Induction of apoptosis by Drosophila *Myc*

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Abstract

Myc proteins are essential regulators of cellular growth and proliferation during normal development. Activating mutations in *myc* genes result in excessive growth and are frequently associated with human cancers. At the same time, forced expression of Myc sensitizes vertebrate cells towards different pro-apoptotic stimuli. Recently, the ability of overexpressed Myc to induce cell-autonomous apoptosis has been shown to be evolutionarily conserved in *Drosophila* Myc (dMyc). Here, we show that dMyc induced apoptosis is accompanied by the induction of *Drosophila* p53 mRNA, but that dp53 activity is not essential for dMyc's ability to induce apoptosis. Conversely, larvae carrying a hypomorphic *dmyc* mutation are more resistant to the apoptosis-promoting effects of X-irradiation. These data suggest that the control of apoptosis is a physiological function of Myc and that dMyc might play a role in the response to DNA damage.

Keywords: Myc / p53 / apoptosis / growth / X-rays / DNA damage / *Drosophila*

Introduction

Mutations in *myc* genes are amongst the most frequently found lesions in human cancer. The resulting deregulation of Myc expression promotes different cellular processes, including most prominently cell cycle progression, cellular growth and apoptosis (Oster *et al.*, 2002; Meyer *et al.*, 2006). The ability of a powerful proto-oncogen such as Myc to induce apoptosis has been hypothesized to serve as a protective anti-tumor function: mutations leading to increased Myc activity would result in apoptotic elimination of mutant cells, rather than allowing them to proliferate and form a tumor (Lowe et al., 2004). The importance of this control mechanism is underlined by experiments where the pro-apoptotic abilities of overexpressed Myc are blocked: co-expression of the anti-apoptotic protein Bcl-2 with c-Myc in pre-B cells of transgenic mice dramatically accelerates the process of tumorigenesis as compared to expression of c-Myc alone (Strasser et al., 1990). Similarly, ectopic expression of c-Myc in the pancreas of transgenic mice results in massive apoptosis and almost eliminates that organ, but co-expression with Bcl-X_L leads to rapid transformation (Pelengaris et al., 2002). These observations also imply that, during the process of Myc-induced transformation, the Myc-dependent apoptosis has to be overcome by mutations in other pro- or anti-apoptotic genes. Indeed, an impairment of the pro-apoptotic p53 pathway is a frequent event in Myc-induced transformation; conversely, mutations in p53, or in upstream components, function similar to Bcl-2 overexpression and co-operate with c-Myc overexpression to accelerate transformation (reviewed by Meyer et al., 2006).

Several pathways have been proposed to mediate the pro-apoptotic activities of overexpressed Myc. The best-studied example involves the tumor suppressor p53. Myc can increase p53 levels both directly (Roy et al., 1994) and via the DAP kinase – $p19^{ARF}$ axis (Zindy *et al.*, 1998; Raveh *et al.*, 2001), whereby $p19^{ARF}$ stabilizes p53 protein by inhibiting the p53-directed E3 ligase MDM2 (reviewed in Sherr, 2001). p53 then induces the transcription of various pro-apoptotic genes (reviewed in Vousden and Lu, 2002). In addition, several other proteins have also been implied in Myc-triggered apoptosis, such as Cytochrome C, CD95 / Fas, Bax, Bim, mtCLIC (reviewed by Meyer et al., 2006).

While the ability of over-expressed Myc to induce apoptosis is well documented, less is known about the involvement of Myc proteins in apoptotic processes during normal development. In a limited number of studies, cultured vertebrate cells treated with anti-sense oligonucleotides or double-stranded RNA directed against *c-myc* were shown to be more resistant to the pro-apoptotic effects of TCR activation (Shi *et al.*, 1992; reviewed by Nilsson and Cleveland, 2003), TNF (Janicke *et al.*, 1994; Klefstrom *et al.*, 1994; Dong *et al.*, 1997), TRAIL (Aza-Blanc et al., 2003), or etoposide (Dong *et al.*, 1997; Adachi *et al.*, 2001). Also, *c-myc*$^{-/-}$ thymocytes are less sensitive to anti-CD95 induced apoptosis (de Alboran et al., 2001) and immortalized *c-myc*$^{-/-}$ rat fibroblasts are less sensitive to etoposide and doxorubicin (Adachi et al., 2001; Grassilli et al., 2004). It is unclear, however, to which extent the behavior of immortalized, and most often transformed, cells that are grown *in vitro* reflects the behavior of normal cells located within the environment of the intact organism. Several publications examining *myc* mutations *in vivo* did not observe any effects on the frequency of apoptosis (Baudino *et al.*, 2002; Knoepfler *et al.*, 2002; Bellmeyer *et al.*, 2003).

Here, we use *Drosophila* to analyze the apoptotic functions of Myc in a physiological setting *in vivo*. *Drosophila melanogaster* contains a single Myc protein, dMyc, which has similar if not identical biochemical and molecular characteristics as its vertebrate homologs (Gallant et al., 1996; Schreiber-Agus et al., 1997). Like vertebrate Myc, dMyc controls growth and cell cycle progression (Johnston et al., 1999) and it can even partially substitute for c-Myc in mouse embryo fibroblasts in which the endogenous c-*myc* locus has been disrupted (Trumpp et al., 2001). Conversely, human c-Myc variants can rescue lethal *dmyc* mutations to viability, attesting to the functional conservation of the proteins (Benassayag et al., 2005). In contrast to the situation in vertebrates however, overexpression of dMyc in flies has so far not been observed to induce any tumors (Johnston *et al.*, 1999; Bellosta *et al.*, 2005), and – in contrast to activated Ras – dMyc over-expression does not confer metastatic behavior onto tumors caused by inactivating mutations in genes that control cellular polarity (Pagliarini and Xu, 2003). Flies also have a comparatively short life span, and hence they are expected to be exposed to a lower general risk of acquiring activating mutations in proto-oncogenes during their lifetime. It could be argued therefore that,

in comparison with vertebrate Myc proteins, *Drosophila* Myc has been under less evolutionary pressure to acquire pro-apoptotic activities. Furthermore, the main pathway by which vertebrate Myc induces apoptosis does not seem to be conserved in *Drosophila*: while the *Drosophila* ortholog of p53 has been identified, it lacks the sequence motifs required for binding to MDM2, and neither MDM2 nor $p19^{ARF}$ are present in the fly genome (Brodsky et al., 2000; Ollmann et al., 2000).

Nevertheless, dMyc has recently been shown to induce apoptosis in *Drosophila* imaginal discs cell-autonomously (De La Cova et al., 2004). In addition, it was found that dMyc plays a role in a process called cell competition, whereby the juxtaposition of cells with different dMyc levels results in the death of the cells expressing *less* dMyc (De La Cova *et al.*, 2004; Moreno and Basler, 2004; reviewed in Gallant, 2005). The molecular pathway leading from a relative decrease in dMyc levels to apoptosis is currently unclear. Here, we examine the process of cell-autonomous apoptosis caused by dMyc in *Drosophila* imaginal discs. This apoptosis likely involves a combination of the proapoptotic genes *rpr*, *grim*, *hid* and *sickle*, but does not require *Drosophila* p53. We further show that a hypomorphic mutation in *dmyc* significantly reduces the extent of apoptotic cell death that can be induced by treatment with ionizing radiation. These results demonstrate that the control of apoptosis is a physiological function of dMyc.

Results

Overexpression of dMyc causes apoptosis

With the aim of establishing a system useable for a genetic modifier screen, we expressed dMyc in the developing eye under the control of the GMR-Gal4 driver. Consistent with dMyc's reported ability to promote growth and proliferation, the resulting adult eyes were bigger than control eyes and contained bigger ommatidia (Fig. 1A and B). In addition, the adult eyes showed a rough exterior which prompted us to investigate the possible involvement of apoptosis in this phenotype. A small, but statistically significant, increase in TUNEL signal (reflecting the number of apoptotic cells) was detected in the GMR-Gal4 expression domain posterior to the morphogenetic furrow in larval 3rd instar imaginal eye discs (Fig. 1C2), and similar results were also obtained with antibodies directed against activated caspase 3 and with acridine orange staining (not shown). To assess the contribution of apoptosis to the dMyc-dependent phenotype, we used the viral caspase inhibitor p35 to block apoptosis (Hay *et al.*, 1994). Expression of p35 alone allows cells to survive that otherwise would have succumbed to developmental apoptosis, resulting in an increase in eye and ommatidial size (Fig. 1A, B). p35 also blocks the apoptosis caused by co-expressed dMyc, indicating that caspases are necessary for such dMyc-induced apoptosis (Fig. 1C3). This co-expression of p35 and dMyc results in eyes and ommatidia that are significantly larger than eyes expressing either p35 or dMyc alone, reflecting the combined abilities of p35 to block death and of dMyc to promote growth (Fig. 1A, B). A similar increase in eye size was also observed when dMyc was co-expressed with the *Drosophila* inhibitor of apoptosis Diap1 (Hay et al., 1995; not shown). Note that the consequences of dMyc overexpression in the developing eye have recently been reported by Secombe and colleagues (Secombe et al., 2007); these authors described the growth promoting effect of dMyc, but in the absence of a detailed statistical analysis and without the co-expression of p35 or Diap1 they failed to detect the increase in apoptosis caused by dMyc.

The observations shown so far demonstrate that overexpressed dMyc can induce ectopic apoptosis in the developing eye. Since this effect was rather weak, we also tested the consequences of dMyc overexpression in a different tissue. When the *ap*-Gal4 driver is used to express dMyc in the dorsal compartment of wing imaginal discs, increased apoptosis can be observed in the dMyc-expression domain as assayed by TUNEL (Figs. 2A, B middle; 2C) and anti-activated caspase staining (not shown). Thus, overexpression of dMyc induces apoptosis both in a population of differentiating cells in the eye, and in proliferating, undifferentiated cells in the wing.

Downstream effectors of dMyc-mediated apoptosis

A recent genome-wide search for dMyc targets has revealed that ectopic expression of dMyc in transgenic larvae under the control of a heat-shock promoter results in a 2-fold up-regulation of *dp53* mRNA (Hulf et al., 2005). To confirm these microarray data, we analyzed *dp53* levels by quantitative

real-time RT-PCR at different times after ectopic heat-shock induced dMyc expression in 3rd instar larvae. Already 1 hour after the end of a 1-hour heat-shock, *dp53* mRNA was found to be increased by 2.2-fold in dMyc transgenic larvae as compared to identically treated isogenic larvae lacking a dMyc transgene, and by 4 hours after the end of the heat-shock elevated p53 levels can still be detected (Fig. 3; for control purposes, the expression level of the *bona fide* dMyc target *nnp-1* [Hulf et al., 2005] is shown, as well as the baseline *dp53* expression observed in a $dp53^{-/-}$ mutant larvae). Note that we have not been able to detect dp53 protein by Western blotting (presumably because of low dp53 abundance, even in the induced state) and therefore we cannot document the effects of dMyc on dp53 protein levels, but we assume that they parallel the induction of *dp53* mRNA. This rapid induction raises the possibility that dMyc directly activates transcription of *dp53*. The *dp53* gene contains a single consensus dMyc-binding site (called E-box) located 978 nt downstream of the transcription start site that could mediate transactivation of *dp53* by dMyc, although we cannot exclude the possibility that dMyc indirectly regulates *dp53* expression.

The effects described above suggest that dp53 plays a role in dMyc-induced apoptosis. Much to our surprise however, this is not the case. Even the complete absence of dp53 (i.e. homozygosity for a null mutation; Rong *et al.*, 2002) does not reduce the number of apoptotic cells in dMyc overexpressing wing imaginal discs (Figs. 2A right and 2C for TUNEL staining, and data not shown for anti-activated caspase 3 staining; note that *dp53* null mutant wing discs that are also heterozygous for *ap*-Gal4 but do not overexpress dMyc show as little apoptosis as the "*ap*-Gal4" control discs in Figs. 2A & B [not shown]). Instead, the number of apoptotic cells even slightly increases in *dp53* mutants overexpressing dMyc as compared to heterozygotes overexpressing dMyc, suggesting that dp53 contributes to cellular survival in the face of excessive dMyc levels. Although such an anti-apoptotic activity of dp53 is unexpected, recent studies have shown that during normal development (in the absence of exogenous stress) dp53 controls a large number of genes that play no obvious role in the induction of apoptosis (Akdemir *et al.*, 2007). It is conceivable that the mis-regulation of these genes in a $dp53^{-/-}$ background impairs the cells' ability to resist the damages inflicted by dMyc overexpression, and hence results in an increased rate of apoptosis. Indeed, it has recently been reported that the activities of dp53 are not restricted to the induction of apoptosis, but that in a particular context dp53 contributes to the stimulation of proliferation (Wells *et al.*, 2006; see also the Discussion).

While dp53 is dispensable for dMyc-induced apoptosis, the pro-apoptotic proteins *hid*, *reaper, grim* and *sickle* play a role in this process, since heterozygosity for the deficiency G870 (which removes all four genes: Bischof et al., 2007, P.Geuking & K.Basler, pers. comm.) reduces dMyc-induced apoptosis in the wing (Figs. 2B and 2C; De La Cova et al., 2004); a reduction in apoptosis was also observed with the smaller deficiency H99 that only affects *hid*, *reaper* and *grim* (not shown). Furthermore, heat-shock controlled expression of dMyc in transgenic third instar larvae induces the expression of *rpr* and the functionally related gene *sickle* within 1 hour by 2.2 ± 1.2 fold (range 0.9- to 4.1-fold) and 1.6 ± 0.5 fold (range 1.0- to 2.4-fold), respectively (average of 6 independent qRT-PCR experiments). Four hours after the induction of dMyc expression the levels of *grim* are also increased by 2.9 ± 1.9 fold (range 1.4- to 5.6-fold in 3 independent qRT-PCR experiments). We do not know to which extent this effect of dMyc on *rpr*, *sickle* and *grim* is direct, and to which extent it is mediated by other transcription factors such as dp53; repeated experiments with dMyc overexpression in a $p53^{-/-}$ background were inconclusive (as they showed large variations). However, the *grim*, *hid* and *sickle* genes contain canonical E-boxes (at -1720 bp, -123 bp, -182 bp relative to the transcription start site, respectively), and they, as well as *rpr*, also contain non-canonical E-boxes. Together with the observed effect of the deficiency G870 (and the absence of an effect of a *dp53* null mutation), these data suggest that dMyc might activate the expression of these genes independently of dp53, and that dMyc and dp53 might to some extent be able to co-operate in the induction of these genes. Consistent with such a hypothesis, ectopic co-expression of dMyc and dp53 under the control of ap-Gal4 leads to an increased level of apoptosis as compared to expression of either protein alone (data not shown). Thus, both overexpression and loss-of-function of dp53 co-operate with dMyc to induce apoptosis, but we believe that the mechanisms are different in the two situations (see above).

dMyc is required for X-ray induced apoptosis

The experiments described so far address the ability of over-expressed dMyc to induce apoptosis. Below, we explore the importance of physiological dMyc levels for the induction of cell death. The hypomorphic alleles $dmyc^{P0}$ and $dmyc^{P1}$ did not affect the negligible baseline apoptosis in unchallenged imaginal discs of wandering third instar larvae (data not shown). However, such *dmyc* mutant cells become more resistant to the pro-apoptotic effects of DNA damage: upon 5 or 10 Gy of X-irradiation, eye discs of wild type larvae show a large number of apoptotic cells (Figs. 4A-C). In contrast, in $dmyc^{P0}$/Y and $dmyc^{P1}$/Y eye imaginal discs, apoptosis is strongly reduced in response to such irradiation, but not at higher doses of X-rays (Figs. 4D-F, and data not shown). A quantitative analysis of eye discs treated with 5 Gy showed 28±15 apoptotic cells in $dmyc^{P1}$/Y discs (n=15) and 146±66 apoptotic cells for wild type (n=15), a highly significant difference ($p<0.001$); similar results were obtained in several independent experiments, comparing either $dmyc^{P1}$ with Oregon R wild-type flies, or $dmyc^{P0}$ with genotype-matched control animals (see Materials). This partial resistance to DNA-damage induced apoptosis cannot be ascribed to the moderately reduced size of *dmyc* mutant eye discs ($dmyc^{P0}$/Y eye discs are ca. 23% smaller than *y w*/Y control discs; Bellosta et al., 2005); it is also not an indirect consequence of a cell cycle block, as only a marginal increase in the fraction of G1 phase cells in the $dmyc^{P1}$/Y mutant discs was observed (Johnston et al., 1999). We also consider it unlikely that this impairment in apoptosis is caused by differences in developmental age, since the experiments were carried out with wandering larvae (i.e. animals of the same developmental age for all genotypes), and besides, the overall duration of development is only moderately extended in $dmyc^{P0}$/Y flies (by one day as compared to control flies). Thus, a reduction of dMyc levels either reduces or delays the apoptotic response to weak doses of DNA-damaging agents.

Discussion

Myc-induced apoptosis is often seen as a protective function that has evolved to reduce the ability of mutationally activated Myc to induce cancer (Lowe et al., 2004). However, although *Drosophila* dMyc has not been linked to any disease, it also plays a role in apoptosis, both cell-autonomously and in the non-autonomous process of cell competition (De La Cova et al., 2004; Moreno and Basler, 2004). Here, we examine the mechanism of cell-autonomous apoptosis by modulating dMyc levels broadly throughout the whole organism or an entire compartment. We confirm earlier observations that dMyc overexpression can induce apoptosis cell-autonomously. The downstream effectors of dMyc-induced apoptosis are not fully elucidated, but a combination of the pro-apoptotic genes *rpr*, *grim*, *hid* and *sickle* is required for this process, and our analysis raises the possibility that some of these genes might be directly activated by dMyc. dp53 is also rapidly induced by overexpressed dMyc, but surprisingly, is not required for dMyc induced apoptosis. It remains possible, though, that dMyc needs dp53 to trigger apoptosis in other tissues that were not analyzed here, e.g. polyploid larval tissues. In vertebrates a similar situation is encountered, where p53 is dispensable for c-Myc induced apoptosis in some settings (e.g. Martins et al., 2006), but required in others (reviewed in Meyer et al., 2006).

Irrespective of the molecular pathway, our studies show that the ability of dMyc to induce cell-autonomous apoptosis is physiologically relevant. This is demonstrated by the observation that a moderate reduction in dMyc levels (to about 29% at the RNA level in $dmyc^{P0}$ vs. wild type eye imaginal discs; data not shown) reduces the ability of imaginal disc cells to undergo apoptosis in response to moderate DNA-damage. Interestingly, preliminary observations suggest that the decreased cellular apoptotic response in *dmyc*-mutant larvae is accompanied by a slightly impaired organismal survival in the face of X-irradiation: whereas 91% and 89% of control animals survived from third instar to adulthood with or without irradiation, respectively, the numbers for $dmyc^{P0}$/Y larvae were reduced to 86% (without irradiation) and 69% (with 10 Gy X-irradiation; n=125 to 173 input larvae from 3 independent experiments), suggesting that the reduction of apoptosis observed in the irradiated mutant animals is associated with a decrease in fitness. A similar sensitivity to X-irradiation has recently been reported for growth impaired flies in general, i.e. animals that were either nutritionally starved or mutant for *dmyc*, *chico* or *cdk4* (Jaklevic et al., 2006). After irradiation with 40 Gy of X-rays such animals show similar levels of apoptosis as wild type larvae (consistent with our observations for *dmyc* mutant larvae

irradiated with 50 Gy). However, wild type animals can compensate for the dead cells by a process called compensatory proliferation, whereby dying cells emit growth factors (such as Wg and Dpp) that stimulate proliferation of the surrounding intact cells (reviewed in Gallant, 2005). These proliferation signals are likely to be present in the irradiated growth impaired larvae as well, but the recipients of these signals cannot proliferate fast enough to compensate for the lost cells, resulting in increased organismal lethality (Jaklevic et al., 2006). A similar defect in compensatory proliferation might also be responsible for the lethality we observe in our experiments. *dmyc* mutant larvae treated with low doses of X-rays (10 Gy) show less apoptosis than wild type larvae, but it is reasonable to assume that the X-rays damage a similar number of cells in the *dmyc* mutant larvae as in the wild type, and that these damaged cells have a reduced proliferative potential. However, since the damaged cells do not die (at least within the time course of our experiment), they may be unable to trigger the signal for compensatory proliferation, which in turn results in a similar lethality as after treatment with higher doses of X-rays. Interestingly, dp53 itself has recently also been shown to have an important role in compensatory proliferation (Wells et al., 2006), and hence its loss is likely to affect compensatory proliferation and thus contribute to the observed lethality. Of course, the limited proliferative potential of *dmyc* mutant cells may very well also contribute to the observed lethality.

The mechanism by which dMyc contributes to X-ray induced apoptosis is unclear. X rays do not cause a significant increase in *dmyc* mRNA levels (as measured by qRT-PCR, within 1 to 2 hours of X-irradiation *dmyc* mRNA levels in wandering 3rd instar larvae are at 91±32% of un-challenged larvae; 4 independent experiments; p=0.67). This suggests that dMyc plays a permissive role, i.e. that normal dMyc levels are needed to allow a maximal apoptotic response to DNA damage. Since dp53 is thought to directly activate pro-apoptotic genes such as *rpr*, *hid* and *skl* (Brodsky *et al.*, 2000; Sogame *et al.*, 2003; Brodsky *et al.*, 2004; Akdemir *et al.*, 2007), it is conceivable that dMyc cooperates with dp53 at the level of gene expression. Alternatively, dMyc might play a more general role in determining the cellular ability to react to incoming signals. dMyc affects the expression of a large number of target genes (possibly thousands of them), albeit to a comparatively moderate extent (Orian *et al.*, 2003; Grewal *et al.*, 2005; Hulf *et al.*, 2005). Therefore, the decreased expression of a large group of genes in the *dmyc* mutants could conspire to reduce the efficiency of transcriptional activation by dp53, or to affect the execution of the apoptotic program downstream of dp53 targets.

A co-operation between dMyc and dp53 might also take place in unchallenged animals, since dp53 also controls the expression of many genes during normal development (Akdemir *et al.*, 2007). Indeed, our preliminary observations indicate that (in the absence of any irradiation) flies doubly mutant for *dp53* and *dmyc* show an increased organismal lethality as compared to singly mutant larvae (only 60% of third instar $dmyc^{P0}$/Y; $dp53^{-/-}$ larvae survive to adulthood, as compared to 90% for +/Y; $dp53^{-/-}$ and 86% for $dmyc^{P0}$/Y; $dp53^{+/-}$; 93 to 178 input larvae from 3 independent experiments). We do not know the cellular basis for this synthetic lethality, but notice a similarity to the genetic interaction between the growth regulators *chico* or *cdk4* on one hand, and the checkpoint kinase *grp (chk1)* on the other hand (Jaklevic et al., 2006): whereas all these single mutants are viable, the double mutant combinations are fully lethal, possibly because such flies are less efficient in dealing with physiologically occurring DNA double strand breaks.

In conclusion, the detailed mechanism of dMyc's effect on cell-autonomous apoptosis still needs to be elaborated, but our observations demonstrate that the ability of Myc to induce apoptosis is evolutionarily conserved. This ability is not restricted to overexpression of Myc but also important at physiological levels of Myc. Our studies suggest that *Drosophila* can be used to dissect this connection between Myc and apoptosis.

Experimental Procedures

Analysis of imaginal discs

Immunocytochemistry was carried out according to standard procedures. Primary anti-activated caspase 3 antiserum (Cell Signaling Technology; Srinivasan et al., 1998) was used at 1:300 dilution to reveal apoptotic cells. Secondary antibodies conjugated to FITC (Jackson ImmunoResearch Laboratories Inc.) were used at 1:200 dilutions. TUNEL staining was performed using the ApopTag Fluorescein in situ

Apoptosis detection kit (Intergen Company)**,** following the manufacturer's protocol. Numerical values of TUNEL staining in the eye are the ratios of green intensity of the signal posterior to anterior of the morphogenetic furrow, as determined with Adobe Photoshop. Discs were mounted in Vectashield Zymed (Vector Laboratories Inc.) or FluoroGuard Antifade (BioRad), and images were obtained on a Leica Nomarski optics microscope.

Examination of adult eyes

Flies were prepared for scanning electron microscopy as described (Wolff and Ready, 1991). To determine the surface of individual ommatidia an area covering approximately 22 ommatidia in the centre of the eye was measured (in pixel, using Adobe Photoshop) and divided by the number of included ommatidia.

Quantitative real-time RT-PCR

To avoid crowding effects, 50 -70 freshly hatched first instar larvae from timed egg lays (max. of 5 h) were transferred into fresh vials and then raised at 25°. Wandering third instar larvae were heat-shocked for 1 h in a 37° water bath and collected 1 h or 4 h after the end of the heat-shock. Ten larvae per genotype and time point were homogenized in 1 ml Trizol (Invitrogen) using a tissue shredder and total RNA was extracted according to the manufacturer's protocol. Ten µg RNA per sample were treated with Turbo DNase (Ambion) to eliminate contaminating genomic DNA and reverse transcription of 1 µg was carried out with the Omniscript RT kit (Qiagen) and random hexamers as primers. Quantitative real-time RT-PCR (qRT-PCR) reactions were carried out in duplicate on an ABI 7900HT machine, using SYBR green PCR master mix (Applied Biosystems); qRT-PCR of mock reverse-transcribed RNA samples (in the absence of RT) confirmed the absence of any genomic DNA contamination.

The following primers were used: actin 5C (reference): 5'-GCCCATCTACGAGGGTTATGC & 5'-AATCGCGACCAGCCAGATC; dp53: 5'-TGCGCAATCCCAACCAA & 5'-CACGTTTCTTAAGGCTCAGCAA; nnp-1: 5'-CTATACACACGAAAGTTTCCATGCTATA & 5'-CAACACGGAACTATCGTAAATTTCA; dmyc: 5'-GAATCGCGCTCGGTTAGTG & 5'-CTACGCCGCCGCTTTAAG; grim: 5'-CCGACGGTCATTCAGATATCCT & 5'-CACGTCGTCCTCATCGTTGT; hid: 5'-TGATGCCACACCATTCCG & 5'-GCGTGTAGCCGGCGTAGT; rpr: 5'-CACCGTCGTCCTGGAAACC & 5'-CGATATTTGCCGGACTTTCTTC; sickle: 5'-CCCGGACATGATCAAGTGG & 5'-TGGTGGCGGGAGTTCCT.

X-irradiation experiments

Wandering third instar larvae were exposed to X-rays using an X-ray cabinet system. The larvae were then returned to their food and the incidence of apoptosis was determined 5 hours after the irradiation. For analysis of transcript levels by qRT-PCR larvae were harvested 1 or 2 hours after irradiation with 10 Gy.

Fly stocks

The following transgenic and mutant flies were used in this study: GMR-*Gal4* (Hay et al., 1995), UAS-*dMyc* (Johnston et al., 1999), UAS-*p35* (Hay et al., 1994), ap-*Gal4* (Milan et al., 1997), $dp53^{-/-\,(11\text{-}1B\text{-}1)}$ and $dp53^{-/-(5A\text{-}1\text{-}4)}$ (Rong et al., 2002), $dmyc^{P0}$ and $dmyc^{P1}$ (Johnston et al., 1999), $dmyc^{P0\text{-rev}}$ (a wild-type revertant of the $dmyc^{P0}$ allele; generated in this study), w^{1118}; hs-dMyc^{29} and the isogenic line w^{1118} (Hulf et al., 2005)**.** Df(3L)G870 is a deletion of cytological bands 75B-C that was kindly provided by K.Basler (Bischof et al., 2007). For the analysis of apoptosis in response to X-irradiation, the $dmyc^{P0}$ line (full genotype: P[$ry^{+t7.2}$=P-Sal]$dmyc^{P0}$/C(1)DX, y^1 f^1; bw^1; st^1) was compared to the genotype-matched line "P[$ry^{+t7.2}$=P-Sal]*I16*; bw^1; st^1", as both originated in the same P-element mobilization screen (Robertson and Engels, 1989).

Statistical analysis

p-values were derived using Student's t-test.

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References

Adachi S, Obaya AJ, Han Z, Ramos-Desimone N, Wyche JH, Sedivy JM. 2001. c-Myc is necessary for DNA damage-induced apoptosis in the G(2) phase of the cell cycle. Mol Cell Biol 21:4929-4937.

Akdemir F, Christich A, Sogame N, Chapo J, Abrams JM. 2007. p53 directs focused genomic responses in Drosophila. Oncogene 26:5184.

Aza-Blanc P, Cooper CL, Wagner K, Batalov S, Deveraux QL, Cooke MP. 2003. Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. Mol Cell 12:627-637.

Baudino TA, McKay C, Pendeville-Samain H, Nilsson JA, Maclean KH, White EL, Davis AC, Ihle JN, Cleveland JL. 2002. c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. Genes Dev 16:2530-2543.

Bellmeyer A, Krase J, Lindgren J, LaBonne C. 2003. The protooncogene c-myc is an essential regulator of neural crest formation in xenopus. Dev Cell 4:827-839.

Bellosta P, Hulf T, Diop SB, Usseglio F, Pradel J, Aragnol D, Gallant P. 2005. Myc interacts genetically with Tip48/Reptin and Tip49/Pontin to control growth and proliferation during Drosophila development. Proc Natl Acad Sci USA 102:11799-11804.

Benassayag C, Montero L, Colombie N, Gallant P, Cribbs D, Morello D. 2005. Human c-Myc isoforms differentially regulate cell growth and apoptosis in Drosophila melanogaster. Mol Cell Biol 25:9897-9909.

Bischof J, Maeda RK, Hediger M, Karch F, Basler K. 2007. An optimized transgenesis system for Drosophila using germ-line-specific {phi}C31 integrases. PNAS 104: 3312-3317.

Brodsky MH, Nordstrom W, Tsang G, Kwan E, Rubin GM, Abrams JM. 2000. Drosophila p53 binds a damage response element at the reaper locus. Cell 101:103-113.

Brodsky MH, Weinert BT, Tsang G, Rong YS, McGinnis NM, Golic KG, Rio DC, Rubin GM. 2004. Drosophila melanogaster MNK/Chk2 and p53 regulate multiple DNA repair and apoptotic pathways following DNA damage. Mol Cell Biol 24:1219-1231.

de Alboran IM, O'Hagan RC, Gartner F, Malynn B, Davidson L, Rickert R, Rajewsky K, DePinho RA, Alt FW. 2001. Analysis of C-MYC function in normal cells via conditional gene-targeted mutation. Immunity 14:45-55.

De La Cova C, Abril M, Bellosta P, Gallant P, Johnston LA. 2004. Drosophila myc regulates organ size by inducing cell competition. Cell 117:107-116.

Dong J, Naito M, Tsuruo T. 1997. c-Myc plays a role in cellular susceptibility to death receptor-mediated and chemotherapy-induced apoptosis in human monocytic leukemia U937 cells. Oncogene 15:639-647.

Gallant P. 2005. Myc, cell competition, and compensatory proliferation. Cancer Res 65:6485-6487.

Gallant P, Shiio Y, Cheng PF, Parkhurst SM, Eisenman RN. 1996. Myc and Max homologs in Drosophila. Science 274:1523-1527.

Grassilli E, Ballabeni A, Maellaro E, Del Bello B, Helin K. 2004. Loss of MYC confers resistance to doxorubicin-induced apoptosis by preventing the activation of multiple serine protease- and caspase-mediated pathways. J Biol Chem 279:21318-21326.

Grewal SS, Li L, Orian A, Eisenman RN, Edgar BA. 2005. Myc-dependent regulation of ribosomal RNA synthesis during Drosophila development. Nat Cell Biol 7:295-302.

Hay BA, Wassarman DA, Rubin GM. 1995. Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. Cell 83:1253-1262.

Hay BA, Wolff T, Rubin GM. 1994. Expression of baculovirus P35 prevents cell death in Drosophila. Development 120:2121-2129.

Hulf T, Bellosta P, Furrer M, Steiger D, Svensson D, Barbour A, Gallant P. 2005. Whole-genome analysis reveals a strong positional bias of conserved dMyc-dependent E-boxes. Mol Cell Biol 25:3401-3410.

Jaklevic B, Uyetake L, Lemstra W, Chang J, Leary W, Edwards A, Vidwans S, Sibon O, Tin Su T. 2006. Contribution of growth and cell cycle checkpoints to radiation survival in Drosophila. Genetics 174:1963-1972.

Janicke RU, Lee FH, Porter AG. 1994. Nuclear c-Myc plays an important role in the cytotoxicity of tumor necrosis factor alpha in tumor cells. Mol Cell Biol 14:5661-5670.

Johnston LA, Prober DA, Edgar BA, Eisenman RN, Gallant P. 1999. Drosophila myc regulates cellular growth during development. Cell 98:779-790.

Klefstrom J, Vastrik I, Saksela E, Valle J, Eilers M, Alitalo K. 1994. c-Myc induces cellular susceptibility to the cytotoxic action of TNF-alpha. EMBO J 13:5442-5450.

Knoepfler PS, Cheng PF, Eisenman RN. 2002. N-myc is essential during neurogenesis for the rapid expansion of progenitor cell populations and the inhibition of neuronal differentiation. Genes Dev 16:2699-2712.

Lowe SW, Cepero E, Evan G. 2004. Intrinsic tumour suppression. Nature 432:307-315.

Martins CP, Brown-Swigart L, Evan GI. 2006. Modeling the therapeutic efficacy of p53 restoration in tumors. Cell 127:1323-1334.

Meyer N, Kim SS, Penn LZ. 2006. The Oscar-worthy role of Myc in apoptosis. Seminars in Cancer Biology 16:275.

Milan M, Campuzano S, Garcia-Bellido A. 1997. Developmental parameters of cell death in the wing disc of Drosophila. Proc Natl Acad Sci USA 94:5691-5696.

Moreno E, Basler K. 2004. dMyc transforms cells into super-competitors. Cell 117:117-129.

Nilsson JA, Cleveland JL. 2003. Myc pathways provoking cell suicide and cancer. Oncogene 22:9007-9021.

Ollmann M, Young LM, Di Como CJ, Karim F, Belvin M, Robertson S, Whittaker K, Demsky M, Fisher WW, Buchman A, Duyk G, Friedman L, Prives C, Kopczynski C. 2000. Drosophila p53 is a structural and functional homolog of the tumor suppressor p53. Cell 101:91-101.

Orian A, Van Steensel B, Delrow J, Bussemaker HJ, Li L, Sawado T, Williams E, Loo LW, Cowley SM, Yost C, Pierce S, Edgar BA, Parkhurst SM, Eisenman RN. 2003. Genomic binding by the Drosophila Myc, Max, Mad/Mnt transcription factor network. Genes Dev 17:1101-1114.

Oster SK, Ho CS, Soucie EL, Penn LZ. 2002. The myc oncogene: MarvelouslY Complex. Adv Cancer Res 84:81-154.

Pagliarini RA, Xu T. 2003. A genetic screen in Drosophila for metastatic behavior. Science 302:1227-1231.

Pelengaris S, Khan M, Evan GI. 2002. Suppression of Myc-induced apoptosis in beta cells exposes multiple oncogenic properties of Myc and triggers carcinogenic progression. Cell 109:321-334.

Raveh T, Droguett G, Horwitz MS, DePinho RA, Kimchi A. 2001. DAP kinase activates a p19ARF/p53-mediated apoptotic checkpoint to suppress oncogenic transformation. Nat Cell Biol 3:1-7.

Robertson HM, Engels WR. 1989. Modified P elements that mimic the P cytotype in Drosophila melanogaster. Genetics 123:815-824.

Rong YS, Titen SW, Xie HB, Golic MM, Bastiani M, Bandyopadhyay P, Olivera BM, Brodsky M, Rubin GM, Golic KG. 2002. Targeted mutagenesis by homologous recombination in D. melanogaster. Genes Dev 16:1568-1581.

Roy B, Beamon J, Balint E, Reisman D. 1994. Transactivation of the human p53 tumor suppressor gene by c-Myc/Max contributes to elevated mutant p53 expression in some tumors. Mol Cell Biol 14:7805-7815.

Schreiber-Agus N, Stein D, Chen K, Goltz JS, Stevens L, DePinho RA. 1997. Drosophila Myc is oncogenic in mammalian cells and plays a role in the diminutive phenotype. Proc Natl Acad Sci USA 94:1235-1240.

Secombe J, Li L, Carlos L, Eisenman RN. 2007. The Trithorax group protein Lid is a trimethyl histone H3K4 demethylase required for dMyc-induced cell growth. Genes Dev 21:537-551.

Sherr CJ. 2001. The INK4a/ARF network in tumour suppression. Nat Rev Mol Cell Biol 2:731-737.

Shi Y, Glynn JM, Guilbert LJ, Cotter TG, Bissonnette RP, Green DR. 1992. Role for c-myc in activation-induced apoptotic cell death in T cell hybridomas. Science 257:212-214.

Sogame N, Kim M, Abrams JM. 2003. Drosophila p53 preserves genomic stability by regulating cell death. Proc Natl Acad Sci USA 100:4696-4701.

Srinivasan A, Roth KA, Sayers RO, Shindler KS, Wong AM, Fritz LC, Tomaselli KJ. 1998. In situ immunodetection of activated caspase-3 in apoptotic neurons in the developing nervous system. Cell Death Differ 5:1004-1016.

Strasser A, Harris AW, Bath ML, Cory S. 1990. Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature 348:331-333.

Trumpp A, Refaeli Y, Oskarsson T, Gasser S, Murphy M, Martin GR, Bishop JM. 2001. c-Myc regulates mammalian body size by controlling cell number but not cell size. Nature 414:768-773.

Vousden KH, Lu X. 2002. Live or let die: the cell's response to p53. Nat Rev Cancer 2:594-604.

Wells BS, Yoshida E, Johnston LA. 2006. Compensatory Proliferation in Drosophila Imaginal Discs Requires Dronc-Dependent p53 Activity. Current Biology 16:1606.

Wolff T, Ready DF. 1991. Cell death in normal and rough eye mutants of Drosophila. Development 113:825-839.

Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ, Roussel MF. 1998. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev 12:2424-2433.

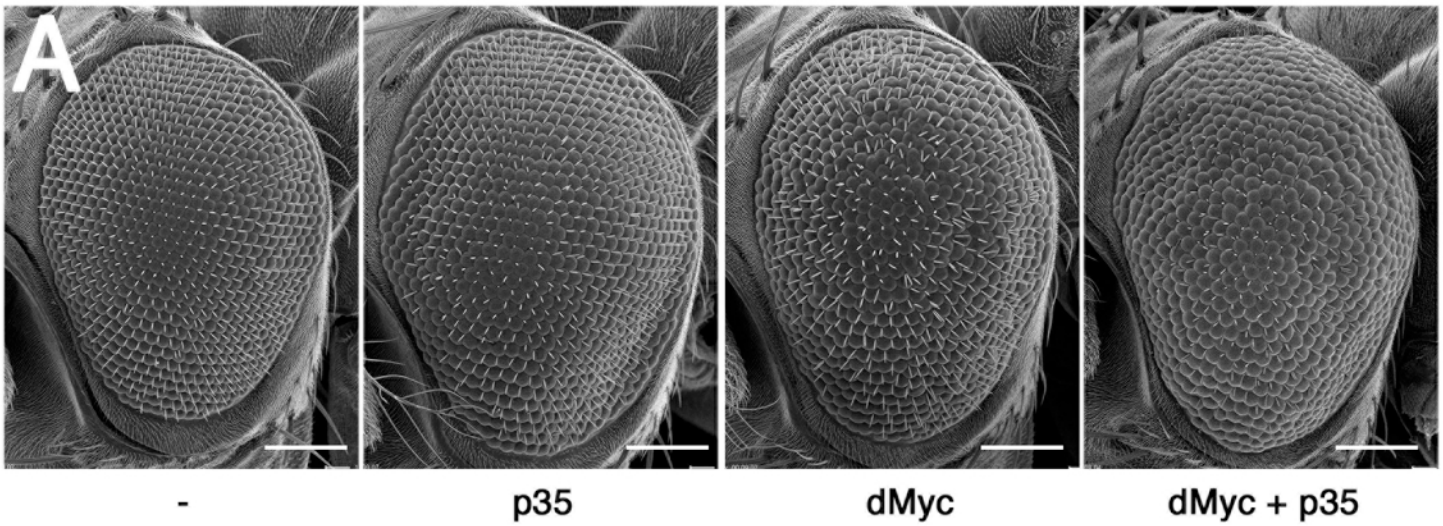
Figures legends

Figure 1. dMyc over-expression promotes growth and apoptosis in the *Drosophila* eye. A, scanning electron micrographs of adult female eyes (SEM). **B**, average size of adult ommatidia (as determined from 6 independent eyes per genotype; bars indicate standard deviation). p-values are: $6*10^{-4}$ (dMyc vs control), $4*10^{-2}$ (p35 vs control), $4*10^{-2}$ (dMyc vs p35), 10^{-6} (dMyc+p35 vs control), $4*10^{-5}$ (dMyc+p35 vs p35), 10^{-4} (dMyc+p35 vs dMyc). **C**, TUNEL staining of third-instar larval eye imaginal discs; the white bar marks the expression domain of GMR-*Gal4*. Numbers indicate the ratios of signal intensities posterior relative to anterior of the morphogenetic furrow ($n > 10$; $p < 0.01$ for the comparison of C1 with C2, $p < 0.001$ for C2 versus C3). Genotypes: all flies are heterozygous for one copy of GMR-Gal4 plus the indicated UAS-transgenes – p35 (UAS-p35), dMyc (3x UAS-dMyc), p35 + dMyc (UAS-p35, 3x UAS-dMyc). The scale bars in panel A correspond to 100 μm. In this and subsequent figures the eyes and imaginal discs are oriented with their posterior to the right.

Figure 2. Dp53 is not required for dMyc-induced apoptosis in the wing. Control wing imaginal discs from wandering 3rd instar larvae show low levels of apoptosis (**A** and **B**, left), but ectopic expression of dMyc in the dorsal compartment strongly induces apoptosis (**A** and **B**, center), which is not reduced by elimination of *dp53* (**A**, right; dp53-/- corresponds to the heteroallelic combination $dp53^{11\text{-}1B\text{-}1/5A\text{-}1\text{-}4}$), but by heterozygosity for Df(3L)G870 (**B,** right); panels A and B correspond to separate experiments. **Panel C**, average number of apoptotic cells in the pouch region of 9 to 10 wing discs of the genotypes shown in panels A (first group of 3 bars) and B (second group of 3 bars); error bars indicate standard deviations. p-values for comparisons are: **A**: $8*10^{-2}$ (dMyc vs control), 10^{-4} (dMyc, $p53^{-/-}$ vs control), $5*10^{-2}$ (dMyc, $p53^{-/-}$ vs dMyc); **B**: $5*10^{-4}$ (dMyc vs control), $7*10^{-5}$ (dMyc vs dMyc, Df), $8*10^{-2}$ (dMyc, Df vs control). All wing discs are oriented with their dorsal sides up. The white line in panel 1A outlines the *ap*-Gal4 expression domain.

Figure 3. Heat-shock induced expression of dMyc rapidly induces *dp53* mRNA. Levels of *dp53* mRNA after heat-shock induced expression of dMyc as assayed by qRT-PCR; for comparison, *nnp-1* levels are shown (a well-characterized direct dMyc target; Hulf et al., 2005). Each column corresponds to the average of two biologically independent samples; the error bars show standard deviations, and "1 h" and "4 h" indicate the time elapsed after the end of the heat-shock. The genotypes are indicated in the legend (whereby "control" corresponds to "w^{1118}").

Figure 4. dMyc plays a role in X-ray-induced apoptosis. TUNEL staining of third instar larval eye imaginal discs irradiated with X-ray doses of 5 Gy (**A**, **D**), 10 Gy (**B**, **E**), or 50 Gy (**C**, **F**). Irradiation of *dmyc* mutant eye imaginal discs with 5 and 10 Gy results in fewer apoptotic cells (**D, E**) than irradiation of discs of wild type flies (**A, B**). The numbers in **A** and **D** show the average (and standard deviations) of apoptotic cells from 15 independent eye discs. Genotypes (all flies are males): **A-C**: *Oregon R*; **D-F**: $dmyc^{P1}$/Y.


A
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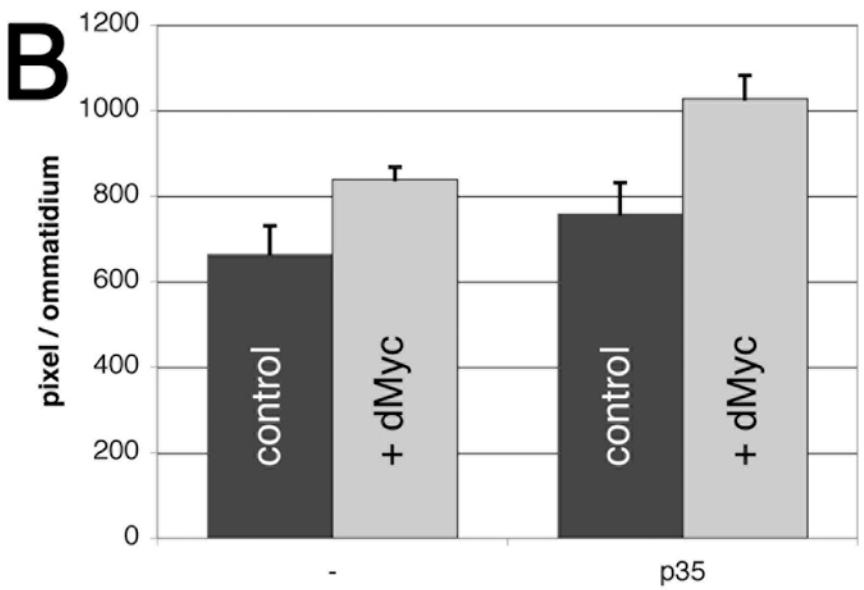
p35
dMyc
dMyc + p35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1000
800
600
400
200
0
pixel / ommatidium
control
+ dMyc
control
+ dMyc
-
p35

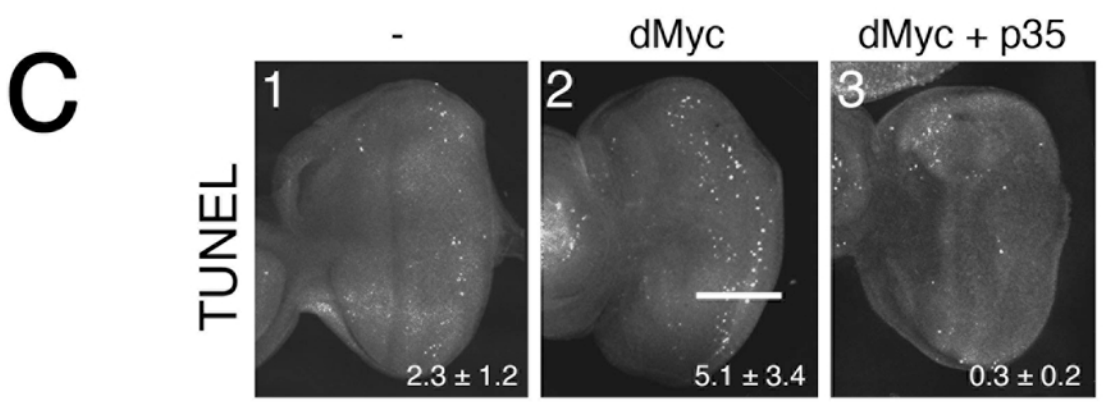
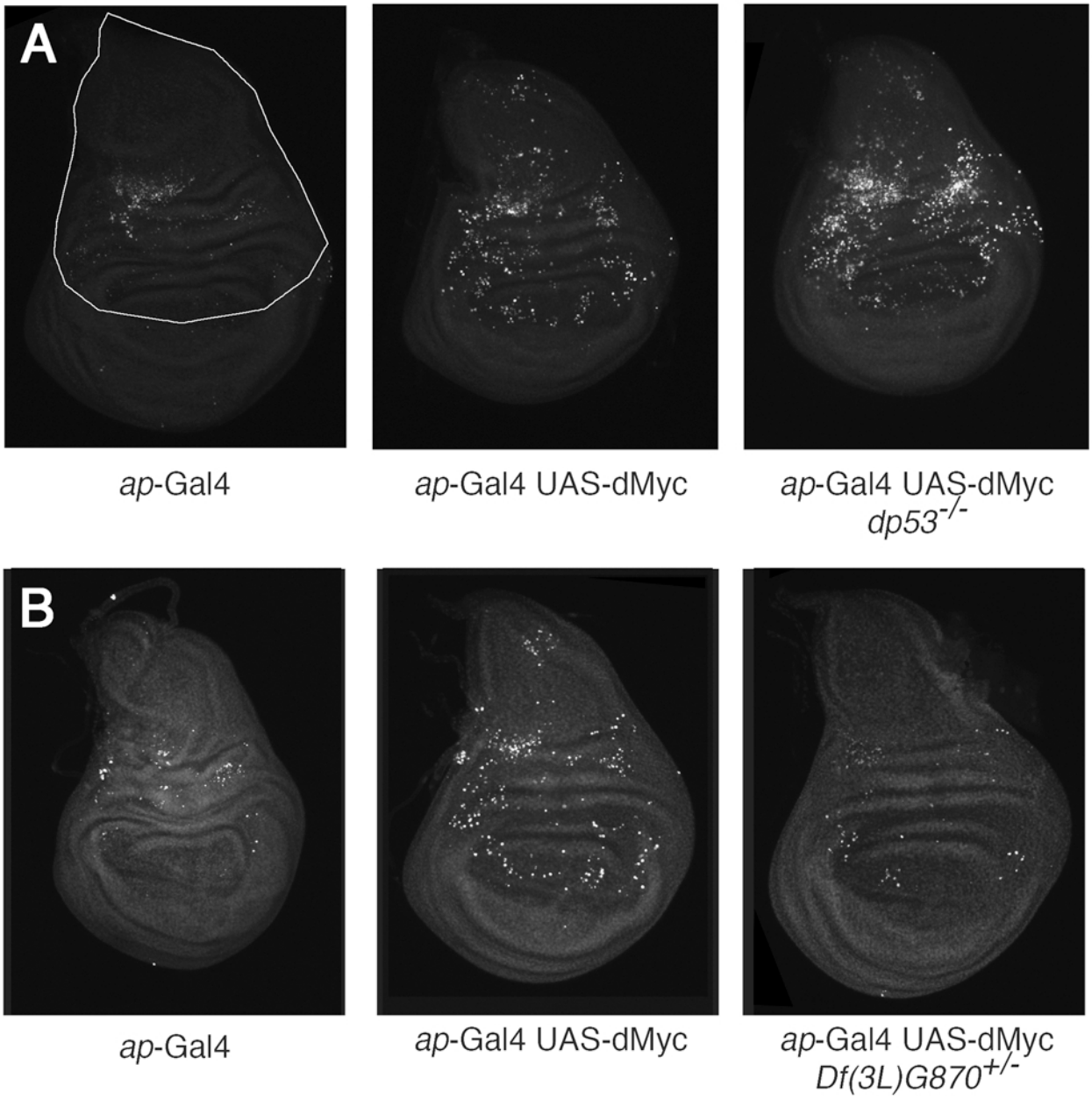

C
-
dMyc
dMyc + p35
TUNEL
1
2.3 ± 1.2
2
5.1 ± 3.4
3
0.3 ± 0.2

Figure 1


A
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dp53-/-
B
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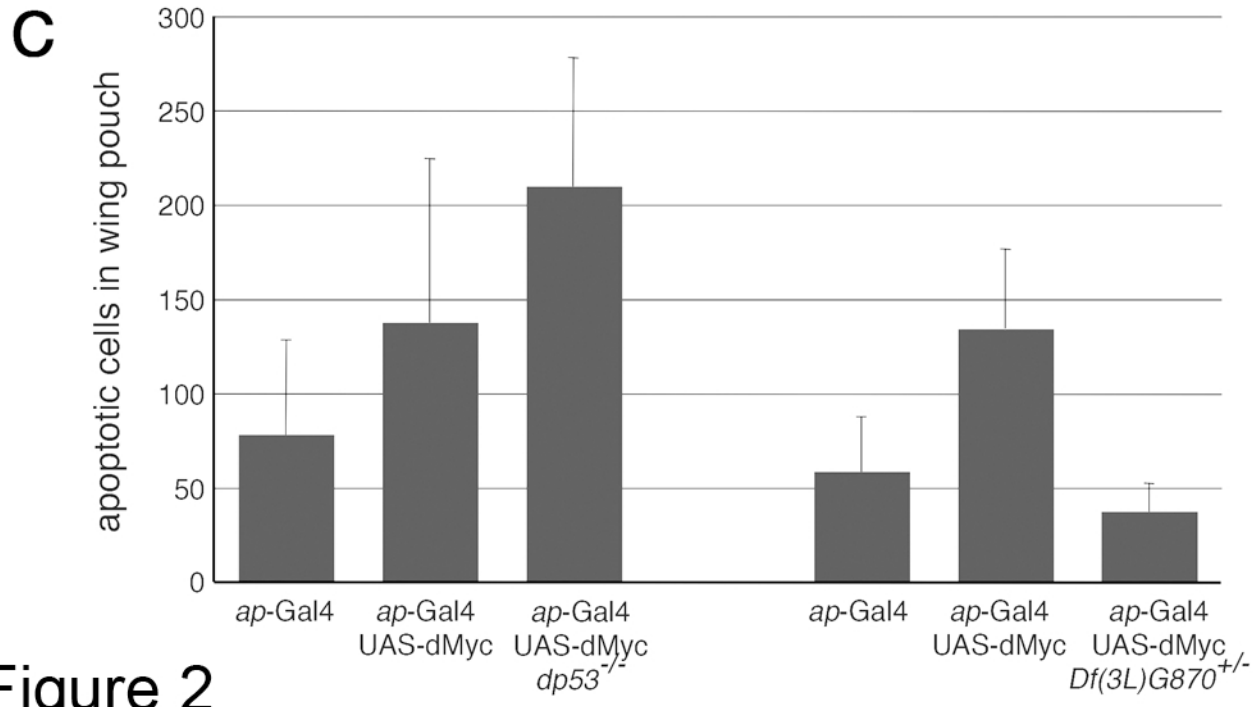
ap-Gal4 UAS-dMyc
Df(3L)G870+/-


C
apoptotic cells in wing pouch
300
250
200
150
100
50
0
ap-Gal4
ap-Gal4 UAS-dMyc
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ap-Gal4
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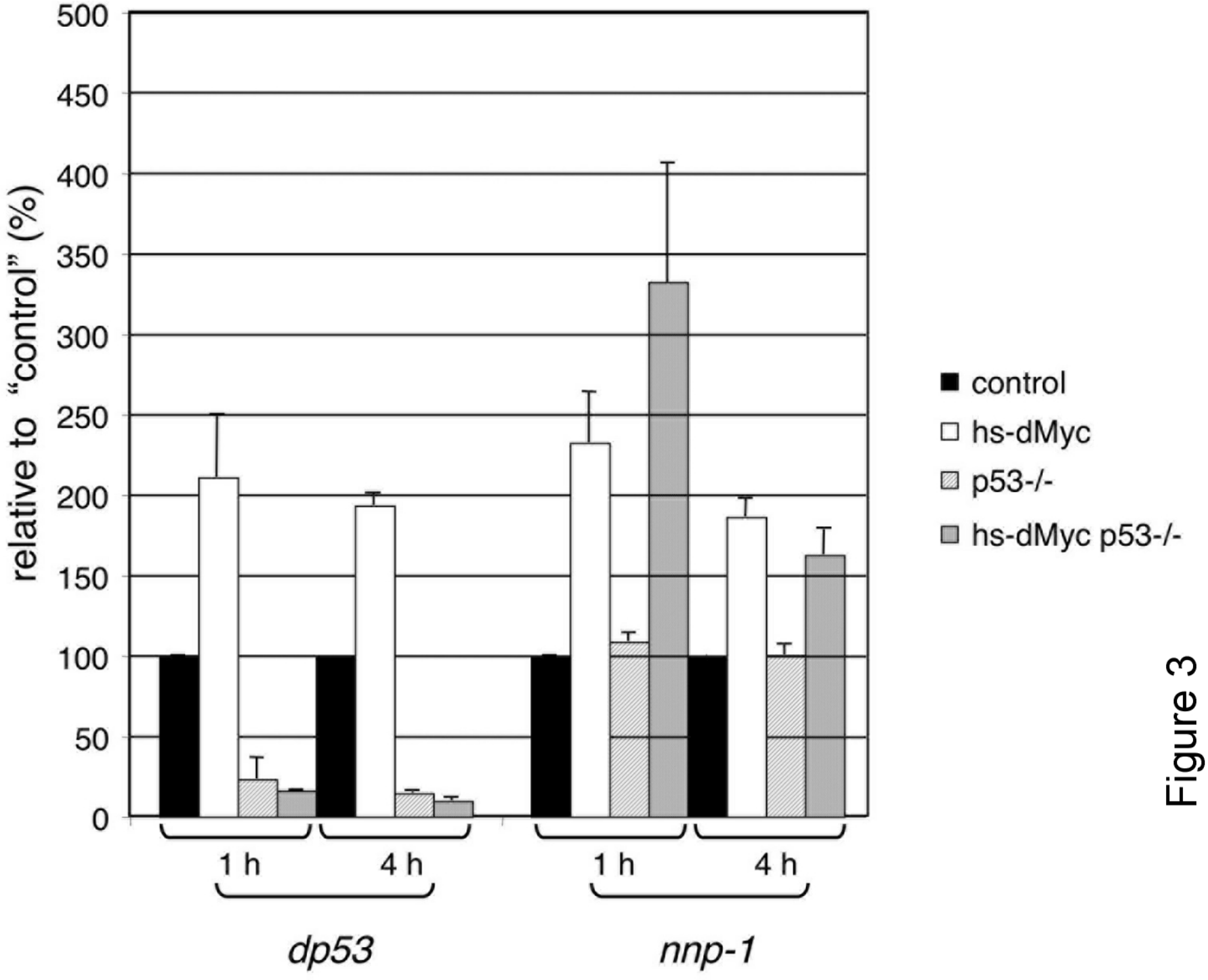
ap-Gal4 UAS-dMyc Df(3L)G870+/-

Figure 2


500
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400
350
300
250
200
150
100
50
0
relative to "control" (%)
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4 h
1 h
4 h
dp53
nnp-1
control
hs-dMyc
p53-/-
hs-dMyc p53-/-

Figure 3

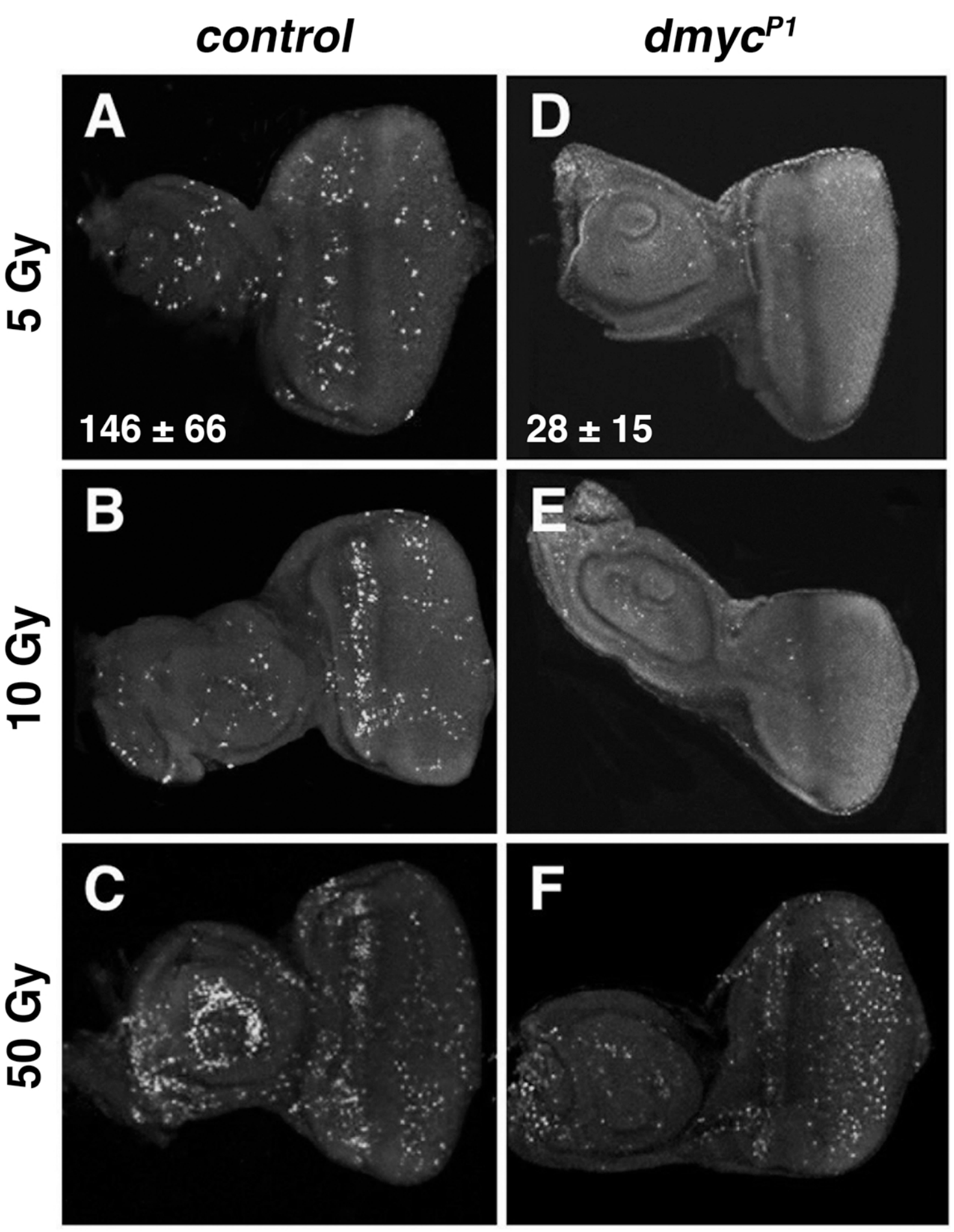

Figure 4