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Analysis of the Otd-dependent transcriptome supports the evolutionary conservation of CRX/OTX/OTD functions in flies and vertebrates

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Abstract

Homeobox transcription factors of the vertebrate CRX/OTX family play critical roles in photoreceptor neurons, the rostral brain and circadian processes. In mouse, the three related proteins CRX, OTX1, and OTX2 fulfill these functions. In *Drosophila*, the single founding-member of this gene family, called *orthodenticle (otd)*, is required during embryonic brain and photoreceptor neuron development. We have used global gene expression analysis in late pupal heads to better characterize the post-embryonic functions of Otd in *Drosophila*. We have identified 61 genes that are differentially expressed between wild type and a viable eye-specific *otd* mutant allele. Among them, about one third represent potentially direct targets of Otd based on their association with evolutionarily conserved Otd-binding sequences. The spectrum of biological functions associated with these gene targets establishes Otd as a critical regulator of photoreceptor morphology and phototransduction, as well as suggests its involvement in circadian processes. Together with the well documented role of *otd* in embryonic patterning, this evidence shows that vertebrate and fly genes contribute to analogous biological processes notwithstanding the significant divergence of the underlying genetic pathways. Our findings underscore the common evolutionary history of photoperception-based functions in vertebrates and invertebrates and support the view that a complex nervous system was already present in the last common ancestor of all bilateria.

Keywords

phototransduction; photoreceptor; circadian rhythms; orthodenticle; ocelliless; oc; visual transduction; eye evolution; photoreceptor development

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INTRODUCTION

Patterning, fate specification and differentiation during development are orchestrated by tissue and cell specific transcription factors that control extensive genetic networks. In vertebrates, distinct, but often structurally-related, transcriptional regulators control these different steps in the developmental program. In mouse, three highly related homeodomain transcription factors have been identified that are critical for many aspects of anterior head patterning and neuronal specification: OTX1, OTX2, and OTX5/CRX. OTX1 and OTX2 function early during embryonic development in the specification and regionalization of the anterior nervous system (Suda et al., 1996; Boyl et al., 2001). In addition, OTX2 is required for cell specification in the pineal gland and CRX/OTX5 controls circadian gene expression in this organ (Nishida et al., 2003; Gamse et al, 2002; Furukawa et al., 1999). All three factors contribute to the development of distinct cell types in the retina as well. CRX, in particular, is a critical regulator of terminal differentiation in vertebrate photoreceptor cells (Freund et al., 1997, 1998; Viczian et al., 2003; Plouhinec et al., 2003; Fey et al., 1999; Bobola et al, 1999; Fong and Fong, 1999; Nishida et al., 2003; Martinez-Morales et al., 2001). Several OTX2 and CRX allelic variants are linked to congenital eye diseases, underscoring the importance of these proteins for eye development in humans (Freund et al., 1998; Jacobson et al., 1998; Sohocki et al., 1998).

In *Drosophila*, the sole representative of the OTX/CRX family is the Orthodenticle (Otd) transcription factor (synonym: Ocelliless, Oc). Its embryonic expression is essential for patterning of the anterior region of the body plan and the central nervous system (Finkelstein et al., 1990a and b; Wieschaus et al., 1992; Hirth et al., 1995; Younossi-Hartenstein et al., 1997). Consistent with this early role, severe or null *otd* mutant alleles are embryonic lethal. Nonetheless, the postembryonic functions of Otd can be investigated using the viable mutant allele otd^{uvi}. This strong hypomorphic allele was identified in a phototactic behavior screen, and significantly reduces otd expression in the developing and adult eye, but not in the embryo. In otd^{uvi} animals, the photoreceptor neurons of the eye (R-cells R1 through R8) have aberrant morphology and the expression of several *Rhodopsin (Rh)* genes is mis-regulated. Specifically, i) the light-sensing organelles (called rhabdomeres) are misshapen, duplicated or shortened, ii) the opsin-encoding genes *Rh3* and *Rh5* fail to be expressed in a subset of the R7 and R8 cells, and iii) *Rh6* transcription is ectopically activated in the R1–R6 photoreceptors (Vandendries et al., 1996; Tahayato et al., 2003). These phenotypes implicate Otd in the terminal differentiation of the photoreceptor neurons of the fly eye.

The otd^{uvi} mutant phenotype can be largely rescued by inducing Otd expression (*hs-otd*) from 12% to 75% of pupal development (PD) (Vandendries et al., 1996; Tahayato et al., 2003), marking a critical interval during which *otd* is required for the terminal differentiation of R-cells. Two phases of Otd function have been identified: a phase prior to 40% PD that is critical for the development of proper R-cell morphology and a later phase (from ~50% to ~75–80% PD) that is required for opsin genes activation (Vandendries et al., 1996; Tahayato et al., 2003). Despite the crucial roles played by this factor in photoreceptor neurons, the only gene targets identified to date are Rh3, Rh5 d Rh6 (Tahayato et al., 2003). Thus, our understanding of how Otd contributes to the morphological and functional specialization of photoreceptor cells remains very limited. Moreover, whether Otd fulfills other photoperception-related roles has not been determined.

In this work, we sought to identify direct and indirect targets of Otd during the late stage of retinal differentiation. Toward this end, we carried out a comparative global gene expression analysis of otd^{uvi} mutant and wild type fly heads during late pupal stage 12 (~80% PD), soon after the critical interval for Otd function in photoreceptor morphogenesis and *Rh* gene expression. We find that 61 genes are differentially expressed. A substantial fraction of these

genes (~1/3) contain, within 1 kb of their transcription start sites, one or more putative Otd binding sites that are evolutionarily conserved and, thus, represent potentially direct targets of Otd. The spectrum of direct and indirect gene targets identified establishes Otd's role as a critical regulator of morphogenesis and phototransduction in photoreceptor cells, as well as suggests its involvement in circadian processes.

MATERIALS AND METHODS

Drosophila *stocks and target preparation*

The *Drosophila* strain *Canton S (CS)* was used as wild type for comparative gene expression analysis with the mutant otd^{uvi} (Vandendries et al., 1996) strain. For each biological replicate, samples were derived from independent RNA extractions consisting of 15–20 heads. CS and otd^{uvi} flies were raised side by side under identical conditions (incubator at 25°C). An equal mix of male and female pupae staged to late P12 as per Ashburner (1989) (~80% PD) were selected for RNA extraction. Fly heads were immediately transferred to 600μl Trizol (GIBCO/BRL), and stored at −80°C. RNA extraction was performed according to manufacturer's instructions. Qualitative and quantitative analysis of the total RNA was carried out using capillary electrophoresis on a RNA 6000 Nano Assay chip, with the Agilent 2100 Bioanalyzer (Agilent Technologies USA). Probes were generated from 10 μg total RNA according to the Enzo BioArray HighYield RNA Transcript Labeling Kit (Affymetrix, P/N900182). 15 μg synthesized biotin-labeled cRNA target was fragmented and hybridized to arrays according to the standard Affymetrix protocol. Other fly stocks include *Oregon-R(OR)*, the enhancer-trap line *R32* (Shafer et al., 2006), and the line otd^{uvi}*; p[WIZ]8; rh4-lacZ* (Tahayato et al, 2003). *p[WIZ8]* is an RNAi P-element insertion that eliminates expression of the white pigmentation gene and provides the same advantages as using a *w* mutant genetic background in immunofluorescent stainings or β-Galactosidase activity measurements (Lee and Carthew, 2003).

Microarray analysis

Microarray data have been deposited in NCBI's Gene Expression Omnibus with the GEO Series accession number GSE5321 (http://www.ncbi.nlm.nih.gov/geo/). High density oligonucleotide microarrays covering the *Drosophila melanogaster* genome (DrosGenome1) from Affymetrix, Santa Clara, CA, were used in this study. The DrosGenome1 array includes 14,090 probe sets representing 13,369 transcripts encoding *Drosophila* proteins deposited in SWISS-PROT/TrEMBL databases (Celera Genome/BDGP Release 1). Each probe set is represented on the array by a set of 14 oligonucleotide probes of perfect matching sequence and 14 probes with a single nucleotide mismatch. The oligonucleotides are 25mers. The Average Difference between the perfect match hybridization signal and the mismatch signal is proportional to the abundance of a given transcript (Lipshutz et al., 1999). Microarray data were normalized at the probe level with S-plus / Array Analyzer version 2 (Insightful, Seattle) using the invariant set algorithm. Background correction and expression index calculations were carried out using the MAS5 Statistical Algorithm (details described in Affymetrix technical manual). Five GeneChips were used in this experiment, 2 replicates for *CS* and 3 for otd^{uvi}. Statistical score and fold change criteria were applied to identify differentially expressed genes. The statistical scores were assessed using the local pooled error (LPE) test (Jain et al., 2003). The raw statistical scores were corrected for multiple comparisons using the Benjamini and Hochberg (1995) method. The fold change and its confidence intervals were calculated using dChip version 1.3 (Li and Wong, 2001). The list of differentially-expressed genes was generated by selecting genes that fulfilled both statistical score and fold change criteria, and showed at least a 2 fold change of the lower bound of confidence intervals. One hundred and twenty one genes showed at least 2 fold change in expression between CS and otd^{uvi} at a

significance level of corrected *p*-value ≤ 0.01 (Supplementary Table 1). Further testing was carried out by RT-PCR as detailed below.

RT-PCR microarray data validation and genetic background testing

Validation of microarray - Microarray data were validated using semi-quantitative end-point reverse-transcription PCR (RT-PCR) with densitometry. Fly husbandry and RNA extraction were as described above. First strand synthesis was carried out on 2 μg total RNA using Superscript RT (Invitrogen) and PCR amplification was performed with High Fidelity PCR Supermix (Invitrogen). Cycle number, primers and template concentrations were individually optimized for each gene. Amplification products were visualized on a 1% agarose gel stained with ethidium bromide. Band intensity was estimated using Image-J (http://rsb.info.nih.gov/ij). GAPDH (a constitutively expressed gene encoding a glycolytic enzyme) and CG7214 (a gene consistently unchanged in all mutant and wild type comparisons of the microarrays with a P-value > 0.0001) were used as controls in parallel, and GAPDH as an internal control when needed (Fig. 1A and B). The mRNA levels in mutant (*otd*^{uvi}) and wild type (*CS*) samples could be reliably evaluated for 112 candidate genes identified by microarray. This number does not include 9 genes that gave inconsistent results with the selected primers and were scored as not determined, ND, in Supplemental Table 1. Among the 112 that could be evaluated, the RT-PCR results for 22 genes (XX in Supplementary Table 1) were repeatedly in disagreement with the microarray findings, whereas 90 genes (80%) repeatedly showed a change in transcript levels consistent with the microarray findings. These results confirm the high level of reliability of the microarray experiment. *Genetic background comparison* - To correct for differences in genetic background (independent of the *otd* locus), we also analyzed expression of the 90 validated genes in the wild type strain *OR*. In the case of 29 (32%) genes, the changes in expression observed in *CS* versus *otd*^{uvi} were not supported in the *OR* versus *otd*^{uvi} comparison (X in Supplementary Table 1). Hence, the expression of these 29 genes is significantly influenced by genetic background and may or may not also be affected by the *otd*^{uvi} mutation. Lastly, we compared expression levels for the genes involved in phototransduction, expressed in the eye, and/or regulated by circadian rhythms (*Arr2*, *boss*, *CdsA*, *CG15630*, *CG7033*, *CG8889*, *chp*, *Cpn*, *Cyp6a17*, *Cyp6a2*, *GstE1*, *Rh3*, *Rh4*, *Rh5*, *Slo*, *Slob*, *trx*) between the original *otd*^{uvi} line and a derived *otd*^{uvi} line. This derived line was generated by crossing mutant males from the original *otd*^{uvi} stock to attached-X C(1)Dx females; thereafter, male progeny was backcrossed to C(1)Dx females from the original stock for four consecutive generations. *Rh1 testing* - Rh1 was mildly decreased in our microarray analysis (−1.49; *p*-value 0.00312) but essentially unchanged by RT-PCR with GADPH as internal control (levels vary between 0.94 and 1.04 of wild type). List of all primer pairs used will be provided upon request.

β-Galactosidase activity measurement

Assays were carried out using either o-nitrophenyl β-D-galactoside (OPNG) or chlorophenol red-β-D-galactopyranoside (CPRG) as substrates (Simon and Lis, 1987). OPNG protocol: heads were homogenized in 110 μl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). Cell debris was removed by centrifugation for 15 min at 4 °C. 10 μl of extract was used to measure total protein concentration at 280 nm. 90 μl of extract was mixed with 410 μl Z-buffer, 100 μl ONPG (4 mg/ml), and incubated at 37 °C until yellow color developed. The reaction was stopped by adding 500 μl 1 M Na_2CO_3, and an optical density reading was taken at 420 nm. β-Galactosidase activity was calculated in Miller units as follows: $1000 \times OD_{420}/(t \times \text{volume in } \mu l \times OD_{280})$, where t is reaction time in min. CPRG protocol: heads were homogenized in 100 μl of assay buffer (50 mM potassium phosphate, 10 mM magnesium chloride, pH 7.5) and the final volume was brought to 1 ml. The extracts were vortexed and centrifuged for 10 minutes at 12000, 4°C. 200 μl extracts of each sample were used in the Bradford assay to determine protein concentration

(OD_{595}). 350 µl of the wild type and 700 µl of *otd*^{uvi} head extracts were mixed with CPRG (Roche) in assay buffer to a final concentration of 1 mM CPRG in 1 ml total volume. Reactions were incubated for 1 hr at 37 °C before optical density readings. β-Galactosidase activity was calculated as OD_{574}/[reaction time (min) × OD_{595} × extract volume (µl)]. For each sample, at least five heads from male flies of the following genotypes were processed: 1) *CS*$_X$*/Y; p*$^{[Wiz8]/CS_{II}}$*; p[Rh4-lacZ]/CS*$_{III}$ (WT); *2) otd*^{uvi}*/Y; p[Wiz8]/CS*$_{II}$*; p[Rh4-lacZ]/CS*$_{III}$(mutant); 3) *OR*$_X$*/Y; p[Wiz8]/OR*$_{II}$*; p[Rh4-lacZ]/OR*$_{III}$ (WT); *4) otd*^{uvi}*/Y; p[Wiz8]/ OR*$_{II}$*; p[Rh4-lacZ]/ OR*$_{III}$ (mutant); *otd*^{uvi}*/Y; p[Wiz8]/* +; *p[Rh4-lacZ]*/+ [from a cross of two *otd*^{uvi} lines: *otd*^{uvi}*/ otd*^{uvi} females to *otd*^{uvi}*/Y; p[Wiz8]/ p[Wiz8]; p[Rh4-lacZ]/p[Rh4-lacZ]* males] Both experiments were repeated at least twice using duplicate or triplicate samples. In all experiments, β-Galactosidase activity was reduced in the *otd*^{uiv} mutants measuring from 17% to 40% of matched wild type backgrounds.

Antibodies and immunofluorescent staining

The full-length *otd* coding sequence was cloned into pET28b plasmid and transformed into BL21-CodonPlus (DE3)-RP competent cells (Stratagene). Protein expression was induced with 0.1mM IPTG for 4 hr, and bacteria were lysed for 2 hours at room temperature in 8M urea lysis buffer (ULB) (100 mM NaH_2PO4, 10 mM Tris-HCl, pH8.0, 10 mM imidazole, 8M urea, 10mM β-mercaptoethanol, 0.5% NP-40). Samples were centrifuged for 30 min at 16,000 × g, and the supernatant was mixed with Ni-NTA beads (Qiagen) for 4 hr at room temperature. Beads were washed five times with wash buffer (ULB+250 mM NaCl), and the fusion protein was eluted from the beads with elution buffer (ULB+300 mM imidazole). This solution was used to immunize guinea pigs (Cocalico Biologicals, Inc). Specificity for 'GP6 anti-Otd' was tested in an *otd*^{uvi} mutant background. For cryosections, heads from *y w*^{67} or R32 flies were embedded and frozen in OCT, sectioned (10 µm), processed and stained as previously described (Cook et al., 2003) and then mounted with or without DAPI to visualize cell nuclei in the brain. Primary antibodies: GP6 anti-Otd (1:750), rabbit anti-Rh6 (1:2500, Tahayato et al, 2003), rabbit anti-β-Galactosidase (1:4000, Cappel) and mouse anti-PDF (1: 5, Developmental Studies Hybridoma Bank, Iowa). Secondary antibodies: AlexaFluor-conjuated secondary antibodies were used at 1:1500 (Fig. 2A) and 1:500 (Fig. 2B) (Invitrogen/Molecular Probes). Digital images were taken with the Apotome deconvolution system (Zeiss) and processed with Axiovision 4.5 (Zeiss) (Fig. 2A) and on a LeicaTS confocal microscope (Fig. 2B).

Putative binding sites identification

1 kb of 5' upstream genomic DNA from each of the 61 differentially expressed genes identified by microarray and confirmed by RT-PCR was obtained using a batch query from the Ensembl Genome browser at http://may2005.archive.ensembl.org/. Sequences were scanned for Otd binding sites using Target Explorer (Sosinsky et al., 2003) (http://luna.bioc.columbia.edu/Target_Explorer) based on the Otd position weight matrix (PWM) described by Lifanov et al (2003) (see Supplemental Figure 2). The comparison of DNA sequences from various *Drosophilids* was carried out through VISTA (Frazer et al., 2004) (http://pipeline.lbl.gov/cgi-bin/gateway2?bg=dm2&selector=vista) and in a few cases by BLAST alignment.

RESULTS AND DISCUSSION

The comparative analysis of gene expression in wild type and *otd*^{uvi} mutant heads was carried out at the late P12 stage of pupal development (or ~80% PD), at a time in the terminal differentiation of photoreceptor neurons characterized by the establishment of *Rhodopsin* genes expression (Earl and Britt, 2006). Genes that were found to be differentially expressed between *CS* and *otd*^{uvi} in our microarray analysis were further investigated by RT-PCR in two

wild type strains (*CS* and *OR*) and two *otd*^{uvi} fly lines (see Materials and Methods). Through this analysis, we arrived at a list of 61 genes that show at least a 2-fold change in mRNA levels by microarray ($p \leq 0.01$), and consistently display analogous changes in gene expression by RT-PCR (Table 1 and Supplementary Table 1). This is equivalent to <0.5% of the 13,369 genes represented on the Affymetrix Drosgenome1 array.

Among the 61 differentially expressed genes, 37 are downregulated and 24 are upregulated in *otd*^{uvi} mutant heads (Table 1). Forty-six genes are presently annotated for a number of biological processes and functions (Table 1 and Supplemental Table 2), and 15 have unknown functions. Although the *otd*^{uvi} allele is hypomorphic, and thus does not result in a complete loss of *otd* activity, the expression of 40% of the genes (24/61) was strongly affected (> 4 fold). Among these, four genes that are robustly expressed in the wild type appeared to be transcriptionally inactive in the mutant (*Cyp6a17, Rh3, Acyp2, and Rh5*), whereas 4 genes that are normally expressed at low levels or not at all were found to be strongly induced in *otd*^{uvi} (*CG14743, Try29F, mthl8, and Cyp4p3*) (Table 1, Figure 1A and not shown).

The absence of *Rh3* and *Rh5* mRNA is consistent with the direct transcriptional regulation of both genes by Otd (Tahayato et al., 2003). However, we did not detect a significant change in the only other known direct target in fly heads, *Rh6* (Tahayato et al., 2003). This is likely due to the developmental stage selected for our analysis. Earl and Britt (2006) have recently shown that *Rh6* is the last opsin to be expressed in the pupal retina beginning around ~79% PD and reaching 70% of the adult *Rh6* mRNA levels by 82% PD. Since we sampled gene expression at ~80% PD, increased levels of *Rh6* mRNA due to ectopic expression in the *otd*^{uvi} R1–R6 photoreceptors may not be detectable until later in pupal development or in the adult.

Due to the time point chosen for our analysis, the use of the strong but not null *otd*^{uvi} allele, as well as the stringent criteria applied in the selection of differentially expressed genes, this study can not result in the identification of all genes regulated by Otd in the head. Nonetheless, since the critical interval for Otd function in the differentiating retina extends from ~12% to ~75–80% PD, the list of genes identified in this study should include critical downstream targets of Otd during photoreceptor morphogenesis.

Otd regulates multiple components of the phototransduction cascade

A number of genes involved in phototransduction were down-regulated in *otd*^{uvi} mutant tissue as compared to wild type (Table 1). These include the *Rhodopsins Rh3, Rh4,* and *Rh5, CDP diglyceride synthetase (CdsA),* and *Arrestin2 (Arr2)* (Chou et al., 1996;Papatsenko et al., 1997;Wu et al., 1995;Dolph et al., 1993). As mentioned above, the observed downregulation of *Rh3* and *Rh5* was expected (Tahayato et al., 2003), while the changes in *Rh4, CdsA* and *Arr2* expression identify new direct or indirect targets of Otd.

Although previous work suggested that *Rh4* expression is unchanged in *otd*^{uvi} mutant retinas (Tahayato et al., 2003), we found *Rh4* mRNA levels to be reduced by more than 4-fold in our microarray analysis (Table 1). *Rh4* transcript levels were confirmed to be lower at both the pupal and adult stages in two separate *otd*^{uvi} lines as compared to *CS* and *OR* by RT-PCR (Fig. 1A and not shown). Furthermore, we detected a reduction in β-Galactosidase activity encoded by an *Rh4-lacZ* transgene in the *otd*^{uvi} mutant background as compared to the wild type (Fig. 1C). Thus, it appears that *Rh4* transcript levels are in fact significantly reduced in *otd*^{uvi} R7 cells even though, as reported by Tahayato and colleagues (2003), the spatial pattern of Rh4 expression remains essentially unchanged. The decrease in *Rh4* expression does not reflect a general downregulation of all opsins in mutant photoreceptors. In fact, expression of Rh1, the major rhodopsin expressed in the R1–R6 cells, is not similar affected (see Materials and Methods; Tahayato et al., 2003). However, since the regulatory region included in the *Rh4-*

lacZ transgene does not contain canonical Otd binding sites (Tahayato et al. 2003), the regulation of *Rh4* by Otd is most likely indirect.

Interestingly, the *otd*^{uvi} mutant was originally identified based on its abnormal phototactic behavior in a visible-light (VIS) versus ultraviolet-light (UV) choice test (Vandendries et al., 1996). Rh3 and Rh4 are the two UV-sensitive opsins expressed in the fly eye: Rh4 mediates UV detection in 70% of the R7 neurons, whereas Rh3 does so in the remaining 30%. Rh4 is therefore the predominant UV-sensitive opsin and the downregulation observed in this study is consistent with, and likely contributes to, the abnormal phototactic behavior of *otd*^{uvi} mutant flies (Vandendries et al., 1996).

The *CdsA* and *Arr2* genes also encode critical components of the phototransduction cascade. CdsA is required to regenerate PIP_2, which is the source of the intracellular signals for the visual transduction cascade (Wu et al., 1995; Zuker 1996). Arr2 is involved in the deactivation of the Rhodopsins, and the regulated light-dependent trafficking of the Arr2 protein is essential for light adaptation of photoreceptor cells (Lee et al., 2003). Both are downregulated in *otd*^{uvi} mutant tissue and had not been previously identified as potential Otd targets.

Thus, in addition to *Rh3* and *Rh5*, one more opsin receptor, *Rh4*, and at least two other critical components of the visual transduction cascade, *CdsA* and *Arr2*, are positively regulated by Otd.

Otd regulates known structural components of the photoreceptor cell

Several other genes that are known to function and/or to be transcribed in the eye are also differentially expressed between *otd*^{uvi} and wild type, including *boss, CG8889, chp, Cpn, slo, Slob, trx*. Two of these, *chaoptic (chp)* and *Calphotin (Cpn)*, are known to be required for the differentiation of photoreceptor neurons, and mutations in either gene result in morphological defects similar to those observed in *otd*^{uvi} mutants.

The *chp* gene encodes an adhesion protein that is thought to mediate inter-microvillar stacking within the rhabdomere (Van Vactor et al., 1988). The *Cpn* gene encodes a Ca^{++} ion binding protein (Yang and Ballinger, 1994). As observed by Yang and Ballinger (1994), the *Cpn* mutant phenotype is very similar to the *chp* phenotype as both display distorted, reduced and split rhabdomeres. However, the most severe *Cpn* alleles also lead to photoreceptor cell death, whereas *chp* is dispensable for photoreceptor cell viability (Yang and Ballinger; 1994; Van Vactor et al., 1988). The aberrant rhabdomere morphology observed in *otd*^{uvi} flies (Vandendries et al., 1996) is similar to phenotypes seen in strong *chp* alleles and hypomorphic *Cpn* alleles (Van Vactor et al., 1988; Yang and Ballinger; 1994). Accordingly, *Cpn* and *chp* expression is not abolished in *otd*^{uvi} mutant flies but reduced by about 3-fold in our microarray analysis (Table 1 and Fig. 1A).

Two other genes, *trithorax* or *trx* (transcription regulation) and *bride of sevenless* or *boss* (cell-cell signaling) are required in the early stages of photoreceptor cell development, primarily at the time of cell fate acquisition (Janody et al., 2004; Reinke and Zipursky, 1988; Hart et al., 1990). Although no changes in cell fate have been reported in *otd*^{uvi} mutants, these genes may continue to be expressed and function during later stages of retinal development. Indeed, *boss* expression has been detected in multiple retinal cell types during pupation, including in all photoreceptors and the neurons associated with the bristles of the eye (Kramer et al., 1991), consistent with a potential role for *boss* in later aspects of photoreceptor morphogenesis.

Lastly, several other genes are associated, either experimentally and/or through electronic annotation, with biological processes that could be relevant to the *otd*^{uvi} mutant phenotype,

including factors related to cytoskeleton organization (3), protein processing (6) or signaling/cell-adhesion (7 in addition to *boss*) (Table 1).

In summary, the regulation of *chp* and *Cpn* directly ties Otd to the control of R-cell morphology and several other *otd*-dependent loci identified in this study may contribute to specific aspects of R-cell development and function.

Otd regulates genes whose expression cycles with circadian rhythmicity

The Otd homologue *CRX/OTX5* has been linked to circadian-regulated processes in vertebrates, including photic entrainment and circadian gene expression in the pineal gland (Furukawa et al., 1999; Gamse et al., 2002). To explore whether Otd may also contribute to the regulation of metabolic, physiological and/or behavioral processes under the control of the circadian clock, we compared our set of differentially expressed genes with a list of loci previously identified as cycling in fly heads (Claridge-Chang et al., 2001; McDonald and Roshbash 2001; Lin et al., 2002; Ueda et al., 2002; Ceriani et al., 2002).

We found that 13 of the 61 genes differentially expressed in the otd^{uvi} mutant background are included in this 'circadian gene list' (Table 2). All but one are downregulated in mutant tissue and therefore, would be positively regulated by Otd in wild type flies. Twelve of the genes are reported to show altered expression in circadian mutants (Table 2) (Claridge-Chang et al., 2001;McDonald and Roshbash 2001;Lin et al., 2002;Ueda et al., 2002;Ceriani et al., 2002). Moreover, as proposed by Ceriani et al. (2002),Claridge-Chang et al. (2001) and/or Ueda et al. (2002), some of these genes may mediate the circadian regulation of visual sensitivity (*Rh3*, *Rh4* and *Rh5*), detoxification (*Cytochrome P450–6a2* or *Cyp6a2*, *Cyp6a17*, *Glutathione S transferase E1* or *GstE1*), and locomotor behavior (*slowpoke or slo* and *Slowpoke binding protein* or *Slob*).

In the case of the calcium-activated potassium channel Slo and its modulator Slob, analyses of mutant phenotypes more directly implicate these factors in the circadian control of locomotor activity. Wild type flies entrained to a 24 hour light-dark (LD) cycle are more active at dawn and dusk and are quiescent during the day. Once entrained, they maintain this behavioral rhythm even if moved to constant darkness (DD). Flies mutant for *slo* exhibit an arrhythmic locomotion phenotype lacking clear peaks of activity but displaying overall activity levels similar to wild type (Ceriani et al., 2002). Similarly, flies with neuron-targeted expression of *UAS-Slob* (under the control of the pan-neural driver *elav-Gal4*) exhibit a loss of photic entrainment when shifted from LD to DD as suggested by the breakdown of rest:activity patterns over time (Fernandez et al., 2007).

The contribution of the other cycling genes (Table 2) to circadian rhythms has not been investigated, and in all but two cases (*Cyp6a17* and *Rh3*), gene expression is reduced rather than abolished in the otd^{uvi} hypomorphic background (Table 2, Fig. 1A and not shown). Since stronger *otd* mutant alleles are embryonic lethal and therefore less easily analyzed, it is currently difficult to evaluate the role of *otd* in regulating biological rhythms. However, we did investigate whether the Otd transcription factor would exercise its influence exclusively at the level of the retina, where it is known to be broadly expressed (Vandendries et al., 1996), or whether it may also function elsewhere in the head, particularly in the other circadian centers of the fly (specifically in the Hofbauer-Buchner eyelet and/or in pacemaker cells of the central brain) (Hofbauer and Buchner 1989;Yasuyama and Meinertzhagen 1999,Helfrich-Forster et al., 2002).

As previously shown, Rh6 is expressed in the eyelet (Yasuyama and Meinertzhagen, 1999), and the enhancer-trap line *R32-lacZ* in all pacemaker neurons (Shafer et al., 2006). Using these molecular markers, we found that Otd is expressed in all 4 cells of the eyelet (Fig. 2A) and in

group 3 of the dorsal pacemaker neurons (DN3) (Fig. 2B). We estimate that about half of the ca. 40 DN3 cells express Otd. Interestingly, the DN3 neurons can synchronize molecular rhythms in the absence of external photoreceptors and appear to be non-homogeneous based on variations in cellular size and in *R32-lacZ* expression level (Valeri et al., 2003; Rieger et al., 2006; Shafer et al., 2006). The presence of Otd in only a subset of these neurons confirms this observation and provides the first endogenous molecular marker for a distinct DN3 subtype.

Since the retina, eyelet, and pacemaker neurons contribute somewhat redundantly to the entrainment of circadian rhythms, understanding the consequences of the loss of *otd* function in the various specific cell types will require extensive analyses. Nonetheless, the expression of Otd in cells of all three circadian centers as well as the potentially direct control of *slo* and *slob* expression (see below) suggests that *otd* contributes to the regulation of circadian-related gene networks.

Direct and indirect targets of Otd

The Otd/OTX/CRX transcription factors belong to a subgroup of homeodomain proteins known as the K50-type based on the presence of a lysine at the critical amino acid 50 of the homeodomain. In the case of the only known direct targets of Otd in the fly (*Rh3*, *Rh5* and *Rh6*), gene transcription is regulated through TAATCC (GGATTA) sites located within the first few hundred base pairs upstream of the start of transcription (Tahayato et al., 2003). Although Otd binding characteristics have not been extensively studied, the availability of these sites and their variable conservation in other *Drosophila* species (*D. pseudoobscura* and *D. virilis*) permit the generation of an Otd-binding-site position weighted matrix (PWM) (Supplementary Fig. 1) (Lifanov et al., 2003; Sosinsky et al 2003). Based on this PWM, we searched for Otd-binding sites within each of the differentially expressed genes and investigated their evolutionary conservation in the distantly related *Drosophila* species, *D. pseudoobscura* (ca. 55+ million years; Tamura et al., 2004). Because of the limited characterization of Otd-binding specificity and the short nature of the consensus sequence (6 bp), we introduced four additional constraints to our search: 1) we limited our analysis to the 1000 base pairs (bp) of genomic DNA immediately upstream of each start of transcription (5'-$_{FLANK}$) reasoning that many functional promoters (including for the known Otd targets *Rh3*, *Rh5*, and *Rh6*) are present in this region; 2) we selected a PWM score cut off of 4.5 in order to exclude any sites with more than one mismatch from the TAATCC sequence (see Supplementary Fig. 1); 3) only perfectly matching sites between *D. melanogaster* and *D. pseudoobscura* were considered conserved; and, 4) whenever the 5'-$_{FLANK}$ contained another gene, the DNA within this upstream transcription unit was excluded from consideration because of the potential for additional evolutionary constraints on sequence variation.

Using these criteria, we were able to investigate 60 of the 61 genes. We identified a total of 129 PWM matching sites within the ~54 Kb of DNA analyzed. This constitutes more than twice the site frequency expected based on random occurrence (~53 sites at 1 in 1024 bp) (Supplementary Figure 1 and Supplementary Table 3). Among the 60 genes, 19 (31%) have 1 or more putative Otd-binding sites that are perfectly conserved between *D. melanogaster* and *D. pseudoobscura* (Table 3 and Supplementary Table 3). The presence of upregulated (6) and downregulated (13) loci among the 19 putative direct targets is consistent with the ability of Otd to function as a repressor (Rh6) as well as an activator (Rh3 and Rh5) (Tahayato et al., 2003). Ten genes have 1 conserved site, four genes contain 2 conserved sites (*CG10924, CG8942, Cpn, Rh5*), four genes have 3 (*CG30492, CG5391, Dyb, Slob*) and one gene, *Rh3*, contains 4 conserved sites (Table 3). Lastly, we were able to further investigate 13 of these 19 loci in the more distantly related species *D. virilis* (ca. 63+ million years; Tamura et al.,

2004) and found evidence of conservation in 10 cases (10/13) (Table 3 and Supplementary Table 3).

As proof of principle, we identified *Rh3* and *Rh5* as candidate Otd targets. In addition, among the other genes implicated in visual transduction, R-cell morphology or circadian rhythms, *Arr2*, *Cpn*, and *slob* are particularly good candidates for direct regulation by Otd (Table 3 and Supplementary Table 3). *Drosophila Arr2* contains 1 TAATCC match at position −305 (bp from the start of transcription) within its 502 bp of 5'-FLANK (Table 3 and Supplementary Table 3). An additional conserved site with a lower score (4.08) is found at −20 bp (Supplementary Table 3). Both sites are also perfectly conserved in *D. virilis*. Interestingly, CRX appears to directly control the expression of arrestins in vertebrate photoreceptors (Chen et al., 1997;Mani et al., 1999;Fujimaki et al., 2004), suggesting a long-standing connection between *otd* and *arrestin* expression in the eye. Similarly, the *Cpn* gene also has multiple sites that are 100% conserved in *pseudoobscura*. The two sites with a PWM score above 4.5 (at −631 and −434) are also perfectly conserved in *D. virilis* (Table 3 and Supplementary Table 3). Lastly, some *slob* transcripts may be directly under Otd control. The gene *Slob* has large transcription units with 2 alternate first exons and two alternatively spliced internal exons. Although the upstream promoter is not associated with potential Otd sites, the 5'-FLANK of the downstream promoter, encoding the A-, C- and D-type transcripts (A&C–D), is preceded by 3 sites that are conserved in *D. pseudoobscura* (at positions −97, −253 and −849). An additional conserved site is found further upstream at −1915 (Table 3 and Supplementary Table 3). In a comparison with *D. virilis*, the sites at −253, −849, and −1915 are perfectly conserved.

Forty-one genes either had no sites above the 4.5 cut-off in the 5'-FLANK (10 genes in the 'no sites' or N category) or none of the sites identified were conserved in *pseudoobscura* by VISTA alignment (31 genes in the 'no match' or NM category) (Table 3 and Supplementary Table 3). However, we believe that additional direct targets are present among these genes and were excluded as a result of the particularly stringent selection criteria applied. For instance, in a few cases, sites scored as non-conserved differed at a single nucleotide in *D. pseudoobscura* but resulted in a site with a higher PWM score than in *melanogaster*. In other cases, the relaxation of the first or second selection criteria (1 kb limit or 4.5 score cutoff) results in the identification of additional conserved sites. An interesting example is provided by the gene *slo* which, based on the four criteria, falls in the NM category but can be shown to have conserved sites when either criterion 1 or 2 is relaxed. A site with a PWM score of 4.08 is present upstream of the first promoter (transcripts B and C) at position - 471 and it is perfectly conserved in both *D. pseudoobscura* and *D. virilis*. Analysis of the second promoter (transcripts A and D through Q = *A&D–Q*) is complicated by the presence of another gene upstream of the start of transcription. Thus, about 620 bp immediately upstream of the *A&D–Q* promoter fall within the transcription unit of the gene *CG31117*. An analysis of further upstream sequences identifies three sites that are conserved in *pseudoobscura* [at −2920 (score 6.19), −2522 (score 6.19) and −1540 (score 4.08) from the *A&D–Q* start of transcription]. The site at −2522 is also perfectly conserved in *virilis*, whereas a modified sequence, but still a PWM match, is found nearby the −2920 position. Hence, one or both *slo* promoters may be targets of direct regulation by Otd. Lastly, VISTA provides a powerful, but not infallible, tool for genome comparison. For example, in the analysis of the *Arr2* 5'-FLANK, a conserved GGATTA site at position –305 appears to be non-conserved in *D. pseudoobscura* based on VISTA alignment. However, alignment by BLAST shows perfect conservation of this site in both *peudoobscura* and *virilis*. Together, these considerations suggest that more of the loci listed in Table 1 may be directly controlled by Otd.

The identification of conserved target sites in the *Arr2*, *Cpn*, *slo* and *Slob* genes supports the direct involvement of Otd in phototransduction and photoreceptor cell morphogenesis and strongly suggests that Otd is involved in aspects of circadian rhythmicity as well.

Otd functions in distinct ways in brain patterning and photoreceptor differentiation

As mentioned in the Introduction, Otd is not only important for photoreceptor neuron differentiation, but also plays a critical role during embryonic development. At this stage, Otd functions in patterning rather than terminal differentiation (Finkelstein et al., 1990a and b; Wieschaus et al., 1992; Hirth et al., 1995; Younossi-Hartenstein et al., 1997). The transcriptome regulated by Otd in the *Drosophila* embryo has been investigated through genome-wide microarray analysis by Montalta-He et al. (2002). In this study, the expression level of 287 annotated genes was found to be significantly changed in response to Otd over-expression.

A comparison of the Otd-regulated transcriptome characterized in our study with data from Montalta-He and colleagues (2002) has allowed us to investigate whether similarities exist between Otd function during embryonic development and R-cell morphogenesis. Whereas we had expected differences in the Otd-regulated transcriptome at embryonic and pupal stages, we were surprised to find a complete lack of overlap between the 'Montalta-He set' of 287 putative Otd targets and our list of 61 loci. The difference in experimental design between the two studies may contribute to this result, as Montalta-he and colleagues relied on a gain-of-function study in whole embryos whereas we analyze the consequences of a tissue-specific loss-of *otd* function. However, the observation that none of the 61 genes identified in our study appears to respond to heat shock induced expression of Otd at the embryonic stage is nonetheless surprising, and suggests that the Otd transcription factor regulates gene expression in profoundly distinct ways as a patterning factor during embryogenesis and as a differentiation factor in the pupal head. Thus, it will be interesting to investigate how transcriptional regulation by Otd is modified at these different stages at the level of chromatin structure and through interactions with specific cofactors.

CONCLUSIONS

The *CRX/OTX* genes are associated with phototransduction, photoreceptor cell development, circadian gene regulation, photic entrainment, and rostral brain development in mouse and other vertebrates. During development of the embryonic central nervous system, both fly Otd and mouse OTX2 play critical roles in anterior brain development, and *OTX1*, *OTX2*, and *otd* can functionally substitute for each other to a great extent in cross-phylum rescue experiments (Leuzinger et al., 1998; Acampora et al., 1998 and 2005; Nagao et al., 1998). These observations have led to the proposal of a common origin for the tripartite brain in bilateria and an evolutionarily conserved role for Otd and OTX genes in this process (Hirth et al., 2003).

In this work, we have uncovered additional parallels between *otd* in flies and another vertebrate family member, the *CRX* gene. As mentioned in the Introduction, the CRX protein is a prominent regulator of terminal differentiation in vertebrate photoreceptor cells. It controls the expression of multiple components of the phototransduction cascade (e.g. rhodopsin, rod α-transducin, recoverin, rod cGMP-phosphodiesterase α and.β subunits, and arrestins) as well as specialized structural proteins of photoreceptors (e.g. peripherin) (Chen et al, 1997; Furukawa et al., 1997; Mani et al, 1999; Kimura et al, 2000; Livesey et al., 2000; Fujimaki et al., 2004; Pittler et al., 2004). As shown here, in addition to the previously identified *Rh3*, *Rh5* and *Rh6* genes, fly Otd controls the expression of several phototransduction-related genes (*Arr2*, *CdsA* and *Rh4*) and factors that contribute to the morphological differentiation of fly photoreceptor neurons (*chp, Cpn*). These functions are reminiscent of the regulation of visual transduction and structural components of murine photoreceptor cells by CRX. In addition, CRX plays a role in circadian gene expression in the pineal gland and in photic entrainment, as suggested by the phenotype of CRX mutant mice (Gamse et al, 2002; Sohocki et al., 1998; Furukawa et al., 1999). We find that *otd* may also play circadian-rhythmrelated functions in the fly. The Otd protein is expressed in all three circadian centers of the fly (retina, Hofbauer-

Buchner eyelet, pacemaker neurons) and several cycling genes are found among its targets. Its control of the *slo* and *Slob* genes, in particular, suggests circadian locomotion and photic entrainment as possible areas of influence. Lastly, we provide evidence that transcriptional regulation of many targets may be direct since evolutionarily conserved Otd-binding sites are found upstream of their promoters. Intriguingly, examples of related loci directly controlled by Otd in the fly and CRX in mouse can be found among the opsin and arrestin genes.

The striking parallels in the range of biological processes regulated by Otd in the fly and CRX/OTX genes in mouse strongly suggest an evolutionary conservation of functions that pre-date the protostome / deuterostome split. Thus, the single ancestral Otx orthologue (Li et al., 1996; Williams and Holland, 1998) present in *Urbilateria* (the last common ancestor of fly and mouse, De Robertis and Sasai, 1996) would have already played significant roles in central nervous system development, in photoreceptor cells differentiation, in phototransduction, and in photic entrainment or some other circadian process. In this context, the direct regulation of opsin and arrestin genes by CRX and Otd could reflect the retention of an ancestrally determined regulatory relationship. A significant test of this hypothesis may be provided by the analysis of the OTX orthologue and its targets in the ragworm *Platynereis* (Annelida), an organism with both ciliary and rhabdomeric photoreceptors that has been proposed as a model for the *Urbilateria* (Arendt et al., 2002).

Today, the Otd and CRX/OTX genes control analogous biological processes through very different genetic cascades. This is apparent when considering the larger number of Otd or CRX targets specific to either flies (e.g. chaoptic, Calphotin) or mouse (e.g. rod α-transducin, recoverin, rod cGMP phosphodiesterase γ subunit, peripherin) photoreceptors as compared to genes playing related functions in the two species (e.g. opsins or arrestins). Significant differences in the genetic networks controlled by these genes likely emerged through co-option of new genes and genetic modules in the pre-existing ancestral pathway (Garcia-Bellido, 1975; Gehring and Ikeo, 1999). Thus, only traces of their common origin would be detected in extant species after ~600–800 million years of diversification in the regulation of photoreception, brain development and circadian rhythms in metazoan organisms.

The conserved roles of Otd/CRX/OTX in multiple photoperception-related processes and brain development supports the proposed nature of *Urbilateria* as an organism with a complex nervous system with well developed sensory and central processing centers (reviewed by Arendt and Wittbrodt, 2001, Reichert and Simeone, 2001; Arendt, 2003, 2004; Hirth et al., 2003; Ghysen, 2003; Lichtneckert and Reichert, 2005).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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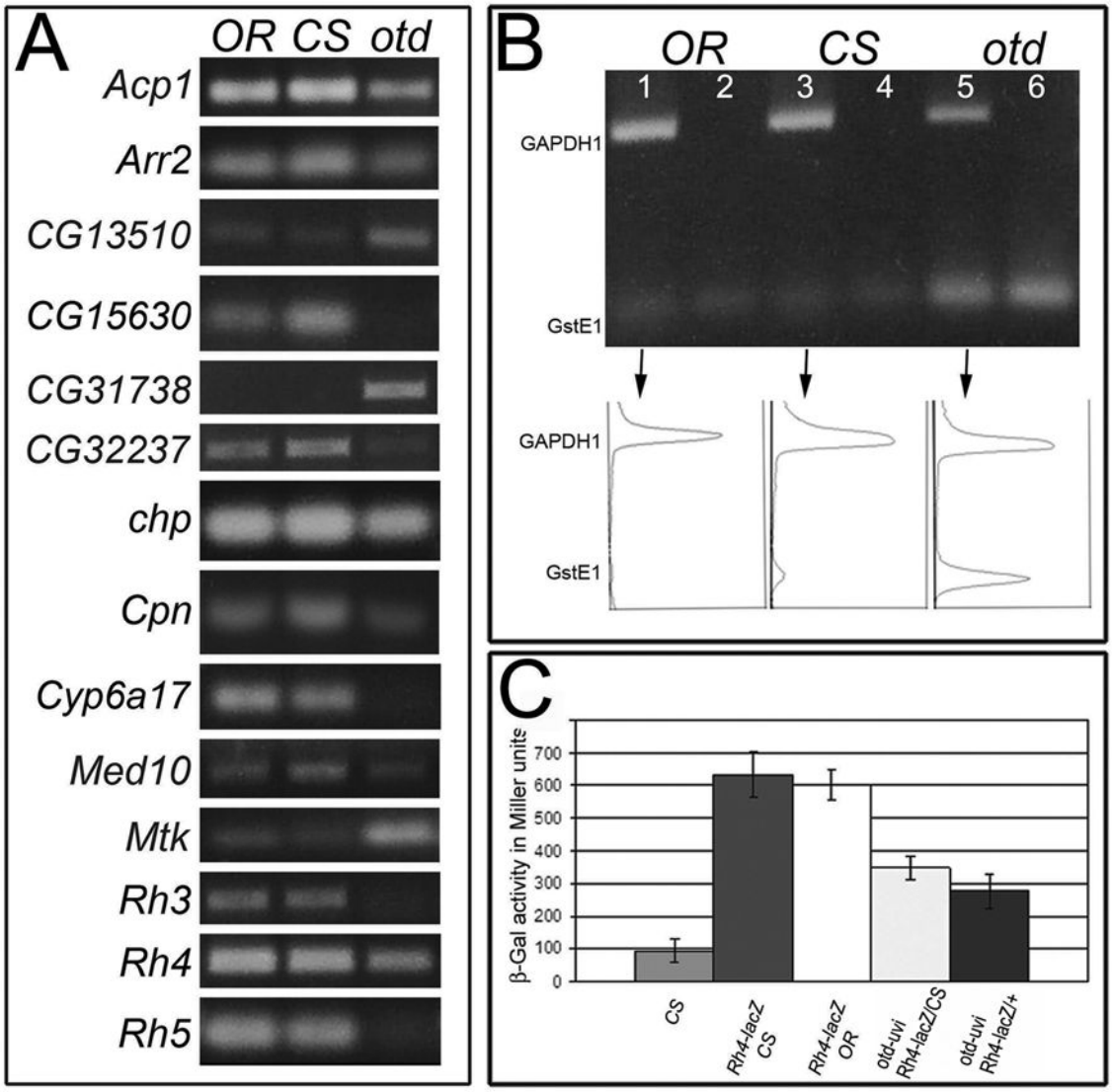

Figure 1. Differential gene expression in pupal heads of wild type and *otd*^{UVI} flies

Validation of differentially expressed genes identified by microarray. A) RT-PCR comparisons of expression levels in pupal heads of *OR*, *CS*, and one of two *otd*^{UVI} mutant lines used in our analysis; B) example of an RT-PCR test run with the internal control *GAPDH1*. Upper panel: gel of RT-PCR for *GstE1* run with (lanes 1, 3, 5) or without (lanes 2, 4, 6) the *GAPDH1* internal control. Lower panel: ImageJ plot showing change in *GstE1* peaks relative to each *GAPDH1* internal control; C) results of one test for β-Galactosidase activity (*Rh4-lacZ*) in adult heads of wild type versus *otd*^{UVI} mutant flies, negative control is wild type *CS* without *Rh4-lacZ*. Lower levels of β-Galactosidase activity in the *otd*^{UVI} mutant samples as compared to wild type were repeatedly observed in this and other tests using two detection methods (see Materials and Methods). In this experiment, n=6, error bars = ± 1 SE or standard error.

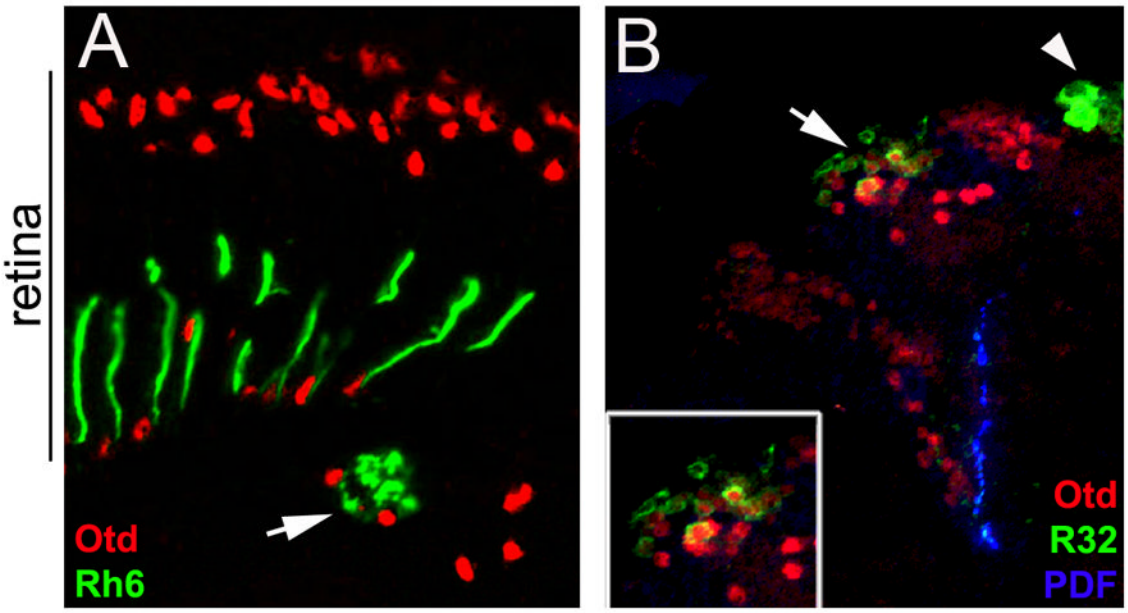

Figure 2. Otd is expressed in the Hofbauer-Buchner eyelet and a subset of the DN3 pacemaker neurons

Confocal microscope image of 10 micron cryostat sections from adult fly heads (wild type). Otd expression is found in the eyelet and in some of the DN3 circadian pacemaker neurons. Images were processed with Adobe Photoshop.

(A): section stained with antibodies to visualize expression of Otd (red) and Rh6 (green). The eyelet (arrow) is located below the retina and expresses both Otd in the nucleus and Rh6 in the rhabdomeric membranes. Two of the 4 cells of the eyelet are visible in this section. In the retina (region marked by the vertical side bar), the Otd protein is found in the nucleus of multiple R-cells and Rh6 is found in the rhabdomere of a subset of the retinal R8 neurons. Additional neurons in the brain also express Otd (such as the three Otd-positive nuclei in the lower right end corner).

(B): section stained with antibodies to visualize expression of Otd (red), the R32 enhancer-trap marker for all circadian pacemaker neurons (green), and the PDF marker for ventro-lateral pacemaker neurons and their projections (blue). Panel shows the projection of an A–Z series that spans part of the DN3 cluster (see inset for higher magnification). Otd is expressed in some but not all of the DN3 neurons (arrow). DN1 neurons are located nearby in a more dorso-medial position (arrowhead) but do not express Otd. The cell bodies of the PDF-positive ventro-lateral pacemaker neurons are outside the field shown in B and do not expressed Otd, but their projections help identify the DN3 and DN1 groups.

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Table 1

61 genes differentially expressed in *otd*^{UVI}

Gene Symbol	Description	RTPCR	Microarray		Otd sites
			Fold Change	Adjusted P-Value	
Cytoskeleton organization, biogenesis, function					
CG31738	structural constituent of cytoskeleton	↑	2.92	7.12e-06	§√
Act88F	actin	↑	2.62	0.00248	§
Dyb	actin binding cytoskeletal protein	↓	−12.67	0.000959	§√
Detoxification, stress, defense response					
Cyp12a4	steroid metabolism	↓	−2.93	0.00729	§
Cyp4p3	steroid metabolism	↑	16.42	0.00198	§√
Cyp6a17	steroid metabolism	↓	−231.42	2.02e-23	§
Cyp6a2	steroid metabolism	↓	−6.74	0.00062	§
GstE1	glutathione transferase	↑	5.42	0	
Mtk	defense response	↑	10.02	0.00324	§√
Metabolism					
Aldh	pyruvate metabolism	↓	−2.96	0.00046	
CG10924	gluconeogenesis	↑	3.56	2.92e-08	§√
Photoreceptors morphogenesis					
chp	adhesion	↓	−3	0.000402	§
Cpn	rhabdomere development	↓	−2.92	0.000647	§√
Phototransduction					
Arr2	metarhodopsin inactivation	↓	−2.65	0.000841	§√
CdsA	phospholipid metabolism	↓	−2.95	0.00343	
Rh3	opsin receptor	↓	−187.2	2.02e-23	§√
Rh4	opsin receptor	↓	−4.42	1.79e-06	§
Rh5	opsin receptor	↓	−9.48	4.63e-20	§√
Protein folding, targeting, degradation					
Trp1	cotranslational membrane targeting	↓	−2.48	0.000957	§
Gtp-bp	cotranslational protein targetting	↑	2.51	0.000804	§
CG17633	metalloprotease	↑	5.84	1.92e-09	§
Try29F	serine protease	↑	19.27	0.000647	
CG7033	chaperonin	↓	−3.45	0.000682	§
CG9086	ubiquitination	↓	−2.01	0.00487	§
Regulation of transcription					
CG13510	other transcription factor	↑	2.71	0.00712	§
MED10	Pol II transcription factor	↓	−3.21	7.34e-09	ND
Sox100B	Pol II transcription factor	↓	−3.48	7.80e-06	§
trx	chromatin packaging / remodeling	↓	−3.86	0.00209	§√
Signaling , cell adhesion					
boss	signaling, ligand/ receptor	↓	−2.47	0.00333	§√
CG15630	cell adhesion	↓	−7.99	1.43e-05	
Src64B	signaling, intracellular	↓	−2.5	1.02e-05	§
CG18208	GPCR signaling, receptor	↓	−4.58	1.18e-07	§
CG4629	cell adhesion & signaling, intracellular serine/ threonine kinase	↓	−2.24	0.00366	
CG8942	Wnt-protein binding	↑	12.67	0.0067	§√
mthl8	GPCR signaling, receptor	↑	16.53	0.00318	§

Gene Symbol	Description	RTPCR	Microarray		Otd sites
			Fold Change	Adjusted P-Value	
Nmda1	glutamate receptor activity	↑	3.3	0.00327	§
Synaptic transmission					
slo	calcium-activated potassium channel	↓	−2.93	0.000884	§
Slob	regulation of slo	↓	−2.93	0.00046	§√
Syn	vesicle traffic regulator	↓	−2.39	0.00108	§
Transport					
CG14743	cation transporter	↑	62.33	0.000482	§√
dro5	ion channel inhibitor activity	↑	3.44	1.54e-08	§
VGlut	phosphate:sodium symporter	↓	−2.79	0.000699	§
Other					
Acp1	adult cuticle constituent	↓	−2.19	0.00729	§√
Acyp2	acylphosphatase activity	↓	−25.53	6.65e-10	
Aph-4	nucleotide phosphatase activity	↓	−3.23	0.000138	§
CG8889	hydrolase/esterase activity	↓	−4.65	0.00118	§
Unknown					
CG10407	unknown	↓	−4.57	3.47e-08	
CG13056	unknown	↓	−3.47	0.000441	§√
CG13060	unknown	↑	2.5	0.00412	§
CG14095	unknown	↑	2.27	0.00599	§
CG14374	unknown	↑	3.54	2.61e-05	§
CG18107	unknown	↑	14.72	0.00296	§
CG18643	unknown	↑	7.65	0.00333	
CG30492	unknown	↓	−2.42	0.00341	§√
CG32237	unknown	↓	−6.91	3.29e-05	§√
CG3259	unknown	↑	12.47	0.00237	
CG5391	unknown	↓	−3.6	1.37e-05	§√
CG6912	unknown	↑	3.42	1.02e-05	§
CG9339	unknown	↓	−2.87	0.00717	§
CG9445	unknown	↑	8.85	0.00536	§
CG9689	unknown	↑	2.62	0.00624	§

↓ confirmed to be down-regulated in otd^{UVI} as compared to *Canton-S* and *Oregon-R* wild-type strains by semi-quantitative RT-PCR

↑ confirmed to be up-regulated in otd^{UVI} as compared to *Canton-S* and *Oregon-R* wild-type strains by semi-quantitative RT-PCR

§ one or more Otd PWM matches are found upstream of the gene promoter(s)

√ one or more of the Otd PWM matches are conserved between *D. melanogaster* and *D. pseudobscura*

Table 2

genes that cycle in a circadian fashion in adult fly heads

#	Gene Symbol	Otd		Circadian Rhythms Related Phenotypes	Cycling pattern*	Expression in circadian mutants	Ref.
		change	site				
1	*boss CG8285*	−2.47 ↓	§√	---	LD	non-rhythmic in Clk^{Jrk}	4
2	*CG15630*	−7.99 ↓		---	LD	non-rhythmic in Clk^{Jrk}	3
3	*CG7033*	−3.45 ↓	§	---	LD & DD	non determined	5
4	*CG8889*	−4.65 ↓	§	---	LD & DD	non-rhythmic in Clk^{Jrk}	4
5	*Cpn CG4795*	−2.92 ↓	§√	---	LD & DD	non-rhythmic & high in Clk^{Jrk} non-rhythmic in per^{01}	3,5
6	*Cyp6a17 CG10241*	−231.42 ↓	§	---	LD & DD	non-rhythmic & high in Clk^{Jrk} non-rhythmic in Clk^{Jrk}	3,4
7	*Cyp6a2 CG9438*	−6.74 ↓	§	---	LD & DD	non-rhythmic & low in Clk^{Jrk}	3
8	*GstE1 CG5164*	5.42 ↑		---	LD & DD	non-rhythmic in Clk^{Jrk}	3
9	*Rh3 CG10888*	−187.2 ↓	§√	---	LD	non-rhythmic in Clk^{Jrk}	4
10	*Rh4 CG9668*	−4.42 ↓		---	LD	non-rhythmic in Clk^{Jrk}	1,4
11	*Rh5 CG5279*	−9.48 ↓	§√	---	LD & DD	non-rhythmic in Clk^{Jrk}	1,4
12	*Slo CG10693*	−2.93 ↓	§	slo^4 displays arrhythmic behavior	LD & DD **	non-rhythmic in Clk^{Jrk}	3,7
13	*Slob CG6772*	−2.93	§√	overexpression causes the breakdown of rest:activity patterns	LD & DD	altered expression in Clk^{Jrk}, per^{01}, and tim^{01}	1,2,3,4,5,6

* three of the studies (3, 4 ,5) were also designed to differentiate between genes that cycle during the 24 hour light-dark (LD) cycle (photic entrainment) versus those that cycle independently of light input (i.e. in constant darkness or DD)

** *slo* was shown to cycle in phase with *slob* by RT-PCR (3)

§ one or more Otd PWM matches are found upstream of the gene promoter(s)

√ one or more of the Otd PWM matches are conserved between *D. melanogaster* and *D. pseudobscura*

References: (1) Claridge-Chang et al., 2001; (2) McDonald and Rosbash, 2001; (3) Ceriani et al., 2002; (4) Ueda et al., 2002; (5) Lin et al., 2002; (6) Jaramillo et al., 2004; (7) Fernandez et al., 2007.

Table 3

putative binding sites and conservation summary

COLUMN	**CONTENT** ***(see Supplementary Table 3 for details)***					
Ch (Change)	direction of change: upregulated (↑) or downregulated (↓) in otd^{UVI} as compared to wild type					
S (Sites)	yes (Y) or no (N) sites (based exclusively on sites that score above 4.5) N no PWM matching sequences are above 4.5 Y one or more PWM matching sequences are above 4.5 ND not determined (5'-flank DNA is part of another gene/exon)					
D.m. D.p.	*D. melanogaster / D. pseudoobscura* comparison (see Supplementary Table 3 for details) C# number of PWM-matching sites conserved between *D. melanogaster* and *D. pseudoobscura* NM none of the sites are perfectly conserved between *D. melanogaster* and *D. pseudoobscura*					
D.m. D.v.	*D. melanogaster / D. virilis* comparison (see Supplementary Table 3 for details) C# number of PWM-matching sites conserved between *D. melanogaster* and *D. virilis* NM none of the sites are perfectly conserved between *D. melanogaster* and *D. virilis* - genes for which *D.virilis* sequences are not currently well characterized within the 5' region					
Gene Name	**Description as in Table 1**	**Ch**	**S**	***D.m. D.p.***	***D.m. D.v.***	*#*
Acp1	***Other*** adult cuticle constituent	↓	*Y*	*C1*	*NM*	*1*
Arr2	***Phototransduction*** metarhodopsin inactivation	↓	*Y*	*C1*	*C*	*2*
boss	***Signaling, cell adhesion*** signaling , ligand/receptor	↓	*Y*	*C1*	*C*	*3*
CG10924	***Metabolism*** gluconeogenesis	↑	*Y*	*C2*	*C1*	*4*
CG13056	***Unknown***	↓	*Y*	*C1*	-	*5*
CG14743	***Transport*** cation transporter	↑	*Y*	*C1*	*NM*	*6*
CG30492	***Unknown***	↓	*Y*	*C3*	*C1*	*7*
CG31738	***Cytoskeleton organization, biogenesis, function*** structural constituent of cytoskeleton	↑	*Y*	*C1*	-	*8*
CG32237	***Unknown***	↓	*Y*	*C1*	*C1*	*9*
CG5391	***Unknown***	↓	*Y*	*C3*	-	*10*
CG8942	***Signaling / cell adhesion*** Wnt-protein binding	↑	*Y*	*C2*	-	*11*
Cpn	***Photoreceptors morphogenesis*** rhabdomere development	↓	*Y*	*C2*	*C2*	*12*
Cyp4p3	***Detoxification, stress, defense response*** steroid metabolism	↑	*Y*	*C1*	-	*13*
Dyb	***Cytoskeleton organization, biogenesis, function*** actin binding cytoskeletal protein	↓	*Y*	*C3*	*C1*	*14*
Mtk	***Detoxification, stress, defense response*** defense response	↑	*Y*	*C1*	*NM*	*15*
slob	***Synaptic transmission*** regulation of the calcium-activated potassium channel slo	↓	*Y*	*C3*	*C2*	*16*
trx	***Regulation of transcription*** chromatin packaging / remodeling	↓	*Y*	*C1*	-	*17*
Rh5	***Phototransduction*** opsin receptor	↓	*Y*	*C2*	*C2*	*18*
Rh3	***Phototransduction*** opsin receptor	↓	*Y*	*C4*	*C3*	*19*
Act88F , Aph-4, CG13060 , CG13510 , *CG14095 , CG14374, CG17633, CG18107, CG18208, CG6912 , CG7033, CG8889, CG9086 , CG9339, CG9445, CG9689, chp , Cyp6a2, Cyp6a17, Cyp12a4, dro5, Gtp-bp, mthl8, Nmda1 , Rh4, slo, Sox100B, Src64B, Syn, Trp1, VGlut*			*Y*	*NM*		*31*
Acyp2 , Aldh, CdsA, CG10407 , CG15630, CG18643 , CG3259 , CG4629, GstE1 , Try29F			*N*			*10*
Med10			*ND*			*1*