Midkine-a Is Required for Cell Cycle Progression of Müller Glia during Neuronal Regeneration in the Vertebrate Retina

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In the retina of zebrafish, Müller glia have the ability to reprogram into stem cells capable of regenerating all classes of retinal neurons and restoring visual function. Understanding the cellular and molecular mechanisms controlling the stem cell properties of Müller glia in zebrafish may provide cues to unlock the regenerative potential in the mammalian nervous system. Midkine is a cytokine/growth factor with multiple roles in neural development, tissue repair, and disease. In midkine-a loss-of-function mutants of both sexes, Müller glia initiate the appropriate reprogramming response to photoreceptor death by increasing expression of stem cell-associated genes, and entering the G1 phase of the cell cycle. However, transition from G1 to S phase is blocked in the absence of Midkine-a, resulting in significantly reduced proliferation and selective failure to regenerate cone photoreceptors. Failing to progress through the cell cycle, Müller glia undergo reactive gliosis, a pathological hallmark in the injured CNS of mammals. Finally, we determined that the Midkine-a receptor, anaplastic lymphoma kinase, is upstream of the HLH regulatory protein, Id2a, and of the retinoblastoma gene, p130, which regulates progression through the cell cycle. These results demonstrate that Midkine-a functions as a core component of the mechanisms that regulate proliferation of stem cells in the injured CNS.

Key words: zebrafish; neurogenesis; photoreceptors; proliferation; reprogramming

Significance Statement
The death of retinal neurons and photoreceptors is a leading cause of vision loss. Regenerating retinal neurons is a therapeutic goal. Zebrafish can regenerate retinal neurons from intrinsic stem cells, Müller glia, and are a powerful model to understand how stem cells might be used therapeutically. Midkine-a, an injury-induced growth factor/cytokine that is expressed by Müller glia following neuronal death, is required for Müller glia to progress through the cell cycle. The absence of Midkine-a suspends proliferation and neuronal regeneration. With cell cycle progression stalled, Müller glia undergo reactive gliosis, a pathological hallmark of the mammalian retina. This work provides a unique insight into mechanisms that control the cell cycle during neuronal regeneration.

Introduction
Cell division is an essential biological process during development, homeostasis, and repair. In the CNS of adult mammals, stem cells reside in specialized niches, and these cells maintain the ability to divide and generate new neurons (Kriegstein and Alvarez-Buylla, 2009; Ming and Song, 2011). In the vertebrate retina, Müller glia harbor molecular features of stem and progenitor cells (Dyer and Cepko, 2000). In mammals, Müller glia respond to injury by partial dedifferentiation and entering the G1 phase of the cell cycle (Bringmann et al., 2006). However, in general, this reprogramming does not lead to cell division, and structural remodeling and the loss of retinal homeostasis are the typical sequelae (Bringmann et al., 2009; Karl and Reh, 2010; Hamon et al., 2016). Importantly, in the limited instances where regeneration does occur, new neurons functionally integrate into existing synaptic circuits (Jorstad et al., 2017; Yao et al., 2018).
indicating that in the mammalian retina the limitations of neuronal regeneration hinge on a more complete neurogenic response in Müller glia.

In zebrafish, Müller glia can adopt the features of stem cells (Karl and Reh, 2010; Goldman, 2014; Gorsch and Hyde, 2014; Lenkowski and Raymond, 2014; Hamon et al., 2016). In uninjured retinas, Müller glia reside in a quiescent state and function to maintain retinal homeostasis. Neuronal death triggers Müller glia to reprogram into a stem cell-like state, enter the cell cycle, and undergo a single asymmetric division to produce rapidly dividing, multipotent retinal progenitors with the ability to regenerate retinal neurons (Nagashima et al., 2013; Lenkowski and Raymond, 2014). Several signaling pathways have been identified that regulate the initial response of Müller glia (Karl and Reh, 2010; Goldman, 2014; Gorsch and Hyde, 2014; Lenkowski and Raymond, 2014; Hamon et al., 2016). Ascl1, Lin28, and Stat3 have been identified as “core” transcriptional regulators that govern signaling cascades required for Müller glia to divide (Fauseett and Goldman, 2006; Ramachandran et al., 2010; Nelson et al., 2012).

Midkine is a growth factor/cytokine that has multiple roles in neural development, repair, and disease (Sakamoto and Kadomatsu, 2012; Winkler and Yao, 2014; Sorrell et al., 2017). In malignant tumors, Midkine promotes proliferation and metastasis (Muramatsu, 2011) and is also involved in CNS inflammation (Muramatsu, 2011; Weckbach et al., 2011; Herradon et al., 2019). The diverse functions of Midkine are transduced through receptors, which may function individually or as members of a multi-protein complex (Muramatsu, 2011; Weckbach et al., 2011; Xu et al., 2014). During retinal development in zebrafish, midkine-a is expressed by retinal progenitors and functions to govern elements of the cell cycle (Calinescu et al., 2009b; Uribe and Gross, 2010; Luo et al., 2012). Postmitotic neurons downregulate midkine-a. Retinal injury rapidly induces midkine-a in Müller glia (Calinescu et al., 2009b; Gramage et al., 2014, 2015). Induction of midkine-a following injury has been reported for a variety of tissues with the capacity to regenerate (Ochiai et al., 2004; Lien et al., 2006), suggesting that Midkine may universally regulate aspects of tissue regeneration. The molecular mechanisms whereby Midkine governs regeneration are not well understood.

Using a Midkine-a loss-of-function mutant, we demonstrate that, following a retinal injury, Midkine-a is required for reprogrammed Müller glia to progress from G1 to S phases of the cell cycle. Following photoreceptor death, Müller glia in Midkine-a mutants reprogram into a stem cell state and enter G1 phase of the cell cycle. However, for the vast majority of Müller glia, subsequent entry into the S phase and mitotic division are blocked, resulting in failure to reorganize cone photoreceptors. Further, Midkine-a is required for the upregulation of id2a, which inhibits the retinoblastoma (Rb) family of cell cycle inhibitors. In addition, the G1-arrested Müller glia undergo reactive gliotic remodeling, hallmark of pathology in the mammalian retina. Finally, we provide evidence that activation of the Midkine receptor, ana-plastic lymphoma kinase (Alk), is required for proliferation in Müller glia.

Materials and Methods

Zebrafish. Fish were maintained at 28°C on a 14:10 h light/dark cycle with standard husbandry procedures. Adult WT, AB-strain zebrafish (Danio rerio; ZIRC, University of Oregon, Eugene, OR) and the transgenic reporter line Tg(gfap:eGFP)mi2002 (Bernardos and Raymond, 2006) were of either sex and used between 6 and 12 months of age. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Michigan.

CRISPR-Cas9-mediated targeted mutation of midkine-a. Targeted mutations in the midkine-a locus were introduced using CRISPR-Cas9 (Hwang et al., 2013). Briefly, ZFIt software (http://zfit.partners.org/ZFIt/) was used to identify guide RNA target sequence for midkine-a. Oligos to the target sequencing (GGC AGC TGC GTG GCC AAC CGG) were annealed and subcloned into the pF7 gRNA vector (Addgene plasmid # 46759; http://n2t.net/Addgene:46759; RRID:Addgene_46759; Nasevic and Ekker, 2000; Hwang et al., 2013; Jao et al., 2013). The subcloned vector was digested with BamHI, and sgRNA was transcribed with MEGAscript T7 kit (Thermo Fisher Scientific). To synthesize cas9 mRNA, pC52-nCas9n plasmid (Addgene plasmid # 47929; http://n2t.net/addgene:47929; RRID:Addgene_47929) and mRNA isolation kit (Thermo Fisher Scientific) and RNeasy Mini Kit (QIAGEN). Single-cell stage embryos were injected with 1 nl solution, containing 150 pg cas9 mRNA and 100 pg sgRNA diluted in 1X Danieux buffer with 2.5% phenol red. F0 embryos were raised to adulthood and then outcrossed with AB-WT animals. To screen for founder mutations in F1 generation, genomic DNA fragment containing the midkine-a target site was amplified with primers (forward: TGCATTTGAAGTATTAGACGCTG; reverse: GTGACGGTGTGTGCA) and was subjected to T7 endonuclease assay. PCR products with potential indel mutation in the midkine-a gene were sequenced and analyzed with National Center for Biotechnology Information Basic Local Alignment Search Tool and ExPaSy translate tool (www.expasy.org). F1 progenies with indel mutation were in-crossed, and homozygous F2 mutants were identified.

Western blots. Western blot analyses were performed as previously described (Calinescu et al., 2009a). Briefly, proteins were extracted from the heads of 30–50 WT and midka-mi2002 embryos or adult retinas (6 retinas from 3 animals per sample) in cold RIPA lysis buffer containing protease and phosphatase inhibitor mixture (Cell Signaling Technology). Proteins were separated in 12% Mini-PROTEIN TGX Precast gel (Bio-Rad) and were transferred to PVDF membranes (GenHunter). After blocking in 5% nonfat dry milk in Tris-buffered saline containing 0.3% Tween 20, membranes were incubated with rabbit anti-Midkine-a antisera or rabbit anti-STAT3 (Nelson et al., 2012) followed by HRP-conjugated secondary antibody (1:1000) (Calinescu et al., 2009a). Immunolabeled proteins were detected using the enhanced ECL detection system for chemiluminescence assay (GE Healthcare). Actin was used as a loading control.

RNAseq. Embryos at 30 hpf were manually dechlorinated. Depolymerization was performed by triturating with glass pipette in cold Ringer’s solution. Total RNA from 30 embryos was extracted using TRIZol (Invitrogen). Purity of RNA was analyzed with Bioanalyzer (Agilent Technologies). Samples with an RNA integrity number of acceptable quality (>7) were used for Illumina RNA-seq library preparation. Deep sequencing was performed on an Illumina GALLIX Sequencer (Illumina).

Read quality trimming and quality assessments. Trim Galore! (version 0.2.7; Babraham Institute) was used to trim adapter sequences and poor-quality bases (below Phred of 20) from the reads while removing any reads that were <20 nt long, using the default parameters. Trim Galore! makes use of cutadapt (version 1.4.2) (-f fastq-e 0.1-q 20-O 1-a AG-ATCGGAAGAGC file.fq.gz). The quality of the reads was assessed before and after trimming with FastQC (version 0.10.1).

Read mapping and gene-level quantitation. Quality trimmed and filtered reads were aligned to release 83 of the GRCz10 Ensembl genome build with bowtie2 (version 2.2.6), and gene-level quantitation was performed with RSEM (version 1.2.2). This was done using the rsem-calculate-expression command from RSEM, which calls bowtie2 (-sensitive -dpad 0 -gbar 99999999 -mp 1,1 -np 1 score-min L,0,– 0.1) and streams reads into RSEM for quantitation.

Differential expression analysis with DESeq2. The gene-level counts output from RSEM were filtered to remove noise before normalization with trimmed means of M, such that only genes with a FPKM (fragments
per kilobase of exon per million reads mapped) value > 1 in all replicates of any genotype were retained. Counts per million were determined using edgeR (version 3.10.2), genes with a counts per million < 1 in all samples were removed, and remaining counts were trimmed means of M normalized. Limma (version 3.24.15) was used to perform the false discovery rate (FDR) correction. Reactome pathways that were positively or negatively enriched at a FDR < 0.05 was performed with zebrafish Entrez gene identifiers to determine the associated molecular pathways. Annotations for each gene were added using biomaRt (version 2.24.0), including both the *D. rerio* Entrez gene identifiers and the corresponding *Mus musculus* Entrez orthologous gene identifiers.

**Gene ontology and pathway analysis overview of workflow.** Gene ontology (GO) enrichment analysis was performed using a log2-fold change (log2FC) ranked list from limma (log2FC > 1) and at a false discovery rate < 0.05 as input into clusterProfiler (version 2.2.4). This analysis determines which Molecular Function, Biological Process, or Cellular Component gene ontology terms are positively or negatively enriched in the mutant embryos compared with WT, at a false discovery rate < 0.05, while taking into account the magnitude and direction of change. Pathway database, Reactome pathway analyses, were used. A log2FC ranked list from limma (log2FC > 1) and at a false discovery rate < 0.05 was input into ReactomePA (1:1.2.3).

All the zebrafish genes in the dataset were manually annotated with their murine orthologs using biomaRt, and a Reactome pathway analyses was performed using zebrafish gene annotations (from zebrafish differential expression data) and zebrafish pathway annotations. The analysis was performed with zebrafish Entrez gene identifiers to determine the Reactome pathways that were positively or negatively enriched at a false discovery rate < 0.05.

**EdU and BrdU labeling.** Proliferating cells were labeled with the S-phase markers, EdU (Thermo Fisher Scientific) or BrdU (Millipore) using a protocol described previously (Bernardos et al., 2007). Briefly, zebrafish were exposed to 120,000 lux light from an EXFO X-Cite 120 W metal halide lamp for 30 min and then returned to the aquaculture system.

**Histology.** Embryos and eye balls were fixed in 4% PFA with 5% sucrose in PB, pH 7.4, at room temperature for 2 h or 4°C overnight, respectively. After rinsing with 5% sucrose in PBS, tissues were cryoprotected, fixed, and sectioned at 6 μm. Immunocytochemistry was performed as described previously (Bernardos et al., 2007). Briefly, sections were blocked with 5% normal goat serum/0.5% Triton X-100 in PBS, pH 7.4, for 2 h. Slides were blocked with 5% normal goat serum/0.5% Triton X-100 in PBS, pH 7.4, for 30 min before blocking.

**Flat-mount retinal immunocytochemistry.** Retinas were isolated from the flat-mounted zebrafish and fixed overnight at 4°C in 4% PFA in PB with 5% sucrose, pH 7.4. Immunocytochemistry was performed as previously described (Nagashima et al., 2017). Briefly, retinas were treated with 10 mM sodium citrate in 0.05% Tween 20, pH 6.0, in boiling water for 5 min. For additional washes, retinas were blocked with 10% normal goat serum/1% Tween 20/1% DMSO in PBS, pH 7.4, with 0.1% sodium azide for 2 h. Primary and secondary antibodies were diluted in 0.5% normal goat serum/1% Tween 20/1% DMSO in PBS, pH 7.4, with 0.1% sodium azide, and incubations were performed at room temperature overnight.

**Microscopy and image analysis.** Retinal cross sections and flat-mount retinas were imaged with DM6000 Upright Microscope System and TCS SP5 confocal microscope (Leica Microsystems), respectively. Adobe Photoshop CS6 Extended (Adobe Systems), Application Suite X (Leica Microsystems, ImageJ (https://imagej.nih.gov/ij/), or Imaris 7.6.1 (Bitplane) were used for image analysis, 3D reconstruction, and movie production.

**qPCR.** Total RNA from whole retinas was extracted using TRIzol (6 retinas from 3 fish per sample) (Invitrogen). RNA was quantified using Nanodrop spectrophotometer (Thermo Fisher Scientific). Reverse transcription and qPCR were performed according to the manufacturer’s instructions using QIAGEN QuantiTec Reverse Transcription kit and Bio-Rad IQ SYBR Green Supermix, respectively. Reactions were performed using a CFX384 Touch Real-Time PCR Detection Systems (Bio-Rad). Primer sequences are listed in the supporting Table 1.

**ALK inhibitor treatment.** Zebrafish were housed from 24 to 72 h post lesion (hpl) in system water containing 10 μM TAE684 (Abcam, ab142082) and 0.1% DMSO. Control groups were housed in system water containing 0.1% DMSO. Solutions were changed daily.

**Experimental design and statistical analysis.** In radial sections, cells counted in three nonadjacent sections in each retina were averaged. Three to seven retinas were analyzed. In flat-mount preparation, ZO1 profiles with perimeter >3.5 μm were identified as cone photoreceptors. For each retina, cones in 5625 μm² area were counted using National Institutes of Health ImageJ (https://imagej.nih.gov/ij/). A total of six retinas were analyzed.

| Primer name | Sequence |
|-------------|----------|
| gppa forward | TCC AAC GAA ACA ACA GAA GC |
| gppa reverse | TTC CAC ATC ACA CCC TGC AC |
| axc1a forward | GGG CCT ATA CGA CCC TCT GA |
| axc1a reverse | GCC CAA GGC AGT GCT GAT TTT |
| stat3 forward | TCG GTG AGG AAA CTC AGT CGT |
| stat3 reverse | CGG GAT GGG CTG CAG ATT TC |
| cdk6 reverse | GCC CCC GCT ACT TGG AAG GC |
| cdk6 forward | GTC TCT TGG GAG CAC GC |
| ccne1 reverse | GCG TTC GGG TGC AGA CCA AT |
| ccne1 forward | AGC AGG CAG CTC AGC CCT TA |
| cdka2 reverse | CCT AAC AAG GGC TGG GGC |
| cdka2 forward | GGT GCT TTC CCA CCA GAG T |
| id2a reverse | AGT CTA ACA CCC ACA GAG T |
| id2a forward | CAC ATC GCC GTC GAC CC |
| p130 forward | CAG GGG TGG TCT GGA TGG CC |
| p130 reverse | AGT CCA GTA ACC GAG CCT GGA |
| gpia forward | TGG GCA CTA ATG AGC GAC AC |

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Results

Loss-of function mutant, \textit{mdka}^{\text{ms5001}}

We generated a CRISPR-Cas9-mediated Midkine-a loss-of-function mutant, \textit{mdka}^{\text{ms5001}}, which carries a 19 bp deletion in the exon three of \textit{midkine-a}. This deletion results in a predicted premature stop codon (Fig. 1A) and absence of protein in Western blot analysis (Fig. 1B). Immunostaining of both larval and adult retinas showed absence of protein in these tissues (Fig. 1C). \textit{Mdka}^{\text{ms5001}} larvae progress normally through early developmental stages and at 48 hpf show only slight reduction in body pigmentation, shortened body length, and smaller eyes (Fig. 1D). The pigmentation defect recovers by 72 hpf (Fig. 1D). Notably,
the \textit{mdka}^{ms5001} mutants replicate the delayed retinal development described previously following morpholino-mediated knockdown of Midkine-a (Fig. 1E) (Luo et al., 2012).

**Transcriptome analysis**

Larvae from adult \textit{mdka}^{ms5001} mutants were initially evaluated using transcriptome analysis of whole embryos at 30 hpf. This identified 638 differentially expressed genes (log2 fold change $\geq 1$ and a false discovery rate $\leq 0.05$) (Table 1-1, available at https://doi.org/10.1523/JNEUROSCI.1675-19.2019.t1-1). Pathway-level analysis with the Reactome tool identified 181 pathways that were differentially regulated (156 upregulated and 25 downregulated; Table 1-2, available at https://doi.org/10.1523/JNEUROSCI.1675-19.2019.t1-2). Of the 156 upregulated pathways, 33 were related to cell cycle regulation (Table 1-2, available at https://doi.org/10.1523/JNEUROSCI.1675-19.2019.t1-2). On the other hand, pathways related to the delayed proliferation in the mutant retinas, can explain the similarity in the number of PCNA positive cells in WT and mutants at 3 dpl. In WT, Müller glia become positive for PCNA at 1 dpl. At 3 dpl, PCNA positive cells form neurogenic clusters. Mutant retinas lack PCNA-labeled cells at 1 dpl. Very few, isolated cells are positive for PCNA in the \textit{mdka}^{ms5001} at 3 dpl. The number of PCNA + cells in WT and \textit{mdka}^{ms5001} in the inner (B: 1 dpl: $p = 0.0017$; 2 dpl: $p < 0.0001$; 3 dpl: $p < 0.0004$, ANOVA with post hoc Tukey; $F$ ratio = 116.1834) and outer (C: 3 dpl: $p = 0.0001$, ANOVA with post hoc Tukey; $F$ ratio = 17.5589) nuclear layers. The \textit{mdka}^{ms5001} retinas have significantly less PCNA + cells at 1, 2, and 3 dpl compared with WT retinas. A total of 3 sections were counted and averaged in each retina. A total of 3 retinas were analyzed. Scale bar, 30 mm. $^*p < 0.01$.

![Figure 2](image)

**Figure 2.** In the \textit{mdka}^{ms5001} mutant, Müller glia fail to proliferate in response to photoreceptor cell death. A. Immunocytochemistry for PCNA (magenta) in WT and \textit{mdka}^{ms5001} at 1 and 3 dpl. In WT, Müller glia become positive for PCNA at 1 dpl. At 3 dpl, PCNA + progenitors form neurogenic clusters. Mutant retinas lack PCNA-labeled cells at 1 dpl. Very few, isolated cells are positive for PCNA in the \textit{mdka}^{ms5001} at 3 dpl. B. The number of PCNA + cells in WT and \textit{mdka}^{ms5001} in the inner (B: 1 dpl: $p = 0.0017$; 2 dpl: $p < 0.0001$; 3 dpl: $p < 0.0004$, ANOVA with post hoc Tukey; $F$ ratio = 116.1834) and outer (C: 3 dpl: $p = 0.0001$, ANOVA with post hoc Tukey; $F$ ratio = 17.5589) nuclear layers. The \textit{mdka}^{ms5001} retinas have significantly less PCNA + cells at 1, 2, and 3 dpl compared with WT retinas. A total of 3 sections were counted and averaged in each retina. A total of 3 retinas were analyzed. Scale bar, 30 mm. $^*p < 0.01$.

**Regeneration of cone photoreceptors is compromised in the \textit{mdka}^{ms5001} mutant**

Persistent, growth-associated neurogenesis is a hallmark of teleost fish (Hitchcock et al., 2004). In the growing eye and retina, stem and progenitor cells at the ciliary marginal zone generate new retinal neurons, with the exception of rod photoreceptors, which rapidly divide, migrate to areas of neural retina, from the cell cycle at 4 dpl (Raymond and Rivlin, 1987; Bernardos et al., 2007; Stenkamp, 2011). This growth-associated neurogenesis occurs normally in the retinas of \textit{mdka}^{ms5001} mutants, and there is no apparent alteration in the maturation or variety of cell types in the \textit{mdka}^{ms5001} retina (Fig. 1E).

In response to neuronal cell death, Müller glia in zebrafish dedifferentiate and undergo a single asymmetric division to produce retinal progenitors, which rapidly divide, migrate to areas of cell loss, and differentiate to replace the ablated nuclei (Nagashima et al., 2013). To assess photoreceptor regeneration in the \textit{mdka}^{ms5001}, we used a photolytic lesion that selectively kills photoreceptors; photoreceptors undergo apoptotic cell death by 1 day post lesion (dpl) (Vlhtelic and Hyde, 2000; Bernardos et al., 2007). In WT retinas, by 1 dpl, Müller glia can be labeled with antibodies against the late G1 or early S phase marker, PCNA and, by 3 dpl, the Müller glia-derived progenitors form radial neurogenic clusters that span the inner nuclear layer (Fig. 2A). In contrast, in the \textit{mdka}^{ms5001} retinas, PCNA labeling was completely absent at 1 dpl, and only a few cells were PCNA + at 3 dpl (Fig. 2A, B). Based on the size and location of their nuclei, we infer that these cells are Müller glia. However, at 5 dpl, there were no differences in the number of PCNA + cells in the outer nuclear layer of WT and mutant retinas (Fig. 2A, C). In WT retinas, photoreceptor progenitors in the outer nuclear layer begin withdrawing from the cell cycle at 4 dpl (Bernardos et al., 2007). This, coupled with the delayed proliferation in the mutant retinas, can explain the similarity in the number of PCNA + cells in the outer nuclear layer at 5 dpl.

We next asked whether PCNA + cells in \textit{mdka}^{ms5001} are capable of progressing further through the cell cycle. We exposed WT and mutants to BrdU between 48 and 72 hpl and killed the animals immediately for histology. In WT retinas at 3 dpl, BrdU + cells were present in the inner and outer nuclear layers, and we
In 3 dpl, the mutant background. This showed that, in 3 dpl, the number of BrdU-labeled cells was significantly lower in the mutants at 3 dpl, whereas in the WT, a few Mu¨ller glia can progress through the cell cycle. Although the initial reactive gliosis is neuroprotective, persistent gliosis results in dysregulation of retinal homeostasis, glial remodeling and scar formation, and the subsequent death of neurons (Bringmann et al., 2006). In zebrafish, the gliotic response of Müller glia is transient and interrupted by cell cycle entry (Thomas et al., 2016). To determine whether the failure of Müller glial proliferation in Midkine-a mutants leads to a mammalian-like gliotic response, the expression of GFAP was compared in the WT and mdka mis5001 retina. In unlesioned retinas, immunostaining for GFAP labels the basal processes of Müller glia (Bernardos and Raymond, 2006). In WT retinas at 28 dpl, GFAP immunolabeling resembles that in unlesioned retinas (Fig. 5A). In contrast, in the mdka mis5001 retinas at 28 dpl, GFAP immunolabeling is present throughout the cytoplasm, extending apically into the inner nuclear layer (Fig. 5A). Enhanced expression of GFAP is a marker of gliosis in mammalian Müller glia (Bringmann et al., 2006). To further characterize the gliotic response in the mutant retinas, we again used the transgenic reporter Tg(gfap:eGFP)mis2002. Computing the planimetric density of Müller glia in flat-mount preparations at 28 dpl showed no significant difference in the number of Müller glia in wildtype and mutant retinas, suggesting that Müller glia do not die. However, in the mdka mis5001; Tg(gfap:eGFP)mis2002 line, Müller glia remained hypertrophic, as evidenced by elevated eGFP levels (Fig. 5B; arrows; Movies 1, 2), and these cells adopt abnormal morphologies, including expanded lateral extensions in the inner plexiform layer and migration of the somata into the outer plexiform layer (Fig. 5C,D; Movies 3, 4). This hypertrophic morphology is also revealed in flat mounts stained with the cell junction marker, ZO1, in which the apical profiles of Müller glia have expanded to fill the planar surface of the outer limiting membrane previously occupied by cones (Fig. 4C, dashed line). The abnormal gliotic remodeling observed in the mutant retinas is a hallmark of persistent reactive gliosis in mammals.

**Müller glia in the mdka mis5001 dedifferentiate in response to photoreceptor death**

In response to neuronal cell death, Müller glia spontaneously reprogram, upregulating stem cell-associated genes before entering the cell cycle (Goldman, 2014; Gorsuch and Hyde, 2014; Lenkowski and Raymond, 2014; Hamon et al., 2016). Immunostaining retinas at 1 and 2 dpl for the stem-cell associated proteins, Rx1 and Sox2, labeled elongated, polygonal nuclei, replenished by 28 dpl (Fig. 4B). Together, these results demonstrate that Midkine-a is required for Müller glial to proliferate in response to cell death, and the absence of Midkine-a leads to a failure of cone photoreceptor regeneration. In contrast, rod photoreceptors regenerate normally in the mutants.
Figure 4. The mdka<sup>−/−</sup> mutant retinas fail to regenerate cone photoreceptors. A, Immunocytochemistry for red/green cone photoreceptor marker, Zpr1. In WT retina, immature cone photoreceptors start to appear at 5 dpl, and regeneration largely completes by 14 dpl. In the mdka<sup>−/−</sup> mutant, regenerating photoreceptors are absent at 5 dpl. At 7 dpl, very few cone photoreceptors appear. The number of cone photoreceptors is less at 14 dpl compared with WT. B, Immunocytochemistry for rod photoreceptor marker Zpr3 following lesion. In WT retina, regenerating rod photoreceptors appear by 7 dpl. In the mdka<sup>−/−</sup> retinas, rod photoreceptors slowly regenerate by 28 dpl. C, Flat-mounted retinal preparation immunostained with ZO1 in unlesioned and 14 dpl. In unlesioned retina of both WT and mdka<sup>−/−</sup>, cone photoreceptors form a crystalline mosaic array in the planar apical surface of the retina (Livak and Schmittgen, 2001; Nagashima et al., 2017). Higher magnification of boxed region indicates the alignment of cones in the mosaic array (asterisks) with flattened cell boundaries (arrowheads). At 14 dpl in WT, cone photoreceptors regenerate (asterisks), although the crystalline mosaic array is not restored. In the mdka<sup>−/−</sup> retina, cone profiles are instead replaced by irregularly shaped, expanded Müller glial apical processes (dotted line). D, Counts of ZO1-labeled cone photoreceptors at 14 and 28 dpl. Significantly fewer cones are regenerated in the mdka<sup>−/−</sup> mutant (white) compared with WT (gray). n = 6. 14 dpl: p = 0.0051; 28 dpl: p = 0.0051, nonparametric Mann-Whitney-Wilcoxon. onl, Outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. Scale bars: A, B, 30 μm; C, 10 μm. *p < 0.01.
characteristic of Müller glia (Nagashima et al., 2013; Gorsuch et al., 2017), in both WT and mutant retinas (Fig. 6A). Further, the Rx1+ and Sox2+ nuclei were displaced apically in both (Fig. 6A), revealing the interkinetic nuclear migration that is associated with cell cycle progression in Müller glia (Fig. 6B) (Nagashima et al., 2013). We also evaluated the reprogramming in Müller glia by qPCR for the core transcriptional factors, ascl1a, stat3, and lin28 (Fausett and Goldman, 2006; Ramachandran et al., 2010; Nelson et al., 2012). At 30 and 36 hpl, which is before when Müller glia divide, ascl1a, stat3, and lin28 are significantly upregulated in both WT and mutant retinas, although the expression level of ascl1a is slightly reduced in the mutants (Fig. 7B, C). These data indicate that, in the absence of Midkine-a, Müller glia respond to photoreceptor death by reprogramming into a stem cell-like state.

Midkine-a is partially responsible for ascl1a expression via phosphorylation of Stat3

Following a retinal lesion, phosphorylation of Stat3 is required for the upregulation of ascl1a in Müller glia (Nelson et al., 2012; Zhao et al., 2014). In WT and mdkam5001 retinas at 1 and 2 dpl, STAT3 protein was induced (Fig. 7D, E). Consistent with previ-
ously published data (Zhao et al., 2014), at 1 and 2 dpl, pStat3 antibody labels Müller glia in WT animals (Fig. 7F). In contrast, in mdka<sup>m15001</sup> retinas at 1 and 2 dpl, pStat3 immunostaining was markedly diminished (Fig. 7E), demonstrating that, in Müller glia, Midkine-a regulates the phosphorylation of Stat3, which can explain the reduced expression of <i>ascl1a</i> in the mutant retinas.

**Absence of cell cycle progression in mutant Müller glia**

Following reprogramming, Müller glia begin entering the cell cycle around 24 hpl and complete the asymmetric cell divisions by 42 hpl (Nagashima et al., 2013). We next asked whether Müller glia in the mdka<sup>m15001</sup> possess the ability to enter the cell cycle by quantifying the expression of G1 phase cyclins, <i>ccn</i> <i>d1</i> (<i>ccnd1</i>) and <i>ccn</i> <i>e1</i> (<i>ccne1</i>). These cyclins are expressed during G1 and function to drive G1-to-S phase transition (Dyer and Cepko, 2001). We isolated mRNA at 30 and 36 hpl, knowing that cell cycle progression is not completely synchronous among the population of Müller glia, but that these time points will allow us to capture gene expression changes in Müller glia and exclude Müller glia-derived progenitors. This analysis showed that mdka<sup>m15001</sup> upregulates <i>ccnd1</i> and <i>ccne1</i> significantly at both 30 and 36 hpl (Fig. 8A, B), indicating that, following photoreceptor death in the mdka<sup>m15001</sup> mutants, Müller glia enter the G1 phase of the cell cycle. In WT retinas, cell cycle entry is followed by upregulation
of S phase cyclin, ccna2 (Fig. 8C). In contrast, there is no upregulation of ccna2 in the mdka<sup>m5001</sup> retinas, indicating that Müller glia in mutants fail to progress from G<sub>1</sub> to S (Fig. 8C). This was confirmed using the S-phase label, BrdU, between 24 and 30 hpl. In WT retinas, Müller glia are uniformly labeled with BrdU; whereas in the in the mdka<sup>m5001</sup> retinas, there are no BrdU-labeled cells (Fig. 8D, n = 6 retinas). Consistent with these results, the expression of the cell cycle regulators, cyclin-dependent kinase 4 and 6, is dysregulated in mutant retinas (Fig. 8E,F). Together, these results indicate that, following photoreceptor cell death in the mdka<sup>m5001</sup> retinas, cell cycle progression of Müller glia is compromised, demonstrating that, in reprogrammed Müller glia, Midkine-a regulates the G<sub>1</sub>-S phase transition.

During retinal development, Midkine-a governs cell cycle kinetics through Id2a (Luo et al., 2012). Id proteins play important roles in cell cycle regulation during development and in cancer (Lasorella et al., 1996; Sikder et al., 2003). In WT retinas, id2a expression is markedly upregulated at 30 hpl, as Müller glia progress through the cell cycle, and rapidly returns to baseline levels by 48 hpl, when the single asymmetric mitotic division is complete (Fig. 8G). This transient induction of id2a is completely absent in the mdka<sup>m5001</sup> retinas (Fig. 8G). In cancer cells, Id2 proteins antagonize the Rb family of cell cycle inhibitors, thereby allowing progression from G<sub>1</sub> to S phase of the cell cycle (Lasorella et al., 2001; Sikder et al., 2003). Previous analyses of the Müller glia specific transcriptome show that p130, one of the Rb gene family, exhibits highest expression among Rb genes in quiescent Müller glia (Sifuentes et al., 2016; Nieto-Arellano and Sánchez-Iranzo, 2019). Consistent with these data, we validated that, in WT retinas, the expression of p130 decreases as Müller glia progress through the cell cycle (Fig. 8H). In contrast, in mdka<sup>m5001</sup> retinas at 30 and 36 hpl, p130 levels are elevated above the those found in quiescent Müller glia (Fig. 8H). These results suggest that Id2a is downstream of Midkine-a, and in Müller glia Id2a functions to inhibit Rb genes.

**Figure 6.** Müller glia in the mdka<sup>m5001</sup> mutant differentiate following photoreceptor death. A. Immunocytochemistry for regeneration-associated genes, Rx1 and Sox2, following photolytic lesion in WT and mdka<sup>m5001</sup> retinas. Lesion induces Rx1 and Sox2 expression in Müller glia both in WT and mdka<sup>m5001</sup> retinas at 2 dpl. B. The mdka<sup>m5001</sup> retinas carrying the Müller glial reporter, Tg(fgfl:EGFP<sup>m5002</sup>). Photoreceptor injury induces interkinetic nuclear migration of Müller glial nuclei in the mdka<sup>m5001</sup> mutant. Arrows indicate cell bodies of Müller glia. The nuclei were stained with Hoechst (gray). OLM, Outer limiting membrane; inl, inner nuclear layer; onl, outer nuclear layer. Scale bars, 30 μm.

**Signaling through the ALK receptor is responsible for Müller glial proliferation**

ALK is a member of the superfamily of receptor tyrosine kinases. ALK is involved in the initiation and progression of many cancers, including neuroblastoma (Morris et al., 1995; Webb et al., 2009; Hallberg and Palmer, 2013). Midkine and its related protein pleiotrophin are the only ligands known to activate ALK (Stoica et al., 2001, 2002). To determine whether Alk functions as a Midkine-a receptor on Müller glia during photoreceptor regeneration, double immunocytochemistry was performed for pALK and PCNA following a photolytic lesion. In WT retinas, pALK colocalizes with PCNA, indicating activation of Alk in dividing Müller glia and Müller glia-derived progenitors (Fig. 9A). In contrast, both pALK and PCNA immunolabeling were absent in mdka<sup>m5001</sup> retinas, indicating that, in the retina, Midkine-a is required for ALK phosphorylation. To test whether activation of ALK is required for proliferation among Müller glia, WT animals were housed from 24 to 72 hpl in the ALK inhibitor, TAE684. Inhibiting the activation of ALK phenocopied the proliferation defect observed in the mdka<sup>m5001</sup> mutants (Fig. 9B,C). These data indicate that phosphorylation of Alk is required for Müller glia to proliferate and identify ALK as a putative receptor for Midkine-a during the initial asymmetric division in Müller glia and the subsequent regeneration of cone photoreceptors.

**Discussion**

In mammals, neuronal damage in the retina stimulates transient entry of Müller glia into the cell cycle; however, any subsequent proliferative response is very limited (Dyer and Cepko, 2000; Hamon et al., 2019; Rueda et al., 2019). In zebrafish, Müller glia respond to neuronal death by spontaneously entering and transiting the cell cycle, giving rise to Müller glia-derived progenitors that amplify in number and functionally replace ablated neurons. Numerous studies have identified transcriptional regulators and signaling cascades that promote Müller glia reprogramming in both mammals and fish (Karl and Reh, 2010; Goldman, 2014; Gorsuch and Hyde, 2014; Lenkowski...
Figure 7. The \textit{mdkam5001} mutant upregulates “core” transcriptional regulators following photoreceptor death. \textbf{A–C}, qPCR for dedifferentiation markers, \textit{ascl1a}, \textit{stat3}, and \textit{lin28}, at 30 and 36 hpl. Both WT and \textit{mdkam5001} upregulate \textit{ascl1a} (\textbf{A}; WT: 30 hpl, \(p < 0.0001\), 36 hpl, \(p < 0.0001\); \textit{mdkam5001}: 30 hpl, \(p = 0.0004\), 36 hpl, \(p = 0.006\), \(p = 0.006\) relative to WT; F-ratio = \textit{37.5606}), \textit{lin28} (\textbf{B}; WT: 30 hpl, \(p < 0.0001\), 36 hpl, \(p = 0.0095\); \textit{mdkam5001}: 30 hpl, \(p < 0.0001\), 36 hpl, \(p = 0.0004\); F-ratio = \textit{32.9337}), and \textit{stat3} (\textbf{C}; WT: 30 hpl, \(p = 0.0048\), 36 hpl, \(p = 0.0016\); \textit{mdkam5001}: 30 hpl, \(p = 0.0004\), 36 hpl, \(p = 0.0016\)), following lesion. ANOVA with post hoc Tukey, relative to unlesioned. \textbf{D}, \textbf{E}, Western blot for \textit{Stat3} in WT and \textit{mdkam5001} retinas at 1 dpl. WT: \(p = 0.0002\), \textit{mdkam5001}: \(p = 0.0004\), ANOVA with post hoc Tukey; F-ratio = \textit{40.8763}. \textbf{F}, Immunocytochemistry for phosphorylated \textit{Stat3} (pStat3) in the WT and \textit{mdkam5001} mutant. In unlesioned retina, immunosignal for pStat3 is not detected in the inner nuclear layer. Following photoreceptor lesion, Müller glia in the inner nuclear layer upregulate pStat3 in WT, whereas \textit{mdkam5001} mutants have reduced phosphorylation of Stat3. Scale bar, 30 \(\mu\)m. inl, Inner nuclear layer; ipl, inner plexiform layer. *\(p < 0.01\).
and Raymond, 2014; Hamon et al., 2016). The molecular mechanisms that govern cell cycle kinetics in Müller glia, while essential, have received relatively little attention. Here we provide the first evidence that Midkine-a, ostensibly acting as an extrinsic regulator of proliferation, governs the transition from G1 to S phases of the cell cycle in injury-induced, reprogrammed Müller glia.

Our data support the mechanistic model shown in Figure 10. In unlesioned retinas, Müller glia remain quiescent in the G0 phase (Fig. 10A). In response to photoreceptor cell death, nearby Müller glia upregulate reprogramming-associated genes, and enter the cell cycle (Fig. 10B). Midkine-a, signaling through Alk receptors, promotes the expression of ascl1a via phosphorylation of Stat3 (Fig. 10B). Midkine-a also induces the brief, transient upregulation of Id2a, which suppresses cell cycle inhibitor p130, thereby allowing Müller glia to enter both S and the subsequent phases of the cell cycle (Fig. 10B, C). The consequence of this cell cycle block is the selective failure in the regeneration of cone photoreceptors.

During retinal development, Müller glia emerge from late-stage retinal progenitors, and cell cycle inhibitors play pivotal

**Figure 8.** Müller glia in the mdkami5001 mutant arrest in the G1 phase of the cell cycle. A–C, qPCR assay for cell cycle regulator cyclins, ccnd1, ccne1, and ccna2 following photolytic lesion. Both WT and mdkami5001 upregulate G1 cyclins, ccnd1 (A; WT: 30 hpl, p = 0.0107, 36 hpl, p = 0.0013; mdkami5001: 30 hpl, p = 0.0048, 36 hpl, p = 0.0213; F-ratio = 12.0576) and ccne1 (B; WT: 30 hpl, p < 0.0001, 36 hpl, p < 0.0001; mdkami5001: 30 hpl, p = 0.0012, 36 hpl, p = 0.0001; F-ratio = 36.5808), following lesion (A, B). The mdkami5001 mutant fails to upregulate S phase cyclin, ccna2 (C; WT: 36 hpl, p = 0.0011; F-ratio = 12.5433). D, S phase assay with BrdU labeling (green) between 24 and 30 hpl. Müller glia in the mdkami5001 mutants did not incorporate BrdU following lesion. E–H, qPCR of additional cell cycle regulators. Expression of cyclin-dependent kinases, cdk4 (E; WT: 36 hpl, p < 0.0001; F-ratio = 15.8783) and cdk6 (F; WT: 36 hpl, p = 0.0087; F-ratio = 8.3300), is dysregulated in the mdkami5001 retinas (E, F). The WT transiently upregulates id2a at 30 hpl (p = 0.0003; F-ratio = 7.2578), whereas expression levels did not change in the mdkami5001 (G). Expression of the cell-cycle inhibitor, p130, decreases in the WT after lesion, whereas mdkami5001 maintains steady levels of expression (H; p = 0.002, relative to WT; F-ratio = 6.3823). ANOVA with post hoc Tukey, relative to unlesioned. Scale bar: D, 30 μm. onl, Outer nuclear layer; inl, inner nuclear layer; gcl, ganglion cell layer. *p < 0.05, #p < 0.01.
Figure 9. Activation of the ALK receptor is required for Müller glial to proliferate. A, Double immunostaining for PCNA (magenta) and pALK (green). PCNA+ cells express pALK in WT retina at 2 dpl, whereas pALK immunostaining was not detected in mdkα−/− retina. Scale bar, 30 μm. B, Pharmacological inhibition of ALK using TAE684 suppresses proliferation in the WT retinas after a lesion. C, Counts of PCNA+ proliferative cells in DMSO- or TAE684-treated WT retinas at 3 dpl. n = 7. *p = 0.0011 (nonparametric Mann-Whitney-Wilcoxon).

Figure 10. Model of Midkine-a-mediated cell cycle regulation in Müller glia. A, In quiescent Müller glia, cell cycle inhibitors keep cells in G0 phase. B, Injury induces cytokines and growth factors to upregulate regeneration-associated reprogramming genes for dedifferentiation and cell cycle reentry. Midkine-a-ALK signaling participates in the induction of the regeneration-associated gene, ascl1a, via phosphorylation of Stat3. Midkine-a signaling also induces expression of the cell cycle regulator, id2a, that inhibits cell cycle inhibitors, such as p130. C, In the absence of Midkine-a, Müller glia fail to suppress cell cycle inhibition, resulting in compromised progression of the cell cycle.
roles in their fate determination (Turner and Cepko, 1987; Furu-
ka et al., 2000; Levine et al., 2000; Ueki et al., 2012; Del Debbo-
et al., 2016). In adult retinas, Müller glia retain an intrinsic ge-
netic program that is shared with these retinal progenitors (Roe-
sch et al., 2008). Müller glia in mammalian retina respond to
neuronal death by entry into the cell cycle (Joly et al., 2011;
Nomura-Komoike et al., 2016). Importantly, in the mouse, ge-
netic modifications that allow Müller glia to persistently express
cyclin genes are sufficient to promote their mitotic division (Ham-
on et al., 2019; Rueda et al., 2019). Our data, together with
these observations, suggest that Müller glia in both mammals and
zebrafish possess similar mechanisms that function to integrate
injury-related signals from dying neurons and promote entry
into the cell cycle, but in zebrafish, Midkine-a serves as a unique
extrinsic signal that allows these cells to proliferate.

Following photoreceptor death, reprogrammed Müller glia
enter the cell cycle within 24 hpl and undergo a single division by
42 hpl (Nagashima et al., 2013). This temporal sequence is closely
coordinated, but not completely synchronized. We timed exper-
iments using RNA isolated from whole retinas such that cell
cycle-related gene expression could be evaluated in Müller glial
stem cells while excluding Müller glia-derived progenitors.
Following photoreceptor death in zebrafish, id2a is upregulated
transiently at 30 hpl and returns to baseline levels by 36 hpl. This
indicates that Midkine-a-dependent Id2a expression is required
for the proliferation of Müller glia, but not Müller glia-derived
progenitors. The family of Id proteins is involved in intrinsic
control of proliferation during development and in cancer (Ru-
zinova and Benezra, 2003; Sikder et al., 2003). Id2 regulates the
Rb proteins, and high levels of Id2 can suppress the Rb tumor
suppressor pathway, which blocks progression from G1 to
S phase of the cell cycle (Lasorella et al., 2000, 2001). In adult mice,
the Rb-family member p130 maintains quiescence in muscle sat-
elite cells, which retain the capacity to self-renew and regenerate
myoblasts (Carnac et al., 2000). During sensory hair cell regener-
ation in zebrafish inner ear, p130 is downregulated immediately
following injury (Jiang et al., 2014). Consistent with these data,
our in silico screening identified p130 as a highly expressed gene
in quiescent Müller glia, suggesting that p130 expressed in Müller
glia of uninjured retinas functions to restrict their proliferation
(Sifuentes et al., 2016; Nieto-Arellano and Sánchez-Irango,
2019). Our data suggest that, in response to cell death, Midkine-a
signaling blocks p130 through upregulating Id2a, allowing Müller
 glia to progress through the cell cycle. We suggest that the brief
upregulation and downregulation of id2a are a mechanism that
allows Müller glia to divide, but restricts these cells to a single
mitotic cycle. Further, our data suggest that Midkine-a is re-
quired for the rising phase of this transient id2a expression. Not-
tably, increased levels of Id2 are also present in anaplastic large
cell lymphomas that result from constitutive activation of the
Midkine receptor, ALK (Mathas et al., 2009). It is not known
whether ALK in Müller glia functions alone or as a member of
multiprotein complex to relay the Midkine-a signal. Receptor
protein tyrosine phosphatase-ζ (RPTP-ζ) is also a known recep-
tor for Midkine and can activate the intracellular kinase domain
of ALK, and may function as a coreceptor to transduce Midkine-a
signaling in Müller glia (Mathas et al., 2009; Hallberg and Palmer,
2013).

Following photoreceptor death in the mdkami5001 mutants,
Müller glia initially fail to progress through the cell cycle, al-
though a small number of Müller glia eventually do so. As a
consequence, the regeneration of cone photoreceptors is perma-
nently compromised, whereas the regeneration of rod photore-
ceptors is not. This suggests the initial proliferative response of
the Müller glia gives rise to cone progenitors. Previous reports
suggest that separate lineages give rise to regenerated cone and
rod photoreceptors, respectively (Morris et al., 2008; Thummel
et al., 2010; Gorsuch et al., 2017), and our results are consistent
with these reports. We favor the interpretation that fate-restricted rod
precursors persist in the outer nuclear layer and contribute to the
regeneration of rod photoreceptors. However, we cannot exclude
the possibility that regenerated rods in the mdkami5001 mutants
originate from the few latent Müller glia that progress through the
cell cycle.

Heterogeneity of stem and progenitor populations is a clinical
challenge when treating malignant tumors, where Midkine is
highly expressed (Muramatsu, 2011; Sakamoto and Kadomatsu,
2012). Müller glia share common features of quiescence, self
renewal, and multipotency with cancer stem cells. In unlesioned
retina, Müller glia are quiescent and sporadically divide and pro-
duce fate-restricted rod precursor (Raymond and Rivlin, 1987;
Stenkamp, 2011). Cell death reprograms Müller glia to a stem
cell-like state (Karl and Reh, 2010; Goldman, 2014; Gorsuch and
Hyde, 2014; Lenkowski and Raymond, 2014; Hamon et al., 2016).
In vitro experiments demonstrated that inhibition of Midkine
successfully suppresses proliferation of cancer stem cells (Mirkin
et al., 2005; Erdogan et al., 2017). Therefore, Midkine silencing is
proposed as a potential therapy for limiting cell cycle progression
in cancer stem cells (Muramatsu and Kadomatsu, 2014). Our data
also provide molecular insights into the potential role of
Midkine in tumorigenesis, especially in regulating the cell cycle
among cancer stem cells.

Constitutive activation of glial cells and formation of a glial
scar are detrimental to the function of the CNS. An intriguing
phenotype in the mdkami5001 mutants is the cell death-induced
gliotic remodeling of Müller glia. A previous report showed that
pharmacological suppression of cell cycle progression following
photoreceptor death results in hypertrophy and increased GFAP
in Müller glia (Thomas et al., 2016). Together, these results sug-
gest that, in zebrafish Müller glia, the molecular mechanisms that
promote cell cycle progression are required to limit the initial
gliotic response. Although it is not clear whether entry into the
cell cycle initiates reactive gliosis in mammalian retinas, levels of
cell cycle proteins appear to be a critical variable in the gliotic
response (Dyer and Cepko, 2000; Levine et al., 2000; Vázquez-
Chona et al., 2011; Ueki et al., 2012).

Our data significantly expand the understanding of retinal
regeneration in zebrafish and more fully define the function of
Midkine-a in governing the eukaryotic cell cycle. We provide
convincing evidence that Midkine-a regulates proliferation of re-
programmed Müller glial tissue during the regeneration of cone pho-
toreceptors. In the absence of Midkine-a, zebrafish Müller glia
respond similarly to Müller glia in mammals, with only a limited
ability to regenerate neurons. In developing mammalian retinas,
Midkine has been identified as component in the core transcrip-
tional repertoire of retinal progenitors (Livesey et al., 2004).
It remains to be determined whether Midkine-dependent cell
cycle machinery is present in the Müller glia of adult mammals or
whether manipulation of Midkine signaling in adult mammals
could promote neuronal regeneration.

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