Supplemental Information

The Hippo Pathway Blocks Mammalian Retinal Müller Glial Cell Reprogramming

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SUPPLEMENTAL FIGURES

Figure S1 (related to Figure 1 and 2). The YAP antibody labels both the MG nuclei in the INL and the cytoplasmic processes spanning the adult retina, but the pYAP antibody does not label MG nuclei (A). In the MG cytoplasmic processes, YAP and pYAP signals overlap (B-C). TEAD1 immunofluorescence on adult retinal cryosections showed clear localization to SOX9+ MG nuclei (D). Whole adult retinal lysates were immunoprecipitated with antibodies against YAP or an IgG negative control. Western blot with anti-TEAD1 and YAP antibodies showed that YAP and TEAD1 co-immunoprecipitate (E). By 24 hours post NMDA damage, wild type retinae exhibited a significant increase in Cyclin D1 mRNA and protein expression (see Figure 2A, 2D-F). To verify that the increase in CYCLIN D1 occurs in MGs, we performed co-immunofluorescence with antibodies against MG markers GS and SOX2 and showed that the CYCLIN D1 immunofluorescent signal is within MGs (F-G). N = 3 per sample. ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer.
Figure S2 (related to Figure 2). In the adult retina, *Glast-CreERT2* expression and activity is specific to MG cells. In the absence of tamoxifen, *Glast* promoter-driven CreERT2 protein is unable to translocate to the nucleus to mediate recombination between loxP sites (white cell in A). Upon intraperitoneal tamoxifen administration, CreERT2 is now able to translocate to the nucleus leading to recombination between loxP sites. Shown here (red cell in A) is an example of recombination of a loxP-flanked STOP cassette within *ROSA26R-tdTomato* Cre reporter mouse. *Glast-CreERT2<sup>+/tg</sup>; R26R-<sup>tdTomato</sup>+/tg* mice show Cre-mediated recombination specifically in tdTomato<sup>+</sup> MGs spanning the retina and this identity was confirmed by overlapping SOX9 immunofluorescence in the nuclei of tdTomato<sup>+</sup> MGs within the INL (B). *Glast-CreERT2<sup>+/tg</sup>* specificity was further validated by crossing to the *ROSA26R-nTnG<sup>+/tg</sup>* Cre reporter (Prigge et al., 2013; Schmidt, 2013). Nuclear GFP (indicative of Cre-mediated recombination) was observed specifically in the INL and co-localized to SOX9<sup>+</sup> MGs (C, see magnified insets). We never observed GFP expression adjacent to the GCL among the retinal astrocyte population. N = 3 per sample. ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer.
mRNA was extracted from total retinal lysates from three independent tamoxifen-induced CKO and corn oil-injected control retinae. We performed qrtPCR analysis and found statistically significant reductions in Yap, Taz, Cyclin D1, and Cyclin D3 (A). It is important to note that the varying levels of mRNA reduction between mice likely reflect a combination of Cre mosaicism, variation in tamoxifen induction, and inefficiency in recombining four independent floxed loci in a given cell. Furthermore, Yap and Taz are also expressed in retinal endothelial cells, which are expected to contribute to mRNA transcripts within total retinal lysate preparations (Neto et al., 2018; Sakabe et al., 2017). For qrtPCR, mRNA levels are relative to the corn oil control (set to 1) and depicted as fold change ±SEM (n = 3 independent pooled samples per group; Student’s t test). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001.

To further illustrate that the loss of Cyclin D1 in MGs is coincident with loss of Yap and Taz, we generated Chx10-Cre+/tg; Yaplox/lox; Tazlox/lox; ROSA26R-mTmG+/tg mice. Chx10-Cre (Rowan and Cepko, 2004) is expressed broadly in retinal progenitor cells during development and provides large regions of Cre-mediated recombination that can be easily visualized by GFP expression from the R26R-mTmG Cre reporter. In GFP+ regions of the retina, we observed coincident loss of YAP and CYCLIN D1 immunofluorescence in the INL (white arrowheads in B). In adjacent tdTomato+ regions, which did not experience Cre-mediated recombination, CYCLIN D1 and YAP co-expression persisted (yellow arrowheads in B). Importantly, immunofluorescence also showed that SOX9+/YAP-MGs are still present in the CKO retinae indicating that loss of CYCLIN D1 expression in MGs is not due to loss of MGs, but rather loss of Yap and Taz expression (yellow arrowheads in C). The same results were observed for CYCLIN D3 expression (data not shown). N = 3.

Map of the Cyclin D1 gene showing the location of two putative TEAD binding sites (red arrowheads in D). Immunofluorescence showed that the ImM10 MG-like cell line expresses YAP, TEAD1, CYCLIN D1 and SOX9 (E). N = 3 per sample. ImM10 cell lysates were immunoprecipitated with antibodies against YAP or an IgG negative control. Western blot with anti-TEAD1 and YAP antibodies showed that YAP and TEAD1 co-immunoprecipitate (F). N = 3 per sample. YAP chromatin immunoprecipitation was performed on ImM10 cells followed by qPCR analysis of the 2 putative TEAD motifs. YAP was enriched on motif #1 that lies 3.7 kb upstream of the Cyclin D1 start site, but not on motif #2 in intron 3 (G). N = 3 per sample.
mRNA was extracted from total retinal lysates from three independent tamoxifen-induced CKO and corn oil-injected control retinae. We performed qRT-PCR analysis and found that one out of three CKO samples showed a significant reduction in Lats1 while all three showed a modest but significant reduction in Lats2 (A). Importantly, in contrast to the Yap/Taz CKOs (see Figure S3A), the Lats1/2 CKO retinae showed a significant increase in Cyclin D1 mRNA. This finding raised the possibility that loss of Lats1/2 in a subset of MGs results in more active YAP/TAZ, which drives an increase in Cyclin D1 and spontaneous MG cell cycle entry (further supported by Figure 3). Interestingly, the increases in Cyclin D3 levels were less significant suggesting that...
loss of Lats1/2 has a greater impact on Cyclin D1 expression (A). Similar to the Yap/Taz CKOs, the varying levels of mRNA reduction (Lats1/2) between mice likely reflect a combination of Cre mosaicism, variation in tamoxifen induction, and inefficiency in recombining four independent floxed loci in a given cell. Also, Lats1, and Lats2 are expressed in retinal endothelial cells, which are expected to contribute to mRNA transcripts within total retinal lysate preparations (Neto et al., 2018; Sakabe et al., 2017). For qrtPCR, mRNA levels are relative to the corn oil control (set to 1) and depicted as fold change ±SEM (n = 3 independent pooled samples per group; Student’s t test). N = 3 per group. ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001, ns = not significant.

For visual resolution of the Lats1/2 CKO MGs, we generated Glast-CreERT2+/tg; Lats1flox/flox; Lats2flox/flox mice that carry the Rosa26R-nTnG+/tg Cre reporter (Prigge et al., 2013; Schmidt, 2013). We chose this approach because LATS1/2 antibodies proved ineffective for immunofluorescence. A subset of MGs in the Lats1/2 CKO retinae exhibit Cre activity indicated by patches of GFP expression from the ROSA26R-nTnG Cre reporter (see green channel in B-D). Within this population of MGs, we also observed a more intense CYCLIN D1 immunofluorescent signal as compared to corn oil-injected controls (see magenta channel in B-D and quantification in E). It is important to note that the increase in CYCLIN D1 immunofluorescence frequently coincides with GFP (Cre reporter) expression (white arrows in D). However, we also observed CYCLIN D1 upregulation in MGs that do not express GFP (blue arrows in D) and GFP+ MGs that do not upregulate CYCLIN D1 (yellow arrow in D). These data underscore the inefficiencies Cre-mediated recombination of 5 independent loci in a single cell and is the reason why we turned to the more straightforward Yap5SA transgenic model to further investigate bypass of the Hippo pathway in MGs. N > 3 per sample.

We next sought to determine whether MGs within the Lats1/2 CKO retinae also exhibited more nuclear YAP immunofluorescence, which would indicate loss of the Hippo pathway’s negative regulation of YAP. We were able to locate discrete regions of the CKO retinal INL that had MGs with more intense nuclear staining of YAP (compare F-G). Importantly, this increase in fluorescence occurred in the same MGs that exhibited an increase in CYCLIN D1 (H-I, measurements were taken across the red line in H). Therefore, these data suggest that the increase in CYCLIN D1 observed in MGs of the Lats1/2 CKOs is a consequence of deregulated YAP. These data also support our earlier conclusion (Figures 2, S3) that Cyclin D1 is transcriptionally regulated by YAP. N > 3 per sample. EdU labeling of adult Lats1/2 CKO retinae showed EdU+/SOX2+ MGs in the INL of the central retina (arrowhead in J). Panel J is an expanded view of the insets shown in Figure 3F. These results were further confirmed by SOX9 immunofluorescence. EdU+/SOX9+ MGs were observed throughout the retina including peripheral regions (arrowheads in K). N > 3 per group. ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer.
Figure S5 (related to Figure 3). Schematic of the core Hippo signaling pathway and the consequence of YAP5SA expressed by the Yap5SA transgenic mouse (A). Expression of the Yap5SA transgene in the adult mouse retina (B). The transgene, indicated by GFP, is primarily expressed in the ONL. However, we observed mosaic expression among the MGs in the INL that was confirmed by SOX2 immunofluorescence (see inset from boxed region in B). Upon tamoxifen injection (IP), YAP5SA (detected with an anti-FLAG antibody) is expressed in a subset of MGs that also express LacZ from the transgene’s IRES-LacZ cassette (C). N = 3 per group. ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer.
Figure S6 (related to Figure 3 and 4). LacZ fate mapping of the YAP5SA+ MGs. To track the fate of YAP5SA+ MGs, we utilized the IRES-LacZ cassette that, upon Cre recombination, is expressed from the Yap5SA transgene. LacZ and CYCLIN D1 immunofluorescence showed a dramatic, cell-autonomous increase in CYCLIN D1 that was well above the level of endogenous expression in the neighboring LacZ/YAP5SA-negative MGs (compare the white arrowheads to the blue arrowheads in A). Analysis of NMDA damaged transgenic retinas showed a dramatic expansion of the LacZ+ cells with high CYCLIN D1 immunofluorescence (B). When we analyzed tamoxifen-induced and non-induced transgenic retinas for GFAP upregulation in response NMDA damage, we found that the YAP5SA+ MGs failed to express GFAP, but the adjacent non-transgenic cells did (compare C and D and see boxed regions and insets in D). This was further illustrated by determining that, in the MG cytoplasmic processes (across the magenta line in D), GFAP and LacZ signals do not overlap (E). These data suggested that YAP5SA+ MGs lost MG cell identity. N = 3 per group. ONL = outer nuclear layer, INL = inner nuclear layer, GCL = ganglion cell layer.
Figure S7 (related to Figure 3 and 4). Heat map of differential gene expression showing heterogeneity among the YAP5SA+ and YAP5SA- MG cell clusters (A). The YAP5SA+ clusters (YAP5SA-1 to 4) expressed many canonical YAP target genes, like Amotl2, Birc5, and Vgl13. Interestingly, the YAP5SA- control MGs displayed a high degree of heterogeneity, with a total of 7 unique clusters identified (MGC-1 to 7). The most obvious source of heterogeneity was derived from the immune response being sensed by MGs. Clusters MGC-5, MGC-6, and MGC-7 appeared to be undergoing different levels of immune activation and the interferon response. MGC-6 expressed high levels of the
NF-κB target gene Trib3, as well as Prok2 and the Notch signaling regulator Chac1. MGC-7 expressed many interferon response genes, including ligp1 and Zbp1. Moreover, MGC-7 expressed high levels of chemokines like the monocyte-chemotactic protein 3 gene, Ccl7. Thus, MGs respond to wounding by eliciting a robust transcriptional immune response, although they do so in a heterogenous manner.

Cyclin D1 is normally expressed at low levels in MGs but is dramatically upregulated after YAP5SA induction (see Figure 4D). To further illustrate how potent the transgene is at activating Cyclin D1 expression, we grouped all YAP5SA+ clusters together and all YAP5SA- clusters (control) together. This is depicted in the tSNE plot showing the YAP5SA+ cluster in red and the control cluster in blue (B). As shown in the dot plot, transcript levels were quantified to each cluster and we uncovered that over 99% of YAP5SA+ cells expressed Cyclin D1, compared to only 77% of control transcriptomes (C). Importantly, as shown in both the dot and violin plot, the average expression level of Cyclin D1 was also much higher in YAP5SA+ MGs (C-D). The size of the dot represents the percent of cells expressing Cyclin D1 and the color represents expression levels (high=red and low=blue) (C). We also performed cell cycle analysis for every single cell transcriptome within each MG cluster and determined that high Cyclin D1 expression coincides with cell cycle initiation as the proportion of YAP5SA+ MGs found to be proliferating (in S-phase, or G2M) was dramatically higher than control cells (E).

Relative Glul and Gfap expression between YAP5SA+ and YAP5SA- (control) MGs is shown in feature plots (F). Dot plots of transcript levels show that the YAP5SA+ MG population has much lower expression levels of Glul and Gfap compared to control MGs and less than 32% of YAP5SA+ MGs were found to express Gfap transcript (G). These data indicate a shift of YAP5SA+ cells away from MG identity. The size of the dot represents the percent of cells expressing Glul or Gfap and the color represents expression levels (high=red and low=blue).
Figure S8 (related to Figures 1-4). Model of Hippo-mediated block to Müller glia reprogramming to a proliferative, progenitor-like state. During retinal homeostasis, MGs co-express Cyclin D1, Cyclin D3, and p27Kip1. P27KIP1 normally represses the ability of CYCLIN D1/D3 to drive S-phase entry thereby maintaining MGs in a quiescent state (A). In this current study, we show that Cyclin D1 and Cyclin D3 gene expression in MGs is under the control of the Hippo effector YAP (A’). Upon retinal damage, through an unknown mechanism, P27Kip1 expression is reduced and this presumably leads to de-repression of CYCLIN D1/D3 activity, an increase in Cyclin D1 transcription, and MG S-phase entry (B-B’). However, 12 to 24 hours post-damage, due to Hippo pathway repression of YAP, Cyclin D1/D3 gene expression is not maintained, and subsequent rounds of MG cell cycle entry are blocked (C-C’). In the case of the Yap5SA transgenic MGs, the MGs are non-responsive to Hippo signaling resulting in sustained YAP-driven Cyclin D1 expression, successive rounds of MG proliferation, and reprogramming to a progenitor-like state (D-D’).