Duplication and maintenance of the *Myb* genes of vertebrate animals

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Summary

Gene duplication is an important means of generating new genes. The major mechanisms by which duplicated genes are preserved in the face of purifying selection are thought to be neofunctionalization, subfunctionalization, and increased gene dosage. However, very few duplicated gene families in vertebrate species have been analyzed by functional tests *in vivo*. We have therefore examined the three vertebrate *Myb* genes (c-*Myb*, A-*Myb*, and B-*Myb*) by cytogenetic map analysis, by sequence analysis, and by ectopic expression in *Drosophila*. We provide evidence that the vertebrate *Myb* genes arose by two rounds of regional genomic duplication. We found that ubiquitous expression of c-*Myb* and A-*Myb*, but not of B-*Myb* or *Drosophila Myb*, was lethal in *Drosophila*. Expression of any of these genes during early larval eye development was well tolerated. However, expression of c-*Myb* and A-*Myb*, but not of B-*Myb* or *Drosophila Myb*, during late larval eye development caused drastic alterations in adult eye morphology. Mosaic analysis implied that this eye phenotype was cell-autonomous. Interestingly, some of the eye phenotypes caused by the retroviral v-*Myb* oncogene and the normal c-*Myb* proto-oncogene from which v-*Myb* arose were quite distinct. Finally, we found that post-translational modifications of c-Myb by the GSK-3 protein kinase and by the Ubc9 SUMO-conjugating enzyme that normally occur in vertebrate cells can modify the eye phenotype caused by c-Myb in *Drosophila*. These results support a model in which the three Myb genes of vertebrates arose by two sequential duplications. The first duplication was followed by a subfunctionalization of gene expression, then neofunctionalization of protein function to yield a c/A-*Myb* progenitor. The duplication of this progenitor was followed by subfunctionalization of gene expression to give rise to tissue-specific c-*Myb* and A-*Myb* genes.

Key words: Gene duplication, Neofunctionalization, Subfunctionalization, Evolution, *Myb*

Introduction

The duplication of existing genes has been proposed to be an important source of new genes (Bridges, 1936; Muller, 1935; Ohno, 1970). Two general questions about this process have been debated in the literature (Hahn, 2009). The first question concerns the mechanisms by which duplicate genes arise (Kaessmann, 2010). Examples of such mechanisms include tandem duplications of individual genes, retrotransposition of individual genes, regional duplication of chromosomal regions, and duplication of entire genomes. The second question concerns the mechanisms by which duplicated genes survive purifying selection (Conant and Wolfe, 2008; Innan and Kondrashov, 2010; Prince and Pickett, 2002). Three general mechanisms have been proposed: (i) neofunctionalization, in which one of the duplicates acquires a novel function; (ii) subfunctionalization, in which essential functions of the ancestral gene are partitioned between the duplicates; (iii) increased gene dosage, in which more copies of an identical gene confer a selective advantage. Because most neomorphic mutations are likely to be deleterious, the means by which neofunctionalization might evolve has been particularly puzzling. There have been numerous theoretical analyses of these questions and, more recently, genome-wide computational approaches have been used to argue for the relative importance of different mechanisms in different species (Hahn, 2009). However, thus far only a small number of duplicated gene families in vertebrate animals have been analyzed in detail by functional tests *in vivo*.

The genomes of vertebrate animals each contain three related *Myb* genes (c-*Myb*, A-*Myb*, and B-*Myb*), whereas the genomes of most invertebrate animals each contain a single *Myb* gene (Fig. 1) (Coffman et al., 1997; Katzen et al., 1985; Klempnauer et al., 1982; Lipsick, 1996; Nomura et al., 1988; Roussel et al., 1979; Souza et al., 1980). The presence of a single *Myb* gene in urochordate (*Ciona*) and cephalochordate (*Amphioxus*) species implies that the three *Myb* genes of vertebrate animals arose via two duplications that occurred in a vertebrate ancestor. Phylogenetic analyses of *Myb* genes from mammals, birds, amphibians, and bony fish imply that these two duplications occurred prior to the divergence of these classes of modern vertebrates (Davidson et al., 2005). These observations are consistent with the "2R hypothesis" that two genome-wide duplications occurred during the evolution of the last common ancestor of modern vertebrates (Holland et al., 1994; Meyer and Schartl, 1999; Wolfe, 2001). However, the 2R hypothesis has remained contentious (Hokamp et al., 2003; Hughes and Friedman, 2003).

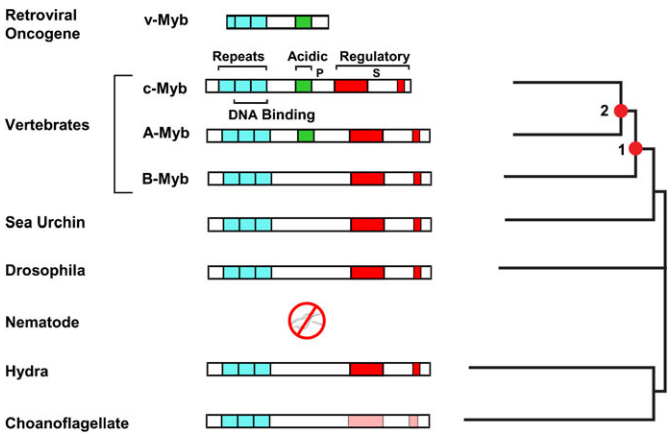

Fig. 1. Myb proteins of selected animal species. Schematic drawings of Myb protein sequences are based on local alignments, global alignments, and visual inspection as previously described (Ganter and Lipsick, 1999; Larkin et al., 2007; Schuler et al., 1991). No closely related *Myb* gene is present in the nematode *C elegans*. The Myb-related protein of the choanoflagellate *Salpingoeca* (GenBank: EGD77245.1) was used as an outgroup. Conserved regions are indicated as colored boxes (blue = N-terminal Myb repeats of the DNA-binding domain; red = animal-specific C-terminal regulatory domains; green = central acidic transcriptional activation domain; pink = regions with patchy similarity to C-terminal regulatory domains of animal Myb proteins). P indicates the clustered GSK-3 phosphorylation sites in c-Myb and v-Myb^{AMV}. S indicates the two major SUMOylation sites in c-Myb. On the right is a bootstrapped phylogenetic tree generated by alignment of deduced protein sequences using CLUSTALX followed by tree rendering with TREEVIEW (Larkin et al., 2007; Page, 1996). Numbered red circles indicate putative duplications.

The three-repeat Myb proteins of animals, plants, protists, and fungi contain a highly conserved DNA-binding domain near their amino terminus (Biedenkapp et al., 1988; Lipsick, 1996). In most animals, these proteins also contain a conserved regulatory domain near their carboxyl terminus (Fig. 1). A central transcriptional activation domain is present in the c-Myb and A-Myb proteins of vertebrates, but not in the B-Myb proteins of vertebrates or in the Myb proteins of invertebrates (Ibanez and Lipsick, 1990; Sakura et al., 1989; Weston and Bishop, 1989). The v-*Myb* oncogene of the avian myeloblastosis virus (AMV) encodes a doubly truncated form of the chicken c-Myb that lacks part of the DNA-binding domain and most of the conserved C-terminal regulatory domain (Lipsick and Wang, 1999). c-*Myb* and A-*Myb* are tissue-restricted in their expression, whereas B-*Myb* is expressed in mitotically active cells of all tissues (Amaravadi and King, 1994; Bouwmeester et al., 1992; Desbiens et al., 1991; Mettus et al., 1994; Sitzmann et al., 1996; Sitzmann et al., 1995; Sleeman, 1993; Trauth et al., 1994).

Mice with null mutations of c-*Myb* and A-*Myb* initiate development normally, but eventually display tissue-specific phenotypes as late embryos or adults (Mucenski et al., 1991; Toscani et al., 1997). c-*Myb* deficient mice die in mid-gestation due to a failure of fetal liver hematopoiesis. A-*Myb* deficient mice are viable, but the males are sterile due to a failure of spermatogenesis and the females cannot nurse their young due to a failure of mammary gland proliferation in response to pregnancy. In contrast, mice with a null mutation of B-*Myb* display very early embryonic lethality prior to implantation in the uterine wall (Tanaka et al., 1999). Conditional knockout mice have revealed additional tissue-specific roles for c-*Myb* (Bender et al., 2004; Malaterre et al., 2007; Malaterre et al., 2008; Thomas et al., 2005). Studies of c-*Myb* and B-*Myb* mutants in bony fish have led to similar conclusions (Lipsick, 2010; Moriyama et al., 2010; Shepard et al., 2005; Soza-Ried et al., 2010). *Drosophila Myb* null mutants die as third instar larvae and display mitotic defects (Manak et al., 2002; Manak et al., 2007; Wen et al., 2008). These results are consistent with the phenotypes of temperature-sensitive *Drosophila Myb* mutants that have been shifted to the restrictive temperature (Fung et al., 2002; Katzen and Bishop, 1996; Katzen et al., 1998; Okada et al., 2002).

We have previously reported that B-*Myb*, but neither c-*Myb* nor A-*Myb*, can partially complement the *Drosophila Myb* null mutant phenotype (Davidson et al., 2005). Furthermore, both the B-Myb and *Drosophila* Myb proteins are subunits of closely related multiprotein complexes (Myb-MuvB/DREAM) that regulate gene expression and cell cycle progression (Beall et al., 2002; Georlette et al., 2007; Korenjak et al., 2004; Lewis et al., 2004; Lipsick, 2004; Litovchick et al., 2007; Pilkinton et al., 2007; Schmit et al., 2007; Wen et al., 2008). Surprisingly, the animal-specific C-terminus of *Drosophila* Myb is sufficient to rescue lethality, interaction with the MuvB core proteins, transcriptional regulatory defects, and the chromosomal condensation defects of a *Myb* null mutant (Andrejka et al., 2011; Wen et al., 2008). We have now sought to answer several additional questions about the evolution of this gene family. What mechanism(s) generated the three *Myb* genes of vertebrates? Are any of the vertebrate *Myb* genes deleterious in *Drosophila*? Do any of the vertebrate *Myb* genes cause specific neomorphic phenotypes in *Drosophila*?

Materials and Methods

Drosophila stocks and genetics

The UAS-chicken B-*Myb* transgenic line *y,w^{67};+;P{w[+mC]=UAS-B-Myb}*, the UAS-chicken c-*Myb* transgene line *y,w^{67};+;P{w[+mC]=UAS-c-Myb}*, and the UAS-chicken A-Myb transgenic line *y,w^{67};+;P{w[+mC]=UAS-A-Myb}* have been previously described (Davidson et al., 2005). A UAS-v-Myb transgenic line *y,w^{67};+;P{w[+mC]=UAS-v-Myb}* was constructed in a similar fashion by subcloning an XbaI-resistant restriction fragment containing the v-*Myb* open reading frame of the N-v-Myb-1151 avian retrovirus into the pSP73 plasmid, and then subcloning a *Bam*HI/XhoI fragment into pUAST plasmid DNA that had been digested with *Bgl*II and XhoI (Brand and Perrimon, 1993; Fu and Lipsick, 1996). A GMR-c-Myb transgene was contructed by subcloning the chicken c-*Myb* ORF into the pGMR plasmid kindly provided by G. Rubin (UC Berkeley) (Hay et al., 1994). Flies containing these transgenes were crossed to flies containing *actin5C-GAL4*, *eyeless-GAL4*, *GMR-GAL4*, or *lozenge-GAL4* transgenes. F1 progeny were then analyzed for survival and/or eye morphology. Transgenic flies containing the *GMR-c-Myb* transgene were obtained by injecting *w^{1118}* embryos with plasmid DNA as previously described (Sullivan et al., 2000). A third chromosome insertion of this *GMR-c-Myb* transgene was then recombined with a third chromosome insertion of the *GMR-GAL4* transgene in order to test the effect of UAS-modifier genes in F1 crosses.

The eye-specific flip-out expression line *P{hsFLP}1, w[*]; P{ GMR >FRT w$^+$* STOP *FRT >Gal4}* was kindly provided by E. Hafen (University of Zurich) (Rintelen et al., 2001). UAS-Dm*Ubc9*, or *lwr*, flies were kindly provided by S. Tanda (Ohio University) (Apionishev et al., 2001). UAS-*dpias537* flies were kindly provided by J.E. Darnell (The Rockefeller University) (Betz et al., 2001). All other fly stocks were obtained from the Bloomington Drosophila Stock Center. Stocks were cultured on standard cornmeal, molasses, yeast, agar medium and maintained at 25℃ except where indicated.

Mosaic analysis

To generate marked clones that express *UAS-c-Myb* in the adult eye, 24- to 48-hour-old larvae containing a heat-shock-inducible *Flp* recombinase, a flip-out transgene (*GMR >FRT w$^+$* STOP *FRT >Gal4*), and a *UAS-c-Myb* construct were subjected to a heat shock for 3 hours at 37℃. Heat-shock expression of the *Flp* recombinase induces recombination between the *FRT* sites of *GMR >FRT w$^+$* STOP *FRT >Gal4* and removes the intervening *w$^+$* STOP cassette in clones, thus allowing expression of *UAS-c-Myb* under the control of *GMR-Gal4*.

Microscopy

Adult fly heads and eye were analyzed by light microscopy, scanning electron microscopy, and by light microscopy of toluidine blue-stained thick sections as previously described (Sullivan et al., 2000).

Cell culture and immunoblotting

Drosophila embryonic S2 cells and the S2-dervived cell line, 529SU, were grown at 25℃ in Schneider's *Drosophila* medium (Gibco/Invitrogen) supplemented with 10% heat-inactivated fetal calf serum. The 529SU cell line and the pPAC-FLAG-Ulp1 vector were gifts from A. J. Courey (UCLA) (Smith et al., 2004). Plasmid DNAs encoding tubulin-*GAL4*, and UAS-c-*Myb*, or UAS-v-*Myb* were transfected using Fugene (Promega) according to the manufacturer's instructions. For the copper-induction experiments in 529SU cells, 500 µM $CuSO_4$ was added to the culture medium ~18–24 hours after transfection. Following incubation for an additional 48 hours, cells were washed with phosphate-buffer saline (PBS) and lysed directly in SDS-PAGE sample buffer. Samples were resolved by electrophoresis in 4–12% NuPAGE Novec Bis-Tris gels (Invitrogen) with MOPS SDS Running buffer. Following electrophoretic transfer to nitrocellulose membranes, Myb proteins were detected using primary anti-Myb mouse 5E11 monoclonal antibodies, anti-mouse HRP-conjugated secondary antibodies, and chemiluminescent substrate (Pierce/Thermo) as previously described (Wen et al., 2008).

Results

Vertebrate *Myb* genes arose by regional duplications

Gene duplications occur by a variety of mechanisms ranging from local tandem duplication of individual genes to global duplication of entire genomes. To explore the nature of the duplications that gave rise to the three *Myb* genes of modern vertebrates, we searched databases of paralogous regions within the human genome (Ding et al., 2008; McLysaght et al., 2002). We also performed manual genome browser searches of the regions surrounding the human *Myb* genes. Our goal was to identify genes that may have been co-duplicated together with the *Myb* family. Near each of the three human *MYB* genes, we identified members of four other gene families – *SGK*, *PLAG1*, *EYA*, and the *SRC*-related tyrosine kinases (Fig. 2).

The *SGK* and *PLAG* gene families were similar to the *MYB* gene family, in that they all consist of three members located in similar regions of the human genome (near 6q23, 8q13, and 20q13). Phylogenetic analysis of the proteins encoded by the human, chicken, and *Drosophila SGK* genes (supplementary material Fig. S1) was consistent with a model in which the regions at 6q23 (*MYB*/c-*Myb* and *SGK1*) and at 8q13 (*MYBL1*/A-*Myb* and *SGK3*) arose via the most recent duplication. These results are similar to those previously obtained for the *MYB* gene family (Fig. 1) (Davidson et al., 2005; Lipsick, 1996). Phylogenetic analysis of the *PLAG1* gene family was complicated by the absence of a *PLAGL1* ortholog in birds and by the absence of a clear homolog in *Drosophila*.

The human *EYA* gene family contains four members. Three *EYA* genes are present at or near same chromosomal locations as the three human *MYB* genes. An additional *EYA* gene is located at 1p35. Phylogenetic analysis of the proteins encoded by the human, chicken, and *Drosophila EYA* genes (supplementary material Fig. S2) was consistent with a model in which the regions at 6q23 (*MYB*/c-*Myb* and *EYA4*) and at 8q13 (*MYBL1*/A-*Myb* and *EYA1*) arose via the most recent duplication. Interestingly, *EYA3*, which is not linked to a MYB gene at 1p35, appears to be most closely related to the sole *eya* gene of *Drosophila*. This result suggests that either: (i) a fourth *MYB* gene once resided near *EYA3* and was lost during evolution; or (ii) the linkage of *EYA* and *MYB* genes occurred after the duplication that gave rise to *EYA3* (unlinked to a *MYB* gene) and *EYA2* (linked to *MYBL2*/B-*Myb*), but prior to the two additional duplications that gave rise to *EYA4* (linked to *MYB*/c-*Myb*) and *EYA1* (linked to *MYBL1*/A-*Myb*).

The presence of the closely related *PDE7A* and *PDE7B* genes adjacent to MYB/c-Myb (6q23) and MYBL1/A-Myb (8q13) is consistent with a linkage between *MYB* and *PDE7* that occurred after the regional duplication that gave rise to a common ancestor of *MYB*/c-*Myb* and *MYBL1*/A-*Myb*, but prior to the most recent regional duplication that gave rise to these two genes. The *SRC*-related tyrosine kinase gene family is far more complex (Manning et al., 2002). In humans, an entire clade of *SRC*-related genes is located adjacent to the four *EYA* genes (Fig. 2). This observation is consistent with a linkage between the *SRC* and *EYA* genes that predates the regional duplications that gave rise to the *EYA* gene family. However, unlike the *EYA* and *MYB* gene families, the *SRC* gene family appears to have undergone additional duplications. Rather than showing a one-to-one correspondence between *SRC* and *EYA* genes, a greater number of human *SRC*-related genes are distributed near the four *EYA* chromosomal locations (1p35, 6q23, 8q13, 20q13). Furthermore, the lack of a clear one-to-one correspondence between the *SRC*-related genes of humans (e.g. *FGR*) and chickens (e.g. *YES* and *YRK*) is consistent with ongoing duplication and selection of this gene family.

The paralogous linkage blocks at the four *EYA* chromosomal locations are generally conserved between the human genome and that of the laboratory mouse (1p35=>4D2; 6q23=>10A3; 8q13=>1A3; 20q13=>2H2). The corresponding members of the murine *EYA*, *MYB*, and *SGK* gene families are linked in a fashion similar to that in humans. With the exception of *LYN*, the corresponding murine *SRC* gene family members are also present within these syntenic regions. This exception appears to have resulted from relatively recent chromosomal rearrangements within the genome of the mouse, because another gene linked to the 8q13 region of the human genome (*PLAG1*) remains linked to *LYN* (4A1) rather than to *EYA1*, *MYBL1*/A-*Myb*, and *SGK3* within the mouse genome. *PDE7A*, another gene linked to the 8q13 region of the human genome, has been dispersed to yet another mouse chromosomal location (3A2).

Taken together these analyses of cytogenetic maps and phylogenetic trees provide strong support for a model in which the three *Myb* genes of vertebrates arose by at least two regional duplication events that occurred prior to the divergence of modern vertebrate animal species (Davidson et al., 2005). The first regional duplication gave rise to B-*Myb* and to a common ancestor of c-*Myb* and A-*Myb*. A second regional duplication that included this common ancestor gave rise to the c-*Myb* and A-*Myb* genes.

A-*Myb*, c-*Myb*, and v-*Myb* are lethal in *Drosophila*

We previously reported that vertebrate B-*Myb*, but neither A-*Myb* nor c-*Myb* could rescue specific aspects of the *Drosophila Myb* null phenotype, including the failure of larval hemocyte proliferation and differentiation (Davidson et al., 2005). Those results suggested that c-*Myb* and A-*Myb* had been retained in vertebrates as a result of neofunctionalization. We therefore wished to test whether this putative neomorphic protein function might at least in part have been deleterious. To test this hypothesis, we drove the expression of various *Myb* genes under control of the *Actin5C* promoter via the *GAL4-UAS* system (Ito et al., 1997). In these experiments the GAL4 transcriptional activator from budding yeast is used to drive expression of the cDNA of interest via multimerized GAL4 DNA-binding sites similar to those present in the upstream activating sequence (UAS) of the GAL1 and GAL10 genes that are normally

~1p35	EYA3					FGR LCK
~6q23	EYA4	MYB/ c-Myb	SGK1	PLAGL1	PDE7B	FRK FYN
~8q13	EYA1	MYBL1/ A-Myb	SGK3	PLAG1	PDE7A	LYN
~20q13	EYA2	MYBL2/ B-Myb	SGK2	PLAGL2		HCK SRC

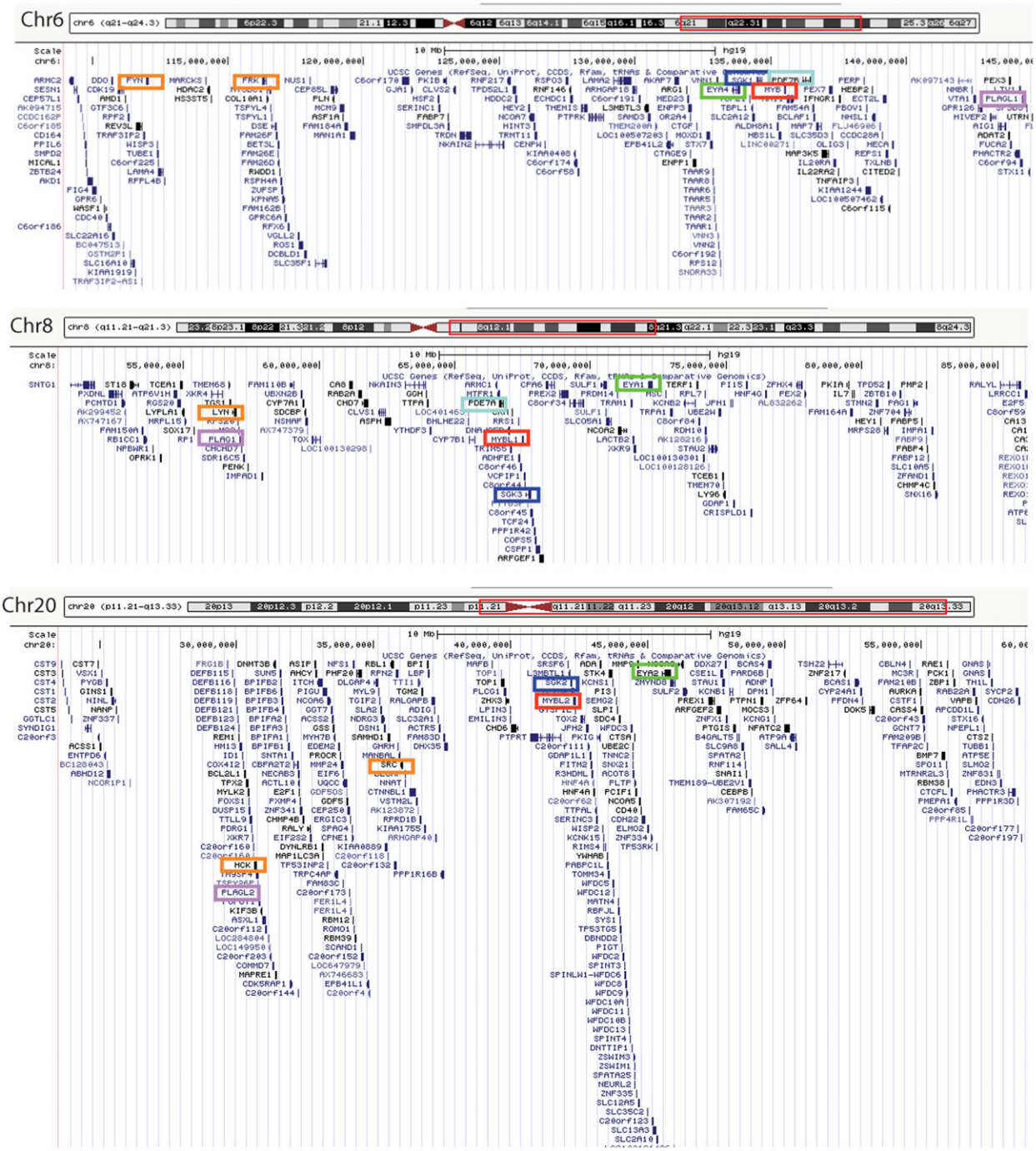

Fig. 2. See next page for legend.

activated by GAL4 (Brand and Perrimon, 1993). There were no adult F1 progeny that had *Actin-GAL4* and A-*Myb*, *Actin-GAL4* and c-*Myb*, or *Actin-GAL4* and v-*Myb* (Table 1). In contrast, *Actin-GAL4*-driven expression of either *Drosophila Myb* or B-*Myb* was compatible with adult viability. Indeed, an even greater than expected percentage of progeny with ectopically expressed *Drosophila Myb* or B-*Myb* were present, presumably due to the presence of one or two balancer chromosomes in the other classes of F1 progeny (Ashburner, 1989). These results show that A-*Myb*, c-*Myb*, and v-*Myb* display a neomorphic lethal effect in *Drosophila*, whereas B-*Myb* does not.

Early expression of A-*Myb*, c-*Myb*, and v-*Myb* is compatible with *Drosophila* eye development

We wished to determine whether the lethality caused by A-Myb, c-Myb, and v-Myb was due to a lethal effect in all cells, or whether these neomorphic proteins might cause specific defects in cell viability, proliferation, and differentiation. To address this question we turned to *Drosophila* eye development, which has become a powerful tool for analyzing the effects of both endogenous and exogenous gene function (Thomas and Wassarman, 1999). The eye develops as a larval imaginal disc in two main steps (Wolff and Ready, 1993). First, there is a massive proliferation of undifferentiated precursor cells within an epithelial sheet. Second, a wave of cell differentiation occurs behind the morphogenetic furrow as it passes from the posterior to the anterior of the imaginal disc epithelium.

GAL4 expressed under control of the *eyeless* promoter (*ey-GAL4*) can be used to drive expression of a gene of interest in all cells in the eye imaginal disc during the early period of cell proliferation and anterior to the morphogenetic furrow during differentiation (Lai and Rubin, 2001). We found that *ey-GAL4*-driven expression of *Drosophila* Myb had no discernable effect upon eye development (Fig. 3). Similar expression of B-Myb, A-Myb, or c-Myb caused a variable reduction in overall size of the eye, but did not alter the overall architecture. Furthermore, microscopic examination of sections of these eyes revealed a normal arrangement of photoreceptors, pigment cells, and cone cells. Expression of viral v-Myb (Fig. 3) or of high levels of c-Myb via increased copy number (data not shown) resulted in a greater reduction in size of the adult eye, but again did not substantially alter the gross or microscopic architecture of the eye. These results imply that expression of vertebrate Myb proteins during early eye development does not cause uniform cell death, nor does it interfere with normal differentiation and development. Large-scale genetic screens have previously shown that a similar small eye phenotype is frequently associated with alterations in cell cycle regulatory genes (Tseng and Hariharan, 2002)

Late expression of A-*Myb*, c-*Myb*, and v-*Myb* severely disrupts *Drosophila* eye development

Cellular differentiation occurs posterior to the morphogenetic furrow within the larval eye imaginal disc of *Drosophila*. GAL4 expressed under control of the *glass* enhancer (*GMR-GAL4*) can be used to drive expression of a gene of interest in all cells within and posterior to the morphogenetic furrow (Freeman, 1996). We found that *GMR-GAL4*-driven expression of *Drosophila* Myb or of vertebrate B-Myb had little if any effect upon eye development (Fig. 4). In contrast, GMR-GAL4-driven expression of vertebrate A-Myb or c-Myb caused a similar drastic alteration in eye phenotype. The gross alterations included a narrowing of the eye in the anterior–posterior dimension, a blurring of ommatidial boundaries with facet fusion, a variable loss of pigmentation, and a variable loss of sensory bristles. Microscopic examination of sections revealed a variable loss and/or rearrangement of photoreceptor cells, pigment cells, and cone cells. GMR-GAL4-driven expression of viral v-Myb (Fig. 4) or of high levels of c-Myb (data not shown) caused a more severe phenotype reminiscent of the *spectacle* loss-of-function allele of the *lozenge* gene (Batterham et al., 1996). Notable aspects of this phenotype included a smoothened eye surface, a central loss of pigment, and preservation of an outer rim of pigmented cells. At the microscopic level, there was a greater disorganization of photoreceptor cells. Similar to the more severe mutant alleles of *lozenge*, there appeared to be a loss of the fenestrated membrane at the base of the eye that is formed by the pigment cells and that maintains the photoreceptor neurons in their proper orientation (supplementary material Fig. S3). The loss of the fenestrated membrane is thought to lead to the collapsed appearance of the eye in scanning electron micrographs due to the lack of structural strength under vacuum.

We wished to determine whether or not the defects caused by *GMR-GAL4*-driven expression of c-Myb were cell-autonomous. To answer this question, we used a "flip out" strategy in which the GMR enhancer/promoter was separated from the GAL4 open reading frame (ORF) by an intervening *white*$^{+}$ gene ORF, which itself was flanked by Flippase (FLP) recognition targets (FRTs) (Rintelen et al., 2001). The *white*$^{+}$ ORF can be removed by the induction of a heat shock promoter-driven FLP recombinase. This results in GAL4 expression via GMR and simultaneous loss of red eye pigment in patches of cells that result from successive mitoses following FLP induction. Under the dissecting microscope, we observed patches of white cells in an otherwise red background. The appearance of these "flip out" clones varied from animal to animal, but in many cases we observed a localized phenotype similar to that described above with *GMR-GAL4* driving c-*Myb* (Fig. 5). The affected ommatidia displayed an irregular arrangement, fused facets, and loss or duplication of sensory bristles. Microscopic examination revealed the expected loss and/or rearrangement of photoreceptor cells, pigment cells, and cone cells. Importantly, these phenotypic changes were restricted to cells within the "flip out" clone as marked by the absence of red pigment. These results imply that the phenotype caused by *GMR-GAL4*-driven c-*Myb* is cell-autonomous.

Because of the superficial similarity between the eye phenotypes caused by *GMR-GAL4*-driven v-*Myb* and the *lozenge* loss-of-function mutant, we wished to ask whether expression of v-Myb in lozenge-expressing cells was sufficient to cause this phenotype. We therefore used a *lozenge-GAL4* (*lz-GAL4*) driver to express various Myb proteins. This driver is

Fig. 2. Regional duplications generated the three *Myb* genes of vertebrate animals. Top: human gene families with members mapping close to the three human Myb genes were identified using databases of paralogous regions and by visual inspection using the USCS Genome Browser (Ding et al., 2008; McLysaght et al., 2002). The approximate cytogenetic location for each row of genes is indicated in the left-most column. **Bottom**: schematic representations of paralogous regions including the three human *Myb* and *SGK* genes were generated using the USCS Genome Browser. Members of each gene family of interest are highlighted by colored boxes. Each region contains ~35 megabases of DNA (~1% of the entire human genome).

Table 1. Lethality of vertebrate A-Myb, c-Myb and v-Myb in *Drosophila*.

	TM3/TM6	GAL4/TM3	GAL4/UAS-Myb	UAS-Myb/TM6	Total	GAL4/UAS-Myb (Percent)
D-Myb	20	40	48	25	133	36%
A-Myb	48	81	0	64	193	0%
B-Myb	13	41	81	67	202	40%
c-Myb	57	59	0	68	184	0%
v-Myb	41	58	0	53	152	0%

Actin5C-GAL4/TM6B, *Tb* virgin females were crossed to *UAS-Myb/TM3*, *Sb* males. Phenotypes of the F1 progeny were scored and tabulated.
The expected yield of *Actin5C-GAL4/UAS-Myb* progeny was 25%.
D-Myb signifies *Drosophila* Myb.

initially expressed in the eye imaginal disc posterior to the morphogenetic furrow in an array of apparently undifferentiated cells surrounding the clusters of differentiated R8, R2/5, and R3/4 photoreceptor cells (Crew et al., 1997). Expression then progresses to include R1/6 cells, R7 cells, cone cells, and pigment cells. *lz-GAL4*-driven expression of *Drosophila* Myb, B-Myb, A-Myb, or c-Myb caused no reproducible development abnormalities of the adult eye (Fig. 6; data not shown). However, expression of higher levels of c-Myb via *lz-GAL4* caused a rough-eye phenotype with occasional facet fusion, preservation of sensory bristles, and a mild-to-moderate microscopic disorganization of the photoreceptor cells (Fig. 6).

Unexpectedly, *lz-GAL4*-driven expression of viral v-Myb protein caused a rather different phenotype. Individual ommatidia displayed a white central region surrounded by red pigmentation most noticeable in the center of the eye. There was also a loss of sensory bristles. Microscopic examination revealed an unusual organization and orientation of the photoreceptor cells within each ommatidium. We hypothesize that this abnormal orientation may be the cause of the apparent lack of pigmentation. Although the surface of the eye appeared very irregular by scanning electron microscopy, microscopic sections confirmed the presence of relatively normal lenses, implying the presence of functional cone and pigment cells. These results demonstrate that expression of c-Myb in the normal *lozenge* pattern is not sufficient to cause the GMR-GAL4-driven eye phenotype. Furthermore, these results show that the oncogenically activated v-Myb protein can disrupt eye development in a different manner than does the normal c-Myb protein.

GSK3 and by SUMOylation are modifiers of the c-Myb *Drosophila* eye phenotype

We hypothesized that the neomorphic c-Myb and A-Myb proteins must have survived purifying selection via novel functions within a common ancestor of modern vertebrates. Presumably these novel functions would require interactions with existing biochemical pathways. We therefore wished to test whether post-translational modifications of c-Myb that are known to occur in vertebrate cells might also affect the phenotype caused by c-Myb in the *Drosophila* eye. A peptide motif present in c-Myb and v-Myb, but neither B-Myb nor *Drosophila* Myb, is a target for phosphorylation by glycogen synthase kinase 3 (GSK-3) *in vitro* and is the major site of v-Myb phosphorylation *in vivo* (Fig. 1) (Boyle et al., 1991; Fu and Lipsick, 1996).

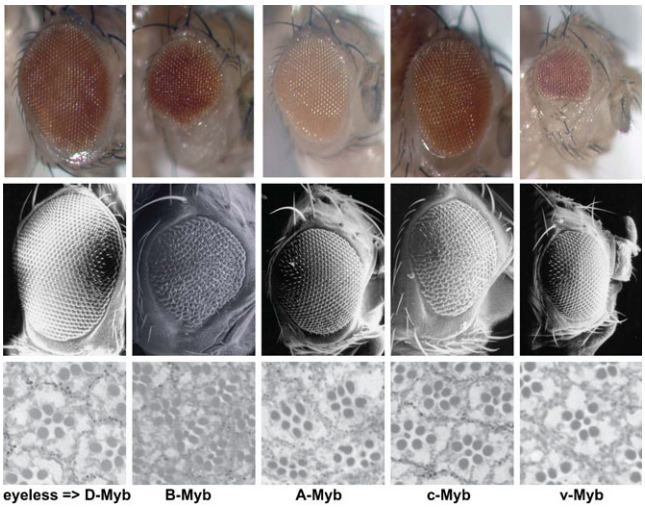

Fig. 3. Expression of exogenous Myb proteins during early *Drosophila* eye development. The *eyeless-GAL4* driver was used to drive the expression of *Drosophila* Myb (D-Myb), B-Myb, A-Myb, c-Myb, or v-Myb.
Top: photomicrographs of eyes of anesthetized flies using a dissecting light microscope. Eye color varies between flies of different genotypes due to transgene insertion sites. **Middle**: scanning electron micrographs of glutaraldehyde-fixed fly heads. **Bottom**: photomicrographs of toluidine blue-stained thick sections of glutaraldehyde-fixed, plastic-embedded fly eyes.

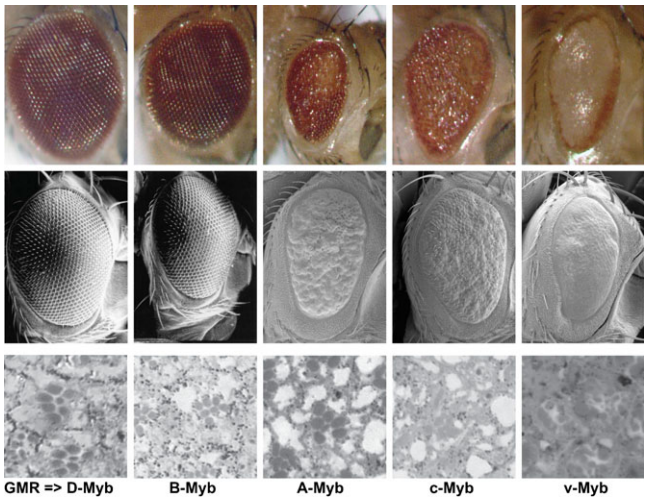

Fig. 4. Expression of exogenous Myb proteins during late *Drosophila* eye development. The *GMR-GAL4* driver was used to drive the expression of *Drosophila* Myb (D-Myb), B-Myb, A-Myb, c-Myb, or v-Myb.
Top: photomicrographs of eyes of anesthetized flies using a dissecting light microscope. **Middle**: scanning electron micrographs of glutaraldehyde-fixed fly heads. **Bottom**: photomicrographs of toluidine blue-stained thick sections of glutaraldehyde-fixed, plastic-embedded fly eyes.

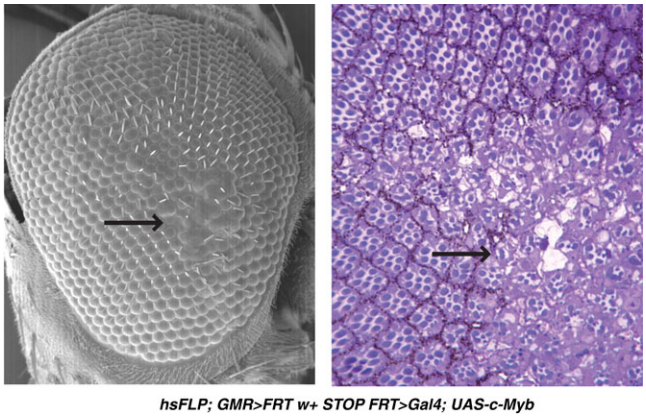

Fig. 5. Cell autonomous nature of the *GMR-GAL4*-driven c-Myb eye phenotype. Flip out clones were induced during larval development, resulting in the expression of c-Myb in discrete patches of adjacent cells. **Left**: scanning electron micrograph of glutaraldehyde-fixed fly head. Note the fusion of adjacent facets, the absence of sensory bristles, and occasional multiple sensory bristles in the mosaic patch (arrow). **Right**: photomicrograph of toluidine blue-stained thick section of a glutaraldehyde-fixed, plastic-embedded fly eye. Note the disorganization of ommatidia within the mosaic patch that is marked by the absence of red pigment cells (arrow).

Phosphorylation by GSK-3 within the C-terminal region of c-Myb that was deleted in v-Myb has also been reported (Kitagawa et al., 2009). In *Drosophila*, GSK-3 is encoded by the s*haggy* (*sgg*) gene, which is also known as *zeste-white 3* (*zw3*) (Bourouis et al., 1990; Hughes et al., 1992). To test for an interaction between c-Myb and Sgg/GSK-3 *in vivo*, we expressed one or both under control of the GMR enhancer (Fig. 7). Sgg/GSK-3 alone expressed under control of *GMR-GAL4* caused a slightly rough, but otherwise normal appearing eye. When placed under direct control of the GMR enhancer, c-Myb displayed an eye phenotype similar to that described above with fused facets, loss of bristles, but generally uniform pigmentation. Co-expression of *GMR-GAL4*=>Sgg/GSK-3 and *GMR*=>c-Myb caused a more severe eye phenotype reminiscent of that caused by *lz-GAL4*=>v-Myb, with a smooth surface, loss of sensory bristles, and apparent absence of central pigmentation within individual ommatidia. These results imply that the neomorphic c-Myb protein can respond to signaling via GSK-3 pathways *in vivo* in *Drosophila*.

The c-Myb protein has also been shown to be post-translationally modified by SUMOylation in vertebrate cells (Bies et al., 2002; Dahle et al., 2003). Ligation of SUMO to c-Myb has been reported to increase protein stability. The major sites of this modification are two lysine residues located near conserved regions within the C-terminus of c-Myb. However, these sites are not well conserved in A-Myb, B-Myb, or *Drosophila* Myb (Ganter and Lipsick, 1999). To test whether SUMOylation might modify the function of c-Myb in *Drosophila*, we initially focused on Ubc9, a highly conserved E2-like SUMO conjugating protein (Johnson and Blobel, 1997). We found that *GMR-GAL4*-driven Ubc9 itself caused no eye phenotype, but that Ubc9 enhanced the phenotype caused by GMR-driven c-Myb (Fig. 8). A similar enhancement of the GMR-driven c-Myb phenotype was caused by the protein inhibitor of activated Stat (Pias), a highly conserved E3-like SUMO ligase (Fig. 8) (Johnson and Gupta, 2001; Takahashi et al., 2001). In contrast to the strong enhancement of GMR-driven c-Myb caused by Ubc9, little if any enhancement of GMR-driven v-Myb was caused by Ubc9 (Fig. 8). This result is consistent with the absence of the major sites of SUMOylation in v-Myb due to C-terminal truncation of the protein (Fig. 1). Consistent with these genetic observations, we found that vertebrate c-Myb but not *Drosophila* Myb could be SUMOylated by Ubc9 in *Drosophila* S2 cells, causing a corresponding stabilization of c-Myb. Furthermore, this SUMOylation could be readily reversed by increasing doses of Ulp1, a conserved enzyme capable of deconjugating SUMO on targeted proteins (Fig. 9) (Li and Hochstrasser, 1999). These results imply that the neomorphic c-Myb protein can also respond to signaling via SUMOylation in *Drosophila*.

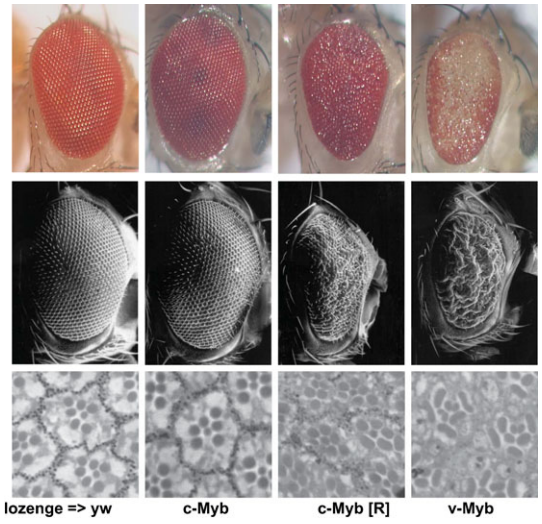

Fig. 6. Expression of c-Myb and v-Myb proteins in a *lozenge* pattern. The *lozenge-GAL4* driver was used to drive the expression of c-Myb or v-Myb. [R] indicates the presence of multiple copies of the UAS-c-Myb transgene to provide increased dosage. yw indicates F1 progeny of control flies of the y^1 w^{67} genotype lacking a UAS transgene crossed to *lozenge-GAL4* flies. **Top**: photomicrographs of eyes of anesthetized flies using a dissecting light microscope. **Middle**: scanning electron micrographs of glutaraldehyde-fixed fly heads. **Bottom**: photomicrographs of toluidine blue-stained thick sections of glutaraldehyde-fixed, plastic-embedded fly eyes.

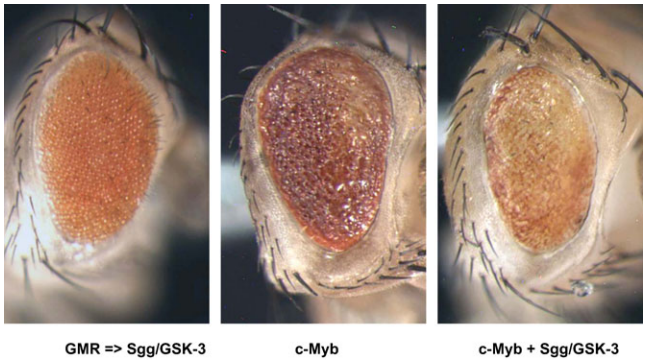

Fig. 7. Shaggy/GSK-3 enhances the *GMR-GAL4*-driven c-Myb eye phenotype. The *GMR-GAL4* driver was used to express the indicated UAS transgenes. Photomicrographs of eyes of anesthetized flies using a dissecting light microscope.

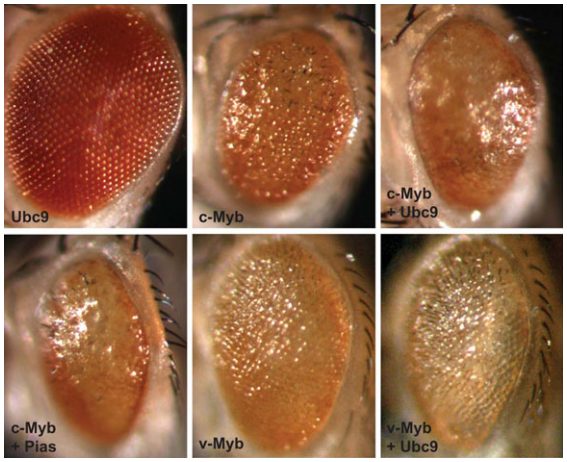

Fig. 8. SUMOylation enhances the *GMR-GAL4*-driven c-Myb but not the v-Myb eye phenotype. The *GMR-GAL4* driver was used to express the indicated UAS transgenes. Photomicrographs of eyes of anesthetized flies using a dissecting light microscope.

Discussion

Cytogenetic map and phylogenetic sequence analyses imply that the three *Myb* genes of vertebrate animals arose by regional chromosomal duplications (or possibly by whole genome duplications), rather than by tandem gene duplications or retrotransposition events (Figs 1, 2; supplementary material Figs S1, S2). The evidence is consistent with a model that proposes an initial duplication of a B-*Myb*-like ancestral gene, followed by the evolution of a central transcriptional activation in one of the duplicates, followed by a second duplication of the proto-c/A-*Myb* gene to generate the c-*Myb* and A-*Myb* genes of existing vertebrates (Davidson et al., 2005).

Drosophila Myb and vertebrate B-*Myb* are normally expressed in most tissues during development. Increased expression of either of these proteins in *Drosophila* is compatible with normal development, cell differentiation, cell proliferation, and organismal viability (Table 1; Figs 3, 4). Others have reported that increased levels of *Drosophila Myb* can result in lethality, mitotic defects, and replication defects in endocycling cells (Fitzpatrick et al., 2002). Perhaps these phenotypes were due to a much greater dosage of gene expression. Nevertheless, a variety of experiments with genomic rescue *Myb* constructs and with a variety of GAL4 drivers support the conclusion that moderately increased levels of *Drosophila Myb*, as are predicted to occur immediately following gene duplication, are unlikely to have any deleterious effects (Andrejka et al., 2011; Davidson et al., 2005; Manak et al., 2002; Manak et al., 2007; Wen et al., 2008).

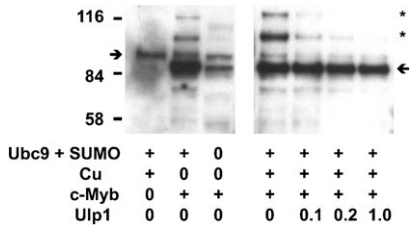

Fig. 9. c-Myb but not *Drosophila* Myb protein is SUMOylated by Ubc9. Parental *Drosophila* S2 cells or S2 cells with integrated copper-inducible Ubc9 and SUMO transgenes (Ubc9 + SUMO) were transected with plasmid DNAs encoding c-Myb and/or Ulp1. Copper was used to induce Ubc9 and SUMO expression. + and 0 signify the presence or absence of indicated transgenes or copper (Cu). Numbers in the Ulp1 row signify micrograms of transfected plasmid DNA. Cell lysates were analyzed by immunoblotting with an anti-Myb antibody that recognizes both *Drosophila* Myb (arrow at left) and c-Myb (arrow at right). Asterisks signify the mobility of SUMOylated forms of c-Myb. Number to the left of the blot indicate relative mobility of co-electrophoresed molecular weight standards ($\times 10^{-3}$).

The lethality of c-*Myb* and A-*Myb* in *Drosophila* when expressed widely, but not when expressed in a tissue-specific manner, imply that alterations in transcriptional regulation are very likely to have preceded the evolution of the neomorphic central transcriptional activation domains of c-Myb and A-Myb (Fig. 1). Without a more restricted pattern of gene expression, the deleterious effects of these neomorphic c-Myb and A-Myb proteins would almost certainly have led to extinction without rapid pseudogenization and/or gene loss. Furthermore, the drastic differences in adult eye phenotypes caused by early versus late expression of c-*Myb* and A-*Myb* during *Drosophila* larval development argue that specific alterations of the transcriptional regulation of these genes are likely to have been required to permit these neomorphic duplicates to survive purifying selection.

The adult eye phenotypes caused by expression of c-*Myb* during late larval development can be modified by the overexpression of *Drosophila* genes that encode homologs of proteins previously reported to modify the function of vertebrate c-Myb protein via phosphorylation and SUMOylation (Figs 7, 8). These results are consistent with a model in which the new protein coding sequences within c-Myb and A-Myb plugged into existing pathways of protein function. Furthermore, the dramatic difference in eye phenotypes caused by *lz-GAL4*-driven expression of c-Myb versus v-Myb argues that existing pathways in *Drosophila* can distinguish between the functions of wild type c-Myb and oncogenically activated forms of this protein (Lipsick and Wang, 1999; Ramsay and Gonda, 2008).

The central activation domain conserved in c-Myb and A-Myb proteins interacts specifically with the CBP/p300 transcriptional coactivator proteins (Dai et al., 1996; Facchinetti et al., 1997; Oelgeschläger et al., 1996; Zor et al., 2004). *Drosophila* Myb and B-Myb have no significant sequence homology to this central activation domain (Ganter and Lipsick, 1999). Furthermore, the central region of B-Myb appears to be under much less evolutionary constraint than the corresponding regions of c-Myb and A-Myb (Simon et al., 2002). Nevertheless, *Drosophila* Myb has been reported to interact biochemically and genetically with *Drosophila* CBP (Fung et al., 2003; Hou et al., 1997). Interestingly, the N-terminal DNA-binding domain and the C-terminal regulatory domain of c-Myb have also been reported to be required for interactions with CBP (Pattabiraman et al., 2009). These results lead us to speculate that one of driving forces for the preservation of a neomorphic c/A-Myb ancestral gene duplicate may have been the strengthening of existing weak interactions between *Drosophila*/B-Myb and CBP. It is interesting in this regard that either increases or decreases in the levels of *Drosophila* CBP can also cause dramatic eye phenotypes, some of which are superficially similar to those caused by c-Myb, v-Myb, and A-Myb (Anderson et al., 2005; Kumar et al., 2004).

A close examination of the functional evolution of this small gene family has implications for more general models of gene duplication and for the survival of duplicated genes in the face of purifying selection (Hahn, 2009). Although neofunctionalization

and subfunctionalization have often been presented as alternative fates of duplicated genes, our results imply that alternating rounds of subfunctionalization, neofunctionalization, and subfunctionalization are most likely to have led to the modern *Myb* genes of vertebrates. In this regard, our findings are supportive of models in which subfunctionalization and neofunctionalization have been proposed to work in concert during the evolution of duplicated genes (He and Zhang, 2005; Rastogi and Liberles, 2005).

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Competing Interests

The authors have no competing interests to declare.

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