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Ptch2 shares overlapping functions with Ptch1 in Smo regulation and limb development

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ABSTRACT

Ptch1 and Ptch2 are highly conserved vertebrate homologs of *Drosophila* ptc, the receptor of the Hedgehog (Hh) signaling pathway. The vertebrate *Ptch1* gene encodes a potent tumor suppressor and is well established for its role in embryonic development. In contrast, *Ptch2* is poorly characterized and dispensable for embryogenesis. In flies and mice, *ptc*/*Ptch1* controls Hh signaling through the regulation of Smoothened (Smo). In addition, Hh pathway activation also up-regulates *ptc*/*Ptch1* expression to restrict the diffusion of the ligand. Recent studies have implicated *Ptch2* in this ligand dependent antagonism, however whether *Ptch2* encodes a functional Shh receptor remains unclear. In this report, we demonstrate that Ptch2 is a functional Shh receptor, which regulates Smo localization and activity *in vitro*. We also show that *Ptch1* and *Ptch2* are co-expressed in the developing mouse limb bud and loss of *Ptch2* exacerbates the outgrowth defect in the limb-specific *Ptch1* knockout mutants, demonstrating that Ptch1 and Ptch2 co-operate in regulating cellular responses to Shh *in vivo*.

Introduction

Sonic hedgehog (Shh) is an important regulator of patterning, development and homeostasis in the embryo and adult (Hui and Angers, 2011; Jiang and Hui, 2008). The Shh signal is transduced by the transmembrane protein Patched 1 (Ptch1) through regulation of Smoothened (Smo) (Chen and Struhl, 1996; Hooper and Scott, 1989; Ingham et al., 2000). In the absence of Shh, Ptch1 inhibits Smo activity. Binding of Shh to Ptch1 alleviates this inhibition resulting in Smo activation. The precise mechanism of Smo activation is not known, but it requires the translocation of Smo from the plasma membrane into the primary cilium (Corbit et al., 2005; Dorn et al., 2012; Milenkovic et al., 2009; Rana et al., 2013; Rohatgi et al., 2007, 2009; Wang et al., 2009; Wilson et al., 2009). Accumulation of Smo in the primary cilium promotes the activation of transcription factors Gli1, Gli2 and Gli3, the effectors of Shh signaling, and Gli target gene expression (Hui and Angers, 2011).

Ptch1 regulates Shh signaling through two distinct mechanisms – *ligand dependent antagonism* (LDA) and *ligand independent antagonism* (LIA) – which are conserved from flies to mice (Briscoe et al., 2001; Chen and Struhl, 1996; Goodrich et al., 1996; Holtz et al., 2013). LIA refers to the ability of Ptch1 to constitutively inhibit Smo in the absence of Shh. In contrast, LDA involves the transcriptional up-regulation of *Ptch1* mRNA and the accumulation of Ptch1 protein at the cell surface in response to Shh. It is believed that LDA serves to restrict the diffusion range of the Shh ligand, thereby regulating the Shh gradient required for patterning (Briscoe et al., 2001; Chuang and Mcmahon, 1999; Holtz et al., 2013; Jeong and McMahon, 2005).

Elegant studies in the mouse neural tube demonstrated that the Shh-target gene *Hip* (Hedgehog interacting protein) also accumulates at the cell surface in response to pathway activation and co-operates with Ptch1 to restrict the diffusion of Shh (Chuang and Mcmahon, 1999; Holtz et al., 2013; Jeong and McMahon, 2005). This confirmed that LDA is critical for establishment of neuronal cell fate which is specified by discrete levels of Shh pathway activity along the dorsoventral axis (Briscoe et al., 2001; Chuang and Mcmahon, 1999; Jeong and McMahon, 2005).

Recent studies in the chick limb revealed that diffusion of Shh is dependent on transport along stabilized filopodia and direct transfer, from signal secreting to signal receiving cells. The membrane glycoproteins Cdo and Boc are required for Shh signal transduction and were shown to intercept the ligand on signal receiving cells in the limb (Allen et al., 2011; Izzi et al., 2011;

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Kavran et al., 2010; Sanders et al., 2013). *Cdo* and *Boc* show both overlapping and distinct expression patterns in Hh-responsive tissues and their loss has tissue-specific effects, which partly recapitulate the phenotype of the *Shh*$^{-/-}$ mutants (Chiang et al., 2001; Kraus et al., 2001; Okada et al., 2006; Tenzen et al., 2006; Zhang et al., 2006, 2011). Similarly, *Gas1* is highly expressed in Hh-responsive tissues of the chick and mouse where it binds and promotes Hh transduction in signal receiving cells, particularly in regions where ligand concentration is low (Lee and Fan, 2001; Lee et al., 2001; Martinelli and Fan, 2007; Seppala et al., 2007). Notably, Cdo, Boc and Gas1 form distinct complexes with Ptch1 and both *in vivo* and *in vitro* studies have demonstrated that these glycoproteins are critical components of the signal transduction machinery (Allen et al., 2007, 2011; Holtz et al., 2013; Izzi et al., 2011; Seppala et al., 2007).

In addition to these proteins, vertebrates also encode a *Ptch1* homologue – *Ptch2* – which is expressed in many Shh-responsive tissues, including the neural tube, the limb and the skin (Motoyama et al., 1998a, 1998b; Pearse et al., 2001). Until recently, the function of Ptch2 was poorly understood. *Ptch2* mutant mice (*Ptch2*$^{-/-}$ and *Ptch2*$^{lacZ/lacZ}$), generated in our laboratory, display no overt developmental defects, and are viable and fertile (Adolphe et al., 2014; Nieuwenhuis et al., 2006). Similarly, *Ptch2* mutants independently generated by others are also grossly normal (Holtz et al., 2013; Lee et al., 2006). This is in stark contrast to *Ptch1*$^{-/-}$ mutant mice, which are embryonic lethal by mid-gestation (E9.5) and exhibit severe defects consistent with activated Shh signaling, including exencephaly, open neural tube and cardiac defects (Ellis et al., 2003; Goodrich et al., 1997).

Recent work by Holtz et al. (2013) revealed that Ptch2 interacts with Cdo, Boc and Gas1 *in vitro* and cooperates with Hip and Ptch1 to regulate the Shh gradient in the embryonic neural tube through LDA. It remains unclear whether Ptch2 contributes to LIA as evidence for a role for Ptch2 in Smo regulation and Shh signal transduction is limited. In particular, over-expression studies utilizing human *PTCH1* and *PTCH2* isoforms suggested that while both homologs can bind and internalize Shh, only *PTCH1* is able to regulate the expression of a Shh-dependent luciferase reporter (Carpenter et al., 1998; Motoyama et al., 1998a; Rahnama et al., 2004). However, work from our laboratory and others has shown that murine *Ptch2* is able to inhibit the activity of Shh/Gli-responsive reporters and that Ptch2 responds to Shh in transfection assays (Holtz et al., 2013; Nieuwenhuis et al., 2006). Furthermore, studies of zebrafish somite and fin development as well as mouse skin development and brain tumorigenesis have suggested that *Ptch1* and *Ptch2* play overlapping roles in pathway regulation (Adolphe et al., 2014; Koudijs et al., 2008; Lee et al., 2006). Thus, it is important to establish if *Ptch2* functions as a receptor by transducing the Shh signal and regulating Smo through LIA. To address this question, we performed biochemical and genetic experiments to determine whether Ptch2 is a functional Shh receptor *in vitro* and *in vivo*. Our results indicate that, in the absence of *Ptch1*, *Ptch2* plays a critical role in the regulation of Smo at the primary cilium. This LIA function of Ptch2 complements its role in LDA and gradient regulation in Shh signaling (Holtz et al., 2013).

Results and discussion

Ptch2 over-expression reconstitutes normal Shh signaling in Ptch1$^{-/-}$ *MEFs*

Although Ptch1 and Ptch2 share 56% identity at the amino acid level, it is not clear if their function is biochemically similar (Kawamura et al., 2008; Motoyama et al., 1998a). The two proteins differ mostly in the C-terminal region, which is truncated in *Ptch2* (Carpenter et al., 1998). *In vitro* analysis suggests that both human PTCH1 and PTCH2 bind to Shh and the Shh co-receptors Gas1, Cdo and Boc (Bae et al., 2011; Carpenter et al., 1998; Holtz et al., 2013; Izzi et al., 2011). However, mutant analysis revealed that their requirement in mouse development is drastically different – while *Ptch2*$^{-/-}$ mutants are viable and fertile, *Ptch1*$^{-/-}$ mutants are lethal at E9.5 and exhibit severe defects consistent with hyper-activation of Shh signaling (Goodrich et al., 1997; Lee et al., 2006; Nieuwenhuis et al., 2006).

Holtz et al. (2013) recently demonstrated that *Ptch2* inhibits Shh signaling using luciferase reporter and chick neural tube electroporation assays. However, it is unclear if *Ptch1* and *Ptch2* use the same mechanism to inhibit the pathway and if they work together. It is well established that *Ptch1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) exhibit constitutive pathway activation (Rohatgi et al., 2007; Taipale et al., 2000). *Ptch2* is transcriptionally upregulated in response to Shh and primary limb fibroblasts, derived from *Prx1-Cre;Ptch1*$^{f/-}$ mutant forelimbs (E12.5), exhibit transcriptional upregulation of *Ptch2* (Fig. S1A) suggesting that these cells may be responsive to Shh. To address this question, we treated *Ptch1*$^{-/-}$ MEFs with recombinant Shh protein or carrier (BSA) and assessed pathway activation. It was previously shown that loss of *Ptch1* results in constitutive Smo localization to the primary cilium and pathway activation in MEFs (Rohatgi et al., 2007). Consistent with this, we observe Smo localization in more than 80% of primary cilia in *Ptch1*$^{-/-}$ MEFs. Treatment with Shh, but not BSA (carrier), results in a $>10\%$ increase in the number of Smo^{+} primary cilia (Fig. 1A–C', quantified in Fig. 1D). This is associated with a 1.4-fold increase in *Gli1* (Fig. 1E) and 1.6-fold increase in *Hip* (Fig. 1F) mRNA expression indicating that *Ptch1*$^{-/-}$ MEFs remain sensitive to Shh ligand. Similarly, we demonstrated that *Ptch1*-mutant fibroblast cells, derived from limbs of *Prx1-Cre*; *Ptch1*$^{f/-}$ mutant E12.5 embryos, remain sensitive to Shh-conditioned media as shown by upregulation of *Gli2* (Fig. 1G) and *Gli1* mRNA (Fig. 1H). These findings are consistent with the recent work of Alfaro et al. (2014), which demonstrated that *Ptch1*$^{-/-}$ cells remain sensitive to Shh independent of Ptch1 antiporter activity.

Previous studies showed that over-expression of *Ptch1* is able to partly rescue the phenotypes of *Ptch1*$^{-/-}$ mouse mutants (Milenkovic et al., 1999). To address the functional similarity between *Ptch1* and *Ptch2*, and to determine if *Ptch2* mediates Smo localization and Shh responsiveness in *Ptch*$^{-/-}$ MEFs, we tested if retroviral infection with *Ptch1HA* and *Ptch2myc* constructs, can suppress constitutive pathway activity in *Ptch1*$^{-/-}$ MEFs (Fig. 2A–F). *Ptch1*$^{-/-}$ MEFs were infected with retroviral vectors carrying *Ptch1HA-IRESGFP*, *Ptch2myc-IRESGFP* or with an empty vector (encoding *IRESGFP*) and GFP^{+} cells were selected by flow cytometry. To test if GFP^{+} cells expressed Ptch1HA or Ptch2myc protein, we performed Western blot analysis on cell lines after cell sorting for GFP. Incubation of whole cell lysates with antibody against HA (Fig. S1D) or Ptch1 (Fig. S1E') detected abundant Ptch1 protein in *Ptch1*$^{-/-}$*::Ptch1HA* cells. Similarly, antibody against c-myc (Fig. S1E) and Ptch2 (Fig. S1D') detected Ptch2 protein in *Ptch1*$^{-/-}$*::Ptch2myc* cells. Similar expression was observed in membrane extracts from *Ptch1*$^{-/-}$*::Ptch1HA* and *Ptch1*$^{-/-}$*::Ptch2myc* cells (data not shown). Notably, endogenous Ptch2 level appears low in untreated *Ptch1*$^{-/-}$ MEFs (Fig. S1D'). Anti-Ptch1 antibody could not detect endogenous Ptch1 in *Ptch2*$^{-/-}$ primary limb fibroblast (control) (Fig. S1E'). This suggested that retroviral infection followed by cell sorting successfully enriched for *Ptch1*$^{-/-}$ MEFs expressing high levels of exogenous Ptch1 and Ptch2 protein. To compare the effects of Ptch1 and Ptch2 on Smo regulation, we examined Smo localization. We found that 96% of *Ptch1*$^{-/-}$ MEFs exhibit constitutive localization of Smo to