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Unchaining the beast; insights from structural and evolutionary studies on TGF β secretion, sequestration, and activation

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Abstract

TGF β is secreted in a latent state and must be “activated” by molecules that facilitate its release from a latent complex and allow binding to high affinity cell surface receptors. Numerous molecules have been implicated as potential mediators of this activation process, but only a limited number of these activators have been demonstrated to play a role in TGF β mobilization *in vivo*. Here we review the process of TGF β secretion and activation using evolutionary data, sequence conservation and structural information to examine the molecular mechanisms by which TGF β is secreted, sequestered and released. This allows the separation of more ancient TGF β activators from those factors that emerged more recently, and helps to define a potential hierarchy of activation mechanisms.

Keywords

TGF β ; activation; evolution; LTBP; Extracellular matrix

1. Introduction to TGF β

The three TGF β isoforms encoded by the human genome (TGF β 1, 2 and 3) are part of a much larger group of 38 human growth factors described as the “TGF β superfamily”. This group includes growth and differentiation factors (GDFs), bone morphogenic proteins (BMPs), and several other important cytokines (1, 2). TGF β plays a significant role in the development and homeostasis of many tissues. For example it performs crucial roles in wound healing, immunity, muscle differentiation, palate closure, bone growth, and control of cellular proliferation (3–5). However, TGF β is a beast that can bring devastation to numerous tissues if not properly controlled, and excess TGF β activity is associated with liver cirrhosis (6), pulmonary fibrosis (7), arthritis (8), muscular dystrophy (9), aortic aneurysm (10), alzheimers disease (11), inflammation (12), and cancer (13, 14), making TGF β a major target for therapeutic research (15).

The importance of TGF β in development is clearly demonstrated by the phenotypes of knock out mice (Table 1). TGF β 1 null mice die within three to four weeks of birth due to

multiorgan inflammation (3). TGF β 2 null mice display severe disruption in the development of many tissues, yielding congenital heart defects, skeletal defects, cleft palate, and urogenital defects, with most mice dying at birth (4). TGF β 3 null mice die shortly after birth displaying significantly disrupted lung development and cleft palate (16, 17). Redundancy between TGF β 2 and TGF β 3 has been demonstrated by double knock out mice, which display early embryonic death due to failure of midline fusion (5), while *Tgfb1*^{RGE/RGE} and *Tgfb3*^{-/-} double mutant mice display cerebral haemorrhages and other defects in vasculogenesis (18).

Signalling by TGF β is regulated at several levels; extracellular factors control TGF activation and receptor binding and intracellular factors modulate the downstream signalling pathways. The main features of the TGF β pathway are summarized in Figure 1. Within the ER, pairs of TGF β precursor proteins form a dimeric complex, which is subsequently processed into the mature TGF β dimer and the cleaved latency associated peptide (LAP) dimer by furin cleavage in the trans-Golgi network (19). LAP binds TGF β with high affinity, with the result that LAP and TGF β remain associated after cleavage. This assemblage is referred to as the small latent complex (SLC), as when in this complex TGF β cannot bind to its cell surface receptors (20). Structural studies have shown that TGF β adopts a similar structure in the free, latent, and receptor bound states, but when bound to LAP, the TGF receptor binding sites are shielded by regions of the propeptide (21). Simultaneously with pro-TGF β dimerisation in the ER, a single latent TGF β binding protein (LTBP) also binds the pro-TGF β complex via disulfide bonds with LAP (22, 23). The assemblage of LTBP and the SLC is referred to as the large latent complex (LLC).

Before TGF β signalling occurs, the growth factor must be released from its latent complex; a process referred to as activation, and a variety of molecules have been implicated as catalysing this process (discussed later) (20). Once released from its LAP “straightjacket”, the TGF β dimer forms a complex with two TGF β type II (TGF β RII) and two TGF β type I (TGF β RI) cell surface receptors that are both serine/threonine kinases (24). Complex formation between TGF β and its receptors leads to phosphorylation of the inactive TGF β RI by the constitutively active TGF β RII. TGF β RI then phosphorylates SMAD2 and SMAD3 proteins that transmit the TGF β signal to the nucleus by association with co-SMADs and binding to specific CAGA nucleotide repeats (25). These intracellular signalling pathways and their structural mechanisms have been reviewed elsewhere (10, 26). Other non-canonical signalling pathways may also be activated by TGF β , which trigger phosphorylation events that appear to bypass SMAD signalling (27).

Signalling by free TGF β may be modulated by other molecules, such as collagen and decorin, that bind active TGF β [3]. The “type III” TGF β receptor (beta-glycan) can also facilitate TGF β presentation to type I and II receptors (28). Once activated TGF β does not persist in the ECM and is rapidly cleared from the extracellular space, although the mechanisms mediating this clearance are not well understood.

2. Evolution and diversification of bona fide TGF β ligands

Members of the TGF β superfamily have existed since the early evolution of the animal kingdom and are found in the simplest metazoans, such as the sponge (29) and the comb jelly fish (30). Previous studies have examined the evolution of the superfamily, and identified several potential subfamilies (1, 31). However “bona fide” TGF β growth factors are unique in covalently binding LTBP to form a large latent complex, as well as having several other distinguishing characteristics; such as specifically binding type I and II TGF receptors and being rendered latent by pro-peptide binding.

A phylogenetic tree was constructed using TGF β superfamily protein sequences from a wide variety of organisms (Figure 2) (search methods detailed in supplementary information). Sequences from a broad range of metazoan's were included in the tree, but only proteins from the deuterostome¹ genomes were found to comprise the true TGF β subtree (Figure 2B). The closest TGF β homologues in protostomes², cnidarians³, and other more basal metazoans located to different sub-trees, and often displayed closer homology with the activin or myostatin like proteins. The sea urchins and acorn worms are the most evolutionarily distant organisms from humans in which a true TGF β homologue is found. The urchin TGF β orthologue only displays 31% homology with human TGF β 1, while the acorn worm TGF β displays 38% homology with human TGF β 1, but in phylogenetic trees both are consistently clustered within the bona fide TGF β sub-tree (Figure 2B). Both Sea squirt and lancelet genomes also contain a single bona fide TGF β gene.

Lampreys are more closely related to fish and humans than sea squirts and lancelets, and their genome contains two TGF β sequences, one of which is more closely homologous to TGF β 2 and 3 than TGF β 1 (Figure 2B). Generally the TGF β 2 and 3 isoforms cluster to a separate sub tree (consistent with previous reports (1, 31)). This suggests that these isoforms are more closely related to each other than to TGF β 1, and that TGF β 2 and 3 may have resulted from a secondary gene duplication event that occurred after the divergence of lamprey and jawed vertebrates. Bony fish possess all three TGF β isoforms and even some additional TGF β genes. The abundance of TGF β isoforms in fish may stem from the occurrence of two genome duplication events during bony fish evolution (32), which could have created a number of redundant TGF β isoforms that were subsequently lost in some species but retained in others. Reptile, bird and mammal genomes all have the standard complement of three TGF β isoforms, but the amphibian *Xenopus Tropicalis* appears to lack a TGF β 3-like protein.

As well as providing a timeline for growth factor emergence and diversification, these varied TGF β sequences can be used to examine the biological importance of specific residues based on their conservation, and thereby highlight fundamental elements of TGF β biology.

3. Structure, folding and secretion of TGF β

3.1 Disulfide formation in TGF β

The correct formation of disulfide bonds is a critical factor in the folding and secretion of many extracellular proteins, as unpaired cysteines may disrupt folding or lead to protein aggregation, resulting in the misfolded protein being retained by proof reading elements of the ER (33). The C-terminal growth factor region of pro-TGF β contains nine cysteines, eight of which form intra-molecular disulfides and one of which forms an intermolecular disulfide at the dimerisation interface of the mature growth factor. This pattern of cysteines is conserved in many TGF β superfamily members (shown by sub-tree colours in Figure 2A). However, the pattern of cysteine residues in LAP is considerably more variable.

Each TGF β 1 LAP polypeptide contains three cysteines, one of which forms a disulfide bond with LTBP (discussed later), and the other two form intermolecular dimerisation links in the “bow-tie” region of the LAP1 structure (Figure 3A) (21). Human TGF β 2 and TGF β 3 LAPs

¹Deuterostomes are organisms where the anus develops before the mouth during gastrulation, and includes mammals, other vertebrates, sea squirts, sea urchins, lancelets and acorn worms.

²Protostomes are organisms where the mouth develops before the anus during gastrulation, and includes invertebrates, annelid worms, molluscs and nematodes.

³Cnidarians are organisms with a basic body plan consisting of non-living jelly-like “mesoglea”, sandwiched between two epithelial layers. This group includes corals, jellyfish and anemones.

contain additional cysteine residues for which the disulfide-bonding pattern has not been mapped. Homology models of the TGF β 2 and TGF β 3 growth factor-LAP complexes indicate that the additional cysteines found in β 2 and β 3 LAPs are likely located near LAP dimerisation interfaces (Figure 3A). This suggests that they form additional crosslinks between the LAP monomers. These isoform-specific patterns of cysteine residues are conserved across various species (Figure 3B), and no TGF β 1 protein has a cysteine at a position equivalent to C89/91 of human TGF β 2/3, no TGF β 1 or 3 has a CXCC motif equivalent to TGF β 2, and only TGF β 3 proteins have a cysteine equivalent to Cys 123 in human TGF β 3. Although this pattern could be coincidental, it may act as a mechanism to prevent TGF- β isoform heterodimerization, as it is unlikely that any heterodimeric combination of TGF β 1, 2 or 3 could support disulfide formation between all LAP cysteines. Therefore heterodimeric LAP complexes could end up misfolded and retained in the cell.

TGF β proteins that diverged before the appearance of multiple TGF β isoforms, also have a distinct pattern of cysteine residues in their pro-peptides; urchin, acorn worm, lancelet and sea squirt sequences all possess a cysteine equivalent to the TGF β 2/3 C89/91, but lack the second cysteine of the CXC bow tie motif, suggesting this additional disulfide is not essential for the structural stability of LAP in these more distant species. The biological importance of LAP's structural stability is highlighted by Camurati Engelmann disease, where mutations affecting residues in TGF β 1-LAP cause a dominant disorder characterised by bone thickening and pain, especially in the shafts of the long bones (34–36). Many of the disease causing substitutions replace conserved residues (Data not shown), including cysteines and charged amino acids that form salt bridges, which may therefore disrupt LAP structure and TGF β activation (34–36).

3.2 Interaction of TGF β with LTBP

A key feature that distinguishes true TGF β 's from other TGF β superfamily members is their ability to covalently bind LTBPs. This is thought to occur through disulfide bond formation between cysteine 33 of the TGF β 1 propeptides, and the 2–6 disulfide pair of the third 8-cys/second TB domain of LTBP (37–40). Replacement of cysteine 33 with serine in mouse TGF β 1 produces a phenotype similar to TGF β 1 knockout mice, although less severe (41). All complete bona fide TGF β sequences identified here contain a conserved cysteine in this position (Figure 4A), whereas an equivalent cysteine is not present in related TGF β superfamily members (Figure 4B).

The conservation of a cysteine at a position analogous to human TGF β 1 C33 suggests that covalent attachment of TGF β to LTBP, or another protein, has occurred since the early evolution of the growth factor and represents a fundamental element of TGF β function. Interestingly, LTBP-like sequences are also first seen in the deuterostomes and can be found in sea urchin and acorn worm genomes (42) (Figure 5). This indicates that LTBPs emerged on a very similar timescale to true TGF β s, and that these two proteins may have co-evolved to perform their specific functions.

Mutagenesis studies have identified a number of basic residues in the TGF β 1 propeptide that are important for complex formation with LTBP1 (43). These residues are highly conserved (highlighted with arrows and orange spheres in Figure 4A and 4C). However, in the crystal structure of the SLC, many of these residues are buried by interactions between the pro-peptide and growth factor region of TGF β (Figure 4C). Therefore, it is unclear whether they are conserved due to their importance in binding the growth factor or due to a role in LTBP interaction.

Other studies have identified key sequence features in the 3rd 8-cys/2nd TB domain of LTBPs that facilitate binding to TGF β LAP. One of these features is the insertion of two

amino acids into the sheet region prior to the 7th cysteine (figure 4C) (38, 39). The solution structure of this domain indicates that the two amino acid insertion exposes the 2–6 disulfide bond, making it available to react with the propeptide of TGF β (40). These inserted residues are not found in the equivalent domain in LTBP2, which does not bind TGF β (38, 39). Several acidic residues also surround the exposed 2–6 disulfide bond in LTBP1 8-Cys3/TB2, and substitution of these residues with alanine inhibits interaction with LAP (39), consistent with charge interactions between basic residues in LAP and acidic residues in LTBP1. Analysis of LTBP sequences through evolution shows strict conservation of these sequence features in LTBP1 and LTBP3, as well as in urchin, acorn worm, and lancelet LTBP-like proteins (Figure 6), suggesting a conserved LTBP-TGF β interaction in these species and supporting the co-evolution of these two proteins.

The 3rd 8-cys/ 2nd TB domain of human LTBP4 binds the TGF β 1 pro-peptide weakly, and does not interact with the LAPs of TGF β 2 or 3 (39). Some results also suggest TGF β binding is restricted to the long isoform of LTBP4 (44). This poor binding maybe due to the 3rd 8-cys/ 2nd TB domain of human LTBP4 lacking several key acidic residues (39), which are also absent in LTBP4 sequences from a number of organisms, and in *Xenopus tropicalis* the critical two amino acid insert is also missing (Figure 6). This suggests the primary biological function of LTBP4 may not be TGF β binding. On the other hand LTBP2-like proteins only seem to lose the critical amino acid insertion in mammals, and LTBP2 sequences from birds, reptiles, and fish all have the dipeptide insertion and sufficient acidic residues to support hypothetical TGF β binding.

The biological importance of LTBP1 and LTBP3 is underlined by several mouse model studies. Mice with exons specific to the long isoform of LTBP1 knocked out display lethal defects in cardiovascular development, and defective TGF β signalling (45, 46). LTBP3 knock out mice show a variety of skeletal defects, including kyphosis, and an osteopetrosis-like phenotype accompanied by reductions in phosphorylated SMAD2/3 in bone (47–50). Studies in zebra fish have also implicated an LTBP3-like protein as important in cardiac development (51), while in humans LTBP3 null mutations are associated with oligodontia (52), suggesting variation of the functional niche occupied by this protein through evolution. The phenotypes of LTBP1 and LTBP3 deficient mice do not clearly reflect those seen in TGF β -deficient animals, but as both LTBP1 and LTBP3 can bind all three TGF β isoforms, and are both widely expressed (23), there may be significant redundancy between these LTBPs in TGF β signalling, which complicates interpretations of phenotype.

3.3 Interaction of TGF β with GARP

Recently, the glycoprotein-A repetitions predominant protein (GARP) has been shown to bind to TGF β (53), GARP is a trans membrane leucine rich repeat protein unrelated to the LTBPs. GARP presents TGF β on the surface of regulatory T-cells and platelets (54, 55), and may assist integrin mediated activation of covalently bound TGF β when attached to the cell membrane (53). It is thought that the biological role of the GARP-TGF β complex is focused on immune regulation, as GARP expression appears to be restricted to regulatory T cells and platelets (56), whereas LTBPs are expressed ubiquitously (57). From an evolutionary perspective GARP-like proteins appear limited to gnathostomes⁴ (Figure 5, and data not shown) suggesting that this protein performs a more specialised role in TGF β regulation, which may have evolved along with the more complex vertebrate immune system.

⁴Vertebrates with jaws

4. Storage of latent TGF β in the ECM

As well as assisting secretion of TGF β , LTBP1 localise TGF β to extracellular matrix fibers (58–63). Specific interactions have been demonstrated between N-terminal regions of LTBP1, 2, and 4 and fibronectin (62, 63) and between the C-termini of LTBP1, 2 and 4 and the N-terminus of fibrillin (59–61, 64).

Co-localization of LTBP1 with fibronectin is observed in immature fibroblast cultures (58), and fibronectin is essential for initial matrix incorporation of LTBP1 in culture (65). Pull-down assays have demonstrated an interaction between N-terminal fragments of LTBP1 and full-length fibronectin (62), and heparan sulphate proteoglycans may mediate this interaction (66). A fibronectin network is also essential for integrin α 6-mediated TGF β activation in culture (62) (discussed in more detail later). LTBP is a substrate for crosslinking to the ECM by transglutaminase (67), and the inhibition of tissue transglutaminase can prevent TGF β activation in cell culture (68), although whether covalent crosslinking of LTBP1 to the ECM is essential for activation has not been directly tested. The importance of TGF β linkage to LTBP for activation *in vivo* is inferred from the phenotype of the TGF β 1 C33S mice, which is similar to that seen in TGF β 1 null animals (41). As C33S TGF β is still secreted by cells but gives a TGF β 1 null-like phenotype, attachment to LTBP or other factors must play a critical role in the extracellular activity of TGF β 1.

The most evolutionarily distant fibronectin-like protein, with a complete set of FN type 1, 2 and 3 domains, is found in sea squirts (69), and no true fibronectin-like protein sequences are found in lancelets, urchins or acorn worms (Figure 5) (70, 71). This suggests that fibronectin emerged after the evolution of the LLC. Fibrillin, on the other hand, is a much more ancient ECM protein and has changed very little in its domain organization since its emergence prior to the split of cnidaria and bilateria (42, 72). Fibrillin is structurally related to the LTBPs, and it is likely that the LTBPs evolved from a duplication of an ancestral fibrillin gene (42). The interacting regions of LTBP and fibrillin have many conserved surface residues, which are not found in other structurally homologous domains (42, 73) and could be important for the LTBP-fibrillin interaction (42). From the available sequences we can speculate that an LTBP - fibrillin interaction evolved on a similar timescale to the LLC.

Many observations point towards an *in vivo* connection between ECM integrity and proper TGF β signalling. Specifically, Marfan syndrome is a genetic disease caused by dominant mutations in the fibrillin1 gene that results in excess activation of latent TGF β (74, 75). This condition has a number of symptoms, including aortic dilatation and dissection, long bone overgrowth, and lens dislocation. A number of these symptoms, including aortic dissection, are seen in patients with Loeys-Dietz syndrome, who have heterozygous mutations in TGF receptor genes (76, 77). Patients with heterozygous mutations in the TGF β 2 gene also suffer from aortic dissection and other MFS like symptoms (78, 79). Additionally antagonism of TGF β signalling in mouse models of Marfan syndrome restores normal development in multiple tissues and prevents aortic dissection (80–82), suggesting a clear link between the fibrillin matrix and TGF β signalling.

5. Activation of TGF β

The trigger of the TGF β signalling cascade is not the release of the growth factor from cells, but liberation from its latent extracellular complex by latent TGF β activators. In the following sections a number of activators are reviewed, paying particular attention to their evolutionary history and the availability of *in vivo* data to validate their biological significance. The proposed activation mechanisms are also evaluated in light of the available structural data.

5.1 Integrins

The cell surface integrins $\alpha 6$ and $\alpha 8$ are well-established activators of TGF β (83–85). Other α integrins have also been reported to interact with, and in some cases activate, TGF β -LAP (86–91). Integrins release TGF β by two different mechanisms classified as protease-independent and protease-dependent activation.

Protease-independent TGF β activation was first identified with $\alpha 6$ integrin over-expressing cells that activated latent TGF β 1 even in the presence of protease inhibitors (83). This TGF β 1 activation was dependent on covalent attachment of TGF β 1-LAP to LTBP1 (92), attachment of integrin to the actin cytoskeleton (92), specific ECM binding regions of LTBP1 (92), and the presence of a fibronectin matrix (62). An activation mechanism that explains these results is that traction between the cell surface and the insoluble ECM is transmitted to the large latent complex by LTBP and integrins, and liberates active TGF β by deforming LAP (62, 90, 92, 93) (Figure 7A). This notion is supported by the integrin-dependent release of active TGF β by contracting myofibroblasts (90), and force dependant release of active TGF β by integrin coated ferromagnetic beads added to a cell free matrix containing latent TGF β (93).

Cells expressing integrin $\alpha 8$ also activate latent TGF β , but activation is blocked by the presence of matrix metalloprotease (MMP) inhibitors or when the membrane bound MT1-MMP is knocked down (85). This suggests an alternative protease-dependant mechanism of integrin-based activation involving recruitment of both the latent TGF β complex and metalloproteases by integrins to facilitate LAP cleavage and release of TGF β (Figure 7 B).

The significance of integrin-based activation was demonstrated *in vivo* by the phenotype of a mouse in which the integrin binding RGD motif in TGF β 1 LAP was replaced with an RGE sequence (94, 95). These mice phenocopy TGF β 1 null mice, displaying severe tissue inflammation and a lack of Langerhans cells. As the mutant TGF β protein is secreted normally, this phenocopy demonstrates that the integrin-binding motif in TGF β 1 is essential for proper TGF β function. Knocking out or pharmacologically inhibiting integrins $\alpha 6$ and $\alpha 8$ results in vascular phenotypes similar to those seen with *Tgfb1*^{RGE/RGE} *Tgfb3*^{-/-} double null mice (94, 95). As TGF β 3 also contains an RGD motif, it is possible that $\alpha 6$ and $\alpha 8$ integrins are the primary activators of both cytokines.

The evolutionary significance of integrin binding by TGF β is reflected in the strict conservation of the RGD motif in TGF β proteins from evolutionarily distant organisms such as urchins and acorn worms (Figure 7 C). α and β integrins themselves are extremely ancient molecules, and are present in sponges and some bacteria and fungi. Sponge integrins also bind to RGD-like sequences (96). This evolutionary analysis shows that integrins have the potential to be one of the earliest TGF β activators, as they were present in the common ancestor of deuterostomes, and the functional RGD sequence is conserved in at least one TGF β isoform of every species examined. TGF β 2 proteins, on the other hand, consistently lack an RGD motif in the integrin-binding region, and activation of TGF β 2 by integrins has not been reported. This means TGF β 2 may have its own distinct mechanisms of activation that evolved after its split from TGF β 1.

5.2 Proteases

Numerous proteases activate TGF β *in vitro*, but as many of these proteases cleave multiple substrates and can exhibit significant redundancy it is difficult to delineate their importance for *in vivo* TGF β activation. Proteases may also work in concert with other factors that can provide substrate specificity, as seen with MT1-MMP and integrin $\alpha 8$.

Interpreting the *in vivo* significance of MT1-MMP in TGF β signalling is difficult, as while MT1-MMP knock out mice display osteopenia, dwarfism, and fibrosis, these phenotypes are believed to be due to a deficiency in collagen turn over, another substrate of MT1-MMP (97). Although specific cleavage motifs are not well defined, MT1-MMP shows a preference for PXXL sequences or similar motifs (98) which can be found in TGF β sequences (discussed below). Blast searches located MT1-MMP-like sequences in a number of distantly related organisms, including the cnidarians (Figure 5), suggesting MT1-MMP-like proteases were present when the TGF β -LTBP system first evolved.

MMP2 and MMP9 activate TGF β when localized by CD44 on the surface of tumour cells (99). MMP2 and MMP9 have differing expression patterns during development (100), but both appear to have very similar substrate specificities (98, 101), and PXXL like motifs are particularly favoured as with MT1-MMP. Searches of TGF β sequences reveal numerous potential MMP2/9/MT1-MMP cleavage sites throughout TGF β proteins, but of particular interest is the presence of relatively well-conserved PXXV putative cleavage motifs in the “latency lasso” region of all three TGF β isoforms (Figure 8 Ai). Sites on this loop should be exposed to the solvent (Figure 8 Aii), and cleavage of the latency lasso could split LAP into two low affinity fragments allowing TGF β release. However MMP2 and 9 diverged from the MMP family relatively late in vertebrate evolution (Figure 5), and would not have been present in early deuterostomes to activate TGF β . Also mice lacking both MMP2 and MMP9 display relatively mild phenotypes that do not reflect a TGF β deficiency (100). Interestingly MMP2 and MT1-MMP double knockout mice die immediately after birth, but their defects do not closely resemble those seen in TGF β -deficient animals (102). Together the evolutionary and *in vivo* evidence does not support MMP2 or 9 playing widespread roles in TGF β activation.

BMP1 is another metalloprotease implicated in TGF β activation (103). BMP1 cleavage of LTBP1 is proposed to release the latent complex from the ECM and lead to subsequent TGF β activation (103). Mice that have BMP1 and the closely related tolloid1 (Tll1) protease knocked out display excessive accumulation of LTBP1 in embryonic tissues and reduced pSMAD2/3 staining suggesting defective TGF β activation (103). BMP1 and Tll1 also play important roles in processing other extracellular proteins, such as collagen and chordin, and knockouts of BMP1 or Tll1 are embryonic lethal (104). This makes it difficult to assess the *in vivo* significance of BMP1 or Tll1 for TGF β activation, and leaves the possibility that the observed accumulation of LTBP1 and the reduction in pSMAD2/3 in BMP1/Tll1 null animals is an indirect consequence of the altered ECM environment, since these enzymes are important regulators of matrix assembly. Members of the BMP1/tolloid family are present throughout metazoan evolution (Figure 5), and therefore have the potential to be ancient TGF β activators. However the aspartate residues that define putative BMP1 cleavage sites are not well conserved in LTBP1 (103) (Figure 8 B), making a conserved role for BMP1 seem less likely.

An array of serine, cysteine and aspartyl proteases also activate TGF β . One of the best studied proteolytic activators of TGF β is the serine protease plasmin (105), which is an important blood protease that degrades various plasma proteins, including fibrin. Plasmin mediates TGF β activation in co-cultures of endothelial cells and pericytes (106) as well as with retinoid-treated cells (107). Activation in endothelial-pericyte co-cultures is inhibited by antibodies raised against LTBP1 (108) and is dependant on the presence of the mannose-6-phosphate receptor (109), suggesting a role for the cell surface and LTBP. Activated macrophages also activate TGF β by a mechanism dependent on cell surface localization of plasmin and TGF β by TSP1 and CD36 (110, 111).

BLAST searches only detected plasmin-like proteins in lancelets and higher organisms (Figure 5), suggesting plasmin-like proteases emerged after TGF β and LTBP. Also despite the body of evidence demonstrating the role of plasmin in TGF β activation in cell culture, mice deficient in plasmin develop normally and are viable (112). This lack of TGF β related phenotypes rules out a non-redundant role for this protease in TGF β activation.

Another group of serine proteases implicated in TGF β activation are the kallikreins. These proteases have been suggested to play a role in TGF β mediated immunosuppression in seminal plasma (113), and LPS impairment of liver regeneration (114). However previous analysis of kallikrein evolution has shown these proteases only emerged and diversified with the tetrapods (115) (supported by searches performed here (Figure 5)), making their evolution quite recent.

Many other extracellular and intracellular proteases have been shown to activate TGF β *in vitro*, including cysteine proteases, such as calpain (116), and aspartyl proteases, such as cathepsin D (117), as well as several other serine and metallo-proteases (reviewed elsewhere) (111, 118). Many of these proteases are intracellular and have diverse substrates, and as their *in vivo* role in TGF β activation has not been assessed, their evolution is not reviewed here.

5.3 De-glycosylation

Another group of proteins reported to enhance TGF β activation are the deglycosidases. Early experiments demonstrated that Endoglycosidase F activated TGF β (119), and more recently Influenza neuramidase has been shown to activate TGF β by de-glycosylation (120, 121). Human TGF β 1 possesses three potential N-glycosylation sites, as does TGF β 2, while TGF β 3 has two sites. Sequence alignments demonstrate that only the first glycosylation site is conserved across all TGF β isoforms in all species (Figure 9 A). This site is close to the “latency lasso” region of LAP (Figure 9 B) where there are putative MMP cleavage sites (Figure 8 A). Thus glycosylation might obstruct cleavage of protease sensitive regions of LAP, and sugar removal would enhance protease cleavage. However this conserved glycosylation site may also be important for TGF β folding or cellular trafficking, as its removal caused an 85% decrease in TGF β secretion (122). While de-glycosidase-dependent activation may be biologically relevant in influenza infection, there is currently no data supporting a role for deglycosidases in TGF β activation during developmental or homeostatic processes.

5.4 Other protein factors

As well as integrins, proteases, and deglycosidases, additional proteins have been implicated as TGF β activators. Thrombospondin 1 (TSP1) has been the subject of many conflicting studies on its role in TGF β signalling. Addition of exogenous TSP1 to cell cultures and to latent TGF β *in vitro* has been reported to release the active growth factor (123), and interactions of TSP1 with both active TGF β (124) and LAP (125, 126) have been observed in biochemical assays. Peptide studies have suggested the TGF β activation sequence in TSP1 is an RFK motif between the first and second TSP1 domains (127), as a “KRFK” containing peptide activates TGF β at pM concentrations (128) and inhibits TSP1 binding. Further peptide inhibition experiments defined potential interactions between the RFK motif of TSP1 and the LSKL motif in the N-terminus of LAP (125). The TSP1 RFK motif is absent from TSP2, and TSP2 does not activate TGF β (128). However, some groups have failed to observe TGF β activation in response to exogenous TSP1 (129), and SPR studies have not clearly demonstrated the interaction of TSP1 with LAP, LAP-TGF β or TGF β alone (130). Also platelet α -granules (a rich source of TSP1) have been shown to activate TGF β just as efficiently if derived from WT or TSP1 null animals (131).

TSP1 knockout mice exhibit mostly normal development, although they do suffer from severe lung inflammation (132) and have other histological abnormalities that partially overlap with the phenotype of TGF β 1 null mice (3, 133). Improved histology was seen in *Tsp1*^{-/-} animals treated with the TGF β activating KRFK peptide, while treatment of WT mice with the LAP-like LSKL peptide resulted in increased inflammation (133). However *Tsp1*^{-/-} null mice fail to mimic all of the features of TGF β 1 nulls. Moreover *in vitro* TSP1 and the KRFK peptide also activated TGF β 2 (125), but *Tsp1*^{-/-} animals did not display any of the serious developmental defects seen in TGF β 2^{-/-} animals.

Evolutionary analysis of the TSPs demonstrates that although TSP-like proteins are present in basal deuterostomes, TSP1-like proteins that contain an “RHK” TGF β activation sequence are limited to the bony fish and higher organisms (Figure 5 and also observed in a separate study (134)). Together the conflicting biochemical observations, the limited phenotype of TSP1 null mice, and the late evolution of TGF β activating sequences suggest TSP1 is not a central activator of TGF β *in vivo*, although it may still play a role in regulating inflammation or other specific TGF β activities.

F-spondin (or spondin-1) is another protein containing thrombospondin type 1 domains that has been implicated in TGF β activation. Specifically F-spondin has been shown to increase TGF β activity in cartilage explants (135). F-spondin has not yet been shown to directly activate TGF β in cell-free systems, but its effect on TGF β activation can be attenuated by an antibody specific to the F-spondin thrombospondin type 1 repeats, suggesting parallels with activation by TSP1. Proteins containing a unique N-terminal “Spondin” like domain can be found in all deuterostomes examined, as well as the cnidarians (Figure 5), suggesting they emerged before TGF β . However more information is needed on the mechanism by which F-spondin activates TGF β before any conclusions can be drawn.

Neuropilin (Nrp) has been suggested to act as an activator of latent TGF β , both on the surface of T-cells (136, 137) and in some tumour lines (137). The mechanism of TGF β regulation by neuropilin is unclear, as in solid phase assays neuropilin-1 binds mature TGF β 1, LAP, and type I and II TGF β receptors (137). Nrp is also hypothesized to be a co-receptor for TGF β (136–138), and may initiate its own intra-cellular signalling cascades. However, neuropilin-like proteins were only detected in lampreys and higher vertebrates, and not in lower deuterostomes (Figure 5), which does not support neuropilin being an ancient activator of TGF β .

5.5 Physicochemical factors

TGF β is also released from its latent state by a variety of physicochemical factors, including detergents, UVB and ionising radiation (139–144), reactive oxygen species (145), heat (146), physical shear (147), and extremes of pH (117). The relevance of many of these factors *in vivo* is unclear, and many are unlikely to be encountered in a physiological context, but potential biological roles for some are discussed below.

Reactive oxygen species (ROS) activate TGF β both in cell culture and in cell free systems (145) and may mediate the effects of ionizing (140) and UVB radiation (139). ROS are also implicated in asbestos mediated TGF β activation (148), and in immuno-suppression by HIV infected regulatory T-cells (149). Mechanistically ROS are proposed to release TGF β by catalysing backbone scissions in LAP (145), but this has not been demonstrated directly. One study showed that ROS activation was specific to TGF β 1 (150), and relied on the presence of methionine 253 (150). However, this methionine is confined to mammalian TGF β 1 sequences, which would suggest that ROS sensitivity evolved extremely recently.

Prolonged stirring or physical shear activates a percentage of the TGF β present in platelet releaseates (147). This activation is dependant on LTBP binding and is not seen with C33S TGF β (147). The activation mechanism also appears to involve disulfide isomerisation, and is inhibited by the thiol isomerase binding peptide mastoparan (151). No specific residues have yet been defined in TGF β or LTBP that are responsible for shear-sensing, and the *in vivo* role of this mechanism remains unknown.

TGF β activation by low pH (117) could also be relevant *in vivo*, as physiological micro-environments exist that are sufficiently acidic to activate TGF β . For example bone resorption by osteoclasts reduces extracellular pH to ~4.5 (152), and osteoclasts can activate TGF β (153), as do tumour cells that create an acidic microenvironment (154). However, whether the low pH generated by these cells is essential for activation is unknown. No specific residues have been identified in TGF β that confer pH sensitivity, and without such information it is difficult to assess the physiological significance of pH driven activation.

6. Conclusions

The evolutionary data reviewed here suggests that the first true TGF β -like growth factor emerged during the early evolution of the deuterostomes. LTBP β s appear to have emerged at the same time, and in both TGF β and LTBP sequences amino acids required for their covalent linkage are extremely well conserved. This suggests the functions of these two proteins may have been intimately connected since their early evolution.

Of the plethora of described TGF β activators, only a limited number were present when true TGF β first evolved (Figure 5). This does not dismiss the biological significance of factors that evolved later, but the first TGF β would have needed an activator, and only factors that were present when TGF β first emerged could have performed this role. Potential activation mechanisms were also examined by alignments to test the conservation of key residues, although for some activators this analysis was limited by a lack of molecular understanding of the residues involved. Of the factors for which activation is well understood, integrins appear to be clear front-runners for the role of earliest TGF β activators. As well as being extremely ancient molecules, the RGD integrin-binding motif is found in all the early TGF β sequences and all TGF β 1 like sequences (Figure 7). The consistent absence of an RGD motif from TGF β 2 could reflect a specific adaptation of this growth factor, which may be activated by alternative mechanisms yet to be elucidated.

A number of other activators, including MT1-MMP, BMP1, and F-Spondin were also present when TGF β first evolved, but a lack of mechanistic insight into activation by these factors limits their evolutionary analysis, and *in vivo* data on their role in activation is either absent or inconclusive. Analysis of relatively well-studied activators, such as plasmin and TSP1, demonstrates that these proteins did not emerge until later in deuterostome evolution, suggesting they may have less critical roles in TGF β activation, consistent with knockout mouse studies.

It has previously been suggested that the complex of TGF β -LAP-LTBP acts as a “sensor” (20), with release of active TGF β serving as a readout of the extracellular events that prompted its activation. It is not always clear what events trigger the TGF β sensor in specific tissue contexts *in vivo* or how this “triggering” requirement relates to the physiological roles of TGF β signalling. The evolutionary data presented here suggest that LAP-LTBP, and LAP-integrin interactions may have been the first to evolve when TGF β broke off from the rest of the superfamily. The establishment of these two interactions should have allowed the early LLC to sense traction between cell surface and the extracellular matrix (Figure 7 A), and it is possible that true TGF β first evolved as a readout of this cell-ECM traction.

While TGF β has been the subject of intense research, a clear understanding of the biology of this molecule has been obfuscated by the perplexing array of potential TGF β activators. The volume of literature on TGF β may suggest a system of mind-boggling complexity, but TGF β signalling must have evolved from a simple starting point, and by defining that point we gain a more solid foundation to understand how TGF β performs its multitudinous roles in development and tissue homeostasis. Placing TGF β activators within an evolutionary framework also highlights the physiological processes and evolutionary adaptations for which specific TGF β activators may and may not be essential. A clearer understanding of these processes may suggest specific TGF β activators as drug targets, and this in turn will enable us to better treat the numerous diseases where the shackles of the beast have been broken.

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Supplementary Material

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References

- Herpin A, Lelong C, Favrel P. Transforming growth factor-beta-related proteins: an ancestral and widespread superfamily of cytokines in metazoans. *Dev Comp Immunol*. 2004; 285:461–85. [PubMed: 15062644]
- Kahlem P, Newfeld SJ. Informatics approaches to understanding TGFbeta pathway regulation. *Development*. 2009; 13622:3729–40. [PubMed: 19855015]
- Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, et al. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci U S A*. 1993; 902:770–4. [PubMed: 8421714]
- Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, et al. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development*. 1997; 12413:2659–70. [PubMed: 9217007]
- Dunker N, Kriegelstein K. Tgfbeta2 $-/-$ Tgfbeta3 $-/-$ double knockout mice display severe midline fusion defects and early embryonic lethality. *Anat Embryol (Berl)*. 2002; 2061–2:73–83.
- Dooley S, ten Dijke P. TGF-beta in progression of liver disease. *Cell Tissue Res*. 2012; 3471:245–56. [PubMed: 22006249]
- Coward WR, Saini G, Jenkins G. The pathogenesis of idiopathic pulmonary fibrosis. *Ther Adv Respir Dis*. 2010; 46:367–88. [PubMed: 20952439]
- Pohlers D, Brenmoehl J, Löffler I, Müller CK, Leipner C, Schultze-Mosgau S, et al. TGF-beta and fibrosis in different organs - molecular pathway imprints. *Biochim Biophys Acta*. 2009; 17928:746–56. [PubMed: 19539753]
- Burks TN, Cohn RD. Role of TGF-beta signaling in inherited and acquired myopathies. *Skelet Muscle*. 2011; 11:19. [PubMed: 21798096]
- Doyle JJ, Gerber EE, Dietz HC. Matrix-dependent perturbation of TGFbeta signaling and disease. *FEBS Lett*. 2012; 58614:2003–15. [PubMed: 22641039]
- Wyss-Coray T, Masliah E, Mallory M, McConlogue L, Johnson-Wood K, Lin C, et al. Amyloidogenic role of cytokine TGF-beta1 in transgenic mice and in Alzheimer's disease. *Nature*. 1997; 3896651:603–6. [PubMed: 9335500]
- Han G, Li F, Singh TP, Wolf P, Wang XJ. The pro-inflammatory role of TGFbeta1: a paradox? *Int J Biol Sci*. 2012; 82:228–35. [PubMed: 22253566]
- Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. *N Engl J Med*. 2000; 34218:1350–8. [PubMed: 10793168]
- Massague J. TGFbeta in Cancer. *Cell*. 2008; 1342:215–30. [PubMed: 18662538]

15. Akhurst RJ, Hata A. Targeting the TGFbeta signalling pathway in disease. *Nat Rev Drug Discov.* 2012; 1110:790–811. [PubMed: 23000686]
16. Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N, et al. Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet.* 1995; 114:415–21. [PubMed: 7493022]
17. Taya Y, O'Kane S, Ferguson MW. Pathogenesis of cleft palate in TGF-beta3 knockout mice. *Development.* 1999; 12617:3869–79. [PubMed: 10433915]
18. Mu Z, Yang Z, Yu D, Zhao Z, Munger JS. TGFbeta1 and TGFbeta3 are partially redundant effectors in brain vascular morphogenesis. *Mech Dev.* 2008; 1255–6:508–16.
19. Dubois CM, Blanchette F, Laprise MH, Leduc R, Grondin F, Seidah NG. Evidence that furin is an authentic transforming growth factor-beta1-converting enzyme. *Am J Pathol.* 2001; 1581:305–16. [PubMed: 11141505]
20. Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFbeta activation. *J Cell Sci.* 2003; 116(Pt 2):217–24. [PubMed: 12482908]
21. Shi M, Zhu J, Wang R, Chen X, Mi L, Walz T, et al. Latent TGF-beta structure and activation. *Nature.* 2011; 4747351:343–9. [PubMed: 21677751]
22. Hyytiainen M, Penttinen C, Keski-Oja J. Latent TGF-beta binding proteins: extracellular matrix association and roles in TGF-beta activation. *Crit Rev Clin Lab Sci.* 2004; 413:233–64. [PubMed: 15307633]
23. Saharinen J, Hyytiainen M, Taipale J, Keski-Oja J. Latent transforming growth factor-beta binding proteins (LTBPs)--structural extracellular matrix proteins for targeting TGF-beta action. *Cytokine Growth Factor Rev.* 1999; 102:99–117. [PubMed: 10743502]
24. Radaev S, Zou Z, Huang T, Lafer EM, Hinck AP, Sun PD. Ternary complex of transforming growth factor-beta1 reveals isoform-specific ligand recognition and receptor recruitment in the superfamily. *J Biol Chem.* 2010; 28519:14806–14. [PubMed: 20207738]
25. Massague J, Chen YG. Controlling TGF-beta signaling. *Genes Dev.* 2000; 146:627–44. [PubMed: 10733523]
26. Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell.* 2003; 1136:685–700. [PubMed: 12809600]
27. Habashi JP, Doyle JJ, Holm TM, Aziz H, Schoenhoff F, Bedja D, et al. Angiotensin II type 2 receptor signaling attenuates aortic aneurysm in mice through ERK antagonism. *Science.* 3326027:361–5.
28. Lopez-Casillas F, Wrana JL, Massague J. Betaglycan presents ligand to the TGF beta signaling receptor. *Cell.* 1993; 737:1435–44. [PubMed: 8391934]
29. Adamska M, Degnan SM, Green KM, Adamski M, Craigie A, Larroux C, et al. Wnt and TGF-beta expression in the sponge *Amphimedon queenslandica* and the origin of metazoan embryonic patterning. *PLoS One.* 2007; 210:e1031. [PubMed: 17925879]
30. Pang K, Ryan JF, Baxeavanis AD, Martindale MQ. Evolution of the TGF-beta signaling pathway and its potential role in the ctenophore, *Mnemiopsis leidyi*. *PLoS One.* 2011; 69:e24152. [PubMed: 21931657]
31. Burt DW, Law AS. Evolution of the transforming growth factor-beta superfamily. *Prog Growth Factor Res.* 1994; 51:99–118. [PubMed: 8199356]
32. Braasch I, Salzburger W. In ovo omnia: diversification by duplication in fish and other vertebrates. *J Biol.* 2009; 83:25. [PubMed: 19291270]
33. Hagiwara M, Nagata K. Redox-dependent protein quality control in the endoplasmic reticulum: folding to degradation. *Antioxid Redox Signal.* 2012; 1610:1119–28. [PubMed: 22229892]
34. Janssens K, Vanhoenacker F, Bonduelle M, Verbruggen L, Van Maldergem L, Ralston S, et al. Camurati-Engelmann disease: review of the clinical, radiological, and molecular data of 24 families and implications for diagnosis and treatment. *J Med Genet.* 2006; 431:1–11. [PubMed: 15894597]
35. Janssens K, Gershoni-Baruch R, Guanabens N, Migone N, Ralston S, Bonduelle M, et al. Mutations in the gene encoding the latency-associated peptide of TGF-beta 1 cause Camurati-Engelmann disease. *Nat Genet.* 2000; 263:273–5. [PubMed: 11062463]

36. Kinoshita A, Saito T, Tomita H, Makita Y, Yoshida K, Ghadami M, et al. Domain-specific mutations in TGFB1 result in Camurati-Engelmann disease. *Nat Genet.* 2000; 261:19–20. [PubMed: 10973241]
37. Gleizes PE, Beavis RC, Mazzieri R, Shen B, Rifkin DB. Identification and characterization of an eight-cysteine repeat of the latent transforming growth factor-beta binding protein-1 that mediates bonding to the latent transforming growth factor-beta1. *J Biol Chem.* 1996; 27147:29891–6. [PubMed: 8939931]
38. Saharinen J, Keski-Oja J. Specific sequence motif of 8-Cys repeats of TGF-beta binding proteins, LTBP1s, creates a hydrophobic interaction surface for binding of small latent TGF-beta. *Mol Biol Cell.* 2000; 118:2691–704. [PubMed: 10930463]
39. Chen Y, Ali T, Todorovic V, O'Leary JM, Kristina Downing A, Rifkin DB. Amino acid requirements for formation of the TGF-beta-latent TGF-beta binding protein complexes. *J Mol Biol.* 2005; 3451:175–86. [PubMed: 15567420]
40. Lack J, O'Leary JM, Knott V, Yuan X, Rifkin DB, Handford PA, et al. Solution structure of the third TB domain from LTBP1 provides insight into assembly of the large latent complex that sequesters latent TGF-beta. *J Mol Biol.* 2003; 3342:281–91. [PubMed: 14607119]
41. Yoshinaga K, Obata H, Jurukovski V, Mazzieri R, Chen Y, Zilberberg L, et al. Perturbation of transforming growth factor (TGF)-beta1 association with latent TGF-beta binding protein yields inflammation and tumors. *Proc Natl Acad Sci U S A.* 2008; 10548:18758–63. [PubMed: 19022904]
42. Robertson I, Jensen S, Handford P. TB domain proteins: evolutionary insights into the multifaceted roles of fibrillins and LTBP1s. *Biochem J.* 2011; 4332:263–76. [PubMed: 21175431]
43. Walton KL, Makanji Y, Chen J, Wilce MC, Chan KL, Robertson DM, et al. Two distinct regions of latency-associated peptide coordinate stability of the latent transforming growth factor-beta1 complex. *J Biol Chem.* 2010; 28522:17029–37. [PubMed: 20308061]
44. Kantola AK, Ryyanen MJ, Lhota F, Keski-Oja J, Koli K. Independent regulation of short and long forms of latent TGF-beta binding protein (LTBP)-4 in cultured fibroblasts and human tissues. *J Cell Physiol.* 2010
45. Todorovic V, Frendewey D, Gutstein DE, Chen Y, Freyer L, Finnegan E, et al. Long form of latent TGF-beta binding protein 1 (Ltbp1L) is essential for cardiac outflow tract septation and remodeling. *Development.* 2007; 13420:3723–32. [PubMed: 17804598]
46. Todorovic V, Finnegan E, Freyer L, Zilberberg L, Ota M, Rifkin DB. Long form of latent TGF-beta binding protein 1 (Ltbp1L) regulates cardiac valve development. *Dev Dyn.* 2011; 2401:176–87. [PubMed: 21181942]
47. Chen Y, Dabovic B, Colarossi C, Santori FR, Lilic M, Vukmanovic S, et al. Growth retardation as well as spleen and thymus involution in latent TGF-beta binding protein (Ltbp)-3 null mice. *J Cell Physiol.* 2003; 1962:319–25. [PubMed: 12811825]
48. Dabovic B, Chen Y, Colarossi C, Obata H, Zambuto L, Perle MA, et al. Bone abnormalities in latent TGF-[beta] binding protein (Ltbp)-3-null mice indicate a role for Ltbp-3 in modulating TGF-[beta] bioavailability. *J Cell Biol.* 2002; 1562:227–32. [PubMed: 11790802]
49. Colarossi C, Chen Y, Obata H, Jurukovski V, Fontana L, Dabovic B, et al. Lung alveolar septation defects in Ltbp-3-null mice. *Am J Pathol.* 2005; 1672:419–28. [PubMed: 16049328]
50. Dabovic B, Levasseur R, Zambuto L, Chen Y, Karsenty G, Rifkin DB. Osteopetrosis-like phenotype in latent TGF-beta binding protein 3 deficient mice. *Bone.* 2005; 371:25–31. [PubMed: 15878314]
51. Zhou Y, Cashman TJ, Nevis KR, Obregon P, Carney SA, Liu Y, et al. Latent TGF-beta binding protein 3 identifies a second heart field in zebrafish. *Nature.* 2011; 4747353:645–8. [PubMed: 21623370]
52. Noor A, Windpassinger C, Vitcu I, Orlic M, Rafiq MA, Khalid M, et al. Oligodontia is caused by mutation in LTBP3, the gene encoding latent TGF-beta binding protein 3. *Am J Hum Genet.* 2009; 844:519–23. [PubMed: 19344874]
53. Wang R, Zhu J, Dong X, Shi M, Lu C, Springer TA. GARP regulates the bioavailability and activation of TGFbeta. *Mol Biol Cell.* 2012; 236:1129–39. [PubMed: 22278742]

54. Tran DQ, Andersson J, Wang R, Ramsey H, Unutmaz D, Shevach EM. GARP (LRRC32) is essential for the surface expression of latent TGF-beta on platelets and activated FOXP3+ regulatory T cells. *Proc Natl Acad Sci U S A*. 2009; 10632:13445–50. [PubMed: 19651619]
55. Stockis J, Colau D, Coulie PG, Lucas S. Membrane protein GARP is a receptor for latent TGF-beta on the surface of activated human Treg. *Eur J Immunol*. 2009; 3912:3315–22. [PubMed: 19750484]
56. Wang R, Wan Q, Kozhaya L, Fujii H, Unutmaz D. Identification of a regulatory T cell specific cell surface molecule that mediates suppressive signals and induces Foxp3 expression. *PLoS One*. 2008; 37:e2705. [PubMed: 18628982]
57. Rifkin DB. Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability. *J Biol Chem*. 2005; 2809:7409–12. [PubMed: 15611103]
58. Unsold C, Hyytiainen M, Bruckner-Tuderman L, Keski-Oja J. Latent TGF-beta binding protein LTBP-1 contains three potential extracellular matrix interacting domains. *J Cell Sci*. 2001; 114(Pt 1):187–97. [PubMed: 11112702]
59. Massam-Wu T, Chiu M, Choudhury R, Chaudhry SS, Baldwin AK, McGovern A, et al. Assembly of fibrillin microfibrils governs extracellular deposition of latent TGF beta. *J Cell Sci*. 123(Pt 17): 3006–18. [PubMed: 20699357]
60. Isogai Z, Ono RN, Ushiro S, Keene DR, Chen Y, Mazzieri R, et al. Latent transforming growth factor beta-binding protein 1 interacts with fibrillin and is a microfibril-associated protein. *J Biol Chem*. 2003; 2784:2750–7. [PubMed: 12429738]
61. Ono RN, Sengle G, Charbonneau NL, Carlberg V, Bachinger HP, Sasaki T, et al. Latent transforming growth factor beta-binding proteins and fibulins compete for fibrillin-1 and exhibit exquisite specificities in binding sites. *J Biol Chem*. 2009; 28425:16872–81. [PubMed: 19349279]
62. Fontana L, Chen Y, Prijatelj P, Sakai T, Fassler R, Sakai LY, et al. Fibronectin is required for integrin alphavbeta6-mediated activation of latent TGF-beta complexes containing LTBP-1. *FASEB J*. 2005; 1913:1798–808. [PubMed: 16260650]
63. Kantola AK, Keski-Oja J, Koli K. Fibronectin and heparin binding domains of latent TGF-beta binding protein (LTBP)-4 mediate matrix targeting and cell adhesion. *Exp Cell Res*. 2008; 31413:2488–500. [PubMed: 18585707]
64. Hirani R, Hanssen E, Gibson MA. LTBP-2 specifically interacts with the amino-terminal region of fibrillin-1 and competes with LTBP-1 for binding to this microfibrillar protein. *Matrix Biol*. 2007; 264:213–23. [PubMed: 17293099]
65. Zilberberg L, Todorovic V, Dabovic B, Horiguchi M, Courousse T, Sakai LY, et al. Specificity of latent TGF-beta binding protein (LTBP) incorporation into matrix: role of fibrillins and fibronectin. *J Cell Physiol*. 2012
66. Chen Q, Sivakumar P, Barley C, Peters DM, Gomes RR, Farach-Carson MC, et al. Potential role for heparan sulfate proteoglycans in regulation of transforming growth factor-beta (TGF-beta) by modulating assembly of latent TGF-beta-binding protein-1. *J Biol Chem*. 2007; 28236:26418–30. [PubMed: 17580303]
67. Nunes I, Gleizes PE, Metz CN, Rifkin DB. Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta. *J Cell Biol*. 1997; 1365:1151–63. [PubMed: 9060478]
68. Kojima S, Nara K, Rifkin DB. Requirement for transglutaminase in the activation of latent transforming growth factor-beta in bovine endothelial cells. *J Cell Biol*. 1993; 1212:439–48. [PubMed: 8096847]
69. Tucker RP, Chiquet-Ehrismann R. Evidence for the evolution of tenascin and fibronectin early in the chordate lineage. *Int J Biochem Cell Biol*. 2009; 412:424–34. [PubMed: 18761101]
70. Tucker RP, Drabikowski K, Hess JF, Ferralli J, Chiquet-Ehrismann R, Adams JC. Phylogenetic analysis of the tenascin gene family: evidence of origin early in the chordate lineage. *BMC Evol Biol*. 2006; 6:60. [PubMed: 16893461]
71. Hynes RO. The evolution of metazoan extracellular matrix. *J Cell Biol*. 2012; 1966:671–9. [PubMed: 22431747]
72. Piha-Gossack A, Sossin W, Reinhardt DP. The evolution of extracellular fibrillins and their functional domains. *PLoS One*. 2012; 73:e33560. [PubMed: 22438950]

73. Jensen SA, Robertson IB, Handford PA. Dissecting the fibrillin microfibril: structural insights into organization and function. *Structure*. 2012; 202:215–25. [PubMed: 22325771]
74. Robinson PN, Arteaga-Solis E, Baldock C, Collod-Beroud G, Booms P, De Paepe A, et al. The molecular genetics of Marfan syndrome and related disorders. *J Med Genet*. 2006; 4310:769–87. [PubMed: 16571647]
75. Dietz HC, Loeys B, Carta L, Ramirez F. Recent progress towards a molecular understanding of Marfan syndrome. *Am J Med Genet C Semin Med Genet*. 2005; 139C1:4–9. [PubMed: 16273535]
76. Attias D, Stheneur C, Roy C, Collod-Beroud G, Detaint D, Faivre L, et al. Comparison of clinical presentations and outcomes between patients with TGFBR2 and FBN1 mutations in Marfan syndrome and related disorders. *Circulation*. 2009; 12025:2541–9. [PubMed: 19996017]
77. Loeys BL, Chen J, Neptune ER, Judge DP, Podowski M, Holm T, et al. A syndrome of altered cardiovascular, craniofacial, neurocognitive and skeletal development caused by mutations in TGFBR1 or TGFBR2. *Nat Genet*. 2005; 373:275–81. [PubMed: 15731757]
78. Lindsay ME, Schepers D, Bolar NA, Doyle JJ, Gallo E, Fert-Bober J, et al. Loss-of-function mutations in TGFBR2 cause a syndromic presentation of thoracic aortic aneurysm. *Nat Genet*. 2012; 448:922–7. [PubMed: 22772368]
79. Boileau C, Guo DC, Hanna N, Regalado ES, Detaint D, Gong L, et al. TGFBR2 mutations cause familial thoracic aortic aneurysms and dissections associated with mild systemic features of Marfan syndrome. *Nat Genet*. 2012; 448:916–21. [PubMed: 22772371]
80. Neptune ER, Frischmeyer PA, Arking DE, Myers L, Bunton TE, Gayraud B, et al. Dysregulation of TGF-beta activation contributes to pathogenesis in Marfan syndrome. *Nat Genet*. 2003; 333:407–11. [PubMed: 12598898]
81. Cohn RD, van Erp C, Habashi JP, Soleimani AA, Klein EC, Lisi MT, et al. Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic states. *Nat Med*. 2007; 132:204–10. [PubMed: 17237794]
82. Habashi JP, Judge DP, Holm TM, Cohn RD, Loeys BL, Cooper TK, et al. Losartan, an AT1 antagonist, prevents aortic aneurysm in a mouse model of Marfan syndrome. *Science*. 2006; 3125770:117–21. [PubMed: 16601194]
83. Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, et al. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell*. 1999; 963:319–28. [PubMed: 10025398]
84. Annes JP, Rifkin DB, Munger JS. The integrin alphaVbeta6 binds and activates latent TGFbeta3. *FEBS Lett*. 2002; 5111–3:65–8.
85. Mu D, Cambier S, Fjellbirkeland L, Baron JL, Munger JS, Kawakatsu H, et al. The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1. *J Cell Biol*. 2002; 1573:493–507. [PubMed: 11970960]
86. Wipff PJ, Hinz B. Integrins and the activation of latent transforming growth factor beta1 - an intimate relationship. *Eur J Cell Biol*. 2008; 878–9:601–15.
87. Munger JS, Harpel JG, Giancotti FG, Rifkin DB. Interactions between growth factors and integrins: latent forms of transforming growth factor-beta are ligands for the integrin alphavbeta1. *Mol Biol Cell*. 1998; 99:2627–38. [PubMed: 9725916]
88. Lu M, Munger JS, Steadele M, Busald C, Tellier M, Schnapp LM. Integrin alpha8beta1 mediates adhesion to LAP-TGFbeta1. *J Cell Sci*. 2002; 115(Pt 23):4641–8. [PubMed: 12415008]
89. Ludbrook SB, Barry ST, Delves CJ, Horgan CM. The integrin alphavbeta3 is a receptor for the latency-associated peptides of transforming growth factors beta1 and beta3. *Biochem J*. 2003; 369(Pt 2):311–8. [PubMed: 12358597]
90. Wipff PJ, Rifkin DB, Meister JJ, Hinz B. Myofibroblast contraction activates latent TGF-beta1 from the extracellular matrix. *J Cell Biol*. 2007; 1796:1311–23. [PubMed: 18086923]
91. Tatler AL, John AE, Jolly L, Habgood A, Porte J, Brightling C, et al. Integrin alphavbeta5-mediated TGF-beta activation by airway smooth muscle cells in asthma. *J Immunol*. 2011; 18711:6094–107. [PubMed: 22025551]
92. Annes JP, Chen Y, Munger JS, Rifkin DB. Integrin alphaVbeta6-mediated activation of latent TGF-beta requires the latent TGF-beta binding protein-1. *J Cell Biol*. 2004; 1655:723–34. [PubMed: 15184403]

93. Buscemi L, Ramonet D, Klingberg F, Formey A, Smith-Clerc J, Meister JJ, et al. The single-molecule mechanics of the latent TGF-beta1 complex. *Curr Biol.* 2124:2046–54.
94. Yang Z, Mu Z, Dabovic B, Jurukovski V, Yu D, Sung J, et al. Absence of integrin-mediated TGFbeta1 activation in vivo recapitulates the phenotype of TGFbeta1-null mice. *J Cell Biol.* 2007; 1766:787–93. [PubMed: 17353357]
95. Aluwihare P, Mu Z, Zhao Z, Yu D, Weinreb PH, Horan GS, et al. Mice that lack activity of alphavbeta6- and alphavbeta8-integrins reproduce the abnormalities of Tgfb1- and Tgfb3-null mice. *J Cell Sci.* 2009; 122(Pt 2):227–32. [PubMed: 19118215]
96. Wimmer W, Perovic S, Kruse M, Schroder HC, Krasko A, Batel R, et al. Origin of the integrin-mediated signal transduction. Functional studies with cell cultures from the sponge *Suberites domuncula*. *Eur J Biochem.* 1999; 2601:156–65. [PubMed: 10091595]
97. Holmbeck K, Bianco P, Caterina J, Yamada S, Kromer M, Kuznetsov SA, et al. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell.* 1999; 991:81–92. [PubMed: 10520996]
98. Rawlings ND, Barrett AJ, Bateman A. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res.* 2012; 40(Database issue):D343–50. [PubMed: 22086950]
99. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGFbeta and promotes tumor invasion and angiogenesis. *Genes Dev.* 2000; 142:163–76. [PubMed: 10652271]
100. Garg P, Vijay-Kumar M, Wang L, Gewirtz AT, Merlin D, Sitaraman SV. Matrix metalloproteinase-9-mediated tissue injury overrides the protective effect of matrix metalloproteinase-2 during colitis. *Am J Physiol Gastrointest Liver Physiol.* 2009; 2962:G175–84. [PubMed: 19171847]
101. Turk BE, Huang LL, Piro ET, Cantley LC. Determination of protease cleavage site motifs using mixture-based oriented peptide libraries. *Nat Biotechnol.* 2001; 197:661–7. [PubMed: 11433279]
102. Oh J, Takahashi R, Adachi E, Kondo S, Kuratomi S, Noma A, et al. Mutations in two matrix metalloproteinase genes, MMP-2 and MT1-MMP, are synthetic lethal in mice. *Oncogene.* 2004; 2329:5041–8. [PubMed: 15064723]
103. Ge G, Greenspan DS. BMP1 controls TGFbeta1 activation via cleavage of latent TGFbeta-binding protein. *J Cell Biol.* 2006; 1751:111–20. [PubMed: 17015622]
104. Pappano WN, Steiglit BM, Scott IC, Keene DR, Greenspan DS. Use of Bmp1/Tll1 doubly homozygous null mice and proteomics to identify and validate in vivo substrates of bone morphogenetic protein 1/tolloid-like metalloproteinases. *Mol Cell Biol.* 2003; 2313:4428–38. [PubMed: 12808086]
105. Lyons RM, Gentry LE, Purchio AF, Moses HL. Mechanism of activation of latent recombinant transforming growth factor beta 1 by plasmin. *J Cell Biol.* 1990; 1104:1361–7. [PubMed: 2139036]
106. Antonelli-Orlidge A, Saunders KB, Smith SR, D'Amore PA. An activated form of transforming growth factor beta is produced by cocultures of endothelial cells and pericytes. *Proc Natl Acad Sci U S A.* 1989; 8612:4544–8. [PubMed: 2734305]
107. Kojima S, Rifkin DB. Mechanism of retinoid-induced activation of latent transforming growth factor-beta in bovine endothelial cells. *J Cell Physiol.* 1993; 1552:323–32. [PubMed: 8482724]
108. Flaumenhaft R, Abe M, Sato Y, Miyazono K, Harpel J, Heldin CH, et al. Role of the latent TGF-beta binding protein in the activation of latent TGF-beta by co-cultures of endothelial and smooth muscle cells. *J Cell Biol.* 1993; 1204:995–1002. [PubMed: 8432736]
109. Dennis PA, Rifkin DB. Cellular activation of latent transforming growth factor beta requires binding to the cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor. *Proc Natl Acad Sci U S A.* 1991; 882:580–4. [PubMed: 1846448]
110. Yehualaeshet T, O'Connor R, Green-Johnson J, Mai S, Silverstein R, Murphy-Ullrich JE, et al. Activation of rat alveolar macrophage-derived latent transforming growth factor beta-1 by plasmin requires interaction with thrombospondin-1 and its cell surface receptor, CD36. *Am J Pathol.* 1999; 1553:841–51. [PubMed: 10487979]

111. Jenkins G. The role of proteases in transforming growth factor-beta activation. *Int J Biochem Cell Biol.* 2008; 406–7:1068–78.
112. Bugge TH, Flick MJ, Daugherty CC, Degen JL. Plasminogen deficiency causes severe thrombosis but is compatible with development and reproduction. *Genes Dev.* 1995; 97:794–807. [PubMed: 7705657]
113. Emami N, Diamandis EP. Potential role of multiple members of the kallikrein-related peptidase family of serine proteases in activating latent TGF beta 1 in semen. *Biol Chem.* 2010; 391:85–95. [PubMed: 19919178]
114. Akita K, Okuno M, Enya M, Imai S, Moriwaki H, Kawada N, et al. Impaired liver regeneration in mice by lipopolysaccharide via TNF-alpha/kallikrein-mediated activation of latent TGF-beta. *Gastroenterology.* 2002; 123:352–64. [PubMed: 12105863]
115. Pavlopoulou A, Pampalakis G, Michalopoulos I, Sotiropoulou G. Evolutionary history of tissue kallikreins. *PLoS One.* 2010; 511:e13781. [PubMed: 21072173]
116. Abe M, Oda N, Sato Y. Cell-associated activation of latent transforming growth factor-beta by calpain. *J Cell Physiol.* 1998; 174:186–93. [PubMed: 9428805]
117. Lyons RM, Keski-Oja J, Moses HL. Proteolytic activation of latent transforming growth factor-beta from fibroblast-conditioned medium. *J Cell Biol.* 1988; 106:1659–65. [PubMed: 2967299]
118. Maeda S, Dean DD, Gay I, Schwartz Z, Boyan BD. Activation of latent transforming growth factor beta1 by stromelysin 1 in extracts of growth plate chondrocyte-derived matrix vesicles. *J Bone Miner Res.* 2001; 16:1281–90. [PubMed: 11450704]
119. Miyazono K, Heldin CH. Role for carbohydrate structures in TGF-beta 1 latency. *Nature.* 1989; 338:621:158–60. [PubMed: 2493139]
120. Schultz-Cherry S, Hinshaw VS. Influenza virus neuraminidase activates latent transforming growth factor beta. *J Virol.* 1996; 70:2:8624–9. [PubMed: 8970987]
121. Carlson CM, Turpin EA, Moser LA, O'Brien KB, Cline TD, Jones JC, et al. Transforming growth factor-beta: activation by neuraminidase and role in highly pathogenic H5N1 influenza pathogenesis. *PLoS Pathog.* 2010; 6:10:e1001136. [PubMed: 20949074]
122. Brunner AM, Lioubin MN, Marquardt H, Malacko AR, Wang WC, Shapiro RA, et al. Site-directed mutagenesis of glycosylation sites in the transforming growth factor-beta 1 (TGF beta 1) and TGF beta 2 (414) precursors and of cysteine residues within mature TGF beta 1: effects on secretion and bioactivity. *Mol Endocrinol.* 1992; 6:10:1691–700. [PubMed: 1448117]
123. Murphy-Ullrich JE, Poczatek M. Activation of latent TGF-beta by thrombospondin-1: mechanisms and physiology. *Cytokine Growth Factor Rev.* 2000; 11:2:59–69.
124. Murphy-Ullrich JE, Schultz-Cherry S, Hook M. Transforming growth factor-beta complexes with thrombospondin. *Mol Biol Cell.* 1992; 3:2:181–8. [PubMed: 1550960]
125. Ribeiro SM, Poczatek M, Schultz-Cherry S, Villain M, Murphy-Ullrich JE. The activation sequence of thrombospondin-1 interacts with the latency-associated peptide to regulate activation of latent transforming growth factor-beta. *J Biol Chem.* 1999; 274:19:13586–93. [PubMed: 10224129]
126. Yang Y, Dignam JD, Gentry LE. Role of carbohydrate structures in the binding of beta1-latency-associated peptide to ligands. *Biochemistry.* 1997; 36:39:11923–32. [PubMed: 9305986]
127. Schultz-Cherry S, Lawler J, Murphy-Ullrich JE. The type 1 repeats of thrombospondin 1 activate latent transforming growth factor-beta. *J Biol Chem.* 1994; 269:43:26783–8. [PubMed: 7929414]
128. Schultz-Cherry S, Chen H, Mosher DF, Misenheimer TM, Kruttsch HC, Roberts DD, et al. Regulation of transforming growth factor-beta activation by discrete sequences of thrombospondin 1. *J Biol Chem.* 1995; 270:13:7304–10. [PubMed: 7706271]
129. Grainger DJ, Frow EK. Thrombospondin 1 does not activate transforming growth factor beta1 in a chemically defined system or in smooth-muscle-cell cultures. *Biochem J.* 2000; 350(Pt 1):291–8. [PubMed: 10926856]
130. Bailly S, Brand C, Chambaz EM, Feige JJ. Analysis of small latent transforming growth factor-beta complex formation and dissociation by surface plasmon resonance. Absence of direct interaction with thrombospondins. *J Biol Chem.* 1997; 272:26:16329–34. [PubMed: 9195938]

131. Abdelouahed M, Ludlow A, Brunner G, Lawler J. Activation of platelet-transforming growth factor beta-1 in the absence of thrombospondin-1. *J Biol Chem.* 2000; 275:24:17933–6. [PubMed: 10849431]
132. Lawler J, Sunday M, Thibert V, Duquette M, George EL, Rayburn H, et al. Thrombospondin-1 is required for normal murine pulmonary homeostasis and its absence causes pneumonia. *J Clin Invest.* 1998; 101:5:982–92. [PubMed: 9486968]
133. Crawford SE, Stellmach V, Murphy-Ullrich JE, Ribeiro SM, Lawler J, Hynes RO, et al. Thrombospondin-1 is a major activator of TGF-beta1 in vivo. *Cell.* 1998; 93:7:1159–70. [PubMed: 9657149]
134. Bentley AA, Adams JC. The evolution of thrombospondins and their ligand-binding activities. *Mol Biol Evol.* 2010; 27:9:2187–97. [PubMed: 20427418]
135. Attur MG, Palmer GD, Al-Mussawir HE, Dave M, Teixeira CC, Rifkin DB, et al. F-spondin, a neuroregulatory protein, is up-regulated in osteoarthritis and regulates cartilage metabolism via TGF-beta activation. *FASEB J.* 2009; 23:1:79–89. [PubMed: 18780763]
136. Glinka Y, Prud'homme GJ. Neuropilin-1 is a receptor for transforming growth factor beta-1, activates its latent form, and promotes regulatory T cell activity. *J Leukoc Biol.* 2008; 84:1:302–10. [PubMed: 18436584]
137. Glinka Y, Stoilova S, Mohammed N, Prud'homme GJ. Neuropilin-1 exerts co-receptor function for TGF-beta-1 on the membrane of cancer cells and enhances responses to both latent and active TGF-beta. *Carcinogenesis.* 2011; 32:4:613–21. [PubMed: 21186301]
138. Grandclement C, Pallandre JR, Valmary Degano S, Viel E, Bouard A, Balland J, et al. Neuropilin-2 expression promotes TGF-beta1-mediated epithelial to mesenchymal transition in colorectal cancer cells. *PLoS One.* 2011; 6:7:e20444. [PubMed: 21747928]
139. Wang H, Kochevar IE. Involvement of UVB-induced reactive oxygen species in TGF-beta biosynthesis and activation in keratinocytes. *Free Radic Biol Med.* 2005; 38:7:890–7. [PubMed: 15749385]
140. Barcellos-Hoff MH. Latency and activation in the control of TGF-beta. *J Mammary Gland Biol Neoplasia.* 1996; 1:4:353–63.
141. Anscher MS, Thrasher B, Rabbani Z, Teicher B, Vujaskovic Z. Antitransforming growth factor-beta antibody 1D11 ameliorates normal tissue damage caused by high-dose radiation. *Int J Radiat Oncol Biol Phys.* 2006; 65:3:876–81. [PubMed: 16751069]
142. Biswas S, Guix M, Rinehart C, Dugger TC, Chytil A, Moses HL, et al. Inhibition of TGF-beta with neutralizing antibodies prevents radiation-induced acceleration of metastatic cancer progression. *J Clin Invest.* 2007; 117:5:1305–13. [PubMed: 17415413]
143. Barcellos-Hoff MH, Derynck R, Tsang ML, Weatherbee JA. Transforming growth factor-beta activation in irradiated murine mammary gland. *J Clin Invest.* 1994; 93:2:892–9. [PubMed: 8113421]
144. Ehrhart EJ, Segarini P, Tsang ML, Carroll AG, Barcellos-Hoff MH. Latent transforming growth factor beta1 activation in situ: quantitative and functional evidence after low-dose gamma-irradiation. *FASEB J.* 1997; 11:12:991–1002. [PubMed: 9337152]
145. Barcellos-Hoff MH, Dix TA. Redox-mediated activation of latent transforming growth factor-beta 1. *Mol Endocrinol.* 1996; 10:9:1077–83. [PubMed: 8885242]
146. Brown PD, Wakefield LM, Levinson AD, Sporn MB. Physicochemical activation of recombinant latent transforming growth factor-beta's 1, 2, and 3. *Growth Factors.* 1990; 31:35–43. [PubMed: 2200452]
147. Ahamed J, Burg N, Yoshinaga K, Janczak CA, Rifkin DB, Collier BS. In vitro and in vivo evidence for shear-induced activation of latent transforming growth factor-beta1. *Blood.* 2008; 112:9:3650–60. [PubMed: 18544680]
148. Pociask DA, Sime PJ, Brody AR. Asbestos-derived reactive oxygen species activate TGF-beta1. *Lab Invest.* 2004; 84:8:1013–23. [PubMed: 15122306]
149. Amarnath S, Dong L, Li J, Wu Y, Chen W. Endogenous TGF-beta activation by reactive oxygen species is key to Foxp3 induction in TCR-stimulated and HIV-1-infected human CD4+CD25- T cells. *Retrovirology.* 2007; 4:57. [PubMed: 17688698]

150. Jobling MF, Mott JD, Finnegan MT, Jurukovski V, Erickson AC, Walian PJ, et al. Isoform-specific activation of latent transforming growth factor beta (LTGF-beta) by reactive oxygen species. *Radiat Res.* 2006; 166:839–48. [PubMed: 17149983]
151. Brophy TM, Collier BS, Ahamed J. Identification of the Thiol Isomerase-binding Peptide, Mastoparan, as a Novel Inhibitor of Shear-Induced TGF-beta1 Activation. *J Biol Chem.* 2013
152. Teitelbaum SL. Bone resorption by osteoclasts. *Science.* 2000; 289:5484:1504–8. [PubMed: 10968780]
153. Oreffo ROC, Mundy GR, Seyedin SM, Bonewald LF. Activation of the bone-derived latent TGF beta complex by isolated osteoclasts. *Biochemical and Biophysical Research Communications.* 1989; 158:817–23. [PubMed: 2920041]
154. Jullien P, Berg TM, Lawrence DA. Acidic cellular environments: activation of latent TGF-beta and sensitization of cellular responses to TGF-beta and EGF. *Int J Cancer.* 1989; 43:886–91. [PubMed: 2785508]
155. Engle SJ, Ormsby I, Pawlowski S, Boivin GP, Croft J, Balish E, et al. Elimination of colon cancer in germ-free transforming growth factor beta 1-deficient mice. *Cancer Res.* 2002; 62:6362–6. [PubMed: 12438215]
156. Engle SJ, Hoying JB, Boivin GP, Ormsby I, Gartside PS, Doetschman T. Transforming growth factor beta1 suppresses nonmetastatic colon cancer at an early stage of tumorigenesis. *Cancer Res.* 1999; 59:3379–86. [PubMed: 10416598]
157. Bommireddy R, Engle SJ, Ormsby I, Boivin GP, Babcock GF, Doetschman T. Elimination of both CD4+ and CD8+ T cells but not B cells eliminates inflammation and prolongs the survival of TGFbeta1-deficient mice. *Cell Immunol.* 2004; 232:96–104.
158. Yang LT, Kaartinen V. Tgfb1 expressed in the Tgfb3 locus partially rescues the cleft palate phenotype of Tgfb3 null mutants. *Dev Biol.* 2007; 312:384–95. [PubMed: 17967447]

Biographies

Ian Robertson studied for his D. Phil at Oxford University in the lab of Prof Penny Handford, examining the molecular basis for the interaction of LTBP1 and fibrillin1, and more broadly looking at the structure and evolution of these molecules. Having completed his D. Phil in 2012 Ian started a Post doc in the lab of Prof Daniel Rifkin at the New York University school of medicine where he is currently studying a number of aspects of TGF biology, but is particularly interested in the biological significance of the extracellular matrix in TGF activation.

Daniel Rifkin received his Biochemistry Ph.D. from the Rockefeller University in 1968. Throughout his career he has worked on many aspects of TGF signalling and investigated

many novel mechanisms of TGF activation. He is currently the Charles Aden Poindexter Professor of Medicine at the New York University school of Medicine.

Figure 1. Simplified scheme of TGF β secretion and signalling

A summary of key steps in TGF β production and signalling. Starting at the bottom left; TGF β associates with LTBP inside the cell to form the large latent complex (LLC). After secretion the LLC is localized to ECM fibres via LTBP mediated interactions. Active TGF β may be released by a number of factors reviewed here. TGF β can induce downstream signalling by binding its type 1 and type 2 receptors. The canonical signalling pathway involves phosphorylation of R-SMADs by the type I TGF β receptor. Phosphorylated R-SMADs (pSMAD2/3) interact with SMAD4 and are translocated to the nucleus, where they regulate the transcription of many genes through interactions with CAGA repeat elements. Various nuances of TGF β signalling have been excluded here for the purposes of simplicity.

Figure 2. Phylogenetic tree of the TGF β superfamily

A) Phylogenetic tree of 359 TGF β sequences from a variety of organisms (for search and tree construction methods see supplementary information). To save space sub-trees are compressed and labelled according to the predominant superfamily members they contain. Sub-trees are coloured depending on the pattern of cysteines present in the mature growth factor domains (colour code at bottom). The vertical dimension of each sub-tree is dependent on the number of sequences it contains. Bootstrapping values are shown at each node, and the scale bar at the bottom gives the average number of amino acid substitutions per site. **B)** The expanded sub-tree of bona fide TGF β homologues. Sub-trees corresponding to TGF β 1, 2 and 3 isoform groups are highlighted with coloured boxes. Despite the wide range of animal genomes searched, only sequences from the deuterostomes are found in the true TGF β sub tree.

Figure 3. LAP cysteines

A) Structure of TGF 1 and homology models of TGF 2 and 3 with LAP cysteines highlighted as spheres. TGF 1 LAP is coloured orange while the growth factor is coloured dark red, TGF 2 LAP is coloured light green and the growth factor is coloured dark green, and TGF 3 LAP is coloured light blue and the growth factor is coloured dark blue. **B)** Alignments of cysteine containing regions, residues are highlighted blue if >60% conserved or yellow if a cysteine residue. Cysteines not conserved in over 60% of sequences are highlighted in pale orange. Specific cysteines of interest are numbered above human TGF 1, TGF 2 and TGF 3 sequences (as highlighted in A).

Figure 4. Conservation of TGFβ residues interacting with LTBP

A) Alignment of the N-terminus of the TGFβ propeptide showing absolute conservation of cysteine 33, highlighted in yellow. Residues highlighted blue are also conserved in >75% or more of the sequences. Orange arrows at the top of the alignment highlight residues that when mutated disrupt LTBP1 binding. The grey arrow highlights arginine 58, which when mutated to alanine did not affect LTBP binding. **B)** Alignment with other TGFβ superfamily members. Some members do possess cysteines in this region of sequence, but they do not align well with the conserved features of bona fide TGFβ. **C)** Structure of TGFβ-LAP (red and orange) and LTBP1 TB2 (blue). TGFβ residues that when mutated inhibit the LAP-LTBP1 interaction are highlighted as orange spheres. Residues in LTBP1 8-Cys3/TB2 that have been shown to contribute to TGFβ binding are highlighted as dark blue spheres. (Carbon atoms coloured as described, while all oxygen atoms are coloured red, nitrogen atoms coloured blue, and sulphur atoms coloured yellow)

Figure 5. Emergence of TGF β and regulatory factors

Representation of the proteins present in various organisms selected to give a comprehensive overview of bona fide TGF β evolution. The tree (top) illustrates the evolutionary relationships between each organism (according to the tree of life web project). The column below each illustrated organism shows whether or not the gene of interest is present, as indicated by a + or -, or other details such as the names of specific isoforms. Blocks of colour highlight where each gene emerges. Details on the methodologies and data used to construct this figure are described in supplementary material.

Figure 6. Alignment of LTBP 8-cys3/TB2 domains

Black arrowheads highlight acidic residues implicated in binding the TGF β propeptide (numbered according to Chen et al.), and the 2 amino acid insert shown to be important for TGF β binding is surrounded by a black box. Residues present in >60% of all sequences are highlighted as blue, or yellow when cysteine. Sequences from a variety of organisms representing all LTBP isoforms were aligned with the MEGA 5 BLOSUM matrix (Gap opening penalty 5, gap extension penalty 0.5). Alignment divided into blocks by LTBP isoform.

Integrin mediated TGF β activation mechanisms Figure 7. Integrin mediated TGF β activation mechanisms

A) Scheme for traction mediated activation by integrins. On the left the large latent complex is shown in a “resting” state with integrin binding LAP via its RGD motif. On the right the latent complex is shown under traction, which deforms LAP and releases active TGF β . Components are labelled within the figure. **B)** Scheme for protease mediated integrin activation. On the left the integrin is shown binding to LAP at the cell surface. This integrin may then recruit proteases that degrade LAP and release active TGF β , as shown on the right. **C)** Alignment of the RGD containing region of TGF β . Residues conserved in >60% or more of sequences were highlighted blue, or yellow if cysteine, a black line above the alignment highlights the RGD motif.

Figure 8. Conservation of protease cleavage sites in TGF and LTBP1

Ai) Alignments of the “latency lasso” region of LAP. Some potential MMP2/9 cleavage sites are boxed, and pairs of light red or dark red asterisks above the human TGF 2 sequence highlight two potential MMP cleavage PxxV motifs. **Aii)** Homology model of TGF 2, with LAP shown in light green, and the mature growth factor in dark green. The side chains of the two putative PxxV cleavage motifs highlighted in A) are shown as red coloured spheres. **B)** Sequence alignments of LTBP regions where putative BMP1 sites were detected (103). Residues present in 60% or more sequences are highlighted. The aspartate residues that define the putative cleavage sites are highlighted by boxes, but do not appear to be well conserved.

Figure 9. Glycosylation of TGF

A) Alignments of N-glycosylation sites from all human TGF isoforms, residues conserved in 60% or more sequences are highlighted blue. Putative N-glycosylation sites are boxed. Only the first glycosylation site is well conserved. **B)** Glycosylation sites highlighted on the structure of TGF 1. TGF and LAP are coloured as in previous figures and sugar moieties are shown coloured pink and represented as spheres. In the crystallised protein the third glycosylation site, “NNS” has been replaced with “QDS”, but the Q residue is coloured pink here and shown in stick representation labelled “N”176. The position of the RGD motif is also highlighted by a black line, although it is not resolved in the structure.

Table 1

Mouse knock outs of TGF

Mutation / disruption	Heterozygous mouse phenotype	Homozygous mouse phenotype	Other Notes	Refs
TGF 1	<ul style="list-style-type: none"> Normal and gain weight at same rate as WT mice 	<ul style="list-style-type: none"> Die after 3–4 weeks due to multi-organ inflammation Infiltration of lymphocytes and macrophages, especially the heart and lungs. High embryonic lethality Mice exhibit “dishevelled” appearance, and wasting Lack langerhans cells in epidermis Defective haematopoiesis and vasculogenesis 	<ul style="list-style-type: none"> Develop gastrointestinal tumours when exposed to pathogens or combined with Rag2 ^{-/-} mutation (155, 156), Elimination of CD4+ and CD8+ T cells prevents inflammation and prolongs survival (157) No significant inflammation seen in small intestine, thymus, most connective tissues, brain, skin, or bone marrow. 	(3)
TGF 2	<ul style="list-style-type: none"> No immediately apparent defects or embryonic lethality Closer analysis showed aortic root dissection and increased phosphorylation of Smad2, Smad3 and Erk1/2 (78) 	<ul style="list-style-type: none"> Two thirds die shortly before or during birth Congenital heart defects; thin walled aorta, ventricular septation defects, hypercellular myocardium. Skeletal defects; reduction in skull bone size and less ossification, retrognathia, complete cleft palate, shortened forelimbs, spina bifida, abnormal ribs and sternum. Eye defects Inner ear defects Urogenital defects Degeneration of kidney tubular epithelium 	<ul style="list-style-type: none"> Lungs exhibit no major defects, except collapsed airways in PN animals Brain, gut, tooth bud, and Thyroid glands normal 	(4)
TGF 3	<ul style="list-style-type: none"> Normal 	<ul style="list-style-type: none"> No prenatal lethality Offspring did not suckle and died within 20h of birth Similar birth weight to WT animals but deteriorated rapidly and showed respiratory distress Severely disrupted lung development including mesenchymal thickening, 	<ul style="list-style-type: none"> TGF 2^{-/-} TGF 3^{-/-} double nulls display early embryonic death, with failure of midline fusion (5) TGF 2^{-/-} TGF 3^{-/+} also still display severe malformations while TGF 2^{-/+} TGF 3^{-/-} mice do not 	(16) (17)

Mutation / disruption	Heterozygous mouse phenotype	Homozygous mouse phenotype	Other Notes	Refs
		<p>hypercellularity, and pleural haemorrhage</p> <ul style="list-style-type: none"> Cleft palate, (although at E14.5 shelves had grown out and were adherent, but did not fuse.) No skeletal or cardiovascular abnormalities, or changes in tooth bud and whisker follicle development, despite high TGF $\beta 3$ expression in these tissues. 	<ul style="list-style-type: none"> TGF $\beta 1$ knocked into the TGF $\beta 3$ locus gives partial but not complete rescue of palate closure (158) TGF $\beta 1^{rge/rge}$ TGF $\beta 3^{-/-}$ mice have defects in brain vascular morphogenesis (18) 	