Scientific Research Publishing

Journal of Biosciences and Medicines, 2024, 12, 60-75
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

The NORE1A/RASSF5 Tumor Suppressor Forms a Complex with GSK-3β to Regulate β-Catenin

M. Lee Schmidt[1,2], Howard Donninger[3,4], Geoffrey J. Clark[1,4*]

[1]Department Pharmacology and Toxicology, University of Louisville, Louisville, USA
[2]US Worldmeds, Louisville, USA
[3]Department of Medicine, University of Louisville, Louisville, USA
[4]Experimental Therapeutics Program, James Graham Brown Cancer Center, University of Louisville, Louisville, USA
Email: *geoff.clark@louisville.edu

How to cite this paper: Schmidt, M.L., Donninger, H. and Clark, G.J. (2024) The NORE1A/RASSF5 Tumor Suppressor Forms a Complex with GSK-3β to Regulate β-Catenin. *Journal of Biosciences and Medicines*, **12**, 60-75.
https://doi.org/10.4236/jbm.2024.128006

Received: June 28, 2024
Accepted: August 5, 2024
Published: August 8, 2024

Abstract

NORE1A (RASSF5) is a tumor suppressor of the RASSF family that is often down-regulated in human tumors. NORE1A has multiple roles in controlling cellular homeostasis, one of them being regulating levels of β-catenin by binding and modulating the ubiquitin ligase substrate recognition factor β-TrCP. β-catenin is a major executor of the Wnt pathway. The ubiquitin SCF-$^{\beta\text{-TrCP}}$ ligase complex acts on a phospho-degron site in β-catenin that can be phosphorylated by GSK-3β. We now show that in addition to binding β-TrCP, NORE1A also promotes the phosphorylation of the β-catenin phospho-degron by complexing with the kinase GSK-3β. Indeed, NORE1A enhances the formation of a GSK-3β/β-TrCP complex. A structural mutant of NORE1A that retains β-TrCP binding but will no longer interact with GSK-3β inhibits the β-catenin degrading action of NORE1A. The GSK-3β interaction with NORE1A plays an important role in the biology of NORE1A as a GSK-3β inhibitor blocks NORE1A induced senescence. Thus, we identify a new role for the tumor suppressor NORE1A: The regulation of GSK-3β. GSK-3β has many other substrates including multiple transcription factors and co-activators such as p53 and the Hippo component TAZ. The work implies that NORE1A may be able to influence all of them via this new kinase scaffolding interaction.

Keywords

RAS, NORE1A, RASSF5, GSK-3β, Beta Catenin, HIPPO

1. Introduction

NORE1A (Novel Ras Effector 1 or RASSF5) is a member of the RASSF family of

tumor suppressors [1] [2]. In human tumors, NORE1A expression is frequently down-regulated as a result of promoter hyper-methylation or Calpain-mediated proteolysis [3] [4]. Additionally, loss of NORE1A via a genetic translocation results in a familial human cancer syndrome [5]. Studies on NORE1A *in vitro* show that exogenous expression of NORE1A can promote apoptosis, cell cycle arrest or senescence [3] [6]-[9]. In human tumor cell lines deficient for NORE1A expression, the restoration of endogenous levels of NORE1A effectively blocks the tumorigenic phenotype [3] [5].

Similarly to other members of the RASSF family, NORE1A contains a Ras Association (RA) domain and can bind directly to the RAS oncoprotein [10] [11]. Although NORE1A has been confirmed to be a major Ras senescence/apoptosis effector, the mechanisms utilized remain only partially characterized.

NORE1A directly binds the MST kinases and modulates the Hippo pathway [12] [13]. This is a kinase cascade terminating in the phosphorylation and regulation of the oncogenic transcriptional co-activators YAP and TAZ [14] [15]. However, we have previously shown that it also regulates β-catenin [16]. β-catenin is a key component of the Wnt pathway. Signaling by the Wnt family of proteins to modulate β-catenin is one of the core mechanisms that coordinate cell proliferation, cell polarity, and tissue homeostasis [17] [18].

In the canonical Wnt/β-catenin pathway, β-catenin is the terminal executor, serving as both a nuclear transcriptional co-regulator and a key component of adherens junctions [19]-[21]. In the absence of Wnt ligand signaling, a multi-protein complex consisting of APC, Axin, and GSK-3β, phosphorylates β-catenin. This phosphorylation is necessary for the binding of β-TrCP, the substrate recognition component of the SCF-$^{\beta\text{-TrCP}}$ ubiquitin ligase complex [22] [23]. SCF-$^{\beta\text{-TrCP}}$-mediated ubiquitination of β-catenin results in its rapid degradation by the 26S proteasome [24]. Upon Wnt signaling, the phosphorylation complex is destabilized by Disheveled family proteins allowing for unphosphorylated β-catenin levels to quickly increase in the cytoplasm and translocate to the nucleus. Nuclear β-catenin functions as a cofactor for transcription factors of the TCF/LEF family, modulating genes involved in growth and survival [19].

While cells constitutively synthesize β-catenin to maintain a ready response to incoming Wnt signaling, the high turnover rate of β-catenin via the SCF-$^{\beta\text{-TrCP}}$ ubiquitin ligase complex maintains homeostasis [22]. Corruption in the regulatory mechanism of β-catenin has been described in many human cancers and mutated forms of β-catenin, which cannot be ubiquitinated by the SCF-$^{\beta\text{-TrCP}}$ ubiquitin ligase, are oncogenic [25]. By enhancing down-regulation of β-catenin, β-TrCP can serve as a tumor suppressor [18].

We now show that in addition to binding to β-TrCP, NORE1A also forms an endogenous complex with GSK-3β. This allows NORE1A to promote the phosphorylation of the β-catenin phospho-degron by GSK-3β. Identification of a triple point mutant of NORE1A that binds β-TRCP but not GSK-3β allowed us to

determine that the interaction with GSK-3β is essential for NORE1A to modulate β-catenin. Thus, we identify a new signaling partner for NORE1A, GSK-3β, and show that NORE1A scaffolds the kinase and the ubiquitin ligase complex that is required to promote β-catenin degradation and modulation of the Wnt pathway. This may provide a mechanism by which Hippo pathway and Wnt pathway activity can be coordinated by NORE1A.

2. Materials and Methods

2.1. Molecular Biology

β-TrCP1 expression construct was obtained from Addgene (#4489) and sub-cloned into pEGFP-C1 using BamHI and SalI. Full-length human NORE1A cDNA was obtained from Origene (Rockville MD), amplified and subcloned using BglII/EcoRI into a BamHI/EcoRI restriction digest of pCDNA3 (Invitrogen) containing an in-frame 5' HA tag. We developed a triple point mutant of NORE1A mutating the 92 - 94 residues from Arg to Ala using a PCR based approach. The mutant was subjected to full sequencing to confirm fidelity before use. Activated RAS plasmid has been described previously [26]. We obtained the HA-GSK-3β plasmids from Addgene (#14753 and #49491).

2.2. Antibodies

Anti-GFP (Santa Cruz Biotechnology SC-9996), Anti-HA (Covance MMS-101P), Anti-Flag (Sigma-Aldrich #F1804), Anti-β-TrCP (Cell Signaling Technology #4394), Anti-β-catenin (Cell Signaling Technology #8814), Anti-Phospho-β-catenin (Cell Signaling Technology #9561), Rabbit polyclonal Anti-NORE1A (ProSci PAS#71), anti-GSK-3β (Cell Signaling Technology #27C10), anti-NORE1A (Pro-Sci [27]), anti-β-actin (Sigma Aldrich Cat# A5441) and secondary antibodies were from Amersham.

2.3. Cell Culture and Transfections

Cells were obtained from the ATCC (Manassas VA). HEK-293 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). NCI-H1299 and A549 cells were grown in RPMI with 10% FBS. MCF-10A cells were grown in 50/50 DMEM/HAMSF12 medium supplemented with 5% horse serum, 10 ug/ml insulin, 10 ng/ml EGF and hydrocortisone at 0.5 ug/ml. Cells were transfected using jetPRIME® (Polyplus) transfection reagent according to the manufacturers' protocol with 1 ug of plasmid DNA. In the β-catenin stability studies, 24 hours post-transfection, the cells were trypsinized and split into two groups and allowed to rest for an additional 24 hours at 37 degrees in a tissue culture incubator. One group was then treated with the proteasome inhibitor MG132 (Sigma-Aldrich, St. Louis MO) at a final concentration of 5 μM or DMSO and placed back in the incubator for 5 hours. The cells were then lysed and immunoblotted (IB) for levels of β-catenin.

2.4. Immunoprecipitation and Western Blotting

Cellular lysates for immunoprecipitation were prepared using a modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1% NP-40). Precleared lysate was incubated with GFP-Trap® beads (Allele Biotech, BG) or with primary antibody or control IgG followed by protein A/G beads (eBioscience) and was washed with lysis buffer. Proteins were run on a 4% - 15% Tris-Glycine gel (Bio-Rad) and transferred to 0.2 μm Nitrocellulose (Bio-Rad). Blots were developed using West Pico Enhanced ECL (Pierce Biotechnology) or West Femto Enhanced ECL (Pierce).

2.5. Senescence Assays

Senescence assays were performed using a Senescence Detection Kit manufactured and distributed by BioVision, Lilipitas, CA, (Catalog#K320-250).

2.6. Statistical Analysis

GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA) was used to evaluate statistical significance in the senescence assays by Student's *t*-tests. Values of $p < 0.05$ were considered significant. Data are expressed as mean ± SD for each group.

3. Results

3.1. NORE1A Promotes the Phosphorylation of *β*-Catenin via GSK-*β*

We previously showed that NORE1A can negatively regulate *β*-catenin protein levels by interacting with the E3 ubiquitin ligase substrate recognition sub-unit, *β*-TrCP and promoting *β*-catenin degradation [16]. *β*-catenin is regulated by a dual-kinase system consisting of CK1 and GSK-3*β*, the latter actually phosphorylates a phospho-degron sequence (amino acids Thr41, Ser37, and Ser33) on *β*-catenin that is required for recognition and ubiquitination by *β*-TrCP/ubiquitin ligase complex [28]. As RASSF family proteins have been shown to be scaffolding proteins [1], we wondered if NORE1A might play a larger role in the process and also affect the phosphorylation of *β*-catenin by GSK-3*β*.

The NCI-H1299 lung tumor cell line has lost expression of NORE1A. Previously we generated a NORE1A plus/minus cell system by stably transfecting a NORE1A expression plasmid or the empty vector into the cells [16]. We transiently transfected the cells with a *β*-catenin expression construct and examined the phosphorylation status of the *β*-catenin protein using an antibody against specific GSK-3*β* phosphorylated residues. In the lysates from cells expressing NORE1A, there was a substantial increase in phosphorylated *β*-catenin (Ser33/37/Thr41) when compared to lysates expressing empty pZIP-HA-vector (**Figure 1(a)**). As the phosphorylated form of *β*–catenin is rapidly degraded by the proteasome, we used pre-treatment with low levels of the proteasome inhibitor MG132 to stabilize the protein to visualize the phosphorylation.

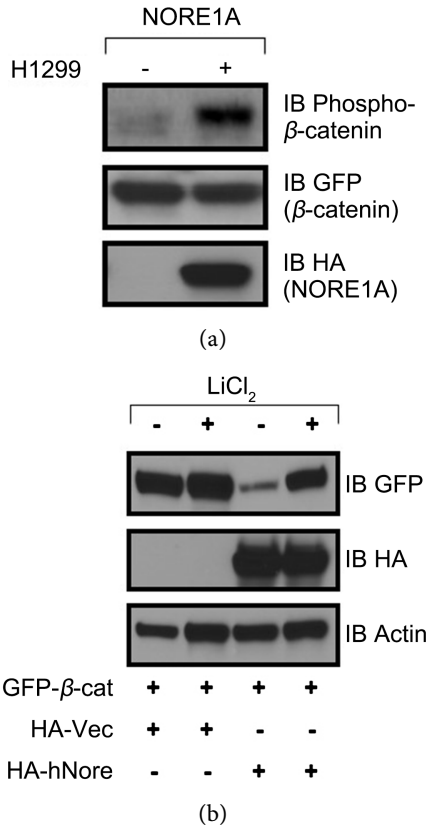

Figure 1. NORE1A Enhances β-catenin Phosphorylation on GSK-3β Target Residues. (a) H1299 cells, null for NORE1A expression, were transfected with either pZIP vector or pZIP-HA-NORE1A. Transformants were selected using G418 yielding a stable population that is either +/− for NORE1A expression. Cells were transfected with GFP-β-catenin. They were then lysed and analyzed on a Western blot for phosphorylated β-catenin. In the cells expressing NORE1A, there is a marked increase in levels of phosphorylated endogenous β-catenin on GSK-3β target residues. Total GFP-β-catenin levels are shown as the control. (b) HEK-293 cells were transfected with expression constructs expressing NORE1A and β-catenin. 24 hours post-transfection, the cells were treated with $LiCl_2$ for another 24 hours. $LiCl_2$ is known to be an effective inhibitor of GSK-3β kinase activity. In the cells treated with $LiCl_2$, NORE1A mediated down-regulation of β-catenin is impaired. These results suggest that NORE1A is regulating β-catenin protein levels by enhancing GSK-3β kinase activity.

To determine if NORE1A is using GSK-3β or another kinase to phosphorylate and drive β-catenin degradation, we used Lithium Chloride to specifically inhibit GSK-3β phosphorylation activity [29]. HEK-293 cells were transfected with expression constructs expressing β-catenin paired with either an empty vector or HA-NORE1A. As expected, in the presence of NORE1A, levels of β-catenin are difficult to detect However, when the GSK-3β inhibitor $LiCl_2$ is added to the cell media for 24 hours, NORE1A loses the ability to suppress β-catenin protein levels (**Figure 1(b)**). NORE1A can bind several cellular kinases, including HIPK2 [30]. HIPK2 is known to be able to regulate β-catenin as well [31]. However, $LiCl_2$ is considered fairly specific for GSK-3β [32] and HIPK2 dominant negatives do not affect β-catenin levels [33].

3.2. NORE1A Forms an Endogenous Complex with GSK-3β

As NORE1A appeared to be modulating the phosphorylation of β-catenin on a GSK-3β phosphorylation site, we sought to determine if NORE1A was interacting with GSK-3β. First, HEK293 cells were transfected with expression constructs expressing HA-GSK-3β and GFP-NORE1A along with an activated H-Ras (12Val). Cells were lysed 24 hours post-transfection and immunoprecipitated against the GFP-epitope tag. NORE1A was found in complex with GSK-3β. However, unlike the case with β-TrCP [16], this interaction did not seem to be affected by the presence of activated Ras (Figure 2(a)). To determine if this result was physiological, MCF-10A cells were lysed and an antibody against endogenous GSK-3β was used to immune-precipitate GSK-3β. Western analysis of the IP with a NORE1A antibody showed that the two proteins were in an endogenous complex (Figure 2(b)).

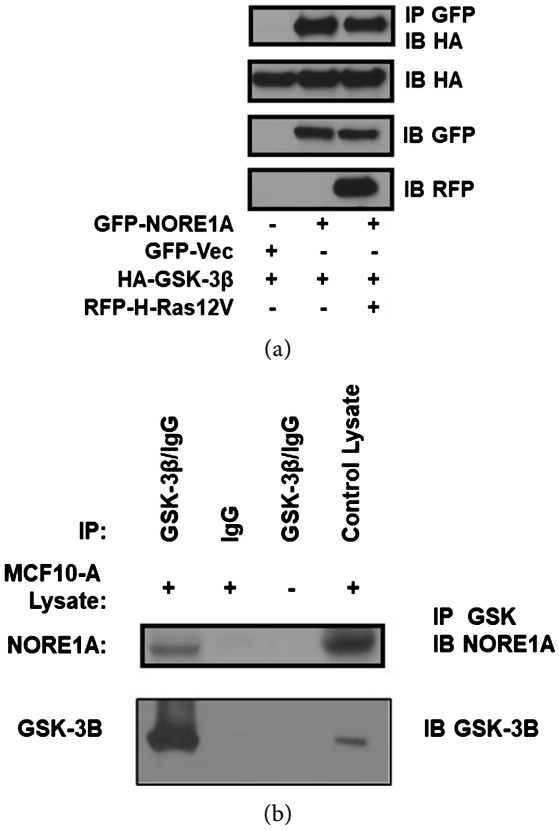

Figure 2. NORE1A forms an endogenous complex with GSK-3β. (a) HEK-293 cells were transfected with protein expression constructs expressing HA-GSK-3β along with an empty vector or GFP-NORE1A in the presence or absence of activated H-RAS (12Val). 24 hours post-transfection, the cells were lysed and the purified lysates were immunoprecipitated using GFP-agarose beads. The immunoprecipitation was analyzed by Western blot. (b) MCF-10A cells were lysed and the purified lysate was separated into equal batches before immunoprecipitating using an antibody against GSK-3β. IgG beads and antibody/IgG beads serve as controls. When the immunoprecipitation was analyzed by Western blot, NORE1A was detected in an endogenous complex with GSK-3β. The blot was then probed with GSK-3β as a control (lower panel).

3.3. NORE1A Functions as a Scaffold Stabilizing the GSK-3β/β-TrCP Protein Degradation Complex

NORE1A has no apparent catalytic activity and appears to function as a scaffold protein. It directly binds β-TrCP [16], and so could be scaffolding GSK-3β and β-TrCP into a complex, allowing for efficient phosphorylation and ubiquitination of target substrates, such as β-catenin. To investigate this, we transfected HEK293 cells with expression constructs for β-TrCP and GSK-3β in the presence or absence of NORE1A. We then performed an immunoprecipitation for GSK-3β and measured the levels of β-TrCP in the IP. We detected a weak interaction between β-TrCP and GSK-3β (**Figure 3**). However, when NORE1A expression was added to the experiment, the interaction between β-TrCP and GSK-3β was significantly increased (**Figure 3**). Therefore, we provide the first evidence that NORE1A does scaffold β-TrCP and GSK-3β into a complex, explaining why we observe such a potent effect on β-catenin protein stability.

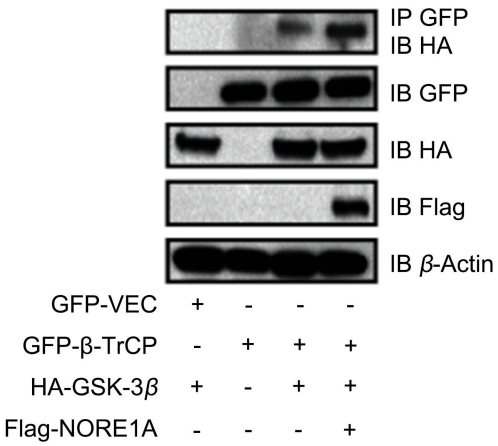

Figure 3. NORE1A Scaffolds GSK-3β to β-TrCP - HEK-293 cells were transfected with expression constructs for GSK-3β, β-TrCP, and NORE1A. 24 hours post-transfection, cells were lysed and immunoprecipitated. GSK-3β and β-TrCP were detected weakly in a complex. However, in the presence of NORE1A, the association between GSK-3β and β-TrCP was enhanced.

3.4. Identification of a NORE1A Mutant Deficient for Binding GSK-3β Demonstrates the Importance of the Interaction for β-Catenin

To further support the Lithium Chloride data, a triple point mutant of NORE1A was developed mutating residues 92 - 94 from Arg to Ala. HEK-293 cells were transfected with expression constructs for GSK-3β paired with empty vector, NORE1A, and the triple point mutant (NORE1A 92-94A mutant). 24 hours post-transfection, the cells were lysed and the purified cell lysate was immune-precipitated using GFP-conjugated agarose beads. The assay was then analyzed on a Western blot where GSK-3β was found to complex strongly with NORE1A; however, GSK-3β failed to complex with the mutant NORE1A (**Figure 4(a)**). When we examined the interaction of β-TrCP, we found that the

NORE1A point mutant still bound (Figure 4(b)). We then examined the effects of the GSK-3β binding mutant on β-catenin. HEK-293 cells were transfected with expression constructs expressing either β-catenin and subsequently paired with an empty vector, wild-type NORE1A, or the mutant NORE1A 92 - 94A. Results show the wild-type NORE1A suppressed β-catenin protein levels, while the mutant NORE1A (92-94A) was unable to do so (Figure 4(c)). This is in agreement with the $LiCl_2$ experiment and again supports the idea that NORE1A is indeed coordinating GSK-3β to regulate β-catenin.

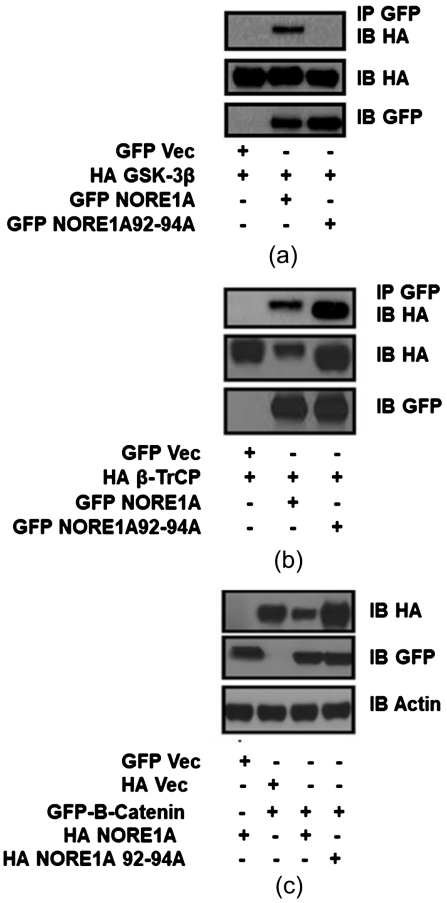

Figure 4. A NORE1A Point Mutant that Fails to Bind GSK-3β fails to suppress β-catenin. (a) Deletion mutagenesis suggested that potential GSK-3β binding region rested in the 92 - 94 amino acid residues of NORE1A. These residues were mutated to Alanine residues via PCR mutagenesis. Then HEK-293 cells were transfected with expression constructs for GSK-3β and either wild-type NORE1A or the mutant NORE1A (92 - 94 A). Cells were lysed 24 hours post-transfection and immunoprecipitated. Wild-type NORE1A was found in complex with GSK-3β. However, the mutant NORE1A (92 - 94 A) failed to bind GSK-3β. (b) To ensure the point mutant had not also lost the ability to interact with β-TrCP we performed similar over expression/pull down studies in HEK-293 cells using a GFP tagged form of β-TrCP. The NORE1A mutant retained binding, indeed, the binding appeared to increase somewhat. (c) HEK293 cells were co-transfected with expression constructs for β-catenin in the presence or absence of wild-type or mutant NORE1A. The levels of β-catenin protein expression were measured by Western blot. Only the wild-type NORE1A suppressed the expression of β-catenin.

3.5. NORE1A Requires GSK-3β Activity to Induce Cellular Senescence

In primary mammalian cells, the expression of oncogenes, such as activated RAS, induces premature senescence rather than transformation [34]. Oncogene Induced Senescence (OIS) can be bypassed by a number of different genetic events including inactivation of the p53/p21/p19ARF pathway, inactivation of the Rb family of proteins, and aberrations in p53, PML, SIR2, KLF4, and YAP/TAZ [34] [35]. Recent research has now identified GSK-3β as a key player in the activation of cellular senescence [34] [36] [37]. These reports demonstrate that loss GSK-3β expression is a vital factor that determines whether a cell transforms or becomes senescent in the presence of constitutively active RAS [34]. Exactly how GSK-3β functions in RAS-mediated senescence is not clear, and likely occurs on multiple levels including regulation of β-catenin, p53, and potentially partial suppression of TEAD genes [34] [35] [38]. NORE1A is a powerful RAS senescence effector that functions through both p53 and Rb pathways [27] [39]. As we had found that NORE1A binds to GSK-3β, we sought to determine whether NORE1A may also use GSK-3β to shift cells into a senescent phenotype. To achieve this, we used A549 lung tumor cells (mutant RAS containing) and transiently transfected them with either an empty vector or an expression construct expressing a GFP-tagged NORE1A. 6 hours post-transfection the cells were then treated with $LiCl_2$ or water, the carrier solution, at a final concentration of 10 mM for 72 hours. The cells were then processed using a β-gal senescence staining kit. The number of positive blue cells for each experimental group was counted and quantified. Results show that NORE1A strongly induced a senescent phenotype in the A549 cells and this effect was suppressed by the $LiCl_2$, suggesting that NORE1A is using GSK-3β in part as a senescence effector (**Figure 5**).

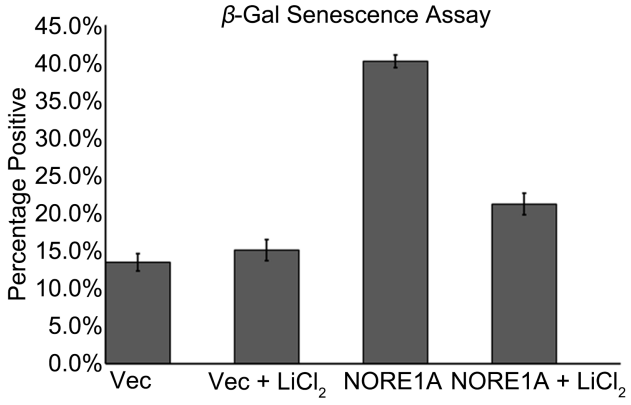

Figure 5. The GSK-3β inhibitor Lithium Chloride Blocks the Ability of NORE1A to Induce Senescence. A549 cells were transfected with vector or NORE1A expression construct. 6-hours post-transfection, the cells were treated with LiCl2 at a final concentration of 10 mM for 72 hours. The cells were then processed for β-gal activity using a senescence detection kit. The cells were then counted and the results were quantified. As expected, NORE1A strongly shifted cells into senescence ($p = 0.05$). This effect was impaired in the presence of $LiCl_2$, supporting the hypothesis that NORE1A is working with GSK-3β to activate senescence.

4. Discussion

NORE1A is a RASSF family member that serves as a direct, pro-apoptotic and senescent effector of the RAS oncoprotein [5]-[8] [10] [30] [40]. It is frequently down-regulated in human tumors both at an epigenetic and at a protein level [3] [4]. Therefore, NORE1A is likely to serve as an important human tumor suppressor in human cancer. Like other RASSF family members, NORE1A binds the MST kinases directly, and thus has the potential to connect RAS to the Hippo pathway [12]. However, deletion mutants of NORE1A that cannot interact with MST kinases retain the ability to inhibit cell growth and suppress the tumorigenic phenotype [41]. Thus, other tumor suppressor pathways independent of the canonical Hippo signaling pathway must be modulated by NORE1A.

One MST-independent function of NORE1A may be the modulation of the ubiquitin proteosome system. The ubiquitin-proteasome system controls the degradation of the majority of regulatory eukaryotic proteins, including proteins that play a key role in tumorigenesis, like β-catenin, IκB, YAP, and TAZ [42]. E3 ubiquitin ligases determine the timing and specificity of ubiquitination for substrates and typically target proteins with specific post-translational modifications like phosphorylation or hydroxylation [43]. We previously showed that NORE1A forms a direct, RAS regulated, endogenous complex with β-TrCP [16]. β-TrCP is the E3 substrate recognition component for the SCF-$^{\beta\text{-TrCP}}$ ubiquitin ligase complex, and is a key element in the regulation of both Wnt and Hippo pathways [44]. We found that RAS can use NORE1A to promote the degradation of β-catenin by directly binding β-TrCP [16]. β-catenin is the terminal executor of the Wnt signaling pathway and must be phosphorylated before it is recognized by β-TrCP [22] [23].

One of the major kinases that phosphorylates the phospho-degron of β-catenin is GSK-3β [28]. This is a serine/threonine kinase that is an important component of diverse signaling pathways involved in the regulation of cell survival, protein synthesis, glycogen metabolism, cell mobility, and proliferation [45] [46]. It has been reported to have both tumor promoting and tumor suppressing effects in different circumstances [47] [48]. Unlike many other protein kinases in the cell, GSK-3β has constitutively active kinase activity [49]. Thus, control of GSK-3β function may rely on targeting proteins, like Axin or APC, that control its coupling to the appropriate substrate. Here we show that NORE1A is likely one of these targeting proteins, as it can complex with the kinase and when we used a GSK-3β inhibitor, or a mutant that does not complex with GSK-3β, we blocked the ability of NORE1A to suppress β-catenin protein. The interaction of NORE1A and GSK-3β may be indirect, as we have never seen the kinase appearing in any of our NORE1A two-hybrid screens. However, we have detected NORE1A in a direct complex with both cdc37 and TRIM25, both of which have been reported to be binding partners of GSK-3β in the BioGRID database. So there are multiple potential mechanisms by which NORE1A could modulate the kinase. (**Figure 6**)

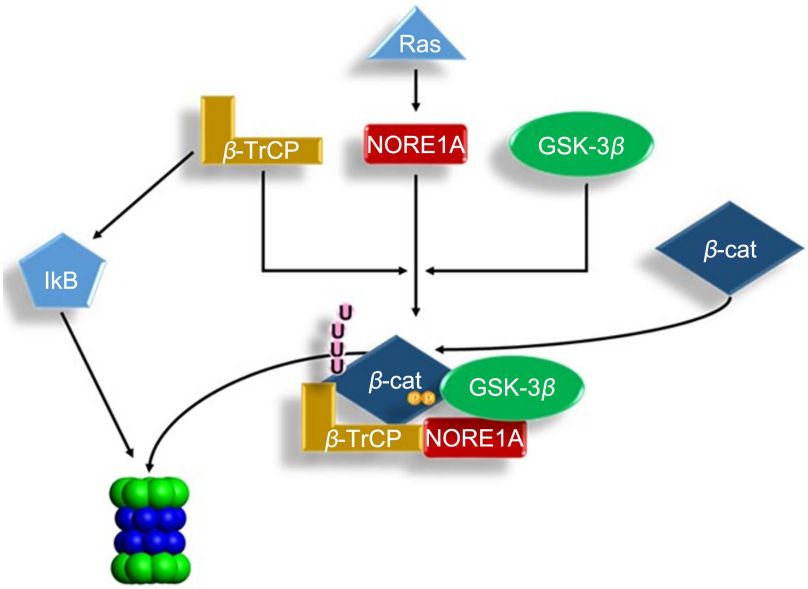

Figure 6. Proposed NORE1A/GSK-3β/β-TrCP Signaling Complex—The schematic, depicts how we hypothesize NORE1A to function as a scaffolding molecule in this protein regulatory complex for β-catenin in contrast to other β-TrCP binding substrates such as IkB.

By identifying NORE1A as a scaffold molecule for the GSK-3β kinase complex, we link NORE1A to the potential control of a broad range of targets in addition to β-catenin, including p53, Snail, Smad1, Smad3, BCL-3, p21CIP1, HIF-1α, and Cyclin D1 [50]. GSK-3β can also phosphorylate the Hippo pathway component TAZ, resulting in its proteosomal degradation [51]. So the NORE1A/GSK-3β connection provides a potential MST independent mechanism by which NORE1A could regulate Hippo.

Moreover, in addition to the SCF-$^{\beta\text{-TrCP}}$ Ubiquitin ligase complex, NORE1A can also modulate the ITCH ubiquitin ligase system [52]. ITCH can also impact components of the Wnt pathway upstream of β-catenin [52] and the Hippo pathway component LATS [53]. So the role of NORE1A in regulating the proteosome and in Hippo/Wnt signaling and coordination has the potential to be quite complex.

NORE1A is a RAS effector and could serve as a connection between RAS and GSK-3β. However, although we found that the interaction of NORE1A with β-TrCP was RAS dependent, [16], its interaction with GSK-3β was not. Therefore, NORE1A may have RAS dependent and RAS independent effects on GSK-3β activity or targeting.

NORE1A is a potent senescence inducer [54]. Indeed, senescence induction rather than apoptosis induction seems to be its major physiological activity [3]. NORE1A acts, in part, by scaffolding the kinase HIPK2 to the key tumor suppressor p53 [27]. Here, we show that full NORE1A induced senescence is dependent upon GSK-3β activation as well. GSK-3β can indirectly regulate p53 via mdm2 regulation [55] and Hippo pathway components LATS1 and 2 kinases

can also modulate p53 [56]. Thus, NORE1A potentially has at least three mechanisms by which it can impact p53 activity, HIPK2 [27], Hippo pathway and now GSK-3β.

Lithium chloride has the potential to have non-GSK-3β dependent effects and the NORE1A point mutant may lose binding to other proteins in addition to GSK-3β. Moreover, over-expressing scaffolding proteins can give misleading results due to the requirements of stoichiometry for their function. Further studies using knockout strategies and more precise inhibitors of GSK-3β may strengthen the conclusions.

Acknowledgements

MLS performed experimental work and writing the paper, HD assisted with writing the paper and performing experiments, GJC conceived the concept, supervised the work and assisted in writing the manuscript.

The work was funded by R01 CA133171-01A2 to GJC.

Conflicts of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

References

[1] Donninger, H., Schmidt, M.L., Mezzanotte, J., Barnoud, T. and Clark, G.J. (2016) Ras Signaling through RASSF Proteins. *Seminars in Cell & Developmental Biology*, **58**, 86-95. https://doi.org/10.1016/j.semcdb.2016.06.007

[2] van der Weyden, L. and Adams, D.J. (2007) The Ras-Association Domain Family (RASSF) Members and Their Role in Human Tumourigenesis. *Biochimica et Biophysica Acta* (*BBA*)—*Reviews on Cancer*, **1776**, 58-85. https://doi.org/10.1016/j.bbcan.2007.06.003

[3] Calvisi, D.F., Donninger, H., Vos, M.D., Birrer, M.J., Gordon, L., Leaner, V., *et al.* (2009) NORE1A Tumor Suppressor Candidate Modulates P21CIP1 via P53. *Cancer Research*, **69**, 4629-4637. https://doi.org/10.1158/0008-5472.can-08-3672

[4] Kuznetsov, S. and Khokhlatchev, A.V. (2008) The Growth and Tumor Suppressors NORE1A and RASSF1A Are Targets for Calpain-Mediated Proteolysis. *PLOS ONE*, **3**, e3997. https://doi.org/10.1371/journal.pone.0003997

[5] Chen, J., Lui, W., Vos, M.D., Clark, G.J., Takahashi, M., Schoumans, J., *et al.* (2003) The T (1; 3) Breakpoint-Spanning Genes LSAMP and NORE1 Are Involved in Clear Cell Renal Cell Carcinomas. *Cancer Cell*, **4**, 405-413. https://doi.org/10.1016/s1535-6108(03)00269-1

[6] Donninger, H., Barnoud, T. and Clark, G.J. (2016) NORE1A Is a Double Barreled Ras Senescence Effector That Activates P53 and Rb. *Cell Cycle*, **15**, 2263-2264. https://doi.org/10.1080/15384101.2016.1152431

[7] Geli, J., Kogner, P., Lanner, F., Natalishvili, N., Juhlin, C., Kiss, N., *et al.* (2008) Assessment of *nore1a* as a Putative Tumor Suppressor in Human Neuroblastoma. *International Journal of Cancer*, **123**, 389-394. https://doi.org/10.1002/ijc.23533

[8] Park, J., Kang, S.I., Lee, S., Zhang, X.F., Kim, M.S., Beers, L.F., *et al.* (2010) Tumor Suppressor Ras Association Domain Family 5 (RASSF5/NORE1) Mediates Death

Receptor Ligand-Induced Apoptosis. *Journal of Biological Chemistry*, **285**, 35029-35038. https://doi.org/10.1074/jbc.m110.165506

[9] Avruch, J., Xavier, R., Bardeesy, N., Zhang, X., Praskova, M., Zhou, D., *et al.* (2009) Rassf Family of Tumor Suppressor Polypeptides. *Journal of Biological Chemistry*, **284**, 11001-11005. https://doi.org/10.1074/jbc.r800073200

[10] Khokhlatchev, A., Rabizadeh, S., Xavier, R., Nedwidek, M., Chen, T., Zhang, X., *et al.* (2002) Identification of a Novel Ras-Regulated Proapoptotic Pathway. *Current Biology*, **12**, 253-265. https://doi.org/10.1016/s0960-9822(02)00683-8

[11] Vos, M.D., Martinez, A., Ellis, C.A., Vallecorsa, T. and Clark, G.J. (2003) The Pro-Apoptotic Ras Effector Nore1 May Serve as a Ras-Regulated Tumor Suppressor in the Lung. *Journal of Biological Chemistry*, **278**, 21938-21943. https://doi.org/10.1074/jbc.m211019200

[12] Avruch, J., Praskova, M., Ortiz-Vega, S., Liu, M. and Zhang, X. (2006) Nore1 and RASSF1 Regulation of Cell Proliferation and of the MST1/2 Kinases. *Methods in Enzymology*, **407**, 290-310. https://doi.org/10.1016/s0076-6879(05)07025-4

[13] Liao, T., Jang, H., Tsai, C., Fushman, D. and Nussinov, R. (2017) The Dynamic Mechanism of RASSF5 and MST Kinase Activation by Ras. *Physical Chemistry Chemical Physics*, **19**, 6470-6480. https://doi.org/10.1039/c6cp08596b

[14] Zhu, N., Yang, R., Wang, X., Yuan, L., Li, X., Wei, F., *et al.* (2023) The Hippo Signaling Pathway: From Multiple Signals to the Hallmarks of Cancers. *Acta Biochimica et Biophysica Sinica*, **55**, 904-913. https://doi.org/10.3724/abbs.2023035

[15] Edgar, B.A. (2006) From Cell Structure to Transcription: Hippo Forges a New Path. *Cell*, **124**, 267-273. https://doi.org/10.1016/j.cell.2006.01.005

[16] Schmidt, M.L., Donninger, H. and Clark, G.J. (2014) Ras Regulates SCF$^{\beta\text{-TrCP}}$ Protein Activity and Specificity via Its Effector Protein NORE1A. *Journal of Biological Chemistry*, **289**, 31102-31110. https://doi.org/10.1074/jbc.m114.594283

[17] MacDonald, B.T., Tamai, K. and He, X. (2009) Wnt/β-Catenin Signaling: Components, Mechanisms, and Diseases. *Developmental Cell*, **17**, 9-26. https://doi.org/10.1016/j.devcel.2009.06.016

[18] Liu, J., Xiao, Q., Xiao, J., Niu, C., Li, Y., Zhang, X., *et al.* (2022) Wnt/β-Catenin Signalling: Function, Biological Mechanisms, and Therapeutic Opportunities. *Signal Transduction and Targeted Therapy*, **7**, Article No. 3. https://doi.org/10.1038/s41392-021-00762-6

[19] Giles, R.H., van Es, J.H. and Clevers, H. (2003) Caught up in a Wnt Storm: Wnt Signaling in Cancer. *Biochimica et Biophysica Acta* (*BBA*)—*Reviews on Cancer*, **1653**, 1-24. https://doi.org/10.1016/s0304-419x(03)00005-2

[20] Song, P., Gao, Z., Bao, Y., Chen, L., Huang, Y., Liu, Y., *et al.* (2024) Wnt/β-Catenin Signaling Pathway in Carcinogenesis and Cancer Therapy. *Journal of Hematology & Oncology*, **17**, Article No. 46. https://doi.org/10.1186/s13045-024-01563-4

[21] Taniguchi, K., Moroishi, T., de Jong, P.R., Krawczyk, M., Grebbin, B.M., Luo, H., *et al.* (2017) YAP–IL-6ST Autoregulatory Loop Activated on APC Loss Controls Colonic Tumorigenesis. *Proceedings of the National Academy of Sciences of the United States of America*, **114**, 1643-1648. https://doi.org/10.1073/pnas.1620290114

[22] Bi, Y., Cui, D., Xiong, X. and Zhao, Y. (2020) The Characteristics and Roles of β-TrCP1/2 in Carcinogenesis. *The FEBS Journal*, **288**, 3351-3374. https://doi.org/10.1111/febs.15585

[23] Winston, J.T., Strack, P., Beer-Romero, P., Chu, C.Y., Elledge, S.J. and Harper, J.W. (1999) The SCF$^{\beta\text{-TRCP}}$-Ubiquitin Ligase Complex Associates Specifically with Phosphorylated Destruction Motifs in IκBα and β-Catenin and Stimulates IκBα Ubiqui-

tination *in Vitro*. *Genes & Development*, **13**, 270-283. https://doi.org/10.1101/gad.13.3.270

[24] Orford, K., Crockett, C., Jensen, J.P., Weissman, A.M. and Byers, S.W. (1997) Serine Phosphorylation-Regulated Ubiquitination and Degradation of B-Catenin. *Journal of Biological Chemistry*, **272**, 24735-24738. https://doi.org/10.1074/jbc.272.40.24735

[25] Bugter, J.M., Fenderico, N. and Maurice, M.M. (2020) Mutations and Mechanisms of WNT Pathway Tumour Suppressors in Cancer. *Nature Reviews Cancer*, **21**, 5-21. https://doi.org/10.1038/s41568-020-00307-z

[26] Vos, M.D., Ellis, C.A., Elam, C., Ülkü, A.S., Taylor, B.J. and Clark, G.J. (2003) RASSF2 Is a Novel K-Ras-Specific Effector and Potential Tumor Suppressor. *Journal of Biological Chemistry*, **278**, 28045-28051. https://doi.org/10.1074/jbc.m300554200

[27] Schmidt M.L., Calvisi D.F. and Clark G.J. (2016) NORE1A Regulates MDM2 via β-TrCP. *Cancers* (*Basel*), **8**, 39. https://pubmed.ncbi.nlm.nih.gov/27023610/

[28] Stamos, J.L. and Weis, W.I. (2012) The *β*-Catenin Destruction Complex. *Cold Spring Harbor Perspectives in Biology*, **5**, a007898. https://doi.org/10.1101/cshperspect.a007898

[29] Noble, W., Planel, E., Zehr, C., Olm, V., Meyerson, J., Suleman, F., *et al.* (2005) Inhibition of Glycogen Synthase Kinase-3 by Lithium Correlates with Reduced Tauopathy and Degeneration *in Vivo*. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 6990-6995. https://doi.org/10.1073/pnas.0500466102

[30] Donninger, H., Calvisi, D.F., Barnoud, T., Clark, J., Schmidt, M.L., Vos, M.D., *et al.* (2015) NORE1A Is a Ras Senescence Effector That Controls the Apoptotic/Senescent Balance of p53 via HIPK2. *Journal of Cell Biology*, **208**, 777-789. https://doi.org/10.1083/jcb.201408087

[31] Kim, E., Kim, J.E., Sung, K.S., Choi, D.W., Lee, B.J. and Choi, C.Y. (2010) Homeodomain-Interacting Protein Kinase 2 (HIPK2) Targets *β*-Catenin for Phosphorylation and Proteasomal Degradation. *Biochemical and Biophysical Research Communications*, **394**, 966-971. https://doi.org/10.1016/j.bbrc.2010.03.099

[32] Davies, S.P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and Mechanism of Action of Some Commonly Used Protein Kinase Inhibitors. *Biochemical Journal*, **351**, 95-105. https://doi.org/10.1042/bj3510095

[33] Hikasa, H. and Sokol, S.Y. (2011) Phosphorylation of TCF Proteins by Homeodomain-Interacting Protein Kinase 2. *Journal of Biological Chemistry*, **286**, 12093-12100. https://doi.org/10.1074/jbc.m110.185280

[34] Liu, S., Fang, X., Hall, H., Yu, S., Smith, D., Lu, Z., *et al.* (2008) Homozygous Deletion of Glycogen Synthase Kinase 3*β* Bypasses Senescence Allowing Ras Transformation of Primary Murine Fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 5248-5253. https://doi.org/10.1073/pnas.0704242105

[35] Xie, Q., Chen, J., Feng, H., Peng, S., Adams, U., Bai, Y., *et al.* (2013) YAP/Tead-Mediated Transcription Controls Cellular Senescence. *Cancer Research*, **73**, 3615-3624. https://doi.org/10.1158/0008-5472.can-12-3793

[36] Kortlever, R.M., Higgins, P.J. and Bernards, R. (2006) Plasminogen Activator Inhibitor-1 Is a Critical Downstream Target of p53 in the Induction of Replicative Senescence. *Nature Cell Biology*, **8**, 877-884. https://doi.org/10.1038/ncb1448

[37] Damalas, A. (2001) Deregulated *β*-Catenin Induces a P53- and ARF-Dependent Growth Arrest and Cooperates with Ras in Transformation. *The EMBO Journal*, **20**,

4912-4922. https://doi.org/10.1093/emboj/20.17.4912

[38] Watcharasit, P., Bijur, G.N., Zmijewski, J.W., Song, L., Zmijewska, A., Chen, X., *et al.* (2002) Direct, Activating Interaction between Glycogen Synthase Kinase-3β and p53 after DNA Damage. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 7951-7955. https://doi.org/10.1073/pnas.122062299

[39] Barnoud, T., Donninger, H. and Clark, G.J. (2016) Ras Regulates Rb via NORE1A. *Journal of Biological Chemistry*, **291**, 3114-3123. https://doi.org/10.1074/jbc.m115.697557

[40] Lee, C.K., Lee, J., Lee, M., Jeong, S., Ha, T., Kang, M., *et al.* (2010) Epigenetic Inactivation of the *NORE*1gene Correlates with Malignant Progression of Colorectal Tumors. *BMC Cancer*, **10**, Article No. 577. https://doi.org/10.1186/1471-2407-10-577

[41] Aoyama, Y., Avruch, J. and Zhang, X. (2004) Nore1 Inhibits Tumor Cell Growth Independent of Ras or the MST1/2 Kinases. *Oncogene*, **23**, 3426-3433. https://doi.org/10.1038/sj.onc.1207486

[42] Pohl, C. and Dikic, I. (2019) Cellular Quality Control by the Ubiquitin-Proteasome System and Autophagy. *Science*, **366**, 818-822. https://doi.org/10.1126/science.aax3769

[43] Zheng, N. and Shabek, N. (2017) Ubiquitin Ligases: Structure, Function, and Regulation. *Annual Review of Biochemistry*, **86**, 129-157. https://doi.org/10.1146/annurev-biochem-060815-014922

[44] Sileo, P., Simonin, C., Melnyk, P., Chartier-Harlin, M. and Cotelle, P. (2022) Crosstalk between the Hippo Pathway and the Wnt Pathway in Huntington's Disease and Other Neurodegenerative Disorders. *Cells*, **11**, Article 3631. https://doi.org/10.3390/cells11223631

[45] Beurel, E., Grieco, S.F. and Jope, R.S. (2015) Glycogen Synthase Kinase-3 (GSK3): Regulation, Actions, and Diseases. *Pharmacology & Therapeutics*, **148**, 114-131. https://doi.org/10.1016/j.pharmthera.2014.11.016

[46] Wang, L., Li, J. and Di, L. (2021) Glycogen Synthesis and Beyond, a Comprehensive Review of GSK3 as a Key Regulator of Metabolic Pathways and a Therapeutic Target for Treating Metabolic Diseases. *Medicinal Research Reviews*, **42**, 946-982. https://doi.org/10.1002/med.21867

[47] He, R., Du, S., Lei, T., Xie, X. and Wang, Y. (2020) Glycogen Synthase Kinase 3β in Tumorigenesis and Oncotherapy (Review). *Oncology Reports*, **44**, 2373-2385. https://doi.org/10.3892/or.2020.7817

[48] Jacobs, K.M., Bhave, S.R., Ferraro, D.J., Jaboin, J.J., Hallahan, D.E. and Thotala, D. (2012) GSK-3: A Bifunctional Role in Cell Death Pathways. *International Journal of Cell Biology*, **2012**, Article ID: 930710. https://doi.org/10.1155/2012/930710

[49] Doble, B.W. and Woodgett, J.R. (2003) GSK-3: Tricks of the Trade for a Multi-Tasking Kinase. *Journal of Cell Science*, **116**, 1175-1186. https://doi.org/10.1242/jcs.00384

[50] Domoto, T., Uehara, M., Bolidong, D. and Minamoto, T. (2020) Glycogen Synthase Kinase 3β in Cancer Biology and Treatment. *Cells*, **9**, Article 1388. https://doi.org/10.3390/cells9061388

[51] Huang, W., Lv, X., Liu, C., Zha, Z., Zhang, H., Jiang, Y., *et al.* (2012) The N-Terminal Phosphodegron Targets TAZ/WWTR1 Protein for $SCF^{\beta\text{-}Trcp}$-Dependent Degradation in Response to Phosphatidylinositol 3-Kinase Inhibition. *Journal of Biological Chemistry*, **287**, 26245-26253. https://doi.org/10.1074/jbc.m112.382036

[52] Wei, W., Li, M., Wang, J., Nie, F. and Li, L. (2012) The E3 Ubiquitin Ligase ITCH Negatively Regulates Canonical Wnt Signaling by Targeting Dishevelled Protein. *Molecular and Cellular Biology*, **32**, 3903-3912. https://doi.org/10.1128/mcb.00251-12

[53] Salah, Z. and Aqeilan, R.I. (2011) WW Domain Interactions Regulate the Hippo Tumor Suppressor Pathway. *Cell Death & Disease*, **2**, e172. https://doi.org/10.1038/cddis.2011.53

[54] Barnoud, T., Schmidt, M.L., Donninger, H. and Clark, G.J. (2017) The Role of the NORE1A Tumor Suppressor in Oncogene-Induced Senescence. *Cancer Letters*, **400**, 30-36. https://doi.org/10.1016/j.canlet.2017.04.030

[55] Kulikov, R., Boehme, K.A. and Blattner, C. (2005) Glycogen Synthase Kinase 3-Dependent Phosphorylation of Mdm2 Regulates p53 Abundance. *Molecular and Cellular Biology*, **25**, 7170-7180. https://doi.org/10.1128/mcb.25.16.7170-7180.2005

[56] Furth, N., Bossel Ben-Moshe, N., Pozniak, Y., Porat, Z., Geiger, T., Domany, E., *et al.* (2015) Down-Regulation of LATS Kinases Alters p53 to Promote Cell Migration. *Genes & Development*, **29**, 2325-2330. https://doi.org/10.1101/gad.268185.115

Abbreviations

GSK-3β	Glycogen synthetase kinase beta
SCF-Skip	Cullin, F-Box containing ligase complex
β-TrCP	Beta transducing repeats proteins, NORE1A (Novel RAS Effector one)