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Characterization of hNek6 interactome reveals an important role for its short N-terminal domain and colocalization with proteins at the centrosome

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Abstract

Background. Physical protein-protein interactions are fundamental to all biological processes, which are integrated by large, complex networks, like the human kinome and its associated proteins. To explain the heterogeneous nature of complex networks, a 'scale-free' model was recently proposed in which the degree distribution in many large networks follows a power-law, characterized by the presence of few highly connected nodes, called hubs. Nek protein kinases are widespread among eukaryotes and represent an evolutionarily conserved family of 11 mammalian serine/threonine kinases related to the *Aspergillus nidulans* mitotic regulator NIMA. Those that have been studied are related to cell cycle-related functions, and diverse human pathologies, which highlight Neks as potential chemotherapeutic targets. Human Nek6 was recently found to be linked to carcinogenesis, but as for the majority of Neks, the interacting partners and signaling pathways are still unknown.

Methodology/ Principal Findings. Here we introduce hNek6 as a highly connected member in the human kinome interactome. We constructed 5 different networks based on the BioGRID database and Osprey software. Our networks include a broad set of novel hNek6 protein partners identified in yeast two-hybrid system screens from different cDNA libraries. For 66 identified proteins we established 18 functional categories and *in vitro* phosphorylation studies showed that CIR, PTN, RAD26L, RBBP6, and TRIP4 are regulators or candidate substrates of the wild-type hNek6 but not of the activation loop mutant hNek6(S206A). *In silico* analysis indicated common properties of the hNek6 interacting partners and link hNek6 to non-canonical Wnt signaling, Notch signaling and actin cytoskeleton regulation. We also obtained new insights on how hNek6 may regulate previously proposed pathways such as NF- κ B signaling and DNA repair response.

Conclusions/Significance. We therefore propose that protein kinases act in general as hubs, participating as vital regulators of different biological processes. Our connectivity analysis remarkably showed that hNek6 is a high confidence hub kinase within a set of likely essential kinase genes and that human kinases are enriched in network hubs. Further studies investigating if hNek6 interactions are stimulated or abrogated in cancer cells may provide useful information and possible implications for drug design.

Introduction

Physical protein-protein interactions (PPIs) are fundamental to all biological processes, which are integrated by large, complex networks. Analysis of PPI networks provides novel insights into protein function as it indicates a possible biological role for a protein regarding the physical interactions in which this protein participate. Moreover, interaction networks are the focus in the systems biology field, as they help to unravel how cellular networks are organized in time and space [1].

Until recently, classical random network theory [2,3] was used to model complex networks. It assumes that any two nodes (proteins) in a homogeneous network are connected with random probability p and the degrees of the nodes follow a Poisson distribution, which has a strong peak at the average degree, K . To explain the heterogeneous nature of complex networks, a 'scale-free' model was recently proposed in which the degree distribution in many large networks follows a power-law [$P(k) \approx k^{-\gamma}$] [4,5]. A remarkable characteristic of this distribution is that most of the nodes within these networks have very few links (k ; interactions), with only a few of them being highly connected (hubs). The existence of hubs and their minor frequency provides two important features to large complex networks: increased robustness with regard to random defects and shorter path length (distances) between any two nodes, i.e. a signal can travel from any node to another by traversing a small number of intervening nodes [6,7].

Within this context, the human kinome and all the interactions made by protein kinases comprise an important network of vital regulators of cellular

functions, since kinase directed phosphorylation is one of the most common forms of post-translational modification in the cell environment [8,9]. Furthermore, the protein kinase gene family is the domain most commonly found among known cancer genes [10] and thus deserves in-depth scrutiny.

The NIMA-related kinases (Neks) are widespread among eukaryotes. In mammalian they represent an evolutionarily conserved family of 11 serine/threonine kinases, containing 40-45% identity to the *Aspergillus nidulans* mitotic regulator NIMA (*Never In Mitosis gene A*) within their catalytic domain. NIMA kinase is involved in multiple aspects of mitotic progression including mitotic entry, chromatin condensation, spindle organization and cytokinesis [11]. Among the mammalian Nek proteins, those that have been studied appear to have cell cycle-related functions [12-20] and are described as related to pathologies [21-27].

Human Nek6 was recently found to be linked to carcinogenesis. It is overexpressed in gastric cancer and upregulation of Nek6 mRNA correlates with the peptidyl-prolyl isomerase Pin1 upregulation in 70% of hepatic cell carcinomas, while the overexpression of a catalytically-inactive Nek6 reduces the growth rate of human breast cancer cells [28,29,14]. Moreover, in a large-scale screening of serine/threonine kinases on different types of human tumors, Nek6 was shown to be upregulated in non-Hodgkin's lymphomas and breast, colorectal and lung tumors [30]. These facts highlight Nek6 as a potential chemotherapeutic target, but as for the majority of the Nek kinases, its interacting partners and signaling pathways are still unknown.

Here we describe that a broad set of novel human Nek6 (hNek6) protein partners were identified by yeast two-hybrid screens, which could be classified

into 18 Gene Ontology (GO) biological processes. 6xHis-hNek6 recombinant protein was purified from bacteria as a phosphorylated protein with the same pattern of phosphopeptides observed for the protein expressed in human cells and used for in vitro pull down and in vitro phosphorylation assays. All interactions were confirmed and the majority of tested substrates were phosphorylated in vitro by Nek6. In a broader context our analyses also showed that in general the human kinome is enriched in network hubs and that hNek6 could be classified as a high confidence hub kinase within a set of likely essential kinase genes. In summary, our newly identified hNek6 interacting protein partners show common functional and structural properties and provide new insights in how Nek6 may be involved in these novel signaling pathways as well as how it may regulate previously proposed pathways.

Results

Identification of Novel Human Nek6 Interacting Partners

To inhibit the observed autoactivation of hNek6 bait, the screens were performed in minimal medium plates without tryptophan, leucine and histidine but containing 30 or 50 mM 3-AT, since cell growth was disrupted in the presence of 30 mM of 3-AT. We employed the yeast two-hybrid system [31] for three human cDNA libraries – fetal brain, bone marrow and leucocyte – using hNek6 as bait, and a total of about 1.5×10^6 transformants for each screen was plated. Streaked colonies showing the best growth in all conditions (from 30 to 100 mM 3-AT) had their prey plasmid DNAs extracted and sequenced.

A total of 371 plasmid DNAs from positive clones were sequenced. 128 different prey proteins were identified, but only 66 were confirmed in yeast cells

under interaction-selective conditions (Figure S2; Table S1). These were classified into 18 functional categories based on the GO (<http://www.geneontology.org/>) [32] biological processes (proteins may be found into more than one category). (Figure 1; Table S3).

Experimental Confirmation of Interactions

In order to eliminate false-positives and check if hNek6 may really interact with the proteins codified by the plasmids isolated in the yeast two-hybrid screens, two experiments were employed. First, we tested all 128 retrieved interacting proteins for their *in vivo* association with Nek6 in yeast cells based on their growth capacity under stringent interaction-selective conditions (Figure S2), yielding 66 confirmed interactions (Table S1), as described above. Second, we selected 20 biologically relevant interacting proteins among the 66 confirmed, to test their *in vitro* association with hNek6 in pull-down assays. However, only 12 of the 20 subcloned cDNAs resulted in the expression of soluble proteins. The interaction of wild-type hNek6 in fusion with a 6xHis tag (6xHis-hNek6wt) with those proteins in fusion with GST (ANKRA2, ATF4, CDC42, CIR, PRAM1, PRDX3, PTN, RAD26L, RBBP6, RPS7, SNX26 and TRIP4) was then tested by *in vitro* pull-down assays, and all 12 interactions were confirmed (Figure 2). The high number of washes suggests that the interactions were strong. The specificity of the observed interactions was demonstrated because no interaction of Nek6 with free GST was observed under the same conditions (Figure 2). Table 1 summarizes the screenings characteristics and principal biological processes of the proteins confirmed to interact with Nek6.

It is also important to point out that hNek9 was retrieved twice (2 clones) in our screenings, helping to validate our study, as hNek6 was described to copurify with hNek9 in a specific interaction between the RCC1 domain and the coiled-coil motif of hNek9 (amino acids 732-891) [17]. Interestingly, one of our retrieved clones codifies a similar protein region, amino acids 806-979, and the other clone, amino acids 635-860.

Human Nek6 is Purified From Bacteria as a Phosphorylated Protein

6xHis-hNek6wt recombinant protein purified from *E. coli* migrates as a doublet on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described for the protein expressed in human cells [15], whereas the activation loop mutant 6xHis-hNek6(S206A) migrates as a single band lower in mobility to the more rapidly migrating band of wild-type Nek6 (Figure 3C) or as a doublet with an additional lower band. As only the slower moving band of wild-type hNek6 exhibits autophosphorylation and only this upper band catalyzes substrate phosphorylation, indicating that the activity of hNek6 is dependent on hNek6 polypeptide phosphorylation [15], we aimed to identify by mass spectrometry possible autophosphorylation sites in 6xHis-hNek6wt and 6xHis-hNek6(S206A) to compare their phosphorylation and activation status.

6xHis-hNek6wt and 6xHis-hNek6(S206A) were subjected to SDS-PAGE in replicate gels for Pro-Q® Diamond (Figure 3A), Sypro® Ruby (Figure 3B) and Coomassie Blue (Figure 3C) staining. Pro-Q® Diamond staining was used for in-gel detection of phosphate groups attached to serine, threonine, or tyrosine residues, showing specific staining for the wild-type doublet and the mutant

single band as the molecular weight standard proteins were not detected. Sypro® Ruby was used after Pro-Q® Diamond staining in the same gel for detection of the total amount of proteins. As both wild-type and mutant hNek6 showed phosphoprotein staining, the corresponding bands were analyzed by liquid chromatography/ MS/MS in order to identify the sites of phosphorylation. 6xHis-Nek6wt showed the same pattern of phosphopeptides as described for the recombinant protein expressed in human cells by [Belham et al. \[15\]](#). For 6xHis-Nek6wt, the phosphorylated Ser²⁰⁶ was confirmed and a Thr residue (Thr²⁰¹ or Thr²⁰²) is also phosphorylated in the same identified phosphopeptide FFSSETTAAHSLVGTPYYMSPER, whereas 6xHis-Nek6(S206A), as expected, yielded this peptide with spectra confirming the mutation S206A, with an additional oxidized Met²¹⁴ and a phosphorylated Thr residue (Thr²⁰¹ or Thr²⁰²) ([Figure 3D](#)).

Therefore, we propose that the more rapid electrophoretic migration of the mutated hNek6 compared to the wild-type protein is due to the lack of phosphorylation at Ser²⁰⁶. In summary, our data show that our bacterially derived 6xHis-hNek6wt presents the same pattern of phosphopeptides described for the recombinant protein expressed in human cells by [Belham et al. \[15\]](#), being probably very similar to the one expressed in HEK293.

Verification Of Human Nek6 Interacting Partners as Possible Substrates

To confirm whether hNek6 identified protein partners could also be possible substrates and if the full-length 6xHis-hNek6wt and 6xHis-hNek6(S206A) have the ability to autophosphorylate, *in vitro* kinase assays were performed. As expected, in the presence of [γ -³²P]ATP, wild-type hNek6

autophosphorylates within a short period of time (15 min) while the mutant hNek6(S206A) showed autophosphorylation only when incubated for a much longer time (Figure 4A). In a longer autoradiography film exposition, the mutant was visualized to autophosphorylate from 30 minutes to 12 hours, although in a considerable lesser degree when compared to the wild-type (data not shown). Furthermore, 6xHis-hNek6wt could also strongly phosphorylate five proteins in fusion with GST (CIR, PTN, RAD26L, RBBP6 and TRIP4) of six tested in total (Figure 4B). Interestingly, all proteins phosphorylated have putative consensus phosphorylation sites for hNek6 (Tables 2, S2). RPS7 seems not to be phosphorylated by hNek6, although also showing a putative phosphorylation site, which might not be the preferable one. On the other hand, 6xHis-hNek6(S206A) could not phosphorylate any of the proteins (except maybe for a little fraction of CIR) (Figure 4B), which confirms the specificity of 6xHis-hNek6wt phosphorylation against these five putative substrates and suggests that they could be *in vivo* substrates as well. As expected, the negative control proteins in the whole lysate (LYSATE lanes) and the GST protein alone (GST lanes) were not phosphorylated by wild-type or mutant hNek6, the phosphorylation being thus specific for the proteins tested. Moreover, wild-type hNek6 was shown to autophosphorylate in all conditions (Figure 4B, ³²P Autorad.), while mutant hNek6 autophosphorylation was detected only at high protein quantities (Figure 4B, ³²P Autorad., INPUT lane).

Human Nek6 Interacting Partners Show Common Functional and Structural Properties

To visualize and compare the functional and structural properties within hNek6 interacting proteins identified by our yeast two-hybrid screens, computational analyses were carried out. The resultant hNek6 Yeast Two-Hybrid Interaction Network (NYTHIN; Figure 1), constructed from the BioGRID (<http://www.thebiogrid.org/>) [33], was analyzed using Osprey 1.2.0. software (<http://biodata.mshri.on.ca/osprey/>) [34] in order to access the GO biological processes and cellular components for each protein in NYTHIN. The analysis showed enrichment in processes categorized into Metabolism (33%), Signal Transduction (21%), Transcription (21%) and Unknown function (21%). Interestingly, the connectivity of a protein is related to its function [35] and high connectivity is often associated with proteins involved in signaling and transcription [36].

Increased similarity of gene expression profiles for genes encoding interacting proteins has been demonstrated in yeast [37], while preliminary evidence in higher eukaryotes has been reported for *Caenorhabditis elegans* [38] and humans [39,40]. Moreover, studies of expression profiles in *Sacharomyces cerevisiae* protein-protein interaction network led to the identification of two types of hub proteins: static hubs (party hubs) and dynamic hubs (date hubs), where party hubs have multiple simultaneous interactions and high levels of coexpression with their partners, while date hubs have multiple sequential interactions separated in time or in space [41]. Here we used a compendium of coexpression measurements generated from hundreds of public microarray datasets [42], to search for coexpression data within the prey

proteins identified to interact with hNek6. Using each prey protein/gene as a search query at Gemma database (<http://www.chibi.ubc.ca/Gemma/>), many were found as coexpressed genes (Table S3), although none was found to coexpress with hNek6. In most cases of coexpression where both genes were involved in the same biological processes, these corresponded to Metabolism or Transcription, i.e. MMP2 and F13A1 in Metabolism; ADI1 and QDPR in Metabolism; ASHL1, MBD2, NR2F2 and RXRB in Transcription; TRIP4 and MYST2 in Transcription. Apparently, Nek6 interacting proteins form groups of coexpressed proteins related in function, which are separated into different biological processes, or modules [43], while hNek6 may interact with them in a non-simultaneous way at different times and/or locations in the cell.

Next, we sought to evaluate if the proteins retrieved by our yeast two-hybrid screens could be targets of hNek6 phosphorylation. The substrate specificities for NIMA [44] and hNek6 [45] have been determined and revealed novel and unique nature of substrate recognition sites by this group of kinases. The recognition sites include a preferable phenylalanine (for NIMA) or leucine (for hNek6) or other hydrophobic residues at position -3 relative to the phosphorylated Ser/Thr residue. We found that 18 proteins have the preferable -3 Leu and a +1 aromatic residue, 50 proteins have a -3 hydrophobic residue and a +1 Phe/Trp/Tyr/Leu/Met/Ile/Val/Arg/Lys residue (as described in a substitution chip by Lizcano et al. [45], and three proteins have the -3 Phe (Table S2). These results are in agreement with our *in vitro* kinase assays (Figure 4B), demonstrating that at least five proteins are likely to be hNek6 substrates *in vivo*.

The retrieved protein sequences were also analyzed for their structural properties concerning their domain composition and percentage of disordered amino acid residues. hNek6 protein partners showed to be organized in a wide variety of domain families (Table S3), some of them being shared by the neighboring proteins, i.e. zing finger (134 proteins), Ras (2 proteins) and AAA domains (2 proteins). Short linear regions (proline-rich, arginine-rich and leucine-rich regions) and repeats (hemopexin, tetratricopeptide, G-beta and ankyrin) were also found to be recurrent. Another predominant characteristic among hNek6 interacting proteins is the presence of disordered regions, that is, regions that lack a clear structure. Table S3 shows the percentage of disordered residues among the proteins. The range of structural types fell into three broad classes (as indicated in the 'Percent disordered residues' column): mostly disordered (85-100% disordered; 3 proteins), partially disordered (15-85%; 64 proteins) and mostly ordered (0-15%; 1 protein). These regions have been suggested to be important for flexible or rapidly reversible binding [46,47] and, interestingly, an investigation of the functions performed by intrinsically disordered regions reveals that they are often involved in molecular recognition and protein modifications, such as phosphorylation [46].

Table 2 summarizes the putative phosphorylation sites by hNek6 and NIMA and Table 3 summarizes the domain composition, percentage of disordered residues, cellular localization, biological processes and gene coexpression *in silico* analysis for the proteins confirmed to interact with hNek6.

Human Nek6 is a New Super-Hub in a Kinome Enriched in Network Hubs

For analysis of the protein interaction data, a first network was constructed from the hNek6 interactions identified by our yeast two-hybrid screens: NYTHIN (hNek6 Yeast Two-Hybrid Interaction Network) (Figure 1). As in the literature hNek6 was described to have other protein partners discovered by large-scale experiments [48,40], we used the hand-curated literature citation interaction database, BioGRID, to construct another network composed of all hNek6 protein partners, including the novel ones identified by our screens: NIN (hNek6 Interaction Network) (Figure S1). Comparing both networks, it is interesting to note that the proteins retrieved in our screens and all others from the BioGRID are distributed in essentially the same GO biological process categories, in a way validating the putative signaling pathways that hNek6 is involved. Next, we constructed three other major networks, aiming at a better comprehension concerning the connectivity among the proteins in each case: PIN (Protein Interaction Network), KIN (Kinase Interaction Network) and NKIN (Non-Kinase Interaction Network). PIN represents the largest dataset, composed of every physical interactions from the BioGRID and the novel ones identified by our screenings for hNek6. KIN was assembled from the concatenation of all kinases present in the BioGRID and all their partners (including the novel hNek6 interacting proteins), consisting of kinase-kinase and kinase–non-kinase interactions only. And last, NKIN groups every node from PIN and their links, except the kinases and their exclusive partners.

We calculated the degree distribution $P(k)$ of the human proteins, for PIN, KIN and NKIN, measuring the probability that a given protein interacts with exactly k other proteins. As KIN was constructed with the purpose to facilitate

the connectivity analysis of the kinase group among all other groups of proteins in PIN, only the degree distribution for kinases are considered in KIN, since potential interactions between non-kinases were filtered. As shown in [Figure 5](#), the degree distributions of the three networks approximate a power-law and hence are scale-free in topology [\[49\]](#), which is in agreement with interaction studies for model organisms [\[50\]](#). On average, proteins in PIN have 6.40 interaction partners. However, 5327 proteins with $k \leq 5$ (non-hubs), 1581 intermediately connected proteins with $5 < k < 15$, 808 hubs with $k \geq 15$, as well as 161 super-hubs (defined here as high confidence hubs [\[51\]](#) with more than 35 partners) were detected. On the other hand, kinases in KIN have a mean of 11.82 interactions, while non-kinases in NKIN have a mean of 5.81 interactions. Notably, approximately 10% of the proteins in PIN are hubs ($k \geq 15$), whereas 25% are hubs in KIN and 9% are hubs in NKIN, reflecting an enrichment of hub proteins among the kinase group. Although the datasets have not sufficient resolution for an accurate statistical calculation of the dispersion around the average degree of each network, these results suggest that kinases have in general almost twice the interactions than the other proteins and therefore behave as putative general network hubs. Moreover, the putative hubs identified by our analysis were compiled into a list including all super-hub kinases ranked by average degree ([Table 4](#)). This ranking showed that hNek6, with 91 protein partners, is a super-hub kinase found within the four highest connected kinases.

Previous studies in yeast have demonstrated that proteins acting as hubs are three times more likely to be essential for cells than proteins with only a small number of links [\[52\]](#). Therefore, the 25 super-hub kinases from KIN were

used as query genes on the Knockout Mouse Project (KOMP) Repository (<http://www.komp.org/>) to identify the kinases with lethal phenotypes. Most of them (17 kinases) were found as essential genes (NEK6 was considered a putative essential gene because its knockdown in HeLa cells resulted in increased apoptosis [14]) while only three kinase genes did not show knockout phenotype in mouse ('unknown' refers to non-tested genes in KOMP) (Table 4). These remarkably results in a 68% probability of lethality attributed to random deletion of one of the 25 high confidence hubs and indicates a likely tendency for hub kinases to be preserved in an evolutionary perspective. Another previous study in yeast revealed a 32% chance of lethality among 19 high confidence kinase hubs [51].

Discussion

Among the proteins retrieved in our screens, there are three described to be related to the nuclear factor κ B (NF- κ B) signaling: transcription factor RelB (RELB), peroxiredoxin-3 (PRXIII; PRDX3) and thyroid receptor-interacting protein 4 (TRIP-4; TRIP4). (Figure 6). NF- κ B plays an important role in inflammation, autoimmune response, cell proliferation, and apoptosis by regulating the expression of genes involved in these processes. Interestingly, NEK6 gene was described as one within dozens of others that activate the NF- κ B signaling pathway, in a large-scale screening where cDNAs were introduced into HEK293 cells and examined whether they activated the transcription of a luciferase reporter gene driven by a promoter containing the consensus NF- κ B binding sites [53]. However to our knowledge, there ain't an explanation of how hNek6 activates NF- κ B and the first possible links to that question are

addressed here. hNek6 may regulate the NF- κ B signaling pathway directly by phosphorylating/ activating RelB, which functions as a transcriptional activator when associated to p50 or p52, or as a repressor when associated to p65 (RelA).

On the other hand, hNek6 could regulate this pathway indirectly by interacting with PRXIII or phosphorylating/ activating TRIP-4. PRXIII is an antioxidant protein that acts synergistically with leucine zipper-bearing kinase (LZK; MAP3K13) protein in the activation of NF- κ B in the cytosol [54]. In the case of TRIP-4, it is a transcription coactivator molecule harboring an autonomous transactivation domain that contains a putative zinc finger motif (Tables 3, S3). It stimulates transactivation by serum response factor (SRF), activating protein 1 (AP-1), and NF- κ B through direct binding to SRF, c-Jun, p50, and p65; and relieves the transrepression between nuclear receptors and either AP-1 or NF- κ B [55]. TRIP-4 plays thus a pivotal role in NF- κ B transactivation in mammalian cells, and as it was confirmed to interact and be phosphorylated *in vitro* by Nek6 (Figure 4B), it represents another possible link to how hNek6 may activate the NF- κ B signaling pathway

Interestingly, an NF- κ B binding site, which starts with the sequence GGGA, can overlap with a recombining binding protein suppressor of hairless (CBF1) binding site, which ends in GGGAA, [56] this protein being associated to CIR, also identified in our screens and confirmed to interact with hNek6 (Table 1, Figure 2). Repression of genes whose promoters contain this sequence overlap could be relieved by displacement of CBF1 as a consequence of the availability of increased levels of nuclear NF- κ B. The mechanism of CBF1-mediated repression implicated the presence of the corepressor CIR in this

activity. CIR participates in the recruitment of the histone deacetylase (HDAC) to DNA-bound CBF1. Furthermore, it is interesting to note that SNW1 is a component of the CBF1 corepressor complex and interacts with CIR [57], since it was also retrieved in our screens using hNek6 as bait (Table S1). These facts suggest a putative role for hNek6 in Notch signaling through interactions with CIR and SNW1 (Figure 6), which might behave as hNek6 substrates, since both have putative phosphorylation sites (Table S2) and CIR was found to be phosphorylated by hNek6 *in vitro* (Figure 4B).

The DNA Repair functional category identified in our screens reiterates the importance of this context among the functions mediated by Neks in general. In a previous yeast two-hybrid screen performed by our group using human Nek1 as bait [23], proteins that take part in the dsDNA repair during the G2/M transition phase of the cell cycle were identified, and two recent studies suggest that Nek1 and Nek6 are important regulators of this biological process [58,59]. In our screenings, we identified the double-strand-break repair protein rad21 homolog (hHR21; RAD21), a cleavable component of the cohesion complex, involved in chromosome cohesion during cell cycle until the onset of anaphase, in apoptosis, and in DNA repair mediated by homologous recombination during the S/G2 phases through the cohesion between sister chromatids [60-62]. Interestingly, a large-scale immunoprecipitation screening for hundreds of baits, including Nek6, identified for this kinase key components of the cohesin and condensin complexes required for condensation, segregation and structural maintenance of chromosomes during mitosis [48]. As we did not find the putative recognition site for hNek6 phosphorylation in hHR21 sequence, it might not be phosphorylated by hNek6 but instead be a regulator

of hNek6 during DNA repair. Notably, a previous study suggests that Nek6 is also required for metaphase-anaphase transition [14] and thus it might possibly interact with hHR231 at this point of the cell cycle.

We also identified the putative DNA repair and recombination protein RAD26-like (RAD26L) in our screenings. Although there is no study to date characterizing the mammalian protein, Rad26 is described in yeast to be the regulatory subunit of the Rad3 phosphatidylinositol 3-kinase related kinase (PIKK), where it is required for two processes dependent on microtubules: chromosome segregation and cell polarity [63]. Therefore, associated to the fact that RAD26-like is a hNek6 substrate *in vitro* (Figure 4B), it is an interesting interacting target to be studied, as it might link hNek6 to the DNA repair function and/ or processes dependent on microtubules.

Another protein retrieved in our screenings is the PHD finger Protein 1 (PHF1), a polycomb group (PcG) chromatin modifier which was recently involved in the response to DNA double-strand breaks (DSBs) in human cells [64]. Following DNA damage, the DNA damage sensors Ataxia telangiectasia mutated (ATM)/ Ataxia telangiectasia and Rad3-related protein (ATR) and the DNA-dependent protein kinase (DNA-PK) phosphorylate the serine/threonine protein kinases Chk1 and Chk2 to regulate this cell cycle checkpoint, phosphorylate p53 to activate the apoptosis signal pathway, and activate double-strand break repair. [65,66]. In this context, PHF1 is recruited rapidly to DSBs sites, that is dependent on the ATP-dependent DNA helicase 2 subunit 1 (Ku70)/ ATP-dependent DNA helicase 2 subunit 2 (Ku80) [64]. PHF1 is thus another putative target of hNek6 among the proteins involved in the DNA damage response pathways, as it also presents putative phosphorylation sites

for hNek6 (Table S2). Moreover, Chk1 and Chk2 were suggested to be activated by Nek1 after IR or UV radiation [58] and to phosphorylate Nek6 N-terminal region *in vitro* [59]. It was also described that Nek6 is phosphorylated upon IR and UV radiation and that it is responsible for its inhibition during mitosis. As Nek6 activation is an essential step for mitotic cell cycle progression [14], it was suggested that one mechanism for DNA damage-induced cell cycle arrest at the G₂/M phase, is the inhibition of Nek6 activation through Chk-mediated phosphorylation [59]. Therefore, hNek6 seems to be a downstream regulator in the DNA Repair signaling pathway, composed also by hNek1 as an upstream effector kinase, where hNek6 putative substrates addressed here might be phosphorylated/ inactivated by hNek6 prior to DNA injuries, and activated upon such events in the cell, parallel to hNek6 inactivation.

Other pathways proposed for hNek6 in our work are the canonical wntless protein (Wnt)- β -catenin and noncanonical Wnt-JNK (c-Jun N-terminal kinase) signaling cascades. The canonical pathway includes the collapsin response mediator protein 1 (CRMP1) classified here in the Metabolism category, which has recently been related to this pathway along with pleiotrophin (PTN; Signal Transduction) and eukaryotic translation elongation factor 1 alpha 1 (EEF1A1; Protein Biosynthesis) [50]. These three proteins were found to interact with proteins in the Wnt signaling pathway that controls embryogenesis and cancer development [50].

On the other hand, the noncanonical pathway involves hNek6 interactors from the Signal Transduction category, like the mitogen-activated protein kinase kinase kinase 4 (MEKK4; MAP3K4), and the Cell Organization and Biogenesis category, like the cell division cycle 42 (Cdc42; CDC42) (Figure 6). This

pathway operates through receptors coupled to G α protein, leading to activation of the small GTPases RhoA, Rac1 and Cdc42, which connects to the MEKK1/MEKK4-dependent cascade and leads to the activation of JNK, triggering processes linked to cell proliferation and cell polarity [67].

Another hNek6 interactor associated to this context is the Sortin nexin-26, which was recently identified as a RhoGAP that specifically interacts via its GAP domain with Cdc42, and plays an important role in the regulation of insulin-induced glucose transport in adipocytes [68]. It contains an N-terminal Phox homology domain, Src homology 3 (SH3) domain, RhoGAP domain, and several proline-rich sequences at the C-terminal region (Table S3). Since the activities of these regulators are frequently modified by phosphorylation [69-72], it is notable that sorting nexin-26 was confirmed to be phosphorylated *in vitro* by hNek6 (Figure 4B).

Two other proteins that deserve attention in our screenings are PTN and 40S ribosomal protein S7 (RPS7); the first was retrieved in 32 clones of a total of 301, covering 10.6%, while the second was retrieved in 127 clones, representing 42.2% of the screenings. PTN, classified here as a Signal Transduction protein, is a heparin binding growth factor of 18kDa, which signals for axon development and neurite extension, and acts in cell adhesion and organization of the cytoskeleton [73,74]. As it is a secreted protein, it might be regulated by hNek6 during its transport to the cell exterior. In the case of the ribosomal protein S7, it is a structural constituent of the 40S ribosome subunit related to Protein Biosynthesis. However, recent studies have shown that it interacts with the E3 ubiquitin-protein ligase Mdm2, modulating the p53-Mdm2 binding through the formation of a stable ternary complex that prevents p53

ubiquitination mediated by Mdm2, inhibiting cell proliferation and activating the apoptosis [75]. Interestingly, we found that RPS7 interacts with hNek6 and seems not to be phosphorylated by it, functioning as a possible hNek6 regulator (Figure 4B).

Another retrieved protein also related to p53 function and confirmed to interact and be phosphorylated *in vitro* by hNek6 (Figures 2, 4B) is the retinoblastoma (Rb) binding protein 6 (RBBP6), which is a 250 kDa splicing-associated protein that has been identified as an E3 ligase due to the presence of a RING finger domain. Structural studies also showed that the N-terminal 81 amino acids of RBBP6 constitute a novel ubiquitin-like domain called DWNN (Table 3) [76]. RBBP6 was described to suppress the binding of p53 to DNA [77], and has been shown to be highly upregulated in oesophageal cancer [78].

hNek6 also retrieved the cancer related promyelocytic leukemia-retinoic acid receptor alpha (PML-RAR α) regulated adaptor molecule 1 (PRAM1), which is an adaptor protein comprised of an N-terminal proline-rich region with several putative SH3 recognition motifs, a central SH2 binding site, and a C-terminal SH3-like domain [79] (Table 3). Notably, PML-RAR α was described to downregulate expression of PRAM-1 mRNA in the absence of all-trans retinoic acid (ATRA), indicating that PRAM-1 may be an oncogenic target of the fusion protein, since the treatment of patients with ATRA leads to complete remission of APL [80,81]. In this context, it is interesting that PRAM-1 was confirmed to interact with hNek6 *in vitro* (Figure 2), and shows a putative phosphorylation site (Table 2), since hNek6 was also found to be upregulated in human promyelocytic leukemia HL-60 cell line (Gene Atlas, [82]).

Another identified protein in our screens is the activating transcription factor 4 (ATF4). Its expression is upregulated in primary human tumours and facilitates tumour growth in xenograft models [83,84]. ATF4 is induced by stress signals including anoxia/hypoxia [83,85], endoplasmic reticulum stress, amino acid deprivation, and oxidative stress [86], and regulates the expression of genes involved in oxidative stress, amino acid synthesis, differentiation, metastasis and angiogenesis [87]. As ATF4 was confirmed to interact with hNek6 *in vitro* (Figure 2) but does not have potential consensus phosphorylation sites for hNek6 (Table 2), it might regulate hNek6 function in pathways related to metabolism, transcription and/ or stress response (Figure 1). hNek6 also retrieved the ankyrin-repeat protein ANKRA2, which has putative hNek6 phosphorylation sites (Table 2), but which functions are largely unknown. It seems to have its role in the nucleus, where it has been shown to regulate class II HDACs function and localization [88,89].

The many interactors identified and confirmed suggest that hNek6 is a hub kinase involved in several cellular pathways (Figure 1), such as NF- κ B, Notch, and Wnt signalings, DNA repair and regulation of the actin cytoskeleton (Figure 6), through interactions with and phosphorylation of proteins composed of disordered regions and diverse domains and repeats (Table S3).

Most notably, among the proteins shown to be phosphorylated by wild-type hNek6 *in vitro*, none was phosphorylated by the mutant hNek6(S206A) (Figure 4B), indicating that wild-type hNek6 phosphorylation of these putative substrates is specific and that the activation loop phosphorylation at S²⁰⁶ is a critical step for the activation of hNek6, which could be achieved by autophosphorylation in the presence of a cellular level of ATP.

Hub proteins are generally expected to have multi-domains, repeats and to be at least partially disordered, as an increase in binding regions and flexibility may result in a higher connectivity [36,90]. We show that hNek6 is a high confidence hub protein kinase within an enriched set of essential kinase genes, although being a mostly ordered and globular protein, revealed by studies on circular dichroism and small-angle x-ray scattering (our unpublished data). Therefore, we carefully analyzed the structure of its putative binding partners and, in most cases, we found repeats and disordered regions (Table S3). In fact, this is an example among many others of mostly ordered hubs, such as kinases like Cdk2, where intrinsically disordered proteins/ substrates bind to structured hub proteins [90].

The main criticism applied to the yeast two-hybrid screen of protein-protein interactions is the possibility of a high number of false positive identifications, estimated as high as 50% [91]. In fact, from 128 different proteins identified using Nek6 as bait, only 66 were confirmed by stringent interaction-selective conditions, and, among these, all 12 proteins tested for *in vitro* interaction with hNek6 were positive, validating that hNek6 behaves as a super-hub kinase.

Thus, giving the importance of hNek6 in regulating a variety of cellular functions through the interaction with several putative relevant partners described here, it would be desirable to determine the consequences of these interactions in human cells, and even if these interactions are stimulated or abrogated in cancer cells. Further studies may provide useful information and possible implications for drug design and cancer therapy.

Materials and Methods

Plasmid Constructions. The full-length hNEK6 gene sequence was amplified from a human fetal brain cDNA library (Clontech). The PCR product was cloned into pGEM-T easy vector (Promega) and subcloned into *EcoRI* restriction site of the modified vector pBTM116KQ [92] in fusion with the LexA DNA binding domain (Clontech). To express full-length hNek6 fused to a 6xHis tag, the full-length nucleotide sequence was PCR-amplified using a specific primer set, 5'-CGGAATTCCATATGGCAGGACAGCCCGGCCAC-3' and 5'-GGGGAAGCTTTCAGGTGCTGGACATCCAGATG-3', and then inserted into *NdeI* and *HindIII* restriction site of the modified bacterial expression vector pET28a-TEV (Novagen/EMD Biosciences, San Diego, CA). Selected nucleotide sequences encoding the full-length or truncated proteins identified to interact with the full-length hNek6 were subcloned from the vector pACT2 into various types of the bacterial expression vector pGEX (GE Healthcare, Waukesha, WI), which allows the expression of the proteins in the form of a Glutathione S-transferase (GST) fusion. The orientation, frame, and correctness of sequence of each insert DNA were confirmed by restriction endonuclease analysis and automated DNA sequencing.

Site-directed Mutagenesis. The hNek6 activation loop mutation S206A was introduced by PCR-based mutagenesis of the pET28a-TEV-hNEK6 construct using the Quick-Change Site-Directed Mutagenesis kit (Stratagene). Mutation was confirmed by DNA sequencing.

Yeast Two-hybrid Screens and DNA Sequence Analysis. The yeast two-hybrid screens [31] of three human cDNA libraries (Clontech) – fetal brain, bone marrow and leucocyte – were performed by using *S. cerevisiae* strain L40 (trp1-901, his3 Δ 200, leu2-3, ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lac GAL4) and human NEK6 gene as bait fused to the yeast LexA DNA binding domain sequence in the modified vector pBTM116KQ [92]. The autonomous activation test for HIS3 was performed for yeast cells transformed with both the bait construct (pBTM116KQ-NEK6) and the empty prey vector (pACT2) in minimal medium plates without tryptophan, leucine and histidine but containing 0, 5, 10, 20, 30, 50, 70 and 100 mM of 3-AT, the inhibitor of His3p. Furthermore, the autonomous activation of LacZ was measured by the β -galactosidase filter assay described before [23,93]. Yeast cells were transformed according to the protocols supplied by Clontech. As long as the bait construction autoactivates the yeast reporter genes, but the cell growth is disrupted in the presence of 30 mM of 3-AT, the screenings were performed in minimal medium plates without tryptophan, leucine and histidine but containing 3-AT. Half of the transformed cells were plated on selective medium containing 30 mM 3-AT, and the other half was plated on selective medium containing 50 mM 3-AT. All grown colonies were streaked in resuspended drops of 4.0 μ L on selective medium containing 0, 30, 50, 70 and 100 mM 3-AT to confirm and compare their growth levels, and only the ones showing the best growth in all conditions were selected positive for further analysis. Recombinant pACT2 plasmids of positive clones were isolated and their insert DNAs sequenced with a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). The obtained DNA sequence data were compared with sequences in the NCBI data bank using the BLASTX 2.2.12

program [94]. Sequences that corresponded to the noncoding region were eliminated. As frameshift mutations can lead to expression of the correct reading frame in yeast [95], these sequences were retained for further analysis.

PPI Confirmation in Yeast Cells. Each prey plasmid DNA was cotransformed in the yeast strain L40 with the bait construct pBTM116KQ-NEK6 or the empty bait vector (pBTM116KQ). The presence of both types of plasmids (prey and bait vectors) was controlled by growth on minimal medium plates without tryptophan and leucine [96]. Yeast clones were then streaked in triplicate drops of 4.0 μ L on minimal medium plates without tryptophan, leucine, and histidine but containing 0, 30, 50 and 100 mM of 3-AT for testing their growth capacity under interaction-selective conditions (Figure S2).

Protein Expression and Purification. The GST fusion proteins identified in the yeast two-hybrid screens were expressed in *E. coli* BL21 (DE3) cells at 37 °C using 0.5 mM isopropyl 1-thio- β -D-galactopyranoside (IPTG) or in BL21 (DE3/ pRARE) cells at 18 °C using 1.0 mM IPTG for 4 h.

Soluble hNek6 wild-type – 6xHis-hNek6wt – and mutant – 6xHis-hNek6(S206A) – fused to a 6xHis tag were purified for *in vitro* analysis from *E. coli* BL21 (DE3/ pRARE) cells that were induced for 4 h at 18 and 37 °C, using 1.0 and 0.5 mM IPTG, respectively. Cells were harvested and lysed in lysis buffer (50 mM HEPES, pH 7.5, 5 mM sodium phosphate, 300 mM NaCl, 5% glycerol, 20 mM imidazole) by sonication. Soluble and insoluble fractions were separated by centrifugation at 28,500 x g for 40 min at 4 °C. The cleared

supernatant was then loaded onto a HiTrap chelating column (GE Healthcare). Bound hNek6 was eluted in lysis buffer plus 300 mM imidazole.

Phosphorylation Sites Determination. For identification of autophosphorylation sites by mass spectrometry, gel bands corresponding to 6xHis-hNek6wt (upper and lower bands) and 6xHis-hNek6(S206A) (single band) were excised and in-gel trypsin digestion was performed according to Hanna et al. [97]. An aliquot (4.5 μ l) of the peptide mixture was separated by C18 (100 μ m x 100 mm) RP-UPLC (nanoAcquity UPLC, Waters) coupled with nano-electrospray tandem mass spectrometry on a Q-ToF Ultima API mass spectrometer (MicroMass/Waters) at a flow rate of 600 nl/min. The gradient was 0-80% acetonitrile in 0.1% formic acid over 45 min. The instrument was operated in the 'top three' mode, in which one MS spectrum is acquired followed by MS/MS of the top three most-intense peaks detected. The resulting spectra were processed using Mascot Distiller 2.2.1.0, 2008, Matrix Science (MassLynx V4.1) and searched against human non-redundant protein database (NCBI) using Mascot, with carbamidomethylation as fixed modification, oxidation of methionine and phosphorylation of Ser, Thr and Tyr as variable modifications, one trypsin missed cleavage and a tolerance of 0.1 Da for both precursor and fragment ions. The phosphorylation sites were manually validated.

***In Vitro* Binding Assays.** 1 mL of soluble bacterial extracts of GST or GST fusion proteins were allowed to bind to 30 μ L of glutathione-Uniflow resin (Clontech) in PBS for 1 h at 4 °C. After incubation, the beads containing bound

recombinant proteins were washed three times with PBS at 4 °C. 12,5 µg of purified full-length 6xHis-hNek6wt fusion protein was added to the resins containing GST or GST fusion proteins and incubated for 2 h at 4 °C to allow protein-protein interactions to occur. The beads were then washed three times with 0.5 ml of PBS, followed by three washes with 0.5 ml of PBS containing 0.1% Triton X-100, then three washes with 0.5 ml of PBS only. Resin-bound proteins were analysed by SDS-PAGE and immunoblotting as previously described [23,93].

In Vitro Kinase Assays. Reaction mixtures in kinase buffer (50 mM MOPS, pH 7.4, 300 mM NaCl, 10 mM MgCl₂, 0.1 mM PMSF) containing 10 µM ATP, 185 kBq [γ -³²P]ATP (222 TBq/mmol; NEM) and 0.1 µg/µL of purified full-length 6xHis-hNek6wt or 6xHis-hNek6(S206A) recombinant proteins were incubated at 30 °C; 20 µL aliquots were removed at the times indicated and the reactions were terminated by the addition of an SDS electrophoresis buffer. In parallel, 1 mL of soluble bacterial extracts containing GST or the indicated GST fusions proteins, and a whole lysate of empty pET28a vector (Novagen/EMD Biosciences, San Diego, CA) transformed BL21 cells also induced with IPTG, were allowed to bind to 30 µL of glutathione-Uniflow resin (Clontech) in PBS for 1 h at 4 °C and then washed three times with kinase buffer. 20 µL of reaction mixtures equally prepared were added to the resins containing GST or unspecific bound proteins from the whole lysate as controls, or GST fusion proteins as substrates, and incubated at 30 °C for 30 min. The reaction products were separated on SDS-PAGE and phosphorylated proteins were detected by autoradiography.

***In silico* PPI Analysis and Network Parameters.** The biological processes and cellular localization of hNek6 novel interacting proteins were based on the Gene Ontology (GO; <http://www.geneontology.org/>) [39] and Uniprot (<http://www.uniprot.org/>) [98,99] databases, and the gene coexpression analysis was accessed by Gemma database and software system (<http://www.chibi.ubc.ca/Gemma/>); the prediction of disordered amino acid residues in the retrieved protein sequences was calculated by PONDR® VL-XT predictor (<http://www.pondr.com/>) [100-102], the domain composition of these sequences was obtained by Pfam (<http://pfam.sanger.ac.uk/>) [103], PROSITE (<http://www.expasy.ch/prosite/>) [104,105] or InterPro (<http://www.ebi.ac.uk/interpro/>) [106,107] databases, and putative phosphorylation sites by hNek6 or NIMA were determined by searching for similar consensus sequences as described by Lizcano et al. [45] or Lu et al. [44], respectively. The lethality phenotypes of human protein kinases were accessed by the Knockout Mouse Project (KOMP) Repository (<http://www.komp.org/>).

The May 1, 2009 release of interaction data (BIOGRID-ORGANISM-Homo_sapiens-2.0.52.tab.txt) was downloaded from the BioGRID (<http://www.thebiogrid.org/>) to be analyzed and visualized by Osprey 1.2.0. software (<http://biodata.mshri.on.ca/osprey/>) [34].

For analysis of physical interactions, five networks were constructed from the BioGRID: (1) NYTHIN (hNek6 Yeast Two-Hybrid Interaction Network; 67 nodes, 78 links), including the hNek6 interactions identified by our yeast two-hybrid screens and the interactions described in the human BioGRID within the

prey proteins; (2) NIN (hNek6 Interaction Network; 92 nodes, 114 links), including the hNek6 interactions identified by our yeast two-hybrid screens and the ones described in the human BioGRID; (3) PIN (Protein Interaction Network; 7,716 nodes and 49,369 links), including every physical interaction from the hand-curated literature citation interaction database and the hNek6 interactions identified by our yeast two-hybrid screens; (4) KIN (Kinase Interaction Network; 1,997 nodes (366 kinases and 1631 non-kinases) and 4,327 links), including only kinases annotated in the human genome [8] and their direct interacting proteins (including the novel hNek6 protein partners); or (5) NKIN (Non-Kinase Interaction Network; 7,164 nodes and 41,639 links), including every node from PIN and their links except the kinases and their exclusive partners.

To statistically compare the connectivity among the proteins in the networks, the degree distribution $P(k)$ and the average degree (K) of each network was calculated as described by Stelzl et al. [50]. For the degree distribution $P(k)$, the probability that a given protein interacts with exactly k other proteins was plotted against the number of links (k) in a log-log plot; the degree exponents (γ) and R-squares were determined by linear fitting where $P(k)$ approximates a power-law: $P(k) \approx k^{-\gamma}$.

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Figure Legends

Figure 1. Human Nek6 Yeast Two-Hybrid Interaction Network (NYTHIN).

The network has 67 proteins (colored nodes, including the bait hNek6) and 78 interactions involving them (purple and grey links). The nodes are colored based on the GO biological processes. The purple links represent the novel interactions connecting the bait hNek6 and the prey proteins identified by our screens; the grey links refer to published interactions within the prey proteins deposited in the human BioGRID. The network was generated using Osprey 1.2.0. software.

Figure 2. *In vitro* confirmation of the interaction between human Nek6 and selected interacting proteins retrieved in the yeast two-hybrid screens.

In vitro pull-down assays between full-length 6xHis-Nek6wt and the indicated protein fragments fused to GST (see Table 1 for details). Free GST control protein or the indicated GST fusion proteins were loaded on glutathione-Sepharose beads, and after washing the beads they were incubated with the purified full-length 6xHis-Nek6wt fusion protein. After high stringency washes using PBS/Triton X-100, the samples were loaded in replicate SDS-PAGE gels for blot transfer. Monoclonal mouse anti-GST antibody 5.3.3 and a mouse anti-5xHis antibody were used for detection of GST- or His-tagged fusion proteins, respectively. The gray arrows indicate the positions of the GST-fusion proteins in anti-GST Western blot.

Figure 3. Human Nek6 autophosphorylates itself when expressed in *E. coli*.

Purified recombinant 6xHis-Nek6wt is resolved as a doublet band in SDS-PAGE and 6xHis-Nek6(S206A) predominantly as a single band. Bands corresponding to 6xHis-Nek6wt and 6xHis-Nek6(S206A) were excised, digested *in situ* with trypsin, and analyzed by liquid chromatography/MS/MS. (A). Pro-Q® Diamond-stained gel shows the phosphorylated proteins. (B) Sypro Ruby-stained gel shows the total amount of proteins. MW: BenchMark™ molecular weight standard; it is not present in Coomassie Blue-stained gel (C). (D). Schematic representation of the wild-type and mutant Nek6 proteins. 6xHis-hNek6wt shows specific Ser²⁰⁶ autophosphorylation in contrast to the mutant 6xHis-hNek6(S206A) as identified by mass spectrometry.

Figure 4. Human Nek6 *in vitro* autophosphorylation and phosphorylation of proteins retrieved in the yeast two-hybrid screens.

(A) Time course of autophosphorylation of wild-type and mutant Nek6. Purified protein was incubated at 30 °C in kinase buffer for the indicated times with 10 µM radiolabelled ATP. Resulting ³²P incorporation monitored by SDS-PAGE coupled with autoradiography and blot transfer. (B) Qualitative phosphorylation by wild-type and mutant Nek6 of selected proteins identified in the yeast two-hybrid screen. Soluble extracts of these protein fragments in fusion with GST bound to glutathione-Uniflow resin (Clontech) were incubated at 30 °C in kinase buffer for 30 minutes with 10 µM ATP as above and 0.1 µg/µL of purified full-length 6xHis-Nek6wt or 6xHis-Nek6(S206A) recombinant proteins. The gray arrows indicate the positions of the GST fusion proteins in the ³²P autoradiography, SDS-PAGE and anti-GST Western blot, while the white

arrows indicate the positions of the 6xHis-tagged proteins in the ³²P autoradiography and SDS-PAGE. The LYSATE lanes refer to the bacterial extract of BL21 cells transformed with empty pET28a vector after induction with IPTG, in order to control for unspecific protein phosphorylation in the whole lysate, and the INPUT lanes refer to the 6xHis-tagged proteins autophosphorylation without being added to the resin. Neither the lysate nor GST negative controls showed unspecific substrate phosphorylation, and all conditions showed wild-type Nek6 autophosphorylation, while mutant Nek6 autophosphorylation was detected only in a greater amount of protein (INPUT lane).

Figure 5. Degree distribution of the network proteins.

(A) PIN (Protein Interaction Network): 7,716 nodes, 49,369 links, $K = 6.40$, $\gamma = 1.96$, R-square = 0.93; (B) KIN (Kinase Interaction Network): 1,997 nodes (366 kinases and 1631 non-kinases), 4,327 links, $K = 11.82$, $\gamma = 1.06$, R-square = 0.83; and (C) NKIN (Non-Kinase Interaction Network): 7,164 nodes, 41,639 links, $K = 5.81$, $\gamma = 1.96$, R-square = 0.94. The three networks approximate a power-law [$P(k) \approx k^{-\gamma}$] and are scale-free in topology.

Figure 6. Human Nek6 putative signaling pathways.

Signaling pathways proposed for hNek6 based on its novel interacting partners identified by our screenings. [See details such as protein nomenclature and abbreviations in the Tables 1 and 3 and the Supplementary Tables S1 and S3.](#)

Supporting Information

Figure S1. Human Nek6 Interaction Network (NIN).

The network has 92 proteins (colored nodes, including the bait hNek6 and its partners identified by our yeast two-hybrid screens and the ones described in the human BioGRID database) and 114 interactions involving them (purple and grey links), including the known interactions within all the proteins. The nodes are colored based on the GO biological processes. The purple links represent the new interactions connecting the bait hNek6 and the prey proteins identified by our screens; the grey links refer to the human BioGRID interactions. The network was generated using Osprey 1.2.0. software.

Figure S2. Growth score relative to the yeast cells growth capacity under interaction-selective conditions.

The numbers (scores) refer to the growth levels of the yeast clones (after cotransformation of each prey plasmid with the bait construct or the empty bait vector) when streaked in triplicate drops on minimal medium plates without tryptophan, leucine and histidine but containing 0, 30, 50 or 100 mM of 3-AT. (0) transparent cell drop, (1) white thin cell drop, (2) white thick cell drop, (3) reddish thin cell drop, (4) reddish thick cell drop, (5) red thick cell drop. The red color indicates a higher cells growth.

Table S1. Human Nek6 interacting proteins identified by the yeast two-hybrid system screen.

¹ Results obtained from BLASTX (GenBank).

² It is depicted the minimum length of the retrieved sequences which could be visualized by forward DNA sequencing only.

³ Absolute number of sequences retrieved from each human library (FB: fetal brain, BM: bone marrow, L: leukocyte).

⁴ Growth assay for yeast cells transfected with each retrieved sequence in pACT2 vector and the construction pBTM116KQ-NEK6 or the empty pBTM116KQ vector (see also Figure 2).

⁵ Biological process based on the GO database (other functions may be known; see also Table S3).

Table S2. Putative phosphorylation sites present in human Nek6 interacting proteins.

¹ It is depicted the minimum length of the retrieved sequences which could be visualized by forward DNA sequencing only.

² The phosphorylated residue (Ser/Thr) is in bold and the residues Leu (for Nek6, [53]), Phe (for NIMA, [52]), or another hydrophobic residue, at position -3, are underlined.

Table S3. Domain composition, cellular localization, biological process and gene coexpression *in silico* analysis of human Nek6 interacting proteins.

¹ It is depicted the minimum length of the retrieved sequences which could be visualized by forward DNA sequencing only.

² Domain composition of the retrieved sequences obtained by Pfam, PROSITE or InterPro databases (other domains may be present).

³ Percentage of disordered amino acid residues in the retrieved sequences obtained by PONDR.

⁴ Cellular components based on the GO and Uniprot databases.

⁵ Biological processes based on the GO database.

⁶ Coexpressed genes obtained by GEMMA database, including only the ones whose products were identified to interact with hNek6 by our two-hybrid screens.

* Domain composition described by Roig et al. [18].

Table 1. Human Nek6 interacting proteins identified by the yeast two-hybrid system screen.

Protein interacting with hNek6 (aliases) ¹	Gene	Accession no.	Coded protein residues (retrieved/ complete sequence) ²	Expressed protein length (kDa) ³	Frame	Redundancy in library ⁴			Growth score ⁵	Biological process (GO) ⁶
						FB	BM	L		
cell division cycle 42 (GTP binding protein, 25kDa)	CDC42	AAH03682.1	1-191/ 191	48	+2	1			2/1	Cell organization and biogenesis
RAD26L hypothetical protein	RAD26L	A4D997.3	1-531/ 531	87	+1	2			3/1	DNA repair
retinoblastoma-binding protein 6 (p53-associated cellular protein Testis-derived, P2P-R, RBQ-1)	RBBP6	BAC77636.1	1-259/ 1792	57	+1			1	4/1	Metabolism
NIMA-related kinase 9 (Nek9)	NEK9	AAH93881.1	806-979/ 979	–	+2	1		1	4/1	Protein amino acid phosphorylation
ribosomal protein S7	RPS7	AAH02866.1	1-194/ 194	49	+1	11	58	58	4/1	Protein biosynthesis
sorting nexin 26 (TC10/CDC42 GTPase-activating protein)	SNX26	O14559.2	115-1287/ 1287	45	+1	1			4/1	Protein transport
CBF1 interacting corepressor	CIR	AAH46098.1	1-240/ 450	55	+1	3			4/1	RNA processing
PML-RARA regulated adaptor molecule 1	PRAM1	AAH28012.1	360-670/ 670	61	+1		2	2	4/1	Signal transduction
pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1), isoform CRA_c	PTN	AAH05916.1	1-168/ 168	49	+1	31	1		4/1	Signal transduction
activating transcription factor 4	ATF4	AAH16855.1	252-351/ 351	38	+1	1	6	3	2/0	Stress response
thyroid hormone receptor interactor 4 (ASC-1, activating signal cointegrator 1)	TRIP4	AAH12448.1	46-581/ 581	87	+1	2		1	2/1	Transcription
ankyrin repeat family A protein 2	ANKRA2	AAH12917.1	78-313/ 313	52	+1	2			4/1	UNKNOWN
peroxiredoxin 3 (AOP-1, HBC189, thio redoxin-dependent peroxide reductase, mitochondrial precursor)	PRDX3	AAH08435.1	183-256/ 256	35	+1		1		4/1	UNKNOWN

¹ Results obtained from BLASTX (GenBank).

² It is depicted the minimum length of the retrieved sequences which could be visualized by forward DNA sequencing only.

³ Approximated length of the proteins expressed as a GST fusion in *E. coli*.

⁴ Absolute number of sequences retrieved from each human library (FB: fetal brain, BM: bone marrow, L: leukocyte).

⁵ Growth assay for yeast cells transfected with each retrieved sequence in pACT2 vector and the construction pBTM116KQ-NEK6 or the empty pBTM116KQ vector (see also Figure S2).

⁶ Biological process based on the GO database (other functions may be known; see also Table 3).

Table 2. Putative phosphorylation sites present in human Nek6 interacting proteins.

Gene	Coded protein residues (retrieved/ complete sequence) ¹	Putative phosphorylation sites by hNek6 (L-X-X-S/T-F/W/Y) ²	Putative phosphorylation sites by NIMA (F-R-X-S/T-X) ²	Other putative phosphorylation sites by hNek6 (L/F/W/Y-X-X-S/T-F/W/Y/M/L/I/V/R/K) ²
CDC42	1-191/ 191	<u>C</u> <u>L</u> <u>L</u> ISYTTN		E <u>Y</u> VPTVFDN <u>Y</u> AVTVMIG
RAD26L	1-531/ 531			H <u>E</u> SF S KQSH FEVD S VSQF T <u>L</u> PH T KKGQ R <u>L</u> ENTMKDQ
RBBP6	1-259/ 1792			G <u>L</u> HISLCDL T <u>Y</u> VISRTEP
NEK9	806-979/ 979			
RPS7	1-194/ 194			K <u>L</u> DG S RLIK
SNX26	1115-1287/ 1287	<u>L</u> NASYGML		D <u>E</u> LLSYPPA
CIR	1-240/ 450			E <u>E</u> LKSLTTK
PRAM1	360-670/ 670			D <u>L</u> RRTRSA A
PTN	1-168/ 168			E <u>W</u> QWSVCVP
ATF4	252-351/ 351			
TRIP4	46-581/ 581			G <u>W</u> CLSVHQP L <u>E</u> CGTLVCT R <u>L</u> DETIQAI E <u>L</u> QATYRLL
ANKRA2	78-313/ 313			L <u>L</u> AN S LSVH S <u>L</u> AC S KGYT L <u>Y</u> LATRIEQ
PRDX3	183-256/ 256			

¹ It is depicted the minimum length of the retrieved sequences which could be visualized by forward DNA sequencing only.

² The phosphorylated residue (Ser/Thr) is in bold and the residues Leu (for Nek6, [53]), Phe (for NIMA, [52]), or another hydrophobic residue, at position -3, are underlined.

Table 3. *In silico* analysis of human Nek6 interacting proteins structural and functional properties.

Gene	Coded protein residues (retrieved/ complete sequence) ¹	Domain composition (Pfam, PROSITE or InterPro) ²	Percent disordered residues (PONDR) ³	Cellular component (GO) ⁴	Biological processes (GO) ⁵	Coexpressed genes (GEMMA) ⁶
CDC42	1-191/ 191	Ras family domain	18.32	Cytoplasm (filopodium), plasma membrane	Cell cycle, Cell organization and biogenesis, Protein transport, Signal transduction	
RAD26L	1-531/ 531	UNDESCRIBED	36.35	Nucleus	DNA repair	
RBBP6	1-259/ 1792	DWNN domain, Zinc knuckle domain	35.14	Nucleus	Metabolism	
NEK9	806-979/ 979	Proline-rich region, Coiled-coil domain*	52.87	Nucleus, cytoplasm	Cell cycle, Protein amino acid phosphorylation	
RPS7	1-194/ 194	Ribosomal protein S7e family domain	53.61	Nucleus (nucleolus) and cytoplasm (ribosome)	Protein biosynthesis	
SNX26	1115-1287/ 1287	CDC42 GTPase-activating protein family domain, Proline-rich region	88.44	Cytoplasm, plasma membrane	Protein transport, Signal transduction	CRMP1, PSMC6
CIR	1-240/ 450	N-terminal domain of CBF1 interacting corepressor CIR	32.64	Nucleus (nuclear speck and HDAC)	RNA processing, Transcription	F13A1
PRAM1	360-670/ 670	SH2 containing adaptor PRAM-1 related family domain, SH3 domain, Proline-rich region	69.45	UNDESCRIBED	Signal transduction	
PTN	1-168/ 168	PTN/ MK heparin-binding protein family N-terminal domain	35.71	Cytoplasm (ER), ECM	Signal transduction	
ATF4	252-351/ 351	bZIP transcription factor family domain	72.00	Nucleus, cytoplasm, plasma membrane	Metabolism, Stress response, Transcription	
TRIP4	46-581/ 581	Putative zinc finger motif (C2HC5-type), ASCH domain	38.25	Nucleus, cytoplasm	Transcription	CRMP1, PSMC6, MYST2
ANKRA2	78-313/ 313	Ankyrin repeat	23.31	Cytoplasm (cytoskeleton)	UNKNOWN	RAD21, RBBP6, SLU7
PRDX3	183-256/ 256	C-terminal domain of 1-Cys peroxiredoxin, AhpC/ TSA family domain	33.78	Cytoplasm (mitochondrion)	UNKNOWN	PSMC6

¹ It is depicted the minimum length of the retrieved sequences which could be visualized by forward DNA sequencing only.

² Domain composition of the retrieved sequences obtained by Pfam, PROSITE or InterPro databases (other domains may be present).

³ Percentage of disordered amino acid residues in the retrieved sequences obtained by PONDR.

⁴ Cellular components based on the GO and Uniprot databases.

⁵ Biological processes based on the GO database.

⁶ Coexpressed genes obtained by GEMMA database, including only the ones whose products were identified to interact with hNek6 by our two-hybrid screens.

* Domain composition described by Roig et al. [18].

Table 4. Super-hub kinases ranked by degree from KIN network.

Gene	BioGRID Node ID	Lethality ¹	Degree
SRC	EG674	yes	110
EGFR	EG1956	yes	94
NEK6	RP11-101K10.6	yes*	91
FYN	RP1-66H14.1	yes	91
RAF1	EG5894	yes	73
ABL1	RP11-83J21.1	yes	63
CDK2	EG1017	yes	61
LYN	EG4067	no	61
MAPK1	EG5594	yes	59
AKT1	EG207	yes	57
JAK2	EG3717	yes	57
PTK2	EG5747	yes	57
PTK2B	EG2185	no	48
MAPK8	EG5599	yes	47
LCK	EG3932	no	44
SYK	EG6850	yes	43
MAPK14	RP1-179N16.5	unknown	42
MAPK3	EG5595	yes	42
JAK1	EG3716	unknown	40
GSK3B	EG2932	yes	39
PRKDC	EG5591	unknown	38
STK24	RP11-111L24.5	unknown	38
ILK	EG3611	yes	37
ATM	EG472	yes	36
PRKCA	EG5578	no	36

¹ Observed lethality phenotypes from the Knockout Mouse Project (KOMP) Repository.

* Knockdown of endogenous Nek6 expression results in mitotic arrest and increased apoptosis [15].