1 **Neuronal apoptosis drives remodeling states of microglia and shifts in survival pathway**
2 **dependence**
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12   **Abstract:**

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14   Microglia serve critical remodeling roles that shape the developing nervous system, responding

15   to the changing neural environment with phagocytosis or soluble factor secretion. Recent single-

16   cell sequencing (scRNAseq) studies have revealed the context-dependent diversity in microglial

17   properties and gene expression, but the cues promoting this diversity are not well defined. Here,

18   we ask how interactions with apoptotic neurons shape microglial state, including lysosomal and

19   lipid metabolism gene expression and independence from Colony-stimulating factor 1 receptor

20   (CSF1R) for survival. Using early postnatal mouse retina, a CNS region undergoing significant

21   developmental remodeling, we performed scRNAseq on microglia from mice that are wild-type,

22   lack neuronal apoptosis (Bax KO), or are treated with CSF1R inhibitor (PLX3397). We find that

23   interactions with apoptotic neurons drives multiple microglial remodeling states, subsets of

24   which are resistant to CSF1R inhibition. We find that TAM receptor Mer and complement

25   receptor 3 are required for clearance of apoptotic neurons, but that Mer does not drive

26   expression of remodeling genes. We show TAM receptor Axl is negligible for phagocytosis or

27   remodeling gene expression but is consequential for microglial survival in the absence of

28   CSF1R signaling. Thus, interactions with apoptotic neurons shift microglia towards distinct

29   remodeling states and through Axl, alters microglial dependence on survival pathway, CSF1R.

Introduction:

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31   Microglia are parenchymal innate immune cells and vital remodelers of the central nervous

32   system (CNS) [1]. They have a myriad of critical functions during development, including

33   elimination of viable or dying cells [2]. Similar to other macrophages, microglia are extremely

34   attuned to changes in their neural niche [3-5] and rapidly respond by phagocytosis or secreting

35   soluble factors. Recent single-cell RNA sequencing (scRNAseq) has demonstrated that

36   microglia are particularly diverse during development [6, 7]. However, we still lack an

37   understanding of the drivers of this heterogeneity, including the impact of environmental cues or

38   the act of phagocytosis itself. Therefore, connecting microglial states to specific developmental

39   events and determining the pathways involved remains a central challenge.

40

41   One developmental process fundamental for nearly every organ in the body is apoptotic death

42   of excess or dysfunctional cells [8]. In the CNS, death of neurons and glia is an essential and

43   well-documented feature of development [9]. Efferocytosis, or the phagocytosis of apoptotic

44   cells, is a rapid and carefully orchestrated process designed to minimize damage to surrounding

45   cells [10, 11]. Largely mediated by microglia, efferocytosis is important for maintaining CNS

46   homeostasis not only in development but in aging and disease as well [12, 13]. The developing

47   mouse retina is an excellent CNS model system to link remodeling events such as efferocytosis

48   and microglial state and function [14]. During the postnatal period in the retina, as circuits are

49   being established, waves of neuronal death occur [15]. For example, retinal ganglion cells

50   (RGCs), the projection neurons connecting retina and brain, undergo a well-characterized

51   culling during the first postnatal week which is dependent on pro-apoptotic factor, Bcl-2-

52   associated X protein (Bax) [16]. An estimated ~50% of RGCs will undergo apoptosis and need

53   to be cleared [17, 18]. We previously found that neuronal death significantly influences microglia

54   properties in postnatal retina, resulting in a majority of microglia expressing a distinct gene

55   signature and showing independence from colony-stimulating factor 1 receptor (CSF1R)

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56   signaling for survival [19]. Relative to adult microglia, homeostatic genes were reduced while

57   genes associated with phagocytosis and lipid metabolism were increased, similar to aging and

58   disease-associated microglia (DAM) [20-22], and to developmental microglia residing in

59   postnatal white matter tracts (PAM) [7] or (ATM) [6] or CD11c microglia [23].

60

61   Consistent with either apoptotic cell recognition or phagocytosis playing a central role in driving

62   this gene signature, microglia in disease states increase select DAM genes in response to

63   apoptotic cells [22], and ATM/PAM populations in the white matter tract engulf oligodendrocyte

64   precursor cells [7, 24]. In disease, the TREM2 receptor is partially required for acquisition of the

65   DAM signature [21, 22], but not for the developmental signature in retina [19] or brain [7]. Thus,

66   it remains unclear to what extent the process of phagocytosis is altering properties of

67   developmental microglia or whether distinct recognition pathways are involved. Microglia, like

68   other macrophages, can recognize and engulf apoptotic (or viable) cells via a variety of ligand-

69   receptor systems including C1q/C3 to complement receptor 3 (CR3) and exposed

70   phosphatidylserine (PtdSer) to TAM family of tyrosine kinase receptors Mer (gene Mertk) and

71   Axl [25], but how these pathways mediate changes in microglia properties and responses is an

72   active area of research.

73

74   Here, we ask how neuronal apoptosis drives key properties of developmental microglia such as

75   CSF1R independence and expression of DAM/ATM/PAM-related genes, and test the role of

76   phagocytosis and specific recognition pathways. Through scRNAseq on postnatal retinal

77   microglia we find considerable transcriptional heterogeneity, with multiple distinct microglial

78   populations expressing DAM/ATM/PAM-related, lysosomal, and lipid metabolism genes. We

79   further show, by analyzing microglia from Bax knockout (KO) retinas, that exposure to dying

80   neurons drives several of these states and that most are more resistant to CSF1R inhibition. We

81   find that CR3 and Mer are required for clearance of dying RGCs, but not for augmenting

82   microglial survival in the absence of CSF1R signaling. Conversely, Axl is not required for

83   clearance of dying RGCs, but for mediating independence from CSF1R signaling for survival.

84   Loss of Mer did not have a widespread impact on expression of microglial remodeling genes,

85   and neither did loss of Axl or Mertk/Axl double KO (dKO), suggesting that TAM receptor-

86   mediated signaling or reduced clearance are not sufficient. Thus, we find that interactions with

87   apoptotic neurons drives developmental microglial diversity, and that distinct recognition

88   receptors mediate phagocytosis of dying cells versus specific microglial properties, including

89   microglial survival.

90

91   **Results:**

92   **Multiple microglial states coexist in postnatal retina**

93   To better understand the influence of environmental cues on microglial states, we first sought to

94   understand the extent of microglial heterogeneity in the postnatal retina and then explicitly

95   determine states that were driven by neuronal apoptosis. Second, we previously linked CSF1R

96   independence to a DAM/ATM/PAM-related signature and neuronal apoptosis [19], so we

97   wanted to identify and characterize microglial states that remained following CSF1R inhibition

98   (PLX3397, PLX). Therefore, we performed scRNAseq on sorted retinal microglia from 4 groups

99   at postnatal day 6 and 7 (P6/P7): Bax WT, Bax KO, CX3CR1-GFP/+ given vehicle (daily for 3

100   days), and CX3CR1-GFP/+ pups dosed with PLX3397 (daily for 3 days) (Figure 1A). Microglia

101   from Bax WT and littermate KOs (CD45$^+$ CD11b$^+$/CX3CR1-GFP$^+$ CCR2$^-$) as well as microglia

102   from PLX and vehicle controls (CD45$^+$ CX3CR1-GFP$^+$ Ly6C$^-$) were sorted (Supplemental 1A-D)

103   and sequenced using the 10X Genomics platform. Following sequencing and manual filtering

104   (Supplemental 2), we used in silico Bax genotyping to sort out and reassign a subset of cells

105   from animals incorrectly assigned to the Bax WT and KO groups (Supplemental 3).

106   Unsupervised clustering was performed on the 15,084 cells from the four groups (4,902 - Bax

107   WT; 4,224 - Bax KO; 4,198 - Vehicle; 1,760 - PLX3397) (Supplemental 4A). Of the 15,084 cells,

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108   1,417 in satellite clusters were deemed non-microglia cells based on expression of established

109   markers and were excluded from further analysis (Supplemental 4B-E). Differences in cell

110   surface markers used to sort Bax WT/KO (CD45$^+$CD11b$^+$CX3CR1$^+$CCR2$^-$) compared to

111   PLX/Vehicle (CD45$^+$CX3CR1$^+$Ly6C$^-$) (Supplemental 1) led to variance in the proportion of

112   macrophage/monocyte-like cells (Supplemental 4F), but this had no major impact on microglial

113   cell number (Gray dots, Supplemental 4C,F).

114

115   Unsupervised re-clustering of 13,667 remaining cells yielded 11 microglia clusters (Figure 1B).

116   Cells from controls (Bax WT and Vehicle) were represented in every cluster (Figure 1C),

117   indicating that microglia in an individual developing CNS region during a specific time window

118   are strikingly diverse. Further, we found that Bax KO and PLX-treated microglia had dramatic

119   and opposing shifts in their distribution compared to controls (Figure 1C). To determine the

120   characteristics of the 11 clusters, we examined the top genes for each (Figure 1D,

121   Supplemental Table 1). While virtually every cell expressed *Apoe* and *Ctsd*, consistent with our

122   previous findings [19], both genes were significantly enriched in cluster 0, which had high

123   expression of other DAM/PAM/ATM genes such as *Lyz2* and *Cd9*, but also intermediate levels

124   of homeostatic gene expression such as *P2ry12* and *Tmem119*. Thus, we called this cluster

125   Apoe-enriched. Cluster 1 had highest expression of *Tmem119* and *P2ry12* as well as other

126   homeostatic genes, including *Itgam, Siglech, Tgfbr1, P2ry13, Selplg, and Adgrg1* and so we

127   named it Homeostatic. Cluster 2 was enriched for chemokines including *Ccl4* and *Ccl3,* two

128   genes previously found in injury-responsive microglia in demyelinated lesions [6] and in PAM of

129   developing WM tracts [7], and had intermediate/high expression of homeostatic genes (e.g.

130   *Tmem119*; Figure 1D) and so we termed it Hom/chemokine cluster. Since *Ccl3* and *Ccl4* can be

131   induced by dissociation [26] we confirmed expression of *Ccl3* by *in situ* hybridization in whole

132   mount retina (Supplemental 5). Cluster 3 was marked by *Npl*, found in lipid-droplet microglia

133   [27], and *Apoc1*, a lipoprotein high in MS-associated human microglia [28]. Since this cluster

134   was substantially increased in PLX-treated retinas, we named this cluster PLX-enriched.

135   Chemokines *Cxcl2* and *Cxcl10* and pro-inflammatory cytokines, such as *Il1b* and *Tnf,* were high

136   in Cluster 4, dubbed the Chemokine/cytokine cluster. Cluster 5 most resembled ATM [6] (43/68

137   genes shared) and PAM [7] (41/68 genes shared) with specific expression of *Spp1* and high

138   expression of *Fabp5, Ctsl, Lpl, Igf1,* and *Csf1* and thus we termed it ATM/PAM-like. Cluster 6,

139   was named Antioxidant-responsive cluster due to high expression of antioxidant responsive

140   genes, *Hmox1* and *Gclm* [29]. Clusters 7, 8, and 9 represented Cycling microglia and expressed

141   *Mki67, Top2a*, and *Mcm* genes. Cluster 10, the smallest, contained 71 cells with high and very

142   specific expression of interferon response genes *Ccl5, Ifit3* and *Ifitm3* [30] and was thus termed

143   Interferon-responsive cluster. Thus, these data suggest that within the developing retina,

144   several microglial states coexist including homeostatic and various subsets of DAM/PAM/ATM-

145   related and chemokine-expressing microglia.

146

Postnatal retinal microglia encompass a spectrum of homeostatic to remodeling states

148   We noted a continuum of expression of the homeostatic genes *Tmem119* and *P2ry12* across

149   the clusters that appeared to be inverse to broadly expressed DAM/PAM/ATM genes such as

150   *Apoe, Ctsd* (Figure 1D). We more closely examined the expression of homeostatic genes, as

151   well as genes associated with lysosomal function, lipid metabolism and transport, and other

152   DAM/PAM/ATM-related genes. As for *Tmem119* and *P2ry12*, we found a gradient of expression

153   of other homeostatic genes including *Siglech, Tgfbr1, Mafb* and *Selplg* which were highest in

154   Homeostatic cluster (1), high/intermediate in Hom/chemokine (2), intermediate in Apoe-enriched

155   (0), Chemokine/cytokine cluster (4), ATM/PAM-like (5), and Antioxidant-responsive (6), with

156   lowest expression in PLX-enriched cluster (3), suggesting a spectrum from more to less

157   homeostatic (Figure 2B).

158

159   To determine whether this related to shifting phagocytic function of microglia, we examined the

160   expression of lysosomal genes *Cst7, Lyz2, Galns* and *Ctsb* and saw roughly the opposite

161   pattern. These genes were either absent or sparse in Homeostatic cluster (1) and

162   Hom/chemokine (2) and more highly expressed in Apoe-enriched (0), PLX-enriched (3),

163   Chemokine/cytokine cluster (4), ATM/PAM-like (5), and Antioxidant-responsive (6) (Figure 2C).

164   Notably, *Lyz2* showed highest levels in PLX-enriched (3) the least homeostatic cluster.

165   Additionally, genes involved with lipid metabolism and cholesterol transport, important

166   processes following the engulfment of apoptotic cells [31], followed the same trend (Figure 2D).

167   Thus, microglia in clusters (0,3,4,5 and 6) are equipped to be actively breaking down

168   phagocytosed material compared to more homeostatic clusters (1,2). Therefore, we conclude

169   that clusters 0,3,4,5, and 6 are involved in active remodeling activities and will refer to them as

170   remodeling clusters (Figure 2A). Since we saw enrichment of *Apoe* and *Ctsd* in these

171   remodeling clusters, we examined the expression of additional DAM/ATM/PAM-related genes,

172   including *Cd9, Igf1*, as well as the recognition receptors *Itgax* and *Axl*. We noted similar low

173   expression in Homeostatic cluster (1) and Hom/chemokine (2) and high expression in the

174   remodeling clusters (0,3,4,5,6) (Figure 2E). Interestingly, DAM/PAM/ATM-related genes had

175   varying cluster specificity. Genes such as *Apoe* were expressed in nearly every cell, whereas

176   *Igf1* and *Spp1* were more restricted to specific clusters, suggesting that regulation of these

177   genes is complex (Figure 1D, 2E). Together, these results suggest that phagolysosomal

178   function defines the spectrum of microglial states in the postnatal retina.

179

180   **Neuronal apoptosis drives multiple remodeling states**

181   We previously showed that neuronal death has a major influence on expression of remodeling

182   genes in postnatal retinal microglia [19] and wondered whether recognition or clearance of dying

183   neurons was a key factor in driving diverse microglial remodeling states. We examined microglia

184   from Bax KO retinas in which apoptotic death programs in RGCs and other neurons are

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185   selectively lost [32] and saw a dramatic shift in cluster distribution with the loss of Bax compared

186   to littermate controls (Figure 3A). We found a 5-fold expansion in the Homeostatic cluster (1) in

187   Bax KO compared to WT (Figure 3A,B,C). This was concurrent with decreases in remodeling

188   clusters Apoe-enriched (0), Chemokine/cytokine expressing (4), and ATM/PAM-like (5), and the

189   minor PLX-enriched cluster (3), which were 4.7-fold, 3.46-fold, 5.86-fold, and 5-fold more

190   abundant in WT, respectively (Figure 3A,B). Clusters that remained largely unaltered included

191   Hom/chemokine (2), Antioxidant-responsive (6), Cycling (7-9), and Interferon-responsive (10),

192   suggesting they were not driven by apoptotic cell interactions or clearance (Figure 3A,B).

193   Therefore, we conclude that the spectrum of homeostatic to more remodeling clusters (0,3,4

194   and 5) in the postnatal retina is driven predominately by neuronal apoptosis.

195

196   To further examine microglial genes dependent on neuronal apoptosis, we compared all cells

197   from each genotype, ignoring cluster membership, and identified genes that were specific to

198   each sample (Figure 3D, Supplemental Table 2). Consistent with a shift to a more homeostatic

199   state, *Tmem119, P2ry12*, and *Siglech* were among the 133 upregulated genes in microglia from

200   Bax KO retina. The 183 downregulated genes included lysosomal and lipid metabolism genes

201   such as *Apoe, Lyz2, Cst7, Ctsb, Lpl*, and *Cd68,* DAM/PAM/ATM-related genes such as *Igf1* and

202   *Spp1,* and recognition receptors *Itgax* and *Axl* (Figure 3C,D). We next performed KEGG

203   analysis using GeneCodis 4 [33] on downregulated genes in Bax KO to identify major pathways

204   driven by interactions with dying neurons (Figure 3E). Consistent with the idea that microglial

205   phagocytosis was a key factor in these gene expression changes, we found metabolic and

206   lysosomal pathways significantly reduced. To determine whether loss of Bax altered microglia

207   density or distribution, we used C1q as a marker for microglia, which we validated by both

208   scRNAseq (Supplemental 4C) and immunostaining (Supplemental 6A,B). We found that

209   microglia remained uniformly spaced but that density was reduced by nearly half in Bax KO

210   retinas (Supplemental 6C,D), consistent with our prior flow cytometry analysis [19]. Altogether,

211   this suggests that neuronal apoptosis regulates important properties of microglia in the postnatal

212   retina, including overall density as well as the emergence of multiple remodeling states.

213

214   **Subsets of remodeling states survive CSF1R inhibition, while homeostatic microglia are**

215   **more vulnerable**

216   A striking property of microglia in postnatal retina is that ~60% survive inhibition of CSF1R

217   signaling [19], while only very small subsets show this property in adult brain [34]. We previously

218   found that surviving microglia had reduced homeostatic gene expression and increased

219   DAM/PAM/ATM gene expression, and this required neuronal apoptosis [19]. Since we found

220   that the less homeostatic remodeling clusters (0,3,4,5) were driven by neuronal apoptosis, we

221   investigated whether these microglial states would be more resistant to CSF1R inhibition. We

222   examined scRNAseq of Vehicle and PLX-treated microglia (dosed for three days) and found a

223   large shift in the distribution of cells across clusters with PLX treatment (Figure 4A). Several

224   remodeling clusters were either enriched or maintained. The least homeostatic cluster, PLX-

225   enriched (3), which was a very small proportion of cells in both Vehicle and Bax WT (Figure

226   3A,B), represented nearly 60% of remaining microglia following PLX3397 (increased 60-fold)

227   and thus seemed to arise, in part, by treatment (Figure 4A,B). This could potentially be due to

228   the further reduction of homeostatic properties of surviving microglia with CSF1R blockade [35].

229   Remodeling cluster Chemokine/cytokine (4) was increased 2-fold with PLX-treatment and

230   ATM/PAM-like (5), Antioxidant-responsive (6), and Interferon-responsive (10) were slightly

231   reduced or unchanged (0.755-fold, 0.654-fold, 0.979-fold, respectively), suggesting they are

232   more resistant than other subsets (Figure 4A,B). Conversely, we found clusters that had high or

233   intermediate expression of homeostatic genes, Apoe-enriched (0), Homeostatic (1),

234   Hom/chemokine (2), and Cycling (7,8,9) were largely absent following PLX treatment,

235   suggesting these clusters are more dependent on CSF1R for survival or that they had shifted to

236   a different state (Figure 4A,B). We noted the overlap between clusters dependent on neuronal

237   apoptosis (0,3,4 and 5) and resistant to loss of CSF1R signaling (3,4,5,6 and 10), illustrating the

238   link between the two and further arguing that certain microglial states driven by neuronal

239   apoptosis confer CSF1R independence. Furthermore, we found that downregulation of *Csf1r*

240   alone does confer independence as more resistant clusters (3,4,5 and 6) had slightly reduced

241   expression of *Csf1r* compared to susceptible clusters (0,1, and 2) but comparable to other

242   susceptible clusters (7,8,9) (Figure 4D), consistent with our previous findings [19].

243

244   To further probe genes and pathways enriched following CSF1R inhibition, we performed a

245   pairwise comparison on all Vehicle and PLX-treated cells, regardless of cluster. We found that

246   DAM/ATM/PAM-related genes *Apoe, Itgax,* and *Fabp5*, lysosomal genes *Lyz2* and *Ctss,* and

247   lipid metabolism genes *Nceh1*, *Soat1, Abca1,* and *Apoc1* were among the 254 upregulated

248   genes in PLX-treated cells (Figure 4C,E, Supplemental Table 3). Consistent with a loss of more

249   homeostatic microglia, homeostatic genes including *Tmem119, P2ry12,* and *Siglech* were

250   significantly reduced (181 genes downregulated) (Figure 4C,E). To uncover pathways

251   associated with CSF1R independence, we performed KEGG analysis [33] on all upregulated

252   genes in PLX cells and found that translation, metabolic pathways, and phagosome processes

253   were enriched (Figure 4F). This suggested that CSF1R independent microglia may be in an

254   altered metabolic state due to phagolysosomal processes. Altogether, we conclude that CSF1R-

255   independence is linked to increased phagocytosis, lysosomal function, and altered microglia

256   metabolic states, and that homeostatic microglia are more dependent on CSF1R signaling for

257   survival.

258

259   **Mer and CR3 are required for apoptotic RGC clearance**

260   To understand the role of apoptotic cell phagocytosis in promoting distinct microglial properties,

261   we sought to test whether recognition receptors were important both for driving microglial

262   remodeling states as well as CSF1R independence. We first wanted to confirm that microglia

263   were important for clearance of dying neurons within the retina and identify the recognition

264   receptors required, focusing on apoptotic RGCs. We performed a screen of genetic KO of

265   receptors previously implicated in the finding, recognition, or phagocytosis of apoptotic cells:

266   find-me pathway, fractalkine receptor CX3CR1 [36], integrin receptor complement receptor 3

267   (integrin $\alpha_M\beta_2$, CD11b, CR3), TAM receptors Mer and Axl (Figure 5A) [25]. We used wildtype

268   animals from the CX3CR1 (CX3CR1-WT) and Mer (Mertk-WT) background as well as CX3CR1-

269   GFP/+ [37] for controls. First, we looked at whether loss of any of these receptors resulted in

270   changes to microglial density. By wholemount immunostaining, we found slight variations at P5

271   but none that reached significance compared to CX3CR1-GFP/+ (Supplemental Figure 7A,B).

272   Next, we analyzed the density of total apoptotic bodies by cleaved caspase 3 (CC3) in the

273   ganglion cell layer shortly after peak RGC death to measure any buildup or failure to clear

274   (Supplemental Figure 7C,D and Supplemental Figure 8A). We found that loss of CX3CR1, CR3,

275   Mer, and both Mer/Axl resulted in increased apoptotic body density compared to WT controls

276   suggesting these pathways were important for microglial phagocytosis of dying cells within the

277   retina. To ask what pathways were important for clearing dying RGCs specifically, we looked at

278   the density of CC3$^+$RBPMS$^+$ double-positive cells at P5 in all KOs (Figure 5B,C,D,

279   Supplemental Figure 8B). We found the CR3 KO, Mertk KO, and Mertk/Axl dKO all had

280   increased density of dying RGCs compared to controls (Figure 5B,C) with no change in overall

281   RGC density or retinal blood vessel development (Supplemental Figure 7E,F,G,H). CX3CR1

282   and Axl were dispensable for clearance of dying RGCs and Mertk/Axl dKOs did not appear to

283   have a further clearance deficit above Mertk KO alone (Figure 5B,C). Thus, both CR3 and Mer

284   receptors, which are broadly expressed in microglia, are important for the timely clearance of

285   RGCs undergoing developmental cell death, consistent with prior studies implicating these

286   pathways in efferocytosis in the CNS.

287

288   **Axl promotes microglial survival in the absence of CSF1R signaling**

289   Having identified receptors important for RGC clearance (CR3 and Mer), we were next able to

290   ask whether these pathways were also important for driving microglial survival following CSF1R

291   inhibition. We administered PLX3397 as previously published [19] (Figure 6A) and, surprisingly,

292   found that loss of CR3, CX3CR1, or Mer did not significantly reduce the proportion of surviving

293   microglia compared to controls (Figure 6B,C). However, loss of Axl allowed for greater

294   depletion, matching levels achieved with loss of developmental apoptosis (Bax KO) that we

295   previously reported [19] (Figure 6B,C). Mertk/Axl dKOs did not have an additive effect,

296   suggesting Axl signaling alone was important for microglial survival in the absence of CSF1R

297   signaling (Figure 6B,C). Altogether, we find that the receptors important for effective clearance

298   (Mer and CR3) do not alter microglial dependence on CSF1R, but that TAM receptor Axl, which

299   is induced by neuronal apoptosis, augments microglial survival in the absence of CSF1R

300   inhibition in retina.

301

302   **Mer and Axl are not required for expression of lysosomal, lipid metabolism, or**

303   **remodeling genes**

304   Since Mer was important for phagocytosis while Axl mediated survival in the absence of CSF1R

305   signaling, we wanted to delineate microglial gene expression changes that were driven by Mer

306   versus Axl. We performed bulk RNA-seq on sorted microglia from P4 Mertk and Axl KOs and

307   compared them to WT controls by DESeq2 (Figure 7A). Compared to WT controls, we found

308   modest changes in gene expression, with 42 downregulated genes and 44 upregulated genes in

309   Mertk KO microglia (Figure 7B,D, Supplemental Table 4). However, despite the fact that Mertk

310   KOs had reduced clearance of dying RGCs, there was no effect on lysosomal or lipid

311   metabolism genes. Further, DAM/ATM/PAM-related genes were also not significantly reduced,

312   but, in fact, *Csf1* and *Itgax* were significantly increased (Figure 7B,D).

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13

314   Comparison of Axl KO to WT microglia revealed even fewer differentially expressed genes, with

315   no change in lysosomal, lipid metabolism, or DAM/ATM/PAM-related gene expression, except

316   the downregulation of *Spp1*, and no change in genes associated with survival pathways (Figure

317   7C,D, Supplemental Table 4). The majority of differentially expressed genes were different in

318   the two KOs (Figure 7D); however, sequencing of microglia from P7 Mertk/Axl dKO retinas

319   again revealed no change in lysosomal, lipid metabolism, or DAM/PAM/ATM-related genes

320   (Supplemental 9A,B, Supplemental Table 5). Based upon these findings, we first conclude that

321   while phagocytosis is altered in Mertk KOs, this is not sufficient to suppress expression of

322   remodeling genes, suggesting that Mer-mediated signaling does not strictly drive microglia

323   remodeling gene expression. Furthermore, while Axl facilitates microglia survival after CSF1R

324   inhibition, it is also not required to drive microglia remodeling gene expression, raising the

325   possibility that Axl itself, through recognition of apoptotic neurons, could be mediating survival.

326

327   **Discussion:**

328   scRNAseq has revealed that microglia in development and disease are transcriptionally diverse

329   across the CNS [38]. Due to their remarkable plasticity, a major challenge is to determine the

330   impact of local cues on microglial state and properties. Here, using the postnatal retina as a

331   model system, we establish neuronal cell death as a key factor driving developmental microglia

332   diversity. We show by scRNAseq that multiple microglial states coexist within this discrete CNS

333   region, encompassing a spectrum from homeostatic to remodeling states with high lysosomal

334   and lipid metabolism gene expression. We establish that multiple distinct remodeling states are

335   driven by neuronal apoptosis, and further show that several of these remodeling states survive

336   CSF1R inhibition, while more homeostatic microglia are susceptible. CR3 and Mer are important

337   for clearance of apoptotic retinal neurons, while Axl facilitates microglial survival following

338   inhibition CSF1R, but neither significantly regulates expression of microglial remodeling genes.

339   Thus, we conclude that cell death is a critical cue for driving diverse microglial properties and

340   that multiple pathways contribute.

341

342   We identify several states of remodeling microglia characterized by reduced homeostatic gene

343   expression and elevated lipid metabolism and lysosomal genes, with more discrete expression

344   of various genes including chemokines/cytokines. Many of the genes in these remodeling states

345   are shared with microglia in developing white matter tracts of the brain (CD11c/ATM/PAM) [6, 7,

346   39] and disease (DAM) [20-22]. Unlike in brain, we find they represent a majority of microglia in

347   the early postnatal retina, with most of them dependent upon neuronal apoptosis. Due to the

348   large proportion of microglia expressing these remodeling genes, we broaden our

349   understanding of microglia subsets present during normal development. For example, we find

350   genes such as *Apoe* and *Ctsd* are widely expressed at varying levels, while *Fabp5* and *Spp1*

351   are confined to more discrete subsets, suggesting that regulation of these genes and microglial

352   states is multifaceted. At present, it remains unclear whether they transition from state to state.

353   Since we find an increase in the proportion of homeostatic microglia in Bax KO retinas, this

354   argues that microglia in the context of neuronal death shift from a homeostatic state to more

355   remodeling ones.

356

357   In other contexts, efferocytosis can have a major impact on the transcriptional profile and state

358   of the phagocyte. For example, phagocytosis promotes distinct transcriptional states in

359   macrophages in a tissue-specific manner throughout the body [40], and tissue macrophages

360   modify their repertoire of phagocytic machinery in response to phagocytosis [41]. In the CNS,

361   there is much to learn about how phagocytosis impacts microglial state and function [13, 42].

362   One study addressing this found engulfment of newborn neurons by microglia in adult

363   neurogenic zones stimulates a microglial secretome that subsequently regulates neurogenesis

364   [43]. Here, in the postnatal retina, phagocytosis and digestion appear to be a major aspect of

365 microglial heterogeneity. We postulate that some remodeling states may even represent distinct

366 stages of the clearance process. Our analysis also identified microglial states that are not

367 regulated by neuronal death, arguing they may be involved in other important microglial

368 functions during postnatal retinal development. For example, astrocytes are also pruned by

369 microglia in the postnatal retina, which has been shown to be Bax-independent and also not

370 require CR3, Mer, or CX3CR1 pathways [44]. Future studies could target these specific

371 microglia subsets and link them to other important remodeling functions in the developing retina.

372

373 Despite the fact that postnatal RGC death has been a well-characterized model of

374 developmental cell death of the CNS [16], the recognition pathways required for the clearance

375 of apoptotic RGCs was previously undefined. CR3 and Mer are well-known recognition

376 receptors in the clearance of stressed or dying neurons in various contexts [25]. Here, we show

377 that both receptors are important for clearance of apoptotic RGCs. Previous work has

378 suggested that complement component C1q may get deposited on exposed PtdSer [45],

379 suggesting the pathways might work together to signal for phagocytosis, but whether

380 complement and TAM pathways are working redundantly or cooperatively is unknown in any

381 context. While receptors Mer and Axl are both members of the TAM receptor family of tyrosine

382 kinases [46], it has been appreciated that Mertk is more stably expressed in microglia, while Axl

383 is more dynamic and upregulated with inflammation or disease [47]. Both have previously been

384 implicated in phagocytosis and in regulating inflammatory responses [48], however this is cell

385 type-dependent [49]. Here, we find Mertk/Axl dKO did not have an enhanced clearance deficit

386 for apoptotic RGCs compared to Mertk KO, although this could be due to a plateau effect since

387 RBPMS likely gets downregulated as apoptotic programs continue [50].

388

389 Although Mer is important for clearance, we did not see a dramatic reduction in lysosomal or

390 lipid metabolism genes in either Mertk or Axl KO microglia. These results are consistent with

16

391   disease contexts where loss of Mertk and Axl do not have a dramatic effect on the DAM

392   signature in mouse model of Alzheimer's disease [51]. This suggests that transcriptional

393   changes in postnatal retinal microglia may depend upon other pathways for apoptotic cell

394   recognition. An alternate possibility is that Mertk KO microglia are still clearing, but at a reduced

395   rate, since live-imaging has shown that loss of Mer results in delayed engagement with

396   apoptotic cells but does not abolish phagocytosis completely [52].

397

398   We previously found that a large proportion of microglia in the postnatal retina were more

399   resistant to inhibition of the important survival pathway CSF1R [19]. Here, we identify specific

400   remodeling states of microglia that persist following CSF1R inhibition showing reduced

401   homeostatic gene expression. Other groups recently found small populations of CSF1R-

402   independent or repopulating microglia in the brain, and we note similar patterns of gene

403   expression, with reduced homeostatic genes such as *Tmem119* and *P2ry12*, and increased

404   expression of genes such as *Lyz2* [34, 53]. In these microglia repopulation studies, it was

405   proposed that more immature microglia survive CSF1R inhibition, but microglia maturation may

406   not be a key factor in the context of development since we previously found greater dependence

407   of retinal microglia on CSF1R at embryonic stages than in postnatal retina [19]. We find that

408   most of these resistant microglia subsets are induced by neuronal apoptosis, consistent with our

409   prior analysis showing increased microglial dependence on CSF1R in Bax KO retina [19].

410   Interestingly, in culture, peritoneal and bone marrow macrophages that phagocytose apoptotic

411   cells also survive in the absence of serum or survival factors [54]. In periods of elevated cell

412   death and possible lack of extrinsic survival factors, such as neuronally expressed CSF1R

413   ligand, IL-34 [55], phagocytes such as microglia might have evolved strategies to survive in

414   order to continue their job of maintaining homeostasis or reducing inflammation [54].

415   Importantly, cholesterol, along with CSF-1/IL-34 and TGF-ß2, has been shown to be an

416   important survival factor for microglia in culture [56]. We observed that PLX-treatment resulted

417   in enrichment of microglia with the lowest levels of homeostatic gene expression and high levels

418   of lysosomal and lipid metabolism genes. Thus, CSF1R signaling may also be important for

419   promoting microglial homeostasis [57]. Consistent with this, mutations in CSF1R can lead to

420   leukoencephalopathy, a neurodegenerative disorder which is associated with loss of

421   homeostatic microglia phenotype [35]. It will be valuable for future studies to explore the

422   contributions of CSF1R signaling to microglial survival and homeostasis during development.

423

424   Here, we find that Axl expression, which is regulated by neuronal apoptosis, enhances

425   microglial survival in the absence of CSF1R signaling. Since loss of Axl did not substantially

426   alter expression of other candidate apoptotic or survival pathways in microglia, one possibility is

427   that signaling directly downstream of Axl is involved. Axl has been shown to inhibit apoptosis in

428   a variety of cell types [58] including fibroblasts [59], oligodendrocytes in contexts of growth

429   factor withdrawal or TNF toxicity [60], and in Gonadotropin-releasing hormone neurons in the

430   brain [61]. Its role in various types of cancer has been well-established for promoting tumor

431   growth and metastasis in part due to its ability to inhibit apoptosis of cancer cells [62]. Axl has

432   diverse downstream signaling cascades depending on context [58], but one candidate signaling

433   cascade is the PI3K-Akt-NfkB-Bcl2 pathway. Activation of PI3K and Akt has shown to be

434   important for survival of cultured macrophages [54], oligodendrocytes [60, 63], neurons [61] as

435   well as cancer cells [58].

436

437   Altogether, we show neuronal cell death is a key factor driving multiple states of microglia in

438   developing retina, with distinct transcriptional profiles and independence from CSF1R signaling

439   for survival. Apoptotic cell recognition by microglia is thus a critical developmental event driving

440   diverse effects on gene expression, clearance and survival, with distinct pathways involved in

441   mediating each of these responses.

442

449 **Materials and Methods:**

450 **Experimental Model and Subject Details**

451

452 Animal husbandry and procedures

453 All animals were treated within the guidelines of the University of Utah Institutional Animal Care

454 and Use Committee (IACUC) and all experiments were IACUC approved. Mice were housed in

455 an AAALAC accredited animal facility with 12h light/12h dark cycles and ad libitum access to

456 food and water. Both sexes were used for all experiments. Information on the ages of mice used

457 for each experiment can be found in the figures/text. Pexidartinib (PLX3397) (AdooQ

458 BioScience A15520) was dissolved in corn oil (Sigma-Aldrich C8267) and 10% DMSO (Fisher

459 Scientific BP231) and administered to postnatal pups by daily intraperitoneal injection P3-P5 or

460 P4-P6 at 0.25 mg/g body weight. Mice were euthanized by isoflurane asphyxiation followed by

461 decapitation.

462

463 Mouse strains

464 The B6.CX3CR1-GFP/+ mice were a gift from Richard Lang with permission from Dr. Steffen

465 Jung [37]. Bax KO mice (JAX 002994) and B6. CD11b KO (JAX 003991) were purchased from

466 the Jackson Laboratory. The B6.Mertk KO and B6.Axl KO strains were a kind gift from Dr. Greg

467 Lemke.

468

469 **Method Details**

470 Tissue processing

471 Following euthanasia, retinas were dissected in ice-cold 0.1M PBS. Whole heads were fixed in

472 4% PFA for 45 minutes to an hour. Heads were washed 3 times for 15 minutes in PBS and

473 underwent 12-16-hour consecutive treatments with 15% and 30% sucrose in PBS at 4°C.

474 Heads were then embedded in OCT compound (Ted Pella 27050), stored at -80°C, and

20

475 sectioned at 16μm thickness. For retinal whole mounts, eyes were removed from the head and

476 retinas were carefully dissected from the rest of the eye (cornea, lens, RPE, hyaloid

477 vasculature, vitreous, ciliary body) in ice-cold PBS. Whole neural retinas were washed in PBS

478 for 10-20 minutes and then fixed in 4% PFA for 30-45 minutes at room temperature. For

479 RNAase-free dissections for qHCR or FACs, retinas were carefully dissected in RNase-free

480 conditions using ice-cold, sterile RNase-free PBS, removing all non-neural eye tissue (ciliary

481 body, pigmented epithelium, vitreous).

482

483 Immunohistochemistry

484 As previously done [19], frozen sections were incubated at 37°C for 30 minutes, post-fixed for

485 15 minutes in 4% PFA and washed in ice cold PBS for 3 times for 5 minutes. Then they were

486 blocked for 1 hour at room temperature (0.2% triton-X, 10% BSA, 10% normal donkey serum in

487 0.01M PBS), and incubated in primary antibody overnight at 4°C in (0.2% triton-X, 5% BSA in

488 0.01M PBS). The following day, sections were washed 3 times with PBS and incubated in

489 secondary antibodies (5% BSA in PBS) for 2 hours at room temperature, washed, and mounted

490 with Fluoroshield mounting medium with DAPI (MilliporeSigma F6057). For whole mount

491 immunostaining, retinas were incubated in primary antibody at 4°C for 2 days. Primary

492 antibodies used: goat GFP (abcam ab5450), rabbit C1q 1:1500 (abcam ab182451), rabbit active

493 caspase-3 1:500 (CC3) (BD Biosciences 559565), guinea pig RBPMS 1:1000 (MilliporeSigma

494 ABN1376MI), FITC IB4-lectin 1:400 (Sigma L9381). Secondary antibodies: 488 Donkey anti-

495 goat 1:400 (Invitrogen A11055), 555 Donkey anti-rabbit (Life Technologies A31572), and 647

496 Donkey anti-guinea pig (Jackson ImmunoResearch 706-605-148).

497

498 In situ hybridization chain reaction (HCR)

499 Wholemount retinas were fixed overnight in 4% PFA in 4°C. Retinas were washed and

500 dehydrated in methanol/PBS at 25%, 50%, 2 times 100% for 15 minutes each, stored in 100%

21

501   methanol overnight at 4°C and 20°C long term. *In situ* hybridization was performed as published

502   [64] using v3.0 reagents from Molecular Instruments (https://www.molecularinstruments.com).

503   Briefly, samples were rehydrated using (75% Methanol/25% PBST, 50% Methanol/50% PBST,

504   25%, Methanol/75% PBST, 2 times 100% PBST), treated with Proteinase K, and post fixed 20

505   minutes at room temperature in 4% PFA, and washed 3 times with PBST. Pre-hybridization was

506   performed in 30% probe hybridization buffer for 30 minutes at 37°C, and retinas were placed in

507   hybridization buffer at 37°C overnight. Retinas were washed, placed in amplification buffer for

508   30 minutes at room temperature. Separately, hairpins used for amplification were denatured at

509   95°C for 90 seconds and cooled to room temperature for 30 minutes. Retinas were placed in

510   amplification buffer with hairpins at room temperature in the dark overnight. Retinas were

511   washed, DAPI stained, and mounted on slides. Probes recognizing all known transcript variants

512   for each of Cx3cr1 and Ccl3 were generated by Molecular Instruments. For Cx3cr1-GFP

513   knockin mice, the fluorescent signal represents detection of Cx3cr1 mRNA by HCR and GFP

514   fluorescence which persists through the procedure.

515

516   Confocal Microscopy

517   Confocal images were acquired on an inverted Nikon A1R Confocal Microscope. Images were

518   acquired at 20X objective with a 3X digital zoom. Multi-points were stitched with a 10% overlap.

519   Images of retinal whole mounts were 144 multi-point images (on average) to obtain the entire

520   dorsal retina. Stacks through the Z plane were at 0.8μm steps of about ~13 μm thickness to

521   capture just the nerve fiber layer (NFL) and ganglion cell layer (GCL) at 0.2μm pixel resolution.

522   Whole mount retina images represent max projections of inner retina (NFL/GCL) from the

523   central retina (optic nerve) to the periphery (edge of retina). In cases of microglial quantification,

524   whole mount imaging and analysis spanned ~25μm thickness from NFL to inner plexiform layer

525   (IPL). Image acquisition settings were consistent across ages and genotypes.

526

527   Fluorescence-Activated Cell Sorting (FACS) and Flow Cytometry

528   Except for SC-sequencing, we pooled 2 retinas from an individual animal for each sample for

529   flow cytometry and FACs. Freshly dissected pure retinas were dissociated in PBS, 50 mM

530   HEPES, 0.05mg/ml DNase I (Sigma D4513), and 0.025 mg/ml Liberase (Sigma 5401119001)

531   for 35 minutes with intermediate trituration at 37°C. Cells were passed through a 70 μm nylon

532   cell strainer, washed with ice-cold staining buffer (1X PBS, 2% BSA, 0.1% sodium azide, 0.05%

533   EDTA), and red blood cells were lysed (eBioscience 00-4333-57). Cell counts were determined

534   using a cell counter (Invitrogen Countess) and Fc block (BD Biosciences 553142) was added at

535   2 μL per 10^6 cells. Antibodies were applied for 30 min on ice. Antibodies used: BV421 CD45

536   1:200 (BD Bioscience 563890), 488 CD11b 1:200 (CD45 (BD Bioscience 557672), PE CCR2

537   1:200 (R&D Systems FAB5538P), and APC Ly6C 1:200 (BD Bioscience 560595). Cells were

538   washed, pelleted, and resuspended in 500 μL staining buffer. FACS was performed using a BD

539   FACS Aria cell sorter at the University of Utah Flow Cytometry Core. Forward and side scatter

540   were used to eliminate debris, and both the width and area of the forward and side scatter was

541   used to discriminate singlets. For flow analysis, roughly 1 million singlet events (300,000 in rare

542   cases) were recorded for flow analysis using FlowJo software (Flowjo, LLC, Ashland, Oregon).

543

544   Single-cell RNA sequencing

545   13 animals/26 retinas were pooled for each of the Bax WT and littermate KO samples. 12

546   animals/24 retinas for PLX3397 CX3CR1-GFP/+ and 11 animals/22 retinas were pooled for

547   Vehicle CX3CR1-GFP/+ controls. FACs protocol was performed as normal, except pooling 6

548   retinas per tube during dissociation. CD45$^+$ CD11b$^+$/GFP$^+$ CCR2$^-$ cells were sorted for Bax

549   samples and CD45$^+$GFP$^+$ Ly6C$^-$ cells were sorted for PLX and Vehicle samples. Cells were

550   sorted using a 5 laser BD FACSAria with a 70μm nozzle at 55psi by the University of Utah Flow

551   Core into a cold, empty tube before library generation. 15,000 cells for each Bax sample and

552   7,000-10,000 cells for PLX and Vehicle were loaded onto 10X chip. Single-cell libraries were

553   generated with 10X Genomics Single Cell 3' Gene Expression Library Prep v3 reagents at the

554   University of Utah High Throughput Genomics core. The libraries were sequenced on a

555   NovaSeq 6000 to generate at least 200 million paired end reads of 150 bp per sample.

556

557   Analysis of Single-cell RNA-seq Data

558   Aligned reads to the mm10 reference from 10X genomics (version 3.0.0 from Ensembl 93) and

559   generated feature-barcode matrices using cellranger count v3.1.0 with expected-cells set to

560   5000. Filtered feature matrices from cellranger were further filtered with Seurat (v3.1.5) [65].

561   High-quality cells were selected by < 10% mitochondrial gene content, and high number of

562   features (genes). Feature cut-off was specific to each sample: > 1,000 for Bax-ko, > 1,500 for

563   Bax-wt, > 1,800 for vehicle, and > 2,500 for PLX-treated. Likely doublets, with high transcript

564   counts, were also eliminated: < 25,000 counts for Bax-ko, < 27,000 for Bax-wt, < 47,000 for

565   vehicle, and < 125,000 for PLX treated. In silico genotyping, as described below, was used to

566   assign cells to the correct groups. Filtered cells from all samples were then merged and

567   processed with the SCTransform pipeline [66]. Mitochondrial percentage was regressed out of

568   the model and Bax, Ftl1, and Ftl1-ps1 were excluded from the variable features list used for

569   dimensionality reduction. From here we subsetted the data to include only microglial cells. We

570   identified non-microglia populations by their expression of Ptprc, Plac8, Clec12a, Ms4a7, Mrc1,

571   and Rorb, and reran the SCTransform pipeline as before with the exception that FindClusters

572   was run with resolution set to 0.5. Differentially expressed genes for cluster comparisons and

573   whole-sample comparisons were identified with the FindMarkers function, using default

574   parameters.

575

576   In Silico Genotyping

577   During single-cell library preparation, a portion of Bax-wt and Bax-ko cells were allocated to the

578   wrong sample. We used the scSplit pipeline [67] to perform in silico genotyping and reassign

24

579   these cells to the correct sample. Beginning with the bam file for each sample generated by

580   cellranger, we used samtools view v1.8 [68] to exclude reads with mapping quality < 10, or

581   flagged as unmapped, not primary alignment, fails quality checks, PCR or optical duplicate, or

582   supplementary alignment. These filtered bam files were used to identify single nucleotide

583   polymorphisms (SNPs) with freebayes v1.3.1(https://arxiv.org/abs/1207.3907v2). Indels, MNPs,

584   and complex alleles were filtered from the input to the algorithm. Region was set to

585   chromosome 7, where Bax is located, minimum allele count was set to 2, use-best-n-alleles was

586   set to 2, and minimum base quality was set to 1. Vcffilter v1.0.1 [69] was used to exclude SNPs

587   with quality score <=30. We found that some crucial SNPs were identified in one sample and

588   not the other, so Bcftools merge v1.9 [68] was used to combine all SNPs into a single file.

589   ScSplit count v1.0.0 [67] was used to the combined SNPs to generate count matrices for the

590   filtered cell barcodes identified by cellranger, and scSplit run, with expected number of mixed

591   samples set to 1, genotyped the individual cells based on these matrices. We assigned Bax-ko

592   and Bax-wt genotypes to the cell populations based on Bax, Ftl1, and Ftl1-ps1 expression.

593

594   Bulk RNA sequencing

595   For P4 WT, Mertk KO, and Axl KO samples, $CD11b^+$ $CD45^+$ $Ly6c^-$ cells from one animal (2

596   retinas) were sorted directly into RLT buffer using a 4 laser BD FACSAria at the University of

597   Utah Flow Core and stored at -80°C. 2 samples were pooled prior to RNA isolation so that each

598   replicate was cells from 2 animals/4 retinas. WT samples had 5470, 6134, 7029 cells, Mertk KO

599   samples had 4245, 4038, and 8591 cells, and AXL KO samples had 5790, 5282, and 5071

600   cells. For P7 WT and Mertk/Axl dKO, $CD11b^+$ $CD45^+$ cells from one animal (2 retinas) were

601   sorted directly into RLT buffer (QIAGEN 79216) and stored at -80°C. P7 samples were not

602   pooled and thus represent 1 animal/2 retinas. For WT samples, 3783, 4083, and 4982 cells

603   were collected. For Mertk/Axl dKO samples, 2408, 2633, and 2388 cells were collected.

604   RNA from all samples was purified using the RNeasy Plus Micro kit (QIAGEN 74034). Total

605   RNA was hybridized with the NEBNext rRNA Depletion Solution human/mouse/rat (E6310L) to

606   substantially diminish cytoplasmic and mitochondrial rRNA from the samples. Stranded RNA

607   sequencing libraries were prepared as described using the NEBNext Ultra II RNA Library Prep

608   Kit for Illumina (E7770L). Purified libraries were qualified on an Agilent Technologies 2200

609   TapeStation using a D1000 ScreenTape assay (cat# 5067-5582 and 5067-5583). The molarity

610   of adapter-modified molecules was defined by quantitative PCR using the Kapa Biosystems

611   Kapa Library Quant Kit (cat#KK4824). Individual libraries were normalized to 10 nM, and equal

612   volumes were pooled in preparation for Illumina sequence analysis. Sequencing libraries were

613   chemically denatured and applied to an Illumina NovaSeq flow cell using the NovaSeq XP

614   workflow (20043131). Following transfer of the flowcell to an Illumina NovaSeq 6000

615   instrument, a 150 x 150 cycle paired end sequence run was performed using a NovaSeq 6000

616   S4 reagent Kit v1.5 (20028312). Samples were sequenced to a depth of 39-76 million reads.

617

618   Analysis of Bulk RNA-seq Data

619   Optical duplicates were removed with clumpify using BBmap v38.34 (https://jgi.doe.gov/data-

620   and-tools/bbtools/bb-tools-user-guide/bbmap-guide/) and default settings, then Illumina

621   adapters were trimmed with cutadapt v1.16 [70] using a minimum overlap of 6 and minimum

622   length of 20. Reference database was generated by STAR v2.7.9a [71] using mouse Ensembl

623   release 104 with overhang set to 124. Trimmed reads were aligned in two pass mode to

624   generate a BAM file, sorted by coordinates. Reads were assigned to the target with the largest

625   overlap, and uniquely aligned, reversely stranded reads were counted with featureCounts v1.6.3

626   [72]. Differentially expressed genes were identified from counts using DESeq2 v1.32.0 [73].

627   Features with fewer than 5 reads in every sample were eliminated before DESeq was run. rlog-

628   transformed values were used for sample visualizations.

629

Quantification and Statistical Analysis

Image analysis

All counts were blinded and manual, performed using Nikon Elements software (Melville, NY). For double-positive CC3$^+$RBPMS$^+$ or single RBPMS$^+$ counts of retinal whole mounts, two ROIs of roughly 0.4 mm^2 of central and periphery of dorsal retina were analyzed. Images were max projected and roughly 13μm thick, spanning the NFL to the GCL. For CC3$^+$ density, the entire dorsal leaf was analyzed, roughly 2-3 mm^2 again spanning the NFL to GCL only. See also Figure S9. For microglial quantification, whole mount imaging and analysis spanned ~25μm thickness from NFL to IPL and 0.5625 mm^2 of the central to mid-peripheral, vascularized retina of the dorsal leaf.

Statistical Methods

Detailed statistical information can be found in the figure legends, including tests used, sample size, and precision measures. All image and flow data were analyzed using Prism 9 software (GraphPad, La Jolla, CA). All data were first tested for normality using four tests: Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk, and Kolmogorov-Smirnov test. If any one test failed, non-parametric tests were used: Mann-Whitney for pair-wise comparisons and Kruskal-Wallis for comparisons of more than three samples. For all t-tests, we applied the Welch's correction if the two samples had unequal variances determined by an F-test. We tested for heteroscedasticity in groups of 3 or more by a Brown-Forsythe test. If not significantly different, we ran an Ordinary one-way ANOVA with post-hoc Tukey's multiple comparison test. If the standard deviations were significantly different between groups, we ran a Welch's ANOVA with post-hoc Dunnett's T3 multiple comparison's test. For all data that is presented as the mean, error bars indicate the standard error of the mean, SEM. We used a 95% confidence interval and a p-value of < 0.05 for rejecting the null hypothesis.

656 **Data and Software Availability**

657 The sequence data reported in this publication have been deposited in NCBI's Gene Expression

658 Omnibus (GEO).

References:

1. Prinz, M., et al., *Microglia and Central Nervous System-Associated Macrophages-From Origin to Disease Modulation.* Annual review of immunology, 2021. **39**: p. 251-277.

2. Anderson, S.R. and M.L. Vetter, *Developmental roles of microglia: A window into mechanisms of disease.* Dev Dyn, 2019. **248**(1): p. 98-117.

3. Bennett, F.C., et al., *A Combination of Ontogeny and CNS Environment Establishes Microglial Identity.* Neuron, 2018. **98**(6): p. 1170-1183.e8.

4. Gosselin, D., et al., *An environment-dependent transcriptional network specifies human microglia identity.* Science (New York, N.Y.), 2017. **356**(6344): p. eaal3222.

5. Lavin, Y., et al., *Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment.* Cell, 2014. **159**(6): p. 1312-1326.

6. Hammond, T.R., et al., *Single-Cell RNA Sequencing of Microglia throughout the Mouse Lifespan and in the Injured Brain Reveals Complex Cell-State Changes.* Immunity, 2019. **50**(1): p. 253-271.e6.

7. Li, Q., et al., *Developmental Heterogeneity of Microglia and Brain Myeloid Cells Revealed by Deep Single-Cell RNA Sequencing.* Neuron, 2019. **101**(2): p. 207-223.e10.

8. Jacobson, M.D., M. Weil, and M.C. Raff, *Programmed Cell Death in Animal Development.* Cell, 1997. **88**(3): p. 347-354.

9. Oppenheim, R.W., *Cell death during development of the nervous system.* Annu Rev Neurosci, 1991. **14**: p. 453-501.

10. Morioka, S., C. Maueröder, and K.S. Ravichandran, *Living on the Edge: Efferocytosis at the Interface of Homeostasis and Pathology.* Immunity, 2019. **50**(5): p. 1149-1162.

11. Boada-Romero, E., et al., *The clearance of dead cells by efferocytosis.* Nature Reviews Molecular Cell Biology, 2020. **21**(7): p. 398-414.

12. Galloway, D.A., et al., *Phagocytosis in the Brain: Homeostasis and Disease.* Frontiers in Immunology, 2019. **10**(790).

13. VanRyzin, J.W., *Phagocytic microglia in development: Are they what they eat?* Brain, Behavior, & Immunity - Health, 2021. **18**: p. 100373.

14. Li, F., D. Jiang, and M.A. Samuel, *Microglia in the developing retina.* Neural Dev, 2019. **14**(1): p. 12.

15. Braunger, B.M., C. Demmer, and E.R. Tamm, *Programmed cell death during retinal development of the mouse eye.* Adv Exp Med Biol, 2014. **801**: p. 9-13.

16. Péquignot, M.O., et al., *Major role of BAX in apoptosis during retinal development and in establishment of a functional postnatal retina.* Dev Dyn, 2003. **228**(2): p. 231-8.

17. Farah, M.H. and S.S. Easter, Jr., *Cell birth and death in the mouse retinal ganglion cell layer.* J Comp Neurol, 2005. **489**(1): p. 120-34.

696   18.   Farah, M.H., *Neurogenesis and cell death in the ganglion cell layer of vertebrate retina.*
697   Brain Res Rev, 2006. **52**(2): p. 264-74.

698   19.   Anderson, S.R., et al., *Developmental Apoptosis Promotes a Disease-Related Gene*
699   *Signature and Independence from CSF1R Signaling in Retinal Microglia.* Cell Rep, 2019.
700   **27**(7): p. 2002-2013.e5.

701   20.   Holtman, I.R., et al., *Induction of a common microglia gene expression signature by*
702   *aging and neurodegenerative conditions: a co-expression meta-analysis.* Acta
703   Neuropathol Commun, 2015. **3**: p. 31.

704   21.   Keren-Shaul, H., et al., *A Unique Microglia Type Associated with Restricting*
705   *Development of Alzheimer's Disease.* Cell, 2017. **169**(7): p. 1276-1290.e17.

706   22.   Krasemann, S., et al., *The TREM2-APOE Pathway Drives the Transcriptional Phenotype*
707   *of Dysfunctional Microglia in Neurodegenerative Diseases.* Immunity, 2017. **47**(3): p.
708   566-581.e9.

709   23.   Benmamar-Badel, A., T. Owens, and A. Wlodarczyk, *Protective Microglial Subset in*
710   *Development, Aging, and Disease: Lessons From Transcriptomic Studies.* Front
711   Immunol, 2020. **11**: p. 430.

712   24.   Nemes-Baran, A.D., D.R. White, and T.M. DeSilva, *Fractalkine-Dependent Microglial*
713   *Pruning of Viable Oligodendrocyte Progenitor Cells Regulates Myelination.* Cell Reports,
714   2020. **32**(7): p. 108047.

715   25.   Lemke, G., *How macrophages deal with death.* Nature Reviews Immunology, 2019.
716   **19**(9): p. 539-549.

717   26.   Marsh, S.E., et al., *Single Cell Sequencing Reveals Glial Specific Responses to Tissue*
718   *Processing &amp; Enzymatic Dissociation in Mice and Humans.* bioRxiv, 2020: p.
719   2020.12.03.408542.

720   27.   Marschallinger, J., et al., *Lipid-droplet-accumulating microglia represent a dysfunctional*
721   *and proinflammatory state in the aging brain.* Nature Neuroscience, 2020. **23**(2): p. 194-
722   208.

723   28.   Masuda, T., et al., *Spatial and temporal heterogeneity of mouse and human microglia at*
724   *single-cell resolution.* Nature, 2019. **566**(7744): p. 388-392.

725   29.   Rojo, A.I., et al., *Redox control of microglial function: molecular mechanisms and*
726   *functional significance.* Antioxidants & redox signaling, 2014. **21**(12): p. 1766-1801.

727   30.   Dorman, L.C., et al., *A type I interferon response defines a conserved microglial state*
728   *required for effective phagocytosis.* bioRxiv, 2021: p. 2021.04.29.441889.

729   31.   Doran, A.C., A. Yurdagul, and I. Tabas, *Efferocytosis in health and disease.* Nature
730   Reviews Immunology, 2020. **20**(4): p. 254-267.

731   32.   Fricker, M., et al., *Neuronal Cell Death.* Physiological Reviews, 2018. **98**(2): p. 813-880.

732   33.   García-Moreno, A., et al., *GeneCodis 4: Expanding the modular enrichment analysis to*
733   *regulatory elements.* bioRxiv, 2021: p. 2021.04.15.439962.

734   34.   Zhan, L., et al., *A MAC2-positive progenitor-like microglial population is resistant to
735   CSF1R inhibition in adult mouse brain.* Elife, 2020. **9**.

736   35.   Kempthorne, L., et al., *Loss of homeostatic microglial phenotype in CSF1R-related
737   Leukoencephalopathy.* Acta Neuropathologica Communications, 2020. **8**(1): p. 72.

738   36.   Wolf, Y., et al., *Microglia, seen from the CX3CR1 angle.* Frontiers in cellular
739   neuroscience, 2013. **7**: p. 26-26.

740   37.   Jung, S., et al., *Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion
741   and green fluorescent protein reporter gene insertion.* Mol Cell Biol, 2000. **20**(11): p.
742   4106-14.

743   38.   Masuda, T., et al., *Microglia Heterogeneity in the Single-Cell Era.* Cell Rep, 2020. **30**(5):
744   p. 1271-1281.

745   39.   Wlodarczyk, A., et al., *A novel microglial subset plays a key role in myelinogenesis in
746   developing brain.* Embo j, 2017. **36**(22): p. 3292-3308.

747   40.   A-Gonzalez, N., et al., *Phagocytosis imprints heterogeneity in tissue-resident
748   macrophages.* Journal of Experimental Medicine, 2017. **214**(5): p. 1281-1296.

749   41.   Zent, C.S. and M.R. Elliott, *Maxed out macs: physiologic cell clearance as a function of
750   macrophage phagocytic capacity.* The FEBS journal, 2017. **284**(7): p. 1021-1039.

751   42.   Márquez-Ropero, M., et al., *Microglial Corpse Clearance: Lessons From Macrophages.*
752   Frontiers in Immunology, 2020. **11**(506).

753   43.   Diaz-Aparicio, I., et al., *Microglia Actively Remodel Adult Hippocampal Neurogenesis
754   through the Phagocytosis Secretome.* The Journal of Neuroscience, 2020. **40**(7): p.
755   1453-1482.

756   44.   Puñal, V.M., et al., *Large-scale death of retinal astrocytes during normal development is
757   non-apoptotic and implemented by microglia.* PLOS Biology, 2019. **17**(10): p. e3000492.

758   45.   Païdassi, H., et al., *C1q binds phosphatidylserine and likely acts as a multiligand-
759   bridging molecule in apoptotic cell recognition.* J Immunol, 2008. **180**(4): p. 2329-38.

760   46.   Lemke, G., *Biology of the TAM receptors.* Cold Spring Harbor perspectives in biology,
761   2013. **5**(11): p. a009076-a009076.

762   47.   Fourgeaud, L., et al., *TAM receptors regulate multiple features of microglial physiology.*
763   Nature, 2016. **532**(7598): p. 240-244.

764   48.   Elliott, M.R., K.M. Koster, and P.S. Murphy, *Efferocytosis Signaling in the Regulation of
765   Macrophage Inflammatory Responses.* J Immunol, 2017. **198**(4): p. 1387-1394.

766   49.   Seitz, H.M., et al., *Macrophages and Dendritic Cells Use Different Axl/Mertk/Tyro3
767   Receptors in Clearance of Apoptotic Cells.* The Journal of Immunology, 2007. **178**(9): p.
768   5635.

769   50.   Elliott, Michael R. and Kodi S. Ravichandran, *The Dynamics of Apoptotic Cell Clearance.*
770   Developmental Cell, 2016. **38**(2): p. 147-160.

771   51.   Huang, Y., et al., *Microglia use TAM receptors to detect and engulf amyloid β plaques.*
772   Nat Immunol, 2021. **22**(5): p. 586-594.

773   52.   Damisah, E.C., et al., *Astrocytes and microglia play orchestrated roles and respect*
774   *phagocytic territories during neuronal corpse removal in vivo.* Sci Adv, 2020. **6**(26): p.
775   eaba3239.

776   53.   Hohsfield, L.A., et al., *Subventricular zone/white matter microglia reconstitute the empty*
777   *adult microglial niche in a dynamic wave.* Elife, 2021. **10**.

778   54.   Reddy, S.M., et al., *Phagocytosis of Apoptotic Cells by Macrophages Induces Novel*
779   *Signaling Events Leading to Cytokine-Independent Survival and Inhibition of*
780   *Proliferation: Activation of Akt and Inhibition of Extracellular Signal-Regulated Kinases 1*
781   *and 2.* The Journal of Immunology, 2002. **169**(2): p. 702.

782   55.   Nandi, S., et al., *The CSF-1 receptor ligands IL-34 and CSF-1 exhibit distinct*
783   *developmental brain expression patterns and regulate neural progenitor cell*
784   *maintenance and maturation.* Dev Biol, 2012. **367**(2): p. 100-13.

785   56.   Bohlen, C.J., et al., *Diverse Requirements for Microglial Survival, Specification, and*
786   *Function Revealed by Defined-Medium Cultures.* Neuron, 2017. **94**(4): p. 759-773.e8.

787   57.   Hu, B., et al., *Insights Into the Role of CSF1R in the Central Nervous System and*
788   *Neurological Disorders.* Frontiers in Aging Neuroscience, 2021. **13**(807).

789   58.   Axelrod, H. and K.J. Pienta, *Axl as a mediator of cellular growth and survival.*
790   Oncotarget, 2014. **5**(19): p. 8818-8852.

791   59.   Goruppi, S., et al., *Requirement of phosphatidylinositol 3-kinase-dependent pathway and*
792   *Src for Gas6-Axl mitogenic and survival activities in NIH 3T3 fibroblasts.* Mol Cell Biol,
793   1997. **17**(8): p. 4442-53.

794   60.   Shankar, S.L., et al., *Gas6/Axl signaling activates the phosphatidylinositol 3-kinase/Akt1*
795   *survival pathway to protect oligodendrocytes from tumor necrosis factor alpha-induced*
796   *apoptosis.* J Neurosci, 2006. **26**(21): p. 5638-48.

797   61.   Pierce, A., et al., *Axl and Tyro3 Modulate Female Reproduction by Influencing*
798   *Gonadotropin-Releasing Hormone Neuron Survival and Migration.* Molecular
799   Endocrinology, 2008. **22**(11): p. 2481-2495.

800   62.   Zhu, C., Y. Wei, and X. Wei, *AXL receptor tyrosine kinase as a promising anti-cancer*
801   *approach: functions, molecular mechanisms and clinical applications.* Molecular Cancer,
802   2019. **18**(1): p. 153.

803   63.   Shankar, S.L., et al., *The growth arrest-specific gene product Gas6 promotes the*
804   *survival of human oligodendrocytes via a phosphatidylinositol 3-kinase-dependent*
805   *pathway.* J Neurosci, 2003. **23**(10): p. 4208-18.

806   64.   Choi, H.M.T., et al., *Third-generation in situ hybridization chain reaction: multiplexed,*
807   *quantitative, sensitive, versatile, robust.* Development, 2018. **145**(12).

808   65.   Stuart, T., et al., *Comprehensive Integration of Single-Cell Data.* Cell, 2019. **177**(7): p.
809   1888-1902.e21.

810   66.   Hafemeister, C. and R. Satija, *Normalization and variance stabilization of single-cell*
811   *RNA-seq data using regularized negative binomial regression.* Genome Biology, 2019.
812   **20**(1): p. 296.

813   67.   Xu, J., et al., *Genotype-free demultiplexing of pooled single-cell RNA-seq.* Genome
814   Biology, 2019. **20**(1): p. 290.

815   68.   Danecek, P., et al., *Twelve years of SAMtools and BCFtools.* Gigascience, 2021. **10**(2).

816   69.   Garrison, E., et al., *Vcflib and tools for processing the VCF variant call format.* bioRxiv,
817   2021: p. 2021.05.21.445151.

818   70.   Martin, M., *Cutadapt removes adapter sequences from high-throughput sequencing*
819   *reads.* 2011, 2011. **17**(1): p. 3.

820   71.   Dobin, A., et al., *STAR: ultrafast universal RNA-seq aligner.* Bioinformatics, 2013. **29**(1):
821   p. 15-21.

822   72.   Liao, Y., G.K. Smyth, and W. Shi, *featureCounts: an efficient general purpose program*
823   *for assigning sequence reads to genomic features.* Bioinformatics, 2013. **30**(7): p. 923-
824   930.

825   73.   Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and*
826   *dispersion for RNA-seq data with DESeq2.* Genome Biology, 2014. **15**(12): p. 550.

827

1. Multiple microglial states coexist in postnatal retina

Figure 1. (A) Workflow for collection, dissociation, sorting, and sequencing of individual microglia from 4 different groups: Bax WT and KO, PLX and vehicle. (B) UMAP plot of 13,667 microglia cells from all 4 samples distributed into 11 clusters by unsupervised clustering. (C) UMAP plots illustrating the distribution of cells for each sample. Number of cells per sample labeled in lower left. (D) UMAP plots of two genes enriched in each cluster. Color scale is based on relative gene expression: dark purple = highest, light yellow = lowest.

2. Postnatal retinal microglia encompass a spectrum of homeostatic to remodeling states

Figure 2. (A) UMAP plot of 11 clusters from all sequenced microglia. Spectrum of homeostatic (bottom) to more remodeling (top). Dashed line demarcates in interface between relatively more homeostatic and remodeling populations. (B) UMAP plots of microglial homeostatic genes. (C) UMAP plots of microglial homeostatic genes important for lysosomal function (D) UMAP plots of genes associated with lipid metabolism. (E) UMAP plots of DAM/PAM/ATM genes. Color scale is based on relative gene expression: dark purple = highest, light yellow = lowest.
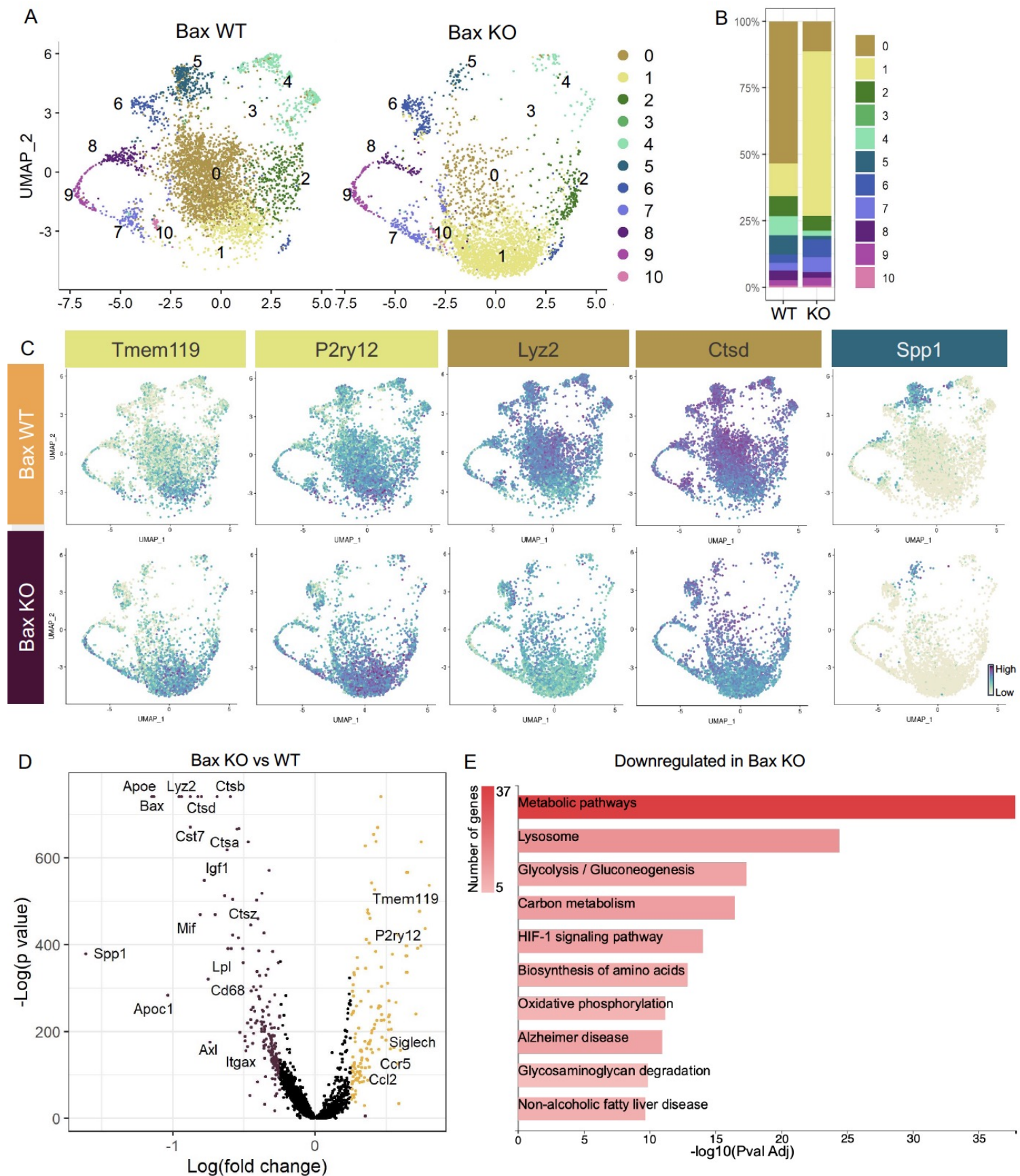
Figure 3. (A) UMAP plot of microglia cells from BAX WT (left) and BAX KO (right) samples distributed into 11 clusters. (B) Bar graph of the proportion of cells in each cluster for each sample. (C) UMAP plots showing expression of representative genes from selected clusters. (D) Volcano plot showing differential gene expression of all BAX KO cells compared to BAX WT cells. Each gene is plotted according to the significance (-Log(p value)) and magnitude (Log(fold change)) of the difference such that those genes enriched in BAX KO have Log(fold change) > 0. Red points indicate genes with p-value ≤0.05 and Log(fold change) > 0.25. (E) KEGG pathway analysis of 183 genes downregulated in BAX KO compared to WT using GeneCodis 4.

4. Subsets of remodeling states survive CSF1R inhibition, while homeostatic microglia are more vulnerable
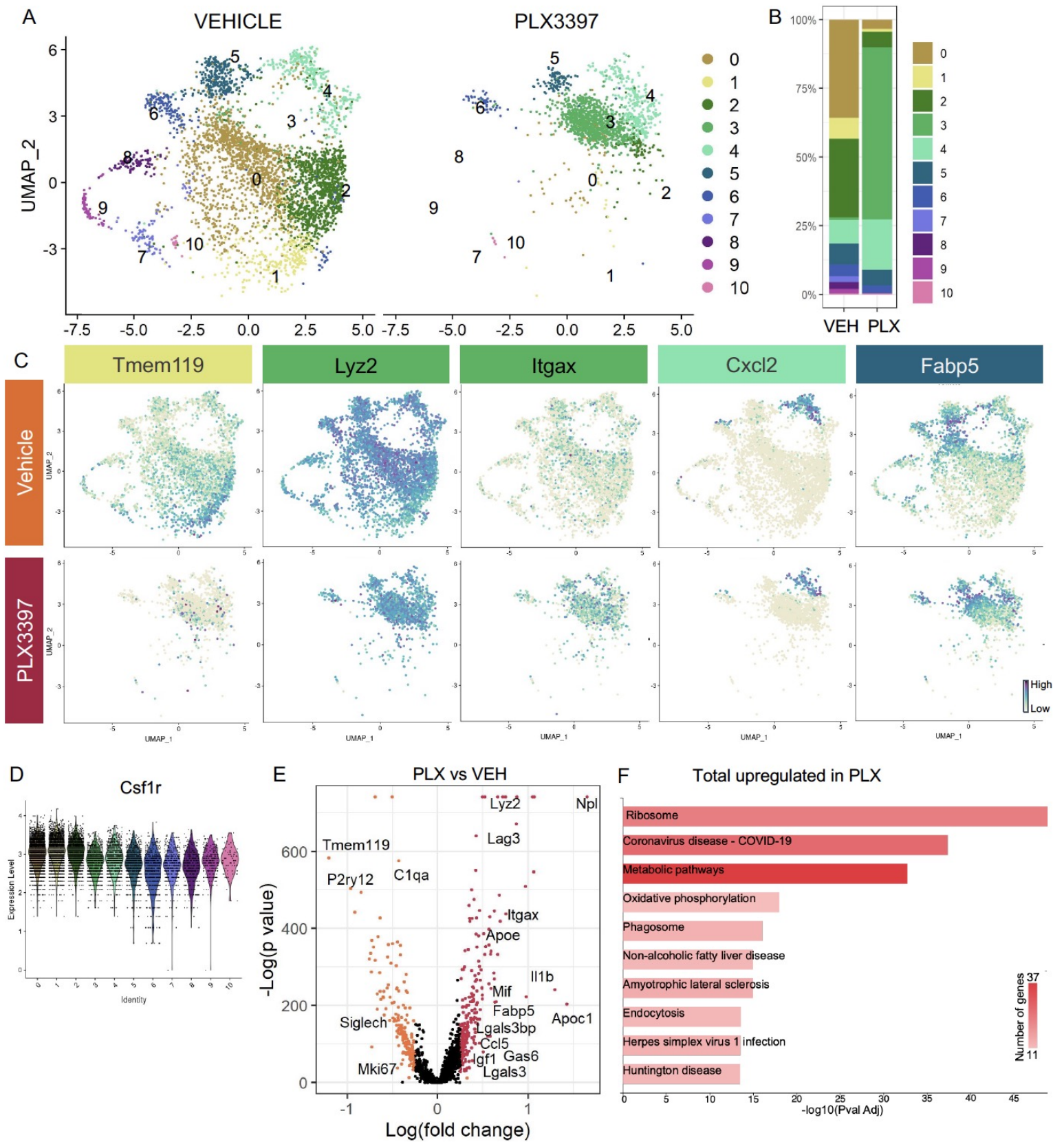
Figure 4. (A) UMAP plot of microglia cells from Vehicle (left) and PLX3397 (right) samples distributed into 11 clusters. (B) Proportion of cells from each sample within each cluster. (C) UMAP plots showing expression of representative genes from selected clusters. (D) Violin plot of Csf1r expression across clusters. (E) Volcano plot showing differential gene expression of pseudobulked PLX cells compared to Vehicle cells. Each gene is plotted according to the significance (-Log(p value)) and magnitude (Log(fold change)) of the difference such that those genes enriched in PLX have Log(fold change) > 0. Red points indicate genes with p-value ≤ 0.05 and Log(fold change) > 0.25. (F) KEGG Pathway analysis of 254 upregulated genes in PLX compared to Vehicle using GeneCodis 4.
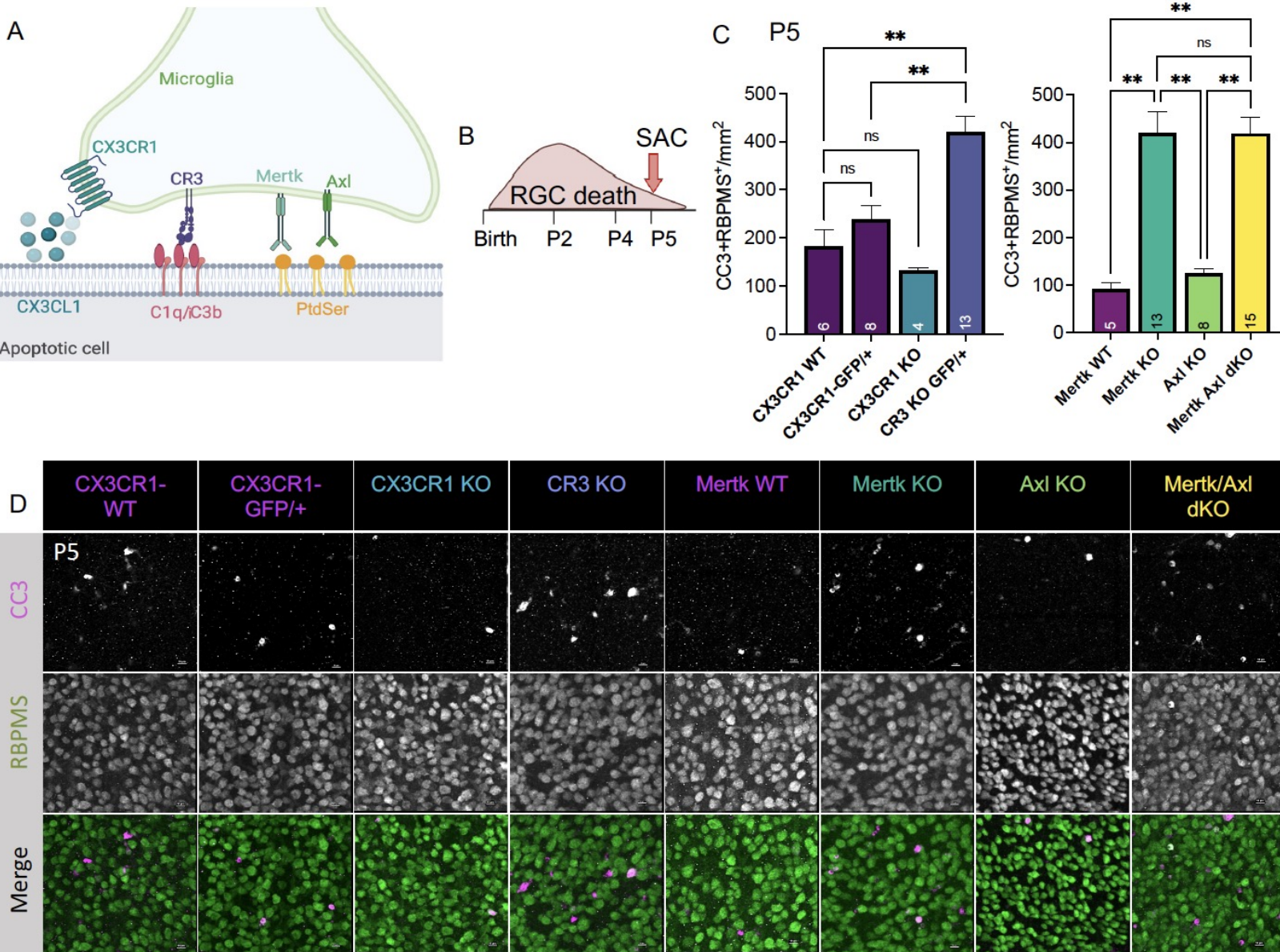
Figure 5. (A) Cartoon schematic of candidate pathways. (B) Schematic for collection at P5 after the bulk of RGC developmental death. (C) Quantification of the average of central and peripheral 0.4 mm^2 RGC death (±SEM) (Figure S9C) in dorsal leaf of all genotypes (n=6 CX3CR1 WT, n=8 CX3CR1-GFP/+, n=4 CX3CR1 KO, n=13 CR3 KO, CX3CR1-GFP/+, n=5 Mertk WT, n=13 Mertk KO, n=8 Axl KO, n=15 Mertk Axl dKO). (left) Welch's ANOVA test W(3,12.58)=23.75, ****p<0.0001 and Dunnett's T3 multiple comparisons test. CX3CR1 WT vs CX3CR1-GFP/+ ns, CX3CR1 WT vs CX3CR1 KO ns, CX3CR1 WT vs CR3 KO **p=0.0016, CX3CR1-GFP/+ vs CX3CR1 KO *p=0.035, CX3CR1-GFP/+ vs CR3 KO **p=0.004, CX3CR1 KO vs CR3 KO ****p<0.0001. (right) Kruskal-Wallis test statistic=25.97 ****p<0.0001 and Dunn's multiple comparisons test. Mertk WT vs Mertk KO **p=0.001, Mertk WT vs Axl KO ns, Mertk WT vs Mertk Axl dKO **p=0.0014, Mertk KO vs Axl KO **p=0.0026, Mertk KO vs Mertk Axl dKO ns, Axl KO vs Mertk Axl dKO **p=0.0036. Not all comparisons shown on graphs. (D) Max projected confocal images of dying RGCs (CC3$^+$RBPMS$^+$) in KOs in the dorsal mid-periphery in the ganglion cell layer. Apoptotic bodies, CC3 (magenta); RGCs, RBPMS (green). Scale bars 10μm.

Figure 6. (A) Dosing regimen of PLX3397 to various genotypes. (B) Confocal images of microglia in the NFL/GCL in all genotypes from central to mid-periphery of the dorsal leaf. C1q (mono). Scale bars 100μm. (C) Ratio of density of CD45$^+$CX3CR1-gfp$^+$ or CD45$^+$CD11b$^+$ (microglia/singlets) in PLX treated retinas over genotype-matched controls by flow cytometry. CX3CR1-GFP/+ and BAX KO data was previously published [19]. (n=17 CX3CR1-GFP/+, n=5 BAX KO, n=11 CX3CR1 KO, n=5 CR3 KO CX3CR1-GFP/+, n=9 Mertk KO, n=8 Axl KO, n=7 Mertk Axl dKO; ±SEM). Welch's ANOVA W(6,18.55)=16.53, ****p<0.0001 and Dunnett's T3 multiple comparisons test. GFP/+ vs BAX KO **p=0.005, GFP/+ vs CR3 KO ns, GFP/+ vs CX3CR1 KO ns, GFP/+ vs Mertk KO ns, GFP/+ vs AXL KO ***p=0.0004, GFP/+ vs Mertk Axl dKO **p=0.001, BAX KO vs CR3 KO *p=0.0168, BAX KO vs CX3CR1 KO *p=0.0151, BAX KO vs Mertk KO ns, BAX KO vs Axl KO ns, BAX KO vs Mertk Axl dKO ns, CR3 KO vs CX3CR1 KO ns, CR3 KO vs Mertk KO ns, CR3 KO vs AXL KO *p=0.0185, CR3 KO vs Mertk Axl dKO *p=0.0175, CX3CR1 KO vs Mertk KO ns, CX3CR1 KO vs Axl KO ***p=0.0008, CX3CR1 KO vs Mertk Axl dKO **p=0.0021, Mertk KO vs Axl KO ****p<0.0002, Mertk KO vs Mertk Axl dKO *p=0.0394. Axl KO vs Mertk Axl dKO ns. Not all comparisons shown.

7. MerTK and Axl are not required for expression of lysosomal, lipid metabolism, or remodeling genes
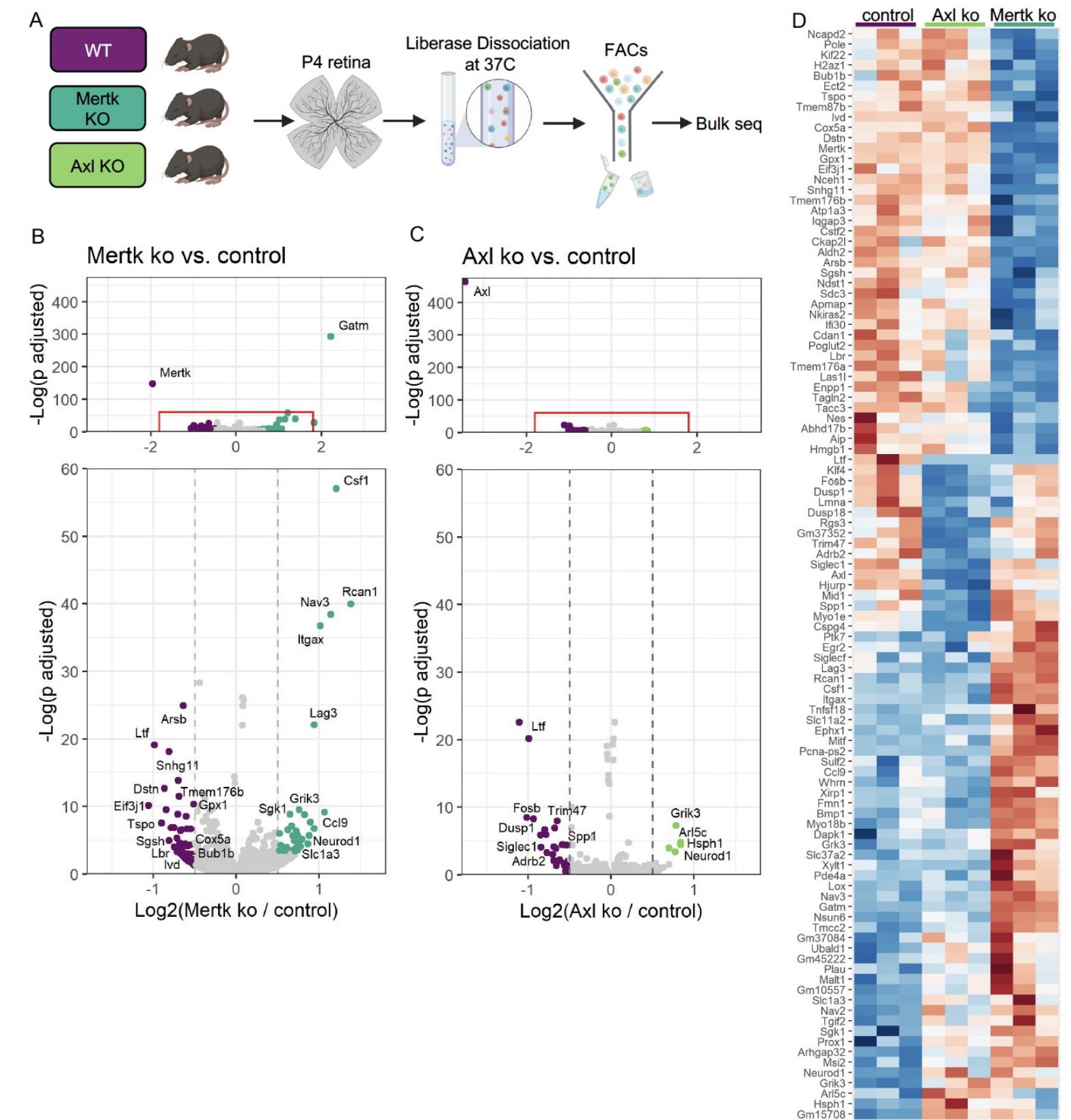
Figure 7. (A) Workflow for P4 retinal collection, dissociation, sorting, and sequencing of individual microglia from 3 different groups: WT, Mertk KO, and Axl KOs (n=3 each). (B,C) Volcano plot of differentially expressed genes. Top: showing all genes and bottom: zoomed in as indicated in red square. Each gene is plotted according to the significance (-Log(p value)) and magnitude (Log(fold change)) of the difference such that those genes enriched in KO have Log(fold change) > 0. Colored points indicate genes with p-value ≤ 0.05 and Log(fold change) > 0.5. (B) Comparison of Mertk KO relative to WT and (C) comparison of Axl KO relative to WT. (D) Heatmap of all differentially expressed genes between WT compared to Mertk KO and WT compared to Axl KO. Color represents z score of normalized gene expression across all samples. Red=high, blue=low.
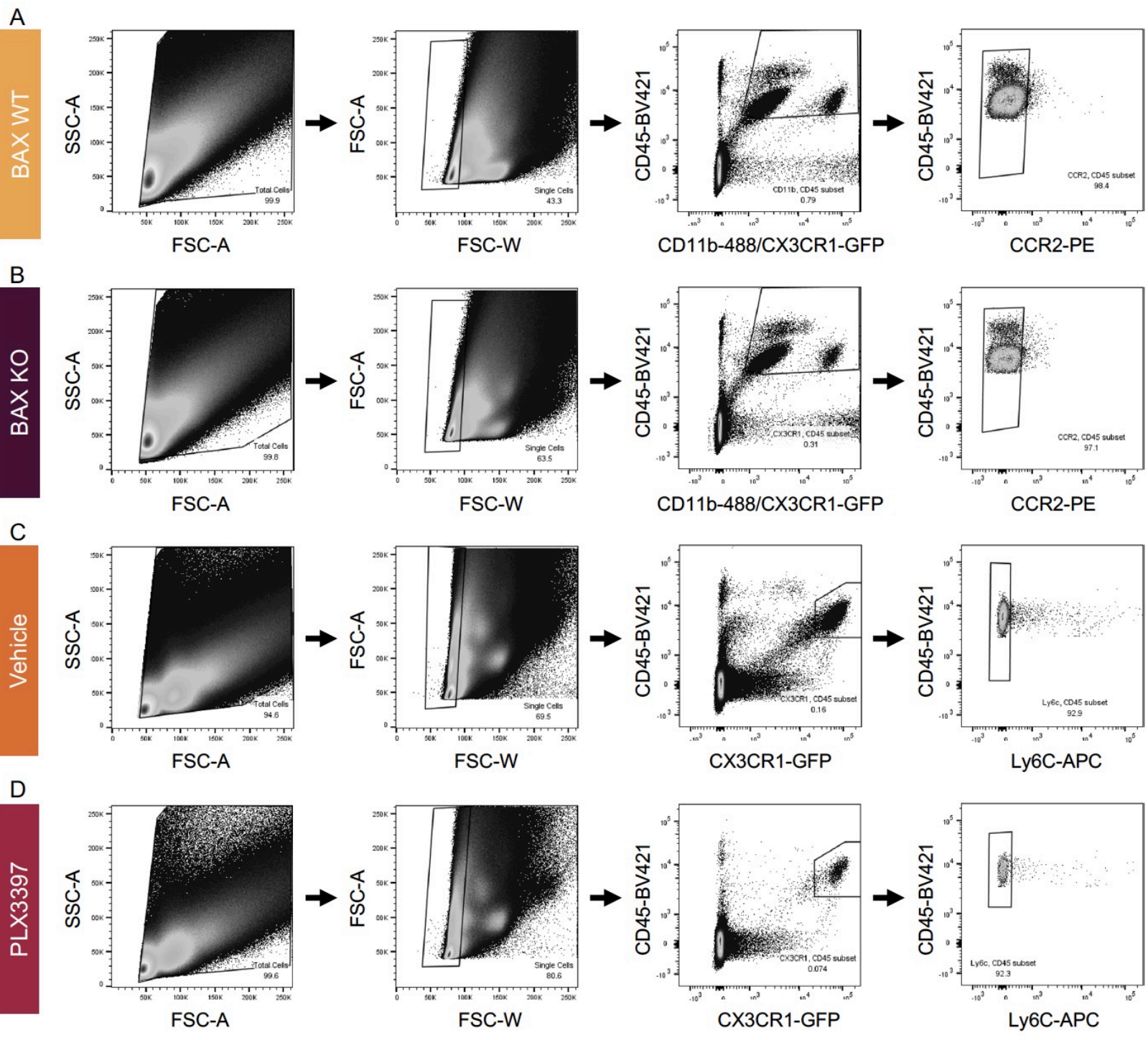
Figure S1. Related to Figure 1. (A) Gating strategy for FACs of BAX WT and (B) KO samples for single-cell sequencing. Animals were a mixture of CX3CR1-GFP/+ and CX3CR1-+/+. CCR2 was used to exclude monocytes and macrophages. (C) Gating strategy for FACs of CX3CR1-GFP/+ Vehicle and (D) PLX3397 treated samples. Ly6C was used to exclude monocytes and macrophages.
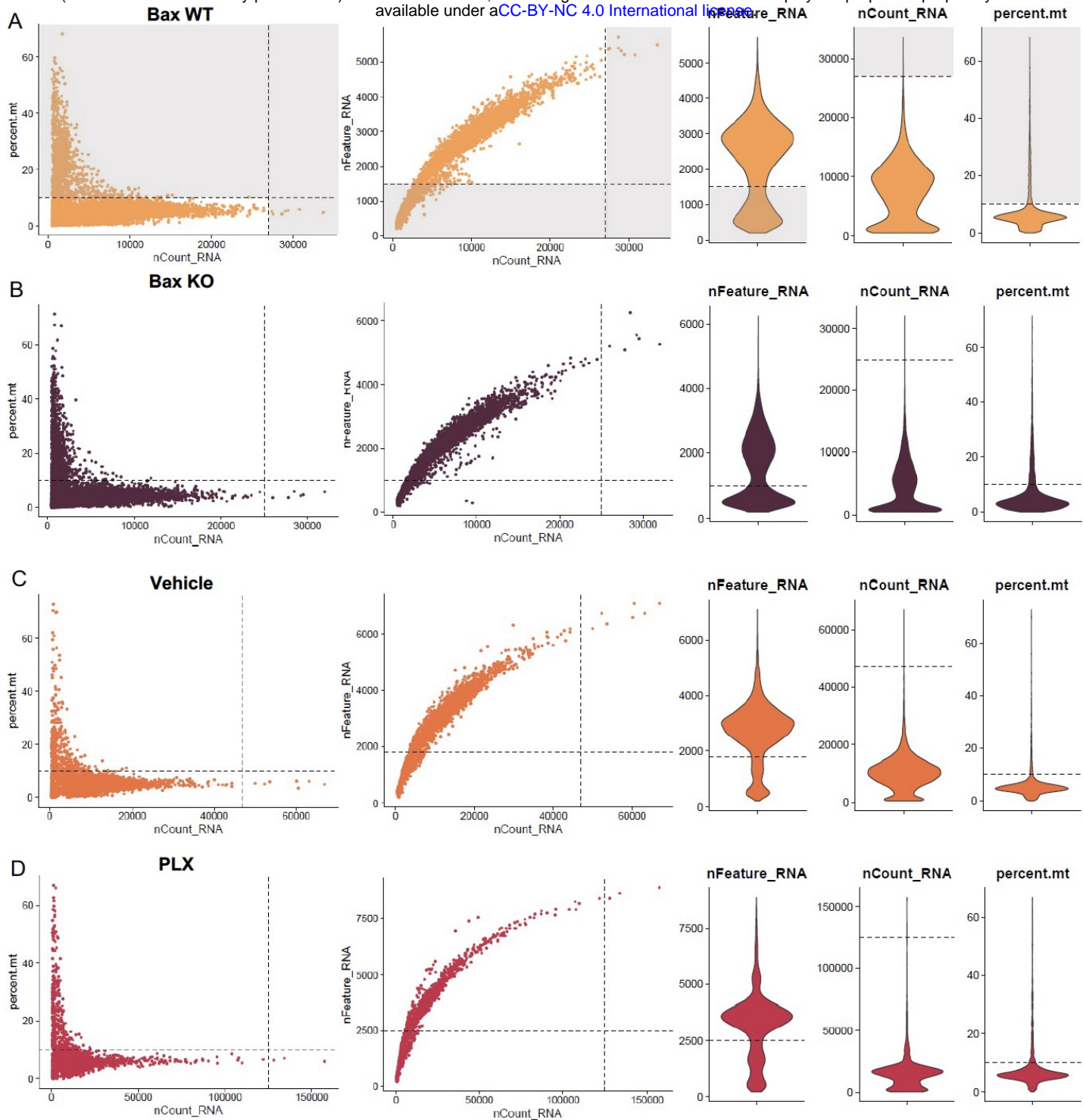
E		Filtered by CellRanger			Manually filtered			
	# cells	Counts per cell	Genes per cell	Mito %	# cells	Counts per cell	Genes per cell	Mito %
Bax WT	7,499	7,532	2,241	7.36	5,237	9,922	2,845	5.24
Bax KO	7,072	4,359	1,531	6.66	3,889	7,012	2,290	3.68
Vehicle	5,117	10,271	2,696	5.84	4,198	11,876	3,056	4.77
PLX	2,437	15,777	3,186	7.90	1,760	20,011	3,862	5.61

Figure S2. Related to Figure 1. (A-D) Cells, as identified by CellRanger, were filtered to exclude low quality cells: those with few features (genes) or high mitochondrial content. Cells with especially high transcript counts were eliminated as potential doublets. Cut-offs were set independently for Bax WT (A), Bax KO (B), Vehicle (C), and PLX (D) samples. (E) Dataset composition before and after cell filtering.

Supplemental Fig. 3. In silico Bax genotyping

A

BAM alignment files:

Bax WT   Bax KO

↓   ↓

Identify SNPs in chromosome 7

↓   ↓

Combine SNPs from each sample

↓

Define cell populations by SNP content

↓   ↓

Identify populations by Bax, Ftl1, and Ftl1-ps1 expression

Assign correct genotype to individual cells

↓   ↓

Bax WT   Bax KO

B

dataset	# wt cells	# ko cells
Bax WT	4,567	670
Bax KO	335	3,554
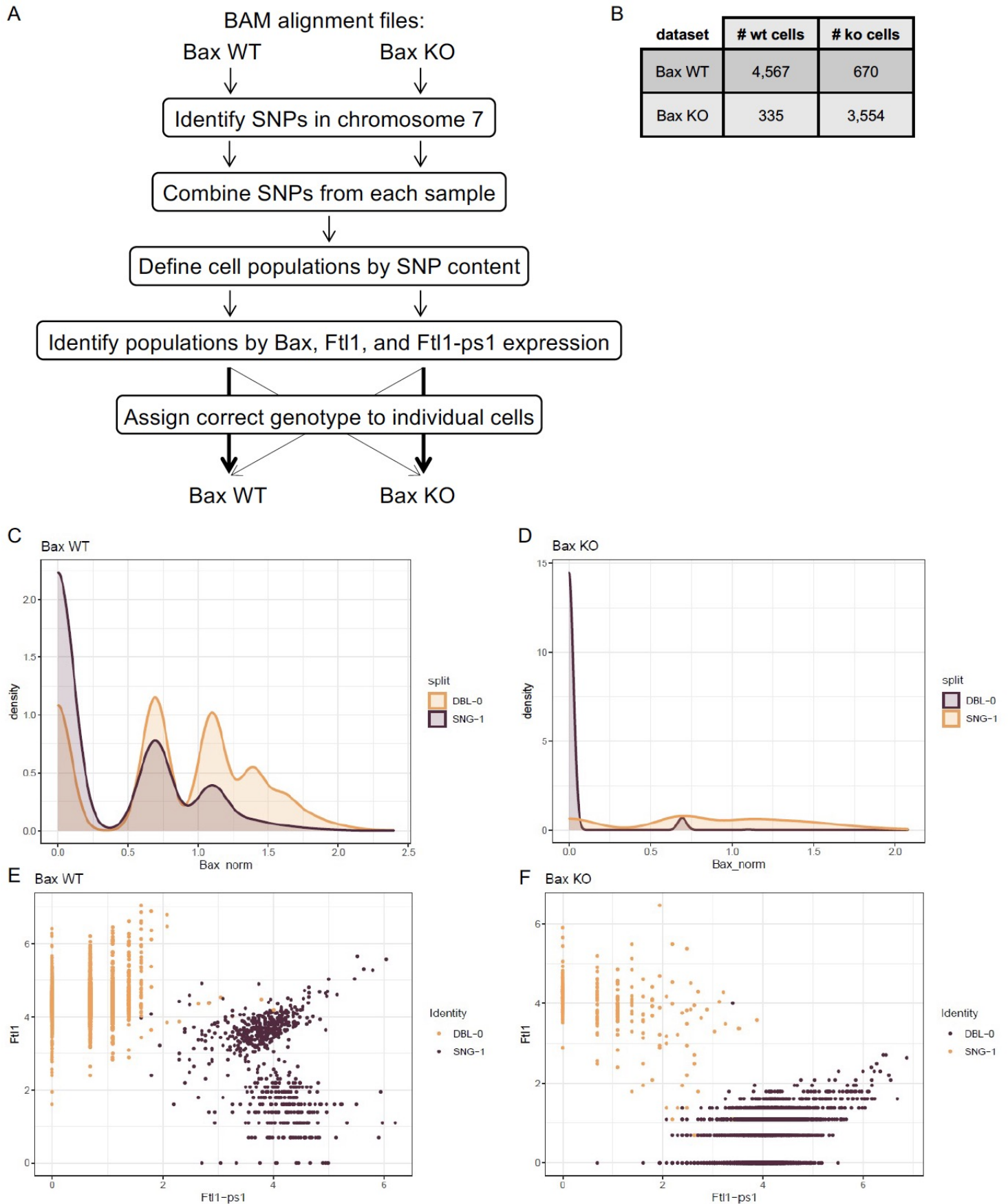
Figure S3. Related to Figure 1. (A) Summary of *in silico* genotyping workflow from alignment files to genotyping of individual cells. (B) Number of cells from the filtered dataset (Bax WT or Bax KO) ultimately assigned to each genotype (# wt or # ko). (C-F) In silico genotyping identified two populations within each dataset, which were classified as wt or ko based on (C, D) Bax, (E, F) Ftl1, and Ftl1-ps1 expression. Data shown are log normalized counts. The two populations are pseudocolored here: yellow for wt and purple for ko.

Supplemental Fig. 4. Identification of non-microglia populations
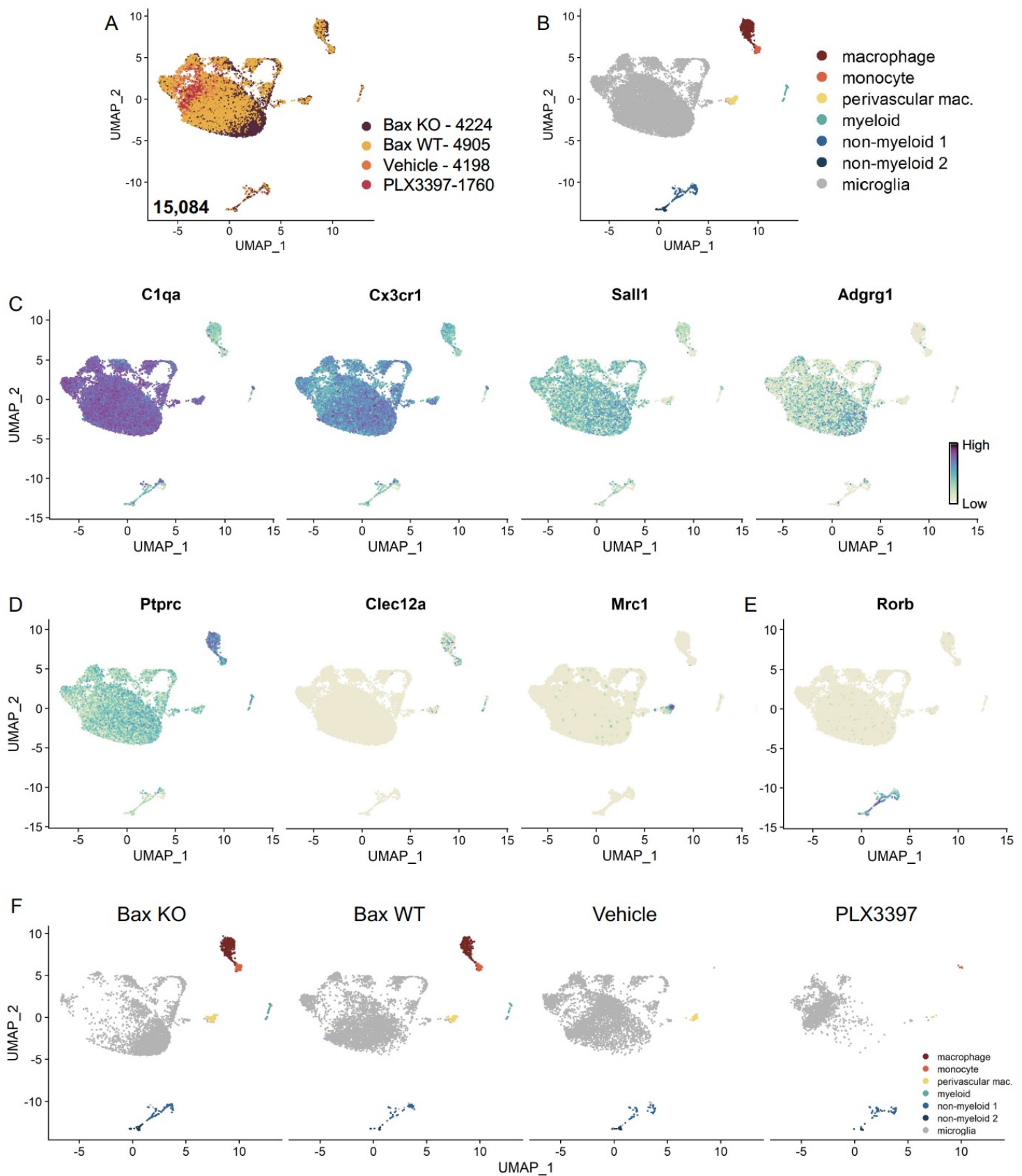
Figure S4. Related to Figure 1. (A) UMAP plot of 15,084 cells from all 4 samples. BAX KO, 4224 cells; BAX WT 4902 cells; Vehicle, 4198 cells; PLX3397, 1760 cells. (B) UMAP plot with clusters labeled based on published markers: macrophages (dark red), monocytes (bright red), perivascular macrophages (yellow), other myeloid-like cells (teal), non-myeloid 1 and 2 (shades of blue), and microglia (gray). (C) UMAP plots colored by relative gene expression (dark purple = highest, light yellow= lowest) of highly expressed or specific microglia genes. (D) UMAP plots of published monocyte or macrophage genes. (E) UMAP plot of neuronal gene expression. (F) UMAP plots illustrating the distribution of cells across clusters for each sample.

Supplemental Fig 5. Postnatal retinal microglia express *Ccl3 in vivo*
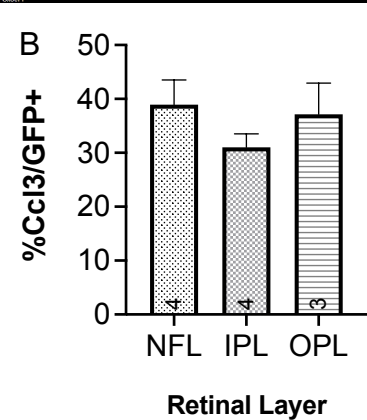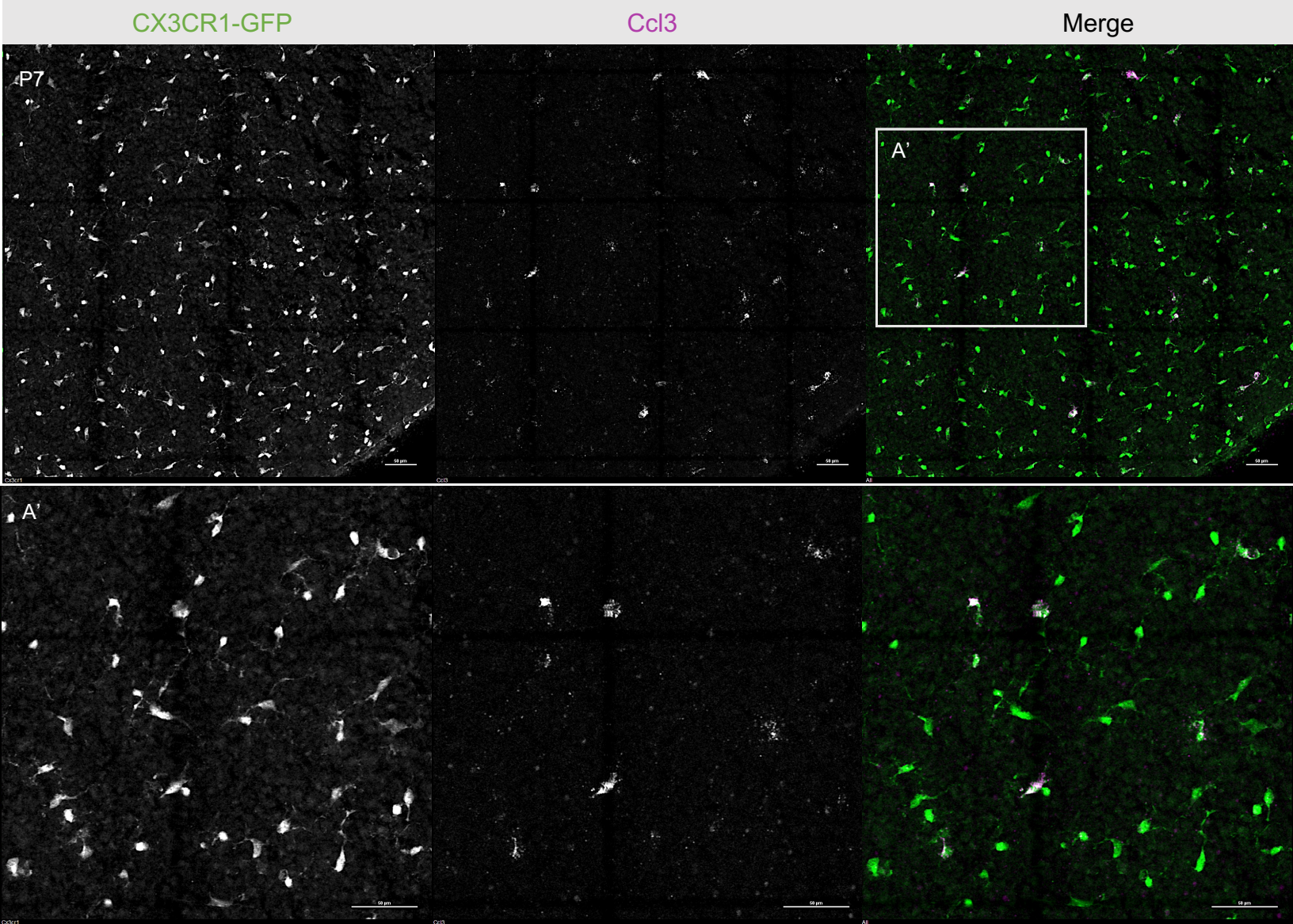
Figure S5. Related to Figure 1. (A) Max projected confocal image of *in situ* hybridization chain reaction (HCR) on P7 whole mount retina probed for *Ccl3*. CX3CR1-GFP (protein, green), Ccl3 (RNA, magenta). A' inset, higher magnification. Scale bar 50 μm (B) Percent *Ccl3*+ of CX3CR1-GFP+ cells in each layer of the P7 retina of the mid-periphery. (n=>3; ±SEM). NFL, nerve fiber layer; IPL, inner plexiform layer; OPL, outer plexiform layer.

Supplemental Fig 6. Bax KO retinas have reduced microglial density
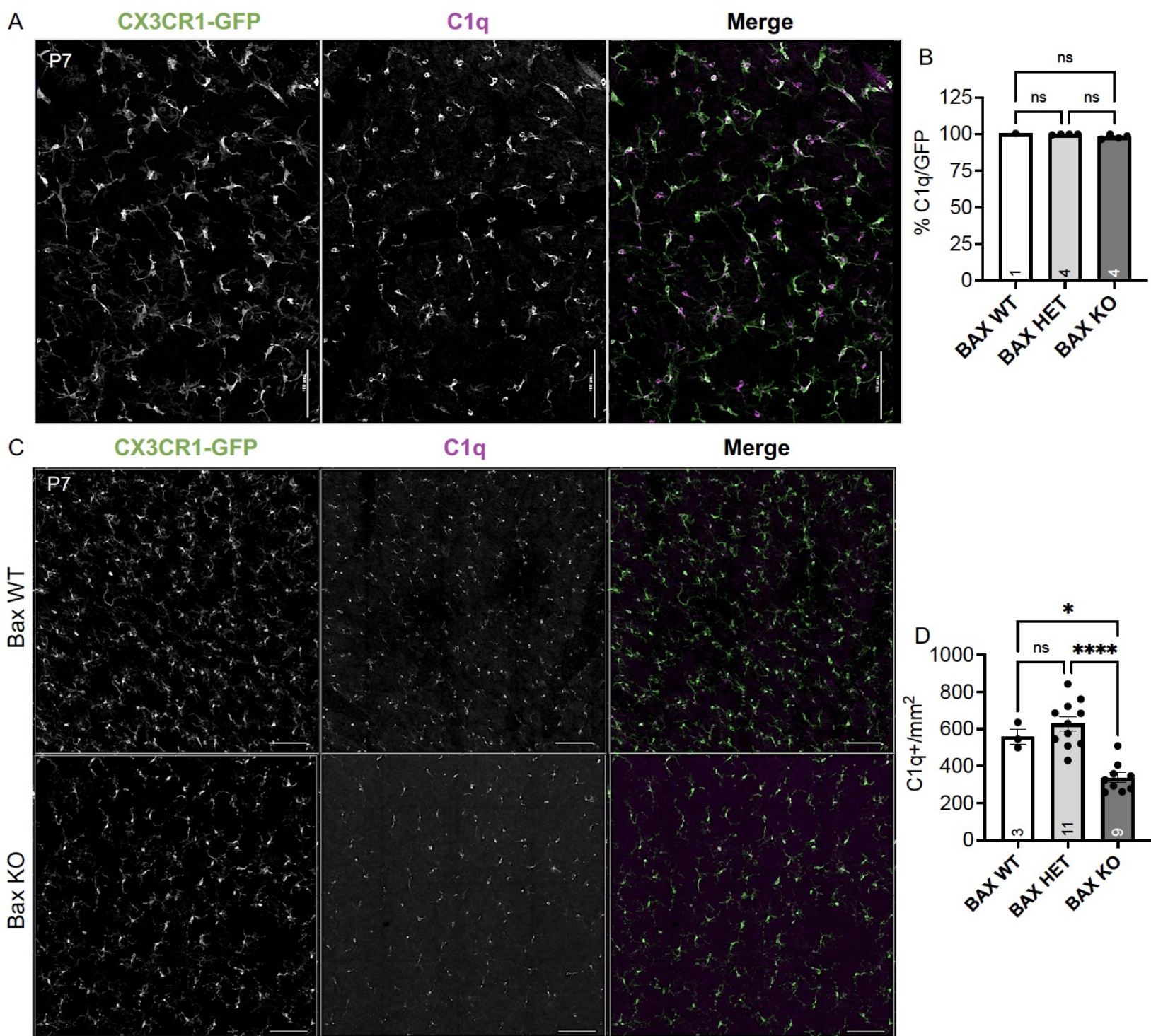
Figure S6. Related to Figure 3. (A) Max projected confocal image of a representative P7 whole mount retinas. CX3CR1-GFP/+ (green); C1q (magenta). Scale bar 100 µm. (B) Percent colocalization of C1q$^+$ cells to CX3CR1-GFP$^+$. (n=1 WT, n=4 HET, n=4 KO ; ±SEM) Kruskal-Wallis test statistic=4.818, p=0.0667. (C) Max projected confocal image of whole mount immunostained retinas of BAX WT and BAX KO. CX3CR1-gfp (green); C1q (magenta). Scale bar 50 µm. (D) Quantification of C1q$^+$ microglia in a 0.526mm^2 area of the dorsal, mid-peripheral, vascularized region of the retina. (n=3 WT, n=11 Het, n=9 KO; ±SEM) One-way ANOVA F(2,20)=19.90 p<0.0001 and Tukey's multiple comparisons test *p=0.0112, ****p<0.0001.
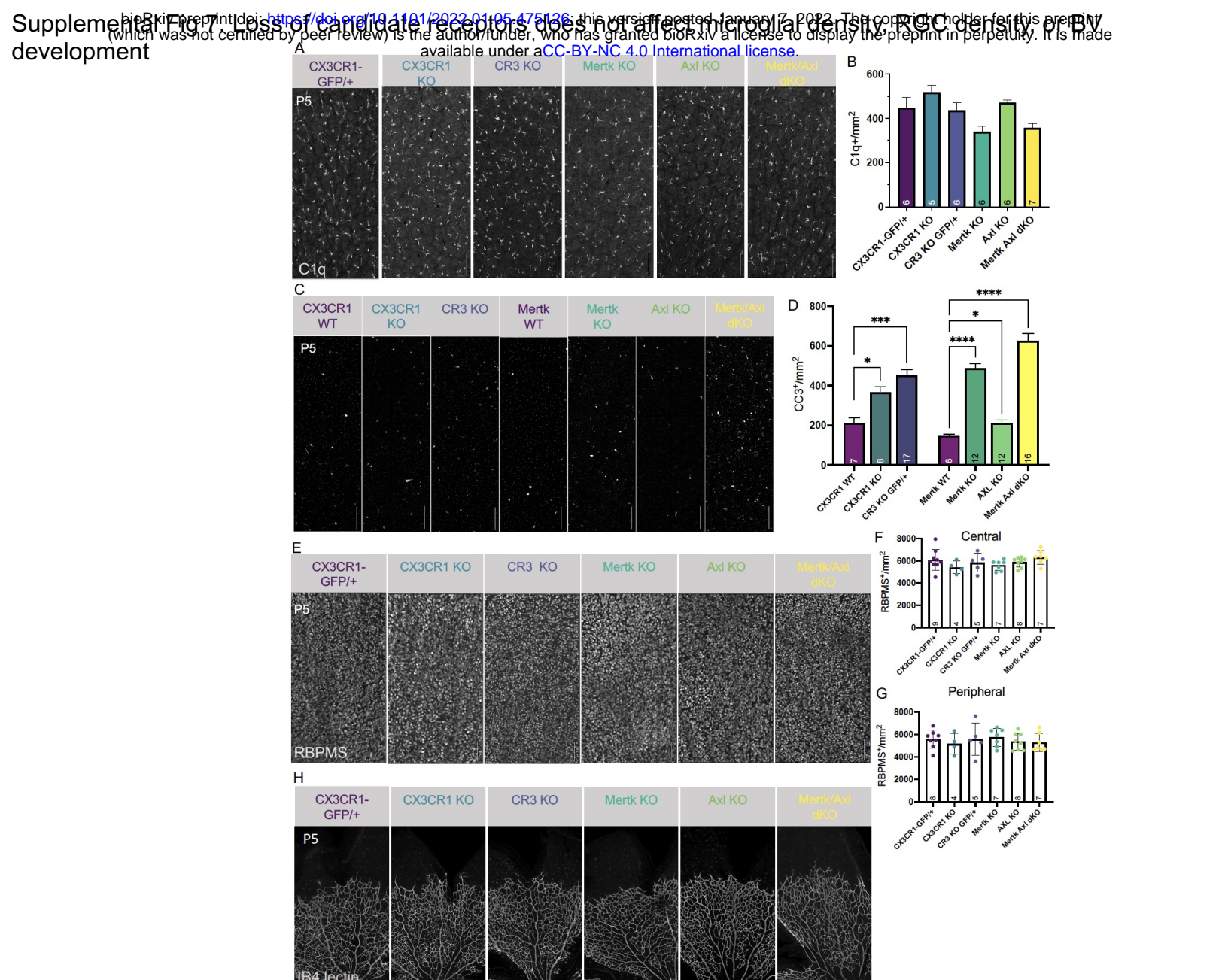
Figure S7. Related to Figure 5. (A) Max projected confocal images of whole mount immunostained retinas of all genotypes at P5 of dorsal leaf, mid-peripheral region. C1q (mono). Scale bar 100µm. (B) Microglia density (C1q$^+$/mm^2) for each genotype in dorsal leaf, mid-peripheral, vascularized region. (n=6 CX3CR1-GFP/+, n=5 CX3CR1 KO, n=6 CR3 KO CX3CR1-GFP/+, n=6 Mertk KO, n=6 Axl KO, n=7 Mertk Axl dKO; ±SEM). Kruskal-Wallis test statistic = 15.91 **p=0.0071 and Dunn's multiple comparisons tests CX3CR1 KO vs Mertk KO *p=0.0489, CX3CR1 KO vs Mertk Axl dKO *p=0.0432, rest of the comparisons, ns. (C) Max projected confocal images of whole mount immunostained retinas of all genotypes at P5 of dorsal leaf, mid-peripheral region. CC3 (mono). Scale bar 100µm. (D) Total apoptotic body density (CC3$^+$/mm^2) for each genotype in entire dorsal leaf (Figure S9A). (n=7 CX3CR1 WT, n=8 CX3CR1 KO, n=17 CR3 KO CX3CR1-GFP/+, n=6 Mertk WT, n=12 Mertk KO, n=12 Axl KO, n=16 Mertk Axl dKO; ±SEM). Welch's ANOVA W(6, 27.82)=54.71 ****p<0.0001 and Dunnett's T3 multiple comparisons test. CX3CR1 WT vs CR3 KO ***p=0.0003, CX3CR1 WT vs CX3CR1 KO *p=0.0328, CX3CR1 WT vs Mertk WT ns, CX3CR1 WT vs Mertk KO ****p<0.0001, CX3CR1 WT vs Axl KO ns, CX3CR1 WT vs Mertk Axl dKO ****p<0.0001, CR3 KO vs CX3CR1 KO ns, CR3 KO vs Mertk WT ****p<0.0001, CR3 KO vs Mertk KO ns, CR3 KO vs Axl KO ****p<0.0001, CR3 KO vs Mertk Axl dKO *p=0.0257, CX3CR1 KO vs Mertk WT ***p=0.0007, CX3CR1 KO vs Mertk KO ns, CX3CR1 KO vs Axl KO *p=0.0089, CX3CR1 KO vs Mertk Axl dKO ***p=0.0004, Mertk WT vs Mertk KO ****p<0.0001, Mertk WT vs Axl KO *p=0.0467, Mertk WT vs Mertk Axl dKO ****p<0.0001, Mertk KO vs Axl KO ****p<0.0001, Mertk KO vs Mertk Axl dKO ns, Axl KO vs Mertk Axl dKO ****p<0.0001. Not all comparisons shown on graph. (E) Max projected confocal images of whole mount immunostained retinas of all genotypes at P5 of dorsal leaf, mid-peripheral region. RBPMS (mono). Scale bar 100µm. (F and G) RBPMS density for each genotype in dorsal leaf, central (F) and peripheral (G). (n=9,8 CX3CR1-GFP/+, n=4 CX3CR1 KO, n=5 CR3 KO CX3CR1-GFP/+, n=7 Mertk KO, n=8 Axl KO, n=7 Mertk Axl dKO; ±SEM). (F) Central: One-way ANOVA F(6,36)=1.251, p=0.3040 and (G) Peripheral: One-way ANOVA F(6,35)=0.4985, p=0.8051. (H) Max projected confocal images of whole mount immunostained retinas of all genotypes at P5 of dorsal leaf. IB4-lectin (mono). Scale bar 100µm.
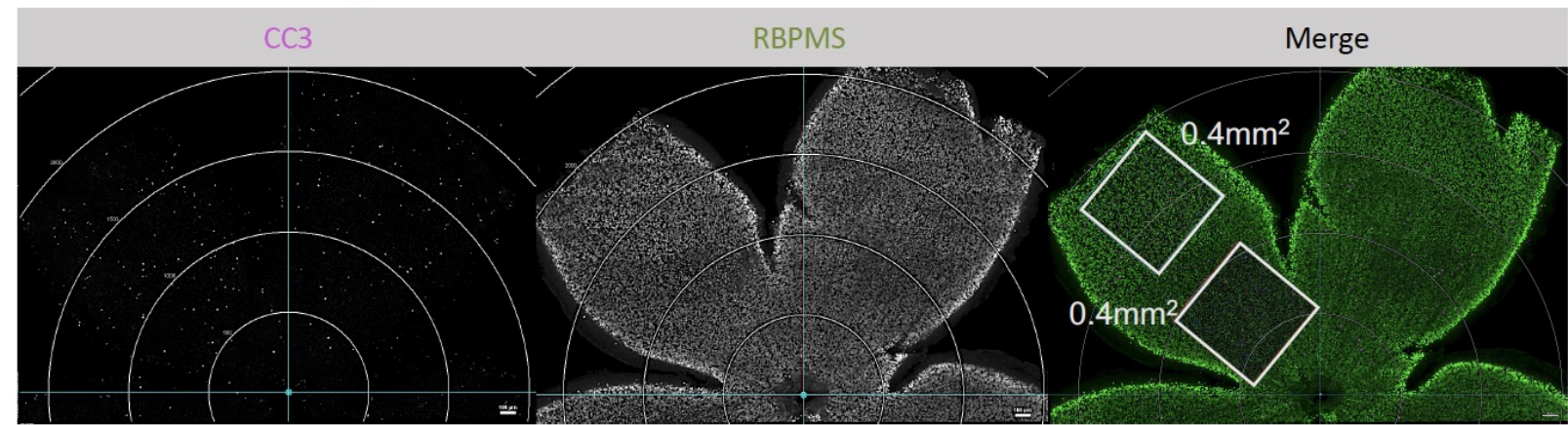
Figure S8. Related to Figure 5 and S7. (A) Max projected confocal images of whole mount immunostained retinas. Apoptotic cells, CC3 (magenta); RGCs, RBPMS (green). Circular grid in increments of 500μm. White line in merge outlines area analyzed for total CC3 analysis. Scale bar 100μm. (B) Confocal of whole mount immunostained retinas. Apoptotic cells, CC3 (magenta); RGCs, RBPMS (green). Circular grid in increments of 500μm. One central and one peripheral ROI of 0.400 mm^2 was analyzed in the dorsal leaf and averaged. Scale bar 100μm.

Supplemental Fig. 9. Mertk Axl dKOs do not have substantially altered lysosomal, lipid metabolism, or remodeling gene expression
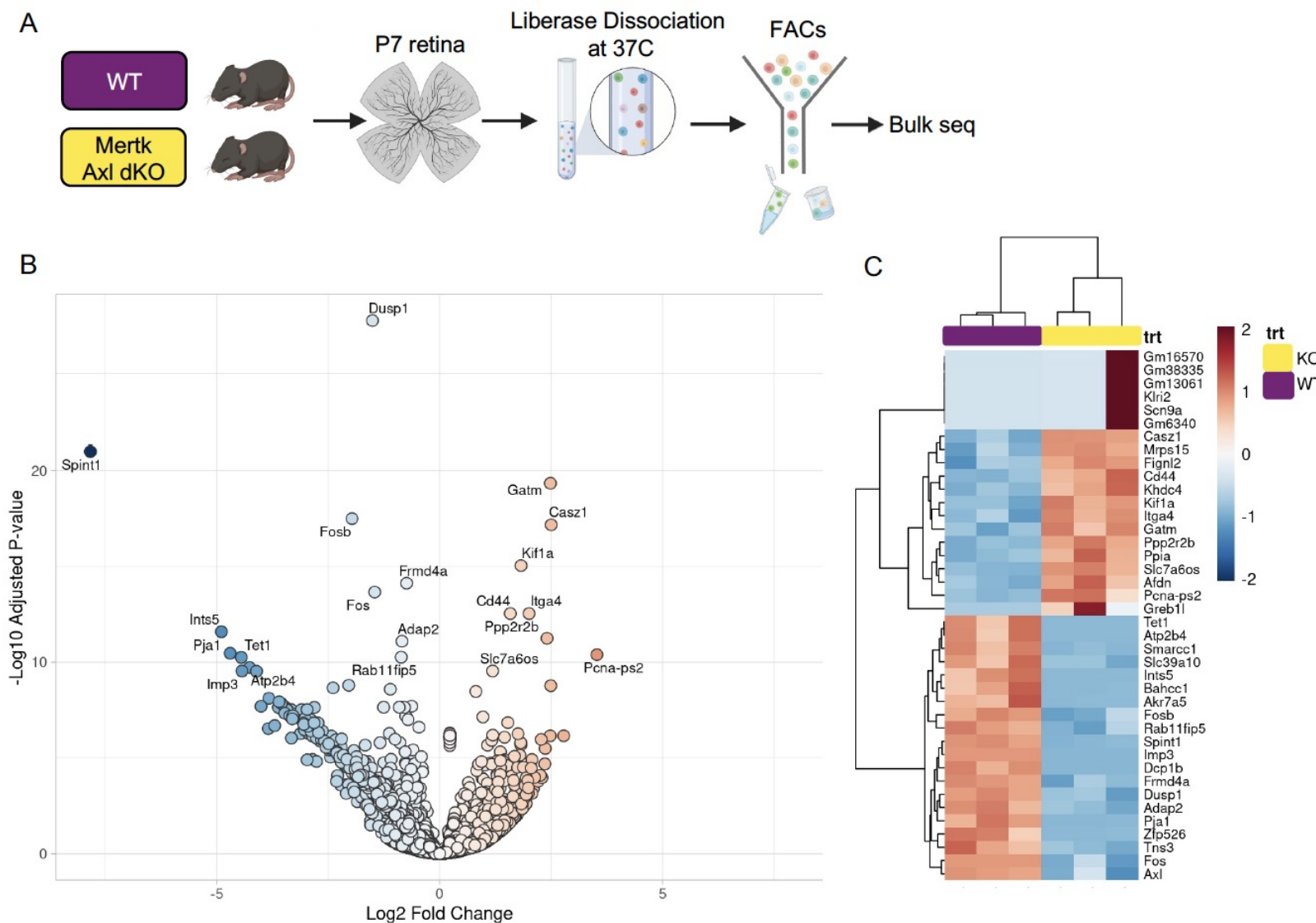
Figure S9. Related to Figure 7. (A) Workflow for P7 retinal collection, dissociation, sorting, and sequencing of individual microglia from 2 groups: WT, Mertk Axl dKO (n=3 each). (B) Volcano plot of differential expression analysis on WT compared to Mertk Axl dKO microglia. 404 upregulated and 1013 downregulated genes in dKO vs WT based on padj <0.05. (B) Heatmap of bulk sequencing on sorted microglia from WT and Mertk Axl dKO retinas top 20 genes upregulated and downregulated in dKO vs WT. Color represents z-score of normalized gene expression across all 6 samples.