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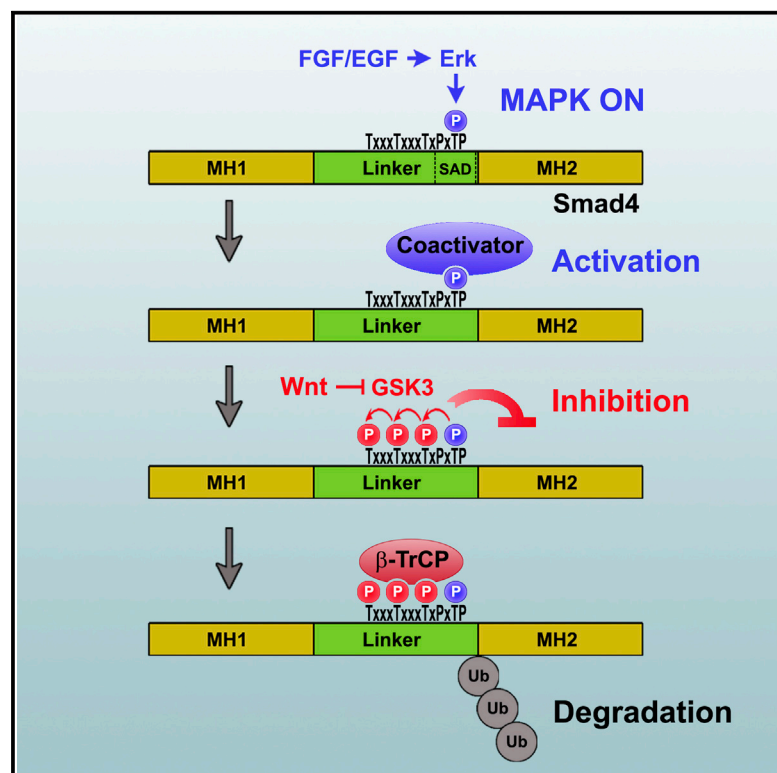
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# Cell Reports

## The Tumor Suppressor Smad4/DPC4 Is Regulated by Phosphorylations that Integrate FGF, Wnt, and TGF- $\beta$ Signaling

### Graphical Abstract



### Highlights

The tumor suppressor Smad4 is regulated by FGF/MAPK and Wnt/GSK3 phosphorylations

Sequential phosphorylations control both Smad4 activity and degradation by  $\beta$ -TrCP

In the presence of FGF, Wnt potentiates TGF- $\beta$  at low physiological concentrations

This mechanism controls germ layer and organizer specification in *Xenopus*

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### In Brief

Demagny et al. show that Smad4 is phosphorylated after FGF primes three inhibitory phosphorylations by GSK3. In the presence of FGF and Wnt, signaling by low concentrations of TGF- $\beta$  is greatly enhanced, helping explain the devastating effects of loss of the tumor suppressor Smad4.

# The Tumor Suppressor Smad4/DPC4 Is Regulated by Phosphorylations that Integrate FGF, Wnt, and TGF- $\beta$ Signaling

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## SUMMARY

Smad4 is a major tumor suppressor currently thought to function constitutively in the transforming growth factor  $\beta$  (TGF- $\beta$ )-signaling pathway. Here, we report that Smad4 activity is directly regulated by the Wnt and fibroblast growth factor (FGF) pathways through GSK3 and mitogen-activated protein kinase (MAPK) phosphorylation sites. FGF activates MAPK, which primes three sequential GSK3 phosphorylations that generate a Wnt-regulated phosphodegron bound by the ubiquitin E3 ligase  $\beta$ -TrCP. In the presence of FGF, Wnt potentiates TGF- $\beta$  signaling by preventing Smad4 GSK3 phosphorylations that inhibit a transcriptional activation domain located in the linker region. When MAPK is not activated, the Wnt and TGF- $\beta$  signaling pathways remain insulated from each other. In *Xenopus* embryos, these Smad4 phosphorylations regulate germ-layer specification and Spemann organizer formation. The results show that three major signaling pathways critical in development and cancer are integrated at the level of Smad4.

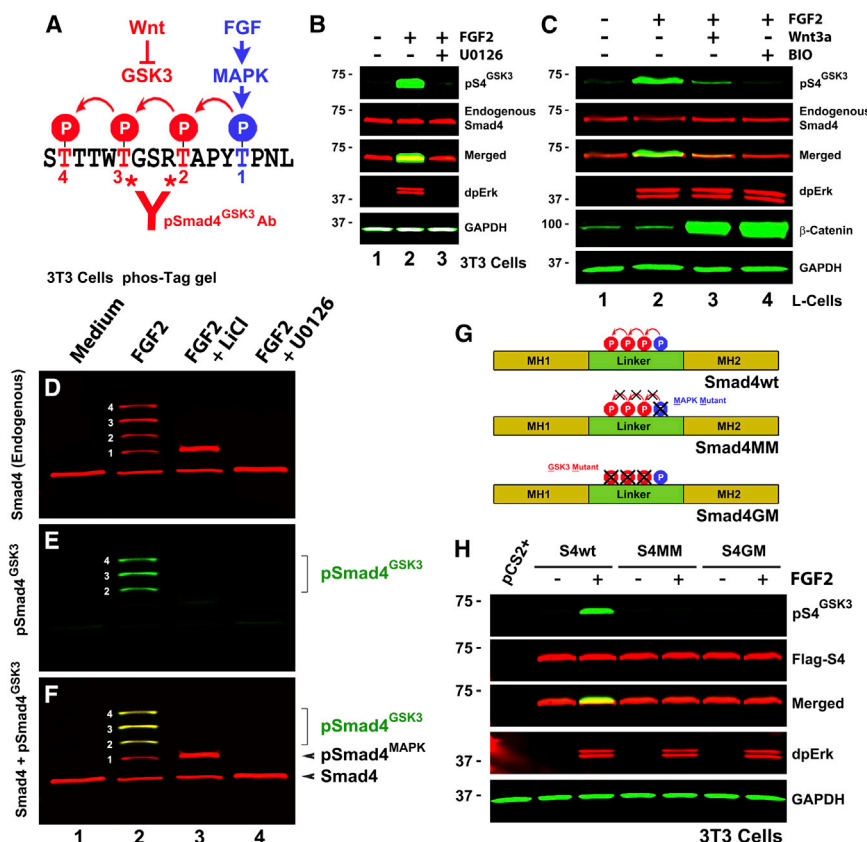
## INTRODUCTION

Smad4, also known as deleted in pancreatic carcinoma 4 (DPC4), is a major tumor suppressor gene that constrains cancer growth. Pancreatic, colorectal, and prostate carcinomas proliferate rapidly and progress toward metastases when Smad4 function is lost (Levy and Hill, 2006; Ding et al., 2011; Massagué, 2012). Transforming growth factor  $\beta$  (TGF- $\beta$ ) receptors signal by phosphorylating carboxy-terminal serines of the transcription factors Smad1/5/8 (for bone morphogenetic proteins [BMPs]) or Smad2/3 (for TGF- $\beta$ /activin). These receptor-activated Smads (R-Smads) then undergo a second set of phosphorylations in the linker region via activation of tyrosine kinase receptors such as those for fibroblast growth factor (FGF) and epidermal growth factor (EGF) (Kretzschmar et al., 1997; Pera et al., 2003; Sapkota et al., 2007; Fuentealba et al., 2007; Millet et al., 2009) or through nuclear CDK8 and CDK9 (Alarcón et al., 2009; Gao et al., 2009;

Aragón et al., 2011). These prime phosphorylations by glycogen synthase kinase-3 (GSK3) that target R-Smads for proteasomal degradation (Fuentealba et al., 2007; Sapkota et al., 2007). The transcription factor Smad4 functions as a co-Smad that binds to R-Smads and has been considered a constitutively active component of the pathway (Massagué, 2012).

The Wnt pathway is activated in the early stages of many tumors, and its transcriptional effects are mediated by the stabilization of  $\beta$ -catenin (Clevers and Nusse, 2012). Canonical Wnt signaling causes the sequestration of cytosolic GSK3, axin, and Dishevelled in multivesicular bodies (Taelman et al., 2010; Vinyoles et al., 2014). In addition to  $\beta$ -catenin, other proteins may be regulated by Wnt signaling through the decrease in GSK3 phosphorylations that are normally recognized as phosphodegrons to be polyubiquitinated and degraded in the proteasome (Kim et al., 2009; Taelman et al., 2010; Vinyoles et al., 2014; Acebron et al., 2014). GSK3 is a kinase that prefers prephosphorylated substrates, introducing phosphorylations on Ser or Thr residues located four amino acids upstream (S/TxxxS/T[PO<sub>3</sub>]; Cohen and Frame, 2001). During a bioinformatic screen of the human proteome, we noticed that Smad4 contains three putative GSK3 phosphorylation sites primed by a mitogen-activated protein kinase (MAPK) site (PxTP; Taelman et al., 2010).

Here, we report that Smad4 activity depends on tyrosine kinase/MAPK- and Wnt/GSK3-regulated phosphorylations, revealing a node of signaling integration between these two main oncogenic pathways and the TGF- $\beta$  tumor suppressor signal. We show that, when cells received an FGF signal, phosphorylation of the MAPK site promoted Smad4 peak transcriptional activity before priming inhibitory GSK3 phosphorylations. Smad4 phosphorylation by GSK3 created a phosphodegron that led to its subsequent polyubiquitination and degradation by the E3-ligase  $\beta$ -TrCP. In the presence of Wnt, Smad4 GSK3 phosphorylations were inhibited and the TGF- $\beta$  signal was prolonged, particularly at low levels of TGF- $\beta$  ligands. Replacing Smad4 with a GSK3-resistant mutant showed that the crosstalk between TGF- $\beta$  and Wnt is mediated by Smad4. This molecular mechanism, in which Wnt and MAPK activation enhance anti-proliferative TGF- $\beta$  signals, may help understand the role of Smad4 as a barrier to tumor progression. In the context of the *Xenopus* embryo, we found that replacing endogenous Smad4 with a GSK3 phosphorylation-resistant mutant converted the



**Figure 1. The Smad4 Linker Region Is Phosphorylated by GSK3**

(A) Smad4 contains MAPK (blue) and GSK3 (red) phosphorylation sites in its linker region. (B) Endogenous FGF-induced pSmad4<sup>GSK3</sup> phosphorylation requires Erk activity in serum-depleted NIH 3T3 cells stimulated with FGF2 for 1 hr. (C) Endogenous pSmad4<sup>GSK3</sup> antigen is induced by a 1 hr FGF2 treatment, inhibited by preincubation with Wnt3a for 5 hr, and blocked by the GSK3 inhibitor BIO. (D–F) Mn<sup>2+</sup>-Phos-tag analysis of endogenous Smad4 in NIH 3T3 cells cultured in the absence of serum. (G) Diagrams of Smad4 constructs encoding Smad4 wild-type (Smad4-WT) or phosphorylation-resistant mutants (Thr to Val) for MAPK (Smad4-MM) and GSK3 (Smad4-GM) sites. (H) GSK3 phosphorylations require an intact MAPK site in transfected 3T3 cells. See also Figure S1.

entire ectoderm into mesoderm and expanded Spemann organizer formation, indicating that the growth-factor-regulated Smad4 phosphorylations play an important role in animal development.

## RESULTS

### Wnt and FGF Regulate Phosphorylation of Smad4 Linker Region

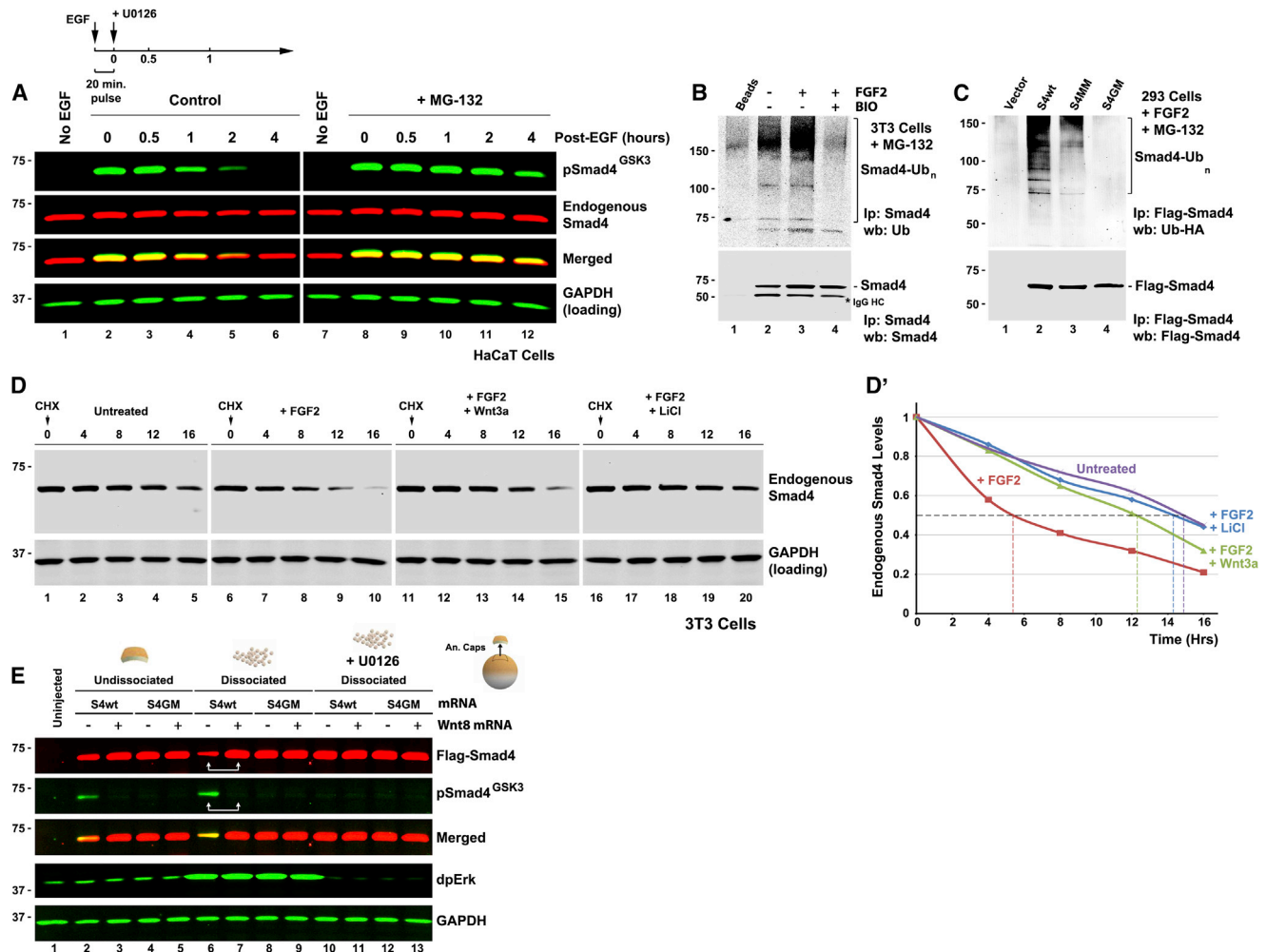
The putative regulatory sites consisted of four threonines located in the linker (middle) region of Smad4 (Figure 1A). To determine whether Smad4 was phosphorylated by GSK3, we generated an antibody raised against phospho-threonines 273 and 269 (pSmad4<sup>GSK3</sup> Ab). Because the priming site was a canonical MAPK/Erk site (PxTP), we treated 3T3 fibroblasts with FGF2 and found that a single band of endogenous pSmad4<sup>GSK3</sup> antigen was increased (Figure 1B, lanes 1 and 2). The pSmad4<sup>GSK3</sup> signal was blocked by treatment with the MEK-specific inhibitor U0126, demonstrating a requirement for Erk/MAPK downstream of FGF stimulation (Figure 1B, lane 3). FGF-induced pSmad4<sup>GSK3</sup> phosphorylation was inhibited by preincubation with Wnt3a protein (Figure 1C, lanes 2 and 3) and blocked by the GSK3 inhibitor BIO (Figure 1C, lane 4). The specificity of the antibody was confirmed by Smad4 small interfering RNA (siRNA) depletion and phosphatase treatment (Figure S1).

To determine the number of sites that were phosphorylated, we separated proteins from untransfected 3T3 cells in polyacryl-

amide SDS gels containing the phosphate-binding compound Mn<sup>2+</sup>-Phos-tag (Kinoshita et al., 2006). In the absence of serum, a single band was detected by a Smad4 monoclonal antibody, whereas upon addition of FGF, four additional bands were displayed (Figure 1D, lanes 1 and 2). The three slower migrating bands were also positive for pSmad4<sup>GSK3</sup> antibody (Figure 1E). GSK3 inhibition by LiCl resulted in the accumulation of the monophosphorylated form, whereas treatment with U0126 eliminated all Smad4 phosphorylations (Figure 1F, lanes 3 and 4). Studies with transfected phosphorylation-resistant MAPK or GSK3 mutants (designated as Smad4-MM and Smad4-GM, respectively, Figure 1G) showed that the priming site (threonine 277) was required for GSK3 phosphorylations (Figure 1H). Taken together, these results demonstrate that Smad4 is regulated by sequential phosphorylations as proposed in Figure 1A.

### Wnt/GSK3 Regulates the Polyubiquitination and Degradation of Smad4

Polyubiquitination of R-Smads is controlled by linker phosphorylations (Sapkota et al., 2007; Fuentealba et al., 2007; Gao et al., 2009), prompting us to investigate the effects of GSK3 phosphorylations on Smad4 stability. HaCaT keratinocytes (which respond well to TGF- $\beta$  and siRNA transfection, but not to FGF) were treated with a 20 min pulse of EGF and found that pSmad4<sup>GSK3</sup> was degraded over a period of 4 hr (Figure 2A). Proteasomal inhibition by MG-132 greatly stabilized the phosphorylated form of Smad4 (Figure 2A), indicating that pSmad4<sup>GSK3</sup> was preferentially degraded by the proteasome. Immunoprecipitation studies showed that endogenous Smad4 polyubiquitination was increased by FGF treatment and required GSK3 activity (Figures 2B and S2C), as well as intact phosphorylation sites for GSK3 and MAPK (Figure 2C).



**Figure 2. Wnt-Regulated GSK3 Phosphorylations Control Smad4 Polyubiquitination and Degradation**

(A) Time course of pSmad4<sup>GSK3</sup> phosphorylation primed by a 20 min pulse of EGF in HaCaT cells, showing that the proteasome inhibitor MG-132 preferentially prolongs the half-life of pSmad4<sup>GSK3</sup>.

(B) Endogenous Smad4 polyubiquitination is increased by FGF and requires GSK3 activity (in the presence of the proteasome inhibitor MG132). Before immunoprecipitation with monoclonal Smad4 antibody, 0.2% SDS was added, samples heated at 95°C for 10 min to break protein-protein interactions, and diluted 10-fold with RIPA buffer to ensure that the polyubiquitinated bands detected were not ubiquitinated Smad4-interacting proteins (Zhu et al., 1999). For 5% input loading, see Figure S2A. Ip, immunoprecipitation; wb, western blotting.

(C) Smad4 polyubiquitination requires intact MAPK and GSK3 sites; Flag-Smad4 or its phosphorylation-resistant mutants were cotransfected with HA-ubiquitin into FGF-treated HEK293 cells and immunoprecipitated (Zhu et al., 1999) with anti-Flag antibodies. For 5% input loading, see Figure S2B.

(D) Wnt3a or LiCl treatment extended the half-life of endogenous Smad4 exclusively in FGF-treated 3T3 cells. Note that, in the absence of FGF, Smad4 has a longer half-life. Similar results were obtained in two independent experiments.

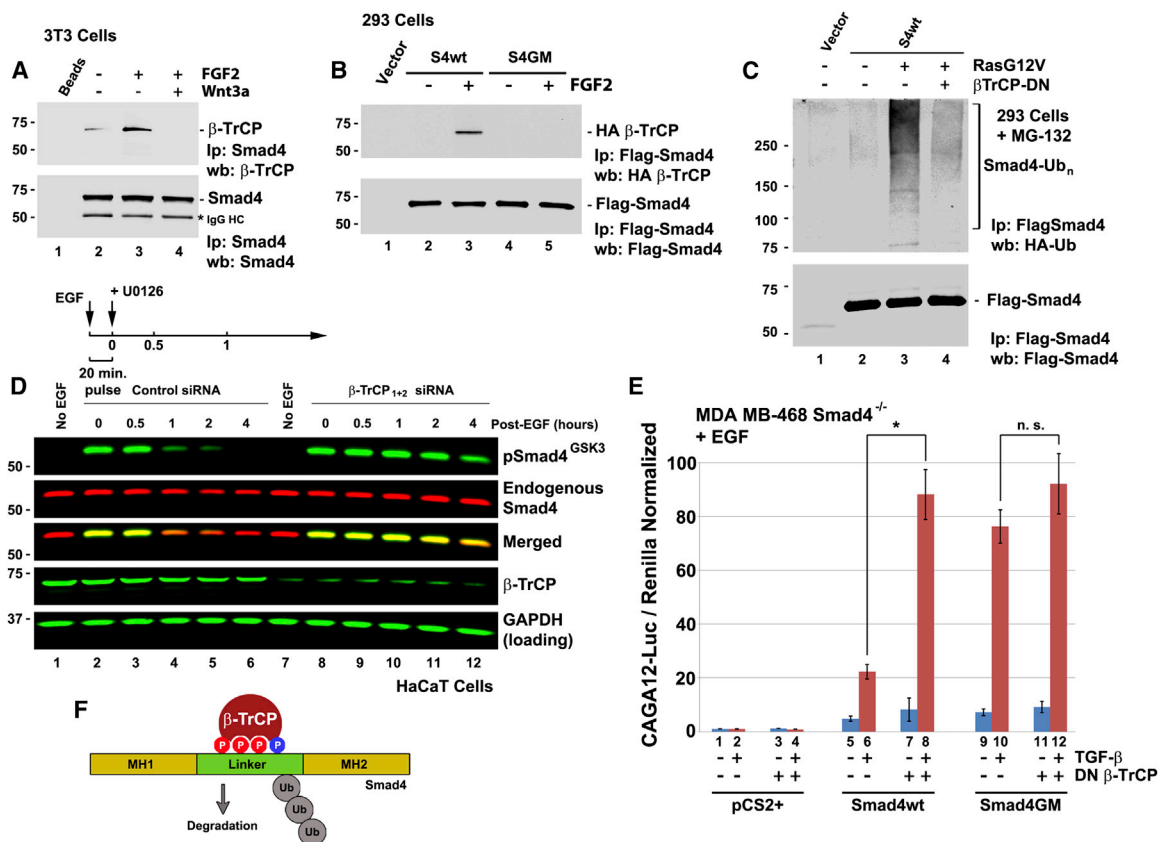
(D') Quantification of western results shown in (D).

(E) Smad4 protein is stabilized by microinjection of xWnt8 mRNA in *Xenopus*-dissociated animal cap cells. In dissociated cells, dpErk is activated, causing increased pSmad4<sup>GSK3</sup> and Flag-Smad4 degradation (lane 6). Both GSK3 phosphorylation and Flag-Smad4 degradation were blocked by coinjection of Wnt8 mRNA (lane 7). Smad4 degradation in microinjected embryos required intact GSK3 phosphorylation sites and was blocked by the Erk pathway inhibitor U0126 (40  $\mu$ M). Cells were harvested at stage 10.5, early gastrula.

See also Figure S2.

Because GSK3 is a Wnt-regulated kinase, we asked whether the Wnt growth factor could regulate Smad4 stability. In cycloheximide time course experiments, endogenous Smad4 was stabilized by addition of Wnt3a or of the GSK3 inhibitor LiCl but only in FGF-treated 3T3 cells (Figures 2D and 2D'). *Xenopus* animal caps explants microinjected with Flag-tagged Smad4

mRNAs were also used to study the degradation of Smad4; because mRNAs were injected, any differences in protein levels should be posttranscriptional. In *Xenopus* ectodermal explants, a potent and sustained activation of the MAPK/Erk pathway is achieved by cell dissociation (Kuroda et al., 2005). We found that, in dissociated animal cap cells, diphospho Erk was



**Figure 3. The Wnt-Regulated Smad4 GSK3 Phosphodegron Is Bound by the Ubiquitin E3-Ligase  $\beta$ -TrCP and Targeted for Degradation**

(A) Endogenous binding between Smad4 and  $\beta$ -TrCP is increased by FGF and blocked by Wnt3a treatment. Immunoprecipitation of endogenous Smad4 from untransfected 3T3 cells using a Smad4 monoclonal antibody bound to protein A/G agarose beads; 5% loading of initial lysate is shown in Figure S3B. (B) FGF-induced binding between Smad4 and  $\beta$ -TrCP requires intact GSK3 phosphorylation sites in transfected 293 cells. Flag-tagged Smad4-WT bound HA-tagged  $\beta$ -TrCP in the presence of FGF, but phosphorylation-resistant Smad4-GM was unable to coprecipitate with  $\beta$ -TrCP; 5% loading is shown in Figure S3C. (C) Smad4 polyubiquitination induced by the oncogenic RasG12V protein is mediated by  $\beta$ -TrCP; 5% loading is shown in Figure S3D. (D)  $\beta$ -TrCP depletion with siRNA prolongs the half-life of pSmad4<sup>GSK3</sup> induced by a 20 min pulse of EGF in HaCaT cells. (E) Transfection of DN- $\beta$ -TrCP significantly increased TGF- $\beta$  signaling in Smad4<sup>-/-</sup> MB-468 cells transfected with hSmad4-WT. DN- $\beta$ -TrCP had no significant effect in Smad4-GM-expressing MB-468 cells. Note that  $\beta$ -TrCP limits TGF- $\beta$  signaling and that this requires the Smad4 GSK3 phosphorylation sites. (F) Diagram of the proposed Smad4 phosphodegron recognition by  $\beta$ -TrCP. See also Figure S3.

activated, the Smad4 GSK3 sites were strongly phosphorylated, and, importantly, Flag-Smad4 was degraded (Figure 2E, lane 6). The phosphorylation by GSK3 and the degradation of Smad4 were dependent on MAPK/Erk activity as they were blocked by U0126 treatment (Figure 2E, lane 10). Coinjection of *Wnt8* mRNA inhibited Smad4 phosphorylation by GSK3 and prevented Flag-Smad4-wild-type (WT) degradation (Figure 2E, compare lanes 6 and 7). Importantly, the GSK3 phosphorylation-resistant Smad4 mutant (Flag-Smad4-GM) was insensitive to stabilization by Wnt8 (Figures 2E, S2D, and S2D').

Taken together, these experiments show that linker phosphorylations regulated by FGF/MAPK and Wnt/GSK3 control Smad4 polyubiquitination and degradation.

#### Wnt/GSK3 Regulates a Smad4 $\beta$ -TrCP Phosphodegron

We next analyzed the molecular mechanism by which Smad4 phosphorylations regulated its polyubiquitination. Smad4 prote-

olysis is mediated by interaction with the F-box E3 ubiquitin ligase  $\beta$ -TrCP but was not previously known to be regulated by growth factor signaling (Wan et al., 2004, 2005; Yang et al., 2006). Because  $\beta$ -TrCP recognizes phosphodegrons (Fuchs et al., 2004), we investigated whether its binding to Smad4 was regulated rather than constitutive. Immunoprecipitation studies with endogenous proteins showed that  $\beta$ -TrCP bound preferentially to Smad4 in the presence of FGF and that Wnt3a treatment prevented this interaction in untransfected 3T3 cells (Figure 3A, lanes 3 and 4). We also found that intact GSK3 phosphorylation sites in Smad4 were essential for the FGF-induced binding of  $\beta$ -TrCP to Smad4 (Figure 3B, lanes 3 and 5). Finally, a dominant-negative form of  $\beta$ -TrCP (DN- $\beta$ -TrCP lacking the F-box domain; Orian et al., 2000) inhibited the polyubiquitination of Smad4 induced by RasG12V (Figure 3C).

To test whether  $\beta$ -TrCP was the E3 ligase responsible for pSmad4<sup>GSK3</sup> degradation, we depleted HaCaT cells of  $\beta$ -TrCP

with an siRNA that targets both  $\beta$ -TrCP1 and  $\beta$ -TrCP2 (Guardavaccaro et al., 2003). GSK3 phosphorylation of Smad4 was primed by a 20 min pulse of EGF, and we found that  $\beta$ -TrCP depletion strongly stabilized the pSmad4<sup>GSK3</sup> form (Figure 3D). Finally, in a functional reporter gene assay, DN- $\beta$ -TrCP increased responsiveness to TGF- $\beta$ , and this effect required intact Smad4 GSK3 sites (Figure 3E).

Taken together, these experiments indicate that MAPK and GSK3 trigger the formation of a phosphodegron bound by the E3 ligase  $\beta$ -TrCP, causing the polyubiquitination of Smad4 as indicated in the model in Figure 3F.

### Wnt and TGF- $\beta$ Signaling Crosstalk via Smad4

A central question is whether the TGF- $\beta$ , FGF, and Wnt signaling pathways are insulated from each other or integrated via the Smad4 phosphorylation sites. To address this, human embryonic kidney 293 (HEK293) cells were transfected with the TGF- $\beta$ -specific reporter CAGA12-luciferase (Denkler et al., 1998) and treated with or without Wnt3a. TGF- $\beta$  signaling was unaffected by Wnt3a (Figure 4A, bars 2 and 4), as expected if the two pathways were distinct and insulated from each other. However, addition of FGF2 reduced TGF- $\beta$  signaling by two thirds (Figure 4A, bar 6), presumably by priming inhibitory GSK3 phosphorylations. Importantly, in the presence of FGF2, Wnt3a was able to stimulate TGF- $\beta$  signaling, reaching signaling levels higher than those of TGF- $\beta$  alone (Figure 4A, see brackets).

Wnt also potentiated expression levels of the endogenous TGF- $\beta$  target genes PAI-1 and Smad7 in HepG2 cells (Figures 4B, S4A, and S4B), and the stimulation of TGF- $\beta$  signaling by Wnt was mimicked by the GSK3 inhibitor LiCl (Figures S4C and S4D). A DN-Tcf3 construct (Molenaar et al., 1996) did not affect the crosstalk between TGF- $\beta$ , FGF, and Wnt3a, indicating that this mechanism is independent of Tcf3/ $\beta$ -catenin-mediated transcription (Figure S4E). We note that the stimulation of TGF- $\beta$  signaling by Wnt was not observed in confluent cell cultures (Figure S4F), as is the case with other TGF- $\beta$  effects (Varelas et al., 2010).

In addition, a BMP reporter gene (BRE-Luc; Korchynskiy and ten Dijke, 2002) was also regulated by Wnt in the presence of FGF (Figures S4G and S4H), indicating that the Smad4 regulatory mechanism described here applies to both the TGF- $\beta$  and BMP branches of the pathway. These experiments showed that Wnt enhances TGF- $\beta$  signaling but only when MAPK/Erk is activated by FGF.

We then investigated the extent to which the observed crosstalk between TGF- $\beta$  and Wnt signaling was mediated by the linker GSK3 phosphorylations in Smad4. The receptor-regulated Smad2/3 contains linker SP sites as well as an unprimed GSK3 site in the DNA-binding domain of Smad3 (Guo et al., 2008; Millet et al., 2009; Abushahba et al., 2012). To assess specifically the role of Smad4, we used mammary carcinoma MDA MB-468 cells, which lack endogenous Smad4 and TGF- $\beta$  responsiveness (de Caestecker et al., 2000). Transfection of Smad4-WT restored TGF- $\beta$  signaling, which was potentiated by Wnt in the presence of EGF (Figure 4C, bars 5 and 6). However, when cells were transfected with the GSK3 phosphorylation-resistant Smad4-GM, TGF- $\beta$  caused a strong signal but lost all regulation by Wnt3a (Figure 4C, bars 8 and 9). Because replacing Smad4 by

a GSK3-insensitive mutant eliminated Wnt potentiation, we conclude that the observed crosstalk between TGF- $\beta$  and Wnt is mediated through the GSK3 phosphorylation sites of Smad4 and not by other components of the signal transduction pathway.

The potent stimulatory effect of Wnt on TGF- $\beta$  signaling was concentration dependent and best revealed in 293 cultured cells treated with FGF and variable amounts of TGF- $\beta$  (Figure 4D). When the same data were displayed as shown in Figure 4E, it was observed that, in the absence of Wnt3a (and presence of FGF), 100 ng/ml TGF- $\beta$  was required for a 240-fold induction of CAGA12-Luc, whereas in the presence of Wnt3a, only 1 ng/ml TGF- $\beta$  was sufficient to reach a similar transcriptional activation.

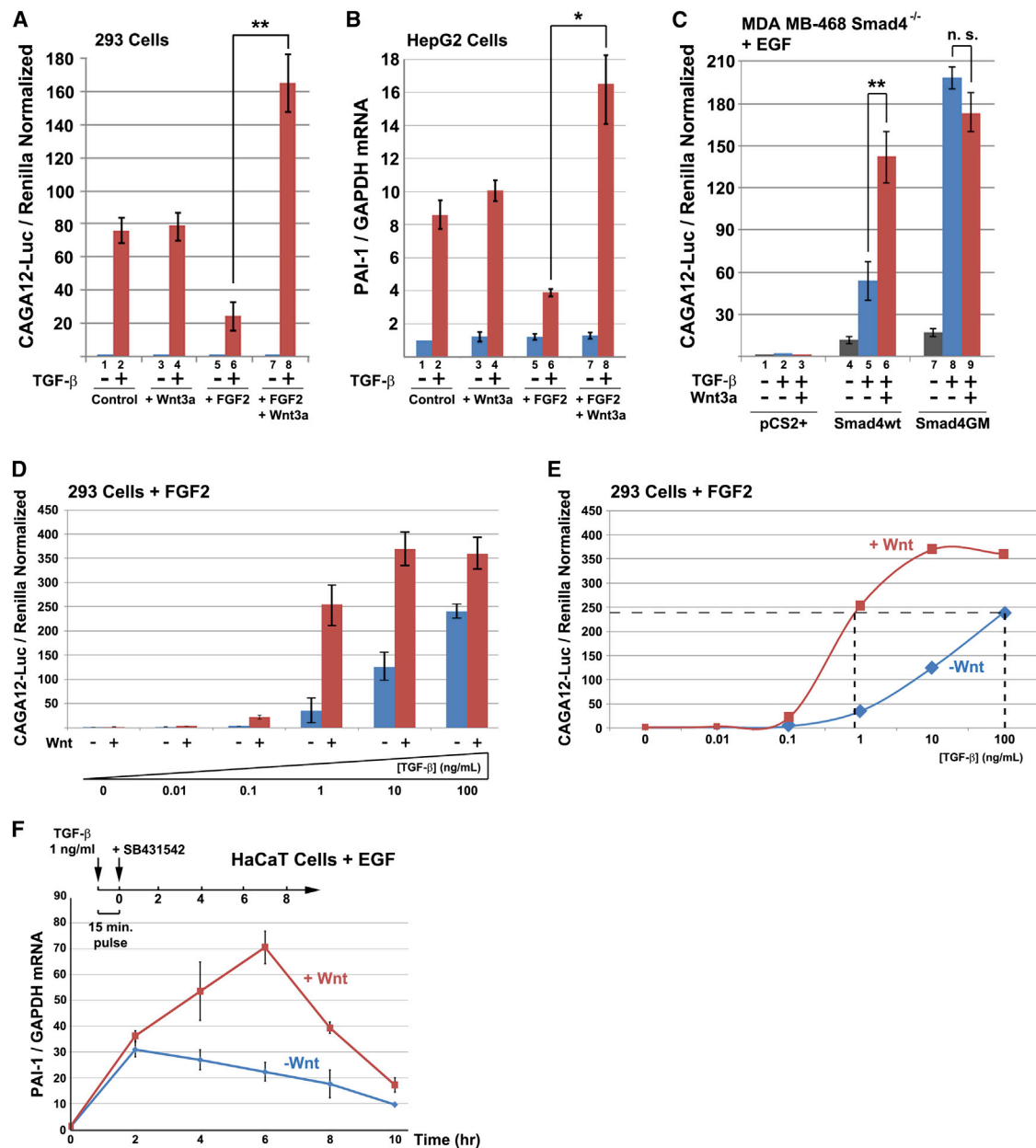
We also examined how Wnt affected the time course of the TGF- $\beta$  transcriptional response (Figure 4F). HaCaT cells were treated with a 15 min pulse of 1 ng/ml TGF- $\beta$ , which was terminated by adding 2  $\mu$ M of the type I TGF- $\beta$  receptor inhibitor SB-431542 (Halder et al., 2005). Analyses of transcripts for the TGF- $\beta$  target gene PAI-1 showed that Wnt significantly prolonged the TGF- $\beta$  transcriptional response (Figure 4F).

From these functional experiments, we conclude that, although TGF- $\beta$  and Wnt signaling are insulated in the absence of FGF, activation of the MAPK pathway causes a robust crosstalk in which canonical Wnt enhances and prolongs signaling by low, presumably the most physiologically relevant, levels of TGF- $\beta$  ligands.

### The Smad4 Linker Contains a Growth-Factor-Regulated Transcriptional Activation Domain

A short region of the linker has been identified as a Smad4 activation domain (SAD) (de Caestecker et al., 2000). This 48-amino-acid sequence binds the transcriptional coactivator p300/CBP and contains the MAPK site, but not the GSK3 sites. We asked if a construct containing the entire linker domain (169 amino acids) could be regulated by FGF and Wnt. The Smad4 linker region was fused to the yeast Gal4 DNA-binding domain (Gal4DBD) (Figure 5A) and used in transcriptional assays with an upstream activating sequence (UAS)-Gal4-luciferase reporter gene (de Caestecker et al., 2000). The linker region of Smad4 was both required and sufficient to drive transcriptional activity in a TGF- $\beta$ -independent way (Figures S5A and S5B). Interestingly, the activity of the transcription activation domain contained in the linker region was repressed by FGF and significantly increased by Wnt3a (Figure 5B, bars 2–4). When the GSK3 sites were mutated (Gal4DBD-S4linker-GM), Wnt lacked any significant effect (Figure 5B, bars 6 and 7). Surprisingly, FGF stimulated the transcriptional activity of the S4linker-GM construct instead of inhibiting it (Figures 5B, bars 5 and 6), indicating that MAPK phosphorylation has a positive effect on the Smad4 transcription factor (in the absence of GSK3 phosphorylations). In agreement with this, mutation of the MAPK-priming site (Gal4DBD-S4linker-MM) had very low levels of transcriptional activity (Figure 5B, bars 8–10).

An important feature of the Gal4DBD-S4linker constructs is that their stability was not affected by FGF or Wnt treatment (Figure 5C). In RasG12V-transfected cells, Gal4DBD-S4linker-WT was heavily phosphorylated by GSK3, but not degraded



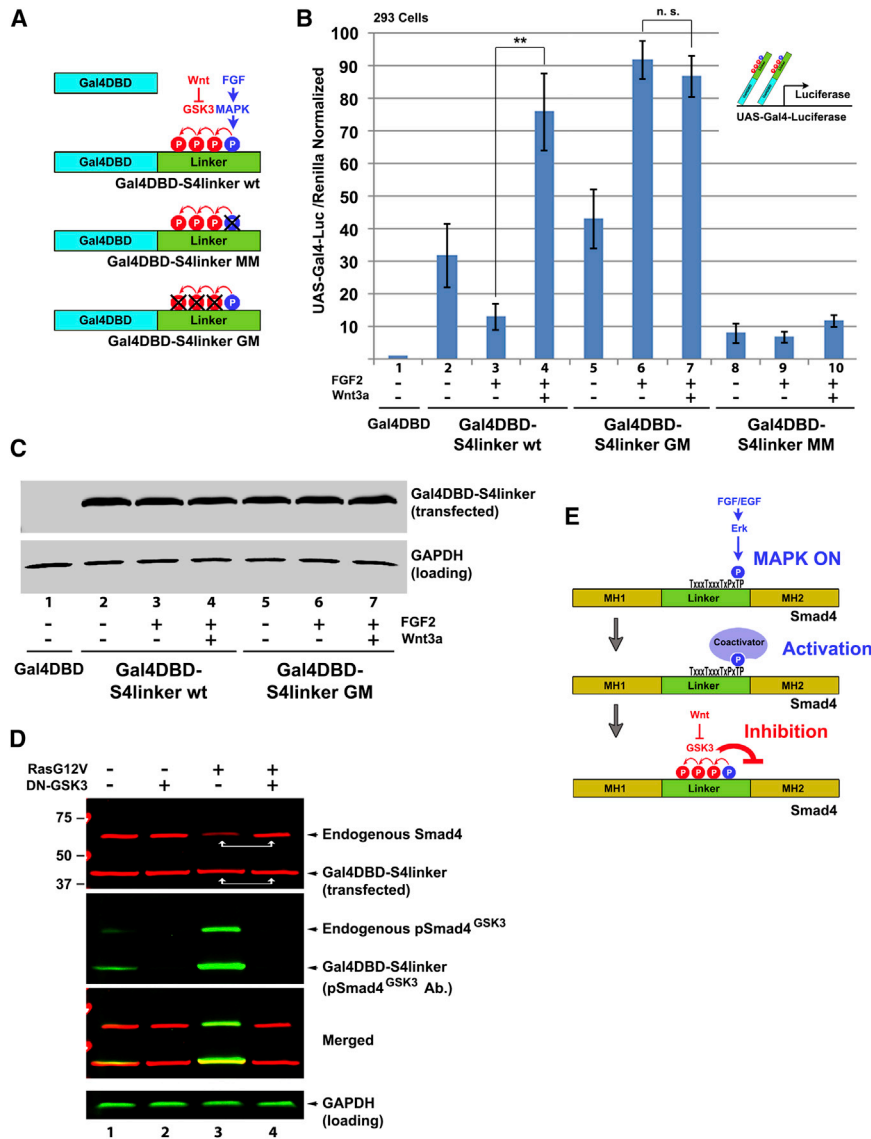
**Figure 4. Wnt and TGF- $\beta$  Signaling Crosstalk via Smad4 GSK3 Phosphorylations**

(A) TGF- $\beta$  CAGA12-Luc reporter gene assays in 293 cells showing that Wnt potentiated TGF- $\beta$  signaling but only in the presence of FGF (brackets). (B) The endogenous TGF- $\beta$  target gene PAI-1 was similarly regulated 3 hr after addition of TGF- $\beta$  and FGF (HepG2 cells). (C) TGF- $\beta$  signaling was restored in MDA-MB-468 (Smad4<sup>-/-</sup>) cells by Smad4-WT. Note that Wnt3a regulation was lost when Smad4-WT was replaced by the GSK3 phosphorylation-resistant mutant Smad4-GM. (D) TGF- $\beta$  concentration dependence of CAGA12-Luc expression in FGF2-treated 293 cells. (E) Same data as in (D) displayed as curves, showing that Wnt3a is a very potent activator at low concentrations of TGF- $\beta$  ligand. (F) Time course analysis of the TGF- $\beta$  transcriptional response after terminating 15 min of a pulse of a low amount of TGF- $\beta$  with SB-431542. The induction of PAI-1 transcripts was prolonged by Wnt3a treatment (in EGF-treated HaCaT cells). See also Figure S4.

(Figure 5D). In the same cells, endogenous Smad4 was destabilized by the sustained Ras activation, and its steady-state levels were restored by a dominant-negative form of GSK3 (Figure 5D, lanes 3 and 4). Because the stability of the Gal4-DBD construct

was unchanged by phosphorylation of the linker sites, the induction of the UAS-luciferase reporter allowed the measurement of transcriptional responses independently of changes in protein stability.





**Figure 5. The Smad4 Linker Domain Contains a Growth-Factor-Regulated Transcriptional Activation Domain**

(A) Diagram of the yeast Gal4 DNA-binding domain (Gal4DBD) fused to Smad4 linker region and of the phosphorylation-resistant mutants used to test transcriptional activation.

(B) UAS-Gal4-luciferase reporter gene assays in 293 cells showing that the linker transcriptional activation domain was regulated by FGF and Wnt.

(C) Western blot showing that Gal4DBD-S4linker protein levels remained unchanged by FGF and Wnt treatment despite the differences observed in transcriptional activity.

(D) Gal4DBD-S4linker was heavily phosphorylated by GSK3 when cotransfected with activated RasG12V, but its stability is not affected. In contrast, endogenous Smad4 was destabilized by the sustained MAPK activation driven by Ras, and this required GSK3 activity.

(E) Model of Smad4 transcriptional activity regulation by FGF and Wnt via the linker phosphorylation sites and binding of an as-yet-unknown coactivator.

See also Figure S5.

signaling (Figure 6B, compare bars 4–6). Smad4GM-MM, which differs by a single amino acid (T277V) from Smad4-GM, also had reduced activity (Figure 6B, bars 8 and 10). These experiments indicated that Thr 277 phosphorylation is required for Smad4 peak activity.

In the absence of priming by EGF stimulation, the crosstalk between TGF- $\beta$  and Wnt3a was not observed in Smad4WT transfected MB-468 cells (Figure 6C, compare bars 5 and 6). However, when a phospho-mimetic amino acid was introduced (T277D) at the MAPK site (MAPK phospho-activated, Smad4-MA), the enhancement of TGF- $\beta$  signaling by

Wnt was restored (Figure 6C, compare bars 8 and 9). Wnt was without effect when the GSK3 sites were also mutated (Smad4GM-MA construct; Figure 6C, bars 11 and 12).

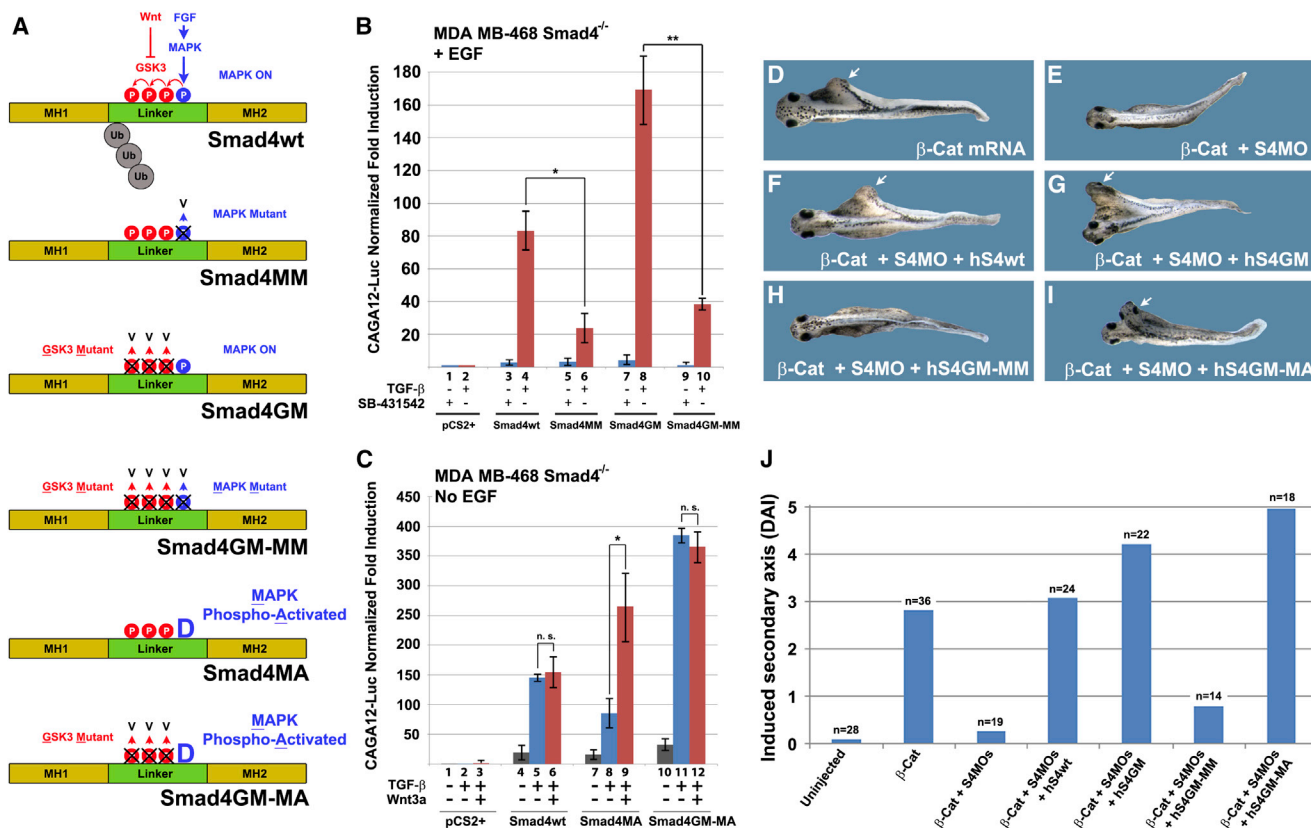
A similar requirement for the Smad4 MAPK site was found in the *Xenopus* embryo using an assay in which a low dose of  $\beta$ -catenin mRNA (20 pg) was injected into a ventral blastomere to induce partial secondary axes (Figure 6D). These axes were blocked by coinjection of Smad4 antisense morpholinos (MOs) (Dupont et al., 2009) but restored by hSmad4-WT mRNA (Figures 6E and 6F). Smad4-GM induced complete secondary axes with heads, and this required a functional or phospho-mimetic 277 site (Figures 6G–6J and S6).

From these experiments, and others shown in this study, we conclude that Smad4 phosphorylation at Thr 277 has a dual function. First, it allows Smad4 to reach peak transcriptional activity. Second, it primes Smad4 for GSK3 phosphorylations that cause transcriptional inhibition and generate a phosphodegron

These results indicate that MAPK and GSK3 phosphorylations regulate the activity of a transcriptional activation domain located in the Smad4 linker region (Figure 5E). The phosphorylation of Smad4 linker region by MAPK and GSK3 initially regulates its transcriptional activation domain and then facilitates its degradation via the E3-ligase  $\beta$ -TrCP.

**Phosphorylation by MAPK/Erk Promotes Smad4 Peak Activity**

To further investigate the function of the MAPK phosphorylation site, we constructed a series of Smad4 mutants mimicking different combinations of signaling events (Figure 6A). The MAPK/Erk site (PxTP) located at position 277 was known to be important for Smad4 nuclear translocation in response to TGF- $\beta$  treatment (Roelen et al., 2003). Using EGF-stimulated MDA MB-468 Smad4<sup>-/-</sup> cells, we found that a MAPK phosphorylation-resistant mutant (Smad4-MM) had lower levels of TGF- $\beta$



**Figure 6. Phosphorylation of Threonine 277 Is Required for Smad4 Peak Activity**

(A) Schematic diagrams of Smad4 phospho-resistant and phospho-mimetic mutants. (B) Phosphorylation of the MAPK site (Thr 277) was required for Smad4 maximal activity in the presence of EGF in Smad4<sup>-/-</sup> cells. Brackets indicate that Smad4-MM had decreased activity in the TGF- $\beta$  pathway. The TGF- $\beta$  receptor inhibitor SB-431542 (2  $\mu$ M) was added in the indicated lanes to block autocrine TGF- $\beta$  derived from MDA-MB-468 cells; for results in the absence of SB-431542, see Figure S6B. (C) In the absence of an EGF signal, mutation of the MAPK-priming site into a phospho-mimetic residue (T277D, Smad4-MA) restored the crosstalk between Wnt and TGF- $\beta$  in transfected Smad4<sup>-/-</sup> cells. Note also that Wnt potentiation of TGF- $\beta$  signaling required functional Smad4 GSK3 sites. (D) Twenty picograms of  $\beta$ -catenin mRNA injected ventrally at the four-cell stage is sufficient to induce a partial duplicated axis lacking head structures. (E) Formation of  $\beta$ -catenin secondary axes required Smad4. (F) Partial axes were rescued by 125 pg of hSmad4-WT mRNA coinjected together with  $\beta$ -catenin mRNA and Smad4 MOs. (G) The same amount of mRNA encoding hSmad4-GM induced complete axis with eyes and cement gland (see arrow). (H) In this assay, 125 pg of hSmad4GM-MM mRNA in which the MAPK site was mutated (T277V) was completely inactive. Note that this construct differs from Smad4-GM by a single amino acid. This suggests that Smad4 activity requires an intact MAPK phosphorylation site in *Xenopus* embryos. (I) hSmad4GM-MA in which the MAPK site was mutated into a phospho-mimetic aspartic acid induced the strongest complete axes, indicating a positive role for the PxTP site. (J) Quantification of the embryos microinjection results using the dorso-anterior index (DAI) (Kao et al., 1986) to measure the completeness of secondary axes; similar results were obtained in three independent experiments. See also Figure S6.

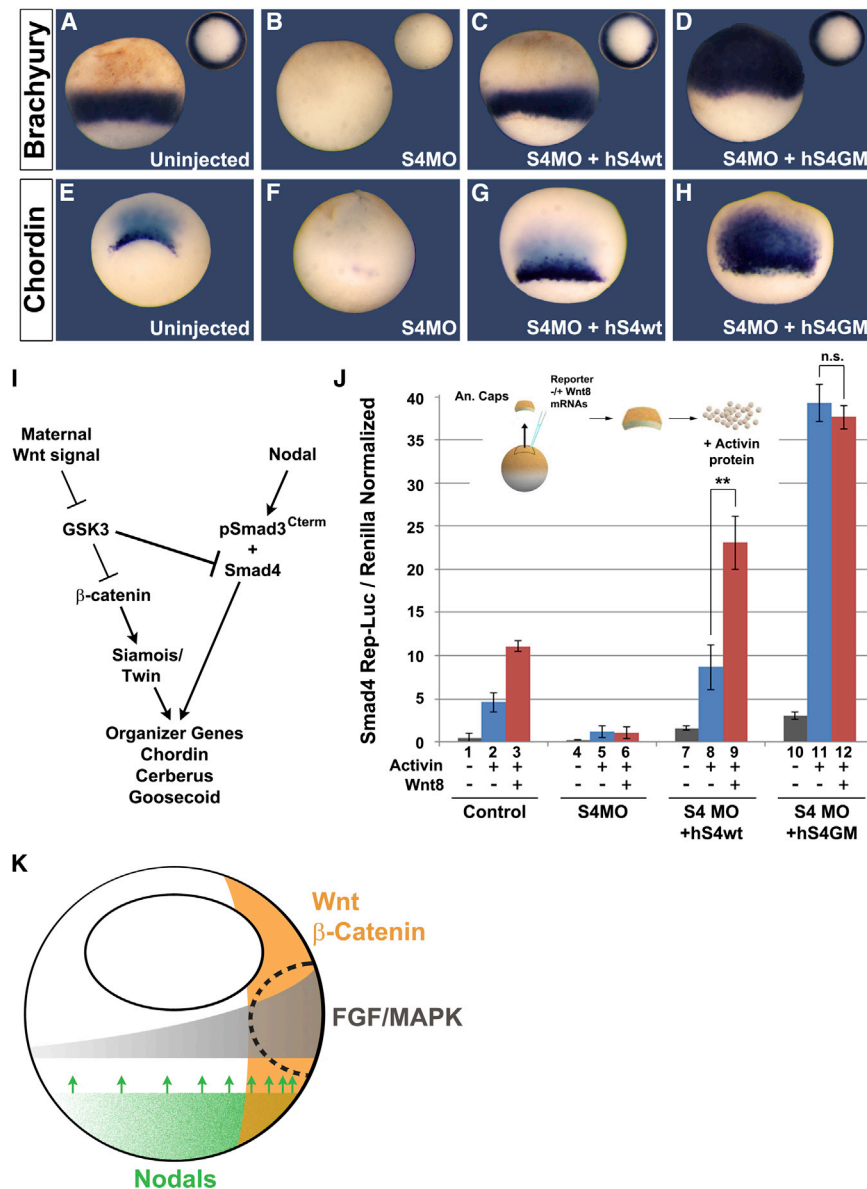
that serves as a docking site for the ubiquitin E3 ligase  $\beta$ -TrCP. Thus, both the activity and the stability of Smad4 are regulated by FGF/EGF and Wnt.

### Smad4 Regulation by GSK3 Determines Germ-Layer Specification

The early *Xenopus* embryo provides an excellent system to study cell signaling. Using embryos depleted of endogenous Smad4 with MOs, we found that hSmad4-WT mRNA rescued expression of *xBrachyury* (a Nodal/TGF- $\beta$  mesodermal target), whereas the same amount of GSK3-resistant Smad4-GM showed a great

increase in signaling (Figures 7A–7D). The replacement of endogenous Smad4 by Smad4-GM mRNA caused the entire embryonic ectoderm to become mesoderm (Figure 7D). This indicated that inhibition of Smad4 by GSK3 plays a crucial role in allowing ectodermal differentiation in vivo.

Smad4-GM also caused a strong increase in Spemann organizer tissue marked by *chordin* mRNA in embryos depleted of endogenous Smad4 (Figures 7E–7H). This suggested that GSK3 activity may normally limit the size of the organizer through Smad4. In *Xenopus*, Spemann organizer formation requires the combined action of the maternal Wnt/ $\beta$ -catenin pathway and



**Figure 7. Smad4 Regulation by GSK3/Wnt Is Involved in Germ-Layer Specification and Organizer Formation in *Xenopus***

(A–D) Endogenous Smad4 replacement by Smad4-GM, showing that GSK3 phosphorylation was required for ectodermal specification in *Xenopus*. The mesodermal marker xBrachyury was greatly expanded when Smad4 GSK3 phosphorylations were prevented.

(E–H) The Spemann organizer gene chordin is expanded by Smad4-GM injection.

(I) Diagram showing how maternal Wnt and zygotic Nodal/TGF- $\beta$  signals converge in early *Xenopus* embryo patterning. GSK3 is proposed to inhibit Nodal/Smad4 activity.

(J) In dissociated *Xenopus* animal cap cells, xWnt8 mRNA potentiates signaling by 5 ng/ml activin through the Smad4 GSK3 phosphorylation sites. This experiment used a novel Smad4-luciferase reporter designed for *Xenopus* assays and shows that Wnt modifies the competence of cells to activin induction through Smad4. Cells were harvested when sibling embryos reached early gastrula (stage 10.5).

(K) Three signaling pathways—Wnt, FGF, and Nodal—converge on the dorsal side of the *Xenopus* embryo (stippled line) during Spemann organizer formation.

See also Figure S7.

three signaling pathways—Wnt/ $\beta$ -catenin, Nodal/pSmad2, and FGF/MAPK—have been shown to be activated in the dorsal region (Schohl and Fagotto, 2002). The convergence of these three signals by the molecular mechanism identified here helps explain the peak Smad4 activity required for Spemann organizer induction (Figure 7K).

## DISCUSSION

The experiments reported here show that Smad4, long thought to act as a constitutively active component of the

of an early zygotic Nodal signal (Labbé et al., 2000; Reid et al., 2012) as indicated in Figure 7I.

To test whether Wnt can directly regulate Smad4 through its GSK3 sites in the embryo, we developed a sensitive synthetic Smad4-luciferase reporter derived from the mouse *chordin* promoter, described in Figure S7. Smad4-depleted embryos were coinjected with the reporter and Smad4-WT or Smad4-GM, animal cap cells dissociated, and treated with activin protein (Figure 7J). Microinjected *Wnt8* mRNA potentiated activin signaling, and this *Wnt8* effect had a complete requirement for the GSK3 sites in Smad4 (Figure 7J, brackets). Because the Smad4 reporter gene does not respond to Wnt or Siamois (Figure S7E), this result shows that the enhanced sensitivity to activin caused by Wnt is mediated, at least in part, through the GSK3 phosphorylation sites in Smad4. In the *Xenopus* early blastula,

TGF- $\beta$  and BMP pathways, is strongly regulated by growth factor signaling through phosphorylation sites in its linker region. We found that Smad4 is phosphorylated by GSK3 in response to FGF. GSK3 phosphorylations have a double effect on Smad4. First, they inhibit a transcription activation domain located in the linker domain. Second, they generate a Wnt-regulated phosphodegron recognized by the E3 ligase  $\beta$ -TrCP. The molecular mechanism discovered here provides a means of integrating distinct pathways, which would otherwise remain insulated, allowing cells to sense FGF and Wnt inputs and adapt TGF- $\beta$  outcome to their context.

### Smad4 Activity Is Regulated by Growth Factors

Although the TGF- $\beta$  pathway has been extensively studied for more than two decades, many efforts have focused on R-Smads

regulation and less is known about Smad4. In this study, we show that four phosphorylation sites located in the linker region of Smad4 control its activity and stability in response to growth factor stimulation. GSK3 phosphorylation is triggered by FGF or EGF through activation of the Erk pathway. Phosphorylation by growth factors via MAPK at Thr 277 allows Smad4 to reach its peak of activity while priming it for subsequent inhibitory GSK3 phosphorylations. The switch operated by GSK3 phosphorylation provides a way of controlling the duration of the Smad4 signal by ensuring that degradation and turnover follow transcriptional activation. Some of our experiments involved phospho-resistant or phospho-mimetic mutations in Smad4, which will cause exaggeration of the physiological effects (e.g., Smad4-GM mimics Smad4 receiving a maximal amount of Wnt); however, all effects reported were also observed by growth factor treatment of untransfected cells. Our observations reconcile previous results in the literature that appeared to be contradictory: it had been proposed that phosphorylation of Thr 277 was required for Smad4 nuclear localization (Roelen et al., 2003) and also for its degradation (Saha et al., 2001). However, regulation by FGF had not been addressed in these studies. In recent work, Smad4 had been found to enter the nucleus in transient pulses of about 30 min during TGF- $\beta$ /BMP signaling (Warmflash et al., 2012; Sorre et al., 2014). It will be very interesting to investigate whether these bursts of nuclear localization are controlled by the Smad4 growth-factor-regulated phosphorylations described here.

Our finding that Wnt signals through Smad4 GSK3 sites and can prolong the duration of a TGF- $\beta$  pulse supports the view that Smad4 phosphorylations are active regulators of TGF- $\beta$  signaling. The stimulatory effects of Wnt on TGF- $\beta$  signaling were entirely lost when Smad4-WT was replaced by the GSK3 phosphorylation-resistant Smad4-GM, both in human cultured cells and in *Xenopus* embryos. This indicates that the crosstalk between Wnt and TGF- $\beta$  described here is mainly mediated by Smad4 GSK3 sites and not by other components of the TGF- $\beta$ -signaling pathway. Perhaps the co-Smad Smad4 evolved a specialized role in the integration of multiple signaling pathways.

Wnt and FGF/EGF growth factors had striking effects on Smad4 transcriptional activity, particularly at low TGF- $\beta$  concentrations (Figure 4). They also had an effect on Smad4 stability by triggering the polyubiquitination and proteasomal degradation of the fraction of Smad4 phosphorylated by MAPK and GSK3 (Figures 2 and 3). A short Smad4 activation domain (SAD) that contains the MAPK site (but not the GSK3 sites) had been described (de Caestecker et al., 2000). We now found that the linker domain of Smad4 acts as a Wnt-stimulated activation domain independently of protein degradation (Figure 5).

### $\beta$ -TrCP Binds to the Smad4 Phosphodegron

Smad4 is polyubiquitinated and degraded by  $\beta$ -TrCP (Wan et al., 2004, 2005; Yang et al., 2006). We now show that the binding of  $\beta$ -TrCP to Smad4 is not constitutive but finely regulated by GSK3 linker phosphorylations triggered by FGF and inhibited by Wnt. In *Drosophila* egg chambers, clonal analysis of *slmb* mutations (the  $\beta$ -TrCP homolog) revealed high levels of Medea protein (the Smad4 homolog), together with a high-BMP phenotype (Muzzopappa and Wappner, 2005). The first two Smad4 GSK3 sites

have been conserved in *Drosophila*, other insects, and even planarians (data not shown), suggesting that linker phosphorylations represent an ancient mechanism that regulates Smad4 activity during embryonic patterning.

The positive effect of threonine 277 phosphorylation on Smad4 activity (Figure 6) and the presence of a transcriptional activation domain in Smad4 suggest that coactivators might bind to the monophosphorylated PxTP site to drive transcription. A prime candidate is p300, which has been shown to bind to the SAD domain of Smad4 (de Caestecker et al., 2000). Recently, it has been found that the mediator of the Hippo pathway YAP binds phosphorylated SP sites in the Smad1 sequence (Alarcón et al., 2009; Aragón et al., 2011) through its WW domain. The other mediator of the Hippo pathway, TAZ, has been shown to bind active Smad2/3/4 complexes and to connect TGF- $\beta$  signaling to cell density (Varelas et al., 2008, 2010). It is therefore tempting to speculate that TAZ or YAP may recognize the phosphorylated TP site in Smad4 acting as coactivators. Alternatively, the Smad4 linker region might recruit other coactivators, depending on cellular context. Future studies will be required to identify Smad4 phospholinker-interacting proteins.

### Signaling Insulation and Crosstalk

Wnt signaling depletes active GSK3 from the cytosol, potentially affecting the phosphorylation of many proteins in addition to  $\beta$ -catenin (Taelman et al., 2010; Vinyoles et al., 2014; Acebron et al., 2014). This raises the general question of how signaling pathways are normally insulated from, or integrated with, each other. The regulation of Smad4 activity by Wnt, which is observed only in the presence of MAPK activation (or by introducing a phospho-mimetic priming site) indicates that the choice between insulation and crosstalk depends on priming kinases regulated by growth factors.

In the *Xenopus* embryo, it has been determined that, shortly after midblastula (stage 8.5), nuclear  $\beta$ -catenin, diphospho Erk, and C-terminal phospho-Smad2 are found in dorsal-marginal cells (Schohl and Fagotto, 2002). These protein distributions result from a maternal Wnt signal, a marginal zone gradient of FGF that starts on the dorsal side, and a Nodal/TGF- $\beta$  gradient emanating from the dorsal-vegetal pole (Figure 7K; De Robertis and Kuroda, 2004). This may generate a perfect storm of growth factor signals that converge on the Smad4 protein to generate maximal transcriptional activation. In this view, the different territories of the embryo would be shaped and defined by Wnt/GSK3 and FGF/MAPK feeding on the Nodal/TGF- $\beta$  morphogen gradient. Other mechanisms including combinations of transcription factors, such as Siamois/Twin and activated Smad2/3/4 at the level of specific promoters, will be important as well (Labbé et al., 2000; Reid et al., 2012).

Replacement of endogenous Smad4 with its GSK3 phosphorylation-resistant mutant in *Xenopus* embryos resulted in the entire ectoderm becoming mesoderm. This suggests that GSK3 inhibition of Smad4 plays an essential role in allowing ectodermal differentiation in vivo and extends previous findings in the field, indicating a key role for Smad4 in ectoderm specification (Dupont et al., 2005, 2009). In addition, phosphorylation of Smad4 by GSK3 serves to constrain the size of Spemann's organizer. The crosstalk between the Wnt and Nodal/TGF- $\beta$

pathways at the level of Smad4 could help explain in part the mysterious “competence modifier” effect observed in *Xenopus*, in which xWnt8 mRNA does not induce mesoderm by itself, yet greatly sensitizes the competence of ectoderm to respond to activin/TGF- $\beta$  (Sokol and Melton, 1992; Moon and Christian, 1992).

### Smad4 Linker Phosphorylation and Tumor Suppression

In cancer, Smad4/DPC4 acts as a barrier for tumor progression (Ding et al., 2011; Vogelstein et al., 2013). TGF- $\beta$  signaling has potent antiproliferative effects in epithelia through the activation of cyclin-dependent kinase inhibitors such as p14<sup>Ink4b</sup> and p21<sup>WAF1</sup> (Hanahan and Weinberg, 2011). At early stages, many tumors are driven by activation of the Ras/Erk and the Wnt oncogenic pathways, which increase proliferation genes such as cyclin D and c-Myc (Hanahan and Weinberg, 2011). In our proposed mechanism, these mitogenic effects will be counterbalanced by the increase in TGF- $\beta$ /Smad4 antiproliferative activity mediated by MAPK and Wnt/GSK3 signaling. This barrier effect of TGF- $\beta$  is lost when the Smad4 tumor suppressor is deleted or inhibited. The discovery that Smad4 activity is not constitutive but instead regulated by growth factors helps understand why its loss has such catastrophic consequences during progression of pancreatic, colorectal, and prostate cancers.

Smad4 is frequently deleted in metastatic tumors, but intragenic point mutations are also found (Levy and Hill, 2006; Xu and Attisano, 2000). Interestingly, several of these point mutations increase Smad4 degradation by facilitating binding to  $\beta$ -TrCP (Wan et al., 2005; Yang et al., 2006). Our finding that  $\beta$ -TrCP binding to Smad4 is regulated by GSK3 phosphorylations suggests that pharmacological GSK3 inhibitors may stabilize Smad4 and restore growth control in such tumors.

### EXPERIMENTAL PROCEDURES

#### Mammalian Cell Culture

NIH 3T3, CAGA12-HaCaT, HEK293 (lacking T antigen, which respond very well to TGF- $\beta$ ), L cells (ATCC no. CRL-2648), as well as L-Wnt3a cells (ATCC no. CRL-2647) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO) and cultured at 37°C in 5% CO<sub>2</sub>. MDA-MB-468 cells (which lack Smad4) were cultured in DMEM:Ham's-F12 (1:1 vol:vol). L cell control-conditioned medium and Wnt3a-conditioned medium were prepared according to the ATCC protocol (Willert et al., 2003), with the exception that 2% serum was used. Wnt3a-conditioned medium was further boosted by adding 200 ng/ml of recombinant murine Wnt3a protein (PeproTech). DNA constructs were transfected with BioT (Bioland) 24 hr after plating cells. siRNAs were transfected with Lipofectamine 2000 using the reverse transfection protocol (Invitrogen) and analyzed after 48 hr. Cycloheximide (Sigma no. C-7698) was dissolved in ethanol and used at a final concentration of 20 mg/ml (Taelman et al., 2010).

#### Antibodies

The following antibodies were used in this study:  $\alpha$ -Smad4 monoclonal (Santa Cruz Biotechnology B-8; 1:250),  $\alpha$ -diphosphorylated ERK-1 and ERK-2 monoclonal (Sigma; 1:500),  $\alpha$ -GAPDH (Cell Signaling Technology 14C10; 1:7,000),  $\alpha$ -Flag mouse (Sigma; 1:3,000), rabbit  $\alpha$ -ubiquitin (Santa Cruz Biotechnology FL-76; 1:200),  $\alpha$ -hemagglutinin (HA) (Sigma; 1:3,000), rabbit  $\alpha$ - $\beta$ -TrCP (Cell Signal D13F10; 1:800), and mouse  $\alpha$ -Gal4DBD (Santa Cruz RK5C1; 1:200). Secondary antibodies used were IRDye 800CW Donkey anti-Rabbit immunoglobulin G (IgG) (LI-COR Biosciences 926-32213; 1:5,000) and IRDye 680RD Donkey anti-Mouse IgG (LI-COR Biosciences 926-68072; 1:5,000). For custom pSmad4<sup>GSK3</sup> antibody, a synthetic peptide ([H]-CKK-Acp-NSTTTWT(PO3)GSRT(PO3)APY-[NH2]) was used to immunize two rabbits

(Covance). The antiserum with the highest ELISA titer was positively affinity purified and was used at a concentration of 1:5,000 for detection of endogenous Smad4 phosphorylations and at 1:25,000 for overexpressed proteins.

#### Statistical Analyses

Results are given as the mean  $\pm$  SEM. Statistical analyses were performed with Excel (Microsoft), applying the two-tailed t test. Differences of means were considered significant at a significance level of 0.05. The following symbols are annotated: n.s., not significant ( $p > 0.05$ ); \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ .

#### Additional Methods

Detailed methods for cell culture, plasmid reagents, polyubiquitination assays, western blotting, Phos-tag analyses, *Xenopus* embryo assays, quantitative RT-PCR, Gal4DBD transcriptional assays, dissociation of *Xenopus* animal cap cells, reporter gene assays, phosphatase treatment, and construction of the new Smad4-Luc reporter gene are provided in Supplemental Experimental Procedures.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2014.09.020>.

### AUTHOR CONTRIBUTIONS

H.D. and E.M.D.R. designed research. H.D. performed all biochemical experiments. T.A. generated the novel Smad4-luc reporter and carried out RT-PCR experiments. H.D. and E.M.D.R. performed the *Xenopus* experiments and wrote the manuscript.

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