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Survey

Integration of BMP and Wnt signaling via vertebrate Smad1/5/8 and *Drosophila* Mad

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ABSTRACT

BMPs pattern the dorsal–ventral axis of vertebrate embryos. Smad1/5/8 transduces the BMP signal, and receives phosphorylation inputs from both MAPK and GSK3. Phosphorylation of Smad1 by MAPK and GSK3 result in its polyubiquitination and transport to the centrosome where it is degraded by the proteasome. These linker phosphorylations inhibit BMP/Smad1signaling by shortening its duration. Wnt, which negatively regulates GSK3 activity, prolongs the BMP/Smad1 signal. Remarkably, linker-phosphorylated Smad1 has been shown to be inherited asymmetrically during cell division. *Drosophila* contains a single Smad1/5/8 homologue, Mad, and is stabilized by phosphorylation-resistant mutations at GSK3 sites, causing Wingless-like effects. We summarize here the significance of linker-phosphorylated Smad1/Mad in relation to signal intensity and duration, and how this integrates the Wnt and BMP pathways during cell differentiation.

Contents

1. Introduction: embryonic axis formation and the double gradient model

Understanding how cells receive and integrate multiple signals is a major challenge in cell and developmental biology. Nowhere is this more apparent than in a rapidly dividing embryo, which has to undergo cell fate decisions in response to a multitude of growth factor signals over narrow periods of time. These extracellular signals are critically regulated both in time and space and are fine

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tuned by a vast network of inhibitors and activators. Two major morphogens exist in developing *Xenopus* embryos, the BMP (bone morphogenetic protein) and the Wnt gradients (Fig. 1). These gradients are perpendicular to each other and are responsible for tissue position and determination along these axes, patterning the embryo from dorsal to ventral (D–V) and anterior to posterior (A–P) [1]. Both these signals are seamlessly integrated and this can be demonstrated experimentally in *Xenopus* embryos. When a blastula embryo is equally cut in half, with each half containing a dorsal and ventral part, the embryo can self-regulate, forming perfectly identical twins [2]. Below we discuss signaling by the BMP and Wnt morphogens and analyze recent advances in our understanding of signal integration along the D–V and A–P gradients at the level of Smad1/5/8 linker phosphorylations [3–5].

1.1. Dorsal–ventral patterning: a morphogenetic gradient of BMP ligands

The earliest requirement for BMP signaling in an embryo is during the patterning of cell fates along its D–V axis. Formation of a D–V gradient of BMP signals has been evolutionary conserved and is utilized by both vertebrates and invertebrates [2,4,6–8]. In vertebrate embryos like *Xenopus* and zebrafish, BMPs pattern ventral cell fates, while BMP repression determines dorsal cell fate (Fig. 1); this D–V polarity is reversed in invertebrate embryos such as *Drosophila*. The gradient of BMP signals subdivides the *Xenopus* ectoderm from ventral to dorsal into epidermis, neural crest, and central nervous system, while the mesoderm is subdivided into blood island, lateral plate mesoderm (kidney), somite, and notochord. Thus, a ventral gradient of extracellular BMPs regulates the initial tissue-type differentiations of the vertebrate embryo.

The main BMPs involved in D–V patterning in the *Xenopus* embryo are the ventrally expressed BMP4 and BMP7 and the dorsally expressed BMP2 and ADMP. Depletion of all four BMPs using injected antisense morpholino oligo nucleotides causes this robust morphogenetic field to collapse, resulting in complete neuralization of the developing embryo [9]. This is a spectacular transformation of the embryo, because the entire ectoderm becomes covered by central nervous system (CNS), in particular brain tissue. If any one of the four BMPs is not depleted, the embryo retains some D–V patterning. This indicates that both the dorsal and the ventral poles of the embryo serve as sources of BMP signals. In zebrafish embryos, mutation of *bmp2b* or *bmp7* result in strong dorsalization or neuralization of the embryo [10–12], demonstrating that the requirement for BMPs in the specification of ventral fates has been evolutionary conserved [13].

The main extracellular regulators of BMP ligands are Chordin and Noggin, two BMP antagonists secreted by the dorsal Spemann organizer at the onset of gastrulation [14,15]. Chordin and Noggin help create and maintain a D–V gradient of BMP and induce dorsal cell fates. Anti-BMPs do not directly signal dorsal fate, but the antagonism of BMP signaling by extracellular binding causes dorsal cell differentiation by decreasing BMP signaling levels [16–18]. The requirement for BMP inhibition was demonstrated by knockdown of Chordin, Noggin and a third BMP antagonist, Follistatin, in *Xenopus tropicalis* embryos. This resulted in severe loss of neural tissue and massive expansion of ventral cell fates [19]. Two opposing ventral and dorsal signaling centers of the gastrula embryo provide the initial basis for D–V patterning, and an elaborate biochemical pathway of extracellular protein–protein interactions has been found to be required to maintain a well-regulated BMP morphogenetic field [4,20].

1.1.1. Intracellular transduction of the BMP signal

BMPs transduce their intracellular signal via BMPR (BMP receptor) activation followed by transcription factor phosphorylation. BMPs first bind to and activate their transmembrane serine/threonine kinase receptors, which in turn phosphorylate the transcription factors Smad1/5/8 at its two C-terminal serines (SVS). Phosphorylated Smad1^{Cter} binds to Smad4 (co-Smad) and translocates and accumulates in the nucleus, activating BMP-responsive genes (Fig. 2) [21,22], such as BMP4/7 and others. A dynamic D–V nuclear gradient of pSmad1^{Cter} has been shown in a number of model organisms such as *Drosophila* [23,24], zebrafish [25] and *Xenopus* [20]. At very low levels of pSmad1^{Cter}, caused by the extracellular inhibitory activity of Chordin and Noggin on BMP ligands, dorsally expressed genes are transcribed. Ventral genes are activated by BMP signals. The dorsal and ventral centers of the gastrula express molecules of similar biochemical activities but under reciprocal transcriptional control. This explains how a self-regulating field is maintained in the early embryo [9]. When the amount of one molecule is lowered in the dorsal side, the gradient can be restored by the expression of ventral counterparts [9,20]. For example, the dorsal organizer expresses Chordin, while the ventral center expresses a Chordin-related BMP-binding molecule called CV2 (Crossveinless-2) [26,27]. When Chordin and CV2 are depleted simultaneously, the embryo reaches very high BMP levels, indicating that CV2 in the ventral side can compensate for loss-of Chordin in the dorsal signaling center [28].

1.2. Anterior–posterior patterning and Wnt signaling

The main determinant of the A–P axis in the early embryo is provided by Wnt signaling [1,29]. A Wnt morphogen gradient is generated by a number of extracellular Wnt ligands, which are modulated by a diverse group of secreted Wnt antagonists such as Dkk-1 (Dickkopf-1) and sFRPs (secreted Frizzled-related proteins) [30]. In *Xenopus* and amphioxious embryos, the Wnt signaling

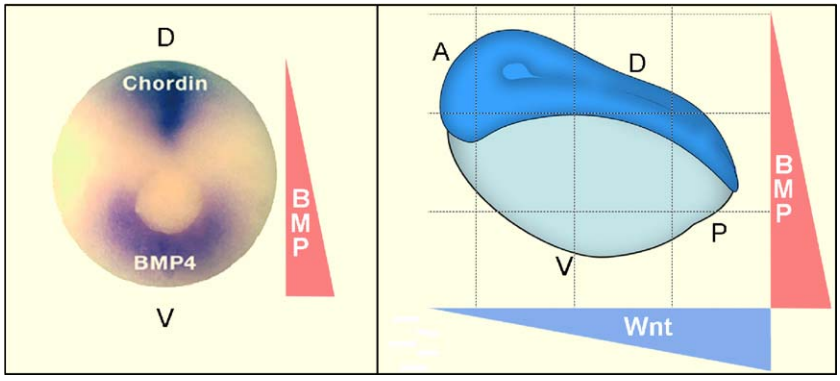

Fig. 1. (Left) Expression of *Chordin* and *BMP4* on opposite centers of a *Xenopus* embryo. (Right) Model illustrating the two perpendicular morphogenetic gradients of BMP and Wnt. Cells sense their position within these Cartesian-coordinates, which specify their fate in the body plan [4].

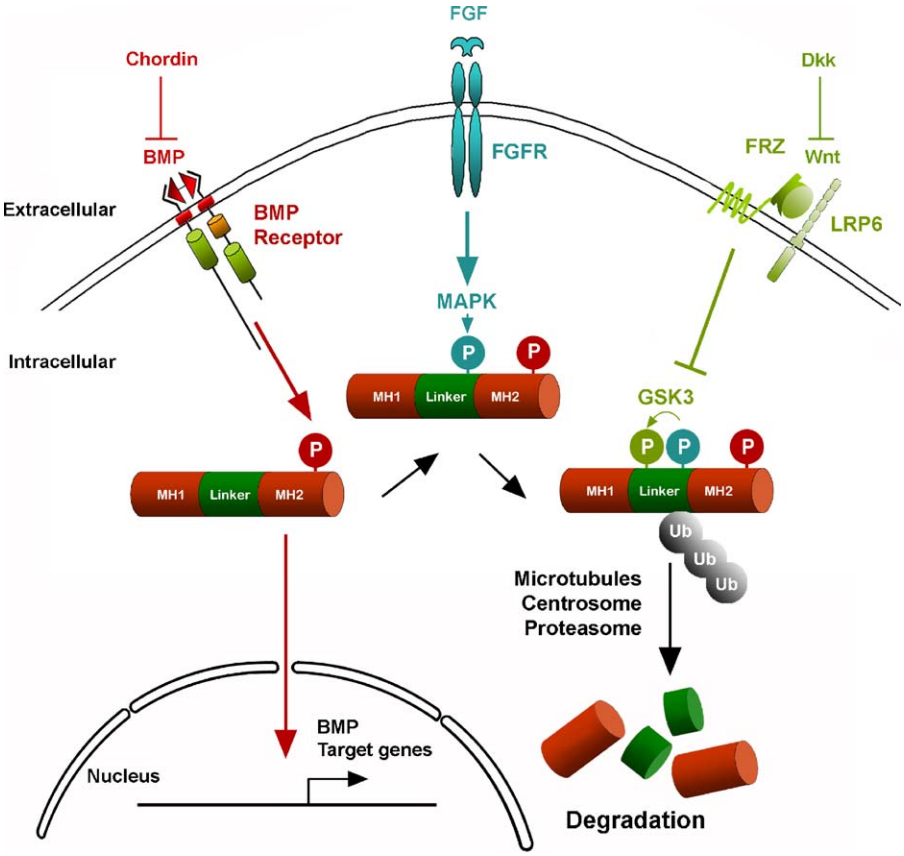

Fig. 2. Integration of multiple extracellular signaling pathways at the level of Smad1/5/8 phosphorylations. BMP-dependent C-terminal phosphorylation of Smad1, activates target gene expression whereas, MAPK and GSK3 linker-phosphorylations promote degradation terminating the BMP/Smad1 signal. Wnt prolongs BMP signals by inhibiting GSK3 phosphorylation.

gradient is maximal at the posterior blastopore [1,31,32], and its signal becomes lower in anterior regions (Fig. 1). When neuralized *Xenopus* ectodermal explants are microinjected with varying doses of Wnt DNA, posterior markers are induced [33]. In planarians, A–P specification is also regulated by Wnt signaling, since inhibition of the canonical Wnt pathway by RNAi causes ectopic regeneration of head structures [34,35]. A–P patterning by a Wnt gradient appears to be a universal property of animal development. At later stages, the A–P axis becomes subdivided into segments in many organisms. The A–P patterning within each segment also requires Wnt signals [4].

The organizer region of the frog embryo not only secretes BMP antagonists, but a cocktail of Wnt inhibitors, which include Frzb-1, sFRP-2, Crescent, and Dkk-1 [36–40]. Inhibition of the Wnt signaling pathway at early gastrula stage plays a vital role in head induction. When Dkk is overexpressed in *Xenopus* embryos it has potent head-inducing activity, resulting in an expanded anterior neural plate at the expense of epidermal tissues [38]. However, the anterior neural inducing activity of anti-Wnts also requires inhibition of BMP signaling to generate head structures [1,37].

Wnt signaling involves ligand binding to its Frizzled/ lipoprotein receptor-related protein 6 (LRP6) co-receptor complex on the extracellular cell surface. These receptors then transduce an intracellular signal through a number of proteins which include Dishevelled, GSK-3 (glycogen synthase kinase-3), Axin, APC (adenomatous polyposis coli), and the transcriptional regulator β-catenin. In the absence of Wnt signaling, β-catenin levels in the cytoplasm are normally kept low by continuous proteasome-mediated degradation, involving a complex containing GSK-3/APC/Axin [30]. When a cell receives a Wnt signal this degradation pathway is inhibited, resulting in nuclear accumulation of β-catenin. Nuclear β-catenin then interacts with other transcription factors such as LEF/TCF (lymphoid enhancer-binding factor1/T cell-specific transcription factor) to initiate transcription of Wnt responsive genes [30].

2. Regulation of Smad1 via linker phosphorylations downstream of BMP

The BMP transcription factor Smad1 is further regulated by inhibitory "linker" phosphorylations. The linker region of Smads lies between its MH1 (Mad homology domain, DNA binding) and MH2 (protein interaction) domains with a large number of potential phosphorylatable Serines and Threonines.

2.1. Inhibitory Smad1 linker phosphorylations by MAPK

Smad1 was first shown to be a target of growth factor signaling through the mitogen-activated protein kinase (MAPK) pathway in human cultured cell lines [41]. MAPK phosphorylations activated by epidermal growth factor receptor (EGFR) occur at four specific MAPK/Erk recognition consensus sites (PXS[PO3]P) within the linker region of Smad1. MAPK phosphorylation prevents nuclear accumulation of Smad1, and therefore inhibits its intracellular transcriptional activity [41]. Mutation of the Serines at the four MAPK sites into Alanines rendered Smad1 resistant to EGFR-induced phosphorylation and inhibition [41]. This discovery provided the first evidence of the antagonistic action of MAPK linker phosphorylation on the BMP signaling pathway.

The opposing BMP and EGFR/MAPK inputs on Smad1 suggested an explanation for the long-standing question of how fibroblast growth factors (FGFs) and insulin-like growth factors (IGFs) induce

neural tissue [42–46]. This was a puzzle, because BMP antagonists such as Chordin and Noggin cause neural differentiations similar to those of FGF and IGF [2,47]. Microinjection experiments and biochemical assays using *Xenopus* embryos demonstrated that MAPK/Erk is activated by both FGF and IGF causing an inhibitory phosphorylation in Smad1 that induces ectoderm to differentiate as neural tissue instead of epidermis [47]. In gain-of-function experiments microinjection of phosphorylation-resistant *Smad1* mRNA induced ectodermal cells to become epidermal tissue at the expense of neural fates. These data demonstrated that the epidermal-inducing activity of Smad1 requires low BMP antagonists, high BMP, and low levels of MAPK signals activated by receptor tyrosine kinases [47–49]. During organ development, there are many situations in which FGF and BMP signals have opposing functions. Examples include: limb development, lung branching morphogenesis, cranial suture fusion, and tooth development [47]. These opposing effects may also involve the integration of FGF/MAPK signals and BMP signals at the level of Smad1/5/8 phosphorylations.

The regulatory cross-talk between the BMP and MAPK pathways was demonstrated in knock-in mice containing Smad1 forms that are resistant to phosphorylation by MAPK in the Smad1 linker region. These studies indicated a requirement for linker phosphorylation in gastrointestinal and reproductive tract development [50]. Using embryonic fibroblasts from these knock-in mice, Sapkota et al. used a BMP reporter gene to show that FGF inhibits signaling by BMP [51]. Importantly, exogenous FGF failed to inhibit BMP signaling in Smad1 linker phosphorylation-resistant knock-in mutant mouse embryonic fibroblasts. In addition, FGF inhibition of BMP signaling has also been reported in rat embryonic dorsal spinal cord precursor cultures, in which FGF2 addition prevents nuclear accumulation of pSmad1^{Cter}, sequestering it in the cytoplasm in a MAPK-dependent manner [52].

Phosphorylation by MAPK has been shown not to be solely restricted to the linker domain. *Drosophila* Mad, which is the homolog of vertebrate Smad1/5/8, is phosphorylated by a MAPK-related kinase called Nlk (Nemo-like kinase) [53]. Nlk, an enzyme known to be involved in the Wingless/Wnt pathway, phosphorylates Mad at a conserved serine residue in its MH1 DNA binding domain. This phosphorylation inhibits BMP signaling by preventing nuclear accumulation of pMad^{Cter}, thus inhibiting the activation of BMP-responsive genes [53].

2.2. GSK3/Wnt regulates BMP/Smad1 signal termination

Extensive sequence analysis within the linker region of Smad1 revealed a number of potential GSK3 phosphorylation sites [3,51]. GSK3 phosphorylation requires a pre-phosphorylated phosphate located four amino acids downstream of a Serine or Threonine (S/TXXXS/T[PO_3)] [54] (Fig. 2). In Smad1, GSK3 is primed by MAPK sites that provide the priming phosphate [3,51]. Linker phosphorylation by GSK3 had an inhibitory effect on BMP signal transduction. Mutation of the GSK3 sites into Alanines resulted in strongly ventralized phenotypes (high BMP signaling) in injected *Xenopus* embryos [3].

Pulse-chase experiments demonstrated that BMP signaling triggered three successive phosphorylations of Smad1 in cultured cell assays [3]. The first phosphorylation caused by BMPR activation, occurs in the C-terminal region of Smad1 (Fig. 2). BMP determines the intensity or amplitude of the BMP signal. The two next phosphorylations in the linker region, provided first by MAPK and then by GSK3, determine the duration of the BMP/pSmad1^{Cter} signal. We deduce that the duration of the signal is a key regulatory step, because inhibition of MAPK or GSK3 in cell culture prolongs the Smad1 C-terminal signal [3].

Once Smad1 is phosphorylated in the linker region by both kinases, signal termination is set in motion. Smurf1 is an E3-ubiquitin protein ligase (of the WW-Hect family), which restricts BMP signaling and is required for the degradation of Smad1 [55–57]. Linker phosphorylation of Smad1 is essential for Smurf1 binding to its recognition motif, PPXY, which is located near the linker phosphorylation sites [3,51]. Smad1 is then polyubiquitinated and degraded in proteasomes (Fig. 2) [3]. Thus, the inhibitory phosphorylations of the MAPK and GSK3 sites regulate the duration of the Smad1/5/8 signal. At high FGF levels the BMP signal will be shorter. Wnt signaling inhibits GSK3 and therefore at high Wnt levels the BMP signal is of longer duration. In this way, BMP determines the intensity of the Smad1/5/8 response, while FGF decreases and Wnt increases its duration (Fig. 2).

3. Asymmetric inheritance of Smad1

Termination of the Smad1/5/8 signal involves linker phosphorylations at the MAPK and GSK3 sites and polyubquitinylation.

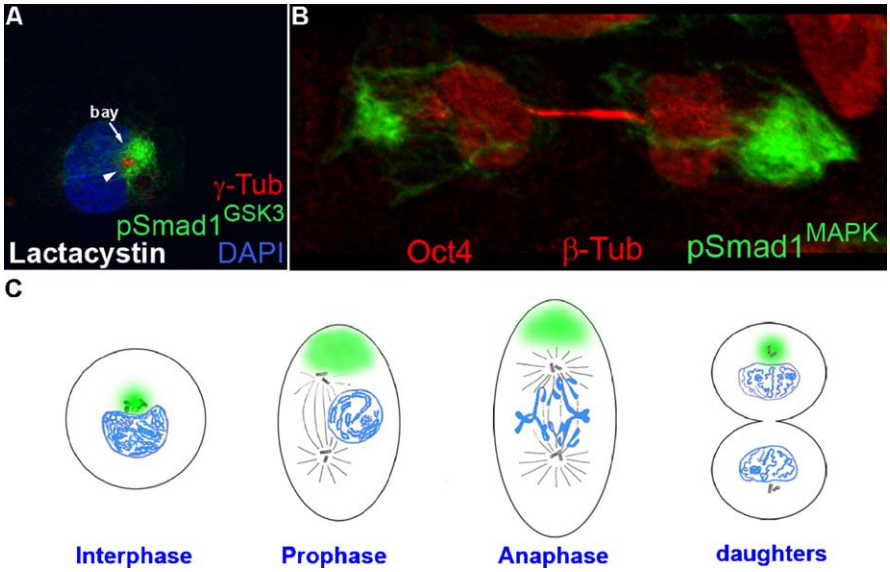

Fig. 3. (A) Asymmetric distribution of pSmad1 targeted for degradation in self-renewing human embryonic stem cells. (B) When the proteosomal machinery is inhibited pSmad1^{GSK3} accumulates in a pericentrosomal nuclear bay. (C) Model illustrating asymmetric inheritance of pericentrosomal material (green) during mitosis [58].

Smad1 targeted for degradation also requires transport along microtubules to be degraded by the proteasomal machinery in the pericentrosomal region of the cell [58]. Inhibition of the proteasomal enzymatic machinery using Lactacystin, a chemical inhibitor, caused accumulation of pSmad1 marked for degradation in the centrosomal region (Fig. 3A).

Unexpectedly, in cultured cells, particularly in human embryonic stem cells, we observed that linker-phosphorylated Smad1 was asymmetrically distributed during mitosis [58] (Fig. 3B). This asymmetry took place in stem cells undergoing self-renewal, which were supposed to be equal divisions. Analysis of Cos7 cells showed a similar tendency to segregate pSmad1^{MAPK} or pSmad1^{GSK3} asymmetrically, with one of the dividing daughter cells retaining the linker-phosphorylated Smad1 [58]. The asymmetric segregation is not a unique property of Smad1, for other proteins targeted for degradation, such as phospho-β-catenin and total polyubiquitinated proteins (which include hundreds of cellular proteins) are also unequally segregated between daughter cells.

This asymmetry appears to be a general property of cell division as shown in the model in Fig. 3C. When the centrioles separate before mitosis to occupy opposite cell poles, the pericentrosomal material is inherited preferentially by one of the daughter cells. This simple cellular mechanism can explain the mitotic asymmetries. To investigate if this remarkable phenomenon occurred in vivo, an antibody was raised against the single MAPK phosphorylation site present in the linker region of *Drosophila* Mad. The pMad^{MAPK} antibody stained a single bright spot in every blastoderm cell and co-localized close to one of the two centrosomes [58].

The asymmetric distribution of phosphorylated proteins targeted for degradation uncovered an interesting phenomenon and raises many questions. Is it a cleansing mechanism so one daughter remains pristine, simply a case of garbage the cell wants to get rid of? Or are the asymmetric proteins targeted for degradation junk the cell might want to reuse? Is this asymmetry regulated by extracellular signals? These and other questions are under active investigation. Since the first description of mitosis by Flemming in 1882, studies on somatic cell division had focused on the equal partition of cellular materials. The new phospho-specific Smad1 MAPK and GSK3 antibodies were of very high titer and marked proteins destined for degradation. These new reagents made possible the discovery of inequalities in many mitoses, which we hope will advance the cell biology of signaling.

4. Smad1 signal duration: phenotypic similarities between BMP and Wnt antagonists in the developing embryo

With the advent of modern molecular embryology it became clear that D–V or A–P pattern formation were intertwined. For example, overexpression of BMP or Wnt antagonists in embryos, such as Chordin or Dkk-1 respectively, generated almost indistinguishable dorsalized phenotypes [3]. These and other experiments suggested some type of cross-talk between the BMP and Wnt pathways. The node of interaction has now been shown to reside at the level of Smad1GSK3 linker phosphorylations. Wnt was shown to inhibit phosphorylation of Smad1 by GSK3 [3]. This stabilizes the Smad1 transcription factor, allowing it to extend the duration of the BMP signal.

The intensity of the BMP signal can be lengthened or shortened via linker phosphorylations [3]. The pSmad1^{Cter} signal intensity will be highest in the ventral region of the gastrula embryo, where BMP is highest. The Wnt pathway, which is strongest in the posterior region of the embryo, provides an extracellular signal that prolongs the duration of the pSmad1^{Cter} signal by inhibiting GSK3 phosphorylation of Smad1, preventing its degradation in the cell (Fig. 2). The implications of these experiments go beyond a simple point of signal convergence. These studies help explain how an embryo reads, processes, and integrates the BMP and Wnt morphogenetic gradients in an embryo, thus determining the overall positional information that determines where the future organs or appendages will develop within the body plan (Fig. 1).

5. Linker regulation of *Drosophila* Mad

Drosophila Mad, like its vertebrate counterparts Smad1/5/8, contains both MAPK and GSK3 phosphorylation sites within its linker domain. However, unlike the vertebrate BMP-Smads, Mad contains just a single canonical MAPK phosphorylation site and two upstream GSK3 sites. With a reduced number of phosphorylation sites and just one gene, *Drosophila* provided an ideal developmental model to study cross-talk between Wg (Wingless) and BMP signaling [59]. We mutated Mad MAPK (MMM) or GSK3 (MGM) Serine phosphorylation sites into Alanines. Expression of these constructs was driven in wing imaginal discs using the UAS-Gal4 system [60]. Large amounts of ectopic vein and crossvein-like tissue were induced in adult wings [59], a sign of increased Dpp (Decapentaplegic, a homologue of BMP2/4) signaling. MGM overexpression also increased expression of Dpp target genes *Spalt* and *Optomotor-blind* in wing discs, without increasing Dpp expression levels. Mutation of either phosphorylation site prevented Smurf-induced ubiquitination and degradation of Mad [59], explaining how point mutations in Mad result in a hyperactive transcription factor.

5.1. Mad linker phosphorylations: BMP dependent or independent?

To determine whether the Mad linker phosphorylations were always BMP-dependent, polyclonal phospho-specific antibodies were raised against both the MAPK and GSK3 phosphorylation sites. Whole-mount immunostaining of blastoderm embryos was carried out to detect in vivo localization of the pMad^{MAPK} and pMad^{GSK3}. A narrow dorsal stripe was present containing strong nuclear accumulation of linker-phosphorylated forms of pMad. These MAPK and GSK3 phospho-stainings tracked pMad^{Cter} and were Dpp-dependent, as they were absent in Dpp null embryos. However, pMad^{MAPK} and pMad^{GSK3} remained in the rest of the blastoderm embryo and therefore appear to be also Dpp-independent. pMad^{MAPK} stained a single bright cytoplasmic spot of antigen usually adjoining one of the cellular centrosomes, where its degradation takes place [58,59]. The persistence of the asymmetric centrosome-associated spots in Dpp null embryos indicates that MAPK and GSK3 phosphorylations can occur independently of Dpp.

The Dpp-independent in vivo staining was also supported by *Drosophila* S2 cell culture experiments using the Mad "null" mutant alleles, Mad^{10} and Mad^{12}. Both these mutants contain point mutations in the MH2 domain of the transcription factor that prevent BMPR phosphorylation of Mad and are described in the literature as nulls. cDNAs encoding MWT, Mad^{10} or Mad^{12} were expressed in *Drosophila* S2 cells, and it was found that MWT had phospho-Mad^{Cter} (as expected), while both mutants did not have any C-terminal phosphorylation. However, the pMad^{MAPK} and pMad^{GSK3} sites were phosphorylated normally in the mutant proteins. These data showed that Mad^{10} and Mad^{12} proteins were stably translated and were nulls for Dpp C-terminal phosphorylation, but were still regulated by MAPK and GSK3 linker phosphorylations, further supporting a Dpp-independent regulation of the linker domain of Mad [59].

In microinjected *Xenopus* embryos, *Mad^{12}* mRNA reduced forebrain structures. When the GSK3 sites of Mad^{12} were mutated (mimicking a protein receiving a maximal Wnt signal), the head region was almost eliminated. These results suggest that Mad

linker phosphorylations can be BMP-dependent or BMP-independent, and that Mad can still function in A–P axis patterning in the absence of C-terminal phosphorylation [59].

5.2. Phospho-resistant Mad mutants display Wg-like phenotypes

The *Drosophila* studies revealed that Wg cannot only determine the stability of Mad by inhibiting GSK3 phosphorylation, as in the vertebrates [3], but that Mad stabilized by Wg is involved in canonical Wg signaling. This is a notable discovery, which places Mad as transducer of both the Dpp and Wg pathways [59].

When Mad resistant to GSK3 phosphorylation (MGM) was driven in wing imaginal discs, additional Wg-dependent sensory bristles were formed along the anterior wing margin, whereas overexpression of MWT had little effect. Analysis of senseless, a Wg target gene required for sensory bristle formation, revealed a marked expansion of the number of cells overexpressing MGM.

MGM cell clones (marked by yellow [61]) induced ectopic wing margins (Fig. 4A), while MWT clones had no effect on the wing margin. In knockdown experiments, UAS-Mad RNAi clones resulted in loss of wing margin tissue (Fig. 4B), a reliable readout for Wg loss-of-function. Thus, overexpression of Mad GSK3 phosphorylation-resistant mutant protein, which mimics Mad receiving a maximal possible dose of Wg, caused Wg-like phenotypes (in the absence of increased Wg signals). Conversely, Mad depletion caused Wg loss-of-function phenotypes. Mad, a protein that is phosphorylated by GSK3 in a Wg-regulated manner, appears to be a component of the Wg signal transduction pathway.

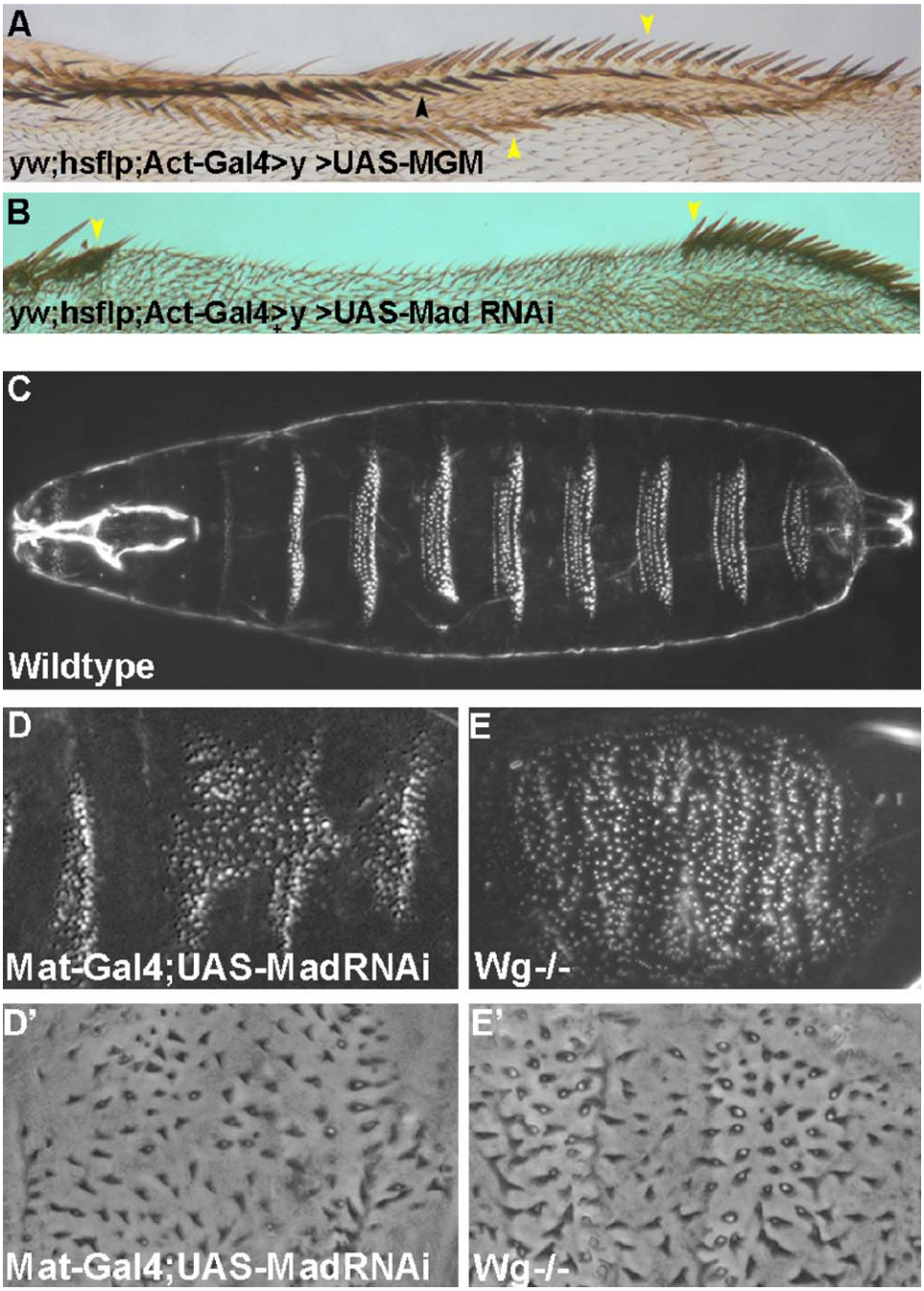

Fig. 4. *Drosophila* Mad transduces Wg signals. (A-B) Overexpression of MGM in clones induce duplications, and Mad depletion resulted in loss-of the margin. (C) Wild-type cuticle. (D–D′ and E–E′) Lawns of row 5 denticles in Mad RNAi or Wg null cuticles [59].

5.3. Mad and Smad1 are required for segment formation

A remarkable finding from the study of linker phosphorylation regulation of *Drosophila* Mad was its involvement in segmental patterning. Maternal depletion of Mad using a pUASp-Mad RNAi (which can be expressed in the oocyte) caused denticle belt fusions at larval stages [59]. These fusions replaced regions of naked cuticle with lawns of large denticles of the same type (row 5) as those seen in Wg null cuticles (Fig. 4C and D) [62]. In gain-of-function experiments, overexpression of Mad GSK3 mutant caused denticle belts to be replaced by regions of naked cuticle, mimicking Wg overexpression [59]. Thus, depletion or overexpression of Mad generated Wg-like phenotypes, indicating that Mad functions in the Wg signaling pathway during segmental patterning.

Finding a role for Mad in segmentation was surprising, since the segmentation process had previously been extensively studied in classical *Drosophila* genetic screens [63,64] and Mad had not been implicated as part of the segmentation machinery. The discovery of this new role for Mad after so many years, may be explained by the fact that Mad appears to also function independently of Dpp, and that the Mad^{10} and Mad^{12} null alleles are nulls only for the BMP pathway, retaining linker regulation.

5.4. The ancestry of segmentation

The role of Mad in segmentation appears to be evolutionary conserved, as it was found that Smads have a role in somite border formation in *Xenopus* embryos. When the main maternally expressed Smad, Smad8 [65], was depleted in the C3 blastomere (using a specific morpholino oligonucleotide), segmental somite borders were erased in the injected side at the tadpole stage (Fig. 5A–C). The Smad8 transcription factor designated as Smad8 in *Xenopus* probably corresponds to Smad5 of other vertebrates [25,65]. Thus, the Mad/Smad5/8 transcription factors are required for segmentation both in *Drosophila* and *Xenopus*. These results are of considerable evolutionary interest.

Many developmental mechanisms have been shown to be conserved throughout evolution [4]. However, no common genes required for segment formation have been found between vertebrates and *Drosophila*. Segmentation in vertebrates depends on the cyclic oscillation of *Notch* pathway transcripts in the posterior paraxial mesoderm of the embryo [66]. Given that BMP/Smad signals have a duration of 1–2 h in cultured cells [3], and are regulated by GSK3 phosphorylations, Smad1/5/8 could be a potential regulator of the segmentation clock. Wnt pathway genes cycle rhythmically during segmentation in vertebrates [66], offering an interesting possibility for regulating Smad5/8 activity by modulating GSK3 activity. Notch, which is involved in spider and cockroach segmentation [67,68], is not required for *Drosophila* segmental formation. We have now found that Smad5/8 is required for the formation of segmental boundaries in *Xenopus* somites and that Mad is required for *Drosophila* segment patterning. This unexpected conserved role for Mad/Smad is important from an Evo-Devo perspective because it suggests that

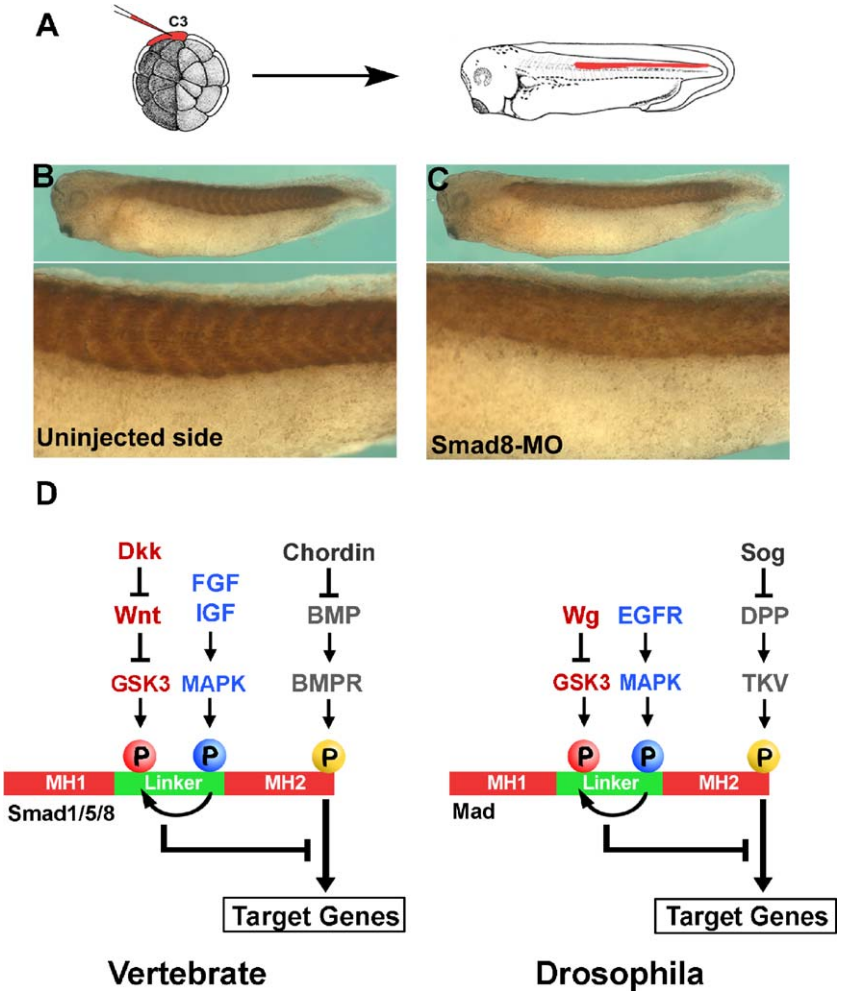

Fig. 5. (A) C3 *Xenopus* blastomere injection targets somites in *Xenopus*. (B) Normal somite border pattern on injected side. (C) Microinjection of Smad8-MO erase segmental somite borders [59]. (D) Comparison of Smad/Mad linker regulation in vertebrate and *Drosophila*.

the last common ancestor shared between *Drosophila* and vertebrates, Urbilateria, might have been segmented [69].

6. Conclusions

Finding new nodes of integration is essential to understanding how the multitude of signals received by each cell is read and interpreted in embryos and adult tissues. The discovery that Smad1/5/8 and *Drosophila* Mad receive negative linker phosphorylations from both MAPK and GSK3 not only explains how the BMP signal is terminated, but also uncovered a novel cell biological pathway of how Smad1/Mad is transported to and degraded in the centrosomal region of a cell. Linker-phosphorylated forms of Smad1 are asymmetrically distributed in dividing cells and inherited unequally by daughter cells after cell division. The demonstration that active Wnt signals, which inhibit GSK3 activity, prolong the duration of the BMP/pSmad1^{Cter} signal helps explain the similarities between the dorsalizing phenotypes of anti-BMPs and anti-Wnts when overexpressed in *Xenopus* embryos. The intensity of the BMP signal is transduced by Smad1/5/8 in the form of C-terminal phosphorylations that determine the D–V axis. The duration of the BMP signal [4] is regulated by the Wnt morphogenetic gradient that specifies the A–P axis. The finding that three major signaling pathways – MAPK, Wnt/GSK3 and BMP – are integrated at the level of Mad/Smad1/5/8 both in the vertebrates and *Drosophila* (Fig. 5D) has interesting implications for the evolution of animal forms through variations on an ancestral gene tool-kit (Fig. 5D) [4]. Although much has been learned about the role of Smad1/Mad as a mode of signaling integration, many open questions remain to be answered before we understand the function of these remarkable transcription factors.

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