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RESEARCH ARTICLE

Simplet/Fam53b is required for Wnt signal transduction by regulating β-catenin nuclear localization

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ABSTRACT

Canonical β-catenin-dependent Wnt signal transduction is important for several biological phenomena, such as cell fate determination, cell proliferation, stem cell maintenance and anterior-posterior axis formation. The hallmark of canonical Wnt signaling is the translocation of β-catenin into the nucleus where it activates gene transcription. However, the mechanisms regulating β-catenin nuclear localization are poorly understood. We show that Simplet/Fam53B (Smp) is required for Wnt signaling by positively regulating β-catenin nuclear localization. In the zebrafish embryo, the loss of *smp* blocks the activity of two β-catenin-dependent reporters and the expression of Wnt target genes, and prevents nuclear accumulation of β-catenin. Conversely, overexpression of *smp* increases β-catenin nuclear localization and transcriptional activity *in vitro* and *in vivo*. Expression of mutant Smp proteins lacking either the nuclear localization signal or the β-catenin interaction domain reveal that the translocation of Smp into the nucleus is essential for β-catenin nuclear localization and Wnt signaling *in vivo*. We also provide evidence that mammalian Smp is involved in regulating β-catenin nuclear localization: the protein colocalizes with β-catenin-dependent gene expression in mouse intestinal crypts; siRNA knockdown of Smp reduces β-catenin nuclear localization and transcriptional activity; human SMP mediates β-catenin transcriptional activity in a dose-dependent manner; and the human SMP protein interacts with human β-catenin primarily in the nucleus. Thus, our findings identify the evolutionary conserved SMP protein as a regulator of β-catenin-dependent Wnt signal transduction.

KEY WORDS: Simplet/Fam53b, Wnt signaling, β-Catenin, Embryogenesis, Nuclear localization, Zebrafish

INTRODUCTION

β-Catenin-dependent Wnt signal transduction is important for several biological phenomena, including anterior-posterior axis formation (Petersen and Reddien, 2009; Cavodeassi, 2013). In the embryo mouse, targeted disruption of Wnt3a results in the loss of caudal somites and the tailbud (Takada et al., 1993). In *Xenopus* embryos, antagonism of Wnt signaling allows the formation of anterior structures at the expense of posterior ones (Leyns et al., 1997; Glinka et al., 1998). This is also true for zebrafish: morpholino knockdown of *wnt8a* results in embryos that predominantly form head but lack posterior structures (Erter et al., 2001; Lekven et al., 2001; Rhinn et al., 2005). Conversely, ectopic activation of Wnt signaling in mouse, *Xenopus* or zebrafish embryos promotes the formation of posterior structures and the loss of anterior ones (Christian and Moon, 1993; Kelly et al., 1995; Popperl et al., 1997; Kiecker and Niehrs, 2001; Rhinn et al., 2005). Thus, Wnt signaling is required for the induction of posterior structures.

β-Catenin-dependent Wnt signal transduction is a multi-step process that consists of several molecular components. It is initiated by secreted Wnt glycoproteins that bind to transmembrane Frizzled receptors (Angers and Moon, 2009). Ligand-receptor interaction induces receptor oligomerization with the low-density lipoprotein receptor-related proteins LRP5 and LRP6 (Angers and Moon, 2009), and this interaction allows LRP5 and LRP6 to bind to the intracellular protein axin. In turn, this complex activates dishevelled, which prevents phosphorylation-mediated degradation of β-catenin, a transcriptional co-factor involved in the activation of genes that are required for specifying posterior structures, e.g. Tbx6 and Cdx4 (Shimizu et al., 2005; Pilon et al., 2006).

The gene *simplet/fam53b* (*smp*) belongs to a family of proteins (Fam53A, Fam53B and Fam53C), the molecular mechanisms of which are unknown. Smp is required for early vertebrate development by regulating progenitor cell proliferation (Thermes et al., 2006), and is also necessary for zebrafish appendage regeneration by regulating cell proliferation and the expression of genes involved in tissue patterning (Kizil et al., 2009). However, it is not understood how Smp is involved in any of these processes, because the molecular mechanisms through which Smp acts have not been determined.

We show that Smp is required for the formation of posterior structures during zebrafish embryogenesis, and that the *smp* knockdown phenotype is associated with the abrupt inactivation of β-catenin-dependent Wnt signaling at late gastrulation due to the loss of nuclear β-catenin. We also show that the Smp protein interacts with β-catenin and that loss of the nuclear localization signal in Smp inhibits β-catenin-dependent Wnt signaling by preventing nuclear localization of β-catenin. Furthermore, subcellular fractionation experiments indicate that Smp and β-catenin interact in the nucleus, and fluorescence recovery after photobleaching experiments suggest that Smp is involved in retaining β-catenin in the nucleus. Thus, we identify a previously unknown regulator of β-catenin-dependent Wnt signaling.

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RESULTS

smp knockdown results in loss of posterior structures and β-catenin-dependent gene transcription

Previous work has shown that Smp is required for early Medaka embryogenesis and zebrafish fin regeneration (Thermes et al., 2006; Kizil et al., 2009), but the molecular mechanisms through which it functions during these processes are unknown. Smp shares some similarity within two domains with two other genes (Fam53A and Fam53C) (supplementary material Fig. S1A); the two conserved domains in these proteins have no clear similarity to any known protein domain. *smp* is present as a transcript (Fig. 1A-E) and protein (supplementary material Fig. S1B-G) during early zebrafish embryogenesis. To elucidate how *smp* functions, we performed morpholino knockdown in the zebrafish embryo. Compared with mismatch-injected (MM) control embryos (Fig. 1F), the knockdown of *smp* with previously characterized translation (ATG) or splice-blocking morpholinos (Kizil et al., 2009) produced axial defects (Fig. 1G,H; supplementary material Fig. S2A,B).

Loss of zygotic β-catenin-dependent Wnt signaling also results in axial defects (Lekven et al., 2001; Agathon et al., 2003; Shimizu et al., 2005; Petersen and Reddien, 2009). We therefore tested whether loss of *smp* affected the activity of Wnt signaling-dependent transgenic zebrafish reporter lines: Tg(7xTCF-XLa.Siam:nlsmCherry)^{ia5} [hereafter Tg(7xTCF:mCherry)] and Tg(Top:dGFP) (Dorsky et al., 2002; Moro et al., 2012). Although *smp* knockdown did not affect Tg(7xTCF:mCherry) reporter expression at 85% epiboly (Fig. 1I,J), at 95% epiboly and later stages the reporter activity observed in mismatch controls (Fig. 1K,M) was nearly abolished in the *smp* antisense (AS) morphants (Fig. 1L,N). Co-injecting *smp* mRNA with the antisense morpholino targeting the splice site restored the reporter activity (supplementary material Fig. S2C-F) and rescued the axial defects (supplementary material Fig. S2G-J), indicating that the loss of reporter activity is specifically due to the loss of Smp function (Kizil et al., 2009). Similar results were obtained using the transgenic Top:dGFP Wnt-reporter line (Fig. 1O-R). *cdx4*, *tbx6* and *gbx1* are β-catenin-dependent Wnt-regulated genes that are required for axial patterning (Chapman and Papaioannou, 1998; Lekven et al., 2001; White et al., 2003; Rhinn et al., 2005, 2009; Shimizu et al., 2005; Pilon et al., 2006). Compared with controls (Fig. 1S,U,W,Y,AA), *smp* morphants showed significant downregulation of these genes (Fig. 1V,Z,BB), demonstrating that *smp* is required for Wnt-dependent gene expression in the embryo. The emergence of the smp morphant phenotype is likely due to a maternally loaded Smp protein (supplementary material Fig. S2O,P), as detected using an antibody for the zebrafish Smp protein (supplementary material Fig. S2K-M′).

BMP, Nodal, FGF and retinoic acid signaling pathways are also required for axial patterning of the embryo (Schier, 2001; Schier and Talbot, 2005; Rhinn et al., 2006), and BMP, Nodal and FGF appear unaffected during gastrulation of the morphants (supplementary material Fig. S3). Although the retinoic acid-synthesizing enzyme *aldh1a2* (*raldh2*) was unaffected (supplementary material Fig. S4A-B′), *smp* knockdown caused ectopic expression of the retinoic acid-degrading enzyme *cyp26a1* at tailbud stage (supplementary material Fig. S4C-F). This broader expression was subsequent to the loss in activity of the β-catenin-dependent reporter (supplementary material Fig. S4G,H), and similar misregulation was induced by Dkk1 overexpression (supplementary material Fig. S4I,J), indicating that the altered expression of *cyp26a1* in the *smp* morphants is a downstream consequence of Wnt inhibition.

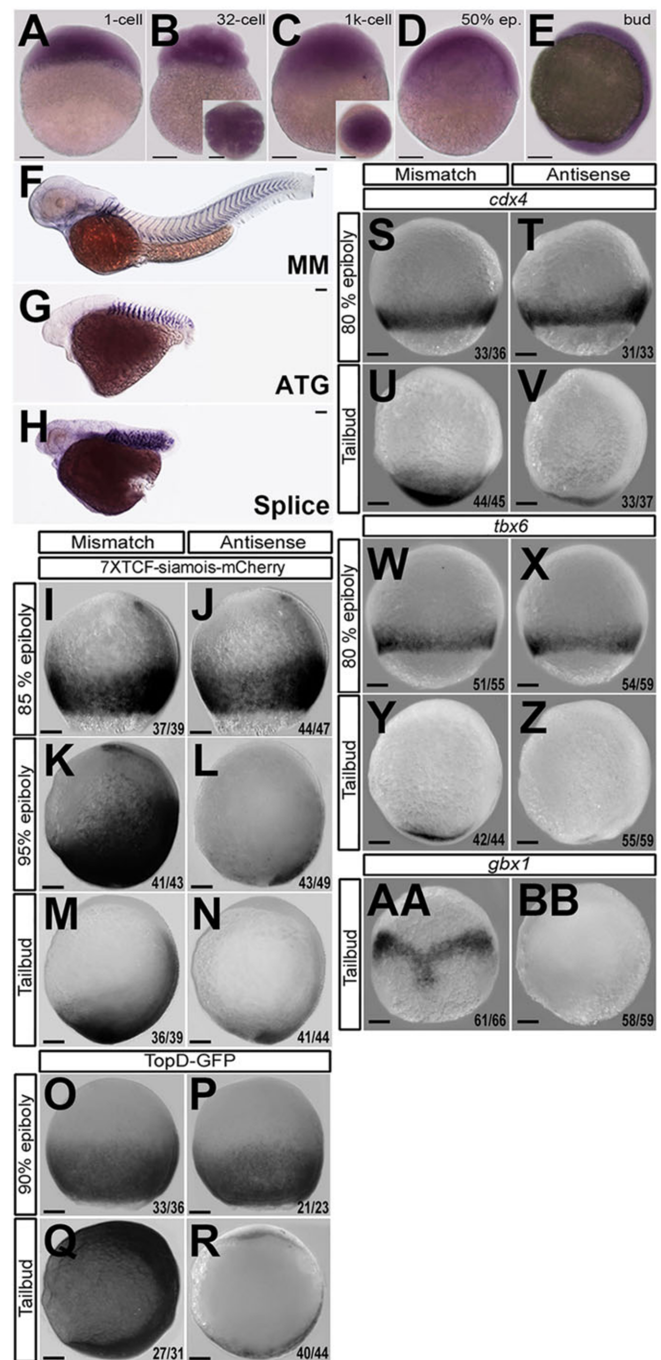

Fig. 1. Loss of *smp* causes Wnt-related developmental phenotype and loss of Wnt-dependent gene expression. (A) *smp* mRNA (dark purple) in the single-cell embryo. (B,C) The *smp* transcript in 32-cell embryo (B) and in 1000-cell embryo (C). Expression remains ubiquitous at 50% epiboly (D) and at tailbud (lateral view) stage (E). (F) Control embryo (MM) 24 h post-fertilization (hpf) after injection with mismatch *smp* morpholino at the one-cell stage. *In situ* hybridization with a *xirp2* probe highlights somatic muscle boundaries. (G,H) Injection of antisense morpholino to the start (ATG) site (G) or first intron-exon (splice) boundary (H) of *smp* in 24 hpf embryos. Activation of 7xTCF:mCherry in mismatch control embryos (I,K,M) compared with *smp* morphants (J,L,N). Activity of Top:dGFP in control embryos (O,Q) compared with *smp* morphants (P,R). *cdx4* expression at indicated stages in mismatch controls (S,U) and in *smp* morphants (T,V). Expression of *tbx6* at indicated stages in controls (W,Y) and *smp* morphants (X,Z). Expression of *gbx1* at tailbud stage in controls (AA) and *smp* morphants (BB). Numbers in lower right corners of the panels indicate the number of embryos with the depicted expression pattern/the total number of animals. Scale bars: 100 µm.

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Subsets of Wnt ligands activate β-catenin-independent planar cell polarity (PCP) signaling, which regulates convergence-extension movements during vertebrate gastrulation (Du et al., 1995; Heisenberg et al., 2000; Tada and Smith, 2000; Kilian et al., 2003; Roszko et al., 2009). We therefore examined the expression of these ligands, the cell contribution to the embryo during PCP-dependent convergence extension and the gene expression associated with PCP signaling. Comparisons between controls and morphants in these experiments indicated no apparent abnormalities normally attributed to defective Wnt-PCP signaling in *smp* morphants (supplementary material Fig. S5). Using markers for neuroectoderm, endoderm, paraxial mesoderm and somatic mesoderm specification, we observed similar expression patterns in control and *smp* knockdown embryos (supplementary material Fig. S6) despite the expected a reduction of proliferating cells in the morphants (supplementary material Fig. S6I-N) (Thermes et al., 2006). However, we observed a slight temporary thickening in the expression pattern of genes transcribed in the axial mesoderm, indicating a short-term (possibly indirect) effect on early axial development before the loss of β-catenin-mediated signaling (supplementary material Fig. S6O-W). Although treatment with hydroxyurea (HU) and aphidicolin (AC) significantly reduced cell proliferation, it did not inhibit β-catenin-dependent Tg(7XTCF:mCherry) reporter activity (supplementary material Fig. S7). Apoptosis was not significantly altered in *smp* morphants (supplementary material Fig. S8A,B), and preventing apoptosis by *p53* knockdown (supplementary material Fig. S8C) did not prevent the loss of Wnt reporter activity (supplementary material Fig. S8D-F). Together, these results suggest that the loss of Wnt signaling is not due to defects in early germ layer formation or to perturbed cell proliferation or death.

Smp is required for β-catenin-dependent signaling by regulating its nuclear localization

We next asked whether *smp* acts up- or downstream of β-catenin in the Wnt signaling pathway. Compared with control (Fig. 2A) and *smp* morphants (Fig. 2B), overexpression of β-catenin expanded the activity of the Tg(7xTCF:mCherry) reporter (Fig. 2C). Interestingly, *smp* knockdown suppressed the β-catenin-mediated expansion of the reporter (Fig. 2D). By contrast, *smp* knockdown did not suppress the activation of the reporter by a β-catenin-independent constitutive active *lef1* construct (*lef1* fused to the VP16 transactivation domain, *lef1*-VP16) (Aoki et al., 1999; Vleminckx et al., 1999) (Fig. 2E,F). In addition, we found that the Wnt ligands required for axis formation are expressed and that Wnt receptor complex activation in unaffected, as evidenced by similar Lrp6 phosphorylation levels in the morphants as in MM controls (supplementary material Fig. S9). These results indicate that *smp* is involved in Wnt signal transduction by regulating β-catenin activity.

β-Catenin transduces Wnt signaling by accumulating in the cytoplasm and translocating to the nucleus (Grigoryan et al., 2008). Immunohistochemistry (IHC) for β-catenin in MM-control embryos showed β-catenin at the plasma membrane and in the nuclei of marginal deep cells (Fig. 2G,H). However, although β-catenin was localized to the plasma membrane (Fig. 2I), it was absent in the nuclei of *smp* morphants (Fig. 2J). Subcellular fractionation experiments also showed that compared with the levels of β-catenin in the nuclear fraction of MM-control embryos, β-catenin was significantly reduced in the nuclear fraction of *smp* morphants and was comparatively higher in the cytoplasmic fraction (Fig. 2K, supplementary material Fig. S10A). The overall levels of β-catenin in the morphants remained unchanged (Fig. 2K), indicating that *smp* is required for β-catenin nuclear localization and not for its stabilization, and that the loss of *smp* results in a shift from nuclear to cytoplasmic distribution of β-catenin.

The dependence of β-catenin nuclear localization on *smp* suggests that the proteins colocalize in the nucleus. Immunohistochemistry with antibodies (supplementary material Fig. S10B,C) against endogenous zebrafish Smp (Fig. 2L) and β-catenin (Fig. 2M) in the dorsal marginal cells showed nuclear co-staining of both proteins (Fig. 2N, white arrowheads). However, several Smp-positive nuclei lacked β-catenin (Fig. 2M, white arrows), indicating that while β-catenin requires Smp for its nuclear localization, the nuclear localization of Smp does not require β-catenin.

We next addressed the ability of Smp to activate β-catenin-dependent Wnt signaling. Because transfection experiments showed that Smp alone failed to activate the pBAR reporter (supplementary material Fig. S11A), Smp appeared to be unable to promote Wnt signaling alone. We compared the distribution of the phenotypic classes from overexpression of *wnt8* with GFP (Fig. 2O) and of *smp* with *wnt8*. We observed that *smp* exacerbated the severity of *wnt8*-induced phenotypes in zebrafish (Fig. 2P). Likewise, *smp* enhanced the activation of the β-catenin-dependent reporter pBAR in HEK293T cells by *wnt8* (Fig. 2Q) as well as by other members of the Wnt signaling cascade (supplementary material Fig. S11B). Furthermore, in zebrafish PAC2 cells, Smp synergized with β-catenin in pBAR activation in a dose-dependent manner (supplementary material Fig. S11C). These results indicate that *smp* mediates β-catenin-dependent Wnt signal transduction.

We next assessed whether the enhancement of β-catenin-dependent Wnt signaling by *smp* is associated with an increase in the nuclear localization of β-catenin. Immunohistochemical staining of embryos overexpressing Smp-GFP showed nuclear localization of the Smp-GFP (Fig. 2S). Whereas endogenous β-catenin was primarily localized at the cell membrane with faint staining in the nucleus of controls (Fig. 2T), overexpression of Smp-GFP increased nuclear β-catenin (Fig. 2U). Smp-GFP and β-catenin co-stains showed that cells with Smp in the nucleus contained nuclear-localized β-catenin (Fig. 2Y). We quantified the distribution of both proteins and observed that when β-catenin was nuclear, Smp was nuclear (>99%) (Fig. 2Z). However, cells lacking nuclear β-catenin displayed nuclear localization of Smp in ∼85% of cells counted (Fig. 2AA), suggesting that Smp nuclear localization is not regulated by Wnt signaling. To examine whether Smp transcription is regulated by Wnt signaling, we used transgenic fish lines that activate or inhibit Wnt signaling and found no change in *smp* expression *in vivo* (supplementary material Fig. S12A-D). Likewise, there was no change in subcellular distribution of the protein in cells cultured with Wnt-conditioned medium *in vitro* (supplementary material Fig. S12E-J). These data associate the nuclear localization of β-catenin with nuclear Smp and argue that Smp itself does not require β-catenin to localize to the nucleus.

Removal of the nuclear localization signal in Smp prevents β-catenin nuclear localization and inhibits Wnt signaling

Smp protein contains a candidate nuclear localization signal (NLS), which could be instrumental in mediating β-catenin nuclear accumulation (Fig. 3A). We therefore tested its importance by generating a NLS mutant that still interacts with β-catenin (Fig. 3A; supplementary material Fig. S13A). Overexpression of the GFP-tagged full-length Smp (Smp-FL-GFP) showed predominant nuclear localization in the dorsal region in zebrafish embryos (Fig. 3B), whereas a Smp deletion construct lacking the NLS (Smp-ΔNLS-GFP) showed a completely cytoplasmic distribution

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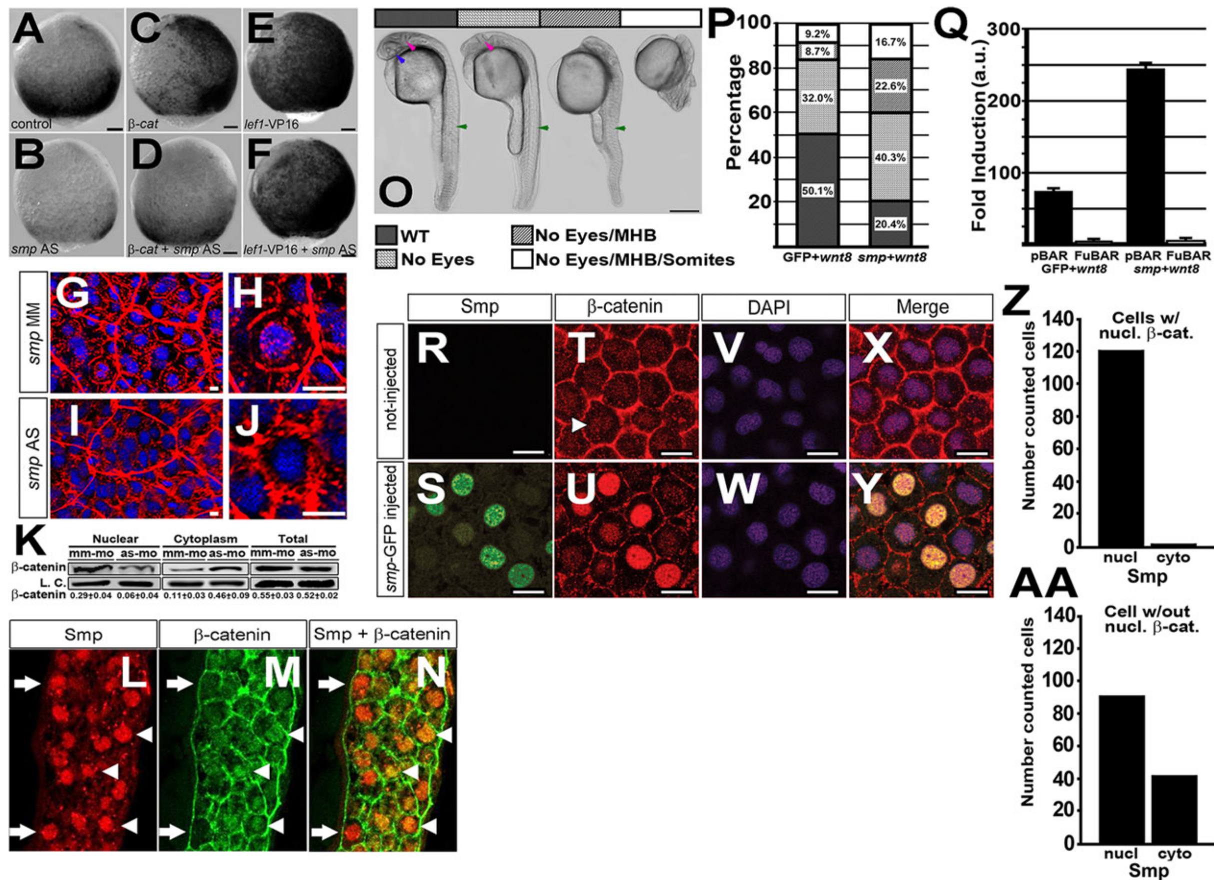

Fig. 2. Smp regulates β-catenin nuclear localization. (A) Activity of the 7xTCF:mCherry transgene at 95% epiboly after mismatch morpholino injection, (B) knockdown of *smp*, (C) overexpression of β-catenin in mismatch controls and (D) overexpression of β-catenin in *smp* morphants. (E) Activity of the 7xTCF:mCherry transgene at 95% epiboly after overexpression of *lef1*-VP16 in mismatch controls and (F) overexpression of *lef1*-VP16 in *smp* morphants. (G,H) Immunohistochemistry staining for β-catenin (red) and staining of nuclei with DAPI (blue) in mismatch control and (I,J) *smp* morphants. (K) Western blots for β-catenin in nuclear and cytoplasmic lysates from mismatch-morpholino controls (mm-mo) and *smp* morphants (as-mo). Total amount of β-catenin levels in the cells was unaltered by the *smp* knockdown. Loading controls were γ-tubulin for the cytoplasmic fraction and H2A for the nuclear fraction. (L) Immunohistochemistry staining for zebrafish Smp shows positive nuclei in the marginal zone where Wnt signaling is active. (M) Immunohistochemistry staining for β-catenin shows localization at the plasma membrane and in distinct nuclei (white arrowheads). (N) Merged stainings show colocalization of Smp and β-catenin in nuclei (white arrowheads) and cells with Smp in nuclei lacking β-catenin (white arrows). (O) Overexpression of *wnt8* during zebrafish development produces phenotype classes that affect normal development of eyes (blue arrow); the midbrain-hindbrain boundary (pink arrow); and the somites and posterior structures (green arrows). (P) Percentage occurrence of phenotypic classes produced by overexpression of *wnt8* alone or overexpression of *wnt8* with *smp*. (Q) Results of luciferase assays of either the pBAR reporter (β-catenin binding sites) or the pFuBAR reporter (mutated β-catenin sites) for Wnt activity in HEK293T cells. (R) Uninjected controls. (S) GFP localization in the nuclei of embryos injected with mRNA encoding Smp-GFP. (T) β-Catenin localization in the dorsal region of control embryos. (U) β-Catenin localization in Smp-GFP-injected embryos. (V,W) DAPI staining labels nuclei. (X,Y) Merged fluorescence for β-catenin, GFP and DAPI. (Z) The number of β-catenin-positive cells with Smp in the nucleus or in the cytoplasm. (AA) The subcellular distribution of Smp in cells lacking β-catenin nuclear staining. Scale bars: 10 μm in A-G,I,L-N; 1 μm in H,J; 300 μm in O; 10 μm in R-Y. All experiments were performed at least three times. Data represent the mean; error bars indicate s.d.

(Fig. 3C). To assess whether β-catenin nuclear localization is perturbed by the cytoplasmic localization of Smp, we compared the subcellular distribution of endogenous β-catenin in the presence either of Smp-FL-GFP or of Smp-ΔNLS-GFP. Compared with β-catenin nuclear localization in Smp-FL-GFP-expressing cells (Fig. 3D,F), the Smp-ΔNLS-GFP-positive cells showed a lack of nuclear β-catenin (Fig. 3E,G). These results indicate that Smp nuclear localization is required for the nuclear accumulation of both Smp and β-catenin.

We next tested whether the Smp-ΔNLS can act as a dominant-negative that interferes with the activation of the Tg(7xTCF:mCherry) reporter and found that, compared with overexpression of GFP (Fig. 3H) or of Smp-FL-GFP (Fig. 3I), overexpression of Smp-ΔNLS-GFP significantly reduced the activation of the reporter (Fig. 3J). Furthermore, we observed posterior truncations similar to those in the *smp* morphants and reminiscent of Wnt loss-of-function phenotypes for Smp-ΔNLS-GFP injected embryos (Fig. 3M,P) when compared with the overexpression of GFP (Fig. 3K,N) and Smp-FL-GFP (Fig. 3L,O). We also tested whether Smp-ΔNLS-GFP is able to antagonize enhanced Wnt signaling. Compared with GFP-expressing controls (Fig. 3Q,Q′), transgenic embryos expressing Wnt8-GFP displayed loss of anterior structures (Fig. 3R,R′). However, injection of Smp-ΔNLS-GFP rescued the Wnt8-induced phenotypes (Fig. 3S,S′), and the extent of rescue was directly associated with the amount of injected Smp-ΔNLS mRNA (Fig. 3T). We also assessed the effects of Smp-ΔNLS on the transcriptional activity of β-catenin. Injection of a stabilized β-catenin (Fig. 3V; supplementary material Fig. S13) or β-catenin with full-length Smp (Fig. 3W; supplementary material Fig. S13) showed increased reporter activity in Tg(7xTCF:mCherry) embryos compared with controls (Fig. 3U; supplementary material

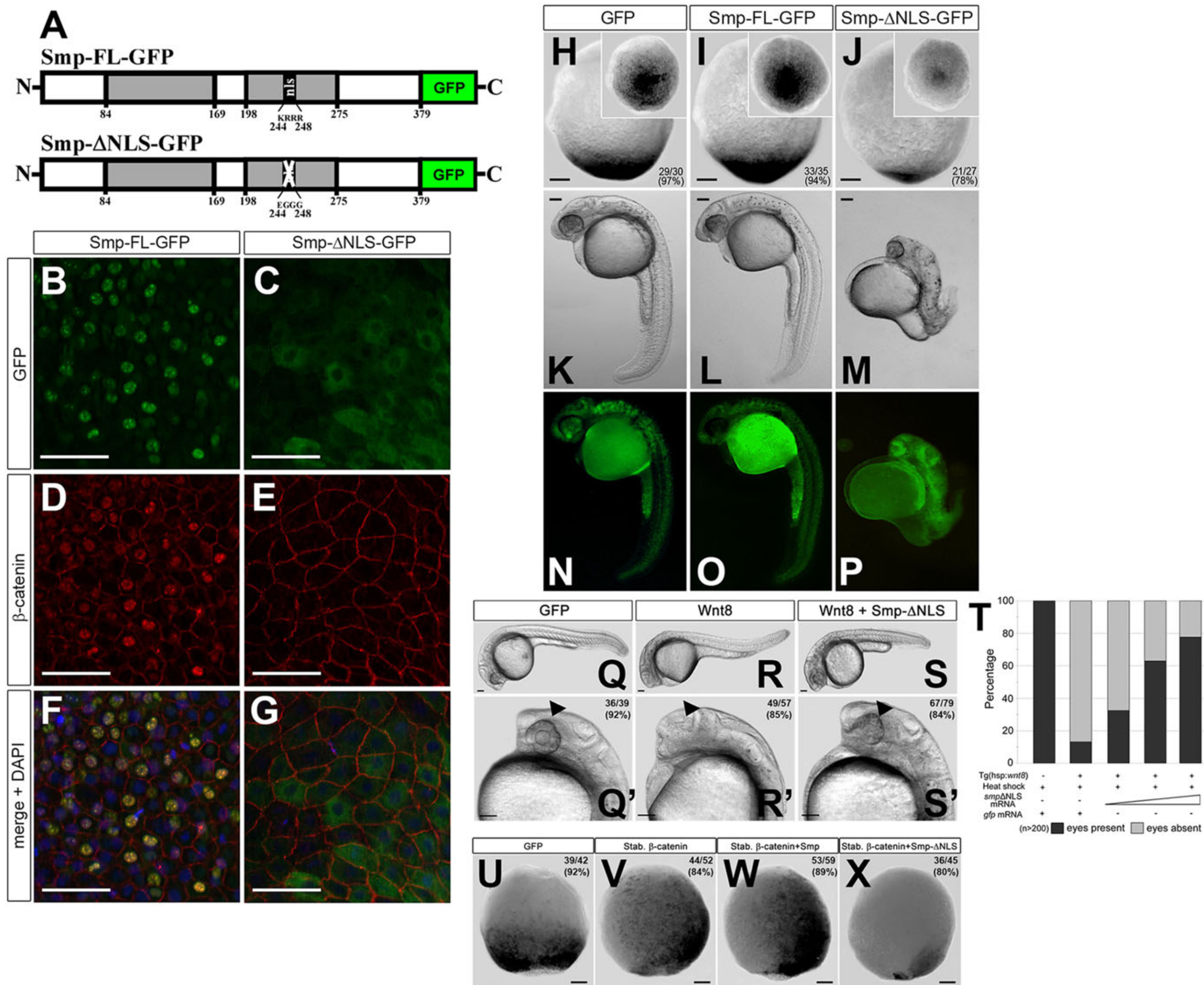

Fig. 3. Nuclear localization of β-catenin requires the Smp nuclear localization signal. (A) The domain structure of Smp. The two grey boxes indicate regions of significant conservation among vertebrates. The numbers indicate the position of the amino acids. 'nls' identifies the nuclear localization signal KRRR. Substitution of these amino acids with EGGG ablates the nuclear localization signal (white cross). (B) Overexpression of Smp-GFP in fish embryos. (C) Deletion of the nuclear localization signal in Smp-GFP. (D) Co-staining for β-catenin in Smp-GFP-injected embryo. (E) Co-staining for β-catenin in Smp-ΔNLS-GFP-injected embryo. (F) Overlay of Smp-GFP and β-catenin immunostainings in Smp-GFP-injected embryo. (G) Overlay of Smp-GFP and β-catenin immunostaining in Smp-ΔNLS-GFP-injected embryo. (H-J) Overexpression of GFP (H), Smp-FL (I) or Smp-ΔNLS (J) in the Tg(7xTCF:mCherry) embryos at tailbud stage. (K) Overexpression of GFP in a 24 hpf embryos. (L) Overexpression of Smp-GFP in 24 hpf embryo. (M) Overexpression of Smp-ΔNLS-GFP in 24 dpf embryo. (N-P) Expression of each GFP-fused construct in the injected embryos. (Q,Q′) Control-injected transgenic embryos at 24 hpf. (R,R′) Transgenic overexpression of *wnt8*. (S,S′) Injection of *smp*-ΔNLS into embryos transgenically overexpressing *wnt8*. Arrowheads indicate the expected location of the developing eye. (T) Graph shows a direct correlation between the rescue from the wnt8-overexpression posteriorization phenotype and the amount of *smp*-ΔNLS mRNA injected. Data are the average percent. (U) Control-injected Tg(7xTCF:mCherry) embryos. (V) Injection of mRNA encoding stabilized β-catenin. (W) Co-injection of mRNAs for stabilized β-catenin and Smp-FL. (X) Overexpression of Smp-ΔNLS with stabilized β-catenin. Numbers in the lower or upper right panel corners represent number of embryos with the observed phenotype/the total number of embryos (also represented as a percentage). Scale bars: 50 µm in B-G; 100 µm in H-X. Numbers in the lower or upper right corners indicate the number of embryos with the depicted expression patterns/the total number of embryos.

Fig. S13). By contrast, co-injection of the mutant Smp-ΔNLS-GFP inhibited the activation of the Wnt reporter (Fig. 3X; supplementary material Fig. S13). Thus, retention of Smp in the cytoplasm inhibits β-catenin signaling, indicating that Smp nuclear localization is essential for nuclear localization and for the transcriptional activity of β-catenin.

The regulation of β-catenin-dependent Wnt signaling by Smp is conserved in mammals

The mammalian ortholog of Smp is family with sequence similarity 53-member B (Fam53b/Smp). The existence of Smp in several vertebrate species suggests that its function is conserved in all vertebrates. Therefore, we assessed whether Smp is present in the mouse intestinal crypts where Wnt signaling is active. Immunohistochemistry for Smp showed staining in the intestinal crypts (supplementary material Fig. S14A) overlapping with Olfm4-postive stem cells (supplementary material Fig. S14B) (van der Flier et al., 2009), where β-catenin-dependent signaling is important (van Es et al., 2012). Immunocytochemistry for human SMP in HEK293T cells showed foci in the nucleus (Fig. 4A,B), indicating that human SMP accumulates and functions in the nucleus. To determine whether human SMP is required for

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β-catenin nuclear localization in human cells, we performed siRNA knockdown of Smp in HEK293T cells (supplementary material Fig. S15A-K) and examined the subcellular distribution of SMP and β-catenin. Compared with unstimulated controls (Fig. 4C,D), stimulation with Wnt3-conditioned medium increased nuclear localization of endogenous β-catenin (Fig. 4E), which was reduced after transfection with *smp* siRNA (Fig. 4F). Subcellular fractionation experiments for β-catenin localization showed significant reduction in nuclear β-catenin upon *smp* knockdown (Fig. 4G; supplementary material Fig. S15L). Likewise, compared with Wnt3-stimulated pBAR activation in control transfected cells, there was a significant reduction in β-catenin-mediated activation of pBAR reporter in Smp siRNA-transfected cells (Fig. 4H). These results support the conclusion from the *in vivo* experiments that Smp is required for β-catenin nuclear localization and transcriptional activity. To assess whether increasing human SMP mediates β-catenin transcriptional activity in a dose-dependent manner, as does zebrafish Smp, we overexpressed SMP with β-catenin in HEK293 cells enhanced the activity of the β-catenin-dependent pBAR reporter in a dose-dependent manner (Fig. 4I), as observed in zebrafish cells (supplementary material Fig. S11C).

The ability of SMP to regulate nuclear localization and transcriptional activity of β-catenin suggests that these two proteins interact. Co-expression and immunoprecipitation of the FLAG-tagged β-catenin with SMP-myc from HEK293T cell lysates showed an interaction between the two proteins (Fig. 4J), suggesting that proteins interact either directly or indirectly as part of a larger protein complex. Immunoprecipitation experiments with different deletion constructs showed that the first homology domain in Smp is required for their interaction (Fig. 4K; supplementary material Fig. S15M). To determine which region in β-catenin is required for its interaction with SMP, we performed co-immunoprecipitation experiments with several β-catenin deletion mutants lacking different stretches of the armadillo repeats or the N terminus preceding the repeats or the C terminus. We observed that the N terminus of β-catenin is required for interaction with SMP (Fig. 4L; supplementary material Fig. S15N,O).

Although co-transfection of full-length Smp enhanced β-catenin activation of the pBAR reporter, the SMP mutant lacking this β-catenin interaction domain (SMPΔHRI) showed a significant reduction in reporter activity after transfection with full-length β-catenin (supplementary material Fig. S15T) or with a stabilized mutant version of β-catenin (Fig. 4M) despite its localization to the nucleus (Fig. 4N). When we injected *smp*ΔHRI mRNA into early embryos, we did not observe any outstanding phenotypes, even after injection of mRNA (supplementary material Fig. S15R,S), unlike injection of *smp*-FL mRNA (supplementary material Fig. S15P,Q,S). We believe that lack of a phenotype from *smp*ΔHRI is due to the inability of the mutant protein to compete with the endogenous protein. We then assessed whether the interaction occurs in the cytoplasm or the nucleus and observed from co-immunoprecipitation experiments that a SMP-β-catenin complex existed primarily in the nucleus with a reduced interaction in the cytoplasm (Fig. 4O), indicating that they are part of a molecular complex that exists primarily in the nucleus.

The regulation of β-catenin activity involves its bidirectional nuclear translocation, and the regulation of its nuclear-cytoplasmic shuttling determines the amount of β-catenin available for transcription (Valenta et al., 2012). To assess whether SMP can alter β-catenin subcellular distribution, we performed fluorescence recovery after photobleaching (FRAP) experiments by bleaching mCherry-tagged β-catenin expressed in HEK293 cells in the presence of GFP or SMP-GFP. The measured decrease in the nuclear fraction of β-catenin in GFP-expressing cells after photobleaching mCherry in the cytoplasm indicates how much β-catenin has mobilized out of the nucleus (Fig. 4P, green nuclear curve). This reduction in nuclear β-catenin in cells transfected with control GFP is associated with an increase in β-catenin in the photobleached cytoplasm (Fig. 4P, green cyto curve). By comparison, photobleaching the cytoplasm of Smp-GFP-expressing cells showed less of a reduction in nuclear β-catenin (Fig. 4P, red nuclear curve) that was accompanied by a reduced cytoplasmic recovery (Fig. 4P, red cyto curve). Conversely, when we photobleach the nucleus, we do not observe statistically significant differences in the recovery of β-catenin into the nucleus between GFP-control transfected and SMP-GFP transfected cells (supplementary material Fig. S15U, black bracket), indicating that SMP is not facilitating β-catenin nuclear import.

DISCUSSION

We identify Smp as a novel regulator of the β-catenin-dependent Wnt signal transduction pathway, because it is required for this pathway, and because it is sufficient to enhance β-catenin nuclear localization. *smp* expression and its subcellular distribution do not appear to be regulated by Wnt signaling, and it is not sufficient to promote β-catenin transcriptional activity without Wnt stimulation, indicating that Smp acts on the pathway after Wnt-mediated accumulation of β-catenin.

Previous work showed that Smp is required for proliferation of the early embryonic cells (Thermes et al., 2006). Although Smp shares stretches of identical amino acid sequence with Fam53A (31% identical) and Fam53C (33% identical), these proteins have not been characterized, so it is unclear what the biological functions of these proteins are and whether they have overlapping functions with Smp or with each other. Our observations that the first homology domain in Smp is required for its interaction with β-catenin and that this domain has conserved stretches of identical amino acid sequences in Fam53A and Fam53C, suggests that they too may regulate the nuclear localization of β-catenin.

Like Smp, Wnt signaling is also required for cell proliferation (Niehrs and Acebron, 2012), but our evidence that inhibiting cell proliferation does not affect β-catenin-dependent transcription (supplementary material Fig. S6) indicates that the regulation of cell proliferation by Smp is either independent of its role in β-catenin nuclear localization or is mediated by β-catenin-dependent signaling. Yeast two-hybrid screens and immunoprecipitation experiments indicate that Smp has other partners in addition to β-catenin: the ski-interacting protein (Skiip) and 14-3-3 (Thermes et al., 2006). The oncogene Ski is involved in cell proliferation, cell differentiation, transformation and tumor progression (Bonnon and Atanasoski, 2012), and one function of this protein is to regulate Tgf signaling by adjusting the downstream activity of Smad (Bonnon and Atanasoski, 2012). In addition to interacting with Ski, Skiip interacts with Epstein Barr virus, NotchIC, Myc and menin of the histone methyltransferase Mll1 (Zhou et al., 2000a,b; Bres et al., 2009). The interaction between Smp and Skiip suggests that Smp may be involved in the activities of these other proteins, but this remains to be determined. Smp has several 14-3-3 binding sites (Thermes et al., 2006), and we confirmed that 14-3-3 isoforms do immunoprecipitate with Smp, suggesting that they may be involved in regulating the interaction of Smp with β-catenin. Co-immunoprecipitation experiments that we performed showed that β-catenin can be pulled down with Smp together with 14-3-3 or independently of it (data not shown), indicating that 14-3-3 presence or absence does not have a role in regulating β-catenin interaction with Smp.

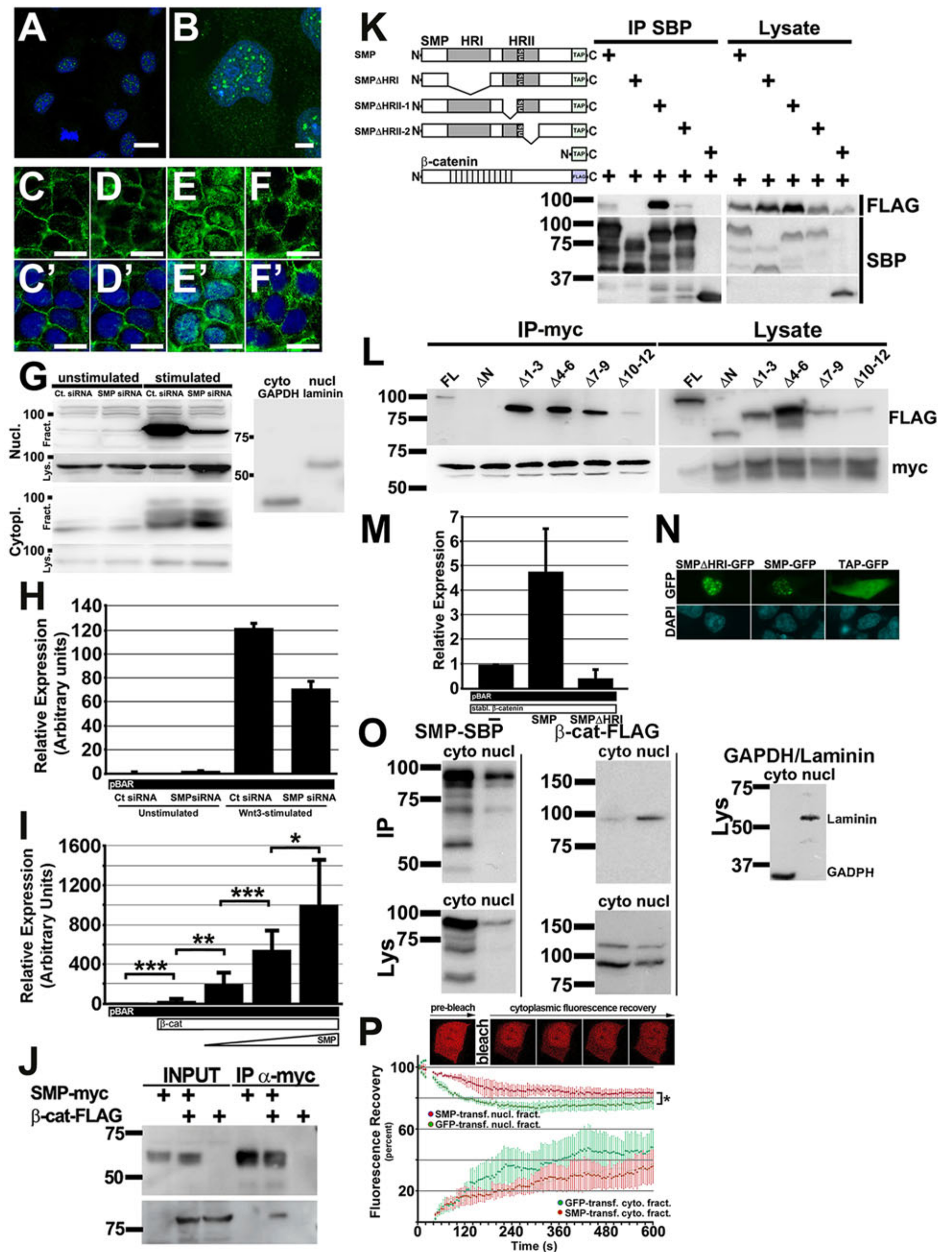

Fig. 4. Mammalian SMP (FAM53B) regulates β-catenin similarly to zebrafish Smp and interacts with β-catenin to retain it in the nucleus. (A) Immunocytochemistry (ICC) for the human SMP (green) in the Hoechst-stained nuclei (blue) of HEK293T cells. (B) Higher magnification shows the protein localizes as foci in Hoechst-stained nuclei. (C-F) β-Catenin ICC (green) of control siRNA-transfected unstimulated HEK293T cells (C), SMP siRNA-transfected HEK293T cells (D). control siRNA-transfected HEK293T cells stimulated with Wnt3-conditioned medium (E) and SMP siRNA-transfected HEK293T cells stimulated with Wnt3-conditioned medium (F). (C′-F′) β-Catenin ICC merged with DAPI (blue). (G) Representative subcellular fractionation blots showing the protein levels of nuclear (Nucl.) and cytoplasmic (Cytopl.) β-Catenin in unstimulated and Wnt3-stimulated HEK293T cells transfected either with control (Ctr) siRNA or Smp siRNA. Fract. denotes nuclear or cytoplasmic fractionation blots. Lys. denotes blots for loading control of total lysates for each fraction. Laminin and GAPDH show clear separation of the fractions. The numbers indicate protein ladder positions in kDa. (H) Fold activation of pBAR luciferase reporter from assays of control and Smp siRNA knockdown HEK293T cells without and with Wnt3-condition medium stimulation (Wnt). P<0.01 between Ct and SMP siRNA Wnt3-stimulated groups. Data are the average±s.d. (I) Dose-dependent activation of β-catenin-responsive promoter by human SMP. *P<0.05, **P<0.001, ***P<0.008. Data are the average±s.d. (J) Immunoprecipitation blot using anti-Myc antibody to pull down Myc-tagged human SMP shows co-immunoprecipitation of Flag-tagged human β-catenin as detected by anti-Flag antibody. (K) Immunoprecipitation experiment using different TAP-Tagged (TAP) SMP deletion mutants to determine which conserved domain interacts with FLAG-tagged β-catenin. (L) Immunoprecipitation experiment of SMP-myc for different FLAG-tagged β-catenin deletion mutants lacking either the N terminus or different sets of armadillo repeats. The lysate gels show the level of expression of each construct. (M) Luciferase assay of HEK293T cells transfected with: pBAR and stabilized β-catenin; pBAR, stabilized β-catenin and full-length SMP; or pBAR, stabilized β-catenin and SMP lacking the first homology domain (SMP-ΔHRI). P<0.01 between all groups. Data are the average±s.d. (N) Transfection of HEK293T cells with TAP-tagged GFP or SMP-GFP or SMP-ΔHRI-GFP shows nuclear localization of both SMP expression constructs. (O) Blot of subcellular fractionation (nuclear versus cytoplasmic) experiment for transfected TAP-tagged Smp and FLAG-tagged β-catenin. Immunoprecipitation blots (IP) are above. Lysate blots (Lys) are below. Lysate blot probed with GAPDH and laminin shows clear separation of each fraction. (P) β-Catenin-mCherry fluorescence before bleaching and at increasing times after photobleaching the cytoplasm of HEK293T cells co-transfected either with GFP or SMP-GFP. The graph shows the decrease in nuclear β-catenin-mCherry and its cytoplasmic recovery. *P<0.05. Scale bars: 20 µm in A,B; 5 µm in C-F′. Data represent at least three or more independent experiments.

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Several phenomena are necessary for the nuclear localization of β-catenin: stabilization of β-catenin in the cytoplasm, and its transport to the nucleus and its subsequent retention there (MacDonald et al., 2009). Previous work has shown that the nuclear export of β-catenin is promoted by the Ran-binding protein 3 (Ranbp3) (Hendriksen et al., 2005), which has been shown to interact with Crm1 and Ran-GTP, and consequently to promote nuclear export of proteins with lysine-rich nuclear export sequences (Nemergut et al., 2002). However, promotion of β-catenin nuclear export by Ranbp3 appears to be independent of Crm1 (Hendriksen et al., 2005). It has also been shown that β-catenin can enter the nucleus independent of Ran GTPase and importin-mediated mechanisms (Fagotto et al., 1998; Yokoya et al., 1999; Eleftheriou et al., 2001; Wieschens and Fagotto, 2001), and it does so using intrinsic nuclear import and export information within specific armadillo repeats (Asally and Yoneda, 2005; Sharma et al., 2012). By contrast, the familial adenomatous polyposis and colon cancer (*Apc*) gene, the nuclear import and export of which involves importin and exportin-mediated transport, has been shown to regulate both nuclear and cytoplasmic shuttling of β-catenin (Henderson, 2000). Although the requirement for Smp for β-catenin nuclear localization could involve either a shuttling or a retention mechanism, the difference in the steady state plateau values after photobleaching the cytoplasm indicate that Smp affects the mobility of β-catenin from the nucleus, and the lack of a significant difference after bleaching the nucleus argues that Smp has less effect on the movement of β-catenin from the cytoplasm into the nucleus. We do not know whether Smp is involved in transporting β-catenin through the nuclear pore or whether it promotes nuclear β-catenin by sequestration in a chromatin or transcription factor complex, as happens with Foxm1 (Zhang et al., 2011).

Other proteins have been shown to regulate β-catenin activity by perturbing its interaction with its transcription partner Lef1/Tcf (e.g. Drapper and Chibby), by shuttling it out of the nucleus (e.g. Drapper, Chibby, Apc) and by promoting its degradation (e.g. Apc) (Ahmed et al., 1998; Gao et al., 2008; Li et al., 2008, 2010). These proteins negatively regulate β-catenin nuclear distribution, while Smp positively regulates it, so it is unlikely that Smp promotes β-catenin nuclear localization through the interaction with these proteins. Both Smp and β-catenin can interact with 14-3-3 proteins (Tian et al., 2004; Thermes et al., 2006); however, we have not observed simultaneous interaction of the three proteins. The interaction between Smp and β-catenin requires the N-terminal sequences of β-catenin, but most known regulators of β-catenin function interact within specific armadillo repeat sequences within β-catenin, so it remains unclear whether Smp regulates nuclear localization of β-catenin by impacting the function of any of the known β-catenin regulators. Future work on defining the co-factors that function together with Smp will provide insight into how Smp promotes β-catenin nuclear localization.

Immunocytochemistry experiments show that endogenous Smp can localize broadly or at foci in the nucleus, and that its nuclear localization overlaps with β-catenin. It is not yet clear whether the location of the foci in the nucleus is arbitrary or targeted to specific sites. Although the protein has conserved domains, there are no similarities to protein domains whose function is known, so the activities of these domains still need to be characterized. Other than the requirement for the first domain to maintain β-catenin in the nucleus, it is not yet clear whether Smp serves simply to keep β-catenin in the nucleus or to promote the localization of β-catenin to specific sites.

The ability of Smp to regulate β-catenin-dependent signaling in the embryo and its presence in the crypts of the intestine support the conclusion that Smp is involved in regulation of β-catenin-dependent Wnt signaling of stem and progenitor cells. Our observation that *smp* mRNA and protein do not change their expression levels or subcellular distribution after modulating Wnt signaling (Fig. 2L-N,Z,AA; supplementary material Fig. S12) indicates that Smp expression and nuclear localization is independent of β-catenin-dependent Wnt signaling. We postulate that Smp is a regulatory node either as an adapter or as a protein with an additional function through which other signal transduction pathway regulate/influence the subcellular distribution of β-catenin. Whether Smp is required in all Wnt signaling contexts or whether there are other factors that perform a similar activity needs to be determined. The broad distribution of the *smp* transcript and the protein (supplementary material Fig. S1) in the progenitor cells of the early embryo and its reactivation in the adult during zebrafish regeneration (Kizil et al., 2009) rather than their strict localization to regions of active β-catenin-dependent Wnt signaling argues that Smp has other functions in addition to regulating β-catenin subcellular distribution. What these functions are still needs to be determined.

In addition to embryonic development, Smp is required for tissue regeneration (Kizil et al., 2009). Based on our findings in the embryo, it is likely that the requirement for Smp during regeneration includes its regulation of Wnt signaling. To date there are no known mutations in Smp associated with disease that aid in understanding the physiological importance of the conserved domains, but there are correlations between increased Smp expression and multiple melanoma (Clevers, 2006; Agnelli et al., 2011). Future experiments will determine what molecular signals are involved in regulating Smp activity and how this regulation modifies the Wnt signal transduction cascade in embryonic and regenerative contexts.

MATERIALS AND METHODS

Fish maintenance and husbandry

Fish were maintained at 28°C (Brand et al., 2002). All procedures were carried out in accordance with the live animal handling and research regulations under protocols approved by the animal welfare committees of the Technische Universität Dresden and the Landesdirektion Sachsen. For heat-shock experiments, embryos were placed at 37°C for 1 h at 60% epiboly.

Morpholino and mRNA injections

Previously characterized morpholino oligonucleotides for *smp* (4 ng) and *p53* (3 ng) (Kizil et al., 2009; Robu et al., 2007) were injected into one-cell stage embryos with glass capillaries (World Precision Instruments, TWF10). Capped *smp* mRNA (20 pg), 20 pg *smp*ΔNLS (Kizil et al., 2009) and/or 2 pg of *wnt8* mRNA (Weidinger et al., 2005), or 2 pg of stabilized β-catenin mRNA were injected similarly.

In situ hybridization

In situ hybridization for fish was performed as described previously (Jowett and Lettice, 1994) using VSi In Situ Robot (Intavis). Probes were transcribed from linearized templates using DIG-labeled NTPs (Roche). Bright-field or DIC images were taken using AxioCam compound microscope (Zeiss). Intestines from adult mice were dissected and flushed gently with PBS prior to fixation in 10% formalin overnight. Samples were then dehydrated and embedded in paraffin. *In situ* hybridization was performed on 5 µm sections as described previously (Gregorieff and Clevers, 2005). The probe against Olfm4 was generated by linearizing pBluescript Olfm4 with *Not*I and *in vitro* transcription with T7 (Roche).

Cell-tracking experiments

Embryos were injected with either *Smp* antisense or *Smp* mismatch morpholino at the one-cell stage. At 50% epiboly, 5 pg tracker dye (CellTrackerTM Red CMTPX, Invitrogen) was injected into the region that

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forms the somitic or head mesoderm, according to the fate map of zebrafish embryo (Kimmel et al., 1990). Labeled cells were detected 10 minutes after injection with red fluorescence. The level of convergent extension was checked at tailbud stage by alignment of labeled cells at the midline and the anterior region.

Calculation of mitotic indices

Mitotic indices were calculated by counting the total number of H3P-positive cells per mm^2. Statistical analyses were performed using Excel software and *t*-test.

Apoptosis assay

Apoptotic cells were detected by TUNEL (Fluorescence In Situ Cell Death Detection Kit, Roche) and AnnexinV-Cy3 (BioVision) stainings, as instructed by the manufacturer. Images were obtained using epifluorescence microscope (Zeiss Apotome).

Hydroxyurea and aphidicolin treatment of embryos

Hydroxyurea (20 mM, Sigma) and aphidicolin (150 µM, Merck) were dissolved in E3 fish water (Brand et al., 2002). Embryos were treated starting at 4 hours post-fertilization in Petri dishes until desired developmental stage.

Immunohistochemistry

Antibody staining was performed using anti-Tbx16 (mouse, 1:100, ZIRC), anti-Dlx3b (mouse, 1:50, ZIRC), anti-Myf5 (rabbit polyclonal, recognizes MyoD, 1:50, Santa Cruz, sc-302), anti-H3p [rabbit polyclonal, 1:100, Upstate (Merck Millipore), 06-570], zebrafish anti-Smp [rabbit polyclonal; 1:600) (for generation of antibody, see the methods in the supplementary material), human anti-SMP (rabbit polyclonal, 1:400, Sigma, SAB1303084) and anti-β-catenin (rabbit polyclonal, 1:200, NEB, #9562) (Kizil et al., 2009). Goat anti-mouse Cy3 (1:500, Dianova, 115-165-146), goat anti-rabbit Alexa-488 (1:200, Molecular Probes, Invitrogen, 111-545-144) secondary antibodies were used. Images were taken with fluorescence ApoTome microscope (Zeiss). For mouse intestines, 7 µm sections were deparaffinized and rehydrated. Antigen retrieval was performed by boiling samples for 20 min in EDTA buffer [1 mM EDTA, 0.05% Tween 20 (pH 8)] and then cooled to room temperature. Endogenous peroxidase was blocked by incubation 15 min in 0.9% H_2O_2. Sections were blocked with 10% goat serum (Vector Laboratories) in 1× PBS for 30 min. The SMP antibody (Sigma) was diluted in 2% BSA in PBS and the HRP anti-rabbit secondary antibody (GE Healthcare) in 10% NGS. Incubation of primary antibody was performed overnight at 4°C and secondary antibody for 1 h at room temperature. The staining was developed using Sigma*FAST* 3,3′-diaminobenzidine tablets following the manufacturer's instructions. Sections were mounted in 70% glycerol for imaging.

Transgenic lines

Transgenic Wnt reporter line Tg(7xTCF-XLa.Siam:nlsmCherry)^{ia5} (Kwan et al., 2007; Moro et al., 2012). Other transgenic lines were Tg(Top:dGFP) (Dorsky et al., 2002), Tg(*hsp70*:*dkk1*-GFP) and Tg(*hsp70*:*wnt8a*-GFP) (Stoick-Cooper et al., 2007), and Tg(*hsp70*:GFP) (Halloran et al., 2000).

Luciferase assays

smp mRNA was generated from the clone IRATp970D074D (ImaGenes). *smp* was subcloned into the pcDNA3 myc-His expression vector (Invitrogen) via *Bam*HI sites. HEK293 cells grown at 37°C in 10% FCS serum (Biochrom AG) in DMEM (Gibco) were transfected with *smp* expression plasmid and with other plasmids containing *wnt8*, *dsh*, β-catenin, and the Renilla and Luciferase reporters (Weidinger et al., 2005) using FuGene 6 (Roche). Firefly and Renilla activities were measured from cell lysates using the Dual-Luciferase Reporter Assay System (Promega) 24 h after transfection.

Immunoprecipitation experiments

HEK293T cells were transfected with pCS2-FLAG-tagged β-catenin, pcDNA6-SMP-Myc or pcDNA6-SMP-SBP (Streptavidin Binding Protein) as designated using the Fugene 6 reagent (Roche) according to manufacturer's instructions with a DNA:Fugene ratio of 1:2. Forty-eight hours after transfection, cells were trypsinized and washed subsequently with PBS and cell buffer [10% glycerol; 75 mM Hepes, KOH 7.4, 150 mM KCl, 2 mM $MgCl_2$, 2 mM EDTA]. Cells were lysed in cell buffer supplemented with 0.1% NP40 and 1× protease and phosphatase Inhibitor cocktails (Roche). Co-immunoprecipitations were performed with the MultiMACS Epitope tag isolation kit (Miltenji Biotec). Cell lysates were centrifuged for 10 min at 16,000 ***g*** at 4°C. An aliquot (50 µl) of the supernatant was boiled with 2× SDS-SB as input. The residual 200 µl were transferred into a new tube and incubated with 50 µl µMACS anti-c-myc MicroBeads (Miltenji Biotec) or 50 µl anti-streptavidin beads (Pierce) for 30 min on ice. Labeled proteins were loaded onto with lysis buffer preconditioned µ Columns (Miltenji Biotec) or centrifuged for 10 min at 16,000 ***g***, and washed with 1 ml lysis buffer and subsequently 100 µl wash buffer 2 (Miltenji Biotec). Elution of proteins was achieved with preheated Elution buffer (Miltenji Biotec).

Nuclear fractionation and western blotting

Nuclei from tailbud embryos were isolated using a glass-glass homogenizer and differential centrifugation ((German and Howe, 2009). Nuclei were lysed [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 2% SDS, 1× proteinase inhibitor cocktail (Roche)] and the lysate was separated by centrifugation (16,000 ***g***, 4°C, 10 min). Samples were loaded to 10% SDS-PAGE gels. Proteins were transferred to a 0.45 µm sieved PVDF membrane (Roth) by electroblotting. Rabbit anti-β-catenin (1:2000, NEB), rabbit anti-FLAG (1:500, Sigma), mouse anti-myc (1:400, Upstate), anti-streptavidin-binding protein (SBP) (1:20,000, Millipore), ECL Plex goat anti-mouse HRP (1:4000, GE Life Sciences) and donkey anti-rabbit HRP (1:4000, GE Life Sciences) were used. Membrane was washed with hybridization buffer [25 mM Tris, 192 mM glycine (pH 8.3)] for 1 h and signal was detected using chemiluminescence kit (ECL Western Blotting Detection Kit, GE Life Sciences). As loading controls, γ-tubulin (1:2000, NEB) and GFP (H2A-GFP transgenic line, 1:2000, Millipore) were used. Band intensities are calculated using Fiji software (http://pacific.mpi-cbg.de/wiki/index.php/Main_Page).

siRNA knockdown experiments

HEK293T cells were incubated for 24 h in reduced serum conditions (1% FBS) and then transfected with Stealth siRNA (Invitrogen) using RNAi MAX lipofectamine (Invitrogen). After 48 h, cells were transfected with plasmid reporter and expression constructs and incubated for 24 h before treating with Wnt3-conditioned medium. Cells were lysed after 8 h and Firefly and Renilla activities were measured from cell lysates using the Dual-Luciferase Reporter Assay System (Promega).

Photobleaching experiments

FRAP analysis was performed with HEK293T cells 24 h post transfection (300 ng/well pCS2+ β-catenin-mCherry and 30 ng/well pCS2+ FAM53B-GFP or equimolar amounts of peGFP) in a Lab-TekII eight-well chamber (ThermoScientific) at 37°C in DMEM (Gibco). Nuclei were counterstained with 1.7 ng/ml Hoechst 33342 before imaging. Photobleaching was performed on a confocal microscope Zeiss LSM780 with an attached ConfoCor3-detection module using Zen 2010 software. EGFP was excited by the 488 nm (Argon laser), Hoechst 33342 by a 405 nm laser diode and β-catenin-mCherry by a 561 DPSS laser. Fluorescence imaging was sequential using three channels: EGFP, 489-559 nm on the GaAsP-detector; Hoechst 33342, a BP 420-475 band using a APD detector of the ConfoCor3 module; mCherry, a LP580 band with a APD detector of the ConfoCor3 module. Imaging used low laser power for all channels (EGFP <1%, 0.263 µW; Hoechst 33342, 0.2%, 14 µW; mCherry, <0.6%, 0.447 µW). Bleaching was with high laser power (100%, 197 µW) and enhanced bleaching using the 488 nm and 514 nm lines of the Argon laser (100%, 129 µW and 69.9 µW, respectively). The cell compartments were bleached with 16 separate point bleaches at a low scanning speed for a period of 15.49 s. Bleaching effects were minimized by using low laser power and fast imaging speed (1.94 s). The fluorescence in the cytoplasm was bleached by 60-70% of the initial images and was taken as a reference. After the bleaching, images were taken every 5 s for 14 min. Average intensities in

regions of interest were measured with ImageJ. The ratio of the average fluorescence in the bleached area (nucleus or cytoplasm) over the unbleached compartment (cytoplasm or nucleus) was plotted over time. The ratio in the pre-bleach image was set to 100% and the first post-bleach image was set to 0%. The recovery curves are averages of four experiments with 15/16 cells (FAM53B/GFP) and three experiments with 11/12 cells (GFP/FAM53B) for cytoplasmic bleaches and nuclear bleaches, respectively.

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Competing interests
The authors declare no competing financial interests.

Author contributions
All authors contributed to the design of the research. Expression analyses were performed by C.K., B.K. and J.-J.Y. The morpholino knockdown experiments were performed by C.K. and J.-J.Y. The subcellular distribution studies were performed by C.K., C.L.A. and J.-J.Y. The transcriptional assays were performed by B.K., C.L.A. and G.Ö. The *in vivo* overexpression studies were performed by B.K. and C.L.A. The immunohistochemistry experiments were performed by B.K. and J.-J.Y. The FRAP experiments were performed by B.K. The siRNA knockdown experiments were performed by J.-J.Y., C.L.A. and C.K. F.A. and E.M. produced the transgenic Wnt reporter line. G.W. supervised transcriptional assays, suggested experiments and contributed to the writing of the manuscript.

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Supplementary material
Supplementary material available online at
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