Molecular signatures that correlate with induction of lens regeneration in newts: lessons from proteomic analysis

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Abstract

Background: Amphibians have the remarkable ability to regenerate missing body parts. After complete removal of the eye lens, the dorsal but not the ventral iris will transdifferentiate to regenerate an exact replica of the lost lens. We used reverse-phase nano-liquid chromatography followed by mass spectrometry to detect protein concentrations in dorsal and ventral iris 0, 4, and 8 days post-lentectomy. We performed gene expression comparisons between regeneration and intact timepoints as well as between dorsal and ventral iris.

Results: Our analysis revealed gene expression patterns associated with the ability of the dorsal iris for transdifferentiation and lens regeneration. Proteins regulating gene expression and various metabolic processes were enriched in regeneration timepoints. Proteins involved in extracellular matrix, gene expression, and DNA-associated functions like DNA repair formed a regeneration-related protein network and were all up-regulated in the dorsal iris. In addition, we investigated protein concentrations in cultured dorsal (transdifferentiation-competent) and ventral (transdifferentiation-incompetent) iris pigmented epithelial (IPE) cells. Our comparative analysis revealed that the ability of dorsal IPE cells to keep memory of their tissue of origin and transdifferentiation is associated with the expression of proteins that specify the dorso-ventral axis of the eye as well as with proteins found highly expressed in regeneration timepoints, especially 8 days post-lentectomy.

Conclusions: The study deepens our understanding in the mechanism of regeneration by providing protein networks and pathways that participate in the process.

Keywords: Regeneration, Lens, Newt, Proteomics, Gene expression, Regeneration program

Background

Several amphibian species own the ability to regenerate multiple different organs during adulthood making them excellent models to study the molecular mechanisms of tissue regeneration. Although regulation of regeneration might diverge among tetrapods, a deeper understanding of regenerative processes in amphibians will provide valuable clues for organ repair and regeneration in other organisms such as mammals [1,2].

Regeneration of the eye lens in newts provides a superb model to study regeneration. After surgical removal of the lens, the whole organ regenerates by transdifferentiation of dorsal iris pigmented epithelial (IPE) cells. Interestingly, the lens is never regenerated from the ventral iris, which provides a number of experimental options [3,4]. Most importantly, gene expression differences between the dorsal and the ventral part of the iris can be identified to unravel the molecular program enabling regeneration. Similarly, changes in the expression profile between the iris while the lens is intact and during lens regeneration allow characterization of regulatory pathways initiating regeneration. In addition, dorsal IPE cells retain their ability to form a lens after in vitro culturing, aggregation, and orthotopic transplantation or implantation into 3-D collagen lattices while ventral IPE aggregates fail to do so. Hence, gene expression profiles of cultured dorsal and ventral IPE cells might provide additional insights into lens regeneration [5].
Recently, the first newt transcriptome was assembled, and RNA sequencing of newt iris has been used to study differences in gene expression between the dorsal and ventral iris. Analysis of gene expression identified genes exclusively expressed in either the dorsal or ventral iris as well as genes expressed in a gradient along the dorsoventral axis of the iris during lens regeneration [6,7]. In another study, custom newt microarrays were used to detect 467 genes that were differentially expressed during lens regeneration [8]. Although these studies provided essential information about the expression of genes during newt lens regeneration, protein data were missing so far. Mass-spectrometry-based protein analysis closes this gap providing information about changes in protein concentrations and potential post-transcriptional regulation during lens regeneration. Here, we computed the newt proteome from the assembled transcriptome and performed liquid chromatography followed by tandem mass spectrometry (LC-MS/MS) to identify proteins differentially expressed between dorsal and ventral iris as well as between regenerating and intact iris. We chose to use dorsal and ventral iris 4 and 8 days post-lentectomy (dpl) since during these timepoints iris cells re-enter the cell cycle and transdifferentiate. We then compared the expression data with the previously reported gene expression data at the mRNA level collected at the same timepoints. In addition, we performed LC-MS/MS with samples collected from in-vitro-cultured dorsal and ventral IPE cells. We compared the expression profiles between the in vitro and in vivo experiments. Lastly, we compared available high-throughput mRNA and protein expression data obtained from amphibian organ model systems undergoing regeneration, identifying a common regeneration program.

Results and Discussion

LC-MS/MS identifies novel newt proteins

Lenses were removed from newt eyes in order to initiate the regeneration process at the dorsal iris. Dorsal and ventral iris samples were collected at 4 and 8 dpl as well as from intact tissue (day 0). At 4 dpl, dorsal and ventral iris cells re-enter the cell cycle while at 8 dpl only dorsal iris cells initiate dedifferentiation. The iris samples were prepared, and LC-MS/MS was performed in order to investigate changes in protein concentrations (Figure 1A). The newly assembled newt transcriptome was used for peptide identification [7]. LC-MS/MS identified 8,167 different proteins. These proteins were uniquely annotated to 4,734 different human proteins (e-value < 1E-10). Direct comparisons with proteins found in previous proteomic studies in newts revealed that 701 of these annotated proteins have not been detected in newts before [7]. Our data set also includes 143 proteins lacking annotations in other species, which raises the possibility that they are unique to newts. These data are summarized as a whole and per sample in Table 1 (Workflow: Figure 1B).

Protein expression patterns during regeneration

The primary interest of our study was to identify proteins that might play a role during tissue regeneration. To achieve this goal, we compared proteins that were present at 4 and 8 dpl (both dorsal and ventral iris) and were expressed at least twofold higher than proteins in the intact iris at day 0 (regeneration group). Likewise, we examined proteins that were down-regulated (at least twofold) during regeneration (control group). To investigate the trends that our analysis yielded, we used the gene ontology (GO) terms...
of the annotated proteins and we performed enrichment analysis using Fisher’s exact test corrected for multiple selections between the regeneration and control groups (false discovery rate, FDR < 0.05; Figure 2A). Metabolic process and gene expression were some of the GO terms enriched during regeneration in both dorsal and ventral iris samples while the GO term “cell periphery” was enriched in the control samples (Figure 2B and Additional file 1). Tables 2 and 3 (for dorsal iris) and Tables 4 and 5 (for ventral iris) list the genes reflecting the GO term enrichments for the regeneration group. These genes determine cellular functions in response to stress and reactive oxygen species. They are also involved in the regulation of translation, RNA maturation, and immune responses. Genes that were induced in the iris post-lentectomy can be grouped per function as follows:

a. Gene expression

Elongation factor 1-delta (EEF1D), elongation factor 1-gamma (EEF1G), valine-tRNA ligase, and ribosomal proteins RPL10, RPL13, RPL18A, RPL27A, RPL3, RPL4, RPL5, RPS23, and RPS8 are all known for their role in the translation of proteins. RNA-processing proteins include poly(U)-specific endoribonuclease (ENDOU), nuclease-sensitive element-binding protein 1 (YBX1), which is also implicated in cell proliferation [9], pre-mRNA-processing-splicing factor 8 (PRPF8), and probable ATP-dependent RNA helicase DDX5. In addition, two members of the ribonucleoprotein complex (ARC), polyadenylate-binding protein 1 (PABPC1) and cold shock domain-containing protein E1 (CSDE1), were detected. Consistent with these results, previous studies in mice have found PABPC1 to be up-regulated during liver regeneration [10]. Similarly, another stress-induced RNA processing protein, the heat shock cognate 71 kDa protein (HSPA8), was found to play a role during rat skeletal muscle regeneration and zebrafish caudal fin regeneration [11,12].

Basic leucine zipper and W2 domain-containing protein 1 (BZW1), protein arginine N-methyltransferase 5 (PRMT5), and SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5 (SMARCA5) are known for regulation of gene expression. Interestingly, BZW1 has been previously found to induce histone H4 gene expression, which aids the progression of the G1/S phase of the cell cycle [13]. In addition, PRMT5 has been shown to play role in cell proliferation in planaria and be up-regulated post-injury in the kidneys [14,15]. Methionine aminopeptidase 1 (METAP1) and receptor-type tyrosine-protein phosphatase C (PTPRC), also known as CD45, have been shown to play a role in cell activation, proliferation, and cell cycle progression [16-18]. The protein expression data implicate regulation of gene expression as an important event for transdifferentiation.

b. ROS balance

Heme oxygenase 1 (HMOX1), redox-regulatory protein FAM213A, cis-aconitate decarboxylase (IRG1), and serpin B10 (SERPINB10) are known for their association with reactive oxygen species (ROS) and redox balance. IRG1 is activated by ROS to prevent infections [19]. SERPINB10 plays a role in protein processing and it is sensitive to redox stress [20]. HMOX1 is induced by ROS and has been linked to wound healing and regeneration in many regeneration model systems with the exception of mouse liver regeneration [21-24]. ROS stress and redox balance has gained a lot of attention since many studies have associated changes related to them with regenerative responses [25]. Our data indicate a potential role of ROS during newt lens regeneration as well.

c. Immune response

Arginosuccinate synthase (ASS1), complement factor B (CFB), acidic mammalian chitinase (CHIA), bis (5’-adenosyl)-triphosphatase ENPP4, eosinophil peroxidase (EPX), coagulation factor XIII A chain (F13A1), and myeloperoxidase (MPO) are up-regulated during lens regeneration and are known for their roles in preventing infections and generally to facilitate host defense and immune response.
d. Metabolic processes

Sterol O-acyltransferase 1 (SOAT1), delta-1-pyrroline-5-carboxylate synthase (ALDH18A1), v-type proton ATPase subunit d 1 (ATP6V0D1), cytochrome b-245 heavy chain (CYBB), n-acylthanolamine-hydrolyzing acid amidase (NAAA), putative neutrophil cytosol factor 1C (NCF1C), neutrophil cytosol factor 2 (NCF2), and 6-phosphogluconate dehydrogenase, decarboxylating (PGD) participate in many metabolic processes and regulation of energy production. SOAT1 is also known to be involved during rat adrenal regeneration [26].

e. Other functions

Additional interesting proteins, potentially involved in the regulation of newt lens regeneration, are msx2-interacting protein (SPEN), which aids wound healing in Drosophila embryos [27]; cathepsin L1 (CTSL1), which mediates proteolysis; DNA topoisomerase 1 (TOP1), which is associated with DNA replication and transcription; epidermal retinol dehydrogenase 2 (SDR16C5), which is involved in making retinoic acid; and ATP-dependent DNA helicase Q1 (RECQL), which mediates DNA repair. Furthermore, we found an up-regulation of unconventional myosin-XVIIIa (MYO18A), which is involved in cell migration, as well as fibronectin (FN1) and integrin beta-2 (ITGB2), which mediate adhesion and cell migration and are essential for zebrafish heart regeneration [28].

The rapid up-regulation of all the aforementioned proteins during lens regeneration clearly underscores the importance of host defense, redox balance, and response to stress for the initiation of regeneration.

Proteins regulated in dorsal versus ventral iris

Since regeneration only proceeds from the dorsal iris, we wanted to find proteins that were specifically up-regulated in this tissue. To achieve this goal, we compared all proteins up-regulated during lens regeneration to proteins only up-regulated in the dorsal or the ventral iris. Proteins identified were imported in VisANT, a program that analyses protein-protein interactions [29]. Proteins that were expressed at higher levels in dorsal samples represented...
complexes associated with extracellular matrix, ribosomes, and DNA (Figure 2C) whereas proteins expressed at higher levels in ventral samples did not exhibit such a pattern (Figure 2D).

a. Extracellular matrix

Collagen proteins such as COL2A1, COL9A2, and COL9A3 are essential for structural support and provide the substrate for surrounding cells. In addition, COL9A2 and COL9A3 compose the vitreous area of the eye. Versican core protein (VCAN) is known for cell-extracellular matrix interactions allowing cell migration and growth. Laminin subunit alpha-3 (LAMA3) promotes migration. Fibrillin-1 (FBN1), fibrillin-2 (FBN2), and syndecan-2 (SDC2) are extracellular matrix proteins regulating the availability of growth factors to nearby cells. SDC2 also plays a role during rat periodontal wound healing [30]. Annexin A5 (ANXA5) is known for its anticoagulant properties and promotes wound healing in the cornea [31]. Previous studies in newt limb and lens regeneration also support these findings. A study in newt limb regeneration suggests that dynamic changes of the extracellular matrix provide a suitable microenvironment for regeneration [32], which is in-line with the up-regulation of several extracellular matrix genes during newt lens regeneration in the dorsal iris as determined by DNA microarray analysis [8]. These results suggest that remodeling an appropriate environment is a fundamental event during lens regeneration.

b. Cell activation

Rho guanine nucleotide exchange factor 1 (ARH-GEF1), ATPase family AAA domain-containing protein 3A (ATAD3A), polypyrimidine tract-binding protein 3 (PTBP3, also known as ROD1), casein kinase 1 isoform epsilon (CSNK1E), src substrate cortactin (CTTN), replication factor C subunit 2 (RFC2), and NEDD8 have all been shown to have roles in cell proliferation, migration, and growth [33-36]. These cellular events are important since the lost tissue needs to be regenerated.

c. Gene expression

Cullin-5 (CUL5), polyadenylate-binding protein 2 (PABPN1), exosome complex exonuclease RRP44 (DIS3), SHC-transforming protein 1 (SHC1), tyrosine-protein kinase SYK, serine/threonine-protein phosphatase 6 regulatory subunit 1 (PPP6R1), chromodomain-helicase-DNA-binding protein 4 (CHD4), and ribosomal proteins RPL15, RPL32, RPL8, RPL28, RPL21, RPL10, and RPL19 are playing roles in regulating gene expression at various levels [37] and are all up-regulated in the dorsal iris. Interestingly, CHD4 has also been shown to be up-regulated during muscle regeneration in mice [38]. RNA sequencing during lens regeneration has previously revealed that genes associated with the regulation of gene expression are enriched in the dorsal iris, a pattern that we also found here at the protein level [6]. These results highlight the importance of rapid and impactful changes in the

| GO:0010467; gene expression and parental GO | Dorsal regeneration | Dorsal control |
|-----------------------------------------------|---------------------|----------------|
| AARS                                          | ENDOU               | RPL10A         |
| ANK3                                          | ETF1                | RPL2           |
| BUD31                                         | FUBP1               | SNRNTP70       |
| BZW1                                          | GARS                | AHCYL2         |
| C                                             | GCN1L1              | RPL3           |
| CSD1                                          | GLG1                | RPS2           |
| DAD1                                          | HBS1L               | RPS23          |
| DX1X7                                         | HCK                 | LUC7L3         |
| DPM1                                          | HM0X1               | RPL13          |
| EEF1D                                         | HRNRPIM             | RPS52          |
| EEF1G                                         | HRNRPNU             | RPS528         |
| EEF2                                          | HP5A8               | RPS1           |
| EEF3B                                         | ILF3                | RPL27A         |
| EEF4G2                                        | IPO9                | RPL28          |
| EIF5A                                         | KHDRBS1             | RPL10          |
| EIF5B                                         | KHDRBS1             | RPL8           |
|                                             |                     |                |
| Table 2 Genes with GO term related to gene expression that are up-regulated in the dorsal iris during regeneration versus control |

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regulation of gene expression that will ultimately lead to transdifferentiation of iris cells to lens cells.

d. DNA repair

Poly [ADP-ribose] polymerase 1 (PARP1), DNA-dependent protein kinase catalytic subunit (PRKDC), and structural maintenance of chromosomes protein 1A (SMC1A) have known roles in DNA repair. DNA repair genes such as RAD1 have been previously found to be up-regulated in the dorsal iris using both microarrays and RNA sequencing during newt lens regeneration [6,8]. Such cellular machinery can play a role in maintaining the integrity of the genome an important aspect of regenerating an exact “clone” of the missing lens.

e. Other functions

Other proteins found to be up-regulated in the dorsal iris during lens regeneration were keratin, type II cytoskeletal 6A (KRT6A), and caspase-3 (CASP3) which have been implicated in wound healing and regeneration [39,40].

All these proteins, grouped in the different functional categories, were up-regulated in the dorsal compared to the ventral iris during lens regeneration. Interestingly, several of these proteins formed interacting networks that can be linked to the regeneration process (Figure 2C).

Validation of changes in expression levels by qPCR

Since only few newt specific antibodies are available, we used quantitative real-time polymerase chain reaction (qPCR) to validate our data. Although qPCR measures mRNA and not protein concentrations, we reasoned that concomitant changes at the mRNA and protein levels might allow us to validate general patterns of gene activity during regeneration. We selected several proteins based on their putative function. Retinal dehydrogenase 1 (ALDH1A1), ephrin-B1 (EFNB1), and ephrin-B2 (EFNB2) were significantly up-regulated in the dorsal iris compared to the ventral irrespective of the timepoint ($p < 0.05$;
Table 4 Genes with GO term related to gene expression that are up-regulated in the ventral iris during regeneration versus control

| GO:00010467; gene expression and parental GO | Ventral regeneration | Ventral control |
|---------------------------------------------|----------------------|----------------|
| AARSD1                                      | EIF3J                | RPL27A         | RQCD1         | THBS1 | CAT | ILK |
| ATR6AP2                                     | ENDOU                | PFDN5          | RPL3          | SEC11A | UBTF | CD44 | KANK2 |
| BZW1                                        | GALNT12              | PRKCI          | RPL38         | SMARCA5 | VARS | CDH13 | KRT17 |
| CARS                                        | HB51L                | PRMT5          | RPL4          | SNF8   | VIPAS39 | CHM1PA | PRKCA |
| CSE11                                      | HMW2X1               | PRPF8          | RPL5          | SNRPE  | VARS | COX2 | PRKDC |
| DD3X9A                                      | HSPA8                | PSME3          | RS13          | SOAT1  | WDR61 | COL4A6 | PURA |
| DNAJC2                                      | KDM1A                | RCL1           | RS15          | SPEN   | WTAP | CTNNB1 | RBCK1 |
| EBNA18B                                     | MARS                 | RPL10          | RS17          | SRSF12 | YBX1 | DEK | SBDS |
| EEF1B2                                     | METAP1               | RPL13          | RS23          | SRR4   | YLPM1 | DMD | SEC31A |
| EEF1D                                      | NPC1                 | RPL17          | RS27          | SPUT6H | EXOSC7 | UGGT2 |
| EEF1G                                      | PABPC1               | RPL18A         | RS58          | TGF81  | GJA1 | VPS36 |

Table 5 Genes with GO term related to metabolic process that are up-regulated in the ventral iris during regeneration versus control

| GO:00008152; metabolic process and parental GO | Ventral regeneration | Ventral control |
|-----------------------------------------------|----------------------|----------------|
| ABCF2                                        | DCN                  | LARP1          | PRRC1         | ABCB5 | ECHDC2 | NIT2 | SLC22A2 |
| ACSBG2                                       | DDX5                 | LYN            | PRSS16        | ADH4  | EHD2  | OXCT1 | SLC9A2R |
| ACY1                                         | DGA1                 | LYZ            | PRTN3         | AGL   | F8    | P4KA | SOD1  |
| ADH1B                                        | DNAJB11              | MCM5           | PTPN6         | AK4   | FAM162A | PLCG1 | SULT1B1 |
| ALDH1A1                                      | DNM2                 | MCM6           | PTPRC         | ALG11 | FBN1  | PNP  | SULT1C2 |
| ALDH1A3                                      | EC12                 | MDN1           | P2P           | ATAD1 | GALE  | PNP  | SYTL2 |
| ALOX5                                        | ECM1                 | MOB1B          | RCC1          | ATG7  | GLUL  | PRKAB1 | TGM1 |
| ALPL                                         | ENPP4                | MPDU1          | RECOl         | C6orf130 | GMPR2 | PRKAR2B | TTP1 |
| ARAP1                                        | EPX                  | MPO            | RFC5          | CAPN5 | GNA14 | P7G1 | TTL1L |
| ASS1                                         | F13A1                | MYO18A         | RNF213        | CDIPT | GNA1 | PTK2 | TUB83 |
| ATP2A1                                       | FAM213A              | MYOSA          | RNLS          | CECR1 | GRHPR | PTPLAD1 | VCP |
| ATP6O1D1                                     | FASN                 | NAAA           | SDR16C5       | CLYBL | HAGH | PTPRD | XPNPE1 |
| ATP6O1F                                      | FBPI                 | NADKD1         | SERPINB10     | COP53 | HIST1H2AG | PYGB |
| C3                                           | FEN1                 | NCF1C          | SERPINB6      | CP    | HMOX2 | RAB27B |
| CFB                                          | FKB4                 | NCF2           | SGFP1         | CTSS  | KLC4 | RAB3D |
| CHA                                          | FN1                  | NOKAP1L        | SLC25A40      | CUL5  | LAN1L | RAB7A |
| CKM                                          | GBF1                 | NMT2           | SMPD3         | CYPA13 | MARCKS | RABGP1 |
| CNEP1R1                                      | GCAT                 | NARS           | SQSTM1        | CYP212 | MTC1 | RGN |
| COX5B                                        | GCLM                 | NUP155         | SYK           | DCTN1 | MT-BY | RTN4P1 |
| CP                                           | GLB1                 | NUP210         | TBC1D9B       | DLG1  | MYH11 | SEPH1 |
| CTSA                                         | GLUL                 | P2R2X4         | TOP1          | DMGDOH | NDUF4B | SERPIN11 |
| CTS1L                                        | HECTD1               | PFAH1B2        | UNC45A        | PNP  | WBSRCR2 |
| CUL2                                         | HIST2H2AB            | PGD            | USP24         | CYB7B |
| CYBB                                          | IDE                  | PLD3           | VW8A          | CYP4F22 |
| CYP7B1                                        | ITGB2                |                |               |        |
Figure 3 (See legend on next page.)
Figure 3A). Interestingly, these genes are also expressed during eye development in the dorsal optic cup [41,42] revealing a persisting pattern of gene expression from embryonic development to adulthood in the iris of newts. It is tempting to speculate that such genes may aid or repress regeneration hence providing the intrinsic regeneration potential of the dorsal iris. COL3A1, glutathione S-transferase omega-1 (GSTO1), galectin-3-binding protein (LGALS3BP), DNA replication licensing factor MCM4, PARP1, and SYK were up-regulated during regeneration both in the ventral and the dorsal parts of the iris ($p < 0.05$; Figure 3B). These genes are related to extracellular matrix, cell adhesion, redox balance, DNA maintenance, and DNA repair, processes required for regeneration and wound repair. S-adenosylmethionine synthase isoform type-2 (MAT2A), DNA replication licensing factor MCM6, MPO, proliferating cell nuclear antigen (PCNA), structural maintenance of chromosomes protein 2 (SMC2), and VCAN are also associated with the above-mentioned cellular processes but showed a higher expression in the dorsal versus the ventral iris and were expressed at higher levels during regeneration compared to undamaged controls ($p < 0.05$; Figure 3C) suggesting that the dorsal iris responds more robustly than the ventral iris to regenerative cues. Desmin (DES) is an intermediate filament found mostly in muscle tissue and has been associated with mitochondrial dysfunction and elevated ROS [43]. Desmin is only up-regulated during regeneration in the ventral iris ($p < 0.05$; Figure 3D). Tropomyosin alpha-1 chain (TPM1) is another cytoskeleton protein up-regulated in the ventral iris compared to the dorsal iris ($p < 0.05$; Figure 3E). Lastly, sulphotransferase family cytosolic 1B member 1 (SULT1B1), an enzyme catalyzing sulfonation, was expressed at higher levels in the intact iris (day 0), an expression pattern also found during liver regeneration in rats (Figure 3F) [44]. Overall, the qPCR data corroborated the expression changes found at the proteome level by mass spectrometry.

Protein expression patterns from the in vitro proteome

IPE cells retain their ability to transdifferentiate to lens cells in vitro, a process that can take up to 2 weeks. After re-aggregation and transplantation into a lentectomized eye, only the dorsal but not ventral iris aggregates transdifferentiate to lens cells [45]. Similarly, only dorsal aggregates will transdifferentiate rapidly within 1–2 weeks to a structured lens when placed in 3-D collagen-based lattices like Matrigel [5]. Intriguingly, even IPE cells from higher animals, including humans, can be induced to transdifferentiate into lentoids (not structured lens) under certain culturing conditions [46]. We therefore examined protein expression in cultured IPE cells from the dorsal and ventral iris to monitor potential changes in the state of IPE cells (Figure 4A). In particular, we wanted to know whether culturing changed the protein profile of IPE cells and to identify markers that reflect transdifferentiation.

In total, we identified 2,269 annotated to known human proteins (e-value < 1E-10) in the cultured IPE cells. Proteins showing more than twofold higher expression either in the dorsal or ventral IPE cells are listed in Additional file 2. Numerous proteins were exclusively found either in the dorsal or ventral IPEs although GO terms analysis only revealed enrichment of cytoskeleton-associated terms in ventral versus dorsal IPE cells (Additional file 1 and Figure 4B).

Next, we compared the in vitro proteome with the in vivo proteome of 0, 4, and 8 dpl. Proteins with a similar expression pattern in respect to the dorsoventral axis under both in vitro and in vivo conditions are shown in Table 6. Some proteins have cell cycle, DNA replication, and splicing functions in the cell. EFN1, DES, ALDH1A1, SMC2, and MCM4 proteins showed differences in expression levels between the dorsal and ventral IPE cells, an exact pattern as of their protein expression in vivo. As potential dorsoventral markers, we further validated these expression data by qPCR (Figure 4C). EFN1, ALDH1A1, SMC2, and MCM4 were significantly up-regulated in dorsal IPE cells, while expression of DES was significantly up-regulated in ventral IPE cells ($p < 0.05$; Figure 4C). ALDH1A1, SMC2, and DES, which are similarly regulated in the iris during lens regeneration in vivo (Figure 3A,C), are involved in retinoic acid synthesis and DNA replication. Such cellular processes have been previously shown to be involved in lens regeneration from the dorsal iris [6,47]. Pearson correlation analysis of in vivo and in vitro datasets revealed that the $R^2$ correlation value increased from 0 to 4 dpl with the highest correlation at 8 dpl, indicating cells activated for tissue regeneration (Figure 4D). In contrast,
Figure 4 (See legend on next page.)
ventral IPE cells did not show such a correlation or trend (Figure 4E). The expression of dorsal markers ALDH1A1 and EFNB1 in dorsal IPE cells showed that they keep a “memory” of their origin, which consequently might be responsible for their ability to transdifferentiate to lens cells. The identified dorsal- or ventral-specific proteins might be used as markers in high-throughput screening using small molecules to identify agents inducing regeneration.

**On the road for a common regeneration program**

During the last two decades, several high-throughput methods including microarrays, next-generation RNA sequencing, and mass spectrometry have been developed to characterize gene expression profiles. We have used datasets from several different studies investigating organ regeneration in amphibians and extracted genes that were expressed at higher levels during tissue regeneration compared to intact controls. We focused on genes that were expressed more than twofold at any regeneration time-point compared to intact tissue (for more information, see the “Methods” section). In addition, we annotated the genes based on human gene names serving as a common reference for the comparisons. Our search included seven microarray datasets from newt brain, spinal cord, hindlimb, forelimb, lens, heart and tail regeneration, one microarray and one RNA-seq dataset from axolotl limb regeneration [48,49], and two LC-MS/MS studies in newt heart regeneration and axolotl hindlimb regeneration [50-52]. We compared these datasets to proteins up-regulated at least twofold in the dorsal iris during lens regeneration compared to intact iris (Figure 5 and Additional file 3). Surprisingly, the highest degree of similarity was found when RNA-seq data from limb regeneration were used (Table 7) [49]. Genes which were jointly activated in these, rather different, tissues during regeneration (Figure 5 and Table 7) most likely represent a part of a canonical regeneration program. Hallmarks of the program include inflammation for host defense and cell activation, proliferation of new cells to replace lost tissue, migration for rearrangement of cells, generation of an appropriate extracellular matrix, regulation of ROS and DNA repair for tissue homeostasis, metabolic processes for cells to meet the needs of energy-consuming cellular processes, and changes in gene expression to shape the newly formed organ. We assume that these functional groups play a decisive role in the majority of all regeneration events.

**Conclusions**

In this study, we employed LC-MS/MS to identify proteins that are highly expressed during newt lens regeneration. Some of these proteins have similar functions and are arranged in protein networks associated with regulation of the extracellular matrix, DNA repair and maintenance, gene expression, and regulation of translation. Comparisons to other datasets collected during regeneration of a variety of different tissues from amphibians species revealed a putative canonical regeneration program, which seems to be required for regeneration to occur. Finally, we showed that cultured dorsal IPE cells *in vitro* maintain a molecular memory of their origin and show similar patterns as the 8-dpl *in vivo* lens regeneration dorsal iris. Taken together, our study provides information about proteins and protein groups that play an important role during tissue regeneration and deepens our understanding of the mechanism of regeneration.

**Methods**

**Animal procedures**

Animal handling and operations have been described previously [6,45]. Adult newts, *Notophthalmus viridescens*, were purchased from the Charles Sullivan Inc. Newt Farm. Anesthesia was performed with 0.1%(w/v) ethyl-3-aminobenzoate methanesulfonic acid (MS222; Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS; 37 mM NaH2PO4 monohydrate, 58 mM Na2HPO4 anhydrous, pH 7.0). All procedures involving animals were approved by the University of Dayton Institutional Animal care and Use Committee.
Sample collection for LC-MS/MS
Newts were anesthetized and whole eye balls were removed and placed in calcium- and magnesium-free (CMF) Hank's solution. Using scissors, eye balls were dissected and iris rings were isolated. Using a scalpel, dorsal and ventral 135° sectors were extracted and kept frozen at −70°C until use.

Sample collection for qPCR, RNA extraction, reverse transcriptase reaction, qPCR reactions, and enrichment analysis
All procedures were performed as described previously [6]. For primers and quantitative real-time polymerase chain reaction (qPCR) settings see Additional file 4. Student's t-test
for independent samples was used to determine statistical significance for qPCR expression data. Equal variances were determined with Levene’s test. Groups for enrichment analysis were selected as follows: For in vivo proteome analysis, protein expression was detected at 0 dpl and at least for one of the 4- and 8-dpl samples. Only differences more than twofold were used for further bioinformatical analysis. Annotation was assigned to newt proteins using BLAST2GO with e-value less than 1E-10. GO mapping and annotation was performed with default settings. GO enrichment analysis was calculated using Fisher’s exact test corrected for multiple selections which is a built-in feature of BLAST2GO [53,54]. GO terms were considered enriched when FDR < 0.05.

Network analysis
Network analysis was performed using VisANT [29]. For construction of the dorsal regeneration network, only proteins with more than twofold change during regeneration (4 and/or 8 dpl) compared to the intact control and more than twofold change compared to the equivalent timepoints in the ventral iris samples were included. The selected proteins were used as input for the program. Human gene names and the human interactome were used for this analysis. Only proteins that had at least one interaction with a different protein of the group were displayed. The same procedure was used for the construction of the ventral regeneration network.

Newt IPE cell culture
Newt dorsal and ventral IPE cell culture was performed as described previously with minor modifications [45]. Dorsal and ventral IPE cells were plated separately in DMEM on collagen I coated plates (Becton Dickinson, Franklin Lakes, NJ). Cells were incubated at 27°C with 2% CO₂. The medium was changed every other day till day 21. On day 21, dispase (Gibco, Life technologies, Grand Island, NY) was added to the medium with a final concentration of 5% (v/v) and incubated overnight at 27°C with 2% CO₂. The collected cells were washed thrice with CMF Hank’s solution and frozen with liquid nitrogen until use.

LC-MS/MS procedures
The iris tissue and cultured cells were isolated as described above. Proteins were isolated as described in [55] and processed for mass spectrometry (reverse-phase nano-LC-MS/MS, Thermo Velos and Q Exactive, Thermo Scientific, Waltham, MA) measurements. In brief, proteins were isolated by homogenizing tissue/cells in a buffer containing 1% Nonidet P-40, 0.1% sodium deoxycholate, 150 mm NaCl, 1 mm EDTA, and 50 mm Tris, pH 7.5 and protease inhibitor mixture (Roche, San Francisco, CA). Next, proteins were separated by 1D SDS-PAGE and stained by Coomassie Blue. The gel was cut into eight slices per lane (each timepoint in vivo, dorsal and ventral in vitro). Gel slices were washed by 100 μL 50 mM ammonium bicarbonate (ABC)/50% ethanol (EtOH) for 20 min at RT and dehydrated by incubating for 10 min in 100 μL absolute EtOH. Protein reduction was performed by incubating the gel pieces in 100 μL 10 mM DTT (in 50 mM ABC) for 45 min at 56°C. Alkylation was done by incubating the gel pieces in 100 μL 55 mM iodoacetamide for 30 min at RT in darkness. After a final washing step, gel pieces were dried and proteins were in-gel digested using trypsin overnight. For desalting, peptides were loaded onto STAGE-tips and eluted with 80% acetonitril for mass spectrometry (MS) analysis [56,57]. Reversed-phase nano-LC-MS/MS was performed by using an Easy nanoflow HPLC system (Thermo Fisher Scientific, Odense Denmark; binary buffer system of A (0.1% (v/v) formic acid in H2O) and B (0.1% (v/v) formic acid in 80% acetonitrile); 50-cm column (75-μm ID) packed in-
house with 1.9-µm diameter C18 resin). The HPLC is coupled to Q Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with an electrospray ionization source (Thermo Fisher Scientific, Bremen, Germany). MS spectra were acquired at a resolution of 70,000 (200 m/z) in a mass range of 350–1,650 m/z and the top 10 most intense ions were selected for fragmentation. To identify mass-spectrometry-derived spectra, a de-novo-assembled transcriptome [7] was utilized by translating it into six reading frames generating an Andromeda search engine [58] compatible database. Only reading frames greater than 50 AA were used. Subsequent protein identification and label-free quantification was performed using MaxQuant software (Version 1.2.0.18) [59]. The maximum false discovery rate was set below 1% for peptide and protein identifications using the DECOY target database approach. Minimum peptide length was set to 7 AA and two peptides per protein group (at least one unique peptide). Carbamidomethyl at cysteine residues was set as a fixed modification. Oxidation at methionine and acetylation (tide).

Protein expression from LC-MS/MS data were normalized together for better correlation of expression levels. Pearson correlation was performed between dorsal IPE cell protein expression and the different timepoints of in vivo dorsal iris. Similar comparisons were performed with ventral samples. Tests were performed using Microsoft Office Excel spreadsheets.

Comparisons with other datasets
Microarray, RNA sequencing, and LC-MS/MS data were extracted from the following papers: newt heart [50], forelimb, hindlimb, spinal cord, tail, brain, heart, tail [51], lens (dorsal iris) [8], and axolotl limb regeneration [48,49,52]. Genes were selected based on two expression criteria: expressed more than twofold in any of the regeneration timepoints compared to the control and not expressed more than twofold in the control compared to any of the regeneration timepoints. Human gene names were assigned to the extracted genes from all the datasets based on the annotation provided in the corresponding papers. Ambiguous annotations were discarded. The extracted genes can be found in Additional file 3. Comparisons, annotation assignments, and data mining were performed using custom Perl scripts.

Comparison between in vitro LC-MS/MS and in vivo LC-MS/MS data
In vitro LC-MS/MS data and in vivo LC-MS/MS data were normalized together for better correlation of expression levels. Pearson correlation was performed between dorsal IPE cell protein expression and the different timepoints of in vivo dorsal iris. Similar comparisons were performed with ventral samples. Tests were performed using Microsoft Office Excel spreadsheets.
10. Hsieh HC, Chen YT, Li JM, Chou TY, Chang MF, Huang SC, Tseng TL, Liu CC, Chen SF: Protein profilomics in mouse liver regeneration after partial hepatectomy using iTRAQ technology. J Proteome Res 2009, 8:1004–1013.

11. Duguez S, Bihan MC, Gouttefangeas D, Freassin L, Freysinet D: Myogenic and nonmyogenic cells differentially express proteinases, Hsp/Hsp70, and BAG-1 during skeletal muscle regeneration. Am J Physiol Endocrinol Metab 2003, 285:C206–C215.

12. Tawk M, Jouille C, Vito Z: Zebrafish Hsp40 and Hsc70 genes are both induced during caudal fin regeneration. Mech Dev 2000, 98:183–186.

13. Mitra P, Vaughan PS, Stein JL, Stein GS, van Wijnen AJ: Promotion of corneal epithelial wound healing in vivo. Invest Ophthalmol Vis Sci 2006, 47:1862–1868.

14. Braun MC, Kelly CN, Prada A, Mishra J, Chand D, Devajaran P, Zahedi K: Human PRRMT5 expression is enhanced during in vitro tubule formation and after in vivo ischemic injury in renal epithelial cells. Am J Nephrol 2004, 24:250–257.

15. Rouhana I, Vieira AP, Roberts-Galbraith RH, Newmark PA: PRRMTs and the role of symmetrical dimethylarginine in chromoatid bodies of planarian stem cells. Development 2012, 139:1083–1094.

16. Morikawa K, Okese F, Morikawa S: The role of CD45 in the activation, proliferation and differentiation of human B lymphocytes. Int J Hematol 1991, 54:495–499.

17. Shivtei S, Lapid K, Kalchenko V, Avigdor A, Goichberg P, Kalinkovich A, Keri G, Stemmann D, Mann M: Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. Mol Cell 2008, 31:436–448.

18. Ueno S, Blanchette P, Yam K, Kamura T, Morrison M, Boivin D, Kaelin WG, Conway RC, Conway JW, Yan Y: Degradation of p53 by adenovirus E1orf4 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. Genes Dev 2001, 15:3104–3117.

19. Li Y, Zhang P, Wang C, Han C, Meng J, Liu X, Xu S, Li N, Wang Q, Shi X, Cao Z: Paracrine control of tissue regeneration and cell proliferation by Caspase-3. Cell Death Dis 2013, 4:e725.

20. Pechter PM, Gil J, Valdes J, Tomic-Canic M, Pastar I, Stojadinovic O, Kinsar RS, Davis SC: Keratin dressings speed epithelialization of deep partial-thickness wounds. Wound Repair Regen 2012, 20:336–242.

21. Fan X, Moltokov A, Manabe S, Dominy CM, Delcourt L, Foglio MH, Cuenca AE, Blain WS, Lipton SA, Duester G: Targeted disruption of Aldh1a1 (Raldh1) provides evidence for a complex mechanism of retinoic acid synthesis in the developing retina. Mol Cell Biol 2003, 23:4637–4648.

22. Triplett JW, Feldheim DA: Eph and ephrin signaling in the formation of retinal topographic maps. Semin Cell Dev Biol 2012, 23:7–15.

23. Maloyan A, Osinko H, Lammerding J, Lee RT, Cingolani OH, Kass DA, Lorenz JN, Robbins J: Biochemical and mechanical dysfunction in a mouse model of desmin-related myopathy. Circ Res 2009, 104:1021–1028.

24. Dunn RT 2nd, Kolaja K, Klassen CD: Effect of partial hepatectomy on the expression of seven rat sulphotransferase mRNAs. Xenobiotica 1999, 29:583–593.

25. Bruns RA, Nakamura K, Tison PA: A system for culturing iris pigment epithelial cells to study lens regeneration in newt. J Vis Exp 2011, 52:e2713.

26. Tison PA, Wang J, Del-Rio-Tsonis K, Equchi G: A unique aged human retinal pigment epithelial cell line useful for studying lens differentiation in vitro. J Int J Dev Biol 2001, 45:753–758.

27. Tison PA, Trombley MT, Rowland T, Chandraratna RA, del Rio-Tsonis K: Role of retinoic acid in lens regeneration. Dev Dyn 2000, 219:588–593.

28. Campbell LJ, Suarez-Castillo EC, Ortiz-Zuazaga H, Knopp D, Tanaka EM, Thomson JA, Dewey CM: Gene expression profile of the regeneration epithelium during axolotl limb regeneration. Dev Dyn 2011, 240:1826–1840.

29. Stewart R, Rascon CA, Tan S, Nie J, Barry C, Chu LF, Ardalani H, Wagner RJ, Prabosko MD, Bolin JM, Leng N, Sengupta S, Volkmer M, Habermann B, Tanaka EM, Thomson JA, Dewey CM: Comparative RNA-seq analysis in the unsequenced axolotl: the oncogene burden highlights early gene expression in the blastema. PLoS Comput Biol 2013, 9:e1002936.

30. Looso M, Michel CS, Konzer A, Bruckskotten M, Borchard T, Kruger M, Braun MW, Sarajani M, Bovin D, Kaelin WG: A unique aged human retinal pigment epithelial cell line useful for studying lens differentiation in vitro. J Int J Dev Biol 2001, 45:753–758.

31. Tison PA, Trombley MT, Rowland T, Chandraratna RA, del Rio-Tsonis K: Role of retinoic acid in lens regeneration. Dev Dyn 2000, 219:588–593.

32. Calcé S, Odelberg SJ, Simon HG: A transient extraocular matrix instructs cell behavior during muscle regeneration. Dev Biol 2010, 344:259–271.

33. Gilquin B, Cannon BR, Hubshenberger A, Moulouel B, Falk E, Merle N, Asanà N, Kieffer S, Rousseau O, Wilder PT, Weber DJ, Baudier J: The calcium-dependent interaction between S100B and the mitochondrial AAA ATPase ATAD3A and the role of this complex in the cytoplasmic processing of ATAD3A. Mol Cell Biol 2010, 30:2724–2736.

34. Sadokavassos G, Dobocan MC, Dilafco MR, Congote LF: Regulator of differentiation 1 (RODI) binds to the amphibiotic C-terminal peptide of thrombomodulin-4 and is involved in its mitogenic activity. J Cell Physiol 2009, 222:672–679.

35. Nitrins K, Mizuno R, Okada T, Nakawai R, Shibato J, Masuo Y, Jiró K, Aimitsu N: MALAT-1 enhances cell motility of lung adenocarcinoma cells by influencing the expression of motility-related genes. FEBS Lett 2010, 584:4575–4580.

36. Hsiao HC, Chen YT, Li JM, Chou TY, Chang MF, Huang SC, Tseng TL, Liu CC, Sousounis et al. Human Genomics http://www.humgenomics.com/content/8/1/22

37. Love NR, Chen Y, Ishibashi S, Nishioka P, Lee R, Koh Y, Gallop JL, Dorney K, Amaya E: Amputation-induced reactive oxygen species are required for successful Xenopus tadpole tail regeneration. Nat Cell Biol 2013, 15:222–228.

38. Tyszczuk M, Rucinski S, Ziolkowka A, Trejter M, Suzyka M, Malendowicz UK: Expression of selected genes involved in steroidogenesis in the course of enucleation-induced rat adrenal regeneration. Int J Mol Med 2014, 33:613–623.

39. Mace KA, Pearson JC, McGinnis W: An epidermal barrier wound repair pathway in Drosophila is mediated by grainy head. Science 2005, 308:381–385.

40. Wang J, Kara R, Dickson AL, Pass KD: Fibronectin is deposited by injury-activated epidermal cells and is necessary for zebrasfish heart regeneration. Dev Bio 2013, 382:427–435.

41. Hu Z, Srinik ES, Deli C, SubANT: an integrative framework for networks in systems biology. Brief Bioinform 2008, 9:317–325.

42. Worapamom W, Xiao Y, Liu Y, Young WG, Bartold PM: Differential expression and distribution of syndecan-1 and -2 in periodontal wound healing of the rat. J Periodontal Res 2003, 37:293–299.

43. Watanabe M, Kondo S, Mizuno K, Yano W, Nakao H, Hattori Y, Kimura K, Nishida T: Promotion of corneal epithelial wound healing in vitro and in vivo by annexin A5. Invest Ophthalmol Vis Sci 2006, 47:1862–1868.
54. Myhre S, Tveit H, Mollestad T, Laegreid A: Additional gene ontology structure for improved biological reasoning. *Bioinformatics* 2006, 22:2020–2027.

55. Looso M, Borchardt T, Kruger M, Braun T: Advanced identification of proteins in uncharacterized proteomes by pulsed in vivo stable isotope labeling-based mass spectrometry. *Mol Cell Proteomics* 2010, 9:1157–1166.

56. Shevchenko A, Wilm M, Vorm O, Mann M: Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal Chem* 1996, 68:850–858.

57. Andersen JS, Lam YW, Leung AK, Ong SE, Lyon CE, Lamond AI, Mann M: Nucleolar proteome dynamics. *Nature* 2005, 433:77–83.

58. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M: Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res* 2011, 10:1794–1805.

59. Cox J, Mann M: MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat Biotechnol* 2008, 26:1367–1372.

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