

Ulk4 promotes Shh signaling by regulating Stk36 ciliary localization and Gli2 phosphorylation

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Abstract

Summary

The Hedgehog (Hh) family of secreted proteins governs embryonic development and adult tissue homeostasis through the Gli family of transcription factors. Gli is thought to be activated at the tip of primary cilium, but the underlying mechanism has remained poorly understood. Here, we show that Unc-51-like kinase 4 (Ulk4), a pseudokinase and a member of the Ulk kinase family, acts in conjunction with another Ulk family member Stk36 to promote Gli2 phosphorylation and Hh pathway activation. Ulk4 interacts with Stk36 through its N-terminal region containing the pseudokinase domain and Gli2 via its regulatory domain to bridge the kinase and substrate. Although dispensable for Hh-induced Stk36 kinase activation, Ulk4 is essential for Stk36 ciliary tip localization, Gli2 phosphorylation and activation. In response to Hh, both Ulk4 and Stk36 colocalize with Gli2 at ciliary tip, and Ulk4 and Stk36 depend on each other for their ciliary tip accumulation. We further show that ciliary localization of Ulk4 depends on Stk36 kinase activity and phosphorylation of Ulk4 on Thr1023, and that ciliary tip accumulation of Ulk4 is essential for its function in the Hh pathway. Taken together, our results suggest that Ulk4 regulates Hh signaling by promoting Stk36-mediated Gli2 phosphorylation and activation at ciliary tip.

eLife assessment

This **fundamental** study substantially advances our understanding of how the pseudokinase ULK4 interacts with an active member of the same kinase subfamily (STK36) to promote GLI phosphorylation and Hedgehog pathway activation. The evidence supporting the proposed mechanism is **compelling**, with rigorous biochemical assays and state-of-the-art cell based imaging techniques. The work will be of broad interest to cell biologists and biochemists.

Pseudokinases, which comprise ~10% of the mammalian kinome, play essential biological functions yet the mechanism of action for many pseudokinases has remained unknown (Jacobsen and Murphy 2017). One such pseudokinase is Unc-51-like kinase 4 (Ulk4), which is a member of Ulk kinase family found in most eukaryotes (Luo et al. 2022). The Ulk family kinases, which include Ulk1 (Atg1 in yeast) and Ulk2 involved in autophagy initiation, contain a conserved kinase domain in their N-terminal regions and a variable regulatory domain in their C-terminal regions (Luo et al. 2022). However, unlike other Ulk family members, Ulk4 contains a pseudokinase domain at its N-terminus and is predicted to be catalytically inactive (Preuss et al. 2020). Ulk4 plays a key role in neurogenesis in mice by regulating neural stem cell pool and neurite branching morphogenesis (Lang et al. 2014; Liu et al. 2016a). In addition, Ulk4 regulates the integrity of white matter by promoting myelination (Liu et al. 2018b). Recent studies have implicated Ulk4 as a risk factor for neuropsychiatric disorders including schizophrenia (Lang et al. 2014; Lang et al. 2016; Liu et al. 2018a; Luo et al. 2022). However, the signaling pathways in which Ulk4 is involved and the substrates it regulates have remained unknown. A recent proteome study has revealed that Ulk4 interacts with Stk36 in addition to many other proteins (Preuss et al. 2020). Stk36 is also a member of the Ulk family and mammalian homolog of *Drosophila* Fused (Fu), a Ser/Thr kinase implicated in Hedgehog (Hh) signaling (Murone et al. 2000; Wilson et al. 2009; Han et al. 2019). A recent study identified Ulk4 as a genetic modifier of the holoprosencephaly phenotype caused by impaired Shh pathway activity (Mecklenburg et al. 2021). In addition, this study showed that Ulk4 was localized to the primary cilia and that Ulk4 knockdown affected Shh induced *Gli-luc* reporter gene expression and Smoothed (Smo) ciliary localization (Mecklenburg et al. 2021). These observations have raised an interesting possibility that Ulk4 may participate in Hh signal transduction.

The Hh family of secreted proteins governs embryonic development and adult tissue homeostasis and deregulation of Hh signaling activity has been implicated in a myriad of human disorders including birth defect and cancer (Villavicencio et al. 2000; Jiang and Hui 2008; Briscoe and Therond 2013; Jiang 2021). Hh exerts its biological influence via an evolutionarily conserved signaling cascade that culminates in the activation of Gli family of Zn-finger transcription factors. The core Hh signal reception system consists of two multi-span transmembrane proteins Patched1 (Ptch1) and Smoothed (Smo) that transduce vertebrate Hh signal at primary cilia (Goetz and Anderson 2010). In quiescent cells, Ptch1 localizes to primary cilia where Smo is barely detectable (Corbit et al. 2005; Rohatgi et al. 2007); the full-length Gli (Gli^F) undergoes ubiquitin/proteasome-mediated proteolytic processing to generate a truncated transcriptional repressor form (Gli^R) (Wang et al. 2000; Jiang 2002; Tempe et al. 2006; Wang and Li 2006; Chen and Jiang 2013). Hh inhibits Ptch1 activity and promotes its ciliary exit, allowing Smo and Gli proteins to accumulate in the cilia to transduce the Hh signal (Corbit et al. 2005; Haycraft et al. 2005; Rohatgi et al. 2007; Chen et al. 2009; Tukachinsky et al. 2010; Chen et al. 2011). Activated Smo blocks the proteolytic processing of Gli into Gli^R and converts Gli^F to an active form (Gli^A) that enters the nucleus to turn on the expression of Hh target genes (Wang et al. 2000; Kim et al. 2009; Tukachinsky et al. 2010; Han et al. 2019; Arveseth et al. 2021).

Although it is generally thought that Smo converts Gli^F into Gli^A at ciliary tip (Goetz and Anderson 2010), the underlying mechanism has remained an enigma. Our recent studies have demonstrated that two Ulk family kinases Ulk3 and Stk36 play a role in converting Gli^F into Gli^A by directly phosphorylating Gli2 at multiple sites in mammalian cells (Han et al. 2019; Zhou et al. 2022; Zhou and Jiang 2022). Here, we provide evidence that Ulk4 acts in conjunction with Stk36 to promote Gli2 phosphorylation and Hh pathway activation. We show that Ulk4 interacts with Stk36 through its N-terminal region containing the pseudokinase domain and with Gli2 via its C-terminal regulatory domain to bring Stk36 and Gli2 into proximity. We find that Ulk4 is not required for Stk36 kinase activation by Shh but is essential for Stk36 ciliary localization and Gli2 phosphorylation. We show that, in response to Shh stimulation, both Ulk4 and Stk36 colocalize

with Gli2 at the tips of primary cilia, and that Ulk4 and Stk36 depend on each other for their ciliary tip accumulation. We further show that ciliary tip localization of Ulk4 depends on Stk36-mediated phosphorylation of Ulk4 on Thr1023, and that ciliary tip localization of Ulk4 is essential for its function in Hh signal transduction.

Results

Ulk4 acts in conjunction with Stk36 to promote Gli2 phosphorylation and Hh pathway activation

To determine whether Ulk4 is required for Hh pathway activation, we depleted Ulk4 by RNA interference (RNAi) in NIH3T3 cells, a well-established system for studying Hh signaling. We found that Ulk4 depletion significantly decreased Shh-induced expression of *Gli1* and *Ptch1* determined by RT-qPCR, which was rescued by the expression of human Ulk4 (hUlk4; **Figure 1A-B**; **Figure 1-figure supplement 1**). The requirement of Ulk4 in Shh-induced expression of *Gli1* and *Ptch1* was confirmed in MEF cells (**Figure 1-figure supplement 2**).

Our previous study revealed that Stk36 and Ulk3, which are Ulk family kinases, act in parallel to promote Gli2 phosphorylation and Hh pathway activation (Han et al. 2019). To determine the relationship between Ulk4 and Ulk3/Stk36, we carried out double RNAi experiments. We found that double knockdown (KD) of Ulk4 and Ulk3 further decreased Shh target gene expression compared with single KD of either Ulk4 or Ulk3 whereas double KD of Ulk4 and Stk36 did not have an additive effect (**Figure 1C-D**; **Figure 1-figure supplement 2**). These results indicated that Ulk4 acts in the same pathway with Stk36 but in parallel with Ulk3 to promote Hh signaling.

Our previous study demonstrated that Ulk3 and Stk36 phosphorylate Gli2 at multiple sites including S230/232 to promote Gli2 activation (Han et al. 2019; Zhou et al. 2022). We found that Ulk4 KD reduced Gli2 phosphorylation on S230/232 stimulated by Shh in NIH3T3 cells, which was determined by western blot analysis using a phospho-specific antibody that recognizes pS230/232 (Han et al. 2019; Han and Jiang 2021), and that expression of hUlk4 restored Gli2 phosphorylation on S230/232 in Ulk4 depleted cells (**Figure 1E-E'**). Consistent with Ulk4 acting in a linear pathway with Stk36 but in parallel with Ulk3, double KD of Ulk4 and Stk36 decreased S230/232 phosphorylation similarly to single KD of either Ulk4 or Stk36 whereas double KD of Ulk3 and Ulk4 further decreased Shh-induced S230/232 phosphorylation compared with single KD of Ulk3 or Ulk4 (**Figure 1F-G**). Taken together, these results demonstrated that Ulk4 and Stk36 act in a linear pathway but in parallel with Ulk3 to participate in Hh transduction by facilitating Gli2 phosphorylation and activation (**Figure 1H**).

Ulk4 forms a complex with Stk36 and Gli2

A previous study using proximity-dependent biotin identification (BioID) identified Stk36 as a proximity interactor (Preuss et al. 2020). To verify that Stk36 formed a complex with Ulk4, we carried out coimmunoprecipitation (Co-IP) experiments and found that a C-terminal HA-tagged Ulk4 (hUlk4-HA) interacted with a N-terminal CFP-tagged Stk36 (CFP-Stk36) in HEK293T cells (**Figure 2A**). To explore whether Ulk4 interacted with Gli2 and Sufu, a Gli binding partner and Hh pathway inhibitor (Dunaeva et al. 2003; Cooper et al. 2005; Han et al. 2015), we co-expressed hUlk4-HA, with a Myc-tagged Gli2 (Myc-Gli2) and a Flag-tagged Sufu (Fg-Sufu) in HEK293T cells. Co-IP experiments showed that both Myc-Gli2 and Fg-Sufu were pulled down by Ulk4-HA (**Figure 2B**), suggesting that Ulk4 could form a complex with Stk36 and Gli2-Sufu.

Ulk4 contains an N-terminal pseudokinase domain and the C-terminal regulatory domain with armadillo (Arm) repeats (**Figure 2C**) (Preuss et al. 2020). We generated two truncated forms of Ulk4: hUlk4N (aa 1-450) that contains the pseudokinase domain and hUlk4C (451-1275) that

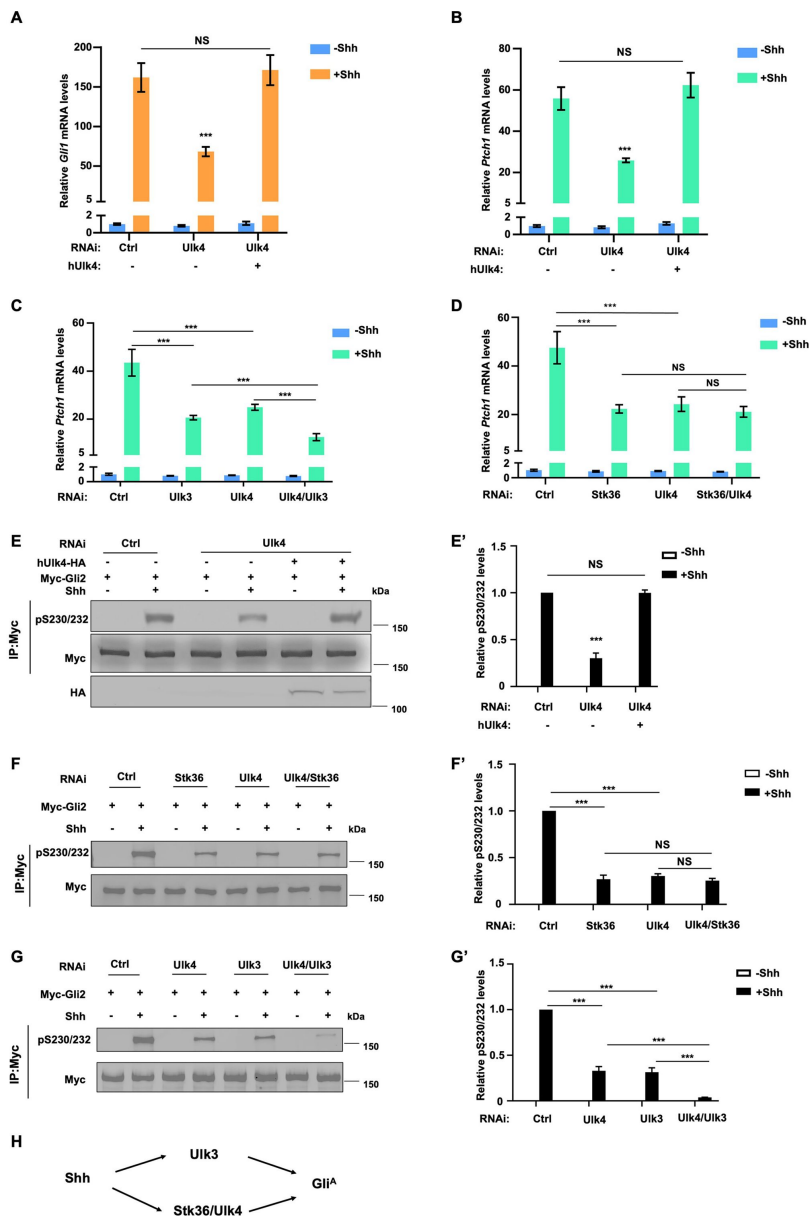


Figure 1.

ULK4 acts in conjunction with Stk36 to promote Shh-mediated Gli activation

(A-B) Relative *Gli1* (A) and *Ptch1* (B) mRNA levels measured by RT-qPCR in NIH3T3 cells expressing the indicated shRNA and a human ULK4 (hULK4) lentiviral construct and treated with or without Shh-N. Of note, hULK4 is insensitive to RNAi, which targets mouse ULK4.

(C-D) Relative *Ptch1* mRNA levels measured by RT-qPCR in NIH3T3 cells treated with the indicated RNAi and with or without Shh-N.

(E-E') Western blot analysis (E) and quantification (E') of Myc-Gli2 phosphorylation in NIH3T3 cells expressing the indicated shRNA and hULK4 lentiviral construct and treated with or without Shh-N.

(F-G') Western blot analysis (F and G) and quantification (F' and G') of Myc-Gli2 phosphorylation in NIH3T3 cells with the indicated RNAi in the presence or absence of Shh-N treatment.

(H) Schematic diagram showing the functional relationship among ULK4, Stk36 and ULK3 in Shh-induced Gli activation.

The cells (A-G) were firstly starved in serum-free medium for 12 hours then cultured in the same medium with or without Shh-N fragment for another 12 hours before they are subjected into RNA preparation (A-D) or Western blot analysis (E-G). Data in (A-D) are mean $\pm$ SD from three independent experiments. Data in (E', F', G') are mean $\pm$ SD from two independent experiments. *** $p < 0.001$ (Student's t test). NS: not significant.

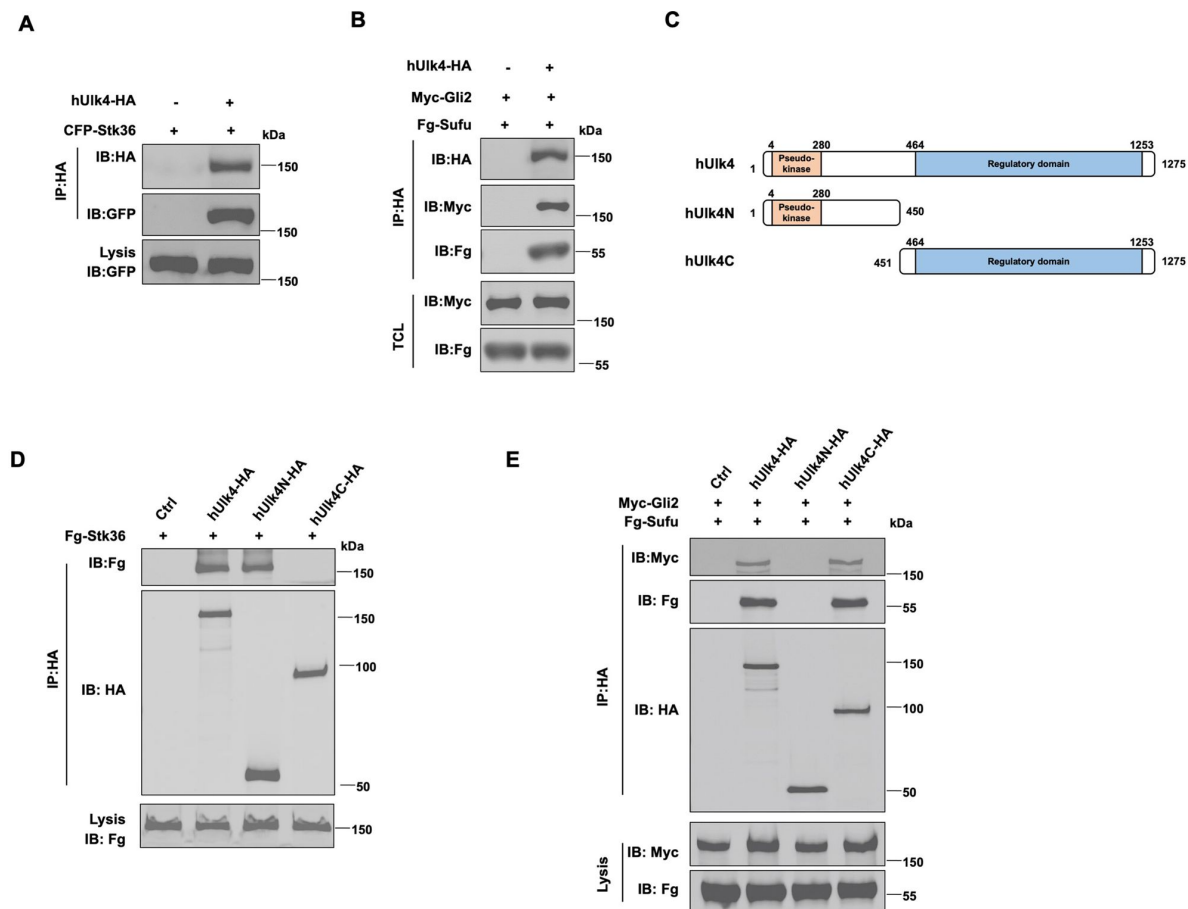


Figure 2.

Ulk4 forms a complex with Stk36 and Gli2

(A) Ulk4 forms a complex with Stk36. HEK293T cells were transfected with hUlk4-HA and CFP-Stk36 constructs, followed by Co-IP and western blot analyses with the indicated antibodies.

(B) Ulk4 forms a complex with Gli2 and Sufu. HEK293T cells were transfected with hUlk4-HA, Myc-Gli2 and Fg-Sufu constructs, followed by Co-IP and western blot analyses with the indicated antibodies.

(C) Ulk4 domain structure and deletion mutants used for Co-IP experiments.

(D-E) Ulk4 interacted with Stk36 through its N-terminal domain (D) and with Myc-Gli2/Fg-Sufu through its C-terminal region (E). HEK293T cells were transfected with the indicated hUlk4, Fg-Stk36, Myc-Gli2 and Fg-Sufu constructs, followed by Co-IP and western blot analyses.

contains the C-terminal regulatory domain. Co-IP experiments showed that hUlk4N-HA interacted with CFP-Stk36 but not with Myc-Gli2/Fg-Sufu whereas hUlk4C-HA formed a complex with Myc-Gli2/Fg-Sufu but not with CFP-Stk36 (**Figure 2D-E**), suggesting that Ulk4 interacted with Stk36 and Gli2-Sufu through its N-terminal pseudokinase domain and C-terminal regulatory domain, respectively. Consistent with our observation, previous BioID experiments using BirA*-Ulk4 pseudokinase domain also identified Stk36 as a proximity binding partner of the Ulk4 pseudokinase domain (Preuss et al. 2020).

Ulk4 is dispensable for Shh-induced Stk36 kinase activation

Pseudokinases could regulate the enzymatic activity of interacting kinases, as has been shown for LKB1 kinase activation by its binding to the pseudokinases STRAD α and STARAD β (Baas et al. 2003). To determine whether Ulk4 regulates Stk36 enzymatic activity, we carried out an *in vitro* kinase assay using GST-Gli2N, which contains the Ulk3/Stk36 phosphorylation site S230, as substrate and either Fg-Stk36 or Fg-Stk36/hUlk4-HA complex immunopurified from HEK293T cells as kinase. GST-Gli2N containing S230A substitution was used as a negative control. As shown in **Figure 3A**, Stk36 and Stk36/Ulk4 phosphorylated GST-Gli2N at similar levels and the phosphorylation was abolished by the S230A mutation. This result suggests binding of Ulk4 to Stk36 does not modulate Stk36 kinase activity.

We next determined whether Ulk4 is required for Stk36 kinase activation by Shh. In *Drosophila*, Hh activates Fu by promoting its kinase domain dimerization and trans-autophosphorylation on multiple S/T residues in its activation loop (AL) including T151, T154, T158 and S159 (Shi et al. 2011; Zhang et al. 2011; Zhou and Kalderon 2011). Interestingly, the AL phosphorylation sites as well as the neighboring residues are highly conserved between Fu and Stk36 (**Figure 3B**) (Shi et al. 2011), raising the possibility that Stk36 could be activated by Shh through a similar mechanism. Indeed, we found that Shh induced phosphorylation of Stk36 on T158/S159 in NIH-3T3 cells as determined by a phospho-specific antibody that recognized pT158/pS159 (**Figure. 3C**) (Shi et al. 2011). To determine whether Shh-induced T158/S159 phosphorylation depends on Stk36 kinase activity, we introduced either wild type Stk36 (CFP-Stk36^{WT}) or its kinase dead form (CFP-Stk36^{KR}) into NIH3T3 cells with endogenous Stk36 and Ulk3 deleted (*Ulk3*^{KO} *Stk36*^{KO}) by CRISPR (Han et al. 2019). As shown in **Figure 3D**, Shh induced phosphorylation of CFP-Stk36^{WT} but not CFP-Stk36^{KR} on T158/S159, suggesting that Shh promotes the phosphorylation of Stk36 AL via its kinase activity, likely through trans-autophosphorylation as are the cases for other kinases. Hence, phosphorylation of T158/S159 can be used as a readout for Stk36 kinase activation.

To determine whether AL phosphorylation promotes Stk36 kinase activation, we converted both S151 and T154 to E (EE) to mimic phosphorylation or to A (AA) to block phosphorylation on these sites (**Figure 3B**). We found that the phospho-mimetic mutation (Stk36^{EE}) increased whereas phospho-deficient mutation (Stk36^{AA}) abolished Stk36 kinase activity as determined by both Stk36 T158/S159 phosphorylation and Gli2 S230/S232 phosphorylation (**Figure 3 E-H**), which is similar to what we have observed previously for Fu (Shi et al. 2011), suggesting that Hh stimulates kinase activation of Stk36 and Fu through a similar mechanism.

To determine whether Ulk4 is required for Shh-induced Stk36 kinase activation, we employed RNAi to deplete Ulk4 from NIH3T3 cells that express CFP-Stk36. As shown in **Figure 3I**, depletion of Ulk4 did not affect Shh-induced phosphorylation of CFP-Stk36 on T158/S159, suggesting that Ulk4 is not required for Stk36 kinase activation in response to Shh stimulation.

Ulk4 mediates the interaction between Stk36 and Gli2

Another possible mechanism for Ulk4 to influence Gli2 phosphorylation by Stk36 is to function as a scaffold that brings Stk36 and Gli2 into the same protein complex. Indeed, we found that Ulk4 depletion diminished the association between exogenously expressed Stk36 and Gli2 in HEK293T cells as determined by Co-IP experiments (**Figure 4A**). By domain mapping, we found that the

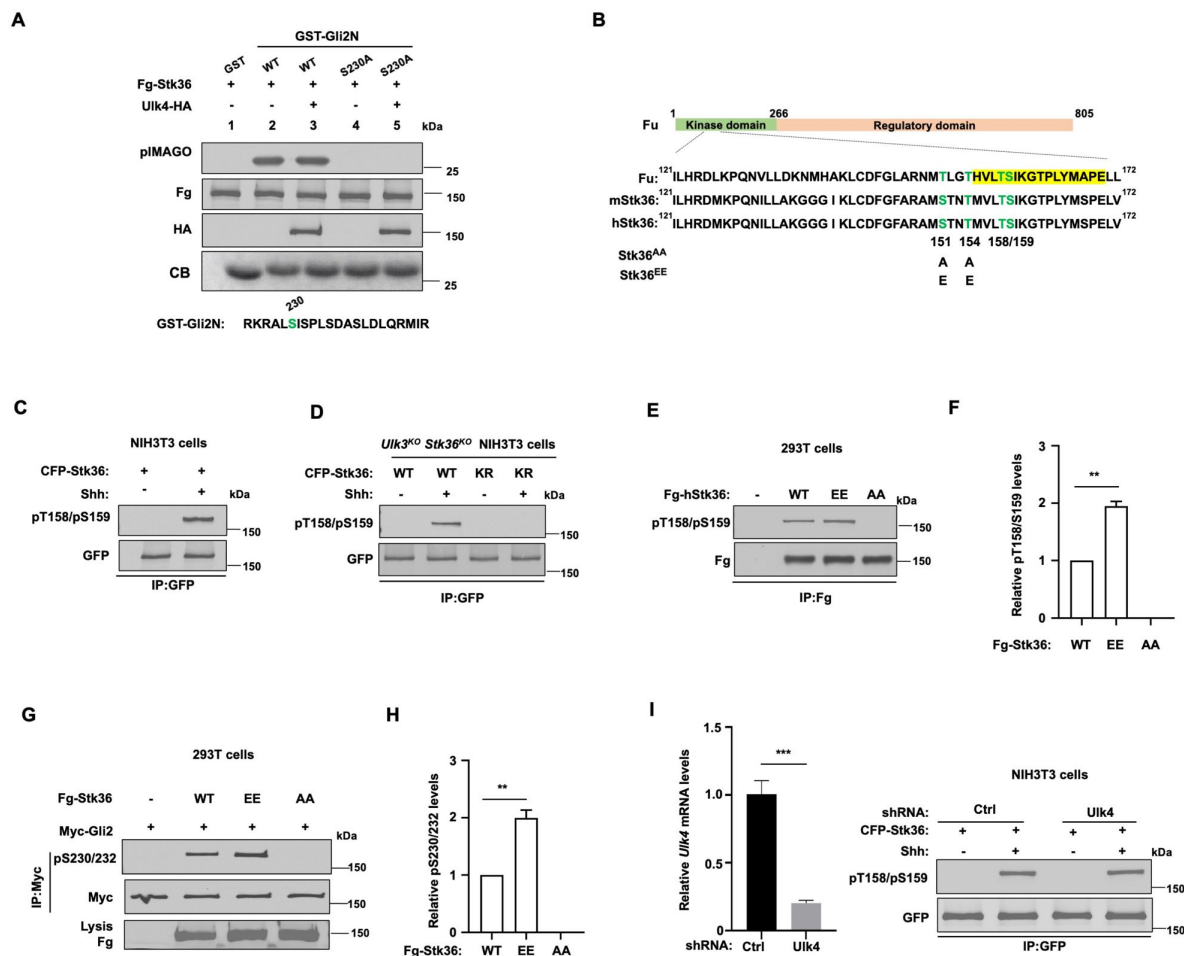


Figure 3.

Ulk4 is dispensable for Stk36 kinase activation.

(A) *In vitro* kinase assay using the immunopurified Fg-Stk36 or Fg-Stk36/hUlk4-HA as kinase and GST-Gli2N^{WT} or GST-Gli2N^{S230A} as substrate. pIMAGO was used to detect GST-Gli2N phosphorylation. The levels of Fg-Stk36/hUlk4-HA and GST/GST-Gli2N were analyzed by western blot and coomassie blue (CB) staining, respectively.

(B) The schematic diagram showing the sequence alignment of the activation segments of *Drosophila* Fu, mouse Stk36 (mStk36) and human Stk36 (hStk36). The conserved Ser/Thr residues in the activation loop are color-coded in green. The residues used as antigen for developing pT158/pS159 are colored in yellow. Amino acid substitutions for Stk36 variants are indicated.

(C) Shh induced Stk36 phosphorylation on T158/S159. NIH3T3 cells expressing CFP-Stk36 lentiviral construct were treated with or without Shh-N, followed by IP with an GFP antibody and western blot analysis using an antibody against the phosphorylated T158/S159 (pT158/pS159).

(D) Shh induced Stk36 phosphorylation on T158/S159 depending on Stk36 kinase activity. Ulk3 and Stk36 double knockout NIH3T3 cells expressing CFP-Stk36^{WT} or CFP-Stk36^{KR} (kinase dead) lentiviral construct were treated with or without Shh-N, followed by IP with an GFP antibody and western blot analysis with the pT158/pS159 antibody.

(E-F) Western blot analysis (E) and quantification (F) of T158/S159 phosphorylation in Fg-Stk36^{WT}, Fg-Stk36^{EE} or Fg-Stk36^{AA}. HEK293T cells were transfected with the indicated Fg-Stk36 constructs, followed by IP with anti-Flag antibody and western blot analysis with the indicated antibodies.

(G-H) Western blot analysis (G) and quantification (H) of Myc-Gli2 phosphorylation on S230/S232 by the wild type (WT) or indicated Stk36 variants in HEK293T cells.

(I) Ulk4 is not required for Shh-induced Stk36 kinase activation. NIH3T3 cells expressing the indicated shRNA and CFP-Stk36 lentiviral construct were treated with or without Shh-N, followed by IP with an GFP antibody and western blot analysis with the pT158/pS159 antibody (right). The knockdown efficiency of Ulk4 was determined by RT-qPCR (left). Data are mean $\pm$ SD from three independent experiments. **p < 0.01, and ***p < 0.001 (Student's t test).

kinase domain of Stk36 (Stk36N: aa1-260) but not its regulatory domain (Stk36C: aa261-1351) interacted with Ulk4 (**Figure 4B-C**). Consistent with Ulk4 mediating the interaction between Stk36 and Gli2, we found that Stk36N formed a complex with Gli2 more effectively than Stk36C (**Figure 4D**).

We next determined whether Ulk4 mediated Stk36/Gli2 interaction in NIH3T3 cells and whether this was regulated by Shh. NIH3T3 cells were infected with lentiviruses expressing low levels of a Flag-tagged Stk36 (Fg-Stk36) and Myc-Gli2 and treated with or without Shh. Co-IP experiments showed that Fg-Stk36 and Myc-Gli2 formed a complex regardless of Shh treatment (**Figure 4E**; lands 1-2). The interaction between Fg-Stk36 and Myc-Gli2 was diminished by Ulk4 RNAi (**Figure 4E**; lands 3-4). Reintroducing full-length hUlk4 (WT) but not hUlk4N or hUlk4C restored Fg-Stk36/Myc-Gli2 association in Ulk4-depleted cells (**Figure 4E**; lands 5-10). Taken together, these results suggest that Ulk4 functions as a molecular scaffold to bring Stk36 and Gli2 into the same complex with its N-terminal region binding to Stk36 and its regulatory domain interacting with Gli2 (**Figure 4F**).

Shh stimulates ciliary tip accumulation of both Ulk4 and Stk36

In response to Shh, Gli2 and Sufu accumulated at the tip of primary cilium in mammal cells (Haycraft et al. 2005; Chen et al. 2009; Tukachinsky et al. 2010), leading to the general view that Gli2 is converted to Gli2^A at the ciliary tip (Goetz and Anderson 2010; Jiang 2021). Indeed, our previous study showed that Shh-stimulated Gli2 phosphorylation by Stk36/Ulk3 depended on ciliary localization of Gli2 (Han et al. 2019). These observations imply that Stk36/Ulk3 may translocate to the primary cilium to phosphorylate Gli2 in response to Shh. To test this hypothesis, we treated NIH3T3 cells stably expressing an N-terminal Myc-tagged Stk36 (Myc-Stk36) with or without Shh and examined ciliary localization of Stk36 by immunostaining with an anti-Myc antibody. Ciliary localization of endogenous Gli2 was examined by immunostaining with an anti-Gli2 antibody. As shown in **Figure 5A-B** and **Figure 5-figure supplement 1A1-B2**, Myc-Stk36 (green signals) and Gli2 (blue signals) were barely detectable in primary cilia in quiescent cells; however, Shh treatment induced ciliary tip localization of both Myc-Stk36 and Gli2.

To determine whether Shh induced ciliary localization of Ulk4, NIH3T3 cells stably expressing hUlk4-HA was treated with or without Shh, followed by immunostaining with anti-HA and anti-Gli2 antibodies. Like Stk36, hUlk4-HA was barely detectable in primary cilia in the absence of Shh but accumulated to the tips of primary cilia and colocalized with Gli2 after Shh stimulation (**Figure 5C-D**; **Figure 5-figure supplement 1C1-D2**). We also examined NIH3T3 cells simultaneously expressing Myc-Stk36 and hUlk4-HA and found that Shh induced the ciliary tip co-localization of Ulk4 and Stk36 (**Figure 5E-F**; **Figure 5-figure supplement 1E1-F2**). We confirmed these results in MEF cells (**Figure 5-figure supplement 1G1-J2**). These observations suggest that Shh induces ciliary tip accumulation of Stk36-Ulk4-Gli2 protein complexes to promote Gli2 phosphorylation and activation.

Ulk4 and Stk36 depend on each other for their ciliary localization

As pseudokinases could regulate the subcellular localization of their interacting kinases, we asked whether Ulk4 is required for ciliary localization of Stk36 in response to Shh. NIH3T3 cells stably expressing Myc-Stk36 were depleted of Ulk4 by shRNA and treated with and without Shh. As shown in **Figure 6A-B**, Ulk4 RNAi diminished Shh-induced ciliary tip localization of Myc-Stk36, suggesting that Ulk4 is required for ciliary localization of Stk36 in response to Shh.

We next asked whether Ulk4 ciliary localization depends on Stk36. We first determined the ciliary location of Ulk4 in NIH3T3 cells with both Stk36 and Ulk3 knocked out (DKO) by the CRISPR-Cas9 system (Han et al. 2019). As shown in **Figure 6C-D**, Shh promoted ciliary tip enrichment of Ulk4-HA in the control cells but not in the Stk36 and Ulk3 DKO cells. Adding back wild type Stk36 (Stk36^{WT}) but not its kinase inactive form (Stk36^{AA}) restored Shh-induced Ulk4-HA ciliary tip

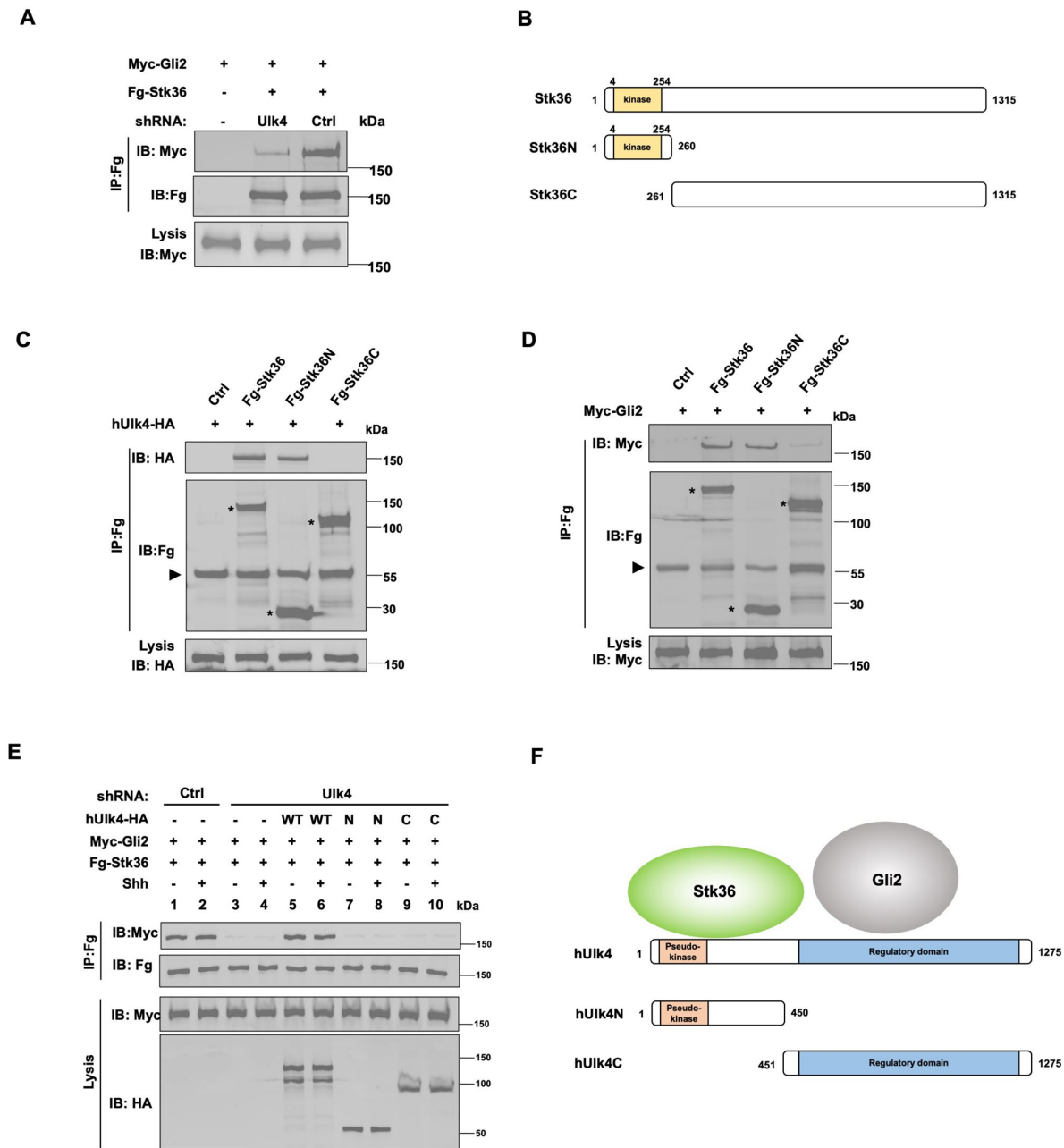


Figure 4.

Ulk4 mediates the interaction between Stk36 and Gli2.

(A) Depletion of Ulk4 diminished the association between Stk36 and Gli2. HEK293T cells were transfected with the indicated shRNA and Fg-Stk36 and Myc-Gli2 constructs, followed by CoIP and western blot analysis with the indicated antibodies.

(B) Domain structure and deletion mutants of human Stk36 used for the Co-IP experiments.

(C-D) Stk36 binds to Ulk4 and Gli2 through its N-terminal kinase domain. HEK293T cells were transfected with Ulk4-HA (C) or Myc-Gli2 (D) and the indicated Fg-Stk36 constructs, followed by Co-IP and western blot analyses with the indicated antibodies. Black triangles indicate the heavy chain of IgG and asterisks indicate the protein bands produced by individual Stk36 constructs.

(E) Both N- and C-terminal domains of Ulk4 are required for mediating Stk36/Gli2 association. NIH3T3 cell lines stably expressing control (Ctrl) or Ulk4 shRNA were infected with lentiviruses expressing the indicated hUlk4 constructs (diagrams shown on F, right), Fg-Stk36 and Myc-Gli2, and treated with or without the Shh-N, followed by CoIP and western blot analysis with the indicated antibodies.

(F) Schematic diagram showing the interaction relationship between Ulk4 and Stk36, Gli2.

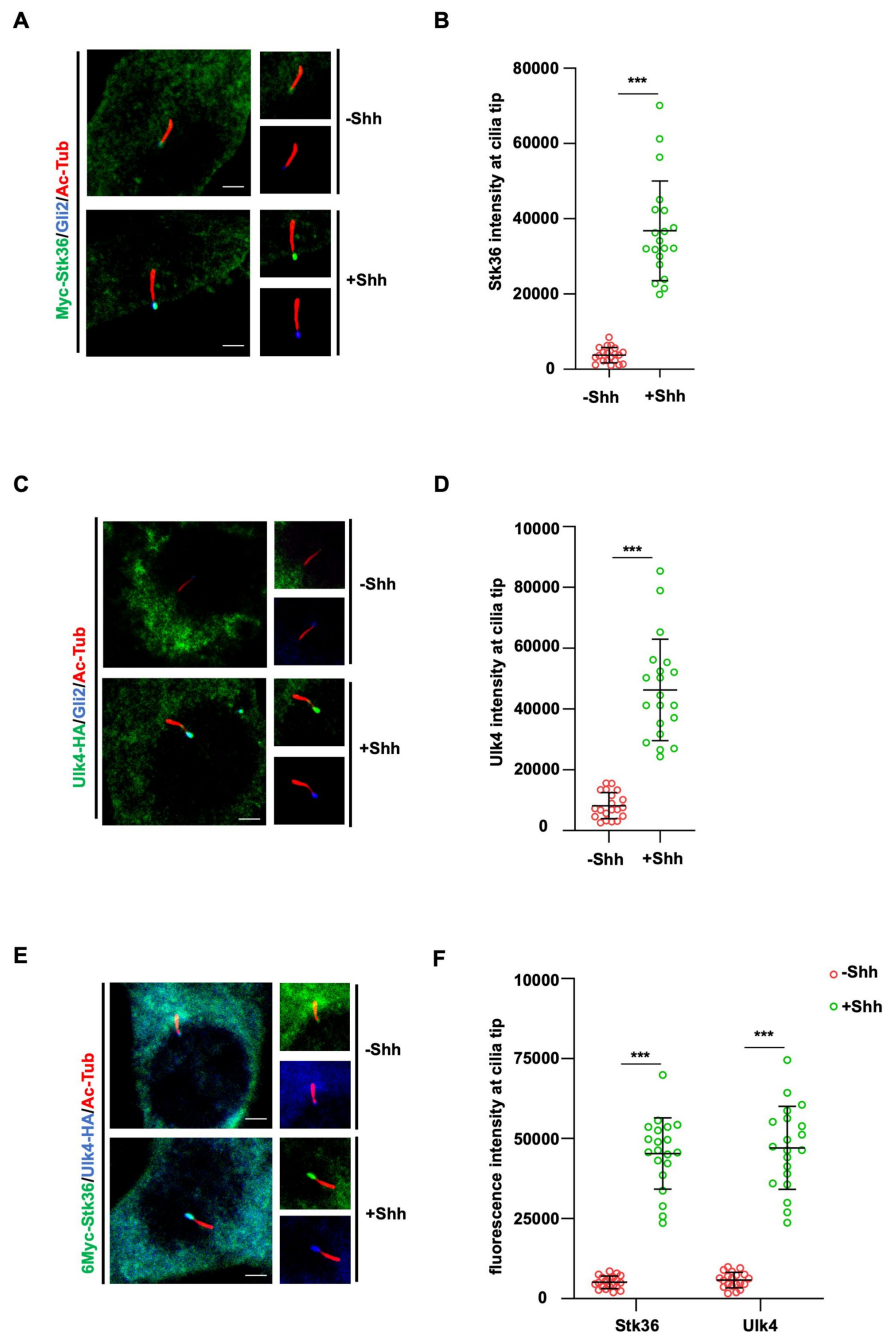


Figure 5.

Shh stimulates ciliary tip accumulation of both Ulk4 and Stk36.

(A-D) Representative images of immunostaining (A, C) and quantification (B, D) of ciliary tip localized Myc-Stk36 (green in A) or Ulk4-HA (green in C) and Gli2 (blue in A and C) in NIH3T3 cells infected with the Myc-Stk36 or Ulk4-HA lentivirus and treated with or without Shh-N. Primary cilia were marked by acetylated tubulin (Ac-tub) staining (red in A and C).

(E-F) Representative images of immunostaining (E) and quantification (F) of ciliary tip localized Myc-Stk36 (green) and Ulk4-HA (blue) in NIH3T3 cells co-infected with Myc-Stk36 and Ulk4-HA lentiviruses and treated with or without Shh-N. Primary cilia were marked by Ac-Tub staining (red).

The cells are starved in serum-free medium for 12 hours to allow ciliation and cultured in the same medium with or without Shh-N fragment for another 12 hours before they are subjected into immunostaining assay. The intensity of ciliary-localized Myc-Stk36, Ulk4-HA and Gli2 was measured by Image J. 20 cells were randomly selected and counted for each group. Data are mean $\pm$ SD from three independent experiments. ***p < 0.001 (Student's t test). Scale bars are 2 μm.

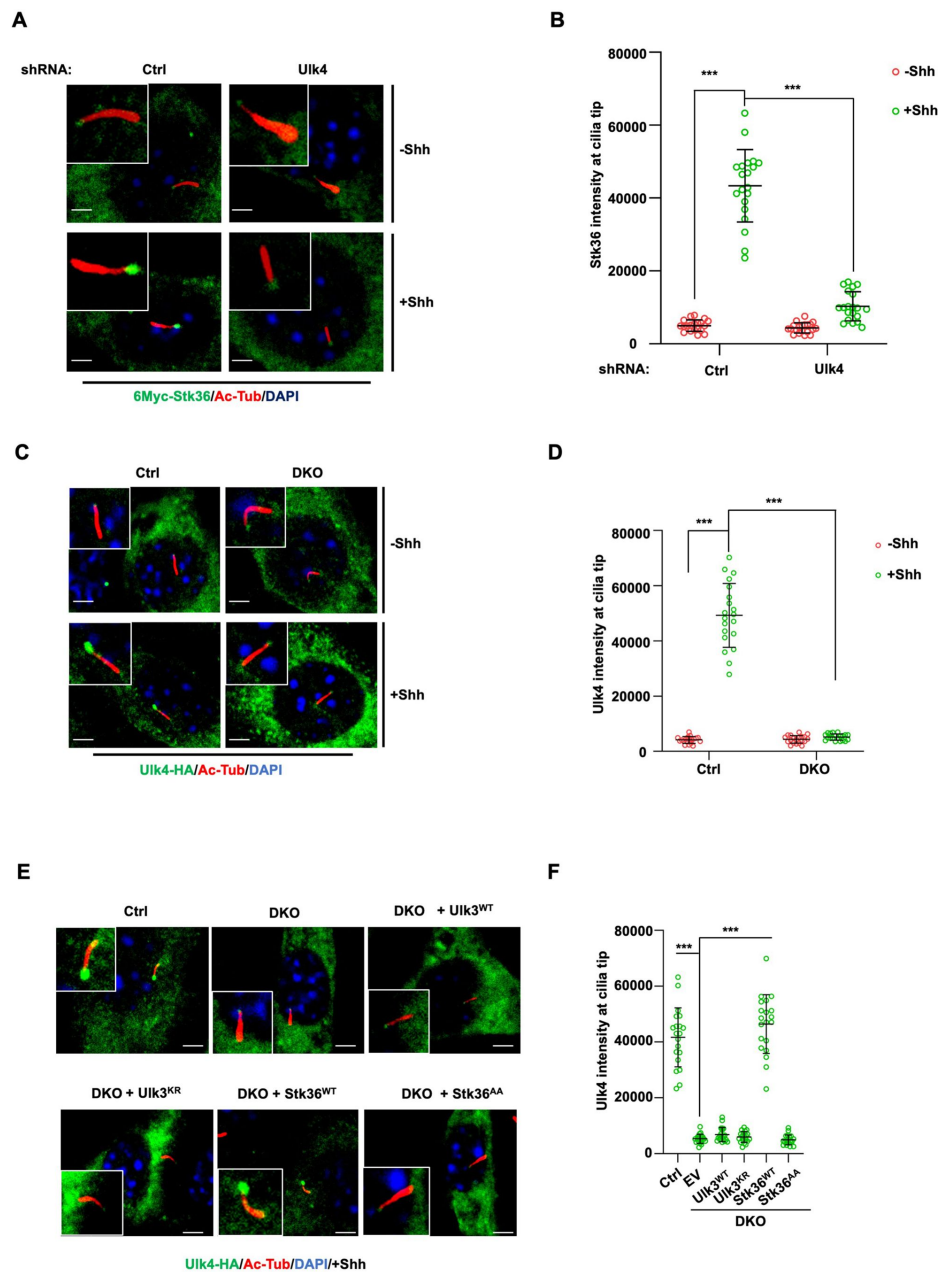


Figure 6.

Stk36 and Ulk4 depend on each other for their ciliary tip accumulation

(A-B) Representative images of immunostaining (A) and quantification (B) of ciliary tip localized Myc-Stk36 in NIH3T3 cells infected with the indicated shRNA and Myc-Stk36 lentivirus in the presence or absence of Shh-N. Primary cilia were marked by Ac-Tub staining (red); Myc-Stk36 was marked by Myc staining (green); nuclei were marked by DAPI (Blue).

(C-D) Representative images of immunostaining (C) and quantification (D) of ciliary tip localized Ulk4-HA (green) in wild type (Ctrl) or Ulk3 and Stk36 double knockout (DKO) NIH3T3 cells treated with or without Shh-N. Primary cilia were marked by Ac-Tub staining (red) and nuclei by DAPI (Blue).

(E-F) Representative images of immunostaining (E) and quantification (F) of ciliary-localized Ulk4-HA (green) in Shh-N treated control or DKO NIH3T3 cells infected with or without lentiviruses expressing the indicated Ulk3 or Stk36 constructs.

The cells are starved in serum-free medium for 12 hours to allow ciliation and cultured in the same medium with or without Shh-N fragment for another 12 hours before they are subjected into immunostaining assay. The intensity of ciliary-localized Myc-Stk36 or Ulk4-HA was measured by Image J. 20 cells were randomly selected and counted for each group. Data are mean $\pm$ SD from three independent experiments. *** $p < 0.001$ (Student's *t* test). Scale bars are 2 μ M.

accumulation in DKO cells (**Figure 6E-F**). By contrast, neither wild type Ulk3 (Ulk3^{WT}) nor its kinase dead form (Ulk3^{KR}) could rescue ciliary tip accumulation of Ulk4-HA in DKO cells (**Figure 6E-F**). These results suggest that ciliary tip localization of Ulk4 depends on Stk36 kinase activity.

Ulk4 ciliary localization is regulated by its C-terminal phosphorylation

Many pseudokinases are also the substrates of their interacting kinases (Kung and Jura 2019). To determine whether Ulk4 is a Stk36 substrate and whether Stk36-mediated phosphorylation of Ulk4 promotes its ciliary tip localization, we sought out to identify the relevant phosphorylation sites. We generated several C-terminally HA-tagged truncated forms of hUlk4 to narrow down the region essential for ciliary tip localization of Ulk4 (**Figure 7A**). These truncated forms were expressed in NIH3T3 cells in which endogenous Ulk4 depleted by RNAi. Deleting the entire regulatory domain abolished Shh-induced ciliary tip localization of the Ulk4 variant (hUlk4₁₋₄₅₀; **Figure 7B-C**). Deleting the Stk36 binding region from Ulk4 (hUlk4₄₅₁₋₁₂₇₅) also abolished its ciliary tip localization in response to Shh, suggesting that binding to Stk36 is essential for Ulk4 ciliary localization. We generated two small deletions from the C-terminus of hUlk4 (hUlk4₁₋₁₀₀₀ and hUlk4₁₋₁₂₀₀; **Figure 7A**). Whereas hUlk4₁₋₁₂₀₀ still localized to the ciliary tip in response to Shh, hUlk4₁₋₁₀₀₀ failed to do so (**Figure 7B-C**), suggesting that the region between aa1000-1200 is essential for Shh-induced hUlk4 ciliary tip localization. Within this region, T1021 and T1088/T1090 fall into the Fu/Ulk3/Stk36 phosphorylation consensus site: S/T [X₅] E/D (**Figure 7D**) (Han et al. 2019; Zhou and Jiang 2022). Furthermore, phosphorylation on T1021 can prime CK1-mediated phosphorylation on T1023 as T1023 falls into the CK1 phosphorylation consensus site: D/E/(p)S/(p)T[X₁₋₃] S/T (Knippschild et al. 2005).

To determine whether phosphorylation on these sites may regulate Ulk4 ciliary localization, we generated hUlk4 variants carrying T1021A/1023A (hUlk4^{T1021A/1023A}) or T1088A/T1090A (hUlk4^{T1088A/T1090A}) substitutions. These hUlk4 variants were expressed in NIH3T3 cells with endogenous Ulk4 depleted by shRNA. We found that hUlk4^{T1088A/T1090A} but not hUlk4^{T1021A/1023A} accumulated at ciliary tip in response to Shh stimulation (**Figure 7E-F**), suggesting phosphorylation on T1021/1023 is essential for Shh-induced ciliary tip localization of Ulk4.

To confirm T1021 phosphorylation by Stk36, we carried out an *in vitro* kinase assay using immunopurified Fg-Stk36 as kinase and GST fusion protein containing hUlk4 sequence from aa1017 to aa1036 (GST-hUlk4C) as substrate. As shown in **Figure 7G**, immunopurified wild type Stk36 (Fg-Stk36^{WT}) but not its kinase inactive variant (Fg-Stk36^{AA}) phosphorylated GST-hUlk4C. Mutating both T1021 and T1023 to A (T1021A/T1023A) abolished the phosphorylation; however, mutating T1021A reduced but not eliminated the phosphorylation, suggesting that in the absence of T1021 phosphorylation, T1023 could also be phosphorylated by Stk36. Interestingly, T1023 but not T1021 is conserved in mouse Ulk4 (mUlk4) and mUlk4 contains an Ala at T1021 (**Figure 7H**), implying that phosphorylation of T1021 might be dispensable for Ulk4 function. Indeed, we found that mutating T1021 to A in hUlk4 (hUlk4^{T1021A}) did not affect its ciliary tip accumulation in response to Shh (**Figure 7E-F**) as well as its activity in the Hh pathway (see below).

Ciliary localization of Ulk4 is essential for its function in Shh signaling

To determine the importance of ciliary localization of Ulk4 for its function in Shh signal transduction, we compared the Hh pathway related activity of ciliary localization deficient hUlk4^{T1021A/1023A} with that of ciliary localization competent hUlk4^{T1021A} (equivalent to mUlk4^{WT}) and hUlk4^{WT} in NIH3T3 cells with endogenous Ulk4 depleted by shRNA. We found that both hUlk4^{T1021A} and hUlk4^{WT} rescued Shh-induced ciliary tip localization of coexpressed Myc-Stk36 (**Figure 8A-B**), phosphorylation of Myc-Gli2 at S230/S232 (**Figure 8C-C'**), and the expression of Shh target genes *Ptch1* and *Gli1* (**Figure 8D-E**). By contrast, hUlk4^{T1021A/1023A} failed to rescue

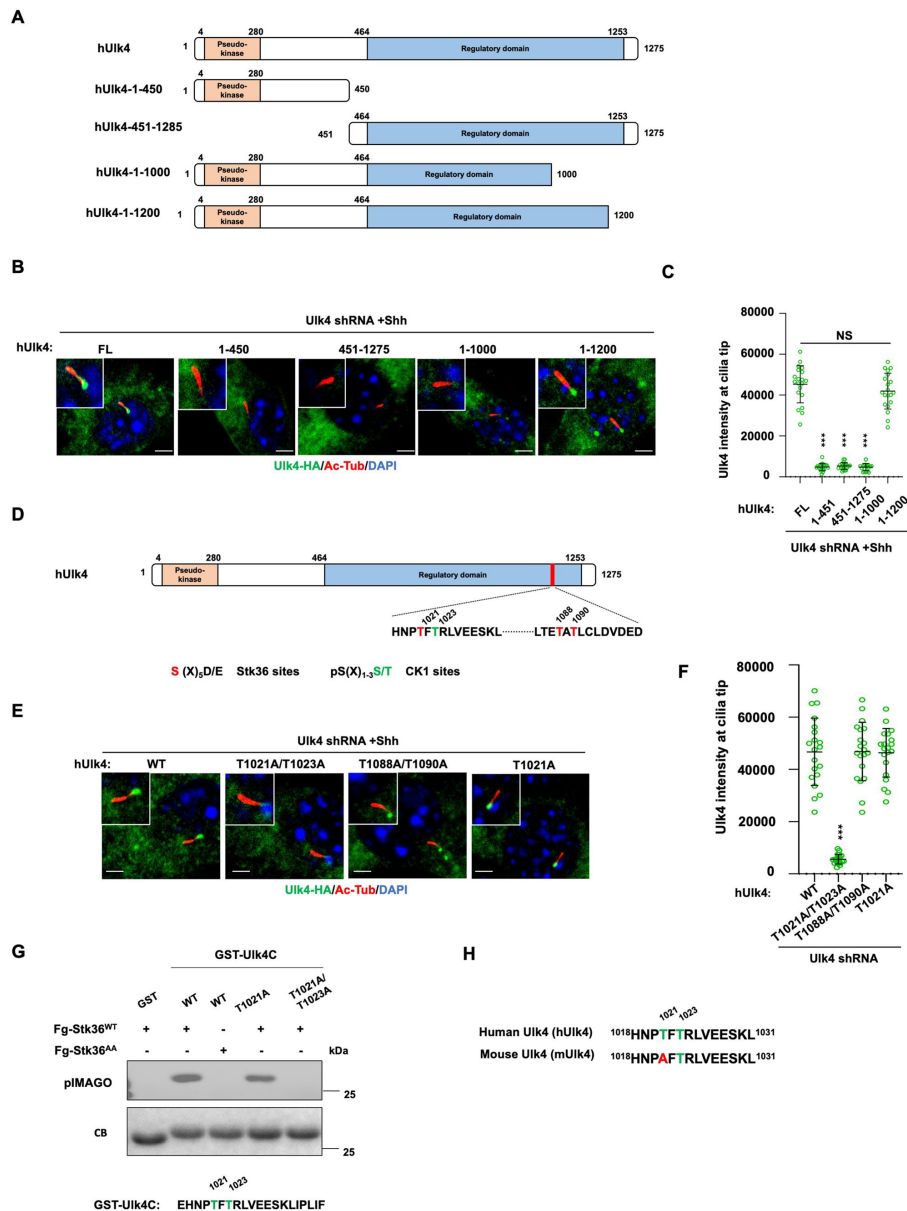


Figure 7.

Ulk4 ciliary tip accumulation is promoted by its phosphorylation by Stk36

(A) Diagrams of hUlk4 deletion constructs.

(B-C) Representative images of immunostaining (B) and quantification (C) of ciliary tip localized full-length or truncated hUlk4-HA in Ulk4 knockdown cells treated with Shh-N.

(D) Stk36 and CK1 phosphorylation sites in the C-terminal region of hUlk4.

(E-F) Representative images of immunostaining (E) and quantification (F) of ciliary tip localized wild type (WT) or mutant hUlk4-HA bearing the indicated amino acid substitutions in Ulk4 knockdown NIH3T3 cells treated with Shh-N.

(G) *In vitro* kinase assay using the immunopurified Fg-Stk36^{WT} or Fg-Stk36^{AA} as kinase and the indicated GST-Ulk4C fusion proteins as substrates. Phosphorylation was detected by the pIMAGO system.

(H) Schematic diagram showing the sequence alignment of C-terminal phosphorylation sites of mouse Ulk4 (mUlk4) and human Ulk4 (hUlk4). The Thr residues phosphorylated by Stk36 are color-coded in green.

The cells are starved in serum-free medium for 12 hours to allow ciliation and cultured in the same medium with or without Shh-N fragment for another 12 hours before they are subjected into immunostaining assay. The intensity of ciliary-localized WT and mutant Ulk4-HA was measured by Image J. 20 cells were randomly selected and counted for each group. Data are mean $\pm$ SD from three independent experiments. *** $p < 0.001$ (Student's *t* test). Scale bars are 2 μ m.

Shh-stimulated Stk36 ciliary tip localization, Gli2 phosphorylation, as well as *Ptch1* and *Gli1* expression in Ulk4 depleted cells (**Figure 8A-E** [↗](#)). These results suggest that ciliary localization of Ulk4 is essential for its ability to promote Stk36 ciliary localization, Gli2 phosphorylation, and Hh pathway activation (**Figure 8F** [↗](#)).

Discussion

The Gli family of transcription factors mediates the transcriptional output of the Hh signaling pathway in species ranging from *Drosophila* to mammals but how Gli is activated by Hh signaling is still poorly understood, especially in vertebrates where Gli activation is thought to occur at the tip of primary cilium. We recently found that the Fu/Ulk family kinases directly phosphorylate Ci and Gli2 on multiple sites to promote their activation (Han et al. 2019 [↗](#); Zhou et al. 2022 [↗](#); Zhou and Jiang 2022 [↗](#)). In mammalian cells, the Ulk family kinases Ulk3 and Stk36 (mammalian homolog of Fu) act in parallel to promote Gli2 phosphorylation and activation in response to Shh (Han et al. 2019 [↗](#)). In addition, Gli2 phosphorylation depends on its ciliary localization (Han et al. 2019 [↗](#)). In this study, we found that Stk36 accumulated to the tip of primary cilium and colocalized with Gli2 in response to Shh stimulation, consistent with Gli2 being converted into Gli2^A at the ciliary tip. In addition, we identified another Ulk family member Ulk4, which is a pseudokinase, as a mediator of Shh-induced Gli2 phosphorylation. We found that Ulk4 and Stk36 act in the same pathway but in parallel with Ulk3 to promote Gli2 phosphorylation and activation in NIH3T3 and MEF cells. The positive role of Ulk4 in Shh signaling could explain why elevated expression of Ulk4 in certain genetic background could suppress the heart outflow tract defects and holoprosencephaly caused by partially reduced Shh signaling activity in LRP2-deficient mice (Mecklenburg et al. 2021 [↗](#)). However, it remains to be determined whether Ulk4 participates in Hh signaling *in vivo* by loss-of-function study. Because knockout of Stk36 in mice did not affect Hh signaling during development, likely due to functional compensation by other kinases such as Ulk3 (Chen et al. 2005 [↗](#); Merchant et al. 2005 [↗](#)), we do not expect that loss-of-function of Ulk4 will affect Hh signaling during development. Therefore, double knockout of Ulk4 (or Stk36) and Ulk3 will be necessary to address the physiological role of the Fu/Ulk family members in mammalian Hh signaling. In addition, it will also be important to determine whether Ulk4/Stk36 and Ulk3 could be differentially required for Hh signal transduction in different cell types/tissues or in different contexts, e.g., development vs. tissue homeostasis or physiological vs pathological settings.

To determine the mechanism by which Ulk4 assists Stk36 to phosphorylate Gli2, we explored whether Ulk4 regulated 1) the enzymatic activity of Stk36; 2) Shh-induced Stk36 kinase activation; 3) the association of Stk36 and its substrate; and 4) ciliary localization Stk36. In an *in vitro* kinase assay, we found that immunopurified Stk36 phosphorylated GST-Gli2N equally well regardless of whether it interacted with Ulk4 or not, suggesting that Ulk4 does not regulate Stk36 kinase activity. Rather, we found that Ulk4 is required for Stk36 to bind to its substrate Gli2. Ulk4 interacts with Stk36 through its N-terminal region that contains the pseudokinase domain and with Gli2 via its C-terminal regulatory domain. Depletion of Ulk4 diminished the physical association between Stk36 and Gli2. As a further support of the notion that Gli2 is brought to Stk36 through Ulk4, we found that the kinase domain of Stk36, which binds Ulk4, also interacted with Gli2 whereas its C-terminal region interacted with neither Ulk4 nor Gli2. Of note, despite being a pseudokinase, Ulk4 contains an unusual ATP binding site that might regulate its ability to bind other proteins such as Stk36 (Preuss et al. 2020 [↗](#)). Therefore, it would be interesting to determine in the future whether ATP binding to Ulk4 could regulate Shh signaling or other processes that Ulk4 is involved.

Although Stk36/Ulk4/Gli2 complex formation appears to be constitutive regardless of the presence or absence of Shh, Stk36 kinase activation depends on Shh stimulation. Like Fu, Stk36 is phosphorylated on multiple sites in the activation loop of its kinase domain in response to Shh likely through trans-autophosphorylation, and activation loop phosphorylation is essential for its

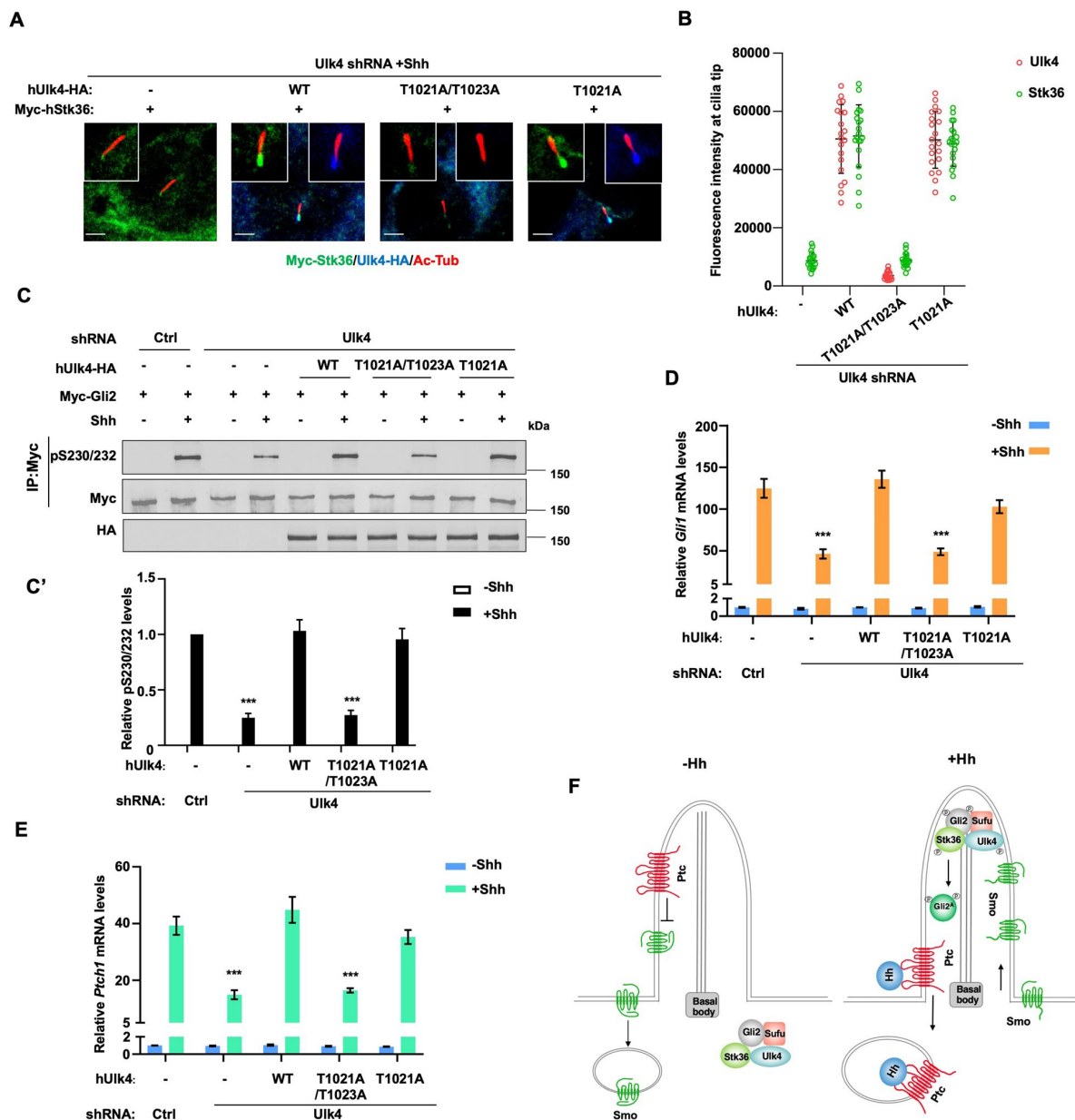


Figure 8.

Ciliary tip localization of Ulk4 is required for Shh signal transduction

(A-B) Representative images of immunostaining (A) and quantification (B) of ciliary tip localized Myc-Stk36 and hUlk4 in Ulk4-depleted NIH3T3 cells expressing the indicated hUlk4 constructs and treated with Shh.

(C-C') Western blot analysis (C) and quantification (C') of Myc-Gli2 phosphorylation on S230/S232 in NIH3T3 cells expressing the indicated shRNA and hUlk4 lentiviral constructs and treated with or without Shh-N. Data are mean $\pm$ SD from two independent experiments. *** $p < 0.001$ (Student's t test).

(D-E) Relative *Gli1* (D) and *Ptch1* (E) mRNA levels in NIH3T3 cells expressing the indicated shRNA and hUlk4 lentiviral constructs treated with or without Shh-N.

(F) Model for how Ulk4 participates in Shh signaling. See text for details.

The cells are starved in serum-free medium for 12 hours to allow ciliation and cultured in the same medium with or without Shh-N fragment for another 12 hours before they are subjected into RNA preparation, Western blot and immunostaining assay. The intensity of ciliary-localized WT and mutant Ulk4-HA was measured by Image J. 20 cells were randomly selected and counted for each group. Data are mean $\pm$ SD from three independent experiments. *** $p < 0.001$ (Student's t test). Scale bars are 2 μ m.

kinase activation. Using a phospho-specific antibody that recognized pT158/pS159 in the kinase activation loop of Fu/Stk36, we found that Ulk4 depletion did not affect Shh-induced phosphorylation of Stk36 on T158/S159, a readout for Stk36 kinase activation, suggesting that Ulk4 is dispensable for Shh signaling to activate Stk36. In *Drosophila*, the kinesin-like protein Costal2 (Cos2) mediates the activation of Fu kinase by Hh. Therefore, it would be interesting to determine whether mammalian homologs Cos2, Kif7 and/or Kif27, participates in Stk36 kinase activation in response to Shh stimulation.

Hh signaling depends on primary cilia in vertebrates. Upon Shh stimulation, key pathway components such as Smo and Gli proteins accumulate in primary cilia, and Gli2 is accumulated to the ciliary tip and thought to be converted into its activator form (Gli2^A) in this ciliary compartment. Consistent with this notion, we found that Shh induced ciliary accumulation of both Ulk4 and Stk36 that colocalized with Gli2 at the ciliary tip. Furthermore, we found that Ulk4 is required for ciliary tip accumulation of Stk36. On the other hand, ciliary tip accumulation of Ulk4 depends on Stk36 kinase activity and phosphorylation of its C-terminal region on T1023. Preventing Ulk4 phosphorylation on T1023 abolished its ciliary tip accumulation and its ability to promote Stk36 ciliary tip accumulation, Gli2 phosphorylation and Hh pathway activation. These results suggest that ciliary tip accumulation of Ulk4 is critical for its function in Hh signal transduction, likely by increasing the local concentration of Stk36 and Gli2 to promote effective phosphorylation of Gli2. It would also be important to examine the localization of endogenous Ulk4 and Stk36 in the future as exogenously expressed epitope-tagged protein might cause artifacts. In addition, the precise mechanism and dynamics of Ulk4 ciliary targeting remain to be determined in the future. e.g., it would be important to determine how phosphorylation of T1023 promotes ciliary accumulation of Ulk4. A recent study identified several motor proteins of the kinesin family (KIF1B, KIF3A, KIF3B, and KAP3) as the interaction partners of Ulk4 (Preuss et al. 2020). KIF3A, KIF3B and KAP3 are components of kinesin-2 motor that act in conjunction with intraflagellar transport (IFT) complexes to transport cargoes into primary cilia (Goetz and Anderson 2010). It would be interesting to determine whether Ulk4 T1023 phosphorylation affects its interaction with KIF3A, KIF3B, KAP3, or components in the IFT complexes. Of note, a previous study suggested that Ulk4 regulated the ciliary localization of Smo although the underlying mechanism was not explored (Mecklenburg et al. 2021). Therefore, it remains possible that Ulk4 could regulate mammalian Hh signaling at multiple steps similar to what Fu does in the *Drosophila* Hh pathway (Claret et al. 2007; Liu et al. 2007).

Both Ulk4 and Stk36 have functions outside the Hh pathway. Stk36 is required for motile ciliogenesis, and its loss of function affects the construction of central pair apparatus of “9+2” motile cilia in both zebrafish and mice (Wilson et al. 2009; Nozawa et al. 2013). Ulk4 malfunction also affects motile ciliogenesis of ependymal cells in mutant mice, leading to hydrocephalus (Vogel et al. 2012; Liu et al. 2016b). A recent study demonstrated that Ulk4 and Stk36 physically interact to mediate the assembly of motile flagella in the flagellated protist *Leishmania Mexicana* (McCoy et al. 2023). However, the biochemical mechanism by which Ulk4/Stk36 regulates motile ciliogenesis has remained unknown. Ulk4 could regulate Stk36 ciliary localization and/or kinase-substrate interaction like what we have discovered for their role in Shh signaling. The relevant substrates for Stk36 in motile ciliogenesis remain to be determined. Knowing the phosphorylation consensus sites for the Fu/Ulk family kinase may facilitate the identification of Stk36 substrates involved in the assembly of motile cilia and flagella. Initially identified Fu/Ulk3/Stk36 phosphorylation sites in Ci/Gli and Cos2 fall into the consensus: S/T(X)₅D/E, where the acidic residue at +6 position is essential (Han et al. 2019; Zhou and Jiang 2022). However, subsequent studies suggested that the spacing between the phospho-acceptor site and the acid residue appears to be flexible. For example, a Ulk3/Stk36 phosphorylation site in the C-terminal region of Gli2 has a spacing of six residues between the phospho-acceptor site and the acid residue (Zhou et al. 2022). Here we found that a Stk36 phosphorylation site in the C-terminal region of Ulk4 contains only four residues between the phospho-acceptor site and the acid residue. Based on these findings, we propose an expanded consensus site for the Fu/Ulk

family kinases as S/T(X)₄₋₆D/E. It would be interesting to determine whether this phosphorylation consensus sequence could be extended to other members of the Ulk kinase family such as Ulk1/Atg1 and Ulk2, which are the initiating kinases for autophagy (Zachari and Ganley 2017 [↗](#)).

Ulk4 has been implicated in several human diseases including hypertension and psychiatric disorder such as schizophrenia (Luo et al. 2022 [↗](#)). Ulk4 also plays a role in neurogenesis in mice by regulating neural stem cell pool and neurite branching morphogenesis (Lang et al. 2014 [↗](#); Lang et al. 2016 [↗](#); Liu et al. 2016a [↗](#)). In addition, Ulk4 regulates the integrity of white matter by promoting myelination (Liu et al. 2018b [↗](#)). How Ulk4 participates in these diverse processes has remained unclear. A recent study revealed that Ulk4 binds several other kinases and phosphatases such as DAPK3, LMTK2, PTPN14 as well as proteins implicated in the regulation of neuronal differentiation and axonal regeneration including CAMSAP family proteins CAMSAP1/3 (Pongrakhananon et al. 2018 [↗](#); Preuss et al. 2020 [↗](#)). Given our findings regarding how Ulk4 regulates Hh signaling, it is tempting to speculate that Ulk4 may also function as a scaffold to bridge kinases or other enzymes to their substrates and/or regulate the subcellular localization of the interacting proteins in other biological processes.

Materials and methods

DNA constructs

Myc-Gli2 and GST-Gli2 were described previously (Han et al. 2019 [↗](#)). All GST-fusion (the GST-tagged human Ulk4C^{WT}, Ulk4C^{T1021A}, Ulk4C^{T1021A/1023A}) proteins were subcloned into the *pGEX 4T-1* vector using EcoRI and XhoI sites. N-terminally CFP-tagged mouse Ulk3^{WT}, Ulk3^{KR}, N-terminally 6XMyC-tagged human Stk36, N-terminally 3XFG-tagged human Stk36^{WT}, Stk36^{AA} and the C-terminally 3XHA-tagged Human Ulk4^{WT}, Ulk4¹⁻⁴⁵¹, Ulk4⁴⁵²⁻¹²⁷⁵, Ulk4¹⁻¹⁰⁰⁰, Ulk4¹⁻¹²⁰⁰, Ulk4^{T1021/1023A}, Ulk4^{T1088/1090A}, Ulk4^{T1021A} were subcloned into the *FUGW* vector with the EcoRI and BamHI sites. N-terminally 3XFG-tagged human Stk36^{WT}, Stk36^{EE}, Stk36^{AA}, Stk36¹⁻²⁶⁰, Stk36²⁶¹⁻¹³¹⁵, and C-terminally 3XHA-tagged Human Ulk4^{WT}, Ulk4¹⁻⁴⁵¹, Ulk4⁴⁵²⁻¹²⁷⁵ were subcloned in the *PLVX* vectors using the EcoRI and MluI sites. The Ulk4^{T1021/1023A}, Ulk4^{T1088/1090A}, Ulk4^{T1021A}, Stk36^{EE}, Stk36^{AA}, were generated by PCR-mediated site-directed mutagenesis.

Cell culture, transfection, and lentiviral production

NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10% bovine calf serum (Gibco) and 1% penicillin/streptomycin (Sigma). The transfection was carried out by using the GenJet Plus *in vitro* DNA transfection kit according to the manufacturer's instruction (SignaGen). HEK293T cells were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Sigma). PolyJet *in vitro* DNA transfection kit (SignaGen) was used to do the transfection according to the manufacturer's instruction. Mouse embryonic fibroblast cells (MEFs) were cultured in DMEM (Sigma) containing 10% FBS (Gibco), and 1% streptomycin / penicillin (Sigma) according to the standard protocol. For Shh-N treatment, cells were cultured at 50%-confluent densities in plate or chamber slides for one day and starved in serum-free medium (0.5% BCS for NIH3T3 cells or 0.5% FBS for MEF cells) for 12 hours to allow ciliation. The recombinant human Shh N-terminal fragment (1 ng/ml; R&D Systems, #8908-SH-005) was then added to the same serum-free medium for 12 hours or overnight.

Immunostaining, immunoprecipitation, and western blotting analysis

The protocol for Ulk4-HA/Myc-Stk36 immunostaining was carried out as previously described (Han et al. 2019 [↗](#)). Immunofluorescence imaging was captured using a Zeiss LSM700 laser scanning microscope. For ciliary Myc-Stk36/Ulk4-HA quantification, the cilia were labelled with the acetylated-Tubulin and the Myc-Stk36/Ulk4-HA fluorescence intensities were measured in the Myc-

Stk36/Ulk4-HA image channel by ImageJ. Twenty cells were randomly selected and calculated for each group. For immunoprecipitation assay, after transfection for 48 hours, cells were washed twice with PBS and then lysed on ice for 10 min with lysis buffer containing 1M Tris pH8.0, 5M NaCl, 1MNaF, 0.1M Na₃VO₄, 1% CA630, 10% glycerol, and 0.5M EDTA (pH 8.0). Cell lysates were incubated with protein A-Sepharose beads (Thermo scientific) for 1 hr at 4°C to eliminate nonspecific binding proteins. After removal of the protein-A beads by centrifugation, the cleared lysates were incubated with Myc (HA or Flag) antibody for 4 h or overnight. Protein complexes were collected by incubation with protein A-Sepharose beads for 1 hr at 4°C, followed by centrifugation. Immunoprecipitates were washed three times for 5 min each with lysis buffer and were separated on SDS-PAGE. Western blot was carried out using standard protocol. Western blot quantification was carried out by employing fluorogenic secondary antibody (IRDye 680LT Goat anti-Mouse:926-68020 or Goat anti-Rabbit: 926-68021) and the blot was scanned and quantified with an Odyssey CLx imaging system (LI-COR). Antibodies used in this study were as follows: Mouse anti-Myc (Santa Cruz Biotechnology; 9E10), Mouse anti-Flag (Sigma; M2), Mouse anti-HA (Santa Cruz Biotechnology; F7), Mouse anti-acetylated Tubulin (Sigma; T7451; staining:1:1000), Rabbit anti-Myc (Abcam; ab9106; staining 1:2000), Rabbit anti-HA (AbCam; ab9110; staining 1:500), Goat anti-Gli2 (R&D system; AF3635; staining 1:1000).

***In vitro* kinase assay**

In vitro kinase assay was performed by incubating 25 µL of reaction mixtures containing 150 mM Tris-HCl (pH 7.5), 0.2 mM Mg²⁺/ATP, 1 µg of purified GST-Ulk4/Gli2 fusion proteins, together with appropriate amount of kinase for 30mins at 30°C. The reaction was terminated by adding 2× SDS loading buffer. The resultant samples were load on SDS-PAGE and subjected to pIMAGO phospho-protein detection kit with fluor-680 (Sigma) detection kit and LI-COR Odyssey platform for western blot analyses of phosphorylated proteins (Millipore Sigma). Fg-Stk36^{WT} and Fg-Stk36^{AA} were immunopurified from HEK293 cells transfected with the Fg-Stk36^{WT} or Fg-Stk36^{AA} construct.

Viral Infection and shRNA

For lentivirus generation, *pLKO.1* vectors expressing shRNA or *FUGW* vectors expressing transgenes were co-transfected with package vectors (PSPAX2 and PMDG2) into HEK293T cells using PolyJet (SignaGen). The supernatants were collected after 48 h and 72 h transfection, filtered through a 0.45 µm filter and centrifuged at 20,000 g for 2 h. The sediment containing the viruses was re-suspended, aliquoted in small volume of culture medium and stored at -80°C for future use. For cell infection, lentiviruses were added to 70% confluent wild type NIH3T3 or MEF cells together with Polybrene (Sigma, Cat. No. H9268) for overnight to make the stable NIH3T3 or MEF cell lines expressing transgene or shRNA. The Mouse Ulk4 shRNA lentiviral vector was purchased from Sigma (TRCN0000328268 for Ulk4 knockdown in NIH3T3 cells and MEFs;TRCN0000002203 for Ulk4 knockdown in HEK293 cells). The Non-Target shRNA Plasmid DNA (sigma; SHC016) was used as control.

RNA interference

For RNAi experiments in NIH3T3 or MEF cells, the siRNAs were purchased from Sigma. The sequences for Ulk3 silencing were: 5'-GUGUACAAGGCCUACGCCA-3' (Cat# SASI_Mm02_00444409) and 5'-CUGAGAAGGUGGCCCGUGU-3' (Cat# SASI_Mm02_00444410). The sequences for Stk36 silencing were: 5'- GGUAUACUGGCUUCAGAAA-3' (Cat# SASI_Mm02_00345637) and 5'-GCCUUAUGUGCUUUGCUGU-3' (Cat# SASI_Mm01_00167751). The target sequence of Mouse Ulk4 shRNA is: CTGCGAAGATTATCGAGAATG. The target sequence of Human Ulk4 shRNA is: CCACTAGGTCCTCTTTCAGA. The sequences for negative control were: 5'-UUCUCCGAACGUGUCACG U-3'. The RNAi MAX reagent (Invitrogen Cat. No. 13778150) was used for the transfection of siRNA. The knockdown efficiency was validated via real-time PCR.

Quantitative RT-PCR

Total RNA was extracted from 1×10^6 cells using the RNeasy Plus Mini Kit (Qiagen), and cDNA was synthesized with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems; 01279158) and qPCR was performed using Fast SYBR Green Master Mix (Applied Biosystems; 2608088) and a Bio-Rad CFX96 real-time PCR system (BIO-RAD; HSP9601). The RT-qPCR was performed in triplicate for each of three independent biological replicates. Quantification of mRNA levels was calculated using the comparative CT method. Primers: Gli1, 5'-GTGCACGTTTGAAGGCTGTC-3' and 5'-GAGTGGGTCCGATTCTGGTG-3'; Ptch1, 5'-GAAGCCACAGAAAACCCTGTC-3' and 5'-GCCGCAAGCCTTCTCTACG-3'; Ulk3, 5'-ACGAAACATCTCTCACTTG-3' and 5'-TGCTGGGCAAAGCCAAAGTC-3'; Ulk4, 5'-ATGCAGAGTGTGATTGCGTTG-3' and 5'-GAGTGGCAGTTTCTGTGAACA-3'; Stk36, 5'-CGCATCCTACACCGAGATATGA-3' and 5'-AAATCAAAGTCACAGAGCTTGA-3'; GAPDH, 5'-GTGGTGAAGCAGGCATCTGA-3' and 5'-GCCATGTAGGCCATGAGGTC-3'.

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Declaration of interests

The authors declare no competing interests.

Supplementary Figure legends

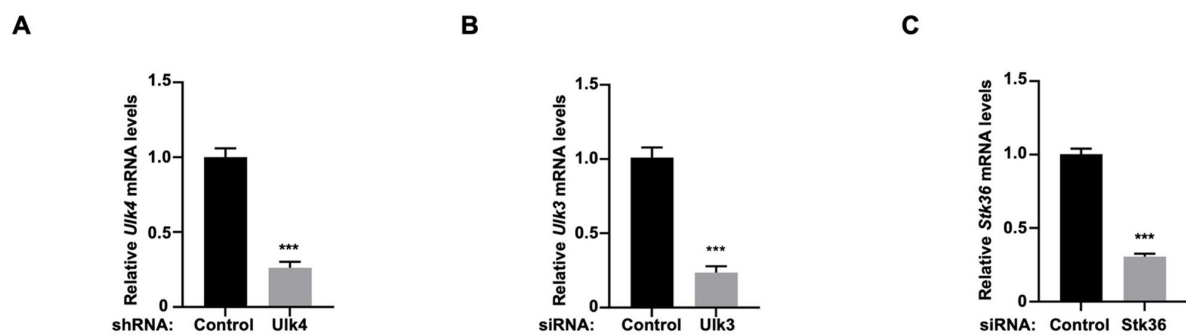


Figure 1- figure supplement 1.

The knockdown efficiency of *Ulk4*, *Ulk3* and *Stk36* in NIH3T3 cells.

(A-C) Relative *Ulk4* (A), *Ulk3* (B), and *Stk36* (C) mRNA levels measured by RT-qPCR in NIH3T3 cells treated with the indicated shRNA or siRNA.

Data are mean $\pm$ SD from three independent experiments. *** $p < 0.001$ (Student's *t* test).

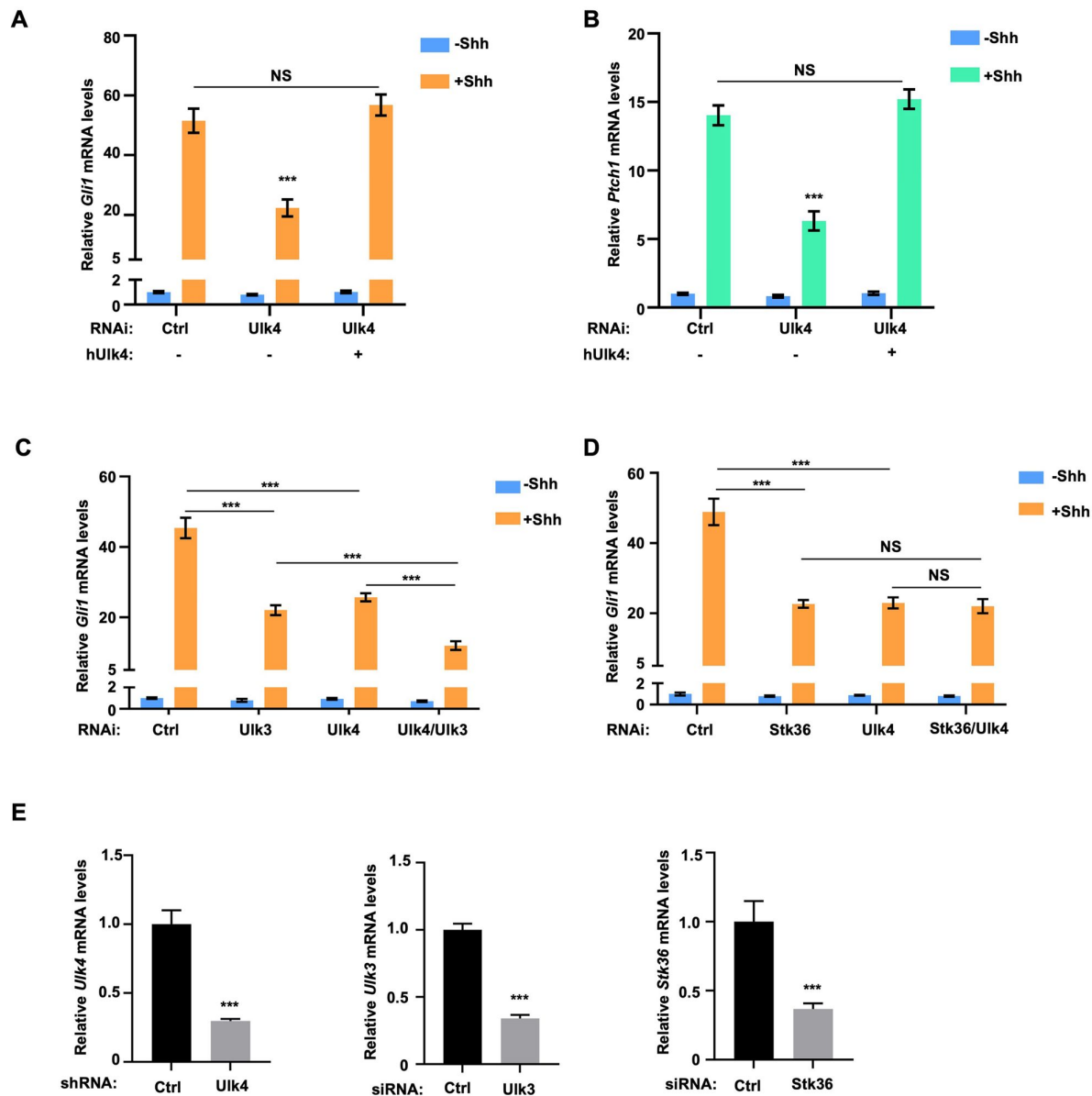


Figure 1- figure supplement 2.

Ulk4 acts in conjunction with Stk36 to regulate Shh-induced expression of *Gli1* and *Ptch1* in MEFs.

(A-B) Relative *Gli1* (A) and *Ptch1* (B) mRNA levels measured by RT-qPCR in MEF cells expressing the indicated shRNA and hUlk4 lentiviral constructs with or without Shh-N treatment.

(C-D) Relative *Gli1* mRNA levels measured by RT-qPCR in MEF cells with the indicated RNAi knockdown in the presence or absence of Shh-N treatment.

(E) Relative *Ulk4*, *Ulk3* and *Stk36* mRNA levels measured by RT-qPCR in MEF cells treated with the indicated shRNA or siRNA. The cells were firstly starved in serum-free medium for 12 hours then cultured in the same medium with or without Shh-N fragment for another 12 hours before they are subjected into RNA preparation. Data are mean $\pm$ SD from three independent experiments. *** $p < 0.001$ (Student's t test).

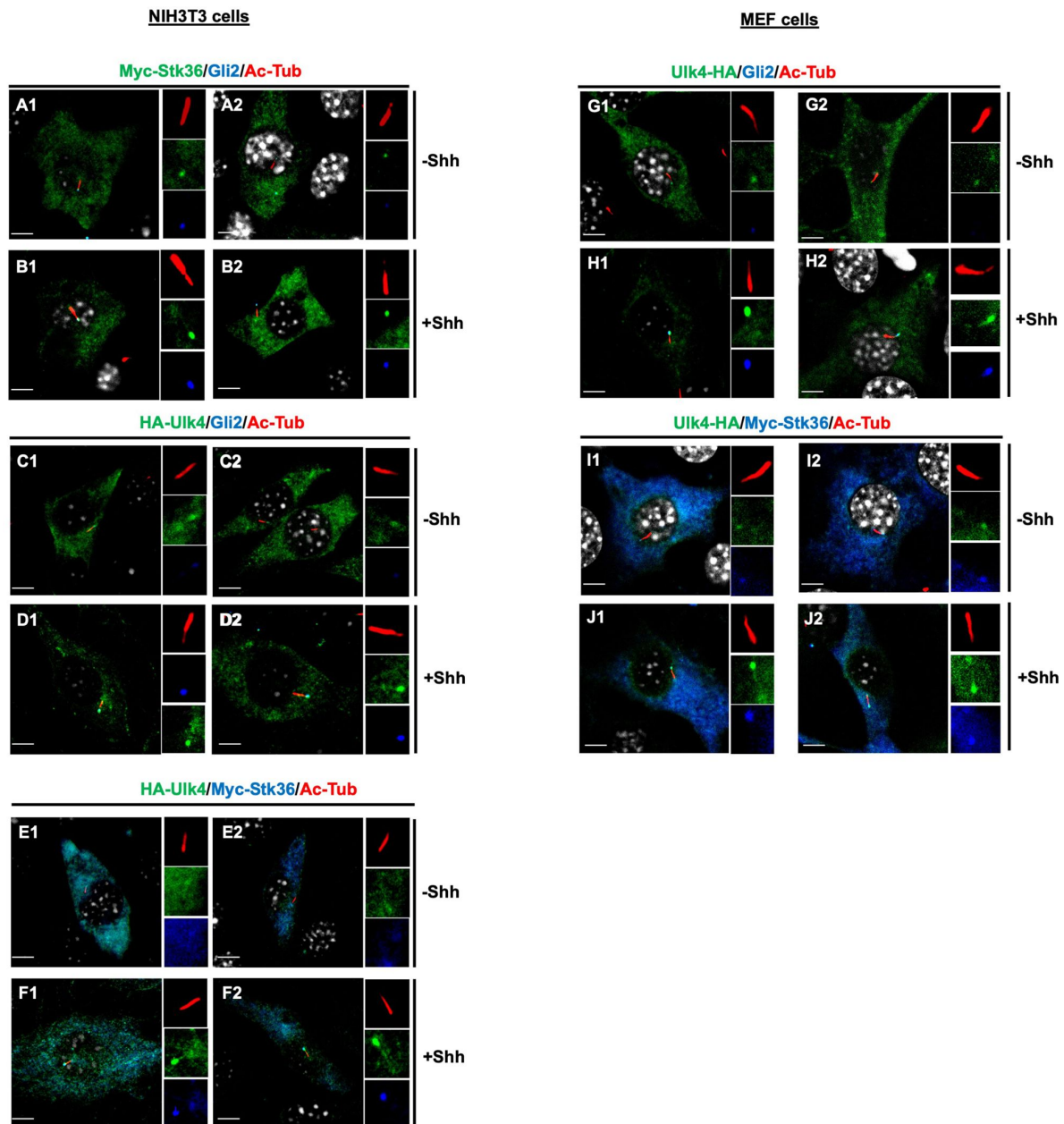


Figure 5- figure supplement 1.

Shh stimulates ciliary tip accumulation of both Ulk4 and Stk36

(A1-D2) Representative images of immunostaining ciliary tip localized Myc-Stk36 (green in A1-B2) or Ulk4-HA (green in C1-D2) and Gli2 (blue) in NIH3T3 cells infected with the Myc-Stk36 or Ulk4-HA lentivirus and treated with or without Shh-N.

(E1-F2) Representative images of immunostaining of ciliary tip localized Myc-Stk36 (blue) and Ulk4-HA (green) in NIH3T3 cells co-infected with Myc-Stk36 and Ulk4-HA lentiviruses and treated with or without Shh-N.

(G1-J2) Representative images of immunostaining of ciliary tip localized Myc-Stk36 (green in G1-H2) and Gli2 (blue in G1-H2) or Ulk4-HA (green in I1-J2) and Myc-Stk36 (blue in I1-J2) in MEFs infected with either Myc-Stk36 (G1-H2) or both Myc-Stk36 and Ulk4-HA lentiviruses and treated with or without Shh-N.

Primary cilia were marked by acetylated tubulin (Ac-tub) staining (red in A1-J2). Insets show the enlarged images of primary cilia with individual channels shown separately. Scale bars are 2 μ M.

References

- Arveseth CD *et al.* (2021) **Smoothened transduces Hedgehog signals via activity-dependent sequestration of PKA catalytic subunits** *PLoS biology* **19**
- Baas AF, Boudeau J, Sapkota GP, Smit L, Medema R, Morrice NA, Alessi DR, Clevers HC (2003) **Activation of the tumour suppressor kinase LKB1 by the STE20-like pseudokinase STRAD** *Embo Journal* **22**:3062–3072
- Briscoe J, Therond PP (2013) **The mechanisms of Hedgehog signalling and its roles in development and disease** *Nat Rev Mol Cell Biol* **14**:418–431
- Chen MH, Gao N, Kawakami T, Chuang PT (2005) **Mice deficient in the fused homolog do not exhibit phenotypes indicative of perturbed hedgehog signaling during embryonic development** *Mol Cell Biol* **25**:7042–7053
- Chen MH, Wilson CW, Li YJ, Law KK, Lu CS, Gacayan R, Zhang X, Hui CC, Chuang PT (2009) **Cilium-independent regulation of Gli protein function by Sufu in Hedgehog signaling is evolutionarily conserved** *Genes Dev* **23**:1910–1928
- Chen Y, Jiang J (2013) **Decoding the phosphorylation code in Hedgehog signal transduction** *Cell Res* **23**:186–200
- Chen Y, Sasai N, Ma G, Yue T, Jia J, Briscoe J, Jiang J (2011) **Sonic Hedgehog dependent phosphorylation by CK1alpha and GRK2 is required for ciliary accumulation and activation of smoothened** *PLoS biology* **9**
- Claret S, Sanial M, Plessis A (2007) **Evidence for a novel feedback loop in the Hedgehog pathway involving Smoothened and Fused** *Curr Biol* **17**:1326–1333
- Cooper AF, Yu KP, Brueckner M, Brailey LL, Johnson L, McGrath JM, Bale AE (2005) **Cardiac and CNS defects in a mouse with targeted disruption of suppressor of fused** *Development* **132**:4407–4417
- Corbit KC, Aanstad P, Singla V, Norman AR, Stainier DY, Reiter JF (2005) **Vertebrate Smoothened functions at the primary cilium** *Nature* **437**:1018–1021
- Dunaeva M, Michelson P, Kogerman P, Toftgard R (2003) **Characterization of the physical interaction of Gli proteins with SUFU proteins** *J Biol Chem* **278**:5116–5122
- Goetz SC, Anderson KV (2010) **The primary cilium: a signalling centre during vertebrate development** *Nat Rev Genet* **11**:331–344
- Han Y, Jiang J (2021) **Expression and purification of fused kinase from insect cells for in vitro kinase assay** *STAR Protoc* **2**
- Han Y, Shi Q, Jiang J (2015) **Multisite interaction with Sufu regulates Ci/Gli activity through distinct mechanisms in Hh signal transduction** *Proc Natl Acad Sci U S A* **112**:6383–6388
- Han Y, Wang B, Cho YS, Zhu J, Wu J, Chen Y, Jiang J (2019) **Phosphorylation of Ci/Gli by Fused Family Kinases Promotes Hedgehog Signaling** *Dev Cell* **50**:610–626

- Haycraft CJ, Banizs B, Aydin-Son Y, Zhang Q, Michaud EJ, Yoder BK (2005) **Gli2 and gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function** *PLoS Genet* **1**
- Jacobsen AV, Murphy JM (2017) **The secret life of kinases: insights into non-catalytic signalling functions from pseudokinases** *Biochemical Society transactions* **45**:665–681
- Jiang J (2002) **Degrading Ci: who is Cul-pable?** *Genes & Dev* **16**:2315–2321
- Jiang J (2021) **Hedgehog signaling mechanism and role in cancer** *Semin Cancer Biol*
- Jiang J, Hui CC (2008) **Hedgehog signaling in development and cancer** *Dev Cell* **15**:801–812
- Kim J, Kato M, Beachy PA (2009) **Gli2 trafficking links Hedgehog-dependent activation of Smoothened in the primary cilium to transcriptional activation in the nucleus** *Proc Natl Acad Sci U S A* **106**:21666–21671
- Knippschild U, Gocht A, Wolff S, Huber N, Lohler J, Stoter M (2005) **The casein kinase 1 family: participation in multiple cellular processes in eukaryotes** *Cellular signalling* **17**:675–689
- Kung JE, Jura N (2019) **Prospects for pharmacological targeting of pseudokinases** *Nat Rev Drug Discov* **18**:501–526
- Lang B *et al.* (2014) **Recurrent deletions of ULK4 in schizophrenia: a gene crucial for neuritogenesis and neuronal motility** *J Cell Sci* **127**:630–640
- Lang B *et al.* (2016) **Control of cortex development by ULK4, a rare risk gene for mental disorders including schizophrenia** *Scientific reports* **6**
- Liu M, Fitzgibbon M, Wang Y, Reilly J, Qian X, O'Brien T, Clapcote S, Shen S, Roche M (2018) **ULK4 regulates GABAergic signaling and anxiety-related behavior** *Transl Psychiatry* **8**
- Liu M, Guan Z, Shen Q, Flinter F, Dominguez L, Ahn JW, Collier DA, O'Brien T, Shen S (2016) **ULK4 Regulates Neural Stem Cell Pool** *Stem Cells* **34**:2318–2331
- Liu M, Guan Z, Shen Q, Lalor P, Fitzgerald U, O'Brien T, Dockery P, Shen S (2016) **ULK4 Is Essential for Ciliogenesis and CSF Flow** *J Neurosci* **36**:7589–7600
- Liu M, Xu P, Guan Z, Qian X, Dockery P, Fitzgerald U, O'Brien T, Shen S (2018) **ULK4 deficiency leads to hypomyelination in mice** *Glia* **66**:175–190
- Liu Y, Cao X, Jiang J, Jia J (2007) **Fused-Costal2 protein complex regulates Hedgehog-induced Smo phosphorylation and cell-surface accumulation** *Genes Dev* **21**:1949–1963
- Luo S, Zheng N, Lang B (2022) **ULK4 in Neurodevelopmental and Neuropsychiatric Disorders** *Front Cell Dev Biol* **10**
- McCoy CJ, Paupelin-Vaucelle H, Gorilak P, Beneke T, Varga V, Gluenz E (2023) **ULK4 and Fused/STK36 interact to mediate assembly of a motile flagellum** *Mol Biol Cell*
- Mecklenburg N *et al.* (2021) **Identification of disease-relevant modulators of the SHH pathway in the developing brain** *Development* **148**

- Merchant M *et al.* (2005) **Loss of the serine/threonine kinase fused results in postnatal growth defects and lethality due to progressive hydrocephalus** *Mol Cell Biol* **25**:7054–7068
- Murone M, Luoh SM, Stone D, Li W, Gurney A, Armanini M, Grey C, Rosenthal A, de Sauvage FJ (2000) **Gli regulation by the opposing activities of fused and suppressor of fused** *Nat Cell Biol* **2**:310–312
- Nozawa YI, Yao E, Lin C, Yang JH, Wilson CW, Gacayan R, Chuang PT (2013) **Fused (Stk36) is a ciliary protein required for central pair assembly and motile cilia orientation in the mammalian oviduct** *Dev Dyn* **242**:1307–1319
- Pongrakhananon V, Saito H, Hiver S, Abe T, Shioi G, Meng W, Takeichi M (2018) **CAMSAP3 maintains neuronal polarity through regulation of microtubule stability** *Proceedings of the National Academy of Sciences* **115**:9750–9755
- Preuss F, Chatterjee D, Mathea S, Shrestha S, St-Germain J, Saha M, Kannan N, Raught B, Rottapel R, Knapp S (2020) **Nucleotide Binding, Evolutionary Insights, and Interaction Partners of the Pseudokinase Unc-51-like Kinase 4** *Structure* **28**:1184–1196
- Rohatgi R, Milenkovic L, Scott MP (2007) **Patched1 regulates hedgehog signaling at the primary cilium** *Science* **317**:372–376
- Shi Q, Li S, Jia J, Jiang J (2011) **The Hedgehog-induced Smoothed conformational switch assembles a signaling complex that activates Fused by promoting its dimerization and phosphorylation** *Development* **138**:4219–4231
- Tempe D, Casas M, Karaz S, Blanchet-Tournier MF, Concordet JP (2006) **Multisite protein kinase A and glycogen synthase kinase 3 β phosphorylation leads to Gli3 ubiquitination by SCF β TrCP** *Mol Cell Biol* **26**:4316–4326
- Tukachinsky H, Lopez LV, Salic A (2010) **A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes** *Journal Of Cell Biology* **191**:415–428
- Villavicencio EH, Walterhouse DO, Iannaccone PM (2000) **The sonic hedgehog-patched-gli pathway in human development and disease** *Am J Hum Genet* **67**:1047–1054
- Vogel P, Read RW, Hansen GM, Payne BJ, Small D, Sands AT, Zambrowicz BP (2012) **Congenital hydrocephalus in genetically engineered mice** *Vet Pathol* **49**:166–181
- Wang B, Fallon JF, Beachy PA (2000) **Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb** *Cell* **100**:423–434
- Wang B, Li Y (2006) **Evidence for the direct involvement of { β }TrCP in Gli3 protein processing** *Proc Natl Acad Sci U S A* **103**:33–38
- Wilson CW, Nguyen CT, Chen MH, Yang JH, Gacayan R, Huang J, Chen JN, Chuang PT (2009) **Fused has evolved divergent roles in vertebrate Hedgehog signalling and motile ciliogenesis** *Nature* **459**:98–102
- Zachari M, Ganley IG (2017) **The mammalian ULK1 complex and autophagy initiation** *Essays Biochem* **61**:585–596

Zhang Y, Mao F, Lu Y, Wu W, Zhang L, Zhao Y (2011) **Transduction of the Hedgehog signal through the dimerization of Fused and the nuclear translocation of Cubitus interruptus** *Cell Res* **21**:1436–1451

Zhou M, Han Y, Wang B, Cho YS, Jiang J (2022) **Dose-dependent phosphorylation and activation of Hh pathway transcription factors** *Life Sci Alliance* **5**

Zhou M, Jiang J (2022) **Gli Phosphorylation Code in Hedgehog Signal Transduction** *Front Cell Dev Biol* **10**

Zhou Q, Kalderon D (2011) **Hedgehog Activates Fused through Phosphorylation to Elicit a Full Spectrum of Pathway Responses** *Dev Cell* **20**:802–814

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Editors

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Reviewer #1 (Public Review):

In their study, Zhou et al. unveil the pivotal role of ULK4 in conjunction with STK36, shedding light on their collective impact on GLI2 phosphorylation and the subsequent activation of the SHH pathway. The research delves deep into the intricate interactions between ULK4 and various components of the SHH pathway within the primary cilium.

The main strength of the study lies in the careful and systematic sequence of logical methods. The authors apply the expression of a range of different deletion and mutation constructs and carry out a comprehensive biochemical study of the consequences of depletion and reintroduction of various components in the context of STK36 and ULK4.

Their findings reveal that ULK4 forms dynamic interactions with a complex composed of STK36 and GLI2. It is proposed that ULK4 acts as a scaffold, facilitating the essential interaction between STK36 and GLI2, thereby driving GLI2 phosphorylation by STK36. Notably, the research reveals that the N-terminal pseudokinase domain of ULK4 binds to Stk36, while the C-terminal regulatory domain of ULK4 interacts with Gli2. Moreover, the study presents compelling evidence for co-localization of ULK4 and STK36 with GLI2 at the ciliary tip within NIH 3T3 cells. Importantly, ULK4 and STK36 mutually rely on each other for their accumulation at this ciliary tip.

This intricate mechanism, orchestrated by ULK4, brings to light the nuanced modulation of the SHH pathway. The research is substantiated by rigorous Co-IP experiments, kinase assays, and confocal imaging localization studies. To unravel the fine details of GLI2 phosphorylation at the primary cilium tip, the authors meticulously employ a diverse array of mutated and wild-type constructs of STK36 and ULK4.

In summary, the study provides compelling insights into the intricate regulation of signaling pathways. Zhou et al.'s work on ULK4 and STK36 in the SHH pathway deepens our understanding of these complex processes, offering potential avenues for drug development, particularly in the context of cancer therapeutics.

Reviewer #2 (Public Review):

The authors provide solid molecular and cellular evidence that ULK4 and STK36 not only interact, but that STK36 is targeted (transported?) to the cilium by ULK4. Their data helps generate a model for ULK4 acting as a scaffold for both STK36 and its substrate, Gli2, which appear to co-localise through mutual binding to ULK4. This makes sense, given the proposed role of most pseudokinases as non-catalytic signaling hubs. There is also an important mechanistic analysis performed, in which ULK4 phosphorylation in an acidic consensus by STK36 is demonstrated using IP'd STK36 or an inactive 'AA' mutant, which suggests this phosphorylation is direct.

The major strength of the study is the well-executed combination of logical approaches taken, including expression of various deletion and mutation constructs and the careful (but not always quantified in immunoblot) effects of depleting and adding back various components in the context of both STK36 and ULK3, which broadens the potential impact of the work. The biochemical analysis of ULK4 phosphorylation appears to be solid, and the mutational study at a particular pair of phosphorylation sites upstream of an acidic residue (notably T2023) is further strong evidence of a functional interaction between ULK4/STK36. The possibility that ULK4 requires ATP binding for these mechanisms is not approached, though would provide significant insight: for example it would be useful to ask if Lys39 in ULK4 is involved in any of these processes, because this residue is likely important for shaping the ULK4 substrate-binding site as a consequence of ATP binding; this was originally shown in PMID 24107129 and discussed more recently in PMID: 33147475 in the context of the large amount of ULK4 proteomics data released.

The discussion is excellent, and raises numerous important future work in terms of potential transportation mechanisms of this complex. It also explains why the ULK4 pseudokinase domain is linked to an extended C-terminal region. Does AF2 predict any structural motifs in this region that might support binding to Gli2?

A weakness in the study, which is most evident in Figure 1, where Ulk4 siRNA is performed in the NIH3T3 model (and effects on Shh targets and Gli2 phosphorylation assessed), is that we do not know if ULK4 protein is originally present in these cells in order to actually be depleted. Also, we are not informed if the ULK4 siRNA has an effect on the 'rescue' by HA-ULK4; perhaps the HA-ULK4 plasmid is RNAi resistant, or if not, this explains why phosphorylation of Gli2 never reaches zero? Given the important findings of this study, it would be useful for the authors to comment on this, and perhaps discuss if they have tried to evaluate endogenous levels of ULK4 (and Stk36) in these cells using antibody-based approaches, ideally in the presence and absence of Shh. The authors note early on the large number of binding partners identified for ULK4, and siRNA may unwittingly deplete some other proteins that could also be involved in ULK4 transport/stability in their cellular model.

The sequence of ULK4 siRNAs is not included in the materials and methods as far as I can see, though this is corrected in the next version of the manuscript.

Reviewer #3 (Public Review):

In this manuscript, Zhou et al. demonstrate that the pseudokinase ULK4 has an important role in Hedgehog signaling by scaffolding the active kinase Stk36 and the transcription factor Gli2, enabling Gli2 to be phosphorylated and activated.

Through nice biochemistry experiments, they show convincingly that the N-terminal pseudokinase domain of ULK4 binds Stk36 and the C-terminal Heat repeats bind Gli2.

Lastly, they show that upon Sonic Hedgehog signaling, ULK4 localizes to the cilia and is needed to localize Stk36 and Gli2 for proper activation.

This manuscript is very solid and methodically shows the role of ULK4 and STK36 throughout the whole paper, with well controlled experiments. The phosphomimetic and incapable mutations are very convincing as well.

I think this manuscript is strong and stands as is, and there is no need for additional experiments.

Overall, the strengths are the rigor of the methods, and the convincing case they bring for the formation of the ULK4-Gli2-Stk36 complex. There are no weaknesses noted. I think a little additional context for what is being observed in the immunofluorescence might benefit readers who are not familiar with these cell types and structures.

The revised manuscript has improved some of the unclear areas.

Author Response

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

The Hedgehog (HH) protein family is important for embryonic development and adult tissue maintenance. Deregulation or even temporal imbalances in the activity of one of the main players in the HH field, sonic hedgehog (SHH), can lead to a variety of human diseases, ranging from congenital brain disorders to diverse forms of cancers. SHH activates the GLI family of transcription factors, yet the mechanisms underlying GLI activation remain poorly understood. Modification and activation of one of the main SHH signalling mediators, GLI2, depends on its localization to the tip of the primary cilium. In

a previous study the lab had provided evidence that SHH activates GLI2 by stimulating its phosphorylation on conserved sites through Unc-51-like kinase 3 (ULK3) and another ULK family member, STK36 (Han et al., 2019). Recently, another ULK family member, ULK4, was identified as a modulator of the SHH pathway (Mecklenburg et al. 2021). However, the underlying mechanisms by which ULK4 enhances SHH signalling remained unknown. To address this question, the authors employed complex biochemistry-based approaches and localization studies in cell culture to examine the mode of ULK4 activity in the primary cilium in response to SHH. The study by Zhou et al. demonstrates that ULK4, in conjunction with STK36, promotes GLI2 phosphorylation and thereby SHH pathway activation. Further experiments were conducted to investigate how ULK4 interacts with SHH pathway components in the primary cilium. The authors show that ULK4 interacts with a complex formed between STK36 and GLI2 and hypothesize that ULK4 functions as a scaffold to facilitate STK36 and GLI2 interaction and thereby GLI2 phosphorylation by STK36. Furthermore, the authors provide evidence that ULK4 and STK36 co-localize with GLI2 at the ciliary tip of NIH 3T3 cells, and that ULK4 and STK36 depend on each other for their ciliary tip accumulation. Overall, the described ULK4-mediated mechanism of SHH pathway modulation is based on detailed and rigorous Co-IP experiments and kinase assays as well as confocal imaging localization studies. The authors used various mutated and wild-type constructs of STK36 and ULK4 to decipher the mechanisms underlying GLI2 phosphorylation at the tip of the primary cilium. These novel results on SHH pathway activation add valuable insight into the complexity of SHH pathway regulation. The data also provide possible new strategies for interfering with SHH signalling which has implications in drug development (e.g., cancer drugs).

However, it will be necessary to explore additional model systems, besides NIH3T3, HEK293 and MEF cell cultures, to conclude on the universality of the mechanisms described in this study. Ultimately, it needs to be addressed whether ULK4 modulates SHH pathway activity in vivo. Is there evidence that genetic ablation of ULK4 in animal models leads to less efficient SHH pathway induction? It also remains to be resolved how ULK3 and ULK4 act in distinct or common manners to promote SHH signalling. Another remaining question is, whether cell type- and tissue-specific features exist, that play a role in ULK3- versus ULK4-dependent SHH pathway modulation. In particular for the studies on ciliary tip localization of factors, relevant for SHH pathway transduction, a higher temporal resolution will be needed in the future as well as a deeper insight into tissue/ cell type-specific mechanisms. These caveats, mentioned here, don't have to be addressed in new experiments for the revision of this manuscript but could be discussed.

We agree with the reviewer that it would be important to investigate in the future the in vivo function Ulk4 in Shh signaling, the relationship between Ulk3 and Ulk4/Stk36, and possible cell type/tissue specificity of these two kinase systems. This will need the generation of single and double knockout mice and examine Hh related phenotypes in different tissues and developmental stages. The precise mechanism by which Ulk4 and Stk36 are translocated to the ciliary tip is also an important and unsolved issue. We include several paragraphs in the “discussion” section to address these outstanding questions for future study.

Reviewer #2 (Public Review):

The authors provide solid molecular and cellular evidence that ULK4 and STK36 not only interact, but that STK36 is targeted (transported?) to the cilium by ULK4. Their data helps generate a model for ULK4 acting as a scaffold for both STK36 and its substrate, Gli2, which appear to co-localise through mutual binding to ULK4. This makes sense, given the proposed role of most pseudokinases as non-catalytic signaling hubs. There is also an important mechanistic analysis performed, in which ULK4 phosphorylation in an acidic consensus by STK36 is demonstrated using IP'd STK36 or an inactive 'AA' mutant, which suggests this phosphorylation is direct.

The major strength of the study is the well-executed combination of logical approaches taken, including expression of various deletion and mutation constructs and the careful (but not always quantified in immunoblot) effects of depleting and adding back various components in the context of both STK36 and ULK3, which broadens the potential impact of the work. The biochemical analysis of ULK4 phosphorylation appears to be solid, and the mutational study at a particular pair of phosphorylation sites upstream of an acidic residue (notably T2023) is further strong evidence of a functional interaction between ULK4/STK36. The possibility that ULK4 requires ATP binding for these mechanisms is not approached, though would provide significant insight: for example it would be useful to ask if Lys39 in ULK4 is involved in any of these processes, because this residue is likely important for shaping the ULK4 substrate-binding site as a consequence of ATP binding; this was originally shown in PMID 24107129 and discussed more recently in PMID: 33147475 in the context of the large amount of ULK4 proteomics data released.

The reviewer raised an interesting question of whether ATP binding to the pseudokinase domain of Ulk4 might be required for its function, i.e., by regulating the interaction with its binding partner. In a recent study (Preuss et al. 2020; PMID: 33147475), the critical Lys39 for ATP binding was converted to Arg (KR mutation); however, unlike in most kinases the KR mutation affect ATP binding, the K39R mutation in the Ulk4 pseudokinase did not affect ATP binding although it slightly increased ADP binding (PMID: 33147475). Another mutation made by Preuss et al (PMID: 33147475), N239L, affected protein stability, making it impossible to determine whether this mutation affect ATP binding. Therefore, in the absence of clear approach to perturb ATP binding without affecting the overall structure of Ulk4, it would be challenging to address whether ATP binding regulates the ability of Ulk4 to bind its substrates. Nevertheless, we discuss the possibility that ATP binding might regulate Ulk4/Stk36 interaction and Shh signaling.

The discussion is excellent, and raises numerous important future work in terms of potential transportation mechanisms of this complex. It also explains why the ULK4 pseudokinase domain is linked to an extended C-terminal region. Does AF2 predict any structural motifs in this region that might support binding to Gli2?

The extended C-terminal domain of Ulk4 contains Arm/HEAT repeats (protein-protein interacting domain), which are predicted by AF2 to form alpha helixes.

A weakness in the study, which is most evident in Figure 1, where Ulk4 siRNA is performed in the NIH3T3 model (and effects on Shh targets and Gli2 phosphorylation assessed), is that we do not know if ULK4 protein is originally present in these cells in order to actually be depleted. Also, we are not informed if the ULK4 siRNA has an effect on the 'rescue' by HA-ULK4; perhaps the HA-ULK4 plasmid is RNAi resistant, or if not, this explains why phosphorylation of Gli2 never reaches zero? Given the important findings of this study, it would be useful for the authors to comment on this, and perhaps discuss if they have tried to evaluate endogenous levels of ULK4 (and Stk36) in these cells using antibody-based approaches, ideally in the presence and absence of Shh. The authors note early on the large number of binding partners identified for ULK4, and siRNA may unwittingly deplete some other proteins that could also be involved in ULK4 transport/stability in their cellular model.

Due to the lack of reliable Ulk4 and Stk36 antibodies, we were unable to confirm knockdown efficiency by western blot analysis. Therefore, we relied on the measure Ulk4 and STK36 mRNA expression by RT-qPCR to estimate the knockdown efficiency (Fig 1- figure supplement 1). We used mouse Ulk4 shRNA to carry out the knockdown experiments in NIH3T3 and MEF cells while the human version of Ulk4 (hUlk4) was used for the rescue experiments (Fig 1-

figure supplement 2; Fig. 8). We have confirmed that the mULK4 shRNA targeting sequence is not conserved in hULK4; therefore, the hULK4 construct is RNAi resistant. The rescue experiments strongly argue that the effect of Ulk4 RNAi on Shh signaling is due to loss of endogenous Ulk4. This argument is further strengthened by the observations that mutations that affected Ulk4 and Stk36 ciliary tip localization also affected Shh signaling such as Gli2 phosphorylation and Ptch1/Gli expression (Fig. 8).

The sequence of ULK4 siRNAs is not included in the materials and methods as far as I can see.

We have added the mouse Ulk4 RNAi target sequence in the revised version.

Reviewer #3 (Public Review):

In this manuscript, Zhou et al. demonstrate that the pseudokinase ULK4 has an important role in Hedgehog signaling by scaffolding the active kinase Stk36 and the transcription factor Gli2, enabling Gli2 to be phosphorylated and activated.

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Overall, the strengths are the rigor of the methods, and the convincing case they bring for the formation of the ULK4-Gli2-Stk36 complex. There are no weaknesses noted. I think a little additional context for what is being observed in the immunofluorescence might benefit readers who are not familiar with these cell types and structures.

We thank this reviewer for the positive comments.

Recommendations For the Authors

Reviewer #1 (Recommendations For The Authors):

This elegant study has been thoroughly and thoughtfully designed and the dataset is solid. The biochemistry results are overall very convincing. Some data lack quantification and there needs to be more information on data analyses and statistics. The following suggestions and comments aim at strengthening the manuscript.

- 1. Please provide quantification normalized to input for IP experiments (Figures 1 E - F; Figure 8 C). More information on data analyses and statistics should be provided and included as information in the figure legends.*

Thanks for the suggestions, we have done the quantification and statistics analyses for Figures 1E-G and Figure 8 C as requested.

- 1. Did the authors investigate whether overexpressing hULK4 in the control NIH3T3 cells leads to an increase in pS230/232 (related to Figure 1E)? This would nicely support the notion of a promoting effect of ULK4 on GLI2 phosphorylation.*

We did not. We speculated that overexpressing hULK4 may not significantly promote GLI2 phosphorylation because Ulk4 is a pseudokinase and endogenous Stk36 (the kinase partner of Ulk4) is limited.

1. The CO-IP experiments to show GLI2 activation were performed in NIH3T3 cells, whereas HEK293 cells were used for the experiments shown in Figure 2. Is there a specific reason for switching between cell lines also for experiments shown in Figures 3 C-I? Did the authors repeat some of the key experiments in both cell lines?

In mammalian cells, Shh-induced activation of GLI2 depends on primary cilia (Han et al., 2019). NIH3T3 cells form the primary cilia but HEK293T cells do not. Therefore, we used NIH3T3 cells to examine the processes that are regulated by the Shh treatment assay (e.g., the Shh-induced phosphorylation of GLI2 and STK36). The HEK293 cells were used to map binding domain between ULK4 and STK36/GLI2/SUFU due to the high transfection efficiency.

1. In Figure 2 D-E the authors nicely showed that hUlk4N-HA interacted with CFP-Stk36 but not with Myc-Gli2/Fg-Sufu whereas hUlk4C-HA formed a complex with Myc-Gli2/Fg-Sufu but not with CFP-Stk36. In Figure 4E the authors showed in their Co-IP experiments that Fg-Stk36 and Myc-Gli2 form a complex independent of SHH treatment. Did the authors see some pull down of Stk36, still in complex with Gli2, using hUlk4C IP and pull down of Gli2, still in complex with Stk36, using hUlk4N IP?

We did not test that. As we have shown in Figures 4A and 4E, knockdown of endogenous ULK4 nearly abolished the interaction between Myc-GLI2 and Fg-Stk36, suggesting that Ulk4 is the major scaffold to bring Stk36 and Gli2 together, and that there is little if any direct interaction between GLI2 and Stk36.

1. Another method to verify hULK4-Stk36-Gli2 complex formation (Figure 4) would be helpful. For example, proximity ligation assays, tripartite split GFP assays, or colocalization based on expansion STED immunofluorescence microscopy could be performed to temporally and spatially resolve localization of Ulk4, Stk36 and Gli2 upon SHH stimulation in the primary cilium

Thanks for the suggestions. We think that our current study using biochemical and cell biology approaches have provide sufficient evidence that Ulk4, Stk36 and Gli2 form complexes. We will keep in mind of those more sophisticated methods in our future endeavors.

1. Please provide more representative images of Ulk4, Stk36 and Gli2 localization in NIH3T3 cells or lower magnification overview images showing more than one cell (Figure 5).

We have provided more representative images in Figure 5- figure supplement 1A-F of the revised manuscript.

1. Confirmation of the results shown in Figure 5 in a second cell line would strengthen the data.

We have confirmed the results in MEFs (see Figure 5- figure supplement 1G-J)

1. Did the authors add immunofluorescence for tubulin as a ciliary base marker to ensure correct assignment of ciliary tip versus ciliary base localization for quantification experiments (Figures 5 - 8)?

It has been well documented that GLi2 is accumulated at the ciliary tip in response to Shh treatment; therefore, we used Gli2 as a marker for ciliary tip where both Ulk4 and Stk36 were also accumulated. γ tubulin staining could be another marker to assign the ciliary tip vs base; however, the antibody combination we have did not allow us to simultaneously stain γ tubulin and acetylated tubulin (Ac-Tub).

1. SMO localization as a further readout of SHH pathway activation might be considered to be added for some of the key results (e.g., Figure 6). Is SMO trafficking affected after depletion or overexpression of ULK4?

Due to the lack of a workable antibody to detect endogenous Smo in our hands, we did not determine whether the trafficking of SMO is affected after depletion or overexpression of ULK4. However, we noticed that a recent study reported that the SHH-induced ciliary SMO accumulation was impaired in Ulk4 siRNA treated cells (Mecklenburg et al. 2021). We include this information and its implication in the discussion section

1. Do the authors see ULK4 only at the ciliary tip after SHH stimulation or is there also a dynamic time-dependent localization along the ciliary shaft? The image in Figure 6E (dKO + Stk36 WT) seems to show ULK4 also in the shaft.

Unlike Smo that is evenly distributed along the axoneme of primary cilia, ULK4 is mainly accumulated at ciliary tips upon Shh stimulation. Ulk4 is also located at low levels outside the cilia and sometimes in the ciliary shaft during its transit to the ciliary tip (e.g., see Figure 5-figure supplement 1F1-2; J1-2).

1. Is the immunofluorescence signal for Ulk4 significantly reduced after shRNA treatment to deplete Ulk4 (Figure 6A)?

We constructed a cell line that stably expressed ULK4 shRNA. The knockdown efficiency was determined by measuring Ulk4 mRNA expression (Fig 1-figure supplement 1). Because we were unable to obtain a reliable ULK4 antibody for immunostaining, we did not examine by whether ULK4 signal was depleted by Ulk4 shRNA.

1. The labelled ciliary tip resembles in some cases images seen for ciliary abscission. The authors could use membrane/ciliary membrane markers to ensure "intraciliary" localization of the investigated factors.

Thanks for the suggestion. We will try that in our future experiments.

1. How many replicates were used in the three independent quantitative RT-PCR experiments (Figure 1 A-D)?

We used 3 replicates in each independent quantitative RT-PCR assay.

1. Please provide p values or statement on no significance for the comparison between *Ulk3* single and *Ulk3/Ulk4* double knockdown (Figure 1C) and between *Stk36* single and *Stk36/Ulk4* double knockdown (Figure 1D; Fig1_Figure Supplement 2).

Thanks for the suggestion, we have added the p value or “ns” as asked.

1. Figure legends in general are a bit short could have some more detailed information.

Thank you for the suggestion, we have revised the Figure legends as asked.

1. What do the asterisks present in Figure 4 C-D?

Thanks for the suggestion. The asterisks in Figure 4C-D indicated the full length STK36 and truncated form STK36N and STK36C fragments. We that included this information in the figure legend.

1. The authors state that a previous study described ULK4 as a genetic modifier for holoprosencephaly and that this raised the possibility that ULK4 may participate in HH signal transduction. Primary ciliary localization of ULK4 in mouse neuronal tissue and SHH pathway modulation by ULK4 in cell culture have been shown by Mecklenburg et al. 2021 before. Maybe the authors could rephrase their introduction and discussion accordingly.

Thanks for the suggestion, we have changed the introduction and discussion accordingly.

1. Overexpression studies in heterologous systems using tagged proteins can potentially have an influence on their subcellular localization and function. Please discuss this caveat.

We have mentioned this caveat in the “discussion” section of the revised manuscript. However, we have tried to express the transgene at low levels using the lentiviral vector containing a weak promoter to ensure that the exogenously expressed proteins are still regulated by Hh signaling. We have also confirmed that the tagged *Ulk4* and *Stk36* can rescue the loss of endogenous genes.

1. More details in the Methods section should be provided on the SHH induction in NIH3T3 cells, HEK293 cells and MEFs.

We have revised the methods section on Shh induction.

1. ULK4 is known to have at least three isoforms that exhibit varying abundance across developmental stages in mice and humans (Lang et al., 2014) (DOI:10.1242/jcs.137604). Can the authors speculate on potential common and distinct functions of the different ULK4 isoforms on SHH pathway modulation based on their present results?

It is interesting that *Ulk4* has multiple isoforms in both mouse and human. Several short isoforms in both mouse and human lack the pseudokinase domain while one short isoform in mouse lacks the C-terminal region essential for *Ulk4* ciliary tip localization. We speculate that the C-terminally deleted isoform may not have a function in the Shh pathway based on our results shown in Fig. 7 and 8 but might still have functions in other cellular processes.

Reviewer #2 (Recommendations For The Authors):

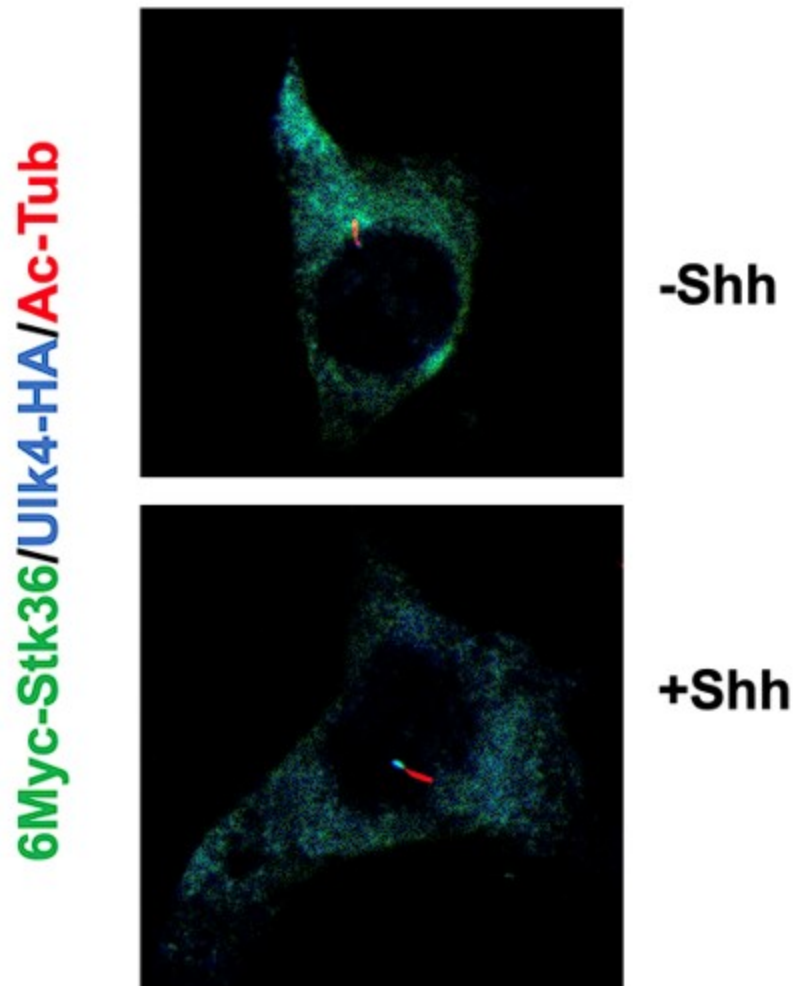
The paper is well written, and clear throughout, with excellent (up-to-date) citations to the field.

We thank reviewer #2 for the positive comments.

Reviewer #3 (Recommendations For The Authors):

My only quibble is that the immunofluorescence images are a little confusing, especially to people outside of the field. Please include an image of the whole field and improve the captions. Is that a single cell for each cilium? Why are there so few cilia? The DAPI makes it seem like What are we looking at? Are those multiple nuclei in Figure 6? They seem a little small if that's the 5 μ M scale bar

We provide uncropped images of Figure 5E to show the entire cells (below). We have added some context to improve the captions. Most of the mammalian cells such as MEF and NIH3T3 cells contain a single primary cilium; however, mutilated cells do exist. The DAPI staining indicated the nuclei. The cells shown in Figure 6 have single nucleus (the scale should be 2 μ M). Due to the unevenness of DAPI signals in the nuclei, only the strong signals (puncta) were shown for individual nuclei.



| One small typo: GLL2 instead of GLI2 on line 363

Thanks, we have corrected this spelling mistake.