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XXVI° CICLO**

***Identification of pericentriolar matrix protein 1
(PCM1) as a novel scaffold for Protein Kinase A
(PKA) and NIMA-related kinase 10 (NEK10).***

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ORIGINAL PUBLICATIONS

1. Carlucci A, **Porpora M**, Garbi C, Galgani M, Santoriello M, Mascolo M, di Lorenzo D, Altieri V, Quarto M, Terracciano L, Gottesman ME, Insabato L, Feliciello A. (2010) *PTPD1 supports receptor stability and mitogenic signaling in bladder cancer cells.* J Biol Chem.

ABSTRACT

The pericentriolar material 1 (PCM1) assembles a molecular platform around the pericentriolar matrix and centrosome that is essential for cell division and microtubule dynamics, and plays a critical role in different aspects of mitosis and ciliogenesis.

Here, we report an additional layer of complexity and found that PCM1 nucleates a signaling complex at the pericentriolar matrix, which includes PKA and NEK10. By co-clustering two distinct signaling enzymes, PCM1 controls critical aspects of mammalian ciliogenesis. Within the PCM1 complex, PKA regulates NEK10 localization and stability, efficiently coupling cAMP signaling to the ubiquitin-proteasome system (UPS). In this context, PCM1 acts as relay that senses, transmits and integrates cAMP and mitogenic signals generated at cell membrane, regulating biological events at the pericentriolar matrix area and primary cilium.

The molecular characterization of relevant protein complexes at specific sub-cellular compartments and understanding the role of scaffold platform in critical aspects of cell biology will likely contribute to the design of novel therapeutic approaches for human diseases.

INTRODUCTION

1.1 The cAMP-dependent signal transduction pathway

Living cells are exposed to many processes and functions that allow the organism to respond and adapt itself to different conditions and needs. To make this possible it is necessary that each cell is capable of responding to different signals translating implementation or shutting down of specific mechanism. There are many molecules that act like extracellular messengers, such as hormones, neurotransmitters and growth factors, that regulate a different cellular processes, as cellular differentiation and division, ionic channels activity, gene transcription and protein translation.

The binding of these molecules to specific cellular receptors elicits cellular responses. Plasma membrane receptors transmit the signals of the specific hydrophilic messengers, generating biophysical modification or increasing intracellular signalling molecules concentration known as second messengers.

The first second messenger identified is the cyclic AMP (cAMP) (*Sutherland et al., 1970*). cAMP-dependent transduction pathway is mediated by G-coupled receptors that activate the adenylyl cyclase (AC), ubiquitous enzyme converting ATP in cAMP. In mammals, the ubiquitous second messenger cAMP is synthesized by two classes of AC: the G protein responsive transmembrane adenylyl cyclases (tmACs) and the widely distributed, bicarbonate-responsive, soluble adenylyl cyclase (sAC) (*Chen et al., 2000*).

tmACs are characterized by a short NH₂-terminal amino-acidic segment and by two cytoplasmic domains (C1 and C2) separated by two highly hydrophobic transmembrane domains (M1 and M2). C1 and C2 constitute catalytic site of adenylyl cyclase.

cAMP mediates its cellular effects via at least three distinct classes of direct effectors; cAMP-dependent protein kinase (PKA), RAP exchange proteins (EPACs), and cAMP gated ion channels (cNGC). cNGCs and a subset of PKA targets are localized to the plasma membrane, near tmACs, in what appear to be macromolecular signaling complexes comprising the G protein coupled receptor, G protein, tmAC, and PKA and its ultimate substrate (*Zippin et al., 2002*) (**Fig. 1**)

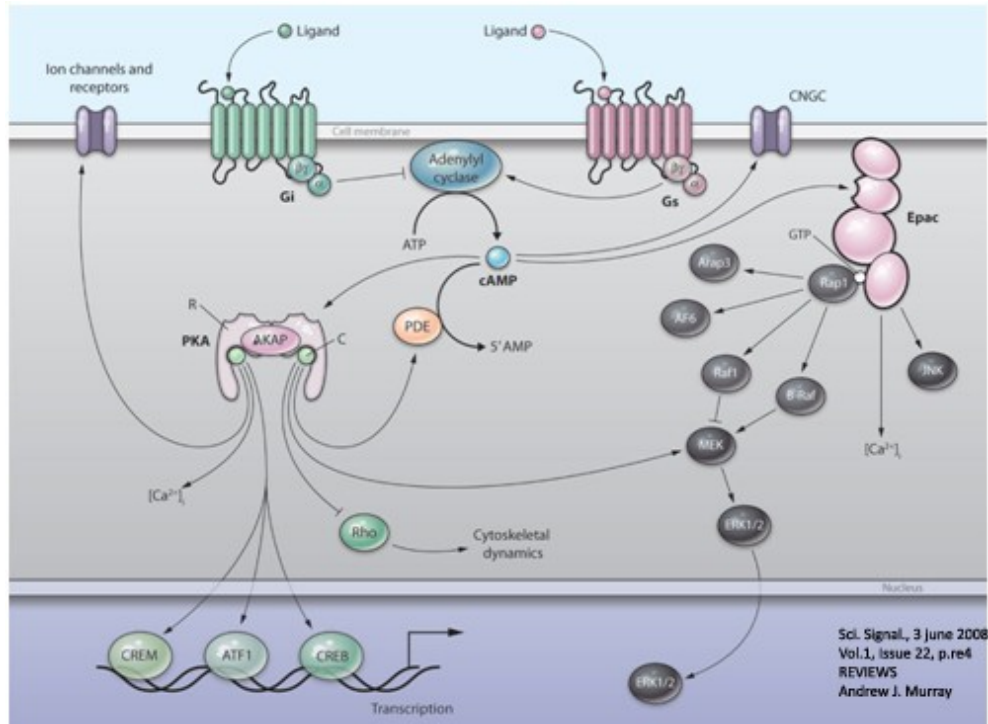


Figure 1. Schematic diagram of cAMP synthesis and downstream effector activation. When an extracellular ligand such as a hormone binds to and activates a seven-transmembrane G-protein-coupled receptor, the signal is passed through the heterotrimeric G protein to adenylyl cyclase. The activated adenylyl cyclase converts ATP into the second messenger cAMP. As the gradient of cAMP concentration diffuses in the cell, various enzymes or effectors are activated. These include PKA, PDE and EPAC. The interplay of each of these effectors as they interact directly or indirectly with each other and other downstream targets is currently an area of intense study.

1.2 Protein Kinase A (PKA)

Activation of PKA is solely accomplished by the major, diffusible secondary messenger cAMP (Su et al., 1995). PKA Holoenzyme is a serine/threonine kinase constituted by two catalytic subunits (PKAc or C) that are held in an inactive complex by a dimer of regulatory subunits (Rs). Binding of cAMP to each R subunit relieves the autoinhibitory contact, allowing the C subunits to dissociate (Wang et al., 1991) thereby resulting in phosphorylation of local substrates (**fig. 2**). PKA-dependent phosphorylation of nuclear and cytoplasmic substrates controls multiple cell functions, including motility, metabolism, differentiation, synaptic transmission, ion channel activities, growth and coordinate gene transcription (Edelman et al., 1987; Taylor et al., 1992; Meinkoth et al., 1993; Montminy et al., 1997).

Two forms of the heterotetrameric PKA holoenzyme exist: type I (RI α and RI β dimer) and type II (RII α and RII β dimer). Type I PKA is predominantly cytoplasmic, whereas type II PKA associates with specific cellular structures and organelles. Catalytic subunits are encoded from three different genes C α , C β and C γ , that show common kinetic features and substrate specificity (Taylor et al., 1992). Four genes encode the R subunits R1 α , R1 β , R2 α and R2 β (Taylor et al., 1992), that confer the different biochemical and biological characteristics to the PKA isoforms. The R subunit is a modular polypeptide containing an

NH₂-terminal dimerization domain, an autophosphorylation site that serves as a principal contact site for the C subunit, and two cAMP binding sites.

The R subunits are differentially distributed in mammalian tissues. RI α and RII α are ubiquitous, whereas RII β is expressed predominantly in endocrine, brain, fat and reproductive tissues (*Edelman et al., 1987; Taylor et al., 1992*). In addition to their distinctive expression and distribution, R subunits differ in their regulation and biochemical properties. The binding affinity to cAMP of RII β in vivo is lower relative to RII α and much lower compared to RI α (*Edelman et al., 1987; Taylor et al., 1992*).

However, there are many AKAPs that bind RII β subunits in a specific manner. These data imply that holoenzymes containing RI subunits or RII subunits (PKAI and PKAII) decode cAMP signals that differ in duration and intensity: PKAI is activated transiently by weak cAMP signals, whereas PKAII responds to high and persistent cAMP stimulation. Neurons and endocrine cells, which express predominantly PKAII, are adapted to persistent high concentrations of cAMP (*Stein et al., 1987*). The specific biochemical properties of PKA isozymes account, in part, for the differential cellular responses to discrete extracellular signals that activate adenylyl cyclase.

The function(s) in vivo of the specific PKA isoforms in gene expression and cell signaling has been probed using knockout (KO) and transgenic mouse models (*Brandon et al., 1995a*). Ablation of the gene encoding the RI β tether to

deficits in hippocampal long-term depression and depotentiation (*Qi et al., 1996; Brandon et al., 1995b*). Despite a compensatory increase in total PKA activity, in the RII β KO mice, hippocampal function is impaired, suggesting a unique role for RII β in synaptic plasticity (*Amieux et al., 1997*). A targeted disruption of the RII α gene yields viable mice with no physiological abnormalities, implying that PKAI and/or PKAII β compensates for the RII α defect (*Burton et al., 1997, 1999*). Targeted disruption of the mouse RII β gene has additional physiological consequences. The mutant mice are lean and have elevated metabolic rates caused by increases in both basal PKA activity and the basal rate of lipolysis (*Cummings et al., 1996; McKnight et al., 1998*). RII β KO mice also display defects in neuronal gene expression, learning and behavior (*Adams et al., 1997; Brandon et al., 1998*).

Living cells developed several regulation system to control the effects of the cAMP/PKA signaling pathway. Termination of cyclic-nucleotide signaling is achieved by PDEs, a superfamily of >70 different isozymes that degrade cAMP and cyclic GMP (cGMP). Distinctive tissue distribution, subcellular compartmentalization and differential regulation of these enzymes contribute to the establishment of local cAMP gradients by limiting the diffusion of cAMP that is generated by adenylyl cyclases (*Houslay et al., 2003*). The type-4 PDEs are a family of >16 distinct isoforms that have a conserved catalytic core. Divergence within the N-terminal region of PDE4 isoforms enables association

with various proteins and, therefore, differential subcellular targeting and regulation. Importantly, the PDE4D3 isozyme has been shown to be part of signaling complexes that target PKA.

The action of PKA, as with many other serine/threonine kinases, is counterbalanced by specific protein phosphatases. In some cases, it has been demonstrated that phosphatases belonging to the PP1 and PP2A families are responsible for dephosphorylation of PKA substrates. In turn, PKA can control phosphatase activity by phosphorylation of specific PP1 inhibitors, such as I-1 and DARPP32 (*Fimia et al., 2000*).

PKA is targeted at specific intracellular microdomains through interactions with A-Kinase-Anchor-Proteins (AKAPs). AKAP forms a local transduction unit, which includes different signalling/metabolic enzymes, receptors, ion channels, adaptor molecules and mRNAs. In this context, the spatio-temporal kinase activation provides a control mechanism to direct, integrate and locally attenuate the cAMP cascade.

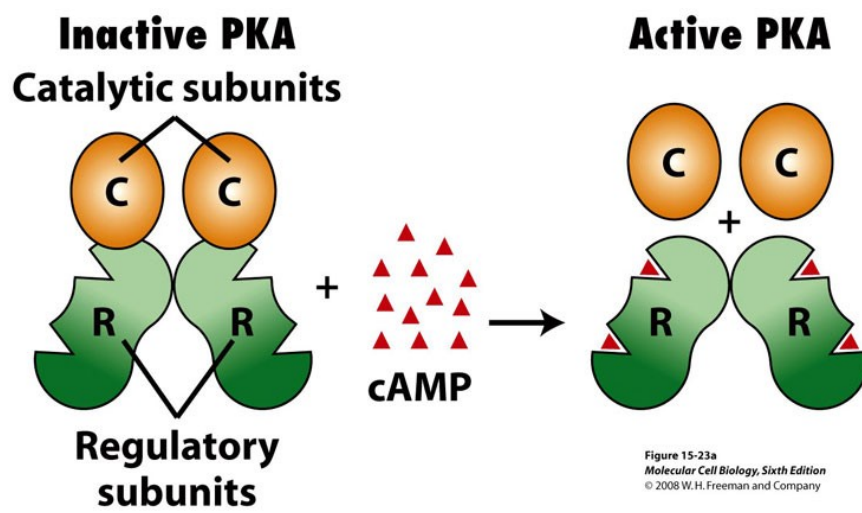


Figure 2. PKA molecular structure and activation mechanism.

1.3 AKAP proteins

Subcellular targeting through association with anchoring proteins has emerged as an important mechanism by which the cells localize signalling enzymes to sites where they can be accessed optimally by activators and, in turn, interact with particular substrates. The PKA is concentrated in particulate membranes and cellular organelles through interaction with a family of non-enzymatic scaffold proteins: AKinase Anchor Proteins (AKAPs) (*Rubin, 1994; Edwards & Scott, 2000; Dodge & Scott, 2000*).

AKAPs are a group (> 50 proteins) of functionally, rather than structurally, related proteins and each contains a common RII-binding site formed by 14–18 amino acid amphipathic aligned along one face of the helix and charged residues and the other side, that bind amino termini of PKA-RII dimer (*Carr et al., 1991; Newlon et al., 1999*) (**Fig. 3**). The first 30 amino acid residues of RII participate in AKAP binding, as shown by site-directed mutagenesis, biochemical analysis and solid-phase binding assays (*Li & Rubin, 1995; Hausken et al., 1994, 1996*). These residues also promote dimerization of RII subunits, which is a prerequisite for binding to AKAP. Although most AKAPs that have been characterized to bind to RII subunits with high affinity, several AKAPs have been reported to interact specifically with RI.

RII subunits bind to AKAPs with nanomolar affinity, by contrast, RI subunits bind to AKAPs with only micromolar affinity. However D-AKAP1 and D-AKAP2 are examples of dual-specificity AKAPs that can anchor both types of R subunit (*Huang et al., 1997; Wang et al., 2001*).

Structural data indicate that there is a single region of multiple contact sites between the RII subunit dimer and the AKAP, which presumably for the high-affinity interaction. Functionally, this suggests that the AKAP–PKA complex is likely to be a constitutive interaction in cells and not subject to regulation. However, the distribution of the PKA holoenzyme can be altered upon induction of AKAP expression. Thereby, regulation of PKA localization might be a function of AKAP targeting.

Each AKAP also contains a subcellular targeting domain that restricts its localization within the cell. A combination of subcellular-fractionation and immunohistochemical studies have identified AKAPs in association with a variety of cellular compartments, including centrosomes, dendrites, endoplasmic reticulum, mitochondria, nuclear membrane, plasma membrane and vesicles. Although AKAPs have been defined on the basis of their interaction with PKA, an additional feature of many of these molecules is their ability to bind to other signalling enzymes, such as protein phosphatases and kinases. AKAPs form a multiproteic complex with the presence of signal transduction and signal termination enzymes in the same network. This

creates focal points of enzyme activity where the bidirectional regulation of signaling events can be controlled and the phosphorylation status of target substrates is precisely regulated. (*Feliciello et al., 2001*)

AKAP forms a "transduceosome" by acting as an autonomous multivalent scaffold that assembles and integrates signals derived from multiple pathways. The transduceosome amplifies cAMP and other signals locally and, by stabilizing and reducing the basal activity of PKA, it also exerts long-distance effects. The AKAP transduceosome thus optimizes the amplitude and the signal/noise ratio of cAMP-PKA stimuli travelling from the membrane to the nucleus and other subcellular compartments (*Feliciello et al., 2001*).

Importantly, besides kinase and phosphatase, reports have demonstrated that phosphodiesterases, the enzymes that catalyze cAMP metabolism, are present in complex with AKAP and PKA. These findings add a novel twist to PKA regulation, as they indicate that an anchored pool of phosphodiesterase may tightly control local cAMP levels.

AKAPs, anchoring the PKA in close proximity of the substrates, optimize the PKA-dependent phosphorylation of a plethora of cellular substrates.

Cells that express high levels of membrane-bound PKA are more sensitive to the cAMP increase (*Feliciello et al., 1998, Feliciello et al., 2000*). This might be explained by the fact that membrane-bound PKA is more stable than the

cytosolic enzyme (*Feliciello et al., 1996*). PKA levels are higher in cells containing a high fraction of membrane-bound PKA, and the effects of PKA activation are more readily transmitted from the point of cAMP generation at the cell membrane (*Lester et al., 1997; Paolillo et al., 1999; Cassano et al. 1999*).

This interaction between AKAPs and cAMP, defines a positive regulatory loop in which the second messenger stimulates AKAP expression that in turn enhances cAMP dependent signaling pathway. In this way anchor proteins are involved in a potent regulatory mechanism that, coordinating and integrating several cellular processes, controls the specificity of signal transduction.

In higher eukaryotes, essential functions such as neurite outgrowth and morphogenesis, synaptic transmission, hormones production and release, require tightly regulated response to PKA stimulation.

S-AKAP84	E	I	K	R	A	A	F	Q	I	I	S	Q	V	I	S	E	A	T
Ht-31	L	I	E	E	A	A	S	R	I	V	D	A	V	I	E	Q	V	K
AKAP350	V	E	E	K	V	A	A	A	L	V	S	Q	I	Q	L	E	A	V
AKAP150	L	L	I	E	T	A	S	S	L	V	K	N	A	I	E	L	S	V
AKAP95	T	P	E	E	V	A	A	E	V	L	A	E	V	I	T	A	A	V
YOTIAO	R	L	E	E	E	V	A	K	V	I	V	S	M	S	I	A	F	A
AKAP-KL	P	L	E	Y	Q	A	G	L	L	V	Q	N	A	I	Q	Q	A	I
MAP-2	T	A	E	E	V	S	A	R	I	V	Q	V	V	T	A	E	A	V
EZRIN	S	Q	E	Q	L	A	A	E	L	A	E	Y	T	A	K	I	A	L
Consensus	X	(L)	X	X	X	(A)	X	X	(I)	(V)	X	X	(V)	(I)	X	X	(A)	(V)
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

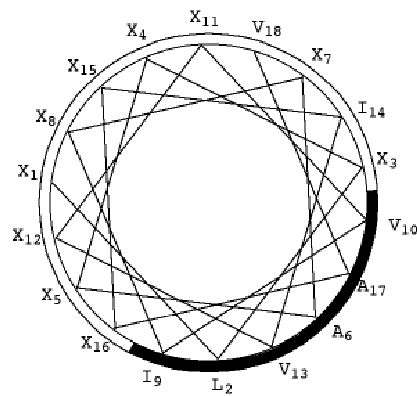


Figure 3. Consensus sequence of AKAP-RII-binding domains.

Consensus sequence derived from the alignment of the primary sequences of several AKAPs (upper). The amphipathic helical wheel and the residues forming it are depicted as a thick line (lower).

1.4 Pericentriolar Material 1 (PCM1)

In mammalian cells, the centrosomes are most important because it is a major microtubule organizing center (MTOC). A centrosome is composed of two orthogonal centrioles, which are surrounded by an electron dense amorphous cloud of pericentriolar matrix, often abbreviated as "PCM". The PCM is essential for nucleation and organization of microtubules. Centriolar satellites are non-membranous 70–100 nm granules scattered around the centrosome of many types of animal cells, and similar structures also exist around the basal bodies (equivalent to the centrosome) of ciliated cells. PCM1 was identified as the major component of centriolar satellites. PCM1 plays a role in the recruitment of centrosomal proteins including centrin, pericentrin and ninein, and it is required for the organization of the cytoplasmic microtubule network (Kim et al., 2008). PCM1 granules are formed by self aggregation of PCM1 in a cell cycle-dependent manner. PCM1-positive granules were not necessarily concentrated around centrioles but are scattered throughout the cytoplasm. PCM1 granules disappear during mitosis, re-appearing when the cells proceed into interphase (Kubo et al., 2003).

PCM1 gene is localized on the short arm of human chromosome 8 (8p22-p21.3). PCM1 gene generates four known transcripts, the longest of which has 39 exons. The 6.8 Kbp transcript encodes for a protein of 2024 residues, with

an estimate weight of 228 Kda (Balczon et al., 1994). PCM1 protein contains coiled coil regions between areas of low complexity as well as an adenosine triphosphate(ATP)/GTPase domain, a nuclear localization domain and an eukaryotic molybdopterin domain. The centriole, when present, influences the organization of PCM1. For example, experimental removal of the centriole causes the PCM1 to disperse (Bobinnec et al., 1998), whereas reduction in the size of the centriole alters the amount of PCM1 associated with the centrosome (Kirkham et al., 2003). There is a specific subset of PCM1 proteins that are positioned along the surface of a mature centriole, integrating the PCM1 with the centriole. The spatial distribution of these proteins is retained throughout interphase, thus producing a stable protein array within the PCM at the surface of the centriole. PCM1 proteins at the surface of the centriole have a distribution that is directly related to the polar nature of the centriole.

The manner in which PCM1 proteins are placed around the centriole integrates PCM1 function with centriole function. Centriole duplication and PCM1 assembly (at least in the vicinity of the centriole) are coordinated, and one of the hallmarks of centrosome duplication is the precise assembly of PCM1 proteins along the surface of the centriole (Young et al., 2004). PCM1 forms a complex with DISC1 and BBS4 through discrete binding domains in each protein. DISC1 and BBS4 are required for targeting PCM1 and other cargo proteins, such as ninein, to the centrosome in a synergistic manner. In

the developing cerebral cortex, suppression of PCM1 leads to neuronal migration defects, which are phenocopied by the suppression of either DISC1 or BBS4 and are exacerbated by the concomitant suppression of both proteins. Furthermore, a nonsense mutation that segregates with SZ spectrum psychosis was found in 1 family.

The centrosomal proteins have an important role in cortical development and the perturbation of centrosomal function contributes to the development of mental diseases, including SZ. (Kamiya et al., 2008). Functions of the PCM1 include microtubule nucleation from γ -tubulin ring complexes (Raynaud-Messina et al., 2007), and influencing the control of centriole number (Loncarek et al., 2008). The expansion in size and microtubule nucleating capacity of the PCM1 during mitosis is influenced by a growing list of proteins, whose depletion or inactivation has been shown to interfere with centrosome maturation (Mahen et al., 2011).

1.5 PCM1 and ciliogenesis

PCM1 is essential role for the ciliogenesis. Primary cilium is an important cell organelle that assembles on basal body and projects from cell surface, and plays critical roles in sensation and cellular signal transduction owing to the enrichment of receptors and channels on ciliary membrane (Berbari et al., 2009; Singla and Reiter, 2006). The defects of primary cilia function lead to numerous cilium-related human diseases, such as Bardet-Biedl Syndrome (BBS), Joubert Syndrome(JS), retinal degeneration, polycystic kidney disease and other disorders (Ishikawa and Marshall, 2011). Primary cilia assemble when cells exit from cell cycle and enter quiescence, while the cilia of proliferating cells often exhibit dynamic probably due to the fact that cilia disassembly liberates centrioles to undergo duplication and separation for bipolar spindle assembly, although centriole duplication can also take place while the mother centriole still acts as the ciliary basal body (Fonte et al., 1971; Quarmby and Parker, 2005; Rieder, 1979; Seeley and Nachury, 2010; Uetake et al., 2007).PCM1 recruits PLK1 to the pericentriolar matrix and promotes primary cilia disassembly before mitotic entry (Wang et al., 2012). In the pericentriolar satellites regions, there are the BBS family proteins, in particular BBS4. BBS4 participates in ciliogenesis through interaction with PCM1 (Kim et al., 2004; Nachury et al., 2007). JS is characterized by mutations

of CEP290 gene. CEP290 binds to PCM-1 and localizes to centriolar satellites in a PCM1 and microtubule-dependent manner. Depletion of CEP290 disrupts subcellular distribution and protein complex formation of PCM1. Down-regulation of CEP290 disorganizes the cytoplasmic microtubule network. Moreover, both CEP290 and PCM-1 are essential regulators of ciliogenesis and are both involved in the ciliary targeting of Rab8, a small GTPase shown to collaborate with BBS protein complex to promote ciliogenesis (Kim et al.,2008).

1.6 NimA related kinase 10 (Nek10)

Early studies in lower Eukaryotes have defined a role for the members of the NimA related kinase (Nek) family of protein kinases in cell cycle control(**Fig.4**). Expansion of the Nek family throughout evolution has been accompanied by their broader involvement in checkpoint regulation and cilia biology. Mutations of Nek family members have been identified as drivers behind the development of ciliopathies and cancer. Recent advances in studying the physiological roles of Nek family members using mouse genetics and RNAi-mediated knockdown are revealing the intricate associations of Nek family members with fundamental biological processes.

Nek protein kinases began to emerge as important regulators of the eukaryotic cell cycle, both during normal cell cycle progression and in response to genotoxic stress. NimA consists of an N-terminal catalytic domain, coiled-coiled domains, which mediate oligomerization, and PEST sequences, which participate in ubiquitin dependent proteolysis. NimA kinase activity exhibits a preference for N-terminal hydrophobic residues and a phenylalanine at position -3 relative to the phosphorylated residue (FR/KR/KS/T, target residue underlined). Despite low overall sequence homology, the organizational features of NimA are broadly conserved among mammalian Nek kinases. For instance, all Nek kinases except Nek10 contain

N-terminal catalytic domains, whereas Nek4, 6 and 7 are the only family members that do not contain coiled-coiled motifs. Moreover, 6 of 11 mammalian Nek kinases contain putative PEST sequence. Outside regions of homology, certain Nek kinases contain unique protein domains that point to the acquisition of novel functions relative to the ancestral NimA protein. Additional unique domains in Nek family members include a predicted DEAD-box helicase-like domain in Nek5 and a cluster of armadillo repeats in Nek10. In addition to the established functions during mitosis, certain Nek kinases also participate in cell cycle regulation following genotoxic stress. All eukaryotic cells have multiple molecular mechanisms to identify and repair damaged DNA and preserve genomic integrity. An important aspect of this process is the activation of a checkpoint with concomitant induction of cell cycle arrest, that would favor repair damaged DNA. Cell cycle arrest can be triggered at G1/S, intra-S and at G2/M phases of the cell cycle following damage caused by endogenous sources, such as stalled replication forks, or by exogenous agents, including ultraviolet (UV) radiation, ionizing radiation (IR), reactive oxygen species (ROS) and certain chemotherapeutic agents. Upon successful repair, the cell will re-enter the cell cycle.

Nek10, a previously uncharacterized Nek family member, has uncovered its role in G2/M checkpoint control. In response to UV irradiation, HEK293 and MCF10A cells depleted for Nek10 displayed an impaired G2/M arrest.

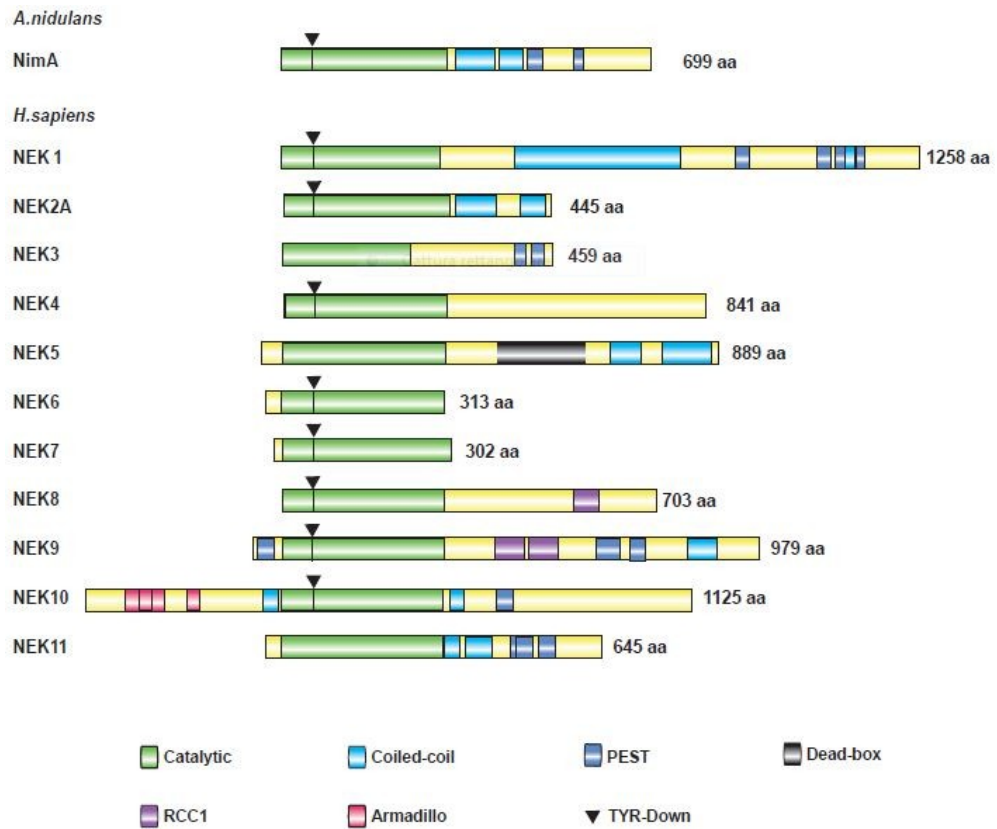


Figure4. Alignment of the key structural features of the 11 mammalian NIMA-related kinases. The relative positions of significant motifs and regions are indicated (Moniz et al., 2011)

Nek10 gene localizes on the short arm of human chromosome 3 (3p24.1). This gene encodes for fourteen transcripts, the longest of which has 39 exons. The 4.25 Kbp transcript encodes a protein of 1172 residues, with an estimate weight of 133 Kda. NEK10 is required for the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) signaling upon UV irradiation, but not in response to mitogens, such as epidermal growth factor stimulation (Moniz et al., 2011). Nek10 physically associated with Raf-1 and MEK1 in a Raf-1-dependent manner, and the formation of this complex was necessary for Nek10-mediated MEK1 activation. The appropriate maintenance of the G2/M checkpoint following UV irradiation required Nek10 expression and ERK1/2 activation, indicating a role for Nek10 in the cellular response to UV irradiation.

AIM OF THE STUDY

By yeast-two hybrid screening, we have identified PCM1 as a novel AKAP that binds a targets PKA in specific subcellular compartments. We also found that PCM1 binds the NIMA-related kinase10 (NEK10). A functional PCM1-based multikinase complex, which includes PKA and NEK10, was characterized and analyzed in mammalian cells. PCM1 is a scaffold protein mostly localized in centriolar satellites and in electron dense granules with a size ranging between 70 and 100 nm in diameter. PCM1 was initially identified for its cell cycle-dependent association with the centrosome complex and microtubules. Functional experiments revealed that PCM1 is essential for cell division and microtubule dynamics, playing a critical role in mitosis and ciliogenesis. Whether PCM1 could be involved in the regulation of PKA and NEK10 signaling at the pericentriolar region and centrosomal area was unknown. During my PhD program, I attempted to address the role of PCM1 as scaffold protein that senses, transmits and integrates signals carried out by PKA and NEK10. Accordingly, the principal aims of my PhD thesis were the following:

- ❖ identify PCM1 as a novel PKA anchor protein (AKAP)
- ❖ map the PKA binding determinants on PCM1
- ❖ characterize the PCM1-PKA complex in mammalian cells.
- ❖ identify NEK10 as novel partner of PCM1
- ❖ study the regulation of PCM1-NEK10 complex formation and stability by PKA
- ❖ determine the role of PCM1-kinase complex in the control of

ciliogenesis.

RESULTS

2.1 PCM1 and RII β form a stable complex

To identify novel PKA interacting proteins, a cDNA library was screened by yeast two-hybrid using mouse full-length RII β as bait. One positive clone encoding for an open reading frame corresponding to human PCM1 was isolated. We investigated whether endogenous PCM1 and RII β subunit interact in cell extracts. Lysates from HEK293 cells were immunoprecipitated with anti-RII β or non-immune IgG. Precipitates were immunoblotted with anti-RII β or anti-PCM1 polyclonal antibody. As shown, we detected an endogenous PCM1-RII β complex (**fig. 5A**). To confirm the binding between PCM1 and RII β subunits, HA-tagged PCM1 and Myc-tagged RII β were co-expressed in HEK293 for 24 hours. As control, we used an empty vector (CMV). Total lysates were immunoprecipitated with anti-Myc antibody. Precipitates were immunoblotted with anti-RII β or with anti-PCM1 polyclonal antibody. The data confirmed the existence of a PCM1-RII β complex in cell lysates (**Fig. 5B**).

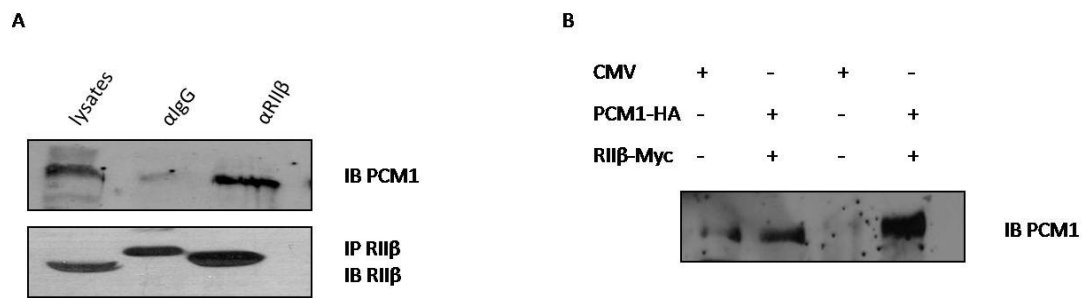


Figure 5. PCM1 binds PKA RIIβ subunit.(A)Lysates (2mg) from HEK293 cells were immunoprecipitated with anti-RIIβ or non-immune IgG. Precipitates were immunoblotted with anti-RIIβ or anti-PCM1 polyclonal antibody.(B) CMV or PCM1-HA and RIIβ-Myc were transiently transfected in HEK293 cells. Lysates were immunoprecipitated with anti-Myc and immunoblotted with anti-PCM1.

2.2 Endogenous PCM1 and RII β colocalize in Human Embryonic Kidney 293 cells.

To demonstrate that PCM1 and RII β are located within the same compartment, we analyzed the localization of both proteins in Human Embryonic Kidney 293 cells (HEK293). To this end, we performed double immunofluorescence using anti-PCM1 and anti-RII β antibodies. As suspected, the results indicate that PCM1 and RII β , in part, colocalize at the Golgi-centrosome region and pericentriolar matrix (**Fig. 6**).

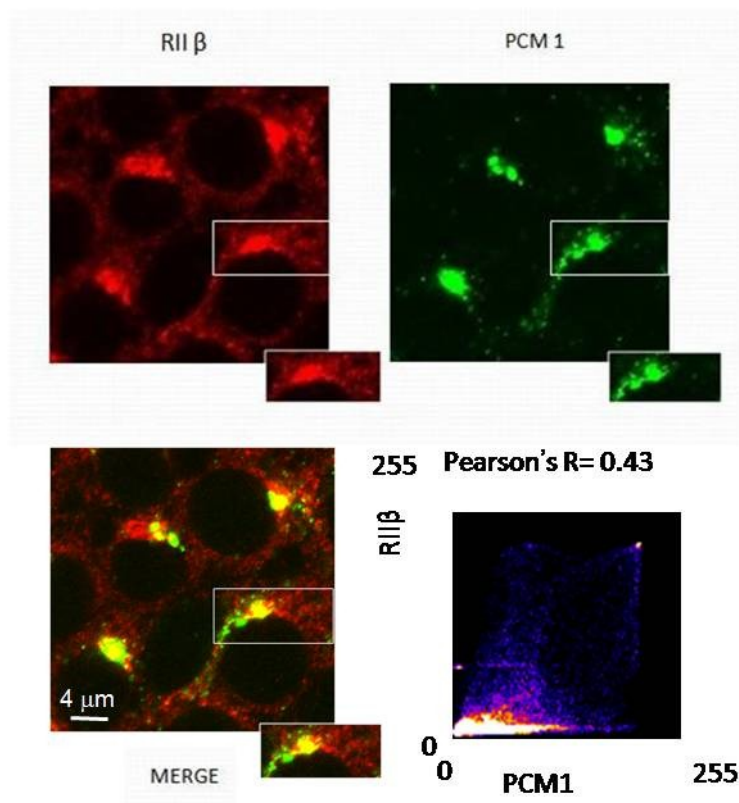


Figure 6. PCM1 and RIIβ colocalize in Human Embryonic Kidney cells (HEK293). HEK293 were subjected to double immunofluorescence with rabbit polyclonal anti-PCM1 and mouse monoclonal anti-RIIβ antibodies. Immunostaining showing colocalization of PCM1 and RIIβ in human embryonic kidney cells HEK293. Colocalization Pearson's coefficient PCM1-RIIβ = 0.43. Scale bar: 20 μm.

2.3 Mapping the PKA binding domain on PCM1

The data above demonstrate that PCM1 and RII β form a stable complex in lysates and partly co-localize within the same subcellular compartment. To identify the domain of PCM1 that mediates the binding to RII β , we used vectors encoding for distinct segments of PCM1. To trace the recombinant polypeptides, the hemoagglutinin epitope (HA) was placed at the NH₂-terminus of each construct. The HA-tagged PCM1 polypeptides used are the following: PCM1(741-1420)-HA, PCM1(1-740)-HA and PCM1(1421-2024)-HA. Recombinant polypeptides were co-expressed with Myc-RII β in HEK293 cells for 24 hours. Lysates were immunoprecipitated with goat anti-RII β . Precipitates were immunoblotted with anti-RII β or anti-HA monoclonal antibody. We found that residues 741 to 1420 of PCM1 are sufficient for optimal binding to RII β (**Fig. 7A**).

To further map the PKA binding domain on PCM1, we generated three polypeptides spanning distinct subdomains of the PCM1(741-1420)-HA construct: mid1-HA (residues 741-1007), mid2-HA (residues 967-1233) and mid3-HA (residues 1193-1420). The polypeptides were transiently expressed in HEK293 and the lysates subjected to cAMP-affinity purification assays. The results revealed that mid1 and mid3 segments both retain the ability to bind RII β (**Fig. 7B**). This suggests the presence of two distinct RII β binding motifs on the middle segment of PCM1.

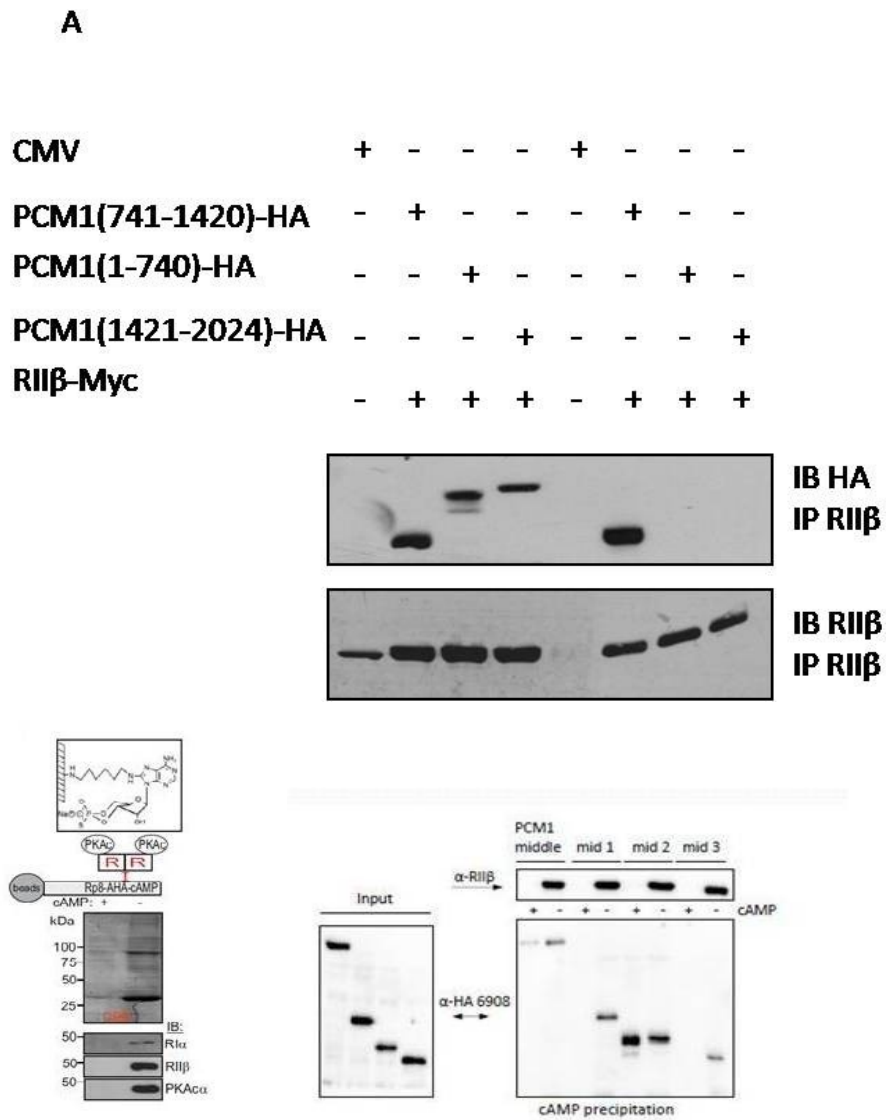
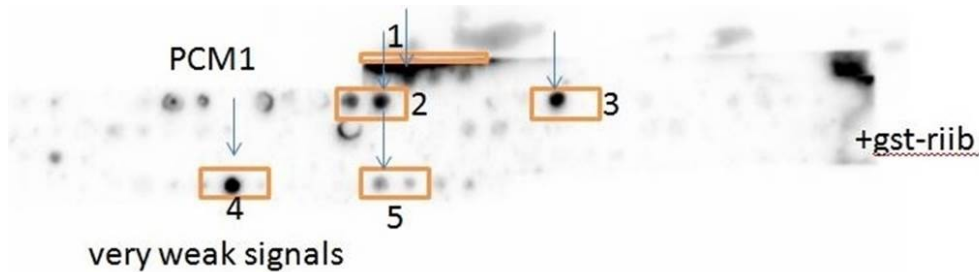


Figure 7. Mapping the RII binding domain on PCM1. A. HA-tagged recombinant fragments of PCM1-HA (741-1420, 1-740, 1421-2024) and RII β were transiently transfected in HEK293 cells. Lysates subjected to immunoprecipitation with anti-RII β . The precipitates were immunoblotted for RII β and HA. **B.** cAMP-agarose affinity purification of PKA complexes using lysates from HEK293 cells transiently transfected with HA-tagged PCM1 constructs and RII β -Myc. The specificity of the binding to cAMP-beads was verified by competing the PKA binding to the cAMP-agarose beads with high concentrations of free, uncoupled cAMP.

2.4 PCM1 is an A-Kinase Anchor Protein

To map the RII β -interacting motif on PCM1, overlapping 25-mer peptides derived from human PCM1 were spotted onto a membrane and overlaid with purified GST-RII β fusion protein, as previously described (*Lignitto et al., 2011, 2013*) We identified five potential binding motifs located at the middle segments of PCM1(**Fig. 8**). Computational analysis of the RII β binding peptides indicates the presence of potential amphipathic α -helical wheels that may coordinate the binding to R subunits(**Fig.8**), as it occurs in prototypical AKAPs.



1-LQEERKKLIDIQEKIQALQTACPDL
 2-TQCALDEEGDEDGYLSEGIVRTDEE
 3- DASSNDNFVCPNSVNHNSYNGKE
 4- ASMSSTCEPCKSRNRHSAQTEEPVQ
 5-AKVFSRKNHEQLEKIIKCNRSTEIS

7LIDIQEKIQALQTACPDL ₂₄		
Physico-chemical properties	Polar residues + GLY	Nonpolar residues
Hydrophobicity $\langle -H \rangle$ 0.545	Polar residues + GLY (n / %) 8 / 44.44	Nonpolar residues (n / %) 10 / 55.56
Hydrophobic moment $\langle -\mu H \rangle$ 0.443	Uncharged residues + GLY GLN 3, THR 1, GLY 0	Aromatic residues
Net charge $\langle - \rangle$ -2	Charged residues LYS 1, GLU 1, ASP 2, Hydrophobic face : none	Special residues CYS 1, PRO 1
Go to screening	Manual mutation	GA mutation

Click to enlarge

Figure 8. Identification of the RII β binding motif on PCM1. Overlapping 25-mer peptides derived from human sequence of PCM1 were spotted onto membranes and overlaid with purified GST-RII β fusion protein. The bound GST-RII β was visualized by immunoblotting the membranes with anti-GST antibody (upper panel). Positive signal present on duplicate membranes were further characterized. The sequence of the RII β binding peptides and the putative α -helical wheels are shown (lower panels)

2.5 PCM1 silencing redistributes endogenous RII β subunits

Next, we attempted to demonstrate that PCM1 is, indeed, mediating the localization of RII β at the Golgi-centrosome compartment. To this end, we monitored the localization of RII β in cells deprived of endogenous PCM1. HEK293 cells were transfected with control siRNAs or with siRNAs targeting endogenous PCM1. Forty-eight hours from transfection, cells were subjected to double immunofluorescence analysis with anti-PCM1 and anti-RII β antibodies. As shown in **Fig. 9**, downregulation of PCM1 diffusely redistributed RII β subunits from the Golgi-centrosome area to the cytoplasm, compared to controls (**Fig. 9**). These findings strongly suggest that PCM1, acting as an AKAP, positions PKAII β at the PCM area.

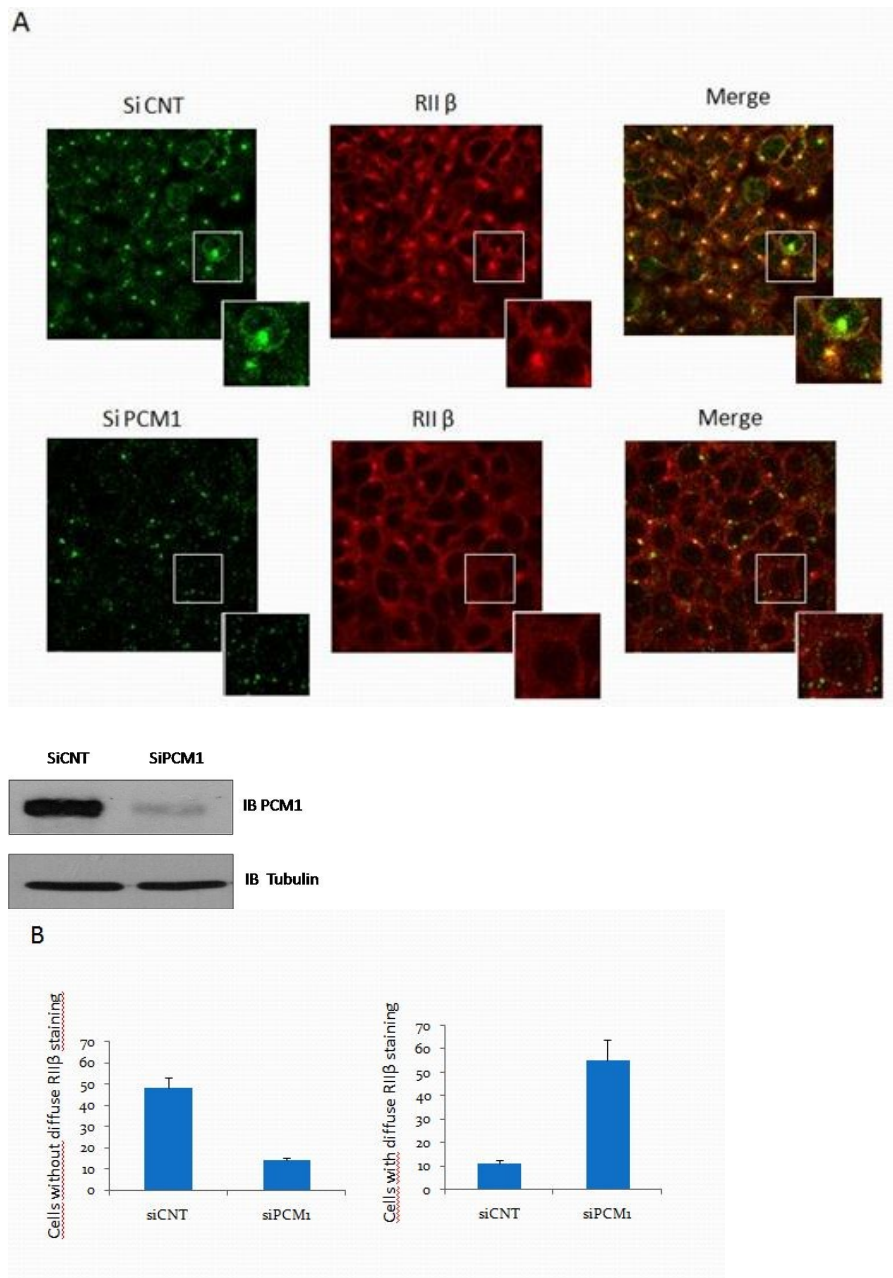


Figure 9. Silencing of PCM1 delocalizes RII β from the PCM area to the cytoplasm. **A.** HEK293 were transiently transfected with control siRNAs (SiCNT) or with siRNA targeting human PCM1 (SiPCM1). 24 hours from transfection, cells were fixed and doubly immunostained with anti-RII β and anti-PCM1 antibodies. Fluorescent images were collected and analyzed by confocal microscopy. **B.** Quantitative analysis of the experiments shown in A. The levels of PCM1 in siRNA transfected cells are shown.

2.6 PCM1 interacts with NimA related kinase 10, Nek10

Members of the NEK protein kinases have been implicated in the regulation of various aspects of centrosome stability, microtubule dynamics and cell cycle progression. In particular, NEK2 is a cell cycle-regulated protein required for centrosome structure at the G2/M transition. Colocalization studies revealed that NEK2 colocalizes with PCM1-containing centriolar satellites and depletion of PCM1 interferes with centrosomal recruitment of NEK2 (Hames et al., 2005). Another member of NEK family, NEK10, is also activated at G2/M transition and its activity is required for proper cell cycle progression. All this led us to investigate if NEKs, and in particular NEK10, might interact to PCM1. This mechanism would position another ser/thr kinase in close proximity of PKA within the same scaffold complex. NEK10-Flag vector was transiently transfected in HEK293 cells. Lysates were immunoprecipitated with anti-flag antibody or with non-immune IgG, and the precipitates were immunoblotted with anti-PCM1 and anti-flag antibodies. As shown in **figure 10**, PCM1 and NEK10 form a stable complex in cell lysates.

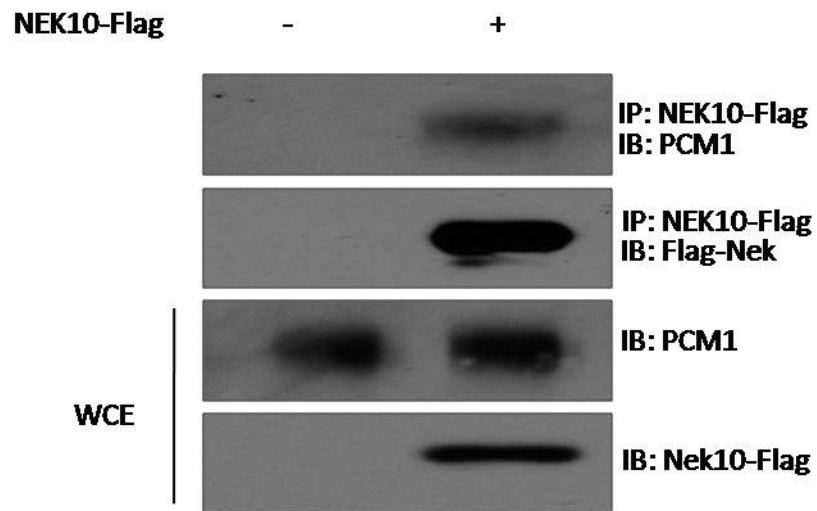


Figure 10. PCM1 interacts with NimA related kinase 10, Nek10. Lysates (2mg) from HEK293 cells transiently transfected with Flag-NEK10 were immunoprecipitated with anti-Flag or with non-immune IgG. Precipitates and a fraction (100 μ g) of lysates (WCE) were immunoblotted with anti-Flag or with anti-PCM1 polyclonal antibody.

2.7 Mapping the NEK10 binding domain on PCM1

To identify the domain of PCM1 that mediates the binding to NEK10, we performed co-immunoprecipitation assays using the three segments of PCM1 (PCM1(741-1420)-HA, PCM1(1-740)-HA and PCM1(1421-2024)-HA), described above. The constructs were co-expressed with Flag-NEK10 in HEK293 cells for 24 hours. Lysates were immunoprecipitated with anti-Flag antibody. Precipitates were immunoblotted with anti-Flag and anti-HA antibodies. The results show that NEK10 binds the middle segment of PCM1 (**Fig. 11**).

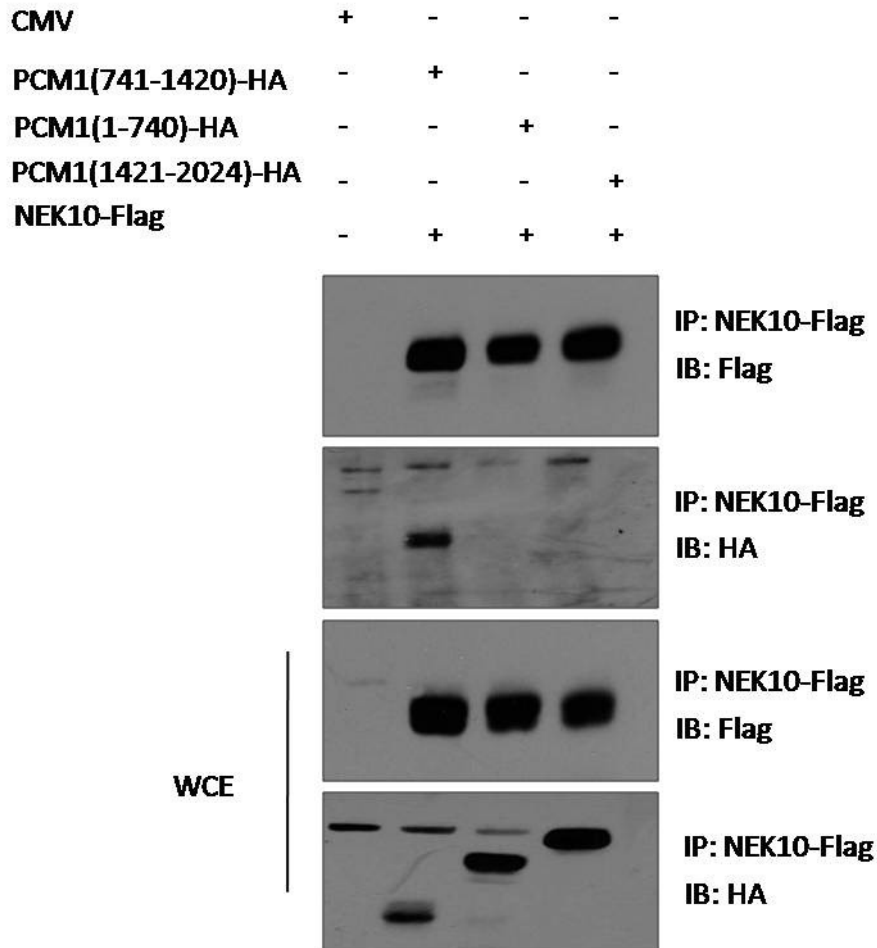


Figure 11. The segment spanning residues 741-1420 of PCM1 binds to NEK10. PCM1-HA constructs and NEK10-Flag were transiently transfected in HEK293 cells. Lysates were subjected to immunoprecipitation with Flag antibody. Precipitates and a fraction of lysates were immunoblotted with anti-Flag or anti-HA.

2.8. PCM1, NEK10 and RII β colocalize at the Golgi-centrosome region

Next, we demonstrated that PCM1, NEK10 and RII β are located within the same intracellular compartment. In particular, we analyzed the localization of the trimeric complex by triple immunofluorescence assay using anti-PCM1, anti-NEK10 and anti-RII β antibodies. As shown in **Fig. 12**, a colocalization signal of the three proteins could be detected at Golgi-centrosome region and pericentriolar matrix.

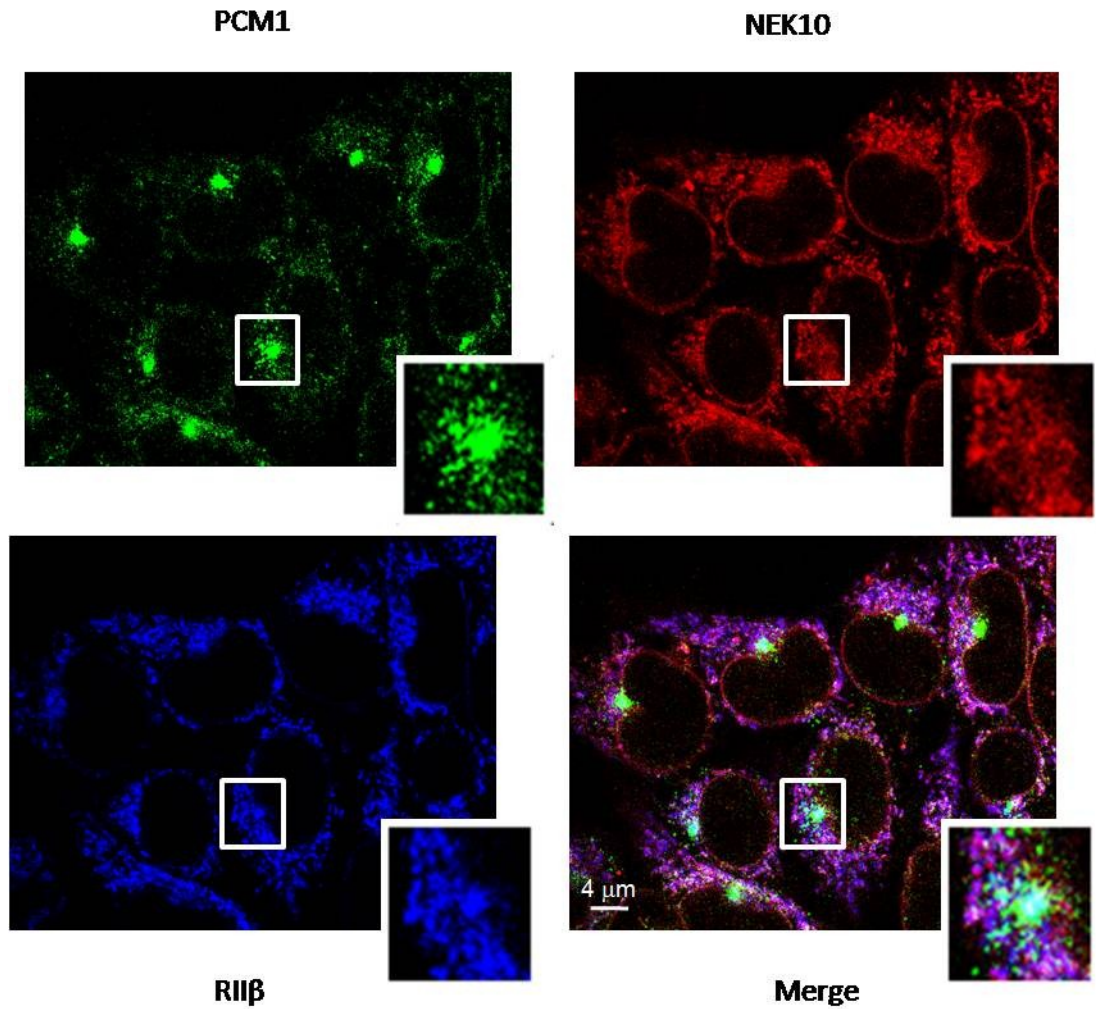
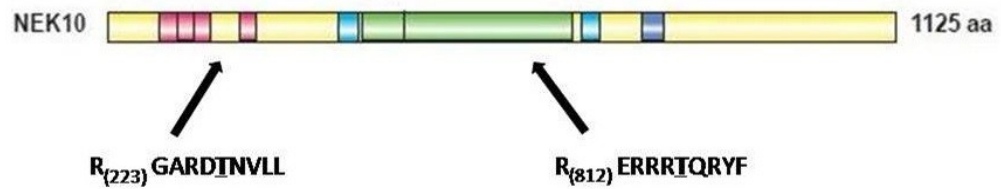


Figure 12. PCM1, NEK10 and RII β colocalize at the PCM region. HEK293 cells were subjected to triple immunofluorescence with rabbit polyclonal anti-PCM1, goat polyclonal anti-NEK10 and mouse monoclonal anti-RII β antibodies. A merge composite of the three fluorescent signals is shown.

2.9 PKA regulates phosphorylation and binding of NEK10 to PCM1

The data indicate that NEK10 and PKA interact with the middle segment of PCM1, suggesting a possible functional interaction between both kinases. Primary sequence analysis of NEK10 predicts the presence of two highly conserved PKA phosphorylation sites (Thr233 and Thr812) within the core region of the kinase (**Fig. 13A**). We postulated that PKA phosphorylates NEK10. We tested this hypothesis, by monitoring NEK10 phosphorylation by PKA in HEK293 cells transiently transfected with NEK10-flag and treated with forskolin, a cAMP elevating agent, for 30' and 60'. Lysates were immunoprecipitated with anti-Flag antibody and the precipitates were, then, immunoblotted with antibodies that recognized phosphoSer/Thr PKA sites. The data indicate that NEK10 is, indeed, phosphorylated *in vivo* by PKA (**Fig. 13B**).

A



B

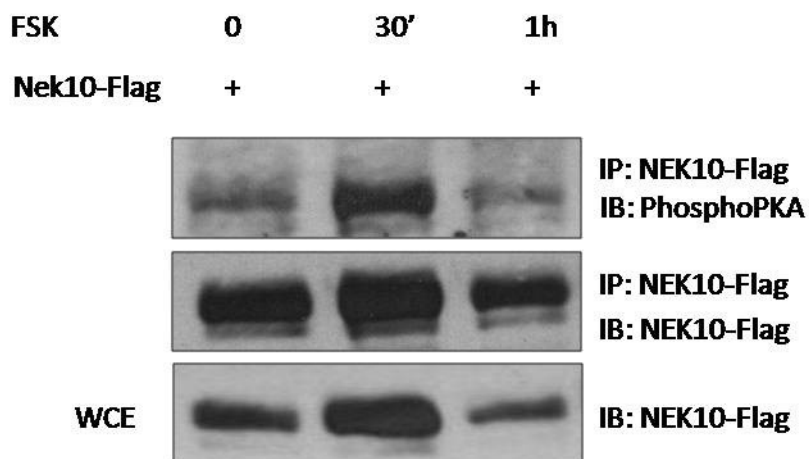


Figure 13. PKA phosphorylates NEK10. A. Schematic diagram of NEK10, including protein sequence surrounding the putative PKA consensus sites. Threonine 223 and Threonine 812 are underlined. B. HEK293 cells transiently transfected with NEK10-flag were serum deprived overnight and treated with forskolin (40 μ M) for 30 min and 1h. Lysates were immunoprecipitated with anti-flag antibody and immunoblotted for the detection of phosphoSer/Thr PKA substrates and flag antibodies.

Next, we analyzed if PKA phosphorylation of NEK modulates its binding to PCM1. To this end, lysates from HEK293 cells, transfected with NEK10-flag and treated with forskolin (15', 30', 1h), were immunoprecipitated with anti-Flag or non-immune IgG. Precipitates were immunoblotted with anti-Flag or anti-PCM1 polyclonal antibody. As shown in **Fig. 14**, PCM1-NEK10 complex formation was significantly affected by PKA stimulation.

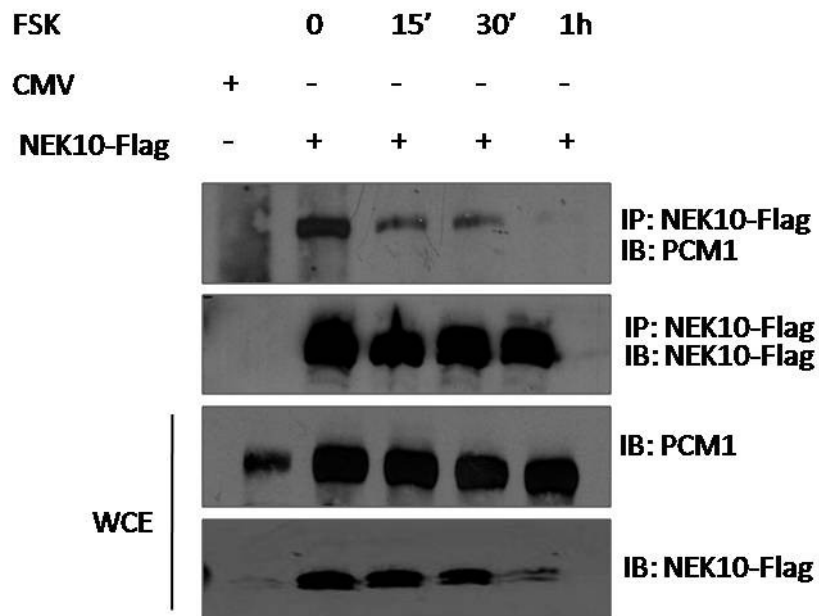


Figure 14. Regulated binding of PCM1 to NimA related kinase 10 (Nek10) by cAMP signaling. NEK10-Flag transiently transfected in HEK293 cells. Cells were serum deprived overnight and then treated with forskolin for 15', 30' and 1h. Lysates were immunoprecipitated with anti-Flag antibody and immunoblotted with anti-PCM1 or anti-Flag antibodies.

We noticed that activation of PKA by forskolin also reduces the levels of NEK10, more evident at the later time points from forskolin stimulation (see **Fig. 13B** and **Fig. 14**). The effects of forskolin on NEK10 levels were abrogated by pre-treating the cells with the proteasomal inhibitor MG132. Moreover, treatment with MG132 increases the levels of NEK10 in unstimulated cells, compared to control. Altogether, these findings suggest that PKA phosphorylation of NEK10 reduces its binding to PCM1, inducing proteolysis of NEK10 through the ubiquitin-proteasome pathway(**Fig.15**).

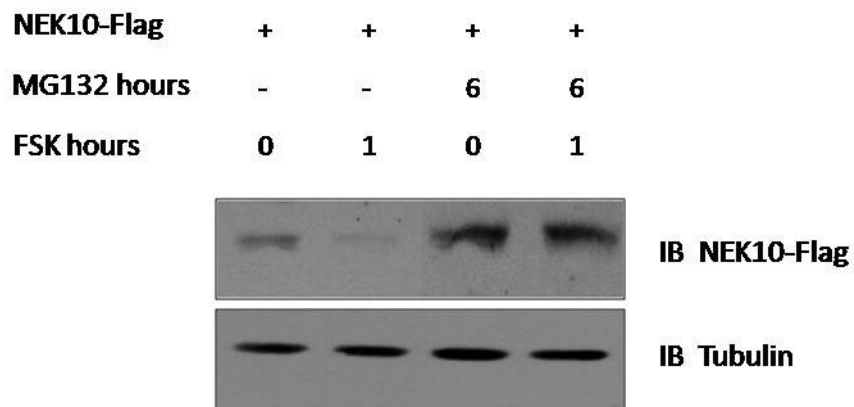


Figure 15. PKA stimulation induces proteolysis of NEK10 through the ubiquitin-proteasome pathway. HEK293 cells were transiently transfected with NEK10-Flag, 24 hours from transfection, cells were treated for 6 hours with MG132 (10 μ M) and then, left untreated, or stimulated with forskolin for 1 hour before harvesting. Lysates were immunoblotted with anti-Flag or anti-Tubulin antibodies.

2.10 NEK10 controls primary cilium formation

Our findings indicate that NEK10 is a novel component of the PCM1-PKA complex and it is also a PKA substrate in living cells. Therefore, we asked if NEK10 contributes to the primary cilium assembly. To this end, HEK293 cells were transiently transfected with siRNA targeting endogenous NEK10 and serum deprived for 36 hours. The presence of cilium was revealed by using an antibody directed against the acetylated tubulin. This is a modified variant of tubulin that selectively accumulates along the cilium. **Figure 16** shows that, in control (siRNAc) cells, serum deprivation significantly increased the number of primary cilia. By contrast, genetic knock-down of NEK10 drastically reduced both the length of primary cilia. As expected, re-expression of NEK10 reversed the effects of the siRNA-NEK10, indicating that NEK10 is, indeed, a biologically relevant player of ciliogenesis.

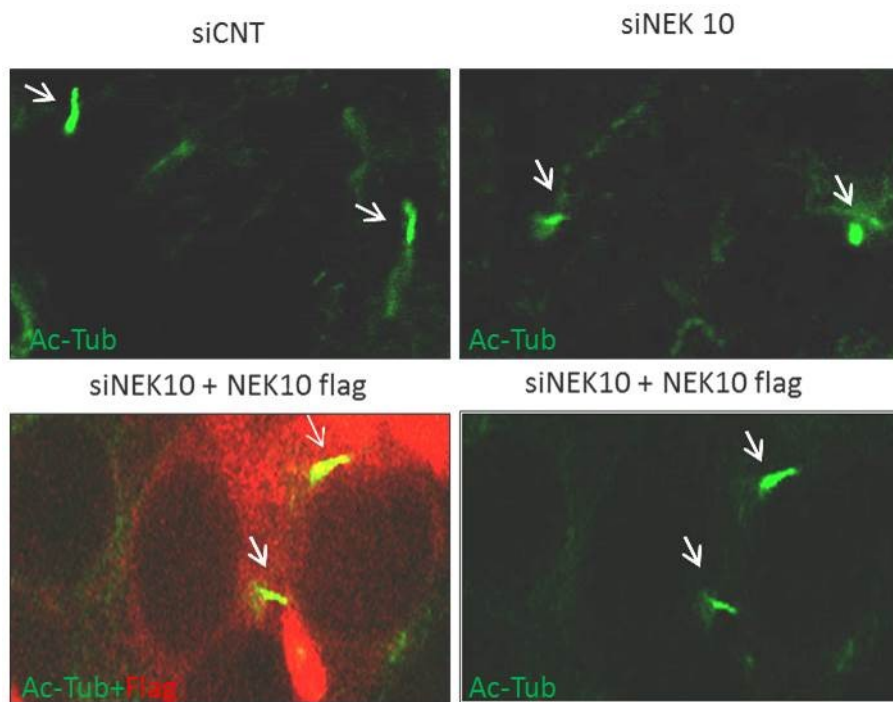


Figure 16. NEK10 controls primary cilium formation. HEK293 were transiently transfected with control siRNA (siCNT) or with siRNA targeting human NEK10 (siNEK10). 24 hours from transfection, cells were serum deprived for 36 hours to induce cilium formation. Cells were fixed and subjected to immunostaining analysis with anti-acetylated alpha tubulin and with anti-Flag antibodies (for NEK10-flag). Fluorescent images were collected and analyzed by confocal microscopy. Arrows indicate the primary cilia.

DISCUSSION

PCM-1 is a scaffold protein that binds different proteins and mostly localizes to small 70–100-nm granules in the cytoplasm of interphase cells (Balczon et al., 1994; Kubo et al., 1999; Li et al., 2001). These granules can move along microtubules in a dynein-dependent manner and they are often concentrated near the microtubule organizing center (Balczon et al., 1999; Kubo et al., 1999). Detailed morphological analysis revealed that the granules containing PCM1 are identical to structures previously described as “centriolar satellites” (Kubo et al., 1999). PCM1 is essential for cell division and microtubule dynamics, playing a critical role in different aspects of mitosis and ciliogenesis. The transition between centrosome and cilium, and the regulation of cell cycle are closely linked in normal dividing cells. Centrosomes, which are composed of two barrel-shaped centrioles surrounded by pericentriolar material, organize the cytoplasmic microtubule network, during interphase, and the bipolar spindle, during mitosis. In postmitotic cells, the centrosome migrates to the cell surface, and one of the centrioles differentiates into a basal body that nucleates microtubules to form a cilium. In normal proliferating cells, the presence of a cilium at the cell surface is transient and it is commonly observed in G1, disappearing during the progression through the cell cycle (Plotnikova et al., 2008).

Several proteins, like PKA and NEK2, have been involved in the assembly/disassembly of primary cilium. Localization of PKA at the base of

primary cilium is essential for cilium growth. At the base of the cilium, PKA negatively regulates hedgehog (Hh) signal transduction pathway, as it is required for the proteolytic processing event that generates the repressor forms of the Ci and Gli transcription factors, keeping target genes off in the absence of Hh. Displacement of PKA from the cilium by PKA-binding peptides that negatively impacts on primary cilium formation (*Barzi et al., 2010*). This strongly suggests the existence of a localized pool of PKA maintained at the base of cilium which targets Hh signaling during the essential steps of ciliogenesis. The question is: how cells maintain the high localized PKA activity at the cilium base? Here, we provide evidence that PCM1 by acting as an AKAP positions a pool of PKA (PKA-RII β) at the pericentriolar matrix, centrosome compartment and most likely at the base of cilium. Genetic knock-down of PCM1 delocalizes PKA from this area, diffusely distributing the kinase throughout the cytoplasm. The PKA binding domain was mapped within the middle segment of PCM1 (residues 741 to 1420). A combinatorial approach of computational analysis and *in vitro* binding assays allowed the identification of peptides mapping within the middle part of PCM1 that specifically interact with RII β . Some of the peptides are predicted to form amphipathic helical wheel that is a prerequisite for most of the PKA binding proteins. The biological role and contribution of the individual PCM1 peptides in PKA binding and ciliogenesis are currently under investigation.

Here, we also report that a member of the Nima-related kinases, NEK10, takes part of the PCM1 assembled complex at the pericentriolar matrix. NEK10 is a kinase that becomes activated at G2/M transition and its activity is required for proper cell cycle progression. We found that the middle segment of PCM1 also binds NEK10, juxtaposing the mitotic kinase in proximity of PKA. Our results show that PCM1, PKA and NEK10 form a trimeric complex at the same subcellular compartment, suggesting that PCM1 works as scaffold protein that receives and integrates signals from PKA and NEK10. Primary sequence analysis of NEK10 predicts the presence of two highly conserved PKA phosphorylation sites (Thr233 and Thr812) within the core region of the kinase. Accordingly, we found that PKA efficiently phosphorylates NEK10 in living cells. Phosphorylation of NEK10 by PKA reduces its binding to PCM1, indicating the existence of a highly dynamic regulation of the multikinase complex assembled by PCM1. Furthermore, we found that PKA activation leads to proteolysis of NEK10 through the ubiquitin-proteasome system, suggesting a potential antagonism between PKA activation and NEK10. Importantly, we found that genetic downregulation of NEK10 severely affected the process of ciliogenesis, reducing the length and the number of mature primary cilia. Our working model predicts that activation of PKA at the pericentriolar matrix, by modulating the localization and the levels of

NEK10, negatively interferes with NEK10-mediated events during microtubule dynamics and ciliogenesis.

In conclusion, we have identified a scaffold platform nucleated by PCM1 at the pericentriolar matrix and centrosome, which includes PKA and NEK10, that controls critical aspects of mammalian ciliogenesis. Within the PCM1 complex, PKA controls NEK10 localization and stability, efficiently coupling cAMP signaling to the ubiquitin-proteasome system (UPS). In this context, PCM1 acts as relay that senses, transmits and integrates cAMP and mitogenic signals generated at cell membrane, regulating biological events at the pericentriolar matrix area and primary cilium.

Identifying relevant scaffold complexes at specific sub-cellular compartments and understanding the role of each component of this multi enzyme signaling cascade in critical aspects of cell biology will undoubtedly contribute to the design of novel approaches and tools for the treatment of several human diseases.

METHODS

Cell lines. Human embryonic kidney cell line (HEK293) was cultured in DMEM containing 10% fetal bovine serum (FBS) supplemented with 2mM L-glutamine, 100 IU/ml penicillin, in an atmosphere of 5% CO₂ at 37°C.

Plasmids, siRNAs and transfection. Vectors encoding for wild type HA-PCM1, HA-PCM1(1-740aa), HA-PCM1(741-1420aa) or HA-PCM1(1421-2024aa) were kindly provided from Dr Kamiya (*Kamiya et al., 2008*); flag-NEK10 were kindly provided from Dr Stambolic (*Stambolic et al., 2011*); epitope Myc tagged RII β vectors were kindly provided by Dr Ginsberg SH (*Lim et al., 2007*). Three different subdomains, mid1-HA (741-1007aa), mid2-HA (967-1233a), mid3-Ha (1193-1420aa) were generated by PCR with specific oligonucleotide primers and subcloned into the same vector as HA-PCM1(741-1420aa) cDNA. SMART pool siRNAs targeting coding regions of distinct segments of PCM1 and NEK10 were purchased from Dharmacon and IDT, Integrated DNA Technologies. The siRNAs were transiently transfected using Lipofectamine 2000 (Invitrogen) at a final concentration of 100 pmol/ml of culture medium.

Antibodies and chemicals. Polyclonal antibodies directed against PCM1 were purchased from ABCAM and Cell Signalling, and used at working 1:1,000; rabbit polyclonal antibodies directed against phosphoPKA was purchased from Cell Signalling, and used at working dilution of 1:1000; goat antibodies directed against RII β was purchased from Santa Cruz at working dilution of

1:1000; monoclonal antibodies directed against RII β was purchased from BD Transduction and used at working 1:2,000; haemagglutinin epitope (HA) was purchased from Covance and used at working dilution of 1:1000; monoclonal antibodies directed against flag and myc epitope used at working dilution 1:3000, were purchased from Sigma; polyclonal antibodies directed against acetylated alpha tubulin was purchased from ABCAM. Forskolin was purchased from Sigma.

Cell lysates and Immunoprecipitation. Cells were washed twice with phosphate-buffered saline and lysed in a RIPA buffer (50mM TRIS–hydrogen chloride, pH 7.4, 150mM sodium chloride, 5mM magnesium chloride, 5mM dithiothreitol, 1mM ethylene diamine tetraacetic acid, 1% Triton X-100, containing aprotinin (5 μ g/ml), leupeptin (10 μ g/ml), pepstatin (2 μ g/ml), Na₃VO₄ and 1mM phenylmethylsulfonyl fluoride and protease inhibitors. The lysates were cleared by centrifugation at 15,000 g for 15 min. Cell lysates (2 mg) were immunoprecipitated in rotation at 4 °C overnight with the indicated antibodies. Pellets were washed four times in lysis RIPA buffer supplemented with NaCl (0.4 M final concentration) and eluted in Laemly buffer. An aliquot of whole cell lysates (WCE) (100 μ g) or immunoprecipitates were resolved on sodium dodecyl sulfate polyacrylamide gel and transferred on nitrocellulose membrane (Biorad, Milan, Italy) for 3 h. Filters were blocked for 1 h at room temperature in Tween-20 Phosphate buffer saline (TPBS) (PBS- Sigma, 0,1%

Tween 20, pH 7.4) containing 5% non-fat dry milk. Blots were then incubated O/N with primary antibody. Blots were washed three times with TPBS buffer and then incubated for 1 h with secondary antibody (peroxidase-coupled anti rabbit (GE-Healthcare) in TPBS. Reactive signals were revealed by enhanced ECL Western Blotting analysis system (Roche).

Immunofluorescence and confocal analysis. For immunofluorescence studies, HEK293 cells transiently transfected with the expression vectors were plated on poly-L-lysine (10 μ g/ml) coated glass coverslips. Cells were fixed with Paraformaldehyde for 20 minutes. After three washes, cells were immunostained with polyclonal antibodies directed against PCM1 was purchased from ABCAM, and used at working dilutions of 1:100; monoclonal antibodies directed against RII β was purchased from BD Transduction and used at working dilution of 1:400 monoclonal antibodies directed against Flag epitope used at working dilution of 1:400; polyclonal antibodies directed against acetylated alpha tubulin was purchased from ABCAM and used at working dilution of 1:100. Immunofluorescence was visualized using a Zeiss LSM 510 Meta argon/krypton laser scanning confocal microscope. Quantification of the immunofluorescent images and correlation (Pearson's) coefficient were calculated by Image-J software.

cAMP precipitation. β 2AR HEK293 cells transiently overexpressing PCM1-mid1-HA (741-1007aa), PCM1-mid2-HA (967-1233a) or PCM1-mid3-Ha (1193-

1420aa) fusion proteins have been treated with indicated stimuli, were lysed and subjected to precipitations with Rp-8-AHA-cAMP agarose resin (Biolog) for two hours. In this assay, binding of R subunits to the resin-coupled cAMP analogue (= PKA inhibitor Rp-cAMP, inhibits holoenzyme dissociation) results in re-association of activated PKAc and R subunits. To test the impact of cAMP on the complex formation of PKA•PCM1-mid1-HA (741-1007aa), PKA•PCM1-mid2-HA (967-1233a) or PKA•PCM1-mid3-Ha (1193-1420aa) we artificially elevated endogenous cAMP levels with forskolin or through activation of the stably expressed beta2-adrenergic receptor (β 2AR) by isoproterenol prior to cell lysis. As control we have added excess of cAMP (5mM) to mask the cAMP binding sites in the R subunits for precipitation. Resin associated complexes have been washed at least four times with the lysis buffer (10 mM sodium phosphate pH 7.2, 150 mM NaCl, 0,5% Triton X100 supplemented with standard protease inhibitors) and eluted with Laemmli sample buffer and subjected to immunoblot analysis.

Statistics Data were analysed using analysis of variance (ANOVA) for each region and *post hoc* repeated-measure comparisons (Least Significant Difference (LSD) test). Rejection level was set at $P < 0.05$.

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