Université de Montréal

Exploring a role for a Par3/CaMKII protein complex in photoreceptor cell polarity and ciliogenesis

Par

Yulia Ezhova

Programme de biologie moléculaire

Faculté de médecine

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Résumé

Le traitement et la propagation de l'information nerveuse repose sur une distribution asymétrique de récepteurs et d'émetteurs à la surface de chaque neurone. Ce cloisonnement en domaines sous-cellulaires distincts est également appelé polarité cellulaire. Dans la rétine, la perte de polarité des photorécepteurs peut entraîner des dystrophies rétiniennes telle que l'amaurose congénitale de Leber, mais les mécanismes moléculaires impliqués restent flous. Un complexe protéique impliqué dans l'établissement de la polarité cellulaire, hautement conservé de *C. elegans* aux mammifères, est le complexe PAR. Localisé au niveau de la région sous-apicale des cellules polarisées, le cœur de ce complexe est constitué des protéines de la famille *partitioning defective* Par3 / Par6 et de la protéine kinase C atypique aPKC. Bien que largement étudié dans les cellules épithéliales, le rôle du complexe Par dans les neurones de mammifères reste mal compris. Nos résultats indiquent que l'inactivation conditionnelle (cKO) de *Par3* dans la rétine de souris en développement interfère avec la croissance polarisée du cil photosensible à la pointe apicale des cellules photoréceptrices (PR), conduisant finalement à une dégénérescence des PRs. Pour découvrir comment Par3 pourrait réguler la ciliogenèse des PRs, nous avons immunoprécipité Par3 à partir d'extraits rétiniens de souris et effectué une analyse par spectrométrie de masse. Nous avons trouvé un ensemble de protéines appartenant à la famille des calcium-calmoduline-dépendantes de la protéine kinase II (CaMKII) comme partenaires potentiels de Par3 dans la rétine. Les CaMKII figurent parmi les protéines les plus abondantes du système nerveux central où elles constituent 1 à 2% des protéines totales. Alors que des études approfondies ont démontré l'importance de CaMKII dans la potentialisation et la dépression à long terme (LTP et LTD), et l'arborisation des dendrites, son rôle dans la polarité cellulaire reste inconnu. En utilisant des versions étiquetées de Par3 et CaMKIID, nous avons validé leur interaction *in vivo* et *in vitro* par co-immunoprécipitation. Nous avons mis en évidence une localisation de CaMKIID dans la région ciliaire des PR, suggérant que Par3 pourrait recruter CaMKIID à la membrane apicale des cellules PR, où il pourrait être impliqué dans la ciliogenèse. Pour explorer cette hypothèse, nous avons étudié si les formes dominantes négatives ou constitutivement actives de CaMKIID pouvaient avoir un impact sur la formation des cils des PRs.

La surexpression des deux formes mutantes au cours du développement des PRs a entrainé un raccourcissement des segments externes, semblable à ce que nous avons observé dans les rétines *Par3* cKO. Cette étude montre qu'un complexe de protéines CaMKIID / Par3 pourrait réguler l'établissement et le maintien de polarité des PRs, suggérant l'implication ce complexe dans le contrôle de la polarité neuronale de l'ensemble du système nerveux central.

Mots-clés : polarité cellulaire, système nerveux, rétine, photoréceptrices, ciliogenèse, Par3, CaMKIID.

Abstract

Cell polarity is an essential property of adult neurons, which rely on asymmetric distribution of receptors and transmitters for proper signal propagation and cell function. In the retina, loss of photoreceptor (PR) polarity can lead to retinal dystrophies such as Leber Congenital Amaurosis, but the molecular mechanisms involved in regulating PR polarity remain unclear. A highly conserved protein complex involved in the establishment of cell polarity from *C. elegans* to mammals is the Par complex. Localized at the subapical region of polarized cells, it is composed of the "partitioning defective" PDZ domain-containing proteins Par3/Par6 and the atypical protein kinase C (aPKC). Although extensively studied in epithelial cells, the role of the Par complex in mammalian neurons remains poorly understood. Our unpublished results indicate that conditional inactivation (cKO) of Par3 in the developing retina interferes with the polarized growth of the photosensitive cilium at the apical tip of PR cells, eventually leading to PR degeneration. To uncover how Par3 might regulate ciliogenesis in PR cells, we immunoprecipitated Par3 from mouse retinal extracts and carried out mass spectrometry analysis. We found a cluster of calcium/calmodulin-dependent protein kinase II (CaMKII) proteins as potential Par3-interacting partners in the retina. CaMKII is one of the most abundant proteins found in the central nervous system, where it constitutes 1-2% of total proteins. While extensive studies have demonstrated the importance of CaMKII in long-term potentiation (LTP), long term depression (LTD) and dendrite arborisation, its role in cell polarity remains unknown. Using tagged versions of Par3 and CaMKIID, we validated their interaction *in vivo* and *in vitro* by co-immunoprecipitation. Interestingly, we found that CaMKIID localizes to the ciliary region of PRs, suggesting that Par3 might recruit CaMKIID at the apical membrane of PR cells, where it could be involved in ciliogenesis. To explore this hypothesis, we investigated whether dominant-negative or constitutively active forms of CaMKIID could impact cilia formation in PRs. Interestingly, overexpression of both mutant forms of CaMKIID during PR development resulted in shortening of the photosensitive cilia (outer segments), similar to what we observed in Par3 cKO retinas. This study suggests that a CaMKIID/Par3 protein complex regulates the establishment of PR cell

polarity, raising the possibility that this complex may be generally involved in controlling neuronal polarity throughout the nervous system.

Keywords : cell polarity, nervous system, retina, photoreceptors, ciliogenesis, Par3, CaMKIID.

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List of abbreviations

CaMKIID : Calcium/calmodulin dependent protein kinase type II

Pard3 : partitioning defective 3 homolog

CCS : Cosmic calf serum

PR : photoreceptors

IS : inner segment

OS : outer segment

ONL : outer nuclear layer

OLM : outer limiting membrane

PFA : paraformaldehyde

shRNA : short hairpin RNA

PEI **:** Polyethylenimine

BSA : bovine serum albumin

NP-40 : Nonidet P40

RT : room temperature

IFL : immunofluorescence

IP : immunoprecepitation

WB : western blot

LTP : long term potentiation

RGC : retinal ganglion cell

aPKC : atypical protein kinase C

AP : anterior-posterior axis

C.elegans : Caenorhabditis elegans

VZ : ventricular zone

SVZ : subventricular zone

KO : knock-out

IFT – intraflagellar transport

GFP : Green Fluorescent Protein

RYFP : Rosa locus - Yellow Fluorescence Protein

PhK : Phosphorylated kinase

Pals1 : Protein associated with Lin seven 1

PATJ : Pals1-associated tight junction protein

DLG : Discs-large

Lgl : Lethal-2-giant larvae

CNS : Central nervous system

IHC : immunohistochemistry

ICC : immunocytochemistry

MDCK : Madin-Darby canine kidney

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Contribution of Authors

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1. Cellular Neurobiology Research Unit, Montreal Clinical Research Institute (IRCM), Montreal, QC, Canada

2. Molecular Biology Program of the Faculty of Medicine, University of Montreal, Montreal, QC, Canada

3. Division of Experimental Medicine, McGill University, Montreal, QC, Canada

4. Department of Medicine, University of Montreal, Montreal QC, Canada

Chapter 1

1.3.1. The schematic diagram presented in **Figure 1** was used from the article by Purves et.al., 2001 (Purves et al., 2001).

1.3.1. The schematic diagram presented in **Figure 2** and in section 1.4. **Figure 3** were used from the article by Ramamurthy and Cayouette, 2009 (Ramamurthy & Cayouette, 2009).

1.5. The schematic diagram presented in **Figure 4** was taken from the article by Lisman et. al., 2002 (Lisman, Schulman, & Cline, 2002).

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2.2. The diagram for the isolation of primary mouse embryonic fibroblasts presented in **Figure 5** was used from ThermoFisher ©, as per their instruction manual

2.8. The diagram for the mouse line crosses presented as **Figure 6** was made by Dr Michael Housset

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In this section, Dr Michael Housset made **Figures 7, 8, 9, 10** representing previous findings in our laboratory, under the supervision of Dr Cayouette.

3.4. The diagram for the cell transfection presented in **Figure 15** was taken from the article Yang et.al., 2017 (Yang, Zhou, Li, Fu, & Sun, 2017).

Chapter 1 – Introduction

Cell polarity is an important property of all eukaryotic cells required for the proper establishment and maintenance of tissues and cellular processes. One important example is the retina, a tissue that absorbs and transmits light to the brain, through its highly polarized laminar architecture defined by a network of appropriately positioned neuronal cells. All vertebrate retinas are composed of three layers of nerve cell bodies and two layers of synapses (Varshney, Hunter, & Brunken, 2015). Although several polarity complexes have been identified, the mechanism of how they establish polarity in the retina has not been elucidated. In general, in the central nervous system (CNS), polarity proteins not only help maintain the tissue morphology, but also contribute to axon extension and dendrite formation, essential for neuronal connections and functional circuitry. Errors in establishing cell polarity are often the cause of photoreceptor death, leading to retina degeneration (Omri et al., 2010; Rich, Figueroa, Zhan, & Blanks, 1995; Stuck, Conley, & Naash, 2012). Thus, we were interested in studying the role of the well-known polarity complex Par during mouse retina development.

To understand the molecular basis of Par3 function in the developing retina, we performed immunoprecipitation and mass spectrometry analysis of retinal extracts and identified proteins that interact with Par3. Among those, in this study, I focused on understanding the functional importance of Par3 and CaMKII (isoform D) interaction for the retinal post-natal development and maintenance in the mouse.

1.1. Cell polarity

Cell polarity is a fundamental feature of all unicellular and multicellular organisms during their development, and it is a reflection of the formation of physically and chemically distinct domains within the cells and tissues (Allam, Charnley, & Russell, 2018; Szu-Yu Ho & Rasband, 2011). Polarity is essential in mediating a variety of cellular processes such as cell division, differentiation, adhesion, protein trafficking and cytoskeletal formation (Arimura & Kaibuchi, 2007; Assémat, Bazellières, Pallesi-Pocachard, Le Bivic, & Massey-Harroche, 2008; Pruyne, Legesse-Miller, Gao, Dong, & Bretscher, 2004; Rodriguez-Boulan & Powell, 1992; Siegrist & Doe,

2007). This is accompanied by changes in the cell shape and structure and it is driven by the associated polarity proteins. There are 3 main types of cell polarity: 1) apico-basal polarity (ABCP), also called epithelial polarity (J. Chen & Zhang, 2013; Tepass, 2012); 2) planar cell polarity (cell organisation in the specific direction in the plane of the cell sheet) (Sebbagh & Borg, 2014; Stephens et al., 2018; Wansleeben & Meijlink, 2011); and 3) front-rear cell polarity that is involved in cell migration (May-Simera & Kelley, 2012; Mayor & Etienne-Manneville, 2016; Yassin & Russell, 2016). Different cell types display a specific type of polarity and this is critical for their formation, migration, lamination and maintenance (Allam et al., 2018; Assémat et al., 2008; Rodriguez-Boulan & Powell, 1992; Singh & Solecki, 2015; Stern, 2006).

To segregate fate determinants, cells use apical-basal or the planar polarity of the surrounding tissue to determine the plane of the cell division during cytokinesis. As a result, the cell can be divided asymmetrically or symmetrically. During the asymmetric division, the fundamental aspect is a production of two daughter cells with a different cellular fates (Prehoda, 2009; Yamashita, Yuan, Cheng, & Hunt, 2010), and it occurs when the plane of division is perpendicular to the apico-basal axis. These cells can be recognized by differences in their size, morphology, gene expression pattern, or the number of subsequent cell divisions undergone by the daughter cells (Knoblich, 2008). As a result, this type of division contributes to an increase of the cell diversity within a tissue where one daughter cell self-renews to maintain the progenitor pool, whereas the other differentiates to populate and maintain tissue homeostasis (Campanale, Sun, & Montell, 2017; Knoblich, 2008; Rose & Gönczy, 2014). In the retina, for example, an asymmetrically dividing progenitor cell can give rise to two neurons of different fates (Chiu et al., 2016; Kechad et al., 2012). On the other hand, a symmetric cell division is when the two daughter cells adopt the same fate as a result of symmetric segregation of the fate determinants (Fraschini, 2020) and it takes place when the division plane is along the apico-basal axis. In the developing retina, for example, the dividing progenitor or neuron cells can produce a new progenitor or a neuron cell, respectively.

1.2. Establishment of polarity in epithelial cells

Three major protein complexes are involved in the establishment of the apical basal polarity in the epithelial cell. The Crumbs complex is required for the establishment of apical membrane; the

Scribble protein complex regulates the establishment of the baso-lateral membrane, and the Par complex is involved in the regulation of apical-lateral membrane border (Assémat et al., 2008; F. & M., 2012; Tepass, 1996; Tepass & Knust, 1993).

1.2.1. Regulation of cell polarity by Par complex

The Par protein complex is evolutionary conserved and was first described in *C. elegans*. Key polarity determinants were identified through a genetic screen for mutants that affected asymmetric sizes of daughter cells during the first division of *C. elegans* embryo. Using this screen, the first members of the "partitioning-defective" family genes were discovered, and their protein products were shown to accumulate at one of the two cell poles before the first cell division (Kemphues, Priess, Morton, & Cheng, 1988). The identified genes were found to play a fundamental role in establishing the anterior-posterior axis in the *C. elegans* zygote. Those were the so-called Bazooka (the orthologue of Par3 in *D. melanogaster*) and its paralogue Par6, belonging to the group of proteins containing PDZ-domains, and the atypical protein kinase C (aPKC), a serine/threonine protein kinase (P. O. Humbert, Dow, & Russell, 2006), and the cell division control protein42 (CDC42).

Early work has shown the importance of the Par complex in the *D. melanogaster* epithelium, where it regulates and maintains apical-basal polarity. Apart from binding to the proteins from the Par complex, Par3 binds to numerous other proteins through its three central PZD-domains and binding motifs in its C- and N- tails (Harris, 2017). However, its active binding to aPKC/Par6 is not necessarily required for all polarity processes in *D. melanogaster.* aPKC-dependent phosphorylation can exclude Par3 from the aPKC/Par6 complex in *D. melanogaster* epithelial cells (Ellenbroek, Iden, & Collard, 2012; Horikoshi et al., 2009; Morais-de-Sá, Mirouse, & St Johnston, 2010). The Par3 complex is shown to interact with other polarity complexes, such as Crumbs, to regulate the membrane identity in epithelial cells (Thompson, Pichaud, & Röper, 2013), but also with the Scribble complex, to control the dendrite morphogenesis, stem cell division and T-cell polarity (P. O. Humbert et al., 2006).

Loss of cell polarity can have a deleterious effect on the tissue structure. Together with a loss of cell proliferation control, it is a hallmark of a complex disease, such as cancer (P. Humbert, Russell, & Richardson, 2003; Rejon, Al-Masri, & McCaffrey, 2016). In addition, in the mammalian CNS, the

loss of *Pard3* in radial glial progenitors (RGPs) causes severe brain cortex malformations, changes in neuronal subtype composition and massive heterotopia (Liu et al., 2018).

1.2.2. The polarity complex - Crumbs

Epithelial cell polarity is regulated by proteins complexes, such as Par and Crumbs that interact with each other directly, and determine the apico-basal axis, positioning and stability of the cell-cell junctions at the apical-lateral side in invertebrates (Bazellières, Aksenova, Barthélémy-Requin, Massey-Harroche, & Le Bivic, 2018). The Crumbs protein complex has been identified in *Drosophila melanogaster* embryo and it consist of Crumbs, Pals1 (Protein associated with Lin seven 1) and Pals1-associated tight junction protein (PATJ) (Ellenbroek et al., 2012). In mammals, the Crumbs protein family consists of four members, Crb1, Crb2, Crb3A and Crb3B. The CRB protein is a transmembrane protein that has a large extracellular domain with epidermal growth factor (EGF) and laminin-globular domains, a single transmembrane domain, and an intracellular tail with PDZ protein-binding motif (Alves, Pellissier, & Wijnholds, 2014; Tepass, Theres, & Knust, 1990) that allows it to interact with Pals 1 and PATJ (Makarova, Roh, Liu, Laurinec, & Margolis, 2003). In epithelial cells, the CRB3 expression is most abundant and its role is to establish a link of the apical membrane with the tight junction (Makarova et al., 2003; Margolis, 2018).

There is a large body of data in the literature regarding the importance of CRB3 in epithelial polarity. Overexpression of CRB3 was demonstrated to cause abnormal overgrowth of the apical surface and imperfection in tight junctions (Lemmers et al., 2004; Roh, Fan, Liu, & Margolis, 2003). Likewise, in *Drosophila*, the alteration in the Crumbs3 gene expression leads to tissue overgrowth (C. L. Chen et al., 2010; Lu & Bilder, 2005; Sotillos, Díaz-Meco, Caminero, Moscat, & Campuzano, 2004), which is directly linked with an aberration in the Hippo pathway (Elbediwy, Vincent-Mistiaen, & Thompson, 2016). On the other hand, knockdown of Crumbs3 in Madin-Darby canine kidney (MDCK) cells causes defects in cilia formation without affecting polarity or tight junctions (S. Fan et al., 2004).

1.2.3. Scribble protein complex

Scribble is a multidomain scaffolding protein complex comprising of Scribble (Scrib), Discs-large (Dlg) and Lethal-2-giant larvae (Lgl) (Stephens et al., 2018). It is involved in several biological

processes such as cell proliferation and migration, neuronal development, asymmetric cell division, the establishment of cell polarity and integrity maintenance.

The scribble complex was first identified in *D. melanogaster* for its role in apico-basal polarity and epithelial integrity. *Dlg, Lgl* and *Scrib* were identified as tumour suppressor genes (P. Humbert et al., 2003), and studies have shown that mutations in these genes lead to a disruption of the cell polarity, cell junctions and induce an uncontrolled cell proliferation (Yamanaka & Ohno, 2008). This complex in *D. melanogaster* and *C. elegans* appears to share conserved functions.

Scribble is a protein of the LAP (LRR and PDZ domain) protein family, that contain leucine-rich repeats (LPPs) (Bryant & Huwe, 2000) and a multi-PDZ domain that is important for the protein-protein interaction (Fanning & Anderson, 1999). The main role of Scribble protein is to facilitate the key molecular interactions that are associated with the maintenance of apical-basal polarity, asymmetric cell division, cell proliferation and migration (Bonello & Peifer, 2019). Dlg is a member of the MAGUK (membrane-associated guanylate kinase) family and consist of two PDZ domains, the function of which is to bind the extreme carboxy-terminal cytoplasmic tail of transmembrane proteins in a sequence-specific fashion and it has a role in junction formation and cell signalling (Anderson, 1996). A characteristic feature of Lgl, on the other hand, is that it has at least 4-5 WD40 motifs involved in the protein-protein and receptor-ligand interactions during signal transduction (Croze et al., 2000; Li & Roberts, 2001), cell cycle regulation (Ohtoshi, Maeda, Higashi, Ashizawa, & Hatakeyama, 2000) and cytoskeleton assembly (Baek, 2004; Su, Mruk, Wong, Lui, & Cheng, 2013).

1.2.4. Polarity in the central nervous system

The complexity of the mature central nervous system (CNS) is a result of a tight balance between cell proliferation and differentiation throughout development (Costa, Wen, Lepier, Schroeder, & Götz, 2008). It is a great example demonstrating a high degree of tissue and cell polarity. Neurogenesis in mammals begins at the early embryonic stage from a pseudostratified neuroepithelium (Götz & Huttner, 2005), and it heavily relies on polarity to differentiate cells into a variety of neuronal subtypes, to migrate to specific cortical layers and maintain synaptic contacts with other neurons for communication (Rodriguez-Boulan & Powell, 1992; Singh & Solecki, 2015; Szu-Yu Ho & Rasband, 2011). After a series of symmetric and asymmetric divisions

of progenitor cells, the newborn neurons reacquire polarity and bipolar morphology, extend the axonal process, migrate, and finally extend their dendritic tree (Namba et al., 2015). Their polarized morphology with dendrites and axons ensures a proper flow of information, on one end receiving and the other transmitting electrical currents. As the polarity proteins are one of the key regulators supporting the architecture of the cellular asymmetry, loss of polarity in neurons could be an underlying cause for developing neuronal diseases and their degeneration. For example, disturbances in the synapses can lead to developing neuropsychiatric disorders, such as schizophrenia, and polymorphism in the Pard3 gene was associated with increased sensibility do develop this disorder (Kim, Lee, Park, Kim, & Chung, 2012). Altogether this makes the CNS an excellent model to study the key regulators of cell polarity formation and maintenance, and here the mammalian retina with its well-defined and polarized architecture can be particularly useful.

1.3. The mouse retina as a model system to study polarity in the central nervous system

The CNS is composed of the brain, retina and spinal cord, whereas the peripheral nervous system includes the spinal nerves that branch from the spinal cord and the autonomous nervous system (Purves et al., 2001; Sharma & Majsak, 2014). The CNS is highly complex and it is a distinctive feature of all vertebrates where the billions of neurons operate in a highly coordinated way (Centanin & Wittbrodt, 2014).

1.3.1. Developmental origin of the retina

The neural retina is the most accessible part of the vertebrate CNS and it is an excellent system to study neurogenesis, at both molecular and cellular levels (Centanin & Wittbrodt, 2014). The pioneers in the vertebrate retinal studies were Ferrucio Tartuferi and Santiago Ramon y Cajal more than 100 years ago, who first described the structure of retina (R. H. Masland, 2001; Ramón y Cajal, 1892). The vertebrate retina is a multilayered tissue, approximately 200 µm thick (in the case of the mouse) located at the posterior part of the eye (Richard H. Masland, 2012). It is composed of eight major different cell types, distributed into three main layers and interconnected by synapsis in the plexiform layers (Figure 1A). Seven cell types (ganglion cells,

amacrines, bipolars, horizontals, rods, cones, and Müller glia) arise from a pool of retinal progenitor cells (RPCs), whereas astrocytes are produced in the brain and migrate into the retina through the optic nerve. RPCs are organized in a neuroepithelium, where each RPC contacts neighbouring RPC in both apical and basal laminae (Centanin & Wittbrodt, 2014). Notably, before neurogenesis the vertebrate embryonic retina is a sheet of epithelial cells, called pseudostratified neuroepithelium, where establishment and maintenance of apicobasal polarity are regulated by Par, Crumbs and Scribble complexes (Malicki, 2004). The studies on zebrafish and medaka revealed that mutations in the genes that regulate the apico-basal polarity cause severe retinal disorganisation (Herder et al., 2013; X. Wei & Malicki, 2002).

In mouse, the retina is made of more than 60 different cell subtypes, where each of them has a specific role in the vision process (Richard H. Masland, 2012). They are generated sequentially during eye development and the beginning of the retina tissue formation starts during the early embryonic day 9 (E9) when the RPCs undergo symmetric and asymmetric divisions (Heavner & Pevny, 2012). While the early-born retinal neurons are ganglion cells, horizontal, amacrine interneurons, and cone photoreceptors, the late-born ones are rod PR, bipolar interneurons, and Müller glia (Figure 1B) (Heavner & Pevny, 2012). In terms of their function, the light-detecting cells are rod and cone, projecting neurons are retinal ganglion cells (RGC) and interneurons (amacrine, horizontal and bipolar). The amacrine cells process the received information from outside of the eye to transmit it to the PRs, horizontal and glial cells (Müller glia). Horizontal cells provide feedback to PRs and bipolar cells, and these are subdivided into rod bipolar and cone, respectively. Both types of bipolar cells transfer PRs output to all amacrine cells and RGC (Heavner & Pevny, 2012; Richard H. Masland, 2012; Sanes & Zipursky, 2010; Wässle, Puller, Müller, & Haverkamp, 2009).

Among mammals, the composition of the retina is conserved, but the total number of cell-types can vary from one species to another. The human retina, for example, contains approximately 6 to 7 million PRs in total, out of which, rod PRs make 95 %, and cones 5% (Mahabadi & Al Khalili, 2019). In diurnal mammals, the total cone cells number can vary from 8 % to 95 % (Ahnelt & Kolb, 2000; Peichl, 2005). For example, the pig retina is rod dominated where only up to 20 % of cells

are cone cells (Hendrickson & Hicks, 2002). In contrast, nocturnal species has a rod-dominated retina, where the ratio of the rod to cone is 12.4 to 1 (van der Merwe et al., 2018).

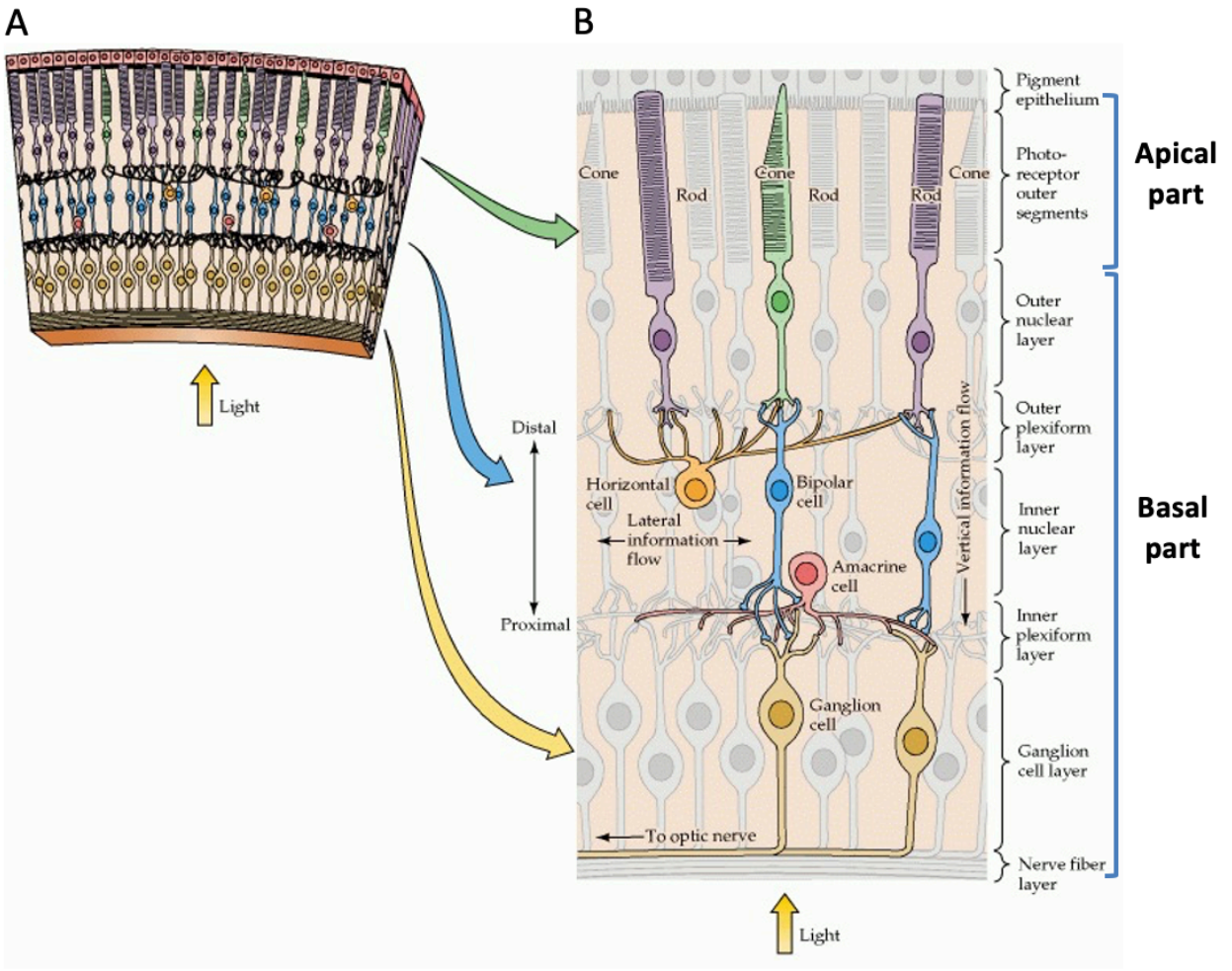


Figure 1. – Retina structure adopted from (Purves et al., 2001). (A) Section of the retina with cellular organisation. (B) Schematic representation of retinal architecture: light detection rod and cone photoreceptors, projection neurons – retinal ganglion cells (RGC) and interneurons – bipolar, amacrine and horizontal cells.

Rod and cone PR cells are neurons that present a remarkable level of sub-compartmentalization of the cell body, related to their specialized function of light detection and contain two different light-sensitive proteins. Rods contain rhodopsin (R. H. Masland, 2001; Sanes & Zipursky, 2010), and the 2 types of cones in mice, the M and S cones are with similar structure and functions and contain opsins (Richard H. Masland, 2012; Thoreson & Dacey, 2019). Rod and cone PRs are active

during different times of the day. While the cones are responsible for colour (photopic) vision during daylight, the rods are accountable for night (scotopic) vision. Additionally, rods have a slow speed response, compared to the rapid one in cones (Mahabadi & Al Khalili, 2019).

PR cells are composed of the inner segment (IS), where the biosynthesis machinery resides to produce the vital important proteins, the outer segment (OS), where the phototransduction processes occur, and the nucleus. The IS and OS are connected between each other by a microtubule structure, named connecting cilium (Figure 2). All produced proteins from IS pass through the connecting cilia to build up the OS of PRs. The OS of PRs consist of stacks of membranous discs, which contain opsin that forms a chromophore when bound to the 11-cis retinal visual pigments necessary for phototransduction. The IS contains most of the cellular organelles and the protein machinery where all proteins are produced and transferred to the OS through the connecting cilia. The connecting cilium is a specialized non-motile cilium, which is an evolutionally conserved structure that has multiple functions in the developing and mature organisms, for example, sensory function (Sedmak & Wolfrum, 2011). Disruption of cilia is associated with several human disorders such as retinal degeneration, hearing impairment, polycystic kidney and liver, hydrocephalus and dyskinesia (Badano, Mitsuma, Beales, & Katsanis, 2006; Rothschild, Francescatto, Drummond, & Tombes, 2011).

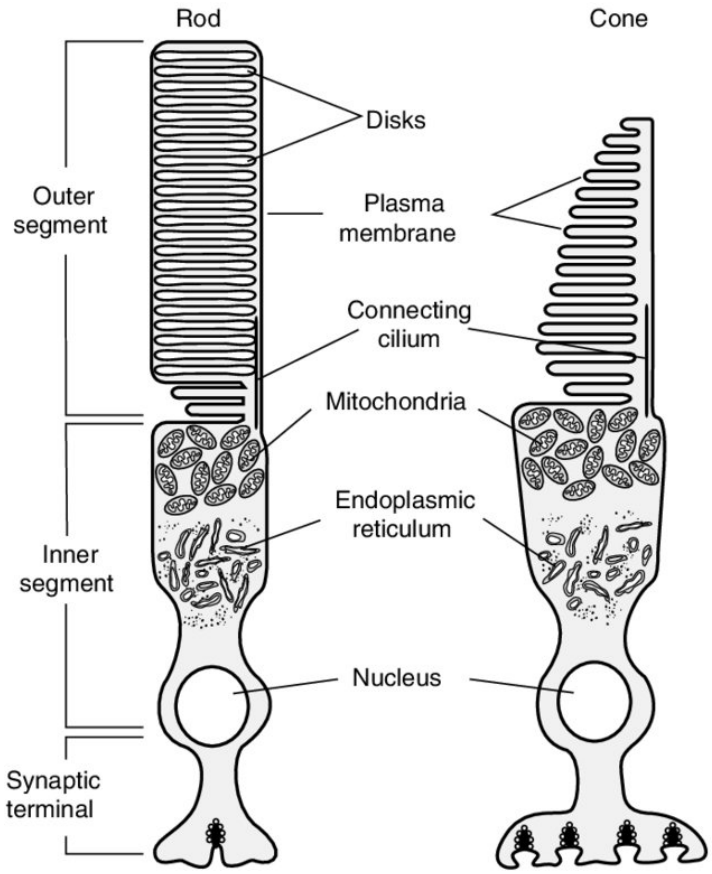


Figure 2. – Schematic diagram of the structure of the photoreceptor. Figure adapted from (Cote, 2019). The phototransducing outer segment (OS) is connected to the inner segment (IS) by the connecting cilium.

1.4. Photoreceptor ciliogenesis

Cilia are a microtubule-based small organelle protruding from the cell surface that play the role of sensory organelles, which help to interpret various environmental signals. Motile cilia were discovered by Antony van Leeuwenhoek in 1670 in protozoa (Dobell & Leeuwenhoek, 2011), and the primary cilia in late 19th century by the Swiss anatomist, KW Zimmerman (Zimmermann, 1898). Cilia are made of a microtubule cytoskeleton that forms the ciliary axoneme that grows from and continues the ninefold structure of the centriole (Satir & Christensen, 2007). Cilia is an evolutionarily conserved structure, and its size can vary from 100-250 nm in diameter and 100-400 nm in length (Roman, Garrido-Jimenez, Diaz-Chamorro, Centeno, & Carvajal-Gonzalez, 2019).

In the animal kingdom, there are different types of cilia, such as motile cilia (9+2) in which nine doublet microtubules surround a central pair of singlet microtubules (Bayless, Navarro, & Winey, 2019), non-motile (primary) cilia (9+0) in which a central pair is missing and nodal cilia, with (9+0) structure that also misses the central pair but has outer dynein arms (ODA) (Satir & Christensen, 2008). Motile cilia are multifunctional organelles that have a function of transporting the extra-cellular fluid. However, immotile (primary) cilia are known for sensing extra-cellular cues to the cell (Bayless et al., 2019; Hua & Ferland, 2018). Malformations of the cilia lead to many human disorders such as primary ciliary dyskinesia, Meckel syndrome, Joubert syndrome, and retinal degeneration, referred to ciliopathies (Reiter & Leroux, 2017). Evolutionally conserved across vertebrates, immotile cilia are exerting a wide range of functions in different organs. For example, the role of cilia in the olfactory epithelium is to detect odorants (Kaupp, 2010). In the kidney epithelium and the ear, primary cilia act as mechanosensors, detecting the fluid flow (Praetorius & Spring, 2003). In PR cells, they generate the light-sensitive OS (Baylor, Lamb, & Yau, 1979), with mutations of cilium genes causing PR cell degeneration.

PRs genesis is a long developmental process that begins at the early embryonic stage and ends postnatally where the cone precursors are born at E14, while rod progenitors are born after birth (Morrow, Furukawa, & Cepko, 1998; Rachel, Li, & Swaroop, 2012). The first step of ciliogenesis occurs postnatally when the basal body docks at the cell cortex, with generation and extension of the rod axoneme (Sedmak & Wolfrum, 2011). Basal body ultrastructure reveals a symmetrical array of nine microtubules in a triplet arrangement of A, B and C tubules (Baehr et al., 2019). Soon after birth, the mother centriole acquires a Golgi-derived ciliary vesicle that mediates docking to the cell membrane (Sorokin, 1962). After basal body docking to the cell membrane, A and B tubules arise from basal body forming the proximal axoneme which matures to the connecting cilia (Baehr et al., 2019). The primary cilia of all ciliated cells lack an *in situ* biosynthesis machinery, meaning that all molecular components forming the cilium are synthetized in the cell prior to transport to the cilium (Ramamurthy & Cayouette, 2009). The formation and maintenance of cilia requires intraflagellar transport (IFT) which refers to the anterograde and retrograde movement of IFT components within the axoneme (Figure 3) (Krock & Perkins, 2014; Rosenbaum & Witman,

2002), initially studied in the unicellular organism *Chlamydomonas* (Kozminski, Johnson, Forscher, & Rosenbaum, 1993; Pedersen, Geimer, & Rosenbaum, 2006).

Both cone and rod PRs develop an OS by growing the connecting cilium at the end of the apical part of the cell (Ramamurthy & Cayouette, 2009). OS is comprised of a stack of membranous disks that contain a high density of opsin (Röhlich, 1975; Young, 1967). The process of disk membrane formation is maintained in fully mature PRs, with approximately 10 % of the whole length of the OS renewed every 24 hours, demonstrating that protein trafficking initiated during maturation of the OS, is prolonged in mature PRs (Young, 1967). For instance, every minute, 2000 molecules are transported from the IS to the OS of a mature mouse rod PR cell (Insinna & Besharse, 2008). The distinctive feature in the morphology of PRs is that in cone PRs, the disc membranes are continuous with, whereas in rod PRs the discs are separated from the plasma membrane (Ramamurthy & Cayouette, 2009). The first discs formation of the mammalian rod PRs, occurs around P9 by the membrane evagination (Burgoyne et al., 2015; Ding, Salinas, & Arshavsky, 2015). By P21, the OS of mouse PRs is fully mature.

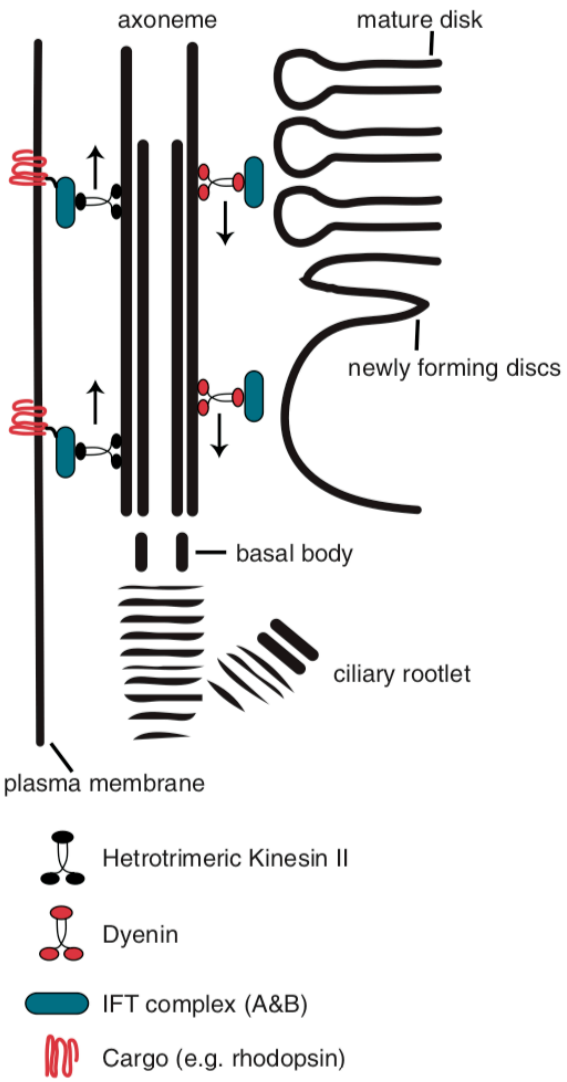


Figure 3. – Protein trafficking in photoreceptors. Figure taken from (Ramamurthy & Cayouette, 2009). Schematic representation the intraflagellar transport (IFT) in the vertebrate photoreceptor. The kinesin II and dynein demonstrate the anterograde and retrograde transport within axoneme. The motor proteins transport the cargo e.g. rhodopsin with IFT complex.

1.5. The role of CaM Kinase family in the CNS

Calcium/calmodulin-dependent protein kinase (CaM Kinase) was identified in nervous tissue and requires for its activation a heat-stable protein factor as well as Ca^{2+} (Huttner & Greengard, 1979).

Later on, CaM was identified as a regulator of myosin light chain (Yagi, Yazawa, Kakiuchi, Ohshima, & Uenishi, 1978) and phosphorylase kinase activity (PhK) (Cohen et al., 1978). Current nomenclature is based on the loading of brain extract into a fractionation column, and includes CaMK I to IV (Yamauchi & Fujisawa, 1983).

Calcium/calmodulin-dependent protein kinase type II (CaMKII) is activated by Ca^{2+} and it is essential for the regulation of gene expression, cell cycle control, neurotransmitter synthesis, synaptic plasticity, long term potentiation (LTP) and long term depression (LTD) (Bayer & Schulman, 2019b; Küry et al., 2017; Puram et al., 2011). CaMKII has many isoforms, which are encoded by four different genes. Each CaMKII gene generates different splice variants depending on the region of expression (Bayer, Koninck, & Schulman, 2002). CaMKII is ubiquitously expressed in many regions of the brain and it exceeds 1-2 % of the total amount of proteins. The synaptic activity in the brain is a critical component of learning and memory, and its impeded function leads to many neurological and psychiatric disorders (Bliss, Collingridge, & Morris, 2014; Grant & Silva, 1994). Apart from the brain, CaMKII is important in kidney development and stabilisation of cilium in the pronephric kidney (Bayer & Schulman, 2019a; Küry et al., 2017; Rothschild et al., 2011).

All Calcium/calmodulin-dependent protein kinase (CaMK) isoforms are closely related to each other. CaMKII family contains seven members: four CaMKII isoforms are encoded by different genes, two PhK (phosphorylase kinase) and CASK (Bayer & Schulman, 2019b). CaMKII α, β, γ and δ are highly homologous and demonstrate differential but overlapping expression patterns in different tissues, brain regions and developing stages. CaMKII α and β subunits are mostly expressed in the brain (especially in hippocampus and neocortex) and they form dodecameric structure containing either one or both subunits (Lisman et al., 2002). On the other hand, CaMKII γ and δ isoforms are expressed ubiquitously in early developmental stages (Bayer, Löhler, Schulman, & Harbers, 1999; Bayer & Schulman, 2019a). Recently, identification of *de novo* rare 19 CaMKIIA and CaMKIIB mutations were shown to affect neuronal migration and cause intellectual disability (Küry et al., 2017).

Each CaMKII isoform contains two regulatory domains, catalytic and autoinhibitory (Figure 4A). The catalytic/regulatory domain contains ATP- and substrate binding sites, as well as site of interaction with anchoring proteins. Catalytic and autoinhibitory domains bind through T and S sites forming a "gate" that regulates protein activity. In the presence of Ca^{2+}/calmodulin, these domains dissociate leading to the kinase activation. Once the T site is phosphorylated the gate cannot be closed even after Ca^{2+}/calmodulin dissociation from the enzyme (Figure 4B) (Lisman et al., 2002). Furthemore, CaMKII can be activated autonomously by autophosphorylation at Thr286 site (Figure 4B). Autophosphorylation occurs as an inter-subunit reaction within the holoenzyme, and it requires 2 molecules of calmodulin. Ca^{2+}/calmodulin activates the "kinase" subunits and presents effectively the "substrate" subunit for autophosphorylation (Hanson, Meyer, Stryer, & Schulman, 1994). Finally, its activity can be also regulated through the NMDA (*N*-methyl-D – aspartate) receptor binding to the T site (Lisman et al., 2002).

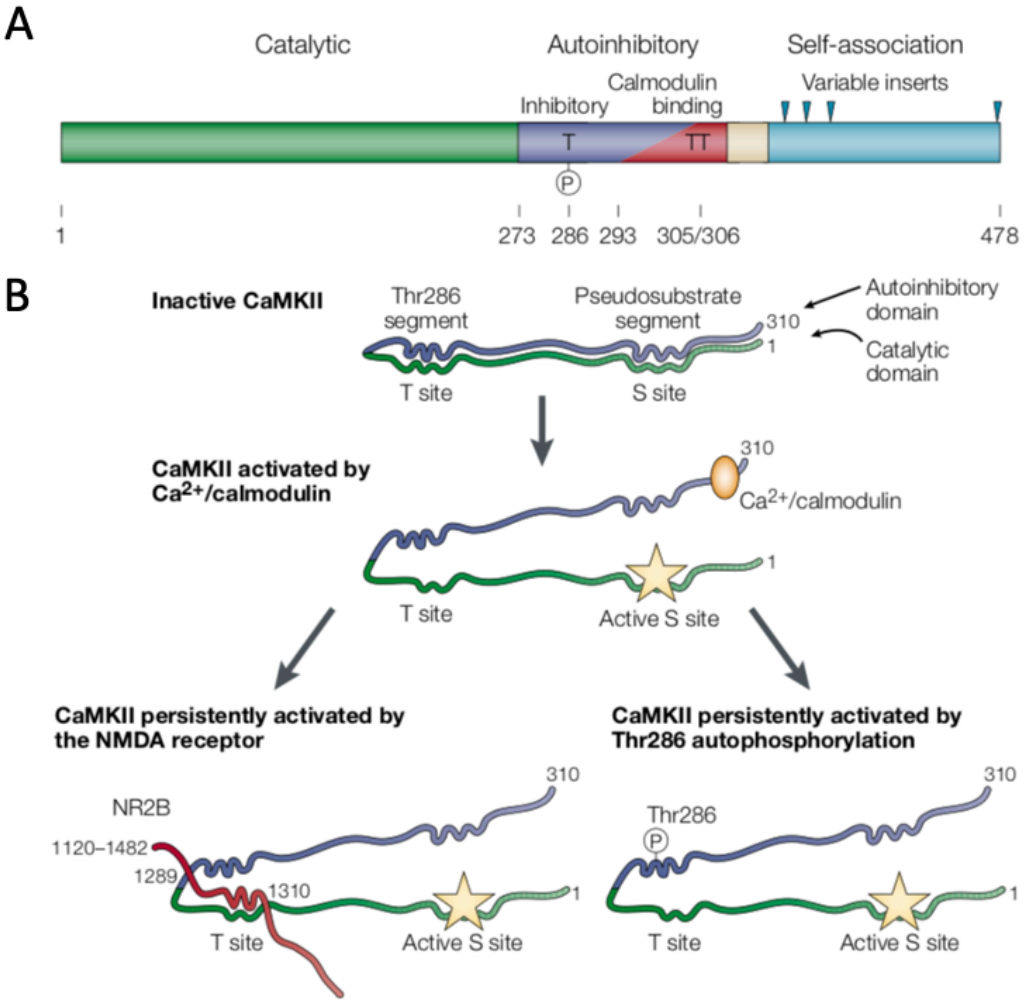


Figure 4. – CaMKII structure was taken from (Lisman et al., 2002). (A) Schematic represents the different protein regulatory domains. (B) Schematic represents inactive and active forms of protein.

In the vertebrate retina, CaMKII isoforms distributions are not described during development, and poorly studied in the adult retina. CaMKIIG is ubiquitously expressed in the entire retina while CaMKIID was present in bipolar and all amacrine cells (Tetenborg et al., 2017). Moreover, the nuclear isoform of CaMKIIαB is highly expressed not only in midbrain and diencephalon but also

in the developing retina regulating ganglion cells survival response (W. Fan, Li, & Cooper, 2007). No function for CaMKII proteins was described in the retina to date.

Given that the role of Calcium/calmodulin-dependent protein kinase II isoform D (CaMKIID) in the mammalian retina is poorly studied, its potential interaction with Par3 allowed us to hypothesise that the Par3 protein complex may recruit CaMKIID to initiate ciliogenesis in the photoreceptor cells.

Hypothesis and aims

Previous work in our lab has shown that conditional ablation of partitioning defective 3 (Pard-3/Par3) gene in retinal progenitor cells of the developing mouse retina leads to a severe disruption of lamination of the retina associated with a defective formation of the apical domain of PRs, leading to their degeneration (unpublished data). To understand the molecular basis of Par3 function in the developing retina, several potential Par3 interacting protein partners were identified by mass spectrometry on retinal protein extracts immunoprecipitated with an antibody directed against the Par3 protein. Interestingly, a cluster of CaMKII was identified among the most abundant Par3 interacting partners, with the isoform D being the most enriched.

The aim of this project is to identify the role of CaMKII in the retinal neurons in order to further elucidate the mechanism by which the loss of the polarity determinant Par3 leads to degeneration. As we identified CaMKIID as a potential binding partner of Par3, we hypothesised that Par3 may require CaMKIID interaction in order to initiate ciliogenesis in PRs. To test this hypothesis, I aimed to characterize the localization of CaMKIID *in vivo* in adult mouse retina and compare with its localization in Par3 conditional knock out retinas (Aim 1). Then, to validate the protein-protein interactions, I used over-expression models *in vitro* (Aim 2). By inducing CaMKIID downregulation with shRNAs my goal was to assess the physiological relevance of this protein *in vivo* in mouse P0 retinal progenitors (Aim 3). Finally, I wanted to understand whether mutations in CaMKIID catalytic/regulatory domain (leading to a constitutively active protein form) and ATP binding domain (leading to a formation of a dominant-negative form) can have any changes in the photoreceptors structure and localization in the retina (Aim 4).

Chapter 2- Material and Methods

2.1. Cloning

CaMKIID coding sequence (Table 1) was cloned in different mammalian expression vectors using Gateway system (Thermo Fischer) together with In-Fusion HD system (Clontech Cat.No.638909) and validated by both sequencing and Western Blot (ATCC; CRL-11268). For amplification of the mus musculus coding sequence of the CaMKIID, we used extracted retinal total RNA from retinas of C57B6J mice retrotranscribed into cDNA. The primers, FOR-GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCTTCGACCACCACC, REV-GGGGACCACTTTGTACAAGAAAGCTGGGTTTTAGTTGATGGGTACTGTGG were used for the *CaMKIID* gene amplification. All primers were designed in SnapGene® software and then synthesized by IDT™ (Integrated DNA Technologies). For the Gateway approach to 5' of both Forward and Reverse primers were added the sequence of attB1 and attB2 sites respectively. PCR fragment was extracted after electrophoresis migration in 1% agarose gel using the Invitrogen Gel extraction kit (Thermo Fisher Scientific Cat.No.K210012) and cloned into *Entry Vector* pcr8-GW-TOPO by using GatewayTM BR Clonase Enzyme Mix (Thermo Fisher Scientific Cat.No.11789013). *Entry vector* containing attL1 and attL2 sites can be recombined with *Destination Vector* by using Gateway™ LR Clonase Enzyme Mix (Thermo Fisher Scientific Cat.No.11791019). Desired constructs were transformed into DH5α E. coli cells for the copy amplification. The transformed cells were heat-shocked, grown in shaking incubator in S.O.C media (Invitrogen) for 1h at +370C, then plated onto LB-agarose plates with proper antibiotics and incubated overnight at 370C. The following day, colonies were picked up for further screening by the restriction enzymes digestion strategy designed in SnapGene®.

2.2. Derivation of mice primary embryonic fibroblast

Primary Mouse Embryonic Fibroblasts (MEFs) were derived from CD1 mouse embryos at embryonic day 13.5 (E13.5). Briefly, the embryos were taken out from the uterus, separated from placenta and yolk sacs. The individual embryo was placed into cold sterile PBS1x, decapitated, desolated and blood and liver tissue were removed. Embryos were treated with 2,5%

Trypsin/EDTA and triturated by up and down mechanical homogenization for cellular dissociation, followed by addition of DMEM complete medium for Trypsin neutralisation. Cells were then pelleted by centrifugation at 180g for 5 minutes. Cellular pellets were resuspended in fresh DMEM complete medium and cells were plated in 10cm petri dish pre-treated in 1:1 poly-L-lysin: Sterile H2O solution (Sigma Andrich, Cat.No.P4707-50ml) and then 0.1% bovine gelatine solution. The cells were cultured in DMEM, 10% heat-inactivated Cosmic Calf Serum, 1% penicillin-streptomycin, 1% MEM Non-Essential Amino acids (NEAA), 1% sodium pyruvate at 370C with 5% CO2. After 4 passages, cells were used for experiments (Figure 5).

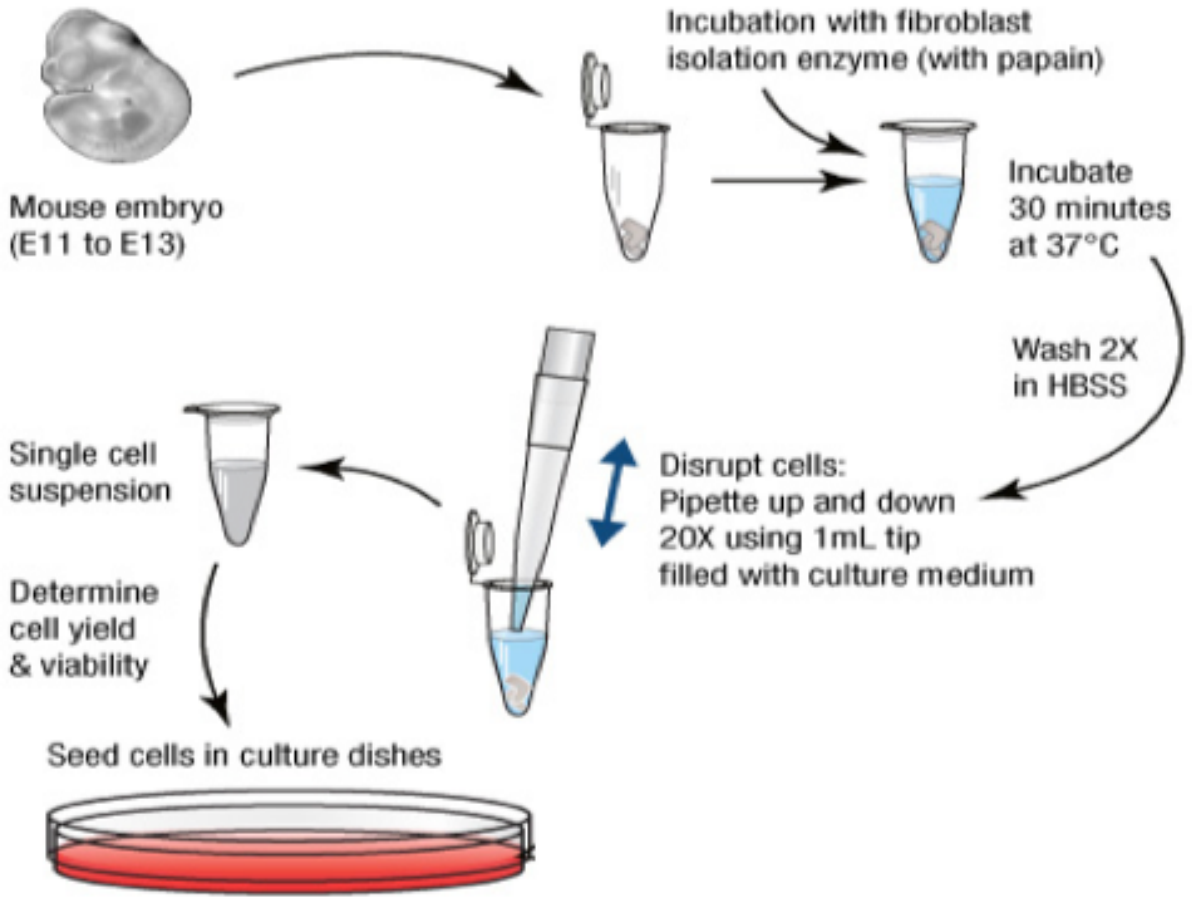


Figure 5. – Experimental outline for the isolation of mouse embryonic fibroblast (MEF) on an embryonic day (E) 13.5 by *in vitro* culture (ThermoFisher Scientific protocol).

2.3. Induction of cilia growth in MEF cells

To induce ciliogenesis in MEF cells, 24h after shRNA transfection, the medium with 10 % serum was replaced by medium containing 1 % serum (starvation medium). Later on, after 24h of starvation, MEFs were fixed in 4% PFA for 10 minutes at RT and stained with the cilium marker, acetylated tubulin. The enumeration of the number of ciliated cells was done using light fluorescent microscopy in four experiments, and the n varied from 20 to 80 cells per group.

2.4. Cells transfection

To validate our cloning, we transfected HEK293 cells with our plasmids (Table 1). Cells were seed in 6-well plates at the density of 300 x 103 and transfected after 24h using Polyethylenimine, PEI (Polysciences, Inc. Cat. No.23966-1) as a transfection reagent (5% (1mg/ml) PEI, 95% Opti-MEM; 0.5-2 ug DNA). The transfected cells were left at +370C and 5% CO2 for 24h. After transfection cells were collected in PBS1x and lysed with NP-40 (Nonidet P40 Substitute Sigma-Aldrich Cat.No. 74385) lysis buffer (Tris-HCL (pH 7.6), 150mM NaCl, 1% NP-40 with Complete Protease Inhibitor Cocktail (Roche REF.11836153001)).

2.5. Protein detection by Western blot

At postnatal day 0(P0), P10 or P30, mice were euthanized by CO2 asphyxia and enucleated. Eyes were dissected individually in cold PBS1x to isolate the neural retina from the eyecup. Isolated retinas were sonicated using 5 pulses of 5 seconds at the low output (2) in cold NP-40 protein lysis buffer (Tris-HCL (pH7.6), 150mM NaCl, 1% Np-40) with Complete Protease Inhibitors Cocktail (Roche)). Proteins lysate were centrifuged at full speed (13K) for 15 minutes to remove non-dissolved proteins and followed by quantification using the Bradford protein assay (BioRad Laboratories). Between 20 and 100 μg of retina lysates in 1X laemli buffer were loaded in 6.5% Acrylamide gels (BioRad Laboratories). After electrophoresis migration, proteins were transferred onto Low-Fluorescence PVDF membranes using Transblot Turbo (BioRad Laboratories). Membranes were blocked in blocking solution (5% dry milk/TBS-T (10mM Tris, pH8; 150mM NaCl and 0,05 % Tween20)) for 1h at room temperature (RT). Membranes were incubated with primary antibodies overnight at +40C in 1% blocking solution. On the next day, the membrane was washed

in TBS-T solution and incubated for 1h at RT with the appropriate HRP-conjugated secondary antibodies (Table 2) in 1% of blocking solution. After 3 washes of the membrane, HRP activity was visualised on ChemiDoc (BioRad Laboratories) by chemiluminescence using the ECL (Fisherscientific Cat. No.45000875) or ECL Prime kit (Fisherscientific Cat. No.45002401). Protein levels were normalised against the level of housekeeping proteins such as glyceraldehyde 3-phosphte dehydrogenase (Gapdh) or Beta-actin (Actb) using ImageJ software.

2.6. Fluorescent immunolabeling

2.6.1. Immunohistochemistry (IHC)

PFA or TCA fixed mouse eyes were embedded in Tissue-Tek® O.C.T.™ Compound, frozen rapidly using liquid nitrogen and kept frozen at -80° C until sectioned. Frozen eyes were cross-sectioned using a cryostat in slices of 14-18 μm and fixed on treated slides (Denville Ultra Clear Microscope Slides Cat. No.M1021). After slices were dried, slides were rinsed in PBS 1X to remove excess embedding medium from the slice. Slides were then blocked by incubation in blocking solution (1% BSA and 0,2 % triton in PBS 1X) for 1h at RT. Slides were incubated at RT overnight with primary antibody (Table 1) diluted in blocking solution. The next day, the sections were washed with PBS1x and incubated with secondary antibodies coupled to Alexa fluorophore (Table 2) diluted 1/1000 in blocking solution for 1 hour at RT. Finally, after 3 washes, slides were stained with Hoechst (Invitrogen Cat. No.H3570) in dilution 1/10000 and mounted using Mowiol.

2.6.2. Immunocytochemistry (ICC)

Mouse embryonic fibroblast (MEFs) cells were seeded on pre-treated sterile glass coverslips in 24-well plate at density 50 x 103 and cultured in DMEM, 10% Cosmic Calf Serum (CCS), 1% penicillin-streptomycin, 1% MEM Non-Essential Amino acids (NEAA), 1% sodium pyruvate. The glass coverslips were treated with poly-L-Lysin (Sigma Andrich, Cat.No.P4707-50ml) for 30 minutes at RT and 0,1% bovine gelatine solution. Twenty-four hours after seeding, cells were transfected with pSIREN plasmid constructs using Lipofectamine 3000 (Invitrogen cat. No. L3000015). 24 hours after transfection cells were fixed with PFA 4% for 10 minutes at RT and followed by 3 washes with PBS1x. Cells were then blocked in blocking solution (1% BSA and 0,2 %

triton in PBS 1X) for 1h at RT. Cells were then incubated with primary antibody diluted in blocking solution (Table 2). The following day cells were rinsed 3 times with PBS1x and incubated with secondary antibody coupled to Alexa fluorophore (Table 3) diluted in 1/1000 in blocking solution for 1 hour at RT. Finally, for labelling the cell nuclei Hoechst was used (Invitrogen Cat.No.H3570) at a dilution 1/10000 in PBS1X and mounted with Mowiol.

2.7. Plasmid electroporation into the eye

P0-P1 CD1 pups were anaesthetized using ice for 2-3 minutes. One to 3 μg of plasmids (Table 3) were delivered in the subretinal space of pups using a glass pipette and then electroporated using electrode pad pulsed 5 times in a unipolar direction (50 ms duration, 950ms Interval, 80 Volts). Pups recovered from surgery under a heat lamp and returned to their mother. After 21 days, mice were euthanized, the retinas were collected and fixed in PFA 4% for 30 minutes at RT, gradually equilibrated in 10 % and 20 % sucrose, and embedded in Tissue-Tek® O.C.T.™ Compound (Sakura Cat.No.4583) and frozen rapidly using liquid nitrogen. Retinas were stored at -80 0C until further processing.

2.8. RNA extraction and cDNA synthesis

Total RNA was extracted from adult mice eyes P120 (postnatal day120) using RNEasy Mini Kit (Qiagen Cat. No.74134). cDNA synthesis was performed by using SuperScript™IV VILO master mix with EZ DNAse (ThernoFisher Cat.No.11766050) on 3 μg of total RNA and stored at -800C. For RT-PCR, 35ng cDNA (equivalent RNA) was used per reaction.

2.9. *In vivo* system/Mouse lines

The animal experiments were performed in agreement with the Canadian Council on Animal Care (CCAC) guidelines and with the IRCM Animal Care Committee and ethical rules. Crossing 3 different mouse lines generated conditional Pard3 knockout mouse line:

-Rosa Yellow fluorescent protein (RYFP) mouse line (Jackson Laboratory) is conditionally expressing the YFP protein under the endogenous Rosa promoter.

-The alpha-Pax6 Cre-Ires-GFP (MGI: 3052661) mouse line is a transgenic mouse line expressing Cre in peripheral progenitor cells from embryonic day 10.5 (E10.5) (Figure 6B).

-The conditional Par3 knockout mouse line, in which *Par3* exons 8 and 9, coding for part of Pard3 domains 1 and 2 are flanked by loxP sites (Floxed) (Figure 6A). Cre recombination generates a shift in the open reading frame, leading to the production of a truncated Pard3 protein. It was generated from the C57BL/6N-Atm1Brd mouse line UC Davis (MGI: 2135608) in which the FRT sites where previously recombined using a mouse line expressing Flippase.

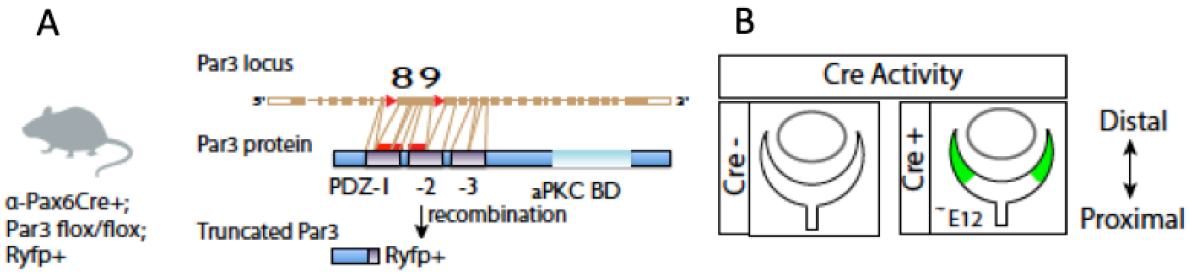


Figure 6. – Par3 conditional knock out (Par3 cKO) mouse line generation. Par3 cKO mouse line was generated by crossing three different mouse lines: alpha Pax6Cre+; Pard3*fl/fl* and RosaYFP. The alpha Pax6Cre+ mouse contains a loxP-STOP-loxP Rosa YFP cassette and it was crossed with Pard3*fl/fl* to induce the *Padr3* gene deletion from progenitor cells at the peripheral retina. (A) Schematic presentation of different domains of *Pard3* gene. (B) Schematic represents the localisation of Cre expression at the peripheral part of the embryonic retina (E12).

2.10. Co-Immunoprecipitation

After transfection, HEK293T cells were lysed in NP-40 buffer plus complete inhibitors, sequentially quantified using Bradford protein assay. Immunoprecipitation was performed by using the superparamagnetic beads Dynabeads® Protein G (Thermo Fisher Scientific Cat. No.1000D3). Briefly, protein G dynabeads were coupled with appropriate primary antibodies in different dilutions (Table 1) in PBS1X-0.05% Tween for 1 hour at +40C. One milligram of total protein extract was added to the beads and incubated overnight at +40C with IpH buffer (50mM Tris pH 8.0, 150 mM NaCl, 5mM EDTA, 0.1% NP-40). Next day, beads were rinsed 3 times with IpH buffer

using the MagnaBind magnet (Pierce). After washes, beads were resuspended in 1x Lameli buffer and boiled for 3 minutes at 950 C. Sequentially samples were loaded in an acrylamide gel.

2.11. Immunoprecipitation Mass-Spectometry (IP-MS)

At postnatal day 10 and 30 (P10 and P30) mice were euthanized by CO2 asphyxia and enucleated. Eyes were dissected individually in cold PBS1x to isolate the neural retina. Isolated retinas were sonicated using 5 pulses of 5 seconds at the low output (2) in cold NP-40 protein lysis buffer (Tris-HCl (pH7.6), 150mM NaCl, 1% Np-40) with Complete Protease Inhibitors Cocktail (Roche). Protein lysates were centrifuged to remove non-dissolved proteins and followed by quantification using the Bradford protein assay (BioRad Laboratories). Immunoprecipitation was performed by using the superparamagnetic beads Dynabeads® Protein G (Thermo Fisher Scientific Cat. No.1000D3). Briefly, protein G beads were coupled with appropriate primary antibodies in different dilutions (Table 1) for 1 hour at +40C. One milligram of total protein extract was added to the beads and incubated overnight at +40C with IpH buffer (50mM Tris pH 8.0, 150 mM NaCl, 5mM EDTA, 0.1% NP-40). Next day, beads were rinsed 3 times with IpH buffer using the MagnaBind magnet (Pierce), which were then replaced by freshly made cold 50mM Ammonium Bicarbonate (Sigma Aldrich Cat.No.A6141) buffer. The on-beads proteins were digested by trypsin overnight at +370C and washed several times with different solutions following the manufacturer's instructions. After elution in 10% ammonium hydroxide/90% methanol (v/v), samples were dried with a Speed-vac, reconstituted under agitation for 15 min in 12 µL of 2%ACN-1%FA and loaded into a 75 µm i.d. × 150 mm Self-Pack C18 column installed in the Easy-nLC II system (Proxeon Biosystems). The peptides were eluted with a two slope gradient at a flow rate of 250 nL/min. Solvent B first increased from 1 to 38% in 105 min and then from 38 to 86% B in 25 min. The HPLC system was coupled to Orbitrap Fusion mass spectrometer (Thermo Scientific) through a Nanospray Flex Ion Source. Nanospray and S-lens voltages were set to 1.3-1.7 kV and 50 V, respectively. The capillary temperature was set to 225 °C. Full scan MS survey spectra (m/z 360-1560) in profile mode were acquired in the Orbitrap with a resolution of 120,000 with a target value at 1e6. The most intense peptide ions were fragmented in the HCD cell and analysed in the linear ion trap with a target value at 2e4 and normalized collision energy at 28 V. A MS3 scanning was performed upon detection of a neutral loss of phosphoric acid (48.99, 32.66 or 24.5 Th) in HCD MS2 scans. The

duty cycle was set to 3 seconds and target ions selected for fragmentation were dynamically excluded for 30 sec after 3 MS/MS events.

The peak list files were generated with Proteome Discoverer (version 2.3) using the following parameters: minimum mass set to 500 Da, maximum mass set to 6000 Da, no grouping of MS/MS spectra, precursor charge set to auto, and the minimum number of fragment ions set to 5. Protein database searching was performed with Mascot 2.6 (Matrix Science) against the Uniprot Mus musculus protein database (April 15th, 2015). The mass tolerances for precursor and fragment ions were set to 10 ppm and 0.6 Da, respectively. Trypsin was used as the enzyme allowing for up to 1 missed cleavage. Cysteine carbamidomethylation was specified as a fixed modification, and methionine oxidation and phosphorylation S/T/Y as variable modifications. Data interpretation was performed using Scaffold (version 4.8).

2.12. Short hairpin RNA generation

shRNAs against murine CaMKIID were designed by using InvivoGEn's siRNA Wizard software. Oligonucleotides were produced by IDT™ (Integrated DNA Technologies). The efficiency of shRNAs was tested in HEK293 together with the overexpression of the gene of interest (CaMKIID) by using (jetPRIME®, DNA and shRNA transfection reagent VWR-114-07 CA89129-922). Immunoblotting analysis was used to assess the potency of shRNA-mediated knockdown against CaMKIID.

2.13. Statistical analysis

All statistics were performed with GraphPad Prism Version 8©. For the multiple comparisons, a Tukey's and Dunnett's tests were applied. For the comparison of three and more groups, one-way and two-way ANOVA was applied. Statistical significance was defined when $P < 0.05$.

2.14. Quantitative analysis of the images

To quantify the number of ciliated MEF cells after serum starvation we used a DM6000 (Leica) microscope. The cells were cultured, fixed and immunostained with appropriate antibodies as described in section 2.5.2. After that, transfected cells of each condition were randomly selected,

counted on the presence of cilium and the numbers were converted in percentage of the total number of counted cells.

The imaging of retinal sections was performed with the confocal microscope SP8 (Leica). To measure the length of the IS and OS of PRs, the retina sections were stained with appropriate primary and secondary antibodies as described above. The length of the IS and OS of GFP positive PRs were individually measured using Volocity® Version 6.0.

To analyse the apico-basal distribution of PRs nuclei, we used ImageJ applying the FIJI macro to divide the outer nuclear layer (ONL) of the retina in three equal compartments (apical, middle and basal). Nuclei were counted separately in each compartment and the number was converted in percentage.

Tableau 1. – List of generated plasmids

Name	**Backbone**	**Insert**	**Primers (IDT)**	**Cloning Method**
pCR8TOPO-CaMKIID var.	pCR8/GW/TOPO	CaMKIID var.1	FOR-GGGGACAAGTTTGTACAAAAA AGCAGGCTTAATGGCTTCGACCACCACC, REV-GGGGACCACTTTGTACAAGAA AGCTGGGTTTTAGTTGATGGGTACTGTGG	Gateway system
pCIG-IRES-GFP-CaMKIID mut.K43A	pCIG-GW-A	CaMKIID mut. K43A	REV-GATAATTGCGGCAGCATACTCTTGTCCA, FOR-GCTGCCGCAATTATCAACACCAAAAAGCTTTCT	In-Fusion HD/mutagenesis
pCIG-CaMKIID mut. T287D	pCIG-GW-A	CaMKIID mut.T287D	FOR-CAGGAGGATGTAGACTGCTTGAAGAAATTTAATGCT, REV- GTCTACATCCTCCTGCCTGTGCATCATG	In-Fusion HD/mutagenesis

pSIREN-shRNA-CaMKIID	pSIREN-RetroQ-Zsgreen	CaMKIID variant1	FOR-GATCCGGATCTGTCAACGCTCTACTGTTTCAAGAGAACAGTAGAGCGTTGACAGATCTTTTTTGCGGCCGCG, REV-AATTCGCGGCCGCAAAAAAGATCTGTCAACGCTCTACTGTTCTCTTGAAACAGTAGAGCGTTGACAGATCCG	Oligo A annealing

Tableau 2. – List of primary antibodies

Antigen	**Species**	**Dilution**	**Sources**
CaMKIID	Rabbit	1/1000 (IFL), 1/500 (WB) 2μg (IP)	LsBio Cat.No. LS-C329304
Ninein	Goat	1/100 (IFL)	Santa Cruz Cat.No. SC-50142
Na/P-ATPase Alpha 3	Mouse	1/100 (IFL)	Novus Biologicals Cat. No. NB300-540SS
Rhodopsin	Rabbit	1/500 (IFL)	GeneTex Cat. No. GTX129910
Par3	Rabbit	1/1000(WB), 1/500(IFL), 2μg (IP)	Millipore Sigma Cat. No. 07-330
GFP	Rabbit	1/5000 (WB), 2μg (IP)	Thermo Fisher Scientific Cat.No.A11122
GFP	Chicken	1/1000 (IFL)	Abcam Cat.No.ab13970
GAPDH	Mouse	1/2000 (WB)	Millipore Sigma Cat.No. MAB374
B-actin	Mouse	1/1000 (WB)	Sigma-Adrich Cat.No.A5441
Myc (9E10)	Mouse	1/500(IFL), 1/1500 (WB), 0.7μg (IP)	Santa Cruz Biotechnology, Sc-40

HA clone F-7	Mouse	1μg (IP, IP-MS)	Santa Cruz Biotechnology Cat.No.SC-7392
Acetylated tubulin	Mouse	1/1000(IFL), 1/1000(WB)	Sigma-Adrich Cat.No.T6793
Pericentrin	Rabbit	1/500	BioLegend Cat.No.923701
Centrin	Mouse	1/500	Millipore Sigma Cat.No.04-1624
Zo-1	Mouse	1/200 (IFL)	Zymed, Cat.No.339100
Sox2	Rabbit	1/500 (IFL)	Abcam Biochemical, Cat.No. ab97959
IgG	Rabbit	2 μg (IP)	Jackson Immunoreserch, Cat.No.111-005-003
IgG	Mouse	2mg (IP)	Invitrogen Cat.No.02-6502
Otx2	Goat	1/500 (IFL)	R&D System
CHT10	Sheep	1/500	Exalpha Biologicals Cat.No. X1180P

IFL-immunofluorescence, WB – western blot, IP - immunoprecipitation

Tableau 3. – List of the secondary antibodies

Fluorochrome	**Species**	**Dilution**	**Sources**
Anti-rabbit HRP	Goat	1/10000 (WB)	Jackson Immunoresearch,Cat.No.111-035-144
Anti-mouse HRP	Goat	1/10000 (WB)	Jackson Immunoresearch,Cat.No.115-035
Protein A, HRP conjugate	Goat	1/5000 (WB)	Millipore Sigma Cat.No.18-160

Anti-mouse AlexaFlour488	Donkey	1/1000	Thermo Fisher Scientific Cat.No.A-21202
Anti-mouse AlexaFlour555	Donkey	1/1000	Thermo Fisher Scientific Cat.No.A-32773
Anti-mouse AlexaFlour647	Donkey	1/1000	Thermo Fisher Scientific Cat.No.A-31571
Anti-rabbit AlexaFlour488	Donkey	1/1000	Molecular Probes Cat.No. A21206
Anti-rabbit AlexaFlour555	Donkey	1/1000	Molecular Probes Cat.No. A31572
Anti-rabbit AlexaFlour647	Donkey	1/1000	Thermo Fisher Scientific Cat.No. A31573
Anti-chicken AlexaFlour488	Donkey	1/1000	Thermo Fisher Scientific Cat.No. A11055

Chapter 3 – Results

To shed light on the role of Par3 in the mouse retina, a conditional Par3 knock out (Par3 cKO) mouse line was crossed with the alpha Pax6-Cre line allowing us to explore the effect of the Par3 deletion in the peripheral retina from embryonic day 10.5 (E10.5) (Figure 6A, 6B, 7). Therefore, the early activation of αPax6 promoter in the peripheral region of retina provides the possibility to study the establishment of cell polarity in the retinal progenitor cells. *Pax6* is a regulatory gene with restricted expression pattern in the developing eye, pancreas and distinct domains of the CNS. In the α-Pax6-Cre mouse line (Marquardt et al., 2001), the gene encoding the Cre recombinase and GFP expression from a single bicistronic mRNA is under control of the "α" retina-specific regulatory element of murine *Pax6* (Kammandel et al., 1999). In this line, Cre activity is detected in the retinal progenitor cells of the peripheral retina starting from E 10.5 (Marquardt et al., 2001). While the staining of the control mouse retina with Par3 antibody showed its localization at the tight junctions in the apical region and in the IS in adult retina (Figure 8), the Par3 cKO demonstrated loss of lamination and induced PRs dislocation from the apical layer, where they normally reside (Figure 9). In order to identify Par3 potential interacting proteins that might be involved in assisting to establish the retinal neurons polarisation, immunoprecipitation followed by mass spectrometry (IP-MS) analysis was performed. Among a number of potential protein partners identified was the KCC2D (CaMKIID) (Figure 10), never reported previously to take a part in the cell signalling that establishes the cell polarity.

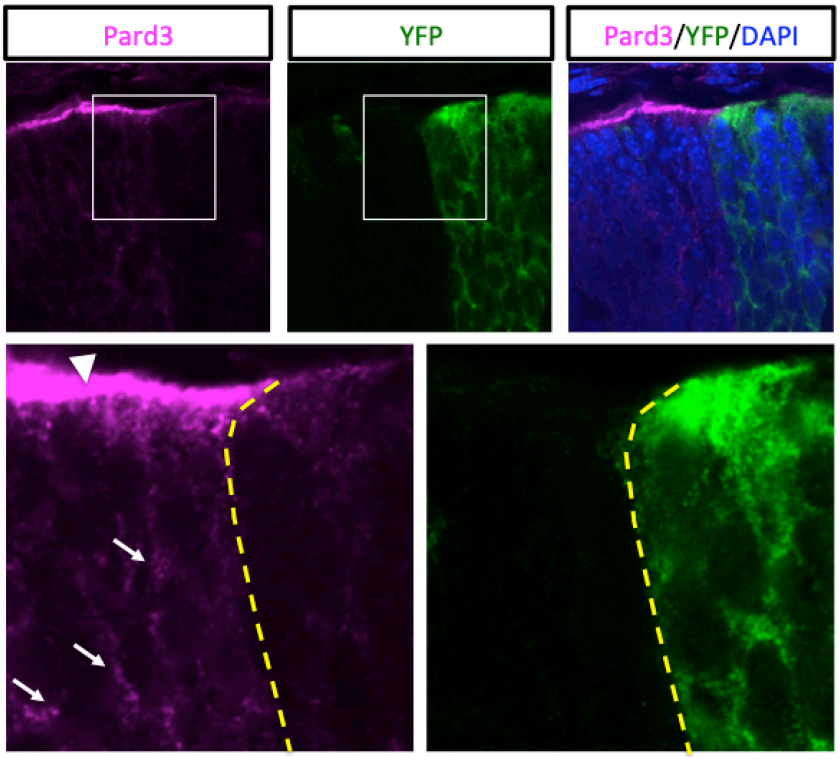


Figure 7. – ***Pard3*** **gene deletion in Par3 cKO mouse line at the peripheral retina.** Immunostaining for Par3 (magenta) and YFP (green) in P0 section of an αPax6-Cre+ ; Par3flox/flox ; RosaYFP/+ mouse retina. In the non-recombined control region of the retina, white arrowhead point to Par3 expression at the outer limiting membrane (OLM) and white arrows indicate Par3 in the cytoplasm of the retinal cells. The yellow dash lines separate the region where the recombination occurred with the region where it did not take place.

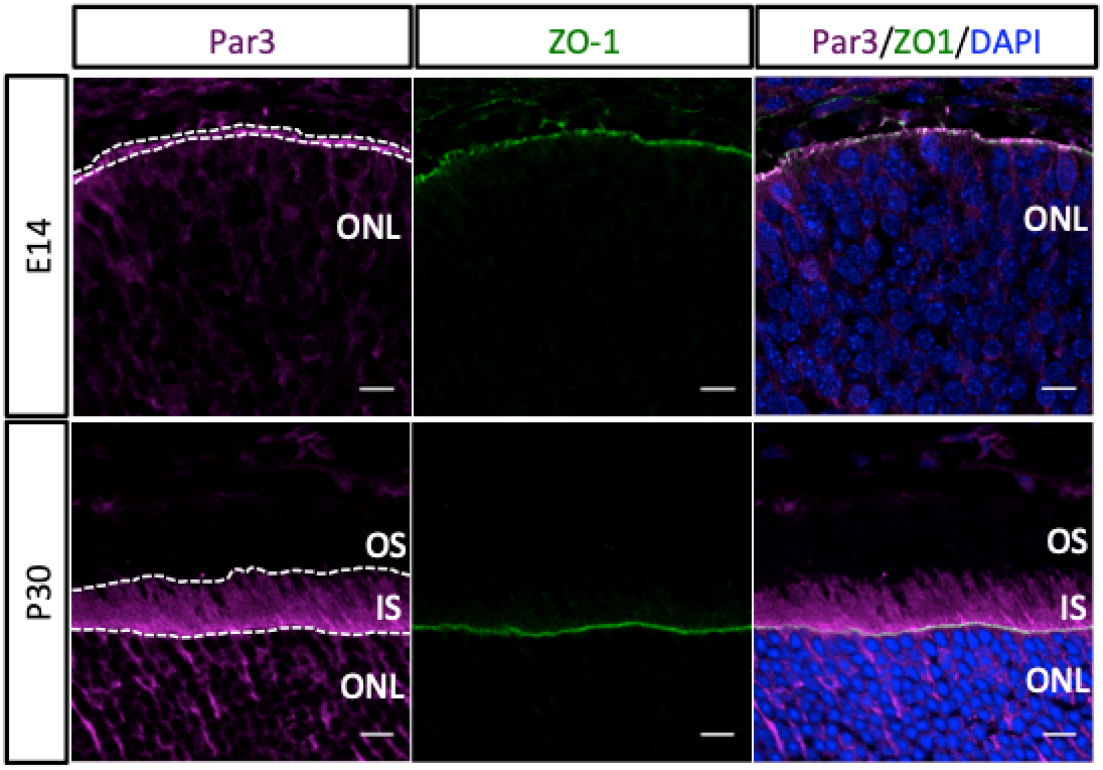


Figure 8. – The localization of Par3 protein expression in embryonic and adult retinas. Immunostaining for Par3 and tight junction marker - ZO1. On the top, section of wild type (WT) retina at embryonic day 14 (E14) shows that Par3 colocalized with ZO1 at the OLM. On the bottom, a section of WT adult retina (P30) indicates the Par3 expression in ONL and the inner segment of photoreceptors. Scale bar for E14 and P30= 10μm.

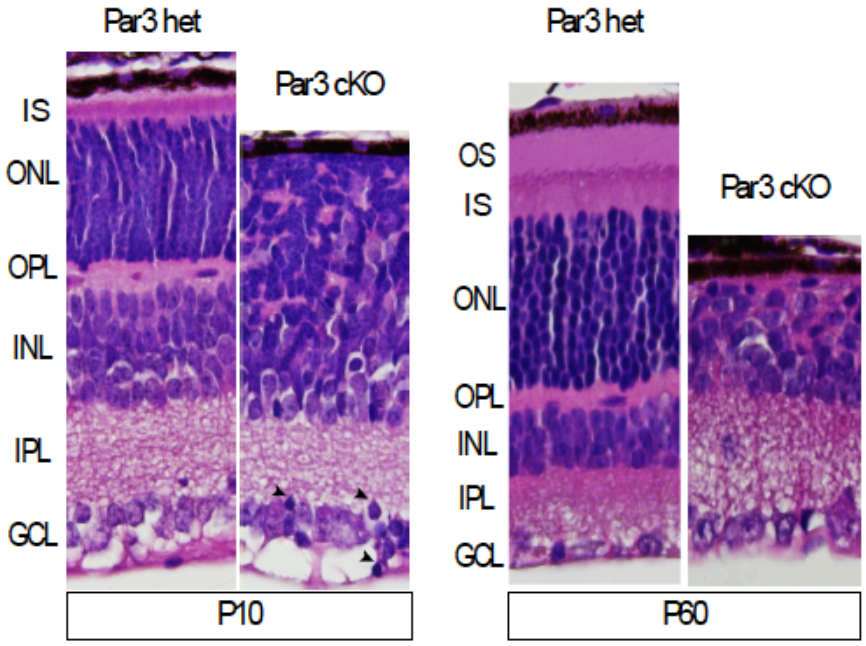


Figure 9. – Par3 function in developing and adult retinas is essential to maintain retinal structure and integrity. Haematoxylin and eosin staining of peripheral retinal sections in control and cPar3 KO at postnatal day P10 and P60. Black arrowheads point at the PRs nuclei localisation in P10 Par3 cKO retina.

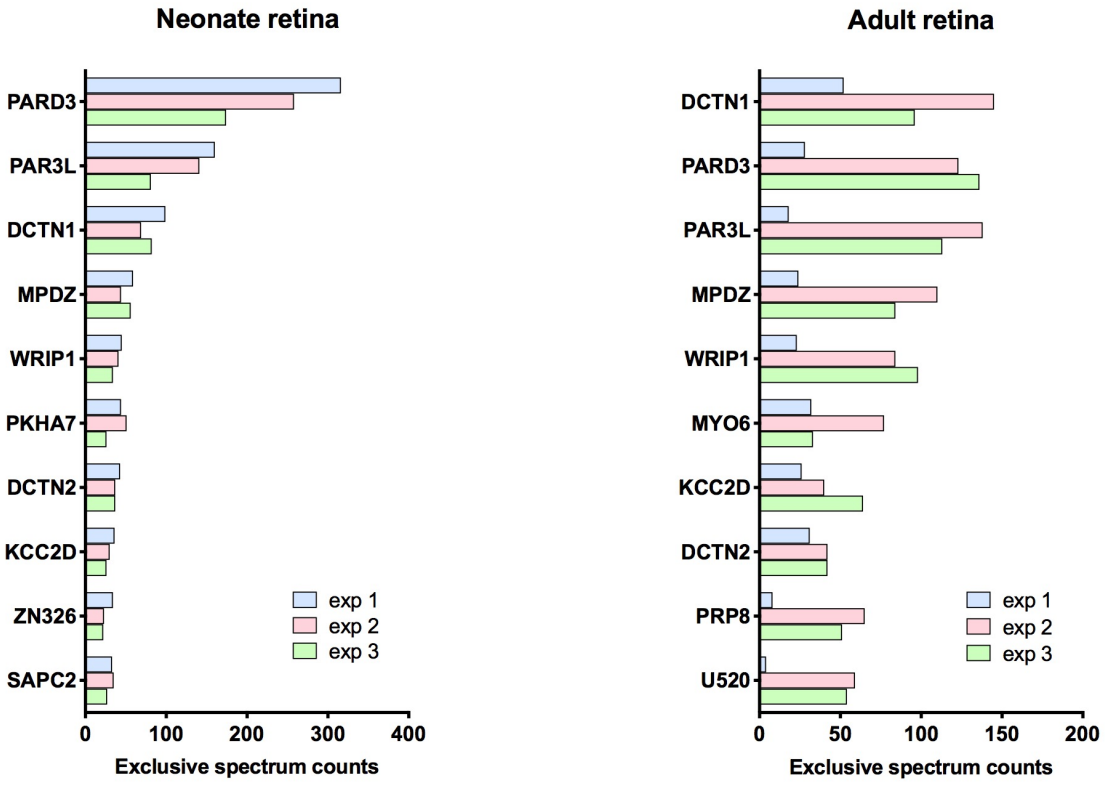


Figure 10. – Top 10 most enriched Par3 interacting proteins in the neonatal (P0) and adult (P30) mouse retinas. Immunoprecipitation of endogenous Par3 on whole retinal protein extracts was followed by mass spectrometry (IP-MS) analysis in 3 different experiments.

3.1. Identification of CaMKIID localization in the adult mouse retina

Given the lack of knowledge in the literature, my goal here was to investigate and characterize the function of CaMKIID in PRs of the mouse retina. In this perspective, I first performed CaMKIID immunofluorescence staining on adult retina sections to identify its cellular localization. Co-staining with Na^+/K^+-ATPase, a marker of IS of PRs, demonstrated CaMKIID expression in PR cells, at the tip of the IS (Figure 11A). Co-immunostaining with Ninein, a centrosomal protein, further showed that CaMKIID is present in the close vicinity to the region, from which emerges the connective cilium of PRs (Figure 11B). These results showed that CaMKIID is expressed in PRs, and more specifically at the base of cilia, suggesting that it might function in ciliogenesis.

In addition to the mass spectrometry analysis data, showing a potential interaction of Par3 and CaMKIID, their expression in the same region in the IS of PRs potentiates the hypothesis that Par3

might recruit CaMKIID at the tip of IS to initiate ciliogenesis. If that was the case, the phenotype observed in the Par3 KO retina with severe polarity defects and loss of lamination could be interpreted as a failure of the Par3 recruitment of CaMKIID to the connecting cilia to form the OS of the PRs.

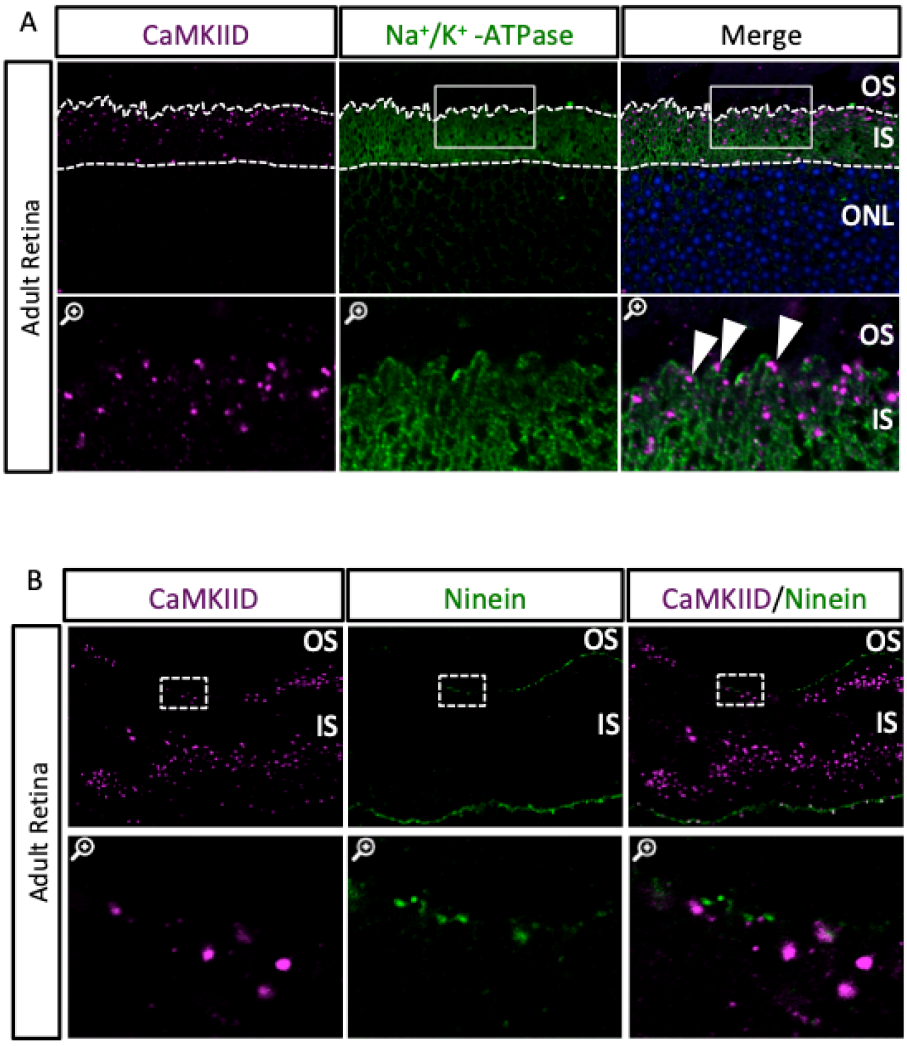


Figure 11. – The localization of CaMKIID in PRs. Confocal imaging of adult retinal section. (A) Immunostaining for CaMKIID (magenta) and Na^+/K^+-ATPase (green) in the adult retina. White arrowheads point to the localization of CaMKIID. (B) Co-immunostaining of CaMKIID (magenta) and Ninein (green) demonstrates localization in the adult retinal section.

3.2. CaMKIID localization in Par3 cKO mouse retina

As we identified CaMKIID as potential Par3 interacting partner in developing and adult retinas, my goal here was to investigate and characterize its localization in the Par3 cKO mouse retina. To this end, I first performed CaMKIID and ZO1 co-immunofluorescence staining on embryonic and postnatal retina sections to identify its localization. Co-staining with ZO1, a marker of tight junctions, showed that CaMKIID is expressed in the outer limiting membrane (OLM) and showed dot-like dispersion in the two time points (Figure 12). Furthermore, immunostaining of CaMKIID in Par3 cKO and Par3 heterozygotes (Par3 HET) in the P0 showed that the protein localizes in OLM in the control retina (P0 Par3 HET), while in the Par3 cKO CaMKIID losses its apical localization in the peripheral retina, from where the Par3 is deleted (Figure 7 and 13). Likewise, in the Par3 cKO, CaMKIID losses its dot-like pattern of expression as opposed to both, in the wild type (Figure 12) and Par3 HET retina. These results clearly showed that Par3 is essential for CaMKIID expression in the tight junction region of the retina. The experiment enforces the hypothesis that Par3 plays an important role for the CaMKIID localization.

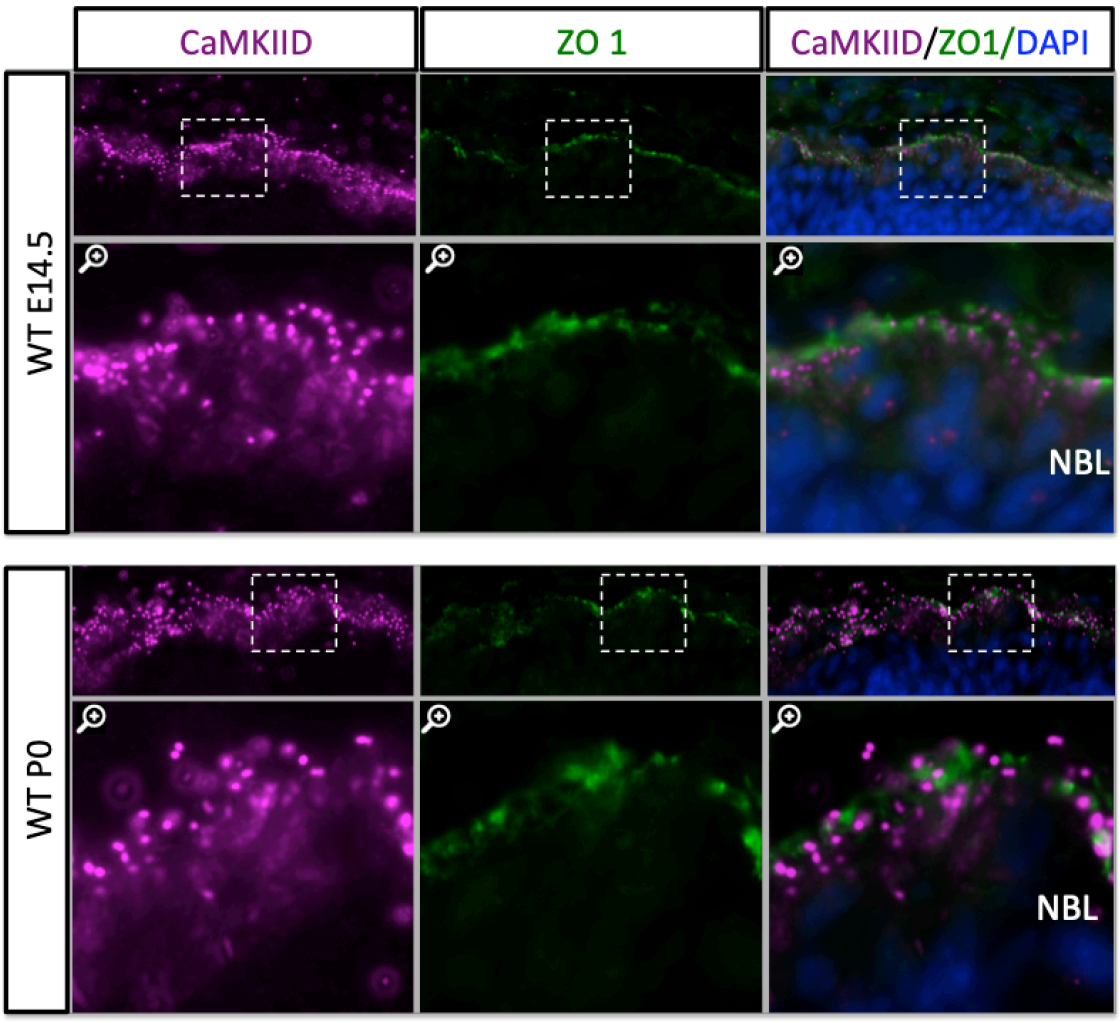


Figure 12. – Localization of CaMKIID in developing wild type retinas. Confocal images of embryonic (E14.5) and postnatal (P0) retinal sections. Immunostaining for CaMKIID (magenta), ZO1 (green) and DAPI-stained nuclei (blue). White dashed squares point to the localization of CaMKIID expression.

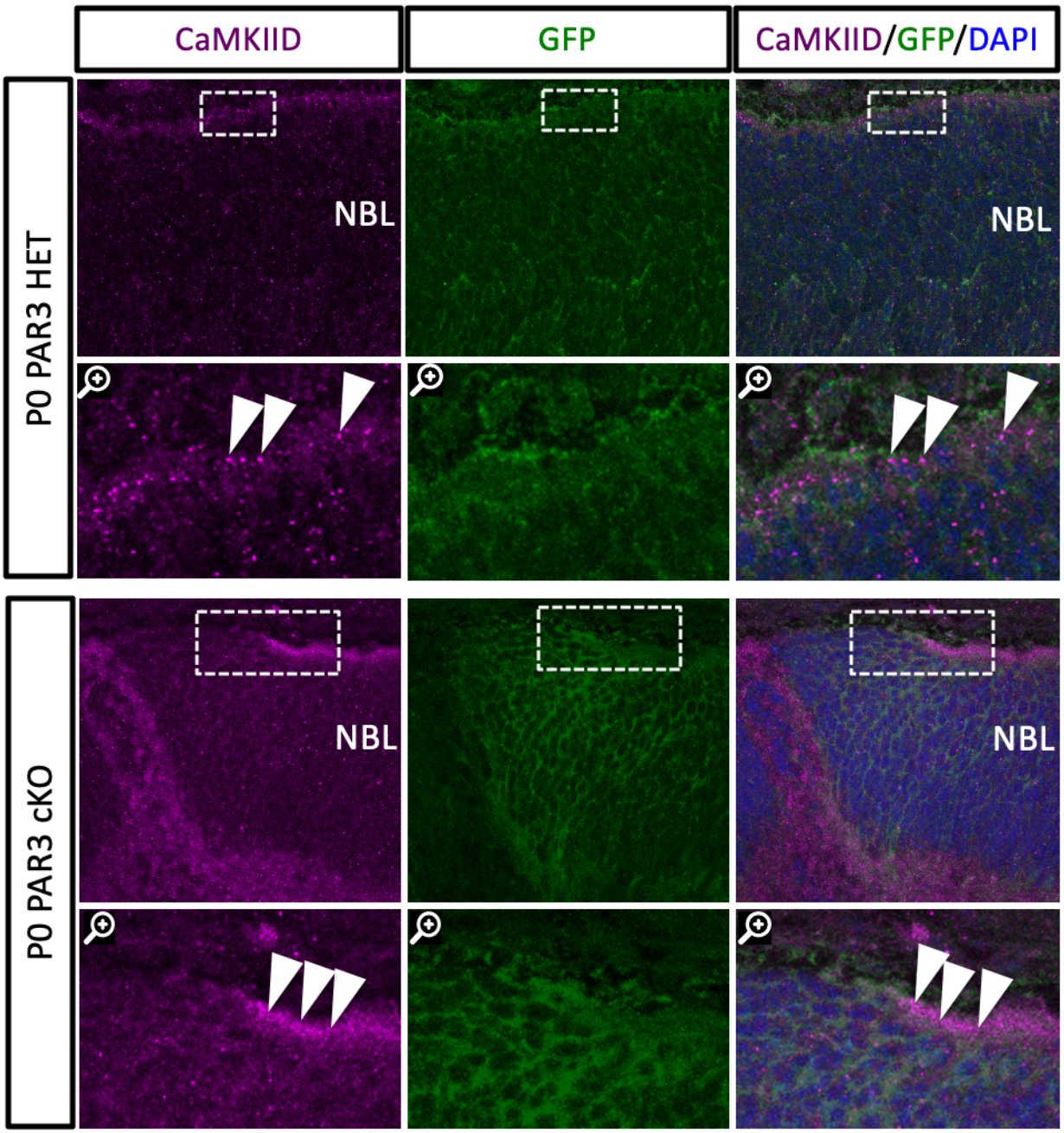


Figure 13. – CaMKIID loss of localization in Par3 cKO mouse retinas. Confocal imaging of Par3 HET and Par3 cKO retinal sections at P0 demonstrates localisation of CaMKIID in OLM in Par3 HET and its absence in the Par3 cKO peripheral retina. Immunostaining for CaMKIID (magenta) and GFP (green). White arrowheads point to the localization of CaMKIID in OLM.

3.3. Identification of Par3 and CaMKIID proteins interaction *in-vitro* and *in vivo*

The preliminary data from the mass spectrometry analysis indicated that CaMKII interacts with Par3 protein in both postnatal day 0 (P0) and adult retina (P60). To validate this interaction, we overexpressed under a CAG (chicken beta-actin) promoter both the YFP-tagged (N-terminus) Par3 and an HA-tagged (C-terminus and at the position 317bp of the coding sequence) CaMKIID in HEK293 cells. CaMKIID:HA and CaMKIID:HA-317 were immunoprecipitated from whole protein lysates with an antibody against the HA tag and analysed by western blot. The immunoblotting using an antibody against Par3 showed a positive signal when the two vectors were co-expressed (YFP-Par3 and CaMKIID-HA; YFP-Par3 and CaMKIID-HA-317) but were absent in all controls (YFP-Par3 alone, or with control-GAS1-HA) (Figure 14 top gel). Hence CaMKIID and Par3 appear to interact when co-expressed in HEK293 cells.

To further validate this interaction takes place in the mouse retina, the CaMKIID: HA expression vector was delivered in neonate P0 retinal progenitor cells by electroporation. The electroporation mainly targets dividing progenitor cells, therefore only cells born from the time of the electroporation will be transfected, including rod photoreceptors, bipolar cells, Müller glia cells, amacrine cells and at very-low-frequency horizontal cells (Matsuda & Cepko, 2004; Venkatesh, Ma, Langellotto, Gao, & Punzo, 2013). At P11, the electroporated retina was processed for immunoprecipitation of CaMKIID though the HA tag. While Par3 immunoblot showed no signal in the control samples (HA- and IgG IP on the contralateral non-electroporated retina), three Par3 isoforms could be identified in the IP for HA of the retina overexpressing CaMKIID: HA (Figure 14, bottom gel). Also, the immunoblot against HA confirmed the expression of CaMKIID from the electroporated vector (Figure 14, bottom gel). These results are in line with the preliminary mass spectrometry data and suggest that in the mouse retina, at P11 the CaMKIID can interact with the polarity protein Par3.

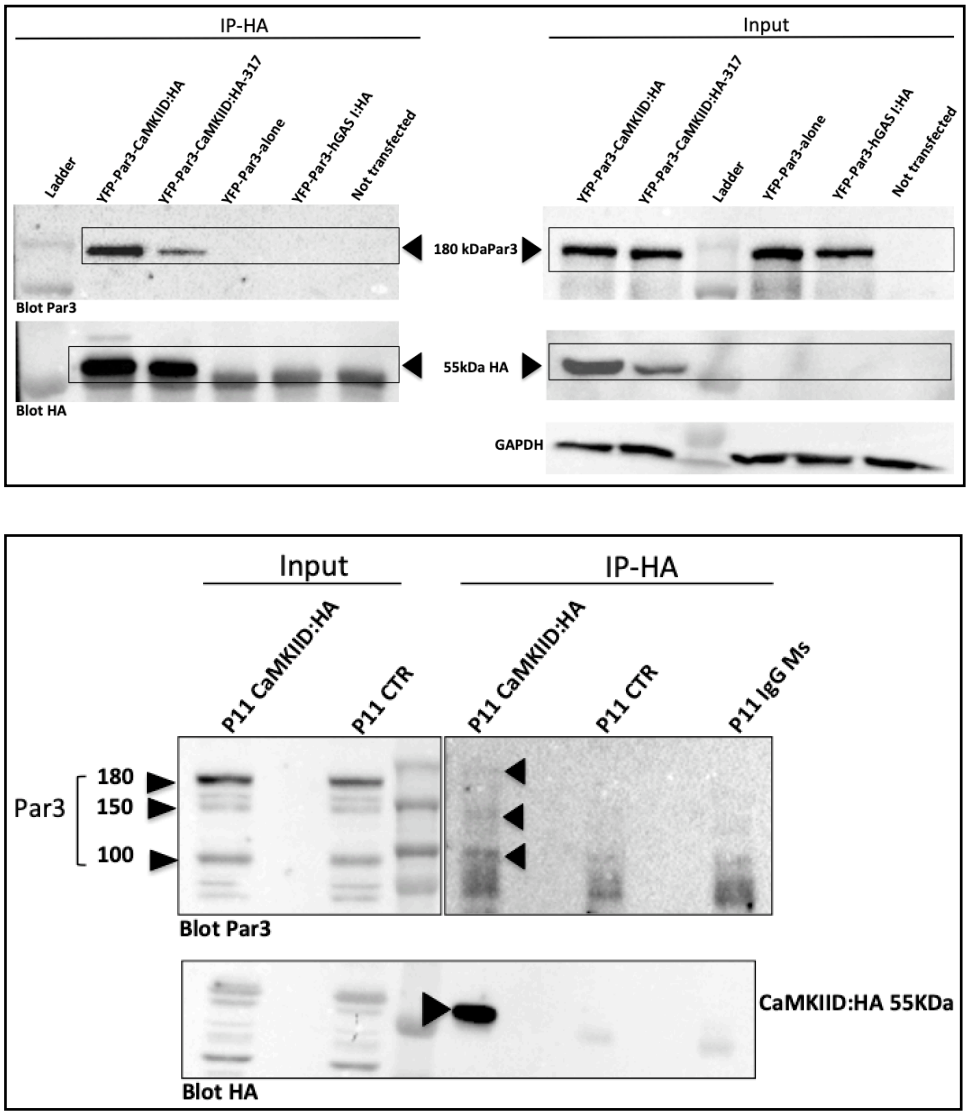


Figure 14. – Validation of CaMKIID and Par3 proteins interaction in HEK293 and in developing retina (P11). Immunoblotting of transfected HEK293 cells (top panel) and retinal lysates (bottom panel) immunoprecipitated for HA tag. Samples were immunoprecipitated for HA tag and blotted for Par3. Arrowheads indicate three isoforms of Par3 in the input and precipitated with CaMKIID: HA.

3.4. Effect of CaMKIID loss-of-function on ciliogenesis in serum-starved mouse embryonic fibroblasts model (MEFs)

Next, I wanted to test if CaMKIID is involved in the generation of cilia, and for that purpose, I used primary MEF cells as they develop cilia under the serum starvation condition. Therefore, I

designed short hairpin RNA (shRNA) in order to downregulate the expression of CaMKIID and assessed the efficiency of protein expression knockdown in HEK293 cells. Three different shRNAs vectors, targeting different regions of CaMKIID, were co-transfected with the plasmid carrying the CaMKIID. Only one of the designed shRNAs had a strong impact (64% reduction) on the protein expression level, as CaMKIID was significantly decreased in cells that co-expressed both plasmids, as opposed to when only CaMKIID was expressed (Figure 15B, C). Given the high efficiency of the tested shRNA, I wanted to examine if CaMKIID downregulation in MEF cells could somehow affect ciliogenesis. MEF cells are particularly interesting in this perspective since ciliogenesis can be robustly induced after twenty-four hours (24h) of serum starvation (Massa et al., 2019; Pampliega et al., 2013; Villalobos et al., 2019). The ciliogenesis in MEF cells was induced by the serum starvation and the enumeration of the number of cells with or without cilia (stained with acetylated tubulin) was done with fluorescent microscopy (Figure 15D and E). It included non-transfected (intact), transfected with the shRNA-scramble (negative control), transfected with empty vector and with a vector carrying shRNA against CaMKIID. The analysis revealed that transfection reduced to 52.8% the proportion of MEF cells harbouring a cilium when compared to non-transfected cells (Figure 15E). However, shRNA against CaMKIID did not show a significant reduction in the number of the ciliated cells when compared to transfected cells from both control groups (P=0.4206 when compared to pSIREN-empty; P=0.9048, when compared to pSIREN-shRNA-scrambled). As we do not technically show if the shRNA is efficient in reducing the CaMKIID level in MEF cells, the analysis was performed on the transfected cells showing low CaMKIID fluorescent signal. Hence, CaMKIID might not be involved in cilia formation in MEF cells, although we cannot exclude that a potential effect was precluded by the effect of transfection *per se*.

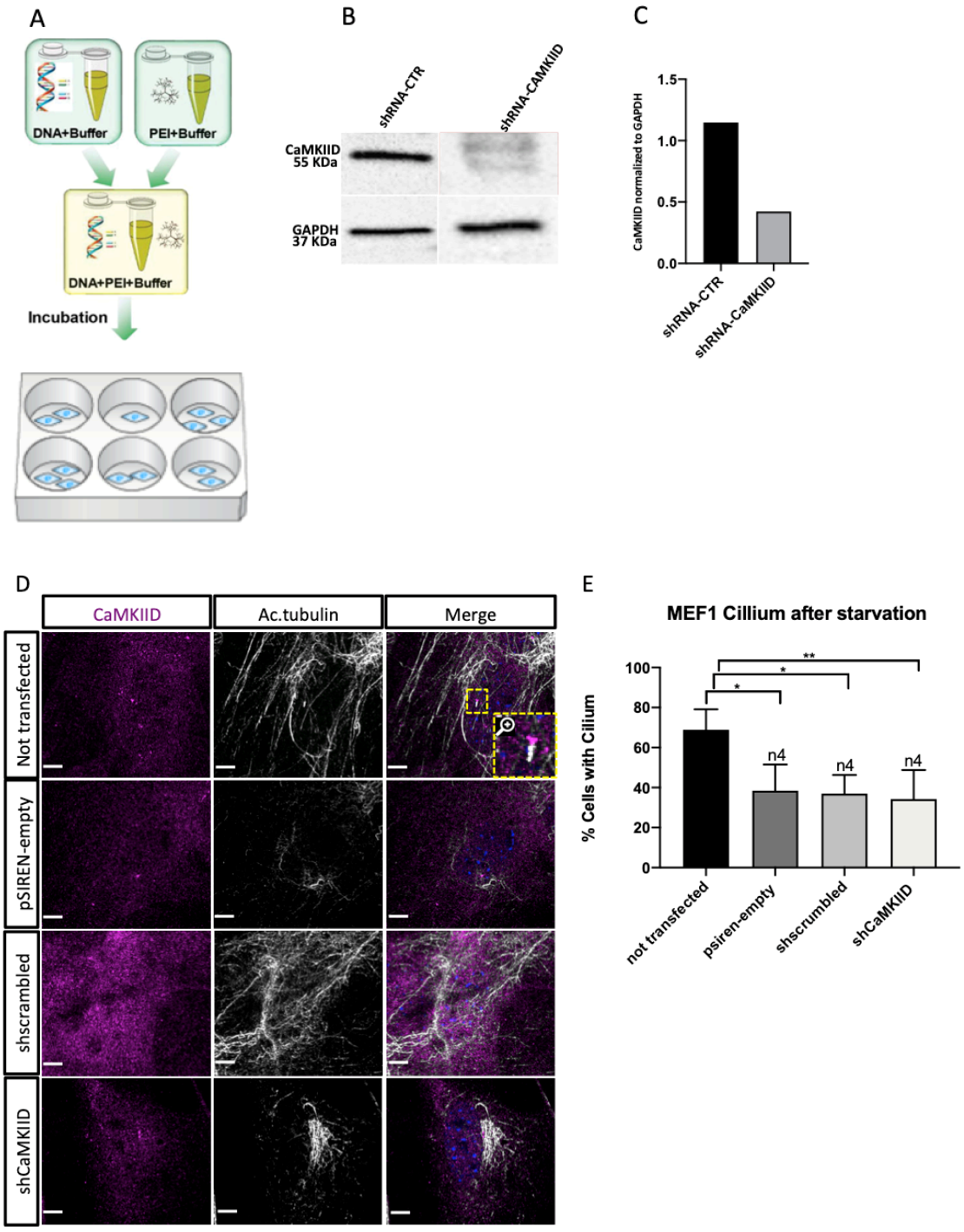


Figure 15. – Loss of function of CaMKIID does not affect ciliogenesis in Mouse Embryonic Fibroblasts (MEFs). (A) Schematic representing the experimental procedure for the transfection of the cells adopted from (Yang et al., 2017). (B) Immunoblotting by using the antibody against CaMKIID, showing the efficiency of designed shRNA to reduce the protein level. (C) Western blot quantification of the CaMKIID signal in the cells transfected with control and shRNA

against CaMKIID. (D) Light microscope imaging of transfected MEFs with pSIREN-shRNA-scrambled, pSIREN-shRNA-CaMKIID and pSIREN-empty after serum starvation. Immunostaining for acetylated tubulin (grey), DAPI (blue) and CaMKIID (magenta) shows a decrease in the number of ciliated MEF cells, 24h after serum starvation when compared to non-transfected cells. (E) The chart represents the percentage of ciliated cells 24 hours after starvation. Statistics: one-way ANOVA, multiple comparisons test. n=biological replicate. *P=0.0111 for not transfected vs. pSIREN-empty; *P=0.0115 for not transfected vs. shRNA-scrambled, **P=0.0043 for not transfected vs. shRNA-CaMKIID. Scale bar=20μm.

3.5. Role of CaMKIID loss-of-function on photoreceptor cells in the mouse retina

To further examine the effect of downregulation of CaMKIID on PRs, we electroporated pups at postnatal day 0 (P0) with shRNA-CaMKIID or with a control shRNA. After 21 days, the mice were euthanized. Retinal sections of PRs were stained with the IS marker, Na+/K+-ATPase, and the OS marker rhodopsin (Figure 16A). All GFP-positive cells (successfully electroporated) were analysed by measuring the length of IS and OS of PRs. The preliminary data suggest that neither the length of the IS nor OS of PRs was affected by CaMKIID KD when compared to control (Figure 16B). We revealed that the decrease in the length of the IS is 16.8 %, whereas the OS is 12.9 % compared to control. Also, it is important to note that the immunofluorescent (IFL) staining for CaMKIID on the shRNA-CaMKIID electroporated retinal sections did not show the reduction of the protein level (data not shown). This could be because the shRNA is not being as efficient in reducing the CaMKIID level in the retina as it is in the HEK293 cells.

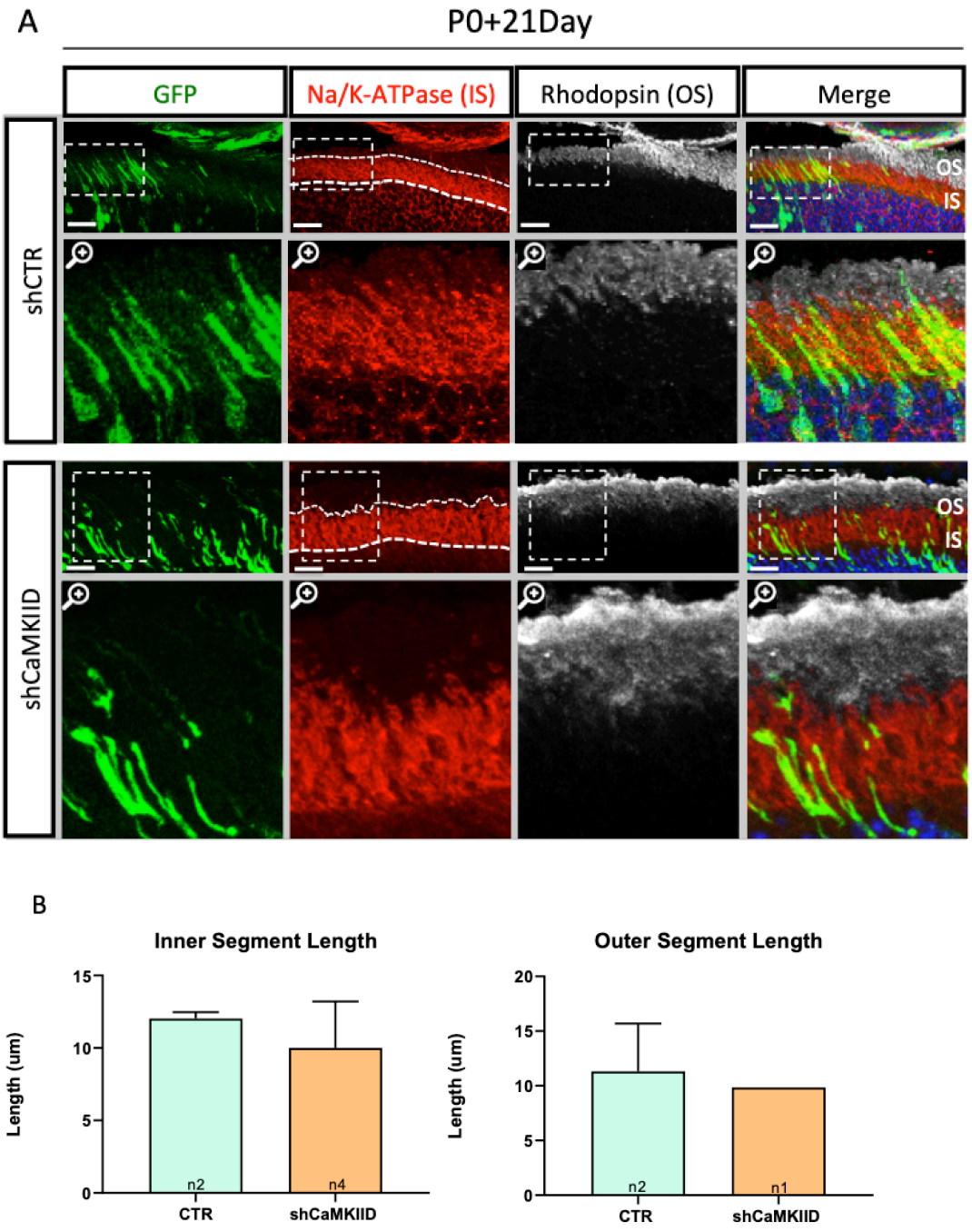


Figure 16. – CaMKIID KD does not appear to affect ciliogenesis of PRs. (A) Confocal imaging of retinal sections in the electroporated area (GFP) with pSIREN-shRNA scrambled and pSIREN-shRNA-CaMKIID. Immunostaining for PRs Na^+/K^+-ATPase (IS) and Rhodopsin (OS) after 21 days of electroporation. Scale bar = 75 μm. GFP - Green Fluorescent Protein. (B) The two charts show

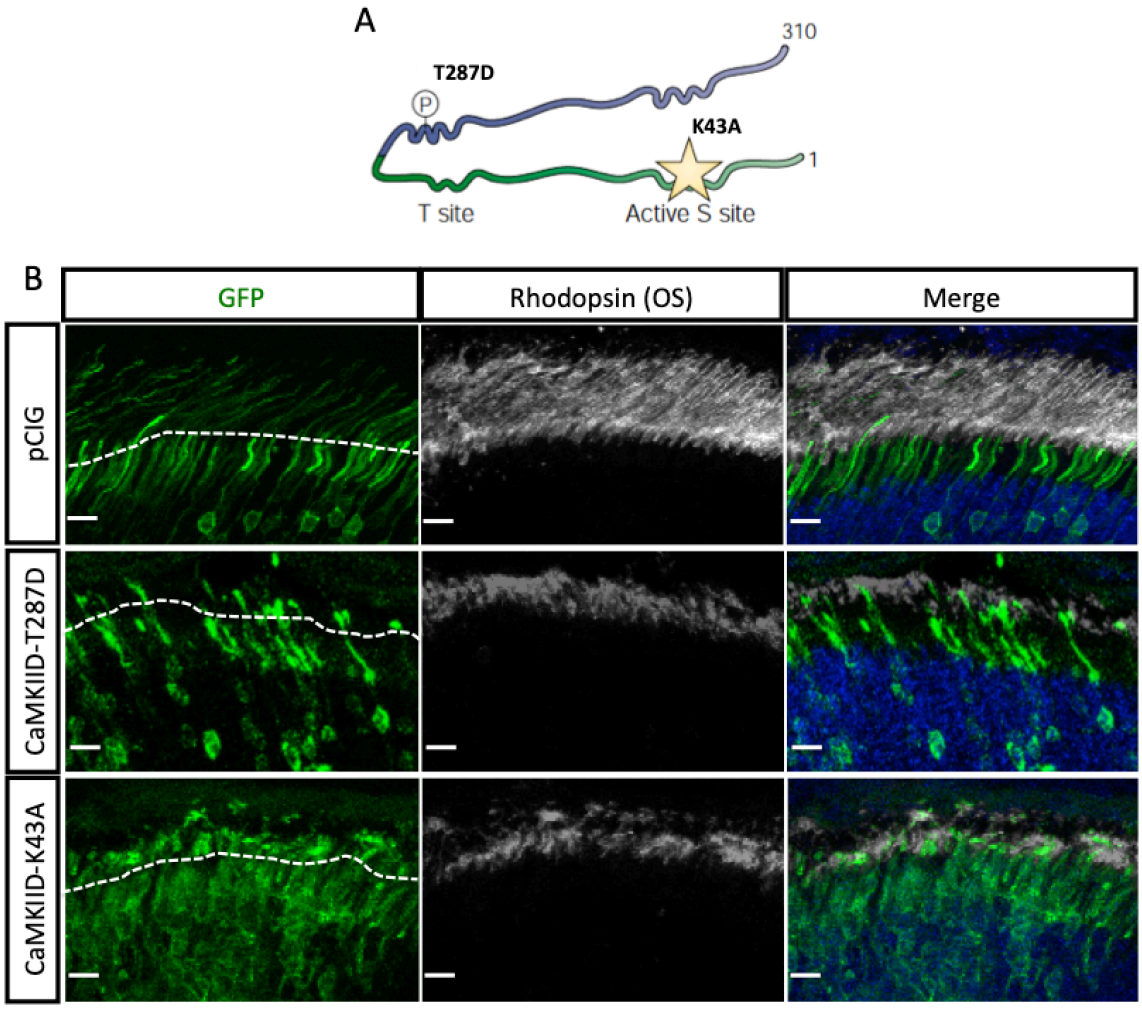
no significant differences in IS and OS length of GFP-positive between CaMKIID and control shRNAs. n=number of biological replicates.

3.6. Expression of dominant-negative (K43A) and constitutively active (T287D) forms of CaMKIID affects the outer, but not the inner segment length of PRs

As an alternative approach to assessing the role of the CaMKIID in the mouse retina, I aimed to express the dominant-negative (K34A) and constitutively active (T287D) forms of the CaMKIID protein in the developing retina and check the potential effect on PRs structure. To produce a dominant-negative form CaMKIID-K34A, a mutation was introduced in the ATP-binding domain by exchanging Lysine (K) to Alanine (A), whereas for generating the constitutively active form CaMKIID-T287D, threonine (T) was substituted with the aspartic acid (D) in the catalytic/regulatory domain mimicking constitutive phosphorylation (Figure 17A) (Pfleiderer, Lu, Crow, Keller, & Singer, 2004).

Both constructs were cloned in an expression vector with a CAG promoter and were delivered separately into the retinas at postnatal day 0 (P0) pups using electroporation. After 21 days, the mice were euthanized, the eyes collected, and fixed. Markers for the IS (Na^+/K^+-ATPase) and the OS (Rhodopsin) were used to quantify the length of individual electroporated cells and measured on retinal cross-sections acquired with a confocal microscope (Figure 17B). Over-expression of either kinase did not affect the IS length when compared to control (Figure 17C). Interestingly, both mutant kinases caused a substantial reduction of the OS length, being 46.2% in the K34A mutant and 49.3% in the T287D mutant (n=3), as opposed to the GFP-expressing cells (n=3) (**P=0.0042 CaMKIID-T287D vs. pCIG; **P=0.0028 CaMKIID-K43A vs. pCIG) (Figure 17C). This effect seemed comparable between the two constructs since no difference in the OS length was elicited between the two mutants (K34A vs. T287D, P=0.9923). Furthermore, our laboratory observation of the retinal sections electroporated with the same mutants shows that the polarity determinants, such as Par3 and alpha PKC reside in the apical domain, suggesting the A-B polarity of the cells is not disrupted. Based on this observation we can hypothesise that the shortness of the OS of PRs is a result of the disrupted connecting cilia formation. All together, these results

suggest that imbalanced CaMKIID activity does not have any impact on the IS formation, while it is very important for developing and maintaining the OS structure, hence the PRs polarity. The data imply a role of the CaMKIID in the formation of the OS, perhaps through the cilia formation.



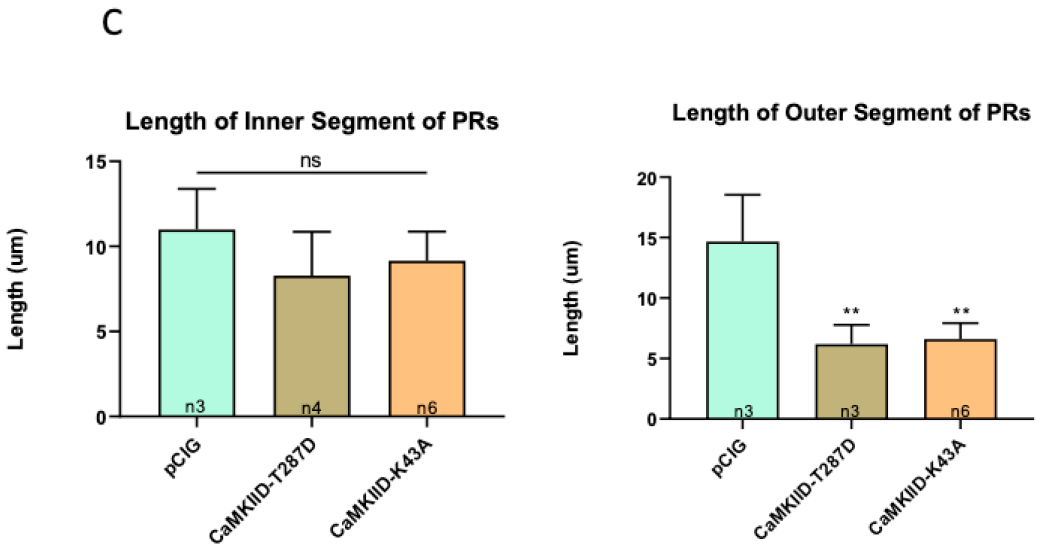
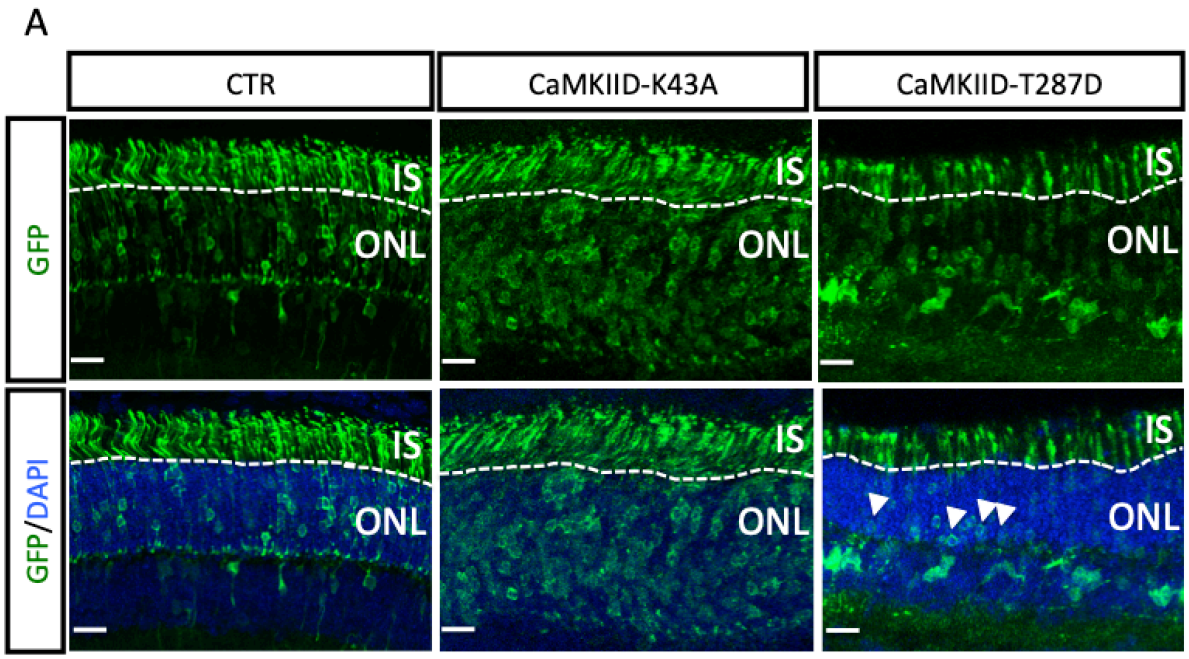


Figure 17. – The proper function of CaMKIID is required for the OS growth of PRs. (A) Schematic of the CaMKIID activity manipulation by introducing mutations in the regulatory domains. K43A mutation in the ATP-binding site represents the dominant-negative form of CaMKIID and the T287D mutation in autophosphorylation site (T site) - constitutively active form of CaMKIID. (B) Confocal imaging of retinal sections electroporated with pCIG-CaMKIID-K43A-IRES-GFP and pCIG-CaMKIID-T287D-IRES-GFP. Immunostaining for GFP and Rhodopsin demonstrates the clear reduction of the OS length of GFP-positive PRs in both K43A and T287D conditions. Scale bar = 75 µm. (C) The left chart represents the measurement of the length of the IS. No significant differences in the IS length of GFP-positive PRs are present. The length in the T287D mutant of 8.276 ± 2.583 µm (n=4) and the K43A mutant of 9.155 ± 1.711 µm (n=6) displays no difference (P=0.2892) when compared to the length (11.00 ± 2.386 µm, n=3) in control cells, expressing GFP only. Statistics: one-way ANOVA, multiple comparisons test. n= biological replicate. The right chart represents the measurement of the length of the OS. The length of the OS being 6.613 ± 1.309 µm (n=5) in the K43A mutant and 6.186 ± 1.593 µm (n=3) in the T287D mutant, as opposed to 14.68 ± 3.859 µm (n=3) in the GFP-expressing cells. **P=0.0042 CaMKIID-T287D vs. pCIG; **P=0.0028 CaMKIID-K43A vs. pCIG.

3.7. Distribution of photoreceptors nuclei

Since the Par3 cKO showed a strong mislocalization of the nuclei along the apico-basal axis in the PRs, I wondered whether overexpression of CaMKIID mutant forms could affect nuclei positioning as well. To analyse the impact of CaMKIID mutants on the apico-basal distribution of PRs nuclei, we divided the outer nuclear layer (ONL) of the retina into three equal compartments (or bins) along the apico-basal axis. The number of nuclei comprised in each compartment of the ONL (apical, middle and basal) was counted and expressed in % of the total (Fig 18A). The overexpression of CaMKIID-K43A does not result in significant changes of nuclei positions in all three compartments when compared to control. However, the PRs expressing the constitutively active mutant (T287D) showed more nuclei located in the basal compartment than in control (pCIG) (Figure 18B). To conclude, expression of CaMKIID constitutively active mutant impacts the nuclei distribution in the PRs at postnatal day 21 (P21), suggesting that the gain, but not the loss of CaMKIID activity, leads to PRs cells relocalization.



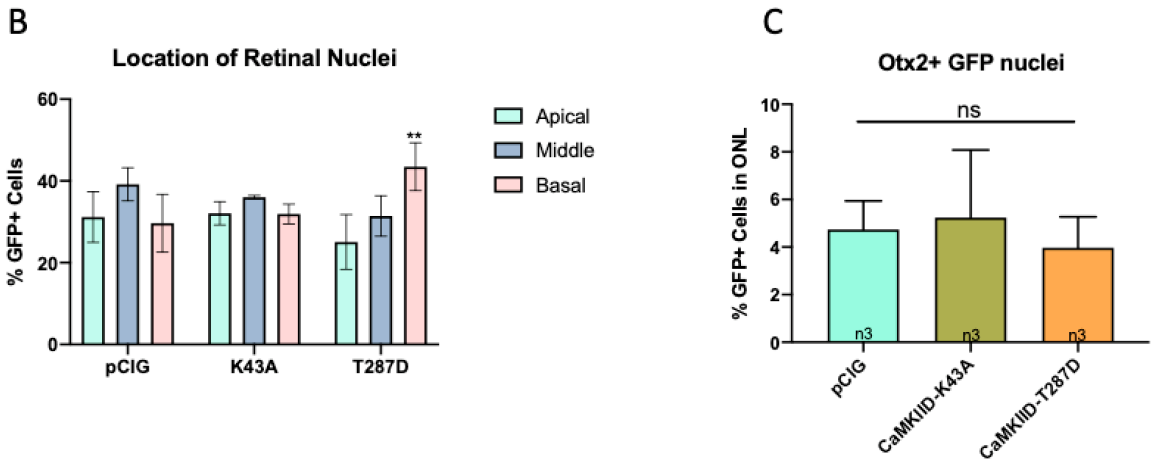


Figure 18. – Overexpression of constitutively active form of CaMKIID changes the nuclei localisation of PRs. (A) Confocal image of retinal sections after electroporation with pCIG-CaMKIID-K43A IRES-GFP and pCIG-CaMKIID-T287D-IRES-GFP. Immunostaining for GFP and DAPI demonstrates a clear concentration of PRs nuclei in the basal part in ONL overexpressing CaMKIID-T287D, P= 0.0052. White arrowheads point to the localisation of PRs nuclei at the basal side of ONL. Scale bar is 75 µm. (B) The relative positioning of GFP-positive PRs nuclei along the apico-basal axis of ONL. (C) Relative numbers of PRs based on Otx2-positive staining and location in the ONL, P=0.7341. Statistics: one-way ANOVA, multiple comparisons test.

3.8. Identification of new CaMKIID interactors in the developing and adult retinas by Mass Spectrometry analysis

Since modulating the CaMKIID activity through overexpression of mutants has a strong impact on PRs polarity, I further wanted to identify its binding protein partners in the developing and adult mouse retinas. Since none of the CaMKIID antibodies we tested were suitable for IP, we decided to immunoprecipitate CaMKIID-HA at P10 and P30 after electroporation of CaMKIID-HA in P0 pups retina. Using the antibody against HA, proteins were immunoprecipitated on whole retina protein lysates and interacting protein partners were identified by mass spectrometry (IP-MS). The result of the analysis identified 91 proteins in P10 and 34 proteins in P30 retinas of CaMKIID-binding partners (Figure 19A), although the most enriched binding protein was CaMKIID in both time points. Interestingly, the Venn diagram shows that only 16 (14.7%) of all identified proteins

are shared between P10 and P30 (Figure 19A). Likewise, comparing the binding proteins of Par3 and CaMKIID, we identified annexin2 (Anxa2) as a shared protein partner between the two analyses. In addition, kinesin-4 family protein (Kif7) and annexin2 (Anxa2) were found to interact with Par3 and CaMKIID at P10, the window during which connecting cilia grow and OS start to form. While Kif7 is an anterograde motor and cilia associated protein (Lewis et al., 2017), Anxa2 is a multifunctional protein plays a role in many cellular processes such as endocytosis, exocytosis, and signal transduction (Grindheim, Saraste, & Vedeler, 2017). The binding of Kif7 and Anxa2 to CaMKIID was detected only in this time point, as in the P30 retina there is no interaction (Figure 19B). At the same time, during both P10 and P30 CaMKIID appears to interact with the SAG (rod arrestin), which is important to regulate signal transduction in PRs (Song et al., 2011) (Figure 19B). Hence, before and after OS formation in the mouse retina, CaMKIID has a preferential binding affinity to different proteins involved in regulating the PRs function and structure.

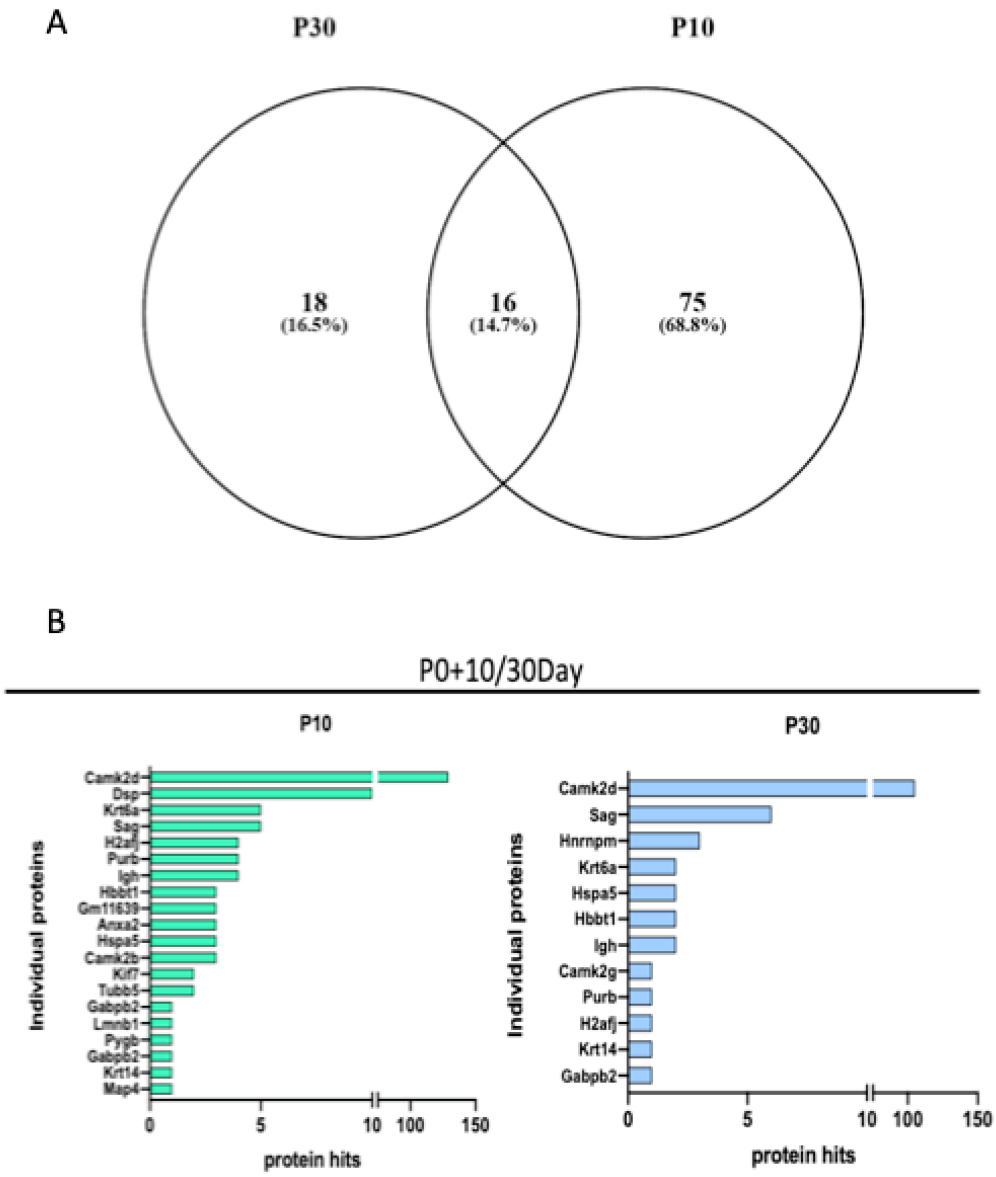


Figure 19. – Identification of CaMKIID interacting proteins in the mammalian retinas in P10 and P30 (n=1 for each). Immunoprecipitation of HA- tag was followed by mass spectrometry (IP-MS) analysis. (A) Venn diagram of total identified proteins at P10 (75) and P30 (18) including those present in the two-time points (16), performed with Mascot software version 2.6. (B) A diagram displaying the most enriched CaMKIID binding partners.

Tableau 4. – List of CaMKIID-interacting partners at P10 and P30.

Name	Exclusive Spectrum Count	Time point	Name	Exclusive Spectrum Count	Time point
Camk2d	129	P10	Lmnb1	1	P10
Camk2d	21	P10	Ighv1-78	1	P10
Sag	5	P10	Hnrnpa3	1	P10
Krt6a	5	P10	Snrpf	1	P10
Hnrnpk	5	P10	Rpl28	1	P10
Ighv6-3	5	P10	Setx	1	P10
H2afj	4	P10	Ndufa4	1	P10
Purb	4	P10	Igkv15-103	1	P10
Hist1h1c	4	P10	Txn	1	P10
Igh	4	P10	Ighv1-56	1	P10
Ighv14-2	4	P10	Ddx17	1	P10
rps14	4	P10	Trim28	1	P10
Hbbt1	3	P10	Ighv1-43	1	P10
Hspa5	3	P10	Krt17	1	P10
Camk2b	3	P10	Camk2d	105	P30
Rps10	3	P10	Camk2d	15	P30
Hnrnpa1	3	P10	Sag	6	P30
Cbx3	3	P10	Krt42	4	P30
Anxa2	3	P10	Ccdc8	4	P30
Igkv8-27	3	P10	Hnrnpm	3	P30
Rpl30	2	P10	Hbbt1	3	P30
Rtcb	2	P10	Igh	2	P30
Ighv14-3	2	P10	Eef1a1	2	P30

Ighv14-1	2	P10	Krt6a	2	P30
Kif7	2	P10	Hspa5	2	P30
Dsg1b	2	P10	Hbbt1	2	P30
Rpl14-ps1	2	P10	Map4	2	P30
Rps3a1	2	P10	Nol3	2	P30
Tcof1	2	P10	OTTMUSPW KG0005939	2	P30
Rpl31	2	P10	Krt36	2	P30
Ighv14-4	2	P10	Ewsr1	2	P30
Ighv1-67	2	P10	Pcbp3	2	P30
Igkv4-50	2	P10	Hmgn2	2	P30
Rpl18	2	P10	Sfpq	2	P30
Fcgr4	2	P10	Jup	1	P30
Rpl8	2	P10	Rpl23a	1	P30
Rpl7a	2	P10	Gm11639	1	P30
Npm1	2	P10	H2afj	1	P30
Eif2s1	2	P10	Purb	1	P30
Nccrp1	2	P10	Gabpb2	1	P30
Rps2	2	P10	Krt14	1	P30
Ddx1	2	P10	Camk2g	1	P30
Tubb5	2	P10	Pcbp3	1	P30
Hbbt1	1	P10	Ecpas	1	P30
Map4	1	P10	Pkp1	1	P30
Gabpb2	1	P10	Rrp9	1	P30
Krt14	1	P10	Pygm	1	P30
Hist1h1e	1	P10	Hspg2	1	P30

Chapter 4 – Discussion

Cell polarity is a fundamental property of adult neurons, which determines their functions and integrity (Barnes, Solecki, & Polleux, 2008; Deretic, 2006). The loss of neuron structure and polarity leads to neuronal dysfunction, and ultimately to neuronal degeneration, such as in retinal dystrophies, with the subsequent vision loss. Our unpublished data showed that polarity determinant Par3 is an essential protein for the retinal lamination and when it is removed early during development (knocked-out in retinal progenitor cells), it interferes with the photosensitive cilia formation resulting in photoreceptor (PR) cell degeneration. Using mass-spectrometry analysis we identified CaMKIID (calcium/calmodulin-dependent protein kinase II delta) as a new interacting protein of Par3 in the developing and adult mouse retina. Although CaMKIID has not been reported previously as a regulator of PR cell polarity, here we provide evidence that CaMKIID may regulate the formation and maintenance of the connecting cilia of PRs and act as a novel regulator of their polarity. We have reached this conclusion based on the ectopic expression of CaMKIID-tagged version, when we validated its interaction with polarity determinant Par3, both *in vitro* and *in vivo* at postnatal day 10, known as a time window of PRs OS genesis. Secondly, overexpression of both negative and constitutively active mutant forms of CaMKIID resulted in shortening of the OS development of PRs.

4.1. The localization of CaMKIID in the mouse retina

We investigated the expression of isoform delta of CaMKII during mouse retinal development. It is noteworthy that all four isoforms of CaMKII family were detected in the retina, specifically at the level of synapses (Del Corsso, Iglesias, Zoidl, Dermietzel, & Spray, 2012; Tetenborg et al., 2017; Wade Kothmann et al., 2012). However, previous reports in the literature focused on the physiological impact rather than on the mechanistic contribution of specific isoforms in the retina.

Immunostaining on cross-sections of mouse retina at different stages revealed a dot-like pattern in the apical region. CaMKIID localizes in close proximity with ZO1, a marker of a tight junction at E14.5 and postnatal day 0 (P0) (Figure 12). Interestingly, in adult wild type (WT) retina, we

observed that CaMKIID has conserved a dots-like pattern and is mostly expressed at the tip of the IS of PR cells, where the basal bodies of PR connecting cilia reside (Figure 11A).

The previous characterization demonstrated a clear colocalization of Par3 with ZO1 during development at the OLM. In the adult retina Par3 localized in the PRs IS in addition to the OLM (Figure 8). Although the pattern of CaMKIID expression does not match perfectly Par3, they both are localized in the same IS sub-compartment, suggesting a synergetic input in the PRs development and their maintenance.

However, if the Par3/CaMKIID protein complex is involved in establishing PRs polarity and ciliogenesis, the question of whether CaMKIID is upstream or downstream of Par3 in the signalling reactions, remained open. To answer this, we used Par3 cKO mice line to see whether the deletion of Par3 would affect CaMKIID localization. Using immunostaining for CaMKIID in the Par3-ablated retinal sections, we observed that the absence of Par3 leads not only to the retinal disorganisation but also to CaMKIID mislocalization (Figure 13). Based on our current findings, we hypothesize that Par3 acts as an "anchor" at the tight junction region for CaMKIID.

4.2. CaMKIID is capable of interaction with Par3 *in vitro* and *in vivo*

Before studying the effect of CaMKIID and Par3 in detail in the mouse retina, we first needed to verify whether the two proteins could interact with each other. To answer this, we carried out *in vitro* experiment using HEK293 cells overexpressing Par3 and CaMKIID tagged with GFP and HA, respectively. We showed that Par3 co-precipitates with CaMKIID (Figure 14 top gel). In *in vivo* experiments conducted at postnatal day 11 (P11), for the first time we showed that CaMKIID is capable of interacting with all three isoforms of endogenous Par3 in mouse retina (Figure 14 bottom gel). Rod and cone PRs genesis in mouse have different timing of development. While cone genesis completes at embryonic day (E) 14, rods are born pre- and postnatally, from E12 to postnatal day P10 (Morrow et al., 1998; Swaroop, Kim, & Forrest, 2010). However, the growth of the OS of both rod and cone PRs are synchronized and begins around P10. Our finding suggests that Par3 and CaMKIID interact during the window of PRs OS maturation (P10-P12). However, because both antibodies are made in rabbit, we could not identify simultaneously where specifically in PRs the interaction of endogenous Par3 and CaMKIID takes place. With specific

antibodies raised in different species, the subcellular localization has been addressed by the immunofluorescence staining of the retinal sections. Moreover, the question of whether Par3 and CaMKIID interact, directly or indirectly remains opened. To address this question, we would need to perform a GST pull-down assay with purified proteins.

4.3. Effect of CaMKIID downregulation on ciliogenesis in MEFs model and mouse retina

It was shown that CaMKII is involved in the stabilization of cilia in the pronephric kidney in zebrafish (Rothschild et al., 2011). To test the effect of down-regulated CaMKIID on the cilia formation in mouse embryonic fibroblasts (MEF), I overexpressed designed shRNA against CaMKIID and two control vectors (scrambled shRNA and pSIREN) and induced the cilia growth by serum starvation. Knowing that CaMKII is expressed in cilia (J. Wei et al., 1998), we, therefore, assumed that changes in its expression would somehow affect cilia growth in MEFs. Quantifications of ciliogenesis induced upon serum starvation, however, demonstrated that transfection by itself strongly inhibited ciliogenesis, and we did not see an additional decrease in ciliogenesis by CaMKIID knockdown (KD) compared to controls. These results suggest either that the shRNA was not effective enough or that the decrease in ciliogenesis by transfection precludes demonstration of any effect of CaMKIID KD. However, it is important to mention that we are limited to study PRs ciliogenesis solely in *in vivo* as the *in vitro* approach does not allow us to address questions of the OS development in cultured PRs. This limitation can be explained by the absence of retinal pigment epithelium in the culture, essential for the OS development.

To look at the physiological impact of the CaMKIID in PRs in more detail, I delivered the designed shRNA against CaMKIID into P0 retina. Similarly, 21 days after CaMKIID KD induced by shRNA electroporation of P0 progenitors, we observed no effect on the length of IS and OS of PRs. Moreover, I could not see the decrease of the amount of the endogenous CaMKIID in the electroporated retina with shRNA against CaMKIID when compared to control. This negative result can be interpreted as a failure of designed shRNA to degrade the CaMKIID transcript due to the lack of specificity and/or stability, or other members of CaMKII family could compensate the absence of CaMKIID. The latter is likely caused by the high homology of CaMKII α, β, γ and δ

isoforms where their C-terminal association domains can form homo- or heteromeric assemblies (Bayer & Schulman, 2019b; Lisman et al., 2002; Myers et al., 2017). Finally, more experiments will be required to formally test the requirement of CaMKIID in PRs ciliogenesis.

4.4. CaMKIID is required for the maintenance of the outer segment length of photoreceptors

To further explore the role of CaMKIID in PRs polarity in the retina, we studied the protein expression in *in vivo* and fixed samples. Based on our current result that CaMKIID and Par3 localize on PRs compartment of the developing and adult retinas, we hypothesized that Par3 recruits CaMKIID apically of PRs IS to participate in the regulation of proteins that are sent to form the photosensitive OS. Briefly, we introduced mutations in the ATP-binding domain (K43A-dominant negative) and in the catalytic domain (T287D- constitutively active) (Pfleiderer et al., 2004) in order to understand whether changes in CaMKIID activity may have an impact on PRs cell structure. Measuring the IS length of GFP-positive PRs, we observed that IS was not affected in both conditions. However, both mutants drastically reduced the OS length of GFP-positive PRs. Taking into account that the OS of rod and cone PRs is formed by the elongation of connecting cilia (Ramamurthy & Cayouette, 2009) one idea is that OS would be affected when the proper balance of CaMKIID function is altered, thus interfering with the signalling pathways. According to our model, the loss- and gain- of CaMKIID activity impacts the length of the OS and the ability to stabilize cilia, resulting in the PRs OS degeneration (Figure 20). This phenotype could be caused either by the lack of the CaMKIID-mediated signalling reactions or, by the absence of the recruitment of Par3 and CaMKIID.

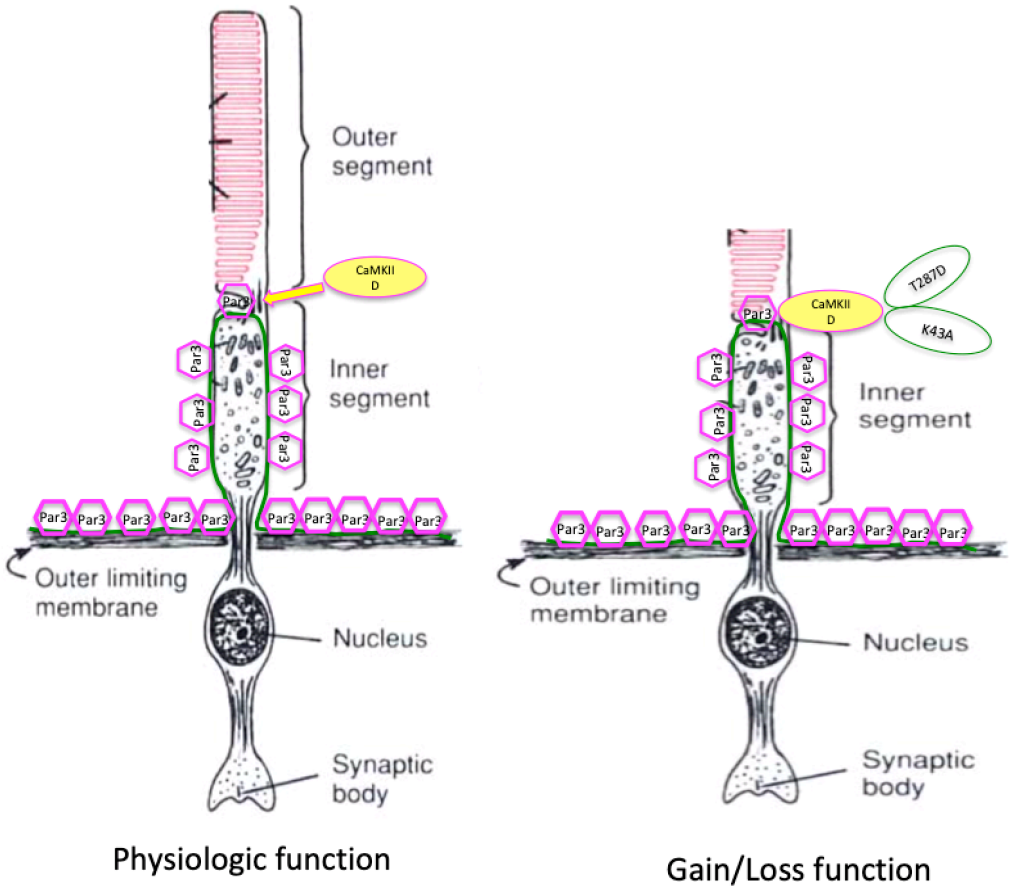


Figure 20. – Model of CaMKIID activity in photoreceptors. Schematics represent the PRs with the physiologic and gain/loss CaMKIID functions. On the left side, schematic indicates the healthy PR with the localization of the expression of Par3 and CaMKIID. On the right side, the schematic represents the affected PR with the shorten OS of PRs. The magenta rhombus point to the Par3 localization in the OLM and in the IS of PR, yellow/green circles point into CaMKIID localization, presumably in the connecting cilia and the green line underline the ONL and IS of PRs.

4.5. Constitutively active CaMKIID promotes the PRs nuclei mislocalization at the basal part of the retina

The previous study reported that nuclei migration is a critical process for both proliferative and post-mitotic phases of neuronal development including retina (Bone & Starr, 2016; Centanin &

Wittbrodt, 2014). The key aspect of apical-basal polarity of retinal progenitors cells (RPC) is known as interkinetic nuclear migration (IKNM) (Baye & Link, 2008) where RPC nuclei perform apico-basal movements in a synchronised fashion during the cell cycle (Frade, 2002). The mitosis is restricted to the apical surface, where the centrosomes are localised (Norden, Young, Link, & Harris, 2009). In this perspective, we wanted to examine the possibility that CaMKIID activity could regulate IKNM and centrosome stabilization in the mouse retina. Importantly, the interplay between the nuclear envelope and microtubules mediates nuclear positioning, disruption of which leads to the human pathologies, in part originating from ciliary defects (Bone & Starr, 2016; Potter et al., 2017). To address this question, the electroporated retinas with vectors that carry CaMKIID mutant forms, such as pCAG-CaMKIID-K43A-IRES-GFP- (dominant-negative), pCAG-CaMKIID-T287D-IRES-GFP (constitutively active) and pCAG-IRES-GFP (control), respectively, were analyzed for the nuclei localisation across the ONL. While in both the dominant-negative mutant and the GFP control nuclei of electroporated PRs were located homogeneously across the ONL, nuclei of PRs overexpressing the constitutively active CaMKIID (T287D) were found to locate more frequently on the basal side of the ONL. This phenotype could be initially caused by the disruption of IKNM and centrosome localization that is essential for the mitosis of the proliferating cells in mouse retina (Centanin & Wittbrodt, 2014). The model of INKM suggests that the mitosis occurs at the apical side which follows by the movement towards the basal surface during G1, and after S phase, nuclei ascend back during G2 for the next mitosis (Miyata, 2008; Murciano, Zamora, López-Sánchez, & Frade, 2002; Nowakowski & Hayes, 2006; Sauer, 1935). As we observed the nuclei mislocalization in the retina with overexpressing constitutively active CAMKIID, we hypothesized that CaMKIID might play an important role in the cell cycle in developing and adult retinas either through the regulating of the nuclei movement from the apical surface so then making a room for other mitotic cells, or, by activating motor proteins that move progenitors from basal part to apical for the further mitosis. However, how CaMKIID could control this process in developing and adult retinas remains unknown. Indeed, in zebrafish retinal neuroepithelia, it was observed that IKNM is an actomysin-dependent process (Norden et al., 2009).

4.6. Identified interaction of CaMKIID with Kif7, annexin2 and SAG (rod arrestin) might play a role in OS formation of PRs

As previously discussed, CaMKIID is regulating the PR cells polarity through the development of OS, and it might play a role in the cell cycle of proliferating progenitors in the retina. However, the molecular mechanism of these processes remains unclear. To gain insights into this mechanism, CaMKIID interacting partners were identified by Mass spectrometry after pulling down CaMKIID-HA at P10 and P30 from mouse retinas that had been electroporated at P0.

Here we show ten of the top CaMKIID-interacting proteins that were identified in P10 and P30 retinas. Notably, the most abundant immunoprecipitated protein was CaMKIID, showing the specificity of the immunoprecipitation. As expected, among all precipitated partners were CaMKIIB in P10 and CaMKIIG at P30, since CaMKII isoforms form the heteromeric complex (Bayer & Schulman, 2019b; Lisman et al., 2002). Interestingly we noticed that two-time points have common interacting proteins that are involved in the intermediate filament organization, protein folding, response to endoplasmic reticulum stress, cellular localization and chaperon-mediated protein folding. Other than these, at P10, we found that CaMKIID is interacting with proteins such as SAG, Kif7, Anxa2, CaMKIIB, Lmnb1, Pygb, Hap4 and Gm11639. At P30 we identified interaction only with CaMKIIG, Hnmpm and SAG. All proteins that appeared as the main interactors with CaMKIID in two-time points might play a specific role in retinal tissue, and this requires further studies. However, my suggestion is that the interaction of CaMKIID with kinesin-4 family protein (Kif7), rod arrestin (SAG) and annexine2 (Anxa2) could be fundamental for the PRs OS development.

It was previously reported that Kif7 regulates mammalian Hedgehog signalling pathway and this controls the cilium architecture (He et al., 2014). We, therefore, posit that both proteins could be involved in the connecting cilium to regulate PRs OS growth in the mouse retina. In addition, other studies showed that Kif7 resides at the tip of the cilia in MEFs and it is not involved into intraflagellar proteins transport into cilia (He et al., 2014), while CaMKIID resides at the base of the cilia in olfactory neurons (J. Wei et al., 1998). As of this, we hypothesized that CaMKIID might be involved in the stabilization of the connecting cilium, knowing its centrosome localization in

olfactory neurons, whereas Kif7, which is localized on the opposite side of the cilium, concludes the chains reaction by controlling the cilium structure. It also might suggest that CaMKIID is the upstream protein of Kif7 cascade reaction, which is essential for cilia function and architecture. However, whether and how this may take place in mammalian retina PRs requires further investigation.

Another interesting CaMKIID-interacting protein we identified is SAG (rod arrestin) in both P10 and P30 retinas. Interestingly, the amount of protein hits at P30 retina is slightly higher compared to P10 retina. Indeed, as was previously mentioned, in wild type (WT) animals arrestin is the second most abundant protein in rods and it regulates the rhodopsin activity (Song et al., 2011; Wu et al., 2006). Moreover, it is essential for the normal photoresponse recovery as well as for healthy rods morphology (Xu et al., 1997). Interestingly, the studies showed that the length of the OS in arrestin mutants mice was dose-dependent (Song et al., 2011). Hence, we assume that the disruption of CaMKII activity might alter the amount of the arrestin found in the OS of rod PRs. One possibility is for this interaction to contribute to the arrestin transport from the IS to the OS of PRs. To address this hypothesis, it would be interesting to examine the arrestin protein level in the presence of CaMKIID mutants, knowing that the dominant-negative and constitutively active CaMKIID affect the length of OS. Besides, it would be interesting to see whether arrestin is CaMKIID's substrate and how arrestin's phosphorylation status can be changed in the presence of CaMKIID mutants.

Anxa2 is a multifunctional protein involved in the cellular processes such as endocytosis, exocytosis, membrane domain organization, actin remodelling, signal transduction, protein assembly, transcription and mRNA transport, as well as DNA replication and repair (Grindheim et al., 2017). A possibility would be that CaMKIID and Anxa2 could facilitate the formation of the vesicles that carry proteins from IS to OS of PRs, through the connecting cilia which are the bridge between two segments (Ramamurthy & Cayouette, 2009). It is noteworthy that the cilia forms from the single Golgi-vesicle that is attached to the end of one centriole (Sorokin, 1962). While CaMKII is involved in the cloacal cilia stability in zebrafish (Rothschild et al., 2011), it stays in centriole in the olfactory neurons (J. Wei et al., 1998). Based on this, we can suggest that this

interaction might determine the connecting cilia organization, where Anxa2 plays a role in the cilia-associated vesicle formation, while CaMKIID in stabilizing cilia.

Conclusion

1. We identified Par3 as a key regulator of the retinal architecture and PR cells polarity. Par3 KO in retina interferes with the photosensitive cilia formation resulting in the PR cells death. In addition, we showed that in mouse retina Par3 co-immunoprecipitated with exogenous CaMKIID suggesting a direct or indirect binding, and involvement of the kinase in the Par complex formation or maintenance.

2. Expression of dominant-negative and constitutively active CaMKIID in P0 retina impedes with the OS growth, but not the IS, suggesting its involvement in the polarity formation of the PRs. Moreover, detecting the CaMKIID in the basal body of adult retina ascribes a potential role in maintaining the PRs polarity.

3. Expression of constitutively active CaMKIID affects the PRs nuclei positioning in the adult retina, resulting in their accumulation in the basal side of the ONL. This result demonstrates that a balance of the protein level is necessary to regulate the movement of nuclei from basal towards the apical sides, and suggest a role in controlling the cell cycle.

4. With the IP-MS experiment on the developing (P10) and adult retinas (P30), we showed that CaMKIID interaction with partner proteins dependent on the timing of development and formation. Among them, the most relevant from the point of view of OS formation of the PRs are the Kif7 and Anxa2, as the CaMKIID interacts with both of them during the retina development at P10. While the interaction with SAG (rod arrestin) at both time points suggests that CaMKIID participates not only during the development but in the maintenance of adult PRs as well.

To highlight the significance of our findings, we believe that unravelling the mechanism behind CaMKIID can lead us to further understand the impact of CaMKIID on the PRs development, in particular the OS growth. With this in mind, we hope to provide further insight toward the establishment and maintenance of PRs polarity and identify additional approaches for preventing

this deregulation. Therefore, we suggest additional experiments and future directions that might shed light on the CaMKIID function in mouse retina.

First, production of CaMKIID cKO mouse line (e.g by using the CRISPR-Cas approach) can give us an additional information about the importance of this protein in the retina development as a whole and in particular could confirm the results of the dominant-negative CaMKIID expression and its relevance for the OS formation in PRs. Second, we could perform a test on the phosphorylation status of proteins in the retina when the constitutively active and dominant negative CaMKIId mutants are overexpressed and correlate the data from the IP-MS analysis. This way we could identify which proteins are potentially activated or inhibited by the CaMKIID in the context of the PRs development. Finally, as we observed that the dominant-negative and constitutively active forms of CaMKIID have negative impact on the OS of PRs formation and on the cell cycle, we think that CaMKIID activity has to be balanced for the normal PRs structure and function. Thus, the clinical relevance could be to successfully control the CaMKIID expression (inhibition or induction) so that PRs could have normal OS development.

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