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Vertebrate Homologues of Drosophila Fused Kinase and Their Roles in Sonic Hedgehog Signalling Pathway

ALLA MALOVERJAN

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TALLINN UNIVERSITY OF TECHNOLOGY
Faculty of Science
Department of Gene Technology

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Supervisors: **Senior Researcher Priit Kogerman, PhD**,
Department of Gene Technology, Tallinn University of Technology, Tallinn, Estonia
Senior Researcher Torben Østerlund, PhD,
Department of Gene Technology, Tallinn University of Technology, Tallinn, Estonia
Docent Marko Piirsoo, **PhD**,
Department of Gene Technology, Tallinn University of Technology, Tallinn, Estonia

Opponents: **Prof. Mart Ustav**, **PhD**,
Institute of Technology, University of Tartu, Tartu, Estonia
Research Associate Viljar Jaks, PhD,
Department of Biosciences and Nutrition, Center for Biosciences, Karolinska Institutet, Huddinge, Sweden

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Declaration:
Hereby I declare that this doctoral thesis, my original investigation and achievement, submitted for the doctoral degree at Tallinn University of Technology has not been submitted for any academic degree.

/Alla Maloverjan/

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CONTENTS

INTRODUCTION

Hedgehog signalling molecules trigger one of the fundamental developmental pathways required for proper formation of body plan. *Hh* was first discovered as a gene responsible for the segment polarity phenotype in fruit fly *Drosophila melanogaster*. At present, it is evident that the Hh signalling pathway is conserved in many aspects from flies to humans. However, severe variations in the Hh signalling cascade generated during evolution by mutations or gene duplication events illustrate divergences of Hh pathway between different species.

Sonic Hedgehog (Shh) is the most widely expressed and best-studied mammalian homologue of Hh proteins and is vitally required for numerous developmental and postnatal processes. However, inappropriate activation and/or deregulation of the Shh pathway are linked to several developmental abnormalities and tumorigenesis in humans. Several molecules implicated in the Shh pathway are proto-oncoproteins or tumour suppressors. Although mechanisms of Shh signal transduction have been investigated for almost two decades, many aspects of intracellular events triggered by Shh are still unclear. Therefore unravelling the molecular mechanisms of Shh signal transduction is a matter of particular interest not only for independent researchers but also for pharmaceutical companies.

One of the most intriguing molecules in the Hh pathway is serine/threonine kinase Fused (Fu). Initially, *fu* was discovered as a segment polarity gene in fruit fly *Drosophila melanogaster*. In *Drosophila,* Fu is maternally required for proper segmentation during embryonic development and for development of wings and legs during the larval stage. Numerous studies have demonstrated that fu participates in hh signal transduction as an obligatory component of multiprotein complex involved in regulation of *cubitus interruptus* (ci), a sole transcription factor mediating the hh activities in *Drosophila*. Ci is a homologue of Gli transcription factors (Gli1, Gli2 and Gli3), the differential regulation of which is the final step of Shh signalling in mammals. In *Drosophila*, fu plays a kinase activity dependent role required for positive regulation of ci, while fu kinase independent regulatory properties are essential for negative regulation of ci in the absence of hh. However, in zebrafish fu participates in hh signal transduction as well as in hh independent genesis of motile cilia.

In 2000, human serine/threonine kinase 36 (STK36) was identified as a protein sharing the highest similarity with fu. Cell culture experiments showed that STK36 (also named as FU) is able to positively regulate Gli proteins. However, in contrast to fu, STK36 acts independently of its functional kinase domain. When the present study was initiated, nothing was known about mouse Stk36 or Fu and its potential function in Shh signalling. In the view of the fact that mouse and human Stk36 homologues share 84% of identity, possible deviations in their roles in regulation of Gli proteins might be expected. At present, it is known that in mice, in contrast to *Drosophila* and zebrafish, Fu is dispensable for Shh-dependent embryonic development but participates only in Shh-independent motile cilia genesis. In addition, even if Stk36 is involved in any pathological or physiological aspect of

postnatal Shh signalling in mammals, it has perhaps only regulatory functions since its catalytic activity seems to be lost during evolution. The more elusive role of mammalian Stk36 indicates that, like for ci/Gli proteins, the different functions of fu have been divided between several proteins, suggesting that other kinases are involved in regulation of Gli proteins.

OUTLINE AND AIMS OF THIS THESIS

The research described in this thesis aims at identifying and describing kinases that regulate Gli transcription factors and other proteins in the Shh signal transduction pathway. Particularly, the roles of mammalian homologues of *Drosophila* serine/threonine kinase fu have been invesigated and are discussed. In the first part of the thesis the current view on *Drosophila* and mammalian Hh signalling pathways is provided by starting with the description of the discovery of Hh proteins and the pathways they trigger. Next, the overview about molecular mechanisms of Hh proteins secretion, diffusion and reception with the emphasis on intracellular events initiated by reception of Hh proteins is presented. Next, the roles of fu and Stk36 kinases in Hh signal transduction in *Drosophila* and mammals, respectively, are characterized and compared. The second part of the thesis addresses the questions about the roles of two mammalian homologues of fu, serine/threonine kinases Stk36 and ULK3, in Shh signalling and positive regulation of Gli transcription factors (**Publications I** and **II**). Besides that, the regulatory role of Ulk3 kinase in Shh pathway is shown (**Publication III**). Additionally, catalytical and self-regulation properties of ULK3 kinase are characterized (**Publications II and III**). Lastly, the presented data are discussed in context with the current literature, and a model for the involvement of Ulk3 in Hh signalling is presented.

ORIGINAL PUBLICATIONS

I Maloveryan, A., Finta, C., Østerlund, T., Kogerman, P. (2007). A possible role of mouse Fused (STK36) in Hedgehog signaling and Gli transcription factor regulation. J Cell Commun Signal. 1(3-4):165-73.

II Maloverjan, A., Piirsoo, M., Michelson, P., Kogerman, P., Østerlund, T. (2010). Identification of a novel serine/threonine kinase ULK3 as a positive regulator of Hedgehog pathway. Exp Cell Res. 316(4):627-37.

III Maloverjan, A., Piirsoo, M., Kasak, L., Peil, L., Østerlund, T., Kogerman, P. (2010). Dual function of unc-51-like kinase 3 (Ulk3) in the Sonic hedgehog signalling pathway. J Biol Chem. 285, 30079-30090.

ABBREVIATIONS

aa	amino acids
Act	activator
A/P	anterior/posterior
BCC	basal cell carcinoma
BMP	Bone Morphogenetic Protein
ci	cubitus interruptus
CK1	Casein Kinase 1
CNS	central nervous system
cos2	costal 2
CTD	C-terminal domain
Disp	Dispatched
Dhh	Desert Hedgehog
dpp	decapentaplegic
en	engrailed
FL	full-length
Fu	Fused
GSK3	Glycogen Synthase Kinase-3
Hh	Hedgehog
HIP	Hedgehog Interacting Protien
HSC	Hedgehog Signalling Complex
HSPGs	Heparan Sulfate Proteoglycans
IFT	intraflagellar transport
Ihh	Indian Hedgehog
KD	kinase domain
Kif	Kinesin family member
lpp	lipophorin
PKA	Protein Kinase A
Ptc	Patched
Rep	repressor
SAG	Smoothened agonist
Shh	Sonic Hedgehog
Smo	Smoothened
Stk36	Serine/threonine kinase 36
Sufu	Suppressor of Fused
Ulk	unc-51-like kinase
unc	uncoordinated (*C.elegance*)
wg	wingless
wt	wild type
ZnF	zinc finger

1. REVIEW OF THE LITERATURE

1.1. Discovery and biological significance of Hh proteins

Hedgehog *(Hh)* genes encode signalling molecules that trigger one of the fundamental developmental pathways responsible for creation of pattern for the developing body plan. Much of what is known of the function of Hh pathway nowadays has been derived from studies in fruit fly *Drosophila melanogaster*, and many of the key elements are conserved from flies through to humans.

In the late 70^{-s} Christiane Nüsslein-Volhard and Eric Wieschaus carried out a large-scale genetic screen for mutations affecting early embryonic development of *Drosophila*. Their work revealed a number of genes required for proper segmentation of *Drosophila* embryo, and they were awarded the Nobel Prize in Physiology or Medicine in 1995. *Hh* was discovered in their screen as **a segment polarity gene** or a gene required for proper establishment of the anterio-posterior axis and spatial pattern within embryonic segments during *Drosophila* embryogenesis (Nusslein-Volhard and Wieschaus 1980).

The name of the gene "hedgehog" cropped up after the specific phenotype of *Drosophila* mutant larvae. Thoracic and abdominal segments of fly larvae are covered with denticles anteriorly and are smooth or naked posteriorly. An Hh-deficient larva completely lacks the posterior naked compartments of the segments and contains only fragments of anterior compartments with denticles. Stuck up denticles of the anterior part resembled "**spines of hedgehog**" and inspired the name for the responsive for this phenotype gene. Loss of function mutations in *hh* gene are lethal (reviewed in (Ingham 1989)).

More than 10 years after initial characterization of the mutant phenotype several groups reported cloning of *Drosophila* and later human *Hh* genes (Lee et al. 1992; Tabata et al. 1992; Echelard et al. 1993; Riddle et al. 1993). Successive studies have revealed that *Hh* genes are evolutionary conserved. Hh proteins have been identified in planaria, fly, leech, sea urchin, zebrafish, mouse, rat, chicken, and human. Moreover, mammals and birds have three Hh homologues: **Indian**, **Desert** and **Sonic Hedgehog** (Ihh, Dhh and Shh, respectively) (Bitgood et al. 1996; Kumar et al. 1996; Vortkamp et al. 1996).

Ihh, Dhh and Shh proteins have distinct expression domains and play unique roles in vertebrate development. Dhh has the highest similarity to the *Drosophila* homologue. *Dhh* is expressed in Schwann and Sertoli cells, and is implicated in germ-cell proliferation, differentiation of germ cells during spermatogenesis, and perineurium-Schwann cell interactions (Bitgood and McMahon 1995; Bitgood et al. 1996). *Ihh* is expressed in gut and cartilage, and is implicated in modulating chondrogenesis in the appendicular skeleton, and acts as a negative regulator of the differentiation of chondrocytes (Vortkamp et al. 1996; Iwasaki et al. 1997).

Shh is the most studied and well-characterized protein of the family. In the biological assays Shh is the most potent member of the family (Pathi et al. 2001). *Shh* is expressed and mediates the signalling activities in notochord, floor plate of

neural tube, gut, and developing limbs. The pathway triggered by Shh is important in mesodermal development, establishment of the dorsal-ventral patterning of neural progenitors in the neural tube, gastrointestinal development and axial skeletal patterning in vertebrates (Jiang and Hui 2008). In the developing cerebellum, Shh stimulates proliferation of granule neuron precursor cells (Rios et al. 2004). The Shh pathway is also active during development of eye, tooth, lung and salivary glands, ovary, skin and hair follicles. In adults the pathway is implicated in tissue homeostasis maintenance. Additionally, Shh regulates both proliferation and differentiation of various types of stem cells and is required for survival, proliferation and differentiation of lymphocytes (Traiffort et al.; Machold et al. 2003; Rowbotham et al. 2007). Shh is also required for postnatal penile morphogenesis (Podlasek et al. 2003).

In humans, disruption or misregulation of the Shh pathway results in various developmental abnormalities including holoprosencephaly, Pallister-Hall syndrome, Gorlin syndrome, Greig cephalopolysyndactyly, Rubinstein-Taybi syndrome and different types of cancer (basal cell carcinoma (BCC), medulloblastoma, glioma, breast, pancreatic and prostate cancers etc) (reviewed in (Walterhouse et al. 1999; Ruiz i Altaba et al. 2002; McMahon et al. 2003; Jiang and Hui 2008) and references therein). **Holoprosencephaly**, a devastating syndrome with variable expressivity from cyclopia to minor midline fusion defects is associated with mutations in the *Shh* gene. **BCC**, the most common skin cancer in humans is associated with mutations leading to up-regulation of the Shh pathway. Greig cephalopolysyndactyly and Pallister-Hall syndromes are associated with mutations and misregulation of one of the Shh-dependent transcription factors. **Greig syndrome** includes facial abnormalities, extra and deformed fingers and toes, and mental retardation. **Pallister-Hall syndrome** is associated with hypothalamic hamartoma, postaxial polydactyly, cleft palate, and malformations in a variety of other systems (larynx, heart, and genitourinary system). All these diseases are very important in humans. Most molecules participating in the Shh pathway are tumor suppressor or oncoproteins, and therefore they are attractive pharmaceutical targets. For this reason, investigation of molecular mechanisms of the Shh signal transduction is of particular interest for many researchers in the world since it may shed light not only on developmental but also on pathological issues.

1.2. Expression and biological roles of Hh proteins

1.2.1. Hh controls segmentation and wings development in *Drosophila*

Hh proteins elicits cell-to-cell, short-range and long-range signalling. During *Drosophila* development *hh* has two phases of activity. First, it is required for the establishment of anterior/posterior domains within parasegments during early embryogenesis. In the embryonic segments *hh* expression is initiated in the cells posteriorly adjacent to the parasegmental (A/P) boundary (or in anterior part of parasegments) (**Figure 1A**). Its expression is induced by a homeodomain-containing transcription factor **engrailed** (en) (Tabata et al. 1992). On the other hand, en contributes negatively to hh signalling by repressing expression of a downstream

effector of the pathway ***cubitus interruptus*** (ci) (Schwartz et al. 1995). Anterior cells are competent to respond to hh, since they express hh receptor ***patch*** (*ptc*) (Chen and Struhl 1996). They also express ci that in the absence of hh serves as a transcriptional repressor (**ci^{Rep}**), and in the hh-stimulated cell ci is converted into transcriptional activator (**ci^{Act}**) (see below). Hh signals antriorily from its expression domain and induces expression of ***wingless*** (wg, a homologue of vertebrates **Wnt** proteins) in the cells adjacent to A/P boundary. Hh also induces expression of *ptc* in a broader stripe of cells (Ingham et al. 1991; Capdevila et al. 1994b). Posterior wg signalling maintains the expression of *hh* by inducing *en* expression thus establishing interdependence and interference of hh and wg signalling pathways during segmentation process (Martinez Arias 1989).

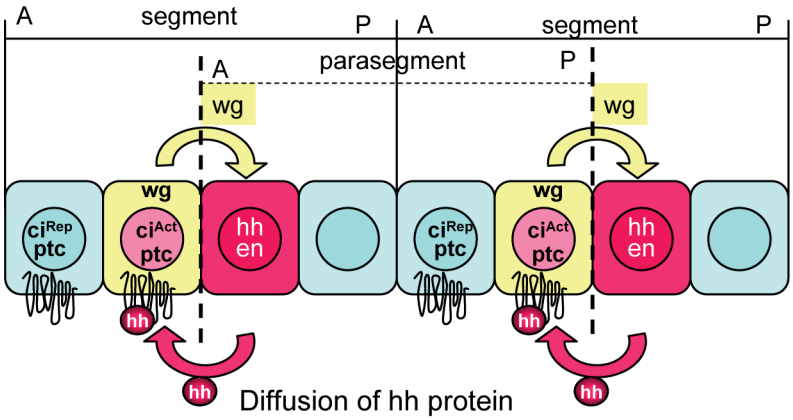


Figure 1. Schematic presentation of cell-to-cell interaction between wg and hh signals during *Drosophila* segmentation. Wg and hh are secreted by adjacent rows of cells located on either side of parasegmental boundaries (dashed thick line). They stabilize each others expression during embryonic development and establish the anterior/posterior axis within parasegments. En protein activates the transcription of *hh* in the posterior cells of embryonic segments. Hh protein diffuses anteriorily and through ci^{Act} induces expression of *ptc* and *wg* in the cells adjacent to A/P boundary. Non-stimulated with hh anterior cells possess ci^{Rep} form. The wg signalling pathway in the posterior cells induces expression of *en* and thus maintains expression of *hh*.

During the second phase of activity (late larval and early pupal stages) hh is required for the correct patterning of various imaginal cuticular structures responsible for the development of legs, wings, eyes and antennas of the adult fly (Mohler 1988). The role of hh signalling is best described in the wing imaginal disk. Thoracic imaginal disk primordia arise in the embryo at positions spanning A/P boundaries. As a consequence, imaginal disks consist of two groups of cells that either do (posterior, P) or do not (anterior, A) express *en* and *hh* genes. Schematic presentation of hh signalling during *Drosophila* wing development is shown in **Figure 2**. During postembryonic wing formation, hh is secreted from cells of the P compartment and acts as a short-range morphogen (Lee et al. 1992; Tabata and Kornberg 1994). Hh signals to A compartment cells near the A/P boundary that, in contrast to P cells,

respond to hh due to expression of *ptc* and *ci*. In the absence of hh, ci^{Rep} blocks *hh* expression and keeps *ptc* expression low. Besides that, ci^{Rep} inhibits expression of *dpp* (Methot and Basler 1999). Hh signalling prevents production of ci^{Rep} and allows ci^{Act} to activate its target genes. Thus, hh signalling induces expression of ***decapentaplegic*** (dpp) (homologue of vertebrate **bone morphogenetic proteins** (BMPs), members of transforming growth factor β (TGF-β) superfamily) in a narrow stripe of cells near the A/P boundary, *wg* in the ventral-anterior stripe, and *ptc* in a broader domain (Capdevila et al. 1994a; Tabata and Kornberg 1994). The *dpp* product acts in the whole wing and controls its growth and patterning, wg determines ventral structures, and hh signalling patterns the central domain of the wing blade primordium (Ferguson and Anderson 1992; Struhl and Basler 1993; Mullor et al. 1997).

In the A compartment close to A/P boundary hh expression is inhibited by a transcriptional repressor complex containing **master of thickveins**, a target of Hh activity encoding a nuclear zinc-finger protein, and transcriptional co-repressor **groucho** (Apidianakis et al. 2001; Bejarano et al. 2007).

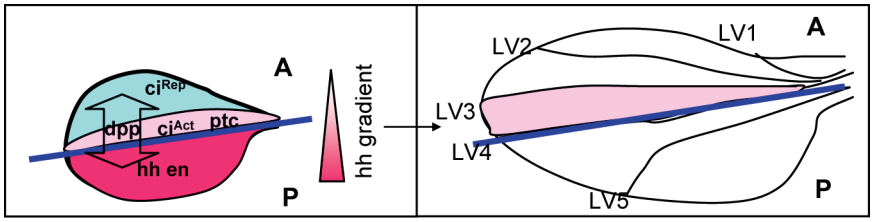


Figure 2. Hh in *Drosophila* wing development. Left panel: hh is expressed in the P compartment cells of a wing imaginal disk (dark-pink colour) and acts as a short-range morphogen inducing *dpp* and *ptc* expression in the cells anteriorily adjacent to A/P boundary (dark-blue line). Those cells express *ptc* and *ci* and are competent to respond to hh. The most anterior cells (light-blue) do not receive hh and contain repressor form of ci (ci^{Rep}) that inhibits *dpp* expression**. Right panel**: hh activity is required for formation, positioning and spacing of longitudinal veins L3 and L4 and for formation of their intervein region (light-pink colour).

1.2.2. Shh controls development of CNS and limbs in vertebrates

During vertebrate development, Shh is expressed in several organizing centres regulating embryonic polarity (Chiang et al. 1996). The morphogenic role of Shh is well described in patterning of ventral neural tube (Briscoe et al. 2001), (reviewed by (Jacob and Briscoe 2003; McMahon et al. 2003). *Shh* expression is initiated during presomitic stage of embryonic development, when its transcripts are detected in the notochord. Shh signalling from the notochord induces the development of the neural floor plate, and *Shh* expression extends into the floor plate region at ventral midline of the developing neural tube. Shh secreted from both the notochord and floor plate, ventralizes the neural tube further acting in a concentration dependent manner. The concentration gradient of Shh induces or represses different transcription factors in the neural progenitor cells, thus regulating differentiation of

distinct subtypes of neurons in the developing neural tube. Shh signalling is mediated by the transcription factors belonging to the **Gli family** (**Gli1, Gli2** and **Gli3**) whereas during development of neural tube Gli2/3 proteins play the most important role (**Figure 3**) ((Hui et al. 1994; Lee et al. 1997; Meyer and Roelink 2003; Motoyama et al. 2003) and see below). *Gli* genes are expressed in the developing neural tube in distinct, but partly overlapping domains. *Gli1* is expressed most ventrally, *Gli3* – most dorsally, and *Gli2* is expressed uniformly between *Gli1* and *Gli3* expression domains. All Gli proteins may serve as transcriptional activators. However, Gli2/3, analogously to ci and in contrast to Gli1, undergo partial proteosomal degradation and may act in the developing neural tube also as transcriptional repressors ($Gli2/3^{Rep}$). The Shh concentration gradient regulates the balance between activator and repressor forms of Gli proteins, and thus induces or represses expression of specific transcription factors that in turn define the fate of neural progenitors. Control of dorso-ventral patterning of the spinal cord is attained by antagonistic cooperation of Shh with **Wnt** and **BMP** signalling pathways (Kuschel et al. 2003; Ulloa et al. 2007; Alvarez-Medina et al. 2008).

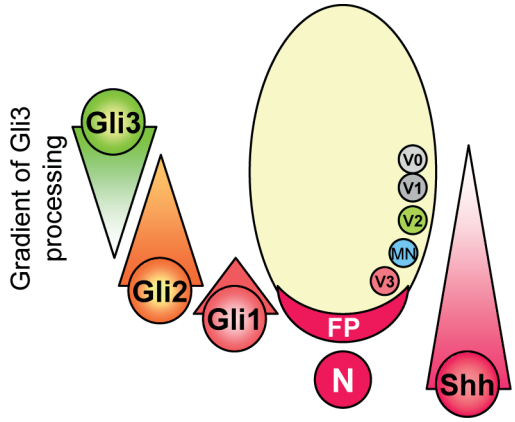


Figure 3. Shh elicits long-range signalling in the developing neural tube. Shh is expressed by notochord (N) and floor plate (FP) cells and forms a dorsally diminishing concentration gradient. Distinct subtypes of interneurons (V0–V3) or motor neurons (MN) are generated in the ventral part of the developing neural tube. Transcription factors Gli1, Gli2 and Gli3 are expressed in partly overlapping domains and mediate Shh activity. In the intermediate region of the developing neural tube where Shh signal is absent, Gli3 is processed to a strong transcriptional repressor ($Gli3^{Rep}$). Concentration of $Gli3^{Rep}$ decreases gradually depending on the strength of Shh signal, and in the most ventral part of its expression domain Gli3 may act as a transcriptional activator $Gli3^{Act}$. Thus, $Gli3^{Act}$ may participate in development of V3 interneuron and motor neurons (MN), and $Gli3^{Rep}$ controls V0 and V1 identity (Persson et al. 2002; Motoyama et al. 2003). Gli1 acts as a strong transcriptional activator in the floor plate (FP) and V3 interneurons. Gli2 participates in control of all neuronal subtypes in the ventral part of developing neural tube, but having the most prominent transcriptional activator role in V3 and MN neuronal precursors (Ruiz i Altaba et al. 2003; Motoyama et al. 2003).

Developing limb represents another widely used experimental model, where Shh signalling has been described in details (Chiang et al. 2001). Prior to activation of Shh signalling, Gli3 and a basic helix-loop-helix transcription factor **HAND2** (heart- and neural crest derivatives-expressed protein 2) prepatterns the limb bud mesenchyme. HAND2 induces expression of *Shh* in the zone of polarizing activity of the developing limb bud, and Shh, in turn, primes the region of the limb competent to form digits (Galli et al. 2010). During limb development Shh antagonizes the repressor function of Gli3, expressed anteriorily, as well as induces expression of *BMP*s in the adjacent mesenchyme and maintains the expression of *Fgfs* in the apical ectodermal ridge (Drossopoulou et al. 2000; te Welscher et al. 2002). Patterning of the limb as a whole is coordinated by complex interactions of Shh, Fgf, Wnt and Bmp signalling pathways. The Shh-Gli3 signalling pathway is required for the establishment of the antero-posterior axis of the limb and specification of vertebrate digit identities (Riddle et al. 1993; Ahn and Joyner 2004; Harfe et al. 2004).

1.3. Maturation and secretion of Hh proteins

Hh is expressed as a precursor protein with a molecular mass of ca 45 kDa. Generation of the biologically active protein requires several post-translational modifications (**Figure 4A**). The crucial steps of maturation and secretion of biologically active Hh proteins from Hh-producing cells are conserved in vertebrates and *Drosophila*. Following cleavage of the N-terminal signal peptide before entering the secretory pathway Hh undergoes **autoproteolytic cleavage**. This reaction is mediated by the carboxyl terminus of Hh and results in the release of ca 20 kDa N-terminal signalling unit (Porter et al. 1995). As a part of this reaction, a **cholesterol** moiety is attached to the new C-terminus (Porter et al. 1996). The second modification is attachment of **palmitic acid** to Cystein-24 exposed after signal peptide cleavage (Pepinsky et al. 1998). Acylation is catalysed by an acyltransferase **skinny hedgehog** (Chamoun 2001). These modifications strongly enhance the signalling activity of Hh and are essential for it secretion as well as modulation of it movement through responsive tissues (Callejo et al. 2006). The lipid-modified Hh protein with highly hydrophobic N- and C- termini is fully active, ready to be secreted and capable of initiating signalling. In vertebrates, Shh lacking cholesterol or palmitic acid shows restricted signalling and impaired diffusion (Lewis et al. 2001; Chen et al. 2004a). In humans, mutations in C-terminus of *Shh* that lead to generation of unprocessed protein or prevent cholesterol modification are associated with holoprosencephaly (Nanni et al. 1999).

Secretion of Hh requires a twelve-pass transmembrane protein **Dispatched** (Disp) that has a sterol-sensing domain and is structurally similar to the Hh receptor Ptc. Disp binds cholesterol-modified Hh and facilitate its transport from the cell (Burke et al. 1999) (**Figure 4B**). *Disp* knockout mice display *Hh* embryonic phenotype since lack of Disp completely blocks Hh secretion (Kawakami et al. 2002).

Movement of Hh from expressing cells and establishment of morphogenic gradients depend on several proteins. It has been shown that cholesterol-modified *Drosophila*

hh associates with lipoprotein **lipophorin** (lpp) that promotes its long-range signalling activity (Panakova et al. 2005). Extracellular matrix proteins, **heparan sulfate proteoglycans** (HSPGs), as well as glycosylphosphatidylinositol -linked HSPGs, **Dally** and **Dally-like**, are essential for hh (and also for other morphogens as wg and dpp) diffusion and establishing of its concentration gradient in the *Drosophila* embryo (Han et al. 2004; Callejo et al. 2006). Moreover, mutations in the genes ***tout velu (ttv), brother of tout velu (btv)*** and ***sister of tout velu (stv),*** products of which are involved in HSPG biosynthesis, lead to arrest of hh movement across hh receiving cells in *Drosophila* embryo (Bellaiche et al., 1998; Bornemann et al., 2004; Takei et al., 2004). The mammalian orthologues of ttv and stv, (**Ext1** and **Ext2**) are also linked to Hh distribution and mediation of its signalling by HSPGs (Lin et al. 2000; Koziel et al. 2004), supporting the view that HSPGs participate in control of the range of Hh signalling also in vertebrates.

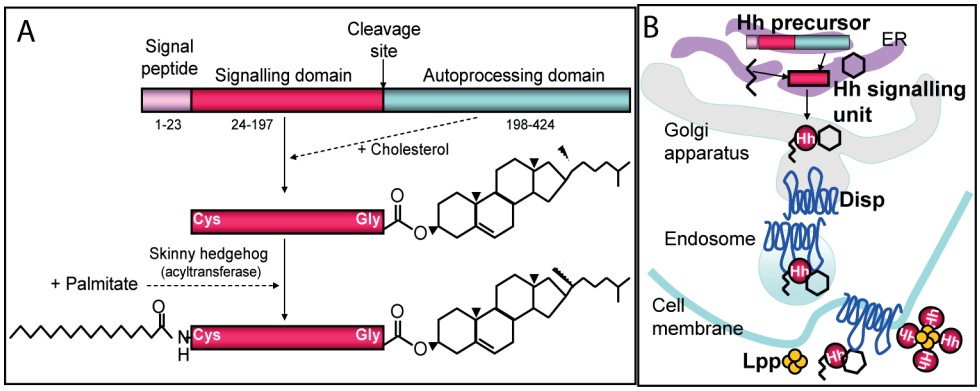


Figure 4. Generation and secretion of biologically active Hh protein. A. Hh is expressed as a precursor protein. During maturation Hh undergoes N-terminal signal sequence trimming, internal proteolysis mediated by its C-terminal domain, addition of a cholesterol moiety and acylation. The signalling unit is modified at its C-terminal glycine residue by cholesterol; the N-terminal cysteine residue becomes palmitoylated. **B.** Hh precursor protein undergoes post-translational modifications in endoplasmic reticulum (ER). Modified Hh is directed to secretion through Golgi apparatus. The twelve-pass membrane protein Disp is required for Hh secretion. Cholesterol-modified Hh associates with lipophorin (lpp) particles that facilitate its diffusion.

1.4. Reception of Hh at the plasma membrane

The mechanism of Hh signal reception at the cell surface is rather conserved between vertebrates and invertebrates. Two membrane-associated proteins, Ptc and **Smoothened** (Smo), are the key components, participating in transmission of the Hh signal into the cell. In humans, *PTC* is a tumor suppressor, and *SMO* is a proto-oncogene; mutations in those genes are detected in several types of cancer.

The main receptor of Hh is the twelve-pass trans-membrane protein **Ptc** (Marigo et al. 1996a). A single *ptc* gene has been identified in *Drosophila*, whereas vertebrate genomes encode two Ptc proteins, Ptc1 and Ptc2, sharing 73% of sequence identity.

Both proteins may bind Hh ligands with similar affinities and form a complex with Smo (Carpenter et al. 1998). However, Ptc1 is a primary Hh receptor, since *Ptc1-/-* mice die at embryonic day 9 with multiple defects in the developing limb, gut, and nervous system, but *Ptc2-/-* mice are viable and do not exhibit perturbations of Hh signalling during embryonic development (Goodrich et al. 1997; Lee et al. 2006; Nieuwenhuis et al. 2006).

Genetic studies in *Drosophila* show that loss of *ptc* induces ectopic expression of hh target genes (Ingham et al. 1991; Forbes et al. 1993). Indeed, functionally Ptc and Hh are reciprocal antagonists: *Ptc* activity inhibits Hh signalling, presence of Hh represses Ptc activity. Ptc binds Hh directly, and serves as a barrier hindering the spread of the secreted Hh (Chen and Struhl 1996; Marigo et al. 1996a). On the other hand, *Ptc* is a transcriptional target of Hh signalling (Goodrich et al. 1996). Thus, Hh signal exploits a self-regulation mechanism, a negative feedback loop where Hh induces *Ptc* expression, while Ptc acts as a receptor which anchors Hh and impedes its further movement.

The second protein involved in Ptc-mediated Hh signal transduction is the seven-pass trans-membrane protein **Smo**, a G-protein-coupled receptor (GPCR) family member. *Smo* was identified in *Drosophila* as a segment polarity gene whose embryonic phenotype resembles that of *hh* (Nusslein-Volhard and Wieschaus 1980; Alcedo et al. 1996). Genetic studies proposed that Smo acts downstream of Hh and downstream or in parallel with Ptc as a positive regulator of Hh pathway required for the transduction of the Hh signal into the cell (Alcedo et al. 1996; Chen and Struhl 1996; Therond et al. 1999).

1.4.1. Reception of hh in Drosophila cells

In the absence of hh, ptc localizes on the plasma membrane, and smo is distributed in the cytoplasm (Denef et al. 2000; Khaliullina et al. 2009). Although ptc is able to interact with smo, biochemical studies suggest that ptc inhibits the intrinsic signalling activity of smo not through direct association (Taipale et al. 2002). Upon hh binding, ptc relieves its inhibitory effect, allowing smo to move to the plasma membrane and activate the downstream components of the signalling. Although overall the process is conserved, vertebrates and invertebrates utilize different molecular mechanisms to activate smo.

The current model of hh signalling in *Drosophila* is illustrated in **Figure 5**. It suggests that in the absence of hh, the cytoplasmic tail of smo is associated with phosphatase **pp4** that dephosphorylates it preventing smo cell-surface accumulation (Jia et al. 2009a). Smo is mostly present in intracellular vesicles, and is able to cycle between plasma membrane and endosomes. Ptc regulates the cycling of smo back to the membrane through promotion of its sequestering in endosomes or by targeting it to the lysosomes (Denef et al. 2000; Nakano et al. 2004). One of the proposed mechanisms by which ptc exerts these effects is utilization of specific lipids contained within lipoprotein lpp (Khaliullina et al. 2009). It has been suggested that ptc via its sterol-sensing domain internalizes lpp-derived particles, sequesters them into ptc-positive endosomes and utilizes them for smo destabilization. The data

showing that lpp contains cholesterol-modified hh, decreased lpp levels reduces hh signalling, and ectopic expression of *ptc* increases lpp level and represses smo, support this model (Panakova et al. 2005; Callejo et al. 2006).

Hh interaction with ptc induces internalization and degradation of ptc as well as accumulation and stabilization of smo at the cell surface. In response to hh, the carboxyl tail of smo is extensively phosphorylated by **protein kinase A** (pka) and **casein kinase 1** (ck1), whereas the degree of phosphorylation controls the activity of smo (Denef et al. 2000; Zhang et al. 2004). Phosphorylation causes conformational changes in the cytoplasmic tail of smo due to stretches of positively charged arginine residues situated opposite the phosphorylation cluster, thus allowing smo to activate downstream signalling evens. Besides that, it has been suggested that hh induces dimerization of smo, which is essential for pathway activation (Zhao et al. 2007).

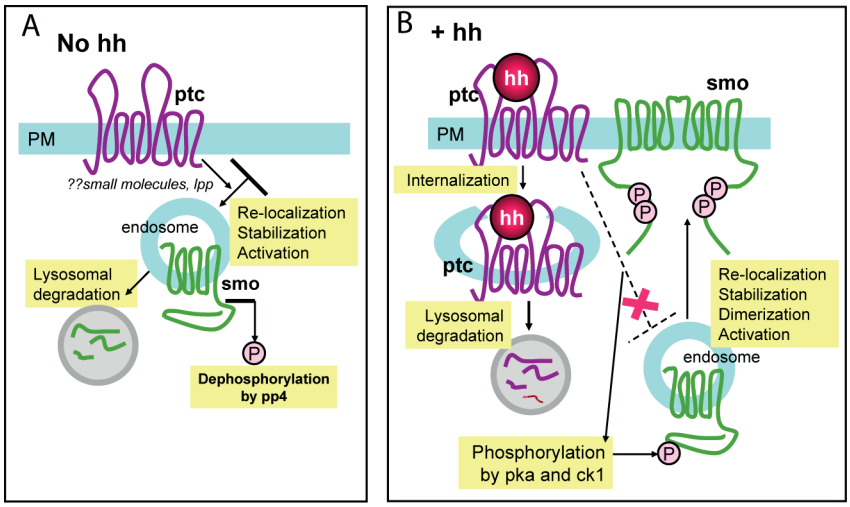


Figure 5. Reception of hh signal at the cell membrane in *Drosophila*. A. In the absence of hh, ptc is localized to the cell membrane and prevents smo accumulation there by keeping smo in the cytoplasm bound to endosomes and/or inducing smo lysosomal degradation. This negative regulation may be achieved through utilization of lpp-derived particles. Smo carboxyl terminus is dephosphorylated by phosphotase pp4. **B.** Binding of hh induces internalization and degradation of ptc. This in turn allows smo to move to the plasma membrane. In response to hh, smo cytoplasmic tail is phosphorylated by pka and ck1 kinases. Phosphorylation induces conformational changes with subsequent translocation to plasma membrane and dimerization. Activated smo initiates downstream intracellular signalling.

It is interesting to note that *Drosophila* smo has a long cytoplasmic tail which is obligatory for its function. It associates with cytosolic proteins **costal 2** (cos2) and **fused** (fu) and contains sites for pka and ck1-mediated phosphorylation that are absolutely required for smo membrane accumulation and activity (Lum et al. 2003a; Ruel et al. 2003; Zhang et al. 2005; Zhao et al. 2007). However, the carboxyl terminus of vertebrate Smo is significantly shorter and Pka and Ck1 sites are not

conserved. Functionally *Drosophila* and vertebrate Smo are also different since *Drosophila* smo fails to rescue the phenotype of Smo-deficient mouse embryonic fibroblasts (Chen et al. 2009). This suggests the implication of alternative mechanisms of Smo activity regulation.

1.4.2. Reception of Shh in vertebrate cells

The vertebrate Hh signalling is regulated at the **primary cilium**, an evolutionary conserved organelle that is found on nearly all mammalian cells (Huangfu and Anderson 2005). Cilia contain a long microtubular axoneme surrounded by an external membrane that is continuous with the plasma membrane. The primary cilium is considered as an extracellular "antenna" required for detection and interpretation of extracellular signals. Maintenance of the primary cilia is mediated by **intraflagellar transport** (IFT) that involves bidirectional movement of molecules anterogradely powered by **kinesins** (**Kif** proteins) and retrogradely by dynein motors (Rosenbaum and Witman 2002). It is noteworthy, that vertebrate Hh signalling requires primary cilia, whereas *Drosophila* does not. Primary cilia are found only in sensory neurons of *Drosophila*, and mutations in either the anterograde kinesin motor or components of the IFT particles do not disrupt Hh signalling (Han et al. 2003).

According to the current model, in the absence of Hh, Ptc1 is localized on the cell surface along the shaft of the cilia (Rohatgi et al. 2007) (**Figure 6**). In contrast, Smo is located distantly from cilia in plasma membrane and in endosomes (Incardona et al. 2002; Milenkovic et al. 2009). Smo, which is able to adopt "inactive" and "active" conformations depending on its localization and trafficking on the primary cilium, is present in an inactive state, and its translocation to the cilia and subsequent activation are blocked in the absence of Hh (Rohatgi et al. 2009; Wilson et al. 2009a). It has been suggested that Ptc1 pumps an inhibitory small molecules into and out of the cell and thus prevents activation of Smo (Bijlsma et al. 2006). Those molecules may be steroid compounds, such as sterols, steroids, vitamin D analogs, and isoprenoids. All those compounds are synthesezed via sterol synthetic pathway. Genetic defects in this pathway is associated with a holoprosencephaly-like malformation, a trait of *Shh* deficiency (Wassif et al. 1998). The data showing that oxysterols may directly stimulate Smo activity, and pro-vitamin D3 secreted by a Ptc-dependent manner directly binds to Smo and inhibits its activity support this point of view (Bijlsma et al. 2006; Corcoran and Scott 2006).

Sequestering of Shh leads to the removal of Ptc1 from the cilia via a Shh-dependent internalization or movement to other regions of the plasma membrane. Smo, in contrast, translocates to the cilia (Rohatgi et al. 2007; Milenkovic et al. 2009). Two distinct mechanisms of Smo relocation have been proposed. First, lateral movement, either via diffusion or active transport of the membrane-associated Smo. This process may be mediated by **cAMP/PKA**-dependent mechanism (Milenkovic et al. 2009). Second, intraflagellar transport of cytoplasmic Smo mediated by a **β-Arrestin/Kif3a** complex (May et al. 2005; Kovacs et al. 2008). Located in cilia, Smo undergoes conformational changes resulting in generation of "active" Smo

competent to activate the Hh cascade. It is notable, that Smo translocated by the β-Arrestin/Kif3a complex is "active" and competent for initiation of downstream signalling, but membrane-associated Smo needs an additional step for its full activation in the cilia (Rohatgi et al. 2009; Wilson et al. 2009a).

Interestingly, treatment of cells with SAG (Smoothened agonist that directly binds to Smo and activates it (Taipale et al. 2000) induces enrichment of Ptc1 in the primary cilia (Rohatgi et al. 2007). On the other hand, binding of SAG changes conformation of Smo, induces its translocation to the cilia and leads to constitutive activation of the pathway. This indicates, firstly, that localization of Ptc in the cilia *per se* neither blocks signalling nor physically prevents Smo to enter the cilia. Secondly, only in the cilia, even in the presence of Ptc, Smo is able to apply such "active" conformation competent to initiate the downstream signalling. Additionally, the data showing that translocation and enrichment of Smo in the cilia are not sufficient for full activation of signalling, suggest the existence of the second step needed for activation of ciliary Smo (Rohatgi et al. 2009; Wilson et al. 2009a). In *Ptc1−/−* mouse embryonic fibroblasts, the Hh signalling pathway is constitutively active, Smo is localized in the primary cilia even in the absence of SAG or Shh. Taken together, these data confirms that the role of Ptc in Smo regulation consists mainly of prevention of Smo "active" form generation.

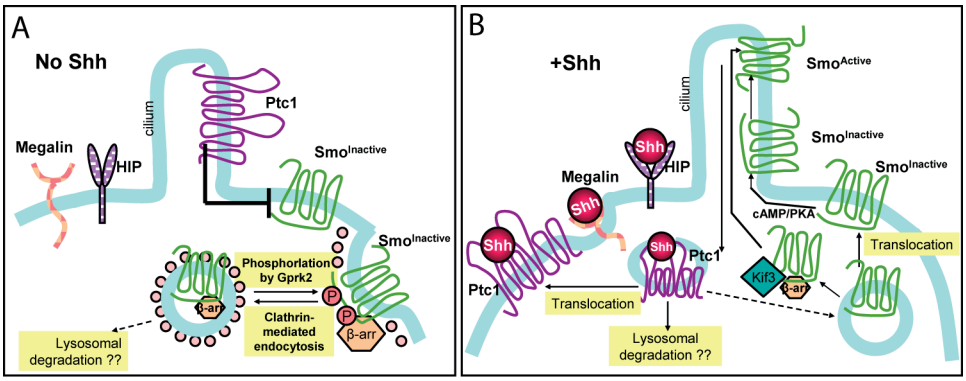


Figure 6. Reception of Shh on membrane of vertebrate cells. A. In the absence of Hh, Ptc1 localizes in the primary cilia and prevents Smo activation. Smo is inactive and presents in the endosomes or on the cellular membrane distantly from the cilia. Smo is phosphorylated by Gprk2 kinase that recruits β-arrestin (β-arr) to Smo inducing it clathrin-mediated internalization with possible subsequent degradation. Shh receptors Megalin and Hip are also present on the cell membrane. **B.** In the presence of Hh, intracellular Smo translocates to the plasma membrane at the tip of cilia by lateral transport or via a Kif3a/β-arrestin dependent mechanism. The first step of membrane-associated Smo translocation is cAMP/PKA- activity dependent. Being in cilia, Smo adopts the "active" conformation and initiates downstream signalling events. Upon binding of Shh, Ptc1 is removed from the cilia by endocytosis with subsequent translocation away from the cilia and/or lysosomal degradation.

Vertebrate cells have at least two more Hh receptors: **Hedgehog Interacting Protein** (HIP) and **Megalin**. The cell-surface protein HIP is involved in generation of negative feedback loop of Hh signalling: Hh induces HIP expression and HIP directly binds and sequesters Hh (Chuang and McMahon 1999). A low density lipoprotein receptor family member endocytic receptor Megalin is able to bind and internalize Shh (McCarthy et al. 2002). In addition, both invertebrates and vertebrates utilize a Ptc-independent mechanism of Smo down-regulation. This involves **G-protein-coupled receptor kinase 2** (Gprk2) that directly posphorylates Smo leading to it association with β-Arrestin and clathrin-mediated internalization (Cheng et al.; Chen et al. 2004b; Meloni et al. 2006; Philipp et al. 2008). Keeping in mind that the cytoplasmic fraction of Smo is positively regulated through β-arrestin/Kif3A complex it may be possible that β-Arrestin promotes cycling of Smo within the cell.

1.5. Transcription factors activities in Hh signalling

1.5.1. Ci/Gli proteins are transcriptional activators and repressors

In *Drosophila* hh aims at controlling the activity of a transcription factor **ci**, whereas vertebrates have three ci homologues named **Gli1**, **Gli2** and **Gli3**. The ci/Gli family of zinc-finger (ZnF) proteins are the nuclear effectors of Hh signalling. *Ci* was identified as a segment polarity gene (Nusslein-Volhard and Wieschaus 1980). The first gene of *Gli* family, *Gli1*, was identified as an amplified gene in human gliomas (Kinzler et al. 1987). At present, it is known that ci/Gli transcription factors mediate all identified Hh signalling activities. Hh regulates ci/Gli transcription factors at three levels: stability of the proteins, nuclear transport and activation. Besides that, unlike *ci*, *Gli* genes are transcriptionally regulated by Hh. Ectopic expression of Shh induces *Gli1*, whereas it represses *Gli3* (Marigo et al. 1996b; Grindley et al. 1997; Lee et al. 1997; Sasaki et al. 1999). Expression of *Gli2* can be induced by SHH in the dorsal CNS (Ruiz i Altaba 1998), but not in mouse lungs (Grindley et al. 1997). Gli proteins have partly overlapping expression domains and possess distinct but partly redundant functions, as indicated by analysis of *Gli* single and double mutant mice (Ding et al. 1998; Park et al. 2000; Bai and Joyner 2001).

Ci and Gli proteins are able to bind DNA through the last three of the five zinc-fingers in a sequence-specific manner (Kinzler and Vogelstein 1990; Pavletich and Pabo 1993). Gli2 and Gli3 as well as ci in *Drosophila* have N-terminal repressor domains and C-terminal activator domains **(Figure 7)**. Therefore, these proteins may serve as full-length transcriptional activators or, if C-terminally truncated, transcriptional repressors in an Hh signalling dependent manner. Gli1 is the most divergent member of the family. It lacks the N-terminal repressor domain, and functions as a strong transcriptional activator. However, recently the C-terminally truncated form of GLI1 has been described and suggested to act as a weak transcriptional repressor (Stecca and Ruiz i Altaba 2009). Normally, *Gli1* is not expressed in the absence of Hh, however its expression is highly activated upon Hh binding to the cell membrane. Thus, during development of neural tube *Gli1* is expressed most proximate to *Shh* expression domains (Walterhouse et al. 1993). The

Gli1 promoter possesses Gli-binding consensus sequence, and Gli2 and Gli3 are able to bind to these and directly activate *Gli1* expression (Dai et al. 1999; Ikram et al. 2004). Although Gli1 is the most potent transcriptional activator, it is dispensable for development in mice, and its function may be completely substituted by Gli2 (Park et al. 2000; Bai et al. 2002). In humans, up-regulation of *GLI1* is associated with many types of cancers caused by elevated SHH signalling, such as BCC (Dahmane et al. 1997). Therefore Gli1 serves as a marker of Hh activity and is very important protein for the diagnostic of the Hh-associated tumors.

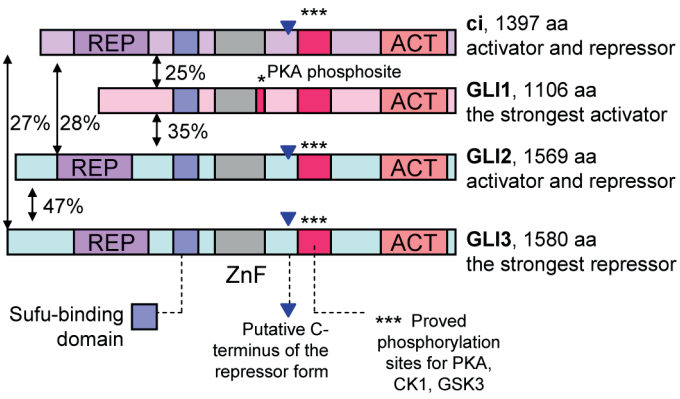


Figure 7. Schematic representation of ci/GLI proteins. Regardless of low overall identity between the proteins, they belong to one family of ci/GLI transcription factors containing DNA-binding zinc-finger domain (ZnF). The percents of identity between the respective proteins have been calculated using pair-wise alignment (ClustalW program). Ci, GLI2 and GLI3 contain repressor (REP) and activator (ACT) domains and may serve as full-length transcriptional activators or C-terminally truncated transcriptional repressors. Ci/Gli proteins contain a highly conserved Sufu binding domain and sites for PKA, GSK3 and CK1 phosphorylation (showed as asterisks) situated in the phosphorylation cluster (colored with patterned pink). The cluster is less conserved in Gli1 that is phosphorylated by PKA at Threonine-374 situated in the end of ZnF domain (Sheng et al. 2006).

Gli2 is a primary activator of Hh target genes; however, it is not as potent as Gli1. After Hh ligand binding, Gli2 induces expression of *Gli1* that afterwards is able to amplify itself and activate expression of its target genes (Sasaki et al. 1999; Aza-Blanc et al. 2000; Bai et al. 2002; Ikram et al. 2004). In contrast to Gli1, Gli2 is required for embryonic development. It has been shown that homozygous *Gli2* mutant mice die at birth with severe skeletal and neural defects (Mo et al. 1997; Ding et al. 1998). However, in mice with one mutant *Gli2* allele, normal expression of *Gli1* can rescue developmental defects caused by *Gli2* deficiency (Park et al. 2000). When expressed from the *Gli2* locus, Gli1 is able to replace embryonic requirement for Gli2, however, that is only in the presence of normal Shh signalling. Gli1 does not have transcriptional repressor activities suggesting that Gli2 repressor function may be completely performed by $Gli3^{Rep}$ during development (Bai and Joyner 2001). *Gli2* has oncogenic properties since over-expression of mouse *Gli2* in the basal epidermal layer of transgenic mice induces the formation of BCC-like

tumours and *Gli2* mRNA level is elevated in some BBC-s (Grachtchouk et al. 2000; Regl et al. 2002).

Gli3 is the most potent transcriptional repressor among Gli proteins. The Gli3 repressor function is most important in the absence of Hh signalling, when the target gene expression must be suppressed, for instance, in the developing vertebrate limb and dorsal part of developing neural tube. The presence of Hh blocks Gli3 processing (Wang et al. 2000a). It has been demonstrated that homozygous Gli3-deficient mice die around birth. They display polydactyly, gastrointestinal and dorsal CNS defects associated with ectopic Shh expression, indicating that Gli3 plays a role in repressing Shh activities (Hui and Joyner 1993; Buscher et al. 1997; Persson et al. 2002). Indeed, in humans, mutations in *GLI3* gene are associated with at least three genetic disorders: Greig cephalopolysyndactyly syndrome, Pallister-Hall syndrome and postaxial polydactyly type A that are all associated with inappropriate up-regulation of SHH signalling. However, in the absence of Gli2, Gli3 can function as a weak mediator of Shh signalling *in vivo* and *in vitro* inducing *Gli1* expression (Motoyama et al. 1998; Dai et al. 1999).

1.5.2. Regulation of ci protein

All of the above described differentiated functions of Gli proteins are performed by ci in insects. Ci is known to be subjected *in vivo* to post-translational modifications that depends on the strength of hh signal and results in three distinct forms of the protein: full-length **ci^{FL}**, activated form of full-length protein **ci^{Act}** and C-teminally truncated ci^{75} or **ci^{Rep}**.

In the absence of hh, ci presumably exists in a short, C-terminally truncated form (ci^{75} or ci^{Rep}). Ci^{Rep} is precent in the nucleus and acts as a transcriptional repressor of its target genes such as *ptc*, *dpp* and *wg* (Aza-Blanc et al. 1997). A cytoplasmic fraction of ci^{FL} is bound by the multimolecular protein complex (Hedgehog Signalling Complex, see below), that is also responsible for ci^{Rep} generation (see below). Processing of ci^{FL} occurs due to its hyper-phosphorylation by **protein kinase A** (pka), **shaggy** (a homologue of mammalian Glycogen synthase kinase 3β (Gsk3β)) and **casein kinase-1** (ck1) serine/threonine kinases at multiple serine residues situated adjacent to the ZnF domain (Chen et al. 1998; Jia et al. 2002; Price and Kalderon 2002). Hyper-phosphorylation provides a high-affinity binding sites for the ubiquitin ligase **Slimb** (orthologue of mammalian β-Transducin repeat-containing F-box proteins **βTrCP)**, which in turn recruits the **E3 SCF (Skp1/Cullin1/F-box) complex of ubiquitin ligases** to ci^{FL} and thus promotes its proteosomal degradation, complete or partial for generation of ci^{Rep} (Jiang and Struhl 1998; Zhang et al. 2005; Smelkinson et al. 2007). The ZnF domain of ci and lysine residue 750 together form a protection signal, that prevents the complete degradation of ci by the proteasome (Wang and Price 2008). In hh stimulated cells, the processing of ci^{FL} is blocked and it is converted into the full-length transcriptional activator form ci^{Act} (Aza-Blanc et al. 1997; Ohlmeyer and Kalderon 1998; Methot and Basler 1999). The process of ci^{Act} generation from ci^{FL} is pka-dependent (Wang et al. 1999), and the potency of ci^{Act} depends on the hh concentration gradient.

Moderate levels of hh cause the release of ci^{FL} from cytoplasmic tethering and it shuttling between cytoplasm and nucleus (Methot and Basler 2000; Wang and Holmgren 2000). However, conversion of ci^{FL} into ci^{Act} requires high levels of hh and additional, yet unknown, activation step. In the proximity to the A/P boundary, where the hh signal is the strongest, ci^{Act} acts as the most potent transcriptional activator (Ohlmeyer and Kalderon 1998). It has been proposed that ci^{Rep} and ci^{Act} have different sets of target genes, and transcriptional outcome depends on a delicate balance between the activator and repressor forms of ci that is regulated by hh.

1.5.3. Regulation of Gli2/3 proteins

Post-translational modifications similar to those of ci occur in Gli2 and Gli3 proteins. In the absence of Hh signals, Gli1 is transcriptionally repressed, Gli2 is phosphorylated for degradation and GLI3 is phosphorylated for processing into the repressor form. The cluster of phosphorylations is conserved in ci/Gli2/Gli3 proteins, and proteosomal generation of $Gli2/3^{Rep}$ forms is preceded by the phosphorylation of full-length Gli2/3 ($Gli2/3^{FL}$) by PKA, GSK3β and CK1 (Wang and Li 2006; Pan et al. 2009). Approximately 83 kDa $Gli3^{Rep}$ form is generated from 190-kDa $Gli3^{FL}$ and ca 78-kDa $Gli2^{Rep}$ is generated from 185-kDa $Gli2^{FL}$ via the **SPOP/Cul3** and **SCF/ßTrCP** ubiquitin-proteasomal pathway (Bhatia et al. 2006; Tempe et al. 2006; Zhang et al. 2009; Wen et al. 2010). In contrast to ci, the ZnF domains of Gli2/3 are not important for preventing their full degradation. The efficiency of $Gli2/3^{Rep}$ generation is regulated by the sequence processing determinant domain situated after ZnF (Pan and Wang 2007). Gli2 is inefficiently processed *in vivo*, and Gli2 protein lacking the C-terminal domain exhibits *in vitro* weaker repressor activity than the respective Gli3 protein (Pan et al. 2006; Pan and Wang 2007). It is noteworthy that, in contrast to Gli3, processing of Gli2 does not depend on Hh activity *in vivo* (Aza-Blanc et al. 2000). Many aspects of Gli proteins regulation depend on primary cilia (see **Figure 10A**), since it has been demonstrated that disruption of IFT, the motor-driven transport mechanism that moves proteins along the cilium, prevents formation of $Gli3^{Rep}$ and reduces $Gli2/3^{Act}$ function in rodent embryos (Huangfu et al. 2003; Huangfu and Anderson 2005; Liu et al. 2005).

In response to Hh signalling all three Gli proteins are found in primary cilia and Gli2/3 are shown to accumulate there (Haycraft et al. 2005). It has recently been demonstrated that only non-PKA phosphorylated $Gli2/3^{FL}$ proteins accumulate at the cilia tip. Therefore generation of $Gli2/3^{Act}$ is believed to occur in response to Shh signalling during anterograde ciliar transport (Huangfu and Anderson 2005; Wen et al. 2010). The data showing that disruption of ITF components attenuates $Gli2/3^{Act}$ function support this view. Transcriptional activator forms of Gli2/3 have not been described yet in molecular terms and the mechanism of $Gli2/3^{Act}$ generation is unknown. However, $Gli3^{Act}$ is found to correspond to the phosphorylated form of $Gli3^{FL}$. This kind of phosphorylation is not mediated by PKA or nuclear kinase **DYRK1A** (dual specificity tyrosine-phosphorylation-regulated kinase 1A) (Mao et al. 2002; Humke et al. 2010). $Gli2/3^{Act}$ proteins move to the nucleus where they induce expression of their target genes (see **Figure 10B**). Besides, in response to Hh signalling, $Gli2/3^{Act}$ were shown to be rapidly degraded in the nucleus, that is

consistent with findings showing that activation of transcription factors is often coupled to their degradation (Collins and Tansey 2006; Humke et al. 2010).

1.5.4. Protein kinases participating in regulation of Gli proteins

The role of PKA, GK3 and CK1 in the regulation of Gli2/3 has been discussed above. Gli1 is known to be negatively regulated by PKA that directly phosphorylates Gli1 at threonine residue 374 and thus prevents it nuclear localization (Sheng et al. 2006). **DYRK1A** directly phophorylates Gli1 retaining it in the nucleus and thus promoting the enhancement its transcriptional activity (Mao et al. 2002). **MAP3K10** (mitogen-activated protein kinase kinase kinase 10) and **DYRK2** (dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2) have been identified in a large-scale kinome screening as protein kinases required for Shh signal transduction *in vitro* (Varjosalo et al. 2008). Both kinases are able to modulate Gli transcriptional activity, **DYRK2** negatively and **MAP3K10** positively. DYRK2 phosphorylates directly Gli2 protein at serine residues 385 and 1011 and promotes its degradation by ubiquitin/proteosome pathway. MAP3K10 modulates the activity of Gli2 via regulation of DYRK2 and GSK3. **Cdc2l1** (cell division cycle 2 like 1) regulate positively Gli transcriptional activity by competing with Sufu for Gli binding site (Evangelista et al. 2008). Shh induces phosphoinositide 3-kinase (**PI3K**)-dependent phosphorylation of **Akt** kinase that in turn positively regulates Gli/Shh signalling antagonizing PKA-dependent degradation of Gli2 (Riobo et al. 2006b). Hh signalling requires also Protein Kinase Cδ (**PKCδ**) and mitogen-activated protein/extracellular signal–regulated kinase-1 (**MEK1**) that both positively regulate Gli transcriptional activity utilizing phorbol esters (Riobo et al. 2006a).

1.6. Intracellular events induced by Hh proteins

1.6.1. Hedgehog Signalling Complex comprises interactions between fu, cos2, sufu and ci^{FL} in *Drosophila*

Activation of smo at the cell membrane stimulates downstream signalling events that are implemented by several proteins. In *Drosophila*, interactions between the serine/threonine kinase **fused** (fu), kinesin-related protein **costal 2** (cos2), PEST motif containing protein **suppressor of fused** (sufu) and transcription factor **ci** play a central role in the intracellular hh signal transduction.

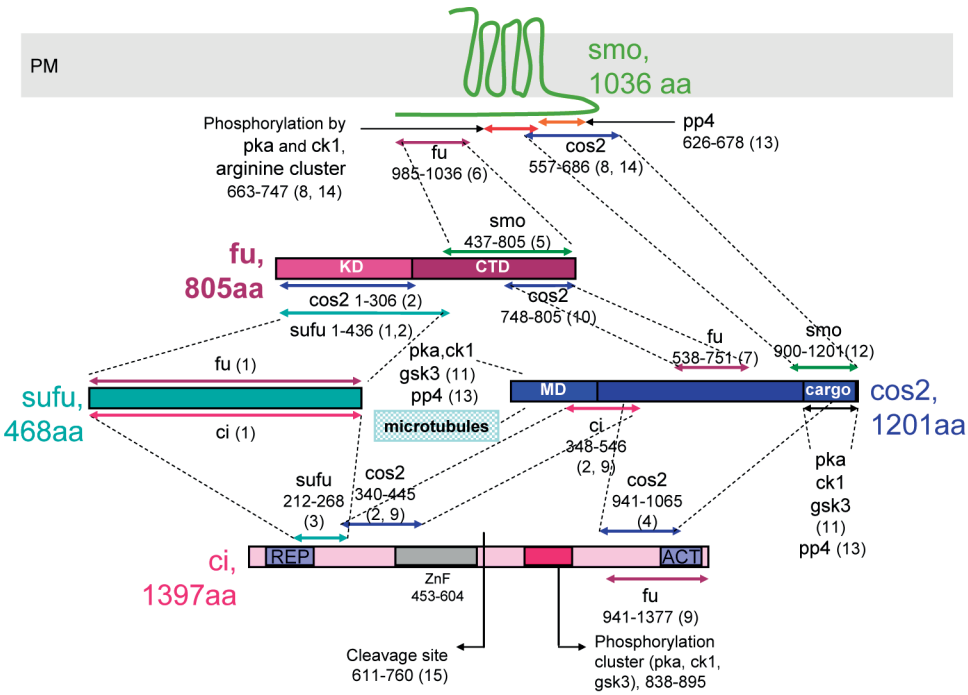
Genetic studies of *fu*, *cos2*, *sufu*, *ptc* and *ci* single and double mutants in *Drosophila* suggest complex relationship and interdependence between the products of these genes. The following observations point to that:

1. *sufu* knock-out mutants have no phenotype, but fully rescue class I *fu* mutants expressing a C-terminal domain of fu (Preat et al. 1993)
2. In a *sufu* mutant background, class II *fu* mutant flies expressing a kinase domain (KD) of wt fu, display a phenotype resembling that of a segment polarity gene *cos2* (Preat et al. 1993)

3. Decrease in *cos2* product leads to a partial suppression of *fu* phenotype (Preat et al. 1993)
4. Mutations in *sufu* strongly increase the *cos2* phenotype (Preat et al. 1993)
5. *fu* mutations do not rescue the *ci* mutant phenotype, but overexpression of ci partly restores the defects of *fu* (Sanchez-Herrero et al. 1996; Alves et al. 1998)
6. *cos2* has a phenotype highly similar to that of *ptc* (Grau and Simpson 1987)
7. Elimination of *sufu* increases the effects of *ptc* (Lefers et al. 2001)
8. Loss of *cos2* de-represses *ptc* transcription (Sanchez-Herrero et al. 1996)
9. *ptc* and *ci* products regulate each other expression, whereas ci acts downstream of ptc (Motzny and Holmgren 1995; Sanchez-Herrero et al. 1996)
10. loss of *ptc* induces ectopic expression of hh target genes (Ingham et al. 1991; Forbes et al. 1993)
11. Ectopic expression of ci partly rescues *fu* phenotype in the 3-4 intervein wing region (Alves et al. 1998)
12. *ptc*, *fu*, *cos2* and *ci* are involved in hh signal transduction (Forbes et al. 1993)

Taken together, these data consolidate hh, ptc, sufu, cos2, fu and ci into one developmental pathway that starts from the reception of the hh-encoded signal at the cell membrane and ends with transcriptional activation in the nucleus. Genetic studies place fu, sufu and cos2 as proteins acting downstream of hh and ptc and upstream of ci. Besides that, genetically, ptc, sufu and cos2 are regarded as negative regulators of the pathway, and fu and ci as positive. Additionally, the data showing that expression patterns of cos2, sufu and fu overlap and proteins cos2 and sufu have synergetic negative effect that is antagonized by fu, point to possible physical interactions between these proteins.

A number of biochemical studies confirm conclusions drawn by genetics. It has been demonstrated that fu, cos2 and sufu physically interact with each other and bind ci^{FL} through cos2 and sufu. These proteins form a tetrameric complex named Hedgehog Signalling Complex (HSC) (Robbins et al. 1997; Stegman et al. 2000). In addition, two proteins of HSC, fu and cos2, have been shown to interact with the carboxyl terminus of smo. The domains responsible for these interactions have been mapped (**Figure 8**) (Robbins et al. 1997; Monnier et al. 1998; Methot and Basler 2000; Wang et al. 2000b; Monnier et al. 2002; Jia et al. 2003; Lum et al. 2003a; Wang and Jiang 2004; Zhang et al. 2005; Liu et al. 2007; Malpel et al. 2007; Ruel et al. 2007). It should be noted, that although biochemical studies show that fu is able to interact with sufu and cos2 through its kinase and regulatory domains, genetic studies strongly propose that the N-terminal KD of fu binds to and counteracts the function of sufu, while the C-terminal regulatory domain (CTD) directly associates with cos2 to oppose its activity (Preat 1992; Ascano et al. 2002; Monnier et al. 2002).



1. Monnier et al, 1998
2. Monnier et al, 2002
3. Methot and Basler, 2000
4. Wang et al, 2000
5. Malpel et al, 2007
6. Lum et al, 2003
7. Ruel et al, 2007
8. Jia et al, 2003
9. Wang and Jiang, 2004
10. Robbins et al, 1997
11. Zhang et al, 2005
12. Ruel et al, 2003
13. Jia et al, 2009
14. Zhao et al, 2007
15. Tian et al, 2005

Figure 8. Domains in ci, cos2, fu, sufu and smo proteins responsible for interactions with each other. The four proteins are denoed by their names and the numbers of residues they have. The interacting sequences are pointed out by the arrows and outlined by residue numbers. Cleavage and phosphorylation sites are shown by thick arrows. MD – motor domain, cargo – cargo domain, ACT – activator domain, REP – repressor domain, KD – kinase domain, CTD – C-terminal domain, ZnF – zinc finger domain.

An alternative model suggests that sufu is not a member of HSC, since even in the absence of sufu a competent trimeric complex containing ci^{FL}, fu and cos2 is formed (Stegman et al. 2000). According to this model, HSC consist of ci^{FL}, fu and cos2, since the presence of those proteins is sufficient for ci^{Rep} generation. Besides, it has been shown that cos2 and fu associate in a stoichiometric manner (Robbins et al. 1997; Ascano et al. 2002) and stabilize each other (Lum et al. 2003a; Ruel et al. 2003; Liu et al. 2007). At the same time sufu is thought to interact with the fraction of ci that is not associated with fu and cos2 ((Monnier et al. 1998; Lum et al. 2003a) and below) and is needed, for instance, for tethering of ci^{FL} in the cytoplasm in order to block its residual activity.

1.6.2. HSC regulates the ci transcription factor in *Drosophila*

The HSC plays a key role in ci activity regulation. In the absence of hh, HSC is responsible for the negative regulation of ci that comprises tethering of ci^{FL} in the cytoplasm and generation of ci^{Rep}. In non-stimulated cells, the complex is bound to microtubules through cos2, and also directly associated with the cytoplasmic tail of smo through CTDs of fu or cos2 or both (Sisson et al. 1997; Lum et al. 2003a; Malpel et al. 2007). Additionally, three protein kinases, pka, ck1 and gsk3, responsible for ci^{FL} hyper-phosphorylation, are sequestered by cos2 (Zhang et al. 2005). It has been proposed that smo-HSC shuttles between the membrane and vesicular compartment (Jia et al. 2003; Ruel et al. 2003).

Upon hh stimulation phosphorylation of fu is induced (Therond et al. 1996b). Fu phosphorylates cos2 and thus induces dissociation of HSC and release of ci^{FL} from its cytoplasmic anchor (Ruel et al. 2007). In the case of HSC dissociation, phosphorylation of ci^{FL} by pka, shaggy and ck1 is blocked. Instead, pka and ck1 phosphorylate specific sites in the cytoplasmic tail of smo, that are required for pathway activation in the presence of hh (Jia et al. 2003; Zhang et al. 2004; Zhao et al. 2007). Dissociation of HSC leads to release of ci^{FL}, ready to be converted into ci^{Act}. Although the precise mechanism of ci^{Act} generation is not clear yet, it is to some extent also dependent on members of HSC that may contribute also to positive regulation of ci and consequently, generation of ci^{Act}. Cos2 is required for the full activation of the hh pathway and tranduction of high levels of hh signal (Wang et al. 2000b). Loss of *sufu* is associated with a decrease in ci^{FL} levels in wing disks (Ohlmeyer and Kalderon 1998; Lefers et al. 2001). Besides that, sufu has been shown to elicit stabilizing effects on ci, because it competes with an ubiquitin-ligase adaptor proteins for binding of ci (Zhang et al. 2006). Thus, sufu contributes in tethering but also in stabilization and conservation of ci^{FL} in the cytoplasm. The kinase activity of fu plays a positive role in regulation of ci in response to hh signal (see below).

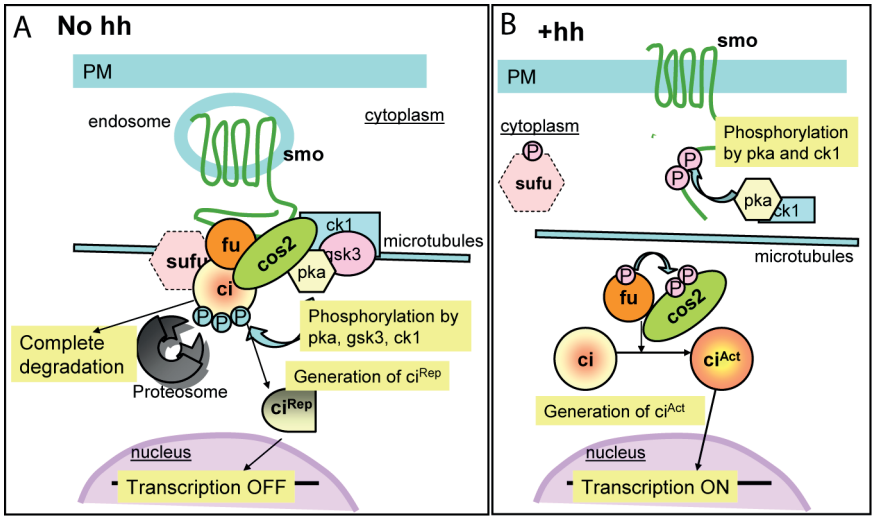


Figure 9. Fu, cos2, probably sufu and ci^{FL} proteins form HSC that is needed for the mediation of hh signal from the cell membrane to nucleus. A. In the absence of hh, the complex is bound to smo cytoplasmic tail and is responsible for the negative regulation of ci – ci^{FL} degradation or/and ci^{Rep} generation. Proteosomal degradation of ci^{FL} is primed by phosphorylation by pka, gsk3 and ck1 kinases sequestered by cos2. The multiprotein complex, bound to smo, may be tethered in the cytoplasm and bound to microtubules via cos2. Sufu is showed by dashed lines since it may not be a member of HSC. **B.** In the presence of hh, fu is (auto?)phosphorylated. Fu phosphorylates cos2, and the complex dissociates. Ci^{FL} escapes the negative phosphorylation and subsequent degradation. By an unknown mechanism ci^{FL} is converted to ci^{Act} that moves to the nucleus and activates transcription of its target genes. Generation of ci^{Act} and full activation of the pathway requires the presence of fu and cos2.

1.6.3. Sufu and Kif7 are main regulators of Gli transcription factors in vertebrates

In contrast to *Drosophila*, a similar Hh-dependent signalling complex has not been described in vertebrates. Genetic studies suggest that Sufu is a major negative regulator of Gli proteins in mammals (Cooper et al. 2005; Svärd et al. 2006). Notably, sufu-/- mutant flies are viable, whereas Sufu-deficient mice die at 9.5 dpc with multiple defects resulting from abnormal up-regulation of Hh signalling. Besides that, recently the role of Kif7, a mammalian homologue of cos2, in transduction of Shh signalling has been described (Endoh-Yamagami et al. 2009). The function of Kif7 appears to be corresponding to the dual function of cos2 in *Drosophila* hh signalling pathway. Mice deficient in Kif7 and Gli3 display very similar phenotypes (Hui and Joyner 1993; Endoh-Yamagami et al. 2009) suggesting that primary function of Kif7 is linked to Gli3 regulation.

The mechanism of Gli regulation by Sufu and Kif7 is complex and seems to occur in different cellular compartments: primary cilia, cytoplasm and nucleus (**Figure 10**). Firstly, in the absence of Hh, Sufu controls Gli proteins by direct binding and sequestering them in cytoplasm (Ding et al. 1999; Kogerman et al. 1999) (**Figure**

10A). It has been shown that in cultured cells Sufu is not able to bind C-terminally truncated Gli2/3^{Rep} and associates only with full-length proteins (Humke et al. 2010). Biochemical analyses suggest that the C-terminus of Sufu binds the N-terminus of Gli, and oppositely, the N-terminus of Sufu binds the C-terminus of Gli (Ding et al. 1999; Dunaeva et al. 2003; Merchant et al. 2004). This interaction on one hand stabilizes Gli2/3^{FL} proteins, protects them from complete proteosomal degradation, since Sufu antagonizes SPOP that promotes Gli2/3^{FL} ubiquitination, and contributes to the accumulation of a pool of Gli2/3 proteins ready to be converted to transcriptional activators (Chen et al. 2009). On the other hand, it has been shown that Sufu contributes to the generation of Gli2/3^{Rep} (Humke et al. 2010). In fact, this complex regulation may be achieved due to the interaction of Sufu with different domains of Gli: Sufu protects Gli when associated with its C-terminus, and exposes to the Gli C-terminus to proteosome when it binds to the N-terminus of Gli. Cytoplasmic tethering of Gli proteins by Sufu seems to be primary cilium-independent, because it has been shown that Sufu is able to inhibit signalling in cells lacking IFT components (Chen et al. 2009; Jia et al. 2009b). Kif7 physically interacts with Sufu and all Gli proteins, and particularly with the amino-terminus of Gli3. In the absence of Shh, Kif7 cooperates with Sufu in negative regulation of Gli proteins and is needed for efficient processing of Gli3^{FL} (Endoh-Yamagami et al. 2009).

Conversion of Gli3^{FL} into a transcriptional activator is inhibited by Sufu. In response to the pathway activation, Gli2/3^{FL} dissociate from Sufu and accumulate in the primary cilia (Haycraft et al. 2005; Humke et al. 2010) (**Figure 10B**). The Gli/Sufu complex dissociation and ciliar translocation of Gli proteins depends on the ciliary motor Kif3a (Humke et al. 2010). Besides that, Kif7 moves to the cilia and is shown to be required for efficient ciliar accumulation of Gli3^{FL} (Endoh-Yamagami et al. 2009). Gli2/3^{FL} activation is believed to occur via direct phosphorylation in the primary cilia in response to Hh binding and requires the presence of "active" Smo there (Wen et al. 2010). Activated Gli2/3 proteins move to the nucleus, and it has been shown that Kif3a is also required for nuclear translocation of Gli3^{Act}. Thus, in response to Hh signal Gli2/3^{FL} proteins translocate to the primary cilia, are phosphorylated there and move to the nucleus (Huangfu and Anderson 2005; Humke et al. 2010; Wen et al. 2010).

Besides that, apart from Gli and Kif7 proteins, Sufu directly interacts with three mammalian proteins, SAP18, Galectin3 and Cdc2l1 (Cheng and Bishop 2002; Paces-Fessy et al. 2004; Evangelista et al. 2008). The role of Sufu interaction with the nuclear protein **Galectin3** is not yet clear. **SAP18** (Sin3A-associated protein 18) is a nuclear protein, a component of the **Sin3** and **histone deacetylase complex**. Sufu, SAP18 and Sin3 are able to form a DNA-protein complex in the presence of Gli1 and oligonucleotide containing Gli-binding element. It has been proposed that Sufu functionally cooperates with SAP18 in the nucleus, where they repress Gli1-regulated transcription by recruiting the Sin3-histone deacetylase co-repressor complex to the promoters containing the Gli-binding element. These data suggest that Gli1 may serve as a transcriptional repressor in certain circumstances. Serine/threonine kinase **Cdc2l1** (cell division cycle 2-like 1) has been shown to act preferably in the nucleus antagonizing the inhibitory function of Sufu on Gli1 thus

serving as a positive regulator of the Shh pathway. It has been suggested that Cdc2l1 may compete with Gli1 for Sufu binding or disrupt Gli-binding site by direct phosphorylation of Sufu.

Thus, Sufu is thought to be able to shuttle within the cell between nucleus and cytoplasm, interacting with different proteins essential for enhancement or suppression of mammalian Hh pathway. The dual function of Sufu suggests the existence of several pools of Sufu, regulating Gli proteins in a context-dependent manner.

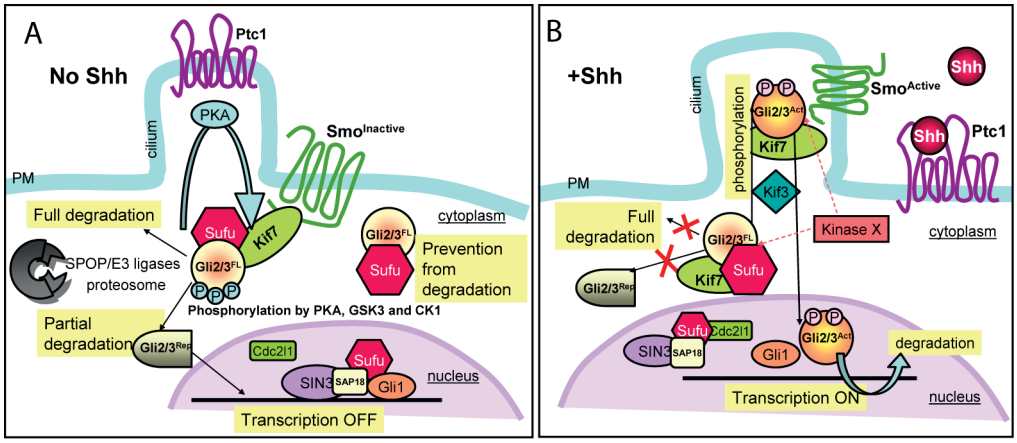


Figure 10. Regulation of Gli2/3 proteins in vertebrate cells. A. In the absence of Shh, Gli2/3^{FL} are tethered by Sufu and Kif7 in the cytoplasm and subjected to proteosomal degradation followed by their phosphorylation with PKA, GSK3 and CK1. Phosphorylation of Gli2/3 by PKA occurs in the cilium suggesting that Sufu/Kif7/Gli complex may shuttle between cilium and cytoplasm. The degradation may be complete or partial resulting in the generation of Gli2/3^{Rep}. Another fraction of Gli2/3 may be bound by Sufu alone and thus protected from the degradation. Sufu may also act in the nucleus in association with SAP18/Sin3 and Gli1 as a part of their co-repressor complex. **B.** Upon binding of Shh ligand, Sufu/Gli2/3 complex dissociates. Gli2/3 bound only by Kif7 translocate to the primary cilia and become phosphorylated there by unknown kinase responsible for generation of Gli2/3^{Act}. This kind of phosphorylation induces dissociation of Gli2/3^{Act} from Kif7 and translocation of Gli2/3^{Act} to the nucleus followed by their rapid degradation. Kif3a and "active" Smo are required for Gli2/3^{Act} generation in the cilium. Cdc2l1 kinase may disrupt nuclear Gli1/Sufu/SAP18/Sin3 inducing dissociation of Gli1 that consecutively acts as a transcriptional activator.

1.7. Serine/threonine kinase Fu in *Drosophila* and mammalian Hh pathways

1.7.1. The *fu* gene encodes a protein kinase maternally required for *Drosophila* development

Fu was identified as a segment-polarity gene in *Drosophila* (Nusslein-Volhard and Wieschaus 1980). The name "fused" originates from a work "Sex-linked inheritance in Drosophila" done by Morgan T.N. and Bridges C.B. in 1916. One of the defects

described by the authors was fusion of the third and fourth wing veins that gave name to the mutation and later also to the respective gene. In 1978 the phenotype was re-interpretated and described as thickening of the third and disappearance of the forth wing veins.

In 1980-90-s different research groups described a variety of phenotypes caused by *fu* mutations. *Fu* is mapped to the X chromosome (1-59.5) and found to be maternally required for proper pattern formation within embryonic segments and imaginal discs. Homozygous *fu* embryos derived from *fu* -/- females are not viable. They display so called segment polarity phenotype that comprises deletion of the posterior part of each thoracic and abdominal segment and a mirror-image duplication of the remaining anterior part. *Fu* -/- embryos derived from heterozygous females develop normally probably due to accumulation of *fu* product (mRNA or protein) in oocytes. However, the adults display several anomalies such as reduced female fertility, tumorous ovaries, expansion of wing vein 3 and disaperence of vein 4 and a posterior expansion of the double-row wing margin bristles (Preat et al. 1990; Therond et al. 1993). These defects occur due to the lack of *fu* product during metamorphosis and adult life, showing the requirement of fu not only during embryonic development.

Analysis of the *fu* expression pattern showed that initiatial expression of *fu* occurs during oogenesis when *fu* mRNA has been detected in both germline (nurse cells and oocytes) and surrounding follicle cells (Therond et al. 1993; Besse et al. 2002). In embryos, *fu* transcripts have been found uniformly distributed in all embryonic cells until the late extended germ band stage. From the beginning of germ band retraction, *fu* transcript abundance is strongly reduced. Later in development *fu* is expressed only in imaginal wing and leg discs. Immunocytochemical analysis of the fu expression pattern in *Drosophila* showed that, although the protein is present both in embryos and imaginal disks in all cells, it accumulates in the cells of anterior compartments known as hh signal responsive ones (Alves et al. 1998; Therond et al. 1999). However, in the ovary, fu protein distribution completely overlaps with that of *fu* transcripts (Besse et al. 2002). Interestingly, fu seems not to be required during development in the majority of the cells where it is expressed and its function in those cells remains unknown (Alves et al. 1998; Therond et al. 1999).

The product of *fu* was identified as an 805 amino acid long protein (Preat et al. 1990). It comprises of an N-terminal catalytic domain (amino acids 1 – 268) similar to the catalytic domains of serine/threonine kinases, and a C-terminal regulatory domain showing no significant homology with other proteins (Therond et al. 1993). Genetic studies show that fu functions *in vivo* as a kinase, since a functional catalytic domain is absolutely required for its activity (Therond et al. 1996a). Besides, the integrity and intramolecular association of two fu domains, kinase and extreme C-terminal (aa 503 – 805), is needed for wt fu function (Ascano and Robbins 2004). The intermolecular region between the kinase and regulatory domains (aa 422 – 502) has been found to be dispensable for fu function (Therond et al. 1996a). This region was deleted in the respective *fu* mutants that had wt phenotype.

1.7.2. fu plays kinase activity dependent and independent roles

Numerous kinases have been shown to have dual functional roles – catalytic and non-catalytic (here refered to as regulatory). In the case of fu the first indication of such a possibility came from the results of genetic analysis of *fu* and *sufu* double mutants and analysis of different *fu* alleles (Preat et al. 1993). Through genetic interactions with *sufu*, three classes of *fu* alleles have been distinguished: *fu0* (amorphic alleles; fu protein is disrupted), *fuI* (the protein is altered in the N-terminal KD; at least 503 – 805 aa of wt fu are expressed) and *fuII* (the protein bears deletions in the CTD). All *fu* alleles display a similar mutant phenotype, affecting the region between veins 3 and 4 of the wing. However, *fu0* and *fuI* mutants may be completely rescued by amorphic *sufu* mutations. But the *fuII* mutant phenotype in *sufu-/-* background resembles the phenotype of a segment polarity gene *costal2,* a kinesin-related negative regulator of hh pathway (Preat et al. 1993; Sisson et al. 1997).

These data have been confirmed and supplemented with the results of studies of the fu role in *Drosophila* wing morphogenesis. Since several studies suggested implication of the fu kinase in hh signalling, the expression pattern of the known target genes of hh, for instance *dpp* and *ptc*, has been examined in different *fu* and *sufu* backgrounds (Alves et al. 1998). *Dpp* is normally expressed only in the posterior compartment of wing disk, and fu protein is required for restriction of *dpp* expression in the anterior compartment. However, *fuII*, but not *fuI*, mutant product, is able to activate *dpp* expression in the anterior compartment away from the A/P boundary in the cells receiving very low or no hh. In the absence of fu product, *ptc* expression near the A/P boundary decreases, indicating a requirement of fu in order to maintain high level of *ptc* expression. The strongest inhibition of *ptc* expression occurs in the case of deletion of fu KD.

Besides that, deletion of the fu CTD in *fuII* mutants results in strong induction of overall ci protein level. In contrast to wt and *fuI* mutants, ci is induced also far anteriorly from the source of hh in *fuII* mutants. Moreover, *fuI* mutants demonstrate decreased level of ci protein in the anterior cells distant from the *hh* expression domain (Alves et al. 1998). These data are consistent with findings showing that high amounts or over-expression of the fu CTD enhances the *fu* mutant phenotype (Therond et al. 1996a).

Thus, investigation of *fuI and fuII* mutants has demonstrated that fu may play kinase activity dependent and independent roles. Positive function of fu depends on its kinase activity and fu extracatalytic domain is needed for down-regulation of the pathway in the absence of hh. Deletion of fu CTD results in stronger positive effects elicited by fu KD on different components of hh pathway. These findings have been confirmed by data showing that the functional KD of fu is needed for the positive regulation of ci transcriptional activity in hh-responsive cultured cells (Fukumoto et al. 2001). Moreover, deletion of fu CTD does not affect fu co-activator properties.

1.7.3. fu is required for the regulation of the hh signalling pathway

In *Drosophila* the hh pathway controls numerous developmental processes that include correct segmentation of imaginal disks during embryonic development and proper wing morphogenesis. Genetic and biochemical studies provide several lines of evidences that fu participates in hh signal transduction and interacts with other components of the hh pathway.

The first evidence that the function of fu may be linked to hh signalling pathway came from results of genetic studies. It has been shown that during embryonic development fu is required for the correct spatial expression of two transcriptional targets of hh activity, *wg* and *en*, at least during the period when the expression of those segment polarity genes is interdependent (Limbourg-Bouchon et al. 1991; van den Heuvel et al. 1993). Besides that, the expression patterns of *ptc* and ci, are affected in *fu* mutants (Motzny and Holmgren 1995; Slusarski et al. 1995; Alves et al. 1998; Therond et al. 1999; Lefers et al. 2001).

Later a role of fu in hh signalling has been specified. It has been shown that during *Drosophila* embryonic development fu is needed to maintain all levels of intracellular Hh signalling in the cells of anterior compartment (Therond et al. 1999; Raisin et al. 2010). In the *Drosophila* wing imaginal disks fu is required in A compartment close to the A/P boundary for up-regulation of genes responsive to high levels of hh, such as *dpp* and *ptc* (Sanchez-Herrero et al. 1996; Alves et al. 1998). Besides that, fu is needed for late *en* expression in A compartment of the wing disk. Absence of this late *en* expression leads to the expansion of *dpp* stripe up to the A/P boundary that is observed in *fu*-deficient discs. It also causes extension of double row bristles seen in wings of *fu* mutants. Hh-dependent somatic cell differentiation during *Drosophila* egg chamber formation requires fu kinase activity (Besse et al. 2002).

In cell culture experiments fu behaves as a positive regulator of hh signalling as assessed by RNAi and ci-induced transcriptional activation assay (Fukumoto et al. 2001; Lum et al. 2003b).

In the absence of hh, fu is in a non-phosphorylated and presumably inactive state, which is potentially mediated by autoinhibition through its regulatory domain (Ascano and Robbins 2004). In response to hh signalling, fu is phosphorylated (Therond et al. 1996b; Robbins et al. 1997). The possible site of hh-dependent phosphorylation has been mapped as Thr-158 was found to be a crucial residue for regulation of fu kinase catalytic activity (Fukumoto et al. 2001). However, it is not yet clear, if fu is subjected to self-phosphorylation or another kinase(s) phosphorylate it in response to hh signal. In the presence of hh, fu phosphorylates another component of hh signalling, cos2, in serine residues 572 and 931 (Nybakken et al. 2002; Ruel et al. 2007). Phosphorylation of cos2 by fu occurs in all hh-responsive cells and matches up the hh gradient (Raisin et al. 2010). Besides that, reception of hh induces phosphorylation of sufu. It is not clear yet, if fu directly phosphorylates sufu, but sufu phosphorylation depends on fu kinase activity (Lum et al. 2003a; Dussillol-Godar et al. 2006). Fu kinase activity is also required for smo phosphorylation and accumulation on the cell surface (Claret et al. 2007). It has been

suggested that in response to hh, fu is anchored to the membrane which is sufficient to make it a potent smo-dependent and ptc-resistant activator of the pathway.

1.7.4. fu is required for post-translational modifications of transcription factor ci

The positive regulation of ci by fu comprises several steps: release of ci^{FL} from a cytoplasmic tethering by sufu and cos2, conversion of ci^{FL} into ci^{Act}, and facilitating ci entry into the nucleus (Ohlmeyer and Kalderon 1998; Methot and Basler 2000; Wang and Holmgren 2000). The positive effects of fu depend on its kinase activity and are restricted by hh signalling region. However, through its CTD fu is required for effective ci processing in the absence of hh signalling.

The first observations hinting a negative feedback between fu and ci came from studies describing the ci protein expression pattern in *fu* mutants. The studies were performed using class II *fu* allele and showed that ci protein distribution was altered and the overall level of ci^{FL} was significantly elevated both in *Drosophila* mutant embryos and wing discs (Motzny and Holmgren 1995; Slusarski et al. 1995; Lefers et al. 2001). The studies performed on *fuI* and *fuII* mutants demonstrated that elevated levels of ci^{FL} protein in mutant wing disks as well as in embryos were not associated with alterations of the *ci* expression pattern (Alves et al. 1998; Therond et al. 1999). These observations suggest that fu is involved in post-translational modifications of the ci protein. Additionally, these studies provide indirect evidence that fu, and particularly its CTD, is needed for ci^{FL} processing in the absence of hh signalling. Generally, ci^{Rep} suppresses *dpp* expression in the anterior cells distant from *hh*-expressing cells. Analysis of the ci protein distribution in *fuI* and *fuII* mutants show that, in contrast to *fuI* mutant disks, *fuII* mutant wing disks demonstrated not only increased level of ci^{FL} protein but also expansion of the ci stripe far anteriorly from the source of hh (Alves et al. 1998; Ohlmeyer and Kalderon 1998). As a result, ectopic *dpp* expression was activated in these cells. The studies in *Drosophila* embryos also suggest that fu function may be linked to ci processing in the cells anterior to the *hh*-secreting cells during late stages of development when the intensity of hh signalling decreases (Therond et al. 1999). These data are in line with the observation that the fu effect on ci protein is not dependent on hh signalling, since alterations of ci expression in *fu* mutants is detectable much earlier than in *hh* mutants.

Immunoblotting analysis of extracts obtained from *fuII* mutant imaginal wing disks demonstrated elevated level of ci^{FL} above that of wt, but also a reduced fraction of the ci^{Rep}. Moreover, the ci processing was completely abolished in *fuII* mutants deficient also for *sufu* (mutants with *cos2*-similar phenotype) (Lefers et al. 2001). In contrast, *fuI* mutants, deficient in KD, did not exhibit reduction of ci^{Rep} in imaginal wing disks (Methot and Basler 2000). Deficiency in fu KD was only accompanied by slightly increased amount of ci^{FL} at the A/P border. Since most of the ci^{FL} is inactive while being in complex with sufu and/or cos2, hh and fu kinase activities are necessary for ci^{FL} to become a transcriptional activator.

Taken together, these results demonstrate that fu CTD is required for effective generation of ci^{Rep}, whereas fu kinase activity elicits positive effects on ci regulation.

1.7.5. Indications of a fu-independent hh pathway

Although the hh pathway includes fu as a component of the signal transduction, several reports produce evidences that fu is not involved in all aspects of hh signalling. Genetic studies in *Drosophila* embryo show that fu is dispensable in the cells of the posterior compartments regardless on its expression there (Therond et al. 1999). In contrast to other components of the hh signalling pathway, fu does not play a role in *Drosophila* somatic stem cells proliferation (Besse et al. 2002). Although hh signalling is required for eye development (Dominguez and Hafen 1997), it does not contain fu as well as ci as downstream components in the process of Bolwig's organ formation (Suzuki and Saigo 2000).

1.7.6. Mammalian serine/threonine kinase Stk36 is a putative *Drosophila* fu homologue

In parallel with *Drosophila* studies, vertebrate homologues of fu have been identified (Murone et al. 2000; Wolff et al. 2003). Serine/threonine kinase 36 (STK36) has been suggested as a *Drosophila* fu homologue, because among mammalian proteins it displays the highest sequence similarity with fu. STK36 or FU is 1315 aa long protein possessing an N-terminal KD (aa 1-276) and a C-terminal regulatory domain which is ca 500 aa longer than the respective domain of fu (1047 vs 537 aa residues). The KDs of fu and STK36 share 54 % identity and 73 % similarity (Daoud and Blanchet-Tournier 2005) (other data suggest 52 % of identity and 72 % of similarity (Merchant et al. 2005)), whereas their regulatory domains are considerably less conserved. Nevertheless, six short regions of fu and STK36 C termini demonstrate limited homology (23 – 47% of similarity) (Daoud and Blanchet-Tournier 2005).

In contrast to the rather narrow expression pattern of Shh, mouse and human homologues of Stk36 are broadly expressed during development. However, expression patterns of Stk36 and Shh overlap in such tissues as developing limbs, neural tube and somites suggesting a potential role for Stk36 in the Shh pathway (Chen et al. 2005). Postnatally, Stk36 is highly expressed in choroid plexus, ependymal lining of the ventricles in the brain, respiratory epithelium, oviduct and testis (Merchant et al. 2005; Wilson et al. 2009b). STK36 is expressed in adult brain, testis, pancreas and kidney (Østerlund et al. 2004).

In cell culture, STK36 is able to activate the transcriptional activity of Gli1 and Gli2 proteins. However, this effect depends on cell type. If STK36 enhances the transcriptional activity of Gli2 in all cell lines tested, it synergizes with GLI1 only in HH signal responsive C3H10T½, NIH3T3 and SW480 cells, but not in HEK293 cells where HH signalling pathway is known to be incomplete (Murone et al. 2000; Østerlund et al. 2004; Daoud and Blanchet-Tournier 2005; Kise et al. 2006).

Further investigations have shown that the functions of fly and vertebrate Fu homologues are not conserved. Although STK36, as its *Drosophila* homologue fu, has a KD, its kinase activity is dispensable for its function. Catalytically dead versions of STK36 carrying the appropriate mutation in the ATP binging pocket or lacking partly the KD as well as STK36 CTD alone show wt activities *in vitro* (Murone et al. 2000; Østerlund et al. 2004). It has been suggested that the positive effect of STK36 on Gli proteins occurs due to their stabilization (Kise et al. 2006). Knockout of fu in zebrafish results in mild hh-related phenotype, and mouse homologue of Stk36 is able to rescue zebrafish *fu-/-* deficiencies (Wolff et al. 2003; Wilson et al. 2009b). However, over-expression of STK36 in wt or *fu* mutant flies shows that STK36 is not able to rescue *fu* phenotype but is able to disturb the endogenous hh signalling (Daoud and Blanchet-Tournier 2005).

Two independent groups have demonstrated that Stk36 is dispensable for mouse embryonic development. If *Hh* knockout mice die at very early stages of development, *Stk36*-deficient mice develop normally until birth and do not display any Hh-related morphological phenotype (Chen et al. 2005; Merchant et al. 2005). All organs defective in Hh-deficient mice develop normally in *Fu* knockouts; the expression pattern of Hh target genes (*Ptch1*, *Gli1* and *Hip1*) is similar to wt. Taken together that indicates that embryonic Hh signalling is not perturbed in *Fu*-deficient mice. However, postnatal Stk36 knockout mice display **growth retardation**, **hydrocephalus**, rhinitis, a massive inflammation in the nasal cavity and **die** within 2-3 weeks after birth. Besides those defects, *Stk36-/-* mice display **atrophy** of **thymus** and **spleen** with massive **apoptosis of lymphocytes** (Merchant et al. 2005). In contrast to *Drosophila* fu and sufu genetic interaction, vertebrate Sufu and Stk36 do not cooperate genetically and loss of Stk36 cannot rescue Hh defects in Sufu-/- mutants (Chen et al. 2009).

As discussed above, Shh signalling requires the primary cilia that are not disrupted in **Stk36-/-** mouse embryonic fibroblasts. However, *Stk36-/-* mice are deficient in (9+2) motile cilia. It has been shown that Stk36 is able to physically interact with **Kif27**, a mammalian orthologue of Cos2, and **Spag16** (sperm associated antigen 16), a protein found in the central apparatus of the axoneme. It has been proposed that Stk36 is not required for Hh signal transduction in mammals but is needed for the generation of the central pair apparatus of motile cilia (Wilson et al. 2009b). It gives a reasonable explanation to hydrocephalus displayed by Stk36-/- mice. **Hydrocephalus** is a disorder affecting the flow, synthesis and absorption of cerebrospinal fluid (CSF) in the brain that lead to the accumulation of CSF and, as a result, to hydrocephalus. One of the factors influencing normal CSF circulation is the presence of functional **motile cilia**. Disruption of motile cilia function in humans leads to primary ciliary dyskinesia, which is associated with recurrent respiratory infection, hydrocephalus and infertility ((Wilson et al. 2009b) and references therein)). Since Stk36 is involved in motile ciliogenesis, hydrocephalus in Stk36-deficient mice may be linked to disturbance of motile cilia in the brain.

Regardless of the fact that Stk36 is dispensable for embryonic development, it plays a role in postnatal developmental events controlled by Shh in cooperation with other regulatory networks. Several groups have reported the role of Shh signalling in the

immune system. It has been shown that during the early postnatal period Shh and/or it downstream effectors are expressed in the adult thymus, spleen and lymph nodes, where they are required for survival of thymocyte progenitors and germinal-centre B cells and formation of mature T and B lymphocytes (Sacedon et al. 2003; El Andaloussi et al. 2006; Dierks et al. 2007). Atrophy of spleen and thymus and apoptosis of lymphocytes in Fu knockout mice in may be linked to defects in Hh signal transduction in those organs. Besides that, a potential oncogenic role of Stk36 in malignant B-cell lymphomas has been described (Dierks et al. 2007). It has been shown that Shh, secreted by bone-marrow, nodal and splenic stromal cells, functions as a survival factor for lymphoma cells and ectopic Shh signalling induces lymphoma growth. Treatment of the lymphoma cells with cyclopamine, an inhibitor of Smo, leads to apoptosis. In this model, forced expression of STK36 overcomes the inhibitory effects of cyclopamine preventing cyclopamine-induced apoptosis of lymphoma cells. These data suggest that STK36 acts downstream of Smo as a positive regulator of oncogenic Shh pathway. Notably, that over-expression of a strong oncogene GLI1 results in weaker rescue. These data suggest the potential role for Stk36 in Shh-related cancers in humans.

1.7.7. Unc51-like kinases (Ulk) are the closest relatives of Stk36

In the light of the fact that Stk36 has lost its kinase activity dependent function during evolution, other serine/threonine kinases are thought to substitute fu in the mammalian Hh pathway. A family of unc51-like protein kinases, particularly Ulk3 and Ulk2, has been hypothetically suggested as candidates for recapitulation of fu catalytic function (Wilson et al. 2009b). There are two reasons that obviously led to this idea:

1. Among other serine/threonine kinases Ulk2 and Ulk3 share higher identity with Stk36 and fu in kinase domain (37% and 38%, respectively) (Chan et al. 2009; Wilson et al. 2009b).

2. Ulk1 and Ulk2 are shown to be active kinases (Yan et al. 1998; Yan et al. 1999).

Unc51 (uncoordinated movement)-51 is an ancient family of serine/threonine kinases conserved from yeast to human. Yeast, *Drosophila* and *C.elegance* genomes have a single *unc51* gene, also called *atg1* or *autophagy-related 1*. In vertebrates, four *Ulk* genes (unc51-like kinases 1, 2, 3 and 4) are annotated to belong to *unc51* family. Among Ulk proteins, Ulk1/2 proteins display the highest homology with each other and unc51/atg1 proteins and represent the functionally conserved members of the atg1/unc51 family (Chan and Tooze 2009).

Several studies have reported that yeast, *Drosophila* and *C.elegance* atg1 kinases, as well as their closest mammalian homologues Ulk1 and Ulk2, are implicated in **autophagocytosis**. Autophagy is a lysosome dependent cytoplasmic degradation of damaged or unwanted cell's own components. It involves generation of **autophagosome** – a structure that envelops the degradation target into a membrane and afterwards fuses with a lysosome. Additionally, atg1/unc51/Ulk1/2 proteins are

shown to be critical for **neuronal development**, where they are involved in neuronal vesicular trafficking, axonal elongation and guidance.

Ulk3 diverges from the members of the family sharing comparable similarity with Ulk1/2 kinases and Stk36 in the KD. Although one study has reported involvement of Ulk3 in special form of autophagy associated with oncogene-induced cell senescence (Young et al. 2009), the common opinion considers a role of Ulk3 in autophagy as controversial and requiring additional investigations (Chan and Tooze 2009).

2. AIMS OF THE STUDY

1. To clone *Stk36* and *ULK3* and analyse their expression in adult tissues, prenatal and adult brain and brain parts
2. To assess the requirement of Stk36 and ULK3 for Shh signal transduction in cultured cells using RNAi
3. To assess the ability of Stk36 and ULK3 to regulate transcriptional activity of Gli1 and Gli2 proteins *in vitro*
4. To estimate the requirement of Stk36 and ULK3 kinase activity in Shh signalling pathway
5. To determine the kinase properties of ULK3
6. To determine ULK3 autophosphorylation sites
7. To analyse the possibility of physical interaction between ULK3 and SUFU
8. To assess the possible biological meaning of ULK3

3. MATERIALS AND METHODS

Detailed description of materials and methods is provided in the publications of this thesis. Briefly, the following methods were used in the present study:

1. Real-Time PCR (Publications I, II and III)
2. Ribonuclease protection assay (Publication I)
3. Nothern blotting (Publication I)
4. FACS analysis (Publication I)
5. Cell culture and transfections (Publications I, II and III)
6. Isolation of rat cerebellar granular cells (Publication III)
7. RNAi and creation of stable cell lines (Publication I and III)
8. Luciferase assay or Transcriptional activation assay (Publications I, II and III)
9. Bacterial expression and purification of proteins (Publications I, II and III)
10. Immunocytochemistry (Publication II)
11. Cellular fractionation (Publication II)
12. Quantitative Real-Time PCR (Publication II and III)
13. Western blotting (Publications II and III)
14. *In vitro* kinase assay (Publications II and III)
15. Mass Spectrometry analysis (Publication III)
16. Immunoprecipitation (Publication III)
17. Bioinformatic analysis (Publications I, II and III)

4. RESULTS AND DISCUSSION

4.1 Stk36 and ULK3 are serine/threonine kinases sharing similarity with fu (Publications I and II)

In order to determine which kinases share similarity with *Drosophila* fu kinase, we performed comparative homology analysis against the human and mouse protein databanks using the sequence of the fu KD as a query. Our bioinformatic analysis revealed that Stk36 shares the highest similarity with fu (51% of identity in the kinase domains). Apart from Stk36, mouse Ulk3 or human ULK3 (unc51-like kinase 3) kinases sharing 95% of identity with each other are also close relatives of the fu protein. The kinase domain of ULK3 shares 37% and 39% of identity with fu and STK36 kinase domains, respectively. Previously, ULK3 has been annotated as belonging to the family of unc-51-like kinases that comprises also ULK1, ULK2 and ULK4 proteins. Although ULK3 shares 39% of identity with ULK1 and 37% of identity with ULK2 in the kinase domains, we have found that ULK3 diverges from the other members of the family. Our analysis suggests that ULK3 may be a homologue of Stk36 and fu.

Drosophila fu is known to encode for a 805 aa long protein with an N-terminal KD (aa 1 – 268) and a C-terminal regulatory domain (aa 503 – 805) (Therond et al. 1993). Sequence analysis predicted that *ULK3* and *Stk36* encode for 472 and 1314 residue long proteins, respectively. The predicted Stk36 and ULK3 proteins possess N-terminal kinase domains (aa 3 – 254 and 14 – 271, respectively) containing theoretically all features required for catalytic activity. However, very low or no similarity has been found in regions outside the KDs of the proteins. Since the length of the CTDs is very different (536 aa in fu, 1060 aa in Stk36 and 201 aa in ULK3), the comparative homology analysis may not be pertinent. Additionally, a MIT domain (the domain contained within Microtubule Interacting and Trafficking molecules) has been detected in ULK3. Neither fu nor Stk36 contain MIT domain. The results of our bioinformatics analysis are illustrated in **Figure 11**. The cDNAs encoding mouse Stk36 and human ULK3 proteins have been cloned.

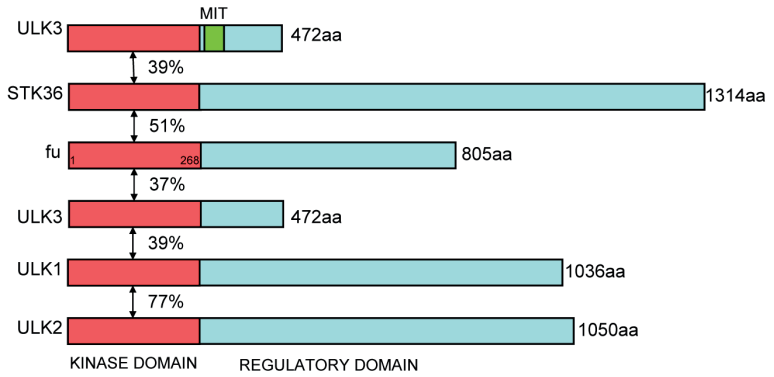


Figure 11. ULK3 shares similarity with ULK1-2 and STK36 serine-threonine kinases. The proteins are denoed by their names and the numbers of residues they have. The percents of identity between the KDs of the proteins were calculated using pair-wise alignment (ClustalW program). MIT - domain contained within Microtubule Interacting and Trafficking molecules.

4.2. Expression pattern of *Stk36* and *ULK3* (Publications I and II)

The expression pattern of *Stk36* was analyzed in different adult tissues using Nothern blot and Ribonuclease Protection Assay (RPA). Besides that, for RPA analysis we used fetal brain at several stages during development. *Stk36* transcripts were detected only in testis, adult and developing brain, where *Stk36* expression was very low on embryonic day 12, gradually increased during embryo brain development but decreased after birth. These results are partly in line with the expression analysis in human tissues where the highest expression of *STK36* was found in the brain, testis, pancreas and kidney (Østerlund et al. 2004). High expression of human and mouse *Stk36* in testis suggests a prominent role of Stk36 in this tissue. It has been shown that *Gli1* and *Ptc1* are expressed in adult mouse testis suggesting a potential role for Hh pathway (Turner et al. 2004). Indeed, an important role for Dhh in testicular development has been elucidated by analysis of the phenotype of *Dhh* knockout mice showing that Sertoli cells derived Dhh is required for the development of adult-type Leydig cells (Clark et al. 2000). However, the question of Stk36 function in testis development has to be addressed. Recently, the expression pattern and a role of Stk36 in postnatal brain have been specified. Stk36 is expressed in choroid plexus and the ependymal lining of the ventricles in the brain where it is required for motile cilia genesis (Wilson et al. 2009b).

The *ULK3* expression pattern was analyzed in postnatal human tissues, fetal brain and fetal liver using qRT-PCR. Additionally, *ULK3* expression was analyzed in 10 adult brain parts and compared with the expression of *GLI1* and *PTC1*. Our analysis reveals that *ULK3* is widely expressed. Its expression is higher in a number of tissues where Shh signalling is known to be active, such as fetal and postnatal brain and liver (Fuccillo et al. 2006; Omenetti and Diehl 2008). In contrast to *Stk36*, only moderate amounts of *ULK3* mRNA has been detected in testis. Within the adult brain, higher expression of *ULK3* is found in hippocampus, olfactory bulb, cerebellum and optic nerve. Also, *GLI1* and *PTC1* mRNA expression levels were higher in the same brain regions. Generally, expression of *ULK3* was in good correlation with the expression of *GLI1* and *PTC1* in the following brain parts: olfactory bulb, thalamus, pons, medulla, cerebellum, spinal cord and optic nerve. Taken together, our data show that SHH-responsive tissues express higher levels of *ULK3* mRNA suggesting that ULK3 may be involved in SHH pathway *in vivo*.

4.3. Stk36 and ULK3 enhance transcriptional activity of GLI1 and GLI2 proteins (Publications I and II)

Genetic and biochemical studies show that fu positively regulates ci transcription factor in a kinase activity dependent manner *in vivo* and *in vitro* (Therond et al. 1999; Methot and Basler 2000; Fukumoto et al. 2001; Lefers et al. 2001). In order to evaluate the potential role of Stk36 and ULK3 in the regulation of Gli proteins, we over-expressed the kinases either alone or together with potent transcriptional activators Gli1 and Gli2 in NIH3T3 derived clonal cells, Shh-L2 (Taipale et al. 2000). Shh-L2 cells contain stably incorporated Firefly luciferase reporter under

control of a Gli-dependent promoter in their genome. ULK3 and Stk36 were able to enhance Gli1 and Gli2 transcriptional activity with a comparable efficiency. However, when over-expressed alone, ULK3, in contrast to Stk36, was able to induce Gli-dependent luciferase reporter activity four times above the control. This effect was enhanced by stimulation of the cells with biologically active Shh protein. These findings indicate that ULK3 potentiates not only over-expressed but also endogenous Gli transcriptional activity. Besides that, we tested in the same assay the closest homologues of ULK3, human proteins STK36 (Østerlund et al. 2004) and ULK1 (kindly provided by Dr. Sharon Tooze (Centre for Cancer Research UK, London)). We have found that ULK3, when compared to ULK1 and STK36, has the strongest effect on GLI-dependent luciferase reporter activity.

It has previously been reported that STK36 is a positive regulator of Gli proteins (Murone et al. 2000; Østerlund et al. 2004). STK36 enhances GLI2 transcriptional activity in HEK293 and C3H10T1/2 cells (Murone et al. 2000; Østerlund et al. 2004) and GLI1 and GLI2 transcriptional activities in C3H10T1/2, NIH3T3 and SW480 cells (Daoud and Blanchet-Tournier 2005; Kise et al. 2006). We show that STK36 fails to induce GLI1 but induces GLI2 transcriptional activity in Shh-L2 cells. However, mouse Stk36 is able to synergize with both proteins in the same cell line. One possible explanation for this phenomenon is cell type specificity suggesting that co-activator properties of Stk36 homologues strongly depend on the particular cell type. Another possible explanation arises from comparative analysis of human and mouse Stk36 sequences. Those proteins share 84% of identity, and the most diverse region sharing 60% of identity is situated in the regulatory domain between amino acids 305 – 402. It may be expected that this particularly region is highly important for the regulation of GLI1 but not essential for the regulation of GLI2. STK36 is able to interact physically with all GLI proteins (Murone et al. 2000). Besides that, it has been shown that STK36 protects the GLI1/2 proteins from degradation and prolonges their half-lives (Kise et al. 2006). Therefore the positive effect of Stk36 on GLI1/2 transcriptional activity may be caused by stabilization of GLI proteins.

4.4. Stk36 and ULK3 regulate transcriptional activity of GLI proteins in a different way: ULK3 does and Stk36 does not require kinase activity (Publications I and II)

In order to analyse the requirement of catalytic activity of Stk36 and ULK3 for regulation of Gli proteins, we mutated the highly conserved lysine residues (K) in positions 33 and 44, respectively, to arginines (R). The resulted constructs are referred as Stk36(K33R) and ULK3(K44R) and are predicted to encode kinase-inactive variants of Stk36 and ULK3. Additionally, in case of ULK3 the conserved K-139 was mutated to R and the resulting construct is referred as ULK3(K139R). Stk36 K-33 and ULK3 K-44 are situated in the ATP binding pocket, and ULK3 K-139 is situated in the substrate binding pocket and catalytic loop regions (BLASTp conserved domain analysis, NCBI). The mutated K residues are conserved in fu (K33 and K127). We over-expressed all constructs together with GLI1 and GLI2 proteins in Shh-L2 cells and assessed the Gli-induced luciferase activity.

Stk36(K33R) is able to enhance the transcriptional activity of GLI1 and GLI2 with the same efficiency as wt Stk36, indicating that kinase activity of Stk36 is not required for the regulation of Gli-induced transcription. These data are in line with previously reported lack of STK36 kinase activity of in regulation of GLI proteins (Murone et al. 2000; Østerlund et al. 2004).

In contrast to Stk36, ULK3 requires kinase activity for the positive regulation of GLI proteins because neither of the mutants have any effect on GLI1 transcriptional activity. However, mutant ULK3(K44R), if co-expressed with GLI2, demonstrates some residual activity, whereas ULK3(K139R) was completely inactive. These data have been confirmed by testing the effect of ULK3 and the mutants on the luciferase reporter when the SHH pathway was activated by cotransfection with pShhN (plasmid expressing biologically active part of SHH). The ULK3 mutants could not activate Gli-luciferase reporter as efficiently as wt ULK3, although ULK3(K44R) had a residual positive effect on the activated pathway. Taken together the data suggest that GLI proteins can be regulated by at least two fu-related kinases - ULK3 and STK36. STK36 is able to enhance Gli-induced transcription in certain cells in a kinase-independent way, whereas ULK3 requires functional kinase domain.

4.5. KD and CTD of Stk36 and ULK3 are involved in GLI regulation (Publications I and III)

Genetic studies have demonstrated that fu KD and CTD are required for positive and negative regulation of ci, respectively. In particular, fu CTD is needed for effective processing of ci^{FL} to ci^{Rep}, and fu KD is one of the factors responsible for conversion of ci^{FL} into ci^{Act} (Alves et al. 1998; Methot and Basler 2000; Lefers et al. 2001; Ascano et al. 2002). In order to test the differentiated requirements of Stk36 and ULK3 KDs and CTDs for GLI proteins regulation, we made expression constructs encoding the respective domains and over-expressed them together with GLI1/2 proteins in Shh-L2 cells. Stk36 KD was only slightly weaker than wt Stk36, and moreover, the CTD alone could moderately enhance GLI1/2 transcriptional activity. These data suggest that both domains of Stk36 are involved in positive GLI regulation, and Stk36 is more likely to play a regulatory rather than catalytic role.

In contrast, ULK3 KD enhanced GLI2 transcriptional activity significantly stronger than wt ULK3, and ULK3 CTD inhibited GLI2-induced luciferase activity. These data suggest that ULK3 through its CTD may participate in negative regulation of GLI proteins. Deletion of ULK3 CTD results in generation of a more potent transcriptional co-activator form of ULK3, ULK3-KD that positively regulates GLI2 via direct phosphorylation (see below). Thus, the transcription activation assay suggests a dual role for ULK3 that resembles to some extend the role of fu in regulation of ci.

4.6. ULK3 is an autophosphorylated kinase that possesses self-regulation properties and is able to phosphorylate GLI proteins in vitro (Publications II and III)

It has been demonstrated that fu is an active kinase able to directly phosphorylate the kinesin-related protein cos2 *in vivo* and *in vitro* (Nybakken et al. 2002; Ruel et al. 2007). ULK3 shares similarity not only with fu but also with ULK1 and ULK2 proteins that have been previously characterized as autophosphorylating kinases *in vitro* (Tomoda et al. 1999; Yan et al. 1999). Our data of the transcription activation assay suggest that ULK3 may be an active kinase since it requires a functional KD for positive regulation of GLI proteins.

Indeed, we found that ULK3 is an autophosphorylated kinase. Mouse Ulk1 and Ulk2 are shown to be autophosphorylated in the conserved central proline/serine rich domains. However, ULK3 lacks such a domain. ULK3 autophosphorylation occurs at four serine residues (Ser-300, Ser-350, Ser-384 and Ser-464) situated outside of the KD.

Besides that, we have found that ULK3 directly phosphorylates all three GLI proteins. Apart from ULK3, only PKA has been shown to directly phosphorylate all Gli proteins, and CK1 and GSK3 have been shown to phosphorylate Gli2 and Gli3. PKA phosphorylates Gli1 in residues Thr-374 and Ser-640 (Sheng et al. 2006). Gli2 and Gli3 are phosphorylated by PKA, CK1, and GSK3 in a cluster between aa 784-855 that corresponds to Gli1 residues 590-658 (Pan et al. 2006; Pan et al. 2009) (**Figure 8**). We have found that ULK3 phosphorylates GLI1 both in the N-terminus (residues 1-426) and C-terminus (residues 754-1126), but the fragment of GLI1 between residues 426-754 is not phosphorylated by ULK3. GLI2 is phosphorylated by ULK3 in residues between aa 650–819 (my unpublished results).

Phosphorylation by PKA, CK1 and GSK3 elicits negative effects on Gli proteins resulting in initiation of their proteosomal degradation. Our analysis of Shh pathway activation in cell culture shows that ULK3 kinase activity is required for positive regulation of GLI1 and GLI2 proteins.

Being in an inactive stage, fu is subjected to autoinhibition through intermolecular association of it KD and CTD (Ascano and Robbins 2004). We demonstrate that ULK3 also has intramolecular self-regulatory properties. Moreover, wt ULK3 is not able to autophosphorylate in *trans*. The sites of ULK3 autophosphorylation are hidden in catalytically inactive ULK3 and unexposed to other ULK3 molecules. Deletion of the KD results in conformational changes making CTD available for intermolecular phosphorylation by ULK3. Deletion of CTD results in generation of catalytically active KD that is not able to autophosphorylate but is able to phosphorylate its substrate, GLI2. Thus, autophosphorylation of ULK3 may involve conformational changes resulting in exposure of CTD to KD and consequently in generation of the catalytically active kinase. A similar mechanism of self-regulation control has been proposed for ULK1 and ULK2 kinases (Chan et al. 2009) and seems to be conserved between members of the ULK family.

4.7. Ulk3 and Stk36 are required for Shh signal transduction (Publications I and III)

It has been shown that protein kinase fu participates in hh signal transduction and is vitally required for normal embryonic development of *Drosophila* (Nusslein-Volhard and Wieschaus 1980; Preat et al. 1990; Therond et al. 1999). In contrast, mice deficient in the closest homologue of fu, Stk36, do not display any Shh-related phenotype during embryogenesis (Chen et al. 2005; Merchant et al. 2005). However, *Stk36-/-* mice die within 3 weeks after birth with brain defects at least partly caused by deficiency in motile cilia (Wilson et al. 2009b). However, over-expressed Stk36 is able to prevent cyclopamine-induced apoptosis of malignant lymphoma cells which *in vivo* require the Shh signal for survival and proliferation (Dierks et al. 2007). Additionally, as discussed above, human and mouse Stk36 differently enhance GLI transcriptional activity in different cell lines suggesting that the function of Stk36 may be restricted to particular cell types.

In order to enhance our understanding of the function of Stk36 and Ulk3 in mammalian cells, we applied RNAi to test the overall requirement of those proteins for Shh signal transduction. We designed siRNA-expressing constructs: one construct encoding siRNA specific for *Stk36* and two constructs encoding siRNAs specific for *Ulk3*. We over-expressed those constructs in Shh-L2 cells and, after selection several clones stably expressing *Stk36*- and *Ulk3*-specific siRNAs were isolated. The efficiency of *Stk36* and *Ulk3* silencing was estimated by RPA and qRT-PCR, respectively. As compared to the parental cell line Shh-L2, two independent clones showed significant reduction of *Stk36* mRNA level – 65% and 84%. The level of Stk36 protein in parental and stable cell lines was measured by FACS analysis using polyclonal anti-Stk36 antibody. Comparing with the parental cell line, the level of Stk36 protein in stable cell lines was 62% and 2%, respectively.

Suppression of *Ulk3* mRNA was achieved in 6 clones. The maximum suppression of endogenous *Ulk3* mRNA expression level was approximately 50%. We failed to get cells with lower level of *Ulk3* mRNA. One possible reason for that may be the fact that reduction of *Ulk3* mRNA level leads to S-phase arrest (Daub et al. 2008). Therefore, it is possible, that the cells with lower level of *Ulk3* mRNA were not able to grow sufficiently to be picked and analyzed. Due to the lack of a working antibody against Ulk3, we were unable to show the endogenous Ulk3 protein levels in the stable cell lines. However, using antibody against the FLAG tag, we estimated the levels of exogenously added Ulk3FLAG protein in the cell clones expressing the lowest level of *Ulk3* mRNA. The level of Ulk3FLAG protein in those clones correlated with reduced *Ulk3* mRNA level.

In parallel with measurements of *Stk36* and *Ulk3* mRNA levels, the respective cells were induced by SHH protein. In this case normal transduction of SHH signal results in moderate increase of Gli-dependent luciferase activity. That was achieved in parental cell line and in the clone expressing 62% of Stk36 protein. The cells with the lowest expression of Stk36 (2%) demonstrated reduced response to SHH protein. Our analysis indicates that Shh signalling may be affected only in the cells expressing very low levels of Stk36 protein.

In contrast, cells expressing the lowest amount of *Ulk3* mRNA demonstrated higher induction of Gli-dependent luciferase activity compared to the parental cell line. The same result was achieved using an independent experimental system where rat cerebellar granular cells (RCGCs) were transiently transfected with *Ulk3*-specific siRNAs, induced by SHH, and the level of *Gli1* mRNA was measured by qRT-PCR. In both cases, reduction of the *Ulk3* mRNA level resulted in elevated response to SHH reflected by an increased levels in either Gli-dependent luciferase activity in Shh-L2 cells or *Gli1* mRNA in RCGCs. In fact, the level of *Gli1* mRNA induction in response to SHH was in correlation with the *Ulk3* mRNA level in the cells. One possible explanation of our RNAi results is that Ulk3 may be involved in the negative regulation of some component(s) of the Shh pathway, for instance in generation of $Gli2/3^{Rep}$ in non-induced cells.

Taken together, our results show that only very strong reduction of *Stk36* mRNA level resulted in loss of Stk36 protein and impaired Shh signalling. Reduction of the *Ulk3* mRNA level in cells triggers a stronger response to SHH. Thus, our data from RNAi experiments suggest that Ulk3 is functioning as a negative regulator of the Shh pathway.

4.8. ULK3 directly interacts with SUFU through its KD that leads to loss of ULK3 autophosphorylation activity (Publication III)

Fu is known to bind directly several proteins – sufu, cos2 and smo. Those interactions are highly important for the negative regulation of ci in the absence of hh (Robbins et al. 1997; Monnier et al. 1998; Monnier et al. 2002; Malpel et al. 2007). In non-stimulated cells, ci^{FL}, fu, cos2 and probably sufu form the so called HCS that dissociates in the presence of hh, releasing ci^{FL} ready to be converted to ci^{Act} (Stegman et al. 2000; Lum et al. 2003a; Ruel et al. 2007). The most comprehensive explanations of biological effects of fu/sufu interaction come from genetic studies, showing that the *fu* phenotype may be completely rescued by additional deletion of *sufu* (Preat 1992). Since ULK3 is similar to fu, and in mammals Sufu is the central negative regulator of Gli proteins (Pearse et al. 1999; Cooper et al. 2005; Svärd et al. 2006), we tested if ULK3 is able to interact with SUFU. We have found that ULK3 interacts not only with over-expressed, but also with endogenous SUFU. Moreover, the ULK3/Sufu complex dissociates under influence of SHH stimulation.

Biochemical studies have shown that fu may interact with sufu through both KD and CTD (Monnier et al. 1998; Monnier et al. 2002). Besides that, the region between aa 306 – 436 has been defined as a minimal domain responsible for sufu binding. However, genetic studies strongly suggest that fu bind sufu through its KD (Preat et al. 1993). Regardless of the overall dissimilarity of the CTDs of fu and ULK3, we mapped two regions of homology between ULK3 and fu CTDs. The first region corresponds to residues 310 – 365 and the second to residues 398 – 433 of ULK3. We have found that ULK3 binds SUFU through it KD, as fu does. ULK3 proteins deficient in intramolecular regions are able to bind SUFU with the comparable with wt ULK3 efficiency. This indicates that Sufu-interaction domain of ULK3 is not

fully equivalent to the corresponding domain of fu. ULK3 CTD does not participate in interaction with SUFU.

To get better understanding about the biological meaning of the ULK3-SUFU interaction and keeping in mind that fu, being in HSC, is catalytically inactive, we tested if ULK3 exhibits autophosphorylation activity in complex with SUFU. We found that the kinase activity of ULK3 was completely blocked by interaction with SUFU. Taken together, our data show that SUFU, the negative regulator of SHH pathway, may bind and block ULK3 catalytical domain thus preventing the positive functioning of ULK3. This effect may be inhibited by SHH signalling that induces dissociation of the SUFU/ULK3 complex and release of catalytically active ULK3.

In over-expression expreriments, STK36 is able to rescue the negative effect of SUFU on Gli-dependent transcription to some extent and physically interact with SUFU (Murone et al. 2000). Apart from Gli and STK36, four mammalian proteins, KIF7, SAP18, Galectin3 and Cdc2l1, have been identified as proteins able to physically interact with Sufu (Cheng and Bishop 2002; Paces-Fessy et al. 2004; Evangelista et al. 2008; Endoh-Yamagami et al. 2009; Humke et al. 2010), suggesting that Sufu may shuttle within the cell, interacting with different proteins essential for enhancement or suppression of its regulatory functions. A key protein interacting with SUFU is likely to be ULK3 that is inactive in respect to auto- and Gli-phosphorylation in this complex.

4.9. The ULK3-SUFU complex promotes generation of GLI2^{Rep} form (Publication III)

Genetic and biochemical studies have demonstrated that fu, and particularly its CTD, is involved in ci^{Rep} generation since the amount of ci^{Rep} is significantly reduced in mutants deficient in fu CTD. Moreover, ci degradation is completely abolished in the mutants lacking not only fu CTD but also sufu (Lefers et al. 2001; Ascano et al. 2002). These data suggest that both proteins, sufu and fu, are required for efficient ci processing. Our data of the transcription activation assay and RNAi suggest that ULK3 participates in both positive (Gli phosphorylation) and negative regulation of SHH pathway, and ULK3 CTD is responsible for negative regulation of at least GLI2 (see p.4.5.). As it has been described in 4.8., ULK3 is able to interact with SUFU, and Sufu contributes to the generation of Gli2/3^{Rep} (Humke et al. 2010). In order to analyse the functional meaning of the ULK3/SUFU interaction, we tested if ULK3 and SUFU, analogically to fu/sufu, act in concert promoting the generation of GLI2^{Rep}. Indeed, we have found that neither SUFU nor ULK3 alone can induce the generation of GLI2^{Rep}, but acting in concert, they promote C-terminal processing of full-length GLI2. As it can be expected, the ULK3 kinase activity is not required for GLI2^{Rep} generation. However, ULK3 CTD is needed for GLI2 processing (my unpublished observations). These data suggest that ULK3/SUFU complex, analogically to fu/sufu complex in *Drosophila*, is involved in negative regulation of HH pathway.

Another protein complex conserved from *Drosophila* to vertebrates is represented by Kif7, the mammalian homologue of cos2, and Sufu (Endoh-Yamagami et al.

2009). Kif7 physically interacts with Sufu and all Gli proteins. In the absence of Shh, Kif7 cooperates with Sufu in negative regulation of Gli proteins and is needed for efficient processing of $Gli3^{FL}$. Besides that, in the presence of Shh, Sufu/Kif7/Gli complex dissociates, and Kif7 contributes to the positive regulation of Gli3 since it is required for the efficient ciliar accumulation of $Gli3^{FL}$, preceded for generation of $Gli3^{Act}$. Although there is no evidence yet that ULK3 functionally or physically interacts with KIF7, it may be speculated that ULK3/SUFU/KIF7/GLI2/3 complex acts similarly to fu/sufu/cos2/ci complex or HSC in *Drosophila*.

4.10. An emerging Shh signalling model involving Ulk3 (Publication III and here)

From the presented data it can be speculated that Ulk3 has at least two distinct roles in the Hh signalling pathway eliciting kinase activity-dependent and -independent effects on Gli transcription factors. The proposed model is depicted in **Figure 12**. In the absence of Shh, Kif7 and Sufu interact with Gli2/3 proteins and dock them in the cytoplasm. Sufu also binds the KD of Ulk3 thus inhibiting its catalytic activity. The $Gli2/3^{FL}$ proteins are sequestered by the Ulk3/Sufu/Kif7 complex and cannot be converted to active forms. Moreover, the complex participates in the generation of C-terminally truncated transcriptional repressors $Gli2/3^{Rep}$. Through its putative MIT domain (domain contained within Microtubule Interacting and Trafficking molecules), Ulk3 may be a link between Sufu/Kif7/GliFL complex and microtubules. In the presence of Shh, the Ulk3/Sufu/Kif7 complex dissociates. Ulk3 becomes an active kinase that autophosphorylates and stabilizes in an active conformation. Ulk3 is now able to phosphorylate the released $Gli2/3^{FL}$ and contribute to $Gli2/3^{Act}$ formation. $Gli2/3^{Act}$ enter the nucleus and activate the expression of their target genes including *Ptc* and *Gli1*. Other molecule(s), for instance Stk36, may participate in the regulation of Gli2/3 activity by converting them to transcriptional activator or repressor forms depending on cellular context and strength of Shh signaling. Whether Ulk3 also interacts with and phosphorylates Kif7 and thereby contributes to the complex dissociation remains to be investigated. It also remains to be investigated if Ulk3 is able to phosphorylate other signalling components like Smo and Ptc further influencing the pathway. However, it is clear from these analyses that Ulk3 plays an important role in regulation of Gli proteins and is likely to be the "missing link" in the Sufu/Gli regulatory complex in Shh signalling.

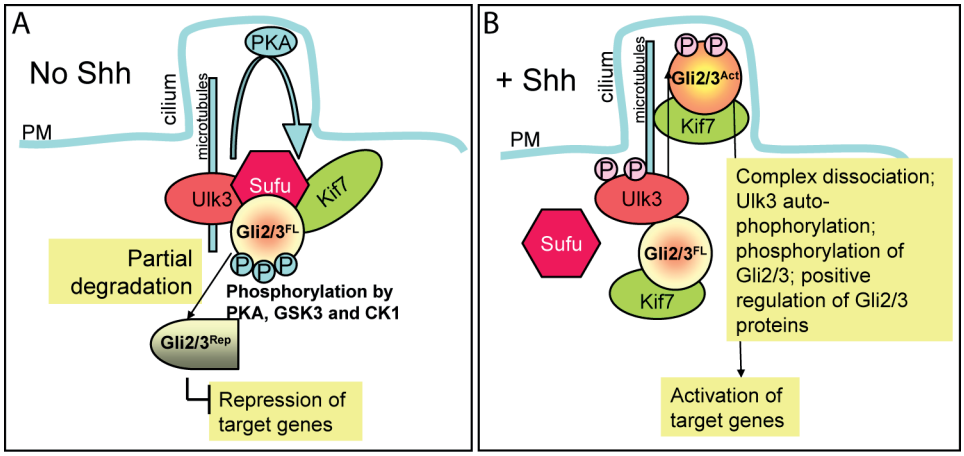


Figure 12. A model for the actions of Ulk3 in the Shh pathway. A. In the absence of Shh, Sufu forms a complex with Kif7 and Ulk3 that possibly interacts with cytoskeleton through its MIT domain. Ulk3 is not catalytically active but plays a regulatory role. The complex binds Gli2/3^{FL} through Sufu and Kif7. Ulk3/Sufu/Kif7 complex contributes to C-terminal processing of Gli2/3 probably through recruiting PKA, GSK3 and CK1 kinases to Gli2/3^{FL}. This results in generation of Gli2/3^{Rep} that may enter the nucleus and inhibit the target gene expression. **B.** In the presence of Shh, Ulk3/Sufu/Kif7/Gli2/3 complex dissociates, Ulk3 activates itself by autophosphorylation and phosphorylates Gli2/3^{FL}. This contributes to generation of Gli2/3^{Act} that translocate to the nucleus and activate their target genes. For simplification, the other proteins participating in Shh signal transduction and Gli proteins regulation (Ptc1, Smo, Kif3 etc) are not shown.

CONCLUSIONS

1. The new active serine/threonine kinase ULK3 has been identified. ULK3 possesses autophosphorylation activity and intramolecular self-regulation properties. ULK3 does not phosphorylate itself in *trans*. Four identified ULK3 autophosphorylation sites are situated in its CTD.
2. Two serine/threonine kinases, Stk36 and ULK3, display similarity to *Drosophila* fu kinase. Stk36 demonstrates higher sequence conservation, but ULK3 exhibits stronger functional similarity
3. Stk36 and Ulk3 participate in Shh signal transduction and act as positive and negative effectors, respectively. However, involvement of Stk36 in Shh pathway may be restricted to a particular cell type.
4. Stk36 and ULK3 are able to regulate positively Gli1 and Gli2 transcriptional activity, however, in a different way. Stk36 acts independently of its functional KD, and ULK3 positively regulates Gli proteins by directly phosphorylating them.
5. ULK3 interacts with SUFU through its KD, loosing thereby its catalytic activity. SHH signalling induces dissociation of ULK3/SUFU complex. ULK3/SUFU interaction is highly similar to fu/sufu interaction in *Drosophila* and represents a new way of GLI proteins regulation.
6. ULK3 KD elicits positive effects on GLI2 protein, whereas ULK3 CTD participates in negative regulation of GLI2. This resembles differentiated involvement fu domains in ci regulation. We suggest that ULK3 is a functional homologue of fu and a part of HSC in mammals.

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SUMMARY

A limited number of signalling pathways coordinate the activities and fate of enormous number of cells during development and participate in homeostasis and regeneration of different organs and tissues during adulthood. Up- or down-regulation of any particular signal transduction pathway leads to serious developmental defects and diseases in adults. In mammals, one of the crucial signalling pathways is triggered by Shh, a secreted protein with morphogenic properties. In the recipient cells, Shh controls the intracellular signalling cascade that enables activation or repression of Gli transcription factors. Combinatorial functions of Gli proteins result in a particular transcriptional outcome thus prescribing the cells fate. Correct regulation of Shh-Gli signalling pathway is one of the key determinants of the proper embryonic development and postnatal life. However, up-regulation of the SHH-GLI pathway is associated with different types of cancer. Therefore getting better understanding about the molecular mechanisms of GLI proteins regulation provides important clue for the development of new therapies of the SHH-related diseases.

Much of the current knowledge about the mechanisms of Shh signal transduction in mammals comes from the investigations of the respective pathway in *Drosophila*. There are many features representing the conserved parts of Hh signalling in vertebrates and invertebrates. However, the mammalian Hh signalling cascade is definitely more complicated due to the existence of several homologues and different isoforms for the majority of proteins involved.

In *Drosophila*, serine/threonine kinase fu is vitally required for embryonic development and is involved in all aspects of ci regulation having both catalytic and regulatory functions. Stk36, the closest homologue of fu in mammals, represents one of the major differences between vertebrate and invertebrate Hh signalling. Being unnecessary for the embryonic development in mice, Stk36 is able to positively regulate Gli transcription factors. However, Stk36 is catalytically inactive and has only regulatory role that occurs in a cell type specific manner and is not dependent on Stk36 functional kinase domain.

Current work identifies a novel component of Shh signalling pathway, serine/threonine kinase Ulk3, that is homologous and functionally similar to *Drosophila* kinase fu. Altogether the results of this work suggest that the current model of Shh signal transduction is incomplete. Novel model, that includes Ulk3 kinase as a member of the cytoplasmic complex involved in regulation of Gli proteins, is proposed. Similarly to *Drosophila* fu, Ulk3 has dual role, regulatory and catalytic. Regulatory properties of Ulk3 are needed for the negative regulation of Gli proteins in the absence of Shh. In this case, Ulk3 kinase activity is blocked by Sufu, the major negative regulator of Shh pathway. Kinase activity of Ulk3 becomes important in the presence of Shh, when Sufu relieves its inhibitory effect on Ulk3 and Gli transcription factors. In Shh stimulated cells, Ulk3 participates in positive regulation of Gli proteins by directly phosphorylating them. In the light of the recent data, showing that the transcriptional activator forms of Gli proteins correspond to

the phosphorylated full-length proteins, we suggest Ulk3 as a kinase responsible for this phosphorylation. Thus, Ulk3 may recapitulate both the positive and negative roles of *Drosophila* fu and probably may substitute in vivo the function of Stk36 in Hh pathway. Therefore we suggest that *Drosophila* fu kinase has at least two homologues in mammals, Stk36 and Ulk3.

KOKKUVÕTE

Multitsellulaarse organismi rakulise mitmekesisuse tekkes ning väljakujunenud organite ja kudede homeostaasi ja regeneratsiooni kontrollis osaleb suhteliselt väike hulk erinevaid signaaliradasid. Nende signaaliradade deregulatsioon viib tõsiste arenguliste häirete ilmnemisele ning haigustele täiskasvanud organismis. Imetajates on üheks selliseks oluliseks signaalirajaks morfogeeni Shh poolt käivitatud signaalirada. Shh signaaliraja aktivatsioon viib signaali vastuvõtvas rakus Gli transkriptsioonifaktorite aktiivsuse aktivatsiooni või repressioonini. Gli valkude aktiivsuse kombinatoorne modulatsioon määrab raku saatuse. Shh-Gli raja korrektne regulatsioon on organismi normaalse embrüonaalse ja postembrüonaalse arengu eelduseks. Shh-Gli raja deregulatsioon on aga seotud mitmete erinevate pahaloomuliste kasvajate tekkega. Seega annavad Shh-Gli raja molekulaarsete uuringute tulemused võimalusi kavandada uusi terapeutilisi meetmeid ravimaks Shh seoselisi haigusi.

Väga suur osa meie teadmistest Shh raja kohta tuleneb vastava raja uuringutest mudelorganismis *Drosophila melanogaster*. Oma põhiosas on Hh signaalirada konserveerunud selgrootutes ja selgroogsetes. Samas on selgroogsete Hh signaalirada oluliselt keerulisem kui vastav rada selgrootutel, kuna enamik raja komponente esineb selgroogsetes mitmete homoloogide ja erinevate valgu isovormidena.

Stk36 on *Drosophila* seriin/treoniin kinaasi fu homoloog imetajates. Stk36 valgu funktsioon märgib üht põhilist erinevust selgroogsete ja selgrootute Hh signaali realiseerimises. Fu kinaas on absoluutselt vajalik *Drosophila* Hh signaali realiseerumises, samas kui Stk36 ei ole vajalik selgroogsete Shh rajas embrüonaalse arengu vältel. Samas on Stk36 võimeline reguleerima positiivselt Gli transkriptsioonifaktorite aktiivsust. Tema positiivne roll Gli faktorite regulatsioonis on sõltumatu tema ensümaatilisest kinaassest aktiivsusest ning on rakutüübi spetsiifiline.

Minu doktoritöö identifitseerib Ulk3 seriin/treoniin kinaasi kui uue imetajate Shh signaaliraja komponendi. Ulk3 on homoloogne ja funktsionaalselt sarnane *Drosophila* fu kinaasile. Uurides Ulk3 kinaasi funktsiooni, pakun ma välja uue mudeli, mis kirjeldab Shh signaaliraja rakusiseseid sündmusi. Uue mudeli üks komponente on tsütoplasmaatiline valgukompleks, mis sisaldab Ulk3 ning on vastutav Gli valkude aktiivsuse regulatsiooni eest. Sarnaselt fu kinaasile on Ulk3 funktsioon Shh signaalirajas duaalne. Ilma Shh manuluseta vastutab Ulk3 Gli valkude aktiivsuse negatiivse regulatsiooni eest, kasutades oma C-terminaalset regulatoorset domeeni. Shh manulusel toimib Ulk3 kui Gli valkude positiivne regulaator, neid otseselt fosforüleerides. Antud Ulk3 valkude funktsioon on sõltuv kinaassest aktiivsusest. Kokkuvõttes võib öelda, et *Drosophila* fu kinaasil on imetajates kaks homoloogi – Stk36 ning Ulk3, kuid vaid Ulk3 suudab sarnaselt fu kinaasile talitleda nii negatiivse kui ka positiivse regulaatorina Shh signaalirajas.

CURRICULUM VITAE

Name: Alla Maloverjan
Date and place of birth: 01.06.1976, Tallinn
Citizenship: Estonian

Contact information
Address: Department of Gene Technology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn
Tel: +372 620 4438
e-mail: alla.maloverjan@ttu.ee

Education
2003 - 2010 PhD student, Tallinn University of Technology, Department of Gene Technology
Area of study: Vertebrate homologues of *Drosophila* fused kinase
1999 - 2001 MSc, Tallinn University of Technology, Department of Gene Technology
Area of study: Application of microbiological, ecotoxicological and chemical methods for environmental risk assessment of polluted soils, sediments and solid wastes
1993 - 2001 Engineer, Tallinn University of Technology, Department of Food Engineering,
Area of study: Investigation of toxicity of food preservatives sodium benzoate and potassium sorbate
1983 - 1993 Tallinn 19 Secondary School

Professional Employment
2005 - .... Tallinn University of Technology, Department of Gene Technology, Chair of Molecular Diagnostics; Researcher
2007 - 2008 FibroTx, Tallinn; Researcher
2006 - 2007 Protobios LLC; Researcher
2002 - 2003 Department of Biosciences, Novum, Karolinska Institute, Huddinge, Sweden; Researcher
2001 - 2005 National Institute of Chemical Physics and Biophysics; Researcher
1999 - 2001 National Institute of Chemical Physics and Biophysics; Engineer

Publications
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ELULOOKIRJELDUS

Nimi: Alla Maloverjan
Sünniaeg ja -koht: 01.06.1976, Tallinn
Kodakondsus: Eesti

Kontakt
Address: Geenitehnoloogia Instituut, Tallinna Tehnikaülikool, Akadeemia tee 15, 12618 Tallinn
Tel: +372 620 4438
e-mail: alla.maloverjan@ttu.ee

Haridustee
2003 - 2010 PhD tudeng, Tallinna Tehnikaülikool, Geenitehnoloogia Instituut
Uurimisvaldkond: Selgroogsete Fused`i homoloogi roll Shh signaalülekanderajas
1999 - 2001 Teadusmagistri kraad, Tallinna Tehnikaülikool, Geenitehnoloogia Instituut
Uurimisvaldkond: Mikrobioloogiliste, ökotoksikoloogiliste ja keemiliste meetodite kasutamine saastatud muldade, setete ja tahkete jäätmete keskkonnaohtlikkuse hindamisel
1993 - 2001 Insenerikraad, Tallinna Tehnikaülikool, Toiduainete Instituut
Uurimisvaldkond: Toidu konservantide naatriumbensoaadi ja kaaliumsorbaadi toksilisuse uurimine
1983 - 1993 Tallinna 19. Keskkool

Teenistuskäik
2008 - ... Tallinna Tehnikaülikool, Matemaatika-loodusteaduskond, Geenitehnoloogia instituut, Molekulaardiagnostika õppetool; teadur
2006 - 2007 OÜ FibroTx, Tallinn; teadur
2006 - 2007 Protobios OÜ; teadur
2002 - 2003 Bioteaduste Instituut, Novum, Karolinska Institute, Huddinge, Sweden; teadur
2001 - 2005 Keemilise ja Bioloogilise Füüsika Instituut; teadur
1999 - 2001 Keemilise ja Bioloogilise Füüsika Instituut; insener

Publikatsioonid

- **Maloverjan, A**.; Piirsoo, M.; Kasak, L.; Peil, L.; Østerlund, T.; Kogerman, P. (2010). Dual function of unc-51-like kinase 3 (Ulk3) in the sonic hedgehog signaling pathway. Journal of Biological Chemistry, 285, 30079 - 30090.
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RESEARCH ARTICLE

A possible role of mouse Fused (STK36) in Hedgehog signaling and Gli transcription factor regulation

Alla Maloveryan · Csaba Finta · Torben Østerlund · Priit Kogerman

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Abstract The segment polarity gene *Fused* (*Fu*) encodes a putative serine–threonine kinase Fu, which has been shown to play a key role in the Hedgehog signaling pathway of *Drosophila*. Human FU (hFU) has been shown to enhance the activity of Gli transcription factors, targets of the signaling pathway. However, $Fu^{-/-}$ mice do not show aberrant embryonic development indicating that mouse Fu (mFu) is dispensable for Hedgehog signaling until birth. In order to investigate if there are important differences between hFU and mFu, we cloned the cDNA, analyzed expression and tested the ability of mFu to regulate Gli proteins. Of the tested tissues only brain and testis showed significant expression. However, in transient overexpression analyses mFu was able to enhance Gli induced transcription in a manner similar to hFU. Thus, we turned to RNAi in order to test if mFu would be important for Hedgehog signaling after all. In one cell line with reduced mFu expression the Hedgehog signaling was severely hampered, indicating that mFu may have a role in Hedgehog signaling and Gli regulation in some cellular situations.

Keywords Fused · Gli · Hedgehog · Signaling · Overexpression · RNA interference

A. Maloveryan · T. Østerlund (✉) · P. Kogerman
Department of Gene Technology,
Tallinn University of Technology,
Akadeemia tee 15,
12618 Tallinn, Estonia
e-mail: torben.osterlund@ttu.ee

C. Finta
Department of Biosciences at Novum,
Karolinska Intitutet,
Hälsovägen 7, 14157 Huddinge, Sweden

Abbreviations

Ci	Cubitus interruptus
FACS	Fluorescence assisted cell sorting
Fu	Fused
FuFL	Full length Fused
GST	Glutathione S-transferase
Hh	Hedgehog
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
RNAi	RNA interference
RPA	Ribonuclease Protection Assay
Shh	Sonic Hedgehog
siRNA	Small interfering RNA
Sufu	Suppressor of Fused

Introduction

Hedgehogs (Hh) are secreted signaling molecules important for numerous biological processes in developing embryos of a variety of multicellular organisms from flies to humans (Hooper and Scott 2005). These proteins are also involved in several postnatal developmental processes. Furthermore, aberrant Hedgehog signaling has been linked to numerous types of cancers and several components of the pathway are either proto-oncoproteins or tumor suppressors (Hooper and Scott 2005). The *Drosophila* segment polarity gene *Fused* (*Fu*) (Preat et al. 1990) is one of the principal constituents in Hedgehog signal transduction, as activator of the transcription factor *Cubitus interruptus* (Ci). The Fused protein (Fu) is likely to activate the pathway in at least two different manners. The predominant role of Fu is being an inhibitor of the Ci inhibitor Sufu. Both fruit fly and vertebrate Fused proteins contain an N-terminal kinase domain of approximately 300 residues and a much longer

C-terminal part with regulatory properties. However, hitherto there have been no reports of phosphorylation substrates of Fused. Vertebrate Fu has also been implicated in the Hedgehog pathway since human FU (hFU) is able to enhance the gene activator function of the Gli transcription factors, orthologues of Ci (Murone et al. 2000; Østerlund et al. 2004; Daoud and Blanchet-Tournier 2005). Most importantly, a recent paper describes the rescue of lymphoma cells by expression of Fu or Gli1 (Dierks et al. 2007). The lymphoma cells were grown in the presence of cyclopamine (an inhibitor of the upstream pathway initiator Smo) that induces apoptosis. The apoptotic induction could be overcome by forced expression of either Fused or Gli1 (Dierks et al. 2007). However, two reports have shown that $Fu^{-/-}$ mice develop normally until birth, suggesting that Fu is not a crucial component of the Hedgehog pathway in mice during embryonic development (Chen et al. 2005; Merchant et al. 2005). There may be several reasons for this difference. (1) Redundancy, another component is able to compensate for the loss of *Fu*, (2) Fused only belongs to a branch of the pathway with limited or no influence during embryogenesis, or (3) Fu is not a component of the mammalian Hh signaling pathway. In fact it is possible that mFu is not able to enhance Gli induced transcription in the way hFU is, though they share 86% sequence identity. We have previously pointed out that there may be significant difference between *Drosophila* and vertebrate Hh signaling (Østerlund and Kogerman 2006). This includes differences in the way Fu and Sufu contribute to the signaling process. The more prominent role of Sufu in Hh signaling in vertebrates (Cooper et al. 2005; Svärd et al. 2006), as opposed to its marginal role in *Drosophila*, may have changed the demands for its regulation. Therefore, mFu may have a more peripheral role as a regulator of Gli proteins. On the other hand, soon after birth the $Fu^{-/-}$ mice develop brain defects and die within 3 weeks (Chen et al. 2005; Merchant et al. 2005). This shows that *Fu* is potentially active in postnatal brain development, which may involve Hh signaling.

In order to investigate if there is a significant functional difference between mouse and human Fu proteins we have cloned mFu cDNA and analyzed its expression pattern in mouse tissues. Unlike with *hFU,* we did not detect ubiquitous expression of mFu, although the expression was seen in the same mouse tissues that had shown high levels of expression in humans. It is thus possible that mFu is expressed in the same tissues as in humans, albeit at a lower level. Overexpression analyses in Hedgehog responsive cells show that mFu is able to enhance Gli induced transcription similar to hFU. Thus, we did mFu silencing by means of RNA interference in the same cell line. We obtained two stable transformants and one of them clearly downregulated the responsiveness to Hh. This observation provide evidence that mFu may have an important but limited role in Hedgehog signaling.

Materials and methods

Isolation of mFused

Adult mouse brain and testis cDNA samples (kindly provided by Dr. Kaia Palm) were used for PCR amplification with primer pair 5′-CCCTGGATCTATAGC TCTT-3′ (mFu exon 3′-specific sequence) and 5′-CAACAGTAGG ACAGTGAAGG-3′ (exon 28-specific sequence) using Expand Long Template PCR System Kit (Roche Applied Science, Basel, Switzerland) according to manufacturer's instructions. The obtained PCR products (full length (FL) and short version (Δ6–26)) were purified from agarose gels using QIAquick Gel Extraction Kit (Qiagen, Valencia CA, USA) and subcloned into pCR-BluntII-TOPO vector using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen, Carlsbad CA, USA).

Generation of cDNA constructs

The Fu cDNAs were subcloned into pCTAP-C (Stratagene, La Jolla CA, USA) using the EcoRI sites to generate FuFLpCTAP and Fu(Δ6–26)pCTAP. Fu(K33R)pCTAP with Lysine 33 substituted by Arginine was generated from FuFLpCTAP by PCR mutagenesis using Expand Long Template PCR System kit (Roche) and a pair of primers carrying the appropriate point mutations. The obtained PCR product was gel-purified using JETquick Gel Extraction Spin Kit (Genomed, St. Louis MO, USA). FuFLpCTAP was digested with Eco52I (Fermentas, Vilnius, Lithuania) that cuts FuFL at position 765 (corresponding to amino acid 255) and pCTAP before and after MCS. The obtained restriction fragments encoding the kinase domain (KD) and C-terminus (CT) of FuFL were gel-purified using JETquick Gel Extraction Kit (Qiagen) and cloned into the Eco52I sites of pCTAP vector resulting in FuKDpCTAP and FuCTpCTAP constructs, respectively. All constructs were verified by DNA sequencing. A Fu-specific siRNApSUPER (FupSUPER) construct was generated according to the pSUPER RNAi System protocol (OligoEngine, Seattle WA, USA). The sequence for small interfering RNA (siRNA) was chosen to be specific for the kinase domain coding region of both isolated mFu isoforms. The primers 5′-GATCCCC*TCCCAACATTGTGCATATG*TTCAAGAG A*CATATGCACAATGTTGGGA*TTTTTA-3′ and 5′-AGCT TAAAAA*TCCCAACATTGTGCATATG*TCTCTTGAA*CATA TGCACAATGTTGGGA*GGG-3′ (the target sequence of mFu is underlined, corresponding nucleotides 180–198) were

annealed with each other and subcloned into pSUPER vector between BamHI and HindIII sites.

The FL human Gli1EGFP construct is described in (Kogerman et al. 1999). The translated region of FL mouse Gli2 was amplified by PCR using pair of oligonucleotides containing BglII (forward) and BamHI (reverse) restriction sites and subcloned to pEGFP-C1 vector (Clontech, Palo Alto CA, USA).

To obtain a ShhC24II encoding construct, the part encoding residues 25–198 of human Shh was subcloned into pGEX-6p-2 (Amersham Biosciences, Little Chalfont, UK). This was achieved by PCR amplification of the particular part using primes also introducing the restriction sites BamHI and EcoR1 used for the subcloning. The 5′ primer also introduced an enterokinase cleavage site and two leucines and the 3′primer introduced a stop codon.

ShhC24II protein purification

The ShhC24II protein is described in (Taylor et al. 2001). The enterokinase-ShhC24II was expressed as GST-fusion protein in *E. coli* strain XL-Blue. Bacteria were lysed by sonication and the lysate was incubated with Glutathione Sepharose 4B Fast Flow slurry (Amersham) during 30 min at room temperature. The enterokinase-ShhC24II protein was cleaved with enterokinase (Invitrogen) overnight at room temperature. After cleavage the enterokinase was removed by EK-away resin (Invitrogen) according to manufacture's instructions and ShhC24II was eluted with 1×PBS.

Northern blot

Northern blot analyses were performed as previously described (Østerlund et al. 2004). Two different cDNA fragments were chosen: one in the 5′end (nucleotides 216–768; numbering of nucleotides is from the translational start site) and another corresponding to exon 26 of the coding region. These fragments were amplified by PCR and radioactively labeled with ^{32}P-ATP using the High Prime DNA labeling kit (Boehringer Mannheim, Manheim, Germany) according to the manufacturer's instructions. The hybridization was performed in ExpressHyb (Clontech) at 68°C for high specificity, to commercially available mouse Multiple Tissue Northern blots (Clontech), according to the recommendations by the manufacturer.

Ribonuclease Protection Assay (RPA)

Two different riboprobes were synthesized (Fig. 2b). Sequence of probe 1 corresponded to nucleotides 1–398 of Fu(Δ6–26). Probe 2 was complementary to mFu cDNA fragment of exon 27 and corresponded to nucleotides 3173–3622 of FuFL or 399–848 of Fu(Δ6–26). Fu(Δ6–26)Topo construct was cut with NcoI and XbaI (probe 1) or XbaI and BamHI (probe 2) and obtained cDNA fragments were subcloned into pTZ57R/T vector (Fermentas) that contains T7 promoter site in antisense orientation 50 nt downstream of the multiple cloning site. Prior to riboprobes synthesis the constructs were linearized with NcoI (probe 1) and XbaI (probe 2) and purified by phenol-chloroform extraction. Linearized mFu templates or pTRI-Actin-Mouse (0.5 μg) (Ambion, Austin TX, USA) were *in vitro* translated using 20 U of T7 RNA polymerase (Fermentas) in the presence of 15 μCi of (α-^{32}P) UTP (Amersham Biosciences) at 37°C for 1 h. After translation, the DNA templates were digested using 10 U of RQ DNaseI (Promega, Madison WI, USA) at 37°C for 1 h. The probes were phenol-chloroform purified and diluted in 100 μl of Hybridization III Buffer (RPA III kit, Ambion). RNA of dissected mouse tissues or cultured cells was isolated using RNAwiz reagent (Ambion) according to the manufacturer's instructions. Total RNA from each sample (10 μg) was ethanol-precipitated, diluted in 5 μl of Hybridization III Buffer and mixed with 5 μl of a radioactively labeled probe. The samples were denatured at 85°C for 10 min and hybridized at 50°C for 16–20 h. Non-hybridized RNA and free probes were digested with RNaseA/T1 mix in 100 μl of Digestion III Buffer (Ambion) for 1 h at 37°C. The reaction was stopped by adding 40 μg of Proteinase K (Fermentas) in the presence of 0.4% (v/v) SDS. The non-digested RNA was purified by phenol-chloroform extraction and resolved on a denaturing 5.5% acrylamide gel. The gel was dried for 1 h at 80°C. Radioactive bands were visualized and quantified using a Bio-Rad GS250 phosphoimager.

Overexpression studies

The NIH-3T3 cell line clone Shh-LIGHT2 (Shh-L2) (Taipale et al. 2000) was used for the overexpression assay. Shh-L2 cells are stably expressing a Gli-dependent Firefly luciferase reporter and the Renilla luciferase. The latter was used for the normalization of data. The cells were propagated in D-MEM growth medium containing 4500 mg/ml glucose, supplemented with 10% Fetal Bovine Serum and 5% penicillin/streptomycin, 0.1 mg/ml zeocin (Invitrogen) and 0.4 mg/ml geneticin (Sigma) at 37°C and 5% CO_2.

The cells were seeded at the density 7×10^4 cells/well on 24-well plates and transfected the next day using Polyethylenimine transfection agent (PEI) (InBio) in ratio PEI: DNA=1.6 μg: 0.8 μg per well. DNA used for cotransfection: 0.2 μg of Gli1/Gli2-EGFP and 0.6 μg of different mFu constructs (or with respective empty vectors). PEI was mixed with DNA in 50 μl of sterile D-MEM medium for each well, incubated for 10 min at room temperature and added to the cells containing 200 μl pr. well of D-MEM. The cells were transfected for 2 h, after that propagated for

24 h in the normal growth medium and for additional 24 h in the Light medium containing 0.5% FBS. Activity was measured by luciferase assay (see below).

RNA interference of mFu expression

RNA interference (RNAi) of the *mFu* gene was induced in Shh-L cell line. The cells were grown as described above and cotransfected with FupSUPER and pBABE-puro constructs in ratio 19:1 using (PEI: DNA=17 μg: 5.6 μg per 10 cm plate). The cells were split 48 h after transfection and 24 h later the normal growth medium was replaced by the selective growth medium supplemented with 2 μg/ml of puromycin. Puromycin-resistant colonies were picked and propagated in 24-well plates, and eight were chosen for subsequent analysis. Efficiency of RNAi was verified by RPA using probe 2. The established cell lines showing reduced level of mFu mRNA and Shh-L2 as a control were chosen for further analysis.

The cells were plated at the density 6×10^4 cells per well on 24-well plates and were transfected the next day with 0.8 μg per well of pShhNpCMV (Stratagene), GLI1-EGFP or empty vector pr. well using PEI as described above. Alternatively the cells were stimulated with the 12 nM ShhC24II. After 24 h post-transfection or adding of the ShhC24II, the normal growth medium was replaced with the Light medium, and cells were incubated for the additional 24 h prior the Luciferase assay.

Luciferase assay

The cells were rinsed in 1×PBS and lysed in 50 μl of Passive Lysis Buffer II (Promega). Aliquots of 10 μl were used for the assay. Firefly and Renilla luciferase activities were measured using Dual Renilla/Firefly Luciferase kit (Promega) on an Ascent Fluoroscan luminometer (Thermo Electron Corporation, Waltham MA, USA) according to manufacturer's instructions. All experiments were performed at least 3 times in 4–6 replicates and the results of representative experiments are shown with standard deviations.

Results

Identification of two mFu isoforms

A comparative analysis of the mouse genomic and hFU cDNA sequences revealed the *mFused* (*mFu*) gene on chromosome 1 region C3 (74.90–74.94 kb by Ensembl annotation (http://www.ensembl.org)) encoding a kinase known as serine/threonine kinase 36. The *mFu* gene is predicted to consist of 28 exons (Merchant et al. 2005) (Fig. 1a). It contains two 5′UTR exons, translation initiator codon ATG in exon 3, and Stop codon TAA in exon 28. Exons 3–9 encode the kinase domain, which is highly homologous to that of hFU and dFu. The remaining exons are anticipated to encode parts important for regulatory functions of Fu (Murone et al. 2000; Østerlund et al. 2004).

Mouse brain and testis cDNAs were chosen for RT-PCR amplification as those, together with pancreas and the kidney, were the tissues showing the highest expression of *hFU* (Murone et al. 2000; Østerlund et al. 2004). Two PCR products (Fig. 1b and c) were obtained using a primer pair complementary to the 19 nt-long unique sequences of the 3rd and 28th exons in the predicted *mFu* gene. Sequence analysis revealed that PCR product 1 (FuFL) contained exons 3–28 of the predicted *mFu* gene encoding the full-length (FL) version of Fu (Fig. 1b). The 2nd product – shorter variant Fu(Δ6–26) – contained exons 3–5 and a part of exon 6 spliced to exon 27 a few nt from the 5′ splice site in the FL clone (Fig. 1b).

Sequence analysis predicts a 1,314 residue long FuFL protein highly homologues to the previously described hFU protein, showing 97% identity in the kinase domain (N-terminal 260 residues) and 80% identity in the regulatory domain. The identity between mFuFL and *Drosophila* Fu (dFu) was lower, showing 50 and 10% identity in kinase and regulatory domain, respectively. The protein encoded by the Fu(Δ6–26) cDNA clone is 133 amino acids long due to a Stop codon generated by a frame shift close to the splicing site in exon 27.

Expression of *mFu* in brain and testis

The expression pattern of mFu isoforms was analyzed in different tissues using Northern blot (Fig. 2a) and Ribonuclease Protection Assay (RPA) (Fig. 2b and c). The Northern analyses of adult tissues only revealed an approximately 5 kb transcript in testis irrespective of the probe that was used. Also a weak band at 5.5 kb is seen, suggesting that *mFu* can encode different mRNA species. It was not possible to detect any band corresponding to the Fu (Δ6–26), showing that this is only produced in small amounts and/or rapidly degraded.

For RPA analyses we used RNA from several adult mouse tissues and brain at 12 dpc and 1 dpp as well as from fetal brain at several stages during development. Three protected fragments were detected using probe 1 (Fig. 2b), full-length (398 nt) protection specific for Fu(Δ6–26) transcripts (indicated with *) and 323 nt (indicated with **) plus 76 nt (not shown) bands for mFuFL. The mFuFL was highly expressed in the testis. Significantly lower levels of mFuFL were detected in the brain. This expression pattern of mFuFL was also observed using an mFuFL-specific probe (3) (data not shown). Fu(Δ6–26) was found only in testis as a minor alternative transcript.

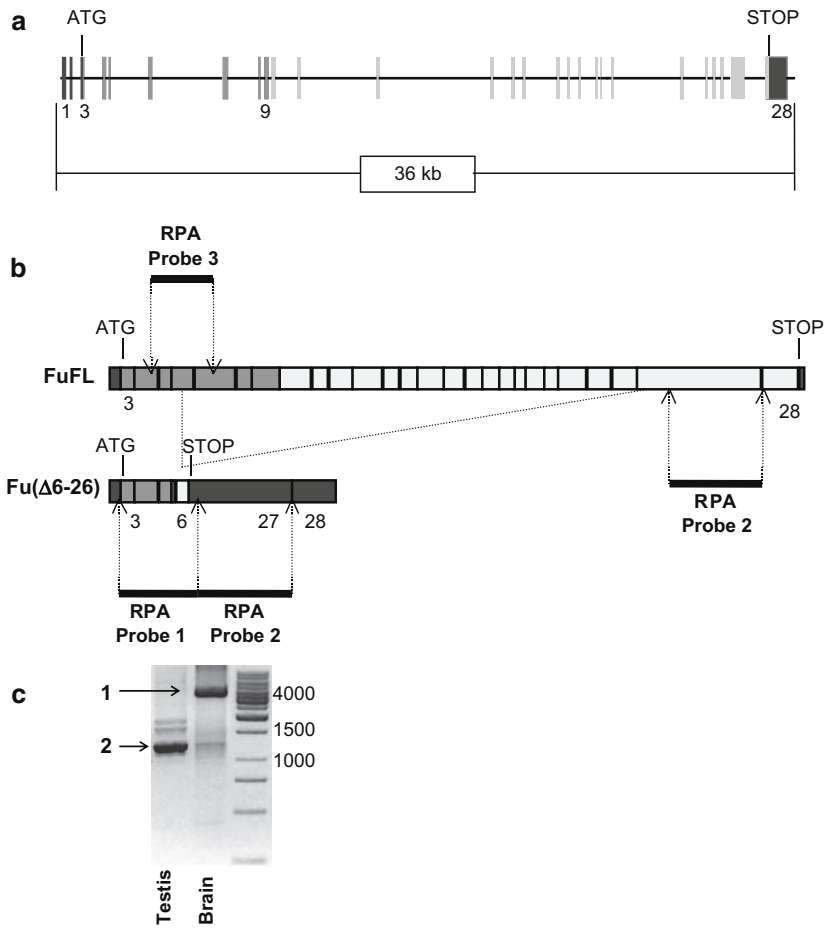

Fig. 1 The structure of *mFu* gene and generated cDNA clones. **a** The *mFu* gene found on Chromosome 1 consists of 28 exons. The translated exons are 3–28 encoding an N-terminal serine/threonine kinase domain (exons 3–9; *dark gray*) and a C-terminal region (exons 10–28; *light gray*) suggested having regulatory functions. Untranslated parts are shown in *black*. **b** Schematic representation of the FuFL and Fu(Δ6–26) cDNA clones obtained by RT-PCR as shown (*arrows*) in (**c**). Compared to the FuFL cDNA the Fu(Δ6–26) lacks a big part from exon 6 to 26 indicated by *dashed lines*. The different RPA hybridizing probes are also indicated in (**b**). Probe 1 consist of 323 nt before and 76 nt after alternative splicing in Fu(Δ6–26) giving a 399 nt long protected fragment. Probe 2 was complimentary to 449 nt of exon 27 present in both FuFL and Fu(Δ6–26). Probe 3 covers 550 nt from the end of exon 4 to the middle of exon 7 and is FuFL specific

Total RNA derived from brain tissue at different stages of development (E12, E14, E16, E18, P4 and adult) was analyzed using probe 2 that recognize both FuFL and Fu (Δ6–26) (Fig. 2c). Expression of mFu was very low on E12, then gradually increased during embryo brain development but decreased after birth.

The mFu protein enhances Gli induced transcription

Human brain and testis are among the tissues with high *FU* expression (Murone et al. 2000; Østerlund et al. 2004). However, *hFU* mRNA was detected in all tested human tissues (Murone et al. 2000; Østerlund et al. 2004) suggesting a functional difference between human and mouse *Fu*. We therefore tested if mFu is able to enhance the transcriptional activity of Gli proteins in a manner similar to hFU (Murone et al. 2000; Østerlund et al. 2004; Daoud and Blanchet-Tournier 2005). FuFL and Fu(Δ6–26) were overexpressed together with Gli proteins and analyzed with respect to the effect on a Gli responsive firefly luciferase reporter gene, which is incorporated in the genome of Shh-LIGHT2 (Shh-L2) cells (Taipale et al. 2000). We also analyzed the effect of a full kinase domain, the regulatory domain and a kinase-inactive variant of mFu (the conserved lysine 33 mutated to arginine (K33R)). The data obtained were normalized with Renilla luciferase values and the results of representative experiments are shown (Fig. 3). Mouse FuFL enhances the transcriptional activity of both Gli1 and Gli2 approximately four times. The same result was achieved using mutant Fu(K33R) indicating that the kinase activity of mFu is not required for the regulation of Gli-induced transcription. Co-activator potency of Fu(Δ6–26) was significantly lower, it enhanced the activity of Gli1 and Gli2 proteins 1.9 and 1.6 times, respectively. However,

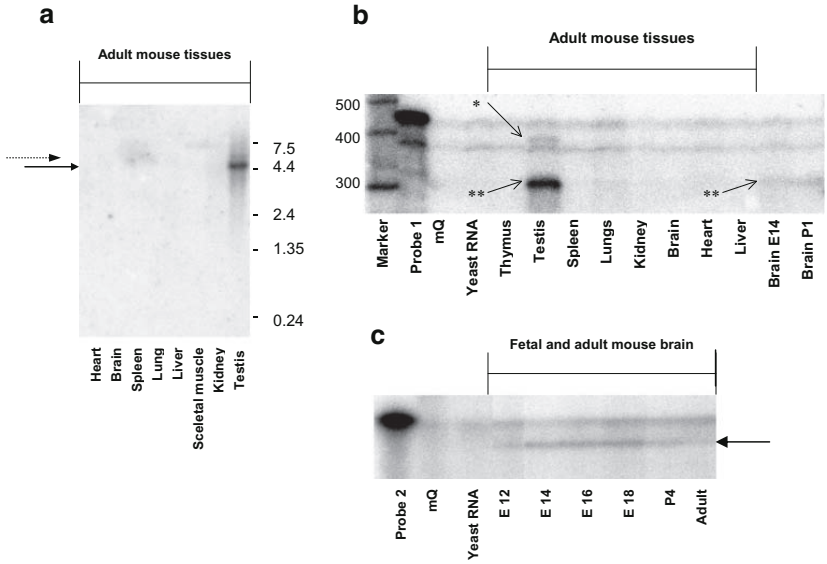

Fig. 2 The *mFu* expression in mouse tissues. **a** Northern blot analysis of mFu expression in eight different tissues as indicated. The major 5 kb band is indicated with a *solid arrow* and the minor 5.5 kb band with a *dotted arrow*. **b** RPA expression analysis of Fu(Δ6–26) (*) and FuFL (**) using probe 1 in eight adult tissues and two stages of brain development. **c** RPA analysis of mouse brain at different developmental stages as indicated (Fused protection indicated by arrow). **b** and **c** Pure water (mQ) and yeast RNA are used as negative controls

the effect of full-length kinase domain of mFu was only slightly weaker than mFuFL and, moreover, the regulatory domain of mFu alone could enhance the Gli activity three times, providing additional evidence that the C-terminal part of mFu is involved in Gli regulation. Taken together, our data demonstrate that mFu could enhance the activity of Gli1 and Gli2 proteins and both the kinase domain and C-terminal regulatory region of mFu are required for the full activation of Gli, though the potential kinase activity appears not to be crucial. These data are similar to those obtained with hFU (Murone et al. 2000; Østerlund et al. 2004; Daoud and Blanchet-Tournier 2005).

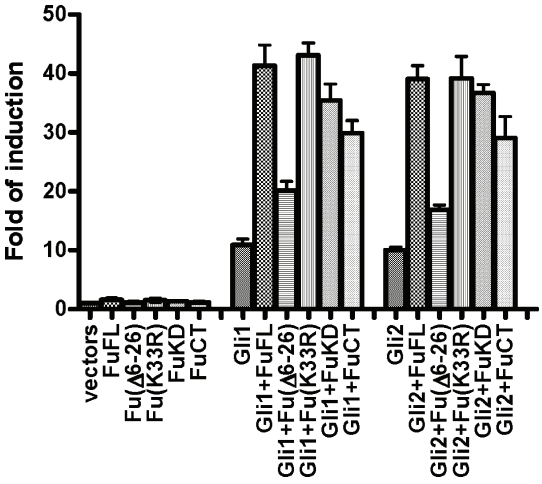

Fig. 3 Regulation of Gli1 and Gli2 by mFu in Shh-L cells. Different construct of mFu were overexpressed either alone or together with either Gli1 or Gli2 in Shh-L cells as shown. Vectors indicate transfection with empty control vectors and the results were calculated proportionally to these (=1). Error-bars represent standard deviations of the calculations

Reduced *mFu* expression impairs Shh signaling

In the light that mFu and hFU are able to regulate Gli proteins in the same manner, we wanted to test the possibility that mFu is of importance for Shh signaling. To this end we used an RNA interference (RNAi) procedure to reduce Fu mRNA in Shh-L2 cells. After selection, several cell lines stably expressing mFu siRNA, were isolated and verified for RNAi efficiency using RPA (Fig. 4a). Compared with the control Shh-L cells two cell lines, Cell line I and Cell line II, showed significant reduction of mFu mRNA level. Cell line I expressed 35% and Cell line II only 16% of the Fu mRNA control level (Fig. 4a). An actin-specific probe was used as control of expression levels (Fig. 4b). In order to assess the mFu protein levels in the RNAi cell lines, we employed a novel mFu affinity purified anti-peptide antibody produced in rabbits (InBio, Tallinn, Estonia). This antibody was not highly specific in Western blots, but could be used for comparison of expression levels of mFu in FACS analysis. Using this method and the mFu antibody, it could be determined that Cell line I expresses mFu almost to the same extent (62%) as control Shh-L2 cells, whereas Cell line II expresses very low amounts (roughly as low as background (2%); Fig. 5).

In order to test the ability of the established cell lines to transduce the Shh signal we either transfected them with the

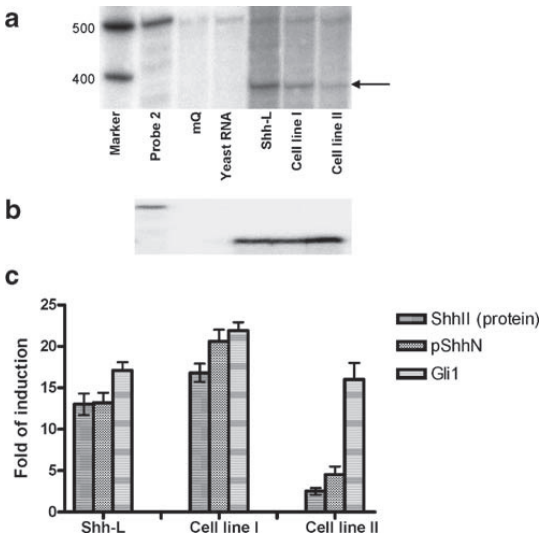

Fig. 4 Analyses of Shh-L clonal cells with reduced mFu mRNA. **a** RPA analysis of using probe 2 of control Shh-L cells and two cell lines (I and II) with significantly lowered mFu expression (Fused protection indicated by *arrow*). **b** Control RPA analysis with actin-specific probe. As negative controls were used Yeast RNA and water (mQ). The intensity of the mFu bands was calculated in relation to that actin bands, showing 35% mFu expression in Cell line I and 16% expression in Cell line II. **c** Control Shh-L, Cell line I and II were stimulated by Shh protein (ShhC24II), by transfection with a Shh construct or by a Gli1 construct. The stimulation of the luciferase reporter is calculated as fold induction compared to non-stimulated control cells (=1). Error-bars represent standard deviations of the calculations

Shh N-terminal encoding (ShhN)-construct or stimulated directly with a highly active Shh protein variant (ShhC24II). Alternatively, luciferase expression was induced directly by transfection of a Gli1 construct. Activation of luciferase in the Clone I cells was achieved in all three cases like for control Shh-L2 cells (Fig. 4c). Cell line II, with lower levels of Fu mRNA expression, was successfully activated by Gli1 whereas Shh protein or the ShhN-construct only had minor effects (Fig. 4c), showing a severely hampered signaling pathway in these cells.

Discussion

In order to investigate the functionality of mFu we have cloned the cDNA and identified the exons in the genomic sequences at Ensembl (http://www.ensembl.org). This corresponds well with the human gene (Østerlund et al. 2004) and the previously published sequences (Chen et al. 2005; Merchant et al. 2005). We did not find the alternatively spliced variants identified by others (Chen et al. 2005). Instead, we identified a short variant lacking a small part of exon 6, exons 7–26 and a small part of exon 27. None of the suggested alternatively spliced variants (this work and (Chen et al. 2005)) corresponds to the variants identified in humans (Østerlund et al. 2004). Such lack of conservation in the pattern of alternative splicing between species, suggests that alternatively spliced variants of vertebrate Fu may not play any profound biological roles. In fact, the short variant identified by us had very little effect on Gli-induced transcription in our cellular overexpression assay.

The analyses of expression in adult tissues only show significant amounts of *mFu* mRNA in the brain and testis. This is partly in line with the expression analyses in human tissues where the highest amounts were found in the brain, testis, pancreas and kidney (Østerlund et al. 2004). However, small amounts of *hFU* mRNA were seen in all analyzed tissues (Østerlund et al. 2004), which we did not see in the mouse. In the embryo, *mFu* was expressed broadly and the highest expression was detected in the brain, limbs, the neural tube and somites (Chen et al. 2005). The high expression of human and mouse *Fu* in the testis indicates a prominent role of Fu in this tissue. However, to assess this issue it is probably necessary to create and analyze conditional *Fu* knock-out mice or perhaps examine the testicular functions of $Fu^{+/-}$ mice.

In order to evaluate the potential role of mFu in the regulation of Gli proteins, we made overexpression analyses in the NIH3T3 cell clone Shh-L2. Expression of mFuFL enhanced the activity of Gli1 and Gli2 4-fold. This corresponds well with the results obtained with hFU. Human FU enhances the transcriptional activity of Gli2 in C3H/10T and HEK293 cells (Murone et al. 2000; Østerlund et al.

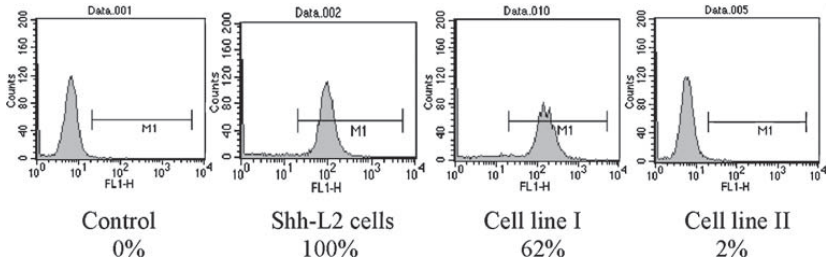

Fig. 5 FACS analyses of mFu RNAi cells. Cell counts of the two Shh-L2 derived RNAi cell lines (I and II), of normal Shh-L2 cells (positive control) and of medium without cells (negative control; control) using an mFu peptide antibody. The percentage of Shh-L2 count is given below

2004) and of GLI1 in NIH3T3C2 and SW480 cells (Daoud and Blanchet-Tournier 2005) to a similar extent. The kinase-inactive version of mFu – mFu(K33R) – exhibited the same effect on Gli activity as the mFuFL which is in line with results previously demonstrated for the kinase-inactive variant of hFU (Murone et al. 2000) and a hFU variant lacking parts of the kinase domain (Østerlund et al. 2004). Taken together, our results are in line with those previously reported for hFU and both Gli1 and Gli2 are regulated by mFu variants in a similar way.

The fact that both human and mouse Fu are able to enhance Gli transcriptional activity suggests that they may influence Hh signaling. To shed more light on this issue we performed RNAi analyses in the same Hedgehog responsive cells as we did the overexpression analyses. Two selected clones showed significantly lower amounts of mFu mRNA (35 and 16% of the normal mFu mRNA level, respectively) and the cells with the lowest expression showed significantly reduced response to Shh. The fact that the two cell lines show very different results and that the previous results with mFu RNAi (Merchant et al. 2005) were similar to those of Cell line I, led us to speculate that the mFu protein levels may not be affected significantly until a very low level of mRNA is reached. Since there were no mFu antibodies available, which could be used for detection of the protein level, this was not done in the previous study (Merchant et al. 2005). Our FACS analyses show that only Cell line II has low mFu levels and this can explain why only these cells respond poorly to Shh. It is possible that Cell line I resembles the previously described Fused RNAi cells (Merchant et al. 2005), although the difference between those cells and our Cell line II may also reflect differences between the cell lines dependence on Fused protein for Hedgehog signaling. In our experiments overexpression of Gli1 served as a positive control and lead to reporter activation like in the control cells. These results also suggest that the level of mFu protein does not follow the RNAi efficiency very well. We conclude that the actual protein levels must be determined in such analyses to verify that the protein levels are in fact affected by the reduction in mRNA.

Mouse embryonic development is not dependent on mFu, showing that neither is prenatal Hh signaling (Chen et al. 2005; Merchant et al. 2005). However, in the light that several reports, including this one, have shown that Fu is able to enhance Gli-induced gene activation and that reducing the mFu levels inhibits Shh signaling in the Shh responsive cells Shh-L2, it is possible that Fu participates in the Hh pathway by regulating Gli proteins in certain cellular contexts. For example inhibition of an upstream pathway component, Smo, by cyclopamine, was overcome by overexpression of either Fu or Gli1 in lymphoma cells (Dierks et al. 2007). An intriguing explanation would be that mammals might exert both Fu-dependent and Fu-independent Hh signaling, where the latter appears to dominate in the developing embryo. Other genes may compensate for the loss of *mFu* (redundancy) or Fu may be a component in a branch of the pathway that is of little importance during embryogenesis. In the light that mFu is vital for postnatal brain development (Chen et al. 2005; Merchant et al. 2005), Fu may have a significant role in the Hh pathway after birth. These results suggest a restricted but identifiable role of Fu as a regulator of Gli proteins. Further investigations are required to assess these possibilities and our mFu RNAi Cell line II could be a helpful tool in this respect. As previously pointed out (Østerlund and Kogerman 2006), it is possible that Hh signaling is significantly different between *Drosophila* and vertebrates. This may also apply to the way Fu regulates Gli proteins. In order to fully understand Gli regulation and the Hh pathway, issues like possible redundancy of Fused-related proteins, as well as Fu-dependent and Fu-independent Hh signaling must be addressed.

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Research Article

Identification of a novel serine/threonine kinase ULK3 as a positive regulator of Hedgehog pathway

Alla Maloverjan, Marko Piirsoo, Piret Michelson, Priit Kogerman, Torben Østerlund*

Department of Gene Technology, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn, Estonia

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ABSTRACT

The Hedgehog (Hh) signaling pathway plays crucial roles in embryonic development and is implicated in tissue homeostasis maintenance and neurogenesis in adults. Aberrant activation of Hh signaling is associated with various developmental abnormalities and several types of cancer. Genetic and biochemical studies ascertain serine/threonine kinase Fused (Fu) as a protein involved in Hh signaling in *Drosophila*. However, the role of Fu is not fully conserved in mammals suggesting involvement of other kinases in the mammalian Hh signaling pathway. In search of potential homologues to *Drosophila* and human Fu, we have cloned human serine/threonine kinase ULK3 and assessed its ability to regulate GLI transcription factors, mediators of SHH signaling. We demonstrate that ULK3 enhances endogenous and over-expressed GLI1 and GLI2 transcriptional activity in cultured cells, as assessed by GLI-luciferase reporter assay. Besides that, ULK3 alters subcellular localization of GLI1, as assessed by immunofluorescent staining and immunoblotting assays. We show that ULK3 is an autophosphorylated kinase and phosphorylates GLI proteins *in vitro*. We also demonstrate that ULK3 catalytical activity is crucial for its function in SHH pathway. We show that *ULK3* is widely expressed and its expression is higher in a number of tissues where Shh signaling is known to be active. Our data suggest that serine/threonine kinase ULK3 is involved in the SHH pathway as a positive regulator of GLI proteins.

Introduction

The Hh pathway is involved in numerous biological processes during embryonic development of many animals ranging from fruit fly to mammals [1]. During postnatal life Hh signaling contributes to tissue homeostasis maintenance and controls neurogenesis and stem cell behavior. In humans, aberrant activation of the Hh signaling is associated with various developmental abnormalities and several types of cancer (reviewed in [2]). Regardless of comprehensive studies, many gaps still exist in understanding the intracellular events initiated by Hh proteins.

Although Hh signaling appears to be conserved between invertebrates and vertebrates in many aspects, there are principal differences among species in intracellular interpretation of Hh signal [3,4]. In *Drosophila* the Hh signaling is mediated through transcription factor *Cubitus interruptus* (Ci) that comprises both gene activator and repressor functions. In vertebrates the function of Ci is divided between three homologous proteins, Gli1, Gli2 and

* *Corresponding author.* Fax: +372 6204401.
E-mail addresses: alla.maloverjan@ttu.ee (A. Maloverjan), marko.piirsoo@ttu.ee (M. Piirsoo), piret.michelson@ttu.ee (P. Michelson), priit.kogerman@ttu.ee (P. Kogerman), toosathome@yahoo.se (T. Østerlund).

Abbreviations: Ci, Cubitus interruptus; CK1, Casein Kinase 1; FCS, fetal-calf serum; Fu, Fused; GSK3, Glycogen Synthase Kinase 3; IP, immunoprecipitation; KB, kinase buffer; NE, nuclear extract; PKA, Protein Kinase A; PEI, polyethylenimine; PIC, protease inhibitor cocktail; Shh, Sonic Hedgehog; ShhL2, Shh-LIGHT2; STK36, Serine/Threonine Kinase 36; qRT-PCR, quantitative Real-Time PCR; SWM, stain wash medium; ULK, unc-51-like kinase; WCE, whole cell extract; wt, wild-type

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Gli3. Gli1 is an obligatory activator, Gli2 and Gli3 carry activator and repressor functions, with Gli3 being the strongest repressor. In the absence of Hh, Gli1 is generally not expressed; Gli2 and Gli3 proteins (as Ci in *Drosophila*) are mostly present in a C-terminally processed transcriptional repressor form, and full-length activator forms are tethered in the cytoplasm or subjected to proteosomal degradation [5]. The signaling is initiated through binding of Hh proteins (Sonic, Desert or Indian Hh in vertebrates) to the 12-pass membrane receptor Patched (Ptch). Binding of ligand allows another transmembrane protein, Smoothened (Smo), to be relieved from the inhibitory effect of Ptch. Through its C-terminal tail Smo triggers the intracellular signaling cascade that culminates in activation, stabilization and nuclear translocation of full-length Ci/Gli proteins that serve as transcriptional activators of Hh target genes, for instance *Ptch1* and *Gli1* (reviewed in [1] and [6]).

Several kinases are shown to be involved in the Hh signaling pathway and in the regulation of Ci/Gli activity. Serine/threonine kinases Fused (Fu), PKA, GSK3, CK1, PI3K, Akt, PKCδ, MEK1, ERK1, MAP3K10 and dual-specificity tyrosine-regulated kinases DYRK1 and DYRK2 have been reported to affect Ci- and/or Gli-dependent Hh signaling [7–15]. However, not all kinases have been found to be functionally conserved between vertebrates and invertebrates. Serine/threonine kinase Fu is, perhaps, one of the most puzzling molecules in Hh signaling.

In *Drosophila* genetic and biochemical studies establish Fu (dFu) as a component of Hh signaling [8,12]. dFu is essential for the embryonic development as homozygous dFu mutants are not viable. Partial loss of dFu activity in *Drosophila* results in a variety of phenotypes including fusion of longitudinal wing veins 3 and 4 that characterizes perturbation of Hh signaling [8,16,17]. The predominant function of dFu is to counteract with Suppressor of Fused (dSufu), known as a cytoplasmic inhibitor of Ci [16]. dFu is able to bind directly to kinesin-like protein Costal-2 (Cos2), dSmo and dSufu [18–20]. According to the accepted model, those proteins down-regulate the pathway in the absence of Hh. dSmo, Cos2, dFu, Ci and, probably, dSufu form a complex that tethers full-length Ci in the cytoplasm preventing its nuclear localization. Besides that, the complex interacts with PKA, Shaggy (*Drosophila* homologue of GSK3) and CK1 through Cos2. These protein kinases are responsible for proteolytic cleavage of Ci in resting cells and phosphorylation followed by subsequent activation of dSmo C-terminus in response to Hh. Activation of the pathway also induces phosphorylation of dFu, dSufu and Cos2, whereas phosphorylation of dSmo, Cos2 and dSufu depends on dFu kinase activity [21–25]. Thus, the kinase activity of dFu is essential for the generation of Ci transcriptional activator form in the presence of Hh ligand.

Hitherto, one mammalian orthologue of dFu has been reported [26,27]. Human serine/threonine kinase STK36 (also known as FUSED) has been identified as a protein sharing the highest homology with dFu. Human and mouse Fu homologues (hFU and mFu, respectively) have been shown to enhance GLI-dependent gene activation, but in contrast to dFu, independently of the functional kinase domain [26,28]. Genetic studies have shown that hFU, over-expressed in fu mutant flies, cannot rescue their phenotype [29]. Besides that, contrary to dFu, mFu is dispensable for embryonic development [30,31]. However, it seems to be highly important later in development, as newborn $mFu^{-/-}$ mice display extensive brain defects and die within 3 weeks after birth [31]. Thus, the role of mammalian Fu in Hh signaling appears to differ from that of dFu.

In the light that there is no a clear Cos2 orthologue in mammalian cells, this is not a surprise. The *Drosophila* Hh pathway centers on Cos2 and in vertebrates this is not the case [4]. The cytoplasmic C-terminus of dSmo, that is extremely important for Hh signaling in *Drosophila*, is not conserved in mammalian Smo, and, moreover, mouse Smo C-terminus is not required for Shh signal transduction [4,22]. In *Drosophila* Sufu gene function is dispensable for Hh signaling and dSufu protein has only a slight negative effect on Ci, whereas mouse $Sufu^{-/-}$ mutants are not viable and inhibiting effect of mammalian Sufu on Gli proteins is very strong [32,33]. The more elusive role of mammalian Fu suggests that, like for Ci/Gli, the different roles have been divided between more proteins. It is in fact possible that other kinases are involved in the regulation of Gli activity.

Here, we report cloning of human serine/threonine kinase ULK3 that has been annotated as belonging to unc-51-like family of serine/threonine kinases, but shares similarity with STK36 and dFu proteins. We show that ULK3 is a kinase with autophosphorylation activity. ULK3 is able to enhance endogenous and over-expressed GLI1 and GLI2 transcriptional activity and to induce nuclear translocation of GLI1 in cultured cells. We show that ULK3 phosphorylates GLI proteins *in vitro*, and GLI1 has at least two phosphorylation sites situated in N- and C-terminus of the protein, respectively. Our data show that the kinase-deficient mutants of ULK3 are inactive indicating that functional kinase domain of ULK3 is required for the regulation of GLI protein activity. Also we show that *ULK3* expression is higher in fetal brain and in a number of postnatal tissues where SHH signaling is known to be active. Our data suggest that ULK3 is involved in Shh pathway as a positive regulator of Gli proteins.

Materials and methods

Expression constructs

ULK3 cDNA was amplified using primers pair sense 5′-AATGGCGGG-GCCCGGCTG-3′ and anti-sense 5′-TCTGCTCCAGATGGCTCACA-3′ from human testis cDNA sample provided by Dr. Tõnis Timmusk (Tallinn University of Technology, Tallinn) using Expand Long Template PCR System Kit (Roche Applied Science, Basel, Switzerland) according to manufacturer's instructions. The obtained PCR product was purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen, Valencia CA, USA) and cloned to pTZ57R/T vector using InsTAclone PCR Cloning Kit (Fermentas INC., Burlington, Ontario, Canada).

ULK3 cDNA was verified by sequencing and subcloned into mammalian expression vectors. ULK3pcDNA3.1 construct was generated by cloning of *ULK3* cDNA into pcDNA3.1 vector linearized with *Kpn*I and *Bam*HI (Invitrogen, Carlsbad CA, USA). ULK3FLAG construct was produced by cloning of ULK3 cDNA into *Eco*RI and *Hin*dIII sites of pFLAG-CMV-4 vector (Sigma-Aldrich, St. Louis MO, USA). ULK3(K44R) construct with the lysine residue at position 44 mutated to arginine and ULK3(K139R) harboring the same mutation at position 139 were generated from ULK3FLAG construct by Quickchange site directed PCR mutagenesis procedure (Stratagene, La Jolla CA, USA) using Expand Long Template PCR System Kit (Roche) and primers carrying the appropriate point mutations. The obtained constructs were verified by DNA sequencing.

N-terminally tagged GLI1GFP and GLI1FLAG constructs have been described [34]. GLI2FLAG and GLI3FLAG constructs were provided by Dr. Illar Pata (Tallinn University of Technology,

Tallinn). GLI2FLAG was generated by subcloning GLI2 cDNA from GLI2pcDNA3 described in [35] into *Hin*dIII and *Xba*I sites of pFLAG-CMV-4 vector. GLI3pcDNA3.1 construct (described in [36]) was used for generation of GLI3FLAG by subcloning GLI3 cDNA to pFLAG-CMV-4 vector. STK36pcDNA3.1 has been described in [27]. ULK1pcDNA3.1 was provided by Dr. Sharon Tooze (Centre for Cancer Research UK, London, U.K.). Construct expressing N-terminal part of mouse Shh (pShhN) has been described in [37]. Construct pSV40-β-gal used for luciferase assay data normalization has been described in [36].

Expression pattern of ULK3

A cDNA panel of 20 human tissues and 10 human brain parts was provided by Dr. Tõnis Timmusk. The levels of *ULK3*, *GLI1* and *PTCH1* mRNAs and mRNA of housekeeping gene *HPRT* used for normalization (provided by Mari Sepp) were detected in triplicates by quantitative Real-Time PCR (qRT-PCR) using qPCR Core kit for SYBR Green (Eurogentec, Oslo, Norway) with Lightcycler 2.0 (Roche Applied Science) according to the manufacturers' instructions. Data were analyzed with Lightcycler 4.05 software (Roche Applied Science). The data are expressed as mean of three replicates ± SEM. The following primers were used for the assay:

ULK3 sense 5′-AAGGAGCAGGTCAAGATGAG-3′
ULK3 antisense 5′-GTGCAAGAGCTACGAACAGA-3′
GLI1 sense 5′- CCTTCAGCAATGCCAGTGA-3′
GLI1 antisense 5′-CTAGGATCTGTATAGCGTTT-3′
PTCH1 sense 5′-ATCCCTTTTGAGGACAGGAC-3′
PTCH1 antisense 5′- ATTGCAGGAAAAATGAGCAG-3′
HPRT sense 5′-GATGATGAACCAGGTTATGAC-3′
HPRT antisense 5′-GTCCTTTTCACCAGCAAGCTTG-3′

Cell culture

HEK293 cells were propagated in Minimum Essential Medium (MEM) (Gibco, Invitrogen). NIH3T3 and their clone Shh-Light2 (Shh-L2) cells [38] were grown in Dulbecco's modified Eagle's medium (D-MEM) containing 4.5 g/l glucose. Cos1 cell line was grown in DMEM containing 1 g/l glucose (Gibco). All growth media were supplemented with 10% FBS (PAA, Pasching, Austria) and 100 μg/ml of penicillin/streptomycin (Invitrogen), and Shh-L2 cells growth medium was additionally supplemented with 400 μg/ml of G 418 (Sigma) and 100 μg/ml of Zeocine (Invitrogen). The cells were grown at 37 °C and 5% CO_2. Approximately 24 h prior to transfection the cells were plated to appropriate growth dishes.

Over-expression studies

Cells were transfected by the expression constructs or respective empty vectors using Polyethylenimine transfection agent (PEI) (Inbio, Tallinn, Estonia) as described [28]. We used 0.25 μg of plasmid per 1 cm^2 of plate surface area for transfections. After 3 h of transfection HEK293, NIH3T3 and Cos1 cells were propagated in the normal growth medium for 48 h. Prior further analysis the cells were washed twice with PBS. Post-transfectional Shh-L2 cells were grown in the normal growth medium for 24 h and for additional 24 h in the Light medium containing 0.25% FBS, washed once with PBS, lysed in Passive Lysis Buffer (Promega, Madison WI, USA) (70 μl per well of 24-well plate format) and subjected to luciferase assay.

Luciferase assay

Luciferase assays was performed as previously described [28,36]. Briefly, firefly luciferase activity was measured using Luciferase Assay Kit and β-galactosidase activity was quantified using Galacto-LightPlus kit (Tropix, Bedford MA, USA) according to the manufacturer's instructions. Chemiluminescence was measured using Ascent FL Fluoroscan (Thermo Electron Corporation, Waltham MA, USA). The normalized data are expressed as mean of at least three replicates obtained from at least three independent experiments ± SEM. Statistical analysis was carried out and *P* values were calculated using *t*-test: Two Sample Assuming Unequal Variances (Excel, Microsoft).

Purification of GLI1 fragments

Three overlapping human GLI1 domains corresponding to amino acids 1–433, 426–754 and 726–1106 were cloned into pET-15b vector containing 6xHis tag (Novagen) between *Nde*I and *Bam*HI sites. The fragments were expressed in BL21(DE3)pLys *Escherichia coli* strain overnight at 21 °C using 0,1 mM IPTG and purified using Ni-CAM HC resin (Sigma) according to the manufacturer's recommendations. The proteins were eluted in PBS and verified by Western Blot (WB).

Immunoprecipitation

HEK293 cells were transfected with 13.5 μg of FLAG-tagged constructs expressing GLI1, GLI2, GLI3, ULK3, ULK3(K44R), ULK3(K139R) or FLAG-CMV-4 empty vector on 10 cm plates. Cells were lysed with 0.5 ml of Lysis Buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing protease inhibitor cocktail (PIC) (Roche). Lysates were centrifuged for 15 min at 4 °C and 15,000 *g*, and supernatants were used for immunoprecipitation (IP). IP was performed using anti-FLAG-M2 affinity gel (Sigma) in batch format according to the manufacturer's instructions. Immune complexes were washed three times with 500 μl of Kinase Buffer (KB) (50 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 25 μM ATP) and resuspended in 12.5 μl of KB. One fifth of the immunocomplexes were subjected to WB and the rest was used for kinase assay.

In vitro kinase assay

Kinase reactions were carried out in KB in a total volume of 20 μl of in the presence of 1 μCi [γ-^{32}P]ATP per reaction. We used 1 μl immunoprecipitated ULK3, ULK3(K44R) or ULK3(K139R) mixed with 5 μl GLI-M2 immunocomplexes or 5 μl bacterially expressed and purified His-GLI1 fragments. Kinase reactions were held at 30 °C for 30 min and terminated by adding of 5 μl 4× Laemmli Sample Buffer. Proteins were resolved by SDS-PAGE. The gel was dried at 80 °C for 2 h, and autoradiography was performed using Bio-Rad Personal Molecular Imager FX.

Immunocytochemistry

NIH3T3 cells were transfected with GLI1GFP and FLAG-tagged expression constructs for ULK3 or ULK3(K139R) in ratio 1:1 on 8-chamber slides (Falcon, BD Biosciences, San Jose CA, USA). The cells were fixed with 4% paraformaldehyde (Sigma) and washed three times for 5 min with Stain Wash Medium (SWM) (0.5% BSA and 0.01% NaN_3 in PBS). Permeabilization of cells was performed at RT

by 10-min incubation in PBS supplemented with 0.5% Triton X-100. Permeabilized cells were washed three times for 5 min with SWM, blocked for 30 min in PBS containing 5% bovine serum albumin (BSA), and incubated with mouse monoclonal M2-anti-FLAG antibody (diluted 1:1000 in SWM) for 1 h at RT with gentle agitation. After three washes of 10 min with SWM, the cells were incubated for 30 min with secondary antibody Alexa-Fluor 568 (Invitrogen) (diluted 1:500 in SWM) mixed with nuclear stain Hoechst 33342 (diluted 1:100). Cells were washed twice with SWM and mounted with Mowiol 4-88 (Sigma). GLI1GFP protein localization was assessed in at least 200 FLAG-positive cells under a fluorescent microscope Olympus BX61 with UPLan SApo 40× objective. Confocal images were obtained with a Zeiss LSM-510 META confocal laser-scanning microscope (Carl Zeiss MicroImaging GmbH, Germany) equipped with Plan-Apochromat 63×/1.4 oil immersion objective. The data are expressed as mean of positive cells obtained from three independent experiments. Obtained data were analyzed and *P* values were calculated using *t*-test: Two Sample Assuming Unequal Variances (Excel, Microsoft).

Subcellular fractionation

Cos1 cells were transfected with FLAG-tagged expression constructs of GLI1 combined with ULK3, ULK3(K139R) or empty vector in ratio 1:1 on 10 cm plates. Nuclear and whole cell extracts (NE and WCE, respectively) were prepared as described [39]. All extracts were normalized for protein amounts measured using BCA kit (Thermo Scientific). Total 10 μg of protein were separated by SDS-PAGE and transferred to the PVDF membrane (Millipore, Billerica MA, USA). The membrane was segmented according to the expected size of the detected proteins. The obtained strips were probed with the appropriate antibodies.

Antibodies and WB procedure

WB was performed using a non-blocking technique as described [40]. Mouse monoclonal M2-anti-FLAG antibody (Sigma) was used for detection of FLAG-tagged GLI1, GLI2, GLI3, ULK3, ULK3(K44R) and ULK3(K139R) by WB in dilution 1:2000. Lamin A/C was detected with mouse monoclonal anti-Lamin A/C antibody (Upstate, Billerica MA, USA) diluted 1:1000. Actin was detected using anti-actin antibody (Santa Cruz, CA, USA) diluted 1:1000. Mouse monoclonal anti-His antibody (Novagen, Darmstadt, Germany) diluted 1:1500 was used for detection of bacterially expressed and purified GLI1 fragments. The secondary antibody used was HRP-conjugated goat-anti-mouse Ig (Jackson, West Grove PA, USA) diluted 1:10000.

Results

ULK3 is the closest homologue of STK36

In order to determine which kinases share significant homology with human STK36 (GenBank accession number NP056505.2), the sequence of its kinase domain was subjected to comparative homology analysis against the human proteins databank using BLASTp algorithm and BLOSUM62 matrix (NCBI). Our analysis revealed that ULK3 (accession number NP001092906) was the protein sharing the highest similarity to STK36 (38% of identity in the kinase domain). Using the sequence of the kinase domain of dFu (accession number NP477499.1) as a query, we found that ULK3 was the closest human relative of dFu protein apart from STK36. The identity between dFu and ULK3 kinase domains was 37%, whereas STK36 shared 51% of identity.

ULK3 was also found to be homologous to ULK1 and ULK2 proteins that belong to unc51 subfamily (39% and 37% of identity in the kinase domain, respectively [41,42]). The sequences of the members of the unc51 subfamily were obtained from NCBI database (accession numbers NP507869 for unc51 (*Caenorhabditis elegans*), NP648601 for Atg1 (*Drosophila melanogaster*), NP003556 for ULK1, NP055498 for ULK2, NP060356 for ULK4). The proteins of Fu and unc51 subfamilies were subjected to multiple sequence alignment and homology analysis using GONNET 250 matrix and ClustalW program (EBI, EMBL), and a homology tree was built using the calculated distances between the aligned proteins (Fig. 1A). The analysis showed that ULK3 shared higher homology to Fu subfamily of serine/threonine kinases than to unc-51-like kinases. Thus, the bioinformatic analysis lets us suppose that ULK3 may be a homologue of STK36 and dFu.

Cloning of ULK3 and generation of kinase-deficient mutants

A comparative analysis of the ULK3 nucleotide sequence against human GenBank was performed using UCSC Genome Browser. The analysis revealed ULK3 gene is situated in chromosome 15 and contains 16 exons with translation initiation codon in the first exon and stop codon in exon 16 (Fig. 1B).

ULK3 cDNA was amplified by RT-PCR from adult human testis cDNA. The obtained 1419 bp long cDNA corresponded to the *ULK3* mRNA sequence (GenBank accession number NM001099436.1). It encodes an expected polypeptide of 472 amino acids with calculated molecular weight of 53 kDa and contains an N-terminal serine/threonine kinase domain (amino acids 14–270).

In order to produce kinase-inactive variants of ULK3, we mutated the highly conserved lysine residues in positions 44 (ULK3(K44R)) and 139 (ULK3(K139R)), to arginines. Lys-44 is situated in the ATP binding pocket, and Lys-139 is situated in the substrate binding pocket and catalytic loop regions (BLASTp conserved domain analysis, NCBI).

ULK3 mRNA is widely expressed in humans with the highest expression in fetal brain

The level of *ULK3* mRNA expression was analyzed in postnatal human tissues, fetal brain and fetal liver using qRT-PCR. *ULK3* mRNA was detected in all tissues analyzed and the data are shown relative to the level in the heart as a tissue with the lowest level of *ULK3* mRNA expression (Fig. 1C). The highest expression of *ULK3* was detected in fetal brain. Postnatal tissues showed high level of *ULK3* mRNA expression in brain, liver and kidney, and moderate amounts of *ULK3* mRNA expression in testis and adrenal gland. Heart, lung, stomach, thymus, prostate and placenta showed low level of *ULK3* expression.

As the expression of *ULK3* in the brain was higher than in most other tissues, we were interested to know if particular regions of the adult human brain showed high level of *ULK3* expression. We analyzed 10 brain regions and found the highest level of *ULK3* expression in hippocampus (Fig. 1D). The data are shown relative to the expression level in the cerebral cortex. High levels of *ULK3*

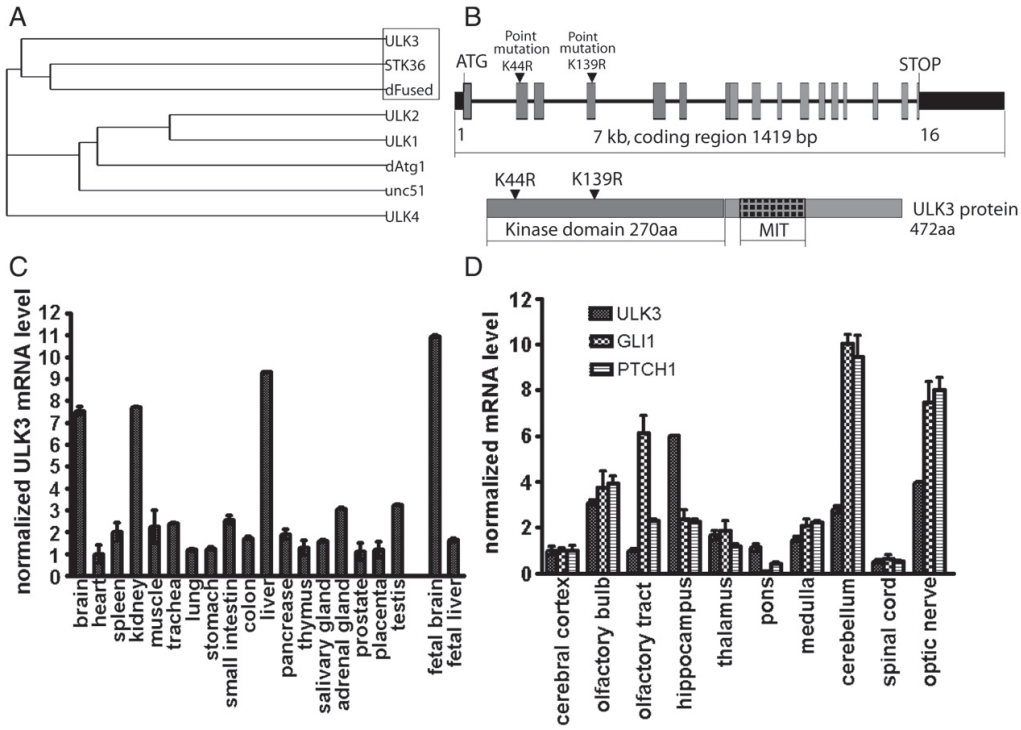

Fig. 1 – Structure, phylogenetic analysis and expression of human ULK3. (A) Phylogram tree of unc51 and Fu subfamilies of protein kinases. The protein sequences of full-length Fu and unc51 subfamilies were obtained from NCBI database and subjected to multiple sequence alignment using ClustalW program (EMBL-EBI). The phylogram tree was built using the algorithm based on GONNET 250 matrix. According to the calculated distances between the proteins, ULK3 belongs to the STK36/Fu subfamily. The same result was obtained using BLOSUM 30 matrix. (B) Structure of ULK3 gene and the expressed ULK3 protein. ULK3 gene is 7 kb, the coding part is 1419 bp. ULK3 gene contains 16 exons whereas first 7 exons encode the putative serine/threonine kinase domain (indicated by darker grey). The point mutations changing Lys-44 and Lys-139 to arginine were generated in exons 2 and 4 in positions 131 and 416, respectively (the numbers are given according to the translation initiation codon ATG). ULK3 gene encodes a polypeptide of 472 amino acids. Amino-terminal serine/threonine kinase domain (amino acids 14–270) and a putative microtubules interacting domain (MIT) (amino acids 279–353) are indicated. (C) qRT-PCR analysis of *ULK3* mRNA expression in human tissues. The data were normalized by *HPRT* mRNA levels and is shown relative to the lowest level of *ULK3* expression detected in the heart. *ULK3* mRNA was detected in all tissues with highest expression in fetal brain. High level of *ULK3* mRNA expression was found in liver, kidney and brain. (D) qRT-PCR analysis of *ULK3*, *GLI1* and *PTCH1* mRNA expression in human postnatal brain parts. Normalized *ULK3*, *GLI1* and *PTCH1* mRNA expression data are shown relative to the expression level in the cerebral cortex. The highest expression of *ULK3* was detected in hippocampus, the lowest in spinal cord. Optic nerve, olfactory bulb and cerebellum showed higher level of *ULK3* expression. *GLI1* expression profile was in good correlation with *PTCH1* expression in all brain parts excepting olfactory tract. Expression of *ULK3* was in good correlation with expression of *GLI1* and *PTCH1* in the following brain parts: olfactory bulb, thalamus, pons, medulla, cerebellum, spinal cord, optic nerve.

mRNA were detected in cerebellum, olfactory bulb and optic nerve. The lowest level of *ULK3* expression was detected in the spinal cord. We also analyzed expression of two known SHH target genes *GLI1* and *PTCH1* mRNA in the same brain parts. *GLI1* expression profile was in good correlation with *PTCH1* expression in all brain parts excepting olfactory tract. Expression of *ULK3* was in good correlation with expression of *GLI1* and *PTCH1* in the following brain parts: olfactory bulb, thalamus, pons, medulla, cerebellum, spinal cord, optic nerve.

Analysis of *ULK3* expression suggests that *ULK3* may contribute to brain development as well as play a role in adult organism.

ULK3 kinase activity is able to enhance GLI-dependent luciferase activity

To investigate if ULK3 plays a role in the Shh pathway, we tested if ULK3 and its closest homologues ULK1 and STK36 are able to activate GLI-dependent luciferase activity in Shh-L2 cells. We cotransfected the constructs together with GLI-expressing plasmids (or respective empty vector) into Shh-L2 cells and assessed their effect on GLI-dependent firefly luciferase activity. The obtained data were normalized with β-galactosidase values. The experiment was repeated 4 times, and results of the representative

experiment are shown (Fig. 2A). Among the tested kinases, ULK3 had the strongest effect on GLI-dependent luciferase reporter activity either alone or cotransfected with GLI1 and GLI2. ULK3 was able to stimulate the luciferase activity 3.8 times and enhanced the transcriptional activity of over-expressed GLI1 and GLI2 approximately 2 and 3.2 times, respectively. The potency of STK36 in the assay was much lower. STK36 alone failed to activate the luciferase activity and exerted no effect on GLI1; however it enhanced GLI2 activity 1.6 times. It is noticeable that ULK1 alone was able to activate luciferase activity 3.4 times; however it had no effect on over-expressed GLI1 and GLI2 indicating that ULK1 may influence the Shh pathway upstream of GLI proteins.

In order to examine if the kinase activity of ULK3 is required for the luciferase reporter activation, we tested the supposed kinase-deficient variants of ULK3, ULK3(K44R) and ULK3(K139R), in the same assay. In contrast to ULK3, neither of the mutants had any effect on GLI1 transcriptional activity. However, mutant ULK3 (K44R), if co-expressed with GLI2, demonstrated some residual activity, whereas ULK3(K139R) was completely inactive (Fig. 2B). We also tested the effect of ULK3 and the mutants on the luciferase reporter when the SHH pathway was activated by cotransfection with pShhN (plasmid expressing biologically active part of SHH). ULK3 significantly affected the activated pathway, inducing the luciferase activity 4.1 times. The mutants could not activate Gli-luciferase reporter as efficiently as wild-type (wt) ULK3, although ULK3(K44R) had a residual positive effect on the activated pathway inducing luciferase activity 1.7 times.

These data indicate that, in contrast to STK36 and ULK1, ULK3 is able to positively regulate the GLI1 and GLI2 transcriptional activity in a manner dependent on its kinase activity.

ULK3 is an active kinase that phosphorylates GLI proteins in vitro

To test if ULK3 has an autophosphorylation activity, we expressed FLAG-tagged wt ULK3 and mutants ULK3(K44R) and ULK3(K139R) in HEK293 cells. The proteins were immunopurified and subjected to *in vitro* kinase assay in the presence of [γ-^{32}P]ATP. ULK3 demonstrated strong autophosphorylation activity, ULK3(K44R) showed reduced efficiency in autophosphorylation, and the autophosphorylation activity was completely absent in ULK3 (K139R) (Fig. 3A). Our data indicate that ULK3 is an autophosphorylated kinase. Its activity is partly inhibited by mutation of Lys-44 and almost abolished by the mutation of Lys-139.

We also assayed whether ULK3 is able to phosphorylate GLI proteins. FLAG-tagged GLI1, GLI2 and GLI3 proteins were expressed and immunoprecipitated from HEK293 cells and used as substrates for immunopurified ULK3 in the *in vitro* kinase assay. ULK3 was able to phosphorylate GLI proteins but with different efficiency (Fig. 3B). The strongest phosphorylation signal was detected in the case of GLI2. The mutant ULK3(K44R) could slightly phosphorylate GLI2 (data not shown) that is consistent with the luciferase assay data. GLI1 and GLI3 were also phosphorylated by ULK3, but with significantly lower intensity compared to GLI2. To support these findings and identify the regions in GLI1 protein that are phosphor-

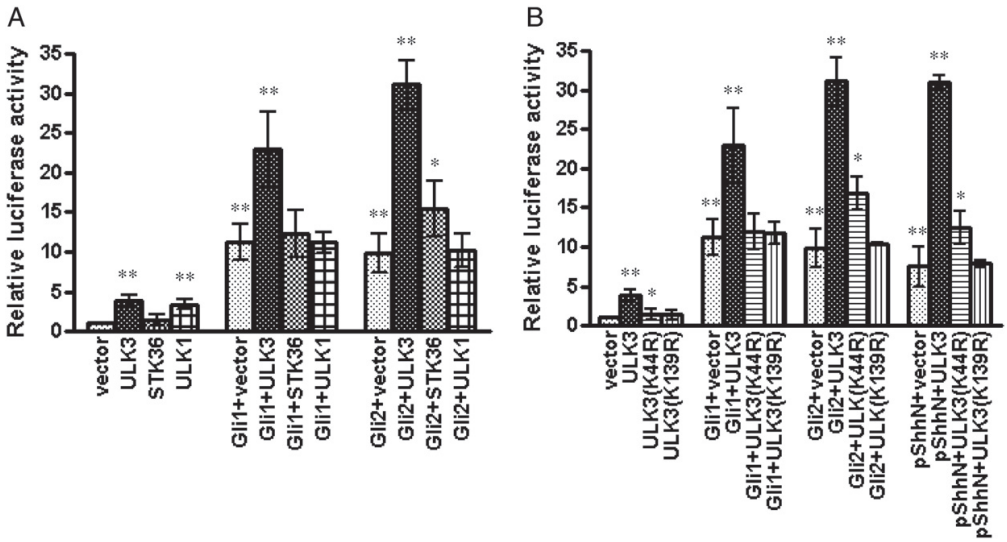

Fig. 2 – ULK3 kinase activity is required for induction of endogenous and over-expressed GLI1 and GLI2 transcriptional activity. (A) ULK3 enhances GLI1- and GLI2-dependent luciferase activity in Shh-L2 cells. ULK3 was able to induce luciferase activity from GLI-dependent promoter either alone or cotransfected with GLI1 or GLI2 in Shh-L2 cells. STK36 failed to stimulate luciferase activity and had no effect on GLI1; however it enhanced GLI2 transcriptional activity. ULK1 alone was able to activate luciferase activity. It had no effect on over-expressed GLI1 and GLI2 indicating that ULK1 may indirectly influence the pathway. (B) ULK3 kinase activity is required for the regulation of GLI protein transcriptional activity. Wt ULK3 was able to activate endogenous and over-expressed GLI1- and GLI2-dependent transcription in the presence or absence of biologically active SHH. Mutant ULK3(K44R) demonstrated residual activity in the case of activated by SHH pathway and in the presence of GLI2, whereas mutant ULK3(K139R) was completely inactive. **P value < 0.001; *P value < 0.05.

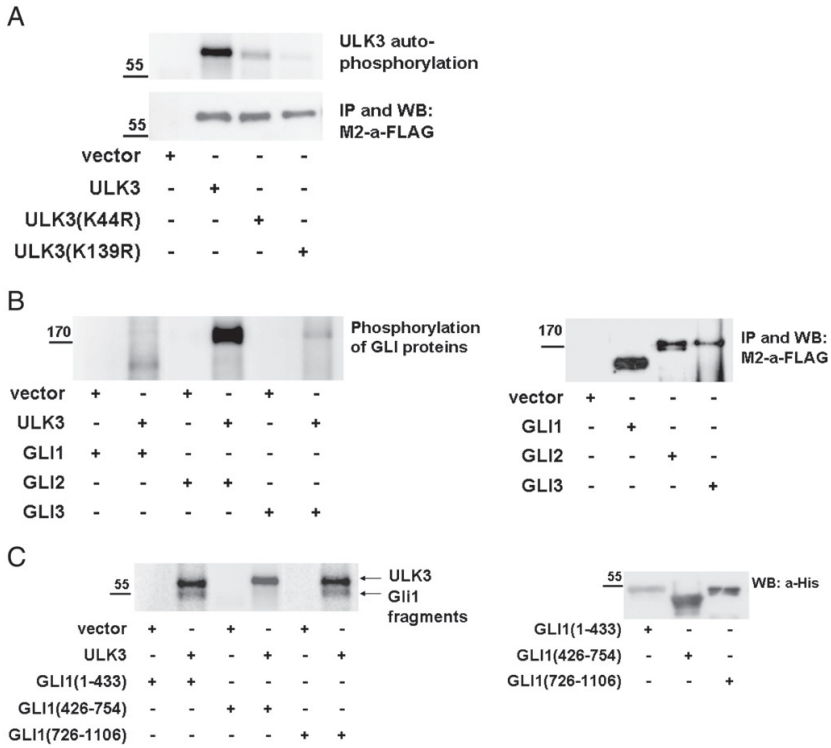

Fig. 3 – ULK3 phosphorylates itself and GLI proteins *in vitro*. (A) Autophosphorylation of ULK3. Wt and mutant ULK3 proteins were expressed in HEK293 cells and immunoprecipitated using M2-a-FLAG affinity gel. Immunocomplexes were detected with WB using M2-a-FLAG antibody and subjected to *in vitro* kinase assay. ULK3 strongly phosphorylated itself. Autophosphorylation activity of ULK3 was partly affected by mutation in Lys-44 and destroyed by mutation in Lys-139. (B) ULK3 phosphorylates GLI proteins. FLAG-tagged GLI proteins were expressed in HEK293 cells, immunoprecipitated using M2-a-FLAG affinity gel and confirmed with WB using M2-a-FLAG antibody. Aliquots of the immunoprecipitated proteins were mixed together as indicated and subjected to *in vitro* kinase assay. ULK3 phosphorylated strongly GLI2 and weakly GLI1 and GLI3. (C) Multiple sites in GLI1 are phosphorylated by ULK3 *in vitro*. His-tagged GLI1 fragments were expressed in *E. coli*, purified, and detected using a-His antibody. Purified GLI1 fragments were mixed with ULK3-M2 or vector-M2 immune complexes and subjected to *in vitro* kinase assay. Phosphorylation of GLI1 by ULK3 was detected using proteins harboring GLI1 amino acids 1–426 and 754–1106.

ylated by ULK3, we used bacterially expressed His-tagged fragments of GLI1 as substrate for ULK3 kinase. Two of them–GLI1(1–433) and GLI1(726–1106)–were phosphorylated by ULK3, whereas the central part of GLI1 (amino acids 426–754) was not. Our data indicate that ULK3 directly phosphorylates GLI proteins, and GLI1 has at least two phosphorylation sites, one of which is situated in the N-terminal and the other in the C-terminal region of GLI1.

ULK3 promotes nuclear localization of GLI1

We then examined if over-expression of ULK3 kinase influences the subcellular localization of GLI1 and GLI2 proteins. As ULK3(K44R) demonstrated some residual activity both in the luciferase and *in vitro* kinase assays and ULK(K139R) was inactive, we preferred the latter as a negative control in the immunofluorescence assay. FLAG-tagged ULK3 and ULK3(K139R) (or empty vector) were co-expressed with GFP-tagged GLI1 or GLI2 in NIH3T3 cells. Subcellular localization of GLI1 and GLI2 was determined, and average values were calculated from three independent experiments. ULK3 and its mutant remained almost completely cytoplasmic. GFP-tagged GLI2 and GLI3 localized predominantly in the nucleus and their localization was not altered by ULK3 or ULK3(K139R) (data not shown). Over-expressed GLI1GFP was detected both in cytoplasm and nucleus (Fig. 4A). The distribution of GLI1 was the following: 27% of the cells showed stronger signal in the nucleus, GLI1 was cytoplasmic in 20% of the cells, and in 53% of the cells GLI1 was distributed uniformly throughout the cell. Expression of ULK3(K139R) did not influence GLI1 subcellular localization. However, ULK3 strongly changed the distribution of GLI1, so that in 70% of the cells GLI1 was mostly nuclear, only in 2% of cells GLI1 retained mostly in the cytoplasm, and 25% of cells demonstrated uniform distribution.

To confirm the data from the immunofluorescent staining, we performed cell fractionation analysis. FLAG-tagged GLI1 and ULK3 or ULK3(K139R) (or the respective empty vector) were co-expressed in Cos1 cells, and the cells were fractionated to whole cell and nuclear extracts. The extracts were used in WB with anti-FLAG, anti-Lamin A/

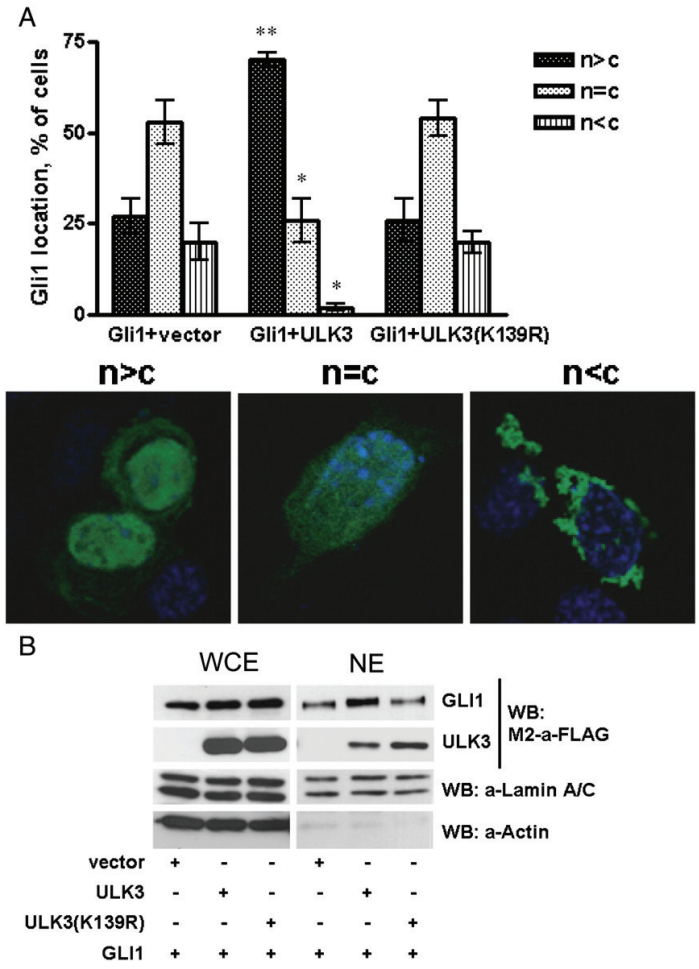

Fig. 4 – ULK3 promotes the nuclear localization of GLI1. (A) ULK3 kinase activity is required for nuclear translocation of GLI1. NIH3T3 were cotransfected with GFP-tagged GLI1 and FLAG- tagged ULK3 constructs or empty vector. Immunostaining was performed using M2-anti-FLAG primary antibody and AlexaFluor-568 secondary antibody mixed with Hoechst. GLI1 localization was estimated in at least 200 FLAG-positive cells in each transfection (n—nuclear localization of GLI1GFP, c—cytoplasmatic localization of GLI1GFP), and the average values from three independent experiments were calculated. Cotransfected with empty vector, GLI1 was distributed uniformly within the cell. GLI1 was shifted to the nuclei in the presence of ULK3. ULK3(K139R) failed to alter the subcellular localization of GLI1. **P value < 0.01; *P value < 0.05. (B) Nuclear translocation of GLI1 in the presence of ULK3 was confirmed by WB. Cos1 cells were cotransfected with FLAG-tagged GLI1 and the constructs indicated. WB analysis of whole cell and nuclear extracts was performed using M2-anti-FLAG, anti-Lamin A/C and anti-actin antibodies. Amount of GLI1 was similar in all whole cell extracts. Cells cotransfected with GLI1 and wt ULK3 retained more GLI1 in the nuclei.

C and anti-Actin antibodies. Three independent experiments were performed showing essentially same results. Data from the representative experiment are shown (Fig. 4B). Expression of lamin A/C was analyzed as a loading control and expression of actin was analyzed to illustrate the purity of the putative nuclear fraction. ULK3 and ULK3(K139R) were detected mostly in whole cell extracts. All whole cell extracts showed similar expression of GLI1. Analysis of nuclear extracts revealed that in cells cotransfected with GLI1 and ULK3, GLI1 is mostly nuclear. Cells cotransfected with GLI1 and ULK3 (K139R) or empty vector demonstrated significantly lower amounts of GLI1 in nuclei. Our data demonstrate that ULK3 alters the subcellular localization of GLI1, and the kinase activity of ULK3 is required to induce the nuclear translocation of GLI1.

Discussion

In this study we report the identification of human ULK3 as a serine/threonine kinase positively regulating Shh pathway by a kinase-dependent mechanism. Apart from STK36, ULK3 shows the

highest homology to dFu, a positive regulator of Hh pathway in *Drosophila*.

It has been shown previously that protein kinase dFu is absolutely required for the activation of Hh pathway in *Drosophila* [8]. dFu works in concert with Cos2, dSmo and dSufu, forming a microtubule-binding complex that controls activity of Ci transcription factor [18–20,22,23]. In spite of overall Hh pathway conservation between vertebrates and invertebrates, the function of those proteins is not fully conserved in mammals. Taking into account the divergence of Hh signaling in *Drosophila* and mammals on molecular level, it is not surprising that the role of mammalian Fu in Hh pathway also differs from that in *Drosophila*.

The mammalian homologue of dFu, STK36, was identified based on sequence homology [26]. While STK36 is able to positively regulate Gli proteins in cultured cells, it is dispensable for Shh signaling during mouse development [30,31]. Therefore, it is tempting to speculate that there is a redundancy of STK36 function and additional kinases are involved in the mammalian Hh pathway. ULK3 appears to be a possible candidate to participate in Gli regulation.

Previously, ULK3 has been annotated as belonging to the family of unc-51-like kinases that comprises also ULK1, ULK2 and ULK4 proteins. We have found that ULK3 diverges from other members of the family. Our bioinformatic analysis suggests that ULK3 is the closest homologue of STK36 in humans.

Analysis of the *ULK3* expression pattern reveals that *ULK3* is widely expressed. Its expression is higher in a number of tissues where Shh signaling is known to be active, such as fetal and postnatal brain and liver [43,44]. Within the adult brain, higher expression of *ULK3* is found in hippocampus, olfactory bulb, cerebellum and optic nerve. Also, *GLI1* and *PTCH1* mRNA expression levels were higher in the same brain regions. Shh signaling has been shown to control stem cells behavior in adult hippocampus and regulate proliferation of the granule cells in postnatal cerebellum [43,45]. Also, the Shh pathway is active in postnatal rodent optic nerve [46]. The role of Shh signaling in olfactory bulb has not been documented yet. However, it has been shown that stem cells in the subventricular zone of forebrain proliferate under the control of Shh. Those cells migrate into the olfactory bulb, where they differentiate into functional interneurons [47]. Taken together, our data show that SHH-responsive tissues express higher levels of *ULK3* mRNA that provides indirect evidence that ULK3 may be involved in SHH pathway *in vivo*.

We show that ULK3 possesses autophosphorylation activity. It has been previously shown that ULK1 and ULK2 are also autophosphorylated kinases *in vitro* [41,42]. Mouse Ulk1 and Ulk2 are autophosphorylated in the conserved central proline/serine rich domain of the protein. However, ULK3 lacks such domain. Instead, according to bioinformatic analysis, ULK3 protein harbors a central domain contained within microtubule interacting and trafficking molecules (MIT). Besides that, the sequence mapped in dFu as Sufu-interacting domain (residues 306–436, [20]) is conserved to a certain extent in ULK3. As expected, C-terminal sequences responsible in dFu for interacting with Cos2 and carboxyl terminus of Smo are absent in ULK3.

We show that ULK3 is able to phosphorylate three mammalian GLI proteins *in vitro*. Although a number of serine/threonine kinases has been proposed to be involved in modulating Hh pathway both in *Drosophila* and in vertebrates, only PKA has been shown to directly phosphorylate all Gli proteins, and CK1 and GSK3 have been shown to phosphorylate Gli2 and Gli3 following primary phosphorylation by PKA [39,48–51]. PKA phosphorylates Gli1 in residues Thr-374 and Ser-640 [39]. Gli2 and Gli3 are phosphorylated by PKA, CK1, and GSK3 in a cluster between amino acids 784–855 that corresponds to Gli1 residues 590–658 [49,51]. We have found that ULK3 phosphorylates GLI1 in both N-terminus (residues 1–426) and C-terminus (residues 754–1126), but the fragment of GLI1 between residues 426–754 is not phosphorylated by ULK3. There are 73 potential serine/threonine sites of phosphorylation in GLI1 (1–433) fragment (50 serine residues and 23 threonine residues), and 52 potential sites of phosphorylation in GLI1 (726–1106) fragment (35 serine residues and 17 threonine residues).

Phosphorylation by PKA, CK1 and GSK3 has been described to elicit mainly negative effects on Gli proteins (however, see [14]). Our analysis of Shh pathway activation in cell culture identifies ULK3 as a positive regulator of the pathway. Cotransfection of ULK3 together with GLI1 or GLI2 in Shh-L2 cells shows that ULK3 is able to potentiate the transcriptional activator function of both of these proteins. Besides, ULK3 also enhances SHH-induced transcription and is able to induce the Gli-dependent luciferase reporter activity on its own.

Also, we have found that ULK3, when compared to its closest homologues ULK1 and STK36, has the strongest effect on GLI-dependent luciferase reporter activity. It has previously been reported that STK36 is a positive regulator of Shh pathway that acts independently on its functional kinase domain [26,27]. STK36 enhances Gli2 but not Gli1 transcriptional activity in C3H10T1/2 and HEK293 cells [26,27] and GLI1 transcriptional activity in NIH3T3C2 and SW480 cells [29]. Here, we show that STK36 fails to induce GLI1 but induces GLI2 transcriptional activity in Shh-L2 cells. Taken together the data suggest that GLI proteins can be regulated by at least two dFu-related kinases - ULK3 and STK36. STK36 is able to enhance Gli-induced transcription in certain cells in a kinase-independent way, whereas ULK3 probably activates GLI proteins by direct phosphorylation. Together these two kinases may recapitulate both the kinase-dependent and kinase-independent functions of dFu.

The fact that ULK3 can phosphorylate the GLI proteins *in vitro*, enhance their transcriptional activity and influence the subcellular distribution of at least GLI1, all in a kinase-dependent manner, points to its important role in GLI regulation. Thus, we suggest that ULK3 could be pan-GLI activating kinase in mammalian cells.

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PUBLICATION III

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Dual Function of UNC-51-like Kinase 3 (Ulk3) in the Sonic Hedgehog Signaling Pathway*[S]

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Alla Maloverjan[‡1], Marko Piirsoo[‡2], Lagle Kasak[‡], Lauri Peil[§], Torben Østerlund[‡], and Priit Kogerman[‡¶]

From the [‡]Department of Gene Technology, Institute of Clinical Medicine, and [§]Technomedicum, Tallinn University of Technology, Akadeemia tee 15, 12618 Tallinn and the [¶]Institute of Technology, University of Tartu, Nooruse 1, 50411 Tartu, Estonia

The Sonic hedgehog (Shh) signaling pathway controls a variety of developmental processes and is implicated in tissue homeostasis maintenance and neurogenesis in adults. Recently, we identified Ulk3 as an active kinase able to positively regulate Gli proteins, mediators of the Shh signaling in mammals. Here, we provide several lines of evidence that Ulk3 participates in the transduction of the Shh signal also independently of its kinase activity. We demonstrate that Ulk3 through its kinase domain interacts with Suppressor of Fused (Sufu), a protein required for negative regulation of Gli proteins. Sufu blocks Ulk3 autophosphorylation and abolishes its ability to phosphorylate and positively regulate Gli proteins. We show that Shh signaling destabilizes the Sufu-Ulk3 complex and induces the release of Ulk3. We demonstrate that the Sufu-Ulk3 complex, when co-expressed with Gli2, promotes generation of the Gli2 repressor form, and that reduction of the *Ulk3* mRNA level in Shh-responsive cells results in higher potency of the cells to transmit the Shh signal. Our data suggests a dual function of Ulk3 in the Shh signal transduction pathway and propose an additional way of regulating Gli proteins by Sufu, through binding to and suppression of Ulk3.

The evolutionarily conserved Hedgehog (Hh)[3] signaling pathway controls a variety of developmental processes through regulation of cell proliferation and differentiation (1). In adults, the pathway is implicated in tissue homeostasis maintenance and stem cell proliferation (2, 3). Because inappropriate activation of the HH pathway contributes to various congenital abnormalities and tumorigenesis in humans, investigations of the molecular signaling mechanisms should shed light not only on developmental but also on important pathological issues. The hh signaling pathway was initially discovered in *Drosophila melanogaster* (4). Molecular mechanisms of the Hh signal transduction have been intensively investigated using fly, fish, chick, and rodent models. Despite well described mechanisms of hh signaling in *Drosophila*, intracellular events induced by Sonic Hedgehog (Shh, one of three mammalian homologues of hh) have remained unclear in many aspects.

The Hh signaling is initiated by binding of the morphogen Hh to its receptor Patched1 (Ptch1), a 12-pass transmembrane protein. This results in the attenuation of the inhibitory effect of Ptch1 on another transmembrane protein, Smoothened (Smo), allowing the latter to transfer the signal into the cell through induction of the signaling machinery responsible for activation of the Hh target genes (3). Initiation of the signaling occurring on the cell membrane is rather similar in invertebrates and vertebrates (5, 6).

In vertebrates the Shh signal aims at controlling the activities of transcription factors Gli1, Gli2, and Gli3 (6, 7). Gli1 is the strongest activator and the *Gli1* gene is a transcriptional target of Shh activity. As Gli1 is generally not expressed in non-stimulated cells, it serves as a marker of Shh activity and is thought to contribute to the maintenance of signaling (8, 9). Both Gli2 and Gli3 contain an N-terminal repressor domain and a C-terminal activator domain, whereas Gli3 is the strongest repressor and Gli2 is a primary activator of the Shh target genes (10). In the absence of Shh, full-length Gli2 and Gli3 are subjected to proteosomal degradation or undergo partial proteolysis resulting in generation of C terminal-truncated repressor forms, $Gli2/3^{Rep}$ (11, 12). In Shh-stimulated cells the proteolysis is repressed and full-length Gli proteins are converted to transcriptional activators, Gli^{Act}, followed by their translocation to the nucleus where they take part in transcriptional activation of target genes. In fact, it has been suggested that the balance between activator and repressor forms of Gli proteins determines the transcriptional outcome (13, 14).

Genetic studies suggest that the PEST domain containing protein Sufu is a major negative regulator of Gli proteins in mammals (15, 16). Notably, $sufu^{-/-}$ mutant flies are viable (17), whereas *Sufu*-deficient mice die at 9.5 days *post coitus* with multiple defects resulting from abnormal up-regulation of Hh signaling (15, 16). Mammalian Sufu controls Gli proteins by direct binding and sequestering them in the cytoplasm (18–20). This interaction is believed to contribute to the generation of $Gli2/3^{Rep}$ that is preceded by the phosphorylation of full-length Gli2/3 by PKA, glycogen synthase kinase 3β, and casein kinase 1 (12, 21–24). However, recent findings have demonstrated that Sufu binding to full-length Gli2 and Gli3 protects them from

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[1] To whom correspondence should be addressed. Tel.: 3726204438; Fax: 3726204401; E-mail: alla.maloverjan@ttu.ee.

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[3] The abbreviations used are: Hh, Hedgehog; CTD, carboxyl-terminal domain; fu, Fused; Gli^{Act}, activator form of Gli proteins; $Gli2/3^{Rep}$, repressor form of Gli2/3 protein; IP, immunoprecipitation; KD, kinase domain; RCGC, rat cerebellar granular cells; Shh, Sonic Hedgehog; Shh-L2, Shh-LIGHT2; Stk36, serine/threonine kinase 36; Sufu, Suppressor of Fused; Ulk3, unc-51-like kinase 3; WB, Western blot; WT, wild-type; HSC, Hedgehog signaling complex; nt, nucleotide.

complete proteosomal degradation, which in turn contributes to the accumulation of a pool of Gli2 and Gli3 proteins ready to be converted to transcriptional activators (25). The dual function of Sufu suggests the existence of several pools of Sufu, regulating Gli proteins context dependently.

In *Drosophila*, the divergent functions of Gli proteins are performed by one hh signal-dependent transcription factor, *Cubitus interruptus* (26). The activity of *C. interruptus* is controlled by a multimolecular complex associated with microtubules (so-called Hedgehog signaling complex or HSC). HSC contains a scaffolding protein costal2 (cos2), and putative serine/threonine kinase fused (fu), and sufu (27–30). HSC, through cos2 and sufu, binds *C. interruptus* and controls its stability, subcellular localization, and activity in an hh signal-dependent way (31, 32). In the absence of hh ligand, HSC is responsible for retaining the full-length *C. interruptus* in an inactive state and also participates in the generation of a C-terminal-truncated repressor form of *C. interruptus*. In the hh-stimulated cells, HSC dissociates, and full-length *C. interruptus* is released to perform transcriptional activation.

Fu is known as one of the central regulators of *C. interruptus* activity. Genetic studies suggest a positive role of fu, as loss of fu leads to Hh pathway activation (33, 34). Indeed, the predominant role of fu is to antagonize the negative effect of sufu (35). Fu and sufu are able to interact, and the fu domain responsible for this interaction has been mapped to amino acids residues 306–436 (27). Fu comprises an N-terminal kinase domain and a C-terminal regulatory domain and has been shown to play both kinase activity-dependent and -independent (regulatory) roles (36–38). In the absence of hh ligand, fu is inactive and subjected to autoinhibition through its regulatory domain (39). This domain is also required for processing of full-length *C. interruptus* into the transcriptional repressor form (37). However, when the pathway is activated, fu becomes phosphorylated (40). Moreover, it has been shown that phosphorylation of cos2, sufu, and smo in response to the pathway activation depends on fu kinase activity and full activation of the hh pathway requires fu kinase (28, 31, 41–43). Thus, fu plays an Hh ligand-dependent dual role in regulation of *C. interruptus* activity.

At present, a similar Gli-containing signaling complex has not been described in vertebrates. In contrast to the comprehensive role of fu, its mammalian homologue Stk36 has no or a limited role in the Shh pathway, as it is dispensable for proper embryonic development in mouse (44, 45). Although Stk36 was shown to some extent rescue the negative effect of Sufu on Gli-dependent transcription (46), no evidence of physical interaction between Sufu and Stk36 has been provided.

Recently, we have demonstrated that serine/threonine kinase ULK3 is able to regulate GLI proteins positively in a kinase activity-dependent manner (47). ULK3 shares sequence similarity with serine/threonine kinases fu and STK36. We have shown that ULK3 directly phosphorylates GLI proteins, enhances their transcriptional activity in cell culture, and promotes the nuclear translocation of GLI1.

In this study, we demonstrate that ULK3 kinase has a intramolecular self-regulation property. In addition to the previously demonstrated phosphorylation/activation of GLI, ULK3 has a kinase-independent regulatory role in the SHH pathway. By RNAi, it is demonstrated that reduction of the *Ulk3* mRNA level results in a higher response of cells to the Shh signal. We show that ULK3 through its kinase domain (KD) physically interacts with SUFU, and that the ULK3-Sufu complex is sensitive to SHH signaling. Interaction with Sufu inhibits the catalytic activity of ULK3, preventing its autophosphorylation and subsequent phosphorylation of GLI proteins. Moreover, the SUFU-ULK3 complex, when co-expressed with GLI2, induces generation of the GLI2 repressor form, whereas none of those proteins alone is able to do that. Finally, we propose a model unraveling the role of Ulk3 in the regulation of Gli proteins.

EXPERIMENTAL PROCEDURES

Expression Constructs—ULK3FLAG, ULK3(K139R), and GLI2FLAG were described in Ref. 47, GLI2GFP was described in Ref. 48, and SUFUmyc described in Ref. 20. The pSV40-β-gal construct used for luciferase assay data normalization was described in Ref. 49. pCI-GFP (Promega, Madison, WI) and pBABEpuro (Addgene, Cambridge, MA) were kindly provided by Dr. Rune Toftgård. The sequence for small interfering RNA1 (siRNA1, 5′-TATCTACCTCATCATGGAG-3′) was chosen to be specific for human, mouse, and rat *Ulk3* nt 258–276 and the sequence of siRNA2 (5′-ACGAAACATCTCTCACTTGGA-3′) was specific for mouse and rat *Ulk3* nt 390–410 (numbering is given relative to translation initiation codon ATG). Expression constructs siRNA1pSUPER and siRNA2pSUPER were generated according to the pSUPER RNAi System Protocol (OligoEngine, Seattle, WA). The part of mouse Ulk3 cDNA containing the sequences specific for siRNAs was amplified by PCR from mouse hippocampus cDNA and cloned into the ULK3FLAG construct between StuI and Eco72I sites (nt 91 and 513, respectively). Constructs ULK3(Δ301–365), ULK3(Δ373–446), ULK3-KD (amino acids 1–270), and ULK3-CT (amino acids 271–472) were generated from ULK3FLAG by PCR using the Pfu Turbo DNA polymerase system (Stratagene, La Jolla, CA) according to the manufacturers' recommendations. Construct pULK3-Ubi was generated by subcloning the ULK3 coding sequence from ULK3FLAG into modified bacterial expression vector pET24d (Novagen, Darmstadt, Germany) containing the sequence encoding decahistidine-tagged *Saccharomyces cerevisiae* ubiquitin protein.

Antibodies—The following antibodies were used for WB: M2 anti-FLAG-HRP (Sigma), anti-GFP-HRP (Rockland), anti-GFP (Clontech, Saint-Germain-en-Laye, France), and H-300 anti-Sufu (Santa Cruz, CA). Antibodies used for IP were C-15 anti-Sufu (Santa Cruz), anti-FLAG-M2 (Sigma), and c-myc 9E10 (Santa Cruz) conjugated with agarose. The secondary antibodies used were HRP-conjugated goat anti-mouse and goat anti-rabbit Ig (Jackson Laboratories, West Grove, PA).

Bacterial Expression and Purification of Proteins—SHH is described in Ref. 50. ULK3-Ubi fusion protein was expressed in *Escherichia coli* strain BL21-CodonPlus™-RP at 30 °C in LB broth containing 10% glycerol and induced overnight with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside. Fusion protein was purified using Ni-CAM HC resin (Sigma) according to the

manufacturer's recommendations except that all buffers contained 10% glycerol.

Cells Culture—HEK293, NIH3T3, and Shh-Light2 (Shh-L2) cells were propagated as previously described (47). Stable cell lines were generated as described in Ref. 50 and propagated in Shh-L2 cell medium supplemented with 2 μg/ml of puromycin (Sigma). Approximately 24 h prior to transfections or induction with SHH, the cells were plated onto appropriate growth dishes. Rat cerebellar granular cells (RCGCs) were isolated from P6 rat pups, as described (51), and plated on dishes precoated with 0.1 mg/cm^2 of poly-L-lysin (Sigma). Cells were propagated in Neurobasal medium supplemented with B-27 (Invitrogen), 78 ng/ml of D-glucose, 20 mM KCl (Sigma), 20 μM glutamine, and 100 μg/ml of penicillin/streptomycin (Invitrogen). All cells were grown at 37 °C and 5% CO_2.

Overexpression Studies—Shh-L2 cells and stable cell lines derived from Shh-L2, HEK293, and NIH3T3 cells were transfected using polyethylenimine transfection agent (PEI) (Inbio, Tallinn, Estonia) as described (50). After transfection, HEK293 and NIH3T3 cells were propagated in the normal growth medium for 48 h. Shh-L2 cells were handled as previously described and subjected to luciferase assay (50). The obtained firefly luciferase data were normalized with β-galactosidase values. For assessment of efficiency of siRNAs, HEK293 cells were co-transfected with the Ulk3FLAG construct, pCI-GFP plasmid, and either siRNA1pSUPER or siRNA2pSUPER constructs (40 + 40 ng + 1500 ng/3-cm plate, respectively). Stable cell clones were co-transfected with 100 ng of Ulk3FLAG and 50 ng of pCI-GFP on a 3-cm plate. RCGCs were transfected using Amaxa Rat Neuron Nucleofector kit (Lonza, Basel, Switzerland) according to the manufacturer's instructions. We used 2 μg of plasmid per ~2 ml of RCGCs. For detection of the GLI2 repressor form, HEK293 cells were transfected with 1 μg of GLI2GFP, 0.6 μg of ULK3FLAG or ULK3(K139R), and 0.4 μg of SUFUmyc plasmids or the respective empty vectors. Alternatively, the FLAG-tagged GLI2 construct was used in combination with His-tagged ULK3 and SUFUmyc encoding plasmids. Cells were incubated 48 h, lysed using RIPA lysis buffer supplemented with Protease inhibitor mixture (Roche Applied Science), and subjected to WB using anti-GFP antibody or M2 anti-FLAG antibody.

Quantitative Real Time PCR—Total RNA from Shh-L2 and stable cell lines was isolated using RNAqueous kit (Ambion, Austin, TX) and total RNA from RCGC was isolated using the RNeasy micro kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using SuperScriptIII reverse transcriptase (Invitrogen) according to the manufacturer's recommendations. The levels of *Ulk3* and *Gli1* mRNAs and mRNA of the housekeeping gene *Hprt* used for normalization were detected in triplicates by quantitative real time PCR (qRT-PCR) using qPCR Core kit for SYBR Green (Eurogentec, Seraing, Belgium) with Lightcycler 2.0 (Roche Applied Science) according to the manufacturer's instructions. Data were analyzed with Lightcycler 4.05 software (Roche). The data are expressed as the average mean ± S.E. of three independent measurements. The following primers were used: *Ulk3* sense, 5′-ACGAAACATCTCTCACTTG-3′; *Ulk3* antisense, 5′-TGCTGGGCAAAGCCAAAGTC-3′; *Gli1* sense, 5′-ACGTTTGAAGGCTGTCGGAA-3′; *Gli1* antisense, 5′-CACACGTATGGCTTCTCATT-3′; *Hprt* sense, 5′-CAGTCCCAGCGTCGTGATTA-3′; and *Hprt* antisense, 5′-AGCAAGTCTTTCAGTCCTGTC-3′.

Statistical Analysis—Statistical analysis was carried out and p values were calculated using t test (Two Sample Assuming Equal Variances).

Immunoprecipitation—Cells were lysed and immunoprecipitation (IP) was performed as previously described (47). HEK293 cells were transfected with FLAG-tagged constructs expressing ULK3, ULK3(K139R), ULK3(Δ301–365), ULK3(Δ373–446), or ULK3-KD and the myc-tagged construct expressing SUFU (each 2.5 μg) on 6-cm plates. For negative controls, expression constructs were substituted with the respective empty vectors. FLAG-tagged constructs were immunoprecipitated using anti-FLAG-M2 affinity gel and myc-tagged SUFU was precipitated using anti-myc affinity gel according to the manufacturer's instructions. Cell lysates were incubated with the antibodies for 2 h at room temperature with gentle agitation. Immunocomplexes were subjected to WB using M2 anti-FLAG-HRP antibody and H-300 anti-myc antibody. Endogenous Sufu was immunoprecipitated from NIH3T3 cells using C-15 anti-Sufu antibody conjugated with agarose beads. Cells were transiently transfected with 10 μg of ULK3FLAG or empty vector and, if indicated, induced with 12 nM SHH for 48 h on 10-cm plates. Cell lysates were incubated with the antibody overnight at 4 °C with gentle agitation. Immunocomplexes were subjected to WB using H-300 anti-Sufu antibody or M2 anti-FLAG-HRP antibody.

In Vitro Kinase Assay—*In vitro* kinase assay was performed as described (47). Briefly, FLAG-tagged ULK3, ULK3(K139R), ULK3-KD, ULK3-CT, and GLI2FLAG proteins were overexpressed in HEK293 cells, immunoprecipitated using anti-FLAG-M2 affinity gel, washed 3 times with TBS, 2 times with kinase buffer and resuspended in 30 μl of kinase buffer. Aliquots of 2 μl were used for *in vitro* kinase assay. One-half of the myc immunocomplexes was washed twice with kinase buffer and subjected to *in vitro* kinase assay in the presence of ULK3-Ubi and separately immunopurified FLAG-tagged ULK3, if indicated.

Mass Spectrometry Analysis—For detection of ULK3 autophosphorylation sites, ULK3FLAG and ULK3(K139R) were expressed in HEK293 cells. The proteins were immunopurified using anti-FLAG-M2 affinity gel. ULK3FLAG and ULK3-Ubi proteins were subjected to *in vitro* kinase assay. The reaction was stopped by adding Laemmli buffer containing 100 mM DTT. ULK3FLAG protein, not subjected to the *in vitro* kinase assay, was used as a control. Proteins were resolved by SDS-PAGE and visualized by Coomassie Blue staining. The bands were excised from the gel and in-gel digested with modified sequencing grade trypsin (Promega), as described previously (52). Peptides from in-gel-digested samples were purified with StageTips1 and analyzed by LC-MS/MS using an Agilent 1200 series nanoflow system (Agilent Technologies, Santa Clara, CA) connected to a LTQ Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) equipped with a nanoelectrospray ion source (Proxeon, Odense, Denmark). Up to five data-dependent MS/MS spectra were acquired in centroid in the linear

ion trap for each FTMS full-scan spectrum. Fragment MS/MS spectra from raw files were extracted as MSM files and then merged to peak lists using Raw2MSM version 1.72 selecting the top six peaks for 100 Da. MSM files were searched with the Mascot 2.2 search engine (Matrix Science, London, UK) against the protein sequence data base composed of ULK3 sequences and common contaminant proteins such as trypsin, keratins etc.

RESULTS

Silencing of Ulk3 Gene Expression by RNAi Suggests a Negative Role of Ulk3 in the Transduction of Shh Signal—To investigate the role of Ulk3 in Shh signal transduction, we suppressed Ulk3 expression by RNAi. We designed two siRNA-expressing constructs, siRNA1pSUPER and siRNA2pSUPER. The effectiveness of the siRNAs was estimated by overexpressing Ulk3FLAG with siRNA1pSUPER and siRNA2pSUPER constructs in HEK293 cells. pCI-GFP plasmid expressing GFP was co-transfected to estimate the efficiency of transfection. Expression of FLAG-tagged Ulk3 and GFP was detected by WB analysis using anti-FLAG and anti-GFP antibodies, respectively. The experiment was repeated three times and the data of a representative experiment are shown in Fig. 1*A*. Both siRNAs were able to suppress Ulk3 expression, whereas siRNA1 demonstrated higher efficiency than siRNA2.

To study the effect of *Ulk3* silencing on Shh signal transduction, we used RCGCs that are known to be Shh responding cells (53). RCGCs were isolated from P6 rat pups and immediately transfected with an empty vector, siRNA1- or siRNA2-encoding constructs in two replicates. One replicate of each transfection was induced by the SHH protein. The level of *Ulk3* and *Gli1* mRNA was measured in triplicates using qRT-PCR and normalized by *Hprt* mRNA expression level. The average mean of three independent experiments ± S.E. is shown in Fig. 1*B*. The normalized level of *Ulk3* mRNA is shown in the *left panel*. The level of *Ulk3* mRNA in non-induced cells transfected with empty vector was set as 100%. Addition of SHH did not affect the level of *Ulk3*. Transient expression of both siRNAs could suppress *Ulk3* mRNA expression by ~30%. Addition of SHH did not significantly affect the extent of *Ulk3* silencing.

Normalized level of *Gli1* mRNA in the same samples is shown in the *right panel* of Fig. 1*B*. As the level of *Gli1* mRNA was below the detection limit in non-induced cells, we concluded that *Gli1* is not expressed in RCGCs. The level of *Gli1* mRNA in cells transfected with empty vector and induced with SHH was considered 100%. Transfection of siRNA1pSUPER and siRNA2pSUPER constructs followed by SHH induction, elevated the *Gli1* mRNA expression levels by 43 and 32%, respectively. It should be noted that siRNA1, as the more potent silencer of Ulk3 expression, triggered higher induction of *Gli1* mRNA expression.

To corroborate our findings in RCGCs and achieve higher levels of suppression of *Ulk3* mRNA expression, we generated stable cell lines expressing the *Ulk3*-specific siRNA1 and siRNA2 in the Shh-responsive cell line Shh-L2. We co-transfected either of the two siRNA constructs together with the pBABEpuro construct into Shh-L2 cells. In total 38 puromycin-resistant clones (23 clones obtained using siRNA1 and 15 clones obtained using siRNA2) from two independent experiments were picked, propagated, and divided in three parts: the first part was plated and induced by SHH to be analyzed using luciferase assay, the second part was frozen for total RNA isolation and the third part was propagated further.

The levels of *Ulk3* mRNA and mRNA of the housekeeping gene *Hprt* used for normalization were measured using qRT-PCR. The level of *Ulk3* mRNA, normalized by *Hprt* mRNA expression, in Shh-L2 cells was taken as 1. Suppression of *Ulk3* mRNA was achieved in 6 clones: clones 1.1, 1.2, and 1.3 were obtained using siRNA1 and clones 2.1, 2.2, and 2.3 were obtained using siRNA2 (Fig. 1*C*, *left panel*). *Ulk3* mRNA was suppressed most effectively (by ~50%) in clones 1.1, 1.2, 2.1, and 2.2. The level of *Ulk3* mRNA in clones 1.4 and 2.4 is shown as an additional control, as the level of *Ulk3* mRNA was similar to that in the parental Shh-L2 cells. We also analyzed the level of *Gli1* mRNA in stable and control cell lines. However, it did not correlate with changes in *Ulk3* mRNA levels (data not shown).

Luciferase activity of three independent replicates was obtained and normalized with alkaline phosphatase values. SHH-dependent induction of luciferase activity in Shh-L2 cells was considered as 1. All clones expressing the lower level of *Ulk3* mRNA had higher potency in the induction of Gli-dependent luciferase gene expression compared with the control cell line Shh-L2, clones 1.4 and 2.4 (Fig. 1*C*, *right panel*). However, although *Ulk3* mRNA levels were similar in clones expressing siRNA1 and siRNA2, clones obtained using the more potent siRNA1 (Fig. 1*A*) demonstrated higher induction of Gli-dependent luciferase activity compared with clones obtained using siRNA2 (3.5 *versus* 2.2 times above the controls, respectively). This suggests that siRNA1 might suppress the expression of *Ulk3* not only at the transcriptional but also at the translational level.

Continuing the analysis of clones stably expressing *Ulk3*-specific siRNAs, we found that during propagation of the cell lines, the *Ulk3* mRNA levels constantly increased with time, reaching the level of the control cell line within 2.5 weeks. A total of 4 clones obtained from two independent experiments were analyzed for *Ulk3* mRNA expression and induction of luciferase activity during 2.5 weeks of culturing. All clones showed the same tendency and the results of representative clone 1.1 are shown in Fig. 1*D*, as compared with parental Shh-L2 cells. The *Ulk3* mRNA level increased from the initial 46 to 120% during 14 days (Fig. 1*D*, *left panel*), in concert with reduction of luciferase activity under the influence of SHH (Fig. 1*D*, *right panel*).

Due to the lack of working antibody against Ulk3, we were unable to show the endogenous Ulk3 protein levels in the stable cell lines. Therefore we transfected Ulk3FLAG and GFP encoding constructs into clones 1.2 and 2.1 and Shh-L2 cells. The levels of exogenously added Ulk3 and GFP proteins were analyzed by WB using antibodies against FLAG tag and GFP, respectively. The experiment was repeated 4 times. The obtained bands were quantified using ImageQuant TL software. Ulk3 protein levels, normalized with GFP levels, are shown in Fig. 1*E* and supplemental Fig. S1. The lowest amount of Ulk3 protein was

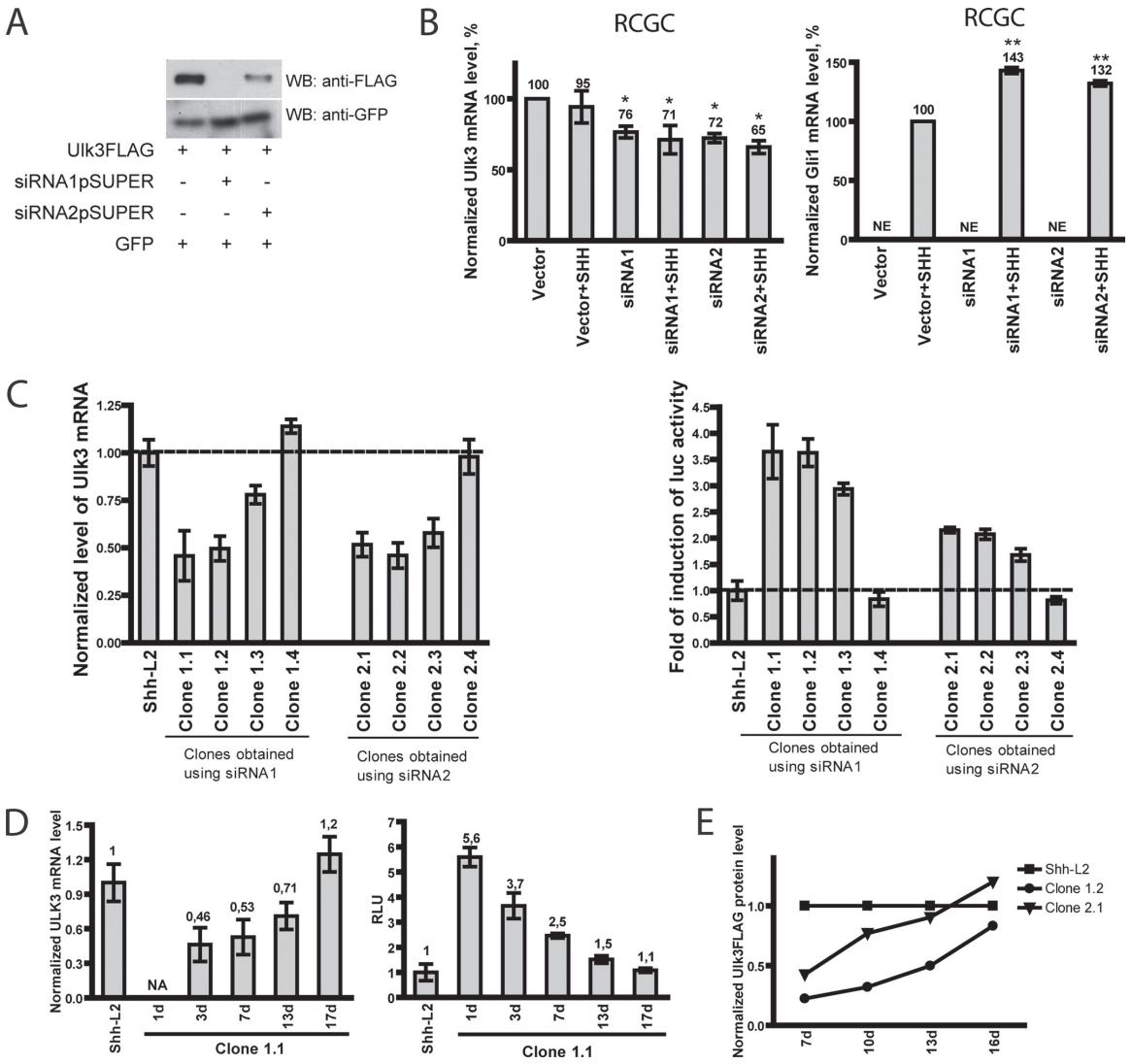

FIGURE 1. **Suppression of *Ulk3* gene expression suggests a negative role of Ulk3 in Shh signal transduction.** *A,* constructs expressing *Ulk3*-specific siRNA1 and siRNA2 were co-expressed with FLAG-tagged Ulk3 and GFP in HEK293 cells and cell lysates were subjected to WB analysis. Both siRNAs suppress expression of Ulk3. *B,* RCGCs were transiently transfected with the construct expressing *Ulk3*-specific siRNA1 or siRNA2 and stimulated with SHH protein. The *Ulk3* mRNA level was measured by qRT-PCR and normalized with the *Hprt* mRNA level (*left panel*) along with measurements of Gli-induced luciferase activity (*right panel*). The cells transfected with the siRNA-expressing constructs were compared with the cells transfected with empty vector *, $p < 0.05$; **, $p < 0.01$ (*NE*, not expressed). The data are presented as average mean ± S.E. of three measurements obtained from three independent experiments. *C,* suppression of *Ulk3* gene expression is achieved in cell lines stably expressing siRNA1 (clones 1.1, 1.2, and 1.3) and siRNA2 (clones 2.1, 2.2, and 2.3) (*left panel*). The *Ulk3* mRNA level is reduced most effectively (~50%) in clones 1.1, 1.2, 2.1, and 2.2. The expression level of *Ulk3* mRNA, normalized with *Hprt* mRNA, in the parental cell line Shh-L2 is set as 1 and the values in other cell lines are normalized accordingly. Clones expressing the lower level of *Ulk3* mRNA demonstrate a higher induction of Gli-dependent luciferase gene expression under influence of SHH compared with control cell lines Shh-L2, clones 1.4 and 2.4 (*right panel*). The data are presented as average mean ± S.E. of three independent measurements. *D,* prolonged propagation of stable cell clone 1.1 expressing the *Ulk3*-specific siRNA1. *Left panel* shows *Ulk3* mRNA levels and the *right panel* shows the luciferase activities in cells induced with SHH protein. *NA*, not analyzed. The data are presented as the average mean ± S.E. of three independent measurements. *E,* cell lines stably expressing *Ulk3*-specific siRNA1 and siRNA2 (clones 1.2 and 2.1, respectively) and Shh-L2 cells were transfected at different time points during propagation with Ulk3FLAG and GFP encoding constructs. Cell lysates were analyzed with WB using anti-FLAG and anti-GFP antibodies. The levels of overexpressed proteins were quantified and the Ulk3FLAG protein level was normalized with the level of GFP expression. Ulk3FLAG protein level in Shh-L2 cells at each time point was calculated as 1.

detected in clone 1.2, whereas clone 2.1 showed a moderately reduced level of Ulk3 and the control cell line demonstrated the highest level of the protein. These results are perfectly in line with data of the transcription assay and higher effectiveness of the siRNA1 construct in suppressing the expression of Ulk3. Similarly to the increase of the *Ulk3* mRNA level in stable cell lines in time, we found that the level of exogenous Ulk3 protein was increased in time, reaching the level in Shh-L2 after 2 weeks.

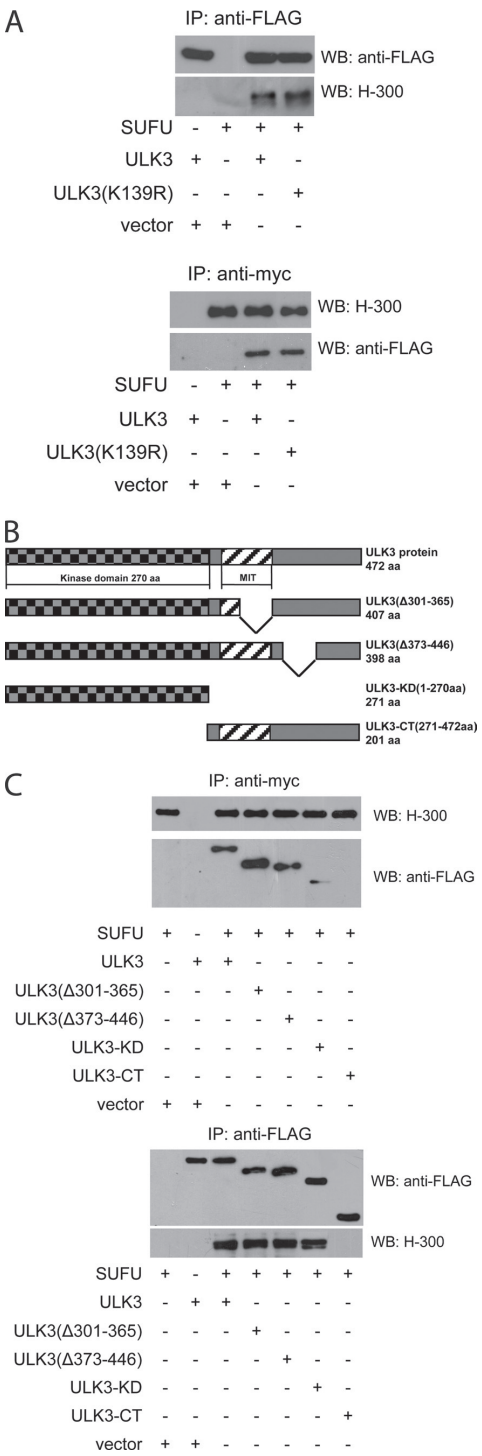

FIGURE 2. **ULK3 physically interacts with SUFU through its KD.** *A,* FLAG-tagged WT and kinase-deficient ULK3 proteins were co-expressed with myc-tagged SUFU in HEK293 cells and immunoprecipitated using M2-a-FLAG or c-myc 9E10 affinity gel, respectively. Immunocomplexes were subjected to WB using a-FLAG and H-300 a-Sufu antibodies. *B,* schematic presentation of ULK3 proteins tested in the current study. *MIT,* domain contained within microtubule interacting and trafficking molecules. *C,* FLAG-tagged WT ULK3 and its deletion mutants were co-expressed with myc-tagged SUFU in HEK293 cells and immunoprecipitated using M2-a-FLAG or c-myc 9E10 affinity gel, respectively. Immunocomplexes were subjected to WB using a-FLAG and H-300 a-Sufu antibodies.

Taken together, our results show that reduction of the *Ulk3* mRNA level in cells triggers a stronger response to SHH signal. Thus, our data from RNAi experiments suggest that Ulk3 is functioning as a negative regulator of the Shh pathway.

ULK3 Binds SUFU through Its Kinase Domain—ULK3 shares similarity with fu, and *Drosophila* proteins fu and sufu form a complex. Because SUFU is the major negative regulator of SHH signaling in mammals, we investigated if ULK3 is able to interact physically with SUFU protein. We overexpressed the FLAG-tagged WT and kinase-deficient ULK3 proteins and myc-tagged SUFU or respective empty vectors in HEK293 cells and immunoprecipitated them. The immunocomplexes were subjected to WB analysis using antibodies against FLAG tag and SUFU protein. The experiment was repeated 3 times, and the results of a representative experiment are shown in Fig. 2*A*. SUFU protein was detected in ULK3 as well as in ULK3(K139R) immunocomplexes (*upper panel*). Also, ULK3 and ULK3(K139R) proteins were detected in SUFU immunocomplexes (*lower panel*). Similar amounts of ULK3 and ULK3(K139R) were coimmunoprecipitated with SUFU suggesting that WT and kinase-deficient ULK3 bind SUFU with equal efficiency.

To investigate further the interaction of SUFU and ULK3, we attempted to determine the domain of ULK3 responsible for this interaction. First, we performed homology analysis of ULK3 and fu protein sequences using the DNAMAN sequence alignment algorithm and ClustalW program (EBI, EMBL). Regardless of the overall dissimilarity of the carboxyl termini of fu and ULK3, the analysis revealed two regions of homology between ULK3 and fu. Homologous regions were mapped to the fu domain, responsible for interaction with sufu (amino acids residues 306–436) (27). The first region corresponded to residues 310–365 and the second to residues 398–433 of ULK3. Bioinformatic analysis of the secondary structure of ULK3 (PredictProtein, EXPASY) predicted several α-helixes in those regions, which might participate in the interaction with Sufu. Based on the bioinformatic analysis, we generated two expression constructs encoding FLAG-tagged ULK3 deletion mutants, ULK3(Δ301–365) and ULK3(Δ373–446). We also generated the expression constructs encoding the kinase domain (KD) and C-terminal domain (CTD) of ULK3 (ULK3-KD and ULK3-CT, respectively). The structure of the resulting proteins is depicted in Fig. 2*B*.

To reveal the domain of ULK3 responsible for interaction with SUFU, we co-expressed the FLAG-tagged ULK3 or its deletion mutants with SUFUmyc in HEK293 cells. The proteins were immunoprecipitated using anti-FLAG and anti-myc antibodies and subjected to WB analysis (Fig. 2*C*). We detected ULK3wt, ULK3(Δ301–365), ULK3(Δ373–446), and ULK3-KD in SUFU immunoprecipitates (*upper panel*) and SUFU

protein in ULK3wt, ULK3(Δ301–365), ULK3(Δ373–446), and ULK3-KD immunocomplexes (*lower panel*). No significant difference was found in the efficiency of interaction of WT ULK3 or deletion mutants ULK3(Δ301–365) and ULK3(Δ373–446) with SUFU. However, we found that ULK3-KD was able to interact with SUFU, but the efficiency of their interaction was lower compared with that of WT ULK3 and SUFU, as indicated on the *upper panel*. ULK3-CT did not interact with SUFU.

Our data indicate that the ULK3 domain responsible for interaction with SUFU is not homologous to the fu interaction domain with sufu. Instead, ULK3 interacts with SUFU at least partly through its KD.

ULK3 C-terminal Autophosphorylation Is Blocked by SUFU Binding—Keeping in mind that fu, being in HSC, is catalytically inactive, we tested if ULK3, being in complex with SUFU, exhibits autophosphorylation activity. Myc-tagged SUFU and FLAG-tagged ULK3 or its catalytically inactive mutant ULK3(K139R) were co-expressed in HEK293 cells, subjected to immunoprecipitation using anti-myc antibody, followed by *in vitro* kinase assay (Fig. 3*A*, *upper panel*). The presence of the respective proteins in the immunoprecipitates was detected by WB and is shown in the *lower panel* of Fig. 3*A*. No ULK3 autophosphorylation activity was detected in the immunocomplexes (Fig. 3*A*, *lane 1*), suggesting that the kinase activity of ULK3 was abolished by interaction with SUFU. In contrast, bacterially expressed ULK3-Ubi or separately immunoprecipitated ULK3FLAG were able to self-phosphorylate themselves, indicating that autophosphorylation *per se* was not affected under these conditions (Fig. 3*A*, *lanes 3–5*). However, ULK3-Ubi neither phosphorylated SUFU-bound ULK3 nor ULK3(K139R) (Fig. 3*A*, *lanes 4* and *5*, respectively). These findings show that the kinase activity of ULK3 may be completely blocked by interaction with SUFU. In addition, we have found that trans-phosphorylation of ULK3 bound by SUFU does not occur. These data suggests that ULK3 phosphorylation takes place only within one molecule.

To further confirm the lack of trans-phosphorylation activity of ULK3 kinase, we expressed FLAG-tagged WT ULK3, its deletion mutants ULK3(Δ301–365) and ULK3(Δ373–446), and kinase-deficient ULK3(K139R) in HEK293 cells and immunoprecipitated the proteins. We mixed the kinase-competent immunoprecipitates with lower molecular mass (either ULK3(Δ301–365) or ULK3(Δ373–446), 46 and 45 kDa, respectively) with the ULK3(K139R) (53 kDa) immunoprecipitate and subjected the complexes to *in vitro* kinase assay. Using this kind of mixing, we were able to distinguish between autophosphorylation and trans-phosphorylation activity. As shown in the *upper panel* of Fig. 3*B*, none of the kinase-competent ULK3 proteins were able to phosphorylate the ULK3(K139R) protein, but retained the autophosphorylation activity. The presence of all proteins tested in the immunocomplexes was confirmed by WB (Fig. 3*B*, *lower panel*).

Because ULK3 loses its autocatalytic activity, when bound to SUFU, we were interested in determining which residues of ULK3 are autophosphorylated. We subjected bacterially expressed and purified protein (ULK3-Ubi), FLAG-tagged ULK3, and ULK3(K139R) proteins immunopurified from mammalian cells to *in vitro* kinase assay. Immunopurified

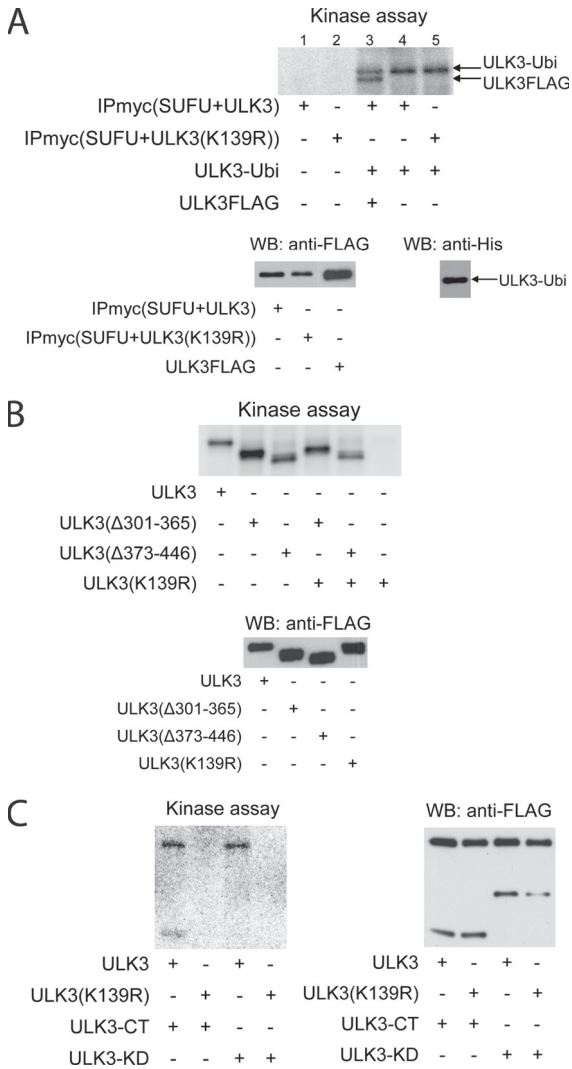

FIGURE 3. **Physical interaction of ULK3 with SUFU abolishes C-terminal autophosphorylation of ULK3.** *A, upper panel*, immunocomplexes obtained using anti-myc antibody and containing myc-tagged SUFU and FLAG-tagged ULK3 or ULK3(K139R) were subjected to *in vitro* kinase assay in the presence of [γ-^{32}P]ATP (*lanes 1* and *2*). Bacterially expressed and purified His-tagged ULK3-Ubi and separately immunopurified FLAG-tagged ULK3 proteins were added to the immunocomplexes prior to the kinase assay (*lanes 3–5*). *Lower panel*, the presence of the proteins in the immunoprecipitates was detected by WB using the respective antibody. *B, upper panel*, FLAG-tagged ULK3 proteins were overexpressed in HEK293 cells, immunopurified using anti-FLAG antibody, and subjected to *in vitro* kinase assay in the presence of [γ-^{32}P]ATP. *Lower panel* shows the presence of the proteins detected by WB using anti-FLAG antibody. *C, left panel*, FLAG-tagged WT ULK3, kinase-deficient ULK3(K139R), ULK3-CT, and ULK3-KD were overexpressed in HEK293 cells, immunopurified using anti-FLAG antibody, and subjected to *in vitro* kinase assay in the presence of [γ-^{32}P]ATP (*CT*, carboxyl terminus). *Right panel*, the presence of the proteins in the *in vitro* kinase reactions was detected by WB using anti-FLAG antibody.

ULK3FLAG protein that did not undergo the *in vitro* kinase reaction was used as a negative control. The proteins were resolved by SDS-PAGE, excised from the gel, and in-gel

TABLE 1

Autophosphorylation sites in ULK3 protein

Bacterially expressed and purified ULK3-Ubi overexpressed in HEK293 cells and immunopurified ULK3FLAG and ULK3(K139R) proteins were subjected to *in vitro* kinase assay. ULK3FLAG protein unexposed to the kinase assay was used as a negative control. The proteins were trypsinized followed by LC-ESI-MS/MS analysis of phosphopeptides. Four phosphorylated serine residues situated in C-terminal part of WT ULK3 proteins are identified. The numbers are given relative to the first methionine residue of ULK3. The Mascot score is given as $S = -10 \times \log(P)$, where P is the probability that the observed match is a random event.

Peptide	Phosphorylated residue	Mascot score
KDQEGDSAAALSLYCK	Ser-300	69
AIVSSSNQALLR	Ser-350	58
LLAALEVASAAMAK	Ser-384	56
EGLSESVR	Ser-464	93

digested with trypsin. Phosphorylated residues were mapped using LC-ESI-MS/MS in two technical replicates. Sequence coverage, representing the percentage of the entire protein sequence within the identified peptides, was 71% in ULK3FLAG and 75% in ULK3-Ubi. In contrast to the negative controls, autophosphorylated ULK3 proteins contained four phosphorylated residues: Ser-300, Ser-350, Ser-384, and Ser-464 (Table 1). Our analysis shows that ULK3 phosphorylates itself in the CTD.

To confirm the MS data, we subjected the immunopurified ULK3-KD and ULK3-CT to *in vitro* kinase assay in the presence of immunopurified WT ULK3 or kinase-deficient ULK3(K139R). No phosphorylation was detected in the ULK3-KD protein when mixed with WT ULK3 or ULK3(K139R) (Fig. 3*C*, *left panel*, *lanes 3* and *4*). However, when we mixed immunopurified WT ULK3 protein with ULK3-CT, we observed phosphorylation of both proteins (Fig. 3*C*, *left panel*, *first lane*). No phosphorylation of ULK3-CT was observed when it was mixed with ULK3(K139R) (Fig. 3*C*, *left panel*, *second lane*). The presence of all proteins tested in the *in vitro* kinase reaction was confirmed using WB (Fig. 3*C*, *right panel*).

These data confirm the MS findings and show that ULK3 autophosphorylation sites lie within the CTD of the protein. Furthermore, these data suggest that the C-terminal-phosphorylated residues of ULK3 are masked, preventing transphosphorylation by another ULK3 molecule. When the KD of ULK3 is removed, the phosphorylation sites become exposed and are readily accessible to another ULK3 molecule.

Luciferase Assay Suggests the Dual Role for ULK3—To get further insight into the biological meaning of the SUFU-ULK3 interaction, we performed an GLI-dependent transcriptional activation assay in Shh-L2 cells. In overexpression studies, SUFU is shown to elicit a strong negative effect on GLI proteins (20). In contrast, ULK3 is able to enhance significantly the transcriptional activity of GLI1 and GLI2 proteins in a kinase activity-dependent way (47). Thus, we assessed the potency of WT ULK3, ULK3(K139R), ULK3-KD, and ULK3-CT to rescue the negative effect of SUFU on GLI2 transcriptional activity. Because deletion mutants ULK3(Δ301–365) and ULK3(Δ373–446) were functionally highly similar to WT ULK3 (Figs. 2*C* and 3*B*, and data not shown), we did not test them in the luciferase assay.

First, we established the concentration curve for GLI2GFP co-expressed with the constant amount of WT ULK3 to find the amount of GLI2GFP plasmid sufficient to achieve the saturated level of Gli-dependent luciferase activity. The total DNA amount was kept constant by co-transfection with a compensatory amount of empty vector. Average induction of GLI2-dependent luciferase activity ± S.E. in the presence or absence of ULK3FLAG is shown in Fig. 4*A*. Our data showed that ULK3 was able to enhance the transcriptional activity of overexpressed GLI2 ~3–2.5 times when co-expressed with low concentrations of GLI2GFP (5–75 ng of GLI2GFP plasmid per well). An increase of GLI2GFP plasmid amount (100–300 ng/well) led to a decrease of additional activation of Gli-dependent luciferase expression by ULK3 (1.6–1.2 times) because the level of induction approached the maximum.

To investigate the role of ULK3 and its KD and CTD in GLI2 regulation in the presence of SUFU, we overexpressed a high amount of GLI2GFP (250 ng of GLI2GFP construct per well) with SUFUmyc and FLAG-tagged ULK3, ULK3(K139R), ULK3-KD, and ULK3-CT proteins (or respective empty vectors) in Shh-L2 cells and analyzed the protein for Gli-dependent transcriptional activation (Fig. 4*B*). GLI2 was able to induce luciferase activity ~46 times above control (induction of luciferase expression by empty vector taken as 1). Co-transfection with SUFU inhibited GLI2-induced luciferase activity 4.7 times. Addition of ULK3, ULK3(K139R), or ULK3-KD could partially rescue the negative effect of SUFU on GLI2 (2.4, 2.4, and 2.9 times, respectively). WT and kinase-deficient ULK3 demonstrated an equal effect in the assay suggesting the existence of a kinase activity-independent function of ULK3 in the regulation of GLI2. Notably, the effectiveness of ULK3-KD in restoring of GLI2 transcriptional activator properties was higher regardless on its lower affinity to SUFU compared with WT ULK3 (Fig. 2*C*, *upper panel*) suggesting the possibility of an inhibitory function of the CTD. Indeed, ULK3-CT could not rescue the inhibitory effect of SUFU on GLI2. Moreover, addition of ULK-CT to GLI2 and SUFU resulted in stronger inhibition of the GLI2 transcriptional activity (6.8 *versus* 4.7 times). ULK3-CT also inhibited GLI2 transcriptional activity 1.6 times (supplemental Fig. 2*A*). Expression of GLI2 with ULK3, ULK3(K139R), or ULK3-KD plasmids could additionally activate Gli-dependent luciferase expression ~1.2 times (supplemental Fig. 2*A*).

To test the effect of ULK3, ULK3-KD, and ULK3-CT on GLI2 transcriptional activity in non-saturated conditions, we co-transfected 50 ng of GLI2GFP and ULK3 constructs (or respective empty vectors) into Shh-L2 cells and analyzed the induction of luciferase activity (Fig. 4*C*). Induction of luciferase expression by empty vector was set as 1. GLI2 induced luciferase activity 16 times. ULK3 was able to enhance GLI2 transcriptional activity ~2.2 times and ULK3-KD enhanced GLI2 transcriptional activity 3.5 times. ULK3-CT, in contrast, inhibited GLI2-induced luciferase activity 2 times. Induction of the Gli-dependent luciferase by ULK3, ULK3-KD, and ULK3-CT in Shh-L2 cells is demonstrated in supplemental Fig. 2*B*. ULK3 was able to stimulate the luciferase activity 2.3 times above the induction by the empty vector, ULK3-KD, 3.4 times, and ULK3-CT had no effect on Gli-dependent luciferase activity.

Previously we have shown that ULK3 directly phosphorylates GLI proteins. Here we tested if ULK3-KD is able to phos-

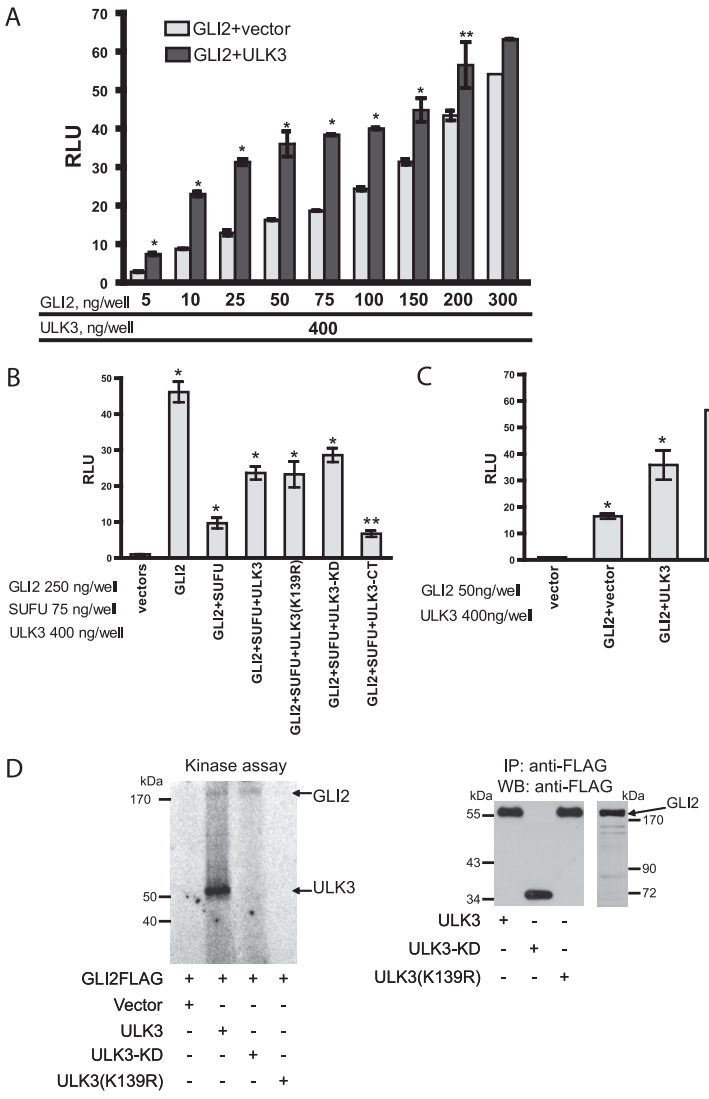

FIGURE 4. **ULK3 relieves the inhibitory effect of SUFU on GLI2 transcriptional activity due to its KD but independently of its kinase activity.** *A,* different amounts of the GLI2GFP-encoding construct and constant amount of the ULK3-encoding construct or respective empty vector were co-overexpressed in Shh-L2 cells and Gli-dependent luciferase activity was measured. Transfected DNA amount was kept constant by compensation of GLI2GFP plasmid with pCI-GFP vector. *, p value < 0.001 and **, p value < 0.05 GLI2 *versus* GLI2 + ULK3. The data are presented as average mean ± S.E. of three replicates obtained from three independent experiments. *B,* SUFU and ULK3 (WT, kinase-deficient, or deletion mutants) and GLI2 (250 ng/well) were co-expressed in Shh-L2 cells. Induction of Gli-dependent luciferase activity by empty vector was set as 1. GLI2 induces luciferase activity 46 times above the control (*, p value < 0.001 GLI2 *versus* empty vector). SUFU represses transcriptional activity of GLI2 (*, p value < 0.001 GLI2 *versus* GLI2 + SUFU). This repression is partly relieved by WT, kinase-deficient ULK3, and ULK3-KD (*, p value < 0.001, GLI2 + SUFU *versus* GLI2+SUFU+ULK3/ULK3(K139R)/ULK3-KD) and is not relieved, but more inhibited, by ULK3-CT (**, p value < 0.05, GLI2 + SUFU *versus* GLI2 + SUFU + ULK3-CT). The data are presented as average mean ± S.E. of three replicates obtained from three independent experiments. *C,* GLI2 (50 ng/well), ULK3, ULK3-KD, and ULK3-CT or the respective empty vector were co-expressed in Shh-L2 cells. Induction of luciferase activity by the empty vector was set as 1. GLI2 induces luciferase activity 16 times above the vector (*, p value < 0.001). ULK3 and ULK3-KD enhance GLI2 transcriptional activity (*, p value < 0.001). ULK3-KD potentiates the transcriptional activator function of GLI2 stronger than ULK3 (♦, p value < 0.001). Overexpression of ULK3-CT leads to inhibition of GLI2 transcriptional activity (*, p value < 0.001). The data are presented as average mean ± S.E. of three replicates obtained from three independent experiments. *D,* FLAG-tagged ULK3, ULK3(K139R), ULK3-KD, and GLI2 were overexpressed in HEK293 cells, immunopurified, and subjected to *in vitro* kinase assay in the presence of [γ-^{32}P]ATP (*left panel*). *Right panel* shows the presence of the proteins detected by WB using anti-FLAG antibody.

phorylate the GLI2 protein. We overexpressed FLAG-tagged ULK3, ULK3(K139R), ULK3-KD, and GLI2 in HEK293 cells, immunopurified them, and subjected them to *in vitro* kinase assay in the presence of [γ-^{32}P]ATP. ULK3-KD, despite its failure to autophosphorylate, was able to phosphorylate the GLI2 protein (Fig. 2*D*). These data suggest that ULK3-KD positively regulates GLI2 transcriptional activity through direct phosphorylation.

Taken together, our overexpression studies in Shh-L2 cells suggests that the ULK3 interaction with SUFU partly restores GLI2 transcriptional activity inhibited by SUFU. Furthermore, we demonstrate that ULK3 may have a dual role in regulation of GLI2 transcriptional activity. KD of ULK3 binds to SUFU and is able to phosphorylate and positively regulate GLI2. The CTD of ULK3 inhibits GLI2 transcriptional activity.

ULK3-SUFU Complex Promotes Generation of GLI2 Repressor Form—Our data showing the inability of ULK3 to entirely restore GLI2 transcriptional activity inhibited by SUFU, lead us to the hypothesis that the function of the ULK3-SUFU complex may be linked to generation of the GLI2^{Rep} form. To test that, we co-expressed GLI2GFP protein with WT or kinase-deficient FLAG-tagged ULK3 and myc-tagged SUFU proteins in HEK293 cells. Alternatively, the constructs encoding FLAG-tagged GLI2 and His-tagged ULK3 were used. Cell lysates were subjected to WB analysis using anti-GFP or anti-FLAG antibodies. The experiment using both GLI2 constructs was repeated three times. The data of representative experiments obtained using anti-GFP and anti-FLAG antibodies are shown in Fig. 5 and supplemental Fig. 3, respectively. GLI2GFP has a molecular mass of 210 kDa (Fig. 5). We failed to detect the GLI2^{Rep}GFP form when full-length GLI2GFP was expressed alone or together with either of the ULK3 proteins or SUFU (Fig. 5, *lanes 2–5*). SUFU significantly sta-

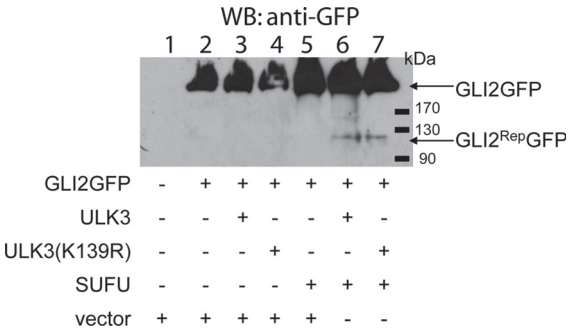

FIGURE 5. **ULK3-SUFU complex induces the generation of GLI2^{Rep}.** GLI2GFP fusion protein was expressed alone (*lane 2*), in combination with WT and kinase-deficient FLAG-tagged ULK3 (*lanes 3* and *4*), or myc-tagged SUFU (*lane 5*) in HEK293 cells. GLI2^{Rep}GFP protein is detected in the case of co-expression of GLI2GFP with SUFU combined with ULK3 or ULK3(K139R) by WB using GFP antibody (*lanes 6* and *7*, respectively).

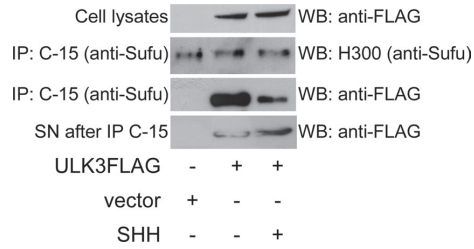

FIGURE 6. **Interaction of ULK3 with endogenous Sufu is regulated by SHH.** FLAG-tagged ULK3 was overexpressed in NIH3T3 cells in the absence or presence of SHH. Endogenous Sufu was immunoprecipitated using C-15 affinity gel. The amount of the precipitated Sufu protein was detected using H-300 a-Sufu antibody. ULK3 protein was detected using a-FLAG antibody. *SN*, supernatant.

bilizes full-length GLI2, which is consistent with previously published results (25) (Fig. 5, *lane 5 versus lane 2*, and supplemental Fig. 3, *right panel*). Proteins with a molecular mass of about 120 kDa were detected in the presence of ULK3 (either WT or kinase-deficient mutant) combined with SUFU (Fig. 5, *lanes 6* and *7*). The molecular weight of the protein corresponded to the expected molecular weight of the GLI2^{Rep}-GFP fusion protein. In the case of FLAG-tagged GLI2 (170 kDa), a repressor form with a molecular mass of about 90 kDa was detected (supplemental Fig. 3, *left panel*). However, using anti-FLAG antibody, we could detect low amounts of GLI2^{Rep}FLAG in the samples obtained from cells transfected with GLI2FLAG alone or together with SUFU. In the case of co-overexpressing of GLI2FLAG with WT or kinase-deficient ULK3, GLI2^{Rep}FLAG was undetectable. Because GLI2^{Rep} was detected in cases when SUFU was co-expressed both with the WT and kinase-deficient mutant of ULK3, we conclude that the kinase activity of ULK3 is not required for the generation of GLI2^{Rep}.

ULK3-Sufu Complex Dissociates under SHH Signal—Next, we asked whether overexpressed ULK3 was able to act in a dominant-negative manner and form a SHH signal-dependent complex with endogenous Sufu. We overexpressed FLAG-tagged ULK3 (or respective empty vector) in Shh-responsive NIH3T3 cells and induced the cells with SHH protein. Endogenous Sufu was immunoprecipitated using an anti-Sufu C-15 antibody. The obtained cell lysates, immunocomplexes, and IP supernatants were subjected to WB using anti-FLAG antibody to detect the presence of ULK3 in the samples. The experiment was repeated 3 times and data of a representative experiment are shown in Fig. 6. The efficiency of IP reactions was estimated using H-300 anti-Sufu antibody. The amount of Sufu protein detected in the immunocomplexes was similar in all samples. Lysates of the transfected cells contained equal amounts of ULK3 protein. ULK3 protein was co-immunoprecipitated with endogenous Sufu from both non-induced and SHH-induced cells, indicating the ability of ULK3 to effectively form a complex with endogenous Sufu. However, immunoprecipitates from non-induced cells contained significantly more ULK3 protein compared with cells induced with SHH (Fig. 6). In contrast, the supernatants obtained after IP with Sufu antibody contained more ULK3 in SHH-induced cells. These data indicate that ULK3 is able to form a complex with endogenous Sufu. This complex is responsive to SHH signaling, because SHH triggers its dissociation.

DISCUSSION

Recently we have reported the identification of serine/threonine kinase ULK3 as a positive regulator of GLI proteins. ULK3 was identified based on its sequence similarity with serine/threonine kinases fu and STK36, a putative homologue of fu. We have previously shown that ULK3 is able to enhance the activity of GLI1 and GLI2 proteins in a kinase activity-dependent manner.

In this article we demonstrate that ULK3 has intramolecular self-regulatory properties. We show that ULK3 autophosphorylation occurs at four serine residues (Ser-300, Ser-350, Ser-384, and Ser-464) situated outside of the KD. Those sites are hidden in catalytically inactive ULK3 and unexposed to other ULK3 molecules. However, deletion of the KD results in conformational changes making CTD available for intermolecular phosphorylation by ULK3. Deletion of CTD results in generation of the catalytically active KD that is able to phosphorylate its substrate, GLI2. Thus, autophosphorylation of ULK3 may involve conformational changes resulted in exposure of CTD to KD and consequently in generation of the catalytically active kinase. A similar mechanism of self-regulation control has been proposed for ULK1 and ULK2 kinases (53) and seems to be conserved between members of the ULK family.

To enhance our understanding of the function of ULK3 in mammalian cells, we applied RNAi to test the overall requirement of Ulk3 for Shh signal transduction. We used two independent experimental models. First, we transiently transfected 2 different expression constructs encoding *Ulk3*-specific siRNAs, the more effective siRNA1 and the less effective siRNA2, into RCGCs. Second, we suppressed *Ulk3* gene expression in the Shh-L2 cell line by stable transfection of the siRNA1 and siRNA2 constructs. The maximum suppression of the endogenous *Ulk3* mRNA expression level was ~30% in RCGC and 50% in stable cell lines. We failed to get cells with a lower level of *Ulk3* mRNA. One possible reason may be the fact that reduction of the *Ulk3* mRNA level leads to S-phase arrest (54). Therefore, it is possible that the cells with a lower level of *Ulk3*

mRNA were not able to grow sufficiently to be picked and analyzed.

In both experimental models the cells were induced by SHH and the induction of the *Gli1* mRNA expression level was assessed, in Shh-L2-derived stable cell lines indirectly by measuring the Gli-dependent luciferase activation, and in RCGCs, directly, using qRT-PCR. Unexpectedly, reduction of the *Ulk3* mRNA level resulted in an elevated response of cells to Shh reflected by an increased level of *Gli1* mRNA. In fact, the level of *Gli1* mRNA induction in response to SHH is in correlation with the *Ulk3* mRNA level in the cells. One possible explanation of these results is that Ulk3 may be involved in the negative regulation of some component(s) of the Shh pathway, for instance, in generation of Gli2/3^{Rep} in non-induced cells.

Because Sufu is known as a negative regulator of Gli proteins and is able to form a complex with fu (19), we tested the possibility that ULK3 acts in concert with SUFU in regulating GLI proteins. Indeed, our coimmunoprecipitation analysis demonstrates a physical interaction of ULK3 with Sufu proteins of human and mouse origin. Moreover, we demonstrate that KD of ULK3 is responsible for the interaction with SUFU. Transcriptional regulation experiments in the SHH-responsive cell line give functional meaning to this interaction, because ULK3 partially rescues the inhibitory effect of SUFU on GLI2-dependent transcription. However, ULK3 could not recover GLI2-induced luciferase activity to the initial level regardless of its molar superiority above SUFU, suggesting, analogically to the RNAi results, the existence of an ULK3-dependent mechanism negatively regulating the GLI2 protein. In fact, we found that CTD of ULK3 elicits a negative effect on GLI2 transcriptional activity. Deletion of this domain results in generation of a more potent transcriptional co-activator form of ULK3, ULK3-KD, that positively regulates GLI2 via direct phosphorylation.

Here, we further demonstrate the involvement of the novel ULK3-SUFU complex in the generation of GLI2^{Rep}, and both proteins are required for this. As expected, the ULK3 kinase activity is not required for GLI2^{Rep} generation.

Although Sufu was previously thought to be merely a negative regulator of Gli proteins by sequestering them in the cytoplasm, its function in protecting the full-length Gli2/3 proteins from proteolytic degradation has been described recently (25). We also observed the stabilizing effect of SUFU on full-length GLI2. However, here we show that SUFU is also needed for GLI2^{Rep} generation. Neither SUFU nor ULK3 alone can induce the generation of GLI2^{Rep}, but acting in concert, they promote C-terminal processing of full-length GLI2 probably through recruiting PKA, glycogen synthase kinase 3β, and casein kinase 1 protein kinases responsible for the initiation of proteolytic cleavage of GLI2/3. However, the precise mechanism of GLI2^{Rep} generation with the help of the SUFU-ULK3 complex remains to be investigated further.

Hitherto, apart from Gli proteins, three mammalian proteins, SAP18, Galectin3, and Cdc2l1, have been identified as proteins able to physically interact with Sufu (55–57). Besides, Sufu has been shown to move to primary cilia in response to Shh signaling (58). Thus, Sufu is thought to shuttle within the cell, interacting with different proteins essential for enhancement or suppression of its regulatory functions.

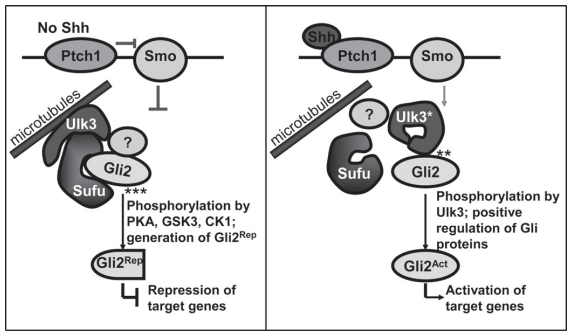

FIGURE 7. **A model of Ulk3 function in Shh signaling pathway.** In the absence of Shh, Sufu forms a complex with Ulk3 that possibly interacts with cytoskeleton through its MIT domain. The complex binds full-length Gli2 through Sufu, protecting it from proteosomal degradation. Ulk3 is not catalytically active but plays a regulatory role. The Ulk3-Sufu complex contributes to C-terminal processing of Gli2 probably through recruiting PKA, glycogen synthase kinase 3β (*GSK3β*), and casein kinase 1 (*CK1*) kinases to full-length Gli2. This results in generation of Gli2^{Rep} that may enter the nucleus and inhibit its target gene expression. In the presence of Shh, Ulk3-Sufu-Gli2 complex dissociates, Ulk3 activates itself by autophosphorylation and phosphorylates full-length Gli2. This contributes to generation of Gli2^{Act}, which translocates to the nucleus and activates its target genes. Other molecule(s) indicated as a question mark (?), for instance, Stk36, may participate in the regulation of Gli2 activity by converting it to transcriptional activator or repressor forms depending on the cellular context and strength of Shh signaling.

We show that ULK3 is able to interact not only with overexpressed, but also with endogenous SUFU protein. Moreover, ULK3 autophosphorylation activity is completely abolished in the complex with SUFU. The rational explanation of this phenomenon is that ULK3 interacts with Sufu through its KD.

In this study we show that the ULK3-Sufu complex is responsive to the SHH signal, which induces its dissociation. An analogous mechanism is described in the case of HSC in *Drosophila* where HSC dissociates under influence of the Hh ligand (31). Taking into account the data showing that catalytically active ULK3 is able to phosphorylate and positively regulate its substrates, GLI proteins (47), we suggest a model for the actions of Ulk3 in the Shh pathway (Fig. 7).

We propose that Ulk3 is part of a Shh signal-dependent cytoplasmic complex regulating the activity and processing of at least the Gli2 protein. In the absence of Shh ligand, the kinase domain of Ulk3 is bound by Sufu, therefore Ulk3 is catalytically inactive and unable to phosphorylate and positively regulate itself and Gli proteins. The complex binds full-length Gli2 through Sufu and induces the generation of the C-terminal-truncated transcriptional repressor Gli2^{Rep}. Through its putative MIT domain (a domain contained within microtubule interacting and trafficking molecules), Ulk3 may be a link between Sufu/Gli2 and the cytoskeleton. In the presence of Shh, the Ulk3-Sufu-Gli2 complex dissociates. Ulk3 is released from the complex and may be able to phosphorylate itself. It may also phosphorylate the full-length Gli2, activating it and promoting its nuclear translocation. Gli2^{Act} activates the transcription of its target genes, for instance *Gli1*. In the case of the activated pathway, Ulk3 plays a kinase activity-dependent positive role.

Our experiments show that the role of Ulk3 in the regulation of the Shh pathway involves eliciting both kinase activity-de-

pendent and -independent effects on Gli transcription factors. The positive regulation is implemented by catalytically active Ulk3, whereas the negative regulation does not depend on Ulk3 kinase activity. That resembles in many aspects the model describing implementation of the fu-sufu-cos2-Ci complex in the *Drosophila* Hh pathway. Taking into account the sequence similarity of fu and Ulk3 proteins and the similarity of their function in *Drosophila* and mammalian Hh pathways, respectively, we suggest that Ulk3 is a structural and functional homologue of fu.

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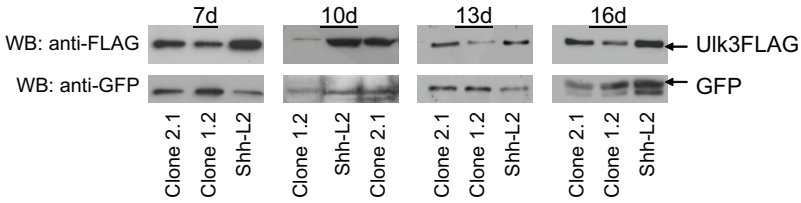
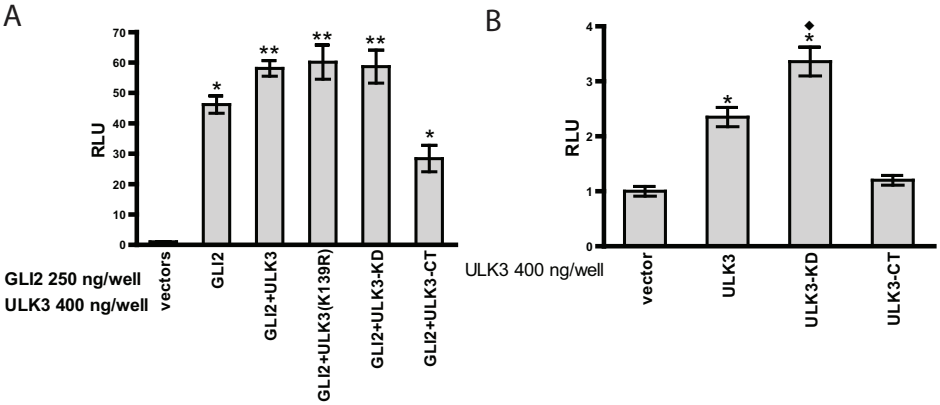
Suppl.Figure 1



Suppl.Fig. 1. WB used for quantification of Ulk3FLAG protein over-expressed in siRNA-expressing clones 1.2 and 2.1, and in Shh-L2 cells. The cells were transfected at different time points during propagation with Ulk3FLAG and GFP encoding constructs. Cell lysates were analyzed with WB using anti-FLAG and anti-GFP antibodies.

Suppl.Figure 2



Suppl.Fig. 2.

A. GLI2 was co-expressed with ULK3, ULK3(K139R), ULK3-KD and ULK3-CT in Shh-L2 cells. Induction of luciferase activity by the empty vector was set as 1. GLI2 induces luciferase activity 46 times above the vector (* - P value < 0.001). ULK3, ULK3(K139R) and ULK3-KD enhances GLI2 transcriptional activity approximately 1.2 times (* - P value < 0.05). ULK3-CT inhibits GLI2 transcriptional activity (* - P value < 0.001). The data are presented as average mean of three replicates obtained from three independent experiments ± SEM.

B. ULK3, ULK-KD and ULK-CT or empty vector were expressed in Shh-L2 cells. ULK3 and ULK3-KD is able to stimulate the luciferase activity (* - P value < 0.001), whereas ULK-KD is more potent activator than ULK3 (♦ - P value < 0.05). ULK3-CT has no effect on Gli-dependent reporter. The data are presented as average mean of three replicates obtained from three independent experiments ± SEM.

Suppl.Figure 3

Suppl.Fig. 3. ULK3-SUFU complex induces the generation of $GLI2^{Rep}$.
FLAG-tagged GLI2 protein was expressed alone, in combination with wt and kinase-deficient His-tagged ULK3 or myc-tagged SUFU in HEK293 cells. WB analysis was performed with 50 µl of cell lysates (left panel) and 5 µl of cell lysates (right panel) using anti-FLAG antibody. Low amount of $GLI2^{Rep}$FLAG protein is detected in the case of co-expression of GLI2 alone or in combination with SUFU. Higher amounts of $GLI2^{Rep}$FLAG protein is detected in the case of co-expression of GLI2 with SUFU combined with ULK3 or ULK3(K139R).

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