

## SHORT COMMUNICATION

## The Yes-associated protein controls the cell density regulation of Hedgehog signaling

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The evolutionarily conserved Hedgehog (Hh) signaling pathway is essential for correct embryogenesis and is misregulated in several malignancies. In cell culture, Hh-sensitive cells display a striking dependence on cell density with active Hh signaling requiring cell-to-cell contact. As the Hippo/YAP system is tightly linked to cell density control and contact inhibition, we investigated the cross-talk between the two pathways. Our data reveal that the suppression of Hh signaling in the absence of cellular contacts is independent of primary cilia and is mediated by the *YAP* oncogene. Overexpression of *YAP* blocks Hh signaling whereas RNA interference-mediated knockdown of *YAP* enhances Hh/GLI activity. Despite this negative regulation, Hh signaling promotes *YAP* activity through post-transcriptional mechanisms, resulting in a negative feedback loop. *In vivo*, we found strong nuclear *YAP* immunoreactivity restricted to compartments with low Hh pathway activity in human and mouse pancreatic cancer. Finally, we identified protease-activated receptors (PARs) as molecules being able to override the inverse Hippo/Hh regulation, potentially giving tumors a mechanism to utilize both oncogenic pathways in parallel.

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## INTRODUCTION

The control of cellular density is of utmost importance for proper embryonic development and for the determination of organ size. In the past years, a serine/threonine kinase cascade (the Hippo pathway) has been identified as a major regulator of cell density control *in vivo* and *in vitro*.<sup>1–4</sup> The Hippo pathway eventually controls the transcriptional co-activator *YAP* (Yes-associated protein) and its paralog *TAZ*. Under conditions of low cell density, the Hippo pathway is inactive and *YAP/TAZ* enter the nucleus to induce target gene transcription.<sup>5</sup> In contrast, under conditions of high cellular density, the Hippo cascade is turned on, resulting in the phosphorylation of *YAP/TAZ* by *LATS* (large tumor suppressor) kinases and their subsequent inactivation by cytoplasmic retention. In order to achieve their biological effects, nuclear *YAP/TAZ* partner with several transcription factors, of which members of the *TEAD* family are best studied.<sup>6</sup> These phenomena are recapitulated in an *in vitro* process termed *contact inhibition*, indicating the growth arrest that normal cells undergo when they have reached full confluency in a culture plate. This process is also dependent on and regulated by Hippo/*YAP* activity. In line with Hippo/*YAP* activity controlling cellular (over-) growth, several cancers have been linked to elevated nuclear *YAP* presence.<sup>1,3,7</sup>

Another developmentally important pathway that is deregulated in cancer is the Hedgehog (Hh) signaling cascade.<sup>8,9</sup> By binding to their *PTCH1/2* receptors, Hh ligands derepress the transmembrane protein *Smoothed* (*SMO*) that initiates additional steps to activate the *GLI* (glioma-associated oncogene) family of transcription factors. At least in culture, Hh signaling is dependent on cell-to-cell contacts and high Hh pathway activity can only be achieved under confluent conditions.<sup>10</sup> Moreover, as

an oncogenic signaling entity, Hh signaling is able to override the contact inhibition of normal fibroblasts.<sup>11,12</sup> Given these density-controlled processes, we investigated the molecular cross-talk between the Hippo/*YAP* and the Hh/*GLI* systems. We could find that *YAP* binds to and negatively controls the activity of *GLI* transcription factors, leading to a repression of Hh pathway target genes. In contrast, Hh pathway activity promotes *YAP* post-transcriptionally by increasing its protein levels, suggestive of a negative feedback loop. In line with a negative Hippo-to-Hh regulation, we found active (nuclear) *YAP* in tumor compartments displaying low Hh pathway activity in pancreatic cancer specimens, but only very little nuclear *YAP* in compartments with high Hh activity.

Assuming that additional potential molecular cues exist that allow for the simultaneous activation of both oncogenic pathways, we identified the protease-activated receptor (PAR) system as a means to overcome the negative Hippo/Hh cross-talk. Thus, the PAR system might represent a promising novel drug target in Hippo/Hh double-positive tumors.

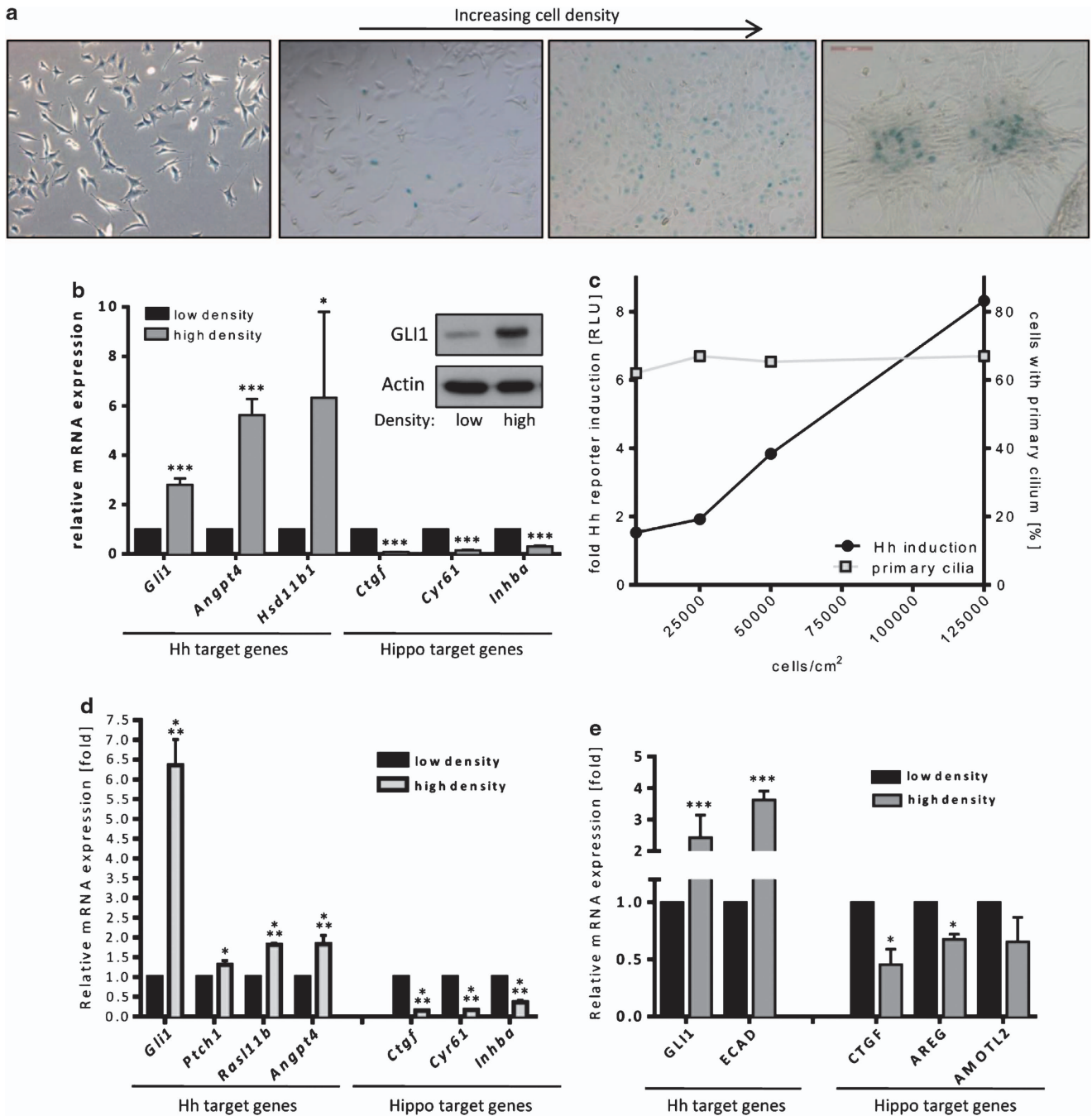
## RESULTS AND DISCUSSION

The Hh pathway is controlled in a density-dependent manner. Previous reports established that Hh signaling in cultured cells is most functional under high cellular densities.<sup>10</sup> We repeated this experiment using *Ptch1*  $-/-$  mouse embryonic fibroblasts (MEFs) that show constitutive high Hh signaling because of the loss of the negative regulator *Ptch1*. The *Ptch1* gene knockout in these cells was achieved by insertion of a *lacZ* reporter sequence into the endogenous *Ptch1* locus. This placed the *lacZ* expression cassette

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**Figure 1.** The expression of Hh target genes is density dependent and is inversely correlated with Hippo target genes. **(a)** X-gal staining for  $\beta$ -galactosidase activity (blue) of *Ptch1*<sup>-/-</sup> MEFs plated in different cellular densities. Blue color indicates active Hh signaling. **(b)** Hh and Hippo target gene expression in *Ptch1*<sup>-/-</sup> MEFs (plated in low (subconfluent) and high (confluent) densities with 1% fetal bovine serum (FBS)) as measured by quantitative PCR (Qpcr). The inset depicts the GLI1 protein levels in relationship to *Ptch1*<sup>-/-</sup> culture confluency. **(c)** Cell density-dependent Hh reporter activity in ShhL2 cells. The cells were plated in different densities (1% FBS) and induced with the SMO agonist SAG (100 nM for 48 h). Also shown is the (cell density independent) frequency of primary cilia in these cells, as measured by acetylated tubulin staining (Sigma-Aldrich, Taufkirchen, Germany, T6793) (1% FBS). **(d)** Hh and Hippo target gene expression in ShhL2 cells plated in different cell densities. **(e)** Hh and Hippo target gene expression in human Panc1 cells plated in different cell densities (1% FBS). \* $P < 0.05$ ; \*\* $P < 0.005$ ; \*\*\* $P < 0.0005$  (two-tailed Student's *t*-test).

under the control of the endogenous *Ptch1* regulatory elements and Hh pathway activity can thus be measured by detecting lacZ enzyme activity.<sup>10</sup> To our surprise, plating *Ptch1*<sup>-/-</sup> MEFs in different cell densities and staining for  $\beta$ -galactosidase activity (blue color in Figure 1a) revealed that only cells that encountered the highest cell densities, such as those in the center of cell foci, displayed clear Hh pathway activation (Figure 1a). The density-

dependent regulation of Hh signaling in *Ptch1*<sup>-/-</sup> MEFs was also verified on the expression level (Figure 1b): Hh target genes (*Gli1*, *Angpt4*, and *Hsd11b1*) were upregulated in cultures with high cell density compared with low density cultures. In contrast, the density-regulated Hippo/YAP transcriptional targets *Ctgf*, *Cyr61* or *Inhba* were downregulated upon higher confluency, suggesting an inverse relationship between Hh and Hippo target gene

expression. Next, we verified our results on density control of the Hh pathway in ShhL2 cells, a clonal NIH3T3 cell line stably expressing a luciferase Hh/GLI reporter construct.<sup>13</sup> As expected, the Hh pathway induction by addition of the SMO agonist SAG<sup>13</sup> was strongly density dependent (Figure 1c). Importantly, primary cilium frequencies remained similar across the different culture densities, demonstrating that the observed density-dependent pathway control was not mediated by altered cilium formation. As before, the density control could be verified on the target gene expression level for the Hh (*Gli1*, *Ptch1*, *Rasl11b*, and *Angpt4*) as well as the Hippo (*Ctgf*, *Cyr61* and *Inhba*) system (Figure 1d). Again, Hh target genes were upregulated by high cell densities, whereas YAP target genes were suppressed under these conditions. A similar inverse mode of density-dependent regulation could also be found in other cell lines, for instance, in human pancreatic adenocarcinoma cells: the Hh target genes (*GLI1* and *E-CADHERIN* (*ECAD*)<sup>14</sup> were induced by high cell density, whereas the YAP target genes (*CTGF*, *AREG*, and *AMOTL2*) were downregulated (Figure 1e). Taken together, these experiments showed that high Hh pathway activity is strongly density regulated, is not mediated by altered primary cilium frequency and is paralleled by an inverse modulation of YAP activity.

#### Increased YAP levels suppress Hh/GLI activity

In order to elucidate whether YAP is causally involved in regulation of the Hh pathway, we generated Hek293T cells stably expressing a dominant active version of YAP (YAP2<sup>(SSA)</sup>), a mutant form of the YAP2 splice variant with increased protein stability and nuclear presence<sup>3</sup>). In agreement with a suppressive functional role of YAP on the Hh pathway, cells expressing YAP2<sup>(SSA)</sup> displayed a significant reduction in Hh pathway target gene expression (*GLI1*, *PTCH1*, and *HIP1*), demonstrating that YAP expression is sufficient for this effect and that no other upstream Hippo pathway elements are required (Figure 2a). Next, we wondered whether the YAP presence directly impinged on the transcriptional activity of GLI transcription factors. To this end, we performed a Hh reporter assay in human fibroblasts (pancreatic stellate cells<sup>15</sup>) expressing *GLI1* (or its naturally occurring splice variant *GLI1ΔN*<sup>16</sup>). As shown in Figure 2b, the expression of the dominant active YAP mutant strongly reduced GLI1 activity, whereas the expression of wild-type YAP had no effect, implying that it was nuclear YAP that repressed GLI1 activity. YAP2<sup>(SSA)</sup> could also potentially inhibit GLI1ΔN that lacks one of the two binding sites for Suppressor of Fused (SUFU, a major negative regulator of the pathway<sup>17</sup>), strongly suggesting that the mechanism of inhibition does not involve SUFU. The antagonistic behavior of YAP2<sup>(SSA)</sup> on GLI1 activity could additionally be verified in other cell lines (Supplementary Figure S1). Furthermore, the repression of the Hh pathway in pancreatic stellate cells was also documented on the mRNA level upon transient transfection with dominant active YAP (Figure 2c). Finally, expression of nuclear YAP inhibited endogenous Hh signaling in SAG-stimulated NIH3T3 cells as measured by a luminometric Hh reporter assay (Figure 2d).

Mechanistically, we found that both exogenous and endogenous GLI1 and YAP proteins could be co-immunoprecipitated from cellular lysates (Figures 2e and f), suggesting a direct interaction. This result was also verified in a second cell line (Supplementary Figure S2A). Moreover, YAP overexpression did not sequester GLI1 away from the nucleus (Supplementary Figure S2B), implying a nuclear YAP/GLI interaction that is responsible for the negative Hh pathway modulation.

#### Knockdown of endogenous YAP increases Hh pathway activity

Next, we went on to investigate whether endogenous YAP levels could regulate endogenous Hh pathway activity. To this end, we knocked down *Yap* mRNA by means of a pool of RNA interference sequences in MEF cells stably expressing the Sonic Hedgehog

(SHH) ligand (MEF<sup>(SHH)</sup> cells<sup>18</sup>). As can be seen in Figure 3a, knockdown of *Yap* increased the protein expression of GLI1, indicative of Hh pathway derepression. A similar finding was made in Hek293T cells stably expressing a short hairpin RNA construct targeting *YAP* (Figure 3b). In addition, human pancreatic stellate cells transfected with small interfering RNA against *YAP* upregulated *GLI1* mRNA. Interestingly, knocking down the YAP paralog *TAZ* also resulted in Hh pathway derepression, suggesting that *YAP* and *TAZ* have overlapping functions with respect to Hh. These findings were verified by separating the small interfering RNA pool into its individual small interfering RNA constructs (Supplementary Figure S3A).

Most importantly, pronounced suppression of Hippo target genes (*CTGF* and *ANKRD1*) was only achieved after dual *YAP/TAZ* knockdown (Figure 3c). In contrast, *GLI1* upregulation was already seen upon single knockdown of either *YAP* or *TAZ*, implying that it was *YAP* or *TAZ* itself—and not their target genes—that was responsible for Hh pathway control (Figure 3c). A similar derepression of *GLI1* expression was also observed in Panc1 cells after combined knockdown of *YAP/TAZ* (Figure 3d).

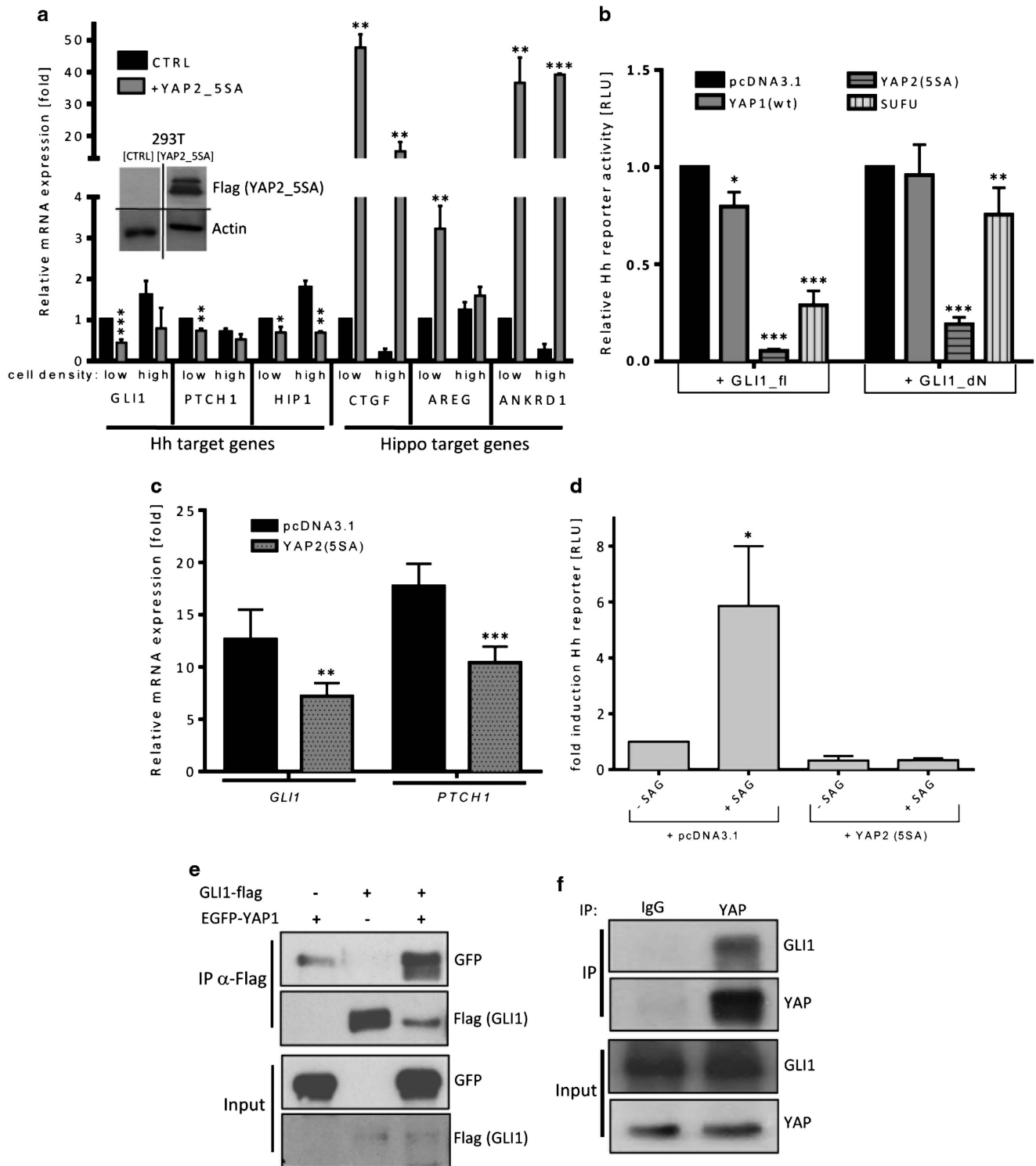
Having analyzed the effects of Hippo signaling on the Hh pathway, we wanted to analyze the effect of Hh signaling on the Hippo pathway. Immunoblot experiments revealed that YAP protein levels were significantly induced in NIH3T3 cells treated with the SMO agonist SAG (Figure 3e). In addition, YAP target genes (*Ctgf* and *Inhba*) were upregulated by SAG exposure (Figure 3f). Intriguingly, the increase in YAP levels was not mediated by a transcriptional upregulation of *Yap* (or *Taz*) mRNA levels (Figure 3f), suggesting post-transcriptional mechanisms. Identical findings were made using NIH3T3<sup>(SHH)</sup> cells in which the active Hh pathway was blocked by addition of the SMO antagonist SANT1 (ref. 13) (Supplementary Figures S3B and C). Following up on these results, we observed that Hh pathway activation by SAG extended the half-life of endogenous YAP protein from ~2.5 h to ~4.5 h (Figure 3g). In general, these findings are in agreement with reports on Hh signaling being able to overcome contact inhibition in culture.<sup>11,12</sup>

Taken together, we postulate that endogenous YAP (and TAZ), which is under the control of cellular density, regulates the Hh pathway at the level of GLI transcription factors.

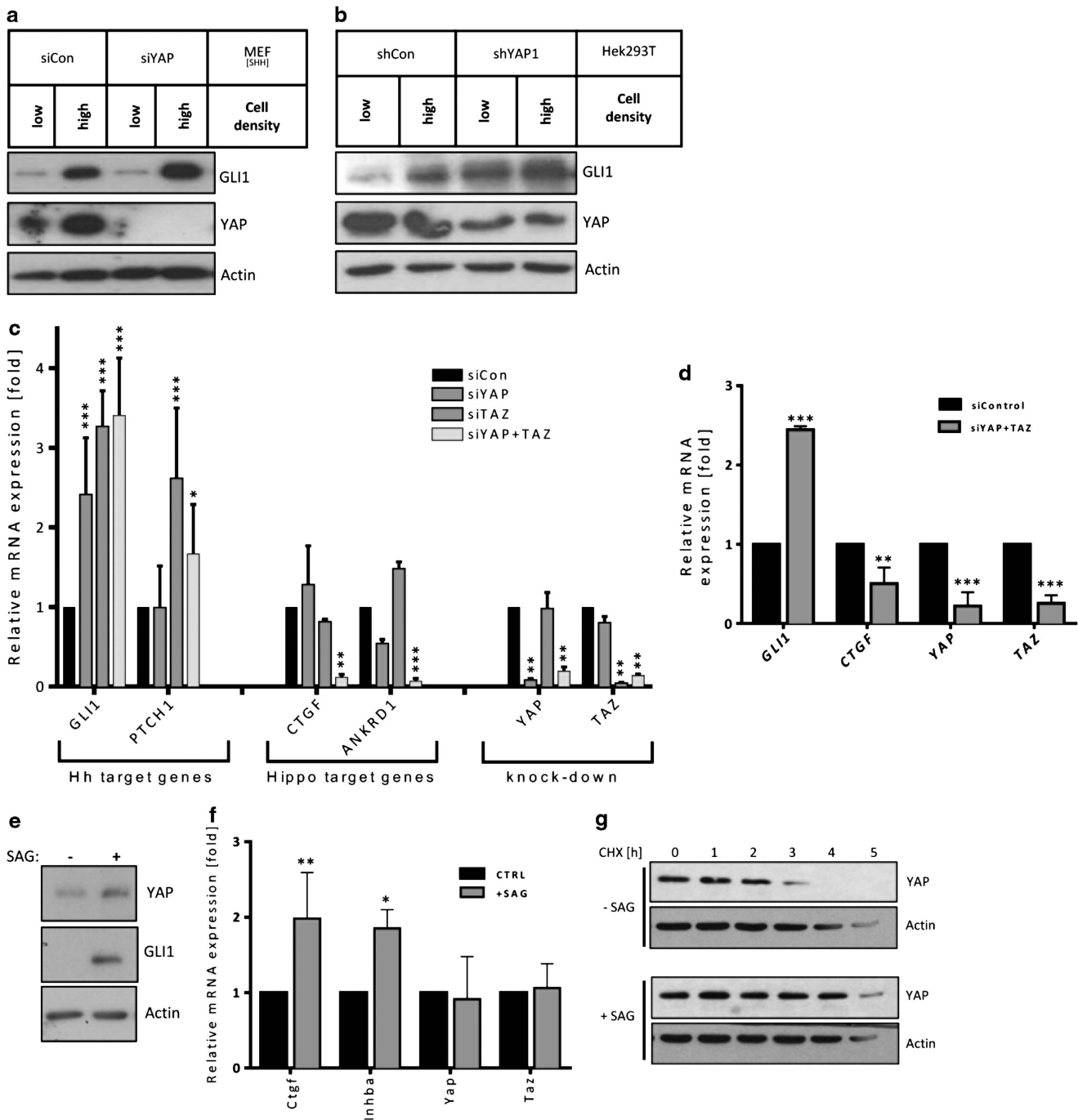
#### PARs can activate YAP and GLI

In light of the inverse correlation between YAP and Hh/GLI activity, we wanted to investigate the distribution of YAP in tumors with active Hh signaling. We focused on ductal pancreatic adenocarcinoma (PDAC), a tumor entity for which the Hh/GLI activity pattern has been well described. Specifically, epithelial PDAC tumor cells secrete Hh ligands, but do not respond to the ligand and thus display a comparatively low Hh pathway activity. In contrast, the desmoplastic stromal fibroblasts surrounding the tumor cells are actively responding to the Hh ligands and therefore express high *GLI1* levels.<sup>19–21</sup> In agreement with our *in vitro* data, the pattern of YAP immunoreactivity was inversely arranged to the Hh pathway activity: whereas there was a strong nuclear signal (indicating YAP activity) in the epithelial PDAC tumor compartment, there was only a weak staining of some cells in the tumor stroma. These findings were made in tissue sections from a PDAC mouse model<sup>22</sup> (Figure 4a) as well as in human PDAC samples (Figure 4b).

Because YAP/TAZ activity had been described in several malignancies in which Hh pathway activation had also been reported,<sup>7,23–27</sup> we searched for potential mechanisms that could bypass the inverse regulation between YAP and GLI and lead to a simultaneous activation of both oncogenes. Here, we focused on PARs that were shown to activate YAP/TAZ through the heterotrimeric G<sub>12/13</sub> proteins.<sup>28</sup> In addition, the  $\alpha$  subunit of G<sub>13</sub> has been shown to promote GLI activation in a SMO-independent manner.<sup>29</sup> In agreement with these reports, we could induce Hh



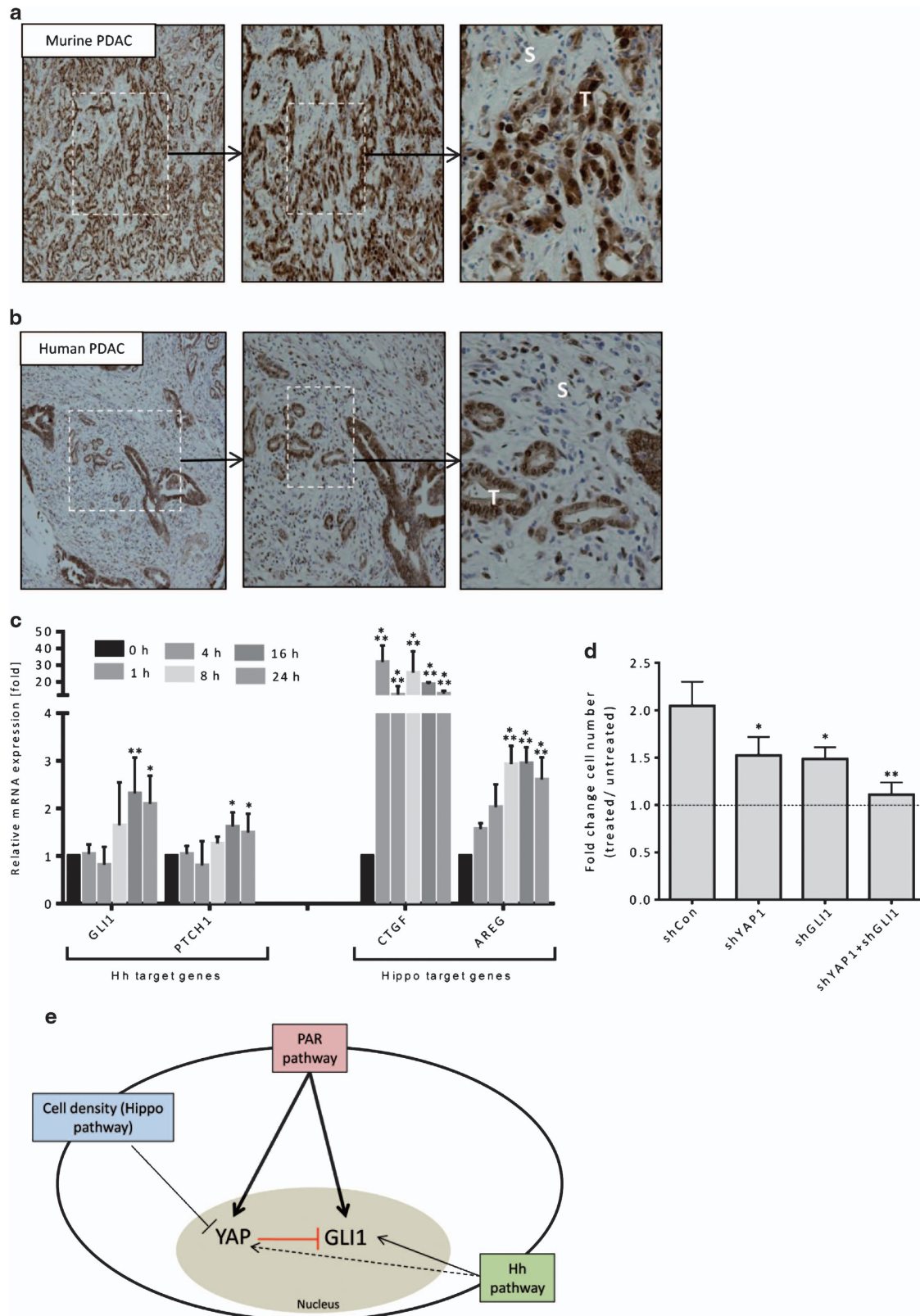
**Figure 2.** YAP overexpression suppresses Hh signaling. **(a)** Hh and Hippo target gene expression in Hek293T cells stably overexpressing a dominant active mutant of YAP (YAP2\_5SA). The inset shows an immunoblot of these cells verifying YAP overexpression (bands were cropped from the same blot). **(b)** Hh reporter activity in transiently transfected human pancreatic stellate cells (PSCs). Cells were transfected with full-length GLI1 (GLI1\_fl) or N-terminally truncated GLI1 (GLI1\_dN) plus the indicated constructs (plasmid ratio GLI1/YAP was 1:3). **(c)** *GLI1* and *PTCH1* mRNA expression in transiently transfected PSCs as measured by quantitative PCR (qPCR). **(d)** Hh reporter assay measuring endogenous Hh pathway activity in NIH3T3 cells. The cells were either transfected with empty vector control (pcDNA3.1) or dominant active YAP (YAP2\_5SA) and were subsequently stimulated with SAG. **(e)** Co-immunoprecipitation of Flag-tagged GLI1 and EGFP-tagged Yes-associated protein 1 (YAP1) in Hek293T cells. Immunoprecipitation (IP): α-Flag; western blot (WB): α-GFP. **(f)** Co-immunoprecipitation of endogenous YAP and endogenous GLI1 in Hek293T cells. IP: α-YAP; WB: α-GLI1. \* $P < 0.05$ ; \*\* $P < 0.005$ ; \*\*\* $P < 0.0005$  (two-tailed Student's *t*-test).



**Figure 3.** RNA interference (RNAi)-mediated knockdown of endogenous YAP promotes Hh signaling. **(a)** Western blot depicting the protein levels of endogenous GLI1 and YAP in MEF<sup>[SHH]</sup> cells after transfection with control small interfering RNA (siRNA) or a pool of four different Yap-specific siRNA constructs (Dharmacon 'SMARTpool'; Dharmacon GE Healthcare, Lafayette, CO, USA). **(b)** Western blot depicting the protein levels of endogenous GLI1 and YAP in Hek293T cells stably expressing control- or YAP-directed short hairpin RNA (shRNA) (293T<sup>[shCon]</sup> and 293T<sup>[shYAP]</sup>, respectively). **(c)** Hh and Hippo target gene expression in pancreatic stellate cells (PSCs) transfected with control siRNA or siRNA against YAP/TAZ. **(d)** Effects of siYAP/siTAZ transfection on the *GLI1* and *CTGF* mRNA expression level in Panc1 cells. The knockdown efficiency is also depicted. **(e)** Protein levels of YAP and GLI1 in NIH3T3 cells treated with SAG. **(f)** Hh pathway activation by means of SAG addition increases the expression of Hippo target genes (*Ctgf* and *Inhba*) without altering the expression levels of *Yap* or *Taz* (NIH3T3 cells). **(g)** Immunoblotting of endogenous YAP protein in NIH3T3 cells exposed to SAG (100 nM) and treated for different time periods with cycloheximide (CHX; 100 µg/ml). \**P* < 0.05; \*\**P* < 0.005; \*\*\**P* < 0.0005 (two-tailed Student's *t*-test).

(*GLI1* and *PTCH1*) as well as Hippo/YAP (*CTGF* and *AREG*) target genes in Hek293A cells by exposure to a synthetic peptide PAR-2 agonist (Figure 4c). Similar results were obtained with Panc1 cells (Supplementary Figure S4A). As both YAP and GLI1 have been associated with enhanced proliferation and survival, we found that PAR-2 activation results roughly in a twofold increase in cell

number within 72 h (Supplementary Figure S4C). Based on these results, we asked whether the experimental removal of YAP and GLI1 would abrogate the proliferation-promoting effects of PAR activation. To this end, we transfected Hek293A cells with short hairpin RNA constructs targeting *YAP1*, *GLI1* (for knockdown efficiency, see Supplementary Figures S4D and E) or the



**Figure 4.** Activation of PAR overcomes the inverse Hippo/Hh cross-talk. **(a)** Anti-YAP immunohistochemistry (Cell Signaling Technologies, Danvers, MA, USA; no. 4912, 1:100) on tissue sections from mouse pancreatic tumors ('KPC' mouse model). S, tumor stroma; T, tumor epithelium. **(b)** Anti-YAP immunohistochemistry on tissue sections from human patients suffering from pancreatic cancer. **(c)** Hh (*GLI1* and *PTCH1*) and Hippo (*CTGF* and *AREG*) target gene expression in Hek293A cells exposed to PAR-2 ligand (SLIGRL-NH<sub>2</sub>, Sigma-Aldrich S9317, 5 μM) for the indicated time periods. **(d)** Cell number change in Hek293A cells transfected with the indicated short hairpin RNA (shRNA) constructs followed by PAR-2 ligand exposure (5 μM for 5 days, 1% fetal bovine serum (FBS)). **(e)** Schematic diagram depicting the major findings of this work. \**P* < 0.05; \*\**P* < 0.005 (two-tailed Student's *t*-test).

combination of both and treated the cells with PAR-2 ligand. As can be seen in Figure 4d, PAR-2 activation doubled the cell number in cells receiving control short hairpin RNA. In cells transfected with shYAP or shGLI1, only a 1.5-fold increase in cell number by PAR-2 ligand treatment was achieved. When knocking down YAP and GLI1 in parallel, the PAR-2 activation was nonfunctional and there was no increase in cell number, indicating that PAR activation promotes simultaneous upregulation of YAP and GLI1 that results in enhanced cellular proliferation.

In summary, we describe an antagonistic impact of Hippo/YAP on Hh/GLI, and a positive effect of Hh on YAP. Regarding the negative regulation, YAP can directly interact with GLI1 and can hypothetically interfere with the correct built-up of transcriptional complexes around GLI1. It is intriguing that Hippo/YAP has been shown to inhibit mammalian WNT signaling, a pathway with close evolutionary ties to Hh.<sup>30,31</sup>

Concerning a positive modulation of YAP by Hh signaling, we found post-transcriptional mechanisms at play that increase the amounts of YAP protein. Although further studies are needed to decipher the exact molecular steps, our data suggest that this process represents a major event in the Hh-driven loss of cellular contact inhibition.

Of note, several reports link YAP/TAZ activity to the formation of a stem cell phenotype<sup>1,5,32</sup> and in mechanosensation. As stem cells are routinely cultured in nonadherent spheres, it will be interesting to learn whether the dense culture conditions within the sphere center differentially affect YAP and GLI functions. Similarly, questions on mechanical stress and Hh/GLI activity will await further investigations.

Eventually, evidence gathered from medulloblastoma<sup>23</sup> argues that the activation of both YAP and GLI oncogenes is feasible. Based on these findings, we anticipated mechanisms that could simultaneously promote both factors and identified the PAR system as a dual driver of YAP/GLI activity. Eliminating YAP plus GLI almost completely abrogated the PAR-2 ligand-induced stimulation in cell proliferation, arguing that these two proteins exert much of the biological growth-promoting impact of PAR.

## ABBREVIATIONS

YAP, Yes-associated protein; Hh, Hedgehog; SHH, Sonic Hedgehog; GLI1, glioma-associated oncogene 1; PDAC, pancreatic ductal adenocarcinoma.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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