STRUCTURE AND REGULATION OF THE HUMAN NEK2 CENTROSOMAL KINASE

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The dimeric Ser/Thr kinase Nek2 regulates centrosome cohesion and separation through phosphorylation of structural components of the centrosome, and aberrant regulation of Nek2 activity can lead to aneuploid defects characteristic of cancer cells. Mutational analysis of autophosphorylation sites within the kinase domain identified by mass spectrometry shows a complex pattern of positive and negative regulatory effects on kinase activity that are correlated with effects on centrosomal splitting efficiency in vivo. The 2.2Å resolution X-ray structure of the Nek2 kinase domain in complex with a pyrrole-indolinone inhibitor reveals an inhibitory helical motif within the activation loop. This helix presents a steric barrier to formation of the active enzyme and generates a surface that may be exploitable in the design of specific inhibitors that selectively target the inactive state. Comparison of this 'auto-inhibitory' conformation with similar arrangements in CDK2 and EGF receptor kinase suggests a role for dimerisation-dependent allostery in the regulation of kinase activity that combines with autophosphorylation and PP1c phosphatase activity to generate the precise spatial and temporal control required for Nek2 function in centrosomal maturation.

Introduction

Protein kinase activity is crucial for the precise regulation of the eukaryotic cell cycle. Although cyclin-dependent kinases remain the master regulators of cell cycle progression, it is clear that a variety of other proteins kinases also play important roles. Nek2 is a member of the NIMA-related kinase - or Nek - family of serine/threonine protein kinases (STKs), so named because all are related to the NIMA kinase of Aspergillus nidulans (reviewed in (1)). In Aspergillus, NIMA is essential for mitotic entry with temperature sensitive nimA mutations leading to cells that are 'never in mitosis' (2). Like Aspergillus, other fungi also express a single NIMA-related kinase, e.g. Kin3 in S. cerevisiae and Fin1 in S. pombe. Whilst the yeast Neks do not appear to be essential for cell cycle progression, elegant studies on Fin1 have revealed key functions in chromosome segregation and mitotic exit (3,4). In contrast, higher eukaryotes express multiple Neks with eleven genes (Nek1 to Nek11) encoded within the human genome (1). With the exception of human Nek6 and Nek7 and Nek10, all Neks have an N-terminal kinase domain followed by a C-terminal non-catalytic regulatory domain. However, the length and composition of motifs present within these C-terminal extensions vary considerably reflecting diverse roles in cell regulation.

Of the human proteins, Nek2 is the most closely related to the fungal kinases being 47% identical to NIMA within the amino acid sequence of their catalytic domains. Nek2 localizes to centrosomes. Here, it contributes to spindle pole formation during mitosis (5) through phosphorylation of proteins involved in centriolar cohesion, including C-Nap1 and rootletin, to allow spindle pole separation (6-8), and others such as Nlp, required for microtubule anchoring and spindle assembly (9). Interestingly, Nek2 homologues in Drosophila
...and Dictyostelium, as well as both NIMA and Fin1, localize to the microtubule organizing centre and functional studies support roles in centrosome organization (4,10-12). Furthermore, it appears that other mammalian Nek family kinases may have microtubule-related functions in cell cycle progression or cilia formation (13,14). In addition to acting at the centrosome, there is some evidence that Nek2 may contribute to other aspects of mitotic progression including chromatin condensation and spindle checkpoint signalling (15-17).

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 (18). At the G1/S transition, vertebrates express two major splice variants, Nek2A and Nek2B (19,20). These variants differ in their extreme C-termini which has important implications for their regulation as the C-terminus of Nek2A, but not Nek2B, contains both a binding site for protein phosphatase 1 (PP1) and motifs which target the protein for ubiquitin-mediated degradation following mitotic entry (21-23). Like many protein kinases, Nek2 activity is also subject to control through its phosphorylation. Downstream of the kinase domain is an unusual leucine zipper motif that mediates dimerisation as a prerequisite for efficient autophosphorylation and thus full Nek2 activity on exogenous substrates (24). Interaction of Nek2A with PP1 can lead to dephosphorylation and inhibition of Nek2 suggesting that Nek2 may only become fully activated once PP1 is inactivated at the onset of mitosis (22,25). Besides autophosphorylation, Nek2 may be regulated by upstream kinases. Indeed, the catalytic domain of Nek2 is phosphorylated in vitro by p90\textsuperscript{Rsk2}, an interesting observation as the activation of chromatin condensation by Nek2 in mouse spermatocytes is under the control of the MAPK/p90\textsuperscript{Rsk2} pathway (26).

In spite of the importance of Nek2 in cell cycle control, its regulation by phosphorylation remains poorly understood. Indeed, no structural information on any Nek kinase family has been reported in spite of the fact that, together, they constitute ~2% of the entire human kinome. Here, we report the structure of the kinase domain of human Nek2 in complex with a pyrrole-indolinone inhibitor SU11652 together with an analysis of the role of autophosphorylation in Nek2 regulation. Given that Nek2 is upregulated in a number of human cancers, and that down-regulation can inhibit cell proliferation (27), our observations have clear significance for the design of Nek2 inhibitors as potential anticancer agents and now provide a structural and mechanistic framework for understanding how this important cell cycle kinase is regulated, with implications for the control of Nek-family kinases as a whole.

**Experimental Procedures**

Nek2 kinase domain expression, purification and crystallisation

For expression of Nek2 constructs, DNA sequences were amplified by PCR, cloned between the Nde1 and Xho1 sites of pET22b and the resulting plasmids used to transform *E. coli* BL21(DE3). Cultures were grown at 37°C to an OD\textsubscript{600} of ~0.7, the temperature was adjusted to 18°C and expression induced for 4 hours using 1 mM IPTG. Cells were collected by centrifugation, resuspended in buffer A (50 mM HEPES pH 7.5, 5 mM sodium phosphate, 300 mM NaCl, 5% glycerol) supplemented with 20 mM imidazole, and lysed by sonication. Clarified lysate was loaded onto a Ni-NTA column equilibrated with lysis buffer and washed with the same buffer supplemented with 1 M NaCl. Bound Nek2 eluted in buffer A plus 150 mM imidazole. The protein sample was concentrated by ultrafiltration and fractionated on a S75 Superdex column equilibrated in 50 mM HEPES pH 7.5, 300 mM NaCl, 10 mM sodium phosphate, 5 mM DTT, 5% glycerol. Eluted fractions were 95% pure as judged by SDS-PAGE. For crystallization, phosphates were removed by treatment with shrimp alkaline phosphatase and λ-phosphatase for 12 h at 4°C.

Crystallisation, structure determination and refinement

Crystals were obtained at 4°C using vapour diffusion sitting drops at a protein concentration of 7.5 mg/ml containing 1 mM of a pyrrole-indolinone compound (5-[(Z)-(5-chloro-2-oxo-1,2-dihydro-3H-indol-3-ylidene) methyl]-N-[2-(diethylamino)ethyl]-2,4 dimethyl-1H-pyrrole-3-carboxamide). The complex was crystallized by mixing with precipitant (10% PEG 6000, 200 mM MgCl\textsubscript{2}) at a ratio of 3:1. Monoclinic plates grew over 3-4 weeks to a maximum size of 75 x 75 x 10 µm\textsuperscript{3}.

Crystals were vitrified in mother liquor supplemented with 25% ethylene glycol and...
diffraction data were collected at 100K on beamline X10A ($\lambda = 0.95\AA$) at the Swiss Light Source (SLS). Data were integrated, merged and scaled using MOSFLM and SCALA. The structure was solved by molecular replacement with PHASER (28) using Aurora-A kinase (PDB ID: 1OL7) as a search model. The C-terminal lobe was well resolved in the initial electron density maps but density for the N-terminal lobe was poor due to conformational changes between the search and target structures. Attempts to place the isolated N-terminal lobe using PHASER failed. Instead, the domain was manually placed in the density and its position optimized using rigid-body refinement. Rounds of model building interspersed with restrained refinement incorporating TLS were carried out using REFMAC5 (29) and COOT (30). The coordinates are available in the Protein Data Bank (Accession code 2CL2).

**Autophosphorylation site mapping**

For identification of autophosphorylation sites by mass spectrometry, an enriched subset of phosphorylated peptides was generated using gallium-chelated IMAC (immobilized metal affinity chromatography) spin columns (Pierce). The phosphorylated protein to be studied was first digested with sequencing grade modified trypsin (Promega) at 1:10 (trypsin:target w/w). The digestion was carried out in 10 mM ammonium bicarbonate overnight at room temperature. The tryptic digest was diluted 1:1 with 20% acetic acid and incubated on the gallium resin for 30 minutes at room temperature. The resin was washed with 10% acetic acid, followed by 10% acetic acid plus 20% acetonitrile. The resin was then equilibrated with water followed by elution in 100 mM ammonium bicarbonate (pH 9.0) plus 10% acetonitrile. Identification of phosphorylated sites was achieved using a combination of ESI-MS, MALDI-TOF and tandem MS-MS methods (Supplementary Methods).

**Site-directed mutagenesis**

Mutations were introduced by PCR-based mutagenesis of the pET22b-Nek2A, pGEM-Nek2A or pRcCMV-myc-Nek2A plasmids using the Gene Tailor™ Site–Directed Mutagenesis System (Invitrogen Ltd., U.K.). All constructs were confirmed by DNA sequencing.

**Kinase assays**

Myc-tagged Nek2 proteins were synthesized from the appropriate pGEM or pRcCMV constructs using coupled in vitro transcription-translation reactions according to manufacturer’s instructions (Promega U.K.). Proteins were immunoprecipitated using anti-myc antibodies (0.4 µg/ml; Cell Signalling) and a fraction of the immune complexes were used for analysis by SDS-PAGE and Western Blotting using an anti-Nek2 antibody (1 µg/ml; Zymed (Fry, et al 1999)). The remaining immune complexes were used for kinase assays. Specifically, 5-10 µl of washed immune complex beads were incubated with 5 µg β-casein or the C-terminal domain of C-Nap1 and 1 µCi [$^{32}$P]-γ-ATP as substrates in 40 µl kinase buffer (50 mM Heps:KOH pH 7.4, 5 mM MnCl$_2$, 5 mM β-glycerophosphate, 5 mM NaF, 4 µM ATP, 1 mM DTT) at 30°C for 30 minutes. Reactions were stopped with 50 µl protein sample buffer and analysed by SDS-PAGE and autoradiography. Western blots were quantified using NIH image software and substrate phosphorylation quantified by scintillation counting of proteins excised from dried gels.

**Cell culture and transfection**

U2OS osteosarcoma and HeLa cervical carcinoma cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen Ltd., U.K.) supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin, at 37°C in a 5% CO$_2$ atmosphere. Transient transfections were performed with either Lipofectamine 2000 (Invitrogen Ltd., U.K. or FuGENE 6 (Roche Diagnostics Ltd., U.K.) according to manufacturer’s instructions.

**Immunofluorescence microscopy**

Immunofluorescence microscopy was performed as previously described (31). Primary antibodies used were anti-γ-tubulin (0.15 µg/ml; Sigma) and anti-myc (0.4 µg/ml) antibodies. Secondary antibodies used were Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 594 goat antimouse IgGs (1 µg/ml; Invitrogen Ltd. U.K.). Fluorescence images were captured on a TE300 inverted microscope (Nikon, U.K.) using an ORCA ER CCD camera (Hamamatsu, Japan) using Openlab 3.5.1 software (Improvision, Coventry, U.K.) and processed using Adobe Photoshop (San Jose, U.S.A.).
Results & Discussion

Bacterial expression of Nek2 constructs and phosphorylation site mapping

Initial attempts to over-express full-length human Nek2A or a 271 residue fragment encompassing just the predicted kinase domain in E. coli were unsuccessful due to apparent toxicity of the recombinant enzymes. However, we were able to circumvent these problems by constructing mutant kinase domains with substitutions at the highly conserved catalytic Asp141 (D141A) or Lys37 (K37R) that could be expressed and purified in high yield. In contrast to the D141A protein, which showed no autophosphorylation at all, ESI-MS analysis of the K37R domain showed auto-phosphorylation at an average of four sites (Supplementary Figure S1A). Of these, one site, Thr175 within the ‘activation’ or T-lobe of Nek2 was unambiguously identified by tandem-MS analysis. Expression of full-length K37R Nek2A yields an unstable product, but a full-length construct containing a T175A substitution showed both low toxicity and increased stability. Purified protein showed phosphorylation on an average of thirteen sites. Two sites (Thr170 or Ser171, Thr179) map to the Nek2 T-lobe region, and a third, Ser241, is located within the kinase C-lobe (see below). A further six phosphorylation sites were assigned within the C-terminal regulatory region (Figure 1A and Supplementary Figure S1B). Four occur around the KEN-box, suggesting a potential autoregulatory role at the level of Nek2 stability. In addition, Ser356 and Ser428 lie within predicted regions of coiled-coil and may therefore be involved in modulating Nek2A dimerisation and localisation. These ideas are currently under investigation.

Structural analysis of the Nek2 kinase domain

In order to maximize yields of recombinant protein and minimise structural perturbations around the ATP-binding site, crystallisation efforts were focussed on the T175A mutant of the Nek2 kinase domain. The purified recombinant protein is heterogeneously phosphorylated and crystallisation required prior dephosphorylation by alkaline and λ phosphatase. Crystallisation of complexes with non-hydrolysable ATP analogues was unsuccessful. However, diffracting crystals were obtained of a complex with a single inhibitor, SU11652, identified using temperature shift assays (Supplementary Methods). The structure was solved by molecular replacement using the Aurora-A kinase structure as a search model and refined against diffraction data extending to 2.1Å resolution. The final model comprises the entire kinase domain (residues 3-271), the C-terminal hexahistidine tag, 107 water molecules and the pyrrole-indolinone ligand. Residues within the loop N-terminal to αC (Thr45-Met51), the loop connecting the two strands β4 and β5 (Arg77-Thr81), the region C-terminal to helix αE (Asp132-Arg140) and the activation segment residues between Asn167-Val177 are not visible in the electron density and presumably disordered. The N-terminal lobe (Ser3-Met86) and the αC helix in particular exhibits significant disorder, presumably due to mobility within the crystal, that is reflected by a high average temperature factor of 75 Å². In comparison, the C-terminal domain (Glu87-His279) is well-ordered with an average temperature factor of 33 Å². Data and refinement statistics are shown in Table 1 and electron density for the inhibitor is shown in Figure 1B.

The Nek2 catalytic domain exhibits a typical bilobal protein kinase fold (Figure 1C). As a family, the Nek kinases form a sub-clade with pairwise sequence identities of 35-42% within the catalytic domains (Figure 1D and Supplementary Figure S2). Their closest homologues in the human kinome are microtubule-associated regulatory kinases (MARK) along with the Polo-like kinases and Aurora-family kinases that are centrosome-associated and known to play important roles in mitotic regulation. Of kinases with known structure, Nek2 is most similar at the sequence level to Aurora-A (31% identity) and MARK2 (31% identity) and a structural overlap of the Nek2 complex with that of the inactive but ATP-bound Aurora A (PDBID 1OL6) is shown in Figure 2A. As expected for kinases that require phosphorylation of specific residues in the activation segment, these regions are not completely ordered in both cases. The overall structures are similar and superimpose with an rmsd of 1.8Å for 202 matched Cα positions. The two structures differ mainly in the length and location of secondary structural elements, particularly in the C-lobes. For example, αG is extended by one helical turn in Nek2 (residues Gly101-Glu105) and the helix inserted between sheet β3 and αC in Aurora-A is not well defined in Nek2 which shows only half a helical turn before the chain becomes disordered.

Perhaps the most notable difference occurs around the highly conserved ‘DFG’ motif that lies at the N-terminal end of the T-loop. Functionally, this...
motif serves to both anchor the N-terminal end of the T-loop and supply the aspartate as a ligand for a bound divalent metal ion. In the ATP-bound Aurora-A structure, the DFG motif adopts an extended conformation as is generally observed in other inactive kinases. However, in Nek2 the DFG motif and the following five residues constitute an extended motif (DFGLARIL) that is largely conserved in Nek-family members and which folds into a short region of α-helix that we refer to as αT. As a consequence of this arrangement the short sheet structure following the DFG motif is missing in Nek2 resulting in disorder of the region at the end of helix αE. Intriguingly, this additional element of secondary structure is wedged between αC, which contains the catalytically important Glu55, and the ATP-binding site (Figure 2B). In this conformation, Glu55 projects away from the active site and is held by a salt-bridging interaction with Arg164 from αT. Clearly, this conformation is incompatible with catalytic activity and the helix must therefore move or be disrupted in the activated form. Although unusual, a helical structure following the DFG motif is not unique to Nek-family kinases and is structurally related to a similar helical ‘insert’ observed previously in structures of cyclin-free CDK2 bound to ATP (32) (Figure 2C upper panel), and in inactive forms of both the EGFR kinase (33) (Figure 2C lower panel) and Src/Hck (34; and references therein). The remarkable similarity in the structure, location and sequence of Nek2 αT to equivalent motifs found in these kinases strongly suggest that αT is a feature of the Nek2 inactive conformation which is selected for, rather than induced by inhibitor binding. In the case of CDK2 activation, binding of an activating cyclin to the characteristic ‘PSTAIRE’ portion of αC pushes it towards the ATP/substrate binding cleft with concomitant disruption of the inhibitory helix. This allows Glu51CDK2 access to the active site, and Arg150 (equivalent to Arg164 in Nek2) to contact pThr160 within the T-loop. The latter interaction is common in kinases activated by T-loop phosphorylation to the extent that the presence of an arginine at a position equivalent to 164 in Nek2, is considered predictive of this mode of activation (35). The fact that Nek2 appears to be activated by T-loop phosphorylation at Thr175 (see below) strongly suggests that αT unfolds into a more extended conformation in the active form.

Pyrrrole-indolinone inhibitor binding to an inactive-like conformation of Nek2

Nek2 expression has been reported to be significantly elevated in a number of tumor cells including Ewing tumor (pediatric osteosarcoma) (36) and primary breast cancer tissues (37). Furthermore, high expression levels of Nek2 have been strongly associated with disease progression in non-Hodgkin lymphomas (38). Moreover, deregulation of Nek2 has been linked to centriosome abnormalities and aneuploidy. These hallmarks of tumor progression are generally associated with poor prognostic outcome since the lack of control over chromosome segregation leads to chromosomal instability - a general mechanism by which tumors select for cells with more malignant phenotypes. The strong link of Nek2 to cellular events critical for tumor progression and a recent report demonstrating that depletion of Nek2 leads to an apparent arrest in HeLa cell proliferation and an increase in apoptosis suggest that Nek2 may be a viable target for the development of novel anticancer drugs (39,40).

We determined the structure of a non-activating Nek2 mutant in complex with the pyrrole-indolinone compound SU11652. Binding of this compound was discovered during screens of a panel of generic kinase inhibitors for their ability to structurally stabilise the Nek2 T175A kinase domain (data not shown). The structure of the Nek2 complex shows the inhibitor bound at the back of the ATP-binding cleft, forming two hydrogen-bonds with main chain atoms from Glu87 and Cys89 within the hinge region between the N- and C-lobes, and making hydrophobic interactions with the active site residues Met86, Val68, Phe148, Cys89 and Leu162 (Figure 3A). In this position, it forms two hydrogen-bonds with main-chain atoms from Glu87 and Cys89 within the hinge. These interactions are common in kinase-inhibitor complexes and mimic the contacts formed by main-chain atoms from hinge residues with the N1 and N6 atoms of the adenine base in ATP-bound complexes. An intramolecular hydrogen bond is also formed between the indole oxygen and the pyrrole ring that stabilises the overall planarity of the extended ring system.

SU11652 has been described as a cell-permeable compound that acts as a potent and ATP-competitive tyrosine kinase receptor and angiogenic inhibitor that exhibits activity for PDGFRβ, VEGFR2, FGFR, EGFR and Kit family members with a broad range of IC_{50} values ranging from 3 nM to 20 µM (41-43). Later reports showed that SU11652 not only inhibits tyrosine kinases for
which it was originally designed but also a number of STKs possibly linked to tumorigenesis (44). SU11652 has been reported to display antiproliferative and pro-apoptotic properties in tumor cells and a closely related compound (SU11248, Sunitinib malate) has been recently approved for the treatment of metastatic renal cancer. We determined IC₅₀ values for both compounds using a substrate peptide GTFRSSIRRLLSRRRY (GTF peptide - phosphorylated residue underlined) derived from analysis of substrate sites of NIMA kinase (45). This substrate is efficiently phosphorylated by the recombinant full-length Nek2 T175A mutant kinase, with a Kₘ of ~90 µM and kₗ of ~17 min⁻¹ (Supplementary Figure S3). This is comparable to the value of 20 µM determined for wild-type Aspergillus NIMA, indicating that the substrate specificity of these kinases is similar and that Nek2 specificity is not significantly perturbed by the T175A mutation. Indeed, the sequence specificity of the full-length Nek2 T175A mutant determined using degenerate peptide libraries (in preparation) shows strong selection for Phe/Leu/Met in the pS -3 position much like that observed for NIMA (45). We determined an apparent Kₘ for ATP to be 115 µM (Supplementary Figure S3) and, using the same peptide substrate, measured IC₅₀ values for SU11652 and SU11248 of ~8 and ~12 µM respectively (Data not shown) indicating that the pyrrole-indolinones are potentially useful lead inhibitors for further optimisation and that some of the efficacy of Sunitinib malate in the treatment of tumors might be associated with Nek2 inhibition.

A number of highly selective inhibitors have been developed targeting the inactive conformation of kinases (reviewed in (46)). These 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 stabilise 'DFG-out' conformations. 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. The structure of Nek2 in complex with SU11652 offers two possibilities for the development of inhibitors that stabilise the inactive conformation. First, 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 that is rather small in the Nek2/SU11652 complex. Secondly, the surface presented by the αT helix could also be targeted to create more stabilising interactions. Indeed, the structure shows that the chlorine of the SU11652 indolinone ring makes favourable interactions with Leu162 of the Nek2 αT helix (Figure 3A). SU11248 contains a fluorine atom in place of chlorine that might be expected to weaken this interaction due to a much smaller van der Waals radius and increased polarity consistent with the small difference in their IC₅₀ values. Since αT prevents access of Glu55 to the active site, inhibitors that interact with and stabilise the helical conformation might be expected to provide increased potency and selectivity.

**Nek2 activation by autophosphorylation**

As described earlier, mass spectrometric analysis identified four sites of autophosphorylation within the catalytic domain. In order to establish their functional significance, we carried out localisation and activity assays in U2OS cells expressing myc-tagged full-length, wild-type and K37R mutant Nek2A, and mutants containing substitutions of Thr170, Ser171, Thr175, Thr179 or Ser241 with alanine, or glutamate/aspartate as phosphomimetics. To assess the activity of Nek2 proteins in cells, we scored transfected cells for premature centrosome splitting (CS) (Figure 4A). When stained with antibodies against γ-tubulin, centrosomes usually appear as two adjacent dots close to the nucleus in interphase cells. However, it has been previously observed that expression of wild-type Nek2A for 24 hours in either HeLa or U2OS cells induces CS by >2 µm in ~50-60% of the cells, compared to untransfected cells in which ~10% show split centrosomes over the same time period (5,47). Since catalytically compromised mutants of Nek2 fail to induce CS, this effect appears to be dependent on phosphorylation of centrosomal components (6-8). By way of comparison, we also wished to assess the relative catalytic activities of the mutant full-length Nek2 proteins in vitro. In order to do this we immunoprecipitated each Nek2 variant from in vitro translation extracts and assayed for kinase activity using β-casein (Figure 4B). Importantly, all Nek2 mutants examined show appropriate centrosomal staining in these experiments demonstrating that any effects of mutation within the kinase domain do not seriously impinge on Nek2 localisation.

As expected, the K37R mutation results in an enzyme with reduced activity in vitro and which
shows CS activity comparable to untransfected cell controls. In contrast, T175E resulted in an activated kinase in vitro that also showed an elevation in splitting in vivo. The overall increase in CS is small, reflecting the fact that splitting efficiency of 50-60% appears to be close to the maximum observable and mechanisms are likely to exist that prevent centrosome separation at higher frequencies. Regardless, the importance of Thr175 suggested by its conservation in many other kinases, is underlined by the T175A null mutant that shows reduced kinase activity and a significant reduction in efficiency of CS.

Like T175E, phosphomimic mutations of either Thr170 or Ser171 produced elevated kinase activity and maximal levels of CS indicating that these sites may have functional significance. However, in both cases, no significant effect of alanine mutation at these positions was observed in either assay. These residues are largely conserved in metazoan Nek2 kinases but are rather variable in other human Nek-family members (Figure 5A). Interestingly, Nek6 activation has been reported to depend primarily on phosphorylation at Thr206, the structural equivalent of Nek2 Thr175, but full activity appears to require phosphorylation of Ser202 (Nek2 Ser171) by Nek9 in a 'Nek-kinase cascade' (48). Thus, it appears that autophosphorylation of Thr170 and/or Ser171 in Nek2 may fine-tune overall activity of Nek2 in vivo. We also note that the sequence context of Ser171 is also highly conserved in Nek2 orthologues (His-Asp-Thr-Ser171-Phe) and matches closely the specificity motif for Plk1 (K/R-[Glu/Asp]-X-p[Ser/Thr]-Φ, where Φ is hydrophobic; (49)). Given that Plk1 phosphorylation efficiency of Nek2 has been proposed to regulate the efficiency of Nek2-mediated CS (50), our data suggest that Ser171 may be a target for phosphorylation of Nek2 by Plk1 at the centrosome.

A role for inhibitory autophosphorylation in Nek2 regulation

In contrast to the activating Thr170/Ser171 and Thr175 phosphorylation described above, our data also indicate that autophosphorylation at Thr179, also within the T-loop, and Ser241 in the C-lobe may act to negatively regulate Nek2. In marked contrast to the Thr170/Ser171 and Thr175 mutants, both T179A and T179E substitutions reduce kinase and CS activity (Figure 4). Thrreonine residues at this position play a conserved role in STKs, serving as an anchor point for the C-terminal end of the T-loop in the activated, substrate-bound kinase. Ternary complex structures of protein kinase A (51) show that this residue stabilises the conformation of catalytically important residues during phosphoryl transfer explaining why phosphorylation of Thr179 is inhibitory (Figure 5B). Whilst autophosphorylation at this position may appear surprising, we note that phosphorylation of the structurally equivalent Thr206 in the related MARK4 by an upstream kinase appears to be important for down-regulation of MARK4 activity in vivo (52). In addition, the DNA damage kinase Chk2 undergoes T-loop autophosphorylation on Thr383, the conserved activating site and on Thr387 (53) that is equivalent to Nek2 Thr179. This suggests that, like Nek2 and MARK4, Chk2 is also controlled positively and negatively by T-loop phosphorylation and supports the notion that this regulatory mechanism may be more common than has previously been appreciated.

The second inhibitory site is Ser241, located at the N-terminal end of αH within the C-terminal domain. Serine or threonine is present at the equivalent structural location in many other kinases but there is also a good deal of variability and we are unaware of any previous study that has demonstrated regulatory phosphorylation of this site. S241A and S241E/D mutations markedly reduce kinase activity and CS (Figure 4) but the extent of these effects is surprising for the following reasons. Firstly, the X-ray structure shows that although loss of the hydrogen-bonding potential, or introduction of a phosphoryl group might be expected to result in local perturbations, αH is, nonetheless, rather remote from the active site. Secondly, Ser241 substitutions do not seem to compromise Nek2 stability since these mutants localise to the centrosome correctly and are as abundant as the wild-type kinase in extracts used for kinase assays. It is possible that the observed effects of Ser241 phosphorylation on catalytic activity are manifested through conformation changes that are propagated through the C-lobe to the active site some 30Å distant. However, Ser241 phosphorylation may play a more direct role in Nek2 regulation as described below.

Kinase-kinase interactions in the Nek2 dimer

Perhaps the most striking feature of the Nek2 kinase domain structure is the presence of the αT helix at the N-terminal end of the T-loop, a conformation that has only been previously been observed in inactive forms of CDK2, EGF receptor
kinase and Src/Hck family kinases. In all cases, the αT helical segment forms a barrier to access of a conserved catalytic glutamate (Nek2 Glu55) on αC to the catalytic site. Furthermore, the interactions that stabilise the position of the inhibitory helix with respect to the N-lobe and αC are also very similar in each of these kinases (Figure 2). The presence of the helical insert in cyclin-free CDK2 is insensitive to Thr160 phosphorylation (32) suggesting that T-loop phosphorylation is unlikely to be responsible for Nek2 αT unfolding.

What, then, is responsible for disruption of αT in the activated form of Nek2? As mentioned, conformational changes in CDK2 required to unwind the 'blocking' helix are effected allosterically through cyclin-binding to the αC/PSTAIRE helix. In the case of Nek2, no activating subunit is known, nor is one necessary since even the purified full-length T175A mutant kinase is highly active (Supplementary Figure S3). However, although the isolated T175A Nek2 kinase domain autophosphorylates efficiently it nevertheless shows significantly lower values for both $K_m$ (190 µM) and $k_{cat}$ (8.4 min$^{-1}$) on the GT peptide substrate and a resulting 4-fold decrease in $k_{cat}/K_m$. Since our mass-spectrometric analysis shows that the isolated and dimerised recombinant T175A kinase domains show rather similar levels of autophosphorylation, it appears that dimerisation may play a direct role in relieving the auto-inhibited conformation observed in the X-ray structure. Interestingly, recent studies of EGFR kinase activation (33) show how ligand-induced receptor dimerisation allows activation through an 'asymmetric' interaction between EGFR kinase domains. Here, αH within the C-lobe of one monomer packs in a cyclin-like fashion against αC of the other thus disrupting the intervening αT-like helix. Such a model is attractive since Nek2 Ser241 that is targeted as a site of inhibitory autophosphorylation, is also located on αH. This would potentially explain the initially surprising observation that alanine or aspartate substitutions of Ser241 are so detrimental to Nek2 activity in vitro and in vivo, in spite of its distance from the active site and the fact that a variety of substitutions are found at the equivalent position in other kinases.

Clearly further work will be necessary to test the veracity of these ideas. Nonetheless, the fact that allosteric effects have been shown to be necessary for formation of active kinase conformations in CDK2, EGFR and Src/Hck, the only other systems where αT-like structures have been observed, together with the overall similarity in αT sequence, structure and interactions between Nek2 and these kinases, suggest that kinase-kinase interactions in dimeric Nek2 may play an important regulatory role.

Concluding remarks

Precise spatial and temporal control of Nek2 activity is necessarily complex, involving autophosphorylation of the kinase domain and its dephosphorylation by PP1c isoforms that form tight complexes with the Nek2A C-terminal region. In addition, PP1 itself is negatively regulated by Nek2 phosphorylation, an interplay of opposing activities that creates a 'bi-stable switch' that maintains the Nek2A dimer in an inactive state until PP1c is released. In the light of the data presented here, we now suggest that extant models of Nek2 control are likely to be inadequate and that regulation acts at many different levels. Firstly, our observation of multiple auto-phosphorylation sites within the C-terminal region may indicate that Nek2 dimerisation and PP1 binding along with stability/turnover and centrosomal localisation may all be subject to phospho-dependent control and these ideas are currently being tested. Secondly, our data now suggest a model in which activating auto-phosphorylation and/or phosphorylation by upstream kinases (pThr170/Ser171 and pThr175) is counterbalanced not only by PP1c activity, but also inhibitory autophosphorylation events that may directly alter stereochemistry at the site of phosphoryl transfer or disrupt potential kinase-kinase interactions within the Nek2 dimer required for allosteric activation. With complexity comes opportunity, and all of the regulatory mechanisms described here may be amenable to therapeutic intervention. The importance of Nek2 in the centrosome cycle, its overexpression in a number of tumor cell types and the prevalence of chromosomal instability and aneuploid defects in cancer cells suggest that it may constitute a valid anti-cancer target. That clinically relevant anti-cancer agents such as Sunitinib and its derivatives are active against Nek2, together with the unexpected features of the inactive Nek2 conformation gives some confidence that effective and specific inhibitors of the Nek-family kinases are likely to be achievable in the future.
References:

1. O'Connell, M. J., Krien, M. J., and Hunter, T. (2003) Trends Cell Biol 13(5), 221-228
2. Morris, N. R., Osmani, S. A., Engle, D. B., and Doonan, J. H. (1989) Bioessays 10(6), 196-201
3. Grallert, A., and Hagan, I. M. (2002) EMBO J 21, 3096-3107
4. Grallert, A., Krapp, A., Bagley, S., Simanis, V., and Hagan, I. M. (2004) Genes Dev 18(9), 1007-1021
5. Fry, A. M., Meraldi, P., and Nigg, E. A. (1998) Embo J 17(2), 470-481
6. Fry, A. M., Mayor, T., Meraldi, P., Stierhof, Y. D., Tanaka, K., and Nigg, E. A. (1998) J Cell Biol 141(7), 1563-1574
7. Mayor, T., Hacker, U., Stierhof, Y. D., and Nigg, E. A. (2002) J Cell Sci 115(Pt 16), 3275-3284
8. Bahe, S., Stierhof, Y. D., Wilkinson, C. J., Leiss, F., and Nigg, E. A. (2005) J Cell Biol 171(1), 27-33
9. Rapley, J., Baxter, J. E., Blot, J., Wattam, S. L., Casenghi, M., Meraldi, P., Nigg, E. A., and Fry, A. M. (2005) Mol Cell Biol 25(4), 1309-1324
10. Prigent, C., Glover, D. M., and Giet, R. (2005) Exp Cell Res 303(1), 1-13
11. Wu, L., Osmani, S. A., and Mirabito, P. M. (1998) J Cell Biol 141(7), 1575-1587
12. Graf, R. (2002) J Cell Sci 115, 1919-1929
13. Roig, J., Groen, A., Caldwell, J., and Avruch, J. (2005) Mol Biol Cell 16(10), 4827-4840
14. Quarmby, L. M., and Mahjoub, M. R. (2005) J Cell Sci 118(Pt 22), 5161-5169
15. Chen, Y., Riley, D. J., Zheng, L., Chen, P. L., and Lee, W. H. (2002) J Biol Chem 277(51), 49408-49416
16. Lou, Y., Yao, J., Zereski, A., Dou, Z., Ahmed, K., Wang, H., Hu, J., Wang, Y., and Yao, X. (2004) J Biol Chem 279(19), 20049-20057
17. Di Agostino, S., Fedele, M., Chieffi, P., Fusco, A., Rossi, P., Geremia, R., and Sette, C. (2004) Mol Biol Cell 15(3), 1224-1232
18. Fry, A. M., Schultz, S. J., Bartek, J., and Nigg, E. A. (1995) J Biol Chem 270(21), 12899-12905
19. Hames, R. S., and Fry, A. M. (2002) Biochem. J. 361, 77-85
20. Uto, K., Nakajo, N., and Sagata, N. (1999) Dev Biol 208, 456-464
21. Hames, R. S., Wattam, S. L., Yamano, H., Bacchieri, R., and Fry, A. M. (2001) EMBO J. 20, 7117-7127
22. Helps, N. R., Luo, X., Barker, H. M., and Cohen, P. T. (2000) Biochem J 349(Pt 2), 509-518
23. Hayes, M. J., Y., K., Wattam, S. L., Lindon, C., Mao, G., Yamano, H., and Fry, A. M. (2006) Nature Cell Biology 8, 607-614
24. Fry, A. M., Arnaud, L., and Nigg, E. A. (1999) J. Biol. Chem. 274, 16304-16310
25. Eto, M., Elliott, E., Prickett, T. D., and Brautigan, D. L. (2002) J Biol Chem 277(46), 44013-44020
26. Di Agostino, S., Rossi, P., Geremia, R., and Sette, C. (2002) Development 129(7), 1715-1727
27. Hayward, D. G., and Fry, A. M. (2006) Cancer Lett 237(2), 155-166
28. Storoni, L. C., McCoy, A. J., and Read, R. J. (2004) Acta Crystallogr D Biol Crystallogr 60(Pt 3), 432-438
29. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Acta Crystallogr D Biol Crystallogr 53(Pt 3), 240-255
30. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr D Biol Crystallogr 60(Pt 12 Pt 1), 2126-2132
31. Faragher, A. J., and Fry, A. M. (2003) Mol Biol Cell 14(7), 2876-2889
32. Brown, N. R., Noble, M. E., Lawrie, A. M., Morris, M. C., Tunnah, P., Divita, G., Johnson, L. N., and Endicott, J. A. (1999) J Biol Chem 274(13), 8746-8756
33. Zhang, X., Gureasko, J., Shen, K., Cole, P. A., and Kuriyan, J. (2006) Cell 125(6), 1137-1149
34. Sicheri, F., and Kuriyan, J. (1997)Curr Opin Struct Biol 7(6), 777-785
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Figure Legends:

Figure 1 Structure and autophosphorylation of human Nek2 - A. Schematic showing the major structural and functional features Nek2A organisation. Sites of autophosphorylation within both the catalytic and C-terminal regions are shown. B. A section of the 2Fo-Fc electron density map around the
SU11652 inhibitor, contoured at 1σ. C. Structure of the Nek2 kinase domain/SU11652 complex. The N- and C-lobes are coloured blue and red respectively, the hinge region is highlighted in yellow and the inhibitor is shown in green. Regions of disorder in the structure are included as dashed lines. D. Phylogenetic tree showing the sequence relationships between the eleven human Nek-family members.

Figure 2 Structural comparisons - A. Least-squares superposition of Nek2 with the inactive Aurora A/ATP complex (PDBID 1OL6) reveals differences distributed throughout the two structures, most obviously the presence of αT in Nek2 (circled) that is absent in Aurora-A. B. The Nek2 αT helix packs against the N-lobe and catalytic helix αC and prevents access of Glu55 to the active site. Glu55 makes salt-bridging interactions with Arg164 that is predicted to contact pThr175 in the active enzyme conformation. C. An inhibitory αT-like helix is observed in CDK2 (top), EGFR kinase (bottom) and Src/Hck family kinases (not shown). In each case, hydrophobic residues at the equivalent positions to Nek2 Leu162, Ile165 and Leu166 positions (see inset) stabilise the inhibitory conformation through non-polar interactions with residues from the kinase N-lobe, including the P-loop, and αC itself.

Figure 3 Structural basis of inhibition - The SU11652 inhibitor interacts with the ATP-binding cleft through a network of hydrogen-bonding interactions with main-chain atoms from the kinase hinge region and van der Waals interactions with residues from the N- and C-lobe together with Leu162 from the αT helix. The structure of the inhibitor is shown in the inset. The (diethylamino)ethyl moiety (highlighted) is disordered in the crystal structure.

Figure 4 Regulation of Nek2A kinase activity by phosphorylation - A. U2OS cells were transfected with myc-tagged Nek2 constructs (as indicated) for 24 hours before being fixed and stained using anti-myc (red) antibodies to detect transfected cells and anti-γ-tubulin (green) antibodies to detect centrosomes. Approximately 100 transfected cells were scored for split centrosomes (>2 μm apart) in three independent experiments; untransfected cells (-ve) were used as a negative control. Standard errors are indicated. Similar results were obtained using HeLa cells (data not shown). Representative merged images, including DNA stained with Hoechst 33258 (blue), are shown in the lower panel with insets showing magnified view of the centrosome staining. Arrowheads indicate centrosomes; scale bar, 5 μm. B. Myc-tagged Nek2A proteins were generated by coupled in vitro transcription-translation reactions and used in kinase assays against β-casein as substrate as described in Materials & Methods. The activity of different Nek2A constructs is expressed as a percentage of wild-type activity normalized to the amount of precipitated protein. Each construct was assayed at least three times and standard errors are indicated. Similar results were obtained using the C-terminal domain of C-Nap1 as a substrate (data not shown).

Figure 5 Autophosphorylation in the T-loop - A. Conservation within the T-loop of vertebrate Nek2 kinases (Hs - Homo sapiens, Mm - Mus musculus, Rn - Rattus norvegicus, Gg - Gallus gallus, Xl - Xenopus laevis), the eleven human Nek-kinases and T-loop sequences of MARK4, Chk2 and protein kinase A (PKA). T-loop phosphorylation at Nek2 position 175 is a conserved feature in many STKs (green circle) whereas phosphorylation at the equivalent position to Thr179 (red circle) has only been previously reported in MARK4 as an inhibitory modification (Thr202) by an upstream kinase (52), and in Chk2 (Thr387) (53) where it has previously been taken as an activating modification. The remaining sites in Nek2 (Thr170 and/or Ser171) are highlighted with a blue circle. B. The structure of a transition-state analogue of phosphoryl transfer in PKA shows that Nek2 Thr179 is likely to play a similar role to PKA Thr201 in stabilising the configuration of catalytic residues. Phosphorylation is incompatible with efficient catalysis due to its proximity to the pentavalent intermediate in the transition-state. Nek2 residue numbers are in parentheses.
Table 1: Crystallographic data and refinement statistics

| **Data Collection** |  |
|---------------------|--|
| Space group        | $P2_1$  |
| Cell dimensions    |  |
| $a$, $b$, $c$ (Å)  | 41.63, 56.92, 66.01 |
| $\alpha$, $\beta$, $\gamma$ (°) | 90, 95.98, 90 |
| Resolution         | 65 – 2.2 |
| Unique observations| 15620 |
| Completeness [%]   | 99.7 (98.1)$^1$ |
| Redundancy         | 8.2 (6.3) |
| $R_{\text{merge}}$ | 0.088 (0.475) |
| $I / \sigma I$     | 16.8 (3.4) |

| **Refinement** |  |
|----------------|--|
| Resolution [Å] | 30 – 2.1 |
| Reflections used (freeR set) | 14833 (796) |
| $R_{\text{work}} / R_{\text{free}}$ [%] | 21.4 / 27.7 |
| No. atoms (protein / ligand / water) | 2053 / 23 / 107 |
| B-factors (protein / ligand / water) [Å$^2$] | 46 / 29 / 35 |
| Rms deviations (bonds, Å; angles, °) | 0.010 / 1.225 |
| Ramachandran (favourable / allowed) [%] | 98.4 / 1.6 |

$^1$Values in parentheses correspond to highest resolution shell
Supplementary Experimental Procedures

MALDI-TOF Mass Spectrometry
A Reflex III MALDI time-of-flight mass spectrometer (Bruker Daltonik GmbH) equipped with a nitrogen laser and a Scout-384 probe was used to obtain positive ion mass spectra of digested protein with pulsed ion extraction in reflectron mode. An accelerating voltage of 26 kV was used with detector bias gating set to 2 kV and a mass cut-off of m/z 650. Analysis of the resulting spectra for potential phosphopeptides was carried out by comparison with an in silico digest of the parent protein with MS-Digest (http://prospector.ucsf.edu). Potential phosphopeptides were confirmed through β-elimination of phosphoric acid (98 Da) by post-source decay.

Electrospray Mass Spectrometry
Protein molecular weight was determined using a stand-alone syringe pump coupled to a Platform electrospray mass spectrometer (Micromass, Manchester, UK). Samples were desalted on-line using a 2 mm x 10 mm guard column packed with 50 micron Poros RII resin (Perseptive Biosystems, Framingham) inserted in place of the sample loop on a rhodyne 7125 valve. Proteins (typically 100 pmol) were injected onto the column in 10% acetonitrile, 0.10% formic acid, washed with the same solvent and then step eluted into the mass spectrometer in 70% acetonitrile, 0.1% formic acid at a flow rate of 10 µl/min.

Nanospray Mass Spectrometry
Samples were loaded onto a 2 mm x 0.8 mm C18 microcolumn (LC Packings, Amsterdam, Netherlands), washed and step eluted with 60% methanol, 0.1% formic acid directly into an Econo12 nanospray needle (New Objective Inc, Cambridge, MA). Nanospray mass spectra were acquired on an LCQ “classic” quadrupole ion trap mass spectrometer (ThermoQuest Corporation, Austin, TX) equipped with a nanospray source (Protana, Odense, Denmark) operated at a spray voltage of 800 V and a capillary temperature of 150°C. Tandem (MS²) mass spectra were acquired at a collision energy of 30% and a parent ion isolation width of 3 Da. The resulting spectra were analyzed for phosphate containing fragments by MS-Product (http://prospector.ucsf.edu/) using the following parameters: mass (monoisotopic); max reported charge (2+); instrument (ESI-ION-TRAP); N-terminal ions (b only); C-terminal ions (y only); neutral-loss ions (-H₃PO₄).

Thermal stability measurements
Thermal melting experiments were carried out using a real time PCR machine Mx3005p (Stratagene, La Jolla, CA). Proteins were buffered in 10 mM HEPES pH 7.5, 150 mM NaCl and assayed in a 96 well plate at a final concentration of 2 µM in 20 µL volume. Inhibitors were added at a final concentration of 10 µM from 500 µM stocks in DMSO. SYPRO-Orange (Molecular Probes, Eugene, OR) was added as a fluorescence probe at a dilution of 1 in 1000. The plate was covered by optical foil, shaken gently for 10 minutes at room temperature and centrifuged at 1000g for 30 sec before starting the experiment. Excitation and emission filters for the SYPRO-Orange dye were set to 465 nm and 590 nm, respectively. Temperature was raised with a step of 1°C per 1.0 min from 25°C to 96°C and fluorescence readings were taken at each interval. The temperature dependence of the fluorescence during the protein denaturation process was approximated by the equation

\[ y(T) = y_F + \frac{y_U - y_F}{1 + e^{\Delta G_{Tm}/RT}} \]

where \(y_F\) and \(y_U\) are the fluorescence intensity of the probe in the presence of completely folded and unfolded protein, respectively. The sloping baselines of these initial and final fluorescent species with increasing temperature were approximated by a linear fit. Data fitting was done using KaleidaGraph (Reading, PA) The observed temperature shifts, \(\Delta T_{m}^{obs}\), for each inhibitor were recorded as the difference between the transition midpoints of sample and reference wells containing protein without inhibitor in the same plate.
**In vitro** coupled kinase assay

Kinetic data for kinase activity were obtained using the coupled pyruvate kinase/lactate dehydrogenase assay (Cook et al., 1982). The standard reaction mixture contained: 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 mM MnCl₂, 1 mM phosphoenolpyruvate (PEP), 9 U pyruvate kinase, 16 U lactate dehydrogenase, 350 µM NADH. All reactions were carried out with 2 µM enzyme in a volume of 100 µL at 25°C. The concentrations used to determine the $K_M$ values for ATP and peptide substrates are shown in Supplementary Figure S3. NADH turnover was monitored at 340 nm, and starting absorbance values ranged from 1.5-2.2. Reactions were recorded for a minimum of 5 minutes at 0.5 second intervals. The maximal rate for each reaction was determined over a 10 second time frame during which the change in absorbance was linear. Data were then fitted to the Michaelis-Menten equation. The $K_M$ value for peptide substrate was determined using 1 mM ATP, and the $K_M$ value for ATP was determined using 250 µM peptide. Control experiments lacking peptide substrate were performed in order to verify that the kinase sample did not contain significant background ATPase contamination. All reactions were initiated with the peptide substrate. IC₅₀ values were determined as the concentration of inhibitor that resulted in half maximal reaction velocity at a kinase concentration of 2 µM in the presence of 250 µM GTF substrate peptide and 1 mM ATP.

**Supplementary Reference:**

Cook, P.F., Neville, M.E., Jr., Vrana, K.E., Hartl, F.T. and Roskoski, R., Jr. (1982) *Biochemistry*, 21, 5794-5799.
Figure 1.

A.

B.

C.

D.
Figure 2.

A. A cartoon representation of Nek2 and Aurora-A. 

B. A detailed view of Nek2 showing key residues.

C. A structural alignment of CDK2, EGFR, and Src. Key residues are highlighted, including Lys836, Glu738, Leu834, Asp831, and Tyr19.
$\alpha_C$ $\alpha_T$

Figure 3.

$R = \text{Cl} \ (SU11652)$
$F \ (SU11248)$
Figure 5.
Supplementary figure S1.

A.

Electrospray mass-spectrometric analysis of bulk autophosphorylation shows a single unphosphorylated species for the D141A mutant (left) and a heterogeneous population for the K37R mutant which shows an average number of four sites (right).

B.

| FL Nek2<sup>1-445</sup> Position | Phospho-peptide Sequence | Number Phosphorylated | Phosphorylated Residue(s) |
|----------------------------------|-------------------------|----------------------|--------------------------|
| 165-190<sup>a</sup>             | ILNHTDFSAKTFVGYMSPEQMN | 2<sup>*</sup>        | T175, unknown            |
| 235-250                          | RIPYRYSDELNEITTR        | 1<sup>*</sup>        | S241                     |
| 348-361                          | AEMLNKSLLKER           | 1                    | S356                     |
| 354-359                          | NYSSKK                 | 1                    | S356                     |
| 397-407                          | SENSEQLTSDK            | 2<sup>*</sup>        | S397, S402               |
| 424-432                          | AQALSDEIK              | 1                    | S428                     |
| 175-190<sup>b</sup>             | AFVGYMSPEQMN           | 1<sup>*</sup>        | T179                     |
| 165-174<sup>c</sup>             | ILNHDJFAAK             | 1<sup>*</sup>        | T170 or S171             |
| 384-396                          | VHFSGESKENIMR          | 2                    | S387, S390               |

Phosphopeptides recovered from Nek2 - Unambiguously assigned sites are indicated in bold and the number of sites within each tryptic peptide is indicated. Peptides requiring nanospray fragmentation for assignment are denoted with asterisks. All phosphopeptides were derived from full-length Nek2 T175A except that used to assign pThr175 (b) which was derived from analysis of the K37R mutant kinase domain. The T-loop peptide for which the phosphorylation site could not be unambiguously assigned is indicated (c).
Figure S3

\[
K_M = 93 \pm 9.2 \text{ } \mu \text{M}
\]
\[
k_{cat} = 17 \pm 1.4 \left( \text{mol} \text{ATP} \text{min}^{-1} \text{mol}^{-1} \text{Kinase} \right)
\]

\[
K_M = 113 \pm 12.4 \text{ } \mu \text{M}
\]
\[
k_{cat} = 12.6 \pm 0.9 \left( \text{mol} \text{ATP} \text{min}^{-1} \text{mol}^{-1} \text{Kinase} \right)
\]
Structure and regulation of the human NEK2 centrosomal kinase

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