NOK/STYK1 interacts with GSK-3β and mediates Ser9 phosphorylation through activated Akt

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ABSTRACT

NOK (also known as STYK1) has been identified as an oncogene. However, its biochemical and biological activities as a molecular regulator are poorly defined. In the present study, we report that NOK overexpression led to enhanced phosphorylation of GSK-3β at its Ser9 residue via Akt phosphorylation at Thr308. NOK could make complexes with both Akt and GSK-3β. Moreover, the expression levels of NOK, p-Akt(Thr308) and p-GSK-3β(Ser9) were positively correlated in cancerous and non-cancerous breast cell lines. Thus, our data identified a novel functional molecular complex formed by NOK, Akt and GSK-3β that may relay a NOK-directed tumourigenic cascade.

Structured summary of protein interactions:
GSK3B physically interacts with **NOK** and **Akt** by anti tag coimmunoprecipitation (View interaction).
GSK3B physically interacts with **NOK** by anti tag coimmunoprecipitation (View interaction).

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1. Introduction

Protein kinases, which are composed of a large number of subfamilies, play significant roles in cancer biology, such as cell proliferation, differentiation, survival, tumourigenesis and metastasis [1]. Like many other kinases, NOK (novel oncogenic kinase, also known as STYK1 (a putative serine/threonine and tyrosine receptor protein kianse)) contains a single transmembrane domain and an intracellular tyrosine kinase domain, possibly for activating downstream signalling cascades. However, this protein lacks an extracellular domain [2]. In cancerous tissues from different organs, including the breast, lung, ovary, endometrium and prostate, expression of NOK is obviously higher than in adjacent non-cancerous tissues [3–8]. Overexpression of NOK promoted cell proliferation of BaF3 cells and also induced rapid tumourigenesis and severe distant metastasis in nude mice [2]. The oncogenic property of NOK critically depends on Tyr327 and Tyr356 phosphorylation [2,9]. Despite the identified physiological function of NOK at the cellular level, its biochemical and molecular functions, interacting partners, and positions in various signalling cascades are largely undefined.

EMT (epithelial–mesenchymal transition) is viewed as a critical intermediate step in tumourigenesis and gives rise to the dissemination of a single cancer cell from primary tumours [10–12]. Cancer cells acquire the mesenchymal phenotype through altered expression of miRNA and EMT-related factors. E-cadherin is one of the most important factors [13]. E-cadherin, as a master regulator of the epithelial phenotype, mediates stable cell–cell contacts and development of adhesive junctions [14]. In the past decade, several transcriptional repressors responsible for the loss of E-cadherin have been identified, such as Snail, Slug, ZEB1 and ZEB2, of which Snail is probably the most important repressor for E-cadherin [15,16]. One critical interacting partner and functional regulator of Snail is GSK-3β. Snail can be stabilised by Ser9-phosphorylated GSK-3β in the nucleus followed by promoter binding to the E-cadherin gene and transcriptional repression [17]. Although in its active form, GSK-3β generally functions as a "tumour suppressor", its phosphorylation at the Ser9 residue would lead to enzymatic inactivation and reversal of tumour-suppressive function. Consequently, the prominent oncogenic kinase Akt directly stimulates the Ser9 phosphorylation of GSK-3β [18]. Importantly, our previous reports demonstrated that the PI3K/Akt pathway was activated in NOK-transformed BaF3 cells and that the PI3K inhibitor LY294002 (LY) significantly inhibited the colony formation capacity of BaF3 cells on soft agar [2]. In addition, we found that overexpression of NOK led to reduced levels of E-cadherin [9]. Together, these findings imply that the Akt-GSK-3β pathway is a potential mediator for NOK-directed biological and physiological functions.

In this report, we tested the hypothesis that NOK is functionally involved in Akt-GSK-3β pathway regulation. We found that overexpression of NOK in stable cell lines led to enhanced

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GSK-3β(Ser9) phosphorylation. We further demonstrated that NOK interacts with both GSK-3β and Akt and is required for the effective phosphorylation of Akt(Thr308) and GSK-3β(Ser9). Additionally, the expression levels of NOK, p-Akt(Thr308) and p-GSK-3β(Ser9) were accordingly elevated in cancerous versus non-cancerous breast cells. Together, our findings indicate that NOK, Akt and GSK-3β form interacting complex in which Akt mediates NOK-dependent GSK-3β Ser9 phosphorylation such that NOK may promote tumourigenesis via enzymatic activation of Akt and inactivation of GSK-3β.

2. Methods and materials

2.1. Co-immunoprecipitation (co-IP) assay

The cells were cultured as described in supplementary materials. HEK293T cells were transfected with the plasmids as indicated in the figures and 36 h after transfection, cells were harvested and lysed on ice with modified RIPA buffer (50 mmol/l Tris–HCl pH7.4, 150 mmol/l NaCl, 1 mmol/l EDTA, 0.5% NP-40, 10% glycerol). The detergent-soluble fraction was recovered by centrifugation at 4 °C for 15 min at 12,000 rmp/min. The supernatant was co-immunoprecipitated with mouse anti-HA monoclonal antibody (ZSGB-Bio, China) followed by further incubation with Protein A/G plus agarose beads (Santa Cruz, USA). When proteins were expressed with flag tag, the supernatant was directly subjected to co-IP with anti-flag M2 affinity beads (Sigma–Aldrich, USA). After 3× washing, the products were eluted with 2 × SDS–PAGE loading buffer and resolved for western blotting.

2.2. SiRNA transfection

Synthetic Akt siRNA corresponds with the following sequence and position on Akt gene backbone: 208(si1208) (5′-ATA-CCGGCAAAGAAGCGATGCTGCA-3′) and 306(si1306) (5′-CAAGATGA CAGCATGGAGTGT-3′). The control siRNA was from GenePharma Co., Ltd. (Genepharma, China). HeLa-HA and HeLa-NOK-HA stable cell lines (30 × 10⁴ cells/well) plated in 6-well plates were transfected with RNAi using Lipofectamine 2000 (Invitrogen, USA) according to the instructions. Briefly, the siRNA-Lipofectamine 2000 mixture was added into a culture medium for 6 h. The medium was subsequently replaced for 48 h of further incubation before being harvested.

2.3. Western blotting

Proteins were resolved on 12% SDS–PAGE and then transferred onto a nitrocellulose membrane in a semi-dry condition. These blots were blotted with 5% skim milk powder or 5% BSA for 1 h at room temperature followed by incubation with primary antibody overnight at 4 °C. After 3× washing, blots were incubated with HRP-conjugated secondary antibody (ZSGB-Bio, China) for 1 h at room temperature. The primary antibodies used are the following: anti-Akt, anti-p-Akt(Thr308) and anti-p-GSK-3β(Ser9) (Cell Signalling, USA), anti-p-Akt (Ser473) (Beyotime, China), anti-GSK-3β (BD, USA), anti-NOK (Sigma–Aldrich, USA), anti-HA (ZSGB-Bio, China), anti-myc, anti-β-actin and anti-GAPDH (YTHX Biotechnology, China). Proteasome inhibitor, MG132 (Selleck, USA), was used to block the protein degradation.

3. Results

3.1. NOK increases GSK-3β Ser9 phosphorylation

As previously mentioned, GSK-3β is a downstream effecter of the PI3K/Akt pathway. GSK-3β is constitutively active in resting cells and functionally inhibits the EMT process [19]. However, the enzymatically inactivated form of GSK-3β (phosphorylated at Ser9 by Akt) markedly increases in both human and murine tumours [20]. In NOK-overexpressing cells, we found increased PI3K activity and decreased E-cadherin expression [2,9], with the latter being regulated by Ser9-phosphorylated GSK-3β. To study if NOK regulates GSK-3β activity, we established HeLa stable cell lines overexpressing HA-tagged NOK (HeLa-NOK-HA) and HA vector (HeLa-HA) respectively (Fig. S1, Fig. 1). Western blotting based on the total proteins from the stable cell lines indicated that normalised p-GSK-3β(Ser9) expression was significantly higher in the HeLa-NOK-HA cells than that in the HeLa-HA cells (Fig. 1A and B). When GSK-3 (Ser9) degradation was blocked by proteasome inhibitor (MG132), the increased amount of phosphorylated GSK-3 (Ser9) in responding to NOK was more clearly observed in the HeLa-NOK-HA cells (Fig. 1C). These findings suggest that NOK can stimulate GSK-3β (Ser9) phosphorylation.

3.2. NOK interacts with GSK-3β and forms a complex with Akt-GSK-3β

Next, we used Co-IP analysis to determine if NOK induces p-GSK-3β(Ser9) via direct interactions. These assays were carried out using both full-length NOK and truncated forms of NOK to identify potential interacting domains. As shown in Fig. 2A, Myc-his-tagged full length NOK (NOK-myc-his) was co-transfected with HA-tagged GSK-3β (GSK-3β-HA) followed by co-IP with an anti-HA antibody. With both anti-his and anti-myc antibodies, the results indicated that full-length NOK was co-immunoprecipitated with GSK-3β-HA but not with the pCDNA3-HA control vector (Fig. 2B; Fig. S2A). Furthermore, two NOK truncation mutants (NOK△ECD: missing the N-terminal ectodomain; NOK-ICD: missing both the N-terminal ectodomain and transmembrane domain) were co-immunoprecipitated by GSK-3β-HA. The NOK△ECD construct produced a lowest yield (Fig. 2B), suggesting that missing both the N-terminal ectodomain and transmembrane domain affects the interaction between NOK and GSK-3β.

To confirm the interaction between NOK and GSK-3β further, we performed additional experiments by introducing Akt into the tests, knowing that PI3K/Akt serves as GSK-3β kinase and can be enhanced by NOK overexpression [2]. We started the testes by co-transfecting NOK-myc-his and GSK-3β-HA into HEK293T cells for co-IP using the anti-HA antibody. The products were examined for the presence of exogenous NOK-myc-his and endogenous Akt. As shown in Fig. 2C, both NOK-myc-his and endogenous Akt were readily detected by GSK-3β-HA-directed co-IP. Moreover, HA-Akt and NOK-HA could be also pulled down by 3flag-GSK-3β (Fig. S2B). For complimentary experiments, we repeated the co-IP analysis using 3flag-NOK to immunoprecipitate GSK-3β-HA and Akt-HA. The results indicated that both GSK-3β-HA and Akt-HA bands were identified (they have different molecular weight) in 3flag-NOK-immunoprecipted product (Fig. 2D). Together, our studies demonstrated that NOK-Akt-GSK-3β can form a 3-way complex and may serve as a molecular complex in vitro and in vivo.

3.3. NOK enhances the phosphorylation of GSK-3β (Ser9) via the induction of p-Akt kinase (Thr308)

It is well documented that Akt is recruited to the membrane and its phosphorylation at Thr308 and Ser473 is critical for Akt activation [21]. In addition, Akt mediates the phosphorylation of GSK-3β on Ser9 [22,23]. Next, we undertook a mechanistic study to address which one of the two Akt phosphorylation sites mediates NOK-dependent GSK-3β Ser9 phosphorylation. As shown in Fig. 3A, transient expression of wild-type Akt led to the increase in p-GSK-3β(Ser9) expression in both the HeLa-HA and HeLa-NOK-HA stable cell lines. The extent of the increase of p-GSK-3β(Ser9) in
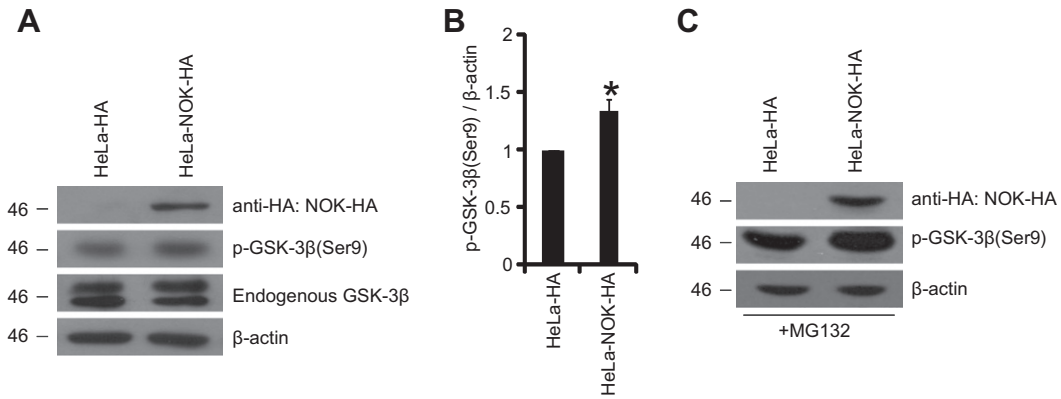
Fig. 1. GSK-3β phosphorylation at Ser9 is enhanced in NOK-overexpressed stable cell lines. (A) Western blotting showing the expression levels of GSK-3β and p-GSK-3β(Ser9) in HeLa-HA HeLa-NOK-HA) stable cell lines. Total proteins were harvested and normalised for western blotting with anti-p-GSK-3β(Ser9) antibody. (B) The ratio of p-GSK-3β(Ser9) to β-actin was analysed based on quantification using the Excel programme and Sigmaplot software, and the results were presented as the mean ± standard error from at least three independent tests. Significant differences were determined by a Student's t test, and statistical significance was indicated as *($P < 0.05$). (C) The proteasome inhibitor, MG132, was added in medium of HeLa-HA and HeLa-NOK-HA cells for 12 h and then the p-GSK-3β(Ser9) was assayed by western blotting.
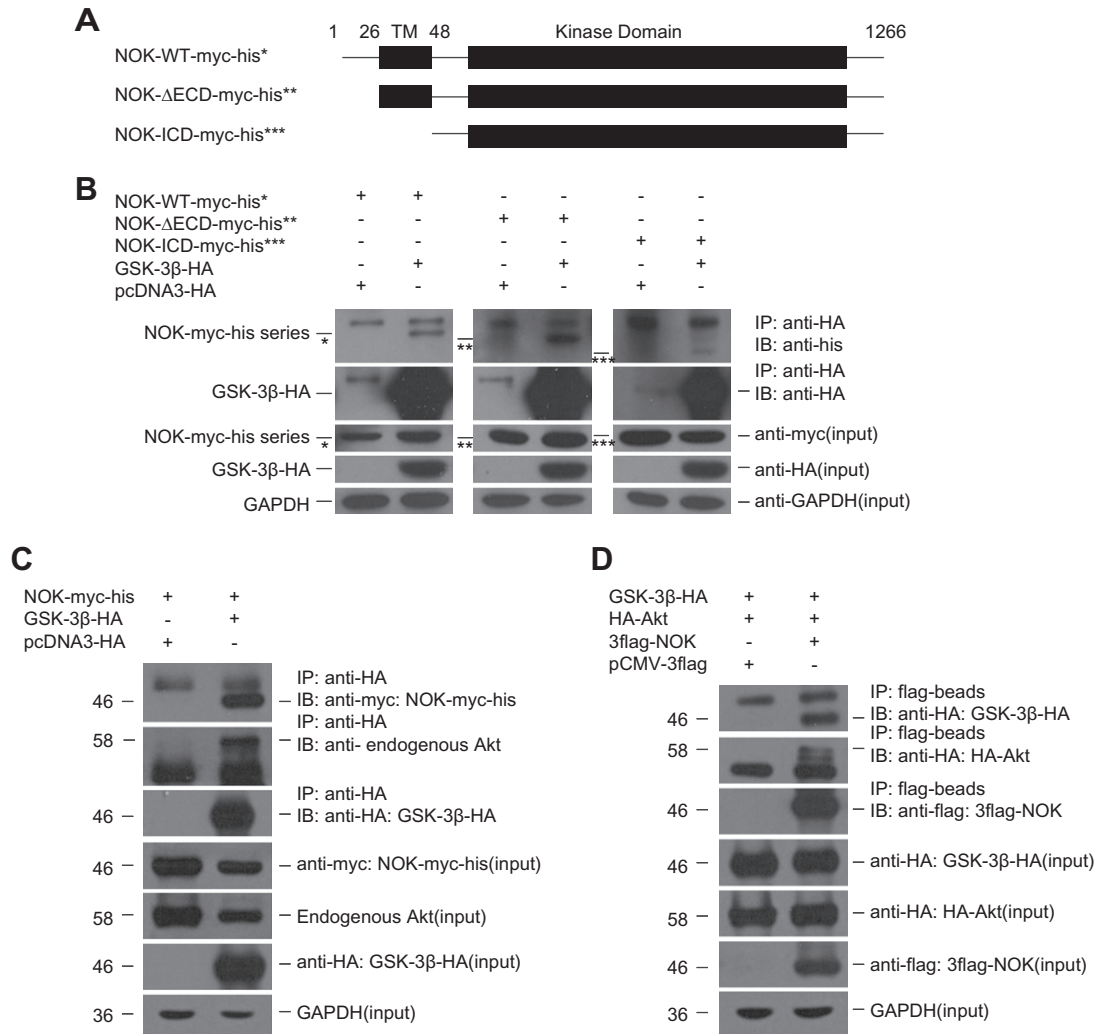

Fig. 2. NOK forms a complex with GSK-3β and Akt. (A) Schematic illustration of NOK constructs used in the co-IP assay: NOK-WT (for full-length NOK), NOK-ICD (for NOK missing intramembrane and extramembrane domain) and NOK-ΔECD (for NOK missing extramembrane domain). (B) HEK293T cells were transfected with myc-his-tagged NOK (NOK-myc-his) together with HA-tagged GSK-3β (GSK-3β-HA). An empty vector was used as a control. Cell lysate was subjected to co-IP with anti-HA antibody and the elute was resolved for western blotting analysis. (C) HEK293T cells transfected with GSK-3β-HA and NOK-myc-his were subjected for co-IP using anti-HA antibody. The products obtained were resolved for blotting with anti-myc (NOK-myc-his) and anti-Akt (for endogenous Akt) antibodies. (D) Cell lysates of HEK293T cells transfected with 3flag-NOK, GSK-3β-HA and HA-Akt were co-immunoprecipitated by anti-flag antibody-coupled beads. The products were resolved for blotting with anti-HA antibody since GSK-3β-HA and HA-Akt have different molecular weight.
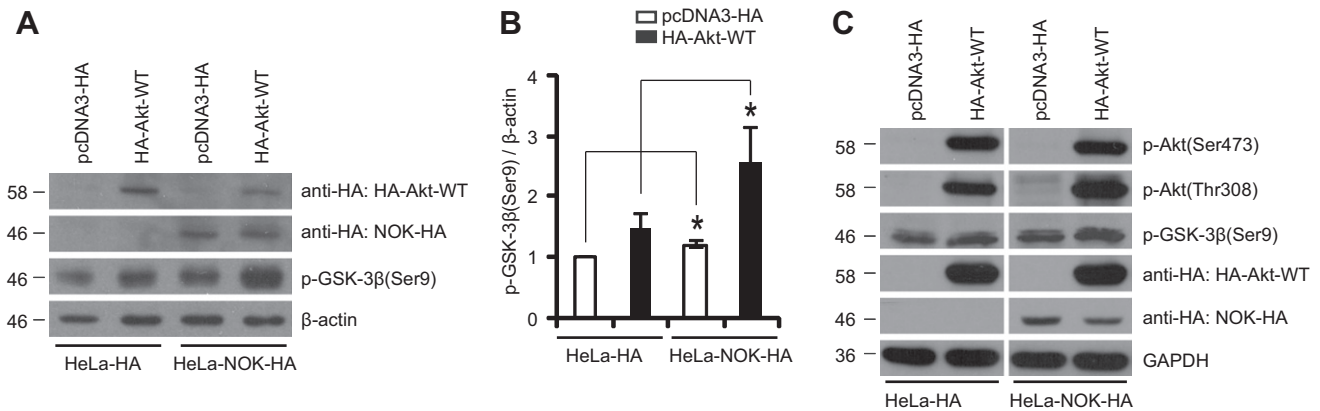
Fig. 3. Akt mediates NOK-dependent GSK-3β phosphorylation. (A) Akt-HA was transiently transfected into HeLa-NOK-HA and HeLa-HA cells. Total proteins were harvested and blotted with anti-p-GSK-3β(Ser9) and anti-HA antibodies. (B) The ratio of p-GSK-3β(Ser9) to β-actin was analysed based on quantification with Excel and Sigmaplot software. This ratio was expressed as the mean ± standard error from at least three independent replicates. Significant differences were determined by Student's test, and statistical significance was displayed as *(P < 0.05). (C) Similarly, plasmids carrying HA-Akt-WT and the HA empty vector were transfected into the above two stable cell lines, respectively. Total proteins were harvested and blotted with anti-p-Akt(Thr308) and anti-p-Akt (Ser473) antibodies.
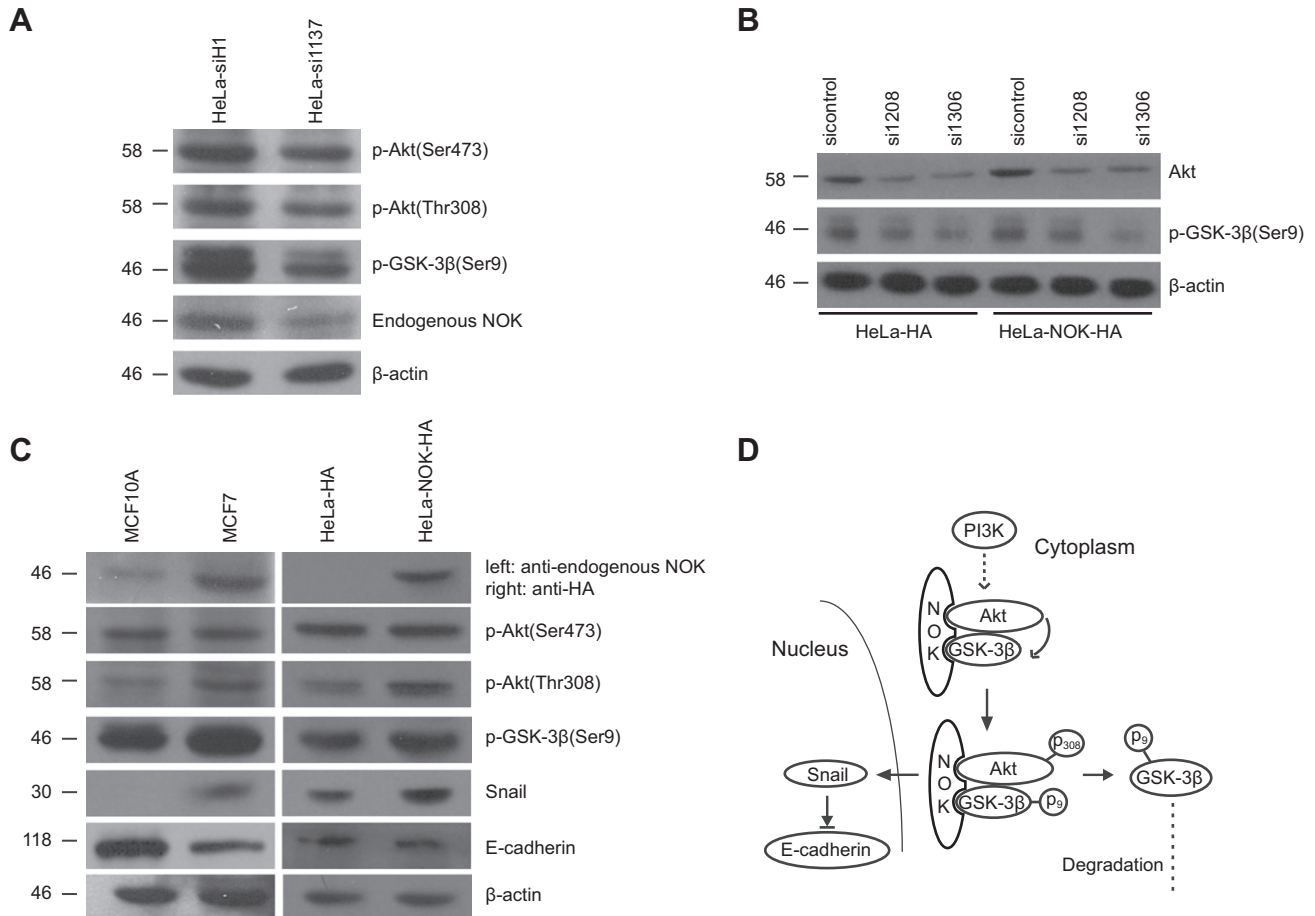

Fig. 4. Akt relays a NOK-directed cascade for effective GSK-3β (Ser9) phosphorylation. (A) Plasmids carrying NOK shRNA (pSilencer3.1-si1137) and the scramble shRNA (pSilencer3.1-siH1) were stably expressed in HeLa cells. Total proteins were harvested and blotted with corresponding antibodies in testing endogenous NOK, p-Akt(Thr308) and p-GSK-3β(Ser9). (B) Synthetic RNAi against endogenous Akt was transfected into HeLa-HA and HeLa-NOK-HA stable cell lines. Total proteins were harvested and blotted with anti-Akt and p-GSK-3β(Ser9) antibodies. (C) Total proteins from MCF10A, MCF7, Hela-HA and Hela-NOK-HA cells were harvested and blotted with anti-NOK, p-Akt(Thr308) and p-GSK-3β(Ser9) antibodies. (D) A schematic diagram of a proposed model that summarises our findings.

the HeLa-NOK-HA cell line was greater than that observed in the HeLa-HA cells (Fig. 3A and B). Importantly, Akt phosphorylation at the Thr308 residue was obviously higher in the HeLa-NOK-HA cells than that in the HeLa-HA cells; however, these two cell lines exhibited no difference in the phosphorylation of Akt at Ser473 (Fig. 3C). These results suggest that NOK stimulates GSK-3β Ser9 phosphorylation by increasing Akt Thr308 phosphorylation. Thus, NOK and Akt function cooperatively to up-regulate GSK-3β Ser9 phosphorylation.

3.4. Down-regulation of either NOK or Akt decreases p-GSK-3β(Ser9)

To validate further the findings that both NOK and Akt are involved in GSK-3β Ser9 phosphorylation, we carried out RNA interference-based knockdown assays. These tests were performed in transfected HeLa cells in which NOK and Akt were individually targeted for down-regulation. Synthetic siRNA (si208 and si360) targeted Akt and vector-based shRNA plasmid (pSilencer3.1–si1137) targeted NOK for down-regulation. As shown in Fig. S2 and Fig. 4A, both exogenous and endogenous NOK protein expression was effectively disrupted by RNA interference and importantly, the reduction in NOK was correlated with a decline in expression of both p-Akt(Thr308) and p-GSK-3β(Ser9). Furthermore, upon repression of endogenous Akt expression by RNA interference, the expression of p-GSK-3β(Ser9) decreased in both NOK-overexpressed cell lines (HeLa-NOK-HA) and control lines (HeLa-HA) (Fig. 4B). It is significant that GSK-3β Ser9 phosphorylation was also similar between HeLa-NOK-HA and HeLa-HA cells when Akt expression was disrupted (Fig. 4B), implying that Akt is the critical mediator for NOK-dependent GSK-3β Ser9 phosphorylation.

3.5. The expression levels of NOK, p-GSK-3β(Ser9) and Akt(Thr308) are positively correlated in breast cell lines

Our abovementioned findings pointed toward a tripartite molecular complex formed among NOK, Akt and GSK-3β that may lead to GSK-3β functional inactivation and tumourigenesis. To study this proposal in physiological settings, we compared the expression of NOK, p-Akt(Thr308), p-GSK-3β(Ser9) and two downstream factors, Snail and E-cadherin, in cancerous (MCF7) versus non-cancerous breast epithelial cells (MCF10A). As shown in Fig. 4C, the level of endogenous NOK in cancerous MCF7 cells was much higher than in non-cancerous MCF10A cells. It is also significant that the activated forms of Akt (p-Akt(Thr308)), inactivated forms of GSK-3β (p-GSK-3β(Ser9)) and Snail were more abundant in MCF7 than in non-cancerous MCF10A cells. The expression level of E-cadherin was lower in MCF7 than in non-cancerous MCF10A cells. All the results were further confirmed in HeLa-NOK-HA and HeLa-HA cells (Fig. 4C). Taken together, the results suggest that the expression levels of NOK, p-GSK-3β(Ser9) and p-Akt(Thr308) are positively correlated in vivo. To this end, we proposed a model to explain our findings (Fig. 4D), which will be further discussed.

4. Discussion

Our own previous studies and studies from other groups have demonstrated that NOK has potent tumourigenic activity, which has not been fully defined mechanistically. All experimental efforts have suggested that NOK has minimal enzymatic activity [7,9], which is a finding that presents an additional obstacle in understanding this protein's functions. In this report, we determined that overexpressed NOK could cause GSK-3β (Ser9) phosphorylation, which can relay a signal cascade for E-cadherin suppression and tumourigenesis. More extensive study at the molecular level led to the identification of a functional complex formed by NOK, Akt and GSK-3β and showed that within this tripartite complex, Akt mediates NOK-dependent GSK-3β phosphorylation. Additional study in cancerous versus non-cancerous breast cell lines suggests that this regulatory complex functions in vivo and has physiological relevance.

As an important inhibitor of the EMT process, active GSK-3β can phosphorylate transcriptional repressors of E-cadherin, such as Snail, and promote their nuclear export and further proteolysis [17]. Phosphorylation of GSK-3β at Ser9 results in its enzymatic inactivation and may thus reverse the protein's capacity to induce EMT

and tumourigenesis. Indeed, p-GSK-3β(Ser9) expression is frequently associated with higher grades of cancers [24–26]. This report was initiated by our observations in HeLa cells that NOK overexpression increased p-GSK-3β(Ser9) expression, suggesting a novel function for NOK that would provide an explanation for its tumour-promoting activity. The next mechanistic study was focused on Akt, a direct GSK-3β kinase that could be activated by overexpressed NOK. Using co-IP assays, we found that NOK can complex with Akt and GSK-3β, in vitro and in vivo, leading to the formation of a tripartite molecular model. Additional tests indicated that NOK-dependent GSK-3β Ser9 phosphorylation is mediated by Akt in its Thr308 (but not Ser473)-phosphorylated forms. Finally, a study in cancerous versus non-cancerous breast epithelial cell lines indicated the existence of such a module in physiological settings.

Our findings uncovered additional layers of NOK function at the biochemical, biological and physiological levels. However, it remains undetermined how NOK increases Akt phosphorylation at Thr308 to activate it. Structural analysis indicated that NOK has a DGF motif deficiency that would limit its kinase activity [7]. NOK was also predicted to have multiple protein binding motifs (data not shown), indicating that there are two possible ways for NOK to participate in the phosphorylation of Akt and GSK-3β: 1. NOK directly phosphorylates Akt through its own weak kinase activity; 2. NOK functions as a scaffold protein to phosphorylate Akt indirectly by bringing other kinases, such as PDK1, to Akt and subsequently linking Akt to GSK-3β. Generally, full activation of Akt activity depends on the phosphorylation of both Thr308 and Ser473 residues, which are located within the activation T-loop and regulation motif, respectively [27]. Akt binds to PIP3 (phosphatidylinositol (3,4,5)-trisphosphate) via its pleckstrin homology (PH) domain in the plasma membrane, where it can be phosphorylated at Thr308 by PDK1 [28–31]. Meanwhile, the NOK molecule contains an intra-membrane domain, leading to its localisation at the cell membrane (including inner membrane) and its high tendency to form aggregates [8]. It is conceivable that membrane localisation can bring NOK and Akt into proximity with each other and that NOK may interfere with Akt's interaction with other partners, including its Thr308 kinase PDK1 and its substrate GSK-3β.

Our findings were summarised in a model (Fig. 4D) to propose that in a tripartite molecular model, NOK interacts with Akt and stimulates phosphorylation of its Thr308 residue. The activated Akt relays the stimulatory effects to GSK-3β, leading to its Ser9 phosphorylation and functional inactivation. The identification of this regulatory pathway provides an explanation for NOK-directed tumourigenesis.

Disclosure summary

The authors have no conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.09.011.

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