

ORIGINAL ARTICLE

LIM domain kinases as potential therapeutic targets for neurofibromatosis type 2

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Neurofibromatosis type 2 (NF2) is caused by mutations in the *NF2* gene that encodes a tumor-suppressor protein called merlin. NF2 is characterized by formation of multiple schwannomas, meningiomas and ependymomas. Merlin loss-of-function is associated with increased activity of Rac and p21-activated kinases (PAKs) and deregulation of cytoskeletal organization. LIM domain kinases (LIMK1 and 2) are substrate for Cdc42/Rac-PAK and modulate actin dynamics by phosphorylating cofilin at serine-3. This modification inactivates the actin severing and depolymerizing activity of cofilin. LIMKs also translocate into the nucleus and regulate cell cycle progression. Significantly, LIMKs are overexpressed in several tumor types, including skin, breast, lung, liver and prostate. Here we report that mouse Schwann cells (MSCs) in which merlin function is lost as a result of *Nf2* exon2 deletion (*Nf2*^{ΔEx2}) exhibited increased levels of LIMK1, LIMK2 and active phospho-Thr508/505-LIMK1/2, as well as phospho-Ser3-cofilin, compared with wild-type normal MSCs. Similarly, levels of LIMK1 and 2 total protein and active phosphorylated forms were elevated in human vestibular schwannomas compared with normal human Schwann cells (SCs). Reintroduction of wild-type *NF2* into *Nf2*^{ΔEx2} MSC reduced LIMK1 and LIMK2 levels. We show that pharmacological inhibition of LIMK with BMS-5 decreased the viability of *Nf2*^{ΔEx2} MSCs in a dose-dependent manner, but did not affect viability of control MSCs. Similarly, LIMK knockdown decreased viability of *Nf2*^{ΔEx2} MSCs. The decreased viability of *Nf2*^{ΔEx2} MSCs was not due to caspase-dependent or -independent apoptosis, but rather due to inhibition of cell cycle progression as evidenced by accumulation of cells in G₂/M phase. Inhibition of LIMKs arrests cells in early mitosis by decreasing aurora A activation. Our results suggest that LIMKs are potential drug targets for NF2 and tumors associated with merlin deficiency.

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INTRODUCTION

Mutations in the tumor-suppressor gene *NF2* cause neurofibromatosis type 2 (NF2), an autosomal-dominant disorder with an incidence of about 1 in 25 000 individuals.¹ The characteristic feature of NF2 is the development of bilateral vestibular schwannomas (VSs) that typically cause deafness, facial paralysis and disequilibrium following surgical removal to prevent life-threatening complications. Frequently, NF2 patients also develop multiple schwannomas in other nerves, as well as meningiomas and ependymomas.² Mutations in the *NF2* gene are also common in malignant mesothelioma.³ Currently, the standard treatment for NF2 schwannomas is microsurgery or stereotactic radiosurgery. Unfortunately, limited operability, poor preservation of hearing, diminished functionality of facial nerves and the small risk of radiation-induced malignant transformation later compromise good clinical outcomes. Only a handful of NF2 preclinical and clinical trials are ongoing, all of which utilize existing anti-cancer drugs.^{4,5}

The *NF2* gene encodes a tumor suppressor called merlin or schwannomin. Merlin regulates a number of processes in Schwann cells (SCs).^{6,7} Normal SCs usually proliferate slowly and adopt a bipolar morphology; however, merlin-deficient human schwannoma cells (HSCs) exhibit abnormalities in proliferation, motility and morphology *in vitro*.⁸ The mechanism of action of

merlin remains poorly understood, but appears to involve regulation of surface receptor activity and turnover by modulation of their interactions with the actin cytoskeleton and transduction of downstream signals.⁷ A potential pathway through which merlin regulates the actin cytoskeleton involves Cdc42/Rac-p21-activated kinase (PAK)-LIM domain kinase (LIMK)-cofilin.^{9–11} Loss of merlin function is associated with elevated levels of active Rac and PAK, suggesting that merlin is a negative regulator of Rac and PAK-dependent pathways. Conversely, PAK phosphorylates and inactivates tumor-suppressor activity of merlin.^{12,13} Work from our laboratory has shown that activation of β1-integrin in normal SCs promotes PAK-dependent phosphorylation of merlin at serine-518¹⁴. Although PAK has been studied as a potential therapeutic target for NF2, its large number of substrates confounds its usefulness.¹⁵

The LIMK family has two members, LIMK1 and LIMK2, which can be phosphorylated by Cdc42/Rac-PAK1, 2 and 4 at threonine-505/508 and converted into active serine/threonine kinases. LIMKs are also regulated by Rho-associated kinases and the myotonic dystrophy kinase-related Cdc42-binding kinase. The major LIMK substrate is the actin depolymerizing and severing protein cofilin/ADF.¹⁶ LIMK-dependent phosphorylation of cofilin at serine-3 inactivates cofilin and promotes stabilization of the actin cytoskeleton.¹⁷ LIMKs also translocate from the cortical

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cytoplasm and focal adhesions, where they modulate cell morphology and motility, to the centrosome and nucleus, where they regulate mitosis and cytokinesis.^{18–24} LIMKs interact with aurora A, a serine/threonine kinase involved in centrosome dynamics, spindle assembly and chromosome alignment and segregation during mitosis.^{25,26} Aurora A is found on duplicated centrosomes and mitotic spindle microtubules. During early mitosis, phospho-LIMKs co-localize with γ -tubulin in the centrosomes. In metaphase, LIMK1 is found at the spindle poles and later redistributes to the cleavage furrow. LIMK2 is located along the spindle and at the cleavage furrow of post-mitotic cells. LIMK1 is a substrate for, and phosphorylates, aurora A to promote its autophosphorylation at Thr288.²⁷ LIMK2 is an aurora A substrate and stabilizes it by kinase and scaffolding actions.²⁸ LIMKs are overexpressed and implicated in various cancers, such as breast, lung, skin, liver and prostate, and several tumor cell lines.^{29–32} Hence, LIMKs are emerging cancer targets; yet, few inhibitors have been identified.³³

We show that the levels of total and phosphorylated LIMK and its phosphorylated substrate are elevated in sporadic VSs compared with normal HSCs. Utilizing *Nf2*^{ΔEx2} mouse SCs (MSCs) as a cellular model of NF2, we show that pharmacological inhibition or genetic silencing of LIMK significantly reduces proliferation of *Nf2*^{ΔEx2} MSCs, but does not substantially affect control *Nf2*^{fl/fl} MSCs. The decreased proliferation is due to cell cycle arrest in the G₂/M phase. Pharmacological inhibition of LIMKs with BMS-5 slows mitotic progression by decreasing phosphorylation of cofilin and aurora A. These studies suggest that LIMKs are potential therapeutic targets for NF2 and other merlin-deficient tumors.

RESULTS

LIMK and phospho-cofilin levels are elevated in *Nf2*^{ΔEx2} MSCs compared with controls

Using two complementary techniques, we assessed the levels of LIMK and phospho-Ser3-cofilin in *Nf2*-deficient (*Nf2*^{ΔEx2}) MSC lines developed by *in vitro* ad-Cre deletion of *Nf2* exon 2 from *Nf2*^{fl/fl} MSCs.³⁴ By immunostaining *Nf2*^{ΔEx2}, MSCs were confirmed to be merlin-deficient and expressed the SC marker S100 (Figure 1a). Merlin-deficient SCs were larger than control *Nf2*^{fl/fl} MSCs and had increased levels of F-actin revealed by phalloidin staining. The intensity of LIMK1 and LIMK2 immunofluorescence was higher in *Nf2*^{ΔEx2} MSCs than in controls and was detected throughout the cell. Consistent with increased LIMK activity, the intensity of phospho-Ser3-cofilin immunofluorescence was also higher in *Nf2*^{ΔEx2} MSCs than in controls (Figures 1a and b).

We also assessed merlin expression in control *Nf2*^{fl/fl} and *Nf2*^{ΔEx2} MSCs by western blotting using an N-terminal merlin antibody. Merlin was detected in *Nf2*^{fl/fl} MSCs but not in *Nf2*^{ΔEx2} MSCs (Figure 1c). The status of the *Nf2* gene was also confirmed by PCR analysis of DNA and revealed deletion of exon 2 in *Nf2*^{ΔEx2} as evidenced by a 338-bp band (Figure 1d).³⁴

In addition, western blotting confirmed that levels of both LIMK1 and LIMK2 protein were higher in *Nf2*^{ΔEx2} MSCs than in *Nf2*^{fl/fl} MSCs (Figure 1e). We detected a coordinate increase in the level of phospho-Ser3-cofilin in *Nf2*^{ΔEx2} MSCs with respect to control consistent with high levels of LIMK activity (Figure 1f).

Reintroduction of wild-type NF2 normalizes LIMK protein levels

To assess whether elevated LIMK levels were linked to merlin inactivation, we reintroduced a halo-tagged wild-type human *NF2* into *Nf2*^{ΔEx2} MSCs by nucleofection. This method yielded ~10% transfection rate, determined by halo-tag fluorescence labeling. Reexpression of merlin in cultured *Nf2*^{ΔEx2} MSCs generally decreased LIMK1, LIMK2 and phospho-Ser3-cofilin levels (Figures

2a and b). This result supports a relationship between merlin inactivation and increased LIMK expression and activity.

Pharmacological inhibition of LIMK1/2 by BMS-5 or genetic silencing of LIMK1/2 reduces *Nf2*^{ΔEx2} MSC viability

We next sought to determine whether LIMK was a potential drug target for NF2. We tested the efficacy of BMS-5, a highly selective small-molecule inhibitor of LIMK1/2, on reducing phosphorylation of cofilin on Ser3.³⁵ BMS-5 inhibited cofilin-Ser3 phosphorylation in a dose-dependent manner in *Nf2*^{ΔEx2} MSCs with an IC₅₀ of ~2 μ M (Figures 3a and b). We next tested the ability of BMS-5 to reduce viability of *Nf2*^{ΔEx2} MSCs. The 100% viability control was 0.1% dimethyl sulfoxide (DMSO), and a positive cell death control was obtained with 50 μ M rapamycin that caused approximately 80% cell death in 24 h. BMS-5 reduced *Nf2*^{ΔEx2} MSC viability in a dose-dependent manner with an IC₅₀ of 3.9 μ M (Figure 3c), but did not significantly reduce the viability of control *Nf2*^{fl/fl} MSCs at equivalent BMS-5 concentrations (Figure 3d). At 10 μ M BMS-5, *Nf2*^{ΔEx2} MSC viability was 40% compared with 83% for controls.

To confirm the validity of LIMK as a drug target, we knocked down LIMK1 and LIMK2 in *Nf2*^{ΔEx2} MSCs using lentiviral shRNA transduction (Supplementary Figure S1). Consistent with the BMS-5 results, LIMK1 and LIMK2 knockdown reduced viability of *Nf2*^{ΔEx2} MSCs compared with scrambled shRNA controls in a 24-h assay (Figure 3e). These results suggest that LIMK inhibition or silencing in merlin-deficient cells reduces their viability either by promoting cell death or hindering proliferation.

Decreased viability of *Nf2*^{ΔEx2} MSCs in response to BMS-5 or LIMK silencing is independent of apoptosis

To determine if BMS-5 promoted *Nf2*^{ΔEx2} MSC apoptosis, we measured caspase 3/7 activity. We found that BMS-5 did not activate caspase 3/7, whereas the positive control, staurosporine, promoted dose-dependent activation of caspase 3/7 (Figure 5a). To determine if BMS-5 promoted caspase-independent apoptosis, we analyzed BMS-5-treated *Nf2*^{ΔEx2} MSCs by flow cytometry using a ratiometric membrane asymmetry probe that detects loss of membrane phospholipid asymmetry associated with apoptosis. We observed only a small increase (3.5%) of apoptotic cells after 7 h of BMS-5 treatment (Figure 4b). Consistent with pharmacological LIMK inhibition, knockdown of LIMK1 or LIMK2 was not associated with caspase 3/7 activity increase compared with scrambled shRNA controls examined 24 h after plating (Figure 4c). Similarly, caspase-independent apoptosis was not observed in *Nf2*^{ΔEx2} MSCs transduced with either LIMK1 or LIMK2 shRNA (Figure 4d). These results suggest that pharmacological inhibition and genetic silencing of LIMK affect *Nf2*^{ΔEx2} MSC viability through an apoptosis-independent mechanism.

Pharmacological inhibition of LIMK1/2 by BMS-5 reduces DNA synthesis and cell cycle progression

We next assessed the ability of BMS-5 to interfere with proliferation of *Nf2*^{ΔEx2} MSCs. The cells were incubated with 1 and 2 μ M BMS-5 for 24, 48 and 72 h, and cell numbers were measured using crystal violet staining. We observed fewer BMS-5-treated *Nf2*^{ΔEx2} MSCs than DMSO-treated controls (Figure 5a). In addition, we found that BMS-5 decreased DNA synthesis in *Nf2*^{ΔEx2} MSCs in a dose-dependent manner (Figure 5b).

To test the possibility that the decrease in proliferation was a consequence of cell cycle inhibition, we performed flow cytometry analysis of cells stained with propidium iodine (PI). BMS-5 interfered with *Nf2*^{ΔEx2} MSCs cell cycle progression as evidenced by an increase in the percentage of diploid cells trapped in the G₂/M phase (Figure 6a). We further analyzed the distribution of cells among the cell cycle phases using a BrdU/7AAD assay. There was a significant increase in the number of cells accumulating in the G₂/M

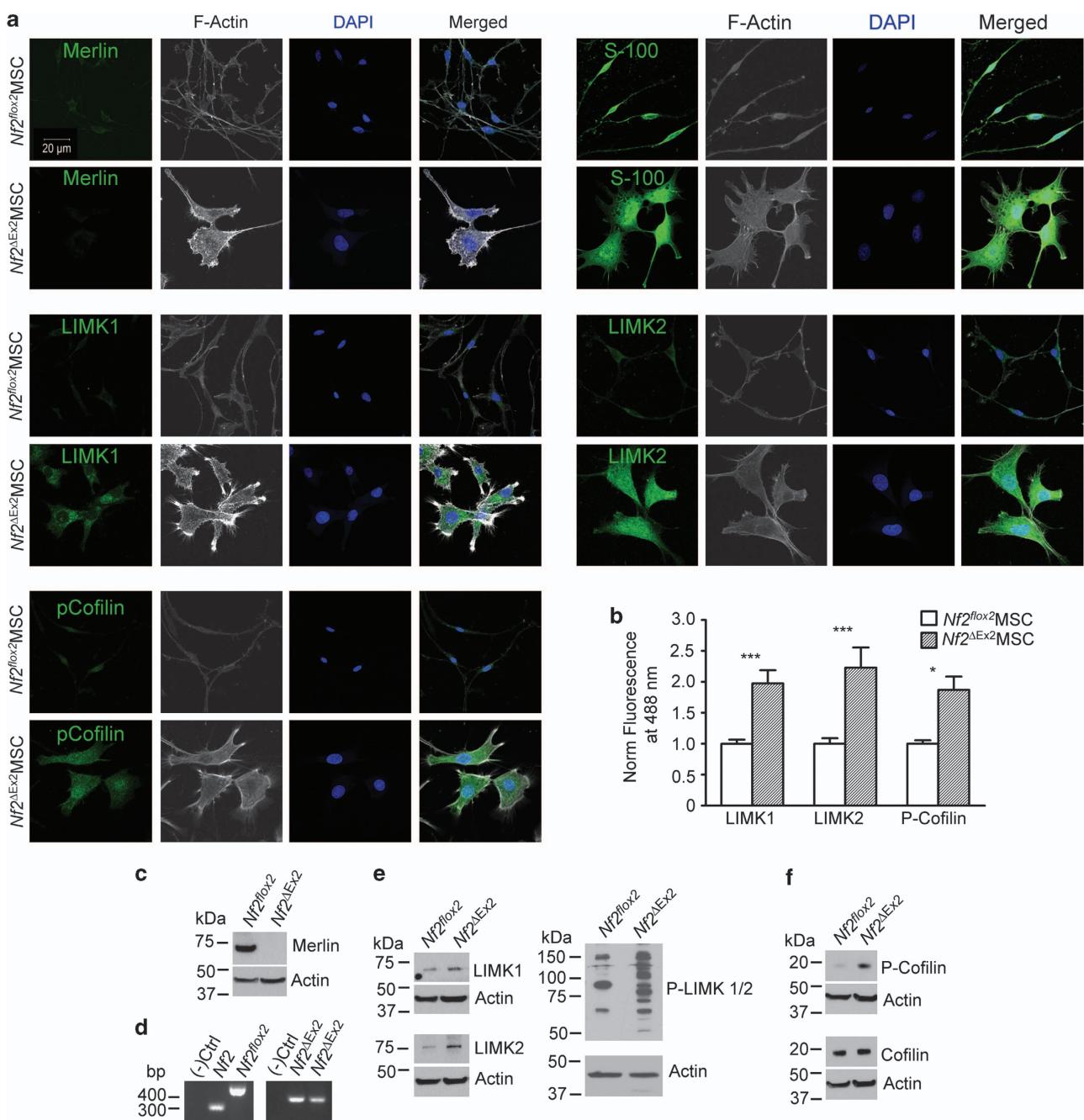


Figure 1. Elevated levels of LIMK and phospho-Ser3-cofilin in *Nf2^{ΔEx2}* MSCs compared with control MSCs. **(a)** Representative confocal images of *Nf2^{ΔEx2}* and *Nf2^{ΔEx2}/flox2/flox2* MSCs grown overnight on glass coverslips, fixed and immunostained with the indicated antibodies (green). F-actin was visualized with phalloidin-Alexa633 (white), and the nucleus was visualized by 4',6-diamidino-2-phenylindole (DAPI) stain (blue). Scale bar: 20 μm. **(b)** Quantitation of the immunofluorescence for the indicated proteins in three independent experiments was performed with Volocity software (Improvision, PerkinElmer, Waltham, MA, USA). ***P<0.001 and *P<0.05 determined by two-way analysis of variance using Bonferroni post-tests. **(c)** Characterization of *Nf2^{ΔEx2}/flox2/flox2* and *Nf2^{ΔEx2}* MSCs. Control *Nf2* with the exon2 was flanked by loxP sites, and *Nf2^{ΔEx2}/flox2/flox2* and *Nf2^{ΔEx2}* MSCs were analyzed using western blotting for N-terminus Merlin and **(d)** PCR analysis of genomic DNA. Primers P4/P5 amplified a 305-bp band for wild-type *Nf2* FVB/N and a 442-bp band for *Nf2^{ΔEx2}/flox2/flox2*, and primers P6/P5 amplified a 338-bp band for *Nf2^{ΔEx2}*. **(e)** *Nf2^{ΔEx2}* and control *Nf2^{ΔEx2}/flox2/flox2* MSCs were analyzed using western blotting for LIMK1, LIMK2, phospho-LIMK1/2 (Thr508/505) and **(f)** phospho-Ser3-cofilin and cofilin. Anti-β-actin was used as a loading control.

phase in samples treated with 5 μM BMS-5 as compared with vehicle control (20% vs 42%). This was accompanied by a decrease in the number of BMS-5-treated cells in G₁ as compared with control (41% vs 26%). This is consistent with the results obtained by analysis of PI staining (Figures 6b and c). Overlay of the 7AAD-area histograms clearly demonstrated a significant increase in the G₂/M population in

BMS-5 compared with DMSO-treated cells (Figure 6d). Furthermore, the increase in the G₂/M population was accompanied by an increase in the number of cells with higher DNA content, consistent with a polyploid or multinucleated cell population. There was also a smaller but substantial change in number of cells detected in the S phase of cell cycle in samples treated with BMS-5 as compared with

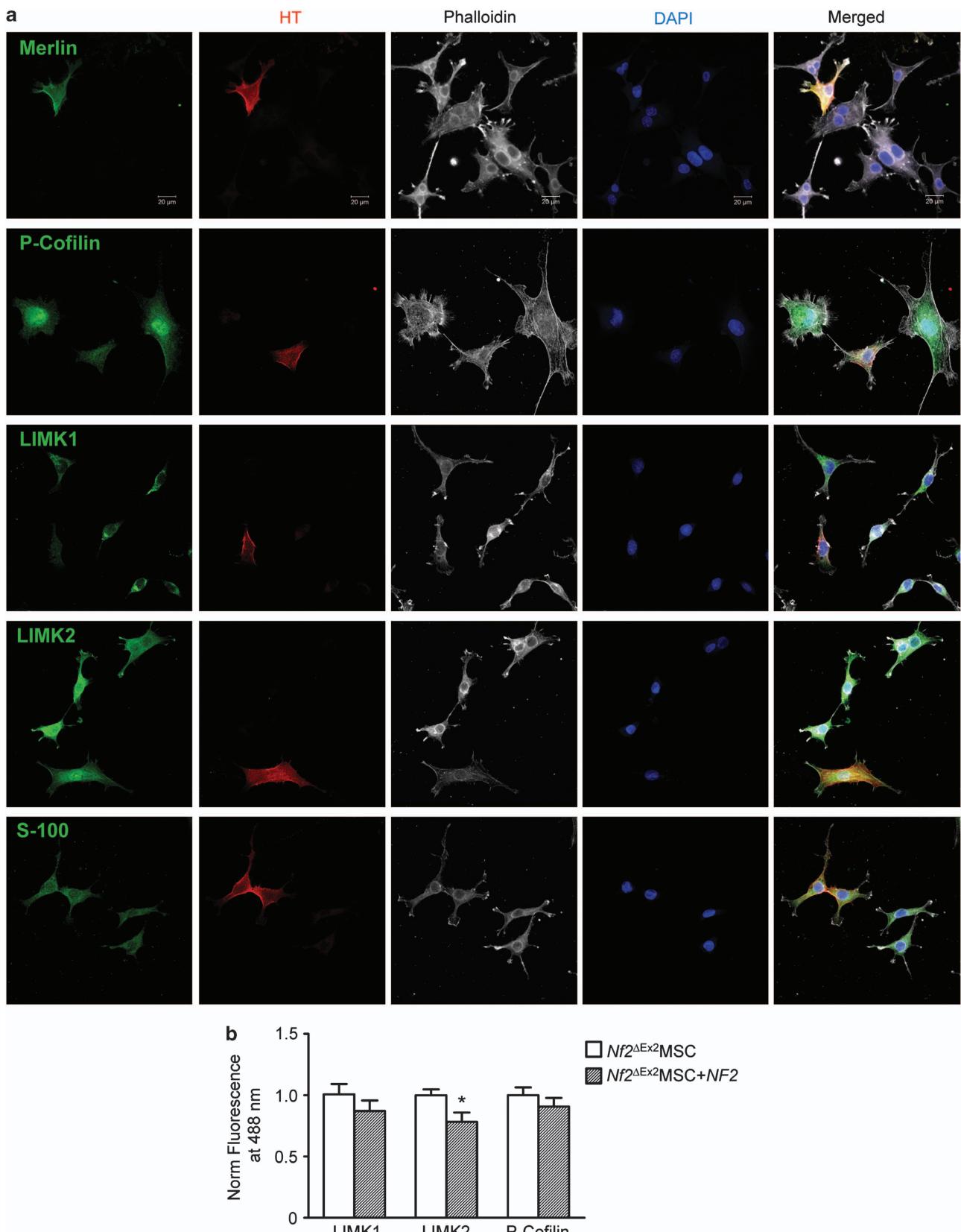


Figure 2. Reintroduction of wild-type merlin normalizes LIMK expression levels. **(a)** *Nf2^{ΔEx2}* MSCs were transfected with Halo-tag-NF2 and incubated for 40 h on glass coverslips coated with 200 µg/ml poly-L-lysine and 10 µg/ml laminin. Transfected cells were identified by halo-tag TMR labeling (red). Cells were fixed and immunostained for the indicated proteins (green). F-actin was visualized with phalloidin-Alexa633 (white) and the nucleus with 4,6-diamidino-2-phenylindole (DAPI) (blue). Confocal images are representative of three independent experiments. Scale bar: 20 µm. **(b)** Quantitation of **a**. Measurements of the mean pixel intensity fluorescence in the green channel of two experiments for the indicated proteins were performed with Volocity software. *P<0.05 determined by two-way analysis of variance using Bonferroni post-tests.

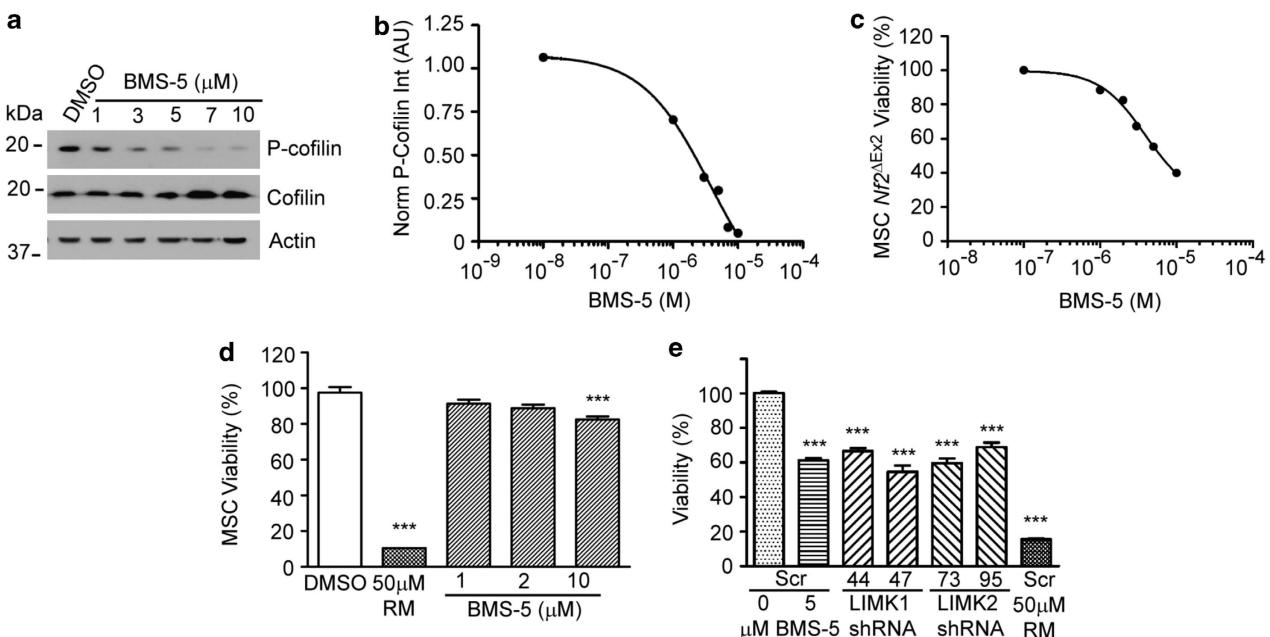


Figure 3. LIMK inhibition by BMS-5 or silencing by shRNA in *Nf2^{ΔEx2}* MSCs decreases cell viability. (a) BMS-5 dose–response western blot. *Nf2^{ΔEx2}* MSCs were plated in 12-well plates. Cultures were treated the next day as indicated for 30 min. Cells were harvested, lysed and analyzed using western blotting for phospho-Ser3-cofilin and total cofilin. β -actin levels were used as loading controls for normalization. Representative blot of three independent experiments. (b) BMS-5 dose–response curve of cofilin phosphorylation in *Nf2^{ΔEx2}* MSCs, analyzed as log (inhibitor) vs response, variable slope (four parameters). (c) BMS-5 dose viability–response curve. *Nf2^{ΔEx2}* MSCs were seeded at 5000 cells/well in 20 μ l growth medium phenol-red free in a 384-well plate and after attachment were incubated with BMS-5 for 24 h. Cell viability was measured with the CellTiter-Fluor assay. Graph represents the mean \pm s.e.m. of three independent experiments analyzed together ($n = 96$) log (inhibitor) vs response, variable slope (four parameters). (d) BMS-5 viability response of control MSCs. Cell viability was measured as in c. Graph represents the mean \pm s.e.m. ($n = 16$). DMSO control was considered 100% viability. Rapamycin (RM) (50 μ M) was a positive control for cell death. *** $P < 0.001$ determined by one-way analysis of variance (ANOVA) using Dunnett's multiple comparison test. (e) Viability of *Nf2^{ΔEx2}* MSCs expressing LIMK1 or LIMK2 shRNAs were compared with cells expressing scrambled shRNA untreated or treated with 5 μ M BMS-5 or 50 μ M RM for 24 h in a 384-well format. Cell viability was measured with the CellTiter-Fluor assay. *Nf2^{ΔEx2}* MSCs expressing scrambled shRNA represented 100% viability, and the 50- μ M RM-treated cells were a positive control for cell death. Graph represents the mean \pm s.e.m. of four independent experiments analyzed together ($n = 128$). *** $P < 0.001$ determined by one-way ANOVA using Dunnett's multiple comparison test.

DMSO control. Lastly, the population of cells in the S phase decreased with increasing BMS-5 concentration (Supplementary Figure S2). These results taken together suggest that inhibition of LIMK activity in *Nf2^{ΔEx2}* MSCs interferes with progression of the G2/M phase of the cell cycle, thereby decreasing the number of viable *Nf2^{ΔEx2}* MSCs.

BMS-5 inhibition of LIMK1/2 activity decreases aurora A phosphorylation and causes formation of abnormal mitotic spindles

To identify additional downstream effects of BMS-5 (beyond inhibition of cofilin-Ser3 phosphorylation) that could cause a G₂/M arrest, we tested the levels of aurora A phosphorylation. Aurora A interacts with both LIMK1 and 2 and is a major regulator of mitotic spindle assembly and chromosomal alignment and segregation. We found that *Nf2^{ΔEx2}* MSCs treated with increasing concentrations of BMS-5 had a dose-dependent decreased phosphorylation of aurora A at Thr288 (Figures 7a and b).

We next studied the effect of BMS-5 on the spindle assembly visualizing the mitotic nuclei and spindle machinery by immunostaining. We found that *Nf2^{ΔEx2}* MSCs had normal spindle assembly and BMS-5-treated *Nf2^{ΔEx2}* MSCs showed abnormal spindle structure (Figure 8a). Analyzing the centrosome positioning by immunostaining for centrin-1, we found that BMS-5-treated cells were not able to organize the centrosomes and generally had more than two with different sizes (Figure 8b). We conducted cell cycle analysis and found that the percentage of cells in late G₂/M, visualized by positive immunostaining for the mitotic marker phospho-Ser10-histone3,

significantly increased from 5 to 17% in the BMS-5-treated *Nf2^{ΔEx2}* MSCs compared with DMSO controls (Figures 8c and d). We observed a highly significant decrease in the percentage BMS-5-treated *Nf2^{ΔEx2}* MSCs that advanced to late mitotic phases (anaphase/telophase). Moreover, BMS-5-treated *Nf2^{ΔEx2}* MSCs had abnormal spindles, and chromosomes did not align at the center as occurs in metaphase (Figures 8a–e). These results suggest that LIMK inhibition exerts its anti-proliferative effect not only by altering organization of the actin cytoskeleton through inhibition of cofilin phosphorylation but also by decreasing LIMK-dependent activation of aurora A.

LIMK levels are elevated in representative samples of human VS compared with HSCs

To examine the relevance of LIMK inhibition to NF2, we compared LIMK1 and LIMK2 protein and phosphorylation levels in a small number of samples of HSCs from normal individuals and sporadic human VSs. We observed a significant increase ($P < 0.05$ determined using unpaired t-test of HSC vs VS populations, two-tailed) in both the protein and phospho-Thr508 levels of LIMK1 in VSs compared with HSCs (Figures 9a and b). Similarly, both LIMK2 and phospho-Thr505-LIMK2 levels showed a tendency to be higher in VSs compared with HSCs (Figures 9c and d). Interestingly, the proportion of phospho-Ser3-cofilin to the total cofilin was higher in VSs than in HSCs (Figure 9e).

We also measured LIMK protein and phosphorylation levels in a HSC line, HEI-193, developed from a NF2 patient by immortalization with HPV E6-E7 genes.³⁶ Although the phospho-Thr508/

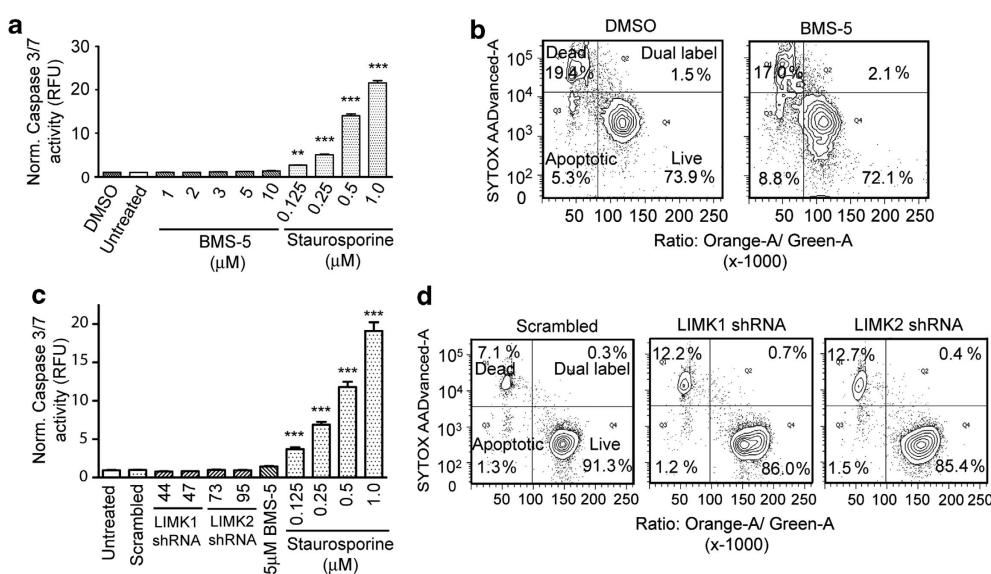


Figure 4. Inhibition or silencing of LIMK does not induce apoptosis. **(a)** Caspase 3/7 activity assay. *Nf2*^{ΔEx2} MSCs were seeded at 5000 cells/well in 20 μl growth media phenol-red free in a 384-well plate and incubated for 16 h at 37 °C, 7% CO₂. Next, 5 μl /well of the indicated solution was added and incubated for 8 h. Caspase 3/7 activity was measured with the Apo-ONE Homogeneous assay. Staurosporine curve was used as positive control. Histogram represent the mean +/- s.e.m. of three independent experiments analyzed together and normalized to untreated controls ($n = 96$). ** $P < 0.01$ and *** $P < 0.001$ determined by one-way analysis of variance (ANOVA) using Dunnett's multiple comparison test. **(b)** *Nf2*^{ΔEx2} MSCs plated in a six-well format were incubated with 2 μM BMS-5 or DMSO vehicle for 24 h. Plasma membrane asymmetry was evaluated with the Violet ratiometric assay by flow cytometry. Densitometry graph illustrates the following quadrants: apoptotic (Q3), dead (Q1), live (Q4) and dual label (Q2). **(c)** Caspase 3/7 activity measured with the Apo-ONE assay of *Nf2*^{ΔEx2} MSCs uninfected or infected with control scrambled or the indicated constructs for LIMK1/2 shRNA silencing. Cells were incubated 24 h in a 384-well format and in the presence of inhibitors for 8 h where indicated. Staurosporine response was used as positive control. Histogram represents five independent experiments normalized to scrambled control and analyzed together ($n = 160$). *** $P < 0.001$ determined by one-way ANOVA using Dunnett's multiple comparison test. **(d)** *Nf2*^{ΔEx2} MSCs infected with scrambled shRNA or shRNAs targeting LIMK1/2 constructs were plated in a six-well format and incubated for 24 h. Plasma membrane asymmetry was assessed with the Violet ratiometric assay by flow cytometry in three independent experiments. Densitometry plots show the quantification of the population distribution into the four quadrants as in **b**.

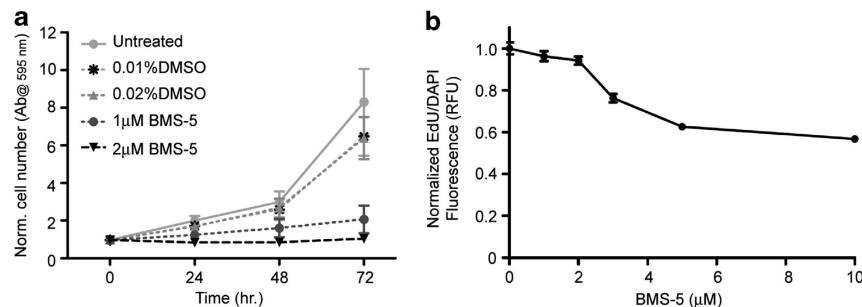


Figure 5. BMS-5 decreases *Nf2*^{ΔEx2} MSC proliferation and DNA synthesis in a dose-dependent manner. **(a)** *Nf2*^{ΔEx2} MSCs were grown in 24-well plates. Cell proliferation was measured at 0, 24, 48 and 72 h time points after incubation with BMS-5 or DMSO vehicle. The number of cells at each time point was assessed with the crystal violet assay. Graph combines three independent experiments in triplicates (mean ± s.e.m., $n = 9$). **(b)** *Nf2*^{ΔEx2} MSCs were treated with the indicated BMS-5 concentrations for 24 h. EdU was added to the culture for the last 6 h. Incorporation of EdU into S-phase cells was assessed with the EdU Click-It microplate assay, and fluorescence was measured on a plate reader. Graph combines three independent experiments (mean ± s.e.m., $n = 44$).

Thr505 LIMK1/2 antibody recognized multiple bands, the relative intensities of the most prominent phospho-LIMK1/2 (Thr508/Thr505) bands were also higher in HEI-193 cells than in control HSCs (Figure 9f).

DISCUSSION

LIMK expression and activity are elevated in merlin-deficient SCs compared with normal SCs

Here we document an association between loss of merlin function and increased levels of LIMK1 and LIMK2 expression and activity.

The increased LIMK activity in *Nf2*-deficient MSCs is consistent with its activation by Cdc42/Rac-PAK1, 2 and 4,¹⁶ which have been well-documented to be increased in schwannomas.^{12,15} Importantly, reintroduction of merlin into *Nf2*^{ΔEx2} MSCs reduced LIMK and phospho-Ser3-cofilin levels, further supporting an association between loss of merlin function and inactivation of the actin severing and depolymerizing activity of cofilin downstream of activation of the Rac/PAK/LIMK pathway. In agreement with previous studies of HSCs, we found that *Nf2*^{ΔEx2} MSCs exhibited an abnormal actin cytoskeleton compared with control MSCs.⁸ Interestingly, similar cytoskeleton characteristics,

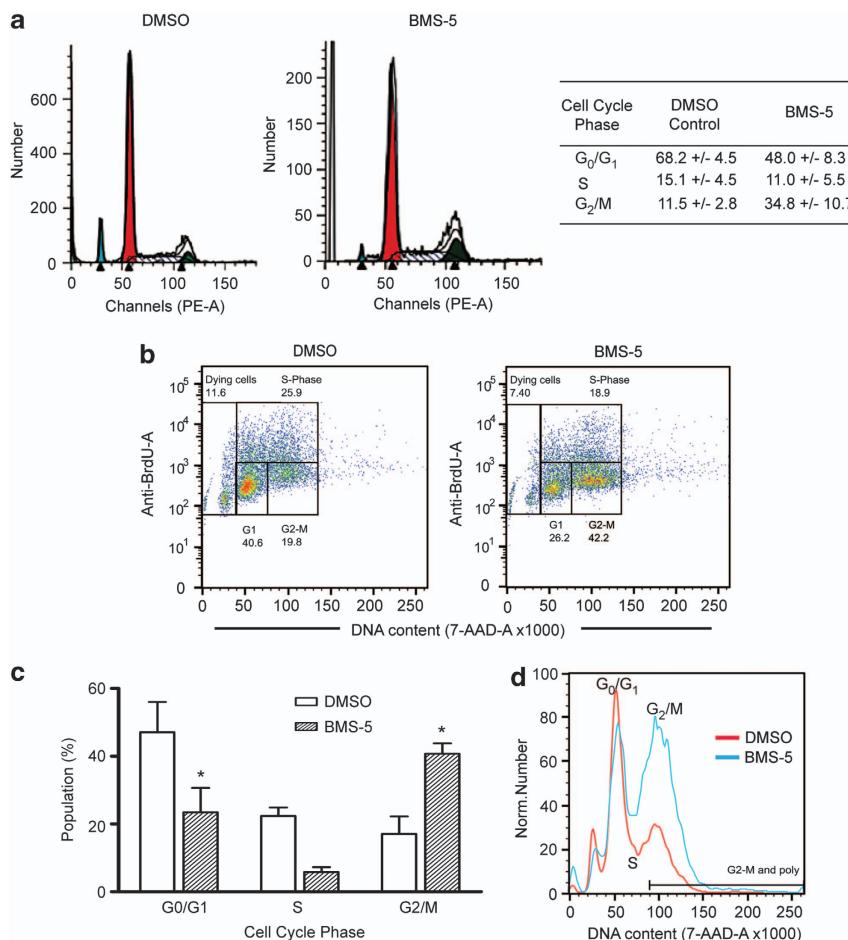


Figure 6. Inhibition of LIMK interferes with cell cycle progression of *Nf2*^{ΔEx2} MSCs. (a) *Nf2*^{ΔEx2} MSCs were grown in a six-well format and treated with 5 μM BMS-5 for 7 h before labeling. Cells were analyzed using flow cytometry. Propidium iodide labeling profile of the diploid cell population analyzed with the ModFit program. Tabulation of the distribution of cell cycle phases of three independent experiments (mean ± s.e.m., n = 3). (b) *Nf2*^{ΔEx2} MSCs were treated with 5 μM BMS-5 or DMSO control overnight before 3 h BrdU labeling and analysis using flow cytometry. Distribution of BrdU- and 7-aminoactinomycin D (7-AAD)-labeled cells analyzed with FlowJo software. These distribution plots are representative of four independent experiments (n = 4). (c) Graph of the distribution of the cell cycle phases of all the experiments as mean ± s.e.m. *P < 0.05 determined by two-way analysis of variance and Bonferroni multiple comparison post-tests. (d) Overlay of histograms of 7-AAD content comparing the vehicle and BMS-5-treated cells shown in b.

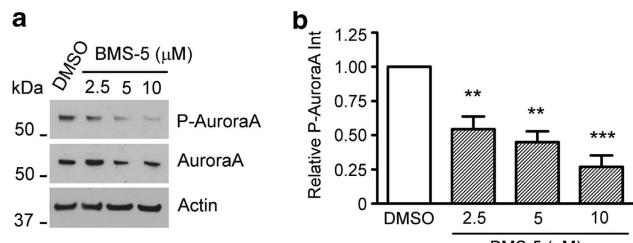


Figure 7. Inhibition of LIMKs decreases aurora A autophosphorylation. (a) Representative western blot of three independent BMS-5 dose-response experiments. *Nf2*^{ΔEx2} MSCs were plated in half of a 12-well plate and treated the next day with increasing BMS-5 concentrations or DMSO for 8 h. Cells were harvested and lysed, and 10 μg of protein was analyzed using western blotting for phospho-Thr288-aurora A and aurora A. β-Actin levels were used as loading controls. (b) Quantification of BMS-5 effect-response on aurora A phosphorylation. Graph represents the mean ± s.e.m. (n = 3). **P < 0.01 and ***P < 0.001 determined by one-way analysis of variance using Dunnett's multiple comparison test.

indicative of F-actin stabilization, have been reported in C2C12 and HeLa cells overexpressing LIMK.^{17,37} We propose that phosphorylation and inactivation of cofilin downstream of elevated Rac/PAK/LIMK activity could account for the reported increased cell size and abundance of actin filaments observed in *Nf2*^{ΔEx2} MSCs and HSCs.³⁸

Inhibition of LIMK activities causes cell cycle arrest of *Nf2*^{ΔEx2} MSCs by preventing aurora A activation

We recently demonstrated using BMS-5 that LIMK-dependent phosphorylation of cofilin is necessary for stable alignment of SC processes along sensory neuron axons in culture.³⁹ Here we showed that BMS-5 decreased *Nf2*-deficient MSC viability in a dose-dependent manner while having little effect on controls. These results suggest a potential therapeutic window for treatment of schwannomas with LIMK inhibitors. Studies using shRNA knockdown of LIMK1 and LIMK2 in *Nf2*^{ΔEx2} MSCs confirmed the specificity of BMS-5 on LIMK activity. Intriguingly, during LIMK silencing in *Nf2*^{ΔEx2} MSCs, we found that these cells did not sustain long-term LIMK knockdown and were able to restore their original

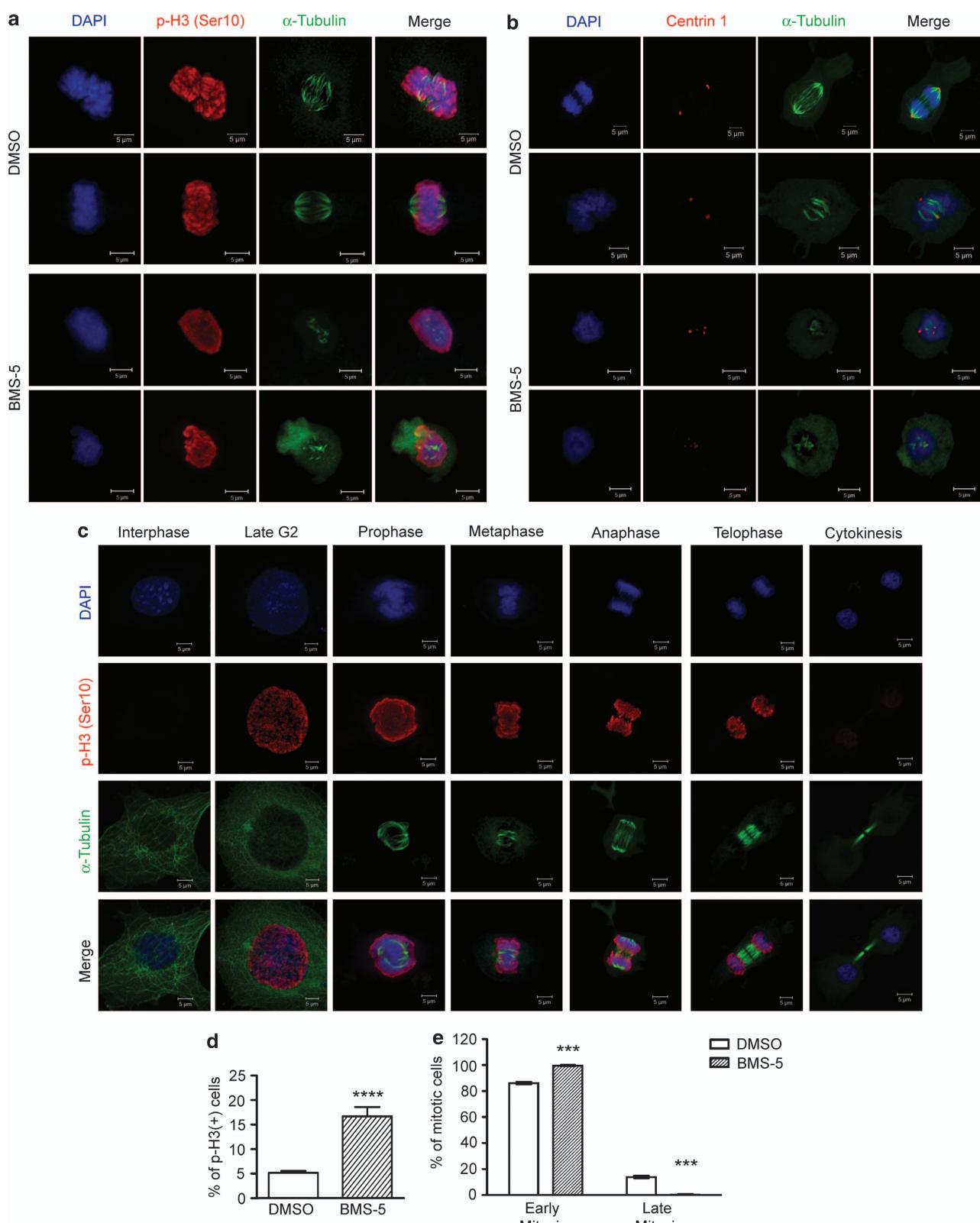


Figure 8. Inhibition of LIMKs produce abnormal spindle and centrosome assemblies. Representative confocal images of *Nf2*^{4Ex2} MSCs grown overnight on glass coverslips and treated with 4 μ M BMS-5 or 0.04% DMSO for 8 h, fixed and immunostained with α -tubulin (green) and (a) P-H3(ser10) or (b) centrin-1 (red). Nuclei were visualized with 4,6-diamidino-2-phenylindole (DAPI) (blue). (c) Representative confocal images of DMSO-treated *Nf2*^{4Ex2} MSCs depicting the cell cycle phases considered for analysis. (d) Quantification of the percentage of cells in late G₂/M phase assessed by positive p-H3 immunostaining. ****P<0.0001 determined by unpaired t-test, two-tailed, from triplicate coverslips of three independent experiments ($n=9$). (e) Quantification of the percentage of mitotic cells in early (pro/metaphase) and late (ana/telophase) mitosis. ***P<0.001 determined by two-way analysis of variance and Bonferroni multiple comparison post-tests. Scale bar: 5 μ m.

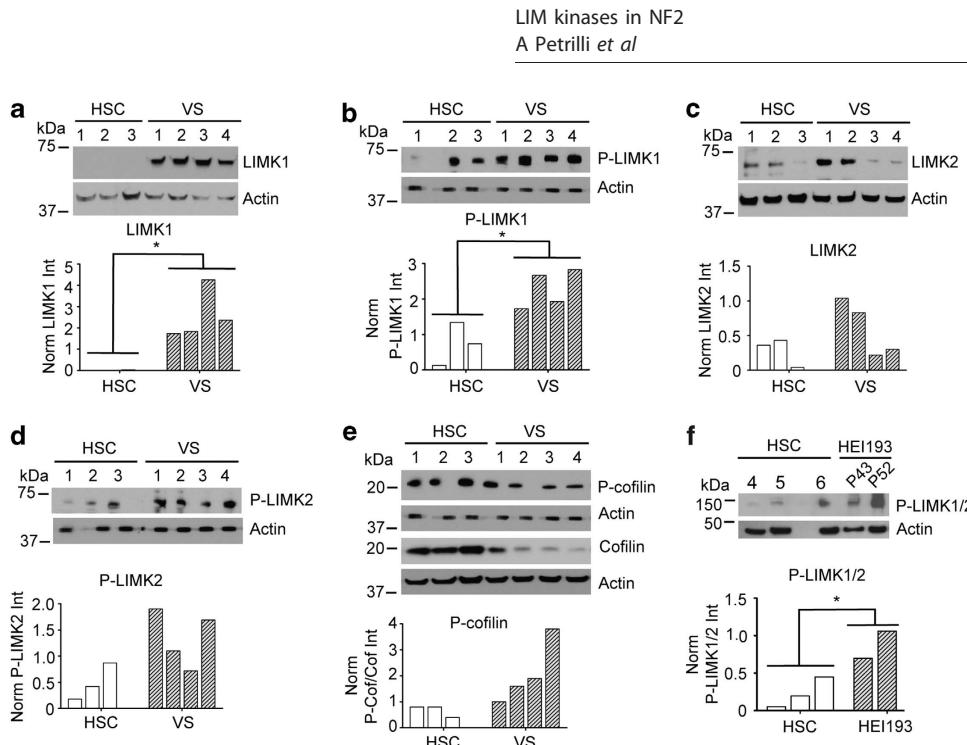


Figure 9. LIMKs are overexpressed in human VSs. Lysed cultured HSCs cultured in 100-mm dishes and homogenized frozen human sporadic VSs were resolved using SDS-PAGE and analyzed using western blot. Quantitation of the western blots was done by densitometry analysis with ImageJ software, normalized to β -actin and is plotted below. Western blots for (a) LIMK1, (b) phospho-Thr508-LIMK1, (c) LIMK2, (d) phospho-Thr505-LIMK2 and (e) phospho-Ser3-cofilin and cofilin. β -actin was used as a loading control. * $P < 0.05$ determined using unpaired t-test of HSC vs VS populations, two-tailed. (f) HSCs from normal individuals (control) and HEI-193 cells at P43 and P52 were cultured in 60-mm dishes and analyzed using western blotting for phospho-Thr508/505-LIMK1/2. β -actin was used as a loading control.

expression levels. Thus, we only used cells within their first two passages after puromycin selection. We speculate that *Nf2*^{1Ex2} MSCs silence LIMK shRNA by a methylation-dependent mechanism as shown for PAK shRNAs in schwannoma cells.¹⁵

Inhibition of LIMK activity with BMS-5 reduced *Nf2*^{1Ex2} MSC viability by arresting them at the M phase of the cell cycle. Others have shown that the localization and activity of LIMK changes during the cell cycle and that cofilin phosphorylation dynamics are necessary for successful cytokinesis.^{21,23,24} Importantly, LIMK1 knockdown in HeLa cells or treatment with a LIMK-inhibitor peptide resulted in delayed mitotic progression and irregular spindle orientation.²² These reports support our conclusion that inhibition of LIMK activity with BMS-5 arrested *Nf2*-deficient cells in the M phase, thereby decreasing the number of cells in S phase and increasing the number of polyploid cells.

The reported interaction and functional cooperation of LIMK1 and 2 with aurora A led us to explore the effect of LIMK inhibition with BMS-5 on aurora A autophosphorylation.²⁵ In agreement with the observations made with LIMK1 knockdown in PC-3 cells, we found that BMS-5 reduced aurora A phosphorylation at Thr288.²⁷ It is important to note that BMS-5 does not directly inhibit aurora A kinase activity, because BMS-5 (compound 3 in Ross-Macdonald *et al.*)³⁵ was tested *in vitro* against a panel of kinases, and aurora A retained 100% activity when tested with 10 μ M BMS-5.³⁵ Interestingly, the abnormal spindle and centrosome organization observed here resembles the abnormal metaphase spindles with multipolar and/or wheel-barrel shape and non-assembled chromosomes observed in a LIMK2 knockdown in SH-EP neuroblastoma cells.²⁴ The integrity of the mitotic spindle is required for progression from metaphase to anaphase, and any abnormality in the spindle assembly checkpoint delays or hinders the onset of anaphase. Hence, inhibition of LIMK activity by BMS-5 leads to inhibition of aurora A kinase activity, and thus provides a molecular pathway for the G₂/M block of merlin-deficient SCs treated with BMS-5.

Recently, LIMK has been shown to promote tumor neovascularization through the VEGF/p38/MK2/LIMK1/annexin1 pathway.⁴⁰ This finding may potentially connect LIMK inhibition with the observed reduction in angiogenesis *in vivo* following treatment with bevacizumab, which reduces tumor volumes and, in some patients, restores some hearing.⁴ Thus, we speculate that inhibition of LIMK might halt tumor progression not only by interfering with cell cycle progression in schwannoma cells but also by inhibiting angiogenesis.

In conclusion, our studies suggest a potential therapeutic target for NF2, the LIMKs, which are recognized substrates of Cdc42/Rac-PAK1,2,4. We show enhanced LIMK expression and activity in merlin-deficient MSCs, as well as VSs, as opposed to controls. Reduction in LIMK activity or protein levels decreases *Nf2*-deficient MSC viability by arresting cells in the prometaphase stage of the cell cycle in association with decreased activation of aurora A necessary for centrosome and mitotic spindle organization. Future studies addressing whether LIMK inhibition is an effective treatment for schwannomas in mouse models of NF2 are warranted.

MATERIALS AND METHODS

Inhibitors

LIMK1/2 inhibitor, BMS-5 (CAS name (N-(192,6-dichlorophenyl)-3-(difluoromethyl)-1H-pyrazol-5-yl)thiazol-2-yl)isobutyramide) was from SynKinase (Shanghai, China). Rapamycin and staurosporine were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Antibodies

Antibodies used were purchased from the following sources: phospho-cofilin-Ser3 (77G2), merlin (D1D8), phospho-LIMK1(Thr508)/LIMK2(Thr505) (used in cell lines), LIMK1, phospho-Histone3(Ser10) (D2C8), α -tubulin (DM1A), phospho-aurora A(Thr288)(D13A11) and β -actin (8H10D10) from Cell Signaling (Danvers, MA, USA); LIMK1 (clone42) mouse monoclonal

antibody from BD Biosciences (San Jose, CA, USA); LIMK2, phospho-LIMK1 (pThr-508) and phospho-LIMK2 (pThr-505) rabbit antibodies from Sigma-Aldrich (St Louis, MO, USA); S-100 rabbit antibody from Dako Cytomation (Glostrup, Denmark); cofilin rabbit antibody from Novus Biologicals (Littleton, CO, USA); centrin-1 rabbit antibody from Abcam (Cambridge, MA, USA); aurora A rabbit antibody from Bethyl Laboratories (Montgomery, TX, USA); peroxidase-conjugated goat anti-rabbit IgG from Pierce, Thermo Fisher Scientific (Rockford, IL, USA); and highly cross-adsorbed Alexa Fluor 488 goat anti-rabbit IgG (H + L) antibody from Invitrogen (Grand Island, NY, USA).

Generation of *Nf2*^{ΔEx2} MSCs

Nf2^{ΔEx2} MSCs were generated *in vitro* by transducing *Nf2*^{flox2/flox2} SCs with an Adeno-Cre virus (University of Iowa Gene Transfer Vector Core) as previously described.⁴¹ The *Nf2*^{ΔEx2} MSCs were used up to passage 20. All protocols are in accordance with the guidelines of and approved by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International-certified University of Central Florida (UCF) Institutional Animal Care and Use Committee.

HSC cultures

Vials of frozen purified HSCs received from Dr Patrick Wood at the University of Miami School of Medicine (Miami, FL, USA) were thawed and cultured on Corning (Corning Inc. Life Sciences, Tewksbury, MA, USA) dishes coated with 200 µg/ml poly-L-lysine hydrobromide (Sigma-Aldrich) and 50 µg/ml laminin (Invitrogen) containing D10M growth media: DMEM (Gibco, Life Technologies, Grand Island, NY, USA) plus 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT, USA), 2 µM Forskolin (Sigma, St Louis, MO, USA), 0.02 mg/ml pituitary extract (Biomedical Technologies Inc, Stoughton, MA, USA) and 1% penicillin/streptomycin (Gibco). Frozen vials from the stock bank of HSC were prepared by the Life Alliance Organ Recovery Agency at the University of Miami School of Medicine and cannot be traced to the specific donor. HSCs were prepared from 10 cm biopsy of sural nerve/cauda equine from cadaveric adult human donors. Nerve fragments were incubated for several days, dissociated and plated. SCs were purified by immunopanning with NGFR (nerve growth factor receptor) p75 antibodies. Culture purity based on S100 immunostaining was 96–99%. HEI-193 cells were purchased from ATCC (Manassas, VA, USA) and grown in DMEM plus 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin.

Human schwannoma samples

Frozen specimens of human sporadic VSs with different types of *NF2* mutations were procured with patient informed consents at the Ohio State University according to the Institutional Review Board regulations.

Western blot analysis

Cultured cells were lysed in modified RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium dodecyl sulfate with protease inhibitor cocktail and phosphatase inhibitor cocktails 2 and 3; Sigma-Aldrich). VS specimens (0.1–0.2 g) were homogenated in 900 µl of modified RIPA buffer with 25–30 strokes in a Wheaton Micro Tissue Grinder glass set no. 528 (Wheaton Scientific, Millville, NJ, USA) on ice. Lysates were rotated for 10 min at 4 °C and centrifuged for 10 min at 4 °C, 15 000 r.p.m. Protein concentration was determined by DC Assay (Bio-Rad, Hercules, CA, USA), and 10–20 µg of protein in lamelli buffer was resolved in 10% or 4–20% polyacrylamide gels (Pierce/Thermo Fisher Scientific), transferred to polyvinylidene difluoride membrane (Immobil-P; Millipore, Bedford, MA, USA) and analyzed using western blotting with anti-LIMK1, (1:500) from BD (San Jose, CA, USA) for human samples and (1:500) from Cell Signaling for mouse samples, LIMK2 (1:500), P-cofilin-Ser3 (1:1500), cofilin (1:70 000), merlin (1:500), S-100 (1:300), P-LIMK1-Thr508 (1:1000), P-LIMK2-Thr505 (1:1000) and P-LIMK1(Thr508)/LIMK2(Thr505) (1:200) used in mouse and HEI-193 cell lines, P-aurora (1:1000), aurora A (1:500) and β-actin (1:15 000), followed by corresponding secondary antibodies (1:20 000). Western blots were quantified by densitometry using NIH ImageJ software. Intensities were normalized to β-actin-loading controls.

LIMK1 and LIMK2 knockdown in *Nf2*^{ΔEx2} MSCs

Nf2^{ΔEx2} MSCs were plated onto poly-L-lysine-coated (200 µg/ml) six-well dishes, cultured at 37 °C, 7% CO₂, in medium containing DMEM/F12 (Invitrogen/Life Technologies, Grand Island, NY, USA) plus N2 supplement (Invitrogen, Burlington, ON, Canada) and 1% penicillin/streptomycin. When cultures reached 40% confluence, lentiviral transduction particles were added at five multiplicity of infection in growth medium plus 8 µg/ml

hexadimethrine bromide (Sigma). MISSION shRNA lentiviral particles for mouse gene silencing were from Sigma-Aldrich. LIMK1 clone IDs: NM_010717.1-1546s1c1 (named construct no. 44); NM_010717.1-1414s1c1 (no. 47) and LIMK2 clone IDs: NM_010718.1-1409s1c1 (no. 73) and NM_010718.3-827s21c1 (no. 95). Negative control was pLKO.1-puro non-mammalian shRNA (SHC002V). Optimum multiplicity of infection determined empirically with pLKO.1-puro-CMV-TurboGFP transduction lentiviral particle (SHC003V) titer. After 18–20 h, infection medium was replaced with fresh medium for 24 h and subsequently replaced with growth medium containing 1 µg/ml puromycin (Sigma-Aldrich) to select transduced cells. The puromycin concentration was determined by plotting a toxicity curve.

Reintroduction of NF2 into *Nf2*^{ΔEx2} MSCs

To reintroduce merlin into *Nf2*^{ΔEx2} MSCs, 1.5–2 million cells were resuspended in 100 µl of basic nucleofector solution for mammalian glial cells with supplements (Lonza, Cologne, Germany) plus 1 µg of pFC15K HaloTagCMVd1-NF2 using an Amaxa Nucleofector II device and Program A-033. One milliliter of warm growth medium was added, and 50 µl of cell suspension was seeded on coated 11-mm round German glass coverslips (Carolina Biology, Burlington, NC, USA) containing 100 µl of equilibrated growth medium and incubated 40 h at 37 °C, 7% CO₂, before analysis.

Immunofluorescence

Cells were plated on coated German glass coverslips and immunostained as previously described.⁴¹ Labeling of Halo-Tag-NF2-transfected cells was done *in vivo* with HaloTag TMR Ligand (Promega, Madison, WI, USA) following the manufacturer's rapid labeling protocol. Confocal images were acquired with a Zeiss (Carl Zeiss MicroImaging Inc., Thornwood, NY, USA) LSM710 microscope with three spectral detection channels, five laser lines—458, 488, 514, 543 and 633 nm, FL filter set 49 DAPI (4',6-diamidino-2-phenylindole), EX G365 shift free, FL filter set 43 CY 3 shift free, FL filter set 38 Endow GFP, shift free, Plan-Apochromat × 63/1.40 oil DIC M27 and EC Plan-Neofluar × 40/1.3 DIC WD = 0.21 M27 objective lenses on an AxioObserver Z1 Stand (Carl Zeiss MicroImaging Inc.) and ZEN2009 software (Carl Zeiss MicroImaging Inc.). Fluorescence was collected from a single plane on separate channels maintaining the same acquisition parameters for each labeled protein. Images from each experiment were processed identically with ZEN2011 software (Carl Zeiss MicroImaging Inc.). For spindle images, Z-stacks were collected with the × 63 oil objective and 3.5 zoom factor. Two-dimensional images were obtained by median filter and maximum intensity projection with ZEN2011.

Cell viability assay

Cell viability was assessed by CellTiter-Fluor assay (Promega) following the manufacturer's specifications. Cells were seeded in 384-well format, centrifuged 1 min at 500 r.p.m. and incubated at 37 °C, 7% CO₂. After cell attachment (2.5–3.5 h), 5 µl/well of compound/vehicle solution was added and plates were quick spun and incubated for 24 h. Fluorescence was measured with a Synergy H1 Hybrid plate reader (BioTek, Winooski, VT, USA).

Caspase activity assay

Caspase activity was assessed by the Apo-ONE homogeneous caspase 3/7 assay (Promega) following the manufacturer's specifications. Fluorescence was measured with a plate reader.

Membrane asymmetry assay

Cell membrane asymmetry was measured with the Violet Ratiometric Membrane Asymmetry Probe/Dead Cell Apoptosis Kit (Invitrogen/Life Technologies). Cells were seeded in 200 µg/ml poly-L-lysine-coated six-well plates and incubated with BMS-5/vehicle for 24 h. Cell were harvested and assayed following the manufacturer's instructions. Samples were analyzed on a BD Canto-II (Becton Dickinson and Co, Franklin Lakes, NJ, USA) flow-cytometer setting excitation and collection emission wavelength recommended by the manufacturer. The ratio parameter was established using BD FACSDiva 6.1.3 software as recommended by the manufacturer.

Proliferation assays

Cell proliferation was studied with two different techniques. *Nf2*^{ΔEx2} MSCs were seeded in a 24-well format and had cell numbers assessed with the Crystal Violet Assay as previously described.⁴² Absorbance at 595 nm was measured with a µQuant plate reader (BioTek).

Rate of DNA synthesis was evaluated at 24 h with the Click-iT EdU Microplate Assay (Invitrogen/Life Technologies). The assay was carried out in 96- and 384-well plate formats following the manufacturer's instructions without the Amplex UltraRed amplification steps. 4',6-Diamidino-2-phenylindole stain was included in the wash after the reaction cocktail incubation step. Oregon green-488 and 4',6-diamidino-2-phenylindole fluorescence was quantified by a plate reader.

Cell cycle analysis

The cell cycle was analyzed by flow cytometry with PI staining and BrdU/7AAD. Ethanol-fixed cells (10^6) were PI stained using 500 µl PI/RNase staining buffer (Beckton Dickinson). Samples were examined on a BD Canto-II flow cytometer, and histograms were analyzed using ModFit LT software (Verity Software House, Topsham, ME, USA). For each sample, 10 000 events were collected.

The BrdU/7AAD assay kit was from Beckton Dickinson. Cells were seeded in six-well plates and treated with inhibitor/vehicle overnight. Next day, cultures were incubated with 10 µM BrdU for 3 h, and the assay was performed according to the manufacturer's instruction. Samples were analyzed with a BD Canto-II flow cytometer using the BD FACSDiva 6.1.3 software. FlowJo software (Tree Star, Inc, Ashland, OR, USA) was used for analysis of acquired data.

Statistical analysis

GraphPad Prism version 5.0 for Windows (GraphPad, La Jolla, CA, USA) was used for statistical analysis and graph generation of experimental data from three independent experiments. Dose-response experiments were analyzed by nonlinear regression (four parameters). Statistical analysis is indicated for each experiment.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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