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The last 59 amino acids of Smoothened cytoplasmic tail directly bind the protein kinase Fused and negatively regulate the Hedgehog pathway

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Abstract

The Hedgehog (HH) signaling pathway is crucial for the development of many organisms and its inappropriate activation is involved in numerous cancers. HH signal controls the traffic and activity of the seven-pass transmembrane protein Smoothened (SMO), leading to the transcriptional regulation of HH-responsive genes. In *Drosophila*, the intracellular transduction events following SMO activation depend on cytoplasmic multimeric complexes that include the Fused (FU) protein kinase. Here we show that the regulatory domain of FU physically interacts with the last 52 amino acids of SMO and that the two proteins colocalize *in vivo* to vesicles. The deletion of this region of SMO leads to a constitutive activation of SMO, promoting the ectopic transcription of HH target genes. This activation is partially dependent of FU activity. Thus, we identify a novel link between SMO and the cytoplasmic complex(es) and reveal a negative role of the SMO C-terminal region that interacts with FU. We propose that FU could act as a switch, activator in presence of HH signal or inhibitor in absence of HH.
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Introduction

The Hedgehog signaling proteins act as key morphogens during the development of many organisms as diverse as flies and human (for review see Huangfu and Anderson, 2006; McMahon et al., 2003). In most cases, HH proteins control cell proliferation, differentiation, migration and death in a dose-dependent fashion. In human, disruption of this pathway is associated with congenital abnormalities, and its inappropriate activation plays a central role in the initiation and progression of numerous forms of cancer (for review see Lau et al., 2006; Pasca di Magliano and Hebrok, 2003). HH exerts its influence on cells through a signaling cascade that ultimately regulates the expression of target genes that encode other signaling proteins such as Decapentaplegic (DPP)/TGFβ or Wingless (WG)/WNT, transcription factors such as Engrailed (EN) and the HH receptor Patched (PTC). These transcriptional effects are mediated by zinc finger transcription factors of the GLI family (such as Cubitus interruptus (CI) in fly), which have both repressing and activating functions (Alves et al., 1998; Aza et al., 1997; Motzny and Holmgren, 1995; Nguyen et al., 2005). CI itself is found in at least three forms: a full-length form (CI-FL), which is a transcriptional activator, a highly potent and labile activator form (CI-A) and a cleaved form (CI-R), which is a transcriptional repressor. A number of proteins are known to be involved in the control of CI, including the HH receptor PTC (a twelve-pass transmembrane protein) (Nakano et al., 1989), the seven-pass protein Smoothened (SMO) (Alcedo et al., 1996; van den Heuvel and Ingham, 1996), the kinesin-related protein Costal 2 (COS2) (Robbins et al., 1997; Sisson et al., 1997), the

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F-box/WD protein SLIMB (Jiang and Struhl, 1998), a number of protein kinases (such as Fused (FU) (Preat et al., 1990), the protein kinase A (PKA), the casein kinase I (CKI) and the glycogen synthase kinase (GSK3) (for review see Price, 2006)) and the pioneer protein Suppressor of Fused (SU(FU)) (Pham et al., 1995).

In the absence of HH signal, the pathway is silenced at multiple levels (for review see Hooper and Scott, 2005; Lum and Beachy, 2004): (i) PTC inhibits SMO, which is endocytosed and undergoes degradation in the lysosome (Denef et al., 2000; Nakano et al., 2004; Zhu et al., 2003); (ii) CI-FL is sequestered in the cytoplasm by both SU(FU) (Methot and Basler, 2000) and microtubule-bound complexes containing FU and COS2 (Stegman et al., 2000; Wang et al., 2000; Wang and Jiang, 2004), leading to its cleavage into CI-R in a proteasome-dependent fashion (Chen et al., 1999). In contrast, in HH receiving cells, HH binding to PTC alleviates its negative effect on SMO, which becomes hyper-phosphorylated and stabilizes at the plasma membrane (Denef et al., 2000). CI is then released from its cytoplasmic tethering, and its cleavage is inhibited, thus allowing it to transactivate HH target genes. Furthermore, in the cells exposed to the highest levels of HH, both FU and a positive input of COS2 promote formation of the highly potent CI-A form (Alves et al., 1998; Sanchez-Herrero et al., 1996; Wang and Holmgren, 2000).

SMO has several structural and functional characteristics in common with G-protein-coupled receptors (GPCR) (Bockaert et al., 2004; Park et al., 2004), such as the ability to interact with β-arrestin (reported in vertebrates only) (Chen et al., 2004) and probably the capacity to dimerize (Hooper, 2003). Nevertheless, SMO displays some atypical features: it does not directly bind any known ligand, and no heterotrimeric G protein has been decisively implicated in the pathway. SMO activity is closely associated with vesicle trafficking, since its targeting to the plasma membrane is sufficient to activate the pathway and endocytosis from the membrane to the lysosome can shut it down (Denef et al., 2000; Nakano et al., 2004; Zhu et al., 2003). Furthermore, SMO reportedly recruits the COS2/CI/FU cytoplasmic complex, probably via a direct interaction with COS2 (Jia et al., 2003; Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2003), suggesting that SMO could directly affect the activity of this complex.

Another positive key member of the HH pathway is the Ser-Thr protein kinase FU. In embryos, FU activity is necessary for the HH-dependent transcription of *wg* during segment polarity establishment (Preat et al., 1990). In wing imaginal discs, it is required along the A/P boundary for the transcription of *en* (Alves et al., 1998; Sanchez-Herrero et al., 1996) and, to a lesser extent, of *ptc* and *dpp* (Glise et al., 2002; Lefers et al., 2001). In all cases, FU antagonizes the negative effects of SU(FU), thus facilitating the entry of CI-FL into the nucleus and allowing its activation in CI-A (Methot and Basler, 2000; Wang et al., 2000). It is composed of two domains: a N-terminal catalytic domain (called FU-KIN) and a C-terminal regulatory domain (called FU-REG) (Fig. 1). FU itself is phosphorylated in response to HH stimulation (Therond et al., 1996b) and it can phosphorylate COS2 (Nybakken et al., 2002) and probably SU(FU) (Dussillol-Godar et al., 2006; Ho et al., 2005; Lum et al., 2003), both of them interacting with FU-REG (Monnier et al., 1998; Monnier et al., 2002). FU-REG is required for FU activity, but complex genetic interactions with *su(fu)* and *cos2* indicate that it also participates in the down-regulation of the pathway in the absence of HH signal (Alves et al., 1998; Sanchez-Herrero et al., 1996).

Here, we report that FU can interact directly with SMO in a HH-independent manner *in vivo*. Furthermore, a version of

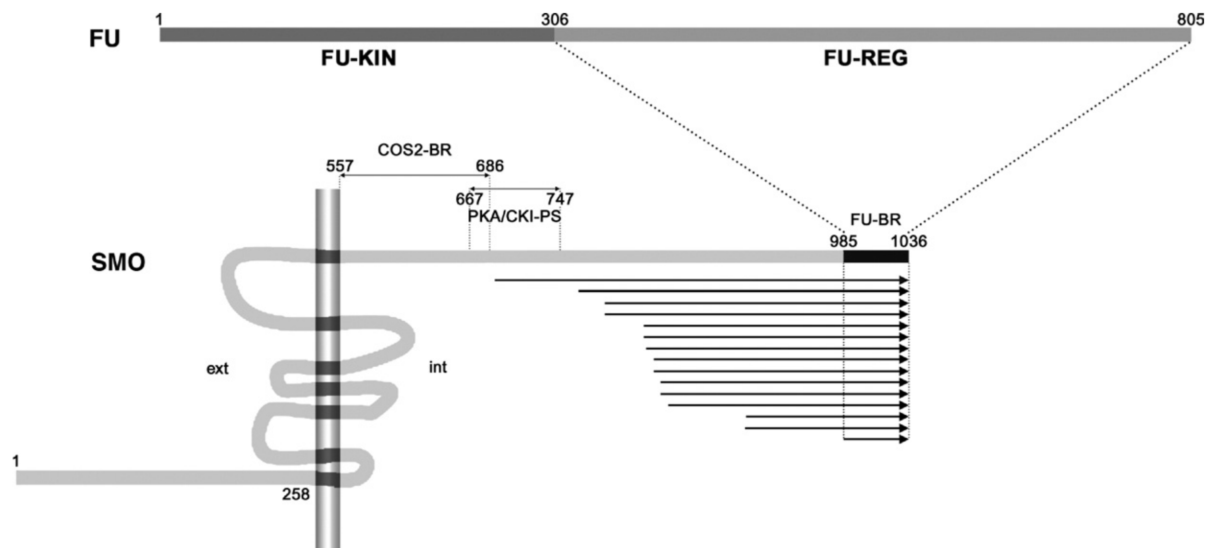

Fig. 1. SMO and FU associate directly. FU is composed of a N-terminal catalytic kinase domain (FU-KIN) followed by a regulatory domain (FU-REG) (aa 306–805, pale grey). A two-hybrid screen with FU-REG as bait led to the identification of 15 different prey clones encoding parts of the SMO C-terminus (horizontal arrows). SMO is a membrane protein with 7 transmembrane domains, an extracellular N-terminus and a cytoplasmic C-terminal tail. The smallest region of SMO that is sufficient for its interaction with FU (FU-binding region or FU-BR) spans amino acids 985 to 1036 and is distinct from the COS2-binding region (COS2-BR, aa 557–686) determined by a two-hybrid assay (Lum et al., 2003), and from a cluster of phosphorylation sites for the PKA and CK1 (PKA/CK1-PS, aa 667–747) that was shown to be involved in SMO activation (Apionishev et al., 2005; Jia et al., 2004; Zhang et al., 2004). ext: Extracellular; int: intracellular.

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