Review

Regulation of TGFβ and related signals by precursor processing☆

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ABSTRACT

Secreted cytokines of the TGFβ family are found in all multicellular organisms and implicated in regulating fundamental cell behaviors such as proliferation, differentiation, migration and survival. Signal transduction involves complexes of specific type I and II receptor kinases that induce the nuclear translocation of Smad transcription factors to regulate target genes. Ligands of the BMP and Nodal subgroups act at a distance to specify distinct cell fates in a concentration-dependent manner. These signaling gradients are shaped by multiple factors, including proteases of the proprotein convertase (PC) family that hydrolyze one or several peptide bonds between an N-terminal prodomain and the C-terminal domain that forms the mature ligand. This review summarizes information on the proteolytic processing of TGFβ and related precursors, and its spatiotemporal regulation by PCs during development and various diseases, including cancer. Available evidence suggests that the unmasking of receptor binding epitopes of TGFβ is only one (and in some cases a non-essential) function of precursor processing. Future studies should consider the impact of proteolytic maturation on protein localization, trafficking and turnover in cells and in the extracellular space.

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1. Introduction

1.1. Mechanisms to control the bioavailability of TGFβ ligands

More than 30 members of the TGFβ family comprising TGFβ isoforms, Activins, Nodal, BMP, GDF, and AMH subgroups bind type I and II serine/threonine receptor kinases and, in some cases, specific co-receptors at the plasma membrane to induce the phosphorylation of Smad transcription factors and other signaling molecules in the cytoplasm. A distant subgroup comprising GDNF and three cousins signal through the tyrosine kinase RET. Several recent reviews describe TGFβ signal transduction pathways and their prominent roles in stem cells and development, cancer and other diseases, and current strategies for therapeutic targeting [1–4]. Much information is also available on the regulation of TGFβ signaling at the level of transcription, ubiquitination and miRNA-mediated silencing of receptors, interactions with co-receptors, endocytosis, Smad-interacting factors, and modification of Smad-target sites in DNA by chromatin-modifying enzymes [1,2]. Of particular interest is the hyperactivation of TGFβ protein production as it is common in many solid cancers and contributes to tumor progression [5]. The bioavailability of Nodal and BMPs also shapes morphogen signaling gradients to pattern whole embryos and tissues during normal development, together with feedback regulators that buffer fluctuations in expression levels and control signal duration and spreading [1,6,7]. In addition, reversible sequestration by inhibitory protein complexes is employed to redistribute and release active ligands within their expression domains [reviewed in 8–10]. Genetic and epigenetic perturbations altering the expression levels of secreted BMP antagonists such as Noggin, Gremlin-1 and Dand5 (Coco/Cerl2) also contribute to the re-wiring of oncogenic signaling in human cancers [reviewed in 1,11–14]. By comparison, little is known about how TGFβ and related ligands are released from seemingly inactive precursors, and no previous review has been dedicated to this topic. This review will therefore first describe molecular mechanisms that govern the release of active TGFβ and related ligands after precursor cleavage, with a focus on the general and specialized functions of individual prodomains in conferring latency (Table 1). I will then survey new roles of intracellular and secreted PCs that have been linked to TGFβ signaling in diverse contexts, followed by reflections on how they regulate proteolytic cleavage and to what end, and what aspects of this process merit further investigation.

1.2. Precursor cleavage is not sufficient to activate TGFβ

TGFβ ligands mature from precursor dimers as proteolytic fragments containing 7–9 conserved cysteines that stabilize a characteristic cystine knot structure through 1 interchain and 3–4 intrachain disulphide bonds [15]. The N-terminal prodomain that is cleaved off usually remains bound in an inactive small latent complex (SLC) as a latency associated peptide (LAP). A crystal structure of latent TGFβ (L-TGFβ) revealed that a homodimer of LAP is held together like two arms at the neck by two interchain disulphide bonds. The forearms consisting of an N-terminal α1-helix and a so-called latency lasso are buckled into a straightjacket around mature ligand to mask receptor-binding epitopes [16] (Fig. 1A). Dissociation from LAP is arguably the most critical step to regulate TGFβ signal activation [3]. Possible mechanisms include dissociation at acidic pH or proteolysis of LAP by matrix metalloproteinases [17]. Alternatively, the SLC is disrupted by tensile forces mediated by specific integrins such as αvβ6 or αvβ8 that interact with an RGD motif in LAP [16,18,19]. Knock-in mice with a substitution of RGD by RGE phenocopy $Tgfb1^{-/-}$ mice, indicating that integrin-mediated activation predominates *in vivo* [20]. Integrin-mediated activation by tensile forces is regulated by L-TGFβ-binding proteins (LTBP) in the extracellular matrix [21]. LTBP-1, -2 or -4 form a disulphide bond with Cys33 at position 4 of the α1-helix of LAP to facilitate the secretion and matrix deposition of TGFβ in a large latent complex (LLC). Mutation of Cys33 in mice attenuates signaling and leads to multiorgan inflammation reminiscent of *Tgfb1* null mutants, suggesting that LLC formation is critical to activate L-TGFβ [22]. On the other hand, inactivation of fibrillin-1, an LTBP-anchoring matrix protein in microfibrils that is mutated in Marfan syndrome [23,24], leads to excess release of active TGFβ and thereby increases the risk of aortic aneurisms [25,26]. Fibrillin also tethers several BMP prodomains and associated mature ligands, including BMP2,4,5,7,10 and GDF5 [27–29]. Presumably, these ligands are deposited in ECM for storage and release upon demand.

In bone marrow, L-TGFβ is activated by αvβ8 integrin of non-myelinating Schwann cells of the autonomic nervous system that are in direct contact with lineage-negative hematopoietic stem cells. Although HSCs produce L-TGFβ, they depend on these novel niche cells as a source of active TGFβ to maintain their quiescence and long-term repopulation potential [30]. Analogous interactions with the microenvironment may also control the bioavailability of active TGFβ in other stem cell compartments. Alternatively, integrins αvβ8 and αvβ86 can activate L-TGFβ that is presented by the spring-shaped transmembrane protein GARP at the plasma membrane, *e.g.* in immunosuppressive T regulatory cells [31,32] (Fig. 1B). Association of LAP with GARP involves Cys33 [32], indicating that some anti-inflammatory TGFβ signals attributed to activation by LTBPs may depend on GARP. Images of LAP and integrins bound to LTBP or GARP obtained by negative stain electron microscopy support the model that integrins pull on LAP subunits to activate L-TGFβ that is held in place by LTBP or GARP [32]. However, the role of precursor cleavage remains obscure because the structure of the loop with the PC cleavage site in crystals of L-TGFβ was disordered (Fig. 1A, stippled line). Since TGFβ precursor can be activated even in its uncleaved form after acidification *in vitro* [33], it would be interesting to know why precursor cleavage is necessary to overcome latency *in vivo*.

Table 1
Prodomains found in complexes with TGFβ-related mature ligands after precursor cleavage.

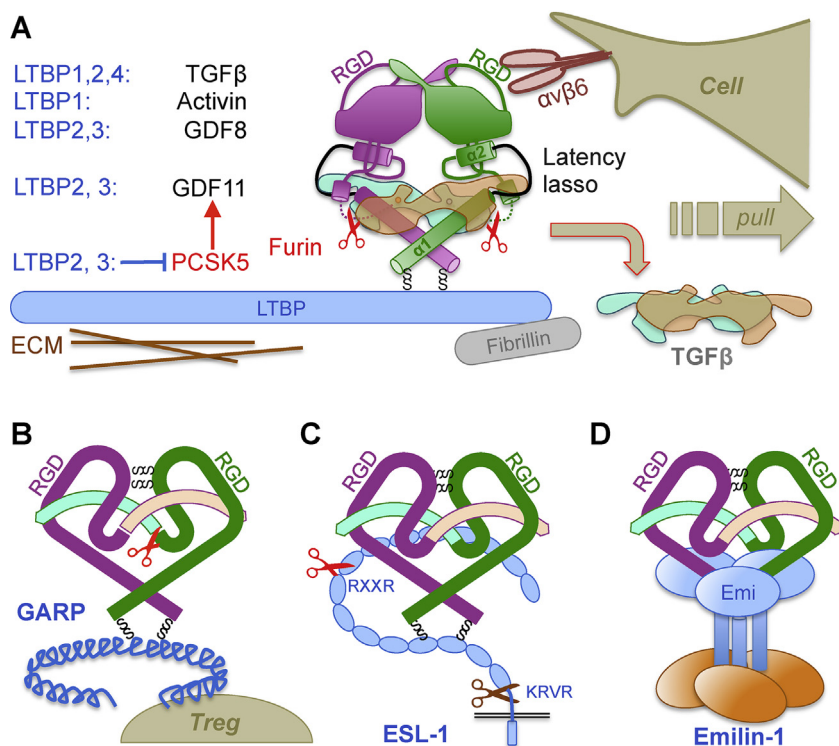|	Inhibitory	References
Activin A	No	[166,180]
BMP2,4,7	No	[28,44]
BMP9	No	[181]
BMP10	Yes	[29]
GDF2 (Dorsalin)	n.d.	[67]
GDF5	No	[28]
GDF8 (Myostatin)	Yes	[45,46]
GDF15 (MIC-1)	n.d.	[38]
GDF11	Yes	[50]

Fig. 1. Regulation of TGFβ bioavailability. (A) Maturation of TGFβ and related ligands requires proteolytic cleavage of the precursor dimer by Furin or a related PC (scissors). Mature TGFβ, Activin, GDF8 and GDF11 can remain bound to cleaved prodomains that are attached to the indicated LTBP for storage in extracellular matrix (ECM). Integrin heterodimers that bind an RGD motif in the prodomain release mature TGFβ from the latent complex to liberate receptor-binding epitopes from a straightjacket formed by the first α-helix (α1) and the latency lasso (black) of the prodomain. Note that LTBP2 and 3 can also bind and inhibit PCSK5. (B) Alternatively, L-TGFβ is linked to the spring-shaped cell-surface molecule GARP. (C and D) The Golgi protein ESL-1 (C), which has 16 cysteine-repeats that can be shed and further processed by PC-like activities, and the EMI domain of the trimeric matrix protein Emilin-1 (D) can inhibit processing of the TGFβ precursor, but their binding sites in TGFβ have not been mapped in detail.

1.3. Roles of prodomains in secretion and latency of TGFβ-related ligands

How prodomains of other TGFβ family members influence the bioavailability of mature ligands is less clear. Generally, prodomains assist in the folding of the precursor in the endoplasmic reticulum [34,35]. Mutagenesis of TGFβ and Inhibin-α revealed that secretion and/or stability depend on the interface between mature ligand and six hydrophobic residues in the C-terminal half of the α1-helix of the prodomain [36]. Only Nodal, GDF6, GDF15 (MIC-1) and AMH lack several of these highly conserved residues (Fig. 2) Interestingly, the same ligands can adopt an active conformation independently of their prodomains [37–41]. Which of these and other prodomains may confer latency remains to be systematically analyzed. In TGFβ1, latency depends on covalent homodimerization of LAP by Cys223 and Cys225 [42]. Analogous regions in BMP and Nodal prodomains lack cysteines [16], explaining perhaps why they do not inhibit binding of mature ligand to receptors [43,44]. BMP and Nodal prodomains also lack cysteines in the α1-helix to bind LTBPs or analogous tethering molecules. By contrast, such cysteines can be found in Inhibin-α, GDF8 (myostatin), GDF11, and BMP8 (Fig. 2). GDF8 and GDF11 also form latent complexes [45,46] and interact with LTBP2,3 [47,48], suggesting that their activation may follow the paradigm of L-TGFβ. Alternatively, the straight jackets that confer latency to GDF8 and GDF11 can be removed by proteolysis. A peptide bond at the C-terminus of the second conserved α-helix in the prodomain of GDF8 proximal to Asp76 can be cleaved by metalloproteases of the BMP1/Tolloid family, leading to the release of mature ligand [49]. BMP1/Tolloid proteases recognize the sequence Y/H-X-Y/F-X-X'D [50]. A similar motif exists in

the prodomains of all TGFβ family members that form latent complexes, including TGFβ1–3, BMP10, GDF2, GDF5 and GDF15, but not in others (Fig. 2), and BMP10 can be cleaved by BMP1 [29]. Cleavage by BMP1/Tolloid thus may be a common alternative strategy to overcome latency.

1.4. Mutations in the prodomains of TGFβ family members associated with human diseases

Human mutations in prodomains of TGFβ and related precursors are found in several diseases. In Camurati-Engelmann disease, a rare genetic disorder characterized by progressive thickening of bones, mutations in the LAP of TGFβ cluster around cysteines 223 and 225 and destabilize the SLC [51–54]. Other skeletal malformations such as shortening of the second and third fingers (brachydactyly type C) and misshapen phalanges are seen in families with a substitution of S204 by arginine in GDF5 [55], and BMP4 prodomain was found mutated in patients with cleft lips [56]. Premature decline in ovarian follicle formation in women is linked to mutations in the BMP15 prodomain [57], and missense mutations in the prodomain of AMH occur in patients with persistent Müllerian duct syndrome [58,59]. Finally, mutations in the prodomain of NODAL associate with congenital left-right patterning defects of the heart outflow tract. In particular, deletion of residues R234 to P241 destroys the NODAL precursor cleavage motif, whereas an E203K substitution may be part of a co-receptor binding site [60–62]. However, to interpret the phenotypes associated with specific mutations, a better understanding of the normal functions of prodomains and of their processing will be needed.

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                    α1                                                    α2          ↓
TGF1po    LSTCKTIDMELVKRKRIEAIRGQILSKLRLASPPSQGDV--------------------PP--------GPLPEAVLALYNSTRDRVAGESVEPE  67
TGF1hu    LSTCKTIDMELVKRKRIEAIRGQILSKLRLASPPSQGEV--------------------PP--------GPLPEAVLALYNSTRDRVAGESAEPE  67
TGF2      SLSTCSTLDMDQFMRKRIEAIRGQILSKLKLTSPPEDY--------------------PE--------PEEVPPEVISIYNSTRDLLQEKASRRA  67
TGF3      ..SLSTCTTLDFGHIKKKRVEAIRGQILSKLRLTSPPE-------------------PT-------VMTHVPYQVLALYNSTRELLEEMHGERE  68
GDF8      ..EGLCNACTWRQNTKSSRLESIKIQILSKLRLETAPNISKDVIRQLL--------------PK--------APPLRELIDQYDVQRDD-SSDGSLED  85
GDF11     ..PDGCPVCVWRQHSRELRLESISKSQILSKLRLKEAPNISREVVKQLL--------------PK--------APPLQQILDLHDFQGDALQPEDFLEE  108
Inh_bA    ..CALAALPKDVPNSQPEMVEAVKKHILNMLHLKKRPDVTQPV--------------PK--------AALLNAIRKLHVGKVGENGYVEI---  83
Inh_bB    ..CGGFRRPEELGRVDGDFLEAVKRHILSRLQMRGRPNITHAV--------------PK--------AAMVTALRKLHAGKVREDGRVEI---  95
Inh_bC    ..CGG--PTLELESQRELLLDLHAKRSILDKLHLTQRPTLNRPV-------------SR--------AALRTALQMLHAGKVPQGALLEDNR---  75
Inh_bE    ..CGG--SKLAPQAERALVLELAKQQILDGLHLTSRPRITHPP-------------PQ--------AALTRALRRLQPGSVAPGN-------  68
Inh_a     ------CQGLELARELVLAKVRALFLDAL--GPPAVTREGGD-------------PG-------------VRRLPRRHALGGFTHRGSEP  56
BMP3      -----------ERPKPPFPELRKAVPGDRTAGGGPDSELQ-------------PQ----------DKVSEHMLRLYDRYSTVQAARTPGSL  57
GDF10     --------SHRAPAWSALPAAADGLQGDRDLQRHPGDAAATLG-----------PS--------AQDMVAVHMHRLYEKYSRQGARPGG---  62
BMP15     MEHRAQMAEGGQSSIALLAEAPTLPLIEEL-LEESPGEQPRK-------------PR------LLGHSLRYMLELYRRSADSHGHPRENRT  71
GDF9      ..SAELESGAMPWSLLQHIDERDRAGLLPALFKVLSVGRGGSPRLQ----------------PD----------SRALHYMKKLYKTYATKEGIPKSNRS  84
GDF1      ---------------PVPPGPAAALLQALGLRDEPQGA---------------PR--------LRPVPPVMWRLFRRRDPQETRSGSRRT  52
GDF3      ----------------QEYVFLQFLGLDKAPS---------------PQ---------KFQPVPYILKKIFQDREAAATTGVSRDL  46
BMP10     ..LFGDVFSEQDGVDFNTLLQSMKDEFLKTLNLSDIPTQDSAKVDP-----------------PE----------YMLELYNKFATDRTS------  77
GDF2      ..GVPGGGLPEHTFNLKMFLENVKVDFLRSLNLSGVPSQDKTRVEP-----------------PQ---------YMIDLYNRYTSDKSTT-----  81
BMP2      ..FAAASSGRPSSQPSDEVLSEFELRLLSMFGLKQRPT-----------------PS-------RDAVVPPYMLDLYRRHSGQPGSPAPDHR  75
BMP4      ..IQGHAGGRRSGQ-SHELLRDFEATLLQMFGLRRRPQ-----------------PS-------AALVPPYMRDLYRLQSGEEEEEQIHST  83
BMP5      DNHVHSSFIYRRLRNHERREIQREILSILGLHRPR-----------------------PF---SPGKQASSAPLFMLDLYNAMTN---EENPEES  67
BMP7      ..DNEVHSSFIHRRLRSQERREMQREILSILGLPHRPR----------------------PH---LQGKH-NSAPMFMLDLYNAMAV---EEGGGPG  70
BMP6      ..SPQSSSGFLYRRLKTQEKREMQKEILSVLGLPHRPRPLHGLQQPQPPALRQQEEQQQQQQLPRGEPPPGRL-KSAPLFMLDLYNALSADNDEDGASEG  135
BMP8      ..GLRPPPGCPQRRLGARERRDVQREILAVLGLPHRPR--------------------PRAPPAASRLPASAPLFMLDLYHAMAGDDDDEDGAPAE  77
GDF5      ..TVTPKGQLPGGKAPPKAGSVPSSFLLKKAREPGPPREPKEPFRPPPIT-------------PH------------EYMLSLYRTLSDADRKGGNSSV  165
GDF7      ..LRAAGAGPVRSPGGGGGGGGGGGRTLAQAAGAAAVPAAAVPRARAARRAAGSGFRNGSVV--PH------------HFMMSLYRSLAGRAPAGAAAVS  92
LEFTY1    --------------LTGEQLLGSLLRQLQLKEVPTLDRADMEELVI-------------PT---------HVRAQYVALLQRSHGDRSRGKRFSQS  60
LEFTY2    --------------LTEEQLLGSLLRQLQLSEVPVLDRADMEKLVI-------------PA---------HVRAQYVVLLRRSHGDRSRGKRFSQS  60
NRTN      ..SHRLGPALVPLHRLPRTLDARIARLAQYRALLQGA-------------------PD----------AMELRELTPWAGRPPGP------  64
ARTN      ..APREGPPPVLASPAGHLPGGRTARWCSGRARRPPP-------------------QP------SRPAPPPPA-------------------  55
PSPN      ------------------WGPDARGVP-------------------------VA----------DGEFSSEQVAKAGGTWLGTHR--------  32
α-GDNF    --------FPLPAGKRPPEAPAEDRSLGRRRAP---------------------FALSS----DSNMPEDYPDQFDDVMDFIQATIKRLKRSPD  61
β-GDNF    --------FPLPA-------------------------------------NMPEDYPDQFDDVMDFIQATIKRLKRSPD  61
Nodal     ------------------TVATALLRTRGQPSSPS---------------------PL-------------AYMLSLYR-------------  27
GDF6      ..RKEGKMQRAPRDSDAGREGQEPQPRPQDEPRAQQPRAQEPPGRGPRVV-------------PH------------EYMLSIYRTYSIAEKLGINASF  96
GDF15     -----------------LSLAEASRASFPG--------------------PS---------ELHSEDSRFRELRKRYEDLLTRLRANQS  43
AMH       ..LGAWLRDPGGQRLVVLHLEEVTWEPTPSLRFQEPP-------------------PG------GAGPPELALLVLYPGPGPEVTVTRAGLP  164
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Fig. 2. Amino-terminal sequences of TGFβ-related prodomains. Numbers indicate amino acid positions relative to the last residue of cleaved signal sequences. Cys33 (fourth residue in the α1-helix of TGFβ1) and analogous cysteines are shaded pink. Conserved hydrophobic residues required for an interaction of the straightjacket with the mature domain are highlighted yellow. An arrow marks the peptide bond proximal to the partially conserved residue Asp76 (red) of GDF8 that is hydrolyzed by Bmp1/Tolloid to overcome latency. Some BMP ligands in *Drosophila*, mammalian Lefty1 and -2 and GDNF have instead a PC motif at this position (green). Both (α) and (β) GDNF prodomains terminate after this first PC motif, and 27 residues of the N-terminal α1-helix are spliced out in (β)GDNF. Alignments are from Ref. [16]. Dashes indicate gaps, dots represent N-terminal extensions that are not shown.

2. Roles of proprotein convertases in TGFβ signaling

2.1. Processing by partially overlapping PC activities

Compared to the regulation of TGFβ latency, relatively little is known about the mechanisms that control precursor processing. Engineered mutations in cleavage sites usually give rise to secreted precursors that can act as dominant negative mutants at least when overexpressed [63–65], suggesting that proteolytic maturation is indispensable. In general, TGFβ and related precursors are only partially cleaved in most cell lines and tissues examined, but the rates of cleavage vary, sometimes in a species-specific manner. Dramatic differences in the steady state levels of mature BMP15 can explain different ovulation quota in mice compared to man [66]. The true extent of cleavage and its regulation cannot be estimated, though, without taking into account the turnover of mature forms by endocytosis or other mechanisms [40,67]. In other words, low steady state concentrations of mature form of a given ligand are no proof that precursor processing is inefficient.

Cleavage site sequences considerably diverge among TGFβ-related precursors and across species [68]. The only conserved feature consists of an RXXR consensus sequence, with X corresponding to any amino acid. The RXXR motif fits the minimal recognition motif of Furin (SPC1, PCSK3) and of the related serine proteases PACE4 (SPC4, PCSK6), PCSK5 (SPC5, PC5/6) and PC7 (SPC7, LPC, PC8, PCSK7) [69–72]. In mammals, these and five additional enzymes represent a family of eukaryotic proprotein convertases (PC) of the subtilisin/kexin type (PCSK) that share similar catalytic domains with bacterial subtilases [reviewed in 73]. A crystal structure of Furin and homology models of related PCs are available [73–75, for a discussion of their therapeutic targeting, see 76]. Furin, Pace4, Pcsk5 and PC7 are widely expressed in most tissues and cell lines [70]. Nevertheless, unique phenotypes of knockout mice suggest that functional redundancy is not unlimited [reviewed in 73, 77–82]. Non-redundant functions may reflect tissue-specific differences in relative expression levels, distinct trafficking itineraries as well as subtle differences in the active sites of individual PCs.

Of many possible PC substrates, only few have been validated *in vivo*. The first study linking TGFβ processing to PCs showed that precursor cleavage is inhibited in cells treated with the pan-PC inhibitor decanoyl-RVKR-chloromethylketone and in the Furin-deficient LoVo colon carcinoma cell line. Conversely, overexpression of Furin or treatment with recombinant Furin in cell-free assays stimulated TGFβ precursor cleavage [83]. Independently, a screen for candidate BMP convertases identified Furin, Pace4, Pcsk5 and the novel PC7 in multiple tissues that are patterned by BMP signals [84]. Overexpression in *Xenopus* and in tissue culture cells confirmed that Furin, PACE4, Pcsk5 and PC7 can promote Bmp4 processing, whereas the Nodal precursor was cleaved by Furin and PACE4 [67,85]. Dissecting the contributions of endogenous PCs to TGFβ signaling turned out to be more complicated owing to functional redundancy. Another limiting factor to validate the role of PCs in BMP processing is the lack of reagents to reliably detect cleaved substrates of interest at physiological expression levels. Genetic analysis showed that neither the loss *of Furin*, *Pace4*, *Pcsk5* or *PC7* alone abolished known functions of Bmp4
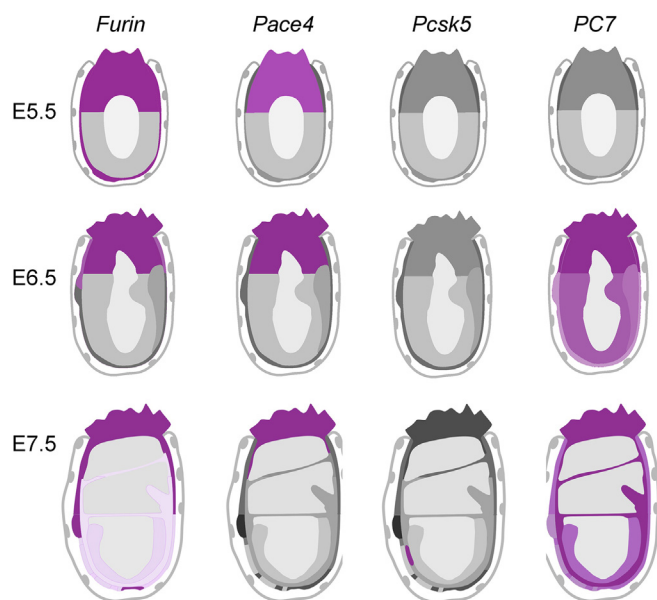
Fig. 3. Summary of PC expression patterns observed in mouse embryos. While Pace4 mRNA specifically marks the extraembryonic ectoderm lineage at all stages between embryonic days E5.5-7.5, Furin is also transiently expressed until E5.5 in visceral endoderm (purple). PC7 is the first PC expressed in the epiblast and its derivatives, starting at E6.5. Pcsk5 is specifically induced in cardiac mesoderm by E7.5. By this stage, epiblast derivatives also express Furin, albeit at low levels.

or Bmp2 in mouse embryos [77,78,81,82], and mature Bmp4 was still detected in *Furin;Pace4* double mutants [86]. However, combined absence of *Furin* and *Pace4* severely inhibited the induction of Nodal target genes [86]. By contrast, combined inactivation of *Furin* and *PC7* selectively blocked BMP, but not Nodal signaling (D.B.C., unpublished). Concurring with these observations, activation of Nodal-related Xnr1, Xnr2 and Xnr3 in *Xenopus* required maternal xPace4 [87,reviewed in 88], whereas combined inactivation of xFurin, xPCSK5 and xPC7 was necessary to abolish the processing of proBmp4 [89]. Exogenous Pcsk5 can also cleave mouse Nodal [86]. However, in the mouse embryo, *Pcsk5* normally is only induced during late gastrulation, explaining why Nodal processing relies on *Furin* and *Pace4* [90] (Fig. 3). Several Furin-like proteases also exist in *Drosophila*, including DFur1, DFur2 and Amon, and DFur1 and DFur2 cleave the BMP4/2 homolog Dpp in S2 cells [68]. Functional overlap among convertases may explain why PCs have not yet emerged from genetic screens for modifiers of BMP signaling in these or other invertebrates.

2.2. Flanking sequences do not predict which PCs will recognize an RXXR motif

PC recognition sequences that contain an additional basic residue (either RXRXXR or RXK/RR) tend to be more rapidly cleaved than the minimal RXXR motif, and RXXR can even entirely resist PC7 in some contexts [89,91], but not in others [72]. Which PCs cleave a given TGFβ family member cannot be predicted. In adult mice, deletion of a conditional *Furin* allele by CD4-Cre in T lymphocytes impairs the induction of T regulatory cells, leading to autoimmunity and inflammatory bowel disease due to severe inhibition of TGFβ1 precursor processing [92]. Why cleavage of the sequence SSRHRR↓ALDT in the TGFβ1 precursor is not rescued by other PCs is unknown. Functional redundancy among PCs is also limited in the case of GDF5 processing: Loss of *Pcsk5* causes late gestation phenotypes that recapitulate defects seen in *Gdf11* mutants, including homeotic transformations of vertebrae, caudal truncations and renal agenesis [80,81,93]. One study reported that PCs other than

Pcsk5 also fail to cleave GDF11 *in vitro*, and this was attributed to a conserved Asn residue (N) in the cleavage motif TKRSRR↓NLGL [80]. However, others reported that GDF11 can also be cleaved *ex vivo* by PACE4 or by soluble Furin [47,48], and processing of the similar sequence SARIRR↓NAKG in the Bmp10 precursor which carries Asn at an analogous position relies mainly on Furin, at least in cultured primary hepatocytes [94]. These examples show that more work is needed to decipher the logic that governs the cleavage specificities of individual PCs *in vivo*.

2.3. Regulation of PC trafficking and autoactivation

In order to be cleaved, substrates must colocalize at some point with active forms of their convertases. The regulation of this colocalization before or after exocytosis emerges as a critical determinant of substrate specificity, since each PC has its unique trafficking itinerary and follows a distinct program of zymogenic maturation [95]. Substrates also travel on distinct secretory and endocytic routes, and their cleavage sites may be masked at some steps along the journey. The trafficking of PCs can be summarized as follows [for in-depth review, see 95]:

- Furin, PC7 and a long splice variant (isoform B) of PCSK5 have trans-membrane and cytosolic domains that enable the cycling between the trans-Golgi network (TGN) and the plasma membrane through endocytic pathways [91,96–99]. Alternatively, a fraction of PC7 can reach the plasma membrane by a TGN-independent route [100].
- Shedding by unidentified proteases can give rise to soluble forms of Furin and PCSK5B [101–103].
- PACE4 and a short splice variant of PCSK5 (isoform A) are soluble by default, but can be thethered to the cell surface by HSPGs [104].
- All PCs derive from inactive zymogens; folding into an active conformation depends on their N-terminal prosegments. Auto-cleavage of the prosegments enables translocation to the Golgi apparatus, but is not sufficient to unmask the active site [70].
- Liberation of Furin from its inhibitory prosegments requires a secondary autocleavage that is regulated by endosomal acidification [105–107]. By contrast, removal of the prosegment of PCSK5A involves an intermolecular cleavage at the cell surface that is induced by cAMP [104].

However, these conclusions are based on overexpression studies. The distribution of endogenous PCs has remained obscure owing to the lack of specific antibodies. An immunoelectron microscopy analysis by Bendayan and colleagues is a notable exception, which detected large amounts of Furin at the plasma membrane of capillary endothelial cells, and in endosomes and basolateral membrane of intestinal and proximal renal tubule epithelial cells, in addition to Golgi stacks and endosomes [99]. Endosomes and the Golgi apparatus also contain Furin in liver hepatocytes, but co-localization with TGN markers has only been reported with overexpressed HA-tagged Furin [99].

2.4. Extracellular or intracellular precursor processing?

When and where a PC substrate matures also depends on its own trafficking. TGFβ itself is generally believed to mature intracellularly. The TGFβ1 precursor acquires complex carbohydrate modifications during exocytosis, indicating transit through the TGN [33]. Pioneering studies detected mature TGFβ alongside uncleaved precursor in conditioned media as well as in lysates of COS-1 and CHO cells [35,108], whereas in a human erythroleukemia cell line the mature form was only found extracellularly [109]. The release of mature form was blocked by pharmacological inhibitors of endosomal acidification such as chloroquine or monensin [109,110].

Therefore, and since overexpressed Furin accumulates in the TGN, it is widely accepted that TGFβ matures in a late Golgi compartment. One caveat is that inhibitors of endosomal acidification also block endocytosis of TGFβ [111] as well as the endocytic recycling and shedding of Furin [62,105]. Mature TGFβ thus will disappear in lysates of chloroquine-treated cells regardless of whether processing normally takes place before or after TGN transit. Since the mere presence of mature TGFβ in cell lysates does not allow to discern whether it was produced in the TGN or at the cell surface or even in endosomes, the case for intracellular processing in our view is not settled. In any case, in systems where the majority of secreted TGFβ is unprocessed, a more relevant question might be whether or not this uncleaved pool can still be used at a later stage and if so, how.

Certain prodomains may be impervious to cleavage by intracellular PCs. In GDNF, which provides neurotrophic support to dopaminergic neurons, alternative splicing gives rise to the isoforms α and β that differ by 27 residues in their short prodomains (Fig. 2). Mature α and β GDNF accumulate together with their precursors in conditioned media of CHO fibroblasts and of neurons derived from PC12 pheochromocytoma cells. By contrast, only uncleaved forms are found in cell lysates, and immunostaining of unpermeabilized cells shows that these accumulate at the cell surface [112]. Immunoelectron microscopy also detected (α)pro-GDNF in the Golgi, whereas (β)pro-GDNF was primarily found in vesicles of the regulated secretory pathway. This suggests that sorting of pro-GDNF to distinct exocytic compartments is regulated by prodomain sequences. No intracellular cleavage was detected, even though the cleavage motif IKRLKR↓SPDK of GDNF perfectly fits a consensus PC recognition sequence.

The importance of prodomain sequences in localizing precursor cleavage is underscored by studies on GDF15. Uncleaved GDF15 precursor is secreted in copious amounts by prostate cancer cells and other tumors, but then matures extracellularly as shown by pulse-chase analysis in undifferentiated U937 monocytoid cells [41,113]. The release of mature GDF15 into the circulation predicts an increased risk of metastasis in human prostate carcinoma [114]. It also leads to systemic effects such as anorexia and weight loss [115]. On the other hand, high levels of uncleaved GDF15 precursor bound to extracellular matrix through its prodomain correlate with increased risk of relapse after surgery [116]. These findings suggest that storage of GDF15 precursor in the ECM and the regulation of its extracellular maturation are likely clinically relevant.

2.5. Specific binding proteins inhibit maturation of selected PC substrates

Cleavage of the TGFβ precursor can be inhibited by the interacting factors E-selectin ligand-1 (ESL-1) or Emilin-1 (Fig. 1C, D). ESL-1 copurified specifically with uncleaved TGFβ1 precursor from CHO cell conditioned medium in a disulphide-linked complex distinct from LLC [117]. ESL-1 is widely expressed and required for normal chondrocyte differentiation and skeletal growth [118]. Loss of ESL-1 in growth plates enhanced pSmad2,3 signaling, whereas overexpression in cultured cells had the opposite effect. Binding to ESL-1 slowed down TGFβ precursor cleavage by Furin in transfected cells and in cell-free assays. Owing to its localization in the Golgi, ESL-1 thus has been proposed to interfere with intracellular TGFβ processing [118]. Interestingly, ESL-1 is itself a PC substrate, and its shedding by a Furin-like convertase generates a soluble form that interacts with heparan sulfate proteoglycans (HSPG) at the cell surface [119,120]. It might be interesting to test, therefore, whether HSPGs influence the localization or processing of an ESL-1/proTGFβ complex.

Uncleaved TGFβ, but not latent or mature forms also associate with Emilin-1 [121], a protein in the ECM of elastic fibers around blood vessels [122]. Deletion of Emilin-1 in mice leads to vasoconstriction and hypertension, which can be rescued by removing one copy of Tgfb1. Biochemical analysis in transfected cells revealed that Emilin-1 directly binds the precursor and blocks its proteolytic maturation. By contrast, signaling of recombinant mature TGFβ was not inhibited, suggesting that Emilin-1 selectively acts on the precursor. Emilin-1 equally inhibited TGFβ precursor processing and signaling regardless of whether it was coexpressed with TGFβ in the same cells or provided in trans. This implies that active TGFβ was mainly derived from extracellular precursor in this experimental setup. Ectopic expression of ESL-1 or Emilin-1 in Xenopus also interfered with the activation of Nodal signaling [118,121]. Other proteins that bind specific precursors to inhibit their proteolytic maturation may include LTBP2 and LTBP3. They attenuated processing of GDF11 when overexpressed in HEK293 cells, and extracellular GDF11 is mainly stored as an uncleaved precursor in mouse hind limb skeletal muscle [47,48]. In addition, recent genetic studies in Drosophila and in mammalian cells revealed that intracellular processing of the unrelated Notch precursor is inhibited by the novel Notch-interacting factor Botch [123]. However, analogous endogenously expressed inhibitors of Nodal or BMP processing remain to be identified.

2.6. Live imaging reveals paracrine activity of extracellular Nodal convertases

Genetic analysis of PC functions in mice suggests that Pace4 and Furin act extracellularly to regulate TGFβ signals mediated by Nodal. In chordates, Nodal proteins induce mesoderm and endoderm formation and determine visceral left-right asymmetry [124]. In addition, deletion of Nodal in mouse embryos leads to premature neural gene expression at the expense of pluripotency markers such as Oct4 and Nanog [125–127], a phenotype that can be recapitulated in human embryonic stem cells by pharmacological inhibition of Nodal/Activin receptors [128–130]. Deletion of Nodal also induces precocious differentiation of germ cells in male embryonic gonads [131], and combined inhibition of Nodal/Activin receptors or germ cell-specific ablation of Smad4 drives male germ cells into meiosis, suggesting that autocrine Nodal signaling cooperates with related ligands to promote male sexual differentiation [132,133]. A growing body of literature also links ectopic Nodal signaling to cancer cell invasiveness in testicular and several other tumors [reviewed in 1,131].

In the early mouse embryo, Nodal expression is primarily required in epiblast cells that derive from the inner cell mass of the blastocyst [134]. A first hint that Nodal matures exracellularly came from the observation that Furin and Pace4 during gastrulation are only transcribed in extraembryonic lineages (Fig. 3). Removal of this extraembryonic source in genetically mosaic chimeras and in cultured explants inhibited autocrine Nodal functions within the epiblast [86]. Pulse-chase experiments in transfected cells later confirmed that Nodal matured after the appearance of the precursor in conditioned medium [40]. However, tissue culture cells lack specific regulatory factors that may control Nodal trafficking in vivo. In particular, they do not express Cripto or the related coreceptor Cryptic which normally associate with Nodal and its type I signaling receptor ActR1B [reviewed in 135]. Cotransfection of Cripto drastically reduces the solubility of Nodal precursor [62]. Furthermore, reconstitution experiments with wild-type or mutant forms of Cripto revealed that a glycosyl-phosphatidylinositol (GPI) modification is indispensable for membrane anchoring, and that substitution of the GPI signal by a transmembrane domain blocks ActR1B/Smad2,3 signaling by Nodal [136]. Smad signaling is also abolished if the GPI anchor is disrupted by a C-terminal Flag epitope, even though Flag-tagged Cripto can still bind both Nodal and ActR1B [137, and our unpublished observation]. So why is the GPI

anchor so important? Biochemical analysis revealed that Cripto recruits uncleaved Nodal to lipid rafts and thereby facilitates access of the mature form to signaling receptors at the limiting membrane of early endosomes [62,138]. Independently, Cripto can bind the Nodal convertases through a conserved sequence flanking their catalytic domains. These observations suggest that GPI-anchoring of Cripto directs the proteolytic maturation of Nodal to membrane domains that have access to signaling-competent endosomes [reviewed in 139].

An important prediction of this model was that Pace4 and Furin *in vivo* would have to act at a certain range to directly activate a functionally relevant pool of Nodal at the surface of distant epiblast cells (Fig. 4A) [140]. Confirming this prediction, expression of transgenic GFP-tagged Furin or Pace4 specifically in the extraembryonic compartment revealed GFP fluorescence reaching the epiblast through the extracellular space [141] (Fig. 4B). Rescue of Nodal signaling in *Furin*−/−;*Pace4*−/− double mutant embryos confirmed that these transgenes were functional. Furthermore, to directly image paracrine PC activity at the cell surface, a Cell-Linked Indicator of Proteolysis (CLIP) was developed. This reporter substrate consists of secreted eCFP and GPI-anchored citrine, fused by a PC cleavage site (Fig. 4C). Proteolytic removal of eCFP revealed endogenous PC activity in the epiblast lineage, and this activity was diminished in *Furin*−/−;*Pace4*−/− double mutants both *in vivo* and in embryo cultures [90]. On the other hand, paracrine PC activity in the epiblast was significantly rescued if transgenic FurinGFP or Pace4GFP was added back specifically from the extraembryonic microenvironment [141]. These observations demonstrate a paracrine function for PCs. Unexpectedly, live imaging detected Pace4GFP and shed FurinGFP on opposite sides of the polarized epiblast epithelium, potentially adding another layer of complexity to the regulation of Nodal processing. Equally surprising, CLIP imaging revealed an additional PC-like activity in the epiblast of *Furin*−/−;*Pace4*−/− double mutants that was inhibited if embryos were removed from the uterus [141]. Whether this activity derives from a maternal source, or whether loss of Furin and Pace4 leads to compensatory upregulation of other family members such as Pcsk5 or PC7 remains to be determined.

3. Precursor cleavage and morphogenetic signaling

3.1. Impact of precursor cleavage on Nodal activity and stability

Removal of the prodomain of TGFβ is essential to unmask receptor binding epitopes. By contrast, the uncleaved prodomain of Nodal does not prevent precursor binding to type I or type II signaling receptors either in transfected cells or in cell-free assays [43]. A PC-resistant mutant Nodal precursor that was expressed at physiological levels from a knock-in allele also retained significant activity *in vivo* and stimulated *e.g.* the expression of Furin and Pace4. This may reflect the fact that the Nodal prodomain lacks 3 of the 5 conserved hydrophobic residues in the N-terminal α1-helix that are all essential for TGFβ1 latency (Fig. 2). Also the stabilizing dimerization interface found in TGFβ1 LAP is not conserved [16,54]. So why is precursor cleavage still required for normal Nodal function? The answer to this unresolved question may be linked to a role of the prodomain in precursor trafficking. If proteolytic maturation is inhibited, Nodal is stabilized at the cell surface, whereas cleavage facilitates access to signaling receptors in early endosomes [62,138]. How the uncleaved prodomain attenuates endocytosis is unclear, but since it can either bind Cripto or the lysosomal sorting receptor Sortilin and perhaps other cell surface molecules, one possibility is that proteolytic maturation influences the choice between alternative interactions with distinct trafficking molecules [62,142]. In keeping with such a model, *ex vivo* studies

on Nodal processing highlighted a role for precursor cleavage in accelerating ligand turnover [40,67].

There may be important species differences, though, since apart from mouse Nodal, only one related protein in *Xenopus* has been shown to retain significant activity after mutation of the PC cleavage motif [65,143]. Nodal prodomains and mature ligands from a given species also differ in their potential to promote long range signaling, as shown by comparisons of Cyc, Sqt and Spw activities in zebrafish [144–146]. The short signaling range of Cyc has been linked to a lysosomal targeting motif in the prodomain, suggesting that this prodomain promotes ligand turnover, rather than inhibiting it [61]. In good agreement, extracellular fractions of deyolked zebrafish blastoderms accumulated much higher levels of Sqt than of Cyc when equal amounts of mRNAs were injected at the 1-cell stage [147]. Similar analysis of correctly processed GFP- or Dendra2 fusions of Sqt or Cyc again detected higher levels of Sqt. A chase of extracellular Sqt-Dendra2 after photoconversion revealed a half life of 95 min [147]. This is comparable to the decay rate of mature mouse Nodal in cultured HEK293T cells, but 3 times faster than that of its precursor [40]. Thus, the high steady state concentration of extracellular mature Sqt points to an increased rate of proteolytic processing compared to that of mouse Nodal in tissue culture.

3.2. Why is extracellular DPP in wing imaginal discs mostly immobile?

In *Drosophila*, patterning and growth of larval wing imaginal discs are coordinated by the morphogen Decapentaplegic (DPP), the homolog of vertebrate BMP2 and BMP4. Whether and how a DPP activity gradient is regulated at the level of precursor processing or by its influence on ligand turnover is unclear. DPP is secreted by a stripe of cells along the anteroposterior compartment boundary and directly induces different target genes at distinct threshold concentrations by activating complexes of the type I and II receptors Thickveins (TKV) and Punt in distant cells [reviewed in 148]. While TKV is expressed at elevated levels at the periphery of the wing primordium to sensitize cells to DPP, downregulation of TKV in the central region facilitates the spreading of DPP activity [149,150]. To test whether receptor-mediated endocytosis shapes a morphogen gradient, a DPP-GFP fusion protein was expressed in the *dpp* stripe. Live imaging outside the *dpp* stripe revealed DPP-GFP fluorescence both between cells and in endosomes containing TKV [151,152]. Biochemical analysis indicated that the extracellular fraction was processed and short-lived, consistent with rapid clearance by receptor-mediated endocytosis [151,152]. However, whether endocytosis restricts the movement of DPP from cell to cell, or whether it promotes gradient formation by a process of planar transcytosis is still debated [7,153–155]. Defining the role of endocytosis remains difficult because disruption of this process will not only affect TKV, but also the trafficking of DPP coreceptors, convertases and other regulatory factors. It also remains a challenge to discern what defines the mobile fraction of DPP that forms the activity gradient. Immunostaining of cross-sectioned third instar imaginal discs revealed that extracellular DPP-GFP and endogenous DPP primarily fill up the lumen above the apical surface of the wing disk epithelium, where free diffusion will rapidly suppress any gradient [156]. By contrast, the graded extracellular signal on the basolateral side is weak, and a recent estimate suggests that less than 3% of the total DPP-GFP within the wing disk epithelium is free to move [155]. Since the mobile fraction is so underrepresented in the total pool of DPP-GFP, it remains technically challenging to ascertain whether it corresponds to mature DPP or a processing intermediate.

A related question is why the mobile fraction of DPP is so small [155]. Deletion or overexpression of DPP receptors in clones of cells
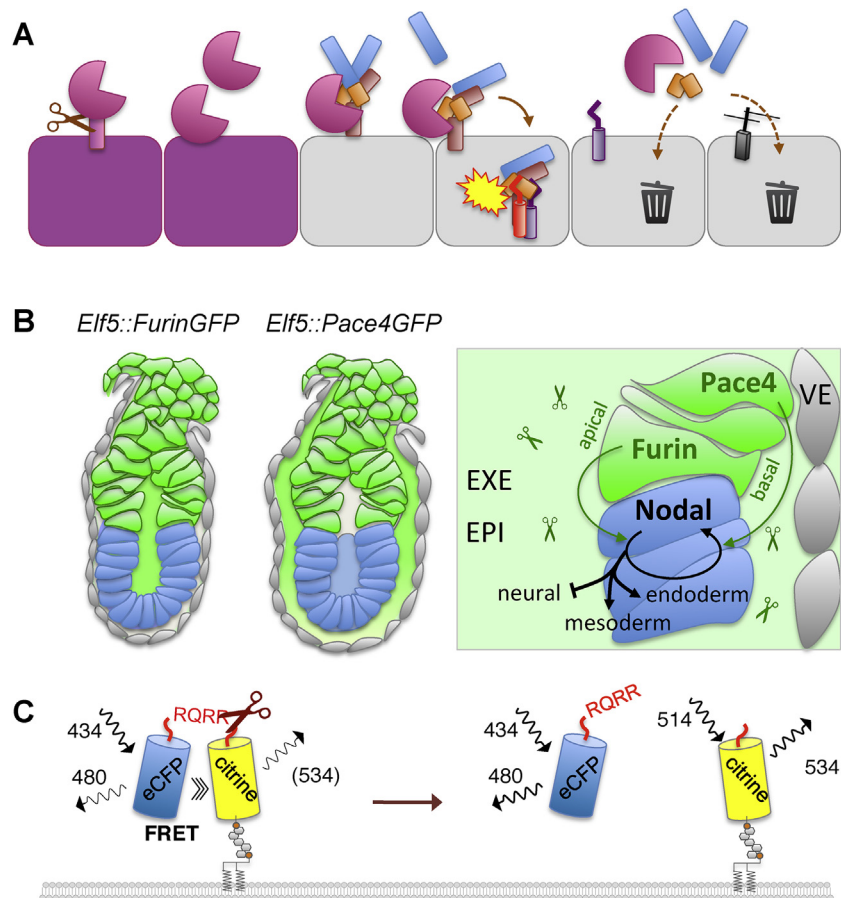
Fig. 4. Analysis of paracrine PC functions. (A) When bound to Cripto (brown), mature Nodal (orange) and cleaved prodomain (blue) can access signaling-competent membrane domains (yellow), whereas Cripto-independent maturation promotes lysosomal targeting (trash). To activate membrane-bound Nodal in epiblast cells (gray), Pace4 and a shed form of Furin (purple) have to travel a certain distance from extraembryonic ectoderm. (B) Transgenic FurinGFP and Pace4GFP expressed in extraembryonic ectoderm (EXE) reach epiblast cells from the apical and basolateral side, respectively, and can rescue Nodal signaling in *Furin⁻/⁻;Pace4⁻/⁻* double mutants. Although epiblast cells (EPI) adhere to visceral endoderm (VE), a space is drawn in between to show the routes of PC delivery (green). (C) Paracrine Furin and Pace4 activities have been imaged in epiblast cells by analyzing eCFP/citrine ratios of the transgenic biosensor CLIP. Sensitized emission analysis of Förster resonance energy transfer (FRET) at the indicated wavelengths is a quantitative readout of CLIP cleavage in cultured cells.

only minimally affected the distribution of DPP-GFP, and 60–80% of extracellular ligand thus was estimated to bind neither TKV nor Punt [154]. So how else is the majority of DPP immobilized? Likely regulators of this process include the GPI-anchored HSPGs Dally and Dally-like which bind a lysine- and arginine-rich basic sequence at the N-terminus of mature DPP next to the PC recognition motif RNKR [157]. While clones overexpressing Dally show increased endogenous DPP signaling [158,159], lack of Dally and Dly, or failure to sulfate their glycosaminoglycan side chains dramatically diminishes the amount of DPP-GFP at the cell surface and inhibits DPP signal transduction [160,161]. Mutations in *dally* and *dly* also inhibit the spreading of DPP activity to wild-type cells [160,161]. It is possible, therefore, that HSPGs inhibit endocytosis and facilitate extracellular diffusion of DPP by a capture-and-release mechanism. In this model, differences in the concentration of DPP between producing and receiving cells drive the movement of DPP from one sulfated side chain to another [157,160]. HSPG binding sites are found at analogous positions in mature BMP2 and BMP4 [162,163]. However, endocytosis of recombinant BMP2 without its prodomain is facilitated by HSPGs in mammalian cells, rather than being decreased [164]. Since HSPGs bind close to the PC recognition motif, potential effects of HSPGs on DPP processing may confound their complex influence on DPP trafficking. In any case, since vertebrate HSPGs also bind related ligands such as GDF8, Activin A and Nodal [29,165,166] as well as several PCs [104],

they emerge as likely candidates to modulate or spatially localize the processing of TGFβ-related precursors in specific membrane microdomains.

3.3. TGFβ family members with multiple PC cleavage sites

All known signaling functions of TGFβ and related factors are associated with the C-terminal mature domains. Nevertheless, some precursors of the BMP subgroup are cleaved at up to three different PC motifs. In pro-BMP4, multiple PCs can cleave both a distal site S1 and a proximal site S2, with the exception of PC7 which only hydrolizes S1 [89,167]. Functional analysis in mice carrying a mutant knock-in allele established that the S2 site is required for normal BMP4 function in some tissues, but not in others [168]. This points to tissue-specific regulation of ligand turnover because the BMP4 prodomain remains bound to mature ligand and promotes its lysosomal degradation when cut only at S1 [169]. *Drosophila* DPP even has three PC cleavage sites. In transgenic constructs that were introduced into *dpp*-deficient embryos for functional analysis of long range signaling, only the proximal-most site that corresponds to S2 of BMP4 was necessary and sufficient for the maturation of functional DPP and for its release into the extracellular space of wing imaginal discs [68,170]. In sharp contrast, cleavage of this proximal site was minimal in embryonic mesoderm and dispensable for short range DPP signaling to the gut epithelium [170].

Distinct cleavage sites thus may be used in a tissue-specific manner according to the demand for short or long range signaling activity. Adding to this versatility, the prodomains of the *Drosphila* BMP ligands Gbb and Scw contain an additional PC cleavage motif, the so-called Pro site, close to their N-termini [171,172,reviewed in 173]. Cleavage at this position also occurs in Lefty proteins [174], and in GDNF (Fig. 2). Superimposed to the structure of L-TGFβ, this position corresponds to a loop after the same conserved α2 helix that is cleaved by Bmp1/Tolloid proteases in GDF8 and GDF11 to overcome latency [16,50]. Biochemical analysis and genetic rescue constructs showed that both the Pro and the Main site have to be cleaved to release Scw from the straighjacket and to unleash its activity. By contrast, cleavage at the Pro site was sufficient to activate Gbb, and the human Gbb homolog BMP7 did not require any functional PC cleavage motif at all to signal [172]. These observations indicate that different ligands can use PC motifs at distinct locations within the prodomain to unmask their receptor binding epitopes. Moreover, the cleavage-independent activity of BMP7 concurs with earlier findings on Nodal processing and corroborates that some prodomains of TGFβ-related precursors do not abolish receptor binding. Proteolytic processing in these instances may be more important to control interactions with specific regulatory factors than to unmask receptor binding sites of mature ligand.

4. Emerging concepts and future directions

In sum, precursor processing can be regulated by several different mechanisms. An obvious first option is to control the expression levels of individual PCs (Fig. 5A). For example, upregulation of Furin and Pace4 expression in the extraembryonic ectoderm of gastrulating mouse embryos, and local repression in the epiblast lineage are likely important to control the activities of Nodal in space and time. Expression of *Furin* is under feedback regulation by Nodal in both

of these lineages [43,127,175]. One of three Furin promoters also directly responds to TGFβ [176], whereas specific microRNAs such as miR-24 can mediate negative feedback [177]. Feedback mechanisms to regulate Furin and other PCs will also likely emerge in other contexts. A second layer of regulation of PCs involves their maturation from inactive zymogens. Even after their autocleavage in the ER, inhibitory prodomains of PCs can mask the catalytic cleft (Fig. 5B). In the case of Furin, a second autocleavage is pH-dependent since the histidine content of the prodomain acts as a pH sensor to mediate a conformational change [178]. As this step is rate-limiting, endogenous Furin may be inactive until a first round of recycling in acidic endosomes. It will be important to quantify the fraction of Furin that is re-cycled from endosomes to the TGN at endogenous expression levels. In cells where shedding is efficient and endogenous expression levels are low, not much Furin may be left to replenish the TGN with active enzyme by means of endosomal recycling (Fig. 5C). Furthermore, the finding that PACE4 and PCSK5 zymogens are activated at the plasma membrane emphasizes the need to investigate how this step is regulated in whole tissues.

Regulated zymogenic activation and modulation of PC expression levels will affect multiple substrates simultaneously. To selectively regulate only a specific substrate, alternative mechanisms are required. Tissue-specific use of alternative PC motifs within the same precursor *e.g.* of DPP has emerged as a conceptually novel regulatory mechanism [170] (Fig. 5D). A related, though poorly understood regulatory determinant involves the actual PC recognition motif and its flanking sequences. One example might be GDF11, which was preferentially cleaved by PCSK5 [80]. By contrast, BMP10 was mainly cleaved by Furin, at least *in vivo*. The fact that these substrates share highly similar cleavage motifs points to complex influences of other features. But while specific flanking residues may influence cleavage rates, they cannot readily explain
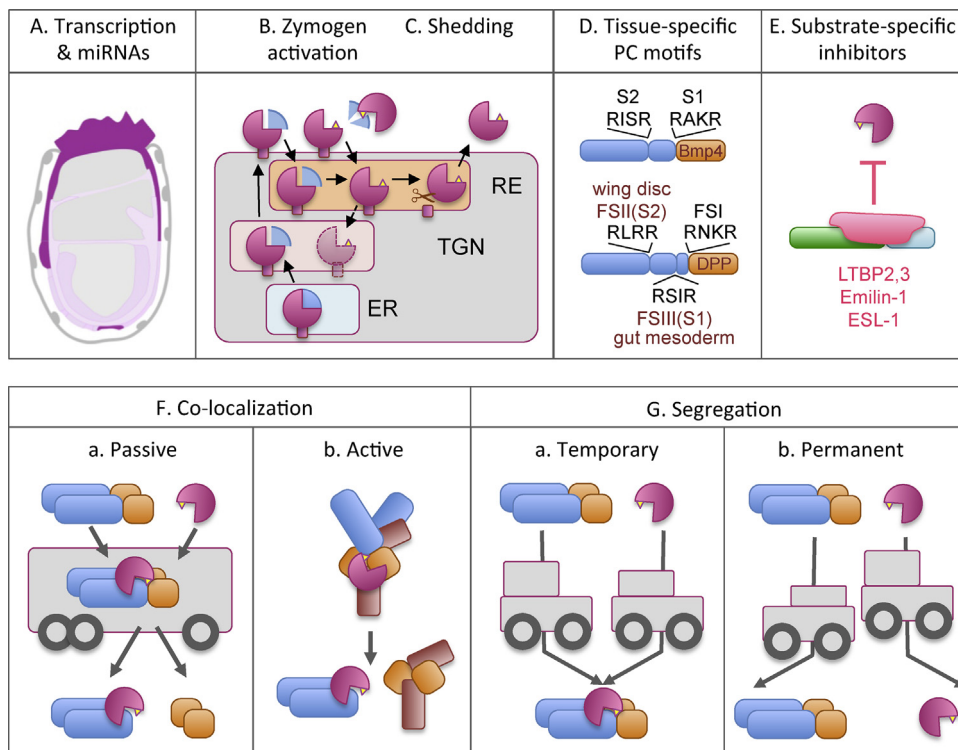

Fig. 5. Mechanisms that regulate PC activities and the processing of TGFβ-related precursors. (A–E) While regulation of the distribution and zymogen maturation of PCs (A–C) will affect multiple substrates, alternative cleavage motifs (D) and interactions of substrates with dedicated inhibitors (E) can modulate processing of a given precursor in a tissue-specific manner. (F and G) Sorting determinants in prodomains can be used to regulate co-localization (F) or the segregation (G) of a given substrate and its convertases in specific membrane and tissue compartments. See text for details.

observed substrate preferences of individual PCs *in vivo*. The studies on Emilin-1 and ESL-1 show that the conformation or sterical accessibility of a given PC cleavage motif can also be regulated by specific interacting factors (Fig. 5E). In their physiological contexts in mice, these factors preferentially inhibit the maturation of TGFβ. They may be important to control not only the rate, but also the localization of proteolytic processing in specific subcellular or tissue compartments. Since the interactions of ESL-1 and LTBP with L-TGFβ are mutually exclusive [117], they likely influence when and where TGFβ meets its convertases. It will be important to learn whether analogous molecules regulate the proteolytic maturation of TGFβ or of related PC substrates in other tissues and in human diseases.

The most versatile, but probably least understood mechanism to control precursor cleavage is spatial compartmentalization, which may be subdivided into several types (Fig. 5F, G). **Passive co-localization** may be compared to two next-door neighbors who commute on the same bus line to the same university: The probability that they meet daily even before arrival at the workplace is high. In this scenario, which corresponds to the traditional view of TGFβ processing by Furin in the TGN, precursor cleavage is limited by the duration of the joint "commute". By contrast, **active co-localization** may be illustrated by co-workers who choose car-pooling as their means of transportation. This pattern likely corresponds to the behavior of Nodal and Furin, which congregate on the co-receptor Cripto to be at the same place at the right time. Depending on whose car they use, they may or may not arrive at work: An unsafe car illustrates the proposed role of Sortilin [142], because it too binds TGFβ-related precursors as well as Furin, but for lysosomal delivery. Since Sortilin itself must first be cleaved before it can load cargo [179], its clearance function may only engage when there is too much Furin (Fig. 4A). **Temporary segregation** is illustrated by neighbors who commute in separate cars and thus only communicate in the work place. Such a relationship appears to exist between Furin and (β)proGDNF as they travel in distinct exocytic vesicles. If the same neighbors develop a more antagonistic relationship, one of them might change shifts, his job or move house to further minimize contact. Though not yet described for any TGFβ family member, this **permanent segregation** would describe a hypothetical substrate and a PC that are secreted on opposite sides of a polarized epithelium. Alternatively, permanent segregation could be achieved by a TGFβ that represses expression of a candidate PC.

Spatial compartmentalization can be implemented and modified by simply altering intrinsic sorting signals in prodomains without the need for special inhibitors that always bear the risk of collateral effects on related ligands. After all, this may be the reason for the dramatic diversification among TGFβ-related prodomains, and why this form of regulation prevails over still elusive PC-specific endogenous inhibitors. However, elucidating this complex form of regulation and its function in diverse contexts is a daunting task that will likely require new and more sensitive technologies to track single molecules and to monitor effects of proteolytic processing on protein-protein interactions, trafficking and protein stability.

Acknowledgements

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