Cell

The Fused/Smurf Complex Controls the Fate of *Drosophila* Germline Stem Cells by Generating a Gradient BMP Response

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DOI 10.1016/j.cell.2010.11.022

SUMMARY

In the *Drosophila* ovary, germline stem cells (GSCs) are maintained primarily by bone morphogenetic protein (BMP) ligands produced by the stromal cells of the niche. This signaling represses GSC differentiation by blocking the transcription of the differentiation factor Bam. Remarkably, *bam* transcription begins only one cell diameter away from the GSC in the daughter cystoblasts (CBs). How this steep gradient of response to BMP signaling is formed has been unclear. Here, we show that Fused (Fu), a serine/threonine kinase that regulates Hedgehog, functions in concert with the E3 ligase Smurf to regulate ubiquitination and proteolysis of the BMP receptor Thickveins in CBs. This regulation generates a steep gradient of BMP activity between GSCs and CBs, allowing for *bam* expression on CBs and concomitant differentiation. We observed similar roles for Fu during embryonic development in zebrafish and in human cell culture, implying broad conservation of this mechanism.

INTRODUCTION

In adult tissues, stem cells execute asymmetric cell divisions to self-renew and produce differentiated daughters for maintaining tissue homeostasis via interaction with their surrounding stromal cells, which form a microenvironment commonly termed as a niche (Nishikawa et al., 2008; Spradling et al., 2008). Although the signaling pathways involved in this interaction have been identified in many stem cell populations, the mechanisms to explain how stem cells and their specialized sisters differentially respond to and interpret the signals from the niche remain poorly understood.

The germline stem cells (GSCs) in the *Drosophila* ovary have provided heuristic examples for understanding the niches that maintain stem cells (Li and Xie, 2005; Ohlstein et al., 2004; Spradling et al., 2001; Yamashita et al., 2005). The asymmetric division of GSCs takes place within a niche made up of a small number of stromal cells (terminal filament, cap cells, and inner sheath cells) at the tip of the germarium (Figures 1A and 1C) to produce two daughter cells along the anterior-posterior axis of the ovary. The anterior daughter cell retains contact with the stromal cap cells and becomes a stem cell, whereas the posterior daughter cell dissociates from the cap cells but associates with inner sheath cells and becomes a cystoblast (CB), which divides four times to produce a cyst of 16 interconnected cells that can sustain oogenesis. The stromal cells form the niche by secreting signaling ligands that direct the fate of GSCs and their immediate daughter cells (King et al., 2001; Song et al., 2004).

Bone morphogenetic protein (BMP) ligands, Decapentaplegic (Dpp) and Glass bottom boat (Gbb), produced from cap cells (Song et al., 2004; Xie and Spradling, 1998), and perhaps other niche cells, maintain GSCs by suppressing GSC differentiation (Figure 1B) (Chen and McKearin, 2003a; Song et al., 2004). In GSCs, BMP signaling activates the *Drosophila* Smads, Mad (the *Drosophila* Smad1/5/8 homolog) and Medea (the *Drosophila* Smad4 homolog), that bind to both the *bag of marbles* (*bam*) transcriptional silencer element and the nuclear membrane protein Otefin, resulting in *bam* transcriptional silencing (Chen and McKearin, 2003a; Jiang et al., 2008; Song et al., 2004). Given that *bam* expression is essential for differentiation of CBs, cells with active BMP signaling cannot differentiate but remain GSCs by default. Thus, *bam* silencing is the hallmark of asymmetry in the *Drosophila* ovarian germline stem cell niche, and its range is restricted to one cell diameter at the most anterior end of the germarium (Chen and McKearin, 2003b).

How is this very steep gradient of BMP response formed? One possible explanation is that Dpp/Gbb ligands are secreted only from one point source, such as cap cells. Previous studies, however, have suggested that the Dpp ligands are present in both cap cells and inner sheath cells (Casanueva and Ferguson, 2004; Song et al., 2004), raising the likelihood that Dpp ligands are not restricted to a single source. An alternative possibility (Figure 1B) is that CBs develop a cell-autonomous mechanism

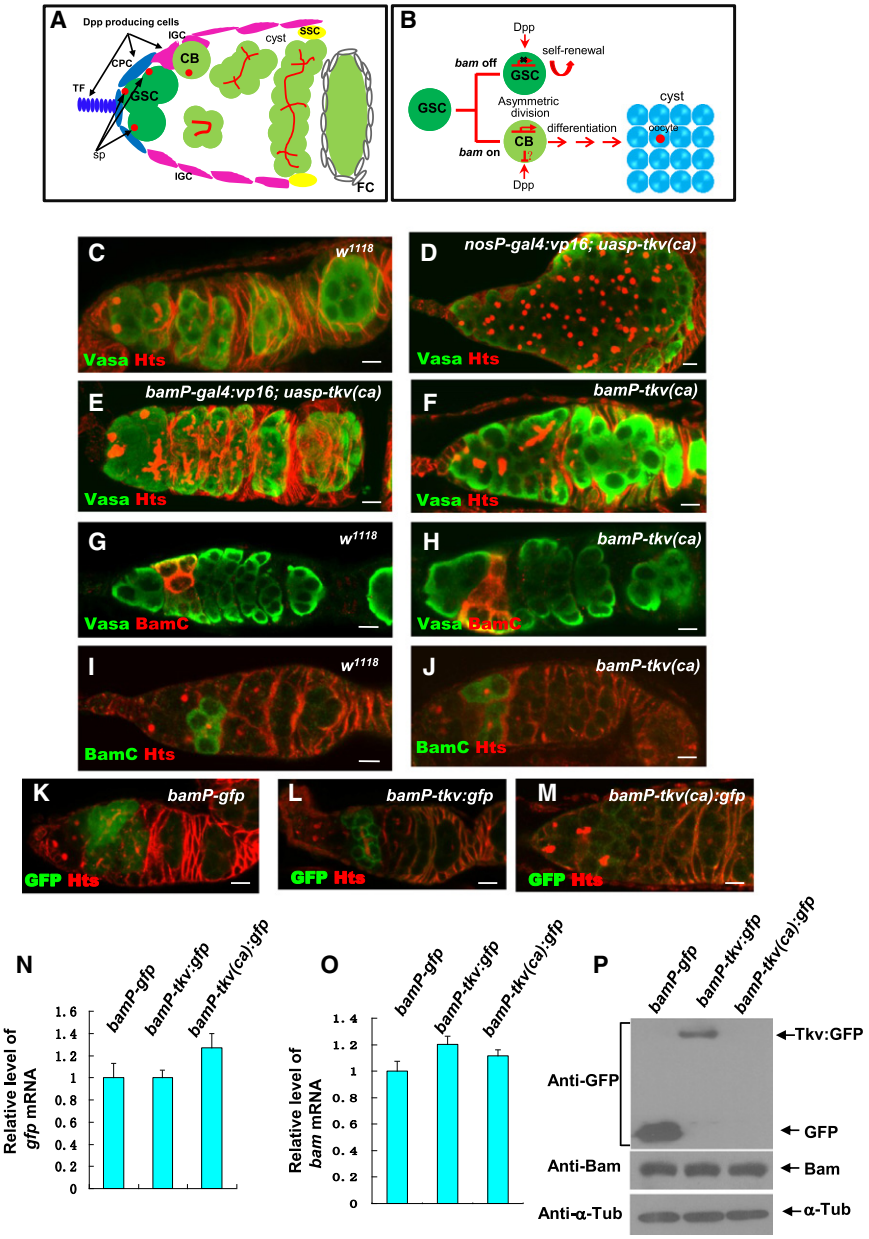

Figure 1. A Dpp Antagonist Is Required for the Proper Differentiation of CBs

(A) A schematic diagram of the germarium, with different cell types and organelles indicated as follows: terminal filament (TF), cap cells (CPC), inner germarium sheath cells (IGC), germline stem cells (GSC), cystoblast cells (CB), follicle cells (FC), somatic stem cells (SSC), cyst (differentiated germ cells with extended or branched fusomes), and spectrosome (Sp). Among these, TFs, CPCs, and IGCs produce Dpp ligands.

(B–M) Schematic diagram summarizing that *dpp* signal from CPCs silences *bam* transcription and is necessary for maintaining the self-renewal of GSCs. CBs are exposed to the Dpp signal but are *bam* active, raising the hypothesis that Dpp antagonism involves CB differentiation. Ovaries collected from wild-type w^{1118} (C), P{*nosP-gal4:vp16*}/P{*uasp-tkv(ca)*} (D), P{*bamP-gal4:vp16*}/P{*uasp-tkv(ca)*} (E), and P{*bamP-tkv(ca)*} (F) flies were stained with anti-Vasa (green) and anti-Hts (red) antibodies. Anti-Hts was used to outline the germarium and the morphology of the fusome, and the staining of anti-Vasa was used to visualize all germ cells in the germarium and egg chambers. Ovaries from wild-type w^{1118} (G) and P{*bamP-tkv(ca)*} (H) flies were stained with anti-Vasa (green) and anti-BamC (red) antibodies. Ovaries from wild-type w^{1118} (I) and P{*bamP-tkv(ca)*} (J) flies were stained with anti-BamC (green) and anti-Hts (red) antibodies. Ovaries from P{*bamP-gfp*} (K), P{*bamP-tkv:gfp*} (L), and P{*bamP-tkv(ca):gfp*} (M) were stained with anti-GFP (green) and anti-Hts (red) antibodies.

(N–P) Quantitative PCR (N and O) and Western blot (P) analysis of *gfp* and *bam* expression in P{*bamP-gfp*}, P{*bamP-tkv:gfp*}, and P{*bamP-tkv(ca):gfp*} ovaries. Scale bar, 10 μm.

The experiments were carried out by duplicates, and the standard deviations were calculated by Excel. See also Figure S1.

to antagonize BMP/Dpp activity and derepress *bam* transcription to promote their differentiation.

The transforming growth factor β (TGFβ) and BMP signals play important roles in controlling diverse normal developmental processes as well as tissue homeostasis (Feng and Derynck, 2005; Wu and Hill, 2009). Dysregulation of TGFβ/BMP signals results in numerous developmental abnormalities and has been linked to many human diseases, including cancer and degenerative diseases. Therefore the precise activity of TGFβ/BMP must be tightly controlled. TGFβ/BMP signaling has been proposed to be balanced through the regulation of Smads and/or their receptors to trigger distinct target gene expression in a spatiotemporal manner (Itoh and ten Dijke, 2007; Kitisin et al., 2007). In *Drosophila* ovary, it has been shown that BMP signaling maintains GSCs, whereas diminished signaling, such as that produced by the action of *Drosophila smurf*, promotes CB differentiation (Casanueva and Ferguson, 2004). However, the molecular mechanisms underlying the Smurf-mediated regulation of BMP in *Drosophila* germline cells remain elusive. In this study, we have identified a mechanism involving Fused (Fu), a serine/threonine kinase, which regulates Hedgehog (Hh) signaling as a core component of Hh-signaling complexes, functions in concert with Smurf to promote the proper turnover of Thickveins (Tkv), and generates a steep gradient of BMP activity between GSCs and CBs. In addition, we find that the roles of Fu in regulating the BMP/TGFβ signaling pathway are conserved in zebrafish during embryonic development and in human cell cultures.

RESULTS

CB Differentiation Involves Antagonism of BMP Signaling

To understand the mechanism underlying the formation of a steep gradient of BMP response between GSCs and differentiated CBs, we used a transgene that expressed the constitutively active Dpp receptor, Tkv(ca) (Wieser et al., 1995), to explore the sensitivity of CBs to BMP signaling. It has been shown that driving Tkv(ca) expression in pole cells, primordial germ cells, and adult germ cells with a *nanos* promoter (Van Doren et al., 1998) blocked *bam* transcription, prevented GSC differentiation, and caused germ cell hyperplasia (Casanueva and Ferguson, 2004; Figure 1D). We were surprised, however, to find that controlling expression of Tkv(ca) with a *bam* promoter (Chen and McKearin, 2003b) permitted normal germline development (Figure 1E). To exclude the possibility that transcriptional delays accounted for the failure of Tkv(ca) to block *bam* expression due to the bipartite strategy, we attempted to transcribe the Tkv(ca) transgene P{*bamP-gal4:vp16*}; P{*uasp-tkv(ca)*}. We therefore repeated the experiment with the new transgenes, P{*bamP-tkv(ca)*} or P{*bamP-tkv(ca):gfp*}, in which either *tkv(ca)* or *tkv(ca):gfp* was placed directly under the control of the *bam* promoter. These transgenes produced normal oogenesis and wild-type expression patterns of Bam and Hts proteins in ovaries (Figures 1F–1J). Whereas females carrying either the P{*bamP-tkv(ca)*} or P{*bamP-tkv(ca):gfp*} transgene were fertile, transgenic males were sterile, and their testes filled with many undifferentiated germ cells lacking Bam expression (Figure S1 available online), indicating that these transgenes were indeed active. Thus, our results suggested that, in contrast to GSCs, CBs become insensitive to BMP signaling.

Tkv(ca) Protein Is Subject to Degradation in CBs

To investigate the mechanism underlying the potential antagonism of BMP signaling in CBs, we examined Tkv(ca):GFP expression driven by the *bam* promoter at both the transcriptional and protein levels. As shown in a quantitative RT-PCR analysis, there was similar *gfp* expression in P{*bamP-gfp*}ovaries and *tkv:gfp* (a wild-type form of *tkv* tagged with *gfp*) expression in P{*bamP-tkv:gfp*} ovaries, with *tkv(ca):gfp* expression present at normal levels in P{*bamP-tkv(ca):gfp*} ovaries (Figure 1N). Consistent with this observation, no difference in the endogenous *bam* expression was detected in ovaries of these transgene flies (Figure 1O), suggesting that the *bam* promoter had normal transcriptional activity in P{*bamP-tkv(ca):gfp*} ovaries. We then performed analysis by both immunostaining and western blot to examine the expression of Tkv(ca):GFP in P{*bamP-tkv(ca):gfp*} ovaries. As shown in Figures 1K–1M and 1P, GFP and Tkv:GFP were easily detected in control ovaries from P{*bamP-gfp*} and P{*bamP-tkv:gfp*} transgene flies, respectively. However, no apparent expression of Tkv (ca):GFP was observed in P{*bamP-tkv(ca):gfp*} ovaries, revealing the existence of a mechanism that negatively regulates the activated form of Tkv at the protein level in CBs.

Identification of Fu as a Tkv-Interacting Factor

To explore how Tkv is regulated, we performed immunoprecipitation followed by mass spectrometry to search for Tkv-interacting factor(s). Mass spectrometry analysis of Flag-Tkv complexes from S2 cells, which were treated with MG132, revealed that Fused (Fu), which has been demonstrated as a positive regulator in Hh signaling, was present in the Tkv complex (Figure 2A). Reciprocal immunoprecipitation experiments showed that Fu and Tkv could be coimmunoprecipitated with each other in transfected S2 cells (Figures 2B and 2C), indicating that Fu and Tkv could form a complex together. Domain mapping of Tkv showed that the fragment lacking extracellular and transmembrane regions exhibited the strongest binding activity to Fu (Figure 2F), although all of the truncation mutants of Tkv (Figure 2D) interacted with Fu. Domain mapping of Fu showed that both the N and C terminus of Fu could associate with Tkv (Figures 2E and 2G). Further detailed domain mapping analysis revealed that the STYKc domain is essential for Tkv interaction with the N terminus of Fu (Figures S2A–S2D).

fu Is Required for CB Differentiation by Antagonizing BMP/Dpp Signaling

To test whether Fu acts in balancing BMP/Dpp signal activity by regulating Tkv to control the fate of GSCs and CBs, we examined the behavior of fu^A mutant germ cells at an early stage by measuring the number of germ cells carrying spectrosomes in ovaries using a previously described method (Cox et al., 2000). We observed that, in contrast to the wild-type control, the fu^A mutant contained multiple types of germaria, with each type carrying different numbers of the spectrosome-containing germ cells. Approximately 10% of germaria (n = 113) contained a normal number of the spectrosome-containing germ cells per germarium (Figure 2H), nearly 60% of germaria (n = 113) contained 5–10 GSC-like cells, and 30% of germaria (n = 113) were tumorous (Figures 2H–2J and 2L), suggesting that loss of *fu* blocks or delays GSC/CB differentiation. Because the defects of GSC/CB differentiation associated with the *fu* mutant can be rescued by the transgene P{*fuP-fu*} (Figures 2K and L), we concluded that *fu* is required for the proper differentiation of GSCs/CBs.

To determine whether *fu* has a cell-autonomous role in promoting germ cell differentiation, we specifically knocked down *fu* in CBs by constructing P{*uasp-shmiR-fu*}; P{*bamP-gal4:vp16*} flies according to a method described previously (Haley et al., 2008). As shown in Figures S3A–S3E, knockdown of *fu* by the *bam* promoter increased the number of GSC-like cells to nearly seven per germarium (n = 72) (Figure S3B). Similarly, in P{*uasp-shmiR-fu*}; P{*nosP-gal4:vp16*} ovaries, ~90% of germaria (n = 111) contained 5–10 GSC-like cells (Figure S3C), and nearly 5% of germaria were tumorous (Figure S3C′). Thus, *fu* has a cell-autonomous role in promoting germ cell differentiation.

We then asked whether the kinase activity was essential for the function of Fu in germ cells by generating a transgene line, P{*fuP-fuKD*}, which expresses a kinase dead form of Fu, FuKD, by the *fu* promoter. As shown in Figures S3F and S3G, in contrast to P{*fuP-fu*}, P{*fuP-fuKD*} completely failed to rescue germ cell defects in *fu* mutant, revealing that *fu* acts in a kinase-dependent manner for germ cell differentiation.

Previous studies have shown that CB differentiation was controlled by either the *bam*-dependent or *bam*-independent pathway (Chen and McKearin, 2005; Szakmary et al., 2005).

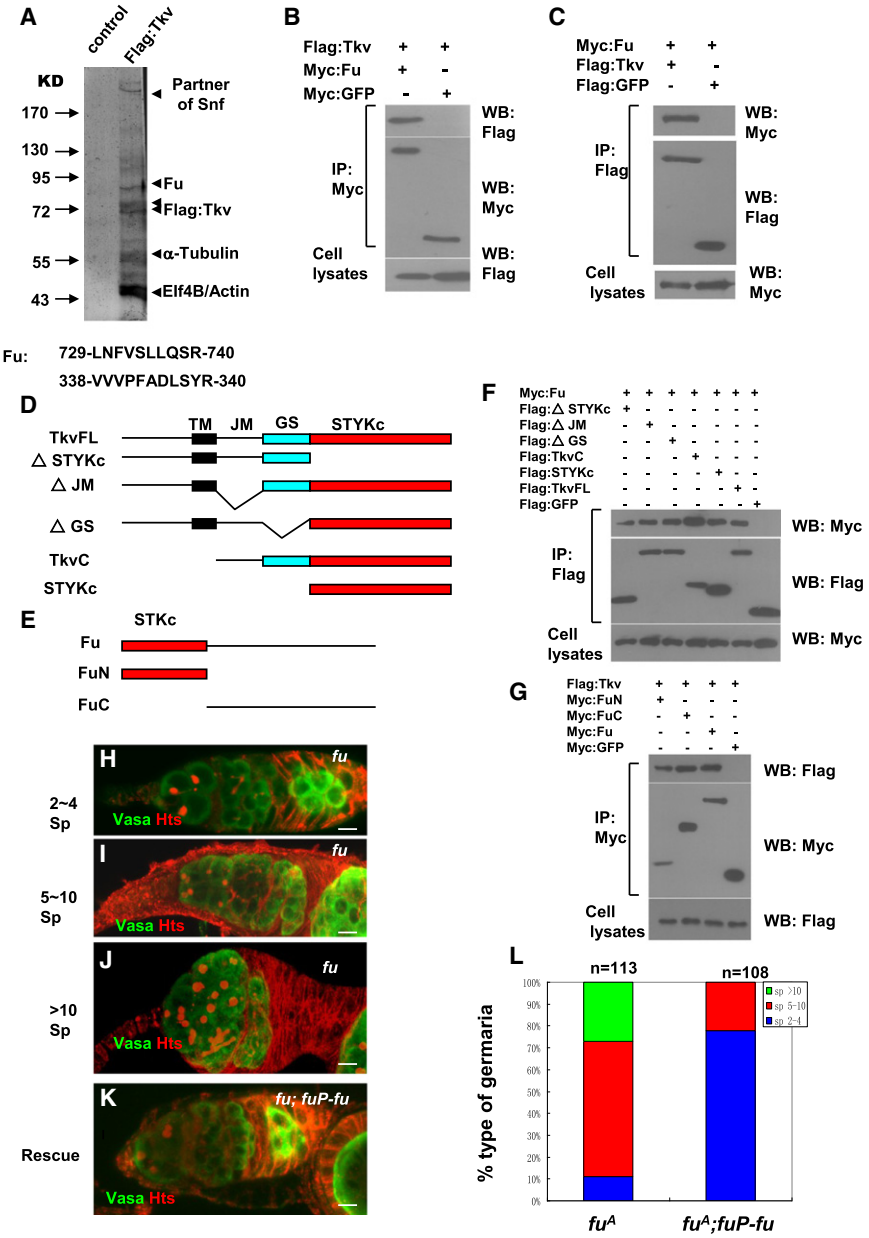

Figure 2. Identification of Fu as a Tkv-Interacting Protein

(A) Lysates from S2 cells expressing Flag-tagged Tkv were immunoprecipitated with Flag beads and then fractionated by electrophoresis through polyacrylamide gels followed by staining with silver. Mass spectrometry analysis showed that the amino acid sequence of two peptides, as indicated, matched the *Drosophila* Fu protein.

(B and C) S2 cells were transfected with combinations of DNA constructs as indicated. At 48 hr posttransfection, lysates from transfected S2 cells were immunoprecipitated with anti-Myc antibody (B) or anti-Flag M2 affinity gel (C). Western blots were performed to analyze the presence of Flag- or Myc-tagged proteins.

(D and E) Schematic drawings of Tkv (D) and Fu (E) and their deletion mutants correspond to (F) and (G).

(F and G) S2 cells were transfected with different combinations of constructs. Lysates from transfected S2 cells were immunoprecipitated with anti-Flag M2 affinity gel (F) or with anti-Myc antibody. Western blots were performed to analyze the presence of Flag- or Myc-tagged protein as indicated.

(H–K) Ovaries from wild-type w^{1118}, *fu* mutant, and *fu* mutant flies carrying the P{*fuP-fu*} transgene were stained with anti-Vasa (green) and anti-Hts (red) antibodies.

(L) Quantitative analysis of the percentage of germaria types in wild-type, *fu* mutants, and *fu* mutants carrying the P{*fuP-fu*} transgene. The x axis shows genotypes of tested flies, whereas the y axis shows the percentage of types of germaria in different genotypes. Scale bar, 10 μm.

See also Figure S2.

To define the pathway through which *fu* acts, we overexpressed *bam* on a *fu* mutant background using the transgene P{*hs-bam*} (Ohlstein and McKearin, 1997). As shown in Figures S3H and S3I, ectopic expression of *bam* completely drove *fu* mutant germ cell differentiation, suggesting that *fu* acts mainly in a *bam*-dependent manner for the differentiation of GSCs and CBs, raising the possibility that *fu* acts as a negative component of the Dpp pathway. We then tested whether the ectopic GSC-like cells in *fu* mutants respond to Dpp signaling by introducing the Dpp-responsive reporters, *bamP-gfp* and *dad-lacZ*, into the *fu* mutant background. In agreement with previous findings (Narbonne-Reveau et al., 2006), we found that many of the *fu*-inducing GSC-like cells behaved as GSCs rather than CBs, given that *gfp* was negative and *lacZ* was positive in these cells (Figures 3D–3G). To test whether the induction of GSC-like cells through the loss of *fu* depends on the activity of the *dpp* signal, we employed the transgene P{*uasp-dad*} (Jiang et al., 2008) to overexpress Dad (the *Drosophila* Smad6/7 homolog), a BMP/Dpp inhibitor. As shown in Figures S3J–S3L, ectopic expression of Dad also completely drove *fu* mutant germ cell differentiation, suggesting that induction of GSC-like cells through the loss of *fu* depends on Dpp signaling. Taken together, our findings strongly argue that *fu* is intrinsically required for GSC and CB differentiation by antagonizing Dpp signaling.

Fu Negatively Regulates BMP/Dpp Signaling by Controlling Tkv Stability

Given that Fu forms a complex with Tkv, we then asked whether *fu* has a direct role in affecting Dpp signaling through regulating the expression of Tkv and established a *bam* transcription-dependent *luciferase* reporter assay in S2 cells. As shown in Figure 3A, the *bam* transcription reporter was silenced by the expression of Tkv(ca) in a dose-dependent manner, which

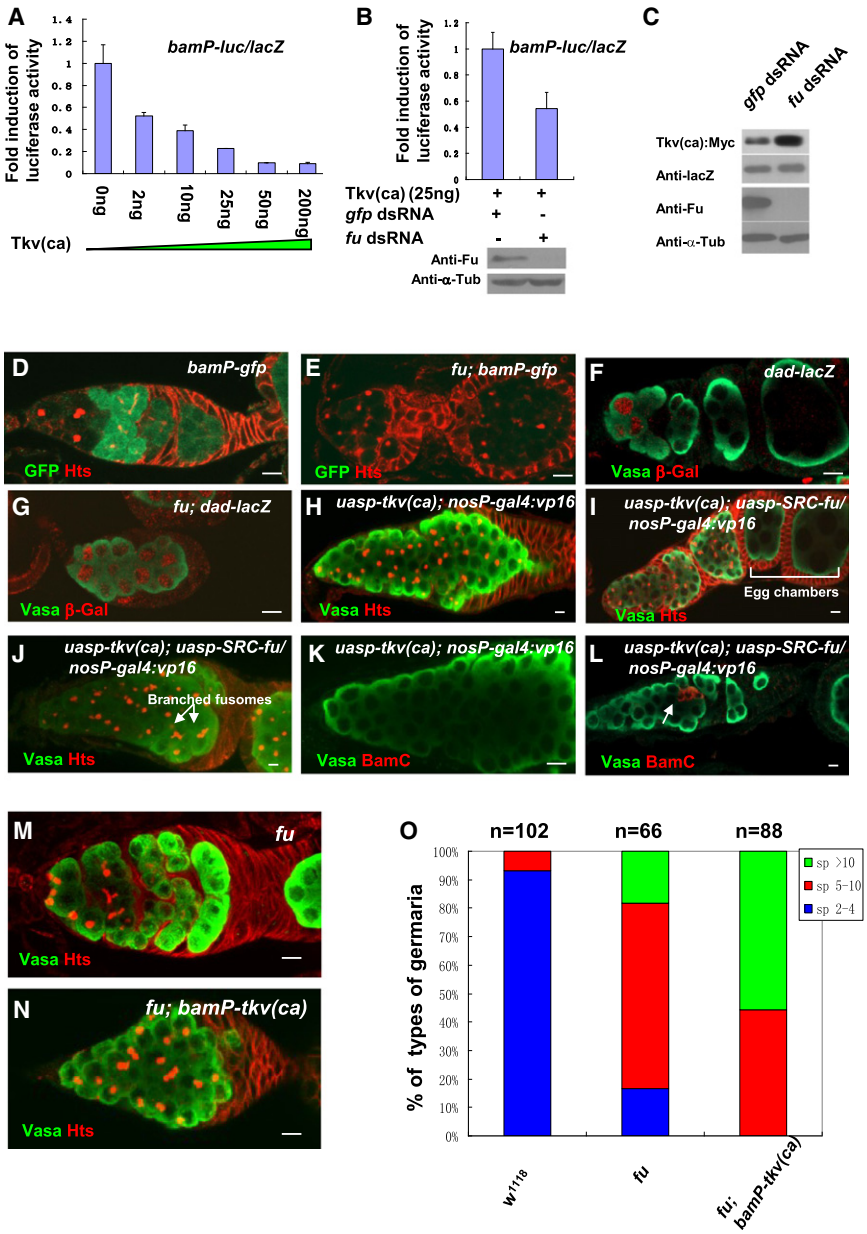

Figure 3. Fu Negatively Regulates BMP/Dpp Signaling by Controlling Tkv Stability

(A) The S2 cells were transfected with the *bamP-luciferase* reporter with gradient concentrations of *actinP-tkv(ca)*. At 48 hr posttransfection, cells were harvested for luciferase analysis.

(B) The S2 cells were transfected with *bamP-luciferase* and *actinP-tkv(ca)* and also treated with dsRNAs of *fu* or *gfp*. Knockdown of *fu* enhanced the repression of the *bam* reporter by Tkv(ca).

(C) The S2 cells were transfected with *pMT-tkv(ca)* and *actinP-lacZ* constructs or were also treated with dsRNAs of *fu* or *gfp*. Western blots were performed to analyze the presence of Myc-tagged Tkv(ca).

(D and E) Ovaries from P{*bamP-gfp*} and *fu* mutant flies carrying P{*bamP-gfp*} were stained with anti-GFP (green) and anti-Hts (red) antibodies.

(F and G) Ovaries from P{*dad-lacZ*} and *fu* mutant flies carrying P{*dad-lacZ*} were stained with anti-Vasa (green) and anti-β-gal (red) antibodies.

(H–J) Ovaries from different genotype flies as indicated were stained with anti-Vasa (green) and anti-Hts (red) antibodies.

(K and L) Ovaries from the indicated flies were stained with anti-Vasa (green) and anti-BamC (red) antibodies.

(M and N) Ovaries from *fu* and *fu* mutant flies carrying P{*bamP-tkv(ca)*} were stained with anti-Vasa (green) and anti-Hts (red) antibodies.

(O) Quantitative analysis of the percentage of germaria types as indicated in wild-type, *fu* mutant, and *fu* mutant carrying the P{*bamP-tkv(ca)*} transgene. Scale bar, 10 μm.

The experiments were carried out by duplicates, and the standard deviations were calculated by Excel. See also Figure S3.

mimics the response of the *bam* promoter to Dpp signaling in the in vivo GSC system. Of interest, we found that knockdown of *fu* in S2 cells increased stability of the Tkv protein (Figure 3C) and accordingly enhanced Tkv-mediated *bam* transcriptional silencing (Figure 3B), indicating that knockdown of *fu* influences the Dpp signal by stabilizing the Tkv protein. To confirm this finding, we performed a genetic assay by constructing the strain *fu*; P{*bamP-tkv(ca):gfp*}/+. As shown in Figures 3M–3O, constitutive *dpp* signaling from the transgene P{*bamP-tkv(ca):gfp*} resulted in a stronger tumorous germarium phenotype in the *fu* mutant background than that in *fu* mutant alone. Consistently, overexpression of an activated form of Fu, in which the Fu protein was tagged with an SRC domain at its N terminus (Jia et al., 2003; Claret et al., 2007), partially suppressed the overexpression of Tkv(ca) driven by the *nanos* promoter, as indicated by the presence of branched fusomes and ectopic Bam expression, as well as 30% of ovarioles (n = 50) carrying normal egg chambers, in P{*uasp-tkv(ca)*}; P{*nosP-gal4:vp16*}/P{*uasp-SRC-fu*} ovaries (Figures 3H–3L). Taken together, we argue that Fu negatively regulates Tkv stability to determine the fate of GSCs and CBs.

Smurf Interacts Physically and Genetically with Tkv

We noted that the phenotype of the GSC-like cells in the *fu* mutant ovary resembled that in the *Drosophila smurf* mutant. It has been shown that *smurf* antagonizes BMP signaling by targeting phosphorylated Mad for degradation in *Drosophila* somatic cells (Liang et al., 2003; Podos et al., 2001). In ovaries, *smurf* transcript is ubiquitously present in the germarium (Figures S4E and S4F), and loss of *smurf* delays the differentiation of CBs (Casanueva and Ferguson, 2004). However, the molecular mechanism underlying the action of *smurf* in CBs remains unknown. To test whether *smurf*

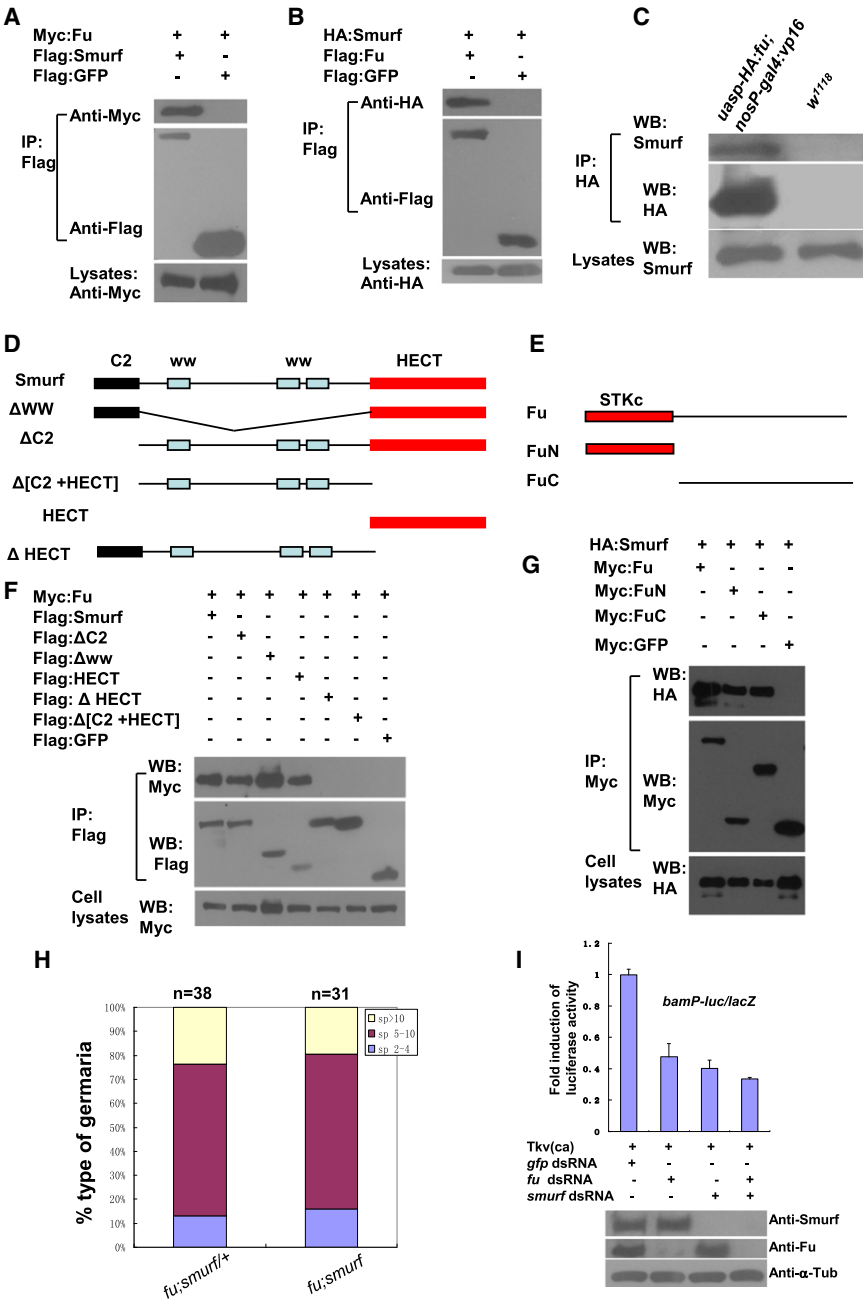

Figure 4. Fu Physically and Genetically Interacts with Smurf

(A and B) S2 cells were transfected with combinations of DNA constructs as indicated. At 48 hr posttransfection, lysates from transfected S2 cells were immunoprecipitated with anti-Flag M2 affinity gel. Western blots were performed to analyze the presence of Myc-tagged (A) or HA-tagged (B) proteins as indicated.

(C) Ovarian extracts from P{*uasp-HA:fu*}; P{*nosP-gal4:vp16*} and w^{1118} flies were immunoprecipitated with anti-HA antibody. Western blots were performed with anti-Smurf and anti-HA antibodies to analyze the presence of Smurf and HA:Fu proteins, respectively, as indicated.

(D and E) Schematic drawings of Smurf (D) and Fu (E) and their deletion mutants correspond to (F) and (G).

(F and G) S2 cells were transfected with different combinations of DNA constructs. Lysates from transfected S2 cells were immunoprecipitated with anti-Flag M2 affinity gel (F) or anti-Myc antibody (G). Western blots were performed to analyze the presence of Myc- or Flag-tagged proteins (F) or the presence of HA- or Myc-tagged proteins (G).

(H) Quantitative analysis of the percentage of germaria types in different genotypes.

(I) The S2 cells were transfected with *bamP-luciferase*, *actinP-lacZ*, and *actinP-tkv(ca)* and were also treated with dsRNAs of either *fu* or *smurf*, or both. The *gfp* dsRNA was used as a control.

The experiments were carried out by duplicates, and the standard deviations were calculated by Excel. See also Figure S4.

is involved in regulating Tkv, we performed coimmunoprecipitation and reporter assays as well as ubiquitination analysis of Tkv in S2 cells. As shown in Figures S4A and S4B, Smurf and Tkv coimmunoprecipitated with each other. Knockdown of *smurf* reduced the ubiquitination of Tkv (Figure 5F) and accordingly enhanced Tkv-mediated *bam* reporter silencing (Figure 4I). To determine the biological importance of this interaction in vivo, we examined the genetic relationship between *smurf* and *tkv* in the ovary. As shown in Figures S4C and S4D, overexpression of Tkv(ca) driven by the *bam* promoter in the *smurf* mutant strongly blocked CB differentiation. Nearly 38% of the ovarioles (n = 84) was composed of a tumorous germarium, and 62% of the ovarioles (n = 84) contained tumorous germaria that were attached to one or several egg chambers, suggesting that, like in the *fu* mutant background, *smurf* mutant germ cells were also much more sensitive to Dpp signaling than were *smurf*+ cells.

Fu Interacts Physically and Genetically with Smurf

To explore whether *fu* acts on a common pathway with *smurf* to regulate Tkv and accordingly control BMP signal activity, we determined whether Smurf physically interacts with the Fu protein by performing reciprocal immunoprecipitation assays in S2 cells. As shown in Figures 4A and 4B, Smurf and Fu coimmunoprecipitated with each other in transfected S2 cells. Consistently, we found that endogenous Smurf physically associated with HA:Fu in P{*uasp-HA:fu*}; P{*nosP-gal4:vp16*} ovaries (Figure 4C). These results suggested that Fu could form a complex with Smurf in both S2 cells and germ cells. To map the essential domain in Smurf that interacts with Fu, we generated truncated forms of Smurf. As shown in Figures 4D and 4F, the HECT domain is an essential domain for Smurf to interact with Fu. We then determined the region of Fu required for interaction with Smurf. As shown in Figures 4E and 4G, both the N and C terminus of Fu could

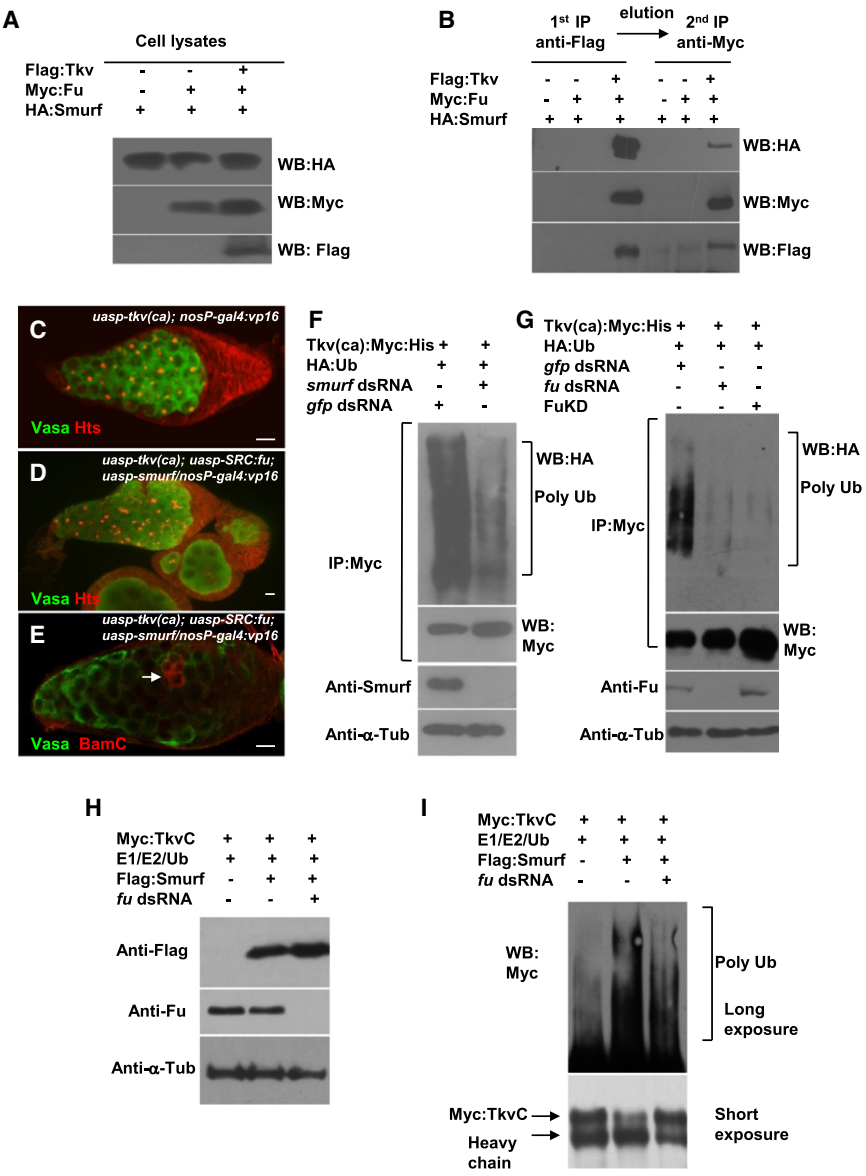

Figure 5. Fu in Concert with Smurf Targets Tkv for Ubiquitination

(A and B) S2 cells were transfected with different combinations of constructs as indicated. Lysates from transfected S2 cells were used in a two-step immunoprecipitation method employing anti-Flag and anti-Myc successively, and western blots were performed to analyze the presence of HA-tagged Smurf, Myc-tagged Fu, or Flag-tagged Tkv as indicated.

(C and D) Ovaries from different genotype flies as indicated were stained with anti-Vasa (green) and anti-Hts (red) antibodies.

(E) Ovaries from the indicated flies were stained with anti-Vasa (green) and anti-BamC (red) antibodies. Scale bar, 10 μm.

(F and G) In vivo assay of Tkv ubiquitination. S2 cells were transfected with DNA combinations, including Myc and His double epitope-tagged Tkv(ca) and HA epitope-tagged Ubiquitin (Ub) with dsRNAs of *gfp* (as a control) or *smurf* (F) or *fu* (G) treatment, or were transfected with FuKD, the kinase dead form of Fu (G). Western blots were performed to analyze the ubiquitination product of Tkv.

(H and I) An in vitro ubiquitin reaction was reconstituted with components that contained HA-Ub, E1, E2, Flag-Smurf complexes purified from S2 cells, and the Myc:TkvC (Figure 2D) produced by in vitro translation as indicated in lane 2 (lane 1 was a control lacking Flag-Smurf complexes). In lane 3, the ubiquitin reaction was the same as that in lane 2 except that Flag-Smurf complexes purified from S2 cells were treated with *fu* dsRNA. Western blots were performed to analyze ubiquitination products using the antibodies indicated.

coimmunoprecipitate with Smurf. To test the genetic relationship between *smurf* and *fu*, we constructed *smurf* and *fu* double mutants and found that the ovaries in these double mutants closely resembled those in the *fu* single-mutant ovaries (Figure 4H). Consistently, as shown in Figure 4I, there was no greater effect on the *bam-luc* reporter by knockdown of both *smurf* and *fu* compared with knockdown of *smurf* or *fu* alone. Together, these data support that Fu and Smurf are functionally dependent upon each other and act in a complex by regulating BMP/Dpp activity.

Fu, Smurf, and Tkv Form a Trimeric Complex to Promote Tkv Ubiquitination

To determine whether Fu, Smurf, and Tkv formed a trimeric complex, we coexpressed Flag-Tkv, Myc-Fu, and HA-Smurf in S2 cells and performed two-step immunoprecipitation (Extended Experimental Procedures). As shown in Figures 5A and 5B, after the two-step immunoprecipitations, both Flag-Tkv and HA-Smurf were present in the Myc-Fu complex, suggesting that Fu, Smurf, and Tkv form a trimeric complex rather than mutually exclusive heterodimers such as Fu/Smurf, Fu/Tkv, and Smurf/Tkv, raising the possibility that Fu, like Smurf, is involved in ubiquitination of Tkv. We then evaluated whether Fu was also involved in ubiquitination of Tkv. As shown in Figure 5G, knockdown of *fu* greatly reduced the conjugation of ubiquitin to Tkv, suggesting that, like Smurf, the Fu protein is also essential for Tkv ubiquitination. Given that Fu is a serine/threonine protein kinase, we then tested whether Fu supports Tkv ubiquitination in a kinase-dependent manner by using the kinase dead form of Fu, FuKD. As shown in Figure 5G, the efficiency of Tkv ubiquitination was greatly reduced when FuKD was overexpressed in S2 cells, indicating that the kinase activity of Fu is important for Fu-mediated ubiquitination of Tkv.

To substantiate the model that Fu functions in concert with Smurf to catalyze the ubiquitination of Tkv, we performed biochemical assays to assess the Smurf E3 ligase activity in the Fu/Smurf complexes by reconstituting Tkv ubiquitination in vitro. Smurf complex from S2 cell lysates efficiently supports

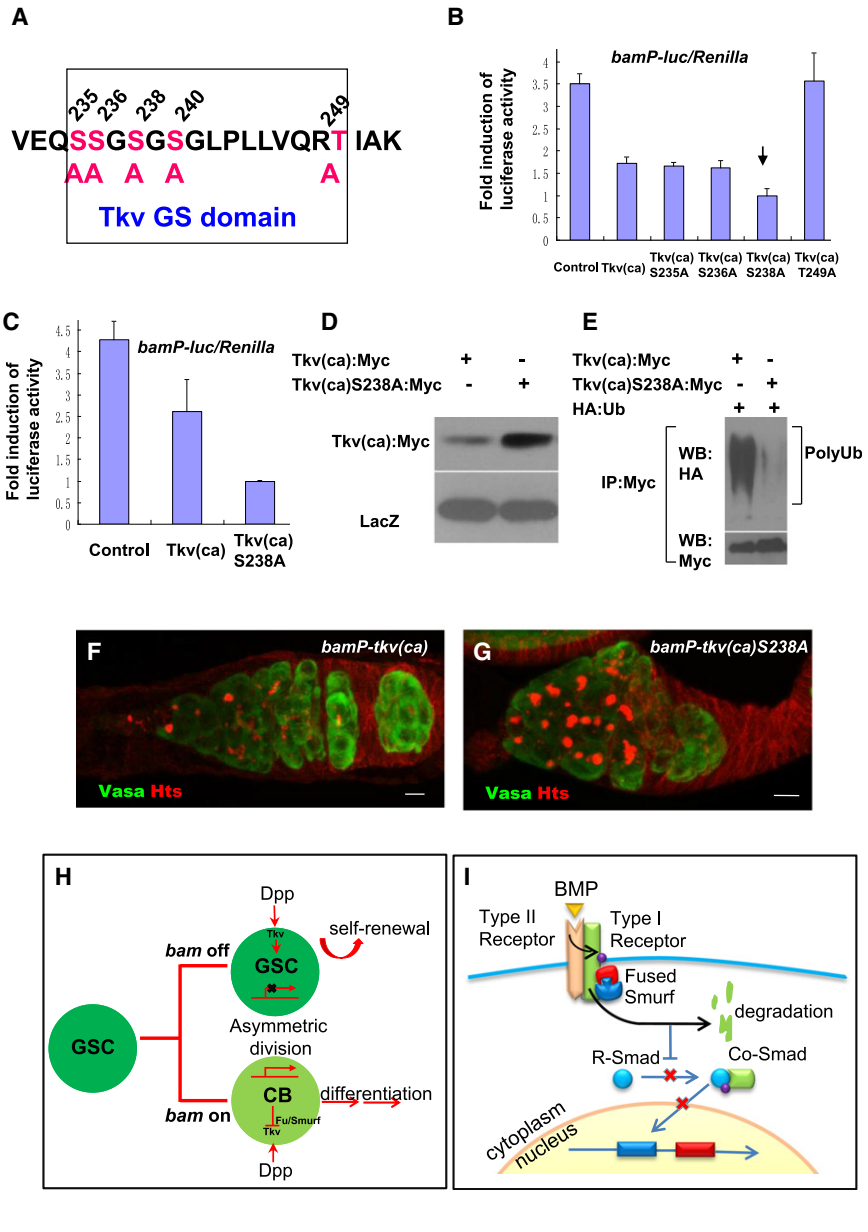

Figure 6. Identification of the S238 Site, a Putative Phosphorylation Site, Is Critical for Tkv(ca) Ubiquitination and Degradation

(A) Schematic diagram showing the sequence of the Tkv GS domain, which contains multiple S/T sites. A series of mutant forms of Tkv(ca) constructs, in which the S/T sites as indicated were individually mutated to A, was generated.

(B) The S2 cells were transfected with *bamP-luciferase*, *actinP-Renilla*, and *actinP-tkv(ca)* or mutant forms of *tkv(ca)* as indicated.

(C and D) *Luciferase* reporter analysis and protein stability assay for Tkv(ca) and Tkv(ca)S238A proteins revealed that Tkv(ca)S238A has stronger stability than Tkv(ca).

(E) Ubiquitination analysis for Tkv(ca) and Tkv(ca) S238A proteins showed that Tkv(ca)S238A protein is resistant to ubiquitin, compared with Tkv(ca).

(F and G) Ovaries from P{*bamP-tkv(ca)*} and P{*bamP-tkv(ca)S238A*} were stained with anti-Vasa (green) and anti-Hts (red) antibodies. Scale bar, 10 μm.

(H) The diagram shows that, in contrast to GSCs that undergo self-renewal, CBs develop a BMP/Dpp antagonistic pathway mediated by a Fu/Smurf complex to degrade Tkv for their differentiation.

(I) Schematic diagram summarizes a conserved mechanism in the regulation of BMP/TGFβ signaling.

The experiments were carried out by duplicates, and the standard deviations were calculated by Excel. See also Figure S5.

ubiquitination of Tkv, whereas those from S2 cells treated with dsRNA of *fu* showed significantly reduced activity toward Tkv ubiquitination (Figures 5H and 5I), suggesting that Smurf ubiquitinates Tkv in a Fu-dependent manner. To verify the importance of the coordination between Fu and Smurf in vivo, we performed a genetic assay and found that co-overexpression of Smurf and SRC-Fu strongly suppressed Tkv(ca) overexpression as indicated by the presence of the branched fusomes and expression of Bam protein, as well as nearly 50% of ovarioles (n > 100) carrying normal egg chambers (Figures 5C–5E).

The Putative Phosphorylation Site of Tkv, S238, Is Responsible for Tkv Ubiquitination and Degradation

Given that Fu regulates Tkv ubiquitination and degradation in a kinase-dependent manner, we then turned our attention to understanding the mechanism of how Tkv is regulated by searching for the specific S/T site(s) in Tkv(ca). Of interest, a previous study has implicated that several S/T sites in the GS domain of TGFβ type I receptor were subjected to phosphorylation in cell culture assays (Wrana et al., 1994). We therefore speculated that one of the corresponding sites in the GS domain of Tkv might be important for Tkv ubiquitination and degradation. To test this hypothesis, we generated a series of mutant forms of Tkv(ca) constructs in which the S/T sites, as indicated in Figure 6A and Figure S5A were individually mutated to A. We investigated whether these mutant forms of Tkv(ca) affected the response of *bamP-luc* reporter in S2 cells. As shown Figures 6B and 6C and Figure S5B, one of the mutant forms of Tkv(ca), Tkv(ca)S238A, exhibited the strongest transcriptional silencing activity on the *bamP-luc* reporter. To evaluate whether the S238 site is responsible for controlling the ubiquitination and stability of Tkv(ca), we performed ubiquitination assays on Tkv (ca) and Tkv(ca)S238A. As shown in Figures 6D and 6E, compared to Tkv(ca), Tkv(ca)S238A showed much stronger stability and appeared resistant to ubiquitination. Together with the data in Figures 3B and 3C and Figure 5G, our findings support the notion that S238, a putative phosphorylation site,

is important for Tkv to respond to Fu and critical for Tkv ubiquitination and degradation.

To determine the biological function of the S238 site, we generated a transgene fly P{*bamP-tkv(ca)S238A*} that expresses a mutant form of Tkv(ca) carrying the S238A mutation by the *bam* promoter. As shown in Figures 6F and 6G, ovaries from P{*bamP-tkv(ca)*} showed normal germline development, whereas in P{*bamP-tkv(ca)S238A*} ovaries, expression of a ubiquitin-resistant form of Tkv(ca), Tkv(ca)S238A, resulted in a tumorous germarium phenotype, demonstrating the biological importance of the S238 site of Tkv in germ cell differentiation.

Fu/STK36 Has a Conserved Role in Regulating the BMP/TGFβ Signaling Pathway in Human Cell Cultures and in Zebrafish during Embryonic Development

Given that FU (also called STK36 in vertebrates) is an evolutionarily conserved protein in flies and vertebrates, we explored whether FU has a role in the regulation of BMP signaling in human cell cultures. As shown in Figures S5C–S5H, in agreement with the data from *Drosophila*, FU/STK36 physically interacts with both SMURF proteins and ALK3, the type I receptor of BMP signaling (Figures S5C and S5D). Knockdown of *FU/STK36* reduced the ubiquitination of ALK3 (Figures S5E and S5F) and accordingly enhanced the transcriptional response of BRE-luciferase (Figures S5G and S5H). These findings suggested that FU/STK36 might have a conserved role in SMURF-mediated regulation of BMP signaling in mammals.

To further explore the in vivo function of Fu/Stk36 in vertebrates, we investigated the developmental roles of *fu* in zebrafish embryos. As shown in Figures S6A–S6F, the *fu* transcripts were present from the one-cell stage up to 24 hr postfertilization (hpf). Knockdown of *fu* with a morpholino (fu-MO) (Wolff et al., 2003) caused severe neural necrosis and growth retardation at 24 hpf (Figure 7B), which was largely due to nonspecific activation of the p53 signaling pathway (Robu et al., 2007) because coinjection with p53MO reduced neural necrosis (Figure 7C). However, in contrast to the fu-cMO/p53MO coinjected embryos (Figure 7A), fu-MO/p53MO coinjection resulted in dorsalized phenotypes that manifested as a shortened trunk (Figure 7C). The expression of *gata1* in ventral mesoderm-derived hematopoietic progenitors was inhibited in the *fu* morphants (Figures 7F, 7G, and 7S), whereas the expression of the dorsal organizer marker *gsc* in the morphants was expanded variably at the shield stage (Figures 7J, 7K, and 7T). On the other hand, embryos injected with *fu* mRNA exhibited a slight expansion of blood island, small or fused eyes, and an abnormal notochord at 24 hpf (Figure 7D), indicative of ventralization. In a high proportion of embryos injected with *fu* mRNA, *gata1* expression was enhanced (Figures 7H and 7S) and *gsc* expression slightly reduced (Figures 7L and 7T). These findings reveal that *fu* may be involved in the dorsoventral (DV) patterning of zebrafish embryos.

We then investigated whether *fu* controls DV patterning by regulating Nodal/BMP signaling. Overexpression of *sqt*, a zebrafish Nodal ligand, caused variable degrees of dorsalized phenotypes at 24 hpf with ~73% of embryos showing severe dorsalization (D1) and 20% showing relatively mild dorsalization (D2) (n = 63; Figures 7N, 7O, and 7U). When *fu* and *sqt* mRNAs were coinjected, 58% of embryos (n = 62) had almost normal morphology, and only 24% and 18% of embryos showed D1 and D2 dorsalization, respectively (Figure 7U). These results indicate that *fu* overexpression is able to inhibit Nodal-induced dorsalization. In contrast, upregulation of BMP signaling activity by injecting *bmp2b* mRNA led to embryonic ventralization at 24 hpf, with 28% (n = 141) exhibiting an onion-like shape, the strongest ventralized phenotype (V1); 27% having an enlarged tail and no head (V2, severely ventralized); and 44% showing a smaller head (V3, moderate ventralization) (Figures 7P–7R and 7U). Coinjection of *fu* and *bmp2b* mRNAs resulted in 81% of embryos (n = 69) developing normally (Figure 7U), indicating that *fu* overexpression also antagonizes *bmp2b*-induced ventralization.

To test whether Fu has a role in the degradation of BMP receptors in zebrafish, we made a zebrafish *alk3a* and *GFP* fusion mRNA (*zalk3a-GFP*). Consistent with the *Drosophila* data that ectopic expression of Src:Fu downregulated Tkv(ca):GFP in the early embryo (Figures S2E and S2F), as shown in Figures S6G–S6J, coinjection with *fu* mRNA resulted in much weaker fluorescence, compared with *zalk3a-GFP* mRNA injection alone, suggesting that *fu* might play a conserved role in degrading BMP receptors.

To further study the genetic relationship between Fu and BMP receptors, we used a well-defined dominant-negative form of BMP type I receptor (tBr). As shown in Figures S6K–S6Y, coinjection of *fu* with *tBr* mRNA partially rescued the *tBr*-induced dorsalized phenotype, whereas coinjection of fu-MO and *tBr* mRNA had no rescue effect. Considering that Nodal and BMP signals have opposite effects in DV patterning (Schier and Talbot, 2005), these results suggest that Fu antagonizes Nodal signaling when BMP signaling is downregulated.

Taken together, our results support that *fu* functions as a modulator in zebrafish DV patterning by antagonizing both BMP and Nodal signaling.

DISCUSSION

Previous studies have demonstrated that BMP/Dpp signals from the niche play primary roles in the self-renewal of GSCs by silencing *bam* transcription (Chen and McKearin, 2003a; Song et al., 2004). However, the mechanism by which the differentiating CBs avoid the control of BMP/Dpp and activate *bam* remains poorly understood. In this study, we have provided direct evidence that the differentiating daughter cells of GSCs, known as CBs, become resistant to BMP signaling through degradation of Tkv in CBs. We showed that Fu functions as an antagonistic factor in BMP/Dpp signaling by regulating Tkv degradation during the differentiation of CBs. Moreover, we provided both genetic and biochemical evidence that Fu acts in concert with Smurf, a HECT domain-containing ubiquitin E3 ligase, to regulate the ubiquitination of Tkv in the CB, thereby generating a steep gradient of response to BMP signaling between GSCs and CBs for their fate determination (Figure 6H). Finally, we showed a conserved role for *fu* in antagonizing BMP/TGFβ signals in zebrafish embryonic development as well as in human cell cultures. Our findings not only reveal a conserved function of *fu* in controlling BMP/TGFβ signal-mediated developmental processes, but also provide a comprehensive view of

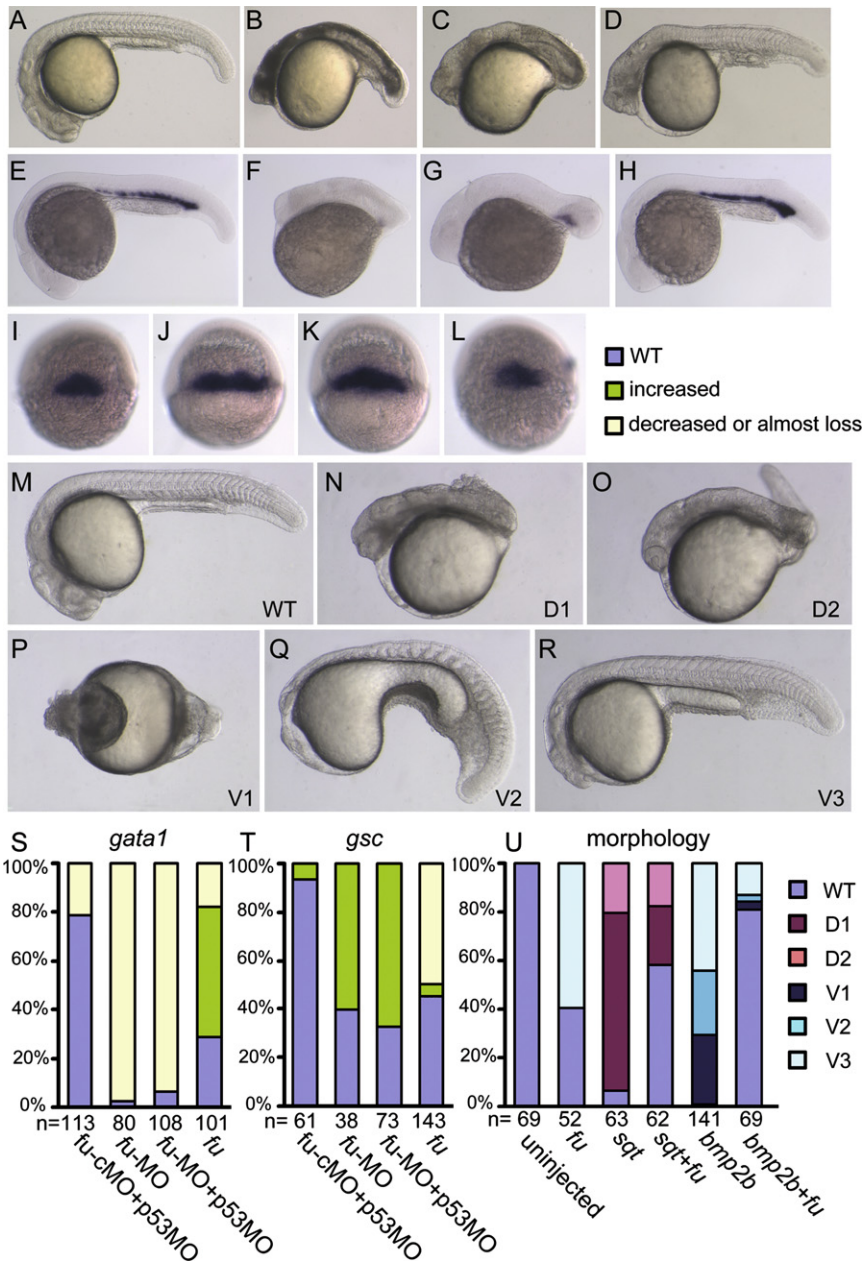

Figure 7. *fu* Participates in Dorsoventral Patterning by Regulating both Nodal and BMP Signaling Pathways in Zebrafish

(A and B) Embryonic morphology at 24 hpf after downregulating or upregulating Fu activity. Embryos injected with 5 ng fu-MO exhibited more severe necrosis (B) than those injected with 5 ng fu-cMO/p53MO (A).

(C) Coinjection of 5 ng p53MO with 5 ng fu-MO alleviated necrosis as observed in (B) but caused dorsalized phenotypes.

(D) Overexpression of 300 pg *fu* mRNA led to ventralized phenotypes.

(E–L) Examination of dorsoventral marker genes *gata1* (24 hpf) and *gsc* (shield stage). Compared to control embryos injected with fu-cMO and p53MO (E and I), 5 ng fu-MO injected alone (F and J) or coinjected with 5 ng p53MO (G and K) led to both *gata1* inhibition and *gsc* expansion. A 300 pg *fu* mRNA injection (H and L) led to an expansion of *gata1* and a slight reduction of *gsc*. Statistical data are shown in (S) and (T). Embryo orientations: lateral views with head to the left for *gata1*; dorsal views with animal pole to the top for *gsc*.

(M–R) Compared with the uninjected control (M), embryos injected with 0.75 pg *sqt* mRNA were classified into D1 and D2 groups of dorsalization (N and O). Embryos injected with 10 pg *bmp2b* mRNA were classified into V1–V3 groups of ventralization (P, Q, and R).

(U) Statistical data for rescue experiments in which 300 pg *fu* mRNA was coinjected with 0.75 pg *sqt* or 10 pg *bmp2b* mRNA. Coinjection of *fu* mRNA rescues *sqt*- or *bmp2b*-induced dorsoventral patterning defects.

See also Figure S6.

mechanisms that produce both self-renewal and asymmetry in the division of stem cells.

A Role for Fu in Smurf-Mediated Ubiquitination of BMP/TGFβ Signaling

Observations of the existence of a BMP resistance mechanism that controls the proper division of GSCs through the regulation of Tkv prompted us to explore how Tkv was regulated. Using immunoprecipitation followed by mass spectrometry analysis, we identified that Fu associates with the Tkv protein. Given that previous studies demonstrated that a loss of *fu* leads to early germ cell proliferation and a tumorous germarium phenotype (Narbonne-Reveau et al., 2006) and that our biochemical evidence showed that Fu forms a complex with Tkv and affects its stability, we subsequently identified that Fu as a component negatively regulates BMP/Dpp signaling by interacting with the BMP/Dpp type I receptor, Tkv.

BMP/TGFβ signals play pivotal roles in controlling diverse normal developmental and cellular processes (Wu and Hill, 2009). In the canonical BMP/TGFβ pathway, the receptors and Smad proteins are the essential components for BMP/TGFβ signal transduction. However, this pathway is known to be modulated by additional factors to reach physiological levels in a cellular context-dependent manner (Kitisin et al., 2007). Smurfs and HECT domain-containing proteins have been shown to antagonize BMP/TGFβ signals through the regulation of the stability of either receptors or Smads in vertebrates (Ebisawa et al., 2001; Murakami et al., 2003). In *Drosophila*, Smurf has previously been implicated in regulating proteolysis of phosphorylated Smad proteins in somatic cells (Liang et al., 2003; Podos

et al., 2001). In the ovary, Smurf was also proposed to downregulate the level of BMP to promote CB differentiation (Casanueva and Ferguson, 2004). The mechanism underlying the action of Smurf in *Drosophila* early germline cells remains elusive. In this study, we showed that Fu, Smurf, and Tkv could form a trimeric complex in S2 cells. Importantly, both Fu and Smurf are required for ubiquitination of Tkv in S2 cells and for turnover of Tkv in germ cells. Combined with our genetic evidence, we proposed that Fu and Smurf likely function in a common biochemical process by controlling Tkv degradation. The present study reveals a mechanism by which Fu serves as an essential component in the Smurf-mediated degradation of the BMP/TGFβ receptor, thereby terminating BMP/TGFβ signaling and negatively regulating the downstream target genes of BMP/TGFβ (Figure 6I).

Because Fu is a putative serine/threonine protein kinase, the question becomes how Fu acts on Tkv regulation in concert with Smurf. Given that knockdown of *fu* does not significantly change the pattern of autoubiquitination of Smurf itself (data not shown), it is therefore likely that Tkv is a strong candidate substrate for Fu kinase. Although there is no assay system for analyzing the kinase activity of Fu presently, in this study, we performed mutagenesis assays and identified that the S238 in Tkv is important for Tkv(ca) to respond to Fu and is critical for Tkv(ca) ubiquitination and degradation. Of note, we found that the ubiquitin-resistant form of Tkv(ca) [Tkv(ca)S238A] blocks CB differentiation. A previous study has shown that the S189 site in TGF-β type-I receptor, the corresponding site of S238 in Tkv, was phosphorylated in the cell culture system (Wrana, et al., 1994). Our results suggest that Fu likely acts on Tkv through targeting and phosphorylating the S238 site and subsequently leads to Tkv ubiquitination and degradation by Smurf. Nevertheless, it would be advantageous to develop a kinase assay system for Fu to determine whether the S238 site in Tkv is an authentic phosphorylation site for Fu kinase in the future.

A Conserved Role for Fused in the Regulation of BMP/TGFβ Signals

Previous genetic analyses revealed that Fu plays an evolutionarily conserved role in the proper activation of the Hh pathway and functions downstream of the Hh receptor (Jiang and Hui, 2008; Sánchez-Herrero et al., 1996; Ruel et al., 2003; Wilson et al., 2009). Increasing evidence has shown that the kinase Fu regulates the Hh-signaling complex by targeting Cos2 (Liu et al., 2007; Nybakken et al., 2002; Ruel et al., 2007; Ruel et al., 2003). However, the function of Fu as a component in the Hh pathway is not consistent with its spatiotemporal expression pattern during development. For example, Hh signaling only plays a role in zebrafish embryonic development at late stages, but Fu is expressed ubiquitously at both the early and the late stages of zebrafish embryonic development. These findings suggest that Fu may have Hh-independent functions in different physiological conditions. In this study, by using several different systems, including *Drosophila* germline, zebrafish embryo, and human tissue cultures, we demonstrated that Fu is indeed required for balancing proper BMP/TGFβ signals in different developmental processes. Given that both Fu and Smurf are evolutionarily conserved proteins, it would be interesting to determine whether the Fu/Smurf complex also plays roles in other signaling pathways.

EXPERIMENTAL PROCEDURES

Drosophila Strains

Fly stocks used in this study were maintained under standard culture conditions. The w^{1118} strain was used as the host for all P element-mediated transformations. Strains P{*bamP-gal4:vp16*}, P{*uasp-tkv(ca)*} P{*bamP-gfp*}, P{*dad-lacZ*}, $smurf^{15c}$, and P{*nosP-gal4:vp16*} have been described previously (Casanueva and Ferguson, 2004; Chen and McKearin, 2003b; Van Doren et al., 1998). Strains P{*uasp-SRC-fu*}, P{*uasp-smurf*}, P{*bamP-tkv(ca)*}, P{*bamP-tkv:gfp*}, and P{*bamP-tkv(ca):gfp*} were made in this study. The fu^{A} mutant and the rescue transgene for the *fu* mutant, P{*fuP-fu*}, were a gift from Dr. Jin Jiang. The transgene line, P{*fuP-fuKD*}, was generated to express the kinase dead form of Fu (Fu^{G13V}) in which the conserved glycine (G13) site of Fu was changed into a valine. The *fu* knockdown transgene line, P{*uasp-shmiR-fu*}, was generated according to the method described previously (Haley et al., 2008). The detailed information of primers was described in the Extended Experimental Procedures.

Immunohistochemistry for *Drosophila* Ovary

Ovaries were prepared for immunohistochemistry as described previously (Chen and McKearin, 2005). The following primary antibody dilutions were used: rabbit anti-GFP (1:5000, Invitrogen); mouse anti-Hts (1:500, DSHB); rabbit and mouse anti-BamC (1:1000); rabbit anti-Vasa (1:1000, Santa Cruz); and mouse anti-β Gal (1:1000 Promega). The following secondary antibodies were used at a 1:200 dilution: goat anti-mouse Alexa568 and goat anti-rabbit Alexa488 (Molecular Probes).

Phenotypic Analysis

Ovaries isolated from 3-day-old flies were incubated with Hts antibody, and images were collected on a Zeiss LSM 510 Meta confocal microscope to count the number of spherical spectrosomes/fusomes and to identify differentiated cysts with branched fusomes. This protocol was described previously (Cox et al., 2000).

Anti-Fu and Anti-Smurf Antibodies

The anti-Fu antibody was generated by immunizing rabbit with the recombinant protein His6-Fu (amino acids 260–431) produced in *E. coli*, and the anti-Smurf antibody was generated by immunizing mice with the recombinant protein His6-Smurf protein (amino acids 1–300) produced in *E. coli*.

Cell Culture, Immunoprecipitation, and Western Blot Analysis

S2 cells were cultured in Schneider's *Drosophila* medium (Sigma). Transfection was performed using the calcium phosphate transfection method. Immunoprecipitation and western blots were performed using protocols previously described (Jiang et al., 2008). The following reagents were used: rabbit and mouse anti-Myc and rabbit anti-HA (Santa Cruz); rabbit and mouse anti-Flag and anti-Flag M2 affinity gel (Sigma); and rabbit anti-α-tubulin (Abcam). A detailed procedure for the two-step immunoprecipitation assay is given in the Extended Experimental Procedures.

S2 Cell Reporter Gene Assay

The *bam* transcription reporter assay in S2 cells was performed by using the *bamP-luciferase* construct in which the luciferase coding sequence was placed under the control of the *bam* promoter. For normalizing the efficiency of the transfection, the *actinP-lacZ* or *actinP-Renilla* construct was used. The luciferase and β-galactosidase assays were performed as standard procedures and measured on a luminometer.

In Vivo and In Vitro Ubiquitination Assays

For the in vivo ubiquitination assay, S2 cells were transfected with DNA constructs and also treated with dsRNA according to the protocols described previously (Chen et al., 2009). In brief, at 48 hr posttransfection, MG132 (final concentration 50 μM) was added into the media. Cells were harvested 4 hr later

and lysed with a lysis buffer (50 mM Tris [pH 7.5], 120 mM NaCl, and 0.5% NP40) containing 1% (w/v) sodium dodecyl sulfate (SDS) that was preheated to 100°C. Before binding with the anti-Myc beads, the concentrations of NaCl and SDS in the binding buffer were adjusted to 500 mM and 0.1%, respectively. After pull-down with anti-Myc beads, the beads were then washed with lysis buffer containing 0.1% SDS and were subjected to immunoblot analysis.

For the in vitro ubiquitination assay, Myc:TkvC protein was synthesized by the in vitro transcription-coupled translation method. To test whether the ubiquitination of Tkv was coordinately supported by Smurf and Fu proteins, E1, E2 (His-UCH5C), E3 (Smurf complexes with Fu or without Fu), substrate (Myc:TkvC), and HA:Ub were then incubated at 30°C for 2 hr in a 40 μl ubiquitination reaction (50 mM Tris-HCl [pH 7.5], 1 mM dithiothreitol, 50 mM NaCl, 5 mM $MgCl_2$, and 2 mM ATP) with 0.2 μg of E1, 10 μg of ubiquitin (both from Upstate). Reactions were terminated with SDS sample buffer and analyzed by western blotting with anti-Myc antibody.

Mammalian Cell Culture, Transient Transfection, and Immunoprecipitation

Human HEK293T and HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator containing 5% CO_2. Calcium phosphate or lipofectine was used for plasmid transfection. For the reporter assay, 36 hr after transfection, cells were fed with fresh medium containing 0.2% FBS and were treated with 10 ng of ligands for another 12 hr. The luciferase and Renilla assays were performed as standard procedures and measured on a luminometer.

Zebrafish Embryo Assay

All of the zebrafish embryos were derived from the Tübingen strain. Embryos were incubated in Holtfreter's solution at 28.5°C and staged. The mRNAs were synthesized in vitro with the mMESSAGE mMACHINE Kit (Ambion). An RNeasy Mini Kit (QIAGEN) was used for mRNA purification. The fu-MO and fu-cMO morpholinos have been described previously (Wolff et al., 2003) with sequences of 5′-TGG TAC TGA TCC ATC TCC AGC GAC G-3′ (fu-MO) and 5′-TGC TA*G* TGA TC*G* ATC TCC A*C*C G*T*C G-3′ (fu-cMO). The fu-cMO was a mismatch (italicized) control for fu-MO. The p53MO used to suppress nonspecific activation of morpholino oligonucleotides (Robu et al., 2007) was purchased from Gene Tools, LLC. The mRNA and morpholino were injected into the yolk of the embryos at the one- or two-cell stage. Digoxigenin-UTP-labeled antisense RNA probes were generated by in vitro transcription. Whole-mount in situ hybridization was carried out following standard procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.cell.2010.11.022.

ACKNOWLEDGMENTS

We thank Drs. Dennis McKearin, Duojia Pan, Peng Jin, and Zongping Xia for critical readings of the manuscript. This work was supported by grants from the National Basic Research Program of China (2007CB947502 and 2007CB507400 to D.C.) and from the NSFC (#30630042 and 30825026 to D.C. and #30830068 to A.M.).

Received: March 5, 2010
Revised: July 27, 2010
Accepted: November 9, 2010
Published: December 9, 2010

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