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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 Marfany[1,2,4]

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

Correspondence to: Gemma Marfany, Dept. de Genètica, Microbiologia i Estadística Facultat de Biologia, Universitat de Barcelona, Avda. Diagonal 643, 08028 Barcelona, Spain; Phone: +34 93 4021502; FAX: +34 93 4034420; email: gmarfany@ub.edu

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

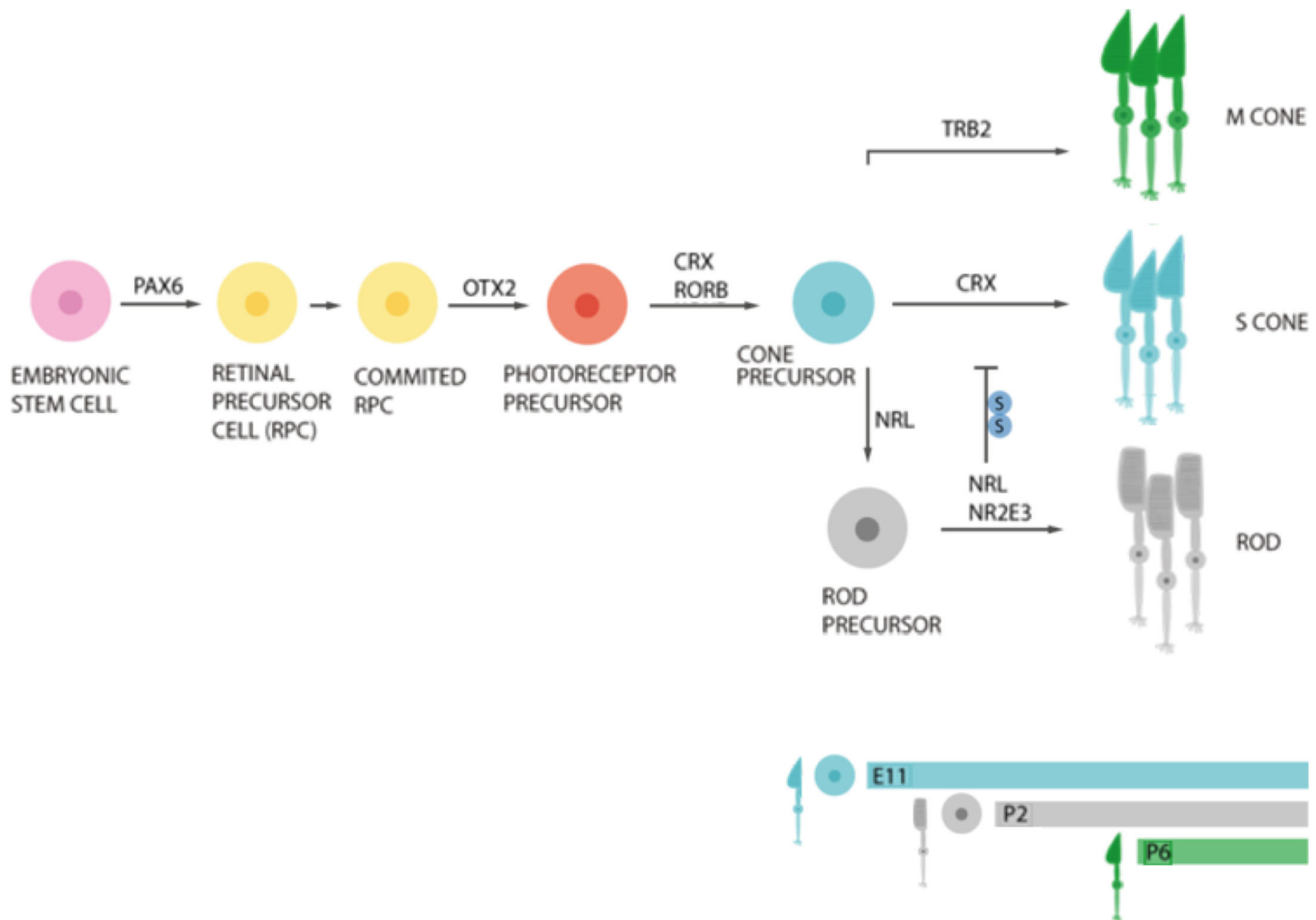

Figure 1. Diagram of murine photoreceptor development with key regulatory transcription factors. From embryonic stems cells, several transcription factors at specific developmental times are required to determine retinal precursor cells and eventually, give rise to fully mature photoreceptors. PAX6, 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 IIx 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.

Table 1. List of human and mouse DUBs used in transcriptomics analysis classified by families and alphabetical name.

USP				JAMM			
USP1	USP3	USP4	USP5	COPS5 (CSN5)	COPS6	EIF3H	MPND
USP6[a]	USP7	USP8	USP9	MYSM1	PRPF8	PSMD7	PSMD14 (POH1)
USP10	USP11	USP12	USP13	STAMBP (AMSH)	STAMBPL1 (AMSH-LIKE)		
USP14	UPS15	USP16	USP17L2[b]				
USP17LE[b]	USP18	USP20	USP21	**MJD**			
USP22	USP24	USP25	USP26	ATXN3	JOSD1	JOSD2	TAF1D (JOSD3)
USP27X	USP28	USP29	USP30				
USP31	USP32	USP33	USP34	**OTU**			
USP35	USP36	USP37	USP38	OTUB1	OTUB2	OTUD1	OTUD3
USP39	USP40	USP41[c]	USP42	OTUD4	OTUD5	OTUD6A	OTUD6B
USP43	USP44	USP45	USP46	OTUD7A	OTUD7B	PARPF11	TNFAIP3
USP47	USP48	USP49	USP50	VCPIP1	ZRANB1		
USP51	USP53	USP54	USPL1				
CYLD	PAN2 (USP52)	TBC1D1		**UCHL**			
				BAP1	UCHL1	UCHL3	UCHL5

[a]Gene identified only in the human genomes[b] Family of retrogenes derived from the USP17 gene [c] Gene identified only in the human genomes, paralogue of USP18

RNA-seq expression data reanalysis: Expression data on mouse tissues were obtained from public databases (ENCODE 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*, *Cns5,* 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): *Uchl1* shows a mirror expression pattern compared to *Uchl3* and *Bap1* in the developing mouse and human fetal retinas (*Uchl1* is highly expressed in human fetal retinas, whereas in mouse fetal retinas, *Uchl3 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*, *Mysm1*, *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 *Tnfaip3, 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

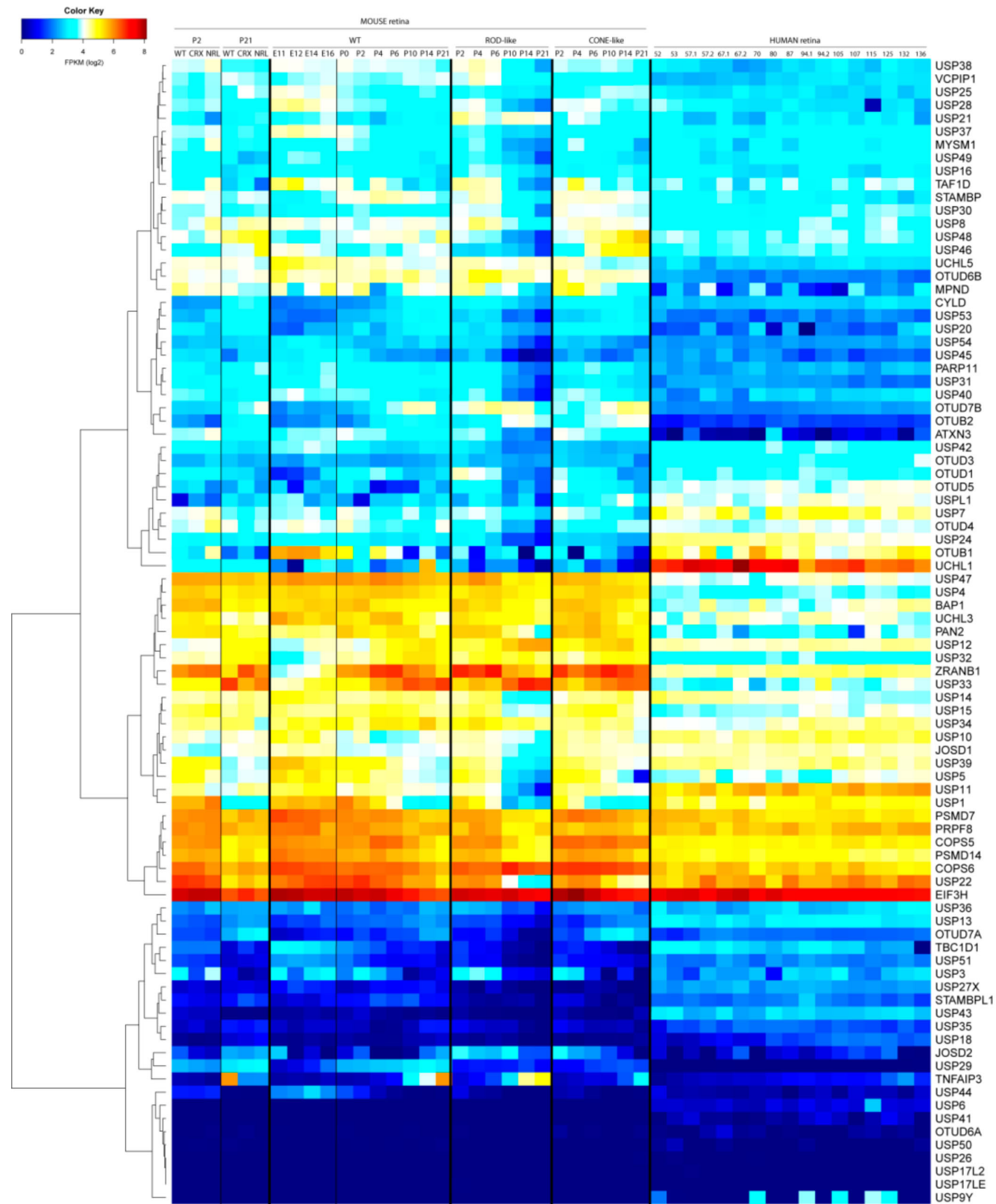

Figure 2. Expression heatmap of deubiquitinating enzyme genes in several human and mouse developmental stages. WT, wild-type mouse retinas; CRX, mouse Crx knockout (KO) retinas; NRL, mouse Nrl knockout retinas. For the mouse WT retinas, the data shown correspond to the embryonic 11 (E11), E12, E14, E16, 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 [a]	ChIP-seq[b]	RNA-seq on TISSUES [c]	PHENOTYPE[d]	BIOLOGIC PROCESS
JOSD1	Decrease during development in whole retina and in rods, but not in cones.	CRX	Adrenal Gland, Cerebellum, Spleen, Thymus	3 dpf: eyes shape, abnormal retino-tectal projection (ZF)	Involved in endocytosis. Almost no bibliography
OTUD7B	Low expression throughout development, small increase from P10. Continuous expression in rods.	—	Testis	—	Involved in NFκB signaling. Role as oncogene: via deubiquitination of EGFR
PAN2 (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 grastro-carcinoma, and Adrenal tumors, Regulation of NFκB 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; NFκB 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, hepatolcellular 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).	NFκB signaling. β2 adrenergic receptor recycling. Tyroid hormone activation.
USP22	Highly expressed throughout development with a shut down in rods from P10.	CRX	Testis	(Neuronal) 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 [a]	ChIP-seq[b]	RNA-seq on TISSUES [c]	PHENOTYPE[d]	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, Mammal 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[e]. Corticoph adenomes[f]

[a]RNA-seq on total retina lists the expression features observed in the RNA-seq data.[b]ChIP-Seq "CRX" and "NRL" indicate that the gene promoter was bound by CRX or NRL, respectively, in ChIP assays. [c]RNA-seq on tissues list tissues in which the DUB gene is mainly expressed. [d]Phenotype includes the phenotypic traits reported in different model organisms caused by mutations in these genes [3,30,34]. [e] [35]. [f] [36]. (D): Drosophila (H): Human; (M): Mouse; (ZF): Zebrafish.

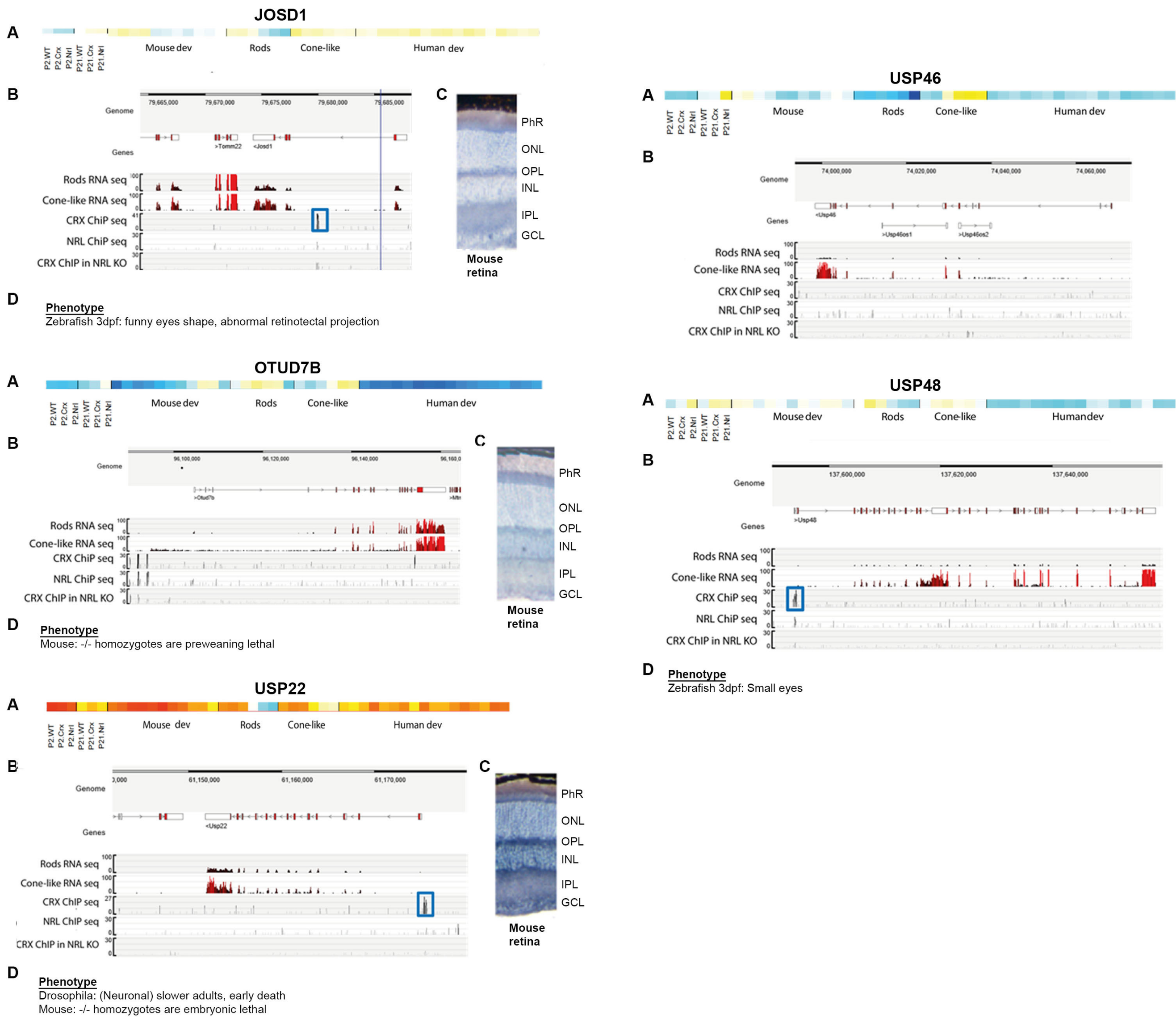

Figure 3. Diagram showing the criteria considered for the selection of five relevant DUB genes in the retina. For each of the selected genes, *Josd1, Otud7b, Usp22, Usp46*, and *Usp48*, the composite images show the following. **A**: Transcriptome analysis of each selected gene, extracted from Figure 2. **B**: Track view of CRX- and NRL-chromatin immunoprecipitation sequencing (ChIP-seq) density profiles (after CRX and NRL immunoprecipitation, respectively) using postnatal day 28 (P28) wild-ty[e (WT) mouse retinas, visualized using the UCSC genome browser (https://neicommons.nei.nih.gov/). **C**: Pattern of expression with in situ hybridization in WT mouse retinas (reported in [30]). PhR, photoreceptor cell layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. **D**: Described phenotypes in either knockout or knockdown animal models (references in Table 2).

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*, *Zranb1*, 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*, *Zranb1,* 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.

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