βA3/A1-crystallin is a critical mediator of STAT3 signaling in optic nerve astrocytes

Mallika Valapala1, Malia Edwards1, Stacey Hose1, Jianfei Hu1, Eric Wawrousek2, Gerard A. Lutty1, J. Samuel Zigler, Jr.1, Jiang Qian1 & Debasish Sinha1

1Wilmer Eye Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21287, USA, 2National Eye Institute, National Institutes of Health, Bethesda, Maryland 20892, USA.

We have previously reported that in the Nuc1 rat, which has a spontaneous mutation in Cryba1 (the gene encoding βA3/A1-crystallin), astrocytes exhibit decreased Notch signaling, leading to reduced promoter activity for glial fibrillary acidic protein (GFAP). Interestingly, in both Nuc1 astrocytes and in wild type astrocytes following knockdown of Cryba1, vascular endothelial growth factor (VEGF) secretion is decreased. This has led us to explore signaling mediators that could be regulated by βA3/A1-crystallin to modulate both GFAP and VEGF. Several studies have shown that the signal transducer and activator of transcription 3 (STAT3) is involved in the co-regulation of GFAP and VEGF. We show that STAT3 and βA3/A1-crystallin may co-regulate each other in astrocytes. Such co-regulation would create a positive feedback circuit; i.e., in the cytosol of astrocytes, βA3/A1-crystallin is necessary for the phosphorylation of STAT3, which then dimerizes and translocates to the nucleus to form DNA-binding complexes, activating transcription of Cryba1. This stoichiometric co-regulation of STAT3 and Cryba1 could potentiate expression of GFAP and secretion of VEGF, both of which are essential for maintaining astrocyte and blood vessel homeostasis in the retina. Consistent with this idea, Cryba1 knockout mice exhibit an abnormal astrocyte pattern and defective remodeling of retinal vessels.

The primary vascular layer in the retina is intimately associated with the underlying meshwork of astrocytes that emerge from the optic nerve1. Although, it has been suggested that the astrocyte template (the honeycomb-like arrangement of astrocytes in the developing retina) guides the endothelial cells to facilitate vessel growth2, the molecular mechanisms underlying this process remain elusive. Previous studies have shown that plasticity of blood vessels is closely associated with the interplay between astrocytes, endothelial cells and pericytes3,4. Indeed, retinal astrocytes are only found in species with vascularized retinas5. We have previously shown that Notch signaling is important for proper astrocyte template formation and for maintenance of vascular homeostasis in the retina6. Although several studies have suggested the importance of the Notch signaling pathway in the development and maintenance of the retinal vasculature7–9, our knowledge of the mechanisms by which the Notch pathway and its downstream effectors mediate vascular homeostasis in the retina is very limited.

Emerging evidence suggests dynamic interactions between Notch signaling and VEGF, an inducer of angiogenesis in multiple cell types10,11. Recent reports show that inhibition of Notch signaling suppresses endothelial tip cell formation by reducing the levels of VEGF11,12. During retinal vascular development, astrocyte-derived VEGF is believed to provide guidance cues to the growing endothelial tip cells; hence, astrocytes are important for the stabilization and maturation of blood vessels13. These results suggest that the Notch signaling pathway and its downstream mediators are involved in astrocyte template formation and the subsequent remodeling of retinal vessels. However, the signaling mediators linking Notch to VEGF expression in astrocytes are unknown. One such signaling intermediate could be STAT3, a member of the signal transducers and activators of transcription (STAT) family, latent gene regulating proteins that are translocated to the nucleus and regulate gene transcription14. Previous studies have shown a direct link between Notch signaling and STAT3 activation to be important for astrogial differentiation and regulation of GFAP expression15,16. It has been shown that Notch signaling induces astrocyte differentiation in gliogenic cells by STAT3-mediated activation of the GFAP promoter17. Thus, Notch and its downstream signaling mediators are involved in the regulation of GFAP, and this pathway is also...
We therefore provide in this study mechanistic insights as to how, in turn, STAT3 regulating the transcription of Cryba1 participating in the phosphorylation of STAT3 in the cytosol and, co-regulation leads to a positive feedback loop, with astrocytes, STAT3 and Cryba1 astrocytes compared to WT cells. Error bars indicate s.d.; Cryba1 is non-functional. This suggests that in astrocytes where Cryba1 is non-functional. This suggests that in astrocytes, STAT3 and Cryba1 are co-regulated. Such co-regulation leads to a positive feedback loop, with βA3/A1-crystallin participating in the phosphorylation of STAT3 in the cytosol and, in turn, STAT3 regulating the transcription of Cryba1 in the nucleus. We therefore provide in this study mechanistic insights as to how βA3/A1-crystallin modulates the Notch/STAT3 signaling axis to regulate normal functions of astrocytes in the retina, including secretion of VEGF and expression of GFAP. Interestingly, Cryba1 knockout mice also exhibit an abnormal astrocyte template and defective remodeling of the retinal vessels, suggesting that βA3/A1-crystallin is essential for normal functioning of astrocytes in the optic nerve and the retina.

**Results**

**Inhibition of Notch signaling reduces secretion of VEGF in optic nerve astrocytes.** Because of the abnormal retinal vasculature in animal models where βA3/A1-crystallin is non-functional in astrocytes, we have recently shown for Notch.

We report here that inhibition of the Notch pathway decreases the level of astrocyte-derived VEGF both in Nuc1 astrocytes and in wild type (WT) cells upon knockdown of Cryba1. The levels of STAT3 phosphorylated at tyrosine 705 (Y705) were reduced significantly in astrocytes lacking functional βA3/A1-crystallin relative to WT. Phosphorylation of STAT3 leads to the formation of homodimers that translocate to the nucleus and are critical for STAT3 dependent transcriptional activation. To investigate whether STAT3 could regulate transcription of Cryba1, we took advantage of the ENCODE project, in which genome-wide binding locations were experimentally determined for a large number of transcription factors using a ChIP-chip or ChIP-seq approach in HeLa and MCF10A cell lines. Synteny mapping of the human to mouse genome identified STAT3 binding sites in the mouse Cryba1 gene, and using quantitative real-time PCR as well as a promoter-based luciferase assay, we confirmed that Cryba1 possesses a functional STAT3 binding site in the promoter region. Moreover, inhibition of Notch with N-[N-(3, 5- difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyler ester (DAPT) reduced phospho-STAT3 in both WT cells and in astrocytes where Cryba1 is non-functional. This suggests that in astrocytes, STAT3 and βA3/A1-crystallin are co-regulated. Such co-regulation leads to a positive feedback loop, with βA3/A1-crystallin participating in the phosphorylation of STAT3 in the cytosol and, in turn, STAT3 regulating the transcription of Cryba1 in the nucleus. We therefore provide in this study mechanistic insights as to how βA3/A1-crystallin modulates the Notch/STAT3 signaling axis to regulate normal functions of astrocytes in the retina, including secretion of VEGF and expression of GFAP. Interestingly, Cryba1 knockout mice also exhibit an abnormal astrocyte template and defective remodeling of the retinal vessels, suggesting that βA3/A1-crystallin is essential for normal functioning of astrocytes in the optic nerve and the retina.
of this pathway in cells with non-functional βA3/1-crystallin has a more pronounced effect on VEGF secretion. Our studies also show that rat optic nerve astrocytes express VEGF receptor 1 (VEGFR1) and the co-receptor Neuropilin-1 (NRP1), whose transcript levels are significantly downregulated in Nuc1 compared to WT astrocytes (Fig. 1, d and e).

Astrocytes deficient in βA3/1-crystallin protein exhibit altered levels of total and activated STAT3. We performed a sandwich ELISA assay using antibodies to total STAT3 and p-STAT3 to quantitate protein levels in WT and Nuc1 astrocytes. Interestingly, no difference in the level of total STAT3 was observed when WT and Nuc1 astrocytes were compared; however, the level of p-STAT3 was reduced by ~50% in Nuc1 astrocytes relative to WT astrocytes (Fig. 1, d and e).

Previous studies have suggested that the Notch pathway is an important upstream regulator of STAT3 signaling and that it plays a crucial role in determining glial cell fate15,16. Since our previous studies have shown impaired Notch signaling in astrocytes lacking functional βA3/1-crystallin6, we wanted to determine if inhibition of the Notch pathway directly affects STAT3 activation. We evaluated the effect of the Notch pathway inhibitor, DAPT, on the activation of STAT3. Our studies show that treatment with DAPT reduced p-STAT3 significantly in both WT (~57%) and Nuc1 (~75%) rat astrocytes compared to vehicle-treated cells. Moreover, in the presence of DAPT, Cryba1°/° and Cryba1 KD astrocytes showed ~50% and ~62% reduction in p-STAT3 levels compared to the respective vehicle controls (Fig. 2, c and d). These results suggest that phosphorylation of endogenous STAT3 is impaired in astrocytes lacking functional βA3/1-crystallin.

βA3/1-crystallin affects STAT3 activation in mouse optic nerve astrocytes. The STAT family of transcription factors is known to be activated in response to cytokines and growth factors via phosphorylation at Y705, leading to subsequent dimerization and translocation to the nucleus21. Interleukin-6 (IL-6) binding to the gp130 receptor stimulates STAT3 phosphorylation by Janus activated kinase 2 (JAK2)22. The activity of STAT3 can also be inhibited by several
Figure 3 | βA3/A1-crystallin modulates the phosphorylation of STAT3 in astrocytes. (a). Immunoblot analysis of total and p-STAT3 in WT astrocytes upon treatment with IL-6 and Static showed a 2.1 fold increase in the levels of p-STAT3 in astrocytes treated with IL-6 and ~70% reduction in p-STAT3 upon treatment with Static compared to vehicle-treated astrocytes. A ~70% reduction in p-STAT3 in astrocytes pre-treated with IL-6 upon treatment with Static compared to astrocytes treated with IL-6 alone was observed. (b). Analysis of p-STAT3 levels by ELISA revealed 5 fold induction of p-STAT3 in the presence of IL-6 and ~76% reduction in the presence of Static compared to the vehicle control. Static significantly decreased the levels of p-STAT3 in the presence of IL-6 (~85%) compared to the astrocytes treated with IL-6 alone. (c). However, in Cryba1 KD astrocytes there was only modest activation (1.3 fold) of p-STAT3 in the presence of IL-6. Significant inhibition of p-STAT3 was observed in the presence of Static. (d). ELISA data revealed that IL-6 treatment induced p-STAT3 by 4.5 fold in Cryba1 KD astrocytes compared to vehicle-treated astrocytes. Static decreased the level of p-STAT3 by ~80% when treated alone and by ~85% when treated in combination with IL-6 compared to vehicle-treated and IL-6 treated Cryba1 KD astrocytes. Error bars indicate s.d.; *P<0.05.
In our studies, we used IL-6 and Statik to activate and selectively inhibit activation, dimerization and nuclear translocation of the STAT3 SH2 domain, thereby preventing phosphorylation and pharmacological inhibitors. Recently, Statik, a small molecule inhibitor of STAT3, has been developed that selectively inhibits the function of the STAT3 SH2 domain, thereby preventing phosphorylation and activation of STAT3. In several cell types, Statik has been shown to selectively inhibit activation, dimerization and nuclear translocation of STAT3. In our studies, we used IL-6 and Statik to activate and inhibit STAT3 phosphorylation, respectively, in vitro. Immunoblot analysis revealed a 2.1 fold increase in the levels of p-STAT3 in astrocytes treated with IL-6 and ~70% reduction in astrocytes treated with Statik compared to vehicle-treated cells. Statik also effectively reduced p-STAT3 levels in cells treated with IL-6 by ~70%, when compared to cells treated with IL-6 alone (Fig. 3, a). We also show by ELISA that IL-6 treatment increased the levels of p-STAT3 protein by 5 fold, while treatment with Statik decreased p-STAT3 by ~70%, compared to the vehicle control. Statik also decreased the levels of p-STAT3 in the presence of IL-6 by ~85% compared to the cells treated with IL-6 alone (Fig. 3, b). Cryba1 KD mouse astrocytes showed only modest increase (1.3 fold) in p-STAT3 in the presence of IL-6 compared to vehicle-treated cells. However, Statik either alone or in the presence of IL-6 decreased the levels of p-STAT3 in Cryba1 KD astrocytes (Fig. 3, c). Similarly, ELISA data show that IL-6 treatment induced the levels of p-STAT3 by 4.5 fold in Cryba1 KD astrocytes compared to vehicle-treated cells. Moreover, in Cryba1 KD astrocytes, Statik decreased the levels of p-STAT3 by ~80% when treated alone and ~85% when treated in combination with IL-6 compared to vehicle-treated and IL-6 treated Cryba1 KD astrocytes, respectively (Fig. 3, d). These results suggest that βA3/Al-crystallin is an important molecule necessary for the phosphorylation of STAT3 in the cytosol of optic nerve astrocytes.

**The expression of Cryba1 in optic nerve astrocytes is regulated by STAT3.** The ENCODE program suggested that there are two potential STAT3 binding sites in the Cryba1 gene, one in the promoter and another within intron 2 (Fig. 4, a). We therefore hypothesized that STAT3 might regulate the expression of Cryba1 and performed quantitative real time PCR (qRT-PCR) on astrocytes treated with IL-6 and Statik. Our data suggest that IL-6 treatment of WT rat astrocytes results in a 4.5 fold increase in the expression of Cryba1 and that Statik treatment decreased the expression of Cryba1 by ~51% compared to vehicle-treated astrocytes. In astrocytes treated with both IL-6 and Statik, the expression of Cryba1 was reduced by ~70% compared to cells treated with IL-6 alone (Fig. 4, b). Our results provide novel evidence that IL-6 increases, and Statik decreases, expression of Cryba1 in WT astrocytes, supporting our hypothesis that STAT3 regulates the expression of Cryba1 in astrocytes.

In order to confirm that STAT3 binds at the sites in the promoter and intron 2 of the Cryba1 mouse gene (Fig. 4, a), we performed luciferase assays. The predicted STAT3 binding site in the Cryba1 promoter was cloned into the pGL3-promoter vector, upstream of the luciferase reporter gene. The STAT3 binding site in intron 2 of the Cryba1 gene was cloned downstream of the luciferase gene in the pGL3 vector. The pGL3 vector constructs containing the promoter and intron STAT3 binding sites were each co-transfected into WT and Nuc1 astrocytes along with the pRL-CMV vector carrying the Renilla luciferase gene under the control of the cytomegalovirus promoter. A significant induction (3.2 fold) of luciferase activity was observed in WT astrocytes transfected with pGL3-Cryba1 intron compared to cells treated with vehicle. We did not detect any significant changes in luciferase activity in cells transfected with pGL3-Cryba1 intron or with empty vector transfected cells. WT cells transfected with pGL3-Cryba1 promoter and pre-treated with IL-6 in the presence of Statik had ~60% reduction in luciferase activity compared to cells treated with IL-6 alone (Fig. 5, a). Furthermore, IL-6 treatment of Nuc1 astrocytes transfected with pGL3-Cryba1 promoter resulted in 1.8 fold induction of luciferase activity. Luciferase activity was not induced in Nuc1 astrocytes transfected with pGL3-Cryba1 intron or with empty vector. Nuc1 astrocytes transfected with pGL3-Cryba1 promoter and pre-treated with IL-6 had a ~44% reduction in luciferase activity in the presence of Statik compared to cells treated with IL-6 alone (Fig. 5, b). In addition, to further validate the existence of a functional STAT3 binding site in the Cryba1 promoter, we generated a Cryba1 STAT3 deletion construct (pGL3-Cryba1ΔSTAT3) by deleting the potential STAT3 binding site in the Cryba1 promoter. As noted above (Fig. 5, a), a significant induction of luciferase activity was observed when astrocytes were transfected with pGL3-Cryba1 promoter vector in the presence of IL-6. However, in astrocytes transfected with the pGL3-Cryba1ΔSTAT3 construct, luciferase activity was close to baseline levels in the presence of IL-6. To confirm that the activity was specific to STAT3, we treated the cells with Statik in addition to IL-6. Statik inhibited the luciferase activity only in astrocytes transfected with the pGL3-Cryba1 promoter vector (Fig. 6). These results confirm the presence of an active STAT3 binding site in the region 2774 to 2756 base pairs upstream of the transcription start site in the Cryba1 promoter (Supplementary Fig. 1).

**Cryba1 knockout mice show abnormal retinal astrocytes and remodeling of the blood vessels.** We generated Cryba1 knockout (KO) mice to further validate that loss of Cryba1 affects astrocyte...
patterning as well as subsequent remodeling of the retinal vessels in vivo. Cryba1 floxed mice were crossed with BEST1 (Bestrophin-1)-cre mice to generate RPE-specific conditional knockout mice. However, it is known that germline deletion of floxed alleles may occur when floxed mice are maintained for multiple generations with the BEST1-cre allele, creating a global knockout of the floxed gene. We used this as a strategy to generate Cryba1 complete KO mice. In contrast to the Nuc1 rat, the lens is not ruptured in the Cryba1 KO mice. Retinal flat mounts from 10 month old Cryba1 fl/fl or Cryba1 KO mice were stained with GFAP (red; astrocytes) and GS isolectin (green; blood vessels). The Cryba1 fl/fl retinas showed the expected close association of retinal vessels and astrocytes (Fig. 7a–c). Astrocytes were observed in an organized honeycomb-like pattern across the entire retina (Fig. 7 b, c). Upon closer investigation, the astrocyte endfeet were clearly visible on retinal vessels (Fig. 7, c). In contrast, Cryba1 KO retina showed an abnormal pattern (Fig. 7d–f), with an apparent increase in the density of astrocytes in focal areas (Fig. 7 e, f). While a few astrocyte endfeet were observed on retinal vessels in Cryba1 KO mice, they were mostly replaced by astrocyte bundles on vessels (Fig. 7, g). Vessels in these areas had unusual budding and pattern (Fig. 7, h). In some areas, large vessels were constricted with disrupted orientation (Fig. 7, i). Together, these data demonstrate that βA3/A1-crystallin is essential for normal astrocyte function in the retina and that loss of βA3/A1-crystallin in astrocytes can affect the normal remodeling of retinal vessels.

**Discussion**

Astrocytes are critical for the proper functioning of the retina. During retinal histogenesis, they migrate from the optic nerve into the inner retina and are closely associated with retinal blood vessels. Several studies have shown that astrocytes and retinal blood vessels are developmentally linked. The basic molecular mechanisms of Notch signaling, and its importance in retinal vascular development, are becoming increasingly clear. Still, a clear gap exists in the literature regarding our knowledge of the cellular and molecular interactions of astrocytes during vascular remodeling in the retina. Our previous studies using genetically engineered animal models and in vitro systems suggested that βA3/A1-crystallin, a lens structural protein and a member of the β/γ-crystallin superfamily, is involved in astrocyte template formation and remodeling of the retinal vessels. We have also shown previously that βA3/A1-crystallin is localized to the lysosomes of astrocytes and retinal pigment epithelium, where it modulates the activity of the lysosomal proton pump vacuolar ATPase (V-ATPase). This novel function of βA3/A1-crystallin in astrocytes is critical for normal astrocyte function and retinal blood vessel remodeling.
βA3/A1-crystallin, mediated through Notch signaling in the endo-
lysosomal system, is required for proper patterning of the hon-
eycomb-like astrocyte template in the retina6. To further delineate
the mechanisms by which βA3/A1-crystallin affects astrocyte tem-
plate formation and to link that process to vascular remodeling of the
retina, we focused on the signaling pathways and cellular processes
possibly modulated by βA3/A1-crystallin. The factors in the signal-
ning pathway linking Notch to VEGF expression in astrocytes are
unknown. JAK/STAT and Notch signaling act synergistically to pro-
mote astrogenesis32. In this study, we provide novel evidence that
STAT3 affects Cryba1 expression in astrocytes, which in turn regu-
lates proper functioning of the Notch signaling pathway and its
downstream activation of STAT3. The pool of βA3/A1-crystallin
in the cytosol regulates the Notch signaling pathway leading to the
phosphorylation and activation of STAT3. While βA3/A1-crystallin
is entirely cytoplasmic in lens fiber cells, the protein appears to be
present in the cell nucleus as well as in the cytoplasm of astrocytes19.
Thus, our data provide direct evidence that in astrocytes
βA3/A1-crystallin is involved in a positive feedback loop with STAT3, which
regulates signaling events essential to the maintenance of cellular
homeostasis in the astrocytes.

Previous studies have suggested that astrocytes secrete factors
essential for vascular remodeling, including VEGF13,33. Our data sug-
ject that βA3/A1-crystallin regulates the secretion of VEGF via the
Notch signaling pathway in astrocytes. In Cryba1 KO mice, the ret-
inal astrocytes are morphologically abnormal, and there is a striking
difference in the organization of the astrocyte template. While vessels
form in the Cryba1 KO retina, the subsequent remodeling process
required to provide a mature vascular network is deficient. The
phenotype in the Cryba1 KO mice and our previous studies on
Notch signaling6, as well as the present study on STAT3 signaling,
suggest that βA3/A1-crystallin is a regulatory protein in astrocytes
that can coordinate interactions among intracellular signaling pro-
teins. For example, we have previously shown that the loss of βA3/
A1-crystallin in astrocytes leads to decreased Notch signaling6. Based
on our data, we also postulated that Notch is involved in juxtacrine
signaling in astrocytes. In Nucl astrocytes, defective juxtacrine
Notch signaling may account for improper template formation and
patterning by retinal astrocytes19. In the present study, loss of βA3/
A1-crystallin affects phosphorylation of STAT3, which inhibits
dimerization and translocation to the nucleus, thereby decreasing
the binding of STAT3 to the Cryba1 promoter to initiate
transcription.

Thus, a positive feedback loop between βA3/A1-crystallin and
STAT3 appears to be crucial for astrocyte patterning in the retina,
as well as for subsequent remodeling of retinal vessels. Support for
such an idea, is obvious in flat-mounts from Cryba1 KO retina, which
show abnormal astrocyte patterning, along with abnormal vascular
branching and budding (Fig. 7). In addition, areas of astrocyte bun-
dles were also observed with increased GFAP staining. The increased
GFAP staining probably results from activation of Müller cells, as
would be expected in response to retinal degenerative changes due to
abnormal blood vessel remodeling. Müller cell processes were evid-
ent in these focal areas with clusters of astrocytes. We have previously

Figure 7 | Cryba1 knockout mice exhibit an abnormal astrocyte template and retinal vessels. Retinal flat mounts from 10 month old Cryba1fl/fl (controls)
or Cryba1 KO mice were stained with GFAP (red; astrocytes) and GS isoelectin (green; blood vessels). Control retina showing normal astrocyte/blood vessel patterning (a, b, c). (c) Higher magnification demonstrated the astrocyte endfeet on retinal vessels (arrow). The Cryba1 KO retinas have
abnormal astrocyte patterning (d–f) with astrocyte aggregates in focal areas (arrows in e and f). In some areas, astrocytes form swirls on retinal vessels
(arrow in g). The retinal vessels of the Cryba1 KO mice show unusual budding and networks (arrow in h) as well as constriction of larger vessels with
abnormal orientation (arrow in i). n= 5 Cryba1fl/fl animals and 5 Cryba1 KO animals. Bar = 500,000 nm (f), 100 μm (a, b, d), and 50 μm (c, e, g, h, i).
reported that astrocytes in the Nuc1 rat, exhibit increased promoter activity for GFAP as well as decreased GFAP protein expression. We speculate that the increased GFAP staining in focal areas of the Cryba1 KO retina is the result of accumulation of both astrocytes and Müller cell processes. It is conceivable that the bundle-like astrocyte aggregates in the Cryba1 KO mice reflect a survival strategy similar to that exhibited by Nuc1 astrocytes in culture when subjected to anoikis-potentiating conditions. We have suggested previously that a subset of Müller cells may be controlled by an anoikis-mediated cell death process. Further, we have shown that loss of β3/A1-crystallin induces IGF-II and increases cell survival by regulating the PI3K/AKT/mTOR and ERK pathways, thereby protecting astrocytes from anoikis-mediated cell death. Therefore, β3/A1-crystallin appears to modulate various intracellular signaling proteins along different signaling pathways, possibly in a juxtapacrine manner amongst astrocytes to maintain cellular homeostasis. Together, these studies demonstrate that β3/A1-crystallin is essential for normal astrocyte function in the retina and that its loss may lead to retinal vascular pathologies.

In summary, our data provide novel evidence that β3/A1-crystallin is a local mediator in astrocytes, modulating the Notch/STAT3 signaling axis, affecting patterning of the astrocyte template, and stimulating the secretion of VEGF required for remodeling of retinal vessels. Such studies provide important insights into the critical interplay between astrocytes and retinal vascular cells, potentially identifying therapeutic targets, such as β3/A1-crystallin, for the treatment of some retinal vascular diseases.

Methods

Antibodies. The following antibodies were used in this study: STAT3 (Cell Signaling Technologies, 9139), p-STAT3 (Cell Signaling Technologies, 9145) and GFAP (Dako, Z0334). Goat anti-rabbit Cy3 (Jackson Immunoresearch, 111-165-003) was used as the secondary antibody. GS isolectin conjugated with Alexa 488 (Life Technologies, ZO334). Goat anti-rabbit Cy3 (Jackson Immunoresearch, 111-165-003) was used as the secondary antibody. GS isolectin conjugated with Alexa 488 (Life Technologies, ZO334).

Quantitative real time PCR. Total RNA was extracted from wild type and Nuc1 cells using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. 2 μg of total RNA was reverse-transcribed to cDNA in a 20 μl reaction volume using Superscript Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA) as described. PCR amplification was performed utilizing the 7500 PCR Fast Real-Time System (Applied Biosystems, Carlsbad, CA) and custom-made TaqMan probes for Cryba1 (Mm00501613_m1), VEGFR1 (Rn00570815_m1), VEGFR2 (Rn00564986_m1), NRPI (Rn00688106_m1), HPRT(Rn01527840_m1) and GAPDH (Rn01775763_g1). All data were analyzed with the ABI 7500 Real-Time PCR system using the data assist software (Applied Biosystems), and the graphs were plotted using Microsoft Excel. All experiments were performed at least three times in triplicate. Data are represented as mean ± standard deviation of the mean (SD). For statistical analysis, Student’s t-test was performed, and a p-value of <0.05 was considered statistically significant.

Luciferase assay. For luciferase assays, lipofectamine 2000 (Invitrogen) was used to transfet cells with the plG3 promoter vector containing the Cryba1 promoter spanning 100 bp on either side of the STAT3 binding site located 2750 bp upstream of the promoter. The vector containing Cryba1 promoter is mentioned as plG3-Cryba1 vector. We also cloned 100 bp on either side of the STAT3 binding site at 1942 bp in intron 2; this vector is referred to as plG3-Cryba1 intron. To generate the Cryba1 promoter deletion construct, the region containing ∼2774 to ∼2756 bp was deleted from the plG3-Cryba1 promoter vector using In-Fusion HD Cloning Kit (Clontech Laboratories Inc., Mountain View, CA) following the manufacturer’s instructions. This vector is referred to as plG3-Cryba1ΔSTAT3. The Notch luciferase reporter construct (2.3 μg) together with pRL-CMV (0.1 μg; Promega, Madison, WI) carrying the Renilla luciferase gene under the control of the cytomegalovirus promoter was co-transfected. Luciferase activity was measured using a luciferase assay system (Promega). The reporter activity was calculated by normalizing the firefly luciferase value with that of the Renilla luciferase control vector and expressed as relative luciferase units. All data were obtained from at least three independent experiments. The relative promoter activities were depicted as the mean ± S.D.

SDS-PAGE and Western blot analysis. SDS-PAGE and Western blot analysis were performed as described previously.

ELISA. Total VEGF in the conditioned medium of cultured cells was measured using quantitative sandwich ELISA kit (R&D Systems) according to the manufacturer’s instructions. Path scan ELISA kit (Cell Signaling Technologies) using STAT3 and p-STAT3 (Tyr 705) coated antibodies was according to the manufacturer’s instructions. The data is represented as absorbance units at 450 nm from at least three independent experiments. Data are represented as mean ± standard deviation of the mean (SD).

Cryba1 knockout (KO) mice. Cryba1 floxed homozygous mice (Cryba1fl/fl) were generated as described previously. Cryba1mice were mated with Best1-c mice that expressCre recombinespecifcally in RPE to generate Cryba1 conditional knockout mice. It is known that germline deletion of floxed alleles may occur when floxed mice are maintained for multiple generations with the BEST1-c allele, creating a global knockout of the floxed gene. We used this as a strategy to generate Cryba1 complete knockout (KO) mice. All animal studies were conducted in accordance with the Guide for the Care and Use of Animals (National Academy Press) and were approved by the Animal Care and Use Committee of Johns Hopkins University.

Retinal flat mounts and Confocal microscopy. Ears were used for flat mounts were fixed and prepared for confocal microscopy to analyze the astrocytes and retinal blood vessels as previously described. GS isolectin conjugated with Alexa 488 (Invitrogen) was used to label blood vessels.

Statistical Analysis. Statistical analysis was performed using Microsoft Excel. The P-values were determined by two-tail Student’s t-test in a triplicate experiment representative of at least three independent experiments. Significance was defined as *P<0.05. Results are presented as mean ± S.D.

1. Garino, R. F. & Gardner, T. W. Retinal angiogenesis in development and disease. Nature 438, 960–966 (2005).
2. Dorrell, M. I., Aguilar, E. & Friedlander, M. Retinal vascular development is mediated by endothelial filopodia, a preexisting astrocyte template and specific R-cadherin adhesion. Invest. OphthalmoL Vis. Sci. 43, 3590–3510 (2002).
3. Benjamin, L. E., Hemo, I. & Keshet, E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development 125, 1591–1598 (1998).
4. Kim, J. H., Kim, J. H., Yu, Y. S., Kim, D. H. & Kim, K. W. Recruitment of pericytes and astrocytes is closely related to the formation of tight junction in developing retinal vessels. J. Neurosci. Res. 87, 653–659 (2009).
5. Stone, J. & Dreher, Z. Relationship between astrocytes, ganglion cells and vasculature of the retina. J. Comp. NeuroL 255, 34–49 (1987).
6. Valapala, M. et al. Impaired endodysplasia function disrupts Notch signaling in optic nerve astrocytes. Nat. Commun. 4, 1629 (2013).
7. Benedito, R. et al. The Notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. Cell. 137, 1124–1135 (2009).
8. Ehling, M., Adams, S., Benedito, R. & Adams, R. H. Notch controls retinal blood vessel maturation and quiescence. Development 140, 3051–3061 (2013).
9. Gridley, T. Notch signaling in vascular development and physiology. Development 134, 2709–2718 (2007).
10. Blanco, R. & Gerhardt, H. VEGF and Notch in tip and stalk cell selection. Cold Spring Harb Perspect. Med. 3, a006569 (2013).
11. Jakobsen, L., Bentley, K. & Gerhardt, H. VEGFRs and Notch: a dynamic collaboration in vascular patterning. Biochem. Soc. Trans. 37, 1233–1236 (2009).
12. Suchting, S. et al. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. Proc. Natl. Acad. Sci. U. S. A. 104, 3225–3230 (2007).
13. Scott, A. et al. Astocyte-derived vascular endothelial growth factor stabilizes vessels in the developing retinal vasculature. PLoS One 5, e11863 (2010).
14. Hirano, T., Ishihara, K. & Hibi, M. Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. Oncogene 19, 2548–2556 (2000).
15. Chen, A. A top-NOTCH way to make astrocytes. Dev. Cell. 16, 158–159 (2009).
16. Nagao, M., Sugimori, M. & Nakafuku, M. Cross talk between notch and growth factor/cytokine signaling pathways in neural stem cells. Mol. Cell. Biol. 27, 3923–3934 (2007).
17. Ge, W. et al. Notch signaling promotes astrogliaogenesis via direct CSL-mediated gial gene activation. J. Neurosci. Res. 69, 848–860 (2002).
18. Rosenblom, K. R. et al. ENCODE data in the UCSC Genome Browser: year 5 update. Nucleic Acids Res. 41, D56–63 (2013).
19. Sinha, D. et al. beta3A1-crystallin in astroglial cells regulates retinal vascular remodeling during development. Mol. Cell. Neurosci. 37, 85–95 (2008).
20. Sinha, D. et al. beta3A1-crystallin is required for proper astrocyte template formation and vascular remodeling in the retina. Transgenic Res. 21, 1033–1042 (2012).
21. Zhong, Z., Wen, Z. & Darnell, J. E. Jr. Stat3: A STAT family member activated by tyrosine phosphorylation and is responsive to epidermal growth factor and interleukin-6. Science 264, 95–98 (1994).
22. Schust, J., Sperl, B., Hollis, A., Mayer, T. U. & Berg, T. Stat3c: a small-molecule inhibitor of STAT3 activation and dimerization. Chem. Biol. 13, 1235–1242 (2006).
23. Pan, Y., Zhou, F., Zhang, R. & Claret, F. X. Stat3 inhibitor Stattic exhibits potent antitumor activity and induces chemo- and radio-sensitivity in nasopharyngeal carcinoma. *PLoS One* **8**, e54565 (2013).

24. Sanseverino, I., Purificato, C., Gauzzi, M. C. & Gessani, S. Revisiting the specificity of small molecule inhibitors: the example of stattic in dendritic cells. *Chem. Biol.* **19**, 1213–1216 (2012).

25. Valapala, M. *et al.* Lysosomal-mediated waste clearance in retinal pigment epithelial cells is regulated by CRYBA1/betaA3/A1-crystallin via V-ATPase-MTORC1 signaling. *Autophagy* **10**, 480–496 (2014).

26. Iacovelli, J. *et al.* Generation of Cre transgenic mice with postnatal RPE-specific ocular expression. *Invest. Ophthalmol. Vis. Sci.* **52**, 1378–1383 (2011).

27. Dorrell, M. I. & Friedlander, M. Mechanisms of endothelial cell guidance and vascular patterning in the developing mouse retina. *Prog. Retin. Eye Res.* **25**, 277–295 (2006).

28. Watanabe, T. & Raff, M. C. Retinal astrocytes are immigrants from the optic nerve. *Nature* **332**, 834–837 (1988).

29. Wechsler-Reya, R. J. & Barres, B. A. Retinal development: communication helps you see the light. *Carr. Biol.* **7**, R433–6 (1997).

30. Hellstrom, M. *et al.* Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature* **445**, 776–780 (2007).

31. Lobov, I. B. *et al.* Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 3219–3224 (2007).

32. Kanski, R., van Strien, M. E., van Tijn, P. & Hol, E. M. A star is born: new insights into the mechanism of astrogenesis. *Cell Mol. Life Sci.* **71**, 433–447 (2014).

33. Gerhardt, H. *et al.* VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J. Cell Biol.* **161**, 1163–1177 (2003).

34. Ma, B. *et al.* betaA3/A1-Crystallin controls anoikis-mediated cell death in astrocytes by modulating PI3K/AKT/mTOR and ERK survival pathways through the PKD/Bit1-signaling axis. *Cell. Death Dis.* **2**, e217 (2011).

35. Zigler, J. S. & Sinha, D. betaA3/A1-crystallin: More than a lens protein. *Prog. Retin. Eye Res.* **44**, 62–85 (2015).

**Acknowledgments**

We thank Dr. Morton F. Goldberg for critical reading and discussion of the manuscript and Dr. Celine Gongora for her help during the course of this study. This research was supported by National Institutes of Health: EY019037 (D.S.), EY019037-S (D.S.), EY009357 (G.L.) and Research to Prevent Blindness (an unrestricted grant to The Wilmer Eye Institute).

**Author contributions**

D.S. designed the study. M.V., M.E. and J.H. conducted the experiments. M.V., M.E., G.L., S.Z., J.Q. and D.S. analyzed the data. S.H. constructed figures for the manuscript and contributed to some experiments. E.F.W., S.Z. and D.S. participated in the generation of Cryba1-floxed and knockout mice. M.V., S.Z. and D.S. wrote the paper. All authors have approved the final manuscript.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

** Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Valapala, M. *et al.* betaA3/A1-crystallin is a critical mediator of STAT3 signaling in optic nerve astrocytes. *Sci. Rep.* **5**, 8755; DOI:10.1038/srep08755 (2015).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder in order to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/