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Article

PAF and EZH2 Induce Wnt/β-Catenin Signaling Hyperactivation

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SUMMARY

Fine control of Wnt signaling is essential for various cellular and developmental decision-making processes. However, deregulation of Wnt signaling leads to pathological consequences, one of which is cancer. Here, we identify a function of PAF, a component of translesion DNA synthesis, in modulating Wnt signaling. PAF is specifically overexpressed in colon cancer cells and intestinal stem cells and is required for colon cancer cell proliferation. In *Xenopus laevis*, ventrovegetal expression of PAF hyperactivates Wnt signaling, developing a secondary axis with β-catenin target gene upregulation. Upon Wnt signaling activation, PAF dissociates from PCNA and binds directly to β-catenin. Then, PAF recruits EZH2 to the β-catenin transcriptional complex and specifically enhances Wnt target gene transactivation, independently of EZH2's methyltransferase activity. In mice, conditional expression of PAF induces intestinal neoplasia via Wnt signaling hyperactivation. Our studies reveal an unexpected role of PAF in regulating Wnt signaling and propose a regulatory mechanism of Wnt signaling during tumorigenesis.

INTRODUCTION

Strict regulation of stem cell proliferation and differentiation is required for mammalian tissue homeostasis and its repair in the setting of tissue damage. These processes are precisely orchestrated by various developmental signaling pathways, and dysregulation contributes to disease and genetic disorders, including cancer (Beachy et al., 2004). Cancer is initiated by the inactivation of tumor-suppressor genes and the activation of oncogenes. For instance, colon cancer cells harbor genetic mutations in Wnt/β-catenin pathway constituents such as adenomatous polyposis coli (*APC*), *Axin*, and *β-catenin* (Polakis, 2007). In mouse models, inactivation of APC or activation of β-catenin results in the development of intestinal hyperplasia and adenocarcinoma (Moser et al., 1990), indicating that hyperactivation of Wnt signaling promotes intestinal tumorigenesis.

In canonical Wnt signaling, Wnt ligand induces stabilization of β-catenin protein via inhibition of the protein destruction complex (glycogen synthase kinase 3, APC, casein kinase I, and Axin). Then, activated β-catenin is translocated into the nucleus and binds to its nuclear interacting partners, TCF and LEF. Finally, β-catenin-TCF/LEF transactivates the expression of its target genes (Clevers and Nusse, 2012).

Although various Wnt/β-catenin modulators have been identified (the Wnt homepage, wnt.stanford.edu), the pathological relevance of these modulators to tumorigenesis remains elusive. Also, many reports have suggested that mutation-driven Wnt-signaling activation can be enhanced further (Goentoro and Kirschner, 2009; He et al., 2005; Suzuki et al., 2004; Vermeulen et al., 2010), which implies the presence of an additional layer of Wnt signaling regulation in cancer beyond genetic mutations in *APC* or *β-catenin*. Here, we unravel a function of the DNA-repair gene, *PAF* (PCNA-associated factor)/*KIAA0101*. PAF was shown to be involved in translesion DNA synthesis (TLS), an error-prone DNA-repair process that permits DNA-replication machinery to replicate DNA lesions with specialized translesion DNA polymerase (Emanuele et al., 2011; Povlsen et al., 2012; Sale et al., 2012). Our comprehensive approaches reveal that PAF expressed specifically in cancer hyperactivates Wnt/β-catenin signaling and induces intestinal tumorigenesis.

RESULTS

Mitogenic Role of PAF via Wnt Signaling

To identify colon-cancer-specific Wnt signaling regulators, we analyzed multiple sets of human colon cancer tissue samples using the publicly available database (www.oncomine.org) and selected genes that are highly expressed in colon cancer cells (fold change >2; $p < 0.0001$; top 10% ranked). Among several genes, we investigated the biological role of *PAF*, based on its significant overexpression in human colon adenocarcinoma with correlated expression of *Axin2*, a well-established specific target gene of β-catenin (Jho et al., 2002; Lustig et al., 2002)(Figure 1A). To validate our in silico analysis, we performed immunostaining of a colon cancer tissue microarray and confirmed that

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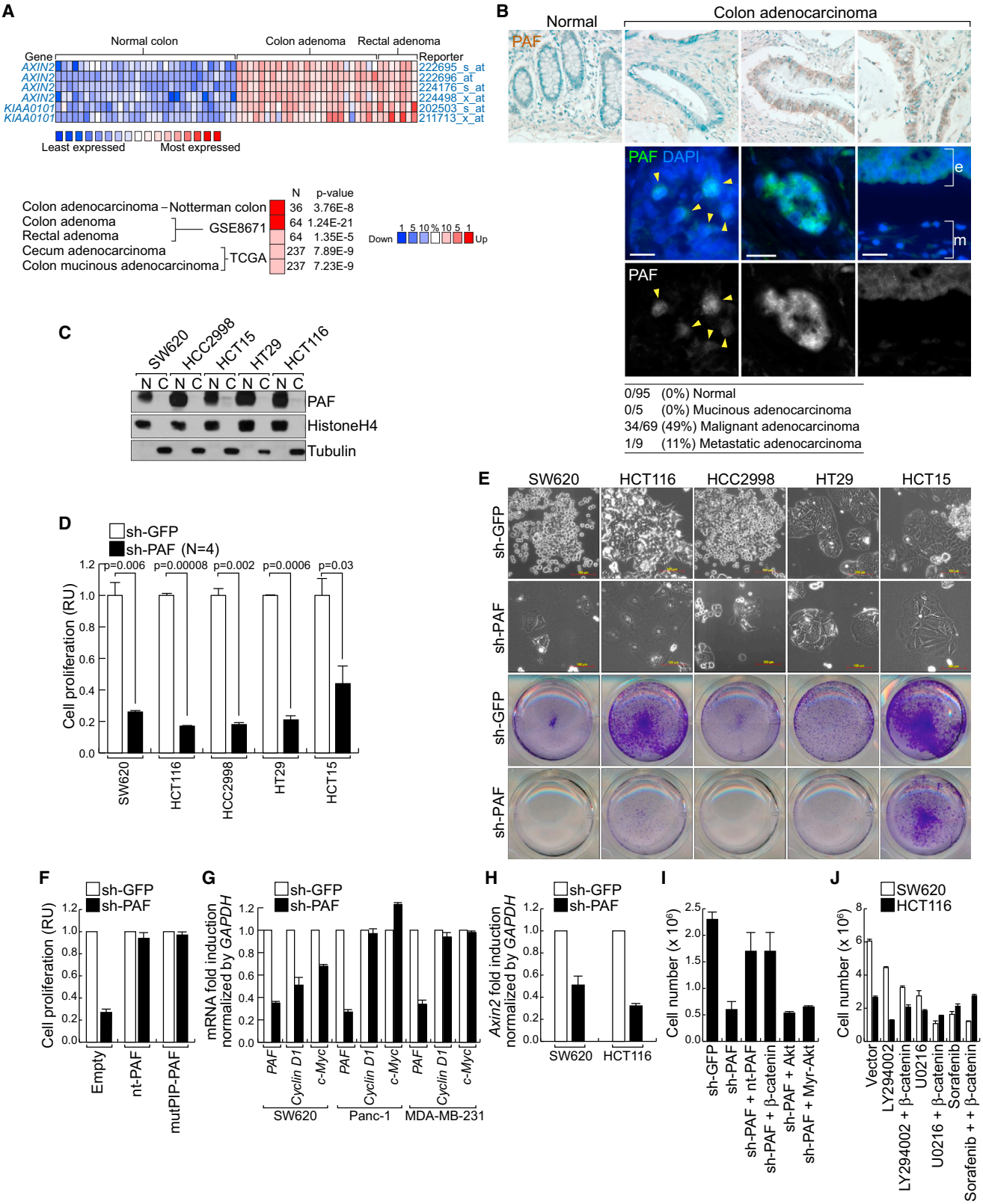

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PAF was highly expressed in colon cancer cells, whereas its expression was barely detectable in the normal intestine (Figure 1B). Consistent with this, PAF was strongly expressed and mainly localized in the nucleus of colon cancer cell lines (Figure 1C). Additionally, we found that PAF was not expressed in nontransformed cells such as NIH 3T3, mouse embryonic fibroblasts, and mammary epithelial cells (data not shown). Next, to assess the relevance of PAF upregulation in colon cancer cell proliferation, we depleted endogenous PAF using short hairpin RNAs (shRNAs) in these cell lines. Intriguingly, *PAF* knockdown (sh-PAF) inhibited colon cancer cell proliferation (Figures 1D and 1E). Given that PAF was shown to interact with PCNA via a PCNA-interacting protein (PIP) box (Yu et al., 2001), we also examined whether PAF-PCNA interaction is required for mitogenic effects of PAF. In reconstitution experiments, sh-PAF-induced cell growth inhibition was rescued by ectopic expression of both shRNA nontargetable wild-type PAF (nt-PAF) and PIP mutant PAF (mutPIP-PAF) (Figure 1F), indicating that the PAF-PCNA interaction is not necessary for PAF-mediated colon cancer cell proliferation. Interestingly, PAF knockdown downregulated cell-proliferation-related genes (*Cyclin D1* and *c-Myc*) (Figure 1G). Given that *Cyclin D1* and *c-Myc* are β-catenin direct target genes (He et al., 1998; Tetsu and McCormick, 1999), PAF probably participates in regulating Wnt/β-catenin signaling. Interestingly, PAF-depletion-induced downregulation of *Cyclin D1* and *c-Myc* was only observed in SW620 colon cancer cells, not in Panc-1 and MDA-MB-231 cells (Figure 1G), indicating the specific effects of PAF on *Cyclin D1* and *c-Myc* expression in colon cancer cells. We also assessed the effects of PAF knockdown on *Axin2*. Indeed, PAF knockdown suppressed *Axin2* transcription in colon cancer cells (Figure 1H). Moreover, as nt-PAF did, β-catenin ectopic expression reverted sh-PAF-induced cell growth arrest (Figure 1I), implying that PAF might be functionally associated with Wnt/β-catenin. We also examined whether other mitogenic signaling pathways mediate PAF's mitogenic role. Of note, except HT29, other colon cancer cell lines (SW620, HCT116, HCC2998, and HCT15) harbor oncogenic mutations in the *K-Ras* gene. Nonetheless, PAF depletion induced the suppression of cell growth on all five colon cancer cells (Figure 1D), indicating that PAF's mitogenic function is independent of Ras/MAPK (mitogen-activated protein kinase) signaling activation. Additionally, overexpression of wild-type Akt or a constitutively active form of Akt (myristoylated form of Akt [Myr-Akt]) did not rescue sh-PAF-induced inhibition of cell proliferation (Figure 1I). Moreover, β-catenin activation did not revert the cell-proliferation suppression resulting from MAPK or PI3K inhibition (Figure 1J), indicating that β-catenin-mediated mitogenic function is independent of MAPK and PI3K signaling pathways. These results suggest that PAF contributes to colon cancer cell proliferation, possibly via Wnt/β-catenin signaling.

PAF Positively Modulates Wnt Signaling

Given that many cancers develop as a result of the deregulation of developmental signals (Beachy et al., 2004), analyzing PAF expression during development may provide insights into the mechanisms of PAF-mediated signaling regulation. Whole-mount immunostaining of mouse embryos showed that PAF was specifically enriched in the apical ectodermal ridge (AER) of the limb bud, midbrain, hindbrain, and somites (Figure 2A and data not shown). During limb development, AER induction is specifically coordinated by active Wnt signaling (Figure 2B)(Kengaku et al., 1998). Using *Axin2-LacZ*, a β-catenin reporter (Lustig et al., 2002), and mouse embryos, we confirmed the specific activation of Wnt signaling in AER (Figure 2C). Intriguingly, Wnt signaling activity, as exhibited in the AER, overlapped with the pattern of PAF expression (Figures 2A and 2C). Given that (1) Wnt signaling is deregulated in most colon cancer, (2) PAF is highly overexpressed in colon cancer cells, (3) PAF is required for colon cancer cell proliferation (Figure 1D), and (4) PAF is enriched in AER where Wnt signaling is active (Figure 2A), we hypothesized that PAF modulates the Wnt signaling pathway. To test this, we first examined the impact of PAF on β-catenin transcriptional activity using TOPFLASH reporter assays. In HeLa cells, PAF knockdown decreased β-catenin reporter activation with the use of 6-bromoindirubin-3′-oxime, a GSK3 inhibitor (Figure 2D). Similarly, Wnt3A-induced transcriptional

Figure 1. Mitogenic Role of PAF in Colon Cancer Cells

(A) Expression of PAF in human colon cancer cells. Oncomine analysis of *PAF* gene expression in human colon cancer cells is shown. Comparison of *Axin2* and *PAF* expression is shown in the upper panel (GSE8671; fold change >2; p value < 0.0001; gene rank < top 10%). Comparison of PAF expression in colon cancer tissues and in normal tissues is shown in the lower panel (fold change >4; average p value = 9.34×10^{-6}; gene rank < top 10%; N = sample number).

(B) Upregulation of PAF in human colon cancer tissues. Human colon cancer tissue microarray samples were analyzed for immunohistochemistry (DAB [brown]: PAF; hematoxylin [blue]: nuclei) and immunofluorescent staining (arrowheads: nuclear PAF; e: epithelial cells; m: mesenchymal cells). The scale bar represents 20 μm.

(C) Expression of PAF in human colon cancer cell lines. Colon cancer cell lines were fractionated into nuclear (N) and cytosolic (C) fractions for immunoblotting (IB). Fractionation controls: histone H4 (nucleus) and tubulin (cytosol).

(D and E) PAF depletion inhibits cell proliferation. Each cell line stably expressing shRNAs (sh-GFP [control] and sh-PAF) were analyzed using cell counting (D) and crystal violet staining (E); RU: relative units.

(F) PCNA-interaction-independent mitogenic role of PAF. SW620 (sh-GFP and sh-PAF) were stably transfected with nt-PAF or mutPIP-PAF for cell-proliferation analysis (cell counting).

(G) Downregulation of *Cyclin D1* and *c-Myc* by PAF knockdown. SW620, Panc-1, and MDA-MB-231 cells stably expressing sh-GFP or sh-PAF were analyzed using qRT-PCR.

(H) *Axin2* downregulation by PAF depletion. SW620 and HCT116 cells were analyzed using qRT-PCR.

(I) β-catenin rescues PAF-depletion-induced cell growth inhibition. SW620 (sh-GFP and sh-PAF) cells were transfected with each plasmid and analyzed by cell counting. N = 3.

(J) β-catenin is not involved in activating MAPK and PI3K signals. SW620 and HCT116 cells stably expressing β-catenin or empty vector were treated with LY294002 (PI3K inhibitor; 10 μm), U0216 (MEK1/2 inhibitor; 10 μm), or Sorafenib (Raf inhibitor; 10 μm). After 3 days, cells were counted.

All error bars indicate SD.

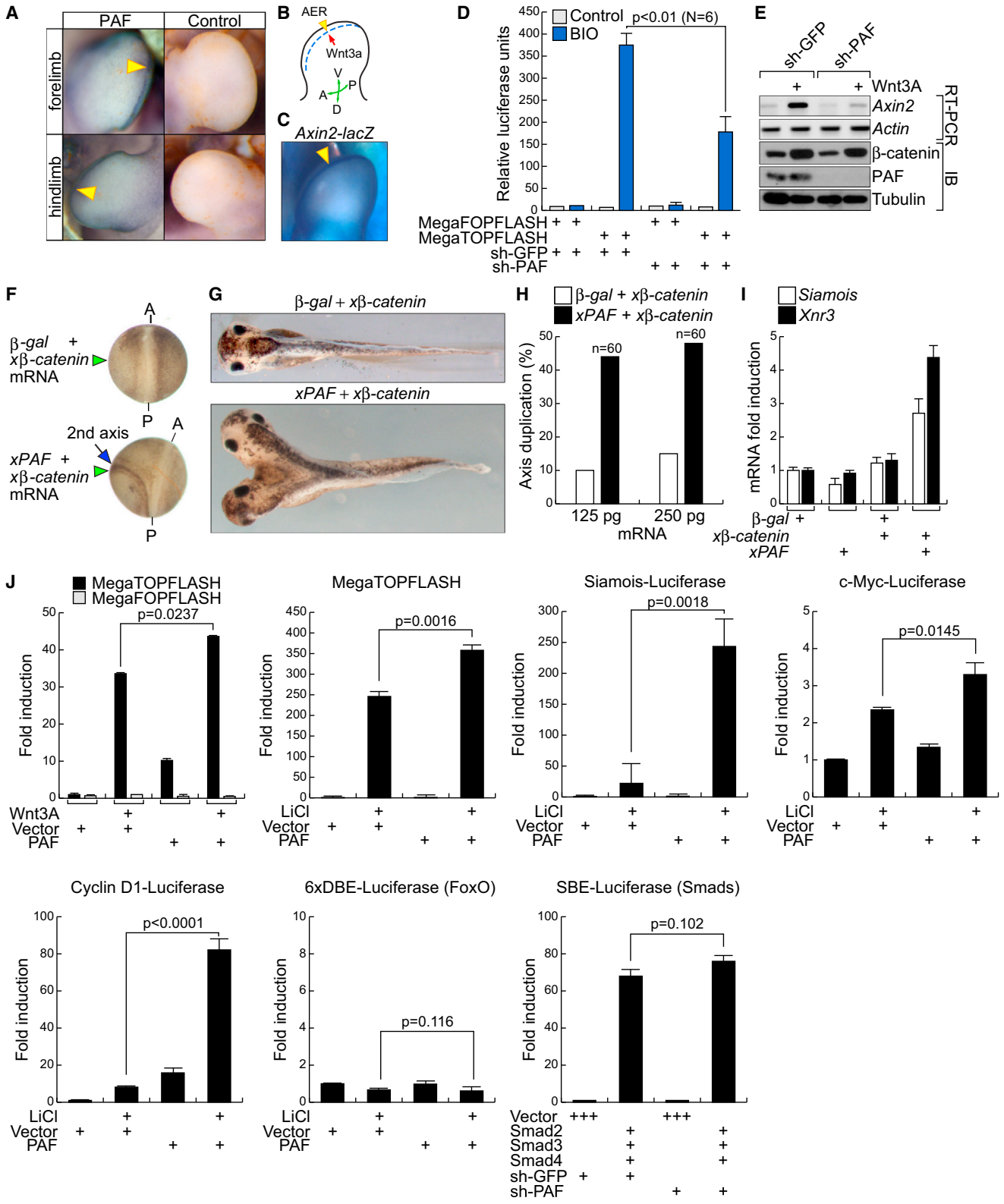

Figure 2. Activation of Wnt Signaling by PAF

(A) PAF expression in AER. Whole-mount immunostaining for PAF in mouse embryos at embryonic day 11. Arrowheads represent PAF.

(B) Schematic diagram of AER in the limb bud. Dotted line, AER; A, anterior; P, posterior; D, dorsal; V, ventral.

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activation of *Axin2* was also inhibited by PAF depletion (Figure 2E). These data suggest that PAF might be required for Wnt target gene expression.

To gain better insight of PAF's role in Wnt signaling regulation, we utilized *Xenopus laevis* embryos for axis-duplication assays (Funayama et al., 1995), as previously performed (Park et al., 2009). Because of Wnt signaling's pivotal role in vertebrate anterior-posterior axis development, the effects of *Xenopus* PAF (xPAF) on Wnt signaling can be monitored and quantified on the basis of secondary axis formation following injection of in vitro transcribed messenger RNAs (mRNAs). *xβ-catenin* mRNA, titrated to a subphenotypic level when expressed in isolation, was coinjected with *xPAF* mRNA into ventrovegetal blastomeres. Unlike the controls (*β-catenin* and *β-galactosidase* mRNA), the experimental group (*β-catenin* and x*PAF* mRNA) displayed axis duplications (Figures 2F–2H). Of note, the ventrovegetal injection of *xPAF* mRNA alone failed to induce secondary axes (data not shown), showing that PAF hyperactivates Wnt/β-catenin signaling only in the presence of active β-catenin. Consistent with the results of axis-duplication assays, quantitative RT-PCR (qRT-PCR) assays showed that xPAF expression upregulated expression of *Siamois* and *Xnr3*, β-catenin targets in frogs (Figure 2I). Furthermore, we examined the specificity of PAF on Wnt/β-catenin signaling activity using various luciferase assays. Ectopic expression of PAF hyperactivates activation of β-catenin target-gene reporter activity (MegaTOPFLASH, *Siamois*, *c-Myc*, and *Cyclin D1*) induced by Wnt3A or LiCl, a GSK3 inhibitor. Of note, the BMP/Smad pathway also plays an essential role in the developing limb AER (Ahn et al., 2001). However, PAF knockdown or overexpression did not affect BMP/Smad or FoxO signals, respectively (Figure 2J), indicating the specificity of PAF in regulating Wnt signaling. These results suggest that PAF positively modulates Wnt/β-catenin signaling in vitro and in vivo.

PAF-EZH2-β-Catenin Transcriptional Complex Formation

Next, we investigated the molecular mechanism underlying PAF hyperactivation of Wnt signaling. Given that stabilization of β-catenin protein is a key process in transducing Wnt signaling, we asked whether PAF affects the β-catenin protein level. However, we found that the level of β-catenin protein was not altered by PAF knockdown or overexpression (Figures 2E and 3A), leading us to test whether PAF controls β-catenin/TCF transcriptional complex activity. Owing to the nuclear-specific localization of PAF in colon cancer cells (Figure 1C), we tested whether PAF interacts with the β-catenin transcriptional complex. Using a glutathione S-transferase (GST) pull-down assay, we found that PAF bound to β-catenin and TCF proteins (Figure 3B). Also, endogenous PAF interacted with β-catenin and TCF3 in SW620 cells that display constitutive hyperactivation of Wnt signaling by *APC* mutation (Figure 3C). Moreover, binding-domain mapping assays showed that the Armadillo repeat domain of β-catenin was essential for its interaction with PAF (Figure 3D). Although PAF is a cell-cycle-regulated anaphase-promoting complex substrate (Emanuele et al., 2011), PAF-β-catenin interaction was not affected (Figure S1). These data suggest that PAF directly binds to the β-catenin transcriptional complex and that this interaction is independent of cell cycle. Next, due to interaction of PAF with β-catenin and TCF, we tested whether PAF is also associated with Wnt/β-catenin target genes. First, we analyzed the subnuclear localization of PAF by chromatin fractionation. We found that PAF was only detected in the chromatin fraction of HCT116 cells (Figure 3E). Second, chromatin immunoprecipitation (ChIP) assays showed that both ectopically expressed and endogenous PAF occupied the TCF-binding element (TBE)-containing proximal promoter of the β-catenin targets (*c-Myc* and *Cyclin D1*) in HCT116 cells (Figures 3F and 3G). These data show that PAF is specifically associated with the promoters of Wnt/β-catenin target genes.

In the intestine, Wnt/β-catenin signaling constitutively activates intestinal stem cells (ISCs) to give rise to progenitor cells, which replenishes the intestinal epithelium (Figure 3H). Given the involvement of PAF in Wnt/β-catenin signaling regulation (Figure 2), we analyzed the spatial expression of PAF in intestinal epithelium. Immunostaining showed that PAF was specifically expressed in B lymphoma Mo-MLV insertion region 1 homolog (Bmi1)-positive ISCs (Figures 3I and 3J). Bmi1 and its associated components in Polycomb-repressive complexes 1 and 2 (PRC1 and PRC2) are shown to epigenetically regulate gene expression (Sparmann and van Lohuizen, 2006). Because of (1) specific association of PAF with TBEs of β-catenin target promoters (Figures 3F and 3G) and (2) colocalization with Bmi1-positive ISCs (Figure 3J), we asked whether PAF is associated with components of PRC1 and PRC2, using coimmunoprecipitation (coIP) assays. Intriguingly, PAF interacted with both Bmi1 and EZH2 (enhancer of zeste homolog 2) in SW620 cells (Figure 3K), which led us to test whether either Bmi1 or EZH2 is functionally associated with PAF-mediated Wnt signaling hyperactivation. To do this, we assessed the effects of Bmi1 and EZH2 on β-catenin

(C) Active Wnt/β-catenin signaling in the AER. *Axin2-LacZ* mouse embryos at embryonic day 12 were stained with X-gal (arrowhead).

(D and E) PAF knockdown suppresses β-catenin transcriptional activity. HeLa (sh-GFP or -PAF) were transiently transfected with β-catenin reporter and renilla plasmids. Twenty-four hours after transfection, cells were treated with 0.5 μM BIO. After 6 hr, luciferase activity was measured (D). 293T cells stably transduced with sh-GFP or sh-PAF lentivirus were treated with Wnt3A (100 ng/ml, 6 hr) for RT-PCR (E).

(F–H) Axis duplication by xPAF-induced β-catenin hyperactivation. *xPAF* or *β-galactosidase* mRNA with *β-catenin* mRNA was injected into the ventrovegetal blastomeres of *X. laevis* embryos. The secondary axis was examined from neurulation (F) to tail-bud stages (G). The *xβ-catenin* mRNA concentration was titrated to prevent induction of axis duplication per se. A: anterior; P: posterior. Two different doses of *xPAF* mRNA with *β-catenin* mRNA were injected into frog embryos for axis-duplication analysis (H).

(I) β-catenin target gene upregulation by xPAF. *X. laevis* embryos injected with each mRNA at the two-cell stage were collected at the gastrulation stage for qRT-PCR. Ornithine decarboxylase (*ODC*) was used as an internal control.

(J) PAF-induced hyperactivation of Wnt/β-catenin target gene reporters. 293T cells transfected with each plasmid were treated with Wnt3A (100 ng/ml, 24 hr) or LiCl (25 mM, 24 hr) and analyzed for luciferase assays. N = 3.

All error bars indicate SD.

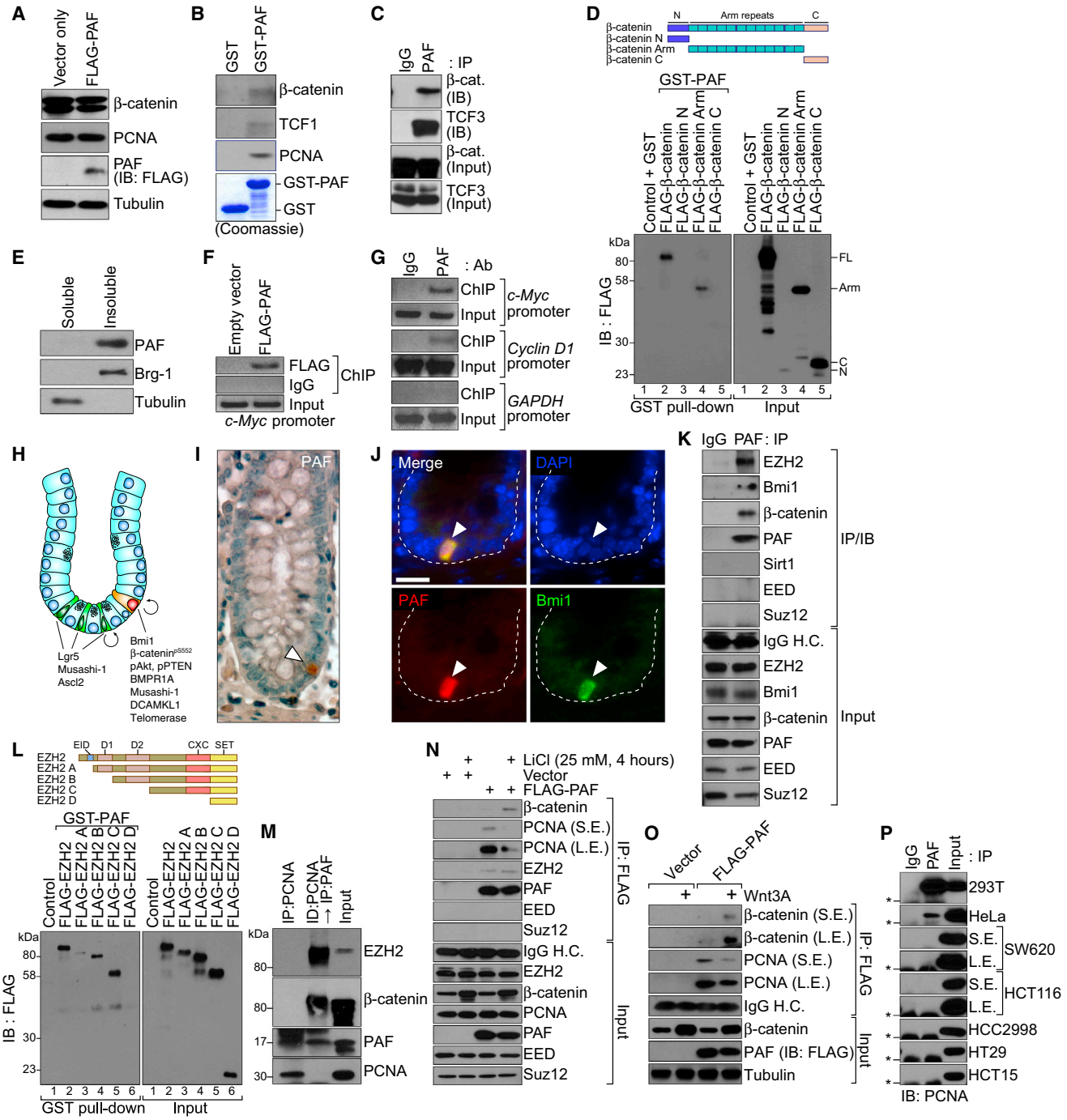

Figure 3. PAF-EZH2-β-Catenin Transcriptional Complex at Target-Gene Promoters

(A) No effect of PAF on β-catenin protein stability. HeLa cells were transiently transfected with FLAG-PAF-pcDNA for IB.

(B and C) Interaction of PAF with β-catenin and TCF/LEFs. GST-PAF was used for pull-down with SW620 cell lysates and IB (B). SW620 cells were analyzed for coIP and IB (C).

(D) PAF-β-catenin interaction via the Armadillo repeat domain. GST-PAF was incubated with in vitro-transcribed and translated FLAG-tagged β-catenin deletion mutants (N, N-terminal domain; Arm, Armadillo repeat domain; C, C-terminal domain; FL, full length) and analyzed using GST pull-down and IB.

(E) PAF is a chromatin-associated protein. A chromatin-associated lysate (insoluble) and a soluble fraction of HCT116 were analyzed for IB. Brg-1: a chromatin-fraction control.

(F and G) PAF occupies TBEs. HCT116 stably expressing FLAG-PAF (F) and parental cells (G) was analyzed using ChIP. *GAPDH* promoter: a negative control. IgG, immunoglobulin G.

(legend continued on next page)

transcriptional activity using β-catenin reporter assays. We observed that ectopic expression of EZH2 upregulated β-catenin transcriptional activity, but Bmi1 overexpression did not (data not shown), implying that EZH2 might be associated with Wnt signaling activation. Binding-domain mapping analysis showed that EZH2 bound to PAF via the middle region of EZH2 including the CXC cysteine-rich domain (Figure 3L). In conjunction with the Bmi1-containing PRC1, the EZH2-containing PRC2 catalyzes histone H3 lysine 27 trimethylation (H3K27me3) via the histone-methyltransferase domain. Despite the crucial role of EZH2 in H3K27me3-mediated gene regulation, we found that other core components of PRC2, EED, and Suz12 were not associated with PAF (Figure 3K). Moreover, although EZH2 overexpression in cancer induces PRC4 formation in association with the NAD^+-dependent histone deacetylase Sirt1 (Kuzmichev et al., 2005), the PAF-EZH2 complex did not contain Sirt1 (Figure 3K). These data indicate that the PAF-EZH2 complex is distinct from the conventional PRCs in cancer cells. Also, we questioned whether PCNA is required for PAF's interaction with either PAF or β-catenin. Interestingly, β-catenin-PAF and EZH2-PAF complexes existed only in PCNA-free fractions (Figure 3M, compare lanes 1 and 2), which is consistent with a PCNA-independent mitogenic role of PAF in colon cancer cell proliferation (Figure 1I). Due to exclusive interaction of PAF with either PCNA or β-catenin, we asked whether Wnt signaling activation affects either PAF-β-catenin or PAF-PCNA interaction. CoIP assays showed that, in HeLa cells, PAF-β-catenin interaction was only detected upon LiCl treatment, whereas PAF-EZH2 interaction remained constant. Moreover, PAF-PCNA association was decreased by LiCl or Wnt3A treatment (Figures 3N and 3O, compare lanes 3 and 4). These data suggest that Wnt signaling activation is required for PAF-β-catenin interaction. Due to the absence of endogenous Wnt signaling activity in HeLa cells, we also assessed the effects of active Wnt/β-catenin signaling on PAF-PCNA binding in colon cancer cell lines that exhibit hyperactivation of Wnt signaling by genetic mutations in *APC* or *β-catenin* alleles. Surprisingly, PAF-PCNA interaction was barely detectable in colon cancer cell lines, whereas 293T and HeLa cells displayed strong PAF-PCNA association (Figure 3P), implying that active β-catenin may sequester PAF from PCNA. In our binding-domain mapping analysis, we also found that N-terminal and PIP regions are required for β-catenin interaction (Figure S2 available online), suggesting that β-catenin competes with PCNA for PAF interaction. These results suggest that, upon Wnt signaling activation, PAF is conditionally associated with the β-catenin transcriptional complex.

PAF Activates β-Catenin Target Genes by Recruiting EZH2 to Promoters

Previous studies showed that EZH2 interacts with β-catenin (Li et al., 2009; Shi et al., 2007). Also, we found that PAF is physically associated with EZH2, independently of PRC2 (Figure 3). This evidence prompted us to ask whether EZH2 mediates PAF-induced Wnt signaling hyperactivation. Given PAF-EZH2-β-catenin complex formation, we tested whether EZH2 is also associated with the promoters of β-catenin target genes. Intriguingly, PAF, EZH2, and β-catenin steadily co-occupied the promoters of *c-Myc*, *Cyclin D1*, and *Axin2* in HCT116 cells carrying a *β-catenin* mutation, whereas PCNA, EED, and Suz12 did not (Figure 4A), which recapitulates PRC2-independent association of EZH2 with PAF (see Figures 3K and 3N). Next, we asked whether PAF, EZH2, and β-catenin are recruited to a β-catenin target gene promoter upon Wnt signaling activation, given that PAF-β-catenin interaction was dependent on Wnt signaling activation (Figure 3N). In HeLa cells, we found that PAF, EZH2, and β-catenin conditionally bound to TBEs in the *c-Myc* and *Axin2* promoters only upon LiCl treatment (Figure 4B), indicating that Wnt signaling activation is a prerequisite for PAF-β-catenin-EZH2's promoter association. To further confirm the specificity of PAF-EZH2-β-catenin's recruitment to β-catenin target promoters, we performed ChIP promoter scanning of 10 kb of the *c-Myc* promoter and found that PAF, EZH2, and β-catenin specifically co-occupied the proximal promoter containing TBEs of the *c-Myc* gene (at −1,037 and −459 bp) (He et al., 1998) in HCT116 cells (Figure 4C). Also, the analysis of EZH2 ChIP-sequencing (ChIP-seq) data from mouse embryonic stem cells showed that EZH2 was specifically enriched in the proximal promoters of β-catenin targets (*Lef1*, *Lgr5*, *c-Myc*, and *Axin2*) (Figure 4D).

Next, we asked whether EZH2 promoter recruitment is necessary for activation of β-catenin target gene transcription. Previously, depletion of EZH2 was shown to inhibit *c-Myc* expression in DLD-1 colon cancer cells (Fussbroich et al., 2011). Consistent with this, EZH2 knockdown downregulated β-catenin target genes *Axin2* and *Cyclin D1* in HCT116 cells (Figure 4E) and decreased LiCl-induced β-catenin reporter activation (Figure 4F), suggesting that EZH2 is required for PAF-mediated Wnt target gene hyperactivation. These results are also supported by previous finding that EZH2 enhances β-catenin transcriptional activity by connecting β-catenin with the Med1/RNA polymerase II (Pol *II*) complex (Shi et al., 2007). Indeed, Med1/TRAAP220 and Pol *II* conditionally bind to *c-Myc* and *Axin2* promoters in LiCl-treated HeLa cells (Figure 4G). Given the PRC2-indepednent

(H) Lgr5-positive and Bmi1-positive ISCs are located in the crypts. ISCs divide into transit-amplifying (TA) cells and differentiate into IECs. Wnt/β-catenin signaling is highly active in crypts containing ISCs and TA cells.

(I) PAF expression in IECs of crypts. A small-intestine tissue was immunostained for PAF (arrowhead); hematoxylin: blue.

(J) PAF expression in Bmi1-positive ISCs. Immunofluorescent staining of murine colon tissue samples for PAF and Bmi1. Scale bars represent 20 μm.

(K) PAF interaction with EZH2. CoIP and IB assays of SW620. H.C.: heavy chain.

(L) PAF-EZH2 interaction via the CXC region. GST-PAF was incubated with HeLa cell lysates expressing each FLAG-EZH2 deletion mutant (A–D), and analyzed for GST pull-down and IB. EID, EED interaction domain; D1 and D2, homologous domains 1 and 2; CXC, cysteine-rich domain; SET, SU(var)3-9, E(z), and trithorax histone methyltransferase domain.

(M) Interaction of PAF with EZH2 and β-catenin, independently of PCNA. CoIP of HCT116 is shown. A PCNA-immunodepleted (ID) supernatant was used for IP and IB.

(N and O) Wnt-dependent PAF-β-catenin interaction. HeLa (vector or FLAG-PAF) were treated with LiCl (25 mM, 4 hr) (N) or Wnt3A (100 ng/ml, 4 hr) (O) and analyzed for coIP and IB. L.E.: long exposure; S.E.: short exposure.

(P) PAF-PCNA interaction in colon cancer cells. CoIP and IB assays. Asterisks: IgG light chain.

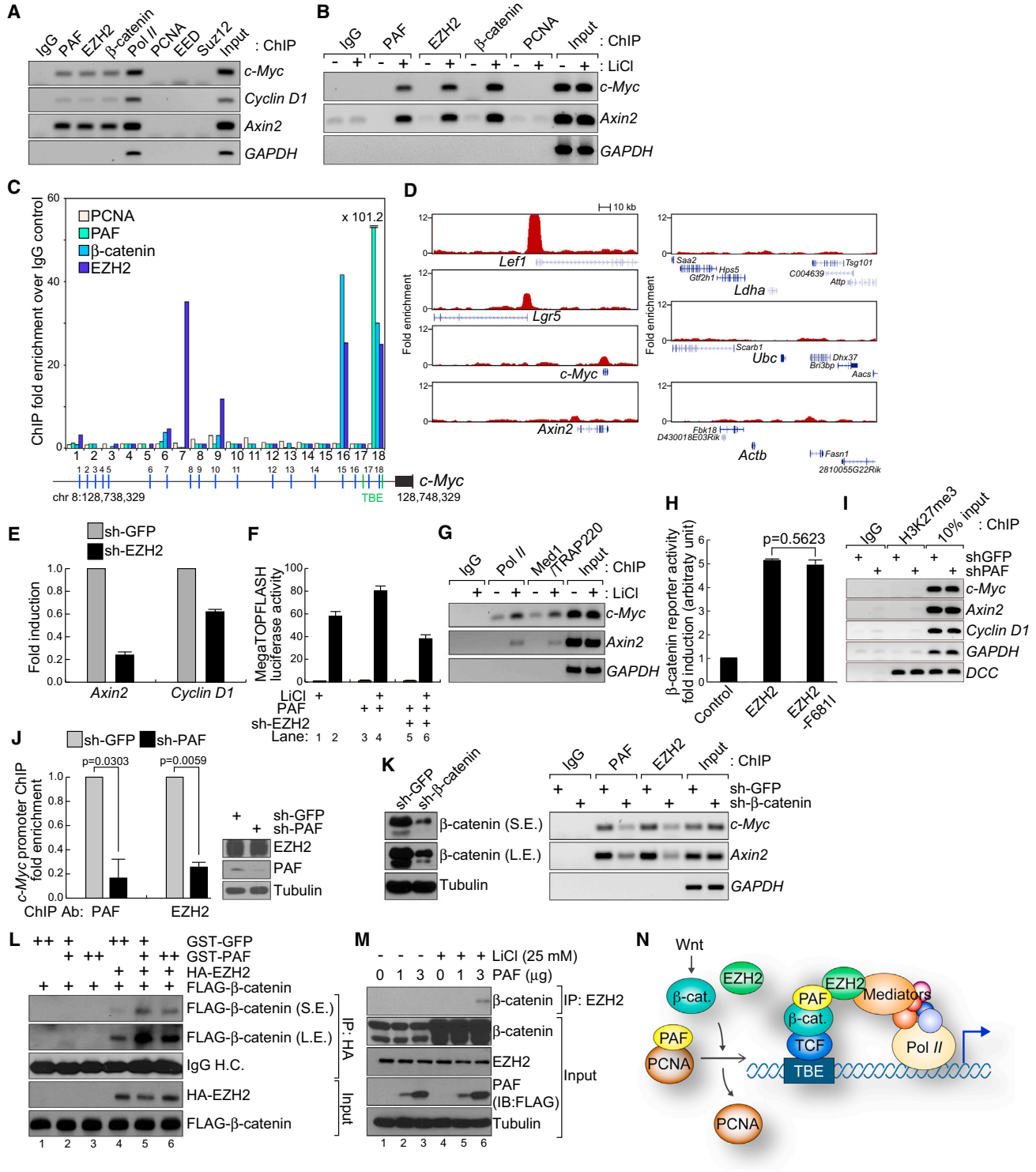

Figure 4. PAF Promotes EZH2-β-Catenin Interaction

(A) Constant association of EZH2, PAF, and β-catenin with the β-catenin target promoters. ChIP assays using HCT116 nuclear lysates. RNA Pol *II*: positive controls for the proximal promoter.

(B) β-catenin-induced recruitment of EZH2 and PAF to promoters. HeLa cells (control and LiCl-treated; 25 mM, 4 hr) were used for ChIP analysis. IgG and *GAPDH* served as negative controls for IP and ChIP-PCR, respectively.

(C) ChIP promoter scanning of *c-Myc* promoter. HCT116 cells were analyzed by ChIP assays on the *c-Myc* promoter with 18 pairs of PCR primers. Average size of amplicons = 150 bp; green vertical bars: TBEs.

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interaction between EZH2 and PAF (Figures 3K and 3N), we asked whether EZH2's histone-methyltransferase activity is dispensable in β-catenin regulation. We utilized an EZH2 point mutant (F681I) that disrupts the contact between the EZH2 hydrophobic pocket and histone-lysine residue H3K27 (Joshi et al., 2008). Ectopic expression of either EZH2 or EZH2-F681I enhanced β-catenin reporter activity (Figure 4H). Also, PAF knockdown did not change the H3K27 methylation status (H3K27me3) of proximal promoters of *c-Myc*, *Axin2*, *Cyclin D1*, and *DCC* in HCT116 cells (Figure 4I). These results indicate a methyltransferase-independent role of EZH2 in transactivating β-catenin targets.

Due to PAF's (1) small size (111 amino acids, one α-helix), (2) lack of a specific catalytic domain, and (3) binding to both β-catenin and EZH2, PAF may facilitate the interaction between EZH2 and β-catenin through recruiting EZH2 to the promoter. We tested this using ChIP assays for EZH2 in the setting of PAF depletion. Indeed, PAF-depleted HCT116 cells displayed decreased EZH2-association at the *c-Myc* promoter (Figure 4J), suggesting that PAF assists or is needed to recruit EZH2 to the β-catenin transcriptional complex. Also, β-catenin knockdown decreased recruitment of PAF and EZH2 to promoters (Figure 4K), showing that PAF and EZH2 occupy target promoters via β-catenin. We then asked whether PAF promotes β-catenin-EZH2 binding. In vitro binding assays showed that the addition of GST-PAF protein increased EZH2-β-catenin association (Figure 4L). Moreover, ectopic expression of PAF promoted the EZH2-β-catenin interaction in HeLa cells treated with LiCl (Figure 4M). Additionally, we tested whether Wnt-signaling-induced posttranslational modification of either β-catenin or PAF is required for EZH2 interaction. However, in GST pull-down assays, we found that either bacterially expressed GST-β-catenin or GST-PAF bound to EZH2 (Figure S3). Due to the lack of posttranslational modification in the GST protein expression system, these data indicate that posttranslation modification of either β-catenin or PAF is not necessary for EZH2 interaction. Together, these results suggest that PAF acts as a molecular adaptor to facilitate the EZH2-β-catenin complex and subsequently enhances the transcriptional activity of the β-catenin transcriptional complex at Wnt target promoters (Figure 4N).

Intestinal Tumorigenesis following PAF Conditional Expression

Having determined that PAF is overexpressed in colon cancer cells and hyperactivates Wnt/β-catenin signaling, we aimed to determine whether mimicking PAF overexpression drives intestinal tumorigenesis, using genetically engineered mouse models. To conditionally express PAF, we generated doxycycline (doxy)-inducible PAF transgenic mice (*TetO-PAF-IRES-emGFP* [*iPAF*]). For intestine-specific expression of PAF, we used *iPAF:Villin-Cre:Rosa26-LSL-rtTA* mouse strains. *Villin-Cre* is specifically expressed in intestinal epithelial cells (IECs), including ISCs and progenitor cells. Cre removes a floxed STOP cassette (loxP-STOP-loxP [LSL]) from the *Rosa26* allele and induces reverse tetracycline transactivator (rtTA) expression. Upon doxy treatment, rtTA drives the transcriptional activation of the tetracycline-responsive element promoter, resulting in conditional transactivation of *PAF* selectively in IECs. We also utilized the *Rosa26-rtTA* strain for ubiquitous expression of PAF (Figure 5A and Figure S4). First, we examined the effects of PAF induction on IEC proliferation using a crypt organoid culture system (Figure S5A). Intriguingly, PAF conditional expression (2 weeks) induced expansion of the crypt organoids (Figures 5B and 5C), which recapitulates the mitogenic function of PAF (Figure 1). In mice, IEC-specific PAF expression (*iPAF:Villin-Cre:Rosa26-LSL-rtTA*; 2 months) developed adenoma in both the small intestine and the colon (Figure 5D). Also, microscopic analysis using hematoxylin and eosin (H&E) staining showed aberrant IEC growth and crypt foci formation (Figures 5E and 5F), with disorganized epithelial cell arrangements (Figure S5B). Consistent with this, PAF-induced IEC hyperproliferation was manifested by increased Ki67 expression, a mitotic marker (Figure 5G). Importantly, these lesions exhibited the upregulation of *CD44*, a β-catenin target gene, whereas *CD44* was expressed strictly in the crypts of normal intestines (Figure 5H). Next, we examined whether PAF directly hyperactivates Wnt/β-catenin in vivo using *BAT-gal*, a β-catenin reporter transgenic mouse carrying multiple TBEs, followed by a *LacZ* reporter. To quantify the early effects of PAF on β-catenin activity, we treated mice with doxy for 1 week and found that short-term induction of PAF increased β-catenin transcriptional activity, as represented

(D) EZH2 association with β-catenin target promoters. EZH2 ChIP-seq data from mouse embryonic stem cells (GSE13084) were analyzed for EZH2 association in the promoters (200 kb) of β-catenin targets (*Lef1*, *Lgr5*, *c-Myc*, and *Axin2*). *Ldha* (lactate dehydrogenase A), *Ubc* (ubiquitin C), and *Actb* (β-actin): negative controls.

(E) Downregulation of β-catenin targets by EZH2 depletion. HCT116 (sh-GFP or -EZH2) were analyzed by qRT-PCR.

(F) EZH2 depletion inhibits PAF-mediated hyperactivation of β-catenin reporter activity. 293T cells (sh-GFP or -EZH2) were transfected with PAF and pMegaTOPFLASH plasmids and treated with LiCl (25 mM, 24 hr) for luciferase assays.

(G) Recruitment of Pol *II* and Med1/TRAP220 to promoters upon β-catenin activation. ChIP assays using HeLa (control or LiCl [25 mM, 4 hr]).

(H) 293T cells transfected with EZH2, EZH2-F681I, and pMegaTOPFLASH were analyzed for luciferase assays (N = 4).

(I) ChIP assays of HCT116 sh-GFP or -PAF using semiquantitative PCR. *DCC* promoter: a positive control for H3K27me3 (Derks et al., 2009).

(J) PAF depletion impairs EZH2 recruitment to the *c-Myc* promoter. ChIP assays of HCT116 sh-GFP or -PAF using qPCR. Of note, PAF knockdown did not downregulate EZH2 (right IB panel).

(K) β-catenin depletion decreases recruitment of PAF and EZH2 to promoters. ChIP assays of HCT116 (sh-GFP or sh-β-catenin).

(L) PAF enhances EZH2-β-catenin interaction in vitro. GST-PAF pull-down of EZH2 and β-catenin protein mixture was performed. EZH2-β-catenin interaction was then analyzed by coIP and IB.

(M) PAF increases β-catenin-EZH2 binding in vivo. HeLa cells (vector or PAF) treated with LiCl (25 mM, 4 hr) were analyzed for coIP and IB.

(N) Illustration of PAF-induced hyperactivation of the β-catenin transcriptional complex. In the setting of Wnt signaling activation, stabilized β-catenin sequesters PAF from PCNA. As a molecular adaptor, PAF facilitates interaction between β-catenin and EZH2. The EZH2/Mediator complex recruits RNA Pol *II*-associated transcriptional machinery to TBEs and transactivates β-catenin target genes.

All error bars indicate SD.

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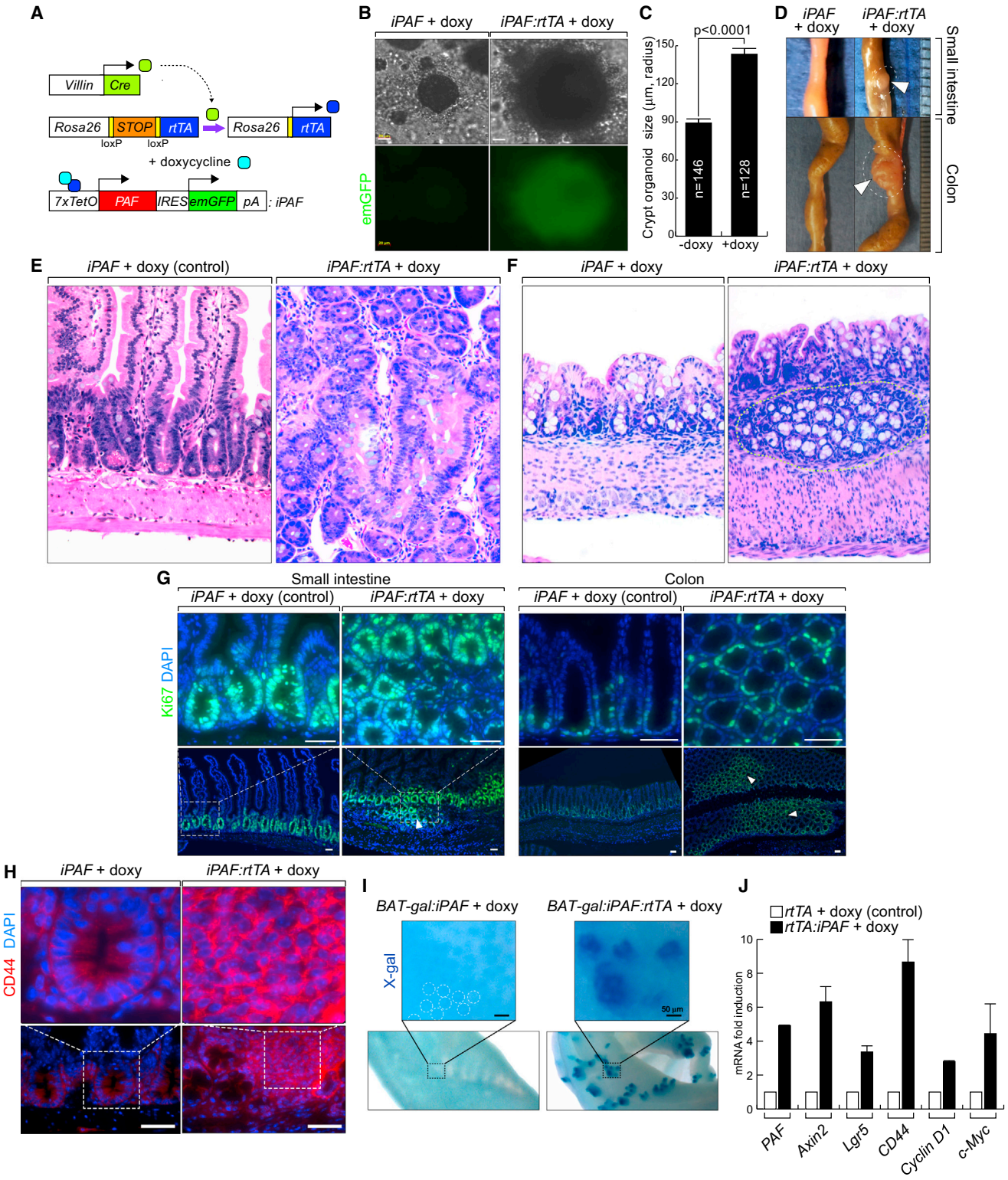

Figure 5. Induction of Intestinal Neoplasia by PAF Expression

(A) PAF-conditional inducible mouse models.

(B and C) Colonic crypts were isolated from *Rosa26-rtTA:iPAF* mice and maintained with or without doxy treatment (2 weeks). Phase-contrast images (B) and quantification of the size of crypt organoids (C) are shown.

(legend continued on next page)

by enhanced 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) staining (Figure 5I). Moreover, conditional PAF expression upregulated the β-catenin target genes *Axin2*, *Lgr5*, *CD44*, *Cyclin D1*, and *c-Myc* in crypt organoids (Figure 5J). Additionally, mice ubiquitously expressing PAF exhibited intestinal hypertrophy (Figure S5C), which is similar to that induced by R-Spondin1, a secreted Wnt agonist (Kim et al., 2005). These data strongly suggest that PAF expression is sufficient to initiate intestinal tumorigenesis via Wnt signaling hyperactivation.

DISCUSSION

Herein we reveal the unexpected role of PAF in modulating Wnt/β-catenin signaling. PAF enhances the transcription of Wnt targets by recruiting EZH2 to the β-catenin transcriptional complex. This is similar to the mechanism by which Lgl/BCL9 binds to β-catenin and thereby recruits the PHD-finger protein Pygopus to bridge the β-catenin/TCF complex to Med12 and Med13 (Carrera et al., 2008). Importantly, given the specific overexpression of PAF in cancer cells, our studies identified an additional layer of the regulatory mechanism of β-catenin target gene transactivation.

In cancer cells, the upregulation of EZH2 contributes to tumorigenesis through the epigenetic repression of various genes including tumor-suppressor genes, Wnt antagonists, and DNA-repair genes (Chang et al., 2011; Cheng et al., 2011; Kondo et al., 2008). Our results propose a noncanonical function of EZH2 in activating β-catenin target genes in conjunction with PAF. Consistent with this, recent study also suggests a methyltransferase-activity-independent function of EZH2 in gene activation (Xu et al., 2012). Moreover, this noncanonical role of EZH2 is supported by several lines of evidence: (1) EZH2 transactivates β-catenin target genes (Li et al., 2009; Shi et al., 2007) (Figures 4E and 4F); (2) EZH2 overexpression in murine mammary epithelium induces ductal hyperplasia (Li et al., 2009), which phenocopies that in a ΔNβ-catenin (constitutively active form of β-catenin) mouse model (Imbert et al., 2001); (3) EZH2 occupies β-catenin target promoters (Figures 4A–4D); and (4) EZH2's methyltransferase activity is dispensable for β-catenin target activation (Figures 4H and 4I). Moreover, similar to PAF expression in the AER (Figure 2A), EZH2 is also specifically expressed there for maintenance of *Hox* cluster gene transcription (Wyngaarden et al., 2011). Thus, it is plausible that EZH2 and PAF cooperatively control *Hox* gene activation in the developing limb. Interestingly, despite the presence of a physical and functional connection between Bmi1 and EZH2 in H3K27me3-mediated gene repression, EZH2 is expressed only in crypt IECs, including ISCs (Figure S6), whereas Bmi1 is expressed in ISCs at position 4 (Figure 3J), implying a Bmi1-independent role for EZH2 in gene regulation. These results demonstrate the function of EZH2 in β-catenin target gene activation independent of the histone-methyltransferase activity of EZH2.

Previously, we found that TERT, a catalytic subunit of telomerase, positively modulates Wnt signaling (Park et al., 2009), elucidating a nontelomeric function of telomerase in development and cancer. Here, our results propose that one component of the DNA-damage bypass process also functions in regulating Wnt signaling, dependent on context. In cancer, PAF overexpression may play a dual role in inducing (1) cell hyperproliferation (via Wnt signaling hyperactivation) and (2) the accumulation of mutations arising from DNA-lesion bypass (by PAF-mediated TLS) (Povlsen et al., 2012). Importantly, PAF is only expressed in cancer cells, but not in normal epithelial cells. Thus, upon DNA damage, instead of cell growth arrest to permit high-fidelity DNA repair, the PAF overexpression in cancer cells is likely to induce DNA-lesion bypass by facilitating TLS. However, in the setting of Wnt signaling deregulation, nuclear β-catenin sequesters PAF from PCNA and utilizes PAF as a cofactor of the transcriptional complex, which induces Wnt signaling hyperactivation and possibly leads to increased mutagenesis.

We observed that PAF marked the stemness of ISCs and mouse embryonic stem cells (Figure S7), implicating its roles in stem cell regulation under physiological conditions. In a previous study, a *PAF* germline knockout mouse model displayed defects in hematopoietic stem cell self-renewal (Amrani et al., 2011), suggesting a crucial role of PAF in stem cell maintenance and activation. In the intestine, β-catenin activation in Lgr5-positive or Bmi1-positive cells is sufficient to develop intestinal adenoma (Barker et al., 2009; Sangiorgi and Capecchi, 2008), suggesting an essential role of tissue stem cells in tumor initiation. Considering PAF expression in Bmi1-positive ISCs, PAF upregulation in ISCs probably hyperactivates the Wnt/β-catenin signaling and contributes to intestinal tumor initiation.

Despite the critical role of Wnt signaling in early vertebrate development, *PAF* germline knockout mice are viable (Amrani et al., 2011). It is noteworthy that, whereas deletion of any core component in the Wnt signaling pathway causes embryonic lethality, mice with germline knockout of Wnt signaling modulators, including *Nkd1/2*, *Pygo1/2*, and *BCL9/9-2*, exhibit no lethal phenotypes (Deka et al., 2010; Schwab et al., 2007; Zhang et al., 2007). This may result from the robustness of Wnt signaling during embryogenesis because of functional compensation, not only via the presence of multiple Wnt signaling regulators per se, but also via other types of signaling crosstalk. Therefore, as

(D–F) Induction of PAF expression develops intestinal microadenoma. *Villin-Cre:Rosa26-LSL-rtTA:iPAF* mice (experimental group) and *iPAF* mice (control) were given doxy (2 mg/ml in drinking water; small intestine [2 months] and colon [4 months]). Arrowheads represent adenomas (D). H&E staining of small intestine (E) and colon (F) is shown. Dotted circles: aberrant crypt foci.

(G) IEC hyperproliferation by PAF. *Villin-Cre:Rosa26-LSL-rtTA:iPAF* and *iPAF* mice were given doxy (2 months). Ki67 immunostaining is shown (arrowheads: hyperplastic lesions). Scale bars represent 50 μm.

(H) Upregulation of CD44 by PAF. CD44 immunostaining of small-intestine specimens from control and PAF-induced (4 months) mice. Scale bars represent 500 μm.

(I) PAF hyperactivates β-catenin reporter activity. X-gal staining of *iPAF:BAT-gal* and *Rosa26-rtTA:iPAF:BAT-gal* mice (doxy; 7 days). Dotted circles: endogenous Wnt signaling activity in crypts.

(J) Upregulation of β-catenin target genes by PAF. Crypts isolated from *Rosa26-rtTA:iPAF* mice were treated with doxy (1 μg/ml, 36 hr) for qRT-PCR.

All error bars indicate SD.

described previously in *pRb* studies (Sage et al., 2003), acute deletion of *PAF* in a conditional knockout mouse model may disrupt the developmental balance or tissue homeostasis and then reveal the full spectrum of the physiological and pathological roles of PAF in tumorigenesis. Taken together, our findings reveal an unexpected function of PAF and EZH2 in modulating Wnt signaling and highlight the impact of PAF-induced Wnt signaling deregulation on tumorigenesis.

EXPERIMENTAL PROCEDURES

Oncomine Database Analysis

Complementary DNA (cDNA) microarray data sets of colon adenocarcinoma and normal tissue samples from Oncomine (www.oncomine.org, September 2012 release) were analyzed for identifying genes that are specifically expressed in colon cancer cells (fold change >2; $p < 0.0001$; top 10% ranked).

Immunohistochemistry

Tissue samples were collected and fixed in 10% formalin and processed for paraffin embedding. Sectioned samples were immunostained according to standard protocols. Information regarding the antibodies used is available in Supplemental Information.

Mammalian Cell Culture

Cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Cell fractionation was performed using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). For gene depletion, at least five different shRNA lentiviruses (sh-PAF, sh-EZH2, and sh- β-catenin; MISSION shRNA, Sigma) were stably transduced into target cells using puromycin selection (1–2 μg/ml). LY294002, U0216, and Sorafenib were purchased from Sigma.

GST Pull-Down Assay

GST, GST-GFP, PAF, and β-catenin proteins were purified from an *Escherichia coli* BL21 strain using a standard procedure. Each protein (0.1 μg) was incubated with HeLa cell lysates expressing binding proteins for 1 hr, precipitated using glutathione sepharose 4B (GE Healthcare), and analyzed by immunoblotting (IB).

Transgenic Animals

A TetO minimal promoter-*mPAF*-IRES-EmGFP-BGHpA DNA fragment was injected into the pronucleus of the zygotes to generate transgenic *iPAF* mice. *iPAF* pups from three independent founder strains were utilized for analysis. *PAF* transgene expression was induced by doxy administration in the late generations crossed with C57BL/6 mice. All mice were maintained according to institutional guidelines and Association for Assessment and Accreditation of Laboratory Animal Care International standards.

Constructs

All constructs were generated from cDNA or open reading frame sources via PCR. Mutants were constructed using PCR-based mutagenesis.

Immunoblotting and Immunoprecipitation

Whole-cell lysates of mammalian cells were prepared and analyzed for IB and IP, as previously performed (Jung et al., 2013). Information regarding antibodies is available in the Supplemental Information.

Axis-Duplication Assays

X. laevis embryos were microinjected with in vitro-transcribed mRNAs into ventrovegetal regions in four-cell-stage embryos, as previously performed (Park et al., 2009).

Statistical Analysis

The Student's t test was used for comparisons of two samples. Calculation of the average was performed using at least three biological replicas. p values < 0.05 were considered significant. Error bars indicate SD.

Full experimental procedures are available in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.08.028.

AUTHOR CONTRIBUTIONS

J.-I.P. conceived the experiments; H.-Y.J., S.J., H.-C.K., M.L., X.W., H.J., and J.-I.P. performed the experiments; J.-I.P. and P.D.M. analyzed the results; and J.-I.P. wrote the manuscript.

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