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Abstract

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Max-independent functions of Myc in *Drosophila melanogaster*

Dominik Steiger[1,2], Michael Furrer[1,3], Daniela Schwinkendorf[1,3] & Peter Gallant[1]

Myc proteins are powerful proto-oncoproteins and important promoters of growth and proliferation during normal development. They are thought to exercise their effects upon binding to their partner protein Max, and their activities are largely antagonized by complexes of Max with Mnt or an Mxd family protein. Although the biological functions of Myc, Mxd and Mnt have been intensively studied, comparatively little is known about the *in vivo* role of Max. Here we generate *Max* loss-of-function and reduction-of-function mutations in *Drosophila melanogaster* to address the contribution of Max to Myc-dependent growth control. We find that many biological activities of Myc do not, or only partly, require the association with Max—for example, the control of endoreplication and cell competition—and that a Myc mutant that does not interact with Max retains substantial biological activity. We further show that Myc can control RNA polymerase III independently of Max, which explains some of Myc's observed biological activities. These studies show the ability of Myc to function independently of Max *in vivo* and thus change the current model of Max network function.

Numerous human tumors are characterized by deregulated expression of c-Myc or the related proteins N-Myc or L-Myc[1]. Myc proteins function as transcription factors to control a large number of protein-coding genes, as well as genes for rRNA and small noncoding RNAs that are transcribed by RNA polymerases I and III, respectively[2]. Myc proteins contain an N-terminal transcription regulatory domain and a C-terminal basic-helix-loop-helix-zipper (BHLHZ) that mediates interaction with the BHLHZ protein Max and is required for binding of the resulting heterodimer to hexameric E-box motifs; at physiological concentrations, Myc proteins cannot homodimerize (reviewed in ref. 2). In addition to activating E-box–containing targets, Myc-Max heterodimers also repress other genes indirectly by binding to, and thereby inhibiting, other transcriptional activators such as Miz-1 (ref. 3). Of note, Max has been shown to be necessary for transcriptional activation and repression by Myc[4–7], as well as for the ability of overexpressed Myc to transform cells, induce apoptosis and trigger cell cycle progression[8,9], and it has been speculated that Max might even be required for the correct folding of the Myc protein[10]. Max also interacts with the BHLHZ-containing Mxd/Mnt proteins (Mxd1–4, Mnt, Mga; reviewed in ref. 11). The resulting heterodimers bind to E-boxes and repress many of the genes that are activated by Myc-Max, thereby functioning as Myc antagonists. As a consequence, the loss of Mnt partially relieves the need for Myc, and cells lacking Mnt show several characteristics of Myc-overexpressing cells[12,13] (reviewed by ref. 11).

The *in vivo* analysis of the Myc-Max-Mxd network in vertebrates is rendered difficult by the redundancy of the *Myc* and *Mxd*/*Mnt* genes. In addition, mice lacking *Max* die before day 6.5 of embryogenesis, and the cellular functions of Max have not been studied in detail[14]. We have therefore initiated a study of this network in *Drosophila melanogaster*. Flies contain a single *Myc* gene (*diminutive*, abbreviated as *dm*; the protein is called Myc) that has an important role in the cell-autonomous control of growth[15,16]. Flies with reduced Myc activity are delayed in their development and ultimately eclose at a reduced size[17], whereas dm^4 animals (null for Myc) experience a normal embryogenesis but then fail to grow and die as small larvae[18]. Myc also has a role in cell-autonomous apoptosis[19] and in a process called cell competition, whereby neighboring cells compare their Myc levels and cells with less Myc are eliminated by apoptosis[20,21] (reviewed in ref. 22). Of note, the function of Myc is evolutionarily conserved such that *Drosophila* Myc can rescue the proliferation defect of $c\text{-}Myc^{-/-}$ murine embryonic fibroblasts[23], and conversely, an isoform of human c-Myc overcomes the lethality of a strong hypomorphic *dm* mutant allele[24]. The transcriptional targets of *Drosophila* Myc fall into similar classes as the vertebrate Myc targets[25,26], and like vertebrate c-Myc[27,28], *Drosophila* Myc stimulates the activity of RNA polymerase I, albeit indirectly by increasing the levels of co-factors for RNA polymerase I[29]. In contrast to Myc, the single Mxd/Mnt homolog in flies, Mnt, is not essential, and flies carrying the null allele Mnt^1 are mainly characterized by a slight increase in body size[30]. Overexpression of Mnt shows the expected growth- and proliferation-inhibiting functions, confirming the role of Mnt as a Myc antagonist.

Here we address the biological function of the central member of the network, Max. Flies contain a single *Max* gene[15,31]. Using RNAi

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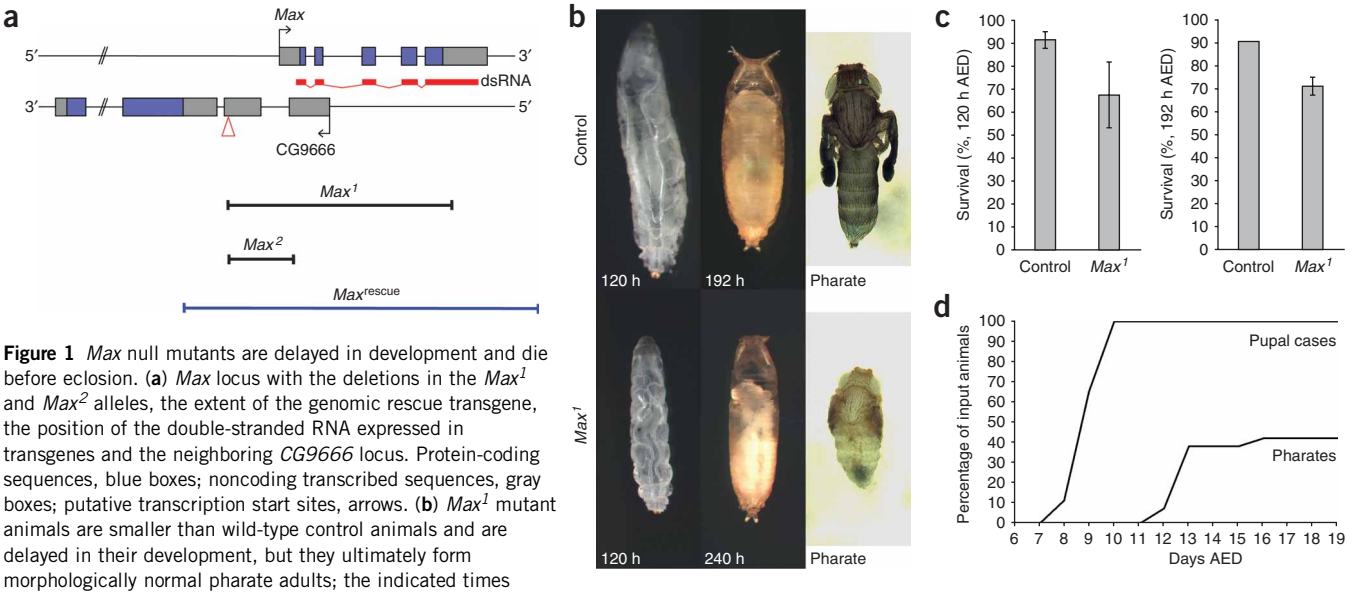

Figure 1 *Max* null mutants are delayed in development and die before eclosion. (**a**) *Max* locus with the deletions in the Max^1 and Max^2 alleles, the extent of the genomic rescue transgene, the position of the double-stranded RNA expressed in transgenes and the neighboring *CG9666* locus. Protein-coding sequences, blue boxes; noncoding transcribed sequences, gray boxes; putative transcription start sites, arrows. (**b**) Max^1 mutant animals are smaller than wild-type control animals and are delayed in their development, but they ultimately form morphologically normal pharate adults; the indicated times correspond to hours after egg deposition (AED). (**c**) Percentage of larvae surviving from the time of collection at 48 h AED to the indicated times (at 120 h AED, $n = 209$ and 131 larvae for control and Max^1, respectively (six independent experiments); at 192 h AED, $n = 60$ and 55 larvae for control and Max^1, respectively (two independent experiments); $P < 0.05$ for both comparisons between Max^1 and control). (**d**) For 26 Max^1 larvae collected at 120 h AED, the graph indicates the percentage that form pupae and pharate adults at the indicated times. Error bars, s.d. between biologically independent replicates.

transgenes and a null mutation in *Max*, we confirm the importance of Max for Myc activity and for the control of growth. We further demonstrate the antagonism between Myc and Mnt and show a partial rescue of the dm^4 mutant phenotype by the Mnt^1 mutation. Surprisingly, however, our analysis reveals the ability of Myc to partially function upon depletion of Max. This Max-independent function of Myc is confirmed by the partial activity of a Myc mutant that lacks the Max-interaction domain and by the overexpression of Myc in the presence of reduced levels of Max. Our experiments also reveal the control of RNA polymerase III as an activity of Myc that does not depend on dimerization with Max. Thus, we provide the first clear *in vivo* evidence for fundamental growth functions of the Myc proto-oncogene that are independent of Max.

RESULTS

Flies lacking *Max* can complete metamorphosis

Using imprecise excision of a P element, we generated a *Max*-null mutant allele, Max^1 (**Fig. 1a** and **Supplementary Note** and **Supplementary Fig. 1** online). Max^1 homozygous animals survive embryogenesis and hatch at the same proportions as controls. However, their growth is impaired and Max^1 animals are smaller than controls at all larval stages, as pupae and as pharate adults (**Fig. 1b** and below); further, they show a reduced viability at larval stages (**Fig. 1c**). Their rate of development is normal up to the molt from second to third instar (at 70–80 h after egg deposition (AED)), but Max^1 third instar larvae pupariate only between days 8 and 10 AED (**Fig. 1d**; wild-type flies pupariate at day 5 AED). Most homozygous mutant animals survive until pupal stages, and 42% undergo metamorphosis to end up as morphologically normal pharate adults, but none of these animals ever eclose (**Fig. 1b**,**d**). Apart from the reduced size, only minor defects were visible in Max^1 animals: third instar larvae had fewer teeth on their mouth hooks and occasionally the tips of these mouth hooks were forked, and most pupae failed to evert their anterior spiracles. No defects were observed in Max^1/+ heterozygous animals, except for a slight developmental delay specifically in animals that were simultaneously mutant for the hypomorphic *Myc* allele dm^{P0}, confirming a genetic interaction between *Max* and *Myc* (**Supplementary Fig. 1d**). Taken together, these defects and the genetic interaction with *Myc* are consistent with a role for Max in the control of growth.

Loss of Max or Myc leads to different phenotypes

Although *Max* is also essential, *Myc* clearly has a more severe mutant phenotype: larvae lacking Myc fail to grow, and most of them die a few days after hatching[18] (see below). This unexpected difference in phenotypic strengths could be explained by three non–mutually exclusive hypotheses. First, maternally deposited Max protein could persist until late in development and mediate some of the observed growth. We consider this explanation unlikely, on the basis of the observed *Max* mRNA abundance and short protein half-life (see **Supplementary Note** and **Supplementary Fig. 2** online), and because the Max^1 phenotypes are not further enhanced by simultaneous targeting of *Max* with RNAi (using a *Max-IR* transgene that otherwise phenocopies a *Max* mutation; **Supplementary Fig. 3** online). Furthermore, the experiments discussed below strongly argue against this possibility, as they demonstrate substantial growth in *Max*-mutant tissues at late times of development and reveal some activity of a mutant form of Myc that is incapable of interacting with Max. Second, loss of *Max* might reflect the simultaneous loss of growth-promoting Myc-Max and growth-inhibiting Mnt-Max complexes. Third, Myc might have functions that do not depend on dimerization with Max and that are lost only in *Myc*- but not in *Max*-mutant animals. To distinguish between these latter two possibilities, we compared Max^1 mutants (lacking Myc-Max and Mnt-Max activities but retaining Max-independent functions of Myc) with animals lacking all Myc and Mnt functions[18,30] (dm^4 Mnt^1 mutants, carrying null alleles for both *Myc* and *Mnt*). dm^4 Mnt^1 mutants, as well as dm^4 Max^1 mutants, grow better and survive to a greater extent than dm^4 mutants (**Fig. 2a**). Thus, elimination of *Mnt* partially relieves the need for Myc in animal development (see also ref. 32). Of note, dm^4 Mnt^1 Max^1 animals behave similarly, demonstrating that Max does not associate with a second repressor protein whose activity would be lost in Max^1 mutants but not in dm^4 Mnt^1.

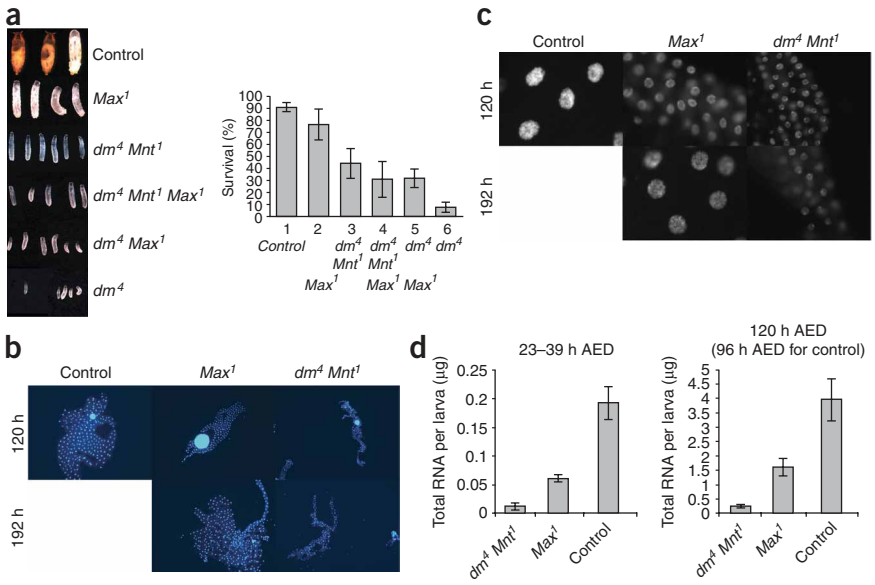

Figure 2 Loss of *Max* leads to milder defects than simultaneous loss of *Myc* and *Mnt*. (**a**) Larvae of the indicated genotypes were collected at 48 h AED into normal food vials and photographed at 120 h AED (left panels); the percentages of surviving animals from 3–6 independent experiments are indicated in the graph on right (Max^1 differs significantly with $P < 0.05$ from all other genotypes except control; number of input larvae were 209, 71, 236, 45, 38 and 147 for genotypes 1 through 6, respectively). (**b**,**c**) Individual fat bodies (**b**) or close-up views of salivary gland nuclei (**c**) at the indicated times of development. All photomicrographs within a panel (**a**,**b** or **c**) are at the same magnification. (**d**) Average total RNA content per larva at the indicated times ($n = 12$ larvae per sample for control at 96 h AED and 20 for all other genotypes; error bars indicate s.d. for 2–8 independent RNA preparations; all genotypes are significantly different from each other at both time points, with $P < 0.05$).

However, Max^1 larvae (as well as Mnt^1 Max^1 larvae; **Supplementary Fig. 4** online) are markedly bigger and show less lethality than any of the other genotypes (**Fig. 2a**). Max^1 animals continue to grow, and the difference compared to the dm^4 Mnt^1 animals becomes even more pronounced at later times (data not shown). The body size differences between these genotypes are reflected in the relative sizes of the polyploid organs and their constituent cells that make up most of the larval mass (**Fig. 2b**,**c**). The Max^1 fat body and salivary gland cells grow markedly between days 5 and 8 (when no more *Max* mRNA can be detected; see below) and reach near wild-type sizes, whereas the growth of dm^4 Mnt^1 cells remains severely stunted (**Fig. 2b**,**c**); this growth defect is cell-autonomous, as it is also seen in clones of mutant cells embedded in a wild-type organ (**Supplementary Fig. 5c** online). The growth defect is paralleled by a strong reduction of total RNA levels in dm^4 Mnt^1 mutants, and a more modest reduction in Max^1 mutants; this effect is observed in young larvae even before overall size differences become apparent, and it persists to later stages (**Fig. 2d**). As most cellular RNA consists of rRNA, this observation suggests a defect in rRNA accumulation in both genotypes, and it further indicates that the Max^1 mutant phenotype is manifest at the very beginning of larval development. Taken together, these observations reveal clear differences between the lack of Max and the simultaneous elimination of Myc and Mnt, strongly suggesting the existence of molecular functions of Myc that do not require the interaction with Max.

Such functions are further supported by the *Myc* allele dm^2 (ref. 33), encoding a Myc protein that is truncated before the leucine zipper. This altered Myc does not bind to Max when ectopically expressed in S2 cells (**Fig. 3a**), consistent with the published requirement for the leucine zipper for the heterodimerization between vertebrate Myc and Max[34–36]. Thus, dm^2 Mnt^1 mutant flies have lost all Max-dependent activities but nevertheless survive better and grow larger than dm^4 Mnt^1 mutants, demonstrating that they retain some Myc function (**Fig. 3b**). To eliminate any potentially remaining Myc-Max functions (potentially originating from translational read-through at the premature stop codon in dm^2), we further combined the dm^2 Mnt^1 chromosome with the Max^1 allele. The resulting dm^2 Mnt^1 Max^1 animals show a less severe growth defect than dm^4 Mnt^1 Max^1 mutants and form larger pupal cases (**Fig. 3c**), again demonstrating that Myc can partially function without binding to Max. Nonetheless, Max^1 mutants grow and survive better than dm^2 Mnt^1 mutant animals (**Fig. 3b**), indicating that a substantial part of the Max-independent functions of Myc requires the presence of the leucine zipper.

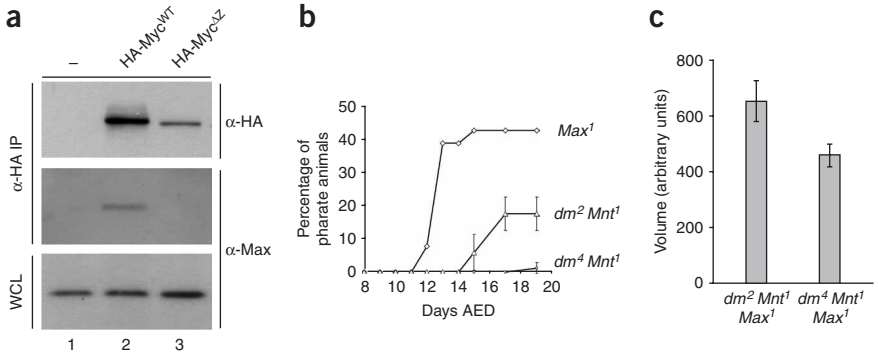

Figure 3 C-terminally truncated Myc does not interact with Max but retains partial function. (**a**) S2 cells were cotransfected with Max and the indicated HA-tagged Myc variants. The two top lanes show immunoblots of anti-HA (α-HA) immunoprecipitates probed with anti-HA or anti-Max antiserum; the bottom lane shows whole-cell lysates probed with anti-Max antiserum. (**b**) Percentage of larvae of the indicated genotypes (collected at 5 d AED) having undergone metamorphosis at the indicated times; on days 17 and 19, the differences between the *Myc*-mutant genotypes are significant with $P < 0.05$ (2 to 3 independent experiments with a total number of 72 and 155 animals for dm^2 Mnt^1 and dm^4 Mnt^1, respectively; the Max^1 data are the same as shown in **Figure 1d**). (**c**) Maximal lengths and widths of nine pupal cases per genotype were measured using Adobe Photoshop and their volumes extrapolated by approximating the pupal shape with a cylinder (the difference is highly significant; $P = 1.4 \times 10^{-5}$). Error bars, s.d.

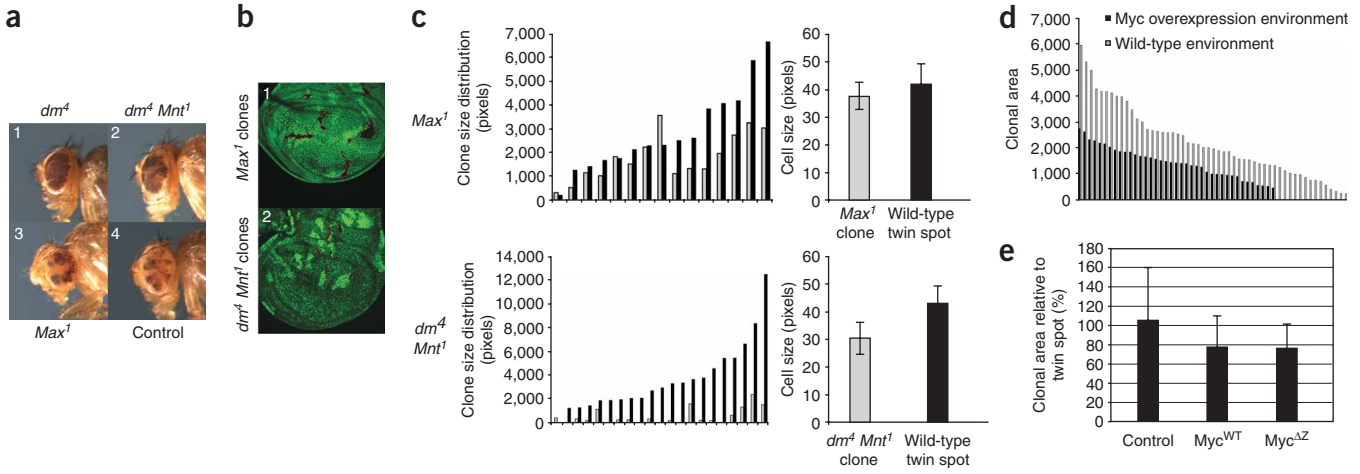

Figure 4 *Max* mutant mitotic clones grow better than *dm Mnt* double-mutant clones. (**a**) Typical adult eyes generated with the "*ey-FLP cell-lethal*" system, containing patches of homozygous mutant ommatidia (white) and heterozygous tissue (red). (**c**) Shown are 72-hour-old mutant clones (black and outlined in red, lacking GFP expression) and their wild-type twin spots (bright color, expressing two copies of a *GFP* transgene) in wing imaginal discs from wandering third instar larvae. (**c**) The left graphs show the size distribution of mutant clones (light bars) and their wild-type twin spots (dark bars); many $dm^4\ Mnt^1$ mutant clones do not survive by 72 h after induction, and therefore their twin spots are unaccompanied. The right graphs depict the average size of the cells making up the indicated clones or twin spots, respectively ($n = 15$–20 clones per sample; twin spot cells differ significantly in size from $dm^4\ Mnt^1$ cells ($P = 1.1 \times 10^{-6}$), but not from Max^1 cells ($P = 0.087$)). (**d**) Sizes of neutral clones (expressing wild-type levels of Myc) in Max^1 wing discs that do (dark bars) or do not (light bars) overexpress Myc; the clones in both genotypes are genetically identical, but their surroundings express different levels of Myc. (**e**) Relative clone areas in discs overexpressing the indicated transgenes (clone area divided by the area of the corresponding twin spot, in percent; average of 20 independent clones per genotype; *P* values for the comparison with control are 0.064 and 0.045 for Myc^{WT} and $Myc^{\Delta Z}$, respectively). Error bars, s.d.

Cellular differences between Max^1 and $dm^4\ Mnt^1$ animals

We also observed clear differences between the different genotypes in diploid imaginal disc cells. Normally patterned mosaic eyes could be generated in which Max^1 homozygous mutant ommatidia occupied a large fraction (using the *ey-FLP cell-lethal* technique[37]; **Fig. 4a**) or even the majority of the eye (using the *ey-FLP Minute* technique; **Supplementary Fig. 5a**), whereas the dm^4 or the $dm^4\ Mnt^1$ mutant ommatidia only ever occupied a small fraction of such mosaic eyes, and the resulting eyes are always rough (**Fig. 4a** and **Supplementary Fig. 5a**). However, occasional bristles produced by Max^1 homozygous cells are tiny (**Supplementary Fig. 5a**,**b**), indicating that Max is essential for the normal development of bristles but not of ommatidia.

We observed similar differences in mitotic clones in the wing disc, where many $dm^4\ Mnt^1$ mutant clones are eliminated by cell competition[20–22] and where the remaining clones are significantly smaller and contain significantly smaller cells than their wild-type sister clones (**Fig. 4b**,**c**). In marked contrast, Max^1 mutant clones persist and are only moderately smaller than their wild-type sister clones, and they are made up of normally sized cells (**Fig. 4b**,**c**). These differences cannot be explained by persisting Max protein because as a consequence of dilution (in average, these clones consist of 47 cells) and degradation (the age of the clones corresponds to more than seven Max half-lives), these clones can only contain negligible amounts of Max protein. These observations confirm the difference between $dm^4\ Mnt^1$ and Max^1 mutants, and they show that cells depleted for Max maintain a normal size and that loss of Max entails less cell competition than loss of Myc.

To further characterize this effect on cell competition, we overexpressed Myc in Max^1 wing imaginal discs and induced clones of cells lacking the overexpression construct; in a wild-type (Max^+) background, such clones suffer from competition by the surrounding, Myc-overexpressing cells[20,21]. Similarly, in a Max^1 background, such clones are smaller than genetically identical control clones of equal age (**Fig. 4d**), indicating that at least some cell competition takes place in Max^1 mutants. In further support of this notion, the Myc derivative $Myc^{\Delta Z}$ is capable of inducing cell competition to the same extent as Myc^{WT}, such that clones lacking a ubiquitously expressed $Myc^{\Delta Z}$ transgene in heterozygous wing discs are smaller than similarly aged neutral clones in control wing discs (**Fig. 4e**). Taken together, these

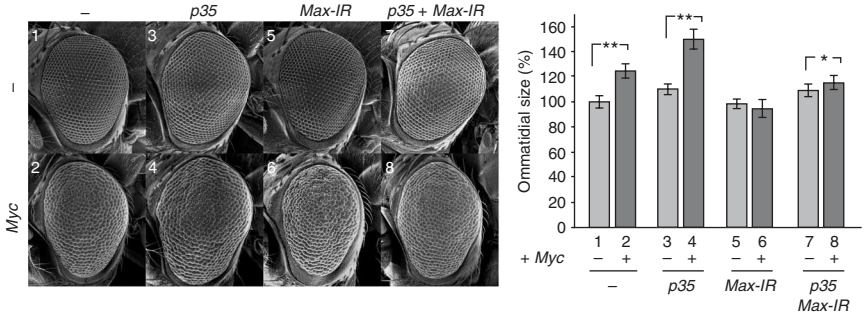

Figure 5 Myc overexpression in the eye induces growth and apoptosis in a largely Max-dependent and Max-independent manner, respectively. Left panel, scanning electron micrographs of adult eyes overexpressing the indicated transgenes. Right panel, average ommatidial sizes for the corresponding genotypes (percentage of genotype 1). For each bar, the sizes of 20 centrally located ommatidia derived from 6 independent eyes were determined; error bars indicate s.d. Significance of differences between genotypes differing only by the ectopic expression of Myc are denoted; $^*P < 0.05$ and $^{**}P < 0.001$.

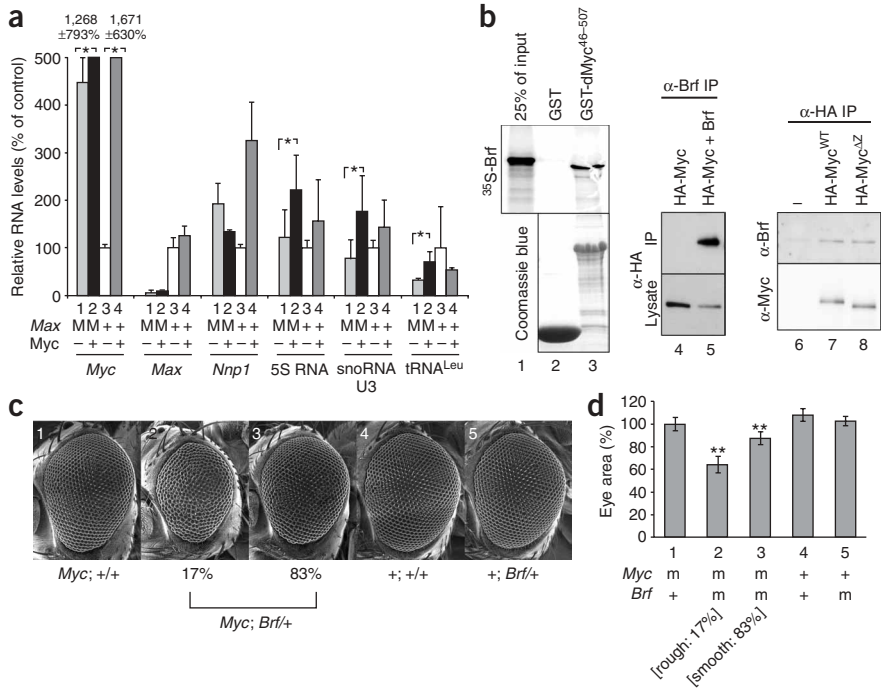

Figure 6 Myc interacts with Brf and can induce RNA Pol III targets independently of Max. (**a**) mRNA levels at 9–11 h after a heat-shock in 4-day-old (*Max*^{1}/+, indicated by "+") or 5-day-old (*Max*^{1}, indicated by "M") larvae ($n = 2$–8 samples; $^{*}P < 0.05$). The low level of signal in the *Max* PCR reaction in the *Max*^{1} genotypes results from unspecific amplification (as ascertained by agarose gel electrophoresis). (**b**) Physical interaction of Brf with Myc. Left panels, *in vitro* translated ^{35}S-labeled Brf specifically binds to a GST-Myc$^{46\text{-}507}$ (upper panel, lane 3); the lower panel shows the GST proteins from the same gel. Center panels, antibodies to Brf specifically retrieve HA-Myc from S2 cells transfected with both HA-Myc and Brf (lane 5). Right panels, anti-HA immoprecipitates from S2 cells expressing HA-Myc^{WT} (lane 7) or HA-Myc$^{\Delta Z}$ (lane 8) both contain endogenous Brf protein. (**c**) heterozygosity for *Brf* causes a defect in eyes that are mutant for *Myc* (genotype "*w dm*^{P0} *tub>Myc>GAL4 ey-FLP*/Y"), as revealed by scanning electron microscopy (eyes labeled *Myc*; *Brf*/+). Such a defect is only rarely seen in control eyes (labeled "*Myc*; +/+" and "+; *Brf*/+", respectively). (**d**) Average eye size ($n = 5$–6 independent eyes; genotype 2: 4 eyes). Samples 2 and 3 have the same genotype, but only eyes in sample 2 have a defective morphology. *t*-tests are indicated for the comparisons between genotypes 1 and 2/3 and between genotypes 4 and 5 with $^{**}P < 0.01$. Error bars, s.d.

experiments strongly argue that association with Max is not necessary for Myc-induced cell competition.

Biological activity of Myc in Max-depleted tissues

As a complementary approach to characterize Max-independent functions of Myc, we overexpressed Myc in the eye and simultaneously targeted *Max* with RNA interference; as described in the **Supplementary Note**, *Max-IR* transgenes (expressing dsRNA against *Max*) phenocopy a *Max* mutation—they strongly decrease *Max* mRNA levels, reduce growth and genetically interact with *Myc* mutants (**Supplementary Fig. 3**). Strong overexpression of Myc with *GMR-GAL4* (using three *UAS-Myc* transgenes) has previously been shown to stimulate growth[38] and cell-autonomous apoptosis[19] (such 'cell-autonomous apoptosis' does not require juxtaposition of cells with different levels of Myc as in cell competition associated apoptosis but only excessive levels of Myc in the apoptotic cell itself; **Fig. 5** and **Supplementary Fig. 6a**,**b** online). Downregulation of *Max* by RNAi (or by titration of the Max protein; **Supplementary Fig. 6c**) abrogates Myc-induced overgrowth (**Fig. 5**) but does not suppress the associated roughness and the loss of pigment cells (**Fig. 5** and **Supplementary Fig. 6a**; similar effects are observed in a *Mnt*^{1} mutant background, indicating that they cannot be ascribed to the loss of Mnt-Max complexes; **Supplementary Fig. 6d**). These latter effects are strongly reduced by co-expression with the viral pan-caspase inhibitor p35, suggesting that they are associated with apoptosis. Simultaneous expression of p35 and interference with *Max* reduces both Myc-induced apoptosis and growth and results in eyes that are (of all genotypes) the most similar to controls (**Fig. 5** and **Supplementary Fig. 6a**,**b**). Thus, strong overexpression of Myc induces both apoptosis, which is largely independent of Max, and growth, which (to a large extent) requires Max. Consistent with this notion, ectopic expression of Myc$^{\Delta Z}$ cannot increase the size of *dm*^{4}-mutant ommatidia but is able to induce apoptosis (**Supplementary Fig. 6e**). Note that moderate Myc overexpression (with one *UAS-Myc* transgene) in wild-type eyes induces no detectable apoptosis but nevertheless a substantial increase in size, which is only partially abolished by RNAi against *Max* (**Supplementary Fig. 6f**), suggesting that the Max-independent functions may also contribute to growth.

Molecular basis of the Max-independent functions of Myc

To characterize the effect of Myc on its transcriptional targets in the absence of Max, we conditionally overexpressed Myc in *Max*^{1} animals and analyzed the consequences on target gene expression (see Methods). As expected, mRNA levels for *Myc* are strongly elevated in the genotypes carrying a Myc transgene, and *Max* mRNA levels are undetectable in the *Max*^{1} mutants (**Fig. 6a**); endogenous *Myc* levels are also increased in *Max*^{1} mutants, consistent with the notion that Myc autorepresses its own transcription[39] and that Max is required for this process[6]. The E-box–dependent Myc-Max target gene *Nnp-1* (ref. 26) is strongly induced by Myc overexpression in control animals but not in *Max*^{1} mutants, demonstrating strict Max dependency of this prototypical Myc target and confirming the absence of functional Max protein in this genotype (the increased *Nnp-1* levels in *Max*^{1} mutants may reflect the simultaneous loss of repression by Mnt-Max complexes or the difficulty of comparing absolute transcript levels between *Max*^{1} and control larvae). Thus, the Max-independent effects of Myc cannot be explained by the activation of classical, E-box–containing Myc targets. We were unable to identify other Max-independent transcriptional targets of Myc by comparing the genome-wide transcriptomes of S2 cells treated with dsRNA against either *Myc* or *Max* (ref. 26 and data not shown). However, recent publications suggest that vertebrate Myc proteins also control RNA polymerase III targets that are not represented on standard genome-wide microarrays[40]. Indeed, we found that RNAi against *Myc* in S2 cells significantly reduced the levels of the Pol III targets tRNA^{Leu} and snoRNA U3 (ref. 41), as assayed by qRT-PCR (by 27 ± 2% and 34 ± 6%, respectively, as compared to control

cells treated with *gfp*-dsRNA; average ± s.d. of two biologically independent replicates). Conversely, overexpression of Myc in wild-type larvae induced the expression of the two Pol III targets 5S RNA and snoRNA U3 (**Fig. 6a**), and they, as well as $tRNA^{Leu}$, were induced even more efficiently by Myc upon depletion of Max (**Fig. 6a**). Taken together, these data constitute the first evidence that Myc stimulates Pol III activity in insects and, furthermore, that Myc can do so upon depletion of Max. In addition, Myc is required for the synthesis of rRNA (see above, **Fig. 2d**)—that is, the stimulation of Pol I. However, this process seems to involve Max, presumably via the Myc-Max–dependent activation of Pol I cofactors such as dTIF-1A[29].

In vertebrates, the effect of Myc on Pol III has been shown to be mediated by the TFIIIB-component Brf[40]. Two lines of evidence suggest that the same holds true for *Drosophila*. First, Myc interacts physically with Brf, both *in vitro* and in transiently transfected S2 cells (**Fig. 6b**). This interaction can be seen with C-terminally truncated forms of Myc that do not bind Max, both in S2 cells and *in vitro* (**Fig. 6b**). Second, *Myc* interacts genetically with *Brf*, as documented for the eye-specific allele $ey>dm^{P0}$ (ref. 42). $ey>dm^{P0}$ flies[42] (**Fig. 6c**,**d**) develop normally and eclose with phenotypically wild-type eyes that contain slightly smaller ommatidia, but simultaneous heterozygosity for *Myc*-interacting genes such as *pont* results in a reduction in ommatidial size and number and in marked eye defects[42]. Of note, heterozygosity for Max^1 does not affect eye development of dm^{P0}/Y animals, consistent with the observation that eyes containing large Max^1 clones develop normally (**Fig. 4a** and **Supplementary Fig. 5a**). In contrast, heterozygosity for a *Brf* mutation leads to a statistically significant reduction in eye size (9%; $P = 0.01$) and induces clear eye defects in 17% of the animals, whereas less than 2% of control animals have such defects (**Fig. 6c**,**d**). Similar eye defects were also observed with heterozygosity for a deficiency uncovering the *Brf* locus (data not shown). Taken together, these data show that Myc can activate RNA polymerase III without requiring association with Max, and they suggest that this effect is mediated by a direct physical interaction between Myc and the Pol III co-factor Brf.

DISCUSSION

Myc is one of the most potent human proto-oncogenes and growth regulators, and Max has been generally accepted to be an indispensable partner for Myc. In this work, we show that a substantial part of the function of *Drosophila* Myc does not require association with Max. This contention is based on three lines of evidence. First, simultaneous loss of Myc and its only known antagonist, Mnt, (in dm^4 Mnt^1 mutants) impairs growth and proliferation at the cellular, tissue and organismal level significantly more than loss of *Max* (in Max^1 mutants). Second, Myc can activate target genes and trigger biological responses even upon depletion of Max. Third, an altered Myc lacking the leucine zipper retains some biological activity, even though it is incapable of binding to Max. These observations cannot be attibuted to maternally deposited Max protein—the massive reduction of total RNA in young Max^1 larvae indicates that Max levels are already strongly reduced at this early time point. Furthermore, clear differences between dm^4 Mnt^1 and Max^1 animals (with respect to organismal growth, cell competition, growth and proliferation of polyploid tissues and of mitotic clones) are manifest at much later times of development when no more *Max* mRNA can be detected and (given its short half-life) most Max protein must have decayed.

These observations strongly argue that Myc can support development to pharate adult stage even upon depletion of Max, which contrasts with the early requirement for Max in mice[14]. We believe that these different outcomes reflect the developmental flexibility of flies, which respond to the growth defect imposed by the loss of Max by extending their development by at least three days, rather than molecular differences between mammalian and insect Myc proteins. Indeed, *Drosophila* Myc and vertebrate c-Myc can largely substitute for each other, and two recent studies in tissue culture also raised the possibility of Max-independent functions of Myc in vertebrates[43,44].

The observation that mutant Myc lacking the C terminus can only partially substitute for wild-type Myc suggests that Myc acts in different Max-independent protein complexes, some of which require the Myc C terminus whereas others do not. The identity of other partners for the C terminus of Myc are currently unknown, but several candidate proteins have been described in vertebrate systems (reviewed by ref. 45). However, our work reveals one partially C terminus–independent activity of Myc: we show that Myc can induce the expression of RNA polymerase III targets; this effect is presumably mediated by an interaction between the RNA Pol III co-factor Brf and a part of the Myc protein that does not include the C terminus. Thus, like its vertebrate counterparts, Myc controls all three RNA polymerases and thereby coordinates the production of ribosome components.

Although at present we can only speculate about the relative importance of the different branches of Myc-dependent processes, our experiments have shown several biological processes that can take place (to a large extent) upon depletion of Max, such as cell-autonomous cell death caused by Myc overexpression in the eye, the development of ommatidial precursor cells, endoreplication of polyploid larval cells, cell competition and the control of cell size. This observation comes as a surprise, as the well-characterized Myc-Max targets have been generally assumed to drive growth and possibly endoreplication. Given the functional conservation of Myc in evolution, we consider it likely that many activities of vertebrate Myc will also not require association with Max.

METHODS

Myc protein derivatives. For ectopic expression of Myc in S2 cells, we cloned a cDNA coding for full-length Myc containing an N-terminal triple HA-tag into the vector pUASTattB[46]. The mutant derivative lacking the leucine zipper ($HA\text{-}Myc^{\Delta Z}$, containing a multiple HA-epitope tag and Myc amino acids 1 to 675; the stop codon is in the same position as in the dm^2 allele) was expressed from an analogous construct.

To express $HA\text{-}Myc^{WT}$ or $HA\text{-}Myc^{\Delta Z}$ ubiquitously *in vivo*, the same cDNAs were cloned under the control of the *αTub84B* promoter in pBSattB or under the control of UAS-sites in pUASTattB and integrated into the attP site zh86Fb[46]; for control purposes, a transgenic line was established containing the empty vector pBSattB at zh86Fb. All sequences are available on request.

Fly culture. Flies were kept on standard *Drosophila* medium. Test crosses were performed in climate-controlled chambers at 25 °C.

Phenotypic analysis. To determine survival rates, 6–10 h egg lays were typically performed on apple agar plates supplemented with yeast paste. At 48 h AED, we transferred defined numbers of larvae to tubes containing standard fly food and determined their survival rates at the indicated time points. For photography, we first froze larvae and then photographed them using a Zeiss AxioCam HRc camera attached to a Zeiss Stemi SV 11 microscope.

Adult fly eyes were recorded on a JEOL JSM-6360 LV scanning electron microscope at ×180 magnification. We determined the area of 20 central ommatidia (from at least 5 independent eyes per genotype) using Adobe Photoshop. Fusions of two ommatidia were counted as two individual ommatidia. We determined eye sizes by multiplying the number of ommatidia in individual eyes (as counted from scanning electron micrographs) with average ommatidial size (as measured in the same eye).

Clonal analysis. For a description of the ey-*FLP* system, see ref. 47.

For mitotic clones in wing discs, 6 h egg lays were performed on standard food, 15–30 min heat shocks (in a 37° water bath) were given at 48 h AED, and larvae dissected and fixed 72 h later. Expression of *hs-GFP* was induced by a 1 h heat-shock 3 h before dissection. Wing discs were recorded on a Leica SP2 confocal microscope (at ×40). Clonal areas and cell sizes (calculated from clonal areas and from the counts of nuclei) were determined with Adobe Photoshop. The frequency of surviving 72 h old clones was determined from 24 and 106 twin spots for dm^4 Mnt^1 and Max^1, respectively.

To assess cell competition in a Max^1 background, we subjected Max^1 larvae carrying the *tub>Myc>GAL4* (">" indicating an FRT site) or the *actin>CD2>GAL4* transgene to heat shock for 12 min and 5 min, respectively, in a 37° water bath at 120 h AED and dissected them at 216 h AED. Clones (53 from 14 discs for *act>CD2>GAL4*; 39 from 6 discs for *tub>Myc>GAL4)* were photographed (at ×10) on a Leica DMRA compound microscope.

Microscopy. Larval imaginal discs and polyploid tissues were dissected, fixed in 4% paraformaldehyde in PBS for 15 min and washed in PBS three times for 10 min. We visualized nuclei with 0.5 µg/ml Hoechst 33342 included in the second washing step. Wing discs were mounted in Vectashield mounting solution.

Molecular analysis. To extract RNA, we homogenized larvae (12 to 20 larvae per sample for qRT-PCR) for 1 min in 1 ml of TRIZOL reagent (Invitrogen) with a Polytron tissue homogenizer. The homogenate was frozen at –80 °C for at least 1 d and then processed according to the manufacturer's protocol. We then redissolved the precipitated RNA in 20 µl ddH_2O and determined its concentration with a Nanodrop ND-1000 spectrophotometer.

We removed contaminating genomic DNA by treatment with the TURBO DNA-free kit (Ambion), and analyzed the purified RNA on a Bioanalyzer chip (Agilent). cDNA was synthesized from 1 µg of template RNA per sample using the Omniscript Reverse Transcription Kit (Qiagen) and random hexamer primers. Parallel control reactions containing only RNA provided templates for "–RT" samples. We carried out qRT-PCR reactions in triplicates on an ABI 7900 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 expression level of *Act5C* as an internal reference for each biological sample. Primer sequences and PCR conditions are described in the **Supplementary Methods** online.

Characterization of Myc targets in Max^1 larvae. We induced ubiquitous Myc overexpression in 4.3- to 5.2-day-old Max^1 larvae (at a stage where *Max* is undetectable) by giving a strong heat-shock to *hs-FLP actin>CD2>GAL4 UAS-Myc* Max^1 larvae. Such a treatment triggers expression of GAL4 and hence expression of Myc in virtually all cells; these animals will continue their development and ultimately die during metamorphosis. To minimize indirect consequences of Myc activation, we extracted and processed total RNA as described above at 9 to 11 h after the induction of Myc and then analyzed it by qRT-PCR. As controls, we used identically treated animals that lacked the Myc transgene and/or were heterozygous for Max^1.

***In vitro* interaction.** Full-length Brf (*Drosophila* Gold Collection clone LD32109 in pOT2a) was *in vitro* translated in 25 µl TNT lysate (Promega) according to the manufacturer's instructions, in the presence of 15 µCi ^{35}S-labeled methionine (GE Amersham). GST and GST-$Myc^{46\text{-}507}$ were produced in bacteria and bound to glutathione beads (detailed protocol available upon request), resuspended in 260 µl Binding Buffer (200 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, 10% glycerol, Complete Mini Protease inhibitors (Roche)) and incubated with 10 µl of *in vitro* translation mixture for 2–3 h at 4°. After four washes in Wash Buffer (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40), bead-bound proteins were analyzed by SDS-PAGE, followed by Coomassie blue staining (to reveal the GST proteins) and exposure to a phosphorimager (to detect the radiolabeled Brf).

Tissue culture experiments. *Drosophila* Schneider S2 cells were cultured and transfected as previously described[42]. Briefly, 5 × 10^6 cells in a 3.5-cm well were transfected in 1 ml of serum-free medium with 10 µl Cellfectin (Gibco) and 10 µg plasmid DNA, containing 3.3–4 µg tubulin-GAL4 and the balance in UAS-plasmids; 16–17 h after transfection, the cells were re-fed with complete medium. After 24–48 h, we lysed the cells in lysis buffer (250 mM NaCl, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.5% NP-40) containing a cocktail of protease inhibitors (Roche). After incubation on ice for 30–60 min, the lysates were precleared with protein G-sepharose beads, then incubated with primary antibodies for 3–6 h at 4°, followed by another incubation with protein G-sepharose beads for 1–2 h. We then washed the beads 3–5× with lysis buffer and resuspended them in SDS-PAGE sample buffer, and analyzed the equivalent of 40–50% of each well by SDS-PAGE followed by immunoblotting. Antibodies for immunoprecipitations were mouse anti-HA (Covance) or rabbit anti-Brf[48]; primary antibodies for protein blotting were rabbit anti-HA (Roche) or mouse anti-Myc[49], rabbit anti-Brf[48], mouse anti-Max.

Reagents. Sources of original fly lines are indicated in the **Supplementary Table 1** online; Brf ^{c07161} is caused by a piggyBac insertion in the third intron. Anti-Myc antibodies were first described by Prober and Edgar[50]; anti-Brf was a gift from S. Takada (MD Anderson)[48].

Fly genotypes used in the figures. Figure 1: panel **b**, "*y w*", "*y w*; Max^1" and "*y w*; Max^1/*TM3, Ser* y^+" (as control for pharates); panel **c**, "Oregon R" and "*w*; Max^1"; panel **d**, "*w*; Max^1".

Figure 2: panel **a**, "Oregon R" (1); "*w*; Max^1" (2); "*w* dm^4 Mnt^1/*Y*" (3); "*w* dm^4 Mnt^1/*Y*; Max^1" (4); "*w* dm^4/*Y*; Max^1" (5); "*w* dm^4/*Y*" (6); panels **b** and **c**: "*y w*", "*w*; Max^1", "*w* dm^4 Mnt^1/*Y*"; panel **d**, "*w* dm^4 Mnt^1/*Y*", "*w*; Max^1" (23–39 h AED) and "*y w*/*Y*, y^+; Max^1" (120 h AED), "*y w*".

Figure 3: panel **b**, "*w*; Max^1"; "*w* dm^4 Mnt^1/*Y*", "*y w* dm^2 Mnt^1/*Y*"; panel **c**, "*y w* dm^2 Mnt^1/*Y*; Max^1", "*w* dm^4 Mnt^1/*Y*; Max^1".

Figure 4: panel **a**, "*w* dm^4 *FRT19/y* cl^{15b} *FRT19; Sp/ey-FLP*" (1); "*w* dm^4 Mnt^1 *FRT19/y* cl^{15b} *FRT19; Sp/ey-FLP*" (2); "*y w ey-FLP/Y; Max*^{1} *FRT80/*cl^{w+} *FRT80*" (3); "*y w FRT19/y* cl^{15b} *FRT19; ey-FLP/+*" (4); panels **b** and **c**, "*w* dm^4 Mnt^1 *FRT19/y w hs-GFP FRT19 hs-FLP*", "*y w hs-FLP; Max*^{1} *FRT80/ubi-GFP FRT80*"; panel **d**, "*y w hs-FLP; actin>CD2>GAL4 UAS-GFP Max*^{1}/Max^1" (light bars), "*y w tub>Myc>GAL4 hs-FLP; UAS-GFP/+; Max*^{1}" (dark bars); panel **e**, "*y w hs-FLP/y w; FRT82B (zh86Fa)/FRT82B ubi-GFP*" (empty vector control), "*y w hs-FLP/y w; FRT82B tub-*Myc^{WT}*(zh86Fa)/FRT82B ubi-GFP*", "*y w hs-FLP/y w; FRT82B tub-*Myc^{AZ}*(zh86Fa)/FRT82B ubi-GFP*".

Figure 5: "*y w/Y; GMR-GAL4/+*" (1); "*y w/Y; GMR-GAL4 UAS-*Myc^{132}*/+; UAS-*Myc^{13} *UAS-*Myc^{42}*/+*" (2); "*y w/Y; GMR-GAL4/+; UAS-p35/+*" (3); "*y w/Y; GMR-GAL4 UAS-*Myc^{132}*/+; UAS-*Myc^{13} *UAS-*Myc^{42}*/UAS-p35*" (4); "*y w/Y; GMR-GAL4/UAS-Max-*$IR^{2\text{–}7}$" (5); "*y w/Y; GMR-GAL4 UAS-*Myc^{132}*/UAS-Max-*$IR^{2\text{–}7}$; *UAS-*Myc^{13} *UAS-*Myc^{42}*/+*" (6); "*y w/Y; GMR-GAL4/UAS-Max-*$IR^{2\text{–}7}$; *UAS-p35/+*" (7); "*y w/Y; GMR-GAL4 UAS-*Myc^{132}*/UAS-Max-*$IR^{2\text{–}7}$; *UAS-*Myc^{13} *UAS-*Myc^{42}*/UAS-p35*" (8).

Figure 6: panel **a**, "*y w hs-FLP; actin>CD2>GAL4 UAS-GFP Max*^{1}/Max^1" (1); "*y w hs-FLP; UAS-*Myc^{132}*/+; actin>CD2>GAL4 UAS-GFP Max*^{1}/Max^1" (2); "*y w hs-FLP; actin>CD2>GAL4 UAS-GFP Max*^{1}*/+*" (3); "*y w hs-FLP; UAS-*Myc^{132}*/+; actin>CD2>GAL4 UAS-GFP Max*^{1}*/+*" (4). Panel **c**, "*w* dm^{P0} *tub>Myc>GAL4 ey-FLP/Y*" (1); "*w* dm^{P0} *tub>Myc>GAL4 ey-FLP/Y; Brf*^{c07161}*/+*" (2,3); "*w* dm^{rev} *tub>Myc>GAL4 ey-FLP/Y*"; (4), "*w* dm^{rev} *tub>Myc>GAL4 ey-FLP/Y; Brf*^{c07161}*/+*" (5). Panel **d**, as in **c**.

Note: Supplementary information is available on the Nature Genetics website.

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1. Vita, M. & Henriksson, M. The Myc oncoprotein as a therapeutic target for human cancer. *Semin. Cancer Biol.* **16**, 318 (2006).
2. Dang, C.V. *et al.* The c-Myc target gene network. *Semin. Cancer Biol.* **16**, 253 (2006).
3. Wanzel, M., Herold, S. & Eilers, M. Transcriptional repression by Myc. *Trends Cell Biol.* **13**, 146–150 (2003).
4. Kretzner, L., Blackwood, E.M. & Eisenman, R.N. Myc and Max proteins possess distinct transcriptional activities. *Nature* **359**, 426–429 (1992).
5. Amati, B. *et al.* Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max. *Nature* **359**, 423–426 (1992).
6. Facchini, L.M., Chen, S.J., Marhin, W.W., Lear, J.N. & Penn, L.Z. The Myc negative autoregulation mechanism requires Myc-Max association and involves the c-Myc P2 minimal promoter. *Mol. Cell. Biol.* **17**, 100–114 (1997).
7. Mao, D.Y. *et al.* Analysis of Myc bound loci identified by CpG island arrays shows that Max is essential for Myc-dependent repression. *Curr. Biol.* **13**, 882–886 (2003).
8. Amati, B. *et al.* Oncogenic activity of the c-Myc protein requires dimerization with Max. *Cell* **72**, 233–245 (1993).
9. Amati, B., Littlewood, T.D., Evan, G.I. & Land, H. The c-Myc protein induces cell cycle progression and apoptosis through dimerization with Max. *EMBO J.* **12**, 5083–5087 (1993).
10. Adhikary, S. & Eilers, M. Transcriptional regulation and transformation by Myc proteins. *Nat. Rev. Mol. Cell Biol.* **6**, 635–645 (2005).
11. Hurlin, P.J. & Huang, J. The MAX-interacting transcription factor network. *Semin. Cancer Biol.* **16**, 265 (2006).
12. Hurlin, P.J. *et al.* Deletion of Mnt leads to disrupted cell cycle control and tumorigenesis. *EMBO J.* **22**, 4584–4596 (2003).
13. Nilsson, J.A. *et al.* Mnt loss triggers Myc transcription targets, proliferation, apoptosis, and transformation. *Mol. Cell. Biol.* **24**, 1560–1569 (2004).
14. Shen-Li, H. *et al.* Essential role for Max in early embryonic growth and development. *Genes Dev.* **14**, 17–22 (2000).
15. Gallant, P., Shiio, Y., Cheng, P.F., Parkhurst, S.M. & Eisenman, R.N. Myc and Max homologs in *Drosophila*. *Science* **274**, 1523–1527 (1996).
16. Schreiber-Agus, N. *et al. Drosophila* Myc is oncogenic in mammalian cells and plays a role in the diminutive phenotype. *Proc. Natl. Acad. Sci. USA* **94**, 1235–1240 (1997).
17. Johnston, L.A., Prober, D.A., Edgar, B.A., Eisenman, R.N. & Gallant, P. *Drosophila* myc regulates cellular growth during development. *Cell* **98**, 779–790 (1999).
18. Pierce, S.B. *et al.* dMyc is required for larval growth and endoreplication in *Drosophila*. *Development* **131**, 2317–2327 (2004).
19. Montero, L., Müller, N. & Gallant, P. Induction of apoptosis by *Drosophila* Myc. *Genesis* **46**, 104–111 (2008).
20. De La Cova, C., Abril, M., Bellosta, P., Gallant, P. & Johnston, L.A. *Drosophila* myc regulates organ size by inducing cell competition. *Cell* **117**, 107–116 (2004).
21. Moreno, E. & Basler, K. dMyc transforms cells into super-competitors. *Cell* **117**, 117–129 (2004).
22. Gallant, P. Myc, cell competition, and compensatory proliferation. *Cancer Res.* **65**, 6485–6487 (2005).
23. Trumpp, A. *et al.* c-Myc regulates mammalian body size by controlling cell number but not cell size. *Nature* **414**, 768–773 (2001).
24. Benassayag, C. *et al.* Human c-Myc isoforms differentially regulate cell growth and apoptosis in *Drosophila melanogaster*. *Mol. Cell. Biol.* **25**, 9897–9909 (2005).
25. Orian, A. *et al.* Genomic binding by the *Drosophila* Myc, Max, Mad/Mnt transcription factor network. *Genes Dev.* **17**, 1101–1114 (2003).
26. Hulf, T. *et al.* Whole-genome analysis reveals a strong positional bias of conserved dMyc-dependent E-boxes. *Mol. Cell. Biol.* **25**, 3401–3410 (2005).
27. Grandori, C. *et al.* c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. *Nat. Cell Biol.* **7**, 311–318 (2005).
28. Arabi, A. *et al.* c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. *Nat. Cell Biol.* **7**, 303–310 (2005).
29. Grewal, S.S., Li, L., Orian, A., Eisenman, R.N. & Edgar, B.A. Myc-dependent regulation of ribosomal RNA synthesis during *Drosophila* development. *Nat. Cell Biol.* **7**, 295–302 (2005).
30. Loo, L.W. *et al.* The transcriptional repressor dMnt is a regulator of growth in *Drosophila melanogaster*. *Mol. Cell. Biol.* **25**, 7078–7091 (2005).
31. Gallant, P. Myc / Max / Mad in invertebrates - the evolution of the Max network. *Curr. Top. Microbiol. Immunol.* **302**, 235–253 (2006).
32. Pierce, S.B. *et al. Drosophila* growth and development in the absence of dMyc and dMnt. *Dev. Biol.* **315**, 303 (2008).
33. Maines, J.Z., Stevens, L.M., Tong, X. & Stein, D. *Drosophila* dMyc is required for ovary cell growth and endoreplication. *Development* **131**, 775–786 (2004).
34. Blackwood, E.M. & Eisenman, R.N. Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science* **251**, 1211–1217 (1991).
35. La Rocca, S.A., Crouch, D.H. & Gillespie, D.A. c-Myc inhibits myogenic differentiation and myoD expression by a mechanism which can be dissociated from cell transformation. *Oncogene* **9**, 3499–3508 (1994).
36. Nair, S.K. & Burley, S.K. X-ray structures of Myc-Max and Mad-Max recognizing DNA. Molecular bases of regulation by proto-oncogenic transcription factors. *Cell* **112**, 193–205 (2003).
37. Newsome, T.P., Asling, B. & Dickson, B.J. Analysis of *Drosophila* photoreceptor axon guidance in eye-specific mosaics. *Development* **127**, 851–860 (2000).
38. Secombe, J., Li, L., Carlos, L. & Eisenman, R.N. The Trithorax group protein Lid is a trimethyl histone H3K4 demethylase required for dMyc-induced cell growth. *Genes Dev.* **21**, 537–551 (2007).
39. Goodliffe, J.M., Wieschaus, E. & Cole, M.D. Polycomb mediates Myc autorepression and its transcriptional control of many loci in *Drosophila*. *Genes Dev.* **19**, 2941–2946 (2005).
40. Gomez-Roman, N., Grandori, C., Eisenman, R.N. & White, R.J. Direct activation of RNA polymerase III transcription by c-Myc. *Nature* **421**, 290–294 (2003).
41. Isogai, Y., Takada, S., Tjian, R. & Keles, S. Novel TRF1/BRF target genes revealed by genome-wide analysis of *Drosophila* Pol III transcription. *EMBO J.* **26**, 79–89 (2007).
42. Bellosta, P. *et al.* 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 (2005).
43. Wert, M., Kennedy, S., Palfrey, H.C. & Hay, N. Myc drives apoptosis in PC12 cells in the absence of Max. *Oncogene* **20**, 3746–3750 (2001).
44. Cowling, V.H. & Cole, M.D. The Myc transactivation domain promotes global phosphorylation of the RNA polymerase II carboxy-terminal domain independently of direct DNA binding. *Mol. Cell. Biol.* **27**, 2059–2073 (2007).
45. Kleine-Kohlbrecher, D., Adhikary, S. & Eilers, M. Mechanisms of transcriptional repression by Myc. *Curr. Top. Microbiol. Immunol.* **302**, 51–62 (2006).
46. Bischof, J., Maeda, R.K., Hediger, M., Karch, F. & Basler, K. An optimized transgenesis system for *Drosophila* using germ-line-specific phiC31 integrases. *Proc. Natl. Acad. Sci. USA* **104**, 3312–3317 (2007).
47. Stocker, H. *et al.* Rheb is an essential regulator of S6K in controlling cell growth in *Drosophila*. *Nat. Cell Biol.* **5**, 559–565 (2003).
48. Takada, S., Lis, J.T., Zhou, S. & Tjian, R.A. TRF1:BRF complex directs *Drosophila* RNA polymerase III transcription. *Cell* **101**, 459 (2000).
49. Prober, D.A. & Edgar, B.A. Growth regulation by oncogenes–new insights from model organisms. *Curr. Opin. Genet. Dev.* **11**, 19–26 (2001).
50. Prober, D.A. & Edgar, B.A. Ras1 promotes cellular growth in the *Drosophila* wing. *Cell* **100**, 435–446 (2000).