Zinc-binding Domain-dependent, Deaminase-independent Actions of Apolipoprotein B mRNA-editing Enzyme, Catalytic Polypeptide 2 (Apobec2), Mediate Its Effect on Zebrafish Retina Regeneration*1

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Background: The mechanism by which Apobec2 stimulates retina regeneration remains unresolved.

Results: Apobec2 lacks cytosine deaminase activity and interacts with proteins that regulate retina regeneration.

Conclusion: Apobec2 subcellular localization is regulated by sumoylation and requires a zinc binding domain and protein interactions to regulate retina regeneration.

Significance: This study delineates previously unidentified biochemical functions of Apobec2 proteins.

The Apobec/AID family of cytosine deaminases can deaminate cytosine and thereby contribute to adaptive and innate immunity, DNA demethylation, and the modification of cellular mRNAs. Unique among this family is Apobec2, whose enzymatic activity has been questioned and whose function remains poorly explored. We recently reported that zebrafish Apobec2a and Apobec2b (Apobec2a,2b) regulate retina regeneration; however, their mechanism of action remained unknown. Here we show that although Apobec2a,2b lack cytosine deaminase activity, they require a conserved zinc-binding domain to stimulate retina regeneration. Interestingly, we found that human ApoBEC2 is able to functionally substitute for Apobec2a,2b during retina regeneration. By identifying Apobec2-interacting proteins, including ubiquitin-conjugating enzyme 9 (Ubc9); topoisomerase I-binding, arginine/serine-rich, E3 ubiquitin protein ligase (Toporsa); and POU class 6 homeobox 2 (Pou6f2), we uncovered that sumoylation regulates Apobec2 subcellular localization and that nuclear Apobec2 controls Pou6f2 binding to DNA. Importantly, mutations in the zinc-binding domain of Apobec2 diminished its ability to stimulate Pou6f2 binding to DNA, and knockdown of Ubc9 or Pou6f2 suppressed retina regeneration.

Apobec proteins are a family of cytosine deaminases capable of introducing mutations into DNA and/or RNA (1). Apobec2 was the second Apobec family member identified (2, 3). Although its overexpression has been correlated with tumor formation, and its deletion correlated with perturbations in muscle development, retina regeneration, and left-right axis specification, the biochemical function of Apobec2 remains unknown (4–8). Unlike other Apobecs, Apobec2 fails to induce cytosine deaminase-dependent mutations in bacterial and yeast-based mutagenesis assays (deamination of cytosine results in its conversion to uracil) (9–11), and the ability of Apobec2 to bind polyadenylates is limited (3, 4). Although biochemical data are lacking, prominent models of Apobec2 function include cytosine deaminase-dependent DNA demethylation (12, 13) and C-to-U mRNA editing (5, 6, 8).

Previously, we demonstrated that Apobec2a,2b are necessary for zebrafish retina and optic nerve regeneration (7). During retina regeneration, Apobec2a,2b regulate Müller glia (MG) activation and the generation of Müller glia-derived progenitor cells (MGPCs). One of the ways they influence these events is by directly or indirectly stimulating expression of the asc1la gene (7), a gene that encodes a transcriptional activator necessary for retina regeneration (14, 15). Interestingly, knockdown of Apobec2a,2b during retina regeneration does not influence the changes in DNA methylation that accompany retina regeneration, indicating that Apobec2 proteins perform an alternate function (16).

Here we build upon our previous work aimed at determining the mechanism by which Apobec2 proteins regulate retina regeneration (7, 16). Through a combination of bacterial expression assays, transgenic fish, and site-directed mutagenesis, we demonstrate that although zebrafish Apobec2 proteins lack detectable zinc-dependent cytosine deaminase activity, their zinc-binding domain is critical for their function during retina regeneration. To understand how Apobec2 proteins regulate retina regeneration, we turned to a yeast two-hybrid screen to identify Apobec2-interacting proteins. This analysis

References:
1. This article contains supplemental Table 1.
2. The abbreviations used are: Apobec2a,2b, Apobec2a and Apobec2b; apobec2a,2b, apobec2a and apobec2b; SUMO, small ubiquitin-like modifier; MG, Müller glia; MGPC, Müller glia-derived progenitor cell; MO, morpholino antisense oligonucleotide; hpi, h postinjury; dpi, days postinjury; PCNA, proliferating cell nuclear antigen; INL, inner nuclear layer; DBD, DNA-binding domain.

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identified Ubc9, Topors, and Pou6f2, which led us to discover that sumoylation controls Apobec2 subcellular localization and that nuclear Apobec2 regulates the propensity of Pou6f2 to bind DNA. Importantly, we also found that Ubc9 and Pou6f2 regulate retina regeneration. This study reveals previously unrecognized functions for Apobec2 proteins and provides new insight into their regulation and cellular actions.

**EXPERIMENTAL PROCEDURES**

*Animals, Retinal Injury, Morpholino Antisense Oligonucleotide (MO) Delivery, BrdU Injections, and Heat Shock—* All experiments involving animals were performed in accordance with institutional guidelines and regulations and approved by the University of Michigan’s Committee on Use and Care of Animals.

Transgenic *gfp:gfp* (17) and 1016 *tubα1α:gfp* (18) fish have been described. In preparation for the construction of *hsp70* transgenic fish, the 1016 *tubα1α:gfp* SV40 cassette (18) was cloned, followed by a second expression cassette encoding 1523 bp of the *hsp70* promoter (19) driving the expression of 1) *myc-zapobec2b-Viral2apeptide(V2a)-flag-zapobec2a(C156A)* (*hsp70:zapobec2mut*), or 3) *myc-hAPOBEC2* (*hsp70:hAPOBEC2*) followed by an SV40 sequence (Fig. 4) into the pT2AL200R150G Tol2 vector (20). The primers and intermediate clones used in the preparation of these constructs are listed in **supplemental Table 1**. Overlap extension PCR was used for the preparation of constructs including V2a peptide sequences (optimized for zebrafish translation) and constructs including mutations. The V2a peptide allows for polycistronic expression (Fig. 3C) (21). Tol2 transposase-mediated integration of the transgene was performed by injection into single-cell zebrafish embryos, which were raised to adulthood and screened for transgenic progeny (7). Multiple independent lines were selected and grown to adulthood, each exhibiting a similar phenotype (Fig. 3).

Retina lesions and BrdU injections have been described previously (7, 16). Briefly, while anesthetized in 0.02% tricaine methane sulfonate, fish were placed under a dissecting microscope for visualization, and the right eye was gently rotated in its socket and steadfastly through the sclera with a 30-gauge needle. Lissamine-tagged MOs (Gene Tools) were delivered at the time of injury by using a Hamilton syringe. MO delivery to cells was facilitated by electroporation as described (7). The control, *apobec2a, apobec2b,* and *ube2i* targeting MOs have been described previously (7, 12, 16, 22, 23). The coding sequence of the *pou6f2* MO is CATGAGAAGCATCTACTCTAAGGTC (5' → 3'). All morpholinos were designed to inhibit protein translation (targeted to the 5’ end of transcript) and were 3’-conjugated to lissamine to facilitate their electroporation and subsequent visualization. Intrapercitoneal BrdU injections were carried out with 20 μl of 20 mm BrdU 3 h before harvesting.

Heat shock was carried out at 36.5 °C as has been described (24). Unless indicated otherwise, in the analyses of uninjured fish, heat shock was carried out every 12 h for 1 h each over a 4-day period. For analyses of injured fish, heat shock was carried out at 9 h postinjury (hpi) (1-h heat shock), 24 hpi (1-h heat shock), 28 hpi (30-min heat shock), and 33 hpi (1-h heat shock) to mimic the injury-dependent induction of endogenous *apobec2a,b*, and fish were harvested at 48 hpi. Analyses were performed using size- and age-matched fish. Transgenic fish analyzed in this study were heterozygous to wild type.

*Site-directed Mutagenesis and Cloning—* Site-directed mutagenesis was carried out using overlap extension PCR and the primers listed in **supplemental Table 1**. Cloning was carried out using Phusion DNA Polymerase (New England Biolabs) and the primers listed in **supplemental Table 1**. Each clone was sequenced by the University of Michigan DNA Sequencing Core.

For this study, clones were used in the following applications: mRNA preparation (pcS2 *flag-zapobec2a-V2a-GFP* and pcS2+MIT *myc-zapobec2b-V2a-GFP*), creation of transgenic fish (pTal *hsp70:zapobec2wt*, pTal *hsp70:zapobec2mut*, and pTal *hsp70:hAPOBEC2*), mutagenesis assays (*pHis, pHis−-rApobe1 (removing the His tag), pHis−-rApobe1*(W905S), *pHis−-hAID*, *pHis−-hAPOBEC2*, *pHis−-hAPOBEC2(T)*, *pHis−-hAPOBEC2(1725W), pHis−-zapobec2a*, *pHis−-zapobec2a(T)*, *pHis−-zapobec2a(1553W)*, *pHis−-zapobec2b(T)*, and *pHis−-zapobec2b(1777W)*), yeast two-hybrid assays (pLexA2DE2Noti(pLexA) hAPOBEC2, pLexA hAPOBEC2(T), pLexA hAPOBEC2(C128A), pLexA zApobec2a, pLexA zApobec2b, pLexA zApobec2b(C180A), pVp16 zApobec2a, pVp16 Pou6f2DBD, pVp16 Pou6f2M1, pVp16 Pou6f2M2, pVp16 Pou6f1DBD, and pVp16 Pou6f3DBD), bacterial sumoylation assays (pT E1E2S1 (25), pT E1E2/C93A1S1, pETDuet flag-Toporsa, pGST hAPOBEC2, pGST hAPOBEC2(T), pGST zApobec2a, pGST zApobec2a(T), pGST zApobec2b, pGST zApobec2b(T), pETDuet GST-Pou6f2DBD, pHis− hAPOBEC2, pHis− zapobec2a, and *pHis−-zapobec2b*), tissue culture analyses (pEGFP, pcS2 hSUMO1-gfp, pcS2 hAPOBEC2-gfp, pcS2 hSUMO1-hAPOBEC2-gfp, pcS2 hAPOBEC2(T)-gfp, pcS2 hAPOBEC2(K4R)-gfp, pcS2 zApobec2a-gfp, pcS2 hSUMO1-zApobec2a-gfp, pcS2 zApobec2a(T)-gfp, pcS2 zApobec2b-gfp, pcS2 hSUMO1-zApobec2b-gfp, pcS2 zApobec2b(T)-gfp), pcS2 mCherry-Ubc9, and pcS2 mCherry-Pou6f2), EMSAs (pGST Pou6f2DBD, pGST hAPOBEC2, pGST hAPOBEC2(C128A), pGST zApobec2a, pGST zApobec2a(T), pGST zApobec2b, and pGST zApobec2b(C180A)), and MO validation (pcS2 ube2iMOs-gfp and pcS2 pou6f2MOs-gfp). pcDNA V5 hAPOBEC2 (provided by Dr. Hongjun Song, Johns Hopkins University), pTrc99a hAID (provided by Dr. Michael Neuberger, Medical Research Council Laboratory of Molecular Biology), pTrc99a rApobe1 (provided by Dr. Michael Neuberger, Medical Research Council Laboratory of Molecular Biology), pCRlITopo zApobec2a (provided by Dr. David Jones, University of Utah), and pCRlITopo zApobec2b (provided by Dr. David Jones, University of Utah) served as templates for cloning. hSUMO1 was cloned using cDNA prepared from HEK293 cells. The pLexA and pVP16 plasmids were provided by Dr. Anne Vojetk (University of Michigan), and pT-E1E2S1 was provided by Dr. Jeremy Henley (University of Bristol).
Insights into the Function of Apobec2 Proteins

Fluorescence-activated Cell Sorting RNA Isolation, RT-PCR, and Real-time PCR—FACS sorting of GFP+ cells from uninjured gfp:gfp (isolation of MG) and 4-day post injury (dpi) 1016 tuba1a:gfp transgenic fish (isolation of MG PCs) was carried out as described previously (16). Briefly, zebrafish retinas were collected in 0.8 ml of Leibovitz’s L15 medium, treated for 15 min with 1 mg/ml hyalurondase (Sigma) at room temperature, and then dissociated in 0.01% (v/v) trypsin with frequent trituration. A single-cell suspension was confirmed by microscopy, and cells were washed in Leibovitz’s L15 medium before sorting. Cell sorting was performed by the University of Michigan Flow Cytometry Core on a BD Biosciences FACSARia triple-laser high speed cell sorter.

Total RNA was isolated using TRIzol (Invitrogen) and was DNase-treated (Invitrogen). cDNA synthesis was performed using random hexamers and either SuperScript-II (Invitrogen) or M-mulv (New England Biolab) reverse transcriptase. PCRs used Taq and gene-specific primers (supplemental Table 1). Real-time PCRs were carried out with ABSolute SYBR Green Fluorescein Master Mix (Thermo Scientific) on an iCycler real-time PCR detection system (Bio-Rad). The Fluorescein Master Mix (Thermo Scientific) on an iCycler real-time PCR—

and Real-time PCR—

Validation of MO functionality was carried out in embryos as described (9, 29). For bacterial mutagenesis assays, colonies were picked and gene-specific primers (supplemental Table 1) were microinjected into single cell embryos at a concentration of 2 ng/μl. 1 ml of LB containing 100 μg/ml ampicillin to isolate MG) and 4-day post injury (dpi) 1016 tuba1a:gfp transgenic fish (isolation of MG PCs) was carried out as described previously (16). Briefly, zebrafish retinas were collected in 0.8 ml of Leibovitz’s L15 medium, treated for 15 min with 1 mg/ml hyalurondase (Sigma) at room temperature, and then dissociated in 0.01% (v/v) trypsin with frequent trituration. A single-cell suspension was confirmed by microscopy, and cells were washed in Leibovitz’s L15 medium before sorting. Cell sorting was performed by the University of Michigan Flow Cytometry Core on a BD Biosciences FACSARia triple-laser high speed cell sorter.

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mRNA Synthesis and Embryo Microinjections—pCS2 flag-zapobec2a:V2a-GFP and pCS3+MT myc-zapobec2b:V2a-GFP plasmids were linearized with NotI (New England Biolab), and capped mRNAs were synthesized using the mMESSAGE mMACHINE (Ambion). Single cell zebrafish embryos were injected with ~200 pl of solution containing 0.125 mM MO or 0.125 mM MO with 2 ng/μl mRNA.

Validation of MO functionality was carried out in embryos as has been done previously (7). Briefly, purified constructs (pCS2 ube2iM0bs:gfp and pCS2 pou6f2M0bs:gfp) were microinjected into single cell embryos at a concentration of 2 ng/μl in conjunction with 0.125 mM lissamine-tagged experimental morpholino or control morpholino. Microinjections were analyzed for GFP expression 2 days postfertilization (Fig. 11, A and B).

Bacterial Mutagenesis and Growth Assays—Bacterial mutagenesis assays were carried out as described (9, 26). Briefly, BL21(DE3) cultures harboring the indicated plasmid were grown at 37 °C in 2 ml of LB containing 100 μg/ml ampicillin to an A600 of 0.5. 1 ml of LB containing 100 μg/ml ampicillin, 3 mM isopropyl-β-D-thiogalactopyranoside was then added, and the culture was grown overnight with shaking at either 37 or 25 °C (27, 28). The next day, 1 ml of the culture was harvested, spun down, and spread onto a plate containing LB and 10 μg/ml rifampicin. Samples were allowed to grow overnight at 37 °C, and the next day, the number of growing colonies was quantified. To identify mutations that were occurring in the rpoB gene, colony PCR was carried out on rifampicin+ colonies using the rpoB primers (supplemental Table 1). PCR products were sequenced by the University of Michigan DNA Sequencing Core. Variants were identified by comparison with the wild type rpoB sequence as has been done by others (9, 29). For bacterial growth assays, protein expression was induced overnight as outlined above for the bacterial mutagenesis assay. The following day, the A600 for each sample was determined. Student’s t tests were performed to determine statistical differences between the control and each experimental sample.

Tissue Preparation and Immunofluorescence—Zebrafish eyes were prepared, sectioned at 12 μm, and stained, as has been done previously (7, 16). The following primary antibodies were used for immunofluorescence: rat anti-BrdU (dividing cell marker, 1:1000; Abcam), mouse anti-PCNA (dividing cell marker, 1:500; Sigma), rabbit anti-GFP (1:1000; Invitrogen), mouse anti-FLAG (1:1000; Rockland), and rabbit anti-Myc (1:1000; Sigma). The following secondary antibodies were used: Alexa Fluor 555 donkey anti-mouse IgG (1:1000; Invitrogen), Alexa Fluor 488 donkey anti-mouse IgG (1:1000; Invitrogen), Alexa Fluor 488 goat anti-rabbit IgG (1:1000; Invitrogen), and Cy3 donkey anti-rat (1:1000; Jackson Immunoresearch). Antigen retrieval for BrdU and PCNA staining was performed by boiling the sections in 10 mM sodium citrate for 20 min, followed by cooling them for another 20 min in solution.

Retinal sections were examined using a Zeiss Axiophot, Axio Observer Z1 microscope (Zeiss LD Plan-Neofluor ×20/0.4 Korr objective). Images were captured at room temperature using a digital camera adapted onto the microscope and were processed and annotated with Adobe Photoshop CS4. For the quantification of PCNA+ cells, injured eyes were sectioned across five slides. The total number of PCNA+ cells per slide was quantified for three individual eyes per condition. Student’s t tests were performed to determine statistical differences between the control and each experimental sample.

Preparation of Full-length Normalized Yeast Two-hybrid Library—RNA was purified from FACS-sorted GFP+ cells isolated from 4-dpi 1016 tuba1a:gfp transgenic fish and treated with DNase (Invitrogen). Library preparation was performed using the EasyClone normalized cDNA library construction package (Dualsystems Biotech) as outlined by the manufacturer. Briefly, 500 ng of RNA was used for the production of cDNA, double-stranded cDNA synthesis was carried out for 21 cycles, and 1200 ng of double-stranded cDNA was used for library normalization. Normalized library amplification was then carried out using Phusion DNA Polymerase (New England Biolabs) and the Y2Hlibraryascl-F and Y2Hlibrarynt-R primers (supplemental Table 1). These were cloned into a modified pVP16ascl construct into the AscI (New England Biolabs) and NotI (New England Biolabs) sites. Library ligation was carried out with 250 ng of template and 250 ng of backbone. To assay the quality of the library, a small aliquot of this ligation was transformed, and the insert size of 20 independent colonies was measured. The average insert size was ~1330 bp. A large scale transformation was then performed using DH10B Electromax Ultracompetent Cells (Invitrogen), resulting in ~2 million independent transformants. Transformants were scraped from their plates, and plasmid and bacterial stocks of the pVP16 4-dpi 1016 tuba1a:gfp yeast two-hybrid library were prepared as outlined by the EasyClone normalized cDNA library construction package.

Yeast Transformations and Yeast Two-hybrid Screens—Yeast transformation and large scale yeast two-hybrid screens for Apobec2a (pLexA zApobec2a) and Apobec2b (pLexA zApobec2b) using the pVP16 4-dpi 1016 tuba1a:gfp yeast
two-hybrid library were used for YC-WHULK assays: growth assays on YC-WHULK plates and β-galactosidase filter assays. The omission of tryptophan (W), leucine (L), uracil (U), or lysine (K) from the media maintained selection for the LexA plasmid, the VP16 plasmid, the integrated lacZ reporter, or the integrated HIS3 reporter, respectively. If proteins interact, L40 yeast can grow in the absence of histidine (H) and stain blue in β-galactosidase filter assays. For the β-galactosidase filter assays, yeast growing on YC-WUL plates (selecting for the plasmids) were transferred to nitrocellulose membranes. To identify interacting VP16 protein fusions, colony PCR was performed using YC-WHULK + L40 colonies (transferred and grown on >4 independent plates for correct plasmid selection in case multiple VP16 clones were transformed in the original screen) using the Y2HVP16PCR primers listed in supplemental Table 1 and Phusion DNA Polymerase (New England Biolabs). PCR products were then sequenced using the Y2HVP16seqp primer (supplemental Table 1) by the University of Michigan DNA Sequencing Core.

**Bacterial Sumoylation Assays and Western Blotting**—Bacterial sumoylation assays were carried out as described (25). Briefly, BL21(DE3) cultures harboring the indicated plasmids were grown at 37 °C in 2 ml of LB containing appropriate antibiotics (100 μg/ml ampicillin, 50 μg/ml kanamycin, and/or 50 μg/ml chloramphenicol) to an A600 of 0.5. 1 ml of LB containing appropriate antibiotics and 3 μl isopropyl-β-d-thiogalactopyranoside was then added, and the culture was grown overnight with shaking at 25 °C. Bacteria were then pelleted and stored at −80 °C until use. Predictions of sumoylation sites were performed using SUMOsp 2.0 (31) and the SUMOplotTM Analysis Program.

In preparation for Western blotting, bacteria were lysed with boiling in 1× denaturing SDS-PAGE loading buffer and spun down to pellet particulate matter. Microinjected zebrafish embryos were lysed by sonication on ice in nuclei lysis buffer (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% SDS) including a protease inhibitor mixture (Thermo). Extract from lysed embryos was then boiled in 1× denaturing SDS-PAGE loading buffer. Proteins were resolved on 8 or 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were blocked for 1 h in 1× PBS, 0.1% Tween containing 3% donkey serum (Sigma) before probing with antibodies. For the detection of GST-tagged proteins, blots were incubated with primary antibody diluted in blocking solution for 1 h at room temperature, followed by washing and protein detection. The probing of all other blots was performed as outlined previously (7). The following primary antibodies were used: mouse anti-FLAG (1:12,000; Rockland), rabbit anti-Myc (1:12,000; Sigma), rabbit anti-GFP (1:12,000; Invitrogen), mouse anti-SUMO1 (1:10,000; Santa Cruz Biotechnology, Inc.), and mouse anti-GST conjugated with HRP (1:5000; Santa Cruz). The following secondary antibodies were used: goat anti-rabbit IgG conjugated to HRP (1:15,000; Rockland) and goat anti-mouse IgG conjugated to HRP (1:15,000). Proteins were detected using Lumi Lights Western blotting Substrate (Roche Applied Science) and exposed using x-ray film or a FluorChem M Digital Darkroom.

**Tissue Culture**—Human embryonic kidney cells (HEK293T) were maintained on 100 mm dishes in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 4.5 g/liter D-glucose, 2 mM glutamine, 10% fetal bovine serum (FBS), and penicillin/streptomycin (Invitrogen) at 37 °C with 5% CO2. Cells were seeded on 12-mm coverslip glass in 24-well plates and allowed to grow to 50–70% confluence before transfection with 200 ng of plasmid DNA using Lipofectamine 2000 (Invitrogen). After transfection, cells were either placed at 37 °C for 24 h or at 37 °C for 4 h and then at 28 °C for 20 h before fixation with 4% (w/v) paraformaldehyde, PBS and followed by DAPI staining.

**EMSA**—In preparation for EMSAs, oligonucleotides (with and without a 5’-biotin tag) were designed with the sequences outlined in supplemental Table 1 and Fig. 10, A and B. Labeled and unlabeled probes were resuspended in 10 mM Tris, pH 7.5–8.0, 50 mM NaCl, 1 mM EDTA and mixed at a ratio of 1:2 (labeled or unlabeled F oligonucleotide/unlabeled R oligonucleotide). Oligonucleotides were annealed by boiling in a heat block for 5 min and cooling slowly to room temperature. Annealed probes were stored in aliquots for further use.

Expression of GST-tagged proteins was induced in BL21(DE3) cells with isopropyl-β-d-thiogalactopyranoside for 4 h at 37 °C and stored at −80 °C for later use. GST-tagged proteins were purified using the MagneGST™ protein purification system as outlined by the manufacturer (Promega) and stored in aliquots at −80 °C. Protein concentrations were measured by Bradford and visualized by SDS-PAGE and Coomassie staining. Apobec2 proteins were used fresh (within 3 days of isolation). EMSAs were carried out using 300 fmoles of probe, the indicated protein amounts, and the Lightshift chemiluminescent EMSA kit as outlined by the manufacturer (Pierce). Separation was carried out at 75 V on a 6% Tris borate-EDTA polyacrylamide gel and was run at 4 °C. Transfers were performed at 70 V for 13 min at 4 °C. EMSAs were quantified by densitometry using ImageJ software. Quantifications were performed using independent replicas and controls run on the same gel. Student’s t tests were performed to determine statistical differences between the control and each experimental sample.

**RESULTS**

**Overexpression of Apobec2a,2b Does Not Increase Survival in Bacterial Mutagenesis Assays**—Mammalian Apobec2 proteins fail to induce cytosine deaminase-dependent mutations in bacterial and yeast based mutagenesis assays (9–11). Here we analyzed zebrafish Apobec2a,2b in a similar assay. Whereas induction of Apobec1 and AID increased the number of rifampicin-resistant bacteria colonies (cytosine deaminase-dependent mutations of select sites within the bacterial rpoB gene leads...
Insights into the Function of Apobec2 Proteins

A

Induce Protein

Potential Mutagenic Protein

Plate to Rif Plates

Control

B

Number of Rif Resistant Colonies at 37°C

| Protein | Wt | Trunc | SSS/SWS |
|---------|----|-------|---------|
| Apobec2a | 18 | 7 | 9 |
| Apobec2b | 12 | 22 | 12 |
| Apobec2a (T) | 8 | 22 | 12 |
| Apobec2b (T) | 12 | 22 | 12 |

C

Number of Rif Resistant Colonies at 25°C

| Protein | Wt | Trunc | SSS/SWS |
|---------|----|-------|---------|
| Apobec2a | 11 | 9 | 7 |
| Apobec2b | 17 | 22 | 7 |
| Apobec2a (T) | 11 | 9 | 7 |
| Apobec2b (T) | 17 | 22 | 7 |

FIGURE 1. Bacterial mutagenesis assays suggest that Apobec2a,2b lack catalytic activity. A, graphic providing an overview of the bacterial mutagenesis assay. B and C, bacterial mutagenesis assays carried out at 37 °C (B) or 25 °C (C). Graphs are on log scale. Compared with pHis (empty vector) the induction of Apobec1 (rat), AID (human), Apobec2a(T) at 37 °C, and Apobec2b(T) at 25 °C resulted in significant increases in bacterial survival. *, p < 0.02369. The number of replicas (n) and the average (A) colony number are listed for each sample. Wt, wild type; T, truncation; Rif, rifampicin. Error bars, S.D.

to bacterial resistance to rifampicin), APOBEC2, Apobec2a, and Apobec2b did not (Fig. 1, A and B). Other work has suggested that temperature impacts the activity of zebrafish AID (27, 32). To test if this is also true of zebrafish Apobec2a,2b, we performed mutagenesis assays at 25 °C instead of 37 °C. Unlike the report of AID, this temperature change did not increase the mutagenic activity of Apobec2a,2b (Fig. 1C).

Comparisons of Apobec proteins revealed at least three features unique to Apobec2: 1) they are the most acidic of all Apobec proteins; 2) they have extended N termini; and 3) unlike previously characterized catalytically active Apobec proteins that contain an SWS motif in their active sites, Apobec2 proteins have an SSS motif (Fig. 2A). The acidic nature of these proteins may negatively influence their ability to bind polynucleotides, which has been demonstrated to be limited (4, 33).

It is less clear how the N termini or SSS motifs could impact potential mutagenic function of Apobec2 proteins in the bacterial mutagenesis assay. Perhaps the N termini of Apobec proteins regulate their oligomerization, which could affect their ability to bind large polynucleotides. Indeed, full-length Apobec2 appears to exist as a monomer, whereas an N-terminal truncation allowed oligomerization (34, 35). Furthermore, the conversion of the SWS motif to an SSS motif in the Apobec1 W90S mutant completely abolishes its mutagenic activity (Fig. 1B) (9).

To investigate whether the SSS motif or the extended N terminus of Apobec2 masked or hindered its mutagenic potential, Apobec2 mutations converting the SSS motif to an SWS motif or N-terminal truncations, Apobec2(T), were generated. For the truncations, the first 41, 73, or 93 amino acids were removed from APOBEC2, Apobec2a, and Apobec2b, respectively (this is the case in all of the truncations described in this work). Bacterial mutagenesis assays revealed no enhancement of mutagenesis when the SSS motif was converted to an SWS motif, regardless of whether the assay was conducted at 25 or 37 °C (Fig. 1, B and C). However, expression of Apobec2 proteins harboring N-terminal deletions suggested that Apobec2a(T) at 37 °C and Apobec2b(T) at 25 °C had a small increase in mutagenic capability (Fig. 1, B and C). To determine whether these increases in rifampicin resistance were due to cytosine deaminase-dependent mutagenesis, we characterized mutations of bacterial rpoB and quantified the percentage of dC/dG transitions occurring after induction of Apobec2a(T) at 37 °C or Apobec2b(T) at 25 °C. In comparison with reports of APOBEC1 (100% dC/dG transitions versus 27% dC/dG transitions in the control) (9) and AID (80% dC/dG transitions versus 31% dC/dG transitions in the control) (26), analysis of Apobec2a(T) and Apobec2b(T) revealed dC/dG transitions of 20 and 40%, respectively (Fig. 2B), indicating that the differences in rifampicin resistance were not due to a preponderance of cytosine deamination.

Although we were unable to identify the exact mechanism whereby these truncations increase the number of rifampicin-resistant colonies, we did note that bacteria expressing Apobec2a(T) and Apobec2b(T) at 37 and 25 °C, respectively, grew to higher densities (Fig. 2D). This may have contributed to the increased number of rifampicin-resistant colonies because more cells would have been added to the plates according to our methods, which were based on volume (see “Experimental Procedures”). Thus, although Apobec2a(T) and Apobec2b(T) slightly increased the number of rifampicin-resistant colonies, they did so in a cytosine deaminase-independent fashion. These results, in combination with those of full-length Apobec2a,2b and APOBEC2 (Fig. 1) and those reported by others (3, 4, 9–11, 16), cast doubt on the hypothesis that Apobec2 proteins function as cytosine deaminases. Rather, they suggest that the function of Apobec2 proteins may be independent of cytosine deamination.

The Function of Apobec2 during Retina Regeneration Is Conserved across Species and Requires a Conserved Zinc-binding Domain—Apobec proteins are zinc-dependent deaminases. Although the coordinated zinc ion is critical for their ability to biochemically function as cytosine deaminases, it is likely that
zinc binding plays multiple roles. Metallprotein structure is dependent on the proper binding of the metal, and changes in structure resulting from improper metal binding or the lack thereof can disrupt the protein’s interactions with other macromolecules and/or alter its stability and/or solubility. Although the catalytic activity of Apobec2 proteins has come into question, their sequences contain the conserved residues needed for zinc binding, and they are bona fide zinc-binding proteins (34, 35). This suggests that zinc binding is important for the function of Apobec2 proteins.

To determine whether Apobec2 function during retina regeneration is conserved across species and requires proper zinc binding, we designed rescue experiments using tagged Apobec2 proteins (Fig. 3A). The myc and flag tags appended to apobec2a,2b mRNAs allow them to escape knockdown by MOs targeting the endogenous apobec2a,2b translation start site. Indeed, the N-terminal tags did not hinder their ability to rescue previously characterized Apobec2a/b knockdown phenotypes (curved body) during zebrafish development (Fig. 3B) (6).

We also confirmed that the viral 2a peptide (V2a) could be used for polycistronic expression (Fig. 3C). Success in these preliminary experiments motivated us to generate transgenic fish that allow the conditional expression, via heat shock, of N-terminally tagged Apobec2a,2b (hsp70: zapobec2wt), mutant Apobec2a,2b (harboring alanine substitutions of a cysteine involved in the coordination of zinc) (hsp70: zapobec2mut) (34), or human APOBEC2 (hsp70: hAPOBEC2) (Fig. 4A). Also included in these transgenic constructs was a tuba1a: gfp expression cassette, which has been shown to label MGPCs after injury and facilitates transgenic screening (Fig. 4A) (18). Analyses of uninjured adult transgenic fish retinas (in the absence of heat shock) revealed basal GFP expres-
sion in a subgroup of cells in the inner nuclear layer (INL) and the retinal ganglion cell layer (Fig. 3, D–F). Similar to wild type fish, they showed minimal cellular proliferation in the uninjured adult retina (Fig. 3, G–I). After injury (in the absence of heat shock), these transgenic fish showed phenotypes highly similar to previously characterized 1016 tuba1a:gfp transgenic fish, with induced GFP expression and proliferation at the site of injury (Fig. 3, D–I).

The ability of these transgenic fish to induce the expression of their respective transgenes following heat shock was demon-
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FIGURE 4. Induction of the zapobec2wt and hAPOBEC2 transgenes, but not the zapobec2mut transgene, rescues regeneration following knockdown of endogenous Apobec2a,2b. A, graphic outlining the hsp70:zapobec2wt, hsp70:zapobec2mut, and hsp70:hAPOBEC2 transgenes. B, quantifications of injury-dependent ascl1a mRNA expression levels (qPCR) or PCNA + cells (C) per injury at 2 dpi following control or endogenous Apobec2a,2b knockdown in combination with heat shock to induce the expression of transgenes. B, ascl1a mRNA levels were normalized to the expression of gapdh and compared with the value of the control MO sample for their respective transgenic line, which was given a value of 1. Data represent means ± S.D. (n = 3 individual cDNA sets). C, PCNA quantifications were normalized to the level in the wild type control MO sample, which was given a value of 1. Data represent means ± S.D. (error bars) (n = 3 independent fish). D, representative images from injured retinas treated with apobec2a,2b lissamine-tagged MO in the indicated fish lines and quantified in C. Arrowheads delineate PCNA +, lissamine + cells within the INL. Scale bar, 50 μm. *p < 0.04132. ONL, outer nuclear layer; GCL, ganglion cell layer.

FIGURE 3. Analyses of feasibility, transgene expression, and proliferation in uninjured and injured transgenic fish, with and without heat shock. A, graphic depicting the makeup of the pCS2 clones used to create myc-apobec2a-V2a-gfp and flag-apobec2a-V2a-gfp mRNAs for microinjection. B, the expression of myc-apobec2b(m2b)-V2a-gfp and flag-apobec2a(V2a)-V2a-gfp mRNAs escape MO knockdown (0.125 μm), as indicated by the expression of GFP and the expression of a functional protein that rescues the curved body axis caused by Apobec2a,b knockdown (6). Scale bar, 400 μm. C, the proper functioning of the viral 2a (V2a) peptide was demonstrated after microinjection of flag-apobec2a-V2a-gfp or myc-apobec2b-V2a-gfp mRNA. Western blotting showed the proper separation of Apobec2 and GFP proteins. D–I, analyses performed on transgenic fish in the absence of heat shock. Scale bar, 50 μm. D–F, GFP immunostaining of uninjured or 4-dpi retinas shows basal and injury-dependent expression of the 1016 tuba1a:gfp expression cassette in hsp70:zapobec2wt (D), hsp70:zapobec2mut (E), and hsp70:hAPOBEC2 (F) transgenic fish. G–I, BrdU immunostaining of uninjured or 4-dpi transgenic fish retinas indicates that hsp70:zapobec2wt (G), hsp70:zapobec2mut (H), and hsp70:hAPOBEC2 (I) transgenic fish retinas show limited basal cellular proliferation in the uninjured retina and induced injury-dependent levels of cellular proliferation at 4 dpi. Fish were given a pulse of BrdU 3 h prior to harvest. J–L, real-time PCR quantifications were carried out to measure basal and 4 h post-heats shock (4hphs) levels of apobec2a (J), apobec2b (K), and APOBEC2 (L) in retinas isolated from the indicated fish backgrounds. The apobec2a and apobec2b primers recognize both endogenous and exogenous mRNAs. The expression levels of apobec2a and apobec2b were normalized to the expression of gapdh and compared with the basal levels of wild type (wt) fish, which were given a value of 1. The expression levels of APOBEC2 were normalized to the expression of gapdh and compared with its basal level in each respective line, which was given a value of 1. Data represent means ± S.D. (error bars) (n = 3 individual cDNA sets). M, heat shock induced the production of transgenic proteins, as measured by immunostaining. Scale bar, 50 μm. N, 4 days of heat shock did not induce proliferation in uninjured zapobec2wt transgenic fish, demonstrating that although Apobec2a,2b are required for retina regeneration, they are not sufficient. Fish were given a pulse of BrdU 3 h prior to harvest. Scale bar, 50 μm. O, real-time PCR quantifications of basal and injury-dependent (with heat shock) ascl1a expression. The expression levels of ascl1a were normalized to the expression of gapdh and compared with the basal levels of wild type (wt) fish, which was given a value of 1. Data represent means ± S.D. (n = 3 individual cDNA sets). P, real-time PCR quantifications of injury-dependent ascl1a expression at 2 dpi following control or Apobec2a,2b knockdown. ascl1a mRNA levels were normalized to the expression of gapdh and compared with the value of the control MO, which was given a value of 1. Data represent means ± S.D. (n = 3 individual cDNA sets). *p < 0.03088. BF, bright field; L, line; ONL, outer nuclear layer; GCL, ganglion cell layer.
An important role for zinc coordination by Apobec2 is also supported by experiments using apobec2a,2b MO to knock down endogenous Apobec2a,2b after injury. For these experiments, we identified the lowest concentration of apobec2a,2b MO that consistently reduced the level of injury-dependent ascl1a induction with heat shock (Fig. 3, P). Rescue experiments were then performed to determine whether transgenic expression of Apobec2a,2b, mutant Apobec2a,2b, or APOBEC2 could rescue the previously characterized Apobec2a,2b knockdown phenotype that consisted of diminished ascl1a expression and reduced MGPC proliferation (7). As expected, heat shock of hsp70:zapobec2wt transgenic fish rescued ascl1a expression and MGPC proliferation after knockdown of endogenous Apobec2a,2b (Fig. 4, B–D). Experiments using hsp70: hAPOBEC2 transgenic fish showed similar results (Fig. 4, B–D), suggesting that Apobec2 function is evolutionarily conserved.

On the other hand, hsp70:zapobec2mut transgenic fish were unable to rescue retina regeneration following Apobec2a,2b knockdown, again suggesting that the conserved zinc-binding domains of Apobec2a,2b are essential for their function (Fig. 4, B–D).

Yeast Two-hybrid Screens Identify Apobec2-interacting Proteins and Suggest That Apobec2a,2b Proteins Do Not Oligomerize—With little evidence to support a role for Apobec2 proteins as cytosine deaminases, we hypothesized that the hsp70:zapobec2mut transgenic fish failed to rescue regeneration due to the disruption of a protein-protein interaction interface that is essential for proper regeneration. To identify Apobec2-interacting proteins, specifically within the context of a proliferating MGPC, a full-length normalized yeast two-hybrid library was generated using RNA isolated from 4-dpi MGPCs, FACS-sorted from 1016 tuba1a:gfp transgenic fish.
fish (16). Using this library (pVP16 4-dpi 1016 tuba1agfp), yeast two-hybrid screens were carried out for Apobec2a- and Apobec2b-interacting proteins.

Interestingly, these screens suggested that Apobec2a and Apobec2b do not form homo- or hetero-oligomers. This result was confirmed through yeast two-hybrid assays (Fig. 5, A and B), bringing into question models proposing a role for Apobec2 dimerization and tetramerization (34). However, these screens did identify Ubc9, Toporsa, and Pou6f2 as novel Apobec2a,2b-interacting partners (Fig. 5, C and D). Furthermore, yeast two-hybrid analyses using APOBEC2 indicated that these protein interactions are conserved between fish and mammals (Fig. 5E).
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Basal and injury-dependent expression analyses of *ube2i* (the gene that encodes for Ubc9), *toporsa*, and *pou6f2* indicated that although all of these genes are expressed basally in MG, only the expression of *ube2i* is regulated with injury, showing almost a 3-fold induction at 4 dpi (Fig. 5, F–I).

It is intriguing that each of these interacting proteins plays a role in retinal development or physiology: Ubc9, the only known E2 SUMO ligase, has been shown to control retinal progenitor cell cycle exit during development (36); TOPORS is an E3 SUMO ligase, and TOPORS mutations cause retinitis pigmentosa (37); and POU6F2 is a homeodomain transcription factor associated with retinal differentiation (38). These reports support the hypothesis that the interaction of these proteins with Apobec2 plays an important role during retina regeneration.

Next, we investigated whether mutations disrupting the Apobec2 zinc-binding domain (as done in the transgenic rescue experiments outlined above) would influence the ability of Apobec2 proteins to interact with Ubc9, Toporsa, or Pou6F2, as hypothesized previously. Surprisingly, yeast two-hybrid analyses of these mutant Apobec2 proteins demonstrated that perturbation of their zinc-binding domains did not diminish these interactions appreciably (Fig. 5, J–L), although moderately subtle changes in affinity undetectable by this method may exist. This suggests that a conserved zinc-binding domain is essential for a different aspect of Apobec2 function, possibly a function acquired through one of these protein interactions. Because of this, we sought to characterize these interactions to identify their potential outcomes.

Apobec2 Proteins Are Sumoylated at Their N Termini by Ubc9 and Toporsa—Because two of the Apobec2-interacting proteins regulate protein sumoylation, predictive analyses were performed, which identified putative sumoylation sites localized to the N termini of Apobec2a,2b and APOBEC2. Although sumoylation was predicted to occur at the N termini of each of these proteins, the lysines thought to be sumoylated are not conserved (Fig. 6A). Consistent with the possibility that Ubc9 and Toporsa regulate Apobec2 sumoylation at these sites, N-terminal truncations (T) of Apobec2a,2b and APOBEC2 showed diminished binding to Ubc9 and Toporsa relative to their full-length (FL) forms (Fig. 6, B and C).

To determine whether Apobec2 proteins have the potential to be modified by N-terminal sumoylation, bacterial sumoylation assays were performed in which Apobec2 proteins were expressed alone or in combination with components of the sumoylation machinery: an E1 SUMO ligase (a fusion of mouse Aos1 and Uba2) (39), an E2 SUMO ligase (*Xenopus* Ubc9), an E3 ligase (zebrafish Toporsa), and hSUMO1 (25) (Fig. 7A). Interestingly, expression of Apobec2a,2b or APOBEC2 in conjunction with components of the sumoylation machinery led to increases in their molecular weights concordant with the mass of hSUMO1 (11.5 kDa) (Fig. 7, B–D). This pattern, indicative of protein sumoylation, was stimulated with inclusion of Toporsa (Fig. 7, B–D). Each Apobec2 protein showed multiple shifts of size, indicating that each can be sumoylated at more than one site because Sumo1 proteins cannot polymerize. Importantly, these bands had corresponding bands that were positive for SUMO1 staining (Fig. 6, D–F). As expected, N-terminal truncations of Apobec2 proteins diminished their sumoylation (Fig. 7, E–G). Furthermore, sumoylation assays performed using a catalytic mutant of Ubc9 (incapable of sumoylation) completely abolished these sumoylation-related banding patterns (Fig. 7, H–J) (23). These results show that Apobec2a,2b and APOBEC2 can be sumoylated by Ubc9 and that Toporsa stimulates this sumoylation.

N-terminal Sumoylation of Apobec2 Proteins Can Regulate Their Subcellular Localization—Protein sumoylation has been shown to regulate a number of processes, including protein subcellular localization, function, and stability (40). To determine whether the N-terminal sumoylation of Apobec2 proteins regulates their subcellular localization, HEK293 cells were transfected with constructs encoding non-sumoylated and sumoylated GFP-tagged (C-terminal) Apobec2 proteins. Although GFP alone was equally abundant in the nucleus and cytoplasm (Fig. 7, K–M), GFP fusions of Apobec2a,2b and APOBEC2, although present in the nucleus, were enriched in the cytoplasm (Fig. 7, L–P). Fusion of an N-terminal SUMO1 to these Apobec2 proteins greatly stimulated this nuclear exclusion (Fig. 7, L–P).

We hypothesized that the cytoplasmic enrichment of GFP-tagged Apobec2 proteins (minus the N-terminal SUMO1 fusion) was partially due to their N-terminal sumoylation that occurred after cellular expression. Consistent with this hypothesis, N-terminally truncated Apobec2 proteins showed less nuclear exclusion than their full-length forms (Fig. 7, L and M). Importantly, the N-terminal regions removed from each of these proteins did not contain any evident nuclear import or export signal. Furthermore, mutation of a putative sumoylation site within the N terminus of APOBEC2 (K4R) showed a similar effect (Fig. 7, L and N). These results strongly suggest that N-terminal sumoylation of Apobec2 proteins can regulate their subcellular localization.

We next investigated whether this N-terminal sumoylation and subsequent cytoplasmic retention of Apobec2 proteins could regulate the subcellular localization of Apobec2-interacting proteins. Ubc9 and Pou6F2 fused N-terminally to mCherry demonstrated nuclear retention in HEK293 cells (Fig. 8, A–D). This nuclear

FIGURE 6. Ubc9 and Toporsa interact with the N termini of Apobec2 proteins and facilitate their sumoylation, and Apobec2s interact with the C terminus of Pou6F2. A, ClustalW alignments of the N termini of zebrafish Apobec2a, zebrafish Apobec2b, and human APOBEC2. Lysines (K) that are predicted to be sumoylated are highlighted. B, C, yeast two-hybrid assays demonstrate that the interaction between N-terminally truncated (T) Apobec2 proteins and Ubc9 (B) or Toporsa (C) is diminished relative to the full-length (FL) Apobec2 proteins. D–F, the banding patterns seen in the Apobec2 bacterial sumoylation assays align with banding of hSUMO1 staining. G and H, yeast two-hybrid assays indicate that Apobec2 proteins specifically interact with the portion of the Pou6F2 DBD encoded in exon 9 (G) and that truncation of Apobec2 proteins does not perturb their binding to Pou6F2 (H). I, graphic describing the plasmids used in the Pou6F2 bacterial sumoylation assays. J, Pou6F2 bacterial sumoylation assays indicate that although the C terminus of Pou6F2 can be sumoylated, Apobec2 proteins do not impact this sumoylation in a positive or negative manner. K, ClustalW alignments of the Pou6F2 DBD encoded in exon 9 with that of Pou6F1. L, graphic describing the plasmids used in the Pou6F1 sumoylation assays.
FIGURE 7. N-terminal sumoylation of Apobec2 proteins, facilitated by Ubc9 and Toporsa, stimulates their nuclear exclusion.

A, graphic describing the plasmids used in the Apobec2 bacterial sumoylation assays. B–J, anti-GST Western blots following Apobec2 bacterial sumoylation assays. B–D, co-expression with Ubc9 increases the mass of a portion of APOBEC2 (B), Apobec2a (C), and Apobec2b (D), indicative of sumoylation. This increased mass is stimulated with co-expression of Toporsa. The presence of multiple bands suggests that these Apobec2s are sumoylated at more than one site. E–G, N-terminal truncation of APOBEC2 (E), Apobec2a (F), and Apobec2b (G) precludes this banding pattern. H–J, the catalytic activity of Ubc9 is necessary for the increased Apobec mass because it is not observed when a catalytically dead mutant of Ubc9 (C93A) is used in the assay.

K and N–P, representative images demonstrating the subcellular localization of GFP (K) and human APOBEC2 (N), Apobec2a (O), or Apobec2b C-terminally tagged with GFP (P) in the absence (top) or presence of an N-terminal fusion to SUMO1 (bottom in K, O, and P; middle in N). The bottom panel of N shows a representative image demonstrating the subcellular localization of C-terminally tagged APOBEC2 harboring a mutation (K4R) preventing its sumoylation at this site. L and M, quantifications of the subcellular localization of the indicated fusion proteins. Experiments with APOBEC2 (L) were performed at 37 °C, and those of Apobec2a/2b (M) were performed at 28 °C. Densitometric means of cytoplasmic and nuclear GFP signal were calculated. The y axis represents their ratio. Scale bar, 10 μm. Data represent means ± S.D. (error bars) (n = 25). *, p < 0.002381. E1, Aos1/Uba2 fusion protein; E2, Ubc9; S1, SUMO1; E3, Toporsa; T, truncation; hA2, APOBEC2; hA2M, APOBEC2(K4R); zA2a, zebrafish Apobec2a; zA2b, zebrafish Apobec2b.
retention was unaltered by the co-expression of APOBEC2 (C-terminal GFP tag) with or without an N-terminal SUMO1 modification (Fig. 8, A-D). Furthermore, co-expression of APOBEC2 (C-terminal GFP tag) with Ubc9 or Pou6f2 (N-terminal mCherry tag) did not stimulate the nuclear inclusion of APOBEC2 (Fig. 8, E and F). Thus, Apobec2 proteins do not regulate the subcellular localization of Ubc9 or Toporsa, and vice versa.

APOBEC2 Proteins Interact with the C Terminus of Pou6f2—POU proteins are transcription factors with positive and negative regulatory roles and are characterized by their bipartite...
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DNA binding domain (DBD) consisting of a POU domain and a homeodomain. To begin to characterize the interaction between Apobec2 proteins and Pou6f2, we cloned pou6f2. Interestingly, we found three independent transcripts, one of which includes an alternative exon (Ex) introduced between Ex6 and Ex7 (pou6f2t2) (Fig. 9A). This exon introduces a premature stop codon, preventing the translation of the DBD located in Ex8 and Ex9. The other two transcripts (pou6f2t1 and pou6f2t3), characterized previously (38), produce proteins that differ in their POU domains (Fig. 9, A and B). This difference has been shown to alter the ability of Pou6f2 to bind to the Oct-1 DNA binding consensus sequence (38).

Expression analyses of each pou6f2 transcript demonstrated basal expression in MG that is not perturbed by injury (Fig. 9, D and E). Insights to each other, pou6f2t2 was the most abundant (Fig. 9F). Interestingly, yeast two-hybrid analyses indicated that Apobec2a,2b and APOBEC2 specifically interact with the region of Pou6f2 DBD encoded in Ex9, suggesting that Apobec2 proteins can interact with proteins encoded by both the pou6f2t1 and pou6f2t2 transcripts (Fig. 6G). These interactions were highly specific because Apobec2 proteins did not interact with the DBD of Pou3f1 or Pou6f1. Comparisons of the DBDs of these POU proteins and subsequent yeast two-hybrid analyses allowed us to map the interaction of Pou6f2 and Apobec2s to the 35 C-terminal amino acids of Pou6f2, KRQALKN-TIKRLKQPEGLVPNAPMDPVDNMEEQH (Fig. 6, K–N).

Because of the interaction of Apobec2 with the sumoylation machinery, we wondered if Apobec2 might help regulate the sumoylation of Pou6f2. Truncation of Apobec2a,2b and APOBEC2 did not perturb their binding to Pou6f2, suggesting that Apobec2s could simultaneously interact with UbC9 and Pou6f2 (Fig. 6H). Predictive analyses of the Pou6f2 DBD revealed multiple putative sites of sumoylation (Fig. 9B). To test whether Apobec2a,2b and APOBEC2 could serve as E3 ligases in the potential sumoylation of Pou6f2, bacterial sumoylation assays were performed (Fig. 6, I and J). Although these results indicated that the DBD of Pou6f2 can be sumoylated, the presence of Apobec2a,2b and APOBEC2 did not affect this sumoylation (Fig. 6J). These results suggest that the interaction of Apobec2 with Pou6f2 performs an alternate function.

Apobec2 Proteins Regulate Pou6f2 Binding to DNA—Although very little is known about Pou6f2, including whether or not it acts as a transcriptional activator or repressor, previous work has demonstrated that Pou6f2t1, but not Pou6f2t2, can bind to the Oct-1 consensus sequence (38). Unfortunately, although pou6f2t2 is the most highly expressed pou6f2 transcript in the retina, little is known about the DNA binding preferences of Pou6f2t2. To verify the binding of Pou6f2t1 to the Oct-1 consensus sequence, probes were generated using Oct-1 consensus and flanking sequences found in the promoters of insm1a and lin28 (Fig. 10, A and B), two genes whose expression is rapidly activated following zebrafish retinal damage and whose products are necessary for retina regeneration (24, 41). Consistent with the previous report, Pou6f2t1 bound to probes containing the Oct-1 consensus sequence in a concentration-dependent fashion (Fig. 10, C and H).

With this foundation, we sought to determine whether Apobec2 proteins could regulate the binding of Pou6f2t1 to these Oct-1 consensus sequences. Interestingly, although Apobec2 proteins did not bind to the Oct-1 consensus sequence in the absence of Pou6f2t1 (Fig. 10D, last lane), they increased the propensity of Pou6f2t1 to bind these probes (Fig. 10, D–F, I, and J). Furthermore, this enhanced binding required an intact Apobec2 zinc-binding motif (Fig. 10, E, F, I, and J). We confirmed equal WT and mutant Apobec2 protein levels in these assays (Fig. 10, G and K). Unlike Apobec2, other soluble proteins, including GST and BSA, did not increase the DNA binding propensity of Pou6f2 (Fig. 10, D and E). Thus, Apobec2-dependent regulation of Pou6f2 DNA binding requires an intact zinc-binding domain, which may be one reason why the heat shock induction of zapobec2mut failed to rescue regeneration following knockdown of endogenous Apobec2a,2b (Fig. 4, B–D).

FIGURE 9. Multiple pou6f2 transcripts are expressed in the retina. A, schematic of the three pou6f2 transcripts (t) identified in its cloning from the retina. pou6f2t2 encodes an alternate exon (Ex), Ex9, and pou6f2t3 encodes an alternate exon, Ex7, which introduces a premature stop codon (red circle). B, protein sequence of the DNA binding domain of Pou6f2t2. The sequence in orange is encoded in Ex9 alt. Lysines (K) predicted to be sumoylated are highlighted. C, graphic of the pou6f2 gene outlining the localization of pou6f2 primers (red) used in gene expression analyses. Some primers span introns and are identified by dotted lines. The transcripts detected using each primer pair are indicated. D, RT-PCR transcript expression comparisons of quiescent MG (gfp), and 4-dpi MGPC (1016 tuba1a gfp) cell populations using the P5/P6, P7/P8, and P9/P10 primer combinations in C. E, quantification of mRNAs in D by qPCR. The expression of gfp served as the control. Data are means ± S.D. (error bars) (n = 3 individual CDNA sets). F, RT-PCR transcript expression comparisons of quiescent MG (gfp), and 4-dpi MGPC (1016 tuba1a gfp) cell populations demonstrating relative abundance comparisons of each pou6f2 transcript using the P1/P2 and P3/P4 primer combinations outlined in C. pou6f1t1 appears to be most abundant, followed by pou6f2t2 and pou6f3t3.
Interestingly, we did not observe supershifted bands in our binding assays involving Pou6f2 and Apobec2 proteins. We hypothesize that Apobec2 proteins bind to the C terminus of Pou6f2, facilitating/stabilizing a conformation of Pou6f2 that is better able to engage DNA and that this ability of Apobec2 proteins is dependent on their conserved zinc binding domains.
Our two-hybrid analyses suggest that the interaction between Apobec2 and Pou6f2 is direct and that it occurs in the absence of DNA. There are at least two explanations for this phenomenon: 1) Apobec2 remains attached to Pou6f2 until it binds to DNA, after which Apobec2 detaches, or 2) the interaction between Apobec2 and Pou6f2 is highly transient, becoming destabilized after Pou6f2 adopts an amenable DNA binding conformation. Additional work will be needed to resolve the intricacies of this interaction.

**Ubc9 and Pou6f2 Regulate MGPC Proliferation during Zebrafish Retina Regeneration**—The above data identified Ubc9 and Pou6f2 as Apobec2-interacting proteins and characterized outcomes of these interactions. Based on these interactions, we suspected that, like Apobec2a,2b, Ubc9 and Pou6f2 would regulate MGPC proliferation and retina regeneration. To test this idea, we knocked down Ubc9 or Pou6f2 in the injured retina and assayed MGPC proliferation by PCNA immunofluorescence (Fig. 11, A–D). We observed a significant reduction in PCNA+ MGPCs at 2 days postinjury following Ubc9 or Pou6f2 knockdown (Fig. 11, C and D). Although it is possible that Ubc9 and Pou6f2 act in an Apobec2-independent fashion, these results are consistent with the hypothesis that these Apobec2 interactions are essential for MGPC proliferation during retina regeneration (Fig. 11E).

**DISCUSSION**

Although phenotypes have been described following manipulations of their expression, the function of Apobec2 proteins has remained unresolved. Previously, we demonstrated that zebrafish Apobec2a,2b are required for MG activation and MGPC proliferation during zebrafish retina regeneration (7). Furthermore, we showed that their function during regeneration is independent of site-specific DNA demethylation (16).

Previous biochemical studies of mammalian Apobec2 proteins have questioned their catalytic activity (9–11) and their ability to bind large polynucleotides (3, 4). Here we show that Apobec2 proteins provide compelling evidence that they function independent of cytosine deamination. Cumulatively, data collected through studies of Apobec2 proteins have questioned their catalytic activity (9–11) and their ability to bind large polynucleotides (3, 4). Here we show that Apobec2 proteins provide compelling evidence that they function independent of cytosine deamination.

To gain further insights into what this function may be, we conditionally expressed Apobec2a,2b, Apobec2a,2b mutants with alanine substitutions of conserved cysteine zinc-binding residues, or human AP0BEC2 in injured retinas of transgenic fish following knockdown of endogenous Apobec2a,2b. These studies revealed that the essential function of Apobec2a,2b during retina regeneration is conserved with human AP0BEC2 and requires a conserved zinc binding domain. The exact reason why the zapobec2mut transgenic fish do not rescue regeneration remains unclear, although one possible explanation is its effect on Pou6f2 DNA binding, described in more detail below.

To gain additional insights into the mechanism of Apobec2a,2b we designed a novel yeast two-hybrid library using RNA isolated from MGPCs. Yeast two-hybrid screens using this library identified Ubc9, Toporsa, and Pou6f2 as conserved Apobec2-interacting proteins. Remarkably, each of these interacting proteins has previously been shown to play a role in retinal development or physiology (36–38).
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We were surprised to find no indication of Apobec2a,2b oligomerization in our yeast two-hybrid assays. The possibility that Apobec2 proteins oligomerize was suggested by analysis of the crystal structure of N-terminally truncated APOBEC2, and this oligomerization was predicted to augment its ability to bind large polynucleotides (34). Other studies using purified Apobec2 seem to support a model of oligomerization (4, 6). In contrast to these reports, an NMR structure of full-length APOBEC2 indicated that it is a monomer in solution and that the N-terminal region removed in the crystal structure occupies the region predicted to be the oligomerization surface (35). Although Apobec2 proteins may have the ability to oligomerize under certain in vitro conditions, our data suggest that Apobec2a and Apobec2b do not oligomerize in vivo, at least in yeast. Further work will be needed to clarify these contradictions.

We next sought to characterize the conserved Apobec2 protein interactions identified in our yeast two-hybrid analyses and began to do so through a number of methods, including bacterial sumoylation assays, analyses of subcellular localization, and DNA binding assays. However, because of the immense scope of the endeavor, we do not describe here a detailed exploration of the function of these interactions within MG and MGPCs during zebrafish retina regeneration but rather provide interesting and definite insights into these interactions (described below) that can serve as a springboard for future more in depth studies.

We demonstrate that Ubc9 and Toporsa interact with the N termini of Apobec2 proteins and facilitate their sumoylation. In addition, we show clear evidence that the N-terminal sumoylation of Apobec2 proteins can stimulate their nuclear exclusion, providing context for at least one purpose of this sumoylation. Other functions for this sumoylation are possible and may include changing the stability of Apobec2 or enhancing/diminishing its ability to interact with other macromolecules. The possible sumoylation of Apobec2 proteins by other SUMO proteins remains to be analyzed, and these modifications may perform additional roles; moreover, the interaction between Apobec2 proteins and Ubc9 or Toporsa may not be limited to sumoylation. Indeed, Ubc9 has been shown to perform sumoylation-independent functions, such as a role in transcriptional regulation (42). In addition to its role as an E3 SUMO ligase, Toporsa, a multidomain protein, has been shown to function as an E3 ubiquitin ligase (43) and may have other uncharacterized functions.

Studies of the interaction between Apobec2 proteins and Pou6f2 indicate that this interaction is occurring between a non-N-terminal region of Apobec2 proteins and the C terminus of Pou6f2. POU proteins have been shown to be both positive and negative regulators of gene expression (44). Interestingly, the expression of Pou6f2 during development is associated with the differentiation of retinal cells (38). Analyses of its DNA binding have identified a preference for sequences containing (A/T)AAT; in particular, it was found that Pou6f2t1 binds to the Oct-1 consensus sequence ATGC(A/T)AAT (38). We demonstrate here that the interaction between Apobec2a,2b and Pou6f2t1 stimulates binding of Pou6f2t1 to the Oct-1 consensus sequence. Although searching promoters for regions containing (A/T)AAT probably is not sufficiently stringent, a superficial analysis of genes recently shown to be necessary for the activation of MG and the proliferation of MGPCs identified the Oct-1 consensus sequence in the promoters of lin28, insm1a, and apobec2a (7, 24, 41). It remains to be seen whether or not the interaction between Apobec2a,2b and Pou6f2t2, whose expression is the highest of the pou6f2 transcripts in MG and MGPCs, also enhances its DNA binding. Furthermore, a complete understanding of the gene expression programs controlled by Pou6f2, with and without injury, remains to be explored.

After identifying these Apobec2-interacting proteins and the outcomes of their interactions, we were curious to assess whether or not these interactions influence MG activation and MGPC proliferation. To begin to address this question, we performed in vivo knockdown experiments of Ubc9 and Pou6f2 during retina regeneration and found that each is necessary for MGPC proliferation. Although these results fall short of clearly delineating the role of these Apobec2 interactions in vivo, they are consistent with the hypothesis that these interactions play an important role during zebrafish retina regeneration.

Finally, we provide a working model of the cytosine deaminase-independent function of Apobec2a,2b proteins during MG activation and the generation of MGPCs (Fig. 11E). In this model, MG possess basal levels of Ubc9, Toporsa, Pou6f2, and Apobec2a,2b. In the absence of injury, Apobec2a,2b are excluded from the nucleus via N-terminal sumoylation facilitated by Ubc9 and Toporsa, preventing the interaction between Apobec2a,2b and Pou6f2. After injury, the expression of apobec2a,2b is induced (7), resulting in high levels of Apobec2a,2b, some of which are able to enter the nucleus, escape sumoylation, and interact with the C terminus of Pou6f2, enhancing its propensity to bind DNA. Pou6f2-DNA interactions regulate a gene expression program that stimulates MG activation and the proliferation of MGPCs. Moreover, we hypothesize that a similar model functions during optic nerve regeneration because we have previously demonstrated that Apobec2a,2b are required for zebrafish axonal regeneration (7).

In all, this work adds greatly to the understanding of Apobec2 proteins and their conserved function and opens up multiple avenues for future research.

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