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Nek1 shares structural and functional similarities with NIMA kinase

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Abstract

The *Aspergillus* NIMA serine/threonine kinase plays a pivotal role in controlling entrance into mitosis. A major function attributed to NIMA is the induction of chromatin condensation. We show here that the founder murine NIMA-related kinase, Nek1, is larger than previously reported, and that the full-length protein conserves the structural hallmarks of NIMA. Even though Nek1 bears two classical nuclear localization signals (NLS), the endogenous protein localizes to the cytoplasm. Ectopic overexpression of various Nek1 constructs suggests that the C-terminus of Nek1 bears cytoplasmic localization signal(s). Overexpression of nuclear constructs of Nek1 resulted in abnormal chromatin condensation, with the DNA mainly confined to the periphery of the nucleus. Advanced condensation phenotype was associated with nuclear pore complex dispersal. The condensation was not accompanied by up-regulation of mitotic or apoptotic markers. A similar phenotype has been described following NIMA overexpression, strengthening the notion that the mammalian Nek1 kinase has functional similarity to NIMA.

Keywords: NIMA; Nek kinase; Chromatin condensation; Cell cycle; Sub-cellular localization

1. Introduction

The *Aspergillus* NIMA kinase, is required by this fungus for entry into mitosis [1,2]. As implied by its name (never in mitosis A), in the conditional absence of NIMA activity, fungal cells cannot enter mitosis and they are arrested in G_2, exhibiting interphase microtubules and uncondensed chromosomes [3,4]. The mitotic activation of NIMA is probably achieved through autophosphorylation, followed by hyperphosphorylation by Cdc2 [5]. Overexpression of NIMA results in premature chromatin condensation from any phase of the cell cycle; this effect is independent of Cdc2 activation. Consistent with this phenotype, it has been demonstrated that overexpression of NIMA in *Aspergillus* triggers histone H3 phosphorylation on Ser-10, a phosphorylation event connected with mitotic chromatin condensation [6]. An additional documented task of NIMA is the nuclear localization of cyclin B [7], possibly by phosphorylation of nuclear pore complex (NPC) proteins [8]. NIMA is localized to the NPC during early mitosis, and is essential for NPC proteins dispersal [9].

Several observations suggest that the NIMA pathway is also evolutionarily conserved. Thus, overexpression of the fungal NIMA kinase in *Xenopus* oocytes induces germinal vesicle breakdown, and overexpression in human HeLa cells results in premature chromatin condensation without mitotic spindle formation [2,10]. In addition, similar to the phenotype in *Aspergillus*, overexpression of dominant-negative variants of NIMA in human cells results in characteristic G_2 arrest [2]. Finally, mammalian genomes encode for a family of NIMA-related kinases (designated Nek1 to Nek11), which are natural candidates for performing NIMA-like mitotic activities, and recent data suggests several common characteristics [11]. Among the mammalian Nek proteins, Nek2 has the greatest sequence similarity, within the kinase domain, to NIMA. Its major activities are associated with centrosomal, spindle and kinetochore-related functions [12,13]. Nek2 has also been shown to be localized to condensing meiotic chromosomes [14], and to be involved in meiotic chromatin condensation, probably by interacting with, and phosphorylation of, the architectural chromatin protein, HMGA2 [15,16]. Recently, mitotic roles have been demonstrated for another Nek member, Nek9/Nercc1, and for its interacting proteins, Nek6 and Nek7

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[17–20]. Involvement of Nercc1/Nek9 in interphase and S-phase progression was suggested by virtue of Nek9 binding to FACT, a nuclear complex active in regulation of the chromatin in relation to DNA replication and transcription [21]. Taken together, the mammalian Nek kinases have been shown to be involved in various aspects of cell cycle control, but there is no evidence for their direct involvement in chromatin condensation.

Inherited mutations in the *nek1* gene in mice result in several developmental defects. The mutants suffer from dwarfism and early mortality. Animals that survive to adulthood are either sterile (males) or subfertile (females), and develop polycystic kidneys [22]. Even though Nek1 was the first mammalian NIMA-related gene to be cloned [23], its cellular or molecular functions are unknown. We report here that Nek1 has greater structural similarity to NIMA than previously reported. Overexpression of truncated Nek1 variants induces chromatin condensation resembling the phenotype induced by NIMA overexpression in vertebrate cells.

2. Materials and methods

2.1. Plasmid construction

For construction of Nek1 expression vectors, we used the Nek1–not12 fragment [23]. A unique *Sal*I site was added by adaptor ligation just upstream to the initiator Met, and the *Sal*I–*Not*I fragment was cloned into corresponding sites of pCMV-Myc (Clontech). This fragment encodes a slightly shorter polypeptide than the full-length protein, encompassing amino acids 1–1146. For construction of the full-length protein, extension of the original Nek1–not12 fragment was performed using a 3′ fragment from the not18 clone [23]. Site-directed mutagenesis at the ATP-binding site (G13R) was carried out using the QuikChange kit (Stratagene, La Jolla, CA), with primers: 5′ CAGAAGATTGGAGAA***C***GTTCATTTGGAAAAG 3′ and 5′ CTTTTCCAAATGAAC***G***TTCTCCAATCTTCTG 3′. Wild type and G13R full-length vectors were then truncated by cutting with *Bgl*II and *Not*I, followed by mung bean nuclease digestion and self-ligation (resulting in constructs N-1001, and N-1001^{G13R}). An additional truncation, using *Xcm*I and *Not*I resulted in construct N-753. Removal of an internal *Xho*I fragment resulted in the $FL^{\Delta 151-438}$ construct. The first NLS mutations (K365 R366/AA) were generated using the QuikChange kit and the primers: CTGAGGAAGCAGCAAAA***GC***A***G***-AAGGTTGGAATTTATTGAGAAAG and CTTTCTCAATAAATTCCAA-CCTT***GC***T***GC***TTTTGCTGCTTCCTCAG, and the second NLS mutation K581R582/AL) using the primers: GAAGACCTGGACCGA***GCTCTG***AAG-***CAGAGC***TCGCTCCAGCTGTTC. NES mutations (L1131G, L1134A) were generated using the primers: 5′ CAGTGTCTTTAACCAT***GG***AGAGGAA***G***-AAGACTTCACTTGGAGCAAG 3′ and 5′ CTTGCTCCAAGTGAAGTC-TT***GC***TTCCTCT***CC***ATGGTTAAAGACACTG 3′ using the QuikChange kit. The 764 NES sequence was mutated (L644G, L767S) using the primer ATGGCTCTCCAAGAAAAGTCTGGGGGAAAAACCCTACAGATTCTG-TG***GG***GAAGATA***TC***TGGAGAAGCTGAATTACAGC in a PCR reaction.

2.2. Sequence analyses

PEST predictions were run online at http://www.at.embnet.org/embnet/tools/bio/PESTfind/, at a window of 10.

Nuclear localization signal (NLS) predictions were performed at http://cubic.bioc.columbia.edu/predictNLS/.

2.3. Transfections

HeLa, NIH3T3 and HEK293 cells were transiently transfected using CellPhect Transfection kit (Amersham, Pharmacia Biotech) according to the manufacturer's instructions.

2.4. Immunocytochemistry

Polyclonal antibodies against Nek1 were raised against two different domains: I. A *Sph*I–*Hin*dIII fragment encoding the N-terminal 187 amino acids (and 2 amino acids upstream of the initiating methionine), inserted into the pQE30 vector (Qiagen,Valencia, CA) downstream and in-frame with a 6XHis tag. II. A *Bgl*II–*Not*I fragment encoding the C-terminal 204 amino acids inserted into the pGEX-4T-2 vector downstream and in-frame with the GST tag. Purified proteins were injected into rabbits, and the resulting antibodies were affinity-purified using the corresponding Nek1 fragment coupled to an activated cyanogen bromide Sepharose column (Sigma). Antiserum was passed over the column and eluted with 100 mM glycine, pH 2.5, into 1 M Tris, pH 9.5.

For immunofluorescence microscopy, cultures grown on coverslips were fixed in 4% paraformaldehyde for 20 min., blocked with 3% BSA/0.5% Triton X-100 in PBS, and incubated overnight with primary antibodies at 4 °C. Primary antibodies were used as follows: Nek1 purified polyclonal antibodies diluted 1:50, Myc-monoclonal antibodies (Zymed Laboratories, CA) 1:250, HA-monoclonal antibodies (BAbCO, Richmond, CA) 1:1000, mouse α/β Tubulin (Biomeda, Foster City, CA) 1:1000, rabbit anti P-S10-Histone-H3 (Upstate Biotechnology, NY) 1:100, and rabbit anti activated Caspase-3 (Cell Signaling) diluted 1:75. Following washes, the coverslip was incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse and/or Rhodamin-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch) (1:150). DNA staining was done with propidium-iodide solution, and the coverslips were mounted on slides with Vectashield mounting solution (Vector, Burlingame, CA) and sealed. Staining was analyzed using an MRC 1024 confocal laser microscope (Bio-Rad).

2.5. Cell fractionation

Cells were washed twice by PBS and harvested by lysis buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris–HCl pH=7.5, 0.5% NP-40). The extracts were homogenized by a syringe needle (21G, 25 times), and centrifuged for 3 s at 20,000×g at room temperature. SDS sample buffer was added to the supernatant that were marked as cytoplasm. The pellet was washed twice by lysis buffer, followed by spin down for 5 s. The pellet was dissolved by SDS sample buffer and marked as nucleus.

3. Results

3.1. Nek1 protein structure is highly similar to that of NIMA

The murine Nek1 kinase was reported to consist of 774 amino acids, with a calculated molecular weight of 88.4 kDa, and limited structural homology to NIMA [23]. However, antibodies raised against the N-terminus portion of Nek1 detected a prominent band at about 180 kDa in Western analysis of various murine tissue extracts, indicating that the translated product is larger than originally predicted (see below). We thus compared the reported *nek1* cDNA sequence to the deposited murine sequences in the various data bases (genomic, cDNAs and ESTs), and sequenced one of the original clones (clone 23, described in [23]). The sequence analysis suggested that two reading mistakes occurred in the original report (T instead of C was reported at nucleotide 2898, and G following nucleotide 3869 was omitted; the corrected sequence has been deposited in NCBI GenBank Accession No. AY850065). According to the corrected open reading frame, the murine Nek1 encodes a 1203 amino acid protein, with a calculated molecular weight of 136.7 kDa (Fig. 1A). Nek1 is the longest documented NIMA-family kinase, bearing a 945 C-terminal non-catalytic tail. As the C-terminus is highly enriched in acidic residues, the overall

A.

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   1  MEKYVRLQKI GEGSFGKAVL VKSTEDGRHY VIKEINISRM SDKERQESRR
  51  EVAVLANMKH PNIVQYKESF EENGSLYIVM DYCEGGDLFK RINAQKGALF
 101  QEDQILDWFV QICLALKHVH DRKILHRDIK SQNIFLIKDG TVQLGDFGIA
 151  RVLNSTVELA RTCIGTPYYL SPEICENKPY NNKSDIWALG CVLYELCTLK
 201  HAFEAGNMKN LVLKIISGSF PPVSPHYSYD LRSLLSQLFK RNPRDRPSVN
 251  SILEKGFIAK RIEKFLSPQL IAEEFCLKTL SKFGPQPLPG KRPASGQGVS
 301  SFVPAQKITK PAAKYGVPLT YKKYGDKKLL EKKPPPKHKQ AHQIPVKKMN
 351  SGEERKKMSE EAAKKRRLEF IEKEKKQKDQ IRFLKAEQMK RQEKQRLERI
 401  NRAREQGWRN VLRAGGSGEV KASFFGIGGA VSPSPCSPRG QYEHYHAIFD
 451  QMQRLRAEDN EARWKGGIYG RWLPERQKGH LAVERANQVE EFLQRKREAM
 501  QNKARAEGHV VYLARLRQIR LQNFNERQQI KAKLRGENKE ADGTKGQEAT
 551  EETDMRLKKM ESLKAQTNAR AAVLKEQLER KRKEAYEREK KVWEEHLVAR
 601  VKSSDVPLPL ELLETGGSPS KQQVKPVISV TSALKEVGLD GSLTDTQEEE
 651  MEKSNSAISS KREILRRLNE NLKAQEDEKE KQHHSGSCET VGHKDEREYE
 701  TENAISSDRK KWEMGGQLVI PLDAVTLDTS FSATEKHTVG EVIKLDSNGS
 751  PRKVWGKNPT DSVLKILGEA ELQLQTELLE NTSFKSEVYA EEENYKPLLT
 801  EEENLQCISK EINPSATVDS TETKSPKFTE VSPQMSEGNV EEPDDLETEV
 851  LQEPSSTHTD GSLPPVLNDV WTREKEAAKE TELEDKVAVQ QSEVCEDRIP
 901  GNVDQSCKDQ RDPAVDDSPQ SGCDVEKSVQ PESIFQKVVH SKDLNLVQAV
 951  HCSPEEPIPI RSHSDSPPKT KSKNSLLIGL STGLFDANNP KMLRTCSLPD
1001  LSKLFRTLMD VPTVGDVHQD SLEIDELEDE PIKEGPSDSE DTVFEETDID
1051  LQELQASMEQ LLREQPGDEY SEEEESVLKS SDVEQTARGT DAPDEEDNPS
1101  SESALNEEWH SDNSDAETTS ECEYDSVFNH LEELRLHLEQ EMGFEKFFEV
1151  YEKVKAIHED EDENIEICST IVENILGNEH QHLYAKILHL VMADGAYQED
1201  NDE
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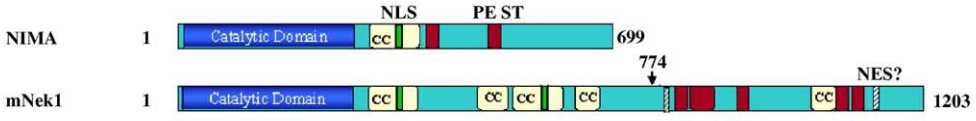
B.



C.

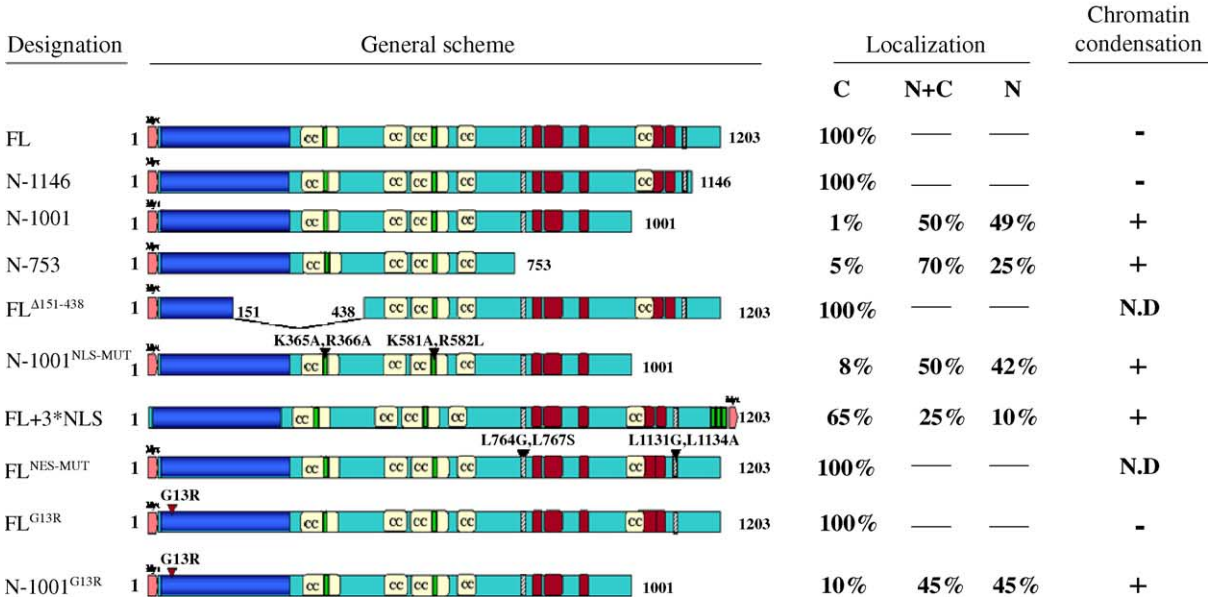


Fig. 1. Nek1 sequence and structure. (A) Complete sequence of the mouse Nek1 protein. The putative NLS motifs are highlighted by bold and underline. The carboxy extension (starting at position 774, marked with an asterisk) described in the text harbors potential PEST-rich sequences (proline, glutamine, serine and threonine-rich) (dashed underscore) and a putative NES (nuclear export sequence) (double underscore). The mutated NES leucines are in bold. (B) Schematic comparison of the structures of NIMA and Nek1. Paircoil analysis of NIMA sequence predicts one coiled-coil region (at a window of 28 residues, cutoff of 0.5), two potential PEST sequences and an NLS. Nek1 contains five predicted coiled-coil regions, five PEST-rich regions, and two classical NLS motifs. C. Major Nek1 constructs used in this study. Nek1 constructs were tagged with Myc at the N- or C-terminus. The subcellular distribution of the constructs 48 h after transfection, and the observed chromatin condensation phenotype are indicated.

calculated isoelectric point of murine Nek1 is 5.34, and not the very basic (9.46), reported previously [23]. According to the corrected sequence, Nek1 and NIMA proteins share several important characteristics (Fig. 1B): (A) As is the case with NIMA, and with several NIMA-related kinases, classical nuclear localization signals follow the catalytic domain. (B) A prominent portion of the Nek1 tail is composed of predicted coiled coil domains (at amino acids 350–410; 475–510; 525–600; 640–685 and 1035–1065). Coiled coil domains have been reported for NIMA and for several Nek kinases including Nek2, Nek8/Nercc1 and Nek11 [24,17]. For Nek2 and Nercc1, the coiled coil domains have been demonstrated to be essential for homodimerization and for autophosphorylation and activation [24,17]. (C) Like NIMA, Nek1 harbors several potential PEST sequences implicated in regulated protein degradation (at amino acids 810–824, 827–858, 911–927, 1018–1079 and 1088–1110). (D) Candidate mitotic phosphorylation sites in NIMA (SP and TP) also prevail in Nek1. Interestingly, SP repeats 13 times (including 3 times in the sequence SPSPCSP, immediately following the first putative NLS), while TP appears only once. These structural similarities strengthen the notion that Nek1 may have NIMA-related activities.

Even though the calculated molecular weight of Nek1 is about 137 kDa Western analyses of extracts from several cell lines and from mouse tissue lysates, with antibodies raised against the C-terminal 203 amino acids of Nek1 detected a prominent band running as a 180-kDa protein, and much fainter band at around 95 kDa (Fig. 2A, and see below). The same slow mobility was observed following overexpression of tagged full length Nek1 protein. The slower migration is probably not due to phosphorylation, as omission of phosphatase inhibitors in the extractions, and incubation with calf intestine phosphatase did not significantly affect Nek1 migration (not shown). The reason for the slow electrophoretic mobility is currently obscure, and it worth noting in this regard that similar slow migration phenomenon has been recently reported for the active Nek9/Nercc1 protein [25].

In accordance with the reported RNA expression patterns [23,26], highest levels of Nek1 protein were found in the testis, while lower levels were detected in ovary, lung, brain, heart and spleen (Fig. 2B, and not shown). Nek1 null Kat2J mice did not exhibit any protein staining (Fig. 2A, B), confirming the specificity of the antibodies, and that this mutant is truly null for Nek1 [22].

3.2. The full-length Nek1 protein is mainly cytoplasmic

The *Aspergillus* NIMA protein bears nuclear localization signals (NLS) and is temporarily localized to the nucleus during fungal mitosis [6]. However, overexpression of NIMA in mammalian cells results in cytoplasmic localization [27]. Nek1 protein contains two classical nuclear localization signals

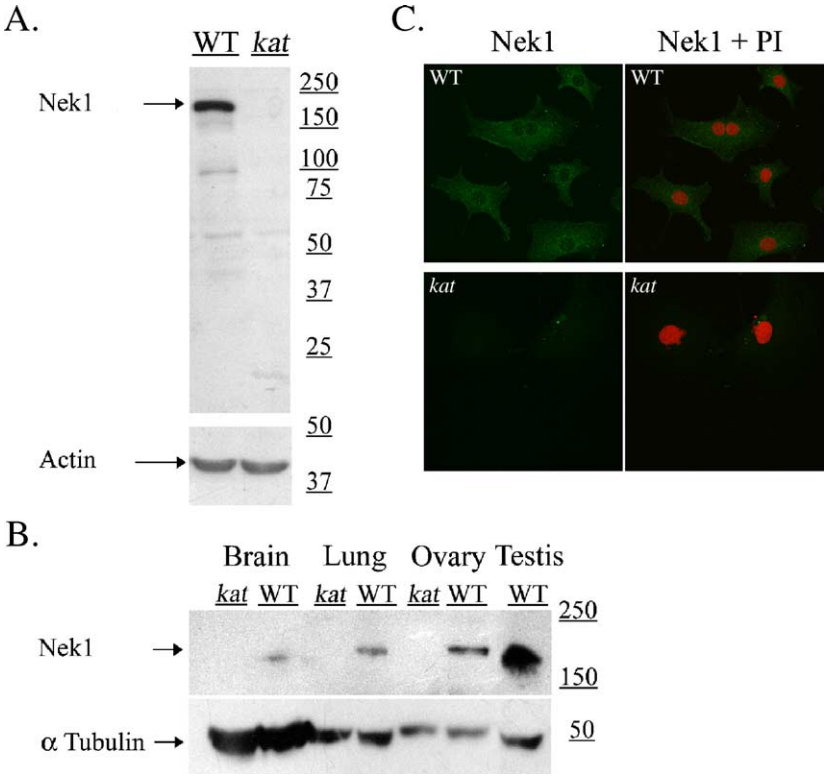


Fig. 2. Endogenous Nek1 expression. (A) Western blot analysis of Nek1 expression in extracts of 12.5 days embryonic heads of *wt* and *kat* mutants, using affinity-purified antibodies directed against the C-terminus of Nek1. The membrane was re-blotted with anti-actin antibodies to assess loading. (B) Western blot analysis of Nek1 expression in extracts of the indicated tissues from *wt* and *kat* mutants, using affinity-purified antibodies directed against the C-terminus of Nek1. The membrane was re-blotted with anti-αβ tubulin antibodies to assess loading. (C) Immunolocalization of endogenous Nek1 (stained with secondary anti-rabbit antibodies coupled to FITC) in embryonic fibroblasts from *wt* mice (upper panel), and *kat* mutants (lower panel). The nuclei were counterstained with propidium-iodide.

(364KKRR367 and 580RKRK583). However, immunohistochemistry with antibodies raised against the C-terminal portion of Nek1 demonstrated cytoplasmic localization of Nek1 in embryonic fibroblasts (Fig. 2C), and in various tissues (not shown).

To probe the sequences responsible for the subcellular localization of Nek1, a series of tagged Nek1 protein deletions and truncations was constructed and their subcellular localizations were examined following transient transfections into the HeLa, NIH3T3 and HEK293 cell lines (for schematic presentation of the various deleted/truncated constructs, and their localizations see Fig. 1C). Overexpression of full-length proteins tagged in either the C- or N-terminus resulted in almost exclusive cytoplasmic staining (Fig. 4A). Constructs containing the N-terminal 753 or 1001 amino acids of Nek1 (N-753 and N-1001, respectively), which include the putative NLSs, were found to shift from being mostly distributed in both the nucleus and the cytoplasm shortly following transfection, to nuclear localization by 48 h after transfection (Fig. 5A). However, the distribution of the full-length (FL) and almost full length Nek1 proteins (to amino acids 753 or 1146, N-753 and N-1146, respectively) were cytoplasmic, and did not change with time. Cell fractionation confirmed the restriction of the FL protein to the cytoplasm, and that the truncated protein are found in both the cytoplasm and the nucleus (Fig. 3C, and not shown).

To test the involvement of the putative NLS signals in the nuclear entrance, we mutated each of the putative NLS signals. Quite surprisingly, the mutations mildly reduced, but did not avoid, the entrance into the nucleus of the N-753 and N-1001 constructs (Fig. 1C). In addition, Nek1 fragment containing the second putative NLS motif (containing a.a. 439–1001) was entirely cytoplasmic, suggesting that this NLS motif (RKRK583) is non functional. Taken together, these results suggest that while Nek1 contains two classical NLSs, the full-length protein is cytoplasmic, as amino acids 1001–1146 contain sequences that inhibit nuclear localization. Truncated proteins, missing the C-terminus, localized to the nucleus following a delay, and their entrance is not solely dependent on their classical NLS motifs.

3.3. Nek1 cytoplasmic localization in not due to nuclear export

Two possible models can explain the contribution of the C-terminus to the cytoplasmic localization. It is possible that the C-terminal tail masks motifs essential for nuclear localization

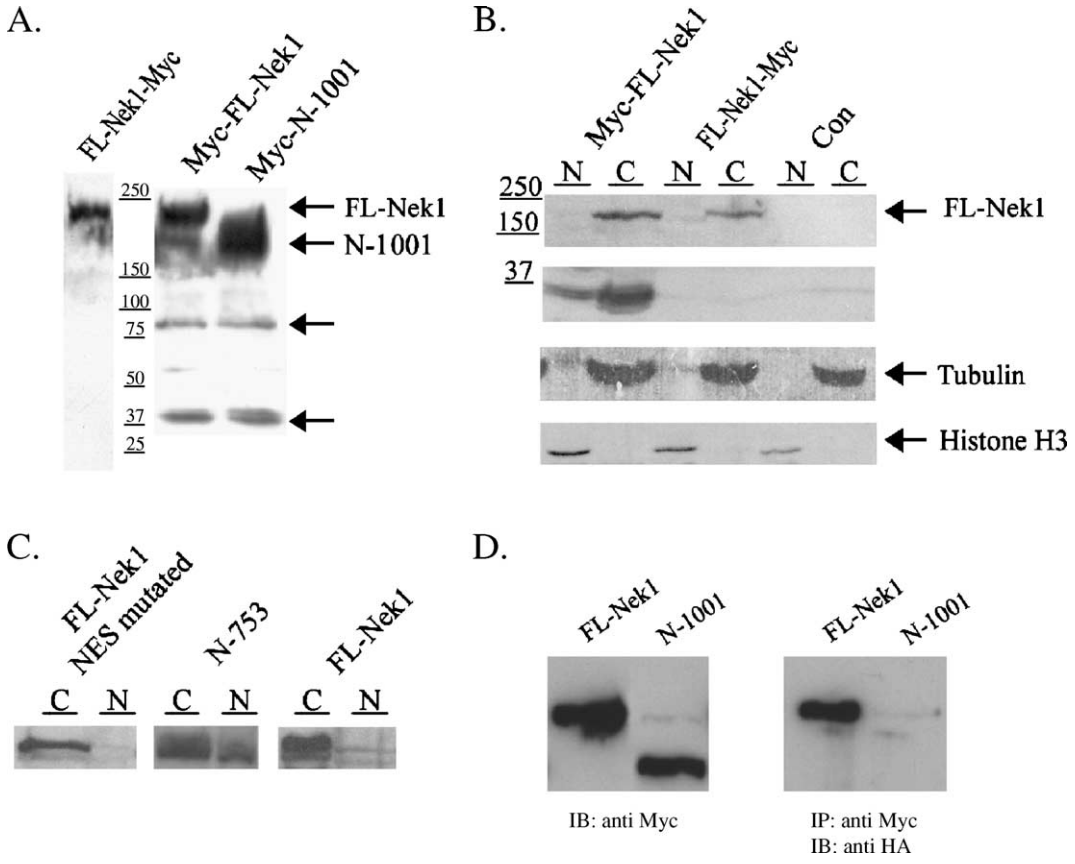


Fig. 3. Nek1 cleavage, cellular localization and co-precipitation. (A) Overexpression of Nek1 constructs harboring myc tag in the N-terminus (myc-FL-Nek1 and myc-N-1001) results in a prominent band of about 40 kDa. No such band is observed following overexpression of FL protein tagged in the C-terminus (FL-myc-Nek1). (B) Cell fractionation of the full-length and the 40-kDa fragment. The 40-kDa is localized both to the cytoplasm and to the nucleus. Tubulin and Histone H3 serve as cytoplasmic and nuclear markers, respectively. (C) Cell fractionation of the truncated and NES-mutated Nek1 constructs. (D) Co-immunoprecipitation of FL and N-1001 proteins. Left panel-myc-tagged FL and N-1001 proteins overexpressed in HEK293 cells and detected by Western blotting with anti-myc antibodies. Right panel-co-immunoprecipitation of HA-tagged FL protein by co-expressed FL myc-tagged protein, but not by co-expressed myc-tagged N-1001 protein.

(e.g. binding to proteins bearing NLS), most probably by intramolecular folding. Alternatively, the C-terminal fragment could contain sequences that retain Nek1 in the cytoplasm, either by docking it to cytoplasmic elements or by inducing nuclear export. Assuming the first explanation, addition of an ectopic NLS signal to Nek1 protein should lead to Nek1 entrance into the nucleus. However, hooking three repeats of the SV40 NLS to the C-terminus of Nek1 (yielding Nek1-myc-NLS) resulted in only low percentage of cells in which Nek1 was localized to the nucleus (Fig. 1C).

Examination of the C-terminal fragment revealed a potential nuclear export sequence (NES): 1127VFNHLEELRLHL1138, which conforms to the NES consensus (and an additional putative NES signal starting at a.a. 764, LKILGEAELQL). To examine the functionality of these plausible NES, the two of the leucine residues were mutated (creating the sequences VFNHGEEARLHL and GKISGEAELQL). Analogous mutations in different NES sequences cripple nuclear export induction by corresponding elements [28]. However, the double NES-mutated FL protein remained in the cytoplasm, suggesting that these sequences are not essential for the cytoplasmic retention of Nek1 (Fig. 3C, and not shown).

We next examined the possible contribution of Nek1 kinase activity to Nek1 localization. Autophosphorylation kinase assays for immunoprecipitated myc-Nek1 constructs overexpressed in HEK293 cells confirmed the activity of the kinase-containing constructs (not shown). To generate a kinase negative version of Nek1, the invariant Gly at a.a.13 was mutated to Arg (designated G13R) (at the ATP-binding site; kinase subdomain I). The cytoplasmic distribution of the full-length construct, as well as the nuclear localization of the N-1001 construct, were not affected by the G13R mutation (Fig. 1C), suggesting that Nek1 localization is not dependent on its enzymatic activity.

Interestingly, in Western analyses of overexpressed Myc-Nek1, we consistently observed a prominent Nek1 band of about 40 kDa (and slighter additional band of about 85 kDa), corresponding to the N-terminal portion of Nek1 (containing the kinase domain and the first NLS) (Fig. 3A). It is thus possible that a specific proteolytic processing step yields a putative nuclear kinase, which could explain the weak nuclear signal observed following overexpression of the full-length Nek1 sequence. Indeed, cell fractionation revealed that while the FL 180 kDa protein is almost exclusively restricted to the cytoplasm, the 40 kDa fragment is equally distributed in the nucleus and the cytoplasm (Fig. 3B).

3.4. Overexpression of truncated Nek1 protein results in chromatin condensation

Forced expression of NIMA in mammalian cells disrupts the cell cycle [2]. Examination of HeLa cells overexpressing the myc-Nek1 constructs revealed an elevated frequency of multinucleated cells. At 72 h following transfection, a significant portion of the transfected cells (~5%) had three or more nuclei, with bulky cytoplasm (Fig. 4B). Many of these giant cells displayed unevenly-sized or lobed nuclei. Interestingly, this phenotype was also observed following transfection with a construct missing most of the catalytic domain ($FL^{\Delta\text{-}151-438}$, Fig. 4C). Thus, the phenotype is not dependent on Nek1 kinase activity. Such multinucleated huge cells were rarely observed in HeLa cells transfected with a construct containing GFP alone, or in cells transfected with a construct containing an internal Nek1 fragment (a.a. 151–438) (<1%, not shown).

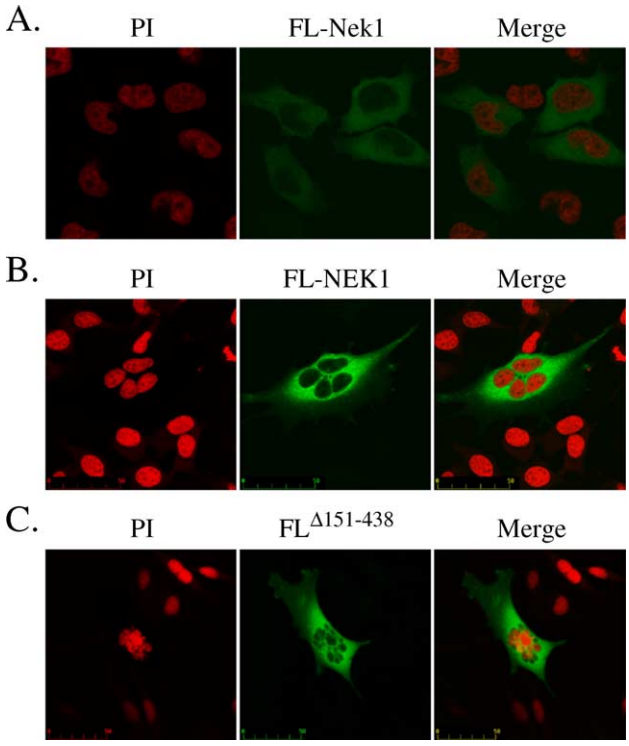


Fig. 4. FL Nek1 is localized to the cytoplasm and induces multinucleation in HeLa cells. (A) Staining of HeLa cells transfected with FL Nek1 construct showing distribution of transfected protein. (B and C) Overexpression of FL Nek1 (B) or a deleted form ($FL^{\Delta 151-438}$) (C), induces the formation of giant, multinucleated cells.

Intriguingly, following transfection of HeLa cells with the construct harboring the first 753 or 1001 amino acids of Nek1, a dramatic change in chromatin staining was noticed. By 24 h after transfection, some of the cells had uneven chromatin distribution, with higher condensation at the periphery of the nucleus (not shown). As noted before, by 24 h the truncated Nek1 constructs were localized to the nucleus in only about 18% of the cells, and the phenotype almost exclusively appeared in the cells exhibiting nuclear Nek1 localization. By 48 h, there was clear correlation between N-1001 localization and the cellular phenotype. In almost all of the cells, N-1001 was found in either the nucleus or in both the nucleus and the cytoplasm. Within this population, in about 60–70% of the cells, DNA staining revealed dense peripheral chromatin ring, concomitant with appearance of large unstained spaces within the nuclei of the transfected cells (Fig. 5A). DNA condensation was also apparent around nucleoli (white arrows in Fig. 5A, and not shown). Nuclear N-1001 localization was restricted to the nucleoplasm, and was absent from nucleoli spaces (Fig. 5A). However, in cases that N-1001 was localized to the cytoplasm, no chromatin condensation was observed.

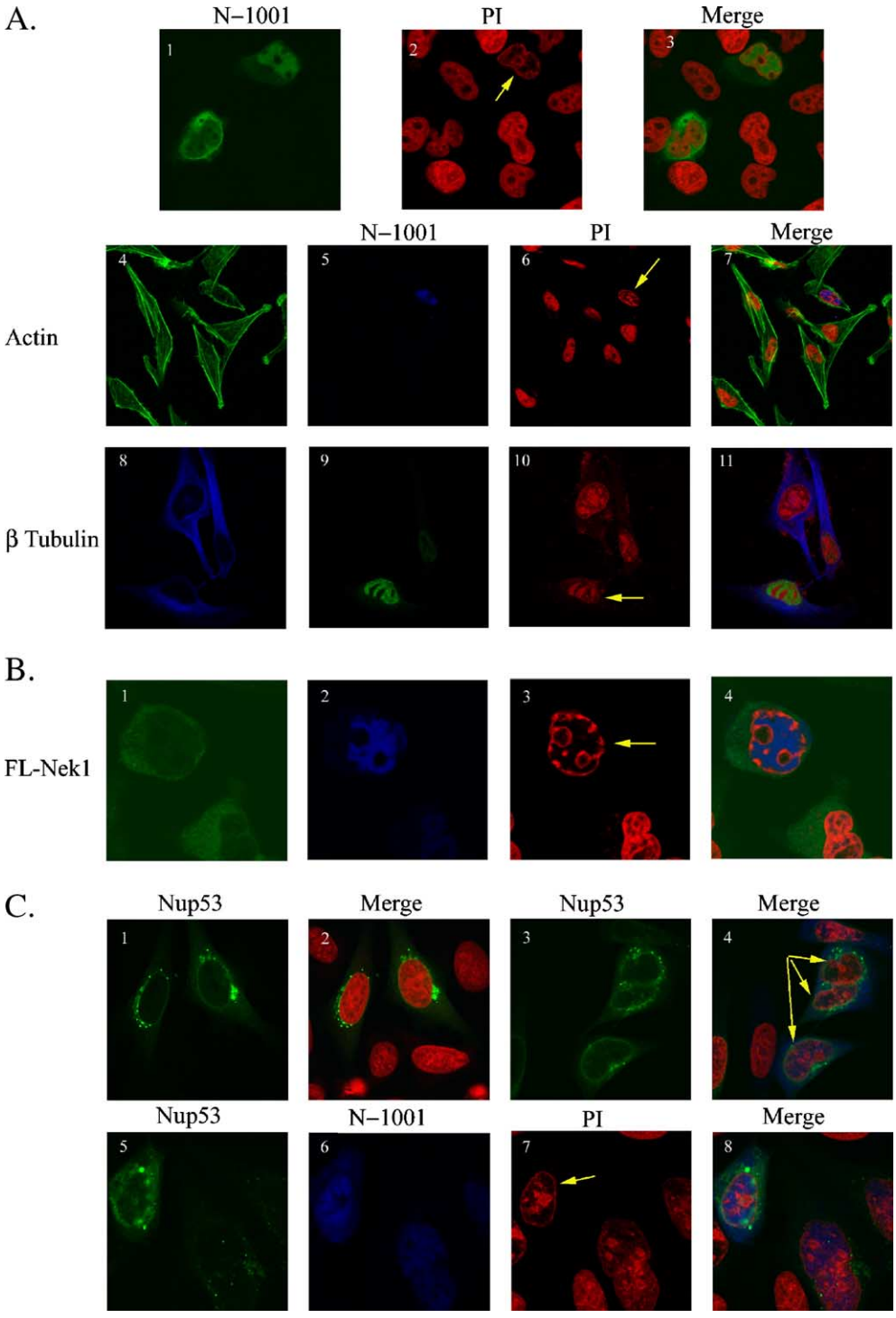


Fig. 5. Nek1 overexpression induces chromatin condensation. (A1–3) Transfection with a construct harboring amino acids 1–1001, followed by staining for propidium-iodide (PI) and Myc-tag (left panel). A merged view is seen in the right panel. Nek1 localization to the nucleus (marked by an arrow) was correlated with induction of chromatin condensation. Note that the chromatin distribution is mostly peripheral and around the nucleoli. Cell displaying N-1001 cytoplasmic localization (marked by arrowhead) does not exhibit the chromatin condensation phenotype. (A4–7). Actin fiber and (A8–11) microtubule staining in N-1001 transfected cells. (B) Co-overexpression of HA-FL Nek1 (green) and myc-N-1001 (blue). Chromatin condensation phenotype did not affect the FL protein localization. (C) Nup53-GFP localization in N-1001 overexpressing cells. (C1–2) Nup53-GFP localization in control cells. (C3–4) Early condensation phenotype of N-1001 transfected cells did not affect Nup53 distribution. (C5–8) Cells exhibiting strong chromatin condensation. Note Nup53 diffused staining.

Immunofluorescence analysis of the cytoskeleton of the cells which demonstrated chromatin condensation revealed decrease in cell extensions and some reduction in actin stress fibers density, and microtubule staining (Fig. 5A4–11). More advanced and stronger condensation was accompanied by cell rounding and sharp reduction in microtubule staining (not shown). In the cases in which N-1001 protein was cytoplasmic, the transfected cells did not exhibit chromatin

condensation, and the cytoskeleton did not show any obvious alteration.

Similar chromatin condensation was also observed in all of the Nek1 constructs exhibiting nuclear localization, including FL-myc-NLS in which the full-length protein was forced into the nucleus. Transfection with a kinase-mutated version of the N-1001 protein (N-1001^{G13R}) yielded much weaker chromatin condensation phenotype, and appeared in smaller fraction of the transfected cells (approx. 40% of the cells expressing the protein in the nucleus).

NIMA has been shown to interact genetically with nucleoporin proteins, and it has been suggested that NIMA-nucleoporin interaction is critical for dispersal of some NPC proteins, and aids in the transport of mitotic regulators into the nucleus during the fungal closed mitosis [6,7,9]. Over-expression of NIMA in mammalian cells induces nuclear membrane and nucleoporin dispersal [2]. We therefore examined whether N-1001 influences nucleoporin localization. Co-transfection of N-1001 and the nucleoporin Nup53-GFP revealed N-1001 nuclear staining in cells with intact nucleoporin localization (Fig. 5C1–4), suggesting that Nek1 nuclear entrance and (initial) chromatin condensation are independent of nuclear membrane breakdown and nuclear pore disassembly. However, advanced chromatin condensation was accompanied with diffuse Nup-53-GFP staining, or its disappearance (Fig. 5C5–8).

The observed correlation between N-1001 nuclear localization and chromatin condensation could reflect the initial effect of the truncated Nek1 on protein traffic through the nuclear pores, which would consequently allow N-1001 entrance into the nucleus. Next, we wondered whether the overexpression of the N-1001 protein could affect the nuclear pore properties thus allowing FL-protein entrance into the nucleus. To this end, we co-transfected the FL-Nek1 (HA-tagged) construct with the N-1001 (myc-tagged) species. Co-transfected cells exhibited the chromatin condensation phenotype, however, the FL-protein localization was not influenced and it remained almost exclusively cytoplasmic (Fig. 5B), suggesting that the different distribution of the FL versus the C-terminally truncated proteins is not solely due to disruption of the nuclear membrane by the truncated proteins.

Overexpression of two different FL-Nek1 constructs tagged with either HA or Myc in HEK293 cells followed by reciprocal co-precipitation suggested that the Nek1 protein is expressed as a dimer (or oligomer) (Fig. 3D). In agreement with the distinct localization of the co-expressed constructs, the FL-Nek1 construct did not precipitate the N-1001 protein. The dimerization motif is thus localized in the C-terminal 203 amino acids, the same region which governs the cytoplasmic retention.

3.5. Chromatin condensation induced by Nek1 does not induce phosphorylation of Histone- H3 Ser10 and is not due to apoptotic events

Chromatin condensation during mitosis is coupled to phosphorylation of histone H3 on serine-10, and this phosphorylation serves as a common marker for mitotic chromatin condensation. To examine whether the change in chromatin structure resulting from Nek1 overexpression is associated with phosphorylation of serine-10, cells were stained with antibodies that specifically recognize P-S^{10}. Whereas mitotic cells stained positively for P-S^{10}, N-1001 transfected cells did not exhibit histone phosphorylation (Fig. 6A).

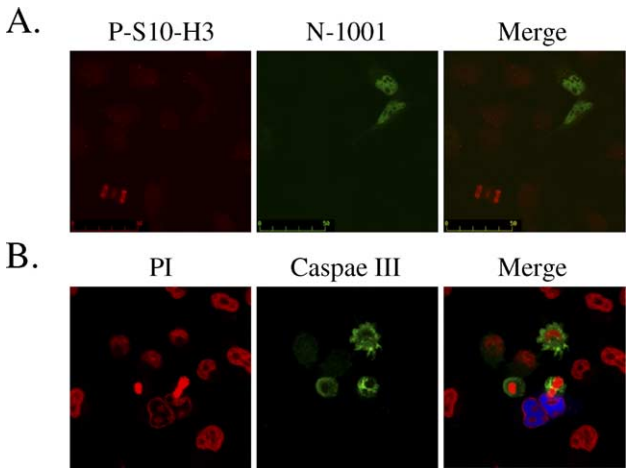


Fig. 6. Chromatin condensation induced by N-1001 overexpression is not coupled to Histone-H3 phosphorylation or Caspase 3 activation. (A) Cells were transfected with N-1001 construct and stained for N-1001 (green) and anti p-Ser10 (red). (B) Cells were transfected with N-1001 construct and stained for DNA (PI-red), for N-1001 construct (blue), and for apoptotic cells with antibodies against activated Caspase 3 (green).

Condensation of the chromatin into a peripheral nuclear ring characterizes some early and transient forms of apoptosis. However, the DNA of spontaneously apoptotic HeLa cells or etoposide-induced apoptotic cells appeared discontinuous and punctuated, while the DNA of N-1001 transfected cells formed an uninterrupted ring. In addition, by 48 h post-transfection, classical apoptotic nuclear morphology of condensed oligonucleosomal chromatin fragments [29] was rarely observed in the N-1001 transfected cells. To rule out the possibility that the abnormal chromatin staining observed following N-1001 overexpression represented early stages of an apoptotic event that was then been stabilized, HeLa cells were harvested 24 or 48 h following N-1001 transfection. Comparison of N-1001 to control-DNA transfected cells, did not show a significant difference in the percentage of sub-G1 phase cells in FACS analysis, or of Annexin-V stained cells (data not shown). Apoptosis is usually correlated with the activation of the caspase cascades, ultimately leading to activation of the effector caspases 3, 6 and 7 [30]. Gene targeting of caspase 3 demonstrated that it is essential for apoptotic chromatin condensation and DNA degradation [31]. To examine possible involvement of caspase 3 in Nek1-induced chromatin condensation, HeLa cells were transfected with N-1001, fixed after 48 h, and stained for DNA (propidium iodide), the Nek1 myc tag, and activated caspase 3. No correlation was found between Nek1 induced chromatin condensation and caspase 3 activity (Fig. 6B). These results suggest that the changes in chromatin structure following N-1001 transfection are not mediated by

caspase 3, and that the chromatin condensation is not due to an apoptotic event.

4. Discussion

While the founding member of the mammalian NIMA-like family, Nek1, was characterized about a dozen years ago, the cellular and molecular roles of Nek1, and their possible relationship to the documented mitotic roles of NIMA are still enigmatic. Absence of Nek1 activity (as in the *kat* mutant mouse) leads to dwarfism, male-specific sterility and polycystic kidneys [22]. No overt mitotic phenotype is observed in the mutants, though the polycystic kidney disease and the dwarfism could result from a cell cycle defect. The corrected full-length Nek1 sequence presented here extends its structural similarity to NIMA, strengthening the likelihood that Nek1 performs NIMA-like activities.

Our results indicate that Nek1 protein bears two classical NLS, yet the full-length protein is cytoplasmic. Deletion analyses identified a segment spanning amino acids 1002–1145 as responsible for the cytoplasmic localization of the full-length protein. Although this segment contains a classical nuclear export signal our experiments suggest that this putative NES sequence is not functional in the native protein. Thus, the 1002–1145 fragment of Nek1 may retain Nek1 in the cytoplasm, as a result of intramolecular folding that masks the NLS, or by binding to a cytoplasmic protein. Alternatively, it is possible that additional, unidentified, nuclear export signals reside in this fragment, which interacts directly with an export protein, or indirectly with a protein bearing NES. According to this possibility, Nek1 may shuttle between the cytoplasm and the nucleus. Interestingly, in *Aspergillus* cells, NIMA enters the nucleus towards mitosis [6], and it has been shown to influence cyclin B localization to the nucleus [8]. This activity could be compensated by a mutation in the nucleocytoplasmic transporter SONA/RAE1 [8]. It has been recently shown that the nucleoporins SONA and SONB control the entrance of NIMA into the nucleus, and it has been proposed that NIMA phosphorylates these nucleoporins and thereby regulates the mitotic nuclear pore complex [7,9]. However, it is not clear whether NIMA itself shuttles between the two compartments, and which determinants control the temporal NIMA localization.

Overexpression of several cytoplasmic Nek1 constructs resulted in an increased frequency of multinucleated cells. The nuclei were usually of uneven size and micronuclei were frequently observed. Highly similar phenotypes have been associated with overexpression of Nek9/Nercc1 [17], and mutated or kinase-defective Nek8 [32]. Inactivation of Nek8 in *jck* mutant mice and of Nek1 in *kat* mutants both result in severe polycystic kidney. Thus, Nek1 and Nek8 could operate in the same signal transduction cascade, or might be components of the same complex. However, coimmunoprecipitation assays of Nek1 and Nek8 failed to detect association between the two proteins, and co-overexpression of Nek1 and mutant Nek8 constructs did not result in a synergistic effect in the frequency of the multinucleation phenotype (unpublished results).

Overexpression of the nuclear N-1001 or N-753 constructs results in a striking phenotype. The chromatin appears condensed and is localized mainly to the periphery of the nucleus. Examination of N-1001 localization suggests that the protein is initially mainly cytoplasmic (or perinuclear), and nuclear entrance is correlated with chromatin condensation. We therefore suggest that cytoplasmic Nek1 expression affects (directly or indirectly) the nuclear membrane or the nuclear pore complex, allowing Nek1 entry into the nucleus. The catalytic activity of Nek1 contributes, but is not critical for this process, as the kinase-dead N-1001 version exhibits a similar, albeit much weakened and less frequent, chromatin condensation phenotype. The lack of chromatin condensation activity of the FL Nek1 protein could be due to its quite strict cytoplasmic localization, or to a difference in the spectrum of cellular proteins with which it interacts. Even though the FL protein did not demonstrate chromatin condensation activity in our experiments, it is possible that under some conditions, either as a result of Nek1 modification, or truncation of the C-terminus, Nek1 may enter the nucleus and become involved in chromatin modifications. Interestingly, it has been recently demonstrated that following ionizing radiation, Nek1 protein could be found in discrete nuclear foci, in colocalization with other DNA damage response proteins [33]. It is notable in this regard, that in addition to the expected full-length Nek1 protein, we identified a major band of about 40 kDa, probably representing truncation at around amino acid 350, C-terminal to the first NLS, and cell fractionation suggests that it could enter the nucleus.

Overexpression of NIMA in mammalian cells has been reported to induce peripheral ‘worm-like’ chromatin condensation [2]. This effect is distinct from the mitotic chromatin condensation in *Aspergillus*. In fungal cells, NIMA activity induces mitotic-like histone-H3 phosphorylation on ser-10, while no such phosphorylation has been documented in mammalian cells following either NIMA [27], or N-1001 overexpression. A similar condensation phenotype was recently reported following MLK3 overexpression, a kinase which shares sequence similarity with NIMA in the non-catalytic domain [27]. It should be noted, however, that the NIMA and MLK3 effect was observed even though the proteins were mainly cytoplasmic [27]. Nevertheless, the nuclear phenotype of NIMA and MLK3 could be due to leakiness, or to shuttling through the nucleus. Alternatively, as NIMA was reported to induce nuclear membrane breakdown and P62 nucleoporin dispersal in HeLa cells [2], it is possible that the chromatin effect is induced following NIMA’s entrance into the nucleus.

Our results indicate that Nek1 could be involved in chromatin condensation. Nevertheless, null mutants for the *nek1* gene, *kat* mice, suffer from developmental abnormalities, but they do not exhibit an obvious cell cycle defect. It is possible that the absence of Nek1 is compensated by the activity of another (Nek) kinase. Alternatively, as the highest expression levels of *nek1* were observed in the developing germ cells, and mutants of Nek1 suffer from sterility, it is possible that Nek1 is involved in meiotic chromatin condensation. Nek1 molecular pathways and cellular targets are yet to be explored.

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