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TGFβ signalling in context

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Abstract

The basic elements of the transforming growth factor-β (TGFβ) pathway were revealed more than a decade ago. Since then, the concept of how the TGFβ signal travels from the membrane to the nucleus has been enriched with additional findings, and its multifunctional nature and medical relevance have relentlessly come to light. However, an old mystery has endured: how does the context determine the cellular response to TGFβ? Solving this question is key to understanding TGFβ biology and its many malfunctions. Recent progress is pointing at answers.

Transforming growth factor-β (TGFβ) signalling provides animal cells with a versatile means of driving developmental programmes and controlling cell behaviour, a role that is evident in the many effects of TGFβ-related cytokines on cell proliferation, differentiation, morphogenesis, tissue homeostasis and regeneration, and the severe diseases that result from their malfunctions.

Pioneers in this field in the 1980s welcomed the multifunctional nature of TGFβ with mixed feelings. Up to that point, endocrinology was forged on the principle that, by and large, a hormone has one main role and this role only. With TGFβ it was clear from the beginning that this paradigm did not quite fit. The effects of TGFβ were different, even opposite, depending on the cell type and the conditions. The cellular context more than the cytokine dictated the nature of the response. To those intent on elucidating the TGFβ pathway, this contextual functionality was sobering news, as it raised the specter of an impossibly complicated signal transduction process. Yet, the work uncovered a pathway that is relatively simple (in hindsight) and with the power to mediate most effects of any TGFβ family member in any cell type. The TGFβ receptor system, its activation mechanism and SMAD proteins, which function both as substrates for TGFβ receptors and signal transducers, came to light in quick succession. Disease-causing mutations in these components stressed the medical relevance of the new findings. Regulators and complementary pathways were also found. TGFβ target genes that trigger differentiation in stem cells, cell cycle arrest in epithelial cells or homeostatic constraint in immune and vascular cells were identified. Crystal structures of the pathway components were emerging

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in the blink of an eye as the century was drawing to a close[1]. The TGFβ pathway had been solved, to a first approximation at least.

Writing on this subject at the time, the phrase “How cells read TGFβ signals” was picked as a title for two reasons[2]. It was an affirmation that a molecular framework for the exploration of TGFβ biology was firmly in hand. Fleshing out the newly defined pathway became the next task, and the field keenly obliged by identifying additional components, regulators, ancillary pathways and biological effects of the TGFβ family. However, that phrase also implied a challenging question: how does the cellular context determine the response to TGFβ? It was not clear how TGFβ can inhibit cell proliferation but also promote cell growth, enhance stem cell pluripotency but also differentiation, regulate muscle genes in myoblasts and neural genes in neuroblasts, or suppress pre-malignant cells but encourage metastatic ones. These paradoxes suggested that cells read TGFβ signals in ways that could not be explained. Non-canonical TGFβ pathways and malignant switches were explored as alternatives but yielded no answers either. Fifty thousand TGFβ papers later, the old enigma carries on.

Interest in solving this puzzle is growing, and it is driven by the importance of TGFβ signalling in medically relevant processes of immunity, inflammation, cancer and fibrosis, as well as bone, muscle, adipose, vascular and haematopoietic homeostasis. At last, recent progress is pointing to a resolution. To cover this progress, the present article provides an overview of the contextual determinants of TGFβ action followed by an update on the signalling, transcriptional and genomic elements of the pathway. Building on this, the final section covers, in broad strokes, the mode of action of TGFβ in various contexts, including embryonic stem (ES) cells, lineage-committed progenitors, cells undergoing epithelial–mesenchymal transition (EMT), induced pluripotent stem (iPS) cells, differentiated cells and cells at various stages of malignancy.

Contextual determinants of TGFβ action

The TGFβ pathway seems to have emerged with the first animal species[3], ostensibly to control multicellular life in metazoans. It does so largely by regulating gene expression. The number of target genes for a given TGFβ family member can range from just a few in pluripotent ES cells[4,5] to hundreds in differentiated cells[4–8]. The effects of TGFβ on transcription can be positive or negative depending on the targeted gene and the cell ular context. For example, TGFβ represses inhibitor of differentiation 1 (*ID1*) in mammary epithelial cells[9] but induces this gene in metastatic breast cancer cells[10]. Few TGFβ target genes are common to all cell types, such exceptions being the negative feedback regulators *SMAD7* and *SKIL* (SKI-like oncogene; also known as SNON)[11,12]. The SMAD signalling pathway is a central `conduit' for all these gene responses and, as such, it recapitulates the context-dependent nature of TGFβ action.

Three types of contextual determinants shape the TGFβ transcriptional response in a cell (FIG. 1). Within a given epigenetic and transcriptional context the response of a cell is determined by the extracellular and intracellular composition of the TGFβ signal transduction system. The abundance and activity of different TGFβ ligands, receptors and

regulators determine the nature and intensity of the TGFβ signal in the nucleus, and coexisting cues further shape the response by regulating SMAD function or activating non-canonical pathways[13–15]. Inputs that affect the intensity of the TGFβ signal may qualitatively influence the cellular response. A dramatic example is the different cell fates that emerge from finely tuned gradients of bone morphogenetic protein (BMP) acting alongside WNT and Hedgehog signals during development[16–18].

A second set of determinants is the factors that co operate with SMAD proteins to regulate transcription (FIG. 1). The role of such factors was originally highlighted with the identification of forkhead box H1 (FOXH1; also known as FAST1) as a factor that allows SMAD proteins to recognize `activin response elements' (AREs) in promoters of genes involved in mesoderm differentiation[19]. This principle was extended to the TGFβ subfamily of BMPs with the identification of zinc-finger protein 423 (ZFP423; also known as OAZ), which is a SMAD cofactor for the activation of ventral mesoderm homeobox genes[20]. Lineage-specific transcription factors direct TGFβ- and BMP-activated SMAD proteins to specific loci genome-wide in myoblasts, pro-B cells, myeloid precursors and erythroid precursors[4,8]. In differentiated cells, diverse transcription factors guide SMAD proteins to distinct subsets of target genes[21]. The resulting SMAD complexes then recruit chromatin readers, modifiers and remodellers to regulate transcription. The availability of all these SMAD partners determines what genes will be targeted and whether they will be activated or repressed.

Last, but not least, the epigenetic landscape of the cell, including DNA methylation marks, histone modifications, nucleosome positioning, non-coding RNAs and other components, shapes the chromatin and dictates what genes are open for expression and thus susceptible to regulation (FIG. 1). For example, under conditions that favour self-renewal, ES cells keep pluripotency-enforcing genes in an open conformation that permits transcriptional activation downstream of TGFβ signals, whereas genes involved in differentiation remain repressed and refractory to these inputs. When conditions are permissive for ES cell differentiation, specific chromatin marks open master differentiation genes to activation driven by Nodal, which is a TGFβ family member that regulates stem cell pluripotency and differentiation[4,5,8,22,23]. EMT and iPS cell generation also incur epigenetic changes that influence TGFβ-mediated gene regulation[22,24,25].

Collectively, these three classes of contextual determinants channel, skew or switch the pleiotropic capacity of TGFβ signalling, thereby giving rise to specific response programmes that are archetypical of particular cell types and conditions.

Activating TGFβ signalling

The TGFβ family includes over 30 members in humans, and orthologues are found in the most primitive metazoan genome sequenced, *Trichoplax adhaerens*. In this organism, the TGFβ pathway is already equipped with four SMAD proteins and four receptors[3]. Sequence similarities define two ligand subfamilies: the TGFβ–activin–Nodal subfamily and the BMP subfamily[1]. The ligands are disulphide-linked dimers, and dimerization is essential for

receptor activation (FIG. 2). Most family members act as paracrine factors on cells near the source.

Receptor combinations

Ligand binding assembles a complex consisting of two type I (that is, signal-propagating) receptor components and two type II (that is, activator) components (FIG. 2a). Both components are Ser/Thr protein kinases. A short cytoplasmic segment is followed by the kinase domain and, only exceptionally, a carboxy-terminal extension (for example, in the type II BMP receptor BMPR2). In the complex, type II receptors phosphorylate the type I components, which then propagate the signal[26]. Phosphorylation switches a region in the type I receptor from a site that binds 12 kDa FK506-binding protein (FKBP12), which silences kinase activity, into a site that binds substrate SMAD proteins for their phosphorylation[27]. These are the sole cell surface receptor Ser/Thr kinases known in humans. Why TGFβ uses receptor Ser/Thr kinases and not Tyr kinases as all other kinase-activating cytokines do remains a mystery.

Seven type I receptors and five type II receptors exist in humans. Interactions with contiguous or non-contiguous molecular surfaces determine the specificity of ligand–receptor pairings[28,29]. Detailed ligand–receptor combinations are reviewed elsewhere[1,30]. Briefly, TGFβ binds exclusively to the type I receptor TGFBR1 (also known as ALK5 and TβRI) and the type II receptor TGFBR2. Activin, Nodal and BMPs share the type II receptors activin receptor type 2A (ACVR2A) and ACVR2B. Activin and Nodal, but not BMPs, share the type I receptors ACVR1 (also known as ALK2), ACVR1B (also known as ALK4) and ACVR1C (also known as ALK7), whereas BMPR1A (also known as ALK3) and BMPR1B (also known as ALK6) primarily act as type I receptors for BMPs and anti-Muellerian hormone (AMH). BMPR2 is another type II receptor for BMPs, and AMHR2 is the type II receptor for AMH. Activin receptor-like 1 (ACVRL1; also known as ALK1) is a BMP9 (also known as GDF2) and BMP10 type I receptor, but it can be collaterally engaged by high TGFβ concentrations[31].

Extracellular regulators

Seven variables outside the target cell determine the extent of stimulation by a TGFβ cytokine (FIG. 2b). First is the level of ligand expression by the source, which is highly regulated by many contextual elements. A good example is *Drosophila melanogaster* BMP Decapentaplegic[16,17]. Second, the ligand subtype (for example, TGFβ1 and TGFβ2) also has a role, as subtype s differ in receptor affinity[32].

Next, various ligand-trapping proteins control the formation of ligand gradients in embryogenesis and depots in adult tissues[1,33,34]. Some traps, for example the BMP trap Noggin, occlude crucial receptor contacting residues in the ligand[35]. Fusion of phalangeal joints in individuals with *NOG* mutations[36] and the involvement of the BMP inhibitor gremlin 1 (GREM1) in basal cell carcinoma[37] highlight the importance of the traps. Fourth, the mediators of ligand release from these traps are relevant, and their action can be nuanced. Latent TGFβ complex activation involves conformational motions driven by contacts with cell surface integrins[38]. Inadequate anchoring of LTBP1 (latent TGFβ-binding

protein 1) owing to mutations in the gene encoding fibrillin 1 in individuals with Marfan syndrome is thought to cause faulty TGFβ release in the aortic wall, which can cause aneurysms[39].

A fifth variable at play is antagonistic ligands. Lefty (also known as left–right determination factor) inhibits Nodal binding to receptors and is essential for the establishment of left–right asymmetry in embryos[40,41]. Inhibin prevents activin binding to its receptors[42,43]. Furthermore, a sixth variable is the presentation of ligands to the signalling receptors by accessory receptors. For example, β-glycan presents TGFβ to its receptors[44] and inhibin to activin receptors[42,43]. Cripto (also known as TDGF1) is essential for Nodal binding to activin receptors[41,45]. Mutations in the accessory receptor endoglin, like mutations in its client receptor ACVRL1, cause haemorrhagic telangectasia[46,47]. Last, the combination of expressed signalling receptors is another determinant of how cells receive TGFβ signals. These seven variables tightly control the initiation of TGFβ signalling.

The SMAD signalling cycle

TGFβ family members regulate gene expression by receptor-mediated activation of SMAD transcription factors (FIG. 2a). Activated SMAD proteins regulate the transcriptional output of active genes and can also open repressive chromatin. Additionally, SMAD proteins serve as hubs for the integration of regulatory inputs and context-dependent modulation of TGFβ signalling.

SMAD activation by TGFβ receptors

SMAD proteins consist of two globular domains (termed MH1 and MH2 domains) coupled by an unstructured linker[1] (FIG. 3a). The amino-terminal MH1 domain contains a hairpin structure with DNA-binding ability. The MH2 domain has a series of hydrophobic surface patches for versatile interactions with cytoplasmic adaptor proteins, activated TGFβ receptors, various partner DNA-binding cofactors, co-activators and co-repressors. BMP type I receptors phosphorylate SMAD1, SMAD5 and SMAD8, and BMP type I receptors for TGFβ, activin and Nodal mainly phosphorylate SMAD2 and SMAD3. Receptor-mediated phosphorylation of this set of SMAD proteins (collectively known as receptor-regulated SMAD proteins (R-SMAD proteins)) targets two C-terminal Ser residues, creating an acidic knob that binds to homologous MH2 domains or to the MH2 domain of SMAD4. SMAD4 itself is not a receptor substrate but functions as a shared partner of all R-SMAD proteins (FIG. 3b). Trimers with two R-SMAD molecules and one SMAD4 are thought to be the principal functional units[1].

In the basal state, SMAD proteins constantly shuttle between the cytoplasm and the nucleus via contact with nucleoporins for transit through the nuclear pore[48]. However, in the receptor-induced oligomeric state, SMAD proteins require nuclear import and export factors[49–51]. In the nucleus, R-SMAD proteins in activated SMAD4–R-SMAD complexes bind other DNA-binding transcription factors as partners for target gene recognition and transcriptional regulation[4,7,8,19,20] (FIG. 3b).

The DNA-binding hairpins in SMAD1, SMAD2, SMAD3 and SMAD5 and are identical in amino acid sequence and all recognize the DNA motif CAGAC (which is the SMAD-binding element (SBE))[1]. However, the positioning of the hairpin in SMAD1 (REF. 52) differs from that in SMAD3 (REF. 53), and this perhaps explains why SMAD1 also bind to GC-rich sequences[54]. These differences are manifest in the genome-wide binding patterns: TGFβ and Nodal-activated SMAD2 and SMAD3 predominantly bind canonical SBEs[55,56], whereas BMP-activated SMAD1 and SMAD5 bind GC-rich elements in addition to canonical SBEs[57,58]. Thus, recognition of specific DNA sequences by different SMAD complexes is dictated by preferences for different SBEs and different DNA-binding partners.

The SMAD action turnover switch

SMAD molecules that are engaged in transcription become rapidly phosphorylated in the linker region by cyclin C–cyclin-dependent kinase 8 (CDK8) and cyclin T–CDK9 (REFS 59,60) (FIG. 3a,b). The phosphorylated motifs recruit factors like YAP (Yes-associated protein) for transcriptional action[59] but also prime the linker for sub-sequent phosphorylation by glycogen synthase kinase 3 (GSK3)[61,62]. GSK3 creates binding sites for the E3 ubiquitin protein ligases SMURF1 (SMAD-specific E3 ubiquitin protein ligase 1) or NEDD4L (neural precursor cell expressed developmentally downregulated protein 4-like), which target SMAD proteins for polyubiquitylation and proteasome-mediated degradation[59,60]. Binding of YAP, SMURF1 and NEDD4L involves two WW domains on these proteins; one WW domain contacts the SMAD phosphorylated linker motif, and the other WW domain interacts with a vicinal Pro/Tyr motif. The order of events, which is SMAD action first and degradation later, is controlled by a GSK3-driven switch in the SMAD motifs that bind WW domains[63]. This SMAD phospho-Ser code and the set of WW code readers provide an efficient solution to the problem of coupling TGFβ signal delivery to turnover of SMAD messenger molecules.

Links to the RNA polymerase II cycle

CDK8 and CDK9 are components of the transcriptional mediator complex and elongation complex, respectively. As such, CDK8 and CDK9 phosphorylate Ser/Pro motifs in the C-terminal domain (CTD) of RNA polymerase II (Pol II) to recruit proteins for DNA transcription, transcript capping and splicing[64]. SMAD linker phosphorylation is therefore coordinated by CDKs that also orchestrate Pol II action. Moreover, the SMAD linker is dephosphorylated by small CTD phosphatases (SCP1, SCP2 and SCP3) that prolong the participation of activated SMAD proteins in transcription before SMURF1 and NEDD4L target them for destruction[65,66] (FIG. 3b). SCPs are structurally related to FCP1 (transcription factor IIF-associated CTD phosphatase 1; also known as CTDP1), which dephosphorylates the Pol II CTD for repeated cycles of transcription[67]. These provocative parallels suggest that SMAD and Pol II transcriptional cycles are coordinated by a common team of linker and CTD kinases and phosphatases (FIG. 3c).

Ending SMAD action

Additional factors have roles in limiting the pool of activated SMAD protein or turning over SMAD molecules that are engaged in transcription (FIG. 3b). Phosphatases remove SMAD

C-terminal phosphorylation; however, which of these phosphatases acts in the nucleus needs clarification[65,68]. The co-activators p300 and CBP (cyclic AMP response element-binding protein) acetylate SMAD proteins on Lys residues that reside in the MH1 domain to enhance SMAD binding to DNA[69], whereas poly(ADP)-ribosylation of this domain inhibits binding to DNA[70]. In addition, SMAD proteins are sumoylated, however, the functional implications of this post-translational modification are still unresolved[30]. Negative feedback is provided by SMAD-induced expression of SMAD7, which recruits SMURF to TGFβ and BMP receptors for polyubiquitylation and degradative endocytosis[11,71]. Receptor ubiquitylation is countered by the deubiquitinases USP4 (ubiquitin-specific processing protease 4), USP11 and USP15 (REFS 72–74). SMAD7 simultaneously recruits USP15 and SMURF2 to TGFβ receptor complexes, and these the two enzymes compete at this site[73]. USP15 can also deubiquitylate R-SMAD proteins[75]. The decoy receptor BAMBI (BMP and activin membrane-bound inhibitor)[76] and SKIL also provide negative feedback, which disrupts SMAD transcriptional complexes[12,77]. Arkadia, a RING-domain E3 ubiquitin ligase, counters SMAD7 and SKIL by ubiquitylating these inhibitors[78,79]. SMAD phosphorylations at other sites provide added control[30].

SMAD proteins as a hub for regulation and integration

The SMAD linker region is a hotspot for the integration of regulatory inputs (FIG. 3). The CDK target sites in SMAD linker regions are phosphorylated by mitogen-activated protein kinases (MAPKs) in response to growth factors (for example, fibroblast growth factor and epithelial growth factor) and stress signals, as well as by CDK4 during cell cycle progression[62,80,81]. GSK3 phosphorylation of CDK-primed or MAPK-primed SMAD linker is a key in this regard. By inhibiting GSK3, WNT augments the useful life of activated SMAD1, thereby providing an entry point for the cooperation between BMP and WNT pathways[61].

WNT also cooperates with BMP and TGFβ through co-occupancy of SMAD target enhancers by WNT-activated LEF1 (lymphoid enhancer-binding factor 1; also known as TCF1α) and TCF7L2 transcription factors[82–84]. SMAD1 and TCF7L2 co-occupy the genome with lineage-identity transcription factors to implement differentiation during regenerative haematopoiesis[8]. Integration with the AKT pathway is provided by FOXO factors as SMAD partners[85] and targets[86]. Pathway integration is also achieved by SMAD target genes, in particular by their protein products that interact with SMAD proteins to regulate other genes. For example, in epithelial cells TGFβ-activated SMAD proteins stimulate the expression of activating transcription factor 3 (ATF3) and SNAIL (also known as SNAI1) and then cooperate with these proteins to repress, respectively, *ID1* and *CDH1* (which encodes epithelial cadherin (E-cadherin))[9,87]. Many other roles of SMAD proteins as a hub for signal regulation and integration are known (reviewed in REFS 14,15,18,88).

SMAD proteins and chromatin

The transcriptional action of signal-activated SMAD proteins involves close interactions with chromatin. Recent insights have shed light on how SMAD proteins remodel chromatin or gain access to loci that are secluded by repressive histone marks.

SMAD-recruited chromatin and DNA modifiers

SMAD proteins recruit the histone acetyl transferases (HATs) p300 and CBP to stimulate transcription[7], leading to acetylation of Lys9, Lys14, Lys18 and Lys23 on histone H3 (REFS 5,89,90). SMAD inhibition of gene expression depends on binding partners that in turn recruit histone deacetylases (HDACs)[7]. Among these partners, TGIF1 (5′-TG-3′-interacting factor 1) and TGIF2 recruit the co-repressor CTBP (C-terminal-binding protein), which in turn binds HDAC1 to limit TGFβ–Nodal signalling[7]. *TGIF1* mutations in humans are associated with holoprosencephaly, which is a devastating defect of craniofacial development. In mice, knockout of *Tgif1* causes a persistent increase in Nodal and TGFβ responses[91] and the occurrence of holoprosencephaly[92].

Recently, studies on TGFβ signalling provided a remarkable example for signal-directed, locus-specific DNA demethylation during gene activation[93]. TGFβ induces expression of *CDKN2B*, which encodes the CDK4 inhibitor $p15^{Ink4b}$. This results in an antiproliferative effect that is mediated by promoter binding of a SMAD4–SMAD2/3–FOXO complex and dissociation of a MYC–MIZ1 (MYC-interacting zinc-finger protein 1; also known as ZBT17) complex[94,95]. In the absence of TGFβ, a complex consisting of zinc-finger protein 217 (ZFN217), co-repressor of RE1-silencing transcription factor (CoREST; also known as RCOR1) and the DNA methyltransferase 3A (DNMT3A) binds to the promoter and methylates a CpG island to mediate repression[93]. Following TGFβ stimulation, SMAD4–SMAD2/3–FOXO binds to the promoter and recruits a DNA excision repair complex that demethylates the promoter, which leads to *CDNK2B* expression. When TGFβ stimulation ends, ZFN217–CoREST–DNMT3A returns the promoter to a methylated, inactive state[93].

Gene activation or repression by TGFβ additionally require, in most cases, a SWI/SNF nucleosome repositioning complex[96]. SWI/SNF complexes mediate nucleosome sliding in an ATP-dependent manner to allow access for the transcriptional machinery to DNA[97]. BRG1 (also known as SMARCA4) is one[98] of two mutually exclusive ATPase subunits that nucleate SWI/SNF complexes. BRG1 binds directly to SMAD2 and SMAD3 (REFS 90,99) and recruits BAF250B (also known as ARID1B), BAF170 and BAF155 to the SMAD complex[96].

A SMAD-associated histone reader for silent chromatin

In ES cells and progenitor cells, signal-activated SMAD2 and SMAD3 bind to the histone-binding protein TRIM33 (tripartite motif containing 33; also known as TIF1γ)[5,99] (FIG. 2b). TRIM proteins contain a RING-finger and various protein interactions domains. TRIM33 additionally contains a plant homeodomain (PHD) and a bromodomain, which are two histone-binding domains essential for its role in TGFβ signal transduction[5]. TRIM33–SMAD2/3 and trimeric SMAD4–SMAD2/3 complexes form with similar abundance and kinetics in response to TGFβ or Nodal stimulation[5,99]. Although TRIM33 was implicated to have a role in inhibitory SMAD4 mono-ubiquitylation[89,100], TRIM33-deficient mouse embryos lack a mesoderm[101], and TRIM33 is essential for Nodal activation of mesendoderm genes in ES cells[5]. TRIM33 mediates erythroid differentiation in human haematopoietic progenitors in response to TGFβ[99]. *Trim33* knockout in the mouse pancreas

pheno copies the tumorigenic effect of *Smad4* knockout, arguing that TRIM33 and SMAD4 converge on tumour suppression[102,103].

The role of TRIM33 in TGFβ signalling was recently elucidated by genetic ablation experiments in ES cells coupled with X-ray crystal structure analysis[5] (FIG. 4a). The TRIM33 PHD–bromodomain cassette binds to N-terminal tails of histone H3 that contain unmodified Lys4 residues, trimethylated Lys9 (H3K9me3) and acetylated Lys18 (H3K18ac). Notably, H3K9me3 is a chromatin mark that represses yet `poises' master differentiation genes for activation[23]. Driven by Nodal, TRIM33–SMAD2/3 recognizes these histone marks and promotes a transition from poise to open chromatin, thereby allowing access for SMAD4–SMAD2/3-dependent transcriptional activation (see below)[5]. However, other target genes, such as *SMAD7* and *SKIL* in ES cells and most of TGFβ target genes in skin and breast epithelial cells[5,99] do not require TRIM33 for their activation. These genes must already be in an active, open chromatin state for SMAD4–SMAD2/3 to bind.

Non-canonical signalling

Although TGFβ signals mainly via the the SMAD pathway, TGFβ can also activate other pathways that are collectively referred to as `non-canonical' TGFβ signalling and complement SMAD action (FIG. 2c,d).

Signalling by type II receptors

Various forms of signal transduction emanate directly from type II receptors. In cells undergoing EMT, ligand-bound TGFBR2 directly phosphorylates the cell polarity regulator partitioning defective 6 (PAR6) (FIG. 2c). Phosphorylated PAR6 recruits SMURF1 to target RHOA GTPase at tight junctions, thereby causing dissolution of the junctions and polarized migration[104,105]. In addition, in neocortical neurons in the developing brain, TGFβ turns naive neurites into axons through TGFBR2-mediated phosphorylation of PAR6 (REF. 106).

BMP type II receptor BMPR2 has a long C-terminal extension that is not required for SMAD signalling, but mutations that truncate this domain cause primary pulmonary hypertension[107]. The C-terminal domain of BMPR2 binds to LIM kinase 1 (LIMK1), which is an inhibitor of the actin-depolymerizing factor cofilin (FIG. 2c). BMP binding prompts LIMK1 to inhibit cofilin, thereby stabilizing actin filaments[108]. BMPR2-bound LIMK1 at the tips of neurites synergizes with the RHO GTPase CDC42 to induce the den-dritic arbor in cortical neurons in response to BMP[109]. Moreover, the BMPR2–LIMK2–cofilin pathway mediates loss of epithelial identity during EMT of the neural crest in the chick embryo[110].

Links to MAPK and PI3K outputs

Besides the negative impact of MAPKs on TGFβ and BMP signalling through phosphorylation of SMAD linker sites (see above), reports over the past two decades have described effects of TGFβ and BMP on the activity of various MAPKs and also on the phosphoinositide 3-kinase (PI3K) pathway. These effects can be either immediate and transient or delayed and secondary, depending on the cell type and the culture conditions[14,15,30]. MAPK and PI3K activation has been proposed to complement and

converge with SMAD signalling[14,15,30,111], although these pathways can also antagonize SMAD signalling in other contexts.

The biochemical links between TGFβ receptors and the MAPK and PI3K pathways are complex and involve TRAF6 (tumour necrosis factor receptor-associated fac-tor 6), mTORC (mammalian target of rapamycin) and other mediators[30,31,112] (FIG. 2d). Due to a lack of structural information it is not known whether activation of the MAPK and PI3K pathways is directly coupled to TGFβ receptors, whether it is the result of collateral activation of other receptors or whether it is a consequence of network-wide signalling crosstalk. Regardless, the MAPK and PI3K pathways on their own are major signalling routes for receptor tyrosine kinases, metabolic inputs and environmental stresses, and in cancer these pathways are often activated by key oncoproteins. These considerations raise questions about what an extra TGFβ input into PI3K or MAPKs can accomplish in this context. It has been suggested that pro-tumorigenic effects of TGFβ such as induction of EMT or enhancement of metastasis involve a pathologic switch of TGFβ signalling from SMAD-mediated tumour suppression to non-canonical malignant pathways. However, canonical SMAD signalling in cancer cells does drive EMT[24,113], tumour-initiating cell stemness[114–116] and pro-metastatic gene expression[10,117].

SMAD proteins as Drosha components in microRNA biogenesis

SMAD proteins have other functions besides their role in transcriptional regulation. A remarkable example is their involvement in micro-RNA (miRNA) biogenesis[118,119]. In human vascular smooth muscle cells, activated R-SMAD proteins stimulate the maturation of a specific set of miRNAs in a SMAD4-independent manner (FIG. 2a). TGFβ-activated SMAD3 or BMP-activated SMAD1 are recruited to the Drosha miRNA processing complex by the RNA helicase DDX5 (DEAD box protein 5), which is a component of the Drosha complex. In the case of miR-21, the R-SMAD proteins promote the processing of primary transcripts (pri-miR-21) into precursor miR-21 (pre-miR-21). The resulting increase in miR-21 levels downregulates *PDCD4* (programmed cell death 4) and induce a contractile phenotype in vascular smooth muscle cells. Most R-SMAD-regulated miRNAs contain a consensus SBE within the stem region of the primary transcript, and this element is required for the incorporation and regulatory effect of R-SMAD proteins in the Drosha complex[118].

TGFβ action in context

TGFβ and BMP regulate pluripotency and differentiation in ES cells and lineage-committed progenitors, reprogramming in EMT and iPS cells, homeostasis in differentiated cells and altered versions of these processes in cancer. The signalling engine in these various contexts is essentially the same. The context more than the proteins involved in the signalling pathway is what shapes the response.

Contexts for ES cell self-renewal and differentiation

The core transcriptional regulators OCT4 (also known as POU5F1 and OCT3), SOX2 and NANOG form an interactive, self-sustaining protein network that induces pluripotency in ES cells[22,23]. This triad directs chromatin-modifying complexes to establish repressive

marks on differentiation genes (FIG. 4b). Polycomb repressive complex 2 (PRC2) promotes accumulation of H3K27me3, which promotes the recruitment of PRC1, and SETDB1 (SET-domain binding 1; also known as KMT1E) catalyses the formation of repressive H3K9me3 marks[23]. These repressive marks may coexist with activating marks that poise chromatin for abrupt transcriptional activation by appropriate signals[23].

Recent work on the genome-wide integration of signalling pathways with the OCT4 network provided fresh insights into the function of SMAD proteins in the context of this network[4,120]. BMP signalling stimulates ES cell self-renewal[121] by directing SMAD1 to co-occupy the genome with leukaemia inhibitory factor (LIF)-activated signal transducer and activator of transcription 3 (STAT3), OCT4, SOX2 and NANOG at sites that contain the H3K4me3 mark of active transcription. The targeted genes include *Oct4*, *Sox2*, *Nanog* and *Id3* (REF. 120), implementing a feed-forward circuit. The OCT4 complex also directs Nodal-activated SMAD3 to neighbouring sites[4], although this results in the activation of only a few genes, including *Nanog* and the Nodal negative feedback regulators, *Lefty1*, *Lefty2* and *Smad7* (REF. 4).

How does this scenario change when conditions are permissive for ES cell differentiation? In the absence of the pluripotency enforcing factor LIF (FIG. 5), as yet unknown changes enable ES cells to respond to autocrine signals and differentiate into mesendodermal cells of the primitive streak and ectodermal cells. Nodal drives mesendodermal differentiation by inducing the expression of the homeobox transcription factors goosecoid homeobox (*GSC*) and mix paired-like homeobox (*MIXL1*)[5]. Certain sites in the regulatory regions of *GSC* and *MIXL1* have H3K9me3 and acetylated Lys18 of histone H3 (H3K18ac)[5] (FIGS 4b,5a). The chromatin compacting protein HP1γ (heterochromatin protein 1γ) bound to H3K9me3 prevents binding of Nodal-activated SMAD4–SMAD2/3 to AREs. The activation of *MIXL1* and *GSC* in response to Nodal and ensuing differentiation require the previous action of a Nodal-activated TRIM33–SMAD2/3 complex. TRIM33 recognizes the dual histone mark H3K9me3–H3K18ac and binds with high affinity, thereby displacing bound HP1γ and opening the downstream AREs to allow access by SMAD4–SMAD2/3 complexes[5]. FOXH1 acts as a primitive mesendoderm identity factor that recruits SMAD4–SMAD2/3 complexes to co-occupy AREs[19,122]. The resulting induction of *GSC* and *MIXL1* commits primitive embryo cells to mesendodermal fates[5] (FIG. 4b). In other words, BMP-activated SMAD1 stimulates ES cell self-renewal by co-occupying the genome with LIF-activated STAT3 and the core pluripotency triad OCT4–SOX2–NANOG. When self-renewal signals fade, the poised chromatin marks that were established by the OCT4 complex provide an entry point for SMAD3 complexes to activate differentiation genes.

Context for lineage regulation in committed progenitors

Recent studies in different types of progenitor cells have shown that lineage identity factors recruit SMAD proteins to many sites in the genome to implement specific differentiation programmes[4,8] (FIG. 5b). TGFβ activated SMAD3 co-occupies the genome with the myogenic identity facto r myoblast determination protein 1 (MYOD1) in mesenchymal progenitors and with the lymphoid identity factor PU.1 (also known as SPI1) in pro-B cells[4]. BMP-activated SMAD1 colocalizes at target genome sites with the myeloid lineage

regulator CCAAT/enhancer-binding protein-α (C/EBPα) or the erythroid lineage regulators GATA1 and GATA2 in haematopoietic progenitors to prompt differentiation towards these two lineages[8]. Ectopic expression of these lineage regulators can redirect SMAD proteins to the corresponding lineage specific loci. Similarly to FOXH1 in ES cells, the master regulators MYOD1, PU.1, C/EBPα and GATA1 and GATA2 in lineage-committed progenitors recruit SMAD4–R-SMAD complexes to enhancer elements in order to implement differentiation. Taken together, core pluripotency factors and line-age identity factors direct signal-driven SMAD complexes to different sets of chosen genes, where SMAD proteins orchestrate transcriptional activation for self-renewal or differentiation.

Context for EMT

EMT, which is the switch of epithelial cells into a mesenchymal migratory phenotype, is a crucial morphogenetic event in gastrulation, embryonic tissue formation and regeneration[24,113]. Pathological forms of EMT participate in fibrosis and cancer. Enforced expression of EMT transcriptional regulators can turn mammary epithelial cells into stem-like cells (FIG. 6a) and provide breast cancer cells with a tumour-initiating phenotype[25]. EMT is driven by an interactive network of transcriptional repressors including SNAIL1, SNAIL2 (also known as SLUG), ZEB1 (zinc-finger E-box binding factor), ZEB2, KLF4 (Krueppel-like factor 4), TCF3 (also known as E47) and TWIST[24,113]. A negative feedback loop is established by miR-200, miR-205 and miR-183, which suppress ZEB1 and ZEB2, whereas ZEB1 represses miR-200 (REFS 24,123) (FIG. 6b).

TGFβ triggers EMT during heart development, palate fusion and renal fibrosis, as well as in breast and hepatic epithelial cells[124]. WNT acts as a competence factor that creates a favourable environment for TGFβ-induced EMT[113] (FIG. 6c), as otherwise epithelial cells would undergo growth arrest in response to TGFβ. In mammary epithelial cells, TGFβ-activated SMAD proteins directly stimulate the expression of SNAIL1 and TWIST1 (TWIST-related protein 1)[125,126]. This is followed by the recruitment of SMAD proteins and SNAIL1 to the *CDH1* promoter to repress the expression of this key epithelial cell junction gene[87] (FIG. 6c). The WNT effector LEF1 also co-occupies the *CDH1* promoter with SMAD proteins[127], providing a molecular mechanism for the observed cooperation of WNT and TGFβ in EMT[25]. BMPs, which are known to induce miR-200 and miR-205 expression[128], antagonize EMT and favour mesenchymal–epithelial transition (MET)[25].

Downregulation of E-cadherin and upregulation of neural cadherin (N-cadherin) and vimentin are markers of EMT and mediators of distinct morphological changes. However, TGFβ-induced EMT proceeds with epigenetic events, which is suggestive of genome-wide reprogramming. These events include a global increase in H3K4me3 and H3K36me3, and a reduction in H3K9me2 within large, organized heterochromatin Lys9 modification regions that depends on the Lys-specific deacetylase LSD1 (REF. 129). The transcriptional effects of TGFβ are complemented by TGFBR2-mediated phosphorylation of PAR6 to dissolve tight junctions and promote a protrusive and invasive phenotype[104,105] (see above) (FIG. 6c). Therefore, TGFβ seems to trigger EMT via a two-pronged TGFBR1–SMAD and TGFBR2–PAR6 pathways, with WNT–TCF providing the right transcriptional context.

iPS cell reprogramming

Ectopic expression of OCT4, Krueppel-like factor 4 (KLF4), SOX2 and MYC (which is termed the `OKSM cocktail') reprogrammes fibroblasts and other differentiated cells into pluripotent iPS cells[130]. As in ES cell self-renewal and EMT transitions, TGFβ and BMP affect iPS cells by signalling to the core reprogramming network[22] (FIG. 6a). In fact, OKSM-driven reprogramming of mouse embryonic fibroblasts is a multistep process that passes first through an MET. BMP facilitates OKSM-driven iPS cell formation by inducing expression of miR-200 and miR-205, which facilitate MET[128] (FIG. 6d). Moreover, OCT4 and SOX2 seem to suppress SNAIL1 expression, and KLF4 enhances E-cadherin expression[131]. TGFβ interferes with iPS cell reprogramming by favouring EMT[131]. Inclusion of TGFBR1 kinase inhibitors in the media eliminates the requirement of ectopic SOX2 and MYC (endogenous SOX2 and MYC may suffice in this context) and increases the rate of iPS cells generation[132,133].

Regulation of homeostasis in differentiated cells

The TGFβ family exerts broad control over many aspects of the biology of differentiated cells, including their proliferation, migration and adhesion, the secretome, extracellular matrix (ECM) production and other functions. The negative regulation of cell cycle progression by TGFβ in epithelial, haematopoietic and neural cells is also of long-standing interest for its implications in cancer. It is largely mediated by the induction of CDK inhibitors (for example, *CDKN1A, CDKN1C* and *CDKN2B*) and repression of *MYC*[14]. TGFβ regulates the expression of components of the ECM, including collagens, fibronectin, tenascins, proteoglycans and their transmembrane receptor integrins[134]. The profound effects of TGFβ on ECM and inflammatory cytokine production and their implications in fibrosis and cancer have long spurred the consideration of targeting TGFβ pathways to treat these conditions. TGFβ is a major inducer of stromal chemokines in wound healing and tumour microenvironments[135]. Its colossal role as a regulator of immune and inflammatory functions depends on balancing acts between positive and negative effects on gene expression[136]. This extends to the regulation of tolerogenic and immunogenic forces in T_H17 lymphocytes, which is a recently identified lineage of intestinal mucosal immunity mediators and suspected culprits in autoimmune diseases[137–139]. BMPs, activins, myostatin and other family members have important roles in the homeostasis of muscle, bone and adipose tissues, blood vessels and haematopoiesis. Such biological impact makes them potential therapeutical targets, which is prompting brisk activity in the pharmaceutical sector.

These diverse effects involve different transcription factors acting as DNA binding partners of SMAD proteins for the recognition of different sets of target genes (FIG. 5c). The sum of these partnerships defines the overall response of a particular cell type to the signal. For example, in skin keratinocytes, the TGFβ-activated SMAD4–SMAD2/3 complex cooperates with FOXO, the transcription factor ETS1 and the adaptor protein AP2 to bind to enhancers in *CDKN1A* and *CDNK2B*[21,85,140] and with E2F4 and E2F5 to bind to an inhibitory element in *MYC*[94,141] to induce antiproliferative gene responses. This distribution of activated SMAD among distinct effector complexes is in contrast with the dedicated interaction of SMAD with a few regulators to enforce differentiation in ES cells and progenitors.

Tumour suppression and tumour progression contexts

TGFβ is a powerful tumour suppressor in the context of pre-malignant cells but an enhancer of invasion and metastasis in the context of more advanced carcinoma cells[14]. This dichotomy may seem paradoxical, but it has its logic. TGFβ has cytostatic effects that are important in tissue regeneration and homeostasis[142,143] (FIG. 7a). When cells incur oncogenic mutations and reach a pre-malignant state TGFβ-mediated suppressive effects become more dramatic, as in this context TGFβ triggers apoptosis[14,103,143] (FIG. 7b). Exactly how pre-malignant cells become programmed to undergo apoptotic cell death is not clear, but the context forces them to avert TGFβ action. In some tumour types, this pressure selects for mutations that eliminate TGFβ signalling altogether (FIG. 7c). Inactivating mutations in *TGFBR2* and *SMAD4* are frequent in the carcinoma transition of gastro-intestinal and pancreatic tumours[14,144]. With the TGFβ pathway eliminated, cancer cells can create with impunity a TGFβ-rich microenvironment that favours tumour progression via effects on the stroma[135].

Things are more devious in breast cancer, melanomas and gliomas. The cancer cell clones that prevail in these tumours retain an intact SMAD signalling machinery. These clones are disabled for TGFβ tumour suppressive responses, but in contrast to their colonic or pancreatic counterparts, the loss of the tumour suppressive responses occurs downstream of SMAD signalling (FIG. 7d). Genetic loss of *CDKN2B* rids cancer cells of this tumour suppressive SMAD target gene, and oncogenic drivers such as human epidermal growth factor receptor 2 (HER2) in breast carcinomas or PI3K in gliomas weaken tumour suppressor SMAD cofactors such as FOXO and C/EBPβ[94,114,145]. Under such conditions, cancer cells can use the remaining signalling capacity of the SMAD pathway to their advantage. Many SMAD-dependent pro-metastatic effects have been identified in such contexts. SMAD-driven EMT enhances stemness and metastatic seeding in breast cancer cells[25,105,146]. TGFβ signalling in breast tumours is associated with lung relapse, partly due to SMAD-dependent expression of angiopoietin-like 4, which enhances extravasation of circulating tumour cells[10]. SMAD-dependent induction of *PTHRP* (parathyroid hormone-like hormone; also known as *PTHLH*), *IL11* (interleukin 11), *CTGF* (connective tissue growth factor) and *JAGGED1* enhances osteolytic metastasis in breast carcinoma[117,147] and melanoma cells[148]. SMAD-dependent activation of *SOX2*, *PDGFB* (platelet-derived growth factor beta polypeptide) and *LIF* supports glioblastoma stem cells[114–116], and these findings provide a rationale for the clinical development of TGFβ inhibitors against glioma[149].

Such SMAD-mediated gene responses would not be oncogenic in a normal cellular context, but in cancer cells they become mediators of malignancy. Aberrations in the TGFβ signalling machinery could certainly also participate in tumorigenesis, as cancer cells will use all the help they can get. However, in cancer cells that retain a functional SMAD pathway, it is the context that uses this pathway to promote cancer.

Outlook

These recent advances have further exposed the logic and power of TGFβ signalling in physiology and disease and moved the field closer to an unambiguous understanding of the context-dependent nature of TGFβ action. Looking ahead, some specific problems seem

particularly worthy of future research. Structural analysis of ligand interactions with traps and co-receptors would aid the pharmaceutical development of mimics and blockers of these interactions. Moreover, structural analysis of receptor interactions with non-canonical mediators, would solve long-standing questions. Similarly, solving the structure of SMAD proteins with their partners bound to DNA would shed light on outstanding questions. A further investigation of SMAD-associated chromatin readers, DNA modifiers and miRNA processors would take our understanding beyond the recent pioneering findings in these promising areas, and quantitative analysis of SMAD dynamics and interactions in live cells would add robustness to this knowledge. It has been known that pathways crosstalk, but the increasingly apparent close interaction between the TGFβ and WNT pathways deserves special attention. Defining the biochemical links between the SMAD cycle and the Pol II cycle could reveal new principles of signal-driven transcriptional action. Genome-wide analysis of SMAD-binding sites, the co-occupying partners and the associated transcriptional outputs, would bring clarity to TGFβ response programmes in contexts of interest. A more vigorous scrutiny of non-transcriptional TGFβ effects on cell contacts, the cytoskeleton and mechanotransduction is warranted, as cell behaviour constantly relies on these processes. It would also be important to elucidate how the responsiveness to TGFβ changes as cells transit from one context to the next (that is, from ES cells to progenitors, from progenitors to differentiated cells, from these to EMT and iPS cells and from pre-malignant cells to metastatic cells). Finally, unravelling how TGFβ signalling activates cell death pathways in aspiring malignant cells would benefit in the development of cancer drugs.

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Glossary

Myoblasts	Mesenchymal progenitor cells that are committed to differentiate into muscle cells.
Epithelial–mesenchymal transition	(EMT). A phenotypic change that is characteristic of some developing tissues and certain forms of cancer. During EMT, cells lose intercellular junctions and apical–basal polarity, become migratory and, in the case of cancer, become invasive.
Homeobox genes	A family of genes encoding transcription factors that are essential for patterning along the anterior–posterior body axis.
Pro-B cells	Cells in the earliest stage of B cell development in the bone marrow. They are characterized by incomplete immunoglobulin heavy-chain rearrangements and are defined as CD19+ cytoplasmic immunoglobulin M (IgM)– or, sometimes, as B220+CD43+ (according the Hardy classification scheme).

Latent TGFβ complex	A complex that includes a bioactive transforming growth factor-β (TGFβ) dimer non-covalently bound to the cleaved propetide of the TGFβ biosynthetic precursor. This cleavage product in turn covalently binds to latent TGFβ-binding protein (LTBP).
Nucleoporins	Family of proteins that constitute the nuclear pore complex, which is a structure that spans the nuclear envelope in eukaryotic cells.
Polyubiquitylation	Post-translational modification of proteins that involves the covalent attachment and polymerization of ubiquitin moieties to Lys chain amino groups.
WW domains	Protein interaction domains that are found in many proteins. The WW domain is characterized by a pair of Trp residues 20–22 amino acids apart, and an invariant Pro residue within a region of 40 amino acids. WW domains interact with Pro-rich regions, including those containing phospho-Ser or phospho-Thr.
Mediator complex	A multiprotein complex that functions as a transcriptional co-activator and binds to the carboxy-terminal domain of the RNA polymerase II (Pol II) holoenzyme. This complex acts as a bridge between the Pol II and transcription factors.
SWI/SNF	(Switch/sucrose nonfermentable). A chromatin-remodelling complex family that was first identified genetically in yeast as a group of genes required for mating type switching and growth on alternative sugar sources to sucrose. This complex is required for the transcriptional activation of~7% of the genome. SWI/SNF complexes exist in multiple forms made up proteins referred to as BRGl-associated factors (BAFs).
MicroRNA	(miRNA). An approximately 21–22 ribonucleotide RNA that arises from the action of the Dicer double-stranded ribonucleases on short stem–loop precursors. miRNAs initiate blocking of the targeted mRNAs, which have nucleotide sequences that are complementary to the miRNA.
Drosha	A ribonuclease III enzyme that initiates processing of microRNAs.
Autocrine	Autocrine signalling refers to when the target cell is the signal-releasing cell itself.
Primitive streak	Structure that forms during early stages of embryonic development. It establishes the first axis of symmetry and marks the beginning of gastrulation.
Melanomas	Malignant tumours derived from melanocyte precursors.

Gliomas The most common types of malignant tumour in the brain.

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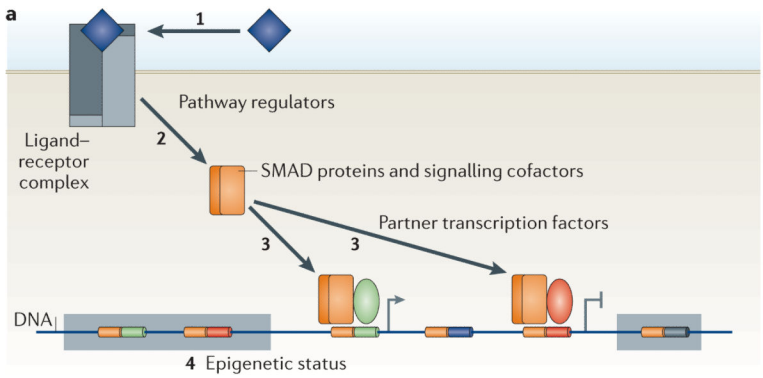

b

Determinants		
Signal transduction	**Transcription**	**Epigenetic status**
Ligand isoforms	Pluripotency factors	Heterochromatin
Ligand traps	Lineage regulators	Pluripotency marks
Co-receptors	DNA-binding cofactors	Lineage marks
Receptor subtypes	HATs and HDACs	EMT marks
Inhibitory SMAD proteins	SWI/SNF	iPS cell marks
Crosstalk inputs	Chromatin readers	Oncogenic marks

Figure 1. Contextual determinants of TGFβ action

a | Three types of contextual determinants shape the transforming growth factor-β (TGFβ)-mediated transcriptional response in a cell. First, a large number of signal transduction regulatory factors determine the access of TGFβ ligands to signalling receptors (**1**), and of receptors to SMAD proteins and other signal delivery factors (**2**). Second, transcription factors, histone readers and modifiers and chromatin remodellers that bind to activated SMAD proteins determine what genes will be targeted by the signal transduction complexes and whether expression of the target genes will be positively or negatively regulated (**3**). The third type of contextual determinants is presented by the epigenetic status of the cell. The epigenetic state dictates whether genes are in an active `open' chromatin conformation (and are therefore accessible for SMAD complexes), in a repressive chromatin conformation (and therefore in a silenced `closed' state that is not accessible for transcriptional regulation), or whether these genes are in a poised chromatin state that is silenced yet responsive to TGFβ signalling and the appropriate chromatin readers (4). Composite enhancer elements are represented as bi-coloured segments on the DNA, inside a shaded box if the genes are in repressive chromatin, and unoccupied if the cell does not express the required DNA-binding SMAD cofactor. **b** | A list of the contextual determinants that affect the signal transduction and transcription steps and the regulators of the epigenetic status. EMT, epithelial–mesenchymal transition; HATs, histone acetyl transferases; HDACs, histone deacetylases; iPS cell, induced pluripotent stem cell; SWI/SNF, Switch/sucrose nonfermentable.

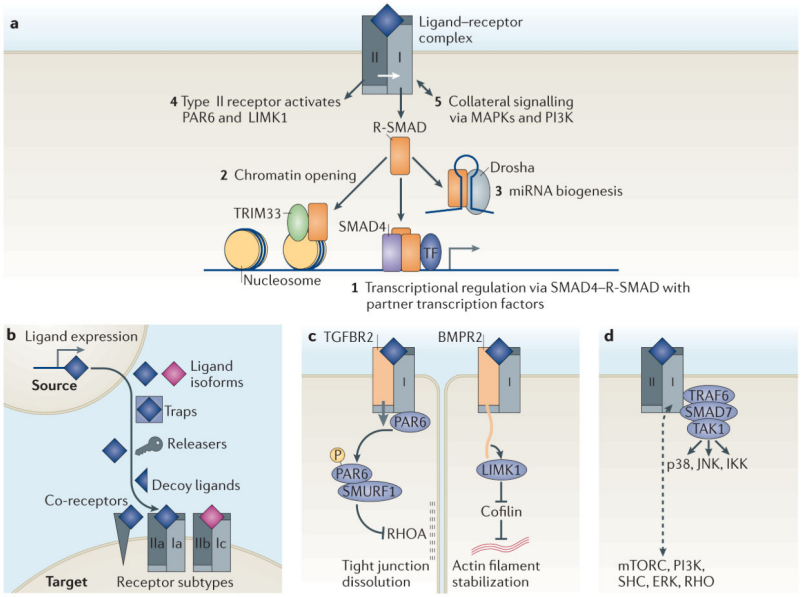

Figure 2. TGFβ receptors and signal transducers

a | Transforming growth factor-β (TGFβ) family ligands signal by assembling a hetero-tetrameric receptor complex with two type I (which are the main signal propagators) receptor components (shown in light grey) and two type II (which are activators) components (shown in dark grey). Signalling is mediated via a cytoplasmic Ser/Thr kinase domain. Signalling modality 1 is the canonical SMAD pathway, and modality 2 its companion. Signalling modalities 3–5 are considered as non-canonical TGFβ signalling pathways. Receptor-phosphorylated SMAD proteins (R-SMAD proteins) form transcriptional complexes that pair with other context-dependent transcription factors to regulate hundreds of genes (**1**) (for details, see FIGS 3,5). Activated R-SMAD proteins can also form a complex with TRIM33 (tripartite motif containing 33) that recognizes certain histone marks and disables their repressive action, which results in chromatin opening and thereby allows access for canonical SMAD complexes (**2**) (for details, see FIG. 4). R-SMAD proteins can participate in microRNA (miRNA) processing by Drosha complexes for the biogenesis of a subset of SMAD-binding miRNA precursors (**3**). TGFβ and bone morphogenetic protein (BMP) type II receptors signal by directly activating partitioning defective 6 (PAR6) and LIM kinase 1 (LIMK1), respectively (**4**). TGFβ and BMP receptors can activate various mitogen-activated protein kinase (MAPKs) and phosphoinositide 3-kinase (PI3K) pathways (**5**). **b** | Seven classes of determinants regulate the access of ligand to TGFβ receptors. These include: the level of expression of a ligand in source cells, the type of ligand isoforms that is available, factors that can sequester the ligands (termed traps) or release them (termed releasers), decoy ligands that occupy the receptors or co-receptors without triggering signalling and the type of receptor and co-receptor that is expressed in target cells. **c** | Direct signalling by type II receptor kinases. TGFβ receptor type II (TGFBR2) phosphorylates the tight junction regulator PAR6 to recruit the E3 ubiquitin ligase SMURF1 and target RHOA for degradation. This leads to dissolution of tight junctions in epithelial cells. BMP receptor type II (BMPR2) contains a long carboxy-terminal tail that binds and activates LIMK1, thereby inhibiting the actin-disassembling

protein cofilin. This results in stabilization of actin filaments. **d** | TRAF6 (tumour necrosis factor receptor-associated factor 6) acts together with SMAD7 and both are known binding partners for TGFβ receptors. The interaction between TRAF6 and SMAD7 is implicated in the activation of TAK1 (TGFβ-activated kinase 1), which is a protein kinase upstream of the signal transduction kinases p38, JNK (Jun amino-terminal kinase) and IKK (inhibitor of κB kinase). TGFβ and BMP receptors can activate several other signal transducers (such as mTORC (mammalian target of rapamycin), PI3K, SHC (SH2 domain-containing transforming protein), ERK (extracellular signal-regulated kinase) and RHO), although the biochemical and structural bases for many of these links remain unknown.

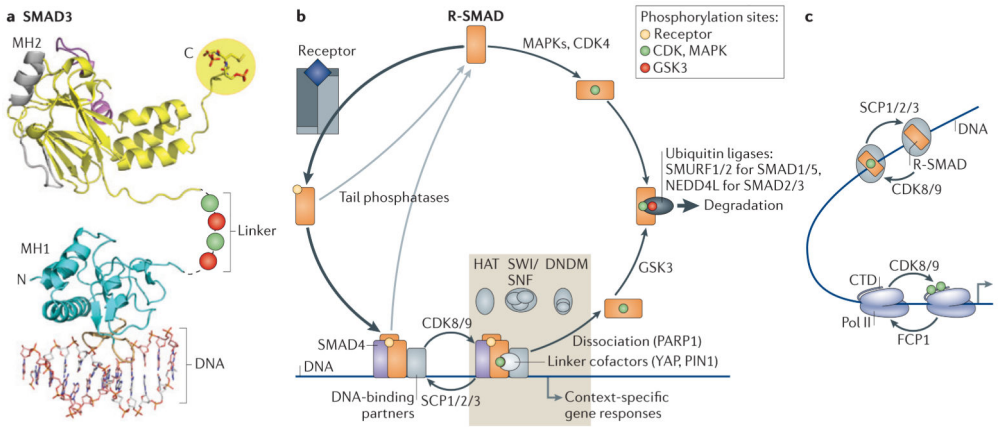

Figure 3. The SMAD signalling cycle

a | Structure of SMAD3 as a representative of receptor-phosphorylated SMAD proteins (R-SMAD proteins). SMAD proteins consist of two globular domains (termed MH1 and MH2) that are coupled by a linker region. The signalling receptors phosphorylate R-SMAD proteins at the carboxy-terminal sequence Ser-X-Ser (where X can be any amino acid), creating an acidic tail that allows binding to SMAD4 (not shown). The linker is phosphorylated by cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MAPKs) (green) and glycogen synthase kinase 3 (GSK3) (red). The phosphorylated linker creates a docking sites for positive and negative regulators of SMAD function. **b** | Following phosphorylation by receptors (yellow), R-SMAD proteins bind SMAD4 and form a hetero-trimeric complex that binds to DNA with partner transcription factors. The transcriptional kinases CDK8 and CDK9 phosphorylate the linker region for peak activation (green). Key participants in the SMAD transcriptional complex include the SWI/SNF nucleosome positioning complex, the HATs (histone acetyl transferases) p300 and CBP (cyclic AMP response element-binding protein) and in certain target genes a DNA demethylating complex (DNDM). Repressive SMAD complexes recruit histone deacetylases (HDACs) through co-repressors (not shown). Various linker-bound factors also participate. Small C-terminal domain (CTD) phosphatases (SCPs) dephosphorylate the linker, allowing repeated utilization of the activated SMAD complex. Poly(ADP-ribose) polymerase 1 (PARP1) and other factors mediate the dissociation of this complex, and tail phosphatases return R-SMAD proteins to the basal state. If R-SMAD proteins are not dephosphorylated, GSK3 recognizes R-SMAD proteins phosphorylated by CDK8 and CDK9 and further increases R-SMAD phosphorylation (red), thereby marking R-SMAD proteins for recognition by E3 ubiquitin ligases and proteasome-mediated degradation. In this manner, SMAD transcriptional action becomes coupled to SMAD turnover. Mitogens and stresses acting through MAPKs, and the cell cycle acting through CDK4, can also phosphorylate the linker to limit the availability of R-SMAD proteins for TGFβ or bone morphogenetic protein (BMP) signalling. **c** | Intriguing parallels exist between the SMAD andRNA polymerase II (Pol II) transcriptional cycles. R-SMAD proteins and Pol II are phosphorylated (green) by the same kinases (which are CDK8 and CDK9) for peak activation and dephosphorylated by structurally related phosphatases (SCP1, SCP2 and SCP3) that reset the basal state. FCP1, transcription factor IIF-associated CTD phosphatase 1; NEDD4L, neural precursor cell expressed developmentally downregulated protein 4-like; PIN1, peptidylprolyl *cis*/*trans* isomerase, NIMA-interacting 1;

SMURF, SMAD-specific E3 ubiquitin protein ligase 1; YAP, Yes-associated protein. Image in part **a** is reproduced, with permission, from REF. 63 © (2011) Cold Spring Harbor Laboratory Press.

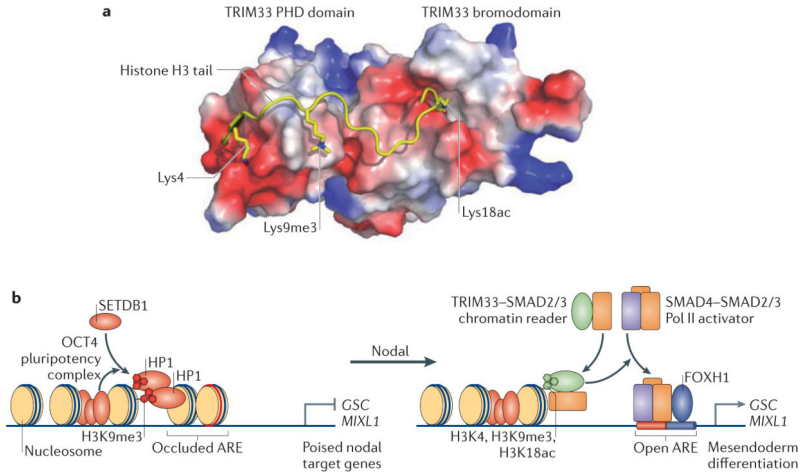

Figure 4. SMAD access to chromatin

a | TRIM33 (tripartite motif containing 33) is a chromatin reader that recognizes a triple feature on histone H3: unmodified Lys4, trimethylated Lys9 (Lys9me3) and acetylated Lys18 (Lys18ac), as seen in the crystal structure of the TRIM33 plant homeodomain PHD-bromo cassette bound to the cognate histone peptide. **b** | The OCT4 pluripotency complex (which contains OCT4, SOX2 and NANOG) in embryonic stem cells prompts SETDB1 (SET-domain binding 1) to trimethylate histone H3 at Lys9 (H3K9me3) at the promoters of the mesendoderm differentiation genes goosecoid homeobox (*GSC*), mix paired-like homeobox (*MIXL1*) and other genes. H3K9me3 recruits the chromatin compacting factor heterochromatin protein 1 (HP1) to implement repression. Under differentiation conditions, *GSC* and *MIXL1* are silent but poised for activation by Nodal signals. The gene promoters present cognate histone marks that are recognized by TRIM33. Nodal drives the formation of two companion complexes, TRIM33–SMAD2/3 and SMAD4–SMAD2/3. The TRIM33–SMAD2/3 complex binds to the poised nucleosomes, displacing HP1, thus enabling SMAD4–SMAD2/3 and their partner in this context, FOXH1 (forkhead box H1), to co-occupy cognate DNA elements (termed activin response elements (AREs)) and activate transcription. Image in part **a** is courtesy of Z. Wang and D. J. Patel, Memorial Sloan-Kettering Cancer Center, New York, New York, USA.

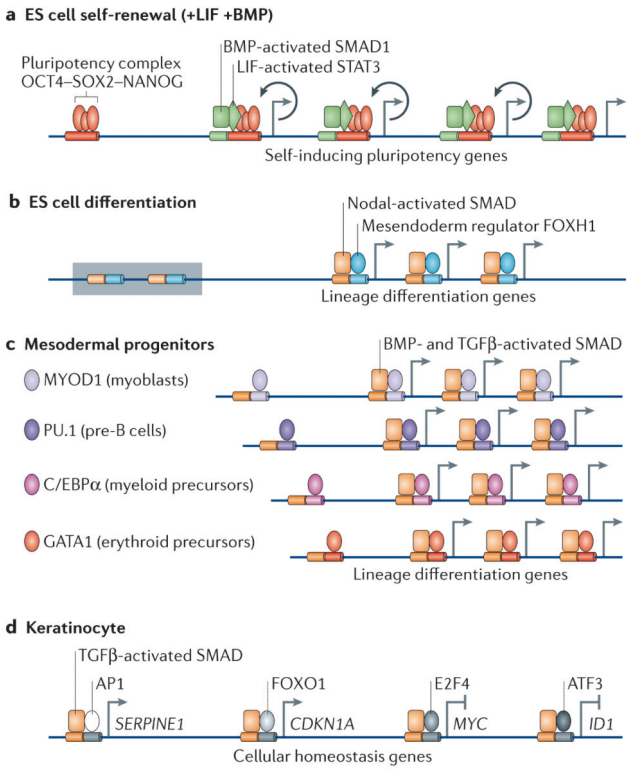

Figure 5. TGFβ action in ES cells, lineage progenitors and differentiated cells

a | In embryonic stem (ES) cells, OCT4, SOX2 and NANOG form the core of a self-renewal network that is stimulated by the bone morphogenetic protein (BMP) mediator SMAD1 and the leukaemia inhibitory factor (LIF) mediator STAT3. SMAD1, STAT3 and the OCT4 complex co-occupy many active sites throughout the genome, including *OCT4*, *SOX2* and *NANOG* themselves, to enforce self-renewal (indicated by circular arrows). **b** | In ES cells that lack self-renewal signals, the Nodal-activated SMAD4–SMAD2/3 complex, together with the TRIM33 (tripartite motif containing 33)–SMAD2/3 complex (see FIG. 4), activates mesendoderm differentiation genes in poised chromatin (indicated by a shaded box). Forkhead box H1 (FOXH1) is a mesendoderm lineage factor that recruits the SMAD4 complex to multiple differentiation genes. **c** | In lineage-restricted progenitors, lineage identity factors are dominant partners of transforming growth factor-β (TGFβ)- or BMP-activated SMAD4 complexes and co-occupy the genome to implement differentiation. **d** | In differentiated cells, TGFβ activated SMAD4–SMAD2/3 complexes are recruited by different partner transcription factors to different subsets of target genes (each subset is represented in the figure by only one gene). The combination of these pathways that lead to transcriptional regulation constitutes the overall TGFβ-mediated transcriptional response for regulation of cell proliferation, adhesion, extracellular matrix properties, the secretome and other cell homeostasis functions in any cell type. The diagram shows the factors that regulate homeostasis of a keratinocyte (that is, a differentiated ectodermal derivative). ATF3, activating transcription factor 3; AP1, adaptor protein 1; C/EBPα, CCAAT/enhancer-binding protein-α; *CDKN1A*, cyclin-dependent kinase inhibitor 1A; *ID1*, inhibitor of DNA binding 1; MYOD1, myoblast determination protein 1.

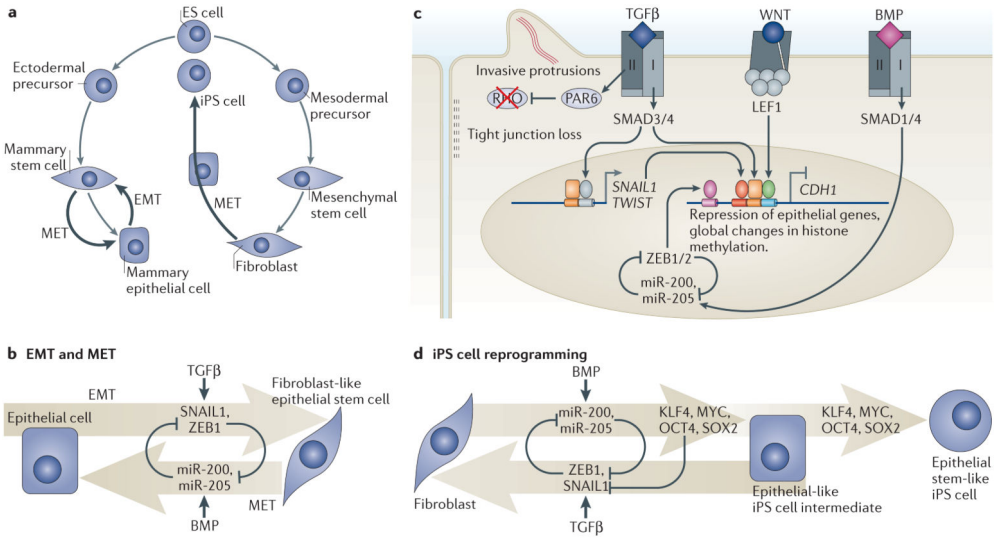

Figure 6. TGFβ action in EMT, MET and iPS cell transition

a | Cell reprogramming processes regulated by transforming growth factor-β (TGFβ) and bone morphogenetic protein (BMP) include epithelial–mesenchymal transition (EMT) in mammary epithelial cells, which can generate epithelial stem-like cells, and the reverse process, mesenchymal–epithelial transition (MET). Reprogramming of fibroblasts into induced pluripotent stem (iPS) cells involves an intermediate MET-like process that is also regulated by BMP and TGFβ. **b** | EMT is driven by the core regulators SNAIL1, SNAIL2, ZEB1 (zinc-finger E-box binding factor 1) and ZEB2 (SNAIL2 and ZEB2 are not shown for simplicity) and other factors. EMT is inhibited by the pro-epithelial microRNAs miR-200 and miR-205, which in turn are suppressed by ZEB1. In mammary epithelial cells, EMT is stimulated by TGFβ and opposed by BMP. **c** | WNT primes epithelial cells to undergo EMT in response to TGFβ. TGFβ triggers SMAD-dependent induction of SNAIL1 and TWIST. SMAD3 and SMAD4 then join SNAIL1 and WNT-activated LEF1 (lymphoid enhancer-binding factor 1) to co-occupy the promoter of *CDH1* (which is the gene encoding epithelial cadherin (E-cadherin)) for repression of this key epithelial gene. This and an associated genome-wide chromatin changes implement the mesenchymal phenotype. In parallel, TGFβ receptor type II (TGFBR2) phosphorylates partitioning defective 6 (PAR6) to mediate dissolution of tight junctions and foster migration (see FIG. 2c). BMP signalling through SMAD1 and SMAD4 upregulates miR-200 and miR-205 to oppose EMT. **d** | Fibroblast reprogramming into iPS cells by ectopic expression of OCT4, Krueppel-like factor 4 (KLF4), SOX2 and MYC requires MET, which is facilitated by BMP-induced miR-200 and miR-205 expression. By interfering with MET, TGFβ suppresses the generation of iPS cells. The use of TGFBR1 inhibitors increases the efficiency of iPS cell generation and reduces the requirement for MYC and SOX2.

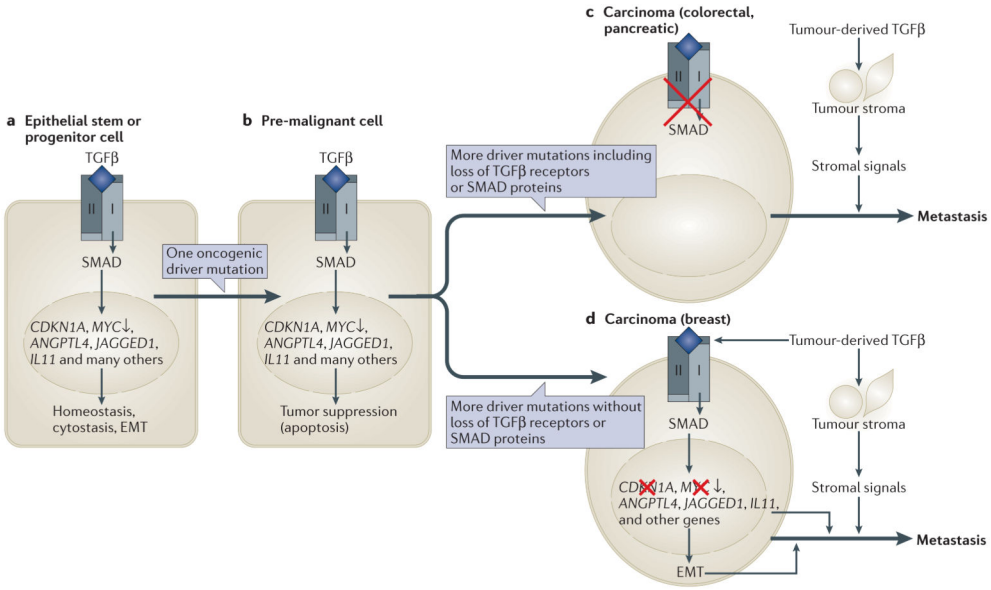

Figure 7. TGFβ action in tumour suppression and tumour progression

a | Transforming growth factor-β (TGFβ)-activated SMAD proteins regulate hundreds of genes in normal epithelial cells, including cytostatic genes and genes involved in homeostasis. The examples include genes that are upregulated by TGFβ, except *MYC*, which is downregulated. **b** | When a stem or progenitor cell incurs an oncogenic mutation (for example, loss of *APC* (adenomatous polyposis coli) tumour suppressor, activation of *KRAS* or amplification of *HER2*) it becomes liable to undergo apoptosis if exposed to TGFβ. To advance in the tumorigenic path, this cell must accumulate additional alterations that, among other achievements, disable the tumour suppressive responsiveness of the cell to TGFβ. **c** | One path to tumour formation involves the selection of malignant clones that have lost TGFβ signalling owing to mutations in *TGFBR1* (TGF beta receptor type II), *TGFBR2* or *SMAD4*. This outcome is frequent in colorectal and pancreatic carcinomas. As a result, cancer cells can withstand a TGFβ-rich tumour stroma and benefit from pro-tumorigenic effects such as TGFβ induction of stroma-derived cytokines that promote cell survival. **d** | Another path involves the selection of clones that have lost tumour suppressive TGFβ responses but retain an intact SMAD signalling machinery. This outcome is frequent in breast carcinomas, gliomas and melanomas. As a result, cancer cells not only withstand a TGFβ-rich microenvironment but also respond to TGFβ, resulting in SMAD-dependent gene responses that in this context are profitable for metastasis. Examples include the induction of angiopoietin-like 4 (*ANGPTL4*) that primes breast cancer cells for extravasation, increased expression of interleukin 11 (*IL11*) and NOTCH ligand *JAGGED1* that allows cancer cells in the bone marrow to activate osteoclasts for osteolytic metastasis, and epithelial–mesenchymal transition (EMT) that provides an invasive and tumour initiating phenotype.