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Research Article

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

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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

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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,