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

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Running title: Ulk4 promotes Gli phosphorylation and Hh signaling

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.

Introduction

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 additional 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

70 and that Ulk4 knockdown affected Shh induced *Gli-luc* reporter gene expression and
71 Smoothened (Smo) ciliary localization (Mecklenburg et al. 2021). These observations
72 have raised an interesting possibility that Ulk4 may participate in Hh signal transduction.
73 The Hh family of secreted proteins governs embryonic development and adult tissue
74 homeostasis and deregulation of Hh signaling activity has been implicated in a myriad
75 of human disorders including birth defect and cancer (Villavicencio et al. 2000; Jiang
76 and Hui 2008; Briscoe and Therond 2013; Jiang 2021). Hh exerts its biological influence
77 via an evolutionarily conserved signaling cascade that culminates in the activation of Gli
78 family of Zn-finger transcription factors. The core Hh signal reception system consists of
79 two multi-span transmembrane proteins Patched1 (Ptch1) and Smoothened (Smo that
80 transduce vertebrate Hh signal at primary cilia (Goetz and Anderson 2010). In quiescent
81 cells, Ptch1 localizes to primary cilia where Smo is barely detectable (Corbit et al. 2005;
82 Rohatgi et al. 2007); the full-length Gli (Gli^F) undergoes ubiquitin/proteasome-mediated
83 proteolytic processing to generate a truncated transcriptional repressor form (Gli^R)
84 (Wang et al. 2000; Jiang 2002; Tempe et al. 2006; Wang and Li 2006; Chen and Jiang
85 2013). Hh inhibits Ptch1 activity and promotes its ciliary exit, allowing Smo and Gli
86 proteins to accumulate in the cilia to transduce the Hh signal (Corbit et al. 2005;
87 Haycraft et al. 2005; Rohatgi et al. 2007; Chen et al. 2009; Tukachinsky et al. 2010;
88 Chen et al. 2011). Activated Smo blocks the proteolytic processing of Gli into Gli^R and
89 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 liner pathway with Stk36 but in parallel with Ulk3, double KD of Ulk4 and Stk36 decreased S230/232

138 phosphorylation similarly to single KD of either Ulk4 or Stk36 whereas double KD of
139 Ulk3 and Ulk4 further decreased Shh-induced S230/232 phosphorylation compared with
140 single KD of Ulk3 or Ulk4 (Figure 1F-G'). Taken together, these results demonstrated
141 that Ulk4 and Stk36 act in a linear pathway but in parallel with Ulk3 to participate in Hh
142 transduction by facilitating Gli2 phosphorylation and activation (Figure 1H).
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145 **Ulk4 forms a complex with Stk36 and Gli2**
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147
148 A previous study using proximity-dependent biotin identification (BioID) identified Stk36
149 as a proximity interactor (Preuss et al. 2020). To verify that Stk36 formed a complex
150 with Ulk4, we carried out coimmunoprecipitation (Co-IP) experiments and found that a
151 C-terminal HA-tagged Ulk4 (hUlk4-HA) interacted with a N-terminal CFP-tagged Stk36
152 (CFP-Stk36) in HEK293T cells (Figure 2A). To explore whether Ulk4 interacted with Gli2
153 and Sufu, a Gli binding partner and Hh pathway inhibitor (Dunaeva et al. 2003; Cooper
154 et al. 2005; Han et al. 2015), we co-expressed hUlk4-HA, with a Myc-tagged Gli2 (Myc-
155 Gli2) and a Flag-tagged Sufu (Fg-Sufu) in HEK293T cells. Co-IP experiments showed
156 that both Myc-Gli2 and Fg-Sufu were pulled down by Ulk4-HA (Figure 2B), suggesting
157 that Ulk4 could form a complex with Stk36 and Gli2-Sufu.
158 Ulk4 contains an N-terminal pseudokinase domain and the C-terminal regulatory
159 domain with armadillo (Arm) repeats (Figure 2C) (Preuss et al. 2020). We generated
160 two truncated forms of Ulk4: hUlk4N (aa 1-450) that contains the pseudokinase domain
161 and hUlk4C (451-1275) that contains the C-terminal regulatory domain. Co-IP
162 experiments showed that hUlk4N-HA interacted with CFP-Stk36 but not with Myc-
163 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 STRADα and STARADβ (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

189 possibility that Stk36 could be activated by Shh through a similar mechanism. Indeed,
190 we found that Shh induced phosphorylation of Stk36 on T158/S159 in NIH-3T3 cells as
191 determined by a phospho-specific antibody that recognized pT158/pS159 (Figure. 3C)
192 (Shi et al. 2011). To determine whether Shh-induced T158/S159 phosphorylation
193 depends on Stk36 kinase activity, we introduced either wild type Stk36 (CFP-Stk36^{WT})
194 or its kinase dead form (CFP-Stk36^{KR}) into NIH3T3 cells with endogenous Stk36 and
195 Ulk3 deleted (*Ulk3^{KO} Stk36^{KO}*) by CRISPR (Han et al. 2019). As shown in Figure 3D,
196 Shh induced phosphorylation of CFP-Stk36^{WT} but not CFP-Stk36^{KR} on T158/S159,
197 suggesting that Shh promotes the phosphorylation of Stk36 AL via its kinase activity,
198 likely through trans-autophosphorylation as are the cases for other kinases. Hence,
199 phosphorylation of T158/S159 can be used as a readout for Stk36 kinase activation.
200 To determine whether AL phosphorylation promotes Stk36 kinase activation, we
201 converted both S151 and T154 to E (EE) to mimic phosphorylation or to A (AA) to block
202 phosphorylation on these sites (Figure 3B). We found that the phospho-mimetic
203 mutation (Stk36^{EE}) increased whereas phospho-deficient mutation (Stk36^{AA}) abolished
204 Stk36 kinase activity as determined by both Stk36 T158/S159 phosphorylation and Gli2
205 S230/S232 phosphorylation (Figure3 E-H), which is similar to what we have observed
206 previously for Fu (Shi et al. 2011), suggesting that Hh stimulates kinase activation of
207 Stk36 and Fu through a similar mechanism.
208 To determine whether Ulk4 is required for Shh-induced Stk36 kinase activation,
209 we employed RNAi to deplete Ulk4 from NIH3T3 cells that express CFP-Stk36. As
210 shown in Figure 3I, depletion of Ulk4 did not affect Shh-induced phosphorylation of

211 CFP-Stk36 on T158/S159, suggesting that Ulk4 is not required for Stk36 kinase
212 activation in response to Shh stimulation.
213
214
215 **Ulk4 mediates the interaction between Stk36 and Gli2**
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217
218 Another possible mechanism for Ulk4 to influence Gli2 phosphorylation by Stk36 is to
219 function as a scaffold that brings Stk36 and Gli2 into the same protein complex. Indeed,
220 we found that Ulk4 depletion diminished the association between exogenously
221 expressed Stk36 and Gli2 in HEK293T cells as determined by Co-IP experiments
222 (Figure 4A). By domain mapping, we found that the kinase domain of Stk36 (Stk36N:
223 aa1-260) but not its regulatory domain (Stk36C: aa261-1351) interacted with Ulk4
224 (Figure 4B-C). Consistent with Ulk4 mediating the interaction between Stk36 and Gli2,
225 we found that Stk36N formed a complex with Gli2 more effectively than Stk36C (Figure
226 4D).
227 We next determined whether Ulk4 mediated Stk36/Gli2 interaction in NIH3T3
228 cells and whether this was regulated by Shh. NIH3T3 cells were infected with
229 lentiviruses expressing low levels of a Flag-tagged Stk36 (Fg-Stk36) and Myc-Gli2 and
230 treated with or without Shh. Co-IP experiments showed that Fg-Stk36 and Myc-Gli2
231 formed a complex regardless of Shh treatment (Figure 4E; lands 1-2). The interaction
232 between Fg-Stk36 and Myc-Gli2 was diminished by Ulk4 RNAi (Figure 4E; lands 3-4).
233 Reintroducing full-length hUlk4 (WT) but not hUlk4N or hUlk4C restored Fg-Stk36/Myc-
234 Gli2 association in Ulk4-depleted cells (Figure 4E; lands 5-10). Taken together, these
235 results suggest that Ulk4 functions as a molecular scaffold to bring Stk36 and Gli2 into

236 the same complex with its N-terminal region binding to Stk36 and its regulatory domain
237 interacting with Gli2 (Figure 4F).
238
239 **Shh stimulates ciliary tip accumulation of both Ulk4 and Stk36**
240
241 In response to Shh, Gli2 and Sufu accumulated at the tip of primary cilium in mammal
242 cells (Haycraft et al. 2005; Chen et al. 2009; Tukachinsky et al. 2010), leading to the
243 general view that Gli2 is converted to $Gli2^A$ at the ciliary tip (Goetz and Anderson 2010;
244 Jiang 2021). Indeed, our previous study showed that Shh-stimulated Gli2
245 phosphorylation by Stk36/Ulk3 depended on ciliary localization of Gli2 (Han et al. 2019).
246 These observations imply that Stk36/Ulk3 may translocate to the primary cilium to
247 phosphorylate Gli2 in response to Shh. To test this hypothesis, we treated NIH3T3 cells
248 stably expressing an N-terminal Myc-tagged Stk36 (Myc-Stk36) with or without Shh and
249 examined ciliary localization of Stk36 by immunostaining with an anti-Myc antibody.
250 Ciliary localization of endogenous Gli2 was examined by immunostaining with an anti-
251 Gli2 antibody. As shown in Figure 5A-B and Figure 5-figure supplement 1A1-B2, Myc-
252 Stk36 (green signals) and Gli2 (blue signals) were barely detectable in primary cilia in
253 quiescent cells; however, Shh treatment induced ciliary tip localization of both Myc-
254 Stk36 and Gli2.
255 To determine whether Shh induced ciliary localization of Ulk4, NIH3T3 cells
256 stably expressing hUlk4-HA was treated with or without Shh, followed by
257 immunostaining with anti-HA and anti-Gli2 antibodies. Like Stk36, hUlk4-HA was barely
258 detectable in primary cilia in the absence of Shh but accumulated to the tips of primary
259 cilia and colocalized with Gli2 after Shh stimulation (Figure 5C-D; Figure 5-figure

260 supplement 1C1-D2). We also examined NIH3T3 cells simultaneously expressing Myc-
261 Stk36 and hUlk4-HA and found that Shh induced the ciliary tip co-localization of Ulk4
262 and Stk36 (Figure 5E-F; Figure 5-figure supplement 1E1-F2). We conformed these
263 results in MEF cells (Figure 5-figure supplement 1G1-J2). These observations suggest
264 that Shh induces ciliary tip accumulation of Stk36-Ulk4-Gli2 protein complexes to
265 promote Gli2 phosphorylation and activation.
266

267 **Ulk4 and Stk36 depend on each other for their ciliary localization.**
268
269 As pseudokinases could regulate the subcellular localization of their interacting kinases,
270 we asked whether Ulk4 is required for ciliary localization of Stk36 in response to Shh.
271 NIH3T3 cells stabling expressing Myc-Stk36 were depleted of Ulk4 by shRNA and
272 treated with and without Shh. As shown in Figure 6A-B, Ulk4 RNAi diminished Shh-
273 induced ciliary tip localization of Myc-Stk36, suggesting that Ulk4 is required for ciliary
274 localization of Stk36 in response to Shh.
275 We next asked whether Ulk4 ciliary localization depends on Stk36. We first
276 determined the ciliary location of Ulk4 in NIH3T3 cells with both Stk36 and Ulk3
277 knocked out (DKO) by the CRISPR-Cas9 system (Han et al. 2019). As shown in Figure
278 6C-D, Shh promoted ciliary tip enrichment of Ulk4-HA in the control cells but not in the
279 Stk36 and Ulk3 DKO cells. Adding back wild type Stk36 ($Stk36^{WT}$) but not its kinase
280 inactive form ($Stk36^{AA}$) restored Shh-induced Ulk4-HA ciliary tip accumulation in DKO
281 cells (Figure 6E-F). By contrast, neither wild type Ulk3 ($Ulk3^{WT}$) nor its kinase dead form
282 ($Ulk3^{KR}$) could rescue ciliary tip accumulation of Ulk4-HA in DKO cells (Figure 6E-F).

283 These results suggest that ciliary tip localization of Ulk4 depends on Stk36 kinase
284 activity.
285
286 **Ulk4 ciliary localization is regulated by its C-terminal phosphorylation**
287 Many pseudokinases are also the substrates of their interacting kinases (Kung and Jura
288 2019). To determine whether Ulk4 is a Stk36 substrate and whether Stk36-mediated
289 phosphorylation of Ulk4 promotes its ciliary tip localization, we sought out to identify the
290 relevant phosphorylation sites. We generated several C-terminally HA-tagged truncated
291 forms of hUlk4 to narrow down the region essential for ciliary tip localization of Ulk4
292 (Figure 7A). These truncated forms were expressed in NIH3T3 cells in which
293 endogenous Ulk4 depleted by RNAi. Deleting the entire regulatory domain abolished
294 Shh-induced ciliary tip localization of the Ulk4 variant (hUlk4$_{1\text{-}450}$; Figure 7B-C). Deleting
295 the Stk36 binding region from Ulk4 (hUlk4$_{451\text{-}1275}$) also abolished its ciliary tip
296 localization in response to Shh, suggesting that binding to Stk36 is essential for Ulk4
297 ciliary localization. We generated two small deletions from the C-terminus of hUlk4
298 (hUlk4$_{1\text{-}1000}$ and hUlk4$_{1\text{-}1200}$; Figure 7A). Whereas hUlk4$_{1\text{-}1200}$ still localized to the ciliary
299 tip in response to Shh, hUlk4$_{1\text{-}1000}$ failed to do so (Figure 7B-C), suggesting that the
300 region between aa1000-1200 is essential for Shh-induced hUlk4 ciliary tip localization.
301 Within this region, T1021 and T1088/T1090 fall into the Fu/Ulk3/Stk36 phosphorylation
302 consensus site: S/T [X_5] E/D (Figure 7D) (Han et al. 2019; Zhou and Jiang 2022).
303 Furthermore, phosphorylation on T1021 can prime CK1-mediated phosphorylation on
304 T1023 as T1023 falls into the CK1 phosphorylation consensus site: D/E/$_{(p)}$S/$_{(P)}$T[$X_{1\text{-}3}$]
305 S/T (Knippschild et al. 2005).

306 To determine whether phosphorylation on these sites may regulate Ulk4 ciliary
307 localization, we generated hUlk4 variants carrying T1021A/1023A (hUlk4$^{T1021A/1023A}$) or
308 T1088A/T1090A (hUlk4$^{T1088A/T1090A}$) substitutions. These hUlk4 variants were expressed
309 in NIH3T3 cells with endogenous Ulk4 depleted by shRNA. We found that
310 hUlk4$^{T1088A/T1090A}$ but not hUlk4$^{T1021A/1023A}$ accumulated at ciliary tip in response to Shh
311 stimulation (Figure 7E-F), suggesting phosphorylation on T1021/1023 is essential for
312 Shh-induced ciliary tip localization of Ulk4.
313 To confirm T1021 phosphorylation by Stk36, we carried out an *in vitro* kinase
314 assay using immunopurified Fg-Stk36 as kinase and GST fusion protein containing
315 hUlk4 sequence from aa1017 to aa1036 (GST-hUlk4C) as substrate. As shown in
316 Figure 7G, immunopurified wild type Stk36 (Fg-Stk36^{WT}) but not its kinase inactive
317 variant (Fg-Stk36^{AA}) phosphorylated GST-hUlk4C. Mutating both T1021 and T1023 to A
318 (T1021A /T1023A) abolished the phosphorylation; however, mutating T1021A reduced
319 but not eliminated the phosphorylation, suggesting that in the absence of T1021
320 phosphorylation, T1023 could also be phosphorylated by Stk36. Interestingly, T1023 but
321 not T1021 is conserved in mouse Ulk4 (mUlk4) and mUlk4 contains an Ala at T1021
322 (Figure 7H), implying that phosphorylation of T1021 might be dispensable for Ulk4
323 function. Indeed, we found that mutating T1021 to A in hUlk4 (hUlk4^{T1021A}) did not affect
324 its ciliary tip accumulation in response to Shh (Figure 7E-F) as well as its activity in the
325 Hh pathway (see below).
326
327
328 **Ciliary localization of Ulk4 is essential for its function in Shh signaling**
329

330 To determine the importance of ciliary localization of Ulk4 for its function in Shh signal
331 transduction, we compared the Hh pathway related activity of ciliary localization
332 deficient hUlk4$^{T1021A/1023A}$ with that of ciliary localization competent hUlk4^{T1021A}
333 (equivalent to mUlk4^{WT}) and hUlk4^{WT} in NIH3T3 cells with endogenous Ulk4 depleted by
334 shRNA. We found that both hUlk4^{T1021A} and hUlk4^{WT} rescued Shh-induced ciliary tip
335 localization of coexpressed Myc-Stk36 (Figure 8A-B), phosphorylation of Myc-Gli2 at
336 S230/S232 (Figure 8C-C'), and the expression of Shh target genes *Ptch1* and *Gli1*
337 (Figure 8D-E). By contrast, hUlk4$^{T1021A/1023A}$ failed to rescue Shh-stimulated Stk36 ciliary
338 tip localization, Gli2 phosphorylation, as well as *Ptch1* and *Gli1* expression in Ulk4
339 depleted cells (Figure 8A-E). These results suggest that ciliary localization of Ulk4 is
340 essential for its ability to promote Stk36 ciliary localization, Gli2 phosphorylation, and Hh
341 pathway activation (Figure 8F).
342

343 **Discussion**
344
345 The Gli family of transcription factors mediates the transcriptional output of the Hh
346 signaling pathway in species ranging from *Drosophila* to mammals but how Gli is
347 activated by Hh signaling is still poorly understood, especially in vertebrates where Gli
348 activation is thought to occur at the tip of primary cilium. We recently found that the
349 Fu/Ulk family kinases directly phosphorylate Ci and Gli2 on multiple sites to promote
350 their activation (Han et al. 2019; Zhou et al. 2022; Zhou and Jiang 2022). In mammalian
351 cells, the Ulk family kinases Ulk3 and Stk36 (mammalian homolog of Fu) act in parallel
352 to promote Gli2 phosphorylation and activation in response to Shh (Han et al. 2019). In
353 addition, Gli2 phosphorylation depends on its ciliary localization (Han et al. 2019). In this

354 study, we found that Stk36 accumulated to the tip of primary cilium and colocalized with
355 Gli2 in response to Shh stimulation, consistent with Gli2 being converted into $Gli2^A$ at
356 the ciliary tip. In addition, we identified another Ulk family member Ulk4, which is a
357 pseudokinase, as a mediator of Shh-induced Gli2 phosphorylation. We found that Ulk4
358 and Stk36 act in the same pathway but in parallel with Ulk3 to promote Gli2
359 phosphorylation and activation in NIH3T3 and MEF cells. The positive role of Ulk4 in
360 Shh signaling could explain why elevated expression of Ulk4 in certain genetic
361 background could suppress the heart outflow tract defects and holoprosencephaly
362 caused by partially reduced Shh signaling activity in LRP2-deficient mice (Mecklenburg
363 et al. 2021). However, it remains to be determined whether Ulk4 participates in Hh
364 signaling *in vivo* by loss-of-function study. Because knockout of Stk36 in mice did not
365 affect Hh signaling during development, likely due to functional compensation by other
366 kinases such as Ulk3 (Chen et al. 2005; Merchant et al. 2005), we do not expect that
367 loss-of-function of Ulk4 will affect Hh signaling during development. Therefore, double
368 knockout of Ulk4 (or Stk36) and Ulk3 will be necessary to address the physiological role
369 of the Fu/Ulk family members in mammalian Hh signaling. In addition, it will also be
370 important to determine whether Ulk4/Stk36 and Ulk3 could be differentially required for
371 Hh signal transduction in different cell types/tissues or in different contexts, e.g.,
372 development vs. tissue homeostasis or physiological vs pathological settings.
373 To determine the mechanism by which Ulk4 assists Stk36 to phosphorylate Gli2,
374 we explored whether Ulk4 regulated 1) the enzymatic activity of Stk36; 2) Shh-induced
375 Stk36 kinase activation; 3) the association of Stk36 and its substrate; and 4) ciliary
376 localization Stk36. In an *in vitro* kinase assay, we found that immunopurified Stk36

377 phosphorylated GST-Gli2N equally well regardless of whether it interacted with Ulk4 or
378 not, suggesting that Ulk4 does not regulate Stk36 kinase activity. Rather, we found that
379 Ulk4 is required for Stk36 to bind to its substrate Gli2. Ulk4 interacts with Stk36 through
380 its N-terminal region that contains the pseudokinase domain and with Gli2 via its C-
381 terminal regulatory domain. Depletion of Ulk4 diminished the physical association
382 between Stk36 and Gli2. As a further support of the notion that Gli2 is brought to Stk36
383 through Ulk4, we found that the kinase domain of Stk36, which binds Ulk4, also
384 interacted with Gli2 whereas its C-terminal region interacted with neither Ulk4 nor Gli2.
385 Of note, despite being a pseudokinase, Ulk4 contains an unusual ATP binding site that
386 might regulate its ability to bind other proteins such as Stk36 (Preuss et al. 2020).
387 Therefore, it would be interesting to determine in the future whether ATP binding to Ulk4
388 could regulate Shh signaling or other processes that Ulk4 is involved.
389 Although Stk36/Ulk4/Gli2 complex formation appears to be constitutive
390 regardless of the presence or absence of Shh, Stk36 kinase activation depends on Shh
391 stimulation. Like Fu, Stk36 is phosphorylated on multiple sites in the activation loop of
392 its kinase domain in response to Shh likely through trans-autophosphorylation, and
393 activation loop phosphorylation is essential for its kinase activation. Using a phospho-
394 specific antibody that recognized pT158/pS159 in the kinase activation loop of
395 Fu/Stk36, we found that Ulk4 depletion did not affect Shh-induced phosphorylation of
396 Stk36 on T158/S159, a readout for Stk36 kinase activation, suggesting that Ulk4 is
397 dispensable for Shh signaling to activate Stk36. In *Drosophila*, the kinesin-like protein
398 Costal2 (Cos2) mediates the activation of Fu kinase by Hh. Therefore, it would be

399 interesting to determine whether mammalian homologs Cos2, Kif7 and/or Kif27,
400 participates in Stk36 kinase activation in response to Shh stimulation.
401 Hh signaling depends on primary cilia in vertebrates. Upon Shh stimulation, key
402 pathway components such as Smo and Gli proteins accumulate in primary cilia, and
403 Gli2 is accumulated to the ciliary tip and thought to be converted into its activator form
404 ($Gli2^A$) in this ciliary compartment. Consistent with this notion, we found that Shh
405 induced ciliary accumulation of both Ulk4 and Stk36 that colocalized with Gli2 at the
406 ciliary tip. Furthermore, we found that Ulk4 is required for ciliary tip accumulation of
407 Stk36. On the other hand, ciliary tip accumulation of Ulk4 depends on Stk36 kinase
408 activity and phosphorylation of its C-terminal region on T1023. Preventing Ulk4
409 phosphorylation on T1023 abolished its ciliary tip accumulation and its ability to promote
410 Stk36 ciliary tip accumulation, Gli2 phosphorylation and Hh pathway activation. These
411 results suggest that ciliary tip accumulation of Ulk4 is critical for its function in Hh signal
412 transduction, likely by increasing the local concentration of Stk36 and Gli2 to promote
413 effective phosphorylation of Gli2. It would also be important to examine the localization
414 of endogenous Ulk4 and Stk36 in the future as exogenously expressed epitope-tagged
415 protein might cause artifacts. In addition, the precise mechanism and dynamics of Ulk4
416 ciliary targeting remain to be determined in the future. e.g., it would be important to
417 determine how phosphorylation of T1023 promotes ciliary accumulation of Ulk4. A
418 recent study identified several motor proteins of the kinesin family (KIF1B, KIF3A,
419 KIF3B, and KAP3) as the interaction partners of Ulk4 (Preuss et al. 2020). KIF3A,
420 KIF3B and KAP3 are components of kinesin-2 motor that act in conjunction with
421 intraflagellar transport (IFT) complexes to transport cargoes into primary cilia (Goetz

422 and Anderson 2010). It would be interesting to determine whether Ulk4 T1023
423 phosphorylation affects its interaction with KIF3A, KIF3B, KAP3, or components in the
424 IFT complexes. Of note, a previous study suggested that Ulk4 regulated the ciliary
425 localization of Smo although the underlying mechanism was not explored (Mecklenburg
426 et al. 2021). Therefore, it remains possible that Ulk4 could regulate mammalian Hh
427 signaling at multiple steps similar to what Fu does in the *Drosophila* Hh pathway (Claret
428 et al. 2007; Liu et al. 2007).

429 Both Ulk4 and Stk36 have functions outside the Hh pathway. Stk36 is required
430 for motile ciliogenesis, and its loss of function affects the construction of central pair
431 apparatus of “9+2” motile cilia in both zebrafish and mice (Wilson et al. 2009; Nozawa et
432 al. 2013). Ulk4 malfunction also affects motile ciliogenesis of ependymal cells in mutant
433 mice, leading to hydrocephalus (Vogel et al. 2012; Liu et al. 2016b). A recent study
434 demonstrated that Ulk4 and Stk36 physically interact to mediate the assembly of motile
435 flagella in the flagellated protist *Leishmania Mexicana* (McCoy et al. 2023). However,
436 the biochemical mechanism by which Ulk4/Stk36 regulates motile ciliogenesis has
437 remained unknown. Ulk4 could regulate Stk36 ciliary localization and/or kinase-
438 substrate interaction like what we have discovered for their role in Shh signaling. The
439 relevant substrates for Stk36 in motile ciliogenesis remain to be determined. Knowing
440 the phosphorylation consensus sites for the Fu/Ulk family kinase may facilitate the
441 identification of Stk36 substrates involved in the assembly of motile cilia and flagella.
442 Initially identified Fu/Ulk3/Stk36 phosphorylation sites in Ci/Gli and Cos2 fall into the
443 consensus: S/T$(X)_5$D/E, where the acidic residue at +6 position is essential (Han et al.
444 2019; Zhou and Jiang 2022). However, subsequent studies suggested that the spacing

445 between the phospho-acceptor site and the acid residue appears to be flexible. For
446 example, a Ulk3/Stk36 phosphorylation site in the C-terminal region of Gli2 has a
447 spacing of six residues between the phospho-acceptor site and the acid residue (Zhou
448 et al. 2022). Here we found that a Stk36 phosphorylation site in the C-terminal region of
449 Ulk4 contains only four residues between the phospho-acceptor site and the acid
450 residue. Based on these findings, we propose an expanded consensus site for the
451 Fu/Ulk family kinases as S/T$(X)_{4\text{-}6}$D/E. It would be interesting to determine whether this
452 phosphorylation consensus sequence could be extended to other members of the Ulk
453 kinase family such as Ulk1/Atg1 and Ulk2, which are the initiating kinases for autophagy
454 (Zachari and Ganley 2017).

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

468 **Materials and methods**

469

470 **DNA constructs**

471 Myc-Gli2 and GST-Gli2 were described previously (Han et al. 2019). All GST-fusion
472 (the GST-tagged human Ulk4C^{WT}, Ulk4C^{T1021A}, Ulk4C$^{T1021A/1023A}$) proteins were
473 subcloned into the *pGEX 4T-1* vector using EcoRI and XhoI sites. N-terminally CFP-
474 tagged mouse Ulk3^{WT}, Ulk3^{KR}, N-terminally 6XMyc-tagged human Stk36, N-terminally
475 3XFg-tagged human Stk36^{WT}, Stk36^{AA} and the C-terminally 3XHA-tagged Human
476 Ulk4^{WT}, Ulk4$^{1-451}$, Ulk4$^{452-1275}$, Ulk4$^{1-1000}$, Ulk4$^{1-1200}$, Ulk4$^{T1021/1023A}$, Ulk4$^{T1088/1090A}$,
477 Ulk4^{T1021A} were subcloned into the *FUGW* vector with the EcoRI and BamHI sites. N-
478 terminally 3XFg-tagged human Stk36^{WT}, Stk36^{EE}, Stk36^{AA}, Stk36$^{1-260}$, Stk36$^{261-1315}$, and
479 C-terminally 3XHA-tagged Human Ulk4^{WT}, Ulk4$^{1-451}$, Ulk4$^{452-1275}$ were subcloned in the
480 *PLVX* vectors using the EcoRI and MluI sites. The Ulk4$^{T1021/1023A}$, Ulk4$^{T1088/1090A}$,
481 Ulk4^{T1021A}, Stk36^{EE}, Stk36^{AA}, were generated by PCR-mediated site-directed
482 mutagenesis.

483

484 **Cell culture, transfection, and lentiviral production**

485 NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma)
486 containing10% bovine calf serum (Gibco) and 1% penicillin/streptomycin (Sigma). The
487 transfection was carried out by using the GenJet Plus *in vitro* DNA transfection kit
488 according to the manufacturer's instruction (SignaGen). HEK293T cells were cultured in
489 DMEM (Sigma) supplemented with 10% fetal bovine serum (Gibco) and 1%
490 penicillin/streptomycin (Sigma). PolyJet *in vitro* DNA transfection kit (SignaGen) was

491 used to do the transfection according to the manufacturer's instruction. Mouse
492 embryonic fibroblast cells (MEFs) were cultured in DMEM (Sigma) containing 10% FBS
493 (Gibco), and 1% streptomycin / penicillin (Sigma) according to the standard protocol.
494 For Shh-N treatment, cells were cultured at 50%-confluent densities in plate or chamber
495 slides for one day and starved in serum-free medium (0.5% BCS for NIH3T3 cells or
496 0.5% FBS for MEF cells) for 12 hours to allow ciliation. The recombinant human Shh N-
497 terminal fragment (1 ng/ml; R&D Systems, #8908-SH-005) was then added to the same
498 serum-free medium for 12 hours or overnight.
499
500 **Immunostaining, immunoprecipitation, and western blotting analysis**
501 The protocol for Ulk4-HA/Myc-Stk36 immunostaining was carried out as previously
502 described (Han et al. 2019). Immunofluorescence imaging was captured using a Zeiss
503 LSM700 laser scanning microscope. For ciliary Myc-Stk36/Ulk4-HA quantification, the
504 cilia were labelled with the acetylated-Tubulin and the Myc-Stk36/Ulk4-HA fluorescence
505 intensities were measured in the Myc-Stk36/Ulk4-HA image channel by ImageJ. Twenty
506 cells were randomly selected and calculated for each group. For immunoprecipitation
507 assay, after transfection for 48 hours, cells were washed twice with PBS and then lysed
508 on ice for 10 min with lysis buffer containing 1M Tris pH8.0, 5M NaCl, 1MNaF, 0.1M
509 Na3VO4, 1% CA630, 10% glycerol, and 0.5M EDTA (pH 8.0). Cell lysates were
510 incubated with protein A-Sepharose beads (Thermo scientific) for 1 hr at 4°C to
511 eliminate nonspecific binding proteins. After removal of the protein-A beads by
512 centrifugation, the cleared lysates were incubated with Myc (HA or Flag) antibody for 4
513 h or overnight. Protein complexes were collected by incubation with protein A-

514 Sepharose beads for 1 hr at 4°C, followed by centrifugation. Immunoprecipitates were
515 washed three times for 5 min each with lysis buffer and were separated on SDS-PAGE.
516 Western blot was carried out using standard protocol. Western blot quantification was
517 carried out by employing fluorogenic secondary antibody (IRDye 680LT Goat anti-
518 Mouse:926-68020 or Goat anti-Rabbit: 926-68021) and the blot was scanned and
519 quantified with an Odyssey CLx imaging system (LI-COR). Antibodies used in this study
520 were as follows: Mouse anti-Myc (Santa Cruz Biotechnology; 9E10), Mouse anti-Flag
521 (Sigma; M2), Mouse anti-HA (Santa Cruz Biotechnology; F7), Mouse anti-acetylated
522 Tubulin (Sigma; T7451; staining:1:1000), Rabbit anti-Myc (Abcam; ab9106; staining
523 1:2000), Rabbit anti-HA (AbCam; ab9110; staining 1:500), Goat anti-Gli2 (R&D system;
524 AF3635; staining 1:1000).

525

526 ***In vitro* kinase assay**

527 *In vitro* kinase assay was performed by incubating 25 µL of reaction mixtures containing
528 150 mM Tris-HCl (pH 7.5), 0.2 mM Mg2+/ATP, 1 µg of purified GST-Ulk4/Gli2 fusion
529 proteins, together with appropriate amount of kinase for 30mins at 30°C. The reaction
530 was terminated by adding 2× SDS loading buffer. The resultant samples were load on
531 SDS-PAGE and subjected to pIMAGO phospho-protein detection kit with fluor-680
532 (Sigma) detection kit and LI-COR Odyssey platform for western blot analyses of
533 phosphorylated proteins (Millipore Sigma). Fg-Stk36^{WT} and Fg-Stk36^{AA} were
534 immunopurified from HEK293 cells transfected with the Fg-Stk36^{WT} or Fg-Stk36^{AA}
535 construct.

536

537 **Viral Infection and shRNA**

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

550

551 **RNA interference**

552 For RNAi experiments in NIH3T3 or MEF cells, the siRNAs were purchased from
553 Sigma. The sequences for Ulk3 silencing were: 5'-GUGUACAAGGCCUACGCCA-3'
554 (Cat# SASI_ Mm02 _ 00444409) and 5'-CUGAGAAGGUGGCCCGUGU-3' (Cat#
555 SASI_Mm02_00444410). The sequences for Stk36 silencing were: 5'-
556 GGUAUACUGGCUUCAGAAA-3' (Cat# SASI_ Mm02 _00345637) and 5'-
557 GCCUUAUGUGCUUUGCUGU-3' (Cat# SASI_Mm01_00167751). The target sequence
558 of Mouse Ulk4 shRNA is: CTGCGAAGATTATCGAGAATG. The target sequence of
559 Human Ulk4 shRNA is: CCACTAGGTCACTCTTTCAGA. The

560 sequences for negative control were: 5'-UUCUCCGAACGUGUCACG U-3'. The RNAi
561 MAX reagent (Invitrogen Cat. No. 13778150) was used for the transfection of siRNA.
562 The knockdown efficiency was validated via real-time PCR.
563

564 **Quantitative RT-PCR**

565 Total RNA was extracted from 1 × 10^6 cells using the RNeasy Plus Mini Kit (Qiagen),
566 and cDNA was synthesized with High-Capacity cDNA Reverse Transcription Kit
567 (Applied Biosystems; 01279158) and qPCR was performed using Fast SYBR Green
568 Master Mix (Applied Biosystems; 2608088) and a Bio-Rad CFX96 real-time PCR
569 system (BIO-RAD; HSP9601). The RT-qPCR was performed in triplicate for each of
570 three independent biological replicates. Quantification of mRNA levels was calculated
571 using the comparative CT method. Primers: Gli1, 5'-GTGCACGTTTGAAGGCTGTC-3'
572 and 5'-GAGTGGGTCCGATTCTGGTG-3'; Ptch1, 5'-GAAGCCACAGAAAACCCTGTC-3'
573 and 5'-GCCGCAAGCCTTCTCTACG-3'; Ulk3, 5'- ACGAAACATCTCTCACTTG-3' and
574 5'- TGCTGGGCAAAGCCAAAGTC-3'; Ulk4, 5'- ATGCAGAGTGTGATTGCGTTG-3' and
575 5'- GAGTGGCAGTTTCTGTGAACA-3'; Stk36, 5'- CGCATCCTACACCGAGATATGA-3'
576 and 5'- AAATCCAAAGTCACAGAGCTTGA-3';GAPDH, 5'-
577 GTGGTGAAGCAGGCATCTGA-3' and 5'-GCCATGTAGGCCATGAGGTC-3'.
578

579 **Acknowledgements**

580 We thank Bing Wang, Xu Li, and Yong Cho for technical assistance, Pancheng Xie and
581 Yi Liu for reagents. J. Jiang is a Eugene McDermott Endowed Scholar in Biomedical
582 Science at the University of Texas Southwestern.

Declaration of interests

The authors declare no competing interests.

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713

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715

716 **Figure legend**
717
718

719 **Figure 1. Ulk4 acts in conjunction with Stk36 to promote Shh-mediated Gli**

720 **activation**

721 (A-B) Relative *Gli1* (A) and *Ptch1* (B) mRNA levels measured by RT-qPCR in NIH3T3

722 cells expressing the indicated shRNA and a human Ulk4 (hUlk4) lentiviral construct and

723 treated with or without Shh-N. Of note, hUlk4 is insensitive to RNAi, which targets

724 mouse Ulk4.

725 (C-D) Relative *Ptch1* mRNA levels measured by RT-qPCR in NIH3T3 cells treated with

726 the indicated RNAi and with or without Shh-N.

727 (E-E') Western blot analysis (E) and quantification (E') of Myc-Gli2 phosphorylation in

728 NIH3T3 cells expressing the indicated shRNA and hUlk4 lentiviral construct and treated

729 with or without Shh-N.

730 (F-G') Western blot analysis (F and G) and quantification (F' and G') of Myc-Gli2

731 phosphorylation in NIH3T3 cells with the indicated RNAi in the presence or absence of

732 Shh-N treatment.

733 (H) Schematic diagram showing the functional relationship among Ulk4, Stk36 and Ulk3

734 in Shh-induced Gli activation.

735 The cells (A-G) were firstly starved in serum-free medium for 12 hours then cultured in

736 the same medium with or without Shh-N fragment for another 12 hours before they are

737 subjected into RNA preparation (A-D) or Western blot analysis (E-G). Data in (A-D) are

738 mean ± SD from three independent experiments. Data in (E', F', G') are mean ± SD

739 from two independent experiments. ***$p < 0.001$ (Student's t test). NS: not significant.

740

741 **Figure 2. Ulk4 forms a complex with Stk36 and Gli2**

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

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

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

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

753

754 **Figure 3. Ulk4 is dispensable for Stk36 kinase activation.**
755
756 (A) *In vitro* kinase assay using the immunopurified Fg-Stk36 or Fg-Stk36/hUlk4-HA as
757 kinase and GST-Gli2N^{WT} or GST-Gli2N^{S230A} as substrate. pIMAGO was used to detect
758 GST-Gli2N phosphorylation. The levels of Fg-Stk36/hUlk4-HA and GST/GST-Gli2N
759 were analyzed by western blot and coomassie blue (CB) staining, respectively.

760 (B) The schematic diagram showing the sequence alignment of the activation segments
761 of *Drosophila* Fu, mouse Stk36 (mStk36) and human Stk36 (hStk36). The conserved
762 Ser/Thr residues in the activation loop are color-coded in green. The residues used as

763 antigen for developing pT158/pS159 are colored in yellow. Amino acid substitutions for
764 Stk36 variants are indicated.
765 (C) Shh induced Stk36 phosphorylation on T158/S159. NIH3T3 cells expressing CFP-
766 Stk36 lentiviral construct were treated with or without Shh-N, followed by
767 IP with an GFP antibody and western blot analysis using an antibody against the
768 phosphorylated T158/S159 (pT158/pS159).
769 (D) Shh induced Stk36 phosphorylation on T158/S159 depending on Stk36 kinase
770 activity. Ulk3 and Stk36 double knockout NIH3T3 cells expressing CFP-Stk36^{WT} or
771 CFP-Stk36^{KR} (kinase dead) lentiviral construct were treated with or without Shh-N,
772 followed by IP with an GFP antibody and western blot analysis with the pT158/pS159
773 antibody.
774 (E-F) Western blot analysis (E) and quantification (F) of T158/S159 phosphorylation in
775 Fg-Stk36^{WT}, Fg-Stk36^{EE} or Fg-Stk36^{AA}. HEK293T cells were transfected with the
776 indicated Fg-Stk36 constructs, followed by IP with anti-Flag antibody and western blot
777 analysis with the indicated antibodies.
778 (G-H) Western blot analysis (G) and quantification (H) of Myc-Gli2 phosphorylation on
779 S230/S232 by the wild type (WT) or indicated Stk36 variants in HEK293T cells.
780 (I) Ulk4 is not required for Shh-induced Stk36 kinase activation. NIH3T3 cells
781 expressing the indicated shRNA and CFP-Stk36 lentiviral construct were treated with or
782 without Shh-N, followed by IP with an GFP antibody and western blot analysis with the
783 pT158/pS159 antibody (right). The knockdown efficiency of Ulk4 was determined by RT-
784 qPCR (left).

785 Data are mean ± SD from three independent experiments. **p < 0.01, and ***p < 0.001

786 (Student's t test).

787

788 **Figure 4. Ulk4 mediates the interaction between Stk36 and Gli2.**

789

790 (A) Depletion of Ulk4 diminished the association between Stk36 and Gli2. HEK293T

791 cells were transfected with the indicated shRNA and Fg-Stk36 and Myc-Gli2 constructs,

792 followed by CoIP and western blot analysis with the indicated antibodies.

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

794 experiments.

795 (C-D) Stk36 binds to Ulk4 and Gli2 through its N-terminal kinase domain. HEK293T

796 cells were transfected with Ulk4-HA (C) or Myc-Gli2 (D) and the indicated Fg-Stk36

797 constructs, followed by Co-IP and western blot analyses with the indicated antibodies.

798 Black triangles indicate the heavy chain of IgG and asterisks indicate the protein bands

799 produced by individual Stk36 constructs.

800 (E) Both N- and C-terminal domains of Ulk4 are required for mediating Stk36/Gli2

801 association. NIH3T3 cell lines stably expressing control (Ctrl) or Ulk4 shRNA were

802 infected with lentiviruses expressing the indicated hUlk4 constructs (diagrams shown on

803 F, right), Fg-Stk36 and Myc-Gli2, and treated with or without the Shh-N, followed by

804 CoIP and western blot analysis with the indicated antibodies.

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

806 Gli2.

807

808 **Figure 5. Shh stimulates ciliary tip accumulation of both Ulk4 and Stk36.**

809

810 (A-D) Representative images of immunostaining (A, C) and quantification (B, D) of
811 ciliary tip localized Myc-Stk36 (green in A) or Ulk4-HA (green in C) and Gli2 (blue in A
812 and C) in NIH3T3 cells infected with the Myc-Stk36 or Ulk4-HA lentivirus and treated
813 with or without Shh-N. Primary cilia were marked by acetylated tubulin (Ac-tub) staining
814 (red in A and C).
815 (E-F) Representative images of immunostaining (E) and quantification (F) of ciliary tip
816 localized Myc-Stk36 (green) and Ulk4-HA (blue) in NIH3T3 cells co-infected with Myc-
817 Stk36 and Ulk4-HA lentiviruses and treated with or without Shh-N. Primary cilia were
818 marked by Ac-Tub staining (red).
819 The cells are starved in serum-free medium for 12 hours to allow ciliation and cultured
820 in the same medium with or without Shh-N fragment for another 12 hours before they
821 are subjected into immunostaining assay. The intensity of ciliary-localized Myc-Stk36,
822 Ulk4-HA and Gli2 was measured by Image J. 20 cells were randomly selected and
823 counted for each group. Data are mean ± SD from three independent experiments. ***p
824 < 0.001 (Student's t test). Scale bars are 2 μM.
825
826 **Figure 6. Stk36 and Ulk4 depend on each other for their ciliary tip accumulation**
827 (A-B) Representative images of immunostaining (A) and quantification (B) of ciliary tip
828 localized Myc-Stk36 in NIH3T3 cells infected with the indicated shRNA and Myc-Stk36
829 lentivirus in the presence or absence of Shh-N. Primary cilia were marked by Ac-Tub
830 staining (red); Myc-Stk36 was marked by Myc staining (green); nuclei were marked by
831 DAPI (Blue).

832 (C-D) Representative images of immunostaining (C) and quantification (D) of ciliary tip
833 localized Ulk4-HA (green) in wild type (Ctrl) or Ulk3 and Stk36 double knockout (DKO)
834 NIH3T3 cells treated with or without Shh-N. Primary cilia were marked by Ac-Tub
835 staining (red) and nuclei by DAPI (Blue).
836 (E-F) Representative images of immunostaining (E) and quantification (F) of ciliary-
837 localized Ulk4-HA (green) in Shh-N treated control or DKO NIH3T3 cells infected with or
838 without lentiviruses expressing the indicated Ulk3 or Stk36 constructs.
839 The cells are starved in serum-free medium for 12 hours to allow ciliation and cultured
840 in the same medium with or without Shh-N fragment for another 12 hours before they
841 are subjected into immunostaining assay. The intensity of ciliary-localized Myc-Stk36 or
842 Ulk4-HA was measured by Image J. 20 cells were randomly selected and counted for
843 each group. Data are mean ± SD from three independent experiments. ***$p < 0.001$
844 (Student's t test). Scale bars are 2 μM.
845
846 **Figure 7. Ulk4 ciliary tip accumulation is promoted by its phosphorylation by**
847 **Stk36**
848 (A) Diagrams of hUlk4 deletion constructs.
849 (B-C) Representative images of immunostaining (B) and quantification (C) of ciliary tip
850 localized full-length or truncated hUlk4-HA in Ulk4 knockdown cells treated with Shh-N.
851 (D) Stk36 and CK1 phosphorylation sites in the C-terminal region of hUlk4.
852 (E-F) Representative images of immunostaining (E) and quantification (F) of ciliary tip
853 localized wild type (WT) or mutant hUlk4-HA baring the indicated amino acid
854 substitutions in Ulk4 knockdown NIH3T3 cells treated with Shh-N.

855 (G) *In vitro* kinase assay using the immunopurified Fg-Stk36^{WT} or Fg-Stk36^{AA} as kinase
856 and the indicated GST-Ulk4C fusion proteins as substrates. Phosphorylation was
857 detected by the pIMAGO system.
858 (H) Schematic diagram showing the sequence alignment of C-terminal phosphorylation
859 sites of mouse Ulk4 (mUlk4) and human Ulk4 (hUlk4). The Thr residues phosphorylated
860 by Stk36 are color-coded in green.
861 The cells are starved in serum-free medium for 12 hours to allow ciliation and cultured
862 in the same medium with or without Shh-N fragment for another 12 hours before they
863 are subjected into immunostaining assay. The intensity of ciliary-localized WT and
864 mutant Ulk4-HA was measured by Image J. 20 cells were randomly selected and
865 counted for each group. Data are mean ± SD from three independent experiments. ***p
866 < 0.001 (Student's t test). Scale bars are 2 μM.

867

868 **Figure 8. Ciliary tip localization of Ulk4 is required for Shh signal transduction**
869
870 (A-B) Representative images of immunostaining (A) and quantification (B) of ciliary tip
871 localized Myc-Stk36 and hUlk4 in Ulk4-depleted NIH3T3 cells expressing the indicated
872 hUlk4 constructs and treated with Shh.
873 (C-C') Western blot analysis (C) and quantification (C') of Myc-Gli2 phosphorylation on
874 S230/S232 in NIH3T3 cells expressing the indicated shRNA and hUlk4 lentiviral
875 constructs and treated with or without Shh-N. Data are mean ± SD from two
876 independent experiments. ***p < 0.001 (Student's t test).
877 (D-E) Relative *Gli1* (D) and *Ptch1* (E) mRNA levels in NIH3T3 cells expressing the
878 indicated shRNA and hUlk4 lentiviral constructs treated with or without Shh-N.

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

880 The cells are starved in serum-free medium for 12 hours to allow ciliation and cultured

881 in the same medium with or without Shh-N fragment for another 12 hours before they

882 are subjected into RNA preparation, Western blot and immunostaining assay. The

883 intensity of ciliary-localized WT and mutant Ulk4-HA was measured by Image J. 20

884 cells were randomly selected and counted for each group. Data are mean ± SD from

885 three independent experiments. ***p < 0.001 (Student's t test). Scale bars are 2 μM.

886

887

888 **Supplementary Figure legends**

889

890 **Figure 1- figure supplement 1. The knockdown efficiency of Ulk4, Ulk3 and Stk36**

891 **in NIH3T3 cells.**

892 (A-C) Relative Ulk4 (A), Ulk3 (B), and Stk36 (C) mRNA levels measured by RT-qPCR in

893 NIH3T3 cells treated with the indicated shRNA or siRNA.

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

895

896 **Figure 1- figure supplement 2. Ulk4 acts in conjunction with Stk36 to regulate**

897 **Shh-induced expression of *Gli1* and *Ptch1* in MEFs.**

898 (A-B) Relative *Gli1* (A) and *Ptch1* (B) mRNA levels measured by RT-qPCR in MEF cells

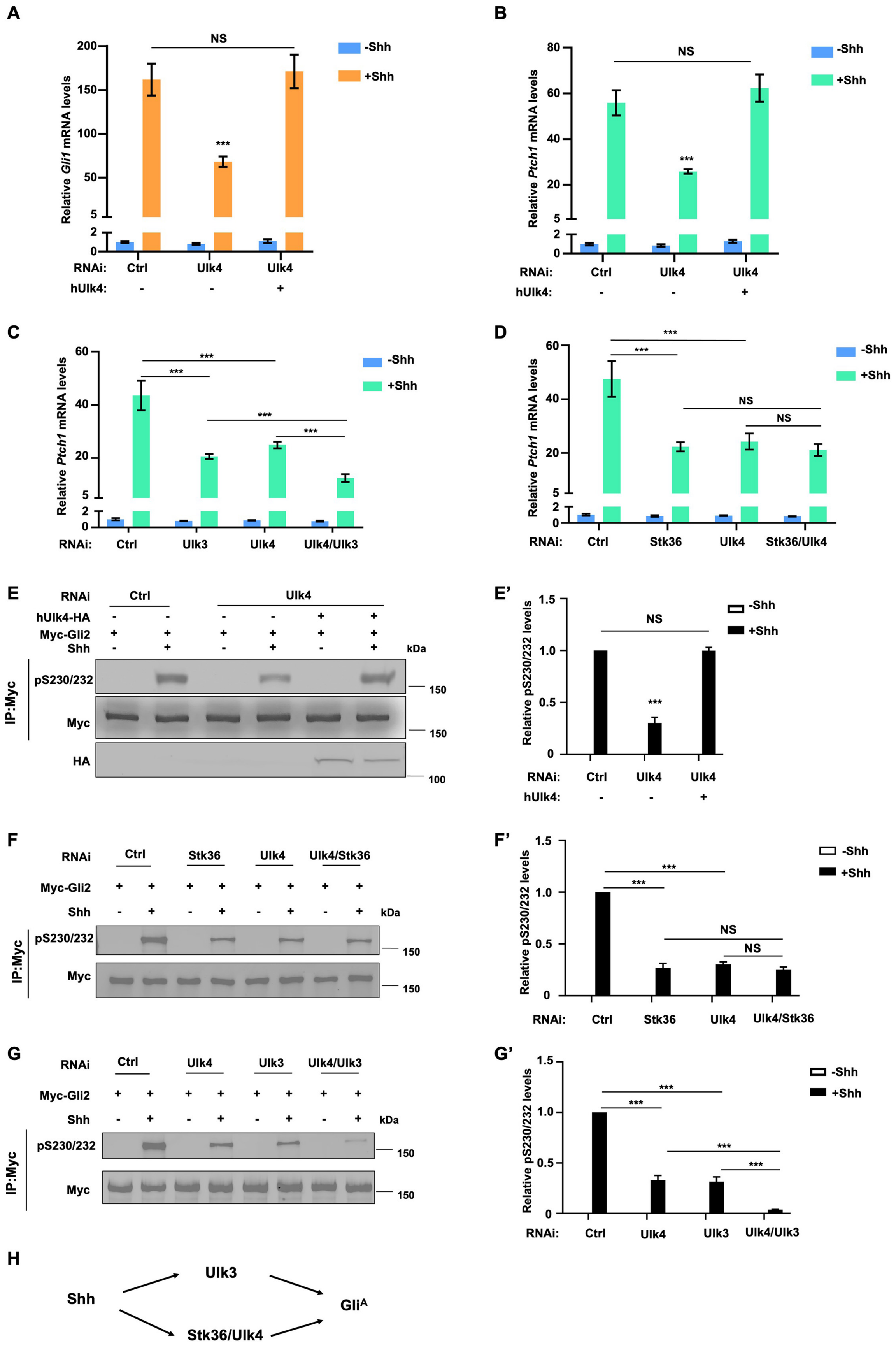
899 expressing the indicated shRNA and hUlk4 lentiviral constructs with or without Shh-N

900 treatment.

901 (C-D) Relative *Gli1* mRNA levels measured by RT-qPCR in MEF cells with the indicated

902 RNAi knockdown in the presence or absence of Shh-N treatment.

903 (E) Relative Ulk4, Ulk3 and Stk36 mRNA levels measured by RT-qPCR in MEF cells
904 treated with the indicated shRNA or siRNA.
905 The cells were firstly starved in serum-free medium for 12 hours then cultured in the
906 same medium with or without Shh-N fragment for another 12 hours before they are
907 subjected into RNA preparation. Data are mean ± SD from three independent
908 experiments. ***p< 0.001 (Student's t test).
909
910
911 **Figure 5- figure supplement1. Shh stimulates ciliary tip accumulation of both Ulk4**
912 **and Stk36**
913 (A1-D2) Representative images of immunostaining ciliary tip localized Myc-Stk36 (green
914 in A1-B2) or Ulk4-HA (green in C1-D2) and Gli2 (blue) in NIH3T3 cells infected with the
915 Myc-Stk36 or Ulk4-HA lentivirus and treated with or without Shh-N.
916 (E1-F2) Representative images of immunostaining of ciliary tip localized Myc-Stk36
917 (blue) and Ulk4-HA (green) in NIH3T3 cells co-infected with Myc-Stk36 and Ulk4-HA
918 lentiviruses and treated with or without Shh-N.
919 (G1-J2) Representative images of immunostaining of ciliary tip localized Myc-Stk36
920 (green in G1-H2) and Gli2 (blue in G1-H2) or Ulk4-HA (green in I1-J2) and Myc-Stk36
921 (blue in I1-J2) in MEFs infected with either Myc-Stk36 (G1-H2) or both Myc-Stk36 and
922 Ulk4-HA lentiviruses and treated with or without Shh-N.
923 Primary cilia were marked by acetylated tubulin (Ac-tub) staining (red in A1-J2). Insets
924 show the enlarged images of primary cilia with individual channels shown separately.
925 Scale bars are 2 μM.
926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5
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hUlk4: - - +
B
NS
-Shh
+Shh
Relative Ptch1 mRNA levels
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60
40
20
5
2
0
***
RNAi: Ctrl Ulk4 Ulk4
hUlk4: - - +
C
-Shh
+Shh
Relative Ptch1 mRNA levels
60
40
20
5
2
0
***
***
***
***
RNAi: Ctrl Ulk3 Ulk4 Ulk4/Ulk3
D
-Shh
+Shh
Relative Ptch1 mRNA levels
60
40
20
5
2
0
***
***
NS
NS
RNAi: Ctrl Stk36 Ulk4 Stk36/Ulk4
E
RNAi Ctrl Ulk4
hUlk4-HA - - - - + +
Myc-Gli2 + + + + + +
Shh - + - + - +
kDa
IP:Myc
pS230/232
Myc
HA
150
150
100
E'
-Shh
+Shh
Relative pS230/232 levels
1.5
1.0
0.5
0
NS
***
RNAi: Ctrl Ulk4 Ulk4
hUlk4: - - +
F
RNAi Ctrl Stk36 Ulk4 Ulk4/Stk36
Myc-Gli2 + + + + + + + +
Shh - + - + - + - +
kDa
IP:Myc
pS230/232
Myc
150
150
F'
-Shh
+Shh
Relative pS230/232 levels
1.5
1.0
0.5
0
***
***
NS
NS
RNAi: Ctrl Stk36 Ulk4 Ulk4/Stk36
G
RNAi Ctrl Ulk4 Ulk3 Ulk4/Ulk3
Myc-Gli2 + + + + + + + +
Shh - + - + - + - +
kDa
IP:Myc
pS230/232
Myc
150
150
G'
-Shh
+Shh
Relative pS230/232 levels
1.5
1.0
0.5
0
***
***
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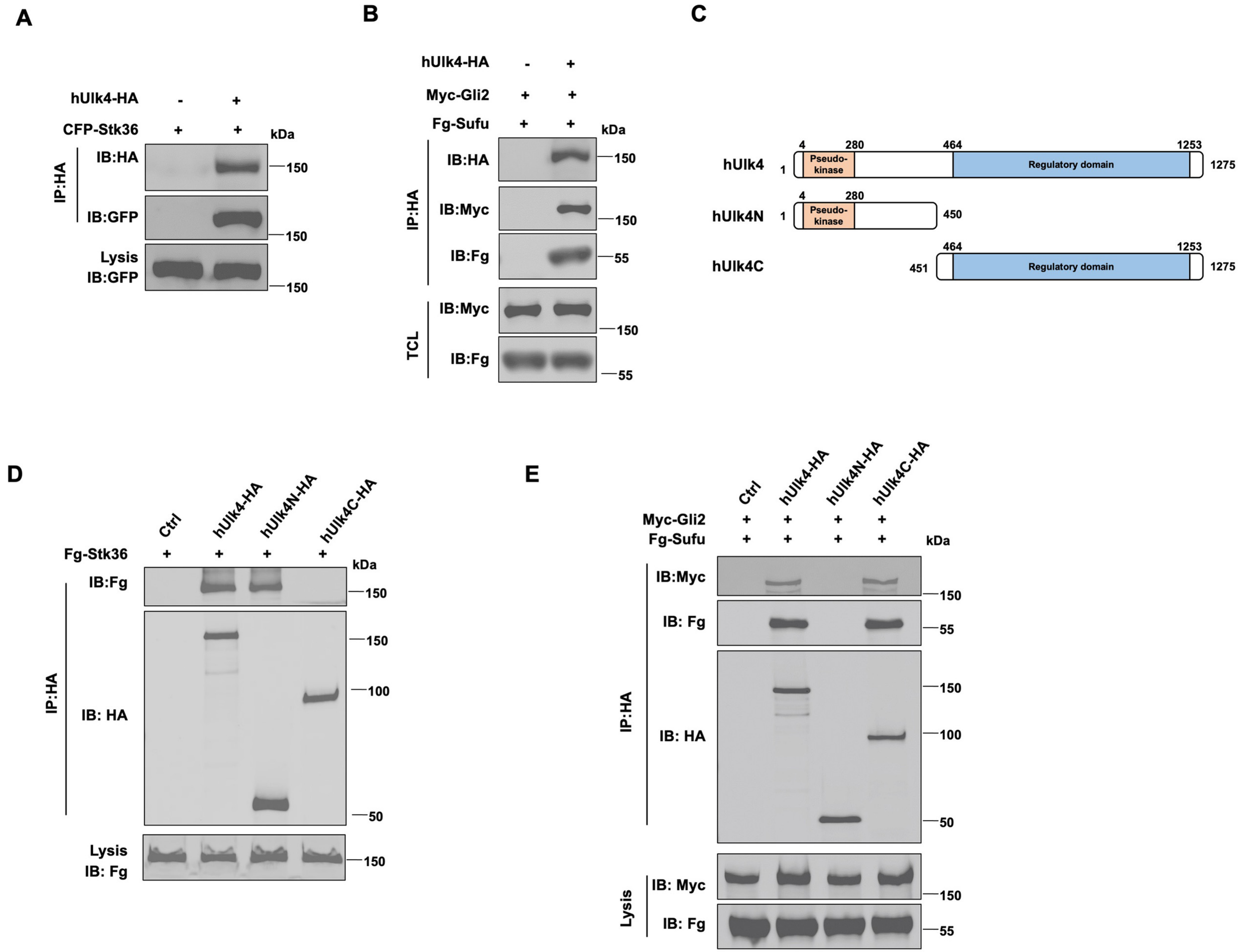
Shh
Ulk3
Stk36/Ulk4
GliA

Zhou_Fig2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Myc-Gli2 + +
Fg-Sufu + +
kDa
IP:HA
IB:HA
150
IB:Myc
150
IB:Fg
55
TCL
IB:Myc
150
IB:Fg
55
C
hUlk4
1
4
280
464
1253
1275
Pseudo-kinase
Regulatory domain
hUlk4N
1
4
280
450
Pseudo-kinase
hUlk4C
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464
1253
1275
Regulatory domain
D
Ctrl
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hUlk4N-HA
hUlk4C-HA
Fg-Stk36 + + + +
kDa
IB:Fg
150
IP:HA
IB: HA
150
100
50
Lysis
IB: Fg
150
E
Ctrl
hUlk4-HA
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Myc-Gli2 + + + +
Fg-Sufu + + + +
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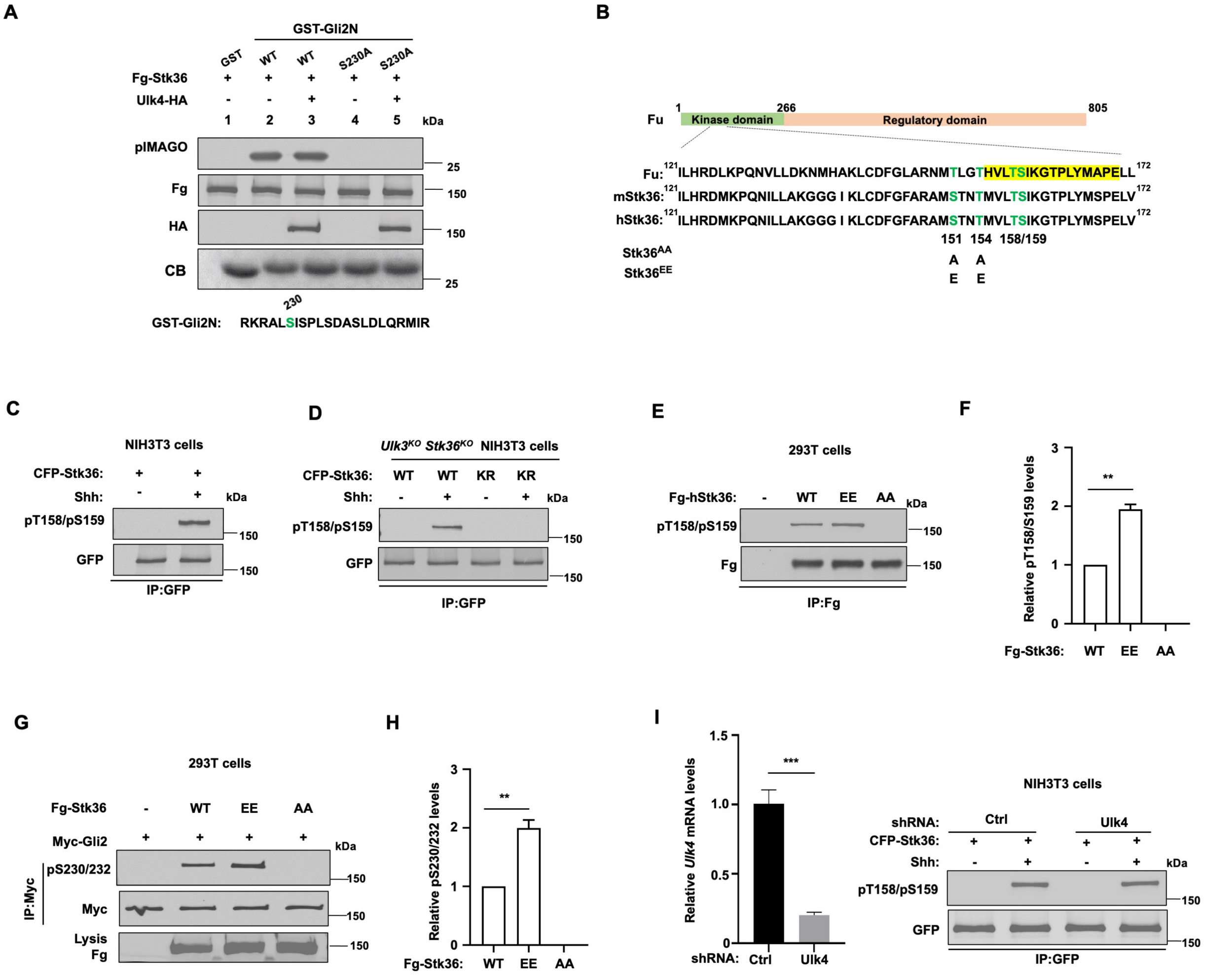
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HA
CB
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150
25
230
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B
Fu
1
266
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Kinase domain
Regulatory domain
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Stk36AA
Stk36EE
A A
E E
C
NIH3T3 cells
CFP-Stk36: + +
Shh: - + kDa
pT158/pS159
GFP
150
150
IP:GFP
D
Ulk3KO Stk36KO NIH3T3 cells
CFP-Stk36: WT WT KR KR
Shh: - + - + kDa
pT158/pS159
GFP
150
150
IP:GFP
E
293T cells
Fg-hStk36: - WT EE AA kDa
pT158/pS159
Fg
150
150
IP:Fg
F
Relative pT158/S159 levels
3
2
1
0
**
Fg-Stk36: WT EE AA
G
293T cells
Fg-Stk36 - WT EE AA
Myc-Gli2 + + + + kDa
IP:Myc
pS230/232
Myc
Lysis Fg
150
150
150
H
Relative pS230/232 levels
3
2
1
0
**
Fg-Stk36: WT EE AA
I
Relative Ulk4 mRNA levels
1.5
1.0
0.5
0
***
shRNA: Ctrl Ulk4
NIH3T3 cells
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pT158/pS159
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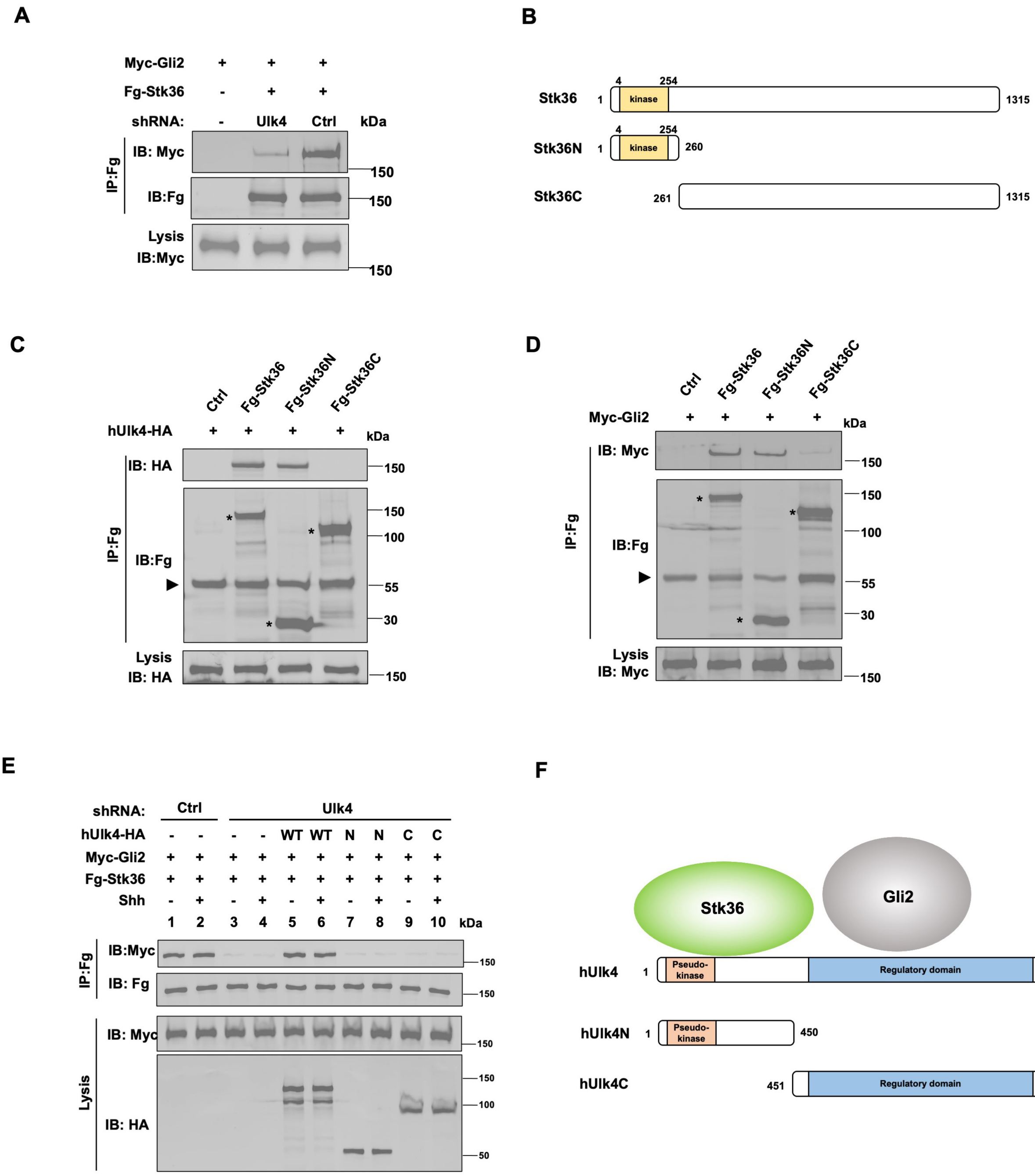
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150
150
B
Stk36
Stk36N
Stk36C
kinase
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254
1
1315
260
261
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Fg-Stk36N
Fg-Stk36C
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kDa
IB: HA
IB:Fg
Lysis
IB: HA
150
100
55
30
D
Myc-Gli2 + + + +
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IB:Fg
Lysis
IB: Myc
E
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hUlk4-HA - - - - WT WT N N C C
Myc-Gli2 + + + + + + + + + +
Fg-Stk36 + + + + + + + + + +
Shh - + - + - + - + - +
1 2 3 4 5 6 7 8 9 10 kDa
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IB: Fg
Lysis
IB: Myc
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150
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Pseudo-kinase
Regulatory domain
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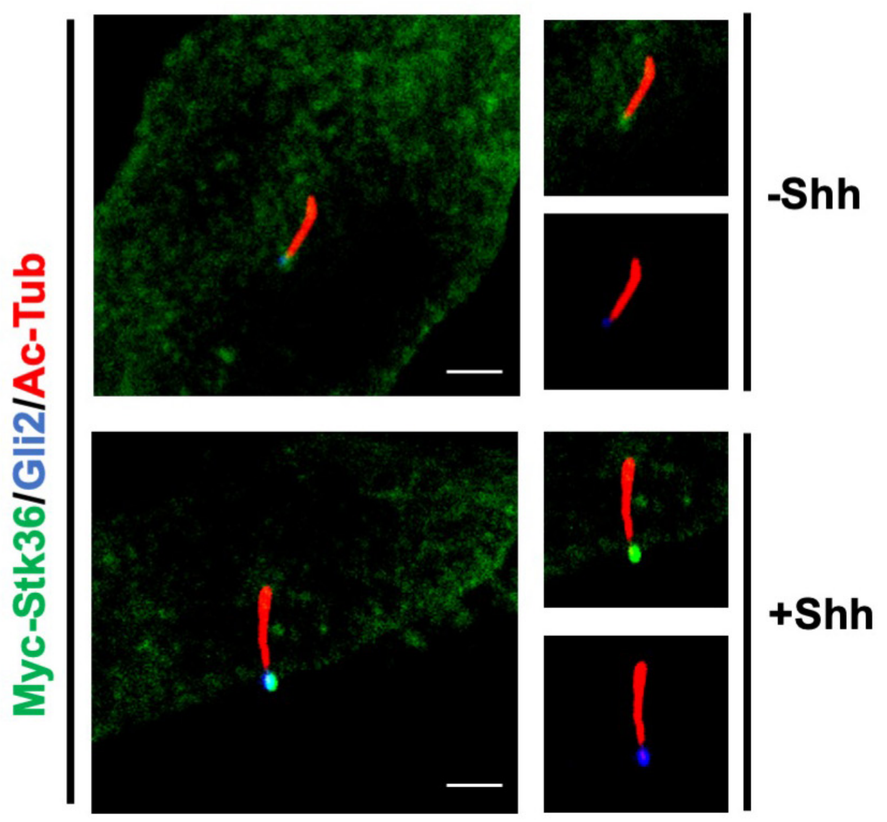
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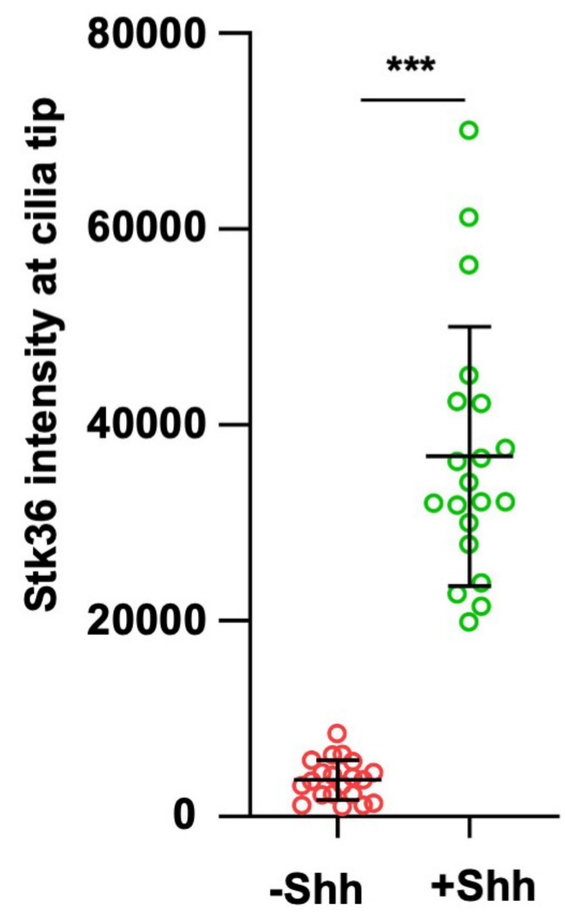
+Shh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Stk36 intensity at cilia tip
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60000
40000
20000
0
***
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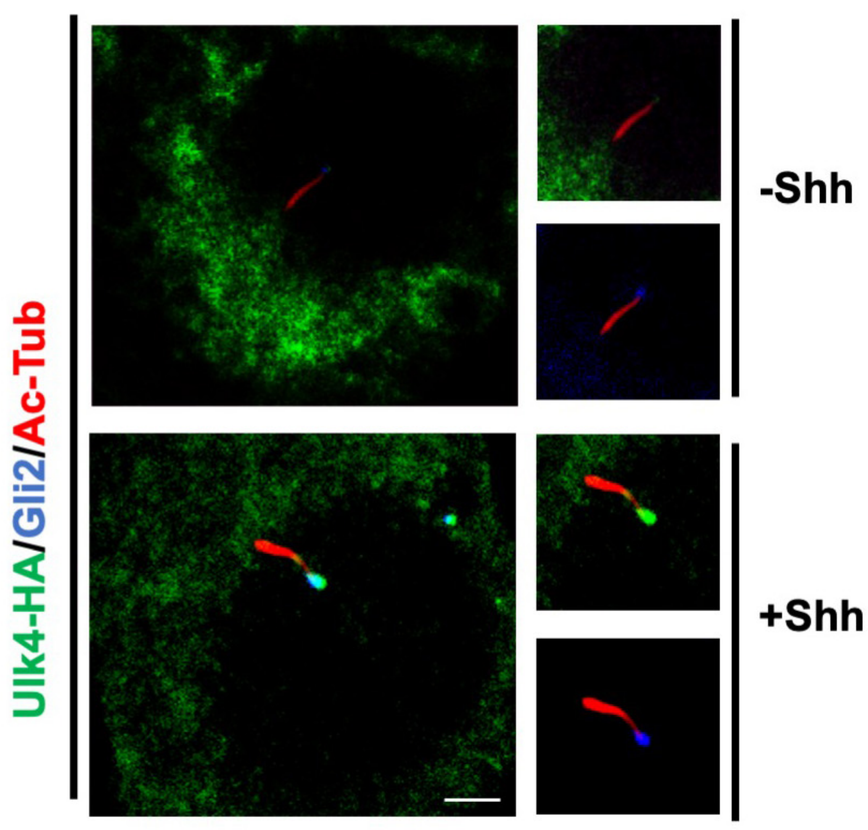
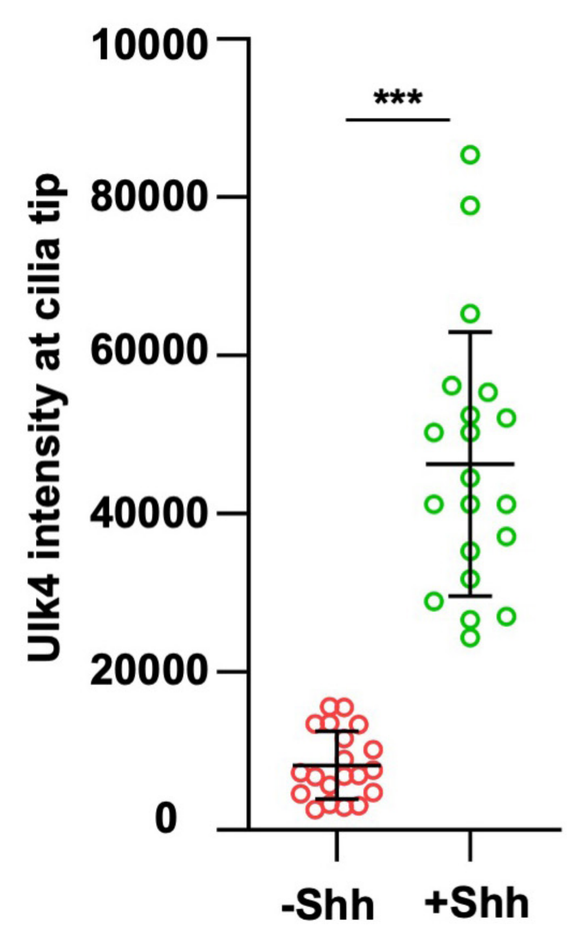
+Shh


C
Ulk4-HA/Gli2/Ac-Tub
-Shh
+Shh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Ulk4 intensity at cilia tip
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80000
60000
40000
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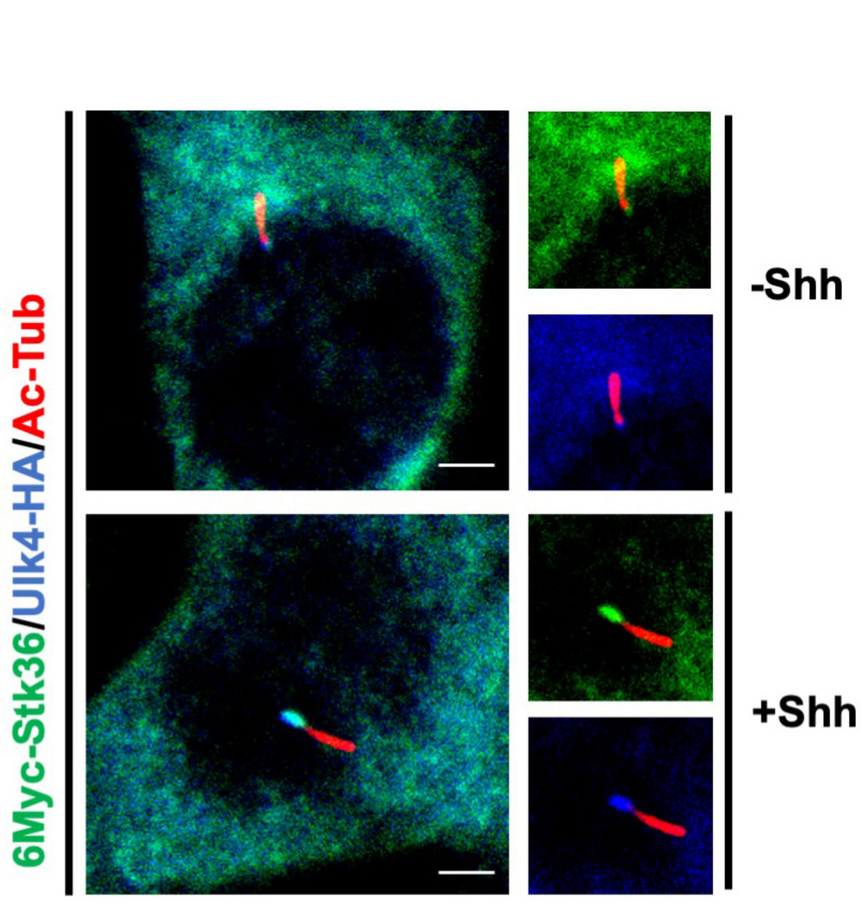
0
***
-Shh
+Shh


E
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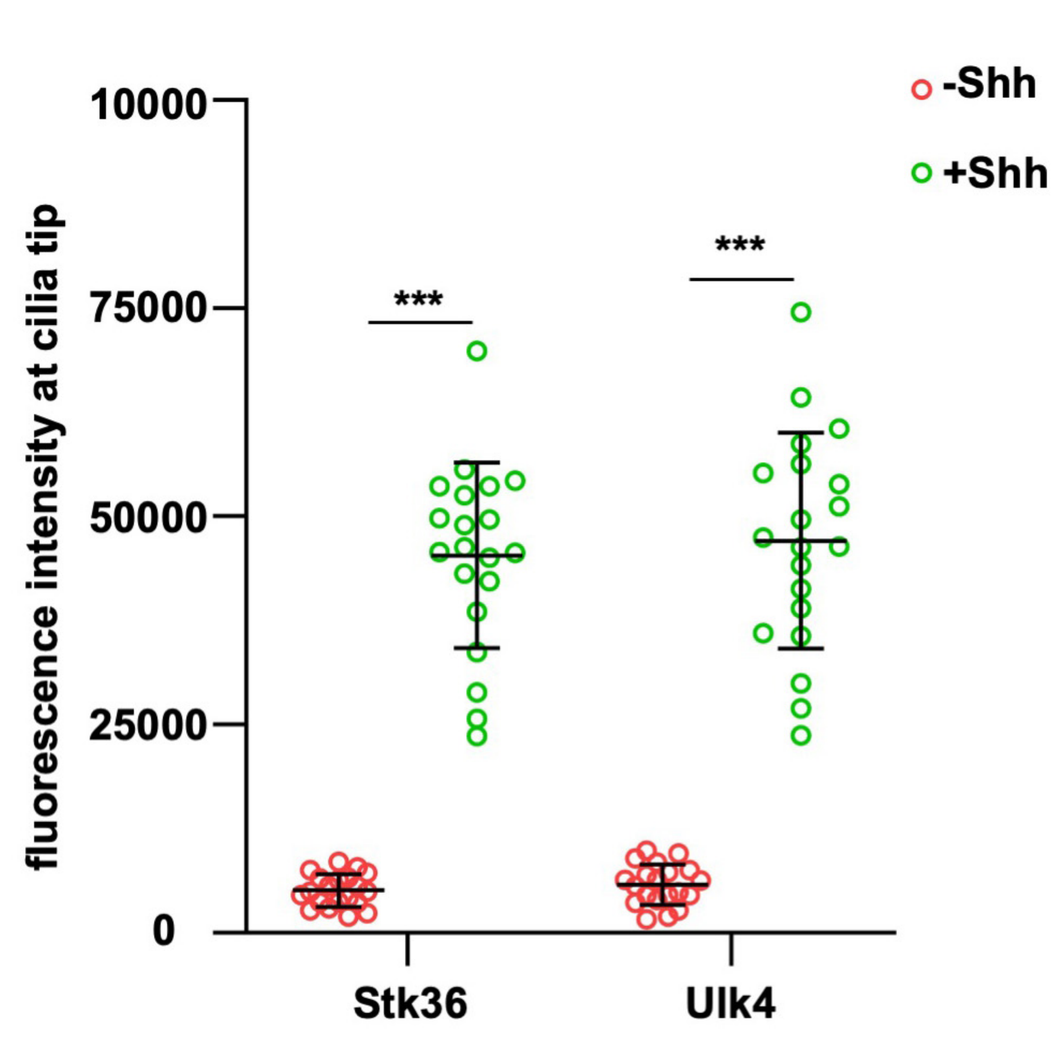
-Shh
+Shh


F
fluorescence intensity at cilia tip
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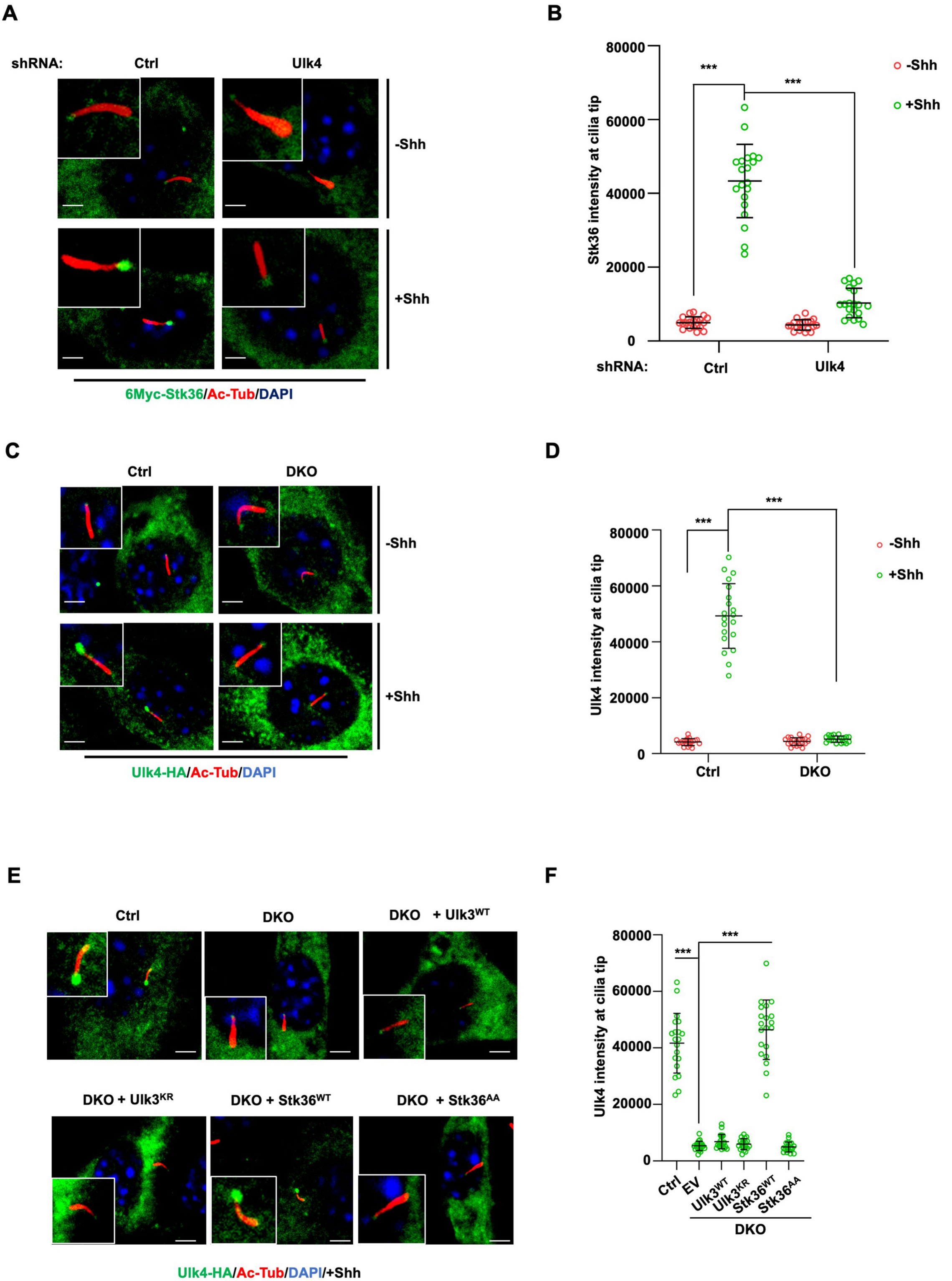
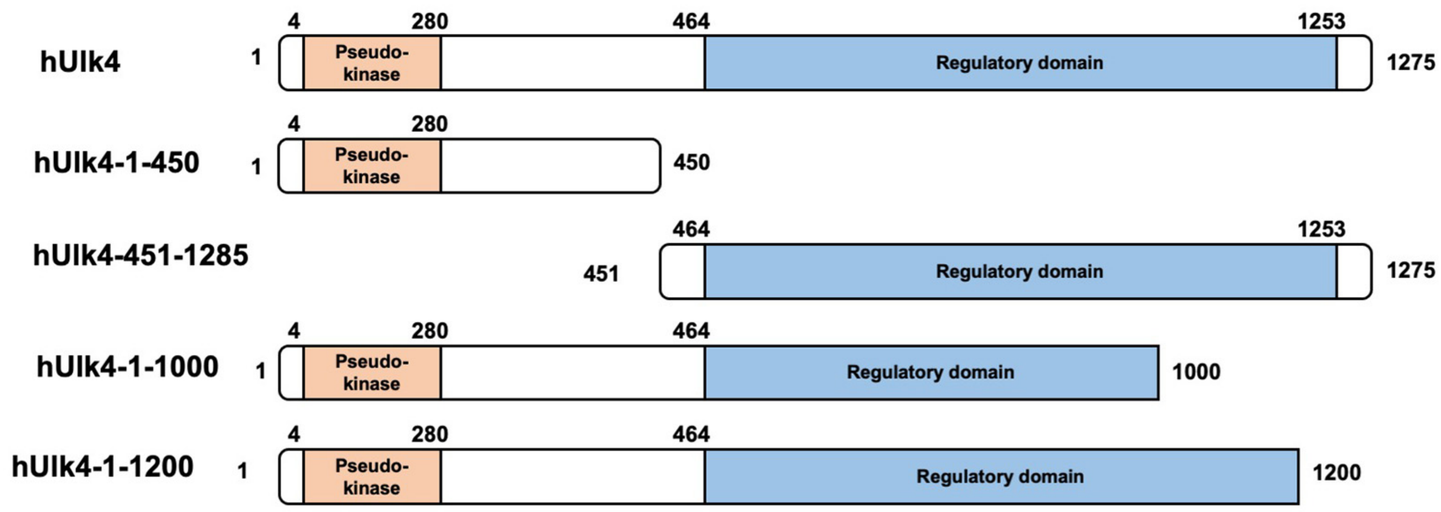
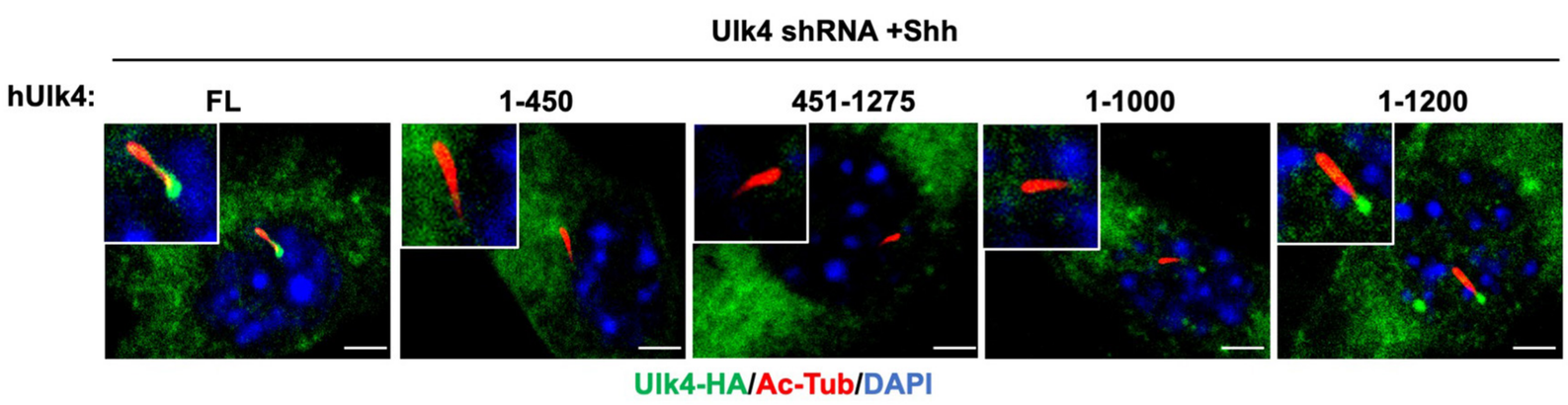
75000
50000
25000
0
***
***
Stk36
Ulk4
-Shh
+Shh


A
shRNA:
Ctrl
Ulk4
-Shh
+Shh
6Myc-Stk36/Ac-Tub/DAPI
B
Stk36 intensity at cilia tip
80000
60000
40000
20000
0
***
***
-Shh
+Shh
shRNA:
Ctrl
Ulk4
C
Ctrl
DKO
-Shh
+Shh
Ulk4-HA/Ac-Tub/DAPI
D
Ulk4 intensity at cilia tip
80000
60000
40000
20000
0
***
***
-Shh
+Shh
Ctrl
DKO
E
Ctrl
DKO
DKO + Ulk3WT
DKO + Ulk3KR
DKO + Stk36WT
DKO + Stk36AA
Ulk4-HA/Ac-Tub/DAPI/+Shh
F
Ulk4 intensity at cilia tip
80000
60000
40000
20000
0
***
***
Ctrl
EV
Ulk3WT
Ulk3KR
Stk36WT
Stk36AA
DKO

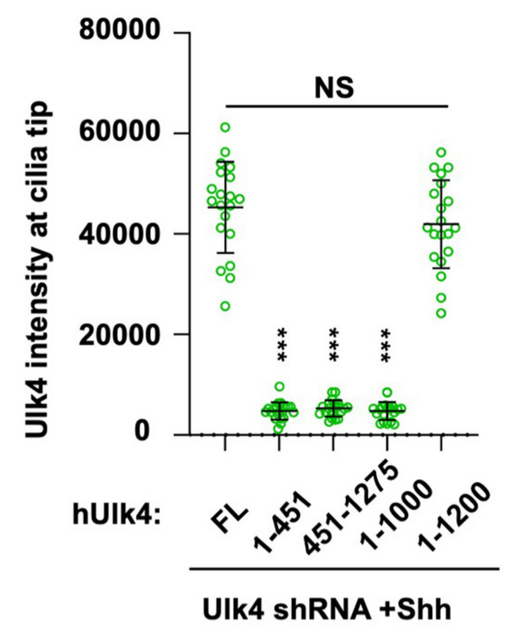
A


hUlk4
1
4
280
464
1253
1275
Pseudo-kinase
Regulatory domain
hUlk4-1-450
450
hUlk4-451-1285
451
hUlk4-1-1000
1000
hUlk4-1-1200
1200

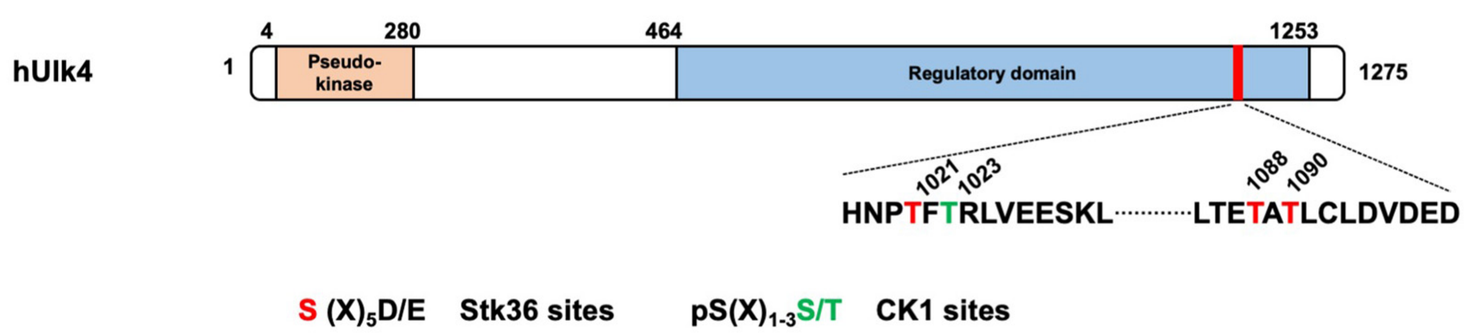
B


Ulk4 shRNA +Shh
hUlk4:
FL
1-450
451-1275
1-1000
1-1200
Ulk4-HA/Ac-Tub/DAPI

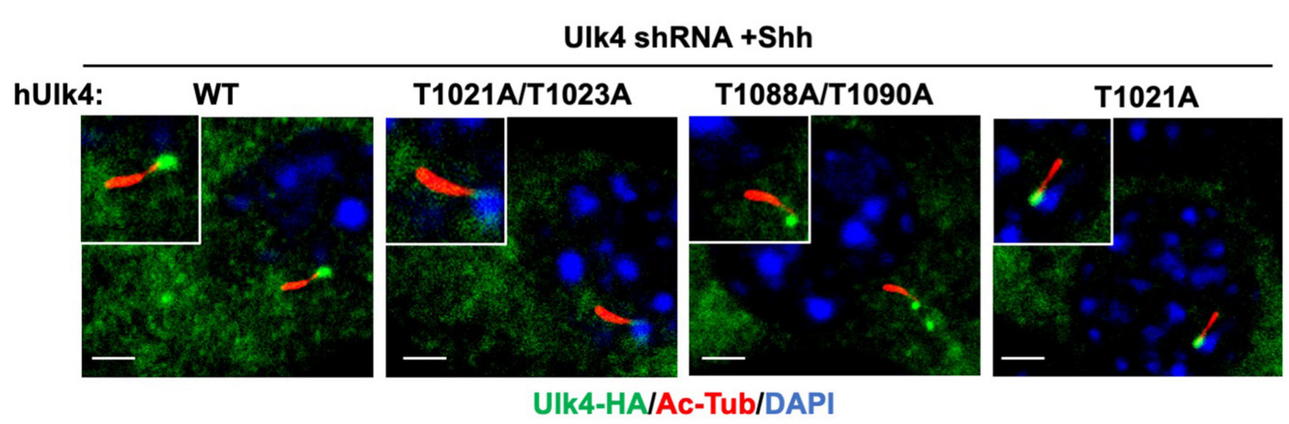
C


Ulk4 intensity at cilia tip
80000
60000
40000
20000
0
NS
***
***
***
hUlk4:
FL
1-451
451-1275
1-1000
1-1200
Ulk4 shRNA +Shh

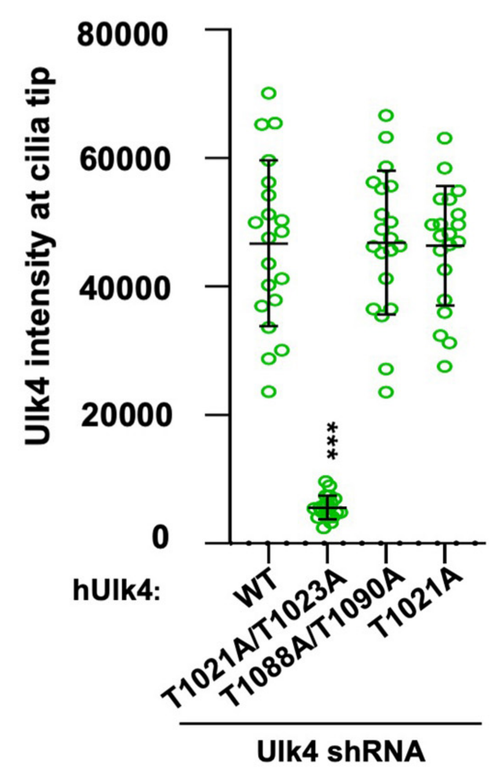
D


hUlk4
1
4
280
464
1253
1275
Pseudo-kinase
Regulatory domain
1021
1023
1088
1090
HNPTFTRLVEESKL
LTETATLCLDVDED
S (X)5D/E Stk36 sites
pS(X)1-3S/T CK1 sites

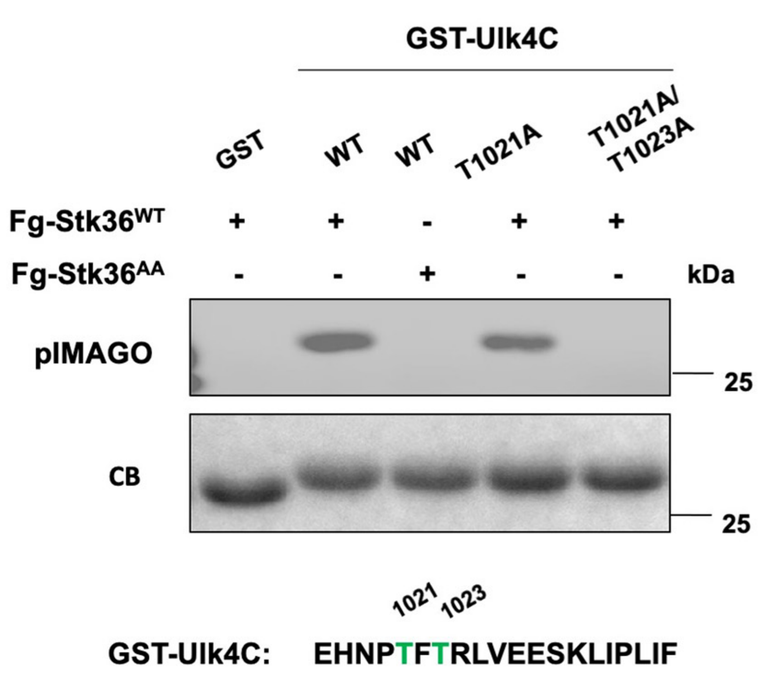
E


Ulk4 shRNA +Shh
hUlk4:
WT
T1021A/T1023A
T1088A/T1090A
T1021A
Ulk4-HA/Ac-Tub/DAPI

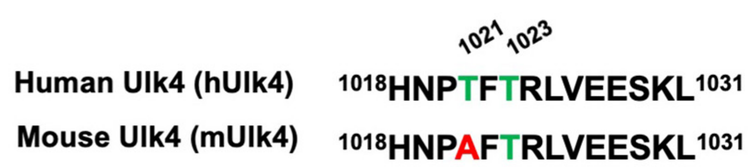
F


Ulk4 intensity at cilia tip
80000
60000
40000
20000
0
***
hUlk4:
WT
T1021A/T1023A
T1088A/T1090A
T1021A
Ulk4 shRNA

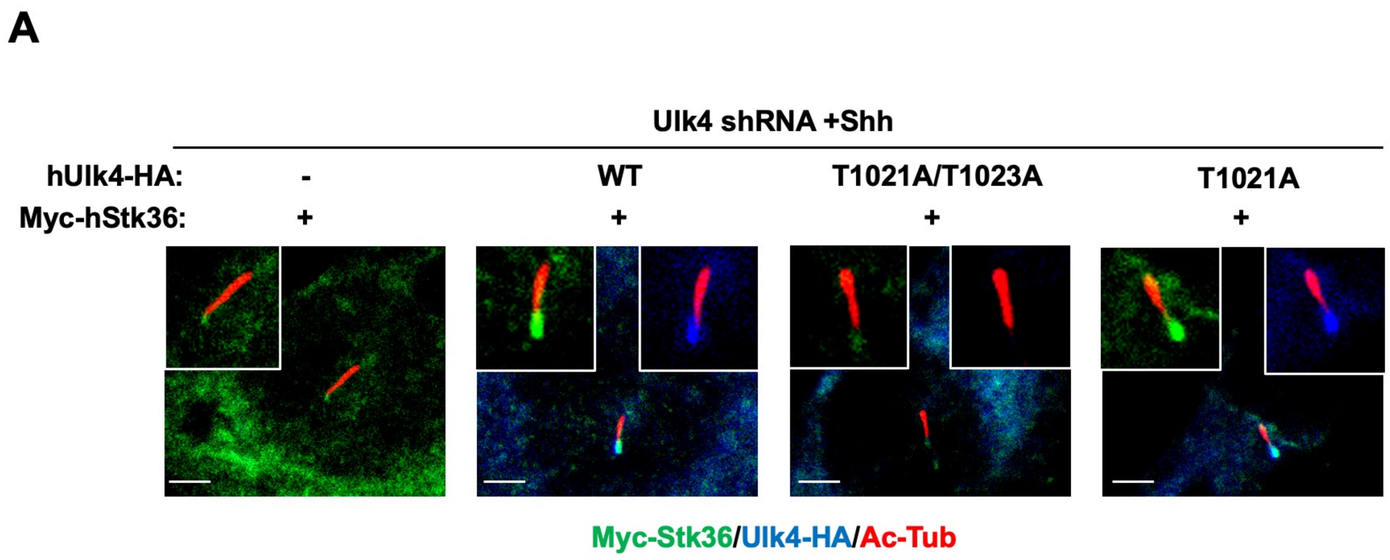
G

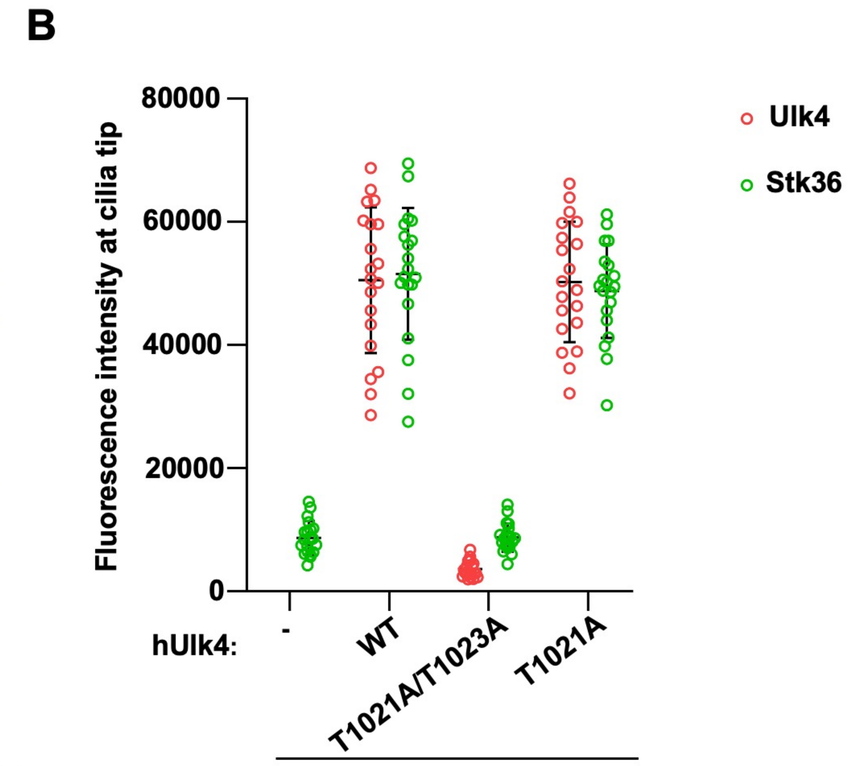

GST-Ulk4C
GST
WT
WT
T1021A
T1021A/T1023A
Fg-Stk36WT + + - + +
Fg-Stk36AA - - + - -
kDa
pIMAGO
25
CB
25
1021
1023
GST-Ulk4C: EHNPTFTRLVEESKLIPLIF

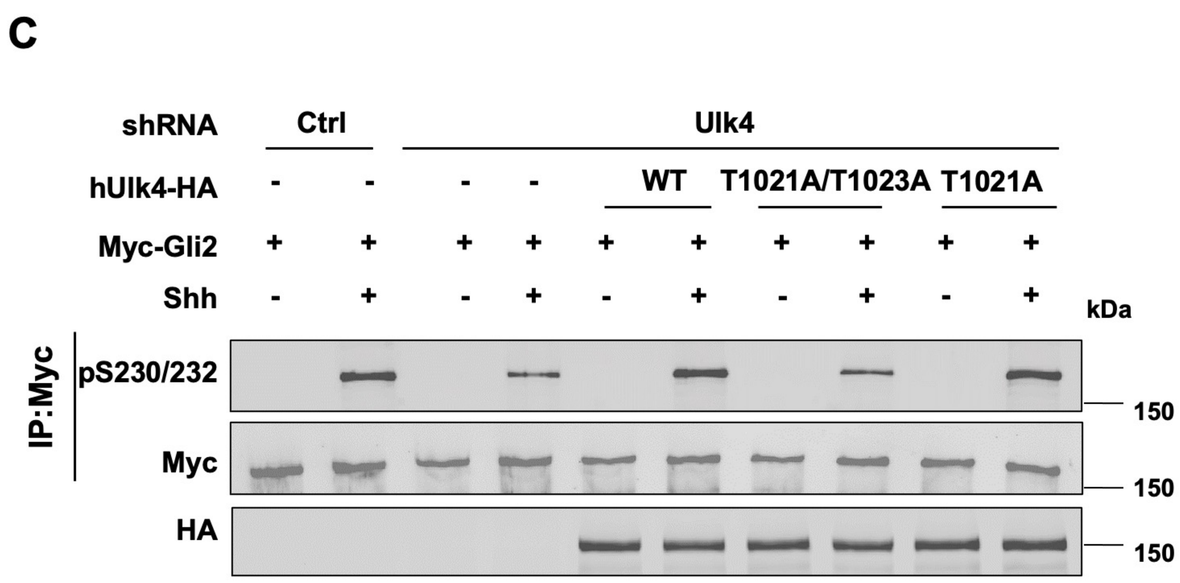
H

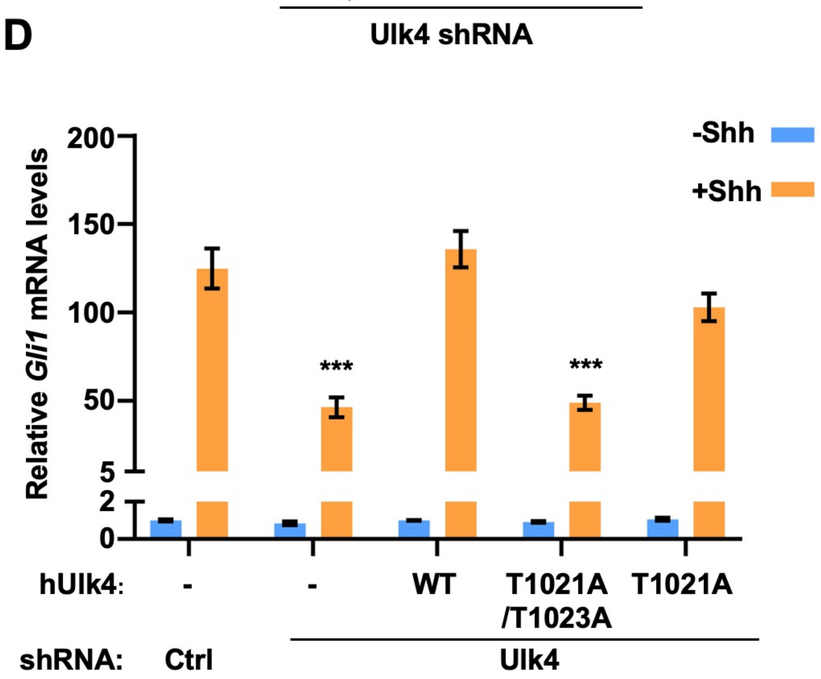

1021
1023
Human Ulk4 (hUlk4) 1018HNPTFTRLVEESKL1031
Mouse Ulk4 (mUlk4) 1018HNPAFTRLVEESKL1031

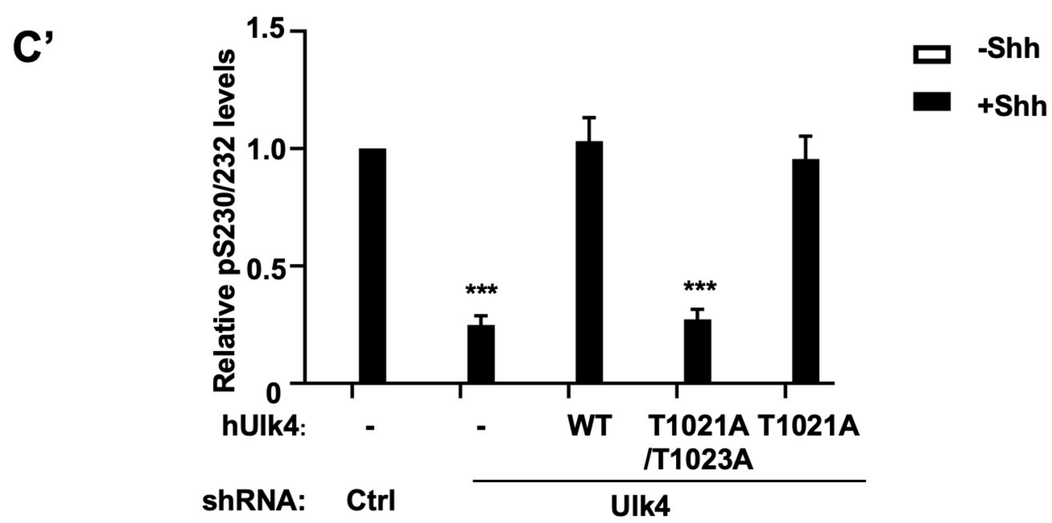
Zhou_Fig8

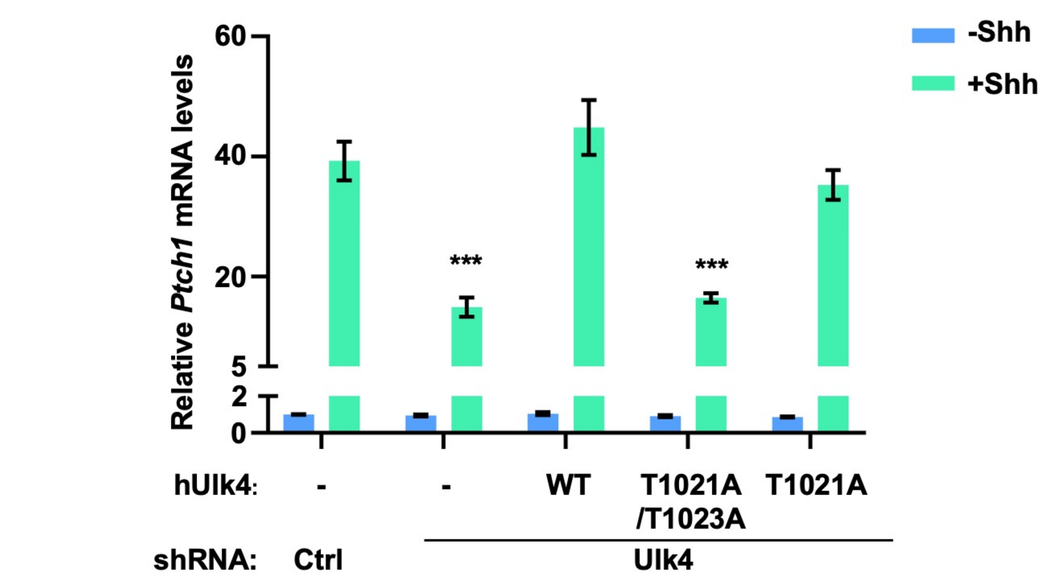

A
Ulk4 shRNA +Shh
hUlk4-HA: - WT T1021A/T1023A T1021A
Myc-hStk36: + + + +
Myc-Stk36/Ulk4-HA/Ac-Tub


B
Fluorescence intensity at cilia tip
80000
60000
40000
20000
0
Ulk4
Stk36
hUlk4: - WT T1021A/T1023A T1021A
Ulk4 shRNA


C
shRNA Ctrl Ulk4
hUlk4-HA - - - - WT T1021A/T1023A T1021A
Myc-Gli2 + + + + + + + + + +
Shh - + - + - + - + - +
kDa
IP:Myc
pS230/232
Myc
HA
150
150
150


D
Relative Gli1 mRNA levels
200
150
100
50
5
2
0
-Shh
+Shh
***
***
hUlk4: - - WT T1021A /T1023A T1021A
shRNA: Ctrl Ulk4


C'
Relative pS230/232 levels
1.5
1.0
0.5
0
-Shh
+Shh
***
***
hUlk4: - - WT T1021A /T1023A T1021A
shRNA: Ctrl Ulk4


E
Relative Ptch1 mRNA levels
60
40
20
5
2
0
-Shh
+Shh
***
***
hUlk4: - - WT T1021A /T1023A T1021A
shRNA: Ctrl Ulk4

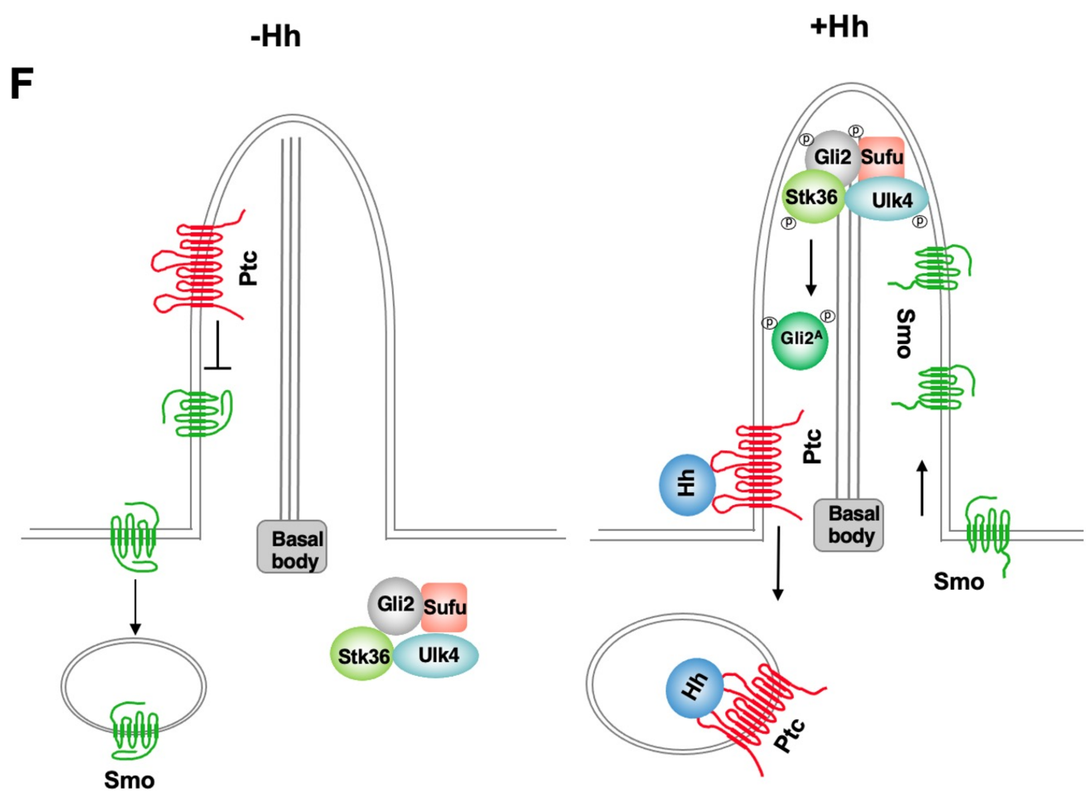

F
-Hh
+Hh
Ptc
Basal body
Smo
Gli2
Sufu
Stk36
Ulk4
Gli2A
Hh
Ptc
Smo
Basal body
Smo
Hh
Ptc

Fig1_figure supplement 1

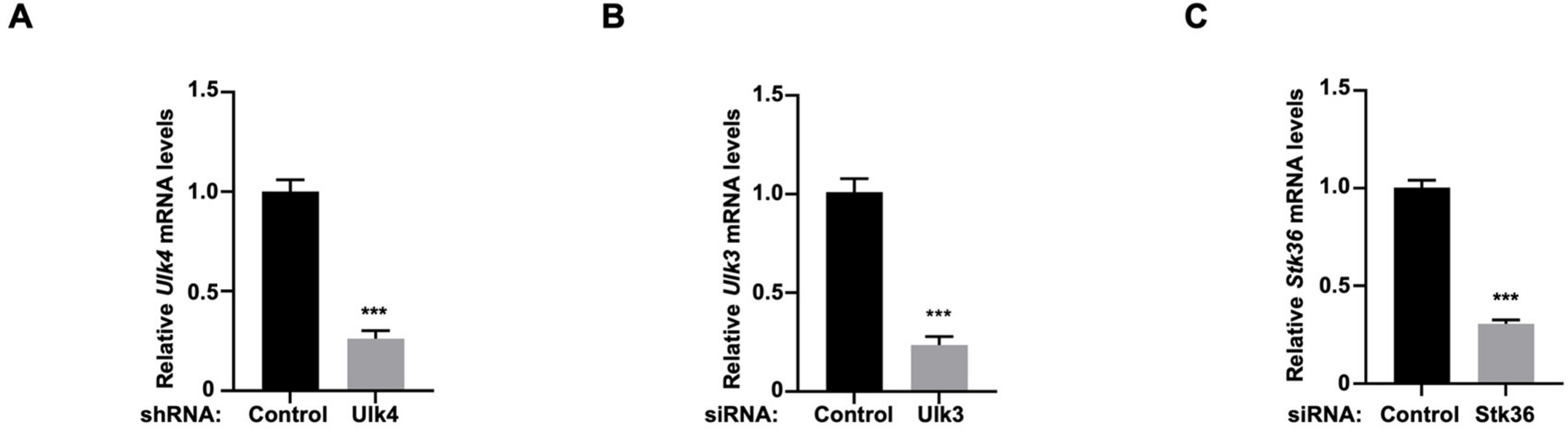
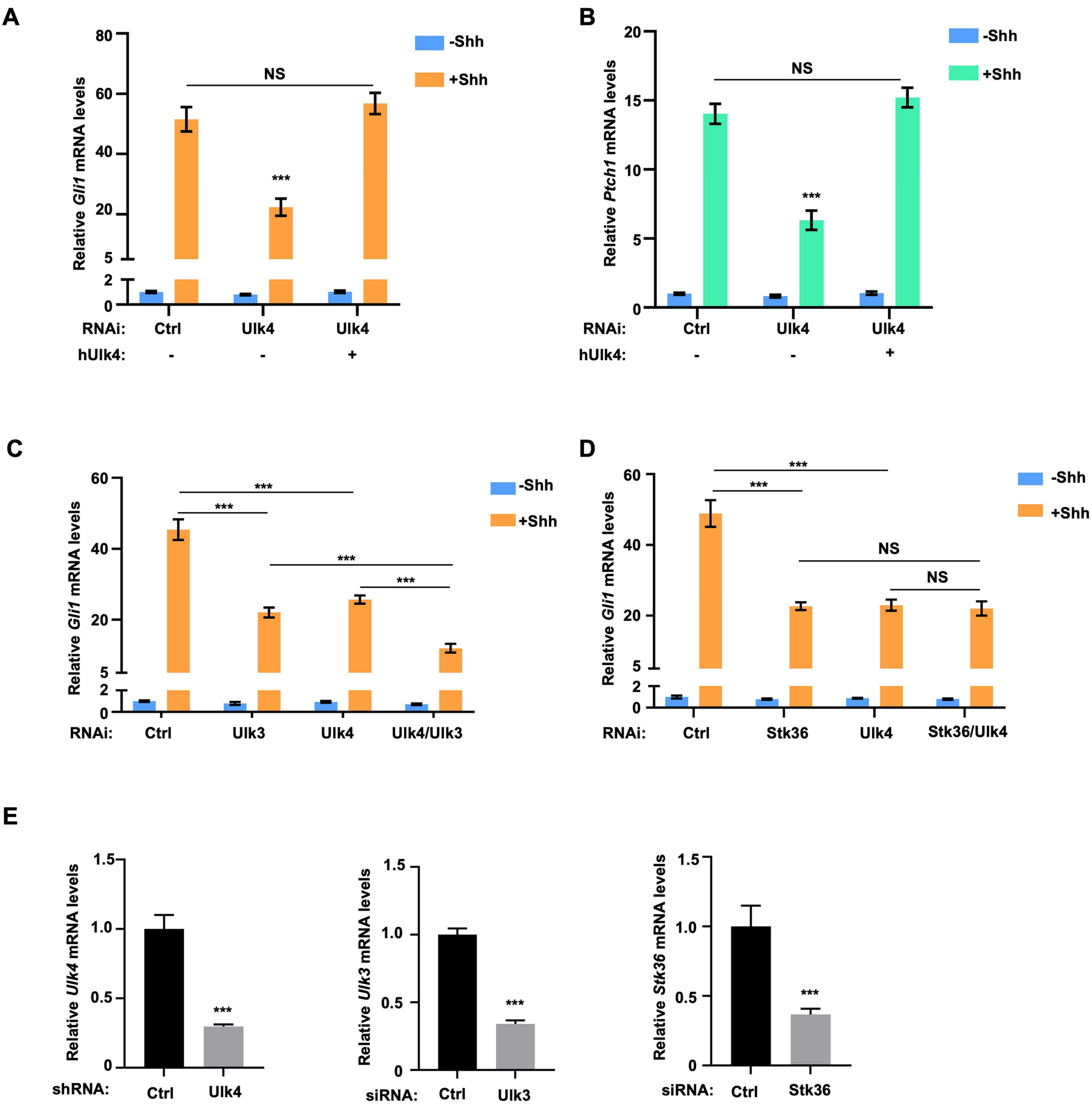
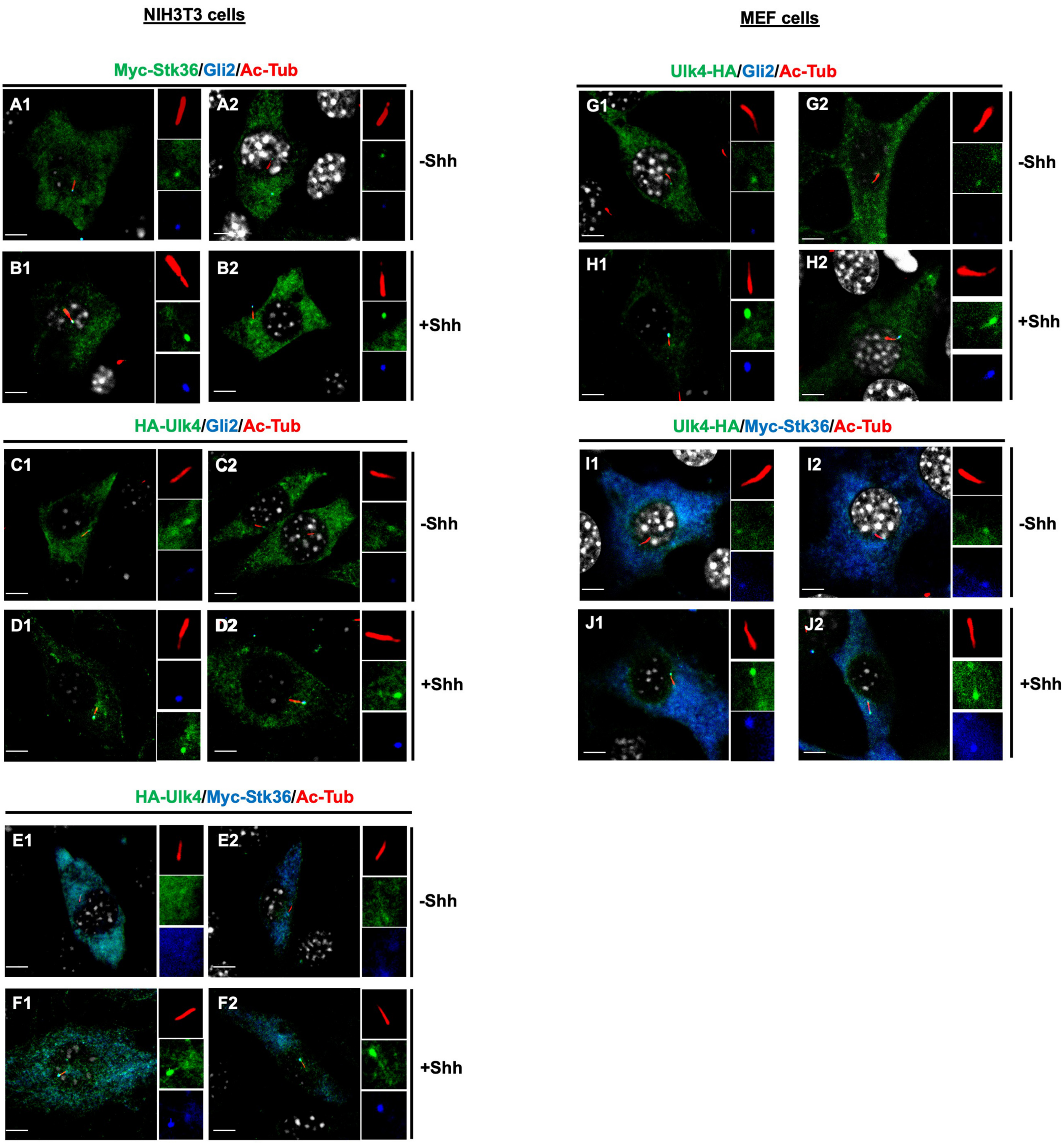


Fig1_figure supplement 2



Zhou_Fig5_figure supplement 1