UNIVERSITY OF NAPLES FEDERICO II

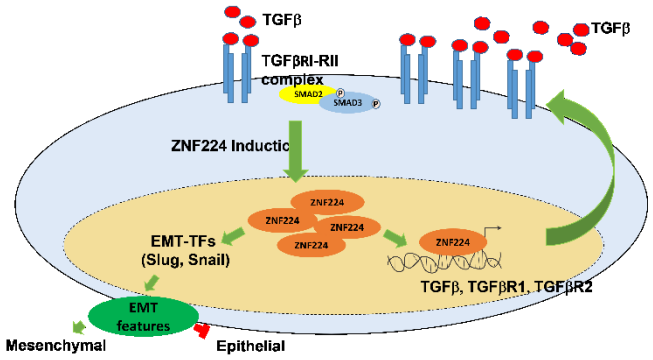
DOCTORATE IN

MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXIV CYCLE

Arianna Pastore

ZNF224 is a mediator of TGF-β pro-oncogenic function in melanoma



2018-2022

UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN

MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXIV CYCLE

ZNF224 is a mediator of TGF-β pro-oncogenic function in melanoma

Tutor

Prof.ssa Paola Costanzo

Candidate

Arianna Pastore

2018-2022

Table of contents

Abstract

ZNF224 is a human pleiotropic KRAB zinc finger protein that plays a crucial role in human cancer. Depending on its cellular context and molecular partners, it can act as both tumour promoter and suppressor.
The Transforming Growth Factor-β (TGF-β) is an essential and pleiotropic cytokine involved in several physiological and pathological processes. In particular, it is a crucial regulator of melanoma progression, a highly invasive and metastatic tumour.
In this thesis work, we investigated the ZNF224 role in melanoma and its involvement in the TGF-β signaling as a mediator of the TGF-β pro-oncogenic function. We first found that ZNF224 expression is increased in melanoma cell lines compared to non-cancerous cells and that its expression is induced by TGF-β stimulation. Then, we showed that ZNF224 potentiated the acquisition of a mesenchymal phenotype and a metastatic behaviour by promoting epithelial-mesenchymal transition (EMT). Indeed, ZNF224 overexpression activated the EMT-associated genes such as Slug, Snail, Vimentin and N-Cadherin, and it acted synergistically with TGF-β to potentiate their activation. Furthermore, ZNF224 overexpression in melanoma cells promotes proliferation, invasiveness, and metastatic potential, while ZNF224 knockdown had the opposite effect. Intriguingly, we found that ZNF224 could sustain the endogenous TGFβ/Smad signaling. Indeed, ZNF224 overexpression prolonged the phosphorylation of the Smad complex and increased the levels of the TGF-β itself and its type 1 and 2 receptors (TβR1 and TβR2). These results unveil the existence of a positive regulatory loop between ZNF224 and TGF-β and suggest that ZNF224 contributes to the constitutive activation of this pathway, thus supporting melanoma progression. However, other experiments are required to investigate the underlying molecular mechanisms between ZNF224 and TGF-β pathway. Identifying ZNF224 as a modulator of TGF-β signaling in melanoma could represent a new important tool in understanding the complex role of the TGF-β pathway in melanoma and could help to identify novel molecular therapeutic targets and suitable treatment options for this deadly disease

1. Background

1.1 Zinc finger proteins

The regulation of gene expression is a finely tuned process that coordinates fundamental biological processes, including development, cell growth, proliferation, differentiation, and cell death (Greber 2019). In response to precise stimuli, specific sets of genes are activated or repressed by selective regulatory transcriptional factors (TFs) that exert their role by recruiting on the DNA cofactors, histones, and chromatin remodelling proteins. In eukaryotic, TFs exert the ability to activate or repress gene expression through the binding to specific DNA elements located in the promoter, enhancer, or silencer region. (Roberts 2000). The binding of TF to target genes relies on specific motifs that interact and stabilize the interaction with DNA. TFs can be classified into classical zinc fingers, homeodomains, and basic helix-loop-helix (Jen 2016). Among these, the C_2H_2 zinc finger protein family (also called Kruppel-related zinc finger protein family) is one of the main groups of proteins with DNA-binding activity in the human genome. Genes encoding C_2H_2 zinc finger protein are clustered in specific regions of the human genome and represent more than 2% of all the human genes (Vaquerizas 2009).

C_2H_2 zinc finger proteins contain from one to over thirty zinc finger motifs. Each C_2H_2 zinc finger motif is a highly conserved element consisting of 21-23 amino acid residues ($CX_2CX_3FX_5LX_2HX_3H$) in which cysteine and histidine residues are folded in two anti-parallel β-strands and one α-helix structure, coordinated a zinc ion, and able to make contact with the major groove of DNA (Wuttke 1997). Zinc finger motifs are able to bind DNA or RNA and also mediate protein-protein interaction (Cassandri 2017).

C_2H_2 zinc finger proteins also contain conserved domains, such as BTB (Broad-Complex, Tramtrack and Bric Brac)/POZ (poxvirus and zinc finger), the Krüppel-associated box (KRAB), and SCAN (SRE-ZBP, CTfin51, AW and Number 18 cDNA) domain (Iuchi 2001; Ladomery 1997). The SCAN domain is involved in mediating protein-protein interactions, while the BTB motif acts as a dimerization domain. SCAN and KRAB domains are vertebrate-specific, whereas the BTB motif is also present in insects. The KRAB repression module is specific to the genomes of vertebrate tetrapods (Shannon 2003).

About one-third of the human C2H2 zinc finger proteins contain the KRAB domain, a potent transcriptional repressor domain, usually located in the NH2 terminus and consisting of an A box and a B box. The A box has a repressor role and binds to specific corepressors, while the B box enhances the repression mediated by the A box through unknown mechanisms (Vissing 1995). Besides the classical KRAB AB domain, other two KRAB domains exist, one carrying the KRAB A box only, and the other having the A box and a divergent “b” box domain (Urrutia 2003) (Figure 1).

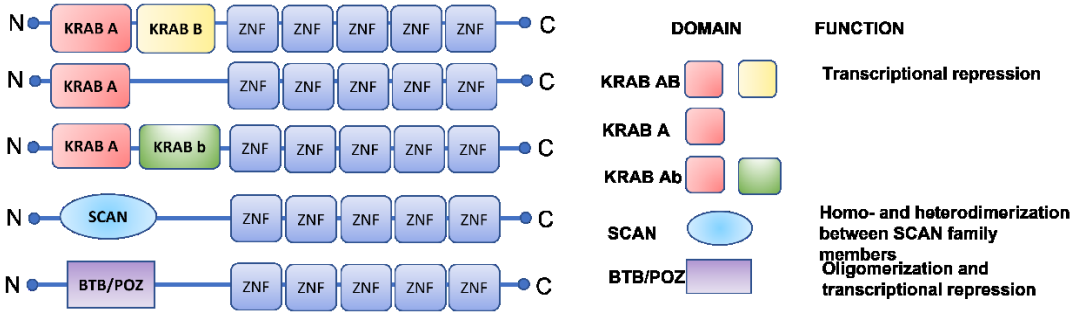


Figure 1. Schematic organization of KRAB, SCAN and BTB/POZ motifs associated with C2H2-type zinc finger domains.

All KRAB variants exert the ability to bind the co-repressor KAP1 and inhibit the transcription. The corepressor KAP-1 contains an RBCC (Ring finger/B box/Coiled-Coil) domain, at N-Terminus, that binds the KRAB module as a homotrimer (Peng 2000). The central region of KAP-1 includes a hydrophobic pentapeptide that interacts with the chromo-shadow domain of heterochromatin protein 1 (HP1) (Lechner 2000). Tandem Plant homeodomain (PHD) and bromodomain of KAP-1, at the C-terminus, act as scaffold domains that recruit histone deacetylases and chromatin remodelling activities (such as NuRD complex), histone lysine-methyl transferase (such as SETDB1) to the promoters of target genes, thus initiating ATP-dependent activities that enhance chromatin condensation and lead to gene expression silencing (Figure 2) (Lupo 2013).

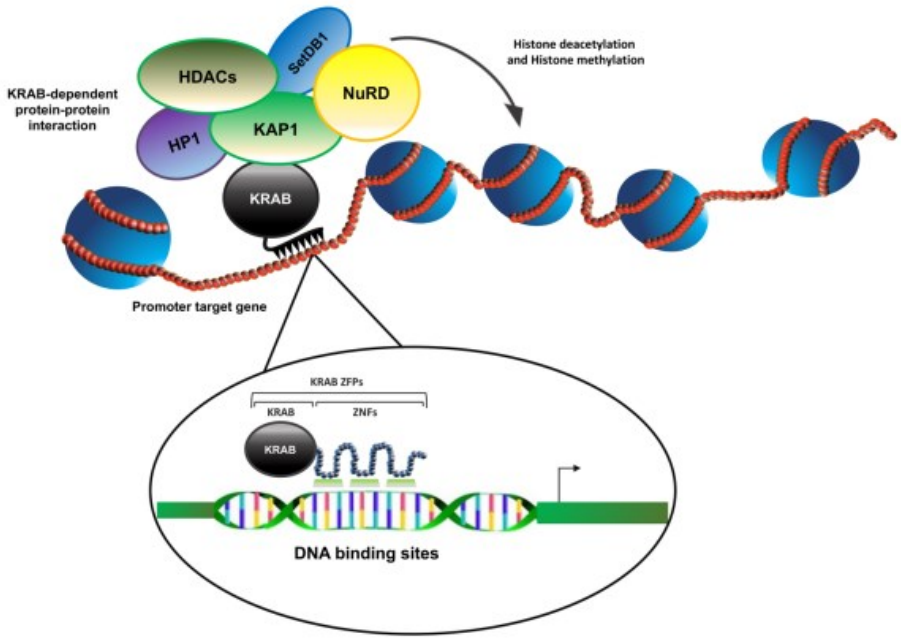


Figure 2. Schematic illustration of KRAB-ZFPs transcriptional repression complex. *KRAB-ZFPs, via the KRAB domain, recruit the corepressor KAP1 and its associated proteins in the transcriptional complex, thus inducing alteration of chromatin structure to repress transcription of target genes. (From Cesaro E. et al., ZNF224 protein: multifaceted functions based on its molecular 2 partners, Molecules, 2021 Oct 18, 26(20):6296)*

KRAB zinc fingers are involved in controlling numerous physiological and pathological processes such as embryonic development, cell differentiation (Shibata 2011), cell proliferation, apoptosis, neoplastic transformation, and cell cycle regulation (Lupo 2013). Since transcriptional regulation abnormalities and epigenetic aberrations are leading events in tumorigenesis (Baylin 2001), it is not surprising that KRAB-ZFPs play an important role in neoplastic transformations, through the modulation of the dynamics of hetero- and euchromatin maintenance (Jen 2016). Accordingly, several KRAB-ZFPs are frequently downregulated in cancer and exhibit tumor-suppressive properties through the regulation of specific genes involved in cell proliferation and apoptosis. It is worthwhile mentioning that the KRAB-ZFP ZNF382 acts as tumour suppressor in a variety of carcinomas, suppressing the expression of many oncogenes and inducing apoptosis through the inhibition of NF-κB and AP-1 signalling (Cheng 2010).
In addition to their role as transcriptional repressor, however, some KRAB-ZFPs act as activators of gene expression in cancer. Notably, the KRAB-ZFP ZNF300

is emerging as a novel oncogene in humans that promotes tumour progression by activating the NF-κB pathway (Wang 2012).
Interestingly, some KRAB-ZFPs have been shown to be involved in both activation and repression of gene transcription in cancer. The KRAB zinc finger transcriptional repressor ZBRK1 (zinc finger and BRCA1-interacting protein with KRAB domain-1) modulates the transcription of genes involved in cell cycle and DNA damage response, by interacting with two different corepressor, KAP1 and BRCA1 (Furuta 2006). It has been shown that ZBRK1 promotes the transcription of numerous genes through an unknown mechanism and ZBRK1 coactivators still remain to be identified (Lin 2010).
In this scenario, several studies have shown the emerging role of ZNF224 in cancer, highlighting its dual function as both tumour suppressor and oncogene. The interaction with different protein partners, recruited in different cellular contexts, may explain ZNF224 differential response (repression or activation) toward specific sets of target genes, eliciting apoptosis or stimulating cell proliferation (Cesaro 2017).

1.2 The transcriptional factor ZNF224

ZNF224 is a transcriptional factor that consists of 707 amino acids and belongs to the KRAB zinc finger proteins (KRAB-ZFPs) family. The gene encoding for ZNF224 maps at the human gene locus19q13.2, in a large gene cluster containing about 200 genes coding for KRAB-ZFPs and no functional orthologue are present in the neighbour species (Huntley 2006).
ZNF224 genomic organization is similar to other genes coding for KRAB-ZFPs. The gene consists of six exons where the first three exons encode for the 5'-UTR region, exon IV and V for KRAB-A box and KRAB-b box, and exon VI for the remaining coding region. A second transcript, encoding the protein ZNF255, originates from the ZNF224 gene. ZNF255 differs from ZNF224 only in the NH2-terminus, as it lacks the KRAB repression module. The biological role of ZNF255, although still poorly understood, is probably linked to RNA maturation and processing (Figure 3) (Florio 2010).

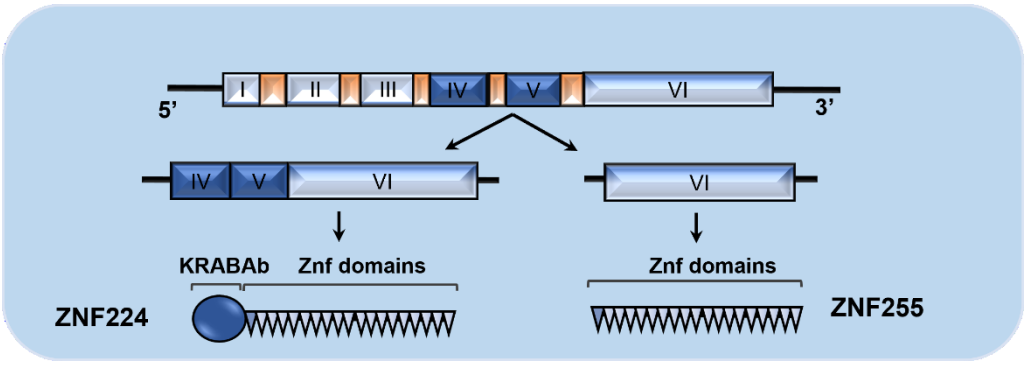


Figure 3. Schematic representation of ZNF224 gene *(from Cesaro E. et al., ZNF224 protein: multifaceted functions based on its molecular 2 partners, Molecules, 2021 Oct 18, 26(20):6296)*

While the role of ZNF255 has been poorly investigated, numerous experimental evidence demonstrated that ZNF224 is a multifaced protein able to exert many different roles both in physiological and pathological molecular pathways. Firstly, it was observed that ZNF224 had a role in the transcriptional repression of important metabolic enzymes. In particular, it was demonstrated that ZNF224 downregulated the expression of the aldolase A and the mitochondrial citrate carrier (CIC) genes (Medugno 2005; Iacobazzi 2009). The repressive activity of ZNF224 required the specific interaction of its KRAB-A module with the N-terminal RBCC (RING finger, B1 box, B2 box and Coiled-Coil domains) domain of the co-repressor KAP-1. Through this interaction, ZNF224 recruited on the promoter of its target genes Histone Deacetylase 1 (HDCA1), the Heterochromatin Protein 1 (HP1) and other chromatin remodelling proteins. It has been reported that the transcriptional repression complex of ZNF224 includes the arginine methyltransferase (PMRT5) (Cesaro 2009) (Figure 4).
PRMT5 is able to methylate the arginine 3 of histone H4 (H4R3) on specific nucleosomes surrounding the promoter region of ZNF224 target genes, representing a key mediator of ZNF224-mediated transcriptional repression. Since the domains of ZNF224 involved in the interaction with PRMT5 are well conserved in other KRAB-ZFPs, it is conceivable that the histone methylation induced by PRMT5 could represent a general mechanism of chromatin modifications required for repression of gene transcription by KRAB-ZFPs (Cesaro 2009).

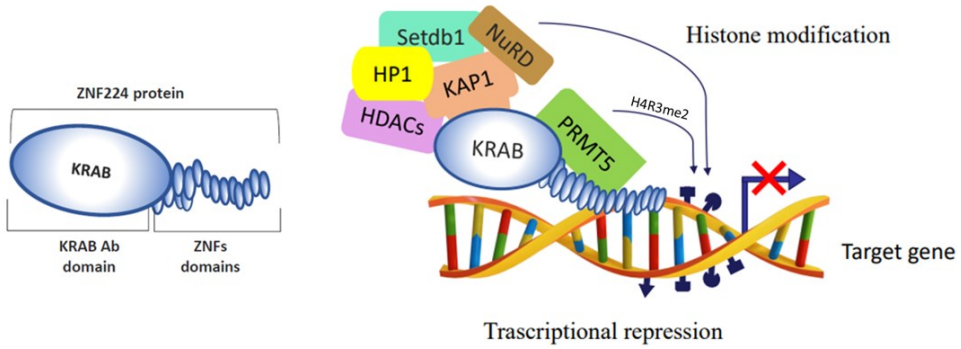


Figure 4. Schematic representation of ZNF224 protein and its transcriptional repression complex. *(From Cesaro E. et al., ZNF224 protein: multifaceted functions based on its molecular 2 partners, Molecules, 2021 Oct 18, 26(20):6296)*

Besides the ZNF224 role as a transcriptional repressor, it has been also reported a role of transcriptional activator, through a molecular mechanism that requires to be investigated more in detail (Helleboid 2019). Previously, our research group has shown that a specific methylation of KAP1 by PRMT5 triggered an impairment of the ZNF224/KAP1 interaction, inducing a switch in the ZNF224 transcriptional complex from repressor to activator (di Caprio 2015).
In the last decades, several studies have focused on ZNF224 and its involvement in cancers. In particular, it has been demonstrated a dual role of ZNF224 as both tumour suppressor and oncogene on the basis of its specific interactors and the cellular context.
Indeed, the interaction with different protein partners, recruited in different cellular contexts, may explain the ZNF224 mediated differential response (repression or activation) toward specific sets of target genes, eliciting apoptosis or stimulating cell proliferation (Cesaro 2017).
For example, in chronic myeloid leukemia (CML), ZNF224 acts as a tumour suppressor. In fact, it is a direct transcriptional repressor of the oncogene c-myc (Sodaro 2018) and of the receptor tyrosine kinase Axl (Sodaro 2018), both involved in oncogenic transformation and imatinib resistance in CML. Of note, in this cancer, ZNF224 can also act independently by its ability to bind DNA, serving as a cofactor of the Wilms' tumour protein 1 (WT1), another zinc finger protein (Florio 2010). ZNF224/WT1 complex stimulates the transcription of proapoptotic WT1-regulated genes and suppresses antiapoptotic genes, thus exerting an onco-suppressive role (Montano 2015) (Figure 5).
More specifically, a combination of overexpression and knockdown experiments in K562 cell line led the authors to highlight the dual effect of ZNF224 on the

expression of WT1 target genes; ZNF224 acts as a coactivator of WT1 in the regulation of proapoptotic genes such as Bak, Bax, and VDR, suppresses WT1-mediated transactivation of antiapoptotic genes such as bag3 and A1/Bfl1 (Montano 2015) and prevent WT1 repression of the tumor suppressor interferon regulatory factor 8 (IRF8) proapoptotic gene (Montano 2016). On the contrary, ZNF224 can also sustain tumorigenesis in other cancer types. In particular, in Chronic Lymphocytic Leukaemia (CLL), ZNF224 binds cyclin D3 gene promoter and positively modulates its expression, thus sustaining tumour growth and inducing apoptosis resistance (Busiello 2017) (Figure 6). In bladder cancer, by recruiting the protein DEPDC1 (DEP domain-containing 1), ZNF224 induces the transcriptional repression of the A20 gene, a negative regulator of the NF-κB antiapoptotic pathway. Interestingly, an inhibitory peptide that mimics the DEPDC1 domain required for ZNF224 interaction, inhibits the formation of the complex and restores A20 activity (Harada 2010). Also, the oncogenic role of the DEPDC1/ZNF224 complex through the activation of the NF-κB signalling pathway was also demonstrated in the human hepatoma cell line HepG2 (Li 2018) (Figure 6). In breast cancer, ZNF224 induces cell cycle progression and affects cell proliferation acting as a transcriptional activator of miR-663 that, in turn, decreases the expression of p53 and p21 (Cho 2016). Furthermore, another mechanism by which ZNF224 may act as an oncogene in breast cancer involves the interaction with MED28, a large protein complex involved in regulating transcription mediated by RNA polymerase II (Allen 2015). In particular, the interaction between ZNF224 and MED28, preventing ZNF224 degradation upon DNA damage, increases the proliferation and survival rate of breast cancer cells due to the ZNF224-mediated p53 and p21 gene repression (Cho 2018) (Figure 6). According to these findings, ZNF224 may be placed among the factors involved in the onset and tumor progression, via cell cycle deregulation and impaired apoptosis. In particular, ZNF224 might influence carcinogenesis by affecting the activity of cell cycle regulatory components. Overexpression of ZNF224 may alter the balance between the various molecular processes required to maintain cellular homeostasis, thus leading to the escape from the control mechanisms involved in normal cellular physiology. All together, these findings highlight the flexibility of ZNF224 both in terms of DNA-binding and in protein–protein interaction. The assembly of different multiprotein complexes in different cell types provides the mechanistic explanation for ZNF224 differential control of gene expression. Elucidating how alterations of these complexes could affect gene expression in tumor cells will shed further light into the complex role of ZNF224 in cancer.

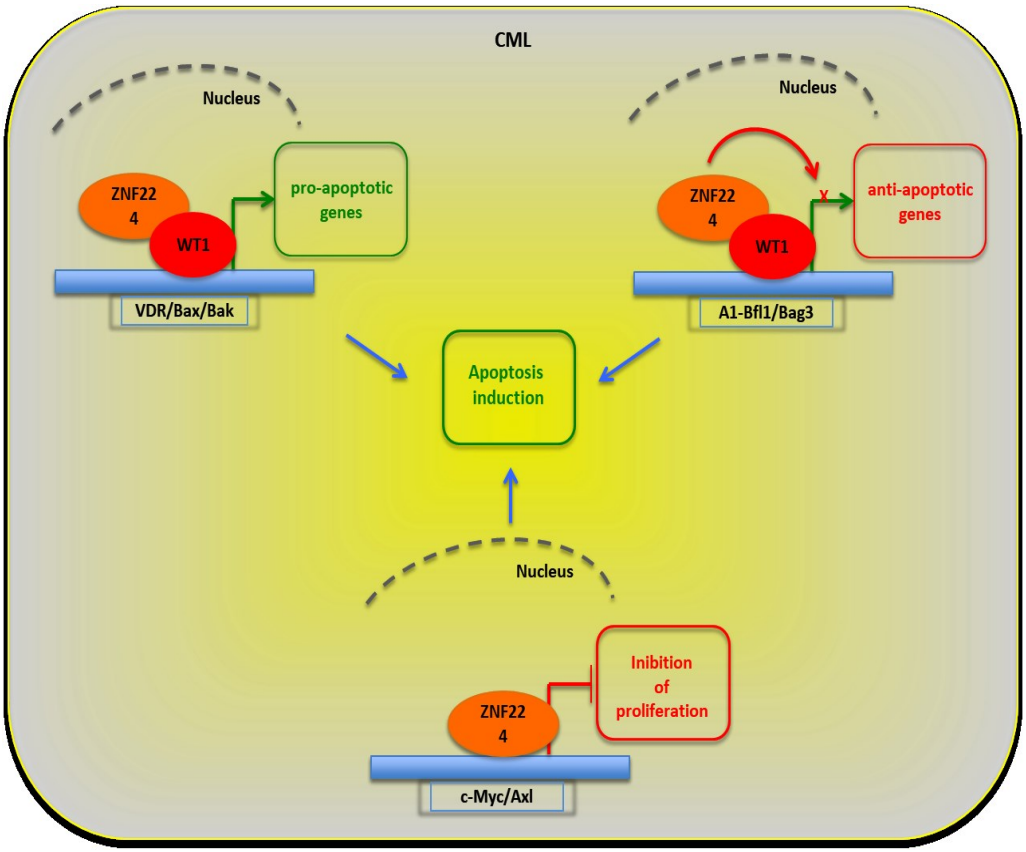


Figure 5. ZNF224 prevents both WT1 activation on its antiapoptotic genes and WT1 repression on the proapoptotic gene in CML. *(Cesaro E, Sodaro G, Montano G, Grosso M, Lupo A, Costanzo P. The Complex Role of the ZNF224 Transcription Factor in Cancer. Adv Protein Chem Struct Biol. 2017; 107:191-222).*

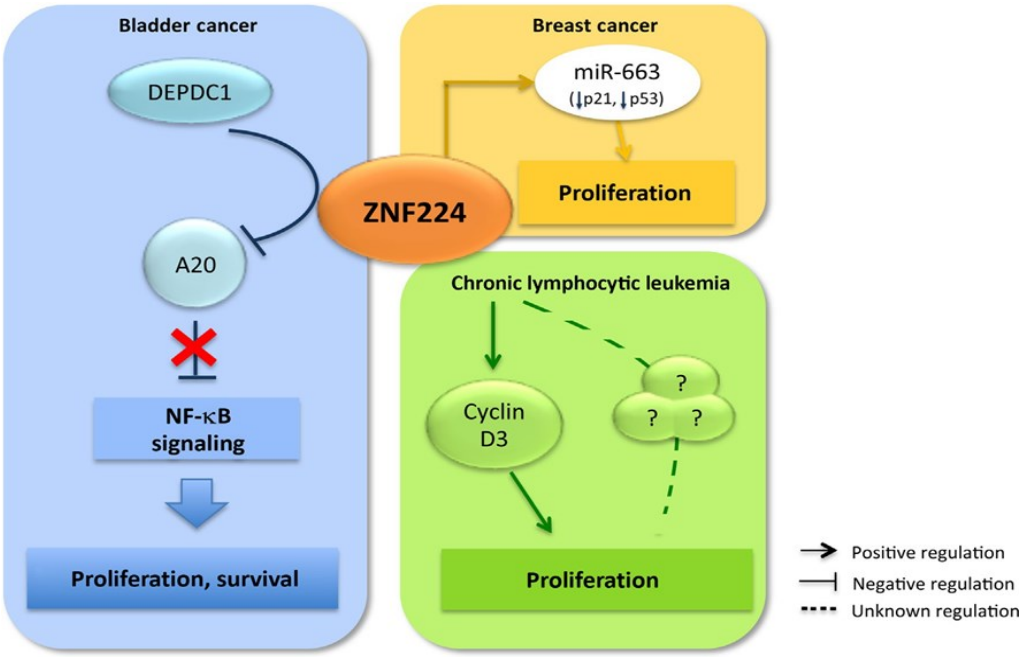


Figure 6. Schematic representation of the three tumoral contexts, so far identified, in which ZNF224 induces proliferation and survival processes by activating different oncogenic pathways. *(Cesaro E, Sodaro G, Montano G, Grosso M, Lupo A, Costanzo P. The Complex Role of the ZNF224 Transcription Factor in Cancer. Adv Protein Chem Struct Biol. 2017; 107:191-222).*

1.3 The TGF-β pathway

The TGF-β is a pleiotropic cytokine involved in embryogenesis and tissue homeostasis, regulating cell proliferation, migration and differentiation, in all tissues of the human body (Poniatowski 2015). The TGF-β superfamily of ligands consist of above 30 members and include TGF-β isoforms, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), activins, inhibins, nodal, and anti-Mullerian hormone (AMH). Three different TGF-β mammalian isoforms (TGF-β1, TGF-β2, TGF-β3) exist, encoded by distinct genes located at different chromosomes, and the three protein isoforms show a high grade of homology, sharing between 75% and 80% of sequence similarity (Massagué 1990). Despite the high level of homology, the three protein isoforms exert some distinct activities confirmed by specific knock out gene studies in mice. More specifically, TGF-β1 knockout causes a severe inflammatory reaction accompanied by tissue necrosis, thus highlighting its involvement in inflammation and immune responses control; TGF-β2 knockout results in severe developmental defects of several organs, including the heart,

while the phenotype of TGF-β3 knockout mice is not fully penetrant and is mainly characterized by severely impaired palatal closure (Böttinger 1997). These evidences suggest that a precise interplay of each isoform is essential to keep the physiologic homeostasis. Firstly, TGF-β family members are synthesized as latent precursors and after multistep maturation events (synthesis, maturation, post translational modifications, and secretion) regulated by specific enzymes, they can bind their cellular receptors, inducing biological responses. TGF-β1, the most common isoform found in human tissues, in its active form is a homodimer consisting of two polypeptide chains, each containing 112 amino acid residues, connected by a disulphide bond (Kingsley 1994). TGF-β transduces its intracellular signalling by binding an heterotetrametric complex, containing two receptor transmembrane subunits type I (TβRI) and two type II (TβRII), characterized by a dual specificity serine/threonine kinase activity (Huang 2011) (Figure 7). Also, a type III receptor (TβRIII or β-glycan) can be distinguished, which is not able to transduce the intracellular signal, due to the lack of the kinase domain, but that can act as a coreceptor (Esparza-Lopez 2001). The type I receptors comprise TβRI, activin receptor 1A, activin receptor 1B, activin receptor 1C, activin receptor L1, BMP receptor 1A and BMP receptor 1B. Instead, type II receptor members are TβRII, activin receptor II, activin receptor IIB, BMP receptor II, and anti-Mullerian hormone receptor II (Heldin 2016).

Before TGF-β binding, TGFβRI is catalytically inactive because the GS region, a peculiar protein segment with characteristic sequence SGSGSG, is inserted into the kinase domain, dislocating the catalytic centre. The binding of the cytokine TGF-β to TGFβRII, leads to the phosphorylation of the GS region within TGFβRI, that in turn causes the stimulation of TGFβRI kinase activity, leading to phosphorylation of its effectors, the Smad proteins (Shi 2003; Huse 1999). Smad proteins are the intracellular mediator of the TGF-β pathway and are classified into three different sub-classes: the first class is represented by Receptor regulated Smads (R-Smad), including Smad 1, 2, 3, 5 and 8. R-Smads are differently recruited to the TGF-β receptor complex by distinct TGF-β ligands. For example, the TGF-β, activin and Nodal active Smad2 and Smad3 while BMP and GDFs active Smad 1, Smad 5 and 8 (Massagué 2000); the second sub-class is the co-mediator Smad (Co-Smad) such as Smad 4, shuttling the R-Smads as complex in the nucleus; the third class is represented by Inhibitory Smads (I-Smads) that include Smad 6, and 7, which are antagonist of R-Smads (Shi 2003). In the best well-known and representative TGF- β /Smad pathway, the activated TβRI phosphorylates the members of the receptor-activated R-Smad family (Moustakas 2009). More specifically, TGF-β stimulation induces the recruitment of Smad 2 and 3 to the TGF-β receptor, through the action of Smad Anchor for Receptor Activation (SARA) protein that interacts with Smad 2 and Smad 3. Once recruited to the receptor, the phosphorylation of Smad 2 and 3 occurs. After phosphorylation, R-Smads are enabled to bind Smad4 and migrate into the nucleus (Tsukazaki 1998; Zeng 2010). The active Smad complex can enhance or inhibit transcription, depending on binding partners that

respectively recruit histone acetyltransferase (HATs), p300, CBP and histone deacetylases (HDACs). Smad 6 and Smad 7 are recruited in the pathways as specific checkpoints, they mediate a feedback negative control leading to different mechanisms. In particular, they can bind R-Smads and Smad 4, preventing their binding to the receptor or targeting the TGF-β receptor for ubiquitin-dependent degradation, and also induce receptor dephosphorylation, inhibiting Smad dependent promoter activation (Shi 2003; Kavsak 2000; Zhang 2007).

Interestingly, binding studies have shown that Smad 3 and Smad 4, but not Smad 2, through the interaction with other DNA-binding factors, are recruited to a specific DNA sequence called SBE (Smad-binding element) characterized by a repeated sequence AGAC or its complementary GTCT (Figure 7). Besides the above described Smad-dependent signalling pathway, also called "canonical", described above, TGF-β can activate a "non-canonical" Smad-independent signalling, including the Erk1/2, JNK, and p38 MAP kinase, the tyrosine kinase Src, phosphatidylinositol-3′ (PI3)-kinase, and Rho GTPases (Heldin 2016).

TGF-β is a potent inhibitor of epithelial cell proliferation, and as such is considered a potent tumour suppressor during early stages of carcinogenesis (Elliott 2005). TGF-β operates through the induction of the cyclin-dependent kinase (CDK) inhibitors p21 and p15, as well as p27 and p57, leading to cell cycle arrest in G1 phase (Massagué 2006). Alterations of TGF-β signalling, including loss-of-function mutations in genes encoding TGFβ receptors or Smad proteins, are often found in human tumours. They may represent mechanisms by which tumour cells escape from the antiproliferative activity of TGF-β (de Caestecker 2000). Remarkably, TGF-β may also exert tumour promoter activities at later stages of carcinogenesis. Most human tumours secrete large amounts of TGF-β, which directly influences the microenvironment and promotes tumour growth, invasiveness and metastases (Leivonen 2007). TGF-β also contributes to the development of peri-tumoral angiogenesis, and as a negative regulator of immune functions, may also favour tumour escape from immune surveillance. TGF-β is considered as an important player repressing the anti-tumour immune response. In particular, TGF-β affects mainly Th-cell polarization, promoting T-reg cells generation through the FOXP3 induction and, in addition, suppresses the proliferation and function of cytotoxic T lymphocytes (CTLs), B-cells and NK- cells. Moreover, TGF-β participates to the resolution of inflammation and tissue repair processes, mediated by specific macrophages (Jang 2011). In addition, TGF-β sustains also motility and invasion exerting its role on tumoral stroma and extracellular matrix components and promotes angiogenesis, stimulating extracellular release of VEGF, a potent chemoattractant, that induce endothelial cell growth and neo vascular formation around tumoral mass (Javelaud 2008). In melanoma progression, TGF-β plays a very important role. While in normal melanocytes TGF-β has antiproliferative activity by inhibiting transcription factors like PAX3 (Yang 2008) and M-MITF (Kim 2004), which are involved in melanocytes differentiation and cell survival, it was documented that TGF-β pathway is activated in melanoma cell lines

(Rodeck 1999). These cells secrete high levels of the three TGFβ isoforms (Javelaud 2008) and their plasma levels are correlated with metastatic progression in patients (Krasagakis 1998; Berking 2001). Further, basal phosphorylation of Smad 3 was also found in melanoma cells, which means that the pathway is constitutively activated. Moreover, high levels of phosphorylated Smad 2 were also found in the nucleus of nevi cells, which implies that the pathway might be important for the progression and the switch from radial to vertical growth (Lo 2008). Several studies highlighted the importance of constitutively active TGF-β pathway through the Smad pathway in controlling the invasive capacity and metastatic potential of melanoma cells (Javelaud 2005).

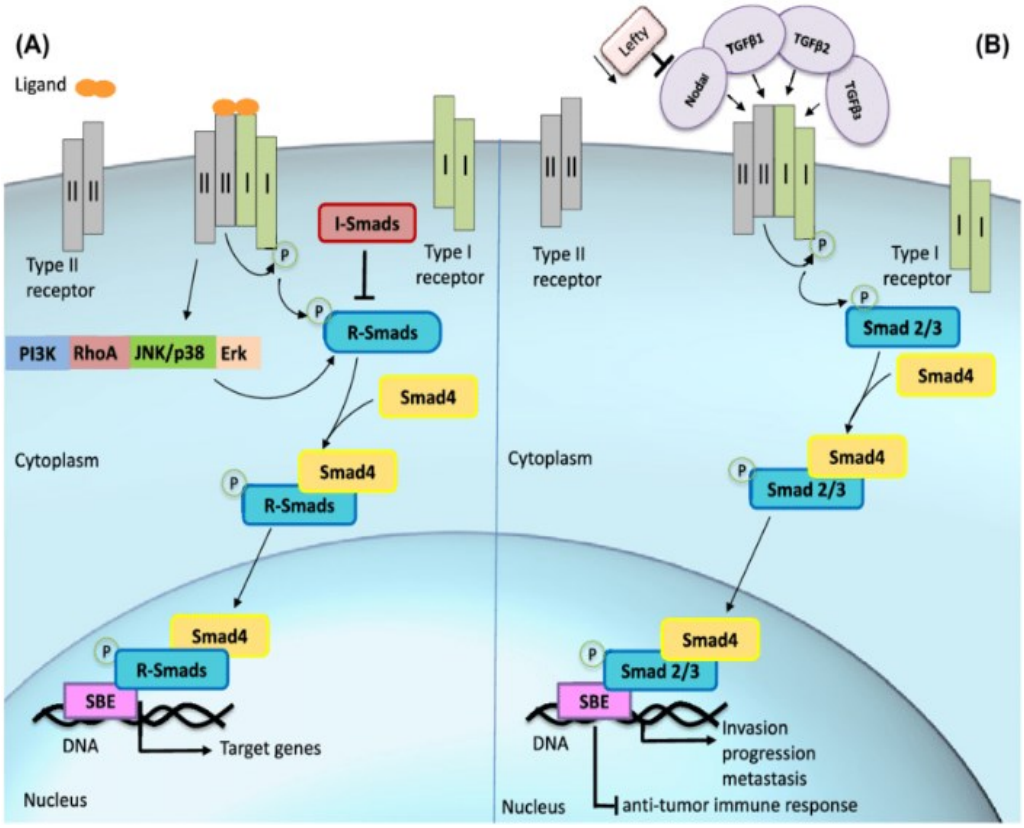


Figure 7. TGF-β pathway cascade in cutaneous melanoma. A) *specific ligands bind to type II receptor which heterodimerizes and phoshporylates type I receptor. This receptor, in turn, recruits and phosphorylates R-Smads. Co-Smad binds p-R-Smads, translocates in the nucleus and associates with DNA-binding cofactors to activate transcription of target genes.* ***B)*** *TGF-β signalling through R-Smads complex can induce invasion, progression, and metastasis and also inhibit immune response (from Dantonio P., Exploring major signaling cascades*

in melanomagenesis: a rationale route for targeted skin cancer therapy, Bioscience Reports 2018)

1.4 Melanoma and TGF-β pathway

Cutaneous melanoma is one of the most aggressive and heterogeneous human cancer with poor clinical outcomes and acquired resistance to treatments. Melanoma affects the melanocyte cells designed to produce melanin. From specific vacuoles named melanosomes, melanocytes spread pigmental melanin to keratinocytes which have a protective role against ultraviolet radiation (UVR), one of the leading causes of melanoma (El Ghissassi 2009). UVR can result in mutations in skin cells by directly interacting with DNA or indirectly generating reactive oxygen species (ROS). In general, white populations have a high risk to develop melanoma and in addition to phenotypic predisposition, 8-12% of melanoma cases arise from genetic susceptibility. Mutations in Cyclin-dependent kinase inhibitor 2A (CDKN2A) and Cyclin-dependent Kinase 4 (CDK4) genes are prevalent in familial melanoma cases.
Melanoma tumorigenesis can develop from benign melanocytic nevus that evolves in hyperplasia and ultimately in dysplasia; from this stage, it faster can progress through invasive lesions, thus degenerating in metastatic melanoma. This multistep tumorigenic process results from an accumulation of genetic alterations that comprise genomic instability and activation of oncogenes such as B-RAF, RAS, and NF1 mutated in almost 50% of melanoma cases (Cancer Genome Atlas Network 2015). Although many melanoma lesions are cured at diagnosis by surgical resection, metastatic melanoma is extremely hard to treat as it is characterized by an impressive resistance to existing therapies, due to the high heterogeneity and plasticity of melanoma caused by several mutations in cell signalling components, affecting several signal transduction and metabolic pathways (Ghosh 2009).
Several studies reported that the pleiotropic cytokine Transforming Growth Factor-β (TGF-β) shows an increased expression in cutaneous melanoma and enhances tumor progression, tissue remodelling, invasiveness, reducing the anti-tumoral immune response (Dantonio 2018).
In normal condition, TGF-β participates to multiple physiological processes, including embryogenesis and tissue homeostasis, regulating cell proliferation, migration and differentiation in all tissues of the human body (Poniatowski 2015). Besides the increased expression of TGF-β, another hallmark of melanoma is represented by a different expression pattern of TGF-β isoform (Krasagakis 1998; Rodeck 1994; Rodeck 1999; Albino 1991). Both normal melanocytes and malignant melanomas express TGF-β1 and TGF-β3 mRNA transcripts but TGF-β2 mRNA transcripts is highly expressed only in melanoma cells (Albino 1991)). An aberrant expression of TGF-β is found in the early

stages of melanoma progression and at these stages TGF-β promotes cycle arrest and apoptosis while at advanced stages, TGF-β actions induce migration and invasiveness abilities, stemness and epithelial-mesenchymal transition of cancer cells, consequently promoting tumor progression and metastasis formation (Jakowlew 2006; Drabsch e ten Dijke 2012). Interestingly, to induce epithelial-mesenchymal transition (EMT) signature, TGF- cooperates with stem cell pathways like Wnt, Ras, Hedgehog and Notch that are involved in the induction and maintenance of stem cell niches, and cells undergoing EMT can acquire a cancer stem cell (CSC)-like phenotype (Fuxe 2010; Singh 2010). These observations suggest that EMT may contribute to the generation of CSC, however, direct evidence that CSC arise in part as a result of EMT is still lacking. CSC are discussed as the only subpopulation of cells within a tumor responsible for tumor growth and metastasis. Moreover, recent evidence now indicates that EMT of tumor cells not only causes increased metastasis, but also contributes to drug resistance, another characteristic of CSC (Singh 2010; 2018). Commonly, the tumor progression requires a downregulation of the tumor-suppressive effects by the signal transduction mediated by TGFβ. Interestingly, in melanoma the anti-tumoral arm of TGF-β signaling is blocked, for example through P15INK4B deletion or C/EBPβ inhibition (Padua e Massagué 2009). Therefore, melanoma exploits the remaining TGFβ activities, promoting tumor progression and metastasis, such as invasion and EMT. In addition, because TGF-β signaling could activate also tumor suppressor genes, tumor promoting activity of TGFβ requires a peculiar cellular context with imbalanced sensitivity towards pro- and anti-growth signals. As example, aberrations in p16INK4 gene are found in up to 90% of several tumor, including HCC and melanoma, which contribute to insensitivity to anti-growth signals by relieving cyclin D/CDK4,6 complex inhibition and lowering p53 activation (Neuzillet 2013). To summarize, the TGFβ pathway has both pro-and anti-tumoral roles in cancer cell, on the basis of tumor stage and genetic alteration background, with various mechanistic interpretations in different models of cancer. This complexity, combined with intratumor genetic heterogeneity, makes the resulting effects of TGFβ inhibition on cancer cell compartment difficultly predictable. Moreover, the fact that TGFβ induces pro-tumoral effects although its signaling is shut down in cancer cells represents a second "paradox" that leads to shift attention to the microenvironment surrounding cancer cell (Neuzillet 2015). The TGFβ pathway exerts most of its pro-tumoral effects mainly by mediating tumor-stroma interactions and remodeling tumor microenvironment (Neuzillet 2014). More specifically, signal transduction mediated by TGFβ ligands and receptors can thus strongly affects tumor microenvironment through the modulation of several processes including fibrosis, angiogenesis, and immune cell infiltration (Neuzillet 2014). Indeed, TGFβ pathway activation represents a pivotal factor in both the generation and the maintenance of a favorable tumoral

microenvironment. During cancer progression TGF-β stimulates the proteolytic activity of cancer cells by increasing the expression of various MMP, including MMP2 and MMP9 (Elliott 2005). Interestingly, the increased expression and activation of these proteolytic enzymes that model the extracellular matrix are strongly linked to the invasive and metastatic phenotypes of the tumors, (Hofmann 2000; Schnaeker 2004). Further, TGF-β mediates the differentiation of fibroblasts within tumor microenvironment into activated cancer associated fibroblasts (CAFs) which display multiple functions during tumor progression and therapy resistance (Xouri 2010), supporting specific adhesive, invasive, and migratory properties of melanoma cells (Alonso 2007). Moreover, TGF-β plays a major role in inducing tumoral angiogenesis. One of the most important protein involved in the angiogenesis of melanoma is endoglin (CD105) - an endothelial-specific accessory TGF-β receptor that binds TGF-β1 and TGF-β3 with high affinity, whose expression is strongly increased in different stages of melanoma progression and regulates endothelial cell proliferation and thereby allowing the establishment of a neovasculature around the tumor (Javelaud 2008). Further, TGF-β1 stimulates VEGF via mitogen-activated protein kinase kinase 3 (MKK3) and activation of p38α (Wang 2004). Indeed, TGF-β plays a fundamental role in the development and progression of Melanoma, exerting pleiotropic effects on tumor microenvironment, inducing angiogenesis and contributing to immune evasion, through the generation of an immunosuppressive niche (Busse 2011). Therefore, TGF-β represents an attractive and versatile therapeutic target. However, being TGF-β signaling is extremely complex with a huge amount of downstream effectors, which are dependent by the cellular context, its use as a therapeutic target requires extreme attention and an extensive study on the function of its interactors (Neuzillet 2015).

1.5 Epithelial Mesenchymal Transition

Epithelial Mesenchymal Transition (EMT) is a cell biological process involved in embryogenesis and morphogenesis, in which epithelial cells acquire mesenchymal features. Generally, it promotes tissue healing, remodelling and repair in response to a variety of stimuli, but it is also involved in many pathological processes such as wound healing, tissue fibrosis, and cancer progression (Thiery 2002) (Figure 8).
During EMT, in response to microenviromental cues, a terminally differentiated epithelial cell enters a series of intermediate phenotypic states, characterizing the Ephitelial-Mesenchimal (E-M) axis, and reaches, at final point, a mesenchymal state (Figure 8). The two cell states, epithelial and mesenchymal, are characterized by marked phenotypical differences. Epithelial cells are featured

by apical-basal polarity and are held together by tight and adherens junctions, that require specific proteins, such as E-cadherin, Claudins and Occludins. Upon EMT activation, E-cadherin expression is repressed, lateral junctions are lost and, in general, epithelial markers are repressed, while cells acquire typical mesenchymal features, such as a strong increase of N-Cadherin, Vimentin and fibronectin protein expression that leads to a more motile phenotype with the typical spindle-shaped mesenchymal morphology (Kalluri 2009).

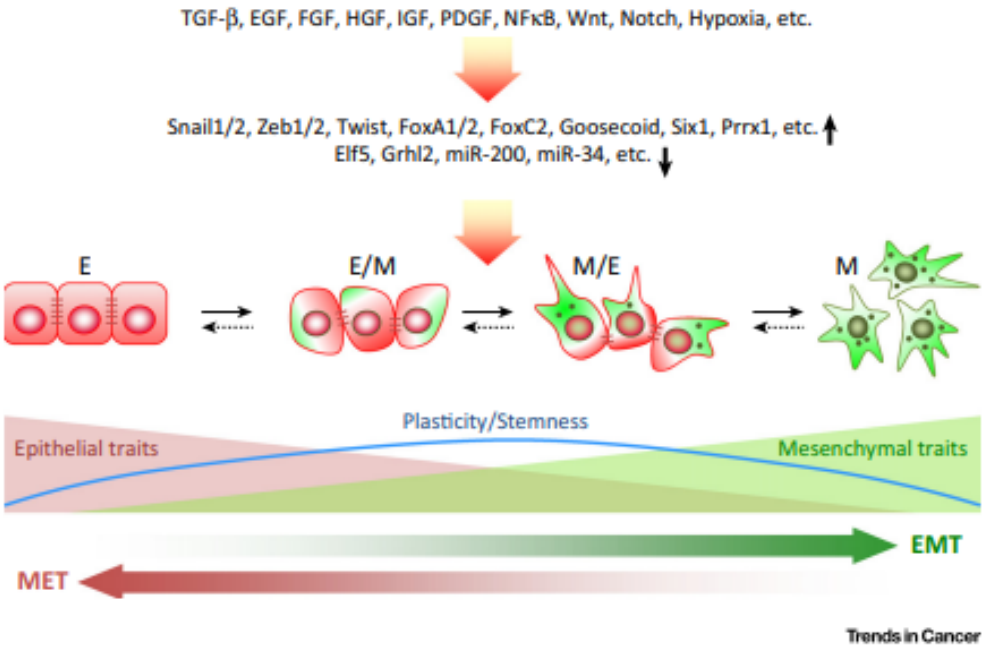


Figure 8. Schematic representation of Ephitelial-Mesenchimal Transition *(from Wenyang Li and Yibin Kang , Probing the Fifty Shades of EMT in Metastasis, Trends in Cancer, Month Year, Vol. xx, No.yy)*

However, in cancers as well as in other pathological conditions, epithelial cells rarely induce a complete EMT. A rare exception is provided by carcinosarcomas, in which distinct epithelial and mesenchymal compartments coexist and are derived from a common cellular precursor (Thompson 1996).
Furthermore, carcinomas usually exert the ability to revert the mesenchymal phenotype to different epithelial states via MET (Mesenchymal-Epithelial transition), depending on cellular contexts (Aiello 2018) (Figure 8). As shown in mice and patient-derived xenograft (PDX) models, carcinomas activate a process termed colonization, where the outgrowth of disseminated micrometastasis is deposited into macroscopic metastasis. Indeed, the activation of EMT program is crucial for the dissemination of tumour cells, whereas the disseminated cells need to undergo MET to efficiently form macroscopic metastases (Tsai 2012; Ocaña 2012; Lawson 2015) (Figure 9).

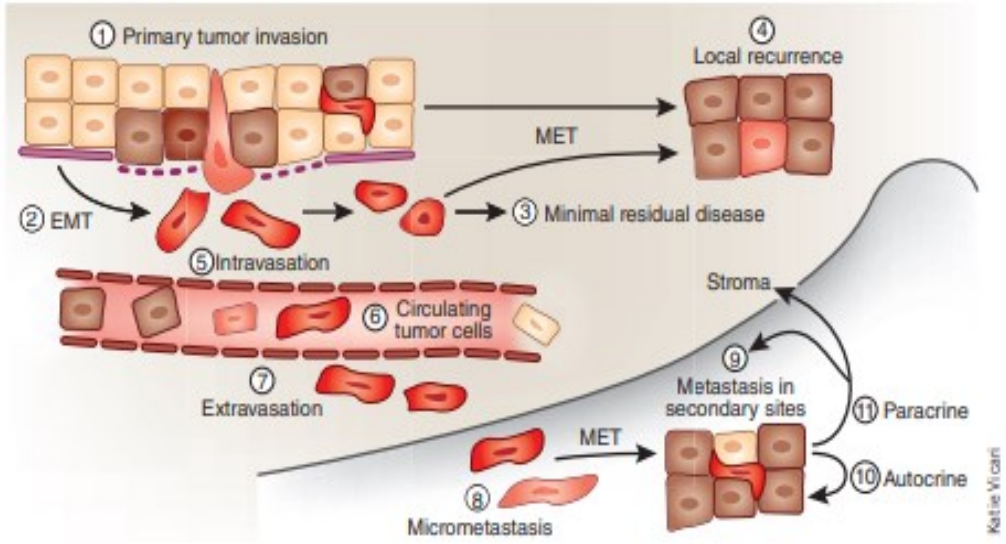


Figure 9. EMT and metastases. *Carcinoma cells usually have a primarily epithelial phenotype but contain small numbers of mesenchymal-like cancer stem cells, and/or they may otherwise undergo EMT induced by genetic, autocrine or paracrine factors (brown and red cells). In primary tumour invasion, the mesenchymal cells escape from the tumour mass, seeding the nearby stroma or vasculature; These cells may form a local recurrence where re-epithelialization occurs through MET to create a new malignancy. From local recurrence, mesenchymal cells can extravasate in the bloodstream and colonize a secondary site where micrometastases seeds and, through MET, establish macrometastasis (from E. W. Thompson E W e Haviv I, The social aspect of EMT-MET plasticity, 2011, Nature medicine, 17, pages1048–1049).*

Mechanistically, EMT is led by a wide network of transcription factors (EMT-TFs), including various families of chromatin interacting proteins, such as Snail (Snai1 and Snai2), bHLH (Twist1 and Twist2), and zinc finger and E-box binding (Zeb1 and Zeb2). EMT-TFs were originally identified as regulators of embryogenesis and cellular differentiation (Postigo 1999; Wang 1997; Fujiwara 1998; Mauhin 1993). But it is now known that they are dysregulated in aggressive cancers (Markiewicz 2012; Shin 2012; Karihtala 2013; Elloul 2005) playing a crucial role in tumorigenesis, resistance to chemotherapy and radiotherapy, and acquisition of stem cell-like properties (Sánchez-Tilló 2012; Peinado 2007). Generally, these EMT TFs work to enhance genes associated with the mesenchymal state. For example, Core-EMT TFs such as Snail, bind E-box domain on E-cadherin gene (CDH1) and recruit polycomb repression

complex, in a similar way Zeb1 also repress E-cadherin and induce N-cadherin and Vimentin, recruiting chromatin remodelling factors (E. Sánchez-Tilló 2010; Dongre 2019). Further, EMT-TFs also directly control the expression of genes associated with cell polarity (Aigner 2007) and induce matrix metalloproteinases (MMPs) required for the degradation of the basement membrane, thus promoting cell invasion (Miyoshi 2005). TGF-β, as EMT master regulator, controls this process through different mechanisms. Specifically, TGFβ-induced Smad complex can activate the transcription of mesenchymal genes, like vimentin and fibronectin, as well as Snail, Slug, Zeb1 and Twist (Xu 2009). These EMT-TFs can, in turn, upregulate the expression of TGF-β ligands, enabling the establishment of a positive feedback loop, that allows cells to sustain EMT once it has been activated (Dhasarathy 2011).

Interestingly, in melanoma progression, ZEB family members, ZEB1 and ZEB2, have opposite effects. ZEB1 is expressed in melanocyte stem cells (melanoblasts) and maintains this state, whereas ZEB2 is necessary for melanocyte differentiation by activating the crucial differentiation factor MITF (Denecker 2014). Several data show that ZEB2 inhibits tumour initiation and metastatic progression in melanoma mice models (Vandamme 2020; Denecker 2014). By contrast, ZEB1 expression is associated with poor clinical outcome and drives melanoma initiation and malignant progression. The switch from ZEB2 to ZEB1 expression in melanoma is induced by mitogen-activated protein kinase (MAPK) signalling, which links the upregulation of ZEB1 to the key driver mutations in the BRAF or NRAS genes (Caramel 2013). TWIST1 cooperates with ZEB1 to support its oncogenic potential, whereas Slug cooperates with ZEB2 with tumour-suppressive effects (Sánchez-Martín 2003). During melanoma tumorigenesis, a progressive loss of ZEB2/SNAIL2 and concomitant TWIST1/ZEB1 overexpression occur (Tang 2020). Strikingly, recent experiments of global gene expression within tumours, at single-cell resolution (scRNA-seq), revealed that tumours display a higher level of intra-tumour heterogeneity with a significant proportion of cells in a mixed/intermediate state that co-express high levels of MITF and ZEB1 (Ennen 2017). However, EMT-TFs are differentially expressed in development, tissues homeostasis and in different tumour types. In particular, in tumours, they are often expressed transiently, and they can regulate each other in a complex, dynamic and interdependent manner (Stemmler 2019). Melanoma cancers cells exert an exceptional capacity to develop resistance to current therapeutic strategies, and despite recent progress, the emergence of resistance and/or toxicities to both targeted- and immunotherapies remains a significant barrier to complete remission. A better understanding of cellular and molecular mechanisms may help define biomarkers of response and new combination therapies.

2. Aim of thesis

ZNF224 is a human pleiotropic KRAB zinc finger that acts as a positive and negative transcriptional modulator, involved in several physiological and pathological molecular mechanisms.
Previously, our research group has shown the dual role of ZNF224 in hematological malignancies, such as Chronic Lymphocytic Leukemia and Chronic Myeloid Leukemia in which ZNF224 acts as an oncosuppressor or an oncogene, respectively, according to the molecular partners recruited on the promoters of its target genes and the cellular context in which it acts.
The Transforming Growth Factor-β (TGF-β) plays an important and pleiotropic role in melanoma progression. TGF-β contributes to several pathological processes, including cell proliferation, migration, invasiveness, and metastasis formation of melanoma, down-regulation of the anti-tumoral immune response, and induction of escape from immune surveillance.
In this study, we aimed to investigate if ZNF224 could be involved in melanoma and could participate in the pro-tumoral functions of TGF-β pathway. Indeed, we observed that ZNF224 expression was higher in melanoma cell lines and was induced by TGF-β.
Starting from these data, we investigated the oncogenic function of ZNF224 in melanoma, mainly focusing on the role played by this zinc finger protein in the TGF-β pro-oncogenic pathway.
Moreover, we investigated if ZNF224 could sustain tumour progression, enhancing melanoma cells' proliferation, migration, and invasiveness
The identification of ZNF224 as a modulator of TGF-β signalling in melanoma could represent a new important tool in understanding the complex role played by TGF-β pathway in this tumour, thus contributing to find new strategies for targeting TGF-β signalling.

3. Materials and Methods

3.1 Cell cultures and treatments

A375 and A2058 melanoma cell lines were provided by the Cell Culture Facility of CEINGE (Naples, Italy). The melanoma cell line SAN was established from a patient's biopsy. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Corning, NY, USA) supplemented with 10 or 15% fetal bovine serum (FBS) (Corning) at 37◦C in 5% CO2. For TGF-β treatments, A375 cells were plated at a density of 1.5×10^5 cells/well in a 12-well plate and 24 hour later, cells were treated with TGF-β1 (10 ng/ml) (Sigma-Aldrich, St. Louis, MO) for 0–9 h or 18–24 h.

3.2 Transient transfection

To obtain the overexpression of ZNF224, A375 and A2058 cells were transient transfected with 4μg and 10μg of p3xFlag-ZNF224 expression vector or p3xFlag empty control vector in 6-well or 10cm plates, respectively, using Metafectene (Biontex, Munchen, Germany), according to manufacturer's instructions. For ZNF224 knockdown, cells were transfected with 50 pmol Dharmacon™ ONTARGET plus HumanZNF224 short interfering RNA (siRNA) - SMART pool or a non-targeting pool as a control at 50 nM final concentrations for 96 hr using Lipofectamine 2000 (Thermo Fisher Scientific Waltham, MA).

3.3 Cell lysates and western blot assays

Total protein extracts were obtained detaching cells with PBS-EDTA, washing with PBS 1% and resuspending in modified RIPA buffer (150 mM NaC, 50 mM Tris-HCl, pH 7.4, 1mM EDTA, 1% Triton X-100, 1% Na-Deoxycholate, 0,1% SDS, 50mM NaF, 1mM Na3VO4, 1mM PMSF, inhibitors protease). Protein samples were quantified using the Bio-Rad protein assay reagent, resolved by Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and then transferred to a nitrocellulose membrane with a RTA Transfer Kit (Bio-Rad Hercules, CA) and Trans-Blot turbo (Bio-Rad), according to the manufacturer's instructions. Non-specific binding sites were blocked for 30' with 3% milk in PBS and after the membranes were incubated with the following antibodies: - anti-ZNF224 (rabbit polyclonal, T3) diluted 1:300 in Super-Block Blocking Buffer (Thermo Fisher Scientific). - anti-p-Smad2, anti-Smad2/3, anti-Slug, anti-Snail, anti-Vimentin, anti-β-Catenin, and anti-N-Cadherin, anti-E-cadherin (Cell Signalling Technology), anti-β-Tubulin (Millipore), anti-α-actin (Sigma-Aldrich), anti-Flag (Sigma-Aldrich), anti-TβRI (Abcam, Cambridge, UK) diluted 1:1000 in 3% PBS-milk. 37 - anti-TGF-β1 and anti-Smad2 (Santa Cruz Biotechnology Inc., Dallas, TX) diluted 1:300 in 3% PBSmilk. The

secondary antibodies were goat-anti-mouse IgG (H + L)-HRP or goat-anti-rabbit IgG (H + L)-HRP conjugated (Bio-Rad) antibodies, (1:5000) in 3% PBS-milk. Signals were detected with ImmunoCruz Western Blotting Luminol Reagent (Santa Cruz Biotechnology) and Clarity Western Blotting Luminol Reagent (Bio-Rad) by enhanced chemiluminescence (ECL). The band intensities were quantified by densitometry using ImageJ software

3.4 RNA extraction, reverse transcription and real-time q-PCR

RNA extraction, reverse transcription, and real-time q-PCR Total RNA was extracted using the Quick-RNA MiniPrep (ZymoSearch Irvine, CA), according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad) as recommended by the manufacturer. Real-time PCR was carried out in a Real-Time CFX 69 System (Bio-Rad) using the SsoAdvanced Universal Sybr Green Supermix (Bio-Rad) and specific primers:

- N-cadherin (Fw: 5'-TCCAGACCCCAATTCAATTAATATTAC-3'; Rw: 5'-AAAATCACCATTAAGCCGAGTGA-3'). –

- β-catenin (Fw: 5'-TGGATGGGCTGCCTCCAGGTGAC-3'; Rw: 5'-ACCAGCCCACCCCTCGAGCCC-3'). –

- E-cadherin (Fw: 5'-GCCTCCTGAAAAGAGAGTGGAAG-3'; Rw: 5'-TGGCAGTGTCTCTCCAAATCCG-3'). –

- Vimentin (Fw: 5'-TACAGGAAGCTGGAAGG-3'; Rw: 5'-ACCAGAGGGAGTGAATCCAG-3').

- TGFβR1 (Fw: 5'-TCCTGGGATTTATAGCAGCAGAC-3'; Rw: 5'-CGTGGACAGAGCAAGTTTTATCA-3'). –

- TGFβR2 (Fw: 5'- TCCTTCAAGCAGACCGATGT-3'; Rw: 5'-GAACCAAATGGAGGCTCATAATC-3'). –

- HPRT (Fw: 5'- TGACACTGGCAAAACAATGCA-3'; Rw: 5'-GGTCCTTTTCACCAGCAAGCT-3'). –

- ZNF224 (Fw: 5'- GGGCTGTCTTGGCACAATTC-3'; Rw: 5'-TTGCCTCCTTGAACGTGGTC-3').

- Snail, Slug, and TGF-β1 were validated primers from QuantiTect (Qiagen, Valencia, CA, USA).

The relative quantification in gene expression was determined using the ΔΔCt method. Normalization: ΔCt = Ct(sample) – Ct(endogenous control); ΔΔCt =

$\Delta Ct(sample1) - \Delta Ct(sample2)$. Relative quantification = $2^{-\Delta\Delta CT}$. HPRT was the housekeeping gene used for relative quantification.

3.5 Immunofluorescence assay

A375 cells were plated on coverslips and transfected with the p3X-Flag ZNF224 expression plasmid or p3X-Flag empty vector. Forty-eight hours post-transfection, the cells were washed with 1× PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. The cells were permeabilized with 0.1% Triton X-100 (AppliChem, Ottoweg, Germany) for 5 min and incubated with the blocking solution for 30 min at room temperature. For antigen detection, the cells were incubated with the primary antibody N-Cadherin (D4R1H) XP® Rabbit mAb #13116 (1:100, Cell Signalling Technology) overnight at 4◦C. The following day, the coverslips were washed with 1× PBS and incubated with the secondary antibody IgG (H + L) Highly Cross-Adsorbed onkey anti-Rabbit, Alexa Fluor™ 488 (Invitrogen, USA) for 1 h at room temperature. The nuclei were stained with DAPI, Dihydrochloride (Calbiochem, San Diego, USA) for 5 min at room temperature. A Leica Thunder Imaging System (Leica Microsystems Wetzlar,Ge rmany) equipped with a LEICA DFC9000 GTC camera, lumencor fluorescence LED light source and 63× oil immersion objective was used to acquire Z-slice images. Small volume computational clearing was used to remove the background signal derived from out-of-focus blur.

3.6 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation was performed as previously described (Busiello, T. 2017). Briefly, 48 h post-transfection with FlagZNF224 plasmid, A375 cells were cross-linked with HCHO (1%) for 10 min at room temperature, lysed and fixed chromatin was sheared using an ultrasonic liquid processor. Chromatin was immunoprecipitated overnight on the wheel at 4◦ with 1 µg anti-Flag antibody (Sigma-Aldrich) or 1 µg IgG (Sigma-Aldrich). On the following day, the immunocomplexes were recovered by protein A/G plus Agarose (Santa Cruz Biotechnology Inc.,). The isolated complexes were washed twice in RIPA buffer [0.1% SDS, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% Na-Deoxycholate, 10 mM Tris– HCl (pH 8) and 140 mM NaCl], four times in 10 mM HEPES (pH 8), 0.1% Igepal, 5 mM EDTA and 250 mM NaCl solution, and once in 10 mM Tris (pH 8) and 1 mM EDTA. Crosslinking was reversed at 65◦C overnight in 10 mM Tris–HCl (pH 8), 1 mM EDTA and 1% SDS. Subsequently, DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The analysis of immunoprecipitated DNA and input controls was performed in triplicate by quantitative real-time PCR using a Master Mix SYBRGreen (Bio-

Rad). The Ct values were calculated by using appropriate Bio-rad software. Relative enrichment was calculated as fold enrichment, obtained by subtracting the Ct value for the IgG antibody background from the Ct value for the antibody of interest (Flag):($2^{\wedge -(\Delta CT\ IP - \Delta CT\ IgG)}$). The negative sample was given a value of 1. Primer sequences were as follows:

-TGFβ1 (Forward: GAACTGTGTTCTGAGGACATGG; Reverse: CCTCTCTGTGTTATCCTCCTCC);

-TGFβR1 (Forward: CTAAAAGCTGGAGGAGGAT; Reverse: TAAATGTCTGGCTCTGCCTTTG);

-TGFβR2 (Forward: AAGGGATAGCTCTGTGTGTGTG; Reverse: AAGAGAGACATCATCCTGAGCC)

- unrelated region (UNR) (Forward: CTGACAAGGTGATGGGCTTATG; Reverse: AAGGATTCGGTGATGGCTCTA).

3.7 Colony formation assay

A375 and A2058 cells overexpressing or silenced for ZN224 and their respective control cells were detached 24 h after transfection and seeded at a density of 5×10^2 in a six-well plate in triplicate and incubated for 15 days. The culture medium was replaced every 2 days. After fixing with 25% methanol and staining with 0.1% crystal violet, the colonies were counted. The average colony count for the three dishes was used to calculate the plating efficiency (plating efficiency = number of colonies counted/number of cells plated). After elution of crystal violet with 1% SDS, absorbance at a 570 nm wavelength was measured using a Microplate Reader-BioTek Synergy H1 (BioTek US, Winooski, VT).

3.8 Migration and invasion assays

The migration of A375 cells was evaluated using Transwell Supports for 24-well plates with an 8-μm pore membrane size (Falcon, Corning Inc.). For the invasion assay, the upper side of the Transwell Supports (Corning) was precoated with 100 μl of Matrigel Basement Membrane Matrix (Corning) diluted 1:5 in DMEM-free medium and allowed to dry out at 37◦C for at least 1 h. In the lower panel, 600 μl of DMEM supplemented with 15% FBS was added. A375 cells overexpressing or silenced for ZN224 were seeded on the upper side of the membrane at a density of 2×10^4 cells/100 μl of DMEM supplemented with FBS 1%. After 20 h of incubation at 37◦C, cells on the upper surface of the membrane were removed using a cotton wool swab and migrated or invasive cells on the lower side of the membrane were fixed with 25% methanol and stained with 0.1% crystal violet. The images of stained cells were captured under a light

microscope (Leica DFC365 FX, Leica Microsystem, Wetzlar, Germany) at a magnification of ×5 to ×10 in five random fields in each well. The percentage of migratory and invasive cells was evaluated by eluting fixed cells with 1% SDS and reading the absorbance at λ570 nm.

3.9 Soft-agar assay

A375 and A2058 cells transiently transfected with 3xFlag-ZNF224 or the 3xFlag empty vector were used to evaluate anchorage-independent growth. Dishes (60 mm) were precoated with a solution containing DMEM 2× (Sigma, St Louis, MO), Tryptose Phosphate Broth Buffer (Difco, BD, Franklin Lakes, NJ) and 1.25% Noble Agar (Difco, BD, Franklin Lakes, NJ) and left to dry for 10 min. Next, 10^4cells were resuspended in 2 ml AGAR DMEM and plated on top of the dried Noble Agar layer in the 60-mm dishes. Cells were grown for 2 weeks in the incubator at 37◦C in 5% CO2, and fresh medium was added once a week. Cell clumps were observed, and their pictures were captured under a light microscope (Leica DFC365 FX, Leica Microsystem, Germany) at a magnification of ×5 in five random fields in each well. Cell colonies were counted using ImageJ software (Version 1.49).

3.10 Wound healing assay

A2058 cells overexpressing ZNF224 and control cells (Flag) were seeded in 60-mm dishes at a density of 4×10^5. After 24 h, a yellow pipette tip was used to make a scratch. Cells were rinsed three times with 1× PBS and once with growth medium to remove the detached cells. Then, 3 ml of fresh DMEM were added. Scratch closure was monitored, and images were captured at 0, 24 and 48 h using a light microscope (Leica DFC365 FX, Leica Microsystem, Germany). Wound closure was measured by calculating the density of the pixels in the area where the cut was made and expressed as a percentage of wound closure in the area. The percentage of wound closure was calculated by Image J software (Version 1.49).

3.11 Statistical analyses

Data were presented as the mean ± standard error of the mean (SEM) from three or more independent experiments unless indicated otherwise. Statistical analysis was performed with Prism 7™ (GraphPad Software Inc. La Jolla, CA). $P \leq 0.05$ was considered a significant difference (*$P \leq 0.05$; **$P \leq 0.01$)

4. Results

4.1 ZNF224 is expressed at high level in melanoma cells, is induced by TGF-β and in turn affects TGF- β pathway

Evaluation of ZNF224 protein levels in different melanoma cells lines (A7, A375, A2058, SAN) showed higher ZNF224 expression compared to a human fibroblast cell line (IMR90) and human primary dermal fibroblasts (Figure 10a). TGF-β plays a pivotal role in the development and progression of melanoma, which produces increasing amounts of the cytokine correlated with disease progression (Krasagakis et al. 1998). To evaluate if ZNF224 high levels in melanoma cell lines could be related to TGF-β, ZNF224 protein levels were measured by western blot analysis in A375 cells treated with TGF-β for different time periods; as shown in Figure 10b, ZNF224 protein expression was induced in a time dependent manner, being already increased 1h after cytokine administration and remaining elevated up to 9 hours after treatment. Smad2 phosphorylation (p-Smad2) was used as control of the activation of signalling pathways downstream of TGF-β. Subsequently, to assess the possible involvement of ZNF224 in the activation of TGF-β/Smad signaling in melanoma cells, we evaluated the phosphorylation status of Smad 2 protein in ZNF224 overexpressing A375 cells. As expected, in cells transfected with an empty control vector (Flag) the phosphorylation of Smad2 was detected 1 h after TGF-β stimulation and gradually decreased. Interestingly, A375 cells transfected with a FlagZNF224 expression vector showed a prolonged Smad2 phosphorylation, up to 9 hours after the activation of TGF-β pathways (Figure 10c). This result suggests that high ZNF224 expression in A375 cells can sustain the TGF/Smad signaling, by promoting the persistence of the phosphorylated, and so activated Smads in the nucleus and the consequent TGF-β-induced transcription.

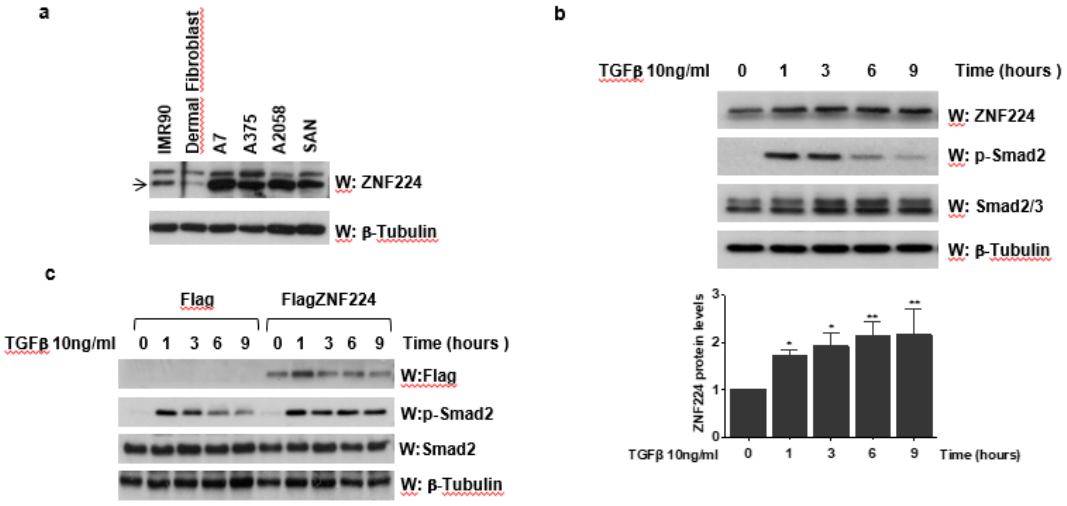


Figure 10. ZNF224 expression was induced by TGF-β treatment and its overexpression prolonged Smad2 phosphorylation. *(a) Western blot analysis of ZNF224 in protein extracts from melanoma cell lines (A7, A375, A2058 and SAN), human fibroblast cell line (IMR90) and human primary dermal fibroblasts. β-tubulin was used as a loading control. (b) Western blot analysis of ZNF224, p-Smad2 and total Smad2/3 levels in A375 cells treated with TGF-β [10ng/mL] for 1, 3, 6 or 9 h. β-tubulin was used as a loading control. Densitometric analysis of ZNF224 protein levels is shown. (c) Western blot analysis of phosphorylated Smad2 and total Smad2 levels in A375 cells transfected with Flag-ZNF224 plasmid or Flag empty vector as a control and treated with TGF-β [10 ng/mL] for 1, 3, 6 or 9 h. β-tubulin was used as a loading control.*

To confirm our hypothesis, we evaluated the effects of ZNF224 overexpression on the expression of some TGF-β target genes. To this aim, A375 cells were transfected with Flag-ZNF224 expression vector or Flag empty control vector, and mRNA and protein levels were analysed by RT-qPCR and western blot, respectively. Interestingly, we observed increased expression of the transcription factors Slug and Snail, regulators of TGF-β-induced EMT, and the mesenchymal markers Vimentin and N-cadherin and a decreased expression of the epithelial marker E-cadherin (Figure 11a; Figure 11b). Interestingly, ZNF224 overexpression increases levels of β-catenin, whose signalling is activated in melanoma progression and promotes growth and survival of melanoma cells (Widlund 2002; Sinnberg 2011).
We performed immunofluorescence staining to confirm the western blot result, showing that ZNF224 induced N-Cadherin expression in A375 cell. To this aim,

A375 cells seeded on coverslips were transfected with Flag-ZNF224 or Flag empty vector, as control. Forty-eight hours post transfection cells were fixed with 4% Paraformaldehyde, permeabilized and then stained with appropriate primary and secondary antibodies conjugated to fluorophore, as indicated in Materials and Methods. The images, acquired by fluorescence microscope, revealed an increased signal of the mesenchymal marker N-cadherin upon ZNF224 overexpression compared to control cells (Figure 11c).
Furthermore, we evaluated the expression levels of some EMT-associated genes in A375 cells silenced for ZNF224. In order to silence ZNF224 expression, A375 cells were transfected with ZNF224 siRNA or scramble siRNA (as a control) and its mRNA and protein levels were analysed by RT-qPCR and western blot, respectively, to confirm the silencing (Figure 11d).
A RT-qPCR analysis revealed that ZNF224 depleted cells failed to activate EMT. Indeed, a reduced mRNA level of N-cadherin, Vimentin, β-catenin, Slug, and Snail was found in A375 cells transfected with ZNF224 siRNA compared to control cells transfected with scramble siRNA (Figure 11e).
These results indicate that ZNF224 is able to mimic the action of TGF-β on the expression of different EMT-associated genes.

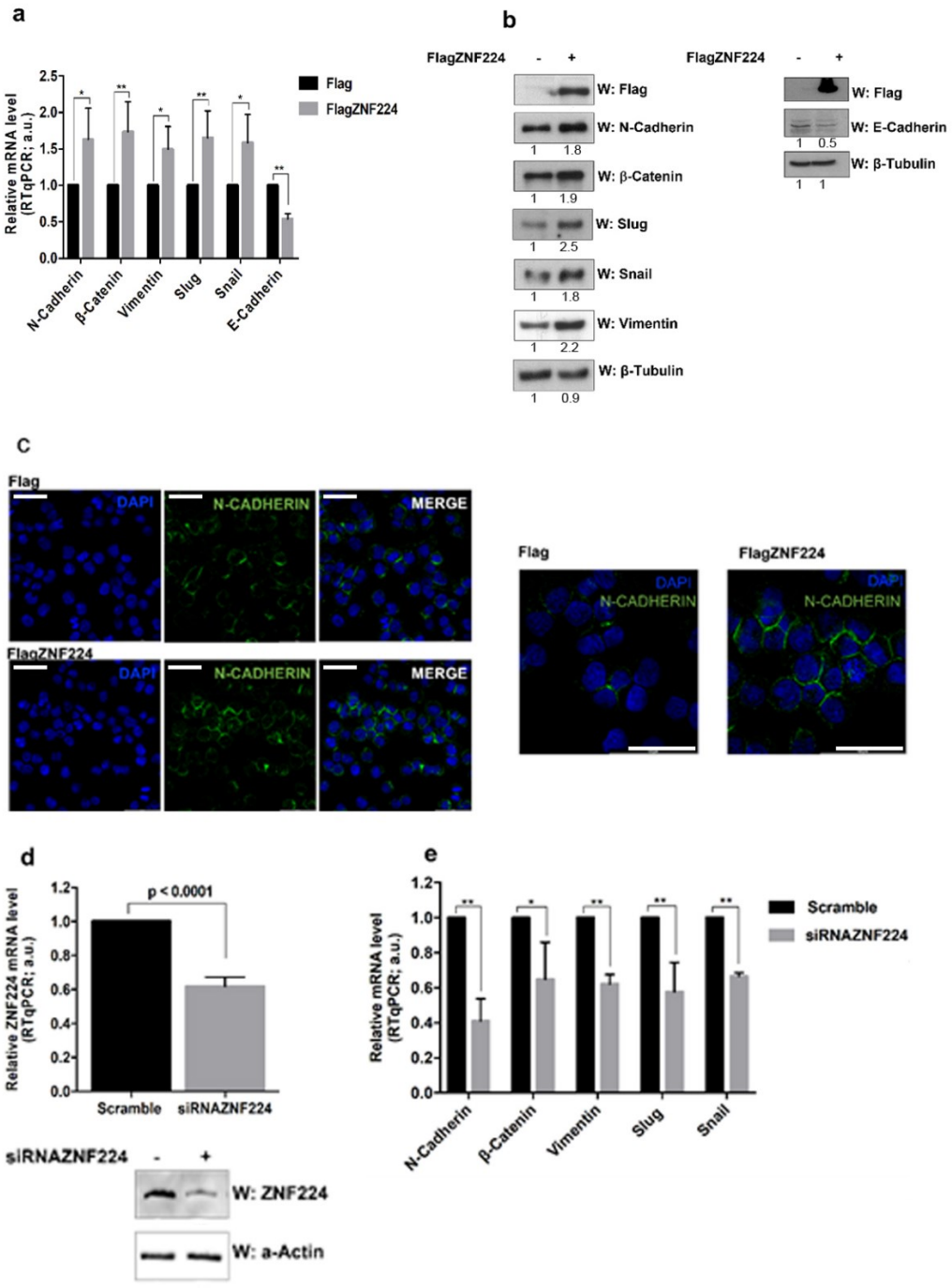


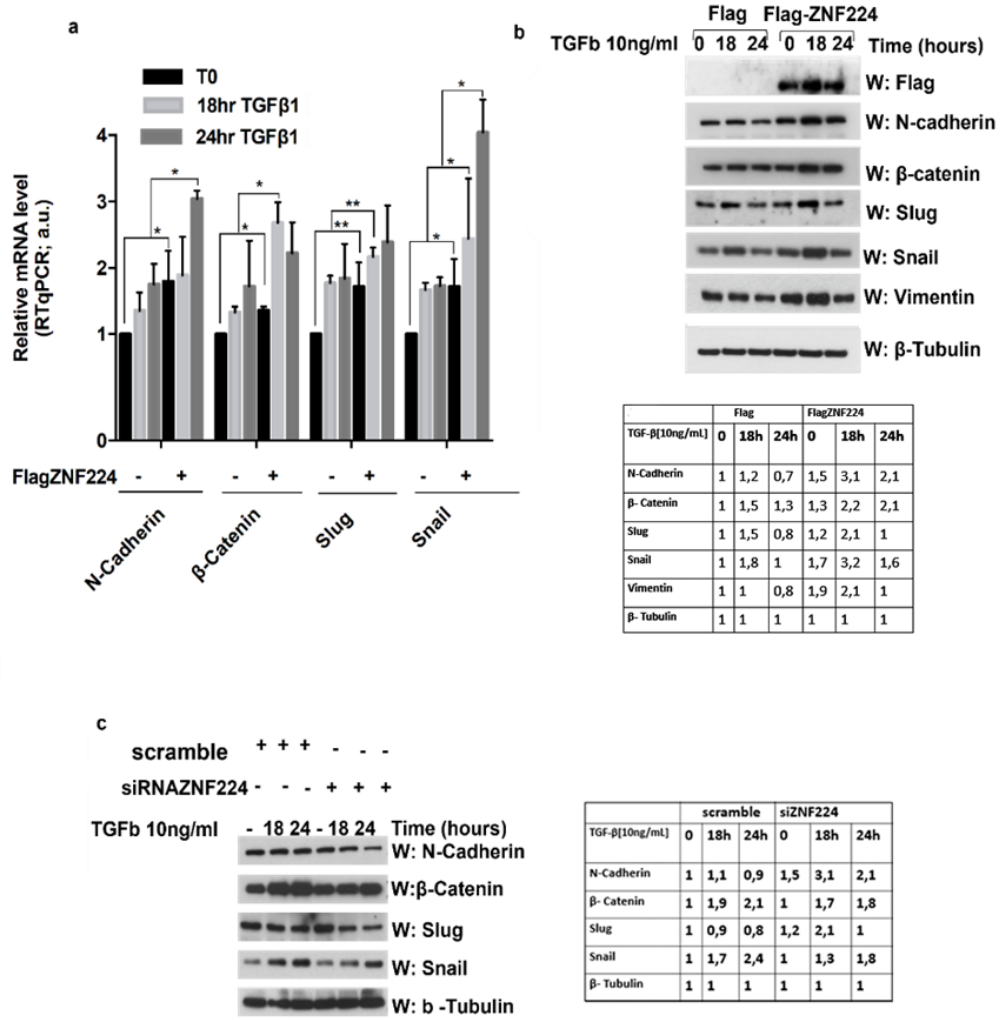
Figure 11. ZNF224 affects the expression of TGF-β target genes. *a) Reverse Transcription quantitative PCR (RT-qPCR) analysis of N-cadherin, β-catenin, Vimentin, Slug, Snail and E-cadherin mRNA expression levels in A375 cells transfected with Flag-ZNF224 plasmid or its empty vector, as control. b) Western blot analyses of N-cadherin, β-catenin, Slug, Snail, Vimentin and E-*

cadherin expression levels in A375 cells transfected with Flag-ZNF224 plasmid or Flag empty vector, as a control. β-tubulin was used as a loading control. Numbers below the bands indicate the related densitometric values obtained by using ImageJ software (c) N-cadherin immunofluorescence in A375 cells transfected with the Flag-ZNF224 construct or Flag empty vector. Microscopy images (Z-slices) showing N-cadherin expression in A375 cells overexpressing Flag empty vector (upper panel) and Flag-ZNF224 constructs (lower panel). Scale bars (white colour) indicate a 50 μm distance. (d) RT-qPCR analysis of ZNF224 mRNA expression levels in A375 cells transfected with ZNF224 siRNA or scramble siRNA (upper panel). Western blot analysis of ZNF224 levels in A375 cells transfected with ZNF224 siRNA or scramble siRNA. α-actin was used as a loading control (lower panel). (e) RT-qPCR analysis of N-cadherin, β-catenin, Vimentin, Slug, and Snail mRNA expression levels in A375 cells transfected with ZNF224 siRNA or scramble siRNA.

4.2 ZNF224 enhances TGF-β induction of EMT-associated genes

Subsequently, we evaluated if ZNF224 could cooperate with TGFβ to induce the expression of TGFβ target genes. In details, we transfected A375 with Flag-ZNF224 or Flag empty vector as a control and then treated with TGF-β for 18 and 24 hours. mRNA and proteins were purified from these cells and analysed by RT-qPCR and western blot, respectively. As expected, the expression of N-cadherin, β-catenin, Slug, and Snail mRNA was induced in control cells by TGF-β treatment alone, but, interestingly, it was significantly increased in ZNF224 overexpressing cells (Figure 12a). We further confirmed the existence of this cooperative effect by western blot analysis (Figure 12b).

These results unveil a synergistic effect between ZNF224 and TGF-β in the regulation of TGF-β target genes involved in EMT. Furthermore, to confirm the effect of ZNF224 in sustaining TGF-β/Smad signalling, we evaluated, by western blot analysis, the TGF-β-induced expression of N-cadherin, β-catenin, Slug and Snail in A375 cells silenced for ZNF224. Figure 12c shows that silencing of ZNF224 before TGF-β treatment counteracted the TGF-induced expression of these targets compared with cells transfected with scrambled siRNA, thus indicating that ZNF224 is required for TGF-β-dependent regulation of its target genes.



	Flag			FlagZNF224		
TGF-β[10ng/mL]	0	18h	24h	0	18h	24h
N-Cadherin	1	1,2	0,7	1,5	3,1	2,1
β- Catenin	1	1,5	1,3	1,3	2,2	2,1
Slug	1	1,5	0,8	1,2	2,1	1
Snail	1	1,8	1	1,7	3,2	1,6
Vimentin	1	1	0,8	1,9	2,1	1
β- Tubulin	1	1	1	1	1	1

	scramble			siZNF224		
TGF-β[10ng/mL]	0	18h	24h	0	18h	24h
N-Cadherin	1	1,1	0,9	1,5	3,1	2,1
β- Catenin	1	1,9	2,1	1	1,7	1,8
Slug	1	0,9	0,8	1,2	2,1	1
Snail	1	1,7	2,4	1	1,3	1,8
β- Tubulin	1	1	1	1	1	1

Figures 12. ZNF224 enhances TGF-β induction of EMT-associated genes*. (a) RT-qPCR analysis of N-cadherin, β-catenin, Slug and Snail mRNA expression levels in A375 cells transfected with the Flag-ZNF224 plasmid or its empty vector as a control and stimulated with TGF-β for 18 or 24 h. (b) Western blot analysis of N-cadherin, β-catenin, Slug, Snail, and Vimentin protein levels in A375 cells transfected with the Flag-ZNF224 plasmid or its empty vector as a control and stimulated with TGF-β for 18 or 24 h. β-tubulin was used as a loading control. Table below the bands indicate the related densitometric values obtained by using ImageJ software. (c) Western blot analysis of N-cadherin, β-catenin, Slug, and Snail levels in A375 cells transfected with ZNF224 siRNA or scramble siRNA as a control and stimulated with TGF-β for 18 or 24 h. β-tubulin was used as a loading control. Table beside the bands indicate the related densitometric values obtained by using ImageJ software.*

4.3 ZNF224 potentiates the expression of TGF-β, Tβ-RI and Tβ-RII

The regulation of TGF-β production and TGF-β receptors activity is critical for the regulation of signal transduction. In particular, to amplify the signalling, TGF-β induced a rapid translocation of its receptors to the cell surface (Duan 2019). Also, it can stimulate its own expression and the expression of its receptors (Menke 1999)

To investigate the molecular mechanism by which ZNF224 can participate in the modulation of TGF-β signalling, we measured the mRNA levels of TGF-β and type 1 and 2 TGF-β receptors in A375 cells overexpressing or silenced for ZNF224. To this purpose, A375 cells were transfected with FlagZNF224 expression vector or empty control vector and with ZNF224 siRNA or scramble siRNA, respectively. mRNA was purified from these cells and analysed by Real-Time PCR. Interestingly, we found an increased expression of TGF-β, TβRI and TβRII mRNA levels in A375 overexpressing ZNF224 (Figure 13a, left panel). Conversely, ZNF224 silencing resulted in reduced expression of these mRNAs (Figure 13a, right panel). Western blot analysis, performed on cells overexpressing ZNF224, confirmed the induced expression of TβRI and TGF-β (Fig. 13b). In addition, RT-qPCR analysis of TGF-β, TGFβR1, and TGFβR2 mRNA expression levels in A375 cells overexpressing ZNF224 and stimulated with TGF-β for 18 or 24 h, showed that ZNF224 overexpression potentiates the expression of TGF-β and its receptors. (Figure 13c). Subsequently, by an *in silico* analysis, we found ZNF224 binding motifs (Sodaro 2018) in the promoter region of TGF-β and in the first intron of TβRI and TβRII genes (Figure 13d, upper panel). To verify if ZNF224 occupies these genomic regions, we performed a chromatin immunoprecipitation assay (X-ChIP). The crosslinked chromatin of A375 cells transfected with the FlagZNF224 plasmid was immunoprecipitated with a Flag antibody. Quantitative PCR (qPCR) analysis confirmed the binding of ZNF224 to the TGF-β, TβRI and TβRII DNA regions containing its consensus sequences (Fig. 13d lower panel). These data indicate that ZNF224 potentiated TGF-β signaling in melanoma cells through the simultaneous activation of the cytokine and its receptors, which are essential components to mediate TGF-β effects (Duan 2019).

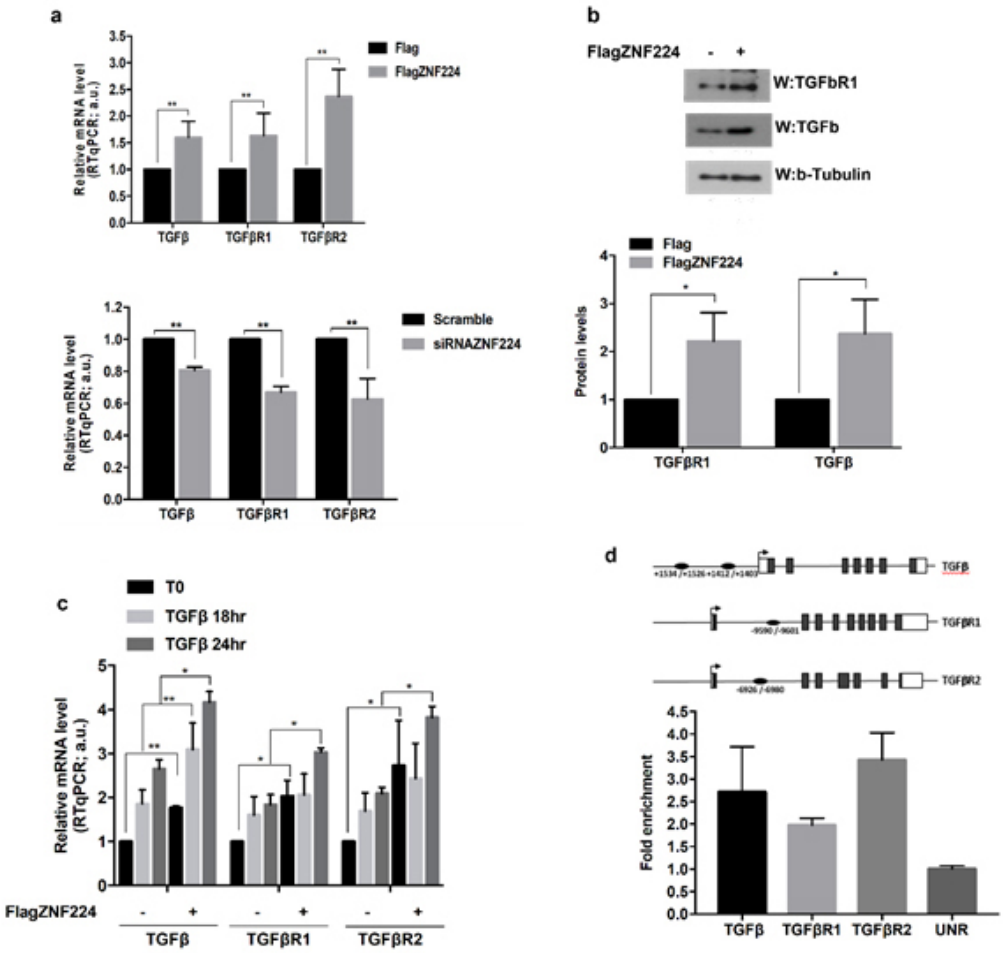


Figure 13. ZNF224 affects the expression of TGF-β, TGFβR1 and TGFβR2. ***(a)*** *RT-qPCR analysis of TGFβ, TGFβR1 and TGFβR2 mRNA levels in A375 cells overexpressing FlagZNF224 compared with cells transfected with the empty vector (Flag) (upper panel) and in A375 cells transfected with ZNF224 siRNA or scramble siRNA (lower panel). (b) western blotting analysis of TGF-β and TβRI in A375 cells overexpressing ZNF224 compared with A375 transfected with its control Flag. β-tubulin was used as a loading control (upper panel). Densitometric analysis of TGFβR1 and TGFβ protein levels is shown in the lower panel (c) RT-qPCR analysis of TGF-β, TGFβR1 and TGFβR2 mRNA expression levels in A375 cells transfected with the Flag-ZNF224 plasmid or its empty vector as a control and stimulated with TGF-β for 18 or 24h. (d) ZNF224 binds in vivo TGFβ, TGFβR1 and TGFβR2 genes. X-ChIp assay was performed in A375 cells overexpressing Flag-ZNF224 with the Flag antibody or IgG as a control. The immunoprecipitated chromatin was analysed by qPCR using specific primers spanning the putative ZNF224 binding sites shown in the upper panel. An unrelated region (UNR) was used as negative control.*

4.4 ZNF224 promotes anchorage-independent growth, migration and invasion of melanoma cells.

To investigate if ZNF224 could modulate some malignant features of melanoma cells, like cell growth and invasiveness, we performed some functional assays in A375 and A2058 cell lines.

At first, the effects of ZNF224 overexpression and depletion were evaluated through a clonogenic assay. A375 and A2058 cells were transfected with a Flag-ZNF224 plasmid or Flag empty vector, as control. Also, in order to downmodulate ZNF224 expression the cells were transfected with two different plasmids, shC3 and shE7, containing ZNF224 short hairpin RNAs (shRNA) and shGFP as negative control. As shown in figures 14a and 14b, both the number of colonies formed and the absorbance measured at 570 nm were significantly increased in A375 cells overexpressing ZNF224 compared to the control cells, in which is shown the expression of ZNF224 by western blotting (Figure 4c); conversely, A375 silenced for ZNF224 showed fewer colonies compared to control cells. Similar effects were found in A2058 cells (Figure 14d-f). These results demonstrated that ZNF224 is able to increase the clonogenic potential of melanoma cells.

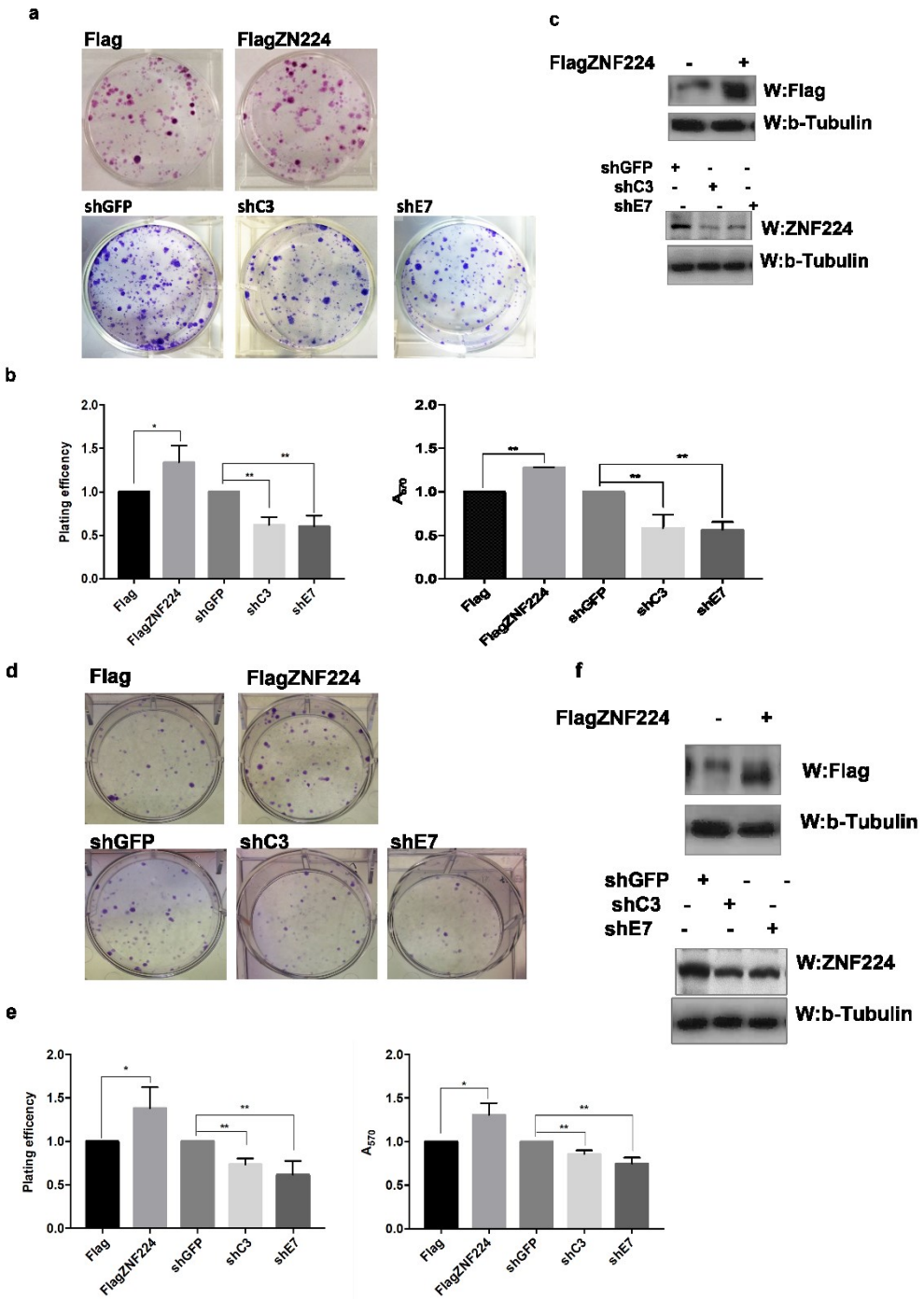


Figure 14. Effects of ZNF224 overexpression and silencing on A375 and A2058 cell growth. *(a) Colony formation assay in A375 cells overexpressing (Flag-ZNF224) or silenced (shC3, shE7) for ZNF224 and their respective control cells (Flag and shGFP). (b) Plating efficiency and A570 after crystal violet elution were evaluated. Flag and shGFP control cells were arbitrarily set as 1. The histograms represent the mean of two independent experiments*

performed in triplicate. (c) Western blot analysis was used to verify Flag-ZNF224 overexpression and ZNF224 silencing in A375 cells. β-tubulin was used as a loading control. (d) Colony formation assay in A2058 cells overexpressing (Flag-ZNF224) or silenced (shC3, shE7) for ZNF224 and their respective control cells (Flag and shGFP). (e) Plating efficiency and A570 after crystal violet elution were measured. Flag and shGFP control cells were arbitrarily set at 1. (f) Western blot analysis was used to verify Flag-ZNF224 overexpression and ZNF224 silencing in A2058 cells. β-tubulin was used as a loading control.

In addition, we assessed the ability of ZNF224 to promote the anchorage-independent growth of melanoma cells by performing a Soft Agar Colony Formation Assay, in A375 and A2058 cells overexpressing ZNF224. A375 and A2058 cells were transfected with Flag-ZNF224 or Flag empty vector, as control. Forty-eight hours after transfection cells were plated in a layer of soft agar mixed with cell culture medium, that prevents the cells adhesion to the culture plate. As expected, in both A375 and A2058 melanoma cell lines overexpressing FlagZNF224 compared to control cells, an evident increase in the colony-forming ability was observed (Figure 15a and 15b).

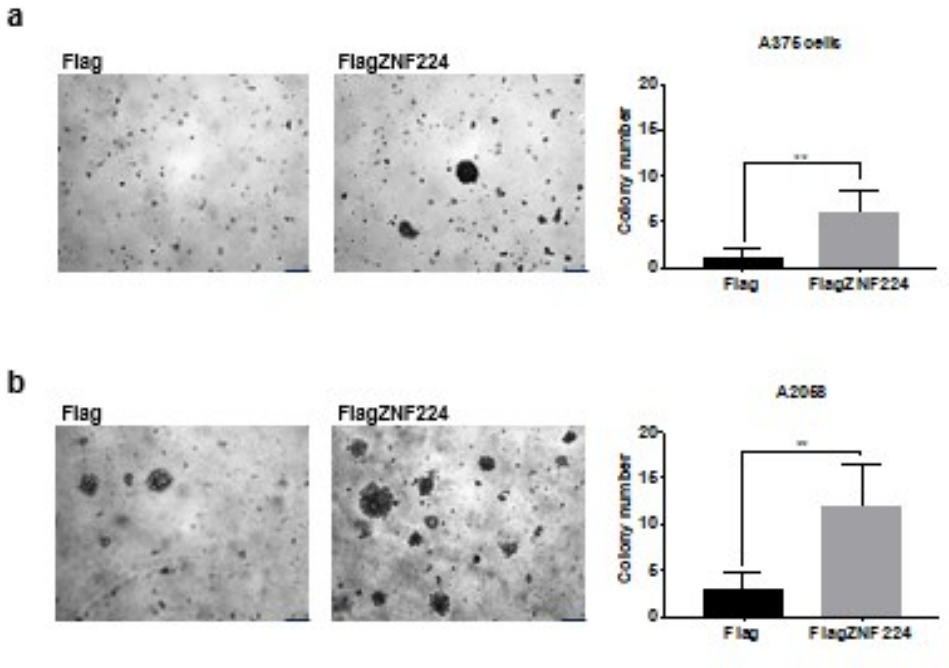


Figure 15. ZNF224 overexpression stimulates anchorage-independent growth of A375 and A2058 cells.
Soft agar colony formation assay was performed in A375 (a) and A2058 (b) cells transfected with p3xFlagZNF224 and with an empty control vector (Flag). Twenty-four hours post-transfection, the cells were cultured in a soft agar medium for two weeks. Then, the colonies were counted, and the images of A375 and A2058 colonies were acquired using a light microscope. Scale bar 250 μm. The experiment was performed once in triplicate.

Next, we asked whether ZNF224 could modulate the migration and invasion abilities of A375 cells, by performing a Transwell Assay. Briefly, we transfected A375 cells with Flag-ZNF224 or ZNF224 siRNA and their respective controls to overexpress or silence ZNF224 expression, respectively. The transfected cells were loaded into a Boyden Chamber, characterized by a chemoattractant gradient of FBS from the top to bottom, in the absence (migration assay) or presence of Matrigel (invasion assay) on the top of the Boyden Chamber. Interestingly, we found that A375 cells overexpressing ZNF224 showed a significant increase in migratory and invasive potential compared to control cells (Figure 16a) while, conversely, A375 silenced for ZNF224 showed significant suppression of cell migration and invasion ability (Figure 16b). The enhanced

migratory ability of cells overexpressing ZNF224 was also evaluated in A2058 cells through a wound healing assay. In this assay, a scratch is generated on a confluent cell monolayer and the speed of wound closure and cell migration can be quantified by taking snapshot pictures with a regular inverted microscope at several time intervals (Justus 2014). Interestingly, we found that A2058 cells overexpressing ZNF224 showed an enhanced migratory ability, compared to the control cells (Figure 16c). Taken together these results indicates that in melanoma cells ZNF224 promotes the acquisition of anchorage-independent cell growth, migration and invasion abilities, which are critical steps in cancer progression and metastasis.

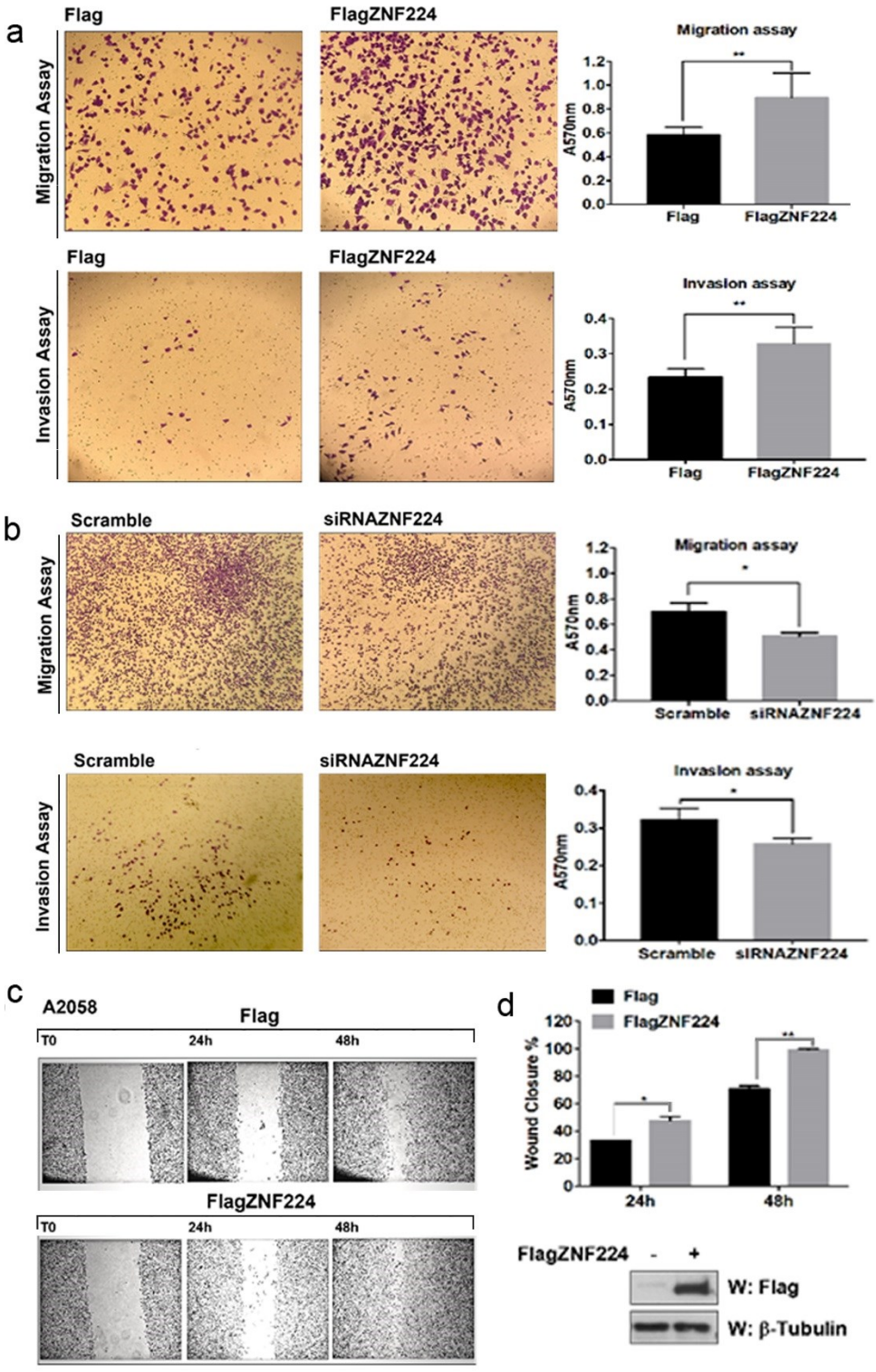


Figure 16. ZNF224 affected the migration and invasion of melanoma cells. *(a) Representative images of the migration and invasion assay performed in A375 cells overexpressing ZNF224 (FlagZNF224) and control cells (Flag). (b)*

Representative images of the migration and invasion assay performed in A375 cells silenced for ZNF224 (siRNAZNF224) and control cells (scramble). The histograms of absorbance measured at 570 nm of eluted crystal violet were obtained from the mean of two independent experiments performed in triplicate. (c) A2058 cells overexpressing Flag-ZNF224 and control cells (Flag) were subjected to an in vitro scratch assay, and the images were captured at 0, 24, and 48 h after the injury using a phase-contrast microscope. The histogram indicates the percentage of wound closure in the area. (d) The expression of the exogenous 3xFlag-ZNF224 protein in transfected cells was verified by western blot analysis. β-tubulin was used as a loading control.

5. Discussion

The KRAB Zinc Finger protein ZNF224 leads a dual role in human cancer; it acts as both a tumour suppressor and tumour promoter. This duality derives from the complex transcriptional regulatory activity depending on the cellular context and the molecular partners recruited (Cesaro 2017).
In this study, we investigated the role of ZNF224 as a mediator of TGF-β pro-oncogenic function in melanoma, a malignant neoplasm of melanocytes, whose development and progression are promoted by TGF-β, a multifunctional cytokine secreted in high amounts by this tumor.
Metastatic melanoma is an aggressive and metastatic cancer accompanied by poor prognosis and resistance to treatments (Adler 2017). TGF-β plays a pivotal role in the development and progression of this tumour, which produces increasing amounts of the cytokine correlated with disease progression (Krasagakis 1998). The cytokine exerts pleiotropic effects on melanoma tumour microenvironment, establishing a tumour friendly niche and promoting cell motility and invasiveness (Marzagalli 2019). TGF-β signalling is modulated by positive and negative feedback regulatory loops (Miyazono 2000; Massagué 2000). TGF-β positively regulates its own expression and induces the upregulation of TGF-β receptors (Duan 2019; Menke 1999); thus amplifying signalling and Smad-mediated gene responses. It is known that TGF-β can also induce the expression of some transcription factors that may cooperate with Smad in the transcriptional regulation of TGF-β target genes (Massagué 2000; Zhang 2009).
Interestingly, we observed that ZNF224 expression is induced by TGF-β in melanoma cell lines and that its expression is accompanied by prolonged phosphorylation of the Smad complex. This evidence prompted us to analyze the molecular mechanisms underlying ZNF224 activity in melanoma, mainly focusing on the examination of its involvement in the regulation of TGF-β pathway.
Firstly, we observed that ZNF224 modulated the levels of some TGF-β target genes, such as Slug, Snail, Vimentin, β-catenin, N-cadherin and in an opposite way E-Cadherin, all involved in the epithelial-mesenchymal transition (EMT), Furthermore, we observed ZNF224 can also potentiate TGF-β effects on these EMT target genes, unveiling a synergistic effect between ZNF224 and TGF-β action that could further enhance melanoma progression.
We did not extensively investigate mechanistically the involvement of ZNF224 in the regulation of the TGF-β pathway and, therefore, we could speculate that ZNF224 DNA-binding activity may facilitate the recruitment of Smad transcriptional complexes to target promoter sites. It is worthwhile mentioning that numerous DNA-binding transcription factors play a crucial role in Smad-controlled target gene selection (Massagué 2000). Smad proteins, through their MAD homology 1 (MH1) domain, recognize their cognate DNA regulatory elements with low affinity and, therefore, require the recruitment of other

cofactors, including FAST, AP-1, AML, TF3 that provide a tight and highly specific recognition of their target genes (Shi 1998). For example, the transcriptional factor TF3, through the binding to p300/CBP coactivator, allows the recruitment of the R-Smad complex to target gene promoter (Stroschein 1999).
However, it is well known that besides the Smad-mediated canonical signal transduction, TGF-β can also activate additional intracellular pathways (Zhang 2009) and we cannot exclude that ZNF224 could also modulate the transcription of some TGF-β target genes in a Smad-independent manner. Indeed, TGF-β is able to activate phosphatidylinositol-3-kinase (PI3K) and several mitogen activated protein kinases (MAPKs), including extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs), and p38 MAPK, which participate in TGF-β induced epithelial-to-mesenchymal differentiation (EMT). In melanoma, the crosstalk between the MEK/ERK and SMAD pathway is much complex. As example, the MEK⁄ERK pathway acts as an upstream activator of the JNK pathway in melanoma cells (Lopez-Bergami 2007), which interferes with TGF-β signaling and supports survival in melanoma cells by controlling cell cycle arrest and apoptosis (Alexaki 2008). Previously, it has been demonstrated that ZNF224 contributes to the regulation of several processes such as apoptosis and proliferation in hematological tumors, acting as a cofactor of WT1 in the modulation of apoptosis-related genes such as Bag-3 and A1/Bfl-1(Montano 2015) and also promoting Cyclin D3 transcription (Busiello 2017). In addition, it is observed the involvement of ZNF224 in multiple pathways associated with cell survival, cellular proliferation, thus inducing tumorigenesis through the interaction with different protein partners. As example, Harada et al. observed that in bladder cancer ZNF224 interacts with the DEPDC1 protein, playing an essential role in carcinogenesis, promoting cell proliferation and suppressing apoptosis. The analysis of the molecular mechanism of the DEPDC1/ZNF224 signaling pathway revealed that this protein complex represses the transcription of the A20 encoding gene. More specifically, the repression of A20, a well-known NF-κB family protein inhibitor, caused an aberrant NF-kB-dependent expression of antiapoptotic genes, affecting the proliferation of bladder cancer cells (Harada 2010). Interestingly, the oncogenic role of the DEPDC1/ZNF224 complex through the activation of the NF-κB signaling pathway was also demonstrate in HepG2 cell lines (Li 2018). NF-κB pathway is aberrantly activated in several hematological and solid malignancies including melanoma (Xia, 2014), and its activation leads to different molecular events implicated in cancer (Chaturvedi 2011), such as I-κB degradation induced by the redox-sensitive activation of the PI3K/PTEN/Akt and p38 MAPK pathways (Trombetti 2021). A tight intersection between NF-κB, MAPK, and PI3K pathways is involved in melanoma pathogenesis. In melanoma, it occurs frequently an aberrant NFκB activation likely due to deregulations in upstream MAPK and PI3K-AKT signaling pathways through different mechanisms, leading to an increase in proliferation and resistance to apoptosis (Teixido 2021). Since our preliminary results show the activation of AKT survival pathway by

ZNF224, we hypothesize that a connection between ZNF224 and NF-κB pathways may occur in melanoma and affect tumor progression. We will aim to a validation of this hypothesis in follow-up studies.
Moreover, ZNF224 may induce and/or sustain TGF-β signaling, activating or repressing other transcriptional regulators of this pathway.
In our study, we identified a possible mechanism of action for ZNF224 as modulator of TGF-β pathway. Specifically, both chromatin immunoprecipitation and RT-qPCR experiments, in condition of ZNF224 overexpression or silencing, showed that ZNF224 is directly associated to the promoter of TGF-βR1, TGF-βR2 and TGF-β and increases their expression (Figure 12), thus demonstrating that these key proteins of TGF-β pathway are targets of ZNF224 mediated transcriptional activation.
The increased expression of TGFβ and its receptors as well as the prolonged Smad2 phosphorylation induced by ZNF224 contribute to the constitutive activation of the pathway, thus resulting in enhanced induction of some TGF-β-responsive genes, that are associated with EMT and, consequently, malignant progression. Furthermore, our data highlight the existence of a positive regulatory loop between TGF-β and ZNF224 in melanoma. Indeed, we also showed that ZNF224 expression was, in turn, induced by TGF-β stimulation (Figure 9), thus further sustaining the altered activation of this pathway. In vitro functional assays, performed by modulating ZNF224 expression, strongly indicate that its high expression in malignant and metastatic melanoma cell lines contributes to the aggressive growth and spread of human malignant melanoma. The medium/high ZNF224 expression in melanoma, reported in the Human Protein Atlas, strengthens our findings on the prooncogenic potential of ZNF224 in this malignancy. Altogether, our data show that ZNF224 is required for the proliferation, migration and invasiveness of melanoma cells. Its overexpression could represent a critical event in the multi-step process that leads to tumour cell invasion and metastasis. Interestingly, it has been previously demonstrated that the arginine methyltransferase PRMT5 is a component of the ZNF224 transcriptional repressor complex. We found that PRMT5 is able to methylate the histone H4 (H4R3me2s) of nucleosomes surrounding the promoter region of ZNF224 target genes, thus acting as a key mediator of the ZNF224-mediated transcriptional repression (Cesaro 2009). Several studies report that PRMT5 is upregulated in several malignancies and contributes to the gene expression deregulation of MITF and p27Kip1 (Nicholas 2013). In addition, it has been reported that PRMT5-MEP50 complex is a critical mediator of TGFβ pathway that affects both EMT and invasive ability of cancer cells through simultaneous histone H3R2 methylation-coupled transcriptional activation and H4R3 methylation-coupled transcriptional repression. Alterations in the histone code signaling of epigenetic information are highly correlated with cancer etiology and Epithelial-to-Mesenchymal Transition (EMT) affecting the abilities of migration and invasiveness as well as metastasis formation of cancer cells (Chen 2017). Therefore, we hypothesize that deregulation of TGF-β response by

ZNF224 may also be dependent by the action of protein complex ZNF224/PRMT5 and we will aim at further investigation in a follow up study.
Epithelial Mesenchymal Transition (EMT) is a metastable process that enables polarized immotile epithelial cells to acquire a motile fibroblastoid phenotype. Physiologically, EMT is essential for proper embryogenesis and morphogenesis and is also engaged to promote tissue healing, remodelling and repair in response to a variety of insults. In contrast, pathophysiological EMT contributes to malignant progression by conferring to cancer cells an increased tumour-initiating and metastatic potential. EMT is orchestrated by EMT-inducing transcription factors (EMT-TFs), which act pleiotropically and, in several combinations, to regulate the expression of specific set of genes, resulting in the switch from a mesenchymal to an epithelial cell state (Thiery 2009). Our results strongly support the idea that ZNF224 could act by inducing the acquisition of mesenchymal and metastatic features of melanoma, by modulating different EMT cancer-related proteins. It is known that EMT contributes to the generation of cancer stem cell (CSC) niches. Several transcription factors associated with EMT interact with Smads and form "EMT promoting Smad complexes" (EPSC) that drive the EMT by both repressing Epithelium-specific gene expression and by activating mesenchymal marker genes, promoting a EMT-like signature (Fuxe 2010). Interestingly, TGF-β cooperates with several proteins belonging to stem cell pathway, including Wnt, Ras, Hedgehog and Notch, that are involved in the induction and maintenance of stem cell niches. Further, cells undergoing EMT, through the action of TGF- β, can acquire a cancer stem cell (CSC)-like phenotype (Fuxe 2010; Singh 2010). In agreement with these observations, by both colony formation and soft-agar colony assays, we have shown that ZNF224 increases independent anchorage growth and clonogenic potential of A375 and A2058 suggesting that ZNF224 could favor the acquisition of a stem-like phenotype by melanoma cells (Figures 14-15).
Great efforts are being made to develop drugs targeting TGFβ in melanoma (Yingling 2004). However, these approaches have proven challenging for rapid application in clinical practice because of the numerous physiological functions in which this signaling pathway is involved (Connolly 2012; Ciardiello 2020).
The identification of ZNF224 as one of the modulators of TGFβ signaling will provide a deeper knowledge of the molecular events involving this pathway in melanoma progression and invasion. In addition, because we have previously demonstrated that in Chronic Lymphocytic Leukemia ZNF224 expression is suppressed by fludarabine and that ZNF224 is involved in apoptosis resistance (Busiello 2017), we can also speculate that ZNF224 could be involved in drug chemoresistance. Therefore, defining the molecular mechanisms underlying the crosstalk between TGF-β and ZNF224 might contribute to clarifying the mechanism of resistance to TGFβ-mediated growth inhibition in melanoma, with relevant implications in the development of new therapeutic approaches in melanoma.

6. Conclusions

In this study, we examined the oncogenic role of the zinc finger protein ZNF224 in melanoma, mainly investigating its role as a mediator of the pro-oncogenic and pro-metastatic functions of TGF-β.

Interestingly, we observed that ZNF224 is overexpressed in different melanoma cell lines compared to non-cancerous cells and that its expression was induced by TGF-β. We also observed that ZNF224 modulated the expression levels of some TGF-β target genes, including N-Cadherin, β -catenin, slug, snail, and vimentin, involved in the epithelial-mesenchymal transition (EMT). Thereby, ZNF224 can promote the acquisition of a mesenchymal phenotype and, consequently, contribute to confer high invasiveness and metastatic potential to melanoma cells.

Moreover, our results unveil a synergistic effect between ZNF224 and TGF-β in the induction of TGF-β target genes involved in EMT, that could further enhance melanoma progression.

Finally, we identified one of the possible mechanisms by which ZNF224 could exert a regulatory role in the TGF-β pathway. Intriguingly, we found out that this zinc finger protein could sustain the endogenous TGFβ/Smad signaling by prolonging the phosphorylation of the Smad complex and increasing the levels of the TGF-β itself and its type 1 and 2 receptors (TβR1 and TβR2). These results unveil the existence of a positive regulatory loop between ZNF224 and TGF-β and suggest that ZNF224 contributes to the constitutive activation of the pathway, thus supporting melanoma progression.

Recently, many studies are aimed at developing drugs targeting TGF-β signalling in cancer (Morris 2014; Uhl 2004). However, due to the numerous physiological functions in which this pathway is involved, these approaches have so far proved difficult to apply in clinical practice (Jenkins 2021).

The identification of ZNF224 as one of the modulators of TGF-β signaling in melanoma can contribute to get new insights on the oncogenic role played by this pathway and to find novel molecular therapeutic targets and suitable treatment options to defeat melanoma.

7. **List of publications**

1) Cesaro E, **Pastore A**, Polverino A, Manna L, Divisato G, Quintavalle C, Sanzo MD, Faniello MC, Grosso M, Costanzo P. ZNF224 is a mediator of TGF-β pro-oncogenic function in melanoma. Hum Mol Genet. 2021 Nov 1;30(22):2100-2109. doi: 10.1093/hmg/ddab173.

2) Cesaro E, Lupo A, Rapuano R, **Pastore A**, Grosso M, Costanzo P. ZNF224 Protein: Multifaceted Functions Based on Its Molecular Partners. Molecules. 2021 Oct 18;26(20):6296. doi: 10.3390/molecules26206296.

3) Cesaro E, Falanga AP, Catapano R, Greco F, Romano S, Borbone N, **Pastore A**, Marzano M, Chiurazzi F, D'Errico S, Piccialli G, Oliviero G, Costanzo P, Grosso M. Exploring a Peptide Nucleic Acid-based Antisense Approach for CD5 Targeting in Chronic Lymphocytic Leukemia. PlosOne, in revision process.

8. References

Adler, Nikki R., Andrew Haydon, Catriona A. McLean, John W. Kelly, e Victoria J. Mar. 2017. «Metastatic Pathways in Patients with Cutaneous Melanoma». *Pigment Cell & Melanoma Research* 30 (1): 13–27. https://doi.org/10.1111/pcmr.12544.

Aiello, Nicole M., Ravikanth Maddipati, Robert J. Norgard, David Balli, Jinyang Li, Salina Yuan, Taiji Yamazoe, et al. 2018. «EMT Subtype Influences Epithelial Plasticity and Mode of Cell Migration». *Developmental Cell* 45 (6): 681-695.e4. https://doi.org/10.1016/j.devcel.2018.05.027.

Aigner, K., B. Dampier, L. Descovich, M. Mikula, A. Sultan, M. Schreiber, W. Mikulits, et al. 2007. «The Transcription Factor ZEB1 (DeltaEF1) Promotes Tumour Cell Dedifferentiation by Repressing Master Regulators of Epithelial Polarity». *Oncogene* 26 (49): 6979–88. https://doi.org/10.1038/sj.onc.1210508.

Albino, A. P., B. M. Davis, e D. M. Nanus. 1991. «Induction of Growth Factor RNA Expression in Human Malignant Melanoma: Markers of Transformation». *Cancer Research* 51 (18): 4815–20.

Alexaki, Vasileia-Ismini, Delphine Javelaud, e Alain Mauviel. 2008. «JNK Supports Survival in Melanoma Cells by Controlling Cell Cycle Arrest and Apoptosis». *Pigment Cell & Melanoma Research* 21 (4): 429–38. https://doi.org/10.1111/j.1755-148X.2008.00466.x.

Allen, Benjamin L., e Dylan J. Taatjes. 2015. «The Mediator complex: a central integrator of transcription». *Nature reviews. Molecular cell biology* 16 (3): 155–66. https://doi.org/10.1038/nrm3951.

Alonso, Soledad R., Lorraine Tracey, Pablo Ortiz, Beatriz Pérez-Gómez, José Palacios, Marina Pollán, Juan Linares, et al. 2007. «A High-Throughput Study in Melanoma Identifies Epithelial-Mesenchymal Transition as a Major Determinant of Metastasis». *Cancer Research* 67 (7): 3450–60. https://doi.org/10.1158/0008-5472.CAN-06-3481.

Baylin, S. B., M. Esteller, M. R. Rountree, K. E. Bachman, K. Schuebel, e J. G. Herman. 2001. «Aberrant Patterns of DNA Methylation, Chromatin Formation and Gene Expression in Cancer». *Human Molecular Genetics* 10 (7): 687–92. https://doi.org/10.1093/hmg/10.7.687.

Berking, C., e M. Herlyn. 2001. «Human Skin Reconstruct Models: A New Application for Studies of Melanocyte and Melanoma Biology». *Histology and Histopathology* 16 (2): 669–74. https://doi.org/10.14670/HH-16.669.

Böttinger, E. P., J. J. Letterio, e A. B. Roberts. 1997. «Biology of TGF-Beta in Knockout and Transgenic Mouse Models». *Kidney International* 51 (5): 1355–60. https://doi.org/10.1038/ki.1997.185.

Busiello, Teresa, Michela Ciano, Simona Romano, Gaetano Sodaro, Olgavalentina Garofalo, Dario Bruzzese, Luigia Simeone, et al. 2017. «Role of ZNF224 in Cell Growth and Chemoresistance of Chronic Lymphocitic Leukemia». *Human Molecular Genetics* 26 (2): 344–53. https://doi.org/10.1093/hmg/ddw427.

Busse, A., e U. Keilholz. 2011. «Role of TGF-β in Melanoma». *Current Pharmaceutical Biotechnology* 12 (12): 2165–75. https://doi.org/10.2174/138920111798808437.

Caestecker, M. P. de, T. Yahata, D. Wang, W. T. Parks, S. Huang, C. S. Hill, T. Shioda, A. B. Roberts, e R. J. Lechleider. 2000. «The Smad4 Activation Domain (SAD) Is a Proline-Rich, P300-Dependent Transcriptional Activation Domain». *The Journal of Biological Chemistry* 275 (3): 2115–22. https://doi.org/10.1074/jbc.275.3.2115.

Cancer Genome Atlas Network. 2015. «Genomic Classification of Cutaneous Melanoma». *Cell* 161 (7): 1681–96. https://doi.org/10.1016/j.cell.2015.05.044.

Caprio, Roberta di, Michela Ciano, Giorgia Montano, Paola Costanzo, e Elena Cesaro. 2015. «KAP1 Is a Novel Substrate for the Arginine Methyltransferase PRMT5». *Biology* 4 (1): 41–49. https://doi.org/10.3390/biology4010041.

Caramel, Julie, Eftychios Papadogeorgakis, Louise Hill, Gareth J. Browne, Geoffrey Richard, Anne Wierinckx, Gerald Saldanha, et al. 2013. «A Switch in the Expression of Embryonic EMT-Inducers Drives the Development of Malignant Melanoma». *Cancer Cell* 24 (4): 466–80. https://doi.org/10.1016/j.ccr.2013.08.018.

Cassandri, Matteo, Artem Smirnov, Flavia Novelli, Consuelo Pitolli, Massimiliano Agostini, Michal Malewicz, Gerry Melino, e Giuseppe Raschellà. 2017. «Zinc-Finger Proteins in Health and Disease». *Cell Death Discovery* 3 (1): 1–12. https://doi.org/10.1038/cddiscovery.2017.71.

Cesaro, E., G. Sodaro, G. Montano, M. Grosso, A. Lupo, e P. Costanzo. 2017. «The Complex Role of the ZNF224 Transcription Factor in Cancer». *Advances in Protein Chemistry and Structural Biology* 107: 191–222. https://doi.org/10.1016/bs.apcsb.2016.11.003.

Cesaro, Elena, Rossella De Cegli, Lina Medugno, Francesca Florio, Michela Grosso, Angelo Lupo, Paola Izzo, e Paola Costanzo. 2009. «The Kruppel-like Zinc Finger Protein ZNF224 Recruits the Arginine Methyltransferase PRMT5 on the Transcriptional Repressor Complex of the Aldolase A Gene». *The Journal of Biological Chemistry* 284 (47): 32321–30. https://doi.org/10.1074/jbc.M109.043349.

Chaturvedi, M. M., B. Sung, V. R. Yadav, R. Kannappan, e B. B. Aggarwal. 2011. «NF-KB Addiction and Its Role in Cancer: "One Size Does Not Fit All"». *Oncogene* 30 (14): 1615–30. https://doi.org/10.1038/onc.2010.566.

Chen, Hongshan, Benjamin Lorton, Varun Gupta, e David Shechter. 2017. «A TGFβ-PRMT5-MEP50 Axis Regulates Cancer Cell Invasion through Histone H3 and H4 Arginine Methylation Coupled Transcriptional Activation and Repression». *Oncogene* 36 (3): 373–86. https://doi.org/10.1038/onc.2016.205.

Cheng, Yingduan, Hua Geng, Suk Hang Cheng, Pei Liang, Yan Bai, Jisheng Li, Gopesh Srivastava, et al. 2010. «KRAB Zinc Finger Protein ZNF382 Is a Proapoptotic Tumor Suppressor That Represses Multiple Oncogenes and Is Commonly Silenced in Multiple Carcinomas». *Cancer Research* 70 (16): 6516–26. https://doi.org/10.1158/0008-5472.CAN-09-4566.

Cho, Jin Gu, Key-Hwan Lim, e Sang Gyu Park. 2018. «MED28 Increases the Colony-Forming Ability of Breast Cancer Cells by Stabilizing the ZNF224 Protein upon DNA Damage». *Oncology Letters* 15 (3): 3147–54. https://doi.org/10.3892/ol.2017.7718.

Cho, Jin Gu, Seho Park, Chae Hyun Lim, Hong Sook Kim, Seung Yong Song, Tae-Young Roh, Jong-Hyuk Sung, et al. 2016. «ZNF224, Krüppel like Zinc Finger Protein, Induces Cell Growth and Apoptosis-Resistance by down-Regulation of P21 and P53 via MiR-663a». *Oncotarget* 7 (21): 31177–90. https://doi.org/10.18632/oncotarget.8870.

Ciardiello, Davide, E. Elez, J. Tabernero, e J. Seoane. 2020. «Clinical development of therapies targeting TGFβ: current knowledge and future perspectives». *Annals of Oncology* 31 (luglio). https://doi.org/10.1016/j.annonc.2020.07.009.

Connolly, Erin C., Julia Freimuth, e Rosemary J. Akhurst. 2012. «Complexities of TGF-β Targeted Cancer Therapy». *International Journal of Biological Sciences* 8 (7): 964–78. https://doi.org/10.7150/ijbs.4564.

Dantonio, Paola M., Marianne O. Klein, Maria Renata V. B. Freire, Camila N. Araujo, Ana Carolina Chiacetti, e Ricardo G. Correa. 2018. «Exploring Major Signaling Cascades in Melanomagenesis: A Rationale Route for Targetted Skin Cancer Therapy». *Bioscience Reports* 38 (5): BSR20180511. https://doi.org/10.1042/BSR20180511.

Denecker, G., N. Vandamme, O. Akay, D. Koludrovic, J. Taminau, K. Lemeire, A. Gheldof, et al. 2014. «Identification of a ZEB2-MITF-ZEB1 Transcriptional Network That Controls Melanogenesis and Melanoma Progression». *Cell Death and Differentiation* 21 (8): 1250–61. https://doi.org/10.1038/cdd.2014.44.

Dhasarathy, Archana, Dhiral Phadke, Deepak Mav, Ruchir R. Shah, e Paul A. Wade. 2011. «The Transcription Factors Snail and Slug Activate the Transforming Growth Factor-Beta Signaling Pathway in Breast Cancer». *PloS One* 6 (10): e26514. https://doi.org/10.1371/journal.pone.0026514.

Dongre, Anushka, e Robert A. Weinberg. 2019. «New Insights into the Mechanisms of Epithelial-Mesenchymal Transition and Implications for Cancer». *Nature Reviews. Molecular Cell Biology* 20 (2): 69–84. https://doi.org/10.1038/s41580-018-0080-4.

Drabsch, Yvette, e Peter ten Dijke. 2012. «TGF-β Signalling and Its Role in Cancer Progression and Metastasis». *Cancer Metastasis Reviews* 31 (3–4): 553–68. https://doi.org/10.1007/s10555-012-9375-7.

Duan, Dana, e Rik Derynck. 2019. «Transforming Growth Factor-β (TGF-β)-Induced up-Regulation of TGF-β Receptors at the Cell Surface Amplifies the TGF-β Response». *The Journal of Biological Chemistry* 294 (21): 8490–8504. https://doi.org/10.1074/jbc.RA118.005763.

El Ghissassi, Fatiha, Robert Baan, Kurt Straif, Yann Grosse, Béatrice Secretan, Véronique Bouvard, Lamia Benbrahim-Tallaa, et al. 2009. «A Review of Human Carcinogens--Part D: Radiation». *The Lancet. Oncology* 10 (8): 751–52. https://doi.org/10.1016/s1470-2045(09)70213-x.

Elliott, Rebecca L., e Gerard C. Blobe. 2005. «Role of Transforming Growth Factor Beta in Human Cancer». *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 23 (9): 2078–93. https://doi.org/10.1200/JCO.2005.02.047.

Elloul, Sivan, Mari Bukholt Elstrand, Jahn M. Nesland, Claes G. Tropé, Gunnar Kvalheim, Iris Goldberg, Reuven Reich, e Ben Davidson. 2005. «Snail, Slug, and Smad-Interacting Protein 1 as Novel Parameters of Disease Aggressiveness in Metastatic Ovarian and Breast Carcinoma». *Cancer* 103 (8): 1631–43. https://doi.org/10.1002/cncr.20946.

Ennen, Marie, Céline Keime, Giovanni Gambi, Alice Kieny, Sebastien Coassolo, Christelle Thibault-Carpentier, Fanny Margerin-Schaller, et al. 2017. «MITF-High and MITF-Low Cells and a Novel Subpopulation Expressing Genes of Both Cell States Contribute to Intra- and Intertumoral Heterogeneity of Primary Melanoma». *Clinical Cancer Research: An Official Journal of the American Association for Cancer Research* 23 (22): 7097–7107. https://doi.org/10.1158/1078-0432.CCR-17-0010.

Esparza-Lopez, J., J. L. Montiel, M. M. Vilchis-Landeros, T. Okadome, K. Miyazono, e F. López-Casillas. 2001. «Ligand Binding and Functional Properties of Betaglycan, a Co-Receptor of the Transforming Growth Factor-Beta Superfamily. Specialized Binding Regions for Transforming Growth Factor-Beta and Inhibin A». *The Journal of Biological Chemistry* 276 (18): 14588–96. https://doi.org/10.1074/jbc.M008866200.

Florio, Francesca, Elena Cesaro, Giorgia Montano, Paola Izzo, Colin Miles, e Paola Costanzo. 2010. «Biochemical and Functional Interaction between ZNF224 and ZNF255, Two Members of the Kruppel-like Zinc-Finger Protein Family and WT1 Protein Isoforms». *Human Molecular Genetics* 19 (18): 3544–56. https://doi.org/10.1093/hmg/ddq270.

Fujiwara, S., J.C. Corbo, e M. Levine. 1998. «The snail repressor establishes a muscle/notochord boundary in the Ciona embryo». *Development* 125 (13): 2511–20. https://doi.org/10.1242/dev.125.13.2511.

Furuta, Saori, Ju-Ming Wang, Shuanzeng Wei, Yung-Ming Jeng, Xianzhi Jiang, Bingnan Gu, Phang-Lang Chen, Eva Y.-H. P. Lee, e Wen-Hwa Lee.

2006. «Removal of BRCA1/CtIP/ZBRK1 Repressor Complex on ANG1 Promoter Leads to Accelerated Mammary Tumor Growth Contributed by Prominent Vasculature». *Cancer Cell* 10 (1): 13–24. https://doi.org/10.1016/j.ccr.2006.05.022.

Fuxe, Jonas, Theresa Vincent, e Antonio Garcia de Herreros. 2010. «Transcriptional Crosstalk between TGF-β and Stem Cell Pathways in Tumor Cell Invasion: Role of EMT Promoting Smad Complexes». *Cell Cycle (Georgetown, Tex.)* 9 (12): 2363–74. https://doi.org/10.4161/cc.9.12.12050.

Ghosh, Papia, e Lynda Chin. 2009. «Genetics and Genomics of Melanoma». *Expert Review of Dermatology* 4 (2): 131. https://doi.org/10.1586/edm.09.2.

Greber, Basil J., e Eva Nogales. 2019. «The Structures of Eukaryotic Transcription Pre-Initiation Complexes and Their Functional Implications». *Sub-Cellular Biochemistry* 93: 143–92. https://doi.org/10.1007/978-3-030-28151-9_5.

Harada, Yosuke, Mitsugu Kanehira, Yoshiko Fujisawa, Ryo Takata, Taro Shuin, Tsuneharu Miki, Tomoaki Fujioka, Yusuke Nakamura, e Toyomasa Katagiri. 2010. «Cell-Permeable Peptide DEPDC1-ZNF224 Interferes with Transcriptional Repression and Oncogenicity in Bladder Cancer Cells». *Cancer Research* 70 (14): 5829–39. https://doi.org/10.1158/0008-5472.CAN-10-0255.

Heldin, Carl-Henrik, e Aristidis Moustakas. 2016. «Signaling Receptors for TGF-β Family Members». *Cold Spring Harbor Perspectives in Biology* 8 (8): a022053. https://doi.org/10.1101/cshperspect.a022053.

Helleboid, Pierre-Yves, Moritz Heusel, Julien Duc, Cécile Piot, Christian W. Thorball, Andrea Coluccio, Julien Pontis, et al. 2019. «The Interactome of KRAB Zinc Finger Proteins Reveals the Evolutionary History of Their Functional Diversification». *The EMBO Journal* 38 (18): e101220. https://doi.org/10.15252/embj.2018101220.

Hofmann, U. B., J. R. Westphal, G. N. Van Muijen, e D. J. Ruiter. 2000. «Matrix Metalloproteinases in Human Melanoma». *The Journal of Investigative Dermatology* 115 (3): 337–44. https://doi.org/10.1046/j.1523-1747.2000.00068.x.

Huang, Tao, Laurent David, Valentín Mendoza, Yong Yang, Maria Villarreal, Keya De, LuZhe Sun, et al. 2011. «TGF-β Signalling Is Mediated by Two Autonomously Functioning TβRI:TβRII Pairs». *The EMBO Journal* 30 (7): 1263–76. https://doi.org/10.1038/emboj.2011.54.

Huntley, Stuart, Daniel M. Baggott, Aaron T. Hamilton, Mary Tran-Gyamfi, Shan Yang, Joomyeong Kim, Laurie Gordon, Elbert Branscomb, e Lisa Stubbs. 2006. «A Comprehensive Catalog of Human KRAB-Associated Zinc Finger Genes: Insights into the Evolutionary History of a Large Family of Transcriptional Repressors». *Genome Research* 16 (5): 669–77. https://doi.org/10.1101/gr.4842106.

Huse, M., Y. G. Chen, J. Massagué, e J. Kuriyan. 1999. «Crystal Structure of the Cytoplasmic Domain of the Type I TGF Beta Receptor in Complex with FKBP12». *Cell* 96 (3): 425–36. https://doi.org/10.1016/s0092-8674(00)80555-3.

Iacobazzi, Vito, Vittoria Infantino, Paolo Convertini, Angelo Vozza, Gennaro Agrimi, e Ferdinando Palmieri. 2009. «Transcription of the Mitochondrial Citrate Carrier Gene: Identification of a Silencer and Its Binding Protein ZNF224». *Biochemical and Biophysical Research Communications* 386 (1): 186–91. https://doi.org/10.1016/j.bbrc.2009.06.003.

Iuchi, S. 2001. «Three Classes of C2H2 Zinc Finger Proteins». *Cellular and Molecular Life Sciences: CMLS* 58 (4): 625–35. https://doi.org/10.1007/PL00000885.

Jakowlew, Sonia B. 2006. «Transforming Growth Factor-Beta in Cancer and Metastasis». *Cancer Metastasis Reviews* 25 (3): 435–57. https://doi.org/10.1007/s10555-006-9006-2.

Jang, Young-Saeng, Jae-Hee Kim, Goo-Young Seo, e Pyeung-Hyeun Kim. 2011. «TGF-B1 Stimulates Mouse Macrophages to Express APRIL through Smad and P38MAPK/CREB Pathways». *Molecules and Cells* 32 (3): 251–55. https://doi.org/10.1007/s10059-011-1040-4.

Javelaud, Delphine, Vasileia-Ismini Alexaki, e Alain Mauviel. 2008. «Transforming Growth Factor-Beta in Cutaneous Melanoma». *Pigment Cell & Melanoma Research* 21 (2): 123–32. https://doi.org/10.1111/j.1755-148X.2008.00450.x.

Javelaud, Delphine, e Alain Mauviel. 2005. «Crosstalk Mechanisms between the Mitogen-Activated Protein Kinase Pathways and Smad Signaling Downstream of TGF-Beta: Implications for Carcinogenesis». *Oncogene* 24 (37): 5742–50. https://doi.org/10.1038/sj.onc.1208928.

Jen, Jayu, e Yi-Ching Wang. 2016. «Zinc Finger Proteins in Cancer Progression». *Journal of Biomedical Science* 23 (1): 53. https://doi.org/10.1186/s12929-016-0269-9.

Jenkins, Russell W., e David E. Fisher. 2021. «Treatment of Advanced Melanoma in 2020 and Beyond». *Journal of Investigative Dermatology* 141 (1): 23–31. https://doi.org/10.1016/j.jid.2020.03.943.

Justus, Calvin R., Nancy Leffler, Maria Ruiz-Echevarria, e Li V. Yang. 2014. «In Vitro Cell Migration and Invasion Assays». *Journal of Visualized Experiments: JoVE*, n. 88 (giugno). https://doi.org/10.3791/51046.

Kalluri, Raghu, e Robert A. Weinberg. 2009. «The Basics of Epithelial-Mesenchymal Transition». *The Journal of Clinical Investigation* 119 (6): 1420–28. https://doi.org/10.1172/JCI39104.

Karihtala, Peeter, Päivi Auvinen, Saila Kauppila, Kirsi-Maria Haapasaari, Arja Jukkola-Vuorinen, e Ylermi Soini. 2013. «Vimentin, Zeb1 and Sip1 Are up-Regulated in Triple-Negative and Basal-like Breast Cancers: Association with an Aggressive Tumour Phenotype». *Breast Cancer*

Research and Treatment 138 (1): 81–90. https://doi.org/10.1007/s10549-013-2442-0.

Kavsak, P., R. K. Rasmussen, C. G. Causing, S. Bonni, H. Zhu, G. H. Thomsen, e J. L. Wrana. 2000. «Smad7 Binds to Smurf2 to Form an E3 Ubiquitin Ligase That Targets the TGF Beta Receptor for Degradation». *Molecular Cell* 6 (6): 1365–75. https://doi.org/10.1016/s1097-2765(00)00134-9.

Kim, Dong-Seok, Seo-Hyoung Park, e Kyoung-Chan Park. 2004. «Transforming Growth Factor-Beta1 Decreases Melanin Synthesis via Delayed Extracellular Signal-Regulated Kinase Activation». *The International Journal of Biochemistry & Cell Biology* 36 (8): 1482–91. https://doi.org/10.1016/j.biocel.2003.10.023.

Kingsley, D. M. 1994. «The TGF-Beta Superfamily: New Members, New Receptors, and New Genetic Tests of Function in Different Organisms». *Genes & Development* 8 (2): 133–46. https://doi.org/10.1101/gad.8.2.133.

Krasagakis, K., D. Thölke, B. Farthmann, J. Eberle, U. Mansmann, e C. E. Orfanos. 1998. «Elevated Plasma Levels of Transforming Growth Factor (TGF)-Beta1 and TGF-Beta2 in Patients with Disseminated Malignant Melanoma». *British Journal of Cancer* 77 (9): 1492–94. https://doi.org/10.1038/bjc.1998.245.

Ladomery, M. 1997. «Multifunctional Proteins Suggest Connections between Transcriptional and Post-Transcriptional Processes». *BioEssays: News and Reviews in Molecular, Cellular and Developmental Biology* 19 (10): 903–9. https://doi.org/10.1002/bies.950191010.

Lawson, Devon A., Nirav R. Bhakta, Kai Kessenbrock, Karin D. Prummel, Ying Yu, Ken Takai, Alicia Zhou, et al. 2015. «Single-Cell Analysis Reveals a Stem-Cell Program in Human Metastatic Breast Cancer Cells». *Nature* 526 (7571): 131–35. https://doi.org/10.1038/nature15260.

Lechner, M. S., G. E. Begg, D. W. Speicher, e F. J. Rauscher. 2000. «Molecular Determinants for Targeting Heterochromatin Protein 1-Mediated Gene Silencing: Direct Chromoshadow Domain-KAP-1 Corepressor Interaction Is Essential». *Molecular and Cellular Biology* 20 (17): 6449–65. https://doi.org/10.1128/MCB.20.17.6449-6465.2000.

Leivonen, Suvi-Katri, e Veli-Matti Kähäri. 2007. «Transforming Growth Factor-Beta Signaling in Cancer Invasion and Metastasis». *International Journal of Cancer* 121 (10): 2119–24. https://doi.org/10.1002/ijc.23113.

Li, Aili, Qingqing Wang, Gaofeng He, Junfei Jin, e Guojin Huang. 2018. «DEP Domain Containing 1 Suppresses Apoptosis via Inhibition of A20 Expression, Which Activates the Nuclear Factor KB Signaling Pathway in HepG2 Cells». *Oncology Letters* 16 (1): 949–55. https://doi.org/10.3892/ol.2018.8770.

Li, Wenyang, e Yibin Kang. 2016. «Probing the Fifty Shades of EMT in Metastasis». *Trends in Cancer* 2 (2): 65–67. https://doi.org/10.1016/j.trecan.2016.01.001.

Lin, Li-Fang, Chih-Hung Chuang, Chien-Feng Li, Ching-Chun Liao, Chun-Pei Cheng, Tian-Lu Cheng, Meng-Ru Shen, et al. 2010. «ZBRK1 Acts as a Metastatic Suppressor by Directly Regulating MMP9 in Cervical Cancer». *Cancer Research* 70 (1): 192–201. https://doi.org/10.1158/0008-5472.CAN-09-2641.

Lo, Roger S., e Owen N. Witte. 2008. «Transforming Growth Factor-Beta Activation Promotes Genetic Context-Dependent Invasion of Immortalized Melanocytes». *Cancer Research* 68 (11): 4248–57. https://doi.org/10.1158/0008-5472.CAN-07-5671.

Lopez-Bergami, Pablo, Conway Huang, James S. Goydos, Dana Yip, Menashe Bar-Eli, Meenhard Herlyn, Keiran S. M. Smalley, et al. 2007. «Rewired ERK-JNK Signaling Pathways in Melanoma». *Cancer Cell* 11 (5): 447–60. https://doi.org/10.1016/j.ccr.2007.03.009.

Lupo, Angelo, Elena Cesaro, Giorgia Montano, Diana Zurlo, Paola Izzo, e Paola Costanzo. 2013. «KRAB-Zinc Finger Proteins: A Repressor Family Displaying Multiple Biological Functions». *Current Genomics* 14 (4): 268–78. https://doi.org/10.2174/13892029113149990002.

Markiewicz, Aleksandra, Tomasz Ahrends, Marzena Wełnicka-Jaśkiewicz, Barbara Seroczyńska, Jarosław Skokowski, Janusz Jaśkiewicz, Jolanta Szade, Wojciech Biernat, e Anna J. Żaczek. 2012. «Expression of epithelial to mesenchymal transition-related markers in lymph node metastases as a surrogate for primary tumor metastatic potential in breast cancer». *Journal of Translational Medicine* 10 (1): 226. https://doi.org/10.1186/1479-5876-10-226.

Marzagalli, Monica, Nancy D. Ebelt, e Edwin R. Manuel. 2019. «Unraveling the Crosstalk between Melanoma and Immune Cells in the Tumor Microenvironment». *Seminars in Cancer Biology* 59 (dicembre): 236–50. https://doi.org/10.1016/j.semcancer.2019.08.002.

Massagué, J. 1990. «The Transforming Growth Factor-Beta Family». *Annual Review of Cell Biology* 6: 597–641. https://doi.org/10.1146/annurev.cb.06.110190.003121.

Massagué, J., e Y. G. Chen. 2000. «Controlling TGF-Beta Signaling». *Genes & Development* 14 (6): 627–44.

Massagué, Joan, e Roger R. Gomis. 2006. «The Logic of TGFbeta Signaling». *FEBS Letters* 580 (12): 2811–20. https://doi.org/10.1016/j.febslet.2006.04.033.

Mauhin, V., Y. Lutz, C. Dennefeld, e A. Alberga. 1993. «Definition of the DNA-binding site repertoire for the Drosophila transcription factor SNAIL.» *Nucleic acids research*. https://doi.org/10.1093/NAR/21.17.3951.

Medugno, Lina, Francesca Florio, Rossella De Cegli, Michela Grosso, Angelo Lupo, Paola Costanzo, e Paola Izzo. 2005. «The Krüppel-like Zinc-Finger Protein ZNF224 Represses Aldolase A Gene Transcription by Interacting with the KAP-1 Co-Repressor Protein». *Gene* 359 (ottobre): 35–43. https://doi.org/10.1016/j.gene.2005.06.021.

Menke, A., I. Geerling, K. Giehl, R. Vogelmann, M. Reinshagen, e G. Adler. 1999. «Transforming Growth Factor-Beta-Induced Upregulation of Transforming Growth Factor-Beta Receptor Expression in Pancreatic Regeneration». *Biochimica Et Biophysica Acta* 1449 (2): 178–85. https://doi.org/10.1016/s0167-4889(99)00011-7.

Miyazono, K., P. ten Dijke, e C. H. Heldin. 2000. «TGF-Beta Signaling by Smad Proteins». *Advances in Immunology* 75: 115–57. https://doi.org/10.1016/s0065-2776(00)75003-6.

Miyoshi, Jun, e Yoshimi Takai. 2005. «Molecular Perspective on Tight-Junction Assembly and Epithelial Polarity». *Advanced Drug Delivery Reviews* 57 (6): 815–55. https://doi.org/10.1016/j.addr.2005.01.008.

Montano, Giorgia, Karina Vidovic, Chiara Palladino, Elena Cesaro, Gaetano Sodaro, Concetta Quintarelli, Biagio De Angelis, et al. 2015. «WT1-Mediated Repression of the Proapoptotic Transcription Factor ZNF224 Is Triggered by the BCR-ABL Oncogene». *Oncotarget* 6 (29): 28223–37. https://doi.org/10.18632/oncotarget.4950.

Morris, John C., Antoinette R. Tan, Thomas E. Olencki, Geoffrey I. Shapiro, Bruce J. Dezube, Michael Reiss, Frank J. Hsu, Jay A. Berzofsky, e Donald P. Lawrence. 2014. «Phase I Study of GC1008 (Fresolimumab): A Human Anti-Transforming Growth Factor-Beta (TGFβ) Monoclonal Antibody in Patients with Advanced Malignant Melanoma or Renal Cell Carcinoma». *PloS One* 9 (3): e90353. https://doi.org/10.1371/journal.pone.0090353.

Moustakas, Aristidis, e Carl-Henrik Heldin. 2009. «The Regulation of TGFbeta Signal Transduction». *Development (Cambridge, England)* 136 (22): 3699–3714. https://doi.org/10.1242/dev.030338.

Neuzillet, Cindy, Armand de Gramont, Annemilaï Tijeras-Raballand, Louis de Mestier, Jérome Cros, Sandrine Faivre, e Eric Raymond. 2014. «Perspectives of TGF-β Inhibition in Pancreatic and Hepatocellular Carcinomas». *Oncotarget* 5 (1): 78–94. https://doi.org/10.18632/oncotarget.1569.

Neuzillet, Cindy, Pascal Hammel, Annemilaï Tijeras-Raballand, Anne Couvelard, e Eric Raymond. 2013. «Targeting the Ras-ERK Pathway in Pancreatic Adenocarcinoma». *Cancer Metastasis Reviews* 32 (1–2): 147–62. https://doi.org/10.1007/s10555-012-9396-2.

Neuzillet, Cindy, Annemilaï Tijeras-Raballand, Romain Cohen, Jérôme Cros, Sandrine Faivre, Eric Raymond, e Armand de Gramont. 2015. «Targeting the TGFβ Pathway for Cancer Therapy». *Pharmacology & Therapeutics* 147 (marzo): 22–31. https://doi.org/10.1016/j.pharmthera.2014.11.001.

Nicholas, Courtney, Jennifer Yang, Sara B. Peters, Matthew A. Bill, Robert A. Baiocchi, Fengting Yan, Saïd Sïf, et al. 2013. «PRMT5 Is Upregulated in Malignant and Metastatic Melanoma and Regulates Expression of MITF and P27(Kip1.)». *PloS One* 8 (9): e74710. https://doi.org/10.1371/journal.pone.0074710.

Ocaña, Oscar H., Rebeca Córcoles, Angels Fabra, Gema Moreno-Bueno, Hervé Acloque, Sonia Vega, Alejandro Barrallo-Gimeno, Amparo Cano, e M. Angela Nieto. 2012. «Metastatic Colonization Requires the Repression of the Epithelial-Mesenchymal Transition Inducer Prrx1». *Cancer Cell* 22 (6): 709–24. https://doi.org/10.1016/j.ccr.2012.10.012.

Padua, David, e Joan Massagué. 2009. «Roles of TGFβ in Metastasis». *Cell Research* 19 (1): 89–102. https://doi.org/10.1038/cr.2008.316.

Peinado, Héctor, David Olmeda, e Amparo Cano. 2007. «Snail, Zeb and BHLH Factors in Tumour Progression: An Alliance against the Epithelial Phenotype?» *Nature Reviews Cancer* 7 (6): 415–28. https://doi.org/10.1038/nrc2131.

Peng, H., G. E. Begg, S. L. Harper, J. R. Friedman, D. W. Speicher, e F. J. Rauscher. 2000. «Biochemical Analysis of the Kruppel-Associated Box (KRAB) Transcriptional Repression Domain». *The Journal of Biological Chemistry* 275 (24): 18000–10. https://doi.org/10.1074/jbc.M001499200.

Poniatowski, Łukasz A., Piotr Wojdasiewicz, Robert Gasik, e Dariusz Szukiewicz. 2015. «Transforming Growth Factor Beta Family: Insight into the Role of Growth Factors in Regulation of Fracture Healing Biology and Potential Clinical Applications». *Mediators of Inflammation* 2015: 137823. https://doi.org/10.1155/2015/137823.

Postigo, A. A., e D. C. Dean. 1999. «Independent Repressor Domains in ZEB Regulate Muscle and T-Cell Differentiation». *Molecular and Cellular Biology* 19 (12): 7961–71. https://doi.org/10.1128/MCB.19.12.7961.

Roberts, S. G. 2000. «Mechanisms of Action of Transcription Activation and Repression Domains». *Cellular and Molecular Life Sciences: CMLS* 57 (8–9): 1149–60. https://doi.org/10.1007/pl00000755.

Rodeck, U., A. Bossler, U. Graeven, F. E. Fox, P. C. Nowell, C. Knabbe, e C. Kari. 1994. «Transforming Growth Factor Beta Production and Responsiveness in Normal Human Melanocytes and Melanoma Cells». *Cancer Research* 54 (2): 575–81.

Rodeck, U., T. Nishiyama, e A. Mauviel. 1999. «Independent Regulation of Growth and SMAD-Mediated Transcription by Transforming Growth Factor Beta in Human Melanoma Cells». *Cancer Research* 59 (3): 547–50.

Sánchez-Martín, Manuel, Jesús Pérez-Losada, Arancha Rodríguez-García, Belén González-Sánchez, Bruce R. Korf, W. Kuster, Celia Moss, Richard A. Spritz, e I. Sánchez-García. 2003. «Deletion of the SLUG (SNAI2) Gene Results in Human Piebaldism». *American Journal of Medical Genetics. Part A* 122A (2): 125–32. https://doi.org/10.1002/ajmg.a.20345.

Sánchez-Tilló, E., A. Lázaro, R. Torrent, M. Cuatrecasas, E. C. Vaquero, A. Castells, P. Engel, e A. Postigo. 2010. «ZEB1 Represses E-Cadherin and Induces an EMT by Recruiting the SWI/SNF Chromatin-Remodeling

Protein BRG1». *Oncogene* 29 (24): 3490–3500. https://doi.org/10.1038/onc.2010.102.

Sánchez-Tilló, Ester, Yongqing Liu, Oriol de Barrios, Laura Siles, Lucia Fanlo, Miriam Cuatrecasas, Douglas S. Darling, Douglas C. Dean, Antoni Castells, e Antonio Postigo. 2012. «EMT-Activating Transcription Factors in Cancer: Beyond EMT and Tumor Invasiveness». *Cellular and Molecular Life Sciences* 69 (20): 3429–56. https://doi.org/10.1007/s00018-012-1122-2.

Schnaeker, Eva-Maria, Rainer Ossig, Thomas Ludwig, Rita Dreier, Hans Oberleithner, M. Wilhelmi, e Stefan W. Schneider. 2004. «Microtubule-Dependent Matrix Metalloproteinase-2/Matrix Metalloproteinase-9 Exocytosis: Prerequisite in Human Melanoma Cell Invasion». *Cancer Research* 64 (24): 8924–31. https://doi.org/10.1158/0008-5472.CAN-04-0324.

Shannon, Mark, Aaron T. Hamilton, Laurie Gordon, Elbert Branscomb, e Lisa Stubbs. 2003. «Differential Expansion of Zinc-Finger Transcription Factor Loci in Homologous Human and Mouse Gene Clusters». *Genome Research* 13 (6A): 1097–1110. https://doi.org/10.1101/gr.963903.

Shi, Y., Y. F. Wang, L. Jayaraman, H. Yang, J. Massagué, e N. P. Pavletich. 1998. «Crystal Structure of a Smad MH1 Domain Bound to DNA: Insights on DNA Binding in TGF-Beta Signaling». *Cell* 94 (5): 585–94. https://doi.org/10.1016/s0092-8674(00)81600-1.

Shi, Yigong, e Joan Massagué. 2003. «Mechanisms of TGF-Beta Signaling from Cell Membrane to the Nucleus». *Cell* 113 (6): 685–700. https://doi.org/10.1016/s0092-8674(03)00432-x.

Shibata, Maho, Kristin E. Blauvelt, Karel F. Liem, e María J. García-García. 2011. «TRIM28 Is Required by the Mouse KRAB Domain Protein ZFP568 to Control Convergent Extension and Morphogenesis of Extra-Embryonic Tissues». *Development (Cambridge, England)* 138 (24): 5333–43. https://doi.org/10.1242/dev.072546.

Shin, Na Ri, Eun Hui Jeong, Chang In Choi, Hyun Jung Moon, Chae Hwa Kwon, In Sun Chu, Gwang Ha Kim, et al. 2012. «Overexpression of Snail is associated with lymph node metastasis and poor prognosis in patients with gastric cancer». *BMC Cancer* 12 (1): 521. https://doi.org/10.1186/1471-2407-12-521.

Singh, A., e J. Settleman. 2010. «EMT, Cancer Stem Cells and Drug Resistance: An Emerging Axis of Evil in the War on Cancer». *Oncogene* 29 (34): 4741–51. https://doi.org/10.1038/onc.2010.215.

Sinnberg, Tobias, Moritz Menzel, Daniel Ewerth, Birgit Sauer, Michael Schwarz, Martin Schaller, Claus Garbe, e Birgit Schittek. 2011. «β-Catenin Signaling Increases during Melanoma Progression and Promotes Tumor Cell Survival and Chemoresistance». *PloS One* 6 (8): e23429. https://doi.org/10.1371/journal.pone.0023429.

Sodaro, Gaetano, Giancarlo Blasio, Federica Fiorentino, Patrick Auberger, Paola Costanzo, e Elena Cesaro. 2018. «ZNF224 Is a Transcriptional Repressor

of AXL in Chronic Myeloid Leukemia Cells». *Biochimie* 154 (novembre): 127–31. https://doi.org/10.1016/j.biochi.2018.08.011.

Sodaro, Gaetano, Elena Cesaro, Giorgia Montano, Giancarlo Blasio, Federica Fiorentino, Simona Romano, Arnaud Jacquel, Patrick Aurberger, e Paola Costanzo. 2018. «Role of ZNF224 in C-Myc Repression and Imatinib Responsiveness in Chronic Myeloid Leukemia». *Oncotarget* 9 (3): 3417–31. https://doi.org/10.18632/oncotarget.23283.

Stemmler, Marc P., Rebecca L. Eccles, Simone Brabletz, e Thomas Brabletz. 2019. «Non-Redundant Functions of EMT Transcription Factors». *Nature Cell Biology* 21 (1): 102–12. https://doi.org/10.1038/s41556-018-0196-y.

Stroschein, S. L., W. Wang, e K. Luo. 1999. «Cooperative Binding of Smad Proteins to Two Adjacent DNA Elements in the Plasminogen Activator Inhibitor-1 Promoter Mediates Transforming Growth Factor Beta-Induced Smad-Dependent Transcriptional Activation». *The Journal of Biological Chemistry* 274 (14): 9431–41. https://doi.org/10.1074/jbc.274.14.9431.

Tang, Yaqi, Simon Durand, Stéphane Dalle, e Julie Caramel. 2020. «EMT-Inducing Transcription Factors, Drivers of Melanoma Phenotype Switching, and Resistance to Treatment». *Cancers* 12 (8): E2154. https://doi.org/10.3390/cancers12082154.

Teixido, Cristina, Paola Castillo, Clara Martinez-Vila, Ana Arance, e Llucia Alos. 2021. «Molecular Markers and Targets in Melanoma». *Cells* 10 (9): 2320. https://doi.org/10.3390/cells10092320.

Thiery, Jean Paul. 2002. «Epithelial-Mesenchymal Transitions in Tumour Progression». *Nature Reviews. Cancer* 2 (6): 442–54. https://doi.org/10.1038/nrc822.

Thiery, Jean Paul, Hervé Acloque, Ruby Y. J. Huang, e M. Angela Nieto. 2009. «Epithelial-Mesenchymal Transitions in Development and Disease». *Cell* 139 (5): 871–90. https://doi.org/10.1016/j.cell.2009.11.007.

Thompson, E. W., e I. Haviv. 2011. «The social aspects of EMT-MET plasticity». *Nature Medicine*. https://doi.org/10.1038/nm.2437.

Thompson, L., B. Chang, e S. H. Barsky. 1996. «Monoclonal Origins of Malignant Mixed Tumors (Carcinosarcomas). Evidence for a Divergent Histogenesis». *The American Journal of Surgical Pathology* 20 (3): 277–85. https://doi.org/10.1097/00000478-199603000-00003.

Trombetti, Silvia, Elena Cesaro, Rosa Catapano, Raffaele Sessa, Alessandra Lo Bianco, Paola Izzo, e Michela Grosso. 2021. «Oxidative Stress and ROS-Mediated Signaling in Leukemia: Novel Promising Perspectives to Eradicate Chemoresistant Cells in Myeloid Leukemia». *International Journal of Molecular Sciences* 22 (5): 2470. https://doi.org/10.3390/ijms22052470.

Tsai, Jeff H., Joana Liu Donaher, Danielle A. Murphy, Sandra Chau, e Jing Yang. 2012. «Spatiotemporal Regulation of Epithelial-Mesenchymal

Transition Is Essential for Squamous Cell Carcinoma Metastasis». *Cancer Cell* 22 (6): 725–36. https://doi.org/10.1016/j.ccr.2012.09.022.

Tsukazaki, T., T. A. Chiang, A. F. Davison, L. Attisano, e J. L. Wrana. 1998. «SARA, a FYVE Domain Protein That Recruits Smad2 to the TGFbeta Receptor». *Cell* 95 (6): 779–91. https://doi.org/10.1016/s0092-8674(00)81701-8.

Uhl, Martin, Steffen Aulwurm, Jörg Wischhusen, Markus Weiler, Jing Ying Ma, Ramona Almirez, Ruban Mangadu, et al. 2004. «SD-208, a novel transforming growth factor β receptor I kinase inhibitor, inhibits growth and invasiveness and enhances immunogenicity of murine and human glioma cells in vitro and in vivo». *Cancer Research* 64 (21): 7954–61. https://doi.org/10.1158/0008-5472.CAN-04-1013.

Urrutia, Raul. 2003. «KRAB-Containing Zinc-Finger Repressor Proteins». *Genome Biology* 4 (10): 231. https://doi.org/10.1186/gb-2003-4-10-231.

Vandamme, Niels, Geertrui Denecker, Kenneth Bruneel, Gillian Blancke, Özden Akay, Joachim Taminau, Jordy De Coninck, et al. 2020. «The EMT Transcription Factor ZEB2 Promotes Proliferation of Primary and Metastatic Melanoma While Suppressing an Invasive, Mesenchymal-Like Phenotype». *Cancer Research* 80 (14): 2983–95. https://doi.org/10.1158/0008-5472.CAN-19-2373.

Vaquerizas, Juan M., Sarah K. Kummerfeld, Sarah A. Teichmann, e Nicholas M. Luscombe. 2009. «A Census of Human Transcription Factors: Function, Expression and Evolution». *Nature Reviews. Genetics* 10 (4): 252–63. https://doi.org/10.1038/nrg2538.

Vissing, H., W. K. Meyer, L. Aagaard, N. Tommerup, e H. J. Thiesen. 1995. «Repression of Transcriptional Activity by Heterologous KRAB Domains Present in Zinc Finger Proteins». *FEBS Letters* 369 (2–3): 153–57. https://doi.org/10.1016/0014-5793(95)00728-r.

Wang, Lin, Joon Hyeok Kwak, Sung Il Kim, Yanjuan He, e Mary E. Choi. 2004. «Transforming Growth Factor-Beta1 Stimulates Vascular Endothelial Growth Factor 164 via Mitogen-Activated Protein Kinase Kinase 3-P38alpha and P38delta Mitogen-Activated Protein Kinase-Dependent Pathway in Murine Mesangial Cells». *The Journal of Biological Chemistry* 279 (32): 33213–19. https://doi.org/10.1074/jbc.M403758200.

Wang, Sherry M., Vincent W. Coljee, Robert J. Pignolo, Mitch O. Rotenberg, Vincent J. Cristofalo, e Felipe Sierra. 1997. «Cloning of the human twist gene: Its expression is retained in adult mesodermally-derived tissues». *Gene* 187 (1): 83–92. https://doi.org/10.1016/S0378-1119(96)00727-5.

Wang, Tao, Xian-guo Wang, Jun-hua Xu, Xiang-Peng Wu, Hong-ling Qiu, Hong Yi, e Wen-Xin Li. 2012. «Overexpression of the Human ZNF300 Gene Enhances Growth and Metastasis of Cancer Cells through Activating NF-KB Pathway». *Journal of Cellular and Molecular Medicine* 16 (5): 1134–45. https://doi.org/10.1111/j.1582-4934.2011.01388.x.

Widlund, Hans R., Martin A. Horstmann, E. Roydon Price, Junqing Cui, Stephen L. Lessnick, Min Wu, Xi He, e David E. Fisher. 2002. «Beta-Catenin-Induced Melanoma Growth Requires the Downstream Target Microphthalmia-Associated Transcription Factor». *The Journal of Cell Biology* 158 (6): 1079–87. https://doi.org/10.1083/jcb.200202049.

Wuttke, D. S., M. P. Foster, D. A. Case, J. M. Gottesfeld, e P. E. Wright. 1997. «Solution Structure of the First Three Zinc Fingers of TFIIIA Bound to the Cognate DNA Sequence: Determinants of Affinity and Sequence Specificity». *Journal of Molecular Biology* 273 (1): 183–206. https://doi.org/10.1006/jmbi.1997.1291.

Xia, Yifeng, Shen Shen, e Inder M. Verma. 2014. «NF-κB, an Active Player in Human Cancers». *Cancer Immunology Research* 2 (9): 823–30. https://doi.org/10.1158/2326-6066.CIR-14-0112.

Xouri, Georgia, e Sven Christian. 2010. «Origin and Function of Tumor Stroma Fibroblasts». *Seminars in Cell & Developmental Biology* 21 (1): 40–46. https://doi.org/10.1016/j.semcdb.2009.11.017.

Xu, Jian, Samy Lamouille, e Rik Derynck. 2009. «TGF-Beta-Induced Epithelial to Mesenchymal Transition». *Cell Research* 19 (2): 156–72. https://doi.org/10.1038/cr.2009.5.

Xu, Mei, Siying Wang, Yongchao Wang, Huaxun Wu, Jacqueline A. Frank, Zhuo Zhang, e Jia Luo. 2018. «Role of P38γ MAPK in Regulation of EMT and Cancer Stem Cells». *Biochimica Et Biophysica Acta. Molecular Basis of Disease* 1864 (11): 3605–17. https://doi.org/10.1016/j.bbadis.2018.08.024.

Yang, Guang, Yitang Li, Emi K. Nishimura, Hong Xin, Anyu Zhou, Yinshi Guo, Liang Dong, Mitchell F. Denning, Brian J. Nickoloff, e Rutao Cui. 2008. «Inhibition of PAX3 by TGF-Beta Modulates Melanocyte Viability». *Molecular Cell* 32 (4): 554–63. https://doi.org/10.1016/j.molcel.2008.11.002.

Yingling, Jonathan M., Kerry L. Blanchard, e J. Scott Sawyer. 2004. «Development of TGF-Beta Signalling Inhibitors for Cancer Therapy». *Nature Reviews. Drug Discovery* 3 (12): 1011–22. https://doi.org/10.1038/nrd1580.

Zeng, Shan, Jia Chen, e Hong Shen. 2010. «Controlling of Bone Morphogenetic Protein Signaling». *Cellular Signalling* 22 (6): 888–93. https://doi.org/10.1016/j.cellsig.2009.12.007.

Zhang, Suping, Teng Fei, Lixia Zhang, Ran Zhang, Feng Chen, Yuanheng Ning, Yuna Han, Xin-Hua Feng, Anming Meng, e Ye-Guang Chen. 2007. «Smad7 Antagonizes Transforming Growth Factor Beta Signaling in the Nucleus by Interfering with Functional Smad-DNA Complex Formation». *Molecular and Cellular Biology* 27 (12): 4488–99. https://doi.org/10.1128/MCB.01636-06.

Zhang, Ying E. 2009. «Non-Smad Pathways in TGF-Beta Signaling». *Cell Research* 19 (1): 128–39. https://doi.org/10.1038/cr.2008.328.