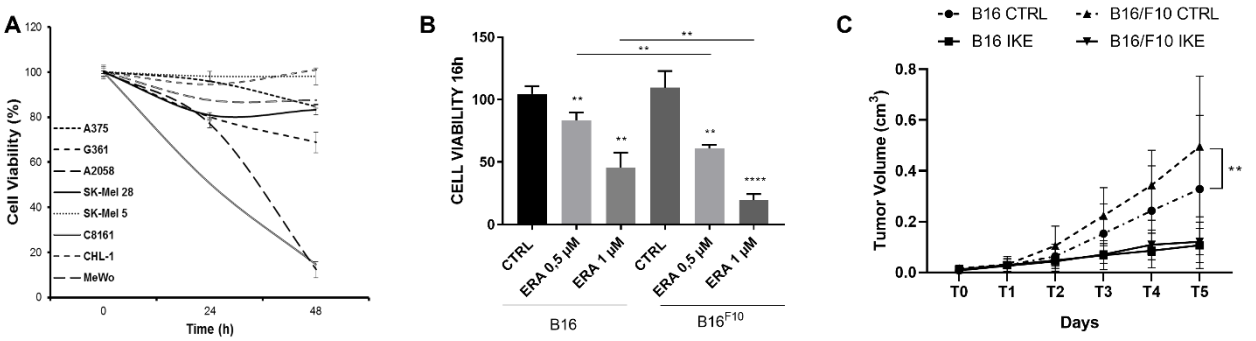

Figure 5.1 Ferroptosis sensitivity *in vitro* and *in vivo* models. A) Melanoma cell lines were treated with erastin (10 μM) for 24-48 h. Cell viability was evaluated by measuring the percentage of $FDA^+/7AAD^-$ cells by flow cytometry. **B**) Murine melanoma cell lines B16 and B16-F10 were untreated or treated with erastin (0,5 μM and 1 μM, 16 h). Cell viability was evaluated by flow cytometry. **C**) C57BL/6 mice were subcutaneously injected with B16 or B16-F10 cells ($1x10^5$ cells) and let the tumor grow for 21 days. Afterward, mice were untreated or treated for 14 days with IKE (20 mg/kg). Histograms represent mean ± s.d.; n = 3; ns = not significant; *p < 0,05; ** p < 0,01; *** p < 0,001; **** p < 0,0001.

5.2. Induction of EMT-LP in metastatic melanoma cells correlates with ferroptosis susceptibility.

A high-mesenchymal cell state observed in human tumors and cancer cell lines has been associated with resistance to multiple treatment modalities, including chemotherapy, target therapy, and immunotherapy, that usually rely on apoptotic pathways. This cell state is characterized by the high expression of specific markers due to EMT in epithelial-derived

carcinomas, but also due to the therapy resistance mediated by TGF-β in melanoma EMT-LP[81]. Moreover, melanoma cells release large quantities of TGF-β as a paracrine factor, promoting a microenvironment that favors invasiveness and metastasis, by inducing EMT in the near keratinocytes[147]. The activity of enzymes promoting the synthesis of polyunsaturated lipids features this high-mesenchymal state, that creates a dependency on pathways converging to GPX4 and results in a vulnerability to ferroptotic cell death[81]. Thus, we focused on two human melanoma cell lines: CHL-1 and A375, characterized by resistance and a moderate sensitivity to ferroptosis, respectively[66]. It was also previously observed that A375 cells have considerably higher basal expression levels of EMT markers than CHL-1 cells. To validate our hypothesis that the resistance to ferroptotic cell death is potentially correlated to the EMT-LP "index", we decided to push forward the EMT-LP of resistant cells and evaluate the impact on ferroptosis sensitivity. Thus, we chronically stimulated CHL-1 (the most resistant to ferroptosis and characterized by a low EMT-LP index) for 14 days and A375 (more sensitive to ferroptosis with enhanced EMT-LP index) for 5 days with TGF-β1 5 ng/ml. Then, the EMT-LP index was evaluated in both cell lines by qPCR. Our data demonstrate that TGF-β1 stimulation induces an epithelial-to-mesenchymal transition (-LP) (Fig. 5.2A) and potentially sensitizes cells to ferroptosis execution.

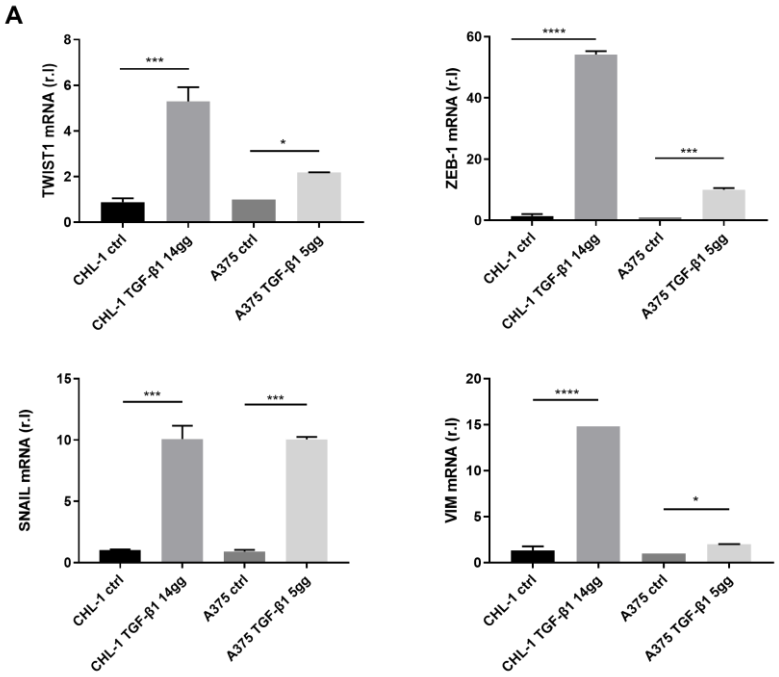

Figure 5.2 TGF-β1 induced EMT-LP in human melanoma cell lines. A) CHL-1 and A375 cell lines were untreated or treated with TGF-β1 (5 ng/ml) for 14 days and 5 days respectively. The mRNA expression of the EMT-LP markers (TWIST1, ZEB1, SNAIL, and Vimentin) was evaluated by qPCR. (In each cell line, the control has been arbitrarily set at 1). Histograms represent mean ± s.d.; n = 3; ns = not significant; *p < 0,05; ** p < 0,01; *** p < 0,001; **** p < 0,0001.

To verify this hypothesis, both cell lines were exposed to TGF-β1 as reported above, and cell viability was evaluated 24h after ERA (10 μM) treatment. As shown in Figure 5.2A, both cell lines chronically exposed to TGF-β1, enhancing their EMT-LP, were more sensitive to ferroptosis than parental (untreated) cell lines (Fig. 5.2B).

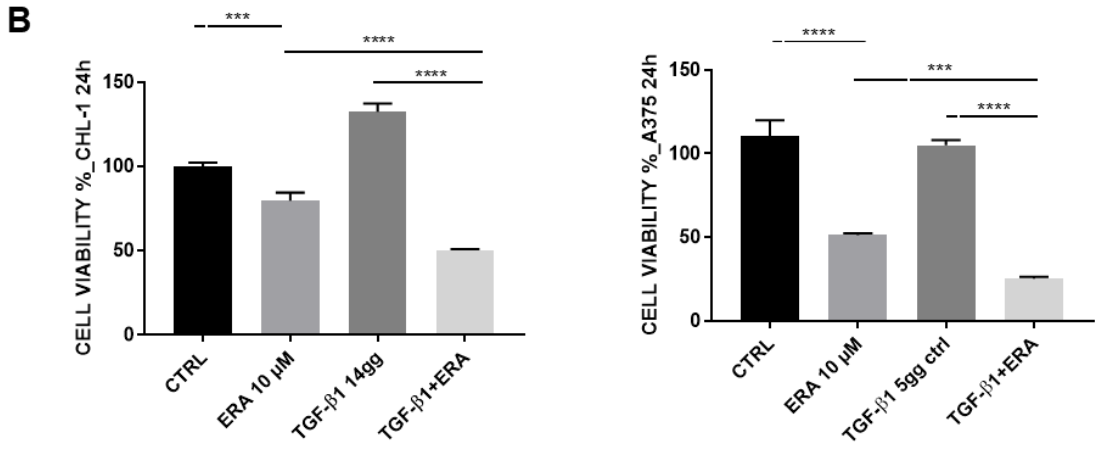

Figure 5.2 TGF-β1 induced EMT-LP in human melanoma cell lines. B) CHL-1 and A375 cell lines were exposed or not to TGF-β1 (5 ng/ml, for 14 and 5 days, respectively) in presence or absence of erastin (10 μM, 24 h). Cell viability was evaluated by measuring the percentage of $FDA^+/7AAD^-$ cells by flow cytometry. Histograms represent mean ± s.d.; n = 3; ns = not significant; *p < 0,05; ** p < 0,01; *** p < 0,001; **** p < 0,0001.

5.3. TN treatment enhances EMT-LP index in human metastatic melanoma cells.

UPR activation is reported in several primary and metastatic tumors, and it allows them to adapt and cope with the cellular demands and the surrounding environment, which is often characterized by hypoxia, nutrient deprivation, and increased protein production[104]. Moreover, chronic induction of the UPR is known to induce an EMT phenotype mainly through PERK and IRE1 signaling pathways and to facilitate tumor invasion by the induction of matrix metalloprotease secretion[109,110]. Therefore, to further verify that the TGF-β1-stimulated EMT-LP in metastatic melanoma cells is mediated by the induction of UPR, CHL-1 cells (characterized by low chronic ER stress) were treated with a sub-lethal concentration (50 ng/ml) of the well-known ER stress inducer Tunicamycin (TN) for 48h, and the expression of ER stress markers (Fig. 5.3A) and EMT-LP index (Fig. 5.3B) were evaluated by qPCR. Our data clearly indicate that TN treatment mimics the exposure of cells to TGF-β1 since TN enhanced the expression of EMT-LP genes (Fig. 5.3B). Finally, to verify that TN-mediated upregulation of EMT-LP markers also results in enhanced cell sensitivity to ferroptosis, CHL-1 cells (with low ER-stress status)[148] were exposed for 48 h to TN (50 ng/ml) and ERA (10 μM) for a further 24 h, and cell viability was measured by flow cytometry.

As shown in Figure 5.3C, the treatment consistently enhanced the sensitivity of CHL-1 cells to erastin-induced ferroptotic cell death, compared to the parental cell line, thus mimicking the exposure to TGF-β1.

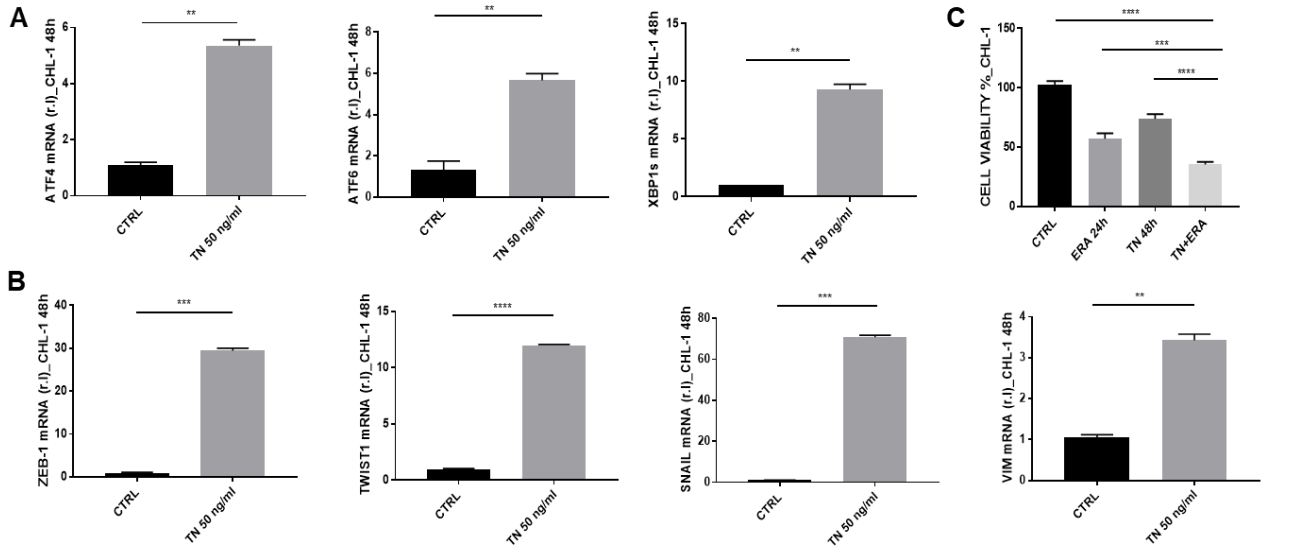

Figure 5.3 UPR induction by TGF-β1 mediates EMT-LP. A) CHL-1 were exposed or not to tunicamycin (TN, 50 ng/ml) for 48 h and UPR expression markers were evaluated by qPCR. **B**) EMT-LP markers were evaluated in the CHL-1 cell line by qPCR after TN (50 ng/ml) 48 h exposure. **C)** A375 cells were untreated, treated with TGF-β1 (5 ng/ml, for 5 days), with 4-PBA (5 μM, for 21 days), or both. The mRNA expression of the UPR markers (ATF4, ATF6, XBP1s) was evaluated by qPCR. Histograms represent mean ± s.d.; n = 3; ns = not significant; *p < 0,05; ** p < 0,01; *** p < 0,001; **** p < 0,0001.

Ultimately, we sought to understand whether ER stress inhibition regulates TGF-β1-mediated epithelial-to-mesenchymal transition. Hence, we chronically treated the A375 (with high chronic ER-stress status)[148] cell line with TGF-β1 for 5 days and 4-PBA (5 μM), a chemical chaperone[149], for 21 days, alone or in combination (Fig. 5.3D). Our results demonstrated that 4-PBA treatment not only decreases the ER-stress markers expression levels (Fig. 5.3D) but in combination with TGF-β1 is able to significantly diminish EMT-LP expression markers (Fig. 5.3E) compared to TGF-β1 that was used as a positive experimental control.

Taken together, the achieved results imply that TGF-β1 relies on UPR activation in melanoma cells to induce EMT-LP. TGF-β1 might cause ER stress and subsequent UPR activation in tumor cells by promoting greater protein synthesis and secretion load through the induction of angiogenesis and extracellular matrix deposition[128].

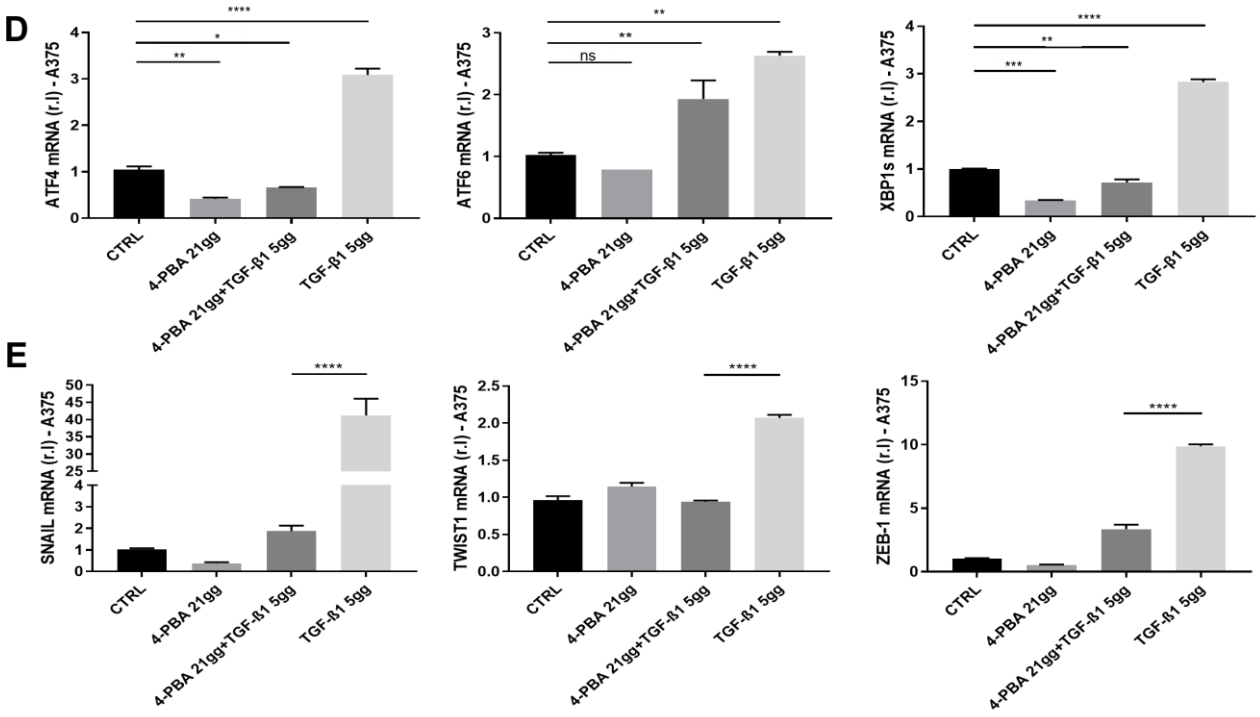

Figure 5.3 UPR induction by TGF-β1 mediates EMT-LP. D) The transcriptional expression of the EMT-LP markers was determined by qPCR in the A375 cell line after exposure to TGF-β1 (5 ng/ml, for 5 days), and 4-PBA (5 μM, for 21 days). **E**) CHL-1 were treated with erastin (10 μM, 24 h) in the presence or absence of TN (50 ng/ml, 48 h), and the cell viability was evaluated by measuring the percentage of FDA+ /7AAD- cells by flow cytometry. Histograms represent mean ± s.d.; n = 3; ns = not significant; *p < 0,05; ** p < 0,01; *** p < 0,001; **** p < 0,0001.

5.4. TG2 overexpression induces EMT-LP and increases the susceptibility of melanoma cells to ferroptosis.

TG2 participates in many biological processes and stressor stimuli, including inflammatory cytokines, such as TGF-β, which regulates its expression and activation[138]. Furthermore, metastasizing cancers show a consistent increase in TG2 biosynthesis than primary tumors, suggesting an involvement in cancer progression[140]. Indeed, calcium-activated TG2 has been reported to induce EMT via the non-canonical activation of NF-κB, due to ER stress[150]. Moreover, NF-κB can be canonically activated by the UPR mediators PERK and IRE1. Interestingly, one of the NF-κB transcriptional targets is TG2 itself[144], which can cause a positive feedback loop in an ER stress context. NF-κB activation leads to the consequent constitutive transcription of HIF-1α, which upregulates the expression of the EMT-TFs[150]. Thus, we measured the TG2 mRNA basal expression level in our cell lines (Fig. 5.4A), resulting in a much higher expression in A375 cells (more sensitive to ferroptosis and with high EMT-LP index) than in CHL-1 cells (more resistant and with low EMT-LP index). Then,

to verify that TG2 expression is related to ER stress induction, we treated both cell lines with TN (50 ng/ml) for 24h (Fig. 5.4B).

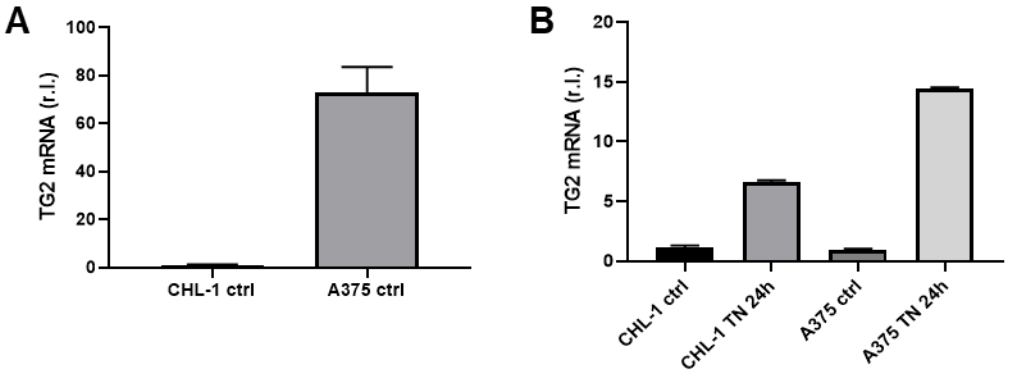

Figure 5.4 TG2 drives the susceptibility to ferroptosis. A) TG2 mRNA expression levels were evaluated in the indicated cell lines at basal conditions by qPCR. **B**) The indicated cell lines were untreated or treated with TN (50 ng/ml) for 24 h. mRNA expression of TG2 was evaluated by qPCR.

Next, to ensure that TG2 upregulation is not just an epiphenomenon of ER stress, but is indeed the driver of EMT-LP, we transfected both cell lines with a TG2 encoding vector, and TG2 overexpression and the increase of EMT-LP markers was verified by qPCR (Fig. 5.4C). Finally, to assess whether TG2 affects the increased susceptibility to ferroptosis of

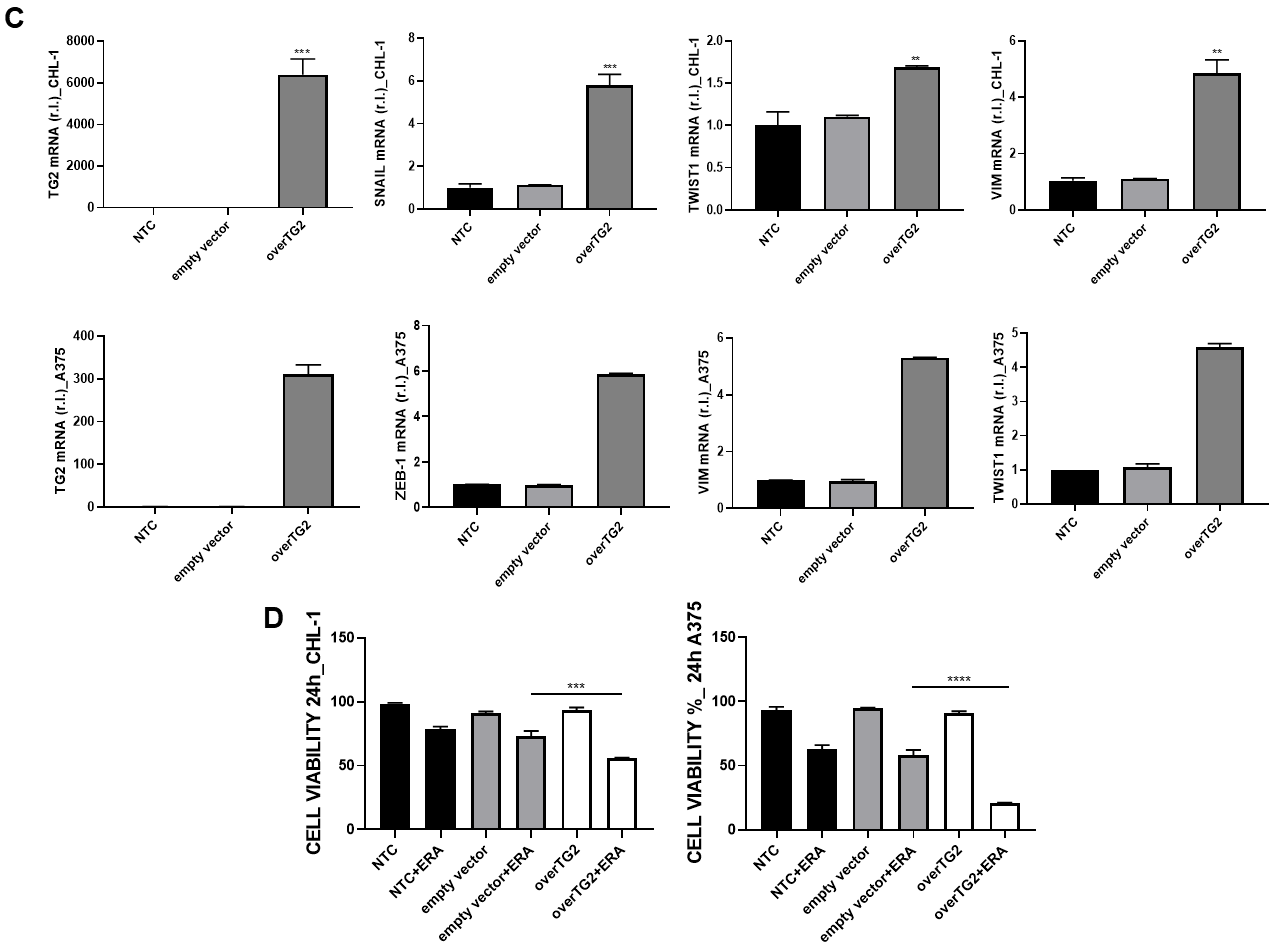

Figure 5.4 TG2 drives the susceptibility to ferroptosis. C) CHL-1 and A375 cells were transfected with an empty vector or a vector containing TG2, and then mRNA expression of TG2 or EMT-LP markers was evaluated by qPCR. **D**) CHL-1 and A375 were transfected with an empty vector or one containing TG2 and were exposed to erastin (10 μM, 24 h). Cell viability was evaluated by measuring the percentage of $FDA^+/7AAD^-$ cells by flow cytometry. Histograms represent mean ± s.d.; n = 3; ns = not significant; *p < 0,05; ** p < 0,01; *** p < 0,001; **** p < 0,0001.

melanomas that underwent EMT-LP, both cell lines were transfected with a TG2 encoding vector and then treated with ERA for 24h. Cell viability analysis was significantly lowered in this condition, compared to the cells treated with erastin and transfected with an empty vector (Fig. 5.4D).

Vice versa, during TG2 silencing we observed a reduction of EMT-LP markers in A375 cell line (with high basal TG2 expression level) (Fig. 5.4E) and an increase of cell viability after erastin treatment (Fig. 5.4F), confirming the pivotal role of TG2 in this process.

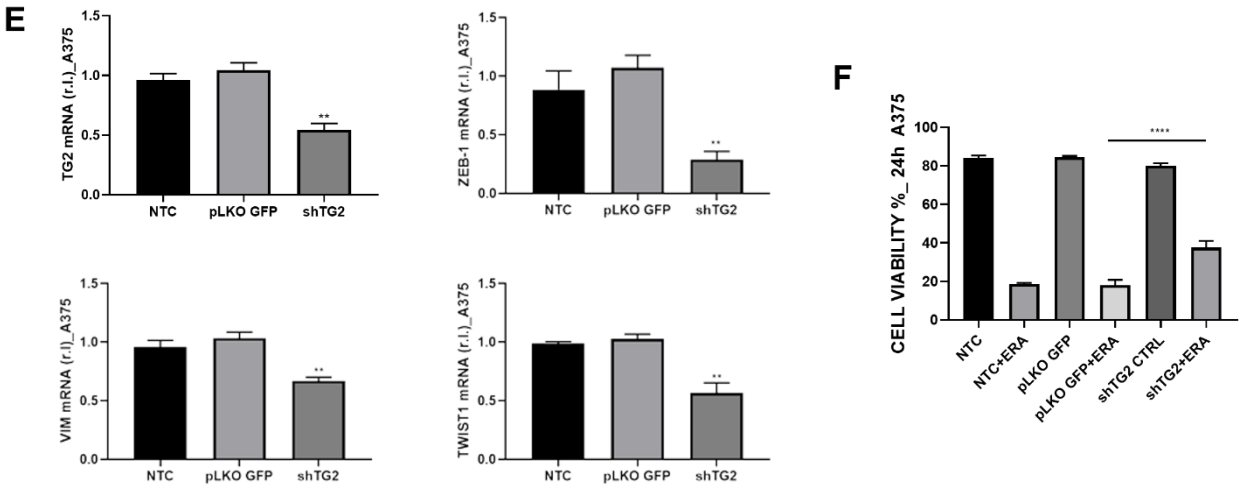

Figure 5.4 TG2 drives the susceptibility to ferroptosis. F) A375 cells were transfected with a pLKO GFP control vector or a vector containing the shRNA of TG2, and then mRNA expression of TG2 or EMT-LP markers was evaluated by qPCR. **E**) A375 transfected with a pLKO GFP control vector and one expressing for the shRNA of TG2 were untreated or treated with erastin (10 μM, 24 h). Cell viability was evaluated by measuring the percentage of $FDA^+/7AAD^-$ cells by flow cytometry. Histograms represent mean ± s.d.; n = 3; ns = not significant; *p < 0,05; ** p < 0,01; *** p < 0,001; **** p < 0,0001.

These results clearly show that TG2 is involved in the EMT-LP, therefore driving the metastasizing process in melanoma cells. Moreover, it is also evident that TG2 plays a role in the impairment of ferroptosis resistance developed by the acquisition of a mesenchymal phenotype. Nevertheless, the molecular mechanism behind TG2 activity needs to be further investigated in order to find new modalities for exploiting ferroptosis to treat metastatic melanoma.

6. CONCLUSIONS

Melanoma is a malignant neoplasm deriving from melanocytes, cells present in different anatomical areas, especially in the epidermis. This malignancy represents almost 50% of lethal skin cancers[1] and, in recent years, the incidence of cutaneous melanoma has increased consistently, showing a very high growth rate compared to other cancers[151]. Thus, high aggressiveness and the absence of effective treatments, due to the onset of primary and/or acquired drug resistance, push forward new therapeutic approaches to cure this neoplasia.

One feature of melanoma cells is the ability to activate a gene expression program (gene reprogramming) similar to the EMT. Melanoma cells that acquire this EMT-like profile become more resistant to chemotherapies and acquire the ability to metastasize. A characteristic of the EMT-LP phenotype is the deregulation of different transcription factors and is also often associated with mutations of NRAS/BRAF that can cause greater aggressiveness, greater invasiveness, and greater resistance to chemotherapeutics[152].

Data previously published by our research group have shown that metastatic melanoma cells show different degrees of sensitivity to the induction of ferroptotic cell death[66]. We expanded this analysis on *in vitro* murine melanoma cell lines (B16 and B16-F10) and also using an *in vivo* model, confirming the different susceptibility of tumor cells to ferroptosis, which correlates with aggressiveness and metastatic tumor profile.

Our study, conducted *in vitro*, allowed us to hypothesize that the degree of sensitivity/resistance of tumor cells to ferroptotic death may be related to the degree of cell differentiation. In fact, our previous data (not shown), evaluating the expression of main EMT markers, showed that the ferroptosis-resistant CHL-1 are characterized by low expression of EMT-LP markers compared to A375, which are more sensitive and with high expression of these markers.

The role of TGF-β1 in the tumor microenvironment is well documented. It can activate different molecular pathways such as SMAD, ERK, and AKT, which ignites the EMT process. The resulting gene reprogramming allows the neoplastic cells to obtain features related to invasiveness, aggressiveness, and motility. Moreover, it is also known that high levels of TGF-β1 in melanoma cells induce a more metastatic profile[153,154]. Therefore, to test the hypothesis by which tumor-dedifferentiation confers sensitivity to ferroptosis, we decided to induce the EMT stimulating melanoma cells with TGF-β1, for 14 days (CHL-1, ferroptosis resistant) and 5 days (A375, ferroptosis sensitive), respectively. The treatment confirmed an increase in EMT-LP response, while the following stimulation with erastin increased the sensitivity of cells to ferroptosis.

Chronic induction of UPR is known to induce an EMT phenotype mainly through PERK and IRE1 signaling pathways and to facilitate tumor invasion by the induction of matrix metalloprotease secretion[109,110]. Therefore, we verified that the TGF-β1-stimulated EMT-LP in metastatic melanoma cells is mediated by the induction of UPR in CHL-1 cells (characterized by low chronic ER stress) through the treatment with a sub-lethal concentration of TN (an ER stress inducer). Our data clearly indicate that TN mimics the exposure of cells to TGF-β1 since TN enhanced the expression of EMT-LP genes. Therefore, our hypothesis is that the induction of UPR mediates the TGF-β-dependent gene reprogramming. This is further sustained by data showing that ER stress inhibition consistently inhibited the induction of EMT markers upon TGF-β stimulation.

Taken together, our results imply that TGF-β relies on UPR activation to induce EMT-LP in melanoma cells, which confers sensitivity to ferroptosis.

Furthermore, metastatic cancers show a consistent increase in TG2 expression than primary tumors, suggesting its involvement in cancer progression[140]. TG2 participates in many biological processes and stressor stimuli, including inflammatory cytokines production, while TGF-β regulates its expression and activation[138]. Moreover, data previously published by our research group have shown that TG2 knockdown confers resistance of mouse embryonic fibroblasts (MEFs) to ferroptosis[141]. Based on this data, we investigated whether the presence or absence of TG2 might affect the resistance/sensitivity of melanoma cells to ferroptosis. Our data confirmed that TG2 expression levels were much higher in ferroptosis-sensitive cells, characterized by enhanced EMT, compared to ferroptosis-resistant cells, with lower EMT profiles. Finally, by modulating the expression of TG2 we were able to modulate the EMT profile of melanoma cells, together with their sensitivity to ferroptosis.

Collectively, our results demonstrate that TG2 clearly appears as a key player in the EMT-LP progression of metastatic melanoma cells, also modulating the sensitivity to ferroptosis execution, although the molecular mechanism is still elusive.

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