Expression of deubiquitinating enzyme genes in the developing mammal retina

Mariona Esquerdo-Barragán,1,2 Matthew J. Brooks,3 Vasileios Toulis,1,2,4 Anand Swaroop,3 Gemma Marfany1,2,4

1Departament de Genètica, Microbiologia i Estadística, Avda. Diagonal 643, Universitat de Barcelona, Barcelona 08028, Spain; 2Institut de Biomedicina (IBUB-IRSJD), Universitat de Barcelona, Barcelona, Spain; 3Neurobiology Neurodegeneration & Repair Laboratory, National Eye Institute, National Institutes of Health, Bethesda, MD; 4CIBERER, ISCIII, Universitat de Barcelona, Barcelona, Spain

Purpose: Genes involved in the development and differentiation of the mammalian retina are also associated with inherited retinal dystrophies (IRDs) and age-related macular degeneration. Transcriptional regulation of retinal cell differentiation has been addressed by genetic and transcriptomic studies. Much less is known about the posttranslational regulation of key regulatory proteins, although mutations in some genes involved in ubiquitination and proteostasis—E3 ligases and deubiquitinating enzymes (DUBs)—cause IRDs. This study intends to provide new data on DUB gene expression during different developmental stages of mouse and human fetal retinas.

Methods: We performed a comprehensive transcriptomic analysis of all the annotated human and mouse DUBs (87) in the developing mouse retina at several embryonic and postnatal time points compared with the transcriptome of the fetal human retina. An integrated comparison of data from transcriptomics, reported chromatin immunoprecipitation sequencing (ChIP-seq) of CRX and NRL transcription factors, and the phenotypic retinal alterations in different animal models is presented.

Results: Several DUB genes are differentially expressed during the development of the mouse and human retinas in relation to proliferation or differentiation stages. Some DUB genes appear to be distinctly expressed during the differentiation stages of rod and cone photoreceptor cells, and their expression is altered in mouse knockout models of relevant photoreceptor transcription factors. We complemented this RNA-sequencing (RNA-seq) analysis with other reported expression and phenotypic data to underscore the involvement of DUBs in cell fate decision and photoreceptor differentiation.

Conclusions: The present results highlight a short list of potential DUB candidates for retinal disorders, which require further study.

Selective degradation of many short-lived proteins in eukaryotic cells is performed by the ubiquitin-proteasome system (UPS). Ubiquitination, a posttranslational modification that consists of the attachment of ubiquitin (Ub) to a protein substrate, is an obligatory step in their degradation via proteasome. Nonetheless, ubiquitination also regulates other protein fates, such as protein subcellular localization or enzymatic activity regulation [1]. Ubiquitination is a dynamic and reversible reaction where ubiquitin is linked and cleaved from substrates by specific ligases and proteases. The proteases that deconjugate ubiquitin from their substrates are named deubiquitinating enzymes (DUBs) [2]. DUBs are classified into six different families: (i) Ub C-terminal hydrolases (UCHs), (ii) Ub-specific proteases (USPs), (iii) Machado-Joseph disease protein domain proteases (MJDs), (iv) ovarian tumor proteases (OTUs), (v) JAMM motif (zinc metallo) proteases, and (vi) the recently described “motif interacting with Ub-containing novel DUB family” (MINDY) [3,4]. The world of ubiquitin conjugation has also expanded to include other ubiquitin-like peptides (e.g., SUMO and NEDD8 [5]), all of which are molecular tags that regulate protein fate.

Disruption of the UPS is associated with many human disorders, mainly cancer and neurodegeneration. However, protein homeostasis is involved not only in the maintenance of cell function but also in developmental decisions and the formation of diverse tissues and organs [3], such as the retina.

The retina develops as an evagination of the central nervous system (CNS) that forms a multilayered neurosensory tissue in the posterior part of the eye. Its formation requires extremely fine regulation at transcriptional and protein level, particularly during photoreceptor differentiation. The photoreceptors, rods and cones, are light-sensitive neurons that capture photons and trigger the visual process. Differentiated photoreceptor cells share a unique morphology, with a highly specialized primary cilium and presynaptic terminals, and express a wide range of cell type-specific...
proteins. The development of these cells follows a tightly controlled genetic program in which multipotent retinal progenitor cells (RPCs) exit the cell cycle and undergo first a process of fate determination and later, commit to a specific photoreceptor subtype (Figure 1) [6,7]. The same post-mitotic precursor cell can become either a rod or a cone, depending on an intricate genetic network of transcription factors (TFs), especially neural retina leucine zipper (NRL) and thyroid hormone receptor β2 (TRβ2) [8]. Fate commitment implicates the expression of genes specific for each photoreceptor type to reach the final differentiation with the expression of the distinct types of cone and rod opsins.

During development, RPC multipotency and proliferation are maintained by the expression of several TFs (e.g., PAX6). RPCs can become lineage specific, and OTX2—together with other TFs, such as RORβ and developmental cues—controls the formation of post-mitotic photoreceptor precursors. As shown in Figure 1, the cone-rod homeobox protein CRX elicits the photoreceptor default pathway, which is to become an S-cone. TRβ2 expression will later determine M-opsin cone identity. In contrast, the determination of the rod fate from the early S-cone requires the expression and activity of NRL, which controls the expression of most rod genes [9,10], including that of the photoreceptor-specific nuclear receptor gene, NR2E3 (Gene ID 10002, OMIM 604485), which also induces and consolidates the rod cell state by activating rod-specific genes and at the same time, suppressing those that are cone-specific [11,12] (Figure 1). The developmental timeframe varies among species: For instance, in humans S-opsin mRNA is detected at fetal week 12, while expression of rhodopsin and M- and L- opsins appears by fetal weeks 15–17 [13]. However, murine cones

Figure 1. Diagram of murine photoreceptor development with key regulatory transcription factors. From embryonic stem cells, several transcription factors at specific developmental times are required to determine retinal precursor cells and eventually, give rise to fully mature photoreceptors. PAX6, OTX2, RORβ, CRX, NRL, NR2E3, and TRβ2 are considered the key regulators of retinal development and photoreceptor differentiation. Blue bullets indicate posttranslational modification of NRL and NR2E3 by SUMO that regulates cone versus rod fate in photoreceptor precursor cells.
start to differentiate by embryonic day 11 (E11), and S-opsin is expressed at later embryonic stages, whereas M-opsin expression is not detected until postnatal day 6 (P6). The genesis of rods peaks at P2, closely preceding rhodopsin transcription [6].

This exquisite transcriptional regulation is further refined by the involvement of ubiquitin and ubiquitin-like molecules: NR2E3 and NRL are posttranslationally modified by SUMO to either activate or suppress cone- and rod-specific genes [14-16]. Other examples of genes related to UPS that participate in retinal development and photoreceptor differentiation are the following: FAF/USP9X and USP5 mutants in Drosophila display defects in photoreceptor differentiation and eye development [17,18], UCH-L1 participates in multiple pathways during eye development in Drosophila [19], and USP45 is important for the correct formation and differentiation of the zebrafish retina [20]. In humans, mutations in several genes related to UPS can cause retinitis pigmentosa and other inherited retinal dystrophies, for instance, TOPORS (Gene ID 10210 , OMIM 609507) [21,22], KLHL7 (Gene ID 55975, OMIM 611119) [23-25], PRPF8 (Gene ID 10594, OMIM 607300) [26] (PRPF8 belongs to the JAMM family of deubiquitinating enzymes, even if it is catalytical core is inactive [27]), and much more recently, USP45 (Gene ID 85015, OMIM 618439) [28]. Moreover, dysfunction of other proteins that belong to the UPS has also been associated with multifactorial retinal disorders, such as age-related macular degeneration, glaucoma, diabetic retinopathy, and retinal inflammation [29].

Therefore, considering the clear implication of the ubiquitin and ubiquitin-related proteins in retinal diseases, we aimed to identify other DUB candidates involved in retinal development and maintenance. We previously provided a screening of the expression of all the DUBs in the mouse adult retina by performing quantitative reverse transcription PCR (qRT-PCR) and in situ hybridization [30]. The results reported allowed us to observe the expression pattern of DUBs in different retinal layers, and their potential role in differentiated retinal cells. However, a detailed comparison of expression during retinal development in the mouse was not feasible unless high-throughput technologies, such as massive sequencing, were used. In this context, whole transcriptome analyses of the retina have already provided a wide overview of gene expression during development in mice as well as in humans [10,31]. Using these transcriptomic data, we present a detailed comparison of DUB expression through many developmental stages in mouse and fetal human retinas, including mutant mouse models that show severe retinal differentiation defects to identify differential DUB expression patterns in rods and cones. By complementing this RNA-sequencing (RNA-seq) analysis with other reported expression and phenotypic data, we highlight interesting DUB candidates to regulate key transcription factors for cell fate decision and photoreceptor differentiation.

METHODS

Retinal transcriptome profiling: For direct comparison of distinct RNA-seq data sets to examine the expression of DUB genes, we reanalyzed the previously published Crx mutant whole retina (GEO accession # GSE52006), flow-sorted photoreceptor transcriptome data sets (GEO accession # GSE74660), and in vivo mouse retina development data (GEO accession # GSE101986) with an analysis pipeline, detailed elsewhere [10,31,32]. Briefly, for mouse samples, RNA was hybridized to GeneChip Mouse Exon 1.0 ST (mouse samples), and strand-specific RNA-seq data were generated using the TruSeq RNA Sample Prep Kit-v2 (Illumina, San Diego, CA). Single end reads (76 bp) were generated on the Genome Analyzer Ix platform (Illumina), and transcript levels were quantified using Ensembl v73 transcriptome annotation [10]. For human fetal samples, strand-specific libraries were constructed with 100 ng of total RNA using the TruSeq Stranded mRNA Library Preparation Kit (Illumina), and paired-end sequencing was performed at a length of 125 bases on the HiSeq2500 (Illumina). Transcript-level quantitation was performed using Kallisto v0.42.4 [31]. All secondary analyses were performed in the R statistical environment. Data sets included in the analysis were Nrl−/− and Crx−/− knockout mouse retinas [9,32,33], flow-sorted rod and S-like cone photoreceptors [10], mouse developmental retinal tissue, and human fetal retinas [31] (websites for data downloading as mentioned). Transcript-level fragments per kilobase of exon model per million reads (FPKM) values were averaged and log 2 transformed before hierarchical clustering using Euclidean distance and Ward’s method. Heatmaps of clustered data were generated using the heatmap.2 function in the gplots package in the R environment. The complete list of DUBs analyzed and their family is shown in Table 1.

ChIP-seq data reanalysis: The chromatin immunoprecipitation sequencing (ChIP-seq) data were reanalyzed from published work [9,33]. It was performed on DNA from the adult mouse retina, which was cross-linked and immunoprecipitated with antibodies against either CRX or NRL, to determine target gene promoters where CRX and NRL were bound. These data are available in public databases (GSE20012) for reanalysis and can be visualized using the UCSC genome browser entourage.
RNA-seq expression data reanalysis: Expression data on mouse tissues were obtained from public databases (ENCODEx mouse project). Of note, the eye is not among the tissues included in ENCODE.

Ethics statement for animal procedures, animal handling, and preparation of samples: All procedures in mice were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, as well as the regulations of the animal care facilities at the Universitat de Barcelona. The protocols and detailed procedures were evaluated and approved by the Animal Research Ethics Committee (CEEA) of the Universitat de Barcelona (our institution) and approved by the Generalitat de Catalunya (local government), with the official permit numbers DAAM 6562 and 7185. Mouse retina samples and eye slides for in situ hybridization were obtained from C57BL/6J (wild-type) animals at the indicated ages (postnatal), as described in [30].

RESULTS

Transcriptome profiling of DUB genes during mouse and human retinal development: One of the aims of our group is understanding the role of DUBs in retinal physiology and their contribution to photoreceptor development. As a first approach, we performed a systematic analysis of the transcriptional levels of all the genes encoding deubiquitinating enzymes described in the mouse genome in the adult mouse retina (P60) with RT-qPCR and in situ hybridization [30]. The age selected for this first analysis was adequate to define a reference working frame in a completely functional retina; however, it gave no insights into the relevance of these enzymes either during the development of this neurosensory organ or in the fate determination (rod versus cone) of the photoreceptors. Thus, further time points were required to properly assess the developmental variations of the DUB expression and consequently, evaluate whether any of them could be involved in the development of the mouse retina.

Taking advantage of published high throughput RNA-sequencing data, we present the results of in silico comparative reanalyses of transcriptomes from i) the developing mouse retina [31]; ii) postnatal retinas from two knockout models, Crx−/− and Nrl−/−, in which relevant retinal transcription factors were ablated [9,32,33]; iii) flow-sorted rod- and cone-like photoreceptors [10]; and iv) human fetal retinas [31]. We selected the expression data for the orthologous DUB genes in the human and mouse genomes. Table 1 shows the complete list of analyzed profiles, ordered by the DUB family and gene symbol.
For mouse wild-type (WT) retinas, RNA-seq data were available at the stages embryonic 11 (E11), E12, E14, E16, P0, P2, P4, P6, P10, P14, and P21. Data from the P2 and P21 stages of the mouse Crx−/− and Nrl−/− knockout retinas were also available, as were the transcriptome profiles from flow-sorted postnatal rod- and cone-like photoreceptor cells, from the P0, P2, P4, P10, P14, and P21 mice. Concerning the human fetal retina, RNA-seq data were available for day 52 postfertilization (D52), D53, D57 (two different samples: D57.1 and D57.2), D67 (two different samples: D67.1 and D67.2), D70, D80, D87, D94 (two different samples: D94.1 and D94.2), D105, D107, D115, D125, D132, and D136.

We performed a comprehensive analysis and comparison of the DUB expression levels, and the results as a heatmap are depicted in Figure 2. The heatmap represents the average log2 of the FPKM values, where dark blue indicates low expression, and dark red indicates high expression of a particular gene at a particular developmental stage.

In a general transversal overview (per genes in all stages and organisms) of the heatmap expression of DUBs in the developing human and mouse retinas, we could observe four main patterns: a) a group of genes with moderate or high expression in embryonic mouse but relatively low expression in human fetal retinas; b) a group of genes with moderate or low expression in mouse retinas with a variable level of expression in human retinas; c) a group of genes with high expression in mouse retinas through all the stages as well as in human fetal retinas, and d) a group of genes with low expression in mouse and in human fetal retinas. The genes that are not expressed in the human and mouse retinas during all the developmental stages are not considered good candidates for retinal function (e.g., genes grouped at the end of Figure 2). However, genes that are highly expressed in all stages and animal models are most likely related to basic cell functions. In this group, we identified Eif3h and Prpf8 (relevant in translation initiation and splicing events), or Cns6, Csn5, and Psmd7 (encoding subunits of the COP9 signalosome or regulators of the proteasome activity). Other genes that belong to the same family may have some redundant function and may swap their roles in different species, as may happen with the ubiquitin C-terminal hydrolase genes (in charge of processing the ubiquitin precursor proteins): Uch1 shows a mirror expression pattern compared to Uch13 and Bap1 in the developing mouse and human fetal retinas (Uch1 is highly expressed in human fetal retinas, whereas in mouse fetal retinas, Uch13 and Bap1 are the UCHs with the highest expression).

Another type of analysis was performed in the comparison of differentially expressed DUBs at P2 and P21 (first lanes in Figure 2) in the WT, Crx−/−, and Nrl−/− mice. Genes that behaved similarly in the WT and the knockout models at P2 and P21 were not related to the photoreceptor types in the retina, but to more general processes of neuronal maturation. For instance, Usp1 and Usp39 are highly expressed in the developmental stages, but they shut down upon retinal differentiation. With a similar behavior, we identified Usp5, in accordance with the potential role in the regulation of Notch and receptor tyrosine kinase (RTK) signaling during eye development in Drosophila [18]. The expression of other genes, such as Usp33, or more moderately, Usp32 and Usp48, increases upon retinal differentiation. Interestingly, at P2 and focusing on the transcriptome differences between the animal models, Nrl−/− showed a clear different pattern with respect to the WT and Crx−/− retinas, for instance, in Usp38, Mysml, Usp11, Usp10, Otud1, and Atxn3, probably detecting differences in the formation of a retina enriched in cone-like photoreceptors. In contrast, at P21, the WT transcriptome clearly stood out with respect to the Crx−/− and Nrl−/− retinas in genes such as Tfnaip3, Zranb1, Otud4, Otud7b, Otud6b, Mpnd, Vcpip1 (all of them from the JAMM family), or Usp8, probably detecting the absence of rod photoreceptors or dysfunction of the retina.

Particularly interesting are the differences between the pattern of expression in DUBs in rod-like versus cone-like cells at different developmental stages. In general, a sharp change in the transcriptome landscape was apparent between days P6 and P10, particularly in rod-like cells but also in cone-like cells (Figure 2), in accordance with the reported expression of relevant eye developmental genes [10]. The expression of several DUB genes was drastically changed, and this shift in expression occurred either in similar or in a different direction in rod- versus cone-like. When the shift in expression was similar in rod- and cone-like cells, the same change was observed during the developing mouse retina and indicated that these genes are developmentally regulated. For instance, upon differentiation, the expression of Taf1d, Atxn3, Bap1, and Usp1 was decreased, whereas that of Usp33 expression was increased. More interesting were the DUB genes whose expression dynamics clearly differed between rod and cone cells. Several genes were moderately expressed in young rod cells (P2–P6), but their expression decreased in later stages, in contrast to the moderate or stable pattern of expression in cones of the same stage: Usp38, Vcpip1, Usp8, Usp14, Usp15, Usp10, Josd1, Usp39, or Usp11. Rarely, some genes were activated through rod differentiation but were barely expressed in cones; such as the case of Usp21 and Tfnaip3. In general, and concerning DUB genes, rod differentiation involves silencing or a steep decrease in expression compared to cone cells. For instance, we observed that Usp7 and Atxn3 were expressed...
Figure 2. Expression heatmap of deubiquitinating enzyme genes in several human and mouse developmental stages. WT, wild-type mouse retinas; CRX, mouse Crx knockout (KO) retinas; NRL, mouse Nrl knockout retinas. For the mouse WT retinas, the data shown correspond to the embryonic 11 (E11), E12, E14, E16, postnatal day 0 (P0), P2, P4, P6, P10, P14, and P21 stages. Rod- and cone-like data correspond to RNA-sequencing (RNA-seq) from flow-sorted rods and cone-like cells from stages P0, P2, P4, P10, P14, and P21. For the human retina, the data shown correspond to day 52 postfertilization (D52), D53, D57 (two different samples: D57.1 and D57.2), D67 (two different samples: D67.1 and D67.2), D70, D80, D87, D94 (two different samples: D94.1 and D94.2), D105, D107, D115, D125, D132, and D136 (details of the RNA-seq libraries and references are in the text). The color key (upper left corner) indicates the relative expression values. Blue indicates low expression; red, high expression; intermediate expression is colored in white and light colors (as described in [10,31]).
at early stages (such as P2, also in Nrl/−/− animals), but later, the expression decreased upon differentiation, whereas Usp48 was maintained at high levels of expression only in cones.

Finally, when we analyzed the expression of DUB genes in the human fetal retinas, no sharp changes in expression were apparent for most genes. Previous whole transcriptome studies revealed three key epochs of expression dynamics, from D52 to D67 (enrichment in genes involved in mitosis and cell proliferation), from D67 to D80 (including genes encoding TFs required for specific neuron differentiation at the retina, and those involved in the formation of synapses and neurotransmitter signaling), and from D90 to D100 (genes required for photoreceptor cells, ganglion cell axon guidance, and synaptogenesis). Overall, few DUB genes reflected a major shift in expression in human fetal retinas, except the expression of Otud5, Usp47, Bap1, and Usp34 at the later analyzed stages, whereas the expression of Pmds7 and Csn6 was moderated.

Selection of DUB candidates potentially involved in retina development: To select putative relevant genes for retinal differentiation, in particular in the determination of cone versus rod fate, we made a short list of relevant, or potentially relevant, DUB genes to be involved and regulate rod versus cone fate. The criteria used to obtain a short list of DUBs were their transcriptome profiling data, as analyzed in Figure 2 (criterion 1), ChIP-seq data for CRX and NRL on adult retinas (criterion 2), and expression pattern and tissue specificity of DUB gene expression in the central nervous system (CNS; criterion 3). These data were checked against previously reported bibliography on their biologic function as well as data on the effect of the knockout or knockdown of particular DUB genes on the eye (and neuronal phenotypes) in different organisms [3,30,34-37] (criterion 4).

Concerning ChIP-seq data, we surmised that DUB genes involved in retinal differentiation pathways would most likely be regulated by key transcription factors, such as CRX or NRL. A promoter or enhancer bound by these transcription factors was considered a good indication of the gene being relevant for retinal function. We contrasted the transcriptome profiles with ChIP-seq data performed on DNA from the adult mouse retina to determine target gene promoters where CRX and NRL were bound, as well as with a possible phenotype. The RNA-seq data of the mouse tissues provided clues on the spatial pattern and tissue specificity of DUB gene expression: For instance, a broadly expressed gene might have a more general role than a gene expressed only in the CNS. Therefore, we preferentially selected genes that were expressed in the CNS. Overall, we preselected a total of 12 genes as possible candidates for involvement in developmental decisions in the retina. The prioritized gene list is shown in Table 2. In summary, one gene was expressed at low levels in the retina (Usp20), eight genes (Josd1, Pan2, Usp11, Usp14, Usp15, Usp10, Usp22, and Usp39) strongly decreased the expression on rod differentiation compared to cones, whereas the expression of three genes (Otud7b, Usp46, and Usp48) increased in late-stage cones. We did not consider Usp45 in this list because we had already proposed it as a candidate gene for retinal dystrophies [20], and it was later confirmed to cause retinal disease in humans [28], thus validating our approach.

To narrow down the list for further assays, we made a second selection with the five most interesting DUB genes: Josd1, Otud7b, Usp22, Usp46, and Usp48 (Figure 3). One of the main criteria for including a gene on this short list was differential expression between cones and rods. We considered their transcriptome profile (Figure 3A), the ChIP-seq data from NRL and CRX binding to their gene promoter as indicative of specific regulation of expression in the retina (Figure 3B), their specific pattern of expression in the retina as detected by in situ hybridization when available (Figure 3C), and the eye phenotypic alteration in mutant organisms (Figure 3D). The five selected genes are silenced or repressed in rods and expressed in cone-like cells of the same stage. Several aspects are worth noting: For instance, CRX strongly binds to the promoter or internal enhancers of Josd1, Usp22, and Usp48. Interestingly, Usp46 and Usp48 are clearly cone-expressed genes, whereas Usp22 is highly expressed in all developmental stages, is dysregulated in many cancers, and when mutated, causes either a pan-neuronal phenotype or lethality. In contrast, the knockdown of Usp48 specifically causes an ocular or retinal phenotype in zebrafish. These results are promising and encourage further work in animal models to determine the functional role of these DUBs in regulating the development of the retina and differentiation of retinal cells.

**DISCUSSION**

The retina is a highly specialized neurosensory organ, and the differentiation process from retinal precursor cells into their final unique morphology and function is regulated by the combination of transcriptional regulatory programs in response to external cues. Particularly interesting is the final differentiation of photoreceptors into rods or cones, with specific membrane structures, distinct gene signatures, and differentiated physiologic role. Among the relevant TFs for retina differentiation, CRX has a primary role in defining the competence of post-mitotic cells to become photoreceptors by regulating most genes, but NRL in concert with NR2E3 are
## Table 2. Data summary of the 12 pre-selected genes as plausible candidates to contribute to retinal development.

| DUB | RNA-seq on total RETINA | ChIP-seq | RNA-seq on TISSUES | PHENOTYPE | BIOLOGIC PROCESS |
|-----|-------------------------|----------|--------------------|-----------|------------------|
| **JOSD1** | Decrease during development in whole retina and in rods, but not in cones. | — | Adrenal Gland, Cerebellum, Spleen, Thymus | 3 dpf: eyes shape, abnormal retinotectal projection (ZF) | Involved in endocytosis. Almost no bibliography |
| **OTUD7B** | Low expression throughout development, small increase from P10. Continuous expression in rods. | — | Testis | — | Involved in NFKB signaling. Role as oncogene: via deubiquitination of EGFR |
| **Pan 2** (USP52) | Highly expressed in the retina. Shut down in rods at P21, but maintenance in cones. | CRX | Cerebellum, CNS, Testis, Cortical Plate, Frontal Cortex, Limb, Liver, Placenta, Testis, Urinary Bladder | — | mRNA Deadenylation. |
| **USP10** | Decrease in rods from P10, but not in cones. | CRX, NRL | Cerebellum, CNS, Cortical Plate, Female Gonad, Frontal Cortex, Limb | CNS necrosis (ZF) | DNA damage. Tumour-associated marker in gastrocarcinoma, and Adrenal tumors. Regulation of NFKB signaling via p53. |
| **USP11** | Strong shut down in rods and not in cones | — | Cerebellum, CNS, Cortical Plate, Frontal Cortex, Gonadal Fat Pad, Kidney, Large Intestine, Placenta, Testis | — | Transport to the Golgi, Protein folding; NFKB signaling; DNA repair after double-strand DNA breaks. Possibly related to X-linked retinal disorders. |
| **USP14** | Stable expression in retina, with a slight shut down only in rods. | — | Cerebellum, CNS, Cortical Plate, Frontal Cortex, Liver, Placenta, Testis, | Neuronal phenotype slower adults, early death (D). Reduced USP14 levels cause tremors, abnormal brain morphology, altered synaptic transmission and increased apoptosis (H) | Involved in Parkinson. Role as oncogene in breast, hepatocellular carcinoma, lung adenocarcinoma. Decreases apoptosis. |
| **USP15** | Stable expression in retina, with a slight shut down only in rods | NRL | Adrenal Gland, Cerebellum, CNS, Colon, Cortical Plate, Heart, Kidney, Large Intestine, Limb, Testis | 2dpf: small eyes, and at 4dpf: deformed eyes (ZF). | Interferon signaling. Involved in mitophagy. Role as oncogene via TGFβ. |
| **USP20** | Very low levels of expression in retina, with a slight shut down in rods from P10. | — | Kidney, Thymus | Earlier adult death (D). 3dpf: small eyes (ZF). | NFKB signaling. β2 adrenergic receptor recycling. Thyroid hormone activation. |
| **USP22** | Highly expressed throughout development with a shut down in rods from P10. | CRX | Testis | (Neuronal) slower adults, early death (D). 3dpf: small eyes (ZF). (Neural) slower adults, early death (D). Homozygotes are embryonic lethal (M) | Role as oncogene in liver, colon, lung, gastric, nasopharyngeal, pancreas (via histone and p53 regulation). |
| **USP39** | Slight shut down both in retina and rods from P10, but not in cones. | CRX | CNS, Limb, Testis | Larval death (D). 2dpf: small eyes (ZF) | Role in cancer, promotes cell proliferation. |
| DUB  | RNA-seq on total RETINA | ChIP-seq | RNA-seq on TISSUES | PHENOTYPE | BIOLOGIC PROCESS |
|------|-------------------------|----------|------------------|-----------|------------------|
| USP46| Low expression in rods with a final strong shut down. Moderate-high expression in postnatal cones. | — | Adrenal Gland, Duodenum, Adipocytes, Thymus, Kidney, Spleen, Female Gonad, Mammary Gland, Colon | — | Role in neurotransmission circuitry, involved in behavior. |
| USP48| Strong difference between rods (low) and cones (high) expression. | CRX | Cerebellum, CNS; Placenta | 3dpf: small eyes (ZF) | DNA repair in Fanconi Anemia<sup>e</sup>. Corticoph adenomes<sup>f</sup>. |

<sup>a</sup>RNA-seq on total retina lists the expression features observed in the RNA-seq data.<sup>b</sup>ChIP-Seq “CRX” and “NRL” indicate that the gene promoter was bound by CRX or NRL, respectively, in ChIP assays.<sup>c</sup>RNA-seq on tissues list tissues in which the DUB gene is mainly expressed.<sup>d</sup>Phenotype includes the phenotypic traits reported in different model organisms caused by mutations in these genes [3,30,34].<sup>e</sup>[35].<sup>f</sup>[36]. (D): Drosophila (H): Human; (M): Mouse; (ZF): Zebrafish.
essential to silence the genes of the default cone differentiation pathway and activate the genes for rod differentiation [6, 8, 38-40]. Mutations in these genes cause severe retinal phenotypes, with alteration of photoreceptor and retinal degeneration.

These key TFs are not only transcriptionally regulated but also by posttranslational modifications, and the SUMOylation state of NR2E3 and NRL defines their role as transcriptional repressors or activators [14-16]. Posttranslational peptide conjugation of ubiquitin and other ubiquitin-like molecules is a versatile and reversible mechanism that allow cells to quickly switch on or switch off particular processes, such as cell proliferation and differentiation. Although ubiquitination has been mainly associated with protein degradation, ubiquitin is a molecular tag for protein fate change. Therefore,
ubiquitin ligases and deubiquitinating enzymes play a role in subtly regulating the availability and the interaction interface of their substrate proteins. Mutations in several genes related to the ubiquitin pathways cause inherited retinal disorders in humans, but also the knockdown of DUB genes in zebrafish embryos indicated that other ubiquitin or proteasome genes are involved in the development and differentiation of the vertebrate retina [34].

Previous work has provided a unique spatial reference map of retinal DUB expression and highlighted that representative genes from all the DUB subfamilies were expressed in the adult retina at different relative expression levels. In addition, the spatial expression pattern of some DUBs is specific to particular layers. For instance, Usp45, Usp53, and Usp54 showed expression restricted to the photoreceptor layer [30]. This work prompted us to perform knockdown assays in zebrafish morpholino-injected embryos, showing that Usp45 was extremely relevant for eye morphogenesis and retinal layer formation [20]. Later, other groups showed that mutations in USP45 in human patients cause Leber congenital amaurosis, a severe form of retinal degeneration [28], overall supporting the validity of this type of analysis to unveil plausible candidates for retinal dystrophies. Nonetheless, a more comprehensive and systematic analysis of DUB genes is required to highlight new potential genes for retinal development. In particular, we wished to focus on identifying plausible DUB candidates to contribute to rod versus cone fate. This is an interesting biological question, as rods are, by far, the most numerous type of photoreceptor in mice and humans even though the default differentiation pathway for a photoreceptor precursor is to become a S-cone. Notably, most rods in mammals are suggested to originate from S-cones to overcome nocturnal bottleneck during evolution [10].

Transcriptomes of the developing retina in humans and mice can detect differential patterns of gene expression through several developmental stages [10, 31] (data accessible at https://neicommons.nei.nih.gov/). We performed a curated in silico expression analysis of the DUB gene superfamily for a more accurate overview of the gene regulatory patterns and correlation with main expression epochs or gene expression transitions. RNA-seq data showed clear variations in the expression levels of DUB genes during mouse retinal development. For instance, Usp28, Usp37, or Otub1, highly expressed in embryonic stages but whose expression was shut down after birth; or Usp12, Zranbl, or Usp32, whose expression was extremely low at embryonic stages but clearly increased around and after the birth date. These differences in gene expression might be due to specific DUBs (e.g., Usp28, Usp37, and Otub1) being important for cell proliferation or the differentiation of certain cell types; thus, when these cells are finally differentiated, those genes are no longer needed, and consequently, their expression levels drop. In this context, the most feasible scenario is that they participate in the differentiation of cells like ganglion, horizontal, or amacrine cells, which fully differentiate in the mouse embryonic stages. Concerning Usp12, Zranbl, and Usp32, their increase from birth might be explained by two different possibilities: 1) either they are important for rod morphogenesis, which peak by P2; or 2) they are rod-specific genes, and thus, as the number of rods increases, their expression levels consequently increase.

Most RNA-seq data and RT-qPCR data have been generated from total retinas, and consequently, genes expressed in most abundant cell types are overrepresented. In this context, the RNA-seq data from flow-sorted rods and cone-like photoreceptors make feasible the identification of differentially expressed genes, identifying candidates that might be important for each type of cell. For instance, and considering that cones are the less numerous type of photoreceptors, the expression of relevant early cone genes may be masked unless the analysis is performed in early cone-like cells or in the Nrl−/− animal model, this might well be the case for Atxn3 and Usp7, whose expression is moderate except in early cones. In addition, the expression of some genes, such as Usp11, is switched off in rods at or after P6, even though their expression in cones is maintained over time. Therefore, this gene might be important for the maintenance of either early photoreceptor or cone differentiated cells, so that it might be no longer required in differentiated rods. In other cases, the gene might be mainly required in differentiated cells, such as Usp8, which has been involved in ciliogenesis regulation—a basic function in all photoreceptors—and proposed as a ciliopathy gene [41]. Tnfaip3, instead, is highly repressed in all developmental stages, but after P10, the gene is highly expressed only in rods, clearly indicating a role in rod differentiation and maintenance. More recently, single cell RNA-seq analyses have been performed for the mouse and human retina [42-45]; however, at this stage, these data sets do not include enough genes and reads for low-expressed genes for evaluation of DUBs.

In accordance with the transcriptome landscape reported for the developing mouse retina, three differential expression profiles can be distinguished, with a sharp transition between P6 and P10 [10], and the pattern of expression of many genes shifted in this transition, for rod and cone cells. The expression of Crx and Nr2e3 increases gradually, whereas Nrl shows a sharp transition as do many other rod-specific genes [10]. In the present DUB transcriptome landscape, only Usp32,
Usp33, and Tnfaip3 made this sharp increase in the whole retina, thus indicating their potential relevance in differentiating rods.

In this context, we propose that the selected short list of genes that show specific expression in cone-rich retinas (Nrl−/−) and in cone-like cells (Figure 3) might regulate relevant cone cell pathways, particularly, Usp48. Usp48 is barely expressed in developing or mature rods but is always expressed in cone cells, thus suggesting a role in cone photoreceptor cells. USP48 has been recently involved in hedgehog signaling and in DNA repair in humans, but with no further roles in non-dividing differentiated cells. As data in favor of a possible involvement of Usp48 in retinal morphogenesis and differentiation, this gene produced 1) a differential expression pattern in cone and rod development, with a shutdown in rods at postnatal stages; 2) a strong ChIP-seq peak with CRX; 3) an altered retinal phenotype when silenced in zebrafish; and 4) it has not been previously assigned any clear physiologic role. For want of stronger evidence, we currently hypothesize that Usp48 is a good candidate for regulating or contributing to cone function. Further work in animal models, for example, with transient knockdown in the mouse retina [46] may shed light on the potential role of this gene and the other candidates in cone dystrophies or age-related macular degeneration.

ACKNOWLEDGMENTS

This research was supported by grants SAF2013–49069-C2–1-R and SAF2016–80937-R (Ministerio de Economía y Competitividad/FEDER), 2017 SGR 738 (Generalitat de Catalunya), and La Marató TV3 (Project Marató 2014,17–30–31–32) to GM, and by the Intramural Research Program of the National Eye Institute (ZIAEY000450 and ZIAEY000546) to AS. VT is fellow of the MINECO (BES-2014–068639, 20648062). Brzezinski JA, Reh TA. Photoreceptor cell fate specification in vertebrates. Development 2015; 142:3263-73. [PMID: 26443631].

REFERENCES

1. Husnjak K, Dikic I. Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. Annu Rev Biochem 2012; 81:291-322. [PMID: 22482907].

2. Hochstrasser M. Ubiquitin-dependent protein degradation. Annu Rev Genet 1996; 30:405-39. [PMID: 8982460].

3. Clague MJ, Barsukov I, Coulson JM, Liu H, Rigden DJ, Urbé S. Deubiquitylases from genes to organism. Physiol Rev 2013; 93:1289-315. [PMID: 23899565].

4. Rehman SA, Kristariyanto YA, Choi S-Y, Nkosi P, Weidlich S, Labib K, Hofmann K, Kalathu Y. MINDY-I is a Member of an Evolutionarily Conserved and Structurally Distinct New Family of Deubiquitinating Enzymes. Mol Cell 2016; 63:146-55. [PMID: 27929798].

5. Kirkin V, Dikic I. Role of ubiquitin-and Ubl-binding proteins in cell signaling. Curr Opin Cell Biol 2007; 19:199-205. [PMID: 17303403].

6. Swaroop A, Kim D, Forrest D. Transcriptional regulation of photoreceptor development and homeostasis in the mammalian retina. Nat Rev Neurosci 2010; 11:563-76. [PMID: 20648062].

7. Ng L, Lu A, Swaroop A, Sharlin DS, Swaroop A, Forrest D. Two transcription factors can direct three photoreceptor outcomes from rod precursor cells in mouse retinal development. J Neurosci 2011; 31:11118-25. [PMID: 21813673].

8. Hao H, Kim DS, Klocke B, Johnson KR, Cui K, Gotoh N, Zang C, Gregorski J, Gieser L, Peng W, Fann Y, Seifert M, Zhao K, Swaroop A. Transcriptional regulation of rod photoreceptor homeostasis revealed by in vivo NRL targetome analysis. PLoS Genet 2012; 8:e1002649. [PMID: 22518866].

9. Kim J-W, Yang H-J, Brooks MJ, Zelinger L, Karakılış G, Gotoh N, Boleda A, Gieser L, Giuste F, Whitaker DT, Walton A, Villasmiño R, Barb JJ, Munson PJ, Kaya KD, Chaitankar V, Cogliati T, Swaroop A. NRL-Regulated Transcriptome Dynamics of Developing Rod Photoreceptors. Cell Reports 2016; 17:2460-73. [PMID: 27880916].

10. Cheng H, Aleman TS, Cideciyan AV, Khanna R, Jacobson SG, Swaroop A. In vivo function of the orphan nuclear receptor NR2E3 in establishing photoreceptor identity during mammalian retinal development. Hum Mol Genet 2006; 15:2588-602. [PMID: 16868010].

11. Oh ECT, Cheng H, Hao H, Jia L, Khan NW, Swaroop A. Rod differentiation factor NRL activatesthe expression of nuclear receptor NR2E3 to suppress the development of cone photoreceptors. Brain Res 2008; 1236:16-29. [PMID: 18294621].

12. Hendrickson A, Bumsted-O’Brien K, Natoli R, Ramamurthy V, Possin D, Provis J. Rod photoreceptor differentiation in fetal and infant human retina. Exp Eye Res 2008; 87:415-26. [PMID: 18778702].

13. Onishi A, Peng G-H, Hsu C, Alexis U, Chen S, Blackshaw S. Pias3-dependent SUMOylation directs rod photoreceptor development. Neuron 2009; 61:234-46. [PMID: 19186166].

14. Roger JE, Nellissey J, Kim DS, Swaroop A. SUMOylation of bZIP transcription factor NRL modulates target gene expression during photoreceptor differentiation. J Biol Chem 2010; 285:25637-44. [PMID: 20551322].

15. Campla CK, Breit H, Dong L, Gumerson JD, Roger JE, Swaroop A. Pias3 is necessary for dorso-ventral patterning and visual response of retinal cones but is not required for rod photoreceptor differentiation. Biol Open 2017; 6:881-90. [PMID: 28495965].
photoreceptor cell number is intimately associated with endocytosis. Development 2000; 127:1727-36. [PMID: 10725248].

18. Ling X, Huang Q, Xu Y, Jin Y, Feng Y, Shi W, Ye X, Lin Y, Hou L, Ling X. The deubiquitinating enzyme Usp5 regulates Notch and RTK signaling during Drosophila eye development. FEBS Lett. 2017; 591:875-88. [PMID: 28140449].

19. Thao DTP, An PNT, Yamaguchi M. LinhThuç T. Overexpression of ubiquitin carboxyl terminal hydrolase impairs multiple pathways during eye development in Drosophila melanogaster. Cell Tissue Res. 2012; 348:453-63. [PMID: 22526625].

20. Toulis V, Garanto A, Marfany G. Combining Zebrafish and Mouse Models to Test the Function of Deubiquitinating Enzyme (Dubs) Genes in Development: Role of USP45 in the Retina. Methods Mol Biol. 2016; 1449:85-101. [PMID: 27613029].

21. Chakarova CF, Papatheoannou MG, Khanna H, Lopez I, Waseem N, Shah A, Theis T, Friedman J, Maukaret C, Bujakowska K, Veraitch B, El-Aziz MMA, Prescott DQ, Paraparam SK, Bickmore WW, Munro PMG, Gal A, Hamel CP, Marigo V, Ponting CP, Wissinger B, Zrenner E, Matter K, Sawaaro A, Koeneekoop RK, Bhattacharya SS. Mutations in TOPORS Cause Autosomal Dominant Retinitis Pigmentosa with Perivascular Retinal Pigment Epithelium Atrophy. Am J Hum Genet 2007; 81:1098-103. [PMID: 17924349].

22. Bowene SJ, Sullivan LS, Gire AI, Birch DG, Hughbanks-Wheaton D, Heckenlively JR, Daiger SP. Mutations in the TOPORS gene cause 1% of autosomal dominant retinitis pigmentosa. Mol Vis 2008; 14:922-7. [PMID: 18509552].

23. Friedman JS, Ray JW, Waseem N, Johnson K, Brooks MJ, Hugosson T, Breuer D, Branham KE, Krauth DS, Bowne SJ, Daiger SP, Heckenlively JR, Andréasson S, Swaroop A. Mutations in a BTB-Kelch Protein, KLHL7, Cause Autosomal Dominant Retinitis Pigmentosa. Am J Hum Genet 2008; 84:792-800. [PMID: 19520207].

24. Hugosson T, Friedman JS, Ponjavic J, Abrahamson M, Sawaaro A, Andréasson S. Phenotype Associated With Mutation in the Recently Identified Autosomal Dominant Retinitis Pigmentosa KLHL7 Gene. Arch Ophthalmol 2009; 127:872-82. [PMID: 20547956].

25. Wen Y, Locke KG, Klein M, Bowene SJ, Sullivan LS, Ray JW, Daiger SP, Birch DG, Hughbanks-Wheaton DK. Phenotypic Characterization of 3 Families With Autosomal Dominant Retinitis Pigmentosa Due to Mutations in KLHL7. Arch Ophthalmol. 2011; 129:1475-82. [PMID: 22084217].

26. Martinez-Gimeno M, Gamundi MJ, Hernan I, Maseras M, Millà E, Ayuso C, Garcia-Sandoval B, Beneyto M, Vilela C, Baiget M, Antiólo G, Carballo M. Mutations in the pre-mRNA splicing-factor genes PRPF3, PRPF8, and PRPF31 in Spanish families with autosomal dominant retinitis pigmentosa. Invest Ophthalmol Vis Sci 2003; 44:2171-7. [PMID: 12714658].

27. Pena V, Liu S, Bujnicki JM, Luhrmann R, Wahl MC. Structure of a multipartite protein-protein interaction domain in splicing factor prp8 and its link to retinitis pigmentosa. Mol Cell 2007; 25:615-24. [PMID: 17317632].

28. Yi Z, Ouyang J, Sun W, Xiao X, Li S, Jia X, Wang P, Zhang Q. Biallelic mutations in USP45,encoding a deubiquitinating enzyme, are associated with Leber congenital amaurosis. J Med Genet 2019; 56:325-31. [PMID: 30573563].

29. Campello L, Esteve-Rudd J, Cuenca N, Martin-Nieto J. The Ubiquitin–Proteasome System in Retinal Health and Disease. Mol Neurobiol 2013; 47:790-810. [PMID: 23339020].

30. Esquadero M, Grau-Bové X, Garanto A, Toulis V, Garcia-Monclús S, Millo E, López-Iniesta MJ, Abad-Morales V, Ruiz-Trillo I, Marfany G. Expression Atlas of the Deubiquitinating Enzymes in the Adult Mouse Retina, Their Evolutionary Diversification and Phenotypic Roles. PLoS One 2016; 11:e0150364. [PMID: 26934049].

31. Hoshino A, Ratnapriya R, Brooks MJ, Chaitankar V, Wilken MS, Zhang C, Starostik MR, Gieser L, La Torre A, Nishio M, Bates O, Walton A, Birmingham-McDonogh O, Glass IA, Wong ROL, Sawaaro A, Reh TA. Molecular Anatomy of the Developing Human Retina. Dev Cell 2017; 43:763-779. e4. [PMID: 29233477].

32. Roger JE, Hiriyanna A, Gotoh N, Hao H, Cheng DF, Ratnapriya R, Kautzmann MA, Chang B, Sawaaro A. OTX2 loss causes rod differentiation defect in CRX-associated congenital blindness. J Clin Invest 2014; 124:631-43. [PMID: 24382533].

33. Corbo JC, Lawrence KA, Karlsetter M, Myers CA, Abdelaziz M, Dirkes W, Weigelt K, Seifert M, Benes V, Fritsche LG, Weber BH, Langmann T. CRX ChIP-seq reveals the cis-regulatory architecture of mouse photoreceptors. Genome Res 2010; 20:1512-25. [PMID: 20693478].

34. Tse WKF, Eisenhaber B, Ho SHK, Ng Q, Eisenhaber F, Jiang Y-J. Genome-wide loss-of-function analysis of deubiquitylating enzymes for zebrafish development. BMC Genomics 2009; 10:637. [PMID: 20040115].

35. Chen J, Jian X, Deng S, Ma Z, Shou X, Shen Y, Zhang Q, Song Z, Li Z, Peng H, Peng C, Chen M, Luo C, Zhao D, Ye Z, Shen M, Zhang Y, Zhou J, Fahira A, Wang Y, Li S, Zhang Z, Ye H, Li Y, Shen J, Chen H, Tang F, Yao Z, Shi Z, Chen C, Xie L, Wang Y, Fu C, Mao Y, Zhou L, Gao D, Yan H, Zhao Y, Huang C, Shi Y. Identification of recurrent USP48 and BRAF mutations in Cushing's disease. Nat Commun 2018; 9:3171. [PMID: 30093687].

36. Velimezi G, Robinson-Garcia L, Muñoz-Martínez F, Wiegant WW, Ferreira da Silva J, Owusu M, Moder M, Wiedner M, Rosenthal SB, Fisch KM, Moffat J, Menche J, van Attikum H, Jackson SP, Loizou JI. Map of synthetic rescue interactions for the Fanconi anemia DNA repair pathway identifies USP48. Nat Commun 2018; 9:2280. [PMID: 29891926].

37. Huo Y, Khatri N, Hou Q, Gilbert J, Wang G, Man H-Y. The deubiquitinating enzyme USP46 regulates AMPA receptor...
ubiquitination and trafficking. J Neurochem 2015; 134:1067-80. [PMID: 26077708].

38. Furukawa T, Morrow EM, Li T, Davis FC, Cepko CL. Retinopathy and attenuated circadian entrainment in Crx-deficient mice. Nat Genet 1999; 23:466-70. [PMID: 10581037].

39. Mears AJ, Kondo M, Swain PK, Takada Y, Bush RA, Saunders TL, Sieving PA, Swaroop A. Nrl is required for rod photoreceptor development. Nat Genet 2001; 29:447-52. [PMID: 11694879].

40. Hennig AK, Peng G-H, Chen S. Regulation of photoreceptor gene expression by Crx-associated transcription factor network. Brain Res 2008; 1192:114-33. [PMID: 17662965].

41. Kasahara K, Aoki H, Kiyono T, Wang S, Kagiwada H, Yuge M, Tanaka T, Nishimura Y, Mizoguchi A, Goshima N, Inagaki M. EGF receptor kinase suppresses ciliogenesis through activation of USP8 deubiquitinase. Nat Commun 2018; 9:758- [PMID: 29472535].

42. Macosko EZ, Basu A, Satija R, Nemesh J, Shekhar K, Goldman M, Tiros H, Bialas AR, Kamitani N, Martersteck EM, Trombetta JJ, Weitz DA, Sanes JR, Shalek AK, Regev A, McCarroll SA. Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. Cell 2015; 161:1202-14. [PMID: 26000488].

43. Bryan JM, Fufa TD, Bharti K, Brooks BP, Hufnagel RB, McGaughey DM. Identifying core biological processes distinguishing human eye tissues with precise systems-level gene expression analyses and weighted correlation networks. Hum Mol Genet 2018; 27:3325-39. [PMID: 30239781].

44. Collin J, Queen R, Zerti D, Dorgau B, Hussain R, Coxhead J, Cockell S, Lako M. Deconstructing Retinal Organoids: Single Cell RNA-Seq Reveals the Cellular Components of Human Pluripotent Stem Cell-Derived Retina. Stem Cells 2019; 37:593-8. [PMID: 30548510].

45. Langer KB, Ohlemacher SK, Phillips MJ, Fligor CM, Jiang P, Gamm DM, Meyer JS. Retinal Ganglion Cell Diversity and Subtype Specification from Human Pluripotent Stem Cells. Stem Cell Reports 2018; 10:1282-93. [PMID: 29576537].

46. Papal S, Monti CE, Tennison ME, Swaroop A. Molecular dissection of cone photoreceptor-enriched genes encoding transmembrane and secretory proteins. J Neurosci Res 2019; 97:16-28. [PMID: 30260491].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 2 December 2019. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.