

THE LONG AND THE SHORT OF IT: THE ROLE OF CELL CYCLE-RELATED KINASE
(CCRK) AND INTESTINAL CELL KINASE (ICK) IN CILIOGENESIS AND HEDGEHOG
SIGNALING

by

ASHLEY SNOUFFER

(Under the Direction of Jonathan Eggenschwiler)

ABSTRACT

Primary cilia are present on nearly every cell in the mammalian body, and this microtubule-based organelle can vary greatly in length depending on the cell type producing it. Each cell type tightly regulates the final length of the primary cilium produced, but the mechanisms controlling length regulation are not fully understood. During embryonic development, it is essential for cells to produce a primary cilium to respond to the developmental signaling protein, Hedgehog (Hh). Disruption of cilia production or function can result in dramatic developmental defects, including exencephaly and polydactyly. In this work, I have studied two protein kinases, Cell cycle-related kinase (CCRK) and Intestinal cell kinase (ICK) and their interactions in cilia length regulation and Hh signaling. Single mutant embryos for both genes have defects in Hh signaling and tissue patterning and are embryonic lethal. I analyzed cilia length of *Ccrk* and *Ick* single mutants in cell culture and in developing embryonic limb buds. Loss of *Ccrk* results in cilia that are both longer and shorter than wild-type cilia. Loss of *Ick*, also results in embryonic developmental defects related to altered Hh signaling, and *Ick* mutant cells have cilia that are longer than wild-type.

Research has shown that CCRK can phosphorylate ICK in vitro. To understand the biological relevance and genetic interaction of these genes in vivo, I generated *Ccrk* and *Ick* double mutant embryos. I found that the double mutant embryos resemble the gross morphology of *Ccrk* embryos, indicating that *Ccrk* is indeed epistatic to *Ick*. However, interestingly, I found that *Ccrk* and *Ick* double mutant embryos seem to have earlier lethality than either single mutant and defects in heart looping not seen in *Ick* single mutants, and only rarely observed in *Ccrk* single mutants. This defect in heart looping is indicative in defects to left right patterning determination and could potentially stem from defects in the cilia production or length at the embryonic node, the tissue that controls the initial break in symmetry in left-right patterning.

INDEX WORDS: Cilia length, Cell-Cycle Related Kinase, Intestinal Cell Kinase, Hedgehog signaling, ciliogenesis, neural tube, limb bud, left-right patterning, heart looping

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B.S., DePaul University, 2012

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2018

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May 2018

ACKNOWLEDGEMENTS

Throughout my time as a graduate student I have received incredible support from my advisor, Jonathan Eggenschwiler. I have been constantly encouraged and motivated in my development as a scientist and have truly benefited from his wealth of scientific knowledge. I greatly appreciate his patience, kindness, and the values of scientific rigor and humbleness he has instilled in me. I am also grateful to the members of my thesis committee, Jim Lauderdale, Jacek Gaertig, Doug Menke, Brian Condie, and former member Jeff Chen, for their invaluable feedback and insights into developing my projects and the research I have done. I also owe a great deal of thanks to Julie Gordon for her help developing and carrying out the work in my third chapter, for all the training and feedback she provided that made all of my research possible, and for her incredible friendship and support throughout these years.

I would like to thank the current and former members of the Eggenschwiler lab, especially Jonathon Walsh for his work on the CCRK project, all of his technical advice and help, and for his constant excitement and discussion of our research and teaching experiences. I truly appreciate all of the help he has given me with molecular and biochemical techniques in the lab. Thank you also to Floria Lupu for sharing her expertise in mouse development, her experiences and stories in science, and for promoting a collegial environment in the lab. Thank you to the undergraduate students Jie Xu and Blake Gowen for their help on these projects and for fostering my interest in training students.

Thank you to Tamara Caspary, for her support, encouragement, and feedback on my research and scientific career. I would also like to thank Tamara, Ben Allen, Amy Ralston, and Camilla Forsberg for the incredible experience as a TA at the Cold Spring Harbor Mouse Course.

Special thanks to all of my wonderful friends who have encouraged me throughout my academic pursuits, especially Alison, Maria, Nikolette, and Katherine for always being encouraging, understanding, fiercely supportive, and for being a constant source of laughter and joy. Thank you to my father and my family for always supporting my interests and promoting the importance of education, learning, and balance. Finally, thank you to John for his unconditional love, support, and understanding. I greatly appreciate his reassurance, kindness, and humor at all times.

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CHAPTER I

GENERAL INTRODUCTION

Hedgehog (Hh) signaling

Embryonic development from a single fertilized cell to an entire developed organism involves complex coordination of cellular proliferation, migration, and differentiation. There are many different signaling molecules that play a role in embryonic development to organize developing tissues and direct cells to their appropriate fates. One of these signaling molecules is the Hedgehog (Hh) ligand. The *Hh* gene and its function were originally described in *Drosophila melanogaster*, where a *Hh* mutant *Drosophila* embryo had clusters of disorganized spine-like bristles resulting in the embryo resembling a hedgehog (Nüsslein-volhard and Wieschaus, 1980). The Hh ligand is a secreted protein that induces proliferation and differentiation within tissues of the developing fly embryo including the wing imaginal disc in *Drosophila* (Ingham and McMahon, 2001). In mammals, there are three *Hh* paralogs, Sonic hedgehog (*Shh*), Indian hedgehog (*Ihh*), and Desert hedgehog (*Dhh*), and these three genes share an evolutionarily conserved signaling mechanism and role in regulating embryonic tissue patterning (Chang et al., 1994; Echelard et al., 1993; Ingham and McMahon, 2001; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994).

The Hh proteins are involved in a broad range of developmental processes within the embryo and are crucial in a number of developing tissues. *Shh* is involved in regulation of tooth and hair development, cerebellar proliferation, and neural tube and limb patterning (Dassule et

al., 2000; Dessaud et al., 2008a; Ericson et al., 1997a; St-Jacques et al., 1998; Wallace, 1999; Wechsler-Reya and Scott, 1999). *Ihh* is partially redundant with *Shh*, but plays an independent and crucial role within the developing cartilage and bones (Karp et al., 2000; St-Jacques et al., 1999; Wu et al., 2001; Zhang et al., 2001). *Dhh* has a distinct role from the other two *Hh* paralogs in germ cell development and formation of peripheral nerve sheaths (Bitgood et al., 1996; Briscoe and Théron, 2013; Lum et al., 2003). All of the Hh ligands are morphogens, where the secreted ligand diffuses across a tissue forming a concentration gradient with the cells nearest the source of Hh are exposed to the highest levels of Hh and the further away a cell is from the source of Hh the successively less Hh they are exposed to. Cells have specific and distinctive responses based on the amount of Hh they are exposed to, resulting in organized domains of cells with different fates within the same tissue (Jessell, 2000).

Before Hh proteins are secreted from a cell, they are cleaved and are post-translationally modified. The Hh proteins start as a 45kDa protein with an amino-terminal peptide linked to a carboxy-terminal peptide (Chen et al., 2011a). The Hh proteins contain a sterol recognition region within the carboxy-terminal peptide which acts to recruit cholesterol and covalently attach it to the amino-terminal peptide. The attachment of cholesterol also results in dissociation of the carboxy-terminal peptide from the amino-terminal peptide, and the carboxy-terminal peptide undergoes proteasomal degradation (Mann and Beachy, 2004; Porter et al., 1996). The amino-terminal peptide is also modified by *skinny hedgehog* (SKI), an acyltransferase, which attaches a palmitic acid group to the most N-terminal residue (Amanai and Jiang, 2001; Chamoun et al., 2001; Hofmann, 2000; Lee and Treisman, 2001; Mann and Beachy, 2004; Micchelli et al., 2002). The two lipid modifications of the Hh protein are required for secretion of Hh from the cell.

Dispatched (DISP) and SCUBE2, proteins within the cellular membrane, bind to the cholesterol of Hh to promote secretion of the Hh protein (Creanga et al., 2012; Tukachinsky et al., 2012). The secreted Hh ligands can be present as monomers and multimers, and the lipid modifications are also essential for forming the multimers and long-range signaling of the Hh ligand (Ayers et al., 2010; Callejo, 2006; Chen et al., 2004; Feng et al., 2004; Gallet et al., 2006; Goetz et al., 2006; Lewis et al., 2001; Zeng et al., 2001). Additionally, there is evidence in the imaginal disc of *Drosophila* that the Hh ligand may be secreted in different forms to have both long-range and short-range activity (Ayers et al., 2010).

The receptor for the Hh ligand is the transmembrane protein Patched (Ptch) (Hooper and Scott, 2005; Nakano and Muramatsu, 1989). Ptch forms a receptor complex along with the co-receptors CAM-related/downregulated by oncogenes (CDO), brother of CDO (BOC), and growth arrest-specific 1 (GAS1). In mice, only the absence of all three co-receptors results in complete loss of Hh activity, suggesting that each co-receptor separately dimerizes with Ptch to promote Hh ligand binding (Allen et al., 2007; Izzi et al., 2011). In the absence of the Hh ligand, Ptch represses the Hh signaling pathway through inhibition of the transmembrane protein Smoothened (Smo). The mechanism of this inhibition remains unclear, however, based on the binding domain of several agonist and antagonists to Smo, it may be possible that a sterol-like molecule regulated by Ptch can control the function of Smo (Corcoran and Scott, 2006; Dwyer et al., 2007; Nachtergaele et al., 2012). Alternatively, Ptch may regulate membrane trafficking of Smo, and Ptch may block Smo function through prevention of translocation to the plasma membrane (Yavari et al., 2010). Furthermore, Ptch may regulate components of endocytosis including LRP2, in certain cell types where LRP2 mediates clathrin-dependent vesicle internalization of Ptch when it is bound to the Hh ligand which then allows Smo to enter the

cellular organelle required for Hh signaling transduction, the primary cilium (Christ et al., 2012; Corbit et al., 2005a). In the presence of the Hh ligand, Ptch mediated inhibition of Smo is relieved, and Smo undergoes a conformational change as a result of phosphorylation of the cytoplasmic tails by CKI α and GPRK2 (Chen et al., 2011a; Meloni et al., 2006).

The activation of Smo then results in regulation of the processing of the Gli transcription factors responsible for activating downstream targets of Hh signaling. Within amniotes, there are three Gli proteins (Gli1, Gli2, and Gli3) that each contains a zinc finger DNA binding domain, and a C-terminal activation domain (Briscoe and Thérond, 2013; Ingham et al., 2011). Additionally, Gli2 and Gli3 contain an N-terminal repressor domain (Jia et al., 2003; Lefers et al., 2001; Lum et al., 2003; Østerlund and Kogerman, 2006). In the absence of the Hh ligand the Glis are associated with Suppressor of fused (Sufu), a negative regulator of the Hh pathway which functions downstream of Smo (Pearse et al., 1999; Stone et al., 1999). Loss of *Sufu* results in hyperactivation of the Hh pathway, similar to that seen in a *Ptch1* mutant mouse embryo (Cooper, 2005; Persson et al., 2002; Svärd et al., 2006). Sufu binding is thought to prevent translocation of the Glis to the nucleus and stabilizes Glis in their full length (in-active) form. Additionally, Sufu inhibition of the Glis is not dependent on the presence of a cilium (Chen et al., 2009; Cheng and Bishop, 2002; Humke et al., 2010; Jia et al., 2009a; Kogerman et al., 1999; Svärd et al., 2006). In the absence of the Hh ligand the GPCR, Gpr161, is enriched within the cilium and promotes the production of cAMP. This in turn promotes protein kinase A (PKA) activity, and the C-terminal activation domain of the Glis is phosphorylated sequentially, first by PKA, then by glycogen synthase kinase 3 β (GSK3 β) and CKI kinase family members with the help of Kif7 (Hui and Angers, 2011; Jiang and Hui, 2008; Mukhopadhyay and Rohatgi, 2014a; Sheng et al., 2005; Singh et al., 2015; Wang et al., 2007a). Sufu is thought to also recruit GSK3 β

to mediate processing of the Gli3 repressor to further suppress Hh signaling in the absence of the ligand (Kise et al., 2009). PKA phosphorylates six conserved serine residues on Gli2/3 which promote Gli repressor formation and inhibits formation of Gli activators. Phosphorylation of the first four sites is sufficient to promote Gli repressor formation, but inhibition of the activator function of Glis requires phosphorylation at all six sites (Niewiadowski et al., 2014; Pak and Segal, 2016). Gli1 degradation is not thought to be regulated by PKA phosphorylation, but rather AMP-activated protein kinase (AMPK) (Li et al., 2015; Magno et al., 2016). PKA also phosphorylates and stabilizes Sufu to promote the complex of Sufu with the Glis (Chen et al., 2011b). The phosphorylation of the Glis recruits β -transducin repeat-containing protein (β TrCP) which further recruits and forms an E3 ubiquitin ligase complex (Jia et al., 2003; Jia et al., 2009b; Smelkinson and Kalderon, 2006; Zhang et al., 2005a). This complex results in the ubiquitylation of the C-terminal end of the Glis and recruitment and partial degradation of the Glis by the proteasome. The remaining N-terminal domain of the Glis can then translocate to the nucleus and acts as a repressor of Hh target gene expression (Gulino et al., 2012; Humke et al., 2010; Kogerman et al., 1999; Zhang et al., 2017).

In the presence of the Hh ligand, activated Smo can enter the cilium and inhibits the phosphorylation, proteolytic processing, and association of Sufu with the Gli proteins. The active full length Glis can then translocate to the nucleus and promote transcriptional activation of Hh target genes (Briscoe and Thérond, 2013; Litingtung and Chiang, 2000; Matise et al., 1998). Additionally, Gpr161 exits the cilium upon Hh induction and Gpr175 can enter the cilium. Gpr175 decreases the cAMP levels within the cilium and stabilizes the full-length activator form of the Glis (Goetz and Anderson, 2010; Mukhopadhyay and Rohatgi, 2014b; Singh et al., 2015). Phosphorylation of the Glis by PKA is reduced and the Glis are phosphorylated at a different site

to promote full transcriptional activation (Niewiadowski et al., 2014). In the presence of the Hh ligand, Gli1, which only contains an activator domain, is thought to be activated by atypical protein kinase C (α PKC I/ γ) (Atwood et al., 2013). Both Gli2 and Gli3 contain activator and repressor domains, however, experimental evidence indicates that Gli2 is primarily an activator of Hh signaling, while Gli3 is primarily a repressor (Bai et al., 2004; Mo et al., 1997). Gli1 lacks the repressor domain, and its minimal activator function is generally redundant with that of Gli2 in mice (Bai et al., 2002; Mo et al., 1997; Park et al., 2000).

Overall, the cellular response to Hh ligand within different tissues depends on the balance of Gli repressor and Gli activator formation. Mice that lack all Shh, and both Gli activator and repressor function, develop a single digit on a truncated limb (Chiang et al., 1996; Chiang et al., 2001; Kraus et al., 2001). Gli2 mutant mice have normally patterned limbs, but decreased expression of the Hh target gene, Gli1 (Bai et al., 2002; Park et al., 2000). Gli2 and Gli3 double mutant mice have no expression of the Hh target gene, Gli1, indicating that both Gli2 and Gli3 have transcriptional activator functions (Bai et al., 2004). Gli3 single mutants, however, do have limb patterning defects with many extra digits (polydactyly), a phenotype that resembles over expression of Hh signaling response, indicating that Gli3 also has a crucial repressive role in Hh signaling (Hui and Joyner, 1993; Mo et al., 1997).

Target genes of Hh signaling in mouse

The Gli proteins mediate the Hh signaling pathway through transcriptional activation of genes involved in proliferation and differentiation. There are general targets of the Hh pathway which create feedback loops to both positively and negatively regulate the cellular response to Hh, and there are also cell type specific gene targets of the Hh pathway that regulate cellular

specification and differentiation. Interactions between different target genes within cells exposed to varying levels of Hh help to generate distinct populations of cells with different fates (Ribes and Briscoe, 2009; Ribes et al., 2010).

General targets of the Hh pathway include *Gli1*, *Hhip*, the Ptch co-receptors, *Cdo*, *Boc*, *Gas1*, and *Ptch1* itself (Allen et al., 2007; Martinelli and Fan, 2007; Ribes and Briscoe, 2009; Tenzen et al., 2006; Yao et al., 2006; Zhang et al., 2006). *Gli1*, *Ptch1*, and *Hhip1* are transcriptionally upregulated when the Hh ligand is present, while *Gas1*, *Boc*, and *Cdo* are transcriptionally downregulated in the presence of the Hh ligand (Chuang et al., 2003; Goodrich and Scott, 1998; Goodrich et al., 1996; Goodrich et al., 1997; Jeong and McMahon, 2005; Marigo et al., 1996a). *Gli1*, *Gas1*, *Boc* and *Cdo* are signals which enhance Hh signaling and upregulation of *Gli1* in the presence of the Hh ligand acts as a positive feedback loop to enhance signaling, while downregulation of *Gas1*, *Boc*, and *Cdo* in the presence of the Hh ligand acts as a negative feedback loop and prevents hyperactivation of the Hh pathway (Allen et al., 2007; Martinelli and Fan, 2007; Tenzen et al., 2006; Yao et al., 2006; Zhang et al., 2006). The promoter region of *Gli1* contains Gli binding sites that are essential for *Gli1* expression, and ectopic expression of *Shh* induces aberrant *Gli1* expression, while *Gli1* is not expressed in the absence of *Shh* (Bai et al., 2002; Dai et al., 1999; Grindley et al., 1997; Hynes et al., 1997; Lee et al., 1997; Marigo et al., 1996b). In addition to downregulation of the Hh signaling enhancers *Gas1*, *Boc*, and *Cdo*, the Hh pathway also transcriptionally upregulates *Ptch1* and *Hhip1* expression, both of which inhibit Hh signaling when the Hh ligand is unbound (Chuang et al., 2003; Holtz et al., 2013; Jeong and McMahon, 2005). A balance between these feedback loops initiated by the Hh signaling pathway are essential in regulating the cellular response to the Hh

ligand in a dose specific and temporal manner within tissues of the developing embryo (Ribes and Briscoe, 2009).

One tissue where Hh signaling has been extensively studied and has been found to be essential in patterning and differentiation is the ventral neural tube in the developing embryo. The neural tube is the precursor to the central nervous system, including the brain and spinal cord, and is where different types of neurons are specified (Dessaud et al., 2008; Eggenschwiler and Anderson, 2007; Goodrich and Scott, 1998). Shh is initially expressed in the notochord, a transient tissue that develops ventral to the neural tube, and this initial production of Shh then induces expression of Shh in the most ventral region of the neural tube. The Shh ligand diffuses in a gradient across the ventral neural tube and acts to specify different neural progenitor cell fates (Guillemot, 2007; Jessell, 2000; Lupo et al., 2006; Ribes and Briscoe, 2009; Zhuang and Sockanathan, 2006). The co-receptors of Ptch1, Cdo, Boc and Gas1, are thought to bind the Hh ligand and enhance Hh signaling by presenting it to Ptch1 thus preventing the inhibition of Smo by Ptch1 (Allen et al., 2007; Martinelli and Fan, 2007; Tenzen et al., 2006; Yao et al., 2006; Zhang et al., 2006).

Within the developing embryo, *Gas1* is initially expressed throughout the ventral neural tube in the neural progenitors of the spinal cord, but as Hh ligand diffuses across the neural tube expression of *Gas1* is downregulated in the most ventral neural tube and is then only expressed in the intermediate and dorsal neural tube (Allen et al., 2007). *Cdo* is transiently expressed in the most ventral region of the neural tube which will become the floor plate, but negative regulation of *Cdo* by Hh signaling reduces its expression at later stages where high levels of Hh are present in the floor plate (Allen et al., 2007; Tenzen et al., 2006). *Cdo* and *Boc* expression are also located in the intermediate and dorsal neural tubes, in regions that are exposed to lower levels of

Hh ligand (Tenzen et al., 2006). Although *Cdo* and *Gas1* expression is negatively regulated by Hh, they are essential for specification and cellular identities, and in *Cdo*^{-/-} and *Gas1*^{-/-} mutant embryos have defects in neural patterning, resembling a decreased level of Hh signaling, suggesting that Cdo and Gas1 are required for enhancing the Hh response in the early ventral neural tube to low levels of Hh ligand, and as Hh levels increase this enhanced response is shut down in the most ventral neural tube (Allen et al., 2007; Martinelli and Fan, 2007; Ribes and Briscoe, 2009; Tenzen et al., 2006).

Ptch and *Hhip1* are inhibitors of Hh signaling and are upregulated in the presence of the Hh ligand within the ventral neural tube, and have a cell-autonomous role in regulating ventral neural tube cell fate (Coulombe et al., 2004; Goodrich and Scott, 1998; Goodrich et al., 1996; Goodrich et al., 1997; Jeong and McMahon, 2005; Johnson et al., 2000; Milenkovic et al., 1999; Ochi et al., 2006; Olsen et al., 2004; Rohatgi et al., 2007; Taipale and Beachy, 2001). Loss of *Ptch1* results in strong derepression of Hh signaling, and the entire neural tube adopts ventral neural progenitor identities (Goodrich et al., 1997). Loss of *Hhip1* also results in ventralization of the neural tube, and ectopic expression of either *Hhip1* or *Ptch1* inhibits the Hh dependent ventral cell specification (Briscoe et al., 2001; Stamatakis et al., 2005). The upregulation of *Ptch* and *Hhip1* may serve to regulate the concentration gradient of Hh in the neural tube in a noncell-autonomous way by binding and sequestering the Hh ligand to prevent the dorsal neural tube from adopting ventral neural tube cell fates and to regulate the concentration gradient of the Hh ligand (Briscoe et al., 2001; Chen and Struhl, 1996; Stamatakis et al., 2005). In support of this hypothesis, embryos that have reduced *Ptch1* and *Hhip1* gene expression have an expansion of ventral neural tube cell fates into the dorsal neural tube domain, and if *Ptch1* and *Hhip1* are not upregulated in the presence of the Hh ligand, such as in a *Smo* mutant, which cannot activate the

pathway, the Hh ligand is able to travel much further (Chamberlain et al., 2008; Jeong and McMahon, 2005). The role of the feedback loops initiated by Hh pathway induction are to ensure robust pathway activation and precise interpretation of the gradient of the ligand to create distinct populations of cells within set domains of the neural tube (Ribes and Briscoe, 2009).

Within the ventral neural tube the gradient of Shh also activates different transcription factors based on the concentration of the Shh ligand the cells are exposed to, and specifies six separate populations of neural progenitor cells, the floor plate, p3, pMotor Neuron (pMN), p2, p1, and p0 (Figure 1.1) (Briscoe et al., 2000; Chiang et al., 1996; Ericson et al., 1996; Ericson et al., 1997a; Marti et al., 1995; Martí et al., 1995; Pierani et al., 1999; Vokes et al., 2007; Vokes et al., 2008; Wijgerde et al., 2002). The populations of neural progenitors are sequentially specified through the concentration gradient of the Hh ligand and through the duration of exposure to the Hh ligand activating different combinations of specific transcription factors within each population of cells (Briscoe et al., 2000; Dessaud et al., 2007; Ericson et al., 1997a).

The transcription factors activated in each population of neural progenitors regulate cell fate identity and generate distinct populations of neural progenitors through cross regulation of transcription factors activated in other neural progenitor populations (Jeong and McMahon, 2005). The transcription factors can be divided into two classes (Class I and Class II) based on how they are regulated by Hh and the pattern of their expression (Briscoe and Ericson, 1999; Briscoe et al., 1999; Briscoe et al., 2000). Class I transcription factors are repressed by different concentrations of Hh and Class II transcription factors are induced at different concentrations of Hh (Briscoe and Ericson, 2001). These two classes of transcription factors interact with one another to form the boundaries of each of the ventral neural tube cell fates. The Class I transcription factor Pax6 is expressed in the dorsal neural tube, and responds to low levels of Hh

signaling while it is inhibited by high levels of Hh signaling found more ventrally in the neural tube. Pax6 inhibits the Class II transcription factor *Nkx2.2* from expanding more dorsally, and *Nkx2.2* inhibits the expansion of *Pax6* more ventrally (Briscoe et al., 2000; Ericson et al., 1997a; Ericson et al., 1997b). Similarly, the Class I transcription factor, *Dbx2* is expressed more dorsally and inhibits expression of the more ventral Class II transcription factor *Nkx6.1* (Briscoe et al., 2000; Sander et al., 2000). In the most ventral neural tube, the transcription factor, *FoxA2*, distinguishes the floor plate of the neural tube which expresses *Shh*. The next most ventral cell fates, p3, pMN, and p2, are controlled through expression of different combinations of the transcription factors *Nkx6.1*, *Nkx2.2*, and *Irx3*. The pMN fate is specified when there is expression of *Nkx6.1*, and while the p3 and p2 domains express *Nkx6.1* they also express *Irx3* and *Nkx2.2*, respectively, which inhibit *Nkx6.1* and prevents the motor neuron cell fate in the p3 and p2 domains (Briscoe et al., 1999; Briscoe et al., 2000; Sander et al., 2000). The different combinations of transcription factors can then activate neural progenitor fate specific genes which promote the specification and differentiation of the neural progenitors into distinct populations of mature neurons (Figure 1.1). *Shh* can also regulate the expression of specific transcription factors in other tissues. The developing somites adjacent to the neural tube also respond to Hh produced in the notochord, but upregulate a completely different transcription factor, *Pax1*, than the neural tube does (Bumcrot and McMahon, 1995; Smith and Tuan, 1995).

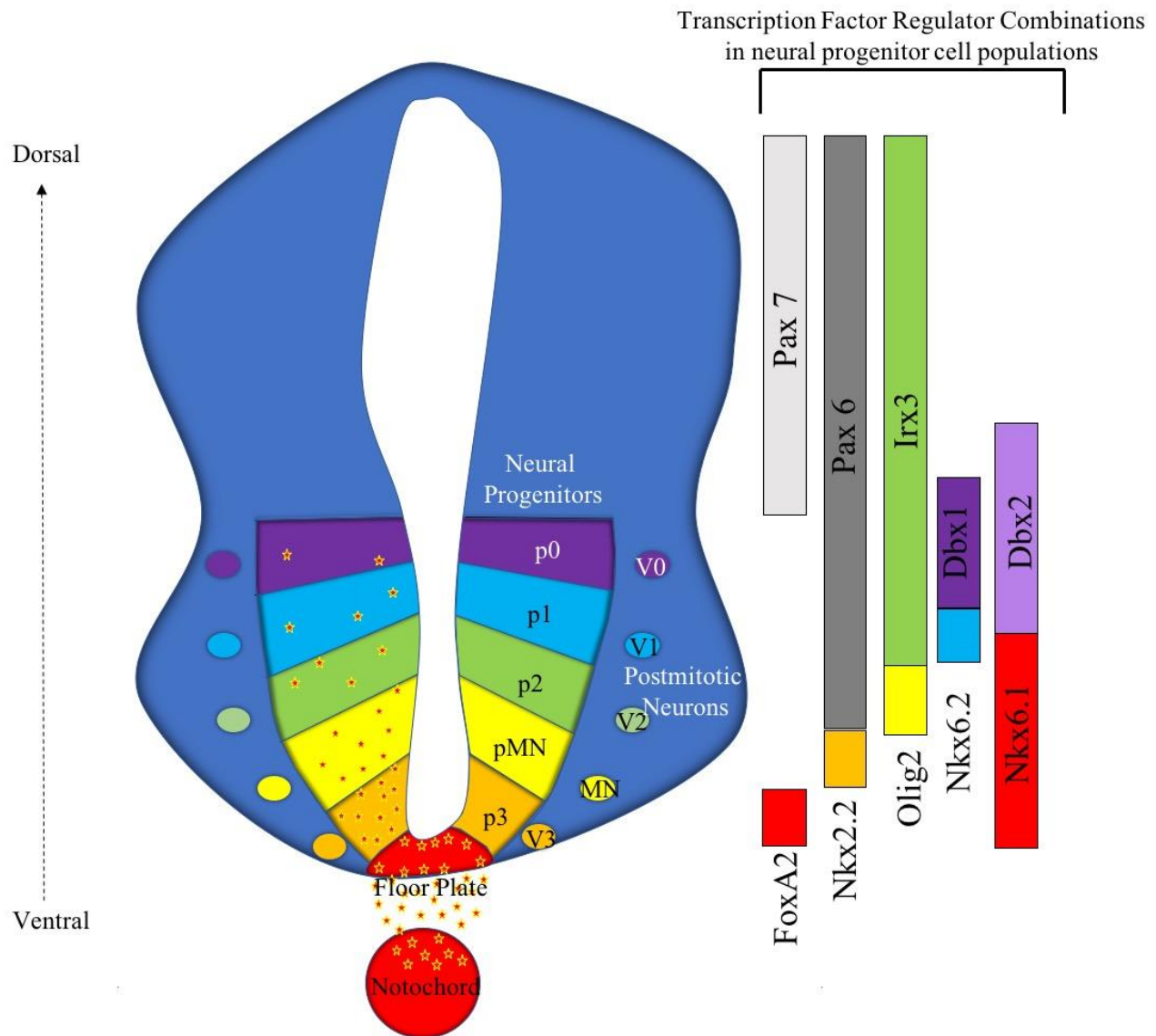
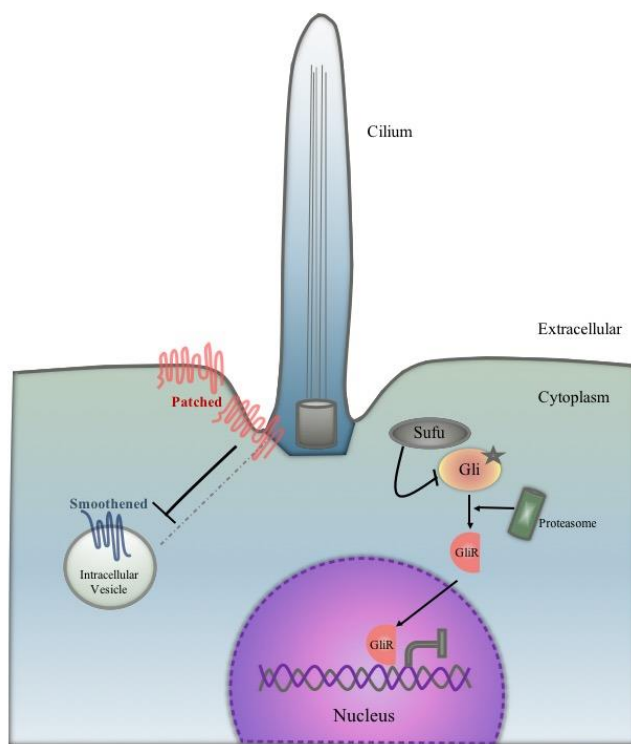


Figure 1.1 Ventral neural tube patterning Shh is produced in the notochord and induces expression of Shh within the floor plate. The gradient of the Hh ligand activates different combinations of transcription factors within each population of neural progenitors. The domain of expression for each transcription factor is dependent on the concentration of the Hh ligand and the duration of exposure to the ligand, and the tightly regulated domains for each transcription factor is regulated through inhibitory interactions with additional transcription factors. Figure modified from (Ribes and Briscoe 2009)

Cilia and Hh signaling

In mammals, a microtubule based cellular projection, called the primary cilium is essential for Hh signaling. Nearly every cell within the mammalian body produces a primary cilium, and mutations that affect the cilium can drastically affect embryonic development, and result in polydactyly, holoprosencephaly, and can be embryonic lethal (Baker and Beales, 2009; Huangfu et al., 2003; Lancaster and Gleeson, 2009). Central components of the Hh pathway localize and are processed within the cilium, and the enrichment of Hh components within the cilium is thought to provide a precise location within the cell to promote interaction between the essential components and ensure rigorous activation of Hh signaling (Corbit et al., 2005b; Goetz and Anderson, 2010; Rohatgi et al., 2007). The Hh receptor Ptch is localized to the base of the cilium when a cell has not been stimulated with the Hh ligand, and Smo is prevented from accumulating in the cilium (Kim et al., 2009a; Ocbina and Anderson, 2008). When a cell is stimulated with Hh, Ptch leaves the cilium and Smo enters the cilium (Corbit et al., 2005b; Rohatgi et al., 2007). Gli2 and Gli3 also localize to the tip of the cilium, and accumulation of Gli2 and Gli3 at the tip is promoted by Hh activation (Haycraft et al., 2005b; Kim et al., 2009a). Sufu acts as a negative regulator of Hh signaling in the absence of cilia and it is thought that Smo inhibits the association of Sufu with the Glis within the tip of a cilium. Then, in the presence of the Hh ligand, the Glis are then able to translocate to the nucleus and promote transcription of Hh target genes (Chen et al., 2009; Haycraft et al., 2005b; Jia et al., 2009b) (Figure 1.2).

Unstimulated



Hh Stimulated

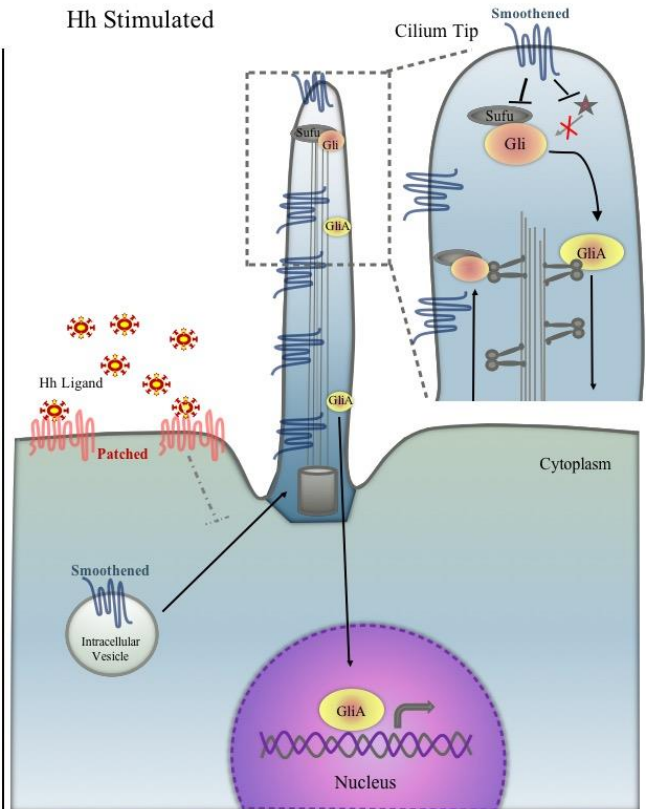


Figure 1.2 Major components of the Hh signaling pathway and the primary cilium In the absence of the Hh ligand the Hh receptor, Patched localizes to the base of the cilium and inhibits Smoothed translocation into the cilium. Sufu associates with the Glis and the Gli proteins are phosphorylated and cleaved into the repressor form of Gli transcription factors which translocate to the nucleus and repress Hh response. In the presence of the Hh ligand, Patched leaves the cilium and Smo enters the cilium and represses Sufu binding of Glis and prevents repressive phosphorylation and cleavage. Glis can exit the cilium and act as transcriptional activators to promote transcription of Hh target genes.

Although mutations in components of cilia generally do not have phenotypes characteristic of mutations in other signaling pathways, the ubiquity of cilia production throughout the embryo calls into question the role of cilia on cells that are not exposed to or do not respond to Hh. A few studies have implicated cilia in other signaling pathways including Wnt signaling and platelet-derived growth factor receptor- α signaling (PDGFR- α). Generally, cilia mutants in both mice and zebrafish do not have evidence of altered Wnt signaling (Huang and Schier, 2009; Ocbina et al., 2009; Tran et al., 2008), but several studies have suggested there is elevated canonical Wnt signaling or disruptions to non-canonical Wnt signaling within culture cells and zebrafish embryos (Corbit et al., 2008; Gerdes et al., 2007; Jonassen et al., 2008; Ross et al., 2005; Simons et al., 2005; Wiens et al., 2010). The majority of the evidence including expression of Wnt reporters in cilia mutants in vivo, suggest that Wnt signaling is not altered in cilia mutants, and that cilia are not required for either canonical or non-canonical Wnt signaling in embryonic development (Ocbina et al., 2009; Tran et al., 2008). PDGFR- α signaling is required in later stages of development in oligodendrocytes and craniofacial development (Klinghoffer et al., 2002; Soriano, 1997). The receptor for this signaling pathway is localized to the primary cilium in adult rat neural stem cells, and cilia have been found to impact PDGFR- α signaling in cultured cells (Danilov et al., 2009; Schneider et al., 2005). More studies analysis the impact of loss of cilia on PDGFR- α signaling in vivo are essential to understanding the role of cilia in this signaling pathway.

Ciliary Structure and trafficking of proteins within the cilium

Primary cilia are made up of a ring of nine pairs of microtubule doublets nucleated from the basal body, a modified centrosome (Kim and Dynlacht, 2013; Kim et al., 2010; Seeley and Nachury, 2010). Motile cilia have an additional central doublet of microtubules, which primary cilia lack. These microtubules make up the axoneme of the cilium and provide the tracks for trafficking of proteins within the cilium. Intraflagellar transport (IFT) is the process of moving proteins through the cilium and is required for ciliary lengthening and maintenance. IFT from the base of the cilium to the tip is called anterograde transport and requires a kinesin-2 motor, made of a complex of two kinesin-2 family proteins, Kif3a and Kif3b, and Kif-associated protein 3 (Kap3) (Figure 1.3). The Kif3 motor complex moves toward the plus end of the microtubules located at the growing tip of the cilium, and mutations that disrupt the kinesin motor function prevents cilia formation (Blacque and Leroux, 2006; Cole, 2003; Iomini et al., 2001; Tran et al., 2008). IFT from the tip of the cilium back towards the cell body requires a dynein motor complex made up of cytoplasmic dynein 2 heavy chain 1 (Dync2h1) and cytoplasmic dynein 2 light intermediate chain 1 (Dync2l1) which moves towards the minus ends of the microtubules. Mutations that disrupt the dynein motor function result in accumulation of proteins at the tip of the cilium, and bulbous or distended ciliary tips (Blacque and Leroux, 2006; Iomini et al., 2001; Pedersen et al., 2008; Tran et al., 2008).

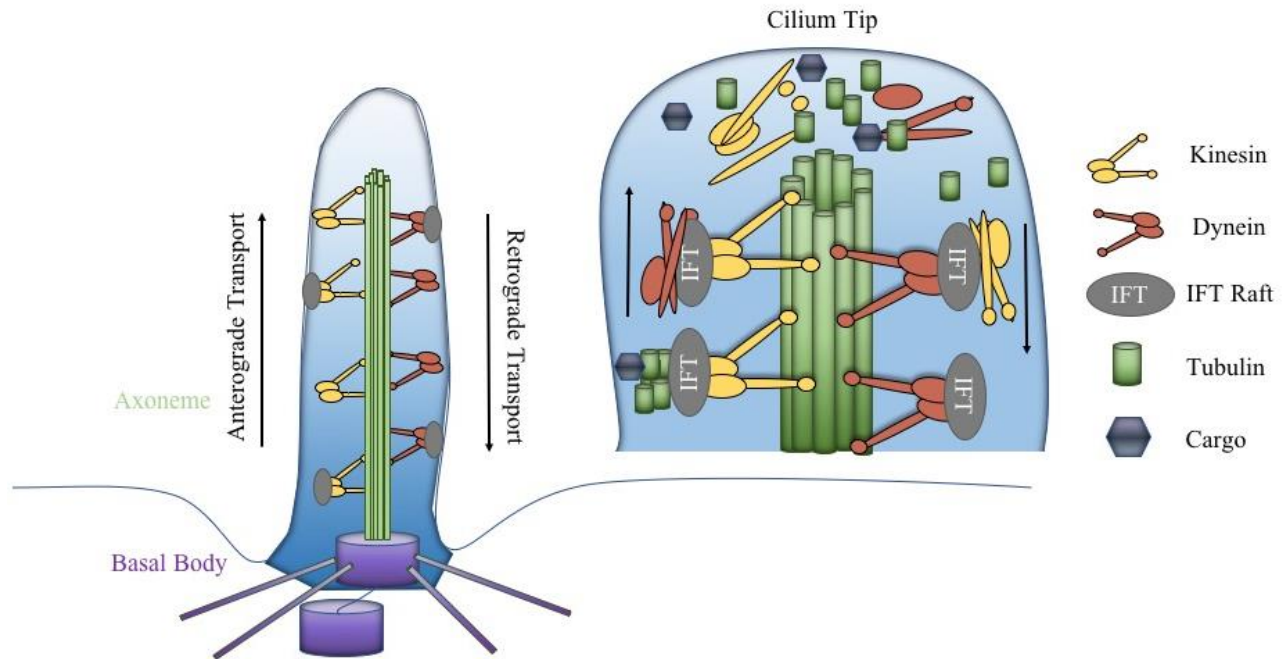


Figure 1.3 Intraflagellar transport within the primary cilium The axoneme of the cilium is made up of a ring of nine microtubule doublets that are nucleated from the basal body. Kinesins facilitate transport of cargo from the base of the cilium to the tip by anterograde transport. Dyneins facilitate transport of cargo from the tip of the cilium toward the base by retrograde transport. IFT rafts are connected to the motor proteins to allow for cargo transport into and out of the cilium.

Two protein complexes, IFTA and IFTB, interact with the motor proteins and act as IFT ferries to enable transport of ciliary cargo (Blacque and Leroux, 2006; Tran et al., 2008). IFTB has been shown to be involved in anterograde transport, although, the IFTB protein complex may have other roles in cilia that are difficult to detect since null mutations in IFTB machinery disrupt anterograde transport and result in the inability to produce cilia thus masking any other ciliary roles (Houde et al., 2006; Huangfu et al., 2003; Liu et al., 2005). The IFTA protein complex is most significantly involved in retrograde transport, but has been shown to also have a role of anterograde transport of specific cargo such as Tulp3, IFT122 and Gpr161 (Alazami et al., 2014; Cameron et al., 2009; Chávez et al., 2015; Cortellino et al., 2010; Garcia-gonzalo and Reiter, 2012; Mukhopadhyay et al., 2010; Norman et al., 2009; Ocbina et al., 2011; Pak and Segal, 2016; Qin et al., 2011; Ropers et al., 2010). Additionally, mutations in components of IFTA can have short bulbous cilia indicating potential additional defects in anterograde transport, and can impact Hh signaling through slowed transport of the essential components (Cortellino et al., 2010). Mutations in IFT machinery and the motor proteins which impact the transport of Hh components have also been implicated in defects in Hh patterning within mouse embryos (Eggenchwiler and Anderson, 2007; Huangfu et al., 2003).

Genetic analysis of mutations in cilia machinery places IFT proteins downstream of Ptch and Smo, but upstream of the Gli transcription factors. Smo is thought to be trafficked from the plasma membrane into the cilium, and single molecule analysis of Smo proteins suggests that Smo primarily diffuses through the cilium rather than actively being transported by IFT (Milenkovic et al., 2009; Milenkovic et al., 2015). It is thought that both active transport and diffusion of proteins, including Smo, from the plasma membrane or cytoplasm into the cilium is tightly regulate, although the mechanism underlying the regulation is not well understood

(Corbit et al., 2005a; Craft et al., 2015; Jia et al., 2003; Milenkovic et al., 2009; Milenkovic et al., 2015). Since IFT is required for both the production of Gli activators and Gli repressors, the resulting phenotypes of Hh signaling defects in IFT mutants is complex (Firestone et al., 2012; Huangfu and Anderson, 2005; Huangfu et al., 2003; Kim et al., 2009a; Liu et al., 2005; May et al., 2005; Ocbina and Anderson, 2008).

In some tissues, such as the developing neural tube, Gli2 activator is primarily responsible for Hh patterning of the tissue. In this case, IFT mutants present with a reduction in Hh response as a result of reduced amounts of Gli activator processed within the cilium. In other tissues, such as the developing limbs, Gli3 repressor has a central role in regulating the domain of Hh expression. Reduction or loss of Gli3 repressor due to a mutation in IFT machinery can result in an expansion of the Hh domain within in the limb and resulting in pre-axial polydactyly and other phenotypes indicative of excessive or ectopic Hh expression within the embryo (Goetz and Anderson, 2010; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005). The transport of Gli2 and Gli3 through the cilium depends on the kinesin, Kif7 (Goetz and Anderson, 2010). Kif7 is localized to the base of the cilium when there is no Hh ligand present, but relocalizes to the tip of the cilium in the presence of Hh ligand (Endoh-Yamagami et al., 2009; Liem et al., 2009). Kif7 has been shown to both positively and negatively regulate the Hh pathway and may do so through actively transporting Glis in the presence of Hh ligand to be processed into transcriptional activators, and by trafficking Glis away from the cilium in the absence of the ligand to prevent processing (Goetz and Anderson, 2010; Liem et al., 2009).

Building and maintaining a cilium

Nearly every cell within the mammalian body produces a primary cilium, but the length of the cilium produced can vary greatly between different tissue types. Length control is essential for cell type specific function of cilia, and to achieve appropriate length each cell type must have a specific mechanism in place (Lechtreck et al., 2017). To generate a cilium, a cell must exit mitosis, since the centrioles used for microtubule production and coordination during cell division are also required and modified for microtubule production within the cilium (Azimzadeh and Marshall, 2010; Ishikawa and Marshall, 2011; Tang and Marshall, 2012). When a cell is not dividing, the unrestricted mother centriole becomes the basal body of the cilium, and anchors to a ciliary vesicle. The ciliary vesicle then fuses with the plasma membrane, and microtubules nucleate from the basal body to initiate ciliogenesis, or the production of the cilium. The microtubules extend the ciliary axoneme and the ciliary membrane, a region of the plasma membrane with slightly altered composition. The ciliary extension is mediated through IFT and diffusion of tubulin into the growing cilium to continue extension at the tip (Avasthi and Marshall, 2012; Marshall and Rosenbaum, 2001). In mammalian cells, the onset of ciliogenesis can occur during centrosome migration or after it has docked at the plasma membrane (Pedersen et al., 2016). In some cell types, such as epithelial cells, the axoneme begins to extend before the centrosome has completely migrated and docked at the plasma membrane (Molla-Herman et al., 2010; Sorokin, 1968). While in other cell types, such as fibroblasts and smooth muscle, ciliogenesis does not begin until the ciliary vesicle docks (Molla-Herman et al., 2010; Sorokin, 1962). These differences in initiation of ciliogenesis may also impact the rate at which a cilium grows and the final length a cilium extends to and may contribute to the differing lengths of cilia found on different cell types.

Much of our understanding of cilia production and maintenance come from work in the small green algae *Chlamydomonas reinhardtii*. Tubulin and other small soluble proteins smaller than 50kDa and transmembrane proteins can diffuse into the cilium, however, it is thought that IFT is required for enrichment of some proteins, including tubulin, within the cilium (Belzile et al., 2013; Craft et al., 2015; Harris et al., 2016; Kee et al., 2012). The amount of tubulin imported into the growing cilium by IFT likely controls both the final length and the rate at which a cilium grows. Cells precisely regulate the length they build their cilium to through feedback loops, where more building materials are imported into a cilium when it is growing, and when cilia grow past the set length for a given cell type a ciliary shortening mechanism is triggered (Hilton et al., 2013; Liang et al., 2016).

The mechanisms for sensing cilia length and regulating cilia length based on the existing length remain unknown. Cells may regulate how the IFT proteins interact with the cargo they carry through modifications to the cargo or the IFT proteins themselves, or cells may regulate the amount and frequency IFT loaded with cargo can enter the cilium (Follit, 2006; Follit et al., 2009). There is evidence for phosphorylation of IFT proteins, but the role of this alteration on cargo binding is unclear (Kubo et al., 2016). In addition to tubulin dynamics, there is also evidence for a role for actin in cilia length regulation. Cortical actin appears to promote ciliogenesis by provide a scaffold for positioning and stabilizing the basal body of cilia, cytoplasmic actin stress fibers, however, seem to inhibit ciliogenesis by disrupting basal body migration (Dawe et al., 2007; Pitaval et al., 2010; Sharma et al., 2011). Additional studies have found that the actin polymerization protein, Cortactin, can be inhibited by MIM, a basal body protein and positive regulator of Hh signaling, and this inhibition of actin polymerization and

branching can promote ciliogenesis (Bershteyn et al., 2010; Kim et al., 2010). Further characterization of the role of actin in ciliogenesis and how it impacts transport is required.

Once a cilium has reached its full length, in most cell types, IFT continues to maintain the cilium and has an additional roles such as promoting Hh signaling within the cilium (Wood et al., 2012). Although, cilia and IFT are essential for Hh signaling, disruption of ciliary length does not always impact Hh signaling. Mice with a mutation in the transcription factor, *Rfx3*, have shortened cilia but do not have alterations to Hh signaling (Bonnafe et al., 2004). IFT likely acts to maintain cilium length through tubulin turnover and to remove small proteins which diffuse into the cilium, but there are multiple models that could explain how this occurs (Kee et al., 2012).

One model for how cilia maintain length at a steady state is through limiting the number or function of IFT trains within the cilium when it is not growing to reduce the amount of tubulin import, however, loss of IFT results in ciliary shortening rather than maintaining a stable length (Marshall and Rosenbaum, 2001). Another model is the limiting precursor model, where a cell only produces a specific number of ciliary subunits required for the full length of its cilium in order to control the length of the cilium. However, studies have shown that there is not a limited amount of precursor to build the cilium, and even after the cilium reaches its full length, the cell maintains a pool of cilia building precursors (Lefebvre and LeBoeuf, 1987; Rosenbaum et al., 1969).

A major model for cilium length maintenance is the balance-point model, where IFT is continuously active in the cilium, but as the cilium reaches full length, it takes a longer length of time for the IFT particles traveling at a set speed to reach the tip of cilia, so it takes longer to accumulate tubulin at the tip to continue to build the cilium. The point at which the cilium

reaches the set length it should be, it reaches a balance in anterograde and retrograde cargo transport, where the rate of ciliary assembly is equal to the rate of ciliary disassembly (Chan and Marshall, 2012; Marshall, 2015; Marshall and Rosenbaum, 2001). Experimental evidence indicates that cells do maintain constant IFT, but the amount of cargo they transport is also decreased as the cilium reaches its set length, indicating that the mechanism is more complex than the length of time it takes an IFT train to reach the tip (Craft et al., 2015; Goehring and Hyman, 2012).

The final model of cilia length regulation considers the observation in the balance-point model that the amount of cargo transported decreases as the cilium reaches a set length and proposes that cilia length maintenance is controlled through differential loading of cargo. Cilia in the process of growing have IFT trains loaded with large amounts of cargo, while cilia that have reached full length decrease the amount of cargo loaded onto IFT particles. The differential amounts of cargo imported on the constant IFT trains would control whether the cilium continues to grow, shortens, or maintains length (Craft et al., 2015; Hilton et al., 2013; Liang et al., 2016).

To re-enter the cell cycle, cells must resorb their cilium, and mutations in proteins which normally facilitate the disassembly of the cilium can delay cell cycle re-entry. Regulation of the retrograde transport, including the function of the dynein motor can impact the speed at which a cilium is resorbed and delay a cells re-entry into the cell cycle. One such example is when the centrosome protein, Nde1, was knockdown in zebrafish embryos ciliary length increased and there was a delayed cell cycle re-entry. Nde1 interacts with the dynein subunit LC8 and presumably affects ciliary length through decreased retrograde transport by the dynein motor (Kim and Tsiokas, 2011).

In mouse, it was also found that phosphorylation of a dynein motor subunit, Tctex-1, can induce resorption of the cilium and promote cell cycle re-entry (Li et al., 2011). In addition to regulation of the dynein motors to promote ciliary resorption, a role for mammalian Aurora A kinase, located at the centrosome, in cilia resorption has been described in human cell culture through regulating the stability and organization of microtubules within the cilium (Pugacheva et al., 2007). Ciliogenesis can be altered in cells which fail to properly exit the cell cycle and over-proliferate, thus most cancer cell lines fail to form cilia (Han and Alvarez-Buylla, 2010; Plotnikova et al., 2009). Further understanding of the mechanism of cilia resorption and its relationship to the cell cycle may come from studies of flagella, an equivalent structure to cilia, in *Chlamydomonas*, *Trypanosoma*, and *Tetrahymena* in addition to mammalian systems.

Regulators of Cilia length (CCRK, ICK and others)

Cilia length can vary greatly between different cell types, fibroblasts have cilia of around 3 μm while olfactory cilia can grow to 200 μm . The length and morphology of a cilium are tightly regulated, and likely have a crucial role in cellular function (Satir and Christensen, 2007; Silverman and Leroux, 2009). Regulation of cilia length likely occurs through the interaction of multiple mechanisms and is not fully understood how a cell interprets the environment and the internal cellular context to produce a cilium of the appropriate length. Studies show that cilia length regulation may depend on a number of factors including, modulation of tubulin, the IFT particles themselves, the motor proteins, regulation of the entrance of proteins into the cilium, different signaling pathway inputs, and a host of different proteins and kinases (Broekhuis et al., 2013).

Cilia length can be modulated through the amount of tubulin transported on the IFT particles, but additional post-translation modifications to tubulin may also impact the final length of cilia, and these posttranslational modifications are required for axonemal stabilization (Pugacheva et al., 2007; Verhey and Gaertig, 2007). For example, the tubulin within the axoneme can be acetylated, and disruption of the acetyltransferase- α TAT1 perturbs ciliary assembly (Shida et al., 2010). Furthermore, it was found that BBIP10, a component of the BBosome, interacts with HDAC6 and is required for acetylation of the axonemal tubulin (Loktev et al., 2008). Posttranslational glycylation and glutamylation of tubulin have also been shown to stabilize ciliary structure in zebrafish and in the motile cilia of the trachea (Ikegami and Setou, 2010; Pathak et al., 2011).

It is likely that distinct sets of proteins have roles in regulating cilia length in different cell types. In mammals the kinesin, Kif17, has an important role in regulating protein localization to the cilium within olfactory cilia (Jenkins et al., 2006). Additionally, Kif7 has been found to function as a regulator of Hh signaling, and is essential for localizing Gli3 to the cilium (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Hsu et al., 2011; Liem et al., 2009). Kif27 has also been found to regulate the Hh pathway, but a potential role for this kinesin in cilia remains to be determined (Liem et al., 2009). More research needs to be done to determine if other kinesins and ciliary proteins have cell type specific roles in cilia length regulation or in specific protein transport into the cilium, and if some proteins may have a more ubiquitous function in ciliary length regulation.

The mechanisms which precisely control the length of primary cilia based on cell type may also involve input from additional signaling pathways, and there is evidence for Fgf, inositol, and mammalian target of rapamycin (mTOR) signaling pathways in regulating cilia

length in vertebrates. Knockdown of the *Fgf* receptor (*Fgfr1*) or the *Fgf* ligands in zebrafish resulted in shorter cilia and the zebrafish node (Kupffer's vesicle) (Neugebauer et al., 2009; Yamauchi et al., 2009). Knockdown of the phosphatidylinositol signaling component, *Ipk1*, in zebrafish also resulted in shortened cilia and reduced cilia frequency (Sarmah et al., 2007). A human inositol signaling component (INPP5E) localizes to the cilium and has also been implicated in the ciliary disease, Joubert syndrome (Bielas et al., 2009). Mice and zebrafish with mutations in the mTOR components *Tsc1* and *Tsc2* had elongated cilia, and when zebrafish were treated with the TOR inhibitor, rapamycin, cilia length decreased (Bonnet et al., 2009; Dibella et al., 2009; Hartman et al., 2009; Yuan et al., 2012). These results suggest that, in addition to the cell autonomous regulation of cilia length, external signals and cellular environment may also have a role in regulating cilia length in certain cell types.

Proteins at the base in and the ciliary membrane may also contribute to ciliary length through regulation of the proteins that can enter and exit the cilium, including tubulin required for ciliary assembly. The ciliary membrane is thought to be a continuation of the cellular plasma membrane, but differs in composition, where the ciliary membrane is enriched in sterols, glycolipids, and sphingolipids compared to the plasmid membrane (Bielas et al., 2009; Bloodgood and Salomonsky, 1995; Jacoby et al., 2009). The differences in the membrane and the presence of septin proteins at the base of the cilium are thought to act as a diffusion barrier, to prevent unregulated entrance and exit of transmembrane proteins into and out of the cilium by diffusion (Hu et al., 2010; Vieira et al., 2006). This selective barrier is also thought to play a role in concentrating signaling proteins, such as those used by the Hh signaling pathway, in the cilium to promote their interaction (Broekhuis et al., 2013). At the base of the cilium there is a region called the transition zone where transition fibers connect the basal body to the ciliary

membrane and Y-links connect the axoneme to the ciliary membrane (Doolin and Birge, 1966; Muresan and Besharse, 1994). It is thought that within the transition zone, IFT particles can be loaded with specific cargo and modified to allow them entrance into the cilium (Broekhuis et al., 2013; Sedmak and Wolfrum, 2010).

The transition zone protein, *Nphp-1*, had been implicated in regulating the amount of IFT complexes in *Caenorhabditis elegans* and in the photoreceptors of mice, and loss of *Nphp1* results in reduction of IFT protein localization and changes in IFT protein amounts (Jauregui and Barr, 2005; Jiang et al., 2008). The transition zone proteins Tctn1, Tctn2, Tmem67, and Cc2d2a form a protein complex, and mutations in these genes results in shorter cilia in cell culture (Garcia-gonzalo and Reiter, 2011). While knockdown of *Nphp-8* in cell culture resulted in longer cilia (Patzke et al., 2010). It is likely that the transition zone proteins and the basal body proteins interact with IFT particles to regulate what is transported within the cilium. Indeed, IFT70 has been shown to interact with the human transition zone protein B9D2/MKS10, and LCA5, a transition protein implicated in the ciliary disease Leber congenital amaurosis, also interacts with several IFT proteins (Boldt et al., 2011; Zhao and Malicki, 2011).

Kinases and phosphatases have also been shown to play an important role in cilia length regulation and ciliary morphology across a number of diverse species. Kinases from the ROS cross-hybridizing kinase (RCK) family have been found to regulate cilia length in a number of species including *Chlamydomonas*, *Leishmania*, *C. elegans*, and mice (Bengs et al., 2005; Berman et al., 2003a; Burghoorn et al., 2007; Omori et al., 2010). The cyclin dependent-related kinase, *long flagella 2 (lf2)* in *Chlamydomonas* also regulates ciliary length and the homolog of LF2p in zebrafish and mice is cell cycle-related kinase CCRK (Asleson and Lefebvre, 1998; Ko et al., 2010; Nguyen et al., 2005; Tam et al., 2003; Tam et al., 2007). Previous work from our lab

found that the mutations in the protein broad-minded (BROMI), which associates with and stabilizes CCRK, resulted in disruption of Hh signaling and ciliary morphology in mice and zebrafish (Ko et al., 2010).

The mechanism by which these kinases control ciliary length is not fully understood, but there is evidence that they may regulate IFT machinery (Burghoorn et al., 2007). In *C. elegans*, DYF-5 is a member of the RCK family of protein kinases and negatively regulates ciliary length (promotes ciliary shortening) and is thought it may do so through phosphorylation mediated regulation of cargo loading onto IFT particles by modulating the affinity of cargo or the removal of kinesin from IFT particles, although direct interaction between DYF-5 and kinesin have not been shown (Guillaud et al., 2008; Hirokawa et al., 2009). Mutations in the homologs of *DYF-5* in *Chlamydomonas* (*lf4*), *Leishmania* (*LmxMPK9*), and mammals (*Ick(Mrk)/Mak/Mok*) all seem to have similar effects on cilia length, and these kinases generally seem to negatively regulate ciliary length (Bladt and Birchmeier, 1993; Miyata and Nishida, 1999; Togawa et al., 2000; Xia and Karin, 2004).

Our data suggests that Cell Cycle-Related Kinase (CCRK) regulates ciliary length in mice and may do so by phosphorylation of targets to regulate protein transport into and out of the cilium. CCRK has been shown to phosphorylate a mammalian member of the RCK family, intestinal cell kinase (ICK/MRK), which generally has a role in ciliary shortening (Fu et al., 2006a; Moon et al., 2014). In *Ccrk* mutant cells, IFT frequencies and velocities are not drastically affected, but the length of cilia is not tightly controlled as observed in wild-type cells, suggesting that through phosphorylation of ICK and other potential targets, CCRK may control the cargo that is carried on the IFT particles to regulate ciliary length rather than regulating the speed or frequency of the IFT particles. ICK has also been implicated in regulating a component

of the mTOR pathway, and may impact cilia length through this mechanism (Bolick et al., 2014; Wu et al., 2012; Yuan et al., 2012). The role of CCRK in cilia length regulation is further discussed in Chapter II and the role of ICK and its interaction with CCRK in cilia length regulation in Chapter III.

Cilia and Hh signaling in tissue patterning and disease

Cilia and Hh signaling are essential for tissues patterning and development throughout the embryo. One of the most well studied tissues which requires Hh is the ventral neural tube. Hh is initially produced in a transient structure ventral to the neural tube called the notochord, and Hh diffuses to the neural tube where it induces its own expression in a group of cells which will become the floor plate. From the floor plate the Hh ligand diffuses across the neural tube forming a concentration gradient, and the varied concentrations specify different neural progenitor populations within the developing neural tube (Ingham and McMahon, 2001).

Cilia are required for localization and activation of the Gli transcription factors in Hh signaling transduction. Within the neural tube, Gli2 is the primary transcriptional activator, in addition to the transcriptional activator function of Gli1. Gli3 can act as both a transcriptional activator and repressor within the neural tube (Eggenchwiler and Anderson, 2007). The first link between Hh signaling and cilia was found in Hh patterning in the neural tube, where ciliary and IFT protein mutants were found to have defects in ventral neural tube patterning (Corbit et al., 2005a; Haycraft et al., 2005a; Huangfu and Anderson, 2005; Liu et al., 2005). In IFT mutants, Gli proteins are not processed into Gli activator form and Gli3 is not processed into the repressor form, resulting in the inability to regulate Hh target genes either by repression or activation. The loss of regulation of Hh target genes results in elevated basal levels of Hh target

genes in cells which would normally repress Hh target genes because of the loss of the Gli repressor, and the inability to mount a full level response by upregulated expression of Hh target genes in the presence of ligand because of loss of the Gli activators (Haycraft et al., 2005b; Huangfu and Anderson, 2005; Huangfu et al., 2003; Liu et al., 2005; May et al., 2005).

Hh is also required within the embryonic limb to regulate growth, digit number and anterior posterior polarity. Within the limb, the Gli3 repressor plays a dominant role, and complete loss of Shh results in the formation of a single digit, whereas Gli3 mutants have additional digits (preaxial polydactyly), even in heterozygous animals. Within the limb, Shh is expressed in the posterior region of the limb and is essential for digit patterning and limb development, but limiting the domain of Hh response is essential to ensure the proper digit number. Expression of Gli3 repressor within the anterior region of the limb prevents expansion of the Shh expression domain, and maintains the proper digit number (Eggenchwiler and Anderson, 2007; Tickle, 2006; Tickle, 2015). As in the neural tube, IFT mutations impact both the formation of Gli activators and Gli repressors. In the developing limb of IFT or cilia mutants there is reduced Gli activator function in the Shh responding posterior domain of the limb bud resulting in decreased Hh response in the posterior limb bud. Additionally, as a result of a cilia or IFT mutation there is also loss of Gli repressor formation, so there is derepression of the pathway and elevated basal level of Hh response. Loss of Gli repressor function results in elevated basal levels of Hh, and the response to Hh becomes ectopically expressed in the anterior region of the limb bud, resulting in polydactyly, a phenotype typically associated with increased levels Hh signaling activity. Within the same mutant, in other tissues where the function of Gli activator is more crucial additional phenotypes may resemble a decrease in Hh signaling (Haycraft et al., 2005b; Liu et al., 2005; May et al., 2005).

Cilia and Hh also play a crucial role in craniofacial development. The distance of craniofacial features from the midline of the face requires Hh, and changes to Hh amounts can result in a spectrum of phenotypes. Loss or severe reduction of Hh results in collapse of craniofacial structures towards the midline, and can result in cyclopia (Chiang et al., 1996; Cordero et al., 2004; Roessler and Muenke, 2003). Increased levels of Hh expands the distance of craniofacial structures away from the midline and can result in duplication of midline facial structures including the nose and also failure to fuse the roof of the mouth (clef palate) (Hu and Helms, 1999; Vortkamp et al., 1991; Vortkamp et al., 1992; Young et al., 2010). As in the neural tube and developing limbs, loss of IFT proteins in the craniofacial tissues also results in disruption of Gli processing, and loss of both Gli2 repressor and Gli3 repressor disrupts craniofacial development and results in widening of the face from the midline and a cleft palate. The widening of the face and cleft palate observed in cilia mutants as a result of the absence of Gli repressors can be further exacerbated by additional expression of a form of Gli2 activator that does not rely on cilia for processing, demonstrating a role for both the repressor and activator function of the Glis in craniofacial development (Chang et al., 2016).

Another important process in embryonic development is establishment of the left and right side of the embryo. Asymmetries in organ placement such as the heart, liver, stomach, and pancreas as well as asymmetries that develop within organs such as the lungs are essential for embryonic development. The initiation of left side determination of the embryo occurs within a small pit of cells at the midline of the posterior embryo called the node. Hh is also expressed within the embryonic node, and is essential for the initial break in left-right symmetry within the mouse embryo (Nakamura and Hamada, 2012). Interestingly, in chicks, Hh is asymmetrically expressed at Hensen's node, a structure equivalent to the mouse node, with more Hh on the left

side of the node than the right, and this asymmetry is instructive in left side determination (Aw and Levin, 2008; Levin et al., 1995; Monsoro-Burq and Le Douarin, 2001). However, in mice Hh is symmetrically expressed at the node and, although expression of Hh is required for left-right patterning, it is not instructive in determining the left-side of the embryo (Burdine and Schier, 2000).

Cells within the node are ciliated and the cilia rotate to create a fluid flow toward the left side of the embryo, and although the mechanism resulting in left side specification remains to be fully elucidated, the flow created by these cilia is essential for left side determination in mice (Essner, 2005; Essner et al., 2002; Nonaka et al., 2005; Schweickert et al., 2007). Mutations that affect the production or positioning of these node cilia result in disruption of left-right patterning. In some cases, where the node cilia are unable to beat, the left side of the embryo is specified randomly where 50% of embryos specify the left side as left (*situs solitus*) while 50% of embryos specify the right side as the left (*situs inversus*). Mutations affecting the cilia of the node can also result in both sides of the embryo being specified as left or neither side (*heterotaxy*). Failure to specify the left side of the embryo properly results in misplacement and disorganization of organs, and can be embryonic lethal especially when affecting the positioning of the heart (Grimes and Burdine, 2017).

Disruption of cilia production or function have also been described in human disease and are collectively referred to as ciliopathies. Syndromes related to disruption of cilia can have a constellation of phenotypes because of the ubiquity of cilia, but can include polycystic kidney disease, retinal degeneration, polydactyly, *situs inversus*, hearing loss, craniofacial defects, and reproductive defects. Other phenotypes including obesity are also frequently observed in these

syndromes, such as Bardet-Biedl syndrome (BBS), although the potential mechanism of cilia in obesity remains to be fully determined (Dawe et al., 2007; Hearn et al., 2005; Kim et al., 2004).

In addition to embryonic tissue specification, Hh remains important in postnatal and adult life to continue to promote cellular proliferation in wound healing, somatic stem cell maintenance, and tissue regeneration in the lung and pancreas (Beachy et al., 2004; Fendrich et al., 2008; Karhadkar et al., 2004; Le et al., 2008; Stecca et al., 2007; Watkins et al., 2003). Aberrant activation of Hh, however, can result in over proliferation and cancer, and has been implicated in a number of cancers including basal cell carcinoma, medulloblastomas, and rhabdomyosarcomas (Hahn et al., 1996a; Taipale and Beachy, 2001).

Tumors caused by disrupted Hh signaling can be either ligand independent (Type I) or ligand dependent (Type II). Mutations in Hh components that disrupt the function of Ptch and Sufu or that activate Smo result in inappropriate hyper activation of the Hh pathway independent of the ligand and are common in medulloblastomas and rhabdomyosarcomas (Hahn et al., 1996a; Reifenberger et al., 2005). Mutations in Gli2 resulting in constitutive activation can also cause medulloblastomas in mice, but only when cilia are also removed to prevent formation of Gli3 repressor (Han et al., 2009). Additionally, mutations that activate Smo only cause tumors in the presence of cilia, indicating that cilia also have a role in regulating Hh mediated tumorigenesis (Wong et al., 2009). Type II ligand dependent tumors can occur when the Hh ligand is overexpressed, or ectopically expressed resulting in expression of Hh target genes and over proliferation of the surrounding tissue. Type II tumors can occur in the stomach, esophagus, pancreas, ovaries, breast, lung, and skin (melanomas) (Skoda et al., 2017).

CHAPTER II

CELL CYCLE-RELATED KINASE (CCRK) IN CILIOTRANSITION AND HH SIGNALING

Introduction

Cell Cycle-Related Kinase (CCRK) and the *Ccrk* null mutation

Cell Cycle-Related Kinase (CCRK), also known as Cdk20 and PNQALRE, is a serine/threonine kinase, and contains all 11 conserved subdomains associated with serine/threonine kinase activity (Hanks and Quinn, 1991). CCRK also has significant homology to members of the Cyclin-dependent Kinase (CDK) family (Wohlbold et al., 2006), and CCRK was originally thought to be a CDK-activating kinase (CAK) involved in cell cycle regulation through Cdk2-activation (Liu et al., 2004). There is evidence that CCRK phosphorylates Cdk2 (Qiu et al., 2008), and CCRK was demonstrated to associate with CAKs in vitro and in vivo (Liu et al., 2004). However, another study suggests that there is no evidence for CCRK mediated CAK activity, and work from our lab suggest that the role of CCRK in cell cycle regulation is minimal (Wohlbold et al., 2006). Our lab sought to resolve the main cellular function of CCRK outside of cell cycle regulation within the mouse.

The *Ccrk* mutation was previously generated in our lab through targeted removal of the first two exons of the *Ccrk* gene. Additionally, the *Ccrk* mutation lacks the promoter sequence, transcriptional and translational start sites, and the ATP binding site required for CCRK kinase activity. Furthermore, Western blot analysis of *Ccrk* homozygous mutant (*Ccrk*^{-/-}) embryos

indicate that this allele of *Ccrk* is a null mutation, and does not produce protein (Snouffer et al., 2017).

Gross Morphology and Neural Patterning in the *Ccrk* mutant embryo

Previous work in our lab demonstrated that homozygous *Ccrk* null mutant mouse embryos survive until late gestation (around e16.5), and are have shortened ulnas, pre-axial polydactyly, eye defects, clef lip and palate, and are exencephalic (Figure 2.1) (Lupu et al., 2017; Snouffer et al., 2017). The constellation of phenotypes is indicative of a Shh signaling defect within the embryo (Caspary et al., 2007; Eggenschwiler et al., 2001; Huangfu and Anderson, 2005; Liu et al., 2005; Park et al., 2000; Wijgerde et al., 2002).

Neural tube patterning within *Ccrk* mutant embryos is altered, where cell fates that require the highest levels and longest duration of exposure to Hh signaling are not specified, and those that require low to intermediate responses are expanded at the expense of the most ventral fates. *Ccrk* mutant embryos fail to specify the most ventral cell fate, the floor plate, but maintain Hh expression within the notochord. *Ccrk* mutant embryos also have a reduced number of Nkx2.2 positive P3 progenitors which are found ectopically in the most ventral domain. Nkx2.2 is a repressor of Pax6, which is normally expressed more dorsally within the neural tube and activated by weak Hh signaling (Briscoe and Ericson, 1999; Ericson et al., 1997a; Ericson et al., 1997b). In *Ccrk* mutants, as predicted by the reduction in Nkx2.2 expressing cells, the Pax6 domain of the neural tube is expanded ventrally. Furthermore, *Ccrk* mutant embryos are able to specify motor neuron progenitors, which require intermediate levels of Hh signaling, and the domain for motor neuron progenitors was expanded both ventrally and dorsally. These data indicate that *Ccrk* mutant embryos are unable to respond to the highest levels of Hh signaling in the most ventral domain of the neural tube, and as a result, have derepression and expansion of

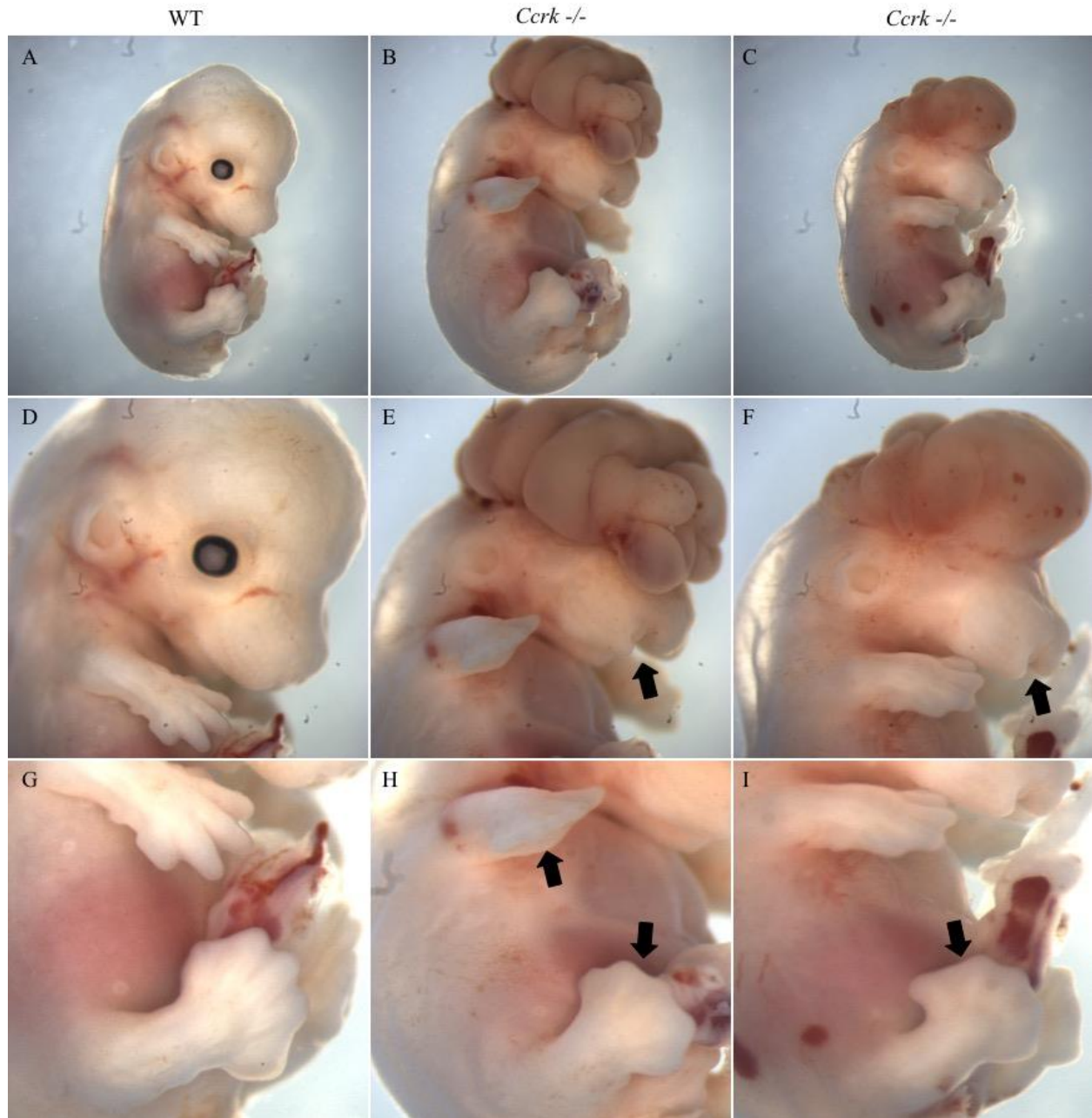


Figure 2.1 Embryonic morphology of e13.5 wild-type and *Ccrk* mutant mouse embryos

(Tails removed for imaging). A,B,C) *Ccrk* mutants present with exencephaly and general edema. D,E,F) *Ccrk* mutants have clef lip and palate and eye defects. G,H,I) *Ccrk* mutants have pre-axial polydactyly, and shortened ulnas.

more dorsal cell markers into more ventral domains. Additionally, *Ccrk* mutant embryos also have ectopic high/intermediate levels of Hh response in the more dorsal regions of the ventral neural tube with expansion of intermediate cell fates into domains which would normally respond to low levels of Hh signaling activity. These results indicated that *Ccrk* may also be involved in repression of the Hh pathway. To genetically test this, embryos double mutant for *Ccrk* and *Smoothed* were generated. Single mutant embryos for *Smoothed* (*Smo*^{-/-}) are unable to respond to Hh signaling and fail to pattern the neural tube (Wijgerde et al., 2002), but if *Ccrk* is involved in repression of the Hh pathway, then the additional absence of *Ccrk* in the *Ccrk/Smo* double mutant embryos would have some derepression of the Hh pathway and would rescue some of the neural patterning defects compared to the *Smo*^{-/-} single mutant. Indeed, there was a restoration of some of the ventral neural progenitors in the *Ccrk/Smo* double mutant compared to *Smo* single mutant, supporting the hypothesis that *Ccrk* is required to suppress weak basal activation of the Hh pathway in the absence of Hh ligand stimulation, in addition to the requirement of *Ccrk* for cells to respond to high levels of Hh in the ventral neural tube (Snouffer et al., 2017).

CCRK mediated posttranslational regulation of the Gli transcription factors

The Gli transcription factors activated by Hh have a crucial role in both activating and repressing Hh target genes within the neural tube (Matise et al., 1998). Gli2 is the primary activator in the neural tube, while Gli3 serves as both an activator and repressor. The activator role of Gli3 is redundant with that of Gli2, and loss of Gli3 activator can be compensated for by Gli2 activator within the neural tube (Bai et al., 2004; Mo et al., 1997; Wijgerde et al., 2002). However, the repressor role of Gli3 is essential, and is the dominant role of Gli3 in Hh signaling

in multiple tissues including the limb bud and neural tube (Persson et al., 2002). Generation of *Ccrk*^{-/-} *Gli2*^{-/-} mutant embryos resulted in more severe dorsalization of the neural tube, indicating that *Ccrk* single mutant embryos maintain some Gli2 function. *Ccrk*^{-/-} *Gli3*^{-/-} double mutant embryos partially rescued the neural patterning defects seen in the *Ccrk* single mutant. These data suggest that *Ccrk* mutants have reduced functional Gli2 activator but maintain some Gli3 repressor. In the absence of Gli3 repressor in the *Ccrk*^{-/-} *Gli3*^{-/-} embryos, the reduced function of Gli2 activator was able partially rescue the neural patterning phenotype, but incomplete rescue indicates that loss of *Ccrk* also impairs the function of the Gli3 repressor (Snouffer et al., 2017).

CCRK may regulate the function of the Gli transcription factors through posttranslational modification. *Ccrk* mutant embryos were found to have two forms of Gli2 detected by Western blot analysis, while wild-type embryos had only one full-length form of Gli2. Furthermore, Gli2 from wild-type samples treated with lambda phosphatase, to remove any posttranslational phosphate groups added on to wild-type Gli2, migrated in a similar manner to *Ccrk* mutant samples not treated with phosphatase, suggesting that in the *Ccrk* mutant embryo there is a decrease or changes in the phosphorylation state of Gli2. Additionally, in *Ccrk* mutant embryos, the full-length form of Gli3, which can go on to act as a transcriptional activator, was found to run at significantly higher levels, while the shorter, proteolytically processed Gli3 repressor was modestly reduced (40%) from wild-type levels (Snouffer et al., 2017).

Although there is an increase in the full-length form of Gli3, the results seen in the *Ccrk*^{-/-} *Gli3*^{-/-} double mutant suggests that the activator function of the form of Gli3 found in *Ccrk*^{-/-} embryos may still be impaired. These data suggest that CCRK is involved in posttranslational modification of the Gli transcription factors, and loss of *Ccrk* results in decreased phosphorylation of Gli2 and reduced activator function, and increases in full length Gli3, which

genetic data suggests is non-functional (Snouffer et al., 2017). It is possible that although there is an increase in full length Gli3, that this is bound to the Hh inhibitor Sufu. Research has found that the Glis are more stable when bound to Sufu and in the absence of Sufu, such as a *Sufu* null embryo, the Glis are rapidly degraded (Humke et al., 2010). There is the potential that *Ccrk* mutants results in a decreased ability of Glis to dissociate from Sufu, thus stabilizing them in an inactive state, which could impact the response to Hh signaling by decreasing the amount of activator present.

Primary cilia in the *Ccrk* null embryos

Primary cilia are essential for Shh signaling transduction, and alteration to the morphology or the transportation system, intraflagellar transport (IFT), within a cilium can impact the ability of a cell to respond to Shh ligand (Corbit et al., 2005; Eggenschwiler and Anderson, 2007; Huangfu and Anderson, 2005; Liu et al., 2005). A previous study analyzing the *broad-minded (bromi)* mutation implicated CCRK in the role of cilia length regulation. Bromi is an uncharacterized TBC domain containing protein. *Bromi* mutant mouse embryos present with similar phenotypes to *Ccrk* mutant embryos, with exencephaly, preaxial polydactyly, and eye development defects. Bromi is required for ventral neural fate specification, and the neural patterning defects observed in the *bromi* mutant embryos phenocopies the neural patterning defects observed in *Ccrk* mutant embryos. *Bromi* mutant embryos fail to specify a floor plate, have reduced numbers of p3 interneuron progenitors, expansion of motor neuron progenitors, and expansion of the Pax6 domain ventrally (Ko et al., 2010).

Bromi mutants were found to have cilia at the same frequency as wild-type embryos within the neuroepithelium, but *bromi* mutant cilia were bulbous and misshapen, forming open

ring like structures. Additionally, *bromi* mutant cilia appear to have ciliary membranes that have expanded away from the ciliary axoneme. Analysis of *bromi* morpholinos in zebrafish supported the ciliary role of *bromi* where cells within the *bromi* morpholino zebrafish kidney tubules had curled ring-like cilia with detached and expanded ciliary membranes. CCRK was found to associate with Bromi using tandem affinity purification and mass spectrometry. Additionally, endogenous CCRK and Bromi co-immunoprecipitate, and it was found that CCRK levels were reduced in *bromi* mutants. These data suggest that Bromi interacts with and promotes CCRK stability, and the function of Bromi in cilia morphology is likely executed by CCRK. Furthermore, morpholino knockdown of both *Ccrk* and *bromi* in zebrafish resulted in more fish with curled cilia within the kidney tubules than single morpholinos for either *Ccrk* or *bromi*, indicating that CCRK and Bromi have interdependent functions within a common mechanism in ciliogenesis (Ko et al., 2010).

Ccrk is the homolog of the *long flagella 2 (lf2)* gene in *Chlamydomonas reinhardtii* (Tam et al., 2007). LF2p along with the long flagella 1 and 3 proteins (LF1p and LF3p, respectively), form the length regulating complex (LRC) and control flagellar length (Barsel et al., 1988; Tam et al., 2007). Zebrafish morpholino knockdown of *Ccrk* indicated that it may also have a role in vertebrate cilia regulation (Ko et al., 2010). CCRK and Bromi localization, determined with tagged versions of CCRK and Bromi in NIH3T3 cells, was found throughout the cytoplasm with no enrichment within the cilium, similar to the localization observed for LF2p in *Chlamydomonas* (Ko et al., 2010; Tam et al., 2007). CCRK protein has also been reported to localize to the nucleus of cells (Ko et al., 2010; Liu et al., 2004).

Ccrk null mouse embryos produce cilia at normal frequencies within the neural epithelium, however, *Ccrk* mutant cilia appear shorter and more bulbous than wild-type cilia

within the neural epithelium (Snouffer et al., 2017). In this chapter, we investigate the role of CCRK in cilia length regulation, ciliogenesis, and in transduction of the Hh pathway.

Results

Embryonic expression of *Ccrk*

Ccrk was predicted to be expressed in a number of tissues throughout the embryo given the constellation of phenotypes observed in the *Ccrk* mutant embryo, including the neural tube, limb bud, and the early craniofacial region. By in situ hybridization, *Ccrk* RNA was ubiquitously expressed in an e10.5 embryo (Figure 2.2A) with expression in tissues that coincide with those observed to have mutant phenotypes, including tissues responsible for exencephaly, pre-axial polydactyly, and craniofacial defects. Additionally, there may potentially be slightly more *Ccrk* RNA present in the forelimb and brachial arches compared to the surrounding tissues (Figure 2.2A).

Sections were taken of *Ccrk* antisense, wild-type embryos e10.5 to observe any tissues specific expression of *Ccrk* within the neural tube or limb bud. *Ccrk* RNA was expressed throughout the neural tube of e10.5 embryo (Figure 2.2D) with no indication of tissues or cell-type specific expression. The *Ccrk* sense *in situ* probe did not react in a wild-type embryo (Figure 2.2B and Figure 2.2E). Additionally, the *Ccrk* antisense in situ hybridization probe developed in a *Ccrk* null embryo in conjunction with the antisense probe in a wild-type embryo was much less reactive, suggesting the absence of *Ccrk* RNA within *Ccrk* null embryos, and supporting the Western blot analysis which detected no CCRK protein in *Ccrk* null embryos.

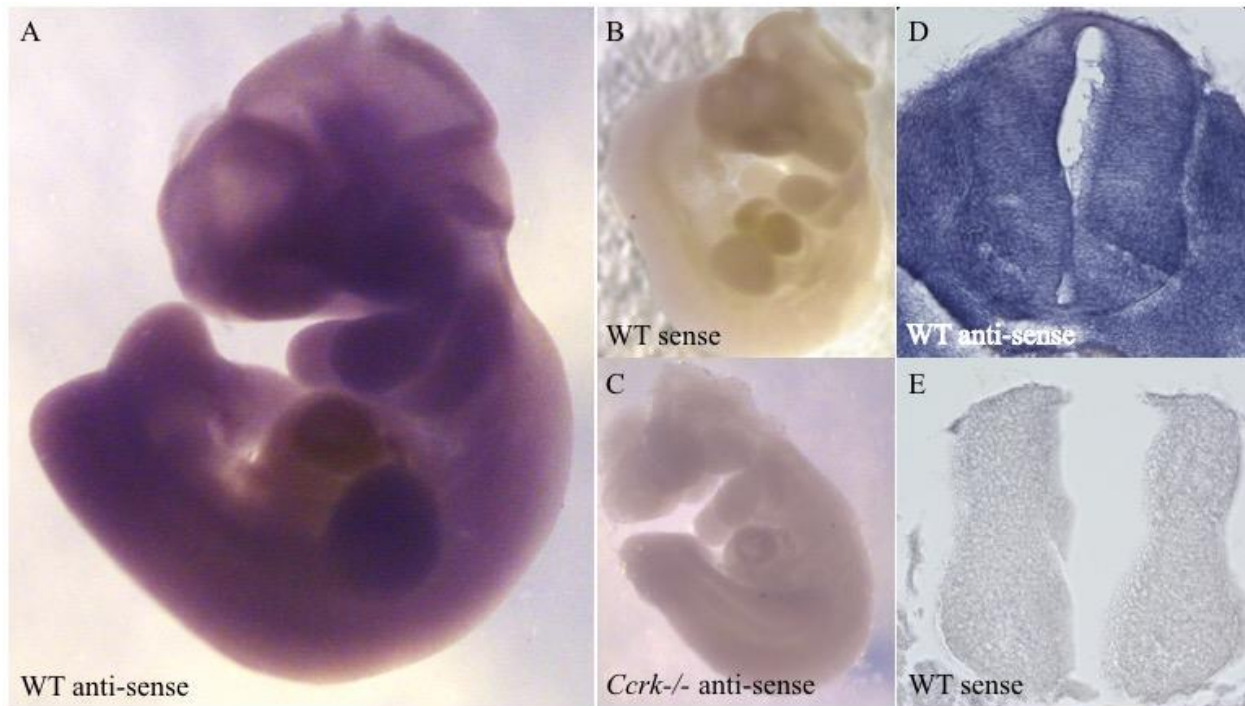


Figure 2.2 Embryonic expression of *Ccrk* detected by *in situ* hybridization in e10.5 embryos

A) *In situ* hybridization with *Ccrk* anti-sense probe detects ubiquitous expression of *Ccrk* throughout the e10.5 wild-type embryo. B,E) *Ccrk* sense probe did not react with a e10.5 wild-type embryo in whole mount nor within sections. C) *Ccrk* anti-sense probe on a *Ccrk* null mutant embryo developed in conjunction with the e10.5 wild-type embryo (A) did not develop signal. D) *Ccrk* RNA is expressed throughout the neural tube and does not exhibit cell type specific expression

The role of CCRK in cilia ultrastructure and cilia length regulation

Inner medullary collecting duct (IMCD) cells homozygous mutant for *Ccrk* analyzed by transmission electron microscopy (TEM) were found to maintain normal ultrastructure with nine doublet rings of microtubules and lack the central pair of microtubules present in motile cilia (Figure 2.3C-F). By scanning electron microscopy (SEM) and TEM *Ccrk* null IMCD cells have bulbous tips (Figure 2.3B and E-F). Bulbous ciliary tips may be a result of accumulation of proteins at the tip of the cilium.

To further investigate the ciliary phenotype of the *Ccrk* null embryo, *Ccrk* null mouse embryonic fibroblasts (MEFs) were labeled by immunofluorescence for ciliary markers (acetylated α -tubulin and Arl13b to label the axonemal, and γ -tubulin to label the basal body). Ciliary length was measured from the basal body to the tip of the cilium. In two wild-type and two *Ccrk* mutant cell lines, the length of *Ccrk* null primary MEFs was, on average, slightly longer than primary cilia of wild-type primary MEFs (Figure 2.4). Furthermore, there was broader variation in cilia length in *Ccrk* null primary MEFs, with more cilia that were shorter than average, as well as, more cilia that were longer than average. Cilia length was also measured in *Ccrk*^{+/-} and *Ccrk*^{-/-} immortalized MEFs. *Ccrk*^{+/-} MEFs appear wild-type in the distribution and average length of primary cilia, but *Ccrk*^{-/-} immortalized MEFs also had a broader distribution of lengths than *Ccrk*^{+/+} or *Ccrk*^{+/-} cells (Figure 2.4). Primary cilia on MEFs were also observed by SEM, and *Ccrk* null MEFs appeared to have expanded, and slightly bulbous cilia (Figure 2.5) similar to the bulbous tip phenotype observed in the *Ccrk*^{-/-} IMCD cells.

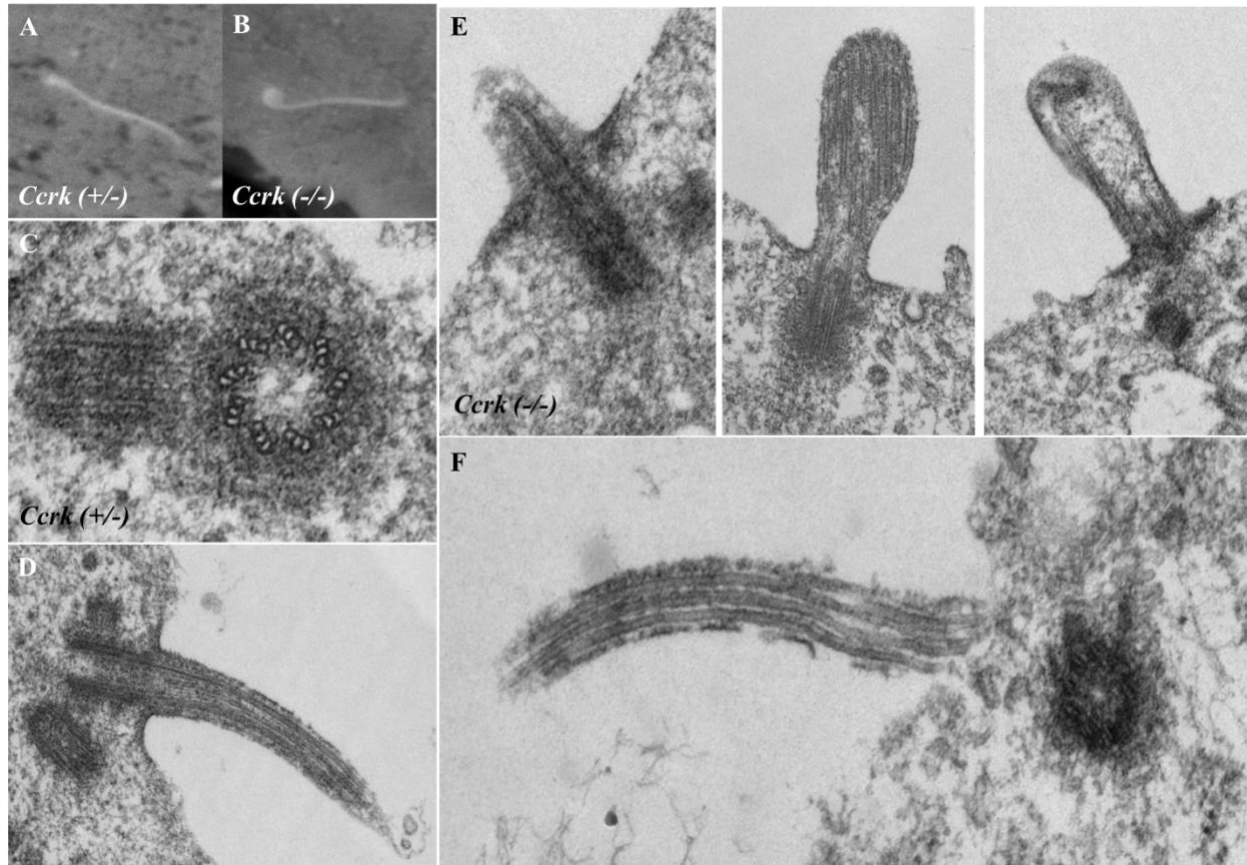


Figure 2.3 Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) of wild-type and *Ccrk*^{-/-} inner medullar collecting duct (IMCD) cells A) SEM of a primary cilium on a wild-type IMCD cell B) SEM of a primary cilium on a *Ccrk*^{-/-} IMCD cell. *Ccrk*^{-/-} frequently presented with bulbous tips on their primary cilia. C) TEM of a section through a wild-type primary cilium basal body showing the triplets of microtubules present within the basal body and the absence of the central pair of microtubules. D) TEM cross section through a wild-type primary cilium. E and F) Four TEM cross section through *Ccrk*^{-/-} primary cilia. *Ccrk*^{-/-} IMCD cilia are generally more bulbous but do not display any defects in microtubules arrangement or other ultrastructural features.

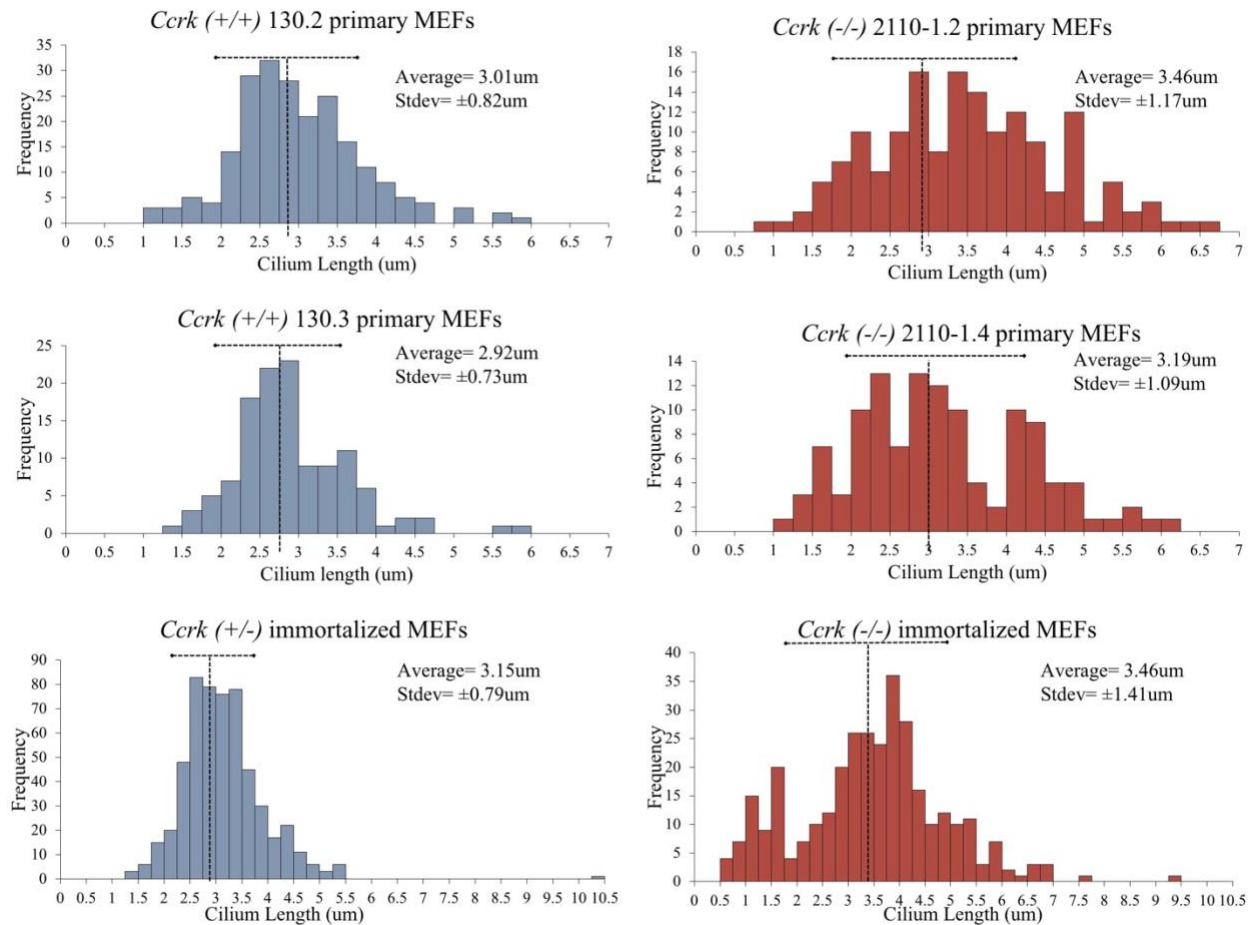


Figure 2.4 Primary Cilia length in primary and immortalized mouse embryonic fibroblasts

(MEFs) Two lines of *Ccrk*^{+/+} primary MEFs have an average length of $3.01 \pm 0.82\mu\text{m}$ and $2.92 \pm 0.73\mu\text{m}$. Two lines of *Ccrk*^{-/-} primary MEFs have an average length of $3.46 \pm 1.17\mu\text{m}$ and $3.19 \pm 1.09\mu\text{m}$. *Ccrk*^{+/-} immortalized cells have a similar length distribution and average length to primary *Ccrk*^{+/+} cells of $3.15 \pm 0.79\mu\text{m}$ while *Ccrk*^{-/-} immortalized cells have an average length of $3.46 \pm 1.41\mu\text{m}$.

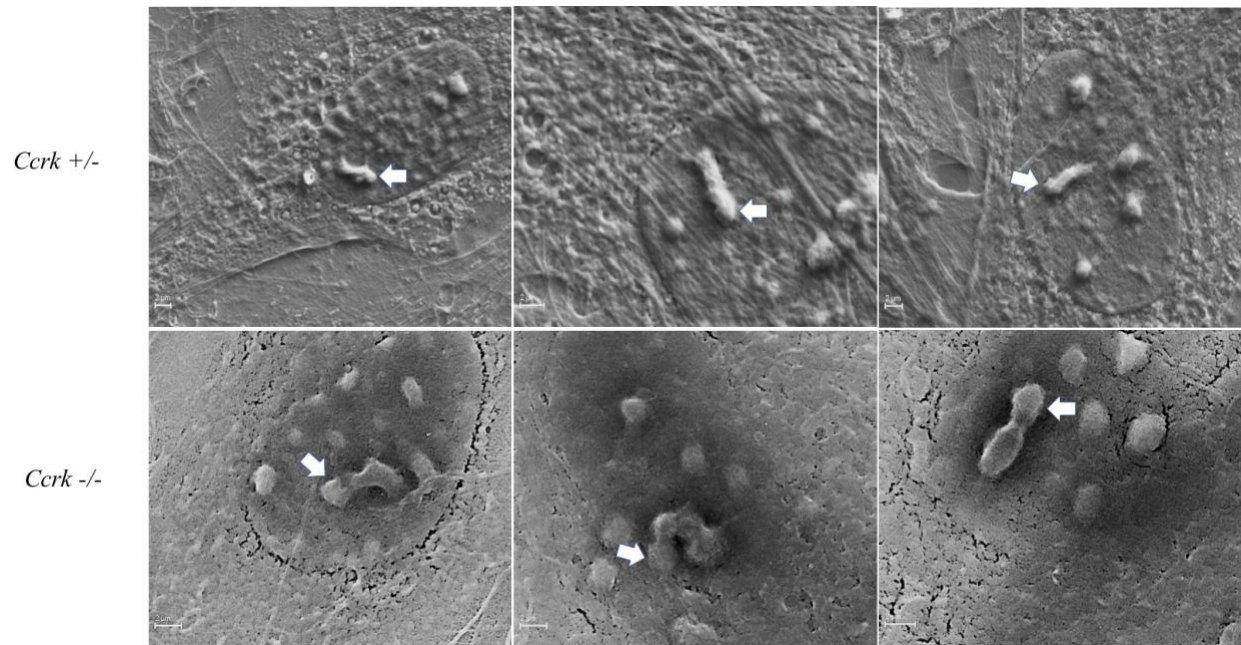


Figure 2.5 Scanning electron microscopy (SEM) of immortalized mouse embryonic fibroblasts (MEFs) *Ccrk* ^{+/−} and *Ccrk* ^{−/−} MEFs have cilia that extend from the cell on the apical surface near or over the nucleus of the cell. *Ccrk* ^{−/−} MEFs have more bulbous cilia than cilia observed in *Ccrk* ^{+/−} MEFs. Arrows pointing to the tip of each cilium.

The role of CCRK in differential dosage response to Hh signaling in MEFs

CCRK may have a role in regulating trafficking of proteins into and out of the cilium based on the phenotypes related to the control of ciliary length regulation (Figure 2.4) and the bulbous cilia (Figure 2.5) seen in cells lacking *Ccrk*. The components of the Hh pathway, including Smoothened (Smo), and the Gli transcription factors, require trafficking through the cilium for activation of the Hh pathway. Neural tube patterning requires a gradient of Hh ligand along the dorsal-ventral axis. Hh ligand is produced within the notochord and then induces the expression of the Hh ligand within the floor plate of the neural tube. The Hh ligand then forms a gradient of differing concentrations through the ventral neural tube, with the highest concentration at the most ventral source and lower concentrations as the ligand is dispersed dorsally (Briscoe and Ericson, 1999; Dessaud et al., 2010; Stamatakis et al., 2005) (Figure 2.6). Previous work in our lab found that neural tube patterning within the *Ccrk* mutant embryos is disrupted, where *Ccrk* mutants fail to specify the most ventral neural precursor cell fates within the neural tube that require the highest levels of Hh. However, other more dorsal neural precursor cell fates, that require intermediate or low levels, have expanded domains (Snouffer et al., 2017).

To further understand the multifaceted phenotype of the Hh patterning defects within the neural tube, immortalized *Ccrk*^{+/-} and *Ccrk*^{-/-} MEFs were induced with different concentrations of Smoothened agonist (SAG), to represent the varying levels of Hh ligand within the neural tube. SAG activates the Hh pathway at the level of Smo, downstream of the receptor Patched (Ptch) (Frank-Kamenetsky et al., 2002). Expression of two downstream targets of the Hh pathway, *Gli1* and *Ptch1*, were analyzed by qRT-PCR after 8 hours and 24 hours of exposure to a given concentration of SAG. After 8 hours of exposure to SAG, *Gli1* levels were relatively low

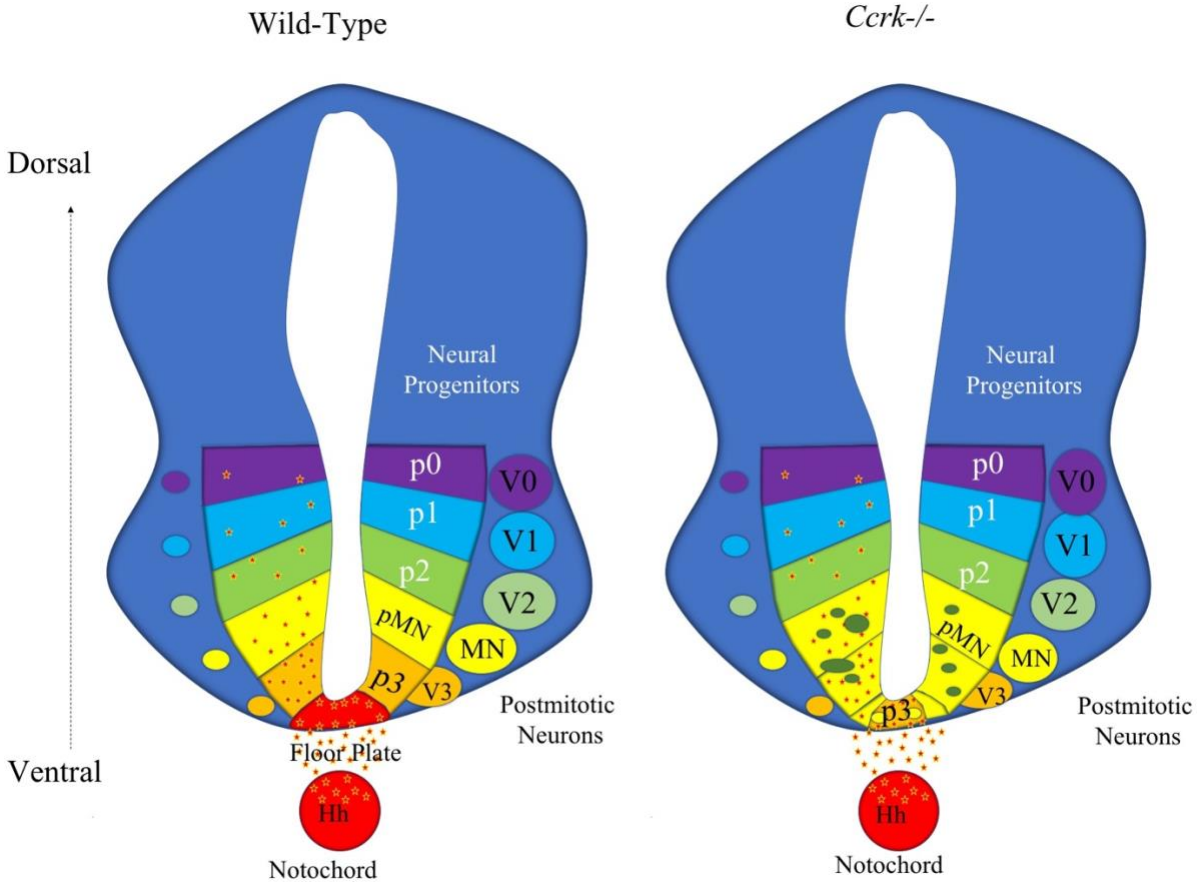


Figure 2.6 Sonic Hedgehog (Shh) Specification of the neural tube Distinct populations of neural progenitors (p3, pMN, p2, p1, and p0) are specified by the concentration gradient of Shh and differentiate into postmitotic neurons (V3, Motor Neuron (MN), V2, V1, and V0), respectively. The highest level of Shh is present within the floor plate, and as the ligand diffuses more dorsally, the intermediate/low levels of Shh will specify the pMN and p2 progenitors. The most dorsal neural progenitors, p1 and p0, require the least amount of Shh for specification. *Ccrk* null embryos fail to specify a floor plate, although there is still Shh expression within the notochord. Additionally, the p3 progenitor domain is greatly reduced to the most ventral portion of the neural tube. The progenitors that require intermediate/low levels of Shh for specification, p2 and pMN, have expanded domains. Figure based on (Ribes and Briscoe, 2009).

overall and lower in the less concentrated doses of SAG. The *Gli1* levels at each concentration were very similar in both the *Ccrk*^{+/-} and *Ccrk*^{-/-} MEFs. After 8 hours of exposure to SAG *Ptch1* levels were also relatively low with less *Ptch1* induction at lower concentrations of SAG. Additionally, the *Ccrk*^{-/-} MEFs had lower levels of *Ptch1* than *Ccrk*^{+/-} MEFs at all the concentrations tested (Figure 2.7).

After 24 hours of exposure to SAG, *Gli1* expression was much higher than that seen at 8 hours, with a range of 10-40X fold more *Gli1* than un-induced MEFs (DMSO). *Gli1* expression was most impacted at the highest concentrations of SAG, with the largest difference between *Ccrk*^{+/-} and *Ccrk*^{-/-} MEFs seen at the highest concentration of 200nM SAG while at lower concentrations of SAG *Ccrk*^{-/-} had a similar level of expression to *Ccrk*^{+/-} MEFs. After 24 hours of exposure to SAG, *Ptch1* relative induction remained low, but was higher than the relative induction seen after 8 hours. Similarly, *Ccrk*^{-/-} MEFs had reduced expression of *Ptch1* at all concentrations of SAG, but again with the most drastic difference seen at the highest concentrations of SAG (Figure 2.7).

These data suggest that *Ccrk* is required for cells to mount a response to the highest levels of Hh signaling, however, in the absence of *Ccrk*, cells are still able to mount a low-level response. These data help to interpret the embryonic neural tube phenotype seen, where neural precursors that require the highest levels of Hh fail to be specified, potentially, because in the absence of *Ccrk*, those cells are unable to mount the large Hh response, or level of downstream target genes, required for that neural precursor fate. However, absence of *Ccrk* does not completely inhibit response to Hh, and at lower concentrations the level of Hh response was similar between *Ccrk*^{+/-} and *Ccrk*^{-/-} cells. These data support the in vivo data were in *Ccrk*^{-/-} embryos, neural progenitors which require low and intermediate levels of Hh signaling are still present, and furthermore, in

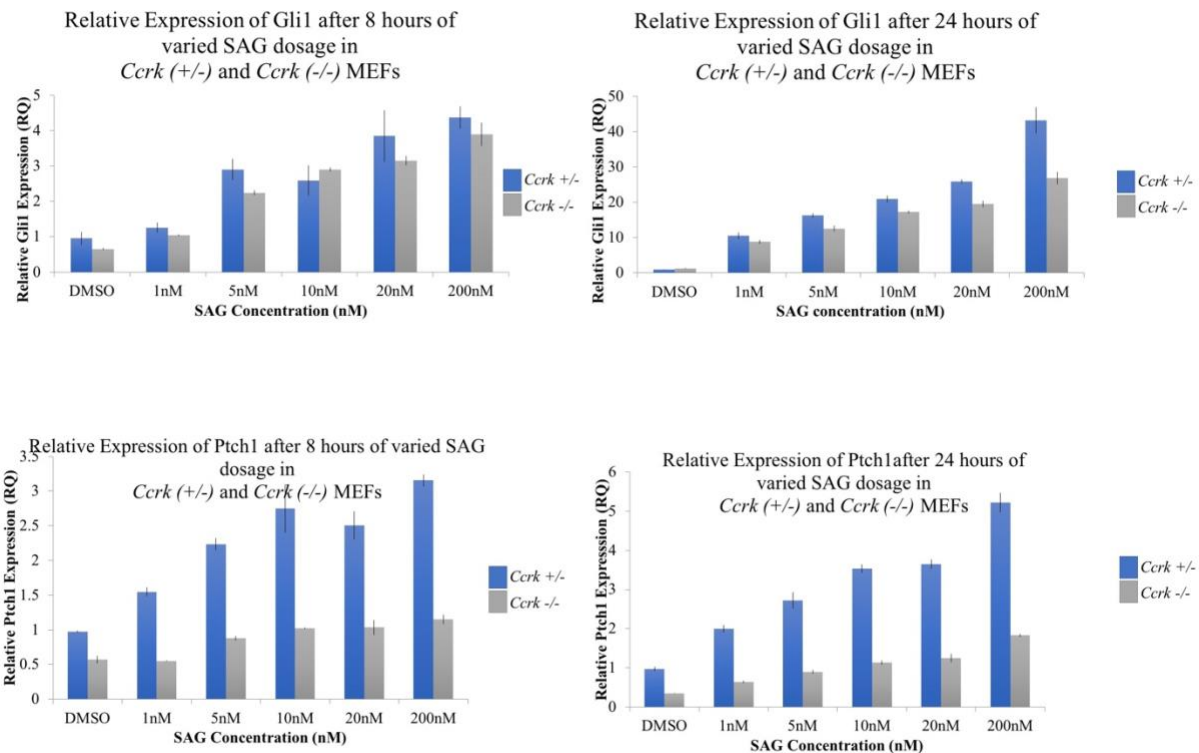


Figure 2.7 Smoothened Agonist (SAG) Dosage response in *Ccrk* ^{+/+} and *Ccrk* ^{-/-} MEFs

Cells were exposed to varying concentrations (1nM, 5nM, 10nM, 20nM, and 200nM) of SAG for either 8 hours (left) or 24 hours (right). Relative expression of *Gli1* (top) and *Ptch1* (bottom) were analyzed by qRT-PCR. Each time point represents biological replicates done in triplicate and are normalized β -Actin expression and to *Gli1* or *Ptch1* expression in un-induced cells (DMSO). *Ccrk* ^{-/-} MEFs had the greatest difference in ability to respond to SAG at the highest concentrations after 24 hours of exposure for both *Gli1* and *Ptch1*. *Ccrk* ^{-/-} cells overall did not express *Ptch1* as much as *Ccrk* ^{+/+} cells. After 8 hours of exposure to SAG *Ccrk* ^{-/-} cells did not vary in their expression of *Gli1* from *Ccrk* ^{+/+}, while *Ccrk* ^{-/-} cells did have lower expression of *Ptch1* at all concentrations.

the absence of *Ccrk* there is an expansion of some of these populations of neural precursors that require intermediate or low levels of Hh response into the more ventral domain of the absent neural precursors that require high levels of Hh in the *Ccrk* mutant neural tube (Figure 2.6) (Snouffer et al., 2017).

The role of CCRK in the temporal response to Hh signaling

Patterning of the neural tube not only involves varying concentrations of Hh ligand along the dorsal-ventral axis, but also depends on the amount of time a cell is exposed to the Hh ligand (Cohen et al.) (Figure 2.8). It takes time for a cell to mount a full level response to Hh, for example, over time, cells at the future floor plate will sequentially activate transcription factors associated with pMN (Olig2), then p3 (Nk2.2) progenitors before expressing the transcription factor associated with floor plate specification (FoxA2) (Figure 2.8A). Furthermore, a population of cells that is exposed to a low level of Hh for a short period of time may specify a different neural progenitor fate than a cell exposed to a low level of Hh for a longer period of time (Figure 2.8B). Time of exposure to Hh, however, works in conjunction with the concentration of Hh a cell is exposed to. For cell fates which require a high concentration of Hh even extended times of exposure to a low or intermediate level of Hh will not activate transcription factors associated with cell fates specified by high levels of Hh if the concentration is still too low (Figure 2.8B). Additional work from our lab found that *Ccrk* mutant embryos have neural tubes patterning defects throughout development and specification of the neural tube where *Ccrk* mutant embryos fail to specify the cell fates that require the highest levels of Hh. Although slight rescue of this phenotype was observed at later stages (E11.5) (Snouffer et al., 2017).

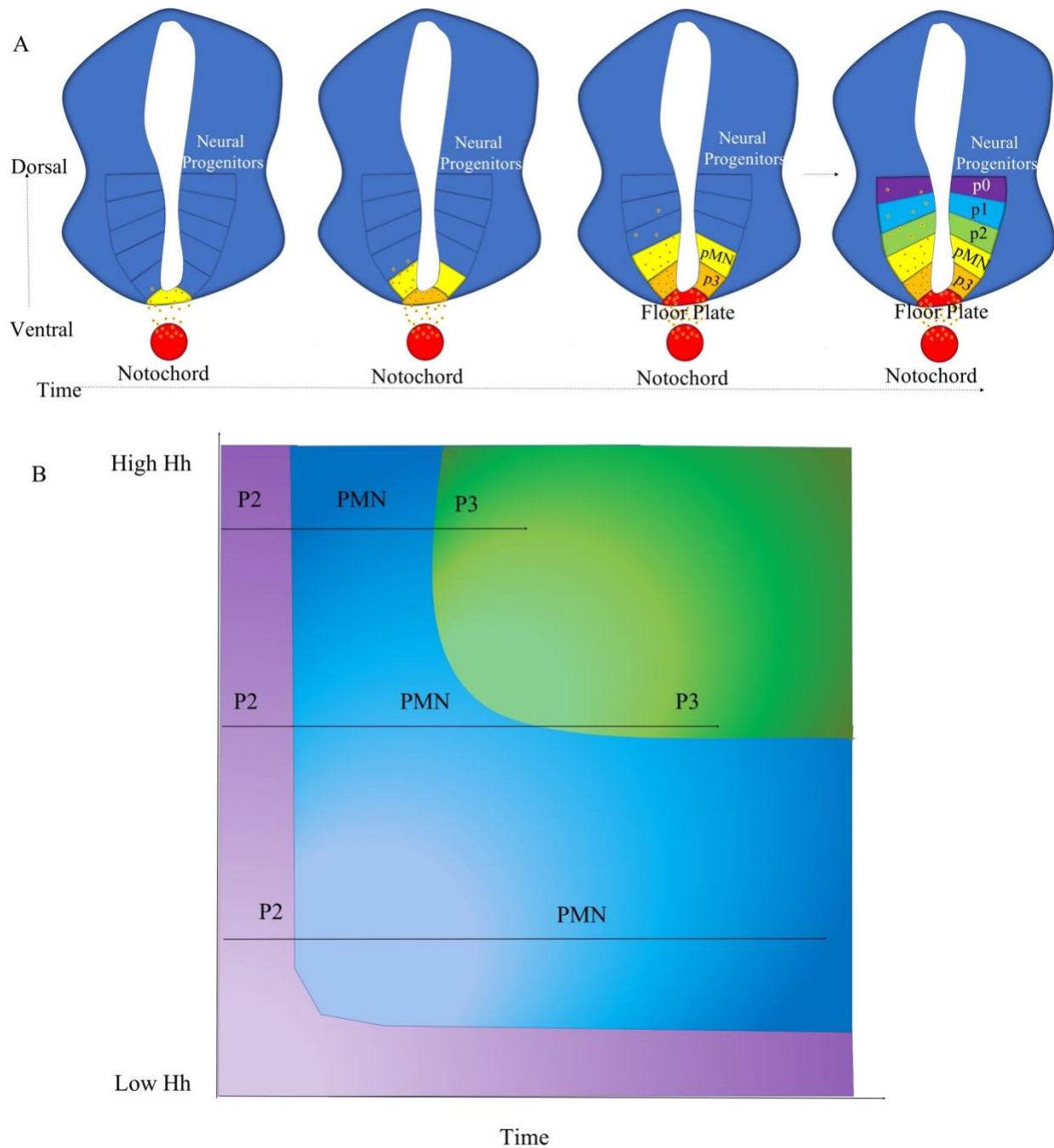


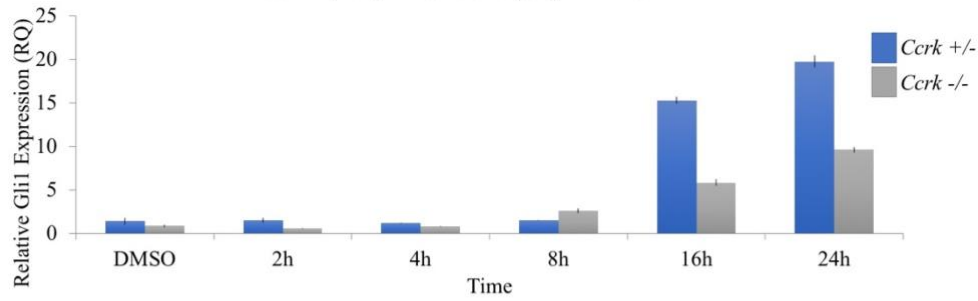
Figure 2.8 Temporal and dosage response to Hh within the neural tube A) Different cell fates are specified in the neural tube based on the concentration of Hh and length of time a cell is exposed to the Hh ligand. At a constant concentration of Hh, populations of neural progenitors may sequentially activate transcription factors until enough time of exposure to the Hh ligand. Initially, exposure to the high levels of will activate the transcription factor associated with pMN

(Olig2) within the cells that will become the floor plate. After longer exposure to Hh these cells will then activate the transcription factor associated with p3 neural progenitors (Nkx2.2). Finally, with longer exposure the cells will then activate the transcription factor associated with floor plate specification (FoxA2). B) The concentration of Hh works in conjunction with the time a cell is exposed to Hh to specify a given fate. Cells exposed to a higher concentration of Hh will require less time to specify the population of neural progenitors that require a higher level of Hh. If the concentration of Hh is low, even with extended periods of exposure to Hh, cells will not mount a response high enough to specify a cell fate that requires a greater concentration of Hh. Figures based on (A) Ribes and Briscoe, 2009 and B) Cohen, et al. 2013.

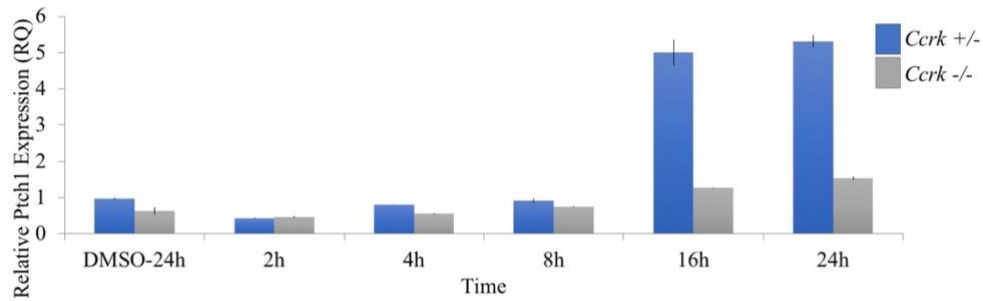
When MEFs were exposed to varying concentrations of SAG for either 8 hours or 24 hours, relative *Gli1* expression was not significantly impacted until 24 hours of SAG exposure. While *Ptch1* expression was impacted at both 8 hours and more substantially at 24 hours. To investigate the temporal differences in response to SAG in *Ccrk*^{-/-} MEFs relative expression of *Gli1* and *Ptch1* was analyzed over time by qRT-PCR. When MEFs were exposed to 5nM and 20nM of SAG over time for 24 hours, cells started to express *Gli1* and *Ptch1* in response to SAG around 8 hours, with much higher responses at 16 hours and 24 hours (Figure 2.9). Interestingly, *Ccrk*^{-/-} MEFs consistently had slightly elevated levels of *Gli1* at 8 hours when compared to *Ccrk*^{+/-}, indicating that *Ccrk*^{-/-} MEFs are at least as capable of mounting the same response to SAG as *Ccrk*^{+/-} MEFs, but may be even more responsive to SAG after 8 hours of exposure, or have an underlying derepression of the pathway at low levels of Hh exposure (Figure 2.9) This result may have implications in the expanded populations of neural progenitors that respond to intermediate levels of Hh within the *Ccrk* mutant embryo. Large differences in SAG response in *Ccrk*^{-/-} and *Ccrk*^{+/-} MEFs were not observed until the later time points (16 hours and 24 hours), where *Ccrk*^{-/-} MEFs expression was still increased for *Gli1* and *Ptch1*, but the cells were unable to mount the larger response seen in *Ccrk*^{+/-} cells (Figure 2.9). This indicates that even at lower concentrations of SAG (5nM and 20nM), *Ccrk*^{-/-} MEFs are unable to mount responses to SAG that require the longest period of exposure.

The response of *Ccrk*^{+/-} and *Ccrk*^{-/-} MEFs to SAG based on *Gli1* and *Ptch1* expression was also analyzed at a higher concentration of SAG, 200nM, over 24 hours (Figure 2.10). *Gli1* expression between *Ccrk*^{+/-} and *Ccrk*^{-/-} MEFs remained relatively similar at earlier time points, and again *Ccrk*^{-/-} MEFs actually had higher expression of *Gli1* at the 4 hour and 8 hour SAG exposure time points. Starting around 14 hours, *Ccrk*^{-/-} MEFs expression of *Gli1* was still

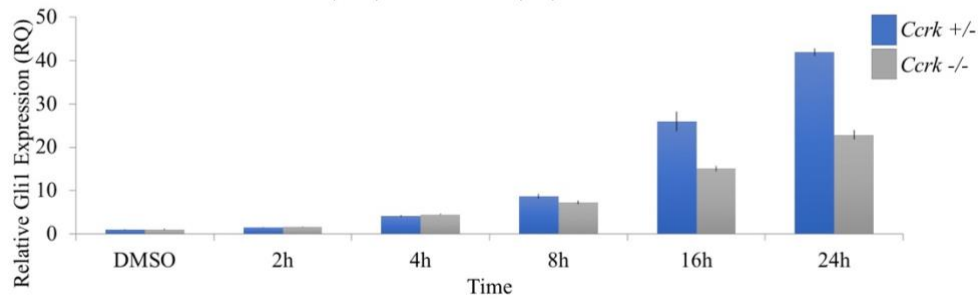
Relative Expression of Gli1 over time after 5nM SAG
induction in
Ccrk (+/-) and *Ccrk* (-/-) MEFs



Relative Expression of Ptch1 over time after 5nM SAG
induction in
Ccrk (+/-) and *Ccrk* (-/-) MEFs



Relative Expression of Gli1 over time after 20nM SAG
induction in
Ccrk (+/-) and *Ccrk* (-/-) MEFs



Relative Expression of Gli1 over time after 20nM SAG
induction in
Ccrk (+/-) and *Ccrk* (-/-) MEFs

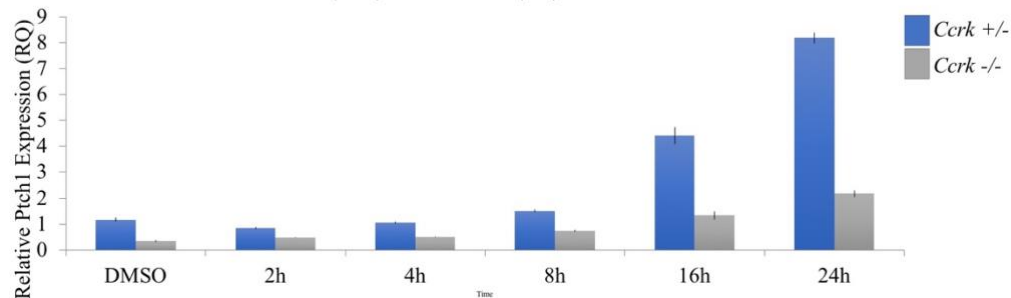
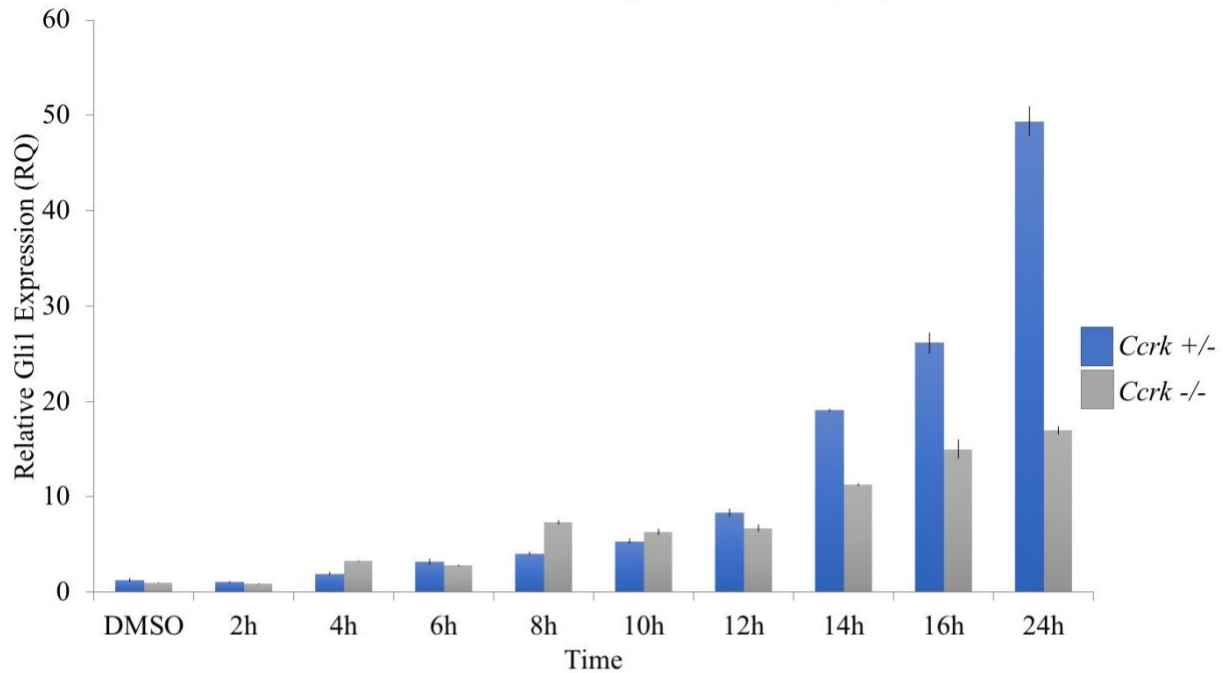


Figure 2.9 Temporal response to SAG at low dosages (5nM and 20nM) in *Ccrk*^{+/-} and *Ccrk*^{-/-} MEFs Relative expression of *Gli1* and *Ptch1* after 2h, 4h, 8h, 16h, and 24h exposure to 5nM and 20nM SAG. *Gli1* and *Ptch1* expression was lower in *Ccrk*^{-/-} MEFs after 16 hours and 24 hours of SAG exposure for both 5nM and 20nM. Each time point represents biological replicates done in triplicate and are normalized β -Actin expression and to *Gli1* or *Ptch1* expression in un-induced cells (DMSO).

Relative Gli1 expression over time after 200nM SAG induction in *Ccrk* (+/-) and *Ccrk* (-/-) MEFs



Relative Ptch1 expression over time after 200nM SAG induction in *Ccrk* (+/-) and *Ccrk* (-/-) MEFs

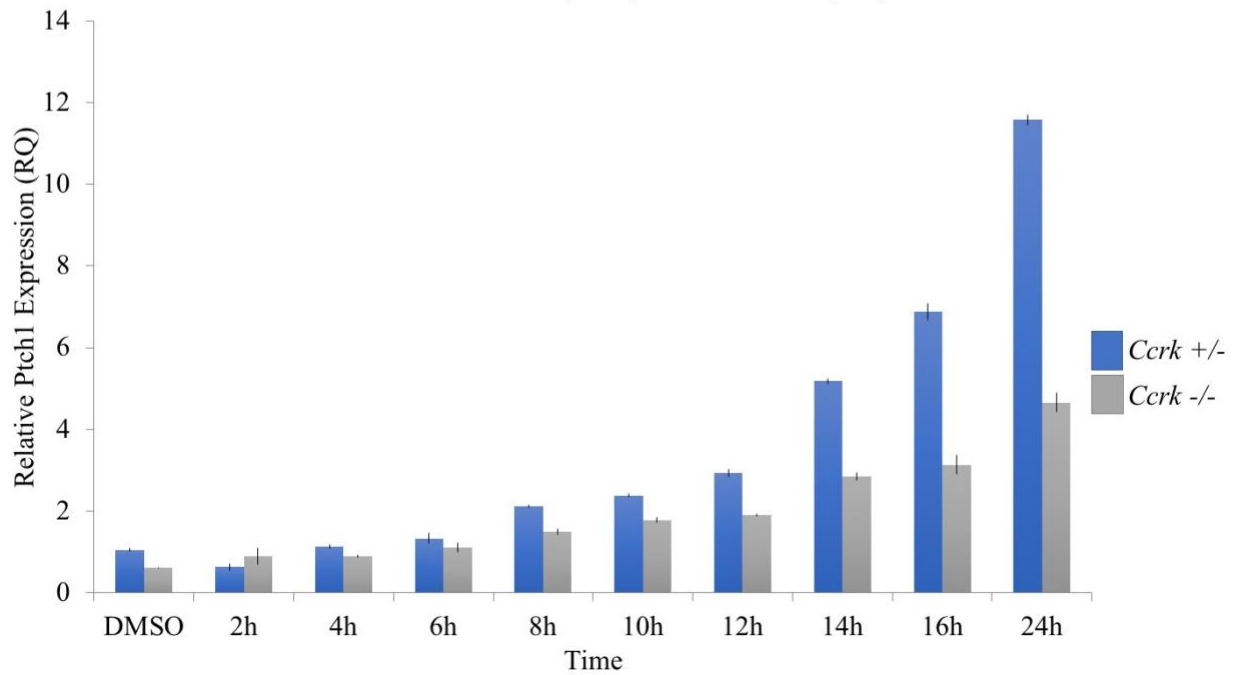


Figure 2.10 Temporal response to SAG at high dosages (200nM) in *Ccrk*^{+/−} and *Ccrk*^{−/−} MEFs over time Relative expression of *Gli1* and *Ptch1* after 2h, 4h, 6h, 8h, 10h, 12h, 14h, 16h, and 24h exposure 200nM SAG. *Gli1* (top) expression was lower in *Ccrk*^{−/−} MEFs after 12 hours and the discrepancy between the expression in *Ccrk*^{+/−} continued to increase with the most extreme difference between *Gli1* expression observed at 24 hours of SAG exposure. Similarly, *Ptch1* expression differences were most drastic at later time points although *Ptch1* levels are lower at all time points with expression higher than that of un-induced cells (4 hours onward). Each time point represents biological replicates done in triplicate and are normalized β-Actin expression and to *Gli1* or *Ptch1* expression in un-induced cells (DMSO).

increasing but does not match the rate at which *Ccrk*^{+/-} MEFs expression increases (Figure 2.10). The expression of *Ptch1* in *Ccrk*^{-/-} MEFs is diminished at every time point where expression is greater than that seen in un-induced cells. The difference in expression between *Ccrk*^{+/-} and *Ccrk*^{-/-} MEFs was greatest at 24 hours for both *Gli1* and *Ptch1*. These data indicate that, in addition to an inability to respond to high concentrations of SAG (Figure 2.7), *Ccrk*^{-/-} cells are also not able to mount high levels of response, like those seen in the *Ccrk*^{+/-} MEFs, after long exposures to SAG as well (Figure 2.9 and 2.10).

The kinase function of CCRK required for Hh response

A *Ccrk* construct carrying a K33R mutation, a mutation that changes a lysine within the catalytic domain essential for kinase function to an arginine rendering CCRK kinase dead was generated and transfected into *Ccrk*^{-/-} MEFs by Jonathon Walsh (Carrera et al., 1993; Snouffer et al., 2017). I analyzed the Shh response of the three kinase dead cell lines by qRT-PCR to look at expression of *Gli1* and *Ptch1* after SAG induction. In the absence of CCRK kinase function, MEFs are unable to mount a wild-type response to 200nM SAG in both *Gli1* expression and *Ptch1* expression compared to *Ccrk*^{+/-} MEFs. The response of the kinase dead cells was similar to *Ccrk*^{-/-} MEFs, implicating CCRK's kinase function in the ability for cells to properly respond to SAG (Figure 2.11). A construct containing a wild-type *Ccrk* was also generated and transfected into *Ccrk*^{-/-} MEFs by Jonathon Walsh (Snouffer et al., 2017). The response to 200nM of SAG after 24 hours of two cell lines resulted in a rescue of Hh response with *Gli1* and *Ptch1* levels higher than those of *Ccrk*^{+/-} MEFs, from over expression of the *Ccrk*^{WT} construct (Figure 2.11). This data supports that within *Ccrk*^{-/-} cells it is indeed the absence of *Ccrk* which is impacting the ability of the cells to respond to SAG.

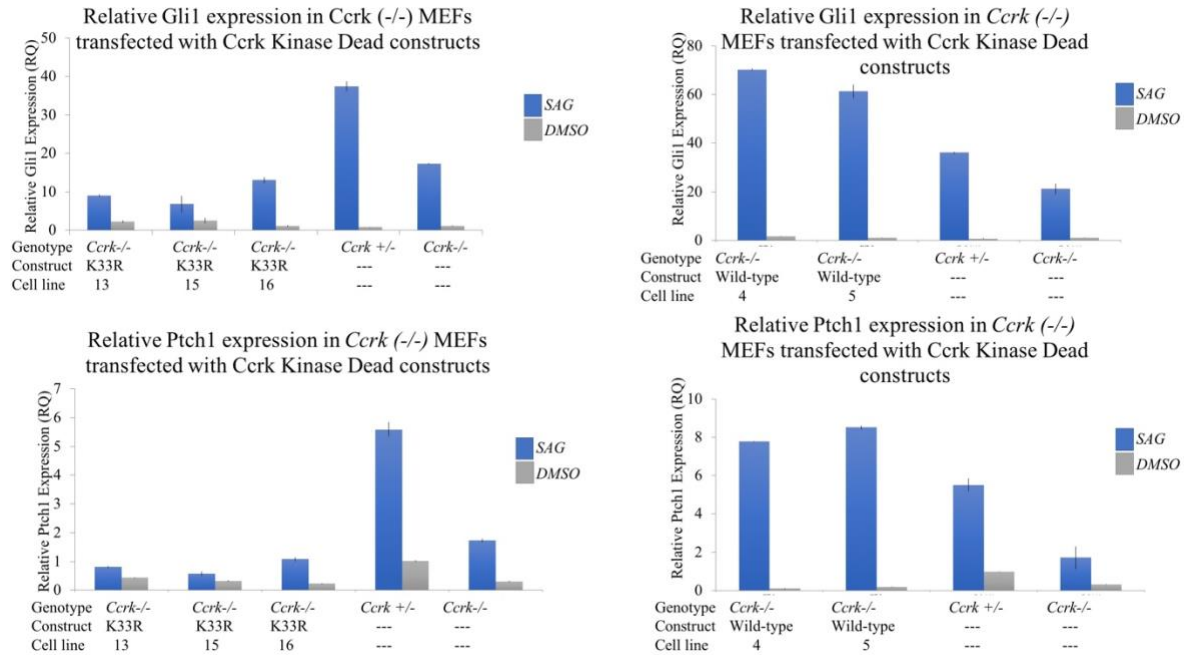


Figure 2.11 *Gli1* and *Ptch1* expression in *Ccrk*^{-/-} MEFs expressing either *Ccrk*^{WT} or *Ccrk*-kinase dead (K33R) constructs Expression of a construct containing a kinase dead version of *Ccrk* (K33R) was analyzed in three MEF lines (left). The K33R mutation fails to rescue the inability of *Ccrk*^{-/-} MEFs to respond to 200nM SAG after 24 hours of exposure based on *Gli1* (top) and *Ptch1* (bottom) expression. *Ccrk*^{-/-} MEFs expressing a construct containing a wild-type version of *Ccrk* (right) are able to respond to 200nM SAG after 24 hours, and indeed surpasses the expression of *Gli1* (top) *Ptch1* (bottom) seen in *Ccrk*^{+/-} most likely due to overexpression of the wild-type *Ccrk* construct.

***Ccrk* in cellular memory of a given Hh signaling state (Hysteresis)**

Ccrk^{-/-} MEFs are able to respond to Hh signaling, but are unable to mount a Hh response comparable to *Ccrk*^{+/-} MEFs at the highest concentrations of SAG and after the longest period of exposure to SAG. To achieve a high-level of activation of Hh target genes, it requires the cell to retain a molecular “memory” of the past state of signaling. For example, a cell exposed to a high level of Hh, would continue to interpret that the state of exposure is a high-level of Hh for a period of time, even after the ligand is removed. To understand if the issue in *Ccrk*^{-/-} cells with mounting a high-level response to Hh is a result of cells being unable to recognize the previous exposure to high levels of Hh, *Ccrk*^{+/-} and *Ccrk*^{-/-} MEFs were exposed to 200nM SAG for 8 hours. The SAG was then washed out, and the Hh inhibitor cyclopamine (5μM) (Rohatgi et al., 2007) was added to prevent residual SAG activation. RNA was isolated from MEFs after 1h, 2h, 4h, and 8 hours after addition of cyclopamine. *Gli1* and *Ptch1* expression were analyzed by qRT-PCR (Figure 2.12). After addition of cyclopamine, the levels of *Gli1* and *Ptch1* continued to increase in *Ccrk*^{+/-} cells. *Ccrk*^{-/-} MEFs also had an increase in *Gli1* and *Ptch1* expression, although less than seen in the *Ccrk*^{+/-} cells. The experiment was also repeated where expression of *Gli1* and *Ptch1* was examined after SAG removal and 8h, 24h, 48h, and 72h after cyclopamine addition. *Gli1* expression after addition of cyclopamine 24h, 48h, and 72 hours after addition of cyclopamine was lower than that seen 8h after addition of cyclopamine, indicating exhaustion of the response to the original 8hours of SAG exposure. Additionally, high levels of Hh over long periods of time end up being repressive because it activates a negative feedback loop by expressing repressive target genes, such as *Ptch*, of the Hh pathway (Ribes and Briscoe, 2009). Expression of *Ptch1* in *Ccrk*^{+/-} cells continues to increase even after 72 hours without SAG exposure. *Ptch1* levels in *Ccrk*^{-/-} cells, however, does not seem altered. Together,

these data indicate that even after removal of SAG, *Ccrk*^{-/-} cells are still able to interpret the previous state of exposure to SAG, and continue to activate Hh target genes. This suggests that *Ccrk*^{-/-} cells do not have an issue with hysteresis, or remembering that they were exposed to a high level of Hh, but rather a real-time defect in mounting a high-level Hh responses at late time points.

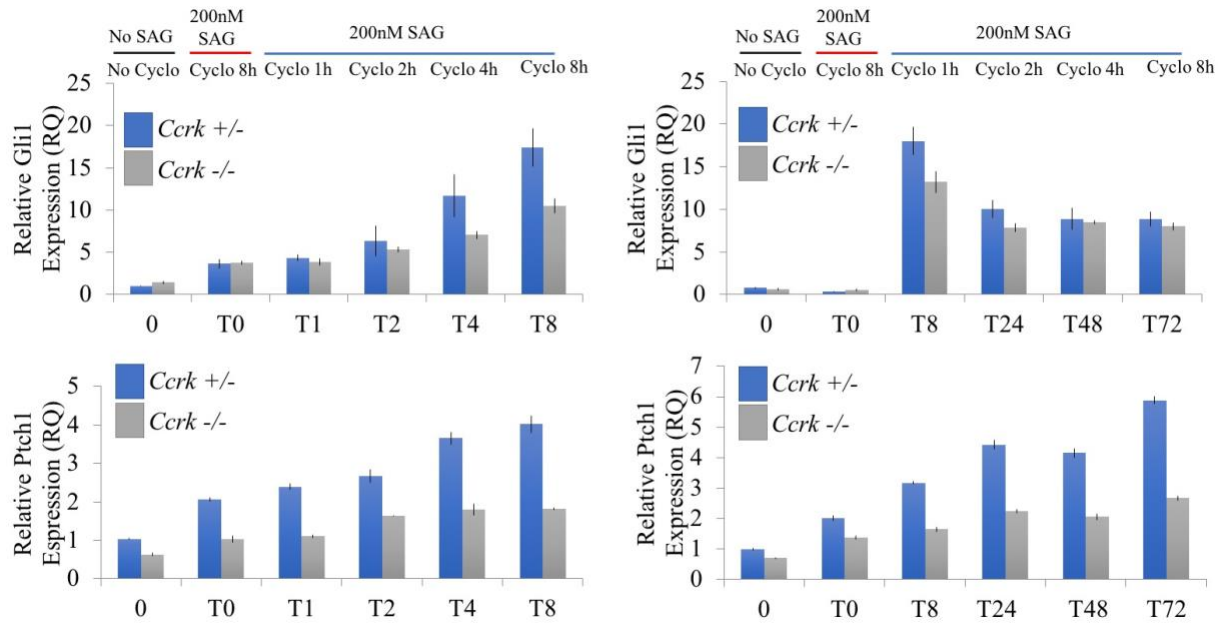


Figure 2.12 Analysis of hysteresis response in *Ccrk*^{+/−} and *Ccrk*^{−/−} MEFs Gli1 (top) and Ptch1(bottom) RNA levels were analyzed by qRT-PCR in the after exposure to 5μM cyclopamine for 8 hours (0), after exposure to 200nM SAG for 8 hours (T0), and after exposure to 200nM SAG for 8 hours + addition of cyclopamine for 2 hours, 4 hours, and 8 hours (T2, T4, and T8, respectively). *Ccrk*^{−/−} Gli1 expression continued to increase, although at lower levels than *Ccrk*^{+/−} expression. *Ccrk*^{−/−} Ptch1 levels remained relatively constant while *Ccrk*^{+/−} levels of Ptch1 increased (Left). The same experiment was repeated but exposure to cyclopamine after 8hours of exposure to SAG was extended to 8hours, 24hours, 48hours, and 72hours (T8, T24, T48, and T72, respectively). In *Ccrk*^{+/−} MEFs Gli1 expression decreases after 8 hours of exposure to cyclopamine and is relatively consistent between *Ccrk*^{+/−} and *Ccrk*^{−/−} MEFs. Ptch1 expression, however, continues to increase up to 72 hours post addition of cyclopamine in *Ccrk*^{+/−} cells, while it remains relatively low in *Ccrk*^{−/−} cells.

Smoothened (Smo) localization in *Ccrk*^{+/-} and *Ccrk*^{-/-} MEFs

To further understand the cellular mechanism underlying the Hh signaling defect within *Ccrk*^{-/-} MEFs the localization of Hh components to the primary cilium of MEFs was analyzed over time. Hh components, including Smo and the Gli transcription factors must localize to the cilium for proper signal transduction. Smo is normally localized throughout the cytosol, but upon Hh induction, Smo localizes to the primary cilium (Corbit et al., 2005b; Kim et al., 2009b; Wang et al., 2007b). Previous work from our lab determined that Gli2 enrichment in the primary cilium occurs more slowly in *Ccrk*^{-/-} MEFs (Snouffer et al., 2017).

To determine if another Hh component, Smo, is also delayed in localization to the primary cilium, MEFs were grown to confluency to promote primary cilium production, and then SAG induced and fixed after a given time of exposure to SAG. The proportion of cilia with Smo localized in *Ccrk*^{+/-} and *Ccrk*^{-/-} MEFs was analyzed by labeling fixed cells with antibodies against Smo and α -tubulin to determine if Smo is localized to the cilium. After 15 of SAG exposure, *Ccrk*^{+/-} MEFs and *Ccrk*^{-/-} MEFs had Smo localized in around 40% of the cilia present. However, at 30 minutes and later time points *Ccrk*^{-/-} cells start to have a much lower proportion of cilia with Smo localized. After 2 hours of SAG induction around 80% of *Ccrk*^{+/-} have Smo localized to the cilium while *Ccrk*^{-/-} only have around 50% of cilia with Smo localized. *Ccrk*^{-/-} MEFs take about 8 hours to reach a steady state, where most cilia have Smo localized, while *Ccrk*^{+/-} MEFs reach this point in about half the time, at 4 hours (Figure 2.13).

The fluorescent intensity of the Smo localized to the primary cilium of *Ccrk*^{+/-} and *Ccrk*^{-/-} MEFs was also analyzed using ImageJ to support the visual determination of Smo localization. Over time, *Ccrk*^{-/-} MEFs also have lower fluorescent intensity than *Ccrk*^{+/-} cells even after they appear reach steady state of Smo localization around 8 hours (Figure 2.14).

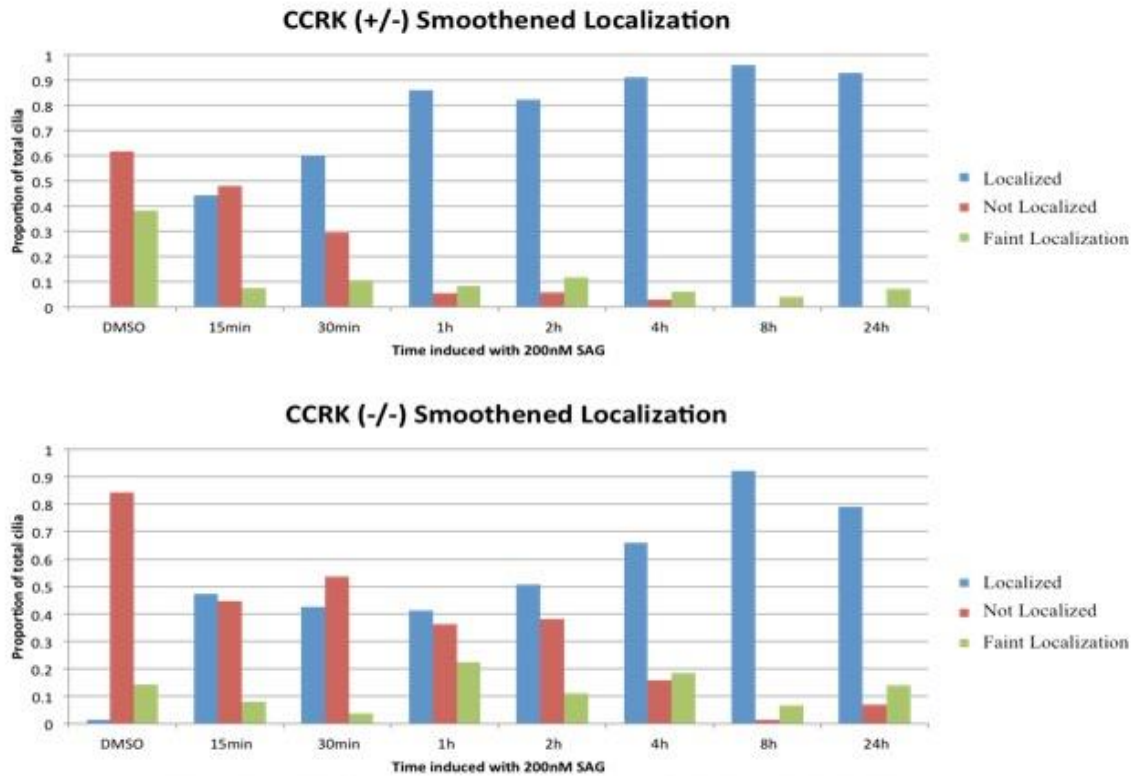


Figure 2.13 The proportion of *Ccrk*^{+/−} and *Ccrk*^{−/−} with Smo localized to the primary cilium over time *Ccrk*^{+/−} (top) and *Ccrk*^{−/−} (bottom) cells were induced with 200nM SAG. The majority of MEFs that were not SAG induced (DMSO) do not have Smo localized to primary cilia, although *Ccrk*^{−/−} MEFs do appear to have lower levels of Smo localization even when un-induced. After 1hour of SAG induction, *Ccrk*^{+/−} MEFs have many more cilia with Smo localized (around 85%) than *Ccrk*^{−/−} MEFs (around 40%). After 8 hours of SAG induction, *Ccrk*^{−/−} cells do have an equivalent number of cilia with Smo localized compared to *Ccrk*^{+/−} cells.

Smoothened Fluorescence Intensity in *Ccrk* (+/-) and *Ccrk* (-/-) MEFs

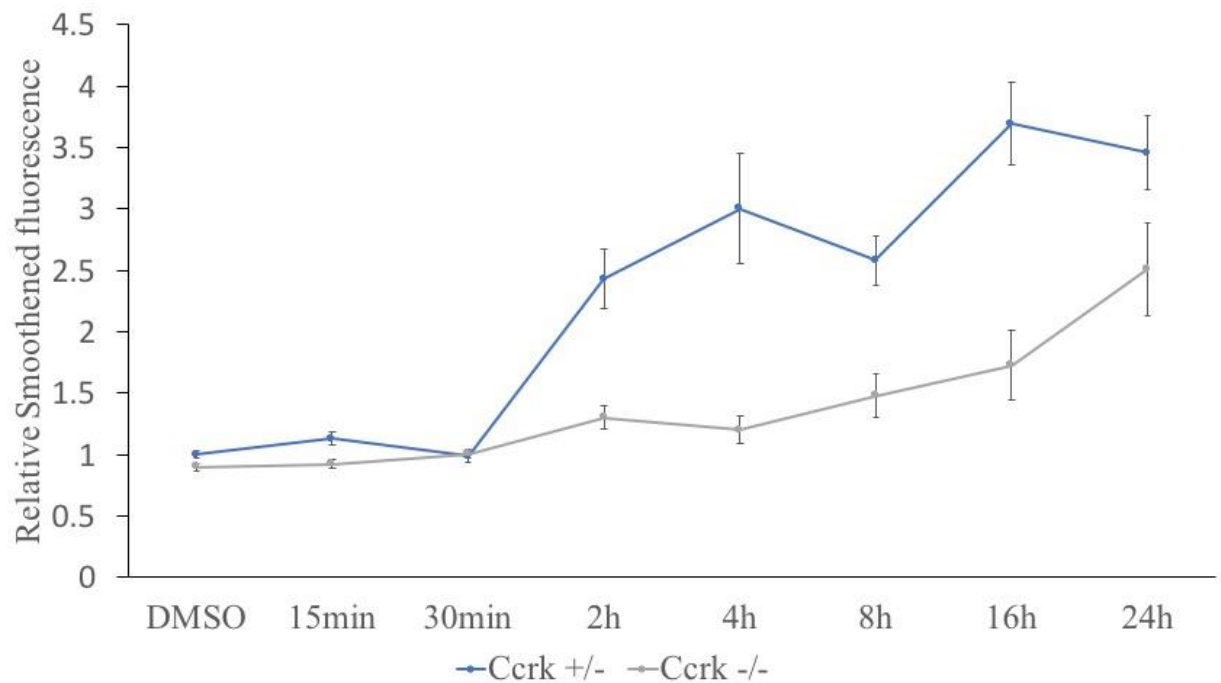
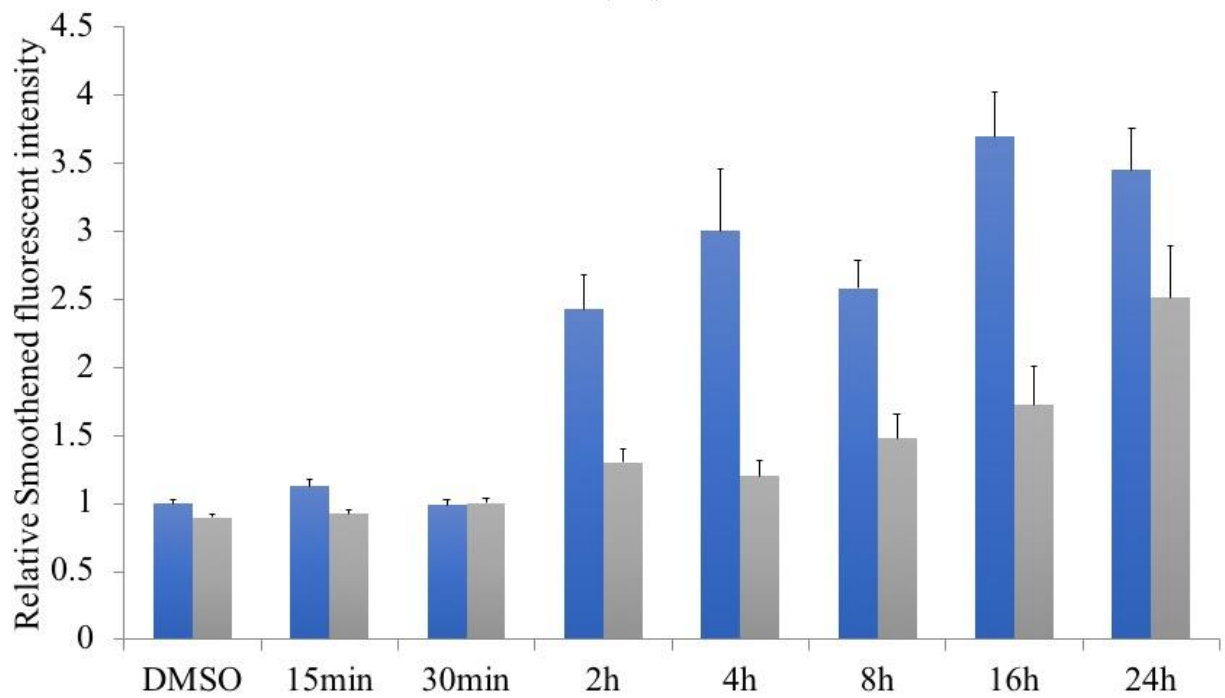


Figure 2.14 Fluorescence Intensity of Smo localization within *Ccrk*^{+/-} and *Ccrk*^{-/-} MEFs

The fluorescence intensity of the Smo antibody localized to primary cilia was measured using ImageJ. After SAG induction *Ccrk*^{-/-} cells consistently have lower fluorescence intensity of Smo within primary cilia that have localized Smo compared to *Ccrk*^{+/-} cells. This may indicate less Smo present within the cilium of *Ccrk*^{-/-} MEFs. Fluorescence intensity is normalized to the intensity observed in DMSO cilia with Smo localized.

Increased Suppressor of fused (SUFU) protein in *Ccrk*^{-/-} MEFs

In the absence of the Hh ligand, suppressor of fused (Sufu) binds to and promotes formation of Gli transcriptional repressors and prevents formation of Gli activators. In the presence of the Hh ligand, the association between Sufu is disrupted and transcriptional activator forms of the Glis can function. (Chen et al., 2011b; Dunaeva et al., 2003; Humke et al., 2010; Kogerman et al., 1999; Svärd et al., 2006; Zhang et al., 2017).

Ccrk^{+/-} and *Ccrk*^{-/-} MEFs were SAG induced for 24 hours and protein was isolated from whole cell lysate and run on a western blot to detect Sufu protein. The amount of Sufu in each well was normalized to the loading control, β -Actin, in ImageJ (Figure 2.15). *Ccrk*^{-/-} cells had more Sufu protein in both un-induced (DMSO) and SAG induced conditions. Increased levels of Sufu present within the cell could potentially result in more Gli proteins bound to and inhibited by Sufu even in the presence of SAG and may contribute to the decreased ability of *Ccrk*^{-/-} cells to respond to SAG. Additionally, the increased levels of Sufu may serve to stabilize more of the full-length form of the Glis while maintaining them in an inactive state. These data suggest that the increased levels of Sufu may have a role in the greatly increased amount of full length Gli3 observed in extracts from *Ccrk* mutant embryos, where the full-length form of Gli3 is stabilized, but inactivated by being bound to the high amount of Sufu (Snouffer et al., 2017)

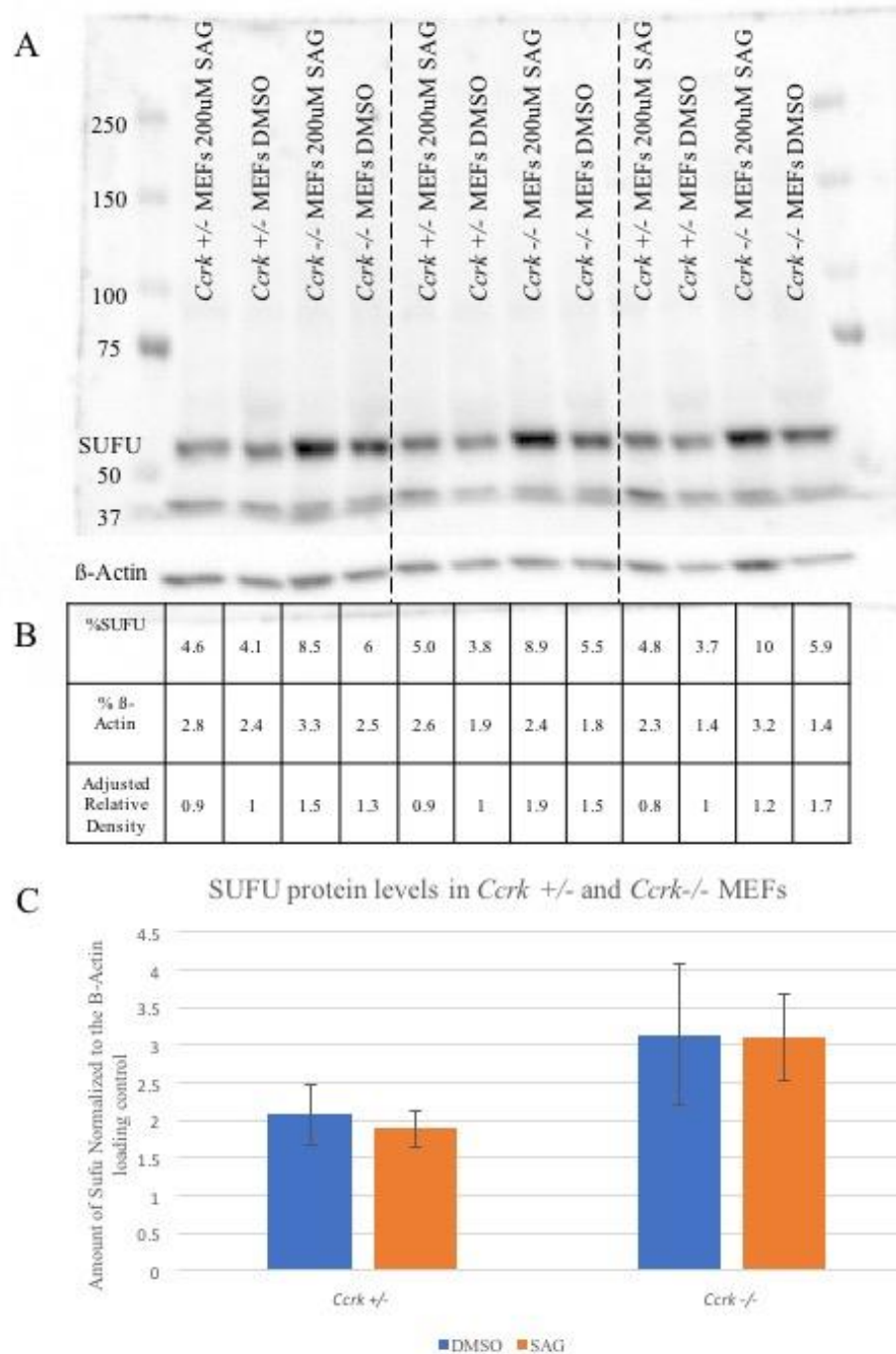


Figure 2.15 Western blot analysis of Suppressor of Fused (SUFU) protein in *Ccrk*^{+/-} and *Ccrk*^{-/-} MEFs A) Three biological replicates of MEFs were grown for 24hours with 200nM Smoothed agonist or DMSO. Cell lysates were extracted and run on a Western blot and blotted

for Suppressor of Fused (58kDa). Membrane was stripped and blotted with B actin 42kDa as the loading control. B) ImageJ gel analysis calculated the density of the SUFU band and the B actin band and then these were normalized to the control condition *Ccrk*^{+/-} DMSO to calculate the relative density. The adjusted relative density normalized the relative density to the amount of protein loaded based on the B actin band density for each well. C) SUFU protein levels only normalized to the amount of protein loaded.

Discussion

Ccrk is required for proper cilia length regulation, Hh response, and Smo localization to the cilium. *Ccrk* RNA is broadly expressed throughout the e10.5 embryo with no evidence of cell-type specific expression in the neural tube indicating that *Ccrk* may be a regulator of cilia in a variety of tissues and cell types, potentially with differential targets in specific tissues to achieve cilia of vastly different lengths seen throughout the embryo.

Ccrk^{-/-} cilia do not have any evidence of ultrastructural defects in microtubule structure but do present with bulbous cilia. These bulbous tips could indicate a transport defect within the cilium resulting in protein accumulation. In support of this hypothesis, work by Jonathon Walsh demonstrated that *Ccrk* null IMCD cells with a fluorescently tagged intraflagellar transport protein (IFT88-YFP) has fluorescent accumulation at the tip of the primary cilia. This accumulation of proteins may be indicative of a protein transport defect within the cilium either from changes in the speed proteins move through the cilium or the frequency at which they are allowed to enter and travel through the cilium. Preliminary evidence indicates that the rate of anterograde (from the cell body to the tip of the cilium) transport is not significantly affected in *Ccrk*^{-/-} IMCD cells, while retrograde (from the tip of the cilium to the cell body) transport rate is significantly slower in *Ccrk*^{-/-} IMCD cells. Additionally, although the rate of anterograde transport does not appear affected, the frequency of anterograde particles traveling through the cilia of *Ccrk*^{-/-} cells is less than the frequency seen in *Ccrk*^{+/+} cilia (Snouffer et al., 2017).

An important component in cilia architecture, tubulin, must be imported into the cilium from the cell body in order for the cilium to extend at the tip. In order for the cilium to be shortened or resorbed, tubulin must be taken off of the tip and sent back to the cell body. This process is largely thought to occur through diffusion of tubulin through the cilium, but how cells

regulate the entrance of proteins to the cilium, even by diffusion, is still not well understood (Lechtreck, 2015; Milenkovic et al., 2015a; Wingfield et al., 2017). Since there is evidence that CCRK is involved to some extent in regulating the rate of transport and the frequency of transport of IFT (Snouffer et al., 2017), it could be more broadly involved in regulation of proteins required for ciliogenesis and length regulation, acting at the level of IFT and/or promoting the entry of proteins and cargo into the cilium (see below).

There is evidence that *Ccrk*^{-/-} MEFs have mis-regulated length, where some cilia are much shorter than the average *Ccrk*^{+/-} cilium, while other cilia are longer than average. The higher proportion of short cilia in *Ccrk*^{-/-} cells than in *Ccrk*^{+/-} cells could be a result of fewer tubulin particles being allowed to enter the cilium, so in the absence of essential building blocks, the cilium ends up being very short. The long cilia could also be explained by transportation issues within the cilium, where the slower rate of retrograde transport could result in slower deconstruction of the cilium. Furthermore, the slower rate of retrograde transport could result in the bulbous tips of *Ccrk*^{-/-} IMCD and MEF cilia, where particles enter the cilium and travel to the tip at a wild-type rate, although lower frequency, but are transported back at a slower rate, resulting in a backup of proteins within the ciliary tip.

Proteins involved in Hh signaling response require localization to the primary cilium for transduction of the Hh signaling pathway (Huangfu and Anderson, 2005; Huangfu et al., 2003; Liu et al., 2005). *Ccrk*^{-/-} MEFs are capable of responding to the Smoothed Agonist (SAG), but are deficient in their ability to mount a wild-type response to SAG at the highest doses and after the longest exposures to SAG, and complementation of *Ccrk*^{-/-} MEFs with a kinase dead version of *Ccrk* indicate that this response to SAG is dependent on the kinase activity of CCRK.

The decreased response to SAG in *Ccrk*^{-/-} MEFs may reflect what occurs *in vivo* within the neural tube of *Ccrk*^{-/-} embryos, where the neural progenitor cell fates that require the highest levels of Hh, and the longest durations of Hh exposure for specification, fail to be specified. Neural progenitor cell fates that require lower or intermediate levels of Hh for specification remain specified within the *Ccrk*^{-/-} embryo and actually have ventrally expanded domains (Snouffer et al., 2017). Additionally, the decreased ability to respond to SAG at high levels and long exposure times does not seem to be a result of the loss of *Ccrk*^{-/-} MEFs hysteresis response, where *Ccrk*^{-/-} cells continue to mount a response by upregulating Hh target genes after the removal of SAG in a similar pattern to *Ccrk*^{+/-} cells, although at decreased levels.

The changes to the rate and frequency of transport of proteins within *Ccrk*^{-/-} cilia could also impact the transport of Hh signaling components and result in a decreased ability to respond to Hh. Previous work demonstrated that there was a change in the kinetics of localizing one of the Hh transcription factors, Gli2, to the primary cilia of *Ccrk*^{-/-} MEFs. Gli2 localization was delayed in *Ccrk*^{-/-} MEFs, although over time the MEFs do reach a steady-state where most cilia have Gli2 localized and have the same fluorescence intensity as *Ccrk*^{+/-} MEFs (Snouffer et al., 2017).

I investigated the rate at which another Hh component required for activation of Hh target genes, Smo, localized to the primary cilia of *Ccrk*^{-/-} cells compared to *Ccrk*^{+/-} cells. The localization of Smo was also delayed, as seen with the localization of Gli2, where *Ccrk*^{-/-} cilia took a longer period of time to localize Smo, but eventually most cilia had localized Smo, just as in *Ccrk*^{+/-} MEFs. I also quantified the fluorescence intensity of the Smo localized to the primary cilia of *Ccrk*^{+/-} and *Ccrk*^{-/-} cilia and found that *Ccrk*^{-/-} cells have significantly less fluorescence intensity of Smo after 30min of SAG induction, indicating that, although the majority of *Ccrk*^{-/-}

cilia localize Smo after a delay in time, there may still be less Smo protein within the cilium when compared to *Ccrk*^{+/-} MEFs. This also suggests that, regardless of the total amount of Smo or Gli2 in the cilium at any one time, the flux of individual molecules into and out of the cilium is affected in the mutants. The delay in localization of Gli2 and Smo may result in slower or reduced ability to mount a response to Hh because processing of the Glis within the cilium to transcriptional activators may also be delayed. This could result in the ability of *Ccrk*^{-/-} cells to still process enough Gli activators to mount a low or intermediate response to Hh, but not be able to process enough Gli activators to mount a high-level response to Hh.

Additionally, *Ccrk*^{-/-} MEFs contain higher amounts of the Hh signaling repressor, Sufu, which binds to the Glis and promotes formation of the repressor form of the Glis (Cooper, 2005; Kogerman et al., 1999; Pearse et al., 1999; Stone et al., 1999; Svärd et al., 2006). The increased amount of Sufu, in combination with slower kinetics of Hh activation may contribute to the decreased response of *Ccrk*^{-/-} cells to SAG at high concentrations and long durations of exposure. Further investigation of the amount of Sufu actually bound to the Glis is required to support the hypothesis that the increased amount of Sufu is indeed affecting the activator/ repressor function of the Glis within *Ccrk*^{-/-} cells.

It has been suggested that Sufu is phosphorylated by GSK3- β and PKA to promote Sufu stability and prevention Hh induced degradation, and promotes Hh inhibition by binding to the Glis (Chen et al., 2011b; Takenaka et al., 2007; Yue et al., 2009). The dual phosphorylation of Sufu promotes localization of Sufu to the cilium along with the Gli transcription factors, and may occur by the same mechanism which promotes Smo localization to the cilium (Chen et al., 2011b; Rohatgi et al., 2007). The decreased ability of *Ccrk* mutants to respond to high levels of Hh may be a result of Sufu, bound to the Glis, are transported more slowly into the cilium, and

may be a result of loss of *Ccrk* may be involved in direct regulation of phosphorylation of the Glis and loss of *Ccrk* makes Sufu bind more tightly to the Glis or reduces the ability of the Glis to be processed into activator form. Alternatively, CCRK phosphorylation could potentially regulate Sufu stability, and in the absence of *Ccrk* there may be increased levels of Sufu as a result of increased stability from direct phosphorylation of Sufu or the Glis by *Ccrk*.

There is no current evidence that CCRK is directly phosphorylating Sufu, Glis, or any other IFT cargo, and our work seems to suggest that *Ccrk* may have a more general role in regulating what is trafficked in the cilium and the potential change in Sufu stability and increase in Sufu levels could be a downstream effect of this role. There is a delay in Smo and Gli2 localization observed in *Ccrk* mutants, and Sufu, bound to Glis, may enter the cilium through a similar mechanism to Smo, through diffusion into the cilium rather than be actively transported by IFT (Milenkovic et al., 2015a). This suggests that CCRK is can also regulate the entrance of Sufu into the cilium. By regulating a gate into and out of the cilium, CCRK, may control the localization of proteins, including Sufu bound to the Glis, and in the absence of *Ccrk* translocation into the cilium is delayed, and the dissociation of Sufu from the Glis, which is thought to occur within the cilium, is also slowed. This could result in an accumulation of Sufu and more full length Gli proteins bound to Sufu and a decreased response to Hh signaling.

The mechanism and direct targets of CCRK phosphorylation, in addition to intestinal cell kinase (ICK), in cilia length regulation remain to be fully elucidated. Additionally, we do not fully understand the stochasticity of the *Ccrk* phenotype, where genetically identical cells grown in the same environment produce both short cilia and long cilia. The general dysregulation of ciliary length may be a result of a single function of *Ccrk*, mediated through *Ick*, in regulating the movement of cargo and other proteins through the cilium. The variable cilia length could result

from dysregulation of cargo import and proteins, such as tubulin, into the cilium when *Ccrk* is absent. Although, there are minor changes to the speed and frequency of IFT particles in *Ccrk* mutant cilia, the differences in amount of cargo may play a larger role in the disrupted length phenotype. In this scenario where *Ccrk* regulates all of cilia length through *Ick*, some *Ccrk* mutant cilia may have less tubulin loaded onto IFT particles, so cannot extend the cilium as much and end up having shorter cilia, while other cilia have increased amounts of tubulin loaded onto IFT particles and end up having longer cilia than wild-type cells.

Overall *Ccrk* may have one function, to regulate the amount of cargo loaded onto IFT particles and the amount of proteins which diffuse into the cilium, and in the absence of *Ccrk*, the cell can no longer balance the amount of proteins entering the cilium, and in some cases more protein than wild-type enter resulting in longer cilia, while in other cases less protein enters resulting in short cilia. There is evidence for altered tubulin transport in the *Chlamydomonas* homolog of *Ccrk*, *lf2*, where *lf2-1* mutants have increased IFT tubulin transport, even when the cilium reaches steady state, although the rate of tubulin transport was not dependent on cilium length (Craft et al., 2015). If *Ccrk* mutants also have an increased rate of tubulin transport independent of the length of the cilium, then the increased amount of tubulin could result in overlengthening of *Ccrk* mutant cilia, but it could also result in protein accumulation if the rate of retrograde transport does not match that of anterograde transport. If the accumulation of proteins at the tip of the cilium is too dense and compact it may prevent elongation machinery from extending microtubules and cilia may be prevented from growing resulting in cells with short cilia.

Although, the model of *Ccrk* having a single function seems to fit for transport of tubulin within the cilium, it is unlikely that it is that simple because if this was generally true, then each

cell would also respond to Hh in different ways, and cells that were able to import larger amounts of Hh signaling components would have a high response while cells that import less Hh components would have a reduced response. This is unlikely as the *Ccrk* mutant embryos do not have any cells within the neural tube which appear to be patterned normally interspersed with miss-patterned cells, suggesting that all of the cells have the same deficiency in Hh response in a *Ccrk* mutant despite having cilia of different lengths.

Another possibility is that *Ccrk* regulates multiple functions, one of which is cilia shortening, mediated by *Ick*, and another function in cilia lengthening mediated through a target yet to be described. These downstream cilia length regulators may control the amount of cargo that can enter the cilium, but in opposite ways. In this case, it is the balance between the activity of the downstream regulators in the absence of *Ccrk* which determines if the cell builds a cilium that is long or of the cell builds a cilium that is short. If in a cell the activity of *Ick*, in the absence of *Ccrk*, predominates, then the cilium is shortened, but if the activity of the lengthening factor predominates then the cilium is lengthened. It could be predicted that the activity of both of these downstream length regulators would be decreased in the absence of *Ccrk*, and the length of the cilium in the absence of *Ccrk* is determined almost randomly, and initiation time of ciliogenesis may increase, and cilia may never reach a steady state where the cilium has grown to the set complete length for a given cell type. Live imaging of ciliary growth would need to be done in *Ccrk* null cells to address the rate at which cilia grow and if they ever reach a steady state length in the absence of *Ccrk* compared to wild-type cells.

Additionally, there is preliminary evidence from our lab which suggests that *Ccrk* may also have a role in regulating the actin cytoskeleton, which can promote or inhibit ciliogenesis by regulating the docking and stability of the basal body (Personal correspondence with Jonathon

Walsh). The multiple roles of *Ccrk* may contribute to the stochastic cilia length and the variability in the cilia length phenotype, as a result of alterations and general dysregulation in different aspects regulating cilia length in the absence of *Ccrk*. Understanding the role *Ccrk* and *Ick* play in cilia length regulation in relationship to each other to further our understanding of how *Ccrk* regulates cilia length is the focus of Chapter III.

Materials and Methods

***Ccrk* Mouse strain**

The *Ccrk* KO mutant allele was previously generated in the lab from mice engineered to contain LoxP sites flanking the first two exons (*Ccrk*Fl allele). Male mice with the *Ccrk* Fl /+ allele were bred to Tg(Ella-Cre)/+ females (Jackson labs) to knockout one copy of *Ccrk* to generate the *Ccrk* KO allele. Full description of the *Ccrk* Fl and *Ccrk* KO allele generation in (Snouffer et al., 2017).

Embryo processing

Timed matings were set up and vaginal plugs were checked each morning. Fertilization (day 0) is assumed to be at midnight the day before a vaginal plug is found. Pregnant females were sacrificed using CO₂ inhalation and cervical dislocation according to guidelines established by the IACUC at UGA and the AVMA guidelines on euthanasia of rodents on the appropriate day of development (embryonic day 10.5-13.5, e10.5-e13.5). Embryos were dissected in phosphate buffered saline and then fixed in 4% Paraformaldehyde at 4C for 2-20 hours.

In situ Hybridization

Ccrk expression was detected by in situ hybridization using sense and antisense riboprobes synthesized from a 506 bp PCR amplified fragment of the 3' UTR region of mouse *Ccrk* (from positions 1169-1676 in the mRNA sequence (ID: NM_053180.2)). (Snouffer et al., 2017) Whole mount *in situs* were done on embryos fixed in 4% PFA

(EMS cat# 15710) overnight using standard methods. Neural tube section in situs were done on embryos fixed in 4% PFA (EMS cat# 15710) overnight using standard methods

Transmission and Scanning Electron Microscopy (TEM and SEM)

Wild-type and *Ccrk* homozygous null IMCD3 cells and MEFs were grown to confluency, followed by fixation in 2% glutaraldehyde/0.01% tannic acid and post-fixation in 1% O₄Os. Samples were embedded in EPON resin for Transmission Electron Microscopy (TEM) or gold-coated (Structure Probe, Inc) for Scanning Electron Microscopy (SEM). Sections for TEM were cut using a RMC MT-X microtome (RMC Products) and placed on copper grids and visualized using a JEOL JEM-1210 Transmission Electron Microscope (JEOL, Inc.). SEM samples were visualized using a Zeiss 1450EP SEM microscope (Carl Zeiss MicroImaging). (Snouffer et al., 2017)

Cell immunohistochemistry and Primary Cilia Length measurements

Control and *Ccrk* mutant MEFs were grown to confluency on glass coverslips and serum starved in 1% FBS for 24 hours to induce ciliogenesis. The cells were processed for staining using standard protocols with the following antibodies: rabbit polyclonal against mouse Smoothed (1:5000), rabbit polyclonal against Arl13b (1:3000), mouse monoclonal against acetylated α -tubulin (1:2000), and a mouse monoclonal against γ -tubulin (1:500). Cells were stained with appropriate secondary antibodies together with DAPI. Images were taken on Zeiss Axioplan 2 microscope with a 63x oil objective and analyzed using AxioVision4.6 and FIJI Software.

MEFs were exposed to 200 nM SAG for time periods ranging from 30 min to 24 hours and then fixed. Experiments were performed in triplicate. Cells were immunostained as described above to visualize the localization of Gli2 and Smoothed to the cilium after different lengths of exposure to SAG. Fluorescent intensity was determined using ImageJ software. Background fluorescence was used to set thresholds for measuring frequency of cilia showing localization. Values were obtained for 50–200 cilia per condition/cover slip.

Ciliary length was determined for control and *Ccrk* null MEFs through immunolabeling to visualize steady-state cilia length. The axonemes of cilia were labeled with Arl13b and acetylated α -tubulin, whereas the centrosome was labeled with γ -tubulin. Cilia in the focal plane were measured from the base of the cilium where it intersected the centrosome to the tip of the Arl13b staining. Length measurements were performed using Zeiss 4.0 and ImageJ Software (Snouffer et al., 2017).

qRT-PCR Quantification of Hh target gene expression in MEFs

Ccrk^{+/−} and *Ccrk*^{−/−} MEFs were grown to confluency on a 6 well tissue culture dish with three wells as replicates for each condition. Cells were then induced with varying concentrations of SAG (5nM, 2nM, or 200nM) for the stated period of time. Cells were then trypsinized and spun at 150 rcf. RNA was extracted from pelleted MEFs using the E.Z.N.A. Total RNA Kit I (Omega R6834-00). cDNA was synthesized using qScript (Quanta). RT-qPCR was performed using *Gli1* (IDT Mm.PT.58.11933824) and *Ptch1* (IDT Mm.PT.58.5305068), and normalized to *Actb* (IDT Mm.PT.58.33540333) levels using an Applied Biosystems 7500 Real Time PCR System.

Normalized values were averaged among triplicates and expressed with standard error (Snouffer et al., 2017).

Western Blot Analysis

Anti-Suppressor of Fused 58kDa (abcam ab28083) used at 1:1000 blocked with 5% BSA and HRP secondary (rabbit) used at 1:2000. Membrane was stripped with Harsh stripping buffer at 55C for 40min. B-actin 42kDa (abcam ab8226) used at 1:5000 with 5% Milk and HRP secondary (mouse) used at 1:5000. Experiment was done in triplicate, and each set represents biological replicates.

Quantification of the Western blot was done in ImageJ gel analyzer was used to calculate the density of the B-actin loading control band and the SUFU band. To calculate the relative density, the percent for each of the Sufu bands was normalized to the percent of the *Ccrk*^{+/-} DMSO control band. This was repeated for the B-actin bands. The adjusted relative density was then calculated by dividing the Sufu band by the loading control B-actin band to account for changes in protein loading.

CHAPTER III

THE EPISTATIC RELATIONSHIP BETWEEN CELL CYCLE-RELATED KINASE (CCRK) AND INTESTINAL CELL KINASE (ICK) IN CILIA LENGTH REGULATION AND HH SIGNALING

Introduction

ICK/MRK and its Paralogs, MOK and MAK

Intestinal cell kinase (ICK), also known as MAK-related kinase (MRK) is a serine/threonine protein kinase originally cloned from the intestine (Togawa et al., 2000). CCRK phosphorylates ICK at Thr-157 residue within the T-loop of the TDY motif, and this phosphorylation is required for ICK activity (Fu et al., 2006a). The catalytic domain of ICK and its paralogs, MOK and MAK, is structurally similar to that of MAPKs (mitogen-activated protein kinases) and CDKs (cyclin-dependent protein kinases) with a TXY motif in the T-loop that is required for kinase activation (Fu et al., 2005; Matsushime et al., 1990; Miyata and Nishida, 1999; Togawa et al., 2000; Wang and Kung, 2012). The non-catalytic domains of these three kinases are more divergent and may have a role in their specific cellular roles, and unlike MAPKs they are not activated by growth factors or MEKs (Chen et al., 2013; Miyata and Nishida, 1999; Togawa et al., 2000).

While *Ick* expression is ubiquitous, the expression of the paralogs of *Ick* seems to be more tissue specific, with overlapping expression observed in some tissues (Chen et al., 2013). The differences in expression of these three kinases may serve to tightly regulate ciliary length in

different tissues. Male germ cell-associated kinase (MAK) is highly expressed in testicular germ cells and in the meiotic phase of spermatogenesis (Matsushime et al., 1990). Mak expression has also been found in the respiratory tract, choroid plexus, prostate and the retina (Bladt and Birchmeier, 1993; Ma et al., 2006; Omori et al., 2010). Within the retina, MAK localizes to the cilium of photoreceptor cells, and these cilia become elongated and the photoreceptors degenerate in *Mak* knockout mice (Omori et al., 2010). MAPK/MAK/MRK-overlapping kinase (MOK) is the other serine/threonine kinase that is closely related to ICK and MAK, and has been found to be expressed in the intestine, brain, lung, and more highly expressed in the testis and kidney (Chen et al., 2013; Miyata and Nishida, 1999; Uesaka and Kageyama, 2004).

Further studies to understand the tissue specific role of expression of these three kinases are required, however, one study has found that all three kinases are expressed in the postnatal intestines, however, ICK, MAK, and MOK have distinct protein expression patterns. ICK is ubiquitously expressed along the entire intestine from the small intestine to the colon, with some variation in expression levels. MOK is also ubiquitously expressed within the intestines, except within the colon. MAK has three protein coding isoforms, and two of the three can be found within the colon (MAK-1 and MAK-2) of the intestines, and some expression of one of the MAK-2 isoform within the duodenum of the small intestine (Chen et al., 2013).

The cellular function of the differential expression of these three kinases within the intestines and other tissues has yet to be fully elucidated, but may involve regulating cellular proliferation and differentiation. Within the intestine, *Mok* and *Ick* are specifically expressed in the crypt cells, and knockdown of *Ick* expression within the intestine resulted in decreased cell proliferation, suggesting *Ick* may have a role in promoting cell cycle progression (Fu et al., 2009; Togawa et al., 2000; Uesaka and Kageyama, 2004). Furthermore, overexpression of ICK, but not

MAK, was found in human primary colon cancer specimens further implicating ICK in cellular proliferation (Chen et al., 2013). *Ick* mouse mutants have also displayed decreased proliferation in lung mesenchyme (Tong et al., 2017). In an *in vitro* study in HT-29 cells, *Mok* was implicated in intestinal epithelial cell differentiation (Uesaka and Kageyama, 2004). An *in vivo* study done in support of this role for *Mok* found that in intestinal adenomas, caused by over-proliferation of the intestinal epithelial cells, had much higher ICK protein levels and greatly reduced MOK protein levels than what was found in the adjacent non-cancerous tissue (Chen et al., 2013).

CCRK and ICK homology in ciliogenesis

CCRK and ICK, both have homologs within *Chlamydomonas reinhardtii* called long flagella 2 and (LF2p) and long flagella 4 (LF4p), and, as their names imply, they control flagellar length within the green algae (Berman et al., 2003a; Tam et al., 2007). *Chlamydomonas* produce two motile flagella, and hypomorphic mutations in the *Ccrk* homolog, *lf2*, result in both flagella being longer than wild-type (Barsel et al., 1988). Null mutations in *lf2* result in *Chlamydomonas* with two flagella on the same cell of green algae that are unequal lengths, where one flagellum is very long and the other is very short (Tam et al., 2007). LF2p localizes within the cytoplasm with two other long flagella proteins (LF1p and LF3p) to form the length regulating complex (LRC) of which LF2 is thought to be the catalytic subunit as a CDK (Barsel et al., 1988; Nguyen et al., 2005; Tam et al., 2003; Tam et al., 2007). In addition to *lf2* null mutants having flagella of unequal lengths, they also have swollen flagellar tips with accumulation of IFT-like particles, and were unable to regenerate flagella longer than a little stump after removal (Tam et al., 2007). The *lf2* mutants were also found to have increased frequency of tubulin transport compared to wild-type flagella, which was independent of length. The inability to regulate tubulin transport

in the absence of *lf2* could contribute to the extended length of the *lf2* flagella compared to wild-type (Craft et al., 2015). The flagellar phenotype of the *lf2* mutant resemble the mouse homolog, *Ccrk*, ciliary phenotype described by our lab, where cilia length is generally disrupted with some cells producing primary cilia that are much shorter than wild-type cilia and cilia (Snouffer et al., 2017) The ability of *Ccrk*^{-/-} cilia to be regenerated has yet to be analyzed in mammalian cells.

LF4p is a MAPK homologous to mammalian ICK. It is localized throughout the cytoplasm, and is enriched in the flagella of *Chlamydomonas* (Berman et al., 2003a). Null mutations in *lf4* result in *Chlamydomonas* with long flagella. Although, LF4p also plays a role in regulating flagellar length, epistatic analysis of double mutants in *Chlamydomonas* suggest that it is not directly downstream of LF2p and the other LRC complex proteins (Berman et al., 2003a; Tam et al., 2003). *Lf1* single mutant *Chlamydomonas*, like *lf2* mutants, cannot regenerate their flagella, and although a *lf1* and *lf4* double mutants can rescue this regeneration defect (Asleson and Lefebvre, 1998), the *lf4* mutation cannot suppress the null *lf2* or *lf3* mutant phenotype. Furthermore, LF4p does not directly interact with any of the other LF proteins by yeast two-hybrid. These data suggest that LF4 may regulate flagella length through a separate pathway from the LRC complex (Tam et al., 2007). The long flagellar phenotype of the *lf4* mutant is very similar to the mammalian homolog, *Ick*, long cilia phenotype reported (Broekhuis et al., 2014; Moon et al., 2014; Taylor et al., 2016; Tong et al., 2017).

The LRC is thought to be involved in regulation of another kinase, LF5, which has homology to the human kinase CDKL5, a cyclin-dependent kinase-like kinase. LF5 localizes to the proximal region of the flagellum, near the base (Tam et al., 2013). A role for CDKL5 in cilia length regulation in mammals has not yet been described, although disruption of CDKL5 has been linked to juvenile epilepsy in humans and mouse neural development, suggesting it may

function within the brain, and based on homology, potentially in cilia length regulation during development (Kalscheuer et al., 2003; Rademacher et al., 2011; Tam et al., 2013; Weaving et al., 2004).

In addition to LF5, two other mammalian homologs of the LF genes that have been identified, LF2 and LF4 (Tam et al., 2007; Tam et al., 2013). CCRK (LF2p) and ICK (LF4p), have both been implicated in cilia length regulation within mammals (Broekhuis et al., 2014; Moon et al., 2014; Snouffer et al., 2017c). Interestingly, both *Ccrk* and *Ick* mutants have phenotypes strongly resembling those seen in *lf2* and *lf4* mutant flagella (Snouffer et al., 2017; Tam et al., 2003; Tam et al., 2007). Also, similar to the *Chlamydomonas* homologs, ICK localizes to the cilium, while CCRK is cytoplasmic (Broekhuis et al., 2014; Moon et al., 2014; Wu et al., 2012). Although, unlike in *Chlamydomonas*, the interaction between CCRK and ICK is direct through phosphorylation of the T157 residue of ICK by CCRK (Fu et al., 2006a). These data suggest that although the mechanism of cilia length regulation has been adapted over time, the components of cilia length regulation are likely very ancient.

Disruption of *Ick* and *Ccrk* alters cilia length regulation and transport of proteins, including those involved in Hh signaling, through the cilium, and bulbous tips observed in both mutants suggests accumulation of proteins including IFT proteins within the cilium (Broekhuis et al., 2014; Chaya et al., 2014; Moon et al., 2014; Oud et al., 2016; Snouffer et al., 2017). However, the cellular mechanism by which these kinases regulate the transport of proteins within the cilium has yet to be fully elucidated. Within the cilium of inner medullary collecting duct cells (IMCD-3), fluorescently tagged ICK travels at about 0.45um/second both in (anterograde) and out (retrograde) of the cilium. The speed at which ICK travels is very similar to fluorescently tagged intra-flagellar transport (IFT) components, suggesting that ICK may be actively

transported by IFT. Additionally, shRNA knockdown of ICK resulted in an increased anterograde IFT speed, while overexpression of ICK resulted in decreased retrograde IFT speed, suggesting that ICK may regulate cilia length by altering IFT transportation speed (Broekhuis et al., 2014). The role for CCRK in cilia length regulation likely lies outside of the cilium, where CCRK may regulate the import of proteins, such as ICK and Hh components, into the cilium. Although CCRK and ICK regulate the length of the cilium, they do not seem to do so through regulating the architecture of the cilium (Moon et al., 2014; Snouffer et al., 2017c). The molecular mechanism precisely regulating cilia length throughout the different mammalian cell types and the role of CCRK and ICK in this regulation need further studying.

Downstream targets of ICK

The molecular mechanisms regulating cilia length have not been fully elucidated, and although several downstream targets of ICK phosphorylation have been proposed, the implications of these interactions in ciliary length have not been fully investigated. The cAMP and target of rapamycin (mTOR) pathways have both been implicated in regulating cilia length and IFT in addition to cell cycle progression and proliferation (Besschetnova et al., 2010; Miyoshi et al., 2009; Yuan et al., 2012). IMCD-w cells with shRNA knockdown of ICK were treated with forskolin to increase cAMP levels. The increased cAMP and decreased ICK levels resulted in increased cilium length, longer than ICK depletion alone, and increased anterograde speed of IFT, suggesting that ICK and cAMP act in the same pathway to regulate IFT, but in parallel pathways to regulate ciliary length in IMCD-3 cells. ICK has also been shown to phosphorylate, *in vitro* and *in vivo*, the Thr-908 of Raptor, the target of the rapamycin (mTOR) complex 1 (mTORC1) in the mTOR signaling pathway (Wu et al., 2012). IMCD-3 cells depleted

for ICK by shRNA and treated with rapamycin suppressed the long cilia and decreased anterograde speed phenotypes seen in ICK knockdown alone cells. These data suggest that ICK inhibits mTORC1 through phosphorylation of Raptor (Broekhuis et al., 2014).

ICK has also been implicated in regulating Glycogen synthase kinase-3 β (GSK-3 β), a protein with many cellular functions, including repressing mTOR signaling, and has been implicated in regulating cilia size (Yuan et al., 2012; Wu et al., 2012). The phosphorylation of GSK-3 β at the Thr-7 residue by ICK is thought to promote GSK-3 β activity and inhibit auto-phosphorylation at Ser-9 which blocks GSK-3 β activity (Wu et al., 2012). ICK may also function more directly to modulate cilia transport by regulating a kinesin involved in anterograde transport of IFT particles. An *in vitro* kinase assay, suggested that ICK can directly phosphorylate Kif3a to regulate ciliary length (Chaya et al., 2014). Although ICK is involved in cilia length regulation, the specific molecular function it has and the role of the direct interaction that have been found are not fully understood in the context of cilia length regulation. ICK may also have different molecular targets within different embryonic tissues or cellular contexts, further complicating our understanding of its function in cilia length regulation.

Discrepancies in the function of *Ick* in ciliogenesis

Analysis of sub-cellular localization of ICK, MAK, and MOK indicate that these three kinases localize to the primary cilium (Omori et al., 2010, Chaya et al., 2014; Moon et al., 2014; Yang et al., 2013; Broekhuis et al., 2014). MAK has been shown to be a negative regulator of ciliary length within the retina (Omori et al., 2010; Özgül et al., 2011), and knockdown of MOK suggests also resulted in increased cilia length (Broekhuis et al., 2014). The majority of studies find that ICK shares this function with its paralogs, and is also a negative regulator of cilia length

(Broekhuis et al., 2014; Moon et al., 2014; Taylor et al., 2016; Tong et al., 2017). The role for ICK in ciliary shortening is further supported by its homolog long flagella 4 protein (LF4p) in *Chlamydomonas*, which produces much longer flagella in the absence of LF4p (Berman et al., 2003). Furthermore, strong over-expression of wild-type ICK or phosphomimetic ICK^{T157E} results in greatly reduced ciliogenesis, while overexpression of Ick with loss of functional kinase activity (Ick E80K or Ick T157A) is a weaker suppressor of ciliogenesis, suggesting that ciliogenesis is particularly sensitive to ICK kinase activity, and particularly the T157 residue phosphorylated by CCRK. Additionally, alterations to the kinase activity of ICK result in ICK accumulation at the tip of the cilium rather than localization throughout the cilium (Taylor et al., 2016; Yang et al., 2013).

A separate allele of *Ick* was generated by Chaya et al 2014 which removed exon 3 by flanking it with loxP sites and was then knocked out within the female germline using CAG-Cre. This allele of *Ick* was found to not produce *Ick* mRNA nor ICK protein. This allele of *Ick* was found to affect cilia formation within the brain, where very few cilia were found in the *Ick*^{-/-Chaya} brain compared to *Ick*^{+/+} E15.5 embryos. They also found fewer cilia in the epidermis and dermis and the intestines of *Ick*^{-/-Chaya} embryos while cilia within the kidney were not affected. They generated an anti-ICK antibody and determined, similar to other reports, that ICK is localized to cilium (Oud et al., 2016; Broekhuis et al., 2014; Taylor et al., 2016), however it was reported that ICK was primarily found at the tip of the cilium, where other studies report only versions of ICK with disrupted kinase function are found primarily at the ciliary tip (Chaya et al., 2014; Taylor et al., 2016; Yang et al., 2013). Further studies suggest that ICK localizes to the base of the cilium, and that mutations that affect the kinase activity (kinase dead K33R or TDY mutated) result in ICK localization primarily at the tip of the cilium (Broekhuis et al., 2014; Moon et al., 2014).

In Chaya et al., 2014 it was found that *Ick*^{-/-Chaya} MEFs had shorter cilia than *Ick*^{+/+} MEFs, and that overexpression of *Ick* resulted in slight ciliary elongation. Additionally, they found that localization of Hh components was disrupted in *Ick*^{-/-Chaya} MEFs where Smoothened (Smo) accumulated within the *Ick*^{-/-Chaya} cilia in the absence of Hh induction. It was also found that only 10% of *Ick*^{-/-Chaya} MEFs have acetylated α -tubulin positive presumptive cilia, and that only 30% of *Ick*^{-/-Chaya} presumptive cilia were positive for Arl13b, where cilia presence was determined by Smo or Gli2 localization to a ciliary shaped structure (Chaya et al., 2014). Absence of Arl13b from the cilium can also result in Smo accumulation within the primary cilium, and has been found to occur in other ciliary mutants (Larkins et al., 2011; Norman et al., 2009). If *Ick* is affecting the localization of Arl13b to the cilium it may also be resulting in the localization of Smo to the cilium even in the absence of SAG. Other alleles of *Ick* analyzed did not report any decreased Arl13b or acetylated α -tubulin localization to *Ick*^{-/-Chaya} primary cilia (Broekhuis et al., 2014; Moon et al., 2014; Taylor et al., 2016; Tong et al., 2017). Although reduced formation of cilia was also reported in MEFs that were isolated from an ECO patient with a novel missense mutation in *Ick*, *Ick*G120C, analysis of the frequency and length of cilia within the ECO patient were not reported, and the MEF cilia that were produced were of normal length (Oud et al., 2016). Interestingly, the same Chaya et al., 2014 *Ick* floxed allele was crossed with *Pax2-Cre* to genetically remove *Ick* from the inner ear during development and found elongated cilia in the supporting cells of the inner ear and that *Ick* is required for positioning the cilia through PCP signaling (Okamoto et al., 2017).

The discrepancies seen in the *Ick* mutant cilia phenotype may be a result of differences in kinase activity levels between the different mutant alleles. Perhaps the very low levels of *Ick* expression in the *Ick*^{tm1a/tm1a} or an incomplete knockdown in culture results in a low level of

kinase activity and promotes abnormal ciliary lengthening. It also remains possible that ICK can have opposing roles in cilia length regulation within differing tissues or molecular contexts, where *Ick* promotes ciliary shortening in one context, such as the limb bud or the inner ear, and in a different context ICK promotes ciliary lengthening and production, such as the brain.

ICK in Hh signaling and Human disease

The *Ick^{tm1a/tm1a}* and *Ick^{-/-} Chaya* alleles both reported shortened ulna, hydrocephalus, preaxial polydactyly, late stage embryonic lethality, and disruption of Hh signaling, within the developing brain and the limb bud (Chaya et al., 2014; Moon et al. 2014). The gross embryonic phenotype along with the reports of alteration to ciliary length and IFT speed are indicative of cilia related Hh signaling defects within *Ick* mutant embryos (Moon et al., 2014; Broekhuis 2014). The primary cilium is essential for Hh signaling (Goetz and Anderson, 2010), and it was found that overexpression of wild type and mutant versions of ICK both decrease the expression of a Sonic Hh induced Gli luciferase reporter compared to cells expressing endogenous levels of ICK. Knockdown of *Ick* by siRNA resulted in changes in Hh component localization within the cilium, where instead of being evenly distributed throughout the cilium, Smo and Gli2 accumulated in patches along the cilium. Within the cilia of the *Ick* mutant embryo the cilia were elongated and Hh target gene expression was decreased in the posterior limb bud. Additionally, *Ick* mutants also have loss of Gli3 repressor activity within the limb bud resulting in ectopic gene expression in the anterior limb bud and polydactyly. In support of loss of *Ick* resulting in reduced Gli3 repressor, it was found that *Ick* mutant embryos had an accumulation of the full length, the inactive, form of the Gli3 protein within limb buds (Moon et al., 2014)

Mutations in *Ick* and its paralog, *Mak*, have been associated with several human syndromes, and are thought to be linked to the disruption of Hh signaling within the cilium. *Mak*

mutations have been found in human patients with the inherited disorder, retinitis pigmentosa, which causes a degeneration of photoreceptors of the retina (Ozgul et al., 2011). *Ick* mutations have been implicated in human patients with endocrine-cerebro-osteodysplasia (ECO) syndrome and short rib-polydactyly syndrome (SRPS) with evidence of disrupted Hh signaling (Lahiry et al., 2009; Moon et al., 2014; Oud et al., 2016; Taylor et al., 2016; Tong et al., 2017). ECO syndrome is a genetic disorder with multiple defects including skeletal development, polydactyly, hydrocephalus, and holoprosencephaly. The neonatal lethality of ECO syndrome is thought to result from the role of *Ick* in lung development. Mice with the loss of function mutation *Ick* R272Q resemble human patients with ECO syndrome, and were found to have disrupted branching within the lungs and reduced mesenchymal proliferation in addition to skeletal defects (Ding et al., 2017; Tong et al., 2017). ECO has also been linked to another missense mutation in *Ick*, G120C (Oud et al., 2016).

SRPS is also neonatal lethal and results in hydrocephalus, craniofacial defects, shortened ribs in addition to other skeletal abnormalities, and polydactyly. SRPS has been linked to a different missense mutation in *Ick*, E80K, and is essential to ATP bonding within the serine/threonine kinase domain of ICK. Similar to what was seen in the limb bud of *Ick* knockout mutant embryos, this mutation in cell culture resulted in a decrease in Gli3 repressor relative to the amount of full length Gli3, and reduced Hh activation after Smoothed Agonist induction (Moon et al., 2014; Taylor et al., 2016). In this chapter, we analyzed two separate alleles of *Ick* to help clarify the differences between the two ciliary phenotypes described in *Ick* mutants. Additionally, we generated mouse embryos double mutant for both *Ccrk* and *Ick* to investigate the epistatic interactions between these two kinases *in vivo*.

Results

General Characterization of the *Icktm1b* allele

Icktm1b was generated by the Knockout Mouse Project (KOMP) (Tate and Skarnes, 2011), and is an allele of *Ick* with exon 6 removed by Cre-lox recombination (Figure 3.1 E). The *Icktm1a* allele, the *Ick* gene with a gene trap cassette insertion before Cre recombination, was previously analyzed in Moon et al. The *Icktm1a* allele was found to produce a truncated nonfunctional RNA transcript by RT-PCR, and overall *Ick* transcript was decreased by more than 90% by qRT-PCR in *Ick^{tm1a/tm1a}* (Moon et al). I analyzed the *Icktm1b* phenotype, and found that it phenocopied the *Icktm1a* allele in all aspects examined, except eye lid closure. Both alleles of *Ick* maintain a wild-type appearance through early embryonic development, and do not have detectable defects in neural tube patterning. However, embryos homozygous for both alleles of *Ick* do not survive the later stages of embryonic development (E17.5-18.5). Later stage embryos, around E15.5-16.5 present with general edema, shortened limbs, preaxial polydactyly, cleft palate, and hydrocephalus (Figure 3.1 A-D) just as the Moon et al., 2014 and Chaya et al., 2014 alleles of *Ick*. The *Ick^{tm1b/tm1b}* will hereafter be referred to as *Ick^{-/-}*.

I sectioned E16.5 *Ick^{+/+}* and *Ick^{-/-}* embryos and found that the *Ick^{-/-}* embryo and had no discernable heart development defects (Figure 3.1 A,B, and E), but did have expanded ventricles and expansion of the craniofacial region with a cleft palate (Figure 3.1 D and Figure 3.2 C). Additionally, one of the *Ick^{-/-}* embryos examined had a bifurcated tongue that was reduced in size (Figure 3.2 D). *Ick^{-/-}* embryos also presented with cyst-like tubule expansions within the kidneys and potential changes to intestinal morphology (Figure 3.2 G and H).

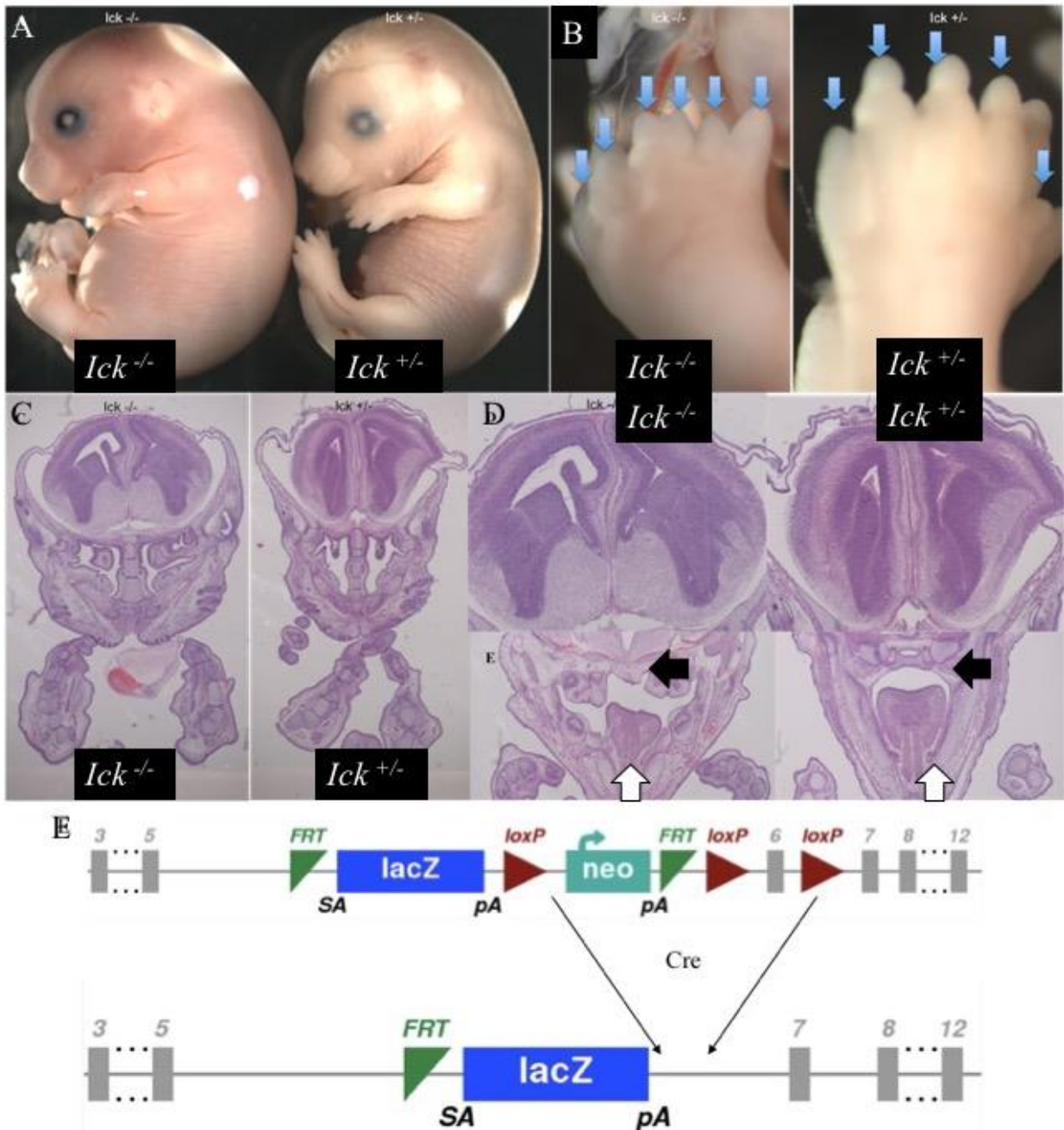


Figure 3.1 General morphology of *Ick*^{tm1b/tm1b} (*Ick*^{-/-}) and *Ick*^{tm1b/+} (*Ick*^{+/-}) E16.5 embryos A)

Ick^{-/-} embryos have general edema, shortened limbs, and open eyelids B) *Ick*^{-/-} embryos have preaxial polydactyly (blue arrows indicate each digit on the hindlimb) C and D) Transverse sections and Hematoxylin and Eosin (H&E) staining demonstrate that *Ick*^{-/-} embryos have dilated ventricles, cleft palate (black arrows), and reduced tongue size (white arrow) E) The

Ick^{tm1a} allele with the gene trap cassette flanking exon 6 of *Ick* (top) and the *Ick^{tm1b}* (bottom) allele after cre-recombination removed exon 6 from the *tm1b* allele of *Ick* from the KOMP Project.

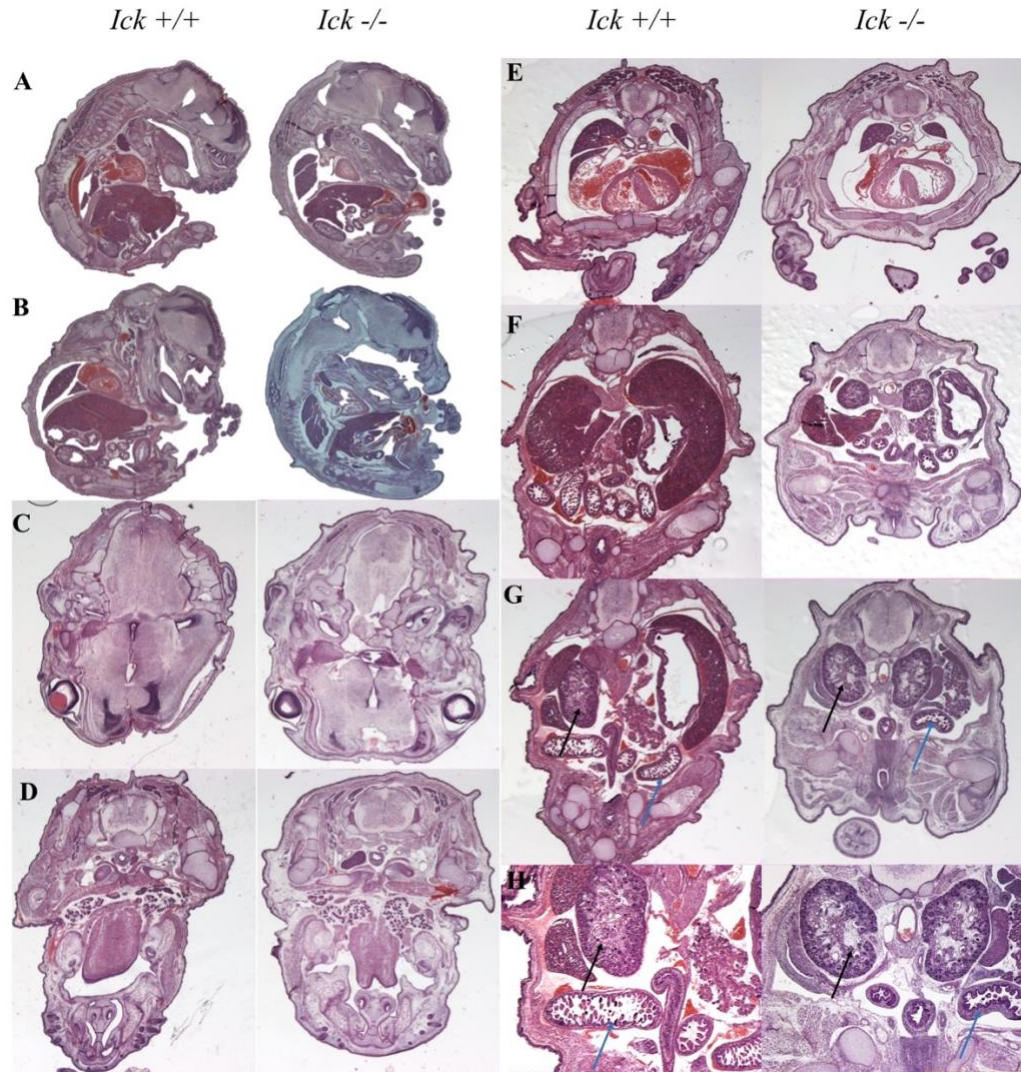


Figure 3.2 Transverse sections of E16.5 with Hematoxylin and Eosin (H&E) Staining of *Ick*^{+/+} and *Ick*^{-/-} Embryos A and B) Sagittal sections of E16.5 *Ick*^{+/+} and *Ick*^{-/-} embryos and demonstrate no discernable heart development defects C and D) *Ick*^{-/-} embryo did have expanded ventricles and expansion of the craniofacial region with a cleft palate and bifurcated tongue E) Transverse section of *Ick*^{-/-} heart demonstrates no phenotype. F,G, and H) *Ick*^{-/-} embryos presented with cyst-like tubule expansions within the kidneys, white spaces within the kidney (black arrows) and potential changes to intestinal morphology with thicker walls present (blue arrows).

We also observed that the *Ick*^{-/-} mutant had eyelids remained open at embryonic stage, E16.5, a time when the eyelids should be close to being fused shut as seen in the *Ick*^{+/+} embryo (Figures 3.1 A and 3.3). An unfused eyelid phenotype was not described in the *Ick*^{tm1a/tm1a} mutant nor in the *Ick*^{-/- Chaya} mutant. In mammalian development, the eyelid grows across the eye and fuses, and is then reopened later in development. Eyelid development begins around E13.0 in the mouse embryo with the formation of two separate sheets of epithelium at the top and bottom of each eye that grow across the eye for several days, and by E17.0 the eyelids are fused shut for the remainder of embryonic development and for around 10 days postnatally (Zhang et al., 2005b). These epithelial sheets are also covered by another cell layer, called the periderm, which originates from the ectoderm, and is involved in temporary fusions throughout the developing embryo including the temporary fusion of the digits (Findlater et al., 1993; Pei and Rhodin, 1970).

We saw no apparent changes to *Ick*^{-/-} eye development beyond the failure of the eyelids to fuse. The *Ick*^{-/-} embryo still developed a lens, optic nerve, and retina. I did not observe any substantial differences between *Ick*^{-/-} and *Ick*^{+/+} retinal thickness at this stage, although there is potential that it could be altered at later stages as observed in Chaya et al 2014. The general edema within the *Ick*^{-/-} embryo may contribute to the inability of the eyelids to fuse, however, sections through the *Ick*^{-/-} eye suggest that the epithelial processes and periderm are not extending to connect and fuse as they do in the *Ick*^{+/+} eye (Figures 3.1A and 3.3). More extensive work would need to be done to determine what is inhibiting eyelid formation in the *Ick*^{-/-} embryos. It could potentially be a result of deficient signaling within the primitive eyelid, including EGFR-ERK signaling which is responsible for F actin polymerization and migration of

the epithelial processes, or TGF α , FGF, and Shh signaling (Harris and McLeod, 1982; Li et al., 2001; Marigo and Tabin, 1996; Mine, 2005).

Ick^{tm1a/tm1a} mutants were shown to have defects in limb patterning which resulted in pre-axial polydactyly. *Ick^{tm1a/tm1a}* patterning differs from wild-type with decreased levels of *Gli1*, *Ptch1*, and *HoxD1* in the posterior region of the E10.5 developing limb bud and ectopic expression of *Gli1*, *Ptch1*, and *HoxD1* in the anterior region of the limb bud. I performed in situ hybridization to detect RNA of *Ptch1*, *Gli1*, and *Gremlin*. One of the functions of the Gremlin protein is to antagonize and restrict the expression of Gli3 repressor to the anterior portion of the limb bud. Gli3 prevents the expression of posterior limb genes within the anterior limb bud (Büscher et al., 1997; Marigo et al., 1996b; Wang et al., 2000). I observed decreased *Ptch1* expression in the posterior limb bud of the *Ick^{-/-}* mutant, although did not observe differences in *Gli1* expression in this experiment, although perhaps a subtle difference would be present if the in situ was developed for less time. I also observed an increased expression and expansion of *Gremlin* within the *Ick^{-/-}* limb bud, suggesting that Gli3 would be unable to inhibit expression of posterior limb genes within the anterior limb resulting in defects in limb development and polydactyly (Figure 3.4). These data suggest that, like the *Ick^{tm1a/tm1a}* allele, *Ick^{-/-}* embryos may have ectopic Hh signaling within the limb bud resulting in extra digits (Moon et al., 2014).

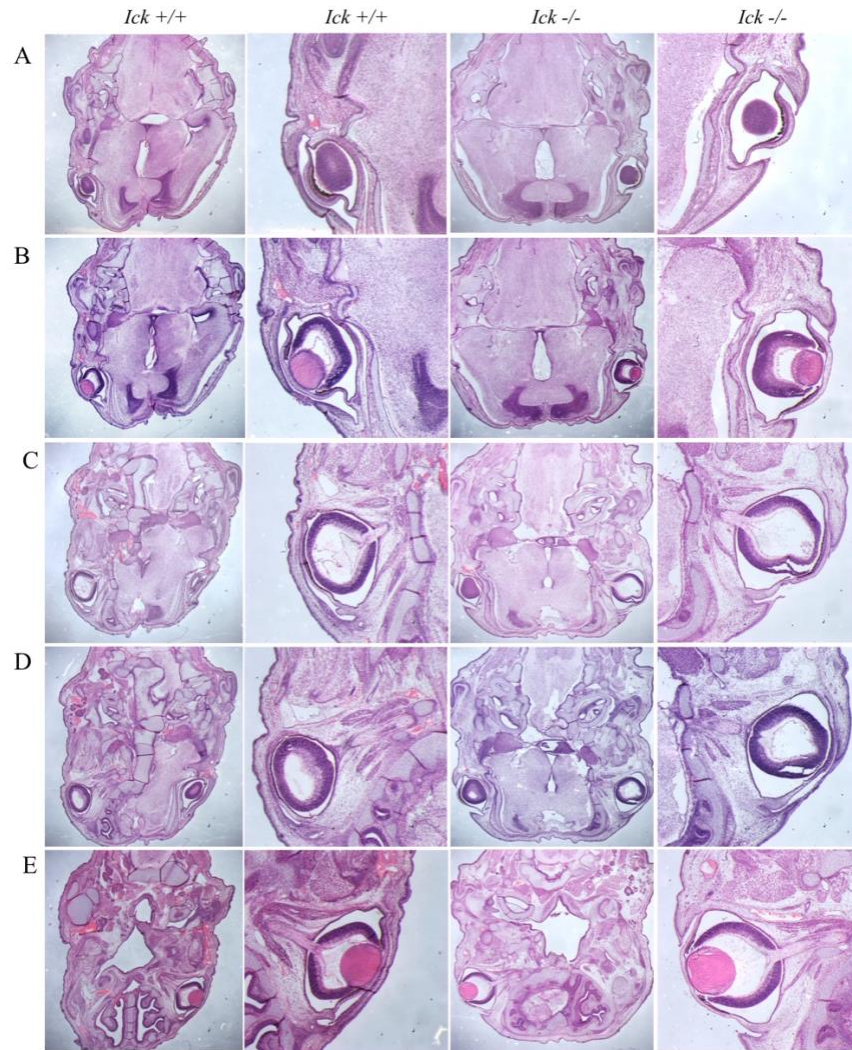


Figure 3.3 Transverse sections through and H&E staining of E16.5 $Ick^{+/+}$ and $Ick^{-/-}$

embryos A) Complete eyelid closure with in the $Ick^{+/+}$ embryo and no eyelid closure in the $Ick^{-/-}$

B) $Ick^{-/-}$ embryos still produce a lens and have the epithelia processes for eyelid closure present,

although they do not meet across theye C-E) $Ick^{-/-}$ embryos produce a normal optic nerve and

have no evidence of decreased retina size throughout the eye.

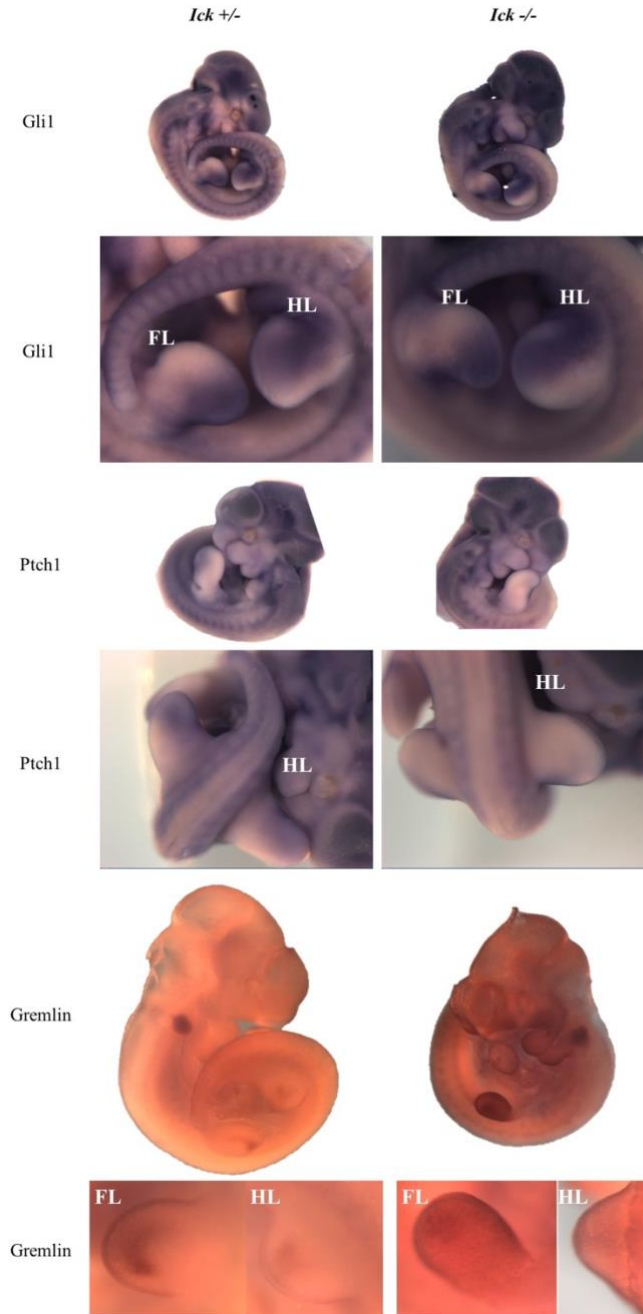


Figure 3.4 *In situ* hybridization to detect *Gli1*, *Ptch1*, and *Gremlin* in *Ick*^{+/+} and *Ick*^{-/-} E10.5

embryos There were no changes in *Gli1* expression between *Ick*^{+/+} and *Ick*^{-/-} within the limb bud. There was a decrease in the level of *Ptch1* expression within the hindlimb bud. *Gremlin* expression was higher throughout the limb bud in the *Ick*^{-/-} embryo and ectopically expressed within the anterior portion of the limb bud of the forelimb bud.

The role of *Ick* in ciliogenesis

Ick is a homolog of the *Chlamydomonas reinhardtii* flagella length regulating gene, long flagella 4 (*lf4*). *Lf4* null mutants have flagella that are longer than wild-type flagella (Berman et al., 2003b; Hilton et al., 2013). *Ick^{tm1a/tm1a}* mutants are also reported to have long cilia within the mutant embryo and cell culture with ShRNA knockdown of *Ick* (Moon et al 2014). Although other reports on an allele of *Ick* with exon 3 removed have indicated that *Ick* ^{-/-} have short cilia (Chaya et al 2014)

To test if our allele of *Ick*, *tm1b*, also produced long cilia, I isolated primary MEFs (pMEFs) from E13.5 *Ick* ^{-/-} and *Ick* ^{+/-} embryos. Cells were grown to confluency and serum starved for 24 hours in 0.5% FBS or 1% FBS to initiate primary cilia production. *Ick* ^{+/+} and *Ick* ^{+/-} pMEFs produced primary cilia, however *Ick* ^{-/-} pMEFs did not produce cilia when isolated and cultured (Figure 3.5). Additionally, *Ick* ^{+/-} pMEFs were able to respond to 200nM SAG induction for 24 hours, but predictably, with the absence of cilia, *Ick* ^{-/-} pMEFs were unable to respond to SAG. I isolated *Ick* pMEFs on two separate occasions, and observed that in both isolations *Ick* ^{-/-} pMEFs did not produce cilia.

To investigate if the tissues used within the *Ick* ^{-/-} E13.5 embryo did not have cilia as well, I examined sections of *Ick* ^{-/-} E13.5 embryos labeled for Arl13b and found the presence of cilia within the tissue isolated for MEFs throughout the trunk (limbs, heart, organs, head, and tail were removed for pMEF isolation). Since the embryos do have Arl13b positive cilia, the inability of *Ick* ^{-/-} pMEFs is most likely a result of the artificial culture conditions, and the interaction between loss of *Ick* and culture conditions was not investigated further. There is the potential that *Ick* ^{-/-} pMEFs are producing cilia that do not localize traditional ciliary components, as seen in the

Ick^{-/-Chaya} MEFs, although this is unlikely since Arl13b positive ciliary were frequently observed within the tissues of the E13.5 embryo used to isolate MEFs.

To further investigate the role of ICK in ciliogenesis in an established cell line, I transfected IMCD-3 IFT88-YFP, cells created by Jonathon Walsh, were co-transfected with plasmids containing hCas9, a gRNA targeting exon 1 of *Ick* (Figure 3.6 A) (Mali et al., 2013), and a plasmid containing the selectable marker puromycin. Individual clones of IMCD-3 cells were picked transfected a second time with the same gRNA and hCas9 and then sequenced. I found that 4 clones out of the 24 sequenced had changes in their *Ick* sequences (Figure 3.6 B). Cilia length was measures by immunostaining cilia with acetylated α -tubulin, Arl13b, and γ -tubulin and measuring from the base of the cilium, labeled by γ -tubulin, to the tip of the cilium, co-labeled by acetylated α -tubulin and Arl13b. The two clones of IMCD-3 IFT88YFP cells with mutations in the first exon of *Ick* measured after being grown for 3 days had longer cilia than IMCD IFT-88YFP wild-type cells (Figure 3.6 C-E).

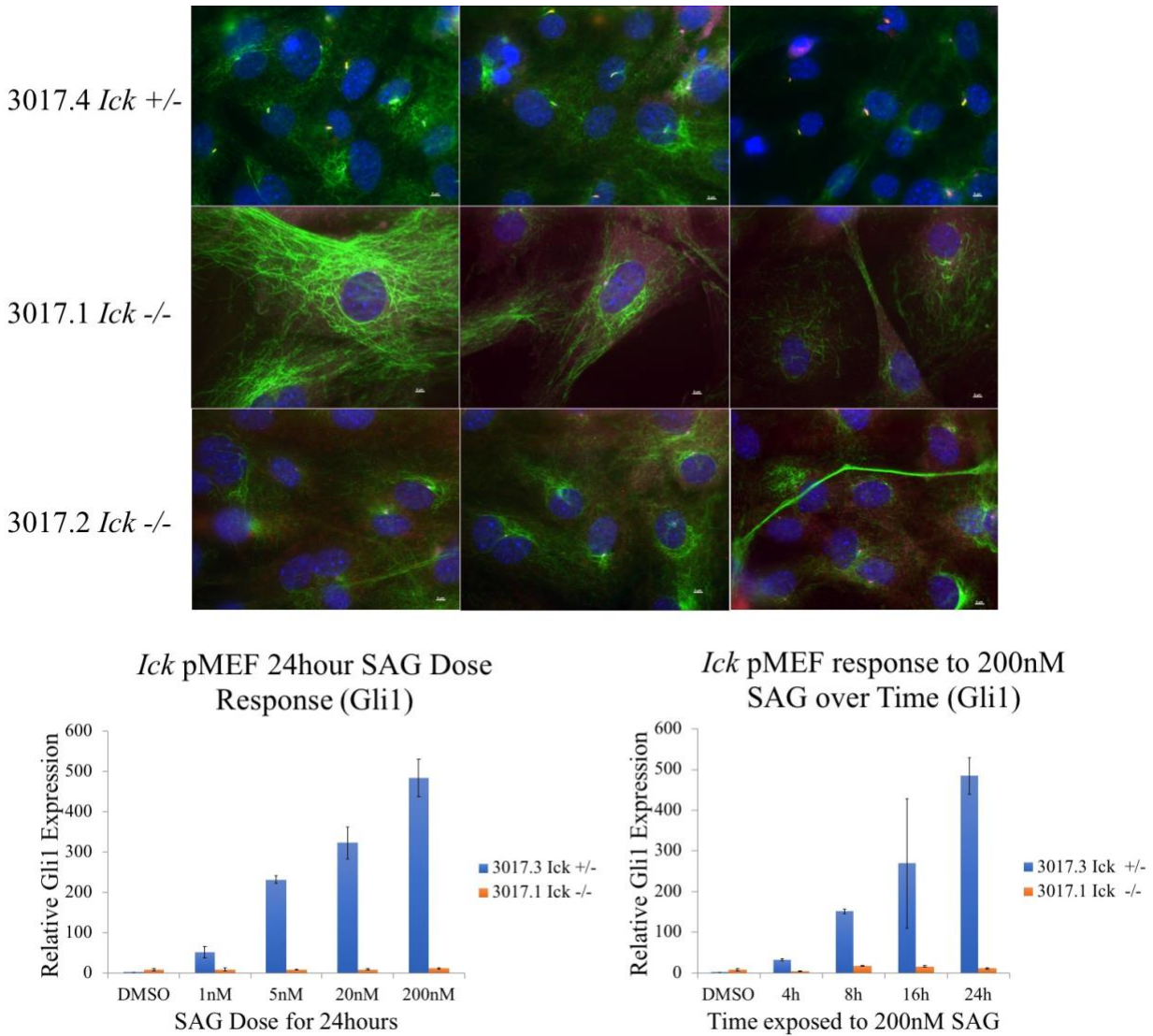


Figure 3.5 *Ick* pMEFs cilia marked by immunofluorescence and SAG response *Ick*^{+/-}

pMEFs produce primary cilia with co-localization of acetylated α -tubulin and (green) and Arl13b (red). Nuclei labeled by DAPI (blue) and the basal body at the base of the cilium is labeled by γ -tubulin (pink), but *Ick*^{-/-} pMEFs do not produce cilia, with no co-localization of acetylated α -tubulin (green) or Arl13b (red), but the basal body is still present (pink). *Ick*^{+/-} pMEFs are response to 200nM SAG after 24 hours of exposure, but *Ick*^{-/-} pMEFs are not.

A

Forward: atgagtgtcccacagagca

Reverse: aggtgattccaactccgtcc

Exon gRNA target

```

ccctgaattggaggtcactcattgtattgtttccaagcatgccatctctagctttataaagacacttattgtattgaaaga
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    *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
65  CTACGGCTCTGCT--TGCTGGGGAGAGCATTGAGTCTGGAGAAGTGAATGCCATTAAAAAGTNAGTG-----1
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    *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
1   ~~~~~1
201  ctgcctatcaaggtgattccaactccgtcctgtccattccctgtctttgaaacgtttctagaggggagtgatggggagcagatgaagaagaa 291
    *      *      *      *      *      *      *      *      *      *      *      *      *      *      *

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    *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
53  CTA-----TGCTGGGGAGAGCATTGAGTCTGGAGAAGTGAATGCCATTAAAAAGTAA-----1
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    *      *      *      *      *      *      *      *      *      *      *      *      *      *      *
1   ~~~~~1
201  ctgcctatcaaggtgattccaactccgtcctgtccattccctgtctttgaaacgtttctagaggggagtgatggggagcagatgaagaagaa 291
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      *      *      *      *      *      *
      *      *      *      *      *      *
70  CTACGGCTCTGTC~TGCTGGGGAGAGCATTGAGTCTGGAGAACTGATTGCCATTAAAAAGTAAGTGTATG~ 1
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      *      *      *      *      *      *
      *      *      *      *      *      *
1   ~~~~~~ 1
201 ctgcctatcaaggtgattccaactccgtcctgtccattccctgtctttgaaacgtttctagaggggagtgaaatgggaggcatgaagaagaa 291
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Thu Jul 14, 2016 11:52 EDT
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      *      *      *      *      *      *
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      |
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      *      *      *      *      *      *
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      |
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      *      *      *      *      *      *
      *      *      *      *      *      *
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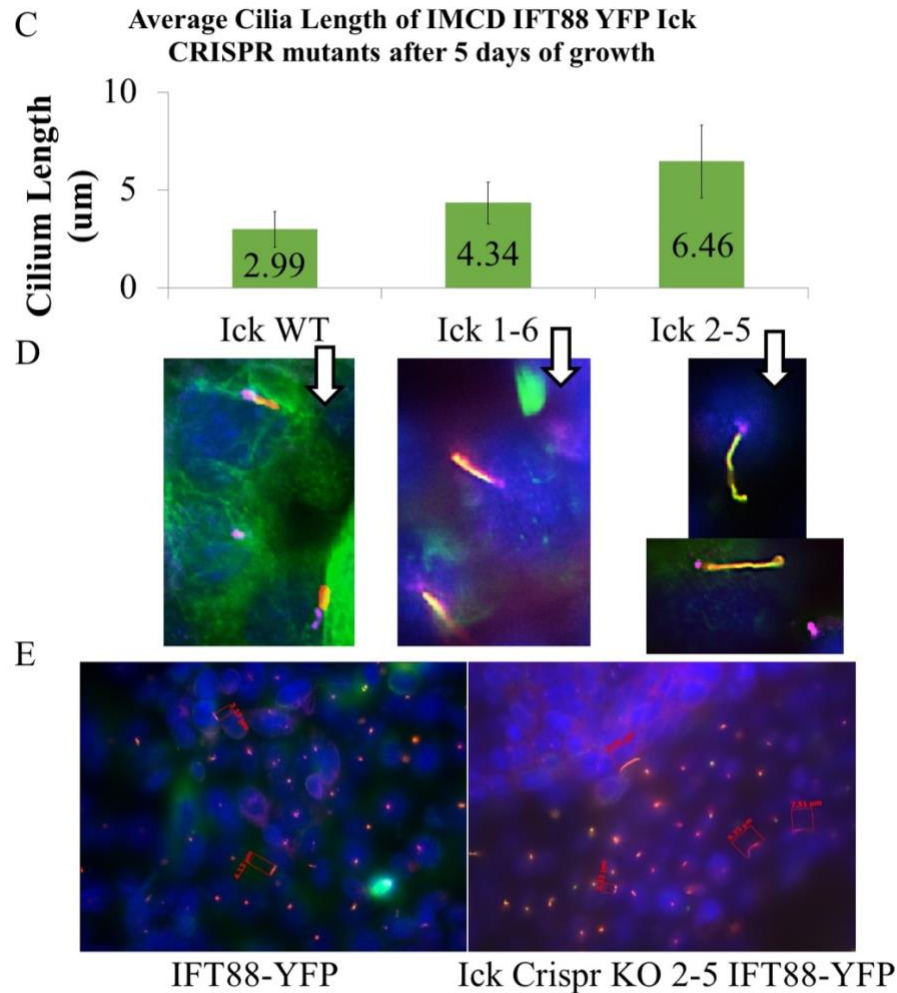


Figure 3.6 CRISPR generated mutation of *Ick* in IMCD-3 IFT88-YFP cells A) IMCD-3 IFT88-YFP, cells created by Jonathon Walsh, were co-transfected with plasmids containing Cas9, a gRNA targeting exon 1 of *Ick* and a plasmid containing the selectable marker puromycin twice. B) 4 clones out of the 24 sequenced had deletions in the *Ick* sequences. C-E) Cilia length was measured by immunostaining cilia with acetylated α -tubulin, Arl13b, and γ -tubulin and measuring from the base of the cilium, labeled by γ -tubulin, to the tip of the cilium, co-labeled by acetylated α -tubulin and Arl13b. The two clones of IMCD-3 IFT88YFP cells with mutations in the first exon of *Ick* measured after being grown for 3 days had longer cilia than IMCD IFT-88YFP wild-type cells

Epistatic interactions between CCRK and ICK *in vivo*

CCRK has been shown to phosphorylate the essential Thr-157 in the T-loop of the TDY motif of ICK *in vivo*. The phosphorylation of ICK by CCRK has been shown to increase ICK kinase activity (Fu et al., 2006a). Additionally, PP5 can dephosphorylate the Thr-157 of Ick to result in deactivation. ICK can also phosphorylate the anti-apoptotic protein, Scythe, *in vitro* and may have functions in regulating cell cycle (Fu et al., 2006a). The *Chlamydomonas reinhardtii* homologs of CCRK and ICK, LF2 and LF4 respectively, have not been shown to interact directly, but both function in the flagella length regulatory pathway (Tam et al., 2003; Tam et al., 2007).

My objective was to start to elucidate the function of the two kinases, CCRK and ICK, in mammalian cilia length regulation within the developing embryo. I first examined *in vivo* cilia length regulation of *Ccrk* and *Ick* single mutant embryos at E10.5. I immuno-labeled cilia within the developing limb buds with acetylated α -tubulin and Arl13b, and found that *Ick*^{-/-} embryos had longer cilia than a wild-type littermate, however, *Ccrk*^{-/-} embryos and wild-type embryos had comparable lengths. Variation between lengths of *Ccrk* and *Ick* single mutants may be a result of different strain backgrounds (*Ccrk*;C3H and *Ick*;B6) (Figure 3.7). Additionally, *Ick* mutants may have reduced cilia numbers within the limb bud mesenchyme, but this observation was not quantified in this experiment (Figure 3.7). *Ccrk* and *Ick* interactions may differ between tissues, and although they are co-expressed in larger portions within the developing E10.5 embryo, including the limb bud (Snouffer et al., 2017; Moon et al., 20014), their interactions may differ based on additional phosphorylation targets within certain tissues.

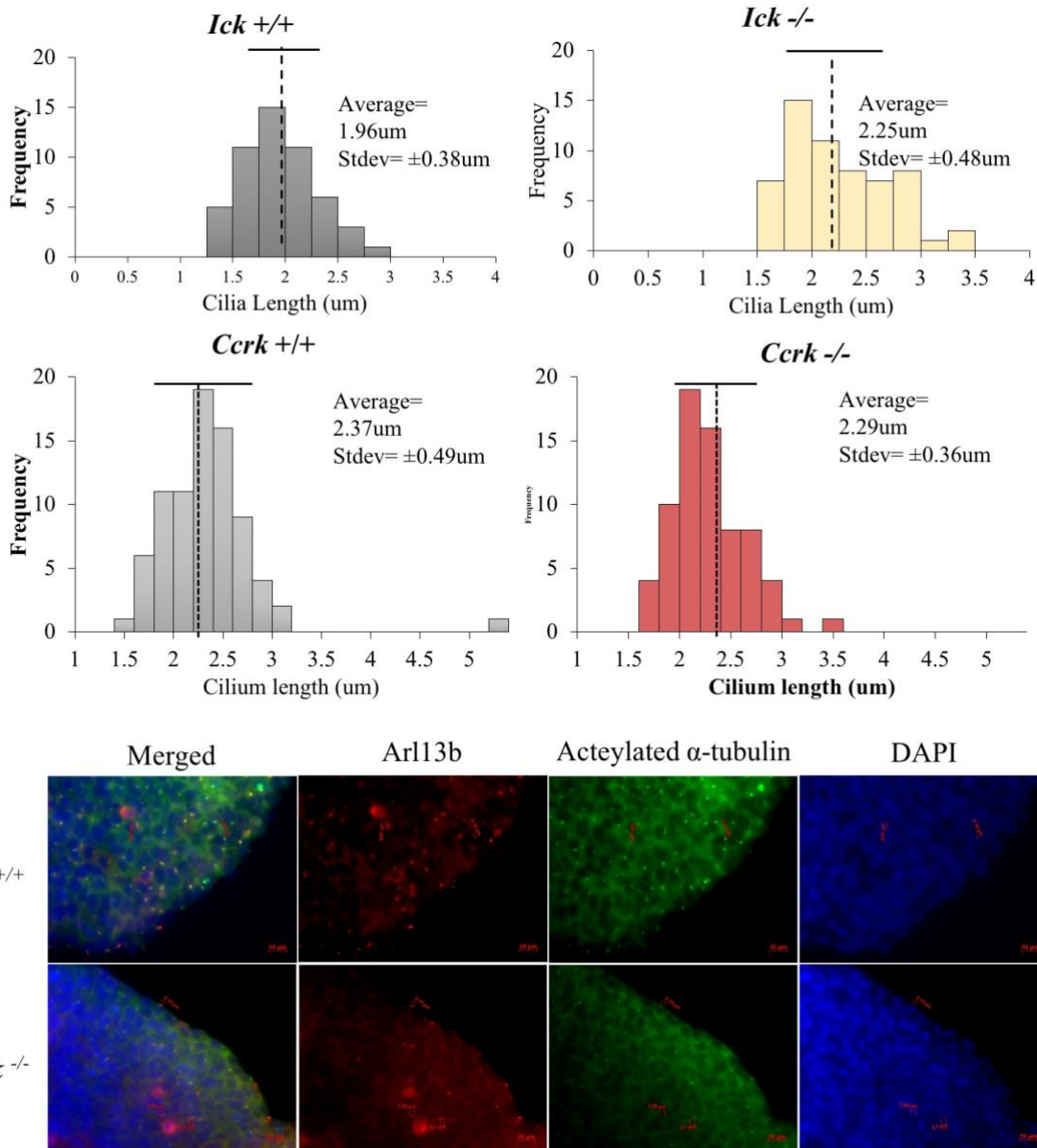


Figure 3.7 Cilia length within the limb bud of E10.5 *Ick*^{-/-} and *Ccrk*^{-/-} embryos

Limb buds were immuno-labeled with acetylated α -tubulin, Arl13b, and DAPI to measure cilia length. *Ick* embryos have slightly longer cilia than wild-type the littermate $P=0.006$ (top) While the *Ccrk* embryo and the wild-type littermate had comparable cilia lengths *Ccrk* $P=0.32$ (bottom). *Ick*^{-/-}, *Ccrk*^{+/+}, and *Ccrk*^{-/-} have comparable lengths within the limb bud potentially due to strain background differences. *Ick*^{-/-} limb buds also appear to have fewer cilia within the limb mesenchyme.

Characterization of *Ccrk/Ick* double mutant embryos

To further understand the interaction between *Ick* and *Ccrk* within the embryo, I generated double mutant embryos by mating male and female double heterozygous (*Ccrk*^{+/-}; *Ick*^{+/-}) mice. I first collected embryos at E13.5 and found 23/109 *Ccrk*^{-/-} single mutant embryos, 30/109 *Ick*^{-/-} single mutant embryos, and 0/109 *Ccrk/Ick*^{-/-} double mutant embryos. The *Ccrk/Ick*^{-/-} double mutant embryos should occur at a frequency of 1 in 16 embryos and within 109 embryos there should have been around 6-7 *Ccrk/Ick*^{-/-} double mutant embryos. This suggested that, although both *Ccrk* and *Ick* single mutant embryos survive until late gestation (Snouffer et al., 2017; Moon et al., 2014), the *Ccrk/Ick*^{-/-} double mutant embryos were not surviving to E13.5.

We decided to collect embryos at the earlier stages of E9.5 and 10.5 to determine if *Ccrk/Ick*^{-/-} embryos can survive to mid-gestation. At E9.5-10.5 I collected 40/155 *Ccrk*^{-/-} single mutant embryos, 32/155 *Ick*^{-/-} single mutant embryos, and 9/155 *Ccrk/Ick*^{-/-} double mutant embryos, which occurred at the predicted frequency of 1 in 16 embryos at these stages. The gross morphology of the *Ccrk/Ick*^{-/-} double mutants resembles that of the *Ccrk*^{-/-} single mutant at E10.5 with exencephaly (Figure 3.8) and has similar neural tube patterning deficiencies to the *Ccrk*^{-/-} litter mates for the neural tube specification markers *Isl1/2* and *Nkx2.2* (Figure 3.9).

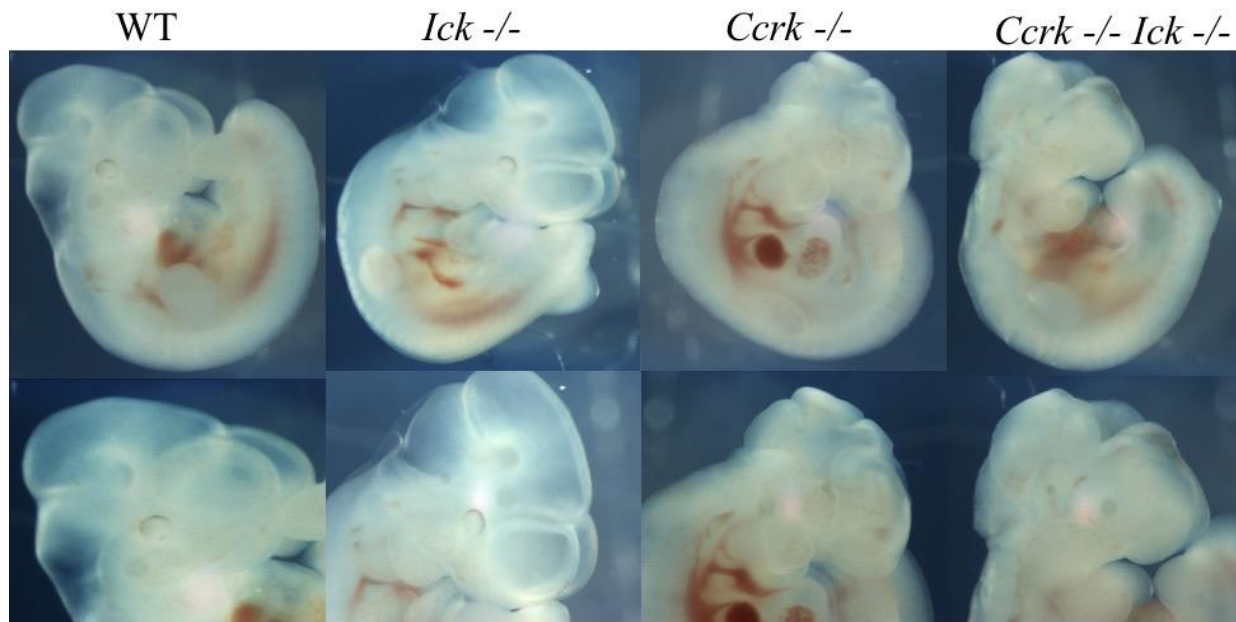


Figure 3.8 Gross morphology of *Ccrk*^{-/-}, *Ick*^{-/-} single mutants and the *Ccrk/Ick*^{-/-} double mutant E10.5 embryos *Ick*^{-/-} embryos resemble wild-type embryos at this stage, while *Ccrk*^{-/-} embryos present with exencephaly. The *Ccrk/Ick*^{-/-} double mutant embryos resemble the gross morphology of the *Ccrk*^{-/-} single mutant embryo and also present with exencephaly. The *Ccrk/Ick*^{-/-} double mutants do appear to have earlier lethality with 0/109 E13.5 embryos being double mutant, while *Ccrk*^{-/-} and *Ick*^{-/-} embryos were present at expected numbers (23/109 and 30/109 respectively). At E9.5-10.5 *Ccrk/Ick*^{-/-} double mutants were recovered at expected numbers (9/155) as well as *Ccrk*^{-/-} (40/155) and *Ick*^{-/-} (32/155) single mutant embryos.

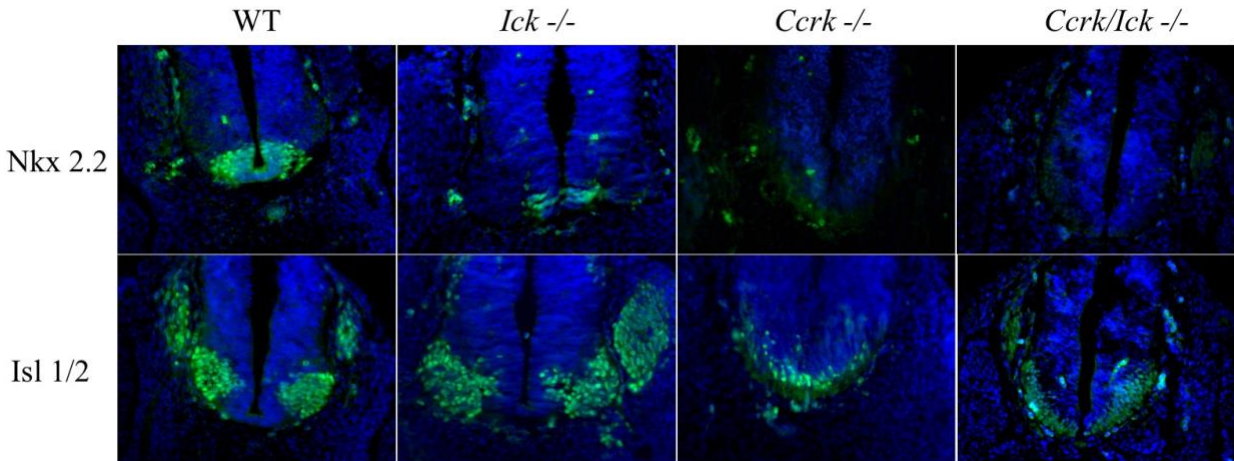


Figure 3.9 Neural tube patterning within E10.5 *Ccrk*^{-/-} *Ick*^{-/-} single mutants and *Ccrk/Ick*^{-/-} double mutants *Ick* mutants are wild-type in neural tube patterning (Nkx2.2 *Ick*^{-/-} neural tube section is off center but patterning is normal). While *Ccrk*^{-/-} and *Ccrk/Ick*^{-/-} double mutant embryos have a dramatic decrease in Nkx2.2 positive cells. Additionally, *Ccrk*^{-/-} and *Ccrk/Ick*^{-/-} double mutant embryos have reduced Isl1/2 positive cells and they are found more ventrally within the neural tube.

Investigation of Heart Morphology within *Ccrk/Ick* double mutant embryos

To investigate the cause of the earlier lethality observed within the *Ccrk/Ick*^{-/-} double mutant embryos, transverse sections stained with H&E were examined for malformations within the heart, a common cause for embryonic lethality around midgestation stages. Heart looping is essential for separation of the heart chambers, and alignment and connectivity of the heart vessels (Kathiriyia and Srivastava, 2000; Ramsdell, 2005). My data suggests that the early lethality observed within the *Ccrk/Ick*^{-/-} double mutant embryos may be the result of failure of heart development due to changes in left-right patterning.

The heart initially starts as a tube around E8.0 where the top of the tube will become the outflow tract of the heart, the bottom the atria and the central part of the tube will form the ventricles. At around E8.5-9.0 the heart tube forms a loop towards the left side of the embryo (*situs solitus*), based on left-right patterning cues previously set up within the embryo, and begins to form the larger left ventricle and smaller right ventricle. At E9.5-10.5 the separate chambers of the heart start to become distinct and form dividing walls (Harrison et al., 2015; Icardo and Sanchez de Vega, 1991; Mercola, 1999; Srivastava and Olson, 2000) (Figure 3.10).

In a *situs inversus* embryo, however, where within the embryo the right side is actually specified as the left side it will reverse the heart looping and will form a heart with the opposite orientation, with the left ventricle and atrium on the right side of the embryo (Figure 3.10). A *situs inversus totalis* (complete reversal of situs) embryo, can survive postnatally and have a relatively unchanged life with all of the organs are completely mirror reversed from the normal left-right position (Levin, 2005). However, if early in development the embryo does not clearly specify either side to be the left or specifies both sides of the embryo to be the left, then the resulting organization of internal organs, including the heart, can be severely disrupted and result

in embryonic death (Kosaki and Casey, 1998). Heart looping and sequential heart development can be complicated by vague specification of the left side (heterotaxy) of the embryo, and can result in the inability for the heart to properly loop and connected the great arteries to the embryonic circulatory system (Harvey, 1998; Mercola, 1999). Additionally, the stomach, liver lobes, spleen, and lungs develop in a disorganized manner within the body cavity, and misplacement and inability to connect organs such as the heart, lungs, and circulatory system properly can result in lethality (Fujinaga, 1997) .

The first break in left-right symmetry occurs in the early embryo, around E8.0 at group of ciliated cells called the node (Conlon et al., 1994; Marszalek et al., 1999; Mcgrath et al., 2003; Yoshida et al., 2012a). A more in-depth review of the molecular signaling underlying left-right patterning within the mouse embryo can be found in Chapter IV. Cilia at the node beat and produce a leftward flow, this flow at the node is essential within the mouse embryo to specify the left side of the embryo (Mcgrath et al., 2003; Nonaka et al., 2002). Mutants which produce no cilia at the node, such as Kif3A and Kif3B knockout mice, end up activating left side genes on both sides of the embryo (bilateral symmetry) (Marszalek et al., 1999; Nonaka et al., 1998; Okada et al., 1999; Supp et al., 1999; Takeda et al., 1999).

Other mutations in cilia can also impact left-right patterning within the embryo, such as the *inversus viscerum* (*iv/iv*) mutants were, instead of bilateral activation of left side genes seen in complete absence of cilia, embryos randomly break symmetry, where some embryos will have left side genes expressed on the left-side (*situs solitus*) while other embryos will have left-side genes expressed on the right side (Brueckner et al., 1989; Burdine and Schier, 2000; Capdevila and Belmonte, 2000; Supp et al., 1997). The *iv/iv* mutation is in the *left-right dynein* gene (*lrd*), these mutants produce cilia at the node, but lack the axonemal dynein that localizes specifically

to node cilia. Without *lrd* the nodal cilia are immotile, resulting in randomized left side specification (Nonaka et al., 2002; Supp et al., 1997; Supp et al., 1999; Tan et al., 2007).

Sections from 3 E10.5 *Ccrk/Ick*^{-/-} double mutants were analyzed for easily discernable changes in heart morphology, such as change in the size or thickness of the heart walls or failure to separate chambers within the developing heart. No obvious changes to heart tissue morphology were observed by us within the sections when compared to the *Ccrk*^{-/-} and *Ick*^{-/-} single mutant and wild-type littermate embryos. However, in the initial analysis, 1 of the 3 double mutant embryos presented with potentially changes in left right patterning within the heart, where the left ventricle was on the right side of the embryo (Figure 3.11). Heart directionality was not assessed at the whole-embryo level for these three embryos, and the other mutants could, potentially, had changes in heart looping that were missed within the sections.

Heart directionality was more closely examined in four more *Ccrk/Ick*^{-/-} double mutant embryos and (4/4) presented with reversed heart symmetry with the heart looping towards the right side of the embryo rather than the left with varying severity. In the most severe case the embryonic heart failed to develop past the heart tube stage, with subtle looping towards the right side of the embryo, although the heart remained able to beat at the time of dissection (Figure 3.12), while 3/4 embryos had well developed hearts that looped to the right (inverted). A low level of reversal of heart looping was observed in *Ccrk*^{-/-} single mutants (4/33), two of which were under/poorly developed compared to litter mates. No changes to heart looping were observed in *Ick*^{-/-} single mutants (0/19) nor *Ccrk*^{+/-} *Ick*^{+/-} double heterozygous embryos (0/28).

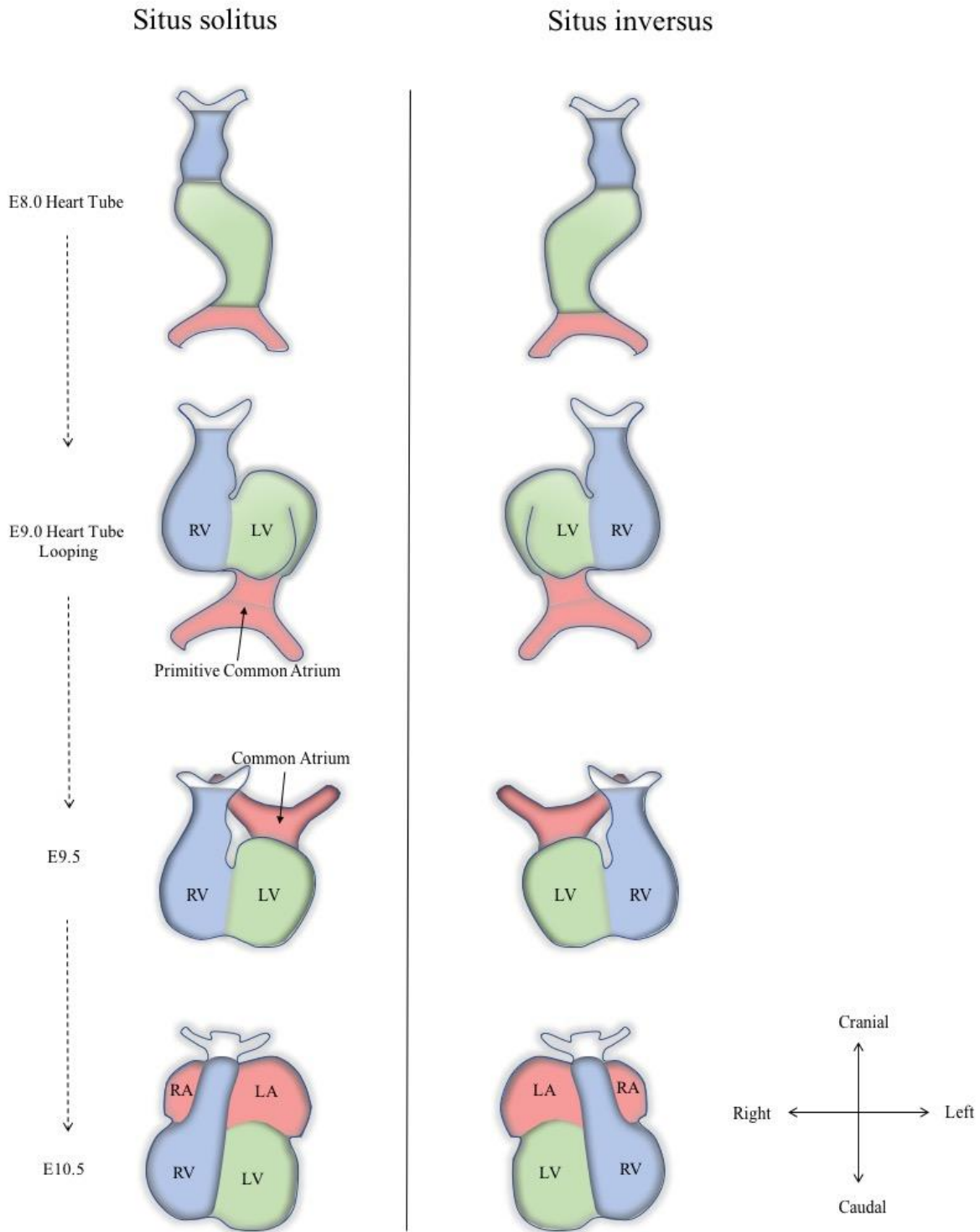


Figure 3.10 Heart morphology and basic development from E8.0 to E10.5 in the embryo

The heart initially starts as a tube around E8.0 where the top of the tube will become the outflow tract of the heart, the bottom the atria and the central part of the tube will form the ventricles. At

around E8.5-9.0 the heart tube forms a loop towards the left side of the embryo (*situs solitus*), based on left-right patterning cues previously set up within the embryo, and begins to form the larger left ventricle and smaller right ventricle. At E9.5-10.5 the separate chambers of the heart start to become distinct and form dividing walls. In a *situs inversus* embryo, however, where within the embryo the right side is actually specified as the left side it will reverse the heart looping and will form a heart with the opposite orientation, with the left ventricle and atrium on the right side of the embryo.

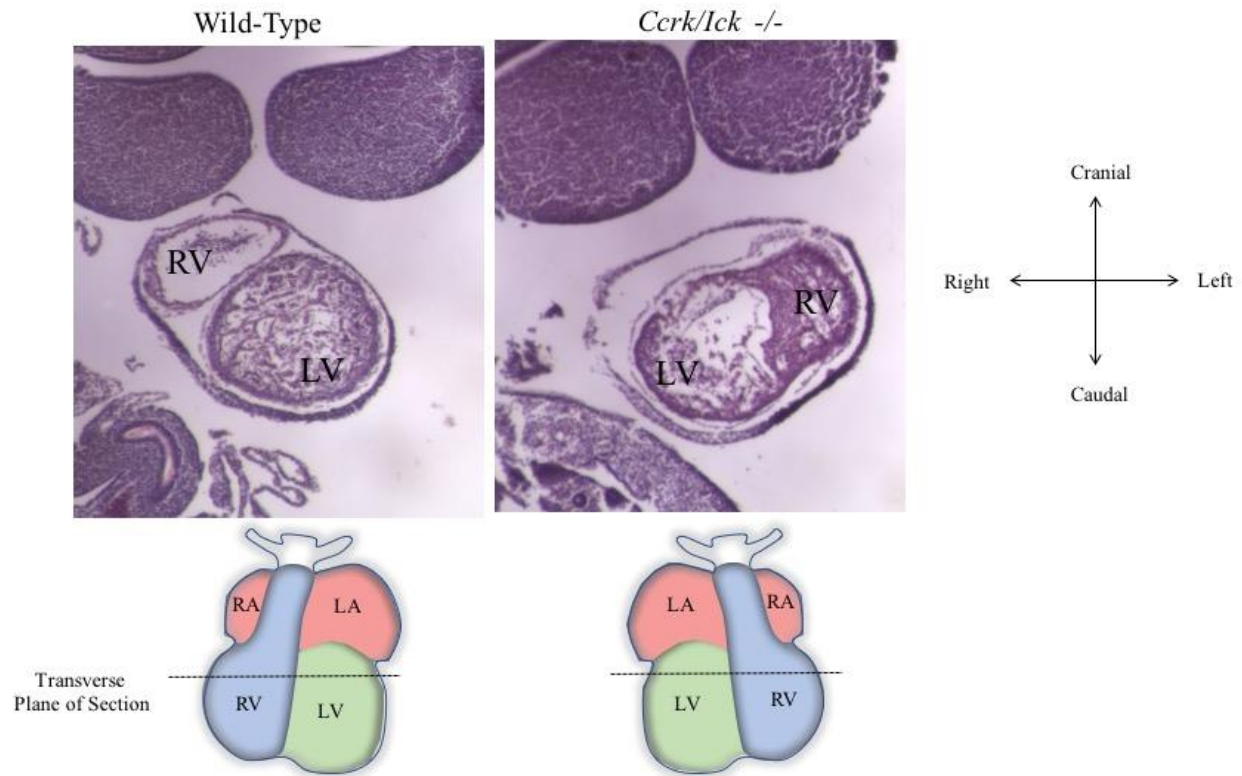


Figure 3.11 H&E staining of transverse sections through a Wild-type heart and *Ccrk/Ick* ^{-/-} double mutant heart The left ventricle of the *Ccrk/Ick* ^{-/-} double mutant is on the right side of the embryo, suggesting that the embryo has reversed heart looping. One out of three double mutant embryos analyzed through sections had reversal of heart symmetry while none of the single mutants presented with changes in heart looping (0/3 *Ccrk* ^{-/-} and 0/3 *Ick* ^{-/-}).

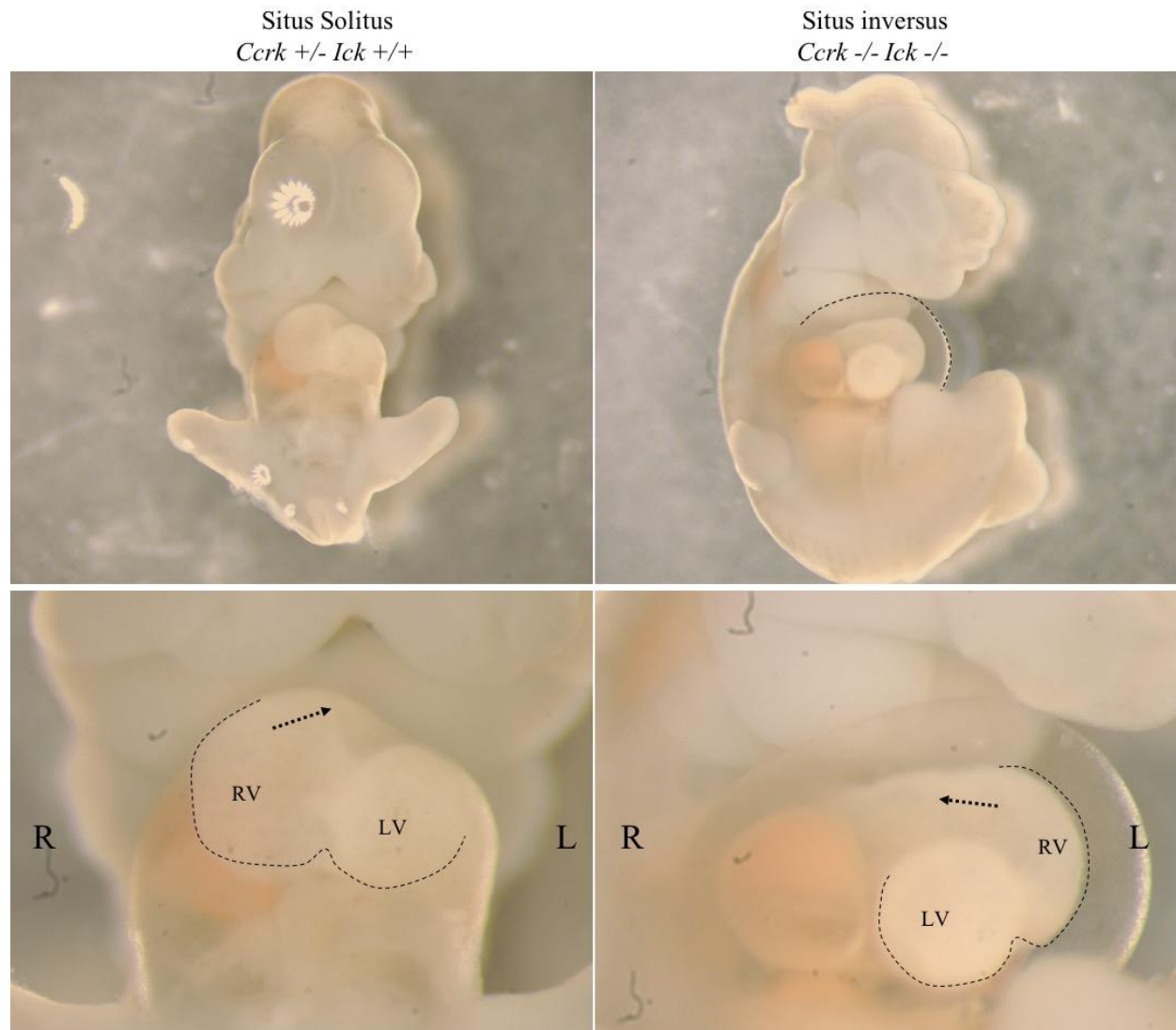


Figure 3.12 Whole mount images of heart looping in wild-type and *Ccrk/Ick* double mutant E10.5 embryos Double mutant *Ccrk/Ick*^{-/-} embryos have heart edema, outlined in top right panel, and reversed heart looping (4/4 embryos). The dotted arrows represent the direction of the outflow track which connects to the right ventricle. 0/19 *Ick*^{-/-} single mutant embryos had defects in heart looping. 4/33 *Ccrk*^{-/-} single mutant embryos had defects in heart looping and 0/28 double heterozygous embryos had defects in heart looping.

Discussion

Ick^{tm1b/tm1b} (*Ick*^{-/-}) resembles gross morphology of *Ick^{tm1a/tm1a}* and *Ick*^{-/- Chaya} alleles with general edema, shortened limbs, dilated ventricles, reduced tongue size, cyst-like structures within the kidney, and potential changes to intestinal morphology. As in the other *Ick* alleles, defects in Hh patterning are likely part of the underlying cause for phenotypes observed within the *Ick*^{-/-} embryos, with reduced but ectopic expression of Hh target genes in the limb bud (Moon et al., 2014). We found an additional phenotype that was not reported in either of the other mutants where the *Ick*^{-/-} embryo failed to close the eyelid.

Ick^{-/-} embryos produce cilia throughout the embryo, and I found that *Ick*^{-/-} embryos have long cilia present within the limb bud, as seen in Moon et al., 2014. *Ick* may also have reduced number of cilia within the limb bud, similar to the phenotype seen in the brain of the *Ick*^{-/- Chaya} allele, although this phenotype was not quantified. However, when pMEFs were isolated from the *Ick*^{-/-} embryo the cells would not induce ciliogenesis in serum starvation conditions, while *Ick*^{+/-} and *Ick*^{+/+} pMEFs isolated from littermates did produce cilia. *Ick*^{-/-} IMCD-3 IFT88-YFP cells were created using CRISPR to target a region within the first coding exon of *Ick*. IMCD-3 IFT88-YFP cells with homozygous mutations in *Ick* produced long cilia, replicating the phenotype observed by Moon et al., 2014.

I generated *Ccrk/Ick*^{-/-} double mutant embryos to further understand the in vivo relationship between CCRK and ICK. I found that the *Ccrk/Ick*^{-/-} double mutant embryos did not survive to the later stages of embryogenesis as the *Ccrk*^{-/-} and *Ick*^{-/-} single mutants do, with 0/109 E13.5 embryos being *Ccrk/Ick*^{-/-} while *Ccrk*^{-/-} and *Ick*^{-/-} single mutants present at expected numbers. The *Ccrk/Ick*^{-/-} embryos do survive until E10.5 and have the gross morphology of a *Ccrk*^{-/-} single mutant, with exencephaly and defects in neural patterning, suggesting that *Ccrk* is

indeed epistatic to *Ick*. However, the *Ccrk/Ick*^{-/-} double mutants presented with heart defects that were not observed in either of the single mutants, where double mutant hearts were incompletely looped and underdeveloped or were looped the opposite direction from a wild-type heart. The earlier lethality observed within the *Ccrk/Ick*^{-/-} double mutants could be a result of the defects in heart looping observed as a result of disrupted left-right patterning. These data suggest that CCRK and ICK may have a more complex relationship than the simple direct phosphorylation of ICK by CCRK within tissues involved in left-right patterning and potentially other tissues not yet examined.

Several models as to how CCRK and ICK interact can be entertained. The first model is where CCRK directs all ciliary length regulation functions through ICK, both shortening and lengthening. At first glance, the phenotype of the single mutants does not seem to support this model, since *Ick* mutants are less severe and do not phenocopy the *Ccrk* single mutant embryo. However, that is assuming that phosphorylation of ICK by CCRK simply determines activity or inactivity of ICK, but it remains possible that CCRK phosphorylation of ICK could in fact change the function of ICK rather than turning it on or off. In the absence of CCRK, ICK may have other ciliary or non-ciliary functions, but in the presence of CCRK, ICK is then directed to function in ciliary length regulation, and may be involved in shortening or lengthening in different context, supporting the different phenotypes seen in *Ick* mutants (Chaya et al., 2014; Moon et al., 2014). If this first model, where ICK's function is changed by phosphorylation by CCRK rather than activated by CCRK then an embryo where ICK is un-phosphorylated by CCRK (a *Ccrk*^{-/-} embryo) would not be equivalent to complete loss of ICK function (an *Ick*^{-/-} embryo), and the function of the un-phosphorylated ICK in a *Ccrk*^{-/-} mutant may result in the more severe phenotype than observed when all of ICK's activity is lost in an *Ick*^{-/-} single mutant.

In this case, it would mean that a double mutant of *Ccrk/Ick*^{-/-} would actually resemble an *Ick* single mutant rather than a *Ccrk* mutant, and double mutants observed did resemble a *Ccrk* single mutant in gross morphology and initial analysis of neural tube patterning. It does remain possible, although unlikely, that ICK may have separate functions when it is not phosphorylated by CCRK, but phosphorylation of the T157A residue by CCRK does seem crucial for activation of ICK kinase function (Broekhuis et al., 2014; Chen et al., 2013; Fu et al., 2006b)

A second model of how CCRK and ICK interact may be where CCRK has multiple targets involved in cilia length regulation where CCRK regulates shortening through phosphorylation of ICK, and CCRK regulates lengthening through other potential targets including MyosinIIa and Map4 may regulate cilia length through altering the cytoskeleton (Personal communication with Jonathon Walsh). This model would support the experimental data where a *Ccrk/Ick*^{-/-} double mutant seems to phenocopy the *Ccrk* single mutant embryo in terms of neural tube patterning and gross morphology but does not fully account for the increased defects in heart looping and earlier lethality observed in the *Ccrk/Ick*^{-/-} double mutant embryos, suggesting a potentially more complex relationship between CCRK and ICK.

A third model is a modification of the second model, where CCRK does phosphorylate ICK, while also having some independent targets, but in this model ICK also has targets which are independent of CCRK phosphorylation. In this case, loss of *Ccrk* results in loss of both the CCRK dependent targets and the targets that require CCRK to phosphorylate ICK, but the ICK independent targets are still able to function. Then loss of *Ick* would result in loss of both the ICK dependent targets and the targets that require CCRK's phosphorylation of ICK. However, in the *Ccrk/Ick*^{-/-} double mutant, all three types of targets would be lost, which could result in a

more severe or synergistic affect within the embryo and lead to defects in heart looping and earlier lethality. The three models have been summarized in Figure 3.13.

The data I have generated by creating *Ccrk* and *Ick* double mutant embryos seems to suggest that *Ccrk* and *Ick* have a complex epistatic relationship in mammals, and the relationship between these two kinases seems to be represented best by the third model based on current experimental data. The loss of both kinases seems to have a synergistic affect, where the double mutant presents with earlier lethality and more frequent reversal of heart looping. The double mutant phenotype also seems to suggest that *Ccrk* and *Ick* may have some redundancy or a feedback loop between their interactions in certain tissues, potentially in the node to impact left-right patterning in a synergistic way. Furthermore, the double mutant phenotype also suggests that the kinases have independent functions in other tissues, such as the neural tube where patterning is not more severely disrupted than in a *Ccrk* mutant and suggests that *Ick* maintains some function even in the absence of *Ccrk*, because otherwise the *Ccrk/Ick* double mutant would resemble the phenotype of the *Ccrk* mutant. In addition to the differences in the single mutant phenotypes, the phenotype of the double mutant embryos suggests that *Ccrk* does indeed function through multiple targets besides *Ick* because if all of the functions of *Ccrk* were mediated through *Ick* then the double mutant embryo would resemble an *Ick* single mutant embryo.

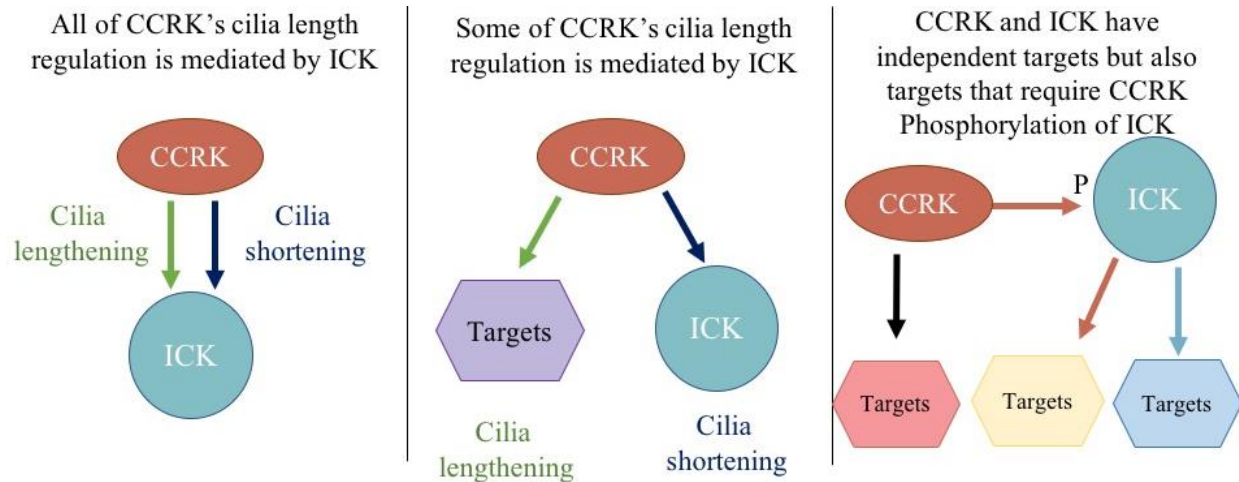


Figure 3.13 Models for the interactions between CCRK and ICK Left to right- Model 1:

CCRK may regulate all of its ciliary length regulation through ICK, while ICK may have separate functions when it is un-phosphorylated and phosphorylated by CCRK. Model 2: CCRK mediates some of its ciliary length functions through ICK but also has other targets that also regulate ciliary length. Model 3: CCRK has specific targets that require CCRK's phosphorylation of ICK but CCRK and ICK also maintain independent targets

Materials and Methods

Embryo processing

Embryos were fixed in 4% PFA overnight and washed with 1X PBS and dehydrated to be stored in EtOH for sectioning or MeOH for in situ or immediately processed for a given application. See Chapter II.

Paraffin sectioning

After fixation, embryos were prepared for paraffin sectioning by placing them in xylene for 30min (E10.5) and 2-5 hours (E13.5) until the embryo had cleared. Embryos were submerged three times for 30 minutes (E10.5) or 2 hours (E13.5) in 65 C paraffin wax. Embryos were then sectioned and slides were left to dry overnight.

Hematoxylin & Eosin Staining

Paraffin sectioned slides were submerged in xylene for 15 minutes to remove wax from the tissue. Slides were then rehydrated in sequential 5 minute washes of EtOH (100%, 90%, 75%, 50%, 25%). Slides were then washed in water twice for 5 minutes. Slides were then submerged in hematoxylin to stain the nuclei for 5-10 minutes. Slides were washed with tap water until the water ran clear and slides were submerged in water until stain turned purple (2-3 minutes). Slides were then submerged in eosin to stain the cytoplasm of the cells within the tissue for 5-10 minutes. Slides were washed with tap water until it ran clear and then quickly and sequentially dehydrated in EtOH (25%, 50%, 75%, 90%, 100%). Slides were then submerged in 2 washes of xylene for 10 minutes and

then coverslips were mounted on the slides with Cytoseal (xylene based mounting media).

In situ hybridization

In situs on E10.5 were done following a standard protocol (Ko et al., 2009). 1ul of each Gremlin (Pearce et al., 1999), Ptch1(Goodrich et al., 1996), and Gli1(Hui et al., 1994) probes was used.

Isolation of pMEFs

E13.5 embryos were dissected into sterile 1X PBS. Embryos were decapitated, eviscerated, and the limbs, tail, and genitals were removed. The carcass was placed in 2.5% Trypsin EDTA for 5 minutes at 37 C. The trypsinized carcass was then pipetted up and down gently and warm MEF media with 15% FBS was added to inactivate the trypsin. Cells were then spun down at 150 rcf and then media was aspirated. Cells were re-suspended in fresh MEF media and plated onto a 10cm tissue culture treated plate.

Cilia length measurement

Method and antibodies used detailed in Chapter II

CRISPR mutation in IMCD-3 cells

General protocol from Mali et al., 2013. A 20 nucleotide CRISPR target was identified in the first exon of Ick with zero off targets off by 1 and 2 nucleotides and six off by 3 nucleotides using crispr.mit.edu created by the Zhang lab at MIT, 2017.

Target sequence and guide RNA scaffold

```
TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTA
CCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCAT
ATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACA
CAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTA
GTTTGCAGTTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAA
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ACC GACCTACGGCTCTGTCCTGC GTTTTAGAGCTAGAAATAGCAAGTTAAAA
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TCTAGACCCAGCTTTCTTGTACAAAGTTGGCATT
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PCR protocol to amplify gRNA (primers from Mali et al., 2013) construct above was run using the following steps 95C for 10seconds [95C for 30seconds, 45C for 30seconds, 72C for 30seconds] cycle for 35X then 72C for 3min. DNA clean and Concentrate (Omega) kit was used to concentrate PCR product.

IMCD cells were grown to 60% confluency and then transfected using Lipofectamine 3000 (Invitrogen). Cells were placed in antibiotic and serum free media (UltraMEM) and then 4ug gRNA, 4ug Cas9, and 0.5ug Puromycin construct were added to 9ul Plus reagent and 70ul Lipofectamine were mixed in 1ml of Ultra MEM. The mixture was allowed to sit for 20min and was then added to the 10cm plate of IMCD cells.

After 24 hours IMCD cells were selected for transfection with puromycin (100ng/ul) for 5 days (until the control plate of cells was killed off by the puromycin). Individual clones of IMCD cells were picked and grown in a 24 well plate.

To analyze potential CRISPR mutant clones primers flanking the first exon were designed and each clone was amplified and then sequenced with the reverse primer to detect any mutations. Sequence were analyzed using ApE software

Forward: **ATGAGTGTCCCGACAGAGCA**

Reverse: **GGACGGAGTTGGAATCACCT**

203bp product

Exon gRNA target

ttgggagataatcacgttctgtgaataacctgtgctggtgctgccatcgaaaaatctggttacagtctggggaggcctgctaccatt
gcaggactgaaccgcctcggccctgagatgagtggtcccgacagagcaggcgacgcaccatgaatagatacacaacgatcaa
gcagcttggggatgggacctacggctctgtcctgctggggagaagcattgagctctggagaactgattgccattaaaaagtaagt
gatgcttgacttcttgaaaattcacagtcgcctctgcctatcaagggtgattccaactccgtcctgtccattccctgtctttgaaacgttt
ctagaggggagtgaaatgggagcatg

Neural tube staining

Embryos were paraffin sectioned (8µm) and sections at the forelimb level were selected for staining. Antigen retrieval was done using sodium citrate buffer and heated for 30minutes before the addition of primary antibodies. Sections were blocked in 10% Heat inactivated Goat Serum, and then Antibodies Isl1/2 (mouse 1:10) and Nkx2.2 (mouse 1:10) were added overnight at 4C. Anti-mouse 488 secondary (Alexaflour) antibody and DAPI were added for 1 hour at room temperature. Images were taken on Zeiss Axioplan 2 microscope with a 20x objective and analyzed using AxioVision4.6 (Ko et al., 2009).

Hh induction and qRT-PCR analysis of Hedgehog target genes

Methods detailed in Chapter I.

CHAPTER IV

INVESTIGATION OF RAB23 MEDIATED TRAFFICKING OF NODAL

Introduction

Establishment of left-right asymmetry

Within the early developing mouse embryo (E7.5) there is a group of cells on the toward the posterior end and on the ventral side of the embryo called the node (Figure 4.1) (Shiratori and Hamada, 2006). The node is the location of the initial break in symmetry within the embryo, and each cell within the node produces a primary cilium (Nonaka et al., 1998). Unlike other primary cilia, the nodal cilia rotate in a clockwise direction, and are tilted towards the posterior side of the embryo (Hirokawa et al., 2006; Shiratori and Hamada, 2006). The posterior positioning of the cilia at the node results in an elliptical rotation pattern where the segment of rotation towards the left side is much stronger than toward the right side. This bias in rotational strength is thought to result in fluid flow towards the left side of the embryo (Cartwright et al., 2004; Cartwright et al., 2008; Nonaka et al., 2005; Okada et al., 2005).

Nodal flow is essential for the initial break in symmetry and specification of the left-side of the embryo, and mutations that impact production, positioning, or movement of the nodal cilia can disrupt the nodal flow (Okada and Hirokawa, 2009; Okada et al., 1999). Mutations that result in a lack of directional flow can result in randomized specification of the left side, as seen in the *inversus viscerum* (*iv/iv*) mutant, which produces immotile cilia at the node due to loss of the left-right dynein required in the ciliary axoneme of node cells specifically (Supp et al., 1997; Supp et al., 1999). A mutation in an intraflagellar transport protein, *Ift122*, can also result in

bilateral left-sidedness (Figure 4.1) (García-García et al., 2005; Huangfu and Anderson, 2005; Huangfu et al., 2003; Marszalek et al., 1999; Murcia et al., 2000; Nonaka et al., 1998). The fluid flow produced at the node is required for left-right determination in the mouse embryo because it results in asymmetrical expression of the secreted TGF- β family member, *Nodal* within the left LPM. However, the mechanism by which the fluid flow results in asymmetric gene expression is not fully understood (Shiratori and Hamada, 2006). One model proposes that a left-side determining molecule is produced in the node and then transported based on nodal flow to the left side of the embryo and generates asymmetric gene expression (Nonaka et al., 1998; Okada et al., 1999; Okada et al., 2005). The determining molecule and the responding element have not yet been identified.

It has also been proposed that the node contains two different types of cilia, the motile cilia that result in fluid flow and non-motile cilia that respond to the mechanical stress of the flow by bending on the left side of the node. The bending of the cilia on the left side of the node may result in asymmetric Ca^{2+} signaling on the left side of the node (Mcgrath et al., 2003; Okada et al., 2005; Tanaka et al., 2005). Additionally, mice mutant for the cation channel *Polycystin 2* (*Pkd2*) gene have normal motile cilia, but fail to express *Nodal* within the left LPM and lack asymmetric expression of Ca^{2+} signaling seen in wild-type mice (Mcgrath et al., 2003; Pennekamp et al., 2002; Tanaka et al., 2007). In support of the idea of mechanical flow inducing Ca^{2+} signaling, it was found that *Pkd2* is required for kidney epithelial cells to respond to mechanical flow (Field et al., 2011; Grimes et al., 2016; Nauli and Zhou, 2004; Nauli et al., 2003). Although the subcellular localization of *Pkd2* is debated it may act as a mechanosensory mechanism within some of the cilia of the node (Mcgrath et al., 2003; Tabin and Vogan, 2003; Yoshida et al., 2012b).

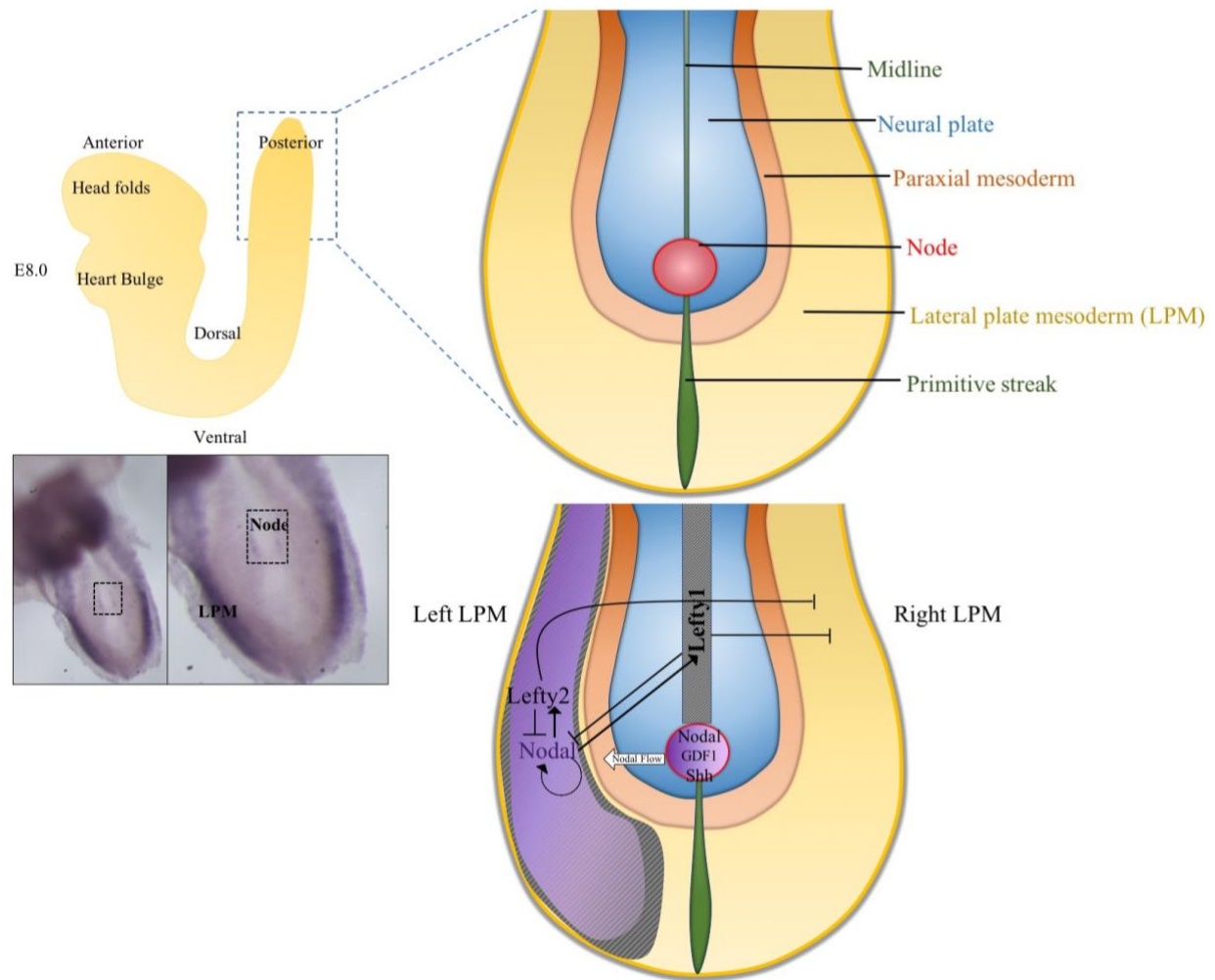


Figure 4.1 Genetic pathway for signaling within the embryonic node and the lateral plate mesoderm (LPM) View from the ventral side of the posterior embryo including only the major components of L/R patterning. *Nodal* is initially expressed in the node and *Nodal* induces expression of *Lefty1* in the midline and *Lefty2* in the left LPM. *Lefty1* and *Lefty2* restrict *Nodal* expression to the left LPM. *Nodal* induces more nodal expression through a positive feedback loop. The *in situ* hybridization for *Nodal* RNA on an E8.0 embryo (*Ift122*^{-/-}) on the left side is an example of a cilia mutant with bilateral nodal expression rather than left-side only.

Nodal Expression in the Lateral Plate Mesoderm

Nodal expression within the node is essential for *Nodal* expression within the LPM (Brennan et al., 2002; Saijoh et al., 2003). However, how *Nodal* expression is relayed from the node to the LPM is still unsure. In chick embryos, Shh in the node activates expression of *Caronte*, a bone morphogenetic protein (BMP) inhibitor, in the paraxial mesoderm which then induces *Nodal* expression in the left LPM (Esteban et al., 1999; Yokouchi et al., 1999). In the mouse embryo, in the absence of BMP effectors (Smad1 and Smad5) *Nodal* is expressed bilaterally in the LPM, suggesting in mouse L/R patterning BMP signaling is also being inhibited from a yet identified negative regulator to prevent bilateral expression of *Nodal* (Chang et al., 2000; Mine et al., 2008; Smith et al., 2011).

Alternatively, in the mouse, it may be that *Nodal* production by the node is able to travel into the LPM and induces its own expression within the LPM. Ectopic *Nodal* has been demonstrated to induce endogenous *Nodal* within the LPM (Kawasumi et al., 2011; Norris and Robertson, 1999; Saijoh et al., 2000; Yamamoto, 2003). Although movement of *Nodal* protein in vivo has not been detected, overexpression of tagged *Nodal* is able to travel over several cell diameters in the embryo and could potentially travel from the node to the LPM (Chen and Schier, 2002; Meno et al., 2001; Tanaka et al., 2007; Yamamoto, 2003). *Gdf1*, a TGF- β family member, is expressed in the node and the LPM that is responsive to *Nodal*. and may interact with *Nodal* to promote *Nodal* transport over longer distances (Rankin et al., 2000; Tanaka et al., 2007).

Nodal production in the LPM induces *Lefty2* expression in the LPM, and *Nodal* is thought to also travel to the midline where it induces *Lefty1* expression. Both *Lefty1* and *Lefty2* are TGF- β family members, and inhibit *Nodal* signaling to limit *Nodal* expression to the left

LPM and to further prevent ectopic expression of Nodal within the right LPM (Meno et al., 2001; Nakamura and Hamada, 2012; Yamamoto, 2003). Lefty1 has been suggested to inhibit Nodal by binding to Nodal directly to prevent receptor activation and by binding additional proteins to prevent the formation of functional receptor complexes (Meno et al., 1996; Saijoh et al., 2000; Smith et al., 2011; Yamamoto, 2003).

Hh signaling in L/R patterning

Sonic hedgehog (Shh) has been proposed to be the left side determinate molecule based on the instructive role of the asymmetrical expression of *Shh* at the node of avian species (Levin, 2005; Tanaka et al., 2005). However, in mammals, *Shh* is symmetrically expressed, and though it seems to have a permissive role in left-right determination, it does not fit the requirements to be the left-side determining molecule. *Shh* is expressed in the midline of the developing embryo, and initially Shh null embryos are able to initiate left side gene expression properly and express Nodal on the left-side. However, because *Shh* null embryos have midline abnormalities, Nodal expression cannot be restricted to the left-side and the Shh null embryos end up with bilateral expression of Nodal (Zhang et al., 2001). The L/R patterning defects in the Shh null embryo are thought to be a result of the lack of Gdf1 expression, a target of Hh signaling and essential for Nodal activation in the LPM (Rankin et al., 2000; Zhang et al., 2001).

Shh is required for L/R patterning because it is essential for formation of a functional midline and node, but does not seem to directly determine the left side of the embryo. The establishment of L/R asymmetries by Nodal allows for L/R patterning within individual organs such as the heart, stomach, spleen, lungs, and liver. The mechanism underlying asymmetric

organogenesis for each organ is not well established, but is dependent on the initial break in symmetry in the early embryo (Shiratori and Hamada, 2006).

Rab23 in Hh signaling and Cilia

Rab23 is a small GTPase and a member of the Rab family and Ras small GTPases superfamily of vesicle transport proteins. Rab proteins localize to the surface of membrane-bound organelles and are involved in membrane transport of other proteins throughout the cell (Bhuin and Roy, 2014). When found in the cytosol, Rab proteins are GDP-bound and in an inactive state. Membrane-associated Rab proteins are GTP-bound and consider in an active state. Cycling between the inactive and active state requires GDP dissociation inhibitor (GDI), guanine nucleotide exchange factor, and GTPase activating proteins. These upstream regulators can result in different active forms of Rab proteins and can impact which downstream effectors through which Rab can function (Simons and Zerial, 1993; Zerial and McBride, 2001; Zheng et al., 2017). Tagged versions of Rab23 have been shown to localize to the plasma membrane and early endosome when Rab23 is GTP bound, and is primarily cytosolic when GDP bound, consistent with the localization of other Rabs (Evans et al., 2003).

Rab23 mutant mouse embryos, also called *Open brain (opb)*, have increased levels of Hh signaling and have defects in neural tube and eye development, polydactyly, and skeletal malformations (Eggenchwiler et al., 2001; Günther et al., 1994). Analysis of the *opb* mutation determined that Rab23 is a negative regulator of the Hh pathway and functions downstream of Smo and upstream of the Gli transcription factors (Eggenchwiler et al., 2001). Rab23 normally functions to suppresses Gli2A activity and is essential in patterning of the dorsal neural tube.

Additional roles for Rab23 in promoting Gli3 repressor and regulates subcellular localization of Hh components have been described (Eggenchwiler et al., 2006).

The components within the Hh pathway that Rab23 is regulating and whether Rab23 is directly or indirectly regulating the Hh components has not been fully elucidated. One study suggests that Rab23 is required in retrograde transport to transport Smo out of the cilium (Boehlke et al., 2010), and another suggests that Rab23 can suppress transcriptional activity of Gli1 in the presence of Sufu (Chi et al., 2012). Additionally, Rab23, in vitro, may regulate Hh signaling in a more indirect way through transport of more general ciliary components to build the cilium, and as a result be required for ciliogenesis (Leaf and Von Zastrow, 2015; Lim and Tang, 2015; Wang et al., 2006; Yoshimura et al., 2007).

There is also evidence to suggest that Rab23 does not have a role in ciliary assembly or function from analysis of Rab23 in *Trypanosoma brucei*. Rab23 localized to the flagella of the protist, however, RNAi knockdown of Rab23 did not impact the flagella (Lumb and Field, 2011). Furthermore, research from our lab suggests that Rab23 has a function in left-right patterning, which is independent of the primary cilium. Analysis of node cilia in Rab23 mutant mice and fish suggested that Rab23 has no apparent role in ciliary assembly or function (Fuller et al., 2014).

Rab23 in Left-Right Patterning

The initial observation that one third *Rab23* mutant embryos have reversed heart looping, led to an investigation of earlier left-right patterning phenotypes in the absence of *Rab23* (Fuller et al., 2014). Although *Rab23* mutants have increased levels of Hh signaling (Eggenchwiler et al., 2001), the left-right patterning defects are thought to be independent of this phenotype since

Ptch mutant embryos, which have extremely elevated levels of Hh signaling, do not have left-right patterning defects and express Nodal on the left side (Goodrich et al., 1997; Zhang et al., 2001).

Early *Rab23* mutant embryos express Nodal within the Node, but fail to induce *Nodal* and *Nodal* target gene expression within the LPM, this results in organ laterality defects within the later embryo, including reversed heart looping and reversal of the right and left lungs. Expression of Nodal in the LPM of *Rab23* mutant embryos can be rescued when exogenous Nodal protein is injected into the LPM, indicating that *Rab23* mutant cells within the LPM can still respond to Nodal, but perhaps the Nodal signal from the node is not being relayed to the LPM in the absence of *Rab23*. This suggests that *Rab23* may be required for the production of Nodal, and given the role of Rab proteins in membrane trafficking, *Rab23* may potentially be involved in transport of Nodal through the cell. Although Hek293 cells transfected with *Rab23* did not have defects in Nodal processing or secretion, this may not be an adequate in vitro system to replicate the node. *Rab23* may indirectly impact nodal transport by regulating the trafficking of another Nodal pathway component such as GDF1 (Fuller et al., 2014). Nodal may also have multiple ways to be processed and secreted from the cell, and in the absence of *Rab23* may utilize the other trafficking pathways (Blanchet et al., 2008).

In this chapter, we sought to further investigate the potential trafficking of Nodal by *Rab23* in an in vitro system, mouse embryonic stem cells (mESC) which potentially resembles the mouse embryonic node more than Hek293 cells.

Results

Rab23 and Nodal Sub-cellular localization is dependent on Rab23 activity in Cos7 cells

We examined the sub-cellular localization of tagged Nodal in the presence of different versions of tagged Rab23 in Cos7 cells, which are large, easily transfectable, and ideal for imaging. Cells were transfected with tagged versions of Rab23-HA and Nodal-FL due to the lack of antibodies against Rab23 and Nodal protein. Cos7 cells were co-transfected with Rab23-HA, Nodal-FL, and GDF1 and we observed that Rab23-HA is broadly dispersed throughout the cell, and Nodal-FL co-localizes most strongly in the organelles just outside the nucleus (Figure 4.2). Cos-7 cells were also co-transfected with a constitutively active version of Rab23, Rab23 Q68L-HA, a mutation which makes Rab23 act as if it is always bound to GTP, Nodal-FL, and GDF1. In the presence of the Rab23Q68L-HA construct, Nodal-FL was localized very similarly within the Cos7 as seen in the wild-type construct of Rab23-HA (Figure 4.2).

When we co-transfected Nodal-FL and GDF1 with a dominant negative version of Rab23, Rab23 S23N-HA, where Rab23 acts as if it is always bound to GDP, a significant change in Nodal sub-cellular localization was observed. In the presence of the dominant negative version of Rab23 S23N-HA, Nodal localization was highly nuclear and co-localization between Nodal and Rab23 was greatly reduced throughout the cytosol (Figure 4.2). This suggests that within Cos7 cells, Nodal sub-cellular localization is dependent on the activity state of Rab23, and in the presence of a dominant negative version of Rab23, Nodal is unable to be dispersed throughout the cell as seen in the presence of wild-type and constitutively active Rab23-HA. RT-PCR using primers designed to detect mouse Rab23 suggest that Cos7 cells may express endogenous Rab23, and may explain why some co-localization is observed in the presence of the dominant negative version of Rab23.

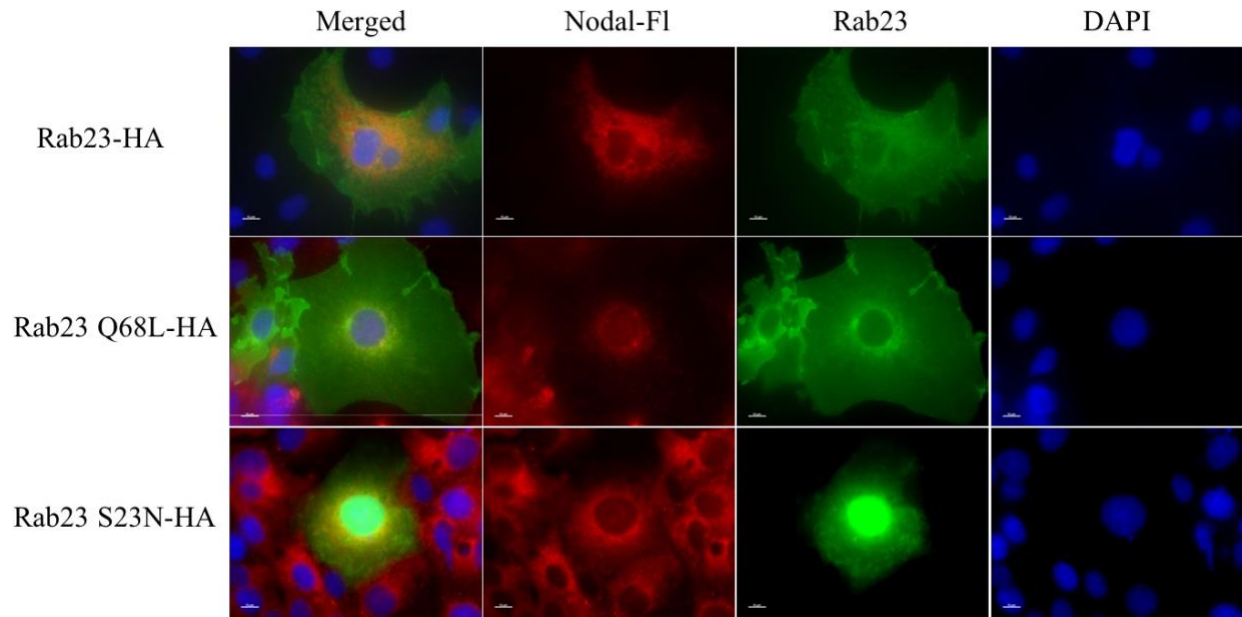


Figure 4.2 Sub-Cellular localization of Rab23-HA and Nodal-FL in Cos7 cells Cos7 cells were co-transfected with one of three Rab23-HA constructs, Nodal-FL and GDF1 and then immuno-labeled with antibodies against HA and FL (GDF1 was unlabeled). In the presence of wild-type Rab23 or constitutively active Rab23 Q68L-HA, Nodal-FL protein was more dispersed throughout the cell with high amounts of co-localization in the organelles near the nucleus. In the presence of the dominant negative Rab23 S23N-HA, Nodal localization was highly restricted to the nucleus and co-localization with Rab23 was greatly reduced.

Sub-cellular localization of Rab23 and Nodal in Cos7 cells

To further distinguish the organelles that Rab23-HA and Nodal-FL were localizing to within the cells, we co-transfected Nodal-FL and Rab23-HA in Cos7 cells (Figure 4.3) and Hek293 cells to further test the robustness of the sub-cellular localization antibodies (data not shown). Nodal-FL and Rab23-HA were shown to strongly co-localize within the golgi (GM130), ER (PDI), and the early endosome (Rab5) in both Cos7 (Figure 4.3) and Hek293 cells. This suggests that both Rab23-HA and Nodal-FL are involved in membrane trafficking within the cell.

Sub-cellular localization of Rab23 and Nodal in mESCs

Previous work from our lab suggests that Rab23 is required for Nodal induction in the left LPM. Rab23 mutant cells within the embryonic LPM are able to respond to exogenous Nodal, but do not express Nodal endogenously. Rab23 may potentially be involved in membrane trafficking of Nodal, or Nodal signaling components within the cells of the LPM, however, Hek293 cells were demonstrated to have no changes in Nodal production or processing (Fuller et al., 2014). We sought to understand Nodal and Rab23 subcellular localization and trafficking in a cell culture system with mouse embryonic stem cells (mESC), which more closely resemble the embryonic node. We first tested the feasibility of imaging sub-cellular markers within mESC, which have vastly reduced cytoplasm compared to Cos7 cells and grow in clusters which can sometimes form multiple layers of cells. Sub-cellular markers were distinguishable within the mESC (Figure 4.4). Additionally, because of the low transfection rate of mESC, a Rab23 antibody was tested, and appeared to localize throughout the cell with strong localization at the plasma membrane, which was not observed in *Rab23*^{-/-} mESC, although further validation is required to draw substantial conclusions.

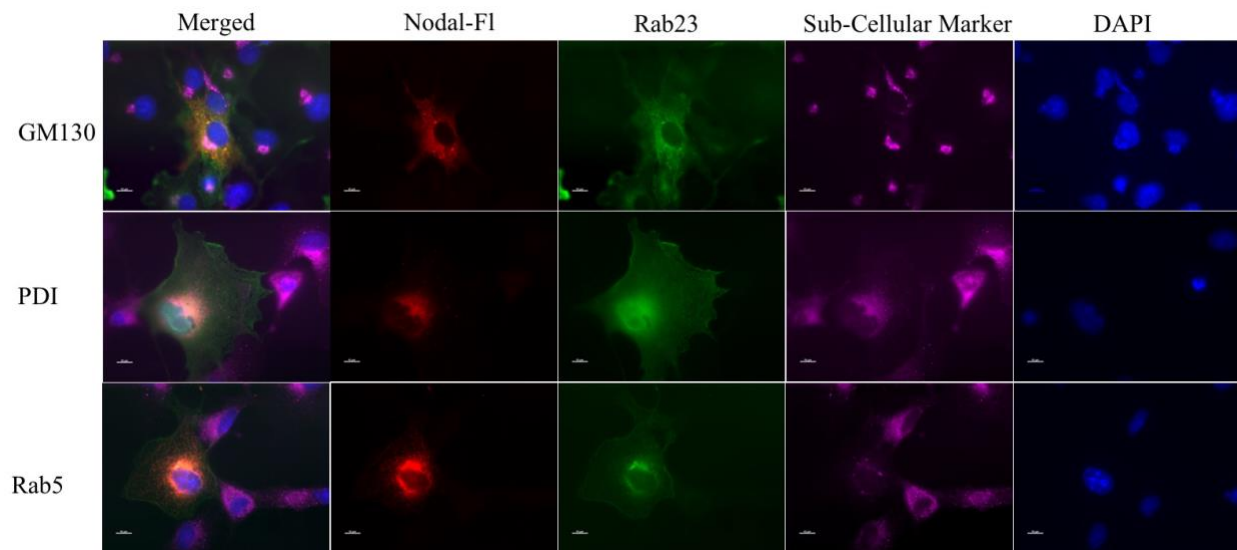


Figure 4.3 Subcellular localization of Rab23-HA and Nodal-FL in Cos7 cells Cos7 cells were transfected with Nodal-FL and Rab23-HA. Cells were then fixed and immuno-labeled with antibodies against FL, HA, and a subcellular marker. Nodal-FL and Rab23-HA were shown to strongly co-localize within the golgi (GM130), ER (PDI), and the early endosome (Rab5)

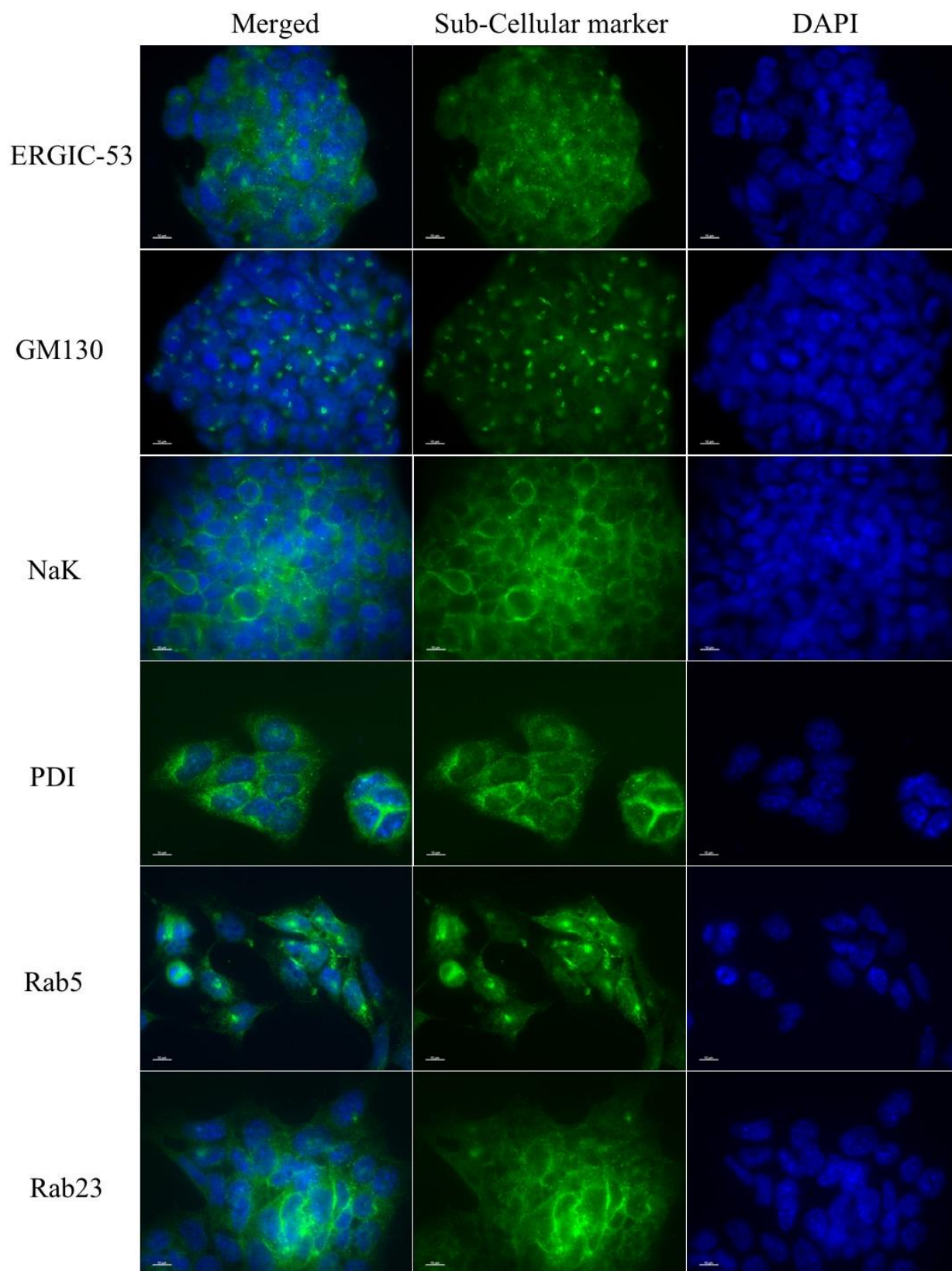


Figure 4.4 Sub-cellular localization markers in mESCs R1 ESC were fixed and labeled with various sub-cellular markers of organelles involved in membrane trafficking. The Golgi-ER intermediate compartment (ERGIC-53), golgi (GM130), plasma membrane (NaK), ER (PDI), early endosome (Rab5) markers, and Rab23 are all visible within ESCs.

Sub-cellular localization of Nodal-FL in *Rab23*^{+/+} and *Rab23*^{-/-} mESCs

ESCs were previously isolated in our lab from *Rab23*^{+/+} and *Rab23*^{-/-} embryos. There are no functional antibodies against Rab23 and Nodal, and, additionally, transient transfections by lipofection or nucleofection have very low efficiency in mESC. We sought to increase the number of mESC expressing tagged Nodal-FL to understand how Nodal is being processed in mESC in the presence and absence of Rab23, we created stable lines of mESC expressing Nodal-FL. To create stable lines, we nucleofected mESCs that were *Rab23*^{+/+} and *Rab23*^{-/-} with Nodal-FL and the selectable marker puromycin. The mESCs were selected and colonies from single clones were selected and tested for Nodal-FL by immunofluorescence. Although the mESC lines were presumably clonal, all clones had heterogeneous expression of Nodal-FL. Lines with around 30% of the mESCs expressing Nodal-FL were analyzed for Nodal-localization in the presence and absence of Rab23 and subcellular localization.

The localization of Nodal-FL in *Rab23*^{+/+} and *Rab23*^{-/-} mESC upon initial investigation appears consistent both genotypes with no obvious changes in Nodal localization (Figure 4.5). In the absence of Rab23, Nodal-FL is still localized throughout the cell body as in *Rab23*^{+/+}, with clear localization at the plasma membrane. Additionally, no strong nuclear localization was observed like that seen with the dominant negative version of Rab23 S23N in Cos7 cells. This suggests that the dominant negative Rab23 S23N may take on a novel function in sequestering Nodal-FL to the nucleus or that Rab23 mediated trafficking impacts Nodal localization differently between different cell types.

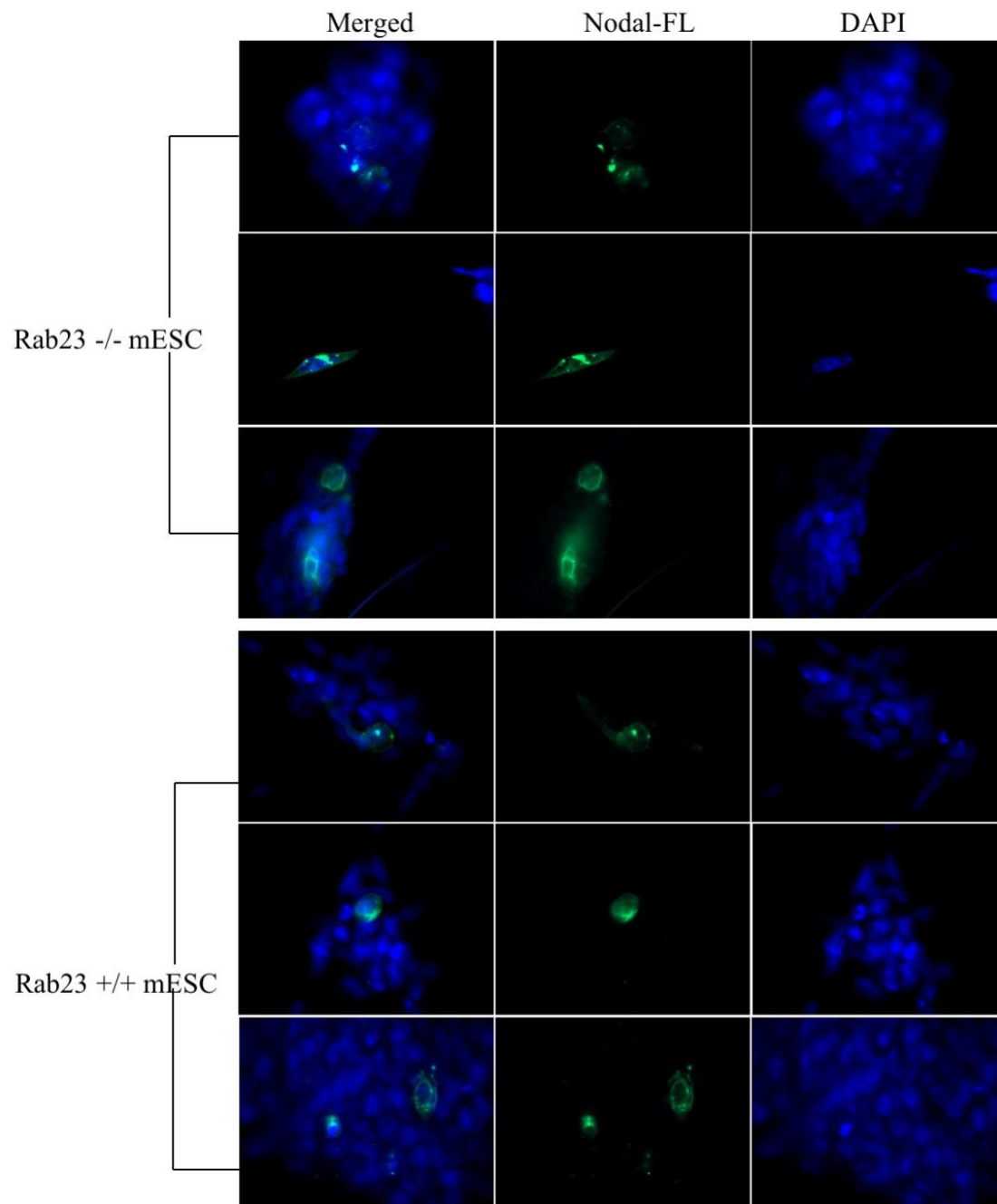


Figure 4.5 Nodal-FL expression in *Rab23^{+/+}* and *Rab23^{-/-}* mESC 63x images of mESC labeled with an antibody against Flag to examine Nodal-FL localization in *Rab23^{+/+}* and *Rab23^{-/-}* ESC. No obvious differences to Nodal-FL localization was observed between *Rab23^{+/+}* and *Rab23^{-/-}* cells, where Nodal-FL is localized throughout the cell and at the plasma membrane.

In wild-type ESC endogenous Rab23 detected by immunofluorescence does not seem enriched in particular organelles or at the cytoplasm, but is diffusely located in the cytoplasm, and antibody staining was not detected in *Rab23*^{-/-} mESC (Figure 4.6). Nodal-FL has strong localization to the plasma membrane in certain cells as well as to other subcellular organelles, although the localization of Nodal-FL may not represent endogenous Nodal as overexpression can often result in sequestration of protein in the ER.

Further investigation of the impact of the dominant negative version of Rab23 on Nodal localization within mESC will be needed to clarify this phenotype and potentially more subtle changes to Nodal localization in the absence of Rab23. Additionally, further Western Blot analysis will need to be done to test if the plasma membrane localization of Nodal-FL even in the absence of Rab23 is indicative of successful processing and secretion of Nodal-FL. There is the potential that the presence of Rab23 is not required for secretion of Nodal, but is required for processing, or that Rab23 does act in the process of secretion, but other membrane trafficking proteins are able to compensate for the loss of Rab23.

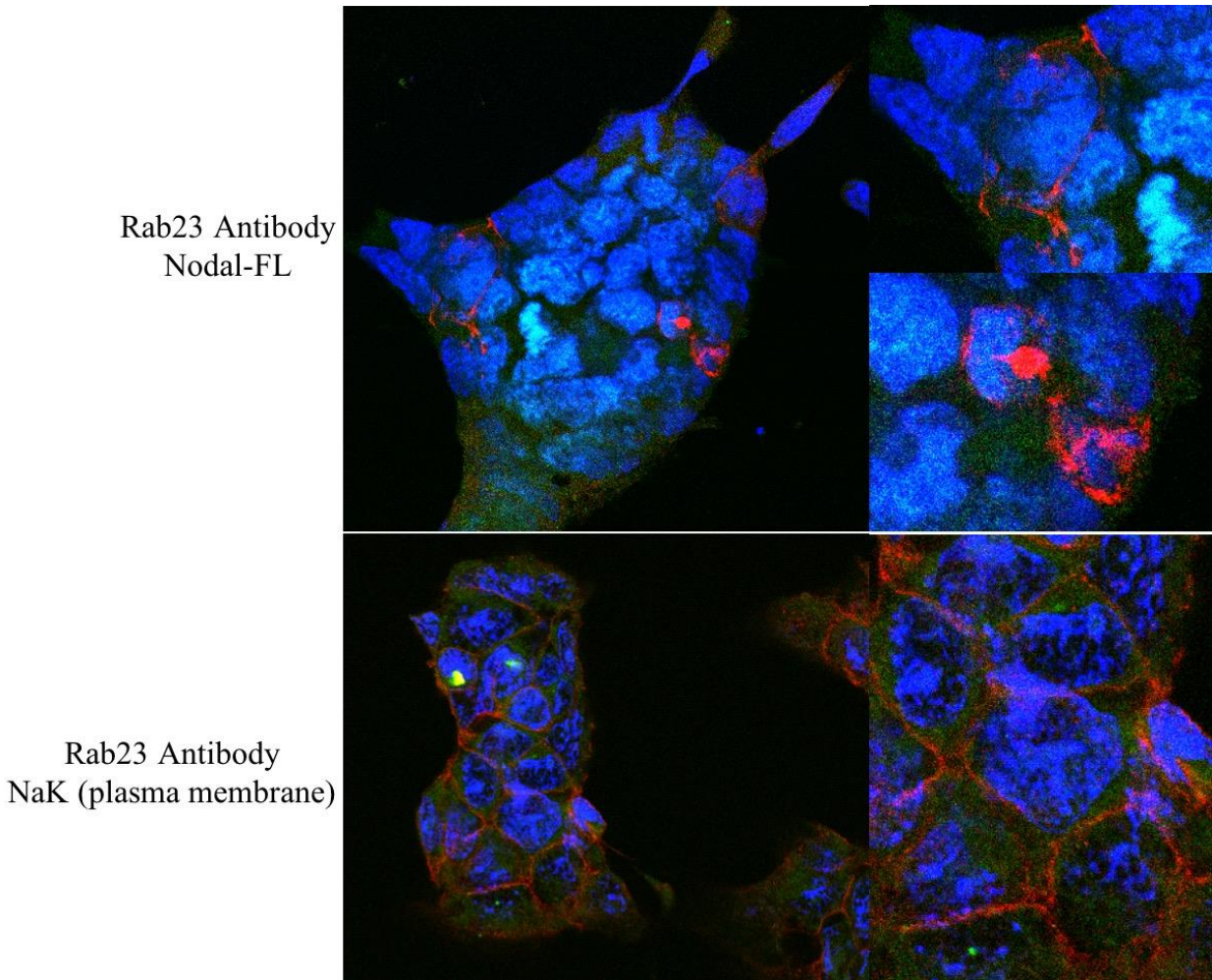


Figure 4.6 Sub-cellular localization of Nodal-FL and endogenous Rab23 in *Rab23*^{+/+} mESC

Endogenous Rab23 (green) in both panels has diffuse localization throughout the cytoplasm, while Nodal-FL overexpression in the top panel (red) localizes to certain organelles and to the presumptive plasma membrane as the staining is similar to that detected by the NaK antibody bottom panel (red).

Injection and lipofection of Rab23-GFP in E8.0 Embryos

To further understand the role and sub-cellular localization of Rab23, in vivo, we injected Rab23-GFP into the LPM of E8.0 embryos and cultured them for 12 or 24 hours. Embryos were then analyzed for Rab23-GFP expression (Figure 4.7), and sectioned to analyze the cellular localization of Rab23-GFP (Figure 4.8). Injection of Rab23-GFP into the presumptive LPM resulted in variable regions of expression within the developing embryo, where injections would frequently result in transfection of the presumptive hindgut or midline (Figure 4.7 C) or within the paraxial mesoderm and presumptive neural plate (Figure 4.7D). This result could be to inaccurate injection sites and/or diffusion of the lipofectamine and Rab23-GFP from the site of injection. Cells within the LPM expressing Rab23-GFP appear to express Rab23 throughout the cytoplasm, but is absent from the nucleus (Figure 4.8). These preliminary data indicate the possibility of further experiments where Nodal is co-injected with Rab23 into the LPM to analyze the subcellular localization of both proteins in *Rab23*^{+/+} and *Rab23*^{-/-} embryos.

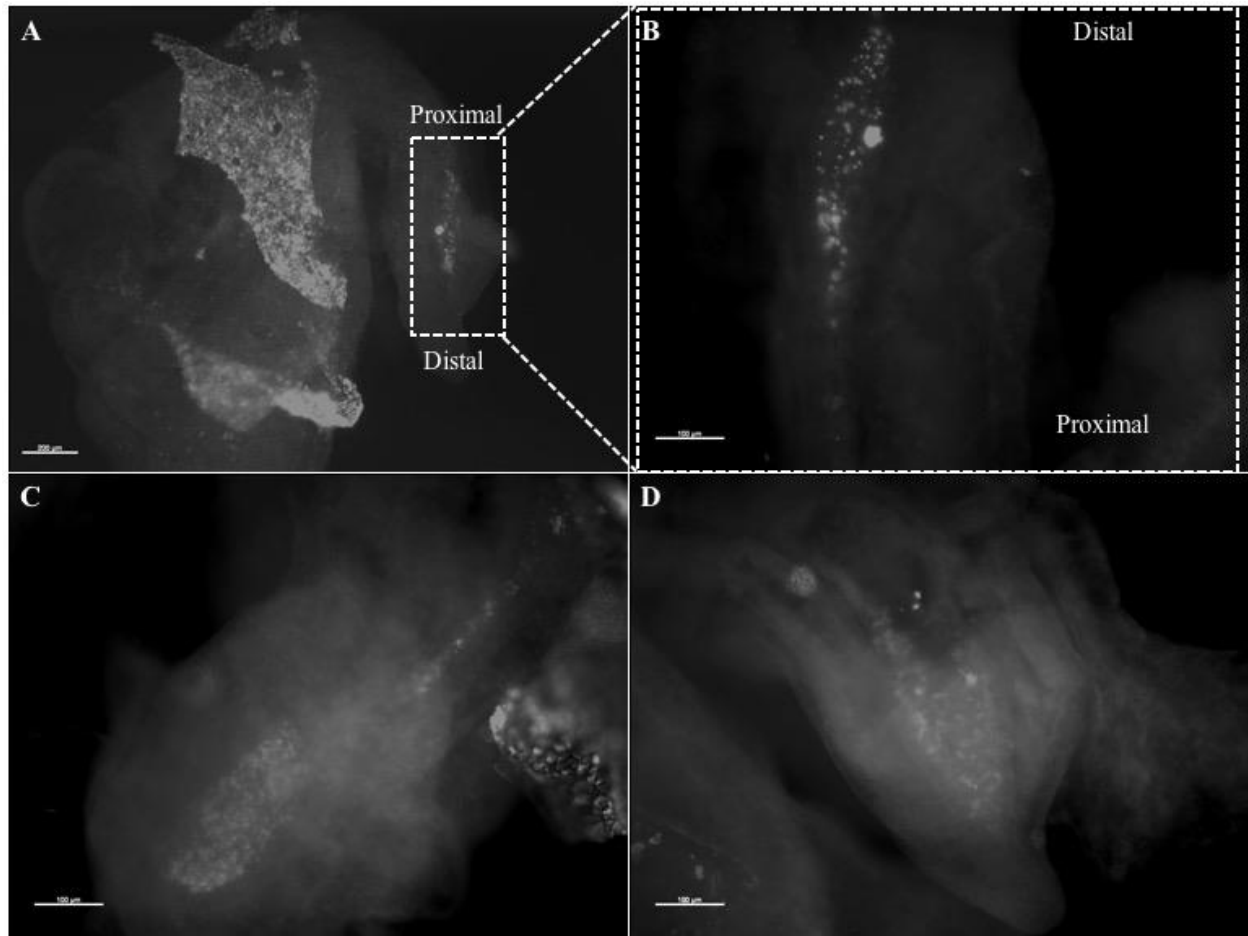


Figure 4.7 Injection and lipofection of Rab23-GFP in the LPM of E8.5 embryos after 12 hours of culture Expression of Rab23-GFP was analyzed by fluorescence microscopy. A and B) Expression of Rab23-GFP in the LPM C) Expression of Rab23-GFP in the posterior embryo, potentially the hindgut and midline D) Expression of Rab23-GFP in the posterior embryo, potentially the neural plate and paraxial mesoderm

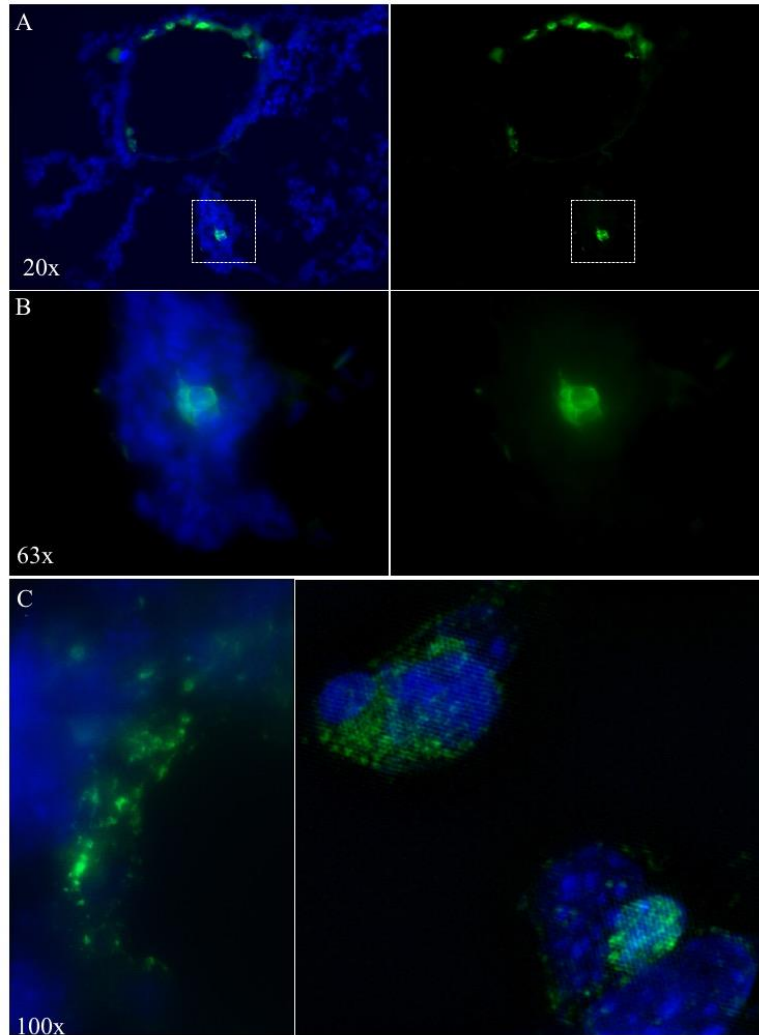


Figure 4.8 Rab23-GFP cellular localization within the LPM Embryo with cells transfected in the LPM was cryo-sectioned and labeled with an anti-GFP antibody A) 20X image of Embryo from panel A in Figure 4.7. The crescent of cells at the top is most likely the hindgut. B) Rab23-GFP expression in cells located in the LPM C) Super resolution images of sectioned embryos injected with Rab23-GFP 100X maximum projection of super resolution images

Rab23 and Megf8 in Nodal transport

Loss of function mutations in *Rab23* have been found in the rare human disorder Carpenter Syndrome (CS). CS patients have a constellation of phenotypes, including polysyndactyl and changes in organ symmetry consistent with defects in left-right patterning (Twigg et al., 2012). The only other gene known to be mutated in CS patients is the multiple EGF-repeat transmembrane protein, *Megf8*. Like, *Rab23* mutant mouse embryos, *Megf8* mutants have expression of *Nodal* in the node, but fail to activate expression of *Nodal* in the LPM. It has been proposed that *Megf8* may be involved in forming the *Nodal*-*Gdf1* interaction thought to promote long-distance transport of *Nodal* (Zhang et al., 2009). *Rab23* may not be directly transporting *Nodal*, although they are co-expressed throughout the membrane trafficking organelles of Cos7 and mESC, *Rab23* may be essential for trafficking another component of *Rab23* signaling, while *Nodal* itself is trafficked through additional mechanisms (Blanchet et al., 2008). The similar phenotypes observed left-right patterning in *Megf8* and *Rab23* mutant embryos and CS patients indicate that these proteins may be involved in a similar pattern regulating *Nodal* signaling within the LPM of the embryo (Twigg et al., 2012; Zhang et al., 2009).

Megf8 is predicted to have a transmembrane domain, however, sub-cellular localization of *Megf8* did not show localization to the cell surface, but did show strong and punctate nuclear staining in MEFs, NIH3T3, and within sections through embryonic cells (Zhang et al., 2009). The nuclear localization and association by yeast-2 hybrid with chromatin regulating protein *Gfi1b* indicate that *Megf8* may have a role in chromatin remodeling (Lim et al., 2006; Vassen et al., 2006). However, *Gfi1b* mutant mouse embryos do not present with left-right patterning defects seen in *Megf8* mutants (Saleque et al., 2002).

As observed in previous studies, our detection of Megf8 using an antibody against it indicates that Megf8 localization is predominately within the nucleus of Cos7 and mESC (Figure 4.9). Although Megf8 expression does not appear to co-localize with Rab23 or Nodal using currently developed antibodies, the similarity between the Megf8 and Rab23 mutant embryos warrants further investigation. Currently tested antibodies may not be able to detect shorter isoforms of Megf8 which may have membrane localization throughout the cell (Zhang et al., 2009). Experiments to tag endogenous Megf8 within mESC would be useful in determining if there is indeed additional localization of Megf8 outside of the nucleus, and to understand the sub-cellular localization in relationship to Rab23 and Nodal.

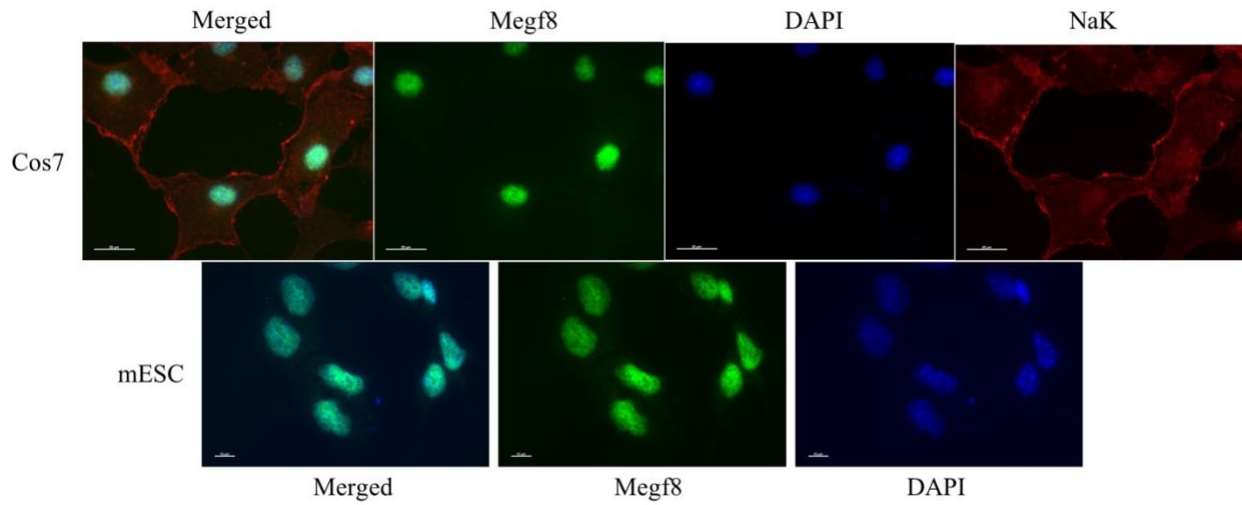


Figure 4.9 Megf8 Antibody localizes to the nucleus in Cos7 and mESC Cos7 cells were stained using a commercially available antibody against Megf8 and a plasma membrane marker (NaK). Megf8 protein appears nuclear in both Cos7 and mESC.

Discussion

Cos7 cells which contain the constitutively active Rab23Q68L-HA construct, Nodal-FL was localized very similarly within the Cos7 as seen in the wild-type construct of Rab23-HA. Cos7 cells transfected with Rab23 S23N-HA, where Rab23 acts as if it is always bound to GDP, is a dominant negative version of Rab23 S23N-HA, Nodal localization was highly nuclear and co-localization between Nodal and Rab23 was greatly reduced throughout the cytosol. This suggests that within Cos7 cells, Nodal sub-cellular localization is dependent on the activity state of Rab23, and in the presence of a dominant negative version of Rab23, Nodal is unable to be dispersed throughout the cell as seen in the presence of wild-type and constitutively active Rab23-HA. It is possible that overexpression of the Rab23 and Nodal constructs alters localization from endogenous expression, and may cause the proteins to be trapped in the ER. Tagging the endogenous Rab23 in mESC would be more ideal to analyze the localization of these proteins. In mESCs no differences in Nodal localization were apparent in *Rab23*^{+/+} and *Rab23*^{-/-} ESC. Nodal localized to sub-cellular markers associated with membrane trafficking within ESCs. Within the embryo, cells within the LPM transfected Rab23-GFP appear to express Rab23 throughout the cytoplasm, but is absent from the nucleus. The Megf8 protein shows nuclear localization, however other isoforms not detected by the antibody may interact with Rab23 to mediate Nodal transport throughout the cell. It will be beneficial to determine endogenous localization of Megf8 and Rab23 to better understand the transport of Nodal in left-right patterning.

Materials and Methods

Lipofection of Cos7 cells

Cos7 cells were grown to 70% confluency in a 6 well dish and transfected using Lipofectamine 3000 (Thermofisher Scientific) with 500ng Nodal-FL and 500ng Rab23-HA

Nucleofection mESCs were nucleofected with Nodal-FL using

Solution I Filter Sterilize and make 80ul aliquots and store at -20C

ATP	0.2g	(stored in blue box in walk-in 4C)
MgCl ₂	0.12g	
Water	up to 1ml	

Solution II Make 4ml aliquots and store at 4C in tissue culture fridge

NH ₂ PO ₄	0.6g
NaHCO ₃	0.06g
Glucose	0.02g
Water	up to 30ml pH to 7.4 then water up to 50ml

80ul of Solution I was mixed with 4ml of Solution II. 100ul of the mixture and 5ug of

Nodal-FL plasmid DNA were added to a cell pellet from a confluent 10cm plate.

Mixture, DNA, and Cells were added to a cuvette (Eppendorf cat# 940001013) and

nucleofected using the program A013. Cells were plated on a 10cm plate and allowed to

recover for 24 hours before selection with 1ug /ml of Puromycin (ThermoFisher

Scientific Cat#A1113803). Single colonies of ESC were picked and grown up to be

analyzed for Nodal-FL expression.

IF of Sub-cellular markers and Nodal-FL Rab23-HA and Megf8

Cells were fixed in 100% MeOH at -20C for 15 minutes. IF was done using standard protocols. Anti-alpha 1 Sodium Potassium ATPase antibody Plasma Membrane Marker (mouse abcam ab7671 used at 1:500). Anti-GM130 antibody (EP892Y) cis Golgi Marker (rabbit abcam ab52649 used at 1:500). Anti-PDI antibody ER marker (rabbit abcam ab31811 used at 1:500). Anti-Rab5 antibody Early endosome marker (rabbit ab18211 used at 1:500). ERGIC-53 (rabbit Santa Cruz Biotechnology, Inc. sc-66880 used at 1:500). Megf8 (rabbit Assay Biotechnology Company, Inc. C16731 used at 1:100). Anti-Rab23 (rabbit JTE-A153 from abcam used at 1:500). Anti-Flag (mouse Sigma-Aldrich catalog number F3165-.2MG used at 1:1000) Anti-HA (rabbit JTE-A178 used at 1:1000). 63X oil immersion images taken on Zeiss 880 Confocal.

Cultured E8.0 embryos injected with Rab23-GFP

A microinjector was used to inject 5ug of Rab23-GFP and phenol red into the LPM of an E8.0 embryo. Embryos were then cultured for either 12 hours or 24 hours in 50:50 DMEM: Rat Serum. Embryos were then fixed overnight in 4% PFA and labeled with an anti-GFP antibody (rabbit from Torrey Pines Biolab #TP401). Embryos were sequentially placed into 15% and 30% sucrose and then embedded in O.C.T (Tissue-Tek O.C.T.). 8µm sections were cut (using which cyrosectioner). Sections were then imaged using the Zeiss Axioplan 2 microscope and Zeiss ELYRA S1 (SR-SIM) super resolution microscope

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APPENDICES

1. Removal of CCRK in Hh induced Medulloblastomas

Introduction

Medulloblastomas are the most common central nervous system (CNS) malignancy in children, and can occur when granule neural precursors (GNPs) within the cerebellum proliferate excessively (Fabo, 2002; Han et al., 2009). GNPs leave their site of proliferation, the rhombic lip, and migrate over the exterior of the cerebellum to form the external granule layer (EGL) around from E13.0-E15.0. The GNPs continue to be mitotically active until P14, however, post birth, the GNPs start to exit the cell cycle and migrate to the interior of the cerebellum forming the internal granule layer (IGL)(Wang and Zoghbi 2001; Corrales et al. 2004; Goldowitz and Hamre 1998) (Appendix Figure 1.1). The proliferation of the GNPs is stimulated by Hh produced by the Purkinje cells (Dahmane and Ruiz, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Concurrently, the primary cilium is required for GNPs proliferation and cerebellum development (Chizhikov et al., 2007; Dahmane and Ruiz, 1999; Wallace, 1999), although, the effect of removing the primary cilia on medulloblastoma formation depends on the where the oncogenic mutations occurs in the Shh pathway.

Mutations upstream in the pathway, such as those that make *Smoothed* (*Smo*) constitutively active, require the primary cilium for tumor formation, and removal of the primary cilium inhibits tumor formation. However, the presence of the primary cilium can also serve to inhibit tumor formation when the oncogenic event occurs in a downstream component of the Shh pathway. Mutations that make *Gli2* constitutively active requires the removal of the primary cilium for tumor formation, while the presence of the primary cilium prevents tumor formation (Han et al., 2009). Although the complete removal of the primary cilium in the presence of oncogenic mutations in *Smo* in the GNPs by *hGFAP-Cre* (*human Glial Fibrillary Acidic Protein promoter driven Cre*) can prevent tumor formation, it also leads to atrophic cerebella. A possible cause of this inhibited cerebellum development is that complete removal of primary cilium

severely reduced the proliferation of the GNPs, and it is known that the primary cilium is required for GNPs proliferation (Han et al., 2009).

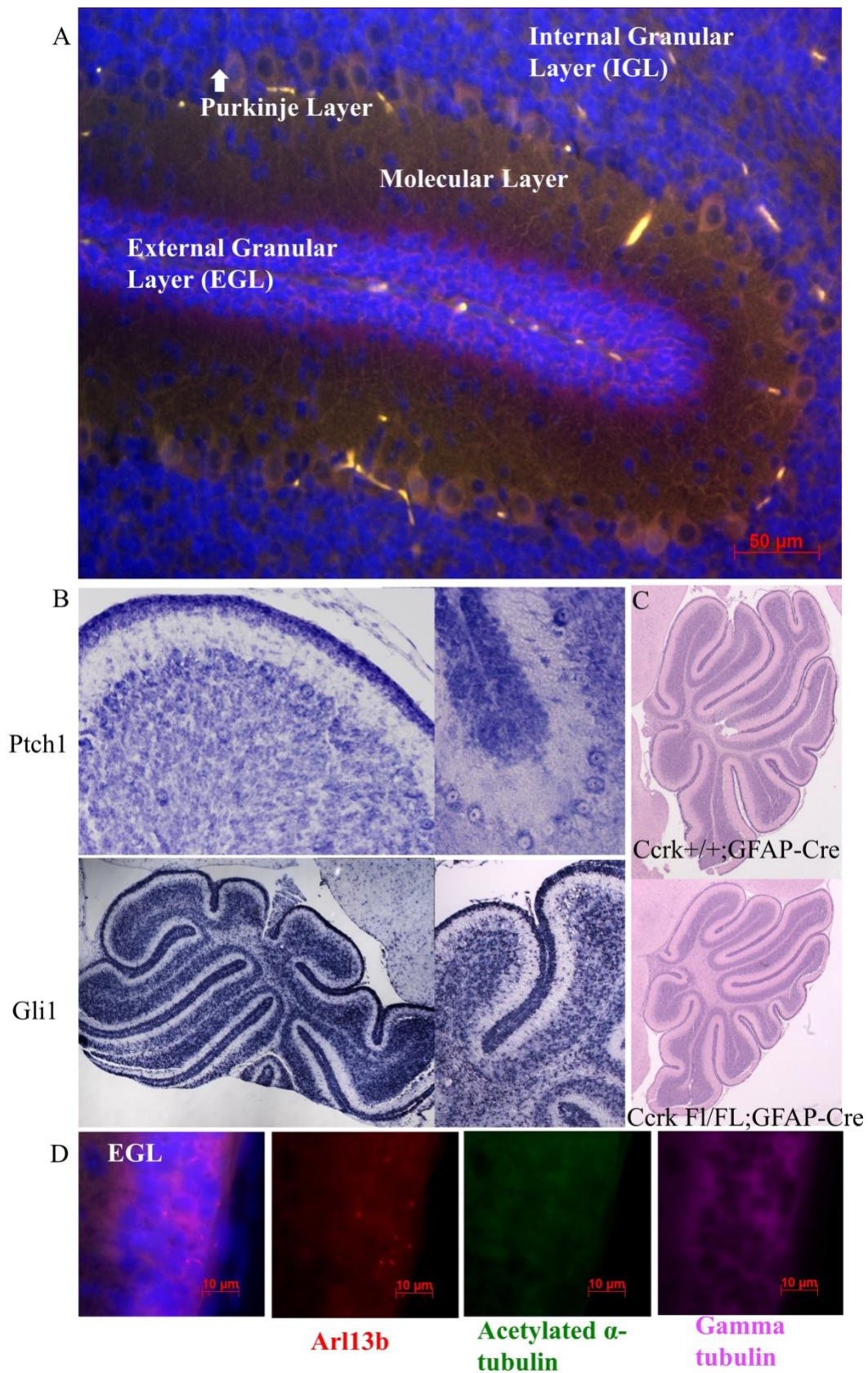
Results

Genetic Removal of *Ccrk* in Medulloblastomas

The *Ccrk* null mice still produce primary cilia, but appear to be unable to respond to high levels of Shh in other neural tissues. This reduced response to Shh could prevent the over proliferation of GNPs caused by hyperactivation of the Shh pathway resulting in medulloblastomas. Tissue specific loss of *Ccrk* within the GNPs of the cerebellum did not result in loss of cilia or changes in expression of essential Hh target genes essential for cerebellar proliferation and development (Appendix Figure 1.1). This indicated that *Ccrk* is not required in the GNP for the normal Shh response required for GNP proliferation. Additionally, CCRK, as a kinase, could be a feasible target of pharmaceuticals in human patients (Noble et al., 2004; Rojanala et al., 2004).

To determine if medulloblastomas caused by upstream hyperactivation of Shh signaling, at the level of Smoothened, can be inhibited or reduced by removal of *Ccrk* from the granule neural precursors (GNPs) I mated *Ccrk*^{Fl/+}; *Smo*^{M2/+} mice to *Ccrk*^{Fl/+}; *Smo*^{M2/+}; *Cre*/+ mice. Mice homozygous for *GFAP-Cre* are embryonic lethal (Zhuo et al., 2001). The constitutively active allele of Smo (*Smo*^{M2}) was also driven using the same *GFAP-Cre* in the GNPs, and when neither allele of *Ccrk* was removed medulloblastomas developed in all mice by P10 (N=6 Appendix Table1.1 red). In mice that were heterozygous for *Ccrk* in the presence of *Smo*^{M2} also all developed medulloblastomas of equal severity to *Smo*^{M2} alone animals by P10 (N=6 Appendix Table1.1 yellow). When both copies of *Ccrk* were removed through cre-mediated

recombination in the presence of *SmoM2*, a range of tumor formation was seen (N=14 Appendix Table1.1 green and Appendix Figure 1.2 C). In most cases (N=9/14) the tumorigenesis appeared just as severe as the *SmoM2* alone animals, however in several cases (N=5/14) there appeared to be a mild rescue, where foliation of the cerebellum was apparent, but the cerebellum was hyperplastic (Appendix Table1.1 and Figure 1.2 D and E). We determined that removal of *Ccrk* from the GNPs was not sufficient to prevent tumorigenesis.

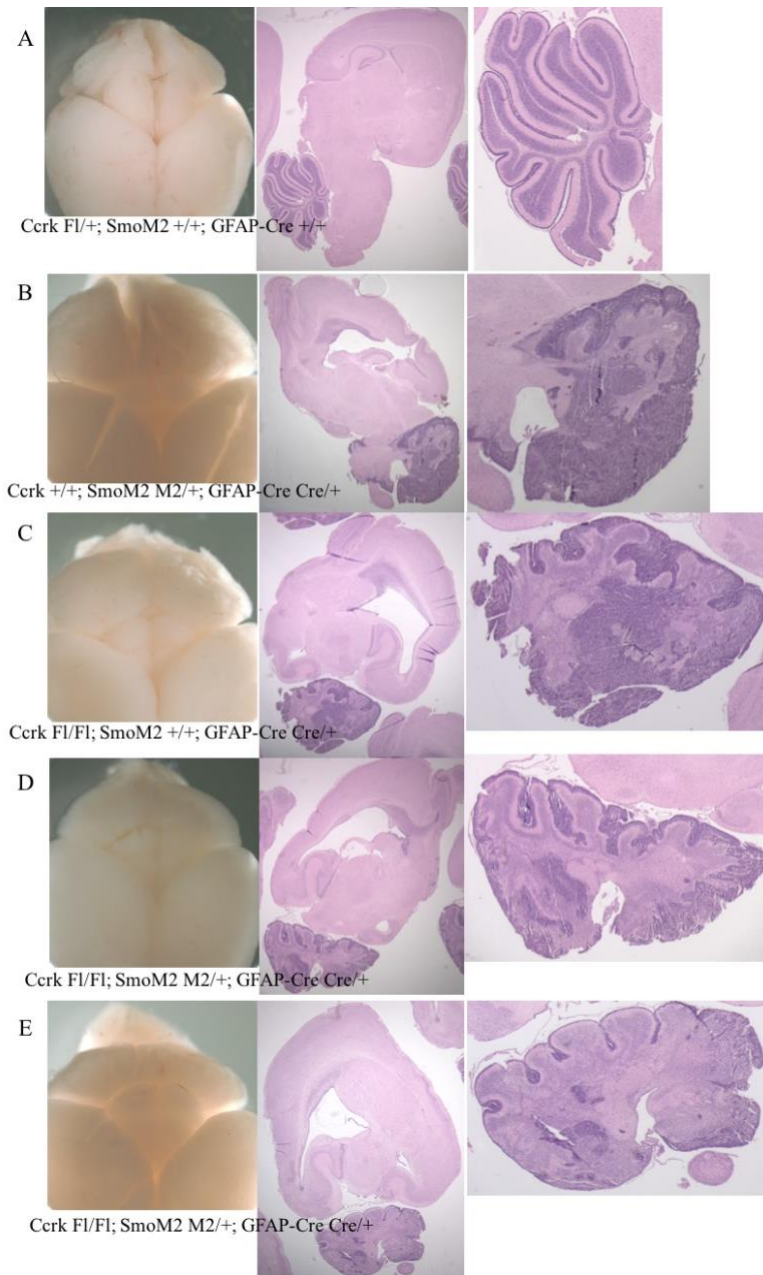


Appendix Figure 1.1 *Ccrk* removal from the GNPs through Cre mediated recombination

A *Ccrk* allele with loxP sites flanking the first two exons was removed from GNPs within the cerebellum using *hGFAP-Cre*. A) GNP migration from the external granular layer to the internal granular layer as the cerebellum develops and the Hh producing Purkinje cells. B) Cerebella with *Ccrk* removed maintain Hh response (*Gli1* and *Ptch1*) within the expected pattern. C) The cerebellum developed normally and appears wild type in the absence of *Ccrk*. D) Primary cilia can still be found within the EGL of cerebella in the absence of *Ccrk*

		<i>Ccrk</i> FL	<i>SmoM2</i>	<i>GFAP-Cre</i>
1592.7	Tumor	+/+	M2/+	Cre/+
1859.7	Tumor	+/+	M2/+	Cre/+
1859.8	Tumor	+/+	M2/+	Cre/+
1860.8	Tumor	+/+	M2/+	Cre/+
1998.3	Tumor	+/+	M2/+	Cre/+
1998.5	Tumor	+/+	M2/+	Cre/+
1594.6	Tumor	FL/+	M2/+	Cre/+
1594.5	Tumor	FL/+	M2/+	Cre/+
1999.6	Tumor	FL/+	M2/+	Cre/+
2022.10	Tumor	FL/+	M2/+	Cre/+
2125.4	Tumor	FL/+	M2/+	Cre/+
2129.6	Tumor	FL/+	M2/+	Cre/+
1592.1	Tumor	FL/FL	M2/+	Cre/+
1594.2	Tumor	FL/FL	M2/+	Cre/+
1860.2	Tumor	FL/FL	M2/+	Cre/+
1860.3	Tumor	FL/FL	M2/+	Cre/+
1860.5	Tumor	FL/FL	M2/+	Cre/+
1998.1	Tumor-some folds	F1/F1	M2/+	Cre/+
1998.7	Tumor	FL/FL	M2/+	Cre/+
1999.3	Tumor	FL/FL	M2/+	Cre/+
1999.7	Tumor	FL/FL	M2/+	Cre/+
2000.3	Tumor-some folds	FL/FL	M2/+	Cre/+
2000.8	Tumor-some folds	FL/FL	M2/+	Cre/+
2001.4	Tumor	FL/FL	M2/+	Cre/+
2019.3	Tumor	FL/FL	M2/+	Cre/+
2125.3	Tumor	FL/FL	M2/+	Cre/+
1594.7	Normal	FL/+	+/+	+/+
1999.4	Normal	FL/FL	+/+	Cre/+
2019.2	Normal	FL/+	+/+	Cre/+
2019.6	Normal	FL/FL	+/+	Cre/+
2022.1	Normal	FL/FL	+/+	Cre/+
2125.2	Normal	+/+	+/+	+/+
2127.4	Normal	FL/FL	+/+	Cre/+
2127.7	Normal	FL/+	+/+	+/+
2129.3	Normal	+/+	+/+	-

Appendix Table 1.1 Experimental Animals Genotypes and tumor description Each number represent a different mouse and a description of the mouse cerebellum. Red boxes represent *SmoM2* positive mice without the potential of rescue with removal of *Ccrk*. Yellow boxes represent *SmoM2* positive mice with only one copy of *Ccrk* removed. Green boxes are *SmoM2* positive animals with both copies of *Ccrk* removed. White boxes are control animals with no *SmoM2* positive animals.



Appendix Figure 1.2 *SmoM2* mediated tumorigenesis in the presence of *Ccrk* removal

Representative selection of experimental results. A) normal cerebellum without *SmoM2* tumorigenesis gene. B) *SmoM2* positive tumor without removal of *Ccrk* C-E) *SmoM2* positive tumors with both copies of *Ccrk* removed did have some folding of the cerebellum in some cases, but most were still tumorigenic and matched the severity of the *SmoM2* alone mice.

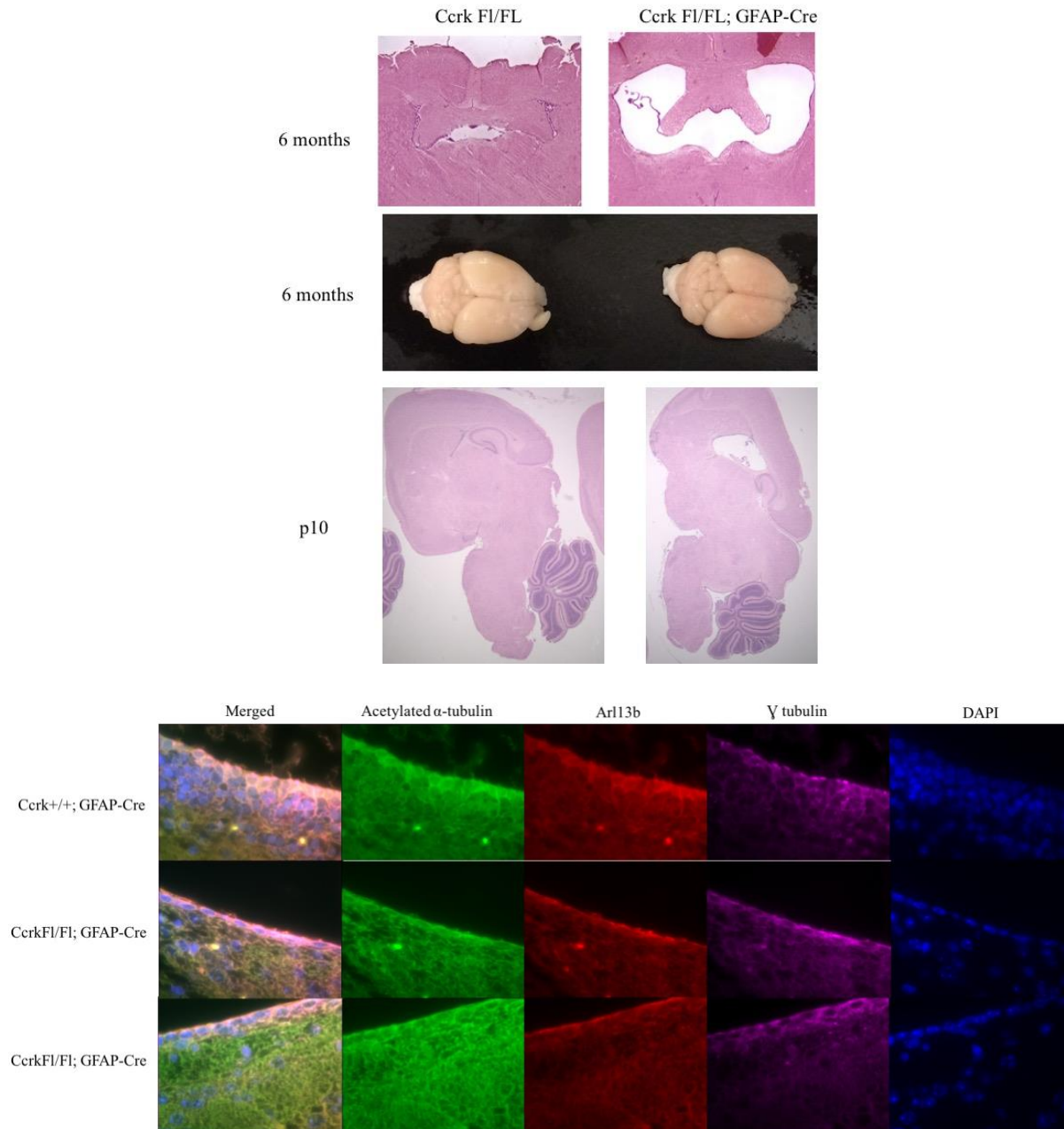
Genetic Removal of *Ccrk* in Hydrocephalus

Although the reduction of proliferation by removal of *Ccrk* does not appear to be significant enough to broadly impact medulloblastoma formation, the removal of *Ccrk* by *hGFAP-Cre* appears to have additional affects within the brain. Hydrocephalous was observed in all of mice that presumably have both copies of *Ccrk* removed by *hGFAP-Cre* and the hydrocephalus persisted into adulthood even at 6 months of age (N=4 and N=6 respectively Appendix Figure 1.3). Additionally, all of the mice with *Ccrk* removed by *hGFAP-Cre* in the presence of the constitutively active *SmoM2* allele had more severe hydrocephalous, clearly visible by P10 (N=12 and). The *hGFAP-Cre* used is known to be expressed in several other cell types within the CNS in addition to GNPs, including astrocytes, oligodenroglia, and ependymal cells (Zhuo et al., 2001).

The ependymal cells line the surface of the ventricles and produce motile cilia on their apical surface. These cilia beat synchronously in a coordinated direction to facilitate the circulation of cerebrospinal fluid (CSF). Improper CSF circulation can lead to fluid accumulation within the ventricles and hydrocephalous (Breunig et al., 2010). *Ccrk* may have a role in production or organization of the motile cilia on ependymal cells with in the ventricles. Antibody labeling of the basal bodies and cilia of ependymal cells demonstrated that in the absence of *Ccrk* the ependymal cells still organize clusters of basal bodies. Interestingly, *Ccrk* may be involved in regulation of both primary and motile ciliary length or may be involved in orientating the production of motile cilia. (Guirao et al., 2010).

I found in mice with both copies of *Ccrk* removed by the presence of *GFAP-Cre*, had hydrocephalus at p10. Mice analyzed at 3 and 6 months of age also presented with hydrocephalus, but not apparent detrimental phenotypes. Ependymal cells that had loss of both

alleles of *Ccrk* through *GFAP-Cre* still had clusters of basal bodies necessary for apical positioning of the ventricle cilia to beat, however the morphology and functionality of the cilia within the ventricles was not analyzed. *Ccrk* may potentially have a role in promoting cilia length within the motile cilia of the ventricles, or be involved in establishment and positioning of these cilia.



Appendix Figure 1.3 *Ccrk* removal using *GFAP-Cre* results in hydrocephalus

Hydrocephalus was first observed in p10 mice and was found to be present in mice up to 6 months of age. Although gross morphology of the brain is not impacted, when sectioned the ventricles appeared expanded. Ependymal cells lacking both copies of *Ccrk* still had clusters of basal bodies as seen in the presence of both copies of *Ccrk*.

2. Genetic Removal of *Ccrk* and Obesity

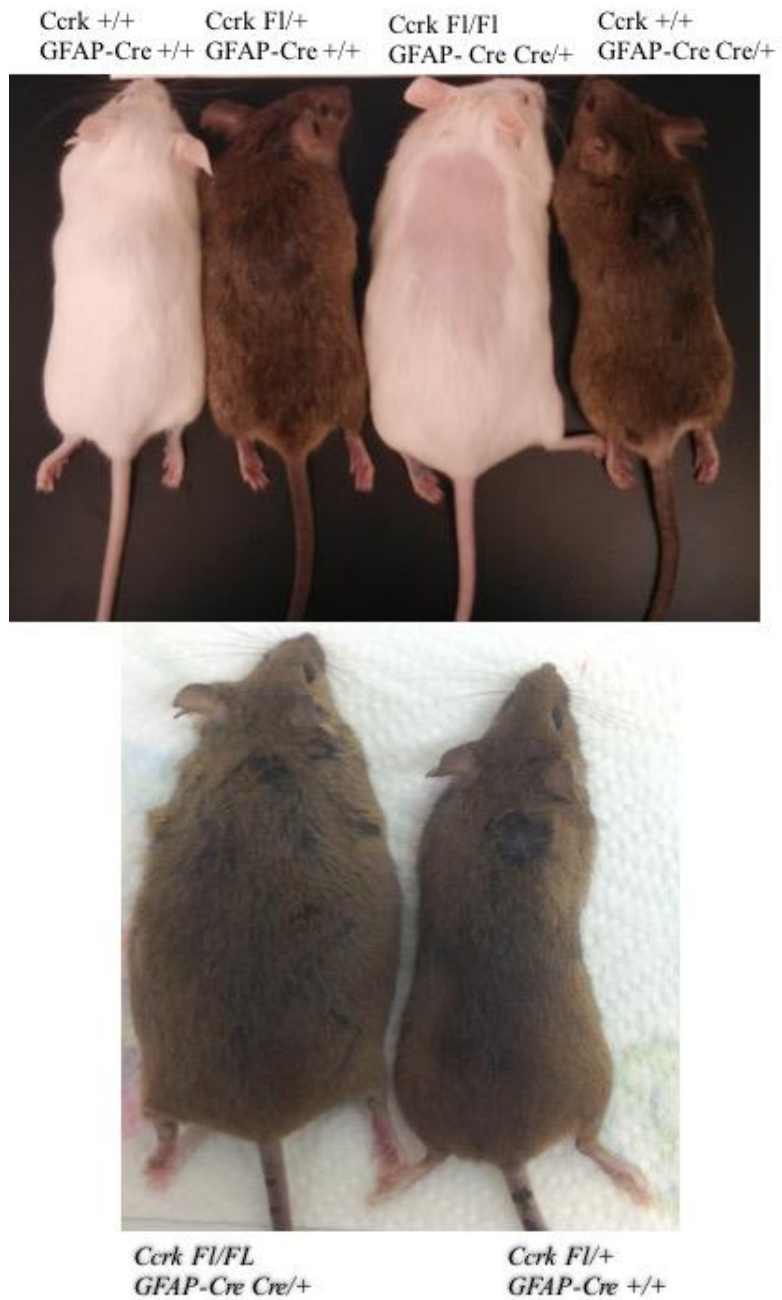
Introduction

Disrupting the production or function of the primary cilium can lead to a number of human disorders with multiple phenotypes. These ciliopathies include retinal degeneration, cystic kidneys, and obesity. There are two populations of neurons within hypothalamus with opposing roles in regulating satiation: pro-opiomelanocortin (POMC)-producing neurons that suppress appetite, and the neuropeptide Y (NPY) and agouti-related peptide (AgRP)-producing neurons that promote appetite (Mok et al., 2010). Loss of primary cilia through disruption of *Kif3a* in POMC neurons alone lead to significant weight increase (Davenport et al., 2007). In the ciliopathy, Bardet-Biedl syndrome (BBS), the phenotypes were due to a mutation in the BBS protein that is required for the assembly and maintenance of the primary cilium. The effects on ciliogenesis in the POMC neurons is thought to lead to defective leptin signaling, leading to improper satiation signaling (Kesterson et al., 2009; Mok et al., 2010; Quinlan et al., 2008). The *GFAP-Cre* is active in multiple cell types throughout the central nervous system, and removal of *Ccrk* may impact other function in the brain including appetite control (Zhuo et al., 2001).

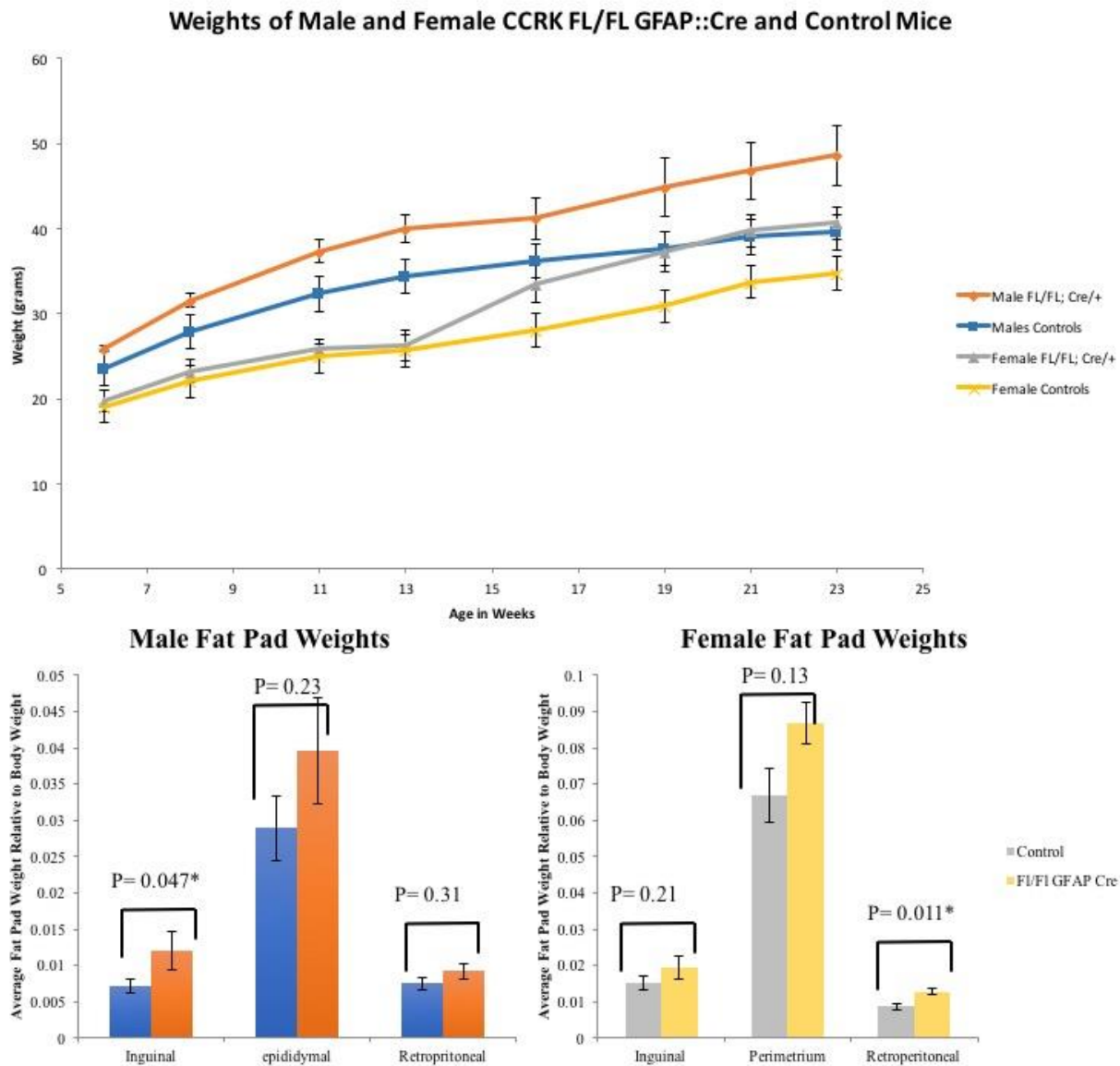
Results

In addition to the hydrocephalous phenotype, preliminary data indicate that mice that have both copies of *Ccrk* removed using the *GFAP-Cre* may also be more obese than those that only have one copy removed (Appendix Figure 2.1). To test if the removal of *Ccrk* using *GFAP-Cre* causes a predisposition to adult onset obesity I monitored the weight of *Ccrk^{Fl/Fl}GFAP-Cre* positive mice over six months and compared their weight to control animals that did not have both copies of *Ccrk Fl*, so maintained at least one functional copy of *Ccrk*.

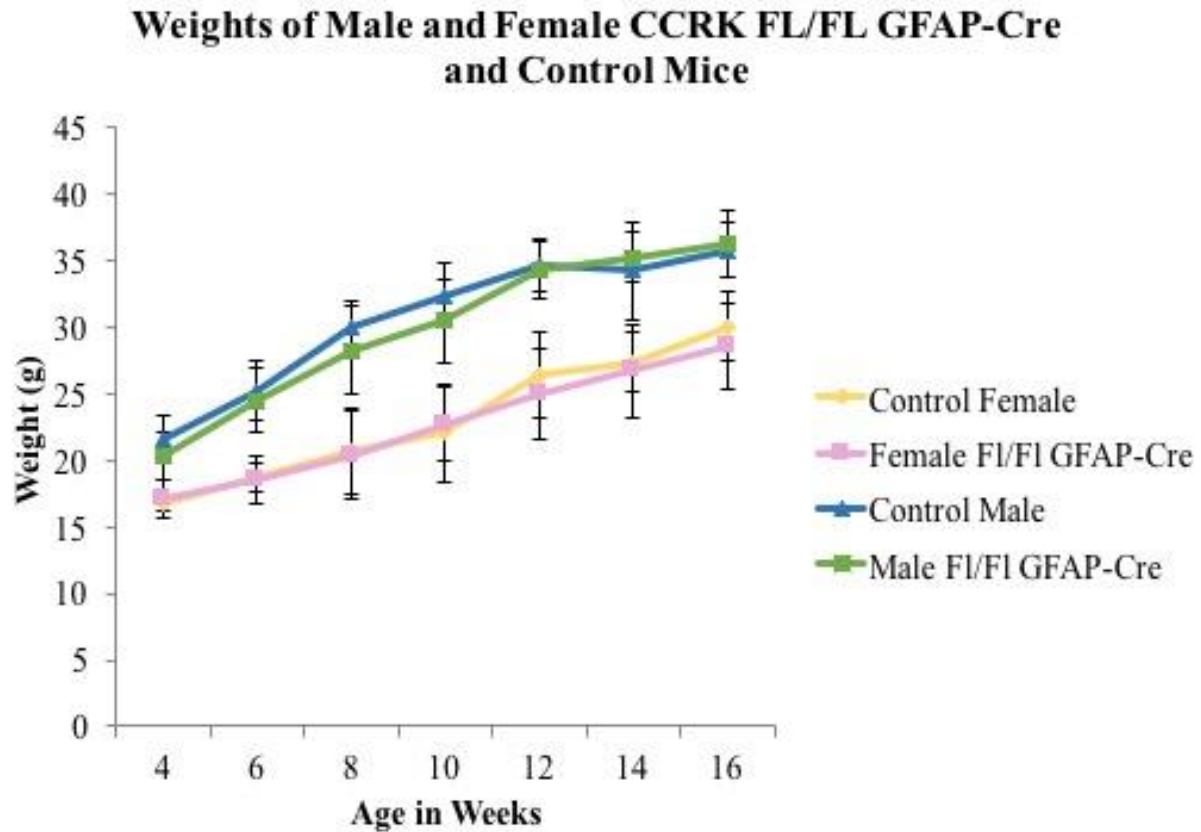
I weighed the mice approximately every two weeks from 6 weeks of age until 6 months of age. The *Ccrk^{Fl/Fl} GFAP-Cre* positive males were always significantly heavier than the control males. While the *Ccrk^{Fl/Fl} GFAP-Cre* positive females did not start to weigh more than the control females until around 3 months of age (Appendix Figure 2.2). At six months of age all experimental mice were sacrificed and fat pads were dissected out and weighed. For male mice, the inguinal epididymal retroperitoneal fat pads were individual dissected and weighed. For female mice, the inguinal perimetrium and retroperitoneal fat pads were dissected and weighed. *Ccrk^{Fl/Fl} GFAP-Cre* positive males contained significantly more fat mass in their inguinal fat pads and *Ccrk^{Fl/Fl} GFAP-Cre* positive females contained significantly more fat mass in their retroperitoneal fat pads. To replicate the experiment on a more consistent mouse strain background, I backcrossed the *GFAP-Cre* to the C3H mouse line for two generations and then crossed the *Ccrk^{Fl}* allele (maintained on a C3H background) back in. I then monitored the weight of the *Ccrk^{Fl/Fl} GFAP-Cre* mice compared to control mice every two week from 4 weeks to 4 months of age. However, this subsequent experimental did not replicate the differences in weight (Appendix Figure 2.3). This could potentially be due to reduced activity of *GFAP-Cre* or the weight difference initial observed was caused by the difference in strain background.



Appendix Figure 2.1 Gross morphology of *Ccrk^{Fl/Fl}*; *GFAP-Cre* mice compared to control genotypes *Ccrk^{Fl/Fl}*; *GFAP-cre* positive mice appear more obese than mice with only once copy of *Ccrk* removed, or mice only positive of *GFAP-Cre*



Appendix Figure 2.2 Weight of female and male mice taken every two weeks for 6 months and weight of fat pads at six months of age *Ccrk*^{FL/FL}; *GFAP-cre* positive male mice were significantly heavier than control mice at all time points starting from 6 weeks of age. They also had significantly more fat within the inguinal fat pad. *Ccrk*^{FL/FL}; *GFAP-Cre* positive female mice were not significantly heavier than control females until 16 weeks of age and remained heavier than the control females. They also had significantly more fat within the retroperitoneal fat pad.



Appendix Figure 2.3 Replication of female and male mice weights taken every two weeks for four months No significant difference was observed between the weights of the *Ccrk^{FI/FI}*; *GFAP-Cre* male or female mice compared to male or female controls, respectively.

3. Ligand dependence in SAG induced MEFs

Introduction

The Hh receptor (Ptch1), in addition to activating the Hh pathway, is thought to also potentially control programmed cell death. This dual role may come from Ptch1 being a dependence receptor, or a receptor that ensures the cell survives as long as it is exposed to the ligand (Thibert, 2003). A dependence receptor would add an additional level of regulation to cells responding to ligands, and may be involved in triggering crucial apoptotic events in proper development (Guerrero, 2003). In addition to specification of different cell fates, Hh is also a strong mitogen, and having a mechanism in place to prevent cells from over proliferating once exposed to the ligand may prevent over-proliferation or tumorigenesis (Goodrich and Scott, 1998; Stecca et al., 2007).

Ptch1 expression in cultured cells can induce apoptosis, and addition of the Hh ligand can block apoptosis. It has been proposed that Ptch1 is cleaved by caspase-3 which then exposes a carboxyl-terminal apoptotic domain on Ptch1, and transfection of just the carboxyl-terminal domain of Ptch1 can induce cell death in culture. It is unclear if Ptch1 has an apoptotic function in vivo, and how the mechanism is regulated. There are cases where cells at a great distance from the Hh ligand source within the neural tube express *Ptch1*, but do not induce programmed cell death. Additionally, there are cells which migrate away from the neural tube, and even after exposure to high levels of the Hh ligand, do not induce apoptosis (Hall et al., 1996). Studies done in the chick neural tube suggest that Ptch1 may be a dependence receptor in vivo, and may induce some ventral neural tube cells to undergo apoptosis to regulate the size and shape of the neural tube (Charrier et al., 2001). Removal of the initial source of Hh, the notochord, from the chick embryo also induces apoptosis within the neural tube and results in a smaller malformed

neural tube. However, the neural tube size and shape was not rescued when a dominant-negative Ptch1 protein, without apoptotic activity was added (Thibert, 2003). Suggesting that the relationship between Ptch1 mediated apoptosis and the Hh ligand is more complex (Guerrero and Ruiz i Altaba, 2003).

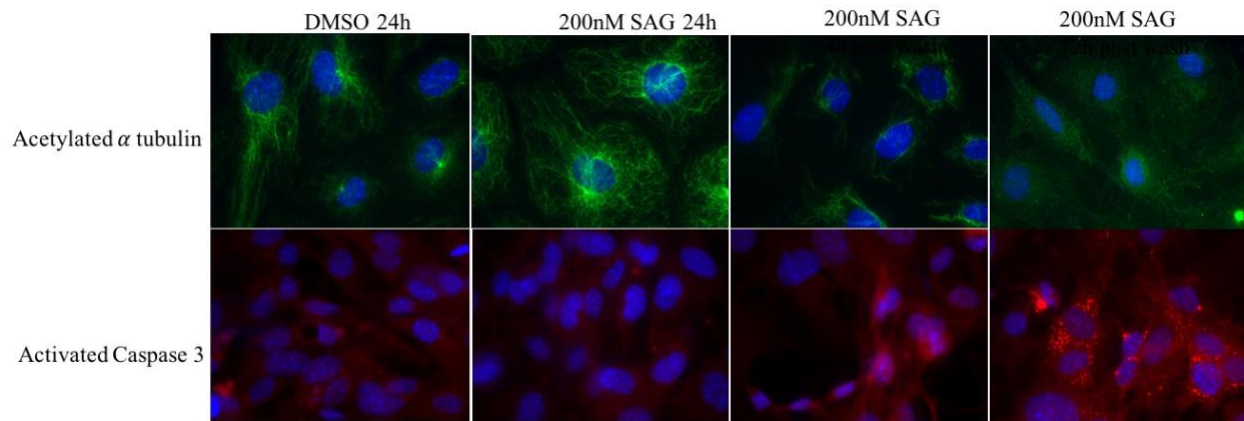
Hh continues to have a role after development, where it controls proliferation of many cell types throughout the body, and mutations which cause hyperactivation of the Hh pathway can result in cancer. Ptch1 is a tumor suppressor which has been implicated in a number of tumors, including medulloblastoma and basal cell carcinomas (Hahn et al., 1996b; Johnson et al., 1996; Ruiz i Altaba et al., 2002). Since the Ptch1 receptor acts as a Hh pathway repressor in the absence of Hh ligand, mutations that prevent the repressor activity lead to activation of the Hh pathway and induction of the Gli transcription factors and cellular proliferation (Briscoe et al., 2001). If Ptch1 also has a role in inducing apoptosis in cells that should no longer be responding to Hh, then mutations in Ptch1 may induce tumorigenesis two-fold. First through hyperactivation of the Hh pathway and secondly by loss of the apoptotic signal to stop a cell from proliferating (Berman et al., 2002; Dahmane et al., 2001; Thibert, 2003; Williams, 2003).

The relationship between exposure to Hh ligand and apoptosis, and may require a certain threshold of Ptch1 expression (Guerrero and Ruiz i Altaba, 2003). I decided to examine the amount of time of exposure to SAG and dosage of SAG required for immortalized mouse embryonic fibroblasts (MEFs) to induce the apoptosis pathway.

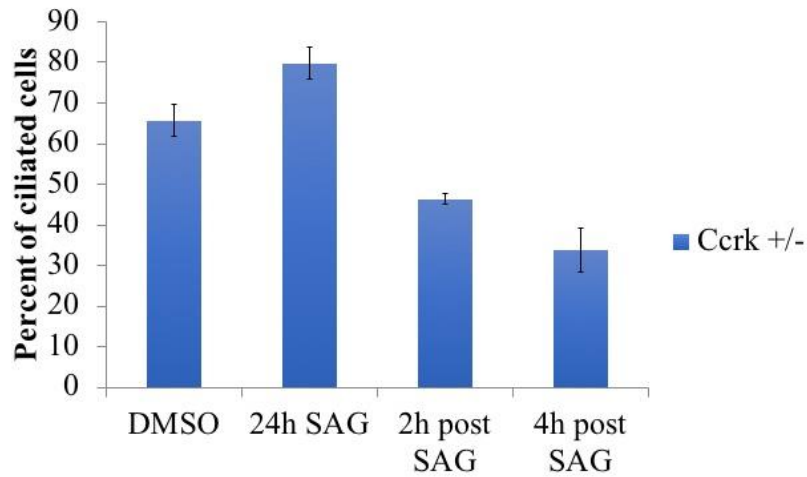
Results

I first exposed MEFs to a high concentration of SAG (200nM) for 24 hours and then fixed cells 4 hours and 12 hours after the SAG was washed out. Cells were then stained for acetylated α -tubulin and activated caspase 3. Cells began to degrade the stable microtubule marker, acetylated α -tubulin, 4 hours post SAG removal and at 12 hours post it no longer appeared associated with microtubules with a dispersed cytoplasmic localization (Appendix Figure 3.1). I also observed a decreased in the percentage of ciliated cells, consistent with loss of stabilized microtubules (Appendix Figure 3.1). In addition to degraded acetylated α -tubulin, MEFs 4 hours and 12 hours post SAG removal had activated caspase 3 proteins within the cytoplasm, indicating the cell had activated the apoptosis pathway (Appendix Figure 3.1).

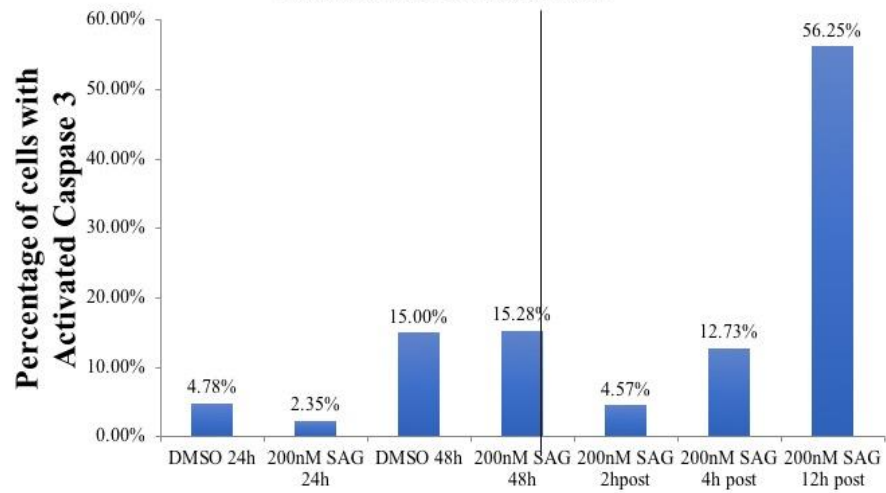
I also tested exposure of cells to lower concentrations of SAG (5nM and 20nM). I saw lower percentages of cells with activated caspase 3 12 hours post SAG removal when cells were exposed to lower concentrations of SAG, but still a significant portion of cells had activated caspase 3 when exposed to 20nM (37%) and 5nM (34%) (Appendix Figure 3.2). The number of cells with activated caspase 3 after 24 hours after the SAG was removed was also examined in several cases, and it was found to be a lower percentage than that seen at 12 hours post SAG removal potentially because many of the cells which did activate caspase-3 underwent apoptosis. In support of this hypothesis was the observation that coverslips which had cells 24 hours post SAG removal were much less confluent compared to the 4hour and 12hour post SAG removal.



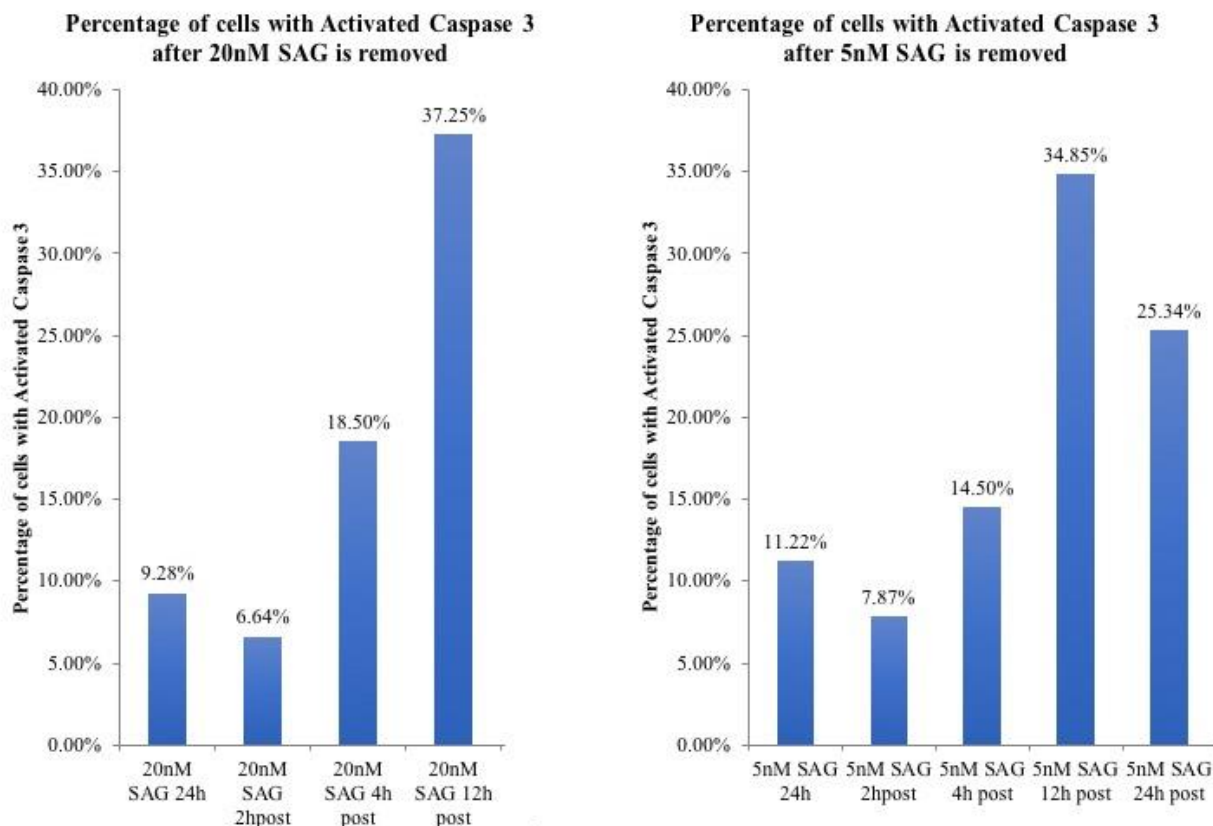
**Percentage of ciliated immortalized MEFs
(Ccrk +/-)**



**Percentage of cells with Activated Caspase 3 After
200nM SAG is removed**



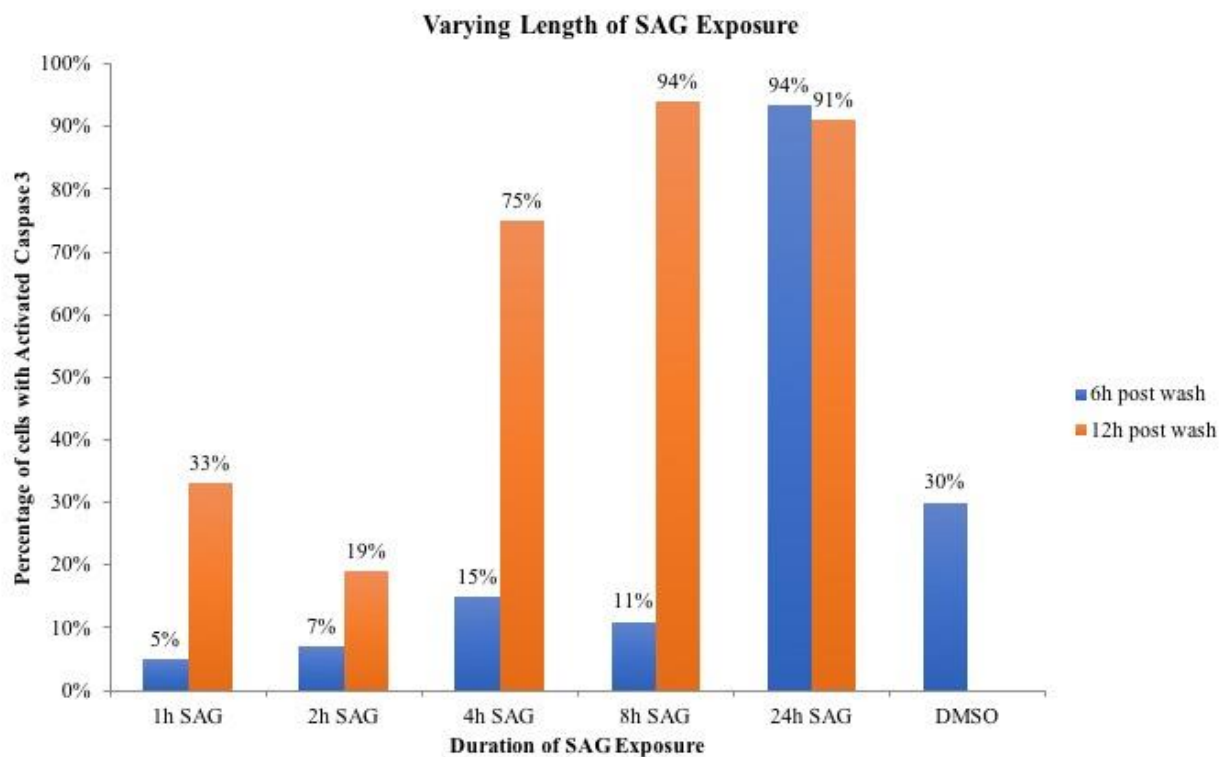
Appendix Figure 3.1 Acetylated α -tubulin degradation and apoptosis response in MEFS after exposure and removal of 200nM SAG Cells exposed to 200nM SAG resorb cilia and degrade acetylated α -tubulin and have activated caspase3 and 4 hours and 12 hours post SAG removal.



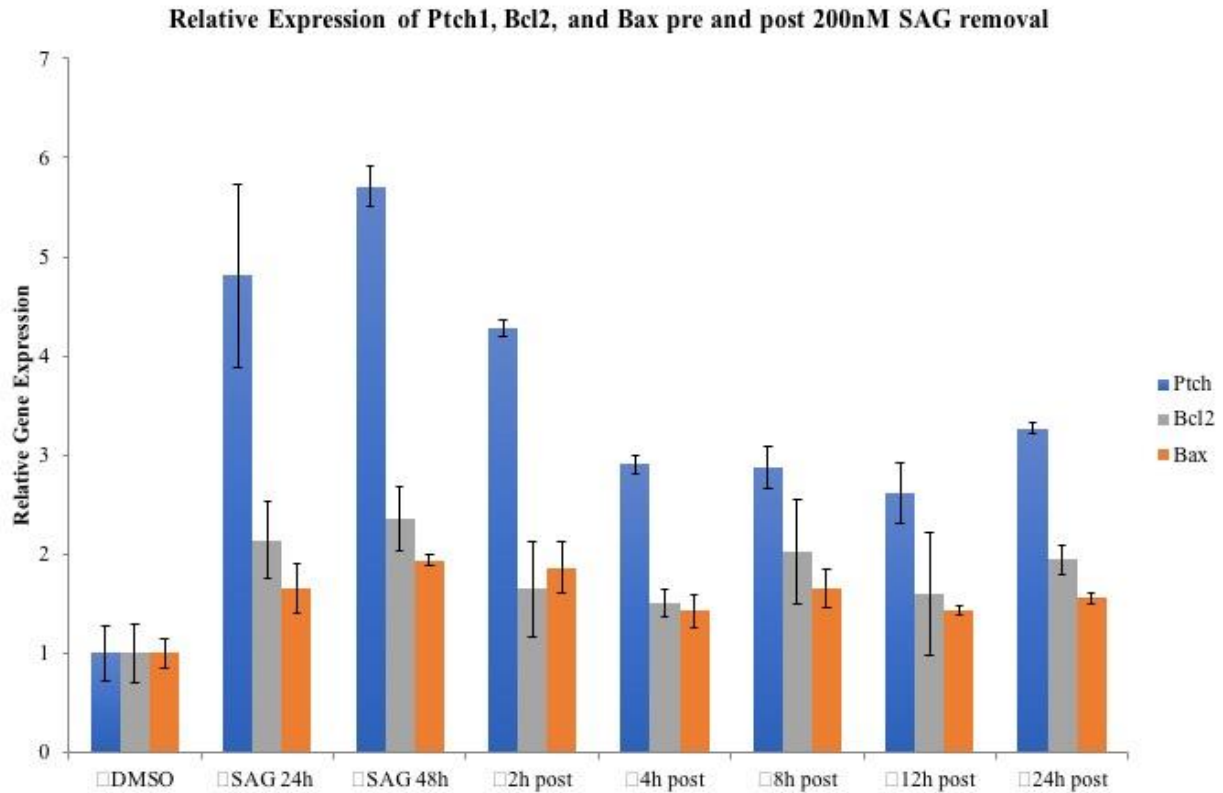
Appendix Figure 3.2 Apoptosis response in MEFS after exposure and removal of 20nM and 5nM SAG Less apoptosis was observed after exposure to a lower dosage of SAG for 24 hours, but activated caspase-3 was still present in a significant portion of the cells 12 hours post removal of SAG. Less apoptosis response was observed 24 hours post SAG removal potentially because many cells which had activated caspase-3 may have already died and would not have been observed.

We also wanted to determine if cells exposed to SAG for less time than 24 hours and then had SAG removed would also induce the apoptosis pathway. Cells were exposed for 1h, 2h, 4h, 8h and 24 hours to 200nM SAG and then the percentage with activated caspase 3 were determined 6 hours and 12 hours after the SAG was washed out. I found that cells exposed to 200nM of SAG for just 1 hour or 2 hours did not have higher activated caspase 3 levels than those seen in the cells not exposed to SAG (DMSO control). However, cells exposed to SAG for 4h and 8h did have elevated caspase 3 levels 12 hours post SAG removal but not 6 hours post SAG removal. Cells exposed to 200nM SAG for 24 hours before SAG was removed had elevated levels of activated caspase 3 even 6 hours post SAG removal.

We then examined the relative expression of *Ptch1* and pro-apoptotic factor (*Bax*) and anti-apoptotic factor (*Bcl2*) to determine if *Ptch1* is upregulated and if it impacts the expression of important apoptotic factors. *Ptch1* may potentially promote apoptosis by upregulating pro-apoptotic factors such as *Bax* or by downregulating anti-apoptotic factors such as *Bcl2*. After 24h and 48h of exposure to SAG *Ptch1* expression was increased. Once SAG was washed out after 24 hours of exposure, *Ptch1* expression decreases and remains relatively consistent from 2 hours post removal to 24 hours post removal (Appendix Figure 3.4). Expression of the pro-apoptotic and anti-apoptotic factors, *Bax* and *Bcl2* respectively, did not seem impacted by the removal of SAG (Appendix Figure 3.4).



Appendix Figure 3.3 Apoptosis response in MEFS after exposure and removal of 200nM SAG after varying times of exposure. Cells exposed to SAG for shorter periods of time had a similar number of cells with activated caspase-3 as seen in the no SAG control (DMSO). However, cells exposed for 4 hours or 8 hours had many cells with activated caspase-3 12 hours post removal of SAG. 24 hours after removal of SAG, very high numbers of cells had activated caspase-3 even 6 hours after post SAG removal.



Appendix Figure 3.4 Relative expression of *Ptch1*, pro-apoptotic *Bax*, and anti-apoptotic *Bcl2* in cells before and after different time points of SAG removal *Ptch1* levels decreased after SAG removal and then remained relatively consistent up to 24 hours post SAG removal. No significant changes were observed in the apoptotic factors *Bax* and *Bcl2* after SAG was removed.

Although increased expression of *Ptch1* was not observed after SAG removal, there is potential that *Ptch1* that is expressed may still take on an apoptotic role within the cell not observed when the cells are exposed to SAG. Additionally, changes in *Bax* and *Bcl2* may not have a role in the potentially *Ptch1* mediated apoptotic response. However, there is potential that SAG is activating another factor, other than *Ptch1* which is involved in the Hh mediated apoptotic response observed. There may be additional complications analyzing gene expression in a population of cells where there is substantial cell death. *Ptch1* expression may indeed be increased in the cells, but since the overall population of cells decreases as they activate apoptosis, the expression may appear decreased or unchanged.

These data suggest that there is indeed a threshold of SAG exposure, and similar to cell type specification induced by the Hh ligand, the apoptotic response may also be dependent on the concentration and the dosage of Hh a cell is exposed to, where high concentrations or long exposure to Hh will result in a quicker apoptotic response in more cells, while lower concentrations or exposure times can still induce apoptosis, but in fewer cells and the response takes a longer period of time. Interestingly, these data suggest that activation of the pathway at the level of Smo, with Smoothed agonist (SAG), will induce apoptosis. SAG will induce greater expression of *Ptch1*, and may be more prone to activating the apoptosis pathway than cells induced with Hh ligand, because in SAG induced cells there is no ligand present to inhibit *Ptch1*, so the expression of *Ptch1* observed may result in a more dramatic apoptotic response than if the cells were induced with Hh. Therefore, these experiments warrant repeating with Hh ligand to see if similar results are obtained. Although these data support the hypothesis that activation of the Hh pathway can induce dependency, it is unclear if *Ptch1* is inducing the

apoptotic response within these cells, and it remains to be determined if there are other factors playing a role in the ligand dependence observed.

Discussion

I. Removal of *Ccrk* within Hh induced medulloblastomas did not significantly reduced tumor frequency or severity. *Ccrk* may have a role in regulation of cilia positioning or function within the ventricles of mice because we observed hydrocephalus in mice with homozygous removal of *Ccrk* with GFAP-Cre.

II. Genetic removal of *Ccrk* with GFAP reveals that *Ccrk* may have a role in regulating obesity. Although, the cell type and the role of *Ccrk* within GFAP positive cells and how it impacts fat pad size remains to be determined.

III. SAG induction does appear to create a dependence on Hh signaling, and removal of SAG resulted in activation of the apoptosis pathway. High concentrations and longer durations of exposure to SAG resulted in more cells activating the apoptosis pathway faster than if cells are exposed SAG at a lower concentration or for a shorter duration of time. However, it remains unclear if this response is mediated through *Ptch1* or another component.

Methods

Mouse strains

Zhuo L; Theis M; Alvarez-Maya I; Brenner M; Willecke K; Messing A. 2001. hGFAP-cre transgenic mice for manipulation of glial and neuronal function in vivo. *Genesis* 31(2):85-94.

FVB-Tg(GFAP-cre)²⁵Mes/J mouse

JAX stock #004600

I. Removal of CCRK in Hh induced Medulloblastomas Methods

H&E Staining

Protocol described in Chapter III

Immunofluorescence on Ventricles

Protocol described in Chapter II for antibodies and Chapter III for antigen retrieval and IF on tissue

II. Genetic Removal of Ccrk and Obesity

Mice were weighed on a scale every two weeks and fat pads were dissected and immediately weighed.

III. Ligand dependence in SAG induced MEFs

Immunofluorescence for activated caspase-3

Activated caspase-3 antibody (rabbit ADD ANTIBODY INFO) was used at 1:1000 using a standard protocol (Chapter II)

q-RT PCR

Done using validated qPCR primers Bax (IDT Mm.PT.58.14012210) and Bcl2 (Mm. PT. 58. 7362966) Ptch1 and Gli1 in Chapter II.

APPENDICES REFERENCES

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