

**Alternative splicing of endothelial fibronectin is induced by disturbed hemodynamics and protects against hemorrhage of the vessel wall**

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**Running title: FN splicing inhibits flow-induced vessel wall hemorrhage**

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## **Abstract:**

### **Objective:**

Abnormally low flow conditions, sensed by the arterial endothelium, promote aneurysm rupture. Fibronectin (FN) is among the most abundant extracellular matrix proteins and is strongly upregulated in human aneurysms, suggesting a possible role in disease progression. Altered FN splicing can result in the inclusion of EIIIA and EIIB exons, generally not expressed in adult tissues. We wished to explore the regulation of FN and its splicing and their possible roles in the vascular response to disturbed flow.

### **Approach and Results:**

We induced low and reversing flow in mice by partial carotid ligation, and assayed *FN* splicing in an endothelium-enriched intimal preparation. Inclusion of EIIIA and EIIB was increased as early as 48hrs, with negligible increases in total *FN* expression. To test the function of EIIIA and EIIB inclusion, we induced disturbed flow in *EIIIA*<sup>-/-</sup> mice unable to include these exons and found that they developed focal lesions with hemorrhage and hypertrophy of the vessel wall. Acute deletion of floxed *FN* caused similar defects in response to disturbed flow, consistent with a requirement for the upregulation of the spliced isoforms, rather than a developmental defect. Recruited macrophages promote *FN* splicing, since their depletion by clodronate liposomes blocked the increase in endothelial EIIIA and EIIB inclusion in the carotid model.

### **Conclusions:**

These results uncover a protective mechanism in the inflamed intima that develops under disturbed flow, by showing that splicing of *FN* mRNA in the endothelium, induced by macrophages, inhibits hemorrhage of the vessel wall.

## **Introduction:**

Risk of aneurysm is determined globally by genetic defects and locally by hemodynamic effects. Genetic risk is often traced to defects in extracellular matrix proteins, such as fibrillin1, collagens, fibulin-4 and elastin in the case of thoracic aneurysm<sup>1</sup>, and collagens in intracranial aneurysm and hemorrhagic stroke<sup>2,3</sup>. Other pathways, such as TGF $\beta$  and lysyl oxidase, are indirectly linked to extracellular matrix (ECM) protein deposition or organization. However, even given pre-existing genetic defects, not all blood vessels develop aneurysms; hemodynamics are a critical determinant of the location of aneurysms. For example intracranial aneurysms are more likely to grow and rupture in regions of the vasculature with disturbed flow<sup>4,5</sup>. The frictional force of flow, or shear stress, is sensed by the endothelium, which plays a critical role in regulating the vessels' response to these flows<sup>6</sup>. Thus, ECM composition and the endothelial response to disturbed flow are key determinants of aneurysm progression.

Fibronectin (FN) is a principal component of the vascular ECM and is highly upregulated in human aneurysm<sup>7-9</sup>. The assembly of FN is considered a crucial step towards the assembly of new vascular ECM, and essential for the matrix integration of many of the aforementioned aneurysm-linked proteins<sup>10</sup>. FN production and deposition by the endothelium is increased *in vitro* by low and disturbed flow, and increased FN has been observed in areas of vasculature chronically exposed to disturbed flow<sup>11</sup>. However, since FN deposition also appears to potentially increase the pro-inflammatory signaling of overlying endothelial cells<sup>11, 12</sup> and play a prominent role in leukocyte recruitment *in vivo*<sup>13</sup>, the role of FN in vasculature exposed to low and disturbed flow is not yet clear. While its structural function might suggest protection, it could also promote pro-inflammatory signaling thought to drive aneurysm progression. Thus, while the role of FN in aneurysm is not yet clear, it is poised to be a major player in regulating disease progression.

FN is alternatively spliced, resulting in variable inclusion of the EIIIA(EDA) and EIIB(EDB) domains and portions of the variable (V or IIICS) domain<sup>9, 14</sup>. Among vertebrates, EIIIA and EIIB domains are as highly conserved at the amino acid level as other FN domains, suggesting strong evolutionary pressure<sup>15</sup>. Their expression patterns are conserved among all vertebrates examined, including human, mouse, rat, cow, chicken, frog and zebrafish, most prominently around the developing heart and blood vessels. Nearly complete inclusion during early development drops to <5-10% post-natally. Although adults express very little EIIIA or EIIB, inclusion is increased in a variety of vascular injuries resulting in damage to the endothelium, such as balloon or wire injury in animal models<sup>16-19</sup>. Increased inclusion of EIIIA and EIIB has also been observed in human aneurysms<sup>20</sup>. But how expression of EIIIA and EIIB is regulated in aneurysm progression remains unclear. Also unclear is how the production of EIIIA+EIIB+ FN affects vasculature damaged by exposure to low and disturbed flow.

We hypothesized that low and disturbed flow might promote the increased inclusion of alternative exons EIIIA and EIIB in the arterial endothelium and, furthermore, that the upregulation of these alternative exons might play a role in preventing the destructive effects of low and disturbed flow on the arterial endothelium. Here we describe experiments supporting these hypotheses.

### **Materials and Methods:**

Materials and Methods are available online.

## **Results:**

### **Low and disturbed arterial flow promotes increased inclusion of FN alternative exons, EIIIA and EIIB.**

To examine alternative splicing in response to low and disturbed arterial flow, we performed near complete carotid ligation on C57BL/6J mice. This severely reduces mean blood velocity in the ligated carotid artery (by ~75%, Figure 1 & 4) and results in the pro-inflammatory activation of the endothelium<sup>21</sup>. We collected RNA from the intima of these mice by a Trizol flush following published methods, yielding a 3000-fold enrichment of endothelial mRNA *Pecam1* (*CD31*) relative to smooth muscle mRNA *acta2* (*alpha-smooth muscle actin*) in the intimal preparation relative to the remainder of the carotid artery (Figure 1A online)<sup>21, 22</sup>. Purified intimal RNA was pooled from three groups of 4-5 vessels for each flow regime: low-flow (ligated) or high-flow (contralateral to the ligated artery). The mRNA pool was sequenced and splicing assessed by MISO<sup>23</sup>. We found that, at 48hrs, the levels of both EIIIA and EIIB inclusion in the intima increased by about 3- to 4-fold over the levels in contralateral carotid intima (Figure 1A&B). Baseline levels of EIIIA inclusion (17%) were higher than EIIB inclusion (5%), as were the levels under disturbed flow (55% for EIIIA and 19% for EIIB).

RNA-seq analysis provided an aggregate assessment of splicing changes in a large pool of mice. To determine splicing changes with greater resolution in the intima of individual carotid arteries, we established a quantitative PCR (qPCR) assay to measure fold changes in the levels of EIIIA+ and EIIB+ mRNAs relative to total *FN* mRNA (Figure II online). We extrapolated the actual inclusion rates from the qPCR data, using cDNA prepared from sequenced samples to calibrate.

Using these methods, we examined splicing in two more cohorts of mice, at 48hrs and at 7 days after nearly complete carotid ligation. We found that the mean changes in inclusion observed by qPCR in these new cohorts replicated the results obtained by RNA-seq. Under low-flow conditions, EIIIA and EIIB inclusion rates increased 2- to 3-fold (from 19% to 47% for EIIIA and from 5% to 11% for EIIB) over the high-flow contralateral carotid at 48hrs (Figure 1C). The high inclusion levels were maintained at the 7-day time-point (Figure 1D). We included sham-operated mice to control for splicing changes induced by the trauma of the surgery in the absence of flow changes. Although surgery alone induced a slight increase in EIIIA and EIIB inclusion at 48hrs, this was less than the change induced by low and disturbed flow [compare Ligated Low(L) vs. Sham noΔ(L) at 48hrs], and the effect disappeared by 7 days. Interestingly, the change in splicing occurred without a significant change in the total *FN* expression at either 48hrs or 7 days (Figure 1E&F). Thus, low and disturbed flow acutely increases intimal EIIIA and EIIB inclusion, as measured by two independent techniques, and this occurs without significant increases in total *FN* expression.

We then asked whether similar changes occurred in the media and adventitia, or whether they were confined to the intima. We used the same qPCR approach, except that analysis was performed on the remainder of the carotid artery, after the intimal flush. We found that, in contrast to the intimal changes, there were no significant differences in splicing between ligated and sham-operated arteries at 48hrs (Figure IIIA online). After exposure to 7 days of low and disturbed flow, inclusion of both EIIIA and EIIB were increased around 2-fold in the media & adventitia relative to the sham-operated carotid (Figure IIIC online). So, EIIIA and EIIB inclusion is also increased in the carotid media & adventitia by low and disturbed flow, but the magnitude of the switch is significantly lower relative to that in the intima. There were no differences in total FN expression (Figure IIIB&D online).

Although enriched for endothelium, the intimal flush includes RNA from adherent leukocytes, as well as fibroblasts and smooth muscle cells. To confirm that the alternatively spliced *FN* transcripts in the intima were endothelium-derived, we used *Cdh5-CreER<sup>T2</sup>* to excise floxed *FN* in the endothelium prior to disturbing flow (Figure IB online). Total *FN* expression was reduced ~90% in the intima at 7 days, without a significant reduction in the media & adventitia. Similarly, there was a ~90% reduction in EIIIA and EIIB (Figure IC online). These results indicate that the endothelium is the primary source of alternatively spliced *FN* in the intimal response to altered shear.

### **Absence of EIIIA and EIIB inclusion promotes hemorrhage of the vessel wall in response to low and disturbed flow.**

To test the function of EIIIA and EIIB inclusion in the vascular response to low flow, we performed carotid ligation in *EIIIA*<sup>-/-</sup>; *EIIB*<sup>-/-</sup> (*EIIAB*<sup>-/-</sup>) mice unable to include EIIIA and EIIB<sup>24</sup>. After 7 days of low flow, carotid arteries of *EIIAB*<sup>-/-</sup> mice showed localized hemorrhage of the vessel wall, typically associated with bulges in the artery (Figure 2A). To quantify these differences further, excised vessels were scored for an “injury” phenotype based on the degree of hemorrhage and/or bulging of the wall (Figure IV online). To confirm the presence of blood beneath the intimal endothelial cell layer, we performed immunofluorescence staining for the endothelial cell marker Pecam1 (CD31) and the erythrocyte marker Ter119. We found large areas of Ter119-labeled blood cells beneath the CD31-labeled endothelium, confirming that blood had leaked into the vessel wall and beneath the endothelium (Figure 2B and Figure V online). Although hemorrhages also occurred in the wild-type littermate controls, they were much less frequent (14% vs. 47%,  $P=0.02$ , Fisher’s exact test). Importantly, we observed no differences in the level of flow reduction induced by carotid ligation in *EIIAB*<sup>-/-</sup> and *EIIAB*<sup>+/+</sup> mice, indicating that the inability to alternatively splice *FN* is the underlying cause of the hemorrhages, rather than a quantitative difference in the hemodynamic stimulus (Figure 2C).

Histological analysis of the arteries suggests that a local expansion of the adventitia may contribute to the appearance of the bulged regions, since significant differences could be observed in thickness of the adventitia across multiple regions of *EIIIAB*<sup>-/-</sup> arteries versus *EIIIAB*<sup>+/+</sup> or *EIIIAB*<sup>+/-</sup> arteries (Figure VI online). In addition to the localized expansion of the adventitia, we also observed focal dilation of the artery lumen *in vivo*, by high-resolution ultrasound images collected at the 7 day endpoint. These focal dilations resulted in regions 30-70% larger than adjacent sections of the same artery, and could be observed in 5/9 arteries with hemorrhage, and 2/9 arteries without obvious hemorrhage. Similar focal dilations did also occur in ligated *EIIIAB*<sup>+/+</sup> control arteries, but the frequency was lower (8/26) overall, and followed a similar trend – appearing more often in arteries with hemorrhage (Figure VII online). Thus, hemorrhage was associated with both increased adventitial growth and lumen enlargement.

Based on proximity of the hemorrhage to the intimal layer, the severe damage to the VE-cadherin-labeled endothelial layer, and the ingrowth of VE-cadherin+ vessels from the intima into the vessel wall in vessels with bleeds (Supplementary Movie 1 and 2), we believe that these bleeds originate from the carotid lumen, rather than from the vasa vasorum. Hemorrhage and bulges were specific to the low-flow carotid artery, and never observed in sham-operated vessels (N=11) or contralateral arteries (N=110). Extravascular bleeding occurred at some point between 48hrs and 7 days, since it was never observed in any vessels harvested at 48hrs, including *EIIIAB*<sup>-/-</sup> (N=5) and a variety of other genotypes (N=101). Therefore, in mice unable to include alternative FN exons EIIIA and EIIB, the intima is at increased risk of hemorrhage and injury when chronically exposed to low and disturbed flow. Notably, the most injured arteries had some of the highest inclusion levels recorded (Figure 1, colored points). Interestingly, this was specific to the intima, changes in medial splicing were not associated with the severity of the phenotype (Figure III online).

To determine whether the flow-induced hemorrhage could be linked to one of the two alternatively spliced domains, we examined *EIIIA*<sup>-/-</sup> mice unable to include EIIIA<sup>18</sup>. These mice appeared to show a milder form of the vessel wall hemorrhage phenotype (Figure IV online) and also showed instances of bleeding beneath the endothelium (Figure VIII online). Thus, the inability to include one of the two alternatively spliced exons may also increase the risk of hemorrhage, but with lower penetrance.

Since the hemorrhage and injury we observed resemble aspects of dissecting aneurysms, we tested whether the absence of EIIIA and EIIB inclusion is increased in another model of aortic dissection, based on the systemic administration of a high-dose of angiotensin II (Ang-II)<sup>25</sup>. In this model, Ang-II results in leukocyte recruitment, elastin degradation, medial apoptosis and partially penetrant dissection of the abdominal and thoracic aorta<sup>26</sup>. Interestingly, despite abundant FN accumulation in the intima of Ang-II-treated mice, EIIIA and EIIB inclusion was not increased to the same extent as in arteries exposed to

low and disturbed flow, nor were mice deficient in EIIIA and EIIB at increased risk of abdominal aorta dissection in this model (Figure IX online). Thus, alternative FN splicing is more regulated and essential to the vascular response to low and disturbed flow than to Ang-II driven aortic dissection.

### **Absence of EIIIA and EIIB does not inhibit FN expression or deposition**

The development of vessel wall hemorrhage in *EIIIAB*<sup>-/-</sup> mice led us to question whether the absence of EIIIA and EIIB resulted in reduced FN expression or deposition. We examined constitutive FN mRNA in the intimal flush and in the remaining media & adventitia of *EIIIAB*<sup>-/-</sup> mice and littermate controls subjected to nearly complete carotid ligation. We found that there was no difference in the level of total FN mRNA in the intima at 7 days after nearly complete carotid ligation in *EIIIAB*<sup>-/-</sup> mice relative to their littermate controls (Figure 3A). So, the absence of EIIIA and EIIB did not impinge on the levels of FN transcript in either the intima or the media & adventitia.

At the protein level, results were similar. Increased FN deposition could be observed in the intima of ligated carotid arteries, relative to sham-operated controls, but the level of staining was not significantly reduced in *EIIIAB*<sup>-/-</sup> mice (Figure 3B). Instead, there was increased deposition of FN in the intima of *EIIIAB*<sup>-/-</sup> mice, as determined by quantitative immunofluorescence (Figure 3C). Although all mice that developed vessel wall hemorrhage had high levels of intimal FN, only in the *EIIIAB*<sup>-/-</sup> group was FN deposition increased in the absence of severe injury (score  $\geq 2$ ). It is not yet clear whether the increased FN deposition represents leak of plasma FN; extravasation of plasma albumin was not significantly increased by the same methods, although it was increased relative to the contralateral artery (Figure X online).

### **Acute deletion of FN promotes hemorrhage of the vessel wall in response to low and disturbed flow**

Hemorrhage in response to low and disturbed flow could be a consequence of the absence of the EIIIA and EIIB isoforms throughout vascular development, the inability to increase the expression of these isoforms under low and disturbed flow, or both. To tease apart these possibilities, we investigated whether there was an acute requirement for FN expression in the response to low and disturbed flow. We had previously observed that the acute deletion of floxed FN in the endothelium by *Cdh5-CreER*<sup>T2</sup> almost entirely ablated endothelial FN mRNA (Figure I online), but did not predispose to flow-induced hemorrhage (Figure 4 and Figure IV online). However, our analysis of FN splicing had revealed increased *EIIIA*<sup>+</sup>*EIIB*<sup>+</sup> FN mRNA in the adjacent media following carotid ligation (Figure III C online), suggesting that smooth muscle cells may be compensating for endothelial FN production. Further supporting this line of reasoning, there was a trend towards increased expression of *EIIIA*<sup>+</sup> and *EIIB*<sup>+</sup>

FN in the media & adventitia of *Cdh5-CreER<sup>T2</sup>; FN<sup>ff</sup>* mice subjected to nearly complete carotid ligation (Figure IC online).

To determine whether FN production by mural cells is able to compensate for the acute deletion of FN in the endothelium we took two complementary approaches, using *Rosa26-CreER<sup>T2</sup>; FN<sup>ff</sup>* mice to delete FN acutely and globally <1 week before carotid ligation, and a combination of *SM22-Cre* and *Cdh5-CreER<sup>T2</sup>* to acutely delete endothelial FN <1 week before carotid ligation in the absence of smooth muscle FN.

As expected, *Rosa26-CreER<sup>T2</sup>* effectively activated a Cre reporter throughout most, but not all, of the endothelial and smooth muscle cells (Figure 4) and ablated plasma FN within 2 days (data not shown), resulting in a nearly complete inhibition of FN protein deposition in the intima of vessels exposed to low and disturbed flow (Figure 4). Without the ability to acutely increase FN production, arteries were susceptible to hemorrhage (2/8 mice and none of the littermate controls) and almost all of the arteries demonstrated a failure in inward remodeling (Figure 4, Figure IV and VII online). In fact, the lumen of many of the arteries enlarged beyond the size of sham-operated controls (Figure VII online). Importantly, neither injury nor hemorrhage were observed in the contralateral arteries or sham-operated *Rosa26-CreER<sup>T2</sup>; FN<sup>ff</sup>* arteries, indicating that enlargement of the artery and hemorrhage were specifically induced by low and disturbed flow conditions, rather than by the acute ablation of FN expression.

However, *Rosa26-CreER<sup>T2</sup>* removes all sources of FN production, including EIIIA-EIIIB- plasma FN. To address more specifically the acute requirement for the EIIIA+ and EIIIB+ FN variants produced by the endothelium, and to delete FN more stringently in the vessel wall, we examined *Cdh5-CreER<sup>T2</sup>; SM22-Cre; FN<sup>ff</sup>* mice after the acute deletion of endothelial FN (<1 week prior to nearly complete carotid ligation). *SM22-Cre* allowed stronger deletion of FN in the vessel wall than *Rosa26-CreER<sup>T2</sup>*, and *Cdh5-CreER<sup>T2</sup>* was more effective in the deletion of endothelial FN than *Rosa26-CreER<sup>T2</sup>*. Despite abundant FN, presumably plasma-derived and EIIIA-EIIIB-, in the intima, arteries unable to produce EIIIA+ and EIIIB+ FN variants in the endothelium developed hemorrhage. Importantly, none of these defects was observed in *SM22-Cre; FN<sup>ff</sup>* littermates, which were born in Mendelian ratios without noticeable vascular defects (Figure 4 and Figure IV online) and resembled littermate *FN<sup>ff</sup>* mice in carotid structure and response to low and disturbed flow, indicating that the defects observed were a result of the absence of endothelial FN.

Therefore, both approaches that acutely blocked the ability to produce new endothelial EIIIA+EIIIB+ FN replicated the hemorrhages in the vessel wall observed in EIIIB-/- mice. Notably, of the two approaches, a stronger phenotype was observed using *Cdh5-CreER<sup>T2</sup>; SM22-Cre; FN<sup>ff</sup>* than *Rosa26-CreER<sup>T2</sup>*, despite the presence of abundant remaining plasma FN in the former case.



**Macrophages are required for the induction of EIIIA and EIIB in the vessel wall.**

Given the importance of the endothelial *FN* splicing switch in preventing the damaging effects of disturbed blood flow on the vessel wall, we investigated how the splicing switch is regulated. We found that macrophages, which are often recruited to sites of injury where EIIIA and EIIB expression is increased, are recruited to the carotid arterial endothelium under disturbed flow conditions (Figure XI online). Therefore, we hypothesized that they might promote EIIIA and EIIB inclusion.

To test this, we used clodronate liposomes to deplete macrophages in mice subjected to carotid ligation, and examined EIIIA and EIIB expression in the intimal flush. Analysis of the macrophage-specific mRNA *Cd68* revealed that the 7-fold increase induced by low and disturbed flow conditions could be completely blocked by clodronate-liposome treatment (Figure 5A). Inhibiting the recruitment of macrophages resulted in a suppression of increased *FN* expression (Figure 5B) and a complete abrogation of the induction of EIIIA and EIIB by low and disturbed flow (Figure 5C&D). Therefore, the switch in endothelial splicing under low and disturbed flow depends on macrophages, which are recruited in large numbers under these conditions.

## **Discussion:**

Low and disturbed arterial flow is known to promote aneurysm growth and rupture<sup>5, 27</sup>. The ECM is profoundly altered in late-stage aneurysms, but how disturbed flow regulates ECM composition *in vivo* remains poorly characterized. Furthermore, how acute changes in individual ECM components during aneurysm formation might contribute to or inhibit further progression is unclear. Here, we show that macrophages, recruited by disturbed flow<sup>28</sup>, promote a rapid change in *FN* splicing resulting in increased expression of EIIIA+ and EIIB+ variants as early as 48hrs after a change in flow. The acute upregulation of these isoforms inhibits hemorrhage of the vessel wall, revealing a protective splicing mechanism in the vascular endothelium of regions exposed to severely disturbed flow.

### **Upregulation of alternative splice variants of *FN* protects against hemorrhage of the vessel wall under low and disturbed flow.**

Our results suggest that alternative *FN* splicing in the vessel wall is an important protective mechanism against hemorrhagic rupture of the intima, and therefore may play a role in the growth and rupture of areas of aneurysm exposed to disturbed flow. Increased EIIIA and EIIB inclusion has been observed in late-stage human aortic aneurysms<sup>20</sup>. Interestingly, EIIIA failed to increase in a subgroup of patients at higher risk of dissecting aneurysm<sup>20</sup>. The same patient group (bicuspid aortic valve) is also at increased risk of intracranial aneurysm, although it is not clear whether this is due to altered *FN* splicing<sup>29</sup>. Given the phenotypic complexity of human samples, these observations remained correlative. Here, we provide genetic evidence that deficient EIIIA and EIIB inclusion directly increases the risk of damage to the vessel wall.

It is not clear why deficiency in EIIIA and EIIB inclusion promotes hemorrhage in response to disturbed flow but not in response to Ang-II. Perhaps one clue lies in the cell types most affected. Low and disturbed flow acts primarily on the endothelium. In the carotid ligation model, macrophage accumulation and changes in *FN* splicing were strongest in the intima. In contrast, it has been proposed that macrophages recruited to the adventitia drive aortic dissection in the Ang-II model<sup>30</sup>. Our data show that both macrophage recruitment and alternative *FN* splicing are reduced in the Ang-II intima, relative to the intima of vessels exposed to low and disturbed flow. Thus, we suggest that alternative *FN* splicing will be of greatest importance in preventing vascular damage in diseases with a strong component of endothelial activation and inflammation.

The mechanisms of protection conferred by EIIIA and EIIB are of considerable interest. Several might be proposed: 1) EIIIA is known to bind and activate TLR4. This could skew macrophage phenotype directly, or affect the presentation of autoantigens from the lesion to innate immune cells (EIIIA has been shown to be a potent adjuvant, and immunization of mice with *FN*

decreases atherosclerotic vascular injury)<sup>31, 32</sup>; 2) EIIIA inclusion in full-length recombinant FN has been shown to increase cell spreading *in vitro* suggesting that it may affect proliferation and apoptosis pathways linked to spreading<sup>33</sup>; 3) Suppressing EIIIA and EIIB inclusion in endothelial cells may affect fibrillogenesis<sup>34</sup>, and thus both endothelial cell-cell junctions and permeability<sup>35</sup>; 4) Mice with increased EIIIA inclusion clot faster under arterial flow rates<sup>36</sup>, suggesting that increasing EIIIA (and perhaps also EIIB) inclusion may help quickly staunch intimal hemorrhage. A better understanding of how EIIIA and EIIB prevent vessel damage should reveal new ways in which the vasculature locally resists flow-induced damage.

### **Macrophages promote the increased EIIIA and EIIB inclusion in the endothelium**

We show that macrophage recruitment is critical for the increased expression of EIIIA and EIIB. In a variety of other fibrotic injury settings, such as organ transplant rejection, and skin, lung, kidney and liver damage and cancer, EIIIA and EIIB inclusion is increased<sup>37</sup>. Among isolated cell types from the liver, endothelial cells showed the strongest increase in inclusion<sup>38</sup>. This switch is thought to be one of the earliest steps in fibrosis, which is EIIIA-dependent in both lung and liver injury models<sup>39, 40</sup>. Our data suggest that recruitment or activation of macrophages could provide signals to promote EIIIA inclusion. The specific mechanisms through which they induce FN splicing are not yet clear, but we have observed a good correlation between PAI1 and the inclusion of EIIIA and EIIB, as well as total FN expression (Figure XII online), perhaps implicating TGF $\beta$  - known to regulate PAI1 and EIIIA inclusion *in vitro*<sup>11</sup>. Conditional deletion of macrophages has revealed a critical role in driving fibrosis in the liver and the lung<sup>41, 42</sup>. So, in a variety of fibrotic settings, macrophages may play a similar role in the induction of alternative FN splicing, with important implications for the composition and signaling of the fibrotic ECM.

Increased numbers of macrophages are found in ruptured intracranial aneurysms, and clodronate depletion experiments suggest macrophages drive the growth of intracranial aneurysms in elastase-induced animal models<sup>43, 44</sup>. We observed that the chronic depletion of macrophages in our carotid ligation model acutely suppresses the flow-mediated activation of the endothelium (indicated by reduced intimal PAI-1 and  $\alpha$ -Smooth muscle actin expression, Figure XIII online) and arterial stiffness (indicated by pulse distension, Figure XIII online), markers of arterial injury. Our current model (summarized in Figure XIV online) is that the exposure of the arterial endothelium to reduced blood flow increases the expression of leukocyte adhesion receptors (e.g., ICAM and VCAM), resulting in the recruitment of blood-derived monocytes to the intima. Monocytes differentiate to macrophages and elicit changes in the endothelium, including the alternative splicing of FN, that are critical in preventing damage to the vessel wall. Since macrophages are also instigators of arterial injury, alternative FN splicing in the

endothelium may be thought of as a mechanism induced by macrophages that protects against their own damaging effects on the vessel wall.

### **Acute flow-induced hemorrhage of the vessel wall**

Hemorrhage in the carotid artery under disturbed flow came as a surprise, since other investigators used the same model, and even inhibited FN assembly (using a small peptide inhibitor) but did not report vessel-wall hemorrhage<sup>45-47</sup>. A likely explanation is that the penetrance of this phenotype on the wild-type background is low (4/27 *AB*<sup>+/+</sup> littermate controls, 0/15 C57 mice +/- clodronate liposomes, 1/9 *EIII*A<sup>+/-</sup> littermate controls, 0/22 *FN*<sup>ff</sup> or *FN*<sup>f/+</sup> littermate controls), and may have escaped the notice of previous investigators. In a typical cohort of 5 to 10 mice, such defects would be rare. However, in the *EIII*A<sup>-/-</sup>, *ROSA-CreER*; *FN*<sup>ff</sup> and *Cdh5-CreER*; *SM22-Cre*; *FN*<sup>ff</sup> mice, hemorrhage within the vessel wall was seen in higher numbers (9/19, 2/8 and 2/8 respectively) and thus was obvious. It is also possible that hemorrhage occurs acutely and is resolved by later, 2-3 week time points; we have not yet examined this in detail. Consistent with the idea that vessel-wall hemorrhage is a partially penetrant phenotype that can be exacerbated by genetic background, hemorrhage has been observed in the carotid plaques of ApoE-deficient mice subjected to nearly complete carotid ligation<sup>21, 48</sup>. Similar to these ApoE<sup>-/-</sup> hemorrhages, we noted endothelial sprouting into the vessel wall<sup>49</sup> (Supplementary Movie 1 & 2). Interestingly, our *en face* imaging suggests that these sprouts originate from the lumen, rather than the vasa vasorum. Therefore, we suggest that the established carotid ligation model can be used as a platform to investigate the contributions of various genetic and epigenetic regulators of disturbed flow on intimal damage and hemorrhage of the vessel wall.

In conclusion, we report that the alternatively spliced *EIII*A and *EIII*B exons of *FN*, long-known to be conserved and highly regulated in all vertebrates but without clearly understood functions, play a significant role in protecting the vasculature from inflammation caused by disturbed flow. They are upregulated in response to disturbed flow by recruited macrophages, and if this upregulation is prevented, hemorrhagic injury of the vessel wall ensues. Since inclusion of these two domains of *FN* occurs in many pathological situations (atherosclerosis, myocardial infarction, organ transplant and other fibroses, angiogenesis and tumor progression), further investigation of the regulation and function of *FN* splicing is warranted. An emerging view is that alternative-splicing programs broadly regulate tissue function<sup>50, 51</sup>. *FN* is likely only one of many genes affected by splicing changes in the activated vascular endothelium. Future work should aim to dissect the regulation and function of these splicing programs.

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**Disclosures:**

None

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**Significance:**

Brain aneurysms are fairly common, but their catastrophic rupture is not. Disturbed flow within the aneurysm is one of the best predictors of rupture, risk of which is strongly modified by extracellular matrix composition. Fibronectin (FN), an essential extracellular matrix protein, is abundant in human aneurysms where it is also alternatively spliced. However, neither the regulation of alternative FN splicing nor its impact on the vasculature have been clear.

Here, we show that disturbed flow promotes alternative splicing of FN in the endothelium. Furthermore, we show that the splicing change is protective, since mutant mice unable to produce alternatively spliced FN are at increased risk of hemorrhage of the vessel wall. Mechanistically, we found that alternative FN splicing was almost entirely dependent on macrophages, recruited to the endothelium by disturbed flow. These results indicate that alternative FN splicing protects against the damaging effects of disturbed flow on the arterial wall.

**Figure 1. Low and disturbed flow promotes inclusion of alternative exons EIIIA and EIIB.**

A) Relative read densities in EIIIA, EIIB and flanking exons from the sequenced intimal RNA of low-flow (ligated) and high-flow (contralateral) carotid arteries 48hrs after surgery. Numbered curves indicate exon-spanning reads. B) Inclusive and exclusive reads were used by MISO to calculate the % inclusion rate (Psi) of alternative FN exons EIIIA and EIIB. Average of three pools (4-5 arteries each) and the standard deviation is shown. (C&D) Exon inclusion in individual carotid arteries as measured by quantitative PCR at 48hrs (C) and 7 days (D) after nearly complete carotid ligation or sham operation. (E&F) Fold-change in FN over sham-operated contralateral artery. Each dot (C-F) represents analysis of the intimal flush from a single carotid artery. Significance of the difference between (B) low flow and high flow pools by Student's t test and (C-F) the ligated left carotid measurements and the other groups by ANOVA and Tukey's multiple comparison test : \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001.

**Figure 2. Mice deficient in EIIIA and EIIB are prone to sub-endothelial hemorrhage in response to low and disturbed flow.**

A) Isolated carotid arteries from *EIIAB*<sup>-/-</sup> and *EIIAB*<sup>+/+</sup> mice after 7 days of low and disturbed flow conditions. Note the visible extravascular blood in the AB<sup>-/-</sup> mouse following saline perfusion (arrowheads). B) Combined staining for CD31 and Ter119 shows blood cells beneath the endothelium. Percent of mice with extravascular blood, following saline perfusion is shown in the graph. P=0.04 by two-tailed Fisher's exact test. C) Mean ultrasound velocity from in vivo measurements at 7 days after nearly complete carotid ligation. Each dot is the measurement of a single carotid artery. Red dots indicate arteries with sub-endothelial hemorrhage. Scale bars = 100µm and 25µm (higher magnification).

**Figure 3. Deficiency in EIIIA and EIIB does not inhibit FN expression or deposition.**

A) Expression of FN mRNA in the intima and media/adventitia of AB<sup>+/+</sup> and AB<sup>-/-</sup> mice 7 days after ligation or sham operation. Each dot represents analysis of the intimal flush from a single carotid artery. B) Immunofluorescence staining for total FN (red) and CD31 (green) in sections of carotid arteries, 7 days after ligation, with and without severe injury. C) Fluorescence intensity in sections of ligated carotid arteries in the intima or in the medial cell layers. Each dot represents a single carotid artery. \*\*P<0.01 by two-tailed Student's t test. Scale bars = 100µm

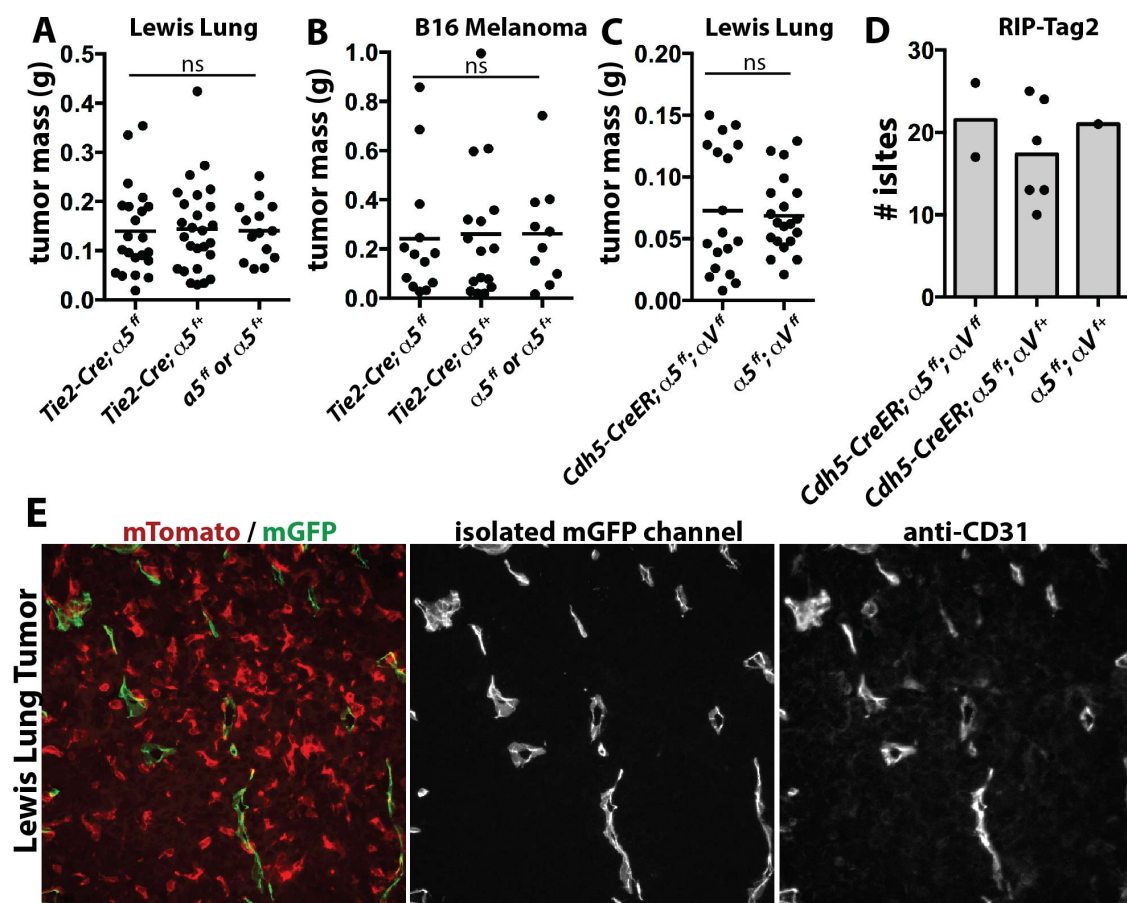
**Figure 4. Acute deletion of FN results in hemorrhage of the vessel wall.**

A) Excised vessels and sections of carotid artery from the indicated genotypes, showing expression of the mT/mG reporter (left panel) which switches from red to green upon Cre excision. FN immunofluorescence staining of the same vessels is shown in the right panel. The ligations have been left on some vessels, to show their location distal to the site of the hemorrhages. Arrowheads point to hemorrhage. B) Hematoxylin and eosin stained sections from the carotid arteries

shown above. Arrows point to blood beneath the endothelium (*ROSA26-CreER<sup>T2</sup>; FN<sup>fl/fl</sup>*) and between elastin layers of the media (*Cdh5-CreER<sup>T2</sup>; SM22-Cre; FN<sup>fl/fl</sup>*). Bottom right panel is immunofluorescence for CD31 (green), Ter-119 (red) and DAPI (blue). C) Mean ultrasound velocity from in vivo measurements at 7 days after nearly complete carotid ligation. Each dot is the measurement of a single carotid artery. Red dots indicate arteries with sub-endothelial hemorrhage, frequency of hemorrhage is indicated above. Scale bars = 100 $\mu$ m

**Figure 5. Endothelial EIIIA and EIIIB inclusion is increased in response to recruited macrophages**

A) Expression of CD68, a marker of macrophages, in the intimal RNA of C57BL/6J mice 48hrs after nearly complete carotid ligation, with or without prior treatment with chlodronate, as determined by quantitative PCR. B) Fold-change in FN expression, relative to the contralateral control from PBS liposome treated mice. C&D) Inclusion frequency of EIIIA (C) and EIIIB (D) in the intimal mRNA of the same C57BL/6J mice, as determined by quantitative PCR. (B-D). Each dot represents analysis of the intimal flush from a single carotid artery. ANOVA and Tukey's multiple comparison test \*\*P<0.01, \*\*\*P<0.001.



**Figure 1. Primary tumor growth is not affected by the endothelial deletion of alphaV and alpha5 integrins.**

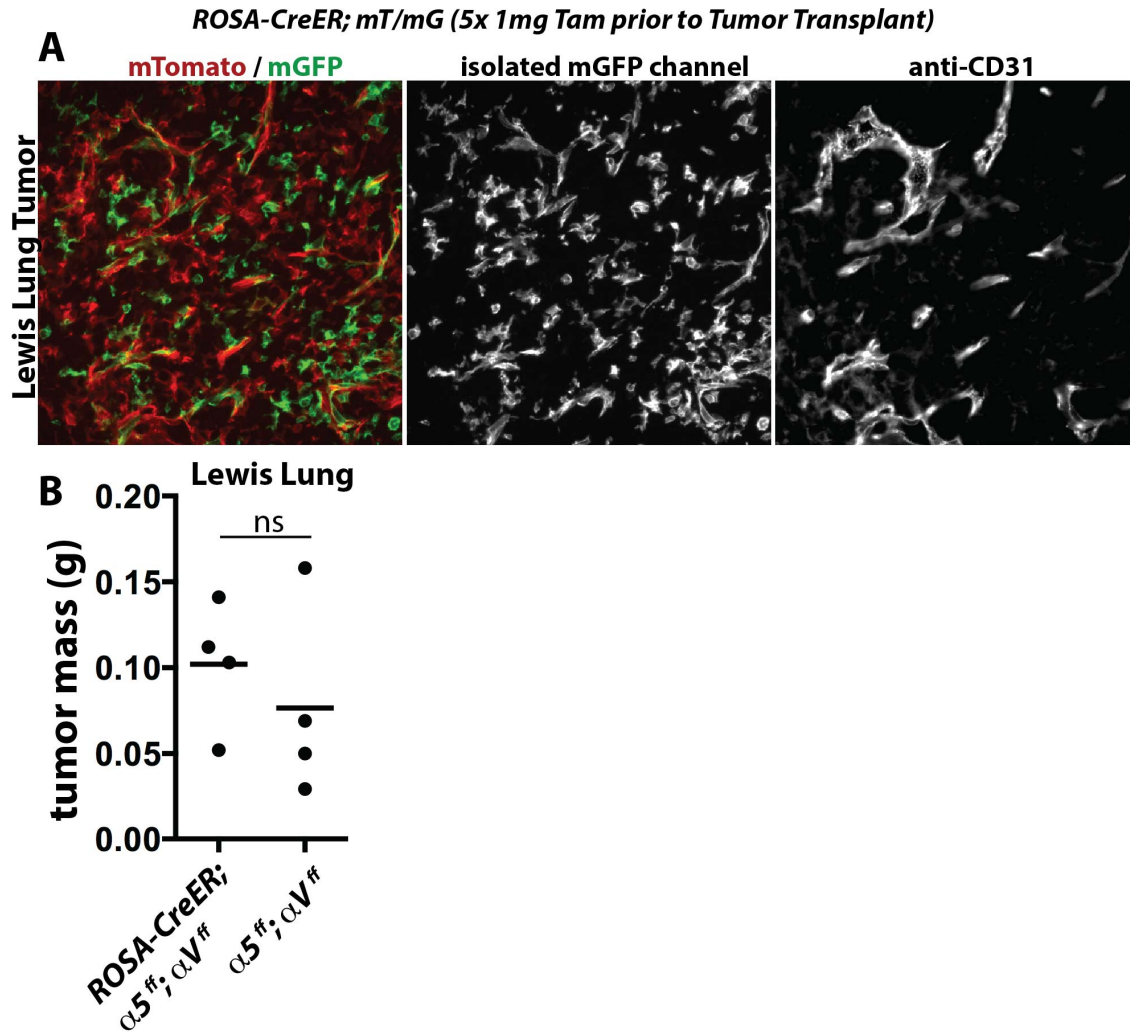
(A&B) Endothelial deletion of floxed alpha5 integrin by Tie2-Cre does not impair the growth of transplanted Lewis Lung (A) or B16 melanoma tumors (B). (C&D) The deletion of both alphaV and alpha5 by Cdh5-CreERT2 does not impair the growth of Lewis Lung transplant tumors (C) or RIP-Tag2 spontaneous pancreatic tumors (D). (E) Efficient excision of the host mT/mG Cre-reporter by Cdh5-CreERT2 in a transplanted Lewis Lung tumor. Note the close overlap of CD31 with the eGFP Cre-reporter. Red cells are host-derived cells in the tumor without Cre-excision.

**NOTE: in these experiments host deletion (by Tam) was performed prior to tumor growth.**

**NOTE: could show images of the same mT/mG reporter with the  $\alpha V$ ;  $\alpha 5$  deletion in the experiment.**

**NOTE: should confirm the deletion in alphaV alpha5 experiment**

- FACS of eGFP+ tumor cells or aortic endothelial cells, qPCR for mRNA ( $\alpha V$ ,  $\alpha 5$ , other integrins  $\alpha 4$ ,  $\alpha 9$  in particular?)
- IF for alpha5 in tumor sections (alphaV is difficult)



**Figure 2. Primary tumor growth is not affected by the host deletion of alphaV and alpha5 integrins.**

(A) Efficient excision of the host mT/mG Cre-reporter by ROSA-CreERT2 in a transplanted Lewis Lung tumor. Note the close overlap of CD31 with the eGFP Cre-reporter. Red cells are host-derived cells in the tumor without Cre-excision. (B) Host deletion of  $\alpha V$  and  $\alpha 5$  by ROSA-CreERT2 does not affect the growth of Lewis Lung tumors.

**NOTE: in these experiments host deletion (by Tam) was performed prior to tumor growth.**

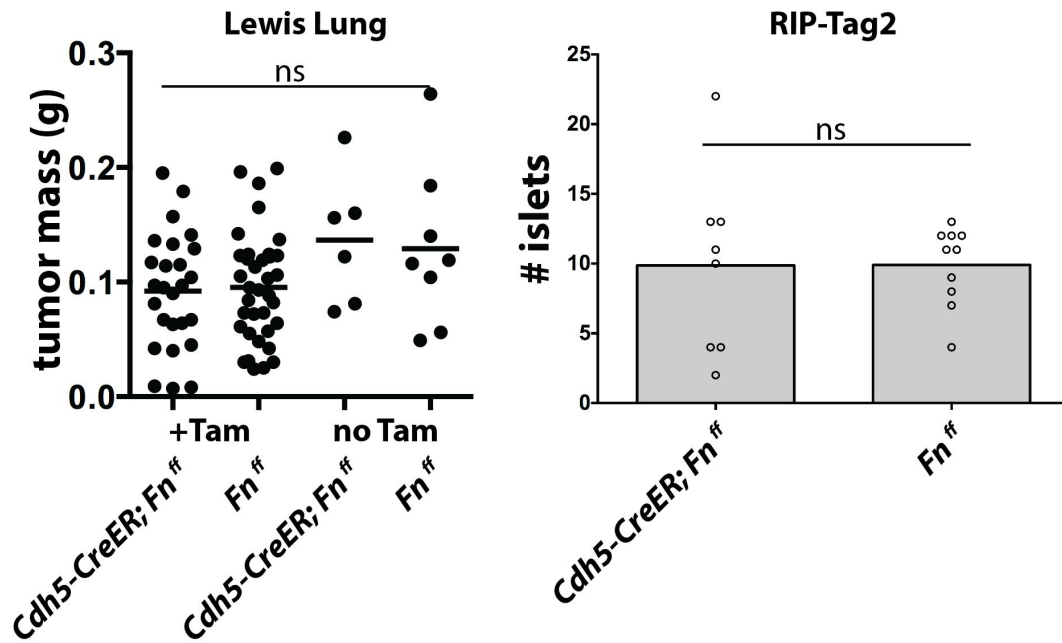
**NOTE: could show images of the same mT/mG reporter with the  $\alpha V$ ;  $\alpha 5$  deletion in the experiment.**

**NOTE: should confirm the deletion in alphaV alpha5 experiment**

- FACs of eGFP+ tumor cells, qPCR for mRNA ( $\alpha V$ ,  $\alpha 5$ , other integrins  $\alpha 4$ ,  $\alpha 9$  in particular?)
- IF for alpha5 in tumor sections (alphaV is difficult)

**Figure 3.**

Compensation by other integrins? From qPCR analysis of isolated cells from LL/2 tumors, test whether other integrins are upregulated in the absence of  $\alpha V$  and  $\alpha 5$ ?



**Figure 4. Endothelial deletion of Fn does not impair the growth of primary tumors**

(A&B) The deletion of both alphaV and alpha5 by Cdh5-CreERT2 does not impair the growth of Lewis Lung transplant tumors (A) or RIP-Tag2 spontaneous pancreatic tumors (B).

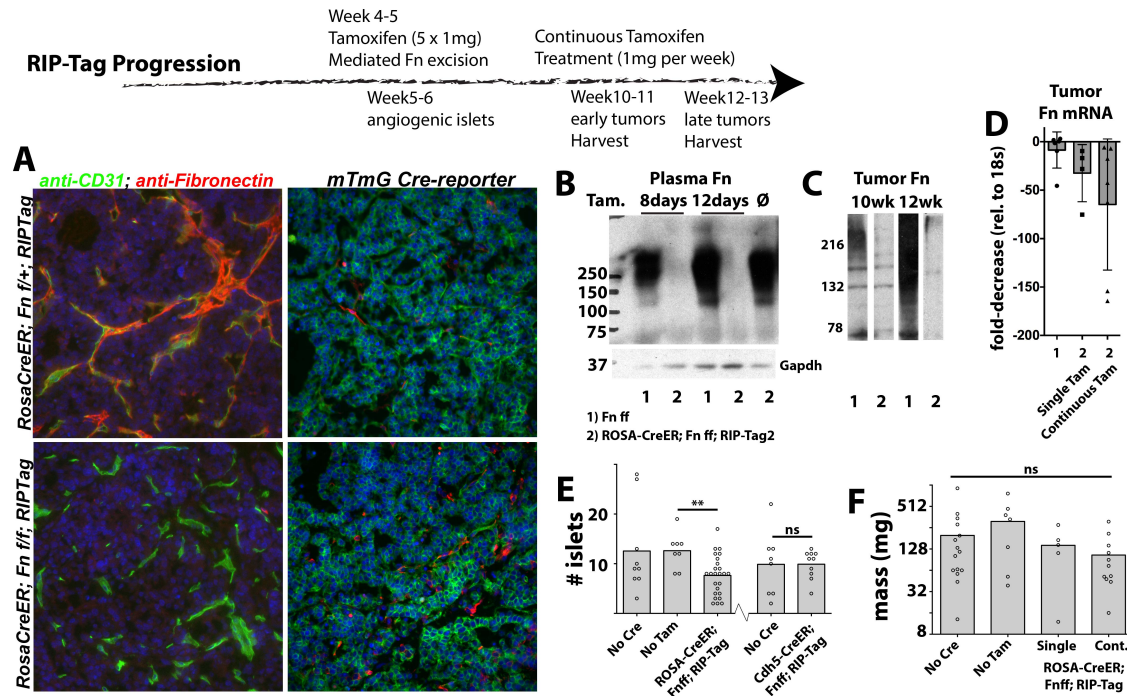
**NOTE: in these experiments host deletion (by Tam) was performed prior to tumor growth.**

**NOTE: could show images of the same mT/mG reporter with the Fn deletion in the experiment.**

**NOTE: should confirm the deletion in alphaV alpha5 experiment**

- FACS of eGFP+ tumor cells, qPCR for mRNA
- IF for Fn in tumor sections (other basement membrane proteins as well?)
- Maybe only if something looks differential in RIP-Tag, with the complete deletion of Fn, since this should be milder.





**Figure 5. Complete deletion of Fn impairs tumor initiation but not final size**  
 (A) Immunofluorescent staining for Fn in 13 week RIP-Tag tumors of mice with and without Fn deletion. Note the absence of Fn staining and abundant CD31 labeled vasculature. (B) Western blot of equivalent protein loads of whole blood from the indicated genotypes, 8 days or 12 days after the first tamoxifen treatment, or with no tamoxifen treatment. (C) Western blot of equivalent protein loads of tumor homogenate at 10 weeks (pool of tumors from 3 mice) or 12 weeks (single large tumor). (D) Results of quantitative PCR, showing the reduction in the level of Fn transcript, relative to 18s, in the indicated genotypes. Single Tam was treated only in the first week with Tamoxifen, Continuous Tam was treated weekly throughout. (E) Number of islets counted at 10-11 weeks in the indicated genotypes. (F) Total pancreatic tumor mass at 12-13 weeks in the indicated genotypes. For D, E and F, each dot represents a single animal.

**NOTE: should clean up the westerns here (8 days and endpoint blood Fn, with no Tam controls; tumor Fn on a single blot).**

**NOTE: should re-run the qPCR, these results do not seem as consistent as I would have predicted, given all the other results. Should be a more consistently strong reduction. Also now have more samples to add.**

## **Figure 6.**

### Compensation by other ECM proteins

#### RNA

- at least, run panel of primers for other ECM proteins in Alexandra's signature (12 week vs. 6 week RIP-Tag)
- Or run entire 250 gene panel on tumors
- this would not detect proteins produced elsewhere (fibrinogen) and deposited locally, which could be substituting in some way for Fn

#### Protein

- at least, run IF for fibrillins 1&2, collIV, LTBPs, vitronectin, laminin a4, a5, b2 (not sure if good antibodies exist for all the laminins, but vitronectin, CollIV and Sakai's Fbn1 and 2 and LTBP1 and 4 are good.
- Or run proteomics experiment (have >5 and 5 late stage Fn deleted tumors).