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The conserved Myc box 2 and Myc box 3 regions are important, but not essential, for Myc function *in vivo*

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Abstract

Myc proto-oncoproteins are important regulators of growth and proliferation in development. Their functions have been evolutionarily conserved from insects to vertebrates, although the sequence conservation is limited to a few short domains. Here, we analyze the requirement for the most highly conserved domains, called Myc boxes 2 and 3 (MB2 and MB3), and for the weakly conserved N-terminus for the biological activity of the single *Drosophila* Myc protein in the animal *in vivo*. We find that a Myc mutant lacking the N-terminus retains very little activity, whereas Myc transgenes carrying a deletion of MB3 have a moderately increased ability to promote growth and apoptosis; mutation of MB2 reduces transcriptional output and the biological activities of Myc. Surprisingly though, Myc without MB2 retains enough activity to partially rescue the lethality of a *Myc* null mutation. Thus, although MB2 and MB3 are highly conserved in evolution, loss of either domain has comparatively mild consequences on Myc activity *in vivo*.

1 Introduction

Myc has been identified as a proto-oncogene that is frequently mutated and overexpressed in human and animal cancers (reviewed by Oster et al., 2002). During normal development and tissue homeostasis, Myc controls several cellular processes, most prominently growth, proliferation and apoptosis (de la Cova and Johnston, 2006; Pirity *et al.*, 2006). These different activities of Myc have been attributed to its ability to stimulate or repress the transcription of many target genes (reviewed by Grandori *et al.*, 2000; Amati *et al.*, 2001; Dang *et al.*, 2006). Up to 15% of all genes are modulated by Myc proteins (Fernandez et al., 2003; Orian et al., 2003), including genes transcribed by RNA polymerases I and III. However, most of its targets are only moderately affected by Myc (reviewed by Dang et al., 2006). In addition, Myc has transcription-independent roles in the control of DNA replication (Dominguez-Sola et al., 2007).

To molecularly understand how Myc controls its target genes and what might distinguish Myc from other transcription factors, several structure/function analyzes of Myc have been carried out. Myc's best understood domain, and the only one for which structural information is available (Nair and Burley, 2003), is the C-terminal basic-helix-loop-helix-leucine zipper region (BHLHZ) which serves as a dimerization interface with a protein called Max (Blackwood and Eisenman, 1991) and also mediates the contact of Myc:Max heterodimers with DNA. In addition, several other proteins have been shown to contact this C-terminus (reviewed by Cowling and Cole, 2006). This region is generally assumed to be essential for all of Myc's activities, although recent reports suggest the existence of some C-terminus independent functions of Myc (Cowling and Cole, 2007; Steiger et al., 2008).

Myc's N-terminal 143 amino acids contain a transcriptional regulatory domain (Kato et al., 1990) that interacts with different co-factors, such as TRRAP, a component of several histone acetyltransferase (HAT) complexes (McMahon et al., 1998), the STAGA HAT (McMahon *et al.*, 2000; Liu *et al.*, 2003; Liu *et al.*, 2008), the DNA helicases Tip48 and Tip49 that also belong to different HAT and chromatin remodeling complexes (Wood et al., 2000), and the elongation factor P-TEFb (Eberhardy and Farnham, 2002). Accordingly, a version of c-Myc that lacks the first 100 amino acids is inefficient in trans-

activating its target genes (Spotts *et al.*, 1997; Xiao *et al.*, 1998; Hirst and Grandori, 2000), and it is unable to transform primary cells in culture (Xiao et al., 1998) or promote cell cycle re-entry of serum-starved fibroblasts (Hirst and Grandori, 2000). Surprisingly, though, such a truncated c-Myc protein retains substantial biological activity: it induces apoptosis in response to limitation of survival factors (Xiao et al., 1998), stimulates the rate of proliferation of exponentially growing fibroblasts (Hirst and Grandori, 2000), even in the complete absence of endogenous c-Myc (Xiao et al., 1998), and it can rescue a lethal mutation of the only *Drosophila Myc* gene, dm^{PG45} (Benassayag et al., 2005).

These properties contrast with those of a short motif called Myc box 2 (MB2) that is located between amino acids 128 and 143 in human c-Myc. The MB2 sequence is unique to Myc and evolutionarily highly conserved in all Myc proteins, down to invertebrates (reviewed in Gallant, 2006). Elimination of MB2 blocks or severely impairs all of Myc's biological activities that have been tested in cultured cells: the ability to promote cellular proliferation (Lee *et al.*, 1997; Conzen *et al.*, 2000; Hirst and Grandori, 2000; Kenney *et al.*, 2003), apoptosis (Evan *et al.*, 1992; Conzen *et al.*, 2000; Dugan *et al.*, 2002; Oster *et al.*, 2003; Cowling *et al.*, 2006), transformation (Sarid *et al.*, 1987; Stone *et al.*, 1987; Li *et al.*, 1994; Brough *et al.*, 1995; McMahon *et al.*, 1998; Conzen *et al.*, 2000; McMahon *et al.*, 2000; Nikiforov *et al.*, 2002; Oster *et al.*, 2003; Cowling *et al.*, 2006), and block differentiation (Freytag et al., 1990). Furthermore, an MB2-mutant Myc very inefficiently rescues the proliferation defect of rat fibroblasts lacking endogenous c-Myc (Bush *et al.*, 1998; Xiao *et al.*, 1998; Nikiforov *et al.*, 2002; Oster *et al.*, 2003). The MB2 motif is required for the efficient interaction with several co-factors such as TRRAP and associated HATs (McMahon *et al.*, 1998; Bouchard *et al.*, 2001; Frank *et al.*, 2001; Nikiforov *et al.*, 2002), the HAT complex component BAF53 (Park et al., 2002), TIP48 & TIP49 (Wood et al., 2000), Skp2 (Kim et al., 2003; von der Lehr et al., 2003), and for the stimulation of pre-mRNA capping (Cowling and Cole, 2007). Nevertheless, it has been shown that a Myc mutant lacking MB2 still efficiently activates the expression of some target genes and artificial reporters (Kato *et al.*, 1990; Bello-Fernandez *et al.*, 1993; Li *et al.*, 1994; Brough *et al.*, 1995; Lee *et al.*, 1997; Xiao *et al.*, 1998; Nikiforov *et al.*, 2002), although many targets are only poorly activated by such a protein (Conzen *et al.*, 2000; Hirst

and Grandori, 2000; Bouchard *et al.*, 2001; Nikiforov *et al.*, 2002; Kenney *et al.*, 2003; Cowling *et al.*, 2006; Cowling and Cole, 2007). Such a MycΔMB2 mutant also fails to repress the genes that are normally down-regulated by wild type Myc (Li *et al.*, 1994; Lee *et al.*, 1997), including the c-*myc* locus itself (Penn *et al.*, 1990; Bush *et al.*, 1998; Oster *et al.*, 2003). Importantly, the biological function of the MB2 cannot be replaced by the strong transactivation domain of the viral transcription factor VP16 (Brough et al., 1995).

Much less is known about the central 240 amino acids of Myc. This region is less conserved, with the exception of a motif called Myc box 3 (MB3) that is found in all Myc proteins from insects to vertebrates (note that the term "Myc box 3" has been applied to a different region by Herbst et al., 2005; Cowling and Cole, 2006). Although MB3 resembles a PEST domain, and hence might be expected to contribute to the degradation of Myc protein, its deletion does not affect Myc's half-life (Herbst et al., 2004); this region also does not seem to contribute to transcriptional regulation by Myc (Kato et al., 1990). To date, MB3 has only been shown to be required for Myc's ability to transform certain cell types (the rat fibroblast line Rat1a and primary chicken hematopoietic cells; Heaney *et al.*, 1986; Biegalke *et al.*, 1987; Stone *et al.*, 1987). However, given its high evolutionary conservation, we consider it likely that MB3 fulfills some additional essential functions that may not be evident in cultured cells *in vitro* - and the same might possibly hold true for other conserved domains as well.

Therefore, we set out to address the importance of the conserved domains for Myc function *in vivo* in the context of intact tissues. We used *Drosophila melanogaster*, which contains a single Myc gene (*diminutive* or *dm*) that is functionally interchangeable with its vertebrate counterparts (Gallant *et al.*, 1996; Schreiber-Agus *et al.*, 1997; Trumpp *et al.*, 2001; Benassayag *et al.*, 2005). Mutations in *Myc* impair growth, such that flies carrying hypomorphic *Myc* alleles (e.g. dm^{P0}) are delayed in their development and eclose as small adults (Johnston et al., 1999), whereas dm^{4} mutant flies (null for *Myc*) fail to grow as larvae and die within a few days of hatching (Pierce *et al.*, 2004; Pierce *et al.*, 2008; Steiger *et al.*, 2008). Conversely, overexpression of Myc promotes cellular growth (Johnston et al., 1999), endoreplication (Maines et al., 2004; Pierce et al., 2004), and apoptosis (De La Cova et al., 2004;

Montero et al., 2008). In the present study, we expressed wild type and mutant forms of Myc (carrying mutations in MB2, MB3, or the N-terminus) *in vivo*. These experiments revealed a strong requirement for the N-terminus for all Myc-dependent activities. Surprisingly, mutation of MB2 only weakened, but did not fully inactivate Myc function, such that an MB2-mutant form of Myc could rescue the survival of a sizable fraction of *Myc*-null mutant flies. Finally, deletion of MB3 only moderately affected several of Myc's activities *in vivo*.

2 Materials and Methods

2.1 Cloning and expression of Myc proteins

A cDNA for wild type Myc with a triple HA-epitope at its N-terminus (Bellosta et al., 2005) was cloned into a *pBS*-vector carrying a single ΦC31 *attB* site, 5 *UAS* repeats, a *hsp70* basal promoter, the SV40 polyA signal, and *mini-white* as a marker (Bischof et al., 2007). By site-directed mutagenesis the following mutant derivatives were created (numbering relative to untagged Myc): MycΔN: lacking amino acids 1 – 65; MycMB2A: containing "AAAA" instead of "DCMW"; MycΔMB2: "GP" substituted for residues 68-84; MycΔMB3: "F" substituted for residues 405-422. The same cDNAs were also cloned directly under the control of the α-*tubulin84B* promoter (see Bellosta et al., 2005) into an analogous *pBS*-vector lacking *UAS*- and *hsp70*-sequences. Full sequences are available upon request.

2.2 Drosophila *lines*

Transgenic flies were established by integrating the different *attB* plasmids into the *attP* landing site *zh-86Fb* (Bischof et al., 2007; http://www.frontiers-in-genetics.org/), and confirmed by PCR. Source of additional flies: GAL4 drivers (*Drosophila* stock center at Bloomington), *act-FRT-CD2-FRT-GAL4* (Neufeld et al., 1998), *GMR-FRT-w^+-FRT-GAL4* (Brogiolo et al., 2001), *tub-FRT-Myc-FRT-GAL4* (De La Cova et al., 2004), dm^{P0} (Johnston et al., 1999), dm^2 (Maines et al., 2004), dm^4 (Pierce et al., 2004), dm^4 mnt^1 (Pierce *et al.*, 2008), dm^{PL35} & dm^{PG45} (Benassayag et al., 2005).

2.3 Western blots & immunocytochemistry

To document transgene expression *in vivo*, 20 wing imaginal discs of uncrowded wandering larvae carrying one copy of *tub-HAMyc^{mut}* were lysed directly in Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting. Primary antibodies were: rabbit anti-HA epitope (Dunn Labortechnik GmbH), mouse anti-α-Tubulin (Sigma). Secondary antibodies were HRP-coupled anti-rabbit (GE

Healthcare) or anti-mouse (Jackson Immuno Research) and for detection the enhanced chemiluminescence kit (Amersham) was used.

For TUNEL staining of wing imaginal discs, wandering third instar larvae were fixed in 4% paraformaldehyde for 20', washed with 1x PBT (0.1% Tween-20 in PBS [130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4]) for 20', post-fixed in ethanol/PBS (2:1) for 5' at –20° C, washed with 3 changes of 0.1% Tween-20/PBS over 15', pre-treated with 10 mM sodium citrate (pH 6) for 30' at 70° C, equilibrated in Equilibration Buffer (ApopTag Red In Situ Apoptosis Detection Kit, Chemicon) for 10', then incubated with TdT enzyme (Chemicon kit) for 1 h at 37° C, Stop/Wash Solution (Chemicon kit) for 10', Rhodamine-coupled anti-DIG antibodies (Chemicon kit) for 30', and 3 changes of 0.1% Tween-20/PBS (containing 0.5 µg/ml Hoechst 33342 for the first step).

For clonal area measurements, larvae carrying the *act-FRT-CD2-FRT-GAL4* construct, *UAS-GFP* and the appropriate *UAS-HAMyc*^{mut} transgene, were heat-shocked for 10' at 37° C in a water bath (at 48h AED), dissected 73 h later (at the wandering stage), fixed in 4% paraformaldehyde for 20', and washed with 3 changes of PBT (containing 0.5 µg/ml Hoechst 33342 for the first step) over 60'.

Wing discs were dissected and mounted in Vectashield Mounting Medium (Vectashield), and photographed on a Leica DMRA compound microscope at a 10x ocular magnification and analyzed with Adobe Photoshop.

2.4 qRT-PCR

Larvae were grown under uncrowded conditions, heat-shocked at wandering stage for 2 h at 37°C in a water bath, collected 8 h later into liquid nitrogen, and stored at -80°. To extract RNA, 10 male whole larvae were homogenized in 1 ml TRIZOL (Invitrogen) with a Polytron tissue homogenizer, following the manufacturers instructions. Upon precipitation, RNA was redissolved in 20 µl RNAse-free water and analyzed for its integrity on a Bioanalyzer (Agilent). Genomic DNA was eliminated using the Turbo DNAse free kit (Ambion) and cDNAs were synthesized from 1 µg of template RNA using the Omniscript kit (Qiagen). Parallel control reactions containing only RNA provided templates for "-RT" samples.

Quantitative real-time PCR (qRT-PCR) reactions were performed in triplicates on an ABI7900 Real Time PCR Instrument (Applied Biosystems), using the SYBR Green PCR Master Mix (Applied Biosystems). Data were analyzed with SDS 2.0 software (Applied Biosystems) and Microsoft Excel, using the ΔΔCt method and the average of the expression levels of *actin5C*, *Rab6* and *Sec24* as internal reference for each biological sample, with the values for "MycWT" set to 100%.

Primer sequences: *Rab6*: 5'-TGCACGTGGCCAAGTCCTA & 5'-CAGCGAACGCGACTGCTA; *Sec24* 5'-CCACTCCCCTGCCATCCT & 5'-ACCCCAAACCCAGCAACA; *CG5033*: 5'-TAACCGCTCGGCTTTAATTCA & 5'-CCCTTGCTCTTGGAGAATGG. The remaining primers and the PCR conditions have been described (Steiger et al., 2008).

2.5 Analysis of adult flies

Flies were kept on standard *Drosophila* medium and test crosses performed in climate-controlled chamber at 25°.

Scanning electron microscope pictures were taken with a JEOL JSM-6360 LV microscope and a magnification of 180x. Flies were frozen at –20° C for at least one day, slowly defrosted at 0°C and directly used for electron microscopy. For determination of ommatidial size, the area of 20 centrally located ommatidia was determined from 5 flies of the same genotype using Adobe Photoshop. The same photomicrographs were also used to determine the total number of ommatidia per eye. Obvious fusions of two ommatidia were counted as two individual ommatidia.

To determine adult weights, 1-2 day adult flies were frozen at –20° C for at least one day, defrosted at room temperature and weighed on a Mettler Toledo MX5 micro balance.

3 Results

3.1 Transactivation potential of Myc mutants

To investigate the *in vivo* roles of the most highly conserved domains of Myc, we mutated or deleted MB2, MB3 or the entire N-terminus and tagged the different variants with a triple HA-epitope at their N-termini (Fig. 1A). Transgenes coding for the different constructs were established using the ΦC31 system for targeted transgenesis, thus ensuring equal mRNA expression levels for all mutants and eliminating potentially confounding integration site effects (Groth *et al.*, 2004; Venken *et al.*, 2006; Bischof *et al.*, 2007). Indeed, the different proteins were mostly expressed at similar levels (Fig. 1B), although repeated Western blots (n=12) revealed some variability in the levels of the different protein variants (with the ΔMB3 mutant occasionally [n=4] accumulating to higher levels than the other forms). As a first step to characterize the biological activities of these mutants, we monitored their ability to activate established endogenous Myc targets in a *Myc*-null mutant background. For this purpose, the transgenes were first combined with the *Myc* null allele dm^4, a "*tub-FRT-Myc-FRT-GAL4*" transgene (which drives ubiquitous expression of a *MycWT* cDNA and thereby rescues the lethality of the dm^4 allele; Bellosta *et al.*, 2005) and *hs-FLP*. Following a massive heat-shock of 2 hours at 37°, virtually all cells have eliminated the rescuing *Myc* cDNA and express GAL4 instead (Hulf et al., 2005), which in turn drives the expression of the UAS-controlled transgenes. Eight hours later (before any overt signs of apoptosis; data not shown), total RNA was isolated and analyzed for the abundance of *Myc* and selected target genes. By this time, the mRNA levels for Myc and its RNA polymerase 2 transcribed targets *Nnp1* and *CG5033* (Hulf et al., 2005) have significantly diminished, whereas the RNA polymerase 3 transcribed Myc targets $tRNA^{Leu}$ and *snoRNA U3* (Steiger *et al.*, 2008) show considerable perdurance (see Sup. Fig. 1A, which also provides an independent partial repeat of Fig. 1C below). Introduction of MycΔN leads to little activation of these genes above background (Fig. 1C, compare with the "-" sample expressing no transgene), but MycΔMB2 partially activates Pol 2 transcribed Myc targets; MycΔMB3 is almost as active as MycWT towards *Nnp1* and *CG5033*, but (like MycΔN and MycΔMB2) has strongly reduced activity towards the Pol 3

transcribed Myc targets. Similar effects were observed in S2 cells, where the transiently expressed MB2 and MB3 mutants were able to partially activate a Myc-dependent reporter, whereas MycΔN was inactive (Sup. Fig. 1B, C). Taken together, these data confirm that N-terminus and MB2 are important for Myc dependent transcription by Pol 2 and Pol 3 (consistent with published data for the vertebrate proteins), and they reveal that MB3 also has a role in transactivation, in particular for the activation of RNA polymerase 3. The molecular basis for this stronger effect on Pol 3 is unclear; the only Myc-interacting protein that is currently known to be specifically involved in Pol 3-dependent transcription in *Drosophila*, Brf (Steiger *et al.*, 2008), binds with similar efficiency to both MycΔMB3 and MycWT (data not shown).

3.2 Overexpression of Myc mutants in vivo

Myc proteins are important both for normal development and tissue homeostasis, when they are expressed at physiological levels, and in the etiology of tumors, where they are frequently overexpressed. While Myc's physiological activities can account for much of its potency as an oncoprotein, it is conceivable that Myc takes on additional, qualitatively different, functions when expressed at supra-physiological levels. For these reasons, we wanted to investigate the activities of our Myc mutants first in overexpression situations, and second in the presence of amorphic *Myc* alleles, where the ability of the mutant proteins to substitute for endogenous Myc could be probed.

First, we analyzed the ability of the different Myc mutants to promote growth upon overexpression. It has previously been shown that overexpression of MycWT under the control of *GMR-GAL4* strongly increases the size of adult ommatidia (Secombe *et al.*, 2007; Montero *et al.*, 2008; Steiger *et al.*, 2008); since this driver expresses GAL4 in eye imaginal discs from the late 3rd larval instar on, it mainly influences the final growth and differentiation phase of the eye cells, but not the proliferation of their precursors and the final number of ommatidia. Consistent with their transactivation abilities, the MB2 mutants are still able to promote growth, albeit to a lesser extent than MycWT, and MycΔN is inactive in this assay (Fig. 2A, B). Surprisingly, MycΔMB3 increases ommatidial size more than MycWT and additionally induces considerable roughness of the eye. Such a morphology (caused by a combination of

excessive growth and apoptosis) is also observed when MycWT is expressed at higher levels (Steiger et al., 2008), indicating that MycΔMB3 is more active than MycWT in this assay, as a consequence of increased specific activity and/or higher levels of MycΔMB3.

The different Myc mutants show similar growth-promoting abilities when over-expressed under the control of *ap-GAL4* in the dorsal compartment of wing imaginal discs. The resulting size difference between the dorsal and the ventral compartments results in a moderate (ΔMB2, MB2A) or strong (WT) bending down of the adult wings (Fig. 2C). MycΔN again shows no activity, even though this bending provides a very sensitive read-out for growth inducers (see Montagne et al., 1999). MycΔMB3 reveals other phenotypes (Fig. 2C) that can also be observed upon overexpression of a different, stronger MycWT transgene (e.g. dissociation of the dorsal and ventral wing surface, necrotic patches; data not shown), and that suggest that the Myc-induced excessive apoptosis overwhelms its growth-promoting ability. Indeed, TUNEL staining of 3rd instar larval wing discs (Fig. 3) reveals that all Myc proteins except for MycΔN induce apoptosis (as previously reported for MycWT; Montero *et al.*, 2008), but that MycΔMB3 clearly has the highest activity. Thus, like in the eye, the MycΔMB3 transgene is more active than MycWT in the wing.

The experiments described so far document the effects of the different Myc mutants on final adult tissue size. To demonstrate directly to which extent Myc proteins promote the rate of cellular growth, we expressed the different proteins in clones of cells in wing imaginal discs carrying an "*act-FRT-stop-FRT-GAL4*" cassette, a *hs-FLP* transgene and a *UAS-GFP* marker. Overexpression clones were induced by a 10' heat-shock at 37°, which drives the expression of FLP recombinase leading to the activation of GAL4 in a few random cells of the disc, and analyzed 73 hours later (after 6 to 7 cell doublings). The coexpressed GFP allowed for the identification of the clones; since larval wing discs consist essentially of a monolayer of undifferentiated cells, the areas of such clones provide a good measure for their volume increase. Consistent with published observations (Johnston et al., 1999), MycWT overexpression strongly promotes clonal growth (Fig. 4A, B), and so does MycMB2A. As expected, MycΔN does not affect the

growth rate of these clones. However, MycΔMB3 significantly reduces clonal area, suggesting that its powerful pro-apoptotic ability (see Fig. 3) overcomes the proliferative capacity of these clones.

Taken together, the overexpression studies demonstrate that the N-terminus is essential for Myc's ability to promote growth, whereas a mutation or deletion of MB2 only moderately impairs Myc activity; loss of MB3 increases the biological activity of Myc (possibly in part by affecting the levels of Myc protein).

3.3 Rescue of Myc mutant eyes by Myc protein mutants

Normal eye development requires Myc, and hypomorphic or null alleles of *Myc* result in smaller eyes, composed of smaller and (for lethal alleles) fewer ommatidia (Bellosta *et al.*, 2005; Steiger *et al.*, 2008; Fig. 5). Therefore the eye constitutes a good organ for analyzing the normal biological activities of Myc proteins. To reveal the phenotype of such *Myc* mutant eyes without lowering Myc activity in the rest of the body (which would interfere with organismal viability), we combined the rescuing transgene "*tub-FRT-Myc-FRT-GAL4*" with "*ey-FLP*", resulting in elimination of the *Myc* cDNA and concomitant expression of GAL4 in precursors of the eye and head capsule. The FLP-mediated replacement of Myc with GAL4 is highly efficient, as shown in Sup. Fig. 2. We used this system to express the different Myc variants in eyes that were wild type for Myc or carried the null allele dm^4. Importantly, transgene expression in this assay is maintained from early development to the terminal differentiation phase, and therefore affects both the final number and size of the resulting ommatidia.

All Myc mutants significantly increase ommatidial size as compared to control, with the effects being largest in the dm^4 background that lacks endogenous *Myc* altogether (Fig. 5A, B). Even MycΔN, which was inactive in all other assays, increases ommatidial size by 10% in a dm^+ background; the difference to the GMR-driven overexpression experiment shown above is presumably due to the different expression dynamics of the two GAL4 drivers [*GMR* is not expressed in all cell types of the differentiating eye (Ellis et al., 1993), but *tub* should be ubiquitously expressed; furthermore level and duration of GAL4 expression are likely to differ between the two systems]. The ΔN mutant also significantly increases the

number of ommatidia in dm^4 mutant eyes (Fig. 5C, compare "-" and "ΔN"), thus demonstrating its activity both during the earlier proliferative and the later differentiation phase. Note that we would not expect any Myc mutant to increase the number of ommatidia in dm^+ eyes, since Myc overexpression does not increase cell division rates in wild type cells (Johnston et al., 1999). Instead, expression of the other Myc forms slightly or significantly reduces the number of ommatidia in this assay, reflecting the pro-apoptotic activity of these proteins (Bellosta et al., 2005). As before, this effect is strongest for MycΔMB3, as can also been seen by the obvious roughness of the eyes expressing this mutant (Fig. 5A). Taken together, this experiment confirms the growth- and apoptosis-promoting capabilities of the different Myc mutants, and it shows that MycΔN also retains some activity.

3.4 Organismal rescue of Myc mutants

As a final, most stringent, functional assay we determined to which extent the different Myc mutants could drive the entire development. None of the Myc transgenes was able to rescue the lethal *Myc* alleles dm^2 or dm^4 when expressed under the control of the ubiquitous GAL4 drivers *arm*, *da*, or *tub*; presumably the resulting Myc expression was either too weak (with *arm*-GAL4) or too strong (when driven by *da-GAL4* or *tub-GAL4* in a wild type background both MycWT and MycΔMB3 caused dominant lethality). Efficient rescue of lethality (to more than 50%) was only observed with the enhancer trap allele dm^{PG45}, which is both mutant for *Myc* and expresses GAL4 in a *Myc*-like pattern (Benassayag *et al.*, 2005), in combination with MycΔN, MycΔMB2 or MycMB2A, indicating that Myc mutants that lack either the N-terminus or MB2 retain sufficient activity to complement the low levels of endogenous Myc in the dm^{PG45} mutant. Neither MycWT nor MycΔMB3 efficiently rescued dm^{PG45}, consistent with previous reports that a *UAS-MycWT* transgene does not rescue this allele but instead causes dominant lethality in a dm^{PG45}/dm^+ background (Benassayag *et al.*, 2005), presumably because the GAL4/UAS system amplifies the transcriptional output of the *Myc* locus to lead to excessive expression of the UAS-transgene. An analogous amplification might explain the lethality of a *MycWT* transgene when expressed under the control of a *tub-GAL4* driver, whereas a transgene expressing MycWT directly under *tubulin* control

causes no harm in flies and even rescues a *Myc* null mutant. Such considerations prompted us to generate a second series of transgenes, in which the expression of the different Myc mutants is controlled directly by *α-tubulin* regulatory sequences; these transgenes were integrated in the same chromosomal location as before using the ΦC31 system. All transgenes could be established, although *tub-MycΔMB3* showed some dominant lethality in a dm^{+} background and could not be maintained as a homozygous line. Both MycWT and MycΔMB3 rescued the lethality of dm^{4} mutant flies (Fig. 6A, B), although the resulting adults were smaller than control (Fig. 6A, C). Expression of MycΔN did not lead to any escapers, but much to our surprise, a substantial fraction of MycΔMB2 expressing dm^{4} flies (15% of the expected number) developed to term when 2 copies of the transgene were present and eclosed as normally patterned adults (Fig. 6). This fraction increases to 39% when the Myc antagonist Mnt is simultaneously mutated, i.e. in a dm^{4} mnt^{1} background, where Myc targets have been shown to be partially derepressed (Pierce et al., 2008). Thus, MB2 is not absolutely required to promote the full development from egg to adult, even in the absence of any endogenous Myc protein.

4 Discussion

4.1 Myc box 2

Myc proteins show a comparatively low overall sequence conservation between the most distant known family members from insects and vertebrates, but they all contain a short, highly conserved sequence motif, the Myc box 2 (MB2). The very fact that this domain is conserved suggests an indispensable function, and MB2 has indeed been shown to play an essential role in different tissue culture based assays. However, the variety of cell types that have been tested in tissue culture (most frequently fibroblasts), as well as the types of biological read-outs that have been used (typically proliferation, apoptosis or transformation), are both limited. As Myc interacts with many different transcriptional co-factors, we hypothesized that the proteins contacting MB2 might be important for the expression of only a subset of Myc's targets, or essential only in certain cell types, or required for only certain biological processes. By expressing MB2-mutant forms of Myc *in vivo*, we exposed the proteins to a variety of different contexts. We expected that the mutants would be inactive in most situations, but possibly retain some activity in a particular assay. Unexpectedly however, MB2 mutants showed an intermediate activity in all our assays (often close to that of MycWT). Most strikingly, MycΔMB2 was able to rescue mutant animals lacking all endogenous Myc, which would otherwise all have died during development. This finding suggests that MB2 does not contribute a qualitatively unique and essential function to Myc, but instead may act to enhance Myc's transcriptional output or modulate it in response to external signals. It further implies that no essential co-factor relies on MB2 for its interaction with Myc, either because no MB2-interacting protein is essential for Myc function, or because all MB2-interacting essential co-factors also contact Myc via additional redundant domains. At present we cannot address these possibilities experimentally, since no protein has been found to contact MB2 in *Drosophila*; two candidates we investigated (dGCN5 and Tip49/Pontin) bound Myc independently of the presence of MB2 (data not shown). Thus, we can only speculate about reasons for the partial dispensability of MB2; for example, the activation of a Myc target that normally relies on an MB2-dependent HAT might be partially taken over

by an MB2-independent HAT, or that such a Myc target gene might instead be activated by a histone acetylation-independent process, or that other targets might supplant the function of such genes. In either case, our observations provide an example of unexpected biological plasticity, considering the exceptional evolutionary conservation of this MB2.

4.2 N-terminus

By comparison, the elimination of the entire N-terminus has substantially more severe effects. Nevertheless, the MycΔN protein (which corresponds to the naturally occurring c-MycS variant in vertebrates) clearly retains some activity. Similar to human c-MycS (Benassayag et al., 2005), MycΔN can rescue the lethality of the dm^{PG45} allele; as dm^{PG45} is a hypomorphic (albeit lethal) *Myc* allele, some Myc activity must still be present in these flies and might cooperate with MycΔN in this rescue. However, MycΔN also significantly enhances the size and number of ommatidia that lack *Myc* altogether (Fig. 5), demonstrating that this mutant has the ability to stimulate cellular growth and proliferation on its own (although, like c-MycS, it cannot induce apoptosis – Fig. 3). This growth effect is comparatively weak and not seen in other overexpression experiments, though, essentially confirming studies of vertebrate Myc that have assigned an important biological role to this part of the protein.

4.3 Myc box 3

The function of the second highly conserved domain in Myc, MB3, is more ambiguous. MB3 resembles PEST motifs, but its deletion has no dramatic effect on Myc levels under normal culture conditions, suggesting that other evolutionary constraints are imposed on this domain. Indeed, the MycΔMB3 mutant has a lower transactivation potential than MycWT, in particular towards Pol3 transcribed genes, raising the possibility that this domain contacts some co-activators (that remain to be identified). In light of its reduced transcriptional activity, it is surprising that MycΔMB3 is more potent than MycWT in inducing apoptosis (in the developing wing) and growth (in the differentiating eye). At present we can only speculate that the deletion of MB3 might differentially affect the expression of certain Myc targets (as suggested by the stronger defect in activating Pol3 targets), and that certain Myc targets that are important

for the terminal size increase of ommatidia are still activated by this mutant (or that their expression is even enhanced at the expense of other targets). Such an "unbalanced" activation of Myc targets might also poise certain cells towards an apoptotic fate. In addition, we cannot exclude the possibility that a slightly increased stability of the MycΔMB3 mutant may also contribute to its increased *in vivo* activities. Finally, it should be noted that the sequence coding for MB3 straddles the junction between exons 2 and 3 in all *Myc* genes (Fig. 1A), raising the possibility that some splice signals or another unknown structure of the Myc gene contribute to the evolutionary maintenance of MB3 – or alternatively, that the conservation of MB3 sequences has concomitantly fixed the exon boundaries in *Myc*.

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6 References

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Figure legends

Figure 1. Transcriptional activity of Myc mutants. ***A****,* schematic representation of the Myc mutants used in this work. All constructs contain an N-terminal triple HA-tag, with the coordinates referring to the untagged Myc. The MB2 and MB3 sequences shown on the top are derived from human c-Myc (upper line) and *Drosophila* Myc (lower line); the dots in c-Myc represent a 6 residue acidic stretch missing in *Drosophila* Myc, and the vertical line shows the junction between exons 2 and 3 that is conserved in *Myc* genes. Uppercase letter designate identically conserved amino acids. In MycMB2A, the amino acids "DCMW" are replaced by "AAAA". ***B***, expression level of the HAMyc variants in wing imaginal discs. Wing imaginal discs of third instar larvae of the genotype "*y w; tub-HAMyc*" were collected at 5 days after egg deposition (AED) and processed for Western blot. Each lane contains the equivalent of 20 wing imaginal discs. The same blot was probed with anti-HA antibodies (top panel) to reveal HAMyc and with anti-α-Tubulin antibodies (bottom panel) as a loading control. ***C***, expression of endogenous Myc targets in *Myc*-mutant larvae in response to the indicated transgenes. The *Myc* null mutation dm^4 was exposed, and the transgene expression triggered by a heat-shock. Shown are the averages (± standard deviations) of two biologically independent samples (except MycΔN: only one sample). Genotypes in C are "*w* dm^4 *tub>Myc>GAL4 hs-FLP*/Y; X", where "X" corresponds to an empty vector transgene (sample "-") or a $UAS\text{-}HAMyc^{mut}$ transgene as indicated.

Figure 2: Effect of Myc overexpression on ommatidial size and wing shape. ***A***, scanning electron micrographs of representative eyes containing single *UAS*-transgenes with the indicated Myc mutants under the control of *GMR-GAL4*. The scale bars correspond to 100 μm. ***B***, average ommatidial area (± standard deviation) for the indicated genotypes (corresponding to the eyes in panel A), calculated from 20 centrally located ommatidia from 5 independent eyes per genotype. "**" indicates genotypes whose ommatidia are significantly larger than "-" ommatidia with $p<0.01$. ***C***, representative frontal views of adult wings of flies heterozygous for *ap-GAL4* (driving GAL4 expression in the dorsal wing compartment) and one *UAS*-transgene coding for the indicated Myc version.

Figure 3: Induction of apoptosis by overexpressed Myc. TUNEL-staining of representative third instar larval wing imaginal discs carrying one copy of *ap-GAL4* and one *UAS*-transgene coding for the indicated Myc version. The TUNEL-positive region in the panel showing a ΔMB3 wing disc provides a good reflection of the GAL4 expressing domain.

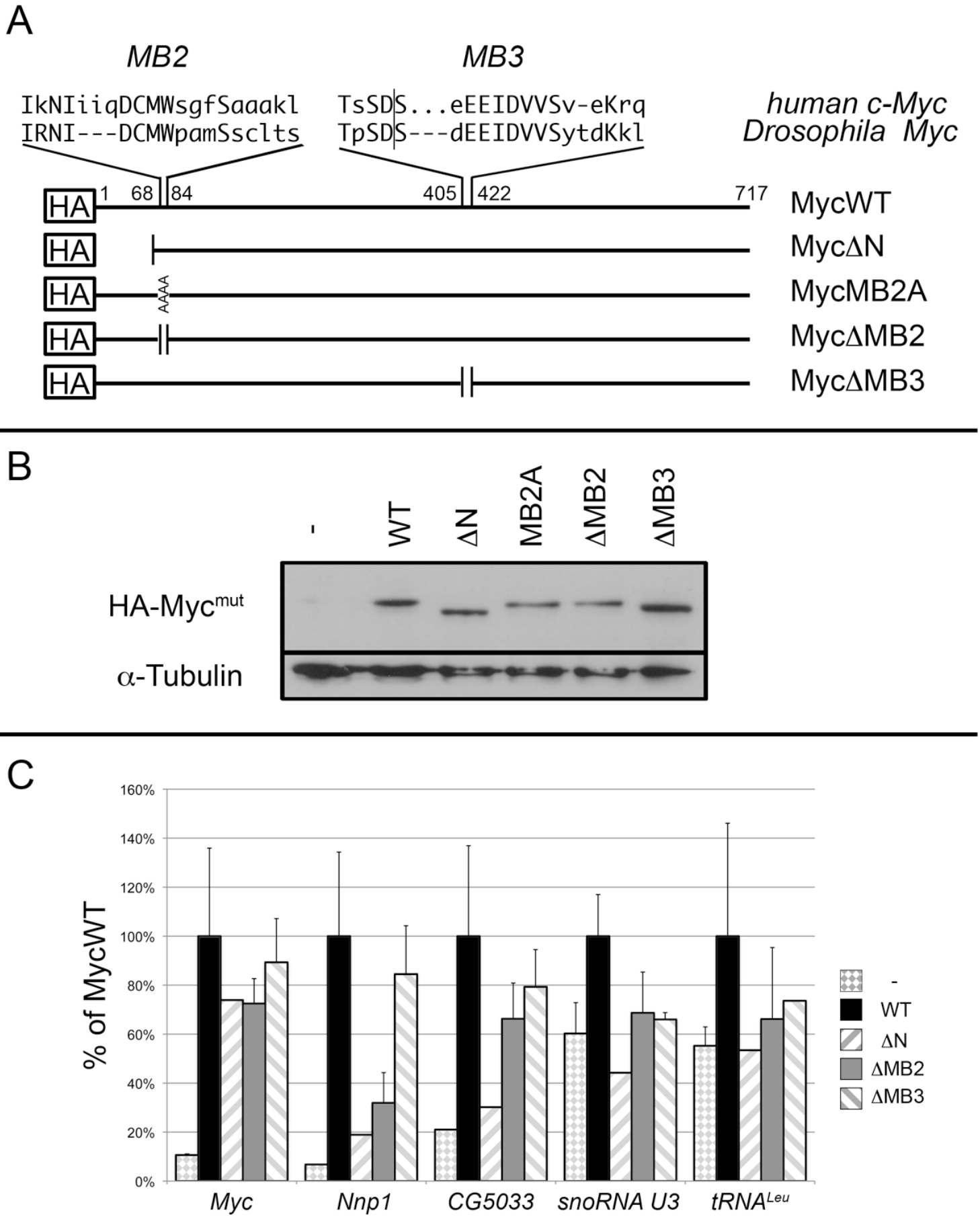
Figure 4: Stimulation of clonal growth by overexpressed Myc. ***A***, third instar larval wing imaginal discs with typical 73 hours old clones (marked in bright white) expressing GFP and the indicated Myc variant. To reveal nuclei, the discs have been stained with Hoechst 33342 dye. ***B***, average clonal area from discs corresponding to the genotypes shown in panel A. Number of analyzed clones (in parentheses the corresponding genotype): 50 (-), 45 (WT), 42 (ΔN), 53 (MB2A), 40 (ΔMB3).

Figure 5: Effects of continuous Myc expression on ommatidial size and number. ***A***, scanning electron micrographs of representative eyes of the indicated genotype. Sex chromosomes are "*y w tub-FRT-Myc stop-FRT-GAL4 ey-FLP/Y*" (labeled "dm^+" on the left) or "*w* dm^4 *tub-FRT-Myc stop-FRT-GAL4 ey-FLP/Y*" (labeled "dm^4"); all flies are males, i.e. contain no additional dm^+ allele; in addition, these flies carry one *UAS*-transgene coding for the Myc variant indicated below the picture. ***B***, average ommatidial area (± standard deviation) for the indicated genotypes (corresponding to the eyes in panel A), calculated from 20 centrally located ommatidia from 5 independent eyes per genotype. The reference genotypes for the "dm^+" and the "dm^4" series are "dm^+; -" and "dm^4; -", respectively; note that "dm^4; -" ommatidia are 23% smaller than "dm^+; -" ommatidia. All areas are significantly larger than the corresponding "-" control with $p<0.01$ (Students t-test), except for "dm^+; ΔN" where $p=0.03$. ***C,*** average ommatidial number (± standard deviation) for the indicated genotypes (corresponding to the eyes in panel A). Asterisks indicate significance of difference to the corresponding "-" genotype, with $p<0.05$ (*) and $p<0.01$ (**).

Figure 6: Rescue of a *Myc*-null mutation by the expression of Myc protein mutants under the direct control of the *tubulin*-promoter. ***A***, dissecting microscope pictures of rescued *Myc*-null mutant adult males expressing two copies of the indicated transgenes. ***B***, relative survival rate for the indicated genotypes (the expected number of "*w* dm^4/*Y*" or "*w* dm^4 $dmnt^1$/Y" males carrying one or two copies of

the indicated "*tub-HAMyc*^{mut}" transgenes was calculated from the number of recovered *dm*^{4}/*dm*$^{+}$ heterozygous females from the same cross, and set to 100%). For each genotype 300-800 flies in total were scored (except for *w dm*^{4}/Y; *tub-HAMycΔMB3/+:* 91 flies, *w dm*^{4} *dmnt*^{1}/Y; *tub-HAMycΔN*: 64 flies, *w dm*^{4} *dmnt*^{1}/Y; *tub-HAMycWT*: 229 flies), of which rescued *Myc*-mutant made up one quarter (one eight for the *tub-HAMycΔMB3* transgenes). ***C***, average weight of at least 13 male flies per genotype (except *dm*^{4}/Y; *tub-HAMycΔMB3/+*: 8 flies); the error bars indicate standard deviation. The control genotype is "*y w*".

Figure 1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MB3
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human c-Myc
Drosophila Myc
HA
1
68
84
405
422
717
AAAA
MycWT
MycΔN
MycMB2A
MycΔMB2
MycΔMB3
B
-
WT
ΔN
MB2A
ΔMB2
ΔMB3
HA-Myc^mut
α-Tubulin
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120%
100%
80%
60%
40%
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WT
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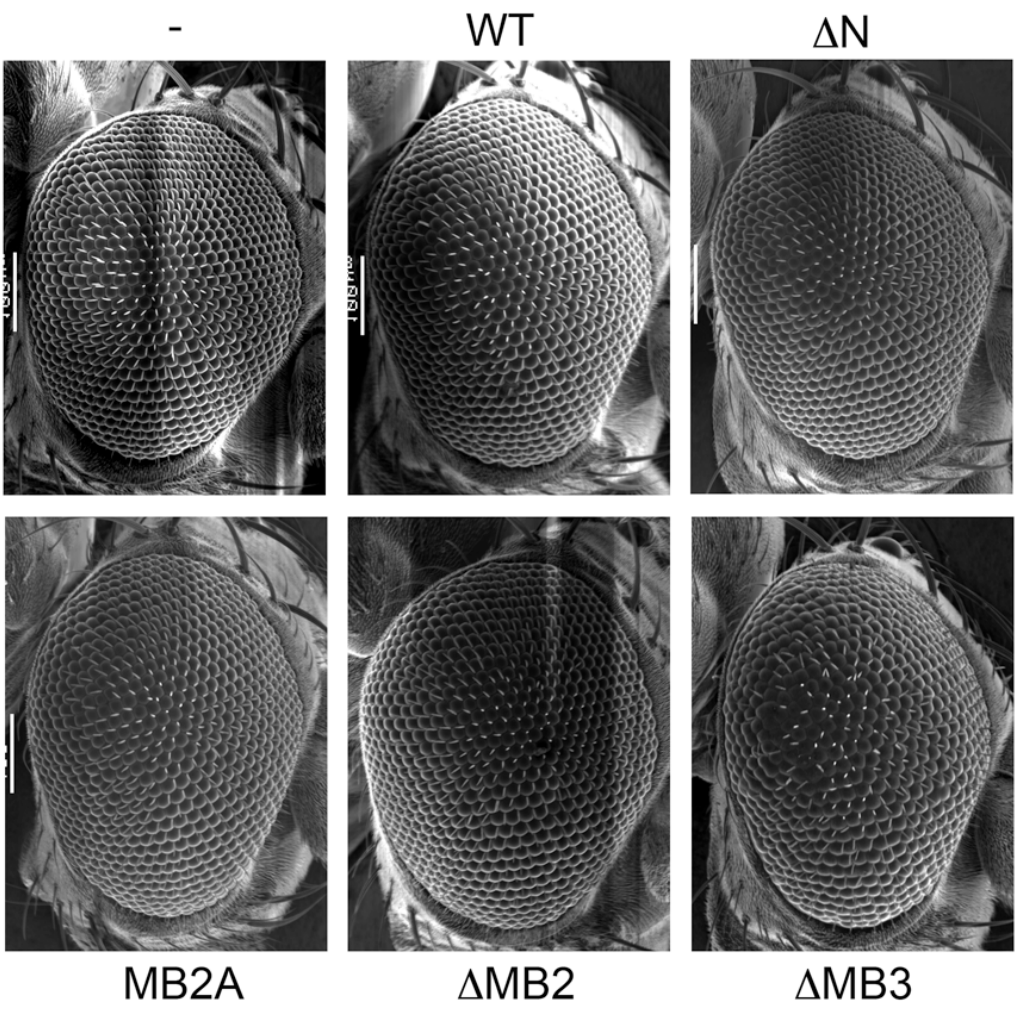
ΔMB2
ΔMB3

Figure 2

A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B

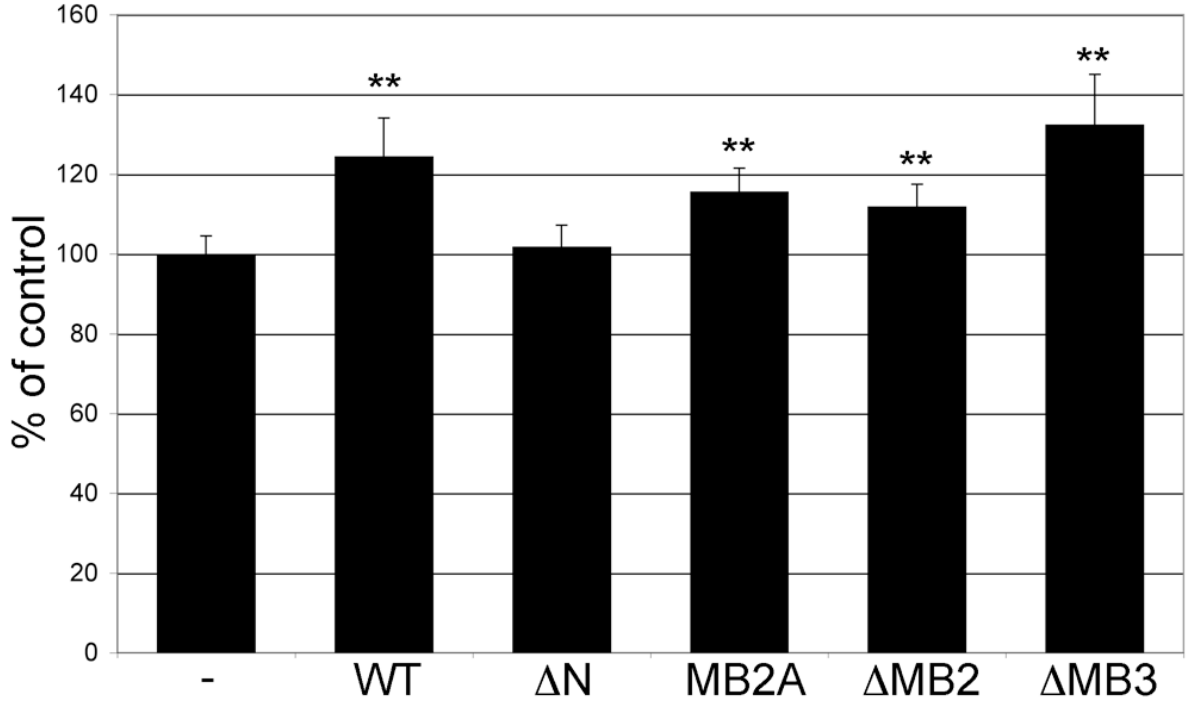
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Figure 2

C

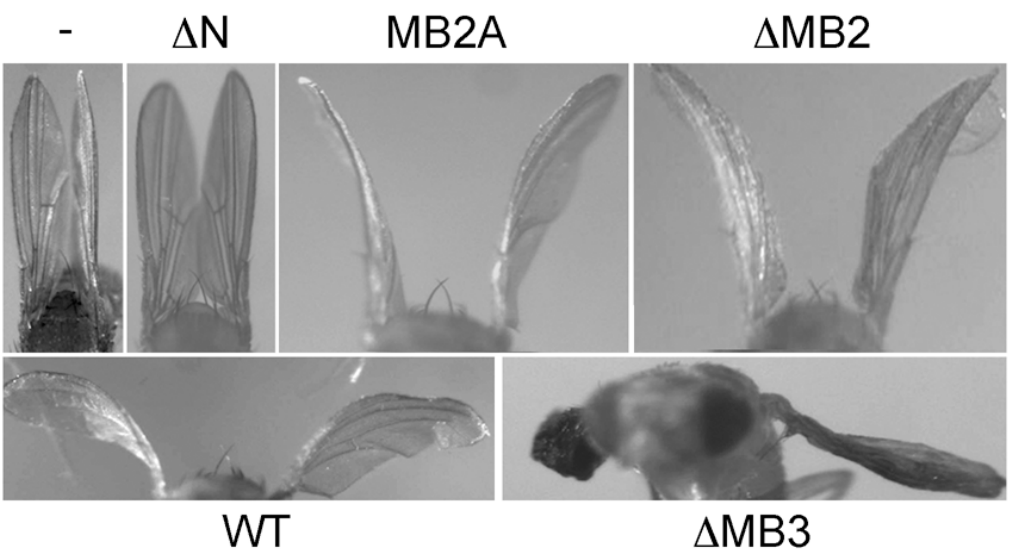


Figure 3

A

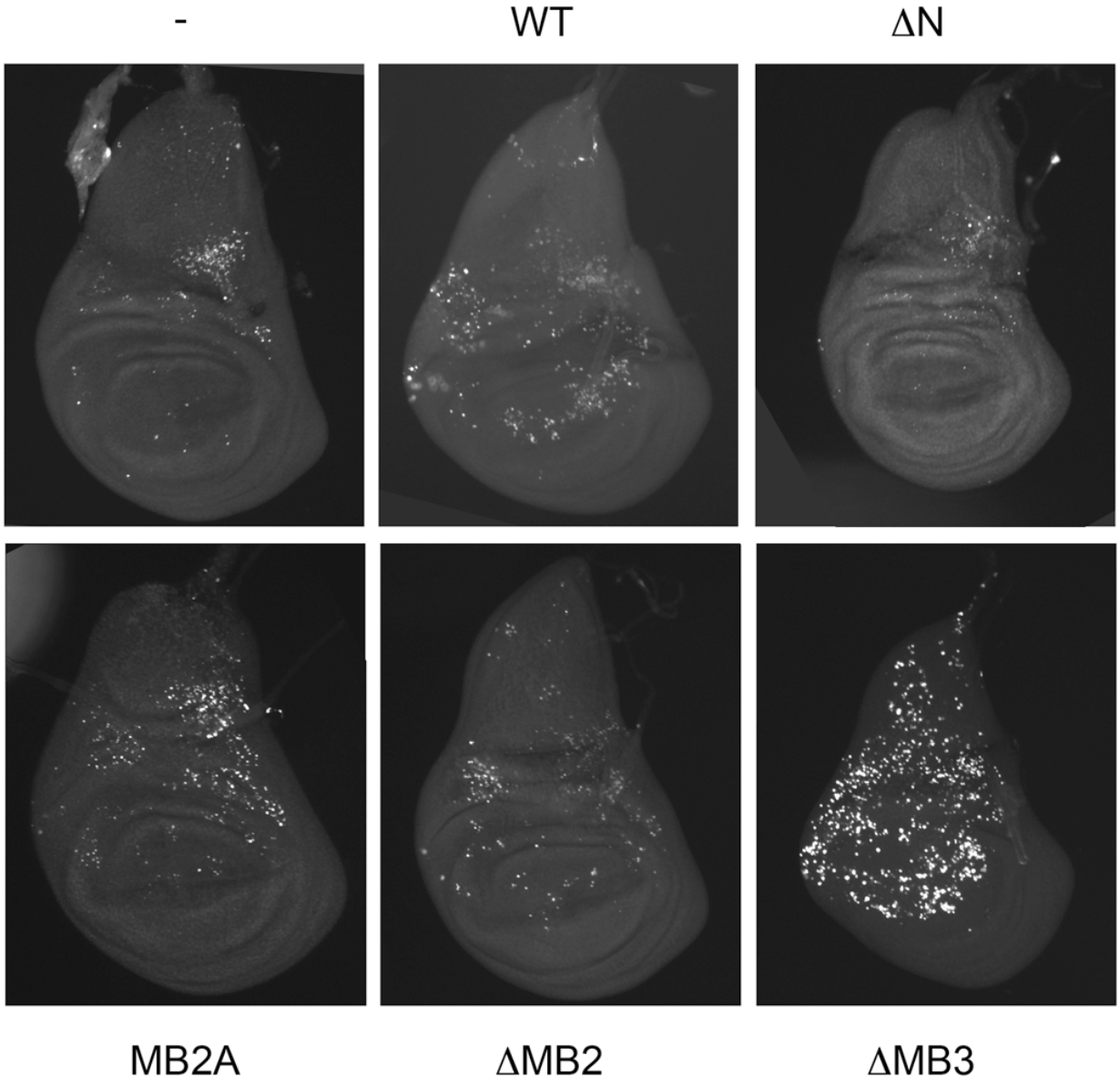
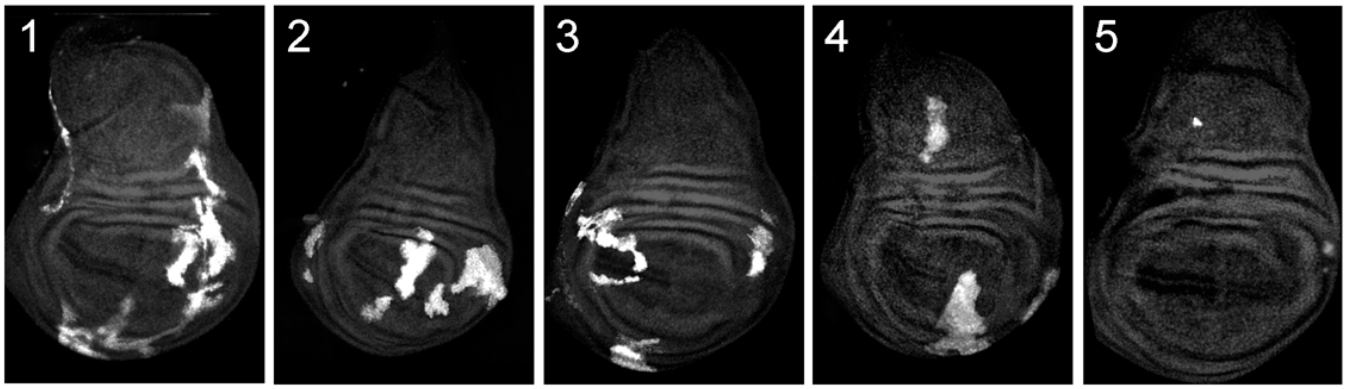


Figure 4

A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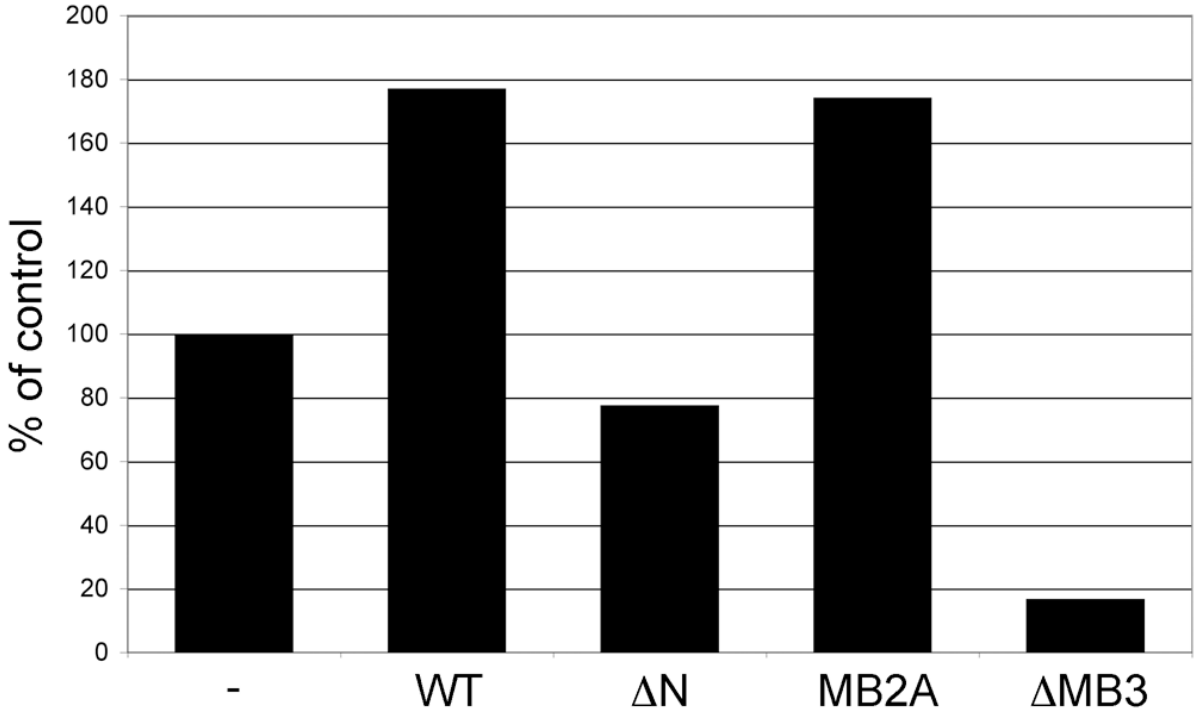
B


% of control
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MB2A
ΔMB3

Figure 5

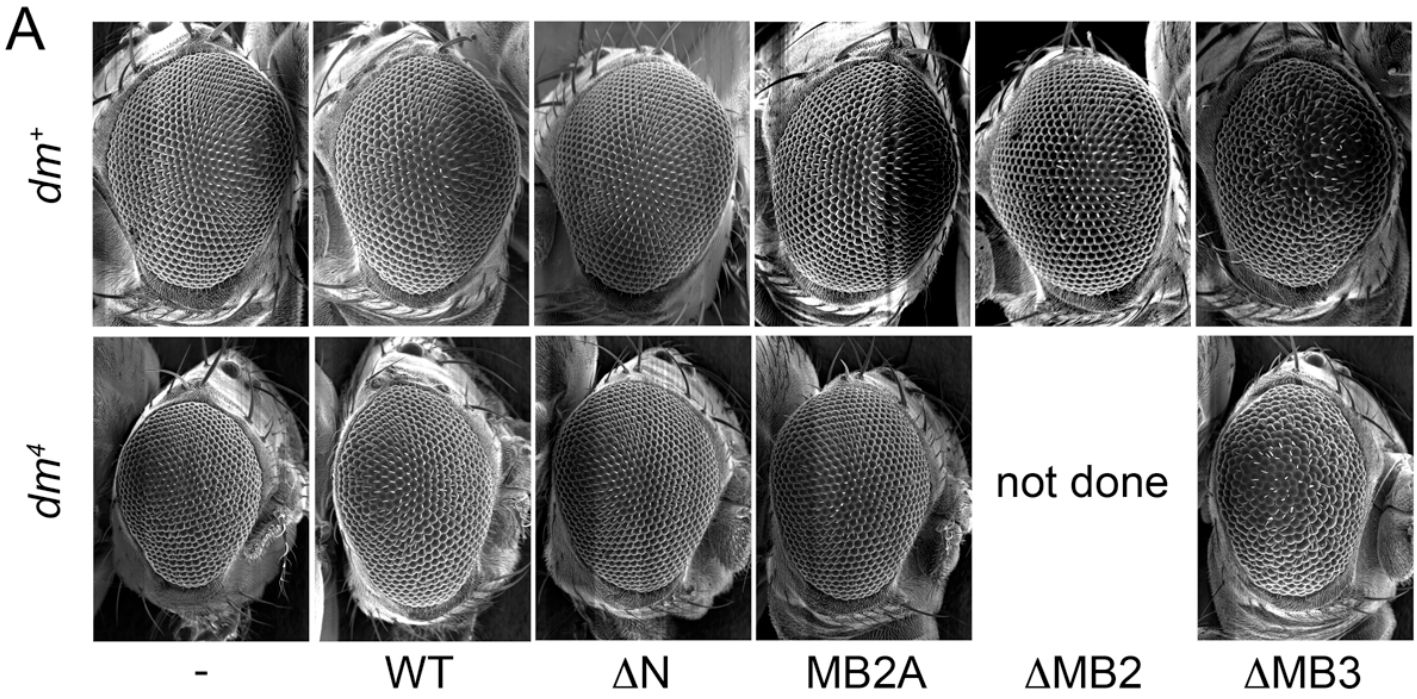
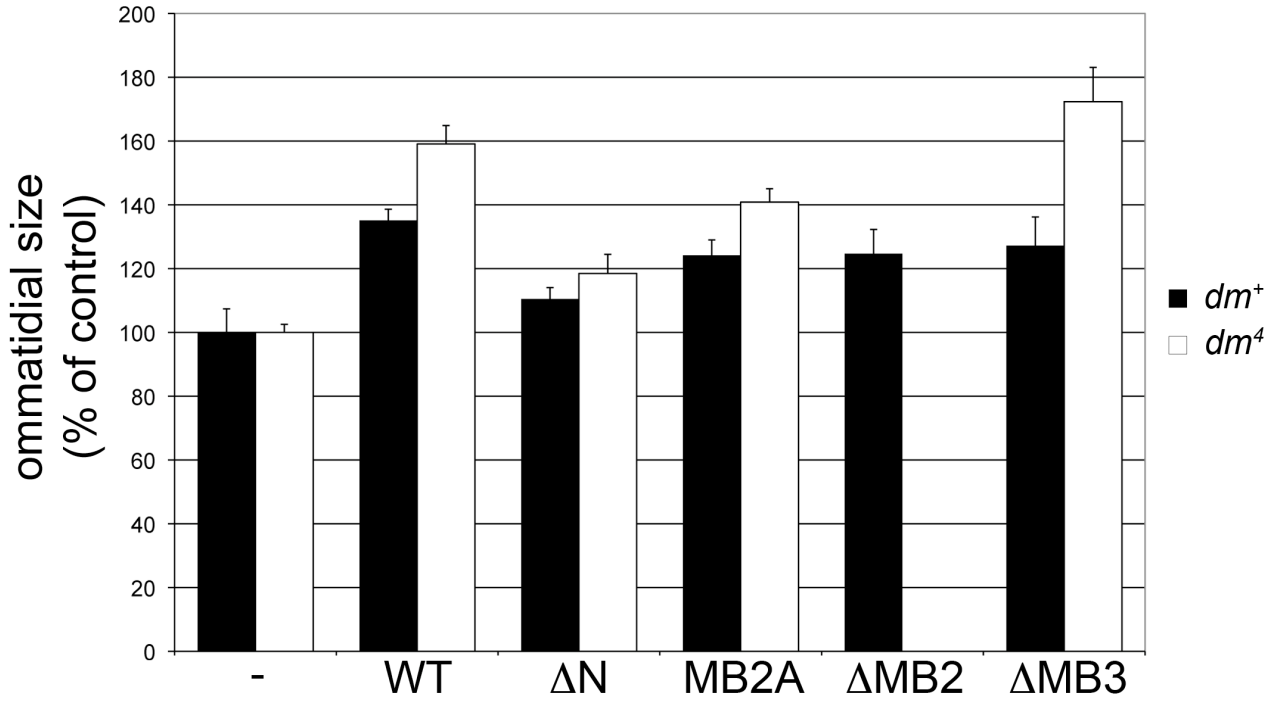


Figure 5

B


ommatidial size
(% of control)
200
180
160
140
120
100
80
60
40
20
0
-
WT
ΔN
MB2A
ΔMB2
ΔMB3
dm+
dm4

C

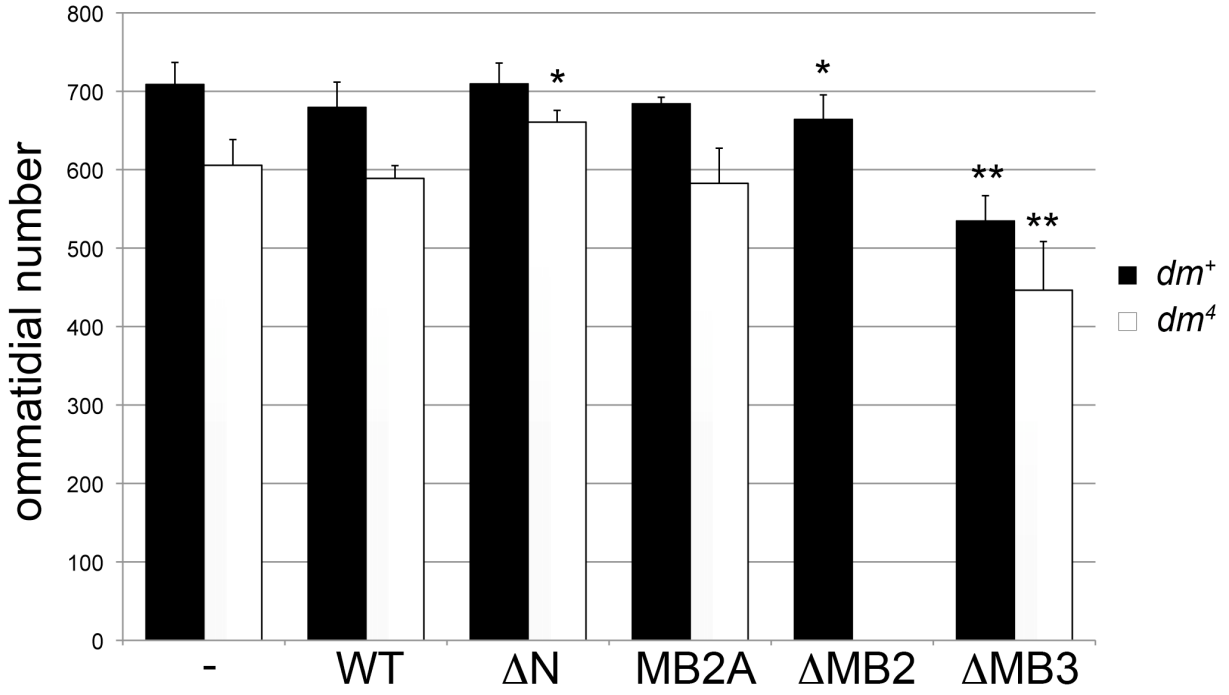
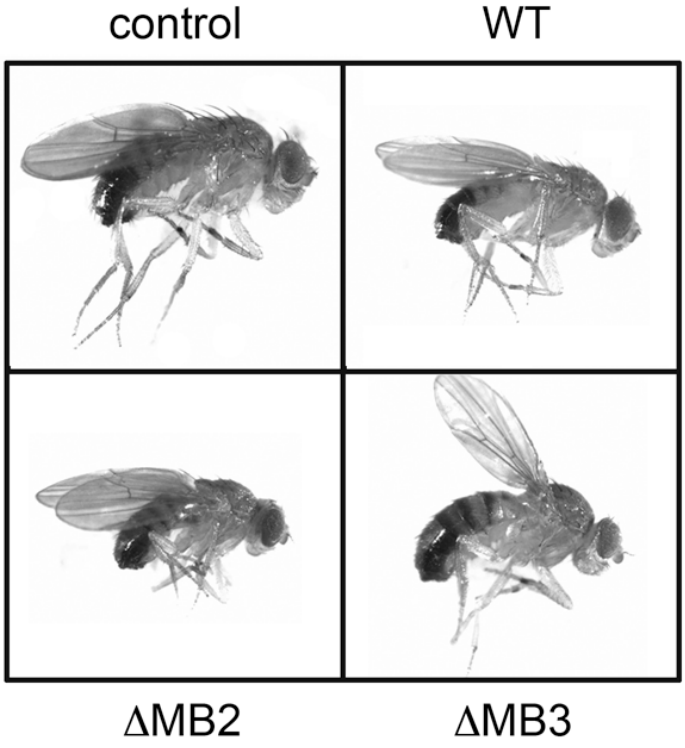

ommatidial number
800
700
600
500
400
300
200
100
0
*
*
**
**
-
WT
ΔN
MB2A
ΔMB2
ΔMB3
dm+
dm4

Figure 6

A


control
WT
ΔMB2
ΔMB3

B

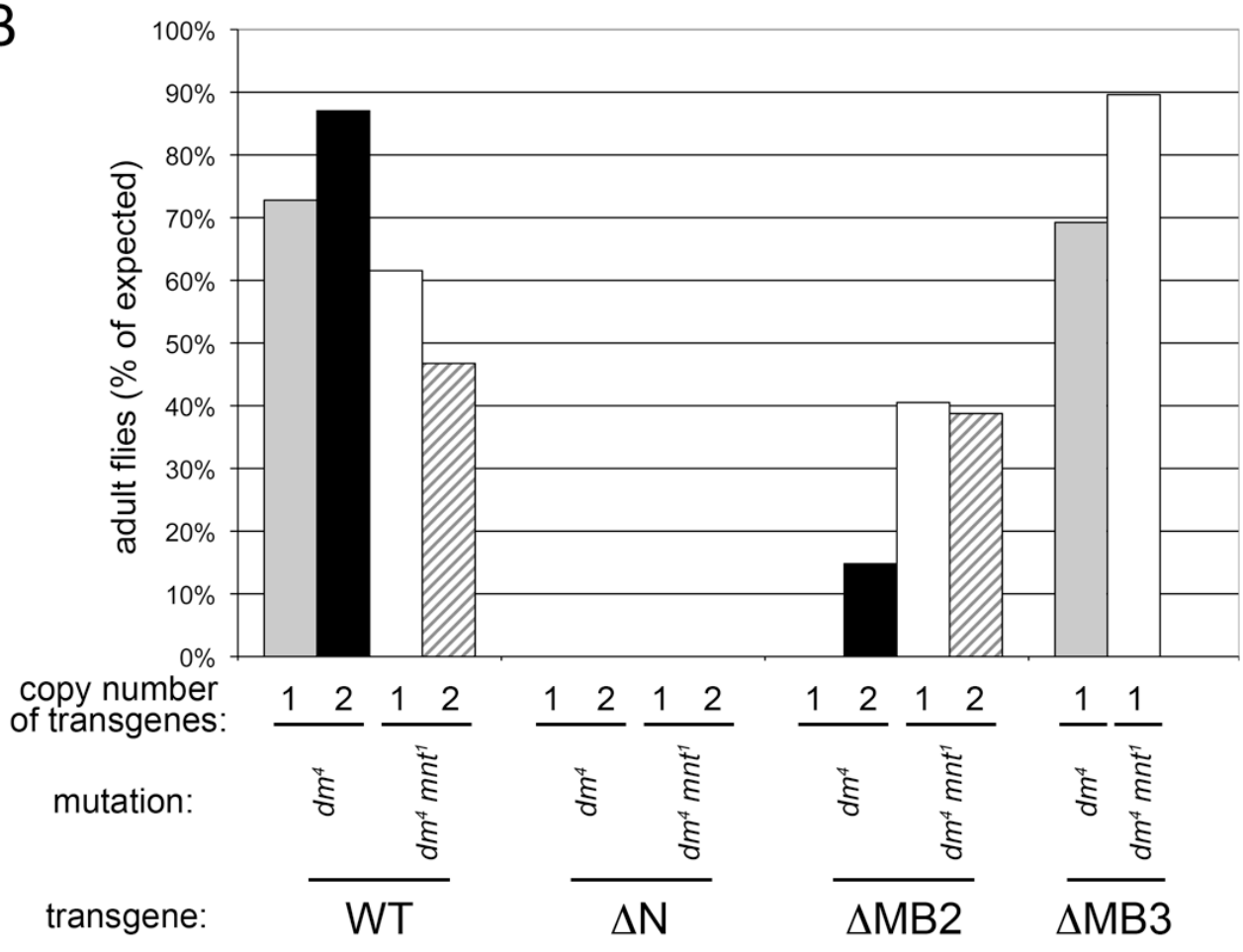
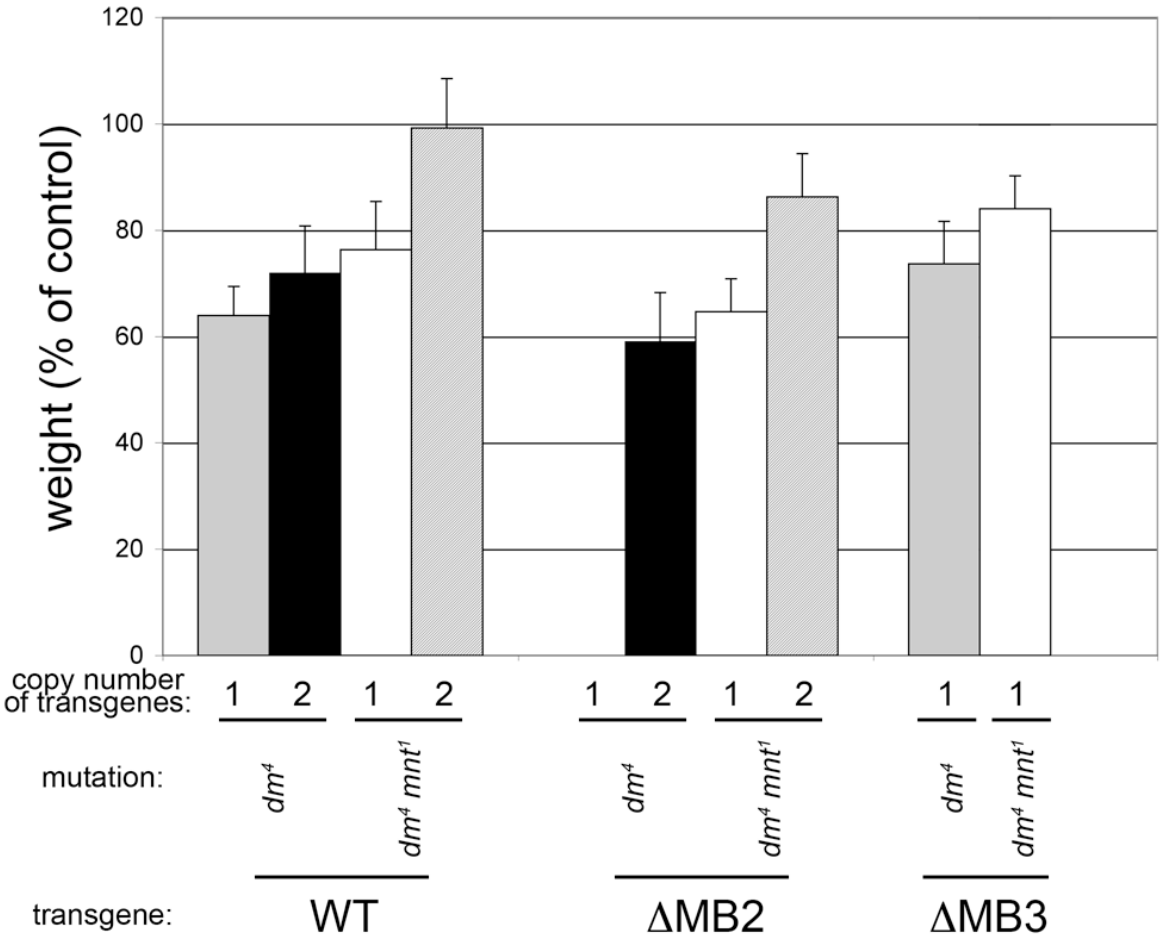

adult flies (% of expected)
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
copy number of transgenes:
1 2 1 2 1 2 1 2 1 2 1 2 1 1
mutation:
dm4
dm4 mnt1
transgene:
WT
ΔN
ΔMB2
ΔMB3

Figure 6

C


120
100
80
60
40
20
0
weight (% of control)
copy number of transgenes:
1 2 1 2 1 2 1 2 1 1
mutation:
dm4
dm4 mnt1
dm4
dm4 mnt1
dm4
dm4 mnt1
transgene:
WT
ΔMB2
ΔMB3

Supplemental Methods

Drosophila S2 cells were cultured at 24°C in 1x Schneider`s *Drosophila* medium (Gibco/BRL), supplemented with 10% fetal bovine serum, 1% Penicillin/Streptomycin. At 48 h after transfection, cells were harvested, washed with 1xPBS, lysed in Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting.

Reporter assays were carried out as previously described (Hulf et al. 2005). Briefly, $1.3x10^6$ cells/well of a 24-well cell culture plate were transfected with 0.65 ml serum-free medium containing 4.2 µl cellfectin (Invitrogen), 0.08 µg dsRNA targeting the 5' UTR of *Myc* (not recognizing the HAMyc-constructs), and 2.6 µg plasmid DNA (consisting of a 1:1 mix of "*CG5033*^{wt}*-Renilla* luciferase" and "*CG5033*$^{\Delta Ebox}$-firefly luciferase" reporters, *tub-GAL4* and *UAS*-plasmids). After 15-16 h, 0.65 ml complete media was added; 24h later, cells were washed in 1xPBS, lysed in 1x Passive Lysis Buffer (Dual-Luciferase kit, Promega), and relative reporter expression determined on a Wallac luminometer.

Supplemental figure legends

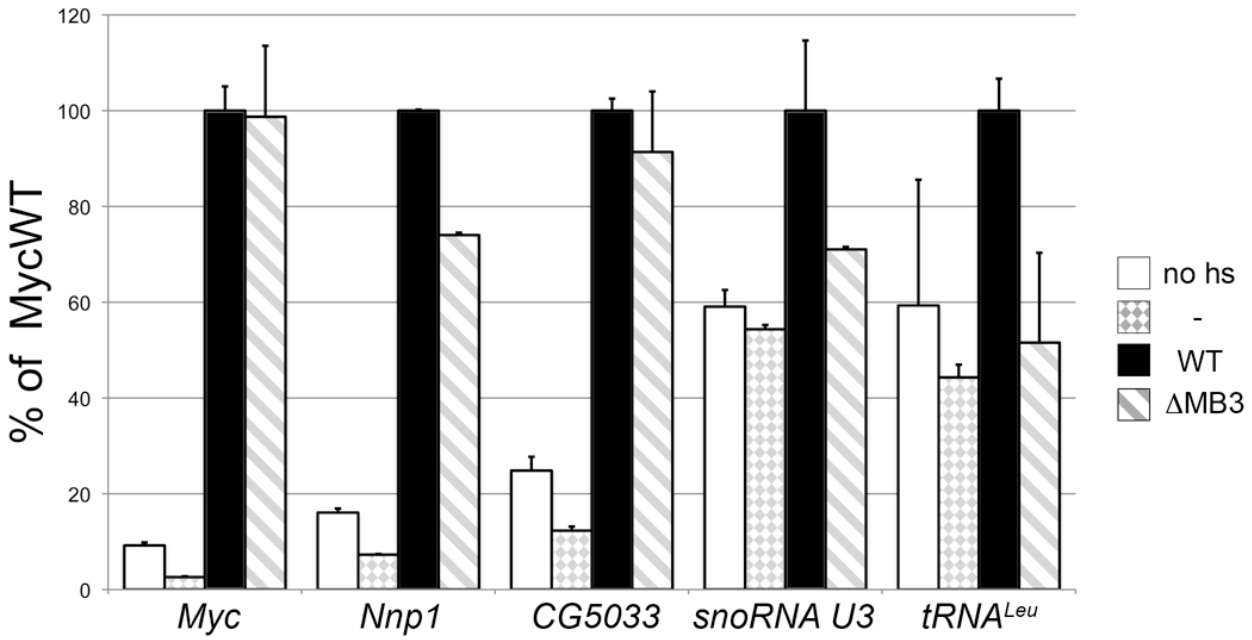
Supplemental figure 1: Transcriptional activity of Myc variants in S2 cells and *in vivo*. *A*, expression of endogenous Myc targets in *Myc*-mutant larvae in response to the indicated transgenes. Analogous experiment to the one shown in Fig. 1C (shown are averages ± standard deviations of two additional biologically independent samples). The "no hs" samples contain *UAS-HAMycWT* but did not receive any heat-shock; the other samples are as indicated in the legend to Fig. 1C. ***B***, Western blot of *Drosophila* S2 cells transiently transfected with *UAS*-plasmids coding for the indicated HAMyc variants together with a *tub-GAL4* plasmid. The cells were lysed at 48 hours after transfection. The same blot was probed with anti-HA antibodies (top panel) to reveal HAMyc and with anti-α-Tubulin antibodies (bottom panel) as a loading control. ***C***, Effect of Myc variants on a reporter gene in S2 cells. Endogenous Myc was down-regulated with RNAi in S2 cells and the indicated Myc mutants were expressed instead. The graph shows the relative activity of the co-transfected luciferase reporter (*Renilla* luciferase activity, controlled by the wild type *CG5033* promoter, divided by firefly luciferase activity, controlled by a

mutant *CG5033* promoter lacking the sole Myc binding site), with the relative activity of MycWT set to 100%; shown are the averages (± standard deviations) of 2 independent transfections from a representative experiment.

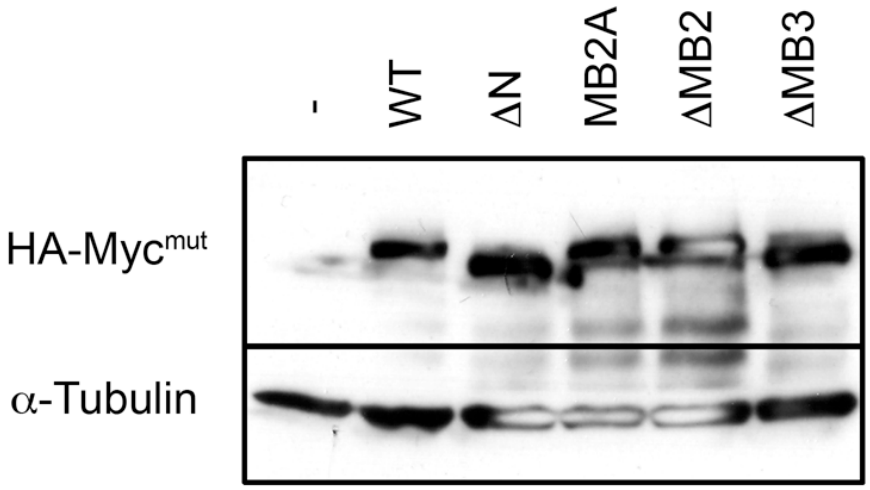
Supplemental Figure 2: Efficiency of the *ey*-FLP system. Red ommatidia in either panel retain the "*FRT-w$^+$ stop-FRT*" cassette and express the rescuing Myc cDNA, whereas the white ommatidia lack this cassette and the Myc cDNA, and hence expose the *dm^4* mutant. This picture also reveals the eye size reduction caused by the loss of *Myc*. Genotypes: 1, "*w dm^4 tub-FRT-Myc stop-FRT-GAL4 ey-FLP/Y*; *GMR-FRT-w$^+$ stop-FRT-GAL4/+*"; 2, "*w dm^4 tub-FRT-Myc stop-FRT-GAL4 hs-FLP/Y; GMR-FRT-w$^+$ stop-FRT-GAL4/+*".

Figure S1

A



B



C

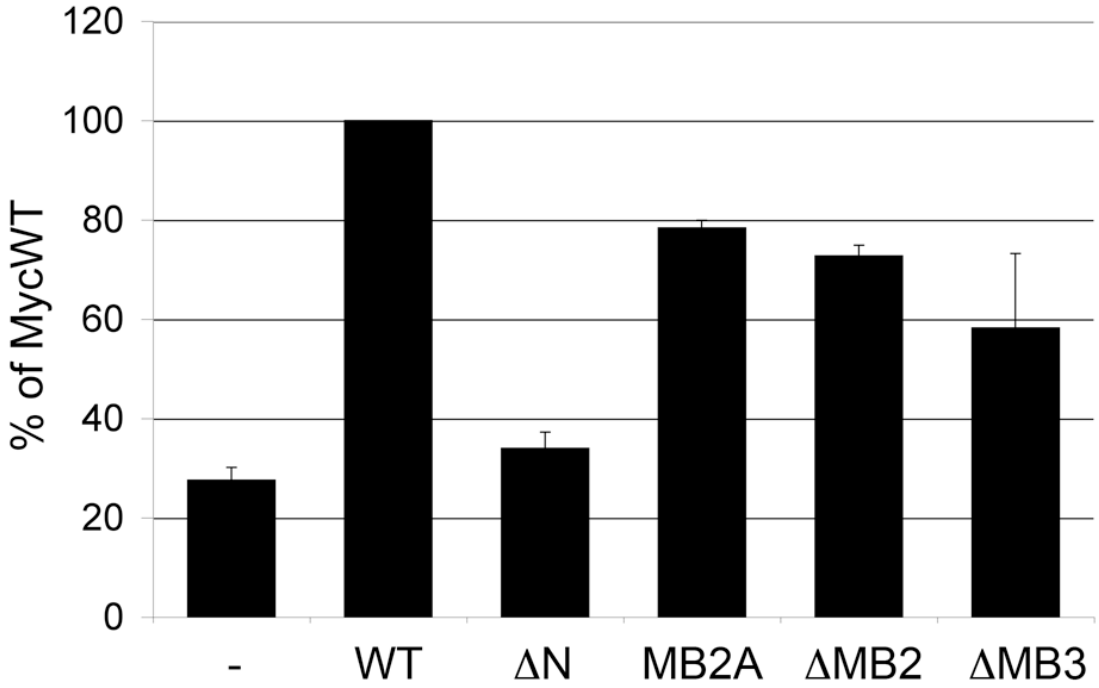
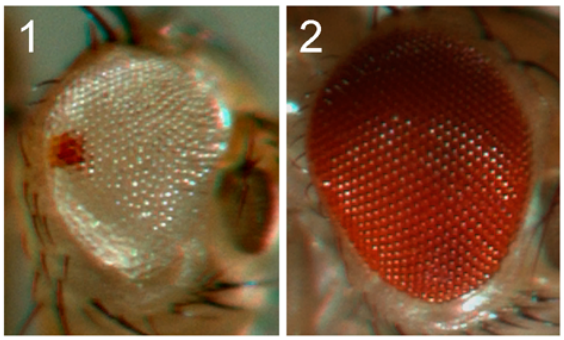


Figure S2

A



ey>dm⁴ *wt*