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14. ABSTRACT

We have a manuscript accepted that demonstrates the role of NF1in endothelial cell function. We have validated siRNA, antibodies, and inducible shRNA specific for individual Ras isoforms and used them to measure the regulation of Ras isoforms following loss of NF1 and their contributions to endothelial regulation, as well as the sufficiency of these isoforms to mimic the loss of NF1. As mTOR is critical to NF1 regulation, we investigated the role of this axis in endothelial cells. Lastly our attempts to create an in-vivo model of NF1 disease in the vasculature continue. We have succeeded in obtaining highly penetrant endothelial specific recombination. We have also bred this mouse onto the NF1 flox/flox background and evaluation of this phenotype is ongoing. We have in place a number of staining protocols and isolation procedures for biochemical analysis which should accelerate the determination of the effects in these mice. While this aspect of our proposed work has moved slower than anticipated, we are confident we now have the basis for an endothelial cell specific model of NF1.

15. SUBJECT TERMS
Endothelial, NF-1, Ras, vasculopathy, molecular, signaling

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Introduction:

NF1 and Vascular Disease

Mutations in the *NF1* gene cause Neurofibromatosis type 1, an autosomal dominant disease that affects approximately 1 in 3000 individuals, making it one of the most common inherited genetic disorders. The autosomal dominant genetic inheritance pattern, as well as the mutational predisposition of this locus makes it highly likely the prevalence of this disease will continue to increase in the population(McClatchey 2007). NF1 has variable clinical manifestations. Most commonly changes in skin pigmentation (café au-lait spots as well as freckling) and the presence of benign and malignant nerve sheath tumors termed neurofibromas, are observed (McClatchey 2007). Importantly, a significant clinical manifestation of NF1 disease can include vascular disease. Patients with NF1 disease make up a significant portion of all those patients presenting with renal artery stenosis and early-onset cerebral vascular disease (Friedman, Arbiser et al. 2002). Moreover, cardiovascular disease is a significant contributor to premature death in NF1 patients and is a particular risk factor among younger patients. One recent study suggested that occurrence of vasculopathy was over seven times more likely to occur in NF1 patients under 30 compared to their unaffected peers.(Rasmussen, Yang et al. 2001)

The manifestation of vasculopathy in NF1 affected individuals can be quite variable. Perhaps the most common is renal artery stenosis and hypertension. The renal artery is also a site of aneurysms and aortic coarctation (Zochodne 1984). Recently a model was developed in the Epstein lab where NF1 was knocked out specifically in the smooth muscle cells of mice using the conditional excision of a floxed allele. The basal morphology and structure of these blood vessels appeared to be normal in these mice. However, in response to injury, these mice had exaggerated responses to vessel injury characterized by smooth muscle cell proliferation and vascular stenosis. These changes were accompanied by activation of Ras signaling, thus Ras driven smooth hyperproliferation is thought to be an important contribution to large vessel stenosis phenotypes(Xu, Ismat et al. 2007).

Another site of frequent vascular involvement is the cerebral circulation. These lesions are often occlusive in nature, perhaps as a result of a pro-thrombotic vasculature. They can also involve nodules of mesodermal cells that occlude the vessel and/or distort the vessel wall. Moyamoya-type lesions with accompanying telangiaectasia are some times seen, as are artiovenous fistulae and aneurysms(Friedman, Arbiser et al. 2002). A frequent and debilitating consequence of many of these changes is vascular hemorrhage. Intracranial hemorrhage is associated with headaches, seizures, loss of motor control, and other neurological deficits. It is important to point out that intracerebral micro-hemorrhage has also been associated with loss of cognitive function(Schneider 2007) and could plausibly be involved with the learning deficits often observed in NF1 patients, though this remains unstudied. The risk of a compromised vasculature is exacerbated by any underlying hypertension, as hypertension itself is a risk factor for cerebral hemorrhage. Indeed hemorrhage is the cause of death in upwards of 50% of all patients who die with NF1 associated cerebrovascular disease(Friedman, Arbiser et al. 2002).

Thus, a better understanding of the role of NF1 in the vasculature is an essential requirement in designing better tools to diagnose this disease and reduce its morbidity.

Our Hypothesis and Previous work:

As noted above, numerous pieces of evidence suggest that NF1 plays an important role in regulating the vascular endothelium. NF1 is clinically associated with multiple vasculopathies including malformations, aneurysms, hypertension and consequently there is a markedly elevated risk of cerbrovascular accidents(Friedman, Arbiser et al. 2002). Mouse models have shown that endothelial-selective ablation of NF1 results in embryonic lethality(Gitler, Zhu et al. 2003), which can be rescued by expression of the GRD of NF1(Ismat, Xu et al. 2006), suggesting Ras activation is critical. Our published data have determined that Ras is required for endothelial responses to VEGF(Meadows, Bryant et al. 2001) and that overexpression of an activated allele of Ras is sufficient to drive multiple angiogenic phenotypes(Meadows, Bryant et al. 2004). Consistent with this, NF1 haploinsufficient mice show exaggerated angiogenic responses(Wu, Wallace et al. 2006) and data have been published suggesting that shRNA mediated knockdown of NF1 can result in Ras activation and activation of downstream signaling in endothelial cells(Munchhof, Li et al. 2006). *Our central hypothesis is that NF1 mediated activation of Ras is a critical mediator of vasculopathy associated with NF1 disease.* We will present data that suggests Ras activation phenocopies NF1 deficiency in cell culture models. Furthermore, we find that inducible expression of activated Ras in the adult mouse results in intracranial hemorrhage. However, to date mechanistic details regarding how NF1 regulates endothelial cell function are missing. We will investigate the molecular regulation of endothelial cell function by NF1 with two Specific Aims: *1) Investigate the relationship between NF1 and Ras isoforms in primary human endothelial cells in vitro; and 2) Determine the consequences of NF1 loss on the vascular endothelium in vivo.*

Key research accomplishments in the first two years include:

- Creating human endothelial cells with drug inducible knockdown of NF1
- Characterization of signal transduction following knockdown of NF1
- Consequences on endothelial cell functions following knockdown of NF1
- Linkage of NF1 loss to activation of mTOR signaling and a role for this signal in the functional outcomes of NF1 loss
- Validation of isoform specific antibodies
- Identification of siRNA and shRNA sequences targeting individual Ras isoforms
- Establishment of NF1Flox and VE:Cad cre mouse colonies
- Establishment of histological staining techniques

Below is a summary of our significant findings, progress, and problems encountered in Year 3 of support.

Body:

A major objective of our last year, as outlined in our Statement of Work, is the finalization of our experiments investigating the cellular consequences of NF1 loss in endothelial cells. We have continued building on previous data demonstrating effective NF1 knockdown in endothelial cells with multiple independent constructs. This data was organized for publication and submitted. The abstract of this work is as follows:

> *Neurofibromatosis is a well-known familial tumor syndrome, however these patients also suffer from a number of vascular anomalies. The loss of NFl from the endothelium is embryonically lethal in mouse developmental models, however little is known regarding the molecular regulation by NF1 in endothelium. We investigated the consequences of losing NF1 expression on the function of endothelial cells using shRNA. The loss of NF1 was sufficient to elevate levels of active Ras under nonstimulated conditions. These elevations in Ras activity were associated with activation of downstream signaling including activation of ERK, AKT and mTOR. Cells knocked down in NF1 expression exhibited no cellular senescence. Rather, they demonstrated augmented proliferation and autonomous entry into the cell cycle. These proliferative changes were accompanied by enhanced expression of cyclin D, phosphorylation of p27KIP, and decreases in total p27KIP levels, even under growth factor free conditions. In addition, NF1-deficient cells failed to undergo normal branching morphogenesis in a co-culture assay, instead forming planar islands with few tubules and branches. We find the changes induced by the loss of NF1 could be mitigated by co-expression of the GAP-related domain of NF1, suggesting Ras regulation was responsible for these changes. Inducible knockdown of NF1 shows that the morphogenic changes are reversible upon removal of the shRNA induction. Similarly, in fully differentiated and stable vascular-like structures, the silencing of NF1 results in the appearance of abnormal vascular structures. Finally, the proliferative changes and the abnormal vascular morphogenesis are normalized by low-dose Rapamycin treatment. These data provide a detailed analysis of the molecular and functional consequences of NF1 loss in human endothelial cells. These insights may provide new approaches to therapeutically addressing vascular abnormalities in these patients while underscoring a critical role for normal Ras regulation in maintaining the health and function of the vasculature.*

This manuscript has been accepted for publication pending minor revisions and we have attached the manuscript in its entirety to this report (see page 17).

In the course of these studies we conducted many experiments in control cells to understand the mTOR interventions we were employing to inhibit mTOR in the NF1 cells. This generated novel results and findings that we have split off into a separate manuscript that we are preparing to submit in the coming week. The abstract for that paper reads as follows:

> *Angiogenesis is important for embryogenesis, wound healing and is a critical element in cancer progression. The mammalian target of rapamycin (mTOR) is a serine threonine kinase, that exists in two distinct signaling complexes: mTORC1 and mTORC2. Aberrant mTOR signaling is often associated with cancer pathologies including renal cell carcinoma, tuberosclerosis, and*

neurofibromatosis, which are known to have associated hypervascularity and other dysfunctional vasculature. The regulation of this axis is also disturbed by metabolic abnormalities such as diabetes, with known vascular complications. Thus mTOR may be a significant regulator of endothelial cell functions, including angiogenesis. Given the connections between angiogenesis, vascular dysfunction, and mTOR signaling, we investigated the role of mTORC1 in vascular endothelial cells. We identified a dose of rapamycin (an allosteric inhibitor of mTORC1) for selective inhibition of mTORC1/S6 without disrupting mTORC2/Akt signaling. In addition, we have developed inducible shRNA to knockdown the requisite TORC1 component, Raptor. We find that mTORC1 inhibition affects developing vessels but not established vessels. These effects are largely due to an mTORC1-dependent contribution to cellular proliferation. Our data suggest a role for mTORC1 in the phosphorylation and destabilization of the cyclin-dependent kinase inhibitor, $p27^{Kip}$. We find identical results using both methods to interfere with TORC1. These data suggest that drugs acting specifically on mTORC1 may provide selective anti-angiogenic activity on developing vasculature through modulation of endothelial cell proliferation, with minimal direct effect on cell survival. These results are of value in developing strategic therapeutic interventions in inherited and pathological conditions where mTOR signaling is enhanced in endothelial cells and contributes to vascular dysfunction.

The figures for this paper are included in this report and comprise Figures 1-8, found on page 8-9.

Another major objective in Aim one of the proposal was to investigate the role of individual Ras isoforms in the endothelial cells, particularly with respect to the loss of NF1. We have successfully knocked down specific isoforms of Ras in primary endothelial cells and as shown in Figure 9, we find that the N-Ras isoform is the primary isoform regulated by NF1, while knockdown of the H or K-Ras has little effect of Ras-GTP levels in NF1 knockdown cells. This finding was confirmed when we performed double knockdown experiments such as those shown in Figure 10. Similarly, we find that signal transduction that is elevated following the loss of NF1, is only significantly affected by the loss of N-Ras, as shown in Figure 11, and in agreement with the GTP loading experiments. To investigate the Ras isoforms in longer term experiments, and to confirm our findings with siRNA, we also developed lentiviral vectors to knockdown Ras isoforms with shRNA in a stable fashion. An example of the knockdown of these isoforms is shown in Figure 12, alone and in combination. When we performed experiments with these vectors in normal endothelial cells, the formation of vascular structures did not rely on N-Ras or H-Ras, but rather was dependent upon K-Ras, as shown in Figure 13. In the absence of K-Ras, cells did not appear to elongate and anastomose properly. In agreement with these findings we found that even in the presence of double knockdown of H and K-Ras, normal vascular structures could form (Figure 14). As NF1 activated N-Ras preferentially, we also wanted to test the consequences of N-Ras activation compared to other isoforms. We used lentiviral infection with viruses that expressed activated mutants of Ras under inducible control as shown in Figure 15. Endothelial cells, infected with these constructs all made normal tubes under non-induced conditions, however upon expression of the active mutant, changes in vascular morphogenesis were noted, Figure 16. Interestingly activation of H-Ras had the most profound phenotype, completely abrogating vascular morphogenesis.

In contrast K-Ras, which is essential as shown in Figures 13 and 14, had no significant effect. Consistent with our results with NF1, activation of N-Ras gave an intermediate phenotype with significant defects in vascular morphogenesis, but with some capacity to still sprout tubes. Quantitation of numerous images from multiple experiments is ongoing, as is the replication and analysis of a set of experiments investigating the effect of N-Ras knockdown on endothelial cell phenotypes induced by NF1 loss. Collectively these experiments will form the basis for a third manuscript submission this fall, during the extension period.

As noted in our original statement of work, we also were developing an in vivo model of NF1 loss in the endothelium. We have had significant delays and problems with this, largely as a consequence of a published strain of mice that we proposed to use originally to provide the endothelial specific excision of NF1 not performing in adult mice consistent with the published data. After trying different induction protocols , we bred in an integrated reporter strain, in the hope that we could track affected cells. However a preliminary experiment revealed no obvious phenotype and only sparsely detectable cells. We realized this was not going to allow an efficient evaluation. Fortunately, in the last year we have been successful in importing another recently available inducible, endothelial-specific Cre strain and establishing a colony. We have bred that strain onto the integrated Cre-reporter strain, ROSA26-LSL-Td-Tomato, which upon successful recombination expresses the red fluorescent protein td-Tomato. As shown in Figure 17, upon tamoxifen treatment we can demonstrate highly penetrant expression (approximately 80-85%) of td-tomato that co-localizes with endothelium as indicated by CD31 staining. Thus for the first time we have a model where we can induce recombination of a floxed gene with high efficiency in the adult mouse (embryonic recombination of NF1 is known to be lethal). If the loss of endothelial cell loss of NF1 results in a phenotype, we are confident that it will detectable in this mouse model. As such we have begun the cross breeding to the $NF1^{flox}$ and have generated mice with all three desired alleles, as shown in Figure 18. We are hopeful that we will have a least a basic characterization of the $NF1^{flox/flox}$ mouse by the termination of the grant term.

The optimism is at least bolstered by the fact that we are well prepared to characterize the vascular phenotype of the mouse. We have worked out all of the histological staining to detect Ki-67, CD31, phospho-ERK, Phospho-S6, inflammatory cells, red blood cells, basement membrane, and pericytes. We have learned how to prepare retinal whole mounts, a technique that is very sensitive for the detecting abnormalities in vascular patterning. In addition, we learned how to isolate purified endothelial cells from the lung and culture them, as well as generate microvessel fragments from the brain, where we anticipate a phenotype may reveal itself. These microvessels, shown in Figure 19, allow biochemical characterization of a vascular enriched component following rapid isolation. We have verified that these can be used for the detection of active Ras and Ras related signals (as shown in Figure 20 and 21.) These will all be necessary analysis in evaluating the effects of NF1 loss in the vasculature of the mouse model. We have applied for additional funding to continue these studies from both DOD programs as well as NIH.

Key Accomplishments in Year 3:

- Completion a manuscript detailing analysis of cellular signaling and function in response to NF1
- Completion of analysis and preparation of a manuscript detain the role of mTOR in endothelial cell function
- Finalization of studies using shRNA that implicate N-Ras as the responsible Ras isoform downstream of NF1 loss but K-Ras as an essential contributor to normal vascular morphogenesis.
- Determination that N-Ras hyperactivation is sufficient to induce abnormal vascular morphogenesis
- Final determination to abandon the breeding of the VE-Cad:CRE-ERT^2 strain due to poor/hard to document recombination
- Importation of mouse, colony establishment and demonstration that CAD5-CRE^{ERT2} gives high efficiency recombination
- Establishment of techniques such as cell purification and micro-vessel fragment isolation to permit ex vivo analysis of biochemical properties of NF1 flox/flox endothelium

Reportable Outcomes:

Loss of NF1 Expression in Human Endothelial Cells Promotes Autonomous Proliferation and Altered Vascular Morphogenesis Bajaj, A., Li-Q-F, Zheng, Q. and Pumiglia, K. PLOS One (pending minor revisions)

TORC1 has a Critical Role in Endothelial Cell Proliferation and Morphogenesis Hakin, S and Pumiglia, K PLOS One (to be submitted 8/2012)

We plan on attending the North American Vascular Biology Meeting in California where we will present our data on the Ras isoforms. In addition, a manuscript detailing the role of Ras isoforms in the effects seen following loss of NF1 is currently being prepared for submission in the Fall of 2012.

Conclusions:

We have completed essentially all of the experiments originally proposed in the "*Statement of Work*". We continue to replicate these data in a few instances to prepare some of these data for publication or obtain sufficient representative data for quantification. One manuscript has been accepted (pending minor revisions) and another is to be submitted in the week. A third should be submitted this fall. We believe these are the most comprehensive and molecularly sophisticated studies to date on the role of NF1 in endothelial cells or in any cell. Our studies provide a broad view of the range of endothelial cell functions impacted by NF1 knockdown. They directly implicate N-Ras as the critical player in these abnormal signaling events. In addition they directly implicate mTORC1 directly as a critical molecular signal in the pro-growth and vascular dysmorphogenic phenotypes. This information may be highly critical as there are

numerous FDA approved therapeutics available to target this pathway. A more comprehensive understanding of the relationship of mTOR signaling to vascular endothelial dysfunction in NF1 patients may lead to novel ways to intervene medically in NF1 associated vasculopathies. The isoform studies will also prove beneficial to NF1 patients, as Ras isoforms are differentially affected by several classes of drugs including the widely prescribed “statins”, as well as several experimental compounds. A better understanding of the relationship between the Ras isoforms and dysfunction mediated by loss of NF1, will provide guidance on whether some of these regimens might be beneficial (or harmful) to NF1 patients. Lastly, the ultimate test of many of our hypotheses regarding vascular dysfunction in NF1 patients will be in the complex tissue microenvironment. This requires *in vivo* experimentation. We have now have animals capable of high efficiency recombination and have generated breeding stock with all three required alleles. While these studies have progressed slower than we wished, they will provide the first animal model of NF1 loss in the mature vascular endothelium. This animal model will lead to novel insights into this important disease.

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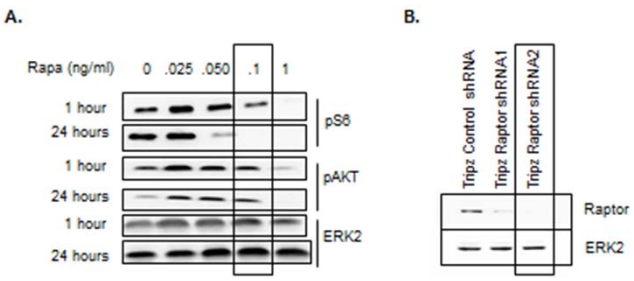

Figure 1. ***Rapamycin selectively inhibits mTORC1 low doses*** A) Huvecs were treated with various doses of rapamycin and cell lysates were probed for p-S6 and p-Akt at indicated times. B) Normalized expression of p-S6 and p-Akt reported as means of +/- standard error of three experiments.

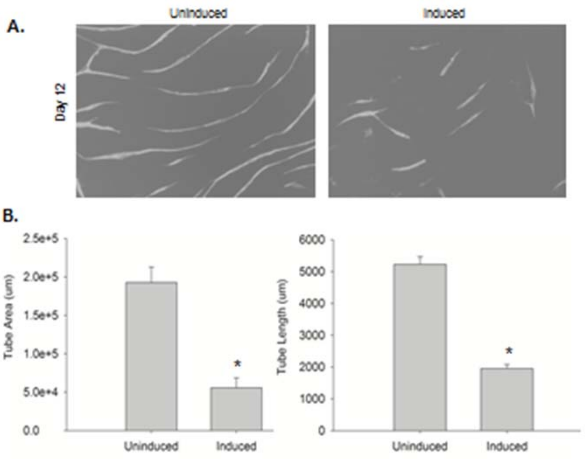

Figure 3. ***Raptor knockdown decreases growth of developing vessels*** A) *In vitro* co-culture assay showing endothelial cells expressing TripZ raptor shRNA in either induced or uninduced conditions. Raptor knockdown was induced with doxycycline from day 0 and developed for 12 days. Images (gray scale, 8 bit) were taken at day 12 for both conditions. C) Total tube area and length in micrometers (µm) from three similar experiments. Columns, reported as mean of +/- standard error of three experiments; bars, SE. *, p < 0.01.

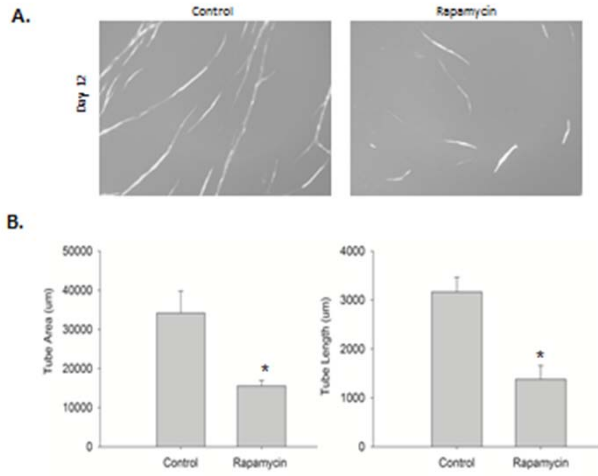

Figure 2 ***Selective inhibition of mTORC1 decreases growth of developing vessels.*** A) *In vitro* co-culture assay showing cells treated with rapamycin from day 0 and developed for 12 days. Endothelial cells expressing RFP were used for this assay. Images (gray scale, 8 bit) were taken at day 12 for both control and rapamycin treated conditions. B) Total tube area and length in micrometers (µm) from three similar experiments. Columns, reported as means of +/- standard error of three experiments; bars, SE. *, p < 0.01.

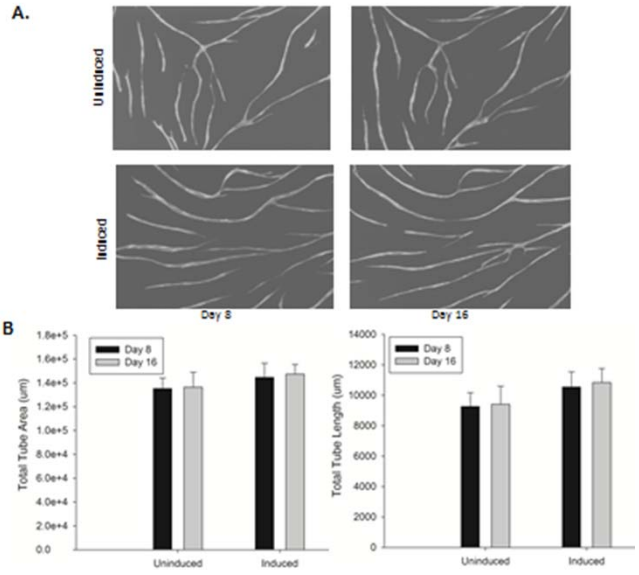

Figure 4 ***TORC1 inhibition does not affect established vessels.*** A) *In vitro* co-culture assay showing vessels developed in normal condition for 8 days and then treated with doxycycline to induce Raptor shRNA, along with un-treated control. Endothelial cells expressing RFP were used for this assay to visualize vascular structures. Images (gray scale, 8 bit) from the same field were taken at day 8 and 16 for both control and Raptor knockdown. B) Total tube area and length in micrometers (µm) from three similar experiments. Columns, reported as mean of +/- standard error of three experiments; bars, SE.

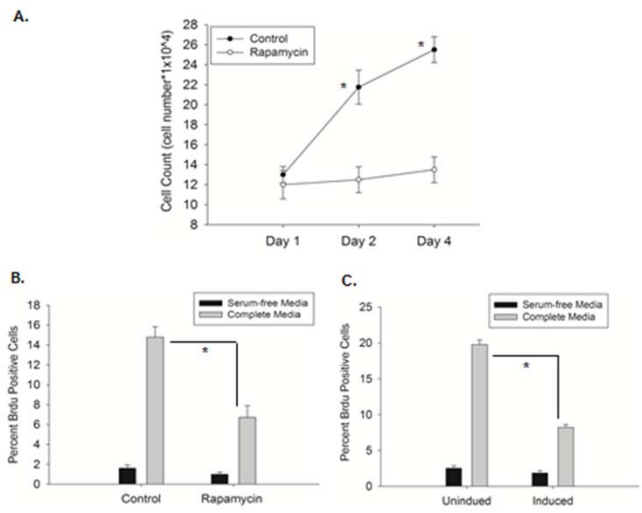

Figure 5 ***TORC1 is required for endothelial cell growth and proliferation***. A) Endothelial cells at indicated times in complete media and counted after trypsinization at day 1, 2 and 4 using cell counter. Points, mean of triplicate determinations from three experiments; bars, SE. *, $p < 0.01$. B) Normalized percent Brdu positive cells in both MCDB serum free media and complete media for both conditions. (C) Identical experiemnt to that in (B) except TORC1 inhbition achieved by induction of Raptor shRNA. Columns, reported as mean of +/- standard error of three experiments; bars, SE. *, $p < 0.01$.

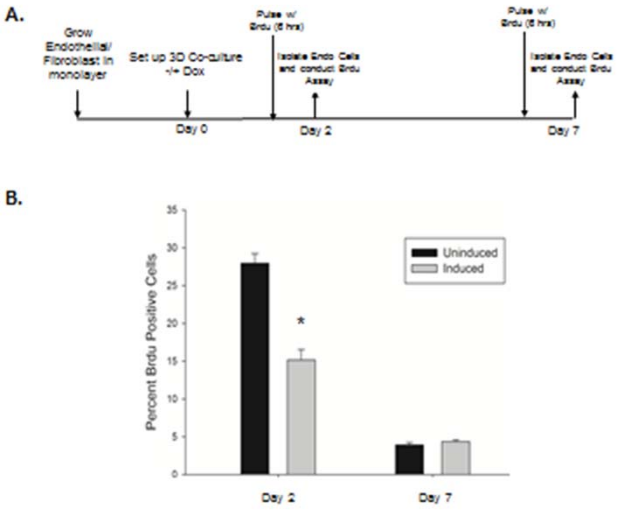

Figure 6 ***Raptor knockdown decreases endothelial cell proliferation in a co-culture model.*** A) A schematic showing timeline of the experiment presented in panel B and C. B) Normalized percent Brdu positive cells at day 2 and 7 for both conditions from three experiments. Columns, reported as mean of +/- standard error of three experiments; bars, SE. *, $p < 0.01$ at day 2.

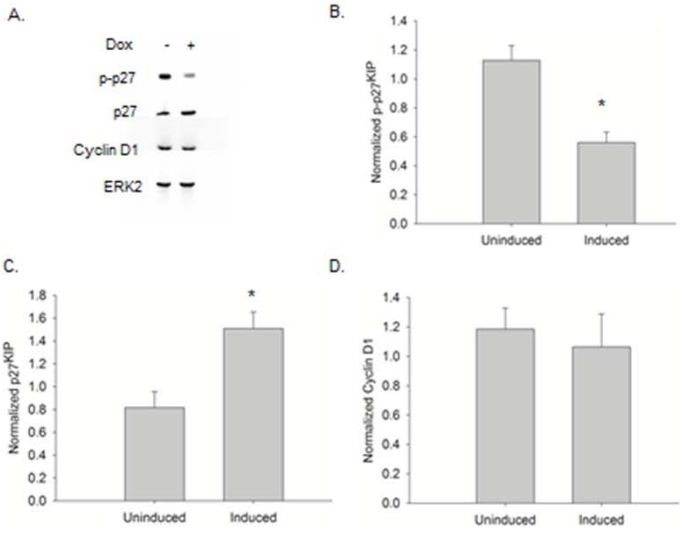

Figure 7 ***Selective mTORC1 inhibition decreases phosphorylation and accompanying stabilization of $p27^{Kip}$ without affecting cyclin D1 level*.** A) Huvecs were treated with doxycycline to induce Raptor shRNA for 48 hours and cell lysates were probed for p-p27, p27 and cyclin D1. B-D) Normalized p-p27, p27 and cyclin D1 reported as means of +/- standard error of three experiments.

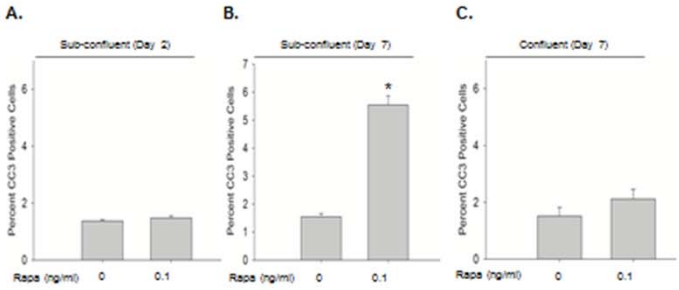

Figure 8 ***Rapamycin induces cleaved caspase 3 (CC3) in exponentially growing cells in a time dependent manner.*** A) Percent of cells positive for CC3 after rapamycin treatment for 2 days when cells were at a sub-confluent state. Columns, reported as mean of +/- standard error of three experiments; bars, SE. B) Percent of cells positive for CC3 after rapamycin treatment for 7 days when cells were at a sub-confluent state. Columns, reported as mean of +/- standard error of three experiments; bars, SE. *, $p < 0.01$ C) Percent of cells positive for CC3 when cells were treated with rapamycin for 7 days at a confluent state. Columns, reported as mean of +/- standard error of three experiments; bars, SE.

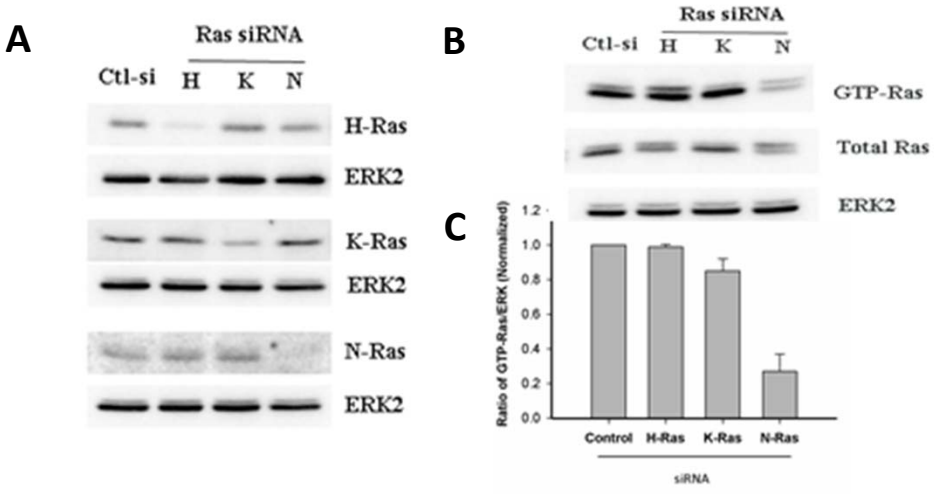

Figure 9. ***Knockdown of Ras isoforms reveals regulation of N-Ras by NF1*** (A) HUVECs expressing NF1 shRNA were electroporated with isoform specific siRNA or control and evaluated for knockdown with isoforms specific antibodies of (B) the presence of active Ras using affinity pull-down assay. (C) Results from three experiments are normalized and quantified.

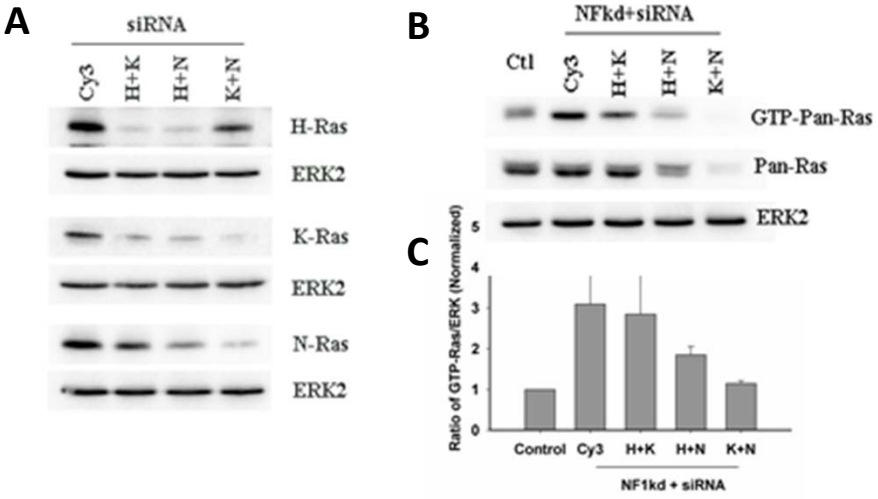

Figure 10. ***Double knockdown of Ras isoforms reveals regulation of N-Ras by NF1*** (A) HUVECs expressing NF1 shRNA were electroporated with combinations of isoform specific siRNA or control and evaluated for knockdown with isoforms specific antibodies of (B) the presence of active Ras using affinity pull-down assay. (C) Results from three experiments are normalized and quantified.

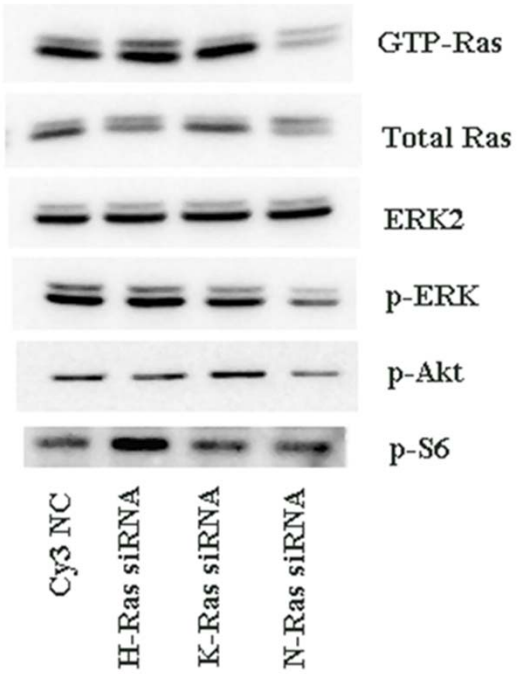

Figure 11. ***Knockdown of N-Ras ablates activation of signals induced by loss of NF1*** (A) HUVECs expressing NF1 shRNA were electroporated isoform specific siRNA or control and evaluated for activation of Ras GTP and downstream effectors such as ERK, AKT and phospho-S6 using phospho-specific antibodies.

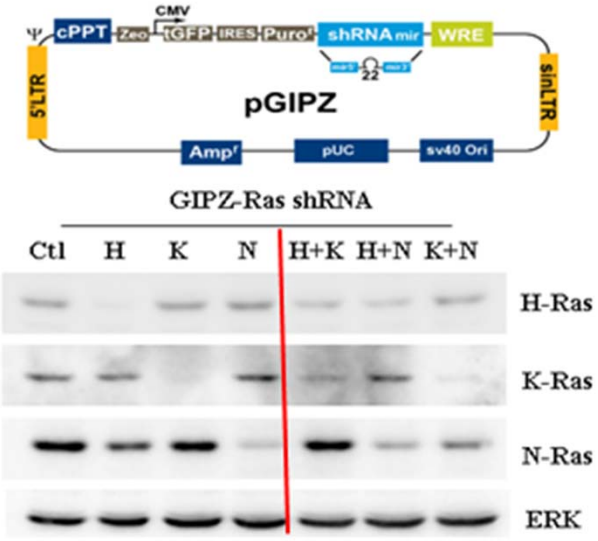

Figure 12 ***Stable knockdown of Ras isoforms in primary HUVECs*** (A) HUVECs were infected with lentiviruses coding for isoform specific shRNA or control and evaluated for knockdown with isoforms specific antibodies alone or in combination (right side of red line).

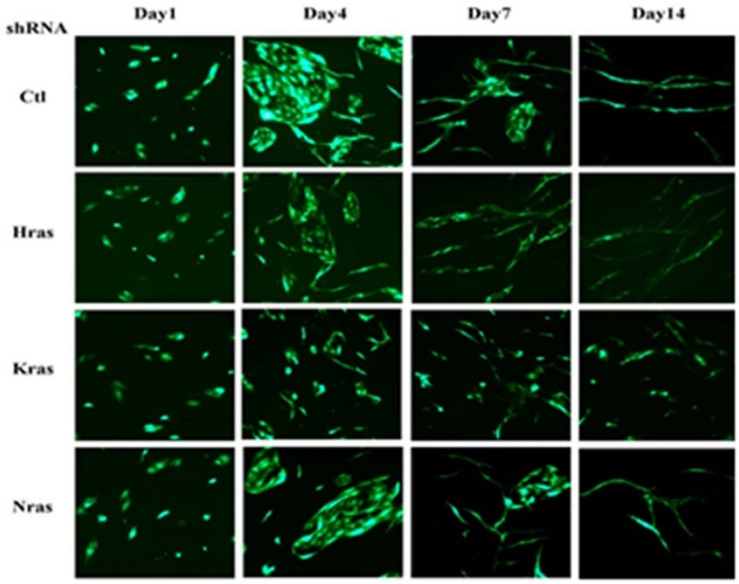

Figure 13. ***K-Ras is required for vascular morphogenesis*** Cells infected with lentiviruses designed with shRNA to knockdown Ras isoforms were analyzed using an endothelial-fibroblast co-culture assay for the times indicated.

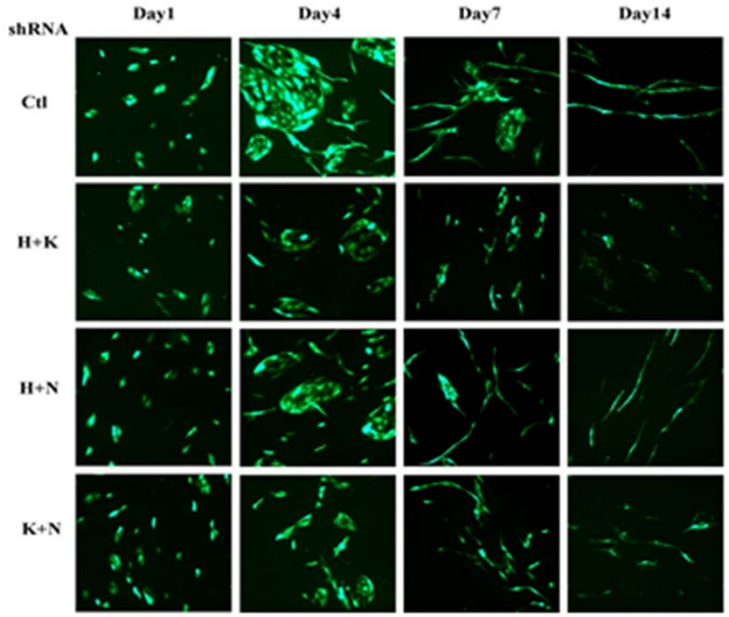

Figure 14. ***K-Ras is sufficient for vascular morphogenesis*** Cells were double infected with lentiviruses designed with shRNA to knockdown Ras isoforms were analyzed using an endothelial-fibroblast co-culture assay for the times indicated. Cells knocked with both H and N isoforms are still capable of forming tubes.

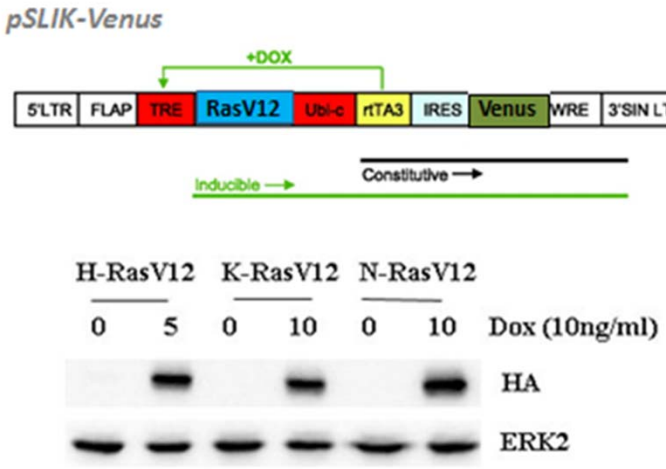

Figure 15. ***Inducible expression of activated isoforms of Ras.*** HUVECs were infected with lentiviruses designed to express specific Ras isoforms under doxycycline inducible control (Dox). All isoforms carry an activating mutation at valine 12 and all are epitope tagged to insure equal expression across isoforms.

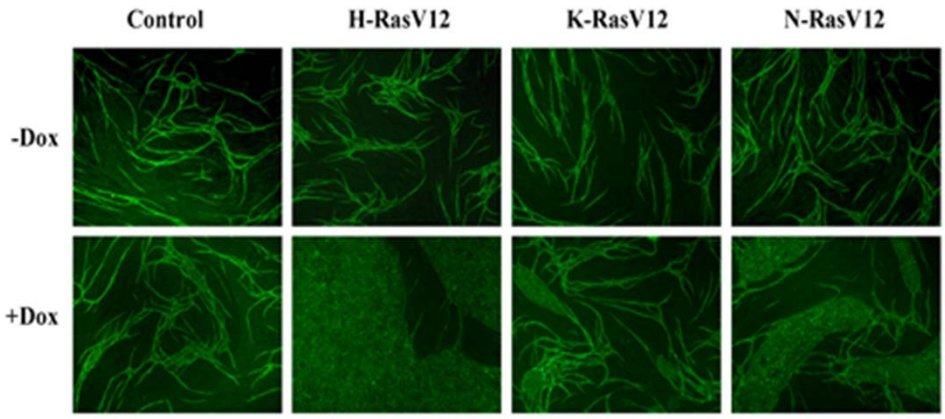

Figure 16. ***H-Ras preferentially alters vascular morphogenesis.*** HUVECs were infected with lentiviruses designed to express specific Ras isoforms under doxycycline inducible control (Dox). Cells were placed in co-culture with primary fibroblasts and induced with doxycycline to express the mutant Ras or left uninduced for 14 days. Detection of vascular structures is by labeling with FITC-UEA-1 lectin. Micrographs were taken at 40X magnification.

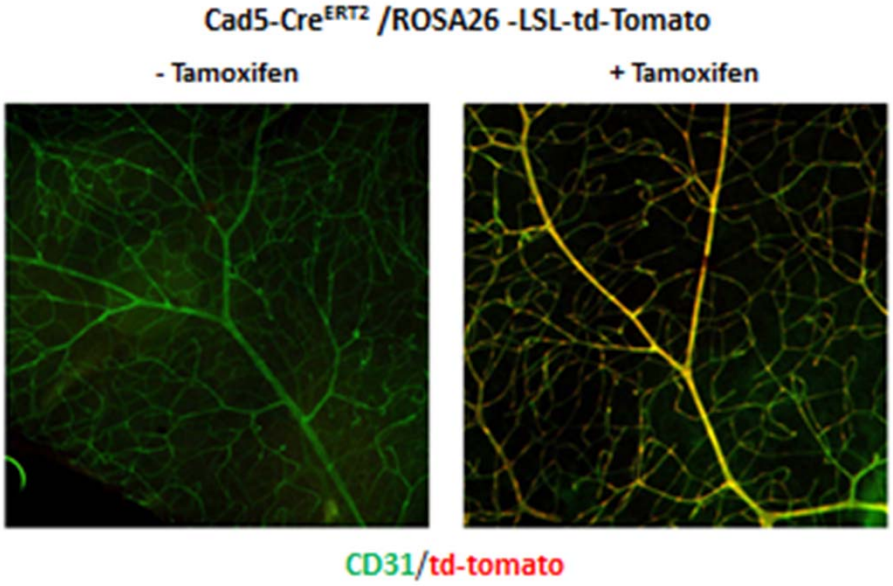

Figure 17 ***Endothelial Specific, Tamoxifen-inducible CRE expression.*** Cad5-Cre^{ERT2} /ROSA26 -LSL-td-Tomato mice were used to test penetrance and specificity of endothelial specific cre expression. Induction with Tamoxifen for 3 days was followed by a waiting for 2 days and then sacrifice. Retinal whole mounts were analyzed by CD31 staining to detect all endothelial cells and red fluorescence from the Cre-responsive reporter, td-Tomato.

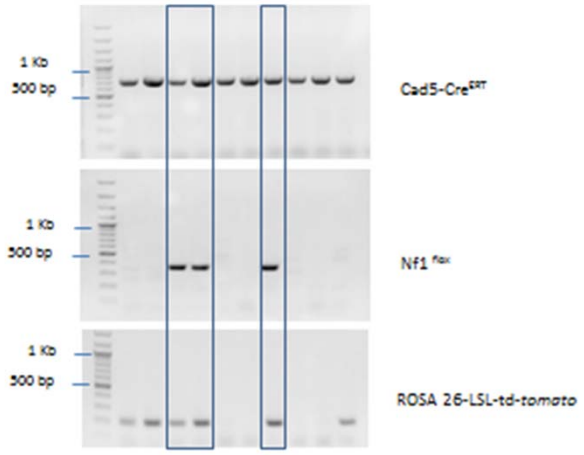

Figure 18 ***Generation of Endothelial Cell Specific, NF1 Deletion Strain***. Endothelial specific Cad5-CreERT was mated with NF1 flox and ROSA26 –td-Tomato mice to generate mice carrying all three alleles, as highlighted by the blue boxes. These mice will be used to breed with similarly produced mice with an NF1 +/- background for the proposed experiments.

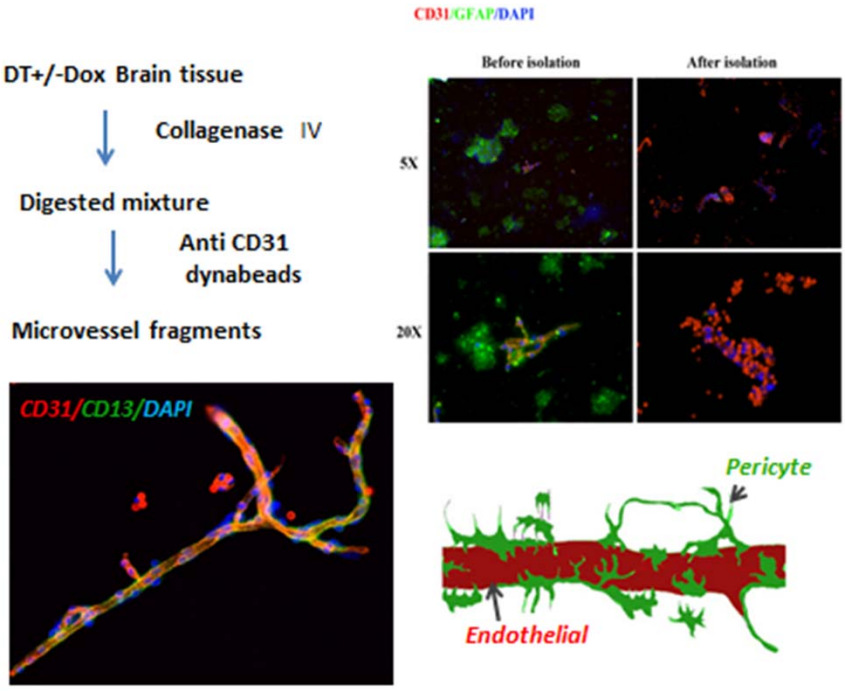

Figure 19 Isolation of microvessel fragments from brain Using a combination of selective digestion and immunoisolation we are to separate intact microvessel fragments away from neurons, astrocytes (GFAP) and microglial cells of the brain. This permits biochemical analysis of signaling and gene transcription.

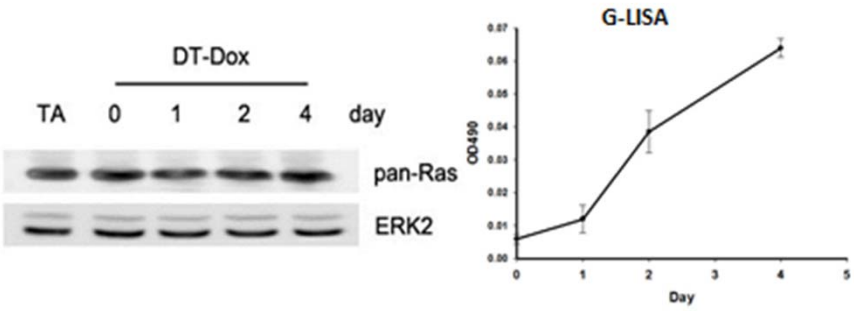

Figure 20 Activation of Ras in microvessel fragments from brain We prepared microvessel fragments from control mice or from bi-transgenic mice expressing activated Ras only in the endothelium upon dox removal. Doxycline was removed from the diet for the days indicated and microvessel fragments were isolated as indicated above. Microvessels were lysed and analyzed for Ras activation using a G-lisa assay, an affinity assay for GTP-Ras in an ELISA format.

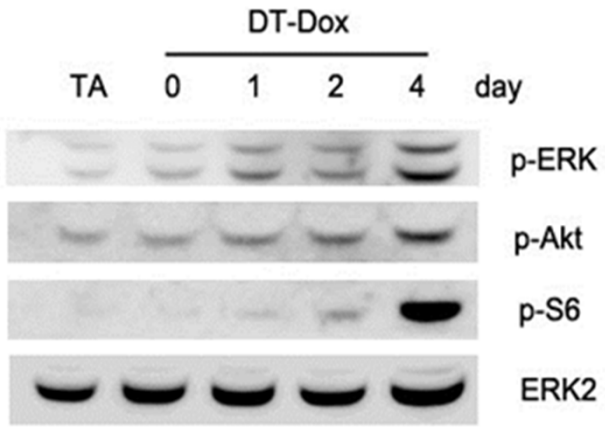

Figure 21 ***Activation of Ras signal transduction in microvessel fragments from brain*** We prepared microvessel fragments from control mice or from bi-transgenic mice expressing activated Ras only in the endothelium upon dox removal. Doxycline was removed from the diet for the days indicated and microvessel fragments were isolated as indicated above. Microvessels were lysed and analyzed for the presence of active Ras-related signaling using phospho-specific antibodies. ERK2 serves as a lysate loading control.

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Loss of NF1 Expression in Human Endothelial Cells Promotes Autonomous Proliferation and Altered Vascular Morphogenesis

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Running title: NF1 is Critical for Endothelial Cell Regulation

Key words: Angiogenesis, Neurofibromin, Ras, Endothelial, Signaling, Morphogenesis, Proliferation

Abstract

Neurofibromatosis is a well known familial tumor syndrome, however these patients also suffer from a number of vascular anomalies. The loss of NFl from the endothelium is embryonically lethal in mouse developmental models, however little is known regarding the molecular regulation by NF1 in endothelium. We investigated the consequences of losing NF1 expression on the function of endothelial cells using shRNA. The loss of NF1 was sufficient to elevate levels of active Ras under non-stimulated conditions. These elevations in Ras activity were associated with activation of downstream signaling including activation of ERK, AKT and mTOR. Cells knocked down in NF1 expression exhibited no cellular senescence. Rather, they demonstrated augmented proliferation and autonomous entry into the cell cycle. These proliferative changes were accompanied by enhanced expression of cyclin D, phosphorylation of $p27^{KIP}$, and decreases in total $p27^{KIP}$ levels, even under growth factor free conditions. In addition, NF1-deficient cells failed to undergo normal branching morphogenesis in a co-culture assay, instead forming planar islands with few tubules and branches. We find the changes induced by the loss of NF1 could be mitigated by co-expression of the GAP-related domain of NF1; suggesting Ras regulation was responsible for these changes. Inducible knockdown of NF1 shows that the morphogenic changes are reversible upon removal of the shRNA induction. Similarly, in fully differentiated and stable vascular-like structures, the silencing of NF1 results in the appearance of abnormal vascular structures. Finally, the proliferative changes and the abnormal vascular morphogenesis are normalized by low-dose Rapamycin treatment. These data provide a detailed analysis of the molecular and functional consequences of NF1 loss in human endothelial cells. These insights may provide new approaches to therapeutically addressing vascular abnormalities in these patients while underscoring a critical role for normal Ras regulation in maintaining the health and function of the vasculature.

Introduction

Mutations in the NF1 gene cause Neurofibromatosis type 1, an autosomal dominant disease that affects approximately 1 in 3000 individuals, making it one of the most common inherited genetic disorders [1]. NF1 has variable clinical manifestations. Most commonly changes in skin pigmentation (café au-lait spots as well as freckling) and the presence of benign and malignant nerve sheath tumors termed neurofibromas, are observed [1]. Importantly, a significant clinical manifestation of NF1 disease includes vascular disease. Patients with NF1 disease make up a significant portion of all those patients presenting with renal artery stenosis and early-onset cerebral vascular disease [2] and cardiovascular disease is a significant contributor to premature death in NF1 patients, particularly among younger patients. One study suggested that vasculopathy was over seven times more likely to occur in NF1 patients under 30 compared to their unaffected peers [3].

NF1 is clinically associated with a pleiotropic array of vascular abnormalities including stenosis, malformations, aneurysms, and hypertension. As a consequence these patients show a markedly elevated risk of cerebrovascular accidents [2]. Previous studies in mice have suggested an important role of smooth muscle cells [4] and bone marrow cells [5] in neointimal hyperplasia, inflammation and exaggerated response to injury including enhanced angiogenesis. However little is currently known about factors contributing to vascular malformations and the role of endothelial cells in regulating these changes. In addition, the endothelium is critically poised to regulate blood vessel formation, vascular tone, inflammation, as well as coagulation, thus a better understanding of the role of NF1 in regulating the function of the vascular endothelium may be critical to understanding many facets of this disease and reducing its morbidity.

Previous studies support the notion that NF1 has a critical role in the vascular endothelium. Deletion of NF1 from the vascular endothelium results in embryonic lethality [6] and NF1 haploinsufficient mice show exaggerated angiogenic responses [7]. As NF1 is a Ras-GTPase activating protein, changes in Ras activation are often associated with the loss of NF1 and data have been published suggesting that shRNA mediated knockdown of NF1 can augment growth factor mediated Ras activation and downstream signaling in endothelial cells [8]. NF1 is a large protein however, with other signaling effects, including changes in cAMP and mTOR, which can be Ras-independent [9]. We have recently published that activation of Ras in primary endothelial cells is sufficient to drive a pro-survival, pro-proliferative phenotype that disrupts normal vascular morphogenesis [10]. It is unclear if loss of NF1 is sufficient to enhance basal activation of Ras and initiate cellular responses in the absence of additional growth factor signaling. We conducted these studies to determine if the loss of NF1 is sufficient to initiate cellular signaling and alter endothelial cell function, to determine the role of Ras and other cellular signals acting downstream of NF1, and to evaluate how these changes affect the behavior of endothelial cells in a complex microenvironment.

Results

Knockdown of *NF1* in primary endothelial cells activates Ras and downstream signaling

To generate endothelial cells lacking neurofibromin a shRNA against NF1 was cloned into pSIREN-RetroQ-ZsGreen retroviral expression vector, as previously described [8] allowing stable knockdown of neurofibromin both at the protein (Fig. 1A) and mRNA levels (not shown). Primary cells have previously demonstrated growth arrest in response to the loss of NF1 [11]. To minimize potential confounding senescence effects, to regulate expression, and also to safeguard against RNAi mediated off-target effects, a different NF1 knockdown sequence was

cloned into a lentiviral pTRIPZ vector that allows for inducible knockdown of the protein in the presence of 0.5 μg/mL doxycycline whereas no knockdown was seen in the absence of doxycycline (Fig. 1B). Using both types of cells we examined the levels of active Ras in quiescent cells. We find that knockdown of NF1 in human endothelial cells using the inducible miR-based shRNA results in increased levels of active Ras under basal conditions (Fig. 1C). Similarly, stable knockdown of NF1 also results in enhanced levels of Ras-GTP (data not shown), even following several passages suggesting limited down-regulation or cellular compensation of this response. The enhanced levels of Ras-GTP were sufficient to trigger downstream signaling, as knockdown of NF1 resulted in enhanced activation of ERK, PI-3'-Kinase/AKT (Fig. 1D), even in the absence of any added growth factors. Another important signaling network linked to tumors and cells lacking NF1 is the mTOR signaling pathway [12,13]. It has been shown that activation of mTOR pathway in nerve sheath tumor cell lines is essential for neurofibroma formation [14]. As the role of NF1 in regulating mTOR signaling in endothelial cells is unknown, we investigated this by examining the phosphorylation of ribosomal S6, a substrate of the TORC1 activated kinase, S6K. We found loss of NF1 expression was sufficient, even the absence of added growth factors, to stimulate mTORC1/S6 signaling. Similar effects were observed with both the inducible and the stable knockdown, suggesting little temporal or kinetic differences between the constructs or approaches. These data provide evidence that knockdown of the Ras-GAP NF1 in human endothelial cells is sufficient elevate cellular GTP-Ras levels and activate downstream signaling in the absence of added growth factors.

Cellular proliferation in endothelial cells lacking NF1

Down-regulation of signaling is part of a feedback response that results in replicative arrest

in primary fibroblasts. However, we see no evidence of signal dampening in endothelial cells lacking NF1 even after prolonged culturing. To determine if the chronic activation of Ras and the related signaling results in replicative senescence, we examined several aspects of cellular growth. Initially, we evaluated population doublings, often used to monitor oncogene-induced senescence. In contrast to the effects observed following NF1-loss in fibroblasts [11], we found no signs of growth arrest in NF1-knockdown cells, rather they showed enhanced proliferative capacity (Fig. 2A). Given the enhanced long-term proliferative capacity of NF1-knockdown cells and the previous report that NF1-knockdown could enhance VEGF-induced proliferation, we next sought to determine if NF1 was sufficient to induce endothelial cell proliferation in the absence of growth factors. As shown in Fig. 2B, even in the absence of added mitogenic factors, endothelial cells lacking NF1 expression demonstrated enhanced entry into S-phase of the cell cycle as measured by BrdU incorporation. The enhanced proliferation was accompanied by upregulation of cyclin D levels, enhanced phosphorylation of p27KIP, and a corresponding decrease in levels of total p27 (Fig. 2C). Thus, the signaling induced by the loss of NF1 is sufficient to stimulate DNA synthesis as a consequence of cyclin D induction and the loss of the Cyclin-dependent kinase inhibitor, p27KIP.

Loss of NF1 is associated with altered vascular morphogenesis

NF1 patients are known to have several distinct types of vascular anomalies associated with the disease, including vascular malformations. At this point it is unclear if this is the result of effects arising in endothelial cells or as a consequence of an altered and pro-angiogenic microenvironment, or both. We reasoned that the altered proliferative control seen upon the loss of NF1 might contribute to altered morphogenic responses by human endothelial cells. To test this we utilized a co-culture assay where human endothelial cells in the presence of human

primary fibroblasts will typically proliferate for a cycle or two followed by a cessation of proliferation and the formation of branched networks of endothelial cell tubules that contain patent lumens [10,15,16]. When we performed this assay we found that endothelial cells lacking NF1 had an altered morphogenic response. In lieu of forming elongated and branched networks, these cells tended to form planar, sheet like structures which showed few elongations and branches, as if an essential signal to "differentiate" into vascular structures was compromised (Fig. 3). Identical phenotypes were seen whether we used constitutive shRNA (pSiren) knockdown or the inducible miR-based construct (TripZ).

Effects on endothelial cells following the loss of NF1 are a consequence of Ras activation

Our data have determined that the loss of NF1 is sufficient to activate cellular Ras. However, NF1 is a large protein with several alternative signaling paradigms through which it can affect cell function in a Ras-independent manner, including changes in cAMP signaling [9,17]. Therefore, we sought to determine if the effects we were seeing were the result of Ras activation. To test this we co- infected endothelial cells with inducible lentiviral vectors to knockdown NF1 and then re-express the GAP-related domain of NF1 (GRD) in order to "rescue" the Ras GAP functions of NF1 (this domain is outside the targeted region). Purified cells were obtained by two-color sorting (Red; TRIP-Z (NF1-KD) co-expressed RFP; Green; pSLIK (GRD) co-expressed YFP). As both vectors are tet-inducible, induction of knockdown also induces the "rescue" in the cells co-infected with the GRD expressing virus. As shown in Fig. 4, the expression of the GRD reverses the Ras activation seen following the loss of NF1, returning it to basal levels. This is accompanied by a similar reversal of the autonomous proliferation (Fig. 4B) that the loss of NF1 promotes. Consistent with this, we found that expression of GRD also reduced the enhanced phosphorylation of p27 that is seen following the loss of NF1 with a

coincident stabilization of this protein (Fig. 4C).

We also used these cells to determine if the Ras modulating properties of NF1 were responsible for the hyperplastic morphogenic responses we observed. As shown in Fig. 5A, co-expression of the GRD reverses the abnormal morphogenesis, with cells forming nicely branched and elongating networks when the GRD domain of Ras is co-expressed. These same cells were used to explore the reversibility of this phenotype. As shown in Fig. 5A, if the NF1-kD cells are allowed to form the hyperplastic structures for 14 days (a time at which normal endothelial cell networks have stabilized) and then the Dox is removed (to cease expression of the silencing shRNA), normal looking tubular branching structures emerge over the next 14 days. We also performed the converse experiment, where normal vessel-like structures were allowed to form and stabilize over 14 days (Fig. 5B). As Dox was added to induce the knockdown of NF1, it can be appreciated that the vessel like structures begin to thicken and fuse, suggesting that abnormal or malformed vascular structures can arise, even from quiescent endothelial cells. Importantly, in cells that were "rescued" by coordinate expression of the GRD, vessel morphology remained intact and unchanged throughout the experimental manipulations. Collectively these data strongly argue that maintenance of appropriate levels of Ras activation are critical for vascular morphogenesis both in developing vessels and in established vascular networks.

mTORC1 activity is essential for endothelial cell proliferation and abnormal morphogenesis following the loss of NF1

We next sought to determine the mechanisms downstream of Ras that are required for the autonomous proliferation seen in response to the loss of NF1 and the accompanying activation of Ras. Given the emerging role of mTOR-related signaling in other aspects of NF1 disease, we

were particularly interested in the role of this enzyme in the endothelial cell phenotype [18,19,20]. We used the minimal concentrations of inhibitors required to effectively inhibit Ras-related signaling back to basal levels and evaluated the effects on cellular signaling following suppression of NF1. We found we could effectively inhibit ERK activation with no significant effects on the S6 activation or AKT activation (Fig. 6A). Proliferation was completely inhibited by inhibition of MAPK, as was the induction of cyclin D1 (data not shown), consistent with previous results from our lab that have consistently found an obligatory role for MAPK signaling in the proliferative response to activated Ras and growth factors in human endothelial cells [10,21,22], as well as those previously reported by Munchhof et al. [8]. The effects of PI-3'-kinase inhibition were difficult to interpret, as inhibition of this signal also partially inhibited mTORC1 activation, likely explaining the intermediate and variable effect we observed with this inhibitor in proliferation assays (data not shown). However we found that at low doses of Rapamycin (0.01 ng/ml), the NF1 mediated activation of AKT and ERK were unaffected while the NF1 mediated S6-phosphorylation was completely inhibited. This dose of Rapamycin was also sufficient to completely abrogate the proliferative response observed with the loss of NF1 expression. These data suggest an unexpected obligatory contribution of this pathway to the enhanced proliferation following suppression of NF1 expression. We next investigated the role mTORC1 in regulating two known modulators of endothelial cell cycle progression, Cyclin D1 and the phosphorylation of the corresponding cyclin dependent kinase inhibitor, $p27^{KIP}$ [23]. We find the induction of cyclin D occurs even under conditions where mTORC1 activation is inhibited; however the phosphorylation of p27 is strongly affected by low dose Rapamycin. Collectively these data suggest that the proliferative response we observe in endothelial cells with reduced NF1 expression is highly sensitive to inhibition by Rapamycin which seems to act

at least in part by suppressing the phosphorylation of p27, a requisite step in its degradation by SKP2 and ultimately cell cycle progression.

As cells lacking NF1 induce a hyperplastic phenotype that appears to be at least partially due to an inability to cease proliferation and induce branching and tubulogenic programs, we reasoned that this abnormal morphogenesis might be altered by low dose Rapamycin. To test this, control and $NF1^{KD}$ cells were co-cultured with primary fibroblasts, with or without low doses of Rapamycin (Fig. 7). We found that in control endothelial cells the presence of Rapamycin inhibited the number of tube-like structures, however the general characteristics of these branching networks were similar to untreated cells. Interestingly, in the NF1-kD cells, the sheet like phenotype was not present, rather the cells underwent normal branching morphogenesis, indicating that the blunting of mTORC1 signaling and endothelial cell proliferation was able to restore a normal phenotype to these cells.

DISCUSSION

Our data strongly support a critical role for NF1 in the regulation of the vascular endothelium. These findings are in agreement with previous studies done in developmental models whereby the endothelial specific deletion of NF1 resulted in embryonic lethality. These data also agree with the data of Munchhof et al. [8], who found that knocking down of expression in endothelial cells augmented VEGF and FGF signaling and induced angiogenesis. Importantly our data extend this previous work in several critical areas both conceptually and mechanistically.

Notably, our data demonstrate that the loss of NF1 is sufficient to induce activation of Ras and initiate downstream signaling. This suggests that NF1 doesn't just play a passive role in

dampening Ras activation but rather is an active regulator of GTP-Ras accumulation. The loss of NF1 triggers the accumulation of Ras-GTP even under serum and growth factor-free conditions. The resulting accumulation of Ras-GTP is sufficient to initiate changes in cell behavior, including entry into the cell cycle and enhanced growth rates under mitogenic conditions. Importantly, we find no evidence of the senescence associated with the loss of NF1 in other cell types [11]. This finding is consistent with our previous findings showing a lack of senescence following expression of H-Ras harboring an activating mutation. The enhanced cellular proliferation, as well as other changes in intracellular signaling, results in a gross perturbation of the endothelial cell vasculogenic program. Under the co-culture conditions we employ, normal primary endothelial cells stop growing, elongate, and form interconnecting tubules; NF1KD cells however, continue to maintain at least a partially proliferative phenotype and fail to branch correctly and form tubular structures reliably. To our knowledge this is the first report that the loss of NF1 is sufficient to alter the morphogenic program of endothelial cells. This finding may help to shed light on the underlying cause of a least some of the vascular anomalies seen in some NF1 patients and may provide an in vitro model to study the molecular basis for these vascular defects.

It is noteworthy that the alteration of the morphogenic phenotype appears to quite plastic. Under conditions where the vessel structures are malformed, a return of normal Ras regulation permits at least a partial normalization. Similarly even in stable and quiescent vessel structures, the loss of NF1 and accompanying Ras activation results in the emergence of an altered phenotype. These data suggest that in NF1 patients, the acquisition of an additional mutation or epigenetic silencing of the remaining copy of NF1 might be sufficient to trigger Ras activation, autonomous proliferation and abnormal vessel formation – even in post-developmental quiescent

vasculature. This data begins to explain the high frequency of moyamoya disease, arteriovenous malformations and possibly even aneurysms in the NF1 patient population. The finding that the Ras-GAP, RASA1 (p120-GAP), is mutated in several distinct vascular anomalies [24,25] including capillary malformation-arterial venous malformations and Vein of Galen aneurysms supports this hypothesis, though it is currently unclear if the loss of RASA1 is sufficient to result in Ras activation. Recent data investigating the role of RASA1 in the endothelium of mice suggests that loss of RASA1 does result in abnormal morphology and lymphatic endothelial proliferation. However the activation of Ras and Ras-related signaling, along with the abnormal vascular phenotype were dependent upon coincident VEGFR3 signaling [26]. Current experiments are determining if the homozygous loss of NF1 in the post-natal vasculature is sufficient to trigger Ras signaling and vascular anomalies, particularly in a haploinsufficient microenvironment which is known to be pro-angiogenic [7].

Our data directly addressed the role of Ras in the endothelial cell regulation by NF1. While some cell types have been reported to have important Ras-independent functions of NF1 [27,28,29], our data suggest that alteration in Ras signaling is essential for the effects seen upon loss of NF1 in endothelial cells. We found that all of the phenotypes and signaling changes observed following the loss of NF1 were rescued by re-expression of the GAP-related domain of NF1 and the restoration of Ras regulation. However it is currently unclear if there is isoform specificity to the Ras activation seen following the loss of NF1 or if particular isoforms are linked to the phenotypic manifestations. Moreover we cannot rule out that other signaling changes are also taking place and contributing to the observed phenotypes.

We find that loss of NF1 triggered several Ras-related signaling pathways including activation of PI-3'-kinase and activation of mTOR signaling. The activation of mTOR was

obligatory for the autonomous proliferation triggered by the loss of NF1. The proliferative dependency appears to arise out of mTOR-dependent phosphorylation of p27 leading to its degradation rather than induction of cyclin D1 which has previously been implicated in NF1 tumors[30]. Previous studies in our lab have determined that p27 degradation is an essential step in endothelial progression to S-Phase [23]. Our data do not address whether mTORC1 is directly phosphorylating p27, however we did not find any significant inhibition of either AKT or ERK (two enzymes known to phosphorylate p27) by the low dose of Rapamycin we employed. It is interesting to note that activation of mTOR has been associated with cutaneous vascular malformations [31]. The sensitivity of the NF1KD endothelial cell proliferation and the normalization of the vascular morphogenesis following treatment with low-dose Rapamycin in our assays suggest that "Rapalogs" currently in clinical trials as anti-tumor medications may be an effective management tool for certain types of vascular dysfunction in NF1 patients. In addition, it seems likely that these patients may be well-positioned to benefit from other currently approved vasculoprotective therapeutics, e.g., statins which can dampen both Ras and mTOR signaling [32,33] as well as metformin which activates AMPK signaling [34] to dampen mTOR.

Materials and Methods

Ethics Statement

Parents and legal guardians of donors provided consent that discarded tissue could be used for research purposes. The collection and use of tissue samples was evaluated and approved by the Institutional Review Board of Albany Medical Center. It was not considered Human subjects research under 45 CFR (Basic HHS Policy for Protection of Human Research Subjects) part 46 because, (1) the specimens were not collected specifically for the currently proposed research project and (2) the investigator(s) cannot readily ascertain the identity of the individual(s), consistent with the guidelines set forth by the Office of Human Research Protection of the US Federal Government.

Cell culture

HUVECs (Human Umbilical Vein Endothelial Cells) were purchased from Cascade Biologics (Portland, Oregon, USA) or Lifeline Cell Technologies (Fredrick MD, USA). Cells were cultured as previously described [21]. Cells were made quiescent by incubation in serum free MCDB-131 supplemented with 1 % penicillin/streptomycin and 2 mM L-glutamine (SF), where noted. Stimulation was performed with complete growth media. Primary fibroblasts were isolated from human foreskins provided as de-identified, discarded tissue from neonatal circumcision procedures at Albany Medical Center and grown in DMEM containing 10% FBS and 1% penicillin/ streptomycin.

Western blotting

Western blotting analysis used the following antibodies: mouse anti-pERK (Santa Cruz

Biotechnology), rabbit anti-ERK2 (Santa Cruz Biotechnology), rabbit anti-NF1 (Bethyl Laboratories), rabbit anti-p27 (Santa Cruz Biotechnology), rabbit anti-phospho-p27 (Zymed), rabbit anti-pS6, rabbit anti-pAKT, mouse anti-cyclin D1, mouse anti-pan Ras (Oncogene Research, Calbiochem). All antibodies were used at a dilution of 1:1000 overnight at 4°C. Other conditions were the same as described in [21] except exposures were captured on a Kodak 4000 MM imager. All figures are representative of at least three independent experiments.

Plasmid construction

To stably knockdown *NF1* expression in HUVECs a shRNA sequence targeting *NF1* (5'gatccGGACACAATGAGATTAGATTTCTCAAGAGAAAATCTAATCTCATTGTGTCCT TTTTTACGCGTg3' sense strand) and (5'aattcACGCGTAAAAAAGGACACAATGAGATTAGATTTTCTCTTGAGAAATCTAATC TCATTGTGTCCg3' antisense strand) into the RNAi-Ready pSIREN-RetroQ-ZsGreen retroviral expression vector from Clontech. The shRNA sequences were synthesized by Operon and had a MluI restriction site in the hairpin loop region with BamHI and EcoRI cut overhangs for cloning. The oligonucleotides were annealed and ligated into the BamHI/EcorI cut pSIREN-ZsGreen according to the "Knockout RNAi Systems User Manual" from Clontech. The same annealed shRNAs were ligated into the *BamHI/EcorI* cut pSIREN-RetroQ-DsRed-Express vector to make a knockdown vector expressing red fluorescent protein. A Negative Control shRNA annealed oligonucleotide provided by Clontech was ligated similarly into the pSIREN-RetroQ-ZsGreen to make a control vector. Inducible knockdown of NF1 was achieved using a lentiviral pTRIPZ vector from Open Biosystems carrying the V2THS_260806 sequence for knocking down NF1. Schematics of these vector constructs are shown in *Supplementary Fig. 1*. To make a lentiviral vector expressing the gap related domain (GRD) of NF1 the MSCV-puro-GRD-V5 plasmid was

purchased from Addgene. A *BglII/NotI* fragment carrying the GRD was ligated into *BamHI/NotI* cut pEN_TRE2 [10]. The Tet promoter along with NF1-GRD was then put into the Gateway compatible destination vector pSLIK-Venus [35] using an LR-*Clonase*™ reaction resulting in the pSLIK-GRD-Venus plasmid.

Production and infection with retroviruses and lentiviruses

The pSIREN-RetroQ-ZsGreen lentiviral vector carrying a negative control or a NF1 shRNA was transfected into retroviral packaging PhoenixA cells [36] using Lipofectamine 2000 reagent. The media from the transfected cells was sterile filtered through a 0.4 μM filter and the viral supernatant was used to infect low passage HUVECs in the presence of 5 μg/mL of polybrene. The HUVECs underwent a second round of infection in a similar manner after 24 h. The infection efficiency was 70-80 % and a pure population of infected cells was obtained by flow cytometry based sorting under sterile conditions, using ZsGreen as a selectable marker. The lentiviral vectors (2 μg) were co-transfected along with the respective packaging plasmids into 60 % confluent 293 FT packaging cells (Invitrogen) cell using Lipofectamine 2000 reagent (Invitrogen). The pSLIK based vectors were co-transfected with three 3rd generation packaging plasmids, 3 μg each of pMDLg/pRRE(Addgene, plasmid 12251), pRSVREV (Addgene, plasmid 12253), the vesicular stomatitis virus (VSV) G envelope plasmid pVSV (Addgene, plasmid 12259) [37]. The pTRIPZ lentiviral vectors were co-transfected along with two 2nd generation packaging plasmids, 3 μg each of pCMV-dR8.2 dvpr (Addgene, plasmid 8455), pCMV-VSVG (Addgene, plasmid 8454). Low passage HUVECs were infected and sorted in a similar manner as above to obtain a pure population of knockdown cells. With both the retroviral and lentiviral vectors, three independent infections were performed on three independent endothelial cell

cultures in order to insure representative results.

Measurement of DNA synthesis and growth assays

Endothelial cells were serum starved for 24 h, after which complete growth media (GM) was added as a mitogenic stimulus for 16 h. Measurements of BrdU incorporation were performed as previously described [22]. In some cases indicated doses of Rapamycin were added to the cells at the time of serum starvation. Growth assays were conducted as described previously [38]. Population doublings were calculated using the formula: Population Doublings = Log(Final cell number/Initial cell number)/Log2. Cumulative population doublings represent the sum of population doublings from all previous passages.

Co-culture assay

This assay was performed as previously described [15] with modifications as we have previously reported [10]. Cell were typically tracked using the expression of fluorescent markers introduced during genetic modification. In some cases, cells were stained live with a FITC-tagged UEA-1 lectin (Sigma-Aldrich) or fixed in 3.7% formaldehyde and visualized with UEA-1 lectin.

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Figure Legends

Figure 1. Knockdown of NF1 in Human Endothelial Cells is Sufficient to Activate Ras and Induce Cellular Signaling. (A) Early passage HUVECs were infected with a pSIREN-GFP retroviral vector carrying either a control or a NF1 shRNA and sorted for GFP expression. Western blot analysis confirmed knockdown of neurofibromin. (B) Early passage endothelial cells were infected with a pTRIPZ lentiviral vector expressing either a non-silencing control or a NF1 miR-based shRNA. The infected cells were induced with 1μg/mL doxycycline for 48 h to induce expression of the microRNA along with red fluorescent protein (expressed in tandem) and sorted for RFP expression. Western blot confirms knockdown of neurofibromin in the presence of doxycycline while no knockdown was seen in the un-induced cells. (C) Uninduced (-Dox) and induced (+Dox) endothelial cells were serum starved for 24 h and levels of active Ras (RasGTP) were determined by using GST-Raf pull-down assay (Pierce), according to the manufacturer's protocol. Total Ras in the total cell lysates confirmed equal amounts of protein input. (D) Cells were treated as described above but western analysis was performed to measure activation of several key signaling proteins including phospho-Akt, phospho-S6, and phospho-ERK. Equal lysate loading was confirmed by monitoring total ERK2 levels. All experiments were performed in at least three independent sets of control and NF1 knockdown HUVECs. Identical results were seen with both knockdown vectors.

Figure 2: Loss of NF1 expression is sufficient to induce endothelial cell proliferation. (A) pSIREN Control and *NF1* knockdown primary endothelial cells were grown in complete media under sub-confluent conditions and cumulative population doublings were recorded over the course of 25 days as described [10]. (B) Control or NF1 knockdown endothelial cells were

serum deprived for 24 h prior to cells being pulsed with BrdU for 3 h. BrdU positive cells were quantified and the data was graphed as the % positive cells compared to total cell number. Data are presented as the mean with error bars representing the standard error (∗$p<0.05$). (C) In parallel to the experiments performed in (B), serum starved cell lysates were made after 24 h and probed with antibodies against cyclin D1, p27, phospho p27, and ERK2. ERK2 is used to insure equal loading of lysate.

Figure 3. Loss of NF1 expression results in abnormal vascular morphogenesis. Top Panel - Primary endothelial cells infected with non-targeting pSIREN lentivirus (Control) or one directed toward knockdown of NF1 (NF1-KD). These cells were plated with primary fibroblasts in an admixed co-culture. After 14 days, endothelial cells were visualized by the vector expressed GFP. Bottom Panel - Endothelial cells infected with TRIPZ-Control or NF1miR-shRNA were plated in co-culture with primary fibroblasts in the presence of doxycycline to induce expression of shRNA and RFP. Vascular structures were visualized at day 14 using RFP co-expressed in the endothelial cells.

Figure 4. Enhanced proliferation following the loss of NF1 is a consequence of Ras activation. HUVECs were double infected with vectors coding for inducible NF1 knockdown shRNA and inducible expression of the GAP-related domain (GRD) of NF1 or empty vector. Cells were incubated for 24 h in the presence of 0.5 μg/ml doxycycline to induce expression of NF1shRNA as well as the co-infected cDNA (GRD or empty). Control cells are cultured in the absence of doxycycline to suppress expression of and cDNA. After 24 h in the presence or absence of doxycycline, cells were switched to serum-free medium. (A) Cells were analyzed for

the presence of active Ras using GST-pull down. Total Ras was used to insure equivalent protein in the input lysates. (B) BrdU incorporation was measured 24 h after changing to serum and growth factor free conditions. (C) Lysates prepared under the conditions described above were probed for changes in phosphorylation of p27 and total p27 levels by western blotting. ERK was monitored as a loading control. All experiments were replicated in an independently generated set of doubly infected cells.

Figure 5. Changes in vascular morphogenesis are reversible and dependent upon active Ras. Cells for these experiments were co-infected with TRIPZ-NF1KD and pSLIK virus that was either empty or expressing the GRD domain of NF1 and sorted by FACS for double positive populations. These cells were then plated in co-culture with primary fibroblasts in the presence or absence of doxycycline as indicated. (A) Admixed cultures were allowed to form structures for 14 days. Representative fields were photographed using expressed RFP at this time and doxycycline was removed from the culture medium. Co-cultures were allowed to persist for an additional 14 days, after which time representative fields were again photographed following visualization of endothelial cells with FITC-labeled UEA-1 lectin. (B) Double positive cells were plated in the absence of doxycycline for 14 days and endothelial cells were visualized by staining live cultures with FITC labeled UEA-1 lectin and representative fields were photographed. Doxycycline was then added to cultures and they were incubated for an additional 14 days followed by visualization again using UEA-1 lectin. Arrows in both (A) and (B) highlight representative changes in morphology.

Figure 6. mTORC1 is critical for enhanced proliferation following the loss of NF1

(pSIREN). Control and NF1 knockdown primary endothelial cells were serum deprived for 24 h prior. Inhibitors were added was added at the time of serum starvation at the following concentrations (U0126, 1 μM; LY29002, 100 nM; Rapamycin, 0.1 ng/ml). At 21 h, some cells were pulsed with BrdU for 3 h. At 24 h cell lysates were made and probed for changes in cellular signaling (A) using phospho-specific antibodies or changes in cellular proliferation (B), visualized with an anti-BrdU antibody. BrdU positive cells were quantified and the data was graphed as the % positive cells compared to total cell number. The error bars represent standard error ($*p<0.001$). In a similar experiment cells was serum starved 24 h in the absence and presence of Rapamycin (0.1 ng/ml) and lysates were made and probed with antibodies against cyclin D1, phospho-p27 and ERK2, the latter used as a loading control. In all cases similar results were observed in at least two additional experiments.

Figure 7. Abnormal vascular morphogenesis is normalized by Rapamycin. Endothelial cells infected with pSIREN expressing either non-targeting (control) or shRNA directed against NF1 (NF1-KD) were plated in co-culture with primary fibroblasts. Co-cultures were incubated in the presence of vehicle (DMSO) or Rapamycin (0.1 ng/ml) from the time of plating. Vascular structures are shown at day 14, visualized by expression of GFP in the endothelial cells by the pSIREN vector.

Supplemental Figure 1. Schematic representation of NF1 knockdown constructs.

Figure 1
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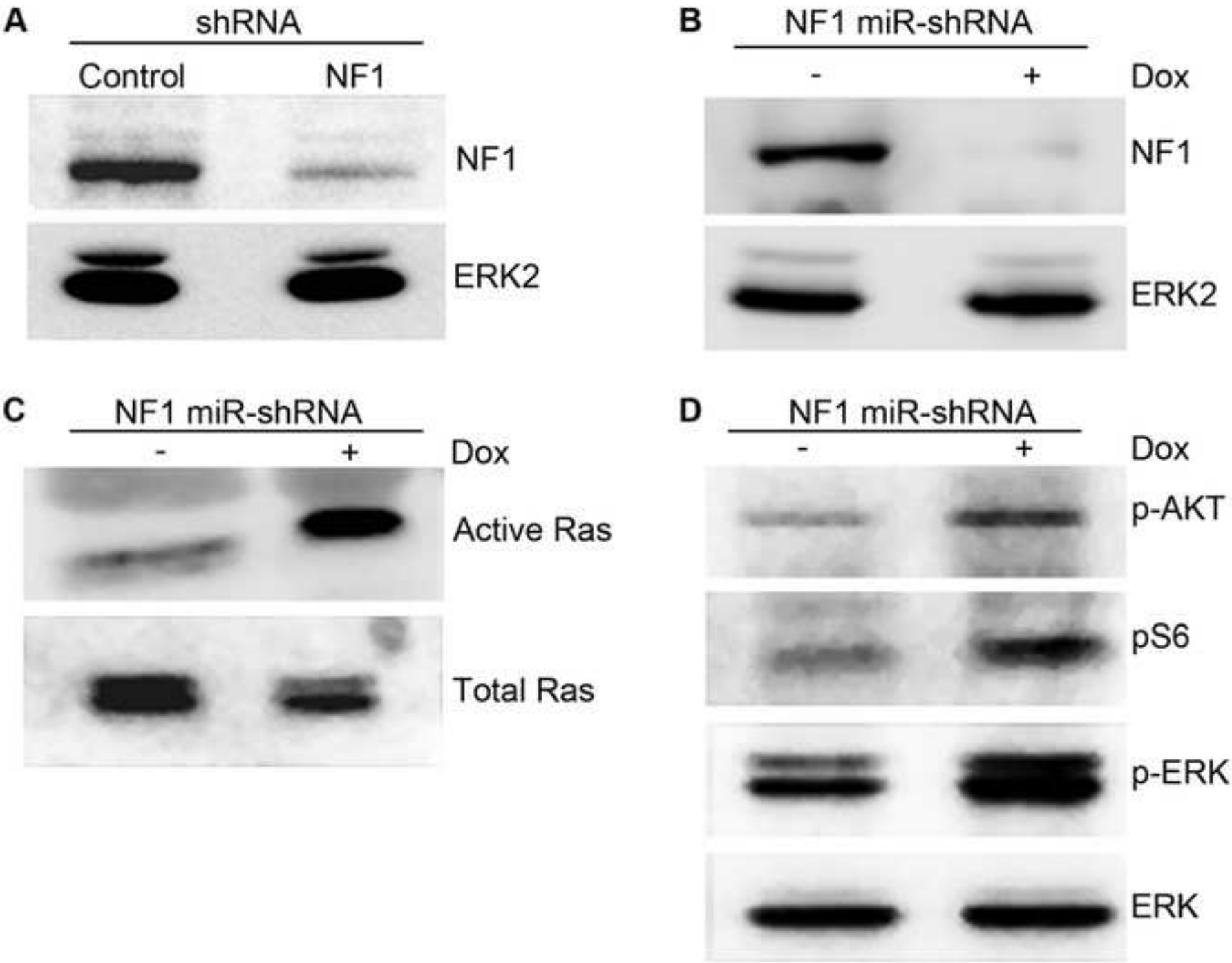

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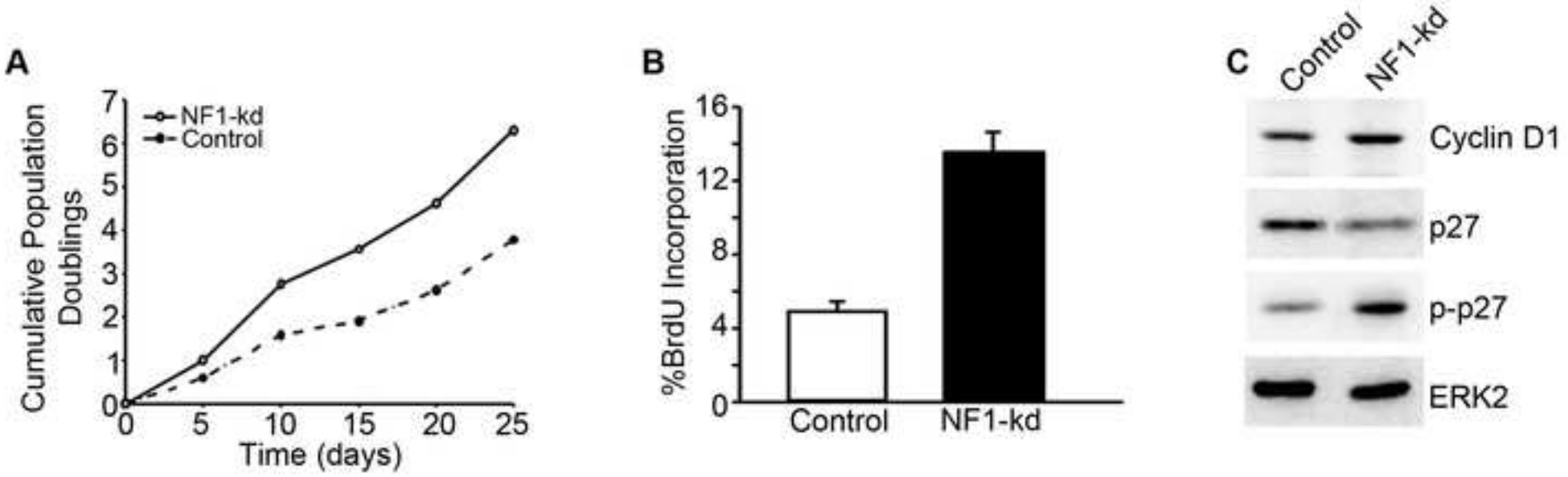

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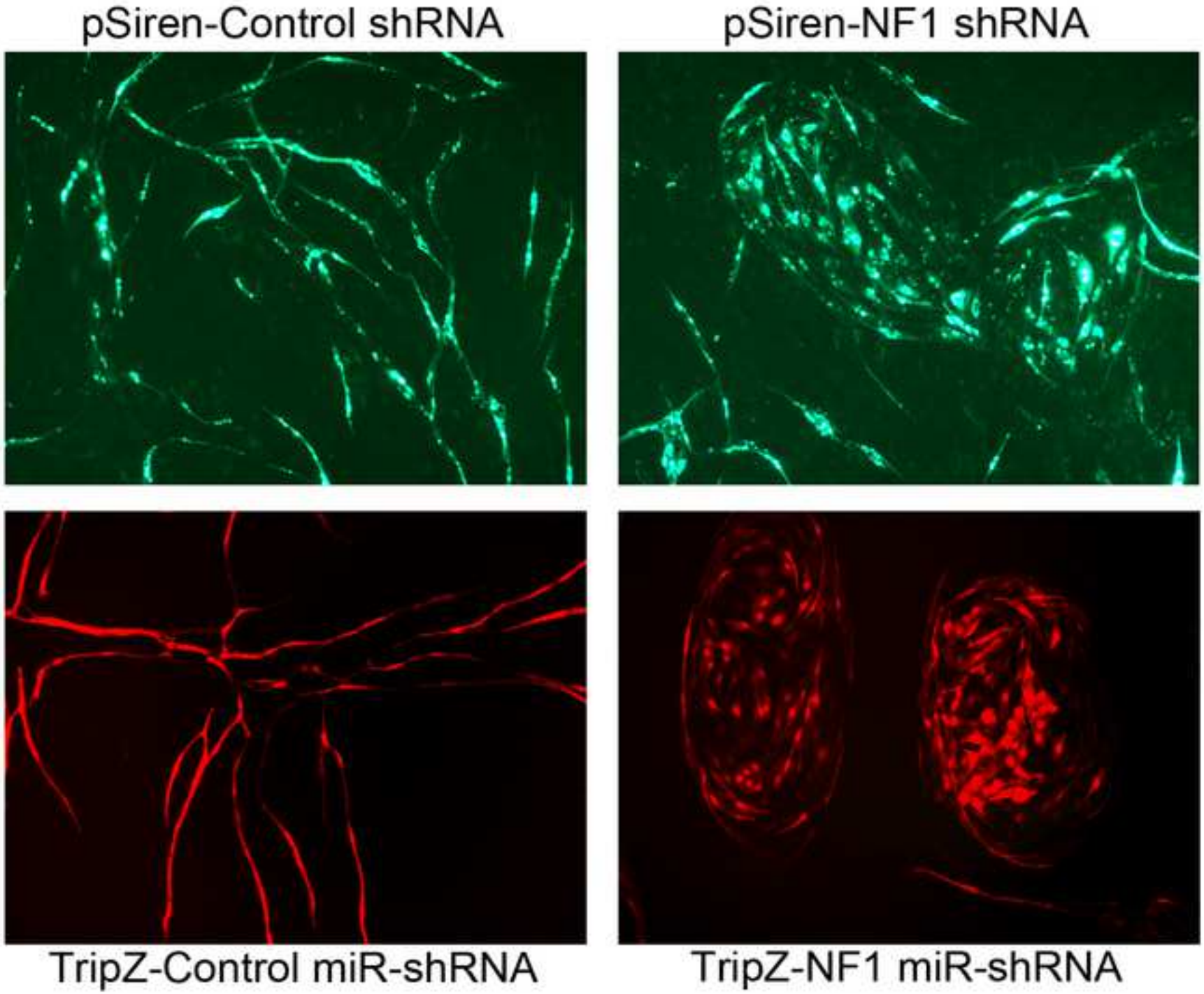

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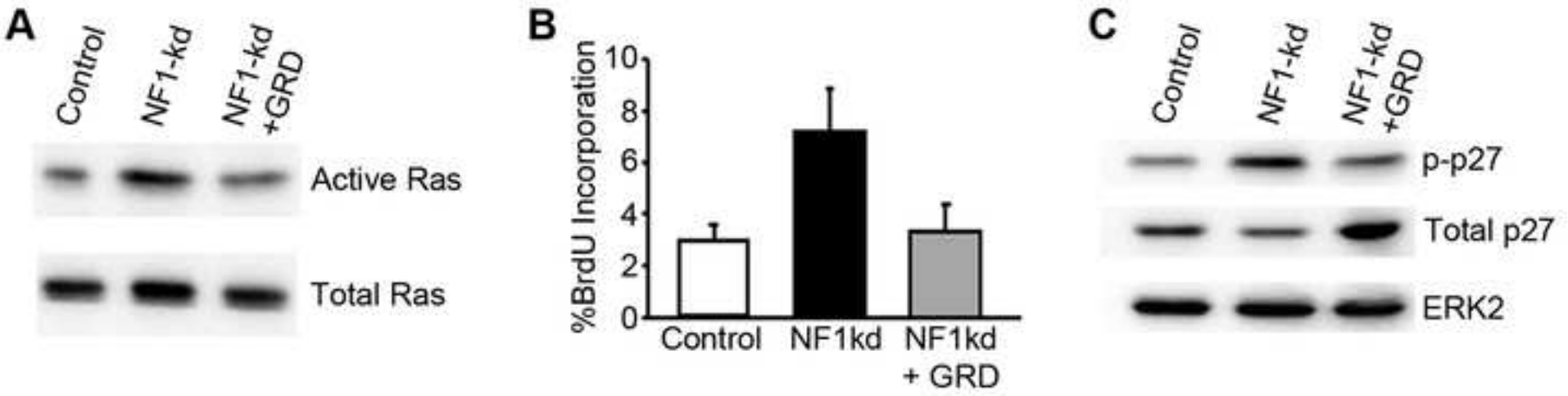

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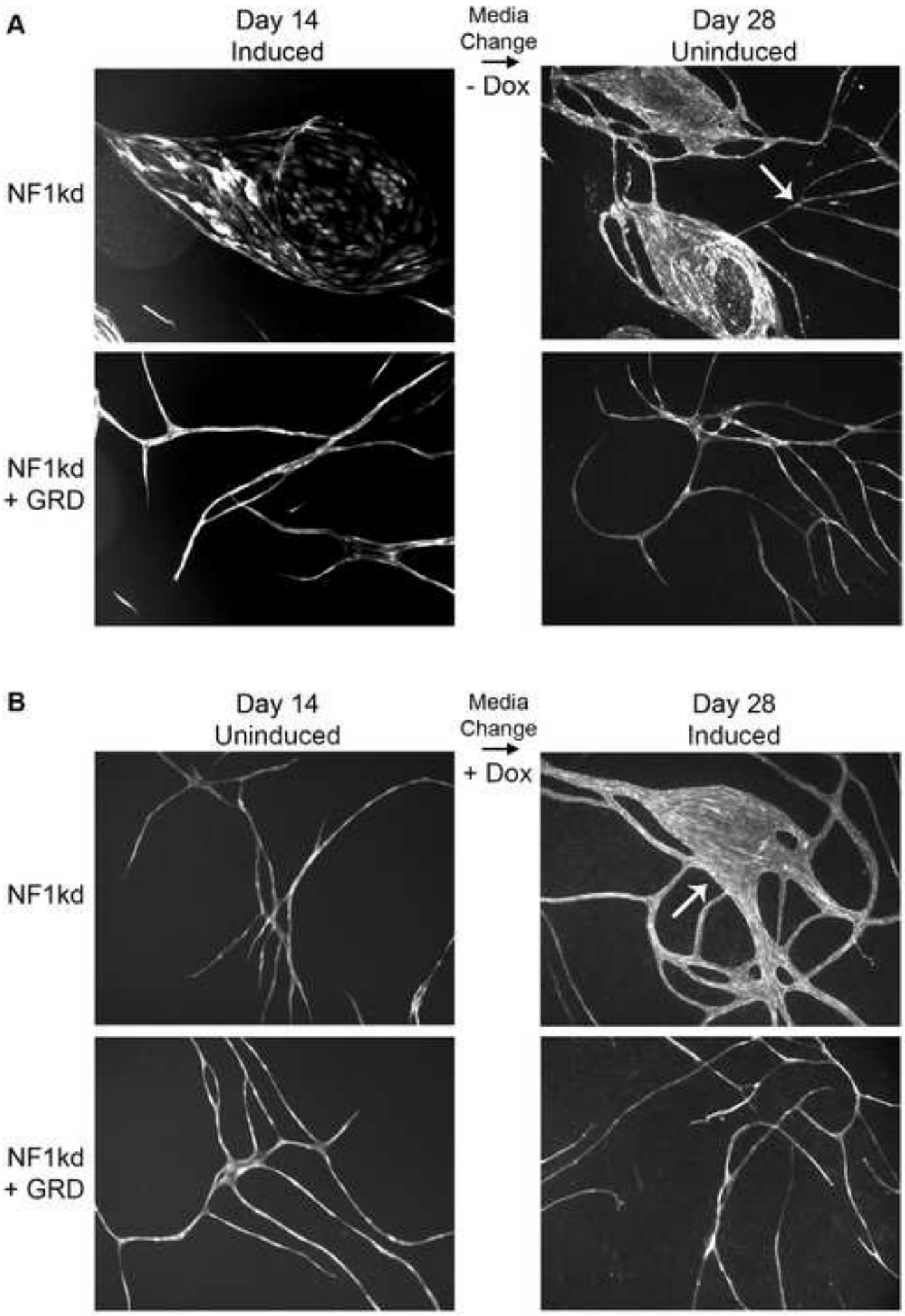

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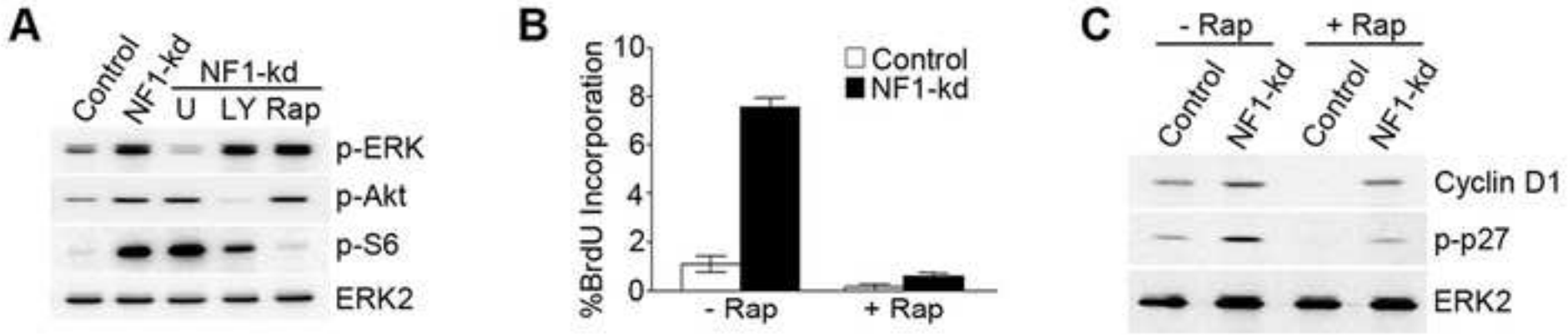
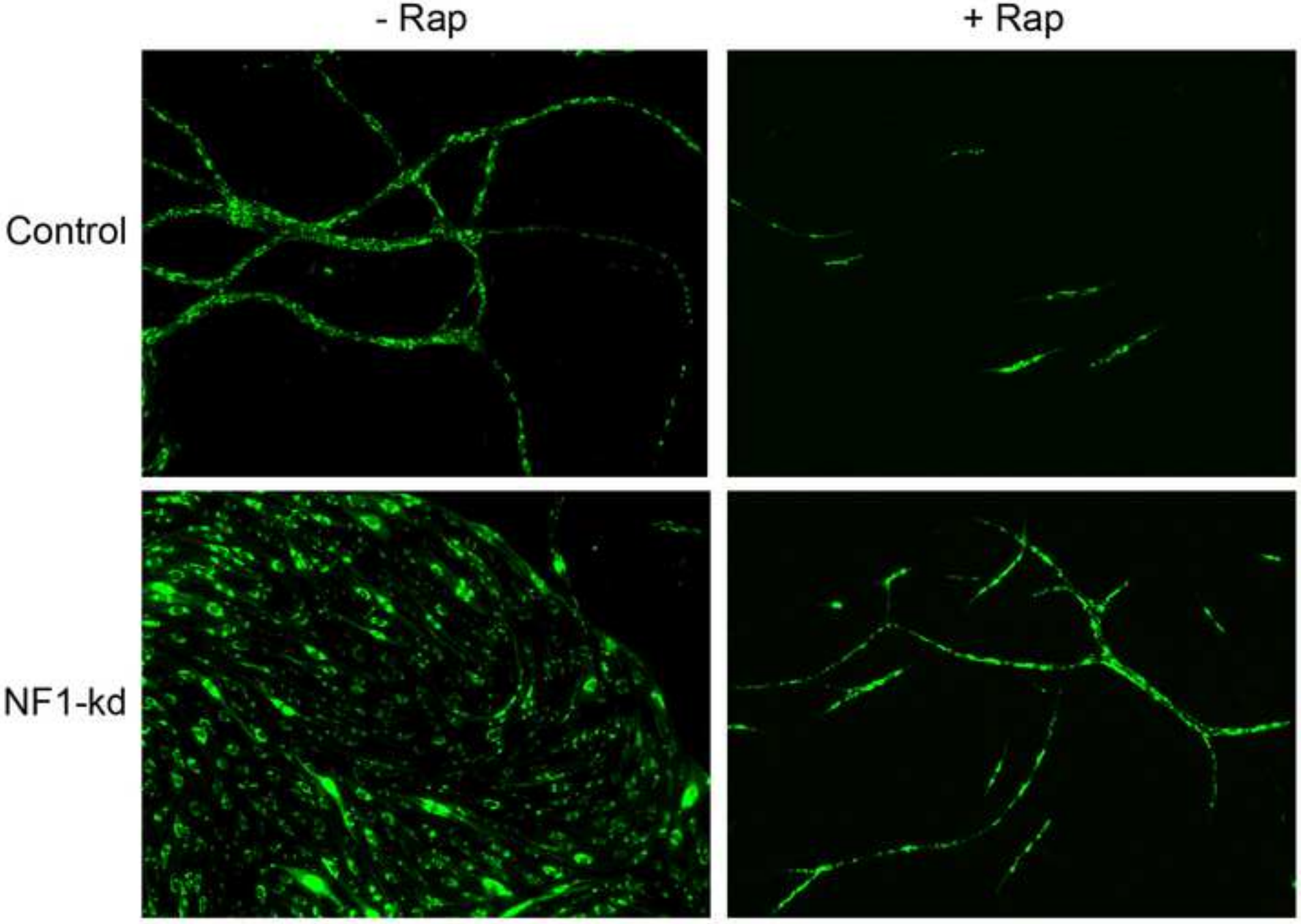
Figure 7
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