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RESEARCH ARTICLE

The non-canonical BMP and Wnt/β-catenin signaling pathways orchestrate early tooth development

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ABSTRACT

BMP and Wnt signaling pathways play a crucial role in organogenesis, including tooth development. Despite extensive studies, the exact functions, as well as if and how these two pathways act coordinately in regulating early tooth development, remain elusive. In this study, we dissected regulatory functions of BMP and Wnt pathways in early tooth development using a transgenic noggin (*Nog*) overexpression model (*K14Cre;pNog*). It exhibits early arrested tooth development, accompanied by reduced cell proliferation and loss of odontogenic fate marker *Pitx2* expression in the dental epithelium. We demonstrated that overexpression of *Nog* disrupted BMP non-canonical activity, which led to a dramatic reduction of cell proliferation rate but did not affect *Pitx2* expression. We further identified a novel function of Nog by inhibiting Wnt/β-catenin signaling, causing loss of *Pitx2* expression. Co-immunoprecipitation and TOPflash assays revealed direct binding of Nog to Wnts to functionally prevent Wnt/β-catenin signaling. *In situ* PLA and immunohistochemistry on *Nog* mutants confirmed *in vivo* interaction between endogenous Nog and Wnts and modulation of Wnt signaling by Nog in tooth germs. Genetic rescue experiments presented evidence that both BMP and Wnt signaling pathways contribute to cell proliferation regulation in the dental epithelium, with Wnt signaling also controlling the odontogenic fate. Reactivation of both BMP and Wnt signaling pathways, but not of only one of them, rescued tooth developmental defects in *K14Cre;pNog* mice, in which Wnt signaling can be substituted by transgenic activation of *Pitx2*. Our results reveal the orchestration of non-canonical BMP and Wnt/β-catenin signaling pathways in the regulation of early tooth development.

KEY WORDS: BMP, Wnt, Noggin, Tooth, Development, Mouse

INTRODUCTION

In mice, tooth development begins at embryonic day (E) 11.5 as a local thickening of the dental epithelium to form the dental placode (the lamina stage), which subsequently proliferates and invaginates into the subjacent mesenchyme, forming the epithelial bud at E12.5 and E13.5 (the bud stage). At E14.5, tooth development progresses to the cap stage, with enamel knot formation in the dental epithelium to control the patterning of tooth cusps. The cap stage is followed by the bell stage, which begins around E16.5, when ameloblasts and odontoblasts start to differentiate. All these developmental processes are under elaborate control of multiple families of signaling molecules, including bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), Shh and Wnt proteins (Tummers and Thesleff, 2009).

The importance of canonical Wnt signaling in odontogenesis has been well illustrated (Liu and Millar, 2010), as evidenced by the expression of multiple Wnt ligands predominantly in the dental epithelium. It is also highlighted by the arrest of tooth development at the bud stage in mice carrying either epithelial or mesenchymal inactivation of *Catnb* (*Ctnnb1* – Mouse Genome Informatics), the gene encoding β-catenin, the master transducer of the canonical Wnt signaling (Sarkar and Sharpe, 1999; Liu et al., 2008; Chen et al., 2009). On the other hand, constitutive activation of Wnt/β-catenin signaling in the oral epithelium induces ectopic tooth formation (Järvinen et al., 2006; Liu et al., 2008; Wang et al., 2009), suggesting that Wnt is probably one of the earliest signaling molecules that regulate initiation of tooth development. However, the exact biological function of Wnt signaling in early dental epithelium development remains unknown.

BMP signaling is transduced into the cell via a heteromeric receptor complex of a type II transmembrane serine-threonine kinase receptor with each of the three type I receptors (BMPR-IA/Bmpr1a – Mouse Genome Informatics, BMPR-IB/Bmpr1b – Mouse Genome Informatics and Alk2/Acvr1 – Mouse Genome Informatics), whereas *Bmpr1a* and *Bmpr1b* have been shown to be expressed and have limited redundant function in the developing tooth (Li et al., 2011). Binding of ligand to the receptor elicits a Smad-dependent canonical pathway and/or Smad-independent mitogen-activated protein kinase (MAPK) pathways, including p38, ERK1/2 and JNK, known as non-canonical pathways (Sieber et al., 2009). Several *Bmp* genes are expressed in either epithelial or mesenchymal components of developing tooth germs, and BMP activity has been implicated in multiple steps of odontogenesis (Vainio et al., 1993; Chen et al., 1996; Zhang et al., 2000; Tummers and Thesleff, 2009; Jia et al., 2013). Whereas *Bmpr1b* null mice develop normal teeth (Yi et al., 2000), inactivation of *Bmpr1a* in either epithelium or mesenchyme or in both results in arrest of tooth development at the bud or early cap stages (Andl et al., 2004; Liu et al., 2005; Li et al., 2011). However, it remains to be identified which BMP-mediated signaling pathways are utilized and what biological functions these pathways exert during early tooth development.

In this study, we used a conditional transgenic *Nog* overexpression mouse model to dissect the biological function of BMP and Wnt signaling in early odontogenesis. We present evidence that transgenic *Nog* expression disrupts BMP-mediated non-canonical signaling pathways in the dental epithelium, leading to inhibition of cell proliferation. Meanwhile, overexpression of *Nog* also attenuates Wnt/β-catenin activity in the dental epithelium by directly binding to

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Wnt ligands, resulting in significantly reduced expression of *Pitx2*, an indication of loss of odontogenic fate. Together with genetic rescue results, our studies reveal an orchestration of non-canonical BMP and Wnt/β-catenin signaling pathways in regulating early tooth development.

RESULTS

Mesenchymal odontogenic program is unaltered in early tooth germs of *K14Cre;pNog* mice

We reported previously that activation of a conditional transgenic *Nog* allele (*pMes-Nog*) by *K14Cre* leads to arrested tooth development at the lamina/early bud stage, accompanied by inhibition of cyclin D1 expression and significantly reduced cell proliferation rate in the dental epithelium (Wang et al., 2012). Meanwhile, the expression of *Pitx2*, a molecular marker for the dental epithelium (Mucchielli et al., 1997; St. Amand et al., 2000), is also downregulated, indicating a deviation of the odontogenic fate. By contrast, the mesenchymal odontogenic markers, including *Bmp4* and *Msx1*, showed comparable expression levels in the binary transgenic mice (named as *K14Cre;pNog*) at E12.5, when the tooth phenotype became discernible (Wang et al., 2012). To determine whether the odontogenic program in the dental mesenchyme was affected and how long the program could be sustained in *K14Cre;pNog* mice, we examined *Bmp4* and *Msx1* expression. This was combined with a subrenal culture of tissue recombinants, consisting of dental mesenchyme of *K14Cre;pNog* mice with the same stage wild-type dental epithelium. In the transgenic animals, the expression levels of *Bmp4* and *Msx1* appeared unaltered at E13.5, became downregulated at E14.5 and were barely detectable at E15.5 compared with controls (Fig. 1A-L). Consistent with the expression of these odontogenic markers, grafts consisting of dental mesenchyme of E12.5-E14.5 *K14Cre;pNog* mice and wild-type dental epithelium did form teeth (E12.5, 5/5; E13.5, 4/5; E14.5, 3/4), whereas E15.5 transgenic dental mesenchyme failed to support tooth formation (0/4) (Fig. 1M-P). These observations indicate that, despite arrest of tooth development at or before E12.5, the odontogenic program in the dental mesenchyme of *K14Cre;pNog* mice does not deviate until E14.5.

Overexpression of *Nog* specifically disrupts BMP non-canonical signaling activities in the dental epithelium

To determine the level of *Nog* overexpression in the transgenic tooth germs, we performed qRT-PCR on control and *K14Cre;pNog* tooth germs at E12.5 and E13.5. The results showed that the relative Nog levels in transgenic tooth were rather constant at both stages, but the endogenous Nog level in controls was much higher at E13.5 than E12.5 (Fig. 2A). To determine which BMP-mediated pathway(s) is implicated in the early arrest of tooth development in *K14Cre;pNog* mice, we examined the activities of BMP canonical and non-canonical signaling pathways, including p38, ERK1/2 and JNK, at E12.5 and E13.5 molar germs by western blot analysis. Similar to previously reported results (Wang et al., 2012), the levels of P-Smad1/5/8 were comparable between transgenic teeth and wild-type controls (Fig. 2B). However, despite an unaltered level of P-JNK, significantly reduced levels of P-p38 and P-ERK1/2 were found in *K14Cre;pNog* tooth germs at both stages examined, as compared with controls (Fig. 2B). Immunohistochemistry confirmed downregulation of P-p38 and P-ERK1/2, particularly in the dental epithelium (Fig. 2C), suggesting that overexpressed *Nog* primarily disrupts BMP-mediated non-canonical pathways.

As either TGFβ or FGF signaling also activate ERK1/2 and p38 pathways, the transgenic *Nog* might affect FGF and/or TGFβ activities directly or indirectly, attributing to the reduced levels of P-p38 and P-ERK1/2. To exclude this possibility, we examined the expression of ligands (*Tgfb1*, *Tgfb2*, *Fgf8* and *Fgf9*) and receptors (*Fgfr1* and *Fgfr2*) of TGFβ or FGF signaling that are

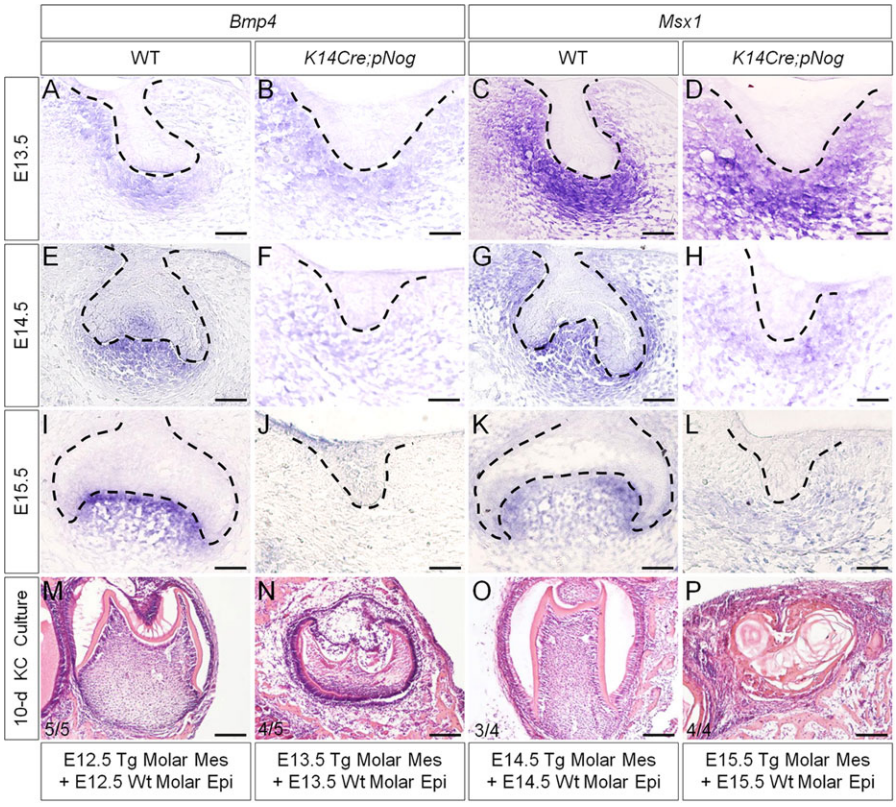

Fig. 1. Loss of *Bmp4* and *Msx1* expression coincides with the loss of odontogenic capability of dental mesenchyme in *K14Cre;pNog* mice. (A-L) *In situ* hybridization of *Bmp4* (A,B,E,F,I,J) and *Msx1* (C,D,G,H,K,L) in controls (WT) and in *K14Cre;pNog* tooth at E13.5 (A-D), E14.5 (E-H) and E15.5 (I-L). Scale bars: 50 µm. (M-P) Tooth formation in tissue recombinants of dental mesenchyme from *K14Cre;pNog* mice and wild-type dental epithelium at E12.5 (M), E13.5 (N) and E14.5 (O), but not in recombinants of E15.5 dental tissues (P) after 10-day kidney capsule culture. Scale bars: 100 µm.

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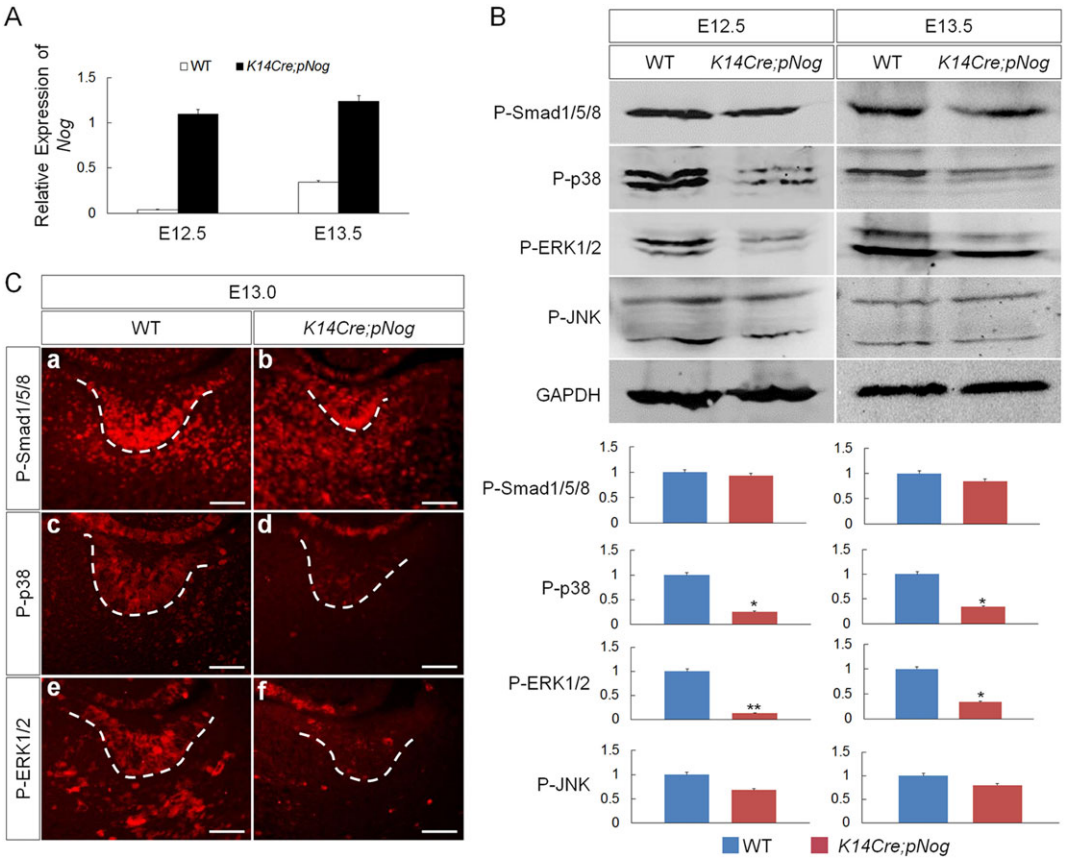

Fig. 2. Inhibition of non-canonical BMP signaling in *K14Cre;pNog* tooth. (A) qRT-PCR shows consistently elevated *Nog* expression levels in *K14Cre;pNog* tooth germs at E12.5 and E13.5 compared with controls (WT). (B) Representative western blot images and band quantification show significant reduction of P-p38 and P-ERK1/2 but not of P-Smad1/5/8 and P-JNK in *K14Cre;pNog* tooth germs at E12.5 and E13.5, as compared with controls. **P*<0.05, ***P*<0.01; *n*=3. (C) Immunofluorescence shows specific inhibition of P-p38 and P-ERK1/2 but not of P-Smad1/5/8 in the epithelium of *K14Cre;pNog* tooth germs at E13.0. Scale bars: 50 µm.

known to be expressed in the early developing tooth in E12.5 and E13.5 *K14Cre;pNog* molar germs (Chai et al., 1994; Pacheco et al., 2008; Liu et al., 2013). The expression levels of *Tgfb1* and *Tgfb2* (supplementary material Fig. S1A), *Fgf8* and *Fgf9* (supplementary material Fig. S2A-H), as well as Fgfr1 and Fgfr2 (supplementary material Fig. S2I-L), showed no change in *K14Cre;pNog* molars compared with controls. We next examined the expression of P-Smad2/3 and the ETS-related factor gene *Etv4* that are downstream readouts for TGFβ and FGF signaling, respectively (Xu et al., 2003; Porntaveetus et al., 2011). As expected, the expression of P-Smad2/3 and *Etv4* appeared comparable in *K14Cre;pNog* molar germs and controls (supplementary material Fig. S1B and Fig. S2M,N). All these results demonstrate unaltered activity of TGFβ or FGF signaling in E12.5 and E13.5 *K14Cre;pNog* molar germs, indicating that inhibition of BMP activity by excess Nog is primarily responsible for the reduced activities of p38 and ERK1/2.

BMP non-canonical pathways regulate cell proliferation in the dental epithelium

To determine whether the inhibited BMP non-canonical signaling pathways are responsible for the inhibition of cyclin D1 and *Pitx2* expression as well as for the reduced cell proliferation rate in the dental epithelium of *K14Cre;pNog* mice, we cultured E12.5 wild-type molar germs in the presence of small inhibitor molecules. The specificity and efficacy of these inhibitors on P-MAPKAPK-2 (the downstream mediator of P-p38), P-ERK1/2 and P-Smad1/5/8 in organ-cultured tooth germs were optimized and are shown in supplementary material Fig. S3. We found that inhibition of p38 alone by SB203580 affected neither tooth development nor the expression of cyclin D1 and *Pitx2* or cell proliferation rate in the dental epithelium, compared with DMSO-treated controls (Fig. 3B,G,L,Q,Z). Whereas inhibition of ERK1/2 pathway by U0126 alone caused dramatic reduction in cyclin D1 expression and statistically significantly reduced rate of cell proliferation in the dental epithelium, *Pitx2* expression was not affected, and tooth germs developed to the early cap stage, comparable to DMSO-treated controls (Fig. 3C,H,M,R,Z). However, despite unaltered *Pitx2* expression, inhibition of both p38 and ERK1/2 pathways resulted in arrested tooth development at the bud stage, accompanied by almost completely abolished cyclin D1 expression and much reduced rate of cell proliferation, resembling the phenotype observed in *K14Cre;pNog* mice (Fig. 3D,I,N,S,Z). Interestingly, in the presence of dorsomorphin, a selective type I BMP receptor inhibitor that inhibits BMP-induced phosphorylation of Smad1/5/8 (Yu et al., 2008), tooth development was arrested at the bud stage, but cyclin D1 and *Pitx2* expression and cell proliferation in the dental epithelium were not affected (Fig. 3E,J,O,T,Z), suggesting that Smad1/5/8-mediated signaling functions primarily in the dental mesenchyme. These results suggest that inhibition of p38 and ERK1/2 pathways are responsible for the downregulation of cyclin D1 expression and the reduced cell proliferation rate, but not for the inhibition of *Pitx2* expression, in the *K14Cre;pNog* tooth germs.

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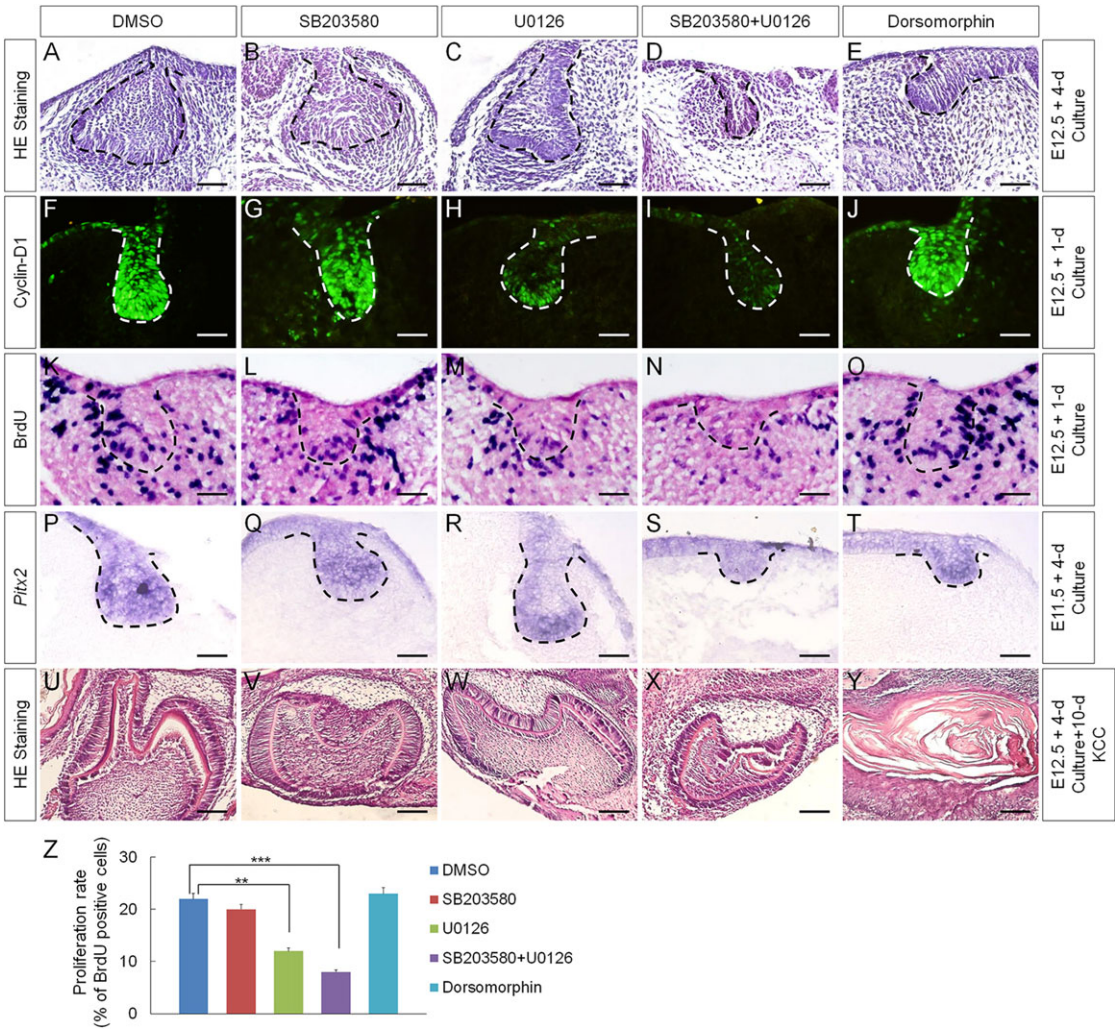

Fig. 3. Inhibition of BMP-mediated signaling pathways in early tooth germ by small inhibitor molecules. (A-E) Histological examination of E12.5 tooth germs after 4 days in organ culture in the presence of (A) DMSO, (B) SB203580, (C) U0126, (D) SB203580 and U0126 and (E) dorsomorphin. (F-O) Immunofluorescence of cyclin D1 (F-J) and BrdU labeling (K-O) of E12.5 tooth germs after 1 day in organ culture in the presence of inhibitors as indicated. (P-T) *In situ* hybridization of *Pitx2* in E11.5 molar germs after 4 days in organ culture in the presence of inhibitors as indicated. Scale bars in A-T: 50 µm. (U-Y) Tooth-forming capability of E12.5 molar germs that were cultured for 4 days *in vitro* with each individual inhibitor prior to being subjected to subrenal culture for 10 days. Scale bars: 250 µm. (Z) Statistic comparison of BrdU-labeled cells in the dental epithelium after treatment with individual inhibitors. Error bars show mean±s.d.; $^{**}P<0.01$, $^{***}P<0.001$.

As *Pitx2* expression was not affected in SB203580 and U0126 treated tooth germs, we reasoned that inhibition of p38 and/or ERK1/2 pathways would not deviate the odontogenic program. To test this hypothesis, we grafted tooth germs after 4-day treatment in organ culture with either SB203580 or U0126 alone, or with both. Well-differentiated tooth structures were found in all samples after 10-day subrenal culture (Fig. 3U-X; n=5 each). By contrast, dorsomorphin-treated grafts failed to form tooth structures (Fig. 3Y; n=6), consistent with a marked downregulation of *Msx1* expression in the mesenchyme of dorsomorphin-treated tooth germs (Yang et al., 2014).

TGFβ signaling is primarily responsible for Smad1/5/8 activation in the dental epithelium

As P-Smad1/5/8 are widely recognized as BMP signaling mediators, the unaffected P-Smad1/5/8 levels in the dental epithelium of *K14Cre;pNog* tooth germs were puzzling. The fact that TGFβ signaling can activate Smad1/5 in a number of cell types (Moustakas and Heldin, 2009), and that TGFβ signaling molecules and activities are not affected in *K14Cre;pNog* tooth (supplementary material Fig. S1), prompted us to test whether TGFβ signaling is responsible for Smad1/5/8 activation in the dental epithelium. We cultured E12.5 tooth germs in the presence of SB525334 (a specific inhibitor of TGFβ type I receptor) or *Tgfb1* and examined P-Smad1/5/8. We found that P-Smad1/5/8 levels were dramatically reduced in the epithelium of E12.5 molar germ after 24-h culture with SB525334, and significantly increased in the presence of *Tgfb1*, as compared with controls (supplementary material Fig. S4A,B,G), suggesting that TGFβ signaling accounts largely for Smad1/5/8 activation in the dental epithelium. In line with this notion is the fact that P-Smad1/5/8 levels were neither affected in the dental epithelium of tooth germs treated with dorsomorphin nor in the dental epithelium of *Msx1* mutants, in which *Bmp4* expression in the dental mesenchyme and *Bmp2* expression in the dental epithelium were diminished (supplementary material Fig. S4C-F; Zhang et al., 2000).

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Suppression of Wnt/β-catenin signaling activity is responsible for *Pitx2* downregulation in the *K14Cre;pNog* dental epithelium

The fact that the arrested tooth development in *K14Cre;pNog* embryos was associated with a dramatic downregulation of *Pitx2*, but that the inhibition of p38 and ERK1/2 pathways failed to alter *Pitx2* expression, suggested an additional role for Nog. Nog can also bind to Activin (Bayramov et al., 2011), which is essential for early tooth development beyond the bud stage by inducing follistatin expression in the dental epithelium (Ferguson et al., 1998). To test whether overexpressed *Nog* disrupts Activin signaling, we examined follistatin expression in *K14Cre;pNog* tooth germs at E12.5 and E13.5, and found comparable follistatin expression in transgenic and control molars (supplementary material Fig. S5), indicating that Activin activity was not affected in *K14Cre;pNog* mice. *Pitx2* is initially induced by FGF8 in the dental epithelium (St. Amand et al., 2000). The observations that *Fgf8* and *Fgf9* expressions were not affected in the dental epithelium of *K14Cre;pNog* mice at E12.5, when *Pitx2* expression became downregulated (supplementary material Fig. S2; Wang et al., 2012), indicated the existence of an alternative regulatory pathway. As the Wnt/β-catenin pathway also induces *Pitx2* expression (Kioussi et al., 2002), we tested whether *Pitx2* downregulation is the consequence of suppressed Wnt/β-catenin signaling activity by breeding *BAT-gal* reporter mice to *K14Cre;pNog* mice. Although *BAT-gal* activity was not affected in the epithelium of *K14Cre;pNog;BAT-gal* tooth germs at E11.5, it became dramatically inhibited at E12.5 and E13.5 compared with controls (Fig. 4A-F). Organ culture experiments further confirmed inhibition of *BAT-gal* activity by exogenously applied Nog in explanted molar germs (Fig. 4G,H). Consistent with these observations is the almost completely abolished active β-catenin in the dental epithelium of *K14Cre;pNog* tooth germs (Fig. 4I,J). Interestingly, in *Nog* mutants ($Nog^{-/-}$), active β-catenin was dramatically increased in the dental epithelium, indicating that endogenous Nog, which is expressed in the dental epithelium (Hu et al., 2012), indeed modulates Wnt signaling activity in the developing tooth (Fig. 4K). To determine whether the inhibition of Wnt/β-catenin signaling activity is responsible for the downregulation of *Pitx2*, we examined *Pitx2* expression in *K14Cre;pNog* molar germs after 2-day organ culture in the presence of LiCl and in tooth germs of *K14Cre;pNog* mice carrying an *Axin2* null allele, a negative regulator of Wnt/β-catenin signaling that is expressed in the developing tooth (Lohi et al., 2010). As expected, *Pitx2* expression was resumed in both cases, but tooth phenotype was not rescued, remaining at the early bud stage (Fig. 4L-S).

Nog physically interacts with and inhibits Wnt/β-catenin signaling activity

To reveal the mechanism underlying the inhibition of Wnt/β-catenin signaling activity by Nog, we first examined whether inhibition of BMP activity would attenuate Wnt/β-catenin

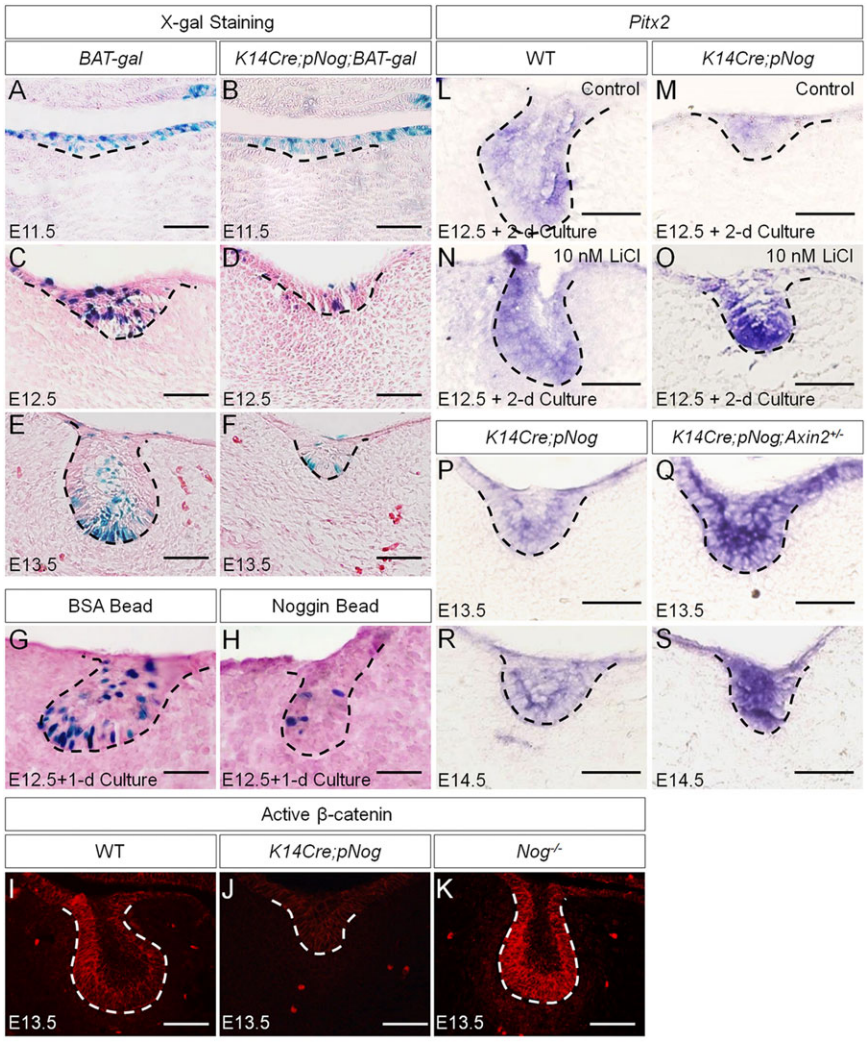

Fig. 4. Attenuated Wnt/β-catenin signaling leads to downregulation of *Pitx2* expression in *K14Cre;pNog* dental epithelium. (A-F) X-gal staining of *BAT-gal* (A,C,E) and *K14Cre;pNog;BAT-gal* (B,D,F) tooth germs at E11.5 (A,B), E12.5 (C,D) and E13.5 (E,F). (G,H) X-gal staining of E12.5 *BAT-gal* molar germs after 1 day organ culture with BSA beads (G) or Nog beads (H). (I-K) Immunofluorescence of active β-catenin in wild type (I), *K14Cre,pNog* (J) and *Nog* mutant (K) tooth germs at E13.5. (L-S) *In situ* hybridization of *Pitx2* in E12.5 wild-type molars (L,N) and E12.5 *K14Cre;pNog* (M,O) molars cultured in the presence or absence of 10 nM LiCl for 2 days. (P-S) *In situ* hybridization of *Pitx2* in *K14Cre;pNog* (P,R) and *K14Cre;pNog;Axin2*$^{+/-}$ (Q,S) molars at E13.5 (P,Q) and E14.5 (R,S). Scale bars: 50 µm.

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signaling by culturing E12.5 *BAT-gal* molar germs in the presence of various inhibitors. Neither inhibition of the Smad-dependent pathway, of p38, or of ERK1/2 alone, nor inhibition of these three pathways simultaneously, decreased Wnt/β-catenin activity in the dental epithelium (supplementary material Fig. S6). This indicated that inhibition of Wnt/β-catenin activity is not the secondary effect of downregulated BMP activity. We next surveyed the expression of Wnt ligands that are known to be expressed in the developing tooth germs, including *Wnt4*, *5a*, *6*, *7b*, *10a* and *10b*, but found unchanged expression levels of all Wnt ligands in *K14Cre;pNog* tooth germs at E12.5 and E13.5 compared with controls (supplementary material Fig. S7).

Although Nog is well known for its function as a BMP antagonist, it has been reported that, in *Xenopus*, Nog was able to physically interact with Wnts at high concentration (Bayramov et al., 2011). To determine whether this is also true in mammals, we conducted co-immunoprecipitation (Co-IP) experiments by co-transfecting into HEK 293T cells the expression vector of *FLAG*-tagged mouse *Nog* and each of *Myc*-tagged *Wnt* genes that are expressed in the developing tooth. Western blot revealed that Nog formed a complex with each individual Wnt tested (Fig. 5A). To determine whether such physical interaction exists *in vivo*, we performed an *in situ* proximity ligation assay (PLA), a technology capable of detecting protein-protein interaction with high specificity and sensitivity *in vivo* (Soderberg et al., 2008). We chose two representative canonical Wnts, Wnt4 and Wnt6, which are highly expressed in the dental epithelium (Sarkar and Sharpe, 1999). The strong PLA signals in the dental epithelium of E13.5 wild-type molar, compared with the complete absence of signals in $Nog^{-/-}$ tooth germs, indicate a direct interaction of endogenous Nog and Wnts under physiological condition (Fig. 5B).

We next tested whether binding of Nog to Wnts could functionally prevent Wnt signaling, by conducting a TOPflash assay in cell culture with FOPflash as negative control. Our results showed that inclusion of *Nog* expression vector attenuated Wnt3 induction of TOPflash activity in a dose-dependent manner. The specific inhibitory effect of Nog on Wnt signaling was confirmed by inclusion of *Bmp4* expression vector that relieved the inhibitory effect by Nog (Fig. 5C). Conversely, the ability of Wnt3 to attenuate the antagonistic effect of Nog on Bmp4 in a reporter assay, using *pGL3-BRE-Luc* construct that harbors BMP-response elements (BRE) from the *Id1* promoter (Korchynskyi and ten Dijke, 2002), further supported this conclusion (Fig. 5D).

The Nog protein contains two conserved domains, the N-terminal clip domain (residues 28-48) that mediates the binding of Nog to BMPs, and the C-terminal cysteine-rich domain (CRD, residues 178-232), the function of which is currently unknown (Fig. 5E; Groppe et al., 2002). A similar CRD was also found in Wnt receptors, including Frizzled and Lrp5/6, as well as the Wnt antagonists sFRPs to mediate their bindings to Wnts (Nathan and Tzahor, 2009; Ke et al., 2013). We sought to determine whether Nog binding to Wnts is mediated by the CRD and whether it is independent of the clip domain, by generating *Nog*(Δ*CRD*) and *Nog*(Δ*clip*) expression constructs that express truncated noggin proteins lacking the CRD or the clip domain, respectively. Co-IP assays showed that Nog(ΔCRD) bound to Bmp4 but not to Wnt3, and that Nog(Δclip) bound to Wnt3 but not to Bmp4 (Fig. 5F). The importance of the CRD in mediating the inhibitory effect of Nog on Wnt signaling was further revealed by TOPflash assays, evidenced by the ability of Nog(Δclip) but not of Nog(ΔCRD) to attenuate Wnt3-induced TOPflash activity (Fig. 5G).

Genetic activation of Wnt and BMP signaling rescues tooth developmental defect in *K14Cre;pNog* mice

To test whether inhibition of either or both Wnt and BMP signaling pathways is responsible for the tooth phenotype observed in *K14Cre;pNog* mice, we performed genetic rescue experiments by compounding *K14Cre;pNog* with either *Axin2* null allele or a conditional, constitutively active *Bmpr1b* transgenic allele (*pcaIB*) (Li et al., 2011). *K14Cre;pNog* mice carrying either homozygous *Axin2* null gene or the *pcaIB* allele exhibited identical tooth phenotype as that seen in *K14Cre;pNog* embryo (Fig. 6B-D). By contrast, mice carrying compounded *K14Cre;pNog;pcaIB;*$Axin2^{+/-}$ alleles presented rescued tooth development (Fig. 6E,F). These rescued teeth formed an enamel knot at the E14.5 cap stage, assessed by *Shh* and *Fgf4* expression (Fig. 6E′,E″), and developed into a bell-like structure at E16.5 (Fig. 6F). The expression of *Pitx2* in the dental epithelium of rescued teeth indicates its retained odontogenic fate (Fig. 6F′). To determine the functional importance of *Pitx2* in mediating Wnt/β-catenin signaling pathway in tooth development, we generated a conditional transgenic mouse line (*pPitx2c*) that expresses *Pitx2c* (Fig. 6G), one of the three *Pitx2* isoforms essential for tooth development (Liu et al., 2003). Whereas *K14Cre;pNog* mice carrying the *pPitx2c* allele did not exhibit rescued tooth development (Fig. 6H), compounding the *pcaIB* allele onto *K14Cre;pNog;pPitx2c* mice rescued tooth development, evidenced by the formation of the cap-like epithelial structure at E14.5 and the bell-like structure at E16.5 with normal expression levels of *Msx1* and *Bmp4* (Fig. 6I-L).

To further dissect the exact function of BMP and Wnt signaling pathways in dental epithelium development, we examined the expression of P-p38, P-ERK1/2, active β-catenin, *Pitx2* and cyclin D1, as well as cell proliferation rate in the dental epithelium of *K14Cre;pNog* mice carrying various transgenic alleles at E12.5. The level of P-p38 was fully restored by the *pCaIB* allele (Fig. 7A-E). P-ERK1/2 expression was mostly restored by *pCaIB*, but fully restored by *pCaIB* and $Axin2^{+/-}$ alleles (Fig. 7F-J). β-catenin was highly activated by $Axin2^{+/-}$ allele, but not by *pCaIB* allele (Fig. 7K-O). Meanwhile, *Pitx2* expression was resumed in *K14Cre;pNog;*$Axin2^{+/-}$ and *K14Cre;pNog;pcaIB;*$Axin2^{+/-}$ mice but not in *K14Cre;pNog;pCaIB* mice (Fig. 7P-T). The level of cyclin D1 was partially restored (Fig. 7V,W) in both *K14Cre;pNog;pCaIB* and *K14Cre;pNog;*$Axin2^{+/-}$ mice compared with controls and *K14Cre;pNog* teeth (Fig. 7U,V′,Y). Accordingly, cell proliferation rate was also partially resumed in mice with each genotype, with *K14Cre;pNog;pCaIB* mice showing a much significantly resumed rate (Fig. 7Z-DD). In *K14Cre;pNog;pcaIB;*$Axin2^{+/-}$ mice, however, *Pitx2* and cyclin D1 expression and cell proliferation rate were fully resumed to levels comparable to controls (Fig. 7S,T,X,Y,CC,DD). These observations indicate that BMP non-canonical signaling plays a major role in regulating cell proliferation, whereas Wnt/β-catenin signaling functions primarily to control the odontogenic fate in the dental epithelium during early odontogenesis.

DISCUSSION

Non-canonical BMP signaling functions in early dental epithelium to regulate cell proliferation

Although the key mediators of BMP canonical and non-canonical signaling pathways, including Smad1/5/8, Smad4, p38, ERK1/2 and JNK, are widely activated in the developing tooth (Xu et al., 2003; Cho et al., 2008; Moriguchi et al., 2011; and this study), the biological function of each pathway during odontogenesis remains elusive. Here, we show that *Nog* overexpression leads to an arrested tooth development at the early bud stage, associated with significantly suppressed cell proliferation and inhibition of p38 and ERK1/2-mediated BMP non-canonical signaling pathways specifically in the

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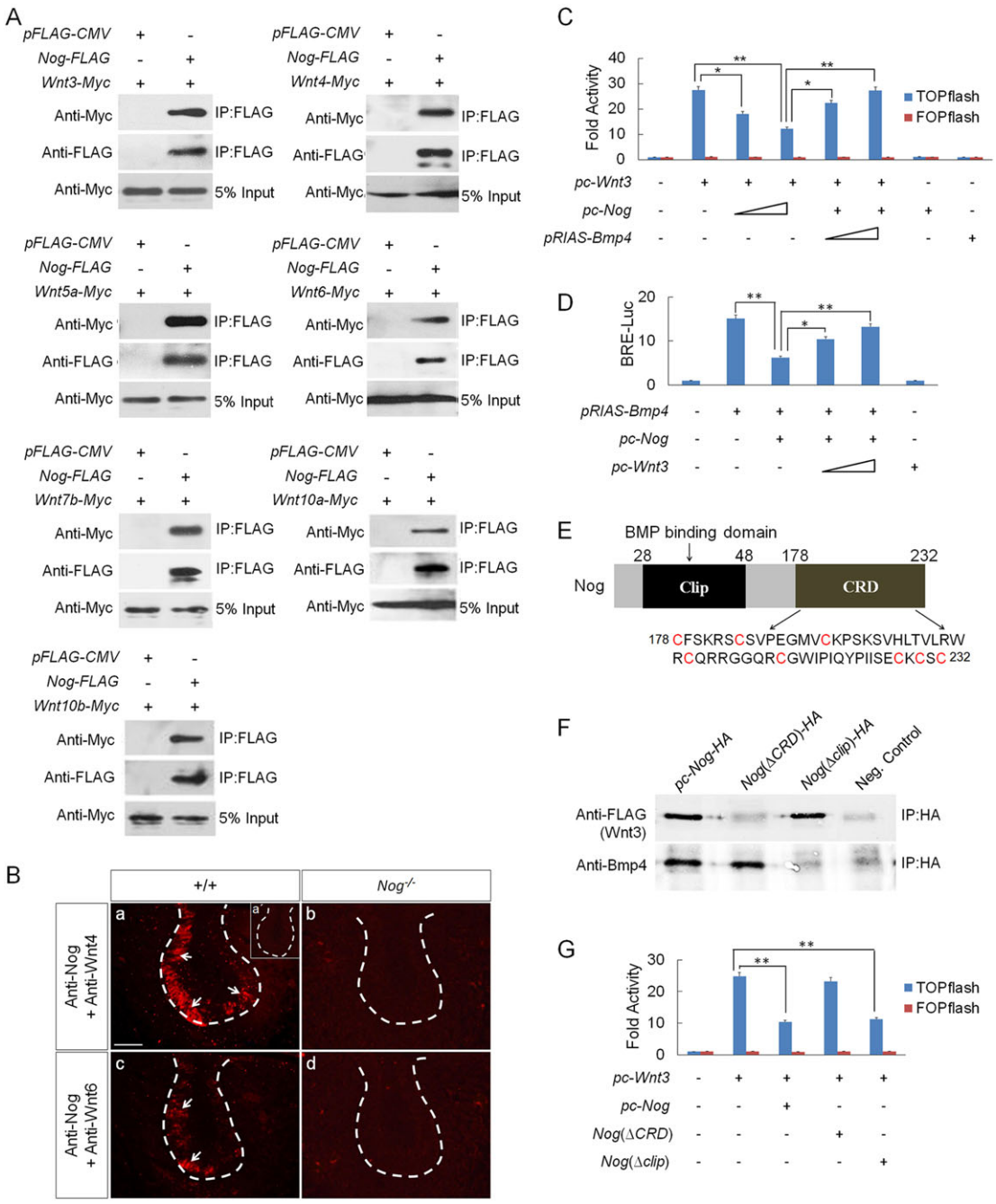

Fig. 5. Physical interaction between Nog and Wnts leads to inhibition of Wnt canonical signaling. (A) Co-IP assays show physical interaction between Nog and Wnt3, 4, 5a, 6, 7b, 10a and 10b. (B) *In situ* PLA shows the bindings of endogenous Nog with Wnt4 (a) and Wnt6 (c) in the dental epithelium of E13.5 wild-type tooth germs, but a lack of signals in $Nog^{-/-}$ tooth germs (b,d). Insert in (a) is a negative control of PLA. White arrows indicate PLA signals. Scale bar: 50 µm. (C) TOPflash reporter assay demonstrates antagonistic effect of Nog on Wnt signaling. TOPflash or FOPflash (0.2 µg), *pRL-SV40* (0.02 µg), *pc-Wnt3* (0.005 µg) vectors with ascending concentrations of *pc-Nog* (0.1-0.4 µg) and *pRIAS-Bmp4* (0.05-0.1 µg) vectors were co-transfected into HEK 293T and TOPflash activity was measured. (D) BRE-luciferase reporter assay shows attenuation of Nog inhibition on BMP signaling by Wnt3. *pGL3-BRE-Luc* (0.002 µg), *pRL-SV40* (0.02 µg), *pTRIS-Bmp4* (0.2 µg) and *pc-Nog* (0.01 µg) vectors with ascending concentrations of *pc-Wnt3* (0.1-0.5 µg) were co-transfected into HEK 293T and luciferase activity was measured. (E) Schematic representation of Nog protein shows the clip domain and CRD domain. The amino acid sequence of CRD is also shown, with cysteine residues highlighted in red. (F) Co-IP assays show physical interactions between truncated Nog and Wnt3 or Bmp4. (G) TOPflash assay shows the effect of truncated Nog on Wnt3 induction of TOPflash activity. TOPflash or FOPflash, *pRL-SV40*, *pc-Wnt3* vectors with *pc-Nog*, *Nog*(ΔCRD) or *Nog* (Δ*clip*) (0.2 µg) were co-transfected into HEK 293T and TOPflash activity was measured. **P*<0.05, ***P*<0.01; *n*=6.

dental epithelium. However, the levels of P-Smad1/5/8 in both the dental epithelium and mesenchyme were not affected. In addition, the expression of the dental mesenchymal markers *Msx1* and *Bmp4*, both downstream targets of BMP signaling (Vainio et al., 1993; Chen et al., 1996), appeared unaltered until E14.5, indicating the sustained BMP activity and odontogenic fate in the dental mesenchyme of *K14Cre;pNog* mice. This could be explained by a potentially limited diffusion of the transgenically expressed Nog into the dental mesenchyme. It has been reported that Nog could be retained at the cell surface by direct binding to cell surface heparin sulfate

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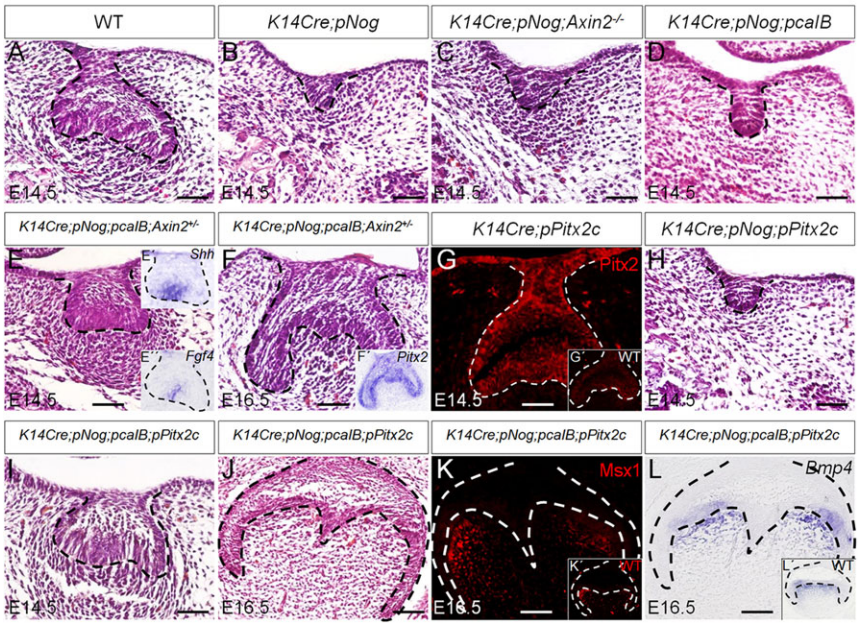

Fig. 6. Genetic rescue of tooth developmental defect in *K14Cre;pNog* mice by simultaneous activation of Wnt and BMP signaling pathways. (A-F,H-J) H&E staining of molar germs from (A) E14.5 wild type, (B) E14.5 *K14Cre;pNog*, (C) E14.5 *K14Cre;pNog;Axin2*$^{-/-}$, (D) E14.5 *K14Cre;pNog;pcaIB*, (E) E14.5 *K14Cre;pNog;pcaIB;Axin2*$^{+/-}$, (F) E16.5 *K14Cre;pNog;pcaIB;Axin2*$^{+/-}$, (H) E14.5 *K14Cre;pNog;pPitx2c*, (I) E14.5 *K14Cre;pNog;pcaIB;pPitx2c* and (J) E16.5 *K14Cre;pNog;pcaIB;pPitx2c* mice. (E′,E″,F′) *In situ* hybridization of *Shh* (E′) and *Fgf4* (E″) in E14.5 *K14Cre;pNog;pcaIB;Axin2*$^{+/-}$ molar germs, and (F′) *Pitx2* in E16.5 *K14Cre;pNog;pcaIB;Axin2*$^{+/-}$ molar germ. (G) Immunofluorescence of Pitx2 in E14.5 *K14Cre;pPitx2c* tooth germ and in control (G′). (K) Immunofluorescence of Msx1 in E16.5 *K14Cre;pNog;pcaIB;pPitx2c* and in control (K′). (L) *In situ* hybridization of *Bmp4* in E16.5 *K14Cre;pNog;pcaIB;pPitx2c* and in control (L′). Scale bars: 50 µm.

proteoglycans (HSPGs) without affecting its function as a BMP antagonist (Paine-Saunders et al., 2002). As dental epithelial cells express multiple HSPGs (Bai et al., 1994), these molecules could confine Nog function within the dental epithelium of *K14Cre;pNog* mice. The ultimate disappearance of the molecular markers and the odontogenic capability in the dental mesenchyme of *K14Cre;pNog* mice apparently is the consequence of an altered odontogenic program in the epithelium.

Our *in vitro* organ culture experiments using inhibitors confirm the function of p38 and ERK1/2, but not of P-Smad1/5/8-mediated BMP signaling in the regulation of dental epithelial cell proliferation by controlling cyclin D1 expression (Fig. 3), which is further supported by the findings of the *in vivo* rescue studies (Fig. 7). Strikingly, despite the presence of abundant P-Smad1/5/8 and Smad4 in the developing tooth germs, the canonical BMP signaling activity, mediated by Smad1/5/8-Smad4 complexes, was not detectable using transgenic alleles harboring the *BMP-response element* (Monteiro et al., 2008; Yang et al., 2014). In line with this notion is the fact that epithelial inactivation of *Smad4* does not affect early tooth development (Xu et al., 2008). The dramatic reduction or increase in P-Smad1/5/8 levels in the dental epithelium of organ-cultured tooth germs in the presence of TGFβ type I receptor inhibitor or Tgfb1, respectively, indicates that TGFβ signaling is responsible for Smad1/5/8 activation in the dental epithelium, consistent with a previous report that TGFβ is able to activate Smad1/5 (Moustakas and Heldin, 2009). TGFβ has been reported to be able to activate Smad1/5 in several cell types, especially in *Alk1* (*Acvrl1* – Mouse Genome Informatics)-expressing endothelial cells (Goumans et al., 2002). In *Alk1* highly expressing cells, Alk1 can be recruited into the TGFβ receptor complex with TGFβ induction and activates Smad1/5 (Goumans et al., 2003). In fact, our preliminary studies identified *Alk1* expression in the dental epithelium (G. Yang, G. Yuan and Y.C., unpublished data). Whereas the biological function of the TGFβ-induced activation of Smad1/5/8 in dental epithelium warrants further investigation, this observation explains the unchanged P-Smad1/5/8 level in the dental epithelium of *K14Cre;pNog* mice. Additionally, TGFβ signaling also inhibits BMP canonical signaling in the dental mesenchyme by limiting Smad4 availability, leading to activation of *Msx1* expression by an atypical canonical BMP signaling (Smad4-independent and Smad1/5/8-dependent) pathway, but not by p38- and ERK1/2-mediated non-canonical BMP signaling (Yang et al., 2014).

A novel function of Nog as Wnt signaling antagonist

In *K14Cre;pNog* tooth germs, the inhibition of cyclin D1 expression and cell proliferation is accompanied by the loss of odontogenic fate in the dental epithelium, as assessed by the loss of *Pitx2* expression. This is in contrast to the inhibition of both p38 and ERK1/2 pathways in organ-cultured tooth germs, which did not affect *Pitx2* expression and the capability of the tooth germ to form tooth organ after removal of the inhibitors, thus suggesting an additional role for Nog. *Pitx2* is a known downstream target of the Wnt/β-catenin signaling pathway (Kioussi et al., 2002). Accordingly, we found that Wnt/β-catenin signaling activity is attenuated significantly in *K14Cre;pNog* tooth germs as well as in Nog-treated tooth germs *in vitro*.

BMPs have dual roles in regulating Wnt signaling activity. For example, BMPs can either antagonize Wnt signaling or induce the expression of Wnt ligands and receptors, and activate the Wnt canonical pathway, depending on the type of tissues and cells (Soshnikova et al., 2003; Chesnutt et al., 2004; Liu et al., 2006; Chen et al., 2007; Ille et al., 2007; Lee et al., 2009). In the developing tooth, however, inhibition of Smad-dependent and -independent BMP pathways did not alter *BAT-gal* activity and *Pitx2* expression, suggesting that the suppression of Wnt/β-catenin signaling in *K14Cre;pNog* tooth is not a secondary effect of BMP inhibition by Nog. This notion is supported by the fact that Nog can bind directly to Wnts and functionally inhibits Wnt signaling activity in TOPflash assays. The *in situ* PLA experiments further revealed a physical interaction between Nog and Wnts in tooth germs under physiological conditions, and the elevated level of active β-catenin in *Nog* mutant tooth germ supports a physiological function of Nog in modulating Wnt signaling activity *in vivo*. Although targeted inactivation of *Nog* causes defects in incisors but not in molars (Hu et al., 2012), it is possible that the enhanced Wnt signaling activities in *Nog*$^{-/-}$ molars do not reach a level that could alter the molar developmental program, similar to that seen in *Axin2* heterozygotes (Lohi et al., 2010). Nevertheless, our results establish a novel function for Nog as a Wnt

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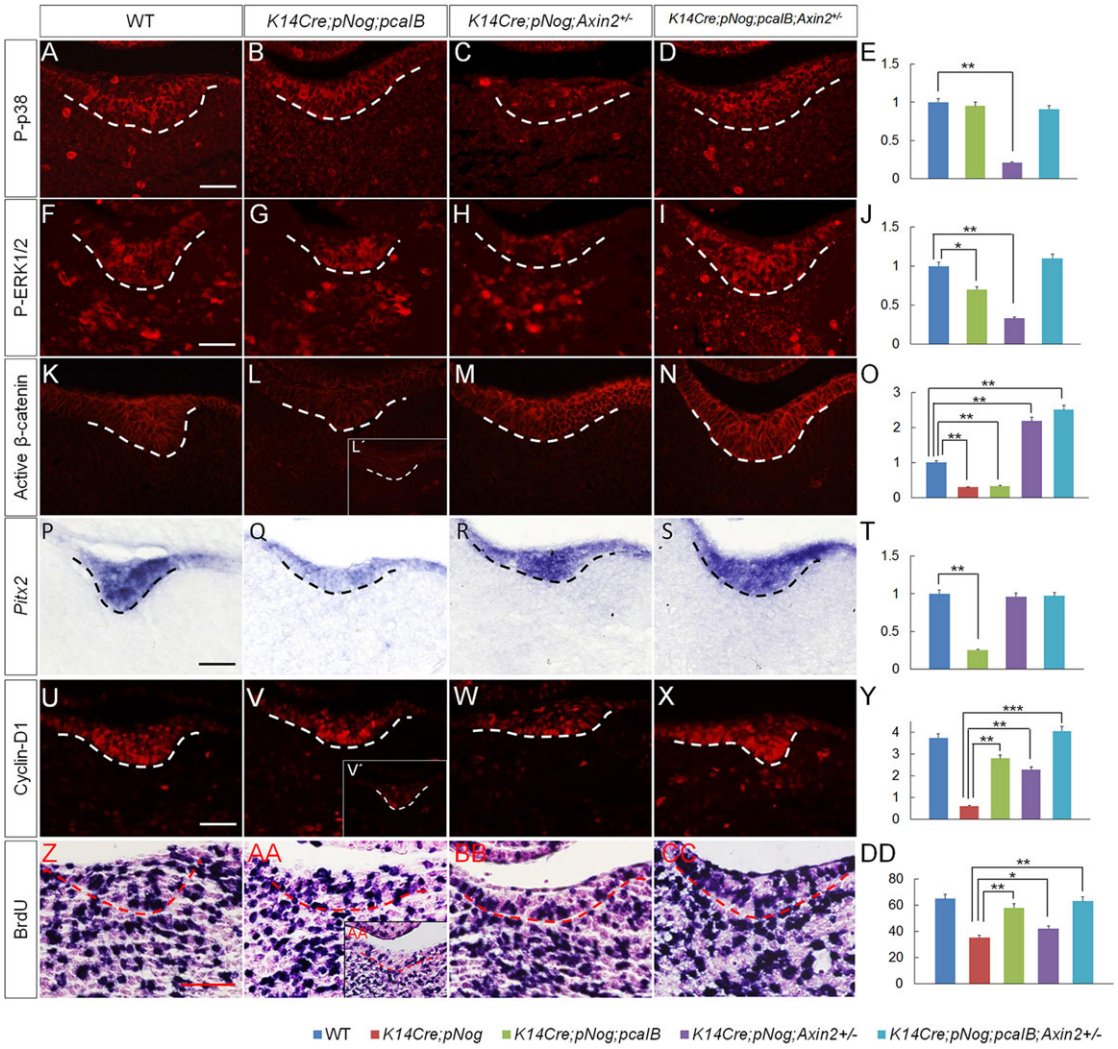

Fig. 7. Rescue of cell fate and proliferation defects in *K14Cre;pNog* mice by activation of BMP and Wnt signaling pathways. (A-D,F-I,K-N,U-X) Immunofluorescence of P-p38 (A-D), P-ERK1/2 (F-I), active β-catenin (K-N) and cyclin D1 (U-X) in E12.5 tooth germs of control and transgenic mice as indicated. (P-S, Z-CC) *In situ* hybridization of *Pitx2* expression (P-S) and BrdU labeling (Z-CC) in E12.5 tooth germs of control and transgenic mice. (L′,V′,AA′) E12.5 *K14Cre;pNog* tooth germ for comparison. Scale bars: 50 µm. (E,J,O,T,Y) Qualification and statistical comparison for expressions of P-p38 (E), P-ERK1/2 (J), active β-catenin (O), *Pitx2* (T) and cyclin D1 (Y). (DD) Statistical comparison of cell proliferation rate (percentage of BrdU-positive cells) in E12.5 molars of control and transgenic mice. **P*<0.05, ***P*<0.01, ****P*<0.001; *n*=3.

antagonist *in vivo*. Several other secreted proteins, such as Cerberus, Coco and Ectodin, could also modulate both Wnt and BMP signals but act through different mechanisms. Similar to Nog, Cerberus and Coco modulate BMP and Wnt signaling pathways by directly binding to BMP and Wnt ligands (Bell et al., 2003; Piccolo et al., 1999). Ectodin antagonizes BMP signaling by binding to BMP ligands, whereas its Wnt modulation function is through binding to the extracellular domain of the Wnt co-receptor, including LRP5/6 and LRP4, thus blocking the binding of Wnts to the receptors (Lintern et al., 2009).

Wnt/β-catenin signaling controls the odontogenic fate of the dental epithelium by sustaining *Pitx2* expression

As the earliest molecular marker for the dental epithelium fate, *Pitx2* is induced by FGF8 (St. Amand et al., 2000), and is essential for tooth development beyond the bud stage (Lin et al., 1999). However, the mechanism that sustains *Pitx2* expression in the dental epithelium remains unknown. In *K14Cre;pNog* tooth, Wnt/β-catenin activity was not altered in the dental epithelium at E11.5, similar to *Pitx2* expression (Wang et al., 2012). However, at E12.5, *Pitx2* expression became downregulated in accordance with the reduced Wnt/β-catenin signaling activity, suggesting that Wnt/β-catenin signaling is responsible for sustaining *Pitx2* expression. This notion is supported by the observations that *Pitx2* expression was resumed in *K14Cre;pNog* tooth germs treated with LiCl or lacking an *Axin2* allele. The functional importance of *Pitx2* in mediating Wnt signaling in early tooth development is further strengthened by the observation that the transgenic *Pitx2* expression in the dental epithelium could substitute for reactivation of Wnt signaling (by deletion of an *Axin2* allele) to rescue tooth development defect in *K14Cre;pNog;pCaIb* mice.

Orchestration of non-canonical BMP and Wnt/β-catenin signaling in regulating early tooth development

Whereas reactivation of BMP signaling by expression of *pcaIB* or reactivation of Wnt signaling by deletion of *Axin2* in the dental epithelium of *K14Cre;pNog* tooth germs partially resumed the expression of cyclin D1 and cell proliferation rate (Fig. 7), the tooth phenotype was not rescued in either case (Fig. 6). However, the early tooth defect was rescued, as associated with fully resumed

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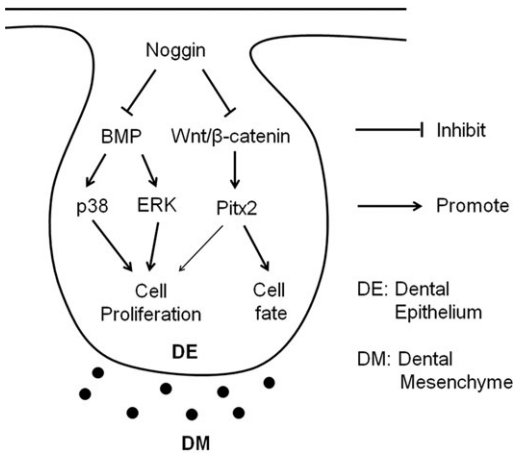

Fig. 8. Model diagram for orchestration of non-canonical BMP and Wnt/β-catenin signaling in regulating early tooth development.

levels of cyclin D1 and *Pitx2* expression as well as cell proliferation rate in the *K14Cre;pNog* dental epithelium, by simultaneous activation of both BMP and Wnt/β-catenin signaling pathways (Figs 6 and 7). This indicates an orchestrated function of BMP and Wnt signaling in regulating early tooth development. The partially rescued cell proliferation rate associated with partially resumed cyclin D1 level, in addition to resumed *Pitx2* expression, in *K14Cre;pNog;Axin2*$^{+/-}$ tooth germs, is consistent with the fact that *Ccnd1* is a direct transcriptional target of Wnt signaling (Shtutman et al., 1999; Tetsu and McCormick, 1999) and its mRNA is stabilized by Wnt/β-catenin-induced Pitx2 (Briata et al., 2003). These results indicate that Wnt/β-catenin signaling also controls cell proliferation in a synergistic manner together with BMP signaling in the dental epithelium.

In summary, using a transgenic animal model combined with *in vitro* organ culture and genetic rescue approaches, we have dissected the distinct and orchestrated function of non-canonical BMP and Wnt/β-catenin signaling pathways during early tooth development (Fig. 8). Here, non-canonical BMP signaling plays a major role in regulating dental epithelial cell proliferation, whereas Wnt/β-catenin signaling functions primarily to control the odontogenic fate and partly to regulate cell proliferation in the dental epithelium during early odontogenesis. We have also established a novel function for Nog as an antagonist of Wnts, which raises an alert for future studies using Nog as a BMP antagonist, and calls for a possible re-evaluation of previous studies involving Nog.

MATERIALS AND METHODS

Animals and embryo collection

The use of animals in this study was approved by the Institutional Animal Care and Use Committee of Tulane University. The generation of *Axin2*^{lacZ} (*Axin2*$^{+/-}$), *Nog*$^{-/-}$, *BAT-gal*, *K14-Cre*, *pMes-Nog* (*pNog*) and *pMes-caBmpr1b* (*pcaIB*) transgenic mice has been described previously (McMahon et al., 1998; Lustig et al., 2002; Maretto et al., 2003; Andl et al., 2004; Xiong et al., 2009; He et al., 2010; Wang et al., 2013). Production of *pMes-Pitx2c* (*pPitx2c*) transgenic mice is described in the methods in the supplementary material. Embryos were collected from timed-pregnant females, and tail samples from each embryo were subjected to PCR-based genotyping.

Histology, *in situ* hybridization, immunofluorescence and X-gal staining

Standard hematoxylin and eosin (H&E) staining was used for histological analysis, and section *in situ* hybridization, using non-radioactive probes, was conducted as described previously (Wang et al., 2012). For immunofluorescence and X-gal staining, samples were fixed in 4% PFA and subsequently dehydrated with 30% sucrose/PBS, embedded in O.C.T. and cryo-sectioned. Immunofluorescence and X-gal staining were conducted as described previously (Ito et al., 2003; He et al., 2010). The following antibodies for immunoflurescence were used: anti-P-Smad1/5/8 (Cell Signaling, #13820, 1:50), anti-P-p38 (R&D Systems, AF869, 1:50), anti-P-ERK1/2 (R&D Systems, AF1018, 1:50), anti-cyclin D1 (Abcam, ab16663, 1:400), anti-active β-catenin (EMD Millipore, 05-665, 1:200), anti-Pitx2 (Capra Science, PA1021-100, 1:100), anti-Msx1 (R&D Systems, AF5045, 1:200), anti-P-Smad2/3 (Santa Cruz Biotechnology, sc-11769, 1:50), anti-Fgfr1 (Abcam, ab823, 1:200) and anti-Fgfr2 (Abcam, ab58201, 1:200).

Organ culture and bead implantation

Mandibular molar germs were carefully isolated from staged embryos and placed in semisolid culture medium as described previously (Hu et al., 2006). For bead implantation, Affi-Gel Blue agarose beads (Bio-Rad) were washed with PBS prior to being incubated with 100 ng/µl Nog protein or 10 ng/µl Tgfb1 (both from R&D Systems) at 37°C for 30 min. Control beads were soaked with 100 or 10 ng/µl BSA. Protein-soaked beads were placed on the top of each tooth germ. For small molecule inhibition experiments, SB203580 (Cell Signaling), U0126 (Cell Signaling) or dorsomorphin (Sigma) at a final concentration of 20 µM, or SB525334 (Sigma) at a final concentration of 10 µM, were added to the culture medium. LiCl was added in culture medium at a final concentration of 10 nM. DMSO was used in control groups.

Tissue recombination and subrenal culture

Tissue recombination and subrenal culture were carried out as described previously (Yuan et al., 2008). Briefly, mandibular molar germs were isolated from staged *K14Cre;pNog* and wild-type embryos, respectively. Dental mesenchyme from *K14Cre;pNog* embryo was recombined with dental epithelium isolated from littermate wild-type embryos. Tissue recombinants were cultured in semisolid culture medium for 1 day prior to being subjected to subrenal culture in adult male CD-1 mice. Samples were harvested after 10 days in subrenal culture and processed for histological analysis.

BrdU labeling

Cell proliferation rate was assessed by a 5-Bromo-2'-deoxyuridine (BrdU) incorporation assay using a BrdU labeling and detection kit II (Roche), as described previously (Xiong et al., 2009). Cell proliferation rates were measured by counting BrdU-positive cells and total cells within defined arbitrary areas, and are presented as percentage of labeled cells against total cells in the area. Three samples were used for BrdU labeling in each group. Data were collected from three continuous sections from each sample.

In situ proximity ligation assay (PLA)

The binding of endogenous Nog with Wnts in wild-type and *Nog*$^{-/-}$ molar germs was conducted using an *in situ* PLA kit (Duolink kit, Sigma-Aldrich). Briefly, tissue sections were blocked with Duolink blocking reagent, and then incubated with anti-Nog (rabbit polyclonal, Abcam, ab16054, 1:100) and anti-Wnt4 (goat polyclonal, R&D Systems, AF475, 1:100) or anti-Wnt6 (goat polyclonal, Santa Cruz Biotechnology, sc-241734, 1:100) antibodies overnight at 4°C. Samples were washed and incubated with secondary antibodies conjugated with oligonucleotides (anti-goat PLA probe Plus or anti-rabbit PLA probe Minus) in a humidity chamber for 1 h at 37°C. Ligation of the oligonucleotide probes was followed by amplification at 37°C for 100 min. PLA signals were detected using a fluorescence microscope. Negative controls were included by replacing anti-Wnt antibodies with goat-negative IgG on wild-type samples.

Co-IP and western blot

The constructions of *Myc*-tagged mouse *Wnt* expression vectors, *Nog-FLAG*, *FLAG*-tagged *pc-Wnt3 and HA*-tagged *pc-Nog* are described in the methods in the supplementary material. Expression vectors were transfected into HEK 293T cells by Lipofectamine 2000 (Invitrogen), and Co-IP and

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western blot were conducted as described previously (Zhu et al., 2013). To examine the level of BMP signaling mediators in tooth germ by western blot, proteins were extracted from molar germs using 2× protein loading buffer (LI-COR Biosciences). Immunoreactive bands were visualized with Odyssey imaging system (LI-COR Biosciences) using IRDye 800 secondary antibody (LI-COR Biosciences). Western blots were performed at least three times independently. The following antibodies were used: anti-P-Smad1/5/8 (1:2000), anti-P-p38 (1:2000), anti-P-ERK1/2 (1:2000), anti-P-JNK (R&D Systems, AF1205, 1:2000), anti-GAPDH (GeneTex, GTX10118, 1:4000), anti-Myc and anti-FLAG (Thermo Scientific, MA1-91878, 1:2000), anti-Bmp4 (Abcam, ab39973, 1:2000), and anti-P-MAPKAPK-2 (Thermo Scientific, PA5-12619, 1:2000).

Reporter assays

HEK 293T cells in 48-well plates were co-transfected by TOPflash or FOPflash (Upstate Biotechnology) or *pGL3-BRE-Luc* (Addgene), with *pc-Wnt3*, *pc-Nog* or *Nog* mutant expression vectors, and/or *pRIAS-Bmp4* (Addgene). *pRL-SV40* (*Renilla* luciferase, Promega) was included as an internal control. Cells were harvested and lysed 48 h after transfection, and luciferase activities were measured with a dual luciferase reporter assay system (Promega) and determined using the GloMax luminometer (Promega). *Firefly* luciferase expression was normalized against *Renilla* luciferase expression to determine relative luciferase activity. Duplicated wells were assayed for each transfection and three independent transfection assays were performed.

Mutagenesis, RT-PCR and quantitative real-time RT-PCR (qRT-PCR)

The methods for the generation of truncated *Nog* expression vectors *Nog*(Δ*CRD*) and *Nog*(Δ*clip*), and primer information for RT-PCR or qRT-PCR are provided in the supplementary material.

Data quantification and statistical analysis

All experiments were repeated at least three times and at least four samples of each phenotype were collected for each genetic rescue experiment. For the western blot, densitometry of immunoblot bands was performed and relative quantification was processed with the ImageJ software (NIH). For histological sections, fluorescence intensity was collected from three continuous sections of each sample with ImageJ software. Quantification data are presented as the mean±s.d. of at least three independent experiments. Statistical analysis was performed using Student's *t*-test or ANOVA, followed by post hoc Bonferroni test, with SPSS (IBM). Significance was defined as $P<0.05$.

Competing interests

The authors declare no competing financial interests.

Author contributions

G.Yuan and G.Yang performed most of the experiments, collected and analyzed data, prepared figures and wrote the manuscript. Y.Z. participated in the initial phase of this study. X.Z. and Z.Z. performed Co-IP assays on binding of Nog with Wnts. Z.C. provided advice on the project and revised the manuscript. Y.C. conceived the project and made a final revision and edit of the manuscript.

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Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.117887/-/DC1

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