University of Cyprus
Department of Biological Sciences

Extracellular Regulated Kinase (ERK): A novel regulator in the development and function of cilia

Chrystalla Nikolaou

A Research-Based Master's Thesis
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ABSTRACT

The role of cilia in biomedicine is becoming more and more recognized since defects in ciliary structure and function induce ciliopathies such as cystic kidney disease, neurological anomalies, obesity, blindness, and skeletal deformities. Here, we examined the possible role of the Extracellular Regulated Kinase 1/2 (ERK1/2) in ciliogenesis and ciliary function. We show that ERK is associated with the basal bodies of primary cilia in cultured cells and in multiciliated cells of *Xenopus Laevis* raising the possibility that it may have a cilia specific function. Immunofluorescence analysis using known ciliary markers and super resolution microscopy revealed that ERK1/2 is localized at the transition fiber region of cilia. Pharmacological inhibition of ERK phosphorylation using a highly selective drug that inhibits the upstream activator of ERK1/2, MEK1/2, decreases the length and number of primary cilia, suggesting a role in ciliogenesis. In *Xenopus* multiciliated cells, inhibition of ERK1/2 leads to decreased ciliary flow, suggesting that ERK1/2 has a role in multiciliated cells development and/or function. Analysis of inhibitor treated embryos revealed a multitude of ciliogenesis defects including defective rotational polarity and basal body spacing, disrupted actin network, and reduced apical cell surface. These are consistent with defects elicited by loss of function approaches targeting JNK, suggesting that ERK1/2 may be acting upstream of JNK in the context of ciliogenesis. We go on to show that inhibition of ERK1/2 affects c-junNH2-terminal kinase's (JNK) activation at the base of the cilia providing evidence for a role of ERK1/2 in the regulation of basal body associated JNK. Overall, the findings of this study reveal a new function of ERK in the development of cilia and their function providing new insights towards our understanding of cilia-related diseases.

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COMPOSITION OF THE EXAMINATION COMMITTEE

Thesis Supervisor (Examination Committee coordinator): Prof. Paris A. Skourides, Dept. of Biological Sciences, UCY

Committee Member: Assoc. Prof. Chrysoula Pitsouli, Dept. of Biological Sciences, UCY

Committee Member: Assoc. Prof. Pantelis Georgiades, Dept. of Biological Sciences, UCY

SEMINAR ANNOUNCEMENT

University of Cyprus
Department of Biological Sciences

Master Research Dissertation in Biomedical Sciences (BIO 830/600)

Student Presentation

Friday, 12 May 2023 at 09:00

Building University of Cyprus Library, Room LRC 012, Panepistimioupoli Campus

This seminar is open to the public.

Chrystalla Nikolaou

Thesis Supervisor: Prof. Paris Skourides

"Extracellular Regulated Kinase (ERK): A novel regulator in the development and function of cilia"

The role of cilia in biomedicine is becoming more and more recognized since defects in ciliary structure and function induce ciliopathies such as cystic kidney disease, neurological anomalies, obesity, blindness, and skeletal deformities. Here, we examined the possible role of the Extracellular Regulated Kinase 1/2 (ERK1/2) in ciliogenesis and ciliary function. We show that ERK is associated with the basal bodies of primary cilia in cultured cells and in multiciliated cells of *Xenopus Laevis* raising the possibility that it may have a cilia specific function. Immunofluorescence analysis using known ciliary markers and super resolution microscopy revealed that ERK1/2 is localized at the transition fiber region of cilia. Pharmacological inhibition of ERK phosphorylation using a highly selective drug that inhibits the upstream activator of ERK1/2, MEK1/2, decreases the length and number of primary cilia, suggesting a role in ciliogenesis. In *Xenopus* multiciliated cells, inhibition of ERK1/2 leads to decreased ciliary flow, suggesting that ERK1/2 has a role in multiciliated cells development and/or function. Analysis of inhibitor treated embryos revealed a multitude of ciliogenesis defects including defective rotational polarity and basal body spacing, disrupted actin network, and reduced apical cell surface. These are consistent with defects elicited by loss of function approaches targeting JNK, suggesting that ERK1/2 may be acting upstream of JNK in the context of ciliogenesis. We go on to show that inhibition of ERK1/2 affects c-junNH2-terminal kinase's (JNK) activation at the base of the cilia providing evidence for a role of ERK1/2 in the regulation of basal body associated JNK. Overall, the findings of this study reveal a new role of ERK in the development of cilia and their function, providing new insights towards our understanding of cilia-related diseases.

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INTRODUCTION

1.Cilia

Cilia are tiny, hair-like protrusions of the cell membrane that can be found across many species, from nematodes to ancient protozoa. Protozoa and their cilia and flagella were first discovered in 1676 by Antony Van Leeuwenhoek, who wrote a letter to the Royal Society of London characterizing cilia as "diverse incredibly thin little feet…which were moved very nimbly.and wherewith they brought off incredibly quick motions" (Haimo and Rosenbaum, 1981). Cilia and flagella are cell organelles with similar structure but different function. When they are present alone or in pairs, are named flagella, while when there are numerous, are called cilia. Both flagella and cilia are synthesized by the assembly of proteins onto a centriole-like basal body and have a similar internal organization of accessory structures, microtubules, and motor proteins (Lindemann and Lesich, 2010).

The length of cilia is between 5-7μm long and less than 1μm in diameter (Keeling, Tsiokas and Maskey, 2016). They are structurally separated into five sub-compartments: the basal body, the transition zone, the axoneme, the ciliary membrane, and the ciliary tip (Fig.1) (Fliegauf, Benzing and Omran, 2007). These dynamic structures are categorized into motile and non-motile subtypes and their axoneme is a 25μm diameter cytoskeletal scaffold made up of proteins (Mitchison and Valente, 2017).

2. Categorization of cilia

Based on their ability to move, cilia are divided into motile and non-motile, primary cilia.

2.1. Motile cilia

Motile cilia are used by many organisms for movement, nutrition, and sensing. Their ability to move is due to dynein which is a molecular motor that hydrolyzes ATP to apply force on microtubules, enabling the cilia to bend repeatedly (Cicuta, 2020). Some of these organisms generate thousands of cilia with a process known as multiciliation. Multiciliated cells may also be

found in the adult brain's spinal cord and ventricles to promote polarized fluid flow for cerebrospinal fluid circulation and neuronal migration (Sawamoto et al., 2006). In addition, the synchronized motion of cilia in the surface epithelium of the airways, provides the required force for mucociliary clearance which is a crucial innate defensive mechanism to maintain the pulmonary system's health and function (L.E Ostrowski and W.D Bennett, 2006). Motile cilia are also found in the fallopian tubes during the female reproductive cycle and when the egg starts to migrate toward the uterus, they oscillate to hit peak flow (Hagiwara et al., 1997). Lastly, the leftward fluid flow of extraembryonic fluid triggered by nodal cilia defines the left-right axis of organ development (Nonaka et al., 1998a).

2.2. Primary cilia

Primary cilia appear both on epithelial cells of endocrine pancreas, kidney tubule, and thyroid, as well as on non-epithelial cells like fibroblasts, neurons, and smooth muscle cells (Satir and Christensen, 2007). Primary cilia obtain multiple roles in chemosensation, mechanosensation, and photosensation that regulate proliferation, cell polarity and developmental signaling (Smith, Lake, and Johnson, 2020). Primary cilia have important functions in the neuronal system since mutations in the genes involved in ciliogenesis cause developmental defects such as abnormal body axis and brain malformation (Goetz and Anderson, 2010). In vertebrate development, the primary cilium plays a crucial role as signaling centers engaging in signal transduction pathways like hedgehog (Hh) which is found to be entirely liable to cilia (Bangs and Anderson, 2017). Moreover, the primary cilium, through the hedgehog signaling pathway, regulates muscle stem cells regeneration and may serve as a potential therapeutic target for the prevention of the aging-related reduction of muscle regeneration ability (Palla et al., 2022). Additionally, primary cilia are implicated in signaling cascades like Wingless (Wnt) and Sonic Hedgehog (SHH) signaling pathway (Lee and Gleeson, 2011).

Hence, cilia abnormalities have the ability to harm numerous organs and cause a wide range of symptoms, including cognitive deficits, neurological disorders, heart, kidney and respiratory dysfunction, and anosmia.

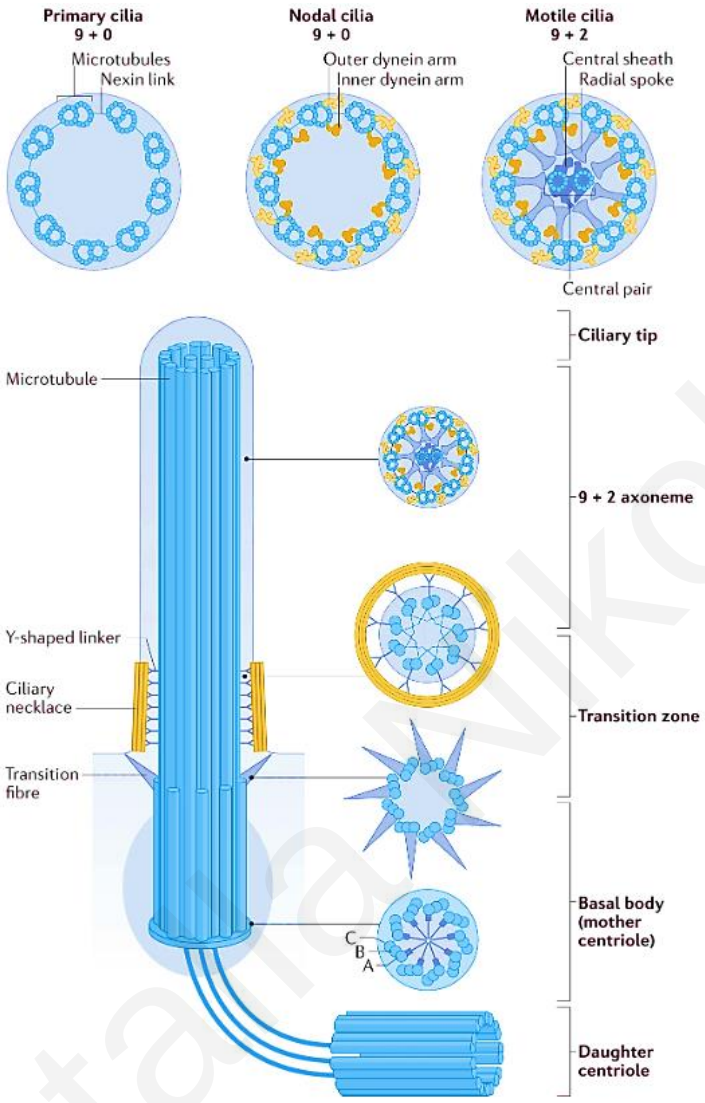


Fig.1 Architecture of Cilia. A cilium contains several pairs of microtubules, running from the basal body and forming the axoneme. At the transition zone, we have the microtubule doublets containing an A-tubule (A) and a B-tubule (B) which necessities radial spokes that extend into the axonemal center to held together. The communication between the doublets is happening via nexin connections (Djenoune et al., 2022).

3. Architecture of cilia

The main compartments of cilium are the ciliary tip, the axoneme, the transition zone and the basal body which is a specialized centriole that serves as the microtubule organizing center that nucleates axonemal microtubules (Fig.1) (Fliegauf, Benzing, and Omran, 2007a). Distal appendages (DAPs) are found on the basal body and are nanoscale, pinwheel-like structures. They are also referred to as transition fibers as well. During ciliogenesis, DAPs protrude from the distal end and mark the base of the cilia around the ciliary gate (Yang et al., 2018). The axonemes extend from the basal body to the apical cell surface and have a 5- to 10-μm length and 300-nm diameter (Ishikawa, 2017).

In motile cilia, the axonemes have nine doublet microtubules of conjoined A tubules and B tubules (Brown and Zhang, 2020). Each microtubule has a complete A tubule which contains thirteen tubulin subunits, and a half B tubule containing 11 subunits (L.E Ostrowski and W.D Bennett, 2006). Motile cilia contains the 9+2 pattern with nine microtubules arranged in a ring around a central pair of microtubules. In order for the doublets to be fixed in position, radial spokes extend into the center of the axoneme and are connected by elastic nexin links (Fig.2). This prevents the microtubules from sliding against one other. Radial spokes operate as spacers, allowing the doublets to form a ring around the core pair of microtubules. The radial spokes and the central pair are important in beat regulation (Fliegauf, Benzing, and Omran, 2007).

Ciliary movement is controlled by axonemal dynein motors. These catalytic dyneins are carried by the inner and outer dynein arms, which are two rows of massive multiprotein complexes that are attached to each entire tubule (Fig.3) Dynein transfers ATP hydrolysis energy into ciliary axoneme and generates bending motion by connecting to a neighboring microtubule and undergoing a conformational shift, forcing the microtubules to move (L.E Ostrowski and W.D Bennett, 2006). These sliding movements between the neighboring microtubules create a well-choreographed beating (Ishikawa, 2017). Primary cilia typically have a 9+0 pattern because of the absence of the central pair of microtubules. In the case of primary cilia, we do not have the phenomenon of multiciliation, that we observe in the Multiciliated motile epithelial cells (Satir and Christensen, 2007).

Cilia have extremely complex structures that contain more than 650 proteins. The movement of proteins over the ciliary compartment dynein boundary and through the length of the axonemes is accomplished through Intraflagellar transport (IFT) (Fliegauf, Benzing, and Omran,

2007). Primary cilia and motile cilia are gathered and maintained by IFT (Drummond, 2012). The axonemal microtubules in both motile and primary cilia serve as a rail for intraflagellar transport (IFT). This includes the movement of ciliary proteins by cargo-laden megadalton complexes known as IFT trains. Two kinds of transportation take place; anterograde transport occurs on the B tubule, while retrograde transport happens on the A tubule. The swap between the anterograde (kinesin) and retrograde (dynein) intraflagellar transfer motors is located at the ciliary tip. The ciliary tip houses also the microtubule plus + ends from which the axoneme is developed (Fliegauf, Benzing, and Omran, 2007a). This partitioning of IFT trains into separate tubules enables effective long-distance transport since it eliminates collision between trains moving in opposing directions. This segregation cannot occur in primary cilia because they contain predominantly singlet microtubules (Brown and Zhang, 2020).

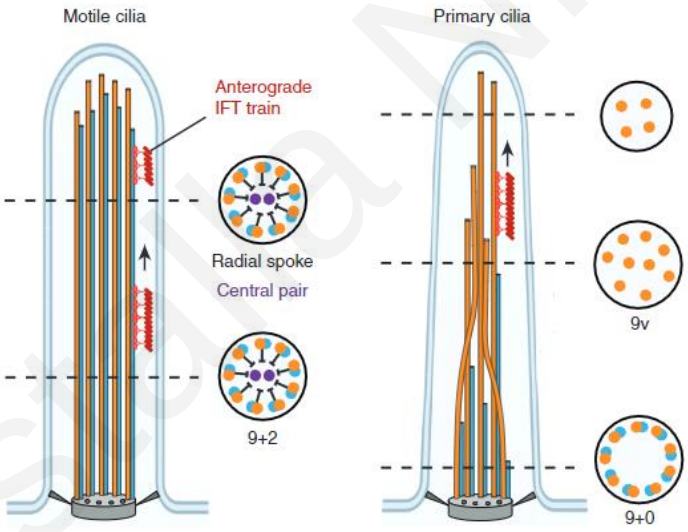


Fig.2 Architecture of motile and primary cilia. Motile cilia contains a 9+2 microtubules structure while the central pair of microtubules is absent in primary cilia (Brown and Zhang, 2020).

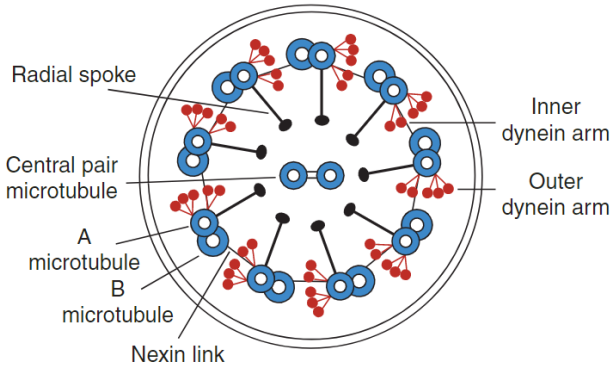


Fig.3 Section of cilia microtubule showing how microtubules are arranged (L.E Ostrowski and W.D Bennett, 2006)

4.Ciliogenesis

Cilia derive from centrioles, which are complex microtubule-based structures. Centrioles form the basal body necessary for the generation of cilia and flagella. Also, a pair of centrioles encased in a protein matrix known as the pericentriolar material PCM constitute the center of the centrosome (Nigg and Raff, 2009). The formation of the cilium takes place during G1 or G0 and disassembles near the time of mitosis. The mother centriole docks at the cell cortex and nucleates the cilium during G1, while the associated daughter centriole from the previous division does not. Then there is the duplication of the mother and daughter centrioles in order to generate the new daughter centrioles during S-Phase. This results in the detachment of the centriole pairs from the cortex, which then move to the spindle poles. Then, cilia reform in the two daughter cells following G1 re-entry (Fig.4) (Ishikawa and Marshall, 2011).

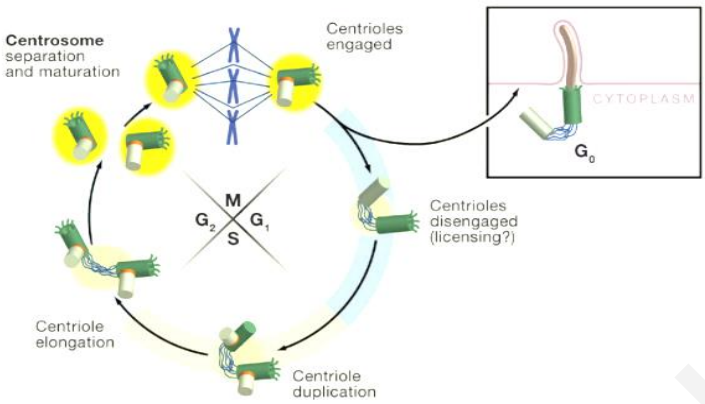


Fig.4 Centriole activity during the cell cycle. At the end of mitosis, each daughter cell carries over a single pair of disengaged centrioles. The cells then enter G1 or alternatively a dormant state G0, during which many cell types generate cilia. Centrioles double in cycling cells during S phase, generating procentrioles, showing in light green, staying closely connected with their mother centrioles (dark green) and steadily elongating during S and G2. The centrioles gather additional pericentriolar material (PCM, yellow) at the G2/M transition. Then, centrosomes begin to split from one another, and becomes the mitotic spindle poles. (Nigg and Raff, 2009)

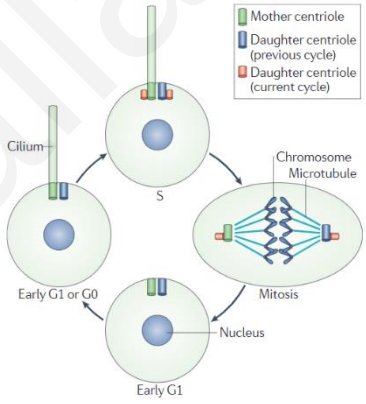


Fig. 5. Ciliary assembly and disassembly with the cell cycle (Ishikawa and Marshall, 2011)

Stages of Ciliogenesis

Axoneme nucleation

The generation of cilium initiates with the docking of a ciliary vesicle (CV) to the mother centriole, most likely via the distal appendages (Reiter, Blacque, and Leroux, 2012). Nucleation of the axoneme requires the exit of centrioles from the mitotic cycle (Avasthi and Marshall, 2012).

Centriole migration

Centrioles are referred to as basal bodies after the attachment of distal appendages and the docking to a CV that merges with the plasma membrane (Lemullois et al., 1988). This process appears to be associated with the actin cytoskeleton (Dawe et al., 2009), and also with membrane-dependent transition zone proteins like MKS1 and MKS3 (Dawe et al., 2007).

Formation of the ciliary membrane

Then, the axoneme elongates to form the ciliary membrane. In multiciliated cells, we observe direct interaction of the basal body with plasma membrane which induces axoneme elongation. In both cases, the developing cilium acquires transition-zone-like characteristics along the expanding axoneme and ciliary membrane (Reiter, Blacque, and Leroux, 2012).

Expansion of cilium

The expansion of the ciliary axoneme and membrane happens due to the intraflagellar transport that includes bi-directional trafficking of IFT protein complexes and microtubule motors (Avasthi and Marshall, 2012)

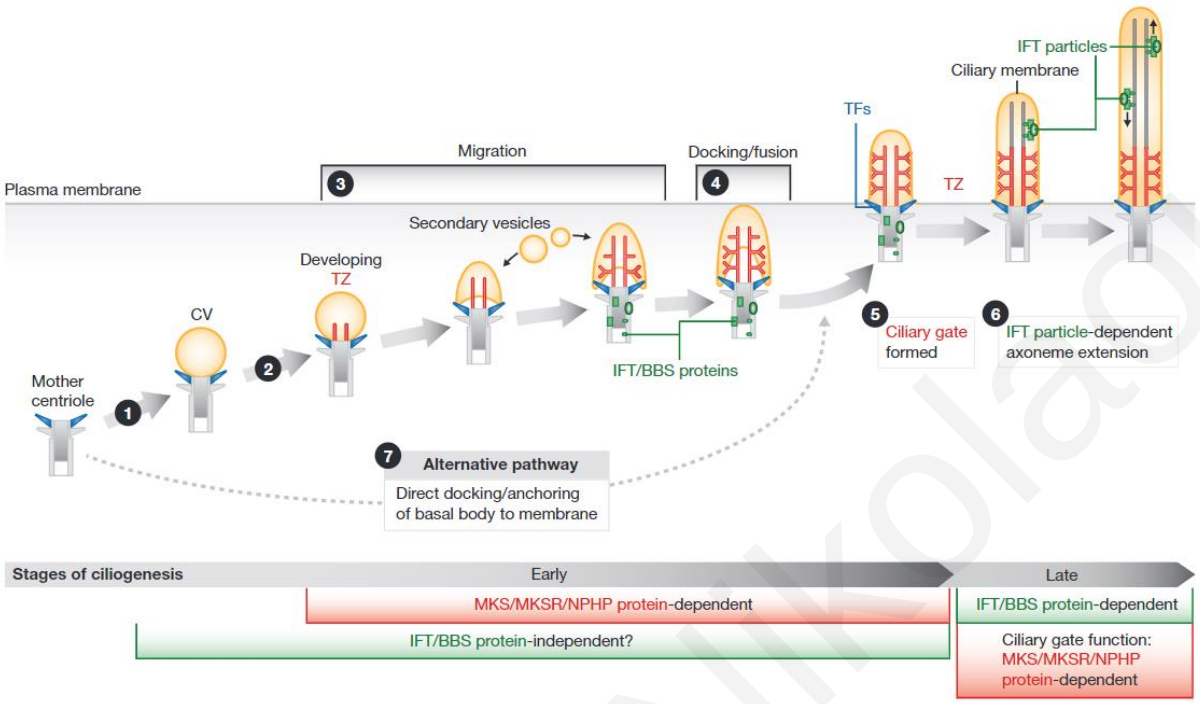


Fig.6 Stages of ciliogenesis: A transition zone region emerges and invades the ciliary vesicle (2). The maturing TZ migrates to the plasma membrane (3) and fuses with it (4). In this point the basal body and the CV forms the ciliary gate (5). Intraflagellar transport/ Bardet–Biedl syndrome IFT/BBS proteins are necessary in order to generate the final form of the axoneme that will result to a functional cilium (6). Different cell types may follow an alternative pathway (7) for this procedure that does not include CV, and the basal body does a direct docking to the membrane (Reiter, Blacque and Leroux, 2012a).

Multiciliated Cell development is more complex process. First, specification of cells occurs in the basal layer of the ectoderm, and then hundreds of centrioles are amplified. Simultaneously, multiciliated cells migrate upward into the outer epithelial layer in a mechanism termed as radial intercalation, and the apical surface expands. Finally, the basal bodies and filamentous actin (F-Actin) form an orderly array to accommodate numerous motile cilia (Fig.7) (Rao and Kulkarni, 2021).

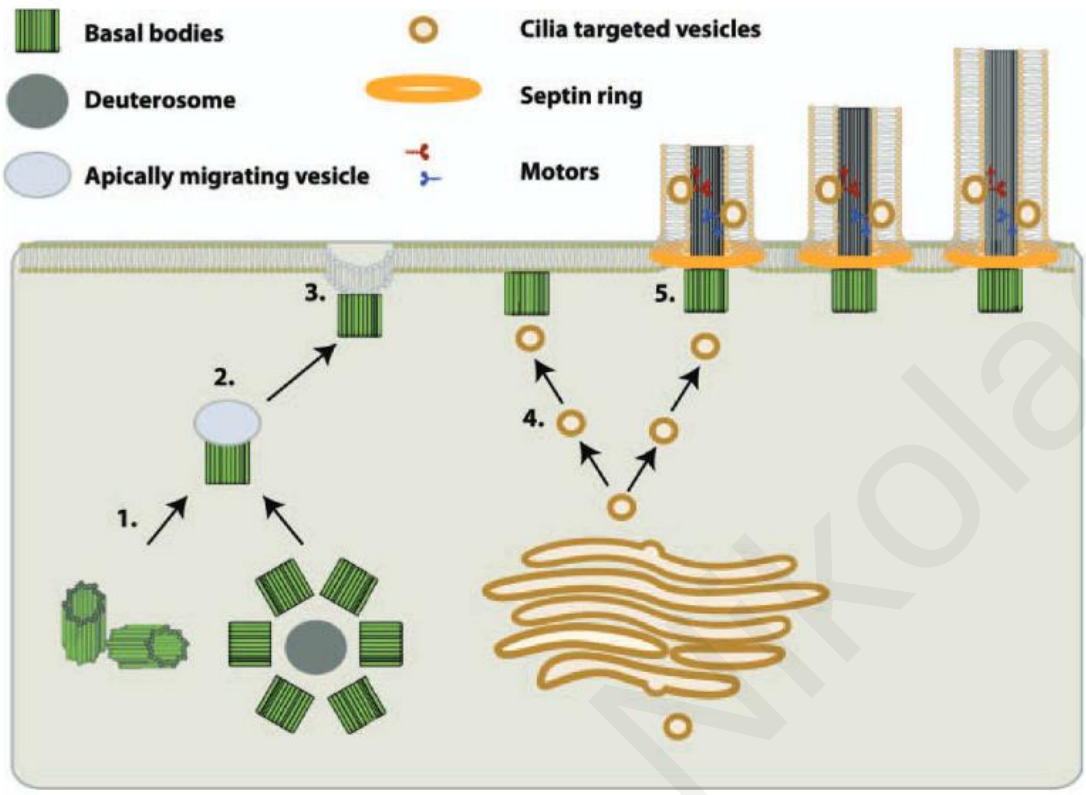


Fig. 7. Ciliogenesis of multiciliated cells. 1. Centriole duplication. 2. Fusing of basal bodies to apically moving vesicles. 3. docking with the apical membrane, 4. targeting of cilia components to the basal body and ciliary membrane, 5. axoneme elongation (Werner and Mitchell, 2012).

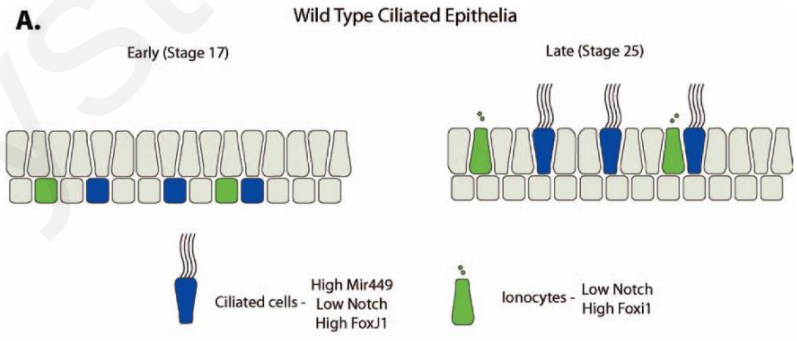


Fig. 8. **Ciliated epithelia.** In wild type ciliated epithelia ciliated cells and ionocytes progenitors reside in an epidermal sublayer and undergo radial intercalation to join the outer epidermis by stage 25. Ciliogenesis is controlled by Notch signaling pathway (Werner and Mitchell, 2012)

5. Diversity in Cilia

In the human body, there are eight types of known cilia or cilia-derived organelles.

Mucus-propelling cilia

Mucus-propelling cilia (respiratory cilia) are motile cilia that are found in the upper and lower airways. Their role is to propel the mucus blanket that is resting on the ciliary tip by pushing the mucus forward with their effective down-stroke (Afzelius, 2004).

Water-propelling cilia

Those are motile cilia including the brain ependymal cilia that circulate cerebrospinal fluid. Also, they are found in the efferent ducts of the testis and the oviduct. Motile sperm floats into the uterus and up into the oviducts using their flagella. During their journey, they meet a flow in the opposite direction caused by oviduct cilia in order to drive the ovum from the ovary to the uterus (Brown and Witman, 2014).

Nodal cilia

Nodal cilia are motile and arise and become activated in the 7.5-day post-coitum embryo. They are found in one cilium per cell and their beating pattern is vortical (Nonaka et al., 1998b).

Monocilia

Monocilia protrude from the apex of the cell in a single direction and are known as primary cilia. Under certain circumstances, monocilia may develop on a cell that will subsequently generate mucus-propelling cilia, or water-propelling cilia (Odor and Blandau, 1985). Monociliated cells are also found in the corneal endothelium (Svedbergh and Bill, 2009). Also, modified primary photoreceptor cilia are found in the outer segments of the retina's rod and cone photoreceptor cells and are responsible for sensing light (Wheway, Parry, and Johnson, 2014). Another type of cilia are rudimentary cilia which are found in neurons and glia cells (Ki, Jeong, and Lee, 2021). Another type of cilia are the olfactory cilia which are found in the olfactory system that gives the ability to smell. The membranes of olfactory cilia are able to detect chemicals and transmit messages to the

brain where they are translated as smell due to the fact that are rich with odorant receptor proteins (Jenkins, McEwen, and Martens, 2009).The kidney collecting duct and tubule cilia are two of the most significant nonmotile cilia. These cilia recognize fluid movement throughout tubules and ducts and assist cells of the kidney in maintaining appropriate cell division patterns (Fliegauf, Benzing, and Omran, 2007b). Defects in ciliary function is linked to pathogenesis of cystic kidney disease, that happens due to abnormal fluid flow and activation of procystogenic pathways (Dell,2015).

6.Mechanisms of beating

The ciliary motion of motile cilia is crucial for optimal respiratory function, and any disruption can result in severe respiratory diseases. Cilia have a synchronized rhythmic beating that moves foreign materials that are trapped in a layer of mucus, which is also responsible for epithelial moisture, out of the upper airway. Then, this mucus is continually pushed toward the throat and is consumed or ejected via the oral cavity (Jing et al., 2017).

In 1965, dynein was identified to be source of the molecular driving force for the flagellar beating. Following, in 1971 it was discovered that the propagating bending movement of sperm tails are caused by forces triggered by ATP between the outer tubules. Specifically, dynein activity in the presence of Mg-ATP causes microtubule doublets to slide across each other's length is a (Summers and Gibbons, 1971). Later on, it was shown that each doublet's dynein moves by acting on the neighboring doublet; the flagellum or the cilium bends in one direction due to the dyneins that are found on one side of the ciliary axoneme, while bending in the opposite way is due to the dyneins on the other side (Lindemann and Lesich, 2010). In order for the axoneme to bend and propagate bends, dyneins on one side must be active while the other side inactive, and these states must flip (Fig.9). Active dyneins move towards minus ends, enabling active sliding between doublets while the dyneins on the inactive position, walk passively in an antithetical direction (Fig.9). The propagation of the beat along the axoneme is regulated spatially and temporally (Mitchison T.J. and Mitchison, 2010). Using computer simulations, it was demonstrated that beating is self-organized by utilizing the inherent physical characteristic of the axoneme. Specifically, by regulating dynein via axoneme local curvature or altering the sliding distance between doublets (Brokaw, 2009).

Uncoupling the mechanism behind the movement of flagellar and cilia; the sliding that is observed modulates motor activity to induce self-organized oscillations, a theory that requires a system of opposing motors and springs (Fig.10) (Mitchison T.J. and Mitchison, 2010). The physical parameters that control the bending forces are the stiffness of the axoneme itself and the viscosity. The axoneme can deform in the direction of an applied force since the deformation activates its internal dyneins and it can be quantified by utilizing negative stiffness and viscosity values (Camalet, Jülicher and Prost, 1999).

Stiffness and friction of the active material inside the axoneme are the modifiable parameters, while the hydrodynamic drag, conventional stiffness, and beat frequency are considered fixed parameters (Hilfinger, Chattopadhyay and Jülicher, 2009). As we observe in Fig.10 b one group of the motors, wins (solid curve) and exerts a force on the losing motors (dotted curve) in the opposite direction of their movements. The presence of the springs is important, and it showed in the model in Fig.5, since in the absence of them, the system is unstable, and it grows the probability that the losing motors will become detached from the microtube (Mitchison T.J. and Mitchison, 2010).

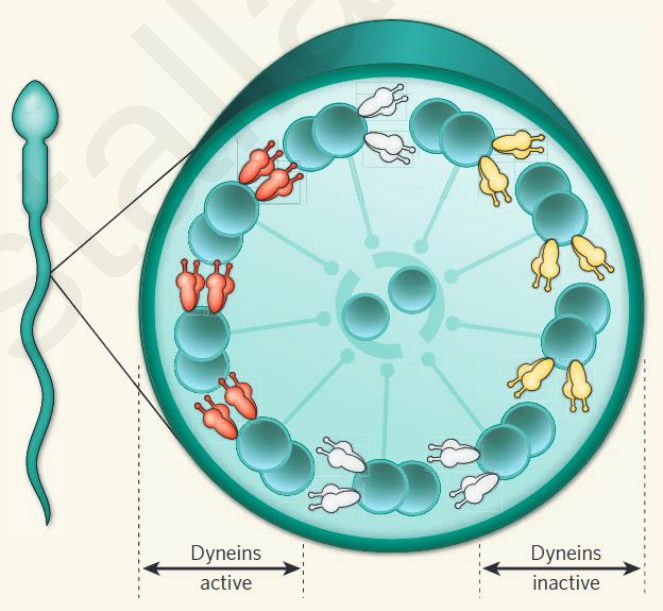


Fig.9 Dynein regulation in axoneme cross-section from a sperm tail Doublets of microtubules are shown as overlapping circles. Whenever dyneins at one side are active (red), and dyneins on the opposing side dormant, axonemes bend (yellow) (Mitchison T.J. and Mitchison, 2010).

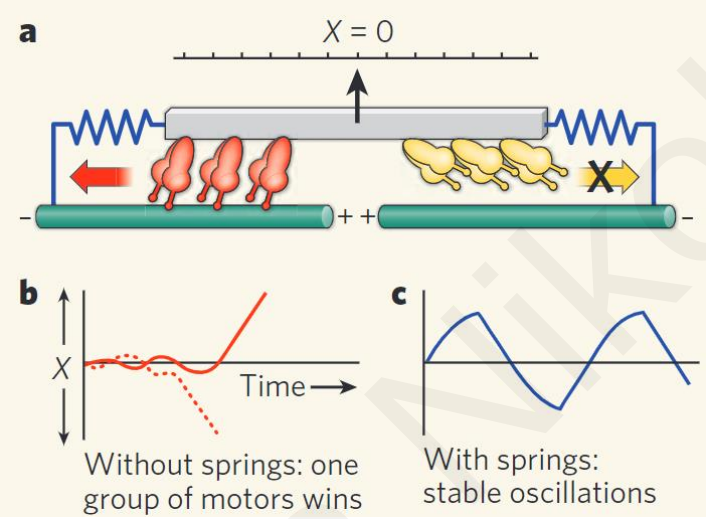


Fig.10 Opposing motors and springs model system showing the self-organization of oscillations. a) Experimental model demonstrating how sliding control leads to oscillations. Two sets of dynein motors (red and yellow) are attached to a rigid scaffold, they move outwardly on two static microtubules with their minus ends pointing outwards. Springs may be excluded (blue zig-zags). b) The springs are missing, resulting in an unstable system c) The springs are present, and the system oscillates in a stable manner. The oscillations are self-organized, and they do not demand external control of the motors (Mitchison T.J. and Mitchison, 2010).

Studies have shown that TH2 cytokines and interferon gamma influence the human respiratory cilia beat frequency (Grosse-Onnebrink et al., 2016). Other parameters that affect the ciliary beat frequency (CBF) are humidity, temperature, medication, and noxious stimuli exposure (Jing et al., 2017). An example of a medication that affects ciliary beating is Tranexamic acid TXA, which is given to patients suffering with recurrent epistaxis and in particular as a result to hereditary hemorrhagic telangiectasia (HHT), to minimize bleeding episodes. Patients receiving

TXA were found to have alterations in the ciliary beating frequency and general impairments of the nasal epithelium function (Behr et al., 2021).

Additionally, researchers found a model in which calcium ions operate as coordinators and catalytic activators of dynein attachments, advocating that the microtubules embedded in axoneme doublets have polyelectrolyte characteristics that allow the production and movement of Ca^{2+} ionic clouds. Those clouds slide down microtubule doublets at a rate equivalent to the ciliary beat's rate of propagation (Satarić et al., 2020).

7.Ciliopathies

Ciliopathies are a family of human pathologies that are associated with mutations in the genes that are necessary for ciliary structure and function. Examples of ciliopathies are polycystic kidney disease (PKD), Meckel-Gruber syndrome (MKS), Bardet-Biedl syndrome (BBS), Senior-Loken syndrome, nephronophthisis (NPHP), Leber congenital amaurosis (LCA), Joubert syndrome (JS) and oral-facial-digital syndrome (OFD) (Waters and Beales, 2011). Symptoms of ciliopathies are blindness, retinal degeneration, cystic kidneys, polydactyly, and other skeletal abnormalities. Also, cognitive difficulties, fluid accumulating in the brain, and cognitive difficulties are effects that were found in patients with ciliopathies (Brown and Witman, 2014).

Dysfunction of motile cilia often leads to Primary Ciliary Dyskinesia (PCD), a group of diseases that causes difficulties in the removal of mucus from airways, a process known as mucociliary clearance (MCC). Defective MCC can contribute to persistent respiratory infections (Jing et al., 2017). Specifically, the motile cilia have defects resulting in failure of the elimination of mucus from airways. PCD patients suffer from chronic bronchitis and sinusitis. Also, male PCD patients suffer from infertility due to the altered mobility of the sperm flagellum. Approximately half of the patients with PCD show a reduction in nodal cilia motility, resulting in situs inversus, where internal organs are growing in opposite placements (Brown and Witman, 2014). Inadequate mucociliary clearance (MCC) is linked to several respiratory disorders, including chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF).

8. Xenopus Laevis as a model in research

Xenopus Laevis presents many advantages as a research model due to their big and robust eggs and a large number of embryos that are available at all developmental stages. Utilizing *Xenopus* embryos can help to understand human development and disease due to the conservation of essential cellular processes as well as developmental processes and the high degree of genetic synteny with mammals (Harland and Grainger, 2011). The first time that *Xenopus laevis* was employed was in clinical human pregnancy tests. In response to mammalian hormones and particularly the chorionic gonadotropin generated during pregnancy, *Xenopus* is capable to produce eggs all year (Gurdon and Hopwood, 2000).

The characteristics that make Xenopus eggs and embryos good candidates for research are as follows:

1. The embryos are able to tolerate intensive surgical manipulations which vary from extremely delicate procedures (single cell transplantations, extensive "cut and paste" techniques that evaluate significant parts of the embryo in several environments).
2. The ability to easily inject material into eggs and embryos. This feature makes them one of the best animal models for studying the functions of gene products. The material can range from nuclei, as in traditional animal cloning studies, to a variety of macromolecules, commonly proteins and nucleic acids.
3. Eggs and embryos offer a generous source of material for biochemical research. For instance, they have been extensively used to uncover the biochemistry of the cell cycle, and signal transduction pathways that control development (Harland and Grainger, 2011). Also, Xenopus laevis has made significant advances to cancer research and in the understanding of early development of tumorigenesis, pathogenesis and metastasis (Hardwick and Philpott, 2015).

Due to the fact that frogs offer the chance to study organ development from the earliest stages, all developmental phases can be easily manipulated, and critical brain development mechanisms are preserved across Xenopus and humans, Xenopus becomes an advantageous model for studying brain development which necessitates comprehending all phases from fertilization to organogenesis and beyond (Exner and Willsey, 2021). Besides developmental and molecular

biology, Xenopus laevis is also used in experimental medicine to explore key components of neuropathology, psychotic and systemic disorders, epilepsy, and pharmacology (Horn, 2006). For instance, using Xenopus oocytes to study hyperkalemia and hypertension disorders (Yang et al., 2003) and test drugs that affect the membrane channels, such as clozapine, an atypical antipsychotic (Kobayashi, Ikeda, and Kumanishi, 1998). Additionally, studies about viral diseases like the human immunodeficiency virus type 1 HIV-1(Hsia and Shi, 2002) , glioma, and the expression of neurotransmitter receptors in astrocytoma (Matute et al., 1992), and investigate the properties of Alzheimer's brain regarding its function, structure and how membrane proteins and channels are affected (Miledi et al., 2003). Impressively, Xenopus was also used for biological research in space in 1975, when flew for the first time, and in 1995 the first successful fertilization occurred and eggs grew into vital tadpoles in microgravity (Horn, 2006).

Xenopus laevis as a model to study cilia

Xenopus larva skin is suitable for studying ciliogenesis and ciliary function due to their ciliated epithelium that transports fluid unidirectionally over their surface (Werner and Mitchell, 2013), and simple to image the whole animal using microscopy (Werner and Mitchell, 2012). *Xenopus* ciliated cells, unlike the mammalian MCCs, are external and are growing rapidly. This creates a vigorous and directed fluid flow within days of fertilization(Rao and Kulkarni, 2021). In addition, *Xenopus* has been used to study both the structure and the function of basal bodies of cilia as well as their transcriptional regulation and polarity (Zhang and Mitchell, 2016). Therefore, studying Xenopus can shed light on molecular mechanisms that cause cilia-related diseases, called ciliopathies and airway diseases (Walentek and Quigley, 2017).

Molecular techniques available for the manipulation of the Xenopus ciliated epithelia are listed below (Werner and Mitchell, 2013).

1. Induction of expression of cilia-related proteins via targeted injection of mRNA containing fluorescent tags.
2. mRNA injection into blastomeres

3. Use of α-tubulin promoter to limit expression to ciliated cells. α-tubulin drives gene expression during the formation of Multiciliated cells beginning at stage 13 and throughout cell differentiation and development.
4. Create knockout phenotypes using morpholino oligonucleotides MOs to attach to the target mRNAs, disrupt the protein translation, and prevent the creation of a functional protein.
5. MOs can also be used to create mosaic embryos (Werner and Mitchell, 2013).

10. Mitogen-activated protein kinase (MAPK) cascades

Intracellular signaling pathways govern cellular behavior by reacting to extracellular stimuli (Zarubin and Han, 2005). The mitogen-activated protein kinase (MAPK) cascades are pivotal signaling pathways that mediate a variety of cellular processes, like cell division, proliferation, stress reactions, and apoptosis (Seger and Krebs, 1995). The pathway consists of 3-5 kinases that transmit signals by sequentially activating and phosphorylating downstream proteins. Those kinases are called MAPK, MAPK-activated protein kinases (MAPKAPK), MAPK kinase kinase kinase (MAPK4K), MAPK kinase kinase (MAP3K, MAPK kinase (MAPKK) (Guo et al., 2020). The three main kinases activate its downstream kinase forming a three-part signal relay. The activated MAPK kinase (MAPKKK or MEKK) activates the MAPK kinase (MAPKK or MEK), which then activates the MAPK. MAPKKKs have an N-terminal regulatory domain and a C-terminal serine/threonine protein kinase domain. When a MAPKKK is phosphorylated then, it activates the downstream target MAPKK by phosphorylating the two serine or threonine residues in its activation loop. The MAPKK is a protein kinase with a dual-specificity (phosphorylating both serine / threonine and tyrosine). Following, the threonine and tyrosine residues of a conserved -*Thr-X-Tyr*- motif in the target MAPK's activation loop are phosphorylated by the activated MAPKK. When there are point mutations that alter the phosphoacceptor residues (Glu or Asp) to acidic residues, MAPK activation is not possible. This happens due to the fact that the phosphorylations that occur arise significant conformational changes. As a result, to extracellular stresses and molecular signals, MAPKs activate a variety of substrates, including translational

regulators, MAPK-activated protein kinases (MAPKAPK), phosphatases, transcription factors that eventually regulate gene expression, metabolism, cell cycle progression, and cellular morphology (Chen and Thorner, 2007).

The mammalian MAPK family includes three subfamilies: p38, c-junNH2-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK). Every protein have multiple isoforms; JNK1 to JNK3, p38-α, -β, -γ, -δ, and ERK1 to ERK8 (Kim and Choi, 2010). The JNK and p38 MAPK pathways play an important role in apoptosis, and cell stress, while ERK/MAPK signaling pathway is involved in differentiation and cell proliferation making the cell signal transduction network essential (Guo et al., 2020). Transcription factors like Elk-1, c-Jun, ATF2, and p53 are among the many substrate proteins that are phosphorylated by activated MAPKs.

There are two ways by which MAPK pathways are regulated:

1. Through a chain of binary interactions between the components of kinases
2. Through a scaffold protein that regulates the creation of a signaling complex involving various kinases.

These scaffold proteins moderate the activation of kinases that are associated with the MAPK signaling pathways. JNK signaling pathway uses JIPs as scaffold proteins. For the ERK signaling pathway, these scaffold proteins are the kinase suppressor of Ras-1 (KSR) and MEK partner 1 (MP1). β-Arrestin 2 is also utilized as a scaffold protein by both ERK and JNK pathways. The JNK and p38 pathways are activated upon the presence of pro-inflammatory cytokines like interleukin (IL)-1β, and tumor necrosis factor (TNF)-α, as well as cellular stresses like genotoxic, osmotic, hypoxic, or oxidative stress. While the ERK pathway is activated by the receptor tyrosine kinase (RTK)-Grb2-SOS signaling axis (Kim and Choi, 2010). After activation, the components of the pathways are categorized into the two groups (MAPKK, MAPKKK) mentioned before.

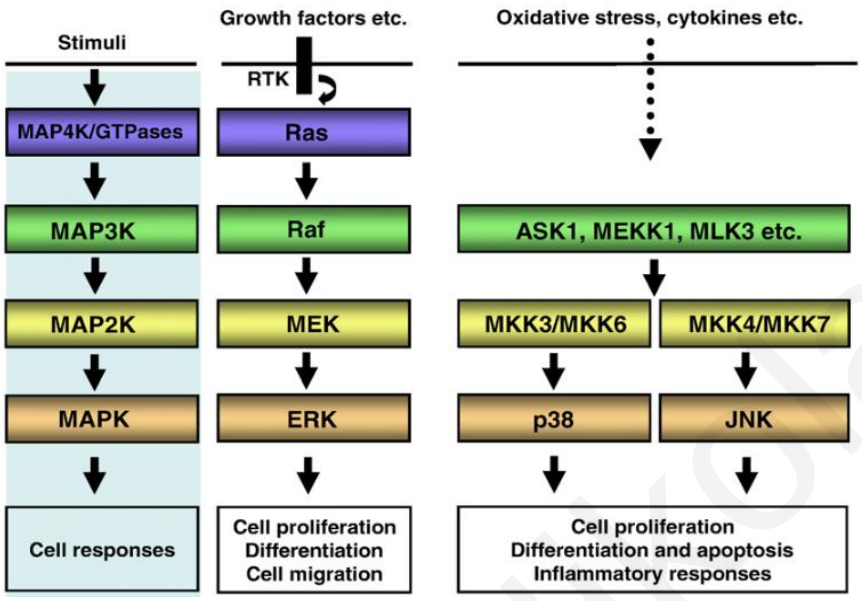


Fig.11 The Mitogen-Activated protein kinase signaling pathways.

11. JNK pathway

JNK is encoded by three genes JNK α, β, γ, and twelve isoforms may result from alternative splicing processes (Ruvolo, 2001). The JNK pathway is activated upon the presence of pro-inflammatory cytokines like tumor necrosis factor (TNF)-α and interleukin (IL)-1β, as well as cellular stresses like hypoxic, osmotic, genotoxic, or oxidative stress.

The Components of the JNK pathway are:

- MAPKK → SEK1 (aka MKK4) or MKK7
- MAPKKK → ASK1, MEKK1, mixed-lineage kinase (MLK), or transforming growth factor-β-activated kinase 1 (TAK1)

After its activation by MAPK3 and MAPK2, JNK phosphorylates c-Jun on Ser-63 and Ser-73 by binding to the NH2-terminal activation domain. This triggers an increase expression of genes that includes an AP-1 transcription factor site in their promoters, resulting in creating a positive feedback loop. C-Jun, p53, ATF-2 (activating transcription factor 2), Sap-1a, Elk-1, NFAT4, and

DPC4 have been identified as JNK substrates (WIDMANN et al., 1999). Interestingly, it has been shown that there is a cross-talk between JNK and ERK. Specifically, JNK kinases can be triggered by ERKs as it was studied in using the growth factor VEGF (Pedram, Razandi, and Levin, 1998). For the activation of JNK, VEGF-induced ERK was both required and sufficient. Also, both kinases were involved in mediating the effects of VEGF on cell proliferation. These results show that JNK serves as ERK's final mediator in the stimulation of cell proliferation (Pedram, Razandi, and Levin, 1998).

The JNK pathway has been linked to both survival and apoptosis signaling (Zhang, Liu, and Tu, 2002). Moreover, during UV-induced apoptosis in fibroblasts, JNK is necessary for the release of cytochrome C from the mitochondria (Tournier et al., 2000). Additionally, studies show that JNK is crucial for insulin resistance and pro-inflammatory macrophage polarization in obesity. Also, JNK activity appears to affect cardiac remodeling following ischemic injury/MI (Craige et al., 2019).

12. p38 pathway

The p38 gene has four splice variants. Based on the type of tissue the different variants p38 γ and p38 δ are expressed whereas the remaining are ubiquitously expressed (Zarubin and Han, 2005). The p38 pathway is activated by cellular stresses such as high osmotic stress, UV irradiation, protein synthesis inhibitors, heat shock, proinflammatory cytokines (eg. IL-1 and TNF-α), specific mitogens, and lipopolysaccharides (Zhang, Liu, and Tu, 2002).

The phosphorylation and thus the activation of all four isoforms of p38 can be triggered by the MAPK kinase MKK6. MKK3 can also phosphorylates p38 α, γ, δ, while MKK4 phosphorylates only p38 α (Raingeaud et al., 1996). The phosphorylation that induce the activation of p38 happens is dual and it happens on Thr180 and Tyr182, which are on the Thr-Gly-Tyr motif found on its activation loop. The phosphorylation alters the conformation of the p38 protein to a more open structure that allows the molecule to rotate giving it the ability to boost the kinase's activity. The activation can also happen in a non-canonical way in the T-lymphocytes when antigen T cell receptor TCR is present. In this case, only Tyr323 is phosphorylated, and an auto-phosphorylation loop is stimulated. In addition, other stimuli like myocardial ischemia,

intracellular infection, or signals associated with the maturation of dendritic cells (DCs) are alternative ways for p38 activation (Fig.12) (Martínez-Limón et al., 2020).

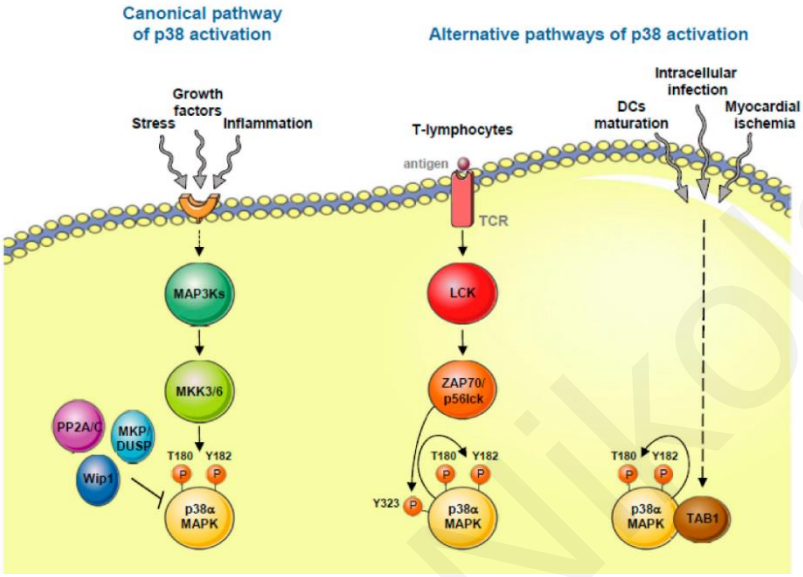


Fig.12 Canonical and alternative pathways of p38 activation

p38 is involved in cell death, inflammation, development, cell cycle, cell differentiation, cancer and, senescence (Zarubin and Han, 2005). Additionally, it appears to play a role in cancer by functioning as an anti-tumorigenic factor due to its pro-apoptotic activity which is related to its capability to inhibit tumor growth (Martínez-Limón et al., 2020). In addition, it has been shown that p38 plays a significant role in Interferon IFN signaling through the STAT1 serine phosphorylation (Goh, 1999).

13. ERK pathway

The ERK/MAPK signaling pathway is crucial to the signaling network since it regulates development, cell growth, and division (Zhang, Liu, and Tu, 2002). It is one of the first cascades that explain how extracellular compounds, like growth factors and hormones, might produce signals that influence gene expression and the behavior and biological programming of the cell (Kolch, 2005). The ERK pathway is activated by a variety of stimuli, such as oncogenes, growth hormones, G-protein-coupled receptor ligands, cytokines viruses. The fundamental signal

transmission stages are as analyzed before, including an upstream activator sequence (Ras), a MAP3K (Raf), a MAP2K (MEK1 or MEK2), and a MAPK (ERK1 or ERK2) constituting the Ras-Raf-MEK-ERK pathway (Guo et al., 2020). The small G proteins Ras activates the downstream Raf kinase, then raf activates MEK1/2 and finally, ERK1/2 is activated via phosphorylation of the two regulatory sites Tyr 204/187 and Thr 202/185. Phosphorylated ERK then translocates to the nucleus and transactivate transcription factors, causing gene expression changes in order to promote differentiation, growth, or mitosis (Zhang, Liu, and Tu, 2002).

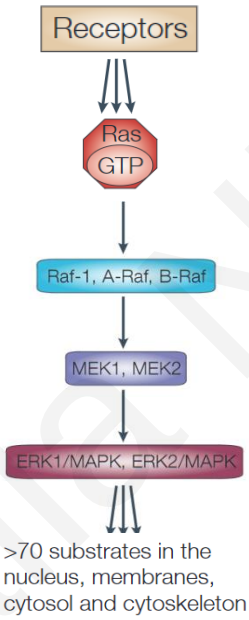


Fig.13 ERK/MAPK pathway

13.1 Properties of the components of the ERK/MAPK signaling pathway

13.1.1 Ras

It is a product of the Ras oncogene (Zhou, Der, and Cox, 2016) and its activation is induced by plenty of stimuli. Some of them are the Src family members, the tumor necrosis factor, protein kinase C (PKS) activators, and the epidermal growth factor (EGF) (Terrell and Morrison, 2019). Ras proteins are considered binary switches since they contain an active GTP-binding

conformation and an inactive GDP-bound state, which allows the switch between the two, to modulate signal transduction (Dohlman and Campbell, 2019). The conversion from GFP to GTP necessitates guanine nucleotide exchange - factors GEFs, to bind to RAS causing conformational alterations in the switch and P loop regions, resulting in GDP release and replacement by GTP (Simanshu, Nissley, and McCormick, 2017). The activated RAS-GTP marks the beginning of the ERK MAP kinase cascade.

In order for the cascade to proceed, the receptor-Grb2-SOS complex must be created as follow; the extracellular molecules bind to the receptors, the growth factor receptor-binding protein 2 (Grb2) binds to the active receptors and then recruits the proline-rich domain at the C-terminus of the son of sevenless (SOS), to the membrane, eventually creating the complex receptor-Grb2-SOS. SOS translocates to the membrane after binding to the Tyr phosphorylation site on the receptor or receptor substrate protein, elevating the SOS concentration nearby Ras (Bandaru, Kondo, and Kuriyan, 2019). Ras proteins remain in their stable inactive GDP-state form until signals induce GDP/GTP exchange.

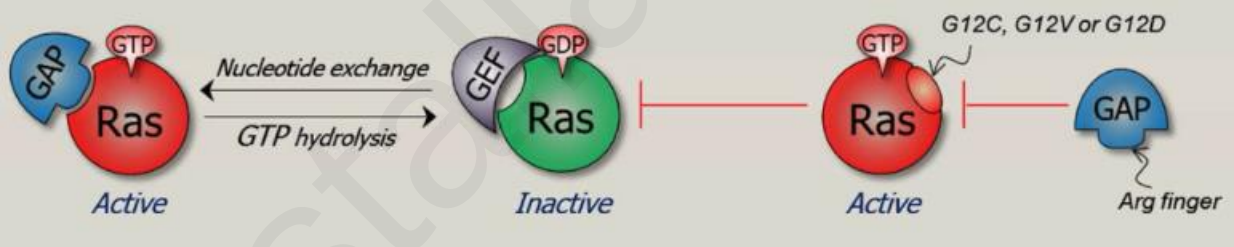


Fig.14 Ras activation and deactivation. Ras activation is due to the binding of guanine exchange factors. The switch back to the stable inactive conformation is modulated by GTPase -activating proteins (GAPs) and happens through hydrolysis of GTP (Nussinov, Jang and Tsai, 2014)**.**

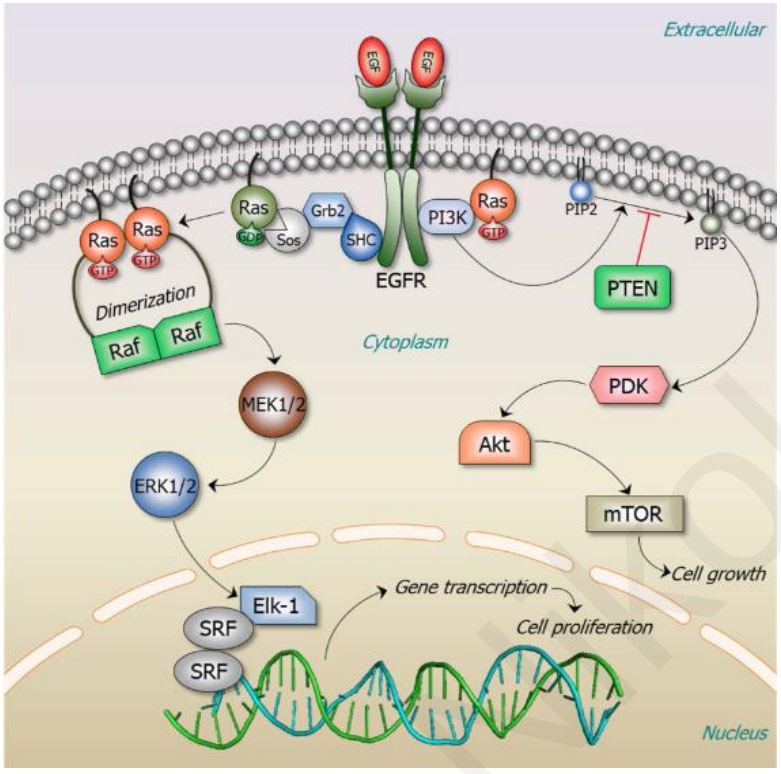


Fig.15 Illustration of the ERK/MAPK cascade

13.1.2 RAF

RAF is a growth factor signaling protein kinase that has a molecular weight of 40-75 kDa (Terrell and Morrison, 2019). The RAF kinase family is divided into three subtypes: A-Raf, B-Raf, and Raf-1 (or C-Raf). The MAPKKK of the ERK/MAPK pathway is Raf-1 and due to its implication in this signaling pathway has been studied in depth (Seger and Krebs, 1995). Raf protein kinase is composed of three regions CR1, CR2, and CR3. The binding domain of RAS is at the CR1 region which is positioned at the NH- terminus, has a zinc finger-like structure, and is abundant in cysteine (Fig.16) (Rukhlenko et al., 2018). CR2 is the regulatory domain, is found towards the NH-terminus, and consists of Ser and Thr residues (Rukhlenko et al., 2018). Specifically, in the CR2 region there is 14-3-3 recognition site. 14-3-3 is a family of conserved regulatory interacting proteins found in all eukaryotic cells, They can bind to a variety of functionally signaling proteins, including transmembrane receptors, kinases, and phosphatases.

(Fu, Subramanian, and Masters, 2000). CR3 is located at the COOH-terminus, and it is responsible for regulating Raf -1 when is phosphorylated on S338, Y340 and Y341. CR3 is the Raf-1 protein kinase's catalytic functional region (Roskoski, 2010) and it includes a second 14-3-3 binding site.

The 14-3-3 dimer is linked to phosphor-serines in CR2 and CR3 regions prior to Raf activation in order to maintain Raf autoinhibited (Park et al., 2019). In Raf-1, the phosphorylation occurs at Y340 and Y341 and is conserved in A-Raf at Y299, and Y300(Diaz et al., 1997). The above phosphorylations are likely mediated by the Src family of kinases (Yeung et al., 1999), whereas B-Raf activation is Src-independent, and the phosphorylation is happening in aspartic acid D492 and D493 (Chang et al., 2003).

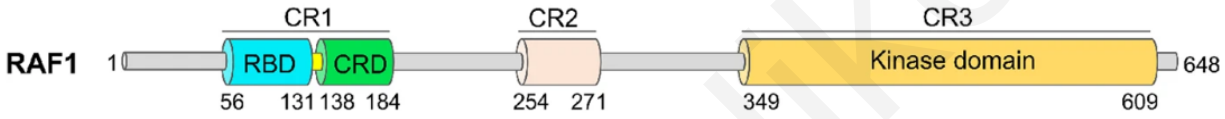


Fig.16. Domain structure of RAF1 protein (Tran et al., 2021)

The activation of Raf kinases follows five mechanisms.

1. Raf protein localization on the inside of the cell membrane via interaction with Ras.
2. Dimerization of Raf protein.
3. Phosphorylation and dephosphorylation at different sites.
4. Dissociation from the Raf kinase inhibitor protein.
5. Binding to Ras kinase inhibitor protein (Roskoski, 2019).

The activation of Raf-1 has two phases. Firstly, on the inner side f the membrane Ras binds to Raf-1, and then, Raf-1 is activated by tyrosine kinases and proceeds to activate the downstream MEK and MAPK. Lastly, it modulates the activity of multiple transcriptional regulators that control gene expression and delivers differentiation and cell proliferation signals to the nucleus (Vandamme et al., 2014).

13.1.3 MEK

The MEK proteins are a serine/threonine kinase family of proteins (Seger and Krebs, 1995). There are two types of MEK in the ERK/MAPK pathway MEK1 and MEK2. MEK1 has a molecular weight of 44kDa while MEK2 weights 45kDa (Muta, Matsuda, and Imajo, 2019). Both are hydrophilic non-receptor proteins, and are widely expressed (Roskoski, 2012a). MEK is considered a unique dual-specificity kinase, since it phosphorylates both Tyr and Thr regulatory sites, resulting in ERK activation (Zheng and Guan, 1994). The dual phosphorylation provides specificity because it increases signal transduction accuracy and minimizes ERK activation mistakes (Yaeger and Corcoran, 2019). Activation of MEK proteins happens through the C-terminal catalytic region of the phosphorylated Raf, which interacts with MEK kinases and phosphorylates their catalytic VIII subregion at the serine residue (Guo et al., 2020). MEK proteins have a trifunctional domain structure consisting of the N-terminal domain, the C-terminal domain, and the protein kinase domain (Fischmann et al., 2009). In the N-terminal domain, there is a nuclear export sequence, the inhibitory region, and a segment that promotes the binding of ERK's substrates (Fig.17A). The activation segment is found in the protein kinase domain, and it interacts directly with the MgADP (Fig.17B).

In the 3D structure of the kinases, we observe a large C-terminal lobe and small N-terminal lobe (Fig. 17B), as expected by all protein kinases (Knighton et al., 1991). The ERK proteins bind MEK at the large C-terminal lobe, while the N-terminus cooperate in the interaction (Roskoski, 2012a). MgADP relies on the catalytic site which is between the small and the large lobe (Fig.17B). Throughout the catalytic cycle, the C-terminal lobe and the N-terminus lobe move relative to one another creating an open and a closed cleft configuration. The opened form allows ATP access and ADP release from the active site, while the closed state offers a catalytically active state allowing phosphoryl-group of ATP to be transferred to the hydroxyl group of the protein (ERK1/2), resulting in its activation (Roskoski, 2012a).

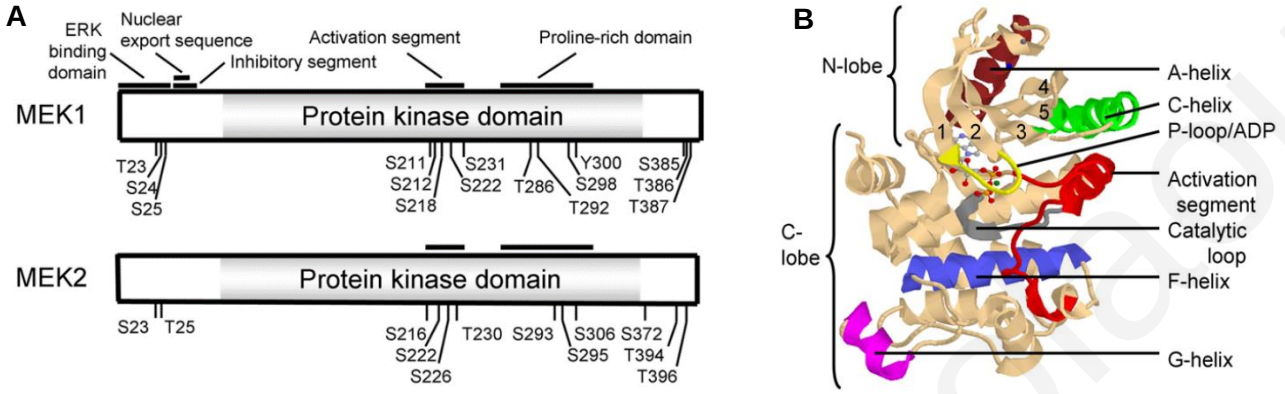


Fig.17 A) Domain structure of MEK 1/2 B) Protein structure of MEK 1 linked to MgADP (Roskoski, 2012a)

13.1.4 ERK

ERK 1, 2, 3, 5, 6, 7, and 8 have been identified as members of the ERK family. In the ERK/MAPK signaling pathway, ERK1 and ERK2 are the two key components, and their molecular weight is 44 and 42 kDa, respectively (Guo et al., 2020). Human ERK1 and ERK2 have approximately 84% sequence similarity, their function is very much alike and for this reason, many studies referred to both as ERK1/2 (Lloyd, 2006). Regarding its domain structure, human ERK 1 has 379 amino acid residues, while ERK 2 has 360 (Fig. 18A). An amino-acid-residue insertion is detected in the N-terminal extension of ERK1/2, and a 31-amino-acid-residue insertion has been identified in the kinase domain, which adds functional specificity (Buscà, Pouysségur and Lenormand, 2016). The structure of ERK1/2 contains a large C-lobe and a small N-lobe. The small lobe has a conserved loop rich in glycine -GxGxxG- (P-loop), which is responsible for positioning for catalysis the β- and γ- phosphates of ATP (Roskoski, 2012b). In the C-lobe, we observe the activation segment and the catalytic loop. The catalytic loop is located in the cleft between the large and the small lobe in order to be accessible to ATP binding. Similarly, with MEK1/2, ERK1/2 also has an active and an inactive state. When the lobes of the enzyme are slightly angled away from each other, then is referred to as open and catalytically inactive state. When the lobes are

close together, the enzyme is catalytically active and in close conformation. However, during the catalytic cycle the two lobes remain capable to move relatively to each other to make sufficient space for ATP to bind. One phosphoryl group will interact with ERK1/2 and the enzyme will be activated, while ADP will be released (Johnson et al., 2001). This reaction is catalyzed by MEK1/2.

ERK demands dual phosphorylation on both activation segment residues, and single phosphorylation does not activate the enzyme (Zheng and Guan, 1994). MEK1/2 initially mediates tyrosine phosphorylation (Tyr190) in the ERK activation region. Then, MEK dissociated from the tyrosine-phosphorylated ERK before reassociating with the same or another active MEK. Two residues upstream from the ERK phosphotyrosine, is the activation segment of threonine 188, which is phosphorylated by the active MEK (Ferrell and Bhatt, 1997). When the MAPK/ERK signaling pathway is inactive, MEK is responsible for maintaining ERK outside of the nucleus. The activation of ERK induces its translocation from the cytoplasm to the nucleus, where it influences the function of other protein kinases or enhances the phosphorylation of cytoplasmic target proteins. Moreover, it mediates transcription factors by phosphorylating them and, eventually affecting the biological processes of cells, such as mobility, survival, and proliferation.

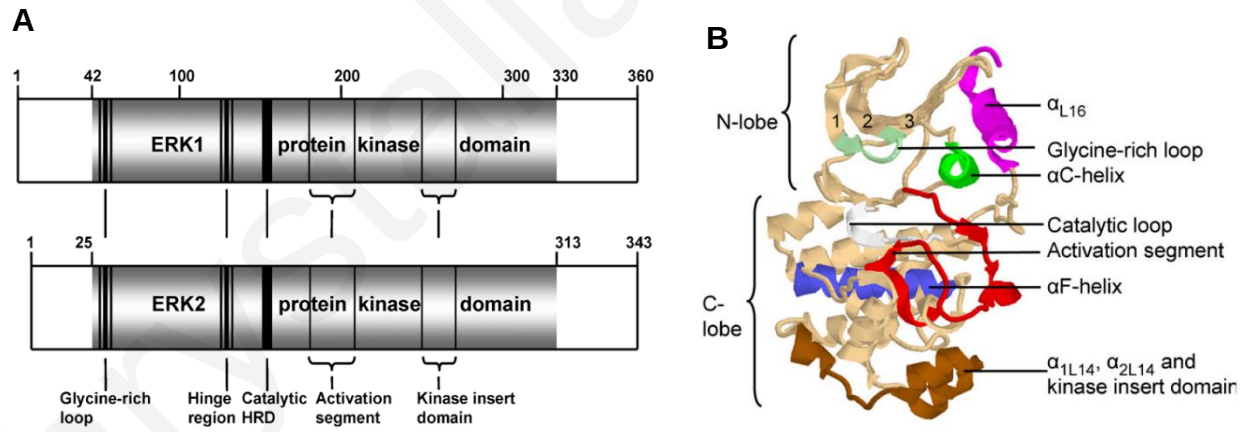


Fig.18 A) Domain structure of ERK1 and ERK2 B) Ribbon diagram of human ERK2

RATIONALE

Recent work from our lab has shown that the MAPK subfamily JNK plays a role in the development and function of multiciliated epithelia. JNK is found to be associated with the basal bodies in motile cilia and specifically is selectively localized at the transition zone of Multiciliated Cells (MCCs). Inhibition of JNK impairs basal body migration and docking, resulting in severe ciliogenesis abnormalities. Moreover, it was demonstrated that JNK has a dual function in ciliogenesis by establishing the intraflagellar transport-B core complex and regulating the actin networks of multiciliated cells.

The ERK/MAPK pathway is a vital signaling pathway that has been studied in depth. Being in the same family of MAPK kinases, we hypothesize that ERK may possibly share some similarities with JNK regarding its function in ciliogenesis. The purpose of this study was to investigate a possible role of ERK in motile ciliogenesis by taking advantage of the vertebrate model *Xenopus Laevis* embryos. Taking it one step forward, we also aimed to examine the function of ERK in primary cilia, using an established cell line and determining ERK's activity in both primary and motile ciliogenesis.

MATERIALS AND METHODS

Cell cultures and transfections

NIH3T3 cells were cultured under standard conditions in DMEM culture media supplemented with 10% FBS and 5% CO_2. For the induction of primary cilia, a serum starvation medium in the presence of DMSO or 10μM Selumetinib inhibitor (Sigma-Aldrich) was utilized for 24 hours. For transfection, cells were seeded in a 12-well plate and incubated with 2μg DNA plasmid together with lipofectamine 2000 in serum-reduced media, OptiMEM. After the transfection, cells were serum starved to induce the generation of cilia.

HCL treatment for coverslips

Coverslips were treated with HCl to coverslips for 10 minutes, washed with distilled water (15-20 washes), and then wash with a solution of 70% ethanol. Then transfer the coverslips one by one on a sprayed with ethanol tissue paper inside a cell culture hood. Wait for 30 minutes or so for them to dry and store them inside the hood.

Drug treatments

Drug treatment in Xenopus laevis with Selumetinib (40μM,) was performed at stage 14, and left in it for 24 hours. Selumetinib was introduced in NIH3T3 cells with starvation serum for 24 hours before fixation.

Immunostaining on cells

Starved NIH3T3 cells and human nasal cells were fixed in 4% PFA for 10 minutes, permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. Otherwise, they were fixed using ice cold methanol for 20 minutes in -20C. Then, the cells were blocked for 30 minutes with PBS containing 10% donkey serum. Primary antibodies were introduced for 1 hour and 30 minutes. After three 10-minutes washes, the secondary antibodies were introduced in the dilutions shown in the table below, following again three 10-minutes washes. Samples were washed and mounted on coverslips with Prolong Diamond Mountant (Invitrogen).

Antibodies	Dilution
Primaries	
Acetylated a tubulin mouse monoclonal Santa Cruz Biotechnology, #23950	1/500
Centrin-1 (Proteintech, #12794-1-AP)	1/500
p44/42 MAPK rabbit (T202/Y204) Cell signaling	1/100
CEP164 (Proteintech, #22227-1-AP).	1/100
Secondaries	
Alexa Fluor Phalloidin 568 (Invitrogen)	1/500
Anti-mouse Alexa 488 or 568(Invitrogen)	1/500
Anti-rabbit Alexa 488 or 568 (Invitrogen)	1/500
Anti-goat Alexa 488 or 569 (Invitrogen)	1/500

Cell lysis

Cells lysis buffer on ice containing 130mM NaCl, 20mM HEPES pH 7.2, 3mM EDTA, 0.3% Triton X-100,10% glycerol and supplemented with protease and phosphatase inhibitors. Then the cells were sonicated using Hielscher Ultrasound Technology and then centrifuged at 10000rpm for 10 minutes to remove debris. 5X laemmli buffer (10% SDS, 50% Glycerol, 0.02% bromphenol

blue, 0.3125M Tris HCl, pH 6.8 supplemented with 25% β-mercaptoethanol) was added to the lysate and then was incubated at 95 ^{o}C for 5 minutes.

Immunoprecipitation and Western blot

In order to segregate the proteins, we use the BlueStar prestained ladder (NIPPON Genetics) Extracts were analyzed by SDS-PAGE on10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (Sigma Aldrich). Membranes were blocked in 5% BSA in PBST (1 PBS buffer and 0.1% Tween 20) and incubated with primary antibodies overnight at 4^{o}C. After three 10-minutes washes with PBS-T, membranes were incubated for 1 hour with horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies (Santa Cruz Biotechnology). The blots were developed using ECL Immobilon Forte HRP substrate and imaged on a ChemiDoc Touch imager (Bio-Rad).

Embryo Manipulations and Microinjections

Human chorionic gonadotropin was injected to female adult Xenopus laevis in order to induce ovulation. The fertilization of the eggs was conducted in vitro. Then, the fertilized embryos were dejellied in 1.8% cysteine (pH=7.8) and reared in 0.1x Marc's modified ringers (MMR) and staged according to Neiukoop and Faber. For microinjections, embryos were placed in 4% ficoll in 1/3 x MMR. For the injection we use a glass capillary pulled needle, a Singer Instrument MK1 micromanipulator, and a Harvard Apparatus pressure injector. The injections were made into the ventral blastomeres at the 4-cell to target the epidermis. Embryos were allowed to grow to the proper stage before being imaged live or fixed in MEMFA at room temperature for 1-2 hours.

DNA plasmids

The DNA plasmids that were used for protein expression in Xenopus laevis and NIH3T3 cells were already available in the lab. The concentrations that were used are shown in the table below.

DNA plasmid	Dilution for injections	Concentration for cell transfections (12-well plate)
RFP-Centrin	60pg	-
GFP-Clamp	50pg	-
GFP-ERK1	50pg	2μg
GFP-IFT52	-	2μg

Fluid flow assay

Embryos of stage 38-40 were anesthetized in 0.01% benzocaine in 0.1 x MMR and fluoSpheres (Invitrogen, F8816). The assay was performed with Zeiss Axio Imager Z1, a Zeiss Axiocam MR3 and Axiovision software 4.8 using time-lapse microscopy. The analysis of the ciliary beating was performed using Imaris Software to track the ciliary speed.

Embryo Immunostaining

Embryos were fixed either for 2 hours in MEMFA at room temperature or for 2 hours in methanol and then rehydrated using MEMFA. If MEMFA is used, then the embryos were permeabilized in PBDT (1 PBS + 0.5% Triton X-100 + 1% DMSO) for 1 hour at room temperature blocking with PBDT + 1% donkey serum for 1 hour at room temperature.

Primary antibodies were mixed into the blocking solution, and the embryos were incubated at 4°C overnight. The next day, the embryos were washed 3 x 10min in PBDT. Then, the secondary antibodies were added PBDT + 1% donkey serum at room temperature for 1 hour. Lastly, embryo were washed again 3 x 10 min in PBDT.

Imaging

The imaging was conducted using Zeiss Axio Imager Z1 microscope equipped with Zeiss Axiocam MR3 and the AxioVision software 4.8. Also, Zeiss LSM 900 Airyscan laser confocal microscope was used with the Zen 2010 software.

Quantifications and statistical analysis

Quantifications were made using AxioVision LE software and Zen (Blue + Black edition) software. Fluorescence intensity was measured using ImageJ software (NIH). Statistical analysis was performed using two-tailed unpaired t-tests, with 95% confidence interval and statistical significance is labelled in the Figures.

RESULTS

ERK is associated with the basal bodies of primary and motile cilia.

Studies have shown that ERK and its upstream activator MEK are abundant on the midbody and mitotic spindle of dividing cells (Willard and Crouch, 2001). Moreover, Weber et al. demonstrated that sustained ERK activity regulates G1 progression by enhancing the expression of cyclin D1 which controls G1 progression (WEBER et al., 1997), indicating that the passage from G1 to S phase required extracellular regulated kinases to regulate gene expression. Evidence showing a possible role of ERK in ciliogenesis has not been reported to our knowledge. Due to the fact that ciliogenesis happens between the passage from G1 to S in an early G1 or G0 phase, we hypothesize that ERK may have a role in this process. To examine this possibility, we co-injected *Xenopus Laevis* embryos ventrally at stage 4 to target epidermis with exogenous Green Fluorescence Protein conjugated with ERK1 (GFP-ERK1) and Red Fluorescence Protein conjugated with Centrin (RFP-Centrin). Centrin is a centrosomal protein which is found at the basal bodies of ciliated cells. In *Xenopus Laevis,* ciliogenesis begins around stage 20.

We studied the localization of ERK with respect to the basal bodies in mature cilia as well as during initial stages of ciliogenesis. ERK and Centrin are found to be co-localized at stage 28 (Fig.19A), when multiciliated cells have mature oriented cilia. Interestingly, we also observed an association of centrin and ERK at stage 19 which is an early stage of ciliogenesis during which multiciliated cells begin to intercalate, indicating that ERK may plays a role in this process (Fig.19B).

Given the association of Multiciliated cells basal bodies we wanted to examine the possibility that ERK is also associated with the basal bodies of primary cilia. We used the mammalian NIH3T3 cells and transfected them with GFP-ERK1 and then induced ciliogenesis with DMEM + 0.3% FBS starving serum. Then, we performed immunostaining of the cells with acetylated a tubulin, a marker for cilia. We observed that ERK1 is localized at the base of ciliary axoneme of primary cilia, in agreement with our results in Multiciliated Cells (Fig.19C).

Lastly, we checked human nasal epithelial cells obtained from transnasal brush biopsies. Immunofluorescence using a specific antibody against phosphorylated ERK1/2 showed that it is enriched at the apical surface of multiciliated cells. Altogether these results show that ERK1/2 is associated with the basal bodies in both motile and primary cilia.

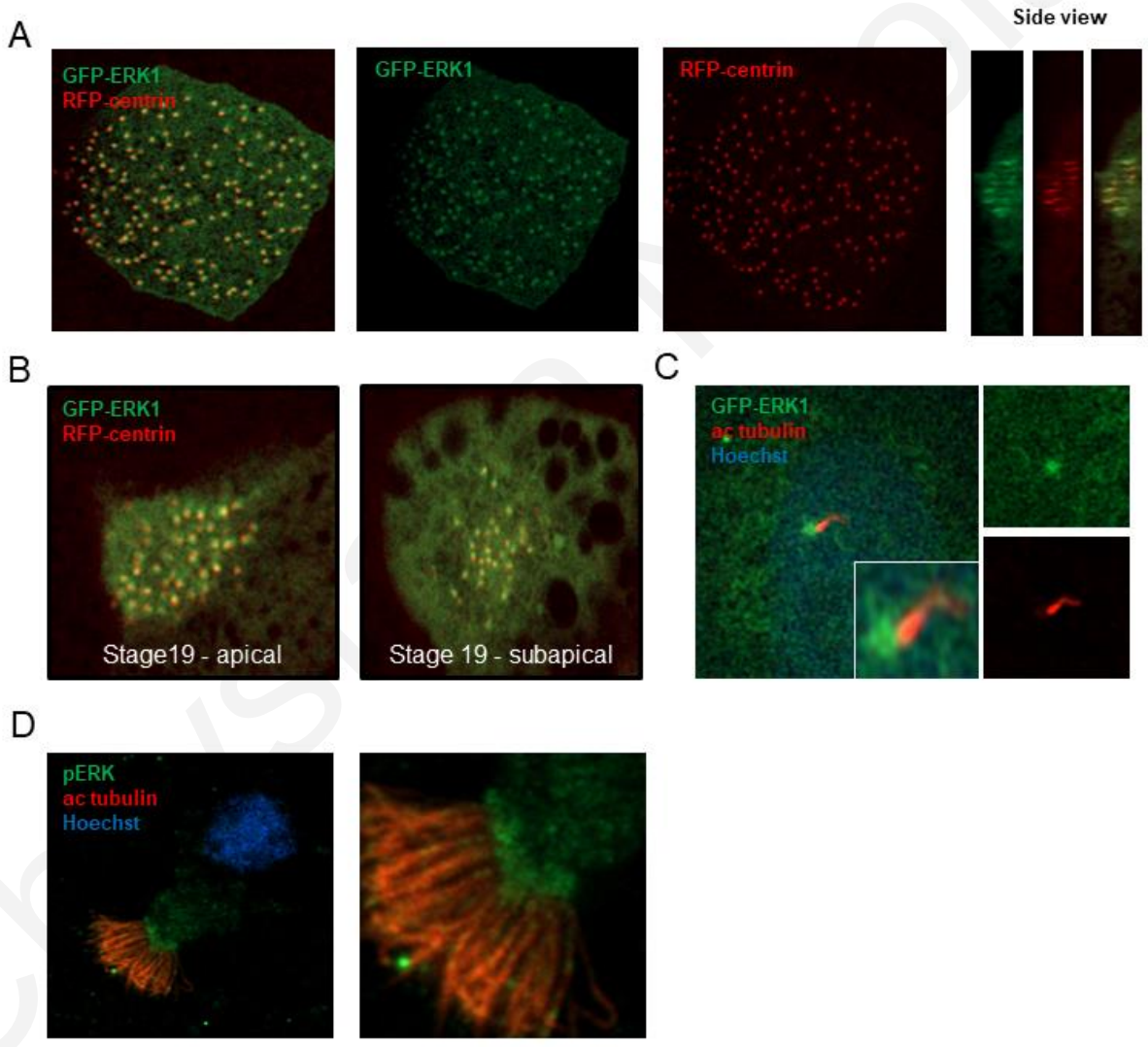


Fig.19 A) Multiciliated cell of Xenopus laevis embryos injected with GFP-ERK1 (green) and RFP-Centrin (red) at 4-cell stage and fixed at stage 29, showing association of ERK with the basal bodies.

B) Stage 19 embryos injected with GFP-ERK (green) and RFP-Centrin (red) showing localization of ERK at the basal bodies.

C) Transfected NIH3T3 cells with GFP-ERK1, immunostained with an antibody against Acetylated a tubulin which is a marker for cilia (red) and Hoechst which is a marker for nucleus (blue).

D) Human nasal epithelial cells immunostained with phosphor-ERK1/2 antibody (green), acetylated a tubulin (red), and Hoechst (blue).

Validation of pERK antibody

We next validated the specific antibody of phosphor-ERK1/2 using a selective inhibitor of ERK1/2 phosphorylation by immunofluorescence and western blotting. The selective inhibitor inhibits the upstream activator of ERK1/2, MEK1/2, and is called Selumetinib. Selumetinib is a medication used for the treatment of neurofibromatosis type I, a genetic disease of the nervous system. It belongs to the family of kinase inhibitors and its function is to prevent abnormal protein from signaling tumor growth. MAPK/ERK signaling pathway is disrupted by treatment with selumetinib, making both MEK1/2 and ERK1/2 unable to phosphorylate downstream substrates. NIH3T3 cells were treated with DMSO or selumetinib, fixed and immunostained with acetylated a tubulin and pERK1/2. Fluorescence microscopy showed that control cells treated with DMSO had pERK1/2 on their basal body, while selumetinib-treated cells lack pERK, indicating that pERK antibody is specific (Fig. 20A).

To verify the previous results, we proceeded with Western blot analysis. We used lysates from three different conditions: 1. cells cultured in DMEM standard media for 48 hours, 2. cells cultured for 24 hours in standard DMEM media, and then starved for 24 hours and 3) cells cultured for 24 hours in standard DMEM media, and then starved for 24 hours in the presence of selumetenib. Immunoblotting analysis showed that in unstarved cells and in starved cells, pERK1/2 was present (Fig.20B). Cells that were treated with selumetenib showed no band of

pERK. These results agree with the previous experiments and proves that pERK1/2 antibody is specific and can be used in our further experiments to detect pERK1/2.

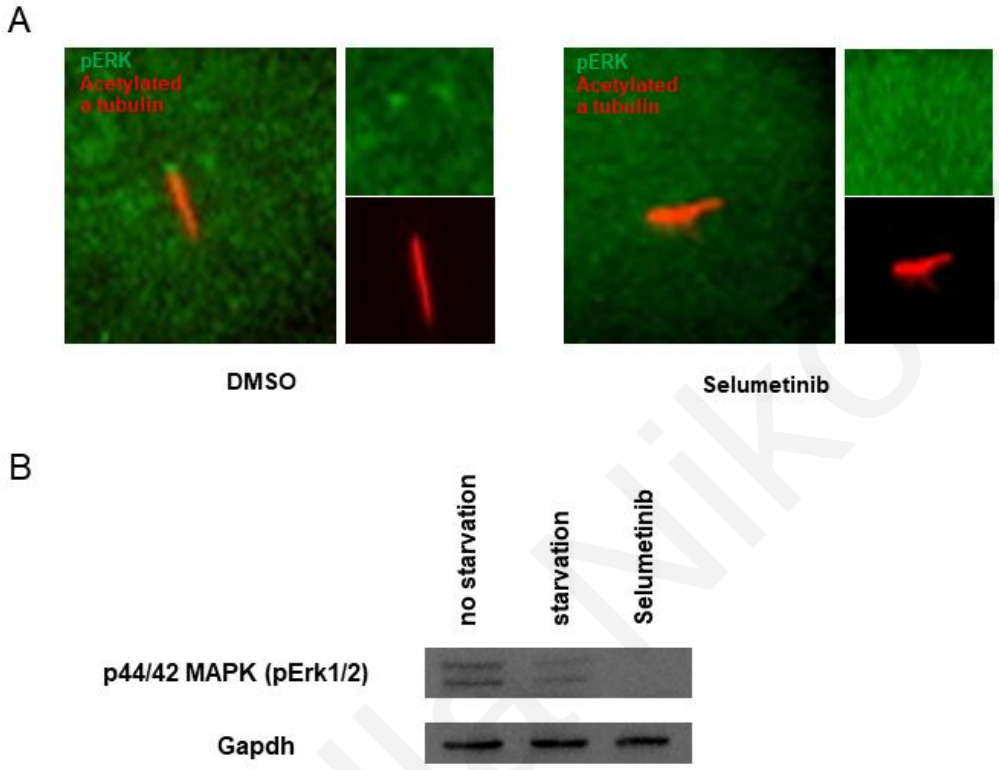


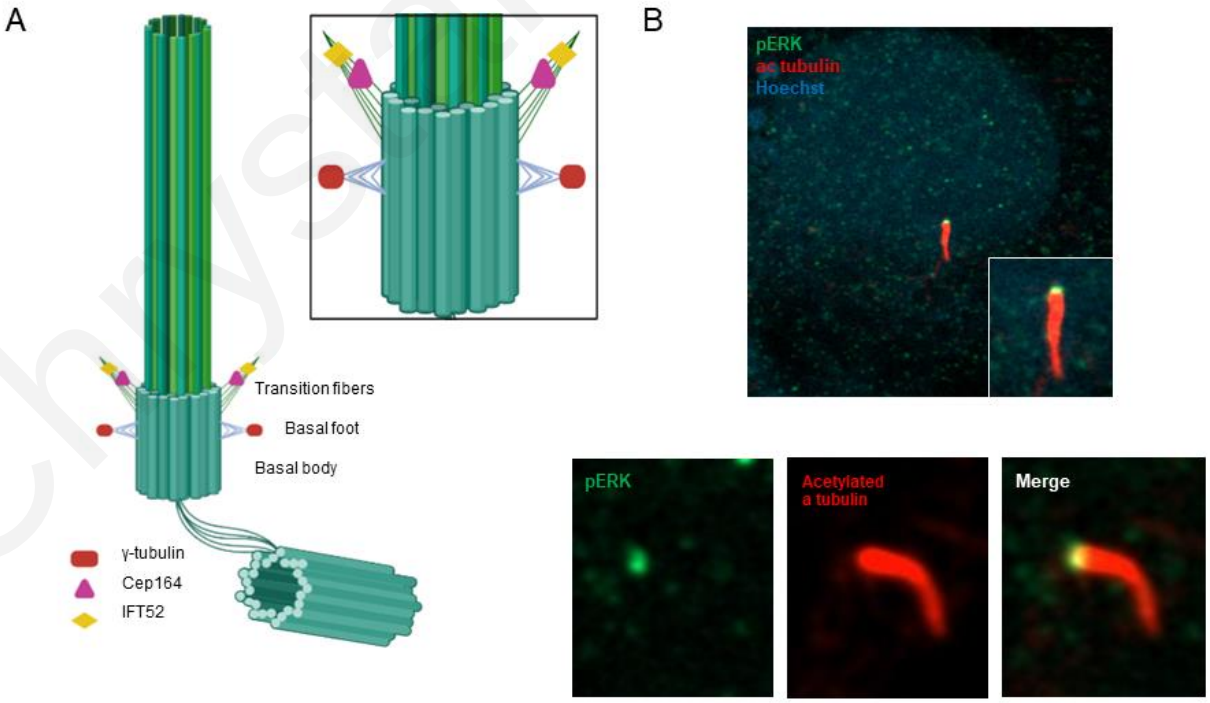
Fig.20 A) NIH3T3 cells were starved and stained with acetylated a tubulin and pERK and imaged using Zeiss Axio Imager Z1 microscope.

B) Western Blot analysis with extracts from three different cell conditions showing complete elimination of ERK1/2 phosphorylation upon inhibition. Cell lysates were supplemented with 2X laemmli and the proteins were resolved on SDS-PAGE gel and electrophoretically transferred from gel onto membrane. Then the membrane were blocked with 5% BSA in PBS tween. Then the membrane was incubated with the pERK antibody (1/5000) left overnight. Next, the membrane was incubated in secondary antibody for 1h. Lastly, we use a chemiluminescence substrate and expose the membrane to a film to view the results in the Bio-Rad touch imager.

ERK is localized at the transition fibers of primary cilia.

In order to further identify the specific localization of ERK at the base of cilia, we decided to examine its localization in association with known ciliary proteins in NIH3T3 cells. First, cultured cells were starved as mentioned in the previous section and immunostained with a specific antibody against Phospho-ERK1/2. Immunofluorescence showed that ERK1/2 is activated at the base of primary cilia (Fig.21B). Next, to determine the localization of ERK in comparison to other ciliary proteins, we selected two proteins; one that localizes at the transition fibers, IFT52, and one at the basal foot, γ-tubulin (Fig.21A). Imaging of immunostained cells showed that phospho-ERK1/2 is not co-localized with γ-tubulin (Fig.20 C). Also, intensity profile of both proteins show no association, indicating that ERK is not localized at the basal foot of the cilium (Fig.21 D).

We next studied Intraflagellar Transport 52 (IFT52) which is a protein that localizes at the transition fibers - also known as distal appendages - of the cilium. NIH3T3 cells were transfected with GFP-IFT52 in OptiMEM media and then, immunostained with a specific antibody against pERK. Imaging showed that pERK signal overlapped with GFP-IFT52 (Fig.21E), and their intensity profiles show significant association, suggesting that ERK1/2 is localized at the transition fibers of primary cilia (Fig.21F).



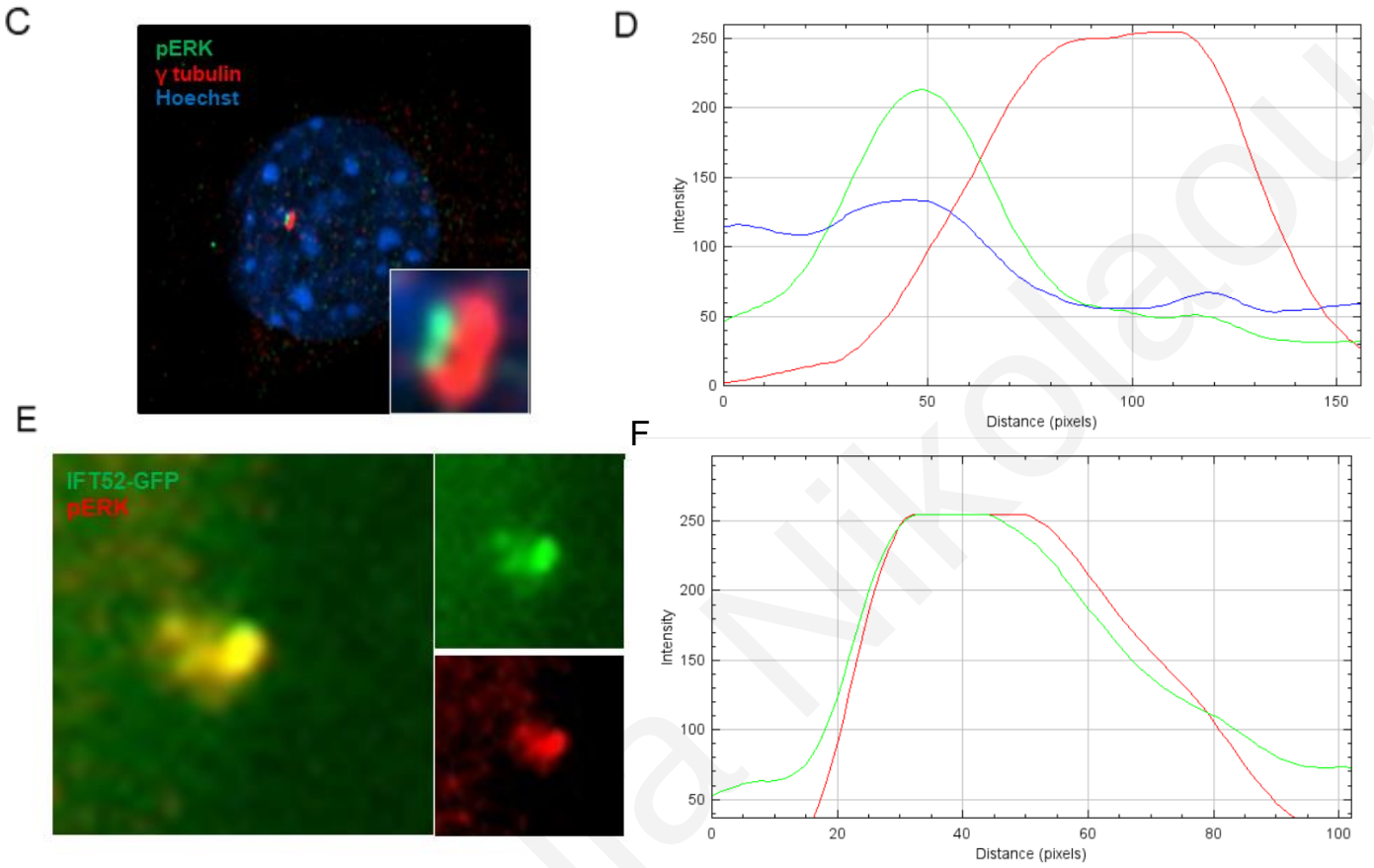


Fig.21 A) A schematic of primary cilium, showing the basal body, the basal foot (in which γ-tubulin is found), and the transition fibers (in which IFT52 and Centrosomal protein 164 are found).

B) NIH3T3 cells were fixed and immunostained with the specific antibody against pERK (green), acetylated a tubulin (red) and Hoechst (blue).

C) NIH3T3 cells stained with pERK (green), γ-tubulin (red) and Hoechst (blue).

D) Intensity profile of pERK, γ-tubulin and Hoechst.

E) NIH3T3 transfected with IFT52-GFP (green) immunostained with the specific antibody of pERK (red).

F) Intensity profile of IFT-52 and pERK.

Inhibition of ERK1/2 phosphorylation decreases ciliated cells and length of cilia.

As we mentioned before, MEK1/2 is the upstream activator of ERK1/2. To address the role of ERK1/2 activity in ciliogenesis, NIH3T3 cells were treated with DMSO or selumetinib and immunostained with acetylated a tubulin. As shown in Fig.22 A, the majority of the control cells possess a cilium on their surface, while in cells exposed to selumetinib, we observed a significant decrease of ciliogenesis. Then, we proceeded to quantify our results by counting cells having a cilium and measuring the length of cilia in each condition.

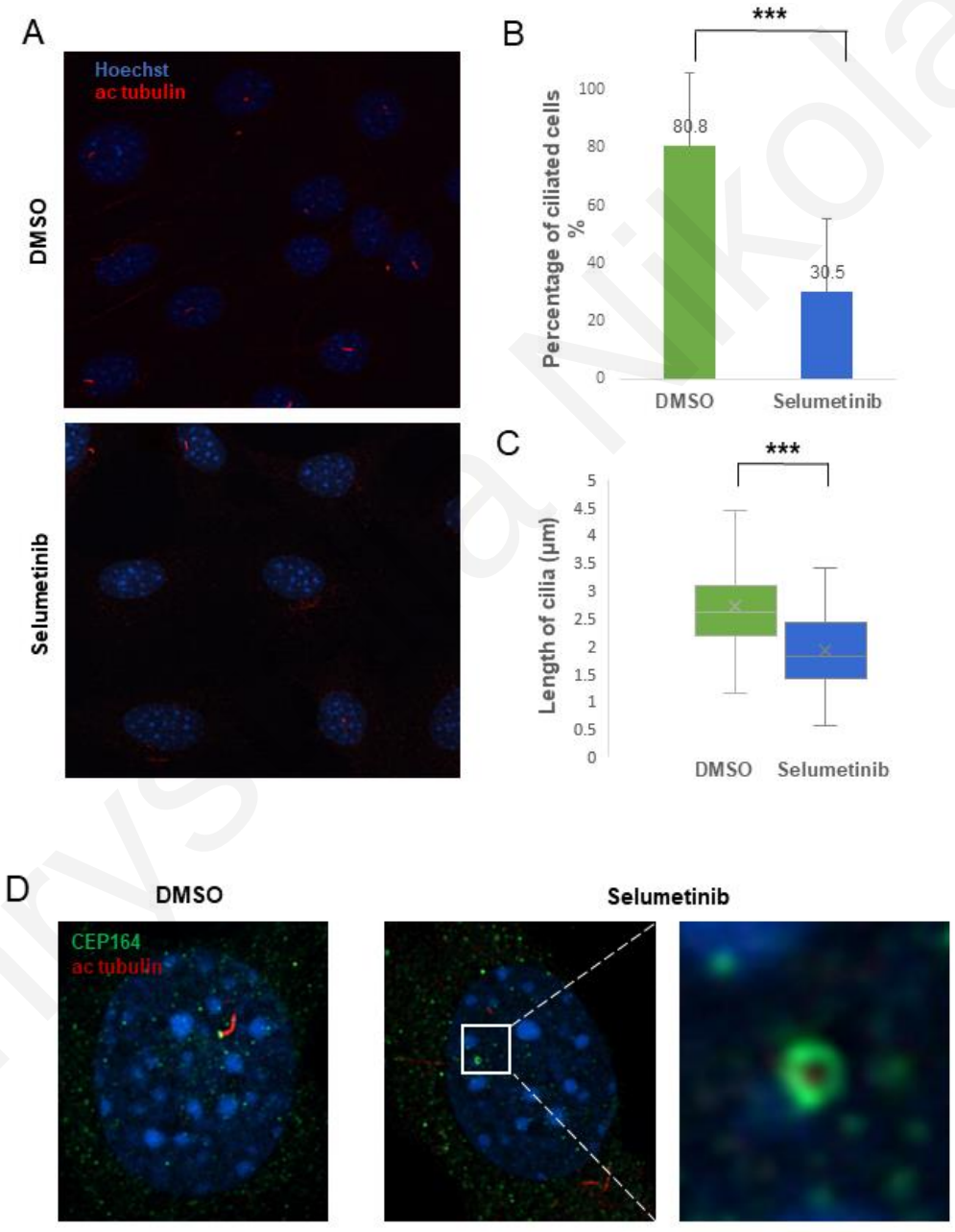
Quantification of the ciliated cells showed that 80.8% of the control cells developed cilia. On the contrary, upon inhibition of ERK1/2 only 30.5% managed to generate a cilium (Fig.22B). This reduction is statistically significant, showing that ERK1/2 activity is critical for ciliogenesis. Moreover, changes in the length of cilia in treated cells were observed. The length of cilia in selumetinib-treated cells that managed to develop a cilium is significantly decreased, suggesting that ERK may be essential for axoneme elongation (Fig.22C).

To investigate whenever known ciliary proteins are influenced by the inhibition of ERK1/2, we cultured starved NIH3T3 cells in the presence of DMSO or selumetinib. After fixation we immunostained control and selumetinib treated cells with a specific antibody against centrosomal protein 164 (CEP164). CEP164 has been identified as one of the mediators of vesicular docking to the mother centriole during early stages of ciliogenesis (Schmidt et al., 2012). It is also known that CEP164 colocalizes with IFT52 on the transition fibers of the basal body as it is illustrated at the Fig.20A. Since ERK1/2 is found to be colocalized with IFT52 we examined if inhibition of ERK1/2 would affect CEP164. Fluorescence microscopy showed that CEP164 localization is not affected after inhibition of the ERK/MAPK pathway (Fig.22D).

Overexpression of GFP-ERK affects ciliogenesis.

Since we established that loss of ERK's function results in defects in primary cilia, we next examined if the gain of function would influence ciliogenesis in an analogous way. We transfected cultured NIH3T3 cells with GFP-ERK1 in 3 different concentrations; 1/500 μg/μL, 2/500 μg/μL and 3/500 μg/μL, and then treated with DMSO or selumetinib.

The cells were immunostained with acetylated a tubulin specific antibody. Fluorescence microscopy showed categorization of cells into two groups: low/medium expressors and high expressors (Fig.22E). The quantification of transfected cells interestingly showed that 39.7% of low-medium expressor cells had cilia, while only 1.8% of the high expressors managed to develop cilia. This demonstrates that alterations of ERK1/2 activity or levels can influence the ciliogenesis process.



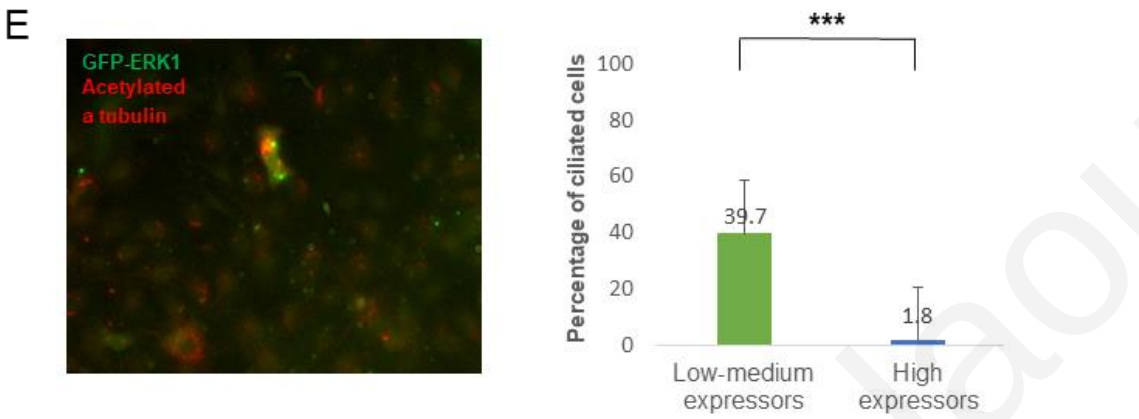


Fig.22 A) A) Starved NIH3T3 cells were treated with DMSO or Selumetinib, fixed and immunostained with acetylated a tubulin and Hoechst.

B) Percentage of ciliated cells in selumetinib-treated cells and controls. DMSO number of cells=212, Selumetenib number of cells=93. Statistical analysis showed that the p-value (0.00018083) is lower than 0.001 showing a significant (***) statistical difference between the means of two samples.

C) Length of cilia in DMSO (number of cilia=280) and upon inhibition (number of cilia=118). The statistical analysis was performed using t-test of two-sample assuming unequal variances. The p-value was $2.29E^{-20}$ which is lower than 0.001 so there is a significant (***) statistical difference between the means of the two samples.

D) NIH3T3 cells cultured in the presence of DMSO and Selumetinib (10μM), immunostained with specific antibodies against CEP164, shown in green in combination with and acetylated a tubulin shown in red.

E) Transfected NIH3T3 cells cultured for 24 hours, fixed, and stained with acetylated a tubulin, clustering into low-medium expressors and high expressors. The graph shows the percentage of ciliated cells in the two categories. The differences of their variances are statistically significant. (P-value = $5.72E^{-06}$).

ERK inhibition affects ciliary function in multiciliated cells.

Since we established that ERK is crucial for ciliogenesis in primary cilia, we then assessed to investigate its role in motile cilia using the ERK1/2 inhibitor. We thus took advantage of *Xenopus laevis* epidermis, which is covered with a multiciliated epithelium. The cilia of epidermal MCCs in *Xenopus* beat a coordinated way to generate a directional fluid flow towards the posterior of the embryos. We first determined the optimized concentration in which selumetinib is not lethal. Embryos were treated with 10μM, 20μM, 30μM, 40μM, and 50μM of selumetinib at stages 13-14. The ideal concentration for our experiments was found to be 40μM. Control and selumetinib treated embryos were fixed at stage 29, then we added fluoSpheres which are fluorescently labeled beads, into the media to track ciliary flow. Using time-lapse microscopy, we tracked the speed of beat movement across the Xenopus epidermis using IMARIS software. By comparing the track speed means of control and treated embryos, we observed a reduction in fluid flow in embryos treated with selumetinib as shown in Fig. 23A-B. This implies that ERK plays a role in regulating ciliary function.

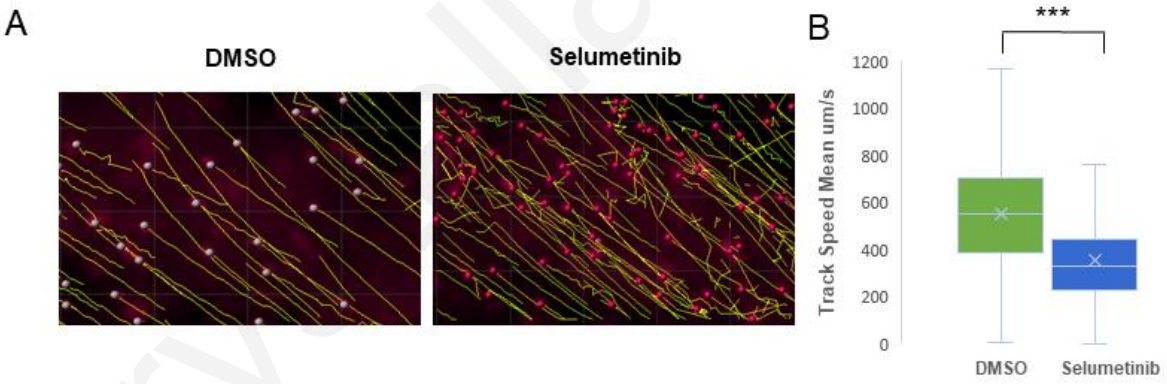


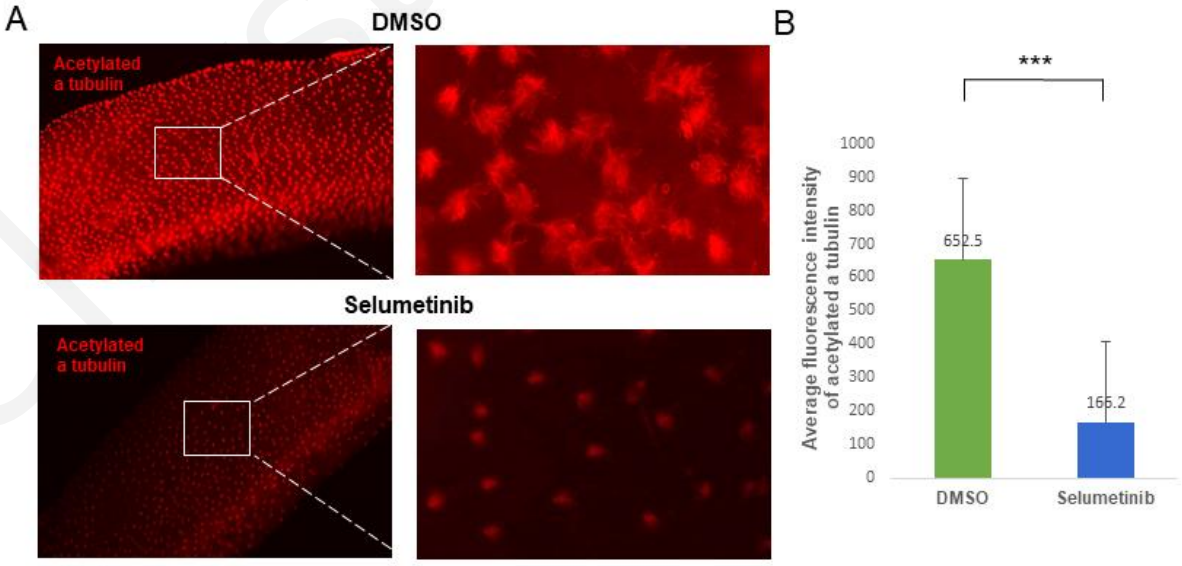
Fig.23 A) Flow assay using fluorescence beads on stage 38-40 of embryos treated with selumetinib or DMSO.

B) Quantification of the mean speed of beads measured with Imaris software.

ERK inhibition decreases the number of motile cilia and alters cilia polarity in multiciliated cells.

To uncover how inhibition of ERK affects the function of ciliary flow, we took advantage of selumetinib, we treated *Xenopus* embryos at stage 13-14 and immunostained them with acetylated a tubulin. With fluorescence microscopy we observed that embryos exposed to selumetinib had fewer ciliated cells (Fig.24A), and a lower acetylated a tubulin fluorescence intensity. To investigate the differences in intensities between control cells and inhibited ones, we measured their fluorescence intensity using ImageJ software. Statistical analysis revealed that the average fluorescence intensity of acetylated a tubulin in DMSO cells is almost 4 times higher than cells exposed to selumetinib. These results showed that inhibition of ERK1/2 activity affects the number of cilia in multiciliated (Fig.24B)

When tracking ciliary flow, we observed defects in the direction of individual beads, and we hypothesized that upon inhibition of ERK1/2, the orientation of cilia is altered. To uncover this, we co-injected 4-cell embryos ventrally with GFP-Clamp and RFP-Centrin to observe the orientation of the rootlet compared to the basal body. Quantification showed that rotational polarity in 82% of multiciliated cells in controls is normal (Fig.25D), while in treated cells 4 types of different directions were observed (Fig.25E). This suggest that cilia polarity is altered upon inhibition of ERK1/2 activity.



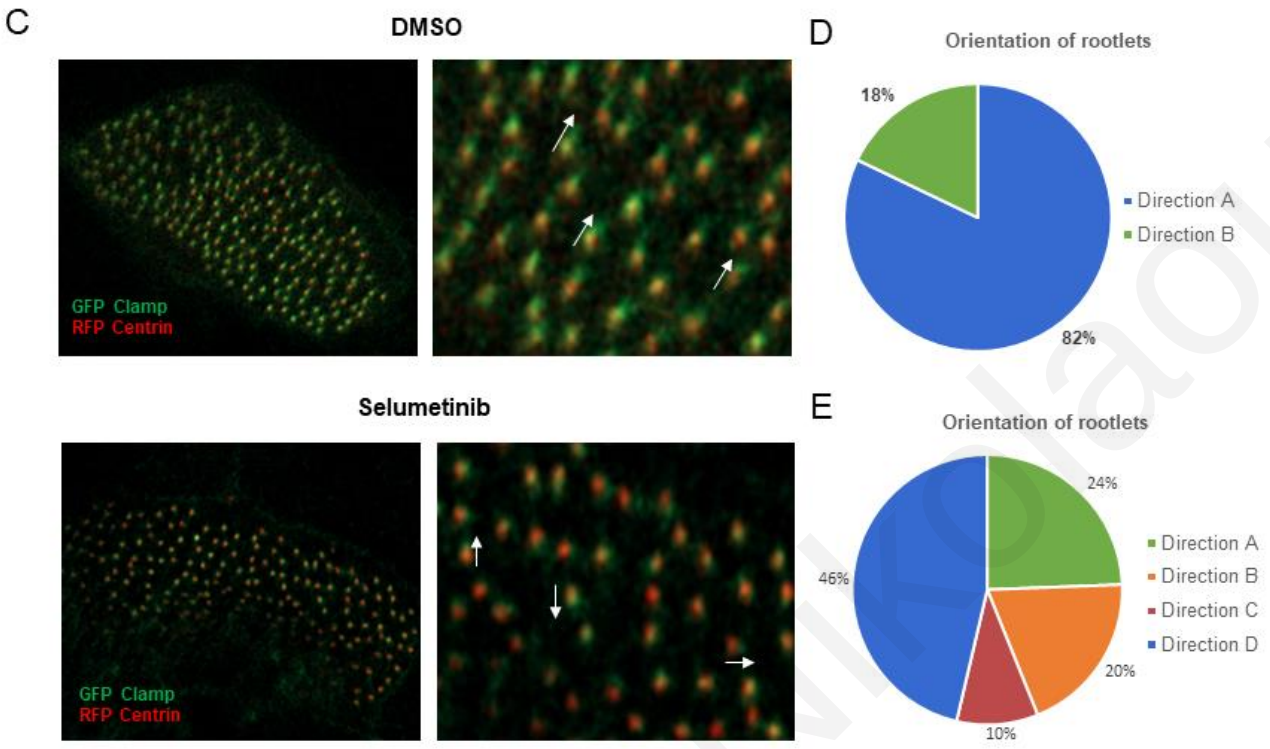


Fig.24 A) *Xenopus laevis* embryos treated with DMSO and Selumetinib at stage 13-14, and stained with Acetylated a tubulin after fixation.

B) Average fluorescence intensity of acetylated a tubulin in multiciliated cells treated with DMSO and Selumetinib. Statistical analysis showed that the p-value (0.000198) is lower than 0.001 showing a significant (***) statistical difference between the means of two samples.

C) Injected embryos with GFP-Clamp and RFP-Centrin at 4-cell stage, treated with selumetinib or DMSO at stage 12-13 and fixed at stage 29.

D-E) Quantification of the orientation of rootlets in DMSO or selumetinib treated multiciliated cells. The quantification was carried out manually.

ERK inhibition and spacing of basal bodies.

The previous results proved that ciliogenesis is affected by the inhibition of ERK1/2 on both motile and primary cilia. Next, we wondered how basal bodies might be influenced by the ERK/MAPK pathway. Xenopus embryos were treated with selumetinib at early stages of ciliogenesis and fixed at stage 29. Then we immunostained with a specific antibody against centrin. Centrin is a calcium-binding phosphoprotein that is found in centrosomes and at the basal bodies of cilia. Upon inhibition of ERK, we noticed differences in spacing between basal bodies (Fig.25A), and we proceeded to measure basal body spacing, to uncover defects caused by selumetinib. Quantification of the distance between individual basal bodies and their nearest neighbor is disturbed (Fig.25B). The decrease of the nearest distance of neighbor in selumetinib treated embryos, is statistically significant as it showed by t-test analysis. These data suggest that ERK contributes to the regulation of basal body migration and that inhibition of ERK induces basal body spacing defects.

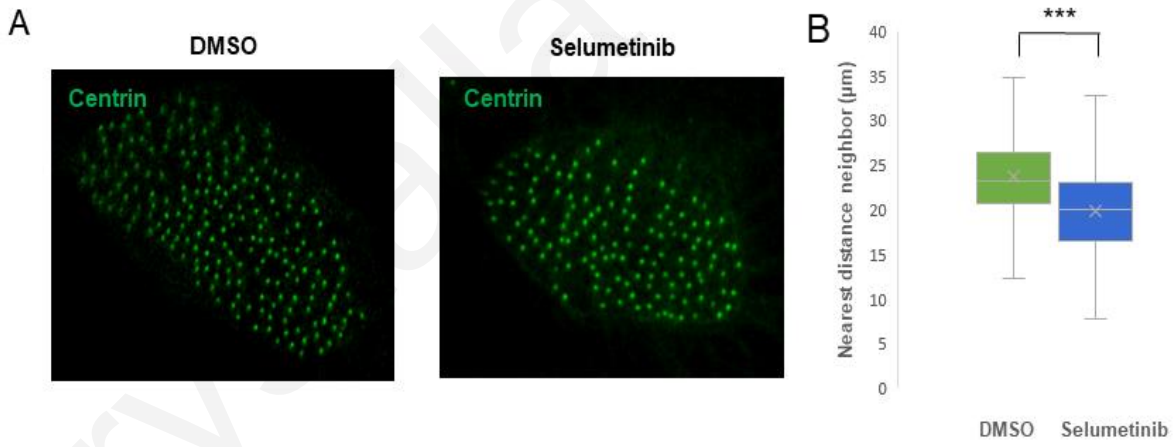


Fig.25 A) Xenopus embryos treated with DMSO and Selumetinib (40µM) at stage 12-13. After fixation, the embryos were stained with centrin and imaged at stage 29.

B) Nearest distance neighbor of individual basal bodies measured with ImageJ. Statistical analysis showed that the p-value ($2.83E^{-20}$) is lower than 0.001 showing a significant (***) statistical difference between the means of two samples.

Actin cytoskeleton is affected by inhibition of ERK.

Previous studies showed that the actin cytoskeleton plays a critical role in ciliogenesis (Sedzinski et al., 2017) . Actin forms two distinct pools that differ in structure and operation. Apical actin is located at the surface of the cell, surrounds each basal body, and is involved in the docking of the basal bodies during ciliogenesis (Boutin and Kodjabachian, 2019). A second actin pool is found beneath the surface (~0.5μm), termed as subapical actin network. In mature MCCS, subapical actin creates bridges that interconnect cilia by linking the basal body of a cilium with the tip of the striated rootlet of the posterior one (Werner et al., 2011).

Given prior studies indicating that ERK1/2 can regulate actin cytoskeleton (Chatzifrangkeskou et al., 2018), and work from our lab showing that another MAPK kinase, JNK, affects ciliogenesis through actin (manuscript in revision), we investigated the effect of ERK inhibition on the actin network of multiciliated cells. Embryos at stage 12-13 were treated with DMSO or Selumetinib overnight and immunostained with specific antibodies against centrin and β-actin. Imaging showed that the actin network is disrupted by ERK inhibition. Specifically, β-actin network at the apical surface was distorted, showing discontinued actin network with gaps. In the subapical surface we also observed altered actin network compared to the control (Fig.26A). Moreover, we observed that due to the disorganized apical actin network the apical cell surface was reduced in treated MCCs (Fig.26B), indicating that inhibition of ERK1/2 negatively affects actin and its function in controlling cell shape and architecture. These results suggest that ERK activity is important for the regulation of actin cytoskeleton networks in multiciliated cells.

Next, we examined whether actin cytoskeleton is also affected in cultured cells upon ERK1/2 inhibition. Phalloidin was used for the staining of actin filaments. Intriguingly, cells that were treated with selumetinib and did not generate a cilium, had much lower fluorescence intensity even though cells' exposure time was identical (Fig.26C). After contrast stretching, we could see some actin fibers, showing that staining was performed correctly and eliminating the possibility of any mistakes have been made during immunostaining. According to literature, reduced actin filaments result in increased in ciliogenesis which is not the case (Smith, Lake, and Johnson, 2020). However, ERK inhibition may have possible actin-independent effects leading to decreased ciliogenesis.

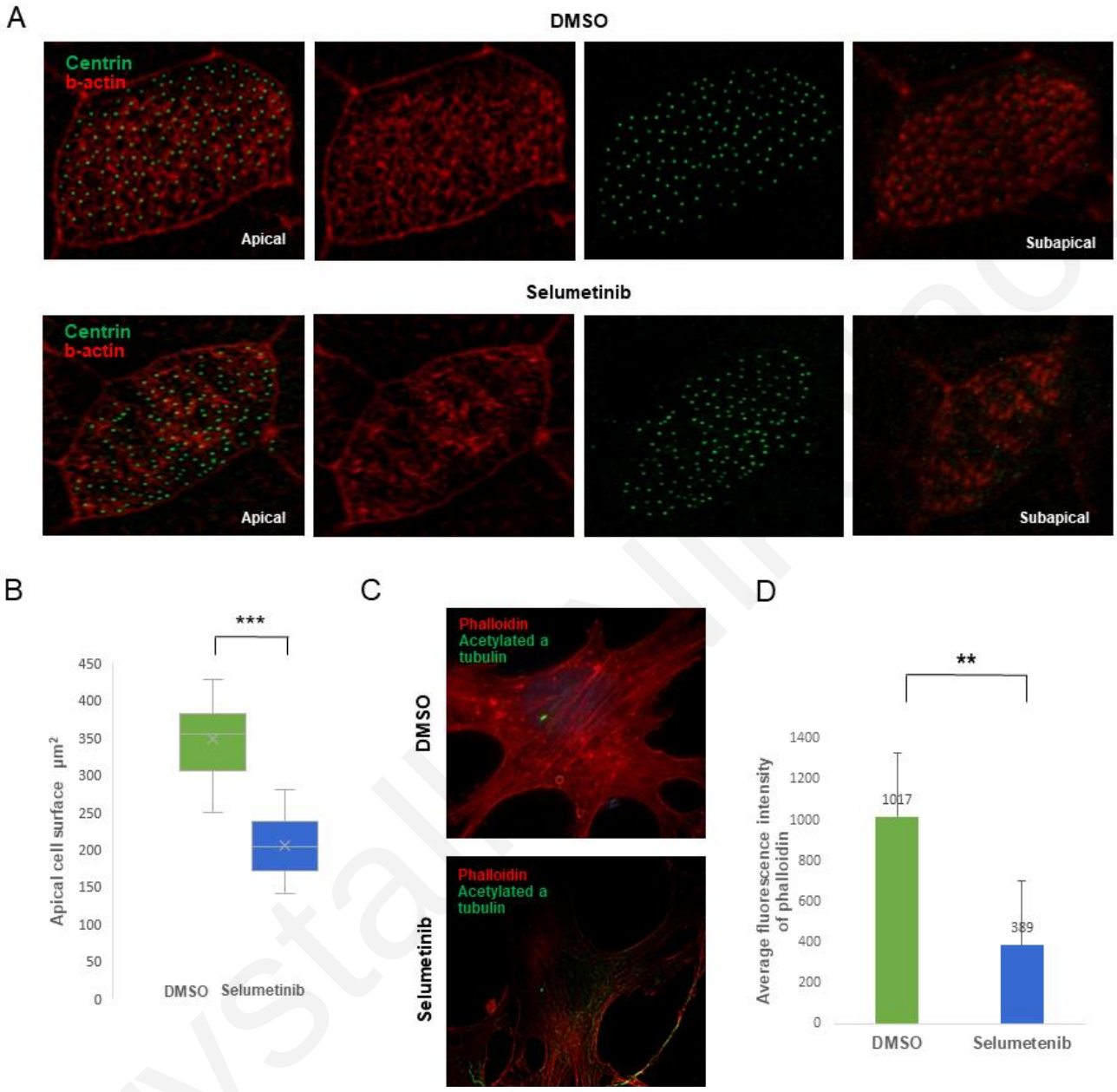


Fig.26 A) Xenopus embryos treated with DMSO and Selumetinib (40µM) at stage 12-13. After fixation at stage 29 the embryos were stained with centrin (green) and phalloidin (red). Fluorescence microscopy images show the apical and subapical actin networks in a single multiciliated cell.

B) Apical cell surface area measured in ImageJ software

C) NIH3T3 cells were cultured in DMEM media for 24 hours and then starved in DMEM+0.3% FBS in the presence of DMSO or Selumetinib for 24 hours. Cells were fixed and immunostained with phalloidin (red) and acetylated a tubulin (green).

D) Average fluorescence intensity of phalloidin measured with ImageJ software.

JNK activation is affected by inhibition of ERK.

Previous work from our lab revealed a new role of JNK in ciliogenesis. Our current data showed similarities of both MAPKs, JNK, and ERK in terms of localization and function. Both JNK and ERK are associated with basal bodies in primary and motile cilia and are localized at the transition zone. By inhibiting the signaling of the two kinases there is a reduction of ciliary flow. Also, JNK and ERK are essential for the regulation of apical and subapical actin networks. Taking these into account we wondered if there is an interplay between the two kinases.

To address this, we used selumetinib to inhibit ERK1/2 and SP600125 to inhibit JNK1/2. Using fluorescence microscopy, we observed no visible differences in the association of ERK1/2 with the basal bodies between control and SP600125 treated cells (Fig.27A). In fact, quantification of the number of cells with pERK associated with the basal bodies and average fluorescence intensity of pERK showed that there is no statistical significance between the mean of the two variances (Fig.27B-C), indicating that JNK is not involved in the association of ERK with the basal bodies.

Further, we investigated the impact of ERK activity in the association of JNK. Intriguingly, immunofluorescence showed that the intensity of pJNK at the base of primary cilia was decreased by 44.2%, in selumetenib-treated cells compared to the control cells (Fig.27F). Lastly, measuring the percentage of cells that had pJNK on their basal body showed a significant reduction in selumetinib treated cells (Fig.27E). Taken together, our data show that ERK1/2 activity may play a role in the activation of JNK at the base of cilia or its association with the basal bodies. However, this needs to be further addressed by examining the total JNK levels upon ERK1/2 inhibition.

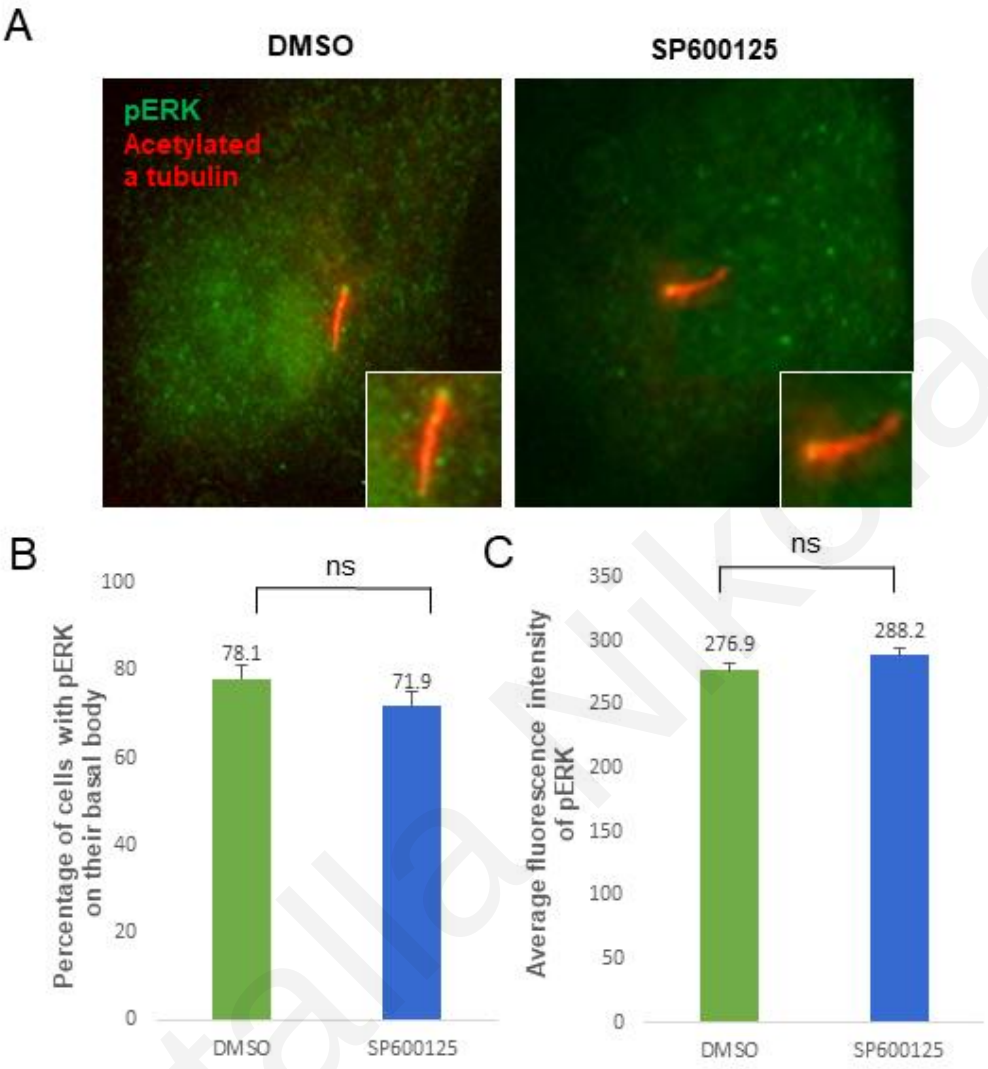


Fig.27 A) NIH3T3 cells cultured for 24 hours in the presence of DMSO and SP600125 (10μM), immunostained with specific antibodies against pERK, shown in green, and acetylated a tubulin shown in red.

B) Graph showing the percentage of cells with pERK on their basal body. Number of DMSO cells = 32, number of cells treated with SP600125=32. T-test statistical analysis revealed no statistical analysis between the mean of the two variances (p-value= 0.3553)

C) Graph showing the average fluorescence intensity of pERK measured with ImageJ software. T-test statistical analysis revealed no significant statistical analysis between the means of control and treated cells. ($p\text{-value}_{pERK}$= 0.1256)

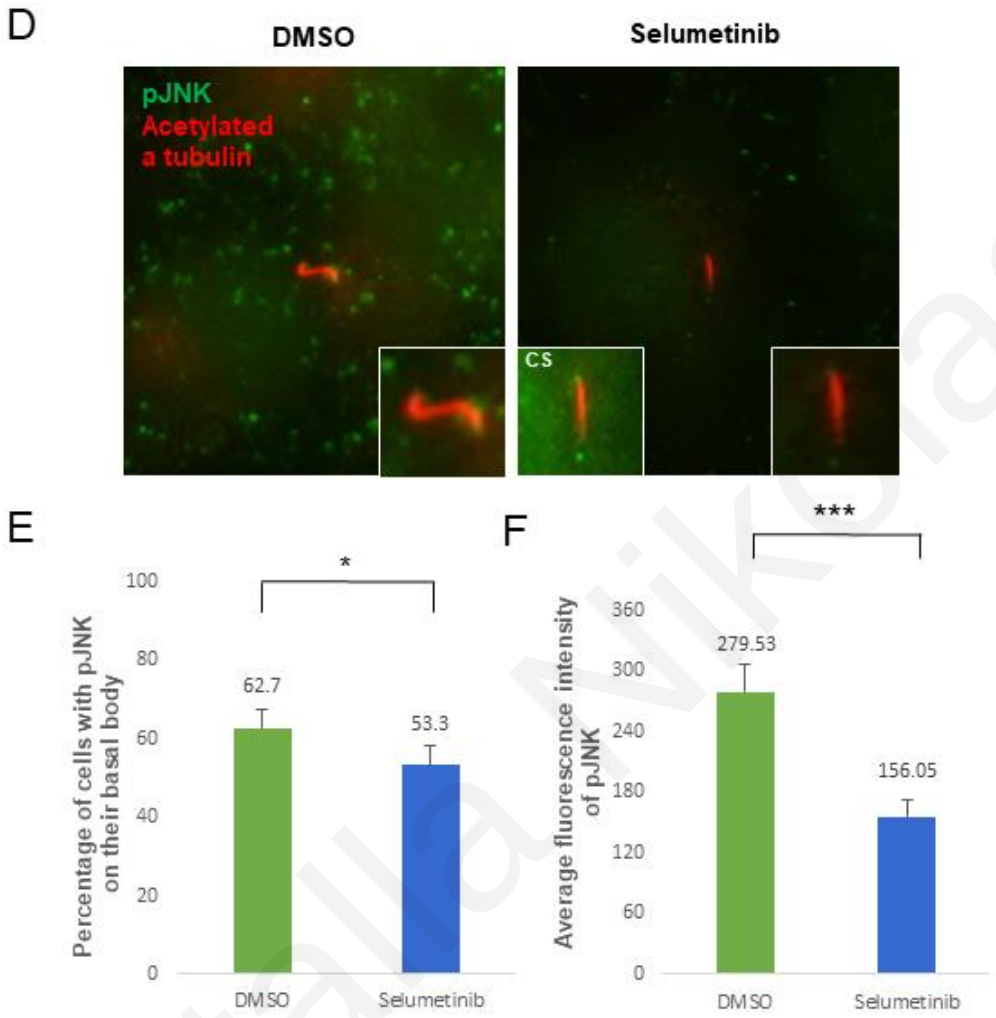


Fig.27 D) NIH3T3 cells cultured for 24 hours in the presence of DMSO and Selumetinib (10μM), immunostained with specific antibodies against pJNK, shown in green, and acetylated a tubulin shown in red.

E) Graph showing the percentage of cells with pJNK on their basal body. Number of DMSO cells = 59, number of cells treated with Selumetinib=15. T-test statistical analysis revealed significant statistical analysis between the mean of the two variances (p-value= 0.03189)

F) Graph showing the average fluorescence intensity of pJNK measured with ImageJ software. T-test statistical analysis revealed significant statistical analysis between the means of control and treated cells. ($p\text{-value}_{pJNK}= 7.17E^{-13}$)

DISCUSSION

Cilia are involved in the transduction of several signaling pathways that are crucial during development and disrupted in genetic disease and cancer such as Hedgehog signaling (Hh), Wingless (Wnt), Platelet Derived Growth Factor Subunit A (PDGFa), Notch and Hippo signaling (Andreu-Cervera, Catala and Schneider-Maunoury, 2021). ERK/MAPK signaling pathway is one the core signaling pathways that incorporate external signals into the cell to promote cell differentiation and proliferation (Shaul and Seger, 2007). Previous studies have shown an association of MAPKs with ciliogenesis in algae. In particular, in Chlamydomonas Reinhardtii, LF4 MAP kinase is required for maintaining wild-type flagellar length (Berman et al., 2003). In addition, MAPK15 (ERK7 or 8) is found to be localized at the basal bodies and cell-cell junctions in Caenorhabditis elegans and human primary cilium. Downregulation of MAPK15 in multiciliated Xenopus and mouse cells induce a reduction of motile cilia quantity and length, while basal body migration and apical cell surface were also altered (Kazatskaya et al., 2017). Interestingly, research on ciliary disruption due to cisplatin chemotherapy uncovers a link between ERK1/2 and cilia. Cilia suppression resulted in the hyperactivation of ERK in HK-2 cells, while suppression of ERK using a MEK inhibitor (U0126) resulted in the maintenance of cilia during cisplatin therapy (Wang et al., 2013). These findings show that there is a connection between cilia and MAPK signaling pathways.

In this study, we uncovered ERK1/2 as a novel regulator of ciliogenesis both *in vivo* in Xenopus multiciliated cells and *in vitro* in NIH3T3 cells. We demonstrated that ERK1/2 is associated with the basal bodies of primary cilia in cultured cells and motile cilia of human nasal epithelial cells and Xenopus laevis epidermis. Moving on, we showed that activated ERK co-localized with the known Intraflagellar Transport protein 52 (IFT-52) suggesting that it is localized at the transition fibers of primary cilia. It is known that Intraflagellar transport particles dock at the transition fibers carrying and transporting protein, and other cargo into and along the ciliary compartment (Ishikawa and Marshall, 2017). Additionally, vesicle docking happens at transition fibers (Reiter, Blacque and Leroux, 2012b), and along with the transition zone work to form the

ciliary gate, which then mediate the entry and exit of cilium proteins and lipids (Garcia-Gonzalo and Reiter, 2017). We propose that ERK may regulate the traffic of transmembrane proteins and lipids along the cilium membrane, but this needs further experimental investigation.

Next, we examined if overexpression of ERK1 influences ciliogenesis by transfecting NIH3T3 cells with GFP-ERK. Overexpression of the ERK/MAPK signaling pathway is activated in many tumors, since many oncogenes, including RAS and RAF trigger the pathway (Mishima et al., 1998). Also, it has been previously reported a relationship between primary cilia and cancer, showing that diverse types of cancer cells do not normally form cilia (Higgins, Obaidi and McMorrow, 2019). Our results showed that overexpression of ERK dramatically decreases the number of ciliated cells, suggesting that it may be an interplay between ciliogenesis in cancer cells and ERK. However, further investigation is required to determine the optimal concentration in which ERK1 overexpression induces the severest effects in normal cells and cancer cell lines.

Further, we investigated the effect of ERK1/2 inhibition on primary cilia by using fluorescence microscopy. The significant reduction of the number of ciliated cells as well as their ciliary length, we observed are in agreement with the results of pharmacological inhibition of the JNK protein from previous work carried out in our lab (manuscript in revision).

We went on and further study the inhibition of MEK1/2 in association with other ciliary proteins. Specifically, we tested CEP164, and γ-tubulin and we showed that they are not affected by ERK1/2 inhibition. Studies have shown that deletion of CEP164 blocks primary cilia formation (Daly et al., 2016), so our results rule out the possibility that the observed defects in ciliogenesis are mediated through CEP164. γ-tubulin is localized at the basal foot, while ERK is localized on the transition fiber, as we proposed. Thus, inhibition of ERK1/2 does not affect proteins localized at the basal foot. More ciliary proteins including IFT52 should be studied upon ERK1/2 inhibition or deletion in both primary and motile cilia in order to uncover any potential interaction.

Our next aim was to investigate the role of ERK in motile cilia taking advantage of the Xenopus epidermis which accommodates a large number of MCCs. Inhibition of ERK1/2 resulted in fewer cilia, spacing defects in basal bodies and altered cilia polarity, as observed by the disorganization of rootlets compared to the basal bodies. To our knowledge, this is the first report that shows ERK playing a role in ciliogenesis of motile cilia.

Actin cytoskeleton dynamics have been proven to have a role in mediating many stages of ciliogenesis in primary and motile cilia, including vesicular trafficking of ciliary components and

basal body docking and position (Hoffman and Prekeris, 2022a). During cell migration, actin filament bundles are generated radially at the leading edge or as axial stress fibers. Studies have shown that mutations in ciliopathy genes increased stress fiber formation and impaired ciliogenesis. Also, by inhibiting actin polymerization, ciliogenesis was enhanced (Smith, Lake, and Johnson, 2020b). Additionally, research has demonstrated that numerous features of ciliogenesis depend on both actin cytoskeleton elements and regulators of actin dynamics. Cilia formation and function are coordinated by both actin and microtubule dynamics. Actin filaments directly modulate the location and docking of the basal body, vesicle trafficking, length of cilia, membrane organization and cilia-dependent signaling (Copeland, 2020). Moreover, studies have shown that actin remodeling is necessary for ciliogenesis (Hoffman and Prekeris, 2022b). Lastly, work from our lab has demonstrated that Focal Adhesion (FA) proteins interact with the cytoskeleton by forming complexes with the basal bodies of motile cilia, and disruption results in cilia defects (Antoniades, Stylianou and Skourides, 2014). Here, we demonstrated a disrupted actin network and a smaller size of apical surface of multiciliated cells, upon ERK1/2 inhibition. We suggest that the inactivation of ERK negatively affects the ability of ciliated cells to form a stable apical actin network. Although a branched actin network was previously observed around basal body of primary cilia (Molla-Herman et al., 2010, Farina et al., 2016), we were not able to visualize it in NIH3T3. However, we observed lower total fluorescence intensity of phalloidin in treated cells suggesting a general effect of ERK1/2 on cytoplasmic F-actin.

As mentioned before, prior research from our lab discovered a novel role of JNK in ciliogenesis. Therefore, our last aim was to examine if there is an interplay between ERK and JNK. Our current findings revealed commonalities between ERK and JNK regarding the localization and function on the basal bodies, leading us to further investigate if their inhibition affects each other's activation. Inhibition of JNK, resulted in ciliogenesis defects as expected, but ERK's activation was not affected, suggesting that ERK activity and association with basal bodies does not depend on JNK. On the other hand, pJNK was affected by inhibition of ERK's activity, since we observed lower fluorescence intensity of pJNK at the base of the axoneme, and fewer cells having pJNK on their basal body. These results indicate that activation of JNK requires ERK's function to optimally operate but this needs to be explored more thoroughly.

Overall, our data provide new evidence of the presence of ERK on basal bodies in both motile and primary cilia, and how essential it is for ciliogenesis to occur normally. Further research should focus on gaining more information about the function of ERK in ciliogenesis that would eventually offer new therapeutic strategies against cilia-related diseases.

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