Chapter 3

Combining Zebrafish and Mouse Models to Test the Function of Deubiquitinating Enzyme (Dubs) Genes in Development: Role of USP45 in the Retina

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Abstract

Ubiquitination is a dynamic and reversible posttranslational modification. Much effort has been devoted to characterize the function of ubiquitin pathway genes in the cell context, but much less is known on their functional role in the development and maintenance of organs and tissues in the organism. In fact, several ubiquitin ligases and deubiquitinating enzymes (DUBs) are implicated in human pathological disorders, from cancer to neurodegeneration. The aim of our work is to explore the relevance of DUBs in retinal function in health and disease, particularly since some genes related to the ubiquitin or SUMO pathways cause retinal dystrophies, a group of rare diseases that affect 1:3000 individuals worldwide. We propose zebrafish as an extremely useful and informative genetic model to characterize the function of any particular gene in the retina, and thus complement the expression data from mouse. A preliminary characterization of gene expression in mouse retinas (RT-PCR and in situ hybridization) was performed to select particularly interesting genes, and we later replicated the experiments in zebrafish. As a proof of concept, we selected *ups45* to be *knocked down* by morpholino injection in zebrafish embryos. Morphant phenotypic analysis showed moderate to severe eye morphological defects, with a defective formation of the retinal structures, therefore supporting the relevance of DUBs in the formation and differentiation of the vertebrate retina, and suggesting that genes encoding ubiquitin pathway enzymes are good candidates for causing hereditary retinal dystrophies.

Key words Deubiquitinating enzymes, USP45, Retina, Morpholino knockdown, Zebrafish Animal Model, Neurodegeneration

1 Introduction

More than 200 genes have been associated with different types of hereditary retinal degeneration (RetNet: https://sph.uth.edu/retnet/). Among them, mutations in genes directly involved in ubiquitin and SUMO pathways, such as *KLHL7* and *TOPORS*, have been identified. Despite the increase in the number of causative genes and mutations, the main challenge in the field is to assess their function in the retina.

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Animal models have played an essential crucial role to discover gene function and test therapeutic approaches for retinal dystrophy genes [1]. The mouse (*Mus musculus*) has been the model per excellence for years because of the easy genetic manipulation, housing and handling, and the conservation of orthologous genes compared to humans [2]. However, the generation of a genetically modified mouse model is rather costly in terms of effort, time, and budget, and there is always the risk that the model does not mimic the human phenotype, as shown for several genes [3–6]. In the last decade, zebrafish (*Danio rerio*) has emerged as an extremely useful tool to rapidly assess candidate genes and mutations at the morphological level, particularly in genes that are involved in organ/tissue development [7], using large numbers of animals that enable confident statistical analyses. Besides, the cone-rich retina of zebrafish is similar to the human retina, and photoreceptor function and phototransduction pathways are highly conserved.

Here we describe some standard techniques to perform an easy and rapid screening of gene expression in the retina of mouse and zebrafish. The information gathered using both models might be extremely useful not only to identify new candidate genes and pathogenic mutations in human but also to evaluate the possibility of generating a costly modified animal model for long-term in vivo studies, ensuring that the gene is expressed in the correct tissue/cell type and shows an altered phenotype.

We have explored the possible role of two DUB genes (*Usp45* and *Usp53*) in the mouse retina, by quantification of the expression levels by real-time reverse-transcriptase PCR (qRT-PCR), determination of their spatial expression pattern by mRNA in situ hybridization in mouse as well as in zebrafish retinas, and finally, by phenotype analysis of gene knockdown in zebrafish embryos. As a proof of principle we focused on *usp45*, whose morphant (morpholino knockdown) resulted in disruption of the normal development of the retina, but also showed a severe reduction of the body size, with an anomalous development of the notochord and nervous system. Therefore, USP45 may be required for the normal development of the nervous system and, particularly, for retinal development.

Overall, this type of complementary phenotypic analysis combining several animal models can shed light on the physiological function of the ubiquitin/proteasome and other post-translational modification (sumoylation) pathways in health and disease.

2 Materials and Solutions

2.1 Dissection of Mouse Retinas and Preparation of Mouse Eye Sections

- Razor blades, scissors, and forceps.
- Stereomicroscope.
- Cryostat.
- Poly-lysine treated slides.
- Petri dishes (for acrylamide embedding).

2.2 RNA Isolation, cDNA Synthesis, qPCR, and PCR

- Polytron or similar blender for tissue samples.
- Agarose and electrophoresis system.
- LightCycler® 480 SYBR green (Roche Diagnostics, Indianapolis, IN) or similar.
- 96 or 384 well plates.
- Thermocycler.
- ImageJ/Fiji software.

2.3 In Situ Hybridization on Mouse and Zebrafish Retinal Cryosections

- Thermocycler.
- LB agar plates with 100 μg/ml ampicillin, 0.1 mM IPTG, and 40 μg/ml X-GAL.
- LB liquid medium.
- Mini-quick spin columns (Roche Diagnostics, Indianapolis, IN).
- Heat incubators at 68, 55, and 37 °C.
- Shaker.
- Microscope and camera.
- A nontransparent box to create the wet chamber for hybridizations.
- Hydrophobic pen, special for high temperatures.

2.4 Zebrafish Embryo Collection, Handling and Fixation, and Morpholino Microinjection

- Adult male and female fish.
- Fish tank with plastic separators.
- Petri dishes.
- Heat Incubator at 28 °C.
- Plastic mold.
- Microinjection equipment, needles, microscope.
- Mineral oil and micrometer.
- Morpholino antisense oligonucleotide (MO).

2.5 Solutions

- Acrylamide monomer solution (for 50 ml): 4.2 g Acrylamide, 0.007 g Bis-acrylamide, 350 μl TEMED, 5 ml 10× PBS, double distilled H2O up to 50 ml.

- *Acrylamide embedding solution*: 50 µl of 10% APS in H_2O added to 10 ml of acrylamide monomer solution.
- 50× Denhardt's solution: 1% (w/v) Ficoll 400, 1% (w/v) Polyvinylpyrrolidone, 1% (w/v) Bovine serum albumin (Fraction V). Dissolved in DEPC-H_2O.
- Prehybridization solution for mouse cryosections: 42% (v/v) Formamide, 10% (w/v) Dextran sulfate, 1× Denhardt's Solution, 0.9 M NaCl, 0.1 M Tris–HCl (pH 8.0), 5 mM EDTA (pH 8.0), 10 mM NaH_2PO_4, 1 mg/ml yeast tRNA in DEPC-treated double distilled water.
- 20× SSC: 3 M NaCl, 300 mM Sodium Citrate, 800 ml of double distilled H_2O. Adjust pH to 7.0 using HCl. Adjust volume to 1 l with double distilled H_2O. Autoclave.
- *NTE*: 0.5 M NaCl, 10 mM Tris–HCl (pH 8.0), 5 mM EDTA (pH 8.0) for this and the next buffers, (*see* **Note 1**).
- *Buffer 1*: 100 mM Tris–HCl (pH 7.5), 150 mM NaCl.
- *Buffer 2*: 100 mM Tris (pH 9.5), 100 mM NaCl.
- *Buffer 3*: 100 mM Tris (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$.
- *E3 medium*: 300 mM NaCl, 10 mM KCl, 20 mM $CaCl_2$, 20 mM $MgSO_4$ in distilled water.
- *Hybridization solution for zebrafish cryosections*: 50% formamide, 1× Denhardt's solution, 10% dextran sulfate, 0.9 M NaCl, 100 mM Tris–HCl (pH 8.0), 5 mM EDTA (pH 8.0), 10 mM NaH_2PO_4, 1 mg/ml yeast tRNA.
- *Wash solution*: 50% formamide, 1× SSC, 0.1% Tween-20.
- *MABT solution*: 100 mM $C_4H_4O_4$ (pH 7.5), 150 mM NaCl, 0.1% Tween-20.
- *Blocking Buffer*: 100 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% BSA, 0.1% Triton X-100.
- *Alkaline phosphatase staining solution*: 100 mM Tris–HCl (pH 9.5), 150 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20.
- *Hematoxylin stock solution*: 1% w/v in alcohol 99%. Let it rest (slow oxidation) during 2 weeks before use. Before use, dilute 1:1 in distilled water and filter (paper filter) to avoid precipitates.
- *Eosin solution*: 1% w/v in distilled water. Filter (paper filter) before use.

3 Methods

3.1 Dissection of Mouse Retinas for RNA Isolation

1. Sacrifice the number of P60 adult mice required (60 postnatal days is a standard age for fully differentiated retina) (*see* **Note 2**).

2. Hold the whole eye with the forceps, make a small cut on the cornea with a razor blade and remove the lens (Fig. 1a).
3. Pull out the neural retina (pink tissue) trying to leave the retinal pigmented epithelium (RPE) out (Fig. 1a), and transfer the retina to a 1.5 ml tube (two retinas per tube, use different tubes for different animals) and freeze immediately in liquid nitrogen. Keep them at −80 °C until use.
4. To obtain the retinal RNA, disrupt and homogenize the tissue in the buffer provided in the kit for tissue RNA isolation, using a polytron or a similar electronic blender.
5. Run 2–3 μl in a 1% w/v agarose/TBE gel to assess RNA quality.
6. Perform the cDNA synthesis reaction, 1 μg of RNA per tube, using a kit that allows to mix an oligodT primer and random hexamers or decamers to ensure the complete coverage of the gene of interest (strongly suggested for large genes).
7. Depending on the protocol and the initial mRNA purity and concentration, the cDNA should be diluted between 1:2 and 1:10 times in H_2O before the qPCR.
8. Prepare the qPCR reaction following the manufacturer's instructions (e.g., Lightcycler® 480 SYBR Green Master protocol) (*see* **Note 3**).

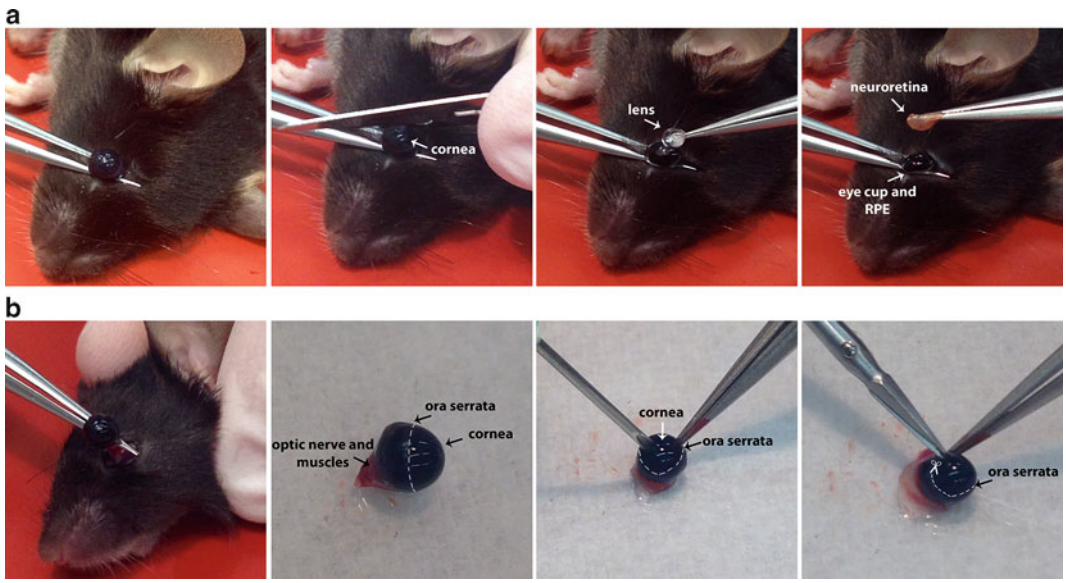

Fig. 1 Procedure for the dissection of the mouse neuroretina and eyecup. (**a**) Images illustrating four steps during the dissection of the mouse neuroretina for the purification of retinal RNA (*see* Subheading 3.1 for a complete description). (**b**) Images illustrating the dissection of the whole eyecup for the embedding and obtention of retina slides (see Subheading 3.2 for a complete description)

3.2 Dissection of Mouse Retinas for Cryosections

1. Enucleate the eye (Fig. 1b) and transfer it to a Petri dish with some drops of 4% PFA in 1× PBS.
2. Perform a small hole or cut in the cornea with a needle (Fig. 1b) to allow the PFA enter into the eye for 10 min.
3. Under the stereomicroscope and using the iridectomy scissors and forceps, cut around the iris to remove the cornea (Fig. 1b).
4. Fix the eyecups for 2 h in 4% PFA at RT.
5. Wash three times in 1× PBS for 15 min each at RT.
6. Embed the eyecups to avoid crystal formation (using either sucrose or acrylamide embedding).

3.2.1 Sucrose Embedding

- Transfer the eyecup to a tube containing a 10% w/v sucrose solution in PBS for 15 min or until the eyecup reaches the bottom at 4 °C. Repeat this step twice.
- Continue the cryoprotection by moving the eyecup to a 20% w/v sucrose solution in PBS for 15 min or until the eyecup settles down at 4 °C. Repeat this step twice.
- Finally, place the eyecup in a tube with 30% w/v sucrose solution in PBS and incubate it o/n at 4 °C.
- Proceed to **step 7**.

3.2.2 Acrylamide Embedding

- Infiltrate the eyecup in acrylamide monomer o/n at 4 °C.
- Polymerize 0.5 ml of fresh prepared acrylamide monomer in a 1.5 ml tube.
- Transfer the infiltrated eyecup on the acrylamide pad of the previous step (one eyecup per tube) and fill the tube with fresh embedding solution.
- Allow the polymerization of the acrylamide in ice (approx: 40–50 min).
- Under a stereomicroscope, remove the acrylamide surrounding the eyecup using a razor blade and iridectomy scissors on a Petri dish with double distilled H_2O.
- Proceed to **step 7**.

7. Cast the embedded tissue in a cryostat mold with OCT and freeze slowly in liquid nitrogen.
8. Using a cryostat, cut the blocks into 10–20 μm sections at −17 °C/−20 °C and place them on poly-lysine treated glass slides. Keep at −80 °C until used.

3.3 Cloning of the Riboprobe

1. Amplify with a standard Taq pol the desired region (between 400 and 800 bp in size) using gDNA or cDNA, depending on whether multiple exons are included. Check the PCR by gel electrophoresis and purify.

2. Ligate the fragment into the pGEM®-T vector following the manufacturer's protocol, and transform by heat shock in DH5α *E. coli* cells.
3. Plate onto LB supplemented with ampicillin/IPTG/XGal plates and incubate overnight at 37 °C for antibiotic and color selection
4. Pick six white colonies and grow o/n in 3 ml LB containing ampicillin (100 μg/ml) (*see* **Notes 4** and **5**).
5. Perform colony screening by plasmid minipreparation using 1.5 ml of the culture. Analyze if the plasmids are recombinant by restriction digestion.
6. Dilute the plasmid DNA down to 10 ng/μl. Use 1 μl to perform the PCR (final volume 50 μl) using the M13 primer (GTAAAACGACGGCCAGT) combined with the forward or reverse primer used in **step 1** (for each miniprep) (*see* **Note 6**).
7. Select two clones (one in the antisense and the other in the sense direction) for each gene, and sequence them for verification.

3.4 Generation of the Riboprobe

From this step onwards all the reagents must be RNase-free (*see* **Note 7**).

1. If PCRs from **step 7** (Subheading 3.3) produced a good yield, the PCR reaction could be directly used for the generation of the riboprobes.
2. Mix 12 μl of the PCR product with 2 μl of T7 RNA polymerase, 2 μl of rNTP mix labeled with digoxigenin, 1 μl DTT (0.1 M), 1 μl RNase Inhibitor, and 2 μl of the T7 pol buffer. Incubate 2–3 h at 37 °C.
3. Add 2 μl of DNAseI and incubate 20 min at 37 °C. Separate 1 μl of the reaction for control.
4. Purify the riboprobe using mini-quick speed columns (Roche) following the manufacturer's protocol. Approximately 25–30 μl will be collected.
5. Test 1 μl of **steps 3** and **4** in a 1% w/v agarose/TBE gel.
6. If the test shows clear riboprobe production and recovery, dilute the riboprobe in 100% formamide (1:1 v/v), final concentration 50% formamide.

3.5 In Situ Hybridization on Mouse Cryosections

3.5.1 Day 1

1. Thaw the cryosections kept at –80 °C (**step 8**, Subheading 3.2) at RT for 1 h.
2. Use the hydrophobic pen (special for in situ hybridization) to surround each retina.
3. Remove OCT by washing the slides three times for 10 min in 1× PBS.
4. Incubate retinas in 2 μg/ml Proteinase K in PBS, for 20 min at 37 °C.

5. Rinse sections twice in 1× PBS for 5 min.
6. Fix retinas in 4% PFA in PBS for 20 min at RT.
7. Wash with 1× PBS.
8. Incubate 5 min in 0.1 M triethanolamine with 0.25% acetic anhydride (in PBS) at RT, followed by 5 min in 0.1 M triethanolamine with 0.5% acetic anhydride (in PBS) at RT.
9. Wash 5 min in 1× PBS at RT. Check that the hydrophobic circle drawn in **step 2** is still in a good condition. Otherwise redraw the circle.
10. Perform a prehybridization step by incubating 2–4 h in prehybridization solution at 55 °C in a nontransparent wet chamber (to avoid evaporation) (**Notes 8** and **9**).
11. Mix 150 µl of prehybridization solution with 5–10 µl of riboprobe (this is the hybridization solution).
12. Remove carefully prehybridization solution and substitute by the hybridization solution. Incubate slides o/n at 55 °C in the wet chamber.

3.5.2 Day 2

From this step onwards RNase-free conditions are not strictly required.

13. Warm 2× SSC and 2× SSC/42% formamide at 55 °C, and warm NTE, some 2× SSC and 0.2× SSC at 37 °C.
14. Wash slides 20 min in 2× SSC at 55 °C, and twice for 5 min in 2× SSC/42% formamide at 55 °C.
15. Wash three times for 5 min in NTE at 37 °C.
16. Incubate 30 min in 10 µg/ml RNaseA (in NTE) at 37 °C.
17. Rinse 15 min in NTE at 37 °C.
18. Wash twice for 15 min in 2× SSC at 37 °C, and twice for 15 min in 0.2× SSC at 37 °C.
19. Incubate 5 min in Buffer 1 at RT.
20. Block 1 h in 1% BSA + 0.1% Triton X-100 in Buffer I at RT.
21. Incubate sections o/n in Buffer I containing anti-DIG-AP (1:1000) at 4 °C.

3.5.3 Day 3

22. Wash twice for 15 min in Buffer 1 at RT, 5 min in Buffer 2 at RT, and 5 min in Buffer 3 at RT.
23. Add the BMP substrate on each slide and incubate at RT in the dark (*see* **Note 10**).
24. After 30 min, check regularly the sections under the microscope (*see* **Note 11**).
25. Stop reaction by washing with PBS, and mount using Fluoprep and a coverslide (Fig. 2, positive mRNA localization is detected in blue).

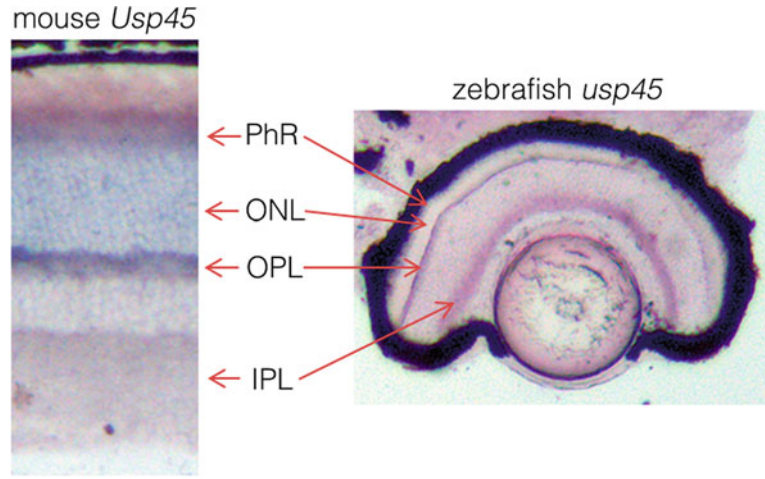

Fig. 2 Comparative *Usp45* in situ hybridizations in P60 mouse and 7 days zebrafish retinal cryosections, showing a strong correspondence of the mRNA localization in the retina of the two animal models. Of note, usp45 mRNA is found in the inner segment of the photoreceptors but also in the outer and inner plexiform layers, where most synapses occur, suggesting a role in the signal transduction pathway or in the regulation of the synaptic signal transmission rather than in photoreceptor differentiation fate. *PhR* photoreceptor cell layer, *ONL* outer nuclear layer, *OPL* outer plexiform layer, *IPL* inner plexiform layer

3.6 Zebrafish Embryo Collection, Handling and Fixation

1. Place several pairs of one male and one female adult zebrafish in a fish tank prepared for fish egg laying, but the male and female of each mating pair should be set close but separated with a plastic separator. Set a cycle of 14-h light/10-h dark cycle room for 24 h (*see* **Note 12**).
2. Next day remove the separator and allow the pair to mate. After fertilization, collect the eggs with a plastic pipette and allow them to develop in a Petri dish with 1× E3 medium.
3. Breed the eggs in an incubator at 28 °C until they reach the desired developmental stages (we obtained embryos at 12 h, 24 h, 36 h, 48 h and 72 hpf.) (*see* **Note 13**) [8].
4. Transfer the selected embryos in an Eppendorf tube. Fixation is performed with a 4% Paraformaldehyde (PFA) solution (w/v) in PBS for 2 h at room temperature.
5. Wash three times with PBS 1× for 10 min each.
6. Immerse successively the fixated embryos into 20 and 30% sucrose in PBS (w/v) solutions for 30 min each at room temperature. Finally, immerse the embryos o/n in 40% sucrose w/v at 4 °C.
7. Embed the embryos in OCT for 1 h, freeze them in liquid nitrogen, and store them at −80 °C.

3.7 Semi-Quantitative PCR

1. Add the components of the PCR to a 50 μl final volume reaction (standard reactions contain: 0.2 μM of each primer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, Taq Buffer 1×, and 1 U Taq DNA Polymerase). The sequences of the primer pairs used, including those of *β-actin*, a normalization control are listed in Table 1.
2. Mix gently the reaction components and set the tubes into a thermocycler. The standard PCR conditions used are shown in Table 2.
3. After agarose gel electrophoresis, the amplified bands are visualized and quantified using appropriate software (e.g., ImageJ/Fiji) to allow comparison between genes and developmental stages (Fig. 3).

3.8 In Situ Hybridization on Zebrafish Retinal Sections

1. Thaw the retinal sections stored at −80 °C, and let them air dry for 1 h at room temperature (RT).
2. Rinse them three times for 10 min with 1× PBS.

Table 1
Sequences and characteristics of the primer pairs used in semi-quantitative PCR and *in-situ* hybridizations

Real-Time qPCR (mouse retinas)			
Gene	**Orientation**	**Sequence (5′-3′)**	**Tm (°C)**
Usp45	Forward	AGCCTCACTGACGGCAGCG	71.5
	Reverse	AGGCTGCTTGGAAGCGATC	66.8
Usp53	Forward	GGAGTCCATGCATGACCCAGG	71.1
	Reverse	TGAACAACTGGACGGGTAGCTG	68.3
Gapdh	Forward	TGACAATGAATACGGCTACAGCAA	67.2
	Reverse	TACTCCTTGGAGGCCATGTAGG	66.1
Rho	Forward	GCCCTTCTCCAACGTCACAG	67.1
	Reverse	GCAGCTTCTTGTGCTGTACGG	67.1
In-situ Hybridization (mouse retinal cryosections)			
Gene	**Orientation**	**Sequence (5′-3′)**	**Tm (°C)**
Usp45	Forward	AGCCTCACTGACGGCAGCG	71.5
	Reverse	GACAGGACTGGACTGAGCAT	62
Usp53	Forward	CATCTGTGAGAACTGCTGGGCT	67.9
	Reverse	TGAACAACTGGACGGGTAGCTG	68.3
Rho	Forward	GCCCTTCTCCAACGTCACAG	67.1
	Reverse	GCAGCTTCTTGTGCTGTACGG	67.1

(continued)

Table 1
(continued)

Semi-quantitative PCR (zebrafish embryos)			
Gene	**Orientation**	**Sequence (5′-3′)**	**Tm (°C)**
usp45	Forward	CAGTCAGGAATTGCTGCATTACC	66.6
	Reverse	TGGGCAGCTAATGAGTCATCATG	68.1
usp53a	Forward	CTGACGCCTGCACGTCCAAG	71.6
	Reverse	AGTGAGGTCGGACTGCTCCGA	70.8
usp53b	Forward	GTCTCATGGATGATGCAGCGGA	71.7
	Reverse	TTGATACTCTGCCCACAGTTAC	60.6
β-actin	Forward	CTACAACGAGCTGCGTGTTGC	68.1
	Reverse	CGGTCAGGATCTTCATGAGGT	65.3
In-situ Hybridization (zebrafish retinal cryosections)			
Gene	**Orientation**	**Sequence (5′-3′)**	**Tm (°C)**
usp45	Forward	TCTCAGACCCACATGCTGAATG	67.4
	Reverse	GTCCACTGAGCCTCCTGCTGT	68.2
crx	Forward	CCTTCCCGAGTCCAGAGTTC	65.1
	Reverse	AAGAGCCATAGCCCTGGCTG	67.6
Control of knockdown			
Gene	**Orientation**	**Sequence (5′-3′)**	**Tm (°C)**
usp45	Forward	TCTCAGACCCACATGCTGAATG	67.4
	Reverse	CCTCCACTCTCATAGAGTCCAG	61.8

!*β-actin* was used as a normalization control

Table 2
Semi-quantitative PCR conditions

Step	Temperature (°C)	Time	
Hot start	94	3 min	×35
Denaturation	94	10 s	
Annealing	58	30 s	
Elongation	72	25 s	
Stop	12	∞	

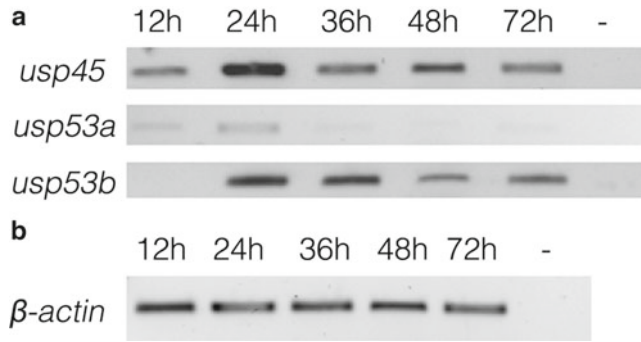

Fig. 3 Semi-quantitative expression analysis during embryonic development in zebrafish. Expression levels of (**a**) three studied genes, *usp45*, *usp53a*, and *usp53b*. There is a single *usp45* gene in zebrafish, which is expressed in the five studied embryonic developmental stages at a high level of expression. *usp53* has two highly similar paralogues in zebrafish, *usp53a* and *usp53b*. Note that *usp53a* is expressed at lower levels than *usp53b*, at the analyzed stages; (**b**) *β-actin*, used for normalization

3. Denature the riboprobes (antisense and sense) for 5 min at 68 °C and add 0.1–1 μg/ml of each one to their corresponding in situ hybridization solution. (Riboprobes are prepared as in Subheadings 3.3 and 3.4). Incubate overnight (at least 16 h) at 68 °C in a wet chamber protected from light.
4. After hybridization, wash the slides thrice for 30 min each at 68 °C in wash solution, and thrice for 30 min at RT in MABT.
5. Block them in blocking buffer for 4 h at RT.
6. Incubate o/n at 4 °C with an anti-digoxigenin-AP conjugate antibody (dilution 1:1000) in Blocking Buffer.
7. Wash the sections once in MABT for 30 min at RT, and twice for 10 min each in staining solution of alkaline phosphatase, in a shaker.
8. Incubate with freshly filtered BMP and allow the reaction to develop until a clear expression signal is obtained in the antisense hybridized sections or if staining appears in the sense sections. The reaction is stopped by washing in 1× PBS.
9. Mount the sections in fluoprep before making photographs with a camera attached to a light microscope (Fig. 2, positive mRNA localization is detected in blue).

3.9 Morpholino Microinjection in Zebrafish Embryos

1. Collect the embryos as described above, and place the embryos in chambers and align them in the same direction. To prepare the chambers set a plastic mold into a Petri dish containing 1.5% (w/v) liquid agarose with 1× E3 medium. Once the agarose is gelified, remove the plastic mold and keep it at 4 °C.
2. Turn on the air source and the microinjector and insert the needle. Pinch off the needle at the point of interest using a

microscope and a pair of sharp forceps. To calculate the volume of each microinjection, use a drop of mineral oil on a micrometer (*see* **Note 14**).

3. Mix the morpholino antisense oligonucleotide (MO) of interest (designed and synthesized by Gene Tools, *see* **Note 15**) with 0.5% phenol red, which serves as a visible marker for the injection of the solution into the embryo.
4. Microinject the MO of interest and the standard scrambled MO (negative control) into the yolk of the aligned 1- to 4-cell stage

Table 3
Injected volumes and final concentrations of MO-USP45

MO	MO injected volumes (pl)	MO final concentration (μM)
MO-USP45 (1)	65	0.036
MO-USP45 (2)	100	0.29
MO-USP45 (3)	150	1

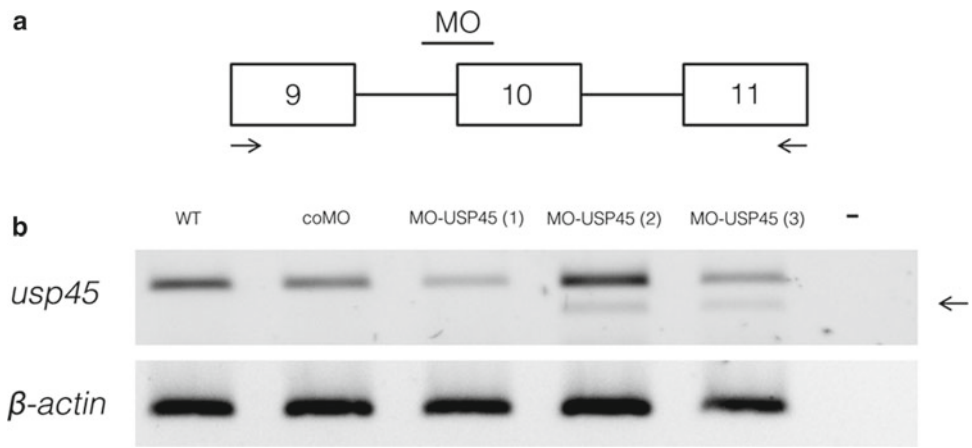

Fig. 4 Knockdown of *usp45* in zebrafish by morpholino microinjection. (**a**) The position of the morpholino in the unprocessed RNA and the primers used for PCR test are indicated. The MO targeted the acceptor splice site of intron 9 and the beginning of exon 10 of *usp45* (MO-USP45: 5′-AATGCGCTGTCAGTGAAAACACAAT-3′). A scrambled MO was used as negative control (coMO: 5′-CCTCTTACCTCAGTTACAATTTATA-3′); (**b**) Effects of the morpholino knockdown on the transcription of *usp45* detected by semi-quantitative RT-PCR, showing inhibition of intron 9 splicing (causing the introduction of a STOP codon, which in turn would result in a premature protein truncation and probably, non-sense-mediated decay of the misprocessed mRNA). Several morpholino concentrations were tested. The lowest tested concentration (0.036 μM) was the most efficient, as it knocked down *usp45* expression to 51.5% while still being compatible with viability. *β-Actin* was used for normalization. coMO: control standard scramble MO; MO-USP45 (1): 0.036 μM; MO-USP45 (2): 0.29 μM; MO-USP45 (3): 1 μM. The *arrow* indicates the band produced when exon 10 is skipped by the morpholino action

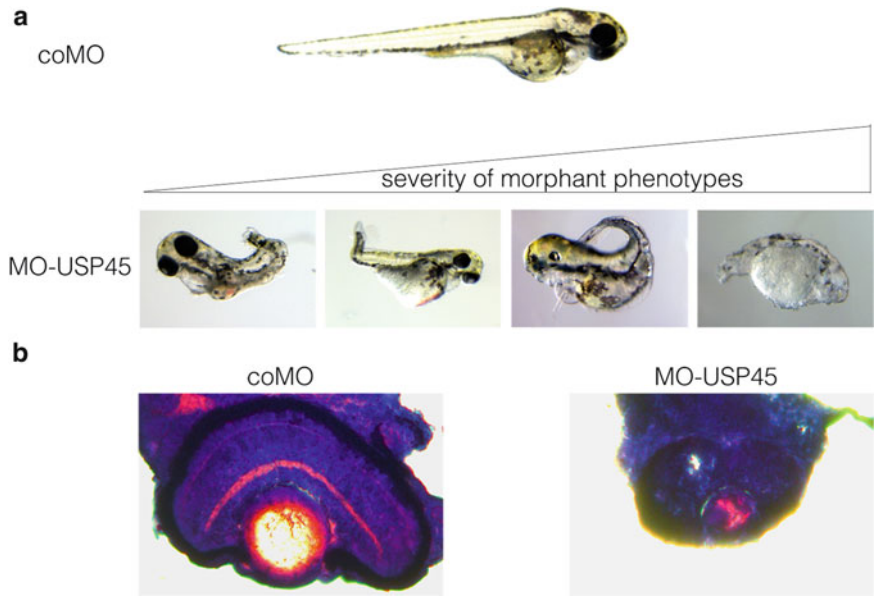

Fig. 5 (**a**) Morphant phenotypes observed in 72 hpf embryos after MO microinjection in eggs (0.036 μM). The main traits are: eye size reduction, small body size with small or no tail, and disruption in the formation of notochord (mild phenotype). 6 % of the embryos show a very severe phenotype with no eyes; (**b**) Hematoxylin and eosin stained eye sections in coMO (control embryos, injected with a standard scramble morpholino) and MO-USP45 injected embryos (72 hpf). MO-USP45 injected morphants show defects in eye formation and the lamination of the retina, with no distinguishable photoreceptors or plexiform layers (IPL and OPL) and with smaller retinas (low number of neurons), compared with the coMO retinas

embryos [9] (*see* **Note 16**). The volumes and inferred final concentrations of injected MO-USP45 are shown in Table 3.

5. Move the injected embryos to a Petri dish with 1× E3 medium and let them develop in an incubator at 28 °C until they reach the desired developmental stage (e.g., 72 h). Every day, the dead embryos should be removed and the E3 medium changed.
6. At the stage(s) of interest (e.g., 72 h), observe the morphant phenotype with a microscope. Anesthetize the embryos by immersion in a tricaine solution (4.2 % v/v in 1× E3 medium) and photograph them with a camera attached to a light microscope (Fig. 5a). After setting them back to 1× E3 medium, the embryos recover from the anesthesia.
7. Select half of the animals to perform a semi-quantitative RT-PCR assay to evaluate the knockdown effect of the *usp45* MO (Fig. 4).
8. Fix the other half of the embryos for histological morphological analysis, as described in the **step 4** (Subheading 3.6), and obtain retinal cryosections (14–16 μm width).

3.10 Hematoxylin/Eosin Staining of Zebrafish Retinal Sections

1. Thaw the retinal sections (14–16 μm width) stored at −80 °C, and let them air dry for 1 h at room temperature (RT).
2. Rinse retinal sections with 1× PBS for 10 min.
3. Stain with freshly diluted and filtered hematoxylin solution for 90 s, and wash with distilled H_2O for 10 min.
4. Stain with freshly filtered eosin solution for 4 min and 30 s, and wash quickly with distilled H_2O.
5. Mount the sections with fluoprep and take photographs with a camera attached to a light microscope.
6. Compare the phenotype qualitatively between scramble and morpholino injected animals (Fig. 5b).
7. Count and compare the number of nuclei rows in the OPL with the help of ImageJ, and compare the relative width of the retina, the outer photoreceptor segment, and the outer plexiform layers (*see* **Note 17**).

4 Notes

1. The pH of Tris buffers of these protocols must be accurate to obtain good results.
2. Due to the inter-individual differences in transcription, we suggest 3–6 animals, depending on the amount of genes to be screened and the number of replicates required for statistical significance.
3. For the qPCR, oligonucleotides to amplify fragments of around 100 bp (preferably, primers should map at different exons to prevent amplification due to genomic contamination) should be used. The annealing (melting) temperature should be close to or higher than 60 °C. Primers of control genes to normalize expression values should be also designed. All primers should be checked first to assess that they amplify a single amplicon.
4. For each gene two riboprobes, sense (negative control) and antisense (assay), are required.
5. Fragments shorter or equal to 300 bp may result in light blue or blue colonies if they are in frame.
6. This will allow the identification of the direction of the probe. If a band with the correct size is observed using the M13 and forward primers the probe is cloned in the antisense direction, while amplification using the M13 and reverse primers indicates sense probes.
7. Before starting, all tubes and stable solutions must be autoclaved twice. Bench must be clean and RNase/DNase-free filter tips are recommended. Non-autoclavable solutions must be freshly prepared and filtered (Ø 22 μm), and only used for RNA-related purposes.

8. As a wet chamber, use a nontransparent flat box, with the bottom covered by a wet paper filter. Place the slides on top, retinas facing up.
9. Never apply any solution directly on the tissue section, morphology might be damaged or the retina section might detach.
10. Use a freshly clarified BM Purple AP (BMP) substrate, either by filtering through a 0.45 μm filter or by centrifuging at maximum speed for 5 min.
11. This step might take from minutes to hours. Replace every 2 h the BMP solution to avoid precipitation. The reaction is faster at RT, but it can also proceed more slowly by incubating at 4 °C using longer incubation times.
12. Three days before mating and egg laying, zebrafish animals should be fed with dry *Artemia salina* pellets to increase the metabolism and favor egg production. Animals should be young and well fed to lay eggs. If the mating pairs do not lay eggs, or the eggs are too fragile and do not survive microinjection, buy fresh younger animals.
13. Several embryonic developmental stages should be analyzed to assess the expression of our genes of interest. Although adulthood and sexual maturity is reached at 90 days, most tissues and organs are developed during the first 72 h of the larval development.
14. For instance, an oil drop of Ø 100 μm contains 520 pl of injection material.
15. Many efficient morpholinos are directed against splice acceptor or donor sites, so that they inhibit proper mRNA splicing.
16. Several concentrations of MO-USP45 (different volumes) were microinjected in order to find the more efficient in knocking down *usp45* (the concentration is calculated as the final DNA amount per embryo).
17. Additionally, confocal microscopy for immunodetection of specific proteins and retinal markers (e.g., rhodopsin for rod photoreceptors) could also be performed for detailed analysis.

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