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RESEARCH ARTICLE

Precise spatial restriction of BMP signaling in developing joints is perturbed upon loss of embryo movement

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ABSTRACT

Dynamic mechanical loading of synovial joints is necessary for normal joint development, as evidenced in certain clinical conditions, congenital disorders and animal models where dynamic muscle contractions are reduced or absent. Although the importance of mechanical forces on joint development is unequivocal, little is known about the molecular mechanisms involved. Here, using chick and mouse embryos, we observed that molecular changes in expression of multiple genes analyzed in the absence of mechanical stimulation are consistent across species. Our results suggest that abnormal joint development in immobilized embryos involves inappropriate regulation of Wnt and BMP signaling during definition of the emerging joint territories, i.e. reduced β-catenin activation and concomitant upregulation of pSMAD1/5/8 signaling. Moreover, dynamic mechanical loading of the developing knee joint activates Smurf1 expression; our data suggest that Smurf1 insulates the joint region from pSMAD1/5/8 signaling and is essential for maintenance of joint progenitor cell fate.

KEY WORDS: Wnt/BMP signaling, Articular cartilage, Immobilization, Joint development, Muscle contraction, Mechanoregulation, Mechanosensitivity

INTRODUCTION

We have previously proposed that joint development requires spatially appropriate specification and differentiation of bi-potential cartilage cells, which take on one of two distinct fates (Ray et al., 2015). The cells at and near the joint line are exposed to canonical Wnt signaling and assume permanent or articular cartilage fate, whereas the cells at a distance from the interzone are exposed to BMP signaling and differentiate as transient cartilage, eventually being replaced by bone cells. It is now well established that dynamic mechanical movement of limbs is not only important for maintenance of articular cartilage in adults but also for embryonic articular cartilage differentiation (Rolfe et al., 2013). Loss of movement during joint development in either embryonic chicken or mouse model systems results in ectopic development of transient cartilage across the presumptive joint region, characterized by Alcian Blue staining, downregulation of joint specific markers and ectopic expression of an early marker of transient cartilage, *Col2a1*, with continuous cartilage formation across the joint site in extreme cases (Kahn et al., 2009; Nowlan et al., 2010; Roddy et al., 2011). Although the early phase of joint specification, including formation of the interzone, is not affected, tissue patterning within the presumptive joint and maintenance of the articular territory is lost.

Little is known about the molecular mechanisms that are perturbed upon immobilization and lead to loss of articular cartilage cells. Using both *in vitro* and *in vivo* model systems, many genes have been identified whose expression levels change upon alteration of the mechanical environment (Bougault et al., 2012; Dowthwaite et al., 1999; Kahn et al., 2009; Roddy et al., 2011; Rolfe et al., 2014a,b; Sironen et al., 2002a,b). The importance of the Wnt pathway in mechanoregulation of joint development was shown by altered expression of multiple Wnt pathway genes in muscle-less mouse embryos (Rolfe et al., 2014b) and reduced canonical pathway read-out in the developing joints of a reporter mouse line (Kahn et al., 2009). A series of prior studies demonstrated that canonical Wnt signaling is crucial for articular cartilage differentiation and maintenance (Guo et al., 2004; Hartmann and Tabin, 2001; Kahn et al., 2009; Ray et al., 2015; Später et al., 2006a,b). However, although knockout of Wnt ligands/β-catenin leads to misexpression of *Col2a1* in the embryonic joint region, unlike the loss of mechanical stimulation, articular cartilage markers continue to be expressed (Später et al., 2006a), suggesting that decreased canonical Wnt signaling is not the only mechanistic explanation for failure to maintain joint progenitor cells in immobile embryos. In several studies, both in chick and mouse, ectopic activation of BMP signaling in the putative interzone/articular cartilage cells led to ectopic expression of *Col2a1* and absence of articular joints (Ray et al., 2015; Zou et al., 1997) – a phenotype strikingly similar to immobilization. We therefore investigated the possibility of misregulation of BMP signaling in the absence of dynamic mechanical loading of joints and possible interplay between BMP and Wnt signaling in the mechanoresponsive definition and maintenance of the joint territory. This possibility was further supported by the identification of altered BMP pathway gene expression in muscle-less embryos (Rolfe et al., 2014b; R.A.R and P.M., unpublished).

Here, by comparing gene expression patterns in decamethonium bromide (DMB)-induced immobilized chick embryos, as well as in muscle-less mouse embryos, we demonstrate that molecular changes in and around developing joints upon immobilization are highly conserved. Investigating the mechanistic basis, we show downregulation of canonical Wnt pathway activity with concomitant upregulation of *Sfrp2*, combined with upregulation of BMP signaling in both chick and mouse embryos. We further show that expression of Smurf1, an intracellular inhibitor of BMP signaling, is not maintained in the absence of dynamic mechanical

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loading and forced expression of mouse Smurf1 in immobilized chick knee joints suppresses *Col2a1* expression. This suggests that modulation of the BMP pathway plays an important part in mechanoregulation, defining a zone that is permissive to the appropriate differentiation of articular cartilage, involving restricted canonical Wnt activation.

RESULTS

Molecular changes at the joint in immobilized chick embryos show similar alterations to muscle-less mouse embryos

To analyze histological and molecular changes in developing chick knee joints upon immobilization *in ovo*, embryos were treated with the neuromuscular blocking agent DMB (Fig. 1A). As previously

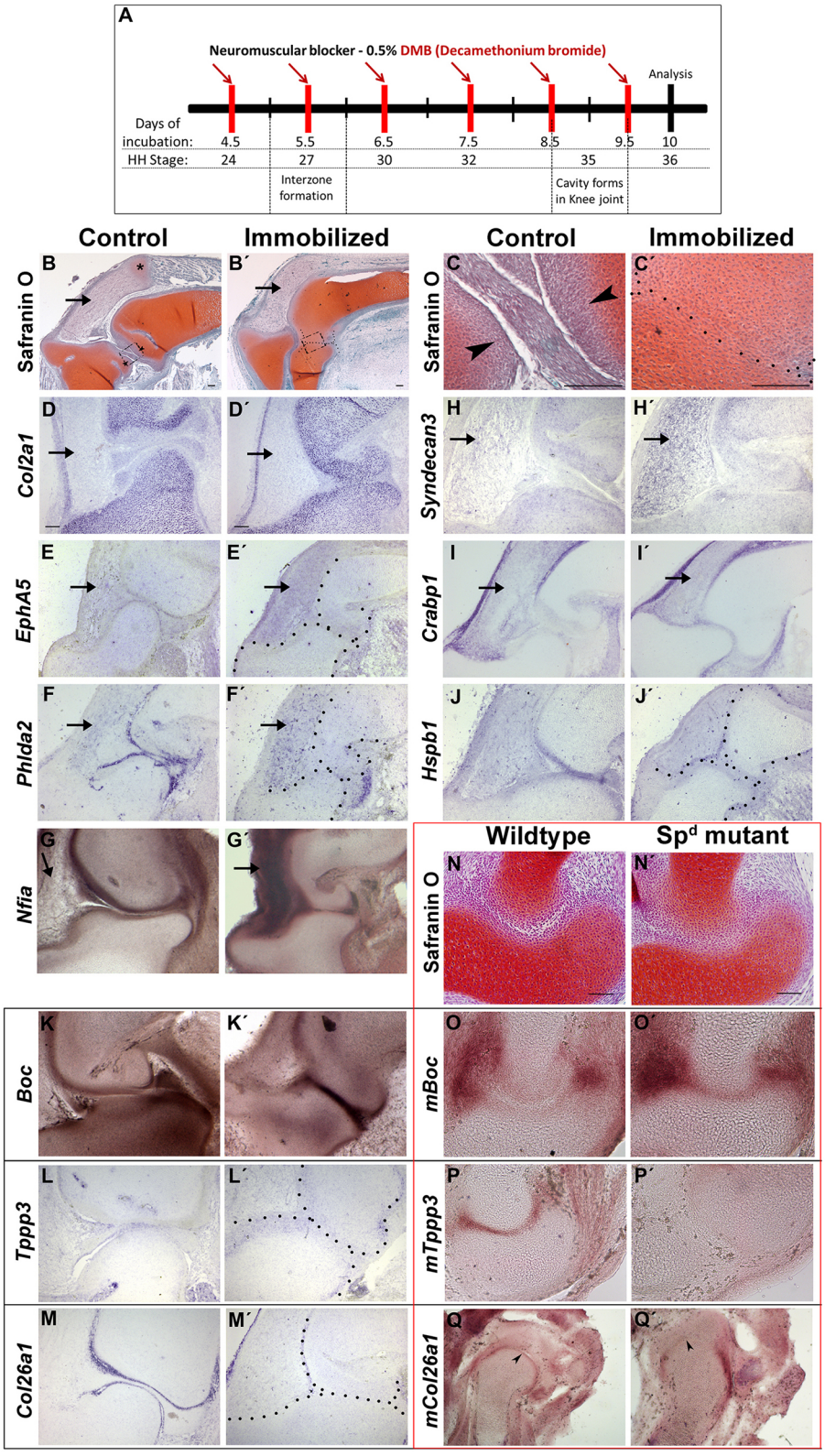

Fig. 1. Molecular changes at the joint in immobilized chick embryos show similar alterations to muscle-less mouse embryos. (A) Schematic of chick embryo immobilization using DMB. Immobilization started at day 4.5 of incubation (HH24), and embryos were analyzed on day 10 (HH36). (B-M′) Longitudinal sections of control (B-M) and immobilized (B′-M′) chick embryos through the knee joint. (B-C′) Safranin O staining; C,C′ show the boxed regions in B,B′ at higher magnification. (D-M′) RNA *in situ* hybridization for genes indicated on the left of each panel. (N-Q′) Longitudinal sections of the control (N-Q) and Sp^d/Sp^d muscle-less (N′-Q′) mouse embryos; (N-P′) humero-ulnar joint, (Q,Q′) shoulder joint. (N,N′) Safranin O staining. (O-Q′) RNA *in situ* hybridization for genes indicated on the left of each panel. Asterisk in B marks the patella. Arrows in B,B′,D-I′ indicate the infrapatellar fat pad (IPFp) and arrowheads in B,C indicate the chondrogenous or future articular cartilage layer. Arrowhead in Q marks the region of *Col26a1* expression; arrowhead in Q′ marks the region where the expression is downregulated. Dotted lines mark the outline of skeletal elements. (G-K′) 100 µm vibratome sections; (O-Q′) 60 µm vibratome sections; (B-F′, H-J′,L-N′) 5 µm paraffin sections. *n*=5. Scale bars: in B-C′, 100 µm; in D, 100 µm for D-M′; in N, 100 µm for N-Q′.

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reported, immobilized embryos exhibited loss of cavitation and lack of clear demarcation of chondrogenous layers (Fig. 1B-C′), along with loss of the *Col2a1*-negative region in the interzone (Fig. 1D-D′) compared with the control (Roddy et al., 2011). This is similar to the phenotype at the elbow joint of muscle-less mouse embryos (Fig. 1N,N′) (Kahn et al., 2009; Nowlan et al., 2010).

To characterize molecular changes in chick knee joints upon immobilization, we selected 10 genes of interest by comparing our previous transcriptomic analysis of genes specifically expressed in the presumptive chick articular cartilage (Singh et al., 2016) with differentially expressed genes in wild-type and Sp^{d} muscle-less mouse humeri (Rolfe et al., 2014b) (Table S1A). All genes showed altered patterns of expression in immobilized chick knee joints that agree with the altered expression (up or downregulated) found in RNAseq analysis of muscle-less mouse embryos (Table S1A). *Epha5* is upregulated in the infrapatellar fat pad (IPFp) (though downregulated in the joint line) (Fig. 1E,E′), *Crabp1* (Fig. 1I,I′), *Boc* (Fig. 1K,K′), *Sfrp2* (Fig. 2D,D′) and *Wnt4* (Fig. 2E,E′) are all upregulated in the joint line, whereas *Fabp3* (Fig. S1A,A′), *Id1* (Fig. S1B,B′), *Scx* (Fig. S1C,C′), *Hspb1* (Fig. 1J,J′) and *Tppp3* (Fig. 1L,L′) are downregulated in the joint line. To further assess whether changes in molecular profile of the developing articular cartilage upon immobilization is conserved between chick and mouse, we examined *Boc* (Fig. 1K,K′,O,O′), *Tppp3* (Fig. 1L,L′,P,P′) and *Col26a1* (Fig. 1M,M′,Q,Q′) expression in Sp^{d} joints, which show similar patterns of upregulation (*Boc*) and downregulation (*Tppp3* and *Col262a1*) in immobilized chick and mouse joints. In addition, we analyzed the expression of a number of other joint-specific genes in immobilized embryos (Table S1B). *Phlda2* (Fig. 1F,F′) expression is dramatically downregulated in the joint line, while upregulated in the IPFp (black arrow), as are *Nfia* (Fig. 1G,G′) and syndecan 3 (*Sdc3*) (Fig. 1H,H′).

Wnt signaling shows complex alteration at the knee joint of immobilized embryos

We examined the Wnt pathway because previous transcriptomics work on muscle-less mouse embryos indicated extensive complex disturbance of Wnt signaling (Rolfe et al., 2014b). In immobilized chick knee joints, we observed downregulation of β-catenin immunoreactivity within the IPFp (Fig. 2B′, arrows) and sub-articular region (Fig. 2C′, asterisks) compared with control embryos (Fig. 2B,C). We similarly saw reduced β-catenin immunoreactivity

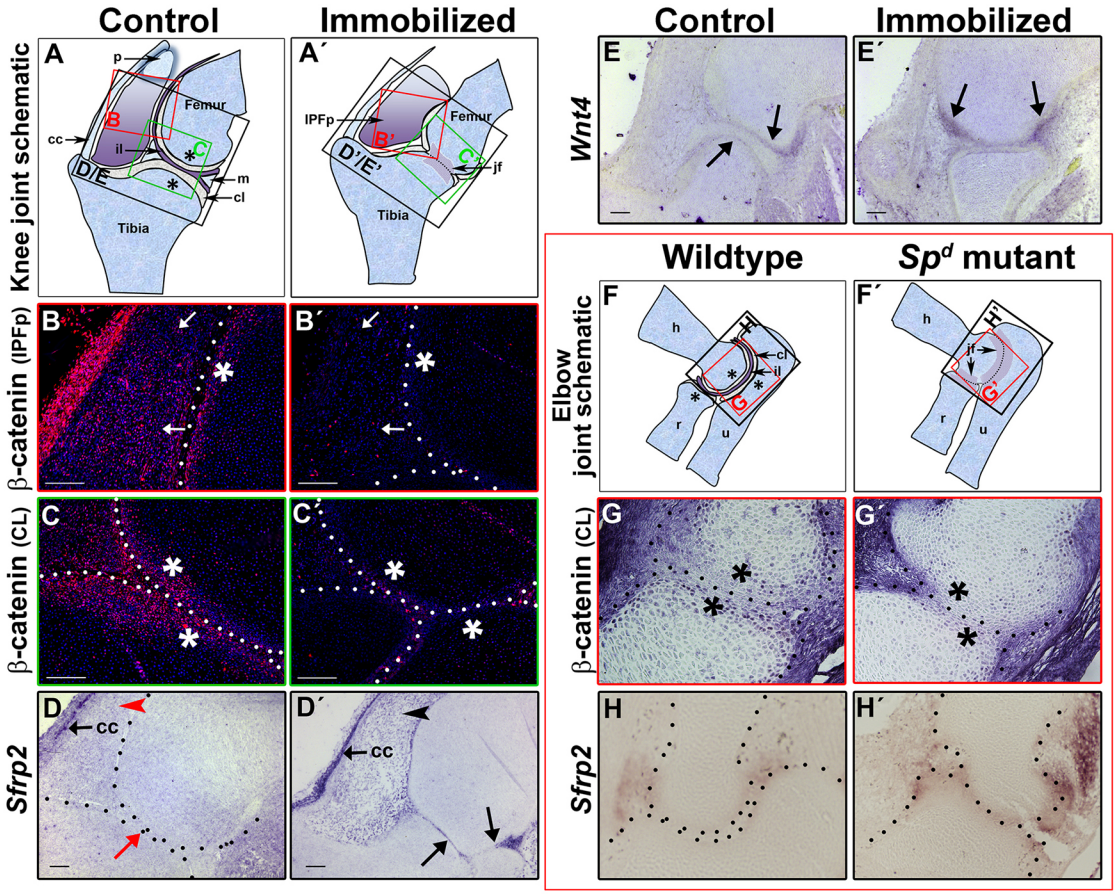

Fig. 2. Canonical Wnt signaling is downregulated in the knee joint of immobilized embryos. (A,A′) Schematic representation of control and immobilized HH36 chick knee joint. Boxes define the regions shown in corresponding lettered panels. Asterisks mark the sub-articular area. (B-E′) Longitudinal sections through chick knee joint of control (B-E) and immobilized (B′-E′) embryos. (B,B′) β-Catenin immunoreactivity in IPFp (white arrows); (C,C′) β-catenin immunoreactivity in the sub-articular region (marked with asterisks). (D,D′) RNA *in situ* hybridization for chick *Sfrp2*, expressed at low levels in IPFp (D, red arrowhead) and in the joint line (D, red arrow), but upregulated in immobilized knee joints (D′, arrows mark the joint line and arrowhead marks IPFp). (E,E′) RNA *in situ* hybridization for chick *Wnt4*; arrows mark expression in the joints. (F,F′) Schematic representation of the TS23 elbow joint of wild-type (F) and Sp^{d}/Sp^{d} muscle-less (F′) mouse embryos. (G-H′) Longitudinal sections through the humero-ulnar joint of control (G,H) and Sp^{d}/Sp^{d} muscle-less (G′,H′) embryos. (G,G′) β-Catenin immunoreactivity in the sub-articular region (marked with asterisks). (H,H′) RNA *in situ* hybridization for mouse *Sfrp2*. Dotted lines mark the outline of skeletal elements. *n*=5. Scale bars 100 µm. IPFp, infrapatellar fat pad; cl, chondrogenous layer; il, intermediate layer; m, meniscus; cc, capsular condensation; p, patella; jf, loss of joint line definition; h, humerus; r, radius; u, ulna. For descriptions of joint tissues and layers, see Jenner et al. (2014) and Roddy et al. (2011).

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at the joint line and sub-articular region of muscle-less mouse embryos (Fig. 2G,G′, asterisks mark the sub-articular region), which agrees with previous reports of reduced Wnt canonical pathway reporter activity (TOPGAL) (Kahn et al., 2009) and downregulation of Wnt pathway target genes (Rolfe et al., 2014b). We have previously reported upregulation of mRNA abundance of *Wnt4* as well as Wnt pathway modulator *Sfrp2* in muscle-less mouse embryos (Rolfe et al., 2014b), indicating complex alteration of pathway components. Here, we show similar upregulation in immobilized chick knee joints of *Sfrp2* (Fig. 2D,D′) and *Wnt4* (Fig. 2E,E′). In particular *Sfrp2*, which is normally expressed in the capsular condensation (cc) and at a low level in and around joint line (red arrow) in control embryos (Fig. 2D) is expressed at an elevated level in immobilized chick knee joints (Fig. 2D′), in particular in the IPFp (arrowheads) and joint line (arrows). In the muscle-less mouse, *Sfrp2* expression is similarly elevated in the joint line and adjacent to the joint (Fig. 2H,H′); it is unclear whether the latter corresponds to the fat pad region in the chick knee. Reduced β-catenin immunoreactivity is reciprocal to Sfrp2 expression changes in the IPFp and joint line (compare corresponding regions of Fig. 2D,D′ with 2B-C′).

BMP signaling at the joint line and adjacent rudiment is altered in immobilized embryos

Previously, we reported that cartilage-specific inactivation of Noggin in chick and mouse embryos resulted in abolition of joint formation and contiguous expression of *Col2a1* through the presumptive articular cartilage region (Ray et al., 2015), similar to effects of immobilization on developing joints reported here (Fig. 1B′-D′). In addition, inactivation of Noggin led to a spread of pSMAD1/5/8 immunoreactivity across the presumptive joint (Ray et al., 2015); i.e. loss of normal restriction at a distance from the joint line. Therefore, we examined the status of BMP signaling in immobilized chick knee joints as well as muscle-less Sp^{d} mouse elbow joints. We used pSMAD1/5/8 immunoreactivity as a read-out of BMP signaling and observed intense upregulation of BMP signaling, not only in the joint line (compare region marked by dotted lines in Fig. 3B,B′ for chick and in Fig. 3I,I′ for mouse), but also in the sub-articular cells (marked by asterisks in Fig. 3B,B′ for chick and Fig. 3I,I′ for mouse) immediately adjoining the joint line, upon loss of mechanical activity in both chick and mouse joints (compare magnified views of the boxed regions from Fig. 3B,B′ and 3I,I′ marked with yellow arrowheads in Fig. 3C,C′ for chick and Fig. 3J,J′ for mouse). Therefore, the sub-articular region that is normally devoid of pSMAD1/5/8 immunoreactivity shows ectopic BMP activity in both chick and mouse immobilized joints.

We have previously suggested that Noggin-expressing cells in the sub-articular region insulate the articular cartilage progenitor cells from exposure to BMP signaling (Ray et al., 2015). Therefore, we investigated whether Noggin expression is abolished upon loss of mechanical stimulation. Contrary to expectation, we observed marked upregulation of Noggin protein not only in the joint line of both chick (Fig. 3D-E′) and mouse joints (Fig. 3K-L′, some cells are marked with white arrowheads in Fig. 3L′) but also in the sub-articular cells (compare the regions marked by asterisks in Fig. 3D,D′ and 3K,K′). The upregulation of Noggin was also observed at the transcript level in chick (Fig. 3F,F′) and mouse (Kahn et al., 2009). Furthermore, we found that the expression of all three BMP ligand-encoding genes, *Bmp2* (Fig. S1D,D′), *Bmp4* (Fig. 3G,G′; Fig. S1F,F′ for mouse) and *Bmp7* (Fig. S1E,E′) were downregulated in the joints of immobilized chick and mouse embryos, although *Bmp2* and *Bmp7* are detected at very low levels in control at these stages (Fig. S1).

We also assessed the status of BMP signaling in developing chick joints at earlier stages (HH31 and HH34) following immobilization. We observed that mRNA expression of BMP ligands was downregulated by HH34 (Fig. S2F,F′) but was not obviously changed at HH31 (Fig. S2B,B′), whereas increased pSMAD1/5/8 immunoreactivity was observed as early as HH31 (Fig. S2D,D′) that persists until later (Fig. S2H,H′, HH34). Noggin mRNA expression was not obviously changed at either HH31 (Fig. S2C,C′) or HH34 (Fig. S2G,G′). So the increase in pSMAD1/5/8 in and around the joint is an early effect of immobilization, whereas downregulation of BMP ligand expression and upregulation of Noggin are later, and possibly secondary, effects in a dysregulated system. It should also be noted that expansion of *Col2a1* expression (Fig. S2A,A′,E,E′) across the joint line was apparent as early as HH31, presumably a direct consequence of ectopic activation of BMP signaling.

Intracellular BMP signaling inhibitors Smurf1 and Smurf2 are downregulated on immobilization, and overexpression can reverse molecular consequences of immobilization

Since BMP and TGFβ signaling can be negatively regulated by E3 ubiquitin ligases known as Smad ubiquitylation regulatory factor 1 (Smurf1) and Smurf2 through proteasomal degradation of pSMAD1/5/8 (Zhang et al., 2001; Zhu et al., 1999), we examined the expression of Smurf1 and Smurf2 in immobilized chick knee joints. At HH36 *Smurf1* and *Smurf2* are normally expressed in the sub-articular region but not within the interzone (Fig. 4B-C) and also in the hypertrophic zone (not shown). The abundance of both *Smurf1* (compare Fig. 4B and 4B′) and *Smurf2* (compare Fig. 4C and 4C′) mRNAs was downregulated in immobilized compared with control embryos. It should also be noted that the area of abundant *Smurf* mRNA expression, i.e. the sub-articular region, is devoid of BMP signaling activity in HH36 knee joints (compare Fig. 4B,C with Fig. 3B, marked with asterisks); and in immobilized limbs, there is a reciprocal relationship between where Smurf expression is reduced and pSMAD1/5/8 activity increased.

It has been demonstrated that Smurf1, and not Smurf2, selectively downregulates BMP signaling activity while sparing TGFβ activity (Zhang et al., 2001; Zhu et al., 1999). We therefore overexpressed mouse Smurf1 (mSmurf1) from a doxycycline inducible bi-directional promoter to investigate whether mSmurf1 can reduce the level of BMP signaling in normal cartilage primordia (experimental design schematically represented in Fig. S3A). mSmurf1-expressing cells were identified by detection of GFP (Fig. S3, compare panels B and E) as both mSmurf1 and GFP are under the control of the same BI-TRE promoter (Sato et al., 2007; Schecterson et al., 2012; Watanabe et al., 2007). Expression of mSmurf1 reduced the level of pSMAD1/5/8 immunoreactivity within its normal domain in the developing chick cartilage (Fig. S3, compare panels C and F). As expected, this downregulation of BMP signaling also reduced the level of *Col2a1* mRNA expression within the domain of mSmurf1 overexpression (Fig. S3, compare panels D and G). To investigate whether overexpression of mSmurf1 in the cartilaginous rudiment of immobilized embryos can reduce the extent of ectopic pSMAD1/5/8 immunoreactivity, we overexpressed mSmurf1 in immobilized chick embryos (experimental design schematically represented in Fig. 4D) and examined domains of pSMAD1/5/8 and Noggin immunoreactivity, and *Col2a1* mRNA expression. mSmurf1-expressing cells were identified by detection of GFP (Fig. 4E′). The GFP-positive cells in Fig. 4E do not express mSmurf1 (vector control). It should be noted that pT2K-m*Smurf1*-

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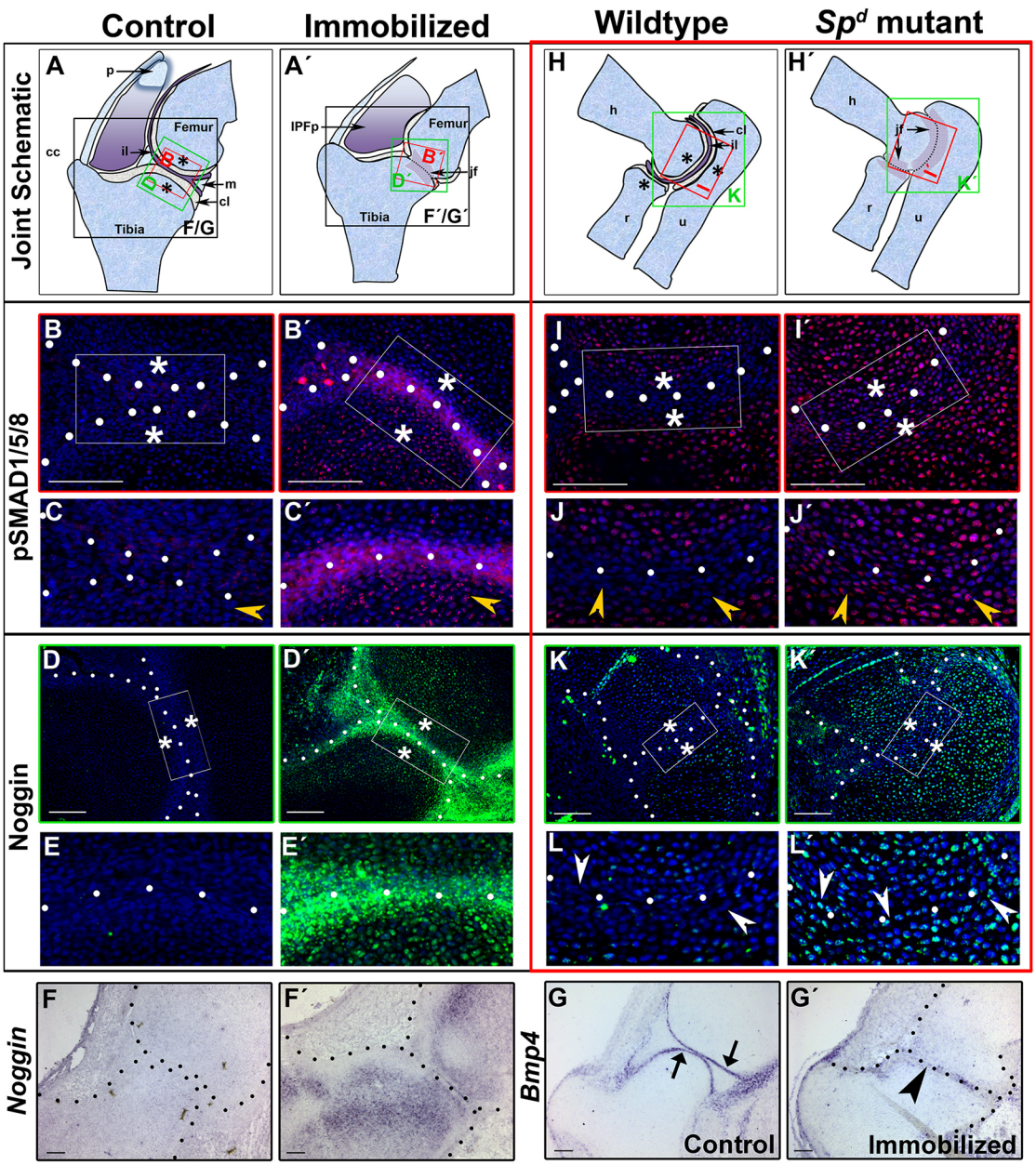

Fig. 3. BMP signaling at the joint is altered in the absence of mechanical stimulation in chick and mouse. (A,A′) Schematic representation of control and immobilized HH36 chick knee joint. Boxes mark regions shown in corresponding lettered panels. (B-G′) Longitudinal sections through the chick knee joint of control (B-G) and immobilized (B′-G′) embryos, *n*=5. (B-E′) Immunohistochemistry for pSMAD1/5/8 (B,B′) and Noggin (D,D′). (C,C′ and E,E′) Higher magnification views of the boxed regions in B,B′ and D,D′, respectively. RNA *in situ* hybridization for chick *Noggin* (F,F′) and *Bmp4* (G,G′). (H-L′) Mouse tissue. (H,H′) Schematic representations of the TS23 elbow joints of wild-type and Sp^d/Sp^d muscle-less mutant mouse embryos. (I-L′) Longitudinal sections through the humero-ulnar joint of control (I-L) and Sp^d/Sp^d muscle-less (I′-L′) embryos, *n*=3. Immunohistochemistry for pSMAD1/5/8 (I,I′) and Noggin (K,K′). (J,J′ and L,L′) Higher magnification views of the boxed regions in I,I′ and K,K′, respectively. Asterisks mark the sub-articular region in A,H,B,B′,D,D′,I,I′,K,K′. Yellow arrowheads mark cells in the sub-articular region in C-C′ and J-J′, whereas white arrowheads in L,L′ mark cells on the joint line. Dotted lines mark the outline of skeletal elements. Black arrows mark the Bmp4 expression domain in the joint line; black arrowheads mark the absence of Bmp4 in the joint line. Scale bars: 100 µm.

BI-TRE-EGFP is expressed within certain patches, which are enclosed with a red outline in Fig. 4E′, of the electroporated cartilage. Adjacent to this region there is an area marked by a yellow outline (Fig. 4E′-H′) that lacks GFP expression and thus mSmurf1 misexpression. The yellow region can be viewed as an internal negative control for this experiment.

This experiment was conducted four times with variation in the precise location of mSmurf1-expressing cells but consistent with respect to effects shown here; Fig. 4 shows analysis of adjacent sections from one typical specimen for direct comparison. We observed an obvious downregulation of pSMAD1/5/8 immunoreactivity in the GFP-expressing cells of immobilized embryos (Fig. 4F′, compare the region outlined in yellow with its adjacent region). We also observed concomitant downregulation of Noggin protein expression (Fig. 4G′) and downregulation of *Col2a1* mRNA expression (Fig. 4H′) in the same region. Interestingly, in the GFP-negative cells within the same rudiment (yellow outline), where mSmurf1 was not misexpressed, pSMAD1/5/8 immunoreactivity, Noggin protein expression and *Col2a1* mRNA expression were not downregulated.

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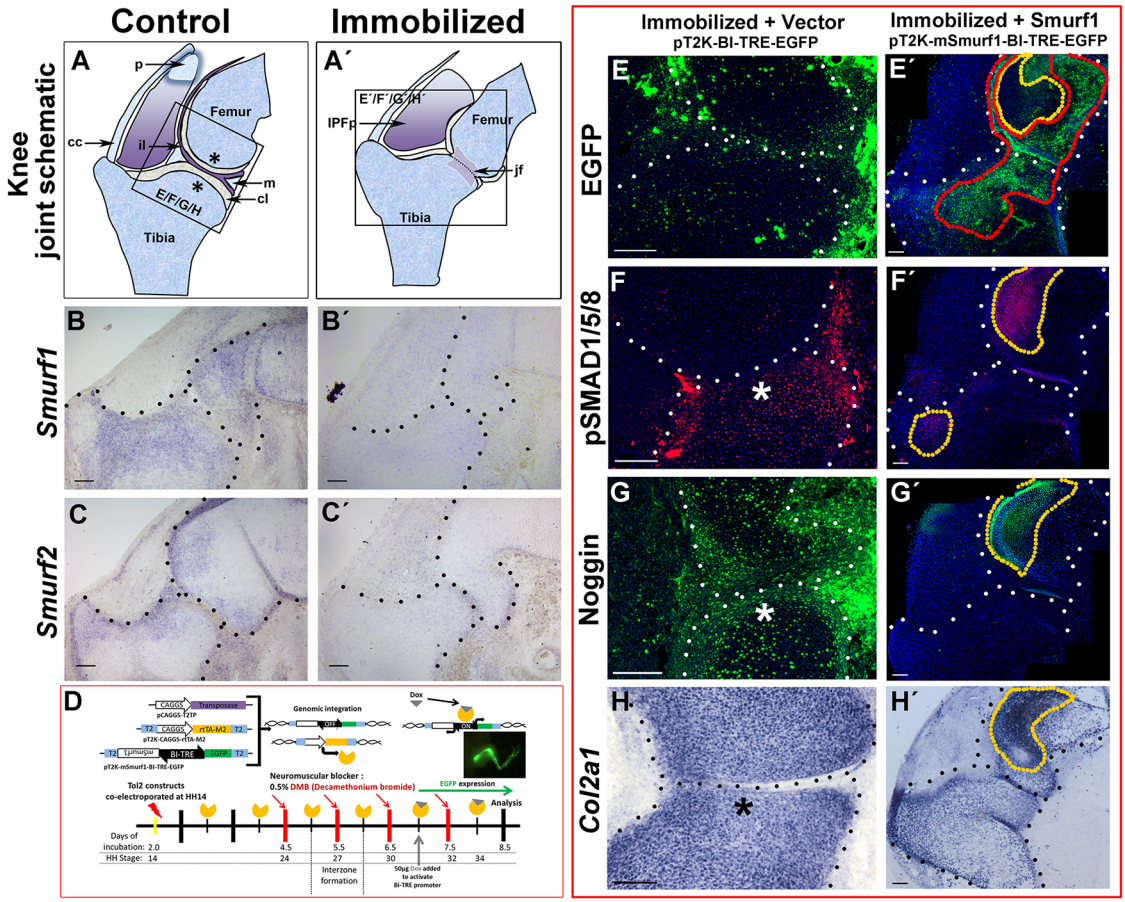

Fig. 4. Misexpression of mouse *Smurf1* in immobilized chick reverses molecular consequences of immobilization. (A,A′) Schematic representation of control and immobilized HH36 chick knee joint. (B-C′) Longitudinal sections through the chick knee joint of control (B,C) and immobilized (B′,C′) embryos. RNA *in situ* hybridization for *Smurf1* (B,B′) and *Smurf2* (C,C′). (D) Schematic of experiments overexpressing mSmurf1 in a doxycycline-dependent manner. Tol2 constructs (described in the Materials and Methods) were electroporated in the limb progenitor cells at day 2 of incubation (HH14). Immobilization was carried out from 4.5 days of incubation and cells were harvested at day 8.5. Doxycycline (50 µg) was added at day 7 to induce expression of either EGFP (control) or both EGFP and mSmurf1 simultaneously from the bi-directional TRE promoter. (E-H) Adjacent sections of immobilized chick knee joint electroporated with control vector (pT2K-BI-TRE-EGFP). (E′-H′) Adjacent sections of immobilized chick knee joint electroporated with a Dox-dependent mouse Smurf1-expressing Tol2 vector (pT2K-mSmurf1-BI-TRE-EGFP); red outlines a domain expressing GFP/mSmurf1 and yellow outlines a non-expressing territory. (E,E′) Immunohistochemistry for GFP (note expression of GFP and mSmurf1 within the red outline); (F,F′) Immunohistochemistry of pSMAD1/5/8 (note yellow domain not electroporated with GFP/mSmurf1 and is positive for canonical BMP signaling); (G,G′) Immunohistochemistry of Noggin (the yellow domain not electroporated continues to express elevated Noggin protein). (H,H′) RNA *in situ* hybridization for *Col2a1* (note the domain not electroporated expresses higher levels of *Col2a1* transcript). Examples shown are typical of four replicate experiments. Black and white dotted lines mark the outline of skeletal elements. Asterisks mark the sub-articular region. Scale bars: 100 µm.

DISCUSSION

In this study, we have uncovered new mechanistic insight into how dynamic mechanical loading due to embryo movement influences the differentiation of articular cartilage from bi-potential cartilage progenitors in developing joints. In the absence of movement, *Col2a1*-expressing transient cartilage extends across the presumptive joint and we found that loss of mechanical stimulus not only leads to downregulation of activated β-catenin in the joint region but also ectopic activation of BMP signaling in both the sub-articular region and presumptive articular cartilage cells in both mouse and chick embryos. Interestingly, this ectopic activation is not accompanied by overexpression of BMP ligands or by downregulation of the secreted BMP inhibitor Noggin. Instead, we implicate a mechanism involving downregulation of an intracellular BMP signaling inhibitor, Smurf1. Finally, through forced expression of mSmurf1 across the joint region in immobilized cartilage rudiments, we could reverse ectopic activation of BMP signaling and reduce expression of the transient cartilage marker *Col2a1*. Our data suggest that downregulation of Smurf transcription following immobilization of the developing joint causes ectopic activation of BMP signaling, thereby creating a non-permissive cellular environment for articular cartilage differentiation and pinpoints Smurf activity as an important modulator of the system that defines an articular cartilage permissive zone where canonical Wnt signaling is active.

It has been reported that, at an intermediate stage between joint specification and cavitation, cells at the joint interface are plastic and co-express transient and articular cartilage markers (Kahn et al., 2009; Ray et al., 2015). However, in the absence of muscle contraction in mice, the co-expression of articular and transient cartilage markers is prolonged beyond the initial stage (Kahn et al., 2009). Eventually, joint-specific markers are lost and transient cartilage markers, *Col2a1*, *Sox9* and *Matn1* are maintained concomitant with joint loss. Kahn et al. suggested that in the absence of mechanical stimulation from movement, cartilage progenitor cells lose their ability to differentiate as articular

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cartilage and exclusively differentiate into transient cartilage cells, primarily due to downregulation of active β-catenin/canonical Wnt signaling, as judged by TOPGAL reporter activity (Kahn et al., 2009). Wnt/β-catenin signaling is known to be required for articular cartilage differentiation (Guo et al., 2004; Später et al., 2006a). However, reduction in Wnt signaling by itself cannot explain the severe phenotype observed upon loss of mechanical stimulus. In fact, the phenotypes observed in response to conditional knockout of β-catenin by Col2- or Gdf5-Cre, conditional knockout of c-Jun by Prx1-Cre, or Wnt9a/Wnt4 double knockout mice are much milder than in immobilized embryos, particularly with respect to transient cartilage differentiation in the putative articular cartilage domain (Kahn et al., 2009; Kan and Tabin, 2013; Koyama et al., 2008; Roddy et al., 2011; Später et al., 2006a). This suggests that immobilization causes greater disturbance to the system than inhibition of Wnt signaling.

Overexpression of BMP signaling in the joints by: (1) upregulation of BMP ligands Bmp2 and Bmp4 (Duprez et al., 1996), (2) misexpression of ca-BMPRIB (Zou et al., 1997) or (3) loss of the BMP inhibitor Noggin (Brunet et al., 1998) leads to formation of transient cartilage at the expense of articular cartilage. This prompted us to examine the status of BMP signaling in immobilized chick knee joints and muscle-less mouse embryos. We have previously reported that BMP signaling is not detectable in a zone covering the joint and adjacent sub-articular region (Ray et al., 2015). Here, in immobilized embryos, we observed clear upregulation of BMP signaling in the presumptive articular cartilage cells and sub-articular region. We were surprised to see concomitant Noggin upregulation in the same cells, in both chick and mouse. We speculate that Noggin upregulation may be a secondary consequence of ectopic BMP signaling (Gazzerro et al., 1998; Merino et al., 1998; Sela-Donenfeld and Kalcheim, 2002). This is supported by the observation that Noggin transcription is not upregulated at HH31 and HH34 when pSMAD1/5/8/ activity is already ectopic across the joint (Fig. S2) [and indeed further supported by downregulation of Noggin as a consequence of Smurf1 overexpression (Fig. 4G,G′)]. We therefore looked for alternative mechanisms to explain ectopic BMP activity and we identified downregulation of *Smurf1* in immobilized chick embryos. As Smurf1 causes proteasomal degradation of pSMAD1/5/8 and hence can have a negative impact on BMP signaling (Zhu et al., 1999), downregulation of *Smurf1* could cause ectopic BMP activation. Smurf genes therefore represent a possible mechanosensitive point in the system where normal mechanical stimulation leads to their persistent expression in the presumptive joint region, reducing BMP activity and distinguishing these cells from the rest of the rudiment, where canonical Wnt signaling can then promote articular cartilage differentiation. It is interesting to note that although Smurf1 is not expressed in the joint line, immobilization causes ectopic activation of BMP signaling across the joint territory. We speculate that downregulation of Smurf1 causes ectopic activation of BMP signaling in the cells adjoining the interzone, and this increased activity spreads through the cells of the interzone by paracrine action. However, downregulation of Smurf1 alone cannot be sufficient to cause the phenotype as joint abnormalities have not been reported in Smurf1 knockout mouse embryos (Yamashita et al., 2005).

Smurf1-deficient mice exhibit increased bone mass and Smurf1 has been shown to negatively regulate osteoblast differentiation *in vivo* and from mesenchymal stem cells (Yamashita et al., 2005; Zhao et al., 2010, 2003). In osteoarthritis (OA), articular chondrocytes exhibit an increase in BMP signaling (Li et al., 2006) and Smurf1 is downregulated in OA meniscal cells (Sun et al., 2010). Conversely, overexpression of Smurf1, in cooperation with Smad6, delays chondrocyte hypertrophy in cartilage (Horiki et al., 2004). Taken together, these studies suggest a central role for Smurf1 in regulating BMP responsiveness in articular cartilage. Unfortunately, Smurf gene knockouts have not produced informative embryonic phenotypes; joint abnormalities are not reported in Smurf1 knockout embryos, but our demonstration of co-expression of Smurf2 in this region (Fig. 4B,C) may mask such an effect, and double knockout embryos die at E12.5 (Narimatsu et al., 2009). A link between Smurf and mechanoregulation is also indicated, with downregulation of Smurf1 expression and accumulation of Smad1/5 proteins in osteoblasts under mechanical strain (Wang et al., 2010). Moreover, ubiquitin ligases have been predicted to be mechanosensitive genes in other contexts (Baskin et al., 2014; Lourenço et al., 2016). We propose that immobilization affects expression of multiple genes involved in successful articular cartilage differentiation, including crucial regulators such as *Sfrp2* and *Smurf1*. *Sfrp2*, an inhibitor of Wnt signaling (the pro-articular cartilage signal), is upregulated. On the other hand, *Smurf1*, an intracellular inhibitor of BMP signaling, is downregulated, resulting in ectopic activation of BMP signaling and creation of a non-permissive environment for articular cartilage differentiation. What remains to be seen though, is how *Smurf* or *Sfrp2* expression are regulated by mechanical stimulation and whether the regulation is direct or indirect.

This is the first study to demonstrate the modulation of both Wnt and BMP signaling pathways in the developing joint in response to altering the mechanical environment, showing conservation across species. Interestingly enough, it appears that these two signals are reciprocally regulated by mechano-transduction where an inhibitor of the Wnt pathway is upregulated and simultaneously an inhibitor for BMP pathway is downregulated. The demonstration that canonical Wnt signaling is altered in the joint line in immobilized chick as well as mouse embryos corroborates our previous finding that components of the Wnt signaling pathway, including multiple Wnt pathway target genes, show altered expression in muscle-less mouse embryos (Rolfe et al., 2014b). Furthermore, similarities in the disturbances caused to joint development across mouse and chick systems are underlined by our analysis of a panel of genes that show joint-specific expression in the chick and transcriptional disturbance in the muscle-less mouse (Table S1A). We found that disturbances were consistent across species (e.g. upregulated or downregulated in reduced mechanical environment) and a subset of genes compared by *in situ* hybridization shows consistent changes to spatial patterns of expression. Upon loss of mechanical stimulus, expression of some articular cartilage specific genes remain unaltered (*Jun* and *Prrx1*; Fig. S4), some are upregulated (*Crabp1* and *Boc*) and some downregulated (*Phlda2*, *Bmp2*, *Bmp4* and *Bmp7*). Even though *Jun*, *Prrx1*, *Crabp1*, *Boc, Phlda2*, *Bmp2*, *Bmp4* and *Bmp7* are expressed in the same cell population (IL), their expression profile changes differently upon loss of mechanical stimulation, suggesting an effect on the regulatory systems controlling subsets of genes rather than a reflection of a global tissue change. In this context, we particularly highlight expression of several genes (e.g. *Nfia* and *EphA5*) lost from the articular cartilage region but upregulated in the IPFp, the significance of which is not immediately apparent.

Fig. 5 presents a model that proposes a point of integration of mechanical cues and molecular signaling in the dynamic environment of the developing joint, when crucial territories are being defined (Fig. 5A). We have revealed the expression domains of key molecules in and around the joint using autotaxin expression in the joint interzone (Fig. 5B) and *Col2a1* expression restricted to future transient cartilage of the

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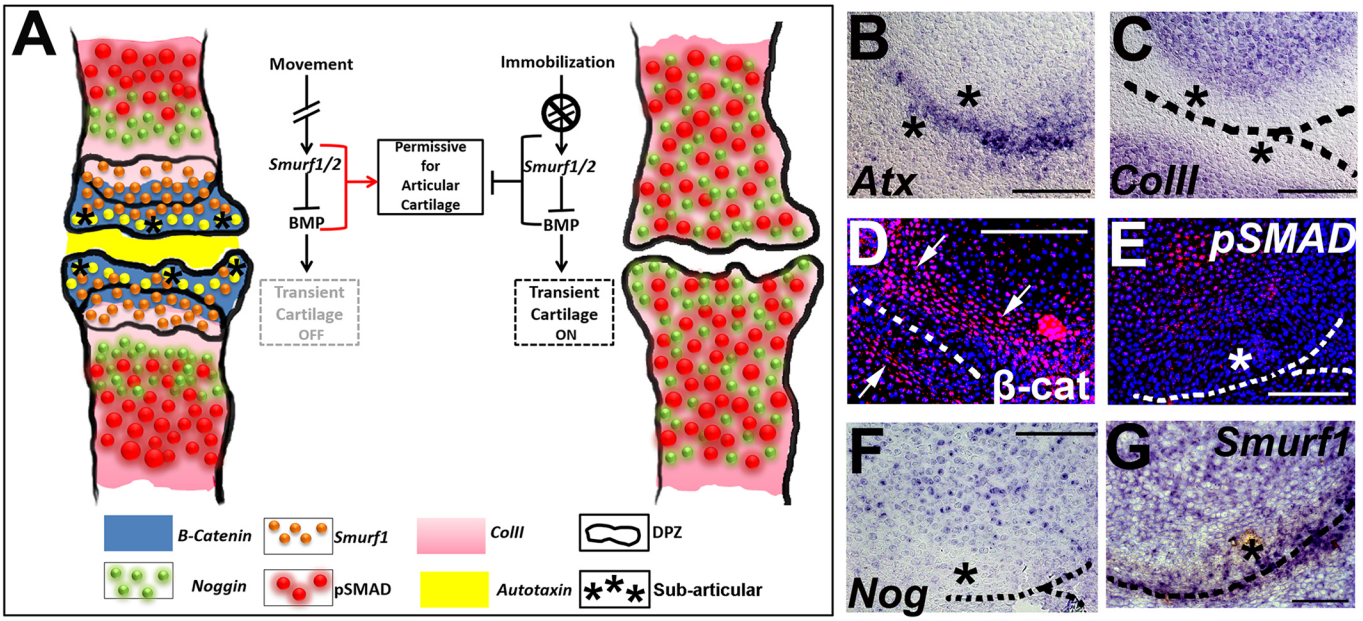

Fig. 5. Definition of joint territories requires integration of molecular and mechanical cues. (A) Schematic overview of findings and proposal of a model that integrates mechanical cues and molecular regulation of joint definition. (B-E) In the normal situation (A, left), joint markers and transient cartilage markers are clearly separated (B,C; autotaxin and *Col2a1* mRNA expression) and canonical Wnt signaling (β-catenin) is restricted to the articular zone (D), whereas BMP/pSMAD1/5/8 activity is detected at a distance (E). In immobilized embryos (A, right), pSMAD1/5/8 activity and transient cartilage characteristics spread through the rudiments, while β-catenin activity is lost. (F) Normal domain of noggin expression is excluded from the sub-articular region (asterisk). (G) We further show normal *Smurf1* RNA expression in the sub-articular region, which is lost on immobilization, where it can no longer restrict BMP signaling. Asterisks mark the sub-articular region; dotted lines demarcate the putative articular joint site. Arrows point to β-catenin immunoreactive cells in the sub-articular region. Scale bars: 100 µm.

rudiment (Fig. 5C). Sub-articular cells close to the joint line experience canonical Wnt signaling (domain of β-catenin immunoreactivity, Fig. 5D), whereas BMP signaling (pSMAD1/5/8 immunoreactivity) is restricted to cells at a distance (Fig. 5E). We have previously proposed that the articular cartilage progenitors, at this stage most of which are present in the DPZ (black marquee area, Fig. 5A), must be insulated from BMP signaling and experience Wnt signaling to undergo articular cartilage differentiation. We also demonstrated that Noggin, expressed in the DPZ, is a crucial part of this regulation (Ray et al., 2015; Fig. 5F). Here, we show another negative regulator of BMP signaling, *Smurf1*, is expressed in the subarticular region (Fig. 5G). It should be noted that the domains of Noggin and Smurf1 mRNA expression are mutually exclusive (compare Fig. 5F with 5G). Smurf1 activity is mechanosensitive, with loss of sub-articular expression in immobilized embryos concomitant with spread of BMP activity and transient cartilage characteristics (e.g. Col2a1 expression) to the termini of the rudiments in immobilized embryos. We further showed that overexpression of Smurf1 in immobilized embryos can reverse pSMAD1/5/8 and *Co2a1* activation.

Taken together, we propose that mechanodependent expression of Smurf1 in the sub-articular cells carries out a pivotal role in defining the articular zone by maintaining these cells free of BMP signaling through a cell-intrinsic mechanism. We do not propose that Smurf1 has a pro-articular cartilage effect, rather it is a molecule that ensures a permissive environment for the articular cartilage progenitor cells to undergo appropriate differentiation by downregulating BMP signaling; immobilization-mediated loss of Smurf1 eliminates this permissive environment and BMP signaling overrides Wnt instruction to stimulate transient cartilage differentiation in the articular cartilage domain (Fig. 5). Overall, our findings suggest that activation of canonical Wnt signaling and downregulation of BMP signaling are two major pathways involved in movement-induced development of joints and this regulation is conserved across vertebrate species.

MATERIALS AND METHODS

Tissues

Fertilized White Leghorn Chicken eggs were obtained from Central Avian Research Institute of India; Chandra Shekhar Azad Agricultural University, Kanpur, UP and Ganesh Enterprises, Nankari, Kanpur, UP. Eggs were incubated at 38°C in a humidified chamber to be treated and/or harvested at specific stages of development as assessed by Hamburger and Hamilton staging criteria (Hamburger and Hamilton, 1951). Homozygous *Splotch-delayed* (*Pax3*$^{Spd/Spd}$) muscle-less mutant mice and either wild-type (*Pax3*$^{+/+}$) or heterozygous (*Pax3*$^{Spd/+}$) littermate controls were generated by mating heterozygous C57BL/6J-Pax3^{Spd} mice. Embryos were staged according to Theiler criteria (Theiler, 1989). Mouse experiments were conducted under personal licenses to C.A.S. and R.A.R., and under the guidelines of Trinity College Dublin Bioresources Unit and Bioethics Committee.

Tissue processing

Embryonic limbs were dissected and fixed overnight in 4% paraformaldehyde at 4°C, embedded in paraffin and 5-10 µm sections cut along the para-sagittal plane using a microtome. Vibratome sections (60 or 100 µm) were generated as described by Roddy et al. (2011).

RNA *in situ* hybridization

cDNA clones used to make digoxigenin-labeled antisense riboprobes generated by *in vitro* transcription are detailed in Table S1. RNA *in situ* hybridization was performed as described previously for chick (Singh et al., 2016) and mouse (Nowlan et al., 2008). Number of specimens analyzed for each gene (*n*) is given in Table S1.

Histology

Safranin-O staining was carried out on de-paraffinized, rehydrated sections that were counterstained in Hematoxylin, rinsed in acetic acid and stained

with Safranin-O (0.1% in water). Sections were subsequently dehydrated and mounted in Aqua-Poly/Mount.

Immunohistochemistry

For pSMAD1/5/8, β-catenin, Noggin and GFP immunohistochemistry, sections were processed and detected as described by Ray et al. (2015) for immunofluorescence. For colorimetric detection, sections were treated with an alkaline phosphatase-conjugated anti-mouse IgG (Santa Cruz, sc-2008) at 1:300 dilution, and developed in an NBT-BCIP [Roche, 87.5 µg/ml each in NMT (pH 9.5)] solution.

Immobilization assay

In ovo immobilization was performed as described by Roddy et al. (2011) and according to the regime illustrated in Fig. 1. For m*Smurf1* misexpression experiment, electroporation of the Tol2 constructs was carried out as described below (*in ovo* electroporation) at day 2 (HH14) of incubation. Embryos were subsequently immobilized from day 4.5 (HH24) and harvested at day 8.5 (HH35) (Fig. 4D).

In ovo electroporation

For m*Smurf1* misexpression, the Tol2 constructs (Sato et al., 2007) pT2K-CAGGS-rtTA-M2, pT2K-BI-TRE-EGFP (control) or pT2K-mSmurf1-BI-TRE-EGFP and pCAGGS-T2TP were mixed with 0.5 µg/µl pCAGGS-mCherry and 1% Fast Green injected between the somatic LPM and splanchnic LPM at a ratio of 1:1:1 using a microinjector. Electroporation was performed as described previously (Suzuki and Ogura, 2008). pT2K-mSmurf1-BI-TRE-EGFP contains a bi-directional Tet-Responsive control region (BI-TRE) flanked by the left and right ends of Tol2, with EGFP expression cassette on one side and full-length mouse *Smurf1* CDS (amplified from IRAV 3660965) on the other, inserted in the *Eco*RV site of the pT2K-BI-TRE-EGFP vector. Control vector (pT2K-BI-TRE-EGFP) contains EGFP expression cassette only. To induce expression, 50 µg doxycycline in HBSS was added at embryonic day 7 (HH31).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: P.N.P.S., P.M., A.B.; Methodology: P.N.P.S., P.M., A.B.; Validation: P.N.P.S., C.A.S., S.K.S., R.A.R., P.M., A.B.; Formal analysis: P.N.P.S., C.A.S., S.K., P.G., S.K.S.; Investigation: P.N.P.S., C.A.S., S.K.S., R.A.R., A.R.; Resources: P.M., A.B.; Data curation: P.N.P.S., C.A.S., S.K.S., A.R.; Writing - original draft: P.N.P.S., P.M., A.B.; Writing - review & editing: P.N.P.S., P.M., A.B.; Visualization: P.N.P.S., C.A.S., S.K.S., P.M., A.B.; Supervision: P.M., A.B.; Project administration: P.M., A.B.; Funding acquisition: P.M., A.B.

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Supplementary information

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