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A new gain-of-function mouse line to study the role of Wnt3a in development and disease

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Summary

Wnt/β-catenin signals are important regulators of embryonic and adult stem cell self-renewal and differentiation and play causative roles in tumorigenesis. Purified recombinant Wnt3a protein, or Wnt3a-conditioned culture medium, has been widely used to study canonical Wnt signaling in vitro or ex vivo. To study the role of Wnt3a in embryogenesis and cancer models, we developed a Cre recombinase activatable *Rosa26*^{Wnt3a} allele, in which a *Wnt3a* cDNA was inserted into the *Rosa26* locus to allow for conditional, spatiotemporally defined expression of Wnt3a ligand for gain-of-function (GOF) studies in mice. To validate this reagent, we ectopically overexpressed Wnt3a in early embryonic progenitors using the *T-Cre* transgene. This resulted in up-regulated expression of a β-catenin/Tcf-Lef reporter and of the universal Wnt/β-catenin pathway target genes, *Axin2* and *Sp5*. Importantly, *T-Cre; Rosa26*^{Wnt3a} mutants have expanded presomitic mesoderm (PSM) and compromised somitogenesis and closely resemble previously studied *T-Cre; Ctnnb1*^{ex3} (*β-catenin*^{GOF}) mutants. These data indicate that the exogenously expressed Wnt3a stimulates the Wnt/β-catenin signaling pathway, as expected. The *Rosa26*^{Wnt3a} mouse line should prove to be an invaluable tool to study the function of Wnt3a *in vivo*.

Keywords

Wnt3a; growth factor; Primitve streak (PS); gastrulation; mouse embryo; pre-somitic mesoderm (PSM); stem cells; development

Introduction, Results and Discussion

Wnt proteins are lipid modified, secreted signaling molecules essential for self-renewal, differentiation, cell motility and cell proliferation in development and disease (Clevers *et al.*,

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2014; Clevers and Nusse, 2012; Kalani *et al.*, 2008; ten Berge *et al.*, 2011; Willert *et al.*, 2003; Zeng and Nusse, 2010). Aberrant Wnt signaling is associated with several human diseases including cancer (Clevers and Nusse, 2012). In mammals there are 19 Wnt genes categorized into 12 conserved subfamilies (Wnt home page<http://wnt.stanford.edu>). Wnt ligands bind to Lrp and Frizzled cell-surface receptors to stabilize β-catenin, which then enters into the nucleus to bind to Tcf-Lef transcription factors to activate Wnt target genes (Logan and Nusse, 2004). The Wnt3a signal is transduced predominantly through β-catenin; however, Wnt3a has recently been shown to also signal through the Yap/Taz pathway, independently of β-catenin, to regulate various biological processes such as osteogenic differentiation, gene expression and cell migration (Park *et al.*, 2015). Thus, in addition to its utility in the study of the Wnt/β-catenin signaling pathway, a transgenic Wnt3a GOF reagent may also prove useful for the study of alternative Wnt3a signaling pathways such as the Hippo pathway.

Wnt proteins are covalently modified by palmitate residue by the palmitoyl transferase enzyme, porcupine (Kadowaki *et al.*, 1996). These post-translational modifications confer hydrophobicity to Wnt proteins, leading to the prediction that Wnts are short-range molecules that principally signal between neighboring cells (Clevers *et al.*, 2014). Indeed, studies tracking the endogenous expression of Wnt3 protein in intestinal crypts suggests that Wnt3 predominantly functions as a short-range, graded, signal and does not freely diffuse (Farin *et al.*, 2016). Wnt/β-catenin signaling is essential for the self renewal of embryonic stem cells (ESC), neural stem cells, haematopoietic stem cells, liver progenitors, skin stem cells, mammary gland stem cells, and intestinal stem cells (Clevers *et al.*, 2014; Kalani *et al.*, 2008; ten Berge *et al.*, 2011; Willert *et al.*, 2003). Wnt/β-catenin signaling also plays an important role in tissue regeneration, replenishing lost cells after normal tissue wear, injury and disease. Amongst the large Wnt protein family, Wnt3a has proven to be a popular choice to study Wnt signaling in mammalian cells. Conventionally, Wnt3a-conditioned media produced from L-cells, or purified recombinant Wnt3a, has been used to activate Wnt signaling in cell lines. Despite the widespread use of Wnt3a to stimulate Wnt/β-catenin signaling in vitro there are no mouse transgenic lines available to express Wnt3a in vivo.

Wnt3a is first expressed in the developing embryo at the blastocyst stage, and is subsequently expressed at PS stages in posteriorly located progenitor/stem cells that build the embryonic axis (Kemp *et al.*, 2005; Takada *et al.*, 1994). Null alleles of *Wnt3a* result in embryonic defects, apparent as early as the eighth day of gestation with a loss of PSM, which later manifests in a dramatic truncation of the embryonic axis (Takada *et al.*, 1994). The defects in PSM development are due to defective β-catenin/Tcf1-Lef1 signaling as embryos lacking β-catenin in the PS or Tcf1/Lef1 double mutants display phenotypes similar to the *Wnt3a* null phenotype (Dunty *et al.*, 2008; Galceran *et al.*, 1999). Furthermore, Wnt3a-like phenotypes also arise when the Wnt/β-catenin target genes, *Brachyury (T),* or *Sp5/Sp8* are mutated (Dunty *et al.*, 2014; Yamaguchi *et al.*, 1999). Conversely, activation of Wnt/β-catenin signaling in the PS using a conditional GOF allele of *Ctnnb1* results in an expanded PSM, elevated expression of the Wnt target genes *Brachyury (T)* and *Sp5*, and the β-catenin/Tcf-Lef β-gal reporter, and can partially rescue the *Wnt3a* mutant phenotype (Aulehla *et al.*, 2008; Dunty *et al.*, 2008; Harada *et al.*, 1999). As so much is known about

the role of Wnt3a signaling in PSM formation, the PSM serves as an ideal tissue to characterize the effects of a transgenic Wnt3a-expressing reagent.

A gain-of-function allele of Wnt3a was generated by targeting a floxed-STOP-Wnt3a cassette into the *Rosa26* locus by homologous recombination in ESCs (Fig. 1A). This allele is hereafter referred to as *Rosa26*^{Wnt3a}. Positively targeted ESC clones were confirmed by Southern blot analysis. The correctly targeted construct generated 5.9kb and 11.6kb fragments, when hybridized with 5′ and 3′ probes respectively, in addition to a 15.6kb WT fragment (Fig. 1B and 1C). A PCR-based genotyping protocol was also developed to detect the WT and *Rosa26*^{Wnt3a} knock-in (KI) allele (Fig. 1D). >20 positively targeted ESC clones were identified. Positive clones were injected into albino C57BL6 blastocysts to generate chimeric mice (F0). Male chimeras were crossed to C57Bl6 females to test for germline transmission of the targeted allele. F1 mice were genotyped by Southern blot analysis. *Rosa26*$^{Wnt3a/+}$ heterozygotes were intercrossed to generate homozygous *Rosa26*$^{Wnt3a/Wnt3a}$ animals. Both heterozygous and homozygous animals were viable and fertile and indistinguishable from WT animals based on morphological features and fertility.

To determine if Wnt3a could be ectopically expressed from the *Rosa26*^{Wnt3a} locus, we crossed *Rosa26*$^{Wnt3a/+}$ mice to the *T-Cre* driver strain to excise the Floxed-STOP cassette and permanently activate *Wnt3a* expression in *T-Cre*-expressing cells. This should include the PS and the neuromesodermal progenitors (NMPs) that give rise to the trunk paraxial mesoderm, spinal cord progenitors and all of their descendants (Garriock *et al.*, 2015; Perantoni *et al.*, 2005) (Fig. 2A). The *T-Cre; Rosa26*$^{Wnt3a/+}$ mutants are easily identified at embryonic day (E) 8.5 by their gross morphology, as they possess an elongated PSM and an enlarged allantois (Fig. 2B). In addition, E9.5 *T-Cre; Rosa26*$^{Wnt3a/+}$ mutants failed to turn and the allantois frequently appeared bulb shaped. Mutant embryos from this intercross were generated at the expected Mendelian ratio and littermate controls appeared phenotypically identical to WT embryos. To verify that the *T-Cre; Rosa26*$^{Wnt3a/+}$ mutant phenotype is due to ectopic expression of *Wnt3a*, we examined *Wnt3a* mRNA expression in control and *T-Cre; Rosa26*$^{Wnt3a/+}$ mutant littermates by whole-mount in situ hybridization (WISH). *Wnt3a* is expressed in the PS and dorsal neural folds in control embryos at E8.5 and E9.5 stages (Fig. 2B). However, in *T-Cre; Rosa26*$^{Wnt3a/+}$ mutants, highly elevated ectopic expression of *Wnt3a* was observed throughout the embryonic trunk in a pattern that is consistent with the activity of *T-Cre* (Perantoni *et al.*, 2005). To confirm that the IRES-AcGFP-Nuc accurately reflects the expression of Wnt3a driven by the *Rosa26* locus (Fig. 1A), we examined embryos for GFP expression. Although direct GFP fluorescence was not readily detectable, GFP was indirectly visualized by immunostaining (Fig. S1). Cross-sections through the trunk region of *T-Cre; Rosa26*$^{Wnt3a/+}$ mutants revealed GFP, and hence Wnt3a, expression in the expected neural and mesodermal domains (Fig. S1). The grossly elongated PSM observed in *T-Cre; Rosa26*$^{Wnt3a/+}$ mutants closely resembled that observed in *T-Cre; β-catenin*^{GOF} mutants (Aulehla *et al.*, 2008; Dunty *et al.*, 2008) suggesting that the *Rosa26*^{Wnt3a} allele is functional and that Wnt3a is likely signaling via the Wnt/β-catenin pathway, at least in *T-Cre* progenitors. Although not investigated directly, it is formally possible that ectopic Wnt3a is also stimulating β-catenin-independent pathways.

To assess whether the β-catenin/Tcf-Lef signaling pathway was stimulated by ectopic expression of Wnt3a, we examined the activity of the transgenic *BATlacZ* β-catenin/Tcf-Lef reporter in *T-Cre; Rosa26*$^{Wnt3a/+}$ mutants (Nakaya *et al.*, 2005). β-galactosidase (β-gal) staining of *BATlacZ; T-Cre; Rosa26*$^{Wnt3a/+}$ mutants revealed expanded and enhanced β-gal expression in the trunk, PSM and PS, compared to *BATlacZ* embryos alone, suggesting that the ectopic overexpression of Wnt3a enhanced β-catenin/Tcf-Lef signaling activity. Remarkably, the enlarged allantois in these mutants was negative for BATlacZ expression despite the ectopic expression of Wnt3a throughout the *T-Cre; Rosa26*$^{Wnt3a/+}$ mutant allantois (Fig. 2B). This result suggests that the allantois phenotype originates in the PS, or that the BATlacZ reporter is not responsive to Wnt signals in the allantois. Nevertheless, these data are consistent with exogenously expressed Wnt3a stimulating the Wnt/β-catenin/Tcf-Lef signaling pathway.

If exogenous Wnt3a is activating the Wnt/β-catenin pathway then the expression of known target genes of Wnt3a and β-catenin should also be ectopically activated. We examined the expression of the well-characterized Wnt/β-catenin target genes *Axin2*, *Sp5* and *T/Brachyury* (Dunty *et al.*, 2014; Ikeda *et al.*, 1998; Kishida *et al.*, 1998; Yamaguchi *et al.*, 1999) in *T-Cre; Rosa26*$^{Wnt3a/+}$ mutants and found that all three genes are up-regulated and anteriorly expanded in the caudal embryo (Fig. 3A and 3B). These expression patterns are highly reminiscent of those observed when β-catenin is constitutively activated by T-Cre and suggests that ectopic Wnt3a may be similarly maintaining PSM progenitors and suppressing somitogenesis (Dunty et al., 2008). Indeed, examination of *T-Cre; Rosa26*$^{Wnt3a/+}$ embryos for expression of the segmentally expressed, somite marker *Uncx4.1* revealed a compressed *Uncx4.1* expression domain and fewer segments, compared to controls (Fig. 3B). Our data suggests that exogenously expressed Wnt3a from our novel *Rosa26*^{Wnt3a} mouse line is behaving similarly to endogenous Wnt3a by stimulating the Wnt/β-catenin pathway.

We conclude that this *Rosa26*^{Wnt3a} mouse line will be a useful reagent for the study of Wnt/β-catenin signaling during early embryonic development, organogenesis and pathogenesis.

Methods

Construction of Wnt3a gain-of-function (*Rosa26*^{Wnt3a}) mice

Construction of targeting vectors, targeting and screening of ESCs were performed as described previously (Cha *et al.*, 2014). CTV plasmid (Klaus Rajewsky, Addgene Plasmid# 15192)(Thai *et al.*, 2007) was modified by replacing IRES-eGFP with IRES2-AcGFP-Nuc cassette isolated from pIRES2-AcGFP-Nuc (Clontech, Cat#632515). mWnt3a cDNA from pCIG-mWnt3a was subcloned and inserted into Asc1 restriction site. For gene targeting, SgfI- linearized targeting vector was electroporated into Bruce-4 C57BL6 ES cells and selected for G418 resistance. Genomic DNA was isolated from positively targeted ES cell colonies, digested with EcoR1 and probed with 5′ probe and 3′ probe (Fig. 1A). For Southern blot analysis, DIG labeled probes were synthesized and detected according to manufacturer's recommendation (Roche). Positively selected ES cells were then injected into albino C57BL6 blastocysts and later transferred into the uteri of pseudopregnant recipients to obtain chimeric mice. For colony expansion, male chimeras were crossed to

albino C57BL6 females and genotyped by Southern blot analysis and PCR genotyping (Fig. 1B–1D). The following primers were used for PCR genotyping:

Rosa10- 5′CTCTGCTGCCTCCTGGCTTCT3′,

Rosa11- 5′CGAGGCGGATCACAAGCAATA3′,

R26R2- 5′GCGAAGAGTTTGTCCTCAACC3′, Product size for WT allele: Rosa10+Rosa11= 322bp, *Rosa26*^{Wnt3a} allele: Rosa10+R26R2=192bp,

StopF- 5′ GCCTTGACTAGAGATCATAATCAGC 3′

mWnt3aR- 5′ ACAGCCAAGGACCACCAGA 3′,

product size for WT allele-no band, *Rosa26*^{Wnt3a} allele: 407bp. Refer to Fig. 1A for location of the primers. We also confirmed that the Frt sites in the targeting vector were functional in vitro. The targeting vector was transformed into EL250 E. coli strain, which has arabinose inducible flpe gene and colonies were picked to isolate plasmids and confirmed that the cassette flanked by FRT sites was removed. This mouse reagent will be made available to the research community upon acceptance of this manuscript.

Mice

Tg(T-Cre)^{1Lwd} and *BATlacZ* mice were previously described (Nakaya *et al.*, 2005; Perantoni *et al.*, 2005). *Rosa26*^{Wnt3a} mice were maintained on C57BL6 background as heterozygotes or homozygotes. To generate *T-Cre; Rosa26*$^{Wnt3a/+}$ mutants, *T-Cre* mice were crossed with *Rosa26*$^{Wnt3a/+}$, and wildtype and mutant littermates were dissected on embryonic day (E) 8.5 and 9.5. Embryos were dissected in PBS, fixed for overnight in 4% Paraformaldehyde at 4°C, and dehydrated in a graded Methanol series and stored in 100% Methanol at −20°C. Although mutant embryos ectopically expressed Wnt3a and AcGFP simultaneously in *T-Cre* progenitors, direct GFP fluorescence was not readily detectable. All animal experiments were performed according to the Guide for the Care and Use of Laboratory Animals (National Academies Press; 8th editions).

Whole mount *In Situ* Hybridization and Immunohistochemistry

For Whole mount In situ Hybridization (WISH), embryos were rehydrated in a graded Methanol series. One-color whole mount *in situ* hybridization was performed as described previously (Biris *et al.*, 2007; Biris and Yamaguchi, 2014). *T, Axin2 and Sp5* DIG labeled antisense RNA probes were synthesized from linearized DNA templates using digoxigenin (DIG) and RNA polymerase. DIG-labeled probes were detected using alkaline phosphate (AP) conjugated secondary antibodies and AP substrate (BM purple, Roche). Embryos were photographed using a Zeiss AxioCam HRc camera on a Leica MZFLIII microscope and processed with Axiovision software. Photographed embryos are representative of at least three replicates. For immunohistochemistry, embryos were fixed briefly in 4% Para-formaldehyde for 30 minutes and passed through 20% sucrose for over-night at 4°C and frozen blocks were prepared in OCT followed by 5μM sections as described previously using standard protocols (Chalamalasetty *et al.*, 2014). For immunohistochemistry, sections were incubated using Rabbit anti-GFP (eBioscience, #14-6774-63, 1:100) and Mouse anti-Sox2 (R&D Systems, #mab2018,1:100) followed by Goat anti-rabbit Alexa flour 488

(1:500) and Goat anti-mouse Alexa flour 567 (1:500) antibodies. Slides were counter stained with 1 nM DAPI (Molecular probes) and mounted using aqua poly/mount (Polysciences Inc.) Images were photographed using Zeiss AxioplanII microscope.

β-galactosidase staining

Whole-mount β-galactosidase staining was performed as previously described (Chalamalasetty *et al.*, 2014; Whiting *et al.*, 1991). Embryos were dissected in PBS and fixed briefly in 1% Formaldehyde, 0.2% Glutaraldehyde, 2mM $MgCl_2$, 5mM EGTA and 0.02% NP-40 for 30 min at room temperature and washed 3 times in PBS plus 0.02% NP-40. Embryos were stained in 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$, 2mM $MgCl_2$, 0.01% Sodium deoxycholate, 0.02% NP-40 and 1mg/ml X-gal solution at 37°C for 5–30 min. Stained embryos were washed and postfixed in 4% Para-formaldehyde plus 0.2% Glutaraldehyde for 30 min at room temperature. Embryos were stored in 80% Glycerol/PBT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

Aulehla A, Wiegraebe W, Baubet V, Wahl MB, Deng C, Taketo M, Lewandoski M, Pourquie O. 2008; A beta-catenin gradient links the clock and wavefront systems in mouse embryo segmentation. Nat Cell Biol. 10:186–193. [PubMed: 18157121]

Biris KK, Dunty WC Jr, Yamaguchi TP. 2007; Mouse Ripply2 is downstream of Wnt3a and is dynamically expressed during somitogenesis. Dev Dyn. 236:3167–3172. [PubMed: 17937396]

Biris KK, Yamaguchi TP. 2014; Two-color in situ hybridization of whole-mount mouse embryos. Methods Mol Biol. 1092:17–30. [PubMed: 24318811]

Cha J, Bartos A, Park C, Sun X, Li Y, Cha SW, Ajima R, Ho HY, Yamaguchi TP, Dey SK. 2014; Appropriate crypt formation in the uterus for embryo homing and implantation requires Wnt5a-ROR signaling. Cell Rep. 8:382–392. [PubMed: 25043182]

Chalamalasetty RB, Garriock RJ, Dunty WC Jr, Kennedy MW, Jailwala P, Si H, Yamaguchi TP. 2014; Mesogenin 1 is a master regulator of paraxial presomitic mesoderm differentiation. Development. 141:4285–4297. [PubMed: 25371364]

Clevers H, Loh KM, Nusse R. 2014; Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. Science. 346:1248012. [PubMed: 25278615]

Clevers H, Nusse R. 2012; Wnt/beta-catenin signaling and disease. Cell. 149:1192–1205. [PubMed: 22682243]

Dunty WC Jr, Biris KK, Chalamalasetty RB, Taketo MM, Lewandoski M, Yamaguchi TP. 2008; Wnt3a/beta-catenin signaling controls posterior body development by coordinating mesoderm formation and segmentation. Development. 135:85–94. [PubMed: 18045842]

Dunty WC Jr, Kennedy MW, Chalamalasetty RB, Campbell K, Yamaguchi TP. 2014; Transcriptional Profiling of Wnt3a Mutants Identifies Sp Transcription Factors as Essential Effectors of the Wnt/beta-catenin Pathway in Neuromesodermal Stem Cells. PLoS One. 9:e87018. [PubMed: 24475213]

Farin HF, Jordens I, Mosa MH, Basak O, Korving J, Tauriello DV, de Punder K, Angers S, Peters PJ, Maurice MM, Clevers H. 2016; Visualization of a short-range Wnt gradient in the intestinal stem-cell niche. Nature. 530:340–343. [PubMed: 26863187]

Galceran J, Farinas I, Depew MJ, Clevers H, Grosschedl R. 1999; Wnt3a−/−-like phenotype and limb deficiency in Lef1(−/−)Tcf1(−/−) mice. Genes Dev. 13:709–717. [PubMed: 10090727]

Garriock RJ, Chalamalasetty RB, Kennedy MW, Canizales LC, Lewandoski M, Yamaguchi TP. 2015; Lineage tracing of neuromesodermal progenitors reveals novel Wnt-dependent roles in trunk progenitor cell maintenance and differentiation. Development. 142:1628–1638. [PubMed: 25922526]

Harada N, Tamai Y, Ishikawa T, Sauer B, Takaku K, Oshima M, Taketo MM. 1999; Intestinal polyposis in mice with a dominant stable mutation of the beta-catenin gene. EMBO J. 18:5931–5942. [PubMed: 10545105]

Ikeda S, Kishida S, Yamamoto H, Murai H, Koyama S, Kikuchi A. 1998; Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3beta and beta-catenin and promotes GSK-3beta-dependent phosphorylation of beta-catenin. EMBO J. 17:1371–1384. [PubMed: 9482734]

Kadowaki T, Wilder E, Klingensmith J, Zachary K, Perrimon N. 1996; The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. Genes Dev. 10:3116–3128. [PubMed: 8985181]

Kalani MY, Cheshier SH, Cord BJ, Bababeygy SR, Vogel H, Weissman IL, Palmer TD, Nusse R. 2008; Wnt-mediated self-renewal of neural stem/progenitor cells. Proc Natl Acad Sci U S A. 105:16970–16975. [PubMed: 18957545]

Kemp C, Willems E, Abdo S, Lambiv L, Leyns L. 2005; Expression of all Wnt genes and their secreted antagonists during mouse blastocyst and postimplantation development. Dev Dyn. 233:1064–1075. [PubMed: 15880404]

Kishida S, Yamamoto H, Ikeda S, Kishida M, Sakamoto I, Koyama S, Kikuchi A. 1998; Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of beta-catenin. J Biol Chem. 273:10823–10826. [PubMed: 9556553]

Logan CY, Nusse R. 2004; The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol. 20:781–810. [PubMed: 15473860]

Nakaya MA, Biris K, Tsukiyama T, Jaime S, Rawls JA, Yamaguchi TP. 2005; Wnt3a links left-right determination with segmentation and anteroposterior axis elongation. Development. 132:5425–5436. [PubMed: 16291790]

Park HW, Kim YC, Yu B, Moroishi T, Mo JS, Plouffe SW, Meng Z, Lin KC, Yu FX, Alexander CM, Wang CY, Guan KL. 2015; Alternative Wnt Signaling Activates YAP/TAZ. Cell. 162:780–794. [PubMed: 26276632]

Perantoni AO, Timofeeva O, Naillat F, Richman C, Pajni-Underwood S, Wilson C, Vainio S, Dove LF, Lewandoski M. 2005; Inactivation of FGF8 in early mesoderm reveals an essential role in kidney development. Development. 132:3859–3871. [PubMed: 16049111]

Takada S, Stark KL, Shea MJ, Vassileva G, McMahon JA, McMahon AP. 1994; Wnt-3a regulates somite and tailbud formation in the mouse embryo. Genes Dev. 8:174–189. [PubMed: 8299937]

ten Berge D, Kurek D, Blauwkamp T, Koole W, Maas A, Eroglu E, Siu RK, Nusse R. 2011; Embryonic stem cells require Wnt proteins to prevent differentiation to epiblast stem cells. Nat Cell Biol. 13:1070–1075. [PubMed: 21841791]

Thai TH, Calado DP, Casola S, Ansel KM, Xiao C, Xue Y, Murphy A, Frendewey D, Valenzuela D, Kutok JL, Schmidt-Supprian M, Rajewsky N, Yancopoulos G, Rao A, Rajewsky K. 2007; Regulation of the germinal center response by microRNA-155. Science. 316:604–608. [PubMed: 17463289]

Whiting J, Marshall H, Cook M, Krumlauf R, Rigby PW, Stott D, Allemann RK. 1991; Multiple spatially specific enhancers are required to reconstruct the pattern of Hox-2.6 gene expression. Genes Dev. 5:2048–2059. [PubMed: 1682218]

Willert K, Brown JD, Danenberg E, Duncan AW, Weissman IL, Reya T, Yates JR 3rd, Nusse R. 2003; Wnt proteins are lipid-modified and can act as stem cell growth factors. Nature. 423:448–452. [PubMed: 12717451]

Yamaguchi TP, Takada S, Yoshikawa Y, Wu N, McMahon AP. 1999; T (Brachyury) is a direct target of Wnt3a during paraxial mesoderm specification. Genes Dev. 13:3185–3190. [PubMed: 10617567]

Zeng YA, Nusse R. 2010; Wnt proteins are self-renewal factors for mammary stem cells and promote their long-term expansion in culture. Cell Stem Cell. 6:568–577. [PubMed: 20569694]

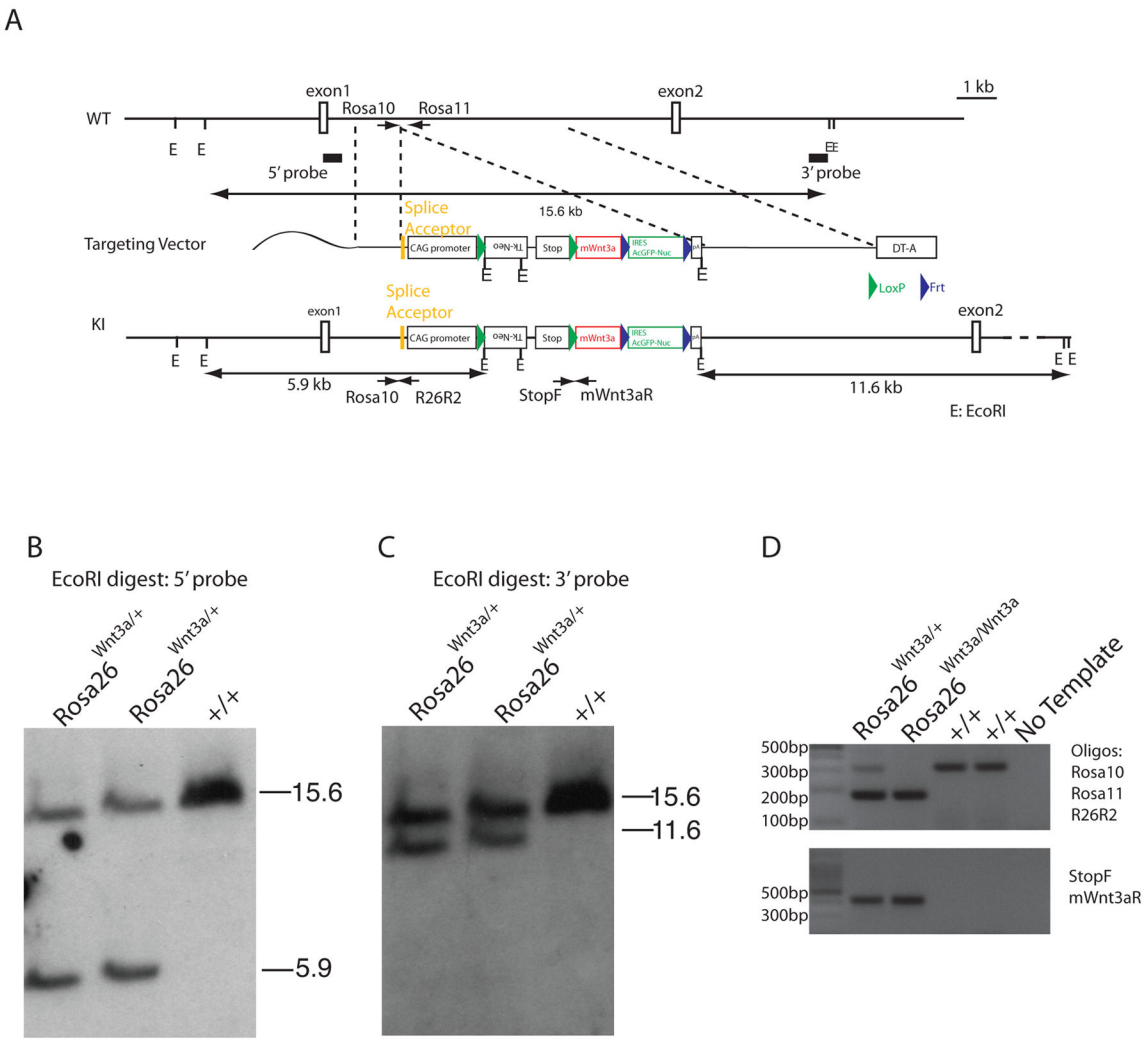

Figure 1. Schematic view of construction of *Rosa26*^{Wnt3a} gain-of-function allele

(A) Schematic diagram of targeting strategy to insert mWnt3a-IRES-AcGFP-Nuc between exons 1 and 2 of *Rosa26* for conditional gene expression. Targeting vector comprised of "Splice acceptor-CAG promoter-LoxP-TK Neo-STOP-LoxP-Wnt3a-FRT-IRES AcGFP-Nuc-FRT-pA" was targeted to intron1 of the *Rosa26* locus by homologous recombination. Black rectangles indicate the site of 5′ and 3′ probes used for Southern blot analysis. For PCR genotyping, oligonucleotide pairs Rosa10; Rosa11 were used to detect the wildtype (WT) allele and Rosa10; R26R2 were used for the knock-in allele (KI). Primer pairs StopF; mWnt3aR were used to specifically detect the KI allele. E; EcoR1 (B,C) Representative Southern blot analysis of positively targeted ESCs. Genomic DNA from *Rosa26*$^{Wnt3a/+}$ and *WT (+/+)* cells was digested with EcoR1 enzyme and Southern blot was performed using 5′ and 3′ probes as shown in (A). Upon EcoR1 digestion, the WT locus generated a 15.6Kb fragment, while the targeted locus generated 5.9Kb and 11.6Kb DNA fragments as detected by 5′ Probe and 3′Probe. (D) Representative PCR genotyping of *Rosa26*$^{Wnt3a/+}$ and *WT (+/+)* alleles using Rosa10, Rosa11 and R26R2 oligos. The WT band migrates at 322bp and

the KI band at 192 bp. As an alternative, the KI allele can also be amplified using StopF and mWnt3aR oligos to generate a 407bp amplicon.

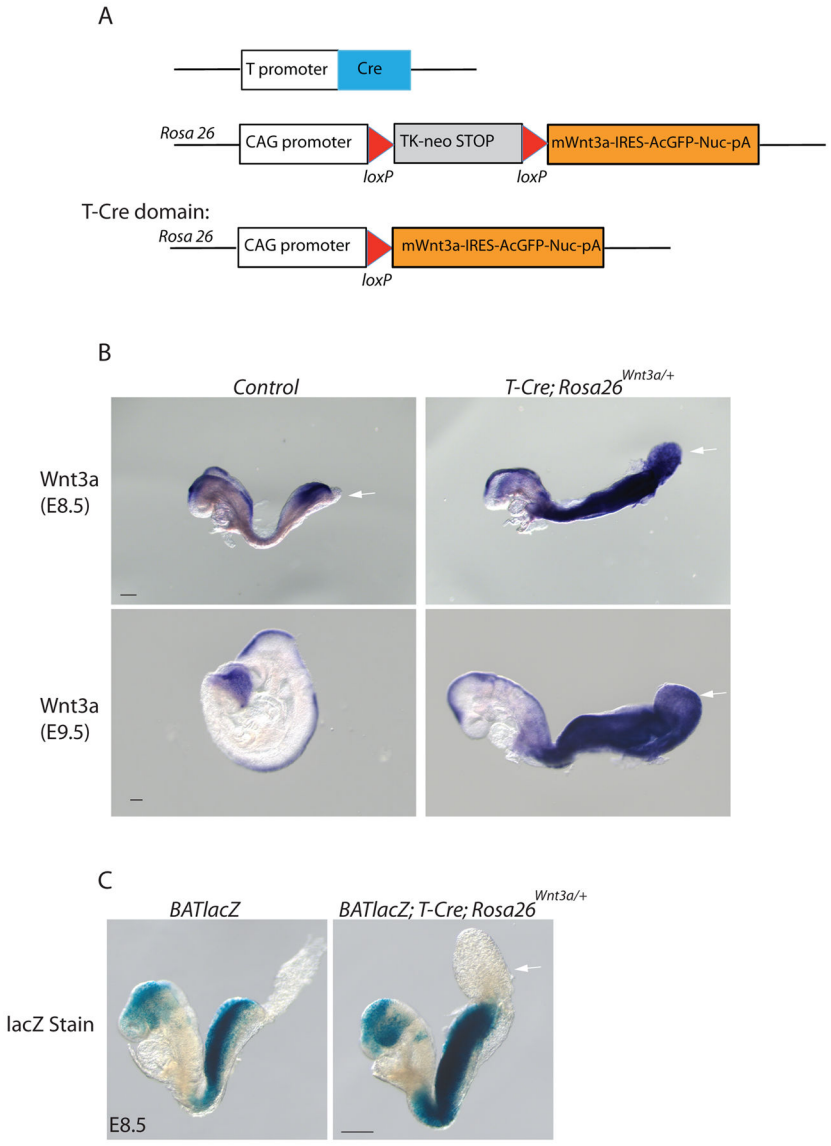

Figure 2. Conditional expression of Wnt3a in T-Cre progenitors and their descendants

(A) Schematic of LoxP-Cre mediated activation of Wnt3a expression in T-Cre-expressing PS progenitors. (B) Lateral views of control and *T-Cre; Rosa26*$^{Wnt3a/+}$ embryos at E8.5 and E9.5 stages analysed by WISH for expression of *Wnt3a* mRNA. (C) β-gal staining of *BATlacZ* control and *BATlacZ; T-Cre; Rosa26*$^{Wnt3a/+}$ embryos at E8.5 stage. Arrows indicate expression in allantois and scale bar is 200μM.

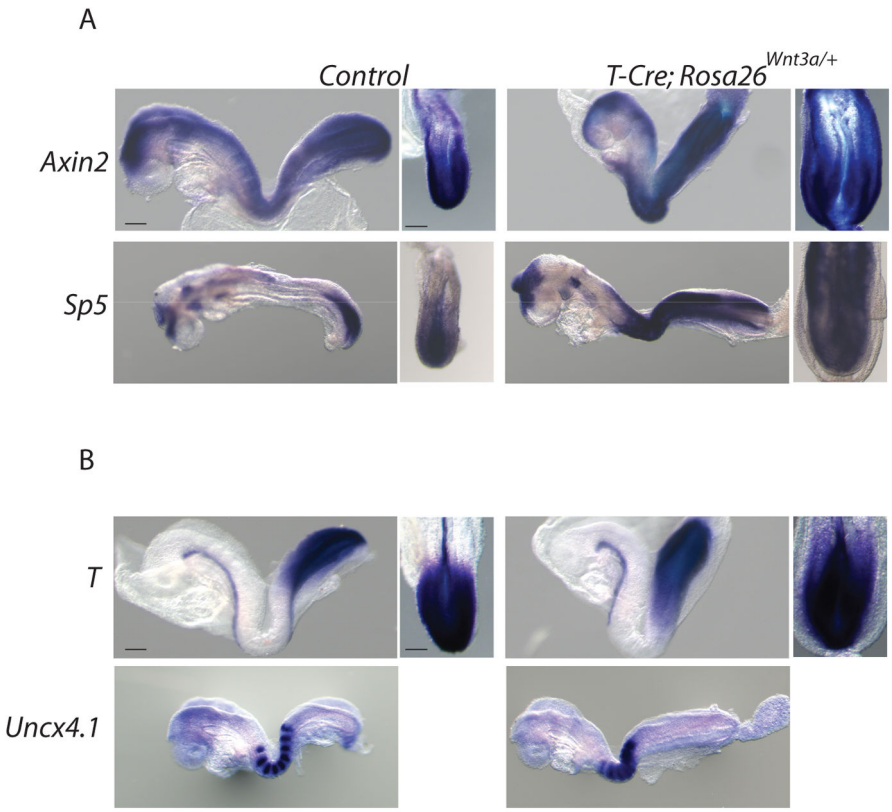

Figure 3. Conditional expression of Wnt3a results in up-regulated Wnt target genes and inhibition of somitogenesis

WISH analysis of *Axin2*, *Sp5, T* and *Uncx4.1* expression in E8.5 control and *T-Cre; Rosa26*$^{Wnt3a/+}$ embryos. Lateral views of whole embryos and dorsal views of the caudal end of the same embryo are shown. Scale bars indicate 200μM.