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Identification of NEK3 interacting proteins and phenotypical characterization of its silencing in HeLa cells

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ABSTRACT

NEKs (NIMA-related kinases) are a group of kinases that share high amino acid sequence identity to NIMA (Never in mitosis gene A), which exists as a single member in the fungi *Aspergillus nidulans* and is functionally involved in the initiation of mitosis. NEK3 is a 506 amino acid serine/threonine kinase, localizes both to the nucleus and cytoplasm, and has an N-terminal catalytic domain and a C-terminal regulatory domain. NEK3 is also involved in cell motility and invasiveness of breast cancer tumor cells through interaction with regulators of the Rho GTPases Rac1 and RhoA, mediated by prolactin induced association of NEK3 to the human Prolactin Receptor (PRLR). A screening for interaction partners was performed and 27 different proteins were found. The identified candidate interacting proteins are functionally involved in processes, which are related with the most important steps of cancer progression and can be used to understand NEK3 function and behavior. One of the most important cytoplasmic NEK3 interactors is RhoGDI2 (RhoGDP-dissociation inhibitor 2). RhoGDI2 as well as VAV2, are regulators of RhoGTPases and inhibit Rac1 and RhoA activation with effects not only in bladder cancer, but also promotes invasion of breast cancer cells. Another GTPase, RhoA, is the main component of the cleavage furrow and is required for proper cytokinesis. In this respect, NEK3 was found in the furrow of ingression and its depletion is associated to cytokinesis delay due to DNA bridges, that is related to aneuploidy, tetraploidy, micronuclei. Also, NEK3 has a role in the promotion of tubulin acetylation, which in turn suggest that the role for NEK3 in DNA bridge formation may be related to microtubule stabilization and consequently, chromosomal instability. According to this, NEK3 also has a role in cell cycle regulation, which help to elucidate one of the roles for NEK3 in cancer.

Keywords: NEK3, Kinases, Yeast Two-Hybrid Screening, RhoGDI2, RhoA.

Abbreviations: NEK3: NIMA-related kinase 3; RhoGDI2: RhoGTPase dissociation inhibitor 2; VAV2: Guanine Exchange Factor 2; HEK293T: Human Embryonic Kidney 293T; GEF: Guanine Exchange Factor; GAP: GTPase Activating Protein; GDI: Guanosine nucleotide dissociation inhibitors; GFP: Green Fluorescent Protein. PIAS1: Protein Inhibitor of Activated STAT 1, SGIP1: SH3-containing GRB2-like protein 3-Interacting Protein 1, CNTN1: Contactin-1, FBN1: Fribrilin-1, MED17: Mediator of RNA polymerase II transcription subunit 17, PA2G4: Proliferation-associated protein 2G4 e COMMD1: COMM domain-containing protein 1.

1. Introduction

NEKs (NIMA-related kinases) are a group of kinases that share high amino acid sequence identity to NIMA (Never in mitosis gene A), which exists as a single member in the fungi *Aspergillus nidulans*, functionally involved in the initiation of mitosis. NIMA is a 79 kDa protein with an N-terminal kinase domain and a C-terminal regulatory domain rich in regulatory motifs: Coiled-coil domain, responsible to mediate protein-protein interactions (as in Nek1 and Nek9) and possibly its dimerization (as found -at least for: Nek1, Nek2, Nek9); and two PEST sequences related to regulated protein degradation [1–4]. Activation of this serine/threonine kinase at the G2/M transition of the cell cycle is required for entry into mitosis. Upon deletion of the gene encoding NIMA, cells

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arrested in G2, whereas when the protein was over-expressed the cells were driven into mitosis from any point in the cell cycle [2,4–8].

Most of the 11 mammalian NEKs already studied, are functionally related to NIMA but some degree of neo-functionalization also took place. Aside their participation in the context of NIMAs function in cell cycle progression and regulation, we can further annotate Neks in associated roles with a) centrosome disjunction b) primary cilia function and c) DNA damage response checkpoints and regulation of repair pathways [9].

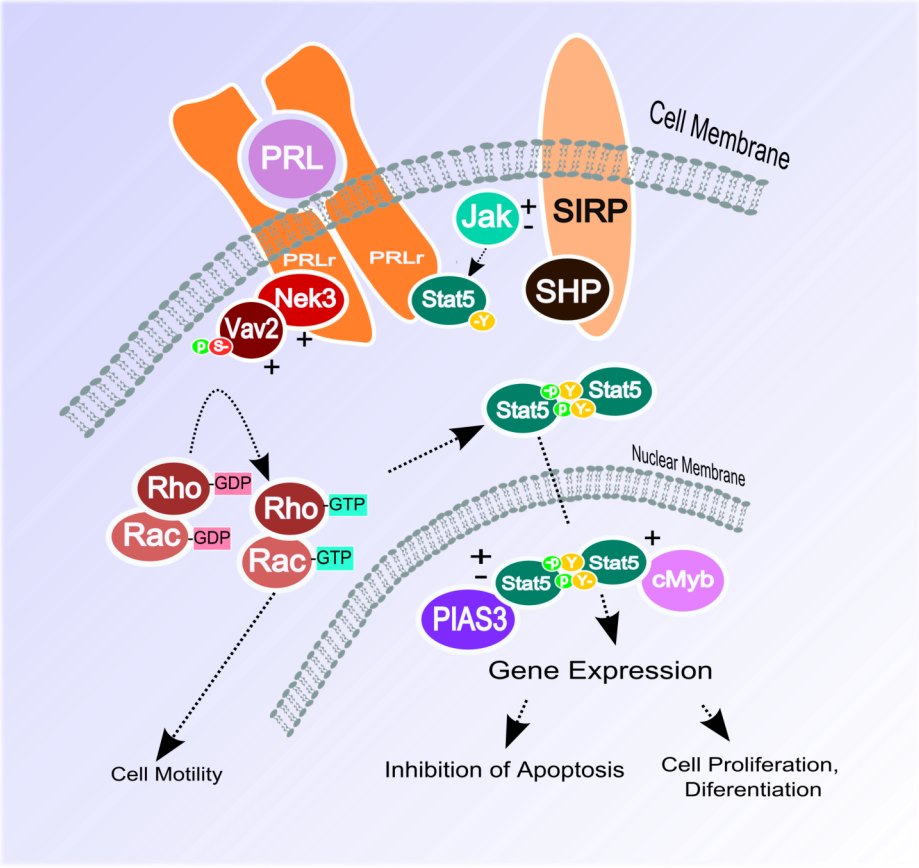
Failure from cell cycle checkpoints results in genomic instability (GIN), a cellular state that leads to an increase in genetic alterations which are classified by: nucleotide instability and chromosomal instability (CIN). Among the causes leading to genomic instability, CIN is the most common. The main consequence of CIN is aneuploidy, that for decades has been related to tumorigenesis [10]. It has been reported that both aneuploidies and DNA alterations (mutation, chromosomal translocation and gene amplification) result in cell heterogeneity, which in turn support tumor adaptation environmental changes, a hallmark of human cancer [11–13].

So far, it is known that the role of NEKs in genomic stability goes beyond their participation in DNA repair. NEK2, due to serine phosphorylation of Hec1, promotes the proper chromosome segregation [14,15]. Cells from NEK1 knockout mice presented errors in DNA repair associated with breakage and irregular size of chromosomes, chromosome lagging and aberrant cytokinesis, which reinforce its role in checkpoint DNA repair. NEK1 knockout cells also presented polyploidy after only a few passages, as a consequence of aberrant cytokinesis [16].

NEK3 is a 506 amino acid long serine/threonine protein kinase that localizes both in the nucleus and cytoplasm [17,18] and which gene localizes to 13q14.2 chromosome. Structurally, NEK3 has an N-terminal catalytic domain and a C-terminal regulatory domain which contains residue Thr475 in its PEST domain that is phosphorylated upon activation [19]. Expression of mutant NEK3 without Thr475 or PEST domain causes changes in cellular morphology and polarity of neuronal cells [20].

Functionally, NEK3 is also involved in cell motility and invasiveness of breast cancer tumor cells through interaction and possibly direct phosphorylation of VAV2, a GEF type regulator of the Rho GTPases. VAV1 and VAV2 are regulators of Rac1, Cdc42 and RhoA GTPases. Proteins of this family are molecular switches that alter between active

Figure 1: The role of NEK3 in prolactin signaling pathway. NEK3 interacts with prolactin receptor (PRLR) and VAV2. Active Vav2 is a GEF for Rac1 and leads to its activation (Rac-GTP). This in turn activates Stat5 that promotes transcriptional activation of several genes in the nucleus that lead to the inhibition of apoptosis, cell proliferation, differentiation and in gall bladder cancer cells to diminished metastatic potential. Adapted from McHale, [42].



(bound to GTP) and inactive states (bound to GDP) and have key functions in cancer cell proliferation, apoptosis/ survival, cell polarity, cell adhesion and plasticity of cell migration [21,22]. A clear connection can be established between Rho proteins over-expression and a large variety of human tumors [23,24]. In turn, Rac1 is described to be involved in prolactin receptor and NEK3 mediated signaling [17] (Figure 1). In this context, the association of NEK3 to the intracellular domain of the human Prolactin Receptor after extracellular binding of the prolactin hormone to its receptor, possibly results in the activation of NEK3. VAV2 on the other hand has been shown to activate RhoGTPase Rac1, leading to activation of the Stat pathway and ultimately causing gene transcription activation in the nucleus [25,26].

In cell proliferation, RhoGTPases contribute through cell cycle progression, which depends on cyclin-dependent protein kinases which activity increases and decreases periodically during the growth and division of cells [27]. RhoA, for example, plays a role during cytokinesis leading to the contraction of the actin ring, through myosin light chain phosphorylation. Once the midbody is formed, RhoA must be inactivated since its excess may cause abscission arrest [53]. Also, to ensure proper cytokinesis progression, RhoA´s cortical localization must be controlled spatially and temporally [54].

In the present study, we identified several new interaction partners for NEK3 by a yeast two hybrid assay, that are related to processes involved in cancer progression and aggressiveness through GTPases regulation. Our experiments suggest that NEK3 has also a role in the cell cycle, specifically in cytokinesis since its suppression is related to DNA bridge formation and consequent delay in cytokinesis.

2. Material and Methods

2.1. Yeast Two-Hybrid Screening

Protein-protein interactions are highly important to understand the role of the protein in cellular context. We cloned the full-length NEK3 wild type cDNA in fusion with the GAL4 DNA Binding Domain (GAL4-BD) in pGBKT7 vector, which also contains *TRP1*, a selection marker for auxotrophic growth. This construct was transformed into Y2H Gold *Saccharomyces cerevisiae* yeast strain that contains heterologous genes *AUR1-C, HIS3, ADE2*, and *MEL1* under GAL4 transcriptional factor control. Another *S. cerevisiae* yeast strain, Y187, contains heterologous genes l*acZ* and *MEL1* previously transformed with the Universal Human (Normalized) cDNA Library constructs in pGADT7 -RecAB with *LEU2* expression marker gene and GAL4 DNA Activation Domain (GAL4-AD). These two haploid yeast strains were mated and plated on a selective quadruple dropout medium containing also Aureobasidin A and X-α-Gal (QDO-XA). Positive blue colonies indicated that proteins were expressed and interacted with Nek3 to reconstitute the functional complete GAL4 transcription factor.

2.2. Confirmation of Positive Interactions

After sequencing and BLAST analysis, *S. cerevisiae* strains were co-transformed with both pGBKT7-NEK3 and pGADT7-prey according to Yeastmaker™ Yeast Transformation System User Manual. The positive control (pGBKT7-p53 and pGADT7-T) and negative control (PGBKT7-Lam and pGADT7-T) were also co-transformed. Blue colonies were compared to control and determined to confirm positive interactions.

2.3. Integrated Interactome System – IIS

Blue colonies from yeast two-hybrid screening were sequenced, processed and integrated to annotated interaction networks using the "Integrated Interactome System" developed at the National Laboratory of Biosciences and State University of Campinas, Brazil (http://bioinfo03.ibi.unicamp.br/lnbio/IIS2/) [28]. Biological processes and cellular components were obtained from the Gene Ontology database (http://www.geneontology.org) integrated to IIS. The enriched biological processes from the Gene Ontology GO database were calculated in each network using the hypergeometric distribution [28]. The final layout and cellular component allocation were obtained with the Cytoscape online software [29] and the CellNetVis software (http://www.lge.ibi.unicamp.br/cellnetvis).

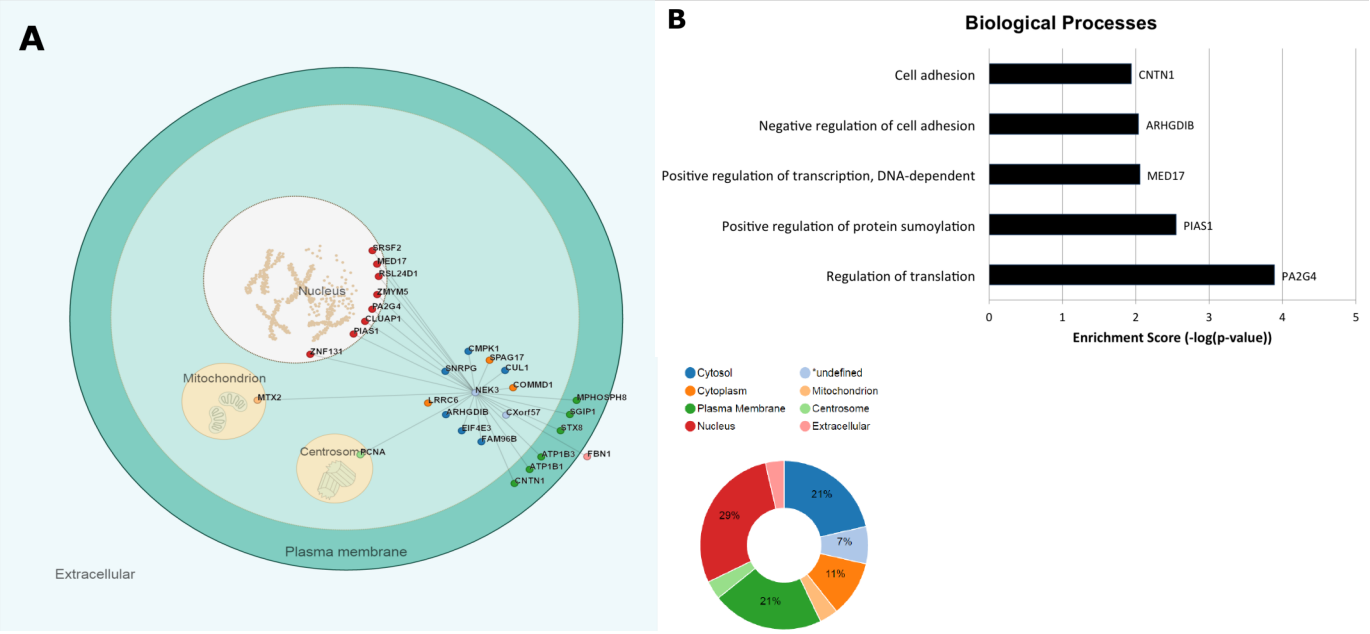
2.4. Analysis of NEK3-depletion phenotypes by short hairpin RNA

Viral transduction was performed using the lentiviral system with short-interfering rRNAs (shRNAs) designed for NEK3 (shRNA-Nek3.1: TRCN0000001471, 5'-GCAGTCCCATAGAACAGAAAT-3', for CDS of NEK3); (shRNA-Nek3.2: TRCN0000001473, 5'-CGAAGCATAACACACCAAGAA -3', for 3'UTR of NEK3). shRNA-GFP was used as a control plasmid. shRNAs sequences were obtained from The RNAi Consortium (TRC, IRB-Barcelona). Lentiviral particles were transduced with 1μg/mL polybrene in HeLa cells and stable lines were selected with puromycin 3μg/mL.

HeLa cells or HeLa stable cell line of shRNA-GFP, shRNA -Nek3.1 or shRNA-Nek3.2 were cultivated onto coverslips in 6-well plate for immunofluorescence assay under incubation in a humidified chamber at 37ºC with 5% CO2 in Dulbecco´s modified Eagle´s medium, DMEM (Gibco), supplemented with 10% certified fetal bovine serum (Gibco) and penicillin/streptomycin (100 units/mL, Gibco). All cells were mycoplasma tested regularly and before all experiments were proceeded.

Cells were fixed with ice-cold 100% methanol for 30 min,

Figure 2: NEK3 yeast two-hybrid interactome network and subcellular localization. (A) Most of the NEK3 interactors are localized in the nucleus, cytosol and plasma membrane. (B) The main cellular processes associated to NEK3 interactors. The enriched biological processes from the Gene Ontology GO database were calculated in each network using the hypergeometric distribution [28].



gently washed with 1xPBS (Phosphate buffer saline) and fixed specimens were permeabilized and blocked for 30 min at room temperature in blocking solution containing 1% BSA; 0.5% Triton X-100 into PBS.

Cells were incubated for 1 hour with the following primary antibodies diluted in blocking solution: rabbit anti-β-Tubulin (1:1000; ab15568; Abcam); rabbit Nek3 (1:200; AP8075C; Abgent); mouse RhoA; sc-418; Santa Cruz Biotechnology), all diluted in blocking solution. Next, the cells were incubated for 40 min in secondary fluorescent-labeled antibody: chicken anti-rabbit Alexa Fluor 488; chicken anti-mouse Alexa Fluor 647 (Life Technologies) diluted in blocking solution for concentration of 1:500. DNA was stained with Hoechst 33258 dye and coverslips were mounted on glass slides using ProLong Gold mounting media (Life Technologies).

Image data were collected in a Zeiss LSM 780 NLO Confocal Microscope (Carl Zeiss AG, Germany) using 40X or 100X lens. Series of Z stack images were captured from 0.7 µm thick sections and the entire cell volume was processed for 3-D rendering using Imaris (Bitplane). The numbers of HeLa cells were manually counted under a fluorescence microscope Leica DM2500 (Leica Microsystems). Statistical analysis was performed by the Student t-test and graphics plotted using GraphPad Prism software.

3. Results and Discussion

3.1. The construction of a NEK3 protein interaction map

The *in silico* analysis at the IIS and BLAST search resulted in 27 different proteins (Supplementary Table S1). The identified candidate interacting proteins are functionally involved in sumoylation, ubiquitinylation, transcriptional regulation, RNA processing, and the regulation of cell proliferation. A yeast co-transformation assay with NEK3-pGBKT7 and the prey proteins analyzed by the *Integrated Interactome* System was performed and, from 27 proteins initially found, some had their interaction confirmed in the yeast two-hybrid system itself like PIAS1, CNTN1, MED17 (CRSP77), PA2G4 (EBP1) (Supplementary Figure S1).

Using the Cytoscape software and CellNetVis, the Nek3 protein and its interaction network with subcellular localizations were performed (Figure 2A). Some interaction partners for NEK3 are mostly localized to the nucleus (29%) such as MED17 and PIAS1, but also to cytosol and to the cell membrane (21%). Importantly, the preys localized to the cell membrane are lipid anchored proteins or attached proteins, such as VAVs and SGIP1, which thus are proteins oscillating between the plasma membrane and cytosolic localizations.

One of the most expected Nek interactors, from the signaling context of Nek3, was RhoGDI2 / ARHGDIB (RhoGDP-dissociation inhibitor 2) (Figure 2). RhoGDI2 as well as VAV2, are negative regulators of the RhoGTPases Rac1 and RhoA which has been reported to suppress metastasis in bladder cancer, but also increased invasion of breast cancer cells [20]. A biphasic expression pattern of RhoGDI2 itself in breast cancer was found, where a correlation of its decreased expression in lymph node metastasis was reported [21]. Furthermore, another work found RhoGDI2 expression was up-regulated in human ovarian tumors of different histological subtypes [22]. These findings suggest that RhoGDI2 biology in cancer may depend on its interaction partners, including NEK3. RhoGDI2 may be alongside VAV2 and Rac1/RhoA an

additional new player in the signaling pathways regulated through the PRLR/Nek3.

PIAS1 (protein inhibitor of activated STAT-1) is an important component of SUMO (small ubiquitin modification protein) complex and acts as an E3 ubiquitin ligase adding SUMO to the substrate [30]. Recent works have shown that SUMOylation is also involved with Ran GTPase regulation and has important functions in nuclear traffic at interphase and mitotic spindle assembly [31]. The interaction between Nek3 and PIAS1 raise the possibility of an involvement of Nek3 in the SUMOylation process.

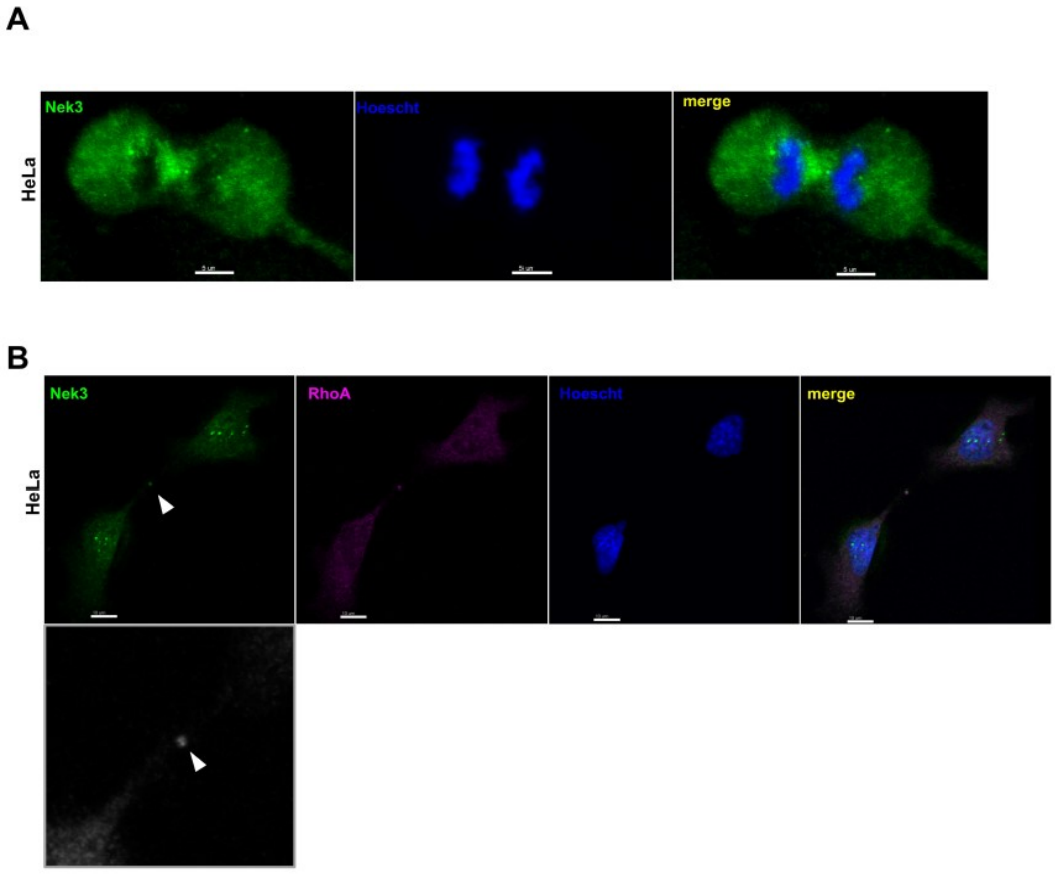
MED17 (CRSP77) is a member of mediator complex, a co-activator involved in transcription regulation of almost all RNA polymerase II-dependent genes. MED17 works as a bridge to transmit information from gene-specific regulatory proteins to RNA polymerase II transcription basal machinery. In this regard, MED17 is recruited by promotors through direct interactions with regulatory proteins and acts as a scaffold of pre-initiation complex with RNA polymerase II and its transcription factors [24]. Jeon and colleagues demonstrated for the first time a possible involvement of Neks in transcription. NEK6 phosphorylate STAT3 on Ser727 affecting the maximal transcription of a cancer cells [32].

Proliferation-associated protein 2G4 (PA2G4) or ErbB3-binding protein 1 (EBP1), is a modulator of the ErbB-3 transduction pathway – member of Epidermal Growth Factor (EGF) family – by interaction with its cytoplasmic domain [25]. EBP1 is also involved in first and late steps of RNA processing by association to 28S, 18S e 5.8S rRNA subunits [27].

Contactin1 (CNTN1) is a cell adhesion GPI-anchored protein, belonging to immunoglobulin superfamily [33]. This protein associates with other cell surface proteins like L1 or NCAM via homophylic interactions, and acts in signal transduction in neurons [34]. Contactin 1 is also related to invasion and metastasis in lung adenocarcinoma by RhoA activation [35], and its suppression increases cancer cell invasion and metastasis [24].

All for all, the NEK3 preys were related to a variety of processes involving cancer progression and aggressiveness, from cell cycle regulation to cell migration and invasion. Since molecular interactions help us to understand protein function and behavior, we can propose a complex role for NEK3 in different steps of cancer development.

Figure 3: Localization of NEK3 during cytokinesis of HeLa cells. Confocal images showing NEK3 accumulation at the furrow of ingression during anaphase (A) and at the midbody ring along with RhoA during cytokinesis (B). Images are presented as maximal intensity projections of multiple z-slices. Scale bar is indicated in the images (A: 5 μM, B: 10 μM). At least 170 randomly selected anaphase or cytokinesis cells were observed and all analyzed cells showed the NEK3 localization pattern represented in the images.



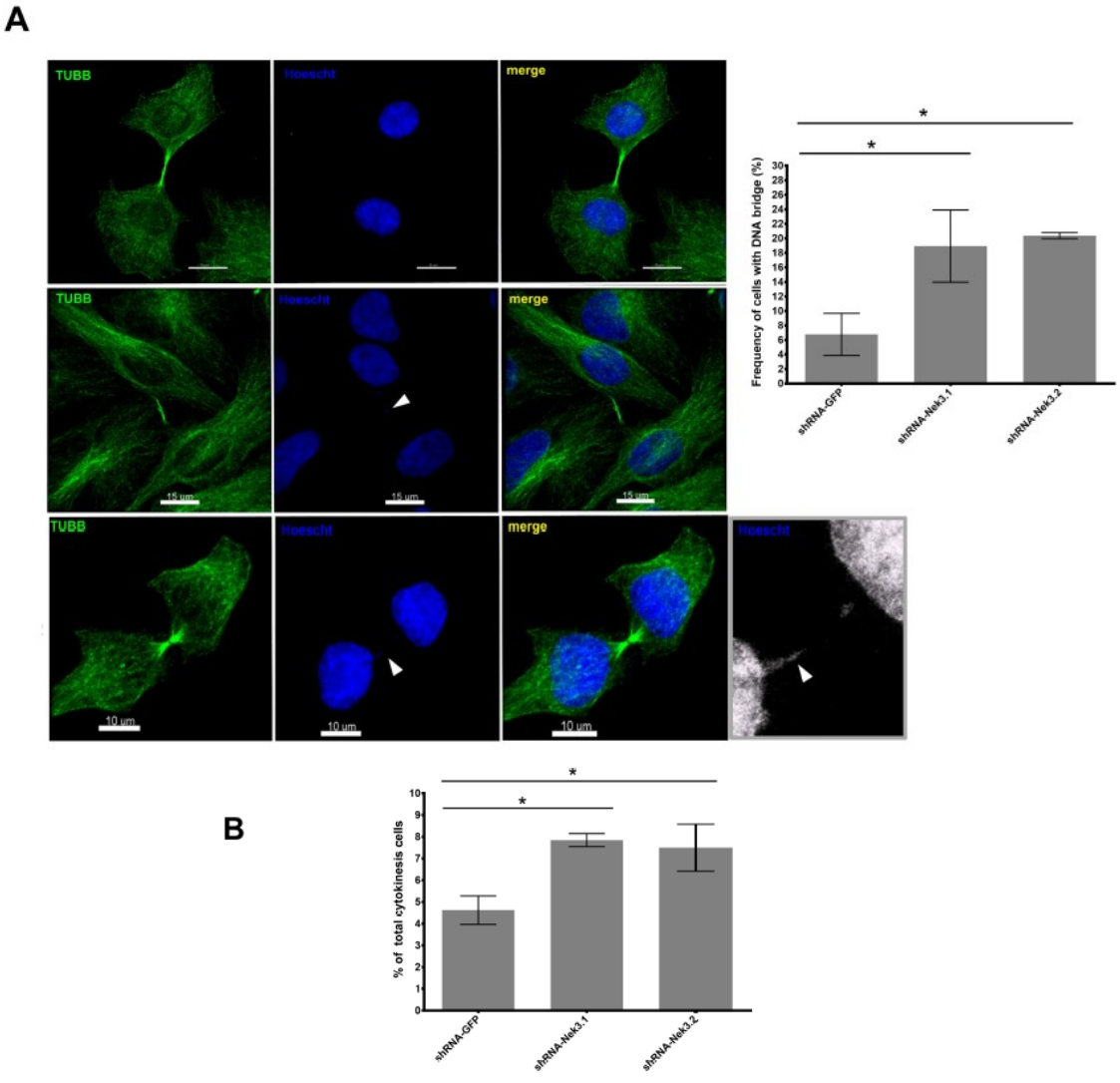
3.2. Study of cell cycle phenotypes in NEK3 knock-down HeLa cells

Formation of the mitotic cleavage furrow is dependent upon both microtubules and activity of the small GTPase RhoA [36]. Since NEK3 interact with RhoA we raised the hypothesis that NEK3 may have functions during cell division.

To explore this idea, we characterized the localization of NEK3 during cytokinesis. To this end, HeLa cells were stained for immunofluorescence of β-tubulin and NEK3 and analyzed by confocal microscopy. NEK3 was found enriched at the furrow of ingression during anaphase (Figure 3A), and at the midbody along with RhoA (Figure 3B) during cytokinesis pointing to their potential functional interplay during cell division.

These results led us to further investigate functional roles of NEK3 during cytokinesis. For this purpose, we employed lentivirus-delivered NEK3 silencing by RNAi in HeLa. The NEK3 suppression resulted in cytoplasmic bridges containing DNA (Figure 4A) Accordingly, there was an increase in the frequency of cells blocked in cytokinesis under these conditions indicating that NEK3 suppression delays cells at cytokinesis (Figure 4B). The cytokinesis delay due to DNA bridges may be related to aneuploidy, tetraploidy and micronuclei formation, which can lead to

Figure 4: Nek3-depletion induces DNA bridges during cytokinesis. (A) Confocal images show bridges containing DNA connecting the cells. DNA bridge are indicated by arrowheads in the images and enlargements of the cytoplasmic bridge region. Images are presented as maximal intensity projections of multiple z-slices in 3D rendering. Scale bars (lower row: 10 μM or 15 μM in the upper two rows) are indicated in the images. HeLa cells were counted through the microscope, and plotted in the graphic (right) using GraphPad Prism software . At least 65 cytokinesis cells per condition were counted in each duplicated of two independent experiments. *P<0.05 was considered as statistically significant. (B) Nek3-depleted cells delay for completion of cytokinesis**.** The number of HeLa cells were manually counted through the fluorescence microscope from immunofluorescence coverslips and plotted in the graphic. More than 400 cells per condition were counted in each duplicate of two independent experiments. Graphs were created and analysis of statistical significance was performed by the Student t-test using GraphPad Prism software. *P<0.05= statistically significant.



both cancer mutations and chromosomal instability [37].

Some interactors for NEK3 found in its yeast two hybrid assay are also related to the observed cell cycle phenotypes. Cullin 1 is involved in ubiquitination, degradation pathway that regulate proteins which controls cell division by two complexes: SCF and APC. This NEK3 partner together with SKP1, RBX1 and F-Box forms the SCF complex, which stimulates ubiquitination of cell cycle proteins such as CDKs in addition to control the transition between G1/S and G2/M.

Roles in cytokinesis for NEKs have been widely proposed. For example, Nek6 and Nek7 may similarly regulate the localization of factors required for cytokinesis. In this respect, either NEK6 or NEK7 is localized at the midbody and depletion or expression of a kinase dead mutant protein together with mitotic spindle checkpoint inhibitors resulted in cytokinesis arrest for both kinases, [38]. In addition, NEK7 mouse knockout cells presented chromosome lagging, micronuclei formation, cytokinesis delay and aneuploidy [39]. In HeLa cells, genistein treatment, responsible for tubulin polymerization inhibition, also promotes DNA bridges and cytokinesis delay [40].

NEK3 has been described to be involved in neuronal morphology due to tubulin acetylation [20]. The suggested new role for NEK3 in DNA bridge formation and chromosomal instability may be also related to microtubule stabilization. Since chromosomal instability leads to mutations which result in tumor progression and also in cancer cell heterogeneity that drive multidrug resistance [41], the results shown in this work reinforce the roles for NEK3 in all stages of carcinogenesis.

4. Concluding Remarks

In this work, we performed a large scale screening for interaction partners of NEK3 by using the yeast two-hybrid system. We identified candidates interacting proteins that were functionally involved in SUMOylation, ubiquitinylation, transcriptional regulation, RNA processing, regulation of cell proliferation, invasiveness and metastasis. These processes are also involved with several steps of cancer progression and can be used to understand NEK3 function and behavior. NEK3 was found at the furrow of ingression and its depletion is associated to cytokinesis delay due to DNA bridges, that is related to aneuploidy, tetraploidy and micronuclei formation [37]. The role of NEK3 in DNA bridge formation may be related to microtubule stabilization, possibly via tubulin acetylation as observed for Nek3 signaling in neuronal cells [21].

In resume, this work has shown that NEK3 and its interaction partners are involved in multiple cancer stages through regulation of GTPase functions, among other signaling events. Different from other NEKs, NEK3 levels do not increase during cell cycle and its localization is not related to cilia function, but it is involved with DNA bridges formation and its knock down causes a cytokinesis delay. Also, this kinase localizes along with RhoA in the midbody, which has been previously described as essential for the cleavage furrow formation. Therefore, NEK3 may have novel important roles in the context of cytokinesis and the relevance of this new function in the context of cancer biology should be further explored.

5. Supplementary material

Supplementary data and information is available at http://www.jiomics.com/index.php/jio/rt/suppFiles/195/0

Supplementary Figure S1: NEK3 interactors in Yeast Two Hybrid Screening.

Supplementary Table S1: NEK3 interactome after Yeast two hybrid screen

Acknowledgments

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