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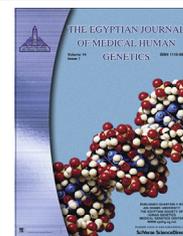
REVIEW

# Insight into Nek2A activity regulation and its pharmacological prospects

## Ambuj Kumar [a], Vidya Rajendran [a], Rao sethumadhavan [a], Rituraj Purohit [a,b,*]

[a] *Bioinformatics Division, School of Bio Sciences and Technology, Vellore Institute of Technology University, Vellore 632014, Tamil Nadu, India*
[b] *Human Genetics Foundation, Torino, Via Nizza 52, I-10126 Torino, Italy*

**Abstract** Nek2A is an essential component of cell cycle progression. It regulates the reorganization of the microtubule network at the G2/M transition and controls the centriole–centriole linkage of the cells entering mitosis. The overexpression of Nek2A coding gene has been widely reported in several cancer-associated disorders. In order to design a potent inhibitor and to control its expression mechanism, it is important to understand the structural orientation and the underlying molecular mechanisms associated with its activity regulation. In this study we have summarized the important information that will help in understanding the functional and activity regulation of Nek2A splice variants, which will further facilitate in designing a potent inhibitor against the cancer associated cases. We have also presented previously reported studies on the domain specifications and inhibitor biosynthesis that provide an insight into its specific target residue regions for developing active and more potent inhibitors.

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## Contents

* Corresponding author at: Bioinformatics Division, School of Bio
Sciences and Technology, Vellore Institute of Technology University,
Vellore 632014, Tamil Nadu, India.
E-mail address: riturajpurohit@gmail.com (R. Purohit).

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## 1. Introduction

Centrosome is an essential component of the cell cycle regulation mechanism. Alteration in its function primarily leads to several cell cycle disorders, including aneuploidy, which is considered as the hallmark of cancer. Centrosomes are composed of pairs of centrioles and the pericentriolar matrix that determines the geometry of microtubule arrays throughout the cell cycle, and thus influences the cell shape, polarity, motility, spindle formation, chromosome segregation and regulates the overall cell cycle progression mechanism [1]. The centrioles are considered among the most essential elements of cilia and microtubule organization in the cell [1]. These in turn play important roles in locomotion, transport and signalling. Aberrations in centriole/basal body formation and function are associated with a plethora of human diseases, including ciliopathies, brain diseases and cancer [2]. The growing line evidence has further suggested the direct involvement of centriole associated proteins in several cell cycle aberrations and the associated disorders [3–5]. The biogenesis of centrioles is strictly controlled by a set of proteins, the foremost including the rootelin, SAS-6, Plk1, CEP68, CEP135, CEP164, CEP215, Mst1/Mst2, hSav1, C-Nap1 and several other associated kinases such as Nek2 and Aurora-A [1,2,7]. Abnormal chromosome content, in other words aneuploidy, is a hallmark of human cancers which is largely affected by centrosomal dysfunction.

Kinases are vital for the execution and regulation of cell cycle progression events. It is interesting that a single cell cycle associated kinase is often involved in multiple cellular processes during mitosis [8]. These mitotic kinases are usually located at their specific sites within the cell and undertake their designated roles. Nek2 is a cell cycle regulated kinase belonging to never in mitosis A (NIMA) family and is highly enriched at the centrosome, preferentially located at the proximal ends of the centrioles [9,10]. It was initially described as a mammalian structural homologue of Aspergillus NIMA whose function was found to be linked with chromosome condensation in mitosis [11,12]. Nek2 was found to be localized on the mid body of the telophase cells [13]. Dynamic changes in subcellular localization of Nek2 have been observed during the mitotic stages [13,14]. There is strong evidence that in mitotic cells Nek2 contributes to centrosome separation and perhaps in reorganization of the microtubule network at the G2/M transition [9,14]. Further, it controls the centriole–centriole linkage of the cells entering mitosis by phosphorylating C-Nap1 [15]. Association of Nek2 with chromosomes is shown to be evident once the cell enters mitosis and maintains until the end of metaphase [12]. In line with functions in mitotic entry, Nek2 is a cell cycle-regulated kinase with activity low in G1, increased in S and G2, and diminished after mitotic onset [1]. It has different splice variants that show a diverse pattern of expression and activity regulations and has been discussed below.

## 2. Nek2 splice variants

Two well known C-terminal splicing variants of Nek2, namely Nek2A and Nek2B, were identified in Xenopus embryos [16,17] and in cultured human cells [18]. The combined abundance and activity of the two forms peak in S and G2 phases of the cell cycle, whereas Nek2A is specifically targeted for proteosomal destruction in mitosis [18]. The Nek2B splice variant lacks a destruction box, thus it is resistant to mitotic destruction mediated by the anaphase-promoting complex and withstands destruction until the early G1 phase [18,19]. It was shown to be present exclusively in the early Xenopus embryos whereas the Nek2A splice variant appeared only after the neurula stage [16]. It was recently suggested that Nek2B plays an active role in promoting the assembly of the functional zygotic centrosome in fertilized Xenopus eggs [20]. Nek2A and Nek2B were shown to be expressed throughout early embryogenesis but Nek2A showed a distinct feature by not being destroyed after the cell has entered the mitotic stage [21]. Recently, a third splice variant, Nek2C, was identified that lacks an eight-amino acid internal sequence within the C-terminal domain of Nek2A [22]. Nek2C shares many properties with Nek2A including kinase activity, dimerization, protein phosphatase 1 interaction, mitotic degradation, microtubule binding, and centrosome localization [22]. The biochemical and physiological properties of the Nek2C splice variant are the subject of further investigation. The variation in activity regulation within these splice variants is the central point of research. Fletcher et al. has shown that the depletion of Nek2A and Nek2B has different effects on the mitotic delay [23]. Upon examination, 84% of the control and 63% of the Nek2A depleted cells showed mitotic exit in less than 2 h, whereas only 30% of Nek2B depleted cells showed mitotic exit during this time. Upon exiting mitosis 15–20% of the Nek2B depleted cells was unable to complete cytokinesis resulting in the formation of multinucleated cells [23]. They observed that the mitotic delay and defects in exiting mitosis were not shown after the downregulation of Nek2A alone. Authors further demonstrated that the Nek2A and Nek2B have distinct roles in regulating cell cycle progression and Nek2A but not Nek2B is involved in the regulation of centrosome splitting prior to mitotic entry. These observations clearly suggest the role of C terminal region in determining the diverse range of functions shown by these two splice variants. According to the authors observation, down-regulation of Nek2B alone actually resulted in a mitotic delay that can lead to mitotic defects, whereas the same phenomena were not observed at a significant level in the Nek2A case [23]. Furthermore, the explanation of activity regulation for Nek2C has not yet been proposed. Individual as well as the group examination of these splice variants, will provide a detailed insight into how they coordinate the cell cycle progression and will help in elucidating their inter-regulatory mechanisms.
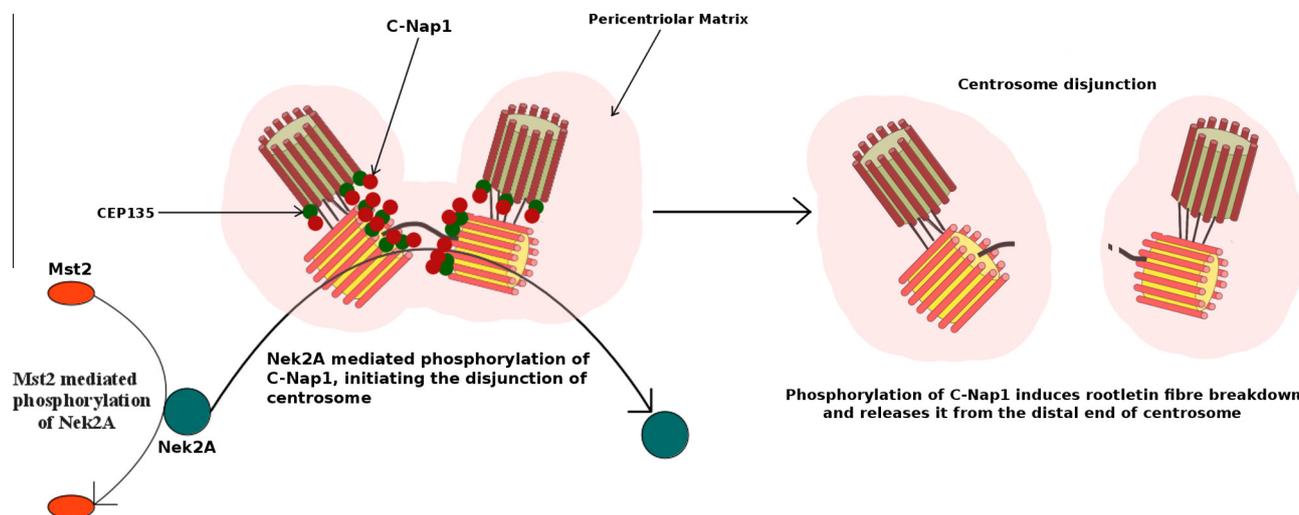
**Figure 1** Figure shows the Nek2A mediated regulation of centrosome disjunction pathway.

## 3. Nek2A activity regulation and associated pathological outcomes

Nek2A governs a wide range of activities that determine the fate of cell cycle progression ranging from centrosome disjunction to spindle checkpoint signal regulation. The overall regulation of Nek2A-mediated centrosome disjunction mechanism is shown in Fig 1. Hec1, a kinetochore protein, is essentially required for maintaining the proper spindle checkpoint cascades and its depletion leads to the impairment of chromosome congression and causes persistent activation of the spindle checkpoint [24]. Hec1 is shown to be an active substrate of Nek2, suggesting the possible involvement of Nek2 in the regulation of spindle checkpoint mechanism [25]. Recently, it was also reported that Nek2A interacts physically with Mad1 and is required for proper response to spindle damage [8]. The hSav1, Mst1/Mst2 and Nek2 shares a common interaction motif known as the SARAH domain. The SARAH domain of Nek2A is shown to interact with the components of the Hippo pathway, which aids in proper centrosome disjunction [6]. In 60% of hSav1 or Mst1/Mst2-depleted cells, Nek2 was no longer detected at centrosomes, whereas the other cells also showed notable loss in Nek2 signals [6]. Moreover, the Eg5-dependent microtubule-pushing forces cooperated jointly with the hSav1–Mst1/Mst2–Nek2A pathway to enable centrosome disjunction and spindle formation [6]. These interactions suggest a significant role of Nek2A in C-NAP1/rootelin disjuction, and are considered as vital for centrosome disjunction. Furthermore, Nek2A also has a binding site for the catalytic subunit of PP1, and hyperactivation of Nek2A at the onset of mitosis is thought to be dependent on inactivation of PP1 [26]. The mechanism of Nek2A dynamics was proposed where the phosphorylation of HsSgo1 by Nek2A is shown to be essential for faithful chromosome congression and the proper attachment of the spindle microtubule to the kinetochore [27]. It has been proposed that the Nek2A-mediated phosphorylation of HsSgo1 links kinetochore-microtubule attachment to chromosome segregation dynamics [27]. Ser14 and Ser507 residues on HsSgo1 are substrates of Nek2A in vitro [27]. Nek2 phosphorylates the chromatin-associated protein, high mobility protein A2, and is activated during the extracellular signal-regulated kinase 1/p90Rsk2-stimulated condensation of chromatin in mouse pachytene spermatocytes, suggesting its active role in chromatin condensation [28,29]. All this evidence collectively suggests that the Nek2A has a significant role in regulating the centrosome dynamics as well as in maintaining the spindle checkpoint signals, thus becoming an important factor for regulating the cell cycle progression. Though the knockout or depletion of Nek2A did not show any significant effect on G2/M progression the above observation makes it evident that the alteration in its functional mechanism may alter the mitotic progressions.

Nek2A has been shown to be actively involved in several cancer cases [3–5]. It has been shown that the ectopic expression of kinase-inactive Nek2A interfered with proper bipolar spindle formation and eventually resulted in chromosomal segregation defects such as unaligned chromosomes in the metaphase, lagging chromosomes in the anaphase and thin chromatin bridges in the telophase [14]. Furthermore, the development of the Nek2-suppressed mouse embryos stopped at the second mitotic division, whereas the development of the Nek2-depleted Xenopus embryos stopped at the 16- or 32-cell stage with irregularly sized blastomeres [30]. Its overexpression induced premature centrosome splitting [9,31], whereas the overexpression of kinase-dead Nek2A led to the formation of centrosomal abnormalities, monopolar spindles, and aneuploidy [14]. Nek2 mRNA was significantly elevated in Ewing tumour (paediatric osteosarcoma) cell lines and upon transformation of low-grade follicular lymphoma to the more aggressive diffuse large B-cell lymphoma [32,33]. Nek2 was also shown to display elevated levels of protein expression in human breast cancer [34]. There was a reduced amount in G1 and M phases compared with S and G2 phases. Growing line of evidence has strongly suggested that the Nek2A may act as a potential candidate for anti-tumour drug discovery.

## 4. Targeting Nek2A: inhibitor based approach

The structural conformation of proteins determines their activity regulation mechanisms as well as their response to a

particular drug inhibitor. Understanding the structural orientations of the corresponding protein, and their respective properties, will help in determining the corresponding drug response cascades and will eventually facilitate in the drug discovery process. When we talk about drug discovery, the most leading factor that governs the efficiency level of the drug molecule is the fine-tuning of the molecular properties of drugs to the corresponding structures of their protein targets, which promotes the binding interactions of high affinity and specificity [35]. The protein-drug interaction events are governed by the enthalpic and entropic contributions. The enthalpy calculations correspond to the stabilizing interactions, including hydrogen bond and salt bridge formations whereas the entropy corresponds to regulating the losses of conformational fluctuations occurring in the protein–drug complexes. These calculations together regulate the efficiency and the stability of drug interactions with target proteins. Shape complementarity and the physicochemical complementarity are the two governing factors of enthalpy contributions in protein ligand interactions. Shape complementarity permits the protein and small molecules to achieve sufficient proximity and contact surface area to form stabilizing interactions, while physicochemical complementarity determines the nature of these interactions [35]. The most complementary conformation from an ensemble of equilibrium structures is selected by the ligand molecules. This mode of interaction has been implicated in the binding selectivity of ligand to tyrosine kinases [36]. A recent comparison of two protein structure datasets, one comprising drug targets and the other of non-drug targets, revealed drug targets to be more hydrophobic, have lower isoelectric values, be composed of more amino acids and have a higher frequency of beta-sheet secondary structure compared with other proteins [37]. Inhibitor based targeting of Nek2A, thus requires proper functional domain knowledge and the proper complementarity for ligand–protein interactions.

Nek2 is a serine/threonine kinase, dimerizes and is rapidly activated by autophosphorylation. Besides autophosphorylation, Nek2 may be regulated by upstream kinases. Indeed, the catalytic domain of Nek2 is phosphorylated in vitro by p90$^{Rsk2}$, an interesting observation as the activation of chromatin condensation by Nek2 in mouse spermatocytes is under the control of the mitogen-activated protein kinase/p90$^{Rsk2}$ pathway [29]. Recent studies suggest that RNAi depletion of Nek2 leads to antiproliferative effects in HeLa, MDA-MB-231, HuCCT1, and MCF7 cells in vitro and in vivo [23] and cholangiocarcinoma cell lines [38]. The genetic knockdown of Nek2 resulted in an antiproliferative and antimigratory phenotype in MDA-MB-231 breast cancer cells and an antitumor effect in a MDA-MB-231 xenograft model when the silencing oligonucleotides were injected intratumorally [39]. It is found that the depletion of Nek2 synergized with cisplatin in inhibiting the growth of colorectal cancer cells in vitro and in vivo [40], although the mechanism for this remains unclear. This provides a possibility to exploit the structural composition of Nek2 protein for anti-tumour drug development. Nek2 is composed of several pharmacologically active domains which can be a good prospect for drug target discovery. Amino acid region from 1 to 271 contains a highly essential kinase domain with an activation loop segment from 159 to 186 [1]. Downstream of the kinase domain is an unusual leucine zipper motif that mediates dimerization as a prerequisite for efficient autophosphorylation and, thus, full Nek2

activity on exogenous substrates [9]. APC/C targets different proteins for degradation at different times in the cell cycle. The Nek2A protein associates tightly with the APC/C, via its C-terminal MR tail [41]. This interaction is considered as essential for ubiquitylation and degradation of Nek2A in prometaphase [41]. The KEN box of Nek2A consists of four phosphorylation sites, suggesting a potential autoregulatory role at the level of Nek2 stability [1]. This region also acts as a recognition site for APC/C$^{Cdc20}$-mediated degradation of Nek2A in early mitotic stages [41]. The amino acid region between 399 and 445 is required for its proper neucleolar localization [1]. Two sites (Thr-170 or Ser-171, Thr-179) which map to the Nek2 T-loop region, and a third, Ser-241, located within the kinase C-lobe, indicate their potential role in regulating kinase activity [1]. Moreover, the Ser-356 and Ser-428 residues lying within the coiled-coil region are reported to be involved in modulating Nek2A dimerization and localization [1]. The DFG and HRD motifs of Nek2A are highly conserved and contain catalytically vital aspartic acid residues. The activation loop is immediately C-terminal to the DFG motif, contributes to substrate binding and helps to organize other motifs, and can be considered as the potential target for drug inhibitors. Its C-helix contains a conserved glutamic acid that forms a salt bridge with a conserved lysine residue. The hinge region of Nek2A formed two hydrogen bonds with the aminopyrazine ring of an active drug molecule [4].

The crystallographic study reported few unstable regions in the Nek2A structure, forecasting multiple disordered regions within the functional domains [1]. Residues within the loop N-terminal to αC (Thr-45–Met-51), the loop connecting the two strands β4 and β5 (Arg-77–Thr-81), the region C-terminal to helix αE (Asp-132–Arg-140), and the activation segment residues between Asn-167 and Val-177 were presumably found to be disordered [1]. The N-terminal lobe (Ser-3–Met-86) and the αC helix in particular have been shown to exhibit disordered conformation, presumably due to mobility within the crystal [1]. It shows that the regions are highly unstable and could be possibly involved in rapid conformational changes for regulating the kinase activity of protein. Rellos et al. further showed that the amino acid residues Met-86, Val-68, Phe-148, Cys-89, and Leu-162 acted as active site residues forming hydrophobic interactions with the inhibitor molecule [1]. Furthermore, Glu-87 and Cys-89, which are the main chain residues, formed two hydrogen bonds with the inhibitor [1]. In another study, Ile14, Gly91 and Gly92 also showed that a promising result is obtained by proper positioning and hydrophobic interactions with the drug molecule [4]. The piperidine rings of compound 2 and 5 and the phenyl ring of the benzoic acid moiety of compound 12 were shown to be sandwiched between the gatekeeper residue Met86 and Phe148 [4]. Furthermore, the carboxylic acid group of compounds 2, 5, and 12 participated in a number of interactions, including hydrogen bonds with Tyr70 and Asp159 and a salt bridge with the catalytic Lys37 [4]. The Tyr70 residue is remarkable for two reasons: first, it is rare among kinases for Tyr to be present in this position 23 and second, it shows an unusual conformation in the structures with compound 2, 5, and 12. Presence of a Phe (Phe148) at the base of the ATP pocket is also a notable feature [4]. Only 39 of the human kinase catalytic domains contain a Phe in this position which is usually occupied by Leu in most kinases [4]. The larger Phe has a profound influence on the shape of the ATP pocket [4]. It protrudes into

the adenine subpocket creating steric hindrance in the plane of the hinge binding motif [4]. The methoxy group of compound 15 formed contact with Val68 and Tyr70 [4]. In another study, the aminopyridine group of rac-17j compound was observed to form two hydrogen bonds with the hinge region at Glu87 and Cys89 [42]. Moreover, the imidazole ring of rac-3 compound formed one hydrogen bond with Cys89 residue. Met86 and Phe148 form the gatekeeper component whereas the amide group engages in two hydrogen bonds with the backbone NH and the side chain carbonyl group of Asp159 of the DFG motif [42]. The thiophene ring engages in hydrophobic contacts with Ile14 and Gly92 [42]. This evidence collectively reports the significance of these functionally active amino acid residues in the exploitation of target based drug design approaches.

Nek2 dimerizes through a C-terminal coiled-coil domain that is essential for efficient cellular activity [17]. The autophosphorylation of the Nek2A dimer is mediated by the Thr175 residue [4]. There are different ranges of inhibitor molecules that can be exploited against the Nek2A activation. The so-called "type II" inhibitors exploit an allosteric site present only in the inactive "DFG-out" conformation, and their clinical success has initiated efforts to develop new families of kinase inhibitors that combine classical type I scaffolds with chemical moieties that are known to stabilize DFG-out conformations [1]. This particular conformation of the activation loop is not accessible for every kinase and opens a binding pocket with much larger sequence variability, making the development of specific inhibitors more likely. Rellos et al. have suggested two possibilities for the development of inhibitors that stabilize the inactive conformation [1]. They reported that the type I pyrrole-indolinone scaffold in combination with more bulky type II inhibitor moieties in place of the exocyclic chlorine could be used to enlarge the allosteric binding pocket [1]. Secondly, since αT helix prevents the access of Glu-55 to the active site, they suggested that the surface presented by αT helix could also be targeted to create more stabilizing interactions [1]. Furthermore, it has been proposed that the autophosphorylation of Thr-179, within the T loop, and Ser-241, within the C lobe, may act to negatively regulate the Nek2A activation. The C lobe Ser-241 residue regulates the dimerization and thus plays an autophosphorylation mediated inhibitory target. It has been shown that the C-terminal domains are among the top 10 commonly observed folds that are used as a target for the drug discovery process [35]. Stabilizing the C-lobe with a type II inhibitor and then targeting it with a kinase mimicking drug molecule may form an effective combination for inhibiting the dimerization and thus in negative regulation of its functional mechanism. Molecular dynamics' (MD) studies reveal that kinases with high ligand affinity spend more time in conformations compatible with drug binding compared with kinases with low ligand affinity [35]. MD simulation is a useful approach for generating protein structural ensembles and capturing alternative conformations that may be relevant for protein function or small-molecule binding [43,44]. Further, the application of MD simulations has shown useful outcomes in studying conformational changes in protein and its effect on its ligand binding affinity [45–54]. Protein dynamics information enhances structure-based binding site prediction methods and reduces their dependence on experimentally determined target protein structures [35]. Thus by MD analysis we can further examine the effectiveness of the proposed inhibitor drug conformation.

## 5. Concluding remarks

Due to the recent advancements in genomic technology, the area of therapeutic development has raised the possibility for the treatment of several diseases [55,56]. Three dimensional protein structure information is frequently used in drug development practices, from the selection of a therapeutic target, to the determination of underlying molecular mechanisms of action, and their possible clinical outcomes on the target patient population. As a result, great improvements have been made in understanding the structure–activity relationship of inhibitor-protein complexes and thus has facilitated in understanding the core insights of drug–target interactions and in the development of highly efficient therapeutic agents. There has been a recent case implicating the overexpression of centrosome associated genes and their correlation with cancer associated evidence, which has now attracted the genomics and pharmacological researchers in elucidating their underlying molecular mechanisms of activity regulation and further in developing effective inhibitor drug molecules. Nek2A is in special focus since it has been observed in a wide range of breast cancer, ewing tumour and in folicular lymphoma cases. Previously few researches have been carried out on the development of effective drug inhibitors to control the activity of Nek2A protein but they have further argued on the requirement of further investigation to derive more effective drugs. Release of several conformations of Nek2A protein has now provided an easier way of determining the potential target domains. Although the available protein structures may not display a conformation compatible with the inhibitor binding for the proposed conformation, probably due to the protein structural preferences or sometimes due to the random chances and crystallization artefacts, these errors can be resolved by incorporating the MD simulation approaches. The collective information that we presented in this manuscript strongly pinpoint to several amino acid residues that lie in the out conformation attaining domains and can be easily targeted by drug molecules. Further the previous reports also depict the use of kinase mimicking inhibitors that can negatively regulate the activity of Nek2A by phosphorylating few essential amino acid residues, such as the Ser-241 present in its C-lobe. This information will be furthermore valuable to the in silico and wet lab researchers for developing highly potent inhibitors against the Nek2A protein.

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