Stabilization of Speckle-type POZ Protein (Spop) by Daz Interacting Protein 1 (Dzip1) Is Essential for Gli Turnover and the Proper Output of Hedgehog Signaling*

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Background: Although Dzip1 positively influences Hedgehog signaling by regulating ciliogenesis, it inhibits the Hedgehog pathway through an unclear mechanism.
Results: Dzip1 knockdown destabilizes Spop E3 ubiquitin ligase, leading to increased Gli transcription factor levels and phenotypes resembling Hedgehog signaling activation.
Conclusion: Dzip1 inhibits Hedgehog signaling through stabilizing Spop.
Significance: We uncover a novel Gli regulatory mechanism.

The Hedgehog (Hh) pathway is essential for embryonic development and adult tissue homeostasis. The Gli/Cubitus interruptus (Ci) family of transcription factors acts at the downstream end of the pathway to mediate Hh signaling. Both Hh-dependent and -independent Gli regulatory mechanisms are important for the output of Hh signaling. Daz interacting protein 1 (Dzip1) has bipartite positive and negative functions in the Hh pathway. The positive Hh regulatory function appears to be attributed to a requirement for Dzip1 during ciliogenesis. The mechanism by which Dzip1 inhibits Hh signaling, however, remains largely unclear. We recently found that Dzip1 is required for Gli turnover, which may account for its inhibitory function in Hh signaling. Here, we report that Dzip1 regulates Gli/Ci turnover by preventing degradation of speckle-type POZ protein (Spop), a protein that promotes proteasome-dependent turnover of Gli proteins. We provide evidence that Dzip1 regulates the stability of Spop independent of its function in ciliogenesis. Partial knockdown of Dzip1 to levels insufficient for perturbing ciliogenesis, sensitized *Xenopus* embryos to Hh signaling, leading to phenotypes that resemble activation of Hh signaling. Importantly, overexpression of Spop was able to restore proper Gli protein turnover and rescue phenotypes in Dzip1-depleted embryos. Consistently, depletion of Dzip1 in *Drosophila* S2 cells destabilized Hh-induced BTB protein (HIB), the *Drosophila* homolog of Spop, and increased the level of Ci. Thus, Dzip1-dependent stabilization of Spop/HIB is evolutionarily conserved and essential for proper regulation of Gli/Ci proteins in the Hh pathway.

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Members of the highly conserved Hedgehog (Hh)[2] family of secreted proteins are essential in a variety of developmental processes. In many cases Hh ligands function as morphogens to regulate cell fate determination in a multicellular target field or as mitogens to control cellular proliferation and survival. Understanding the many orchestrated events that transduce the Hh signal is of considerable interest, as perturbations to the Hh pathway lead to a variety of developmental disorders (1–3). In addition, a wide range of cancers are caused by unchecked Hh signaling (4–6). At the molecular level the Cubitus interruptus (Ci)/Gli family of zinc finger transcription factors are key downstream effectors of Hh signaling. Before pathway activation, Ci/Gli are proteolytically processed, leading to truncated repressor forms that suppress the expression of Hh target genes (7–9). Hh proteins initiate the signaling by binding to its receptor Patched (Ptc) (10–12) and relieving the inhibition of Smoothened (Smo) by Ptc (13, 14). Activation of Smo leads to a series of intracellular signal transduction events that ultimately prevent proteolytic processing of Ci/Gli and convert Ci/Gli to transcriptional activators that, in turn, promote Hh target gene expression. In vertebrates, activation of Smo requires translocation of Smo into the primary cilium. Interestingly, the cilium is dispensable for *Drosophila* Hh signaling, wherein the pathway becomes activated once Smo is accumulated on the cell membrane (15–20).

In addition to Hh-dependent regulation of Ci/Gli processing, Hh-independent Ci/Gli regulatory mechanisms also influence the output of Hh signaling. It has been shown that Speckle-type POZ protein (Spop) and its *Drosophila* homolog Hh induced BTB protein (HIB, also known as Roadkill (Rdx)), substrate

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[2] The abbreviations used are: Hh, Hedgehog; Ci, Cubitus interruptus; Ptc, Patched; Smo, Smoothened; Dzip1, Daz interacting protein 1; Spop, Speckle-type POZ protein; BTB, Bric-a brac/Tramtrack/Broad complex protein domain; HIB, Hh induced BTB protein; Sufu, Suppressor of Fused; DMO, Dzip1 morpholino; Shh, Sonic Hedgehog; SSF, superficial slow fibers; IFT88, intraflagellar transport protein 88 homolog; MT, myc-tagged; *yot*, *you-too* allele.

binding adaptors of cullin3 E3 ubiquitin ligase (21), directly interact with Ci/Gli and promote their degradation by the proteasome (22–24). In vertebrates, the function of Spop/Cullin3 ubiquitin ligase is antagonized by Suppressor of Fused (Sufu), another Gli interacting protein (25–27). It is believed that Sufu competes with Spop for Gli binding and protects Gli2 and Gli3 from proteasome degradation (22, 23). In *Drosophila*, HIB functions to promote Ci degradation (24, 28, 29). Loss of *Hib* leads to increased Ci and elevated Hh signaling, whereas its overexpression inhibits Hh signaling by decreasing Ci levels (28, 29). Thus, the function of HIB/Spop in Ci/Gli turnover is conserved between vertebrates and invertebrates. Nevertheless, Sufu is dispensable for *Drosophila* Hh signaling (30).

Daz interacting protein 1 (Dzip1) has been implicated in the Hh pathway. Dzip1 is a C2H2 zinc finger domain protein with coiled-coil domains and a nuclear localization signal. Requirements for Dzip1 during embryonic development have been best studied using zebrafish *iguana* mutants, which harbor specific mutations in the *Dzip1* gene (31, 32). *Iguana* mutants exhibit midline and somitic muscle defects, both hallmark phenotypes of Hh loss of function in zebrafish. Interestingly, although global Hh signaling activity is reduced in *iguana* mutant embryos, low levels of ectopic expression of Hh-responsive genes, *ptc1* and *engrailed 1*, were also observed. This suggests that Dzip1 can regulate the Hh signaling pathway in a positive and negative manner (31–33). Subsequent investigations revealed that the positive role of Dzip1 in the Hh pathway is due to its requirement for ciliogenesis (34–37). Regarding the inhibitory function of Dzip1, we recently provided evidence that Dzip1 is required for Gli turnover (38). In this study we extend our previous findings by demonstrating that Dzip1 regulates Gli turnover through stabilizing Spop. We provide evidence that Dzip1 regulates Spop independent of its role in ciliogenesis. We show that decreased Spop protein levels in response to knockdown of Dzip1 can account for elevated Gli protein levels and increased Hh target gene expression. Collectively, these findings provide mechanistic insight to explain the ectopic Hh pathway activation in Dzip1-deficient embryos. Furthermore, we show that Dzip1 stabilizes HIB and regulates Ci turnover in *Drosophila* S2 cells. Taken together, our work demonstrates that the novel regulatory mechanism involving Dzip1-Spop-Gli is evolutionarily conserved and essential for the output of Hh signaling.

EXPERIMENTAL PROCEDURES

Xenopus Embryos and Manipulations—*Xenopus* embryos were obtained and injected as described (39). Embryo sectioning and staining were performed as described (39). The dosage of RNA or morpholino for microinjection is indicated in the text or figure legend. For experiments in which RNA and morpholino was injected into the same embryo, injections were performed sequentially. Morpholinos against Dzip1 (DMO1 and DMO2) (38), Gli1 and -2 (40), and intraflagellar transport protein 88 (IFT88)/Polaris (41) were described. For lactacystin treatment, animal caps were dissected at the late blastula stage and cultured in 1× Marc's modified Ringers with or without 2 μg/ml lactacystin. Caps were harvested at stage 15 for Western blotting. For purmorphamine treatments, a 10 mM stock solution in 100% ethanol was diluted to 2.5–10 μM in 0.5× Marc's modified Ringers. Embryos were transferred into 0.5× Marc's modified Ringers containing purmorphamine or vehicle at stage 14 and harvested at stage 35.

Expression Constructs—*Xenopus* Sonic hedgehog (Shh) (42), *Xenopus* Gli1, *Xenopus* Gli2, and human Gli3 (38) expression constructs were described. The full-length Spop and Spop deletion constructs were constructed by standard PCR/cloning methods. These constructs were derived from mouse Spop (IMAGE: 6825425). The Gli2$_{1–799}$ mutant was generated by site-directed mutagenesis. FLAG-HIB and FLAG-dDzip were constructed by cloning individual cDNA sequence in-frame with the 2XFLAG-UAST backbone. FLAG-Ci has been described (43). Cloning details will be provided upon request.

Quantitative Real Time PCR, Whole Mount in Situ Hybridization, and Immunostaining—RNA extraction and RT-PCR method were described (44). Real-time PCR reactions were performed in triplicate using SYBR Green master mix (Applied Biosystems) on the Applied Biosystems 7500 Real-time PCR System. PCR primers for *odc*, *ptc1*, and *gli1* were described (38). Whole mount *in situ* hybridization was performed as described (39). For sectioning embryos after whole-mount *in situ* hybridization, embryos were washed 3–5× in PBS, suspended in 4% low-melt agarose, 0.5× PBS, and sectioned at 100 μm using a Vibratome (Series 100, Electon Beam Sciences). Cilia were stained by labeling acetylated tubulin (anti-acetylated tubulin, Sigma). Superficial slow fibers and total muscle fibers were stained using monoclonal antibodies BA-F8 and 12/101 (Developmental Studies Hybridoma Bank), respectively.

Immunoprecipitation and Western Blots—Immunoprecipitations and Western blots were performed as described (45). To detect ubiquitinated Spop, an antigen retrieval step was added after proteins were transferred to the PVDF membrane. Membranes were washed 3× in TBS-Tween, placed protein side up on a water-soaked filter paper, wrapped in saran wrap, and heated in a pressure cooker for 25 min. After antigen retrieval, the membrane was washed 3× in TBS-Tween and used immediately for Western blotting. Antibodies were anti-Myc (9E10, Sigma, 1:1,000), anti-FLAG (M2, Sigma, 1:1,000), anti-Spop (Santa Cruz, 1:200), anti-ubiquitin (Santa Cruz, 1:500), and anti-β-tubulin (Sigma, 1:2,000).

Cell Culture, Transfection, RNAi, and Treatments—NIH3T3 cells were cultured and transfected as described (46). S2 cell culture and transfection were performed with standard protocols, and the use of dsRNA has been described (43). *dDzip1* dsRNA was synthesized against the region of nucleotide 91–676 of *dDzip1* coding sequence. MG132 (Calbiochem) was added to inhibit proteasome activity at a final concentration of 50 μM for 4 h before harvesting the cells.

RESULTS

Partial Knockdown of Dzip1 Sensitizes Xenopus Animal Caps to Shh—Gli regulatory mechanisms have an important impact on the output of Hh signaling. Dzip1 is required for Gli turnover (38) and ciliogenesis (34–36, 38). We previously reported that these functions of Dzip1 could be uncoupled. Stabilization of Gli proteins could be detected even when Dzip1 was partially knocked down. In contrast, ciliogenesis defects occurred only
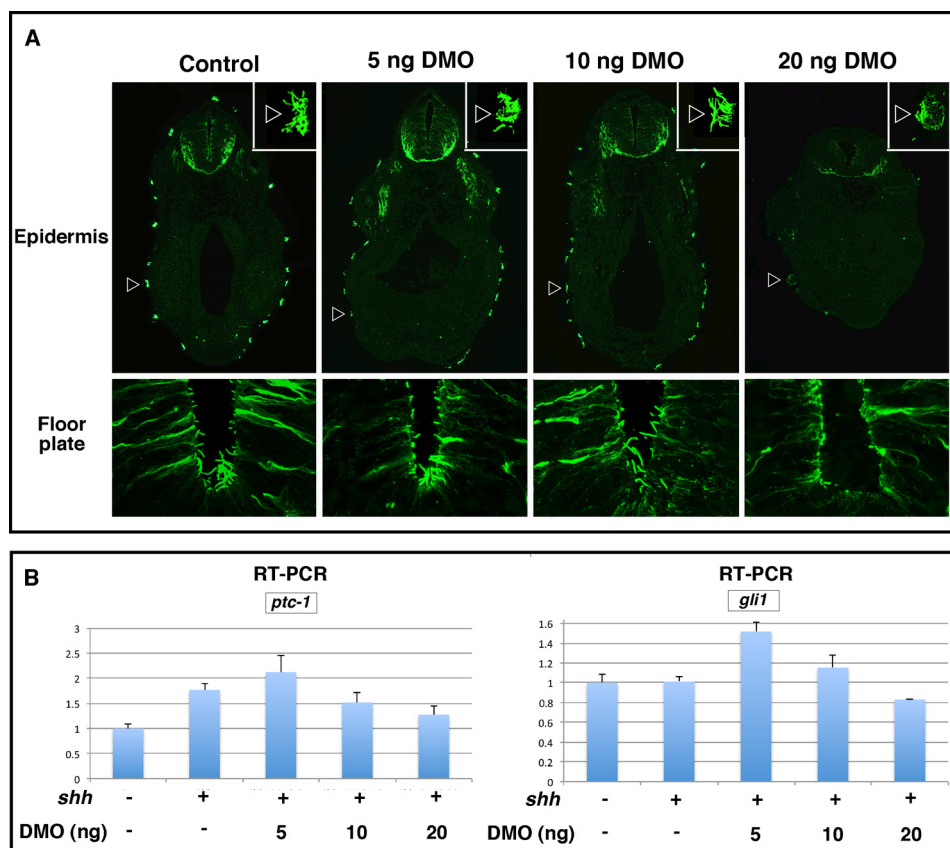
FIGURE 1. **Sensitization of *Xenopus* animal caps to Shh by partial knockdown of Dzip1.** *A*, immunofluorescence showing primary cilia in the floor plate (*bottom panel*) and epidermis motile cilia (*top panel*, *arrowheads*) in control (*left column*) and embryos injected with 5, 10, or 20 ng of DMOs (from *left* to *right*). *B*, real-time RT-PCR showing the expression of *ptc1* and *gli1* in animal caps from controls and embryos injected with *shh* mRNA (500pg) alone or in combination with 5, 10, or 20 ng of DMOs. All samples were normalized to *odc* levels.

when the depletion of Dzip1 was more complete (38). Because different levels of Dzip1 are required for its functions in Gli turnover and ciliogenesis, we set out to investigate how the Dzip1-dependent Gli regulatory mechanism influences the output of Hh signaling by partially depleting Dzip1.

Using a combination of two Dzip1 morpholinos (DMO, described in Jin *et al.* (38)), we knocked down Dzip1 to various degrees and examined the response of cells to Shh in an animal cap assay. In parallel, we examined the formation of floor plate primary cilia and skin motile cilia in tadpoles. We found that injection of 5 ng of DMO, which had no effect on the formation of cilia (Fig. 1*A*), caused a weak but reproducible increase in Shh-induced expression of *gli1* and *ptc1* in animal caps (Fig. 1*B*). Embryos receiving 10 ng of DMO displayed intermediate phenotypes (Fig. 1, *A* and *B*). Injection of higher doses of DMO (20 ng) caused defective primary and motile cilia (Fig. 1*B*) and reduced Shh-induced expression of *gli1* and *ptc1* (Fig. 1*A*). These data indicate that partial depletion of Dzip1 sensitizes animal caps to Shh.

Partial Knockdown of Dzip1 Leads to Ectopic Hh Signaling in Whole Embryo—In light of the above findings, we next sought to perform a detailed phenotypic characterization of embryos after partial Dzip1 depletion. We speculated that partially depleting Dzip1 levels, below the threshold to perturb ciliogenesis, would result in phenotypes characteristic solely of Hh activation in the embryo. To do so, a low concentration (5 ng) of

DMO was injected at the one cell stage, and phenotypic characterization was carried out carefully at the tadpole stage.

To both confirm results from the animal cap assay, which showed that a low DMO dose could enhance Shh-induced target gene expression, and investigate how Hh signaling was spatially regulated in DMO injected embryos, we examined *ptc1* expression in the neural tube and somitic mesoderm, two tissues whose development is regulated by Hh signaling. As shown in Fig. 2*A*, *ptc1* was markedly enhanced throughout the neural tube and somitic tissues after DMO injection (78%, $n = 36$) compared with controls, indicating that Hh signaling is enhanced throughout both tissues. Importantly, the enhanced *ptc1* expression phenotype was visualized when DMO1 or DMO2, which target different sequences of *Dzip1* mRNA, was injected individually or in combination, indicating that increased *ptc1* expression was not an off-target effect. In addition, injection of a five-base pair (bp)-mismatch control morpholino yielded *ptc1* staining patterns that were indistinguishable from uninjected controls (Fig. 2*B*), further supporting the specificity of Dzip1 knockdown by DMOs.

To determine how partial Dzip1 depletion impacts Hh pathway-dependent developmental processes, we analyzed markers of neural tube, somite, and eye in DMO-injected tadpoles. In the neural tube, proper expression of *foxa2* and *nkx2.2*, markers for medial and lateral floor plate respectively, is dependent on Hh signals from the underlying notochord (47–49). In *Xeno-*
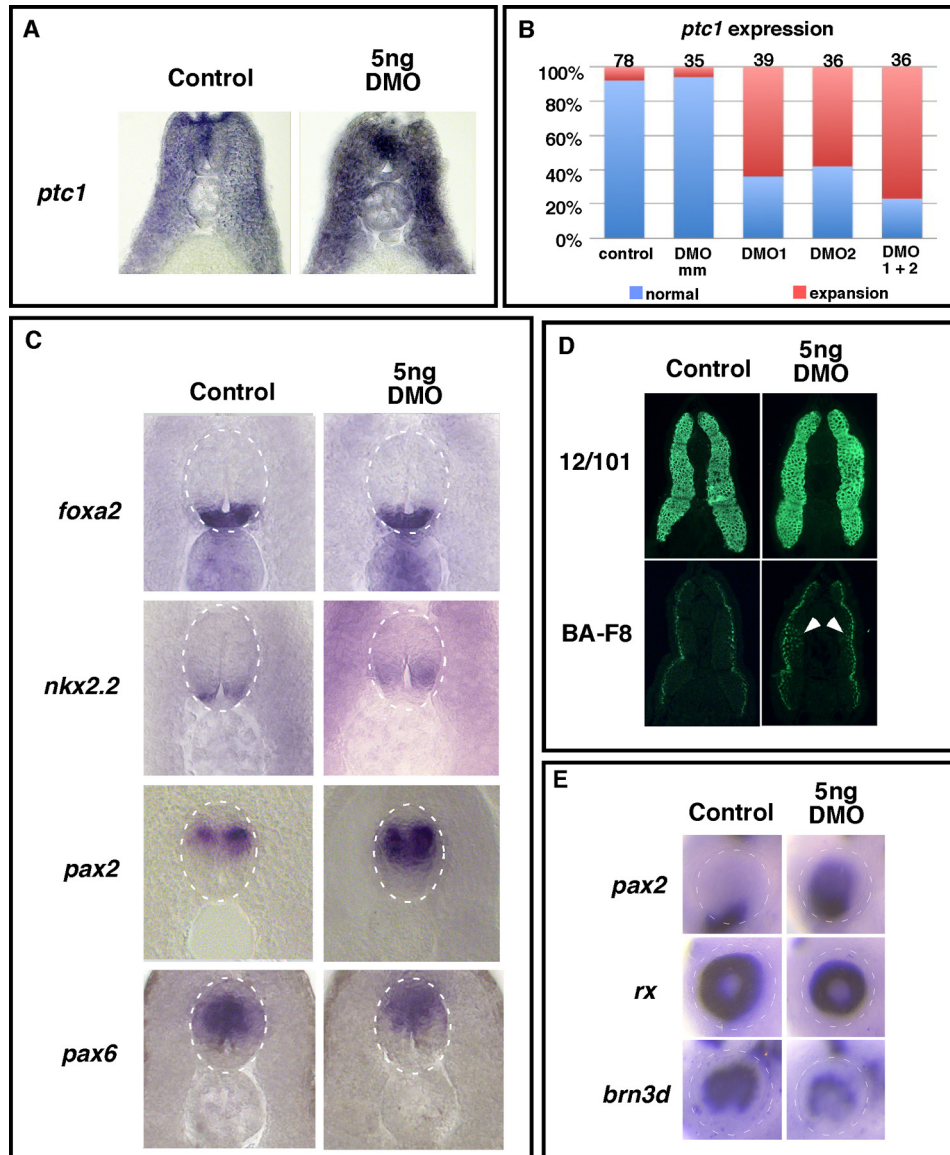
FIGURE 2. **Partial depletion of Dzip1 increased the expression of Hh target genes in embryos.** *A, in situ* hybridization showing that partial Dzip1 depletion (*right*) markedly increased the expression of *ptc1* in the neural tube and somites at stage 35. *B,* summary of the *ptc1* expression phenotypes in control and morpholino-injected embryos. *DMO mm,* 5-bp mismatch control morpholino of DMO2. *C, in situ* hybridization showing the expression of floor plate markers (*foxa2* and *nkx2.2*) and markers for more dorsal neural tube cells (*pax2* and *pax6*) in controls (*left*) and embryos injected with DMO (*right*). The *white dotted lines* outline the neural tube borders. *D,* immunofluorescence showing total muscle (12/101 reactivity; *top panel*) and slow muscle cells (BA-F8 reactivity; *bottom panel*) in controls (*left column*) and DMO-injected embryos (*right column*). *Arrowheads* denote ectopic BA-F8 reactive slow muscle cells evident in DMO injected embryos. *E, in situ* hybridization showing eye patterning in controls (*left*) and DMO injected (*right*) embryos. Markers for optic stalk (*pax2, top panel*), total retina (*rx, middle panel*), and dorsal retina (*brn3d, bottom panel*) were analyzed. *Dotted lines* outline the eye.

pus, these markers are differentially sensitive to increased Hh signaling, with only *nkx2.2* being affected after up-regulation of Hh signaling (50). We found that there was no distinguishable difference in *foxa2* expression between DMO injected ($n = 30$) and control embryos ($n = 30$). The expression of *nkx2.2*, however, was expanded dorsally in DMO-injected embryos (58%, $n = 36$) compared with controls ($n = 33$). The expression of *pax2*, which is localized to an adjacent dorsal region to the *nkx2.2* domain and is positively regulated by Hh signaling (50), was enhanced and expanded dorsally in DMO injected embryos (59%, $n = 32$) compared with controls ($n = 25$). The expression of *pax6*, a gene that is restricted from the ventral neural tube and negatively regulated by Hh signaling (47, 51, 52), was reduced and shifted dorsally in DMO-injected embryos (50%,

$n = 42$) compared with controls ($n = 27$) (Fig. 2*C*). Collectively, marker expression in the neural tubes of DMO injected embryos is suggestive of enhanced Hh signaling, in accordance with the increased *ptc1* expression in morphant neural tubes.

Hh signaling regulates cell fate determination in the somitic mesoderm. In *Xenopus* and zebrafish embryos, superficial slow fibers (SSF) arise at the lateral border of the somite. Specification of SSF depends on low levels of Hh signals from the notochord (53–55). Ectopic Hh signaling in *Xenopus* embryos results in a mis-positioning and an increased incidence of SSF within more medial positions of the posterior somite (55). To determine how partial Dzip1 depletion affected somitic mesoderm development, we examined total muscle fiber and SSF formation in posterior trunk sections of DMO-injected embryos. The

monoclonal antibodies, BA-F8 and 12/101, that react with SSF and all muscle fibers (55), respectively, were utilized for this analysis. In controls, BA-F8 reactivity was confined to a single layer of cells at the lateral edge of 12/101 staining field ($n = 20$). In contrast, DMO-injected embryos contained more slow muscle fibers (57%, $n = 14$), with ectopic BA-F8-reactive cells arising in more medial layers of the 12/101 staining field (*arrowheads*) (Fig. 2*D*). The SSF expansion in DMO-injected embryos is reminiscent of Hh activation phenotypes, consistent with enhanced *ptc1* expression throughout the somitic mesoderm in these embryos.

Hh signaling regulates multiple aspects of eye development, including dorsoventral patterning in the eye. During eye patterning, the optic vesicle becomes specified into a ventral domain, which gives rise to the optic nerve, and a dorsal domain that forms the retina. During zebrafish, *Xenopus*, and chick development, elevated Hh signaling represses eye field transcription factors and activates forebrain differentiation markers. This results in an expansion of the ventral forebrain, which includes the optic nerve, at the expense of retinal primordia, leading to a small eye phenotype with reduced numbers of retinal cells (51, 56–62). We assessed eye-patterning phenotypes in DMO-injected embryos by examining expression of *pax2*, a ventral optic stalk marker, as well as retinal markers *rx* and *brn3d*. In controls, *pax2* expression was confined to the ventral extent of the eye ($n = 67$), whereas *rx* and *brn3d* were expressed throughout the dorsoventral extent of the eyefield ($n = 23$, *rx*; $n = 47$, *brn3d*). Partial Dzip1 depletion disrupted the dorsoventral organization of the eye. In DMO-injected eyes, *pax2* expression was expanded dorsally (59%, $n = 90$), and the ventral expression domain of *brn3d* was significantly reduced (54%, $n = 48$). Partial knockdown of Dzip1 was not sufficient to down-regulate *rx* expression. However, the size of the *rx* expression domain was smaller than that in controls (57%, $n = 28$) (Fig. 2*E*), indicating that DMO-injected embryos had smaller eyes than their siblings. These observations are again consistent with the notion that partial knockdown of Dzip1 elevates Hh signaling. Collectively, partial knockdown of Dzip1 resulted in phenotypes reminiscent of Hh activation during neural tube, somite, and eye patterning.

Partial Knockdown of Dzip1 Sensitizes Embryos to Smo Agonist Purmorphamine—To further strengthen the conclusion that partial depletion of Dzip1 sensitizes cells to Hh signaling, we determined whether partial Dzip1 knockdown enhances the response of embryos to purmorphamine, a Smo agonist. Thus, uninjected and DMO-injected embryos were treated with various doses of purmorphamine. The expression of *ptc1* and *pax6* in the neural tube and that of *pax2* during eye development was analyzed at the tadpole stage.

As expected, in embryos treated with a high dose of purmorphamine (10 μm), we observed an expansion of *ptc1* (86%, $n = 36$), a reduction and dorsal shift in the *pax6* expression domain in the neural tube (57%, $n = 33$), and dorsally expanded *pax2* expression in the eye (78%, $n = 46$) (Fig. 3*A*) in a similar manner to DMO-injected embryos (Fig. 2). By comparison, the expression of *ptc1* ($n = 47$), *pax6* ($n = 20$), or *pax2* ($n = 37$) in vehicle-treated (ethanol) controls (Fig. 3*A*) was similar to nontreated controls (Fig. 2). To test if partial knockdown of Dzip1 sensi-

tizes embryos to purmorphamine, we performed titration experiments to determine doses of both purmorphamine and DMO that were below the threshold capable of altering marker gene expression when delivered on their own. We found that the majority of 3 ng of DMO injections were indistinguishable from controls with respect to *ptc1* (85%, $n = 46$), *pax6* (78%, $n = 30$), and *pax2* (93%, $n = 42$) expression. Similarly, a purmorphamine dose of 2.5–5.0 μm often led to normal phenotypes with respect to the expression of *ptc1* (78%, $n = 64$), *pax6* (82%, $n = 34$), and *pax2* (72%, $n = 55$) (Fig. 3*A*). Notably, low doses of DMO (3 ng) and purmorphamine (2.5–5.0 μm) synergized when delivered in combination, resulting in enhanced expression of *ptc1* (63%, $n = 52$), reduction and dorsal expansion of *pax6* (65%, $n = 30$), and elevated *pax2* (62%, $n = 53$) expression, in a similar fashion to higher doses of purmorphamine or Dzip1 knockdown (Fig. 3*A*). The gene expression changes for all treatments/injection groups are summarized in Fig. 3, *B--D*. Taken together, our results demonstrate that partial depletion of Dzip1 to levels insufficient for perturbing ciliogenesis sensitizes cells to Hh signaling.

Knockdown of Gli1 or Gli2 Rescues Phenotypes Induced by Partial Knockdown of Dzip1—Dzip1 regulates Gli turnover (38). To determine if partial knockdown of Dzip1 sensitizes cells to Hh signaling by stabilizing Gli proteins, we performed double knockdown experiments in which morpholino against Gli1 or Gli2 was injected together with DMO. We then assayed the expression of *ptc1*, *pax6*, and *pax2* in these embryos.

As expected, knockdown of either Gli1 or Gli2 alone suppressed Hh signaling, as evidenced by reduced *ptc1* expression throughout the neural tube and somites (Gli1-MO, 69%, $n = 45$; Gli2-MO, 65%, $n = 40$), ventral expansion of *pax6* in the neural tube (Gli1-MO, 60%, $n = 20$; Gli2-MO, 65%, $n = 20$), and reduction of *pax2* in the ventral eye (Gli1-MO, 43%, $n = 49$; Gli2-MO, 58%, $n = 31$). Next, we compared double knockdown embryos to DMO-injected embryos. Consistent with findings presented in Fig. 2, injection of DMO alone enhanced *ptc1* expression (44%, $n = 41$) (Fig. 4*A*). In contrast, injection of DMO/Gli1-MO or DMO/Gli2-MO reduced *ptc1* expression in 58% ($n = 29$) and 52% ($n = 33$) of embryos (Fig. 4*A*), respectively. In accordance with this, knockdown of Gli1 or Gli2 in combination with DMO was capable of rescuing the dorsal shift of *pax6* expression that is evident in the neural tube after DMO injection alone (52%, $n = 33$) (Fig. 4*A*). Moreover, double knockdown often caused a reversal of the phenotype, leading to ventral expansion of *pax6* within the neural tube after either DMO/Gli1-MO (57%, $n = 30$) or DMO/Gli2-MO (49%, $n = 35$) injection (Fig. 4*A*). As previously reported in Fig. 2, DMO-injected embryos display enhanced *pax2* expression throughout the eye (77%, $n = 56$) (Fig. 4*A*). By comparison, knockdown of Gli1 or Gli2 in DMO-injected embryos prevented DMO-induced dorsal expansion of *pax2*. Moreover, double knockdown often led to an overall reduction of *pax2* expression in the eye (43%, $n = 37$, DMO/Gli1-MO; 41%, $n = 41$, DMO/Gli2-MO). These results are summarized in Fig. 4, *B--D*. Collectively, these data support the notion that Dzip1 influences the sensitivity of cells to Hh signaling by stabilizing Gli proteins.

Dzip1 Regulates Spop Stability in a Cilia-independent Manner—The Spop/HIB-dependent regulation of Gli/Ci is one of the
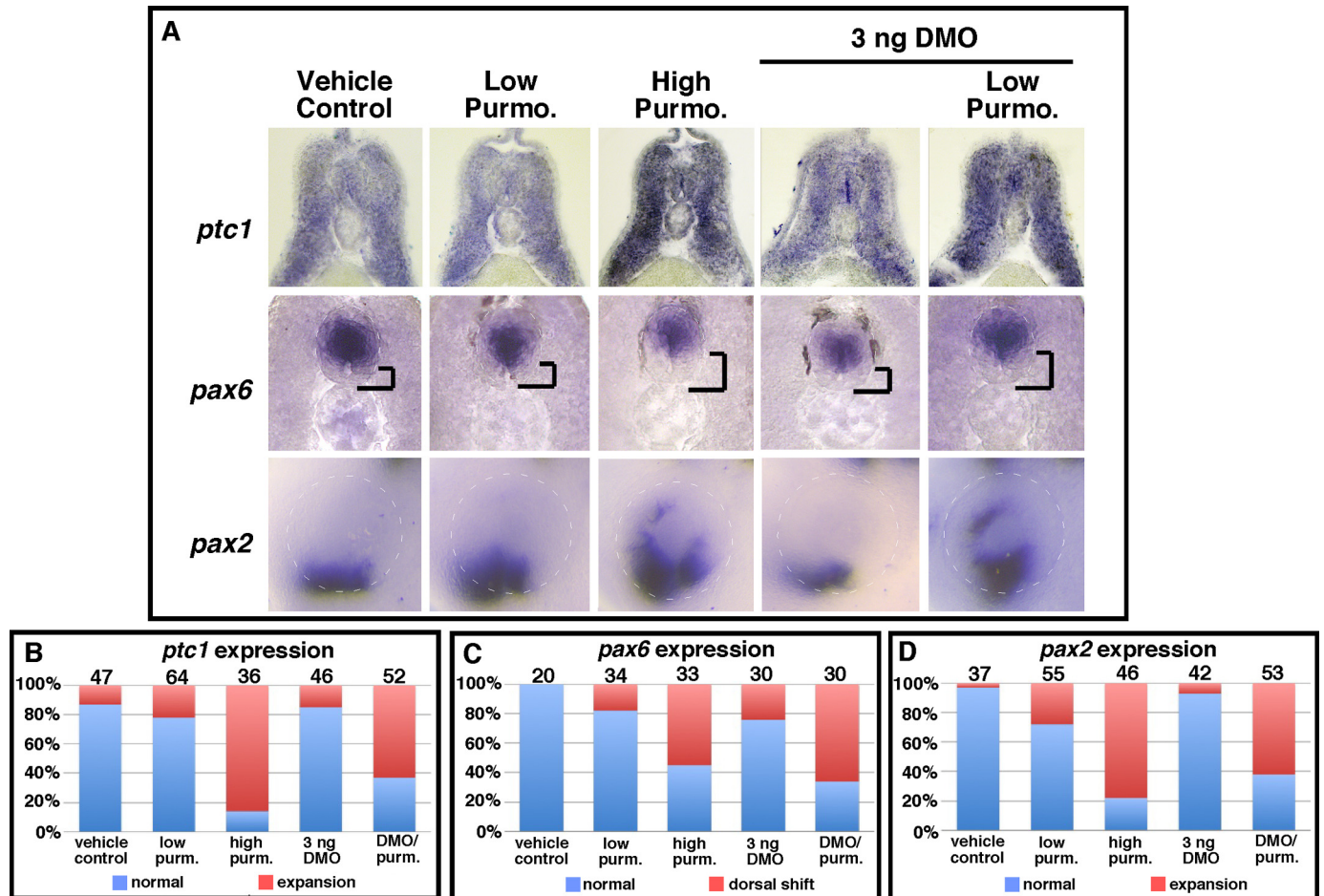
FIGURE 3. **Partial depletion of Dzip1 sensitizes embryos to the Smo agonist purmorphamine.** *A*, whole mount *in situ* hybridization of *ptc1* (*top*), *pax6* (*middle*), and *pax2* (*bottom*) in vehicle-treated (ethanol) controls and embryos treated with either a low dose of purmorphamine (2.5–5.0 μm), a high dose of purmorphamine (10 μm), injected with a low concentration of DMO (3 ng), or receiving a combination of a low dose of purmorphamine (*Purmo.* or *purm.*) and an injection with a low concentration of DMO. In the *middle panels*, the distance between the floor plate and the ventral border of the *pax6* expression domain was highlighted. *B–D*, summary of *ptc1* expression phenotypes (*B*), *pax6* expression phenotypes (*C*), or *pax2* expression phenotypes (*D*) for vehicle-treated (ethanol) controls and the different groups of embryos subjected to purmorphamine, DMO, or both.

best-characterized Gli/Ci regulatory mechanisms. Spop/HIB promotes Gli/Ci ubiquitination and degradation through recruiting Gli/Ci to the Cullin3 E3 ligase (24, 28, 29). In vertebrates, the function of Spop is antagonized by Sufu, which stabilizes Gli by competing with Spop for Gli binding (22, 23).

Our motivation to understand the mechanism by which Dzip1 regulates Gli turnover led us to examine whether Dzip1 regulates Gli turnover by controlling Spop or Sufu. Strikingly, we found that the stability of overexpressed Spop was decreased dramatically in DMO-injected embryos. By contrast, Sufu remained unaltered in Dzip1-depleted embryos (Fig. 5*A*). Interestingly, the level of Spop remained unaffected in IFT88/Polaris-depleted embryos (Fig. 5*A*), which exhibited severe ciliogenesis defects (Fig. 5*B*). Moreover, reduced levels of Spop were detected in embryos injected with 5 ng of DMO (Fig. 5*C*), a dose of DMO insufficient for disturbing ciliogenesis. Importantly, we found that endogenous Spop was destabilized after knockdown of Dzip1 but was unaffected by IFT88 knockdown (Fig. 5*D*). These results demonstrate that Dzip1-mediated regulation of Spop occurs in a cilia-independent fashion.

Spop has two functional domains, an N-terminal MATH domain and a C-terminal BTB (Bric-a-brac/Tramtrack/Broad complex) domain (63). We sought to determine whether individual functional domains mediate Dzip1-dependent regulation of Spop. Several Spop deletion constructs (Fig. 5*E*) were generated and expressed in embryos. We found that the expression of all deletion constructs was unchanged after Dzip1 depletion (Fig. 5*F*), suggesting that both MATH and BTB domains are required for Spop to be regulated by Dzip1. In full, these data indicate a novel function of Dzip1 in controlling Spop stability.

Dzip1 Protects Spop/HIB from Proteasome-dependent Degradation—To better understand how Spop protein stability is regulated, we examined whether Spop is ubiquitinated. Thus, we purified myc-tagged (MT)-Spop from embryo lysate by immunoprecipitation using an anti-myc antibody. Purified Spop was separated on SDS-PAGE and analyzed by immunoblotting using anti-myc and anti-ubiquitin antibodies.

The predicted size of MT-Spop is ~68 kDa. As shown in the anti-myc immunoblot in Fig. 6*A*, the majority of MT-Spop proteins are ~68 kDa. Interestingly, we could detect the presence of slower migrating bands ranging in size from 85 to 170 kDa, well above the unmodified MT-Spop protein at 68 kDa, indicating that Spop protein becomes modified in embryos. Con-
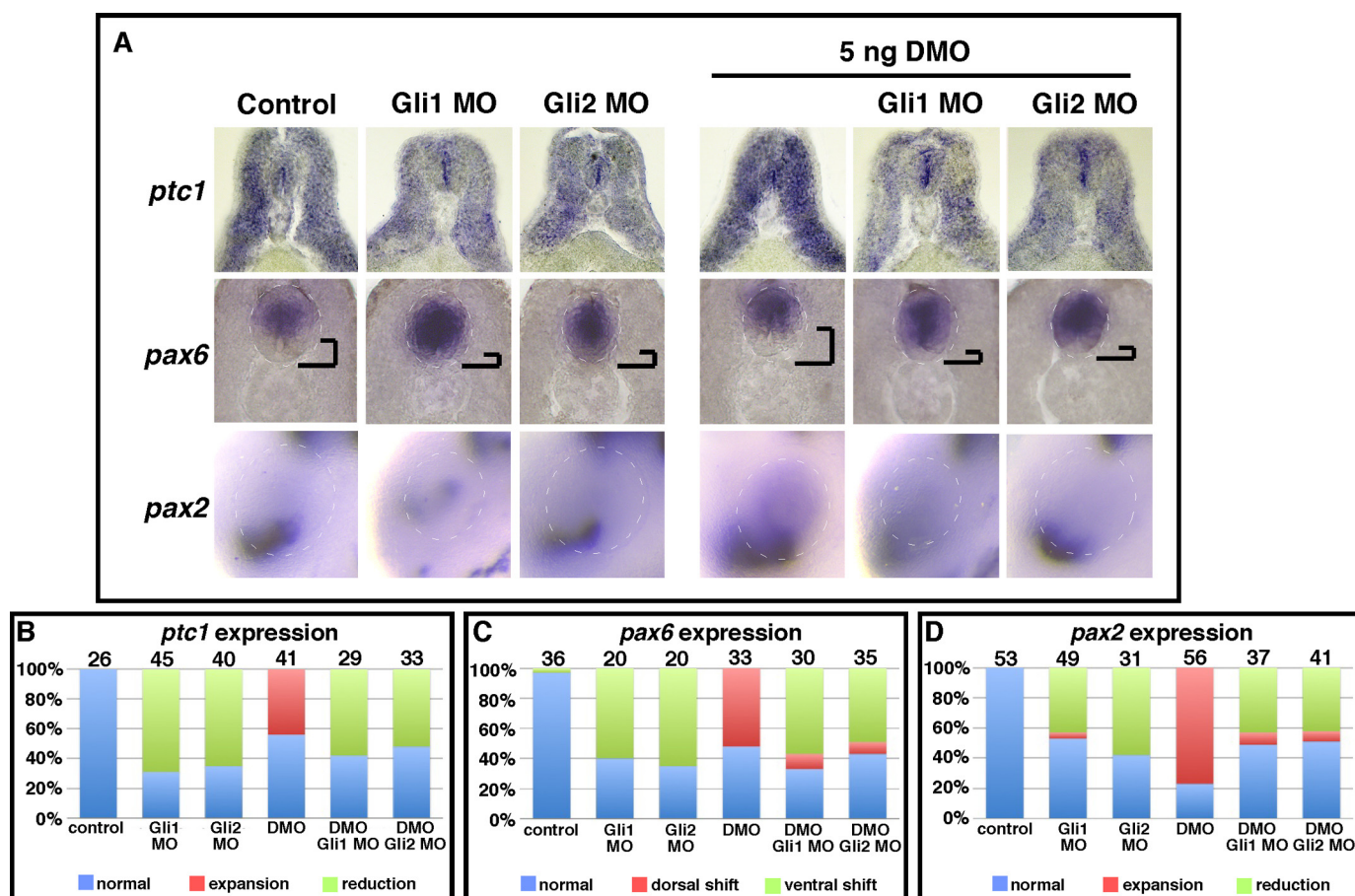
FIGURE 4. **Knockdown of Gli1 or Gli2 rescues phenotypes induced by partial depletion of Dzip1.** *A*, whole mount *in situ* hybridization of *ptc1* (*top*), *pax6* (*middle*), and *pax2* (*bottom*) in controls and embryos receiving either a single injection of Gli1 MO, Gli2 MO, or DMO and double knockdown embryos injected with Gli1 MO/DMO or Gli2 MO/DMO. In the *middle panels*, the distance between the floor plate and the ventral border of the *pax6* expression domain was highlighted. *B–D*, summary of *ptc1* expression phenotypes (*B*), *pax6* expression phenotypes (*C*), or *pax2* expression phenotypes (*D*) in controls and the different groups of embryos receiving either a single or double morpholino injection against Gli1, Gli2, or Dzip1.

sistent with this, myc immunoprecipitates probed with an anti-ubiquitin antibody revealed a smear of high molecular weight forms, ranging in size from 85 to 170 kDa (Fig. 6*A*). Importantly, these bands were only present in immunoprecipitates from embryos expressing MT-Spop, indicating that they represent ubiquitinated Spop. This suggests that Spop undergoes ubiquitin/proteasome-dependent degradation.

To determine if Dzip1 prevents proteasome-dependent degradation of Spop, we examined the effect of the proteasome inhibitor lactacystin on Spop protein levels in an animal cap assay. By dissecting animal caps from embryos, the vitelline membrane was removed, allowing MT-Spop-expressing cells to become exposed to lactacystin in the culture media. As shown in Fig. 6*B*, DMO-induced degradation of Spop was blocked by treatment of lactacystin. These data indicate that Dzip1 prevents Spop from proteasome-dependent degradation.

We next addressed whether regulation of Spop by Dzip1 is evolutionarily conserved. To achieve this, we knocked down *dDzip1* in *Drosophila* S2 cells by RNAi (Fig. 6*C*) and examined the stability of HIB, the fly homolog of Spop. Indeed, knockdown of *dDzip* in S2 cells decreased the levels of HIB protein. The effect of *dDzip1* RNAi on HIB was blocked by MG-132, a proteasome inhibitor (Fig. 6*D*), demonstrating that dDzip1 prevents proteasome-dependent turnover of HIB protein. As

predicted, the level of Ci protein was increased in *dDzip1* RNAi-treated cells (Fig. 6*E*). It appears that Dzip1-dependent regulation of Spop is evolutionarily conserved and essential for controlling the stability of Ci/Gli.

Overexpression of Spop Rescues Phenotypes Induced by Partial Knockdown of Dzip1—Knowing Dzip1 prevents proteasome-dependent turnover of Spop, we set out to determine whether phenotypes induced by partial Dzip1 knockdown could be rescued by overexpression of Spop.

First, we tested the potential of Spop to rescue Gli turnover in Dzip1 knockdown embryos. As shown in Fig. 7*A*, forced Spop expression in Dzip1 morphants abolished the DMO-induced stabilization of xGli1, xGli2, and hGli3. Previous studies reveal that the stability of mammalian Gli1 is not sensitive to Spop (23, 24). Because Spop prevented DMO-induced stabilization of *Xenopus* Gli1 in our experiments, we tested the effects of Spop overexpression on xGli1, xGli2, and hGli3 in NIH3T3 cells. We found that xGli1, xGli2, and hGli3 were all destabilized upon Spop expression (Fig. 7*B*). This suggests that loss of Spop activity in DMO-injected embryos accounts for stabilization of all three Glis.

Next we sought to determine whether restoration of Spop expression in DMO-injected embryos was sufficient to rescue phenotypes induced by partial Dzip1 depletion. To achieve this,
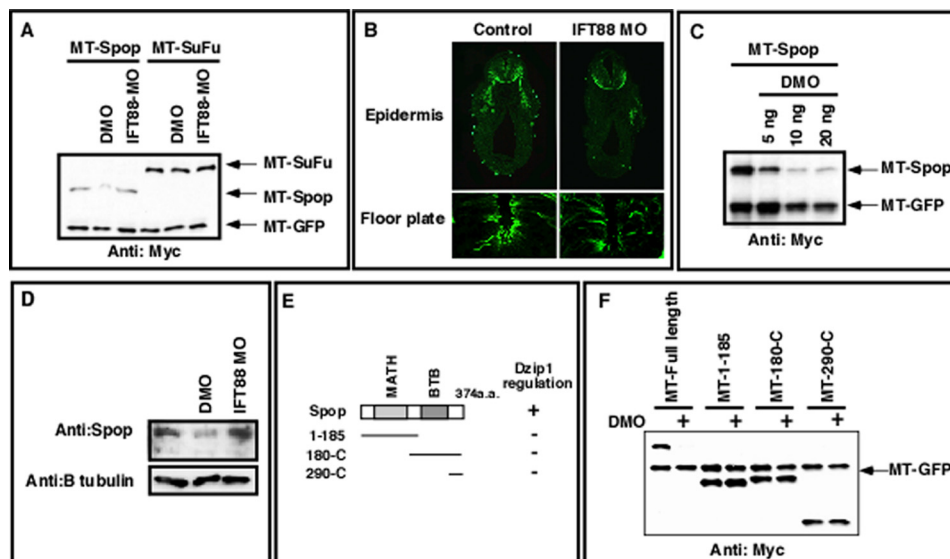
FIGURE 5. **Dzip1 regulates the stability of Spop independent of its role in ciliogenesis.** *A*, Western blot showing that the stability of Spop, but not that of Sufu, was decreased in Dzip1-depleted embryos. RNAs encoding MT-Spop (250 pg) or MT-Sufu (250 pg) were co-injected with MT-GFP (50 pg) into controls or embryos that had received a prior injection of DMO or IFT88 morpholino (*MO*). Embryos were harvested at the neurula stage. MT-GFP serves as injection and loading control. *B*, immunofluorescence showing a significant reduction of motile cilia on the epidermis (*top panel*) and primary cilia in the floor plate (*bottom panel*) within embryos injected with 40 ng IFT88 morpholino (*right column*) compared with controls (*left column*). *C*, DMO injection negatively impacted Spop stability in a dose-dependent fashion. MT-Spop was injected with MT-GFP into controls or embryos that had received increasing concentrations of DMO (5–20 ng). Expression of Spop was assessed by Western blotting. *D*, endogenous Spop protein was reduced in Dzip1-depleted embryos and unchanged in IFT88-depleted embryos. Embryos were harvested at stage 18 and subjected to Western blot with the anti-Spop antibody to detect endogenous Spop levels. Detection of β-tubulin levels serves as a loading control. *E*, schematic presentation of Spop deletion constructs. All deletion constructs were N-terminally myc-tagged. *F*, Western blot results showing the effects of Dzip1 knockdown on the stability of full-length and deletions of Spop. All constructs were co-injected with MT-GFP. Embryo lysates were subjected to Western blot with the anti-myc antibody.
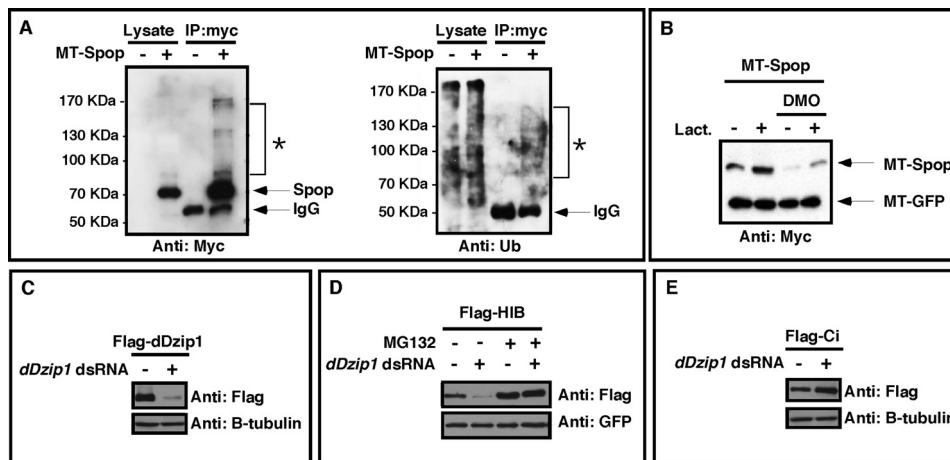

FIGURE 6. **Dzip1 prevents ubiquitin/proteasome-dependent degradation of Spop/HIB.** *A*, Spop is ubiquitinated *in vivo*. MT-Spop was injected into *Xenopus* embryos. Spop protein was purified by immunoprecipitation using an anti-myc antibody. Lysates and immunoprecipitates (*IP*) from injected and uninjected embryos were subjected to Western blot with the anti-myc antibody (*left panel*) and anti-ubiquitin antibody (*right panel*). The unmodified MT-Spop band migrated to 68 kDa. A smear of high molecular weight proteins (*) migrating slower than unmodified MT-Spop could be detected in both myc and ubiquitin Westerns. *B*, MT-Spop was injected into control or embryos that had received a prior injection of DMO. Animal caps were dissected at stage 9. Subsequently, caps were cultured in media with or without 2 μg of lactacystin (*Lact.*). Animal caps were harvested at stage 15 and subjected to Western blot to detect the levels of Spop. *C*, Western blot results showing severe reduction of FLAG-dDzip1 by *dDzip1* dsRNA in S2 cells. β-Tubulin serves as a loading control. *D*, Dzip1 knockdown promoted proteasome-dependent degradation of HIB in S2 cells. S2 cells were cotransfected with FLAG-HIB and GFP constructs and treated with *dDzip1* dsRNA and/or MG132. Cell extracts were subjected to Western blot with the anti-FLAG antibody to detect the levels of HIB. GFP serves as the transfection and loading control. *E*, Dzip1 knockdown promotes Ci stability in S2 cells. S2 cells were transfected with FLAG-Ci and treated with *dDzip1* dsRNA. Western blots were carried out with the cell lysates to determine the levels of Ci. Tubulin serves as a loading control.

embryos were injected with DMO at the one-cell stage. Subsequently, RNAs encoding Spop and the lineage tracer were injected into one side of DMO-injected embryos. Embryos were harvested at the tadpole stage and subjected to *in situ* hybridization for *ptc1*, *pax6*, and *pax2*. The marker gene expression was compared between the *Spop*-injected and -uninjected sides.

As seen in Fig. 7*C*, *ptc1* expression on the *Spop*-injected side was markedly reduced compared with the side that did not receive an injection of *Spop* mRNA. Regarding *pax6*, overexpression of Spop was sufficient to reverse dorsal expansion of *pax6* in the neural tube. *Pax6* expression on the *Spop*-injected side often resembled control embryos and occasionally dis-
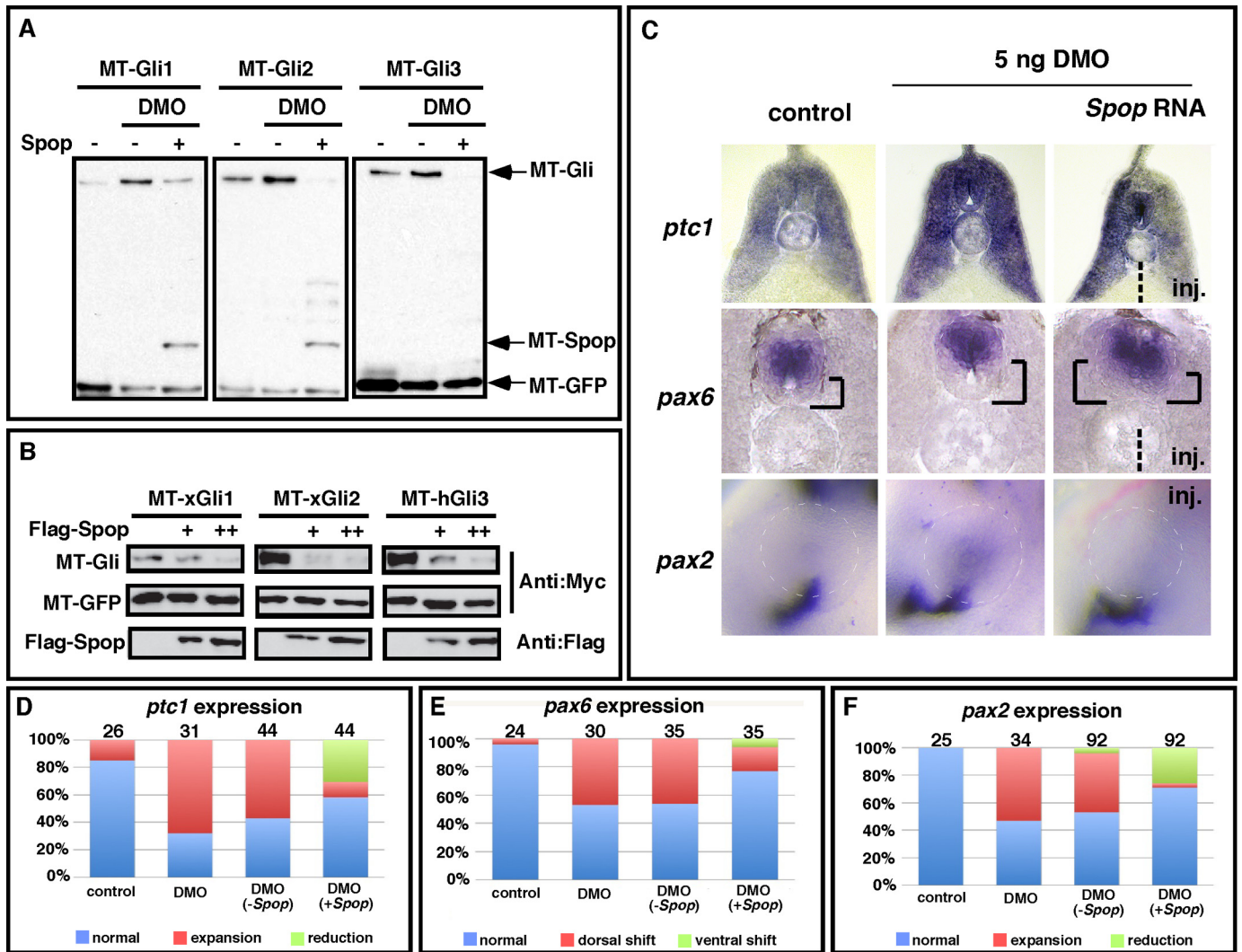
FIGURE 7. **Overexpression of Spop rescues phenotypes caused by partial knockdown of Dzip1.** *A*, Western blot results showing that increases to Gli stability after Dzip1 depletion were reversed by Spop overexpression. RNAs encoding MT-Gli1–3 (2 ng) and MT-GFP (50 pg) were injected alone or in combination with MT-Spop (100 pg) into controls or embryos that had received a prior injection of DMO. Embryo were harvested at the neurula stage and subjected to Western blot with the anti-myc antibody to detect the levels of Spop. *B*, forced Spop expression decreases the stability of xGli1, xGli2, and hGli3 in NIH3T3 cells. NIH3T3 cells were transfected with MT-Glis and MT-GFP alone or in combination with increasing levels of FLAG-Spop. Western blots were carried out with the cell lysates to determine the levels of Gli1, -2, and -3. GFP serves as a transfection and loading control. *C*, *in situ* hybridization results showing the expression of *ptc1*, *pax6*, and *pax2* in controls or embryos injected with DMO alone or DMO and *Spop* RNA. *inj.* indicates the side that received *Spop* RNA injection. *D–F*, summary of the *ptc1* (*D*), *pax6* (*E*), and *pax2* (*F*) expression phenotypes in controls and injected embryos.

played a ventral shift, similar to Gli1- and Gli2-depleted embryos. Consistent with these data, the expansion of *pax2* in the eye field of DMO-injected embryos was reversed by Spop overexpression (Fig. 7*C*). These results are summarized in Fig. 7, *D–F*. Collectively, these data indicate that Dzip1 exerts its inhibitory effect on Hh signaling through stabilizing Spop, which targets Gli for proteasome-dependent degradation.

In zebrafish, the Hh signaling inhibitory effect of the *you-too* (*yot*) allele, which encodes a dominant negative mutant of Gli2 due to a nonsense mutation at Arg-929 (64), is markedly enhanced in *iguana* mutants (31, 32). The molecular mechanisms underlying this perplexing finding remain unclear. In light of our findings above, we hypothesize that loss of Dzip1 enhances the dominant negative function of the *yot* allele through stabilizing the mutated Gli2 protein. We thus generated $Gli2_{1-799}$, which is analogous to *Yot119* (64). We found that the stability of $Gli2_{1-799}$ was markedly enhanced after

depletion of Dzip1 in *Xenopus* embryos (Fig. 8*A*). Consistently, overexpression of Spop destabilized $Gli2_{1-799}$ in NIH3T3 cells (Fig. 8*B*). These findings thus provide a mechanistic explanation for the synergistic relationship between *iguana* and *yot* mutant alleles that negatively impacts Hh signaling. They also provide further evidence that Dzip1 promotes the turnover of Gli transcription factors through a Spop-dependent mechanism.

DISCUSSION

Phenotypic characterization of zebrafish *iguana* mutants reveals that Dzip1 is required for cells to respond to Shh. In *iguana* mutants, there is a global reduction of Hh target gene expression (31, 32). More recent studies indicate that the reduced Hh signaling in *iguana* mutants is likely a consequence of impaired ciliogenesis (34–36). Strikingly, in *iguana* mutants, the expression of some Hh target genes is increased in cells located at a distance from the source of Shh, for example, cells
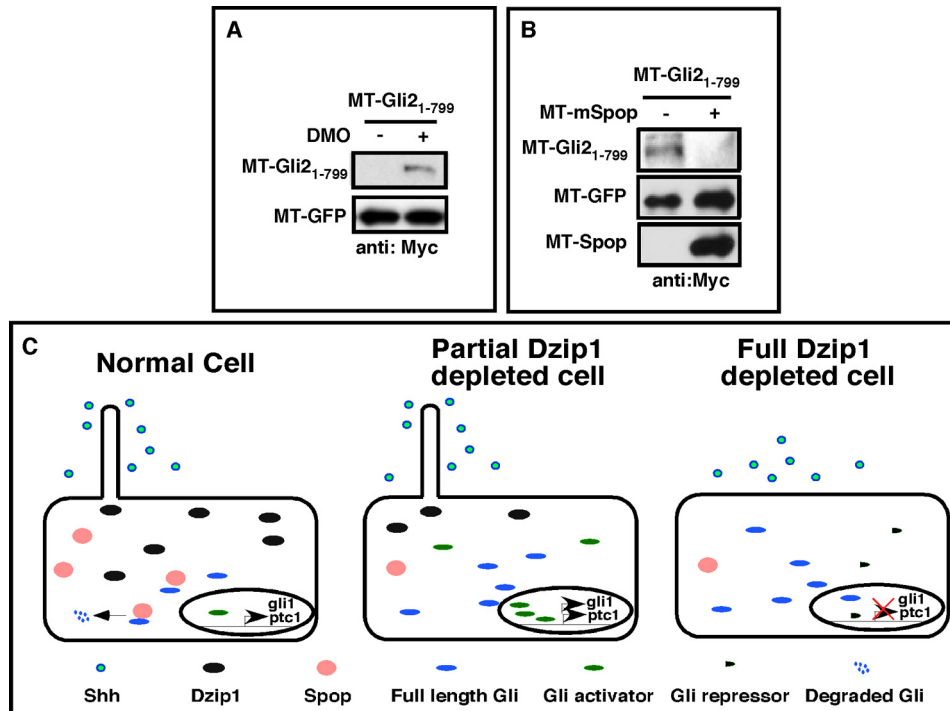
FIGURE 8. **Regulation of Gli2$_{1-799}$, an analog Gli2 mutant of zebrafish Yot, by Dzip1 and Spop.** *A*, Western blot results showing that knockdown of Dzip1 stabilized Gli2$_{1-799}$ in *Xenopus* embryos. RNAs encoding Gli2$_{1-799}$ and MT-GFP were injected into controls and embryos that had received a prior injection of DMO. Embryos were harvested at the neurula stage and subjected to Western blotting. MT-GFP served as an injection and loading control. *B*, forced Spop expression decreased the protein stability of Gli2$_{1-799}$. MT-Gli2$_{1-799}$ and MT-GFP were transfected alone or in combination with MT-Spop into NIH3T3 cells. Cell lysates were subjected to Western blot with the anti-myc antibody to detect the levels of Spop. *C*, model of Dzip1 functions during Hh signaling. Dzip1 supports ciliogenesis and maintains the proper expression level of Gli proteins through controlling the stability of Spop (*left panel*). Partial knockdown of Dzip1 has no effect on the formation of cilia but induces degradation of Spop, leading to increased levels of Gli proteins. When exposed to Shh, these cells produce more Gli activators, resulting in a more robust Hh target gene expression (*middle panel*). When Dzip1 was depleted more completely, ciliogenesis was disrupted. Although these cells have increased levels of Gli, they cannot receive Shh. This prevents the formation of Gli activators. Consequently, gene expression induced by Shh were inhibited (*right panel*). Under normal conditions, cells located at a distance from the source of Shh are not exposed to Shh. Whether these cells form cilia or not are not important. However, when Dizp1 is depleted, destabilization of Spop will induce accumulation of full-length Gli proteins (not Gli activators). This will lead to weak ectopic Hh target gene expression in embryos.

in somites (31, 32). This argues for an inhibitory role of Dzip1 in the Hh pathway. We recently reported that, in addition to controlling ciliogenesis, Dzip1 is required for Gli turnover in *Xenopus* embryos (38), providing important clues to the mechanism through which Dzip1 inhibits Hh signaling. Because we were able to disrupt Dzip1-dependent Gli turnover without perturbing ciliogenesis by injection of low concentrations of DMO, we knocked down Dzip1 partially and specifically investigated the inhibitory function of Dzip1 in the Hh pathway.

Results presented here indicate that partial knockdown of Dzip1 sensitizes *Xenopus* embryos to Hh signaling (Figs. 1 and 3). We found that partial knockdown of Dizp1-induced phenotypes indicative of elevated Hh signaling, including ventralized neural tubes and eyes, expanded slow muscle fibers, and ectopic *ptc1* expression in multiple groups of tissues (Fig. 2). These phenotypes were rescued by depletion of Gli1 or Gli2 (Fig. 4). Thus, the phenotypes associated with partial Dzip1 depletion are likely attributed to elevated Hh signaling as a direct consequence of increased Gli stability.

Intriguingly, some phenotypes observed in Dzip1-depleted *Xenopus* embryos were in stark contrast to that of zebrafish *iguana* mutants. Partial knockdown of Dzip1 in *Xenopus* embryos results in ventralization of the neural tube, whereas the *iguana* mutant displays neural tube patterning defects indicative of an Hh signaling reduction, including the absence

of lateral floor plate specification and reduced *ptc1* expression within the floor plate and ventrally located inter- and motoneurons (31). We argue that the phenotypic differences between the Dzip1 morphants in this study and those reported in *iguana* mutants lies in the differential outcome to cilia formation. In our studies we chose to knock down Dzip1 partially, allowing normal ciliogenesis in embryos. Due to stabilization of Gli proteins, neural tube cells become sensitized and generated a more robust response to Shh from the notochord and floor plate. In *iguana*, however, although the levels of Gli proteins are likely increased, cells in embryos are deficient in primary cilium (34–36), the organelle essential for cells to receive Shh. Consequently, Hh signaling is impaired, and the neural tube of *iguana* becomes dorsalized (31).

Our results further reveal that Dzip1 regulates Gli turnover by preventing proteasome-dependent degradation of Spop/HIB, adaptors of Cullin3 E3 ligase that promotes Gli/Ci turnover. We were able to show that forced expression of Spop could prevent DMO-induced Gli stabilization and rescue phenotypes caused by partial knockdown of Dzip1 in *Xenopus* embryos. This demonstrates that Dzip1 exerts its inhibitory effect on the Hh pathway by preventing degradation of Spop/HIB.

In agreement with the notion that Dzip1 regulates Gli turnover independent of its role in ciliogenesis, we found that Dzip1

stabilizes Spop independent of its function in ciliogenesis. We showed that knockdown of IFT88, which disrupted ciliogenesis in embryos, had no effect on the stability of Spop. Furthermore, reduction of Spop was detected in embryos injected with 5 ng of DMO, a dose of DMO insufficient for causing ciliogenesis defects. It is worth noting that Dzip1 overexpression did not lead to any discernable effects on Spop protein stability. In addition, we could not detect physical interaction between Dzip1 and Spop proteins (data not shown). Thus, further analysis will be needed to fully elucidate the mechanism by which Dzip1 prevents proteasome-dependent degradation of Spop/HIB.

Based on our studies and works from others (31, 32, 34–37), we now propose that vertebrate Dzip1 has at least two independent functions during vertebrate Hh signaling (Fig. 8*C*). First, Dzip1 plays critical roles during ciliogenesis, which in turn positively influences the Hh pathway. Additionally, Dzip1 is required for stabilizing Spop. Because Spop promotes degradation of Gli proteins, the latter function of Dzip1 acts negatively to regulate Hh signaling. Collectively, these dual actions likely account for the contradictory combination of activation and depletion of Hh signaling in zebrafish and *Xenopus* embryos. In *Drosophila*, in which cilia are dispensable for Hh signaling, Dzip1 action is likely restricted to its regulation of HIB stability. In the absence of Dzip1, HIB becomes destabilized, leading to an increased level of Ci. Our work presented here demonstrates that regulation of the stability of Spop/HIB by Dzip1 is an evolutionarily conserved mechanism important for the output of Hh signaling.

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