Delineating the Molecular Mechanisms of KIF7 in Vertebrate Hedgehog Signal Transduction.

By

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A thesis submitted in conformity with the requirements for degree of Doctor of Philosophy

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Abstract

The Hedgehog (Hh) signalling pathway plays important roles in embryonic development and for adult tissue homeostasis and regeneration of multi-cellular organisms. Mutations in components of this pathway often result in human developmental disorders and cancer. The primary cilium, a microtubule based extension from the cell surface is required for Hh signalling in vertebrates. Hh ligand binding to the Patched (Ptch1) receptor leads to the ciliary accumulation and activation of the seven transmembrane protein Smoothened (Smo) and changes the localization of several pathway components within primary cilia subdomains. How Smo activation affects the localization of effector proteins in cilia and how this relates to the activation of target genes is unclear. Genetic and biochemical analysis revealed that the kinesin family protein 7 (Kif7) trafficks to the primary cilia upon pathway activation and fulfills dual positive and negative roles during mouse Hh signalling. Through proteomic and biochemical analysis, we aim to better understand the molecular mechanisms underlying the functions of Kif7 in vertebrate Hedgehog signalling. Using a mass spectrometry-based proteomic approach, we identified Liprin-α1 (PPFIA1) and the PP2A phosphatase as Kif7-interacting proteins, and showed that these proteins regulate Kif7 phosphorylation and its activity. We further identified DYRK2 as a
protein kinase that phosphorylates Kif7. Overall, our study identified phosphorylation as a post-translational modification that dictates Kif7 localization within primary cilia and its control of Gli transcriptional activity. Lastly, through biochemical assays we examined the relationship between SuFu and Kif7, two key regulators of the Hh pathway. Collectively, these studies highlight the importance of Kif7 in vertebrate Hh signalling.
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Abbreviations

Hh  Hedgehog
Shh Sonic Hedgehog
Ihh Indian Hedgehog
Dhh Desert Hedgehog
Smo Smoothened
SuFu Suppresor of Fused
Fu Fused
Cos2 Costal-2
Ptch1 Patched1
Kif7  Kinesin family 7
Kif27 Kinesin family 27
PPFIA1 Liprin-α1
PPFIA2 Liprin-α2
PPFIA3 Liprin-α3
PPFIA4 Liprin-α4
LAR  LAR receptor protein tyrosine phosphatase
OA Okadic acid
NT Non-treated
PP2A protein phosphatase 2A
MRM Multiple reaction monitoring
SWATH Sequential window acquisition of all theoretical fragment ion spectra
AP Affinity purification
MS Mass spectrometry
DIA Data-independent acquisition
DDA Data-dependent acquisition
CAMK2 Calcium/calmodulin-dependent protein kinase II
DYRK2 dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 2
DYRK1A dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A
DYRK1B dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1B
Ihog Interference Hedgehog
BOI Brother of Ihog
BOC brother of CDO
CDO CAM-related/downregulated by oncogenes
GAS1 Growth arrested 1
Chapter 1 Introduction

1.1 Hedgehog Signalling Pathway

1.1.1 Overview of Hedgehog signalling

The Hedgehog (Hh) signalling pathway plays important roles in the embryonic development of multi-cellular organisms. In adults, the Hh pathway is required for tissue homeostasis and regeneration but is often abnormally activated in diseases (Beachy et al. 2004). The *Drosophila hedgehog* (*hh*) gene was first discovered from genetic mutations studies in *Drosophila* embryogenesis. It was found to be one of several genes important for body segmentation in flies. The *Drosophila* gene was named after the mutant larval phenotype which resembled a hedgehog (Nusslein-Volhard and Wieschaus 1980). Three vertebrate Hedgehog genes, *Indian Hh* (*Ihh*), *Desert Hh* (*Dhh*) and *Sonic Hh* (*Shh*) were identified years later to have conserved role in body organization. Hh proteins are morphogens that form a concentration gradient to direct tissue patterning. Their general physiological functions are the same but they differ in their pattern of expression. *Ihh* is important for bone and cartilage development, while *Dhh* is important for germ cell development in the testis and peripheral nerve sheath formation. *Shh* is the most studied and best characterized gene of this family. It is important for the specification of neuronal identities along the dorsal-ventral axis in the neural tube, as well as for the proper patterning of limbs (Briscoe and Therond 2013). Disruption of the Hh signalling cascade leads to neural tube development disorders, limb malformations, as well as tumourigenesis (Jacob and Briscoe 2003, Motoyama 2006). For example, the Hh pathway is mutationally activated in almost all basal cell carcinomas (BCC) and in the Shh subtype of medulloblastoma. Recent reports have also shown the importance of the tumour microenvironment and paracrine Hh signalling in promoting tumourigenesis in endodermal tissues (Beachy, Karhadkar et al. 2004, Yauch et al. 2008).
1.1.2 Hedgehog signalling in *Drosophila*

In *Drosophila* embryos, Hh signalling is required for the patterning of several tissues. Misregulation of Hh signalling leads to the duplication of segmental boundaries and mis-patterning of wing discs (Briscoe and Therond 2013, Hartl and Scott 2014). The intracellular signalling events triggered following Hh binding to its receptor leading to target gene activation are integral to the cellular response. The *hh* gene encodes a 45-kDa protein that undergoes autocatalytic cleavage and modification to give a 20-kDa, active N-terminal fragment covalently bound to cholesterol and a palmitoyl adduct (Briscoe and Therond 2013). Post-translational modifications of Hh proteins are necessary for their secretion from cells and for their function as morphogens (Briscoe and Therond 2013). Hh binds to the 12-transmembrane protein Patched (Ptch), and to the single transmembrane co-receptors Interference Hedgehog (Ihog) and Brother of Ihog (Boi) to initiate intracellular signalling (Yao et al. 2006, Camp et al. 2010). In addition, Hh also interacts with glypicans, such as Dally-like to promote signalling but the mechanism of this interaction is unclear (Yan and Lin 2009). In the resting state, Ptch restricts the activity of seven transmembrane protein Smoothened (Smo) by inhibiting its phosphorylation and promoting its endocytosis and degradation. The kinesin-like protein Costal 2 (Cos2) acts as a scaffolding protein in the cytoplasm to recruit PKA, GSK3, and CKI to Cubitus Interuptus (Ci) (155kDa), a zinc finger protein recognized as the main transcriptional effector of the pathway. Recruitment of kinases to Ci leads to the phosphorylation of multiple sites within its carboxy terminus. When highly phosphorylated, Ci is recognized by the E3 ubiquitin ligase Slimb leading to its partial degradation to generate Ci\(^R\) a truncated (75kDa) transcriptional repressor form (Figure 1-1 left). During the activation of the pathway, Hh secreted from producing cells binds to its cognate Ptch receptor on the surface of responding cells. This leads to the phosphorylation of Smo on several serine residues within its carboxy terminal tail that results in conformational changes promoting its membrane localization. This phosphorylation of Smo by PKA, CK1, and GRK2 (Gprk2) kinases enhances its association with Cos2 and thereby leads to its sequestration away from Ci. This change in localization of Cos2 inhibits the processing of Ci by the proteasome and allows Ci to become a transcriptional activator Ci\(^A\) (Figure 1-1 right). Ci\(^A\) is unstable, and is degraded post-transcriptionally by Hh-induced MATH and BTB protein (Hib) a nuclear Cul3 E3 ubiquitin ligase (Zhang et al. 2006, Briscoe and Therond 2013). The cellular integration of an extracellular Hh gradient into an intracellular Ci gradient establishes
distinct cell fates. In *Drosophila*, SuFu negatively regulates signalling by tethering full length Ci in the cytoplasm and participating in Ci<sup>R</sup> formation. In contrast, the serine/threonine kinase Fused (Fu) is important for high level of Hh signalling and acts to antagonize SuFu (Methot and Basler 2000, Lefers et al. 2001, Ruel et al. 2007, Aikin et al. 2008).

**Figure 1-1. *Drosophila* Hh signal transduction.** In *Drosophila*, the transmembrane proteins Interference Hedgehog (Ihog) and Brother of Ihog (Boi) promote Hedgehog (Hh)–Patched (Ptc) binding. Hh also interacts with glypicans. Ligand-free Ptc represses Smo by triggering its rapid degradation and/or its confinement to an intracellular compartment (left panel, ‘off’). Furthermore, in the absence of Hh, Costal 2 (Cos2), Fused (Fu), Suppressor of Fu (SuFu) and Cubitus interruptus (Ci) form a complex in association with microtubules. This complex promotes, through the activity of protein kinase A (PKA), casein kinase 1α (Ck1α) and glycogen synthase kinase 3β (GSK3β), the formation of the Ci repressor form (Ci<sup>R</sup>). Binding of Hh to Ptc relieves Smo repression. Smo translocates to the membrane and is activated by phosphorylation on its carboxy terminal tail, by PKA, Ck1α and G protein-coupled receptor kinase 2 (Gprk2), which induces a conformational change compatible with plasma membrane localization. Smo phosphorylation also promotes its association with the Cos2-Fu-SuFu protein complex and results in the sequential activation of Fu and Cos2, which leads to activation of full length Ci<sup>A</sup>. Adapted from Briscoe & Thérond 2013 Rev Mol Cell Biol.
1.1.3 Hedgehog signalling in vertebrates

Of the three Hh vertebrate genes, *Shh* is the most broadly expressed and best studied. Deletion of *Shh* leads to defects in neural tube and limb patterning (Chiang et al. 1996, Litingtung et al. 1998, Pepicelli et al. 1998). One important difference for vertebrate Hh signalling is the requirement of primary cilia. The primary cilium is a microtubule based non-motile protrusion from the cell membrane. The structure and roles of primary cilia in Hh signalling will be explained in more details in the next section. In the absence of Hh ligand, the pathway is inactive and the receptor Ptch1 localizes within primary cilia where it actively represses the accumulation of Smo (Rohatgi et al. 2007). Upon Hh binding to Ptch1 on the receiving cells, Ptch1 is activated and de-represses Smo. This de-repression results in Smo translocation into the primary cilium hereby initiating a signal transduction cascade that leads to the activation of Gli family of transcription factors, the homolog of *Drosophila* Ci (Corbit et al. 2005). In addition to Ptch1, Fibronectin type III and immunoglobulin repeat-containing surface proteins BOC and CDO (*Drosophila* homologues of iHog and Cdo, respectively), and the GPI-linked cell surface protein Gas1 are Hh co-receptors that act together to bind Hh on receiving cells (Briscoe and Therond 2013). There are three members in the Gli family proteins in vertebrates: Gli1, Gli2 and Gli3. Gli1 functions primarily as a transcriptional activator (Aza-Blanc et al. 2000, Bai and Joyner 2001). Since *Gli1* is also a universal target gene of the pathway, it functions in a positive feedback loop amplifying the transcriptional response. In contrasts, Gli2 and Gli3 exist in two forms: a full-length (190kDa) activator (GliA) and a truncated (85kDa) transcriptional repressor (GliR) that lacks the C-terminal activation domain. In most contexts, Gli2 is the main transcriptional activator and Gli3 is the main transcriptional repressor (Wang et al. 2000). The repressor activity of Gli3 is especially important in embryonic patterning of the neural tube and limbs (Jiang and Hui 2008). In the development of limb buds, Gli3R exhibits an anterior-posterior gradient inversely proportional to Shh levels, which suggests that regulation of Gli3 processing into Gli3R is a direct readout of dose-dependent signalling by Shh. In the neural tube, Gli3 is also critical to establish a double Gli gradient with opposite polarities of activators (high to low from ventral to dorsal) and repressors (high to low from dorsal to ventral) to regulate sets of Shh responsive genes important for the specification of individual neural subtypes (Jiang and Hui 2008, Briscoe and Therond 2013). Overall, three vertebrate Gli transcription factors act
together in responding cells to integrate Hh signalling inputs, resulting in the regulation of tissue pattern, size and shape (Ruiz i Altaba et al. 2007, Wang et al. 2007, Hui and Angers 2011). The exact mechanism of how Smo leads to inhibition of Gli processing and promotes their activation remains poorly understood. Recent studies have shown that during pathway activation, Smo triggers the dissociation of SuFu from Gli in the primary cilia (Humke et al. 2010, Tukachinsky et al. 2010). The dissociation of SuFu from Gli proteins prevents the formation of GliR. Additionally, SuFu also plays important roles in the absence of Hh ligand when the pathway is in its resting state. SuFu restricts Gli3 in the cytoplasm and promotes its processing by multiple kinases (PKA, CKI, and GSK3β) (Humke, Dorn et al. 2010). Extensive sequential phosphorylation of serines by these kinases on the C-terminus of Gli3 is recognized and bound by the E3 ubiquitin ligase component beta-TrCP. The binding of beta-TrCP leads to ubiquitination of Gli3. Similar to Ci, Gli3 is not fully degraded but instead is specifically processed and cleaved by the proteasome. The mechanism of partial proteolysis is poorly understood, but results in the degradation of the C-terminal activation domain to form a potent transcriptional repressor (Wang, Fallon et al. 2000, Pan and Wang 2007). Activation of the Hh pathway inhibits beta-TrCP binding, thus preventing ubiquitination and proteolysis of the full length Gli3 protein (Tempe et al. 2006, Wang and Li 2006). The GliA translocates to the nucleus and activates the transcription of several target genes including Gli1, for positive feedback or Ptch1 for negative feedback signalling events. Another target gene Hhip (Hh-interacting protein), is a transmembrane protein that functions within a negative regulatory feedback loop to attenuate signalling by binding and restricting the diffusion of Hh proteins (Chuang and McMahon 1999). When GliA is in the nucleus it is not stable and subjected to ubiquitin-mediated degradation by the proteasome through SPOP, a subunit of an E3-ubiquitin ligase and mammalian homolog of Drosophila hib (Figure 1-2) (Zhang, Zhang et al. 2006, Chen et al. 2009).

1.1.4 Evolutionary differences in Hh signalling

Although the requirement for Hh signalling is conserved in all multicellular organisms, the molecular mechanisms underlying the intracellular signalling pathways differ between Drosophila and mice (Aikin, Ayers et al. 2008). Main differences lie in the functions of Smo, Fu and SuFu. The differences in signalling might in part be attributed to the specific requirement of
primary cilia for Hh signalling in vertebrates. Firstly, unlike dSmo (*Drosophila* Smoothened), which is phosphorylated by PKA and CK1, vertebrate Smo does not have conserved PKA and CKI phosphorylation sites. Rather, vertebrate Smo is phosphorylated by G-protein-coupled receptor kinase 2 (GRK2 or Gprk2) (Chen et al. 2004). Furthermore the small molecule antagonist cyclopamine inhibits vertebrate Smo activity but is ineffective in blocking dSmo (Chen et al. 2002). This suggests important differences between the mechanism of action of *Drosophila* and vertebrate Smo. Secondly, the kinase Fu, which is an important negative regulator of Hh signalling in flies, is dispensable for vertebrate Hh signalling. Knockout of *Stk36*, the mouse homolog of Fu, exhibits no Hh signalling defects (Chen et al. 2005, Merchant et al. 2005). However, careful examination of the *Stk36*–/– animals unveiled that Fu is important for motile cilia formation (Wilson et al. 2009). Lastly, whereas SuFu is dispensable for *Drosophila* Hh signalling (Cooper et al. 2005), its function is required for mouse Hh signalling. Loss of SuFu in vertebrates leads to ectopic activation of the pathway and to strong Hh defects similar to phenotypes observed in Ptc1 deficient animals. Overall, in *Drosophila* the complex of Fu-Cos2 plays important role in regulating Ci processing, whereas in mice it is the complex of SuFu-Kif7 that regulates Gli processing and activity. Although the functional relationship between SuFu and Kif7 remains poorly understood, current evidence suggests that Kif7 is important for Gli<sup>R</sup> formation under resting conditions and functions to antagonize SuFu during pathway activation. Kif7 functions will be discussed in more details in later sections.
Figure 1-2. Schematic representations of vertebrate Hh signal transduction. In the pathway OFF state, without ligand binding to Ptch1 receptor, Ptch1 inhibits the ciliary accumulation of Smo, this results in cytoplasmic retention of Gli proteins by SuFu and sequential phosphorylation of Glis by multiple kinases. Phosphorylated Glis are then partially degraded by the proteasome forming Gli\textsuperscript{R} that translocates to the nucleus and represses Hh-dependent transcription. In the pathway ON state, Shh ligand binds to Ptch1, which releases its inhibition on Smo, allowing Smo to accumulate in cilia. Pathway activation also leads to accumulation of Kif7, SuFu and Gli at ciliary tips. However, during pathway activation Gli dissociates from SuFu within the cilia, becoming Gli\textsuperscript{A} and translocates to the nucleus to activate Hh-dependent target genes. Nuclear Glis are eventually degraded by the proteasome through Spop-mediated ubiquitination.
1.1.5 Hedgehog signalling in zebrafish

In zebrafish, Hh signalling is best characterized in the context of neural tube and muscle development (Stickney et al. 2000, Ochi and Westerfield 2007). During myotome development, Shh signalling is required to specify the fate of individual cells in the myotome. High level of Shh signalling is required to form muscle pioneer cells, a slow twitch muscle cell type, while lower concentrations of Shh specify the non-pioneer slow twitch muscle fibers. Defects in Shh signalling during myotome development lead to characteristic U-shaped somites instead of the normal chevron-shaped somites. Somites are segmented mesodermal muscle and bone precursors boundaries. Defects in somite angle and shape are an indication of defect in muscle pioneer migration and consequently developmental delays. These phenotypes are observed in zebrafish loss of function mutant of \textit{shh}, \textit{smo}, \textit{gli2}, and \textit{ptch1} (van Eeden et al. 1996, Karlstrom et al. 1999, Barresi et al. 2000, Kawakami et al. 2005, Woods and Talbot 2005, Hollway et al. 2006). In term of signal transduction, zebrafish Hh signalling shares similarities with both \textit{Drosophila} and mouse Hh signalling, with the expectation that there are five Hh genes: \textit{Shh A}, \textit{Shh B}, \textit{Ihh A}, \textit{Ihh B}, \textit{Dhh} in fish. The basic pathway components and mechanisms of pathway activation are also conserved in zebrafish, from ligand binding to Ptch receptor to Smo translocation in cilia and activation of Gli proteins. Zebrafish Shh signalling therefore requires primary cilia and SuFu (also required in mouse), as well as Fu (required in \textit{Drosophila}) for maximum signalling (Wolff et al. 2003). Despite core components of Hh signalling being conserved in zebrafish, differences between fish and mouse have been observed. For example Gli1 rather than Gli2 is the principal activator of Hh signalling in early zebrafish embryos (Karlstrom et al. 2003). Hence \textit{gli1} is not only expressed as a function of pathway activation as it is observed in other cells from other vertebrate animals (Karlstrom, Tyurina et al. 2003, Ninkovic et al. 2008). A recent genetic study has also revealed important functions of Kif7 in zebrafish Hh signalling (Maurya et al. 2013). This will be discussed in details in later sections.

1.1.6 Hedgehog signalling and developmental disorders

Loss of Hh signalling activity during vertebrate embryogenesis leads to multiple developmental disorders including holoprosencephaly (HPE), polydactyly, craniofacial defects, and skeletal malformations (Muenke and Beachy 2000, McMahon et al. 2003, Zhang et al. 2006, Sweeney et
In HPE, mutations have been found in SHH (Nanni et al. 1999), PTCH1 (Ming et al. 2002), and GLI2 (Roessler et al. 2003). For example patients with SHH loss of function mutations exhibit abnormal development of the forebrain. HPE included phenotypes ranging from pituitary abnormalities, polydactyly, craniofacial defects to skeletal malformations. Furthermore, mutations in GLI3 have been found in disorders associated with congenital malformations such as Greig cephalopolysyndactyly, Pallister–Hall syndrome, postaxial polydactyly type-A, autosomal dominant preaxial polydactyly type-IV, and acrocallosal syndrome (Nieuwenhuis and Hui 2005). All these patients exhibit a common phenotype of digit malformation. Lastly, Gorlin syndrome also known as Nevoid basal cell carcinoma syndrome (NBCC) is an autosomal dominant disorder with germline mutation in PTCH1. Patients carrying this mutation are pre-disposed to develop basal cell carcinoma (BCC), other tumours, as well as congenital anomalies of the brain and skeletal system (Johnson et al. 1996, Wicking and Bale 1997, Ming et al. 1998). These developmental disorders further highlight the importance of Hh signalling for embryonic development and adult tissue homeostasis.

1.1.7 Hedgehog signalling and cancer

Inappropriate activation of Hh pathway can lead to various cancers. Hh signalling related cancers can be divided between cancers with mutations in pathway components and cancers with deregulated Hh signalling through paracrine/autocrine mechanisms. Firstly, mutations within Hh pathway components that result in cell autonomous ligand-independent activation of the pathway have been found in basal cell carcinomas (BCC), Shh-subtype of medulloblastomas, and rhabdomyosarcomas (Gailani et al. 1996, Goodrich et al. 1997, Xie et al. 1998, Kappler et al. 2004, Yang et al. 2008). In BCC, 90% of patients have loss-of-function mutation in PTCH1 (Unden et al. 1996) or gain-of-function mutation in SMO (Xie, Murone et al. 1998), whereas in medulloblastomas, mutations in PTCH1 (Raffel et al. 1997), SMO (Yauch et al. 2009), and SUFU (Taylor et al. 2002) have been identified. In all cases, mutations result in hyperactivation of the pathway and uncontrolled proliferation of progenitor cells (Petrova and Joyner 2014). However, in cancers with increased Hh signalling (autocrine or paracrine) there is ligand-dependent deregulation of Hh pathway activity. This has been observed in various cancers such as lung, stomach, esophagus, pancreas, prostate, breast, liver and brain (Berman et al. 2003, Thayer et al. 2003, Watkins et al. 2003, Karhadkar et al. 2004, Kubo et al. 2004,
Sicklick et al. 2006, Clement et al. 2007). Several of these tumours have elevated expression of Hh ligands and/or ectopic Glis expression without apparent mutation within pathway components. Dysregulation in autocrine/paracrine Hh signalling that contributes to tumour growth can be suppressed by pathway inhibitors. Paracrine Hh signalling appears to play an especially important role in endodermal cancers. In this setting, tumour cells activate Hh signalling in the surrounding stroma, resulting in the expression of soluble factors and extracellular matrix components that, in turns, act upon the tumour epithelium to promote tumour growth (Yang, Ellis et al. 2008, Theunissen and de Sauvage 2009). Understanding Hh secretion and activation in a given tumour microenvironment is critical for the rational design of new therapies. Overall, Hh signalling plays important role in adult life and misregulation of the pathway can give rise to tumours.

1.1.8 Small molecules regulating Hedgehog signalling

Given the implication of Hh pathway in human diseases such as cancer, there has been vast interest in identifying small molecule modulators of the pathway. The first compounds identified to regulate the Hh pathway were the naturally occurring alkaloids, cyclopamine and jervine extracted from the plant *Veratrum californicum*. These compounds function by inhibiting the activity of Smo, but they have poor pharmacokinetics properties (Taipale et al. 2000, Chen et al. 2002). Further screening campaigns to identify Smo inhibitors identified several classes of antagonists, including cyclopamine analogs as well as other inhibitors that bind to different Smo binding sites. The structural similarity between cyclopamine and sterols led to the further findings that sterols like oxysterols can modulate Smo activity (Nachtergaele et al. 2012, Myers et al. 2013, Nachtergaele et al. 2013, Nedelcu et al. 2013). Currently, only one Smo inhibitor GDC-449/Vismodgib is FDA approved for the treatment of advanced BCC (Von Hoff et al. 2009, Sekulic et al. 2012). Vismodegib and other Smo inhibitors are still in different clinical trials for the treatments of other Hh-related cancers. However, resistance to Smo inhibitors rapidly arises (Yauch, Dijkgraaf et al. 2009) and efforts to develop Smo inhibitors with different mode of actions or drugs targeting downstream pathway components are underway. GANT-58 and GANT-61 for example have shown to be inhibitors of Gli activity in cell-based assays (Lauth et al. 2007).
1.2 Primary cilium

1.2.1 General introduction
The primary cilium is a microtubule-based protrusion emanating from the plasma membrane of most eukaryotic cells during interphase. There are two types of cilia, motile and non-motile cilia. Motile cilia are better studied, they are important for fluid flow, mechanosensing and motility. Non-motile cilia, on the other hand, are less characterized. Non-motile cilia were first observed by Zimmerman in 1898 (Zimmermann 1898), and was later termed primary cilia in 1968 by Sorokin (Sorokin 1968) because the primary cilium was the first "cilium" to form in a cell. For the longest time, primary cilium was believed to be vestigial organelle with no important biological roles (Garcia-Gonzalo and Reiter 2012). However, research from the past 10 years has highlighted the importance of primary cilia for cell signalling, cell cycle entry, migration and differentiation during development. Specifically, they are important for chemosensing in olfactory neurons, photo-sensing in rods and cones, and mechanosensing in renal epithelia. Defects in cilia proteins or cilia structure contribute to ciliopathies, a group of genetic disorders with multi-organ defects (Quinlan et al. 2008, Hildebrandt et al. 2011). How such a small organelle came to be so important for signalling and development is only starting to be understood.

1.2.2 Primary cilium structure
Primary cilium originates from the basal body derived from the mother centriole, and is enclosed in ciliary membrane, a bilayer lipid membrane that is continuous with the plasma membrane of the cell. Ciliogenesis starts with the docking of basal body to the plasma membrane. Vesicles containing structural proteins dock at the basal body and move toward the distal tip of the cilium where axoneme elongation occurs. The axoneme is a microtubule-based cytoskeletal structure that is enclosed by the ciliary membrane. Within cilia, microtubules orient with their plus-ends pointing in the direction of the cilium tip. The basal body anchors the minus ends of microtubules and connects the axoneme to the rest of the cell (Kozminski et al. 1993, Scholey 2008). A mature cilium is made of 9 pairs of peripheral microtubules without a central pair, forming a 9+0 axoneme structure. This is different from motile cilium that are of 9+2 in structure, with a central pair of microtubule. Structurally, the primary cilium is comprised of 2 large intraflagellar transport complexes, (IFT) A and B. IFT proteins transport cargos along the
axoneme. Heterotrimeric microtubule motor kinesin II (KIF3) family of proteins facilitates anterograde (toward the tip) transport in association with IFT-B, whereas dynein mediates retrograde transport (toward the basal body) with IFT-A. Disruption of kinesin II eliminates the primary cilium, resulting in diverse developmental and cell signalling defects (Huangfu and Anderson 2005, Liu et al. 2005, Houde et al. 2006, Ocbina and Anderson 2008). Mutations in proteins implicated in retrograde transport such as dynein result in abnormal stumpy cilia with ciliary proteins accumulating at the tip of the cilium including proteins involved in Hedgehog signalling (Huangfu and Anderson 2005, May et al. 2005). This indicates that Hh pathway components are dynamically transported in primary cilia. Recent work has further identified a compartment near the base of the cilium, termed the transition zone (TZ) as an important region for ciliary gating and signalling. TZ is the region between the basal body and the axoneme, it is composed of transition fibers, ciliary necklace, and Y-fibers (Figure 1-3). The TZ houses multiple protein complexes that are implicated in ciliopathies, such as the Meckel–Nephronophthisis-Joubert syndrome complexes (Garcia-Gonzalo et al. 2011, Novarino et al. 2011, Chih et al. 2012, Czarnecki and Shah 2012). Lastly, the ciliary tip is a specialized segment of membrane that extends from the axoneme that appears to have specific signalling roles, in particular in Hh signalling.

**Figure 1-3. Schematic representation of the primary cilium.** Modified from Reiter, Blacque, Leroux EMBO reports 2012.
1.2.3 Primary cilium and signalling

In 2003, Kathryn Anderson’s lab published results of a forward genetic screen in mouse that identified several mutations within cilia genes leading to phenotypes consistent with defective Hh signalling (Huangfu et al. 2003). This was the first study to implicate primary cilia in Hh signalling. Four mutants, wimple (\textit{Ift172}), Polaris (\textit{Ift88}), \textit{Kif3A}, and \textit{Dhc2} were identified with characteristics of Shh signalling defects (neural tube and limb defects and changes in Gli transcriptional activity) and impairment in the formation of primary cilia (Huangfu, Liu et al. 2003). This study led to the realization that primary cilia could be important for vertebrate Hh signalling. Further studies showed that several Hh pathway components including Ptch1, Smo, SuFu, Kif7, Gli2 and Gli3 localize or accumulate in and out of cilia during signalling and that this is important for the control of pathway activity. Additional research established that Ptch1 localizes to cilia in the absence of Hh ligand and moves out of cilia after ligand binding (Rohatgi, Milenkovic et al. 2007). In contrast, upon pathway activation, Smo, SuFu, Kif7, and Glis are enriched in the cilium. A recent study has also demonstrated that the balance between anterograde and retrograde movement controls the overall ciliary architecture, which in turn controls proper Shh signalling (Ocbina et al. 2011). However, the exact role of the primary cilium and ciliary proteins in Gli processing and Hh signal transduction is still unclear but current data suggests that cilia act as a signalling centre where Hh proteins concentrate and interact to integrate the downstream signalling response. Since the publication of the landmark paper from the Anderson lab, other signalling pathways have been found to involve cilia such as Notch and Wnt signalling (Ross et al. 2005, Gerdes et al. 2007, Corbit et al. 2008, Ferrante et al. 2009), and platelet-derived growth factor signalling (Schneider et al. 2005). GPCRs such as Rhodopsin have long been known to localize in cilia of photoreceptors. Other GPCRs like, Somatostatin receptor 3 (SSTR3), melanin-concentrating hormone receptor 1(Mchr1), and serotonin subtype 6 receptor (5HTR6) localize to the cilia in neurons (Handel et al. 1999, Brailov et al. 2000, Berbari et al. 2008). These GPCRs play a role in hormonal regulation and feeding behaviors. Defects in cilia structure results in mis-localization and function of these proteins which often leads to obesity related disorders (Hildebrandt, Benzing et al. 2011).
Recently, orphan GPCR, Gpr161 was shown to localize in cilia and to play roles in Hh signalling (Mukhopadhyay et al. 2013). Similarly, Gpr39, was identified in a screen for chemical inhibitors of the Hh pathway, and was found to act downstream of Smo to inhibit Gli activity (Bassilana et al. 2014). Overall, primary cilia are essential for proper Hh signalling in vertebrates.

1.2.4 Protein trafficking in cilia

Given that there is no protein synthesis within the cilia, all ciliary proteins are translocated to the cilia. The transition zone functions in part as a gate for recognition of ciliary proteins. In general, there are three ways proteins can enter the cilia. First, small proteins (<60kD) can enter through diffusion, whereas larger proteins are kept out because of size limitation (Kee et al. 2012). The ciliary diffusion barrier was found to behave like a molecular sieve that regulates entry of proteins based on size. Second, membrane protein can enter the cilia through lateral diffusion from plasma membrane to ciliary membrane. Smo was found to enter the cilia in part through lateral diffusion (Milenkovic et al. 2009). Third, larger protein with the correct ciliary localization sequence (CLS) traffic into the cilia via transporter proteins such as IFTs. IFT complexes with the cargo destined for the cilium localize at the base of the cilium where they connect with ciliary motor proteins. Only cargos with CLS are targeted into the cilia. Although no universal CLS exists, few common CLS have been identified. For example polycystin-2, a ciliary protein mutated in polycystic kidney disease (Geng et al. 2006), has an amino terminal RVxP motif whereas the GPCR Rhodopsin (Tam et al. 2000) has a c-terminal VxPx sequence that were characterized as CLS. Other GPCRs (SSTR3, 5HTR6, and Mchr1) localizing to cilia in neurons have AxS/AxQ motifs that is required for ciliary trafficking (Berbari, Johnson et al. 2008). The recently identified orphan GPCR GPR161 also localizes to cilia and a CLS within its third intracellular loop, (I/V)KARK, that was previously identified in other GPCRs was found to be important (Berbari, Johnson et al. 2008). Smo on the other hand has a conserved FR motif in the c-terminal cytoplasmic region that was characterized as a CLS (Corbit, Aanstad et al. 2005). Similarly, FR motifs have also been reported to mediate cilia localization of other GPCRs (Dwyer et al. 2001, Corbit, Aanstad et al. 2005). Lastly, nuclear localization motifs have also been recognized as another class of ciliary localization signal in other contexts (Dishinger et al.
Kinesin-17 (Kif17) for instance requires the classical nuclear localization signal KRKK for ciliary localization. When mutated within this sequence, Kif17 fails to localize in the cilia. Interestingly, a Ran-GTP gradient known to control nuclear import similarly acts in a gating mechanism for ciliary proteins (Kee, Dishinger et al. 2012). Several distinct mechanisms have thus been uncovered to direct localization of proteins to cilia.

1.2.5 Ciliopathies

Ciliopathies comprise a group of polygenic disorders that result from defects in cilia ontogeny or function. Examples of ciliopathies include Bardet-Biedl syndrome (BBS), Meckel-Gruber syndrome (MKS), Joubert syndrome (JBTS) and Ellis-van Creveld syndrome (Evc). BBS is characterized by obesity, retino-degeneration, renal abnormalities, polydactyly and cognitive impairment. In BBS, patients have mutations within genes coding for proteins belonging to a protein complex called the BBSome. To date, 12 BBSome proteins (BBS1-12) have been identified. Some BBS (e.g. BBS1 and BBS3) proteins localize to the basal body and are implicated in trafficking cargo to the ciliary base (Su et al. 2014). In MKS, patients exhibit occipital encephalocele, kidney cysts and polydactyly. MKS is considered one of the more severe ciliopathies and involves genes implicated in TZ function and ciliogenesis. In JBTS, patients exhibit cerebellar and brainstem malformations, ataxia, cognitive impairment, renal and retinal symptoms (Hildebrandt, Benzing et al. 2011). Mutations in KIF7 have been identified in JBTS patients (Dafinger et al. 2011). Lastly, Evc patients have mutation in EVC/EVC2 proteins. Evc proteins localize in a region distal to the TZ, termed the Evc-zone. Evc proteins are positive regulator of Hh signalling, they are required for proper Smo localization in the Evc zone. Mutated forms of EVC/EVC2 found in patients act as dominant negative proteins, disrupt normal Hh signalling and consequently lead to a subset of Hh-dependent ciliopathy phenotypes, most prominently skeletal abnormalities (Dorn et al. 2012, Yang et al. 2012, Caparros-Martin et al. 2013). Overall, ciliopathies exhibit extensive allelism and pleiotropy. Different recessive mutations in many different ciliopathy genes cause a wide spectrum of phenotypes, from renal cysts, retinal degeneration, and mental retardation to polydactyly. Severity of ciliopathy depends on whether ciliogenesis is affected or not. Disease is more severe if cilia architecture is impaired. Defects in cilia indirectly affects Hh signalling, hence, a subset of disease phenotypes
are due to defective Hh signalling. More on Kif7 and ciliopathy will be discussed in later sections.

### 1.2.6 Kinesins and cilia

Kinesin motor proteins such as Kif7, Kif3, Kif17, and Kif19 have been found to contribute to cilia structure and trafficking. Kinesins in general and Kif7 more specifically will be covered extensively in the next section. First, Kif3 is part of the kinesin-2 family, are heterotrimeric kinesin comprised of Kif3A, Kif3B, and KAP3. Kif3s are the only known kinesins to heterodimerize to form a functioning complex. Kif3A and B have NH2-terminal motor domain and form a complex with the non-motor protein KAP3. Kif3 functions with IFT-B complex in anterograde trafficking in the primary cilia (Reiter et al. 2012, Bhogaraju et al. 2013). Kif17 is also part of the kinesin-2 family, but it is a homodimeric motor protein. It was first found to be involved in the transport of cargos (cyclic nucleotide-gated channels, NMDA receptor subunit 2B) in the cilia of sensory neurons (Jenkins et al. 2006). Kif17 has further been shown to function as a ciliary motor in zebrafish photoreceptors and mammalian olfactory sensory neurons (Jenkins, Hurd et al. 2006, Insinna et al. 2008, Insinna et al. 2009). Kif17 is not needed for ciliogenesis or cilia structure maintenance. Lastly, Kif19, which is part of the kinesin-8 family, was recently showed to localize to the tips of cilia and function as a microtubule depolymerizing kinesin involved in ciliary length control (Niwa et al. 2012). Overall, it has long been recognized that kinesins play important roles in cellular function, however their roles in primary cilia is just starting to be understood. More research is needed to examine if other kinesins localize and/or contribute to primary cilia functions.

### 1.3 KIF7

#### 1.3.1 Kinesins, classification and overall function

The Kinesin (KIF) family of motor proteins is an essential part of cellular transport. In mammals, there are more than 40 KIF genes (Miki et al. 2005, Hirokawa et al. 2009) categorized in 15 families based on sequence alignment (Miki, Okada et al. 2005). Structurally, kinesins typically have a globular kinesin motor/head domain for ATP-dependent hydrolysis and microtubule binding, a neck/stalk region that allows for flexibility of the motor domain and plays a role in directionality. Lastly, the tail region is composed of 2 coiled-coil helices where
cargos typically bind. The motor domain is highly conserved within each kinesin family, whereas the neck and tail regions are highly diverse, likely to accommodate various cargos in several intracellular processes. Kinesins convert chemical energy to mechanical work by coupling ATP binding and hydrolysis to conformational changes in order to transport cargo along microtubules (Hirokawa, Noda et al. 2009). Many kinesins function as homo-dimer, with the motor domain serving as the site of homo-dimerization, or heterodimerization as in the case of Kif3 (Kif3A dimerizes with Kif3B). Most Kinesins have their motor domain at the N-terminus and have microtubule plus end directed motility (e.g. Kif3, Kif7). However some kinesins, such as c-kinesins, Kif1C, have motor domain on the C-terminus and moves toward the microtubule minus end. Furthermore, kinesins are phospho-proteins, where phosphorylation dictates the ability of kinesin to associate or dissociate with their cargos or regulate their binding to microtubules (Hirokawa, Noda et al. 2009). For example Kif17, which localizes in primary cilia, is phosphorylated by Calcium/calmodulin-dependent protein kinase II (CaMKII). In this case, phosphorylation disrupts the binding of Kif17 from an adaptor protein leading to release of cargo vesicles (Guillaud et al. 2008). Recently, Liang et al showed that in Chlamydomonas, the KIF3B homolog FLA8 is phosphorylated on the tail domain at Ser\textsuperscript{663} by CaMKII. This phosphorylation inhibits KIF3B binding to its cargo IFT-B, and prevents the unloading of IFT-B at the tips of the cilia (Liang et al. 2014). These two studies highlight the importance of post-translational modification for kinesins activity and for their function within cilia.

1.3.2 Costal-2, the Drosophila homolog of Kif7

Costal2 (Cos2), the Drosophila homolog of Kif7 plays multiple roles in Drosophila Hh signalling (Robbins et al. 1997, Sisson et al. 1997, Lum et al. 2003). Cos2 mutants are zygotic lethal and they display a similar cuticle phenotype as ptc homozygous embryos. They also exhibit expanded transcription domains of Hh target genes, and mirror image pattern duplications in the wing (Grau and Simpson 1987). Genetic studies suggest Cos2 is a negative regulator of the pathway, however, Cos2 has both positive and negative functions in the pathway to regulate Ci\textsuperscript{R} and Ci\textsuperscript{A} activity (Lum, Zhang et al. 2003, Zhou and Kalderon 2010). In the absence of Hh ligands when the signalling pathway is in the resting state, Cos2 serves as a scaffolding protein anchoring Ci and the proteins kinases PKA and CK1. Cos2 thereby promotes Ci phosphorylation and this leads to increase Ci proteolytic processing into Ci\textsuperscript{R} and repression
of target genes. In this context Cos2 serves a negative regulatory role (Lum and Beachy 2004). In the presence of Hh ligands however, Cos2 is phosphorylated by Fu (on Ser\(^{572}\) & Ser\(^{931}\)) and this changes Cos2 distribution from the cytoplasm to the plasma membrane, where it associates with Smo (Ranieri et al. 2012). This association with Smo sequesters Cos2 away from Ci and promotes the formation of Ci\(^A\), its translocation to the nucleus and transcriptional activation of target genes. Therefore, during Hh pathway activation, Cos2 plays a positive regulatory role. Overall, in Drosophila, Cos2 performs dual negative and positive functions dependent on the state of pathway activation.

1.3.3 Kif27, the vertebrate paralog of Kif7

In vertebrates, there are two orthologs of Cos2, Kif7 and Kif27, and both belong to the kinesin-4 family of motor proteins. Kif7 and Kif27 share 43.6% of sequence homology (Katoh and Katoh 2004). In vertebrates, Kif27 was shown to be dispensable for Hh signalling or primary cilia function. In contrast, Kif27 was found to mediate important roles in motile cilia formation. Kif27 is involved with STK36, the vertebrate homolog of Drosophila Fu, to assemble the central pair of microtubules in motile cilia. Similar to Kif27, Stk36 is also dispensable for mammalian Hh signalling (Chen, Gao et al. 2005, Merchant, Evangelista et al. 2005, Wilson, Nguyen et al. 2009).

1.3.4 Vertebrate Kif7

1.3.4.1 Kif7 structure and motor activity

Structurally, Kif7 has a motor domain at its N-terminus that exhibits nucleotide and microtubule binding functions. Kif7 also has a neck region that is thought to be the site mediating homodimerization, and a C-terminal tail domain (He et al. 2014) (Figure 1-4). Like most kinesins, the motor domain of Kif7 exhibits the highest sequence homology with the other kinesins (Klejnot and Kozielski 2012). Studies have shown that Kif7 requires its motor domain for function (Liem et al. 2009, He, Subramanian et al. 2014). Forward genetic screens identified mouse embryos with mutation in the Kif7 motor domain (L130P)(motor dead mutant) that exhibited similar phenotypes as Kif7 knock-out mice. In addition, the motor dead Kif7 mutant failed to accumulate at the tips of cilia upon pathway activation, and instead accumulated in the
TZ. This suggests that the motor domain is needed for Kif7 trafficking to the distal tip compartments of the cilia. Despite the functional importance of the motor domain, Kif7 does not exhibit any movement along microtubules in vitro, suggesting it may not have a functional motor domain (He, Subramanian et al. 2014). However, He et al. showed in both Kif7/− MEFs and in cells expressing the L130P mutant, that cilia are longer in length and are unstable, suggesting that the motor domain is needed for the control of cilia length and stability. The increased microtubules catastrophe at distal cilia was taken as evidence that Kif7 is important for primary cilia integrity by regulating microtubule growth at the distal tip.

1.3.4.2. Requirement of cilia for Kif7 functions

Studies of Kif7 null mice crossed with Ift172 null mice (which have no cilia since IFT172 is an integral protein of the IFT-B complex) showed that Kif7's activity depends on IFT proteins in that double mutant mice exhibited the same phenotype as Ift172 mutant embryos. This result suggests that Kif7 requires cilia for its function. However, Kif7 may also have cilia independent functions. A study of Kif7 in retinal epithelial cells, a non-Hh responsive human cell line showed that loss of Kif7 by siRNA result in centrosome duplication, Golgi fragmentation, abnormal tubulin acetylation and microtubular dynamic defects, all of which depends on Kif7's motor domain (Dafinger, Liebau et al. 2011). Kif7 may therefore have roles in many cellular processes in addition of Hedgehog signalling.

1.3.4.3 Kif7 knockout mice

Kif7 was first implicated in mouse Hh signalling in 2009, when three independent studies reported the characterization of Kif7 mutant mice (Cheung et al. 2009, Endoh-Yamagami et al. 2009, Liem, He et al. 2009). Kif7 knockout mice die at birth and exhibit phenotypes consistent with impaired Hh signalling such as polydactyly and exencephaly, similar to phenotypes observed in Gli3/− mice. Loss of Kif7 leads to modest increase in Shh signalling in the neural
tube and decreased Gli3\textsuperscript{R} formation. While these results support a negative function for Kif7, further analysis of the role of Kif7 in development showed that Kif7 also fulfills a positive regulatory role. In $Ptch1^{-/-}$; $Kif7^{-/-}$ double knock-out mice, the strong gain of function phenotypes observed upon deletion of $Ptch1$ are largely rescued (Liem, He et al. 2009). This suggests that Kif7 has a positive role in Shh signalling. Specifically, Kif7 appears to be crucial for Shh signalling in the floor plate, where highest levels of Shh signalling are needed. Thus, in the developing neural tube, Kif7 fulfils a positive role in cells with high levels of Shh signalling whereas it plays a negative role in cells of low signalling activity or when the pathway is not activated.

1.3.4.4 Kif7 in Zebrafish

Zebrafish Kif7 was first characterized in 2005 (Tay et al. 2005) and was found expressed at low level throughout development. At the cellular level, zebrafish Kif7 exhibits cytoplasmic localization and co-localization with microtubules. Knockdown of $kif7$ using morpholino antisense oligonucleotides leads to de-repression of Hh target genes in the myotome leading to characteristics defects in somite morphogenesis, which are typical of Hh signalling deregulation (Tay, Ingham et al. 2005, Putoux et al. 2011). In the original study, Kif7 was characterized as a negative regulator of Hh signalling in zebrafish embryo. In a follow-up study in 2013, Phil Ingham's group generated maternal zygotic $kif7$ knockout in zebrafish using zinc finger nuclease and further characterized the function of fish $kif7$ (Maurya, Ben et al. 2013). Knockout of $kif7$ leads to de-repression of Hh target genes in the myotome but not in the neural tube, indicating a tissue-specific requirement for Kif7. Furthermore, in contrast to mice where Kif7 is essential for normal development, $kif7^{-/-}$ fish are viable. Maurya et al. further showed that Kif7 localizes in the primary cilia and within cytoplasmic puncta and physically interacts with Gli1 and Gli2. In this context, Kif7 is needed to potentiate Gli2\textsuperscript{A} activity in a Smo-dependent fashion by promoting Gli2 dissociation from SuFu. Loss of Kif7 impairs Gli2 accumulation at the tips of cilia and affects Gli2 processing in younger embryos. These results are consistent with Kif7 fulfilling a positive role for Hh signalling in zebrafish. Overall, the dual positive and negative roles of Kif7 are therefore evolutionarily conserved.
1.3.4.5 Kif7 and binding partners

Through binding assays, it was found that Gli proteins bind to Kif7 on the N-terminal motor domain (Cheung, Zhang et al. 2009). In addition, data from co-immunoprecipitation experiments suggest that Kif7 interacts directly with SuFu and Smo, when proteins are overexpressed (Endoh-Yamagami, Evangelista et al. 2009, Chong et al. 2015). These results are similar to what is observed in Drosophila since Cos2 binds to Smo (Jia et al. 2003). Intriguingly, the Cos2 binding site on dSmo does not appear to be conserved in vertebrate Smo suggesting that an alternative mode of binding exist or that Kif7 does not interact directly with Smo. Recently, Evc and Evc2 proteins were described to interact with activated and phosphorylated Smo in primary cilia to enable the recruitment of Kif7 within this complex and to promote Gli protein trafficking and Hh signalling (Yang, Chen et al. 2012). Mechanistically how Evc couples Smo to Gli trafficking and in turns contributes to pathway activation is unknown. Whether Evc proteins are required for Kif7 trafficking in cilia during pathway activation remains to be determined. Considering that Evc expression is highly tissue dependent, a general role for Hedgehog signalling is unlikely. Whether other ciliary proteins serve similar functions in regulating Kif7 activity in the cilia also remains to be determined. Recently, Discs large5 (Dlg5), a scaffolding protein localized at the basal body, and an interactor of activated Smo was found to be important for Kif7 ciliary tips accumulation (Chong, Mann et al. 2015). However, the precise role of Dlg5 on Kif7 trafficking was not studied.

1.3.4.6 Kif7 in chondrocytes

In chondrocytes, Ihh is the main Hh ligand (Hsu et al. 2011). It is needed for the proliferation of chondrocytes and normal bone growth. Kif7 has both positive and negative function on Ihh signalling. In the growth plate, loss of Kif7 in chondrocytes results in reduced Hh pathway activity and increased SuFu protein levels as a possible compensatory mechanism. In proliferating chondrocytes, when the Hh pathway is active, Kif7 localizes at the tips of the cilia, but SuFu and Gli are excluded from the tips. Since loss of Kif7 in this context leads to increase SuFu-Gli localization to the tips of cilia, Kif7 therefore functions to actively prevent SuFu-Gli complexes from accumulating (Hsu, Zhang et al. 2011). Ciliary localization of SuFu, Gli and Kif7 in chondrocytes differs from other cells such as MEFs, where Kif7, SuFu, and Glis all
show basal level of cilia accumulation but are enriched in cilia tips in response to pathway activation. The reasons for this difference in distribution remain to be uncovered.

1.3.4.7 Kif7 and skin cells
In embryonic skin cells, Kif7 is predominantly expressed in proliferating keratinocytes of the basal layer and hair follicles, where Kif7 functions to promote Hh pathway activity during hair follicle development (Li et al. 2012). Loss of Kif7 results in reduction of hair follicle number and delays their development. In keratinocytes, Kif7 promotes the dissociation of SuFu-Gli2 complexes and increases the nuclear accumulation of Gli2A. Hence, Kif7 is a positive regulator of Gli activity in hair follicles when the Hh pathway is activated (Li, Nieuwenhuis et al. 2012).

Overall, the precise molecular and biochemical mechanisms underlying the positive and negative roles of Kif7 are poorly understood. More work is needed to further understand how Hh pathway activation and Smo influence Kif7 trafficking in cilia and how this, in turn, affects Gli localization, processing and function to regulate expression of target genes.

1.3.5 Kif7 in ciliopathies and other genetic disorders.
Recent genetic studies have identified KIF7 mutations in patients with ciliopathies. Frameshift or missense mutations in KIF7 were reported to cause hydrolethalus (HLS), acrocallosal (ACLS), Joubert (JBTS) and Bardet-Biedl (BBS) syndromes (Dafinger, Liebau et al. 2011, Putoux, Thomas et al. 2011, Putoux et al. 2012, Walsh et al. 2013). In 2011, Putoux et al. identified KIF7 mutations in in patients with HLS and ACLS, two rare genetic recessive disorders characterized with polydactyly, brain abnormalities and cleft palate. In the study by Putoux et al., authors identified deletion or frameshift mutations within KIF7 exons resulting in ACLS or HLS, and identified eight other missense changes within the KIF7 coiled-coil domain contributing to other ciliopathies. Consistent with the roles of Kif7 in Gli trafficking and Hh signal transduction, cells from patient with mutation in KIF7 had de-regulation of Gli target genes and impaired Gli3 processing. Other KIF7 mutations have since been identified in ACLS patients (Putoux, Nampoothiri et al. 2012, Walsh, Shalev et al. 2013, Karaer et al. 2015). KIF7 mutations have also been identified in JBTS, an autosomal recessive ciliopathy disorder characterized by brain malformation, cerebellar hypoplasia, ataxia, psychomotor delay, retinal
degeneration, renal cyst, and skeletal abnormalities. Ciliary proteins such as NPHP1, CEP290, MKS3, ARL13B are also known to contribute to JBTS (Dafinger, Liebau et al. 2011). Three different deletions in KIF7 exons were found in JBTS, and were characterized to cause instability of cilia and microtubules, Golgi fragmentation and centrosome duplications. This suggests that KIF7 may have additional Hh-independent roles contributing to disease (Dafinger, Liebau et al. 2011). In addition, a homozygous p.N1060S missense KIF7 mutation was identified in a patient with a rare autosomal recessive syndrome leading to macrocephaly and multiple epiphyseal dysplasia, a clinical condition characterized with defect in cartilage formation that results in skeletal abnormalities and short stature (Ali et al. 2012). Lastly, a recent genetic study of copy number variation in patients with typical orofacial clefts found KIF7 to be one of the contributing genes (Simioni et al. 2015). All human KIF7 mutations identified to date are listed in Table 1. How these disease-causing mutations perturb KIF7 functions is still largely unknown, more research is thus needed to understand the molecular mechanisms of Kif7 to better understand the impact of disease causing mutations.

**Table 1. List of Kif7 mutations found in ciliopathies**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Description</th>
<th>Ciliopathy</th>
<th>Other mutated allele(s)</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>p.Arg33X</td>
<td>truncation, c.67C&gt;T</td>
<td>ACLS</td>
<td></td>
<td>Poutex et al, 2011</td>
</tr>
<tr>
<td>p.Leu78ProfxX2</td>
<td>frameshift, c233_234del</td>
<td>ACLS</td>
<td></td>
<td>Poutex et al, 2011</td>
</tr>
<tr>
<td>p.Glu197GlyfsX19</td>
<td>frame shift, c.587dupT</td>
<td>ACLS</td>
<td></td>
<td>Poutex et al, 2011</td>
</tr>
<tr>
<td>p.Gly547SerfsX5</td>
<td>frame shift, c.1639_1640delinsT</td>
<td>ACLS</td>
<td></td>
<td>Poutex et al, 2011</td>
</tr>
<tr>
<td>p.Ala966Profs*81</td>
<td>frame shift, c.2896_2897del,</td>
<td>HLS</td>
<td></td>
<td>Poutex et al, 2011</td>
</tr>
<tr>
<td></td>
<td>homozygous deletion of the first two base-pairs of exon 15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Genetic Change</td>
<td>Phenotype</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-------------------------</td>
<td>-----------------------------------------------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>p.Leu759Pro, c.2276T&gt;C</td>
<td>heterozygous variation</td>
<td>BBS</td>
<td>Poutex et al, 2011</td>
<td></td>
</tr>
<tr>
<td>p.His1115Gln, c.3345C&gt;G</td>
<td>heterozygous variation</td>
<td>MKS, HLS</td>
<td>Poutex et al, 2011</td>
<td></td>
</tr>
<tr>
<td>p.A73PfsX109</td>
<td>c.217delG exon1, truncation</td>
<td>JBTS</td>
<td>Dafinger et al, 2011</td>
<td></td>
</tr>
<tr>
<td>c.529+2T&gt;C</td>
<td>at donor splice site, intron 3</td>
<td>ACLS</td>
<td>Poutex et al, 2014</td>
<td></td>
</tr>
<tr>
<td>c.1019dupT, p.Asn341GlnfsX122,</td>
<td>T insertion in exon 17, frameshift, premature termination</td>
<td>ACLS</td>
<td>Poutex et al, 2014</td>
<td></td>
</tr>
<tr>
<td>Genomic Position</td>
<td>Mutation</td>
<td>Description</td>
<td>Source</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>----------</td>
<td>-------------</td>
<td>--------</td>
<td></td>
</tr>
<tr>
<td>c.3331C&gt;T, p.Arg1111X</td>
<td>non-sense mutation in exon 17</td>
<td>ACLS</td>
<td>Poutex et al, 2014</td>
<td></td>
</tr>
<tr>
<td>c.2593-3C&gt;G</td>
<td>substitution at acceptor splice site at intron 12</td>
<td>ACLS</td>
<td>Poutex et al, 2014</td>
<td></td>
</tr>
<tr>
<td>c.1054_1061del p.Arg352GlyfsX108</td>
<td>8bp deletion exon 5</td>
<td>ACLS</td>
<td>Poutex et al, 2014</td>
<td></td>
</tr>
<tr>
<td>c.1167_1204del, p.Arg390HisfsX60</td>
<td>38bp deletion exon 5</td>
<td>ACLS</td>
<td>Poutex et al, 2014</td>
<td></td>
</tr>
<tr>
<td>c.2593-2A&gt;C</td>
<td>acceptor splice site of intron 12 (IVS12-2A&gt;C)</td>
<td>ACLS</td>
<td>Karaer et al, 2015</td>
<td></td>
</tr>
</tbody>
</table>

1.3.6 Kif7 in other contexts

In addition to Kif7’s role in cilia and Hh signalling, Kif7 has been implicated in other diseases. Recently Kif7 and Gli proteins were found to be down regulated in choriocarcinoma cell lines, and loss of Kif7 resulted in increased migration of the trophoblast cell line (Ho et al. 2014). Furthermore, Kif7 was found to interact with toxin ExoS secreted from *P. aeruginosa*, leading to bronchial epithelial cells cytotoxicity (Okuda et al. 2014). In addition, Kif7 has been implicated in congenital diaphragmatic hernia through regulation of retinoic acid (Coles and Ackerman 2013). Lastly, in a study of kinesins in breast cancer, Kif7 expression was found to be linked to estrogen levels in estrogen receptor positive breast cancer cells (Zou et al. 2014). Overall, since kinesins have pervasive roles in cell function and signalling, it is predictable that Kif7 may have multiple cellular functions other than Hh signalling.

1.4 PPFIA1

1.4.1 Liprin family: general introduction and function

Liprin proteins are also known as transmembrane tyrosine-phosphatase LAR-interacting proteins. Liprin-α was originally identified as a cytosolic binding partner of the LAR family of
receptor protein tyrosine phosphatases (LAR) and acts as a scaffold for their focal adhesion recruitment (Serra-Pages et al. 1995). In vertebrates, the Liprin family consists of Liprin-α (1, 2, 3, 4), and Liprin-β (1, 2). C. elegans and Drosophila have only one Liprin gene, SYD-2 or dLiprin, respectively. In vertebrates, the 4 Liprin-α genes show differential expression, with Liprin-α2 (PPFIA2) and α3 (PPFIA3) expressed exclusively in mammalian brains, whereas Liprin-α1 (PPFIA1) and α4 (PPFIA4) are found in multiple tissues. Of the four, PPFIA1 shows the broadest expression across many tissue types (Serra-Pages et al. 1998). Liprin-α are well conserved with 50% amino acid identity between human Liprin-α and worm SYD-2. Liprin proteins are characterized by an N-terminal coiled-coil region that mediates homo- and hetero-multimerization and C-terminal SAM domains. Liprin-α proteins also have a carboxy-terminal PDZ-binding domain, and a PEST sequence important to regulate protein degradation that is present between the coiled-coil and the SAM motifs (Serra-Pages, Medley et al. 1998, Spangler and Hoogenraad 2007) (Figure 1-5). Liprin-α are best characterized in synapse formation, where they are required for the assembly of presynaptic active zone and postsynaptic sites, as well as synaptic cargo transport (Spangler and Hoogenraad 2007, de Curtis 2011).

![Figure 1-5. Schematic representation of the protein domains of PPFIA1.](coiled-coil-SAM-SAM-SAM)

1.4.2 PPFIA1

Of the Liprin-α genes, PPFIA1 is the best studied. PPFIA1 is important for synaptic formation in the nerves system. In C. elegans, Syd-2 (Liprin-α homolog) is needed for pre-synaptic development. Loss of Syd-2 function results in mis-localized synaptic vesicles and pre-synaptic structural defects (Zhen and Jin 1999). In Drosophila, dLiprin is also needed for synaptic function since dLiprin mutants show defect in active zone (site of neurotransmitter release) morphology (Kaufmann et al. 2002). In vertebrates, PPFIA has also been shown to regulate synapse formation and function. One study showed that PPFIA1 is negatively regulated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) in synapse morphogenesis. PPFIA1 is degraded in response to CaMKII phosphorylation, and this in turn regulates synaptic vesicles level (Hoogenraad et al. 2007). In non-neuronal contexts, PPFIA1 is best characterized in cell
migration and motility. PPFIA1 is needed for cell motility and spreading, partly through regulation of integrins. Loss of PPFIA1 results in unstable lamellipodia, inhibits cell spreading, and reduces the degradation of ECM (Asperti et al. 2009, Asperti et al. 2010, Asperti et al. 2011, Astro et al. 2014). PPFIA1 is highly expressed in human breast cancer cells and it has been suggested that PPFIA1 may be required for migration and invasion of tumour cells (Astro et al. 2011). Overall, the Liprin-α family of scaffolding proteins have diverse functions in cellular signalling.

1.4.2 Liprin and kinesins

PPFIA1 plays important roles in transporting cargoes in neurons. Research from mammalian cells, Drosophila, and C. elegans all showed that PPFIA1 interacts with the neuron specific motor protein Kif1A and stimulates the trafficking of synaptic vesicles (Shin et al. 2003, Miller et al. 2005, Sieburth et al. 2005). The coiled-coil domain of PPFIA1 binds Kif1A and acts as an adaptor protein linking synaptic cargos to kinesins (Shin, Wyszynski et al. 2003) (Figure 1-6). In Drosophila, dLiprin is also implicated in Kif1A-dependent trafficking in neurons. Fly mutant for dLiprin exhibits defective synaptic vesicle movement, characterized by a decrease in anterograde movement, and an increase in retrograde movement of synaptic vesicles (Miller, DeProto et al. 2005). Similarly, in C. elegans, Syd-2 (Liprin-α) is required for the bidirectional movement of UNC-104 (Kif1A). In Syd-2 mutant, anterograde movement is reduced, resulting in reduced cargo transport (Sieburth, Ch'ng et al. 2005, Wagner et al. 2009). A recent study further showed that Liprin (α, β) interacts with Kif21A to regulate cortical microtubule growth.(van der Vaart et al. 2013). In this context, Liprin acts as a scaffolding protein for protein clustering, whereas Kif21A functions to inhibit cortical microtubule growth. Overall, PPFIA1 acts as a molecular adaptor to link vesicles and cargos to kinesins.

![Figure 1-6. Model of kinesin and Liprin interaction.](image_url)
1.4.3 PPFIA1 and protein phosphatases

The interaction between Liprin-α and protein phosphatase was first identified in proteomic studies. In a proteomic screen of Liprin-α interacting proteins in *Drosophila*, the PP2A subunit B' (Wrd, *Drosophila* homolog) was identified. Liprin-α and PP2A interact in the synapse and PP2A subunit mutants phenocopy Liprin-α mutants with defects in synaptic vesicles accumulation. The Liprin-α:wrd protein complex was described to antagonize Gsk3b kinase activity and to prevent ectopic accumulation of synaptic materials (Li et al. 2014). Proteomic-based screens in mammalian systems have also identified the interaction between PP2A and PPFIA1. Mass spectrometry analysis identified PPFIA1 in immunoprecipitates of PP2A regulatory subunit (B56γ or B') from HEK293T cell lysates (Arroyo et al. 2008). Two other large scale proteomic analysis of PP2A structural (A) and catalytic (C) subunits in human cell lines, revealed PPFIA1 interacts preferentially with PPP2R1A, PPP2CB, PPP2CA (Glatter et al. 2009, Goudreault et al. 2009). Although a physical interaction between PPFIA1 and the PP2A phosphatase is well established, the specific roles of this complex are poorly understood and further biochemical and functional analysis are required.

1.4.5 PPFIA1 and primary cilia

Large scale mass spectrometry based proteomic analysis in kidney cells (Ishikawa et al. 2012), or in sensory cilium of rod and cone photoreceptor cells of the mouse retina (Liu et al. 2007) both identified PPFIA1 as part of the primary cilia proteome. PPFIA1 was also identified in a RNAi functional genomics screen performed in human retinal pigmented epithelial cells to identify human genes involved in ciliogenesis (Kim et al. 2010). These large-scale studies highlight the possible role of PPFIA1 in primary cilia.

1.5 PP2A Phosphatases

1.5.1 Family, classification and general function

The Serine/threonine phosphoprotein phosphatase family has 7 members (PP1, PP2A, PP2B, PP3, PP4, PP5, PP6, and PP7). PP1 and PP2 are the most abundant and ubiquitous phosphatases in this family. In the PP2 family, Protein phosphatase 2A (PP2A) regulates many key physiological processes, including mRNA translation, cell cycle progression, apoptosis and
differentiation (Janssens and Goris 2001). Each PP2A is a heterotrimer that consists of a structural A subunit (PPP2R1A, PPP2R1B), a regulatory B subunit, and a catalytic C subunit (PPP2CA, PPP2CB) (Figure 1-7). The PP2A catalytic (PP2Ac) subunit binds directly to the A subunit to form the PP2A dimeric core. The core serves as platform to associate with regulatory B subunit to form a trimeric complex. Human genome encodes at least 15 known regulatory B subunits that allows for many different combinations of trimeric complexes (Janssens and Goris 2001, Lechward et al. 2001). Hence, it is the B subunit that determines the specificity of each PP2A complex. The B subunits are classified into four families based on sequence homology: B (B55), B’ (B56), B”, and B’” (striatins). B’ (B56) family further consists of 5 members PPP2R5A (B56 α), PPP2R5B (B56 β), PPP2R5C (B56 γ), PPP2R5D (B56 δ), and PPP2R5E (B56ε).

Given the diverse function of PP2A in cellular activities, it is not surprising that mis-regulation or mutation in PP2A results in human diseases. Mutations in PP2A have been found in various cancers, such as ovarian (Nagendra et al. 2012) and uterine cancers (Shih Ie et al. 2011). PP2A itself is a tumour suppressor. Simian virus 40 (SV40) is known to inhibit PP2A leading to malignant transformation of normal human cells (Yang et al. 1991). In addition, PP2A is also involved in the de-phosphorylation of oncogenes such as Myc and Akt and inhibition of PP2A activity is known to induce cancer cell death (Arnold and Sears 2006, Sablina et al. 2010). However, given the ubiquitous function of PP2A, a major drawback of inhibiting PP2A is lack of specificity to cancer cells, resulting in too much cytotoxicity. Currently, all PP2A inhibitors target the phosphatase catalytic subunit and cannot distinguish between different holoenzyme complexes. Okadaic acid (OA), a polyether fatty acid produced by marine dinoflagellates, is one of the most potent inhibitor of PP2A. It is widely used in research to inhibit PP2A function. OA acts through direct binding to PP2Ac via a hydrophobic cage that is not conserved in other Ser/Thr phosphatases providing OA with high affinity for PP2A. However, at higher dose OA can inhibit PP1 (Mumby and Walter 1993, Virshup 2000, Janssens and Goris 2001).
1.5.2 PP2A and Hh signalling

Multiple kinases have been identified to play important roles in *Drosophila* and vertebrate Hh signalling. However, the roles of protein phosphatases in Hh signal transduction have been underscrutinized. In *Drosophila*, PP2A has been identified as a positive regulator of Hh signalling (Nybakken et al. 2005, Casso et al. 2008). PP2A was first identified as an important regulator of Hh signalling in a genome wide RNAi screen performed in *Drosophila* cells (Nybakken, Vokes et al. 2005). Loss of the *Drosophila* gene *microtubule star (mts)*, which encodes the PP2A catalytic subunit, results in reduced Hh signalling (Casso, Liu et al. 2008). Furthermore, loss of B subunit of PP2A (*Widerborst Wdb*) in flies also reduced Hh signalling (Nybakken, Vokes et al. 2005). In 2009, Ci was shown to be a direct substrate of PP2A in flies. PP2A acts to de-phosphorylate Ci to prevent its processing to CiR (Jia et al. 2009). Additional studies revealed that PP2A functions in conjunction with PP1 to de-phosphorylate Smo. Specifically, PP2A dephosphorylates PKA primed, CKI-phosphorylated Smo (Su et al. 2011). In mammalian systems, the role of PP2A in the context of Hh signalling is less studied. In Xenopus, B56e, the vertebrate ortholog of Wdb, was found to be important for Hh signalling in Xenopus eye field separation (Rorick et al. 2007). PP2A also appears to be required for Hh-dependent activation of COUP-TFII. In this case, PP2A is needed to dephosphorylate an unknown target that is required for activation of COUP-TFII and Gli response element–dependent gene expression (Krishnan et al. 1997).

1.6 Dual Specific Tyrosine Kinase 2 (DYRK2)
1.6.1 DYRKs general classification and function

The dual-specificity tyrosine phosphorylation-regulated kinase (DYRK) family is part of the CMGC family of serine/threonine kinases with sequence similarity to cyclin-dependent kinases. DYRKs have conserved catalytic kinase domain and DYRK-characteristic DYRK homology (DH) box. In vertebrate cells, there are 5 members of the DYRK family (1A, 1B, 2, 3, and 4). DYRK1A and DYRK1B belong to class I DYRKs, and DYRK2, DYRK3, and DYRK4 belong to class II. DYRKs have conserved YXY activation sequence, they are known to auto-phosphorylate their own tyrosines for activation. DYRKs are proline directed serine-threonine kinases, with a preference for proline at the P+1 position on the substrate (Campbell and Proud 2002, Aranda et al. 2011). For example, DYRK1A has a consensus phosphorylation sequence of (RPXS/TP) (Himpel et al. 2000) and DYRK2 has a consensus of (RXS/TP) (Campbell and Proud 2002). DYRKs in general have broad cellular functions but they have been most characterized in the context of cell death (Aranda, Laguna et al. 2011). DYRK1A was the first DYRK protein implicated in Hh signalling in 2002 (Mao et al. 2002), since then, DYRK1B (Lauth et al. 2010) and DYRK2 (Varjosalo et al. 2008) have been implicated in vertebrate Hh signalling with different roles.

1.6.2 DYRK1A and DYRK1B and Hedgehog signalling

DYRK1A was first implicated in Hh signalling in 2002 when the kinase activity of DYRK1A was found to promote the transcriptional activity of Gli1 in reporter assays. DYRK1A promoted phosphorylation of Gli transcriptional activity was also shown to be mediated through nuclear retention (Mao, Maye et al. 2002). In 2010, Lauth et al showed that DYRK1B is also important for Hh signalling in the context of pancreatic cancer. DYRK1B functions as a negative regulator of Hh signalling in this context since overexpression of DYRK1B inhibits, whereas knockdown of DYRK1B increases pathway activity. A working model by which autocrine Hh signalling in pancreatic cancer cells is suppressed by a KRAS-DYRK1B signalling axis was proposed (Lauth, Bergstrom et al. 2010). Recently, a genetic study identified a DYRK1B mutation in patients with early onset coronary artery disease and metabolic syndrome (an inherited disease). The adipocytes of these patients exhibit lower Gli2 protein levels, suggesting a further role of DYRK1B and Hh signalling (Keramati et al. 2014). Overall, the exact roles that DYRK1 family
kinases play during Hh signalling are still unclear. Similarly, the substrates regulated by DYRK1 are incompletely defined.

1.6.3 DYRK2 and Hedgehog signalling

DYRK2 was first identified as a negative regulator of Hh signalling in a functional expression screen designed to identify kinases regulating Gli luciferase reporter activity (Varjosalo, Bjorklund et al. 2008). Overexpression of DYRK2 inhibits Shh-induced Gli reporter activity and blocks the expression of Hh-dependent neural tube marker \textit{in vivo}. Knock-down of DYRK2 activates the Shh pathway in the absence of ligand stimulation. DYRK2 was shown to phosphorylate Gli2 and to promote Gli2 degradation by the proteasome. DYRK2 therefore regulates cytoplasmic Gli2 stability (Varjosalo, Bjorklund et al. 2008). In this thesis, I will further investigate the role of DYRK2 within the Hh pathway, specifically in the regulation of Kif7 function.

1.7 Mass Spectrometry

1.7.1 General introduction

Affinity purification coupled mass spectrometry (AP-MS) based proteomic study is the study of protein complexes or protein-protein interactions by isolating target proteins and their complexes by immuno- or affinity- purification and analyzing them by liquid chromatography followed by mass spectrometry. This method allows for unbiased screen of protein-protein interactions. In addition to protein complex identification, MS can also be used to identify post-translational modifications. This has been very useful in examining targets of kinases and phosphatases (Knight et al. 2013). Recently, quantitative and data independent MS analysis has been used to study the entire cell proteome under different treatments (Collins, Gillet et al. 2013, Lambert, Ivosev et al. 2013). With the advances in MS technology and data processing software, proteome level analysis is becoming possible and affordable.

1.7.2 Affinity purifications-coupled LC-MS/MS work flow
The basic workflow of mass spectrometry is: (1) ionization, (2) mass analyzer, and (3) detection. However, given the complexity of peptide mixtures, samples are usually separated through a liquid chromatography (LC) before ionization. A commonly used LC method is reversed-phase liquid chromatography that separates peptides based on their hydrophobicity. By running organic solvents (e.g., acetonitrile) through the chromatography column, the polarity of the mobile phase of the column is gradually reduced, leading to "release" of peptides from the support in decreasing order of hydrophobicity. Resolution is further enhanced through the use of tandem mass spectrometry (MS/MS). Tandem MS is directly coupled to high performance liquid chromatography using nano-scale reversed-phase columns. Peptide mixtures in liquid phase are loaded on HPLC columns, eluted during the LC, and then ionized by electrospray as they enter the mass spectrometer. Raw MS/MS spectra are then analyzed by database search engines to identify peptides and proteins present in the sample (Figure 1-8). Further data analysis by statistical software can help identify which of these proteins are likely interactors. Each step of the LC-MS/MS will be explained in more details below.

Firstly, in the ionization step, peptides are ionized into the gas phase. In general there are two ways of ionization, electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI). ESI is the conversion of liquid containing peptides to gas phase through high electrical field at the tip of a capillary. Ions are then analyzed in the mass spectrometer. In contrast, MALDI generates ions from samples on a solid phase. The sample is pulsed with UV laser thus vaporizing the solid support together with the analytes to gaseous ions, which can then be analyzed by the mass spectrometer.

After the ionization process, ionized molecules are separated and resolved in mass analyzers according to their mass to charge (m/z) ratio. Specifically, the mass spectrometer measures the molecular weight of ions based on their m/z ratios. Commonly used mass analyzers are ion trap, time-of-flight (TOF), and orbitrap. In ion trap, ions are captured because of surrounding electric or magnetic fields. An electrical current orthogonal to the flight-path of the charged molecules expel them to the detector. From the detection of an expelled peptide, the m/z can be determined. Ions are filtered base on their m/z ratio and sequentially ejected for MS/MS. Time-of-flight (TOF) analyzers use electric fields to accelerate ionized molecules and measure the time it takes for a charged molecule to move through a high vacuum flight tube and to reach the
detector. The measured velocity of the ion correlates with its \( m/z \) ratio. Orbitraps trap ions in an orbital motion around a central electrode. \( m/z \) values are measured from the frequency of harmonic ion oscillations along the axis of the electric field.

In tandem MS, after the first MS mass scan, ions with given \( m/z \) are selected and fragmented by collision induced dissociation (CID), where peptide ions are bombarded with neutral gas (e.g. helium), breaking peptide ions along their backbone into small fragments. Then a second MS will scan and analyze the \( m/z \) of the fragmented ions. This generates a CID spectrum or MS/MS spectrum that is used for identification of the peptide.

Once the experimental mass spectra are generated, they are compared with theoretical peptide fragment masses in sequence databases such as Mascot to find the best matching peptide. The degree of matches is translated into a probability score. However, these databases are known to produce incorrect peptide assignments. Therefore, statistical models such as PeptideProphet are used for validation of peptide assignments (Nesvizhskii et al. 2003).

The mass spectrometer used for the studies described in this thesis is the LTQ-XL ion trap, coupled to nano-RPLC-ESI, and C18 reverse phase column (fused silica capillary column packed with C18 reverse phase material). The mass spectrometer is programmed to acquire MS/MS spectra in a data-dependent mode where the first MS scan records all the different ion species within a specific mass window of 400-1400 \( m/z \), followed by MS/MS analysis of the five most abundant ions at a given time point. This sequence is repeated over 2 hours of the HPLC gradient. All data collected are uploaded to ProHits (Protein High-throughput Solution), an open source software package for MS data storage and analysis (Liu et al. 2010). ProHits allows users to compare multiple AP-MS analyses and to identify background contaminants. In ProHits, MS spectra are searched against Mascot and Comet databases, searches are individually processed, and results are scored by TransProteomics pipeline (TPP) where all hits with a probability of \( P>0.05 \) (significance threshold) are selected. The accuracy of searches is estimated by the PeptideProphet\textsuperscript{TM} and ProteinPhophet\textsuperscript{TM} statistical softwares (Keller et al. 2002). ProteinProphet\textsuperscript{TM} provides a probability score that denotes the likelihood of the given protein being correctly or incorrectly identified (Nesvizhskii, Keller et al. 2003). Next, Significance analysis of INTeractome or SAINT analysis was used to calculate the probability
for each protein-protein interaction. SAINT uses statistical modeling based on spectral counts to calculate the probability of true interaction. Lastly, background contaminants of the APs were manually removed after comparison to CRAPome (a web-based contaminant repository for affinity purification). These are proteins that are not real-interactors of the bait protein but interacts non-specifically with the solid-phase support, the antibody or the epitope tag (Peng and Gygi 2001, Keller, Nesvizhskii et al. 2002, Gingras et al. 2005, Gingras et al. 2007, Deutsch et al. 2010, Choi et al. 2011, Choi et al. 2012, Dunham et al. 2012, Eng et al. 2013, Mellacheruvu et al. 2013).

Figure 1-8. MS work flow. The biological sample is subjected to affinity purification to isolate proteins and interacting proteins. The isolated protein complexes are then eluted and digested with MS grade trypsin. The peptides mixture is then loaded on a HPLC column for reverse-phase liquid chromatography followed by ESI-MS/MS. Ions are then subjected to MS1 scan and the 5 most abundant peptides are selected for CID and MS2 scans. The MS2 acquired spectrum is recorded and compared to database to identify peptides and associated proteins.

1.7.3 Sample preparation for AP-MS

The basic work-flow for sample preparation for MS from mammalian cells are (1) generate cell lines that express an epitope-tagged protein of interest, (2) purification of protein complexes, (3) proteolytic digestion of the isolated sample and (4) identification of co-precipitating proteins by MS. Protein complexes are purified through affinity purification methods via immuno- or affinity purifications (i.e. using antibodies directed against an epitope tag such as FLAG). Epitope tagging allows for standardization of the purification protocol without the need to
optimize for every antibody. Background contaminants are also more consistent across all purifications. Epitope tags such as FLAG are conjugated to the protein of interest and expressed in cells. Cell lysates are extracted and tagged proteins are purified with their associated proteins. The purified precipitates are treated with trypsin to digest the proteins to peptides, and the resultant peptides are analyzed by LC-MS/MS. The use of reciprocal purifications, using the identified proteins as baits, can then be used to validate the interactions (Dunham, Mullin et al. 2012, Kean et al. 2012).

1.7.4. Data acquisition methods
As mentioned above, traditional affinity-purification coupled MS (AP-MS) is performed using data-dependent acquisition (DDA), where peptides are ionized and selected for fragmentation based on signal intensity, usually 4 or 5 most intense precursor ions are selected for fragmentation. In contrast, in data independent acquisition (DIA), many peptide ions are concurrently selected and fragmented, and all the spectra are recorded. An example of DIA is Sequential window acquisition of all theoretical spectra (SWATH) MS where peptide ions are sequentially isolated in smaller mass windows and fragmented so that virtually all ions are recorded. Another acquisition method is targeted data acquisition where a pre-determined peptide ion is selected and fragmented, and the intensities of this ion are recorded. An example of targeted data acquisition is multiple reaction monitoring (MRM). MRM and SWATH MS are used in this thesis and will be covered in more details in the following sections (Mann and Jensen 2003).

1.7.5 Multiple Reaction Monitoring (MRM)
MS has long been used to identify phosphorylation sites because the addition of a phosphate group changes the mass of the protein by 80 Da. Recent advances in targeted MS has allowed for label-free phospho-proteomic studies. MRM has been applied for the targeted detection and quantification of phosphopeptides. By inhibiting kinases or phosphatases, with either RNAi mediated knockdown or with chemical inhibitors, changes in ion abundance corresponding to a specific potential phospho-peptide can be monitored (Mann and Jensen 2003). MRM is a label-free MS2 targeted quantification approach. In MRM mode, first MS scans the full mass range
and this is followed by full MS2 scans on a fixed precursor, to quantify the abundance of the target ion of interest. MRM allows for high selectivity and sensitivity for the target ion, however, it requires manual input (initial discovery phase) in precursor selection and it can only quantify what is expected to be present in the sample. After data acquisition, the chromatographs of sequence specific ions are collected, and by comparing the chromatographic peak ratios between different treatments the relative abundance of a peptide can be assessed. (Mann and Jensen 2003, Domon and Aebersold 2006).

1.7.6 Sequential window acquisition of all theoretical spectra (SWATH)
In SWATH acquisition mode, fragment ion spectra of all precursor ions are collected. Data are then mined retrospectively using data-dependent acquisition spectra of bait of interest (also called reference spectral libraries). SWATH employs a wider mass-isolation range at MS step but a tighter extraction window at the MS2 step to maintain high specificity. SWATH-MS offers higher throughput but lower sensitivity as compared to MRM. It allows for high proteomic coverage from a single LC-MS run and requires no assay development at the MS steps. All data is acquired and targets are mined post-acquisition. Data generated provides a permanent record of the whole sample, enabling future re-analysis. SWATH is especially ideal for study of protein-protein interactions associated with disease-associated mutations on a systematic level. It allows the monitoring of changes in protein interaction in a quantitative manner. In traditional AP-MS/MS used for identifying protein interaction (data dependent approach) peptide ions sequenced are selected based on the relative abundance of precursor ions in a given sample (from MS1 to MS2, the top 5 most abundant ions are usually selected). In contrast, SWATH is not limited to abundance of ions in MS1. In this thesis, SWATH was used to study changes in protein-protein interactions for KIF7 mutations that have been associated with human diseases. Statistical analysis used for SWATH in this thesis is based on the protocol from Lambert et al. (Gillet et al. 2012, Collins et al. 2013, Lambert et al. 2013).
1.8 Objectives of thesis

In vertebrates, Kif7 has been described to have both negative and positive roles during Hh signalling for neural tube patterning, limb morphogenesis, chondrogenesis and hair follicle development. Mutations in *KIF7* have been identified in various human ciliopathies and developmental disorders. However, the precise molecular mechanisms underlying the positive and negative roles of Kif7, its biochemical and regulatory mechanisms and how the disease causing mutations affect Kif7-dependent processes are poorly understood. More work is thus needed to further understand how Hh pathway activation influences Kif7 trafficking in the cilia and how this, in turn, affects Gli localization, processing and function to regulate Hh-target genes expression. The overall aim of this thesis project is to better understand how Kif7 and its associated proteins function within the vertebrate Hh signalling pathway. A mass spectrometry-based proteomic analysis was first conducted to identify novel proteins associated with Kif7. Novel interactors of Kif7, Liprin-α1 (PPFIA1) and PP2A were identified. Biochemical and functional characterization of the Kif7-PPFIA1-PP2A interaction was subsequently carried out to better understand the role PPFIA1 and PP2A in controlling Kif7 localization in primary cilia and Hh pathway activation. Furthermore, comparative proteomic analyses were conducted to determine whether disease causing *KIF7* mutations led to change in the interaction with interacting proteins. Secondly, through a candidate identification approach, we identified DYRK2 as a kinase that plays a role in Kif7 function. Through biochemical assays, we further examined gain and loss of functions of DYRK2 on Kif7 function. Lastly, we examined the functional relationship between Kif7 and SuFu, two key negative regulators of the pathway. Overall, this thesis aims to study Kif7 functions using proteomic and biochemical approaches.
Chapter 2: Kif7 phosphorylation regulates its ciliary trafficking and Hedgehog signalling


All experiments and data analysis were performed by Y.C Liu except for the following. MRM and SWATH mass spectrometry analysis were performed by A.L Couzens and A-C Gingras. L.D.B. McBroom-Cerajewski and A-C Gingras performed LC-MS/MS on PPFIA1. A.R. Deshwar and I.C Scott performed the in vivo Zebrafish work. X. Zhang, V. Puvindran, and C-C Hui generated plasmids, cell lines and antibodies, and participated in discussion of data. Y.C Liu and S. Angers wrote the manuscript together with input from all authors.
2.1 Abstract

The Hedgehog (Hh) signalling pathway is most studied in flies and mice through genetic analysis. The biochemical and molecular mechanism of how the pathway functions is poorly defined. One key effector of the pathway Kif7 was shown to have both positive and negative functions through genetic studies. To better study Kif7’s function in mechanistic detail, we used a mass spectrometry based proteomic approach and identified Liprin-α1 (PPFIA1) and the PP2A protein phosphatase as Kif7-interacting proteins. We further showed that PPFIA1-PP2A function to regulate Kif7 phosphorylation, a modification that is important for the trafficking of Kif7 and Gli to the tips of cilia and for the transcriptional output of Hh signalling. Furthermore, mutations in KIF7 have been identified in ciliopathies. Using mass spectrometry based proteomic approach we examined the defects in protein interactions in disease mutations. We found one mutation of KIF7 exhibited defects in PPFIA1 binding, Kif7 ciliary trafficking and Hh target gene activation. Collectively, these studies highlight the importance of Kif7 and ciliary trafficking in mammalian Hh signalling and in human diseases.

2.2 Introduction

Kinesin family member 7 (Kif7) was first identified as important for mammalian Hh signalling in 2009 with genetic studies published from three labs showing Hh defects in Kif7 knock-out mice (Cheung, Zhang et al. 2009, Endoh-Yamagami, Evangelista et al. 2009, Liem, He et al. 2009). Subsequently, KIF7 mutations affecting Hh signalling and cilia functions were found in human ciliopathies (Dafinger, Liebau et al. 2011, Putoux, Thomas et al. 2011). Genetic studies suggest Kif7 has both positive and negative roles in neural tube patterning, limb morphogenesis (Cheung, Zhang et al. 2009, Endoh-Yamagami, Evangelista et al. 2009, Liem, He et al. 2009, Law et al. 2012), chondrogenesis (Hsu, Zhang et al. 2011) and hair follicle development (Li, Nieuwenhuis et al. 2012). These studies revealed that Kif7 interacts with Gli proteins and accumulates at the tips of primary cilia when the Hh pathway is active. Examination of Kif7−/− mouse embryonic fibroblasts (MEFs) revealed reduced Gli3R (Cheung, Zhang et al. 2009, Endoh-Yamagami, Evangelista et al. 2009, Liem, He et al. 2009) and decreased Hh-promoted accumulation of Gli at the tips of primary cilia (Endoh-Yamagami, Evangelista et al. 2009).
suggesting that Kif7 may be important for these processes. The precise molecular mechanisms underlying the positive and negative roles of Kif7, its biochemical and regulatory mechanisms and how the disease causing mutations affect Kif7 dependent processes are poorly understood. More work is thus needed to further understand how pathway activation influences Kif7 trafficking in cilia and how this, in turn, affects Gli localization and Hh-target genes expression.

Here we used \textit{Kif7}^{+/−} and \textit{Ptch1}^{+/−} MEFs to study the mechanisms of Kif7 during Hh signalling. We report that in MEFs, Kif7 fulfilled a predominantly positive role downstream of Smo activation as it promoted the ciliary tips accumulation of Gli proteins and the induction of \textit{Gli} target genes. We identified post-translational modification, phosphorylation on Kif7 as important for its function. We further identified PPFIA1 and PP2A as proteins involved in Kif7 phosphorylation. Our results indicated that Kif7 phosphorylation dictated its subcellular localization at the tips of primary cilia and determined whether it inhibited or activated Hh signalling. Our findings are consistent with a model in which Smo activation is coupled to the trafficking of Kif7 and Gli proteins at cilia tips and to their transcriptional output through the de-phosphorylation of Kif7 mediated by PP2A.

2.3 Methods

2.3.1 Antibodies and reagents

Antibodies were purchased from the following vendors: mouse anti-HA.11 clone 16B12 (Covance); mouse anti-FLAG, mouse anti–beta-Tubulin clone TUB 2.1, mouse anti-acetylated-Tubulin clone 6-11B-1, anti-gamma Tubulin clone GTU-88 (Sigma-Aldrich); rabbit anti-Gli3 (Santa Cruz, Sc20688, for immunofluorescence), rabbit anti-GFP (Santa Cruz, Sc8334), goat anti-PPFIA1 (Santa Cruz, Sc54039), goat anti-Vangl1 (sc-46557, Santa Cruz) was used as IgG control. Goat anti-Gli3 (EMD Millipore, AF3690, for Western blot); rabbit anti-Pericentrin (Abcam, ab4448); rabbit anti-Smo (LSBio, LS-A2668). Goat anti-Gli2 (R&D AF3635, for Western blot). Rabbit anti-Kif7 (Cheung, Zhang et al. 2009). Rabbit anti-Gli2 (for immunofluorescence) (Li, Nieuwenhuis et al. 2012). Rat anti-PPP2AC (Abcam ab77830). Rabbit anti-PPP2R1A (Cell Signalling, #2041). Secondary antibodies conjugated to Alexa
Fluor 488, 594, and 647 were purchased from Life Technologies. Secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories, Inc. Okadaic acid sodium salt was purchased from Bioshop Canada Inc (209266-80-8) and was used at 50nM for 3h for all experiment, except for MRM experiments where cells were treated at 150nM for 2.5h. GANT-61 (Tocris) was used at 5μM for 6h, Cyclopa mine was used at 1μM for 24h, and SAG (Sc-212905 Santa Cruz) was used at 200nM for 24h for all experiments except for endogenous co-immunoprecipitation experiment where SAG was added for 1.5h. Phos-tag Acrylamide Aqueous solution (Wako, 304-93526) was used according to manufacturer’s instructions.

2.3.2 Tissue culture, transfections, and lentiviral transductions

HEK293T, HEK293-Flp-in-Trex, MEF, and C3H10T1/2 cells were cultured in DMEM supplemented with 10% FBS (Sigma-Aldrich), and penicillin/streptomycin. HEK293T and HEK293-Flp-in-Trex cells were transfected using PEI (Polysciences Inc. Cat #23966). All lentiviral particles were produced in HEK293T cells by cotransfection of VSV-G (3μg), psPAX2 (8μg), and lentiviral plasmids (8μg) in 30–40% confluent monolayer cell culture grown in 10-cm plates. Media containing virus particles were collected after 24 and 48h. C3H10T1/12 or MEF cells were subsequently transduced in the presence of 10 μg/ml polybrene (Sigma-Aldrich). 24h after infection the viral media was replaced with fresh DMEM 10% FBS media where appropriate cells were selected with puromycin (2μg/ml) or hygromycin (0.2mg/ml) 48h after viral infection. For experiments with MEF or C3H10T1/2 cells, cells were grown to confluency then serum starved with 0.5% FBS in DMEM to induce ciliogenesis for 24-48h. Primary Kif7−/− MEFs were immortalized through overexpression of SV40 large T antigen by retroviral transduction.

2.3.3 shRNAs

MISSION shRNA clones in the lentiviral plasmid pLKO.1-puro were acquired from Sigma-Aldrich. Kif7 shRNA clones TRCN0000090438 and TRCN0000090439 labeled as Kif7
shRNAs #1 and #2 respectively. PPFIA1 shRNA clones TRCN0000251544 and TRCN0000251546 labeled as PPFIA1 #1 and #2. Smo shRNA clone TRCN0000026312 labeled as Smo was also from Sigma-Aldrich. Sequences of shRNAs used are listed in Table S3.

2.3.4 Plasmids

pEGPF-mKif7, pEGFP-mKif7delN (647-1348), and pEGFP-mKif7delC(1-724) have been previously described (Cheung, Zhang et al. 2009). Kif7GFP was cloned into the pLenti-puro plasmid. Human Kif7 delN (674-1349) was PCR-amplified from a brain cDNA library and was cloned into the pIRES-puro-FLAG plasmid. The Lenti-mKif7GFP and Lenti-FLAG-mKif7 mutants were generated by QuikChange PCR mutagenesis. Ciliopathy mutations are named according to human KIF7 mutations; for the corresponding mouse Kif7 mutations see Table S3. pCDNA5.1-FLAG-hPPFIA1 and FLAG-PPP2R1A were obtained from the Gingras Lab. The respective cDNAs were PCR amplified from Mammalian Gene Collection clones and Gateway cloned into pDEST 5’ Triple FLAG and pcDNA5/FRT/TO. hPPFIA1 was also subcloned into the pIRES-puro-Strep-HA plasmid. For truncation mutations of Kif7 and PPFIA1 see Table S3. All PCR-amplified regions were verified by sequencing. pBABE-neo largeT cDNA was obtained from Addgene and used to immortalize the Kif7−/− MEFs. Primers used for cloning are listed in Table S1. Detailed descriptions of the different plasmids and sequences are provided upon request.

2.3.5 Generation of C3H10T1/2 knock-out cell lines with CRISPR-Cas9 system

sgRNAs targeting mKif7 and mPpfia1 were selected and cloned in px330 according to instructions from (http://www.genome-engineering.org/crispr/) (Cong et al. 2013). Kif7 or PPFIA1 homology arms surrounding the predicted sgRNA cut location were amplified by PCR from mouse genomic DNA and cloned into a donor plasmid designed to introduce a puromycin resistance expression cassette through homologous recombination (HR). C3H10T1/2 cells were transfected with px330 and the HR donor plasmid with Lipofectamine 2000 (Life Technologies). Cells were selected with puromycin 48h post transfection. Single-cells were
isolated by serial dilution and screened by PCR for homozygous disruption of the targeted alleles. Sequences for the sgRNA and the primers used for cloning are listed in Table S1.

2.3.6 RT-PCR and quantitative real-time PCR

Total RNA was extracted from cells using TRIzol (Life Technologies) according to the manufacturer’s instructions. For cDNA synthesis, 2µg of total RNA was reverse transcribed using random primers and Superscript II reverse transcription (Life Technologies). Real-time PCR was performed in a 7900HT Fast Real-Time PCR system using 2.5µl of the synthesized cDNA product plus the SYBR Green and primers mix in a final volume of 20µl (Life Technologies). SYBR gene expression assays (Applied Biosystems) were used to measure the mRNA expression of genes in triplicate. Mean relative gene expression was determined using the ΔΔ Ct method normalized to Cyclophilin mRNA (Bookout et al. 2006). Primers used are listed in Table S1. qPCR figures present averages and SEM of 3 independent experiments.

2.3.7 Affinity purification, immunoprecipitation, and Western blot analysis

Cells were lysed and protein complexes affinity purified using Streptavidin sepharose beads (GE Healthcare) or anti-FLAG M2 agarose beads (Sigma-Aldrich), or immunoprecipitated with the indicated antibodies. In brief, cells were solubilized in lysis buffer containing 0.1% NP-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 10 mM NaF, 0.25 mM Na3VO3, 100 mM β-glycerophosphate, and protease inhibitor cocktail (Sigma) for 1h at 4°C. Lysates were spun down at 20,000g for 10min at 4°C. Supernatants were collected and affinity purified with antibodies or with appropriate affinity resins for 24h, followed by extensive washing of the beads with lysis buffer. Co-purified proteins were eluted from the beads at 95°C for 10min using 4× Laemmli buffer containing β-mercaptoethanol (Sigma-Aldrich), resolved by SDS-PAGE, and transferred onto nitrocellulose or PVDF membranes (Pall) for Western blot analysis.

2.3.8 Zebrafish morpholino experiments
Zebrafish microinjection and handling were carried out using standard techniques. Translation blocking morpholinos were obtained for both Kif7 (5’ GCCGACTCCTTTTGGAGACATAGCT 3’) and PPFIA1 (5’GGTGCGCATACCTCGACATCATC 3’) from Gene-Tools. The Kif7 MO has been previously characterized (Putoux, Thomas et al. 2011). 8ng of either Kif7 or Ppfia1 MO were injected per embryo. Embryos were imaged at 30hpf and for each embryo a single somite in the middle of the yolk extension was chosen for quantification. Somite angles were measured using ImageJ software. Two batches of embryos were analyzed.

2.3.9 Immunofluorescence microscopy and image acquisition

Cells grown on coverslips were fixed with cold 100% methanol for 5 min. Coverslips were covered with antibodies in 1% normal donkey serum in PBS overnight at 4°C. Slides were mounted on coverslips using Vectashield mounting media (Vector Laboratories). Laser scanning confocal images were acquired using a Plan-Apochromat 63×/1.4 NA oil immersion objective on a confocal microscope (LSM700; Carl Zeiss) operated ZEN software. eGFP/Alexa Fluor 488, Alexa Fluor 594, and Alexa Fluor 647 fluorophores were excited individually with 488-, 543-, 638-nm lasers, respectively, with appropriate filter sets. Uncompressed images were processed with Zeiss ZEN software Black edition. To quantify cilium tip localization, images were taken as Z-stacks and rendered as 3D projections. Cilia with Kif7 or Gli at the tips were counted (30 cells in 10-15 independent images were used for each condition). Cilia tips localization was expressed as the percentage of cilia exhibiting Kif7 or Gli proteins localization at the tips. Average and SEM were calculated from 3 independent experiments. % of protein of interest at cilium tip was analyzed with Fisher's Exact Test.

2.3.10 FLAG Affinity purification protocol for mass spectrometry

Lysates of HEK293T cells expressing FLAG-hKif7, FLAG- hPPFIA1, FLAG-hGli3 were processed as previously described using the M2-FLAG magnetic bead affinity purification protocol and on-bead-digest (Kean, Couzens et al. 2012). 25% of the volume of the digested sample was analyzed on a TripleTOF® 5600 (AB SCIEX), using a Nanoflex cHiPLCsystem at 200 nL/min (Eksigent ChromXP C18 3 μm x 75μm x 15 cm column chip). Buffer A was 0.1%
formic acid in water; buffer B was 0.1% formic acid in ACN. The HPLC delivered an acetonitrile gradient over 120 min (2-35% buffer B over 85 min, 40-60% buffer B over 5 min, 60-90% buffer B over 5 min, hold buffer B at 90% 8 min, and return to 2% B at 105 min). The parameters for acquisition were 1 MS scan (250 ms; mass range 400-1250) followed by up to 50 MS/MS scans (50 ms each). Candidate ions between charge states 2-5 and above a minimum threshold of 200 counts per second were isolated using a window of 0.7 amu. Previous candidate ions were dynamically excluded for 20 sec with a 50 mDa window.

2.3.11 Mass spectrometry data acquisition and analysis

TripleTOF 5600 .wiff files were converted to .mgf format using ProteinPilot software before being saved into ProHits (Liu, Zhang et al. 2010). Files were analyzed with the iProphet pipeline (Shteynberg et al. 2011) implemented within ProHits as follows. The database consisted in the human and adenovirus complements of the RefSeq protein database (version 57) supplemented with “common contaminants” from the Max Planck Institute (http://maxquant.org/downloads.htm) and the Global Proteome Machine (GPM; http://www.thegpm.org/crap/index.html). The search database consisted of forward and reverse sequences (labeled “gi|9999” and more recently “DECOY”); in total 72226 entries were searched. The search engines used were Mascot (2.3.02; Matrix Science) and Comet (2012.01 rev.3), with trypsin specificity (2 missed cleavages were allowed) and deamidation (NQ) and oxidation as variable modifications. Charges +1, +2 and +3 were allowed, and the precursor mass tolerance was set at 50ppm while the fragment bin tolerance was set at 0.6 amu. The resulting Comet and Mascot search results were individually processed by PeptideProphet (Keller, Nesvizhskii et al. 2002), and combined into a final iProphet output using the Trans-Proteomic Pipeline (Linux version, v0.0 Development trunk rev 0, Build 201303061711). TPP options were as follows: general options are -p0.05 -x20 -d"gi9999", iProphet options are –ipPRIME and PeptideProphet options are –OpdP. The general TPP options were -p0.05 -x20 -PPM -d"DECOY". All proteins with a minimal iProphet probability of 0.05 were parsed to the relational module of ProHits. Note that for analysis with SAINT, only proteins with iProphet protein probability > 0.95 are considered. This corresponds to an estimated FDR of ~0.5%. To identify significant interaction partners from the affinity purification data, the data were
subjected to SAINT analysis (Choi et al. 2012) implemented in ProHits using SAINTexpress. Proteins with average SAINT score (AvgP) greater than 0.98 were considered to be statistically significant. Artifact proteins (such as trypsin and keratin) were manually curated from the final interaction partner list (Table 2-1).

2.3.12 MRM- Affinity purification for mass spectrometry

Affinity purification was performed as described above using the on-bead-digest protocol, with minor modifications. Briefly, cell pellets were lysed by resuspension in 1:4 (pellet weight/volume) ratio of lysis buffer (50mM HEPES-KOH (pH 8.0), 100mM KCl, 2mM EDTA, 0.1% NP40, 10% glycerol, 1mM PMSF, 1mM DTT, 15nM Ca, 15nM OA and 1X protease inhibitor cocktail (Sigma; P8340) followed by two freeze/thaw cycles. Clarified lysate was immunoprecipitated with 25μL of magnetic anti-FLAG M2 beads (Sigma) for 2 hours at 4°C. One wash with 1mL of lysis buffer was then performed followed by an additional wash with 1mL of 20mM Tris-HCl pH8 2mM CaCl₂. For mass spectrometry analysis, 7.5μL of 20mM Tris-HCl pH8 containing 750ng of trypsin (Sigma; resuspended at 100ng/μl in Tris buffer) was added, and the mixture was incubated at 37°C with agitation for 15 hours. The next morning, the tubes were quickly centrifuged, the beads the magnetized and the partially digested sample transferred to a fresh tube before addition of an extra 2.5μL of 20mM Tris-HCl pH8 containing 250ng trypsin. The samples were incubated for 3 hours at 37°C before addition of 1μL of 50% formic acid. The samples were stored at -80°C until analysis.

2.3.13 MRM- Mass spectrometry data acquisition

Samples were analyzed on the 5600 TripleTOF in two phases: (i) Data dependent acquisition (DDA) and (ii) high resolution multiple reaction monitoring (MRM\textsuperscript{HR}) using the same gradient conditions and the same amounts of sample. For DDA, one quarter of the volume of the digested sample was analyzed using a packed tip emitter at 200 nL/min. Buffer A was 0.1% formic acid in water; buffer B was 0.1% formic acid in ACN. The HPLC delivered an acetonitrile gradient over 120 min (2-35% buffer B over 85 min, 40-60% buffer B over 5 min, 60-90% buffer B over
5 min, hold buffer B at 90% 8 min, and return to 2% B at 105min). The DDA parameters for acquisition on the TripleTOF 5600: were 1 MS scan (250ms; mass range 400-1250) followed by up to 20 MS/MS scans (50ms each). Candidate ions between a 2-5 charge state and above a minimum threshold of 200 counts per second were isolated using a window of 0.7amu. Previous candidate ions were dynamically excluded for 20sec with a 50mDa window. Data from the DDA analysis were searched with (2.3.02; Matrix Science) with trypsin specificity (3 missed cleavages were allowed) and deamidation (NQ), oxidation and phosphorylation (STY) as variable modifications. Charges +2, +3 and +4 were allowed, and the parent mass tolerance was set at 50 ppm while the fragment bin tolerance was set at 0.15Da. Kif7 peptides containing sites of phosphorylation, along with several control peptides (Table S1) were included in a subsequent targeted MRM$^{HR}$ analysis. MS/MS resulting from the MRM$^{HR}$ acquisition was used to quantify intensities between OA treated and control samples.

### 2.3.14 SWATH

SWATH sample preparation and data analysis were done according to Lamert et al (Lambert, Ivosev et al. 2013). Briefly, 2x15cm plates of HEK293T cells stably expressing FLAG-mKif7 wild-type or point mutant were collected. FLAG-GFP cells were used as control.

### 2.3.15 Statistical analysis

The quantification of cilium tip localization was analyzed by Fisher's Exact Test. qPCR and somite angles data were analyzed by Student's T test (GraphPad). All statistical analysis was considered significant at p < 0.05.
2.4 Results

2.4.1 Kif7 promotes Gli ciliary localization and Hh signalling

Previous work has shown that Kif7 accumulates at the tips of primary cilia upon Hh pathway activation, and that in \( Kif7^{-/-} \) MEFs there is a decreased Hh-promoted accumulation of Gli3 at the tips of primary cilia. This suggests that Kif7 may be required for the ciliary trafficking of Gli proteins (Endoh-Yamagami, Evangelista et al. 2009). To directly test this, we showed that upon activation of Hedgehog signalling with the Smo agonist SAG, the trafficking of Gli2 and Gli3 to the tips of cilia, but not the accumulation of Smo, was decreased in \( Kif7^{-/-} \) MEFs (Figure 2-1A). The lentiviral delivery of Kif7-GFP in \( Kif7^{-/-} \) MEFs rescued Kif7 and Gli ciliary localization to the tips of cilia upon SAG treatment (Figure 2-1B, C). In the absence of stimulation with SAG, overexpression of Kif7GFP was not sufficient to promote localization of Gli proteins to the tips of cilia or to induce \( Gli1 \) mRNA expression (Figure 2-1B, D). In \( Kif7^{-/-} \) MEFs, the basal expression of the Hh target gene \( Gli1 \) was minimally affected but its induction by SAG treatment was compromised when compared to MEFs reconstituted with Kif7 (Figure 2-1E). This result suggested that the formation of Gli\(^A\) is impaired in the absence of Kif7. We conclude that, during Hh pathway activation, Kif7 promotes the localization of Gli proteins at the tips of cilia and that in MEFs Kif7 chiefly exerts a positive signalling role.
Figure 2-1. Loss of Kif7 impairs Gli trafficking and Gli1 mRNA induction. A) The SAG-induced accumulation of Gli2 and Gli3 at the tips of cilia is impaired in Kif7−/− MEFs, whereas the SAG-induced ciliary accumulation of Smo is unaffected. B) Stable expression of Kif7-GFP in Kif7−/− MEFs (Kif7−/− MEFs+Kif7GFP) rescues the normal localization of Kif7, Gli2 and Gli3 to the tips of cilia following SAG treatment. C) Western blot confirming expression of Kif7GFP in the rescued cells (Left) and qPCR confirming absence of Kif7 mRNA in Kif7−/− MEFs and rescued levels in Kif7−/− MEFs+Kif7GFP (Right). D) Western blot comparing expression level of endogenous Kif7 in MEFs and overexpression of Kif7GFP in Kif7−/− MEFs (Left) and qPCR comparing expression level of Gli1 mRNA in MEFs and in overexpression of Kif7GFP in Kif7−/− MEFs (Right). E) The time and SAG dependent induction of the Hh target gene Gli1 is impaired in Kif7−/− MEFs but is rescued in Kif7−/− MEFs+Kif7GFP. Gli1 mRNA expression was calculated relative to that in Kif7−/− MEFs+Kif7GFP treated with SAG for 24 h. ΔCT value is calculated by subtracting CT value of cyclophilin from CT value of Kif7. qPCR data are represented as mean ± SEM across 3 biological replicates. Western blot figures are representative of 3 independent repeats. N.D, not detectable. Quantification of ciliary tips localization and qPCR
2.4.2 Proteomic analysis of Kif7 identified PPFIA1 and PP2A as novel interactors

To further understand the regulation and functional importance of protein trafficking during Hh signalling, we used mass spectrometry to sample the composition of Gli3- and Kif7-containing protein complexes purified from mammalian cells (Figure 2-2A, B and Table 2-1). Gli3 interacted with the Hh signalling regulators SuFu and Kif7. Consistent with the localization of Kif7 in cilia, several centrosome and basal body proteins were identified in Kif7 complexes. We also identified Liprin-α1 (PPFIA1), a member of the Liprin family of scaffolding proteins previously recognized for their role in synaptogenesis, in Kif7 purifications (Stryker and Johnson 2007). In support of this finding, Kif7 was detected in PPFIA1 immunoprecipitates by LC-MS/MS and endogenous Kif7 and PPFIA1 coimmunoprecipitated (Figure 2-2 C). Stimulation of cells with the Smo agonist SAG increased the interaction between PPFIA1 and Kif7 (Figure 2-2C), hinting that formation of this complex is important during Hh signalling. Domain mapping experiments using different Kif7 and PPFIA1 mutants showed that these proteins interacted through their coiled-coil domains (Figure 2-8). Consistent with previous reports (Arroyo, Lee et al. 2008, Glatter, Wepf et al. 2009, Goudreault, D’Ambrosio et al. 2009), several peptides assigned to regulatory and catalytic subunits of PP2A were identified in PPFIA1 immunoprecipitates. Endogenous regulatory PP2A subunit PPP2R1A could only be co-immunoprecipitated together with FLAG-Kif7 when PPFIA1 was over-expressed in cells (Figure 2-2 D). These results suggested that Kif7 interacts indirectly with the PP2A phosphatase through binding to PPFIA1. We conclude that PPFIA1 and the PP2A phosphatase form a protein complex with Kif7.
Figure 2-2. Mass spectrometry analysis of Kif7, Gli3, and PPFIA1 protein complexes. A) Heat map representation of high-confidence Kif7, Gli3, and PPFIA1 interacting proteins identified by FLAG IP-MS. Sum of the spectral counts across two biological replicates for each of the bait is represented as a colour code and sorted from highest to lowest spectral counts for the PPFIA1 bait. B) Cytoscape representation of Kif7, Gli3, and PPFIA1 interactomes. Red edges indicate that the protein-protein interaction has been previously reported. See the inset for the legend. C) Co-immunoprecipitation of endogenous Kif7 with PPFIA1 from lysates of MEFs treated or not with SAG. Right, quantification of 3 independent experiments. D) Co-immunoprecipitation of regulatory subunit of PP2A (PPP2R1A) and FLAG-Kif7 with or without overexpression of Strep-HA-PPFIA1. The interaction of Kif7 with PPP2R1A was only detected by western blot when PPFIA1 was overexpressed. Western blots are representative of 3 biological replicates. NT: non-treated.

2.4.3 PPFIA1 is required for efficient trafficking of Kif7, Gli2 and Gli3 to the tips of cilia and for Hh signalling

PPFIA1 plays important roles in transporting cargoes in neurons (Wyszynski et al. 2002). In this context, PPFIA1 interacts with and recruits cargo vesicles to the neuron specific kinesin motor protein Kif1A (Shin et al. 2003). Drosophila and C. elegans PPFIA1 homologs stimulate kinesins by promoting their activity and/or specific subcellular localization (Miller et al. 2005, Wagner et al. 2009, Hsu et al. 2011). This led us to hypothesize that PPFIA1 could promote Kif7 trafficking in cilia and consequently impinge on the localization of Gli proteins and on Hh signalling. Consistent with this notion and confirming previous large-scale proteomic results (Liu, Tan et al. 2007, Ishikawa et al. 2012), we found that PPFIA1 localized to the basal body and to the cilia axoneme (Figure 2-3A). Specificity of the PPFIA1 antibody is shown in Figure 2-9A. PPFIA1 basal body/cilia localization did not change in response to Smo modulation, suggesting its localization is not Hh responsive (Figure 2-3B).

We found that 42% of MEFs stably expressing Kif7GFP and stimulated with SAG exhibited Kif7 localization at the tips of cilia (Figure 2-3C and D, left panel). We next determined that, of the four Liprin-alpha genes (Ppfia1-Ppfia4), MEFs express predominantly Ppfia1 with trace
amounts of the other 3 *Ppfia* genes (Figure 2-9B). Depletion of *Ppfial* using two independent shRNAs (Figure 2-9C) significantly reduced the percentage of cells with Kif7 localization at the tips of cilia upon stimulation of the pathway with SAG (Figure 2-3C, quantification in D). Knockdown of *Smo* resulted in a similar reduction in ciliary tips localization of Kif7 (Figure 2-3C and D). To rule out off-target effects, we stably expressed human PPFIA1 following infection of cells with the shRNA that targets mouse *Ppfial*, which rescued Kif7 trafficking to the tips of cilia (Figure 2-3H, left). Efficiency of rescue is shown in Figure 2-9D. We also introduced Kif7-GFP into MEF cells derived from *Ptch1*−/− knockout mice (*Ptch1*−/−MEFs), which exhibit constitutive Hh pathway activity (Vorechovsky et al. 1997), and found that 57% of cells had constitutive Kif7 localization at the tips of cilia (Figure 2-3C and D, right panel). Depletion of *Ppfial* or *Smo* using shRNAs or inhibition of pathway activity using the Smo antagonist cyclopamine similarly decreased the proportion of *Ptch1*−/− MEFs with Kif7 at the tips of cilia (Figure 2-3D, right panel). These results suggest that PPFIA1 promotes the localization of Kif7 to the tips of cilia during Hh signalling.

Our results demonstrated that Kif7 was required for robust ciliary trafficking of Gli proteins and target gene induction during activation of the Hh pathway (Figure 2-1). Since depletion of *Ppfial* inhibited Kif7 trafficking to the tips of cilia during pathway activation, we predicted that this would also affect Gli2 and Gli3 localization and consequently Hh target gene activation. Knockdown of *Kif7* or *Ppfial* in MEFs and *Ptch1*−/−MEFs both repressed the SAG-induced and the constitutive localization of Gli2 and Gli3 to the tips of cilia in these cells, respectively (Figure 2-3E and quantification in F). Depletion of *Ppfial* inhibited the SAG-induced *Gli1* mRNA expression in MEFs and its constitutive activation in *Ptch1*−/− MEFs to similar amounts obtained with *Kif7* knockdown (Figure 2-3G), and this was rescued upon overexpression of hPPFIA1 (Figure 2-3H, right), thereby supporting a requirement for PPFIA1 during Hh pathway activation. Furthermore, C3H10T1/2 cell lines with homozygous disruption of *Ppfial* or *Kif7* using the CRISPR-Cas9 (Cong, Ran et al. 2013) gene editing system showed an attenuated response to SAG that was partially rescued upon expression of cDNAs encoding PPFIA1 or Kif7 (Figure 2-3I). *Ppfial*−/− cells exhibited a reduction in Gli3R formation similar to that seen in *Kif7*−/− cells (Cheung, Zhang et al. 2009, Endoh-Yamagami, Evangelista et al. 2009, Liem, He et al. 2009)(Figure 2-3J).
Figure 2-3. PPFIA1 is required for localization of Kif7 and Gli proteins to the tips of cilia and for Hh signalling. A) PPFIA1 localizes to the primary cilium. Indirect immunofluorescence images of MEFs stained for PPFIA1 (green), acetylated Tubulin (red) and Pericentrin (blue). B) PPFIA1 cilia localization quantification in Ptch1+/− MEFs, treated or not with cyclopamine. C) Immunofluorescence images of MEFs (top and middle) and Ptch1+/− MEFs (bottom) expressing Kif7GFP (green) stained for acetylated Tubulin (red) and Pericentrin (blue). D) shRNA-mediated knockdown of Ppfia1 or Smo impaired the ciliary tips localization of Kif7 during activation of the Hh pathway in MEFs (left) and in Ptch1+/− MEFs (right). E) Immunofluorescence images of fixed MEFs and Ptch1+/− MEFs stained for Gli2 and Gli3 (green) and acetylated Tubulin (red). F) Knockdown of Kif7 or Ppfia1 with shRNAs impaired the SAG-promoted and constitutive localization of Gli2 and Gli3 at the tips of cilia in MEFs (left) cells and in Ptch1+/− MEFs (right) respectively. G) shRNA-mediated depletion of Kif7 or Ppfia1 impairs Gli1 mRNA induction by SAG (left) or in Ptch1+/− MEFs (right). Control is the max activation of Gli1 by SAG. H) The Kif7 ciliary tips localization defects seen upon knockdown of mPpfia1 are rescued upon over-expression of hPPFIA1 (left). Decrease in SAG-promoted Gli1 mRNA expression in cells expressing knockdown of mPpfia1 is rescued upon overexpression of hPPFIA1 (Right). I) CRISPR-Cas9 mediated knockout (KO) of Kif7 (left) or Ppfia1 (right) in C3H10T1/2 cells impaired Gli1 mRNA induction with SAG. Gli1 mRNA expression was calculated relative to that in SAG-treated wild-type cells. Western blots showing expressing of Kif7 or PPFIA1 are at the bottom. J) Western blot for Gli2 and Gli3 in C3H10T1/2 WT, Kif7 KO and Ppfia1 KO. Quantification of ciliary tips localization, qPCR data are presented as mean ± SEM across 3 biological replicates. *p<0.05. Bars, 1µm. Western blots are representative of 3 biological replicates.

2.4.4 Loss of Kif7 or PPFIA1 results in somite angle defect in zebrafish embryos

Lastly, to further examine the role of PPFIA1 in Hh signal transduction, we asked if morpholino-mediated knockdown of Ppfia1 in zebrafish would phenocopy the somitogenesis defects caused by interference with Kif7 function or with other Hh pathway components (Wolff et al. 2003, Putoux, Thomas et al. 2011). In zebrafish the amount of Hh signalling influences the
fates of skeletal muscle cells. High amounts of Hh signalling is required for specification of muscle pioneer cells, slow muscle cells require intermediate amounts of Hh activity, and fast muscle cells form in the absence of Hh activity (Wolff, Roy et al. 2003). Improper regulation of Hh signalling therefore leads to defective somitogenesis with individual somite losing their characteristic chevron shape. Injection of morpholinos targeting Kif7 or Ppfia1 into embryos led to similar phenotypes (Figure 2-4, quantification on the right) with morphant embryos having significantly wider somite angles (Kif7: 96.6°, Ppfia1: 104.6°) than control morpholino-injected embryos (83.7°). Our data suggest that loss of Ppfia1 phenocopies loss of Kif7 in vivo. We conclude that PPFIA1 positively affects Hh signalling by promoting the localization of Kif7 and Gli proteins in the tips of cilia.

**Figure 2-4. Loss of Kif7 or PPFIA1 results in somite angle defects.** Representative images of whole embryos (top) or magnified views of dotted area (bottom) injected with control, Kif7, or Ppfia1 morpholinos at the 1 cell stage. Images were taken at 30h post-fertilization. In control-injected embryos, somites have a stereotypic chevron shape whereas they are abnormal in Kif7 or Ppfia1 morphant embryos. Quantification of somite angles (bottom panel). n=20 embryos per condition, two batches of embryos were analyzed. Data are presented as mean ± SEM, *p<0.05.

### 2.4.5 The PP2A inhibitor okadaic acid inhibits Kif7 and Gli trafficking in cilia and blocks Hh signalling

Endogenous PPFIA1 coimmunoprecipitated with the catalytic subunit of PP2A (PP2Ac) from MEF lysates (Figure 2-5A), thus supporting our mass spectrometry data. We used okadaic acid, an inhibitor of the PP1 and PP2A-family of serine threonine phosphatases, to test for the
potential involvement of PP2A in ciliary trafficking of Kif7, Gli2, and Gli3 as well as for Hh pathway activity. Okadaic acid treatment inhibited the SAG-induced or constitutive accumulation of Kif7 (Figure 2-5B) and Gli2 and Gli3 (Figure 2-5C) at the tips of cilia in MEFs and $Ptch1^{-/-}$ MEFs respectively. As a control, we showed that the ciliary accumulation of Smo was not affected by okadaic acid in both $Ptch1^{-/-}$ MEF (left) and MEF (right) cells (Figure 2-5D). Consistent with a requirement of Kif7 and Gli proteins trafficking in cilia for pathway activation, okadaic acid treatment inhibited the induction of Gli1 mRNA in response to SAG treatment (Figure 2-5E). Consistent with the role of Kif7 in Gli$^{R}$ formation, okadaic acid treatment slightly decreased Gli3$^{R}$ abundance without altering the amount of the full-length forms of Gli2 or Gli3 (Figure 2-5F). These results suggest the possibility that PP2A activity is required during Hh signalling to promote the ciliary tip trafficking of Kif7 and Gli proteins.
Figure 2-5. The protein phosphatase PP2A promotes Hh signalling through control of Kif7 and Gli proteins localization at the tips of cilia. A) Co-immunoprecipitation of PPFIA1 and the catalytic subunit of PP2A (PP2Ac) from MEF lysate. The PP2A inhibitor okadaic acid inhibits the localization of Kif7 (B) or Gli2 and Gli3 (C) to the tips of cilia in SAG-treated MEFs or in Ptch1<sup>+/−</sup> MEFs. D) Treatment of Ptch1<sup>+/−</sup> MEFs with okadaic acid, Kif7 shRNA #1 or PPFIA shRNA #1 has no effect on the constitutive localization of Smo in cilia (left), okadaic acid does not inhibit the SAG-induced ciliary localization of Smo in MEFs (right). E) Okadaic acid inhibits the activation of the Hh target gene Gli1 by SAG in MEFs or its constitutive activation in Ptch1<sup>+/−</sup>MEFs. Gli1 mRNA expression was calculated relative to that in SAG-treated MEFs or untreated Ptch1<sup>+/−</sup>MEFs. F) Western blot of Gli2 and Gli3 in MEF cells treated with okadaic acid. Okadaic acid treatment slightly inhibits Gli3<sup>R</sup> formation. Quantification of cilia tips or Smo cilia localization and qPCR data are presented as mean ± SEM across 3 biological replicates. Western blot is representative of 3 biological replicates. NT: non-treated.*p<0.05.

2.4.6 Kif7 de-phosphorylation promotes its localization at the tip of primary cilium and Hh signalling.

The above results identify Kif7 as a putative target of PP2A and suggest that post-translational control of Kif7 phosphorylation may be important for its localization at the tips of cilia and for Hh signalling. Stimulation of C3H10T1/2 cells with SAG resulted in a decrease in Kif7 phosphorylation as determined by faster migration of the Kif7 immunoreactive band on phosphatase-containing gels (Figure 2-6A, left). In contrast, treatment of Ptch1<sup>+/−</sup>MEFs with the Smo antagonist cyclopamine led to an upward shift of Kif7, suggesting that Kif7 is phosphorylated when the pathway is inhibited (Figure 2-6A, right). Mass spectrometry of FLAG-Kif7 immunopurified from HEK293T cells identified 3 phosphorylation sites that corresponded to mouse Ser<sup>897</sup>, Ser<sup>969</sup>, and Ser<sup>1337</sup>. We then designed a targeted high-resolution multiple reaction monitoring (MRM<sup>HR</sup>) mass spectrometry method to quantify the abundance of each of these phosphopeptides in FLAG-Kif7 immunoprecipitates. The abundance of the C-terminal 1330-1339 phosphopeptide that contains phosphorylated Ser<sup>1332</sup> (which corresponds to phosphorylated Ser<sup>1337</sup> in mouse Kif7) was the only phosphopeptide that showed varying
abundance according to okadaic acid treatment suggesting that phosphorylation of this residue might be dependent on PP2A (Figure 2-6B, Table 2-2). The abundance of the non-phosphorylated version of these peptides and of three other non-phosphorylated control peptides used as internal standards did not substantially vary (Figure 2-6B, 2-10A and Table 2-2). We then expressed Kif7-wild-type and Kif7-S1337A in MEFs and determined that the electrophoretic mobility of the Kif7-S1337A mutant was faster than Kif7-wild-type when using phos-tag-containing gels suggesting that this site was indeed targeted by phosphorylation (Figure 2-6C).

To investigate the functional importance of phosphorylation of Ser\(^{1337}\) for Kif7 function, we generated MEFs stably expressing the Kif7-S1337A-GFP or Kif7-S1337D-GFP point mutants that mimic the un-phosphorylated and phosphorylated states of this residue respectively. Expressions of the GFP fusion proteins are shown in Figure 2-10B. A large proportion of cells (~44%) exhibited constitutive, ligand-independent localization of Kif7-S1337A to the tips of cilia (Figure 2-6D, quantification on the right). Conversely, Kif7-S1337D did not localize at the tips of cilia in a ligand-independent fashion but its localization to cilia tips was impaired in SAG-treated MEFs when compared to wild type Kif7 (Figure 2-6F). Since we showed that Gli proteins were cargoes of Kif7 in cilia (Figure 2-1), we reasoned that their ciliary tips localization should also be affected in cells expressing the Kif7 mutants. Expression of Kif7-S1337A was indeed sufficient to induce the accumulation of Gli2 and Gli3 at the tips of cilia (Figure 2-7D, middle graph), whereas expression of Kif7-S1337D reduced the localization of Gli2 and Gli3 at the tips of cilia in SAG-stimulated MEFs (Figure 2-7F, second panel).

Because the Kif7-S1337A mutant is constitutively at the tips of cilia, a localization that correlates with the state of activation of the Hh pathway, we next tested whether its expression is sufficient to activate Hh signalling. In MEFs, stable expression of Kif7-S1337A, but not wild-type Kif7, led to robust 30 fold induction of Gli1 mRNA abundance (Figure 2-7D, third panel). Other Hh target genes, such as PtcH1 and Hhip, were also induced upon Kif7-S1337A expression (Figure 2-6E). Moreover, the constitutive ciliary localization of Kif7-S1337A (left) and its ability to activate Hh signalling (right) were Smo-independent since the Smo antagonist cyclopamine had no effect (Figures 2-6G). However, the Gli inhibitor GANT-61 (Lauth, Bergstrom et al. 2007) partially inhibited the Kif7-S1337A dependent Gli1 mRNA induction (Figure 2-6G left). Reciprocally, expression of Kif7-S1337D decreased SAG-induced
localization of Gli proteins at the tips of cilia and Gli1 mRNA induction (Figure 2-6F, middle and right panel). A form of Kif7 with similar mutations at another phosphosite Ser969 did not show altered ciliary tips localization and expression of this mutant did not affect the induction of Gli1 (Figure 2-10C and D). Furthermore, expression of Kif7-S1337A in either Kif7−/− or Ppfia1−/− cells was also sufficient to increase Gli1 mRNA (Figure 2-6H).

Our mass spectrometry analysis thus identified Ser1337 as an okadaic acid-sensitive Kif7 phosphorylation site. Since Hh signalling is inhibited by okadaic acid and Kif7-S1337D expression and activated by Kif7-S1337A expression, we surmised that PP2A inhibition could lead to phosphorylation of Ser1337 and that PP2A-mediated de-phosphorylation of Kif7 at this residue could be required for Kif7 localization to the tips of cilia and Hh target gene activation. Our results showed that Kif7-S1337A-mediated activation of Gli1 transcription or its ligand-independent localization at the tips of cilia were unaffected by okadaic acid treatment (Figure 2-6I). Thus, Kif7-Ser1337 de-phosphorylation appears to be a key step in Kif7 trafficking to the tips of cilia and our results suggest that PP2A-mediated de-phosphorylation of this residue is required for Gli-mediated transcriptional output.
Figure 2-6. Kif7 phosphorylation dictates its ciliary tip localization and Hh signalling. A) Western blots of lysates from C3H10T1/2 cells treated or not with SAG (Left), or from Ptch1\(^{-/-}\) MEFs treated or not with cyclopamine (Right) using phos-tag gels and anti-Kif7 antibodies. B) The abundance of three Kif7 phosphorylation sites was quantified by MRM\(^{HR}\) in cells treated or not with okadaic acid. Both the non-phosphorylated (grey) and phosphorylated peptides (blue) were quantified. Numbering refers to the amino acid positions in human Kif7. The dashed box indicates the only peptide (containing Ser\(^{1332}\) in human and Ser\(^{1337}\) in mouse Kif7) whose abundance was increased by okadaic acid treatment. *Denotes phospho serine. The amounts indicated are from a representative MRM experiment; for the results of the second experiment see Table 2-2. C) Western blots of lysates from MEFs over-expressing Kif7-WT or Kif7-S1337A mutant using phos-tag gel and anti-Kif7 antibodies. D) Kif7-S1337A mutant exhibits constitutive localization at the tips of cilia in MEFs (first and second panels). Expression of Kif7-S1337A also leads to ligand-independent localization of Gli2 and Gli3 to the tips of cilia (third panel). Kif7-S1337A expression is sufficient to induce Gli1 mRNA expression, which was calculated relative to that in cells expressing wild-type Kif7 (fourth panel). E) Kif7-S1337A expression activates Hh target genes Hhip1 and Ptch1. F) The Kif7-S1337D mutant displays impaired ciliary tips localization in response to SAG treatment compared to Kif7 wild-type (left). Expression of this mutant inhibits the ciliary tips localization of Gli proteins (middle) and the SAG-mediated activation of Gli1 mRNA expression, which was calculated relative to SAG-treated cells expressing wild-type Kif7 (right). G) Kif7-S1337A ciliary tips localization is independent of Gli signalling (no effect of GANT-61) or Smo activation (no effect of cyclopamine) (left), activation of Hh signalling by Kif7-S1337A expression requires Gli (inhibited by GANT-61) but not Smo (cyclopamine has no effect)(right). H) Kif7-S1337A expression activates Gli1 mRNA in Ppfia1\(^{-/-}\) C3H10T1/2 (left) and in Kif7\(^{-/-}\) MEFs (right). I) Okadaic acid treatment does not inhibit the induction of Gli1 mRNA expression in cells expressing the Kif7-S1337A mutant (left) or its constitutive ciliary tips localization (right) suggesting that PP2A could be modifying this residue. Gli1 mRNA expression was calculated relative to untreated cells. Quantification of ciliary tips localization and qPCR data are presented as means ± SEM across 3 biological replicates. Western blot figure is representative of 3 biological replicates. NT: non-treated control. *p<0.05. Bar, 1µm.
2.4.7 Increased PPFIA- Kif7 association in Kif7 ciliopathy mutant

Mutations in Kif7 have been identified in various human ciliopathies including acrocallosal syndrome, Bardet–Biedl syndrome, Meckel syndrome and hydrolethalus (Putoux, Thomas et al. 2011, Putoux, Nampoothiri et al. 2012). Some of these mutations occur in the coil-coiled domain of Kif7 that we mapped as the PPFIA1 binding site. We derived stable cell lines expressing four randomly selected coiled-coil mutant Kif7 proteins and performed data dependent (FLAG-AP/MS) and data independent SWATH-MS to determine whether the mutations affected the association of Kif7-interacting proteins (Figure 2-7A). The immunoprecipitates for Kif7-L759P (mouse Kif7-L764P), a mutation found in Bardet–Biedl syndrome, contained an increased number of peptides corresponding to PPFIA1 and PP2A subunits as compared to wild type kif7 suggesting increased association of this mutant Kif7 for the PPFIA1-PP2A complex (Figure 2-7B). This result was further confirmed by SWATH-MS (Lambert, Ivosev et al. 2013), cells stably expressing Kif7-L759P showed increase peptide abundance of Liprin and PP2A family proteins as compare to wild-type Kif7 or other Kif7 mutants (Figure 2-7C & Figure 2-11). I hypothesized that in the mutant L759P cell line, Kif7-PPFIA1 interaction might be enhanced. Indeed, by immunoprecipitation, Kif7-L759P showed increased precipitation of endogenous PPFIA1 when compared to wild-type Kif7 (Figure 2-7D). We hypothesized that enhanced binding of Kif7-L759P to the PPFIA1-PP2A complex would promote its de-phosphorylation and localization at ciliary tips. 32% of MEFs expressing Kif7-L759P-GFP exhibited ligand-independent localization of Kif7-L759P at the tips of cilia (Figure 2-7E). Expression of Kif7-L759P was sufficient to induce Gli1 expression 7-fold compared to wild-type although it was expressed at lower amounts (Figure 2-7F). Disease-causing Kif7 mutations result in decreased Gli3R production (Putoux, Thomas et al. 2011) and we found that cells expressing the L759P mutant also exhibited decreased Gli3R abundance (Figure 2-7G). Together these results suggest that some of the Kif7 human mutations may directly or indirectly affect ciliary tips localization and activity by altering its phosphorylation state.
**Figure 2-7.** Disease causing *Kif7* mutations lead to increased interaction of *Kif7* with the PPFIA1-PP2A complex. A) Four disease causing *KIF7* mutants found in human ciliopathies. B) The protein-protein interaction profiles of the *Kif7* mutants were compared to *Kif7*-WT using FLAG AP-MS. More peptides attributed to PPFIA1 and subunits of PP2A were identified in *Kif7*-L759P immunoprecipitates than in *Kif7*-WT immunoprecipitates. Mean sum of total peptide ±SEM across 3 independent experiments are listed in the table. C) SWATH-MS analysis of 293T cells expressing FLAG-*Kif7*-WT and four disease causing mutants. Data were mined in retrospective using FLAG-*Kif7* spectra reference libraries generated before. D) Lysates of cells stably expressing FLAG-*Kif7*-WT or FLAG-*Kif7*-L759P were immunoprecipitated with FLAG antibodies and probed with anti-PPFIA1 antibodies using western blotting. E) The *Kif7*-L759P mutant stably expressed in MEFs localizes to the tips of primary cilia in a ligand-independent manner. F) *Kif7*-L759P expression leads to intermediate amounts of ligand independent *Gli1* activation compared to the phosphorylation-deficient *Kif7*-S1337A mutant. G) Western blots of *Gli2* (right) and *Gli3* (left) in *Kif7* WT, S1337A, S1337D, and L759P mutants in MEFs. *Gli1* mRNA expression was calculated relative to that in cells expressing wild-type *Kif7*. Quantification of ciliary tip localization and qPCR data are presented as mean ± SEM across 3 independent experiments. Western blot figures are representative of 3 biological replicates. *p<0.05.
2.5 Discussion

How the signalling information carried by Hh ligands is transduced from active Smo to regulation of Gli proteins is poorly understood. Trafficking of Gli proteins from the base to the tip of the primary cilium, a process thought to involve Kif7, leads to dissociation of Gli proteins from the negative regulator SuFu to allow for their nuclear translocation and activation of target genes. Kif7 is important for the localization of Gli proteins at the tips of cilia during pathway activation (Endoh-Yamagami, Evangelista et al. 2009), although it has been unclear whether Kif7 is directly required for Hh-ligand activation of Gli signalling. In the present study, we found that in Kif7<sup>-/-</sup> MEFs or Kif7<sup>-/-</sup>C3H10T1/2 cells, the ability of SAG to promote the accumulation of Gli proteins at the tips of cilia and to induce expression of the Hh target gene Gli<sub>1</sub> is compromised. In the absence of ligand, Kif7 promotes Gli<sup>R</sup> formation and redundantly functions with SuFu to repress Gli activity (Cheung, Zhang et al. 2009, Endoh-Yamagami, Evangelista et al. 2009, Liem, He et al. 2009). The dual negative and positive roles of Kif7 thus reflect its function in trafficking Gli proteins in cilia under resting conditions and during Hh-dependent activation to underlie Gli<sup>R</sup> and Gli<sup>A</sup> formation respectively. We note that Kif7 deficiency in Kif7<sup>-/-</sup> MEFs or Kif7<sup>-/-</sup>C3H10T1/2 cells does not result in increased ligand-independent pathway activation suggesting that Kif7 principally fulfills a positive role in these contexts and that SuFu amounts are likely sufficient to inhibit Gli. The use of these cell systems enabled us to specifically study the mechanisms by which Kif7 positively promotes Hh signalling. Our results differ from similar experiments that suggest the induction of Gli<sub>1</sub> expression by Hh is relatively normal in MEFs derived from Kif7<sup>-/-</sup> mice (Endoh-Yamagami, Evangelista et al. 2009). The reason for this discrepancy is unclear but could be due to heterogeneity in Hh responsiveness of different MEF cultures (wild type MEFs compared to Kif7<sup>-/-</sup> MEFs) or differences in Shh compared to SAG treatment. Nevertheless, our results that Gli protein trafficking to tips of cilia and robust SAG-induced Gli<sub>1</sub> activation were restored when Kif7 was reintroduced into Kif7<sup>-/-</sup> MEFs combined with the blunting of the SAG-mediated response when we knock out Kif7 in C3H10T1/2 cells indicate that Kif7 is required for efficient Hh signal transduction in these contexts. However, since the ligand-promoted response is not completely eliminated, possible redundant mechanisms supporting Gli activation may exist.

Kif7 has been implicated in the organization of the cilium tip (He, Subramanian et al. 2014). In this study, loss of Kif7 results in redistribution of Gli2 from the cilium tip to the axoneme.
Although we observed a strong reduction of Gli2 and Gli3 localization at the tips of cilia in Kif7 mutant cells, we do not observe a localization of these proteins to the axoneme. A difference in antibody sensitivity is a possible explanation for these differences.

Using mass spectrometry, we identified PPFIA1 and PP2A as Kif7-interacting proteins. Using loss of function experiments, we showed that PPFIA1 is required for Kif7 function. Indeed, in addition to inhibiting localization of Kif7 at the cilium tip during pathway activation, knockdown of Ppfia1 phenocopied several defects seen upon loss of Kif7 function, including reduced accumulation of Gli proteins at the tips of cilia, lower induction of the Gli1 target gene and defective somitogenesis in zebrafish embryos.

The protein phosphatase PP2A has previously been linked to Hh signalling in Drosophila where it appears to have multiple roles in dictating signalling output. For instance mts, which encodes the PP2A catalytic subunit in flies, has been identified as a gene required for Hh signalling in a siRNA screen (Casso, Liu et al. 2008). Subsequent studies have shown that mts interacts with and de-phosphorylates Smo (Su et al. 2011) and Ci (Jia, Liu et al. 2009), leading to inhibition and activation of Hh signalling respectively. PP2A also appears to be required for mammalian Hh signalling as it is required for Hh-dependent activation of COUP-TFI (Krishnan, Pereira et al. 1997). In the present study, treatment of MEFs with the PP1 and PP2A inhibitor okadaic acid inhibited the trafficking of Kif7 and Gli proteins to the tips of primary cilia, and the activation of Hh-dependent target genes without affecting Hh-dependent localization of Smo in cilia. These results suggest that PP2A acts downstream of Smo but upstream of Gli to activate vertebrate Hh signalling. Whether other phosphatases are involved in Hh signalling and Kif7 trafficking remain to be tested. Combined with the identification of the Kif7-PPFIA1-PP2A interaction, this led us to test Kif7 as a possible substrate of PP2A. PPFIA1 interacts with the neuron specific motor protein Kif1A (Shin, Wyszynski et al. 2003, Hsu, Moncaleano et al. 2011) and stimulates the trafficking of synaptic vesicles (Miller, DeProto et al. 2005, Wagner, Esposito et al. 2009). Our discovery that the phosphorylation dictates localization of Kif7 raises the possibility that this may represents a more general mechanism that controls kinesin function in diverse contexts. Similar to Kif7, the C-terminus of Kif1A contains a serine residue flanked by several arginine amino acids, suggesting that a similar regulation may occur. However, unlike conventional kinesins, which directionally move along microtubule tracks, the motor domain of Kif7 inhibits microtubule growth by influencing microtubule plus-end dynamics (He, Subramanian et al.
Whether Kif7 phosphorylation influences its tubulin dynamics activity remains to be evaluated.

Although several aspects are conserved between Drosophila and vertebrate Hh signalling, differences exist. The evolutionary adaptation of the primary cilium as a hub directing Gli proteins processing and Hh signalling in vertebrate is likely a reason underlying these differences. In flies, the Kif7 homologue Cos2 also mediates both positive and negative functions during Hh signalling (Lum, Zhang et al. 2003, Zhou and Kalderon 2010). Upon pathway activation, Cos2 is rapidly phosphorylated by Fused, a phosphorylation event that triggers the redistribution of Cos2 from the cytoplasm to the plasma membrane, as well as the magnitude of pathway activation through Ci nuclear translocation and transcriptional activity (Ranieri et al. 2012). Analogous to Drosophila Hh signalling, we report here that Kif7 phosphorylation also directs its subcellular localization and the transcriptional output of the pathway. Unlike flies however, vertebrate Kif7 is phosphorylated under basal conditions and is de-phosphorylated during Hh signalling; thus, phosphorylation is a post-translational modification that controls Kif7 accumulation at the tips of primary cilia. Since Fused is dispensable for mammalian Hh signalling, the kinase that is responsible for Kif7 phosphorylation remains to be determined.

Genetic studies have identified several KIF7 mutations in patients with hydrolethalus, Joubert, Bardet-Biedl and Acrocallosal syndromes. Defects in Gli target gene expression and impaired Gli3 processing confirm that Hh signalling is defective in these disorders. KIF7 mutations also induce Golgi fragmentation, centrosome duplications and ciliogenesis defects suggesting that Kif7 may also have additional Hh-independent roles contributing to disease (Dafinger, Liebau et al. 2011). How these disease-causing mutations perturb Kif7 functions is unknown. Results from our study suggest that in the case of at least one mutation Kif7-L759P, found in Bardet-Biedl Syndrome, the association with the PPFIA1-PP2A complex is promoted and this leads to increased localization of Kif7 at the cilium tip and ligand-independent activation of Gli1 transcription. In the light of these findings, whether other disease-causing mutations affect Hh pathway activity by influencing protein-protein interactions, Kif7 phosphorylation and/or ciliary localization needs to be examined.
2.6 Supplementary Data

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Figure 2-8. PPFIA1 and Kif7 interact through their coiled-coil domains. HEK293T cells were transfected with the indicated expression plasmids and cell lysates were affinity purified using Streptavidin beads followed by Western blotting. A) PPFIA1 interacts with the C-terminal domain (647-1348) of Kif7 that contains coiled-coil domains. B) PPFIA1 interacts with amino acids 698-1057 of Kif7 C-terminal domain. C) Deletion of PPFIA1 C-terminal SAM domains does not interfere with Kif7 binding. D) Deletion of PPFIA1 N-terminal coiled-coil domain impairs Kif7 binding. All figures are representative of 3 independent repeats.
**A**

MEF Ppfia1 shRNA

Merge

PPFIA1 only

Acet tubulin
Pericentrin
PPFIA1

**B**

Relative Ppfia1 mRNA expression (%)

MEF C3H10T1/2

Liprin-alpha:

Ppfia1  Ppfia2  Ppfia3  Ppfia4

C3H10T1/2

0 50 100 150

**C**

Relative Ppfia1 mRNA expression (%)

Scrambled
PPFIA1 #2

**D**

Relative Ppfia1 mRNA expression (%)

1. Scrambled
2. PPFIA1 shRNA #2
3. PPFIA1 shRNA #2 + hPPFIA1

WB: α-PPFIA1
WB: α-Tubulin
**Figure 2-9. Control experiments for Figure 2-3.** A) Immunofluorescent staining of PPFIA1 (green) in MEF cells with shRNA knock down of *Ppfia1*. Acetylated Tubulin(red) and Pericentrin (blue). B) mRNA expression of *Ppfia* isoforms in C3H10T1/2 and MEF cells. Control is the expression of *Ppfia1*. C) shRNA-mediated depletion of *Kif7* or *Ppfia1* impairs *Gli1* mRNA induction by SAG (left) or in *Ptch1*−/− MEFs (right). Control is the max activation of *Gli1* by SAG. D) Validation of efficiencies of knock-down by shRNAs and rescue by qPCR(left) and Western blot (right). qPCR are represented as mean ± SEM across 3 biological replicates. Western blot is representative of 3 independent experiments. Scale bar, 1.5µm. *p<0.05.*
A non-treated 150nM Okadaic acid

hKif7 peptide sequences

Kif7: WT-GFP S1337A-GFP S1337D-GFP
WB: α-GFP
WB: α-Tubulin

C MEFs

% of cells with Kif7 at the tips of cilia

Kif7: WT S1337A S969A

% of cells with Kif7 at the tips of cilia

Kif7: WT S1337D S1337D S969D S969D

% of cells with Kif7 at the tips of cilia

Kif7: WT S1337D S969D

D MEFs

Relative Gli1 mRNA expression (%)

Kif7: WT S969A

Relative Gli1 mRNA expression (%)

Kif7: WT S969D

Relative Gli1 mRNA expression (%)

Kif7: WT S1337D S969D

**Note:** The text is not clearly visible, but it appears to describe experiments involving MEFs (Mouse Embryonic Fibroblasts) and specific Kif7 mutations, with graphs showing the percentage of cells with Kif7 at the tips of cilia and relative Gli1 mRNA expression under different conditions. The graphs also compare non-treated and treated samples with various concentrations of Okadaic acid and other treatments.
Figure 2-10. Control experiments for Figure 2-6. A) Kif7 tryptic peptides used as internal controls for MRM experiment show no change in abundance following okadaic acid treatment. B) a phospho residue in Kif7 (Ser^{969}) that showed no modulation by okadaic acid do not influence the ciliary tips localization of Kif7 (C) or Hh signalling as measured with Gli1 mRNA level (D). Cilia tips counts and qPCR are represented as mean ± SEM across 3 biological replicates. Western blot is representative of 3 independent experiments. *p<0.05.
Figure 2-11. SWATH-MS analysis of Kif7 mutants. Data are normalized to Kif7-WT.
Table 2-1. IP-MS/MS high confidence dataset reported in this study.

The "Bait", "Prey ", "Prey Gene" are from NCBI. "Spectra" are the individual spectral counts across biological replicates ("|") serves as a delimiter between experiments), and "SumSpec" is the sum of the spectral counts and "AvgSpec" is the spectral count averaged for the prey across the highest two purifications of the bait. The spectral counts in negative controls are used for scoring purposes with SAINT. Bait-prey interactions have passed at least one of the following filters: SAINT FDR $\leq$ 1\% or SAINT (AvgP) $\geq$ 0.98. Dashes within the SAINT columns represent values that can’t be calculated.

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Abbreviations: dea = deamidated, P-S = phosphorylated, cntrl = control
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Chapter 3: DYRK2 regulates Hedgehog signalling through phosphorylation of Kif7

All experiments and data analysis in this chapter were performed by Y.C Liu.
3.1 Abstract

Kinesin family member 7 (Kif7) is required for proper Hedgehog (Hh) signalling in vertebrates. Along with several pathway members, Kif7 localizes to primary cilia and accumulates at the tips of cilia upon pathway activation. This pathway activity dependent localization is crucial for the activation of the Gli family of transcription factors and for regulation of target genes. We previously uncovered a role for the post-translational phosphorylation of Kif7 for the control of its ciliary localization and the activation of Hedgehog signalling. The PPFIA1-PP2A protein complex regulates the de-phosphorylation of Kif7. Herein, we identified Dual specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2) as a protein kinase regulating Kif7 phosphorylation. Our results indicate that DYRK2 functions to inhibit the Hedgehog pathway through Kif7 phosphorylation.

3.2 Introduction


Previous work from our group uncovered an important role for Kif7 phosphorylation in dictating its subcellular localization within primary cilia and to regulate Hh pathway activity. Our results showed that, de-phosphorylation of Kif7 by the PPFIA1-PP2A complex is sufficient to promote the localization of Kif7 and Gli proteins at the tips of cilia and to activate Hh target
genes (Liu, Couzens et al. 2014). The nature of the kinase that phosphorylates Kif7 and inhibits its trafficking to the distal ends of cilia in the absence of ligands is still unknown.

Dual-specificity tyrosine phosphorylation-regulated kinase 2 (DYRK2) was previously identified as a negative regulator of Gli transcriptional activity in a cDNA overexpression screen (Varjosalo, Bjorklund et al. 2008). DYRK2 was shown to phosphorylate Gli proteins and to regulate Gli stability. The DYRK family kinases are part of the CMGC family of serine/threonine kinases. DYRKs have conserved catalytic kinase domain and characteristic DYRK homology (DH) box. In vertebrates, the DYRK family is composed of 5 members (1A, 1B, 2, 3, and 4). DYRK 1A and DYRK1B belong to class I DYRKs whereas DYRK2, DYRK3 and DYRK4 belong to class II. DYRK1A and DYRK1B have previously been implicated in Hh signalling (Mao, Maye et al. 2002, Lauth, Bergstrom et al. 2010). DYRKs have conserved YXY activation sequence, they are known to auto-phosphorylate their own tyrosines for activation. DYRKs are proline directed kinases, with a preference for proline at the P+1 position on the substrate (Campbell and Proud 2002, Aranda, Laguna et al. 2011). In this study, we identified Kif7 as a DYRK2 substrate and showed that DYRK2 inhibits Hh signalling by restraining Kif7 trafficking in cilia.

3.3 Methods

3.3.1 Antibodies and reagents

Antibodies were purchased from the following vendors: mouse anti-FLAG, mouse anti–beta-Tubulin clone TUB 2.1, and mouse anti-acetylated-Tubulin clone 6-11B-1, gamma-Tubulin (Sigma-Aldrich); rabbit anti-Gli3 (Santa Cruz, Sc20688, for immunofluorescence), rabbit anti-GFP (Santa Cruz, Sc8334), anti-DYRK2 (Sc-66867, for Western blot use 1:50). Goat anti-Gli2 (R&D AF3635, for Western blot). Goat anti-Gli3 (EMD Millipore, AF3690, Western blot). Secondary antibodies conjugated to Alexa Fluor 488, 594, and 647 were purchased from Life Technologies. Secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories, Inc. SAG (Sc-212905 Santa Cruz) [200nM] was treated for 24h for all experiments. Bio (50nM) was treated overnight (BT-1870199, R&D). Phos-tag
3.3.2 Tissue culture, transfections, and lentiviral transductions

HEK293T, MEF, and C3H10T1/2 cells were cultured in DMEM supplemented with 10% FBS (Sigma-Aldrich), and penicillin/streptomycin. HEK293T cells were transfected using PEI (Polysciences Inc. Cat #23966). All lentiviral particles were produced in HEK293T cells by cotransfection of VSV-G (3µg), psPAX2 (8µg), and lentiviral plasmids (8µg) in 30–40% confluent monolayer cell culture grown in 10-cm plates. Media containing virus particles were collected after 24 and 48h. MEF cells were subsequently transduced in the presence of 10 µg/ml polybrene (Sigma-Aldrich). 24h after infection the viral media was replaced with fresh DMEM 10% FBS media where appropriate cells were selected with puromycin (2µg/ml) 48h after viral infection. All experiments done with MEF or C3H10T1/2 cells, cells were grown to confluency then serum starved with 0.5% FBS in DMEM to induce ciliogenesis for 24-48h. Primary Kif7^-/- MEFs were immortalized by over-expressing SV40 large T antigen.

3.3.3 Plasmids

pEGPF-mKif7 was a gift from C-C Hui (Hospital for Sick Children, Toronto, Canada). mKif7GFP was then subcloned into the pLenti-puro plasmid. FLAG-hDYRK2 and FLAG-hDYRK1B were obtained from the Gingras Lab. The respective cDNAs were PCR amplified from Mammalian Gene Collection clones and cloned into Lenti-FLAG vectors. PcDNA5.1-FLAG-hDYRK1A as obtained from Durocher Lab. Lenti-FLAG-hDYRK2 kinase dead (KD) (K251R) mutant was generated by QuikChange PCR mutagenesis. All PCR-amplified regions were verified by sequencing. Primers used for cloning are listed in Table S1.
3.3.4 Generation of knock-out cell lines with CRISPR-Cas9 system

sgRNAs targeting \( mDyrk1A, mDyrk1B, mDyrk2 \) and \( hDYRK2 \) were selected and cloned in px459 according to instructions from (http://www.genome-engineering.org/crispr/)(Cong, Ran et al. 2013). MEF or C3H10T1/2 cells were transfected with px459 with Lipofectamine 2000 (Life Technologies). Cells were selected with puromycin 48h post transfection. Single-cells were isolated by serial dilution and screened by T7 endonuclease assay for homozygous disruption of the targeted alleles. Sequences for the sgRNA and the primers used for cloning are listed in Table S1.

3.3.5 RT-PCR and quantitative real-time PCR

Total RNA was extracted from cells using TRIzol (Life Technologies) according to the manufacturer’s instructions. For cDNA synthesis, 2\( \mu \)g of total RNA was reverse transcribed using random primers and Superscript II reverse transcription (Life Technologies). Real-time PCR was performed in a 7900HT Fast Real-Time PCR system using 2.5\( \mu \)l of the synthesized cDNA product plus the SYBR Green and primers mix in a final volume of 20\( \mu \)l (Life Technologies). SYBR gene expression assays (Applied Biosystems) were used to measure the mRNA expression of genes in triplicate. Mean relative gene expression was determined using the \( \Delta\Delta Ct \) method normalized to \( Cyclophilin \) mRNA. Primers used are listed in Table S1. qPCR figures represent average and SEM of 3 independent experiments.

3.3.6 Affinity purification, immunoprecipitation, and Western blot analysis

Cells were lysed and protein complexes affinity purified using anti-FLAG M2 agarose beads (Sigma-Aldrich). In brief, cells were solubilized in lysis buffer containing, 50 mM Hepes-NaOH pH 8.0, 100 mM KCl, 2 mM EDTA, 0.1% NP40, 10% glycerol, 1 mM PMSF, 1 mM DTT and Sigma protease inhibitor cocktail for 1h at 4°C. Lysates were spun down at 20,000g for 10min at 4°C. Supernatants were collected and affinity purified for 2h, followed by extensive washing of the beads in lysis buffer. Co-purified proteins were eluted from the beads at 95°C for 10min
using 4× Laemmli buffer containing beta-mercaptoethanol (Sigma-Aldrich), resolved by SDS-PAGE, and transferred onto nitrocellulose or PVDF membranes (Pall) for Western blot analysis.

3.3.7 Immunofluorescence microscopy and image acquisition

Cells grown on coverslips were fixed with cold 100% methanol for 5 min. Coverslips were covered with antibodies in 1% normal donkey serum in PBS overnight at 4°C. Slides were mounted on coverslips using Vectashield mounting media (Vector Laboratories). Laser scanning confocal images were acquired using a Plan-Apochromat 63×/1.4 NA oil immersion objective on a confocal microscope (LSM700; Carl Zeiss) operated ZEN software. eGFP/Alexa Fluor 488, Alexa Fluor 594, and Alexa Fluor 647 fluorophores were excited individually with 488-, 543-, 638-nm lasers, respectively, with appropriate filter sets. Uncompressed images were processed with Zeiss ZEN software Black edition. To quantify cilium tip localization, numbers of cilia with Kif7 or Gli3 at the tips were counted (~100 cilia per experiment) and were normalized to total number of cilia counted. Average and SEM were calculated from 3 independent experiments. % of protein of interest at cilium tip was analyzed with Fisher's Exact Test. Fluorescence intensity was calculated using ImageJ, where fluorescence intensity = integrated density - (area of selected cell * mean fluorescence of background). Average intensity was then calculated from 3 independent repeats, significance was analyzed with Student's T test.

3.3.8 Statistical analysis

The quantification of cilium tip localization was analyzed by Fisher's Exact Test. qPCR and average fluorescence intensity data were analyzed by Student's T test (GraphPad). All statistical analysis was considered significant at p < 0.05.
3.4 Results

3.4.1. Kif7 physically interacts with DYRK2.

We previously identified Ser\textsuperscript{1337} of Kif7 as a functionally relevant phosphorylation site controlling the ciliary localization of Kif7 and Hh pathway activity (Liu, Couzens et al. 2014) (Figure 3A). Using a candidate approach, we hypothesized that DYRK2 could be a Kif7 kinase since it is a proline directed kinase with a consensus sequence (RXS/TP) (Campbell and Proud 2002) that matches the context of Ser\textsuperscript{1337} (RRTSP) on Kif7. Furthermore, DYRK2 was previously recognized as a negative regulator of the Hedgehog pathway in a kinase expression screen (Varjosalo, Bjorklund et al. 2008). Since we showed that Kif7 phosphorylation inhibits Hedgehog signalling, DYRK2 was an ideal candidate. Using co-immunoprecipitation, we first confirmed that DYRK2 interacted with Kif7 when the proteins are co-expressed in HEK293T cells. In contrast, no association could be detected between DYRK2 and Gli2 or Gli3 under the same conditions (Figure 3-1B).

**Figure 3-1. Kif7 physically interacts with DYRK2.** A) Schematic representation of DYRK2 (top) and Kif7 phosphorylation site (bottom in red). B) Co-immunoprecipitation of FLAG-DYRK2 with Kif7GFP, Venus-Gli2, and Venus-Gli3 in HEK293T cells. Western blots are representative of 3 independent experiments.
3.4.2. Loss of Dyrk2 promotes trafficking of Kif7 to the tips of cilia and Hedgehog signalling.

To study the role of Dyrk2 in vertebrate Hedgehog signalling we generated Dyrk2\(^{-/-}\) MEFs cells expressing Kif7GFP using the CRISPR-Cas9 technology. We found that loss of Dyrk2 was sufficient to induce ligand-independent Kif7 trafficking to the tips of cilia and to activate expression of the Hedgehog target gene Gli1 (Figure 3-2A & 3-5A). Given that loss of Dyrk2 promoted Kif7 cilia tips accumulation, we predicted that Gli3 would also be found constitutively at the tips of cilia since its trafficking depends on Kif7 (Endoh-Yamagami, Evangelista et al. 2009, Liu, Couzens et al. 2014). Using Dyrk2\(^{-/-}\) C3H10T1/2 cells (generated through CRISPR-Cas9), we found that endogenous Gli3 showed increased accumulation at the tips of cilia in the absence of any ligand (Figure 3-2B). Similar to MEFs, Gli1 mRNA levels were also increased in Dyrk2\(^{-/-}\) C3H10T1/2 cells (Figure 3-5B). At the protein level, loss of Dyrk2 did not change SuFu or Kif7 expression, but led to a weak increase in Gli2 and a reduction in Gli3\(^{R}\), which is consistent with a de-repression of Hedgehog signalling (Figure 3-2C).
3.4.3. Gain of DYRK2 function inhibits Kif7 ciliary trafficking and Hedgehog signalling.

Previous results implicated DYRK2 as a negative regulator of Hedgehog signal transduction (Varjosalo, Bjorklund et al. 2008) but the mechanism by which DYRK2 function remains incomplete. We first confirmed these findings using over-expression experiments. Expression of DYRK2 partly inhibited the SAG-promoted pathway activation and the constitutive pathway activation observed in \( Ptch1^{-/-} \) MEFs as measured by a reduction in the expression of the Hh target gene \( Gli1 \). The kinase activity of DYRK2 is required for this inhibitory activity since expression of a DYRK2 kinase dead mutant (K251R) (KD) (Gwack et al. 2006) at similar levels had no impact on \( Gli1 \) levels (Figure 3-3A & 3-5C). Since Kif7 contains a consensus DYRK2 phosphorylation site and these proteins interact, we hypothesized that the negative function of DYRK2 could be mediated, in part, through inhibition of Kif7 trafficking in cilia. Confirming this, DYRK2 overexpression inhibited Kif7 accumulation to the tips of cilia promoted by Hh pathway activation whereas catalytically inactive DYRK2 had no effect (Figure 3-3B & 3-5C).
Figure 3-3. Overexpression of DYRK2 inhibits Kif7 cilia tips trafficking and Hedgehog signalling. A) Overexpression of DYRK2 inhibits the SAG promoted in MEFs (right) or constitutive activation of the Hedgehog pathway observed in Ptch1<sup>−/−</sup> MEFs (left) as quantified by measuring levels of the Hh target gene Gli1 by qPCR. Kinase dead mutant (KD) of DYRK2 had no effect on Gli1 level. B) Overexpression of FLAG-DYRK2 inhibits the accumulation of Kif7 to the tips of cilia observed upon pathway activation, whereas the kinase-dead mutant did not. Control is empty vector. Quantification of ciliary tips localization and qPCR data are presented as mean ± SEM across 3 independent experiments. *p<0.05.

3.4.4. DYRK2 controls Kif7 phosphorylation.

We previously showed that the PPFIA1-PP2A protein complex interacts with Kif7 and de-phosphorylates Kif7 on Ser<sup>1337</sup>. De-phosphorylation of Kif7 is required for the accumulation of Kif7 and Gli proteins at the tips of cilia and for the activation of Hh target genes. To determine if Kif7 phosphorylation is affected upon loss of DYRK2, we used phos-tag gels and western blots to analyze Kif7 phosphorylation status in lysates extracted from wild-type and Dyrk2<sup>−/−</sup> MEFs and Dyrk2<sup>−/−</sup> HEK293T cells (generated using CRISPR-Cas9 gene editing). In the absence of DYRK2, immunoreactive bands corresponding to Kif7 have faster mobility when compared to Kif7 extracted from wild-type cells (Figure 3-4A&B). This suggests that Kif7 may be a DYRK2 substrate. The electrophoretic mobility of Kif7 in Dyrk2<sup>−/−</sup> MEFs was similar to Kif7-
S1337A, mutated within a residue previously identified to be a functionally relevant KIF7 phosphorylation site targeted by the PPFIA1-PP2A complex (Liu, Couzens et al. 2014), suggesting that without DYRK2 Kif7 is hypo-phosphorylated (Figure 3-4A). To confirm these findings, we used the pIMAGO reagent that specifically detects phospho-proteins (Iliuk et al. 2012). Briefly, we immunoprecipitated FLAG-Kif7 from wild-type or \textit{DYRK2}\textsuperscript{-/-} HEK293T lysates and separated proteins using SDS-PAGE. Detection using the pIMAGO reagent (a non-antibody based, phospho-specific nanopolymer conjugated to avidin) revealed a phospho-Kif7 band in the wild-type cells that was inhibited in the \textit{DYRK2} mutant cells (Figure 3-4B). These data are consistent with DYRK2 being a protein kinase that phosphorylates Kif7.

\textbf{Figure 3-4. Loss of \textit{DYRK2} impairs Kif7 phosphorylation.} A) Proteins were extracted from the indicated cells and were separated by SDS-PAGE gels containing the phos-tag reagent. Kif7 in \textit{DYRK2}\textsuperscript{-/-} MEFs shows similar electrophoretic mobility as the Kif7-S1337A mutant, which is mutated at a phosphorylation site important for the ciliary trafficking of Kif7 during pathway activation. B) Detection of Kif7 phosphorylation using the pIMAGO reagent (left). FLAG-Kif7 was immunoprecipitated from wild-type or \textit{DYRK2}\textsuperscript{-/-} HEK293T cells and the immunoprecipitated were analysed by SDS-PAGE and pIMAGO detection. Western blot showing loss of \textit{DYRK2} in HEK293T cells is on the right. Western blots are representative of 2 independent experiments.

\section*{3.5 Discussion}

Our previous findings identified an important role for the post-translational control of Kif7 phosphorylation for determining its localization within primary cilia and for regulating Hedgehog signalling. This study also uncovered that the PP2A protein phosphatase regulates phosphorylation of Kif7 at Ser\textsuperscript{1337} and that this was sufficient to promote its localization at the tips of cilia. Expression of the Kif7-S1337A mutant is also sufficient to promote the localization
of Gli proteins at the tips of cilia and to activate Hedgehog signalling. The nature of the protein kinase regulating phosphorylation of this site was however undetermined.

DYRK2 is a kinase that was previously identified as a negative regulator of the Hedgehog pathway (Varjosalo, Bjorklund et al. 2008) that has a consensus phosphorylation site matching the context of Ser\textsuperscript{1337} in Kif7. We therefore hypothesized and confirmed that DYRK2 functions as a negative regulator of the pathway through regulation of Kif7 phosphorylation and cilia localization.

A previous study established Gli2 as a DYRK2 substrate and showed that Gli2 phosphorylation controls its cytoplasmic stability and therefore decreased its transcriptional activity (Varjosalo, Bjorklund et al. 2008). Although we confirmed that gain and loss of DYRK2 function inhibited and activated Gli transcription respectively, we failed to demonstrate an interaction of DYRK2 with Gli proteins. It is however possible that the DYRK2-Gli interaction is transient and that DYRK2 regulates both Kif7 and Gli proteins during Hedgehog signal transduction.

In addition to DYRK2, DYRK1A (Mao, Maye et al. 2002) and DYRK1B (Lauth, Bergstrom et al. 2007) have also been implicated in Hh signalling. We therefore also derived \textit{Dyrk1A}\textsuperscript{−/−} and \textit{Dyrk1B}\textsuperscript{−/−} MEFs (through CRISPR-Cas9) and found that Kif7 cilia tips trafficking and \textit{Gli1} mRNA levels were not significantly altered (Figure 3-5D). In some contexts DYRK kinases are known to be priming kinases for GSK3 phosphorylation (Aranda, Laguna et al. 2011), we therefore tested whether GSK3 could be involved in Kif7 trafficking. Treatment with Bio, a known GSK3 inhibitor did not change Kif7 cilia tips trafficking in MEFs (Figure 3-5E). In \textit{Dyrk2}\textsuperscript{−/−} cells, Kif7 shows increased accumulation at the tips of cilia, additional treatment with Bio did not result in any additive effect on Kif7 trafficking as compared to non-treated \textit{Dyrk2}\textsuperscript{−/−} cells (Figure 3-5E). This suggests that GSK3 may not be involved in for Kif7 trafficking. Overall, our current results are consistent with a role for DYRK2 in regulating Kif7 ciliary tips trafficking by regulating its phosphorylation.
3.6 Supplementary Data

A

Relative expression of Dyrk2 mRNA

B

Relative expression of Gli1 mRNA

C

MEFs

1) Control
2) FLAG-DYRK2-WT
3) FLAG-DYRK2-KD

WB: α-Tubulin

WB: α-FLAG

Ptc1−/− MEFs

1) Control
2) FLAG-DYRK2-WT
3) FLAG-DYRK2-KD

WB: α-Tubulin

WB: α-FLAG

D

% of cells with Kif7 at the tips of cilia

Relative expression of Dyrk1A mRNA

Relative expression of Dyrk1B mRNA

E

% of cells with Kif7 at the tips of cilia
Figure 3-5. Control experiments for this chapter. A) Relative expression of *Dyrk2* mRNA in MEFs. B) Relative expression of *Gli1* (left) or *Dyrk2* (right) mRNAs in C3H10T1/2 cells. C) Western blots showing expressing of FLAG-DYRK2 or KD in MEFs (left) or *Ptch1*−/− MEFs (right). D) Kif7 cilia tips localization and *Gli1* mRNA levels in *Dyrk1A* and *Dyrk1B* knock-out MEFs. Relative expression of *Dyrk1A* or *Dyrk1B* mRNA are shown in the last two panels. E) Quantification of % cells with Kif7GFP at the tips of cilia in *Dyrk2*−/− MEFs with or without treatment with Bio. Cilia tips quantifications and qPCR are represented as mean ± SEM across 3 biological replicates. Western blot is representative of 3 independent experiments. *p<0.05.*
Chapter 4 Biochemical study of Kif7-SuFu interaction

All experiments and data analysis in this chapter were performed by Y.C Liu.
4.1 Abstract

Kif7 and SuFu are two conserved negative regulators of the Hh pathway. Studies in mice have revealed a genetic interaction between Kif7 and SuFu however the mechanistic interplay between these proteins is still unclear. Using proximity-based protein labeling and mass spectrometry analysis (BioID), Kif7 and SuFu were found biotinylated in cells expressing SuFu-BirA and Kif7-BirA respectively, suggesting that these proteins exist in close proximity. Further biochemical analysis showed that SuFu localization at the tips of cilia during Hh pathway activation depends on Kif7, but Kif7 trafficks in cilia independently of SuFu. In addition, experiments performed in MEF knockout cells revealed that loss of Kif7 in SuFu$^{-/-}$ MEFs further activated the pathway when compared to SuFu$^{-/-}$ MEF cells. These results suggest that Kif7 functions as a negative regulator in the absence of SuFu and that it has SuFu-independent function in the Hh pathway.

4.2 Introduction

SuFu is an essential negative regulator of the vertebrate Hh pathway. Loss of SuFu results in derepression of the pathway and ligand-independent activation of Gli transcription (Ding et al. 1999, Kogerman et al. 1999, Pearse et al. 1999, Stone et al. 1999, Chen, Wilson et al. 2009, Jia et al. 2009). Under resting conditions SuFu constitutively trafficks in and out of primary cilia and accumulates at the tips of cilia upon pathway activation (Tukachinsky, Lopez et al. 2010, Zeng et al. 2010). SuFu trafficking in cilia has been shown to be dependent on Gli3 (Zeng, Jia et al. 2010).

Genetic studies uncovered a functional interplay between Kif7 and SuFu in bone (Hsu, Zhang et al. 2011), skin (Li, Nieuwenhuis et al. 2012) and neural tube development (Law, Makino et al. 2012). These studies highlight the dual functions of Kif7, in particular the negative function of Kif7 in the absence of SuFu. Although SuFu and Kif7 were previously shown to physically interact when overexpressed (Endoh-Yamagami, Evangelista et al. 2009), how they functionally interact remains unknown. We previously performed mass spectrometry analysis of Kif7 immunoprecipitates (Liu, Couzens et al. 2014) and failed to identify SuFu as a stable interactor of Kif7. Similarly, mass spectrometry analysis of SuFu immunoprecipitates, carried in our
laboratory, also failed to show Kif7 as an interactor (data not shown). In addition, Kif7 and SuFu fail to interact in endogenous co-immunoprecipitation (Hui lab, personal communication). However, since Kif7 and SuFu both interact with Glis and localize to the primary cilia, we further examined the biochemical and functional link between these two regulators of the Hedgehog pathway. In this study, we used a novel proximity labeling method combined with mass spectrometry and show that Kif7 and SuFu exist in close proximity possibly within a transient protein complex. Functional analysis demonstrated that Kif7 is required for SuFu accumulation at the tips of primary cilia upon Hh pathway activation. Our data further highlights the important interaction between Kif7 and SuFu during Hh signal transduction.

4.3 Experimental procedures

4.3.1 BioID or proximity labeling Mass spectrometry

mKif7 and hSuFu cDNAs were subcloned into PcDNA5/FRT/TO-hBirA, with BirA fused at the N-terminus. The plasmid (1µg) containing the bait of interest was transfected with POG44 (9µg) in HEK293-Flp-in-Trex tetracyclin inducible cell line. Stable cell lines were generated with hygromycin B selection (0.1mg/ml). Sample preparation was similar to Lambert et al (Lambert et al. 2014) with some modification. Briefly, after selection, cells were incubated for 24h in complete media supplemented with 1 µg/ml tetracycline (Sigma), 50µM biotin (BioShop). Cells were collected and pelleted (500g, 5 min), the pellet was washed with PBS, and dried pellets were snap frozen. Pellets were lysed in RIPA lysis buffer (1:4 pellet weight/volume) (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% SDS, 1:500 protease inhibitor cocktail (Sigma-Aldrich), 250U for 10ml Benzonase (Sigma)) at 4°C for 1h, then sonicated for 30 seconds. The lysate was centrifuged at 16,000 rpm for 10 min. Clarified supernatants were incubated with 30 µl packed, pre-equilibrated Streptavidin-sepharose beads (GE) at 4°C for 3 h. Beads were collected by centrifugation (2,000 rpm, 1 min), washed 3 times with 50 mM ammonium bicarbonate pH 8.2, and treated with trypsin (Promega, overnight at 37°C). The supernatant containing the tryptic peptides was then collected and lyophilized. Peptides were resuspended in 0.1% formic acid, 95% water and 5% ACN. Samples
were analyzed with LTX Ion Trap (Thermo Scientific). Data files were uploaded on to ProHits and analyzed with TPP and SAINT as previously described (Liu, Couzens et al. 2014).

4.3.2 Antibodies and reagents
Antibodies were purchased from the following vendors: anti-acetylated-Tubulin clone 6-11B-1 (Sigma-Aldrich); rabbit anti-Pericentrin (Abcam, ab4448); Goat anti-SuFu (Santa Cruz, sc-10933). Secondary antibodies conjugated to Alexa Fluor 488, 594, and 647 were purchased from Life Technologies. Secondary antibodies conjugated to horseradish peroxidase were purchased from Jackson ImmunoResearch Laboratories, Inc. SAG (Sc-212905 Santa Cruz) was used at 200nM for 24h for all experiments.

4.3.3 Plasmids
hSuFu was further subcloned into the pLenti-Venus plasmid to generate pLenti-Venus-hSuFu. pEGPF-mKif7 was previously described (Cheung, Zhang et al. 2009). Kif7GFP was cloned into the pLenti-puro plasmid. The pLenti-mKif7GFP mutants (S1337A & S1337D) were generated by QuikChange PCR mutagenesis.

4.3.4 Tissue culture, transfections, and lentiviral transductions
HEK293T, HEK293-Flp-in-Trex, and MEF cells were cultured in DMEM supplemented with 10% FBS (Sigma-Aldrich), and penicillin/streptomycin. HEK293T and HEK293-Flp-in-Trex cells were transfected using PEI (Polysciences Inc. Cat #23966). All lentiviral particles were produced in HEK293T cells by cotransfection of VSV-G (3µg), psPAX2 (8µg), and lentiviral plasmids (8µg) in 30–40% confluent monolayer cell culture grown in 10-cm plates. Media containing virus particles were collected after 24 and 48h. MEF cells were subsequently transduced in the presence of 10 µg/ml polybrene (Sigma-Aldrich). 24h after infection the viral media was replaced with fresh DMEM 10% FBS media where appropriate cells were selected with puromycin (2µg/ml) or hygromycin (0.2mg/ml) 48h after viral infection. To study Hh
signalling, cells were grown to confluency then serum starved with 0.5% FBS in DMEM to induce ciliogenesis for 24-48h.

### 4.3.5 shRNAs

MISSION shRNA clones in the lentiviral plasmid pLKO.1-puro were acquired from Sigma-Aldrich. Kif7 shRNA clone TRCN0000090438 labeled as Kif7 shRNAs #1. PPFIA1 shRNA clone TRCN0000251544 labeled as PPFIA1 #1. Smo shRNA clone TRCN0000026312 labeled as Smo was also from Sigma-Aldrich. Sequences of shRNAs used are listed in Table S1.

### 4.3.6 Generation of knock-out cell lines with CRISPR-Cas9 system

sgRNAs targeting *mKif7* and *mPtch1* were selected and cloned in px330 according to instructions from [http://www.genome-engineering.org/crispr/](http://www.genome-engineering.org/crispr/) (Cong, Ran et al. 2013). Kif7 or Ptch1 homology arms surrounding the predicted sgRNA cut location were amplified by PCR from mouse genomic DNA and cloned into a donor plasmid designed to introduce a puromycin resistance expression cassette through homologous recombination (HR). *SuFu*−/− MEFs were transfected with px330 and the HR donor plasmid with Lipofectamine 2000 (Life Technologies). Cells were selected with puromycin 48h post transfection. Single-cells were isolated by serial dilution and screened by PCR for homozygous disruption of the targeted alleles. Sequences for the sgRNA and the primers used for cloning are listed in Table S3.

### 4.3.7 RT-PCR and quantitative real-time PCR

Total RNA was extracted from cells using TRIzol (Life Technologies) according to the manufacturer’s instructions. For cDNA synthesis, 2µg of total RNA was reverse transcribed using random primers and Superscript II reverse transcription (Life Technologies). Real-time PCR was performed in a 7900HT Fast Real-Time PCR system using 2.5µl of the synthesized cDNA product plus the SYBR Green and primers mix in a final volume of 20µl (Life
Technologies). SYBR gene expression assays (Applied Biosystems) were used to measure the mRNA expression of genes in triplicate. Mean relative gene expression was determined using the ΔΔ Ct method normalized to Cyclophilin mRNA (Bookout, Cummins et al. 2006). Primers used are listed in Table S3.

4.3.8 Immunofluorescence microscopy and image acquisition

Cells grown on coverslips were fixed with cold 100% methanol for 5 min. Coverslips were covered with antibodies in 1% normal donkey serum in PBS overnight at 4°C. Slides were mounted on coverslips using Vectashield mounting media (Vector Laboratories). Laser scanning confocal images were acquired using a Plan-Apochromat 63×/1.4 NA oil immersion objective on a confocal microscope (LSM700; Carl Zeiss) operated ZEN software. Venus/eGFP/Alexa Fluor 488, Alexa Fluor 594, and Alexa Fluor 647 fluorophores were excited individually with 488-, 543-, 638-nm lasers, respectively, with appropriate filter sets. Uncompressed images were processed with Zeiss ZEN software Black edition. To quantify cilium tip localization, images were taken as Z-stacks and rendered as 3D projections. Cilia with Kif7 or SuFu at the tips were counted (30 cells in 10-15 independent images were used for each condition). Cilia tips localization was expressed as the percentage of cilia exhibiting Kif7 or SuFu proteins localization at the tips. Average and SEM were calculated from 3 independent experiments. % of protein of interest at cilium tip was analyzed with Fisher's Exact Test.

4.3.9 Statistical analysis

The quantification of cilium tip localization was analyzed by Fisher's Exact Test. qPCR data were analyzed by Student's T test (GraphPad). All statistical analysis was considered significant at p < 0.05.
4.4 Results

4.4.1. BioID analysis of Kif7 and SuFu.

To further characterize Kif7 and SuFu interacting proteins, we used the recently developed proximity-dependent labeling of proteins termed BioID (proximity dependent biotin identification) (Roux et al. 2012) method coupled to mass spectrometry (MS). Briefly, for the BioID method, a protein of interest is fused to a mutated form of the *E. coli* biotin protein ligase BirA (R118G), this mutation results in the promiscuous biotinylation of proteins that are near the bait fusion protein (Roux, Kim et al. 2012). The biotinylated proteins are then isolated by Streptavidin affinity purification and identified by MS. HEK293 cells stably expressing BirA-Kif7 and BirA-SuFu were derived and BioID MS was conducted as previously described (Lambert, Tucholska et al. 2014). Full list of interactors found in Kif7 and SuFu BioID are listed in Figure 4-5. In contrast to previous MS results performed on FLAG immunoprecipitates, BioID revealed SuFu and Kif7 peptides in Kif7 and SuFu pull-downs respectively (Figure 4-1A &B). These results raise a few possibilities: 1) the SuFu-Kif7 interaction is transient in nature 2) SuFu and Kif7 interact in a subcellular domain not extracted by the gentle lysis conditions used when performing FLAG immunoprecipitation but revealed with the harsher conditions used for BioID 3) SuFu and Kif7 are not part of the same protein complex but are found in close enough proximity during the biotin labeling period required for BioID.
### Figure 4-1. BioID of Kif7 and SuFu.

A) Mean total peptides ± S.D of 2-3 independent MS of Kif7 and SuFu peptides identified. B) Heatmap representation of some of the interactors identified. For full list of interactors see Figure 4-5. BioID MS for BirA-Kif7 (N=3) and BirA-SuFu (N=2).

#### 4.4.2 Kif7 is required for accumulation of SuFu at the tips of cilia during Hh signalling.

It is known that Kif7 is required for trafficking Gli proteins to the tips of cilia during Hh signalling (Endoh-Yamagami, Evangelista et al. 2009, Liu, Couzens et al. 2014). Since SuFu...
also localizes with Gli proteins at the tips of cilia (Tukachinsky, Lopez et al. 2010, Zeng, Jia et al. 2010), we next were interested to determine whether Kif7 was involved in this process. To monitor SuFu localization we expressed Venus-SuFu in SuFu<sup>−/−</sup> MEF cells and showed that the fusion protein rescued the constitutive activation of Gli1 mRNA observed in these cells validating the proper functioning of the fusion protein (Figure 4-3A, 4-4A). We then knock-down Kif7 expression using shRNA and determined whether this modulated the efficiency of the Smoothened agonist SAG to promote SuFu accumulation at the tips of cilia. Whereas SAG treatment led 56% of cells expressing scrambled shRNA to accumulate Venus-SuFu at the tips of cilia, knock-down of Kif7 or Ppfia1 reduced the percentage of cells to 16% and 14% respectively, which is similar to what is seen upon Smo depletion (Figure 4-2A; 4-4B). Reciprocally, we assessed the requirement of SuFu for the Kif7 translocation to the tips of cilia upon SAG treatment. To do so, we expressed Kif7-GFP into SuFu<sup>−/−</sup> MEFs and determined the percentage of cells exhibiting Kif7 localization at the tips of cilia under resting conditions or upon SAG treatment for 24h. Stimulation of SuFu<sup>−/−</sup> MEFs with SAG led to 37% of cells with Kif7 at the tips of cilia, levels that are similar to what is obtained in wild-type MEFs (Figure 4-2B). We conclude that Kif7 is required for the accumulation of SuFu at the tips of cilia in response to pathway activation but that SuFu is dispensable for Kif7 cilia tips trafficking.

**Figure 4-2. SuFu and Kif7 ciliary tips trafficking.** A) Immunofluorescence images of Venus-SuFu in SuFu<sup>−/−</sup> MEFs. Venus-SuFu (green), acetylated Tubulin (red) and Pericentrin (blue). SAG promoted trafficking of Venus-SuFu to the tips of cilia is reduced upon shRNA-mediated knock-down of Kif7, Ppfia1 or Smo. Quantification is on the right. B) Trafficking of Kif7 to the tips of cilia is normal in SuFu<sup>−/−</sup> MEFs (left) with or without SAG treatment. Trafficking of Kif7GFP in wild type MEFs is on the right. Quantification of ciliary tips localization is presented as mean ± SEM across 3 independent experiments. *p<0.05. Scale bar: 1µm.
4.4.3 Gain and loss of function of Kif7 in SuFu\(^{-/-}\) MEFs

Previous genetic studies in mice showed that compound SuFu\(^{-/-}\):Kif7\(^{-/-}\) double mutant mice exhibit more pronounced de-repression of the Hh pathway and more severe phenotypes in the neural tube and in the skin than either single mutant mice (Law, Makino et al. 2012, Li, Nieuwenhuis et al. 2012). These results suggest non-redundant functions of Kif7 and SuFu as negative regulators of Hh signalling. How these two negative regulators cooperate to repress Gli-mediated signalling is still poorly understood. As previously demonstrated, expression of Venus-SuFu in SuFu\(^{-/-}\) cells rescues the constitutive activation of the pathway (Figure 4-3A)(Varjosalo, Bjorklund et al. 2008, Lauth, Bergstrom et al. 2010). In contrast expression of Kif7-WT or of the S1337A or S1337D Kif7 mutants did not have any significant effect on Gli1 mRNA level (Figure 4-3A). Since Kif7 overexpression cannot rescue the loss of SuFu function, we conclude that Kif7 activity is upstream or at the level of SuFu. Using CRISPR-Cas9, we then knocked-out Kif7 in SuFu\(^{-/-}\) MEF cells, and found a weak increase in Gli1 mRNA (Figure 4-3B) when compare to SuFu\(^{-/-}\) MEFs. This recapitulates what is seen in mouse tissues and suggests that Kif7 has SuFu-independent function, as a weak negative regulator of Hedgehog signalling in the absence of ligands.

**Figure 4-3. Gain and loss of function of Kif7 in SuFu\(^{-/-}\) MEFs.** A) Gli1 mRNA levels in SuFu\(^{-/-}\) MEFs overexpressing Venus-SuFu, Kif7-WT, Kif7-S1337A, or Kif7-S1337D. B) Gli1 mRNA levels in SuFu\(^{-/-}\) MEFs with knockout of Kif7 or Ptch1. qPCR data are presented as mean ± SEM across 3 independent experiments. *p<0.05.
4.5 Discussions

Overall, our BioID study revealed that Kif7 and SuFu exist in close proximity to each other in resting cells, an interaction that we were unable to capture using conventional immunoprecipitation/mass spectrometry methods. Our current understanding of Hedgehog signalling suggests that Kif7 promotes Gli repressor formation under resting conditions (Cheung, Zhang et al. 2009, Endoh-Yamagami, Evangelista et al. 2009, Liem, He et al. 2009). Interestingly, overexpression of Kif7 in SuFu\(^{-/-}\) cells was not sufficient to rescue the constitutive Gli signalling activity observed in the cells (Figure 4-3A). Kif7 knockout in SuFu\(^{+/+}\) cells\(^{,}\) on the other hand, potentiated Gli-mediated signalling indicating that Kif7 inhibits Gli-mediated transcription independently of SuFu. How Kif7 and SuFu cooperate to maintain low levels of Hedgehog signalling under resting conditions remains to be precisely determined.

Previous studies indicated that the localization of SuFu at the tips of cilia during Hh signalling depends on Gli proteins since these proteins appear to be tightly associated (Tukachinsky, Lopez et al. 2010, Zeng, Jia et al. 2010). It is in fact at the tips of cilia that Gli is thought to dissociate from SuFu when the pathway is active to generate Gli\(^{A}\) and translocate to the nucleus (Humke, Dorn et al. 2010, Tukachinsky, Lopez et al. 2010). Since we and others have shown that Kif7 is required for trafficking of Gli proteins to the tips, we predicted and confirmed that Kif7 is also required for SuFu localization in cilia (Figure 4-2A). SuFu on the other hand is dispensable for the trafficking of Kif7 to cilia tips upon pathway activation (Figure 4-2B). Kif7 therefore acts downstream of Smo and upstream of the SuFu-Gli complex in Hedgehog signal transduction.

These findings raise a number of important questions. Does Kif7 interact simultaneously with Gli and SuFu proteins to traffic these proteins to the tips of cilia? Why are Hh pathway components (Smo, Kif7, SuFu and Gli) all enriched at the tips of cilia at steady state when the pathway is activated? How is Smo activation leading to Kif7 translocation to the tips of cilia? How is Kif7 then acting to promote the dissociation of Gli from SuFu when Hh ligands are present? These questions may be answered by real-time imaging studies and/or biophysical approaches to further understand the kinetics of protein dynamics and the nature of protein-protein interaction in the absence or presence of Hh ligands.
4.6 Supplementary Data

Figure 4-4. Control experiments for this chapter. A) Western blot confirm the expression of Venus-SuFu in SuFu\(^{-/-}\) MEFs. B) Knock-down efficiencies of shRNAs in SuFu\(^{-/-}\) + Venus-SuFu. qPCR are represented as mean ± SEM across 3 biological replicates. Western blot is representative of 3 independent experiments. *p<0.05.
Figure 4-5. Full list of SuFu & Kif7 BioID mass spectrometry.
Chapter 5: General discussions and future directions

5.1 Summary of key findings and significance

In summary, this thesis focuses on the molecular mechanisms underlying Kif7 functions in vertebrate Hedgehog signalling. I found that in MEFs, Kif7 fulfills predominantly a positive regulatory role downstream of Smo activation as it promotes the ciliary tips accumulation of Gli proteins and the induction of Gli target genes. I further described a protein complex, consisting of Liprin-α1 (PPFIA1) and the serine threonine protein phosphatase PP2A, which interacts with Kif7. My results indicate that the PPFIA1-PP2A complex regulates Kif7 phosphorylation and promotes the Hh signalling dependent localization of Kif7 and Gli proteins at the tips of cilia and the activation of Hh target genes (Chapter 2). In addition, I identified DYRK2 as one kinase responsible for Kif7 phosphorylation and showed that this post-translational modification dictates its subcellular localization at the tips of primary cilia and determine whether Kif7 inhibits or activates Hedgehog signalling (Chapter 3). These findings are consistent with a model in which Smo activation is coupled to the trafficking of Kif7 and Gli proteins in primary cilia via the regulation of Kif7 phosphorylation (Figure 5). Of significance, some KIF7 mutations found in human developmental syndromes were found to affect the interaction with the PPFIA1-PP2A complex. These mutations lead to ligand-independent localization of Kif7 and Gli proteins to the tips of cilia and constitutively activated Hedgehog signalling. Lastly, we examined Kif7 function in relation to SuFu, and found that SuFu depends on Kif7 for ciliary tips accumulation upon pathway activation, and that Kif7 has SuFu independent functions in the pathway (Chapter 4).
**Figure 5. Working model.** When the Hedgehog pathway is inactive, DYRK2 phosphorylates Kif7 and inhibits its ciliary trafficking and consequently prevents Gli proteins accumulation at cilia tips. At steady state Kif7 and Gli proteins are therefore enriched at the base of cilia but a basal flux of Kif7-Gli trafficking in cilia occurs to allow processing of Gli3 into Gli3R (Left). During pathway activation, Smo accumulates in cilia and promotes the recruitment of the PPF1A-PP2A complex to Kif7. This leads to increased de-phosphorylation of Kif7 on Ser\(^{1337}\) and increased localization of Kif7 and Gli proteins at the cilia tips leading to SuFu dissociation from Gli2 and Gli3 proteins resulting in Gli2 activation and reduction of Gli3R (Right).

### 5.2 General discussions

This work provides important new insights into the function of Kif7 in Hedgehog signal transduction but also raises new questions. For example, we found that Kif7 functions to promote Gli and SuFu accumulation at the distal tips of cilia, a process that is crucial for the activation of downstream target genes but work from He et al. showed that Kif7 has no motor activity *in vitro* (He, Subramanian et al. 2014). It is therefore unclear how Kif7 promotes ciliary trafficking of Gli and SuFu during pathway activation. Recent work demonstrates that Gli proteins form a complex with Kif3A (Carpenter et al. 2015) and our own work identified Kif3A in Kif7 immunoprecipitates by MS. This raises the possibility that Kif3A may contribute to
ciliary localization of the Kif7-SuFu-Gli protein complex. Other kinesins may also play a role in ciliary trafficking of Hh proteins and this remains to be systematically tested.

Additionally, we identified protein phosphatase PP2A as a key regulator of Kif7 function. In flies, PP2A was found to regulate Ci phosphorylation (Jia, Liu et al. 2009). Whether PP2A also plays a role in Gli phosphorylation has not been directly demonstrated. We identified the Kif7 phosphorylation site (Ser\textsuperscript{1337}) as a PP2A sensitive site following treatment of cells with okadaic acid (OA). OA is known to inhibit both PP2A and PP1 (at higher dose). Although we consistently used low dose of OA in our experiments, and we only identified subunits of PP2A in Kif7 AP-MS, we can’t rule out that PP1 or another phosphatase contribute to Kif7 function. Furthermore, similar to flies where multiple phosphorylation sites were identified on Cos2 (Ranieri, Ruel et al. 2012) we also identified at least three additional Kif7 phosphorylation sites that could be targeted by other phosphatase and be important for Kif7 function.

In this thesis we also identified DYRK2 as a kinase implicated in the functional regulation of Kif7. In Drosophila, Cos2 is phosphorylated by Fused but STK36 the vertebrate homolog of Fused is not involved in vertebrate Hh signalling (Chen, Gao et al. 2005, Merchant, Evangelista et al. 2005). Another kinase with sequence homology to Fused is Ulk3, which was found to positively regulate Gli (Maloverjan et al. 2010, Maloverjan et al. 2010). Whether Ulk3 or other kinases also contribute to Kif7 regulation in addition to DYRK2 remains to be tested.

Furthermore, through biochemical approaches, we identified the phosphatase and the kinase regulating Kif7 phosphorylation. However, how Kif7 phosphorylation occurs temporally with changes in pathway activity is still unknown. Additional experiments examining Kif7 phosphorylation \textit{in vivo} is also needed. Developing a phospho-specific Kif7 antibody against the Ser\textsuperscript{1337} site will be one way to address these questions.

Finally, many mutations in \textit{KIF7} have been identified in ciliopathies and other rare genetic disorders, how these mutations affect Kif7 function remains to be elucidated. We identified one Kif7 mutant Kif7-L759P showing increased association with PP2A-PPFIA1. Whether phosphorylation and/or binding to DYRK2 are affected in this mutant remain to be further tested. In addition, we analyzed three other point mutants of \textit{KIF7} using SWATH-MS. The \textit{KIF7}-P632L mutation found in patients with Pallister-Hall syndrome shows decreased binding to Kif5B. Whether Kif5B is involved in protein trafficking in the cilia or Hh signalling
represents an interesting possibility. Lastly, given that Kif7 and SuFu are two key regulators of vertebrate Hh signalling, understanding the formation and dissociation of Kif7-Gli-SuFu complex within the cilia, especially at the distal tips of cilia will be important to continue understand signal transduction in the Hh pathway. Overall, this thesis provides novel insights into the regulation of Kif7 activity during Hedgehog signalling.

5.3 Future directions and perspectives

Over the past 20 years, details of Hh signal transduction have been revealed through genetic and biochemical analysis. Evolutionarily conserved Hh signalling components were discovered between Drosophila, zebrafish and mice. Divergence between these model organisms was also observed. However, much is still unknown about the pathway. Firstly, the biochemical underpinnings of many steps in the pathway are still poorly defined. For example, the central question of how Ptch1 regulates Smo in the absence and presence of Hh ligands is still unknown. In addition, how Hh pathway activation is transmitted from Smo to Kif7, the process triggering dissociation of SuFu from Gli during pathway activation and the nature of Gli modifications required for Gli\(^A\) formation are all poorly answered questions. Secondly, given the importance of primary cilia in cell signalling, more work is needed to better understand how Hh pathway components flux in and out of the cilia during Hh signalling. Lastly, Hh signalling is activated in many cancers, both via ligand-dependent and independent mechanisms. Recent studies showing paracrine activation of Hh signalling in the stroma from Hh ligands secreted by tumour cells shed new light on the complexity of the pathway in tumourigenesis (Yauch, Gould et al. 2008). Enormous efforts have been placed in developing Hh pathway inhibitors, most commonly Smo antagonists, to treat tumours exhibiting Hh pathway activation. Mutations in Smo leading to resistance to Smo inhibitors rapidly arise and therefore in order to develop more effective therapeutics, the molecular basis underlying Hh pathway activation in non-cancerous cells and in tumour cells needs to be determined. Addressing the missing questions in pathway signal transduction is the first step towards understanding this pathway and for developing therapeutics.
References


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Wagner, O. I., A. Esposito, B. Kohler, C. W. Chen, C. P. Shen, G. H. Wu, E. Butkevich, S.

Wagner, O. I., A. Esposito, B. Kohler, C. W. Chen, C. P. Shen, G. H. Wu, E. Butkevich, S.


### Table S1 List of primers used in this thesis

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<th>base pair change</th>
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#### Sequencing primers for Kif7

For S1337

- Forward: atgaggccccggatcagctct

For S969

- Forward: ttcagaggaagagccgagcag

### Mouse Kif7 mutation base pair change Forward primer Reverse Primer

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**LiprinA1 deletion**

** Constructs primers**

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**C′ serial truncations of PPFIA1**

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**hKif7 C′ truncation constructs primers**

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Cloning primers

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sgRNAs

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mPtch1

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Mutagenesis

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Copyright Acknowledgements

Figure 1-1 was adapted from: Briscoe, J. and P. P. Therond (2013). "The mechanisms of Hedgehog signalling and its roles in development and disease." Nat Rev Mol Cell Biol 14(7): 416-429, with permission from Macmillan Publishers Ltd. Copyright 2013 doi: 10.1038/nrm3598.

Figure 1-3 was adapted from: Reiter, J. F., O. E. Blacque and M. R. Leroux (2012). "The base of the cilium: roles for transition fibres and the transition zone in ciliary formation, maintenance and compartmentalization." EMBO Rep 13(7): 608-618, with permission from European Molecular Biology Organization. Copyright 2012 doi: 10.1038/embor.2012.73.