Biochemical and Biophysical Research Communications 464 (2015) 901–907

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Anks3 alters the sub-cellular localization of the Nek7 kinase

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ARTICLE INFO

Article history:
Received 4 July 2015
Accepted 13 July 2015
Available online 15 July 2015

Keywords:
Nephronophthisis
Ankyrin repeat proteins
Ciliopathies
Kinase

ABSTRACT

Nephronophthisis (NPH) is an autosomal recessive cystic kidney disease, and a frequent cause of end-stage renal failure in children. To date, 17 NPH-associated gene products (NPHPs) have been identified. Most NPHPs participate in large multi-protein complexes that localize to the cilium and/or basal body; however, the precise composition of these complexes and their biological function remain largely unknown. We recently observed that the ankyrin repeat protein Anks3 interacts with the NPH family member Anks6. Both Anks3 and Anks6 form complexes with multiple other NPHPs, suggesting that both proteins function in similar or overlapping signaling pathways. Here, we show that Anks3, but not Anks6 interacted with the NIMA-related kinase Nek7, and was heavily modified in the presence of Nek7, resulting in an approximately 20 kD increase in molecular weight. Although mass spectrometry revealed increased serine and threonine phosphorylation of Anks3 primarily within the N-terminal ankyrin repeats also required for Nek7 interaction, the molecular weight increase occurred even in the presence of a kinase-dead Nek7 mutant, indicating that this modification was not caused by Nek7-dependent Anks3 phosphorylation. Furthermore, the Anks3 modification was specific for Nek7, and did not occur in the presence of Nek8. Importantly, Anks3 retained Nek7 in the cytoplasm, suggesting that, Nek7 triggers the modification of Anks3, which in turn prevents the nuclear localization of Nek7.

1. Introduction

Nephronophthisis (NPH) is an autosomal recessive kidney disease that causes renal failure and is associated with multiple extra-renal manifestations [1,2]. Mutations in 17 genes, collectively termed NPHPs, have been associated with nephronophthisis; most of them localize to the cilium or its associated structures, and cause cilia-related phenotypes [3]. Despite the large number of causative genes, two out of every three nephronophthisis patients do not have mutations in any of the known NPHPs [4]. Since most NPHPs contain protein–protein interaction domains and participate in large tissue-specific and developmentally regulated protein complexes, identification of additional components of these complexes will further our understanding of how NPHPs exert their function and could potentially lead to the identification of novel NPHPs.

We have recently identified Anks3 as a binding partner of Anks6/NPHP16, and characterized Anks3 as a molecule associated nephronophthisis-like phenotypes in model organisms [5]. Mutations in ANKS6 were identified in six families affected by nephronophthisis, and *anks6* down-regulation in zebrafish and *Xenopus laevis* caused cyst formation, ciliary phenotypes and *situs inversus* [6]. Anks3 and Anks6 are related molecules that share a number of features, including similar domain architecture. In both proteins, the N-terminal ankyrin repeats are followed by an SAM domain and a short coiled-coil domain. Anks3, Anks6 and NPHP2 are all substrates for the HIF1AN hydroxylase [5,6]. Furthermore, *anks6* or *anks3* depletion in zebrafish resulted in overlapping ciliary phenotypes [5]. Anks3 and Anks6 form heteromeric protein complex that interacts with the SAM domain containing RNA binding protein BICC1 [5,6]. While Anks6 associates only with the NPHP2-NPHP3-NPHP9 module, Anks3 preferentially binds the NPHP1-NPHP4-NPHP8 module, but also associates with the Anks6 containing NPHP2-NPHP3-NPHP9 module [5]. The Anks3 containing NPHP1-NPHP4-NPHP8 module appears to function at the transition zone of cilia and in apical organization of polarized epithelial cells [7].

Nek7 belongs to the conserved NIMA-related family of serine/threonine kinases (Nek) that have been implicated in cell cycle

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http://dx.doi.org/10.1016/j.bbrc.2015.07.063
0006-291X/

progression, and in cilia formation. Nek kinases share a well-conserved N-terminal kinase domain, but differ in their C-terminal tails; the highly similar Nek6 and Nek7 lack the C-terminal tail completely [8]. Nek7 has been best characterized for its role in cell cycle progression. Nek7, Nek6 and Nek9 form a signaling cascade, in which Nek9 phosphorylates and thus activates both Nek6 and Nek7. Activated Nek6/Nek7 in turn phosphorylate the kinesin Eg5, which leads to its accumulation at the centrosomes and controls centrosome separation and normal mitotic spindle formation [9]. Besides the shared role in spindle formation, characterization of the Nek7 proteome suggests that Nek7 acts in different cellular processes independently of Nek6 [10]. Indeed, it was recently shown that microtubule dynamic instability, which is critical for accurate shaping of the mitotic spindle and for cilium formation, depends on Nek7 levels [11]. Currently, there is no evidence that Nek7 directly controls cilium formation or function; however, Nek8/NPHP9, another member of the Nek family, is mutated in nephronophthisis. Nek8 is recruited to the cilium by Inv/NPHP2, interacts directly with Anks6/NPHP16, while mutants of Nek8 show ciliary phenotypes in model organisms [6]. Since Nek7 and Anks3 are components of the Anks6 interactome [6], we decided to investigate the functional consequences of the Anks3/Nek7 interaction.

2. Material and methods

2.1. Reagents and plasmids

F.CD2AP, rat V5.Anks6 and mouse V5.Anks3 have been previously described [5]. YFP.Anks3 was generated by fusing full length mAnks3 cDNA to an YFP vector (eYFP-C1 Clonetech). Truncated versions of Anks3 in pcDNA6 vector were created by polymerase chain reaction (PCR) using appropriate primers from full length Anks3 followed by standard cloning techniques. The point mutation constructs V5.Anks3.S243A, V5.Anks3.S5A, V5.Anks3.S243A, V5.Anks3.TS318, V5.Anks3.S366A and V5.Anks3.S369A were generated by Quick change PCR using V5.Anks3 as a template. Full length Nek7 in pcDNA6 with different N-terminal tags were generated using Nek7 cDNA clone purchased from Source Bioscience. Nek7 kinase-dead (KD) mutant was described elsewhere [12]. Antibodies used in this study included Anks3 antibody (1:1000, Rabbit, Abcam), Nek7 antibody (1:1000,Rabbit, Cell signaling), anti-FLAG M2 (1:3000, Mouse, Sigma–Aldrich), anti-V5 (1:6000, Mouse, Serotec), anti-GFP(1:1000, Rabbit, MBL) anti-β-actin (1:1000, Mouse, Sigma–Aldrich) and anti-HA 12CA5 (1:1000, Mouse, Roche Applied Sciences). Secondary horseradish peroxidase (HRP) coupled antibodies against rabbit and mouse IgG were from Dako and GE Healthcare and were used at dilution of 1:10,000. Cy3 and Cy5 conjugated antibodies (1:500) were from Jackson ImmunoResearch. The lambda protein phosphatase was purchased from New England Bioloabs, Ipswich, USA. The P4D1 antibody (1:1000) was purchased from Covance, Princeton, USA.

2.2. Cell culture, transfection and imaging

Human embryonic kidney (HEK 293T) cells were cultured using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS, Biochrome, Berlin, Germany). The cells were transfected using calcium phosphate or TransPEI transfection method (Eurogentec, Cologne, Germany). HEK 293T cells were cultured on six well plates containing coverslips pre-treated with poly-L-lysine in order to ensure the tight attachment of the cells. After 24 h of transfection, cells were fixed with 4% PFA for 15 min, stained with Hoechst for nuclei and analyzed on an Axiovert 200M with ApoTome microscope (Zeiss, Germany).

2.3. Co-immunoprecipitation (Co-IP) and western blot

After 24 h of transfection, cells were washed with phosphate buffered saline (PBS) and lysed in lysis buffer (20 mM Tris, pH 7.5, 1% Triton X-100, 50 mM NaCl, 50 mM NaF, 15 mM $Na_4P_2O_7$, 0.1 mM EDTA) supplemented with protease inhibitor cocktail complete (Roche Diagnostics, Mannheim, Germany). After centrifugation (15000 × g, 15 min, 4 °C) followed by ultracentrifugation (100,000 × g, 30 min, 4 °C), the lysates were incubated with 30 μl of Flag M2 sepharose beads (Sigma–Aldrich Corporation, St. Louis, USA) or V5 antibody-coated sepharose beads (Abcam, Cambridge, UK) for 2 h at 4 °C in an overhead shaker. Next, the beads were washed with lysis buffer and the bound proteins were eluted in 2× Laemmeli buffer supplemented with dithiothreitol (DTT). The bound proteins were resolved by SDS-PAGE, and the interactions were checked by western blotting using the appropriate antibodies.

3. Results

To identify additional interaction partners of the ankyrin repeats protein Anks3, we carried out a mass spectrometry screen in human embryonic kidney (HEK 293T) cells that were transiently transfected with Flag-tagged mouse Anks3 (F.Anks3), or with control Flag-tagged GFP (F.GFP), and confirmed Anks6 as an Anks3-binding partner (Supplemental Fig. 1A). Among the other Anks3-binding proteins with the most identified peptides was the NIMA-related Nek7 kinase. We validated the interaction by co-expression and co-immunoprecipitation analysis in HEK 293T cells. Using tagged proteins, we found that Flag-tagged mouse Nek7 (F.Nek7) immobilized V5-tagged mouse Anks3 (V5.Anks3) (Fig. 1A, second lane). No interaction with V5.Anks3 was detected, when Flag-tagged CD2AP (F.CD2AP) was used as bait (Fig. 1A, third lane).

We next wanted to demonstrate the endogenous interaction between Anks3 and Nek7. Using a commercially available anti-Anks3 antibody, the endogenous levels of Anks3 appeared to be below the detection limit by Western blot analysis in the lysates of HeLA cells (Supplemental Fig. 1B, first lane). However, we detected a specific band after immunoprecipitation of Anks3 with an anti-Anks3 antibody, but not in the control anti-IgG precipitate (Supplemental Fig. 1B, second and third lanes). Confirming endogenous interaction, precipitation with anti-Anks3 antibody immobilized Nek7 (Supplemental Fig. 1C, third lane). In comparison, Nek7 could not be detected after co-immmunoprecipitation with control anti-IgG (Supplemental Fig. 1C, second lane). The reciprocal co-immunoprecipitation experiment was technically not possible due to the lack of appropriate anti-Nek7 antibody.

Ankyrin repeats are often found in proteins, and are known to mediate protein–protein interactions [13]. To exclude the possibility that any ankyrin repeat-containing proteins would bind Nek7, we examined whether Nek7 interacts with Inversin (Inv), another ankyrin repeat-containing NPHP [6]. In a co-immunoprecipitation experiment, F.Nek7 failed to immobilize mouse V5-tagged Inversin (V5.Inv), confirming the specificity of the Anks3/Nek7 interaction (Fig. 1A, first lane). Since we previously found Nek7 in two independent mass spectrometry-based screens using Anks6 or Anks3 as baits, respectively [5,6], we examined if Nek7 also interacts with Anks6. After co-immunoprecipitation with F.Nek7, no interaction with V5-tagged rat Anks6 (V5.Anks6) was detected, implying that the Nek7 interaction with Anks6 is possibly indirect (Supplemental Fig. 2A).

We noticed that co-transfection of Anks3 and Nek7 always results in the appearance of a second Anks3 band of approximately 20 kD higher molecular weight, either in a straight Western blot or after co-immunoprecipitation (Fig. 1B, second lane). Thus, it appeared that Anks3 is modified in the presence of Nek7. Since the

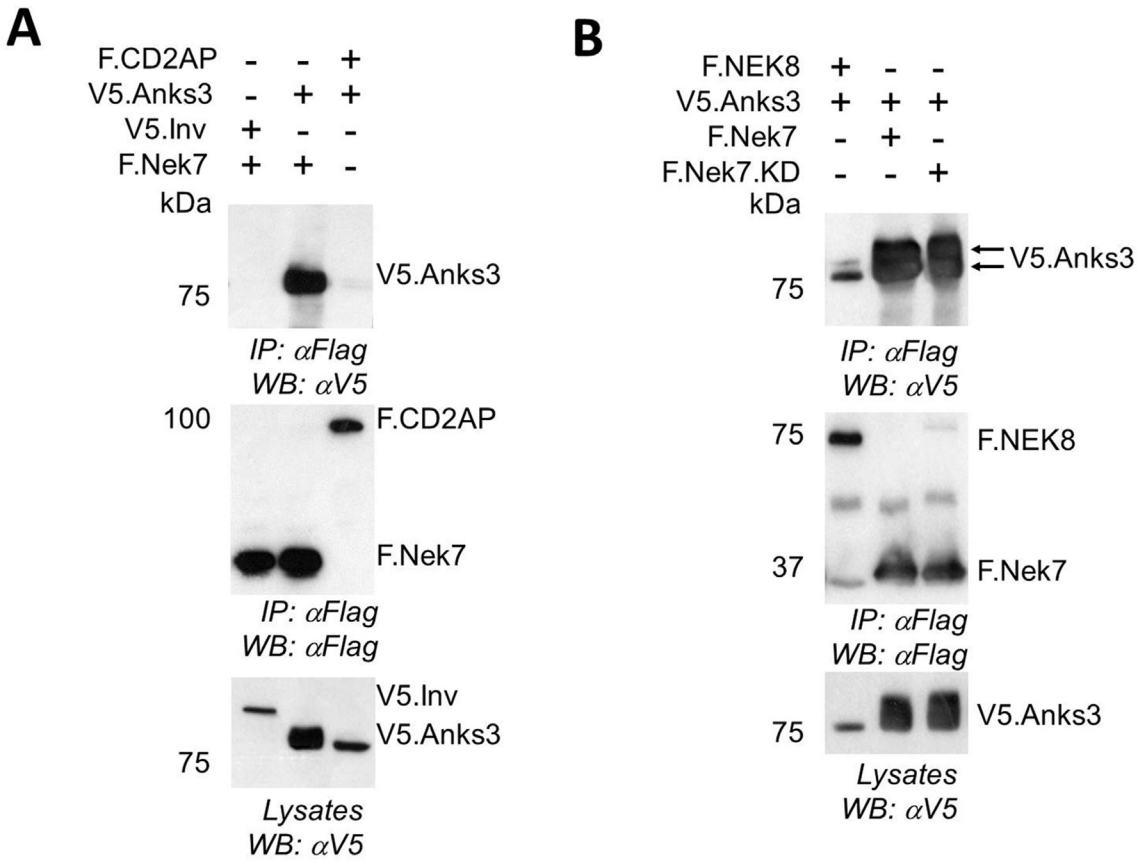

Fig. 1. I Nek7 interacts with and modifies Anks3. **(A)** HEK 293T cells were transiently co-transfected with Flag- and V5-tagged proteins as indicated, and Flag-tagged proteins were precipitated using anti-Flag M2 beads. Precipitation of F.Nek7 (first and second lane) immobilized V5.Anks3 (second lane), but not V5.Inv (first lane). V5.Anks3 did not co-immunoprecipitate with control F.CD2AP (third lane). Expression of V5.Inv and V5.Anks3 is shown on the bottom panel. **(B)** Precipitation with both F.Nek7 (second lane) and a kinase-dead version of Nek7 (F.Nek7.KD, third lane) immobilized V5.Anks3. F.NEK8 weakly interacted with V5.Anks3 (first lane). Expression and F.Nek7-dependent modification of V5.mAnks3 is shown on the bottom panel.

Nek family of kinases consists of eleven distinct gene products [14], we wondered, if Anks3 also interacts with and is modified in a similar fashion by other Nek kinases. We co-expressed Flag-tagged human NEK8 (F.NEK8) together with V5.Anks3, and used an anti-Flag antibody to precipitate F.NEK8. Indeed, F.NEK8 immobilized some V5.Anks3; however, the interaction appeared to be much weaker than the one with Nek7 (Fig. 1B, first lane). Importantly, there was no additional Anks3 band after co-transfection with F.NEK8 (Fig. 1B, first lane) supporting the notion that Anks3 is specifically modified in the presence of Nek7. To further explore the specificity of this modification, we transfected HEK 293T cells with increasing amount of F.Nek7 and fixed concentration of V5.Anks3, and observed that the intensity of the modified V5.Anks3 band in the lysates increased at higher F.Nek7 concentrations (Supplemental Fig. 2B).

We next asked whether modified Anks3 can still interact with Anks6. For that, we co-transfected HEK 293T cells with F.Anks3 and V5.Anks6 in the presence of either V5.NEK7 or control V5.GFP. In a co-immunoprecipitation experiment with an anti-Flag antibody, both the modified and the unmodified version of F.Anks3 immobilized V5.Anks6, indicating that the Anks3 modification does not interfere with the Anks3/Anks6 interaction (Fig. 2A).

To study the nature of modified Anks3, we first asked whether there is a change in the phosphorylation profile of Anks3 in the presence of increased amounts of Nek7. We used mass spectrometry to identify V5.Anks3 phosphorylated serine (S) and threonine (T) residues in HEK 293T cells after transfection either alone or together with F.Nek7 (Supplemental Fig. 3A,B). We found that even without transfected F.Nek7, V5.Anks3 was phosphorylated at positions S201, S243, S244, S245 and S540 (Supplemental Fig. 3C). After co-transfection with F.Nek7, additional Anks3 phosphorylated residues at positions S5, S225, T318, S319, S366 and S369 were detected (Supplemental Fig. 3C). To study the effect of these phosphorylations on the Anks3/Nek7 interaction, we generated single mutants for S5A (V5.Anks3.S5A), S243A (V5.Anks3.S243A), S366A (V5.Anks3.S366A), S369A (V5.Anks3.S369A) and the double mutant T318A, S319A (V5.Anks3.TS318). However, we found that all mutant Anks3 proteins were modified in the presence of NEK7 (Supplemental Fig. 4A). The Anks3 S243, S244, S245 triple phosphorylation site is contained within a consensus Nek kinase recognition site (F/LxxS/T) [15], which is a potential direct Nek7 target (Supplemental Fig. 3C). Thus, we generated the triple S243A, S244A and S245A Anks3 mutant (V5.Anks3.S243A), but found that V5.Anks3.S243A was also modified in the presence of F.Nek7 (Fig. 2B). Since we could not explain the Anks3 modification by mutating potential Nek7 target sites, we asked whether phosphorylation *per se* was responsible for the observed Anks3 modification. To address this question, we co-transfected V5.Anks3 and F.Nek7, and treated the lysates with lambda protein phosphatase, which removes phosphate groups from serine, threonine and tyrosine residues. While this treatment strongly reduced the phosphorylation of endogenous S6 kinase, it had virtually no effect on the V5.Anks3 modification band (Supplemental Fig. 4B). We next asked whether V5.Anks3 can be modified in the presence of a

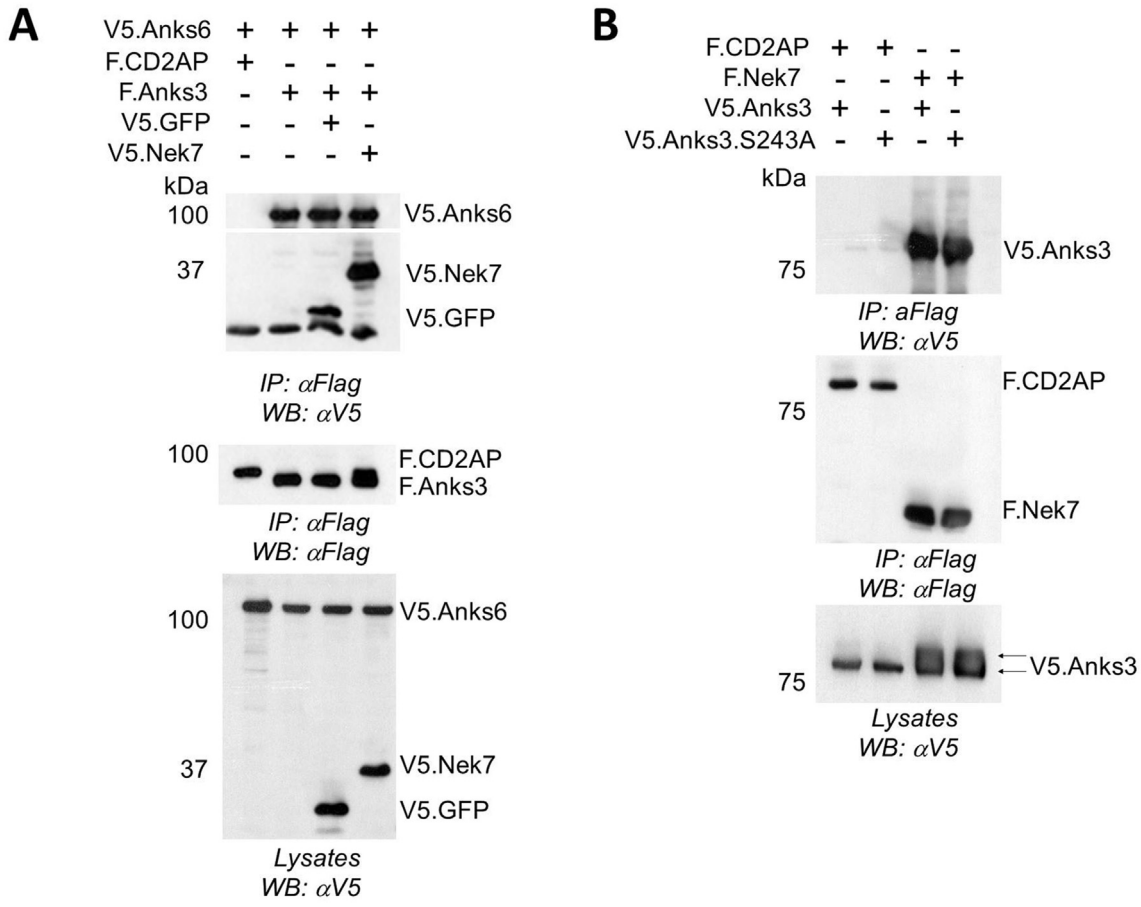

Fig. 2. I Modified Anks3 still interacts with Anks6 and the Anks3 modification is not dependent on the consensus NIMA binding sites. **(A)** V5-tagged Anks6 co-precipitated with Flag-tagged Anks3 both alone (second lane) and in the presence of V5.Nek7 (fourth lane) or V5.GFP (third lane). In the presence of V5.Nek7, F.Anks3 was modified. Precipitated F.CD2AP and F.Anks3 are shown in the middle panel. Expression of V5.mAnks3, V5.Nek7 and V5.GFP is shown on the bottom panel. **(B)** No change of the Anks3 modification status after mutating consensus NIMA binding sites. V5.Anks3.S243A is still modified when co-transfected together with F.Nek7 (fourth lane, bottom panel). Furthermore, V5.Anks3.S243A can be immobilized by precipitated F.Nek7 (fourth lane, first panel), but not by control F.CD2AP (second lane, first panel).

Nek7 kinase-dead version (F.Nek7.KD). Indeed, after co-immunoprecipitation with an anti-Flag antibody, we found that V5.Anks3 was still modified (Fig. 1B). These data indicate that the Anks3 modification band was not caused by Nek7-dependent phosphorylation; however, the modification is dependent on the Anks3/Nek7 interaction.

Another explanation for the Anks3 modification could be mono-ubiquitination. To test this possibility, we co-transfected V5.Anks3 with F.Nek7, immunoprecipitated V5.Anks3 with an anti-V5 antibody and probed for ubiquitination with an anti-P4D1 antibody. This antibody recognizes mono-as well as poly-ubiquitination. We found that V5.Anks3 is poly-ubiquitinated and its poly-ubiquitination status increases in the presence of F.Nek7 (Supplemental Fig. 4C), however no specific increase in mono-ubiquitination was observed. Thus, mono-ubiquitination also could not explain the 20 kDa increase in Anks3 molecular weight.

Anks3 contains an N-terminal ankyrin repeats domain and a C-terminal SAM domain, both of which could participate in protein–protein interactions [4,13]. To determine whether the ankyrin repeats or the SAM domain mediate the Anks3/Nek7 interaction, we generated four Anks3 truncations spanning the N-terminal, ankyrin repeat containing region (V5.Anks3.Tr1, amino acids (aa) 1–221); the central portion of the protein between the two domains (V5.Anks3.Tr2, aa 222–421); the ankyrin repeat and the central portion together (V5.Anks3.Tr.(1 + 2), aa 1–421); and the C-terminal SAM domain (Anks3.Tr3, aa 421–655) (Fig. 3A). We co-transfected the Anks3 truncations with F.Nek7 in HEK 293T cells, and tested for interactions with co-immunoprecipitation assays. We did not detect the V5.Anks3.Tr1 in the cell lysate, suggesting that the protein was not expressed or is unstable (Fig. 3B, third panel, third lane); the V5.Anks3.Tr2 and V5.Anks3.Tr(1 + 2) were expressed (Fig. 3B,C). Immunoprecipitated F.Nek7 did not immobilize V5.Anks3.Tr3 (Fig. 3B, fifth lane), suggesting that the C-terminal SAM-domain was not sufficient for the Anks3/Nek7 interaction. In contrast, the V5.Anks3.Tr(1 + 2) showed a strong interaction with F.Nek7 (Fig. 3C, third lane), while F.Nek7 immobilized only small amounts of V5.Anks3.Tr2 (Fig. 3B, fourth lane). These data suggest that the N-terminal ankyrin repeats domain is essential for the interaction with Nek7, and that the central portion of Anks3 might be contributing to this interaction. We further observed that V5.Anks3.Tr2 and V5.Anks3Tr(1 + 2) displayed the modified band (Fig. 3B,C, third panels), but no additional band was observed with the V5.Anks3.Tr3 version (Fig. 3B).

We were interested to understand how the Anks3/Nek7 interaction affects the subcellular localization of both proteins. Using YFP-tagged Anks3 (YFP.Anks3) and RFP-tagged Nek7 (RFP.Nek7), we observed that YFP.Anks3 alone in HEK 293T cells localized predominantly to the cytoplasm, and was largely excluded from the nucleus (Fig. 4A). In contrast, RFP.Nek7 was ubiquitously distributed, localizing to both the nucleus and the cytoplasm (Fig. 4B).

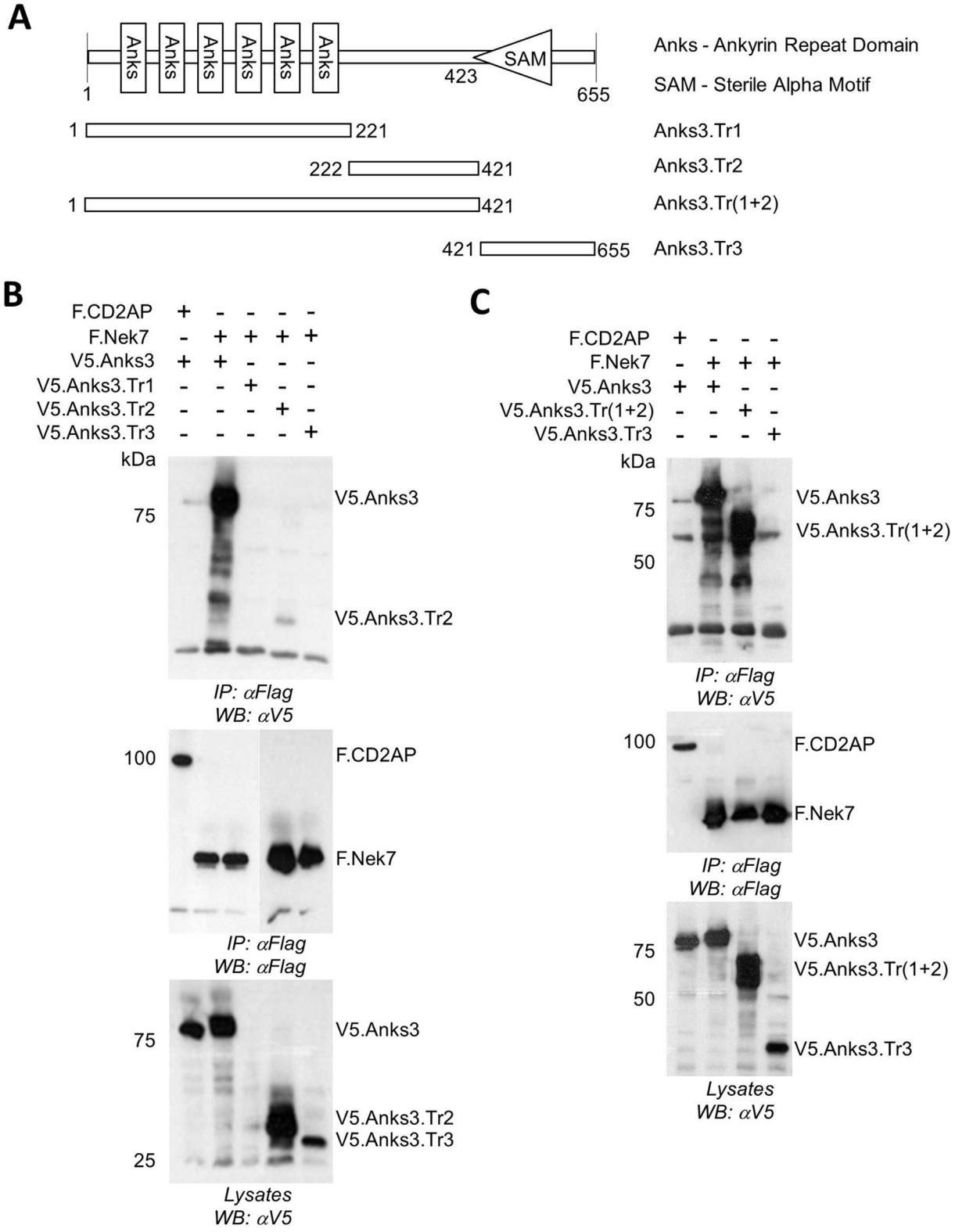

Fig. 3. I Anks3 interaction with Nek7 is mediated by the Anks3 N-terminus. **(A)** Schematic representation of Anks3 structure and truncations. Anks3 harbors six ankyrin repeats N-terminally and one SAM (Sterile Alpha Motif) C-terminally. Anks3.Tr1 comprises the N-terminus including the ankyrin repeats. Anks3.Tr2 includes the middle portion of the protein. Anks3.Tr(1 + 2) includes the ankyrin repeats and the middle portion without the C-terminal SAM domain. Anks3.Tr3 comprises the middle portion and the C-terminus of Anks3, without the ankyrin repeats. **(B)** Full-length and truncated versions of F.Anks3 were co-expressed in HEK 293T cells together with Flag-tagged F.Nek7. V5.Anks3.Tr2 and V5.Anks3.Tr3 were well expressed after transfection in HEK 293T cells (fourth and fifth lane, bottom panel), whereas the protein from V5.Anks3.Tr1 was not detectable after cell lysis (third lane, bottom panel). F.Nek7 weakly interacted with V5.Anks3.Tr2, but not with V5.Anks3.Tr3. **(C)** Precipitated F.Nek7 immobilized large amounts of V5.Anks3.Tr(1 + 2) (third lane, top panel), suggesting that the Anks3 ankyrin repeats are required for the Anks3/Nek7 interaction. Co-precipitation of F.CD2AP and V5.Anks3 serves as a negative control (first lane, top panel) and co-precipitation of full-length V5.Anks3 with F.Nek7 as a positive control (second lane, top panel). Expression of V5.Anks3, V5.Anks3.Tr(1 + 2) and V5.Anks3.Tr3 is shown on the bottom panel.

Interestingly, co-expression of both proteins resulted in nuclear exclusion and cytoplasmic retention of RFP.Nek7. To determine whether the Anks3/Nek7 interaction is important for the Nek7 localization, we co-expressed RFP.Nek7 with either YFP.Anks3.Tr(1 + 2) or with YFP.Anks3.Tr3. Indeed, co-expression of RFP.Nek7 with YFP.Anks3.Tr(1 + 2), which showed strong Nek7 binding, resulted in RFP.Nek7 nuclear exclusion (Fig. 4D). Interestingly, the YFP.Anks3.Tr3 showed a different localization pattern than YFP.Anks3 and YFP.Anks3.Tr(1 + 2). YFP.Anks3.Tr3 localized both to the nucleus and the cytoplasm, similar to RFP.Nek7 (Fig. 4E). Furthermore, co-expression of YFP.Anks3.Tr3 with RFP.NEK7 had no effect on the nuclear/cytoplasmic distribution of RFP.Nek7 (Fig. 4E). Thus, the Anks3 N-terminus is sufficient for the Anks3 cytoplasmic localization, whereas the C-terminal SAM domain is dispensable. In addition, interaction with Anks3 alters the subcellular distribution of Nek7 by preventing its nuclear translocation.

4. Discussion

Terminally differentiated epithelial cells exit the cell cycle and their deregulated proliferation is usually associated with tumorigenesis. Kinases, such as Cyclin-dependent kinases, Aurora kinases, Polo-like kinases and the Nek kinases, control various aspects of cell cycle and mitosis. Whereas not a single human NEK is absolutely required for mitotic entry, it has been established that NEK2, NEK6, NEK7 and NEK9 participate in the structural changes that occur as cells move from interphase into mitosis. In particular, human NEK2, NEK6, NEK7 and NEK9 cooperate to ensure formation of a robust bipolar spindle; they might also contribute to other aspects of

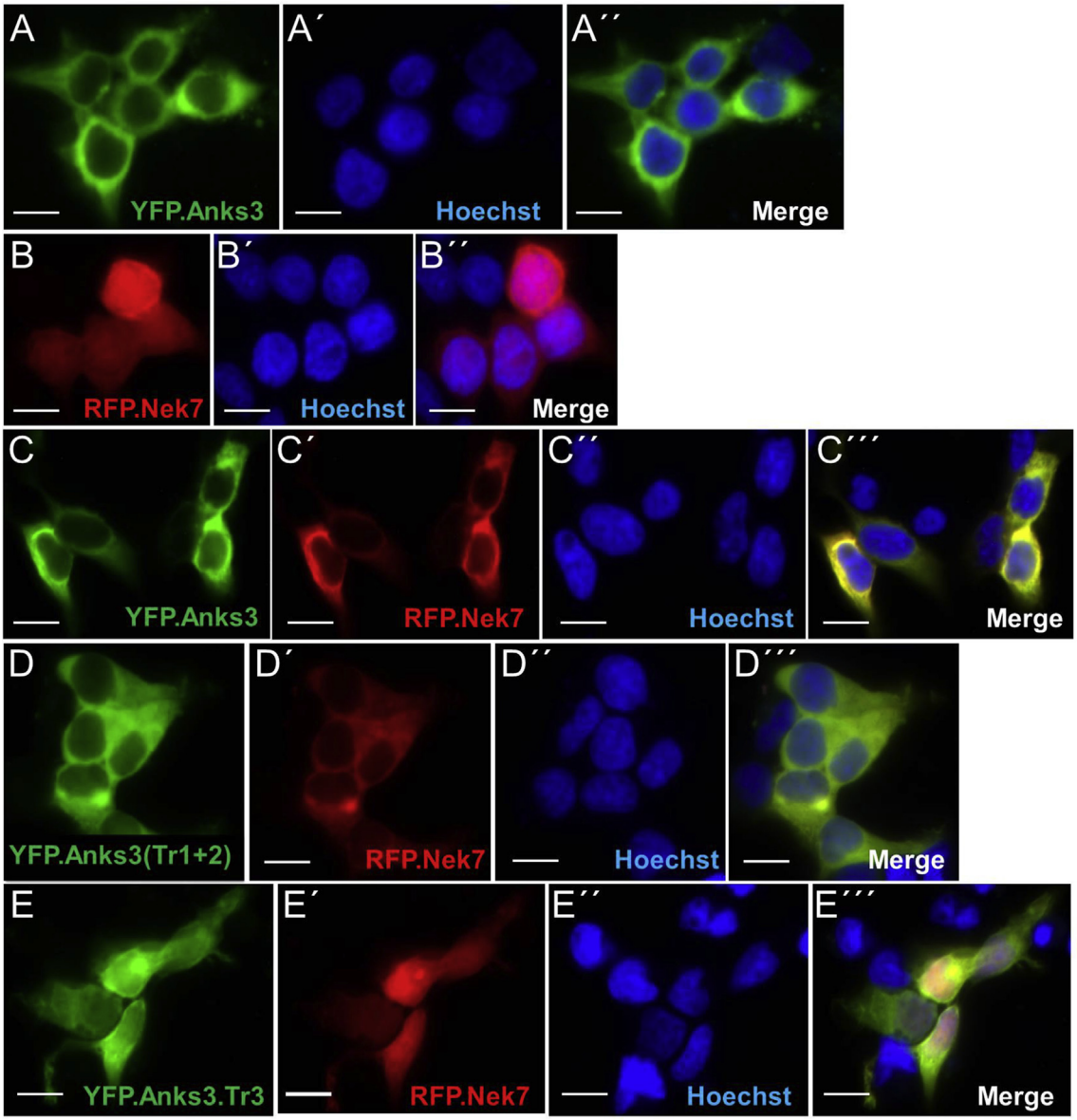

Fig. 4. I Interaction with Anks3 changes the subcellular localization of Nek7. HEK 293T cells were transfected with YFP.Anks3, YFP fusions of the respective Anks3 truncations and RFP.Nek7 as described. **(A)** YFP.Anks3 localized exclusively to the cytoplasm. Hoechst stains the DNA (A′). **(B)** RFP.Nek7 was ubiquitously distributed in the cell, both in the nucleus and in the cytoplasm (B′). **(C)** After co-expression of YFP.Anks3 and RFP.Nek7, the RFP.Nek7 protein was excluded from the nucleus (C′, Hoechst) and localized exclusively to the cytoplasm (C′). **(D)** Co-expression of RFP.Nek7 with YFP.Anks3.(Tr1+2) led to cytoplasmic localization of RFP.Nek7. **(E)** In contrast, RFP.Anks3.Tr3 localized to both the nucleus and the cytoplasm and it did not alter the RFP.Nek7 subcellular localization. Scale bars in all panels represent 10 μm.

mitotic progression, including chromatin condensation, nuclear envelope breakdown and cytokinesis [16]. Thus, it is likely that Neks expression and localization are tightly regulated. Indeed, we found that the interaction with Anks3 altered the subcellular localization of Nek7. Previously it was shown that NEK7 in HeLa cells is largely cytoplasmic and strongly enriched at the centrosomes throughout the cell cycle [17]. In our experiments, overexpressed Nek7 in HEK 293T cells localized both to the nucleus and to the cytoplasm. However, upon co-expression with Anks3, Nek7 was virtually excluded from the nucleus. Given the importance of the Nek kinases in mitotic progression, the cytoplasmic retention of Nek7 by Anks3 could represent a possible mechanism to prevent cell cycle re-entry of differentiated epithelial cells.

Nek7, in turn, had a pronounced effect on the Anks3 post-translational modification status. In the presence of Nek7, the molecular weight of Anks3 was higher by approximately 20 kDa on Western blots. Interestingly, Anks3 S/T phosphorylation was increased in a Nek7-dependent manner, and the majority of the phosphorylation sites mapped to the N-terminal ankyrin domain of Anks3. However, phosphorylation alone could not explain the Anks3 modification band, since mutating single or multiple phosphorylated residues, or treatment with phosphatase did not prevent the Anks3 modification. Importantly, Anks3 was modified even in the presence of a kinase-dead version of Nek7. One plausible explanation is that Nek7 recruits other proteins that modify Anks3. From the various possible modifications, we could exclude mono-ubiquitination. However, one other potential modification, which could explain the Anks3 molecular weight shift, is SUMOylation. The SUMOplot program (Abgent, San Diego, USA) predicts the presence of one highly probable site at amino acid 193–196 (VKVD) as well as four additional motifs (Supplemental Fig. 5).

While our study did not identify the nature of the Anks3 post-translational modification, the presence of Nek7 increases S/T phosphorylation preferentially within the N-terminal ankyrin repeat-containing domain of Anks3, which could lead to the recruitment of SUMO E3 ligases, or other protein modifying enzymatic activities. This modification may contribute to the nuclear exclusion of Nek7, and prevent an undesired re-entry of interphase cells into the cell cycle.

Conflict of interest

None.

Acknowledgments

We are grateful to members of the Renal Division for helpful discussions. This work was funded by the collaborative research center SFB 1140 (G.W.) of the Deutsche Forschungsgemeinschaft (DFG).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.063.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.07.063.

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