Proliferative and Survival Effects of PUMA Promote Angiogenesis

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SUMMARY

The p53 upregulated modulator of apoptosis (PUMA) is known as an essential apoptosis inducer. Here, we report the seemingly paradoxical finding that PUMA is a proangiogenic factor critically required for the proliferation and survival of vascular and microglia cells. Strikingly, *Puma* deficiency by genetic deletion or small hairpin RNA knockdown inhibited developmental and pathological angiogenesis and reduced microglia numbers in vivo, whereas *Puma* gene delivery increased angiogenesis and cell survival. Mechanistically, we revealed that PUMA plays a critical role in regulating autophagy by modulating Erk activation and intracellular calcium level. Our findings revealed an unexpected function of PUMA in promoting angiogenesis and warrant more careful investigations into the therapeutic potential of PUMA in treating cancer and degenerative diseases.

INTRODUCTION

The p53 upregulated modulator of apoptosis (PUMA) is a member of the BH3-only protein family and has been known as an essential apoptosis inducer for many years (Chipuk et al., 2005; Vousden, 2005; Yu and Zhang, 2003). The apoptosis-inducing activity of PUMA has been shown in different cell types, such as mouse embryonic fibroblasts, developing neurons, lymphocytes, tumor cells, and intestinal progenitor/stem cells (Michalak et al., 2010; Villunger et al., 2003). Due to its potent apoptotic activity, PUMA has received considerable research interest and has been considered as an important therapeutic candidate gene for the prevention and treatment of cancer and degenerative diseases (Ito et al., 2005; Yu, 2009).

Despite many years of intensive studies on PUMA, outstanding questions remain. Numerous studies have shown that enhanced PUMA expression is not always linked to apoptosis. Interaction of human umbilical vein endothelial cells (HUVECs) with monocytes increased PUMA protein level in the former (Gawad et al., 2009), yet the elevated PUMA protein level did not result in apoptosis of the cells (Gawad et al., 2009). In brain ischemia, PUMA expression was markedly upregulated (Kuroki et al., 2009). However, apoptosis was not increased, and the brain stroke volume was unchanged in the *Puma*-deficient mice (Kuroki et al., 2009). The biological consequences of such increased PUMA expression remain thus far unclear, indicating potentially unknown functions of PUMA other than induction of apoptosis. In addition, studies have shown that the proapoptotic action of PUMA may be cell specific. For example, although *Puma* deficiency reduced apoptosis in intestinal progenitor/ stem cells, it did not affect apoptosis of endothelial cells (Qiu et al., 2008). Moreover, PUMA is highly expressed in vascular and microglia cells (Tables S1 and S2), yet it has not been shown to induce apoptosis in these cells thus far. Instead, little is known about the function of PUMA in vascular and microglia cells. Of note, PUMA was recently reported to be required for tumor development, whereas Noxa, another proapoptotic gene of the BH3-only protein family, inhibited tumorigenesis (Labi et al., 2010; Michalak et al., 2010; Qiu et al., 2011), demonstrating a distinct proliferative effect of PUMA compared with the other

proapoptotic genes. Together, accumulating data have indicated a potential unknown function of PUMA not related to apoptosis, at least in certain types of cells.

Microglia are resident macrophages of the CNS. Although the immune function of microglia has been widely studied, their role in angiogenesis is not well understood. Some studies reported an angiogenic effect of microglia (Checchin et al., 2006; Liu et al., 2005; Yamaguchi et al., 2011), whereas others reported an antiangiogenic effect (Stefater Iii et al., 2011). Interestingly, in the developing mouse retina, the microglia density increases during the first postnatal week and decreases gradually (Santos et al., 2008). The appearance of retinal microglia precedes and overlaps with the formation of retinal blood vessels, indicating a potential role of microglia in retinal angiogenesis. Further studies are needed to better understand the function of microglia in blood vessel growth.

In the present study, we investigated the effect of PUMA on vascular and microglia cells. We provide evidence that PUMA is an angiogenic factor critically required for the proliferation and survival of vascular and microglia cells by modulating autophagy. At the molecular level, we revealed that PUMA plays a crucial role in regulating Erk activation and intracellular calcium level. Our data revealed an unexpected function of PUMA as an angiogenic factor by promoting survival and proliferation, which contradicts its known role as an essential apoptosis inducer.

RESULTS

PUMA Is Expressed by Retinal Microglia and Blood Vessels

Although PUMA has been intensively studied for many years, its role in the retina remains unexplored. Therefore, we investigated the retinal expression pattern of PUMA. Real-time PCR displayed a postnatal increase in PUMA expression and a subsequent decrease after postnatal day 18 (P18) (Figure 1A). This was confirmed at the protein level by western blot (Figure 1B). Immunofluorescence staining detected abundant PUMA expression in blood vessels (Figure 1C) and CD11b^{+} microglia in the developing mouse retinae (Figure 1D). PUMA expression was also identified in primary retinal microglia isolated from neonatal mice (Figure 1D). Notably, PUMA expression levels in the developing mouse retinae coincided with the time window of retinal blood vessel development, which occurs mainly within the first postnatal 2 weeks. Moreover, the retinal PUMA expression levels paralleled the time window of the emergence of retinal microglia cells, the number of which increases in the retina during the first postnatal week and decreases gradually thereafter (Santos et al., 2008). In neonatal mice, the retinal microglia cells are mainly present in the retinal ganglion cell (RGC) layer and the inner nuclear layer (INL) (Santos et al., 2008), where PUMA expression is abundant (Figure 1C). Thus, even though the changes in retinal PUMA expression (Figures 1A and 1B) may reflect changes in PUMA expression in retinal neurons, the spatiotemporal correlation of the expression level and distribution of PUMA with retinal blood vessel formation and the appearance and location of retinal microglia cells suggest a potential effect of PUMA on retinal microglia and blood vessels.

Puma Deficiency Decreased Microglia and Blood Vessel Density in the Retina

We subsequently investigated the role of PUMA in the retina using *Puma*-deficient mice. Because PUMA is known as an essential apoptosis inducer (Chipuk et al., 2005; Vousden, 2005; Yu and Zhang, 2003), we hypothesized that *Puma* deficiency might lead to decrease apoptosis in the retina. Surprisingly, we found a marked decrease of retinal microglia numbers in the *Puma*-deficient mice (Figures 1E, 1F, S1A, and S1B). Importantly, loss of one *Puma* allele, i.e., loss of half of its normal expression level (Figure S1C), decreased retinal microglia density at different time points (Figures S1D–S1F). *Puma* deficiency also decreased retinal blood vessel branch points, vessel areas, and diameter (Figures 1F, S1G, and S1H).

To further verify these findings, we intercrossed *Puma*-deficient mice with Cx3cr1[GFP/+] transgenic mice, in which microglia are GFP labeled (GFP expression under the control of a Cx3cr1 promoter) with a normal density and morphology (Davalos et al., 2005). We found that the retinal microglia and blood vessel densities decreased in the *Puma*-deficient Cx3cr1[GFP/+] mice at different time points (Figures 1G and S1I). The decreased retinal blood vessel branch points, vessel areas, and diameters were also observed in the retinae of 6-month-old *Puma*-deficient mice (Figure 1H). We next investigated microglia and blood vessel density in the brains of *Puma*-deficient mice to verify whether the effect of PUMA was retina specific. We found reduced microglia (Figure S1J) and blood vessel density in the brains of *Puma*-deficient mice as well (Figure S1K).

Puma Knockdown by Small Hairpin RNA Reduced Microglia and Blood Vessel Density in the Retina

The observation of decreased microglia and blood vessel density in the *Puma*-deficient retina and brain was unexpected, given the known apoptotic activity of PUMA (Chipuk et al., 2005; Vousden, 2005; Yu and Zhang, 2003). To substantiate our findings, we performed a loss-of-function assay using *Puma* small hairpin RNA (shRNA). Intravitreal injection of mouse *Puma* shRNA at P1 decreased PUMA expression level by about 60% in the retinae (Figures S2A and S2B) and decreased retinal microglia number, blood vessel branch points, and vessel areas at P6 (Figure 2A).

Puma Deficiency Did Not Affect Retinal Astrocytes or Decrease Retinal Thickness

Because retinal astrocytes are known to contribute to retinal angiogenesis, we investigated whether *Puma* deficiency affected the retinal astrocyte network and found no significant difference in astrocyte area or morphology between wild-type and *Puma*-deficient retinae (Figure S2C). We further assessed vascular endothelial growth factor (VEGF) expression levels in *Puma*-deficient and wild-type retinal astrocytes and found a slightly higher VEGF protein level in *Puma*-deficient astrocytes (Figure S2D). Similarly, real-time PCR and western blot showed that VEGF expression level was somewhat higher in the *Puma*-deficient retinae (Figures S2E and S2F). Thus, the vascular defects in the *Puma*-deficient retinae were unlikely due to a potential astrocyte or VEGF defect.
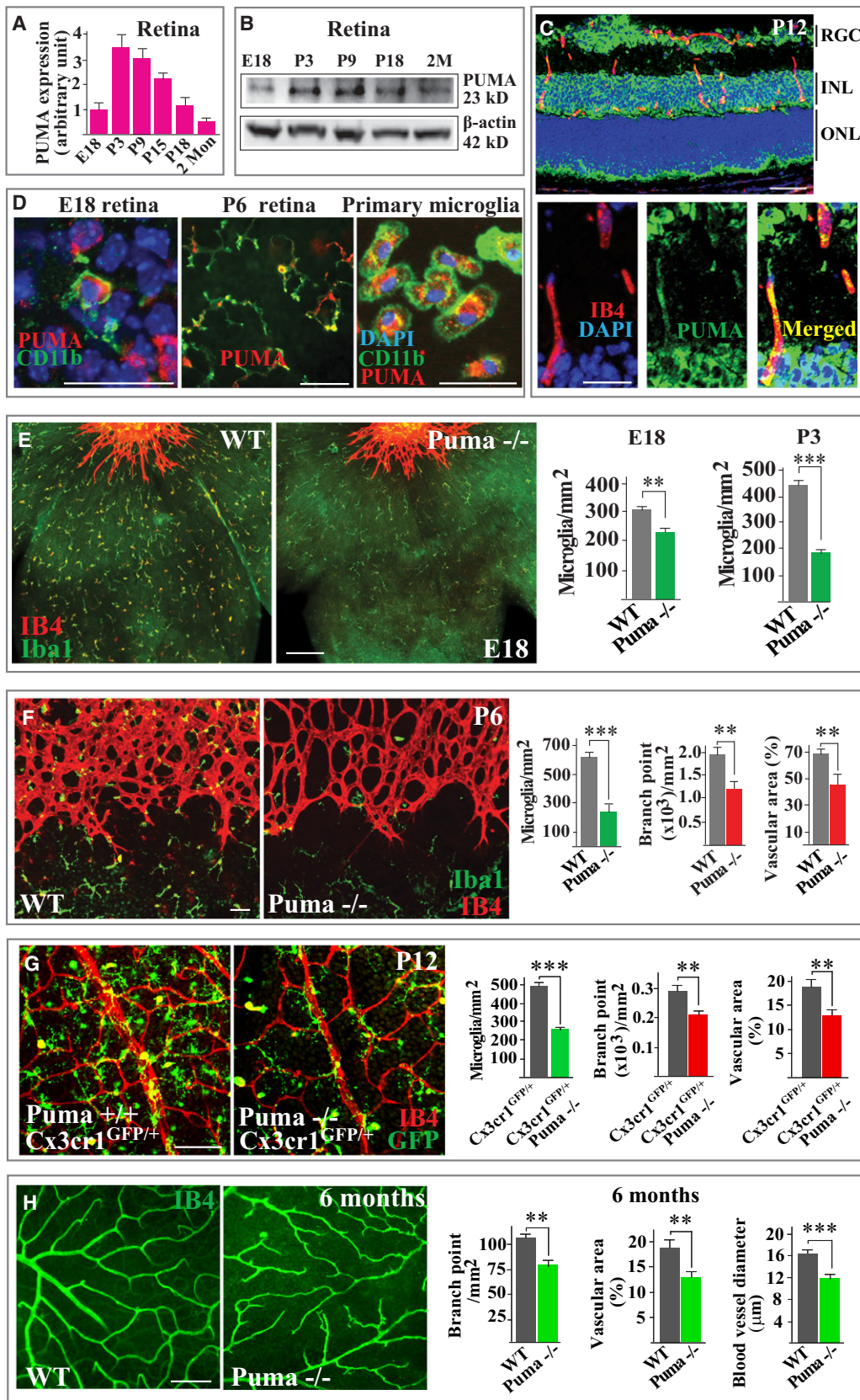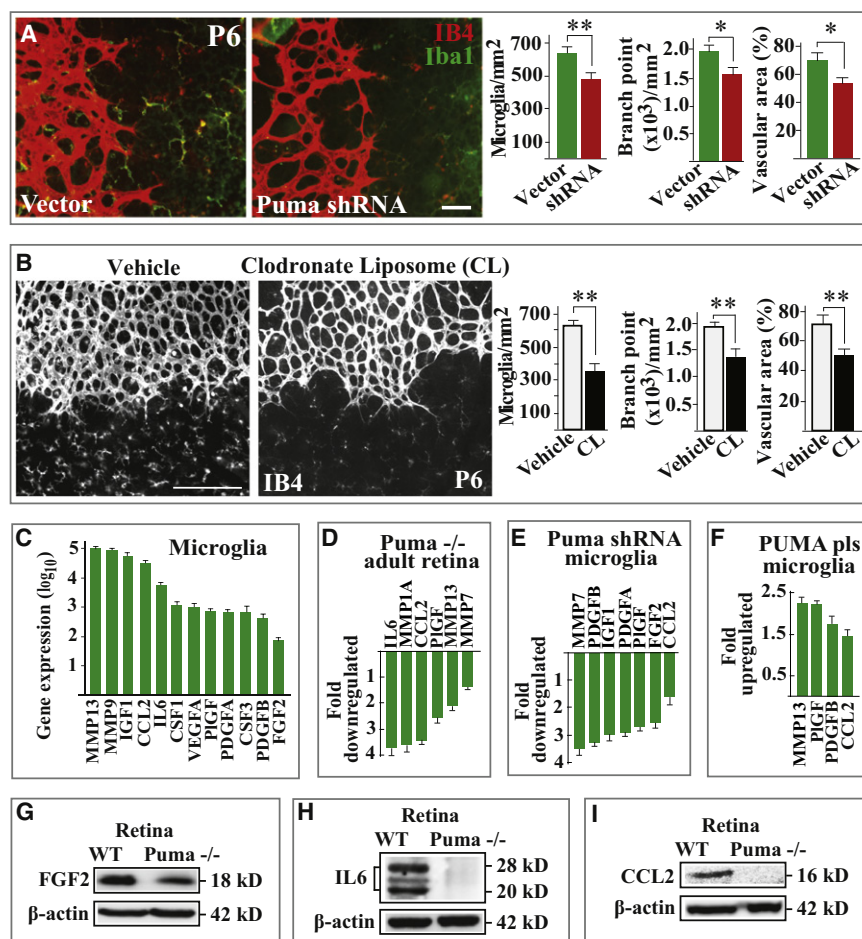
Figure 2. *Puma* Knockdown by shRNA Reduced Microglia Number and Blood Vessel Density, and PUMA Is Required for the Expression of Important Angiogenic Genes

(A) Immunofluorescence staining showed that intravitreal injection of mouse *Puma* shRNA at P1 reduced retinal microglia (Ib1+, green) number, blood vessel (IB4+, red) branch points, and vessel areas at P6 (n = 8, p < 0.01 or 0.05).

(B) Depletion of retinal microglia by intravitreal and intraperitoneal injection of clodronate liposome decreased retinal blood vessel (IB4+, white) branch points and vessel areas (n = 8, p < 0.01).

(C) Real-time PCR detected the expression of numerous important angiogenic genes in the isolated primary retinal microglia.

(D and E) Real-time PCR showed that *Puma* deficiency by shRNA knockdown (D) or genetic deletion (E) inhibited the expression of numerous angiogenic genes in isolated primary retinal microglia in vitro and in the retina in vivo.

(F) Real-time PCR showed that PUMA overexpression by gene delivery upregulated the expression of many angiogenic genes in microglia cells.

(G–I) Western blot showed that *Puma* deficiency by genetic deletion reduced the protein levels of several important angiogenic factors in mouse retinae, such as FGF2, IL6, and CCL2.

Scale bars represent 50 μm (A) or 200 μm (B). Data are represented as mean ± SEM; *p < 0.05, **p < 0.01. See also Figure S2 and Table S3.

retina were increased (Figures S2H–S2J). There was no significant difference in the thickness of the other retinal layers in *Puma*-deficient mice (data not shown). Moreover, PUMA overexpression in the retinal ganglion cell line (RGC5) markedly induced apoptosis (Figures S2K–S2M), consistent with the in vivo observation that *Puma* deficiency increased thickness of the retinal ganglion cell layer (Figures S2H–S2J). In summary, *Puma* deficiency did not lead to retinal neuronal loss.

To address whether the effect of PUMA on retinal microglia and blood vessels was a common event of the BH3-only protein family genes, we utilized *Noxa*-deficient mice. Like PUMA, Noxa

Loss of retinal neurons can subsequently lead to loss of retinal blood vessels (Zheng et al., 2007). We next investigated whether *Puma* deficiency resulted in retinal neuronal loss, which could in turn explain the angiogenesis defect. We measured the thickness of the retinal layers where the retinal blood vessels exist, i.e., the RGC layer, the inner plexiform layer (IPL), and the INL. We found no significant difference in the thickness of the retinal layers before birth at E18 (Figure S2G). After birth, in *Puma*-deficient mice, the thicknesses of the RGC, IPL, INL, and the whole

Figure 1. *Puma* Deficiency Decreased Microglia Number and Blood Vessel Density in the Retina

(A) Real-time PCR showed that PUMA expression level in the developing mouse retinae increased after birth and decreased after postnatal day 18 (P18).

(B) Western blot showed at a protein level that PUMA expression in the developing mouse retinae increased after birth and decreased after P18.

(C) Immunofluorescence staining detected PUMA expression (green) in the retinal blood vessels (red), RGC layer, INL, and the outer part of the outer nuclear layer (ONL).

(D) Immunofluorescence staining detected PUMA expression (red) in CD11b+ (green) resident and isolated primary retinal microglia in the developing mouse retinae. DAPI, diamidino-2-phenylindole fluorescent staining of nuclei.

(E) Immunofluorescence staining showed decreased Iba1+ (green) retina microglia density at embryonic day 18 (E18) and P3 (n = 7 or 8, p < 0.001 or 0.01).

(F) Immunofluorescence staining showed decreased Iba1+ (green) retina microglia density, blood vessel (IB4+, red) branch points, and vessel areas at P6 (n = 7 or 8, p < 0.001 or 0.01).

(G) In the *Puma*-deficient *Cx3cr1*GFP/+ mice with GFP-labeled microglia, retinal microglia (green) density, blood vessel (IB4+, red) branch points, and areas were decreased (n = 10, p < 0.001 or 0.01).

(H) In 6-month-old *Puma*-deficient mice, retinal blood vessel (IB4+, green) branch points, vessel areas, and diameters were decreased (n = 7, p < 0.001 or 0.01). Scale bars represent 50 μm (C, upper; D, F, G); 20 μm (C, lower); 200 μm (E); or 100 μm (H). Data are represented as mean ± SEM; **p < 0.01, ***p < 0.001. See also Figure S1 and Tables S1, S2, and S3.

is also a p53-upregulated proapoptotic gene of the BH3-only protein family. However, no significant microglia or vascular defects were observed in *Noxa*-deficient mice (Figure S2N), demonstrating a functional uniqueness of PUMA.

Retina Microglia Cells Are Required for Retinal Angiogenesis

Microglia cells are present in the mouse retina as early as at embryonic day 11.5 (E11.5) (Santos et al., 2008), before the mouse retinal vasculature starts to develop at postnatal day 1 (P1). Because the observed retinal microglia cell defect precedes the retinal blood vessel defect in the *Puma*-deficient mice, we hypothesized that microglia loss in the *Puma*-deficient retinae might relate to the vascular defect. To test this, we depleted the mouse retinal microglia at P1 (a time point when retinal angiogenesis just begins) by intravitreal and intraperitoneal injection of clodronate liposomes, which reduced retinal microglia numbers by about 50% (Figure 2B). Microglia depletion decreased retinal blood vessel branch points and vessel areas (Figure 2B), demonstrating that retinal microglia are required for retinal angiogenesis. Consistently, real-time PCR showed that isolated primary retinal microglia expressed numerous angiogenic factors (Figure 2C). Importantly, *Puma* deficiency by genetic deletion or shRNA knockdown markedly decreased the expression of many proangiogenic genes in primary retinal microglia in vitro and in the retina in vivo (Figures 2D and 2E). Vice versa, overexpression of PUMA upregulated the expression of some angiogenic genes (Figure 2F). Western blot confirmed the downregulation of some important angiogenic factors, such as fibroblast growth factor 2 (FGF2), IL6, and CCL2, in *Puma*-deficient retinae (Figures 2G–2I). Together, these data demonstrate that retinal microglia contribute to retinal angiogenesis by producing important angiogenic factors.

Impaired Angiogenic Response in *Puma*-Deficient Aortic Ring and Cornea

We subsequently used an aortic ring and a cornea pocket assay to investigate whether *Puma* deficiency affected angiogenesis in other tissues. In the aortic ring assay, *Puma* deficiency nearly completely abolished VEGF- and FGF2-induced angiogenesis (Figures 3A and 3B). Immunofluorescence staining revealed less CD11b+ cells in *Puma*-deficient aortic rings (Figure 3C). In the cornea pocket assay, *Puma* deficiency impaired VEGF-induced angiogenesis (Figures 3D and S3A–S3C). Immunofluorescence staining revealed fewer CD11b+ cells in *Puma*-deficient corneas (Figure 3E). Thus, *Puma* deficiency impaired angiogenesis in other tissues than the retina.

Puma Deficiency Inhibited Choroidal Neovascularization

We next tested whether *Puma* deficiency affected choroidal neovascularization (CNV) using *Puma*-deficient mice and *Puma* shRNA in a laser-induced CNV model. *Puma* deficiency decreased CNV area by more than 50% (Figure 3F). Moreover, intravitreous injection of *Puma* shRNA, which decreased PUMA expression by about 60% in the retinae and choroids (Figure S3D), inhibited CNV formation (Figure 3G) and decreased the number of CNV-associated Iba1+ microglia (Figure 3G). In-

deed, real-time PCR displayed decreased expression of different microglia markers, including Iba1 (Figure 3H), CD11b, and CD68 (Figure S3E) in the *Puma* shRNA-treated choroids and retinae. Consistent with the reduced CNV areas by *Puma* shRNA treatment, the expression of many important angiogenic genes markedly decreased in the *Puma* shRNA-treated choroids and retinae (Figure S3F).

PUMA Is Required for Vascular and Microglia Cell Proliferation and Survival

To investigate the cellular mechanisms underlying the effect of PUMA, we explored whether PUMA affected vascular and microglia cell proliferation and survival. In cultured primary retinal microglia, brain endothelial cells (ECs), and aortic artery smooth muscle cells (SMCs), *Puma* deficiency decreased cell proliferation and survival under different conditions (Figures 4A–4C). Consistently, *Puma* knockdown by shRNA, which decreased PUMA expression in primary microglia by about 40% (Figures S4A and S4B), decreased viability of primary retinal and brain microglia (Figures 4D and S4C). On the other hand, PUMA overexpression increased survival of primary retinal microglia cells (Figures 4E, 4F, and S4D) and proliferation of rat retinal vascular endothelial cells (TR-iBRB) and HUVECs (Figures 4G, 4H, S4E, and S4F). PUMA overexpression did not induce, but instead inhibited, apoptosis in the rat retinal vascular endothelial cells (TR-iBRB, Figures S4G and S4H).

We next engaged several more direct methods than the MTT assay to further verify the proliferative and survival effect of PUMA, such as the BrdU assay, the trypan blue exclusion assay, and the LIVE/DEAD viability/cytotoxicity assay. Using the BrdU assay, we found that overexpression of PUMA increased cell proliferation in the primary microglia cells (Figures 4I and S4I), TR-iBRB, and HUVECs at different time points (Figures 4J, 4K, S4E, and S4F), whereas PUMA deficiency by shRNA knockdown inhibited proliferation of primary microglia (Figures 4L and S4J). To investigate the survival effect of PUMA, we treated the cells with mitomycin C to suppress cell proliferation, and performed a trypan blue exclusion assay and a LIVE/DEAD viability/cytotoxicity assay. Using these two methods, we found that overexpression of PUMA increased survival of primary microglia (Figures 4M and S4I), whereas *Puma* shRNA treatment decreased their survival (Figures 4N, 4O, and S4J). Thus, apart from the MTT assay, several additional assays demonstrated a proliferative and survival effect of PUMA on microglia and vascular cells. Furthermore, *Puma* deficiency impaired retinal microglia migration induced by ATP, CCL2, and CCL3 (Figure 5A).

Puma Deficiency Impairs Erk Activation and M-CSFR Expression

We subsequently investigated the molecular mechanisms underlying the actions of PUMA. *Puma* deficiency markedly attenuated Erk activation under different conditions in primary brain vascular endothelial (Figure 5B) and retinal microglia cells (Figure 5C). Consistently, *Puma* knockdown by shRNA inhibited Erk activation in primary microglia (Figures 5D, S4A, and S4B). In addition, the expression level of macrophage colony stimulating factor receptor (M-CSFR), a factor mainly expressed by microglia/macrophages and critical for angiogenesis (Kubota et al.,
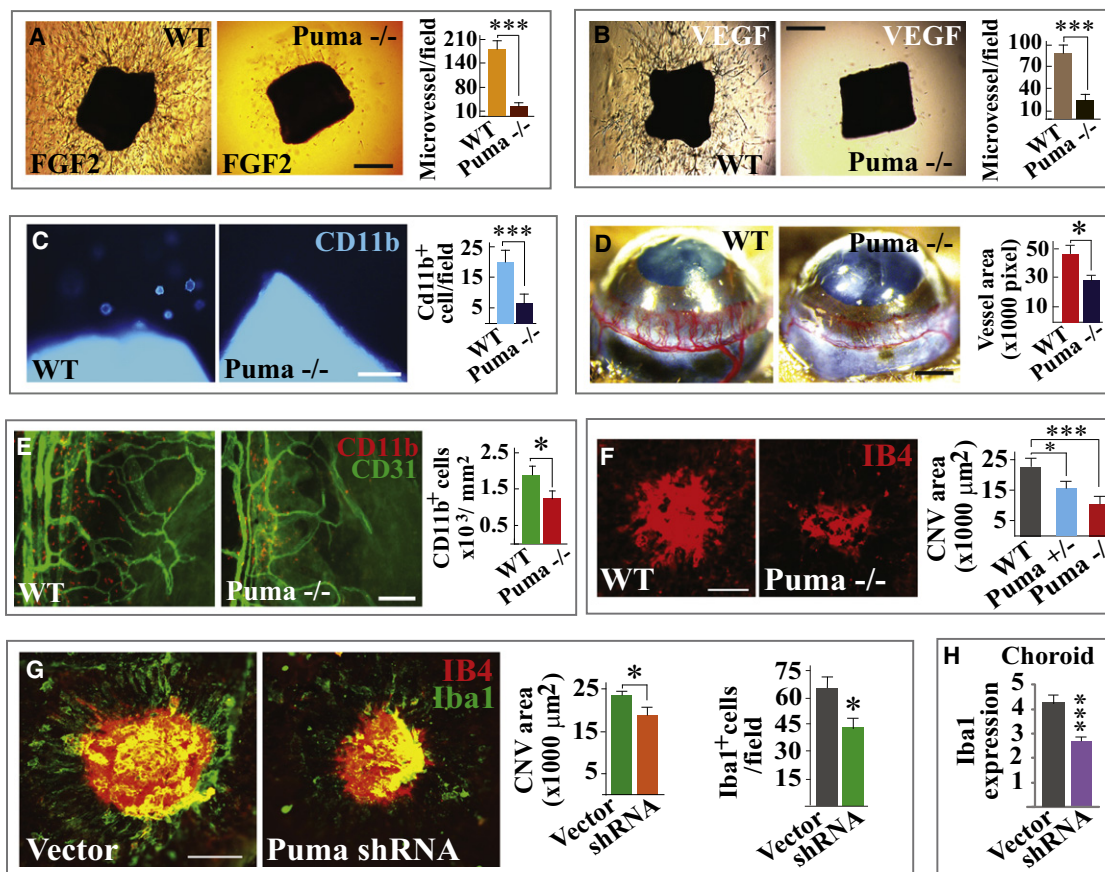
Figure 3. Impaired Angiogenesis in *Puma*-Deficient Aorta, Cornea and Choroidal Neovascularization

(A and B) *Puma* deficiency by genetic deletion nearly completely abolished VEGF- and FGF2-induced angiogenesis in an aortic ring assay (n = 5–7, p < 0.001).

(C) Immunofluorescence staining showed less CD11b^{+} cells in the *Puma*-deficient aortic rings (n = 4, p < 0.001).

(D) *Puma* deficiency by genetic deletion impaired VEGF-induced angiogenesis in a cornea pocket assay (n = 8, p < 0.05).

(E) Immunofluorescence staining showed fewer CD11b^{+} cells in the *Puma*-deficient corneas (n = 8 or 6, p < 0.05).

(F) IB4 immunofluorescence staining (red) showed that *Puma* deficiency decreased CNV area by more than 50% in a laser-induced CNV model (n = 8, p < 0.001).

(G) IB4 immunofluorescence staining (red) showed that *Puma* shRNA treatment inhibited CNV formation (n = 9, p < 0.05) and decreased the number of Iba1^{+} CNV-associated microglia (n = 8, p < 0.05).

(H) Real-time PCR revealed decreased expression of the microglia marker Iba1 in *Puma* shRNA-treated choroids (n = 6, p < 0.001).

Scale bars represent 200 μm (A, B), 100 μm (C, E–G), or 500 μm (D). Data are represented as mean ± SEM; *p < 0.05, ***p < 0.001. See also Figure S3 and Table S3.

2009), considerably decreased in *Puma*-deficient microglia and retinae (Figures 5E and 5F). This observation is consistent with the finding of less microglia in *Puma*-deficient retinae. Moreover, the expression levels of M-CSFR in cultured primary microglia in vitro and in mouse retinae in vivo decreased after *Puma* shRNA treatment (Figures 5G, 5H, S2A, and S2B). Importantly, *Puma* gene delivery by in vivo transfection of PUMA-expressing DNA plasmids into P1 *Puma*-deficient mouse retinae increased retinal microglia numbers (Figures 5I, 5J, and S5A–S5C), retinal vascular branch points and vascular area (Figures 5K and 5L), and restored M-CSFR expression and Erk activation at P6 (Figure 5M). Thus, PUMA is critically required for Erk activation and M-CSFR expression, both of which are important for angiogenesis and microglia proliferation, survival, and migration (Liva

et al., 1999; Otero et al., 2009; Reddy et al., 2009; Sasaki et al., 2000).

PUMA Is Required for Vascular and Microglia Cell Autophagy

Our finding that PUMA is required for vascular and microglia cell proliferation and survival was unexpected, as it contradicted the common belief that PUMA is an essential apoptosis inducer. To understand this paradox, we investigated whether PUMA affected autophagy, a critical regulatory process of cell survival and proliferation, under conditions of metabolic stress (Rabinowitz and White, 2010). The intensity of the LC3II band (16 kDa) was used as a marker for autophagy using western blots. *Puma* deficiency markedly impaired autophagy under various
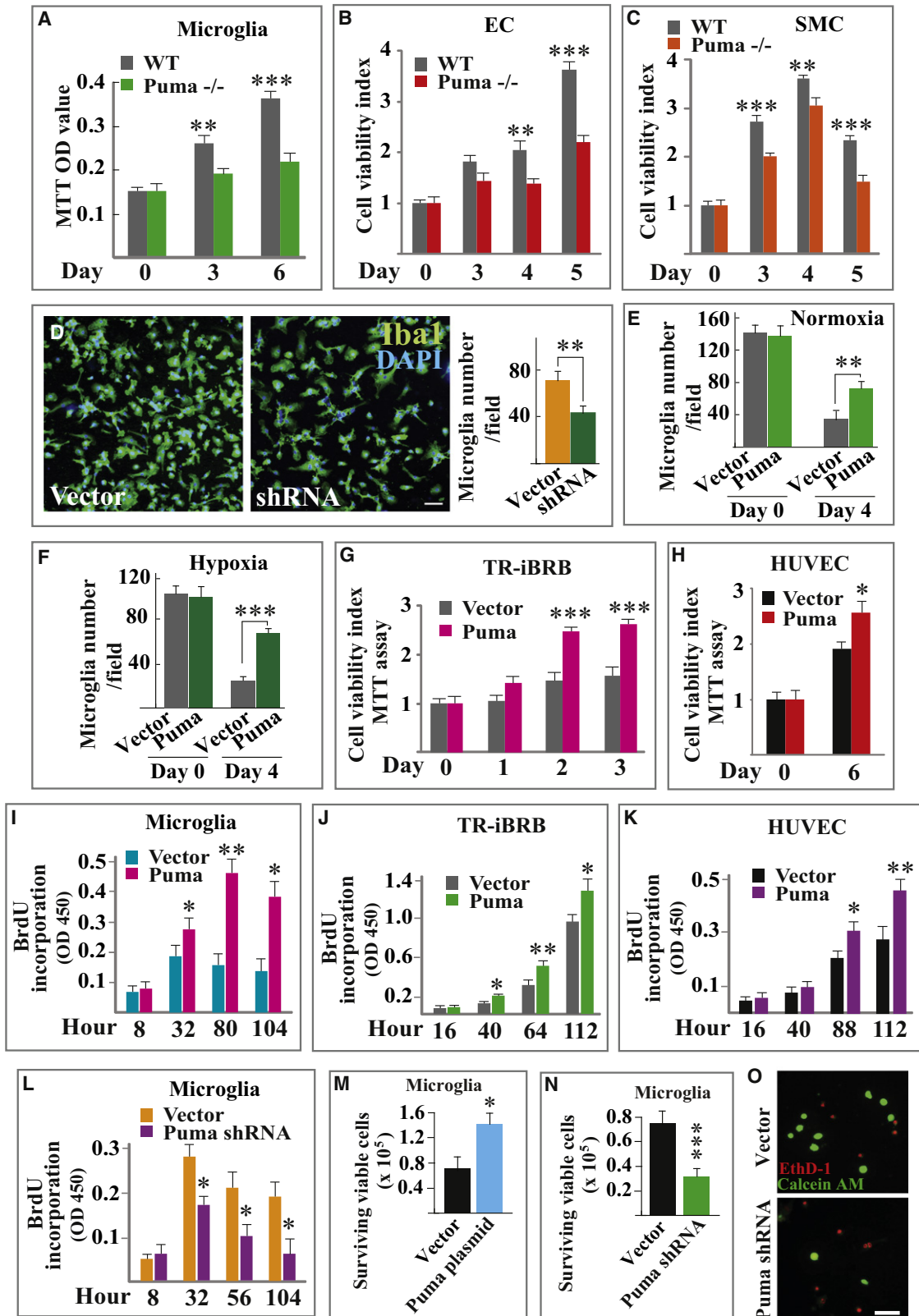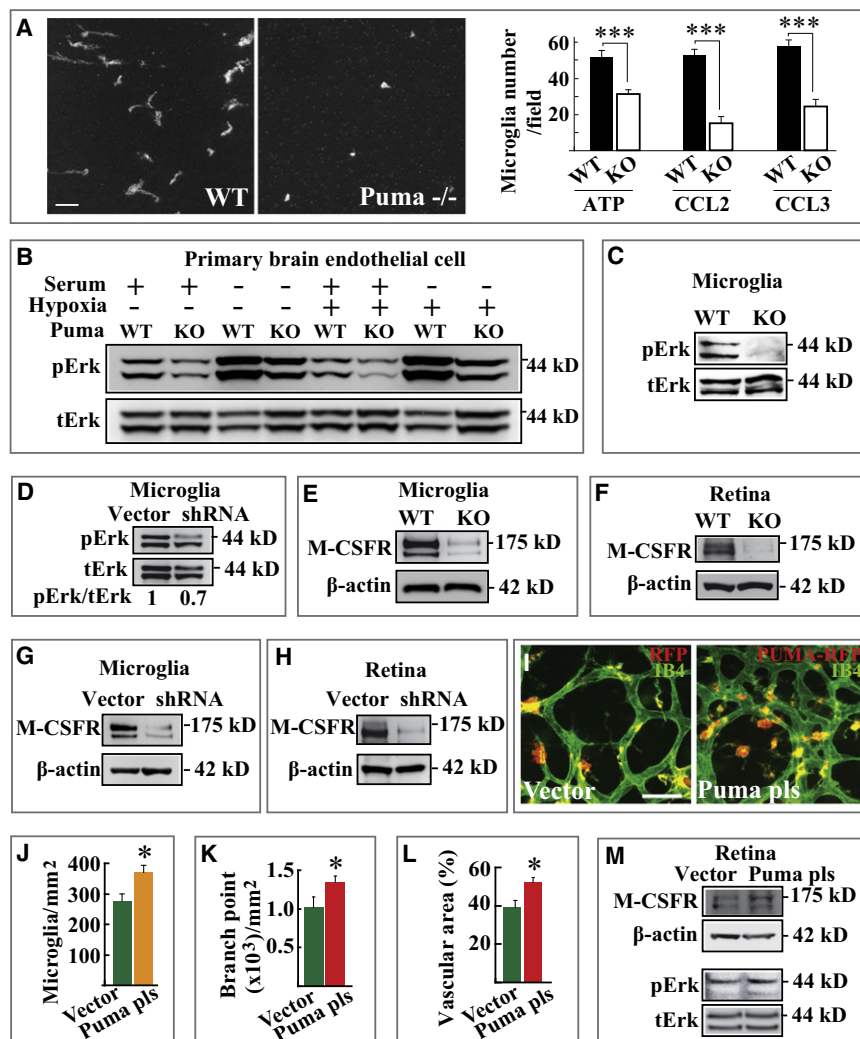
Figure 5. PUMA Is Required for Erk Activation and M-CSFR Expression

(A) *Puma* deficiency markedly decreased retinal microglia migration induced by ATP, CCL2 and CCL3 (n = 7–10, p < 0.001).

(B and C) *Puma* deficiency markedly attenuated Erk activation under different conditions in primary brain vascular endothelial (B) and retinal microglia cells (C).

(D) *Puma* knockdown by shRNA inhibited Erk activation in primary microglia cells.

(E and F) The expression level of macrophage colony stimulating factor receptor (M-CSFR) markedly decreased in the *Puma*-deficient (KO) microglia and retinae (n = 6).

(G and H) *Puma* knockdown by shRNA decreased M-CSFR expression in cultured primary microglia in vitro and in mouse retina in vivo.

(I–L) *Puma* gene delivery by in vivo transfection of a PUMA-expressing DNA plasmid (PUMA pls) into P1 *Puma*-deficient mouse retinae increased retinal microglia number (I, J n = 6, p < 0.05), retinal blood vessel branch points (K, n = 7 or 8, p < 0.05) and vascular areas (L, n = 7 or 8, p < 0.05).

(M) *Puma* gene delivery by in vivo transfection of a PUMA-expressing DNA plasmid (PUMA pls) into P1 *Puma*-deficient mouse retinae increased M-CSFR expression and Erk activation at P6.

Scale bars in A and H represent 50 μm. Data are represented as mean ± SEM; *p < 0.05. See also Figure S5.

16 kDa LC3II band (Figures 6A–6C). Consistently, *Puma* shRNA treatment markedly inhibited autophagy in the RAW264.7 macrophage cells (Figures 6D and S6A), whereas overexpression of PUMA increased autophagy in RAW264.7 cells and primary retinal microglia (Figures 6E, 6F, S6B, and S6C). Moreover, immunofluorescence staining showed that overexpression of PUMA in primary retinal microglia increased autophagy as demonstrated by the increased LC3+ dots

conditions in primary retinal microglia (Figure 6A), brain vascular ECs (Figure 6B) and aortic artery SMCs (Figure 6C), as demonstrated by the disappearance or reduced intensity of the

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Figure 4. PUMA Is Required for Microglia and Vascular Cell Proliferation, Survival, and Migration

(A–C) MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cell proliferation/survival assay showed that *Puma* deficiency decreased cell proliferation and survival in cultured primary retinal microglia (A) and brain endothelial cells (B) in hypoxia and aortic artery smooth muscle cells in normoxia (C) (n = 4, p < 0.001 or 0.01). In (B) and (C), fold change is presented using values of day 0 as 1.

(D) *Puma* knockdown by shRNA decreased the viability of Iba1+ primary retinal microglia cells in hypoxia (n = 8, p < 0.01). DAPI: diamidino-2-phenylindole fluorescent staining of nuclei.

(E and F) *Puma* gene delivery increased the survival of primary retinal microglia cells in normoxia (E, n = 6, p < 0.01) and with a greater effect in hypoxia (F, n = 6, p < 0.001).

(G and H) *Puma* gene delivery increased proliferation/survival of rat retinal vascular endothelial cells (G, TR-iBRB, n = 4, p < 0.001) and HUVECs (H, n = 4, p < 0.05).

(I–K) BrdU assay showed that overexpression of PUMA increased cell proliferation of primary microglia cells (I, n = 3, p < 0.05 or 0.01), retina-derived endothelial cells (TR-iBRB, J, n = 3, p < 0.05 or 0.01), and HUVECs (K, n = 3, p < 0.05 or 0.01).

(L) BrdU assay showed that PUMA deficiency by shRNA knockdown inhibited the proliferation of primary microglia cells (n = 3, p < 0.05).

(M) Trypan blue exclusion assay showed that overexpression of PUMA increased the survival of primary microglia cultured in serum-free medium and treated with mitomycin C to suppress cell proliferation (n = 3, p < 0.05).

(N and O) A LIVE/DEAD viability/cytotoxicity assay that selectively labels viable cell bodies with the calcein AM green fluorescence and dead cell nucleus with the EthD-1 red fluorescence showed that PUMA deficiency by shRNA knockdown decreased the survival of primary microglia cells cultured in serum-free medium and treated with mitomycin C to suppress cell proliferation (n = 3, p < 0.001).

Scale bars in (D) and (O) represent 50 μm. Data are represented as mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S4.
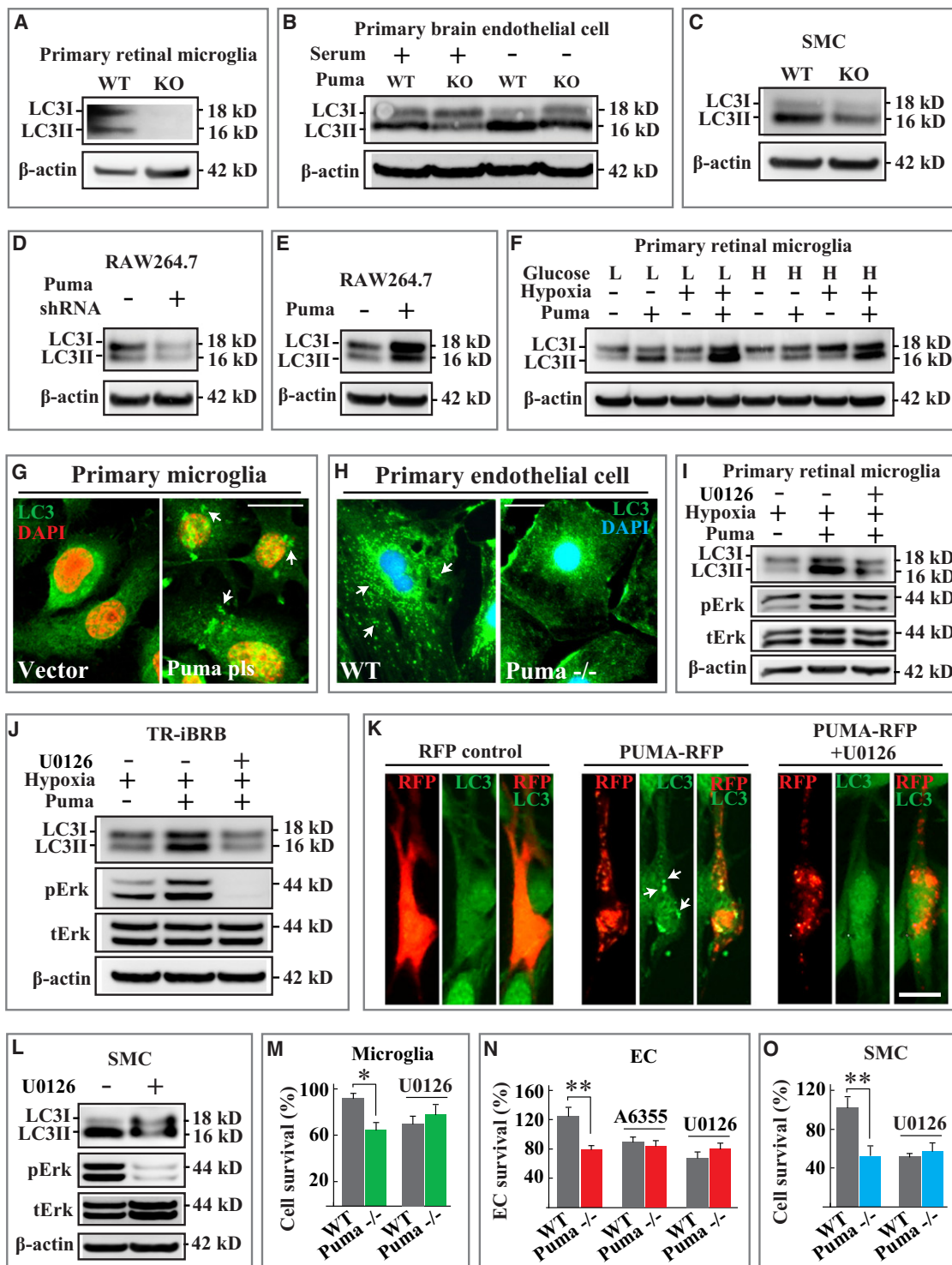
Figure 6. PUMA Is Required for Microglia and Vascular Cell Autophagy

(A–C) Western blot showed that *Puma* deficiency (KO) markedly impaired autophagy under different conditions in primary retinal microglia (A), brain vascular endothelial cells (B), and aortic artery smooth muscle cells (SMC, C), as demonstrated by the disappearance or decreased intensity of the 16 kDa LC3II band.

(D) Western blot showed that *Puma* knockdown by shRNA markedly inhibited autophagy in the RAW264.7 macrophage cells as demonstrated by the decreased intensity of the 16 kDa LC3II band.

(E and F) Western blot showed that PUMA overexpression increased autophagy in both RAW264.7 macrophage cells and primary retinal microglia under different conditions as demonstrated by the increased intensity of the 16 kDa LC3II band.

(Figures 6G [arrows] and S6C), whereas *Puma* deficiency decreased autophagy in primary brain ECs (less LC3$^+$ dots) (Figure 6H, arrows).

PUMA-Induced Autophagy Requires Erk Activation

Because *Puma* deficiency attenuated Erk activation (Figures 5B–5D), we hypothesized that PUMA-induced autophagy might require Erk activation and tested this hypothesis. Treatment with an Erk1/2 inhibitor U0126 inhibited Erk activation and PUMA-induced autophagy in primary microglia (Figures 6I and S6C) and TR-iBRB (Figures 6J and S4E). Consistently, immunofluorescence staining showed that while PUMA overexpression induced autophagy in the TR-iBRB cells (Figure 6K, middle panel, arrows showing the increased LC3$^+$ dots), U0126 treatment abolished PUMA-induced autophagy (Figure 6K, right panel, decreased LC3$^+$ dots). Moreover, western blot showed that U0126 treatment inhibited Erk activation and autophagy (reduced intensity of the LC3II band) in the aortic SMCs (Figure 6L). We next investigated whether Erk activation was responsible for the greater survival rate of the wild-type microglia and vascular cells. We found that U0126 and A6355 (another Erk inhibitor) treatment completely abolished the increased survival rate of the wild-type microglia and vascular cells (Figures 6M–6O). Thus, our data demonstrated that Erk activation was required for PUMA-induced autophagy and survival of vascular and microglia cells.

PUMA Deficiency Decreased Vascular and Microglia Cell Cytoplasmic Calcium Levels

It is known that in fibroblasts PUMA, but not Noxa, increases cytosolic calcium levels (Shibue et al., 2006). Importantly, cytoplasmic calcium plays a critical role in inducing autophagy (Høyer-Hansen et al., 2007; Swerdlow and Distelhorst, 2007) and Erk activation (Agell et al., 2002; Schmitt et al., 2004). We therefore studied whether PUMA affected intracellular calcium levels in vascular and microglia cells. Using fluorescent Fluo-4 acetoxymethyl (Fluo-4AM) as an indicator of cytosolic calcium, we found that *Puma* deficiency reduced cytosolic calcium levels under a steady-state condition and upon ATP or mechanical stimulation in primary retinal microglia (Figure 7A), primary vascular ECs (Figure 7B), and primary vascular SMCs (Figure 7C). Consistently, *Puma* knockdown by RFP-*Puma* shRNA decreased cytosolic calcium levels in primary retinal microglia in the RFP-*Puma* shRNA expressing cells (Figure 7D, arrows in the right panel). These data demonstrated that PUMA plays a critical role in regulating cytosolic calcium levels, which is essential in modulating Erk activation and autophagy.

DISCUSSION

Our findings that PUMA is critically required for the survival and proliferation of vascular and microglia cells and plays important roles in angiogenesis are highly unexpected, given that PUMA has been considered as an essential apoptosis inducer for more than 10 years (Yu and Zhang, 2008). We further revealed that the survival/proliferative effect of PUMA was achieved by induction of autophagy, a critical mechanism for cells to respond to metabolic stress in order to survive, proliferate, and migrate (Fan et al., 2010). Mechanistically, we revealed that PUMA plays a critical role in regulating Erk activation and intracellular calcium level. Our data thus revealed an unexpected function of PUMA as a survival, proliferative, and angiogenic factor that contradicts the known apoptosis-inducing role of PUMA.

Little was known about the function of PUMA in vascular and microglia cells, even though the proapoptotic effect of PUMA on other types of cells has been studied intensively. Indeed, although *Puma* deficiency in mice decreased apoptosis in fibroblasts and lymphocytes (Villunger et al., 2003), the same apoptotic effect has not been observed on vascular or microglia cells. In this study, we found that PUMA is abundantly expressed in vascular and microglia cells. In vitro, *Puma* deficiency impaired proliferation and survival of cultured vascular and microglia cells, and nearly completely abolished the angiogenic response of mouse aortic rings. In vivo, *Puma* deficiency by genetic deletion or shRNA treatment decreased blood vessel and microglia density in retina and brain, and suppressed angiogenesis in different animal models. Consistently, *Puma* gene delivery promoted cell proliferation and survival in vitro, and increased blood vessel and microglia density in vivo. Interestingly, PUMA was recently reported to be critically required for tumorigenesis (Michalak et al., 2010), whereas other apoptotic genes, such as p53 and Noxa, inhibited tumorigenesis (Michalak et al., 2010). Although a different mechanism underlying the tumorigenic activity of PUMA was suggested, it nevertheless indicated a unique growth-promoting function of PUMA, at least under certain conditions. In addition, while retinal microglia density and angiogenesis were reduced in *Puma*-deficient mice, the retinal astrocytes appeared to be normal, demonstrating a cell-specific effect of PUMA. Indeed, other investigators have also reported a cell-specific effect of PUMA (Qiu et al., 2008).

(G and H) Immunofluorescence staining showed that overexpression of PUMA in primary retinal microglia increased autophagy as demonstrated by the increased LC3$^+$ dots (A, arrows), whereas *Puma* deficiency decreased autophagy in the primary brain endothelial cells as shown by the decreased LC3$^+$ dots (B, arrows). DAPI, diamidino-2-phenylindole fluorescent staining of nuclei.

(I and J) Western blot showed that U0126 (an Erk inhibitor) treatment inhibited Erk activation and PUMA-induced autophagy in primary microglia (I) and rat retinal endothelial cells (TR-iBRB, J) as demonstrated by the decreased intensity of the 16 kDa LC3II band.

(K) Immunofluorescence staining showed that while PUMA overexpression induced autophagy in the TR-iBRB cells as shown by the increased LC3$^+$ dots (middle panel, arrows), U0126 treatment abolished PUMA-induced autophagy as shown by the disappearance of the LC3$^+$ dots (right panel).

(L) Western blot showed that U0126 treatment inhibited Erk activation and autophagy in aortic artery smooth muscle cells (SMCs) as demonstrated by the decreased intensity of the 16 kDa LC3II band.

(M–O) MTT assay showed that U0126 and A6355 (another Erk inhibitor) treatment abolished the greater survival rate of the wild-type (WT) microglia and vascular ECs and SMCs.

Scale bars represent 10 μm (G, H, and K). Data are represented as mean ± SEM; *p < 0.05, **p < 0.01. See also Figure S6.
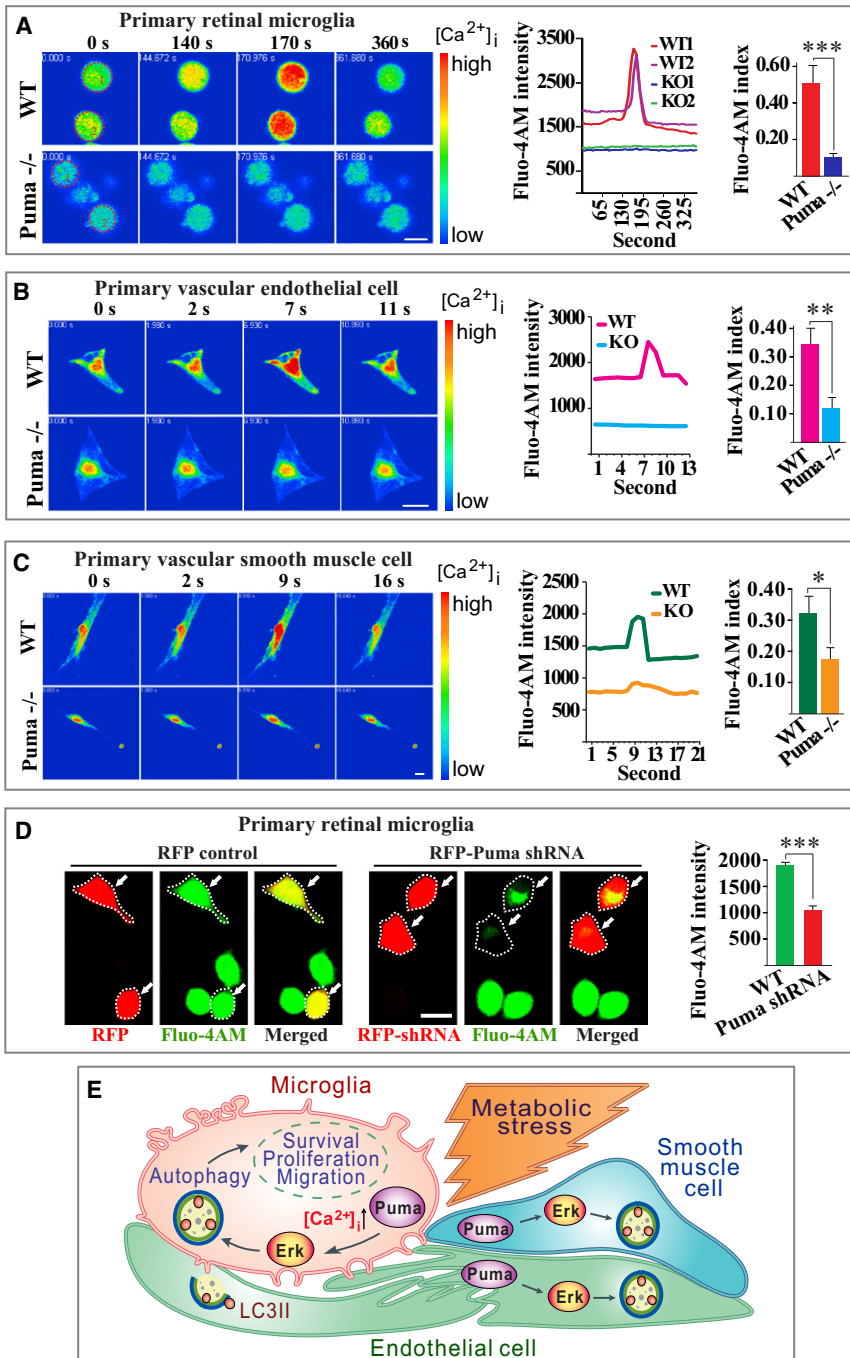
Figure 7. Critical Role of PUMA in Regulating Cytosolic Calcium Levels of Microglia and Vascular Cells

(A) Fluorescent Fluo-4 acetoxymethyl (Fluo-4AM, green) was used as an indicator of cytosolic calcium. Time-lapse microscopy showed that PUMA deficiency decreased cytoplasm calcium levels under a steady-state condition and upon ATP stimulation in primary retinal microglia (n = 8, p < 0.001).

(B) Time-lapse microscopy showed that PUMA deficiency decreased cytoplasm calcium levels under a steady-state condition and upon mechanical stimulation in primary vascular endothelial cells (n = 6, p < 0.01).

(C) Time-lapse microscopy showed that PUMA deficiency decreased cytoplasm calcium levels under a steady-state condition and upon mechanical stimulation in primary vascular smooth muscle cells (n = 8, p < 0.05).

(D) *Puma* knockdown by shRNA (RFP-Puma shRNA) markedly decreased cytosolic calcium levels in primary retinal microglia as demonstrated by the diminished green fluorescence in the RFP-Puma shRNA expressing cells (arrows in the right panel).

(E) PUMA is required for the survival and proliferation of vascular and microglia cells by regulating intracellular calcium level, Erk activation, and autophagy, which is essential for cell survival, proliferation and migration, particularly, under conditions of metabolic stress.

Scale bars represent 20 μm. Data are represented as mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001.

required for macrophage migration and T cell proliferation (Kadandale et al., 2010). In this study, loss of PUMA by gene deletion or shRNA markedly inhibited autophagy and impaired Erk activation in microglia, vascular ECs, and SMCs, whereas *Puma* gene delivery increased autophagy and Erk activation. Calcium signaling plays a critical role in Erk activation (Agell et al., 2002; Schmitt et al., 2004) and autophagy induction (Høyer-Hansen et al., 2007; Swerdlow and Distelhorst, 2007). Because shRNA-mediated loss of PUMA markedly decreased cytosolic calcium levels in microglia, the effect of PUMA on Erk activation and autophagy may be mediated by its regulatory effect on calcium signaling.

The previously unrecognized angiogenic effect of PUMA is surprising, because no other BH3-only protein family member has been shown to display such a function. It is reported that microglia play crucial roles in angiogenesis, at least in part, by producing potent angiogenic factors (Checchin et al., 2006; Liu et al., 2005; Yamaguchi et al., 2011). In this study, we found that the expression levels of many important angiogenic factors

Another important finding in this study is the critical role of PUMA in inducing vascular and microglia cell autophagy by regulating Erk activation and cytosolic calcium levels (Figure 7E). Autophagy is a homeostatic cellular recycling mechanism essential for cell survival, proliferation, and migration (Fan et al., 2010; Rabinowitz and White, 2010). It is especially crucial when the cells are under metabolic stress, such as hypoxia, even though uncontrolled autophagy can eventually lead to cell death. Autophagy has been shown to be critically

were significantly reduced. Thus, apart from its direct effect on vascular cells, PUMA plays an important role in angiogenesis also by ensuring adequate microglia density and sufficient amount of angiogenic factors.

Our finding that PUMA is a survival, proliferative, and angiogenic factor may have important therapeutic implications. Because of its potent apoptotic activity, PUMA has been considered as a promising therapeutic molecule for cancer treatment (Ito et al., 2005). In addition, the therapeutic potential of targeted PUMA inhibition to suppress apoptosis for the treatment of degenerative diseases has attracted considerable research interest (Yu and Zhang, 2008). However, our current findings suggest that *Puma* gene delivery or inhibition may lead to undesired effects on vascular and immune systems and warrant more careful investigations.

Although we have demonstrated an unexpected function of PUMA using comprehensive systems and approaches, outstanding questions remain. For example, we currently cannot explain how PUMA regulates cytosolic calcium levels and Erk activation. Also, it remains elusive how the functional property of PUMA is regulated to fulfill its autophagy or apoptotic activities in different cells or under different conditions. It is possible that there may be intermediate context-dependent molecules mediating the different activities of PUMA. Moreover, although our data demonstrated a cell-autonomous function of PUMA in microglia and vascular cells, this does not rule out the possibility that programed elimination of certain types of retinal cells (e.g., neurons) by PUMA during development might signal the proliferation of retinal microglia and vascular cells. Future studies are needed to answer these questions. In summary, our data revealed an angiogenic activity of PUMA, which can act as a survival and proliferative factor by modulating autophagy, and demonstrated an unexpected complexity of PUMA function.

EXPERIMENTAL PROCEDURES

Animals, Intraocular Injections, and Tissues
The mice used and the eye tissue isolation are described in the Extended Experimental Procedures. For *Puma* knockdown in vivo, 2 μg/0.5 μl/eye of pLKO.1 DNA plasmid encoding the mouse *Puma* shRNA or the pLKO.1 empty vector (Open Biosystems) together with the in vivo-jetPEI reagent (Polyplus-transfection) was injected into P1 mouse vitreous according to the manufacturer's instructions. For *Puma* gene delivery in vivo, 2 μg/0.5 μl/eye of pCMV6-AC-RFP vector encoding mouse PUMA or the pCMV6-AC-RFP vector without PUMA (OriGene Technologies) with the in vivo transfection reagent was injected into P1 mouse vitreous. In vivo microglia depletion was described previously (Checchin et al., 2006).

Immunofluorescent Staining and Retinal Image Analysis
Immunofluorescent staining and retinal image analysis are described in the Extended Experimental Procedures. The reagents used for staining were: isolectin GS-B4 (IB4, Invitrogen), DAPI (Invitrogen), rabbit anti-mouse CD11b (BD PharMingen), rat anti-CD31 (PharMingen), rabbit anti-iba1 (Wako), rabbit anti-PUMA (Cell Signaling), goat anti-mouse PDGFRα (R&D), mouse anti-GFAP (Sigma-Aldrich), rabbit anti-LC3B (Novus Biologicals), and the Alexa Fluor fluorescence-conjugated IgGs (Invitrogen).

Western Blot and Real-Time PCR
Western blot was performed as described (Tang et al., 2010). The antibodies used were: rabbit anti-PUMA (Cell Signaling, Sigma-Aldrich), rabbit anti-FGF2 (Santa Cruz), rat anti-IL6 (Biolegend), armenian hamster anti-CCL2 (Bio-

legend), rabbit anti-LC3B (Novus Biologicals), rabbit anti-M-CSFR (Cell Signaling), mouse anti-p44/42 MAPK (Erk1/2) (Cell Signaling), and rabbit anti-Phospho-p44/42 MAPK (Erk1/2) (Cell Signaling). Monoclonal anti-β-actin-peroxidase (Sigma-Aldrich) was used as an internal control. Real-time PCRs were performed in triplicates as described (Tang et al., 2010) and repeated at least once. Primers used are listed in Table S3.

Cornea Pocket and Aortic Ring Assays
The mouse cornea pocket assay was performed as described (Li et al., 2008; Zhang et al., 2009). Human $VEGF_{165}$ (PeproTech) of 160 ng/pellet/cornea was used in the experiments. $CD11b^+$ microglia density within the active angiogenic area was assessed using an Imager Z1 microscope and AxioVision software (Carl Zeiss). The aortic ring assay was performed as described (Kumar et al., 2010) using human $VEGF_{165}$ and FGF2 (100 ng/ml, R&D Systems). Images of the aortic rings were taken on day 7 after implantation and microvessels analyzed as described (Kumar et al., 2010). $CD11b^+$ cells were analyzed by immunostaining.

Laser-Induced Choroidal Neovascularization Model
The CNV model was performed as described (Zhang et al., 2009). Eight to ten-week-old mice were used for experiments. A total of 1 μg/eye of pLKO.1 mouse *Puma* shRNA plasmid or the control pLKO.1 plasmid without the *Puma* shRNA (Open Biosystems) together with the transfection reagent in vivo-jetPEI (Polyplus-transfection) was injected into the mouse vitreous according to the manufacturer's instructions. CNV area was analyzed 1 week after laser treatment using IB4 staining. Microglia density was analyzed by immunofluorescence staining using Iba1 as a marker.

Primary Cell Isolation and Cell Culture
Primary mouse retina and brain microglia cells, astrocyte, brain ECs, and aortic artery SMCs were isolated and cultured as described (Matsubara et al., 2000; Saijo et al., 2009; Seidel et al., 1991). Cells within ten passages were used for experiment. The immortalized TR-iBRB and RGC5 were cultured as described (Li et al., 2008). The HUVEC (Lonza) and RAW264.7 macrophage cells (ATCC) were cultured according to the manufacturer's instructions.

Intracellular Calcium Assay
The intracellular calcium assay was performed according to the manufacturer's instructions (Invitrogen) and is described in the Extended Experimental Procedures. The primary retinal microglia cells transfected with a pRFP-C-RS vector encoding the mouse *Puma* shRNA or the pRFP-C-RS vector encoding a noneffective 29-mer scrambled shRNA (OriGene Technologies) were used for experiments 3 days after transfection.

Proliferation, Survival, and Cell Chemotaxis Assay
Several methods were used to assess cell proliferation and survival, including an MTT assay, a DAPI-TUNEL staining method, a BrdU assay, a trypan blue exclusion assay, and a LIVE/DEAD viability/cytotoxicity assay (Invitrogen). Primary cells isolated from six to eight mice were pooled and cultured for experiments. Serum-free medium was used for EC, SMC, and 293FT cell starvation. The Hank's balanced salt solution (Invitrogen) was used for microglia starvation. For the MTT assay, experiments were performed according to the manufacturer's protocol (Invitrogen). The Erk inhibitor A6355 and Mek inhibitor U0126 (10 μM each, Sigma) were used to block the Erk pathway using 0.1% DMSO (Sigma-Aldrich) as a control. A DAPI (Invitrogen) and a TUNEL (Roche) staining method was used to count the number of viable and apoptotic cells in 24 fields (450 μm × 350 μm each) each assay and the mean value calculated. Experiments were performed in triplicate under a normoxic or hypoxic condition (1% oxygen) and repeated at least once, and representative data presented. For the BrdU proliferation assay, cells were cultured in complete medium and cell proliferation assessed using a cell proliferation ELISA BrdU colorimetric kit according to the manufacturer's protocol (Roche). For cell survival assay, primary retinal microglia cells were treated with mitomycin C (Sigma, 50 μg/ml) in serum-free medium for 2 hr to inhibit cell proliferation and then cultured in serum-free medium with repeated mitomycin C treatment every 24 hr. After 3 days, the viable cells were counted using a trypan blue exclusion method. The viable and dead cells were also stained and

analyzed using a LIVE/DEAD viability/cytotoxicity assay kit (Invitrogen). The pLKO.1 *Puma* shRNA plasmid and the control pLKO.1 plasmid without the *Puma* shRNA (Open Biosystems), and the pCMV6 *Puma* gene expression plasmid and the control pCMV6 plasmid without the *Puma* gene (OriGene Technologies), were transfected into the cells using a SuperFect reagent (-QIAGEN) according to the manufacturer's protocol. Puromycin (Sigma) or G418 (Sigma) was used for selection according to the manufacturer's protocol. Three biological repeats were performed for each experiment and the mean value from the repeats is presented. Microglia chemotaxis assay was performed as described (Ma et al., 2009) using the chemoattractants ATP (50 μM, Sigma), CCL2 (100 ng/ml, R&D), and CCL3 (50 ng/ml, R&D).

Statistical Analysis

Two-tailed Student's t test was used for statistical analysis. Difference was considered statistically significant when $p < 0.05$. Results are represented as mean $\pm$ SEM of the number of determinations.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, six figures, and three tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.09.023.

LICENSING INFORMATION

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REFERENCES

Agell, N., Bachs, O., Rocamora, N., and Villalonga, P. (2002). Modulation of the Ras/Raf/MEK/ERK pathway by Ca(2+), and calmodulin. Cell. Signal. *14*, 649–654.

Checchin, D., Sennlaub, F., Levavasseur, E., Leduc, M., and Chemtob, S. (2006). Potential role of microglia in retinal blood vessel formation. Invest. Ophthalmol. Vis. Sci. *47*, 3595–3602.

Chipuk, J.E., Bouchier-Hayes, L., Kuwana, T., Newmeyer, D.D., and Green, D.R. (2005). PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. Science *309*, 1732–1735.

Davalos, D., Grutzendler, J., Yang, G., Kim, J.V., Zuo, Y., Jung, S., Littman, D.R., Dustin, M.L., and Gan, W.B. (2005). ATP mediates rapid microglial response to local brain injury in vivo. Nat. Neurosci. *8*, 752–758.

Fan, Q.W., Cheng, C., Hackett, C., Feldman, M., Houseman, B.T., Nicolaides, T., Haas-Kogan, D., James, C.D., Oakes, S.A., Debnath, J., et al. (2010). Akt and autophagy cooperate to promote survival of drug-resistant glioma. Sci. Signal. *3*, ra81.

Gawad, A., Ptak-Belowska, A., Brzozowski, T., and Pawlik, W.W. (2009). Monocytes and vascular endothelial cells apoptosis. Role of p-HSP27. J. Physiol. Pharmacol. *60*, 55–61.

Høyer-Hansen, M., Bastholm, L., Szyniarowski, P., Campanella, M., Szabadkai, G., Farkas, T., Bianchi, K., Fehrenbacher, N., Elling, F., Rizzuto, R., et al. (2007). Control of macroautophagy by calcium, calmodulin-dependent kinase kinase-beta, and Bcl-2. Mol. Cell *25*, 193–205.

Ito, H., Kanzawa, T., Miyoshi, T., Hirohata, S., Kyo, S., Iwamaru, A., Aoki, H., Kondo, Y., and Kondo, S. (2005). Therapeutic efficacy of PUMA for malignant glioma cells regardless of p53 status. Hum. Gene Ther. *16*, 685–698.

Kadandale, P., Stender, J.D., Glass, C.K., and Kiger, A.A. (2010). Conserved role for autophagy in Rho1-mediated cortical remodeling and blood cell recruitment. Proc. Natl. Acad. Sci. USA *107*, 10502–10507.

Kubota, Y., Takubo, K., Shimizu, T., Ohno, H., Kishi, K., Shibuya, M., Saya, H., and Suda, T. (2009). M-CSF inhibition selectively targets pathological angiogenesis and lymphangiogenesis. J. Exp. Med. *206*, 1089–1102.

Kumar, A., Hou, X., Lee, C., Li, Y., Maminishkis, A., Tang, Z., Zhang, F., Langer, H.F., Arjunan, P., Dong, L., et al. (2010). Platelet-derived growth factor-DD targeting arrests pathological angiogenesis by modulating glycogen synthase kinase-3beta phosphorylation. J. Biol. Chem. *285*, 15500–15510.

Kuroki, K., Virard, I., Concannon, C.G., Engel, T., Woods, I., Taki, W., Plesnila, N., Henshall, D.C., and Prehn, J.H. (2009). Effects of transient focal cerebral ischemia in mice deficient in puma. Neurosci. Lett. *451*, 237–240.

Labi, V., Erlacher, M., Krumschnabel, G., Manzl, C., Tzankov, A., Pinon, J., Egle, A., and Villunger, A. (2010). Apoptosis of leukocytes triggered by acute DNA damage promotes lymphoma formation. Genes Dev. *24*, 1602–1607.

Li, Y., Zhang, F., Nagai, N., Tang, Z., Zhang, S., Scotney, P., Lennartsson, J., Zhu, C., Qu, Y., Fang, C., et al. (2008). VEGF-B inhibits apoptosis via VEGFR-1-mediated suppression of the expression of BH3-only protein genes in mice and rats. J. Clin. Invest. *118*, 913–923.

Liu, A., Arbiser, J.L., Holmgren, A., Klein, G., and Klein, E. (2005). PSK and Trx80 inhibit B-cell growth in EBV-infected cord blood mononuclear cells through T cells activated by the monocyte products IL-15 and IL-12. Blood *105*, 1606–1613.

Liva, S.M., Kahn, M.A., Dopp, J.M., and de Vellis, J. (1999). Signal transduction pathways induced by GM-CSF in microglia: significance in the control of proliferation. Glia *26*, 344–352.

Ma, W., Zhao, L., Fontainhas, A.M., Fariss, R.N., and Wong, W.T. (2009). Microglia in the mouse retina alter the structure and function of retinal pigmented epithelial cells: a potential cellular interaction relevant to AMD. PLoS ONE *4*, e7945.

Matsubara, T.A., Murata, T.A., Wu, G.S., Barron, E.A., and Rao, N.A. (2000). Isolation and culture of rat retinal microvessel endothelial cells using magnetic beads coated with antibodies to PECAM-1. Curr. Eye Res. *20*, 1–7.

Michalak, E.M., Vandenberg, C.J., Delbridge, A.R., Wu, L., Scott, C.L., Adams, J.M., and Strasser, A. (2010). Apoptosis-promoted tumorigenesis: gamma-irradiation-induced thymic lymphomagenesis requires Puma-driven leukocyte death. Genes Dev. *24*, 1608–1613.

Otero, K., Turnbull, I.R., Poliani, P.L., Vermi, W., Cerutti, E., Aoshi, T., Tassi, I., Takai, T., Stanley, S.L., Miller, M., et al. (2009). Macrophage colony-stimulating factor induces the proliferation and survival of macrophages via a pathway involving DAP12 and beta-catenin. Nat. Immunol. *10*, 734–743.

Qiu, W., Carson-Walter, E.B., Liu, H., Epperly, M., Greenberger, J.S., Zambetti, G.P., Zhang, L., and Yu, J. (2008). PUMA regulates intestinal progenitor cell radiosensitivity and gastrointestinal syndrome. Cell Stem Cell *2*, 576–583.

Qiu, W., Wang, X., Leibowitz, B., Yang, W., Zhang, L., and Yu, J. (2011). PUMA-mediated apoptosis drives chemical hepatocarcinogenesis in mice. Hepatology *54*, 1249–1258.

Rabinowitz, J.D., and White, E. (2010). Autophagy and metabolism. Science *330*, 1344–1348.

Reddy, P.H., Manczak, M., Zhao, W., Nakamura, K., Bebbington, C., Yarranton, G., and Mao, P. (2009). Granulocyte-macrophage colony-stimulating factor antibody suppresses microglial activity: implications for anti-inflammatory effects in Alzheimer's disease and multiple sclerosis. J. Neurochem. *111*, 1514–1528.

Saijo, K., Winner, B., Carson, C.T., Collier, J.G., Boyer, L., Rosenfeld, M.G., Gage, F.H., and Glass, C.K. (2009). A Nurr1/CoREST pathway in microglia and astrocytes protects dopaminergic neurons from inflammation-induced death. Cell *137*, 47–59.

Santos, A.M., Calvente, R., Tassi, M., Carrasco, M.C., Martín-Oliva, D., Marín-Teva, J.L., Navascués, J., and Cuadros, M.A. (2008). Embryonic and postnatal development of microglial cells in the mouse retina. J. Comp. Neurol. *506*, 224–239.

Sasaki, A., Yokoo, H., Naito, M., Kaizu, C., Shultz, L.D., and Nakazato, Y. (2000). Effects of macrophage-colony-stimulating factor deficiency on the maturation of microglia and brain macrophages and on their expression of scavenger receptor. Neuropathology *20*, 134–142.

Schmitt, J.M., Wayman, G.A., Nozaki, N., and Soderling, T.R. (2004). Calcium activation of ERK mediated by calmodulin kinase I. J. Biol. Chem. *279*, 24064–24072.

Seidel, M.F., Simard, J.M., Hunter, S.F., and Campbell, G.A. (1991). Isolation of arteriolar microvessels and culture of smooth muscle cells from cerebral cortex of guinea pig. Cell Tissue Res. *265*, 579–587.

Shibue, T., Suzuki, S., Okamoto, H., Yoshida, H., Ohba, Y., Takaoka, A., and Taniguchi, T. (2006). Differential contribution of Puma and Noxa in dual regulation of p53-mediated apoptotic pathways. EMBO J. *25*, 4952–4962.

Stefater Iii, J.A., Lewkowich, I., Rao, S., Mariggi, G., Carpenter, A.C., Burr, A.R., Fan, J., Ajima, R., Molkentin, J.D., Williams, B.O., et al. (2011). Regulation of angiogenesis by a non-canonical Wnt-Flt1 pathway in myeloid cells. Nature *474*, 511–515.

Swerdlow, S., and Distelhorst, C.W. (2007). Bcl-2-regulated calcium signals as common mediators of both apoptosis and autophagy. Dev. Cell *12*, 178–179.

Tang, Z., Arjunan, P., Lee, C., Li, Y., Kumar, A., Hou, X., Wang, B., Wardega, P., Zhang, F., Dong, L., et al. (2010). Survival effect of PDGF-CC rescues neurons from apoptosis in both brain and retina by regulating GSK3beta phosphorylation. J. Exp. Med. *207*, 867–880.

Villunger, A., Michalak, E.M., Coultas, L., Müllauer, F., Böck, G., Ausserlechner, M.J., Adams, J.M., and Strasser, A. (2003). p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science *302*, 1036–1038.

Vousden, K.H. (2005). Apoptosis. p53 and PUMA: a deadly duo. Science *309*, 1685–1686.

Yamaguchi, M., Arbiser, J.L., and Weitzmann, M.N. (2011). Honokiol stimulates osteoblastogenesis by suppressing NF-κB activation. Int. J. Mol. Med. *28*, 1049–1053.

Yu, J. (2009). PUMA kills stem cells to stall cancer? Mol. Cell Pharmacol. *1*, 112–118.

Yu, J., and Zhang, L. (2003). No PUMA, no death: implications for p53-dependent apoptosis. Cancer Cell *4*, 248–249.

Yu, J., and Zhang, L. (2008). PUMA, a potent killer with or without p53. Oncogene *27*(Suppl 1), S71–S83.

Zhang, F., Tang, Z., Hou, X., Lennartsson, J., Li, Y., Koch, A.W., Scotney, P., Lee, C., Arjunan, P., Dong, L., et al. (2009). VEGF-B is dispensable for blood vessel growth but critical for their survival, and VEGF-B targeting inhibits pathological angiogenesis. Proc. Natl. Acad. Sci. USA *106*, 6152–6157.

Zheng, L., Gong, B., Hatala, D.A., and Kern, T.S. (2007). Retinal ischemia and reperfusion causes capillary degeneration: similarities to diabetes. Invest. Ophthalmol. Vis. Sci. *48*, 361–367.