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14. ABSTRACT

We have made strong progress on each of the proposed Aims. We have continued our evaluation of the signals and mechanisms involved in dysfunctional morphogenesis with strong evidence both chemical and genetic accumulating that the PI3Kinase and mTOR axis play a critical role. While this is associated with proliferative augmentation it is not clear that enhnaced proliferation is required. Rapmycin was suprisingly unique in its potency to inhibit the NF1 induced mTOR activation and to reverse the dysfunctional morphogenesis. Data continues to accumulate suggesting an inetrplay between TGFb-related signaling and the morphogenic defects seen following loss of NF1. Finally the development of a novel mouse model of induced loss of NF1 in the adult mouse vasculature on a haploinsufficient background led to a suprise finding of rapid development of leukemia. This may suggest important control of the HSC niche by NF1.

15. SUBJECT TERMS
None Listed

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Introduction*:*

NF1 plays an important role in regulating the vascular endothelium. NF1 is clinically associated with multiple vasculopathies including malformations, aneurysms, and hypertension. Consequently there is a markedly elevated risk of cerbrovascular accidents(Friedman et al., 2002). NF1 haploinsufficient mice show exaggerated angiogenic responses(Wu et al., 2006) 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(Munchhof et al., 2006). 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(Bajaj et al., 2010). Our data also document that loss of NF1 in primary human endothelial cells results in autonomous proliferation and activation of multiple signaling networks. These changes result in abnormal vascular morphogenesis. These effects are driven by the specific activation of Ras and are reversed by expression of the GRD of NF1 and by low doses of the mTOR inhibitor rapamycin (Bajaj et al., 2012) Our central hypothesis is that mTOR activation is a crucial component of vascular abnormalities associated with NF1 and contributes to an altered vascular microenvironment that promotes much of the pathology associated with the disease. Moreover we hypothesize that a mechanistic consequence of the loss of NF1 is an alteration in the TGF-b signaling axis, that promotes abnormal vascular differentiation and may alter the tumor microenvironment. We address these central hypotheses in this project with three Specific Aims:

1. ***Investigate the molecular regulation of mTOR-related signaling in NF1-deficient human endothelial cells and its role in altered endothelial cell function.*** Using molecular modulation of componenets of the mTOR pathway as well as clinically available pharmaceuticals, we are determining the critical regulatory pathways that drive the changes seen following the loss of NF1 in endothelial cells.
2. ***Determine if NF1-deficient human endothelial cells have altered responses to TGF-b.*** We are investigating whether the loss of NF1 alters the response to the TGF-b signaling axis and determining if this alters the process of Endothelial-Mesechymal Transition (EndMT).

3. ***Evaluate the consequences of bi-allelic loss of NF1in the vascular endothelium.*** Using endothelial specific, tamoxifen inducible Cre-lox technology, we are determining the effects of losing a second allele of NF1 in the vascular endothelium of the adult mouse. This will be the first model of NF1 loss in the adult endothelium and can serve as a model system for investigation of both cardiovascular effects and the tumor microenvironment.

Body:

Aim 1.

As outlined above, a principal objective of the first aim is to better understand the relationship of mTOR signaling in endothelial cells and determine which aspects of this complicated regulatory network were being dysregulated. As outlined in our SOW and reported largely last year a first priority was developing and characterizing reagents to manipulate signal transduction related to NF1 and mTOR. This past year much of our attention has been focused on repeating, solidifying

and extending the use of these reagents and to investigate the role of the mTOR pathway in the vascular morphogenesis. Some of the significant advancements are highlighted below.

Vascular Morphogenesis: Originally we were using a fibroblast co-culture model to assess vascular morphogenesis but we have realized that this assay is complicated both kinetically and morphologically as it lacks true sprouting behavior, and in addition because of the interdigitation of the endothelial cells and fibroblasts it can be difficult to perform immunofluorescence experiments. In addition we wanted to confirm that the morphogenic defect we observed in planar co-cultures was reproduced in an independent morphogenic assay performed in 3D. Thus we have used an assay where endothelial cells are coated on the surface of beads and suspended in fibrin gels with a layer of fibroblasts placed on the top. Over the course of 10-14 days, sprouts elongate off the beads under control conditions as shown in **Figure 1**. We found using this assay, we could reproduce a very analogous phenotype to that seen in planar co-culture, when NF1 was knocked down. In the place of branching sprout like structures there is an outgrowth of cells maintaining a sheet-like planar morphology, even in 3D. Interestingly, similar to the planar co-culture model, treatment with low-dose rapamycin restores the cells to a near normal morphology with extensive elongated individual sprouts, as shown in **Figure 1A-C**. A benefit of this methodology is that the fibroblast layer is easily removed and the remaining gel is easily analyzed by a modified immunofluorescence protocol. Using this we could clearly demonstrate that knockdown of NF1 results in a numerous cells with activated mTOR in the sheet-like structures as shown in **Figure 1B**, staining for the mTOR substrate phospho-S6. In addition, as we reported last year, loss of NF1 augments proliferation in cell culture and this is sensitive to rapamycin. Using this assay we have extended these data to the 3D morphogenic state. As shown in **Figure 1C**, cells with knocked down NF1 had readily visible Ki67 staining associated with cells on the bead, in the sheets and even occasionally in the few tubular sprouts these cells made. However treatment with rapamycin results in a near absence of cells staining positive for Ki67. These data collectively affirm the data we have previously obtained in two dimensional assays and extend it to a 3D morphological context.

In addition to rapamycin a goal of this Aim was to evaluate other agents in clinical use which were known to have the ability to modulate the mTOR axis. We determined concentrations of metformin, zolendronic acid, and Simvastatin that modulated activation of mTOR as measured by phosphoS6. Similar to rapamycin, metformin and simvastatin had very modest effects on proliferation of control cells, while zolendronic acid was a potent inhibitor of all Ras related signaling and proliferation. Metformin was ineffective at inhibiting the proliferation of NF1-kd cells (indeed it is a poor inhibitor of mTOR in NF1kd cells). Simvastatin was intermediate in its effects and zolendronic acid and rapamycin completely inhibited proliferation. Interestingly, as shown in **Figure 2**, only rapamycin was able to restore the normal phenotype when assayed in co-culture assay. Metformin and simvastatin were without significant effects. Zolendronic acid inhibited the outgrowth of the sheets but did not restore the tubulogenic phenotype.

As stated in Aim1 and the SOW, a goal of this aim was also to investigate these agents for the presence of any paradoxical signaling that might result from feedback regulation. These agents have largely behaved as predicted. Zolendronic acid inhibits all Ras signaling, consistent with a site of action at the level of Ras lipid modification. No rebound signals are evident under the conditions we have measured. Simvastatin when inhibiting mTOR, has weak effects on AKT and eNOS phosphorylation that change in parallel as expected. Effects on this axis seem to take

at least 24 hours of treatment to develop and are likely indirect in nature. Rapamycin is nearly immediately (15 minutes) effective at inhibiting mTORC1 activity, as assessed by phospho-S6 but over 48 hours begins to impact AKT. As dose escalates effects on the AKT axis and ENOS also increase. We found no evidence of effects on the Erk pathway or any rebound signaling. However as shown in **Figure 3A**, we did observe a paradoxical effect on the morphogenic phenotype of the inhibitor metformin at high doses. As we found that doses normally used for the control cells were ineffective in NF1-kd cells, we began to escalate the dose. Interestingly when we did this we found that high doses of Metformin alone seemed to disrupt normal vascular morphogenesis and produce the sheet like phenotype. Importantly as the dose of metformin escalates, so does the level of AKT activation (**Figure 3B**). It seems likely that Metformin at higher doses is triggering modest activation of PI3'-kinase, through a mechanism not previously described. This may explain why this agent is ineffective at dampening the NF1 signaling or phenotypes.

The finding that Rapamycin was unique among the mTOR modulating agents in its ability to modulate vessel morphogenesis was somewhat surprising. To validate that the effect was truly due to mTOR modulation we engineered cells that express NF1-kd shRNA (or non-targeting control) under the pSiren vector which co-expresses GFP, simultaneously with a tet-inducible shRNA targeting RAPTOR. When this vector is activated by doxycycline, it co-expresses RFP. These double infected and selected cells were evaluated for cellular signaling and phenotype. As shown in **Figure 4**, these cells when induction with Doxycycline knocks RAPTOR down, have decreased mTOR activation, decreased AKT activation and the sheet-like phenotype of NF1-kd cells is abrogated.

Consistent with the notion that PI-3'Kinase is a critical player in vascular morphogenesis, we have evaluated the effects of PTEN in the fibrin bead and co-culture assay. As shown in **Figure 5**, the loss of PTEN is sufficient to induce the planar-sheet phenotype in the 3D assay and we have developed methods to quantify this assay quite robustly. Loss of PTEN also augment proliferation (**Figure 6**) and the effects of PTEN are reversed by low dose Rapamycin as shown in **Figure 7**.

Summary of Aim 1 progress: Our evaluation of the experiments proposed in Aim1 is nearly complete. We have found that mTOR modulation plays a critical role both the pro-proliferative phenotype associated with NF1 as well as in the morphogenic defects. Interestingly, the modulation of the mTOR axis however is most effectively achieved by allosteric direct regulators of mTOR such as Rapamycin, as a variety of indirect modulators such a zolendronic acid, simvastatin, and metformin were ineffective in reversing the NF1 –induced morphologic defects. We revealed an interesting, though paradoxical effect of metformin on vascular morphogenesis. In summary these experiments would suggest that a careful balance between signals must be maintained for proper morphogenesis. Genetic manipulation by simultaneous knockdown of the RAPTOR axis did phenocopy the effects of Rapamycin. To complete this analysis we are still awaiting suitable data from Rictor. We have been able to knockdown Rictor, however to date the construct which is most effective at knocking down Rictor, is also affecting Raptor. While possible, this makes interpretation difficult and we are seeking additional sequences to confirm. This construct is also unusually unstable in the cells making progress slow. In addition, the relationship between PI-3Kinase and mTOR is still somewhat undefined with both playing an important role. A mutant Rheb construct has been made with

which we hope to hyperactivate the mTOR axis downstream of PI-3-kinase to test sufficiency. This virus has been made, cells have been successfully infected and analysis is ongoing.

Aim 2. Determine if NF1-deficient human endothelial cells have altered responses to TGF-b.

We reported last year that there appeared to be minor variations in SMAD phosphorylation. Repeated experiments revealed variable results that were inconsistent and added little to the data we have already reported. To determine if TGFb signaling was a feasible approach to continue to pursue, we performed a direct reversal experiment. In this experiment we incubated the NF-kd cells under co-culture conditions where morphogenesis is impaired. We also added low doses of TGFb2 (0.1 ng/ml) to some wells to determine if we could reverse the morphogenic dysfunction by augmenting TGFb signaling. As shown in **Figure 8**, we find that the addition of TGFb was sufficient to mitigate most of the sheet formation and restore normal tubulogenesis. We have also repeated this experiment in the Fibrin 3D system with similar results. The experiments in 3D are in the process of being repeated and quantified as that is a strength of this system. In addition the Fibrin bead system will allow us to investigate the translocation of phospho-SMADS and expression of target genes in situ.

Since TGFb signaling seems to play a role, we turned our attention to whether there were differences in the expression of known TGFb target genes. An example of a gene known to be induced following TGFb stimulation is PAI-1. This gene is important for modulation of pericellular proteolysis, as well as migration – processes that are likely involved in morphogenesis. We found that NF1-kd cells appear to have lower levels of PAI under basal conditions. In addition, stimulation with TGFb2 stimulated production of PAI-1 in control cells but in NF1-kd cells PAI-1 levels were only at 30% of control levels following TGF-b stimulation (**Figure 9**). PAI-1 may be only an indicator of a whole host of genes that may show altered modulation, as TGFb controls the expression of numerous pro-migratory and adhesion proteins including the production of collagens, including the critical vascular basement protein Collagen IV. Along these lines we have developed methods to stain for the deposition of collagen IV in our in situ morphogenesis assays, including the co-culture assay, as this may be a crucial inducer of tubulogenesis. An example of this is shown in **Figure 10.**

Aim 2 Summary: We have continued to make progress on this Aim, which by design was somewhat open-ended in its approach. We have developed further evidence that TGFb is a critical modulator of the morphogenic program which the loss of NF1 is affecting. At this point, the mechanism is unclear. It may be a direct effect or it may be the result of parallel biological antagonism. Several initiatives are underway to investigate this further: 1) we are isolating RNA from endothelial cell re-isolated from the co-culture assay to evaluate genes expression changes across an entire TGFb related gene array; 2) we are creating lentiviruses for the inducible expression of activated ALK5 and a mutant ALK5 which is SMAD-independent; 3) we are evaluating the deposition of basement protein in the co-culture models to see if this is altered; lastly, we have recently observed a subtle condition of cell culture which seems to alter TGFb responsiveness. Thus we plan on repeating some of the SMAD signaling and EndMT experiments paying close attention to making sure cell density is significantly subconfluent (below 50%), as this may have confounded earlier results.

Aim 3: ***Evaluate the consequences of bi-allelic loss of NF1in the vascular endothelium.***

As shown in **Figure 11**, we have successfully generated mice as outlined in the experimental plan and SOW which have a genotype that will allow for inducible, bi-allelic loss of NF1in the endothelium, on a haploinsufficient genetic background, these mice also carry a marker allele for Cre-recombinase, such when cre is active, and RFP variant (td-tomato) will be expressed. A cohort of these mice were induced with tamoxifen to remove the NF1 gene in the endothelium. We note at day 14 that several of the flox/del mice appeared to be losing weight and by 21 days after the induction we needed to sacrifice the mice due to obvious distress. Upon necropsy we observed no evidence of vascular malformations or hemorrhage. However as outlined in the statement of work we evaluated the vasculature using immunostaining. An example is shown in **Figure 12**. We found no visible defects in the morphogenesis of the vasculature we have evaluated at this time including the brain, retina, kidney, lung, and liver. All tissues showed normal vascular patterning. An observation we did make, which was unexpected, was that the spleens of the NF1 flox/del mice were notably enlarged as evidenced in **Figure 13A.** Concerned that perhaps IP injection of Tamoxifen was inducing an inflammatory reaction in the peritoneum that was exacerbating some sort of condition, we repeated the experiment using oral administration of tamoxifen for induction of the Cre. The phenotype we observed was very similar with all mice succumbing to deleterious effects within three weeks. Weighing the spleens revealed a significant difference in splenic weight (**Figure 13B**). As we examined the tissues of these mice, we revealed evarl aspects of their histology that revealed additional details. Most notable the spleens had evidence of extramedullary hematopoiesis or accumulation of white blood cells, with a near obliteration of the normal red pulp architecture. In addition, we found significant peri-aveolar and peri-vascular inflammatory infiltrates in the lung. Lastly, the liver also had evidence of enhanced inflammation, with diffuse inflammation in the parenchyma and readily observable peri-vascular cuffing. Examples of these changes are shown in **Figure 14**. In order to better understand this phenotype we performed some follow-up experiments where another small cohort of animals was treated and cells were harvested from the spleen and bone marrow. Using FACs analysis we probed the phenotypic nature of the white blood cell expansion in the spleen and determined if there was a similar expansion in the bone marrow, the site of hematopoiesis. Our date showed across genders there was an expansion in both the monocyte and granulocyte populations with enhancement of neutrophils being particularly enhanced, in both the bone marrow and the spleen. These data are shown in **Figure 15**. In a preliminary effort to better understand the nature of this expansion we also probed cells from the spleen for the presence of markers of progenitor cells. Our data revealed that induced NF1 flox/del mice had significantly elevated levels of the normally rare progenitor cells across all subtypes of progenitor populations. These data, shown in **Figure 16**, suggest that there may be either an expansion or mobilization of hematopoietic stems cells.

Aim 3 Summary: We have succeeded in developing the mouse model of NF1 disease in the endothelium we proposed and have done experiments investigating the loss of endothelial NF1 in the adult. Unexpectedly these mice developed a lethal phenotype quite rapidly. Interestingly our characterization of this phenotype to date suggests that the primary defect in these mice is not in blood vessel morphogenesis, leak, or hyper-proliferation as proposed. Rather these mice seem to suffer from the rapid development of a leukemia-like condition which involves expansion of the myeloid component, apparently at the progenitor cell level. Interestingly, a

significant complication of NF1 disease in the human population is the development of juvenile leukemia. These data raise the intriguing possibility that this might be triggered by an activation of bone marrow or splenic endothelium which triggers the expansion of WBC progenitor expansion through changes in the local cytokine microenvironment.

Key Research and Accomplishments:

- Incorporation of a 3D angiogenesis assay into the evaluation of vascular morphogenesis affected by the loss of NF1
- Development of Immunofluorescence methods to evaluate vascular morphogenesis in 2D and 3D co-culture assays.
- Determination that dual knockdown of NF1 and Raptor reverts the abnormal morphogenic and proliferative phenotypes, strongly implicating a role for mTOR
- Development of methods to accurately quantify morphogenic changes
- Demonstration that dysfunctional morphogenesis induced by loss of NF1 has a proliferative component
- Demonstration that the proliferative component of dysfunctional vascular formations in situ are sensitive to inhibition of mTOR by low dose rapamycin
- Evaluation of several additional inhibitors of mTOR used clinically for their ability to mitigate the dysfunctional morphogenesis induced by the loss of NF1
- Discovery of the paradoxical induction of dysfunctional morphogenesis by the drug metformin at high doses
- Confirmation and solidification of data suggesting a sufficiency of elevated PI3'-kinase to drive dysfunctional morphogenesis and enhance cellular proliferation.
- Mutagenesis and cloning of a mutant Rheb allele into inducible lentiviral vector
- Determination of the ability of TGFb to reverse the dysfunctional morphogenesis induced by NF1
- Discovery that loss of NF1 can dampen the expression of TGFb responsive gene PAI-1
- Creation of a novel mouse model whereby bi-allelic loss of NF1 in the vascular endothelium can be induced on a haploinsufficient background
- Determination that the vasculature in the induced mouse appears to be normal at three weeks post-induction
- Discovery of the novel and unexpected induction of a leukemia-like state following the removal of NF1 expression from the vascular endothelium
- Preliminary characterization of the myeloid expansion triggered ny NF1 loss in the endothelium

Reportable Outcomes:

There were no reportable outcomes during this period. We are currently organizing our data to evaluate our publication options in the coming months. We plan to present our data at the North American Vascular Biology Meeting in the Fall of 2015.

Conclusion:

We continue to make excellent progress on achieving our goals and planned experiments as outlined in the SOW. At this point we have competed nearly all of the experiments envisioned for Specific Aim 1. Two areas requiring additional resolution in the coming year are the role of Rictor and the sufficiency of mTOR hyper-activation to drive the morphogenic defects. This will be a focus of experiments in the coming months. We also continue to accumulate data testing the proposed notion that dynamic regulation of TGFb signaling or related pathways may be playing a critical role in the vascular dysmorphogenesis. We have several new techniques and approaches which when combined with some of the technical advances we have made in the last year should help bring some clarity and resolution. Lastly, we have made the surprise finding that the loss of NF1 in the endothelium seems to trigger a rapid leukemic crisis. This observation is quite unexpected but consistent with the notion that the endothelial cells play a critical role in the regulatory microenvironment of many stem cell niches(Shen et al., 2004). While beyond the scope of the current proposal we hope to study this possibility in future investigations. To evaluate the role of the endothelial loss of NF1 on vascular morphogenesis, we are currently testing local induction using tamoxifen application on the skin. This should permit evaluation of the effects at longer times as proposed in the local vasculature, without triggering the lethal phenotype

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Wu, M., Wallace, M. R., and Muir, D. (2006). Nf1 haploinsufficiency augments angiogenesis. Oncogene *25*, 2297-2303.

Supporting Material: see supporting figures below

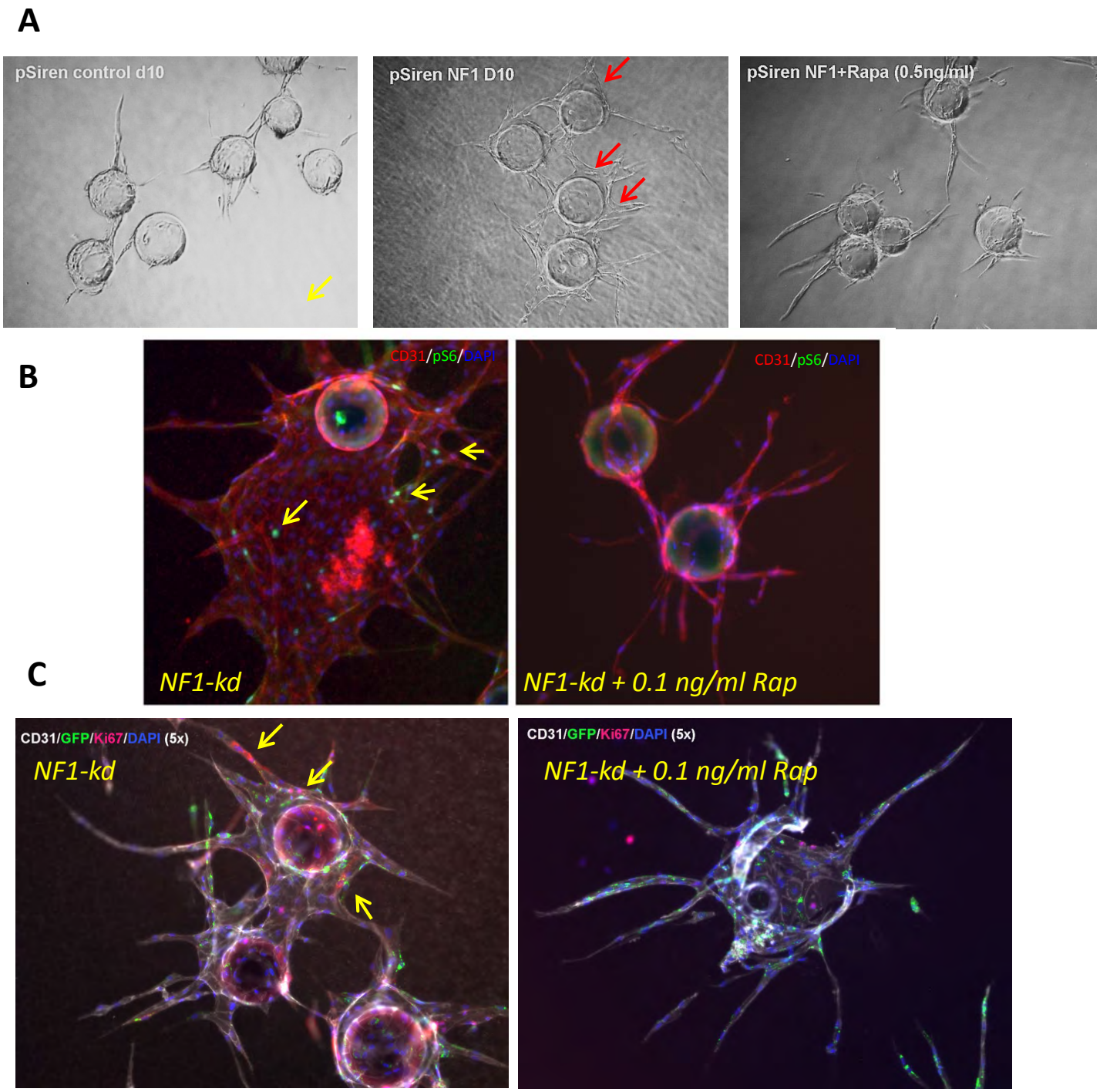


Figure 1. *Loss of NF1 induces alterations in vessel morphogenesis which can be corrected with low-dose rapamycin* **(A)** A fibrin bead angiogenesis assay was used to assess the effects of NF1 loss on vascular morphogenesis. The knockdown of NF1 resulted in the appearance of lamellar or sheet-like structures of fused cells protruding off the beads. Incubation of these cells with low dose rapamycin reversed these findings. **(B)** Immuno staining revealed that phospho-S6, a marker of activated mTOR, can be visualized in the sheet regions. This staining is lost following rapamycin treatment. **(C)** Staining with Ki-67 (red) demonstrated significant positive staining, particularly around the bead, in the sheet and and extending into the tubular structures (yellow arrows). Treatment with Rapamycin significantly reduced but did not eliminate Ki-67 staining

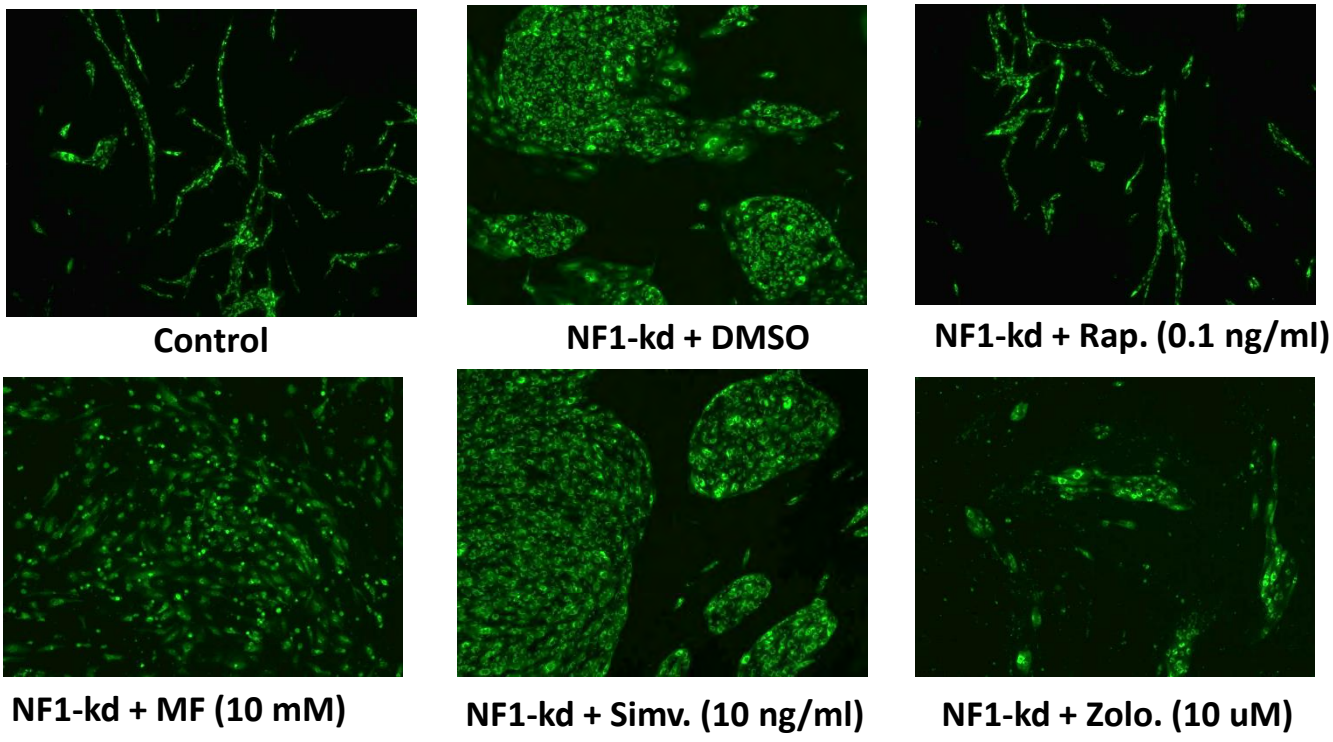


Figure 2. *Selective role for Rapamycin in normalizing NF1 morphogenic defect.* Cells were treated with several other clinically available compounds which can inhibit mTOR signaling and the effect on vascular endothelial cell morphogenesis was assessed in a co-culture assay with primary fibroblasts. Endothelial cells are visualized by the co-expression of GFP in the pSiren vector. Only cells treated with Rapamycin resemble control cells.

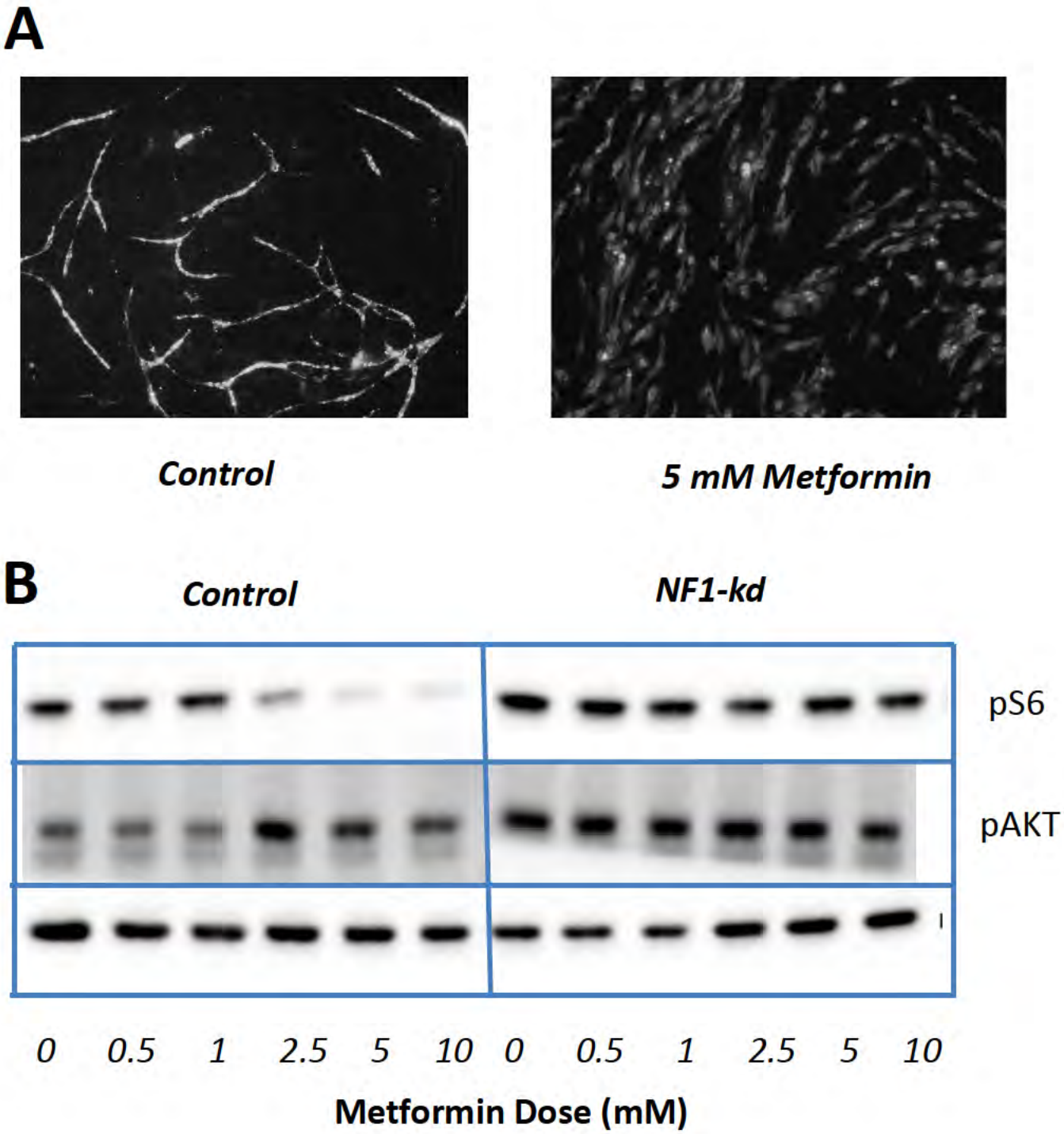


Figure 3 *Paradoxical loss of tube-forming ability following treatment with Metformin may be associated with enhanced PI3'-k OR Akt activation.* **(A)** Treatment of control cells with higher doses of metformin seem to abrogate the ability of cells to form tubular structures. **(B)** These higher doses of Metformin resulted in enhanced AKT activation, similar to levels seen in NF1-kd cells

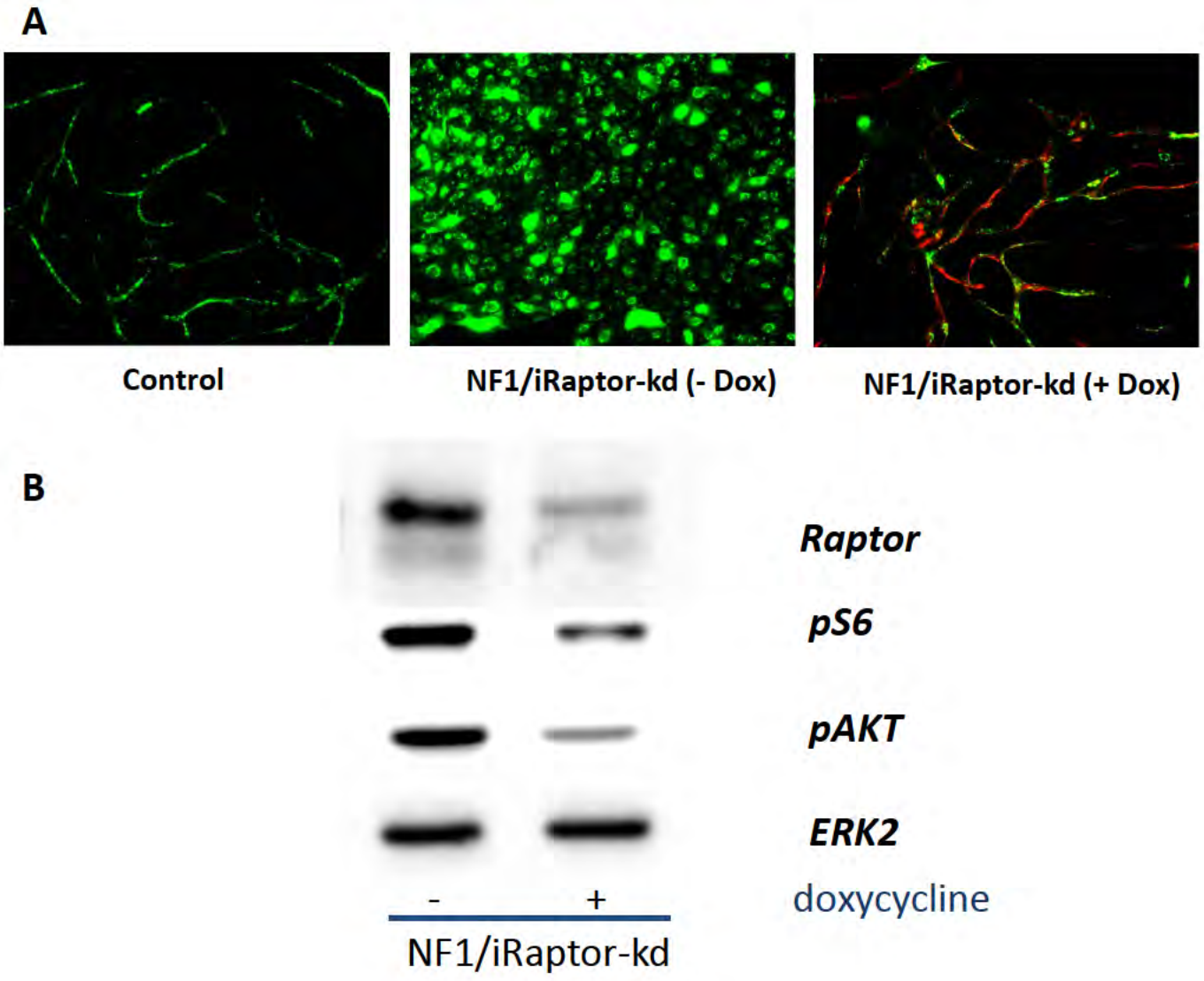
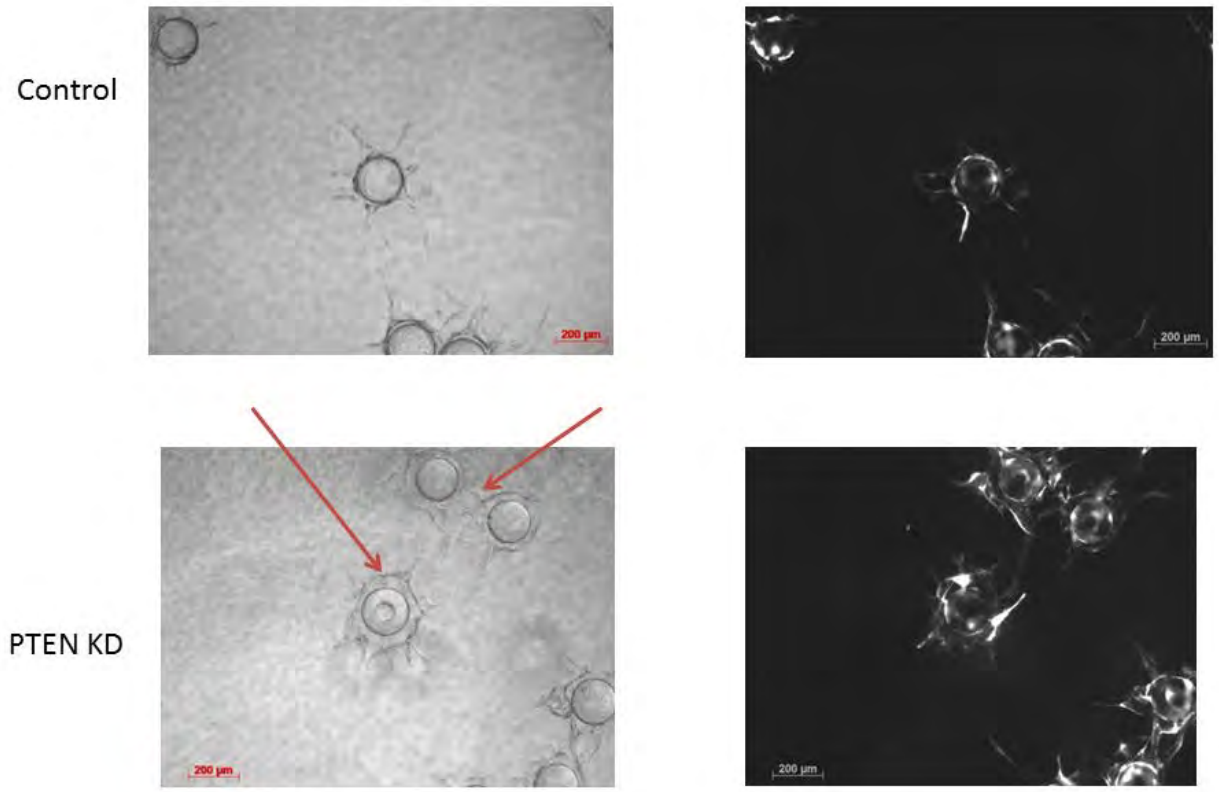


Figure 4. *Simultaneous knockdown of NF1 and Raptor normalizes vessel morphogenesis* **(A)** Cells were created which co-expressed shRNAs targeting NF1 (co-express GFP) and Raptor (inducible with doxycycline and co-express RFP). Expression of iRaptor shRNA in cells, resulted in a normalization of vessel-like morphology when co-cultured with primary fibroblasts. **(B)** Western blot analysis showing doxycycline inducible loss of Raptor and blunting of mTOR associated signaling

A



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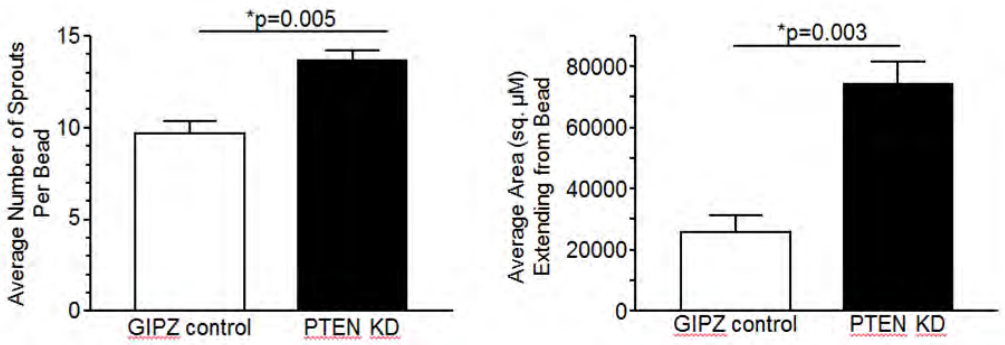


Figure 5 *Upregulation of PI-3'-Kinase activity mimics effects of NF1 loss* HUVECS were infected with a lentiviral vector to knockdown the negative regulator of 3'-phospholipids, PTEN. **(A)** These cells were used in a 3D fibrin-bead vascular morphogenesis assay. The results shown are visualized at day 7 by phase and fluorescence. **(B)** The results from three different experiments were quantified

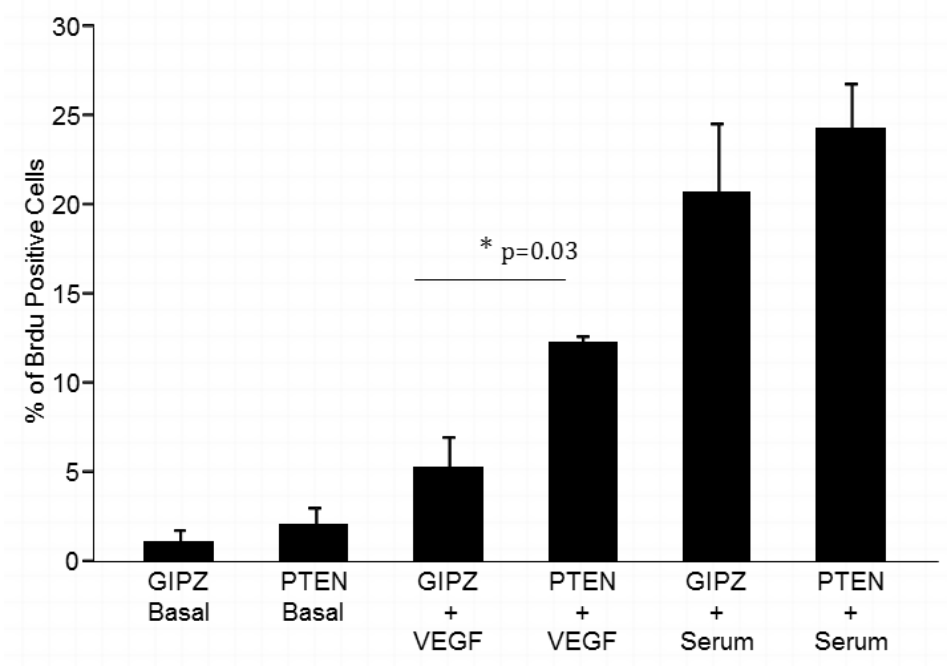


Figure 6. *Loss of PTEN regulation augments submaximal endothelial cell proliferation*. HUVECs infected with Control or PTEN-KD shRNA were analyzed for proliferation following a submaximal stimulus, 25 ng/ml VEGF, or a strong stimulus, Complete Growth Media containing serum.

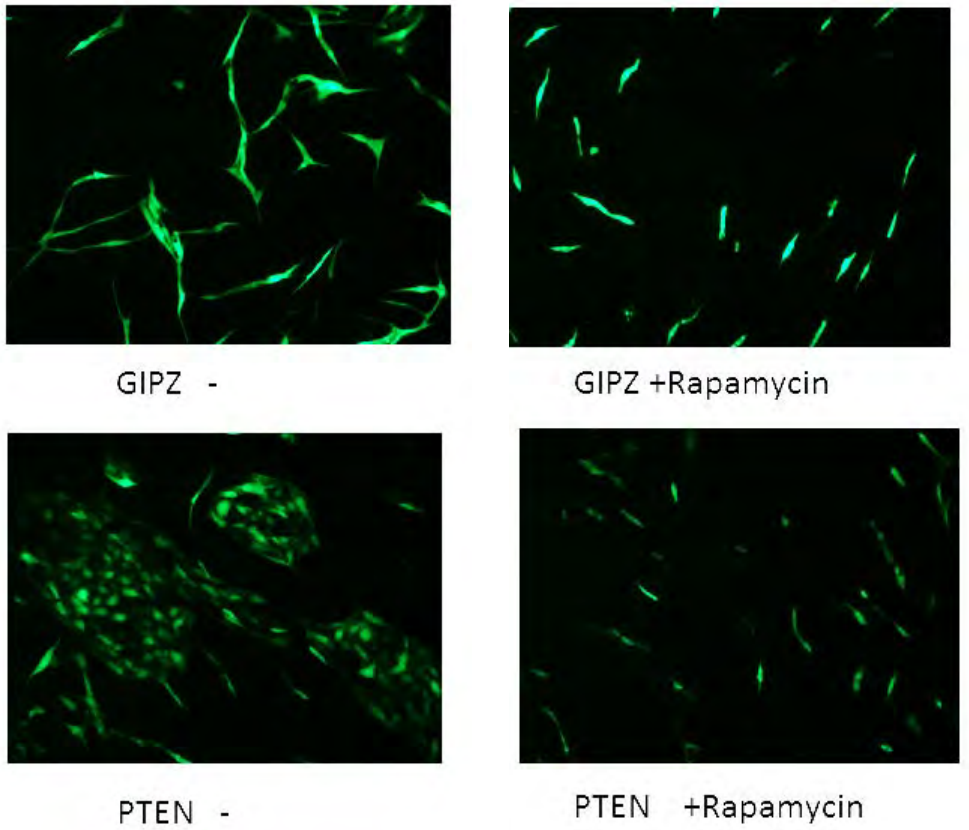


Figure 7. Defective morphogenesis following loss of PTEN is also abrogated by Rapamycin treatment. Cells with expressing Control of PTEN-kd shRNA were analyzed in a co-culture morphogenesis assay, with or without Rapamycin. Cells were visualized by co-expressed GFP in HUVEC cells.

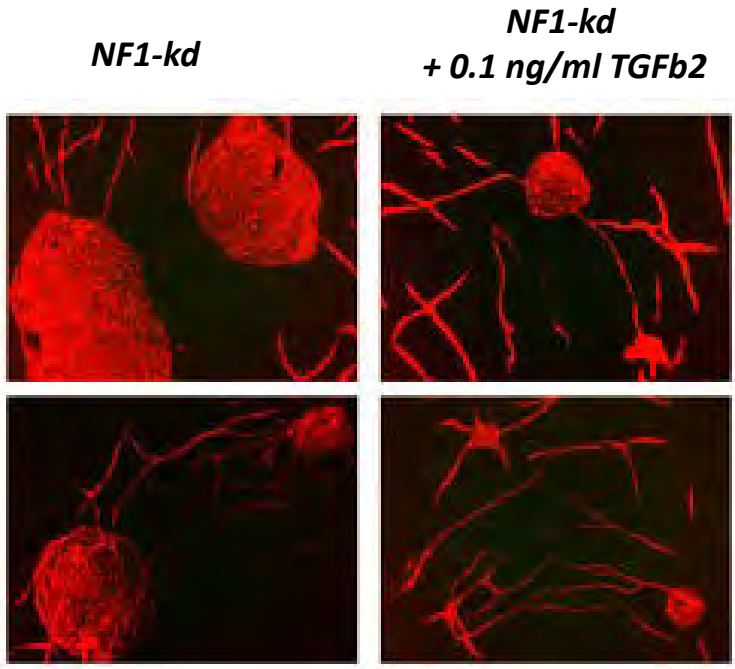


Figure 8. *Exposure to TGFb normalizes vascular morphogenesis in NF1-Kd cells.* NF1-kd HuVECs were co-cultured with primary fibroblasts in the presence or absence of low doseTGFb2. Images represent multiple fields at 10x visualized by the co-expressed RFP present in the TRIPZ vector used for NF1 knockdown.

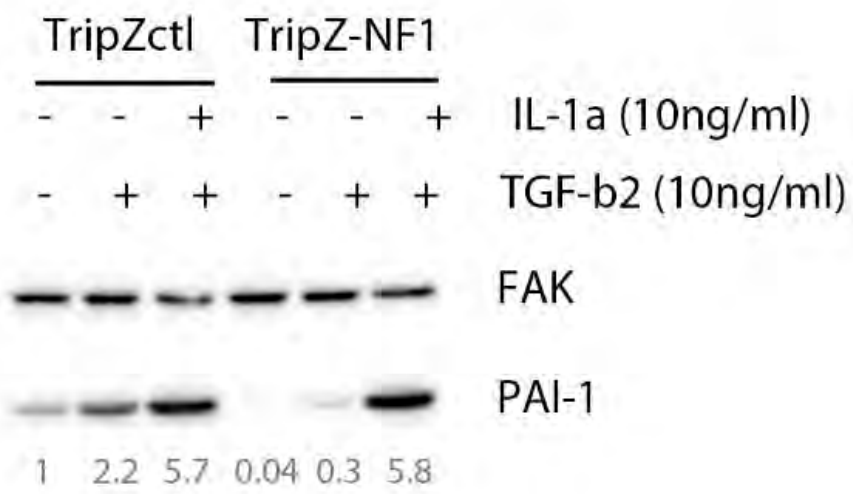
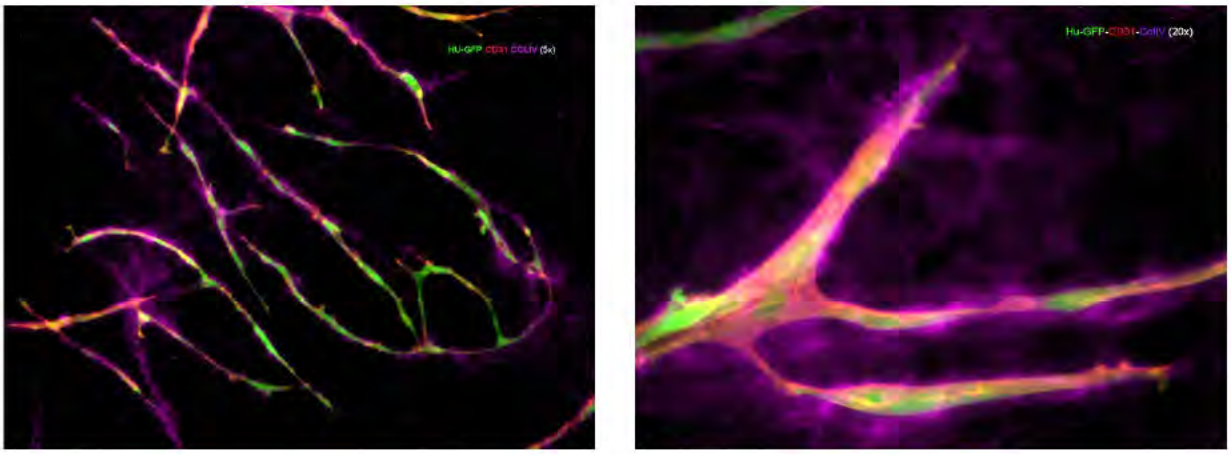
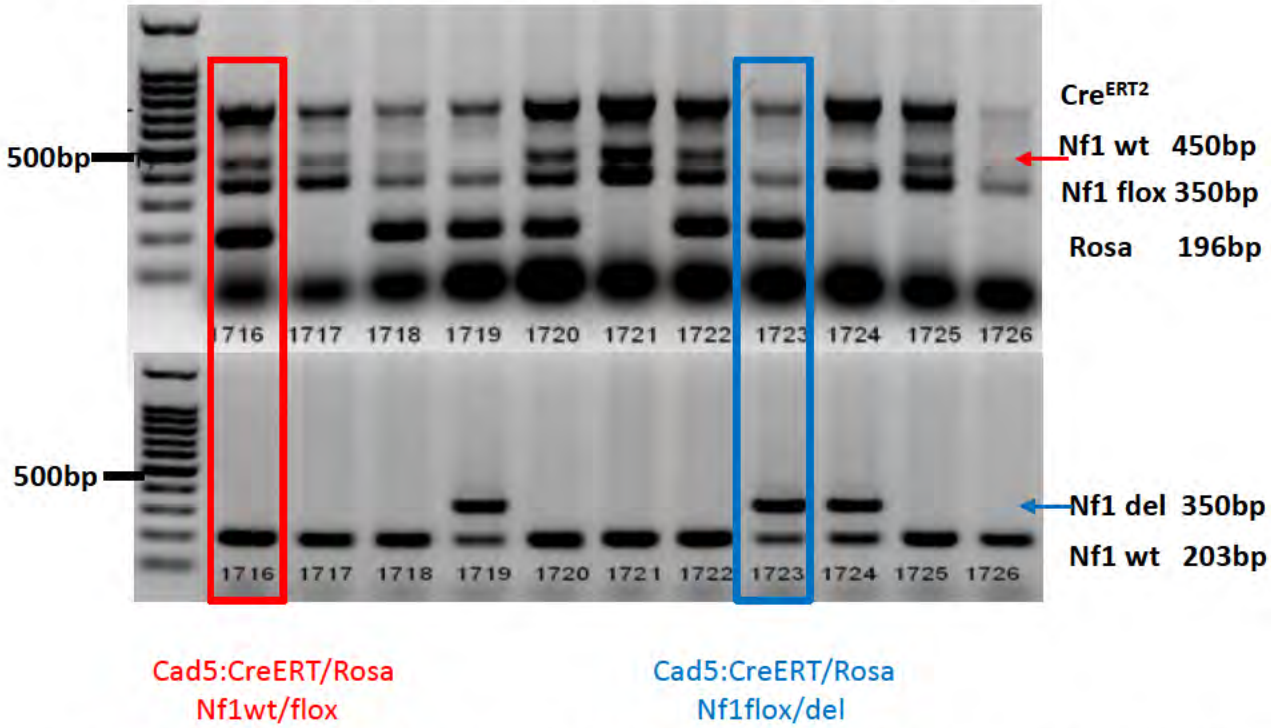


Figure 9. *Knockdown of NF1 may blunt TGFb target gene expression.* HUVECs infected with lentivirus expressing control or NF1 knockdown mir-shRNA sequences were analyzed for ability the expression of PAI-1, a well-studied target gene of TGFb by western blot, following O/N exposure to TGFb or TGFb supplemented with the pro-inflammatory cytokine, IL-1a. Numbers represent normalized expression relative to resting control cells after adjusting for loading using FAK expression.



GFP/CD31/ColIV - 5x GFP/CD31/ColIV - 20x

Figure 10. *Optimization of staining for collagen IV expression.* HUVECs co-cultured with fibroblasts and expressing GFP were allowed to form vascular structures for 14 days after which time they were stained for endothelial cells with anti-CD31, as well as Collagen IV, a component of the vascular basement membrane and anotherTGFb target gene. Multi-color microscopy was used to demonstrate the deposition along the periphery of tube like structures.



Cad5:CreERT/Rosa Nf1wt/flox Cad5:CreERT/Rosa Nf1flox/del

Figure 11. *Breeding a model of human NF1 disease in the endothelium.* Mice were bred that contain a missing functional copy of the NF1 gene as well as a second copy of the NF1 gene that can be rendered inactive by Cre-mediated recombination (Flox). These mutations were bred onto a line of mice that contains a Tamoxifen inducible, endothelial-cell specific Cre recombinase, and a Cre-reporter gene, Td-Tomato, which expresses as a red fluorescent protein when acted upon by Cre. An example of such a mouse is seen in 1723, while an example of a single copy loss is shown in 1716.

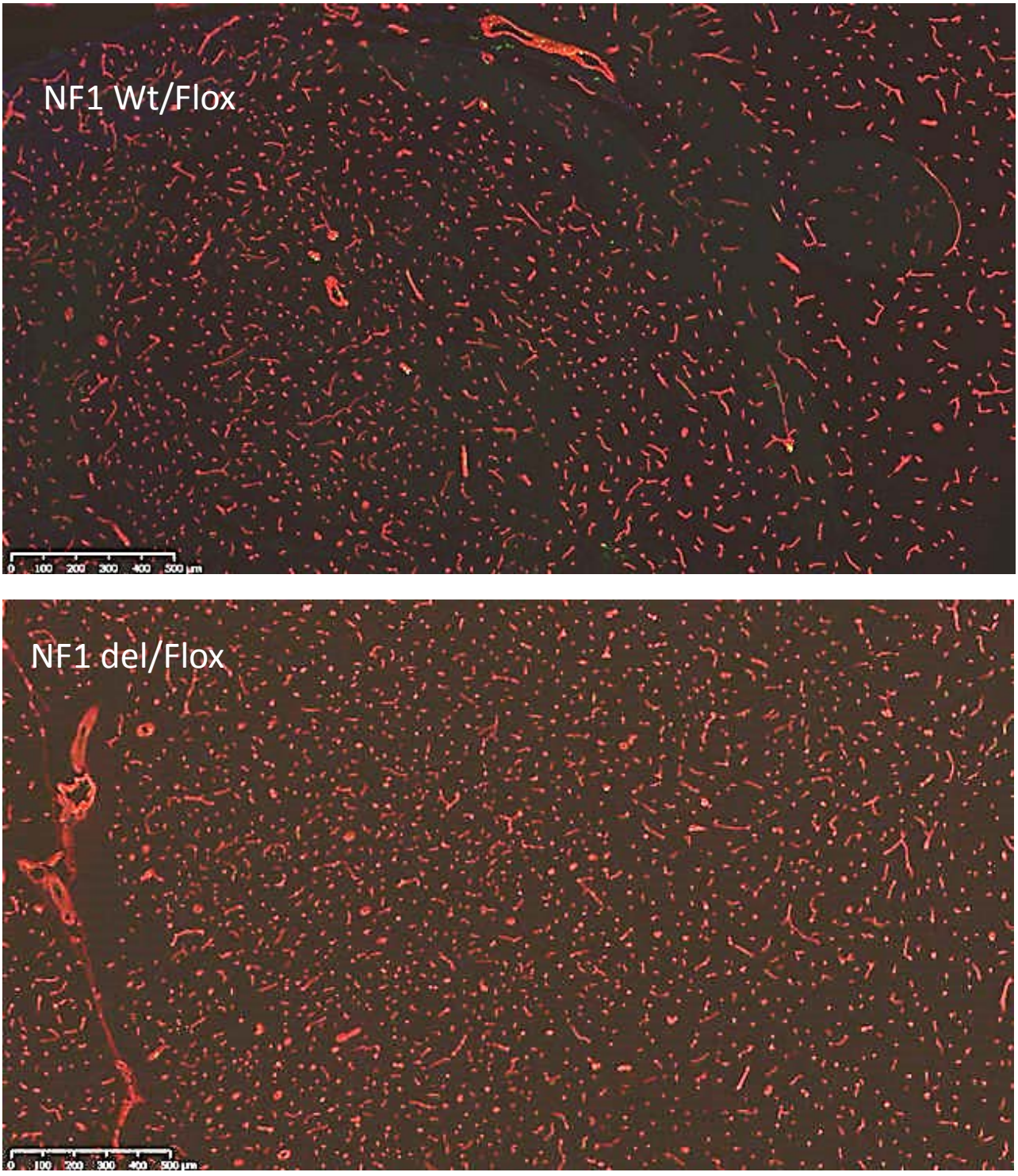


Figure 12. *Vascular patterning in adult mouse brain following endothelial Cre induction*. A mouse model of NF1 disease in the vascular endothelium of human disease was analyzed for apparent vascular abnormalities at three weeks following the induction of Cre-recombinase to drive the loss of the second NF1 allele, using collagen IV (Red)to mark the basement membranes of blood vessels. No apparent dysmorphogenesis was evident in the brain. Image was captured on Nanozoomer and scaled as indicated.

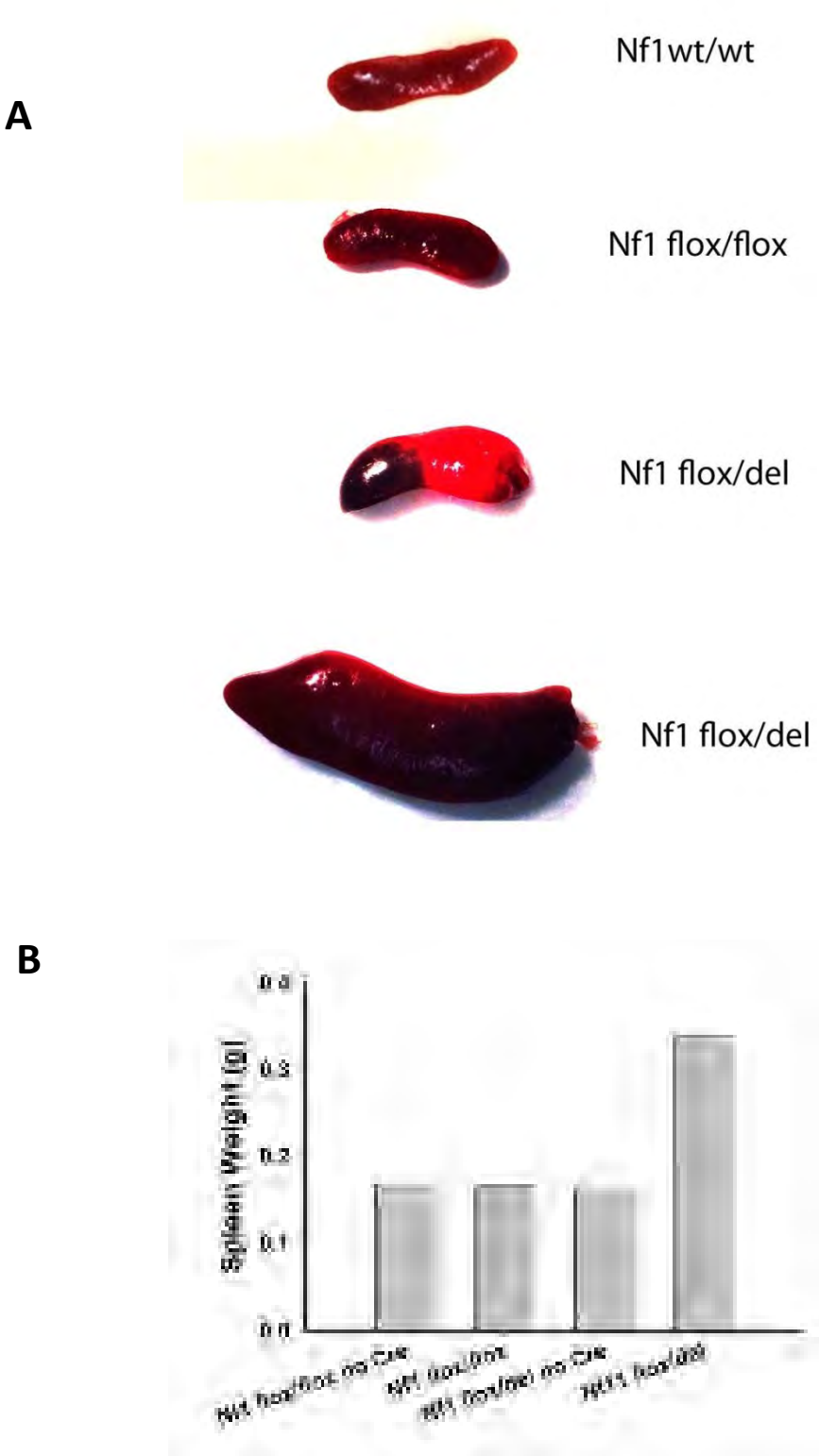


Figure 13. *Loss of NF1 in the endothelium results in splenomegaly* (A) Mice with the genotypes as indicated were treated with Tamoxifen by IP injection. Three weeks following induction mice were sacrificed. Necropsy revealed massively enlarged spleens. (B) a Similar experiment was repeated using Tamoxifen in the diet as an induction regimen. Spleens from these mice were weighed.

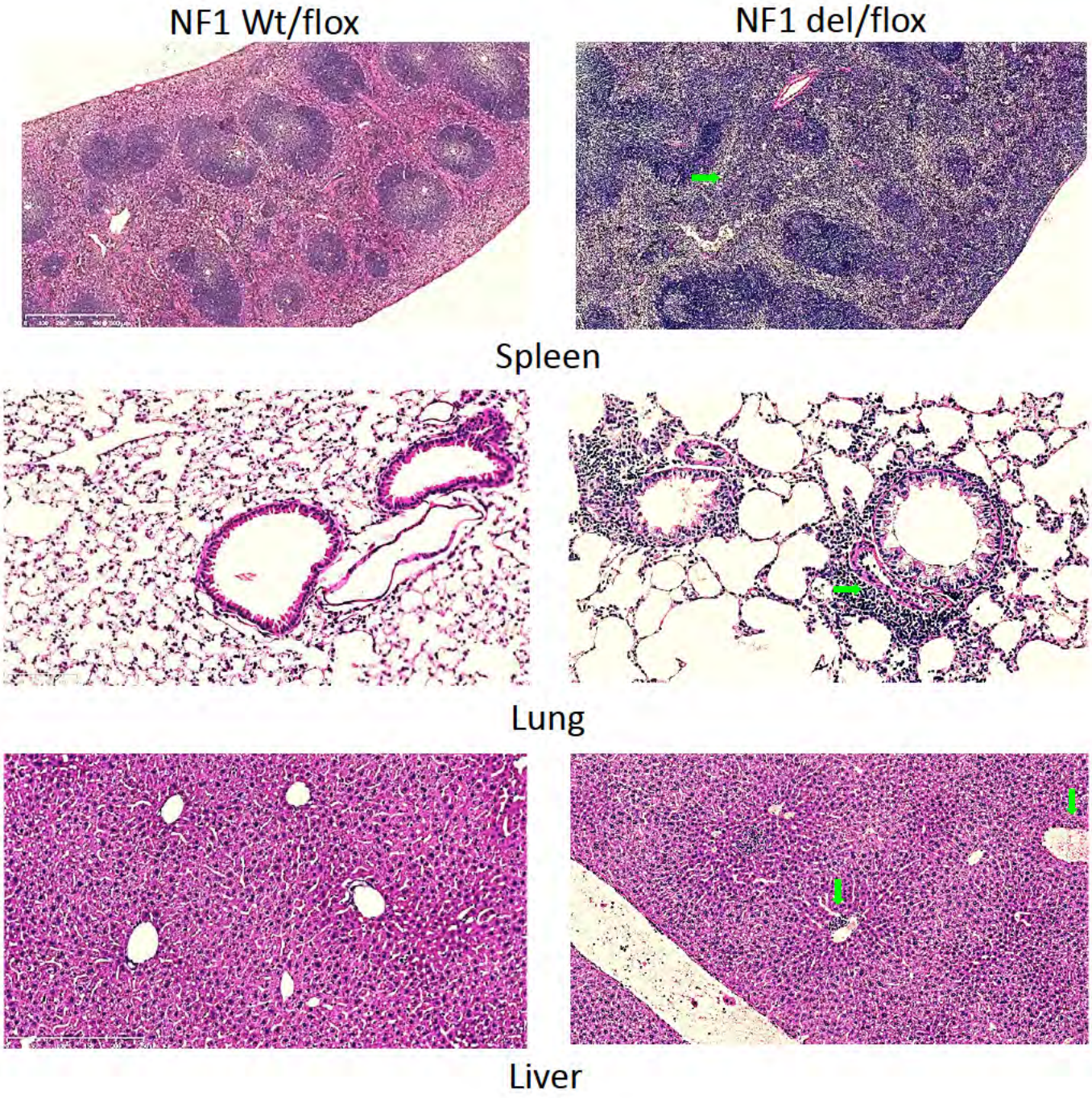


Figure 14. *NF1 del/flox mice develop inflammatory changes following loss of endothelial NF1* Organ tissues were examined by H&E. The spleen demonstrated what appears to be extra-medullary hematopoiesis with a loss of red pulp and massive infiltration of white blood cells. In lung, perivascular and peri-bronchial inflammatory infiltrates are evident. Similarly the liver demonstrates diffuse parenchymal inflammation and visible perivascular accumulation of white blood cells. Green arrows highlight some of the most visible differences.

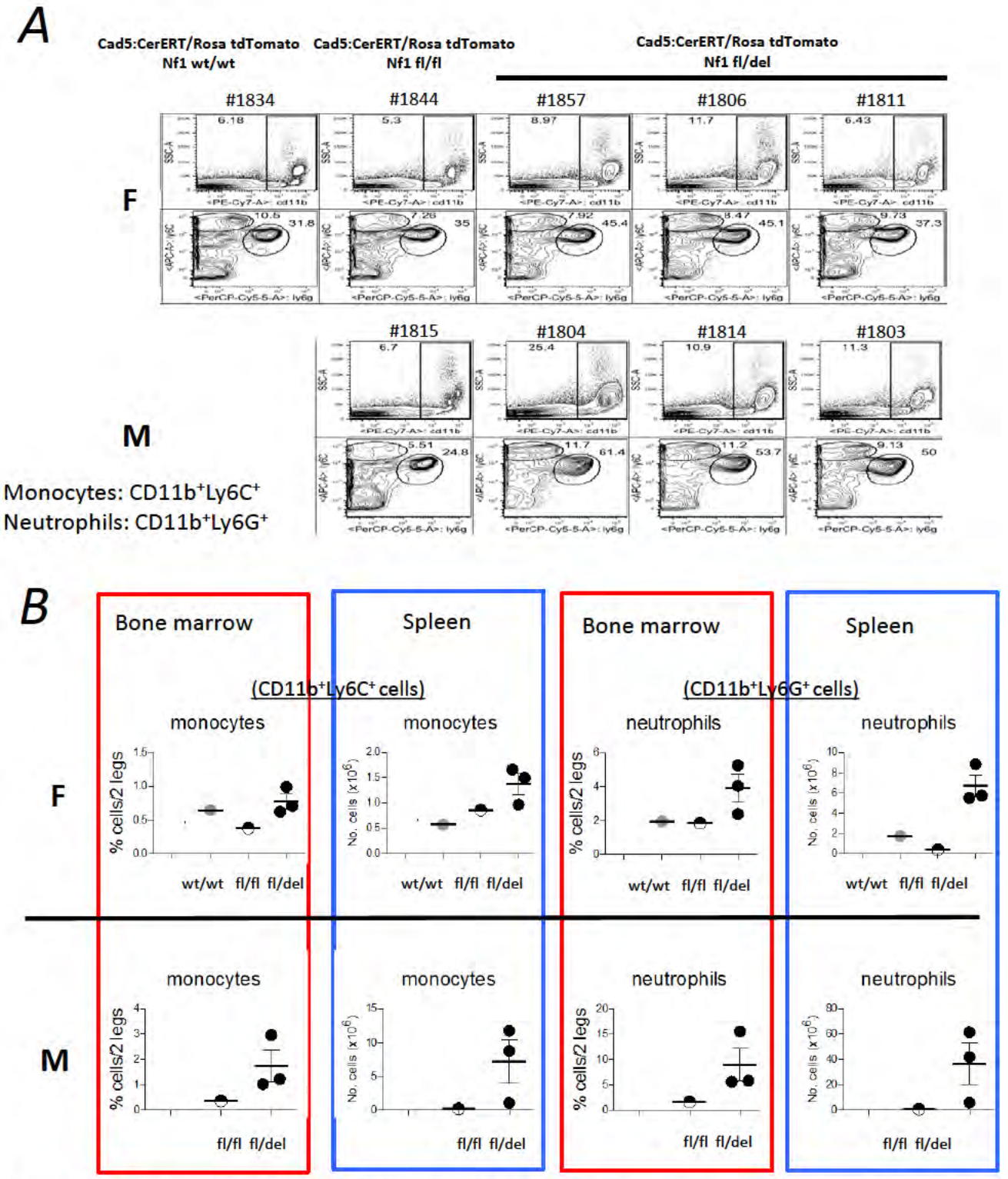
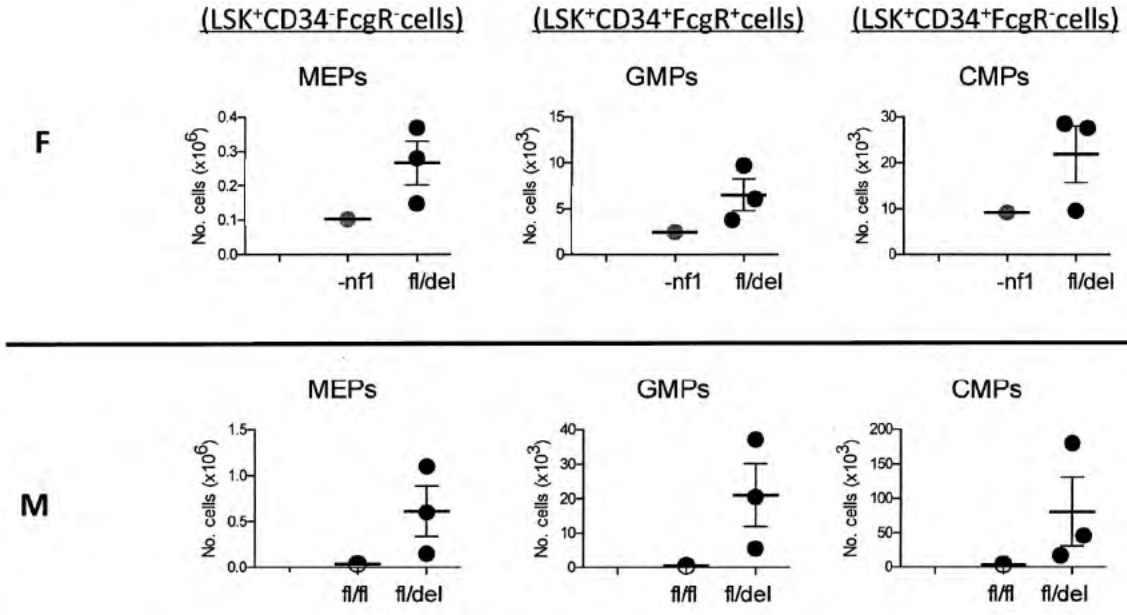


Figure 15. *NF1 loss in the endothelium drives granulocytic expansion in spleen and bone marrow*. Mice of the indicated genotype were treated with tamoxifen to induce loss of NF1 in the endothelium. **(A)** Facs analysis was performed on enumerated cells form the bone marrow and spleen using Ly6C and G to separate the monocytic and granulocytic compartments respectively. **(B)** Results are plotted by sub-type and enumeration location; Bone marrow boxed in red and Spleen in blue.

Splenic myeloid progenitor cells



Common myeloid progenitor-- CMP
Granulocyte-macrophage progenitor---GMP
Megakaryocyte-erythroid progenitor---MEP

Figure 16. *Myeloid expansion may result from enhanced progenitor populations* CD11b populations from the spleen were also sub-typed for the presence of markers of progenitor cells. These populations, while still rare, expand at least two fold in all cases.