PP4 and PP2A regulate Hedgehog signaling by controlling Smo and Ci phosphorylation

Hongge Jia, Yajuan Liu, Wei Yan and Jianhang Jia*

The seven-transmembrane protein Smoothened (Smo) and Zn-finger transcription factor Ci/Gli are crucial components in Hedgehog (Hh) signal transduction that mediates a variety of processes in animal development. In Drosophila, multiple kinases have been identified to regulate Hh signaling by phosphorylating Smo and Ci; however, the phosphatase(s) involved remain obscure. Using an in vivo RNAi screen, we identified PP4 and PP2A as phosphatases that influence Hh signaling by regulating Smo and Ci, respectively. RNAi knockdown of PP4, but not of PP2A, elevates Smo phosphorylation and accumulation, leading to increased Hh signaling activity. Deletion of a PP4-interaction domain (amino acids 626-678) in Smo promotes Smo phosphorylation and signaling activity. We further find that PP4 regulates the Hh-induced Smo cell-surface accumulation. Mechanistically, we show that Hh downregulates Smo-PPI4 interaction that is mediated by Cos2. We also provide evidence that PP2A is a Ci phosphatase. Inactivating PP2A regulatory subunit (Wdb) by RNAi or by loss-of-function mutation downregulates, whereas overexpressing regulatory subunit upregulates, the level and thus signaling activity of full-length Ci. Furthermore, we find that Wdb counteracts kinases to prevent Ci phosphorylation. Finally, we have obtained evidence that Wdb attenuates Ci processing probably by dephosphorylating Ci. Taken together, our results suggest that PP4 and PP2A are two phosphatases that act at different positions of the Hh signaling cascade.

KEY WORDS: Smo, Ci, PP4, PP2A, Hh, Signal transduction, Drosophila

INTRODUCTION

The hedgehog (hh) family members control many aspects of development in both vertebrates and invertebrates (Jia and Jiang, 2006). Abnormal activation of Hh pathway has been observed in several types of human cancers (Pasca di Magliano and Hebrok, 2003; Taipale and Beachy, 2001). The Hh signal is transduced through a receptor system that includes the transmembrane proteins Patched (Ptc) and Smo. The main outcome of Hh signaling is the activation of transcriptional responses via the Ci/Gli family of Zn-finger transcription factors. Hh signaling regulates the balance between the transcriptional activator and repressor forms of Ci/Gli (Lum and Beachy, 2004). In Drosophila, the absence of Hh allows Ptc to inhibit Smo signaling activity. In the cytoplasm, the kinesin-related protein Costal 2 (Cos2) recruits multiple kinases, including PKA, CK1 and GSK3 to sequentially phosphorylate full-length Ci (CiFL) (Zhang et al., 2005), which creates binding sites for the SCF ubiquitin ligase containing the F-Box protein Slimb (Jia et al., 2005; Smelkinson and Kalderon, 2006), leading to Ub/proteasome-mediated processing into a truncated repressor form (CiREP). CiREP functions as a repressor to block the expression of Hh responsive genes such as decapentaplegic (dpp), as well as hh itself (Aza-Blanc et al., 1997; Methot and Basler, 1999). The presence of Hh relieves the inhibition of Ptc on Smo, inducing Smo cell-surface accumulation and phosphorylation (Denef et al., 2000) by kinases including PKA and CK1 (Apionishev et al., 2005; Jia et al., 2004; Zhang et al., 2004). Smo phosphorylation by PKA and CK1 appears to activate Smo by inducing a conformational switch (Zhao et al., 2007), as well as promoting Smo cell surface accumulation (Jia et al., 2004). In addition, peak levels of Hh promote Smo hyperphosphorylation that is modulated by a feedback loop involving downstream components Cos2 and Fusion (Fu) (Claret et al., 2007; Liu et al., 2007). Activated Smo blocks CiFL phosphorylation and proteolytic processing required for generating CiREP, and further promotes nuclear translocation and activation of accumulated CiFL (Chen et al., 1999; Methot and Basler, 2000; Wang et al., 2000; Wang and Holmgren, 2000).

The Drosophila wing disc has been used as an excellent model to study the Hh signal transduction. Posterior (P) compartment cells in the wing discs secrete Hh protein that moves into the anterior (A) compartment and induces the expression of Hh target genes, such as dpp, ptc and en (en), which can be used to monitor the levels of Hh signaling activity (Jia and Jiang, 2006). Ci is produced in A-compartment cells but not P-compartment cells, whereas Smo is expressed in the whole wing but accumulated in P-compartment cells as well as A-compartment cells near the AP boundary, where there is Hh-mediated stimulation. Phosphorylation of Smo and Ci has been shown to be the major post-translational event that regulates their signaling activities, but how their phosphorylation is regulated is still poorly understood.

Levels of cellular protein phosphorylation are often modulated by the opposing action of protein kinases and phosphatases. Phosphatases are typically classified into two main groups, the Serine/Threonine (Ser/Thr) protein phosphatases (STPs) and protein tyrosine phosphatases (PTPs). STPs can be subdivided into the PPP and PPM families based on distinct amino acid sequences and crystal structures (Cohen, 1997). In the Hh signaling cascade, multiple Ser/Thr kinases are involved, including PKA, GSK3 and CK1 family members. Even though PP2A has been implicated as a positive regulator in Hh signaling (Casso et al., 2008; Nybakken et al., 2005), its relevant substrates remained undetermined. Thus, it is not clear whether phosphatases are involved in regulating Smo and Ci phosphorylation, and if so which phosphatases are responsible.
In this study, we performed an in vivo RNAi screen with the RNAi library from VDRC (Vienna Drosophila RNAi Center) (Dietzl et al., 2007) targeting the catalytic subunits of the STPs in the Drosophila genome (Morrison et al., 2000), in which we identified PP4 and PP2A as phosphatases that regulate Smo and CiP3 phosphorylation, respectively. We found that Smo phosphorylation is elevated by RNAi knockdown of PP4 or by abolishing Smo-PP4 interaction. We also found that the signaling activity of CiP3 is positively regulated by PP2A. We provided evidence that PP2A prevents CiP3 proteolytic processing by dephosphorylating CiP3.

MATERIALS AND METHODS

Mutants and transgenes

Wdbb/+ is a hypomorphic allele (Hannus et al., 2002), smo3 is a null allele (Chen and Struhl, 1998). In vivo RNAi library has been described (Dietzl et al., 2007). MSL1096, act–-CD2–Gal4, ap-Gal4, UAS-P35, UAS-HA-CiP3, UAS-mc4, UAS-CRL, dpp-lacZ, ptc-lacZ and hh-lacZ have been described (Jia et al., 2003; Jia et al., 2005; Liu et al., 2007; Zhang et al., 2006). We obtained full-length cDNAs either (CG18339), PP4R (PP4 regulatory subunit, CG2890), Mts (PP2A catalytic subunit, CG6235). We obtained full-length cDNAs either from DGR or by RT-PCR from fly embryonic RNA. Myc-Smo, Myc-SmoCT, Myc-SmoΔCT and HA-Cos2 have been described (Jia et al., 2003; Wang et al., 2000). We swapped each corresponding sequence of SmoCT truncations and internal deletions (Jia et al., 2003) into the 3XFlag-UAST backbone to raise the affinity for western blot. We constructed Flag-CiP3 by swapping the Ci coding sequence from HA-CiP3 (Wang et al., 1999) into the 3XFlag-UAST. HA-PP4 transformants were generated by standard P-element mediated transformation. Multiple independent lines were examined. EP5559, UAS-Wdb, UAS-Tws, UAS-Mts, UAS-DN-Mts fly strains have been examined (Hannus et al., 2002; Mayer-Jaekel et al., 1993; Sathyanarayanan et al., 2004). To construct attB-UAS-Myc-Smo and attB-UAS-Myc-SmoΔ626-678, the attB sequence (Bateman et al., 2006) was inserted in upstream of the UAS-binding sites in pUAST, then Myc-Smo or Myc-SmoΔ626-678 sequences were inserted. The vas-philh24a-VKS flies (gift from Dr Hugo Benne) were used to generate UAS-Myc-Smo and UAS-Myc-SmoΔ626-678 transgenes at the 75B1 attP locus. Genotypes for generating clones are as follows: wdbR7 clones, yw hap-flp1/+; or Y; FRT82 wdbR7/FRT82 hs-FFP; smo clones expressing CiP3 or co-expressing CiP3 with Wdb, y MS1096 hap-flp1/yw or Y; smoR FRT40/hs-FFP FRT40; UAS-HA-Ci or UAS-HA-Ci with Wdb/Wdb/hh-lacZ.

Cell culture, transfection, immunoprecipitation, western blot analysis and GST fusion protein pull-down

S2 cell culture, transfection, immunoprecipitation and immunoblot analysis were performed with standard protocols (Liu et al., 2007). S2 cell surface accumulation was detected by immunostaining with anti-Smo antibody before cell permeabilization (Jia et al., 2004). The intensity of cell-surface or total Smo was analyzed by Metamorph software. To target each phosphatase gene with less than 17 nucleotide contiguous off-target sequence, we synthesized Ms, Wdb, Tws, Pp4 and PP4R dsRNA against the cDNA regions of 301-900, 681-1236, 761-1360, 304-921 and 791-1288, respectively (Chen et al., 2007). Cos2 and GFP dsRNA synthesis and the method for RNAi in S2 cells have been described (Liu et al., 2007). OA (Calbiochem) treatment was used to inhibit both PP4 and PP2A (Cohen et al., 1990) at a final concentration of 50 nM for 3 hours before harvesting the cells. GST-Smo557-686 fusion protein pull-down has been described (Liu et al., 2007). His-Cos2MB and His-Cos2CT were constructed by fusing Cos2 corresponding sequence to the pET30 vector, expressed in E. coli, and purified with the His resins (Clontech). Antibodies used in this study were mouse anti-Cos2 (gift from D. Robbins), anti-Flag, M2 (Sigma), anti-GFP (Chemicon), anti-HA, F7 (Santa Cruz), anti-His, 4D11 (Upstate), anti-Myc, 9E10 (Santa Cruz), anti-SmoN (DSHB), anti-β-tubulin (DSHB); and rabbit anti-HA, Y-11 (Santa Cruz) and anti-GST (Santa Cruz).

Immunostaining of imaginal discs

Standard protocols for immunofluorescence staining of imaginal discs were used with the antibodies mouse anti-Myc, 9E10 (Santa Cruz), anti-HA, F7 (Santa Cruz), anti-Flag, M2 (Sigma), anti-SmoN (DSHB), anti-Pt (DSHB), anti-CD2 (Serotec); rabbit anti-Flag (ABR), anti-HA, Y-11 (Santa Cruz), anti-βGal (Cappel), anti-GFP (Clontech); and rat anti-Ci 2A (gift from R. Holmgren). MG132 (100 μM; Calbiochem) in M3 medium (Sigma) was used to treat wing discs for up to 6 hours before immunostaining.

RESULTS

Identification of Smo phosphatase by in vivo RNAi screen

In response to Hh stimulation, many Hh pathway components are phosphorylated and a number of kinases have been identified. However, the phosphatase(s) involved have remained elusive. To explore whether phosphatases are also involved, we obtained 45 RNAi lines from the VDRC, targeting 26 catalytic subunits of the STPs (Morrison et al., 2000). We performed in vivo screening by overexpressing individual RNAi lines via the wing-specific MSL1096 Gal4 to determine whether they induced any adult wing phenotypes. We found that RNAi of eight genes affected wing development indicated by wing phenotypes or wing blisters (data not shown). All eight genes encode members of the PPP family of phosphatase. The fact that increased Smo phosphorylation stabilizes Smo in wing discs (Deneff et al., 2000; Jia et al., 2004) suggests that modulation of Smo phosphorylation might alter Smo levels. We thus performed the next round of screening with a direct approach to detect Smo changes when a specific phosphatase was knocked down by RNAi. Each UAS-RNAi line targeting the eight candidates was expressed via the dorsal compartment-specific ap-Gal4, and wing discs from late third instar larvae were immunostained with anti-Smo antibody. Knockdown of PP4 by RNAi induced Smo accumulation (Fig. 1B, compared with wild-type disc staining in Fig. 1A). Moreover, expressing UAS-PP4RNAi caused elevated expression and anterior expansion of Hh target genes such as ptc-lacZ in dorsal but not ventral compartment cells (Fig. 1B’), a phenotype similar to that caused by overexpressing PKA (Jia et al., 2004; Liu et al., 2007). These observations indicate that PP4 exerts a negative influence on Hh signaling in responding cells, probably by reducing Smo phosphorylation. RNAi of other phosphatases including Mts did not elevate Smo (Fig. 1C-C’, data not shown).

UAS-PP4 RNAi shares a 22-nucleotide contiguous sequence with CanA1 phosphatase (CG1455; see supplementary information). However, CanA1RNAi had no effects on Smo accumulation in wing disc (see Fig. S1 in the supplementary material), suggesting the accumulation of Smo by PP4 RNAi in wing discs (Fig. 1B) was due to the downregulation of PP4 activity. To further examine the specificity of PP4 RNAi, we tested whether the overexpressed PP4 could rescue its RNAi phenotype. As shown in Fig. 1E, co-expressing UAS-HA-PP4 attenuated the Smo elevation caused by PP4 RNAi. By contrast, co-expressing UAS-Mts did not alleviate PP4 RNAi-induced Smo accumulation (Fig. 1G), indicating the specificity of PP4 RNAi. Overexpression of UAS-HA-PP4 or UAS-Mts alone did not downregulate Smo accumulation in wing discs (Fig. 1D,F). Taken together, our data suggest that PP4 blocks Smo accumulation and downregulates Hh signaling activity.

PP4 downregulates Smo phosphorylation

PP4 is a highly conserved carboxymethylated protein that belongs to the PP2A family of STPs. Drosophila PP4 shares 91.5% identity with human PP4. To further determine whether Smo elevation induced by PP4 RNAi (Fig. 1B) was due to enhanced Smo...
phosphorylation, we examined the levels of Smo phosphorylation in S2 cells with our previously established assay (Jia et al., 2004; Liu et al., 2007). Consistent with previous findings (Apionishev et al., 2005; Denef et al., 2000; Jia et al., 2004; Liu et al., 2007; Zhang et al., 2004), Hh stabilized Smo and induced an electrophoretic mobility shift of Myc-Smo, indicative of Smo phosphorylation (Fig. 2A, lane 2, top panel). The hyperphosphorylated form of Smo was accumulated by either the phosphatase inhibitor okadaic acid (OA) (lane 3, top panel), PP4 dsRNA (lane 4, top panel) or PP4R dsRNA (lane 5, top panel) treatment, but not by Mts, Wdb and GFP dsRNA treatments (lanes 6, 7 and 8, respectively). This is consistent with the observation that PP2A RNAi did not promote Smo accumulation in wing discs (Fig. 1C). In parallel, we examined whether overexpressing PP4 could attenuate Smo phosphorylation in S2 cells. We found that expressing HA-PP4, but not HA-Mts, diminished the Hh-induced Smo mobility shift (Fig. 2B, lanes 3 and 4, top panel).

To test whether PP4 regulates Smo phosphorylation by interacting with Smo, we carried out co-immunoprecipitation experiments. As shown in Fig. 2C, both Myc-Smo and Myc-SmoCT (Smo C-tail), but not Myc-SmoΔCT (Smo lacking its C-tail), co-immunoprecipitated with HA-PP4. To further map the Smo domain responsible for interacting with PP4, we tested various SmoCT truncations or internal deletions we previously generated (Fig. 2D) (Jia et al., 2003) for their ability to bind PP4. We found that SmoCT deletion variants lacking amino acids 626-678, which include Smo556-625, Smo679-1035 and SmoCTΔ626-678, did not pull down HA-tagged PP4 when co-expressed in S2 cells (see Fig. S3A, lanes 2, 5 and 6, top panel in the supplementary material), suggesting that amino acids 626-678 of Smo is responsible for either direct or indirect association between SmoCT and PP4.

We reasoned that if PP4 inhibits Smo phosphorylation through amino acids 626-678, deletion of this Smo-PP4 interacting domain should abolish Smo-PP4 interaction thus may lead to high basal phosphorylation of Smo. Indeed, we found that deletion of amino acids 626-678 in the full-length Smo background (Myc-SmoΔ626-678) elevated Smo phosphorylation and stabilized Smo even in the absence of Hh (Fig. 2E, lane 3, compared to lane 1, top panel), indicating an elevation in Smo basal phosphorylation. In addition, we found that deletion of amino acids 626-678 potentiated Hh-induced Smo phosphorylation (Fig. 2E, lane 4; compare with lane 2, top panel) and rendered Smo resistant to dephosphorylation induced by overexpression of PP4 (see Fig. S4 in the supplementary material). Our data indicate that amino acids 626-678 are responsible for PP4-mediated Smo dephosphorylation.

To determine the precise activity of the exogenously expressed Smo, we developed an in vivo assay by taking advantage of P{C31 integrase-mediated transgenesis (Bischof et al., 2007) in combination with the attP sites in the fly genome (Venken et al., 2006). We generated UAS-VK5-Myc-Smo and UAS-VK3-Myc-SmoΔ626-678 transgenes at the 75B1 attP locus (Venken et al., 2006) to ensure equal expression of Myc-Smo and Myc-SmoΔ626-678. We found that the activity of Myc-SmoΔ626-678 is higher than Myc-Smo as it induced higher level of ectopic dpp-lacZ expression (Fig. 2G, compare with 2F), indicating that amino acids 626-678 negatively regulate Smo activity. Meanwhile, we found that Myc-SmoΔ626-678 is stabilized in A-compartment cells (Fig. 2G’, compare with 2F’), suggesting that deletion of amino acids 626-678 of Smo promotes its stability probably owing to enhanced phosphorylation.

**Cos2 mediates the interaction between Smo and PP4**

Although the role of Smo phosphorylation has been broadly studied, how Smo phosphorylation is regulated remains an enigma. Hh might promote Smo phosphorylation by regulating the phosphatase. It is unlikely that PP4 activity per se is regulated by Hh as PP4 has been shown to be involved in essential cellular process (Zhou et al., 2002), and also in the nucleation of microtubules (Helps et al., Development, 2003).
Our finding that Smo and PP4 exist in the same protein complex led to the hypothesis that Hh might control the accessibility of Smo to the phosphatase. To test this, we used a GST pull-down assay described earlier (Liu et al., 2007; Lum et al., 2003b). We found that Flag-PP4 from S2 cells treated with Hh was barely precipitated by GST-Smo557-686 (Fig. 2H, lane 3, compared with lane 2, top panel), suggesting that Hh regulates the accessibility of PP4 to Smo.

**Fig. 2. PP4 regulates Smo phosphorylation.** (A) PP4RNAi elevates Smo phosphorylation. S2 cells were co-transfected with UAS-Myc-Smo and UAS-GFP, and treated with Hh-conditioned or control medium, or treated with OA, PP4 dsRNA, PP4R dsRNA, Mts dsRNA, Wdb dsRNA or GFP dsRNA. Cell extracts were immunoprecipitated and blotted with anti-Myc to detect Smo phosphorylation. Arrow indicates hyperphosphorylated forms of Smo and arrowhead indicates hypophosphorylated and unphosphorylated forms. The efficiency of knockdown of individual phosphatase is shown in Fig. S2A in the supplementary material. (B) PP4 downregulates the Hh-induced Smo phosphorylation. S2 cells were transfected with indicated constructs and treated with or without Hh. The levels of Smo phosphorylation were examined by immunoblotting. The expression levels of HA-PP4 or HA-Mts were shown by probing cell lysates with HA antibody. (C) PP4 interacts with Smo C-tail. Extracts from S2 cells expressing indicated constructs were immunoprecipitated with Myc antibody followed by western blot with HA antibody. The expressed proteins were shown by probing immunoprecipitates with anti-Myc, or probing the cell lysates with anti-HA. The asterisk indicates the slow mobility of the Myc-Smo/CT (Jia et al., 2003). (D) SmoCT truncations interacting with PP4. See immunoprecipitation results in Fig. S3A in the supplementary material. (E) Deletion of the PP4-binding domain in Smo elevates its phosphorylation. Extracts from S2 cells expressing Myc-Smo or Myc-SmoΔ626-678 and treated with or without Hh were immunoprecipitated and blotted with the anti-Myc antibody. (F-G') Wing discs expressing UAS-Myc-Smo or UAS-Myc-SmoΔ626-678 by ap-Gal4 were immunostained to show Myc expression in F' and G', and ectopic dpp-lacZ expression in F and G. Expressing Myc-SmoΔ626-678 induced higher level of ectopic dpp-lacZ expression (arrowhead in G), compared with expressing Myc-Smo (arrowhead in F). (H) Hh downregulates Smo-PP4 interaction. Extracts from S2 cells expressing Flag-PP4 with or without Hh treatment were incubated with the bacterially expressed GST or GST-Smo557-686 fusion proteins. The bound PP4 proteins were analyzed by western blot with Flag antibody. The middle panel shows the GST and GST-Smo fusion proteins. The lower panel indicates the equal amount of input PP4. (I) Smo-PP4 interaction is attenuated by Cos2 RNAi. S2 cells were transfected with HA-PP4 and Flag-SmoCT and treated with or without Cos2 dsRNA. The SmoCT-bound PP4 was examined by immunoprecipitation with anti-Flag and western blot with anti-HA. Equally expressed PP4 proteins were ensured by probing the lysates with anti-HA. The efficiency of Cos2 RNAi is shown in Fig. S2B in the supplementary material. (J) PP4 directly interacts with Cos2MB and Cos2CT. Bacterially expressed and purified GST, GST-PP4, His-Cos2MB and His-Cos2CT were used for pull-down assay. Antibodies used for western blots are indicated.
The Smo domain responsible for PP4 association falls into the Cos2-binding domains (amino acids 557-686) (Liu et al., 2007; Lum et al., 2003b). In addition, we have shown that Cos2 has a negative role in Smo phosphorylation (Liu et al., 2007). These observations raised the possibility that Smo may serve as a scaffold to bridge PP4 and Smo. If so, removing Cos2 should decrease the amount of Smo-bound PP4, thus attenuating Smo inhibition by PP4. In support of this model, we have found that SmoCT-PP4 interaction is attenuated by Cos2 RNAi (Fig. 2I, lane 2, compare with lane 1, top panel), suggesting that Cos2 may downregulate Smo phosphorylation by recruiting the phosphatase or facilitating the interaction between Smo and its phosphatase. We then examined the interaction between Cos2 and PP4 and we found that both Cos2 microtubule-binding (MB) and cargo domain (CT) bind PP4 in our immunoprecipitation assay (see Fig. S3B in the supplementary material, data not shown). We verified PP4 interaction with Cos2 by using an in vitro GST pull-down assay. As shown in Fig. 2J, GST-PP4 fusion protein pulled down both His-Cos2MB and His-Cos2CT (lanes 2 and 4, top panel), whereas GST protein failed to pull down any His-Cos2 proteins, suggesting that PP4 directly binds Cos2 N- and C-terminal domains.

**PP4 regulates Smo cell-surface accumulation**

Hh-induced Smo phosphorylation promotes its cell-surface accumulation (Jia et al., 2004); we therefore investigated whether PP4 regulates Smo cell-surface localization using a cell-based assay (Jia et al., 2004; Liu et al., 2007). Consistent with our previous observations (Jia et al., 2004), Smo accumulated on the cell surface upon Hh stimulation (Fig. 3B, compare with Fig. 3A without Hh treatment). The Hh-induced Smo cell-surface accumulation was enhanced by OA treatment (Fig. 3D), even though OA treatment alone did not promote Smo cell-surface expression (Fig. 3C), suggesting that the blockade of phosphatase activity elevates the Hh-induced Smo cell-surface accumulation. Knockdown of PP4 but not of Mts elevated the Hh-induced Smo cell-surface accumulation (Fig. 3F,H). Furthermore, RNAi of PP4 regulatory subunit promoted Hh-induced Smo cell-surface accumulation (see Fig. S5D in the supplementary material) but RNAi of PP2A regulatory subunit did not (see Fig. S5F in the supplementary material). Without Hh treatment, neither PP4 RNAi (Fig. 3E) nor Mts RNAi (Fig. 3G) promoted Smo cell-surface accumulation, similar to the effect of OA treatment in the absence of Hh (Fig. 3C). However, overexpression of PP4 but not Mts blocked the Hh-induced Smo cell-surface accumulation (see Fig. S5G,H in the supplementary material). These data suggest that PP4 attenuates the Hh-induced Smo cell-surface accumulation. In the absence of PP4, Smo is not fully phosphorylated and its activation still depends on Hh, which may explain why the accumulation of Smo by PP4 RNAi in anterior-most regions of wing disc is unable to induce ectopic ptc-lacZ expression (Fig. 1B).

Smo intracellular domain has a total of 26 Ser/Thr sites that are phosphorylated upon Hh stimulation (Zhang et al., 2004). We have previously shown that SmoSD12, or SmoSD123, in which the two or three clusters of PKA and CK1 phosphorylation sites are replaced by Asp to mimic phosphorylation, has elevated cell-surface expression and signaling activity (Jia et al., 2004). We wondered whether PP4 regulates the cell-surface accumulation of SmoSD12 or SmoSD123. Consistent with our previous findings (Jia et al., 2004), Hh induced further cell-surface accumulation of both SmoSD12 and SmoSD123 (Fig. 3J,M, respectively). Importantly, the cell-surface accumulation of SmoSD12 or SmoSD123 was elevated by PP4 RNAi (Fig. 3K,N) in a manner similar to Hh treatment, suggesting that PP4 may regulate Smo cell-surface accumulation by controlling the phosphorylation of other sites in addition to the three PKA-CK1 phosphorylation clusters. By contrast, neither RNAi of the PP2A catalytic nor of its regulatory subunit enhanced the cell-surface accumulation of SmoSD123 (see Fig. S5K-L in the supplementary material). Consistent with the above observations, we found that, upon Hh stimulation, deletion of the PP4-binding region in Smo (Smo Δ626-678) elevated Hh-induced Smo cell-surface accumulation (Fig. 3P, compared with 3B). We also found that Smo Δ626-678 did not accumulate on the cell surface in the absence of Hh (Fig. 3O), which was consistent with the observation that PP4 RNAi alone was not sufficient to promote Smo cell-surface expression (Fig. 3E). The above finding, that PP4 regulates Smo cell-surface accumulation, was further supported by quantification analysis of the cell-surface localized Smo (Fig. 3Q and Fig. S5N in the supplementary material).

**PP2A is essential for Hh signaling**

An RNAi screen in cultured cells implicated PP2A as a potential phosphatase involved in Hh signaling (Nybakken et al., 2005). We found that Mts RNAi affected neither Smo accumulation in wing discs (Fig. 1C-C") nor Smo phosphorylation in S2 cells (Fig. 2A, lane 6, top panel). However, when UAS-MtsRNAi was expressed in...
Fig. 4. PP2A is essential for Hh signaling. (A–A') A wild-type wing disc was stained to show the expression of CiFL and dpp-lacZ. The Ci antibody only recognizes CiFL in wing discs. (B-B') A wing disc co-expressing UAS-MtsRNAi with UAS-P35 by MS1096 Gal4 was immunostained with Ci and β-gal antibodies. Arrowhead in B indicates the decreased CiFL levels. Arrowhead in B' indicates the downregulated dpp-lacZ expression. MS1096 Gal4 is expressed at lower level in the ventral region than in the dorsal region of the wing disc (Jia et al., 2003). (C-C') A wing disc expressing UAS-WdbRNAi by MS1096 Gal4. Arrowhead in C indicates the decreased CiFL and arrowhead in C' indicates the downregulated dpp-lacZ. (D-E') Wing discs expressing UAS-Mts or UAS-Wdb by ap-Gal4. Arrowheads in D and E show the elevation of CiFL and arrowheads in D' and E' show the induced ectopic dpp-LacZ expression. (F-F') A wing disc bearing wdb0 homozygous clones that were marked by the lack of GFP expression was immunostained with anti-Ci and anti-SmoN antibodies. Arrowhead in F shows the reduction of CiFL. Arrowhead in F' shows the unaffected Smo accumulation in wdb mutant cells. (G-H) Wing discs expressing UAS-HA-CiFL alone, or along with UAS-Wdb by C765 Gal4. Arrowhead in H indicates the ectopic Ptc-lacZ expression in A-compartment cells.
expression of Hh target genes such as ptc-lacZ (Fig. 5B'), compare with wild-type ptc-lacZ expression in Fig. 5A'). Expressing UAS-Wdb by MS1096 Gal4 elevated Cifl levels (Fig. 5C) but did not affect ptc-lacZ expression (Fig. 5C'). Strikingly, co-expressing UAS-Wdb with UAS-mc* attenuated the effect of mc*, as evident by the rescue of Cifl levels (Fig. 5D) and ptc-lacZ expression near the AP boundary (Fig. 5D'), suggesting that Wdb counteracts PKA to regulate Cifl and Hh target genes. We previously generated CK1 RNAi transformant, UAS-CRL, which produces dsRNA that efficiently interferes with the activity of endogenous CK1 (Jia et al., 2005). Expressing UAS-CRL in wing discs caused accumulation of Cifl (Fig. 5E) (Jia et al., 2005). To determine whether PP2A also counteracts CK1 to regulate Ci, we co-expressed UAS-CRL with UAS-MtsRNAi by MS1096 Gal4. We found that the elevation of Cifl caused by CRL expression was severely restricted by co-expressing UAS-MtsRNAi (Fig. 5F), suggesting that PP2A also counteracts CK1 activity in regulating Cifl.

**PP2A dephosphorylates Ci and attenuates Ci processing**

To determine whether PP2A regulates Ci phosphorylation, we then examined Cifl phosphorylation status when PP2A was knocked down by RNAi in S2 cells. As shown in Fig. 6, upon OA treatment, Cifl underwent rapid changes in phosphorylation indicated by an electrophoretic mobility shift (Fig. 6A, lane 2, compare with lane 1, top panel). We found that Flag-Cifl showed low mobility when endogenous Mts or Wdb was knocked down by RNAi (lanes 3 and 4, top panel). By contrast, Flag-Cifl did not exhibit a mobility shift by PP4 or GFP RNAi (lanes 5 and 6, top panel). These data suggest that Cifl is specifically regulated by PP2A. To further determine whether the elevated levels of Cifl produced by overexpressing Mts or Wdb in wing discs (Fig 4D,E; see Fig. S8A,B in the supplementary material) were due to changes in Cifl phosphorylation, we examined whether overexpressing phosphatases could attenuate Cifl phosphorylation in S2 cells. OA treatment consistently induced the mobility shift of Cifl (Fig. 6B, lane 2; compare with lane 1, top panel). Expressing HA-Mts (Fig. 6B, lane3, top panel) or HA-Wdb (lane 4), but not HA-PP4 (lane 5), alleviated the OA-induced Cifl mobility shift, suggesting that PP2A, but not PP4, prevents Cifl phosphorylation and, thus, the accumulation in wing discs.

We next asked whether downregulation of Ci phosphorylation affects its processing. The proteolytic processing of Cifl requires the activity of the proteasome. The levels of Cifl in wing discs were elevated by treatment with the proteasome inhibitor MG132 (Fig. 6D, compare with 6C). We found that overexpressing UAS-WdbRNAi by ap-Gal4 destabilized Cifl in dorsal compartment cells (Fig. 6E). However, downregulation of Cifl by Wdb RNAi was prevented by the treatment with MG132 (Fig. 6F), suggesting that PP2A acts upstream of the proteasome to dephosphorylate Cifl and promote Cifl accumulation. Similarly, MG132 treatment prevented the downregulation of Cifl by Mts RNAi (not shown).

To determine whether the accumulation of Cifl was due to the blockade of Cifl processing, we assessed Cifl processing using an in vivo function assay, in which UAS-HA-Cifl or UAS-HA-Cifl plus UAS-Wdb were misexpressed in the P-compartment wing discs carrying smo mutant clones and hh-lacZ reporter gene. Consistent with previous findings (Jia et al., 2005), P-compartment smo mutant cells expressing HA-Cifl blocked hh-lacZ expression (Fig. 6G-G'), indicating that Cifl was processed to generate Cifl. By contrast, P-compartment smo mutant cells co-expressing HA-Cifl with Wdb partially suppressed hh-lacZ expression (Fig. 6H-H'), suggesting that HA-Cifl is partially blocked to produce Cifl in the presence of Wdb. We also assessed Cifl processing by immunoprecipitation and western blot analysis. UAS-HA-Cifl was expressed either alone or along with UAS-Wdb by MS1096 Gal4 in wing discs. HA-Cifl did not give rise to detectable Cifl when Wdb was co-expressed, whereas HA-Cifl alone was partially processed into Cifl (Fig. 6I). Taken together, our data suggest that PP2A is a positive regulator in Hh signaling by inhibiting the phosphorylation and processing of Cifl.

**DISCUSSION**

Regulated phosphorylation of Smo and Ci are crucial events in mediating Hh signal transduction. Previous studies have identified multiple Ser/Thr kinases that regulate Hh signaling by phosphorylating Smo and Ci; however, the Smo and Ci phosphatases remain unknown. We are able to identify the phosphatase that regulates Smo phosphorylation by using an in vivo screen. Our screen differs from the previous screens because we use an in vivo assay to examine Smo expression levels, which is a more direct readout, and because knockdown of specific phosphatase
gene(s) involved in Smo dephosphorylation might not affect the pathway activity in a significant way and such gene(s) could have been missed in the previous RNAi screens with cultured cells (Lum et al., 2003a; Nybakken et al., 2005). In this study, we have identified PP4 as a novel Hh signaling component that regulates Smo phosphorylation. Our study provides the first evidence for the physiological Smo and Ci phosphatases, and uncovers the underlying mechanism of Smo regulation by phosphatase (Fig. 6J).

PP4 and PP2A play distinct roles in the Hh pathway

In this study, we have identified PP4 and PP2A to be negative and positive regulators in the Hh pathway, and we showed that they exert their roles through Smo and Ci, respectively. Are PP4 and PP2A the only phosphatases in the Hh pathway? Although our data suggest that PP4 is a phosphatase for Smo, we cannot exclude the possibility of the involvement of other phosphatase(s). Hh induces extensive Smo phosphorylation at numerous Ser/Thr sites, and multiple kinases are involved in these phosphorylation events (Apionishev et al., 2005; Jia et al., 2004; Zhang et al., 2004). It might be possible that multiple phosphatases could be involved. In addition, our loss-of-function studies on PP2A regulating Ci are not based on null mutations. This was due to the fact that genetic null mutations of the catalytic and regulatory subunits cause cell lethality (Sathyanarayanan et al., 2004). Thus, the results might not be exclusive.

Fig. 6. PP2A downregulates Ci phosphorylation and blocks Ci proteolytic processing. (A) Ciβ4 phosphorylation is upregulated by PP2A RNAi. S2 cells were transfected with Flag-Ci and treated with OA or indicated dsRNA. Cell extracts were subjected to direct western blot with anti-Flag antibody. Arrow indicates hyperphosphorylated forms of Ci and arrowhead indicates the hypophosphorylated or unphosphorylated forms. β-Tubulin serves as loading control. The knockdown efficiency of individual phosphatase was estimated by the method used for Fig. 2A. (B) PP2A downregulates Ciβ4 phosphorylation. S2 cells were transfected with Flag-Ci alone or along with indicated HA-tagged phosphatase and treated with or without OA. Cell lysates were probed with anti-Flag or anti-HA antibodies. (C) A disc shows the wild-type Ciβ4 staining. (D) A wing disc shows the Ciβ4 stabilization by the treatment of proteasome inhibitor MG132. (E,F) Wing discs expressing UAS-WdbRNAi ap-Gal4 were transfected with or without MG132 and stained to show Ciβ4. Arrowhead in E indicates the destabilized Ciβ4 by Wdb RNAi. Arrowhead in F indicates that the destabilized Ciβ4 by Wdb RNAi was restored by MG132 treatment. (G-H) Wing discs bearing smo3 clones and expressing UAS-HA-Ciβ4 alone or along with UAS-Wdb by MS1096 Gal4 were stained to show the expression of GFP (green) and hh-lacZ (red). Arrowheads in G and H indicate smo3 clones that are marked by the lack of GFP expression. Arrowheads in G’ and H indicate the hh-lacZ expression in smo3 cells. (I) Western blot analysis of protein extracts from wing discs expressing UAS-HA-Ciβ4 or co-expressing UAS-HA-Ciβ4 with UAS-Wdb using the MS1096 Gal4. Protein extracts were prepared from 400 wing discs, immunoprecipitated and blotted with HA antibody. (J) A model for the involvement of PP4 and PP2A in Hh signaling. PP4 negatively regulates Hh signal transduction by antagonizing the phosphorylation of Smo. PP2A positively regulates Hh pathway by counteracting kinases to downregulate Ciβ4 phosphorylation and attenuate its proteasome-mediated processing.

PP4 and PP2A play distinct roles in the Hh pathway

In this study, we have identified PP4 and PP2A to be negative and positive regulators in the Hh pathway, and we showed that they exert their roles through Smo and Ci, respectively. Are PP4 and PP2A the only phosphatases in the Hh pathway? Although our data suggest that PP4 is a phosphatase for Smo, we cannot exclude the possibility of the involvement of other phosphatase(s). Hh induces extensive Smo phosphorylation at numerous Ser/Thr sites, and multiple kinases are involved in these phosphorylation events (Apionishev et al., 2005; Jia et al., 2004; Zhang et al., 2004). It might be possible that multiple phosphatases could be involved. In addition, our loss-of-function studies on PP2A regulating Ci are not based on null mutations. This was due to the fact that genetic null mutations of the catalytic and regulatory subunits cause cell lethality (Sathyanarayanan et al., 2004). Thus, the results might not be exclusive.

**PP4 and regulation of Smo phosphorylation**

We showed that removal of PP4 by RNAi in wing discs induced Smo accumulation in A-compartment cells both near and away from the AP boundary (Fig. 1B). In addition, PP4 RNAi induced the elevation and anterior expansion of Hh target gene expression (Fig. 1B'). However, the accumulated Smo caused by PP4 RNAi did not ectopically activate Hh target genes in cells away from the AP boundary (Fig. 1B', data not shown). In addition, although Smo phosphorylation was potentiated by knocking down PP4 or abolishing Smo-PP4 interaction (Fig. 2A,E), the elevated
phosphorylation did not suffice to promote Smo cell-surface accumulation (Fig. 3E,O). These data suggest that the basal phosphorylation of Smo regulated by PP4 is not sufficient to activate Smo, and that de novo Smo activation still depends on Hh.

Previous studies have shown that PKA and CK1 are required for Hh-induced Smo accumulation and signaling activity. Phosphorylation-deficient forms of Smo (with PKA or CK1 sites mutated to Ala) are defective in Hh signaling, whereas SmoSD123, the phosphorylation-mimicking Smo, has potent signaling activity and high level of cell-surface accumulation (Jia et al., 2004; Zhang et al., 2004). Thus, the PKA and CK1 sites are apparently crucial in mediating Smo phosphorylation and activation. Hh treatment may cause increased phosphorylation at these sites. In addition to PKA and CK1 sites, there are many other Ser/Thr residues that are phosphorylated upon Hh stimulation (Zhang et al., 2004). Although phosphorylation-mimicking mutations at these sites alone did not have discernible effect on Smo, their phosphorylation could modulate the cell-surface accumulation and activity of Smo phosphorylated at the three PKA/CK1 sites, which may at least in part explain why cell-surface accumulation and activity of SmoSD123 is still regulated by Hh (Jia et al., 2004) (Fig. 3M). Here, we found that removing PP4 alone promoted Smo phosphorylation but did not elevate the cell-surface accumulation of Smo. It is possible that high levels of basal Smo phosphorylation in the absence of PP4 do not reach the threshold for promoting Smo cell-surface accumulation. It is also possible that basal Smo phosphorylation mainly occurs at sites other than the crucial PKA/CK1 phosphorylation clusters. In support of this notion, we found that knockdown PP4 by RNAi promoted SmoSD123 to further accumulate on the cell surface in the absence of Hh (Fig. 3N).

How is Smo phosphorylation regulated? Hh may regulate Smo phosphorylation by regulating the accessibility of its kinase and/or phosphatase. In this study, we found that Smo interacts with PP4 through amino acids 626-678, a region we previously mapped to be a Cos2-interacting domain (Liu et al., 2007). We further found that Smo-PP4 association diminished when Cos2 was knocked down by RNAi (Fig. 2I). Our previous study revealed that Cos2 impedes Hh-induced Smo phosphorylation by interacting with amino acids 626-678 of Smo and Hh-induced phosphorylation of Cos2 at Ser572 dissociates Cos2 from amino acids 626-678 of Smo, thereby alleviating its inhibition on Smo phosphorylation (Liu et al., 2007). In this study, we found that Cos2 inhibits Smo phosphorylation by recruiting PP4 and Hh promotes Smo phosphorylation by preventing Cos2-PP4 complex from binding to amino acids 626-678 of Smo. SmoΔ626-678, when not interacting with PP4, could still interact with Cos2 via a Cos2-interaction domain near the Smo C terminus (Jia et al., 2003). The Cos2-binding Smo C terminus might not recruit PP4. Taken together, our findings suggest that Hh may promote Smo phosphorylation at least in part by reducing the accessibility of a phosphatase.

**PP2A and regulation of Ci phosphorylation**

As stated in the Introduction, phosphorylation of Ci/Gli controls the balance of its activator and repressor activity. Here, we demonstrate a role of PP2A in dephosphorylating Ci and attenuating Ci processing. However, it is not known whether Hh regulates PP2A to dephosphorylate Ci protein. Previous studies have shown that Hh interacts with the Cos2-Ci-kinase protein complex (Zhang et al., 2005). It is possibly that Hh also regulates Ci phosphatase, or the accessibility of the phosphatase. Future studies should determine whether PP2A interacts with Cos2-Ci and whether such interaction is regulated by Hh.

Many aspects of Smo and Ci/Gli regulation are conserved across species. For example, both Drosophila and mammalian Smo proteins undergo a conformational switch in response to Hh stimulation (Zhao et al., 2007). Ci/Gli proteolysis is mediated by the same set of kinases and E3 ubiquitin ligases (Jiang, 2006). In addition, it has been shown that PP2A is involved in vertebrate Hh signaling, probably by regulating Gli nuclear localization and activity (Krauss et al., 2008; Rorick et al., 2007). Therefore, it would be interesting to determine whether PP4 and PP2A play similar roles in regulating phosphorylation of vertebrate Smo and Gli.

We thank Drs Suzanne Eaton, David Glover, Amita Sehgal, David Robbins, Robert Holmgren, C.-ting Wu and Jin Jiang for providing valuable reagents; VDRC for fly strains; DGRC for cDNA clones; and DSHB for antibodies. We thank Dr Hugo Bellen and Koen Venken for vas-phi-zh2A-VK5 fly stock. We are grateful to Drs Jin Jiang for comments on the manuscript, Leoncio Vergara for assistance with cell-surface Smo quantification and Tianyan Gao for helpful discussions. This work was supported by grants from the National Institute of Health (RO1 GM 079684), the American Heart Association and the American Cancer Society Institutional Research Award to J.J. Deposited in PMC for release after 12 months.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/2/307/DC1

**References**


