



The NIMA-related kinase NEK1 cycles through the nucleus

Laura K. Hilton, Mark C. White, Lynne M. Quarmby*

Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, Canada

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ABSTRACT

Mutations in *NEK1* in mice are causal for cystic kidneys, and model the ciliopathy polycystic kidney disease caused by abnormal ciliary structure or signaling. *NEK1* has previously been shown to localize near centrosomes and to play a role in centrosomal stability and ciliogenesis. Recent data suggest that the etiology of kidney cysts involves aberrant signaling from the primary cilium to the nucleus. Here we demonstrate that *NEK1* contains functional nuclear localization signals, is exported from the nucleus via a nuclear export signal-dependent pathway and that the protein cycles through the nucleus. Our data suggest that *NEK1* is a candidate to transduce messages from the ciliary-basal body region to the regulation of nuclear gene expression.

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Introduction

NEK1 is a member of the NIMA-related kinase (Nek) family, members of which are defined by similarity in their kinase domains to that of the essential *Aspergillus nidulans* cell cycle kinase NIMA (never in mitosis A) [1,2]. Two mouse models for polycystic kidney disease (PKD), *kat* and *kat^{2J}*, are caused by mutations in *NEK1*, and therefore implicate *NEK1* in the etiology of PKD [3]. The *kat* strains present a recessive, pleiotropic phenotype that includes progressive cystic kidneys, runting, facial dysmorphism, hydrocephalus, and anemia [3–5]. Despite extensive phenotypic characterization, little is known of the cellular functions of *NEK1*.

NEK1 is found associated with centrosomes, where it remains throughout the cell cycle [6–8]. It has recently been implicated in the maintenance of centrosomes and in the formation of the primary cilium [7–9]. It remains unknown, however, whether defects in *NEK1* cause cystic kidneys directly, by failing to relay a particular signal to the cell; or indirectly, by interfering with the structure of the primary cilium.

Recent evidence indicates that signals for both proliferation and differentiation are received by the primary cilium [10,11]; therefore it is not surprising that some cystogenic proteins are active in both cilium and nucleus. Autosomal Dominant PKD (ADPKD) is

caused by mutations in either gene encoding polycystin-1 (PC1) or polycystin-2 (PC2), large membrane proteins that localize to the primary cilia in renal epithelia (reviewed in [12]). In response to mechanical stimuli proteolytic cleavage releases the C-terminal tail of PC1, which then translocates to the nucleus to alter gene expression independently or in association with the transcription factor STAT-6 [13,14]. PC2 helps stimulate this cleavage [15]; thus the polycystins function co-operatively in altering gene expression through cilium-to-nucleus signaling. Autosomal Recessive PKD (ARPKD) is caused by mutations in the gene encoding fibrocystin, another membrane protein that localizes to cilia. Fibrocystin similarly undergoes proteolytic cleavage in response to Ca^{2+} signals, and the released C-terminal tail translocates to the nucleus [16]. Similarly, mutations in *inversin* cause an autosomal recessive cystic kidney disease (NPHP2 [17]) and *inversin* affects nuclear signaling through the Wnt pathway [18].

The presence of one or more predicted nuclear localization signals (NLSs) and nuclear export signals (NESs) in *NEK1* suggest that *NEK1* might also be directly involved in communicating a change in state to the transcriptional machinery of the cell. Dysfunctional communication with the nucleus is likely to be an important aspect of cystogenesis, accounting for changes in proliferation and differentiation status [14,18]. Therefore, we set out to test whether *NEK1* is capable of translocating to the nucleus, and if so, to define the functional nuclear transit signals. In this paper we demonstrate that endogenous *NEK1* cycles through the nucleus, indicating that *NEK1* may be capable of carrying signals between the primary cilium and the nucleus. In addition, we report the

* Corresponding author. Address: Department of Molecular Biology and Biochemistry, Simon Fraser University, 8888 University Drive, Burnaby, BC, Canada V5A 1S6.

E-mail address: quarmby@sfu.ca (L.M. Quarmby).

functional definition of two NLSs and the use of an NES-dependent nuclear export pathway.

Materials and methods

Cell lines and cell culture. Inner medullary collecting duct (IMCD3) murine renal epithelial cells were maintained in a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 10% fetal bovine serum (DMEM-F12 (+), Invitrogen, Carlsbad, CA). All experiments were carried out on cells that had been passaged fewer than ten times, and grown to confluence on coverslips. Transient transfections were carried out with 4 µg of plasmid and Lipofectamine 2000 (Invitrogen) in OPTI-MEM reduced-serum media according to the manufacturer's protocol. After 6 h, cells were washed with PBS and incubated in DMEM-F12 (+) overnight. For nuclear export experiments, cells were treated with either 5 ng/µl Leptomycin B or a solvent control for 6 h, 24 h post-transfection.

Antibodies and microscopy. For microscopy, cells grown on coverslips were fixed with methanol at −20 °C for ten minutes and rehydrated in PBS. If immunofluorescence was required, coverslips were incubated with primary antibody diluted in PBS for 1 h, and then washed twice for 15 min in PBS. This was repeated for secondary antibody. Coverslips were then incubated with 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) for 10 min at room-temperature to stain nuclei. Coverslips were mounted with MOWIOL 4-88 (Calbiochem, San Diego, CA). Microscopy was carried out on the Delta Vision system (Applied Precision, Issaquah, WA) as described previously [19,20]. By visual inspection, we considered the distribution of NEK1 signal to be "nuclear/cytoplasmic" when there was a clear signal in both compartments; a substantially greater distribution of NEK1 signal to either the nucleus or cytoplasm was counted as "nuclear" or "cytoplasmic", respectively. At least 300 cells were scored for each construct. Primary antibodies include mouse monoclonal anti-myc (Clone 9E10; 1:2000; Clontech,

Palo Alto, CA) and rabbit polyclonal anti-NEK1 (1:100; from Dr. Y. Chen, University of Texas Health Science Center, San Antonio; [21]). Secondary antibodies include Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500; Molecular Probes, Eugene, OR) and Alexa Fluor 594-conjugated goat anti-mouse IgG (1:2000; Molecular Probes).

Molecular constructs. Sequence representing the full-length NEK1 cDNA (GenBank Accession No. AY850065) was cloned into the SacI–SalI sites of pEGFP-C2 (Clontech), and truncations were made using standard molecular biology techniques. To generate myc-NEK1, the full-length NEK1 sequence was cloned into the SalI site of the pCMV-myc plasmid.

Results

NEK1 cycles through the nucleus in IMCD3 cells

Although two classical NLSs have been previously predicted for NEK1 (364KKRRR367 and 580RKRRK583) using the PredictNLS tool (<http://cubic.bioc.columbia.edu/predictNLS/> [20]), mutating one or both of these NLSs does not interfere with the nuclear localization of C-terminally truncated NEK1 (our unpublished data, [22]). These data indicated that an additional sequence participated in the transport of NEK1 into the nucleus, either an NLS or a region that interacted with another protein that itself had an NLS. We re-examined the NEK1 sequence for new NLS predictions using the updated PredictNLS database and uncovered a single bipartite predicted NLS between residues 355–378 (NLS in Fig. 1). The NES prediction tool NetNES v1.1 (<http://www.cbs.dtu.dk/services/NetNES/> [23]) predicts two leucine-rich NESs for NEK1: NES1 at residues 764–774, and NES2 at residues 1131–1138 (NES1 and NES2 in Fig. 1).

While full-length NEK1 is predominantly cytoplasmic, the presence of multiple predicted NLS and NES in NEK1 led us to investigate the nuclear translocation of NEK1. The nuclear

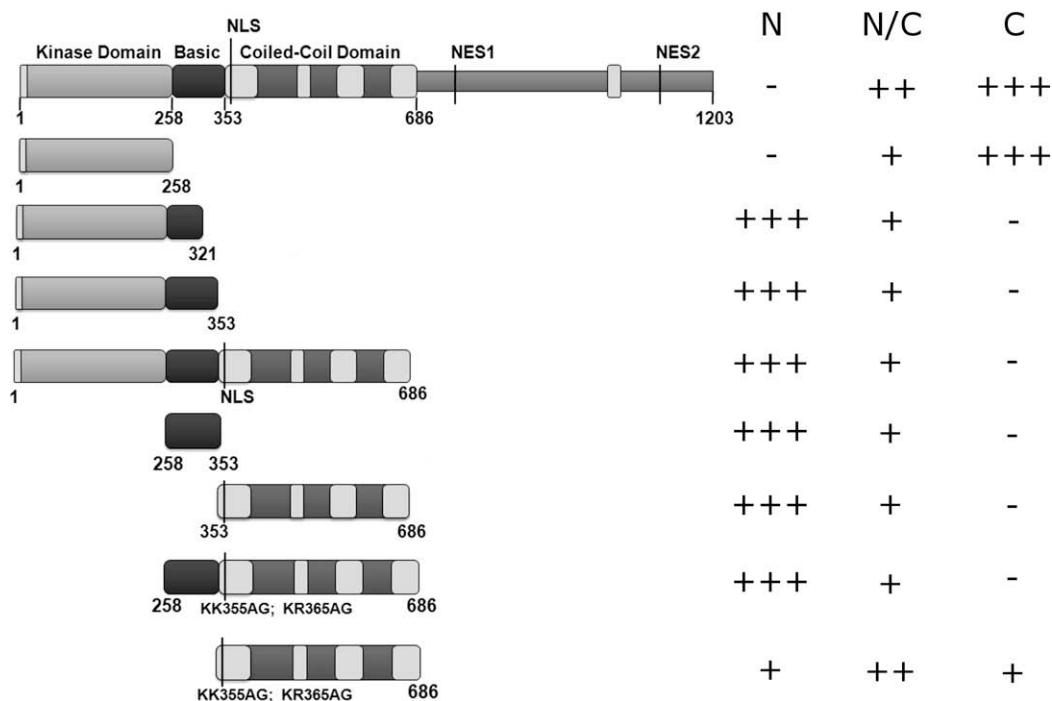


Fig. 1. Predominant localization of different NEK1 constructs. This schematic shows the different domains of the NEK1 protein, and the distribution of predicted nuclear translocation signals. The various NEK1 truncations shown were expressed as GFP fusions in IMCD3 cells, and their predominant localization is indicated as nuclear (N), cytoplasmic (C), or both (N/C). +++: strong localization; ++: moderate localization; +: weak localization; -: no localization.

export-blocking drug Leptomycin B (LMB) binds to CRM1, an essential factor in NES-dependent nuclear export in mammalian cells, and prevents the interaction of CRM1 with NES-containing proteins [24]. To test the effects of LMB on the sub-cellular localization of NEK1, we transfected IMCD3 cells with myc-NEK1 and treated cells with LMB for 6 h beginning 24 h post-transfection. In cells treated with LMB, but not in control cells, full-length myc-NEK1 accumulates in the nucleus (Fig. 2A and B). When untransfected cells are treated with LMB, endogenous NEK1 also accumulates in the nucleus (Fig. 2C and D). This indicates that NEK1 has at least one functional NES that is blocked by LMB.

In attempts to identify the NES, we mutated both predicted NESs individually and together (LQL772AQA and LRL1134AQA). These mutations did not cause nuclear accumulation of the protein (data not shown). Several different scenarios could explain these negative results. It is possible that the mutations disrupted protein structure in such a way as to interfere with import. Alternatively, the functional NES may be cryptic. Finally, it is possible that NEK1 exits the nucleus via interaction with another NES-bearing protein.

The LMB experiments demonstrated the existence of NES-dependent nuclear export of NEK1. However, in tissue culture cells we always observe a predominantly cytoplasmic localization of endogenous NEK1. In order to obtain the result that we did in the LMB experiment, NEK1 must be cycling through the nucleus, accumulating in the nucleus only when export is blocked. This implies the existence of functional NLSs in NEK1.

NEK1 has at least two functional NLSs

To define functional NLSs within NEK1, we generated a series of EGFP-tagged NEK1 truncations (Fig. 1) and transfected them into IMCD3 cells. EGFP alone distributes to both nucleus and cytoplasm (Fig. 3A) because it is smaller than the passive diffusion threshold of the nuclear pore complex [25]. The EGFP-tagged kinase domain (residues 1–258) is predominantly cytoplasmic (Fig. 3B), while EGFP-NEK1 1–321, 1–352 and 1–686 are predominantly nuclear (Fig. 3C, D and E). The EGFP-tagged basic domain (residues 258–353) and the EGFP-coiled-coil domain (residues 353–686) are also predominantly nuclear, indicating that there is a functional NLS in

both the basic and the coiled-coil domains (Fig. 3F and G). The ability of the 1–321 construct to localize to the nucleus indicates that the functional NLS within the basic domain lies within residues 258–321. However, no stretches of basic amino acids occur within this region and consequently it is clear that although this region can direct nuclear import, no canonical NLS exists within this sequence.

We proceeded to test functionality of the predicted bipartite NLS, which is in the coiled-coil domain, by mutating two stretches of basic amino acids in the NLS, KK357AG and KR366AG, in the 353–686 construct. As predicted, nuclear localization of this construct is severely disrupted (Fig. 3H). Including the basic domain in NLS-mutated EGFP-NEK1 (residues 258–686, Fig. 3I) rescues nuclear localization of the construct. Taken together, these data confirm that the predicted NLS is functional, and that the basic domain harbors a second cryptic NLS. Our data demonstrate that each of the two NLSs is sufficient on its own to direct nuclear localization of NEK1.

Discussion

We have established the presence of two functional NLSs and the nuclear export of NEK1 via an NES-dependent pathway. Only one NLS, located at the beginning of the coiled-coil region of NEK1, functions as predicted on the basis of sequence. A second, cryptic NLS lies within the basic region C-terminal to the kinase domain. The NES-dependent export of NEK1 is confirmed through experiments with Leptomycin B (LMB), which causes both transiently expressed myc-NEK1 and endogenous NEK1 to accumulate in the nucleus (Fig. 3). Furthermore, the LMB experiments demonstrate that endogenous NEK1 cycles through the nucleus, making it an excellent candidate for conveying signals between the primary cilium and the nucleus.

While NEK1 is continually cycling through the nucleus, it remains unknown what physiological signals will cause the protein to accumulate in the nucleus. A recent report demonstrated that NEK1 translocates to the nucleus in response to nuclear DNA damage [26]; however, the upstream signals that regulate this activity with respect to functional nuclear transit signals are unknown. Recent reports have identified three phosphorylated residues on

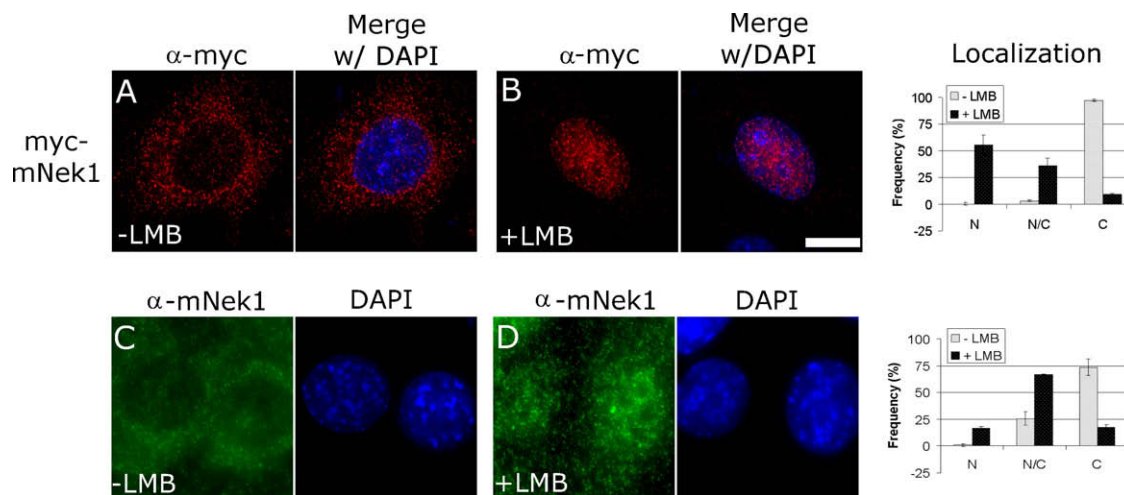


Fig. 2. NEK1 cycles through the nucleus. (A, B) IMCD3 cells were transfected with myc-NEK1. Twenty-four hours post-transfection, cells were left untreated (LMB–) or treated with Leptomycin B (LMB+) for 6 h, then fixed for immunofluorescence and stained with anti-myc (red) and DAPI (blue). (C, D) Un-transfected IMCD3 cells were subjected to the same LMB experiment, then cells were fixed and stained with anti-NEK1 antibody (green) and DAPI (blue). The frequency of the observed localization of myc-NEK1 or endogenous NEK1 [nuclear (N), cytoplasmic (C), or both (N/C)] is indicated. Scale bar represents 5 μ m.

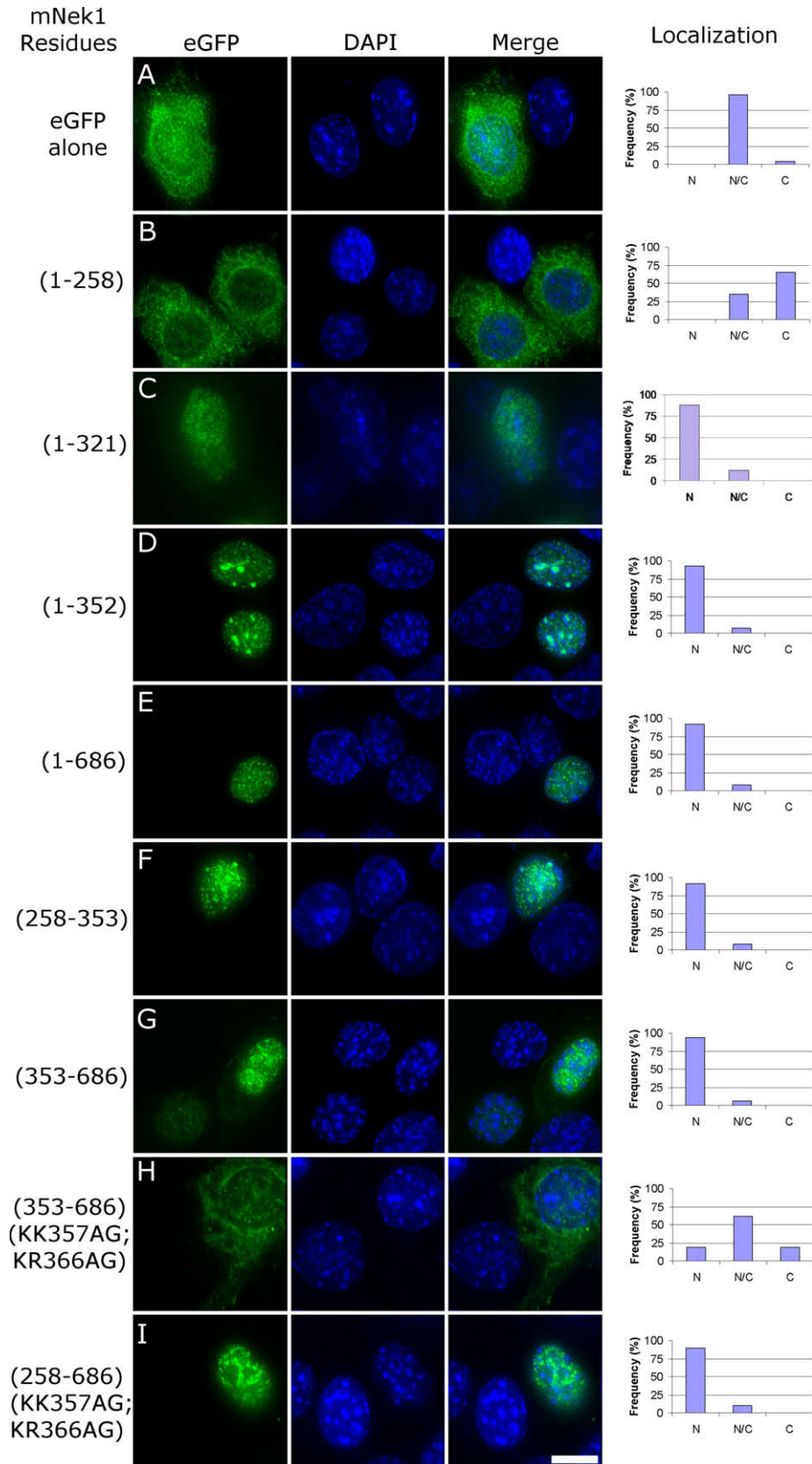


Fig. 3. NEK1 has two functional nuclear localization signals. IMCD3 cells were transfected with GFP-NEK1 fusion constructs (green) using the indicated residues of NEK1, then fixed for immunofluorescence and stained with DAPI (blue). The frequency of the observed localization of each construct [nuclear (N), cytoplasmic (C), or both (N/C)] is indicated. Scale bar represents 5 μ m.

mouse NEK1, which could help regulate the activity of NEK1 nuclear transit signals [27,28]. Aberrant regulation of NEK1 nuclear

localization and its activity within the nucleus could contribute to cystogenesis.

We have recently shown that NEK1 affects ciliogenesis [8]. In the current paper, we have shown that NEK1 cycles through the nucleus. NEK1 therefore may be one of a cohort of cystogenic proteins that affects both ciliary and nuclear signaling. Our data add support to the idea that defective ciliary signaling is transduced into aberrant regulation of nuclear gene expression, which may be an important component of the etiology of kidney cysts.

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