Developmental Biology 390 (2014) 170–180

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

The Drosophila Wilms' Tumor 1-Associating Protein (WTAP) homolog is required for eye development

Abigail M. Anderson, Brandon P. Weasner, Bonnie M. Weasner, Justin P. Kumar *

Department of Biology, Indiana University, Bloomington, IN 47405, United States

ARTICLE INFO

Article history:
Received 9 April 2013
Received in revised form
4 March 2014
Accepted 19 March 2014
Available online 29 March 2014

Keywords:
Sine oculis
Fl(2)d
Elav
Lozenge
Eye development
Drosophila

ABSTRACT

Sine Oculis (So), the founding member of the SIX family of homeobox transcription factors, binds to sequence specific DNA elements and regulates transcription of downstream target genes. It does so, in part, through the formation of distinct biochemical complexes with Eyes Absent (Eya) and Groucho (Gro). While these complexes play significant roles during development, they do not account for all So-dependent activities in *Drosophila*. It is thought that additional So-containing complexes make important contributions as well. This contention is supported by the identification of nearly two-dozen additional proteins that complex with So. However, very little is known about the roles that these additional complexes play in development. In this report we have used yeast two-hybrid screens and co-immunoprecipitation assays from Kc167 cells to identify a biochemical complex consisting of So and Fl(2)d, the *Drosophila* homolog of human Wilms' Tumor 1-Associating Protein (WTAP). We show that Fl(2)d protein is distributed throughout the entire eye-antennal imaginal disc and that loss-of-function mutations lead to perturbations in retinal development. The eye defects are manifested behind the morphogenetic furrow and result in part from increased levels of the pan-neuronal RNA binding protein Embryonic Lethal Abnormal Vision (Elav) and the RUNX class transcription factor Lozenge (Lz). We also provide evidence that So and Fl(2)d interact genetically in the developing eye. Wilms' tumor-1 (WT1), a binding partner of WTAP, is required for normal eye formation in mammals and loss-of-function mutations are associated with some versions of retinoblastoma. In contrast, WTAP and its homologs have not been implicated in eye development. To our knowledge, the results presented in this report are the first description of a role for WTAP in the retina of any seeing animal.

Introduction

In *Drosophila*, the Sine Oculis (So) homeobox transcription factor is a critical member of the retinal determination (RD) network and it plays a central role in the development of the eye (Cheyette et al., 1994; Serikaku and O'Tousa, 1994; Pignoni et al., 1997; Weasner et al., 2007; Kumar, 2009; Anderson et al., 2012; Atkins et al., 2013; Weasner and Kumar, 2013). It appears to have dual roles in regulating gene expression within the retina. On the one hand, So promotes eye development via transcriptional activation of several RD genes including itself, *eyeless* (*ey*), *eyes absent* (*eya*) and *dachshund* (*dac*: Halder et al., 1998; Pauli et al., 2005; Pappu et al., 2005), the patterning gene *hedgehog* (*hh*: Pauli et al., 2005) and several cell fate genes such as *atonal* (*ato*) and *lozenge* (*lz*: Yan et al., 2003; Zhang et al., 2006). However, So is simultaneously required to repress the expression of head capsule and antennal selector genes such as *cut* (*ct*) and *Lim1* during regional specification of the eye-antennal disc (Salzer and Kumar, 2009; Anderson et al., 2012; Wang and Sun, 2012; Weasner and Kumar, 2013). And behind the morphogenetic furrow, So stops promoting *ey* expression and instead is required to inhibits its transcription (Atkins et al., 2013). The ability of So to modulate transcription of downstream target genes is dependent upon interactions with Eyes Absent (Eya) and Groucho (Gro) (Pignoni et al., 1997; Kenyon et al., 2005; Anderson et al., 2012). These interactions are conserved in vertebrate systems as well (Ohto et al., 1999; Kobayashi et al., 2001; Zhu et al., 2002). However, the So-Eya and So–Gro complexes do not fully account for all So-dependent activities in either *Drosophila* or vertebrates. Over the last decade several yeast two-hybrid screens have identified approximately 25 additional factors that could also form biochemical complexes with So (Pignoni et al., 1997; Giot et al., 2003; Kenyon et al., 2005; Neilson et al., 2010). While these complexes are likely to make significant contributions to tissue specification and pattern formation, very little is know about their roles in regulating development in any experimental system.

Here, we report the identification of a biochemical complex containing So and Fl(2)d, the fly homolog of Wilms' Tumor 1-Associating Protein (WTAP: Penalva et al., 2000). During sex determination, Fl(2)d plays an important role in the female-specific splicing of both *Sex-lethal* (*Sxl*) and *transformer* (*tra*) pre-mRNA transcripts (Granadino et al., 1990; 1992; 1996; Ortega et al., 2003).

* Corresponding author.
E-mail address: jkumar@indiana.edu (J.P. Kumar).

http://dx.doi.org/10.1016/j.ydbio.2014.03.012
0012-1606/

Outside of the sex determination pathway, Fl(2)d is also required for the proper alternate splicing of *Ultrabithorax* (*Ubx*) pre-mRNA transcripts in both sexes (Burnette et al., 1999). Mechanistically, Fl(2)d physically interacts with several early splicing factors to promote the alternate splicing of these mRNAs (Penn et al., 2008). This function appears to be evolutionarily conserved, as human WTAP has been isolated from spliceosome complexes (Zhou et al., 2002). Sequence analysis of the Fl(2)d protein has identified long stretches of histidine and glutamine residues with the N-terminal region of the protein. Similar stretches are found within the activation domains of many transcription factors (Ptashne and Gann, 1997; Penalva et al., 2000). Therefore it is possible that, in addition to its role in splicing, Fl(2)d may also function to co-regulate transcription of target genes.

Mammalian WTAP was first identified in a yeast two-hybrid screen for proteins that interact with Wilms' tumor-1 (WT1: Little et al., 2000). Mice lacking WTAP die between embryonic day 6.5 and 10.5 and show dramatic defects in cell proliferation, which in turn leads to defects in endoderm and mesoderm formation (Horiuchi et al., 2006; Naruse et al., 2007; Fukusumi et al., 2008). At least one of its roles in proliferation appears to prevent the degradation of *cyclin A2* mRNA transcripts. In cultured cells depletion of WTAP leads to a dramatic reduction in Cyclin A2 protein levels and as a consequence the cells are arrested in G2 (Horiuchi et al., 2006). Consistent with a role in blocking degradation of *cyclin A2* transcripts, murine WTAP is found within a complex that contains proteins involved in mRNA stabilization, polyadenylation and mRNA transcript export (Horiuchi et al., 2013). Murine WTAP is likely to also play its traditional role in splicing as it was found to interact with serine/arginine (SR) proteins and members of the general splicing machinery (Horiuchi et al., 2013).

WT1 is expressed within the mammalian retina and is required for the expression of Pou4f2/Brn3-b, which is essential for the specification of retinal ganglion cells (Armstrong et al., 1993 K.D. Wagner et al., 2002; 2003). The retinas of mice that lack WT1 display increased levels of cell death and are thus thinner and contain fewer retinal ganglion cells (K.D. Wagner et al., 2002). Certain WT1 mutant alleles are also associated with some versions of retinoblastoma (N. Wagner et al., 2002; Punnett et al., 2003). *klumpfuss* (*klu*), the *Drosophila* homolog of WT1, contributes to the development of the *Drosophila* retina by regulating cell death levels (Rusconi et al., 2004; Wildonger et al., 2005). In contrast, prior to this report neither WTAP nor any of its homologs have been previously implicated in retinal development within any seeing animal. Here, for the first time, we demonstrate a role for a WTAP homolog in the eye. We used yeast two-hybrid assays and immunoprecipitations from Kc167 cells to detect the formation of a So–Fl(2)d complex and to identify the domains within both proteins that mediate the physical interaction. We further show that Fl(2)d is distributed throughout the developing eye disc and that reductions in protein levels results in defects in photoreceptor number, cell fate and rhabdomere structure. Our data indicates that Fl(2)d regulates the levels of the pan-neuronal RNA binding protein Embryonic Lethal Abnormal Vision (Elav) and the RUNX class transcription factor, Lozenge (Lz).The structural defects that are seen in the adult eyes of *fl(2)d* mutants are caused in part by increased levels of both Elav and Lz proteins.

Materials and methods

Fly strains and genetic crosses

The following 20 stocks were used in this study: (1) y w ey-flp; (2) FRT42D fl(2)d^{f01270}/CyO; (3) FRT42D so^{3}/CyO; (4) FRT42D Ubi-GFP/CyO; (5) UAS-fl(2)d RNAi; (6) UAS-dicer2; (7) UAS-fl(2)d; (8) UAS-so; (9) UAS-eya; (10) UAS-elav; (11) UAS-lz; (12) ey-GAL4; (13) UAS-lacZ; (14) GMR-GAL4; (15) DE-GAL4; (16) elav-GAL4; (17) lz-lacZ; (18) w^{1118}; (19) y w ey-flp; FRT42D cl P[w+]; (20) UAS-GFP. All flies and genetic crosses were maintained at 25 °C. GAL4 crosses that involved UAS-dicer2 and UAS-fl(2)d RNAi were compared to control crosses that contained UAS-GFP and UAS-fl(2)d RNAi constructs in order to ensure that any observed effect was not due to a dilution of the GAL4 protein. In all cases the control crosses looked nearly identical to the experimental crosses.

Antibodies and microscopy

The following 17 antibodies were used in this study: (1) guinea pig anti-So (1:50, gift of Ilaria Rebay); (2) rat anti-Elav (1:100, DSHB); (3) mouse anti-Fl(2)d (1:100, DHSB); (4) mouse anti-Ct (1:100, DSHB); (5) mouse anti-Dac (1:5, DSHB); (6) mouse anti-Eya (1:5, DSHB); (7) mouse anti-Ey (1:250, DSHB); (8) mouse anti-22C10 (1:100, DSHB); (9) mouse anti-Lz (1:100, DSHB); (10) mouse anti-Gl (1:20, DSHB); (11) mouse anti-Pros (1:20, DSHB) (12) mouse anti-β-galactosidase (1:100, Promega); (13) chicken anti-β-galactosidase (1:100, Abcam); (14) guinea pig anti-Sens (1:100, gift of Hugo Bellen); (15) mouse anti-Yan (1:5, DHSB); (16) mouse anti-HA (1:1000, Santa Cruz Biotechnology); and (17) mouse anti-Myc (1:1000, Santa Cruz Biotechnology). Secondary antibodies and phalloidin were obtained from Jackson Laboratories and Invitrogen. Imaginal discs and adult flies were prepared as described in Anderson et al. (2012).

Comparison of in vivo Elav and Lz protein levels between normal and fl(2)d mutant cells

Third instar larval eye-antennal discs containing *fl(2)d* mutant clones were stained with antibodies against Elav and Lz, viewed and photographed on a Zeiss Axioplan II fluorescent compound microscope. The image files were imported into Adobe Photoshop and the rectangular marquee tool was then used to select regions of the *fl(2)d* loss-of-function clones. The Analysis Tool within Adobe Photoshop was used to determine the mean pixel intensity of Elav staining within the *fl(2)d* loss-of-function clones and the neighboring wild type tissue. In order to compare the relative level of Elav expression in the clone to that of the surrounding wild type tissue, the mean pixel intensity measurements for the clone was divided by that of the wild type tissue to yield a fold difference ratio. We examined and determined the pixel intensity ratio for clones in multiple discs. In order to determine the average fold difference for a single disc the fold differences for all clones within an individual disc were added and then averaged. In order to determine the average fold difference between *fl(2)d* clones and wild type tissue for the entire experiment we added and averaged the fold differences for the discs that we had examined. These methods allowed us to eliminate any experimental differences (such as antibody penetration) that may have existed between discs. Similar methods were used to determine the fold difference in Lz levels between *fl(2)d* clones and wild type tissue.

DNA constructs

Fl(2)d encodes a protein that is 536 amino acids in length (Penalva et al., 2000). Fl(2)d NT contains amino acids 1–100 (contains the histidine and glutamine stretches) fused to GFP while Fl(2)d CT contains amino acids 101–536 (contains predicted three coiled coil motifs). The So FL, So ΔSD and Optix FL proteins are described in Weasner et al. (2007) and diagramed in Fig. 1A (see figure legend for details on nomenclature).

Yeast 2-hybrid, Kc167 immunoprecipitation and transcriptional activation assays

Full-length *so*, *optix* and *DSix4* cDNAs were cloned into the pDEST32 vector and used to screen a yeast two-hybrid library (Life