

## **B55 $\alpha$ /PP2A Limits Endothelial Cell Apoptosis During Vascular Remodeling: A Complementary Approach To Kill Pathological Vessels?**

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**Running title:** B55 $\alpha$  in Vascular Biology



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## ABSTRACT

**Rationale:** How endothelial cells (ECs) migrate and form an immature vascular plexus has been extensively studied. Yet, mechanisms underlying vascular remodeling remain poorly established. A better understanding of these processes may lead to the design of novel therapeutic strategies complementary to current angiogenesis inhibitors.

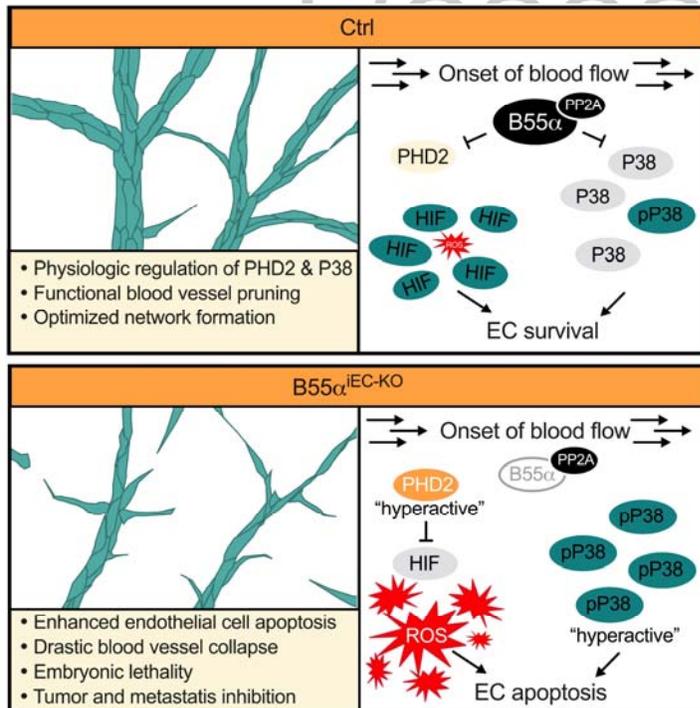
**Objective:** Starting from our previous observations that the PP2A phosphatase regulates the HIF/PHD2-constituted oxygen machinery, we hypothesized that this axis could play an important role during blood vessel formation, tissue perfusion and oxygen restoration.

**Methods and Results:** We show that the regulatory PP2A-phosphatase subunit B55 $\alpha$  is at the crossroad between vessel pruning and vessel maturation. Blood vessels with high B55 $\alpha$  will counter cell stress conditions and thrive for stabilization and maturation. When B55 $\alpha$  is inhibited, ECs cannot cope with cell stress and undergo apoptosis, leading to massive pruning of nascent blood vessels. Mechanistically, we found that the B55 $\alpha$ /PP2A complex restrains PHD2 activity, promoting EC survival in a HIF-dependent manner, and furthermore dephosphorylates p38, altogether protecting ECs against cell stress occurring, for example, during the onset of blood flow. In tumors, EC-specific B55 $\alpha$  deficiency induces pruning of immature-like tumor blood vessels resulting in delayed tumor growth and metastasis, without affecting non-pathological vessels. Consistently, systemic administration of a pan-PP2A inhibitor disrupts vascular network formation and tumor progression *in vivo* without additional effects on B55 $\alpha$ -deficient vessels.

**Conclusions:** Our data underline a unique role of the B55 $\alpha$ /PP2A phosphatase complex in vessel remodeling and suggest the use of PP2A-inhibitors as potent anti-angiogenic drugs targeting specifically nascent blood vessels with a mode-of-action complementary to VEGF(R)-targeted therapies.

### Keywords:

Angiogenesis, B55 $\alpha$ /PP2A-phosphatase, development, tumor progression, apoptosis, transgenic model, tumor, apoptosis.



## Nonstandard Abbreviations and Acronyms:

AKT1	AKT Serine/Threonine Kinase 1
AMPK	AMP-activated protein kinase
ATF4	activating transcription factor 4
CD31	cluster of differentiation 31
cl.Casp3	cleaved Caspase 3
ColIV	Collagen IV
DMOG	dimethyloxalylglycine
EC	endothelial cell
EdU	5-ethynyl-2'-deoxyuridine
eIF2 $\alpha$	eukaryotic initiation factor 2 alpha
ERK	extracellular signal-regulated kinases
FOXO1	forkhead box protein O1
NT	non treated
HIF	hypoxia-inducible factor
HMVECs	human microvascular ECs
HUVECs	human umbilical vein endothelial cells
IsoB4	isolectin griffonia simplicifolia B4
JNK	c-Jun N-terminal kinases
KD	knockdown
KO	knockout
LEC	lymphatic endothelial cell
LLC	lewis lung carcinoma
MnTBAP	Mn(III)tetrakis(4-benzoic acid)porphyrin chloride
Nec1	necrostatin-1
NF $\kappa$ B	nuclear factor kappa-light-chain-enhancer of activated B cells
NRF2	nuclear factor erythroid 2-related factor 2
OE	overexpression
o/n	over night
PHD	hypoxia-inducible factor prolyl hydroxylase 2
PKR	protein kinase R
PP2A	protein phosphatase 2
qVD	5-(2,6-Difluorophenoxy)-3-[[3-methyl-1-oxo-2-[(2-quinolinylcarbonyl)amino]butyl]amino]-4-oxo-pentanoic acid hydrate
ROS	reactive oxygen species
VEGF-R	vascular endothelial growth factor receptor
zIETDS	L-Threoninamide, N-[(phenylmethoxy)carbonyl]-L-isoleucyl-L- $\alpha$ -glutamyl-N-[(1S)-3-fluoro-1-(2-methoxy-2-oxoethyl)-2-oxopropyl]-, methyl ester
zVAD	z-Val-Ala-DL-Asp(OMe)-fluoromethylketone



## INTRODUCTION

The formation of the first vascular network is characterized by fusion of tip cells and the subsequent start of blood vessel perfusion<sup>1</sup>. However, this immature plexus is still inefficient and lacks a clear distinction between arteries, capillaries and venous structures<sup>2,3</sup>.

To create a mature network, this plexus undergoes several remodeling processes, including the removal of supernumerary capillaries. The latter is mediated either by coordinated cell migration or

induction of apoptosis<sup>4-6</sup>, which have to be tightly regulated to avoid an inadequate blood flow supply to organs<sup>7</sup>.

In contrast to sprouting angiogenesis, mechanisms controlling vascular remodeling and apoptosis-mediated blood vessel pruning remain less clear, especially in regard to blood flow induced cell stress<sup>8-11</sup>. As one example, HIF-signaling protects ECs against ROS arising upon vessel perfusion<sup>9, 12-14</sup>. Moreover, also the p38 pathway mediates a tightly balanced cell stress response. While short term activation of p38 promotes cell survival and differentiation, prolonged p38 activation induces apoptosis in ECs<sup>15</sup>.

A better understanding of how these remodeling processes are regulated, could reveal novel therapeutic intervention points for clinical applications. Given the pivotal role of VEGF signaling in vessel sprouting<sup>16</sup> and maintenance<sup>17</sup>, most currently used angiogenesis inhibitors target VEGF or its receptors<sup>18</sup>. However, these drugs only prolong the survival of cancer patients without cure and often display severe side effects<sup>19,20</sup>. Novel agents, with alternative mechanisms of action, could overcome the limits of VEGF-targeted therapies.

Here, we analyzed the role of the PP2A phosphatase subunit B55 $\alpha$  during vascular development and cancer progression. B55 $\alpha$  is one of 26 regulatory subunits which orchestrate the activity and substrate specificity of the trimeric PP2A phosphatase complex<sup>21</sup>. Inhibition of B55 $\alpha$  in cancer cells alleviates tumor progression by reducing cell viability in hypoxia and glucose starvation<sup>22, 23</sup>. However, besides one publication in zebrafish<sup>24</sup>, the role of B55 $\alpha$  in stromal cells remains unknown. Here, we found that genetic deletion of B55 $\alpha$  unleashes apoptosis-mediated blood vessel pruning during development and cancer progression. In two different tumor models, we show that genetic loss of B55 $\alpha$  in ECs or pharmacologic inhibition of the PP2A phosphatase has a strong anti-angiogenic effect specifically targeting immature tumor vessels, thereby delaying tumor and metastatic progression.

## METHODS

A detailed Methods section can be found in the Online Data Supplement. Major Resources Table in the Supplemental Materials contain the list of all the main reagents used.

The authors declare that the majority of supporting data are presented within this article and its Online Data Supplement. Data that are not directly available are available from the corresponding author upon reasonable request.

All sequencing data are deposited in the ArrayExpress database under accession code E-MTAB-9002.

## RESULTS

### *B55 $\alpha$ controls EC survival during developmental vascular remodeling.*

Our initial analysis revealed a conservation rate of 100% between human and murine B55 $\alpha$  protein (Fig. 1A), pointing to a very unique role of this regulatory subunit.

We then analyzed the expression pattern of *Ppp2r2a* (the gene coding for B55 $\alpha$ ) using RNAscope. In mouse embryos, RNA expression of B55 $\alpha$  was detectable in several cell types including CD31<sup>+</sup> blood

vessels (Fig. 1B). In adult mice, expression of B55 $\alpha$  was strongly diminished if not absent, while markedly detectable in sites of active angiogenesis such as progressing tumors (Online Fig. I A-E). We therefore hypothesized that B55 $\alpha$  might be required in the immature vascular plexus while being dispensable in the quiescent vasculature of adults (see text further below and Online Fig. IV).

To analyze the functional role of B55 $\alpha$ , we generated embryos with homo- and heterozygous deletion for the *Ppp2r2a* gene (Online Fig. I G). Deletion efficiency was confirmed at embryonic stage E12.5 on RNA and protein levels (Fig. 1C-E). In line with the high conservation rate, *Ppp2r2a* gene deletion led to embryonic lethality around E14.5 not only in homozygous, but also in 60% of heterozygous knockout (KO) embryos (Fig. 1F). However, surviving heterozygous mice reached the adult stage, were fertile and did not show any obvious defects at steady conditions.

In contrast, homozygous KO embryos displayed severe bleedings in their skin (Fig. 1G), suggesting that these mutants suffered from vascular defects. Analysis of skin samples at E14.5 allowed us to differentiate between vascular defects in areas of initial vascularization and blood vessel remodeling (Online Fig. I K-N).

Although remodeling blood vessels were strongly affected (Fig. 1H-K), we did not observe any changes in the initially formed immature vascular plexus (Online Fig. II A,B). In addition, mutant embryos displayed aberrant lymphatic vessel structures (Fig. 1L,M), which appeared less organized and even completely disrupted in homozygous KO mice.

To analyze the selective role of B55 $\alpha$  in ECs, *Ppp2r2a(lox/lox)* conditional KO mice were intercrossed with the tamoxifen-inducible VE-cadherin.CreERT2 deleter strain (henceforward B55 $\square$ <sup>iEC-KO</sup>) (Online Fig. I G,I). In line with the results in Full-KO embryos, genetic deletion of B55 $\square$  in ECs led to bleedings in the skin of mutant B55 $\alpha$ <sup>iEC-KO</sup> embryos (Fig. 2A) accompanied by vascular and lymphatic defects in regions of vascular remodeling (Fig. 2B-F).

In contrast, there was no obvious phenotype during the initial steps of blood vessel formation neither in embryonic skin nor in postnatal retina samples (Online Fig. II C-J). B55 $\alpha$  deletion drastically increased the frequency of apoptotic ECs in mutant embryos (Fig. 2G,I) and retina samples (Online Fig. II K-O) where the majority of apoptotic events occurred in luminized (*Icam2*<sup>+</sup>) blood vessels<sup>25</sup> and thus before the collapse of these vessels (Online Fig. II P). This is in line with our results in the embryonic skin, showing that the induction of apoptosis preceded the morphological changes in more mature areas of the same vascular plexus (Online Fig. II E,F). Altogether, our data suggest that EC apoptosis in mutants precedes and might even cause the drastically enhanced blood vessel pruning in the remodeling plexi (Fig. 2H,J). As a final result of the progressing vascular defects, EC-specific deletion of B55 $\alpha$  led to embryonic lethality by E16.5 (Fig. 2K-O).

Similar to the defects in full KO embryos (Fig. 1L,M), pan-endothelial deletion of B55 $\alpha$  resulted in enlarged and activated lymph vessels in embryonic skin samples (Fig. 2C,E,N,O). However, B55 $\alpha$  deletion specifically in lymphatic ECs (using *Prox1.CreERT2* mice) did not recapitulate these defects (Online Fig. III A-F), indicating that the lymphatic phenotype in B55 $\alpha$ <sup>iEC-KO</sup> mice might be secondary to the strong vascular defects.

#### *Lack of endothelial B55 $\alpha$ delays tumor progression.*

In contrast to the developmental phenotype, inducible EC-specific (B55 $\alpha$ <sup>iEC-KO</sup>) or ubiquitous (B55 $\alpha$ <sup>iR26-KO</sup>) deletion of B55 $\alpha$  in 8-10 weeks old mice did not lead to lethality or any overt organ defects (Online Fig. IV A-U). Equally treated mice reached an age beyond 50 weeks without displaying any



pathological signs (Online Fig. IV A,C). The histological analysis of different organs from adult B55 $\alpha$ <sup>IEC-KO</sup> mice did not reveal any vascular or lymphatic defect (Online Fig. IV F-U). This is consistent with the downregulated *Ppp2r2a* expression in quiescent blood vessels of adult mice (Online Fig. I A-C) and indicates that B55 $\alpha$  is dispensable for the maintenance of vessel quiescence.

Since tumor blood vessels are mainly immature<sup>26</sup> and express high levels of *Ppp2r2a* (Online Fig. I D,E), we wondered whether B55 $\alpha$  inhibition would also induce pruning of these vessels. We thus injected either LLC cells subcutaneously or E0771 breast cancer cells orthotopically in tamoxifen-treated control and B55 $\alpha$ <sup>IEC-KO</sup> mice. We observed that EC-specific deletion of B55 $\alpha$  resulted in a strongly delayed tumor progression and metastasis formation in both models (Fig. 3A-F), which was also recapitulated by ubiquitous deletion of B55 $\alpha$  (Online Fig. IV X). The decrease in tumor size and weight (Fig. 3B,E) was accompanied by enhanced tumor hypoxia and necrosis (Fig. 3G-N), while cancer cell extravasation and lodging to the lung was not altered by lack of B55 $\alpha$  (Online Fig. IV Y).

Analysis of blood vessels in progressing tumors of B55 $\alpha$ <sup>IEC-KO</sup> mutant mice revealed that these had an altered shape, were less dense and less well connected when compared to controls (Fig. 3O-S,V). Reminiscent to the developmental phenotype, lack of B55 $\alpha$  led to an increased number of apoptotic ECs within the tumor vasculature (Fig. 3S-U,V-X). At the same time, the retinal vasculature of tumor-bearing mice appeared normal and showed no significant differences between the two genotypes (Online Fig. IV V,W).

*B55 $\alpha$  deletion after tumor resection inhibits metastatic growth and prolongs survival.*



Our results in cancer cells<sup>22,23</sup> and the current data in ECs suggest that pharmacologic inhibition of B55 $\alpha$ /PP2A might represent an interesting approach in cancer therapy. However, many patients relapse at a distant site long after the primary tumor has been surgically resected. Thus, approaches inhibiting metastasis progression, accounting for the majority of cancer-related deaths<sup>27</sup>, are currently needed.

To mimic this clinical setting, we injected wildtype LLC cancer cells in mice not yet treated with tamoxifen (Fig. 4A). In line with the non-recombined *Ppp2r2a* gene, tumors in these mice progressed identical (Fig. 4B). Tumors were then surgically removed and after 3 days of recovery tamoxifen treatment was initiated in order to induce EC-specific *Ppp2r2a* deletion. As compared to control mice, EC-specific *Ppp2r2a* deletion conferred longer survival and a drastic reduction in lung metastasis (Fig. 4C-H). Albeit having similar numbers of lung metastasis (Fig. 4D), lack of endothelial B55 $\alpha$  led to a delayed progression of these metastatic lesions (Fig. 4E-H). Similar to the effects observed in primary tumors, blood vessel density within the angiogenic active metastatic site was strongly impaired (Fig. 4I-J).

*Pharmacologic PP2A inhibition induces vessel pruning and delays tumor progression.*

Although specific inhibitors for the B55 $\alpha$ /PP2A are not currently available yet, the pan-PP2A inhibitor LB100, which blocks the catalytic PP2A subunit, was recently approved in a clinical phase I study for use in patients with 2 additional phase II clinical trials still ongoing<sup>28-30</sup>.

In order to test whether LB100-mediated pharmacologic inhibition of PP2A had an anti-angiogenic effect, without affecting cancer or stromal cells directly, we implanted sponges conditioned with tumor-conditioned medium to monitor angiogenesis (Fig. 5A)<sup>31</sup>. Even at low doses, LB100 treatment disrupted the immature vascular network formation in sponges of wildtype mice but had no additional effects in B55 $\alpha$ <sup>IEC-KO</sup> mice (Fig. 5B,C).

We then analyzed the effects of low dose LB100 treatment on tumor progression and found that LLC tumor growth was reduced to a similar extent as in B55 $\alpha$ <sup>IEC-KO</sup> (Fig. 5D,E) without showing any overt signs of toxicity (Fig. 5F). The reduced blood vessel density was comparable in B55 $\alpha$ <sup>IEC-KO</sup> and LB100-treated mice (Fig. 5G,H) and resulted in increased tumor hypoxia under both conditions (Fig. 5I,J). As observed in B55 $\alpha$ <sup>IEC-KO</sup> tumor-bearing mice, inhibition of PP2A by LB100 did not alter the ratio of pericyte-covered tumor blood vessels, suggesting that only vessel numbers, but not blood vessel maturation *per se* was affected (Online Fig. IV Z).

#### *B55 $\alpha$ deficiency induces apoptosis in maturing ECs but not in other cell types.*

We then used either sparse or densely seeded HUVECs to study the role of B55 $\alpha$  in EC apoptosis *in vitro*. As shown by others<sup>32, 33</sup> and by us (Online Fig. V A,B), this is a simplified system comparing proliferative *versus* contact-inhibited (non-proliferative) ECs.

Compared to sparse ECs, B55 $\alpha$  was strongly upregulated in densely-seeded ECs at both mRNA and protein levels (Fig. 6A,B). In contrast, B55b was barely detectable in HUVECs, while B55c showed the highest expression in sparse ECs and, similar to B55,  $\alpha$ B55d was upregulated in dense HUVECs (Online Fig. V C-E). However, only KD of B55 $\alpha$ , but not of any other B55-subunit, led to an apoptosis-mediated decrease of densely seeded HUVECs (Fig. 6C-F, Online Fig. V F-N). Furthermore, only treatment with the pan-Caspase inhibitors zVAD or qVD (Fig. 6G-I), but not the inhibition of necroptosis or extrinsic apoptosis (via block of Caspase8) counteracted cell death in B55 $\alpha$ -KD HUVECs (Online Fig. V O,P), suggesting that loss of B55 $\alpha$  activates the intrinsic apoptotic pathway in ECs.

This effect was phenocopied by treatment with the pan-PP2A-inhibitor LB100 but far less pronounced in sparse HUVECs (Fig. 6E, J-M). PP2A inhibition in different cancer cell lines, macrophages or LECs did not induce apoptosis (Online Fig. V Q,R; Online Fig. III G-K).

Altogether, our *in vivo* and *in vitro* data suggest that B55 $\alpha$ /PP2A promotes an anti-apoptotic program which is relevant for the survival of vascular ECs in neoformed vessels but not of sprouting ECs, LECs or other cell types.

#### *B55 $\alpha$ counteracts ROS in a PHD/HIF dependent manner.*

Expression of B55 $\alpha$  in densely cultured ECs was further increased by flow conditions (Online Fig. V S,T). Moreover, the presence of the ROS-scavenging molecule MnTBAP<sup>34, 35</sup> during growth of HUVECs from sparse to dense conditions, strongly prevented B55 $\alpha$  upregulation (Fig. 7A). We thus hypothesized that B55 $\alpha$  might counteract ROS in newly perfused vessels. Given the involvement of PHD2/HIF in vessel remodeling<sup>22, 36</sup> and ROS scavenging<sup>9, 12-14</sup>, as well as our previous work showing a B55 $\alpha$ -mediated regulation of PHD2 activity and vice versa<sup>22, 23</sup>, we initially focused on this signaling axis.

We found that densely-seeded HUVECs were indeed protected against H<sub>2</sub>O<sub>2</sub> induced ROS-stress in a B55 $\alpha$ -dependent manner (Fig. 7B,C; Online Fig. VI A). In line with our work in cancer cells, protein levels of HIF1 $\alpha$  and HIF2 $\alpha$  were strongly reduced upon B55 $\alpha$ -KD (Fig. 7D), arguing that B55 $\alpha$  accumulation in dense HUVECs stabilized HIF1 $\alpha$  and HIF2 $\alpha$  proteins under normoxia. In contrast, there was a slight increase at the mRNA level upon KD of B55 $\alpha$  (Online Fig. VI B-G), which might be an attempt of the cell to counteract the decreased HIF-protein levels. In line with our previous data in cancer cells, this suggests that the reduced stability of HIF proteins was mediated by increased PHD activity in B55 $\alpha$ -KD HUVECs<sup>22</sup>.

Functionally, overexpression (OE) of HIF1 $\alpha$  or HIF2 $\alpha$  counteracted the induction of apoptosis in B55 $\alpha$ -KD HUVECs (Online Fig. VI H,I). Furthermore, also the pan-PHD inhibitor DMOG reduced cell stress in B55 $\alpha$ -KD HUVECs, indicated by decrease in phospho-eIF2 $\alpha$  and p21 and decreased apoptosis induction, indicated by cleaved Caspase3 (Fig. 7E). At the same time, phosphorylation levels of p38 were not normalized upon DMOG treatment (Fig. 7F), suggesting that this pathway might be regulated independent of the PHD/HIF signaling axis.

Exposure of sparse HUVECs to low doses of H<sub>2</sub>O<sub>2</sub> led to an upregulation of B55 $\alpha$  while not affecting ROS-protected, dense ECs (Fig. 7G). In contrast, exposure to high doses of H<sub>2</sub>O<sub>2</sub>, (500 $\mu$ M), which was lethal for sparse HUVECs, increased B55 $\alpha$  expression in dense HUVECs. This supports the idea that ECs upregulate B55 $\alpha$  in order to counteract otherwise lethal levels of ROS. In line, DMOG treatment in B55 $\alpha$ -KD HUVECs (Fig. 7H) restored the expression of ROS-scavenging genes (Fig. 7I; Online Fig. VI J-M). As a consequence, DMOG-treated B55 $\alpha$ -KD HUVECs showed a restored capacity to counteract ROS and a partial but significant rescue in cell survival (Fig. 7J-L).

To show the direct effect of this signaling axis on ECs *in vivo* (without involvement of stroma or cancer cells), we performed sponge assays in control and B55 $\alpha$ <sup>iEC-KO</sup> mice. In line with our *in vitro* results, sponges implanted in B55 $\alpha$ <sup>iEC-KO</sup> mice had very few blood vessels and DMOG treatment led to a significant rescue (Fig. 7M,N).

Though DMOG is a pan-PHD inhibitor, based on our previous findings<sup>22,23</sup> we can speculate that B55 $\alpha$  is upregulated in ECs in order to elicit a HIF-mediated anti-oxidative program via PHD2 inhibition<sup>22</sup> thereby protecting the nascent endothelium against oxidative stress that may occur at the onset of blood flow<sup>9,12-14</sup>.

#### *B55 $\alpha$ /PP2A modulates a cell stress response by dephosphorylating p38.*

Given that block of PHD only partially rescued survival of ECs lacking B55 $\alpha$ , we analyzed the regulation of other signaling pathways involved in cell stress responses, such as NF $\kappa$ B, NRF2, Foxo1 and ATF4. However, we could not detect any change (Online Fig. VI N-Q).

We therefore performed RNAseq on dense control and B55 $\alpha$ -KD HUVECs treated with the pan-Caspase inhibitor qVD, avoiding artefacts resulting from apoptosis induction. In line with the above results, KD of B55 $\alpha$  led to a strongly diminished response to oxidative stress (Online Fig. VII A). In addition, we found a strong increase in p38 signaling as well as other cell stress pathways such as NRF2, ATF signaling upon KD of B55 $\alpha$ . We decided to focus on the p38 pathway since (i) many of these pathways are regulated in a p38-dependent manner<sup>22,37-39</sup>, (ii) nuclear localization of the main executors of the other pathways did not change (Online Fig. VI N-Q), (iii) PP2A has been previously reported to dephosphorylate p38<sup>15,40</sup>. We found that KD of B55 $\alpha$  strongly increased p38 phosphorylation (Fig. 8A), an effect persistent after inhibition of PHD2 (Fig. 7F). In contrast, there were no changes in activation or total protein levels of other stress sensors such as ERK or JNK (Online Fig. VI R,S).

We thus hypothesized that B55 $\alpha$ /PP2A might be able to regulate p38 $\alpha$  signaling either directly or indirectly by modulating upstream activators such as AMPK and PKR<sup>15,41,42</sup>. By performing co-immunoprecipitation experiments we found a direct binding of B55 $\alpha$  to p38 (Fig. 8B, Online Fig. VI T,U), but not with AMPK, PKR or other cell stress kinases such as JNK and ERK (Online Fig. VI V,W,X).

Prolonged phosphorylation and hyper-activation of p38 induces apoptosis in ECs<sup>15</sup>. Consistently, chemical inhibition of p38 improved survival in B55 $\alpha$ -KD ECs by normalizing p38 activity levels in these

cells (Fig. 8C-E). In control ECs p38 inhibition promoted cell death as well, by generating too low levels of p38 activation (Fig. 8C-E)<sup>15</sup>.

Using the sponge implantation model, we found that chemical inhibition of p38 led to a dose-dependent improvement of blood vessel density in B55 $\alpha$ <sup>iEC-KO</sup> mice while having the opposite effect in control mice (Fig. 8F-H). This supported the idea that p38 activity, tightly controlled by the B55 $\alpha$ /PP2A phosphatase, is relevant for the optimal formation of blood vessels.

## DISCUSSION

To generate a highly effective and functional vascular network, a plethora of well-coordinated and tightly regulated remodeling processes occur at the same time. In contrast to early steps of angiogenesis, these later maturation processes are only poorly understood.

Here, we found that B55 $\alpha$  plays an important role specifically in the remodeling vasculature. Consistently, genetic deletion of B55 $\alpha$  led to enhanced EC apoptosis within the remodeling plexus and drastically increased pruning of immature blood vessels. However, chemical pan-PP2A inhibition had no additional effect on blood vessel pruning in B55 $\alpha$ <sup>iEC-KO</sup> mice, suggesting that vascular remodeling is mainly regulated by the B55 $\alpha$ /PP2A holocomplex but none of the other 26 regulatory subunits.



Although we also observed strong lymphatic defects following pan-endothelial B55 $\alpha$  deletion, selective deletion in lymphatic ECs only, did not recapitulate these defects. This suggests that the lymphatic phenotype in B55 $\alpha$ <sup>iEC-KO</sup> mice is secondary to the strong vascular defects. The subsequent increase of hypoxic areas could either affect LECs either directly or via lymphatic growth factors secreted by activated perivascular stromal cells<sup>43-45</sup>. Alternatively, a reduced vascular density may increase capillary pressure and cause chronic edema, augmenting intralymphatic pressure and lymphatic vessel dilatation<sup>46</sup>.

Vascular B55 $\alpha$  expression is important when some of the newly formed vessels are stabilized while others are pruned away, overall increasing the efficiency of tissue perfusion. The expression of B55 $\alpha$  allows immature blood vessels to undergo maturation while ECs lacking B55 $\alpha$  would be unprotected against cell stress induced by blood flow or the harsh tumor microenvironment. As a result, deletion of B55 $\alpha$  decreased the density of the vascular network by inducing EC apoptosis and hyperpruning of nascent blood vessels. However, lack of B55 $\alpha$  did not affect angiogenic sprouting or ECs in fully mature blood vessels.

Upregulation of B55 $\alpha$  in ECs was recently linked to activated integrin  $\alpha 5\beta 1$ <sup>47</sup>. In addition, our data show that not only cell density, accompanied by stabilized junctions, but also flow increased B55 $\alpha$  expression. It is therefore tempting to speculate whether the mechanosensory complex might play a role in the upregulation of B55 $\alpha$  in these contexts.

The role of B55 $\alpha$  in vessel stabilization is supported by another publication performed in zebrafish embryos<sup>24</sup>. Mechanistically, this previous study suggested that EC apoptosis might be secondary to reduced blood flow after blood vessel collapse<sup>48</sup>. In contrast, the combination of our *in vitro* and *in vivo* data show that EC apoptosis in B55 $\alpha$ -deficient remodeling blood vessels precedes the collapse and retraction of immature blood vessels. The lack of sprouting defects and identical vessel densities in the immature vascular plexus also suggest that ECs did not just become less responsive to angiogenic stimuli *per se*.

Since the onset of blood flow leads to a burst of ROS, ECs in newly perfused vessels have to counteract this sudden cell stress. Our previous work showed an interaction between B55 $\alpha$  and PHD2/HIF,

with the latter being involved in ROS-mediated cell stress scavenging<sup>9,10,12-14</sup>. In the current work, we found that B55 $\alpha$  engaged vasoprotective HIF<sup>12, 49</sup> which induced expression of genes counteracting ROS. We show that the downmodulation of these HIF-mediated pathways following B55 $\alpha$ /PP2A knockdown occurs in a condition of dense, but normoxic EC cultures. However, in our previous work inhibition of B55 $\alpha$ /PP2A phosphatase led to cancer cell apoptosis in response to oxygen or glucose restriction<sup>22,23</sup>. Our current model in ECs suggests that B55 $\alpha$  is upregulated and plays a role in the remodeling of the primitive vascular network during the initial onset of blood flow. Therefore, it is possible that under *in vivo* conditions, limited oxygen and nutrient availability, characterizing the tumor microenvironment, could exacerbate the proapoptotic signaling observed in B55 $\alpha$ -KD ECs even more.

PHD inhibition only partially rescued improved survival of B55 $\alpha$ -KD ECs *in vitro* and *in vivo*, thus implying the involvement of PHD-independent mechanisms. In this context, a proper balance of p38 activation (a known interactor of PP2A) is crucial for EC survival under cell stress conditions<sup>15</sup>. While the initial activation of p38 can be triggered by multiple stimuli such as cell stress or growth factors<sup>50</sup>, complete loss of p38 as well as overactivation leads to vascular malformations and induction of EC apoptosis<sup>15,51,52</sup>. Here we show that B55 $\alpha$ /PP2A maintains this fine balance of p38 activation by dephosphorylating and thus normalizing p38 signaling.

Our *in vitro* RNAseq data showed that a plethora of genes were changed upon KD of B55 $\alpha$  in HUVECs. Although many of these changes can be explained by modulation of p38 and PHD2, we are not excluding that B55 $\alpha$ /PP2A could dephosphorylate additional substrates<sup>47</sup>. In the context of blood vessel remodeling however, our rescue experiments performed *in vitro* and *in vivo* strongly support the idea that p38 and PHD2 are at least highly important, if not the main targets of B55 $\alpha$ .

From a therapeutic point-of-view, we show that both genetic deletion of B55 $\alpha$  and PP2A inhibition hinder tumor growth by inducing EC apoptosis in immature blood vessels. Furthermore, genetic deletion of B55 $\alpha$  reduced the metastatic burden and extended survival in a model of metastatic relapse in the absence of primary tumor. Finally, of the tested E0771 and LLC cancer models, the latter is known to be unresponsive to traditional anti-angiogenic therapies such as VEGF(R) inhibitors<sup>53,54</sup>.

The observation that B55 $\alpha$  was not detectable in fully mature and stabilized blood vessels of adult organs, and that mural cell-covered tumor blood vessels were still detectable in B55 $\alpha$ <sup>iEC-KO</sup> (or LB100-treated) mice, lead to additional clinical speculations. Firstly, B55 $\alpha$ /PP2A blockers or pan-PP2A inhibitors affect only remodeling blood vessels whereas VEGF(R) inhibitors mainly target hyperactive ECs. Yet, B55 $\alpha$ /PP2A blockers or pan-PP2A inhibitors would spare mature vessels, which are the routes for anti-cancer drugs into the tumor<sup>12</sup>. In healthy organs, disturbance of blood vessel homeostasis causes severe side effects<sup>17</sup> as observed in the clinical use of anti-VEGF(R) drugs<sup>20, 26, 55</sup>. Thus, B55 $\alpha$ /PP2A inhibition could tackle pathological blood vessels more selectively than current anti-angiogenic drugs. In an opposite scenario, our data predicts that overactivation of the B55 $\alpha$ /PP2A complex might protect blood vessels against oxidative stress, an important issue which could be explored in disease settings such as ischemic reperfusion injury.

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## DISCLOSURES

None.

## SUPPLEMENTAL MATERIALS

Expanded Materials & Methods

Online Figures I - VIII

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## FIGURE LEGENDS

### Figure 1: B55 $\alpha$ expression is essential for embryonic development.

(A) Analysis of B55 $\alpha$  protein and mRNA sequences between species. (B) Expression of B55 $\alpha$  in embryonic tissue at day 14.5 post fertilization (=E14.5) assessed by RNAscope. Scale bars: 1000 $\mu$ m in the overviews and 50 $\mu$ m in the higher resolution images. (C-E) Analysis of B55 $\alpha$  protein (n=5 per group) and mRNA expression (Ctrl n=4; Het n=6; Hom n=4) levels in control and KO embryos at E12.5. (F) Ratio of surviving B55 $\alpha$ -KO embryos at different stages of development. (G) Representative images of controls and *Ppp2r2a* Full-KO embryos at embryonic stage E14.5. White arrows indicate visible bleedings in the skin of mutant embryos. (H,J,L) Representative images of embryonic skin samples from *Ppp2r2a* Full-KO and littermate controls stained for CD31<sup>+</sup> blood vessels (H) Collagen IV<sup>+</sup> / CD31<sup>-</sup> empty sleeves as a marker for pruned blood vessels (J) and Lyve1<sup>+</sup> lymph vessels (L). Scale bars: 100  $\mu$ m. (I,K,M) Quantitative analysis of CD31<sup>+</sup> blood vessel area (Ctrl n=5; Het n=5; Hom n=3) (I) Collagen IV<sup>+</sup> / CD31<sup>-</sup> pruning blood vessels (Ctrl n=7; Het n=7; Hom n=6) (K), and Lyve1<sup>+</sup> lymph vessel area (Ctrl n=7; Het n=7; Hom n=6) (M) in surviving embryos at stage E14.5. One-way ANOVA followed by Tukey test was performed in (D), (E), (I), (K) and (M). Graphs show standard SEM.

### Figure 2: Endothelial loss of B55 $\alpha$ leads to vascular and lymphatic defects resulting in embryonic lethality.

(A) Representative images of E14.5 embryos lacking endothelial B55 $\alpha$  and littermate controls. Black arrows indicate bleedings in the skin of mutant embryos. (B-D) Whole mount skin samples stained for lymph vessel marker Lyve1 (C) and the blood vessel marker CD31 (D). Scale bars: 200 $\mu$ m. (E,F) Quantitative analysis of lymph vessel diameter (n=4 embryos per group) (E) and blood vessel density in the remodeling vascular plexus (Ctrl n=26; B55 $\alpha$ <sup>iEC-KO</sup> n=12) (F). (G,I) Apoptotic ECs in the remodeling vascular plexus indicated by immunostainings against CD31 and cleaved Caspase3 (G) and quantitative analysis thereof (Ctrl n=18; B55 $\alpha$ <sup>iEC-KO</sup> n=9) (I) Scale bar: 100 $\mu$ m. (H,J) Pruning blood vessels, visualized by Co-staining of CD31<sup>+</sup> and CollagenIV<sup>+</sup> / CD31<sup>-</sup> empty sleeves (H) and quantitative analysis thereof (n=4 per group) (J) Scale bar: 100 $\mu$ m. (K) Representative images of whole embryos at stage E16.5 and statistical quantification thereof. (L-O) Representative images of embryonic skin samples at stage E16.5 stained for CD31<sup>+</sup> blood vessels (L) and Lyve1<sup>+</sup> lymph vessels (N) and statistical analysis thereof (n=9 per group in (M) and n=4 per group in (O)). Scale bars: 200 $\mu$ m. Unpaired t-tests were performed in (E), (F), (I), (J), (M) and (O). Graphs show SEM.

### Figure 3: Deletion of B55 $\alpha$ in the tumor vasculature delays tumor progression, by reducing the blood vessel density.

(A-F) Tumor progression after EC specific deletion of *Ppp2r2a* following the subcutaneous injection of LLC cancer cells (Ctrl n=19; B55 $\alpha$ <sup>iEC-KO</sup> n=8) (A), tumor weight (Ctrl n=18; B55 $\alpha$ <sup>iEC-KO</sup> n=11) (B) and lung metastasis (Ctrl n=40; B55 $\alpha$ <sup>iEC-KO</sup> n=30) (C). Tumor progression after EC specific deletion of *Ppp2r2a* following orthotopically injected E0771 breast cancer cells (Ctrl n=9; B55 $\alpha$ <sup>iEC-KO</sup> n=14) (D), tumor weight (Ctrl n=12; B55 $\alpha$ <sup>iEC-KO</sup> n=7) (E) and lung metastasis (Ctrl n=33; B55 $\alpha$ <sup>iEC-KO</sup> n=36) (F). (G-J) Representative images and quantifications of hypoxic areas in LLC (Ctrl n=13; B55 $\alpha$ <sup>iEC-KO</sup> n=8) (G,H) and E0771 tumors (Ctrl n=8; B55 $\alpha$ <sup>iEC-KO</sup> n=9). (I,J) Scale bars: 100 $\mu$ m. (K-N) Representative images and quantifications and of necrotic areas in LLC (n=19 per group) (K,L) and E0771 tumors (Ctrl n=8; B55 $\alpha$ <sup>iEC-KO</sup> n=7) (M,N). Scale bars: 400 $\mu$ m. (O-X) 70 $\mu$ m thick cryosections of LLC (O,S) and E0771 (Q,V) tumors stained for CD31<sup>+</sup> blood vessels and the apoptosis marker cleaved Caspase3 and statistical analysis of blood vessel density (P,R), total area of apoptotic (tumor) areas (T,W) and cleaved Caspase3<sup>+</sup> ECs (U,X); (n=7 per group) (P,T,U) and (Ctrl n=6; B55 $\alpha$ <sup>iEC-KO</sup> n=7) (R,W,X). Scale bars: 100 $\mu$ m (O,Q), 10 $\mu$ m (S,V). Unpaired t-tests were performed in (B), (E), (F), (H), (J), (N), (P), (R), (T), (U), (W) a non-parametric Mann-Whitney tests in (C), (F), (L) (X) and a two-way repeated measures ANOVA in (A) and (D). Graphs show SEM.

**Figure 4: Deletion of endothelial B55 $\alpha$  after tumor resection delays metastatic growth and prolongs survival of mutant mice.**

(A) Schematic layout of the depicted experiment. (B) Tumor size at the day of tumor resection. (C) Quantitative analysis showing survival of littermate controls and mutant mice after tumor resection and subsequent tamoxifen induced depletion of endothelial *Ppp2r2a*. (D) Quantitative analysis of the number of lung metastasis. (E,F) Representative images of metastatic lungs from controls and mutant mice (E) and quantitative analysis thereof (F). Scale bar: 5mm. (G,H) Representative images of H&E stained lungs showing dense metastatic nodules within normal lung tissue (G) and the quantitative analysis thereof (H). Scale bar: 5mm. (I,J) Representative images showing the vasculature within lung metastasis in controls and mutant mice (I) and quantitative analysis thereof (J). Scale bar: 200 $\mu$ m. (Ctrl n=11; B55 $\alpha$ <sup>iEC-KO</sup> n=8 for all panels). Unpaired t-tests were performed in (B), (D), (F), (H) and (J). Graphs show SEM.

**Figure 5: Chemical pan-PP2A inhibition induces blood vessel pruning and delays tumor progression.**

(A) Schematic representation of the sponge implantation assay shown in (B,C) (B,C) Representative images of sponges stained for CD31<sup>+</sup> blood vessels. Scale bar: 100 $\mu$ m. (B) and quantitative analysis thereof (Ctrl n=7; B55 $\alpha$ <sup>iEC-KO</sup> n=9 for each dose of LB100) (C). (D-F) Subcutaneous LLC tumor growth (D), final tumor burden (E) and body weight (F) in B55 $\alpha$ <sup>iEC-KO</sup> mutant or after systemic LB100 treatment (alone and in combination). (G, H) 6 $\mu$ m thin paraffin sections of tumor samples stained for CD31<sup>+</sup> blood vessels (G) and statistical analysis of blood vessel density (H). Scale bars: 100 $\mu$ m. (I, J) Representative images (I) and quantifications (J) of pimonidazole-positive tumor areas, indicating hypoxic regions. Scale bar: 200 $\mu$ m. Ctrl(PBS) n=5; Ctrl(LB100) n=5; B55 $\alpha$ <sup>iEC-KO</sup> (PBS) n=4; B55 $\alpha$ <sup>iEC-KO</sup> (LB100) n=5 in (D-H). One-way ANOVA followed by Tukey test was performed in (C), (E), (H), (J) and a two-way repeated measures ANOVA in (D) and (F). Graphs show SEM.

**Figure 6: B55 $\alpha$  is upregulated in densely seeded HUVECs and prevents apoptosis induction.**

(A,B) Analysis of B55 $\alpha$  RNA (A) and protein level (B) in HUVECs cultured at different cell densities. (C) Survival analysis of HUVECs after KD of B55 $\alpha$ . (D,E) Analysis of apoptosis in B55 $\alpha$ -KD and LB100 treated HUVECs by subG1 FACS assay performed on fixed samples (D) and AnnexinV/PI FACS assay performed on living ECs (E). (F) WB analysis of cleaved PARP in B55 $\alpha$ -KD HUVECs. (G-I) Immunostainings of B55 $\alpha$ -KD HUVECs treated with vehicle control (NT), pan-Caspase inhibitors (qVD or zVAD) for Phalloidin, cleaved Caspase3 and Hoechst (G) and quantitative analysis thereof (H-I). Scale bar: 100 $\mu$ m. (J) Schematic representation of the experiments depicted in (K) and (L). (K,L) Analysis of cell survival after KD of B55 $\alpha$  in spares and dense HUVECs. (M) Analysis of cell survival in spares and dense HUVECs treated with the pan-PP2A inhibitor LB100.

n=3 per group in (A), (E), (K), (L), (M), n=5 per group in (H), (I) and n=6 per group in (C) and (D). Unpaired t-tests were performed in (D), one-way ANOVA followed by Tukey test in (A), (E), (F), (H), (I), (K), (L), (M) and a two-way repeated measures ANOVA in (C). Graphs show SEM.

**Figure 7: B55 $\alpha$  expression counteracts ROS and cell stress in a PHD dependent manner.**

(A) WBs showing B55 $\alpha$  expression in presence of the ROS scavenger MnTBAP. (B, C) CM-H2DCFDA-based ROS assay analyzing H<sub>2</sub>O<sub>2</sub>-induced ROS on sparse, confluent and dense HUVECs (n=3 per group) (B) qVD-treated B55 $\alpha$ -KD HUVECs under dense conditions (n=6 per group) (C). (D-F) WB analysis of HIF1 $\alpha$ , HIF2 $\alpha$ , p-AKT (D), the cell stress indicators p-eIF2 $\alpha$ , p21, the apoptosis marker cleaved Caspase3 (E), B55 $\alpha$  and p-p38 (F) in B55 $\alpha$ -KD and controls upon DMOG treatment. (G) qPCR-based expression analysis of B55 $\alpha$  in sparse and dense HUVECs upon H<sub>2</sub>O<sub>2</sub> treatment (Sparse n=5; Dense n=6 per treatment group). (H,I) qPCR-based expression analysis of B55 $\alpha$  (H) and genes counteracting ROS and EC maturation markers such as total FLT1 and CDH5 (I) in control and B55 $\alpha$ -KD cells and in combination with DMOG treatment (n=3 per group). Significance was calculated on log<sub>2</sub> transformed data followed by

two-way ANOVA. **(J-L)** Staining of Phalloidin and Hoechst in DMOG treated B55 $\alpha$ -KD and control HUVECs **(J)**, quantification of resistance to H<sub>2</sub>O<sub>2</sub> (n=3 per group) **(K)** and cell survival (n=15 per group) **(L)**. Scale bar: 100 $\mu$ m. **(M,N)** Representative images of sponges stained for CD31<sup>+</sup> blood vessels **(M)** and quantification thereof (n=6 per group) **(N)**. Scale bar: 300 $\mu$ m. One-way ANOVA followed by Tukey test was performed in **(G)**, **(H)**, **(K)**, **(L)**, **(N)** and a two-way repeated measures ANOVA in **(B)** and **(C)**. Graphs show SEM.

**Figure 8: B55 $\alpha$  blocks the prolonged and apoptosis inducing activity of p38.**

**(A)** WB analysis of p-p38 after KD of B55 $\alpha$ . **(B)** Co-IP experiments of B55 $\alpha$  and p38 performed in dense HUVECs. **(C,D)** Ctrl and B55 $\alpha$ -KD HUVECs treated with p38 inhibitor and stained for Phalloidin and Hoechst **(C)** and quantification thereof (n $\geq$ 11 per group) **(D)**. Scale bar: 100 $\mu$ m. **(E)** Schematic representation of p38 control on EC apoptosis, explaining the results of the experiments depicted in **(D)** and **(G)**. **(F-H)** Immunostainings for CD31<sup>+</sup> blood vessels of implanted sponges derived from control and B55 $\alpha$ <sup>iEC-KO</sup> mice treated with p38 inhibitor **(F)**, and quantification thereof (Ctrl n=9; B55 $\alpha$ <sup>iEC-KO</sup> n=7 for each dose of p38 inhibitor) **(G,H)**. Scale bar: 300 $\mu$ m. **(I)** Graphical abstract summarizing our findings and the proposed mechanism. An unpaired t-test was performed in **(H)** and One-way-Anova followed by Tukey test in **(D)** and **(G)**. Graphs show SEM.



# Circulation Research

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## NOVELTY AND SIGNIFICANCE

### *What Is Known?*

- VEGF and other signaling molecules have been implicated in vessel growth. Though the signaling that regulates remodeling of nascent vascular networks is poorly characterized.
- Many progressing tumors do not respond or develop resistance to VEGF-targeted antiangiogenic therapies, resulting in limited success during clinical applications.
- Inhibition of the B55 $\alpha$ /PP2A complex in cancer cells alleviates tumor progression by reducing cell viability in response to hypoxia and glucose starvation.

### *What New Information Does This Article Contribute?*

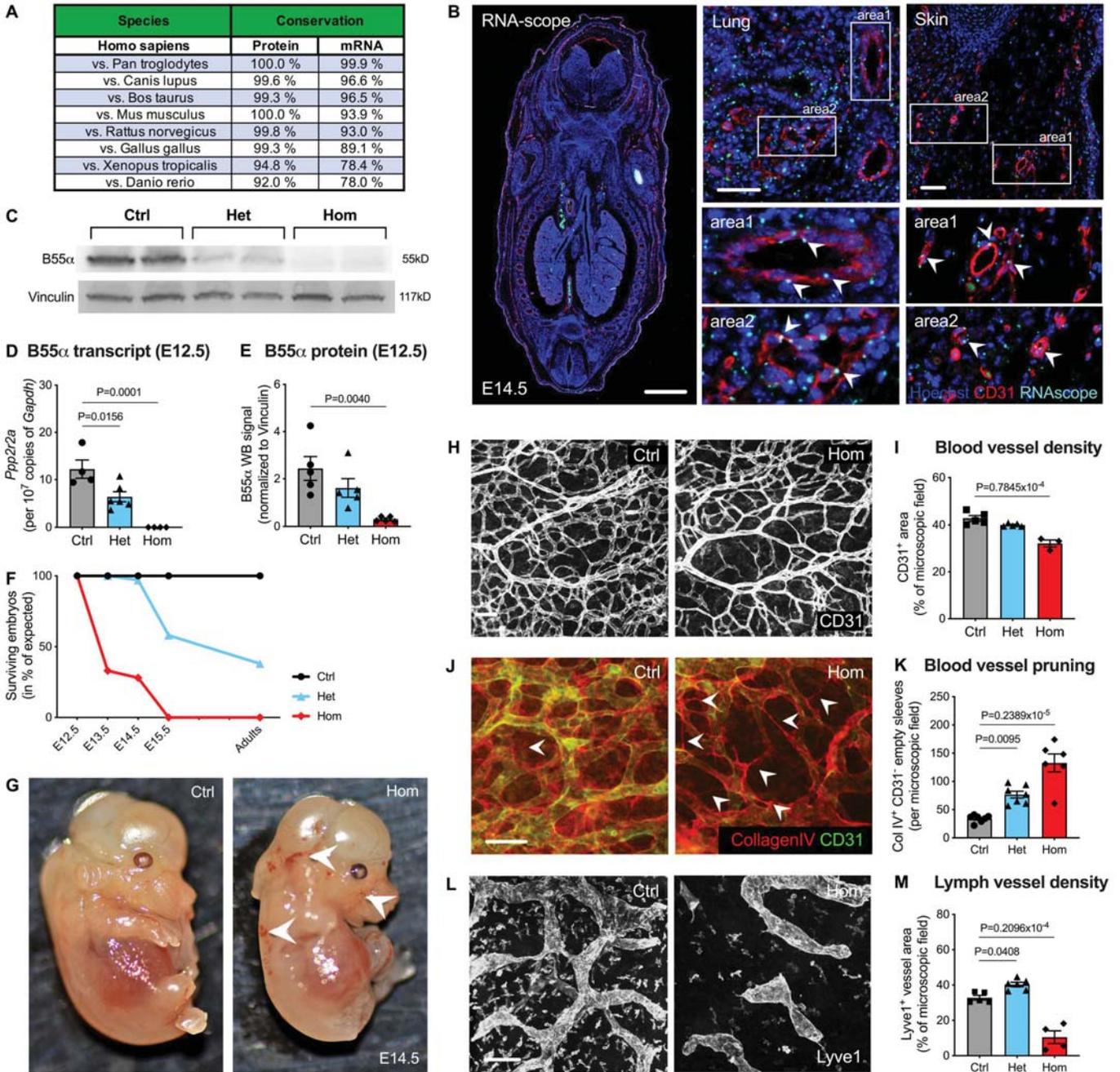
- During both development and adult life (*i.e.* in cancer), B55 $\alpha$ /PP2A elicits a HIF- and p38-dependent survival program during vascular remodeling that protects ECs in nascent blood vessels against oxidative and other flow-related cell stress.
- Deletion of B55 $\alpha$ /PP2A has no effect in healthy adult mice or fully mature blood vessels.
- B55 $\alpha$ /PP2A inhibitors can be exploited as potential anti-angiogenic cancer therapies that work in a complementary manner to anti-VEGF drugs, offering an alternative in case of resistance to these therapies.

We have previously shown that inhibition of the B55 $\alpha$ /PP2A phosphatase complex delays tumor progression by inducing cancer cell apoptosis in response to oxygen or glucose restriction. These findings underline the potential use of B55 $\alpha$ /PP2A phosphatase blockers for cancer therapy. Yet, little is known on the role of B55 $\alpha$ /PP2A in the tumor stroma and particularly in the blood vessels. Moreover, possible side effects due to systemic inhibition of B55 $\alpha$ /PP2A remain understudied.

Here we generated novel transgenic mice to induce genetic deletion of B55 $\alpha$  in different cell types and at specific timepoints of cancer progression. We found that neither ubiquitous nor endothelial cell specific deletion of B55 $\alpha$  had effects in healthy adult mice or more specifically, on established, quiescent blood vessels. In contrast, genetic deletion of B55 $\alpha$  or chemical inhibition of PP2A unleashed strong pruning effects of the immature tumor vasculature and thereby delayed cancer and metastatic progression. The identified mechanism is complementary to currently used anti-angiogenic drugs, suggesting that B55 $\alpha$ /PP2A phosphatase inhibition could serve as an alternative strategy in case tumors become resistant or do not respond to state-of-the-art anti-angiogenic agents.

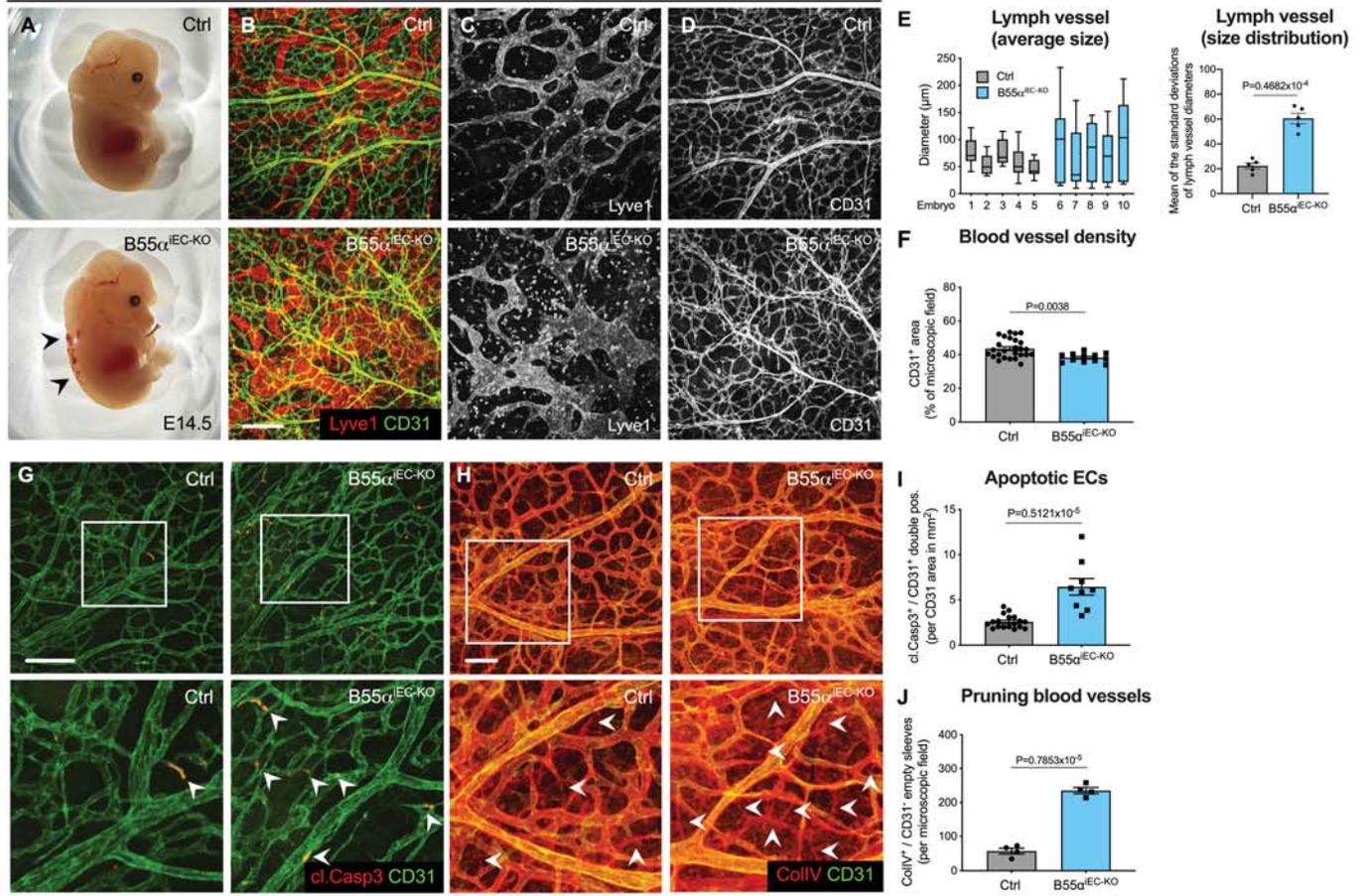
Taken together, our study argues that targeting the B55 $\alpha$ /PP2A complex might be a safe and powerful approach for clinical applications.

**Figure 1**

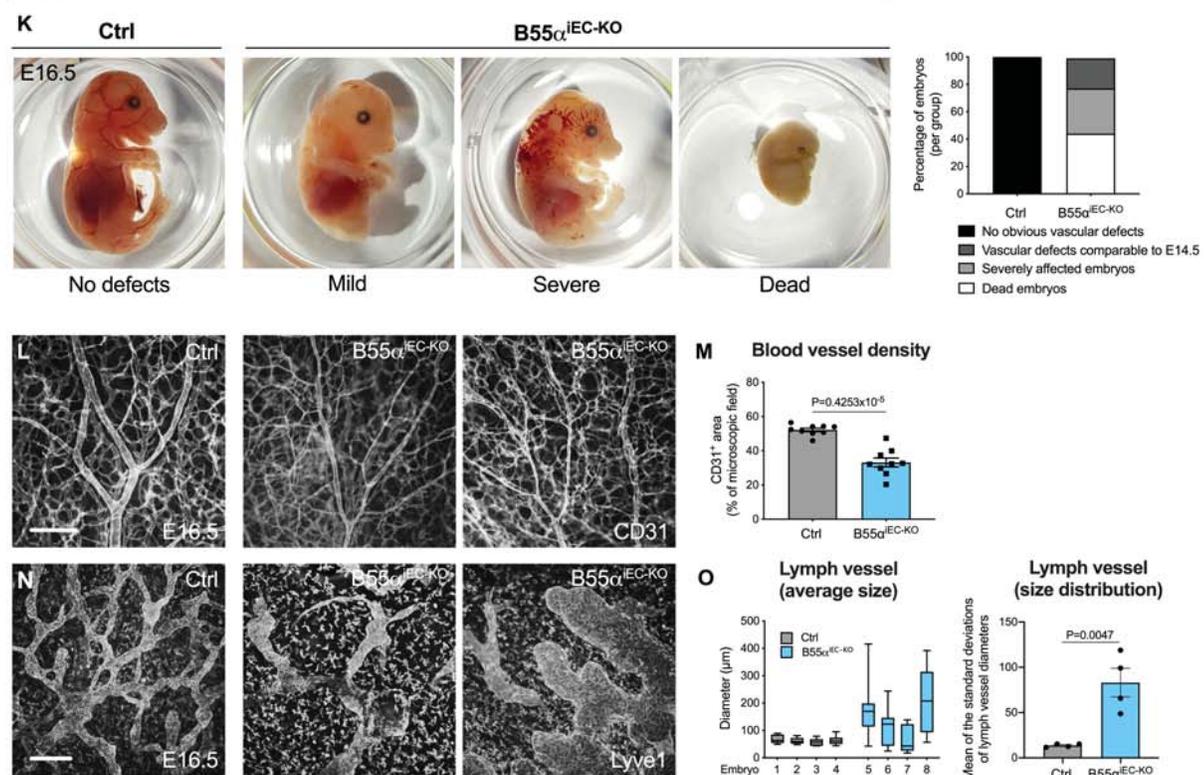


**Figure 2**

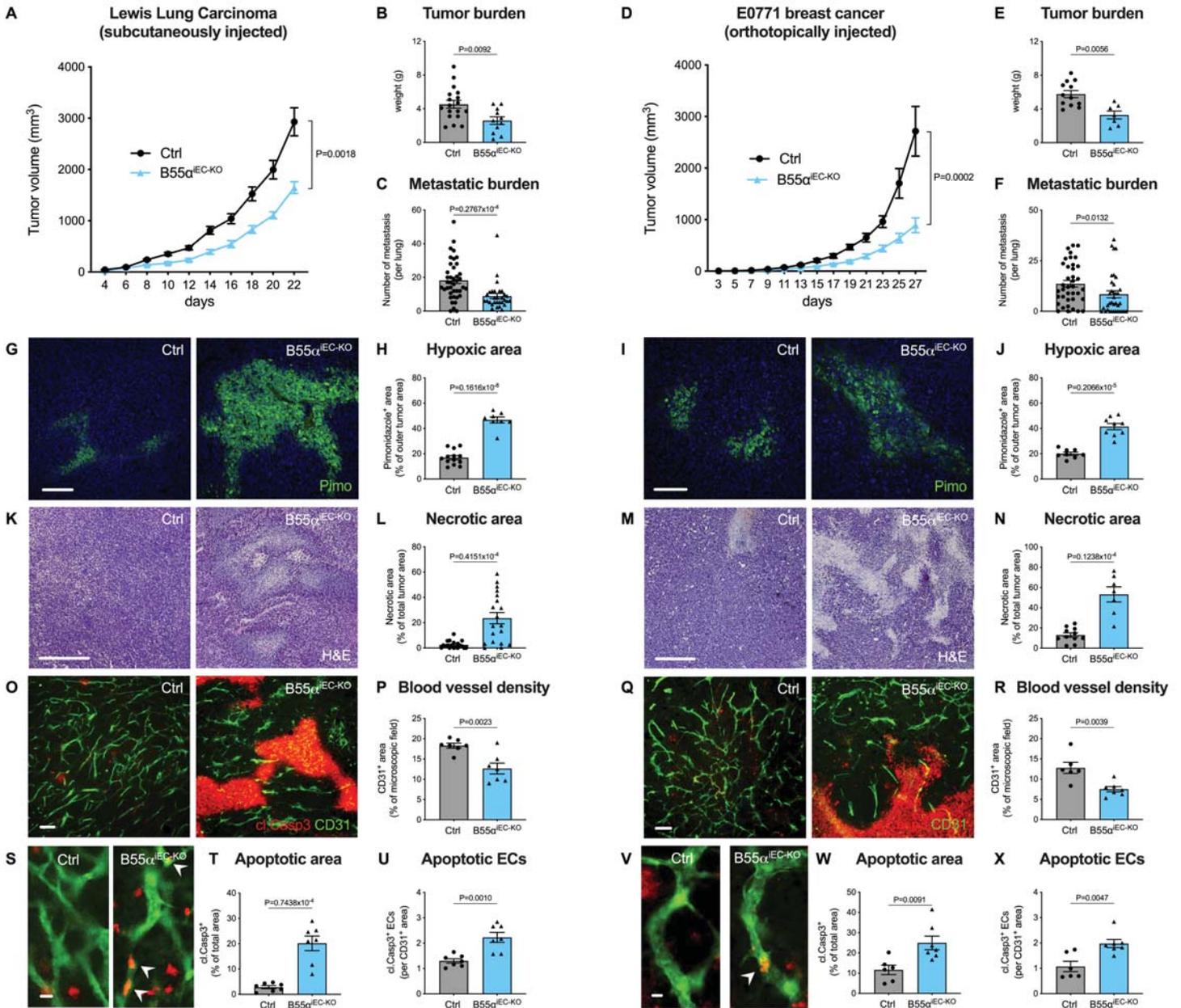
**E14.5**



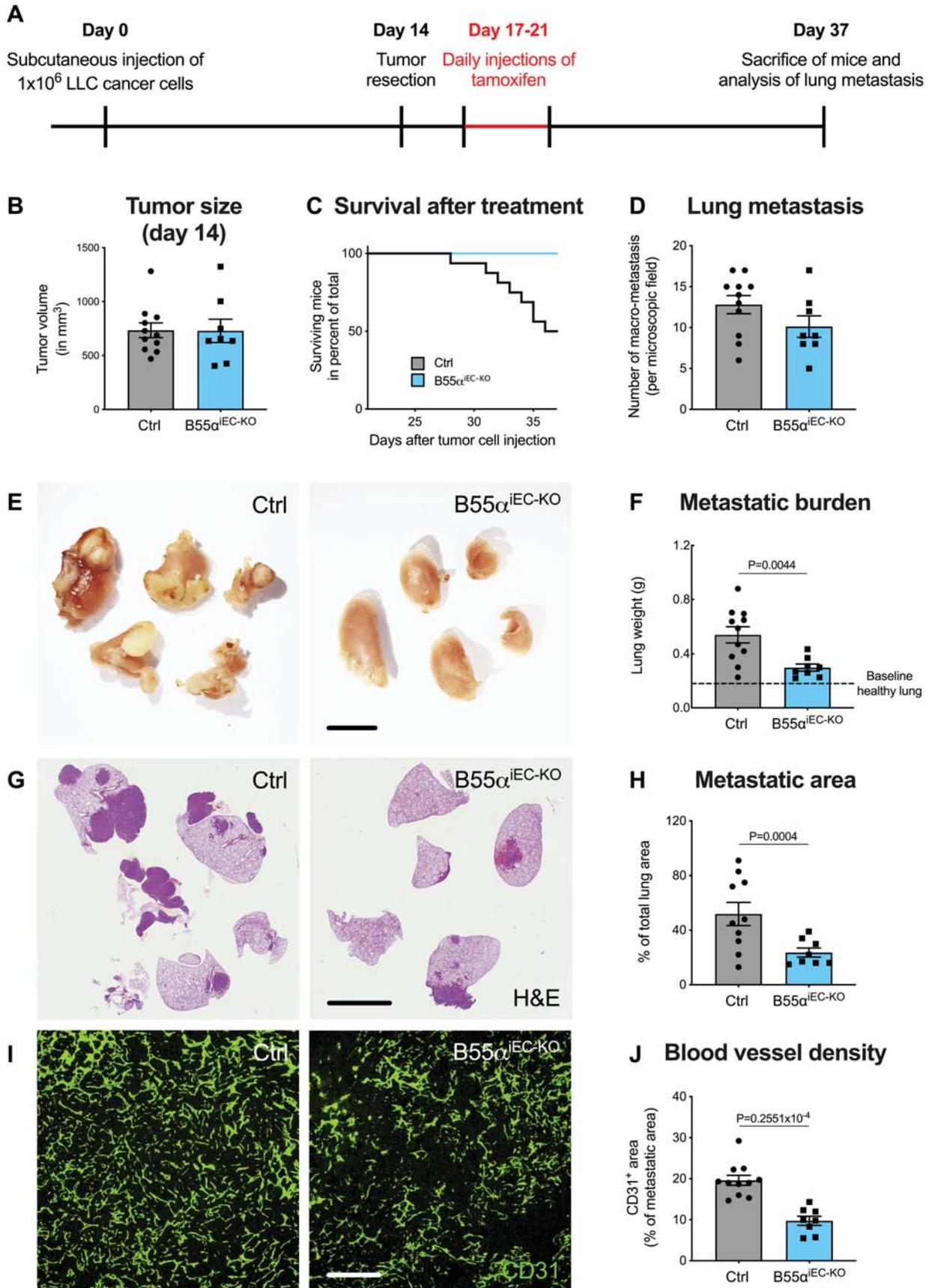
**E16.5**



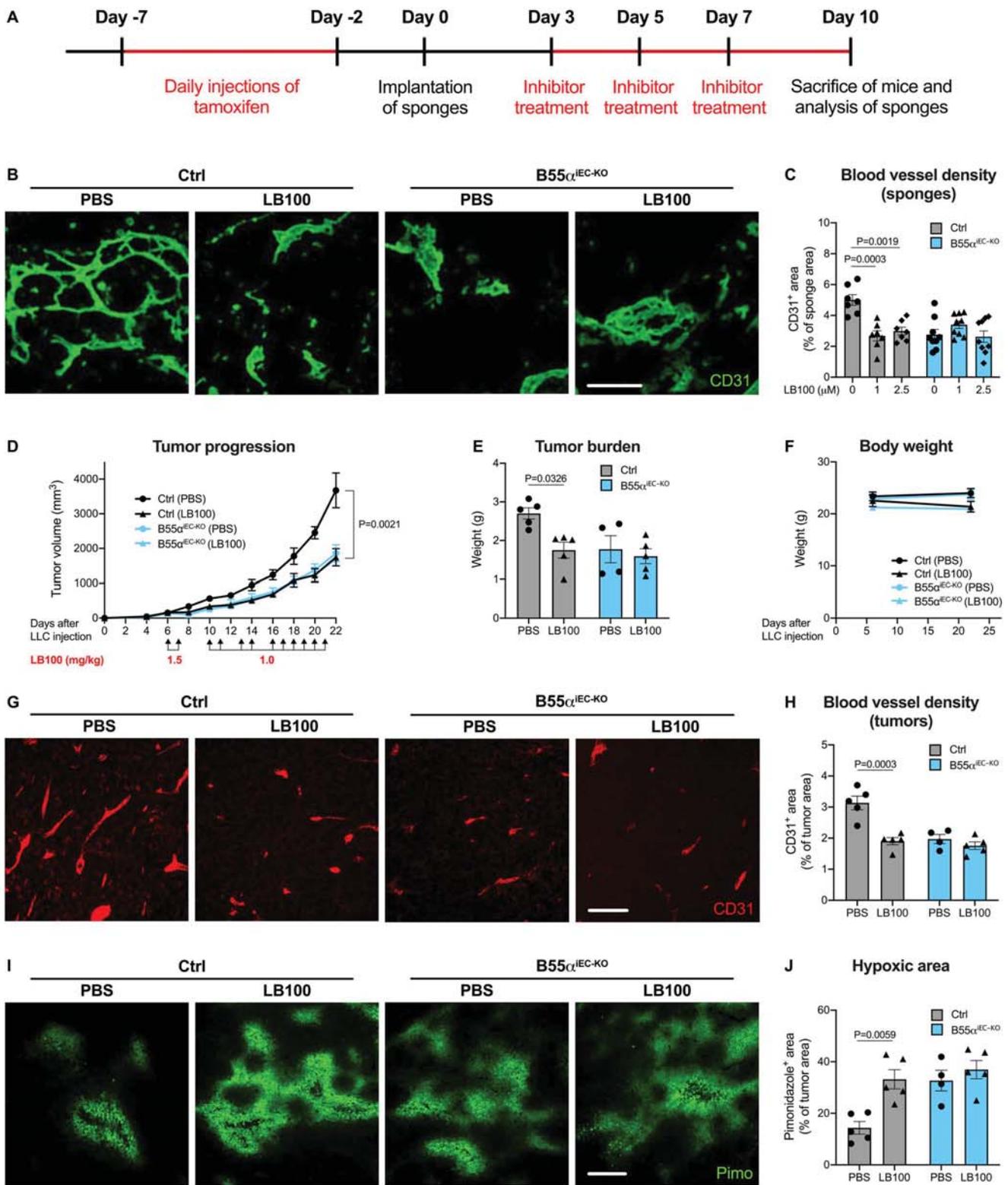
**Figure 3**



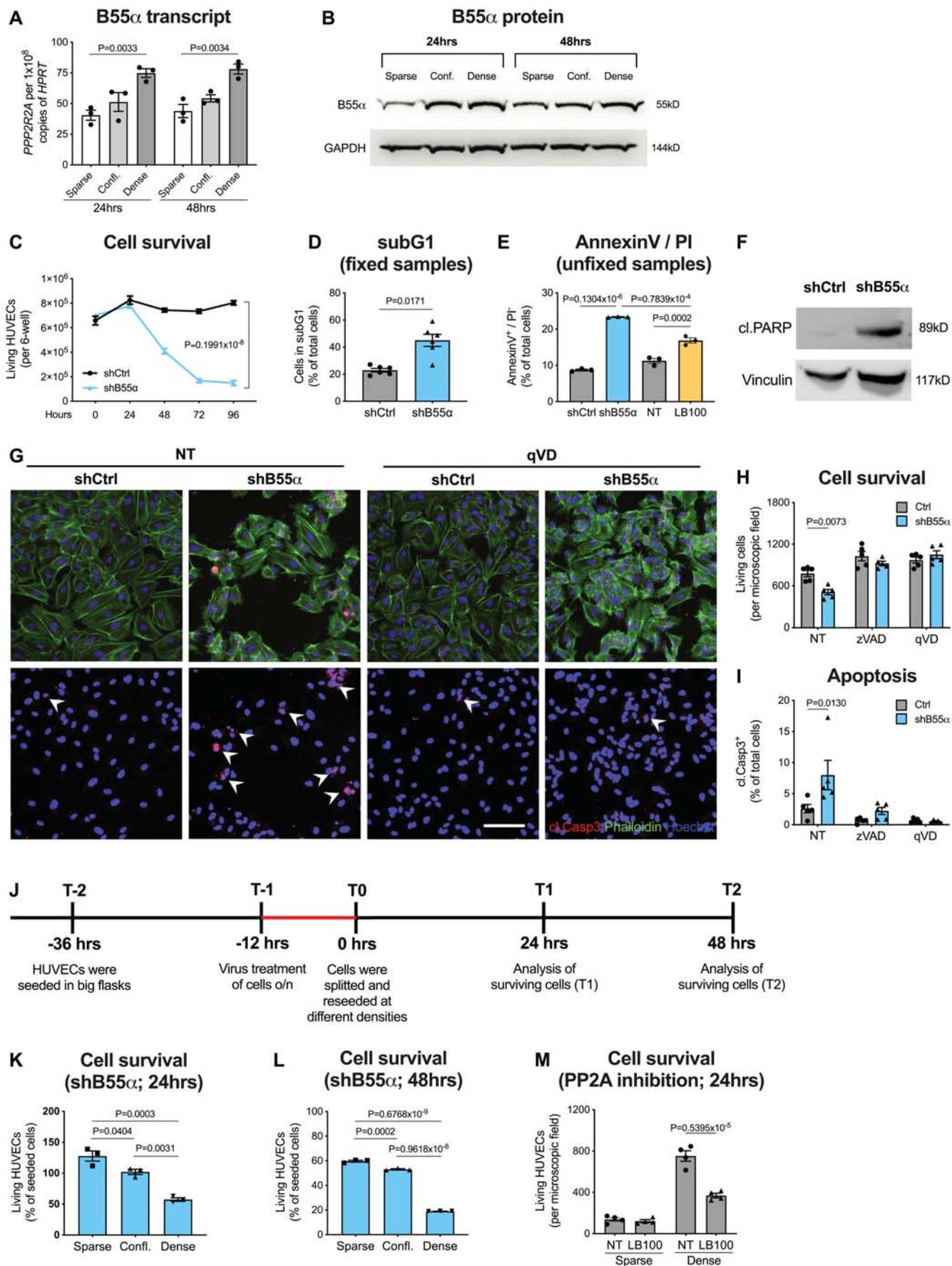
# Figure 4



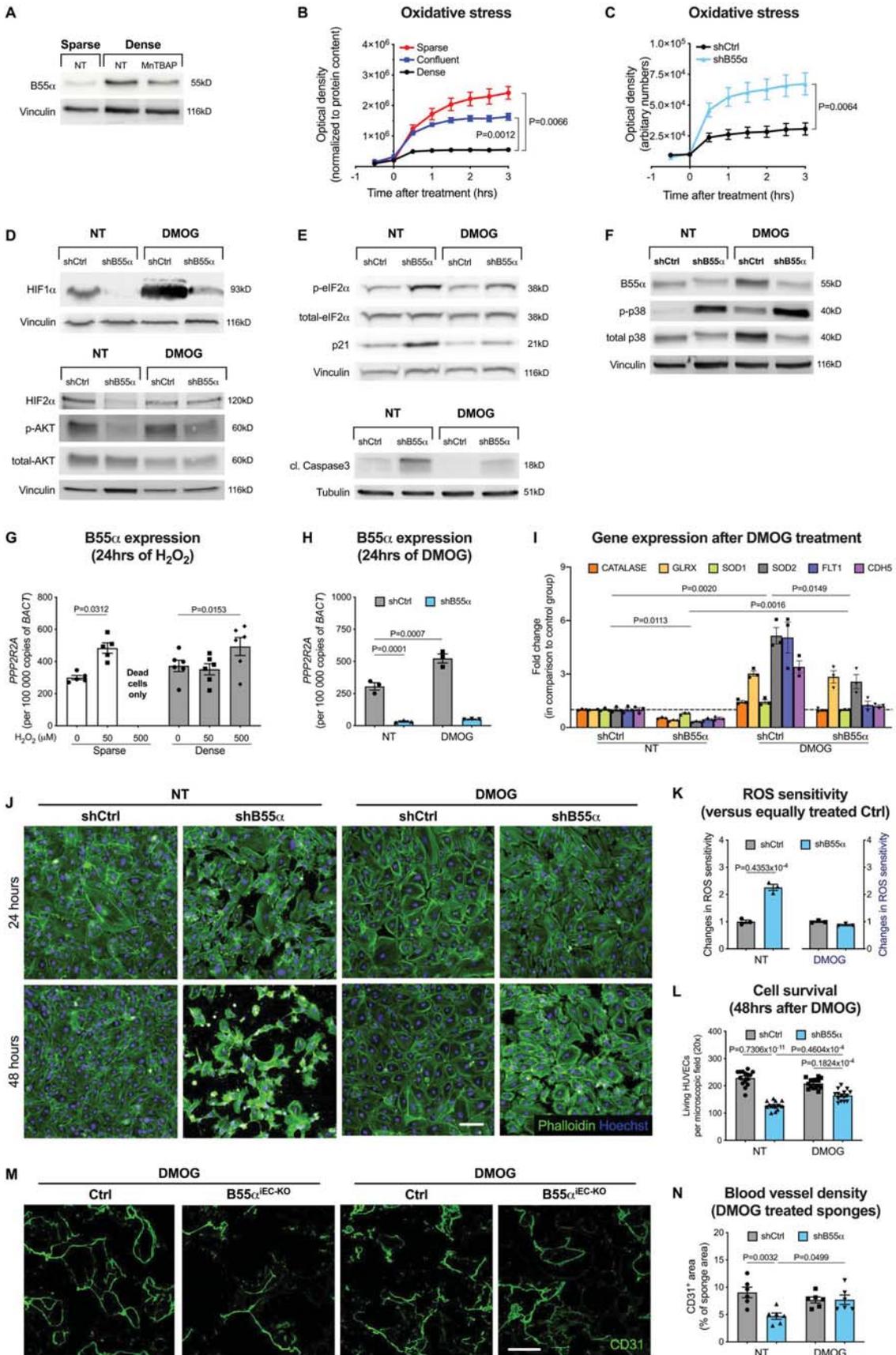
**Figure 5**



**Figure 6**



**Figure 7**



**Figure 8**

