



CELL INJURY, REPAIR, AGING, AND APOPTOSIS

# The Eyes Absent Proteins in Developmental and Pathological Angiogenesis



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Management of neoangiogenesis remains a high-value therapeutic goal. A recently uncovered association between the DNA damage repair pathway and pathological angiogenesis could open previously unexplored possibilities for intervention. An attractive and novel target is the Eyes absent (EYA) tyrosine phosphatase, which plays a critical role in the repair versus apoptosis decision after DNA damage. This study examines the role of EYA in the postnatal development of the retinal vasculature and under conditions of ischemia-reperfusion encountered in proliferative retinopathies. We find that the ability of the EYA proteins to promote endothelial cell (EC) migration contributes to a delay in postnatal development of the retinal vasculature when *Eya3* is deleted specifically in ECs. By using genetic and chemical biology tools, we show that EYA contributes to pathological angiogenesis in a model of oxygen-induced retinopathy. Both *in vivo* and *in vitro*, loss of EYA tyrosine phosphatase activity leads to defective assembly of  $\gamma$ -H2AX foci and thus to DNA damage repair in ECs under oxidative stress. These data reveal the potential utility of EYA tyrosine phosphatase inhibitors as therapeutic agents in inhibiting pathological neovascularization with a range of clinical applications. (*Am J Pathol* 2016, 186: 568–578; <http://dx.doi.org/10.1016/j.ajpath.2015.10.031>)

Pathological neovascularization (NV) is associated with common human diseases such as proliferative retinopathies, wet age-related macular degeneration, cancer, atherosclerotic plaque rupture, and rheumatoid arthritis.<sup>1</sup> Much evidence points to a role for vascular endothelial growth factor (VEGF) signaling in both pathological and developmental angiogenesis, and drugs targeting the VEGF pathway have been extensively tested as potential anti-angiogenic agents.<sup>2,3</sup> Antibodies to VEGF have achieved clinical success in the treatment of wet age-related macular degeneration, clinically significant macular edema, and retinopathy of prematurity, as well as in improving the odds of progression-free survival in certain cancers. However, intrinsic and induced resistance to anti-VEGF treatment poses limitations to their use in cancer treatment.<sup>3,4</sup> Furthermore, side effects associated with normal angiogenic processes such as wound healing are a concern. It is in these clinical contexts that novel anti-angiogenic agents capable of discriminating between developmental and pathological NV and complementing anti-VEGF treatment

would be valuable. Several recent studies have begun to delineate mechanisms that differentiate between developmental and pathological angiogenesis.<sup>5–9</sup> Notably, Okuno et al<sup>5</sup> showed that the ataxia telangiectasia mutated (ATM) pathway is activated by tissue hypoxia but is not active during normal angiogenesis, and Economopoulou et al<sup>6</sup> report that the H2AX-dependent DNA damage response (DDR) is activated and promotes NV specifically under hypoxic conditions. The Eyes absent (EYA) proteins are protein tyrosine phosphatases (PTPs) that hydrolyze the terminal phosphotyrosine of H2AX, permitting the assembly of the DNA damage response machinery and preventing apoptosis.<sup>10</sup> The tyrosine phosphatase activity of EYA is also necessary for endothelial cell (EC) migration and tube

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formation.<sup>11,12</sup> Taken together, these observations led us to postulate that the EYA proteins could contribute to angiogenesis by multiple mechanisms. They have a specific role in DDR-dependent pathological NV following ischemia-associated DNA damage and an EC migration-associated role in early development of the retinal vasculature. Hence, inhibition of the EYA tyrosine phosphatase activity could be a novel target for development of anti-angiogenic drugs. Here we report on the viability of this new therapeutic mechanism by using both genetic and chemical biology tools. These studies open the door for the development of EYA inhibitors with potential uses in the treatment of a host of vascularization-associated disorders, including cancer and proliferative retinopathies.

## Materials and Methods

### Antibodies and Reagents

The following antibodies were used in these studies: anti-cleaved caspase-3 (9664S; Cell Signaling Technology, Danvers, MA), anti-CD31 (550274, BD Biosciences, San Jose, CA), isolectin 488 (121411; Life Technologies, Carlsbad CA), bromodeoxyuridine (BrdU) mouse monoclonal Alexa 647 (B35133, Invitrogen, Carlsbad, CA), donkey anti-rabbit Alexa Fluor-594 and goat anti-mouse Alexa Fluor-488 (A21207 and A-11001, Life Technologies, Carlsbad, CA), anti-MDC1 (AB1169; Abcam, Cambridge, MA), anti- $\gamma$ H2AX (MABE205; Millipore, Billerica, MA), VEGF antibody (VG1, Thermo Fisher, Rockford, IL). Human retinal microvascular ECs (HRMECs) were obtained from Cell Systems (Kirkland, WA), and mouse retinal microvascular ECs (MRMECs) were obtained from Cell Biologics (Chicago, IL).

### Animals

This study adhered to the Association for Research in Vision and Ophthalmology statement for the use of animals in ophthalmic and vision research. C57Bl/6 mice obtained from Jackson Laboratories (Bar Harbor, ME) were bred in the Cincinnati Children's Hospital Medical Center facility (animal protocol 3D09062).

### Generation of *Eya3<sup>fllox</sup>* Allele

We generated a conditional loss-of-function allele, *Eya3<sup>fllox</sup>*, by conventional gene targeting. We targeted exon 6 for conditional deletion. This design left open the possibility that exons 1 to 5 might produce a functional truncated protein. Incorporation of sequences from the C-terminus of ornithine decarboxylase as a signal for rapid degradation<sup>13</sup> was designed to eliminate any expressed *Eya3* N-terminal sequence. The allele design incorporated loxP sites into an artificial exon that is initially in reverse orientation. Through two steps of recombination mediated by a pair of wild-type and a pair of variant loxP sequences that are incompatible,

the C-terminus of ornithine decarboxylase degradation signal was spliced into the mRNA, simultaneously deleting exon 6. This allele design strategy has been used previously.<sup>14</sup> Successful FLP recombination was confirmed by PCR analysis with *Eya3*fl forward, 5'-CCACTTGGAGT-AAGCATCCAGTC-3' and reverse, 5'-AAGCAAAC-GTCCTAGGTGCTC-3'.

### Oxygen-Induced Retinopathy Model

The protocol for oxygen-induced retinopathy was one previously described in studies associating DNA damage repair pathway with NV.<sup>5,6</sup> Briefly, neonatal mice and their nursing mothers were kept in room air from postnatal day 0 (P0) to P8. At P8 each pup and its nursing mother were moved to a chamber maintained at 85% oxygen. At P11 the pups and their nursing mothers were returned to room air (surrogate mothers were used if necessary). When appropriate, intravitreal injection of EYA inhibitors [1  $\mu$ L of either 100  $\mu$ mol/L stock solution of benzarone (BZ) or 250  $\mu$ mol/L stock solution of benzbromarone (BBR)] was performed at P12. To examine the effect of genetic deletion of *Eya3* in the oxygen-induced retinopathy (OIR) protocol, tamoxifen was administered i.p. at 300  $\mu$ g per pup daily between P11 and P13 to both *Eya3<sup>fllox/fllox</sup>*, *pdgf-iCre*, and *Eya3<sup>fllox/fllox</sup>* mice. At various time-points the eyes were enucleated while the pups were maintained under anesthesia. Immunofluorescence imaging and Western blot analysis of ECs demonstrated the effectiveness of this protocol in lowering EYA3 levels in ECs. There was no compensatory up-regulation of other *Eyas* (Supplemental Figure S1).

### Immunostaining of Whole Mount Retinas

Enucleated eyes were fixed for 1 hour in 4% paraformaldehyde/phosphate-buffered saline at room temperature and dissected. Retinas were permeabilized at room temperature for 30 minutes and then incubated with specific antibodies ( $\gamma$ -H2AX, EYA3, or cleaved caspase-3) overnight at 4°C. Antigen was detected with secondary antibodies conjugated to Alexa Fluor 594 or Alexa Fluor 488. To visualize vasculature, retinas were stained with fluoresceinated *Griffonia (Bandeiraea) simplicifolia* (isolectin B<sub>4</sub>-Alexa Fluor 594 conjugate, 1:500 dilution; Invitrogen) in phosphate-buffered saline with Tween (PBST). For the BrdU incorporation studies, 10 mg/kg BrdU was administered i.p. at P13. Isolated retinas were treated with 2N HCl for 30 minutes and then extensively washed with PBS, blocked with 10% fetal bovine serum, and incubated with BrdU Alexa Fluor 647 for 1 hour at room temperature. Images were taken at  $\times$ 400 magnification on a Zeiss microscope (Zeiss, Jena, Germany).

### Quantification of NV and VO

Standard published protocols were used to quantitate NV and VO. The number of pixels in the pathological tufts was quantified and compared with the number of pixels in the entire

retinal area by a computer-aided method (SWIFT-NV<sup>15</sup>) that utilizes a series of macros in ImageJ version 1.48 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>). Percentage of NV and VO in total retinas was compared between inhibitor- and vehicle-treated eyes, or *Eya3*<sup>VEC-KO</sup> and *Eya3*<sup>fl/fl</sup> mice. In each case, *n* was the number of eyes quantified. Each experiment included three independent litters.

### RT-PCR Analysis

To determine expression of *Eya* transcripts in mouse retinal microvascular ECs or retinal ECs from genetically engineered mice, total RNA was extracted from 1 million ECs, and cDNA was synthesized with the Primescript RT reagent kit (Takara Bio, Shiga, Japan). PCR product was analyzed on a 1.5% agarose gel to confirm that amplified products were of the expected sizes. Primers used included EYA1 forward 5'-CATAGCCGACTGAGTGGTAGT-3' and reverse 5'-GCTCTGTTTTAACTTCGGTGCC-3'; EYA2 forward 5'-CACCGCTGGGCTCTATCAAG-3' and reverse 5'-GGGGTAGGACGGATAATCCTG-3'; EYA3 forward 5'-CTCAAACCAGGATTATCCCACC-3' and reverse 5'-CAGCATCACTGTTAGTCTGACC-3'; EYA4 forward 5'-TCCTTGGCCCTGCTAAGAG-3' and reverse 5'-TGCCTATTTTTGTTGCGCTGT-3'; GAPDH forward 5'-AAGGCCGGGGCCCACTTGAA-3' and reverse: 5'-CGGCCATCACGCCACAGCTT-3'.

### EC Culture, Immunostaining, Transwell Migration, and Proliferation Assays

HRMECs were cultured in Complete Medium (Cell Systems) and used in the first nine passages. ECs from *Eya3*<sup>VEC-KO</sup> and control mice were isolated by using magnetic Dynabeads (Life Technologies) coated with anti-PECAM-1 antibody as previously described.<sup>16</sup> For analysis of the formation of DNA repair complexes, cells were fixed with 4% paraformaldehyde at room temperature for 15 minutes, and then the coverslips were immunostained for DDR proteins with anti- $\gamma$ H2AX and anti-MDC1 antibodies. Cell proliferation was measured at 72 hours with the WST-8 assay (CKK-8 Kit, Dojindo Molecular Technologies, Rockville, MD) as previously described.<sup>12</sup> Proliferation studies were performed in Epithelial Cell Growth Medium 2 (Lonza, Allendale, NJ). Vehicle control contained 0.1% dimethyl sulfoxide, and both EYA inhibitors were dissolved in 0.1% dimethyl sulfoxide. For hypoxia experiments, the cells were maintained at 1% O<sub>2</sub> with BioSpherix ProCO<sub>2</sub> Model P120 and ProOx Model 110 controllers for C chambers (Biospherix, Parish, NY). Transwell migration experiments were performed as previously described.<sup>12</sup>

### Statistics

Results are presented as the means  $\pm$  SEM for the *in vivo* experiments and as means  $\pm$  SD for the *in vitro* studies. Statistical analyses were performed with Graphpad PRISM

version 5.0 for Mac OSX, (GraphPad Software, La Jolla, CA). A *t*-test was used when two samples and conditions were compared, and analysis of variance was used for more than two groups. Significance represents *P* < 0.05.

## Results

### Targeted Deletion of *Eya3* in ECs Reduces Extension and Branching of the Early Postnatal Retinal Vasculature

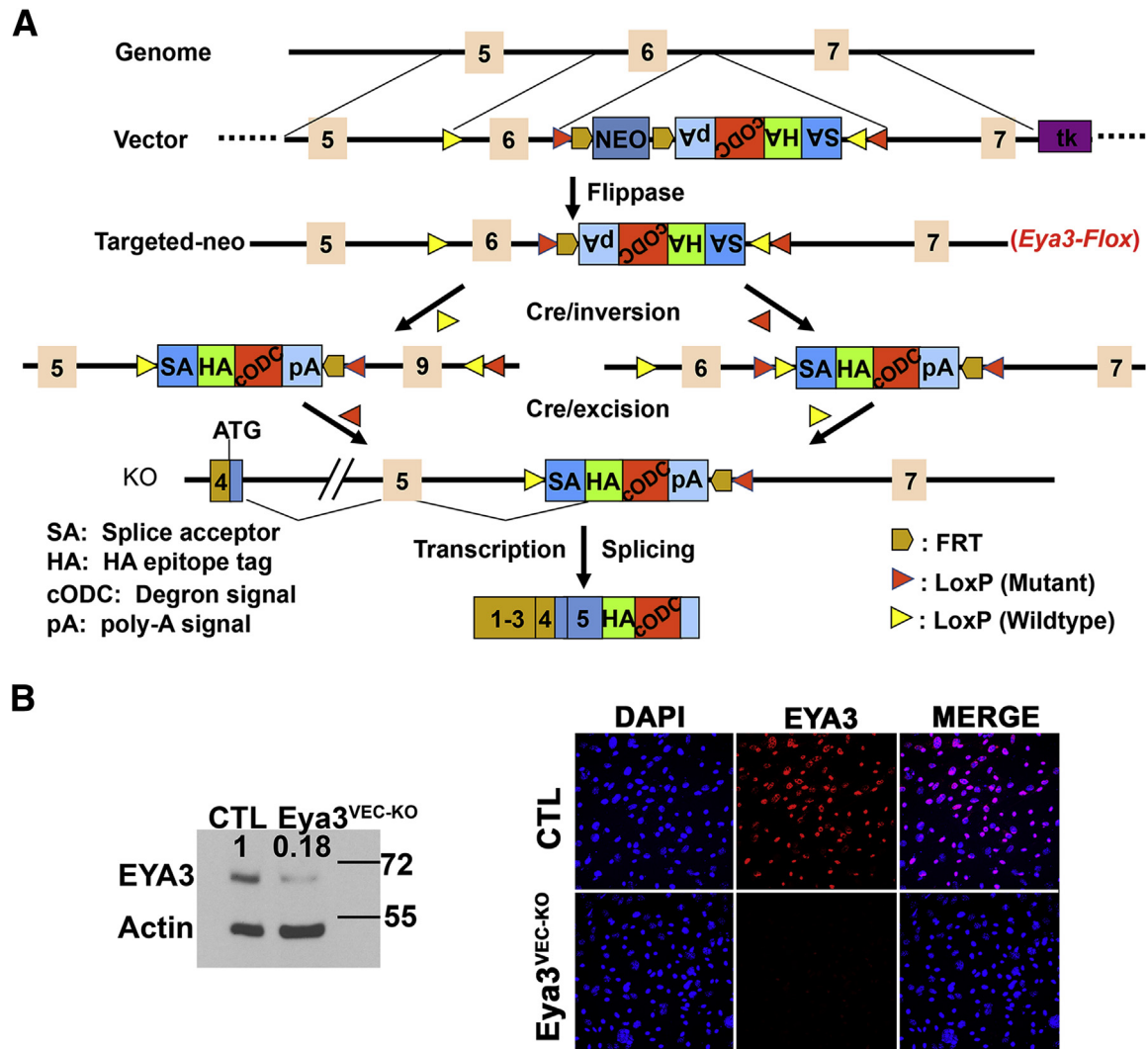
The mouse retina is avascular at birth, with a monolayer of vessels extending out from the center to the periphery between birth and P7, providing an accessible and well-characterized system<sup>17</sup> to examine the role of EYA in developmental angiogenesis. RT-PCR analysis for *Eya1*, *Eya2*, *Eya3*, and *Eya4* in mouse retinal ECs showed a transcript for only *Eya3* (Supplemental Figure S1). To investigate the cell-autonomous function of EYA3 in NV, we deleted *Eya3* from ECs by using the *loxP* recombination strategy and the EC-specific tamoxifen-inducible Cre-recombinase *Pdgfb-iCreER*<sup>18</sup> (*Eya3*<sup>VEC-KO</sup>). The targeting strategy and the resulting reduction in EYA3 protein levels in endothelial cells is shown in Figure 1. The developing retinal vasculature was examined at P5 and P8 by using flat mounts and staining with isolectin B4.

Endothelial deletion of *Eya3* caused a significant reduction in the extension of the superficial vascular plexus at P5, as measured by the ratio of the vascular plexus radius to the radius of the total retina (Figure 2, A and B). Branching density was quantitated by counting the number of branch points per unit area of vasculature at the angiogenic front. On average, 24% fewer branch points were observed in the *Eya3*<sup>VEC-KO</sup> retinas relative to control littermates (Figure 2, C and D).

Both EC proliferation and migration could contribute to the vascular defects observed here. Each of these was examined in retinas of *Eya3*<sup>VEC-KO</sup> and control *cre*-negative littermates at P5. The proliferation of stalk cells plays a key role in the angiogenic process at the vascular front.<sup>19</sup> When using bromo-deoxyuridine (BrdU) incorporation by isolectin B4-positive cells at P5 as a measure of EC proliferation, we did not observe a significant difference between retinas from *Eya3*<sup>VEC-KO</sup> and their *cre*-negative littermates (Figure 2E). In retinas from *Eya3*<sup>VEC-KO</sup> pups, however, we observed a 27% reduction in the number of filopodia per millimeter of vessel length at the vascular front, suggesting a likely defect in EC migration (Figure 2F).

### Targeted Deletion of *Eya3* in ECs Attenuates NV in the Oxygen-Induced Retinopathy Model

Despite the early phenotype observed at P5, analysis of the superficial retinal vascular plexus at P8 shows recovery of both extension and branching defects in *Eya3*<sup>VEC-KO</sup> animals (Figure 3A), suggesting that lack of EYA3 only delays development of the superficial vascular layer.



**Figure 1** Strategy for *Eya3* deletion. **A:** Generation of the *Eya3* conditional allele. Orange boxes correspond to exons. Red and yellow arrowheads are *LoxP* sites. Brown arrowheads are *Frt* sites used to delete the Neo-positive selectable marker; tk represents the thymidine kinase gene that is selectable against after homologous recombination. **B:** EYA3 levels are lower in the *Eya3*<sup>VEC-KO</sup> endothelial cells. Western blot analysis of lung endothelial cells, and immunostaining of retinal endothelial cells extracted from *Eya3*<sup>fl/fl</sup> (CTL) and *Eya3*<sup>VEC-KO</sup> mice showing EYA3 (red), and DAPI (blue). CTL, control.

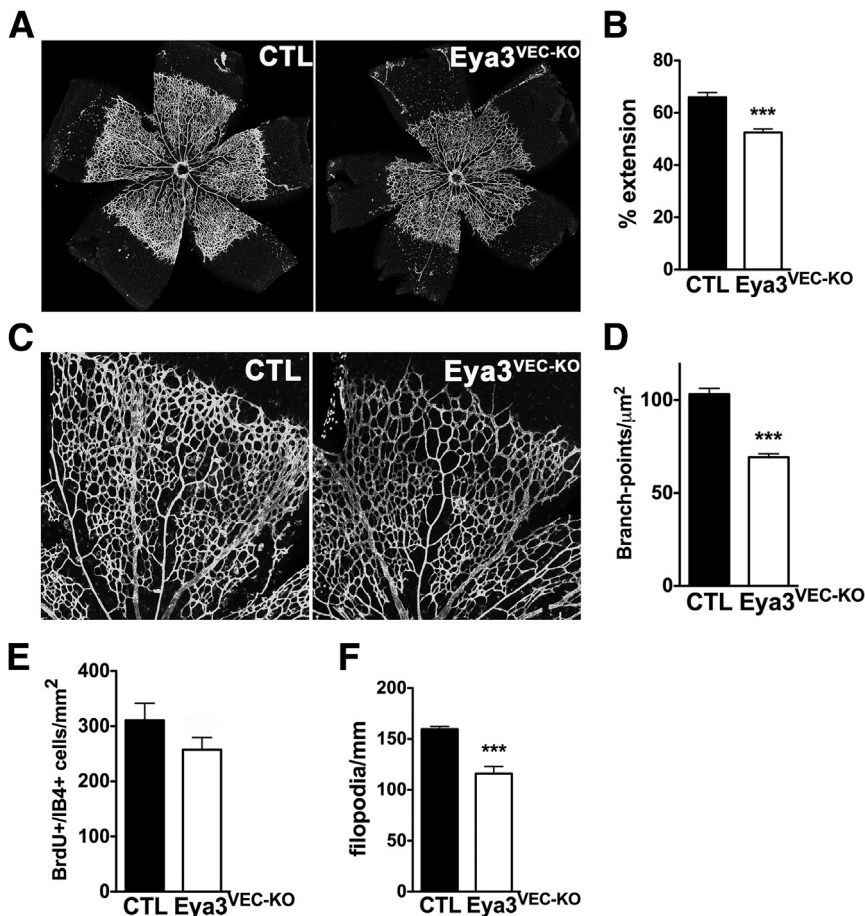
To elucidate the role of EYA in pathological angiogenesis we used a modification of the well-established OIR model<sup>20</sup> in which pups are subjected to hyperoxia (75% O<sub>2</sub> between P7 and P12) and then returned to room air for 4 to 5 days before analysis. This modified protocol (85% O<sub>2</sub> chamber between P8 and P11) was previously described in the study showing that NV requires the DDR-associated minor histone protein H2AX.<sup>6</sup> This protocol was validated by the observation of characteristic regression of immature capillaries in the center of the retina leading to a central area of VO upon return to room air (P11-OIR) (Figure 3B). The resulting effective hypoxia leads to EC proliferation and the formation of neovascular tufts (P17-OIR) (Figure 3B), similar to that seen with the 5-day hyperoxia protocol. Maximum severity of NV, the proliferative phase, typically occurs 5 days after return to room air (P16 in this protocol and P17 in the

5-day protocol). Clinically, NV is considered pathological, whereas revascularization is desirable.

*Eya3*<sup>fl/fl</sup> (control) and *Eya3*<sup>VEC-KO</sup> pups were subjected to hyperoxia from P8 to P11 and injected with tamoxifen from P11 to P13 to induce *Eya3* deletion. NV at P17 was quantitated by using an automated protocol.<sup>15</sup> *Eya3*<sup>VEC-KO</sup> retinas had nearly 40% less NV than did control retinas (Figure 3C). Interestingly, residual VO in the central avascular area was comparable in the control and *Eya3*<sup>VEC-KO</sup> retinas (Figure 3C).

The OIR protocol is a VEGF-dependent model in which VEGF levels are suppressed during hyperoxia and elevated upon return to room air. Maximal VEGF levels are typically observed within 3 days after return to room air (P14 in the protocol used here) and remain high until P17. Interestingly, VEGF protein levels were comparable in both the control and *Eya3*<sup>VEC-KO</sup> retinas (Figure 3D), although reduced cell proliferation, as measured by BrdU incorporation, was seen





**Figure 2** Loss of EYA3 from VECs attenuates development of the superficial vascular plexus in the retina. **A:** Flat-mounted P5 retina from *Eya3*<sup>VEC-KO</sup> conditional mutant mice and control littermates (*Eya3*<sup>fl/fl</sup>) labeled with isolectin B4. Deletion of *Eya3* was induced by tamoxifen injection between P1 and P3. **B:** Reduced extension of the vascular plexus as quantitated in both retina of six mice per group. **C:** Higher magnification images of flat-mounted retina highlight reduced vascular density in *Eya3*<sup>VEC-KO</sup> retinas relative to controls. **D:** Vessel density measured by counting branch points at the advancing vascular front (four fields per retina) shows a significant decrease in branching density upon VEC-specific *Eya3* deletion. **E:** Quantification of BrdU-positive/isolectin B4-positive cells in *Eya3*<sup>VEC-KO</sup> and control retina (*Eya3*<sup>fl/fl</sup>) at P5. No statistically significant difference is observed. **F:** Filopodia number was quantified and normalized to the outline of the angiogenic front. Fewer filopodia per millimeter are observed in *Eya3*<sup>VEC-KO</sup> retinas. Data presented as means ± SEM (**B**, **D**, and **F**).  $n = 6$  (**D**);  $n = 12$  (**E**),  $5$  (**F**), control;  $n = 5$  (**E**),  $8$  (**F**), *Eya3*<sup>VEC-KO</sup>. \*\*\* $p < 0.001$ . BrdU, bromodeoxyuridine; CTL, control; P1, postnatal day 1; VECs, vascular endothelial cells.

in the *Eya3*<sup>VEC-KO</sup> retinas at P14 relative to the controls (Figure 3E).

### Pharmacological Inhibition of the EYA Tyrosine Phosphatase Activity Attenuates NV in the OIR Model

EYA proteins have multiple and separable biochemical activities (tyrosine phosphatase, threonine phosphatase, and transactivation).<sup>21</sup> We hypothesized that only the tyrosine phosphatase activity contributes to pathological angiogenesis through its role in DNA damage repair. To specifically target this enzymatic activity of the EYA proteins, we used a chemical biology approach. We have previously identified and validated EYA tyrosine phosphatase inhibitors that showed *in vitro* anti-angiogenic activity.<sup>11,12</sup> Two of the best-characterized members of this inhibitor series, BBR and BZ, were used in these studies. These compounds show selectivity toward the EYA family of PTPs, and previous cell-based studies support their ability to target EYA3 in cells.<sup>12</sup>

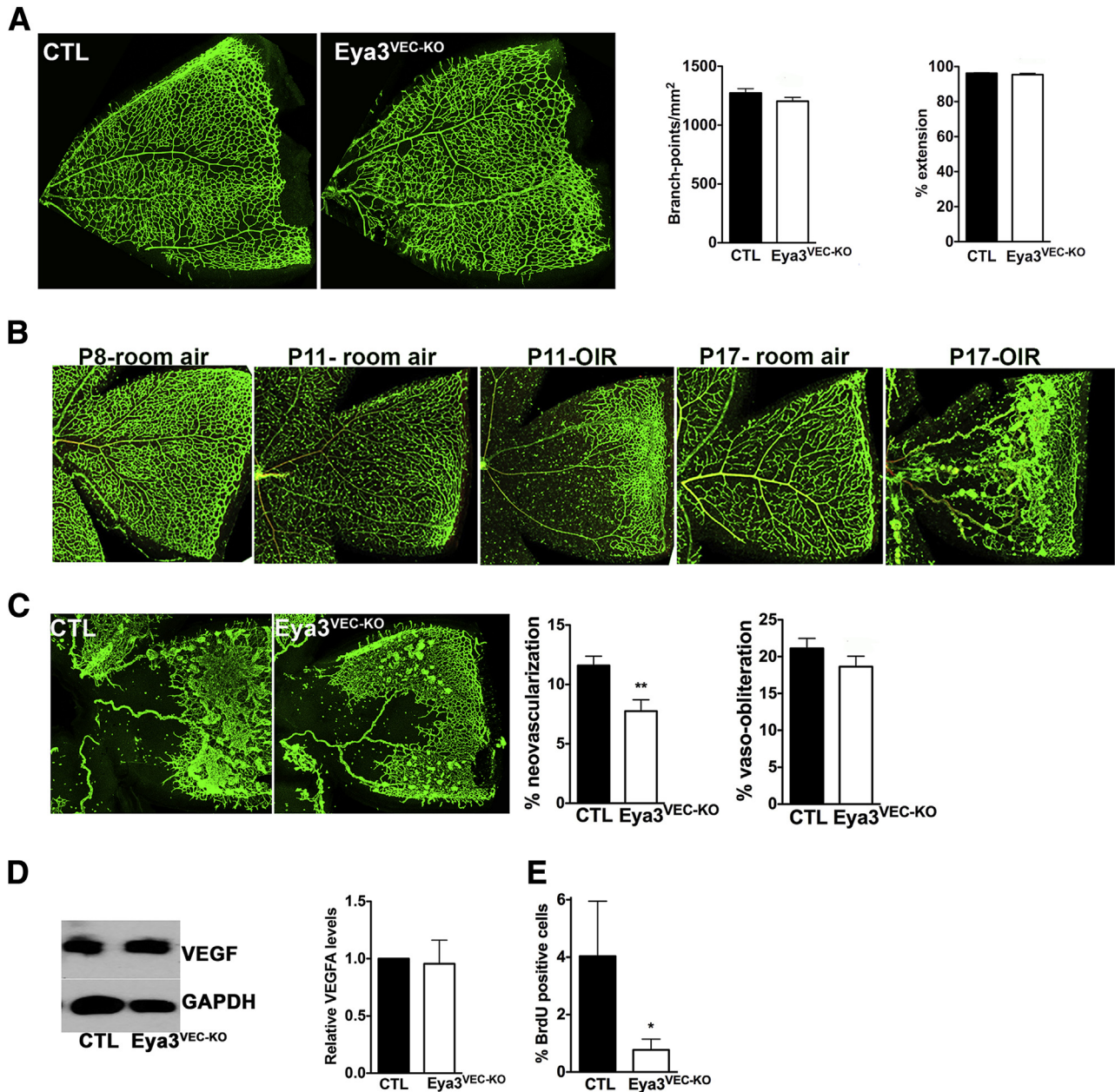
At P12 (24 hours after return to room air), each pup subjected to the OIR protocol was injected in one eye with an EYA inhibitor and in the other with a vehicle control. Retinas were dissected and flat-mounted at P17 and stained with isolectin B4 to visualize the vasculature (Figure 4A). There was significant normalization of the vasculature in the eyes treated with

either BZ or BBR relative to the vehicle controls (54% and 49% reduction in NV, respectively) (Figure 4, A and B); however, VO was comparable to that in the vehicle-treated eye, suggesting a preferential effect on pathological NV (Figure 4C).

Increased EC proliferation is known to contribute to NV as well as to vessel tortuosity in the OIR protocol.<sup>22</sup> To determine whether EYA inhibitors had an effect on proliferation, we measured incorporation of the nucleotide analog BrdU. Low levels of BrdU incorporation were detected in control vehicle-treated OIR retinas in the proliferative phase (measured here at P14), with a 60% decrease in either the BZ- or BBR-treated retinas (Figure 4D).

### EYA-PTP Inhibition or *Eya3* Deletion Attenuates the DNA Damage Response in the Oxygen-Induced Retinopathy Model

To establish the kinetics of DNA-damage repair subsequent to ischemic insult induced by the OIR protocol, we monitored  $\gamma$ -H2AX foci in the retina of mice at 48, 72, 96, 120, and 144 hours after return to room air. Widespread  $\gamma$ -H2AX foci containing nuclei were observed within 48 hours and peaked between 120 and 144 hours (P16 to P17); further analyses were conducted at P17 to coincide with evaluation of NV.



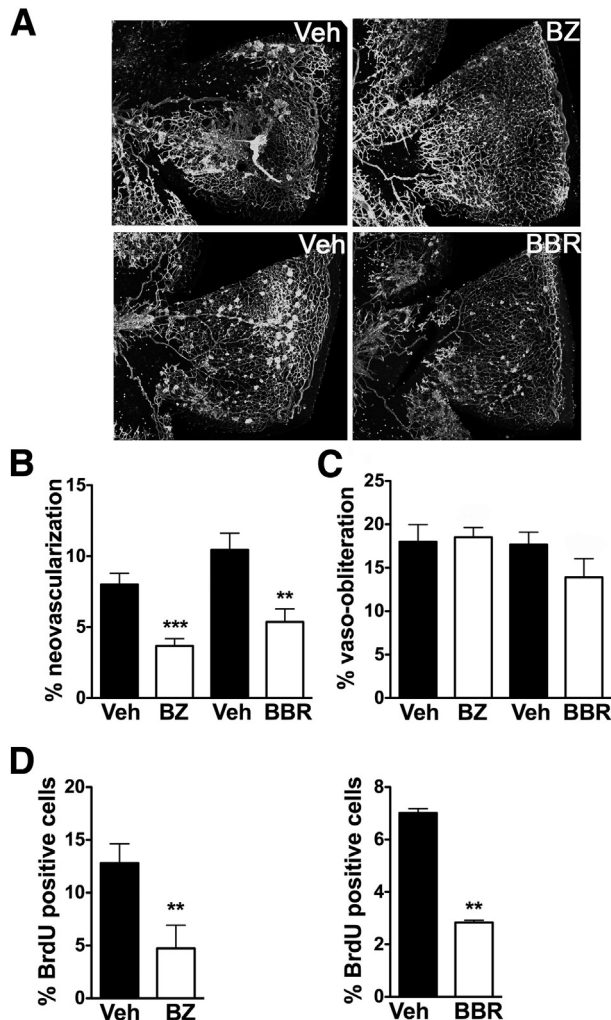
**Figure 3** VEC-specific deletion of *Eya3* in an OIR model results in reduced neovascularization. **A:** Flat-mounted P8 retinas from *Eya3<sup>fl/fl</sup>* (CTL) and *Eya3<sup>VEC-KO</sup>* mice show that development of the vascular plexus in the mutant animals is comparable to that in the controls (tamoxifen injection between P1 and P3). **B:** Flat-mounted retinas from mice maintained in room air at P8, P11, and P17 compared with mice placed in 85% O<sub>2</sub> between P8 and P11 and returned to room air until P17. After hyperoxia treatment, a central zone of vaso-obliteration is evident at P11 and neovascular tufts can be seen at P17. **C:** P17 retinas of *Eya3<sup>VEC-KO</sup>* and control mice subjected to hyperoxia between P8 and P11 with *Eya3* deletion induced by tamoxifen injection between P11 and P13. Significantly less neovascularization but comparable vaso-obliteration are seen in *Eya3<sup>VEC-KO</sup>* retinas relative to CTL retinas. **D:** Representative Western blot analysis of extracts from P14 retinas after hyperoxia (left). Quantitation of normalized vascular endothelial growth factor antibody levels from Western blot analyses (three retinas; right). **E:** Quantitation of the percentage of BrdU-positive cells at P14 in OIR retinas. Data are expressed as means  $\pm$  SEM (**D** and **E**).  $n = 6$  retinas in each group (**A**);  $n = 17$  CTLs,  $n = 16$  *Eya3<sup>VEC-KO</sup>* from three independent litters (**C**);  $n = 2$  CTLs,  $n = 4$  *Eya3<sup>VEC-KO</sup>* (**E**). \* $P < 0.05$ , \*\* $P < 0.01$ . CTL, control; OIR, oxygen-induced retinopathy; P8, postnatal day 8; VECs, vascular endothelial cells.

Although DNA foci were generally more frequent in non-ECs, foci were also observed in ECs at sites of NV (Figure 5A), which is consistent with previous reports that DNA damage repair is coincident with proliferating ECs.<sup>6</sup>

Genetic and chemical biology tools were used to examine the effect of either *Eya3* deletion or EYA-PTP inhibition on

the presence of  $\gamma$ -H2AX foci at P17 as a marker of DNA damage repair. *Eya3<sup>VEC-KO</sup>* and control mice were subjected to the P8 to P11 OIR protocol, and tamoxifen was administered after return to room air. There was more than a 50% reduction in the number of nuclei with  $\gamma$ -H2AX foci in the retinas of the *Eya3<sup>VEC-KO</sup>* mice relative to in the retinas of





**Figure 4** EYA tyrosine phosphatase inhibition suppresses neovascularization in retinopathy. **A:** Representative retinal flat mounts at P17 from mice subjected to the OIR protocol and injected at P12 in one eye each with vehicle or EYA inhibitors BZ or BBR. Vessels were visualized with isolectin B4 staining. **B:** Neovascularization quantitated as percentages of total retinal area. **C:** Areas of vaso-obliteration quantitated as percentages of total retinal area; nonsignificant. **D:** Quantification of percentage of BrdU-positive cells at P14 in OIR retinas treated with either vehicle or EYA inhibitors BZ (left) or BBR (right); \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .  $n = 12$  BZ,  $n = 13$  BBR and represent three independent litters (**B** and **C**);  $n = 2$  BBR,  $n = 3$  BZ (**D**). BBR, benzobromarone; BrdU, bromodeoxyuridine; BZ, benzarone; OIR, oxygen-induced retinopathy; P17, postnatal day 17.

their *cre*-negative littermates (Figure 5C). Similarly, retinas treated with the EYA inhibitors BZ or BBR had significantly fewer nuclei with  $\gamma$ -H2AX foci relative to the vehicle-treated controls, which is suggestive of an impaired DNA damage response (Figure 5, A and B).

#### EYA Inhibition Attenuates EC Migration, Proliferation under Hypoxia, and the Formation of $\gamma$ -H2AX–MDC1 Foci under Oxidative Stress

To better define the molecular mechanism(s) by which the EYAs might play a role in retinal angiogenesis, we used

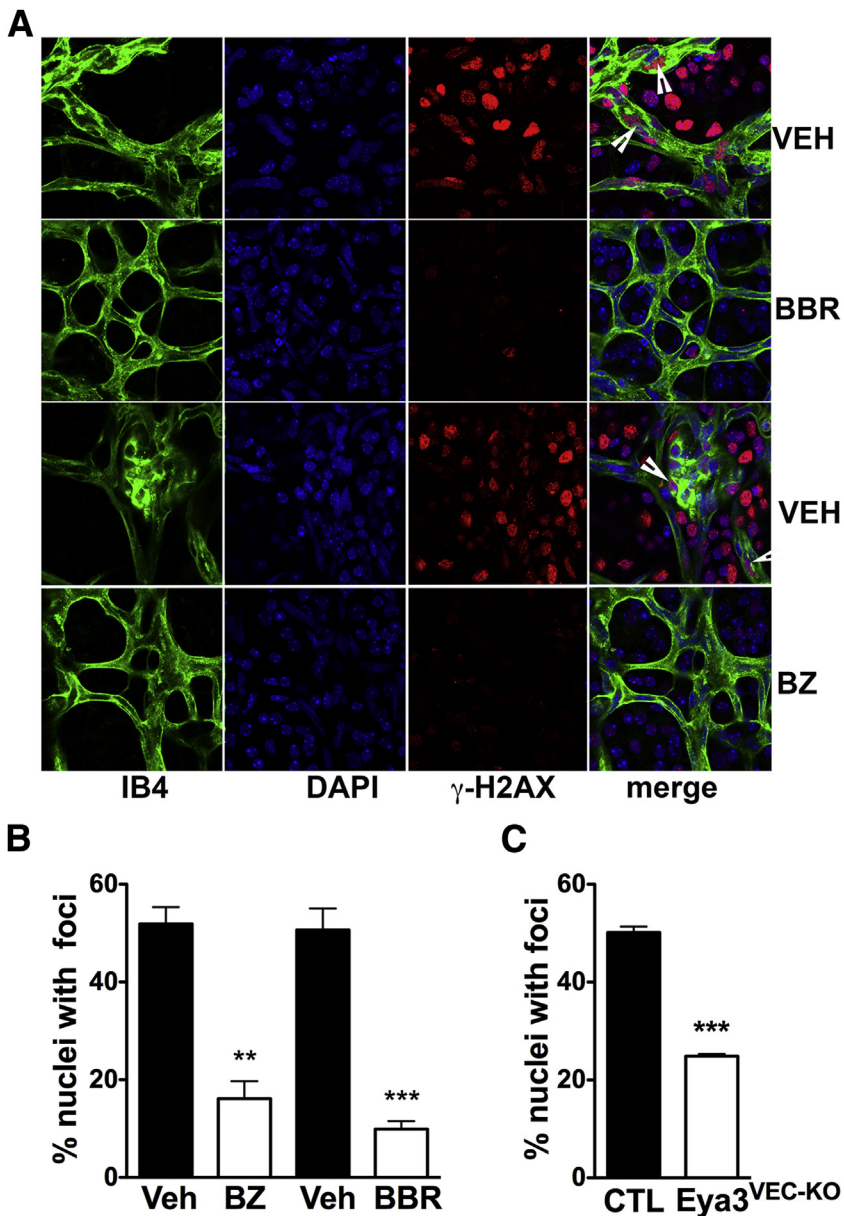
primary HRMECs. We have previously reported that the EYA-PTP inhibitors BZ and BBR inhibit human umbilical vein EC migration *in vitro*.<sup>12</sup> Similarly, BZ and BBR inhibited Transwell migration of HRMECs in a dose-dependent manner (Figure 6A), confirming a role for the EYA-PTP in the motility of retinal ECs as seen in the P5 retina.

We next examined the effect of EYA-PTP inhibition on HRMEC proliferation under hypoxic conditions. As expected, HRMECs were more proliferative under hypoxic conditions (1% O<sub>2</sub>) than in 21% O<sub>2</sub>. Increased proliferation under hypoxic stress distinguishes ECs from most other cell types that undergo cell cycle arrest and apoptosis in hypoxic conditions because of stalled replication forks. Both BZ and BBR attenuated proliferation in a dose-dependent fashion (Figure 6B). Analogous results were previously reported for human umbilical vein endothelial cells transfected with siRNA targeting H2AX: H2AX was required to maintain proliferation only under hypoxic conditions.<sup>6</sup> Because the EYA tyrosine phosphatase targets the C-terminal tyrosine 142 of H2AX, these observations were consistent with a model in which the tyrosine 142–dephosphorylated form of H2AX supports EC proliferation in hypoxia.

ATM/ATR-dependent stabilization of stalled replication forks is generally initiated by formation of  $\gamma$ -H2AX foci.<sup>23</sup> In ECs this process is ATR and H2AX dependent, permits survival and proliferation under conditions of pathological hypoxia, and consequently promotes NV.<sup>5</sup> Critical for the sustenance of  $\gamma$ -H2AX foci is the recruitment of the mediator MDC1, which will only bind to Y142-dephosphorylated  $\gamma$ -H2AX.<sup>24–26</sup> To determine the effect of EYA inhibition on hypoxia-induced DNA damage repair, HRMECs (with or without EYA inhibitors) were maintained in either 0.2% O<sub>2</sub> or 21% O<sub>2</sub> for 12 hours. Previous studies have established that treatment with such extreme hypoxia is required for the detection of  $\gamma$ -H2AX *in vitro*.<sup>23</sup> In control cells  $\gamma$ -H2AX foci that colocalize with MDC1 were observed, suggesting the formation of DNA repair complexes. Significantly fewer  $\gamma$ -H2AX foci were present in EYA inhibitor–treated HRMECs relative to control cells (Figure 6C).

## Discussion

The EYA proteins are part of a conserved developmental cassette most often associated with development of the fly eye; loss of *eya* function in *Drosophila* leads to severe defects, including loss of the compound eye. However, no ocular phenotype has been reported for any mammalian *Eya* knockout (there are four mammalian orthologs, *Eya1* to *Eya4*). The *Eya3*-deficient mouse was reported to have minor functional deficits, including reduced movement and changes in respiratory, heart, and muscle function.<sup>27</sup> We have previously reported a role for the EYA-PTP activity in endothelial and cancer cell motility.<sup>12,28</sup> Because EC migration is a critical component of angiogenesis, the role of



**Figure 5**  $\gamma$ -H2AX foci formation is attenuated in retinas of mice subjected to the OIR protocol. **A:** Isolectin B4 (green) and  $\gamma$ -H2AX (red) staining of whole mount retinas (P17) from mice subjected to the OIR protocol and injected intravitreally with either vehicle or the EYA inhibitors BZ or BBR. Nuclei are stained blue with DAPI. **White arrowheads** highlight  $\gamma$ -H2AX staining in endothelial cells. **B:** Quantification of nuclei with  $\gamma$ -H2AX foci in retinal flat mounts of OIR mice at P17. One eye of each mouse was injected with vehicle and the other with BZ or BBR. **C:** Quantification of nuclei with  $\gamma$ -H2AX foci in the retinal flat mounts from *Eya3<sup>fl/fl</sup>* (CTL), and *Eya3<sup>VEC-KO</sup>* mice subjected to the OIR protocol and with *Eya3* deletion induced by tamoxifen injections between P11 and P13. Data are expressed as means  $\pm$  SEM (**B** and **C**).  $n = 4$  retinal segments per retina of three mice per group (**B**);  $n = 3$  three representative segments from eight retina (**C**). \*\*\* $P < 0.01$ , \*\*\*\* $P < 0.001$ . BBR, benzobromarone; BZ, benzarone; CTL, control;  $\gamma$ -H2AX, Ser139-phosphorylated form of the DNA damage sensor H2AX; OIR, oxygen-induced retinopathy.

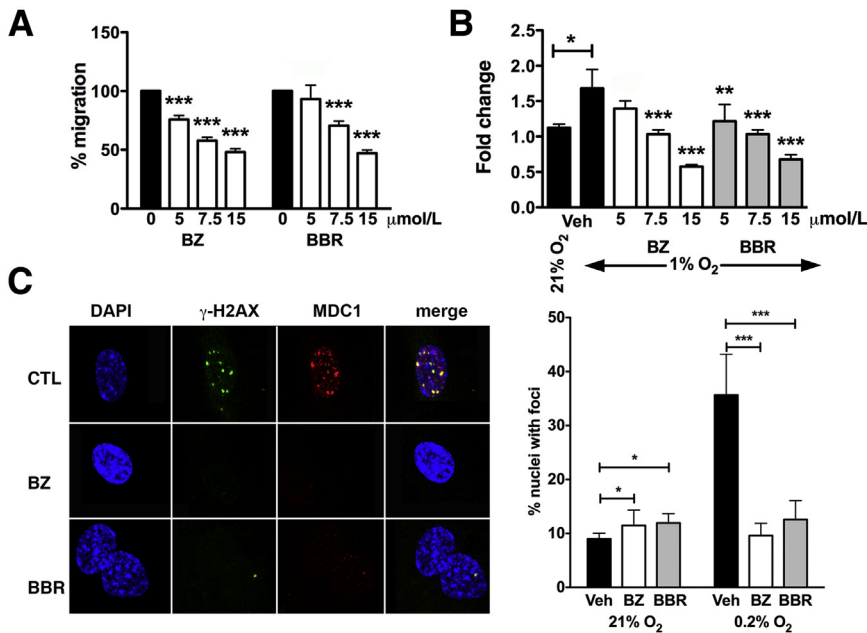
EYA in the development of the retinal vascular plexus of neonatal mice was examined. Developmental angiogenesis involves, at a minimum, proliferation of endothelial stalk cells, migration of tip cells, tube or lumen formation, and degradation of the extracellular matrix. Analysis of retinal vasculature upon EC-specific *Eya3* deletion reveals reduced extension and branching density at P5. The observations that there is little change in EC proliferation upon *Eya3* deletion at this stage and that fewer filopodial extensions are seen on tip cells at the vascular front are consistent with a role for EYA3 in EC migration. Reduced filopodia upon *Eya3* deletion was also previously observed in breast cancer cells,<sup>28</sup> supporting the possibility that the EYA proteins contribute to cell migration by altering the actin cytoskeleton.<sup>21,28</sup>

Despite the early phenotype observed in the superficial retinal vasculature, the plexus in *Eya3<sup>VEC-KO</sup>* extends to

the periphery and is comparable to control retinas by P8. This suggests that loss of *Eya3* only delays vascular development, which is commensurate with the observation that EC migration and not EC proliferation is affected by loss of *Eya3* in EC. A previous report describing germline deletion of *Eya3* noted no defects in eye development or function, although the mutant mice were smaller than the controls.<sup>27</sup> No examination of vascular development *in vivo* has been previously reported. Although the present study focused on the role of endothelial EYA3, it is expressed in other relevant cells, including smooth muscle cells and retinal microglia. The role of nonendothelial EYA3 in angiogenesis is the subject of independent ongoing investigations.

Unlike in the early postnatal days of developmental angiogenesis, in established vascular plexus such as the late





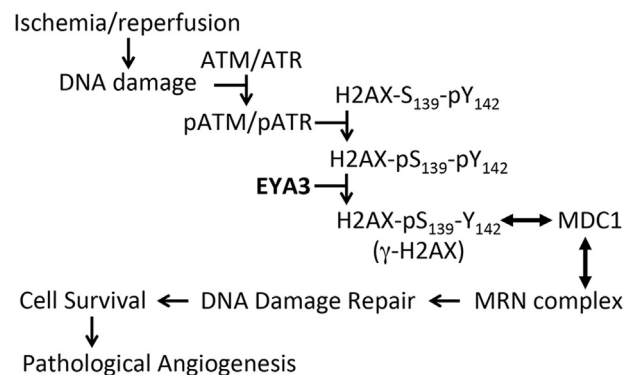
**Figure 6** Lowering EYA3 levels or EYA-PTP inhibition in HRMECs leads to reduced cell motility, proliferation after hypoxia, and an attenuated DNA damage response. **A:** Transwell migration of HRMECs is inhibited in a dose-dependent manner by the EYA-PTP inhibitors BZ and BBR. **B:** HRMECs were treated with either vehicle or the EYA inhibitors BZ or BBR and maintained in either hypoxic conditions (1% oxygen) or 21% oxygen for 72 hours. Cell proliferation was measured by an MTT assay. **C:** Representative immunohistochemical staining for  $\gamma$ -H2AX (green), MDC1 (red), and DAPI (blue) in HRMEC maintained at 0.2% O<sub>2</sub> for 12 hours in the presence of either vehicle or the EYA inhibitors BZ and BBR. Quantification of the number of nuclei with  $\gamma$ -H2AX foci in three independent experiments. Data plotted are means  $\pm$  SD (A, B, and C). \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001. BBR, benzobromarone; BZ, benzarzone; CTL, control; HRMEC, human retinal endothelial cell; PTP, protein tyrosine phosphatase.

postnatal retinal vasculature, little EC proliferation is expected. Under hypoxic stress, as is seen in pathological conditions such as retinopathies and tumors, however, ECs are stimulated to proliferate (Figure 6).<sup>6</sup> Conditions of oxidative stress have been linked to DNA damage and repair through various mechanisms: hypoxia can cause low levels of DNA damage subsequent to replicative stress, as well as through direct repression of DNA damage repair genes. Additionally, reactive oxygen species released by the mitochondria of hypoxic cells could cause DNA damage. Survival of ECs would then be contingent on an active DNA damage repair pathway (Figure 7), which in turn has been linked to the ability of the EYA tyrosine phosphatase activity to dephosphorylate the terminal tyrosine of H2AX and thus permit interaction with MDC1 and the assembly of the DNA repair complex.<sup>10,24,30</sup>  $\gamma$ -H2AX foci can be a measure of either DNA double-strand breaks or stabilized, stalled replication forks.

In the context of the OIR model, hypoxia-induced stalled replication forks are thought to signal an ATR-dependent form of DNA damage response that leads to fork stabilization.<sup>5</sup> As a consequence, cell viability is maintained and hypoxia-induced growth factors can promote proliferation and pathological angiogenesis (Figure 7). Such an interaction makes sense in the context of disease or injury in which DNA repair mechanisms need to be up-regulated and angiogenesis needs to be activated. By using both chemical inhibition and genetic methods, we have shown that inhibition of EYA tyrosine phosphatase activity affects the assembly of  $\gamma$ -H2AX foci, recruitment of the DNA damage repair protein MDC1, survival following hypoxic stress, and pathological NV in an animal model of proliferative retinopathy. Although the possibility of off-target effects of small molecules cannot be completely excluded in chemical

biology approaches, the similar outcomes obtained with both the genetic and chemical inhibition methods increases the likelihood that EYA-PTP inhibition plays a role in the reduced NV described here.

The OIR protocol appears to induce DNA damage in multiple cell types in the retina, with a relatively low frequency in ECs. This is in agreement with previous reports suggesting that  $\gamma$ -H2AX staining is observed only in proliferating, PCNA-positive cells of the retinal vasculature in a similar experiment.<sup>6</sup> Interestingly, the time course of formation and resolution of  $\gamma$ -H2AX foci



**Figure 7** Schematic showing the proposed role of the EYA3-PTP in DNA damage repair-linked pathological angiogenesis. Oxidative stress is known to induce DNA damage. DNA damage repair is contingent on the assembly of a multiprotein complex nucleated by  $\gamma$ -H2AX. H2AX is constitutively phosphorylated on its terminal tyrosine.<sup>29</sup> The EYA3-PTP can dephosphorylate this tyrosine<sup>10</sup> and permit interaction with MDC1 (**double-headed arrow**), an essential step in the assembly of DNA damage repair foci (represented here by the Mre11-Rad50-Nbs1 MRN complex). Repair of DNA damage permits endothelial cell survival and angiogenesis.  $\gamma$ -H2AX, Ser139-phosphorylated form of the DNA damage sensor H2AX; PTP, protein tyrosine phosphatase.

in the OIR protocol parallels that of NV, reaching a maximum 5 days after return to room air and then declining as NV is resolved. Furthermore, continuous angiogenic factor stimulation has been associated with replication stress and  $\gamma$ -H2AX formation independent of hypoxia.

Although either EYA tyrosine phosphatase inhibition or deletion of *Eya3* in ECs significantly reduced the formation of neovascular tufts, we observed little effect on revascularization into the vaso-obiterated central zone. This is reminiscent of the previously reported effect of *Atm* deletion<sup>5</sup> and unlike the suppression of both NV and revascularization seen upon blocking either VEGF or VEGFR2.<sup>31</sup> Although ATM appears to play a role in angiogenesis through oxidative defense and independent of the canonical DDR pathway,<sup>5</sup> our data support a role for the assembly of the DDR-associated  $\gamma$ -H2AX–MDC1 complex in pathological neoangiogenesis.

The EYA proteins are multidomain and have several separable biochemical and cellular functions.<sup>21</sup> The tyrosine phosphatase activity examined here is localized to the conserved C-terminal domain of the proteins.<sup>32</sup> Independent of this PTP domain, the N-terminal domain has trans-activation and threonine phosphatase activities.<sup>33,34</sup> The tyrosine phosphatase activity not only contributes to EYA's role in DDR but also promotes motility of tumor and ECs.<sup>11,12,28,35</sup> Attenuation of EC proliferation by EYA PTP inhibitors under hypoxia demonstrates that this catalytic activity contributes to cell proliferation in DNA damage–inducing conditions. It remains possible that the trans-activation activity of the EYAs also plays a role. Indeed, published *in vitro* studies suggest that the PTP and trans-activation activities of EYA are linked in some contexts; the tyrosine phosphatase activity of EYA contributes to transcriptional activation of a SIX1-EYA complex and cell proliferation.<sup>36</sup>

## Conclusions

EYA contributes to angiogenesis by multiple mechanisms. They promote angiogenic sprouting and tube formation, possibly as a result of their role in promoting EC migration. Herein, we show that as participants in the DNA damage response pathway, EYAs also promote survival, proliferation, and NV under hypoxic conditions. Together, these results validate the EYA tyrosine phosphatase as a target for the development of anti-angiogenic agents and suggest the BBR family of compounds as leads for the development of potent and effective antiangiogenics. EYA-targeted therapeutics could be synergistic with VEGF blockade, as well as with genotoxic regimens in cancer treatment. EYA inhibition offers a specific mechanism to inhibit pathological angiogenesis while permitting physiological revascularization. This distinction from VEGF inhibitors may prove to be of significant clinical benefit.

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Y.W., E.T., and R.N.P. performed the experiments; J.A.S. and R.A.L. designed the allele for conditional deletion of *Eya3*; L.E.H.S. helped with quantitation of NV and interpretation of OIR results; Y.W., R.N.P., J.A.S., L.E.H.S., and R.A.L. edited the manuscript; and R.S.H. designed the experiments, supervised the research, and wrote the manuscript.

## Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.10.031>.

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