Transcriptome Analysis of Newt Lens Regeneration Reveals Distinct Gradients in Gene Expression Patterns

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Introduction

Amphibians, especially newts, possess regenerative capabilities that are missing in higher vertebrates. Newts can regenerate their limbs, brain, heart, tail with spinal cord and other tissues. Interestingly, newts can also regenerate the lens after its complete removal (lentectomy). This system provides many advantages for regenerative studies because the whole organ (lens) is being removed. Lens regeneration occurs from the iris by a process that involves the transdifferentiation of pigmented epithelial cells (PECs) to lens cells. Another interesting aspect of this process is that lens regeneration occurs only from the dorsal and never from the ventral iris. This allows the use of the ventral iris as a natural non-regenerative control in lens regeneration experiments [1,2,3]. Two major hallmarks of lens regeneration are the re-entry of the cell cycle, 4 days post-lentectomy (dpl) and the formation of a dedifferentiated vesicle 8 dpl [4]. Interestingly, both dorsal and ventral iris cells re-enter the cell cycle [5]. In the past, limited expression studies, either using individual gene probes or small-scale microarray analysis have indicated that dorsal and ventral expression show no major differences in gene expression. In other words, most of the examined genes were expressed in both irises.

Thus, to date no clear expression pattern has emerged to account for the ability of the dorsal iris to be the source of the regenerating lens. More recently a microarray analysis during early stages of lens regeneration was performed. In that study expression in 1, 3 and 5 dpl from the dorsal or the ventral iris was compared with the corresponding intact iris (0 day). While that study indicated regulation in genes related to DNA repair, extracellular matrix and redox homeostasis, direct comparisons between gene expression in dorsal and ventral iris could not be assessed. Thus, a direct comparison of transcriptomes was needed to delineate global gene expression differences between dorsal and ventral iris during lens regeneration in newts [6,7].

Next-generation high-throughput techniques allow transcriptome analysis based on de novo assemblies, making them extremely useful for non-model systems like the newt. Here, we investigate transcriptional changes during newt lens regeneration in an attempt to identify patterns that provide clues for the ability of dorsal iris but not the ventral to transdifferentiate. We focused on 4 dpl and 8 dpl for both dorsal and ventral iris as these time points are crucial stages for lens regeneration because these time points encompass the events of cell cycle re-entry and dedifferentiation. For RNA-seq we used a de novo assembled transcriptome making use of short Illumina and longer 454 and Sanger reads [8].

Abstract

Regeneration of the lens in newts is quite a unique process. The lens is removed in its entirety and regeneration ensues from the pigment epithelial cells of the dorsal iris via transdifferentiation. The same type of cells from the ventral iris are not capable of regenerating a lens. It is, thus, expected that differences between dorsal and ventral iris during the process of regeneration might provide important clues pertaining to the mechanism of regeneration. In this paper, we employed next generation RNA-seq to determine gene expression patterns during lens regeneration in Notophthalmus viridescens. The expression of more than 38,000 transcripts was compared between dorsal and ventral iris. Although very few genes were found to be dorsal- or ventral-specific, certain groups of genes were up-regulated specifically in the dorsal iris. These genes are involved in cell cycle, gene regulation, cytoskeleton and immune response. In addition, the expression of six highly regulated genes, TBX5, FGF10, UNC5B, VAX2, NR2F5, and NTN1, was verified using qRT-PCR. These graded gene expression patterns provide insight into the mechanism of lens regeneration, the markers that are specific to dorsal or ventral iris, and layout a map for future studies in the field.

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Competing Interests: The authors would like to state that P.A. Tsonis is a PLOS ONE Academic Editor. This does not alter their adherence to all the PLOS ONE policies on sharing data and materials as detailed in our guide for authors.
Here we report and for the first time the expression of more than 38,000 annotated transcripts in dorsal and ventral iris during lens regeneration. The analysis has been focused on the quantitative and qualitative differences between dorsal and ventral iris of those transcripts. We found very few genes to be dorsal or ventral iris-specific. However, certain cohorts of genes grouped according to their function were found to be preferentially up-regulated in the dorsal iris. Genes involved in the cell cycle, transcriptional apparatus, cytoskeleton and immune response are among those with much higher expression in the dorsal than the ventral iris. This graded expression might provide robust regulation that allows the dorsal iris to “win” over the ventral iris.

Methods

Animals - Lentectomy

Handle and operations on Notophthalmus viridescens have been described previously [6]. Briefly, newts were purchased from Charles Sullivan Inc. Newt Farm. Newts were anesthetized in 0.1%(w/v) ethyl-3-aminobenzoate methanesulfonic acid (MS222; Sigma) in phosphate buffered saline. Lentectomy was performed using a scalpel to incise the cornea and tweezers to pull out the lens through the incision. For the present study newts were kept for 4 and 8 dpl before tissue harvest.

Ethics Statement

All procedures involving animals were approved by the University of Dayton Institutional Animal Care and Use Committee (IACUC; Protocol ID: 011-02). All surgical procedures were performed in anesthetized with MS222 newts. All appropriate procedures were used in order to alleviate pain and distress while working with newts.

Tissue Harvest and RNA Extraction for qRT-PCR

4 or 8 dpl newts were anesthetized in MS222. Whole eye balls were removed and placed in dishes filled with RNAlater® Solution (Applied Biosciences). Using fine scissors and tweezers, eye balls were dissected first by separating the anterior from the posterior part (Figure 1A) and then by removing remaining neural retina and the ciliary body from the anterior part (Figure 1C). Dorsal or ventral iris sectors were collected in approximately 135° of the whole iris leaving out a board area between dorsal and ventral side which has a black-colored pigmentation (Figure 1B). Dorsal and ventral sectors were then collected in microcentrifuge tubes filled with RNAlater® Solution. The tubes were briefly centrifuged and RNAlater® Solution was completely removed. RNA extraction was performed following TRIzol® Reagent protocol (Applied Biosciences) for 500 μl of reagent or the aqueous phase was transferred to RNA Clean & Concentrator™ (Zymo Research) columns and the recommended protocol was followed. Quality of isolated RNA was determined by Nanodrop 2000 spectrophotometer (Thermo Scientific). Good quality samples had A260/A280 ratio greater than 2 and a peak at 260 nm.

Reverse Transcription Reaction (RT)

200 ng total RNA was used for RT reactions. First-strand cDNA synthesis kit (GE healthcare) was used following the recommended protocol for oligo(dT) primers. Half the volumes were used for negative RT reaction without using oligo(dT) primers and the samples were incubated for 5 min at 98°C for enzyme inactivation. All the samples had a clear band after Polymerase Chain Reaction (PCR) with RPL27 gene (housekeeping gene). Dorsal samples needed to be positive for TBX5 and negative for VAX2. Ventral samples needed to be positive for VAX2 and negative for TBX5.

Figure 1. Diagram for collecting iris pieces. A. Whole eye ball with anterior side facing up and ventral facing the screen. Iris appears in the anterior side. Red dashed line indicates the plane that anterior and posterior sides are separated. B. Anterior view of a newt’s anterior part separated previously. Arrow head indicates black pigments present in the dorsal side of the eye. Arrow indicates the v-shaped pupil in the ventral side. These marks are indicative of the dorsoventral axis of the iris. Red dash lines indicate the separation of dorsal and ventral iris pieces performed while in the anterior view of the eye. C. Posterior view of a newt’s anterior part separated previously. Red dash lines indicate the separation of ciliary body and iris performed in this view. Transparent white dash lines indicate the separation of dorsal and ventral iris sectors performed in the anterior view. di: Dorsal iris sectors that have been isolated for the experiment, vi: Ventral iris sectors that have been isolated for the experiment. m: pigmented midline, cb: ciliary body, pu: pupil. Orientation in each panel is indicated above the illustrated eye parts, a: anterior side, d: dorsal side, v: ventral side, p: posterior side.

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Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

qRT-PCR was performed using IQ™ SYBR® Green Supermix (Bio-Rad) and Bio-Rad iCycler (Bio-Rad) following company’s protocol for 25 ml. Primer specificity was determined using melt curve analysis. An extra cycle of 6 sec was added to genes that were showing detectable signal from primer dimers and the temperature was determined by the melt curve (usually 2–4°C lower than the melting temperature; see methods below). Amplification cycles (Ct) of samples were compared to Ct of a standard curve created by the cDNA of the gene used. Gene expressions were then normalized to the expression of the reference gene (RPL27).

Primers, PCR and qRT-PCR Settings

For the present study the following primers were used (written from 5’ to 3’): TBX5 Forward: CTGCCATGCCAGGG-CGGTTG. TBX5 Reverse: GGTCGTGGGCAGGAGGTCCT. VAX2 Forward: TGAGTGCCAGCGCCACCTAACC. VAX2 Reverse: AGGTCCCCAAGCCGTACCCC. FGF10 Forward: GTGTGTCGTCACCAACTACT. FGF10 Reverse: TTGCTTTCTACGCCCCTCAC. NR2F5 Forward: CGGAACCTGAGCTACACCTG. NR2F5 Reverse: GGGAGATGAACCCCGTCAAG. UNC5B Forward: AGTCCAACCGGGGTGATCCTG. UNC5B Reverse: CATCTCGCTCTTGCCCATCTCC. NTN1 Forward: GGTTGCTCCACCCACTACAG. NTN1 Reverse: ACCATTCTCCAGCCTTGTCAG. RPL27 Forward: ATTATTGATGAAACCCGGGAAGG. RPL27 Reverse: CCAGGGCATGACTGTAAGGT. PCR performed with Premix Taq™ DNA polymerase (TaKaRa)) settings for TBX5: 40 cycles including 95°C for 30 sec, 65°C for 30 sec and 72°C for 30 sec. VAX2: 40 cycles including 95°C for 30 sec, 64°C for 30 sec, 72°C for 30 sec. RPL27: 40 cycles including 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec. Last extension was 72°C for 10 mins for all the genes.

qRT-PCR settings for TBX5: 95°C for 3 mins, 40 cycles of 95°C for 30 sec, 65°C for 30 sec, 72°C for 30 sec and 85.5°C for 6 sec. VAX2: 95°C for 3 mins, 40 cycles of 95°C for 30 sec, 64°C for 30 sec and 86.5°C for 6 sec. FGF10: 95°C for 3 mins, 40 cycles of 95°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec and 84.5°C for 6 sec. NR2F5: 95°C for 3 mins, 40 cycles of 95°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec and 82°C for 6 sec. NTN1: 95°C for 3 mins, 40 cycles of 95°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec and 82°C for 6 sec. RPL27: 95°C for 3 mins, 40 cycles of 95°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec and 82°C for 6 sec. NTN1: 95°C for 3 mins, 40 cycles of 95°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec and 82°C for 6 sec. RPL27: 95°C for 3 mins, 40 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec.

Statistical Analysis for qRT-PCR Results

Statistical analysis was performed using two-way analysis of variance (ANOVA) and Student’s t-test for independent samples. Samples were run in triplicates (n = 3). Statistical significance was determined with 95% confidence (p<0.05). Equal variances for student’s t-test were assumed when Levene’s test p value was greater than 0.05.
**Table 1.** List of up-regulated (>2 times) transcripts related to cell cycle*.

| Function       | Dorsal                       | Ventral                      |
|----------------|------------------------------|------------------------------|
| Mitosis        | CNTRL PLK1 CLASP1 RAD21 NUSAP1 SMC4 NCO1 |                             |
|                | KIF11 Asun STAG1 KATNB1 PAR3 TPX2 |                             |
|                | TACC3 CDCA8 CEP120 KIF2C ZW1 CH1 UBE_E1 |                             |
|                | TUBB CENPF NCAPD2 NDC80 NEK3 ASPM |                             |
|                | RAB35 CENPF NCAPG SPC24 SGOL1 SMC3 |                             |
|                | ROCK1 CEP192 ERC6L SPC25 DSCC1 AURKB |                             |
|                | NUP43 NEDD1 NCAPG2 KNTC1 SKA1 MADL21 |                             |
|                | NUF2 NNUMA1                  |                             |
| tumor suppressor| PSMD10 XRN1 HBP1 MDM4 CENPF MRPL41 PTEN |                             |
|                | CCA1 APC LIN9 E4F1 LIN9 TRRAP |                             |
| Interphase     | MNAT1 CUL4B CDA2 POLA1 MCM3 MCM7 LIN9 NRRF2 |                             |
|                | CDC25A CCA2 CDA2 POLA1 MCM4 UHRF1 HECD3 |                             |
|                | CDA1 CCNB1 POLE MCM5 HEXIM1 MRPL41 |                             |
|                | CENPF CCNE2 MCM2 MCM6 DLTAP5 SMAD6 |                             |
| DNA repair     | HERC2 LIG1 GTF2H1 RAD1 TOL1 TOP2A RNF8 |                             |
|                | CLSN FANCI H2AFX CHEK1 RADS |                             |
| APC/C complex  | ANAPC1 ANAPC7 FZR1+ CDA20 UBE2C UBE2S FZR1 |                             |
|                | ANAPC13 CDC27               |                             |
| proliferation  | BOP1 CGRRF1 GST HBP1 PHIP SMARCA2 VASH1 |                             |
|                | BMP2 NR2F2 FGM10 GTBP1 PRDM4 STRADA |                             |
|                | BAX DTYMK CDC67             |                             |

GO:0007049 cell cycle; GO:0022403 cell cycle phase; GO:0002278 mitotic cell cycle; GO:0022402 cell cycle process; GO:0000087 M phase of mitotic cell cycle; GO:0051301 cell division; GO:0000279 M phase; GO:0007067 mitosis.
*Transcript names are from their human homologs.
†Potential isoforms.
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Newt Transcriptome, Data Mining and Functional Annotation

The newt transcriptome [8] was annotated with the BLAST2GO tool [9] using the nr database. We used a cutoff of (e<10) for sequence assignments, collected annotations and corresponding GO terms [10]. Transcripts were selected depending on their expression and location in the iris as described in Figure 2 for comparing dorsal iris versus ventral iris groups and as described in Figure 4 for comparing day 4 and day 8 groups. Fisher’s exact test corrected for multiple selections (feature available in BLAST2GO tool) was used for the different groups and statistically significant enriched GO terms were identified (FDR <0.05). Transcripts assigned to enriched terms were selected. Human homologues of those transcripts were found using the BLAST tool [11].

RNA Expression Calculation

Illumina reads were mapped to the newt transcriptome using BWA [12]. Reads per kilobase per million mapped reads (RPKM) values were calculated for each transcript having at least one unique mapping read. Since intron/exon data are missing for the newt, we used a recently published microarray experiment for RPKM cutoff estimation [6]. We selected transcripts presented on the microarray having a valid spot structure (value for circularity >80%, intensity variation within the spot very small, flagged as valid) but lacking a significant signal during microarray analysis (signal to noise ratio (snr) <1, significant spots are used that have a snr ratio >3). We detected 101 spots to be valid for these parameters. Mapping of array coordinates to the transcriptome resulted in 81 non redundant individual transcripts. We assumed these candidates to be a good estimate for RPKM cutoff selection and calculated the average RPKM value for this transcript set for each investigated timepoint. We received a RPKM value of 0.64 for 4dv, 1.14 for 4dd, 1.13 for 8dv and 1.1 for 8dd. Illumina sequencing raw reads can be found in the NCBI sequence read archive under the accession: ERP001353 [8]. Assembled transcripts and annotation are located in the newtomics database [8,13].

Results and Discussion

Transcriptome and Analysis Overview

The recently assembled newt transcriptome contains nearly 38,000 annotated genes [8]. After Illumina sequencing, the reads from the dorsal and ventral iris 4 and 8 dpl were mapped to the new transcriptome and RPKM values were calculated. Since the newt genome is not yet available and reads that map to non-coding areas can not been found, we calculated the RPKM cutoff using microarrays that were previously used during lens regeneration (see methods for more details). The cutoff in the RPKM values were 0.64 for ventral iris 4 dpl, 1.14 for dorsal iris 4 dpl, 1.13 for ventral iris 8 dpl and 1.1 for dorsal iris 8 dpl. Transcripts
Table 2. List of up-regulated (>2 times) transcripts related to gene regulation*.

| Function     | Dorsal       | Ventral       |
|--------------|--------------|---------------|
| Transcription| ATF6, CNBP,  | ZNF3, SMAD6,  |
|              | POLR2A, FOXN2| NRR2F1        |
|              | APP, CBY1,   |               |
|              | POLR2J, GATA1| MYCN, POLR1A* |
|              | ARNTL, NR2F1|               |
|              | TF2H1, KLFL  |               |
|              | ZNFX1, HIPK2 |               |
|              | ARID4A, CBX1 |               |
|              | DPF2, TF2H2  |               |
|              | LANC2L, NFBK2|               |
|              | KIAA201B, CHD7|             |
|              | E2F3, RAD54L2|               |
|              | LOR5, NR4A1  |               |
|              | BRWD1        |               |
|              | BRMS1L, NR2F2|               |
|              | RN20, HEXM1  |               |
|              | LIMD1, NR6A1 |               |
|              | MYPOP        |               |
|              | BAHD1, CRT2C |               |
|              | UHRF1, HBP1  |               |
|              | LMX1B, NR1H2 |               |
|              | NRR2F2       |               |
|              | MYCBP, CREG3L2|              |
|              | IK3KAP, HMX1 |               |
|              | MEF2B, NRR2C2|               |
|              | DENND4A, CYLD|               |
|              | EL3, HIPK3   |               |
|              | MED12, NFBY  |               |
|              | NFE2L1       |               |
|              | MNTA1, POLR1A*|             |
|              | EL2, HIPK2   |               |
|              | MED23, ESI1  |               |
|              | NR2F1, POLR1D|               |
|              | POLR1C       |               |
|              | PF60, PHTF2  |               |
|              | PHF6, PPARG  |               |
|              | PWP1, PRMD2  |               |
|              | PRDM4, PQBP1 |               |
|              | POLR1B, PHRF1|               |
|              | PHF12, PPARA |               |
|              | MLLT3        |               |
|              | XAB2, BUD31  |               |
|              | RCR02, SMARC4|               |
|              | RELB, BRF1   |               |
|              | ETS2         |               |
|              | PFDN5, LBH   |               |
|              | RARA, TBX5   |               |
|              | TAF2P2, TCF4 |               |
|              | RAX          |               |
|              | CIAO1, MDM4  |               |
|              | RXRA, BFA1   |               |
|              | TF2P2C, TF81M|               |
|              | RGMB         |               |
|              | PHB2, RREB1  |               |
|              | STAT6, TEAD1 |               |
|              | E4F1, TAF1F  |               |
|              | LEO1         |               |
|              | ZEB1, ZGAPAT|               |
|              | TWISTNB, TRM33|             |
|              | TLE3, TAF2   |               |
|              | SPEN         |               |
|              | ZFHX4, ZBTB5 |               |
|              | WWR1, TP53BP1|               |
|              | TLE4, TADA1  |               |
|              | SOX6         |               |
|              | ZKSCAN1, ZIC1|               |
|              | RL, SLC30A9  |               |
|              | CNOT6, CHAF1A|               |
| Histone      | BRMS1L, RN20 |               |
| Modifications| KAT5*, SETD81| KDM2A, MLL3   |
|              |              | ARID1A*       |
|              | ARID4A, TOPORS|              |
|              | MYSM1, SUV420H1| KDM2B, NAA16 |
|              | KDM6B, KDM18 |               |
|              | NCOA3, MLL2  |               |
|              | CHD4, EZH2   |               |
|              | MLL, ARID1A* |               |
|              | MBTD1, NCOA3 |               |
|              | ARID4A, KAT2B|               |
|              | WHSC1L1, MLL5|               |
|              | MTA3, PHF10  |               |
|              | KDM5C        |               |
|              | WHSC1, SMARCA2|              |
|              | TRIM28, VPS72|               |
|              | PHF2, PHF12  |               |
|              | NCOA1        |               |
|              | RBBP5, SMARCA4| TADA3, YEATS2 |
|              | ASXL3, ZGAPAT|               |
|              | SIRT6, SMARC2C|              |
|              | TRRAP, YY1   |               |
|              | --           |               |
| RNA          | XR11, CPSF3  |               |
|              | HEATR1, INTS6| WDR77, PPW1D  |
|              | DX23, HNRPNPA|               |
|              | AQR*, MBNLA2 |               |
|              | PUF60, AQR*  |               |
|              | DBR1, RB1    |               |
|              | RBBP5, SMARCA4| TADA3, YEATS2 |
|              | ASXL3, ZGAPAT|               |
|              | SIRT6, SMARC2C|              |
|              | TRRAP, YY1   |               |
|              | --           |               |
|              | SF1, SRST11  |               |
|              | SRRM1, RBM28 |               |
|              | RP9, DDX41   |               |
|              | SF5WAP, SNRPA1|              |
|              | RSC1, RBM5   |               |
|              | NOP2, UTP11L |               |
|              | SCA1, UTP6   |               |
|              | SRF12, SARNP|               |
|              | RBFOX2, PUM1 |               |
|              | --           |               |
|              | SNIRPN, THOC2|               |
|              | THOC6, TFB1M |               |
|              | TF1P11, MPP6OH10 |           |
|              | --           |               |
| translation   | MRPS18B, MRPL14|              |
|              | GFM1, EIF2B5 |               |
|              | MRPL17, GTPBP4|              |
|              | MRP14        |               |
|              | RPS27L, EEF2K|               |
|              | QRS1L, RPS23 |               |
|              | RPS5K2B2*    |               |
|              | MRPS5        |               |
|              | CPEB2, EEF1E1|               |
|              | IARS, MRPS1 |               |
|              | MRPL41, PDDC4|               |
|              | --           |               |
|              | MRPL15, DUS3L|               |
|              | EIF2D, MRPL12|               |
|              | MRPS9, TRUB2 |               |
|              | --           |               |
|              | PUS10, PUS7  |               |
|              | TRM61A, TAR5 |               |
|              | GM2, SRP14  |               |
|              | --           |               |
|              | TRNAU1AP, RPL3|               |
|              | NSP2U, EIF2B1*|              |
|              | EFSEC, EIF2B2|               |
|              | --           |               |
|              | DUS2L, RPS6KB2*|              |
|              | TRM72A, --   |               |
|              | --           |               |
| miRNA        | DICER1, MOV10|               |
|              | PNPT1, EIF2C3| TNRC6A, TNRC6C|
| Other        | NEO1, AHS1A  |               |
|              | AARSD1, A2M  |               |
|              | DYNC2H1, SIRT5|              |
|              | PRKDC        |               |
|              | TACC3, PPIH  |               |
|              | BAX, AKT2    |               |
|              | EIF4ENIF1, PCSK1|            |
|              | HTT*         |               |

*These are genes involved in gene regulation and their functions are related to transcription and histone modifications.
found to have RPKM more than the cutoff were considered to be expressed and were taken into account for the comparisons below.

Dorsal Iris Shows Enrichment of Up-regulated Genes Involved in Cell Cycle, Cytoskeleton, Gene Expression and Immune Response

Since the major purpose of this study is to identify patterns of gene expression in the dorsal and ventral iris that might correlate with the ability of the dorsal iris for transdifferentiation, we first looked into transcripts that were regulated either in the dorsal or in the ventral iris at both collection points (4 dpl and 8 dpl). Analysis of the results was performed as outlined in Figure 2.

Interestingly, we only saw a handful of genes that are either exclusively expressed (no reads were mapped to them) in the dorsal or the ventral iris. This finding was not surprising because in the past our laboratory had seen, using limited expression data, that

Table 2. Cont.

| Function | Dorsal | Ventral |
|----------|--------|---------|
| CENPF | PPWD1 | FG10 | TNFSF13B | MYO6 | SERPINE1 | INPPL1* |
| C3 | PFDN5 | HTT* | BMP2 | PEX1 | NDFIP2 | PTEN |
| CCNA2 | CDK2 | MAPKAPK2 | CCAR1 | SORT1 | NLK | RPS6KA4 |
| PSEN1 | DISP1 | RRAGC | RIP1 | MTO1 | PHP | PCM1 |
| EIF1AD | PPP3CB | RASSF8 | ATP6AP2 | TRIP11 | INPPL1* | XPO5 |
| TBL3 | TLR2 | — | — | — | — | — |

GO:0010467 gene expression; GO:0010468 regulation of gene expression; GO:0006350 transcription; GO:0045449 regulation of transcription.

*Transcript names are from their human homologs.

Potential isoforms.

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Table 3. List of up-regulated (>2 times) transcripts related to cytoskeleton*.

| Function | Dorsal | Ventral |
|----------|--------|---------|
| microtubule | CNTRL | TUBA1A | CEP120 | DNAH7 | KIF22 | KNTC1 | CLIC5 |
|            | SSNA1 | BBS2 | CYLD | TUBGCP6 | KIF23 | DYNL1 | MARK1 |
|            | CHEK1 | TUBB | DYNC1I2 | HTT* | KIF2C | AURKB | HTT* |
|            | CENPF | TUBB4B | DYNC2H1 | IFT57 | KIF14 | LYST | PCMI |
|            | KIF11 | CAM5AP3 | DYNC1LI1 | KATNB1 | KIF20A | MAP1B | KIF1B |
|            | PLK1 | CEP170 | CLIP2 | KIAA1279 | KIF13A | MACF1 | KIA0284 |
|            | APC | CLASP1 | DNA2 | KIF13B | NDC80 | MAST2 | NOCR1 |
|            | P5K1H | SM3 | CCNB1 | CDC27 | TUBB3 | PLK4 | — |
|            | ARL2BP | RIF1 | MAD2L1 | CEP350 | TUBD1 | SNB2 | — |
|            | TPX2 | ASPA | NUP85 | CEP192 | ZNF415 | SKA1 | — |
|            | CDCA8 | CENPE | RANGAP1 | CBX1 | NEDD1 | DCX | — |
|            | NIN | NINL | NUMA1 | NUSAP1 | SHROOM2 | SHROOM3 | — |
|            | RAB3IP | AURKA | — | — | — | — | — |
| actin | WASHI | CTNNA1 | MYO1E | MYO5A | PLEK2 | VASP | ACTA2 |
|          | ARPC1B | INT56 | MYO9A | MYO7A | DIAPH1 | WDR1 | INPPL1* |
|          | ARPC5L | IQGAP1 | MT551 | PPP1R9A | SSH2* | INPPL1* | SSH2* |
|          | ACTR6 | KLHL3 | MYO1G | MYO1D | RAB3IP | SNB2 | RDX |
|          | SCIN | MYO10 | MYO6 | PDLIM5 | IQGAP2 | SYNE1 | TPM1 |
| Other | CN22 | LANCL2 | MYO9B | MACF1 | ROCK1 | — | UTRN* |
|          | MPP1 | GAN | LMNB1 | SYNM | NE5 | BAG1 | DES |
|          | FERMT3 | DLGAP5 | NF2 | UTRN* | TNS1 | UBR4 | RA14 |
|          | GNE | RAPH1 | NF1 | CORO1A | ANK3 | SLC26A5 | SLC4A1 |
|          | APP | FRMD6 | TRIB2 | PTPN14 | SLC30A9 | STML2 | MICALL2 |
|          | PSEN1 | — | — | — | — | — | — |

GO:0005856 cytoskeleton; GO:0015630 microtubule cytoskeleton; GO:0044430 cytoskeletal part.

*Transcript names are from their human homologs.

Potential isoforms.

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the ventral and dorsal irises show similar patterns of gene expression (see below).

Next, we investigated potential patterns of up-regulated genes. Specifically we asked which genes are consistently up-regulated in the dorsal or the ventral iris (both at 4 and 8 dpl). Fisher’s exact test with multiple testing correction for Gene Ontology (GO) from transcripts that are up-regulated at least 2 fold in dorsal iris 4 and 8 dpl compared to the ventral iris, and transcripts that are up-regulated at least 2 fold in ventral iris 4 and 8 dpl compared to the dorsal iris are shown in Table S1. Interestingly, we found that more GO terms are enriched in the dorsal samples than in the ventral. In particular, GO terms related to cell cycle, regulation of gene expression, cytoskeleton and immune response were over-represented. In contrast, ventral samples, which generally generated fewer enriched GO terms, primarily showed GO terms related to transposons like RNA-directed DNA polymerase family. These include a number of proteins that play a role in functions during mitosis [16], and AURKB and CDCA8 are up-regulated. CPC is related to centromere formation [14], and NDC80, SPC24, SPC25 and NUF2 are up-regulated including NDC80 complex, cohesin complex and chromosomal passenger complex (CPC). NDC80 and SGOL1 are up-regulated. Anaphase promoting complex/cyclosome (APC/C) is a complex that is instrumental for mitosis progression and division [17]. There are 8 APC/C-related transcripts which are up-regulated in dorsal samples: ANAPC1, ANAPC13, ANAPC7, CDC27, FZR1, UBE2S, CDC20 and UBE2C.

Interphase: Factors related to all phases of the interphase are up-regulated in dorsal samples. Cyclins and cyclin-dependent kinases that play a key role in G1/S, G2/M, G1 and S phases are up-regulated including CCNA2 [18], CCNB1 [19], CCNE2 [20], CDK1 [21] and CDK2 [22]. Proteins that act upon cyclins and cyclin-dependent kinases are up-regulated too, including MNAT1 [23], CDC25A [24] and HEXIM1 [25]. Factors that play a role in DNA synthesis during the S phase are up-regulated including POLA1, POLE [26], all the MCM complex (MCM2-7) [27], NASP [28], DSCC1 [29], CHAF1A and CHAF1B [30].

Tumor suppression, proliferation-related, p53/TP53-associated and RB1-associated proteins are up-regulated in dorsal iris. These proteins promote or repress proliferation and cell cycle. It has been previously shown that newt muscle cells re-enter the cell cycle after inactivation of RB. After entering the S phase these cells were resting in G2 phase. So, it was hypothesized that factors should be expressed, which promote the G2/M checkpoint after phosphorylation of RB [31]. Our data suggests that transcripts that regulate the G2/M transition and thereby proliferation include CDC25A, CDC1, CCNB1, the APC/C complex and PLK1.

Factors playing a role in DNA repair need to be seen in the context of cell proliferation and cell cycle, since the failure to repair DNA damage will prevent proliferation. In previous studies using qRT-PCR, it was shown that rad1 is up-regulated at 3 and 5 dpl in the dorsal compared to ventral iris showing activation of robust DNA repair mechanism to prevent accumulation of mutations in dividing cells [6]. A similar pattern emerged in the current RNA-seq analysis: many factors that play a role in DNA repair are at least 2 fold up-regulated in the dorsal compared to the ventral irises. Among them RAD1 along with CHEK1, a kinase that has a key role in cell cycle arrest and apoptosis decisions after DNA damage [32].

**Gene Regulation-related Transcripts**

It is expected that the process of transdifferentiation is marked by activation and regulation of many genes of the transcriptional apparatus. Indeed, we do find many genes related to gene regulation. Such genes are listed and categorized in Table 2 depending on the level of gene expression that they regulate (transcription, histone modification, RNA, translation). Again the majority of these genes show up-regulation in the dorsal iris (314 transcripts) and only a few show up-regulation in the ventral (39 transcripts). These include a number of proteins that play a role in

| Dorsal | Ventral |
|--------|--------|
| A2M | TNFSF13B |
| SIVA1 | TUBB |
| RELB | ENPP2 |
| SYK | FCN1 |
| ZEB1 | GPR1B3 |
| TUBB4B | CTNBL1 |
| CADM1 | STAT6 |
| PPP3CB | INPP5D |
| C15 | RARA |
| INPP11* | TLR7 |
| C1QB | PSEN1 |
| CHUK | INPPL1 |
| TOPORS | -- |
| DDC20 | -- |
| TIMM50 | -- |

**Table 4. List of up-regulated (≥2 times) transcripts related to immune response***.

**Note:** Transcripts are from their human homologs.

**Table 5. List of up-regulated (≥2 times) GO terms related to immune response***.

**Table 6. List of up-regulated (≥2 times) GO terms related to transposons***.
### Table 5.
A selected list of 50 transcripts highly up-regulated in the dorsal iris.

| Transcript ID | Annotation (Blastx against nr) | v4 | d4 | v8 | d8 | log2Fc4 | log2Fc8 | log2Fc | log2Fc |
|---------------|---------------------------------|----|----|----|----|---------|---------|--------|--------|
| transcript114225 | ras-associated and pleckstrin homology domains-containing protein 1-like | 0.000 | 2.987 | 0.000 | 1.296 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript89311 | protein L (Lacoracetus jenynsi 296-3) | 0.000 | 6.490 | 0.000 | 1.292 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript53947 | similar to Chromatin assembly factor 1 subunit B | 0.000 | 5.182 | 0.000 | 1.986 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript75898 | pG1 protein | 0.331 | 5.828 | 0.151 | 19.812 | 4.140 | 7.038 | 5.735 | #DIV/0! |
| transcript41516 | probable E3 ubiquitin-protein ligase HERC2-like | 0.000 | 4.070 | 0.193 | 2.527 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript28206 | regeneration blastema forelimb-specific Tbx | 0.272 | 4.549 | 0.248 | 12.007 | 4.062 | 5.595 | 4.990 | #DIV/0! |
| transcript14449 | similar to TMEM116 protein | 0.335 | 1.492 | 0.485 | 20.092 | 2.156 | 5.373 | 4.719 | #DIV/0! |
| transcript12545 | hypothetical protein LOC432274 | 0.000 | 3.858 | 0.253 | 1.988 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript63962 | hypothetical protein LOC495396 | 0.290 | 4.756 | 0.000 | 1.765 | 4.038 | 4.493 | 4.332 | #DIV/0! |
| transcript80306 | ephrin-B2 | 0.302 | 4.602 | 0.358 | 8.955 | 3.930 | 4.645 | 4.361 | #DIV/0! |
| transcript87396 | programmed cell death 2 | 0.140 | 2.293 | 0.127 | 3.168 | 4.038 | 4.637 | 4.354 | #DIV/0! |
| transcript89253 | transmembrane protein 116-like | 0.163 | 1.691 | 0.653 | 14.078 | 3.374 | 4.430 | 4.272 | #DIV/0! |
| transcript59308 | DEP domain-containing protein 1B-like isoform 1 | 0.106 | 3.145 | 0.194 | 2.270 | 4.886 | 3.548 | 4.172 | #DIV/0! |
| transcript15984 | nuclear pore membrane glycoprotein 210 precursor | 0.000 | 1.977 | 0.183 | 1.155 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript88594 | similar to Thimet oligopeptidase | 0.000 | 3.005 | 0.298 | 1.991 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript82136 | WD repeat and FYVE domain-containing protein 3 isoform 1 | 0.000 | 2.541 | 0.252 | 1.684 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript92162 | netrin receptor UNC5B-like | 0.000 | 2.333 | 0.476 | 5.587 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript89179 | apoptosis-inducing factor 2-like | 0.000 | 4.743 | 0.387 | 1.246 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript22057 | dihydroxyacetone kinase 2 | 0.000 | 2.147 | 0.282 | 2.094 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript103122 | laminin subunit alpha-4-like | 0.000 | 5.539 | 0.513 | 2.030 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript57122 | calmodulin-regulated spectrin-associated protein 3 | 0.000 | 4.118 | 0.497 | 2.953 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript65968 | biphenyl hydrolase-like (serine hydrolase) | 0.000 | 3.044 | 0.476 | 2.953 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript87765 | ras and Rab interactor 2-like | 0.278 | 4.391 | 0.127 | 1.320 | 3.979 | 3.378 | 3.816 | #DIV/0! |
| transcript115898 | serine/threonine-protein kinase PLK4 | 0.000 | 3.900 | 0.467 | 2.426 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript105338 | epithelial cell transforming sequence 2 oncogene | 0.000 | 2.945 | 0.303 | 1.124 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript55938 | hypothetical protein | 0.000 | 7.267 | 0.811 | 3.277 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript23291 | serine/threonine-protein kinase WNK1-like | 0.210 | 3.866 | 0.240 | 1.850 | 4.201 | 2.949 | 3.668 | #DIV/0! |
| transcript93294 | 39S ribosomal protein L15, mitochondrial precursor | 0.155 | 4.783 | 0.353 | 1.574 | 4.948 | 2.015 | 3.636 | #DIV/0! |
| transcript11364 | glycerol kinase | 4.046 | 7.288 | 0.580 | 50.265 | 0.849 | 6.436 | 3.637 | #DIV/0! |
| transcript111723 | adenylate cyclase type 6 isoform 2 | 0.285 | 7.856 | 0.519 | 3.150 | 4.786 | 1.738 | 3.517 | #DIV/0! |
| transcript63503 | coiled-coil domain-containing protein 85C-like | 0.000 | 3.369 | 0.567 | 1.351 | 4.786 | 1.378 | 3.501 | #DIV/0! |
| transcript15530 | signal peptide, CUB and EGF-like domain-containing protein 3 | 0.000 | 2.314 | 0.270 | 1.124 | 4.786 | 1.738 | 3.517 | #DIV/0! |
| transcript50561 | cell cycle regulator Matk80 | 0.000 | 8.811 | 1.121 | 3.103 | #DIV/0! | #DIV/0! | #DIV/0! | #DIV/0! |
| transcript53997 | cell cycle regulator, homework | 0.000 | 1.982 | 0.270 | 1.124 | 4.786 | 1.738 | 3.517 | #DIV/0! |
Table 5. Cont.

| Transcript ID | Annotation (Blastx against nr) | v4  | d4  | v8  | d8  | log2Fc4 | log2Fc8 | log2Fc |
|---------------|--------------------------------|-----|-----|-----|-----|---------|---------|--------|
| transcript95772 | hypothetical protein [Gallus gallus] | 0.113 | 2.902 | 0.413 | 2.646 | 4.679 | 2.679 | 3.398 |
| transcript23822 | UPF0679 protein C14orf101-like [Sus scrofa] | 0.000 | 6.341 | 0.880 | 2.745 | #DIV/0! | 1.641 | 3.368 |
| transcript109593 | mesoderm development candidate 1 [Taeniopygia guttata] | 0.261 | 3.091 | 0.298 | 2.522 | 3.564 | 3.081 | 3.327 |
| transcript84524 | unnamed protein product [Tetraodon nigroviridis] | 0.000 | 1.879 | 0.356 | 1.497 | #DIV/0! | 2.073 | 3.246 |
| transcript25525 | fas apoptotic inhibitory molecule 1-like [Anolis carolinensis] | 0.000 | 7.291 | 0.973 | 1.783 | #DIV/0! | 0.857 | 3.218 |
| transcript47004 | LOW QUALITY PROTEIN: matrix-remodeling-associated protein 5-like [Pongo abelii] | 0.000 | 4.089 | 0.690 | 2.278 | #DIV/0! | 1.722 | 3.205 |
| transcript83773 | hypothetical protein [Ornithorhynchus anatinus] | 0.205 | 6.858 | 0.933 | 3.327 | 5.066 | 1.833 | 3.162 |
| transcript80809 | HIV-1 tat interactive protein [Danio rerio] | 0.221 | 3.551 | 0.402 | 2.017 | 4.009 | 2.325 | 3.160 |
| transcript101040 | LOW QUALITY PROTEIN: protein NEDD1-like [Anolis carolinensis] | 0.000 | 2.837 | 0.525 | 1.755 | #DIV/0! | 1.740 | 3.128 |
| transcript83315 | ras GTPase-activating-like protein IQGAP2 [Xenopus (Silurana) tropicalis] | 0.000 | 3.094 | 0.537 | 1.424 | #DIV/0! | 1.407 | 3.073 |
| transcript48784 | 30S ribosomal protein S14 [Chryseobacterium gleum ATCC 35910] | 0.761 | 10.280 | 0.644 | 1.509 | 3.756 | 1.228 | 3.069 |
| transcript19175 | hyaluronan synthase 2-like [Anolis carolinensis] | 0.205 | 7.412 | 1.975 | 10.789 | 5.175 | 2.450 | 3.062 |
| transcript90710 | vaccinia related kinase 1 [Xenopus laevis] | 0.000 | 5.855 | 0.889 | 1.320 | #DIV/0! | 0.570 | 3.013 |
| transcript119283 | neurobeachin-like protein 1-like [Anolis carolinensis] | 0.000 | 4.137 | 0.47 | 1.884 | 3.802 | 2.011 | 2.985 |

v4: RPKM value of ventral iris 4 dpl, d4: RPKM value of dorsal iris 4 dpl, v8: RPKM value of ventral 8 dpl, d8: RPKM value of dorsal 8 dpl, log2Fc4: fold expression at 4 dpl between dorsal and ventral iris, log2Fc8: fold expression at 8 dpl between dorsal and ventral iris, log2Fc: fold expression between dorsal and ventral iris at both the days.
Table 6. A selected list of 50 transcripts highly up-regulated in the ventral irises.

| Transcript ID | Annotation (Blasx against nr) | v4 d4 | v8 d8 | log2Fc4 | Log2Fc8 | Log2Fc |
|---------------|--------------------------------|-------|-------|---------|---------|--------|
| transcript93602 | retrotransposable element T2 155 KDa protein type 1-like, partial (Xenopus (Silurana) tropicalis) | 4.678 2 | 5.437 2 | 4.155 4 | 2.356 4 | 2.845 4 |
| transcript32521 | ventral anterior homeobox 2a-like (Xenopus (Silurana) tropicalis) | 8.640 2 | 4.081 2 | 3.304 2 | 1.029 2 | 3.413 2 |
| transcript26555 | nuclear receptor subfamily 2 group F member 5-like (Xenopus (Silurana) tropicalis) | 4.516 2 | 2.059 2 | 2.016 2 | 0.424 2 | 2.819 2 |
| transcript106073 | sorting nexin-25 (Monodelphis domestica) | 1.431 2 | 1.305 2 | 1.319 2 | 0.081 2 | 1.305 2 |
| transcript67285 | zinc finger protein 850-like (Monodelphis domestica) | 8.679 2 | 4.885 2 | 4.707 2 | 1.029 2 | 4.707 2 |
| transcript44589 | reverse transcriptase (Anguilla japonica) | 2.819 2 | 2.690 2 | 2.040 2 | 1.281 2 | 2.690 2 |
| transcript5182 | similar to LReO_3 (Strongylocentrotus purpuratus) | 2.802 2 | 2.800 2 | 2.802 2 | 1.709 2 | 2.802 2 |
| transcript19227 | similar to reverse transcriptase-like protein, partial (Strongylocentrotus purpuratus) | 8.679 2 | 4.885 2 | 4.691 2 | 1.029 2 | 4.885 2 |
| transcript67066 | phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 2-like (Anolis carolinensis) | 2.459 2 | 2.059 2 | 2.059 2 | 1.029 2 | 2.059 2 |
| Transcript ID | Annotation (Blastx against nr) | v4  | d4  | v8  | d8  | log2Fc4 | log2Fc8 | Log2Fc |
|---------------|--------------------------------|-----|-----|-----|-----|---------|---------|--------|
| transcript2222 | reverse transcriptase-like protein [Paralichthys olivaceus] | 14.076 | 1.403 | 3.805 | 1.237 | –3.327 | –1.622 | –2.760 |
| transcript5593 | Myb protein P42POP, isoform CRA_a [Mus musculus] | 13.400 | 1.539 | 3.506 | 0.967 | –3.122 | –1.858 | –2.754 |
| transcript899 | hypothetical protein TcasGA2_TCO15886 [Tribolium castaneum] | 10.490 | 0.880 | 2.036 | 0.982 | –3.576 | –1.051 | –2.750 |
| transcript51721 | hypothetical protein LOC100488716 [Xenopus (Silurana) tropicalis] | 10.490 | 0.880 | 2.036 | 0.982 | –3.576 | –1.051 | –2.750 |
| transcript7725 | hypothetical protein LOC734400 [Xenopus laevis] | 9.043 | 0.907 | 2.479 | 0.815 | –3.117 | –1.605 | –2.742 |
| transcript108620 | hypothetical protein LOC100497892 [Xenopus (Silurana) tropicalis] | 17.822 | 2.260 | 3.565 | 0.953 | –2.980 | –1.904 | –2.735 |
| transcript70652 | hypothetical protein LOC100492542 [Xenopus (Silurana) tropicalis] | 7.011 | 0.825 | 2.004 | 0.532 | –3.087 | –1.915 | –2.733 |
| transcript15129 | similar to transposase [Strongylocentrotus purpuratus] | 7.680 | 0.917 | 4.192 | 0.886 | –3.066 | –2.242 | –2.718 |
| transcript1001 | hypothetical protein LOC100490320 [Xenopus (Silurana) tropicalis] | 44.292 | 5.312 | 9.908 | 2.944 | –3.060 | –1.751 | –2.715 |
| transcript7879 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4 b [Xenopus laevis] | 1521.4 | 153.4 | 1306.0 | 98.732 | –3.310 | –0.403 | –2.712 |
| transcript112029 | similar to CR1 Danio rerio 2 reverse transcriptase isofform 3 [Strongylocentrotus purpuratus] | 17.674 | 1.804 | 3.208 | 1.394 | –3.293 | –1.202 | –2.707 |
| transcript56820 | hypothetical protein LOC100497926 [Xenopus (Silurana) tropicalis] | 5.412 | 0.790 | 3.565 | 0.611 | –2.776 | –2.545 | –2.680 |
| transcript1148 | hypothetical protein LOC100493982 [Xenopus (Silurana) tropicalis] | 17.683 | 2.600 | 5.544 | 1.056 | –2.766 | –2.392 | –2.667 |
| transcript338 | reverse transcriptase-like protein [Takifugu rubripes] | 364.357 | 50.283 | 86.144 | 20.794 | –2.857 | –2.051 | –2.664 |
| transcript1216 | hypothetical protein LOC100495475, partial [Xenopus (Silurana) tropicalis] | 8.246 | 0.355 | 2.898 | 1.419 | –4.537 | –1.030 | –2.651 |

v4: RPKM value of ventral iris 4 dpl; d4: RPKM value of dorsal iris 4 dpl; v8: RPKM value of ventral 8 dpl; d8: RPKM value of dorsal 8 dpl; log2Fc4: fold expression at 4 dpl between dorsal and ventral iris; log2Fc8: fold expression at 8 dpl between dorsal and ventral iris; log2Fc: fold expression between dorsal and ventral iris at both the days.

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the general transcriptional apparatus that transcribe all types of RNA including POLR1A, POLR2A, POLR1B, POLR1C, POLR1D, POLR2J, ZNRD1, GTF2H1, GTF2H2, MED12, MED23, MED24, ESF1, BRF1, TWISTNB and some factors from the CCR4-NOT complex (CNOT6, CNOT6L, CNOT4, CHAF1A, CHAF1B) [33]. Transcriptional factors and factors that link them to the basal transcriptional apparatus to regulate specific types or families of genes include ATF6 and CREB3L2, which activate certain genes upon stress [34,35]. Furthermore, MYCBP and CDCA7 regulate MYC activity [36,37], CBY1 inhibits Wnt via beta-catenin [38], NR2F2, RARA and RXRA are involved in gene activation after binding to retinoic acid [39,40,41], NR2C2 is a receptor that represses retinoic acid receptors [42], CYLD, RELB and NFKB2 are related to NF-kappaB pathway [43,44,45], RNF20 is involved in Hox gene activation [46], SMAD6 is a TGF-beta signaling-induced inhibitor of BMP signaling [47,48], TBX5 is involved in dorsal eye patterning and limb regeneration [49,50], NR4A1 is a receptor found to play a role in liver regeneration [51], MDM4 inhibits p53 [52], and TEAD1 is involved in hippo pathway [53], are all up-regulated in the dorsal iris. It is noteworthy that VAX2 is up-regulated in the ventral iris, which is a major player in formation of ventral eye axis during embryogenesis [54]. It is interesting to speculate that the differential regulation of TBX5 and VAX2 in the dorsal and
ventral iris demarcates their regenerative ability. TBX5 is expressed over 32-fold higher in the dorsal and VAX2 is expressed 32 fold higher in the ventral iris (see also below).

Factors that are involved in post-transcriptional regulation and in pre-mRNA maturation like splicing and alternative splicing and are up-regulated in the dorsal group include: GEMIN4 [55], DDX23, DDX41, AQR, PPWD1, XAB2, SRRM1, TFIP11 [56], DDX46 [57], CLASRP, CDK11B [58], DBR1 [59], WDR77 [60], MBNL2, MBNL1 [61], PPH [62], PF60 [63], PRPF8 [64], RBFOX2 [65], RBM28 [66], RBM5 [67], RSR1C1 [68], SRSF12 [69], SRSF11 [70], SFSWAP [71], SCAF1 [72] and SMNDC1 [73], or other ways of pre-mRNA maturation using CSTF1 [74], CPSF3 [75] and EXOSC10 [76]. Factors that play a role in ribosomal RNA maturation include BOP1 [77], EBNA1BP2 [78], HEATR1 [79], PDCD11 [80], UTP11L, UTP6 [81] and MTHOSPI10 [82]. Factors that play role in the stability and transport of the RNA include CARHSP1 [83], PUM1 [84] and part of the TREX complex (THOC2, THOC5, and THOC6) [85].

Protein complexes and related factors involved in histone modifications and are up-regulated in dorsal group include the SIN3A/HDAC1 complex (BRMS1L, ARID4A, TOPO1, RBBP7, NCO1 and SMARCC2), the NuRD complex (CHD4, RBBP7, MTA3, TRIM28 and ZGAPAT) [86], the NuA4 complex (DAP1, KAT5, TRRAP and VPS72) [87], the PRC2/EED-EZH2 complex (EZH2 and RBBP7) [88], the MLL1/MLL complex (MLL and RBBP5) [89], and the SWI/SNF complex (BAF complexes) (ARID1A, PHF10, SMARCA2, SMARCA4 and SMARCC2) [90], and are all up-regulated in the dorsal iris.

Likewise, most of the transcripts that were identified to act on translation (and miRNA processing and function) are up-regulated in the dorsal iris. Only 1 transcript, eIF2B, was found to be up-regulated in the ventral iris.

Cytoskeleton-related Transcripts

Table 3 shows transcripts that are up-regulated in dorsal iris at least 2 fold than the ventral iris and the opposite. Most of the transcripts are shown to be up-regulated in the dorsal iris (134/18).

Microtubules-associated: In this category some of the transcripts are related to the cell cycle like in spindle formation and chromosome movement and we have discussed them previously. Other transcripts that are up-regulated in the dorsal group have a role during signal transduction: APC negatively regulates Wnt signaling [91], MACF1 positively regulates Wnt signaling [92], CYLD and MAST2 positively regulates NF-kappaB pathway [93]. Transcripts involved in microtubule organization and stability include CAMSAP3 [94], DNAH7 [95] and IFT57 [96], in cell shape and movement include ELMO2 [97], GAN [98].

Actin-related: Transcripts up-regulated in the dorsal group and included in this category play roles in actin polymerization and organization in order to support cell shape, movement and cell adhesion with the extracellular matrix, and the linkage of actin
with other proteins in order to facilitate transport or contraction. WASH1 [116], ARPC1B, ARPC5L [117], SSH2 [110] SCIN [119], IQGAP1 [120], PPP1R9A [121] and DIAPH1 [122] are playing roles in actin polymerization and organization. CNN2, MYO10, MYO1E, MYO9A, MYO1G, MYO6, MYO9B, MYO7A, MYO1D and ROCK1 are involved in contraction [123,124]. CTNNA1 and MTSS1 are related to cell adhesion with the extracellular matrix and cell-cell contact [125,126]. KLHL3 and MYO5A are playing roles in molecular transport [127,128]. PLEK2 and VASP are related to cell movement [129,130] and SYNE1 and WDR1 are linking cytoskeleton with other proteins [131,132].

In addition to factors that are actin or tubulin-related, Table 3 shows other proteins that are related to cell adhesion, movement and linkage of plasma membrane with cytoskeleton that are up-regulated in the dorsal iris. For lens regeneration, cell adhesion and locomotion is very important since PECs need to change their environmental behavior to transdifferentiate and change their cell fate. Previous studies have found that extracellular matrix is being remodeled and matrix metalloproteinases are up-regulated already 1 day post-lentectomy to prepare the environment for the onset of lens regeneration [6]. Our data clearly show changes in the molecules that determine the interaction of PECs with the environment and remodeling of cytoskeleton components and networks of PECs. Another interesting aspect is that many factors involved in tumor metastasis are up-regulated in the dorsal iris which indicates a role of these molecules at the onset of cell locomotion.

Immunity-related Transcripts

Most of the transcripts related to immune response are up-regulated in the dorsal iris samples (Table 4). Only 2 transcripts were up-regulated in the ventral iris versus 37 that were up-regulated in the dorsal iris. Factors in this category regulate NF-kappaB activity among others and include SIVA1 [133], CHUK, NFKB2, TLR7 [134] and RELB. Factors involved in immune cells activation and migration include TNFSF13B [135], CD97 [136], GPR183 [137], ENPP2 [138], DCLRE1C [139] and TLR2 [140]. Complement component -related transcripts include C1S, C3, C1QB and C1QBP and other factors involved in cytokine secretion and inflammation include CCL5, DDX38, STAT6 and ZEB1.

The role of immune response and its involvement in the initiation of regeneration has been extensively investigated in the past [141,142]. It has been hypothesized that molecules involved in the regulation of the immune response have a novel role in regeneration or that the immune response itself is crucial for regeneration. The issue has not been settled yet. Nevertheless, complement components seem to be important for liver regeneration [143] and have also found to be expressed in limb and lens regeneration [144,145]. The present results provide strong evidence of a crucial role of injury response in regeneration, which needs to be investigated further.

Transposon-related Transcripts

Interestingly, transposons are the only transcripts that are enriched in ventral compared to dorsal samples (Table S2). Transposons have many types and they do not have an assigned biological function. They can be transcribed, reverse-transcribed and integrated back to the genome (retrotransposons) or not. So far, we have no specific role of transposons in regeneration (or rather, inhibition of it?). Since transposon-related transcripts are enriched in ventral samples it would be interesting to learn more about a specific role in repressing specific programs.

Highly Regulated Transcripts

Summing up, we have identified patterns of gene expression that are predominant in the dorsal iris. Genes that are involved in cell cycle, gene regulation, cytoskeleton and immune response show a graded expression along the dorsal/ventral axis. Thus, our study is the first to show how the dorsal iris differs from the ventral iris, and how specific patterns of gene expression correlates with the dorsal iris’ regenerative ability. Comparisons comprised two critical time points, 4 and 8 dpl. Interestingly, comparison of gene expression patterns at each time point separately, recapitulates our primary finding that most transcripts of these gene categories are up-regulated at 4 dpl in the dorsal iris in comparison to 4 dpl in the ventral iris, or in 0 dpl in the dorsal iris in relation to 0 dpl in the ventral iris. In Tables 5 and 6 we show a selected group of genes to exemplify this point. The tables also allow a view on the top regulated transcripts. It becomes clear that a few of them are either dorsal-specific or ventral-specific. Only 3 transcripts were found to be exclusively present in the dorsal iris. These transcripts correspond to protein-1 like (ras associated and pleckstrin domains-containing), transmembrane protein 185A-like (TMEM family) and to chromatin assembly factor 1 (CAF1). Other transcripts that show very high expression in dorsal iris were TBX5, TMEM185A, E3 ubiquitin-protein ligase HERC2-like (HERC2) (>32 times), TMEM116, ephrin-B2, and netrin receptor (UNC5B) (>16 times). In the ventral iris, except transposons, we find that netrin-1 (NTN1), nuclear receptor 2F5-like (NR2F5), and VAX2 are expressed 32 times higher than in the dorsal iris. The function of TBX5 and VAX2 were discussed above, but it is interesting to note here that they might provide a dorsal or ventral identity to the adult iris. Currently, it is not known to what extent these genes control regeneration but functional assays will settle this issue. Nevertheless, TBX5 and VAX2 can be used as markers for dorsal and ventral iris, respectively. Interestingly, we find NTN1 in the ventral iris but its receptor (UNC5B) in the dorsal iris. Likewise, we find ephrin-B2 in the dorsal and its receptor in the ventral iris, which might reveal a so-far unsuspected communication between dorsal and ventral iris. Despite its role in axon guidance, NTN1 has also been shown to inhibit leukocyte migration. Thus, NTN1 up-regulation might protect injured tissues [146]. UNC5B is responsible for apoptosis and because NTN1 is up-regulated by p53 it is considered as an oncogene [147]. Ephrin receptors activated by ephrins have been shown to inhibit signaling by oncogenes. The case of TMEM proteins is interesting as well. Even though not much is known for TMEM185A and TMEM116, TMEM16F is known to form a Ca<sup>2+</sup>-activated channel, which plays a role in blood coagulation that is also mediated by thrombin activation [148]. In turn, blood coagulation has been implicated in the induction of lens regeneration from the dorsal iris [142].

We have also verified some of these patterns via qRT-PCR, which confirm this remarkable difference in expression along the dorsal/ventral axis. The up-regulation of TBX5, FGF10 and UNC5B in the dorsal iris and the up-regulation of VAX2, NR2F5 and NTN1 in the ventral iris are shown in Figure 3. Interestingly, the expression of these genes is also dependent on time, suggesting a potential role of those genes during regeneration (ANOVA; α<0.05). In addition, we further verified the up-regulation of NTN1 in the ventral iris and its receptor (UNC5B) in the dorsal iris.

What Genes are Regulated Specifically at 4 or 8 dpl?

We showed only minor qualitative differences in gene expression between the dorsal and ventral iris. Quantitative changes are dominant and seem to correlate with regenerative abilities. To
answer the question whether there are any differences at day 4 (both dorsal and ventral) versus day 8 (dorsal and ventral), which might uncover the importance of timing rather than spatial regulation, we compared the 4 day group with the 8 day group as shown in Figure 4.

Fisher’s exact test with multiple testing corrections for GO from transcripts that are up-regulated in both dorsal and ventral iris 4 dpl compared to 8 dpl versus the whole reference transcripts reveals that DNA polymerase activity and nucleotidytransferase activity GO terms to be significantly enriched (Table S3). This indicates the initiation of the cell cycle re-entry which has been found to be the major event 4 dpl [149].

Fisher’s exact test with multiple testing corrections for GO from transcripts that are up-regulated in both dorsal and ventral iris 8 dpl compared to 4 dpl versus the whole reference transcripts revealed many interesting patterns (Table S4): as expected GO terms in the cellular component category and related to extracellular matrix are over-represented in the group. Furthermore, over-represented terms include extracellular matrix structural constituents and metalloendopeptidase activity in the molecular function category (Table S4): Collagen catabolic process, collagen metabolic process, cell adhesion, extracellular matrix and structure organization, and peptide secretion are over-represented in the biological process category (Table S4). In addition, many of the GO terms in the biological process category related to differentiation, movement, development and patterning are over-represented in the group. Finally, many GO terms that are over-represented in the group are related to macromolecule transport and synthesis. These results indicate active remodeling, transcription and metabolism at day 8, which was expected because the process of dedifferentiation and specification of the lens vesicle peak at this time point.

Conclusion

The transcriptome analysis during lens regeneration revealed much needed and useful information. First, we were able to identify quantitative patterns of gene expression that create gradients along the dorsal/ventral iris. This finding is of particular importance since it establishes a molecular framework that drives the ability of the dorsal iris for lens regeneration. Second, our analysis identified genes that might be critical for the induction of lens regeneration. For the first time, we now know factors that can be studied in functional assays, such as in transogenesis or knockdown [150,151], to establish their potency in inducing/inhibiting regeneration. In addition, this knowledge allows us to perform comprehensive comparisons to other animal models that lack the ability for lens regeneration, which might unveil fundamental differences and similarities between regenerating and non-regenerating species.

Supporting Information

Table S1 GO terms that are over-represented in dorsal iris 4 and 8 dpl versus ventral iris 4 and 8 dpl. (XLSX)

Table S2 GO terms that are over-represented in ventral iris 4 and 8 dpl versus dorsal iris 4 and 8 dpl. (XLSX)

Table S3 GO terms that are over-represented in transcripts commonly up-regulated (>2 fold) in dorsal and ventral iris at 4 dpl compared to 8 dpl versus whole reference transcriptome. (XLSX)

Table S4 GO terms that are over-represented in transcripts that are commonly up-regulated (>2 fold) in dorsal and ventral iris 8 dpl compared to 4 dpl versus whole reference transcriptome. (XLSX)

Author Contributions

Conceived and designed the experiments: PAT TB. Performed the experiments: KS ML NM CJI. Analyzed the data: KS ML TB PAT. Contributed reagents/materials/analysis tools: KS ML TB PAT. Wrote the paper: KS ML TB PAT.

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