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Regulation of cell differentiation and invasion by members of the TGFß family

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Abstract

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Transforming growth factor β (TGFβ) and bone morphogenetic protein (BMP) signaling pathways are important in embryonic development and tissue homeostasis, but also have complex roles in the context of cancer. TGFβ promotes epithelial to mesenchymal transition (EMT) a physiological developmental process, often hijacked in different types of cancer, eventually leading to cancer cell invasion and metastasis. BMP signaling is involved in bone formation, angiogenesis and neural cell differentiation, but also regulates cancer by inducing EMT and its reversion.

Liver kinase B1 (LKB1) is a tumor suppressor protein kinase involved in the regulation of cell metabolism, proliferation and polarity. First, we investigated how LKB1 negatively regulates BMP signaling and we demonstrated that LKB1 interacts with one of the BMP type I receptors and mediates its degradation, leading to the inhibition of BMP-induced cell differentiation.

We then focused on the role of LKB1 in the establishment of mammary epithelial polarity. Upon LKB1 depletion, normal mammary epithelial cells lost the ability to form polarized acini, and displayed enhanced TGFβ responses. The use of a chemical inhibitor targeting TGFβ type I receptor restored the formation of acini, therefore we concluded that the contribution of LKB1 to mammary epithelial polarity is dependent on the regulation of autogenous TGFβ signaling.

Glioblastoma (GBM) is a brain malignancy, that is highly invasive and heterogeneous in terms of cell differentiation. TGFβ enhances the self-renewal potential of glioblastoma stem cells (GSCs), while BMP promotes their differentiation towards the astrocytic lineage. In the second part of this thesis, we investigated the role of different effectors downstream of TGFβ/BMP signaling in GBM.

Snail is a well-established inducer of EMT in carcinomas but in the context of GBM, we demonstrated that Snail was induced by BMP7, and via its interaction with Smad signaling effectors, enhanced BMP while it suppressed TGFβ signaling, thus promoting the astrocytic differentiation of GSCs and suppressing stemness.

Finally, the role of the TGFβ/BMP target gene, CXXC5, was investigated in GBM. CXXC5 expression was enriched in GSCs that express high levels of stem cell markers, and depletion of CXXC5 led to reduced self-renewal capacity of GBM cells. Further analysis indicated that CXXC5 epigenetically regulates stemness-related genes by counteracting the activity of the polycomb repressor complex 2 (PRC2), thus affecting the histone modification pattern on the regulatory elements of these genes.

Collectively, the thesis provides evidence on mechanisms that regulate cell differentiation by interfering with TGFβ/BMP signaling.

Keywords: TGFβ, BMP, LKB1, signal transduction, cancer

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To Raffaello, for the love and the support along the way

To Dafni, for making me stronger and better every day

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Raja, E., **Tzavlaki, K.***, Vuilleumier, R. *, Edlund, K., Kahata, K., Zieba, A., Morén, A., Watanabe, Y., Voytyuk, I., Botling, J., Söderberg, O., Micke, P., Pyrowolakis, G., Heldin, C-H., Moustakas, A. (2016) The protein kinase LKB1 negatively regulates bone morphogenetic protein receptor signaling. *Oncotarget*, 7(2): 1120–43.

II **Tzavlaki, K.**, Morén, A., Watanabe, Y., Eriksson, J., Sellin, ME, Kato, M., Caja, L., Heldin, C-H., Moustakas, A. (2020) The liver kinase B1 inhibits transforming growth factor β signaling during mammary epithelial morphogenesis. *Manuscript*.

III Caja, L., **Tzavlaki, K.***, Shahidi Dadras, M.*, Tan, E-J., Hatem, G., Maturi, P., Morén, A., Wik, L., Watanabe, Y., Savary, K., Kamali-Moghaddan, M., Uhrbom, L., Heldin, C-H., Moustakas, A. (2018) Snail regulates BMP and TGFβ pathways to control the differentiation status of glioma-initiating cells. *Oncogene*, 37(19): 2515–31.

IV **Tzavlaki, K.**, Shahidi Dadras, M., Mezheyeuski, A., Morén, A., Heldin, C-H., Caja, L., Moustakas, A. (2020) Epigenetic coupling of transcription factor CXXC5 regulates stemness genes in glioblastoma. *Manuscript under revision.*

* indicates that these authors contributed equally to work.

Related publications

I. Savary, K, Caglayan, D, Caja, L, **Tzavlaki, K**., Bin Nayeem, S., Bergström, T., Jiang, Y., Uhrbom, L., Forsberg-Nilsson, K., Westermark, B., Heldin, C-H., Ferletta, M., Moustakas A. (2013) Snail depletes the tumorigenic potential of glioblastoma. *Oncogene* 32, (47), 5409-20.

II. **Tzavlaki, K**. and Moustakas, A. (2020) TGFβ signaling. *Biomolecules*, 10(3):487. *Review*

Contents

Abbreviations

ALK5: activin receptor-like kinase 5

AMPK: AMP-activated protein kinase

BAMBI: BMP and activin membrane-bound inhibitor

bHLH: basic helix-loop-helix

BMP: bone morphogenetic protein

BMPRI: bone morphogenetic protein receptor type I

BMPRII: bone morphogenetic protein receptor type II

CDK: cyclin dependent kinase

CDKI: cyclin dependent kinase inhibitor

CSC: cancer stem cell

EED: embryonic ectoderm development

EMT: epithelial-mesenchymal transition

EZH2: enhancer of zeste homologue 2

FN1: fibronectin 1

GBM: glioblastoma

GEF: guanine nucleotide exchange factor

GSC: glioblastoma stem cell

GSK3: glycogen synthase kinase 3

HDAC: histone deacetylase

I-Smad: inhibitory Smad

ID: inhibitor of differentiation

JNK: c-Jun N-terminal kinase

KDM: histone lysine demethylase

KMT: histone lysine methyltransferase

LIF: leukemia inhibitory factor

LKB1: liver kinase B1

MAPK: mitogen-activated protein kinase

MLL: mixed lineage leukemia

MH1 or 2: Mad homology 1 or 2

MO25: mouse protein 25

mTOR: mammalian target of rapamycin

NF-κB: nuclear factor κ-light-chain-enhancer of activated B cells

NSCs: neural stem cells

PARP1: poly-ADP ribose polymerase 1

PJS: Peutz-Jeghers syndrome

PI3K: phosphatidylinositol-3' kinase

PKC: Protein kinase C

PRC2: polycomb repressive complex 2

R-Smad: receptor activated Smad

SARA: Smad anchor for receptor activation

SCP: small C-terminal domain phosphatase

SMAD: Small mothers against decapentaplegic

STAT: signal transducer and activator of transcription

STK11: serine/threonine kinase 11

STRAD: STE20-related adaptor

TAK1: TGFβ-activated kinase 1

TF: transcription factor

TGFβ: transforming growth factor β

TRAF6: tumor necrosis factor α receptor associated factor 6

TSC2: tuberous sclerosis complex 2

TSS: transcription start site

TβRI: transforming growth factor β receptor type I

TβRII: transforming growth factor β receptor type II

Introduction

1. Transforming growth factor β (TGFβ) and bone morphogenetic (BMP) signaling pathways

1.1 Canonical TGFβ/BMP signaling

The TGFβ family consists of 33 different secreted cytokines, which are synthesized as precursor molecules and are then proteolytically cleaved to generate the mature dimeric ligands (Tzavlaki and Moustakas, 2020). The family includes the three TGFβ isoforms, the BMPs, the activins, the growth differentiation factors, the nodal and the müllerian inhibiting substance.

TGFβ and BMP signaling initiate when TGFβ and BMP ligands bind to type I and type II receptors that have Ser/Thr kinase activity and form heterotetrameric complexes in the presence of ligand. More specifically, TGFβ ligands (TGFβ1, 2 and 3) signal via receptor complexes that consist of the TGFβ type II receptor (TβRII) and the TGFβ type I receptor (TβRI), also known as activin receptor-like kinase 5 (ALK5), which are ubiquitously expressed. TGFβ ligands signal also via ALK1, another TβRI that is selectively expressed in endothelial cells (Goumans et al., 2003; Heldin and Moustakas, 2016). In the case of BMP signaling, BMP ligands act via receptor complexes composed of BMP type II receptors (BMPRIIs), which are BMPRII, ActRII and ActRIIB, in combination with the type I receptors (BMPRIs) ALK2/ACVR1, ALK3/BMPRIA and ALK6/BMPRIB (Heldin and Moustakas, 2016).

Upon ligand binding, the constitutively active type II receptor phosphorylates and activates the dormant type I receptor, turning on its kinase activity. TGFβ ligands associate with high affinity with TβRII, an interaction that promotes the recruitment of TβRI, leading to the formation of a heterotetrameric receptor complex consisting of two TβRII and two TβRI units, and this complex formation allows TβRI to be phosphorylated by TβRII (Tzavlaki and Moustakas, 2020). On the other hand, BMP ligands show higher affinity for their respective type I receptors (Kirsch et al., 2000). In general, BMP ligands bind to their respective receptor complexes with lower affinity and in a less specific manner compared to what TGFβ ligands do, a fact the explains the increased flexibility by which BMP ligands

interact with a more diverse group of receptors (Heldin and Moustakas, 2016).

Upon activation, the type I receptor recruits and phosphorylates the carboxy-terminal region of the small mothers against decapentaplegic (SMAD) transcription factors, which are predominantly Smad2 and Smad3 in the case of TGFβ signaling and Smad1, Smad5 and Smad9 in the case of BMP signaling (Massagué et al., 2005). The recruitment of Smad2 and Smad3 to the receptor complex is facilitated by the Smad anchor for receptor activation (SARA) protein, and the hepatocyte growth factor-regulated tyrosine kinase substrate (Miura et al., 2000; Tsukazaki et al., 1998; Wu et al., 2000), while Endofin, which interacts with Smad1, probably has a similar role in the initiation of BMP signaling (Shi et al., 2007). The conformational changes triggered by the phosphorylation of R-Smads by the type I receptor, lead to the dissociation of R-Smads from the receptor complex and their association with Smad4, the common mediator for both TGFβ and BMP signaling pathways. The resulting trimeric Smad complexes are then shuttled to the nucleus where they regulate the expression of TGFβ and BMP target genes (Figure 1).

Smad4 and R-Smad proteins, consist of two highly conserved domains, the amino-terminal Mad homology 1 (MH1) and the carboxy-terminal Mad homology 2 (MH2) domain. The MH1 domain contains a β-hairpin structure that mediates the binding of Smads to DNA (Shi et al., 1998). The MH2 domain mediates the association of R-Smads with the type I receptors, as well as the interaction among R-Smads and Smad4 for the formation of trimeric complexes (Chaikuad and Bullock, 2016). The MH2 domain of R-Smads contains also a Ser-X-Ser motif, which serves as the phosphorylation site for type I receptors, leading to Smad activation. MH1 and MH2 domains are separated by the linker region, which is not conserved among Smads and contains multiple phosphorylation sites for several kinases such us the mitogen-activated protein kinases (MAPKs), the cyclin-dependent kinases (CDKs) and the glycogen synthase kinase 3 (GSK3). Linker phosphorylation often regulates Smad subcellular localization, activity and stability, as it leads, for example, to the recruitment of E3 ubiquitin ligases that ubiquitinate Smad proteins and guide them to proteasomal degradation (Xu et al., 2016).

Smad complexes regulate gene expression by recognizing and binding specific DNA sequences in the regulatory elements of target genes. More specifically, it has been described that Smad3 and Smad4 recognize and bind half of the palindromic octamer 5'-GTCTAGAC-3', also known as Smad-binding element, while BMP-activated Smads bind preferentially on a 5'-GGCGCC-3' sequence (Hill, 2016; Morikawa et al., 2011). However,

according to more recent evidence, both TGFβ and BMP R-Smads, as well as Smad4, can bind GC-rich regulatory elements, and more precisely the 5'-GGC(GC)|(CG)-3' consensus sequence (Martin-Malpartida et al., 2017). In contrast to the rest of R-Smads, the most prevalent isoform of Smad2 cannot bind directly on DNA, because of a unique sequence (known as the E3 insert) in the MH1 domain, which affects Smad2 protein conformation and it interferes with its DNA-binding capacity (Zawel et al., 1998).

Smad complexes have low DNA-binding affinity, therefore they interact with a wide variety of transcription factors and chromatin modifying enzymes, in order to positively or negatively regulate the expression of target genes. Many of the Smad-interacting partners are cell-type specific and/or controlled by other signaling pathway proteins that can direct Smad complexes to different gene regulatory elements, thus determining the context-dependent responses of TGFβ/BMP signaling (Mullen et al., 2011).

The above description presents a classic overview of how TGFβ and BMP signaling pathways are activated (Figure 1). To add an extra level of diversity, TGFβ can also promote Smad1 and Smad5 activation via heteromeric complexes consisting of receptors from both TGFβ and BMP branches (Daly et al., 2008; Liu et al., 1998). Interestingly, the coordinated activation of Smad2/3 and Smad1/5 can lead to the formation of mixed R-Smad complexes that regulate distinct subsets of genes, and this combinatorial signaling seems to be required so that the complete repertoire of TGFβ-dependent transcriptional and physiological responses is elicited (Daly et al., 2008; Ramachandran et al., 2018).

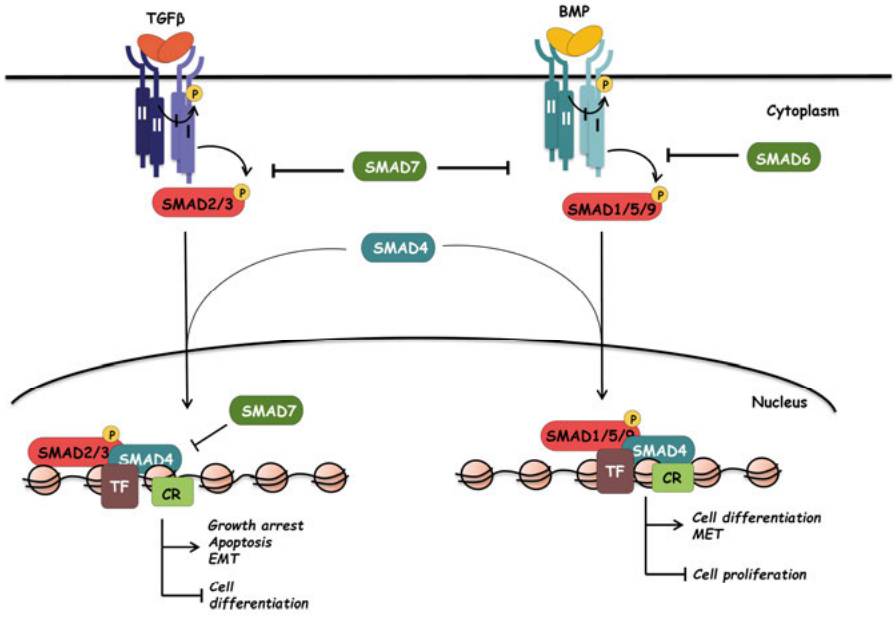

Figure 1. Schematic representation of Smad-dependent TGFβ and BMP signaling pathways. *TF*: transcription factor, *CR*: chromatin regulator.

1.2 Negative regulation of TGFβ/BMP signaling

TGFβ family signaling controls a variety of biological processes, from embryonic patterning, to adult tissue homeostasis, and from stem cell renewal to cell differentiation. As expected, these pathways are tightly regulated both temporally and spatially at many different levels, and by a variety of mechanisms (Figure 2).

1.2.1 Regulation at the extracellular level

At the extracellular level, a variety of antagonists that act by sequestering TGFβ family ligands from the receptors have been identified, including the Chordin/Noggin and the DAN/Cerberus families, which prevent BMP ligands from binding to their receptors, as well as Decorin and α-macroglobulin which act as antagonists for TGFβ ligands. Other negative regulators act by competing with TGFβ ligands for binding on the receptors, thus leading to the formation of non-functional complexes (e.g. Follistatin), or modulate the processing, the secretion and the stability of the ligands (Chang, 2016).

1.2.2 Regulation at the receptor level

At the receptor level, TGFβ family signaling is modulated by co-receptors that interfere with physiological complex formation or promote ligand sequestration. One example is the pseudoreceptor BMP and activin membrane-bound inhibitor (BAMBI), which has an extracellular domain resembling the type I receptors and a short intracellular domain that does not possess kinase activity. BAMBI competes with both TGFβ and BMP type I receptors for associating with the type II receptor, thus preventing the formation of physiological receptor complexes (Onichtchouk et al., 1999).

Immunophilin FKBP12 is a chaperone protein that protects TGFβ signaling from spontaneous ligand-independent activation by binding to TGFβRI in the absence of ligand and inhibiting functional receptor complex formation (Wang et al., 1996).

Initiation of TGFβ/BMP signaling is also counteracted by the activity of the inhibitory Smads (I-Smads), Smad6 and Smad7. In terms of structure, I-Smads share homology with R-Smads at the MH2 domain, even though they lack the Ser-X-Ser motif, which is the phosphorylation site for TβRI. The amino-terminal regions of I-Smads diverge from the MH1 domain and linker region that the rest of Smad proteins have, and actually, they are only partially conserved in between Smad6 and Smad7. I-Smads act at various levels to attenuate TGFβ/BMP signaling, and one way is by promoting the ubiquitination and degradation of type I receptors. To this end, Smad7 recruits members of the HECT-domain ubiquitin ligases, such as Smurf1, Smurf2 and NEDD4-2, which add ubiquitin moieties to the receptors, guiding them to proteasomal degradation (Kamiya et al., 2010; Kavsak et al., 2000; Kuratomi et al., 2005). Both BMP and TGFβ signaling induce the expression of *SMAD6* and *SMAD7* genes, thus creating a negative feedback loop that tightly controls signaling (Afrakhte et al., 1998; Nakao et al., 1997).

Receptor activation, is negatively regulated also by phosphatase activity. Smad7 recruits the protein phosphatase 1 complex (PP1c) to TβRI, thus promoting the dephosphorylation of the receptor (Shi et al., 2004). The PP1 complex can also associate with Endofin, (the Smad anchor protein in the case of BMP signaling), thus promoting the dephosphorylation of BMPRI in a Smad7-independent manner (Shi et al., 2007). The phosphatase Dullard is also implicated in the negative regulation of BMP signaling during neural development in *X. laevis*, as it dephosphorylates BMPRI, eventually leading to its ubiquitination and proteasomal degradation (Satow et al., 2006).

1.2.3 Regulation of Smad activation and complex formation

Besides promoting receptor degradation, I-Smads inhibit also the activation of R-Smads as they antagonize them for binding to type I receptors (Hayashi et al., 1997; Imamura et al., 1997). The amino-terminal domain of Smad7 facilitates the interaction of the Smad7 MH2 domain with the TβRI, therefore making Smad7 a more potent inhibitor of TGFβ signaling compared to Smad6, which targets mostly BMP signaling (Hanyu et al., 2001; Kamiya et al., 2010).

I-Smads interact also with activated R-Smads (Smad6 with Smad1, and Smad7 with Smad2 and Smad3), thus preventing complex formation between R-Smads and Smad4. In addition to that, I-Smads via their association with the E3 ligases Smurf1 and NEDD4-2, facilitate the ubiquitination and subsequent degradation of the Smad1-Smad5 and the Smad2-Smad3 complexes respectively (Miyazawa and Miyazono, 2017).

The linker domain of Smad proteins contains phosphorylation sites for various kinases that generate a platform for recruitment of several ubiquitin ligases, that in turn regulate Smad stability (Xu et al., 2016). Smurf2 targets for proteasomal degradation Smad2, while it inhibits Smad complex formation by promoting multiple mono-ubiquitination of Smad3 (Tang et al., 2011; Zhang et al., 2001). Other E3 ligases that negatively regulate TGFβ or BMP signaling by controlling Smad stability are CHIP, SCF/ROC, and the RING-domain Arkadia which, enhances Smad transcriptional activity while at the same time promotes Smad degradation, thus providing an efficient mechanism of gene activation followed by termination of the signaling at the end of the cascade (Heldin and Moustakas, 2016). Smad activity is negatively regulated also by phosphatases that mediate dephosphorylation of the carboxy-terminal serines, leading to the deactivation of R-Smads and the attenuation of TGFβ/BMP signaling. PPM1A/PP2Cα dephosphorylates Smad2 and Smad3, while small C-terminal domain phosphatases (SCP) target Smad1 C-terminal phosphorylation, leading to attenuation of TGFβ and BMP signaling respectively (Knockaert et al., 2006; Lin et al., 2006).

1.2.4 Regulation of SMAD transcriptional activity

The nuclear pool of Smad7 contributes to the negative regulation of TGFβ signaling at the transcriptional level, by competing with functional Smad complexes for binding on the regulatory elements of target genes (Zhang et al., 2007). Moreover, nuclear I-Smads interact also with histone deacetylases (HDACs), which suggests that they can act as transcriptional repressors for target genes (Bai and Cao, 2002).

The nuclear proteins SKIL (previously known as SnoN) and SKI are important negative regulators of TGFβ signaling. They interact with R-Smads and Smad4, disrupting the formation of functional heteromeric SMAD complexes, eventually inhibiting Smad transcriptional activity (Deheuninck and Luo, 2009). SKI is a negative regulator of BMP signaling as well, as it can associate with Smad1-Smad5 complexes, leading to the inhibition of BMP-dependent transcription (Wang et al., 2000).

Smad transcriptional activity is repressed also by zinc finger protein 451 (ZNF451), which blocks the recruitment of the histone acetyltransferase p300 to Smad complexes, leading to reduced acetylation at Lys9 on histone H3 and transcriptional repression (Feng et al., 2014). Evi-1 is another example of protein that interferes with Smad-mediated transcription activation, by recruiting the C-terminal binding protein (CtBP) to Smad complexes and blocking TGFβ- as well as BMP-inducible gene expression (Alliston et al., 2005; Izutsu et al., 2001).

Additionally, Smad-mediated transcription is negatively regulated by posttranslational modifications on Smads that influence their DNA-binding activity. One such example is the phosphorylation of Smad4 on the MH1 domain by the cell polarity and metabolism regulator liver kinase B1 (LKB1), which inhibits Smad4 binding on gene regulatory sequences and attenuates TGFβ- and BMP-dependent transcriptional responses (Morén et al., 2011). Similarly, phosphorylation by protein kinase C (PKC), abrogates the DNA binding activity of Smad3, thus inhibiting Smad3-mediated transcription (Yakymovych et al., 2001). Another example is TRIM33, a histone-binding protein that has also E3 ligase activity and mono-ubiquitinates DNA-bound Smad4, thus leading to the disruption of Smad complexes and their dissociation from promoters (Agricola et al., 2011). ADP-ribosylation of Smad1, Smad3 and Smad4 by the poly-ADP ribose polymerase 1 (PARP1) also promotes their dissociation from DNA, thus suppressing TGFβ-induced epithelial-mesenchymal transition (EMT) and BMP-induced differentiation (Lönn et al., 2010; Watanabe et al., 2016). The process of sumoylation is also involved in the negative regulation of Smad transcriptional activity, as the protein inhibitor of activated STATy (PIASy) sumoylates Smad3, thus inhibiting Smad3-mediated transcription (Imoto et al., 2003).

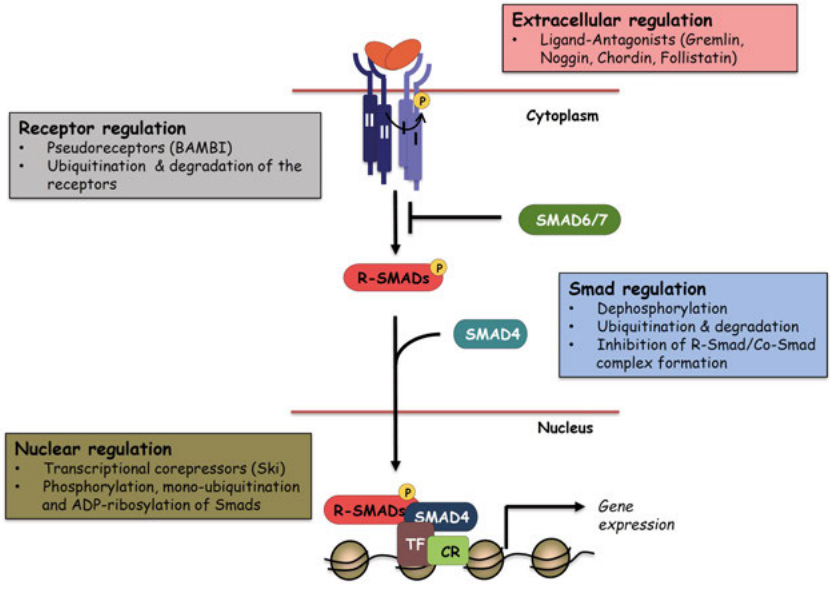

Figure 2. TGFβ/BMP signaling is tightly regulated at different levels starting from the extracellular level till the regulation of Smad transcriptional activity. *TF*: transcription factor, *CR*: chromatin regulator.

1.3 Non-Smad TGFβ/BMP signaling

In addition to Smad-mediated signaling, TGFβ and BMP ligands activate also alternative pathways, including the MAP kinase, the Rho-(like) GTPase and the phosphatidylinositol-3' kinase(PI3K)/AKT pathways in order to regulate downstream cellular responses (Zhang, 2017).

1.3.1 ERK signaling

The rapid TGFβ-dependent induction of ERK signaling depends on the weak yet detectable tyrosine kinase activity of TβRI, and initiates with the phosphorylation of the adaptor protein ShcA, which then forms a complex with the downstream signaling mediators Grb2 and Sos2. The ShcA/Grb2/Sos2 complex activates the Ras GTPase, leading to the subsequent activation of c-Raf, and eventually to the activation of ERK1/2 kinases (Lee et al., 2007). The activation of ERK1/2 is crucial for the TGFβ-dependent induction of EMT as it is involved in the disassembly of adherens junctions and the regulation of cell motility (Zhang, 2017).

1.3.2 JNK and p38 MAPK signaling:

c-Jun N-terminal kinase (JNK) and p38 MAP kinase signaling cascades are rapidly activated in a TGFβ-activated kinase 1 (TAK1)-dependent manner. More specifically, TβRI binds to the E3 ubiquitin ligase tumor necrosis factor α receptor associated factor 6 (TRAF6) or its relative TRAF4 in a ligand-dependent manner, inducing their activation via autoubiquitination. Activated TRAF6 and TRAF4 mediate then TAK1 Lys^{63} poly-ubiquitination and activation, which finally leads to p38 and JNK pathway activation (Sorrentino et al., 2008; Yamashita et al., 2008). TRAF6-dependent poly-ubiquitination of TAK1 is required also for the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling in response to TGFβ (Hamidi et al., 2012). Interestingly, even though TAK1 was firstly characterized as a TGFβ-regulated kinase, it can also be activated by BMP2, leading to p38 activation and induction of apoptosis (Kimura et al., 2000).

To add an extra level of complexity, TRAF6 polyubiquitinates TβRI, thus promoting the cleavage of the receptor by ADAM17 metalloprotease in a protein kinase C ζ (PKCζ)-dependent manner. In response to TGFβ, the TβRI-TRAF6 complex may also recruit and polyubiquitinate the protease presenilin-1, which introduces a second proteolytic cleavage to the receptor, leading to the release of the TβRI intracellular domain (ICD) and its nuclear translocation. There, TβRI ICD associates with chromatin and transcriptional regulators such as p300 to regulate gene expression (Gudey et al., 2014; Mu et al., 2011).

1.3.3 PI3K/AKT signaling

TGFβ and BMP ligands activate also the PI3K/AKT signaling in several cell types. TGFβ promotes the TRAF6-mediated polyubiquitination of the PI3K regulatory subunit p85a, leading to TβRI-p85α complex formation, and the subsequent recruitment and phosphorylation of AKT (Hamidi et al., 2017). The p85 subunit interacts also with TβRII but in a constitutive and ligand-independent fashion, and both TβRI and TβRII are required for the activation of the PI3K (Yi et al., 2005). Moreover, TGFβ promotes AKT activation via the mammalian target of rapamycin complex 2 (mTORC2), a mechanism that contributes to cell motility during EMT in mouse mammary NMuMG cells (Lamouille et al., 2012). The activation of PI3K/AKT pathway in response to BMP2 has also been reported to be important for BMP-induced cytoskeletal rearrangements and migration in mesenchymal cells, although the exact mechanism has not yet been elucidated (Gamell et al., 2008).

1.3.4 Rho GTPase signaling

Rho GTPases are important regulators of cytoskeletal reorganization and cell motility and they can be activated by TGFβ/BMP signaling in a Smad-dependent or -independent manner. TGFβ-induced activation of RhoA promotes actin reorganization, membrane ruffling, and stress fiber formation, all key events of the EMT process, while BMP2 promotes rapid RhoA activation in mesenchymal cells, during osteogenic differentiation (Bhowmick et al., 2001; Edlund et al., 2002; Wang et al., 2011). Apart from the rapid Smad-independent activation, TGFβ promotes also a late wave of RhoA activation at later EMT stages via the Smad-induced expression of the RhoA-specific guanine nucleotide exchange factor (GEF) NET1 (Shen et al., 2001). Prolonged TGFβ activity leads to the upregulation of miR-24 which targets NET1 post-transcriptionally, thus creating a negative feedback loop that contributes to TGFβ-mediated EMT (Papadimitriou et al., 2011).

1.3.5 JAK/STAT signaling

TGFβ can activate the Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signaling, to mediate its profibrotic effects. In fibroblasts, TGFβ promotes STAT3 phosphorylation via JAK2 activation, while in hepatic stellate cells, the constitutive interaction of JAK1 with TβRI leads to STAT3 phosphorylation in response to TGFβ (Dees et al., 2012; Tang et al., 2017).

1.4 TGFβ and BMP signaling in cancer

1.4.1 TGFβ signaling in cancer

TGFβ is known to have two interconnected roles during cancer progression, acting as a tumor suppressor in early stages of tumorigenesis and promoting tumor cell invasion and metastasis at later stages.

One of the main tumor suppressive functions of TGFβ is the induction of cell cycle arrest at the early G1 phase in different cell types, mainly through upregulation of the cyclin-dependent kinase inhibitors (CDKIs) *p15/CDKN2B*, *p21/CDKN1A* and *p27/CDKN1B*, and via transcriptional repression of members of the inhibitor of differentiation (Id) family of helix-loop-helix (HLH) transcription factors (*Id1*, *Id2* and *Id3*). TGFβ negatively regulates also the expression of *c-Myc*, which promotes cell proliferation partly by inhibiting the expression of *p21* and promoting the degradation of p27 (Bretones et al., 2015; Heldin et al., 2009). TGFβ exerts its tumor-suppressive role also by promoting apoptosis (programmed cell death) via upregulation of pro-apoptotic genes such as the *TGFβ-inducible early gene* (*TIEG1*), the *growth arrest and DNA damage-inducible 45β* (*GADD45β*) and the Bcl-2 homology domain-only factor *Bim*. Moreover, at the post-

transcriptional level, the interaction of TβRI with the apoptosis-mediating protein Daxx, is another mechanism of the pro-apoptotic role of TGFβ (Pardali and Moustakas, 2007). In prostate cancer cells, the apoptotic program is induced via the TRAF6-TAK1-p38 signaling (Edlund et al., 2002; Sorrentino et al., 2008). Loss-of-function mutations or allelic loss of TGFβ type I and type II receptors or Smad4 have been identified in many tumor types confirming the tumor suppressive role of TGFβ signaling (Padua and Massagué, 2009). There are however cases, where tumor cells bypass the cytostatic effects of TGFβ, as in the case of glioblastoma (GBM), which is an aggressive adult brain tumor, where the higher expression of the transcription factor FoxG1, results in blocking the TGFβ-induced expression of *p21* (Seoane et al., 2004).

During cancer progression, tumor cells develop resistance to the cytostatic effects of TGFβ, shifting the balance towards the pro-metastatic and pro-invasive role of this pathway. The pro-tumorigenic role of TGFβ can be manifested via a number of cellular mechanisms that affect cancer cells themselves, their extracellular matrix or the tumor stromal cells e.g. cancer-associated fibroblasts, immune cells and vascular cells.

One of the most well-established tumor-promoting responses to TGFβ is the induction of EMT (described more extensively in chapter 3), a process during which, epithelial cells lose their polarity, break their contacts with neighboring cells and acquire mesenchymal characteristics that allow them to migrate to distant tissues (Nieto et al., 2016).

The regulation of immune responses is another function via which TGFβ can exert its pro-tumorigenic role. Some examples include the inhibition of proliferation of T cells, due to the TGFβ-mediated induction of cell cycle inhibitors *p21* and *p27,* and downregulation of *c-myc*, as well as the inhibition of T cell differentiation to T helper (Th) cells, which eventually lead to the suppression of anti-tumor Th1-mediated responses. Moreover, TGFβ signaling inhibits natural killer cells, which normally respond rapidly to tumor cells, and also interferes with the antigen-presenting function of dendritic cells, thus leading to immune suppression (Batlle and Massagué, 2019).

The role of TGFβ in the maintenance of cancer stem cells (CSCs) is of a dual nature and is context-dependent. In some cases, it deprives CSCs of their tumorigenic activities by promoting a less proliferative and more differentiated state, while in other contexts, it sustains stem-cell like characteristics (Bellomo et al., 2016). For example, TGFβ mediates a negative effect on the stem cell-like properties of breast CSCs, by downregulating *Id1* as well as by inducing the expression of differentiation

markers *Mucin-1* and *cytokeratin-18* (Tang et al., 2007). On the other hand, TGFβ promotes stem cell-like properties of the CSC population in hepatocellular carcinoma by promoting *CD133* expression and in glioblastoma by promoting the expression of *leukemia inhibitor factor* (*LIF*) and *Sox4* (Ikushima et al., 2009; Peñuelas et al., 2009; You et al., 2010).

1.4.2 BMP signaling in cancer

Similar to TGFβ, BMPs may have pro-tumorigenic or tumor suppressive roles in different types of cancer (Wakefield and Hill, 2013). BMPs act as growth inhibitory molecules in breast, prostate, thyroid and gastric cancer cells via upregulation of *CDKN1A* (Franzén and Heldin, 2001; Ghosh-Choudhury et al., 2000; Miyazaki et al., 2004; Pardali et al., 2005; Wen et al., 2004). BMPs have also pro-apoptotic functions in myeloma cells, where they induce the expression of pro-apoptotic proteins in a p53-dependent manner (Fukuda et al., 2006; Kawamura et al., 2002). BMP signaling has also been shown to play a tumor suppressive role in sporadic colorectal cancer, where the pathway is often inactivated due to loss-of-function mutations in *ALK3* and *Smad4* genes. Loss-of-function mutations in these genes are also linked to the juvenile polyposis syndrome (JPS), which is characterized by the development of intestinal hamartomatous polyps and is associated with an elevated risk for cancer development (Wakefield and Hill, 2013). In the case of GBM, it has been shown that BMPs can act as tumor suppressors as they deplete the tumorigenic potential of GBM stem cells (GSCs) and promote their differentiation towards astrocyte-like cells (Piccirillo et al., 2006; Savary et al., 2013). However, BMP activity has been also linked to enhanced tumor metastasis and invasiveness in some cancer types, such as the triple-negative breast cancer, where active BMP signaling enhances bone metastasis *in vivo* (Katsuno et al., 2008). BMPs can exert their pro-metastatic functions by promoting EMT, as in the case of pancreatic cancer cells, where BMPs promote the expression of the matrix metalloprotease 2 (MMP2) (Gordon et al., 2009). Another mechanism involves the BMP2-enhanced cellular motility and invasion of gastric cancer cells, via a PI3K/AKT signaling-dependent mechanism (Kang et al., 2010). Finally, even though BMPs have been described as tumor suppressors in GBM, there is also evidence that, BMP7 promotes cell migration and invasion by inducing the expression of the transcription factor *SNAI1* (Savary et al., 2013).

2. LKB1 signaling

2.1 The tumor suppressor kinase LKB1: Structure and function

Liver kinase B1 (LKB1) or serine/threonine kinase 11 (STK11) is a tumor suppressor kinase that is involved in the regulation of multiple biological processes such as cell metabolism, cell polarity and cell proliferation (Alessi et al., 2006). It is ubiquitously expressed in all fetal and adult tissues. LKB1 has been classified as tumor suppressor as loss-of-function mutations in the kinase domain have been identified in sporadic lung and ovarian carcinomas as well as in cases of the Peutz-Jeghers syndrome (PJS) that predisposes patients to cancer development (Alessi et al., 2006; Jansen et al., 2009). The majority of the identified mutations are located in the catalytic domain of LKB1, suggesting that the kinase activity is required for the tumor suppressive functions of LKB1 (Alessi et al., 2006). However, there are also mutations located on the C-terminal non-catalytic region of the protein that impair the function of LKB1 to establish and maintain cell polarity (Forcet et al., 2005). $Lkb1^{-/-}$ mice die at midgestation displaying severe vascular defects but heterozygous $Lkb1^{+/-}$ mice survive and develop gastrointestinal hamartomas and tumors that recapitulate the pathophysiology of PJS patients (Boudeau et al., 2003).

In order to be catalytically active, LKB1 is assembled in a heterotrimeric complex together with the pseudokinase STE20-related adaptor (STRAD) and the adaptor mouse protein 25 (Mo25). Mo25 acts as a scaffolding protein that binds to STRAD to keep it in an active/closed conformation. STRAD binds then to LKB1 and allows it to change to its active conformation, which is further stabilized by Mo25 (Zeqiraj et al., 2009).

LKB1 acts as a master kinase and directly phosphorylates fourteen downstream kinases of the AMP-activated protein kinase (AMPK) family (AMPK1-2, MARK1-4, NUAK1-2, SIK1-3 and SNRK), leading to cell growth, polarity and metabolism regulation (Jaleel et al., 2005; Lizcano et al., 2004) (Figure 3). The prototype AMPK family members AMPK1-2, are activated by LKB1 in response to changes in the intracellular energy levels, when cellular AMP levels are high, eventually leading to the induction of ATP-producing pathways (Shaw et al., 2004).

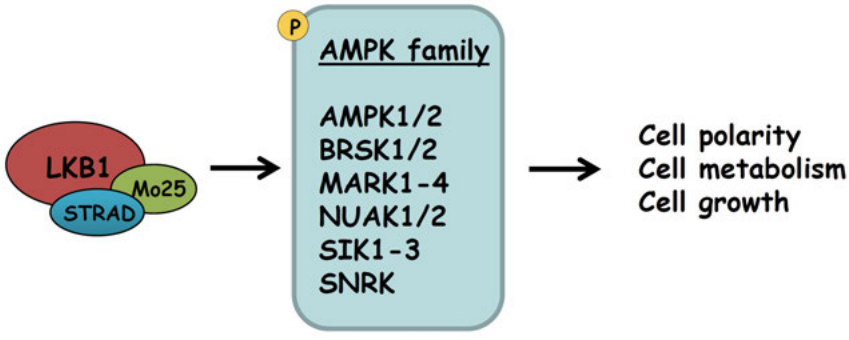

Figure 3. LKB1 is master kinase that activates members of the AMPK family, in order to regulate key biological processes.

2.2 The role of LKB1 in key biological processes

2.2.1 LKB1 as a cell polarity regulator

Cell polarity is a fundamental attribute in cellular architecture that occurs in diverse cell types such as neurons or epithelial cells. The apical-basolateral polarity in epithelial cells is of major importance for tissue organization and function and is characterized by the differential positioning and composition of the plasma membrane domains. Misregulation of cell polarity is characteristic of tumorigenesis. In *C. elegans*, the LKB1 orthologue PAR-4 (partitioning defective 4) regulates asymmetric cell divisions during early embryogenesis (Watts et al., 2000). In a conserved manner, the LKB1 counterpart in *D. melanogaster*, *dLkb1*, regulates anterior-posterior polarity during oogenesis and embryogenesis (Martin and St Johnston, 2003). In mammalian systems, inducible activation of LKB1, promoted the correct positioning of junctional proteins and led to complete polarization of single intestinal epithelial cells in a cell-autonomous manner (Baas et al., 2004). The LKB1-dependent maintenance of epithelial integrity during acinar morphogenesis in mammary epithelial cells restrains the oncogenic activity of c-Myc, a mechanism that is compromised upon LKB1 depletion and leads to c-Myc-dependent cell cycle activation and hyperproliferation (Partanen et al., 2007). This 3D mammary model was also confirmed *in vivo* where conditional deletion of *Lkb1* in the mouse mammary gland resulted in mislocalization of tight junctional proteins and deterioration of desmosomes followed by hyperbranching of mammary ducts (Partanen et al., 2012). In *D. melanogaster,* dLkb1 regulates polarity via Par1 kinase, the homologue of the MARK1-4 kinases that regulate the microtubule network (Martin and St Johnston, 2003). However, the studies performed in mammalian cells

suggest that cell polarity is regulated by LKB1 mainly via activation of the AMPKs, which are involved in the assembly of tight junctions (Zheng and Cantley, 2007).

In mammals, the crucial role of LKB1 in the establishment and maintenance of cell polarity has been studied in a variety of tissues: For example, cellular polarity is physiologically critical also in liver tissue, where LKB1 via AMPKs regulates the formation and maintenance of the canalicular network, which is comprised of hepatocytes (Fu et al., 2010). The role of LKB1 in hepatocyte polarization was also confirmed *in* vivo, as liver-specific *Lkb1* deletion in mice led to mislocalization of tight junctions and impaired paracellular permeability (Porat-Shliom et al., 2016). LKB1 regulates apical junction assembly also during lung epithelial morphogenesis in a kinase activity-independent manner, by directly interacting with p114 RhoA GEF, which in turn activates RhoA GTPase (Xu et al., 2013). Lkb1 deficiency in the pancreatic epithelium of mice resulted in impaired acinar polarity, abnormal cytoskeletal organization and mislocalization of tight junctions (Hezel et al., 2008). Recently, the loss of epithelial cell polarity has been connected to the dysregulation of endosomal trafficking of LKB1, and it has been demonstrated that spatially restricted LKB1 activity is actually essential for the maintenance of epithelial integrity (O'Farrell et al., 2017).

LKB1 is also implicated in the establishment and maintenance of neuronal polarization by promoting axon initiation in the embryonic cortex through the downstream kinases BRSK1/2 (Barnes et al., 2007; Kishi et al., 2005; Shelly et al., 2007).

2.2.2 LKB1 as a cell growth and metabolism regulator.

One of the earliest observations regarding the tumor suppressive function of LKB1, was the fact that it can promote G1 cell cycle arrest via activation of the tumor suppressor p53 and its target gene, the CDKI, *CDKN1A* (Karuman et al., 2001; Tiainen et al.). Moreover, there is evidence that LKB1 inhibits cell proliferation and survival by regulating the expression levels and the activity of the tumor suppressor PTEN, which in turn regulates the AKT pro-survival pathway (Jimenez et al., 2003; Mehenni et al., 2005).

Under conditions of cellular stress, such as nutrient deprivation, low glucose or hypoxia, a major regulatory pathway suppressed by LKB1-AMPK signaling is the mTOR pathway, which positively regulates protein biosynthesis and cell growth (Shaw et al., 2004). AMPK activation leads to tuberous sclerosis complex 2 (TSC2) phosphorylation and subsequent inhibition of the mTOR complex 1 (mTORC1). The negative regulation of mTOR by LKB1/AMPK signaling is also mediated via the inhibitory

phosphorylation of Raptor, which is the binding subunit of mTORC1 (Gwinn et al., 2008).

LKB1 deficiency in tumors triggers a metabolic shift from oxidative phosphorylation to aerobic glycolysis, also known as the Warburg effect (Shackelford et al., 2009). This LKB1-dependent metabolic reprogramming is driven by activation of mTORC1 signaling, which leads to increased hypoxia-inducible factor 1a (HIF1a) levels and subsequent expression of HIF1a target genes (Faubert et al., 2014).

In addition to regulating ATP homeostasis, it has been demonstrated that LKB1/AMPK signaling is also implicated in the maintenance of NADPH levels generated from the pentose phosphate pathway, by inhibiting acetyl-CoA carboxylases 1 and 2 (ACC1-2) under conditions of energetic stress (Jeon et al., 2012). This mechanism is perturbed by the 6-phosphogluconate dehydrogenase-mediated production of ribulose-5-phosphate which disrupts the active LKB1 complex, leading to ACC1 activation and lipogenesis (Lin et al., 2015).

3. Epithelial-mesenchymal transition

Epithelial-mesenchymal transition or EMT, is a highly dynamic developmental process during which, epithelial cells lose the apico-basal polarity and disassemble cell-cell junctions, while they acquire a more migratory phenotype that allows them to move to sites distant from their initial location. EMT is crucial during embryogenesis, organ development and adult tissue homeostasis but is also hijacked in pathological conditions such as organ fibrosis and cancer progression. Key features of EMT include: (i) the downregulation of the epithelial gene expression signature and the upregulation of mesenchymal genes, (ii) the remodeling of the extracellular matrix (ECM), (iii) the loss of cell polarity and the alteration of adhesion and junctional complexes, and (iv) the rearrangement of the cytoskeleton. The acquisition of mesenchymal traits, allows tumor cells at the edge of a primary carcinoma to invade the reactive tumor-associated stroma, intravasate and travel through the blood circulation, and finally extravasate in the parenchyma of a distant tissue. The disseminated cells, can revert back to an epithelial state via the inverse process of mesenchymal-epithelial transition (MET), as they do not receive any EMT-inducing signals by the normal stroma of the new tissue, a fact that highlights the plasticity and the reversibility of the EMT process (Nieto et al., 2016).

3.1 TGFβ-induced EMT

The EMT program can be initiated in cells in response to various signaling factors, TGFβ being one of the most prominent among them. The mechanisms via which TGFβ promotes EMT are diverse.

At the transcriptional level, TGFβ, via Smad activation, promotes an extensive reprogramming of the gene expression profile in epithelial cells. More specifically, TGFβ induces the expression of specific transcription factors that coordinate this nuclear reprogramming and facilitate the establishment of EMT: the zing finger proteins Snail (*SNAI1*) and Slug (*SNAI2*), the ZEB zinc finger homeodomain proteins ZEB1 and ZEB2 and the basic HLH (bHLH) transcription factor Twist. These EMT-inducing transcription factors (EMT-TFs) are known to control the expression of each other and cooperate (in between them as well as with Smads) to downregulate the expression of epithelial genes such as *E-cadherin (CDH1)*, or upregulate mesenchymal genes such as *Fibronectin 1 (FN1)* (Moustakas and Heldin, 2016).

At the post-translational level, the TGFβ-induced activation of RhoA and Cdc42 GTPases regulates cytoskeletal rearrangement and stress fiber formation (Bhowmick et al., 2001; Edlund et al., 2002), while the dissolution

of tight junctions depends on the TGFβ-induced phosphorylation of the polarity protein Par6 that leads to RhoA degradation (Ozdamar et al., 2005).

EMT is also modulated by microRNA (miRNA) networks that are regulated by TGFβ. TGFβ downregulates miRNAs of the miR-200 family, which negatively regulate ZEB1 and ZEB2 during EMT. Other examples include the TGFβ-induced expression of miR-155 and miR-24, which target RhoA and the RhoA-specific GEF NET1 respectively, thus affecting the dissolution of tight junctions during EMT (Lamouille et al., 2013; Papadimitriou et al., 2011).

BMPs counteract the TGFβ-induced EMT and contribute to the inverse MET, partly by enhancing the levels of miRNAs of the miR-200 family. Moreover, BMPs induce the expression of the Id family transcription factors, which can bind and inhibit the transcriptional activity of the bHLH EMT-TF Twist1 (Tan et al., 2015). However, the induction of EMT by BMPs in breast cancer and gastric cancer cells, has also been addressed in recent studies (Kang et al., 2010; Katsuno et al., 2008).

3.2 The role of EMT-TFs in non-epithelial tumors

Apart from activating the classical EMT-associated properties in tumors of epithelial origin (carcinomas), EMT-TFs play important roles also during the progression of non-epithelial tumors such as hematopoietic malignancies, sarcomas and brain tumors, and they affect not only invasion and motility but also cancer cell stemness, survival and resistance to chemotherapy (Kahlert et al., 2017). Snail expression promotes invasiveness while it suppresses the tumorigenic potential of tumor cells in GBM, thus dissociating two usually interlinked processes (Savary et al., 2013). Again in GBM, the most common and aggressive type of adult brain tumors, ZEB1 promotes chemoresistance by positively regulating O^6-alkylguanine DNA alkyltransferase (MGMT) (Siebzehnrubl et al., 2013). ZEB1 and ZEB2 have been linked to aggressive phenotypes in certain leukemias and lymphomas (Sayan, 2014; Stavropoulou et al., 2016). Melanoma is a type of cancer that originates from melanocytes, which are specialized cells derived from neural crest cells that migrate from the neural tube during gastrulation, representing a strong example of developmental EMT. Aberrant expression of EMT-TFs contributes also to melanoma progression, where ZEB1 has an oncogenic role, while ZEB2 and SNAIL act as tumor suppressors (Caramel et al., 2013).

4. Cell differentiation

TGFβ family members are key factors in the regulation of fate commitment in embryonic and adult stem cells. Here we will focus on specific models of cell differentiation and on how TGFβ and BMP signaling pathways are implicated.

4.1 Glioblastoma as a model of cell differentiation

4.1.1 The role of BMPs during normal differentiation of neural stem cells

BMP signaling, by inducing the expression of the Id family of HLH transcription factors, strongly promotes the differentiation of neural stem cells (NSCs) of the adult subventricular zone (SVZ) towards the astrocytic lineage, while repressing neuronal and oligodendrocyte differentiation. More specifically, Id1 and Id3, inhibit the transcriptional activity of the bHLH neurogenic transcription factors *Mash1* and *Neurogenin*, thus blocking neural fate commitment (Nakashima et al., 2001). Similarly, the inhibition of oligodendrocyte differentiation is mediated by Id2 and Id4, which associate and inhibit the bHLH transcription factors Olig1 and Olig2 (Samanta and Kessler, 2004).

4.1.2 Actions of BMP signaling in GBM

Mimicking its function during normal brain development, BMP signaling has a pro-differentiation role also in brain tumor development, and more specifically in glioblastoma or GBM. GBM is characterized by a high proliferation rate, a large degree of heterogeneity, increased invasiveness, microvascular proliferation, a hypoxic and necrotic component, and chemoresistance (Vartanian et al., 2014; Westphal and Lamszus, 2011). BMPs, similar to their effect on NSCs, reduce the proliferation of GSCs and promote their differentiation towards the astrocytic lineage, thus depleting the tumorigenic potential of these cells (Piccirillo et al., 2006; Savary et al., 2013).

4.1.3 Actions of TGFβ signaling in GBM

In GBM, TGFβ promotes the expression of *LIF* in a Smad-dependent manner, which is followed by the subsequent activation of JAK-STAT pathway. As a result, the self-renewal potential of GSCs is enhanced *in vitro,* and the tumor incidence and size when tumor cells are transplanted to the mouse brain, are increased *in vivo* (Peñuelas et al., 2009). Autocrine production of TGFβ is characteristic and essential for GSCs to maintain their self-renewal, and this is achieved also via the TGFβ-dependent induction of the transcription factor *Sox4*, which then cooperates with Oct4 in order to upregulate the stemness-related transcription factor *Sox2* (Ikushima et al.,

2009, 2011). Moreover, high levels of TGFβ activity correlate with an increased $CD44^{high}/Id1^{high}$ subpopulation that is enriched in GSCs. Treatment with a TβRI inhibitor diminished the pool of $CD44^{high}/Id1^{high}$ cells and led to reduced tumor initiating capacity of GSCs (Anido et al., 2010).

4.2 Morphogenesis of mammary epithelial cells

4.2.1 Normal mammary epithelial morphogenesis

Epithelial morphogenesis is a key process for the development of various ductal organs. During morphogenesis, epithelial cells undergo extensive rearrangements in response to various signaling molecules, in order to generate the epithelium that lines the lumen of different glandular organs, such as the prostate gland, the pancreas and the mammary gland.

The adult mammary gland is composed of various cell types, epithelial, immune, adipose and vascular cells, as well as fibroblasts, all having crucial roles in the development and the maintenance of a functional organ.

The mammary epithelial bilayer that lines the ducts, consists of apically oriented luminal epithelial cells and basally oriented myoepithelial cells that are attached to the underlying basement membrane (Figure 4). The myoepithelial cells are derived from cap cells that arise in the mammary end bud during puberty, and actually drive the invasion of the buds into the mammary fat pad in response to hormonal regulation (growth hormone, estrogen, insulin-like growth factor 1), in a process called branching morphogenesis (Macias and Hinck, 2012). The body cells, which at the beginning fill in the end bud can have two different fates: the central body cells undergo apoptosis so that a lumen is formed, while the rest of them differentiate into the luminal epithelial cells (Inman et al., 2015). Finally, during pregnancy, and in response to hormonal changes, we have the terminal differentiation of epithelial alveolar cells, which are capable of milk protein synthesis (Macias and Hinck, 2012).

4.2.2 TGFβ and BMP signaling involvement in mammary epithelial cell morphogenesis

Ductal differentiation is driven by the action and the crosstalk of many different signaling pathways, and TGFβ family members are also involved.

TGFβ is a potent inhibitor of proliferation in mammary epithelial cells, an effect mediated by TβRII. Loss-of-function mutations in TβRII, result in increased mammary cell proliferation, leading to tissue hyperplasia (Gorska et al., 1998). TGFβ acts also as a negative regulator at the late stages of mammary gland morphogenesis by inhibiting STAT5-regulated gene

expression (Cocolakis et al., 2008). Furthermore, TGFβ has also pro-apoptotic effects during the post-lactational involution, when the mammary gland undergoes epithelial cell death and extensive tissue remodeling to return to a similar state as the pre-lactation (Kahata et al., 2018).

On the other hand, BMP signaling has been described to have a positive role during late stages of mammary duct differentiation (Forsman et al., 2013). BMP signaling is also important for maintaining the apico-basal polarity of epithelial cells, by controlling the expression of tight junctional proteins (Kahata et al., 2018).

4.2.3 Mammary epithelial cells as an in vitro model of acinar morphogenesis

When grown in three dimensional (3D) cultures, on a reconstituted basement membrane, untransformed mammary epithelial cells undergo a morphogenetic process that mimics the process of epithelial cell differentiation in the mammary gland, and give rise to growth-arrested, acini-like spheroids that recapitulate several characteristics of the mammary epithelium *in vivo.* These characteristics include the establishment of apico-basal polarity, the formation of a hollow lumen that depends on apoptotic mechanisms as well as autophagy, and the deposition of basement membrane components such as collagen IV and laminin V (Figure 4) (Debnath et al., 2002, 2003; Fung et al., 2007). More specifically, the mammary epithelial cell line MCF10A, has proven to be an important *in vitro* model, used for studying the role of various signaling pathways, oncogenes and tumor suppressors during acinar morphogenesis, and elucidating mechanisms that disrupt glandular architecture and eventually lead to breast carcinoma development. The overexpression of oncogenes such as *ErbB2,* disrupts acinar morphogenesis and enhances cell proliferation, eventually leading to multi-acinar structures with filled lumen (Muthuswamy et al., 2001). Loss of cell polarity proteins such as PKCζ or Par3 disrupts apico-basal polarity of epithelial cells and not only results in over-proliferation of cells and generation of multi-acinar structures, but also promotes EMT and invasion (Jung et al., 2019; Whyte et al., 2010).

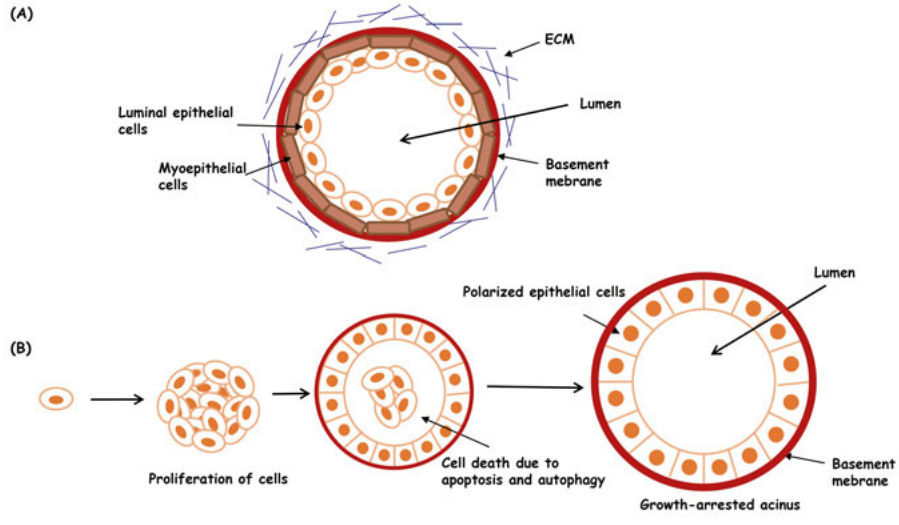

Figure 4. Schematic representation of (A) a breast acinus *in vivo* and (B) of the acinar morphogenesis mammary epithelial cells undergo *in vitro.*

4.3 BMP-regulated bone differentiation

A well-characterized function of BMPs is their ability to promote differentiation of mesenchymal progenitor cells to osteoblasts or chondrocytes, thus contributing to bone formation. BMPs can actually induce ectopic bone formation in muscle tissue *in vivo,* while *in vitro*, they inhibit the myogenic differentiation that the mouse myoblast cell line C2C12 undergoes in low-serum conditions, and promote the differentiation of these cells to osteoblasts (Katagiri et al., 1994).

5. Epigenetic balance

5.1 Chromatin structure and histone modifications

The eukaryotic genome is organized in a highly ordered structure called chromatin. The fundamental unit of chromatin is the nucleosome, which consists of 2 molecules of each of the core histone proteins H2A, H2B, H3 and H4, wrapped around by approximately 147 base pairs (bp) of DNA. The histone N-terminal tails protrude from the nucleosome, and are subjected to a variety of post-translational modifications that change chromatin dynamics and conformation, eventually affecting several cellular processes such as gene transcription, DNA replication and genome stability (Morgan and Shilatifard, 2015). Acetylation, methylation, phosphorylation, ubiquitination, and citrullination, are several examples of histone modifications that have physiological importance. Apart from directly influencing the overall chromatin structure, these modifications are also recognized by adaptor proteins that in turn recruit additional chromatin-modifying and -remodeling protein complexes (Tessarz and Kouzarides, 2014). Some histone marks are associated with a more loose or "open" chromatin structure that is accessible to transcription factors and the whole transcriptional machinery, whereas other histone modifications are enriched in transcriptionally silenced regions where chromatin has a more compact or "closed" conformation. Chromatin conformation is rather important for efficient gene regulation as it ensures that the proper gene expression programs are elicited during cell differentiation.

Among the aforementioned modifications, histone methylation is one of the most well-studied ones. Histone methylation occurs mainly on lysine and arginine residues, on histones H3 and H4. When it comes to lysines, they can be mono- di- or tri-methylated and these modifications can either positively or negatively contribute to the regulation of gene expression, depending on which lysine residue they occur. Among the most well-characterized histone methylation marks is methylation of lysine 4 on histone H3 (H3K4me), which is associated with actively transcribed genes and is found enriched in active promoters, while methylation on lysine 27 (H3K27me) is a characteristic mark of inactive promoters and repressed chromatin regions (Kouzarides, 2007). Lysine methylation is generated by histone lysine methyltransferases (KMTs), that catalyze the transfer of one, two, or three methyl groups from S-adenosyl-L-methionine (SAM) to the ε-amino group of a lysine residue on a histone, whereas methyl groups are removed by histone lysine demethylases (KDMs) (Black et al., 2012). KMTs as well as KDMs, exhibit high degree of specificity regarding the lysine residues they target, as well as the degree of methylation.

5.2 The H3K4 histone methyltransferases

Based on their specificity, KMTs are divided into different groups. One of them, the KMT2 family, also known as mixed lineage leukemia (MLL) family, consists of KMT2A, KMT2B, KMT2C, KMT2D (or MLL1-4), and KMT2F and KMT2G (also known as SET1A, SET1B). KMT2 methyltansferases catalyze mono- di- and tri-methylation on H3K4 (H3K4me, H3K4me^2 and H3K4me^3 respectively), which suggests that they are involved in the positive regulation of transcription (Kouzarides, 2007; Shilatifard, 2012). The propensity of these enzymes to mono- di- or tri-methylate H3K4 residues, as well as their largely variable pattern of genome-wide distribution, both reflect the functional diversity within the KMT2 family. KMT2 enzymes are part of large macromolecular protein complexes and the different subunit composition of each one of these complexes (also known as COMPASS and COMPASS-like complexes), is responsible to some extent for this functional specificity (Shilatifard, 2012). KMT2A and KMT2B contain a CXXC domain that allows them to bind to unmethylated CpG dinucleotides, a classical feature of a large subset of gene promoters (Allen et al., 2006; Saxonov et al., 2006). Similarly, one of the unique components of the KMT2F and KMT2G complexes, the protein CXXC1, mediates their binding on CpG enriched promoter sequences (Lee and Skalnik, 2005). These four complexes catalyze H3K4me^3 on promoter sequences while on the other hand, KMT2C and KMT2D are highly enriched at enhancers, where they catalyze H3K4me^1 (Figure 5A) (Rao and Dou, 2015). Interestingly, KMT2C promotes also long range chromatin interactions between promoters and enhancers by recruiting the cohesin complex (Yan et al., 2018).

5.3 The polycomb repressive complex 2

Polycomb repressive complex 2 (PRC2) is a macromolecular complex that mediates gene silencing by establishing trimethylation on H3K27 (H3K27me^3) mainly at promoters of developmental genes (Figure 5A) (Margueron and Reinberg, 2011). PRC2 actually controls also the other levels of methylation on H3K27, H3K27me^1 and H3K27me^2, which are normally deposited on gene bodies and intergenic regions (e.g. enhancers) respectively. The catalytic activity of PRC2 is mediated by the methyltransferase enhancer of zeste homologue 2 (EZH2). The other core components of the complex, suppressor of zeste 12 (SUZ12), embryonic ectoderm development (EED) and RBAP46/48 are essential for its integrity and catalytic activity (Piunti and Shilatifard, 2016). Apart from these essential subunits, there are additional proteins that have been identified as non-core components of the PRC2 complex: JARID2, AEBP2 and PCL1, 2 and 3, which contribute to PRC2 recruitment to DNA, and/or enhance the

enzymatic activity of the complex. It is worth mentioning here, that the EED subunit, has high affinity for H3K27me^3, the catalytic product of PRC2, thus creating a positive feedback loop that leads to the propagation of this histone mark and the maintenance of transcriptional repression (Holoch and Margueron, 2017).

In *D. melanogaster,* PRC2 is known to be recruited to specific DNA sequences known as polycomb response elements (PREs), but in mammals no consensus sequence has yet been identified. It has been demonstrated though, that PRC2 is associated with specific genomic regions, highly enriched in C and G, which often are CpG islands of transcriptionally inactive genes (Piunti and Shilatifard, 2016). Recent studies have revealed that during the differentiation of mouse embryonic stem cells (mESCs), PRC2 is required for the maintenance, but not for the initiation of the transcriptional silencing (Riising et al., 2014; Yuan et al., 2012). The fact that PRC2-mediated deposition of H3K27me^3 promotes the binding of the closely related PRC1 complex on the chromatin, had previously encouraged a model where PRC2 activity is required as a first step for the PRC1 complex to be recruited. However, according to a recent model that has been proposed, the recruitment of PRC2 is dependent on the enzymatic activity of PRC1, which monoubiquitinates H2AK119 in order to establish gene silencing and to facilitate PRC2 binding. Moreover, it was recently demonstrated that PRC1 binding is in many cases independent of H3K27me^3 deposition (Holoch and Margueron, 2017).

5.4 The role of KMT2 and PRC2 complexes in cancer

The opposing activities of PRC2 and KMT2 complexes establish a balanced regulation of gene expression that if perturbed, results in a deregulated epigenetic state, which has been linked to the pathogenesis of different human diseases including cancer. PRC2 components, as well as members of KMT2 protein complexes are among the most commonly mutated chromatin modifiers in various types of cancer. In the case of KMT2 proteins that are characterized as tumor suppressors, gene rearrangements that lead to KMT2A chimeric oncoproteins are commonly found in hematopoietic and lymphoid malignancies, whereas frameshift mutations that lead to truncated forms of KMT2C and KMT2D proteins that may have or not lost their methyltransferase activity, have been identified in lung and colon adenocarcinomas, and breast cancer, as well as in medulloblastoma and other primitive neuroectodermal tumors. The oncogenic activity of the chimeric KMT2A proteins is often dependent on the CXXC domain that remains intact and recruits them to unmethylated CpGs (Ayton et al., 2004). Moreover, it has been described that the oncogenic potential of the KMT2A chimeric proteins is reduced when the KMT2A complex assembly is

targeted (Cao et al., 2014). In the case of KMT2C and KMT2D, mutations in the plant homeodomain (PHD) and the SET domain, which has methyltransferase activity, are important for their tumor suppressive function (Rao and Dou, 2015). Recently it was demonstrated that mutations in the PHD domain disrupt the interaction of KMT2C with the histone deubiquitinating enzyme BAP1 that actually recruits KMT2C to enhancer chromatin, thus leading to an unbalanced epigenetic state due to increased $H3K27me^3$ on enhancers and subsequent silencing of genes (Wang et al., 2018).

PRC2 has a complex role in the context of cancer, as it can resume both oncogenic and tumor suppressive functions. Gain-of-function alterations leading to increased expression or enhanced activity of EZH2 have been linked to enhanced cell proliferation and increased invasion, initially assigned an oncogenic function to PRC2. However, there is also evidence that loss-of-function mutations in PRC2 subunits, as well as defects in PRC2 recruitment can function together with mutations targeting other genes in order to enhance tumor development. For example, loss of SUZ12 or EED is often detected together with *neurofibromin 1* (*NF1*) or *CDKN2A* mutations in peripheral sheath nerve tumors (Comet et al., 2016; Morgan and Shilatifard, 2015). Even within a specific type of cancer, the outcome of PRC2 gain- or loss-of-function alterations is highly-context dependent, as it depends on the mutation status of other genes (Comet et al., 2016).

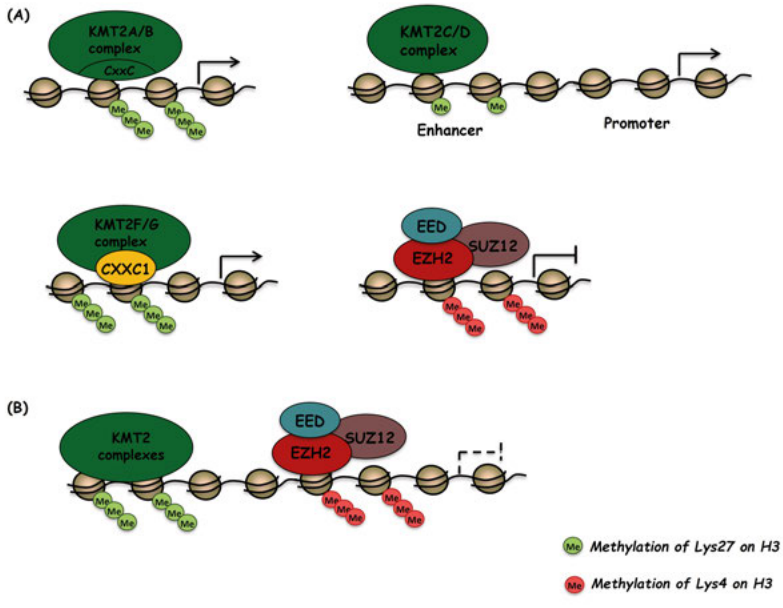

Figure 5. (A) Schematic representation of KMT2 and PRC2 complexes that catalyze H3K4 and H3K27 methylation respectively. (B) Co-existence of H3K4me^3 activating together with H3K27me^3 repressive histone marks, keeps promoters at a poised state, ready to be activated upon pro-differentiation signaling.

5.5 Bivalent chromatin domains

According to studies performed in ESCs, there are subsets of gene promoters that are marked by both the H3K4me^3 activating histone mark and the H3K27me^3 repressive histone mark. The areas co-occupied by these marks are referred to as "bivalent" domains, and are enriched in the promoters of developmental genes that are kept in a poised transcription state (displaying low or no expression), being ready to be activated or repressed when pro-differentiation signals are perceived (Figure 5B). Even though, many bivalent domains are resolved upon differentiation as expected, still, the existence of such domains in non-stem cell lines has been observed, supporting a hypothesis that bivalency may be a chromatin state existing in various cell types and is not restricted to pluripotent cells (Harikumar and Meshorer, 2015; Piunti and Shilatifard, 2016).

The role of bivalency in cancer has not yet been completely clarified, even though the presence of bivalent promoters in cancer cells has been reported. It has been suggested that bivalency predisposes genes to hypermethylation in cancer cells, and in many cases, genes found hypermethylated in different tumor types, are developmental regulators, that are physiologically under the control of bivalent histone marks in ESCs (Rodriguez et al., 2008). Other

studies have proposed that the recapitulation of bivalent chromatin modifications, allows cancer cells to have more plasticity and may be indicative of an un-, or more precisely, a de-differentiated state, closer to a stem-cell like phenotype (Blanco et al., 2020).

5.6 Epigenetic regulation of EMT

The dynamic and reversible nature of EMT is the result of changes in the epigenetic regulation as cells shift within a series of intermediate phenotypes between the epithelial and the mesenchymal state, in response to microenvironmental cues (Tam and Weinberg, 2013).

EMT-TFs interact with chromatin modifying enzymes in order to regulate gene expression. Snail mediates *CDH1* repression by interacting with the demethylase LSD1, which removes methyl groups from the $H3K4me^3$ marked histones, but also by interacting with SUV39H1 methyltransferase which catalyzes the $H3K9me^3$ repressive mark (Dong et al., 2013; Lin et al., 2010).

Increased expression of the EZH2 subunit of the PRC2 complex has been observed in carcinomas that carry an EMT gene expression signature and a redistribution of the repressive $H3K27me^3$ histone mark has been described for mouse mammary cells that undergo EMT (Tam and Weinberg, 2013; Tiwari et al., 2013). The gain of $H3K27me^3$ on promoters such as the *CDH1* promoter creates a plastic state that can be either reverted or can lead to more stable gene repression due to the accumulation of more stable repressive histone marks ($H3K9me^3$) or the establishment of DNA methylation.

The presence of both $H3K4me^3$ and $H3K27me^3$ modifications on the promoter of EMT-associated genes could suggest that this bivalent state of the chromatin allows the dynamic regulation of these genes and contributes to the plasticity of the EMT process. *ZEB1* promoter bears bivalent histone modifications in basal breast cancer $CD44^{low}$ cells, and remains at a poised state, ready to respond to extracellular stimuli such as TGFβ, and shift cells to a more tumorigenic state (Chaffer et al., 2013). However, it is worth mentioning that in order to answer to what extent the regulation of bivalently marked genes is actually responsible for cell plasticity, further investigation is required (Tam and Weinberg, 2013).

6. Present Investigation

TGFβ and BMP signaling pathways are important in embryonic development and adult tissue homeostasis, but they also have complex roles in the context of cancer. During embryogenesis, signaling by TGFβ family members promotes EMT, a developmental process often hijacked in different types of cancer, eventually leading to cancer cell invasion and metastasis. BMP signaling promotes cell differentiation during development and is involved in processes such as bone formation, angiogenesis and neural cell differentiation. The aim of this thesis is to study how TGFβ and BMP signaling regulate cell differentiation in different cancer models and try to elucidate new mechanisms that interfere with TGFβ/BMP signaling to eventually promote or inhibit cell differentiation.

Paper I: The protein kinase LKB1 negatively regulates BMP receptor signaling

LKB1 is a tumor suppressor kinase with well-established roles in cell polarity, proliferation and metabolism. An earlier study in our group, had revealed that LKB1 can modulate TGFβ and BMP signaling by negatively regulating the activity of the common mediator of the two pathways, Smad4 (Morén et al., 2011). In this study a new role for LKB1 in the regulation of BMP signaling was uncovered. LKB1 physically associates with the ALK2 receptor and promotes the ubiquitination and the degradation of the receptor by recruiting Smad7 to the complex. By performing loss-of-function and overexpression assays, we demonstrated that the LKB1-induced downregulation of the receptor led to decreased levels of Smad1/5/9 phosphorylation, reduced BMP-dependent gene expression, repressed BMP-induced osteoblast differentiation, and it also affected wing longitudinal vein morphogenesis in *D. melanogaster*. Immunohistochemical analysis in tissues of non-small cell lung cancer patients revealed that for specific tumor subsets, there is an inverse correlation, where high LKB1 expression was combined with low Smad1 phosphorylation levels, or low LKB1 expression was combined with high Smad1 phosphorylation levels, with the latter correlation being predominantly enriched in adenocarcinomas.

Whether LKB1 phosphorylates the ALK2 receptor in order to promote its ubiquitination is a question that has not yet been answered and is possibly worth investigating further.

Paper II: LKB1 inhibits TGFβ signaling during mammary epithelial morphogenesis

As previously mentioned, a mechanism of negative regulation of TGFβ signaling by LKB1 has already been described (Morén et al., 2011). In this study our aim was to investigate in more depth the contribution of the crosstalk between LKB1 and TGFβ signaling in the establishment and/or maintenance of cell polarity in epithelial cells and to this end, we used the immortalized mammary epithelial cells MCF10A, which can form acinar structures that consist of an outer layer of polarized epithelial cells and a central hollow lumen, when cultured on a reconstituted basement membrane. Crispr/Cas9-mediated loss-of-function mutations of *LKB1,* disrupted the ability of MCF10A cells to form acinar organoids and led to the formation of structures of an uneven morphology, having multiple side-growths and invasive protrusions. Moreover, these structures had lost the capacity to rotate, that normal acinar structures have during the first days of acinar morphogenesis. Loss of *LKB1* expression was also associated with increased TGFβ auto-induction, enhanced TGFβ signaling, as well as enhanced TGFβ-mediated induction of EMT transcription factors. Moreover, LKB1 depleted cells exhibited an EMT-like phenotype characterized by increased levels of Fibronectin and mislocalization of E-cadherin.

Treatment of LKB1-depleted acini with a TβRI inhibitor restored the defective acinar phenotype to a great extent, as we observed structures with an outer layer of polarized cells and a hollow lumen in the center. Inhibiting the activity of the EMT regulator tank-binding kinase 1 (TBK1) partially restored the defective phenotype, and also inhibited the TGFβ-mediated induction of *SNAI1* and *SNAI2*. Similar results were obtained when LKB1 depleted acini were treated with the BMPRI inhibitor DMH1 in terms of morphology, even though this time the formation of a hollow lumen was not clear.

Our results suggest that normal mammary acinar morphogenesis is dependent on a crosstalk between LKB1 and TGFβ signaling, where the hyperactivity of TGFβ signaling is limited by LKB1.

The enhanced TGFβ signaling can partly explain the strong phenotype of LKB1 KO acini, therefore it would be interesting to investigate which other molecules/pathways downstream of LKB1 are implicated in acinar morphogenesis. As LKB1 is a master kinase, we could perform a mass-spectrometry based analysis of the phosphoproteome in LKB1 depleted and parental cells, in order to address this open question.

To correlate our findings to cancer progression and different breast and lung cancer subtypes, we could also perform multiplex immunohistochemical analysis of breast and lung cancer tissue microarrays, where LKB1 expression could be correlated to active TGFβ signaling.

Paper III: Snail regulates BMP and TGF-β pathways to control the differentiation status of glioma-initiating cells

In a previous work, we had demonstrated that BMPs promote astrocytic differentiation in glioma-initiating cells or glioma stem cells (GSCs) and deplete their tumorigenic potential, by inducing the expression of *SNAI1* (Savary et al., 2013). Here, our aim was to elucidate the mechanisms by which Snail blocks the sphere-formation capacity of GSCs, while at the same time promotes their astrocytic fate.

We demonstrated that stable overexpression of Snail was associated with increased expression of astrocytic markers such as *glial acidic fibrillary protein* (*GFAP*) and *secreted protein acidic cysteine rich-like-1* (*SPARCL1*), and with decreased expression of genes related to stem-cell properties such as *LIF* and *SOX2,* and genes related to drug resistance. Snail overexpression resulted also in reduced *TGFB1* and *SERPINE* expression and reduced secreted levels of mature TGFβ1, while at the same time, Snail-overxpressing cells had increased secreted levels of BMP4. Moreover, we found that Snail interacts with the Smad signaling mediators and binds on the *TGFB1* promoter in order to repress *TGFB1* expression. Treatment with BMP7 and TβRI inhibitor mimicked partially the effects of Snail overexpression on the sphere formation capacity and the gene expression profile of GSCs. On the other hand, treatment with the natural BMP antagonist Noggin, abrogated the Snail-dependent astrocytic lineage commitment of GSCs. Exogenous TGFβ combined with BMP receptor inhibitor DMH1 treatment also counteracted Snail function by recovering the expression of stem cell markers and rescuing the sphere formation capacity of GSCs.

Overall, our findings suggest that Snail controls the differentiation status of GSCs by repressing TGFβ1 signaling on one hand and blocking their self-renewal potential, while on the other, promotes BMP signaling by generating a positive feedback loop, thus turning the fate switch towards the astrocytic lineage.

A question that could be addressed in the future is whether Snail, as a target of both BMP and TGFβ signaling pathways, regulates different subsets of genes and cooperates with different co-factors depending on the extracellular

signals cells receive. We could perform a ChIP sequencing analysis in order to see whether in response to different growth factors (TGFβ or BMP), Snail binds and regulates different sets of genes. Moreover, we could perform a mass spectrometry analysis to identify Snail-binding proteins, in order to better elucidate Snail function in GBM.

Paper IV: Epigenetic coupling of transcription factor CXXC5 regulates stemness-related genes in glioblastoma

Signaling by members of the TGFβ family controls fate decisions between self-renewal and differentiation in GSCs. TGFβ promotes the self-renewal potential of GSCs while BMP promotes their differentiation towards the astrocytic lineage. In this study, we were interested in identifying new TGFβ and/or BMP target genes in the context of glioblastoma. Our analysis revealed that TGFβ and BMP signaling both regulate the expression of *CXXC5*, a zinc finger-CxxC-domain containing transcription factor. To understand the role of CXXC5 in GBM, we performed multiplex immunohistochemistry on a tissue microarray of human GBM samples, where we demonstrated that CXXC5 expression was enriched in subpopulations of cells that were characterized by the expression of the stemness markers SOX2 and NESTIN.

Silencing of endogenous CXXC5 reduced the sphere formation capacity of GSCs, and reduced the TGFβ-mediated induction of the stemness related genes *LIF, NESTIN*. A transcriptomic analysis performed in GSCs revealed that silencing CXXC5 expression alters the expression profile of subsets of TGFβ and BMP target genes acting either as an activator or as a repressor. To gain mechanistic insight, we also performed a mass-spectrometry analysis in order to identify CXXC5-interacting partners. The list of CXXC5-interacting proteins included the chromatin remodeler KMT2C methyltransferase, and histone chaperone complexes, suggesting that CXXC5 is involved in the epigenetic regulation of target genes. By performing chromatin immunoprecipitation assays, we demonstrated that CXXC5 and its interacting partner KMT2C regulate *LIF* and *NESTIN* by modifying the histone methylation pattern on CpG motifs located close to the transcription start site (TSS) of these genes. More specifically, silencing CXXC5 increased the levels of the H3K27me^3 repressive histone mark, while levels of the active H3K4me^3 were decreased. CXXC5 depletion led to increased binding of the PRC2 central component SUZ12 on these specific CpG motifs, while we also observed that silencing SUZ2 expression rescued the effect of CXXC5 depletion on the TGFβ-mediated induction of *LIF* and *NESTIN*.

Our results, favor a model where the recruitment of CXXC5 coupled to the interaction with histone modifying enzymes, fine-tunes the expression of genes related to self-renewal by modifying the histone methylation pattern around CpG motifs close to TSS.

The fact that KMT2C is normally localized at enhancers, raises the question whether CXXC5 can regulate other histone modifications (e.g. H3K4me^{1} or H3K27ac) at the enhancers of target genes. In this context, a ChIP-seq analysis for CXXC5 in GBM cells in response to TGFβ and BMP, followed by a comparative analysis, would generate a correlation between CXXC5 binding and histone marks on different gene regulatory elements. Furthermore, according to our mass-spectrometry analysis, CXXC5 associates also with three core components of the cohesin complex, which based on recent evidence, is recruited by KMT2C in order to facilitate long range chromatin interactions between promoters and enhancer elements (Yan et al., 2018). Therefore, the involvement of CXXC5 in long-range chromatin interactions and whether it regulates gene expression by affecting the 3D chromatin architecture is worth investigating in the future.

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