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NIMA-related kinase 7 interacts with Mat1 and is involved in the UV-induced DNA Damage Response

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ABSTRACT

Nek7 is a serine/threonine kinase of the mammalian NIMA-related kinases (Neks) family, which members are involved in the regulation of the progression of the cell cycle. Although several Nek members have been associated with a range of cell cycle-related tasks, including DNA repair, a possible role of Nek7 in the DNA-damage response is so far unknown. Here, we employed in vitro and in vivo interaction assays to identify Mat1 as a specific Nek7 binding partner and substrate. In addition, we showed that Nek7 pulled down both CDK7 and cyclin H and directly phosphorylated Mat1, indicating that Nek7 may play a role in the regulation of the CAK complex. Furthermore, we showed that both Nek7 and Mat1 depletion led to an accumulation of cells in the S-phase, decreased cell proliferation and increased apoptosis. Notably, the mutational ablation of kinase Nek7 activity also induced increased apoptosis upon DNA damage. Collectively, our findings support the notion that Nek7 may cooperate with Mat1 in signal pathways that govern the cell cycle machinery including DDR, S-phase progression and apoptosis, and thereby can constitute an important novel player for in the context of cellular transformation and tumorigenesis.

Keywords: Nek7, Mat1, CAK complex, apoptosis, DNA damage response

1. Introduction

The human Nek7 belongs to the NIMA-related kinases or "Neks" family, that comprises a family of eleven proteins named Nek1 to Nek 11, which have been functionally associated to mitosis, cilium regulation and DNA damage response (DDR) [1].

Nek7 interacts with Nek6 and Nek9, both of which are implicated in cell cycle progression and spindle assembly [1-6]. Specifically, silencing of Nek7 causes alterations in levels of γ-tubulin in interphase cells and results in an arrest in prometaphase, whereas its over-expression results in multinucleated cells and a high proportion of apoptotic cells [4]. In addition, reduced interphase-microtubules growth and contraction speed were observed after Nek7 suppression, showing that Nek7 influences microtubule dynamics [6]. Salem and colleagues [7] showed that Nek7 absence is lethal during embryogenesis, indicating the importance of Nek7 in the development and survival of the organism. Moreover, additional studies have found higher Nek7 expression levels in cancers of larynx, breast, colorectal and gallbladder [8]. Recently, studies showed that NEK7 plays an important role in the regulation of NLRP3 inflammasome activation [9] and its overexpression induces the production of abnormal cells, including the multinucleated cells and apoptotic cells which are closely linked to inflammation [10]. Moreover, Nek7 might be involved in hepatocarcinoma progression by regulating cyclin B1 expression [11]." More recently, the recruitment of Nek7 was shown in TR1 regulation, in response to oxidative telomeric DNA damage [12], demonstrating once more the typical multi-functionality

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found for many protein kinases. Thus, these findings suggest that Nek7 is an essential component to cell division regulation and consequently a possible involvement in tumorigenesis maybe predicted.

We have previously shown in yeast two-hybrid screens that Nek7 interacts with Cyclin-dependent kinase (CDK)-activating kinase assembly factor Mat1 [13], which along with cyclin H and CDK7 forms the CDK-activating kinase (CAK) complex [14]. CAK complex composes the kinase subunit of the basal Transcription factor IIH (TFIIH), that participates in nucleotide excision repair (NER) [15], transcriptional and cell cycle regulation [16]. Recently Patel and co-workers [17] found that expression of CDK7, Cyclin H and MAT1 is elevated in breast cancer, suggesting that this tumor type may be especially sensitive to CDK7 inhibition and that the CDK7 over-expression may contribute to an elevated chemo-resistance of cancer cells in comparison to normal tissues. In essence, Mat1 plays a role in the cell cycle control by modulating the expression of CDK7 and cyclin H [18], determining the CAK substrate specificity towards important cell cycle players such as p53 [19] and pRb [20], and monitoring the TFIIH-DNA damage response [21]. However, no record exists about upstream Mat1 players in regard to cell cycle regulation. In addition, although we previously speculated about a possible connection of Nek7 with the DDR, based on in silico analyses [1], no experimental data so far reported an involvement of Nek7 in the DDR.

Here, we provide the first evidence of functional involvement of Nek7 in the DDR. Through in vitro and in vivo interaction studies, we demonstrate that Mat1 is a novel Nek7 interactor and substrate. Our observations also indicate that Nek7 associates to the other CAK proteins CDK7 and Cyclin H, indicating that Nek7 could participate in the regulation of CAKs tasks. Since Mat1 is a member of the CAK kinase that is crucial for DNA repair and cell cycle progression, we investigated the Nek7 involvement along with Mat1 in DDR and cell cycle regulation. In this regard, we showed that Nek7 or Mat1 depletion led to an accumulation of cells in the S-phase and decreased cell proliferation. Importantly, we further found that expression of a kinase inactive variant of Nek7 leads to increased levels of apoptosis, upon UV-light induced DNA damage. Based on our results we propose that Nek7 and Mat1 are involved in monitoring the integrity of the genome and to protect cells from accumulating genetic damage.

2. Materials and Methods

2.1. Immunoprecipitation

Cells were lysed in lysis buffer [20 mM Hepes (pH 7.4), 150 mM NaCl, 1% Triton, 10% Glycerol, 1 mM EGTA, 1mM EDTA, protease and phosphatase inhibitor cocktail (1:100, SIGMA)]. Cell lysates were incubated and gently rocked for 1h, at 4 °C, in ANTI-FLAG® M2 Affinity gel. Resin and them the immune complexes were precipitated, washed using TBS1X and were subjected to immunoblot analysis.

2.2. Recombinant protein purification and Pull Down assay

Full-length Nek7 (6×His-Nek7) construct was obtained as previously reported by de Souza [13]. For pull down assay, 100 µl Ni-NTA Agarose (Qiagen), was washed twice with 1 mL of wash buffer (50 mM Tris-HCl, pH 8,0; 1% NP40 and 1% protease inhibitor). Then, 100 µg of 6×His-Nek7 was bound to the Ni-NTA agarose resin and incubated for four hours, by gentle rocking at 4°C. The resin containing the 6×His-Nek7 was washed three times with 1 mL of wash buffer, and incubated over-night at 4°C with 800 µg HEK293T lysate. Afterwards, the resin containing the protein-protein complex was washed three times with 1mL of wash buffer, and analyzed by SDS-PAGE and Western blotting, using the indicated specific antibodies against the tagged proteins.

2.3. In vitro kinase assay

For In vitro kinase assay, 0.5 µg of GST-Nek7 and 0.5 µg of each substrate were suspended in kinase assay buffer (20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1 mM NaF, 2 mM β-glycerophosphate, 100 µM ATP, 1 mM dithiothreitol), supplemented with 1 µCi of [γ-P_{32}]-ATP, and incubated at room temperature for 1 hour. SDS-PAGE sample buffer was then added, to stop the reaction, followed by incubation at 100°C for 5 minutes. Proteins were then separated by SDS-PAGE, and gel was dried and autoradiographed.

2.4. Cell culture and plasmid transfections

HEK293T and HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were transfected using Lipofectamine reagent. 4 µg DNA was added to 700 µl of optiMEM and 30 µl of lipofectamine and this mixture was incubated for 20 minutes at room temperature. The mixture was subsequently added to the cell growth medium, and cells were incubated for 48 h prior to medium exchange.

2.5. Viral transduction

The Nek7 and Mat1 knock down lines were produced by viral transfection using a lentiviral system carrying short-interfering RNAs (shRNAs) designed to target human Nek7 or Mat1: - shRNANek7 = TRCN0000001967,5′-CTTTAGTTGGTACGCCTTATT-3′ (generating clone Nek7-N767), - shRNANek7 = TRCN0000001969, 5′-GAAGAGTGTAACCAAAGTAAT-3′ (generating clone Nek7-N769); - shRNAMat1= TRCN0000019944, 5′-CCTAGTCTAAGAGAATACAAT-3′ (generating clone

Mat1.1), - shRNAMat1 = TRCN0000019945, 5′-GCTATACTTCTTCTCTTGCTT-3′(generating clone Mat1.2), all purchased from The RNAi Consortium (TRC) (UMASSmed core, Worchester, USA). The lentiviral particles were introduced into HeLa cells according to the manufacturer's instructions and stable cell lines were generated by selection with 3.5 µg/ml puromycin (Sigma-Aldrich). Two stably transfected clones named N769, N767 (for Nek7) and two others for Mat1 (named MAT1.1 and MAT1.2) were obtained and analyzed. The efficacy of Nek7 and Mat1-depletion was assessed by Western Blotting (WB).

2.6. UV irradiation

For UV irradiation, HEK293T or HeLa cells were seeded at 70% confluence and then subjected to different doses of UV- C irradiation for different times, using a UV source ENF-260C/FE Spectroline® UV lamp (Spectronics Corporation, Westbury, NY, USA). The UV dose used for all experiments was 50 J/m^2, followed by a recovery period of for 2 h, in a CO_2 incubator at 37°C and protected from further light.

2.7. Flow cytometry and EdU labeling for fluorescence microscopy

Flow cytometry was used for cell cycle and apoptosis analysis. For cell cycle analysis, cells were fixed with 70% ethanol, stained with 100 µg propidium iodide and analyzed by flow cytometry using FACS Canto II (Becton Dickinson). For apoptosis determination we used the FITC Anexin V Apoptosis Detection Kit (BD Pharmingen™) and followed the manufacturer's instructions. To detect DNA synthesis (S -phase), cells were incubated for 2 hours with 30 µM thymidine analog EdU (5-ethynyl-2'-deoxyuridine). EdU incorporation was detected using click Chemistry reaction and azide labelled with Alexa Fluor 488, according to manufacturer instructions (Click-iT Edu Image kit, Invitrogen). The images were captured by confocal laser scanning microscope (Leica TCS SP8) and analyzed and processed using Image J 1.43

2.8. Immunofluorescence microscopy

To immunofluorescence assay, cells irradiated or not with UV-C were fixed and permeabilized with 3.7% formaldehyde solution (Sigma-Aldrich, F1635), containing 0.2% Triton X-100 in PBS 1X, then blocked for 30 min in blocking buffer containing 3% bovine serum albumin, and 0.1% Triton X-100 (Sigma-Aldrich) in PBS. The cells were incubated for 1 hour with primary antibodies, diluted in blocking buffer, containing mouse anti-phospho-Histone H2A.X (EMD Millipore 05-636; 1:500 dilution). Then, cells were washed with 1x PBS and incubated with secondary antibody chicken anti-mouse Alexa Fluor 488 - (dilution 1:500) for 40 min. Hoechst was used to stain DNA.- Data image were collected on a Zeiss LSM 780 NLO Confocal Microscope (Carl Zeiss AG, Germany) using 40X or 100X lens. Series of Z stack images were captured from 0.5 µm thick sections and images were processed using Image J software program (http://rsb.info.nih.gov/ij/).

3. Results and discussion

3.1. Nek7 is associated to CAK complex and phosphorylates Mat1

In response to genotoxic stress, cells protect their genomes integrity by activating a conserved DDR pathway that coordinates DNA repair and cell cycle progression [26, 27]. Clear roles for Nek7 in regulating the cell cycle [4, 28], as well as for Mat1 in the cell cycle response to DNA-damaging agents have been established [29, 30, 21, 14]. However, there has so far been no evidence to support an involvement of Nek7 in DNA damage-induced cell-cycle regulation, via Mat1. We have earlier reported a yeast two-hybrid screens for Nek7 that resulted in the identification of the CAK assembly factor Mat1 as a Nek7 interactor [13]. This interaction prompted us to investigate if Nek7 can associate with the others CAK components and if Mat1 is phosphorylated by Nek7, to obtain clues about possible new functions for this protein. Toward this end, we performed pull down assay using recombinant Nek7 as bait and endogenous CDK7, Cyclin H and Mat1 as prey from HEK293T cells. As shown in the Western blots in Fig. 1A, Nek7 was able to associate specifically with all CAK components, but not with the control protein RAR, indicating that it can be found in association with the CAK

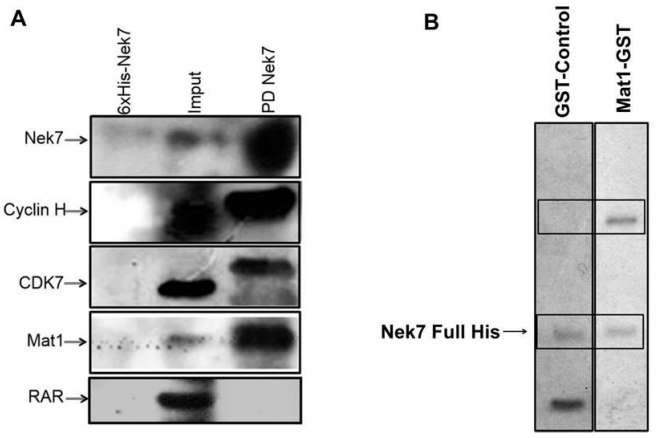

Figure 1 | Nek7 is associated to CAK complex and phosphorylates Mat1. A) Imuno blot detection (anti Nek7, Cyclin H, CDK7, Mat1 or RAR) of co-precipitated proteins from pull-down (PD) of recombinant Nek7 (6xHis-Full-Length-Nek7) from Hek293T (detection of endogenous Cyclin H, Cdk7 and Mat1). RAR was used as a negative control. The pull-down assay results are based on three independent experiments. B) Autoradiography of P_{32} labelled proteins. Recombinant 6xHis-Nek7 phosphorylates GST-full-length-Mat1, but not the negative control protein GST.

complex. In order to determine whether the CAK assembly factor Mat1 can serve as a substrate for Nek7, an in vitro kinase assay was performed, using recombinant Nek7 and Mat1. The results show that Mat1 is indeed phosphorylated by Nek7 (Fig. 1B) in vitro. These findings are in line with our previous report of Mat1 being an Nek7 interactor and substrate [13] and supply further evidence that different CAK components could be regulated by Nek7 to mediate CAK functions in the context of the cell cycle, apoptosis and potentially DNA repair.

Thus, we speculated that the interaction and phosphorylation of Mat1 by Nek7 may influence the equilibrium between the free ternary CAK and the core TFIIH-related CAK. In this context, Nek7 could contribute to regulating Mat1 functions, including mechanisms that involve the substrate specificity of CDK7 [31, 32], cyclin H expression regulation [18] or Mat1 participating in nucleotide excision repair [30,14].

3.2. Nek7 and Mat1 regulate UV-induced DDR during S-phase

Recent data have shown that CAK proteins are involved in DNA repair mechanisms, particularly in UV damage response, with Mat1 being rapidly recruited to the injury site [14]. UV radiation is among the most frequent causes of DNA damage to cells that lead to a DNA damage repair, cell cycle arrest and apoptosis [22]. Since Mat1 has been previously implicated in DNA damage repair [14], our detection of the Nek7-Mat1 interaction and phosphorylation, raised the hypothesis that both proteins may cooperate in the UV-induced DDR.

To explore this connection, Mat1 or Nek7 were depleted by shRNA from HeLa cells (Fig. 2A-B), irradiated or not with 50 J/m2 UV and examined by immunofluorescence staining of phosphorylated histone γH2AX (Suppl. Fig.1), a key player of the UV-induced DDR that when detected in the form of nuclear foci, is considered to be a marker of formation of DNA double-strand breaks (DSBs) [23, 24]. The results show that the UV treatment promoted a nuclear accumulation of γH2AX suggesting that Nek7 or Mat1 inhibition somehow is involved in either inducing or increasing the UV-derived DNA damage. Increased levels of γH2AX may result in the activation of DDR and the phosphorylation of the ATM and ATR kinases, which in turn activate the mitotic checkpoints Chk1, Chk2 and the pro-apoptotic p53 protein, to induce cell cycle arrest with accumulation of cells in the S-phase and apoptosis [35]. Thus the possible relation of Nek7/Mat1 with other proteins involved in this pathway should be better characterized in the future.

Under these conditions, Nek7 or Mat1 depleted cells (Fig.2 A,B) were submitted to cell cycle analysis using flow cytometry. According to Fig. 2D, in the non-irradiated condition, after Nek7 depletion the percentage of cells in the S-phase (replicating cells) presented an increase of about 5%, while upon Mat1 depletion, there was an increase of 30% when compared to shRNA-control transfected cells. When these cells were UV irradiated (Fig. 2E) we observed an increase of the percentage of cells in the S-phase of 18.5% (Nek7 depletion) or 28.8% (Mat1 depletion). Therefore, Nek7 or Mat1 inhibition led to decreased accumulation of cells in the S-phase. These findings support the notion, that interference with Nek7 or Mat1 may regulate DNA replication during S-phase upon DNA damage and can potentially affect cell proliferation.

To further explore this hypothesis, we decided to investigate whether Nek7 and Mat1 have roles in cell proliferation. To this end, cells over-expressing Nek7 wild-type or Nek7 or Mat1-depleted (Fig. 2A and 2B, respectively) were irradiated or not with 50 J/m2 of UV-C, and then labeled with EdU (5-ethynyl-2'-deoxyuridine). Then, cell proliferation was assessed by fluorescence microscopy and flow cytometry.

Fig. 3 (A and B) shows that after UV light exposure all cell types tested showed a significant decrease in proliferation. However, in the cells with Nek7-overexpression, the proliferation was significantly higher and in the cells with Nek7 (Fig.3B) or Mat1 (Fig. 3D) depletion, the EdU incorporation was significantly reduced, or abolished, after UV light exposure (Fig. 3A-D). These results are consistent with Nek7 or Mat1 regulating the S-phase (Fig 2D and E), and suggest that inhibition of Nek7 and mainly Mat1 affect cellular proliferation mostly after in DNA damage conditions.

3.3. Nek7 and Mat1 trigger UV-induced apoptosis

Based on the above results, we decided to check if Nek7 or Mat1 could, eventually, increase UV-induced apoptosis as a consequence of both the cell cycle arrest and increase in the number of cells in the S-phase. Therefore, we examined whether UV-induced apoptosis is affected by Nek7 and Mat1. To this end, Nek7 or Mat1-depleted cells as well as wild-type Nek7 or "kinase dead" Nek7 (Nek7K63A and Nek7K63/64A) overexpressing cells (Fig. 2A-C), were irradiated with 50 J/m2 of UV-C to be submitted to an apoptosis assay, using flow cytometry.

Indeed, Nek7 or Mat1 depletion induced a significant increase of the cell´s percentage undergoing apoptosis (Fig. 4A and 4B, respectively and Suppl. Fig. 2A and B), with a higher tendency to additionally increase after UV irradiation.

Interestingly, there was a decreased percentage of cells undergoing apoptosis upon UV irradiation after wild-type Nek7 over-expression, in opposition to what is observed after "kinase dead" Nek7 over-expression (Fig. 4C and Suppl. Fig.3). These results are in line with the UV-induced S -phase arrest following wild-type Nek7-overexpression (Fig. 3 A-B) and suggest that Nek7 and its kinase activity are important to protect the cells of UV-induced apoptosis.

Together, these findings may indicate that Nek7 and Mat1

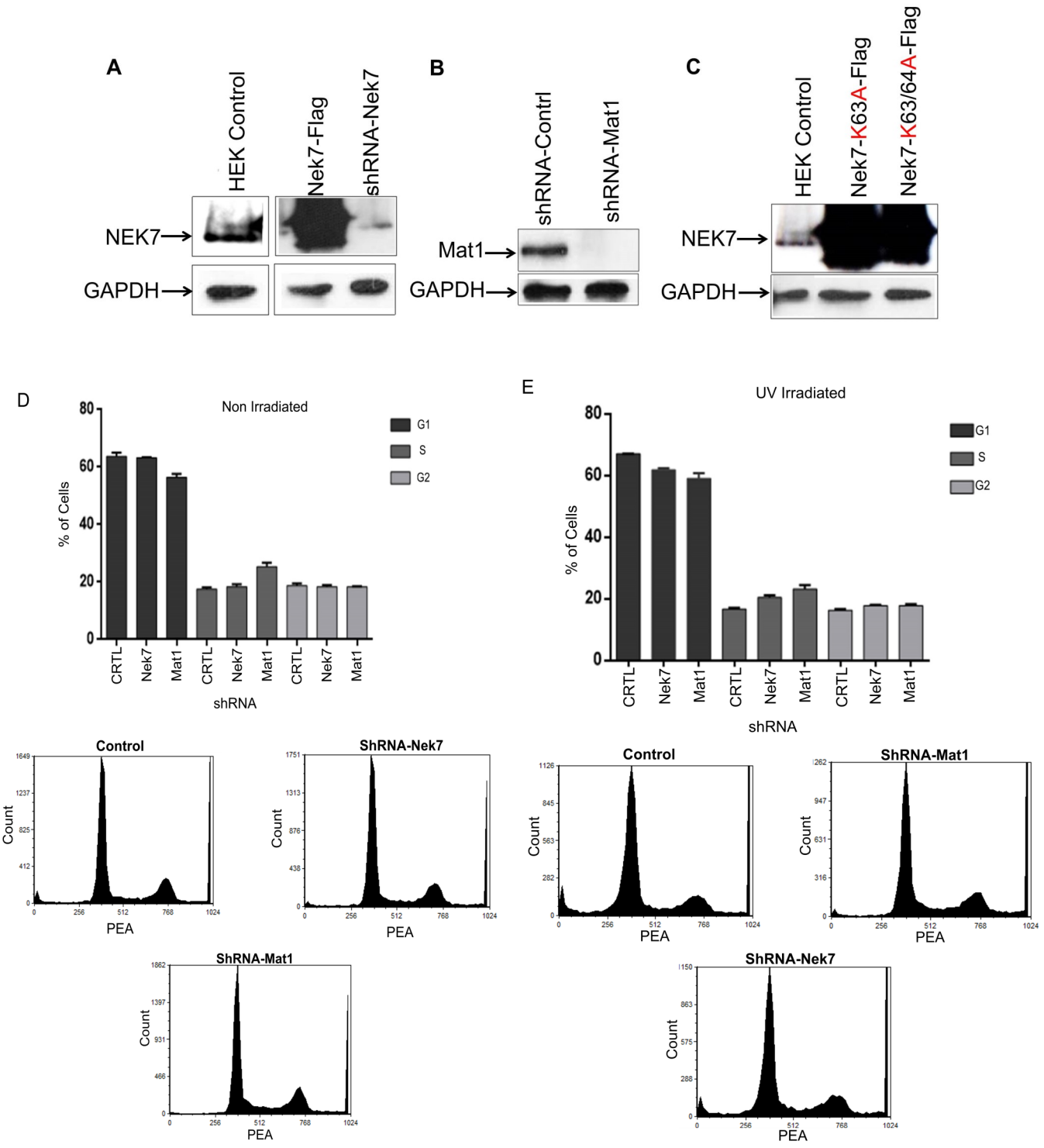

Figure 2 | Nek7 and Mat1 regulate S-phase upon induction of DDR by UV irradiation. A) Western blot (anti-Nek7 imuno blot) of Hek293T cells showing the Nek7 wild type, over-expression of Nek7-Flag and also Nek7 depletion by shRNA; B) Western blot of Hek293T cells showing Mat1 depletion by shRNA; C) Western blot of Hek293T cells showing the over expression of Nek7 with two different point mutations in the kinase domain (Nek7-K63A and Nek7K63/64A). GAPDH antibody was used as loading control. D-E) Graphs represent the FACS analysis showing the cell cycle distribution - The bars correspond to the different phases of the cell cycle (G1, S, G2 fases) for control cells depleted for Nek7 or Mat1 in normal conditions and after UV irradiation respectively. D. Statistical significant differences between columns 1 and 3 and between columns 4 and 6: $P < 0.05$ calculated using two-way ANOVA, Bonferroni post-test (GraphPad Prism). $P< 0{\cdot}05$ calculated using two-way ANOVA, Bonferroni post-test (GraphPad Prism). Histograms represent the FACS analysis showing the cell cycle distribution. Studies were performed in cells control, Nek7 and Mat1 depleted cells (shRNA-control, shRNA-Nek7, shRNA-Mat1) irradiated or not with 50 J/m2 of UV-C light and recovered for 2 hours.

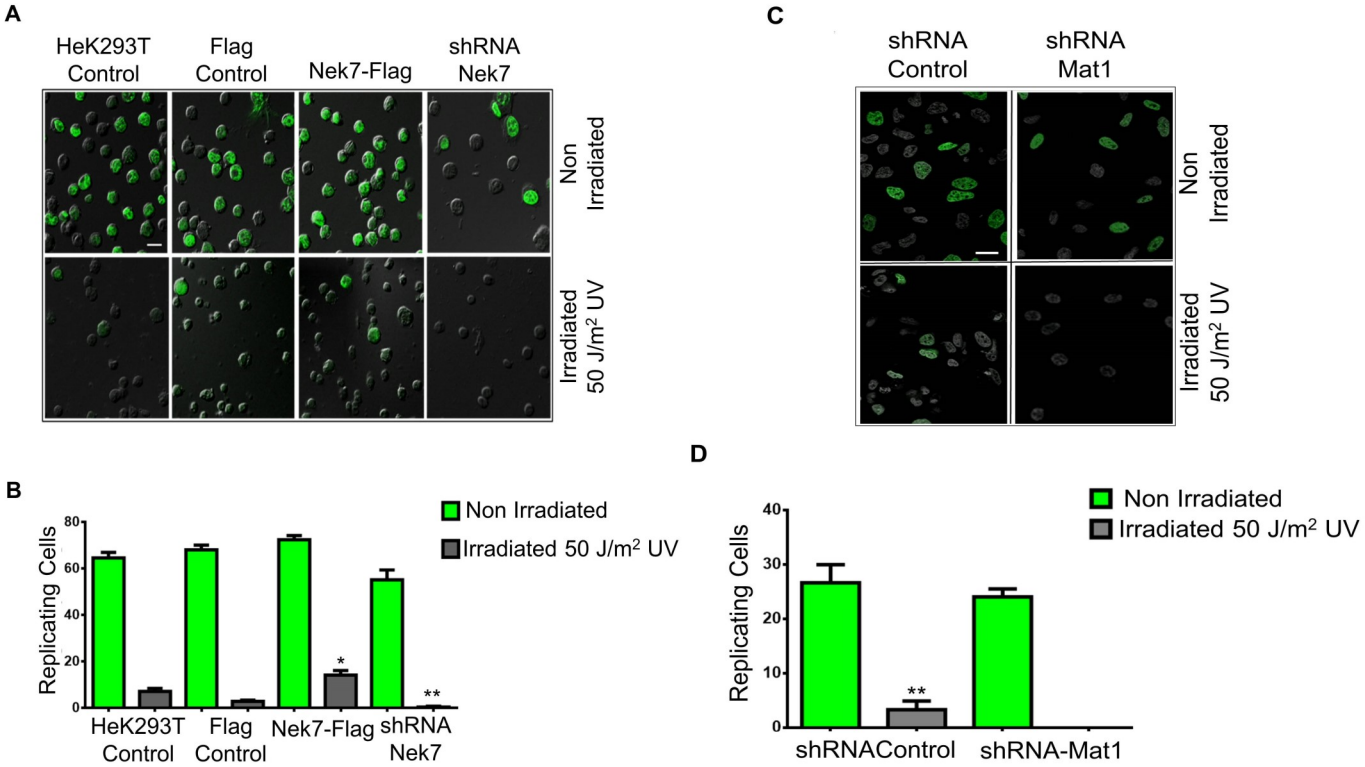

Figure 3 | Nek7 and Mat1 modulate cell proliferation: A) Non transfected Hek293T (control), over-expressing Nek7 (Nek7-Flag) and depleted for Nek7 (shRNA-Nek7), were irradiated or not with 50 J/m2 of UV-C light and recovered for 2 hours. After this the cells were stained with Alexa Fluor 488-EdU for cell replication analysis by fluorescence microscopy. Cell stained green are replicating. B) Representative graphs of the experiment shown in A, indicating the percentage of replicating cells. Cultures irradiated with UV (grey bars) or not (green bars). (*, **) indicate P < 0,05; calculated using two-way ANOVA, Bonferroni post-test (GraphPad Prism). C) The same experiment show in B was done for Mat1 depleted cells (shRNA-Mat1). D) Representative graphs of the experiment shown in C, indicating the percentage of replicating cells. Cultures irradiated with UV (grey bars) or not (green bars). (**) Indicates P < 0,05 calculated using two-way ANOVA, Bonferroni post-test (GraphPad Prism).

cooperate in the DDR and regulate a cell-cycle arrest and even apoptosis to prevent of accumulation of further mutations, genome instability and hence carcinogenesis. This adds with Nek7/Mat1 another pair of protein players to the eukaryotic cells arsenal to respond to DNA damage by activating a network of biochemical pathways that enable damage recognition and initiate responses leading to repair, apoptosis or senescence [25].

In line with these results, the wild-type Nek7 over-expression reduced the apoptosis, in opposition to what is

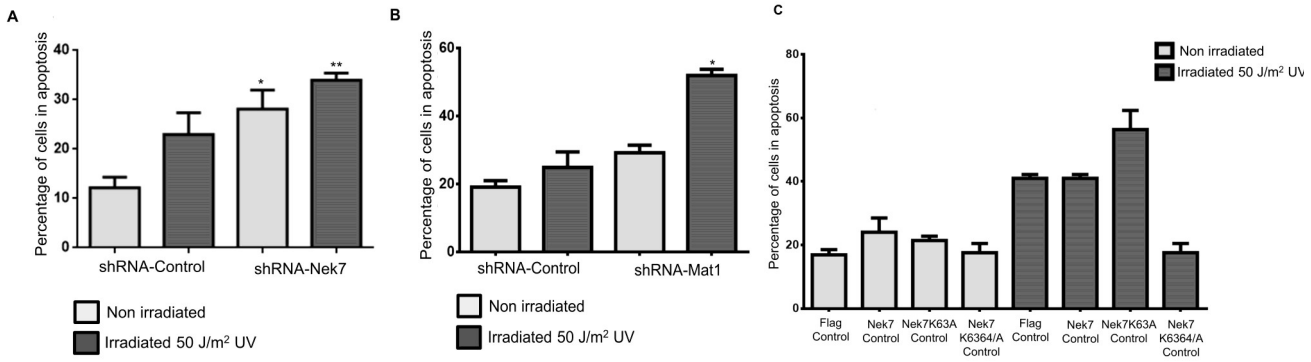

Figure 4 | Nek7 and Mat1 mediate UV-induced apoptosis. shRNA control cells, Nek7 or Mat1 depleted cells and cells over-expressing wide-type Nek7 or "kinase dead" (Nek7-K63A and Nek7K63/64A) were irradiated or not with 50 J/m2 of UV-C light and recovered for 2 hours. After this, the Hela cells were stained with propidium iodide and annexin-FITC and analyzed by flow cytometry. Graph corresponds to percentage of apoptosis cells. A) Graphs represent the percentage of apoptosis cells obtained by FACS analysis of control cells (shRNA-Control) or cells depleted by Nek7 (shRNA-Nek7); B) Control cells (shRNA-Control) and cells depleted of Mat1 (shRNA-Mat1); and C) Control cells and wild type Nek7 or kinase dead over-expressed (Nek7-K63A and Nek7-K63/64A). (*, **) Indicate P < 0,05 calculated using two-way ANOVA, Bonferroni post-test (GraphPad Prism).

observed for inactivated (without kinase activity) Nek7 over-expression. Taken together these findings support the hypothesis that Nek7 and Mat1 can affect cell cycle progression by promoting selective accumulation of cells in the S phase, upon DNA damage.

This study provides evidences that cell cycle arrest and apoptosis mediated through Nek7 and Mat1 may dependent on the activation of the DDR pathway, suggesting that Nek7 and Mat1 carry out their functions by temporarily halting cell proliferation, perhaps by engaging in specific cell cycle checkpoints or may alternatively affect CDK7s activity toward p53 [33, 19, 34]. The latter possibility suggests that triggering of p53 may then indirectly result in activating the response to DNA damage followed by DNA repair.

4.Conclusion

Here, we report that Nek7 interacts with the CAK complex and may cooperate with Mat1 in the S-phase regulation, in response to DNA damage. Thus, we speculated that the interaction and phosphorylation of Mat1 by Nek7 may influence the equilibrium between the free ternary CAK and the core TFIIH-related CAK. In this context, Nek7 could contribute to regulating Mat1 functions, including mechanisms that involve the substrate specificity of CDK7 [31, 32], cyclin H expression regulation [18] or Mat1 participating in nucleotide excision repair [30,14].

Our results show that Nek7 or Mat1 depletion led to a decreased cell proliferation, accumulation of cells in the S-phase and ultimately to an increase in apoptosis.

Alternatively, Nek7 and Mat1 may cooperate in mechanisms to eliminate cells by switching from non-successfully repaired UV–induced DNA damage to apoptosis. This model is consistent with the previous reports that over-expression of a kinase-defective form of Nek7 or its silencing result in both higher mitotic index and higher apoptosis [4,28]. Mat1 defective cells fail to enter S phase [36], and RNA antisense depletion of Mat1 in rat aortic smooth muscle cells induced a G1 arrest followed by apoptosis [37]. Both Nek7 and Mat1 gene knock-out in mice severely affect mitosis and lead both to early embryonic lethality [7, 18]. The details of the functional mechanism involved in the Nek7-Mat1 interplay, yet need to be determined in future experiments.

Briefly, our findings support the notion that Nek7 may cooperate with Mat1 in the signaling pathways that govern the cell cycle regulatory machinery, including DDR, S-phase progression and apoptosis, and thereby can constitute an important natural barrier against cellular transformation and tumorigenesis.

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