Biochimica et Biophysica Acta 1853 (2015) 1738–1748

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Presumed pseudokinase VRK3 functions as a BAF kinase

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ARTICLE INFO

Article history:
Received 28 October 2014
Received in revised form 4 April 2015
Accepted 10 April 2015
Available online 18 April 2015

Keywords:
Pseudokinase
Vaccinia-related kinase 3
Barrier-to-autointegration factor

ABSTRACT

Vaccinia-related kinase 3 (VRK3) is known as a pseudokinase that is catalytically inactive due to changes in motifs that are essential for kinase activity. Although VRK3 has been regarded as a genuine pseudokinase from structural and biochemical studies, recent reports suggest that VRK3 acts as an active kinase as well as a signaling scaffold in cells. Here, we demonstrate that VRK3 phosphorylates the nuclear envelope protein barrier-to-autointegration factor (BAF) on Ser4. Interestingly, VRK3 kinase activity is dependent upon its N-terminal regulatory region, which is excluded from the determination of its crystal structure. Furthermore, the kinase activity of VRK3 is involved in the regulation of the cell cycle. VRK3 expression levels increase during interphase, whereas VRK1 is enriched in late G2 and early M phase. Ectopic expression of VRK3 induces the translocation of BAF from the nucleus to the cytoplasm. In addition, depletion of VRK3 decreases the population of proliferating cells. These data suggest that VRK3-mediated phosphorylation of BAF may facilitate DNA replication or gene expression by facilitating the dissociation of nuclear envelope proteins and chromatin during interphase.

1. Introduction

Protein kinases are key regulators of most cellular processes, including metabolism, transcription, cell cycle progression, apoptosis, and differentiation [14]. Protein kinase superfamilies comprise approximately 2–3% of human genes [23] and are designated as the 'kinome.' Unexpectedly, nearly 10% of the human kinome have been categorized as pseudokinases on the basis of a lack of one or more of the conserved amino acids required for kinase activity. Therefore, pseudokinases are predicted to be enzymatically inactive [3]. Although approximately 10% of protein kinases are predicted to be catalytically inactive pseudokinases, recent biochemical and structural studies have revealed that several of them are enzymatically active, suggesting that atypical mechanisms of protein phosphorylation are at work in the cell [6,7,27,38–40].

Vaccinia-related kinase 3 (VRK3) is a member of mammalian vaccinia-related kinases (VRKs), a novel family of mammalian serine/threonine protein kinases that was initially characterized by its homology to the vaccinia virus B1R kinase [28]. Recent studies identified the essential roles of the VRK family in cell signaling, nuclear envelope dynamics, histone modification, apoptosis, and cell stress responses [18,21,30,32]. Our previous studies demonstrated that VRK3 negatively regulates MAP kinase signaling by promoting the activity of the vaccinia H1-related (VHR) phosphatase, which specifically dephosphorylates and inactivates extracellular signal-regulated kinases (ERKs) in the nucleus [16,17]. It is noteworthy that VRK3 elevates the phosphatase activity of VHR by a mechanism independent of its kinase activity [16]. Sequence analyses predict that VRK3 has mutations in three conserved amino acids in the catalytic domain that is required for kinase activity [3,23]. VRK3 reportedly cannot incorporate ^{32}P into casein in contrast to other VRK family members and casein kinases [28]. In addition, the structure of the VRK3 catalytic domain was revealed to be incapable of binding ATP [34]. These data collectively support the notion that VRK3 is a 'dead' protein kinase. Thus, VRK3 is currently classified as a truly inactive pseudokinase [42]. However, the kinase activity of full-length VRK3, which retains the extracatalytic domain, has not been rigorously examined, especially under physiological conditions. In our previous report, VRK3 showed putative kinase activity in a cellular context [16]. Moreover, it was suggested that VRK3 functions as an active protein kinase like VRK1 and VRK2 while this work was in an initial stage [37]. Therefore, it is unclear whether VRK3 has an unconventional mode of kinase action, like some other active pseudokinases [7].

Barrier-to-autointegration factor (BAF) is an essential nuclear envelope protein that links the nuclear envelope and chromatin [24,35]. BAF is localized in the nucleus and is highly enriched in the nuclear envelope [26]. Phosphorylation and dephosphorylation of BAF are required for disassembly and reassembly, respectively, of the nuclear envelope during mitosis [1,9]. It is well known that mitotic phosphorylation of BAF at its N-terminus by VRK1 strongly reduces the affinity of BAF for chromatin and LEM proteins, thus contributing to nuclear envelope breakdown during early mitosis [10,25,28,29]. Notably, the levels of mitotic VRK1 are enriched from late G2 to prophase, and its residual activity is suppressed by histone variant macroH2A1.2 in the nucleus

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http://dx.doi.org/10.1016/j.bbamcr.2015.04.007
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during interphase [18,20]. However, while the N-terminus of BAF is phosphorylated during interphase, the kinase responsible for this reaction and its role remains elusive [2].

Even though VRK3 has been regarded as an inactive pseudokinase from sequence and structural analyses, we demonstrate that VRK3 functions as an active protein kinase on BAF, which is dependent upon its N-terminal extracatalytic domain. We also present the possibility that VRK3-mediated BAF phosphorylation is associated with cell cycle progression during interphase.

2. Results

2.1. VRK3 interacts with the nuclear envelope protein BAF in cells

Although the kinase domain of VRK3 was demonstrated to be inactive in a study of its protein structure [34], the data presented in our previous study suggest that the kinase activity of full-length recombinant VRK3 protein is retained when it is preincubated with whole-cell lysates [16]. Thus, we hypothesized that the kinase activity of VRK3 may be dependent upon co-regulators or substrates. To systemically discover the cellular binding partners that are required for VRK3 kinase activity, FLAG-tagged human VRK3 was transfected into HEK 293A cells, followed by co-immunoprecipitation with anti-FLAG antibodies. LTQ-orbitrap mass spectrometric analysis was then performed to identify VRK3-interacting proteins [13]. We identified that the nuclear envelope protein BAF was associated with VRK3 in this proteomic analysis (data not shown).

To validate the interaction between VRK3 and BAF, HEK 293A cells were transfected with constructs encoding FLAG-tagged VRK3, and co-immunoprecipitation assays were performed. As a result, ectopically expressed VRK3 formed a complex with endogenous BAF (Fig. 1A) in accordance with mass spectrometric data. In addition, endogenous VRK3 was also co-immunoprecipitated with FLAG-tagged BAF (Fig. 1B).

To confirm the interaction between VRK3 and BAF at endogenous protein levels, HEK 293A cell lysates were prepared and endogenous VRK3 immunoprecipitation was performed using anti-VRK3 antibodies. We found that BAF was co-immunoprecipitated with endogenously expressed VRK3 in HEK 293A cells (Fig. 1C). Furthermore, results of immunofluorescence experiments show that FLAG-tagged VRK3 co-localized with endogenous BAF in the same subcellular compartment of HEK 293A cells. As shown in Fig. 1D, VRK3, which has a nuclear localization signal, and BAF were both prominently present in the nucleus [11]. In addition, a nuclear envelop marker Lamin B is also colocalized with VRK3 in the nucleus (Fig. 1E). These results suggest that nuclear-localized VRK3 associates with nuclear envelope protein BAF in HEK 293A cells.

2.2. VRK3 phosphorylates BAF on Ser4 in vitro

Although recombinant VRK3 failed to phosphorylate casein in vitro [28], the kinase activity of VRK3 has not been rigorously tested using authentic binding partners. Thus, we examined whether VRK3 phosphorylates BAF in the presence of various divalent cations. We used purified GST-VRK3 for an in vitro kinase assay, which showed phosphorylation of histidine-tagged BAF (His BAF) when either Mg^{2+} or Mn^{2+} was used as a divalent cation for the kinase reaction (Fig. 2A). However, autophosphorylation of recombinant VRK3 was not detected in this assay using radiolabeled ATP (Fig. 2B). To further confirm the VRK3 kinase activity, we used other substrates, such as casein and histone H3, in an in vitro kinase assay. As a result, VRK3 only phosphorylated BAF, but not casein or histone H3 (Fig. 2B). In contrast, VRK1 robustly phosphorylated BAF, including casein and histone H3 (Supplementary Fig. 1). These data suggest that VRK3 has a distinct mode of action different from that of VRK1 and other casein kinase family members.

To validate the BAF phosphorylation mediated by VRK3, we constructed BAF mutants, in which the N-terminal serine or threonine residues were substituted with alanine. BAF is phosphorylated on Thr2, Thr3, and Ser4 by VRK1 in a cell cycle-specific manner [2,3]. According to in vitro kinase assay results, the initial phosphorylation of Ser4 induced the subsequent phosphorylation of Thr2 and Thr3. Thus, Ser4 is regarded as a primary VRK1 phosphorylation site [29]. Therefore, we needed to assess whether N-terminal residues are also phosphorylated by VRK3. Notably, the catalytic ability of VRK3 to phosphorylate BAF was completely abrogated when the Ser4 residue was mutated to alanine (Fig. 2C). This result implies that VRK3 also plays a role as a BAF kinase in cells.

As we previously reported, the mutation of Lys182 abolishes the putative autophosphorylation of mouse VRK3 after preincubation with an HT22 cell lysate [16]. This residue of mouse VRK3 is equivalent to Lys203 of human VRK3. Thus, we subsequently tested whether the kinase activity of VRK3 was abrogated when Lys203 is mutated to glutamic acid (K203E). In vitro kinase assay results showed that the VRK3 K203E mutant failed to catalyze the phosphorylation reaction of BAF, suggesting that the K203E mutant is inactive (Fig. 2D).

To analyze the catalytic activity of VRK3 in cells, VRK3 immunoprecipitates were incubated with exogenous recombinant BAF. As expected, phosphorylation of wild-type BAF was also detected in an in vitro kinase assay using FLAG-tagged VRK3, whereas phosphorylation of mutant BAF Ser4A was not detected (Fig. 2E). Collectively, these data suggest that VRK3 is an active protein kinase that phosphorylates BAF in vitro and in vivo.

2.3. N-terminal region of VRK3 is required for its kinase activity

Even though a substantial fraction of all presumed pseudokinases turn out to be catalytically active, VRK3 is regarded as a genuinely inactive pseudokinase, because a structural study showed that it is incapable of binding ATP [23,34,42]. In this study, however, we presented VRK3 as an active BAF kinase. The most notable difference between the previous structural study and our biochemical assay is that the N-terminally truncated, catalytic domain of VRK3 (residues 148–472) was used to elucidate its crystal structure [34], whereas the full-length form of VRK3 (VRK3 FL) was employed to test its kinase activity. Therefore, we constructed a catalytic domain of VRK3 that is devoid of N-terminal residues, designated VRK3 (ΔN1–147). This construct encompasses the entire catalytic domain of VRK3 used in the previous structural study.

Initially, we compared the kinase activities of VRK3 FL and VRK3 (ΔN1–147) towards BAF in vitro. As shown in Fig. 3A, VRK3 FL phosphorylated BAF in a concentration-dependent manner, consistent with previous observations. In contrast, VRK3 (ΔN1–147) was unable to phosphorylate BAF, indicating that the N-terminal region (residues 1–147) is required for VRK3 activity in vitro. Additionally, FLAG-tagged VRK3 (ΔN1–147) immunoprecipitates failed to incorporate radiolabeled ATP into BAF (Fig. 3B). Furthermore, FLAG-tagged VRK3 (ΔN1-147) was unable to form a complex with endogenous BAF in a co-immunoprecipitation assay (Fig. 3C). Actually, VRK3 (ΔN1–147) was localized to the cytosol in cells (Supplementary Fig. 2). In addition, just the N-terminal region of VRK3 (N1-147) is not sufficient to interact with BAF (Supplementary Fig. 4). Taken together, both N-terminal extracatalytic domain and kinase domain of VRK3 appear to be required for proper conformation to interact and phosphorylate BAF.

2.4. BAF phosphorylation mediated by VRK3 modulates its association with nuclear components

BAF interacts with LEM domain-containing proteins, such as LAP2, emerin, and Man1, in the inner nuclear membrane, [24] contributing to the integrity of the nuclear envelope. It was reported that ectopic expression of VRK1 and BAF lessened the association of BAF with the

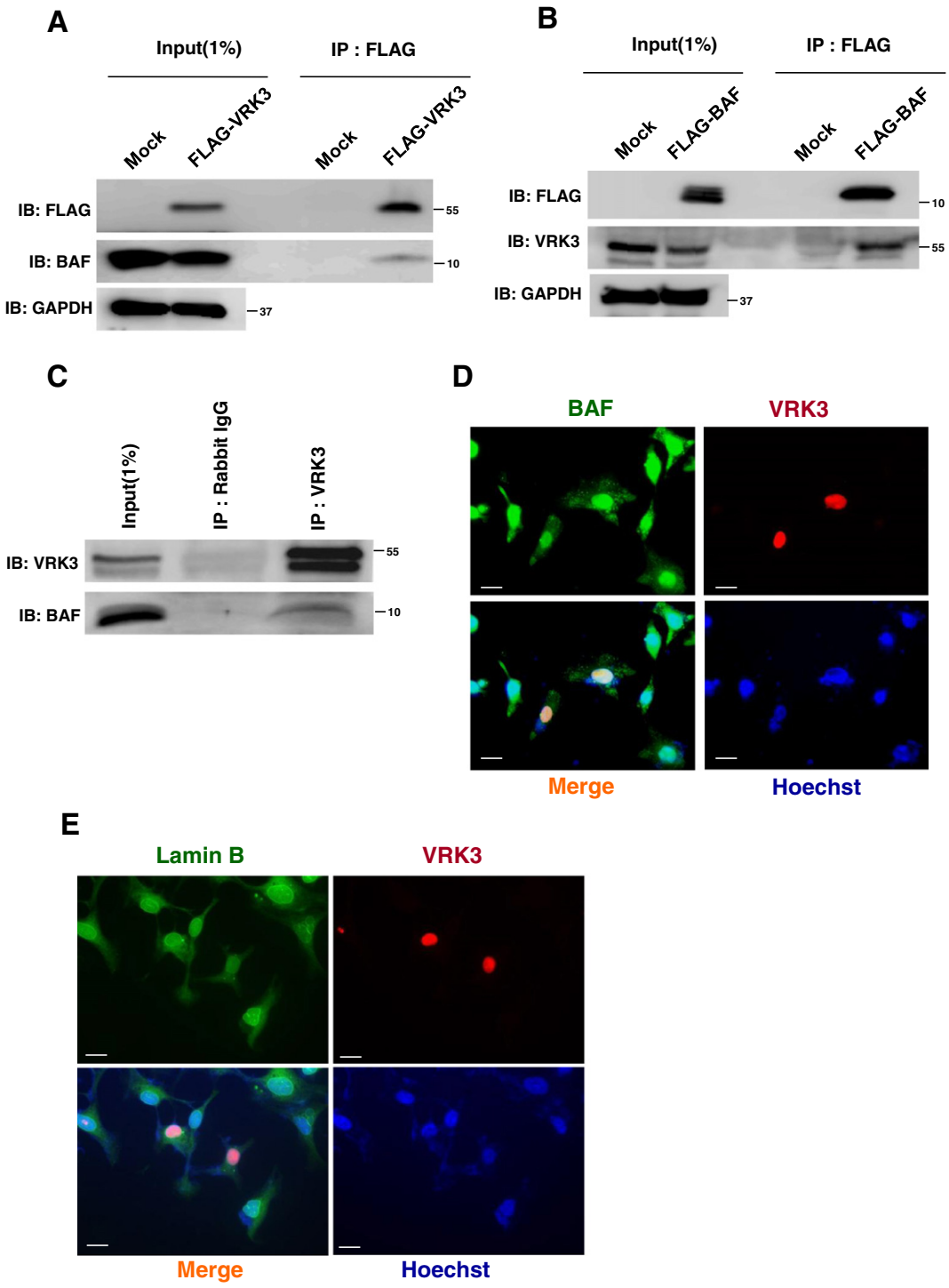


Fig. 1. VRK3 specifically interacts with BAF in cells. (A) Whole-cell lysates from HEK293A cells transfected with pFLAG (Mock) or pFLAG-VRK3 were immunoprecipitated with anti-FLAG antibodies, and the precipitates as well as input sample were immunoblotted with antibodies to FLAG, BAF, or GAPDH. (B) Whole-cell lysates from HEK293A cells transfected with pFLAG (Mock) or pFLAG-BAF were immunoprecipitated with anti-FLAG antibodies, and the precipitates and input sample were immunoblotted with antibodies to FLAG, VRK3, or GAPDH. (C) Whole-cell lysates collected from HEK293A cells were used for endogenous immunoprecipitation with anti-VRK3 antibodies. The VRK3 immunoprecipitates were analyzed by immunoblotting with antibodies to VRK3 or BAF. (D) HEK293A cells were transfected with pFLAG-VRK3. Nuclei were stained with Hoechst, and the localization of BAF and VRK3 was observed with fluorescence microscopy. (E) HEK293A cells were transfected with pFLAG-VRK3. Nuclei were stained with Hoechst, and the localization of Lamin B and VRK3 was observed with fluorescence microscopy. The scale bars in panels D and E represent 10 μm. All immunoblotting results are from at least three separate experiments.

nuclear component, leading to its diffusion from the nucleus to the cytosol [29]. Thus, we assessed whether VRK3 kinase activity can trigger the redistribution of BAF in cells. First, ectopically expressed EGFP-BAF maintained its nuclear localization in A549 cells. However, when FLAG-tagged VRK3 and EGFP-BAF were co-transfected into A549 cells, there was a substantial increase in the cytoplasmic localization of EGFP-BAF (Fig. 4A and C, Supplementary Fig. 5). In contrast, the catalytically inactive VRK3 K203E mutant could not substantially alter the subcellular localization of EGFP-BAF. Furthermore, the VRK3-dependent translocation of BAF was diminished when its Ser4 residue was mutated

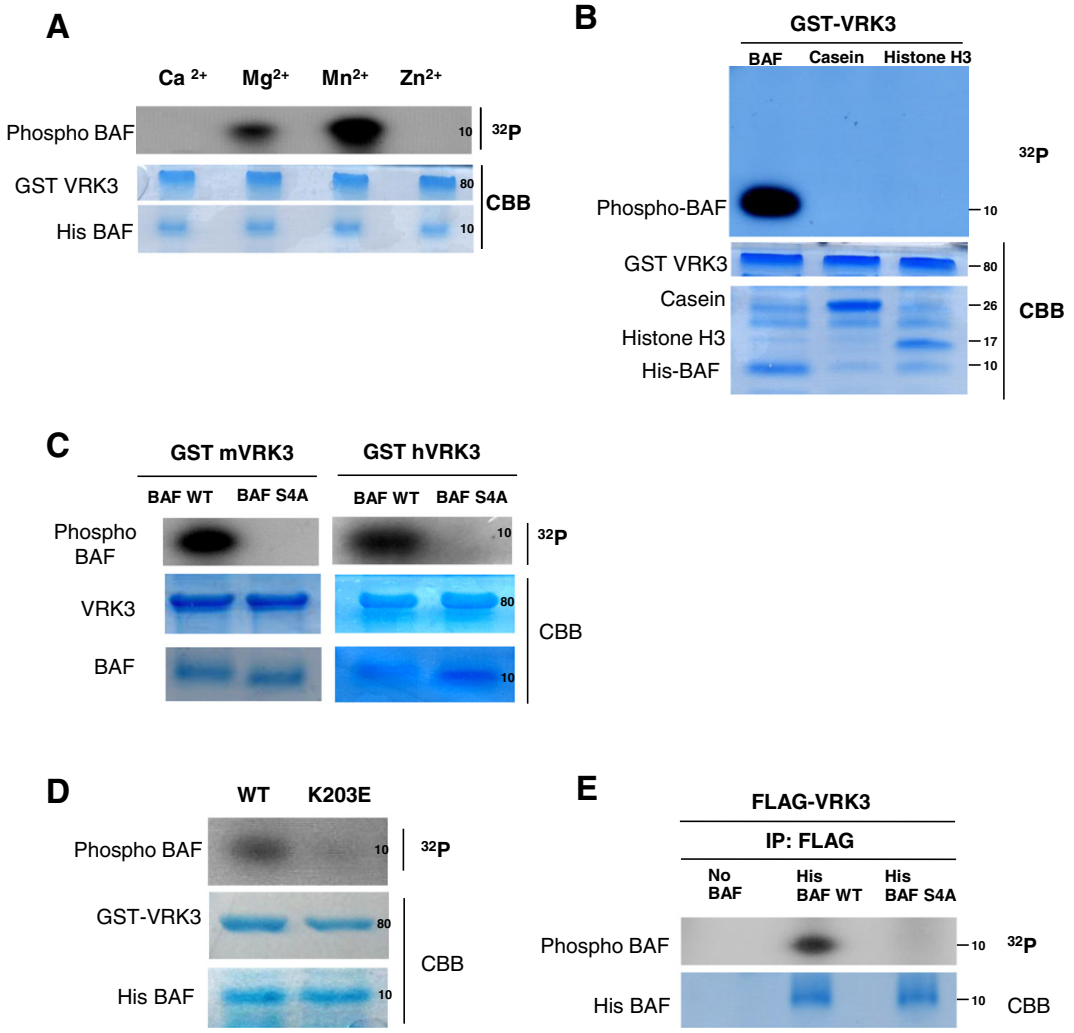


Fig. 2. Identification of VRK3 kinase activity in vitro. (A) A VRK3 kinase assay was performed using recombinant GST-VRK3(2 μg)in the presence of various divalent cations. His-tagged BAF(1 μg) was used as a substrate. (B) A VRK3 kinase assay was performed using recombinant GST-VRK3 (2 μg) in the presence of various substrates. His-tagged BAF (1 μg), casein (1 μg) and histone H3 (1 μg) were used as substrates. (C) Identification of BAF residues phosphorylated by VRK3. Recombinant His-BAF (WT, 1 μg) or the His-BAF S4A mutant (1 μg) was incubated with GST-mouse VRK3 (GST-mVRK3. 2 μg) or GST- human VRK3 (GST-hVRK3, 2 μg). (D) GST-VRK3 (WT, 2 μg) or GST-VRK3 (K203E, 2 μg) was incubated with His-BAF(1 μg) as a substrate. (E) HEK293A cells were transfected with pFLAG-VRK3. After 24 h, whole-cell lysates were immunoprecipitated with anti-FLAG antibodies. In vitro immune complex kinase assays were performed without exogenous protein, or with His-BAF(1 μg) or His-BAF S4A(1 μg) as substrates.

to alanine (Fig. 4B and C). In addition, VRK3 affects the localization of LEM protein emerin, which interacts with BAF.

2.5. Cell cycle-dependent expression of VRK3

VRK1 is known to phosphorylate BAF on Ser4, Thr2, and Thr3 to promote mitotic nuclear envelope disassembly [1,9,10]. Our studies show that VRK3 also catalyzes the phosphorylation of the Ser4 residue of BAF. Thus, both VRK1 and VRK3 are able to phosphorylate the Ser4 residue of BAF. Therefore, we wished to delineate the roles of VRK3 in cell cycle regulation compared to VRK1. To do this, we first examined the levels of VRK3 in HeLa cells at different stages of the cell cycle (Fig. 5A). As we previously reported, the protein levels of VRK1 were enriched when mitotic arrest was induced by treatment with nocodazole [18]. In contrast, VRK3 levels increased in the G1 phase, after cell cycle arrest induced by hydroxyurea. Some cell cycle markers such as cyclin B, cyclin E and phospho-Rb also suggest that the expression of VRK3 is enriched in interphase (Fig. 5A) The cell cycle-dependent accumulation of VRK3 was seen in A549 cells, consistent with results observed in HeLa cells, suggesting that enrichment of VRK3 during interphase is not confined to a specific cell line (Fig. 5B). In addition, HeLa cells were synchronized at the G1/S boundary using the double thymidine block method. As shown in Fig. 4C, at the indicated time points of release, the VRK3 protein gradually decreased, indicating that VRK3 is differentially expressed during cell cycle progression and peaks during the G1/S phase (Fig. 5C).

To further evaluate the role of VRK3 in cell cycle progression, we identified the S-phase cell populations. The portion of BrdU-positive cells increased when HeLa cells were transfected with wild-type VRK3, but not with the kinase-inactive K203E mutant (Fig. 5D). The involvement of VRK3 in cell cycle progression was also examined by reducing VRK3 levels using shRNA (Fig. 5G). Flow cytometric analyses revealed that the population of BrdU-positive cells decreased after VRK3 knockdown (Fig. 5E and F). In addition, when we tested growth of mouse embryonic fibroblast (MEF) isolated from VRK3 depleted mice VRK3$^{-/-}$ MEFs grew noticeably slower than wild-type control (Fig. 5H). Collectively, these results suggest that the expression of VRK3 may be associated with BAF phosphorylation during interphase, which promotes cell cycle progression, especially in the G1/S phase.

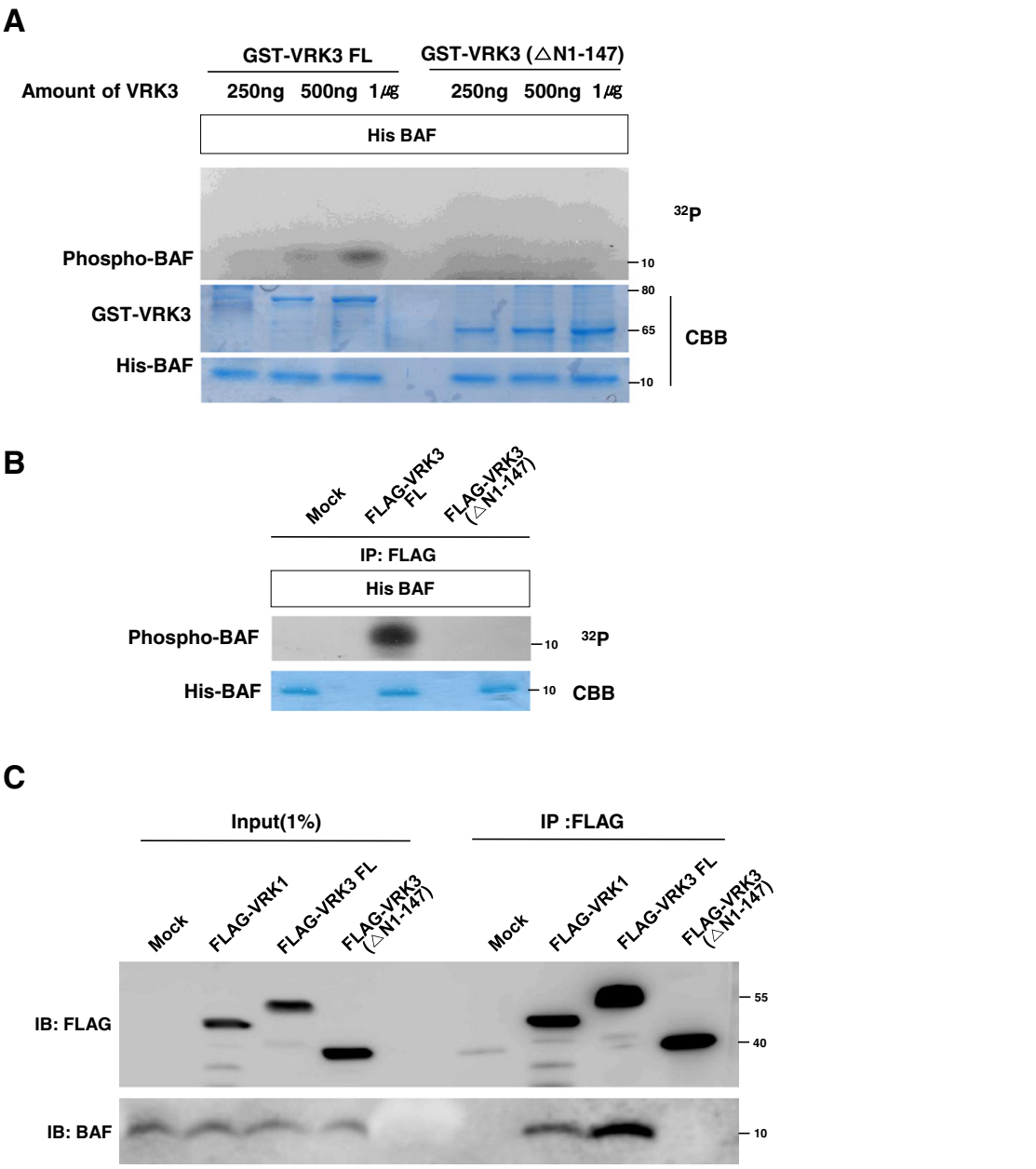


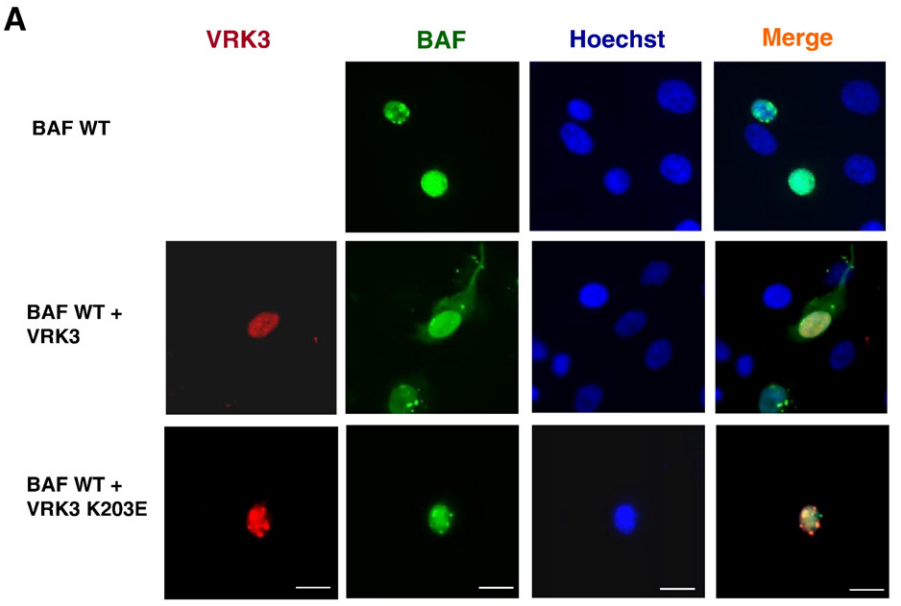
Fig. 3. N-terminus dependent phosphorylation of BAF by VRK3. (A) Comparison of kinase activity of full-length VRK3 and truncated VRK3(Δ1–147). GST-VRK3 full length (FL) or GST-VRK3 (Δ1–147) was incubated with His-BAF(1 μg) as a substrate. (B) HEK293A cells were transfected with pFLAG, pFLAG-VRK3, and pFLAG-VRK3(Δ1–147) respectively. After 24 h, whole-cell lysates were immunoprecipitated with anti-FLAG antibodies. In vitro immune complex kinase assays were performed with His-BAF(1 μg) as a substrate. (C) Whole-cell lysates from HEK293A cells transfected with pFLAG (Mock), pFLAG-VRK1, pFLAG-VRK3, or pFLAG-VRK3(Δ1–147) were immunoprecipitated with anti-FLAG antibodies, and the precipitates and input sample were immunoblotted with antibodies to FLAG or BAF.

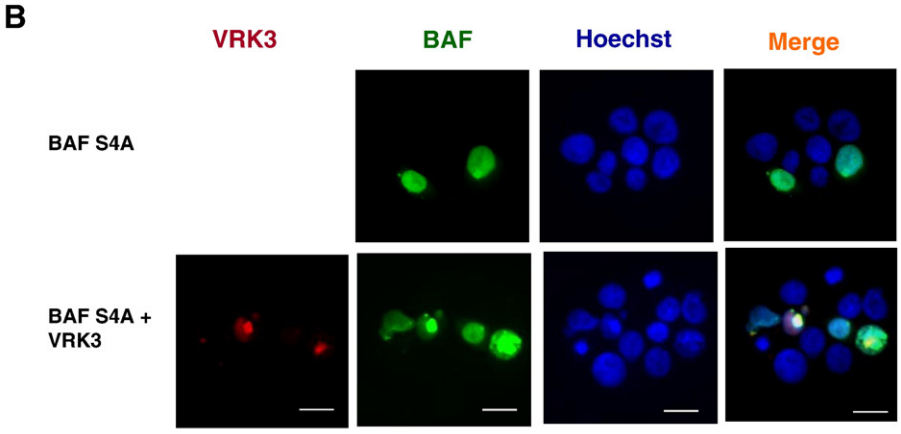
3. Discussion

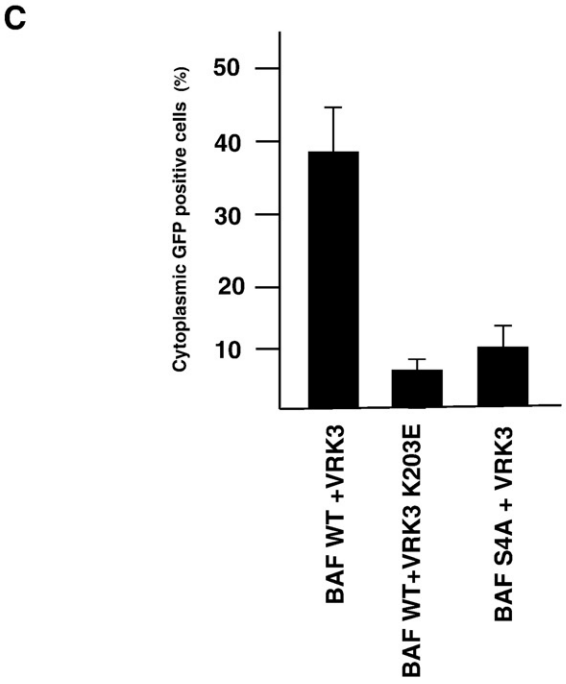
3.1. The N-terminal regulatory region is essential for VRK3 kinase activity

As previously mentioned, a considerable fraction of presumed pseudokinases turn out to be catalytically active, suggesting that noncanonical mechanisms of phosphorylation exist in cells [7]. One of the most remarkable examples of active pseudokinase is the Ca^{2+}/calmodulin-dependent serine/threonine kinase (CASK) [19,27]. Unlike conventional protein kinases, CASK functions in the absence of divalent cations. Intriguingly, its activity is suppressed by Mg^{2+}. Another example is Her3 (human epidermal growth receptor 3), the pseudokinase counterpart of EGFR, which shows mild autophosphorylation activity when it is clustered in reconstituted vesicles [36]. Actually, Her3 has no kinase activity under standard conditions without vesicles [38]. Additionally, KSR (kinase suppressor of ras) provides a remarkable

Fig. 4. Ectopic expression of VRK3 induces BAF translocation. (A) A549 cells were transfected with plasmids encoding EGFP-BAF, with or without pFLAG-VRK3 WT or pFLAG-VRK3 K203E. After 24 h, cells were fixed and stained with anti-FLAG antibodies. The localization of BAF and VRK3 was observed with fluorescence microscopy. (B) A549 cells were transfected with plasmids encoding EGFP-BAF S4A, with or without pFLAG-VRK3 WT. After 24 h, cells were fixed and stained with anti-FLAG antibodies. The localization of BAF and VRK3 was observed with fluorescence microscopy. (C) The percentage of cytoplasmic GFP positive cells are calculated among co-transfected cells with FLAG-VRK3 and EGFP-BAF. The results are expressed as the mean ± SD of four independent experiments. At least 100 hundreds cells are counted at each experiment. (D) A549 cells were transfected with pFLAG-VRK3. Nuclei were stained with Hoechst, and the localization of emerin and VRK3 was observed with fluorescence microscopy. The scale bars in panels A, B and D represent 10 μm.


A
VRK3
BAF
Hoechst
Merge
BAF WT
BAF WT +
VRK3
BAF WT +
VRK3 K203E


B
VRK3
BAF
Hoechst
Merge
BAF S4A
BAF S4A +
VRK3


C
Cytoplasmic GFP positive cells (%)
50
40
30
20
10
BAF WT +VRK3
BAF WT+VRK3 K203E
BAF S4A + VRK3

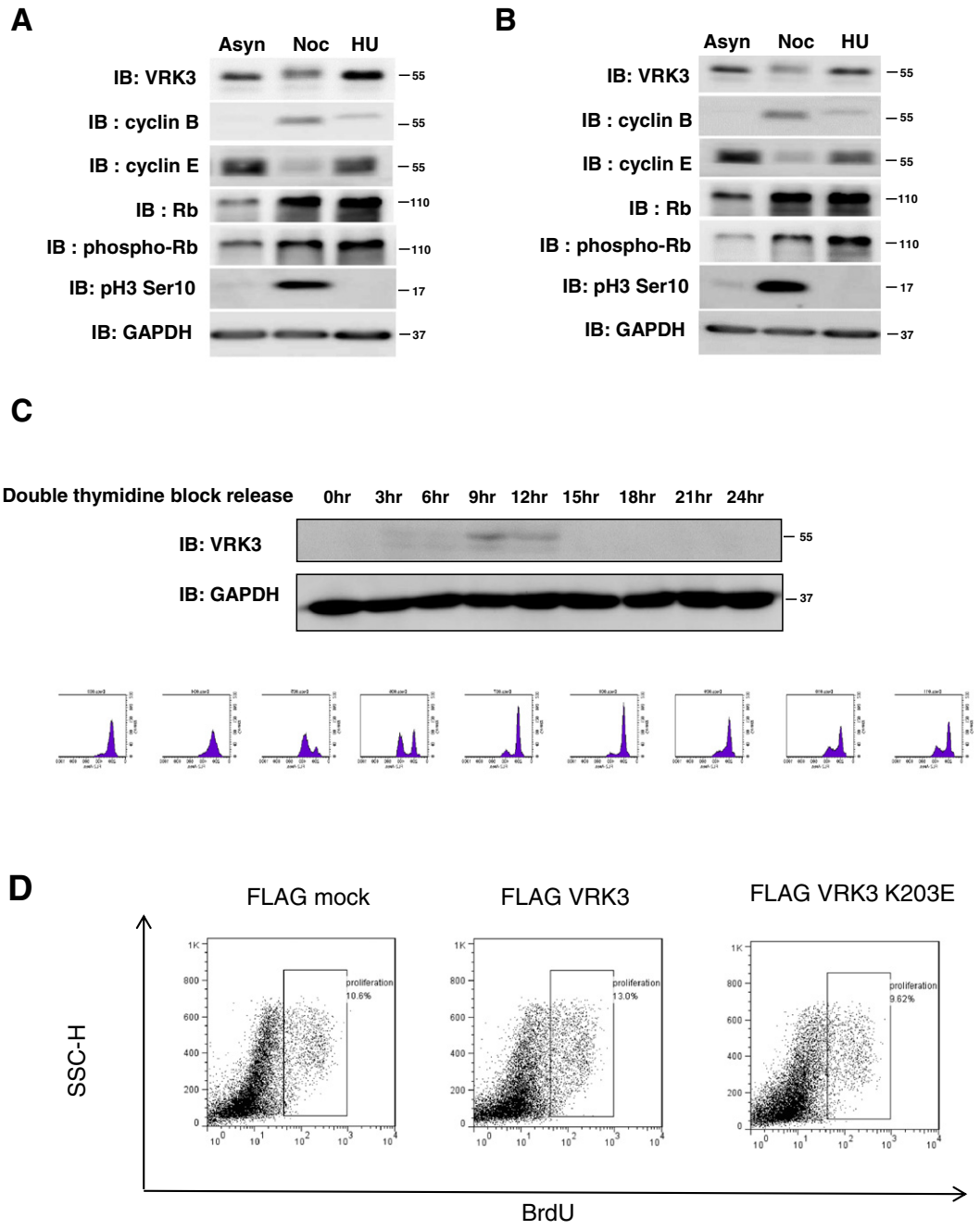


Fig. 5. Role of VRK3 in cell cycle regulation. (A) Whole-cell extracts were prepared from asynchronously growing HeLa cells (Asyn) or cells treated with hydroxyurea (HU) or nocodazole (Noc). The extracts were analyzed by immunoblotting with specified antibodies. (B) Whole-cell extracts were prepared from asynchronously growing A549 cells (Asyn) or cells treated with hydroxyurea (HU) or nocodazole (Noc). The extracts were analyzed by immunoblotting with specified antibodies. All immunoblotting results are from at least three separate experiments. (C) HeLa cells were synchronized by double thymidine block. FACS analyses and immunoblotting were performed at the indicated times after release from thymidine block. (D and E) HeLa cells were pulsed with BrdU following transfection with the indicated VRK3 plasmids or empty vector, then chased in the absence of BrdU. The DNA content of BrdU-positive cells was measured by flow cytometry. (F) Quantitation of BrdU-positive cells is expressed as the mean ± SEM of three independent experiments. (G) HeLa cells were transfected with FLAG mock, FLAG VRK3, sh mock or shVRK3. Knockdown efficiency of endogenous VRK3 is checked by immunoblotting with specified antibodies. (H) Growth curves for VRK3 $^{+/+}$ and VRK3$^{-/-}$ MEF cultures at passage 2.

example of an active pseudokinase through a novel mechanism. It was found that the pseudokinase domains of both KSR1 and KSR2 possess kinase activity through an allosteric mechanism [4]. Taken together, these findings clearly demonstrate that several pseudokinases are catalytically active in a context-dependent manner using novel mechanisms. Therefore, it is conceivable that other presumed pseudokinases may also be active protein kinases with atypical reaction mechanisms.

In this study, we show that VRK3 functions as an active BAF kinase; this activity is dependent on the N-terminal region (N1–147) of VRK3. According to the secondary structure of the protein predicted by its amino acid sequence, the N-terminal extracatalytic domain of VRK3 lacks a stable secondary structure and is expected to be highly flexible (Supplementary Fig. 3). This region may hinder the successful crystallization of full-length VRK3 protein. This might explain why the N-terminus of VRK3 was deleted in the elucidation of its protein structure. However, this region of VRK3 has multiple targets for Pro-directed S/T kinases, such as cyclin-dependent kinases and mitogen-activated protein kinases [8]. In fact, the N-terminus of VRK3 was shown to be highly phosphorylated in several phosphoproteomic studies [5,12,15,41,43], suggesting that this

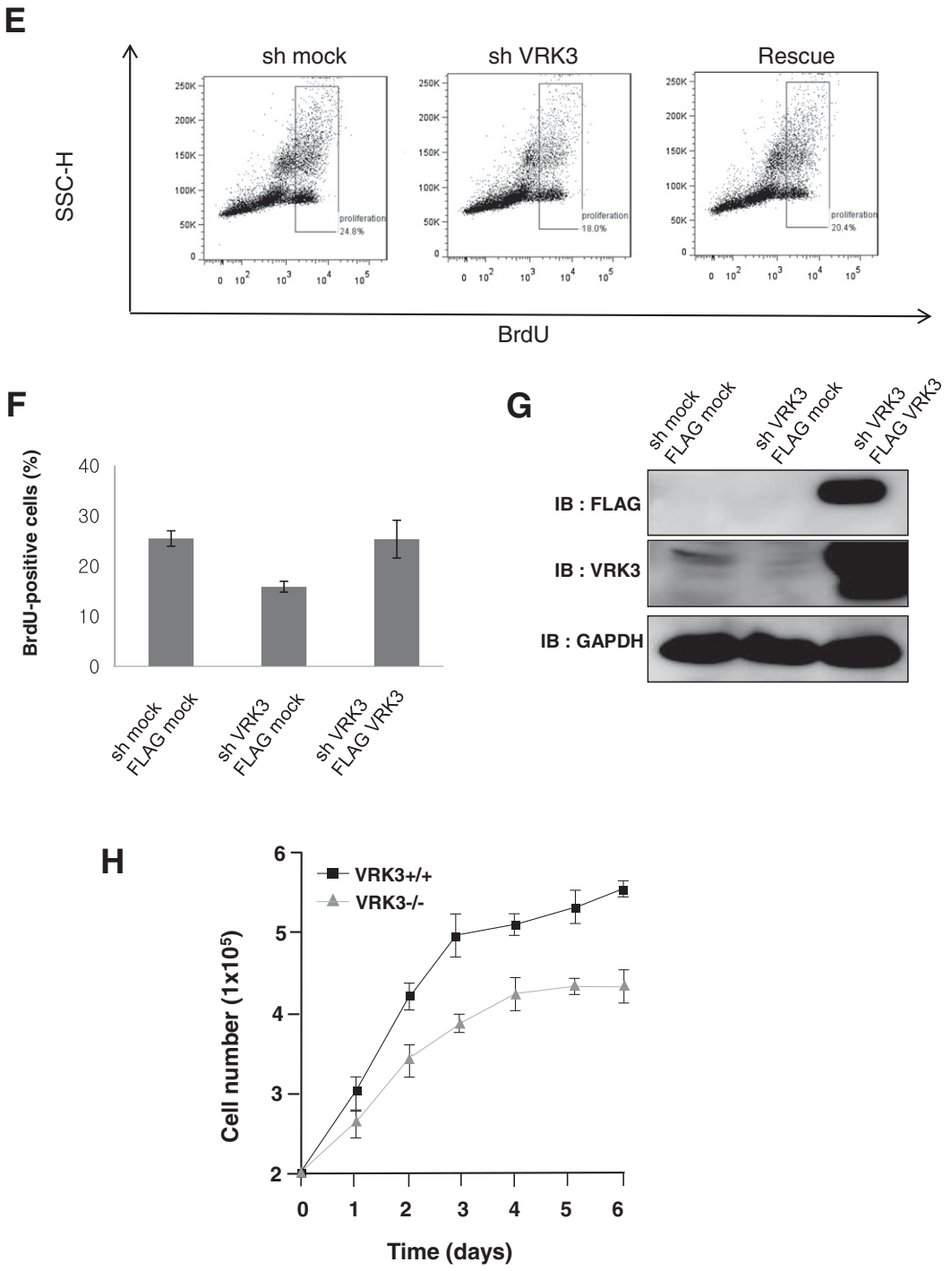


Fig. 5 (*continued*).

region may have a regulatory role for its catalytic activity. According to previous structural studies, the ATP-binding pocket of the VRK3 catalytic domain (ΔN1–147) is blocked by large hydrophobic side chains. For catalytic activity, this pocket must be accessible [22,34]. Thus, we hypothesize that the N-terminal regulatory region of VRK3 contributes to the opening of the closed ATP-binding pocket for its kinase activity. It will be very interesting to determine the entire structure of VRK3, including the N-terminal region. We thus anticipate that unraveling the full structure of VRK3 may reveal another atypical kinase mechanism.

3.2. Role of VRK3 in cell cycle regulation

BAF interacts with various nuclear envelope proteins, and its binding properties are tightly regulated in a cell cycle-dependent manner [1,11, 24,35]. As reported, the localization of BAF fluctuates during the cell cycle [11]. In early mitosis, BAF is released from the nuclear envelope and is evenly dispersed throughout the cytoplasm. Hyperphosphorylation of BAF on Thr2, Thr3, and Ser4 by VRK1 initiates the disassembly of the complex containing LEM proteins, chromatin, and BAF [10,29]. At the end of mitosis, protein phosphatases such as

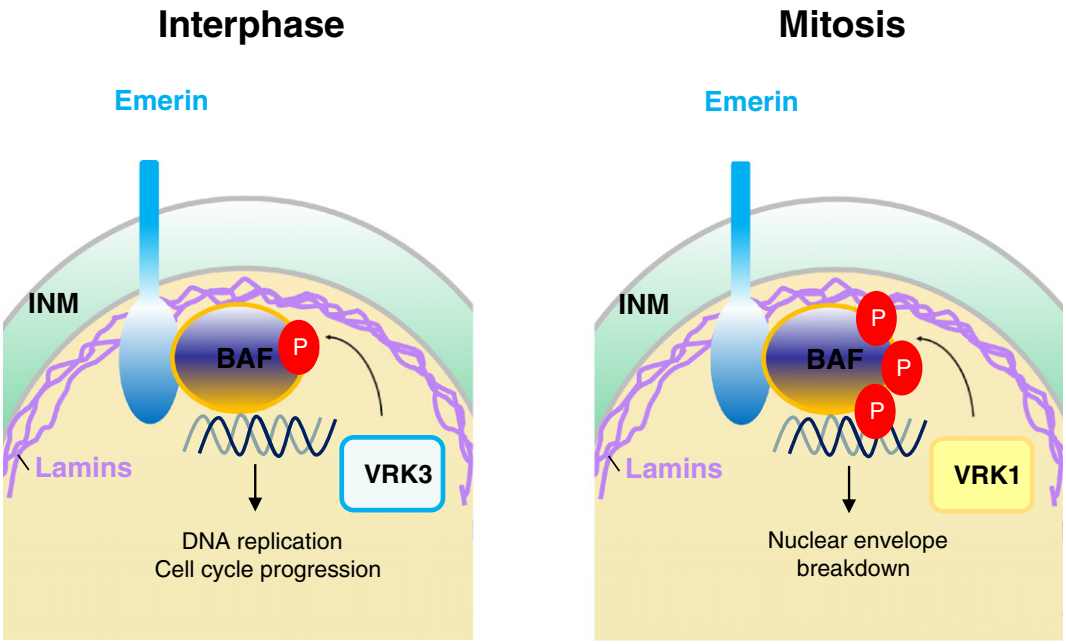


Fig. 6. Hypothetical model. Differential roles of VRK3 and VRK1 in cell cycle regulation.

PP2A or PP4C dephosphorylate BAF [1,44]. This event allows BAF to reassemble with LEM proteins and chromatin at the end of mitosis [9].

As is well known, BAF is robustly phosphorylated on Ser4 by VRK1 during mitosis, yet it is also phosphorylated on Ser4 during interphase [29]. It is not clear why BAF is phosphorylated in interphase as well as in mitosis. One possibility is that BAF must be released from the nuclear envelope and chromatin to facilitate DNA replication. Another possibility is that the interaction of BAF and LEM proteins must be weakened to regulate gene expression associated with emerin and lamin during interphase [2]. In this study, we show that the level of VRK3 is enriched in the G1 phase. Moreover, the overexpression of VRK3 increases the population of proliferating cells, whereas knockdown of VRK3 decreases the population of proliferating cells. These data imply that VRK3 is involved in cell cycle progression during interphase by diminishing the association between BAF and chromatin. In addition, ectopically expressed VRK3 causes the relocalization of nuclear BAF, indicating that VRK3-mediated BAF phosphorylation also releases LEM proteins and subsequently regulates LEM-associated gene expression during interphase.

Collectively, these data suggest that VRK3 mediates BAF hypophosphorylation on Ser4 during interphase, thus promoting DNA replication and regulating gene expression. We thus propose a hypothetical model presenting differential roles for VRK1 and VRK3 in cell cycle regulation (Fig. 6). During mitosis, BAF hyperphosphorylation mediated by VRK1 causes nuclear envelope breakdown required for the progression of mitosis. However, during interphase, BAF hypophosphorylation mediated by VRK3 induces the disassociation of BAF, LEM proteins, and chromatin, thereby contributing to the DNA replication and related gene expression during interphase.

4. Materials and methods

4.1. Antibodies and reagents

Hydroxyurea, thymidine and nocodazole were purchased from Sigma (St. Louis, MO). [γ-^{32}P]ATP was from NEN Life Science Products (Boston, MA). The rabbit polyclonal antibody against BAF was a gift from Dr. Tokuko Haraguchi (Advanced ICT Research Institute Kobe, Japan). The rabbit polyclonal anti-phospho-BAF antibody was a gift from Dr. Robert Craigie (National Institute of Health, Bethesda, MD). Anti-VRK3 (HPA-016040) and anti-FLAG antibody (clone M2) were from Sigma (St. Louis, MO); anti-Lamin B (C-20, SC-6216), anti-emerin (FL-254, SC-15378) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 0411, SC-47724) were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-cyclin B (#4158), anti-cyclin E (#4129), anti-Rb (#9313) and anti-phospho-Rb (#8516) were from Cell Signaling Technology (Danvers, MA); anti-histone H3 (phosphor S10, ab5176) was from Abcam (Cambridge, UK). Antibodies are diluted to 0.2 μg/ml for immunoblotting or 2 μg/ml for immunocytochemical analysis.

4.2. Plasmid construction

Human full-length VRK3 was amplified by PCR from HeLa cell cDNA. For mammalian expression constructs, VRK3 coding region was cloned into pFLAG-CMV (Sigma) vector. Kinase-dead mutant (Lys 203 to Glu) was generated by site-directed mutagenesis and subcloned into pFLAG-CMV (Sigma) vector.

4.3. Preparation of recombinant VRK3 and BAF proteins

Complete VRK3 coding region and the deletion mutant (△N1–147) were cloned into pGEX-4T-3 (Pharmacia Biotech, Uppsala, Sweden). The resulting plasmids were transformed into *Escherichia coli* BL21(DE3) to produce GST tag-VRK3 fusion proteins after treating with 0.1 M isopropyl-1-thio-β-D-galactopyranoside for 24 h at 18 °C. Bacteria were lysed in a phosphate-buffered saline (PBS) containing 1 mM dithiothreitol, 1 mM PMSF, and 1 mM Na_3VO_4. The GST fusion proteins were then purified using glutathione-sepharose resin (Amersham Biosciences, Little Chalfont, UK) and eluted from the beads with reduced glutathione according to the manufacturer's recommendations. The mutant constructs were confirmed by DNA sequencing. Complete BAF coding region is cloned into pProEx HTa (Invitrogen, Carlsbad. CA). His BAF protein was prepared from the insoluble fraction of bacterial cell lysates. BAF containing pellet was extracted under denaturing conditions using guanidine buffer (6 M guanidine-HCl, 150 mM KCl, 20 mM HEPES, pH 7.6, 2 mM β-mercaptoethanol, 0.1 mM EDTA, 5 mM imidazole). After centrifugation to remove any remaining insoluble material, the supernatant is mixed

with Ni-NTA Agarose bead (Invitrogen, Carlsbad. CA). 6× His-tagged BAF protein was purified according to manufacturer's instructions.

4.4. VRK3 in vitro kinase assay

The kinase assay was carried out in a total volume of 30 μl of a kinase reaction buffer containing 50 μM ATP, and affinity-purified GST fusion proteins or 500 μg/ml His-BAF as a substrate for 2 h incubation at 37 °C. The phosphorylated proteins were resolved on 15% Tricine–SDS–polyacrylamide gels [33].

4.5. Cell culture and transfection

HEK293A, HeLa and A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotics in a humidified 5% CO_2 incubator at 37 °C. HEK293A cells were transfected with the plasmid indicated in the specific experiments by using Lipofectamine 2000 (Invitrogen). After incubation for 24 h, the transfected cells were treated as indicated for analysis. The pLL 3.7 LentiLox system (Addgene, Cambridge, MA) was used to generate shRNA. The shRNA sequence targeting VRK3 are 5′-ACTCAA GGCCTGCTGTTTA-3′.

4.6. Immunoblotting and immunoprecipitation

Immunoblotting analysis was performed as we previously described [30,31]. For immunoprecipitation, cell lysates were prepared in lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 1 mM PMSF, 10 μg/ml aprotinin, 10 mM NaF, and 1 mM Na_3VO_4). Equal amounts of proteins were immunoprecipitated using anti-VRK3 or anti-FLAG antibodies and collected with protein G-Sepharose beads (GE healthcare) at 4 °C for 16 h. The immunoprecipitate was then washed four times in cold lysis buffer and subjected to Western blot analysis and VRK3 kinase assay.

4.7. Immunocytochemical analysis

Cells were grown on coated glass coverslips. For immunocytochemistry, cells were fixed with 4% PFA and then incubated with blocking solution (2.5% bovine serum albumin and 2.5% equine serum in PBS) for 1 h at room temperature. The samples were incubated overnight at 4 °C with anti-VRK3 antibody (Sigma), followed by incubation with Alexa546-conjugated anti-rabbit IgG. Slides were mounted and visualized by fluorescence microscopy (Axioplan2, Zeiss; Oberkochen, Germany).

4.8. Cell cycle synchronization and flow cytometry

HeLa cells were synchronized in G1 by treatment for 24 h with 2 mM hydroxyurea, A double thymidine blocking was performed by treating cells 2 mM thymidine for 20 h, released for 6 h in fresh DMEM containing 10% FBS, and treated again with 2 mM thymidine for 12 h. Cells arrested in G1/S were released by switching the medium to fresh DMEM containing 10% FBS. For flow cytometric analysis, the cells were fixed with 70% ethanol for 20 min, stained with 20 μg/ml propidium iodide, and treated with 1 μg/ml RNase A for 10 min at room temperature. Samples containing 10,000 cells were then analyzed on a FACSCalibur system (Becton Dickinson).

4.9. Isolation of VRK3$^{-/-}$ MEFs

VRK3 knockout mice are generated using genetrap ES cell line (YTA189, http//genetrap.org). Wild type and VRK3$^{-/-}$ mouse embryonic fibroblasts (MEFs) were isolated from 12.5 days of embryos. MEFs at passage 2 (p2) were used for growth curves.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

We are grateful to Dr. Tokuko Haraguchi (Advanced ICT Research Institute Kobe, Japan) and Dr. Robert Craigie (National Institute of Health, Bethesda, USA) for providing anti-BAF antibodies and anti-phospho-BAF antibodies, respectively. This work was supported by grants from the Basic Research Laboratory program (No. 2014054324), the Brain Korea 21 plus program (10Z20130012243) through the National Research Foundation (NRF) funded by the Ministry of Education of Korea and the Cooperative Research Program for Agriculture Science & Technology Development (No. PJ01121602) funded by the Rural Development Administration, Republic of Korea.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.04.007.

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