Genetic evidence for shared mechanisms of epimorphic regeneration in zebrafish

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In a microarray-based gene profiling analysis of Müller glia-derived retinal stem cells in light-damaged retinas from adult zebrafish, we found that 2 genes required for regeneration of fin and heart tissues in zebrafish, *hspd1* (*heat shock 60-kDa protein 1*) and *mps1* (*monopolar spindle 1*), were up-regulated. Expression of both genes in the neurogenic Müller glia and progenitors was independently verified by quantitative reverse transcriptase PCR and in situ hybridization. Functional analysis of temperature-sensitive mutants of *hspd1* and *mps1* revealed that both are necessary for Müller glia-based cone photoreceptor regeneration in adult zebrafish retina. In the amputated fin, *hspd1* is required for the induction of mesenchymal stem cells and blastema formation, whereas *mps1* is required at a later step for rapid cell proliferation and outgrowth. This temporal sequence of *hspd1* and *mps1* function is conserved in the regenerating retina. Comparison of gene expression profiles from regenerating zebrafish retina, caudal fin, and heart muscle revealed additional candidate genes potentially implicated in injury-induced epimorphic regeneration in diverse zebrafish tissues.

retina | photoreceptors | neural stem cells | hspd1 | mps1

The study of regeneration has long fascinated biologists and has lately experienced a renaissance associated with growing interest in regenerative medicine and the therapeutic potential of stem cells. Zebrafish (*Danio rerio*) are an ideal genetic model for studying regeneration in vertebrates (1) because they have remarkable capabilities to regenerate fins (2), heart muscle (3), and nervous tissues (4–7) following injury. A forward mutagenesis screen for temperature-sensitive mutations that interfere with regeneration of amputated caudal fin identified several genes whose functions are critical for specific steps in fin regeneration, including *mps1* (*monopolar spindle 1*, also called *ttk*, a kinase required for mitotic checkpoint regulation), *hspd1* (*heat shock 60-kDa protein 1*, a mitochondrial chaperone), and *fgf20* (fibroblast growth factor 20) (8–10). In addition, gene profiling analysis of regenerating tissues has provided lists of candidate genes associated with regeneration in fin (11), heart (12), and neural retina (13–15).

The regeneration of retinal neurons in adult zebrafish is an especially powerful model for studying regeneration of neuronal tissues; laminar retinal architecture and visual function are restored following damage inflicted by surgical lesions, neurotoxins, and laser or photic lesions of retina (16). The neural stem cells in the retina arise from differentiated Müller glia, which respond to local retinal injuries by dedifferentiation, proliferation, and production of multipotent neuronal progenitors (retinal stem cells) that can regenerate all types of retinal neurons (17–19). To discover genes expressed in injury-activated neurogenic Müller glial cells that activate stem cell properties and trigger a neurogenic program, we generated transcriptional profiles of isolated fluorescent-tagged Müller glial cells from light-lesioned adult transgenic zebrafish retinas during the early stages of photoreceptor regeneration. We found that 2 genes required for fin regeneration, *hspd1* and *mps1*, are also up-regulated in the injury-activated Müller glia. Functional analyses of *hspd1* and *mps1* mutants revealed that both genes are required for regeneration of cone photoreceptors. Moreover, consistent with the temporal sequence of mutant phenotypes in regenerating fins (8, 9), we found that *hspd1* is required for an early step in retinal regeneration (formation of retinal stem cells from dedifferentiated proliferating Müller glia), whereas defects in *mps1* function block regeneration at a later step (proliferation of specialized photoreceptor progenitors).

Results

Photoreceptor Regeneration After Ultra-Intense Light Treatment. The injury model we used is a light-lesion paradigm. Freely-swimming adult zebrafish were briefly exposed (20–30 min) to a spot source of ultra-intense light that selectively destroys cone and rod photoreceptors while leaving the inner retina intact (18). Postembryonic generation of rod photoreceptors continues in the differentiated retina of adult teleost fish; thus, we specifically examined regeneration of cone photoreceptors, which are not produced in central differentiated regions in the intact retina (16). To visualize the entire retinal lesion and subsequent regeneration of cones, we examined isolated flat-mounted retinas immunolabeled with zpr-1, a specific marker for red-green double cones in zebrafish (Fig. 1 *A–C*). The lesion is confined to a central region ≈1/4 to 1/3 of the total retinal area in a horizontal band along the nasal-temporal axis (Fig. 1 *D–F*).

Cones completely regenerate by 14 days (Fig. 1 *G* and *H*). In the intact zebrafish retina, cones form a highly regular square mosaic pattern (20), with red-green double cones arranged in rows (Fig. 1*C*). Previous studies have shown that the regular cone mosaic pattern is not restored during regeneration (20), although the photoreceptors are functional and vision is restored (21). The disruption in the arrangement of red-green cones within the lesioned/regenerated area of the retina (Fig. 1*I*) was used in subsequent experiments to identify the regenerated region within the lesioned retina.

Gene Expression Profiling of Isolated Müller Glia from Intact and Regenerating Zebrafish Retinas. Injury-activated Müller glia dedifferentiate, proliferate, and give rise to radial clusters of neuronal progenitors that migrate into the layer of damaged/dying photoreceptors [outer nuclear layer (ONL)], where they differentiate to replace the missing cone and rod photoreceptors (14, 17, 18, 22, 23). By using the transgenic zebrafish reporter line, *Tg(gfap:GFP)mi2002*, in which expression of GFP is controlled by the *cis*-regulatory sequences of a glial-specific gene, *gfap* (24),

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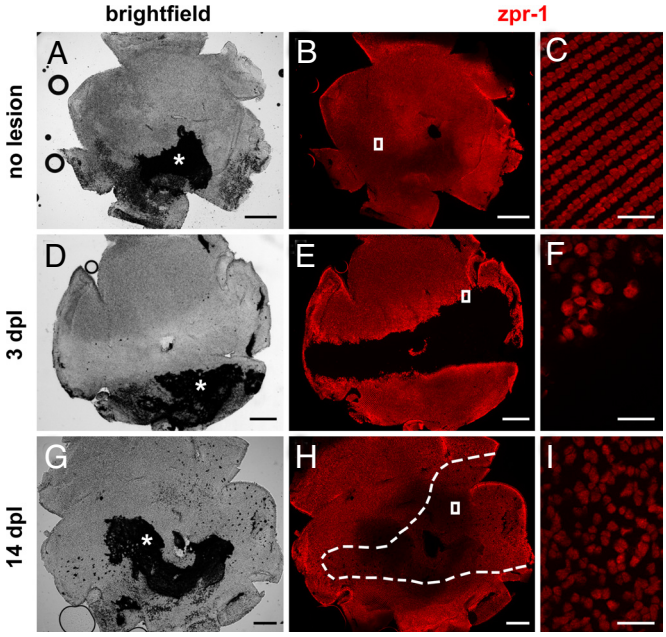

Fig. 1. Cone photoreceptor regeneration in adult zebrafish. Flat-mounted zebrafish retinas are immunolabeled with cone-specific zpr-1 (red). Retinas are oriented dorsal up, ventral down, nasal left, and temporal right. (*A*, *B*) Intact retina. Asterisk, attached retinal pigment epithelium. (*D*, *E*) At 3 days after exposure to intense light, cones are missing in a horizontal band across the retina. (*G*, *H*) By 14 days, cones have regenerated within the lesioned region (dashed lines). (*C*, *F*, and *I*) Magnified images of the boxes in *B*, *E*, and *H*, respectively. (Scale bars: 300 μm in *A*, *B*, *D*, *E*, *G*, and *H*; 20 μm in *C*, *F*, and *I*.)

we previously showed that after destruction of photoreceptors, the progeny of dividing Müller glia differentiate into cone photoreceptors (18). To discover the cell-intrinsic regeneration-responsive factors in the neurogenic Müller glia, we compared gene expression profiles of GFP$^+$ cells isolated from intact and light-lesioned *Tg(gfap:GFP)mi2002* zebrafish retinas at 8, 16, 24, and 36 h following light treatment. These intervals are within the window during which Müller glia are activated but before the "birth" (terminal mitotic division) of the earliest regenerated cone photoreceptors at 2 days postlesion (dpl) (23). We thereby limited our data set to genes regulated at the early stages of regeneration to discover the molecular triggers that mediate the transformation of Müller glia into retinal stem cells. Harvested retinas were dissociated enzymatically, and GFP$^+$ cells were isolated from the resulting cell suspension by fluorescence-activated cell sorting [supporting information (SI) Fig. S1].

With microarray gene profiling, we identified a total of 953 transcripts differentially expressed in at least 1 of the 4 sample times compared with the untreated control. The complete microarray data set is available in the Gene Expression Omnibus database GEO (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE14495. Hierarchical clustering grouped these genes into 3 distinct groups based on their temporal expression patterns (Fig. S2). Expression of genes in clusters I and II (n = 745) was up-regulated but with different time courses: cluster I, immediate up-regulation after the lesion (n = 644); cluster II, delayed up-regulation (n = 101). Cluster III includes all genes that were down-regulated after the lesion (n = 208). For a broad overview of the major biological functions associated with each cluster, we grouped genes according to gene ontology terms: biological process, cellular component, and molecular function (see Fig. S3). In cluster I, the translation/protein biosynthesis group of genes predominates (see Fig. S3*A*); accordingly, genes classified as cellular components and molecular functions of ribosome are highly represented (data not shown). This suggests that an early step in the injury-induced activation of Müller glia is stimulating protein synthesis and metabolism. The enrichment of DNA replication/cell cycle genes in the genes up-regulated with a delayed onset (cluster II, Fig. S3*B*) is consistent with the observation that most or all Müller glia within the lesioned area re-enter the cell cycle by 48 h postlesion (hpl) (18). The down-regulation of genes involved in chromatin assembly and ion homeostasis (cluster III, Fig. S3*C*) is consistent with the dedifferentiation of injury-activated Müller glia described previously (14, 17, 18, 22, 23).

As an initial validation of the microarray data, 8 genes with different temporal expression patterns from distinct gene ontological groups were selected for quantitative reverse transcriptase (qRT) PCR analysis; the 2 methods showed excellent agreement (Fig. 2 *B* and *E* and Fig. S4). Among the genes we investigated, *ascl1a* (*achaete-scute complex-like 1a*, formerly *zash1a*, Fig. S4*A*) and *six3b* (*sine oculis homeobox homolog 3b*, Fig. S4*C*) are transcription factors expressed in retinal progenitors in the ciliary marginal zone of postembryonic zebrafish retina (23). The proneural basic helix-loop-helix (bHLH) gene *ascl1a* is induced in activated Müller glia and their neurogenic progeny following retinal lesions (25). The homeobox transcription factor *six3* plays a crucial role in early eye development and interacts with bHLH proteins (26, 27). Another up-regulated gene, *sox4a*, belongs to the C-group Sox family of high mobility group-box transcription factors, which are expressed in committed neuronal progenitors and operate downstream of proneural bHLH genes to establish neuronal properties (28). Consistent with the increased expression of *ascl1a*, *six3b*, and *sox4a*, a negative regulator of the bHLH genes, *id3* (*inhibitor of DNA binding 3*), was down-regulated (Fig. S4*F*). Interactions among these genes may form a transcription regulatory network to initiate a neurogenic program in the injury-activated Müller glia.

As expected, many cell cycle genes and growth factors were up-regulated during regeneration, such as *pcna* (*proliferating cell nuclear antigen*, Fig. S4*E*) and *pdgfa* (*platelet-derived growth factor a*, Fig. S4*B*). A recent gene expression profiling analysis of regenerating zebrafish heart muscle found that *pdgfa* is also up-regulated during heart regeneration and that PDGF signaling is necessary for cardiomyocyte proliferation (12).

***hspd1* and *mps1* Are Up-Regulated in Injury-Activated Müller Glia with Different Time Courses.** We were intrigued to find that 2 genes required for fin and heart regeneration in zebrafish are up-regulated in injury-activated Müller glia. One is *hspd1*, which encodes heat shock protein 60 (Hsp60), an ancient highly conserved protein that functions in the cellular stress response as a chaperone for protein folding and assembly (29). In the regenerating zebrafish caudal fin, *hspd1* is required for the formation and maintenance of the early/distal blastemal stem cells derived from mesenchyme (9). The other regeneration gene is *mps1*, a protein kinase involved in mitotic checkpoint regulation (30). In the regenerating fin, *mps1* is not required for the activation of mesenchymal stem cells or initial establishment of the blastema but is required later in rapidly proliferating progenitor cells at the outgrowth stage (8). We observed a similar temporal sequence of gene regulation in the light-lesioned retina in that *hspd1* was up-regulated immediately after the light treatment at 8 hpl, whereas *mps1* was not induced until 36 hpl in both our microarray and qRT-PCR analyses (Fig. 2 *B* and *E*).

To define the spatial expression patterns of *hspd1* and *mps1* during retinal regeneration, we performed fluorescent in situ hybridization for both genes on retinal sections of light-lesioned *Tg(gfap:GFP)mi2002* zebrafish. These results confirmed the timing of gene expression following injury: *hspd1* was up-regulated locally within the region of the lesion at both 24 and 48 hpl (Fig. 2*A*), whereas *mps1* was undetectable in unlesioned

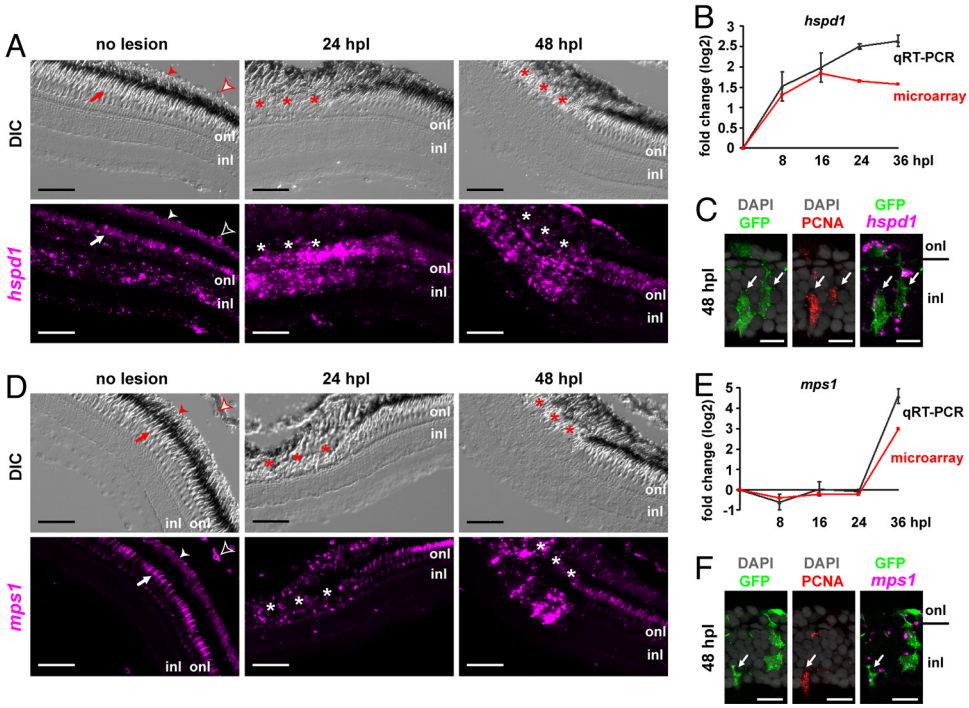

Fig. 2. *hspd1* and *mps1* are up-regulated in injury-activated Müller glia during zebrafish photoreceptor regeneration. Expression patterns of *hspd1* (*A–C*) and *mps1* (*D–F*). (*A* and *D*) Fluorescent in situ hybridization of *hspd1* and *mps1* on retinal sections of *Tg(gfap:GFP)mi2002* zebrafish. Autofluorescence in cones (arrow), rods (solid arrowhead), and red blood cells (empty arrowhead). Asterisks, lesioned area (note the disrupted retinal pigment epithelium). DIC, differential interference contrast. (*B* and *E*) Expression fold changes of *hspd1* and *mps1* in isolated GFP$^+$ cells detected by qRT-PCR (gray) and microarray (red). Error bars represent SEM for 3 independent biological replicates. (*C* and *F*) Within the lesioned region at 48 hpl: in situ hybridization with *hspd1* and *mps1*, respectively (magenta), produces discrete fluorescent dots associated with GFP$^+$ neurogenic Müller glia (green) and anti-PCNA (red). Arrows indicate triple-labeled cells; onl, outer nuclear layer; inl, inner nuclear layer. These are not microglia, which are confined to the onl in the lesioned region (23). (Scale bars: 50 μm in *A* and *D*; 10 μm in *C* and *F*.)

retina and at 24 hpl but was induced within the lesioned area at 48 hpl (Fig. 2*D*). Both genes were up-regulated specifically in the inner nuclear layer (INL) of the retina, where the cell somas of Müller glia reside. In addition, *hspd1* was expressed in the damaged/dying photoreceptors within the lesioned region at 24 hpl (Fig. 2*A*). Colabeling with the GFP transgenic reporter and PCNA at 48 hpl confirmed that both genes were expressed in the injury-activated Müller glia and their progeny (Fig. 2 *C* and *F*).

***hspd1* and *mps1* Are Required for Zebrafish Cone Photoreceptor Regeneration.** We next asked whether *hspd1* and *mps1* are necessary for retinal regeneration. The zebrafish mutant *nbl* (*no blastema*) is a temperature-sensitive null allele of *hspd1* that disrupts chaperone activity (9); *ncp* (*nightcap*) has a missense substitution in the conserved kinase domain of *mps1* and also exhibits a temperature-sensitive phenotype (8). Homozygous *nbl* or *ncp* mutants and their homozygous WT siblings were light-lesioned and allowed to recover at the restrictive temperature (33°C) after the injury. We found that cone photoreceptor regeneration takes place much faster at 33°C than at the standard temperature of 28°C: by 7 dpl, cones were fully regenerated in WT ($nbl^{+/+}$, $n = 4$; $ncp^{+/+}$, $n = 5$; Fig. 3 *A* and *C*), whereas ~14 days were required to achieve a comparable stage of recovery at 28°C (data not shown). In contrast, both mutants did not regenerate cones, or did so only sporadically, at the restrictive temperature of 33°C ($nbl^{-/-}$, $n = 6$; $ncp^{-/-}$, $n = 3$; Fig. 3 *B* and *D* and Fig. S5).

To characterize the cellular nature of the retinal regeneration defects, we collected eyes from light-lesioned *nbl* or *ncp* mutants and WT siblings held at 33°C for 1, 2, or 3 days after the lesion. Retinal regeneration in zebrafish requires mitotic activation of Müller glial cells (31); thus, we quantified the proliferative response of Müller glia at 1 dpl by counting PCNA$^+$ cells in the INL of the lesioned region, nearly all of which appear to be injury-activated Müller glia. We found 6.8 ± 0.8 PCNA$^+$ cells per 100 μm of linear length retina in *nbl* mutants and 6.2 ± 1.1 PCNA$^+$ cells per 100 μm in *ncp* mutants; neither is significantly different from WT: 6.1 ± 1.0 PCNA$^+$ cells per 100 μm ($P = 0.31$) and 6.8 ± 0.9 PCNA$^+$ cells per 100 μm ($P = 0.36$), respectively. At 2 dpl, clusters of proliferating, Müller glia-derived, multipotent retinal progen-

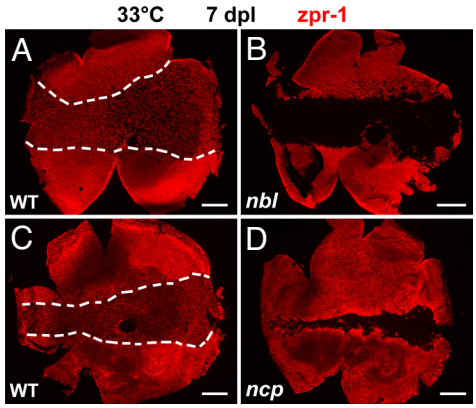

Fig. 3. Cones fail to regenerate in *nbl* and *ncp* mutants at the restrictive temperature. Flat-mounted retinas at 7 dpl immunolabeled with zpr-1 (red). (*A* and *C*) Regenerated cones between dashed lines in WT. (*B* and *D*) Few or no cones are seen in the lesioned central area in *nbl* and *ncp*, respectively. The occasional zpr-1$^+$ profile in the region of the lesion might represent a spared cone photoreceptor. (Scale bars: 300 μm.)

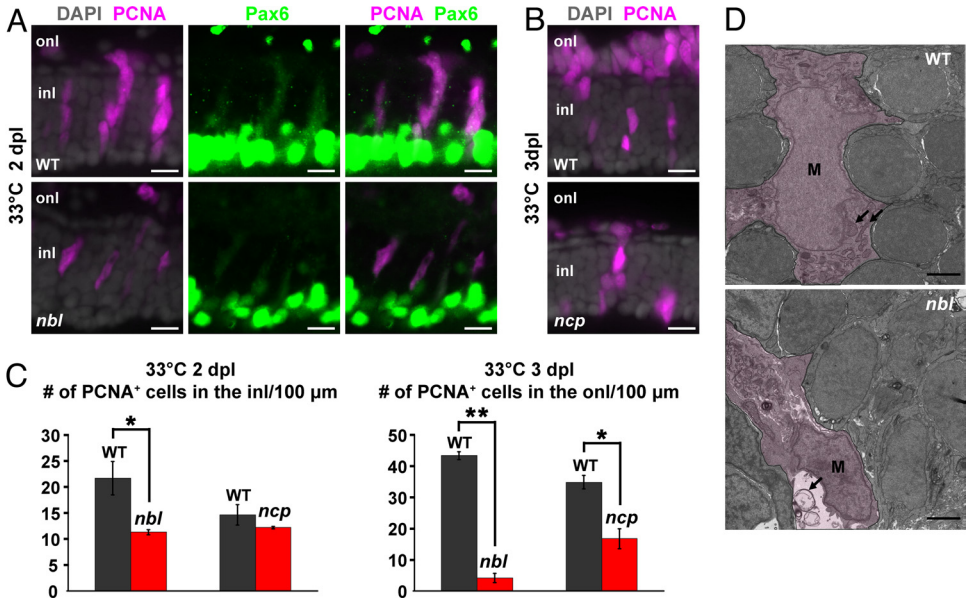

Fig. 4. Retinal regeneration defects of *nbl* and *ncp*. (*A*) Neurogenic clusters at 2 dpl in the inner nuclear layer (inl) immunolabeled with anti-PCNA (magenta) and weakly labeled with anti-Pax6 (green) in WT and *nbl*. Note that Pax6 is also expressed at high levels in amacrine cells at the inner boundary of the inl. (*B*) PNCA$^+$ photoreceptor progenitors at 3 dpl in the outer nuclear layer (onl) of WT and *ncp*. (*C*) Number of PNCA$^+$ cells in the inl or onl per 100 μm of linear length retina at 2 or 3 dpl, respectively. Error bars represent SEM for 3 individuals. *, $P < 0.05$; **, $P < 0.0001$. (*D*) Transmission electron micrographs of injury-activated Müller glia in WT and *nbl*. See text for description of temperature shift paradigm. Müller glia (M) are shown by the magenta wash. Mitochondria (arrows) in Müller glia of WT appear normal after 8 h at 33°C, whereas in *nbl* mutants, Müller glia contain swollen mitochondria. (Scale bars: 10 μm in *A* and *B*; 100 μm in *D*.)

itors weakly immunoreactive for the retinal progenitor marker Pax6 are seen in the INL within the lesioned area in WT. These regularly spaced radially oriented groups of PCNA$^+$/Pax6$^+$ cells associated with Müller glia, called ''neurogenic clusters,'' are characteristic of retinal regeneration in teleost fish (16). The number of proliferating progenitors in the neurogenic clusters of *nbl* was reduced to ~50% of WT: 11.3 ± 0.5 PCNA$^+$/Pax6$^+$ cells per 100 μm in *nbl* compared with 21.7 ± 3.2 PCNA$^+$/Pax6$^+$ cells per 100 μm in WT ($P < 0.05$, Fig. 4 *A* and *C*). The *ncp* mutants showed a slight but not statistically significant reduction in formation of neurogenic clusters: 12.2 ± 0.2 PCNA$^+$/Pax6$^+$ cells per 100 μm in *ncp* compared with 14.6 ± 1.9 PCNA$^+$/Pax6$^+$ cells per 100 μm in WT ($P = 0.17$; Fig. 4*C*).

Proliferation of retinal progenitors in *ncp* mutants was reduced at 3 dpl when the neuronal progenitors have migrated into the ONL and become committed to the photoreceptor lineage, as evidenced by expression of a photoreceptor-specific homeobox gene, Crx (18). At 3 dpl, *ncp* mutants had fewer than half as many photoreceptor progenitors (PCNA$^+$ cells in the ONL) relative to WT: 16.8 ± 3.2 PCNA$^+$ cells per 100 μm in *ncp* compared with 34.9 ± 2.1 PCNA$^+$ cells per 100 μm in WT ($P < 0.05$; Fig. 4 *B* and *C*). Consistent with the reduction in multipotent retinal progenitors at 2 dpl, the *nbl* mutants showed a substantial decrease (~90% reduction) in the number of photoreceptor progenitors at 3 dpl: 4.1 ± 1.6 PCNA$^+$ cells per 100 μm in *nbl* and 43.5 ± 1.2 PCNA$^+$ cells per 100 μm in WT ($P < 0.0001$; Fig. 4*C*). Note that the number of PCNA$^+$ cells in the WT retinas varies between the 2 mutant lines and across experiments; this variability in the absolute rate of cell proliferation in teleost fish retinas is typical (32) and likely reflects environmental modulation of endogenous growth rates. Taken together, these data suggest that *nbl* blocks cone photoreceptor regeneration at an earlier step compared with *ncp* and are consistent with the differential time course of *hspd1* and *mps1* expression during retinal regeneration.

In amputated fins, *nbl* causes structural defects in mitochondria, specifically in the putative blastemal stem cells (9). To determine whether Müller glia-derived retinal stem cells are similarly differentially affected by the *nbl* mutation, we used a temperature shift paradigm and examined retinas with transmission electron microscopy. Regeneration was allowed to proceed normally at 28°C for 2 or 3 days before fish were shifted to 33°C for 4 or 8 h. Müller glia were identified by the position (in the inner half of the INL) and morphological features of their nuclei (polygonal, often lobulated with clumped heterochromatin) and by the presence of cytoplasmic glycogen granules. In *nbl* (but not WT) at 2 dpl following 8 h at 33°C, most of the identified Müller glia within the lesioned area had swollen distorted mitochondria with an empty matrix (Fig. 4*D* and Fig. S6 *A–C*): of 29 Müller glia that we examined, 20 had defective mitochondria and 3 of the 20 also had 1 or more mitochondria with normal morphology. This mitochondrial defect was not seen in the neurogenic progeny of Müller glia (i.e., the radial clusters of neuronal progenitors migrating into the ONL), which were increased in abundance at 3 dpl (Fig. S6 *D–F*). These results suggest that the defect in *nbl* is confined to injury-induced stem cells derived from differentiated cells in both neural retina and mesenchymal tissues in the caudal fin.

Additional Genes Shared in Regenerating Tissues. To identify additional candidate genes that might be involved in epimorphic regeneration independent of the body structure damaged, we compared our microarray data set from isolated injury-induced Müller glia/progenitors with published gene profiling results from 2 other zebrafish regeneration models: amputated caudal tail fins (11) and surgically lesioned hearts (12). Table S1 lists 28 genes whose expression levels changed in the retinal data set and in one or both of the comparison data sets. A large subset of these regeneration-associated genes is involved in the innate immune response to tissue injury, several regulate the immune system by suppressing inflammatory cytokine signaling, and others mediate the stress response. In addition, a number of the

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regeneration genes regulate developmental signaling pathways (e.g., TGF-β, Hedgehog, Notch) or are transcription factors that regulate progenitor cells. Another recently published retinal regeneration microarray data set designed to identify molecular signatures of injured and dying photoreceptors and microglia was generated from tissue obtained by laser-capture microdissection of the photoreceptor layer from light-damaged zebrafish retinas (15); at least 3 of the secreted growth factor signals found—*midkine*, *progranulin*, and *galectin*—are also up-regulated in regenerating heart (12). This provides further support for a common molecular program of injury-induced regeneration in mesodermal and neural tissues.

Discussion

Our study differs from 3 previously published microarray-based gene expression profile studies of retinal regeneration in adult zebrafish (13–15) in 2 fundamental ways: (*i*) we used brief exposures to ultra-intense light to induce widespread and rapid photoreceptor death, whereas the earlier studies exposed fish to continuous light at lower intensities for several days (14, 15) or surgically removed a small piece of retina (13), and (*ii*) we isolated the injury-activated Müller glia for RNA extraction and gene profiling analysis, whereas the other studies harvested RNA from the entire retina (13, 14) or from laser-captured ONL tissue (15). Retinal injury induces a series of complex cellular responses in many cell types, including neurodegeneration and apoptosis of the damaged cells, stress responses in other retinal cells, and activation of microglia/macrophages (6, 14, 18). By purifying the GFP$^+$ Müller glia, we increased the sensitivity of our analysis to identify injury-induced changes in gene expression that activate the retinal stem cell population and initiate a neurogenic program. Although some of the genes whose expression levels changed dramatically in our data set were also identified in previous studies, the magnitude of the changes observed in these other studies was necessarily diluted by the cellular heterogeneity of the samples. For example, the maximum fold change of *hspd1* reported previously was 2.0 (13) or 1.7 (14) compared with 3.6 in our study, and changes in *mps1* were not reported in one of these studies (14). A recent analysis of mechanically injured zebrafish retina in which *ascl1a* (*achaete-scute complex-like 1a*) function was knocked down with morpholino antisense oligonucleotides verified that it is required for the regenerative response (25), which validates the utility of our data set as a tool for discovering genes that induce a neurogenic program in differentiated glial cells. Consistent with the increased expression of *hspd1* that we observed in the ONL by in situ hybridization, the microarray data from laser-captured ONL tissue also showed up-regulation of *hspd1* (15). In contrast, neither *mps1* nor *ascl1a* was up-regulated in that analysis, again consistent with our observation that these genes are specifically induced in injury-activated Müller glia during the initial stages of regeneration (23) (Fig. 2 *D* and *F*).

A model of Müller glia-based photoreceptor regeneration in adult zebrafish (18) is shown in Fig. 5. In response to the light lesion (step 1), Müller glia are activated locally in the region in which photoreceptors were damaged by the intense light treatment (step 2); Müller glia activation is evidenced by apical nuclear migration and up-regulation of GFAP intermediate filaments. This is followed by dedifferentiation of Müller glia and their entry into the mitotic cycle (step 3). Asymmetric division of Müller glia generates neurogenic clusters of multipotent progenitors that proliferate, migrate into the ONL, and differentiate into photoreceptors, resulting in the self-renewal of the Müller "stem cell" (step 4). In this study, we found that *hspd1* is essential for the formation of neurogenic clusters (step 3), whereas *mps1* is required for a later step during photoreceptor progenitor proliferation (step 4).

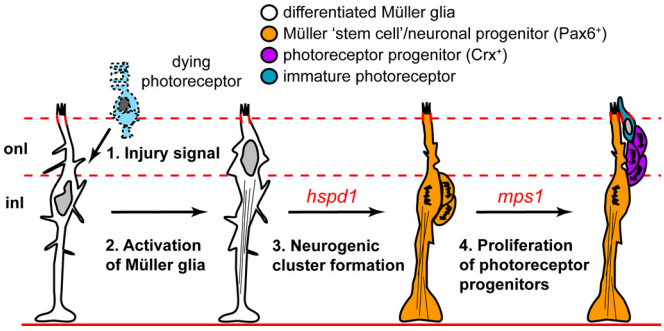

Fig. 5. Model for Müller glia-based photoreceptor regeneration in adult zebrafish retina. Four steps in the regeneration of photoreceptors in the light-damaged retina. In *nbl* mutants, regeneration is blocked at step 3, and in *ncp* mutants, regeneration is blocked at step 4. See text for further description.

Comparison of gene expression profiles from regenerating zebrafish caudal fin, heart muscle, and neural retina revealed a number of shared genes even though different cellular substrates are required for regeneration of these diverse structures: amputated fins regenerate from a blastema derived from dedifferentiated mesenchymal stem cells (33), hearts regenerate by cardiomyocyte proliferation (3), and the neural retina regenerates from progenitors derived from nonneuronal Müller glial cells. What each of these regenerating tissues has in common, however, is that the stem cells responsible for replacing the missing cells and repairing the damaged tissue arise from differentiated cells that respond to injury by dedifferentiation and proliferation. The fundamental nature of the proteins encoded by the 2 genes on which we performed functional analysis—Hsp60, a mitochondrial protein chaperone important in the cellular stress response, and Mps1, a kinase with a function in mitotic checkpoint regulation—hints at a universal mechanism of epimorphic regeneration. These results, together with the comparative analysis of regeneration transcriptomes, suggest that the capacity of diverse cell types to respond to tissue injury by dedifferentiation and acquisition of stem cell properties may require the activation of conserved cellular and molecular mechanisms that regulate choice of cell fate and morphogenetic patterning during embryogenesis.

Materials and Methods

Zebrafish. Zebrafish lines *Tg(gfap:GFP)mi2002* (24), *nbl* (kindly provided by M. Keating, Novartis Institute for BioMedical Research, Cambridge, MA) (9), and *ncp* (kindly provided by K. Poss, Duke University Medical Center, Durham, NC) (8) were maintained according to standard methods. The Committee on Use and Care of Animals in Research at the University of Michigan approved all procedures using animals. Adult fish (3 months to 1 year of age) were used for all experiments. Light lesions were as described previously (18).

Retinal Dissociation and Isolation of Müller Glia. Retinas were dissected from dark-adapted *Tg(gfap:GFP)mi2002* zebrafish at 8, 16, 24, and 36 hpl and from non-light-treated controls (0 hpl). Tissues were minced with a razor blade and dissociated by enzymatic digestion with 16 U/mL papain (Worthington) and 0.2 U/mL Dispase (Worthington) (34) in PBS at pH 6.5 for 30 min at 28°C and triturated. Cells were pelleted at 3,400 $\times$ *g* for 3 min, resuspended in 1 mg/mL papain inhibitor (Worthington) and 100 μg/mL DNase I (Sigma-Aldrich) with 2 mM $MgCl_2$ in PBS at pH 7.4 for 10 min at room temperature, and then put on ice. GFP$^+$ cells were isolated on a Vantage SE cell sorter (BD Biosciences). Gating was based on cell size and fluorescence intensity, with parameters set by reference to a control sample of dissociated retinal cells from WT zebrafish.

Microarray Analysis. At each sample time, retinas from 3 or 4 fish were pooled for cell dissociation and cell sorting. Total RNA was extracted and purified from 1–2 $\times$ 10^5 freshly sorted GFP$^+$ cells using the RNAqueous-4PCR kit (Ambion). The interval between retinal isolation and cell lysis was ~2.5 h. The quality and quantity of RNA were assessed with a 2100 BioAnalyzer

(Agilent Technologies). For microarray gene profiling, 20 ng of total RNA was used for linear amplification with the Ovation Biotin Labeling System (NuGEN) and 2.75 μg of biotin-labeled fragmented cDNA was hybridized to a GeneChip Zebrafish Genome Array (Affymetrix) with 15,617 probe sets. Independent hybridizations of 3 biological replicates were performed for each time interval.

For data analysis, the ''AFFY'' package (www.bioconductor.org) was used to filter probe sets based on absent-present call; the Robust Multichip Average method and a 2-stage filtering procedure based on false discovery rate confidence interval (FDRCI) were used as described (35). Genes differentially expressed at 1 or more time intervals compared with the untreated control were identified by a fold change $\geq$2 and an FDRCI *P*-value $\leq$0.15. Hierarchical clustering was performed as described (36). Gene ontology analysis used the Affymetrix NetAFFX Web interface and the Database for Annotation, Visualization, and Integrated Discovery (DAVID) annotation tool (37). Statistically overrepresented ($P \leq 0.1$) gene ontological groups were identified as described (38).

qRT-PCR. Total RNA was reverse transcribed and linearly amplified with the Ovation Biotin Labeling System (NuGEN). All real-time PCR reactions were carried out in duplicate with iQ SYBR Green Supermix (BioRad) on an iCycler iQ real-time PCR detection system (BioRad). The standard curve method was used to determine levels of expression of the genes of interest relative to *gpia* (*glucose phosphate isomerase a*) and relative fold changes in gene expression after lesioning. Sequences of the gene-specific primer pairs are provided in *SI Text*.

Tissue Processing. Immunohistochemistry was performed as described (18). For in situ hybridization, digoxigenin-labeled cRNA probes for *hspd1* (IMAGE clone identification number: 3819432) and *mps1* (IMAGE clone identification number: 6797095) were prepared and hybridized at 5 μg/mL as described (23). Light microscopy was performed with an AxioImager epifluorescent compound microscope (Carl Zeiss Microimaging); images were processed with Adobe PhotoShop (Adobe Systems) as described previously (18). All adjustments were applied to the entire image. Cells expressing the nuclear cell proliferation marker PCNA were counted in cryosections through the dorsoventral axis in the plane of the optic disc and expressed as number of cells per 100 μm of linear length as described (18). The selection of regions for counting was done ''blind'' (without viewing PCNA immunofluorescence). PCNA$^+$ cells were counted in 10 retinal sections from each of 3 fish for both mutants and WT siblings. An unpaired Student's *t* test was used for statistical analysis. Transmission electron microscopy was performed as described (39), and ultrathin sections were viewed with a Phillips CM-100 transmission electron microscope equipped with an AMT digital camera (Philips).

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