# *Drosophila* Myc

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## *I. Abstract*

Myc genes play a major role in human cancer, and they are important regulators of growth and proliferation during normal development. Despite intense study over the last three decades, many aspects of Myc function remain poorly understood. The identification of a single Myc homolog in the model organism *Drosophila melanogaster* more than 10 years ago has opened new possibilities for addressing these issues. This review summarizes what the last decade has taught us about Myc biology in the fruit fly.

## *II. Abbreviations*

| | |
|---|---|
| BHLHZ | basic region-helix-loop-helix-leucine zipper |
| CNS | central nervous system |
| *dm* | *diminutive* (= *Drosophila melanogaster* Myc gene) |
| Dpp | Decapentaplegic (a *Drosophila melanogaster* TGFβ homolog) |
| FRT | FLP recombinase target |
| GMC | ganglion mother cell |
| GSC | germline stem cell |
| H3K4me3 | histone H3, trimethylated on lysine 4 |
| Hh | Hedgehog |
| Inr | Insulin receptor |
| MB1/2/3 | Myc box 1/2/3 |
| SID | Sin3-interaction domain |
| TOR | target of rapamycin |
| UAS | upstream activating sequence |
| Wg | Wingless (a *Drosophila melanogaster* Wnt homolog) |
| ZNC | zone of non-proliferating cells |

## *III. Introduction: the Myc/Max/Mxd network in vertebrates*

Myc is amongst the most intensely studied genes in biomedicine - more than 19'000 articles dealing with Myc can be found in PubMed (Meyer and Penn, 2008). Several recent publications have extensively reviewed different aspects of Myc function (Dang et al., 2006; Cowling and Cole, 2006; Pirity et al., 2006; Vita and Henriksson, 2006; Cole and Cowling, 2008; Eilers and Eisenman, 2008; Meyer and Penn, 2008). Therefore, I will only briefly summarize some key features of vertebrate Myc proteins. The main part of this review is dedicated to the characterization of Myc in the fruit fly *Drosophila melanogaster*: what this protein does in insects, how it does it and how its activity is controlled.

The "Myc saga" began more than 30 years ago with the identification of the first Myc genes as the transforming principles of different avian retroviruses. Subsequent research identified the cellular homologs c-, N- and L-Myc in vertebrates. The corresponding proteins were found to be frequently overexpressed in human and animal tumors and to causally contribute to the development of cancer, as demonstrated in numerous animal models. The transforming power of Myc could be traced back to Myc's ability for influencing a variety of cellular processes, most notably growth, cell cycle progression, apoptosis, cell migration, cell adhesion, and stem cell behavior. Most of these processes are also controlled by Myc proteins in physiological situations and during normal development. Myc's versatility is explained by its molecular activity as a transcription factor that controls hundreds if not thousands of target genes, including genes transcribed by RNA polymerases I, II and III. However, each of these targets is only moderately affected by Myc, typically by 2- to 3-fold.

Myc proteins consist of an N-terminal transcription regulatory domain containing the highly conserved “Myc boxes” 1 and 2 (MB1 and MB2), an ill-defined central region with another conserved sequence called Myc box 3 (MB3), and a C-terminal basic region-helix-loop-helix-leucine zipper domain (BHLHZ), that mediates heterodimerization with another BHLHZ-domain protein, Max (“Myc-associated protein X”), as well as binding to DNA. Myc:Max heterodimers recognize so-called E-boxes (CACGTG, and variants thereof), and activate the expression of nearby genes. In addition to binding to all members of the Myc family, Max also homodimerizes, and it interacts with the Mxd proteins (Mxd1 – 4, formerly known as Mad1, Mxi2, Mad3, Mad4, respectively), with Mnt and with Mga. All these Max-partners contain BHLHZ domains and their heterodimers with Max control similar genes as Myc:Max dimers, but in contrast to Myc:Max heterodimers, they repress the corresponding targets. Accordingly, these Max partners function as antagonists of Myc. Besides activating many target genes, Myc:Max dimers also repress a distinct set of targets; Myc:Max does not recognize these Myc-repressed genes by directly binding to DNA at E-boxes, but indirectly via the interaction with other DNA-bound transcription factors. Finally, Myc has recently also been shown to control DNA replication independently of transcription.

As diverse as the transcriptional targets of Myc are the co-factors recruited by Myc to control the expression of these targets. They include the histone acetyltransferases GCN5, Tip60 and CBP, the INI1 chromatin remodeling complex, the P-TEFb protein kinase that phosphorylates the C-terminal domain of RNA polymerase II, and several proteins that have no known enzymatic functions or that participate in different multiprotein complexes. For most target genes, it is currently

unclear to which extent individual co-factors contribute to their Myc-dependent regulation.

## *IV. The Myc/Max/Mnt network in flies*

The search for a Myc/Max/Mxd network in invertebrates was initially motivated by the need for a simple model system – a system that contains less gene redundancy than vertebrates, that is genetically tractable and that is more easily accessible at all stages of development. Widely used models such as yeasts and worms turned out to lack Myc genes (although *C.elegans* contains two Max genes and one gene coding for a Mxd-like protein; Yuan et al., 1998), but *Drosophila melanogaster* fit the bill: fruit flies carry one gene each coding for Myc, Max and for a Mad-family member protein. *Drosophila* Myc has even been known to biologists long before the vertebrate Myc genes. In 1935, a mutation was described that results in a small adult body size, disproportionally small bristles and female sterility (Bridges, 1935). Based on these phenotypes, the affected gene was dubbed "*diminutive*", abbreviated as "*dm*". Many years later, molecular cloning revealed the identity of *diminutive* with the *Drosophila* Myc gene (Gallant et al., 1996; Schreiber-Agus et al., 1997). According to *Drosophila* conventions this gene should therefore be called *diminutive/dm*; to minimize confusion I will refer to the gene and protein as "Myc" in the following text and to the mutant alleles as "$dm^X$" (where X is the allele identifier).

### A. Basic properties of the Myc/Max/Mnt proteins in flies

*Drosophila* Myc was identified in yeast 2-hybrid screens with human Max as the bait (Gallant et al., 1996; Schreiber-Agus et al., 1997). Subsequent 2-hybrid screens used first *Drosophila* Myc as the bait to clone *Drosophila* Max (Gallant et al., 1996), and then *Drosophila* Max as the bait to fish out *Drosophila* Mnt (Loo et al., 2005); Mnt was also identified independently based on the published *Drosophila* genome

sequence (Peyrefitte et al., 2001). All three proteins show clear sequence similarity to their vertebrate counterparts. Thus, Myc is 26 % identical in its overall amino acid sequence to human c-, N- and L-Myc, and it contains the conserved sequence motifs MB2 (whose role in transactivation and –repression was demonstrated for vertebrate Myc) and MB3 (of unknown function), as well as a BHLHZ domain at its C-terminus (Figure 1). Furthermore, vertebrate and insect Myc genes have an identical genomic organization: in all cases the major open reading frame starts at the beginning of the second exon and ends in the third exon, and the second intron interrupts the open reading frame at the same codon within the conserved MB3 (reviewed by Gallant, 2006).

*Drosophila* Mnt also shares the functionally identified domains with the vertebrate Mnt and Mxd proteins (although the sequence similarity is higher to vertebrate Mnt): an N-terminally located SID ("Sin3-Interaction Domain" that mediates binding to the transcriptional co-repressor Sin3) and a centrally positioned BHLHZ (Figure 1). Interestingly, two Mnt splice variants have been identified that lack either the SID or the leucine zipper, suggesting the existence of protein variants that either do not repress transcription (MntΔSID) or do not bind to Max and DNA (MntΔZ), and thereby might act as antagonists of the full-length variant of Mnt (Loo et al., 2005). Finally, Max is the most highly conserved component of the whole network, with 52% overall amino acid sequence identity to human Max protein, and an identical genomic organization (reviewed by Gallant, 2006).

The *Drosophila* Myc, Max & Mnt proteins also share biochemical similarities with their vertebrate homologs: in both vertebrates and *Drosophila*, Myc and Mnt only interact with Max, whereas Max is also able to homodimerize (in addition, Myc also

has certain functions that are independent of its dimerization with Max, see below). Furthermore, in band shift assays all possible types of dimers (Myc:Max, Mnt:Max, Max:Max) bind to the same E-box sequence that is also recognized by the corresponding vertebrate complexes (and Myc has also been shown to bind an E-box in a target gene promoter in tissue culture cells; Hulf et al., 2005). Myc:Max dimers activate, and Mnt:Max dimers repress, transcription from artificial reporters (Gallant et al., 1996; Hulf et al., 2005; Loo et al., 2005). Finally, *Drosophila* and vertebrate Myc proteins can even functionally substitute for each other: *Drosophila* Myc can collaborate with activated Ras to transform rat embryo fibroblasts (Schreiber-Agus et al., 1997), and it overcomes the proliferation block in mouse embryonic fibroblasts that lack the endogenous c-Myc gene (Trumpp et al., 2001). Conversely, human c-MycS (a translation variant of c-Myc with a truncated N-terminus) rescues the development of flies carrying the lethal Myc allele $dm^{PG45}$ (Benassayag et al., 2005).

These observations show that the Myc/Max/Mnt network has been conserved during evolution, and they suggest that whatever we learn about Myc function in flies is relevant for our understanding of vertebrate Myc biology. What then is the function of *Drosophila* Myc?

## B. Biological functions

As is the case for its vertebrate homologs, overexpression or down-regulation of *Drosophila* Myc affects several cellular processes (Figure 2). Some of these processes may be dependent on each other, but the molecular nature of such putative connections is as yet unknown, and therefore the individual activities of Myc will be treated separately below. However, if there is any unifying theme behind Myc's different biological activities, it is the control of size. Most of the individual activities listed below somehow conspire to control the size of cells, of organs and of the whole animal.

### *1. Drosophila as an experimental system*

Before delving into the biological properties of Myc and consorts, I need to briefly introduce the model system and some of the principal experimental techniques that made these analyses possible in the first place. For a more detailed description of the biology and experimental analysis of *Drosophila melanogaster* the reader is referred to several excellent treatises (e.g. Greenspan, 2004; Ashburner et al., 2005; Dahmann, 2008).

The fruit fly develops in about 10 days from the egg to the adult (under optimal growth conditions at 25°). Along the way, the fly spends 1 day in embryogenesis, 4 days in larval stages (3 different larval stages, or "instars"), and the last 5 days immobilized in a pupal case where it metamorphoses into an adult. Of particular interest for scientists studying growth and proliferation is the larval phase, since this period is characterized by a massive, 200-fold increase in weight, but as yet little cellular differentiation. Most of the larval mass is found in different polyploid tissues,

e.g. fat body, salivary gland, and muscles. These tissues attain their final cell number already during embryogenesis and afterwards only endoreplicate their genomes without undergoing cell division, reaching ploidies of up to 2000 N and accordingly large nuclear volumes. During metamorphosis, most of these polyploid tissues are histolysed and their contents used by diploid imaginal tissues (abdominal histoblasts and imaginal discs that give rise to adult appendages and body wall structures) for their own growth. These imaginal discs consist of an epithelial monolayer of columnar cells that proliferate near-exponentially during larval phases and are subject to similar regulatory mechanisms as typical vertebrate cells.

A large number of experimental techniques have been developed to manipulate these different cell types. For example, by expressing the yeast recombinase FLP (from a heat-shock inducible or a tissue-specifically expressed transgene) mitotic recombination can be induced between two homologous chromosomes that each carry an FRT site ("FLP-recombinase target"), resulting in two daughter cells that are homozygous for either the corresponding paternal or maternal chromosome, including any mutation that is located on these chromosomes (or more precisely: the part of the chromosome that is distal to the FRT site). By following the descendants of such homozygous mutant cells (i.e. clones), the properties of mutations can be determined *in vivo*, even if such mutations are lethal at the organismic level and do not allow the animals to develop to a stage where they can be analyzed (reviewed in Xu and Harrison, 1994). A large number of reagents also exist that allow controlled overexpression of transgenes. Many of these rely on the temporally or spatially controlled expression of the yeast transactivator GAL4 (by transgenes where specific artificial or endogenous enhancers control the expression of GAL4) together with

transgenes containing a cDNA under the control of GAL4-responsive UAS elements ("upstream activating sequences"). Many hundreds different GAL4 lines and even more different UAS lines currently exist. Hence, by crossing such flies together, an enormous variety of transgene expression patterns can be achieved (reviewed in Brand et al., 1994). The GAL4/UAS- and the FLP/FRT-systems can also be combined such that heat-shock induced FLP expression triggers FRT-mediated recombination within a GAL4-expressing transgene, leading to the constitutive expression of GAL4 (Pignoni and Zipursky, 1997). By keeping the heat-shock conditions mild (i.e. incubating the larvae for only a few minutes at the inducing temperature) FLP is induced in only a few random cells per animal, and hence GAL4 can drive the expression of UAS-transgenes in only these few cells. Such cells then go on to form clones, and the behavior of these clones (most typically size, shape, cell number) can be assayed at freely chosen times after their induction. Such timed induction of GAL4 can also be used for polyploid tissues, although the "clones" in these tissues only consist of one polyploid cell each (if the heat-shock is given after the end of embryogenesis).

This is only a small selection from the vast and ever-growing "*Drosophila* toolkit", but I hope that it facilitates the understanding of the following text.

### *2. Cellular growth*

The observation of the small adult flies carrying the hypomorphic Myc allele $dm^1$ immediately revealed Myc's involvement in size control (see above, Bridges, 1935). In more detailed studies it was later shown that reduction of Myc levels decreases the size of larval diploid cells (Johnston et al., 1999) and of Schneider S2 cells grown in culture, while at the same time slowing down passage through G1 phase (Hulf et al.,

2005). As a consequence, cells depleted of Myc accumulate to lower numbers than untreated cells (Boutros et al., 2004). Conversely, overexpression of Myc in clones of diploid wing imaginal disc cells increases the size of the clones and of the cells constituting these clones, without affecting cell number (i.e. division rates). Myc overexpression is able to accelerate passage through G1 phase, but these cells compensate by extending their G2 phase. When the cell cycle regulator Cdc25/String (which is limiting for entry into M-phase) is co-expressed with Myc, both gap phases are shortened and cell division times are significantly reduced. Such Myc + Cdc25/String co-expressing clones are equally large as clones expressing Myc alone, but the former consist of an increased number of normally sized cells, whereas the latter contain the same number of cells as control clones, albeit these cells are much bigger in size (Johnston et al., 1999). These properties of Myc contrast with those of a typical cell cycle regulator such as Cyclin E: down-regulation of Cyclin E also impairs progression into S-phase and leads to accumulation of G1-phase cells, but at the same time allows growth to continue unabated, thus resulting in bigger than normal cells (Hulf et al., 2005). This demonstration that Myc controls cellular growth in flies was echoed by similar findings in vertebrates, revealing another evolutionary conservation of Myc function (Iritani and Eisenman, 1999; Schuhmacher et al., 1999).

Thus, in addition to its (in vertebrates) long-accepted role in influencing passage from G1- to S-phase, Myc also controls the increase in cellular mass. This effect is likely to be explained by the nature of Myc's transcriptional targets. Like its vertebrate homologs, *Drosophila* Myc controls the expression of a large number of genes, possibly many hundreds of them (Orian et al., 2003; Hulf et al., 2005). These genes

fall into different functional categories, but many of them play a role in ribosome biogenesis, such as the RNA helicase Pitchoune whose vertebrate homolog MrDb/DDX18 is also a Myc target (Zaffran et al., 1998; Grandori et al., 1996) and Modulo, a putative homolog of the vertebrate Myc target Nucleolin (Perrin et al., 2003; Greasley et al., 2000). Myc also contributes to ribosome biogenesis by stimulating RNA polymerases I and III (Grewal et al., 2005; Steiger et al., 2008), as do its vertebrate counterparts (Gomez-Roman et al., 2003; Arabi et al., 2005; Grandori et al., 2005). In contrast to vertebrates, however, the activation of RNA polymerase I by Myc occurs indirectly, presumably via the RNA polymerase II-dependent activation of RNA polymerase I cofactors such as TIF-1A (Grewal et al., 2005). Thus, activation of Myc presumably leads to a general increase in cellular translational capacity, resulting in increased growth.

Interestingly, the different proteins that have been shown to promote an increase in cell size (i.e. “growth”) do so in qualitatively different ways. Thus, the insulin receptor (Inr) pathway differs from Myc in that it has a prominent effect on the cytoplasmic volume of polyploid cells and on the level of the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Britton et al., 2002, and see below). Also, unlike Myc,the growth-promoting Cyclin D/Cdk4 complexes stimulate, and are critically dependent on, mitochondrial activity (Frei et al., 2005). These differences emphasize the different molecular mechanisms that underlie different types of “growth”, and they suggest ways how growth regulators could collaborate even though all ultimately control the rate of cellular size increase.

*3. DNA synthesis*

Myc also strongly influences the nuclear size of polyploid cells in larvae (fat bodies, salivary glands, muscles) and in adult egg chambers (somatic follicle cells and germline-derived nurse cells). In these cells Myc predominantly controls the rate of endoreplication and hence DNA content: whereas overexpression increases DNA content in polyploid larval cells by up to 8-fold (Pierce et al., 2004; Berry and Baehrecke, 2007; Demontis and Perrimon, 2009), mutation of Myc strongly reduces the ploidy of such larval or ovarian cells (Maines et al., 2004; Pierce et al., 2004; Pierce et al., 2008; Steiger et al., 2008; Demontis and Perrimon, 2009). Myc does not seem to affect the onset of endoreplication, since Myc overexpression does not induce premature endocycles (at least in follicle cells; Shcherbata et al., 2004), although forced Myc expression can extend the duration of endoreplication (Pierce et al., 2004). It is not clear whether Myc is also required for sub-genomic polyploidization, i.e. the amplification of specific genes. Thus, chorion genes are amplified in wild type follicle cells after they have become polyploid, and this chorion gene amplification was reported to occur normally in follicle cell clones that are homozygous for a strong Myc-allele $dm^2$ and that are surrounded by phenotypically wild type tissue (Maines et al., 2004). In contrast, females that are homozygous for the weak Myc-allele $dm^{P1}$ show reduced chorion gene amplification in their follicle cells (Quinn et al., 2004). The reason for these differences is unclear, but the $dm^{P1}$ mutant flies clearly suffer from reduced growth rates throughout their body, and it is conceivable that this systemically impacts the behavior of follicle cells (e.g. via reduced levels of circulating growth factors).

Myc activity has less dramatic effects on DNA replication in diploid cells. On one hand, Myc overexpression does not trigger polyploidization in diploid cells (and only shortens the duration of G1-phase). On the other hand, the loss of Myc slows down G1-phase and overall cell division rates, but has a comparatively mild effect on the structure of diploid tissues (imaginal discs). This can be seen in *Myc Mnt* double mutant animals, where polyploid tissues remain severely stunted as compared to wild type animals (and, as a consequence, such double mutant larvae are considerably smaller than the control). In contrast, diploid imaginal discs show normal patterns of proliferation and differentiation, and they develop to comparable sizes as wild type discs, although they do so more slowly and require several days more for this process (Pierce et al., 2008). The same analysis cannot be carried out in *Myc* single mutant animals, since they die before the third larval instar when most of the size increase of imaginal discs takes place. However, a genetic trick allows the generation of *Myc*-mutant eye imaginal discs within an animal that is otherwise functionally wild type for *Myc*. Such flies develop to fully viable adults with surprisingly normal-looking eyes and heads that are composed of Myc-mutant cells, although these organs are clearly smaller than in the control (Steiger et al., 2008; Schwinkendorf and Gallant, 2009).

There are two reports, though, showing dramatic effects of forced Myc expression on cellular proliferation. Ectopic expression of different transcription factors in developing eye-antennal imaginal discs strongly interferes with their development, and often results in flies lacking heads altogether (Jiao et al., 2001). This defect can be largely overcome by co-expression of Myc, but also by co-expression with Cyclin E which specifically controls cell cycle progression, suggesting that in such an

artificial situation Myc is able to stimulate the proliferation of diploid imaginal disc cells (Jiao et al., 2001). Similarly, certain mutations in the transcription factor Prd produce male flies with strongly reduced cellularity in their accessory glands, and these deficits can be overcome by ectopic expression of Myc or of Cyclin E (Xue and Noll, 2002). The molecular basis of these effects has not been analyzed, and it is therefore not known whether Myc directly stimulates the cell cycle machinery or whether the effect is more indirect.

The effects of Myc on DNA replication could be mediated by different transcriptional targets. In genome-wide and directed expression analyses several cell cycle regulators have been found to respond to changes in Myc levels, e.g. dE2F1, RBF, different cyclins, Stg/Cdc25, but it is unclear whether these constitute direct Myc targets (Orian et al., 2003; Duman-Scheel et al., 2004; Hulf et al., 2005). A better characterized, presumably directly Myc activated gene is the "DNA-replication element binding factor" DREF that itself controls the expression of DNA-replication related genes such as dE2F, dPCNA, and Cyclin A (Thao et al., 2008). Interestingly, the DREF-binding site (DRE) is significantly enriched in the promoters of Myc activated genes, raising the possibility that Myc might also cooperate with DREF in controlling the expression of S-phase specific targets (Orian et al., 2003). In addition to directly controlling DNA replication specific genes, Myc may also influence endoreplication rates indirectly, via the same targets that promote growth and overall cell size increases in diploid cells. For example, the S-phase regulator Cyclin E (which is also essential for endoreplication) has been shown to be controlled posttranscriptionally by Myc (at least in imaginal disc cells, but the same may hold true for polyploid cells as well; Prober and Edgar, 2000), possibly via Myc's effect on

ribosome biogenesis and hence protein synthesis (Grewal et al., 2005). Finally, it is conceivable that Myc influences DNA replication directly in a transcription-independent manner, as has been shown for vertebrate Myc (Dominguez-Sola et al., 2007). However, such an activity has not been demonstrated in *Drosophila* so far.

Interestingly, Myc has little (if any) effect on cytoplasmic and overall size in polyploid cells. This contrasts with Myc's command on the size of diploid cells (see above), but also with the ability of another growth-regulator, the insulin signaling pathway, to control polyploid cell size (e.g. Demontis and Perrimon, 2009). It is conceivable that Myc's effect on diploid and on polyploid cells are mediated by different sets of targets and constitute separate biological activities of Myc. Alternatively, the same downstream effectors of Myc control both diploid cell and polyploid cell behaviors, but the two cell types are wired differently to respond either with cytoplasmic growth or with endoreplication, respectively.

### 4. *Apoptosis*

We have seen that overexpression of Myc increases the size of the affected cells and organs, but there are limits to this growth-stimulating activity. Excessive Myc activity triggers apoptosis that can overcome the gain in tissue mass caused by Myc-induced growth (with the definition of "excessive" depending on tissue and developmental stage). Thus, high level Myc overexpression in eye imaginal discs is accompanied by different hallmarks of apoptosis, such as activation of Caspase 3 and DNA fragmentation as revealed by TUNEL- and acridine orange-staining (Montero et al., 2008). The resulting adult eyes are disorganized and rough, they all but lack a particular cell type (pigment cells), and their ommatidia are smaller than those of flies expressing more moderate levels of Myc – attributes that presumably

reflect the death of some cells during ommatidial differentiation, and hence the absence of these cells from the mature ommatidia (Steiger et al., 2008). Signs of apoptosis are also seen upon Myc overexpression in wing imaginal discs (de La Cova et al., 2004; Benassayag et al., 2005; Montero et al., 2008), and expression of a mutant form of Myc (with a presumably slightly higher activity than wild type Myc) in clones of cells leads to their elimination from the wing disc as a consequence of apoptosis (Schwinkendorf and Gallant, 2009). In contrast, Myc overexpression does not stimulate or inhibit the autophagic cell death of 3rd instar larval polyploid salivary gland cells, nor does a Myc mutation induce autophagy, indicating that some tissue types and some modes of cell death are not affected by Myc (Scott et al., 2004; Berry and Baehrecke, 2007).

Importantly, this ability of Myc to induce cell death is not only observed upon overexpression. In hypomorphic Myc mutants, where Myc activity is reduced by three- to five-fold (but not completely eliminated), some forms of cell death are impaired, as would be expected if Myc has a normal role in controlling this process. Thus, $dm^{P0}$ homozygous females do not show the nurse cell death that normally occurs in late-stage egg chambers, and this presumably contributes to the sterility of these flies (Quinn et al., 2004). Also, $dm^{P0}$- and $dm^{P1}$-mutant wing imaginal discs show a significantly reduced incidence of apoptosis upon exposure to low doses of X-rays (up to 10 Gy), although higher doses (50 Gy) evoke similar apoptotic responses in wild type and Myc-mutant cells (Montero et al., 2008).

The molecular pathway by which Myc influences apoptosis is poorly understood. Myc overexpression leads to the upregulation of p53 mRNA within one hour of Myc induction, raising the possibility that Myc directly activates transcription of p53.

However, p53 is not required for the Myc-dependent apoptosis, since Myc equally efficiently triggers cell death in p53 null mutant wing imaginal disc cells (Montero et al., 2008). In contrast, heterozygosity for chromosomal deletions that simultaneously eliminate the four pro-apoptotic genes *hid*, *grim*, *reaper* and *sickle* (or only three of them) strongly reduces Myc-induced apoptosis in wing discs, indicating that these proteins are important for this process (de La Cova et al., 2004; Montero et al., 2008). These four proteins have previously been shown to bind and inactivate the caspase-inhibitor dIAP1, resulting in caspase activation and cell death (Steller, 2008). Their expression is induced by a variety of pro-apoptotic stimuli, including Myc – and the kinetics of induction of *reaper* and *sickle* by Myc is comparably rapid as that of p53. Thus, Myc might transcriptionally activate these genes, presumably by direct binding of Myc:Max heterodimers to E-boxes located in their regulatory regions (Montero et al., 2008). However, Myc can also induce cell death through other pathways that do not involve E-box containing target genes. This was shown in experiments where Myc's partner Max was knocked down (Steiger et al., 2008). Myc requires Max for binding to E-boxes, and down-regulation of Max abrogates Myc's ability to induce E-box dependent targets and promote overgrowth in the eye – but it leaves intact the ability of overexpressed Myc to trigger apoptosis. This suggests that Max-independent activities such as the activation of RNA polymerase III (see below) contribute to Myc's pro-apoptotic actions, but the relative contributions of E-box dependent and independent targets, and possible differences between different tissues and different developmental stages, have not been explored in detail. Furthermore, it is not known whether physiological levels of Myc (that are required for

the normal apoptotic response to DNA damage, as described above) affect apoptosis via the same pathways as overexpressed Myc.

*5. Cell competition*

The notion that Myc affects apoptosis cell-autonomously is familiar to scientists studying Myc in vertebrates. In addition, *Drosophila* Myc also influences cell death non-autonomously in neighboring cells, in a process called “cell competition”.

“Cell competition” was first described 30 years ago in a study of a class of mutants called *Minutes* (Morata and Ripoll, 1975; Simpson and Morata, 1981). There are more than 50 different *Minute* loci in flies, and we now know that most (perhaps all) of them code for ribosomal proteins (Lambertsson, 1998). Homozygous *Minute* mutations are cell-lethal, as would be predicted; even heterozygosity for a *Minute* mutation reduces cellular proliferation rate and extends the overall duration of development, but ultimately such *Minute*/+ animals eclose with a normal morphology, although their bristles are more slender than those of wild type flies (Lambertsson, 1998). The process of cell competition is observed when cell clones are generated during imaginal disc development such that *Minute*/+ cells are juxtaposed to +/+ cells. While it would be expected that the former grow more slowly than the latter and ultimately occupy an accordingly smaller area, the growth defect of *Minute*/+ cells has more dramatic consequences: these cells are killed by the contact with their faster growing, healthier neighbors and tend to disappear altogether from the wing tissue – even though such *Minute*/+ cells would have the potential to give rise to a complete adult animal as we have seen above. The demise of these *Minute*/+ cells is prevented if the growth rate of the surrounding cells is also decreased (e.g. by heterozygosity for a different *Minute* mutation), or if they are separated from the

competing cells by a compartment boundary; i.e. slow-growing cells in the posterior compartment of a wing imaginal disc are not affected by adjacent wild type cells in the anterior compartment. The final size of the resulting wing is not changed by the cell competition taking place during larval wing development, and it has been proposed that cell competition serves as a quality control mechanism to replace "unfit" cells by their healthier neighbors (de La Cova et al., 2004).

Cell competition is thought to arise from differences in growth rates between adjacent cells, and additional growth regulators have been proposed to affect cell competition, e.g. components of the Hippo tumor suppressor pathway (Tyler et al., 2007) and most notably Myc. A moderate reduction of Myc levels still allows for the development of phenotypically normal (albeit small) animals, but the same reduction of Myc levels in clones triggers their elimination if they are surrounded by phenotypically wild-type cells (Johnston et al., 1999). Conversely, overexpression of Myc leads to the death of surrounding *wild type* cells, even though they are perfectly healthy, making these Myc-overexpressing cells "super-competitors" (de La Cova et al., 2004; Moreno and Basler, 2004). This process can be triggered by remarkably small differences in Myc levels between adjacent cells (presumably two-fold or even less), which distinguishes cell competition from the cell-autonomous apoptosis that is induced by comparatively high-level Myc overexpression only (Moreno and Basler, 2004). The study of Myc-dependent cell competition also suggested an additional biological function for this process: when apoptosis (and hence cell competition) was blocked during the development of wing imaginal discs, the resulting adult wings showed considerably higher variability in their sizes, although the average size was

the same as in control. Thus, cell competition might also serve to reduce the consequences of “developmental noise” (de La Cova et al., 2004).

The mechanism that senses the subtle differences in Myc activity is currently under investigation. Some effector components of the “cell competition pathway” have been identified. For example, engulfment of competed Minute/+ cells by their wild-type neighbors was shown to be essential not only for the removal of the dead cells, but also for allowing these cells to die in the first place (Li and Baker, 2007). In the case of Myc-induced competition the pro-apoptotic gene *hid* also plays an important role: competed cells up-regulate *hid*, and heterozygosity for this gene virtually eliminates Myc-dependent cell competition and allows wing disc compartments containing competed cells to overgrow (de La Cova et al., 2004). However, neither *hid* nor the engulfment factors explain how differences in cellular growth rate are sensed in the first place, and the question remains how the competition process is initiated. A candidate upstream factor is the signaling pathway activated by the TGFβ-homolog Dpp. In a competing environment *Minute*/+ cells transduce the Dpp signal with reduced efficiency as compared to their surviving neighbors, leading to excessive expression of the Dpp-repressed gene *Brinker*, followed by activation of the kinase Jnk and subsequent apoptosis (Moreno et al., 2002). The involvement of Jnk signaling downstream of Myc-dependent competition remains controversial, though (de La Cova et al., 2004), and it has been suggested that it is the experimental heat-shock treatment that leads to the activation of Jnk, rather than cell competition *per se* (Tyler et al., 2007). Consistent with a possible involvement of Dpp signaling in cell competition, different mutants that prevented the competition of *Minute*/+ cells also re-established Dpp signaling activity (Tyler et al.,

2007). Conversely, upregulation of the Dpp-pathway in cells suffering from Myc-dependent competition also rescued their survival (Moreno and Basler, 2004), as did the elimination of the Dpp-effector Brinker or its putative transcriptional co-factor dNAB (Ziv et al., 2009). The defect in Dpp signaling in the competed cells has been suggested to result from impaired endocytosis (Moreno and Basler, 2004), but it is still enigmatic which signals could mediate the slight initial differences in Myc activity between neighboring cells and subsequently lead to reduced endocytosis and presumably additional defects that induce a cell to die. Such signals are likely to be diffusible, since cell competition was observed at a distance of up to 8 cell diameters between the competed and the competing cell (de La Cova et al., 2004). To find these signals a cell-culture based system was developed where Myc-overexpressing *Drosophila* Schneider cells induce apoptosis in naïve Schneider cells (Senoo-Matsuda and Johnston, 2007). This system mimics several aspects of the cell competition observed in the animal (e.g. the ability of Myc-expressing “super competitors” to induce apoptosis without direct cell-cell contact), and there is hope that this approach, or a genetic screen similar to the one recently published (Tyler et al., 2007), will soon unravel the molecular basis of cell competition. Investigations of cell competition are fuelled by an interest for its role during normal insect development, but in part also by the speculation that an analogous process might contribute to human cancers that are characterized by overexpression of one of the Myc oncoproteins, although currently no data exist to support this notion (Moreno, 2008).

A discussion of cell competition would be incomplete without mentioning the phenomenon of “compensatory proliferation” (reviewed by Fan and Bergmann,

2008). The term originates from the observation that different types of abuse (e.g. strong irradiation, prolonged heat-shock) will kill the majority of imaginal disc cells, but nevertheless allow the eclosion of normally shaped adults, since the surviving cells increase their proliferation rate and thus replace the dead cells. Before they die, such mortally wounded cells synthesize different patterning factors (Wg, Dpp, Hh, depending on the tissue type) that might induce the compensatory proliferation of the surrounding cells. Whereas the connections between compensatory proliferation and cell competition have not been extensively investigated, it is tempting to speculate that (while they are dying) the competed cells feed back on the competing cells and further stimulate their growth, thus helping to reinforce the "fitness difference" between the "winners" and the "losers". To date there is no evidence for a specific involvement of Myc in compensatory proliferation, but it is interesting to note that larvae carrying a hypomorphic Myc mutation are more sensitive to ionizing irradiation than control animals (Jaklevic et al., 2006), even though their wing disc cells show a reduced rate of apoptosis (Montero et al., 2008). One possible explanation for this observation is that these animals might suffer from a defect in compensatory proliferation. However, the increased sensitivity to irradiation is not restricted to *Myc* mutations, as disruption of other growth regulators (e.g. Cdk4, the Insulin pathway) results in a similar defect (Jaklevic et al., 2006). Given the current interest in cell competition, compensatory proliferation and Myc, it is likely that any missing molecular links between these three will soon be uncovered.

### *6. Asymmetric stem cell division*

Another similarity between vertebrates and *Drosophila* resides in the involvement of Myc in stem cell biology. One tissue where this function of Myc has been studied is

the female germline. Oogenesis in *Drosophila* takes place in about 18 ovarioles per ovary (reviewed in Fuller and Spradling, 2007; Bastock and St Johnston, 2008). At one end of each of these ovarioles resides a stem cell niche harboring 2 to 3 germ-line stem cells (GSCs). These stem cells undergo asymmetric divisions, producing another GSC and a differentiating cystoblast, which will divide four more times to form an egg chamber that then develops into an oocyte. Myc protein is highly expressed in the GSCs, but drops to low levels in their daughter cystoblasts (by a poorly defined mechanism involving the protein Mei-P26), before it rises again during later stages of oogenesis (Neumuller et al., 2008; Rhiner et al., 2009). When Myc levels are kept artificially high by means of a constitutively expressed transgene, the differentiating cystoblasts maintain a stem cell-like morphology and retain the ability to efficiently transduce the Dpp signal (emanating from the stem cell niche), suggesting that the drop in Myc levels contributes to the differentiation of these cells, although it is not clear how (Rhiner et al., 2009). Interestingly, GSCs can also compete with each other for niche occupancy, similar to the cell competition in imaginal discs that was discussed above. The involvement of Myc in this type of competition is controversial, though – two recent publications came to opposite conclusions in this regard. The group of E.Moreno found hypomorphic Myc-mutant GSCs to be driven from the niche by adjacent wild type GSCs, whereas GSCs with higher than normal Myc levels behaved as “super competitors” and chased away the neighboring wild type GSCs (Rhiner et al., 2009). In contrast, T.Xie and coworkers observed no competitive disadvantage in Myc-null mutant GSCs as compared to their wild type neighbors, nor any competitive advantage of Myc-overexpressing GSCs (Jin et al., 2008). It is conceivable that differences in overexpression regimes and in

the examined *Myc* mutant alleles are responsible for this discrepancy. For now, the jury is still out whether Myc is also involved in GSC competition.

However, Myc is likely to play a role in other stem cell divisions as well. Similar to GSCs, larval neuroblasts contain high levels of Myc protein (Betschinger et al., 2006). These cells divide in a stem cell-like manner, producing another neuroblast and a ganglion mother cell (GMC), which then gives rise to differentiated neurons. As in the germline, Myc levels are considerably lower in the differentiating GMCs than in their stem cell mothers. Both the asymmetric neuroblast division and the downregulation of Myc in GMCs require the protein Brat (brain tumor). During the neuroblast division Brat localizes to the GMC where it downregulates Myc post-transcriptionally. In *Brat* mutants neuroblasts divide to produce two additional neuroblasts, and the levels of Myc protein remain high in both of these daughter cells. Interestingly, Brat and Mei-P26 have a similar domain architecture (both containing a "B-Box" and an "NHL domain") and they share at least one interaction partner (the RNase Argonaute1, which is a key component of the miRNA-producing RISC complex), suggesting that both proteins might control Myc levels by a similar mechanism.

These studies did not address a functional requirement for Myc in neuroblast divisions, but two other reports revealed an effect of Myc on neurogenesis. First, the Myc gene was identified as a quantitative trait locus for adult bristle number – a hypomorphic mutation in Myc reduced the number of abdominal and sternopleural bristles (Norga et al., 2003). Second, overexpression of Myc in the embryonic CNS increased the number of neuroblasts, consistent with the idea that Myc might promote neuroblast self-renewal at the expense of producing differentiating daughter

cells (Orian et al., 2007). Myc is normally expressed in these embryonic neuroblasts, where it was proposed to act by binding to the transcriptional co-repressor Groucho and thereby antagonizing Groucho's repressive activity. Some of the common target genes of Myc and Groucho have an established role in the development of the CNS, but interestingly, they lack the typical Myc:Max binding sites (E-boxes) and they have also not been identified as Max or Mnt targets (Orian et al., 2003), suggesting that Myc's action on Groucho and on these targets might be independent of Max (Orian et al., 2007). This is most probably not the only mechanism by which Myc influences stem cell fate. Brat-mutant, Myc-overexpressing larval neuroblasts are characterized by larger nucleoli (Betschinger et al., 2006), as are Myc-overexpressing imaginal disc and salivary gland cells (Grewal et al., 2005), raising the possibility that Myc's general growth-stimulating activity might contribute to "stemness".

*7. Other functions*

The enumeration of Drosophila Myc's biological activities is necessarily incomplete. Several abstracts or short descriptions have been published that suggest additional functions for Myc that are not obviously connected to any of the processes described above. For example, during oogenesis Myc presumably controls the migration of follicle cells, in particular of a subpopulation called "border cells" (King and Vanoucek, 1960; King, 1970). It is to be expected that we will learn more about additional Myc activities in the future.

## C. Molecular mechanism of Myc action – the partners

The genetic tractability of *Drosophila* holds great promise for the functional analysis of proposed transcriptional co-factors of Myc and the identification of novel such co-factors, and hence for the characterization of the mechanism by which Myc controls the expression of its target genes. To date, studies have been published that address the function of the DNA helicases Tip48 and Tip49, of Max, the co-repressor Groucho, several Trithorax- and Polycomb-group proteins, as well as the Myc-antagonist Mnt (Figure 3).

### *1. Max*

The first identified Myc partner, and arguably the best characterized, is the BHLHZ protein Max. Different studies in vertebrate tissue culture cells have convincingly demonstrated that Myc requires the association with Max in order to bind to E-boxes and control the activation of the corresponding targets (Amati et al., 1992; Kretzner et al., 1992), but also for the repression of genes lacking E-boxes (Facchini et al., 1997; Mao et al., 2003). A mutated form of vertebrate c-Myc that cannot associate with Max is incapable of transforming cultured rat embryo fibroblasts, or of stimulating cell cycle progression or inducing apoptosis in established rat fibroblasts (Amati et al., 1993a; Amati et al., 1993b). Based on these and similar observations it was speculated that all functions of Myc might depend on Max, because Myc might require the dimerization with Max for its correct folding (Adhikary and Eilers, 2005). It therefore came as surprise that *Drosophila* Myc retains substantial activity even in the absence of Max (Steiger et al., 2008). This is most strikingly demonstrated by the phenotypic differences between *Myc*- and *Max*-mutant

animals: flies lacking Myc altogether fail to grow and mostly die as small larvae, whereas up to a third of *Max*-null mutant flies initiate metamorphosis and many of them even reach the pharate adult stage (i.e. they develop all adult body structures but they do not manage to leave the pupal case and die at this stage).

Part of this difference can be explained by the Myc antagonist Mnt, whose activity is also lost in *Max* mutants but not in *Myc* mutants: *Myc Mnt* doubly mutant animals survive for longer and grow larger than *Myc* singly mutant animals, presumably because typical Myc-activated genes are expressed at higher levels in *Myc Mnt* larvae than in *Myc* mutants (although still substantially lower than in control animals). This indicates that Myc functions in part to derepress Mnt-repressed genes (Pierce et al., 2008), as has been shown in vertebrate studies (Nilsson et al., 2004; Hurlin et al., 2003). However, Myc retains substantial activity in the absence of Max, and *Myc Mnt* doubly mutant animals clearly do not grow as well and do not develop as far as *Max* mutants. Thus, endoreplication is only partially impaired by the loss of Max but strongly by the loss of Myc, overexpressed Myc is capable of inducing cell-autonomous apoptosis in the absence of Max, and differences in Myc levels still trigger cell competition in *Max*-mutant animals. These observations point to the existence of substantial Max-independent activities of Myc. At least some of these may reside in Myc's interaction with RNA polymerase III (Steiger et al., 2008). It has previously been found that vertebrate Myc can activate RNA polymerase III, and that Myc does so by physically interacting with the polymerase III cofactor Brf (Gomez-Roman et al., 2003). This activity of Myc was shown to be conserved in flies, i.e. *Drosophila* Myc activates RNA polymerase III targets and is required for their full expression, and *Drosophila* Myc physically and genetically interacts with Brf (Steiger

et al., 2008). Importantly, both Myc's effect on Pol III targets and its interaction with Brf are also observed in the absence of Max. Thus, this effect on polymerase III may explain some of the observed differences between *Myc* (or *Myc Mnt*) and *Max* mutants, but there are likely to be additional functions of Myc that do not rely on the association with Max.

*2. Groucho*

One of these may be mediated by the transcriptional co-repressor Groucho (Orian et al., 2007). Groucho was found to associate with several genes that are also bound by Myc but lack known Myc:Max-binding sites (E-boxes). It is possible that Myc and Groucho are recruited to these genes together in the absence of Max, since Myc and Groucho also physically associate *in vivo* and *in vitro*. Several of these common targets play a role in neurogenesis and mitosis, and it was proposed that Groucho and Myc antagonistically control these genes and thereby affect the neuronal development: Groucho mediates the activity of the Notch-signaling pathway in repressing these genes, whereas Myc acts downstream of the EGF-receptor in activating them and promoting neuronal specification (Orian et al., 2007). While this observation suggests an interesting new role for Myc, the mechanistic details of the Myc:Groucho interaction still need to be worked out. In particular, the additional components of the Myc:Groucho complex need to be identified, that determine how the complex gets recruited to its target genes and how it controls their expression.

*3. Tip48 & Tip49*

In contrast to Groucho, the DNA helicases Tip48 and Tip49 have already been identified in studies in vertebrate tissue culture cells as putative co-activators for Myc

(Wood et al., 2000). The analysis of their *Drosophila* homologs (called Pontin and Reptin, respectively) confirmed their physical interaction with Myc and the existence of a ternary Myc:Pontin:Reptin complex, and further showed that Pontin (and to a lesser extent Reptin) is essential for Myc-dependent growth *in vivo* (Bellosta et al., 2005). Unexpectedly, Pontin could not be shown to play a role in Myc-dependent gene activation, but instead in Myc-dependent gene repression. An analogous repressive function was investigated in greater detail for the *Xenopus* homologs of Pontin (and Reptin). Both proteins were demonstrated to be essential for the ability of *Xenopus* Myc to repress the transcriptional activator Miz-1 and prevent it from activating the cell cycle inhibitor p21 (Etard et al., 2005). These observations further confirm the similarity between insect and vertebrate Myc. The mechanistic basis for the action of Pontin and Reptin remains open, though, as both proteins can act in several different transcription-associated complexes and it is not clear which of them is responsible for the observed repressive effects (Gallant, 2007).

*4. Polycomb- and Trithorax-group proteins*

The identification of Polycomb- and Trithorax-group genes in genetic screens emphasizes the potential of *Drosophila* for the discovery of novel Myc co-factors. The Trithorax-group genes ash2 (“Absent, small, or homeotic discs 2”; the homolog of vertebrate ASH2L), brahma (the homolog of human hBrm and Brg1) and lid (“Little imaginal discs”; the homolog of vertebrate Rbp-2/JARID1A and PLU-1/JARID1B) were found to be required for overexpressed Myc to promote overgrowth (Secombe et al., 2007). The three proteins physically interact with Myc in two separate complexes, one containing Ash2 and Lid, the other one containing Brahma. Lid was further shown to be required for the full activation of at least one direct Myc-activated

gene. Such a role in gene activation is consistent with Lid's classification as a Trithorax-group protein (as Trithorax proteins generally play a positive role in transcription), but appears at odds with Lid's molecular activity as a histone H3 lysine 4 trimethyl (H3K4me3) demethylase, as trimethylation on H3K4 is generally associated with active transcription. However, this demethylase activity does not seem to be required for Lid's ability to cooperate with Myc *in vivo*, since a mutant form of Lid lacking the demethylase domain also enhanced a Myc-overexpression phenotype, and since binding to Myc inhibits this demethylase activity. This does not explain how Lid helps Myc in the activation of its targets, but an answer might be found in the recent observation that Lid can associate with, and inhibit, the histone deacetylase Rpd3 in a potentially demethylase-independent manner, and thereby promote the transcription of certain target genes (Lee et al., 2009). The roles of Ash2 and Brahma can more easily be rationalized, as Ash2 is known from other studies to be associated with H3K4 trimethyltransferases and Brahma is a component of the SWI/SNF chromatin remodeling complex, and hence both have a documented function in transcriptional activation.

In an independent screen, Pc ("Polycomb"; the homolog of human CBX2/4/8), Psc ("Posterior sex combs"; the homolog of vertebrate Bmi1), Pho ("Pleiohomeotic"; the homolog of vertebrate YY1), and Ash1 ("Absent, small, or homeotic discs 1"; the homolog of vertebrate ASH1L) were found to affect the expression of some Myc targets during embryogenesis (Goodliffe et al., 2005; Goodliffe et al., 2007). Some of these targets were activated by Myc and by these other proteins, others (including the *Myc* locus itself) were repressed by both, and yet others were repressed by Pc and Pho, but activated by Myc. However, none of these proteins has been shown to

physically associate with Myc so far, and it is possible that their influence on Myc target gene expression is indirect. For example, it has been suggested that Ash1 functions as an H3K4 mono- and dimethyltransferase, thereby creating a substrate for the subsequent H3K4 trimethylation by an Ash2-containing complex (Byrd and Shearn, 2003). It is conceivable that Myc (in conjunction with an Ash2-complex) is involved in such a H3K4 trimethylation, and thereby (indirectly) depends on the prior activity of Ash1. Alternatively, Ash2 might help recruit Myc to genes that are already trimethylated on H3K4, as this post-translational modification has been shown to predate Myc recruitment to its targets in vertebrates (Guccione et al., 2006). It is currently unclear how Pc and Pho (which are both found in the same complex, PRC1; Schuettengruber et al., 2007) affect Myc targets.

Finally, a close functional connection between Myc and Trithorax-/Polycomb-group proteins was also suggested by the recent comparison of Myc targets with those of Trx (“trithorax”, homolog of vertebrate MLL proteins). Many of these genes were found to be arranged in clusters, and most of these target clusters were shared between Myc and Trx (Blanco et al., 2008). Whereas the molecular mechanisms of the interactions between Myc and these Polycomb-/Trithorax-proteins still need to be worked out, there is a good chance that (some of) this mechanism is conserved in vertebrates, since the vertebrate homologs of Lid (Secombe et al., 2007), Ash2 (Luscher-Firzlaff et al., 2008), Brahma (Cheng et al., 1999), Psc/Bmi1 (e.g. Jacobs et al., 1999) and Pho/YY1 (Shrivastava et al., 1993; Austen et al., 1998) all were shown to physically and/or functionally interact with vertebrate Myc.

*5. The Myc protein*

The sections above have addressed different trans-acting factors that collaborate with Myc in the control of gene expression. In addition, the fruit fly has also been used to analyze the requirement of parts of the Myc protein itself for the transcriptional regulation (Schwinkendorf and Gallant, 2009). Previous work in vertebrate tissue culture systems had identified Myc box 2 (MB2) as important for transactivation and –repression, and as generally essential for all biological activities of Myc proteins. This domain is highly conserved in *Drosophila* Myc, and it therefore came as surprise that it is partially dispensable for Myc function *in vivo*. A mutant Myc protein lacking MB2 can rescue the lethality of a substantial fraction of flies lacking all endogenous Myc, indicating that MB2 only modulates Myc activity, but is not essential for it. The co-factors contacting MB2 in *Drosophila* (that are therefore partially dispensable for Myc function *in vivo*) still need to be identified (Schwinkendorf and Gallant, 2009).

It is to be expected that future experiments in *Drosophila* will result in the identification of additional transcriptional co-factors for Myc. It will be important to explore the possible connections between the different Myc partners mentioned above (as well as between these proteins and the sequence motifs within Myc itself). It is likely that Myc recruits different enzymatic activities to control the expression of its target genes, and hence that some of these factors associate separately with Myc, but it is also conceivable that some of these proteins that have been analyzed separately so far are located in the same multi-protein complexes.

## D. Control of Myc activity

A large variety of inputs controls Myc activity in vertebrates (reviewed in Spencer and Groudine, 1991; Liu and Levens, 2006). In *Drosophila*, fewer such signals have been reported to date, simply because this subject has not yet been investigated to the same depth, but the short half-life of *Drosophila* Myc raises the possibility of an equally tight regulation: whereas the stability of *Drosophila* Myc mRNA has not been determined yet, *Drosophila* Myc protein decays with a half-life of 30-60', comparable to that of its vertebrate counterparts (Galletti et al., 2009; Schwinkendorf & Gallant, unpublished data). The pathways currently known to affect this protein stability or Myc's expression are summarized below (Figure 4).

### *1. Control of Myc expression*

During early embryogenesis, maternally deposited Myc mRNA is ubiquitously distributed in all cells (Gallant et al., 1996). Fertilization destabilizes this maternal message (as is the case for 21% of all maternal transcripts), such that its levels are significantly reduced in 4-6 hour old embryos (Tadros et al., 2007). Zygotic Myc transcripts then accumulate in the presumptive mesoderm, presumably under the control of the mesoderm specifying transcription factor Twist, which has been shown to bind to the Myc gene (Sandmann et al., 2007). Later, Myc is induced (by some as yet unknown mechanism) in the cells of the gut and salivary placodes (Gallant et al., 1996).

During larval development, Myc transcripts can be broadly detected in most diploid and polyploid cells. However, in the second half of the 3$^{rd}$ larval instar, a stripe of cells along the future wing margin, called the "zone of non-proliferating cells"

(ZNC), exits from the cell cycle and down-regulates Myc expression. This Myc repression is mediated by the Wingless signaling pathway, as the expression of dominant-negative Pangolin/TCF (the transcription factor at the end of the Wingless cascade) prevents this down-regulation and the cell cycle exit of the ZNC cells (as does forced expression of Myc; Johnston et al., 1999; Duman-Scheel et al., 2004). It is not clear, though, whether TCF directly represses Myc expression. According to one report, Wingless signaling up-regulates a protein called Half-pint (Hfp), which in turn represses Myc (Quinn et al., 2004; interestingly, Hfp is also repressed by the molting hormone ecdysone via the zinc-finger transcripton factor Crooked Legs/Crol, indicating that ecdysone can also positively regulate Myc expression: Mitchell et al., 2008). Mutation of Hfp leads to increased Myc mRNA levels in imaginal disc clones (including clones that extend into the ZNC) and in egg chambers. Consistent with this, heterozygosity for Hfp suppresses the female sterility associated with hypomorphic Myc alleles. Hfp is the *Drosophila* homolog of vertebrate FIR ("FBP interacting protein"), which was shown to repress vertebrate c-Myc through the "far upstream sequence element" (FUSE) (Liu et al., 2000), raising the possibility that Hfp directly binds to and represses the Myc gene – although no FUSE has been identified in Drosophila Myc so far (Quinn et al., 2004).

A separate report showed that Wingless signaling (and TCF) acts by repressing the Notch pathway, which in turn represses Myc (Herranz et al., 2008). An opposite effect of Notch on Myc expression was observed in larval neuroblasts, where a mutation of Aurora A kinase leads to up-regulation of Notch and subsequent induction of Myc (Wang et al., 2006). The molecular basis for either of these Notch effects is currently unknown, but it is interesting to note that a genetic interaction

between the Notch pathway and Myc has been reported (Muller et al., 2005; Orian et al., 2007). It remains possible that Notch also affects Hfp expression, or that Hfp, Notch (and possibly TCF) provide separate and parallel inputs into Myc expression.

As might be expected, Myc expression is also affected by the major growth-regulating axis in *Drosophila*: the insulin receptor (Inr) / target-of-rapamycin (TOR) pathway. This pathway monitors the fly's nutrient status: when food is copious, Inr signaling stimulates protein synthesis and induces the phosphorylation and inactivation of the transcription factor Foxo; at the same time, TOR activity increases translation rates and the transcription of growth-activating genes. On the other hand, upon starvation Inr and TOR are reduced in their activity, Foxo is dephosphorylated, enters the nucleus and binds its target genes – including Myc (Teleman et al., 2008). The consequences of Foxo binding for Myc expression are ambiguous, though, as shown by either site-directed mutation of the Foxo-binding site in the Myc promoter or by mutational inactivation of Foxo itself. Both treatments increase Myc expression in the fat body of fed larvae (i.e. in a situation where Foxo is normally kept inactive by Inr signaling), but they reduce Myc expression in starved larvae (where Foxo is normally active). The situation is different again in larval muscles, where the deletion of the Foxo binding site has no effect on Myc mRNA levels, but a Foxo mutation increases Myc levels specifically in starved larvae. These observations show that the action of Foxo on Myc levels depends on tissue type and nutritional status of the animal, although the basis for these differences is currently not known. Taking into consideration that TOR signaling also controls Myc protein levels (see below), and that Foxo was also proposed to affect Myc *activity* independently of Myc levels

(Demontis and Perrimon, 2009), it is difficult to predict how Inr, TOR and Myc actually cooperate in the control of growth at the organismal level.

Growth is also controlled by the evolutionarily conserved Hippo / Yorkie signaling pathway. One of the upstream regulators of this pathway is the transmembrane protein Fat (reviewed by Reddy and Irvine, 2008). Mutations in Fat induce tissue overgrowth. This overgrowth is accompanied by increased expression of Myc and hypomorphic mutations in Myc strongly reduce the growth-promoting effect of Fat (Garoia et al., 2005). These observations suggest that the Hippo / Yorkie pathway also controls Myc transcription.

Finally, *Drosophila* Myc has been shown to autorepress its own expression (Goodliffe et al., 2005). Like in vertebrates, this autorepression requires dimerization of Myc with Max (Facchini et al., 1997; Steiger et al., 2008), and it involves the Trithorax- and Polycomb-proteins discussed above (Pc, Pho, Psc, Ash2; Goodliffe et al., 2005; Goodliffe et al., 2007), but the relevant cis-acting sequences in the Myc gene have not been analyzed yet.

Thus, Myc transcript levels might be as tightly regulated in flies as they are in vertebrates. Surprisingly, though, such a tight control does not seem to be essential for *Drosophila* development. A transgene directing ubiquitous expression of a Myc cDNA (under the control of the $\alpha$-*Tubulin* promoter) is able to fully rescue the development of *Myc* null-mutant flies, although these rescued animals suffer from a slight growth deficit (Schwinkendorf and Gallant, 2009). This suggests either that the physiological pattern of Myc activity is not required for development, or that (partially redundant) mechanisms control Myc activity at the post-transcriptional stage. Indeed,

several such pathways have been identified in recent years, and they are summarized below.

*2. Control of Myc protein levels*

The stability of vertebrate Myc is regulated by the ubiquitin proteasome pathway. Briefly, the Ras/Raf/ERK kinase cascade leads to the phosphorylation of serine 62 (S62, located within Myc box 1 / MB1). This phosphorylation has a stabilizing effect on Myc, but it is also a prerequisite for the phosphorylation of threonine 58 (T58, also within MB1) by GSK3β. The doubly phosphorylated (T58 S62) protein is then dephosphorylated on S62 by the consecutive actions of the prolyl isomerase Pin1 and protein phosphatase 2A (PP2A), which in turn leads to Myc's ubiquitination by the E3 ubiquitin ligase Fbw7 and subsequent degradation. These different reactions are facilitated by the scaffolding protein Axin, which binds several of the involved proteins, including Myc (reviewed by Sears, 2004; Schulein and Eilers, 2009).

This pathway is (at least partially) conserved in flies. Thus, Myc levels are post-transcriptionally increased in imaginal disc cells expressing activated Ras (RasV12) (Prober and Edgar, 2002; note, though, that a different publication observed no such up-regulation of Myc upon overactivation of the EGF-receptor that acts upstream of Ras: Parker, 2006). On the other hand, the kinase GSK3β (called Shaggy / Sgg in *Drosophila*) triggers ubiquitination of Myc in cultured cells and, as a consequence, decreases Myc stability in tissue culture and in imaginal discs *in vivo* (Galletti et al., 2009). An involvement of Axin has not been demonstrated yet. Interestingly, though, another kinase known to associate with Axin, Casein Kinase 1α (CK1α) (Huang and He, 2008), has similar effects on Myc as GSK3β in cultured cells (and to some extent *in vivo* as well). MB1 and hence the phosphorylation site for GSK3β in vertebrate c-

Myc is not well conserved in *Drosophila* Myc, but two other putative targets for phosphorylation by GSK3β and CK1α have been identified, and their mutation strongly increases Myc stability. One of these sites is located within an acidic stretch that is highly conserved across Myc proteins from different species and that has been dubbed Myc box 3 (MB3), the function of which has remained mysterious in the past (Galletti et al., 2009). Another conserved player in the degradation pathway is the F-box containing E3 ubiquitin ligase Ago ("Archipelago"; homolog of vertebrate Fbw7; Moberg et al., 2004). Ago physically interacts with Myc and targets it for degradation. Loss of Ago in cell clones increases Myc protein levels and the size of these clones; heterozygosity for Ago in entire animals reduces the growth deficit of hypomorphic Myc mutant flies and increases their fertility. It is not known which sequence in the Myc protein contacts Ago, since the Fbw7 interaction site in vertebrate c-Myc (MB1) is only poorly conserved. However, *Drosophila* Myc contains several suboptimal Ago binding sites, and one of them coincides with MB3, suggesting that the phosphorylation of this domain by CK1α and GSK3β triggers recognition by Ago and subsequent degradation of Myc (Moberg et al., 2004; Galletti et al., 2009).

Having identified these proteins that regulate Myc stability, it will be of obvious interest to characterize the upstream inputs that feed into this degradation pathway. GSK3β is known to be controlled by the Inr signaling pathway, but so far no effects of this pathway on Myc stability have been reported. On the other hand, the TOR kinase has been shown to feed back on components of the Inr pathway, including GSK3β (e.g. Sarbassov et al., 2005; Zhang et al., 2006). Since rapamycin-mediated inhibition of TOR has been shown to reduce Myc protein levels post-transcriptionally (Teleman et al., 2008), it is conceivable that this effect is mediated by the pathway outlined

above. This report also identifies Myc as a downstream mediator of TOR's growth-promoting effects. Consistent with this observation, reduced TOR activity (caused by expression of either the negative upstream regulators TSC1 and TSC2 or a dominant-negatively acting TOR itself) can be overcome by ectopic expression of Myc (Tapon et al., 2001; Hennig and Neufeld, 2002).

TOR is certainly not the only regulator that affects the levels of Myc protein. One additional family of proteins that control Myc levels has been identified in asymmetrically dividing stem cells. As mentioned above, in neuroblasts mutation of Brat post-transcriptionally elevates Myc protein levels (Betschinger et al., 2006), and in female germline stem cells, the loss of Mei-P26 has a similar effect (Neumuller et al., 2008; Rhiner et al., 2009). Brat and Mei-P26, as well as a third *Drosophila* protein called Dappled, are related in domain structure, suggesting that they might affect Myc levels through a common mechanism. This mechanism appears to be evolutionarily conserved, as a vertebrate homolog of these proteins, TRIM-32, was recently shown to mediate ubiquitination and subsequent degradation of c-Myc (Schwamborn et al., 2009).

These different observations suggest the existence of several mechanisms that control Myc levels. It will be interesting to determine the molecular details of these pathways, as well as possible connections to the "core degradation machinery" described above.

## E. Outlook

Myc proteins have fascinated biomedical researchers for 30 years. This interest is largely explained by the enormous impact of *Myc* mutations on human health. In addition, Myc's central role in coordinating growth during normal development has become increasingly obvious in recent years. The discovery of the Myc/Max/Mxd network in *Drosophila* has opened a new experimental window for addressing these physiological functions of Myc. Research in the fruit fly has already contributed significantly to our understanding of pathological and physiological Myc function in vertebrates, for example by pinpointing the control of cellular growth as an essential, evolutionarily conserved role of Myc. Additional findings made in *Drosophila* are likely to be valid for the vertebrate system as well, such as the realization of Max-independent functions of Myc and the identification of alternative mechanisms of transcriptional control by Myc. Similarly, I expect the results of the genetic screens in *Drosophila* to play an important role in shaping our molecular understanding of the Max network, in flies as well as in vertebrates.

Beyond the molecular dissection of Myc's transcriptional function, *Drosophila* will be increasingly used to uncover systemic interactions with the different pathways controlling organismal development. These include the Insulin, TOR and Hippo/Salvador/Warts signaling pathways, which have been defined as the major determinants of body size. In addition, the effect of extrinsic factors, such as food availability, on Max network activity need to be addressed. *Drosophila* offers an ideal experimental system for investigating such issues, and we can expect significant advances in the near future. Stay tuned!

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## *VII. Figure legends*

**Figure 1. Domain structure of the *Drosophila* Myc, Max and Mnt proteins.** Domain names are explained in the main text. MB1 is only tentatively indicated as it shows low sequence similarity to the corresponding domain in vertebrate Myc proteins. The exact extents of the regions involved in transactivation, transrepression and protein stability are not known. The BHLHZ domains mediate dimerization with Max and DNA-binding. The numbers to the right show the the protein lengths (in amino acids).

**Figure 2. Activities of Myc.** Myc controls the activity of RNA Polymerases II and III, and (indirectly) of RNA Polymerase I. Their targets (together with possible transcription-independent activities of Myc) affect the indicated cellular processes.

**Figure 3. Myc-interacting proteins.** The depicted proteins have been shown to (directly or indirectly) bind to Myc. The colors indicate whether the corresponding proteins are thought to contact Myc:Max complexes (greenish shades) or interact with Myc independently of Max (reddish shades); no pertinent information exists for the proteins shown in grey. Some putative functions of the interacting proteins are also shown. Full protein names are (in parentheses: vertebrate homologs): Ash2 / "absent, small, or homeotic discs 2" (ASH2L), Brf (BRF1), Brm / Brahma (Brg1, hBrm), Gro / groucho (TLE), Lid / "little imaginal discs" (Rbp-2/JARID1A, PLU-1/JARID1B), Pont / Pontin (TIP49/RUVBL1), Rept / Reptin (TIP48/RUVBL2).

**Figure 4. Upstream regulators of Myc.** Proteins in the top half affect Myc mRNA abundance (presumably transcriptionally), whereas the proteins in the lower half act post-transcriptionally on Myc protein levels. Proteins that are thought to act in the same pathway (e.g. Wg, Hfp and Notch) or use the same molecular mechanism (e.g.

Brat and Mei-P26) are grouped together. The directionality of the effect (increase versus decrease of Myc levels) is reflected in the shape of the arrows and the color of the proteins (red or green, respectively). The effects of Notch and Foxo are ambiguous. Full protein names are (in parentheses: human homologs): Ago / archipelago (FBXW7), Ash1 / “absent, small, or homeotic discs 1” (ASH1L), Brat / “brain tumor” (similar to TRIM32), CK1$\alpha$ / “Casein kinase 1$\alpha$”, Foxo / “forkhead box, sub-group O” (FOXO3), Hfp / pUf68 = “poly U binding factor 68kD” (FIR / PUF60), Mei-P26 (similar to TRIM32), Pc / Polycomb (CBX2/4/8), Pho / pleiohomeotic (YY1), Psc / “Posterior sex combs” (Bmi1, Mel-18), Sgg / shaggy (GSK3$\beta$), TOR / “Target of rapamycin” (mTOR), Twist (TWIST), Wg / Wingless (Wnt).

Figure 1

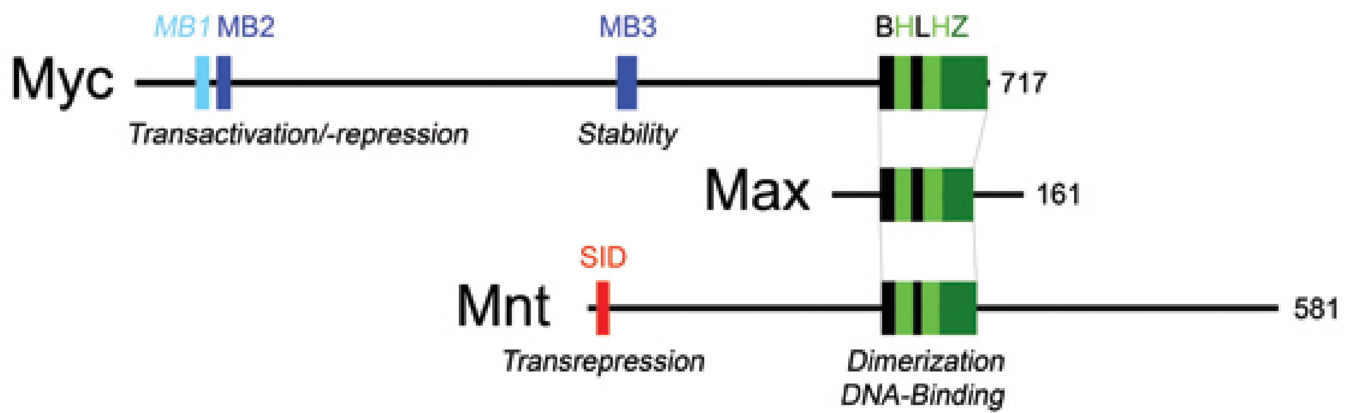

Figure 2

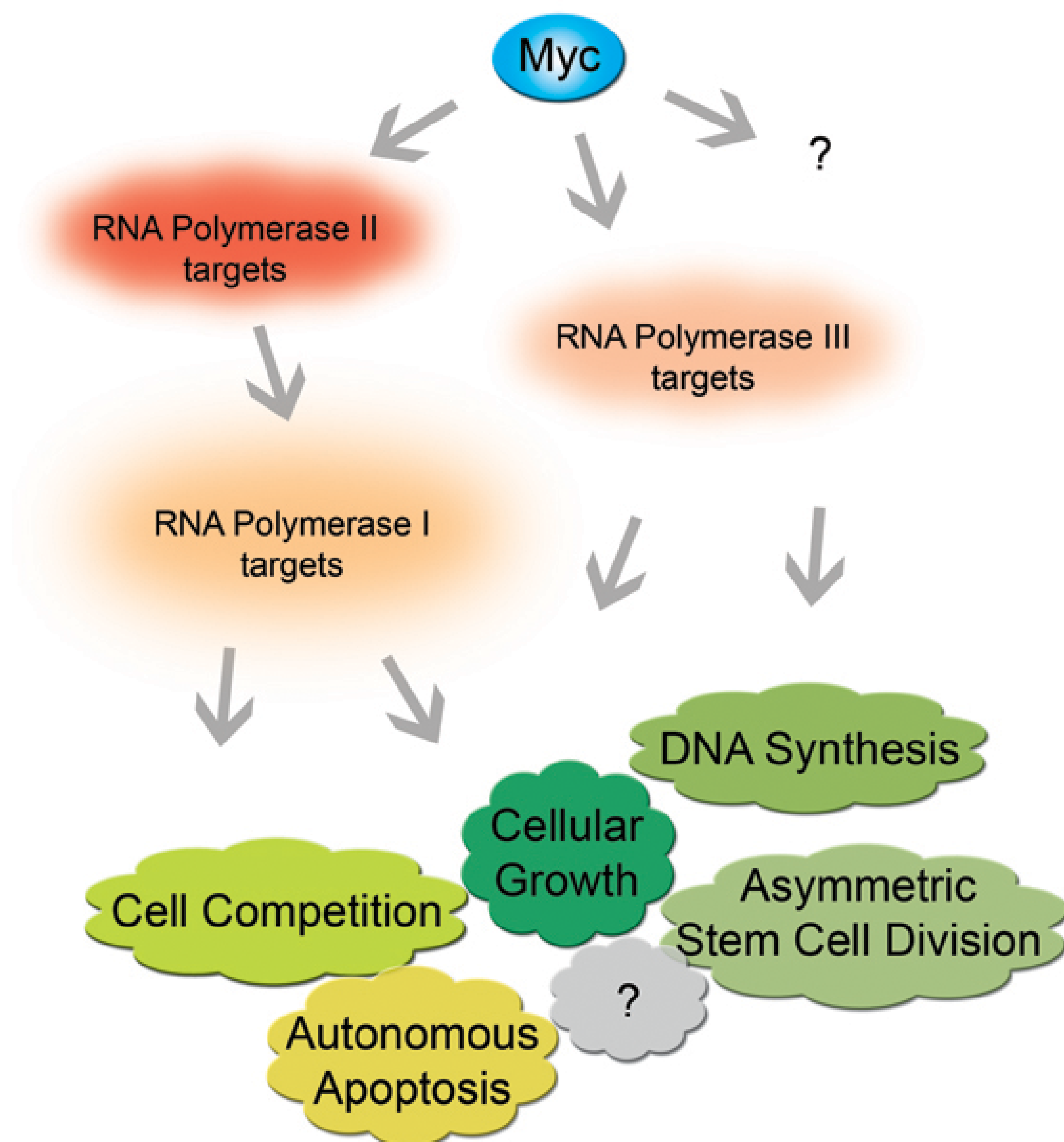

Figure 3

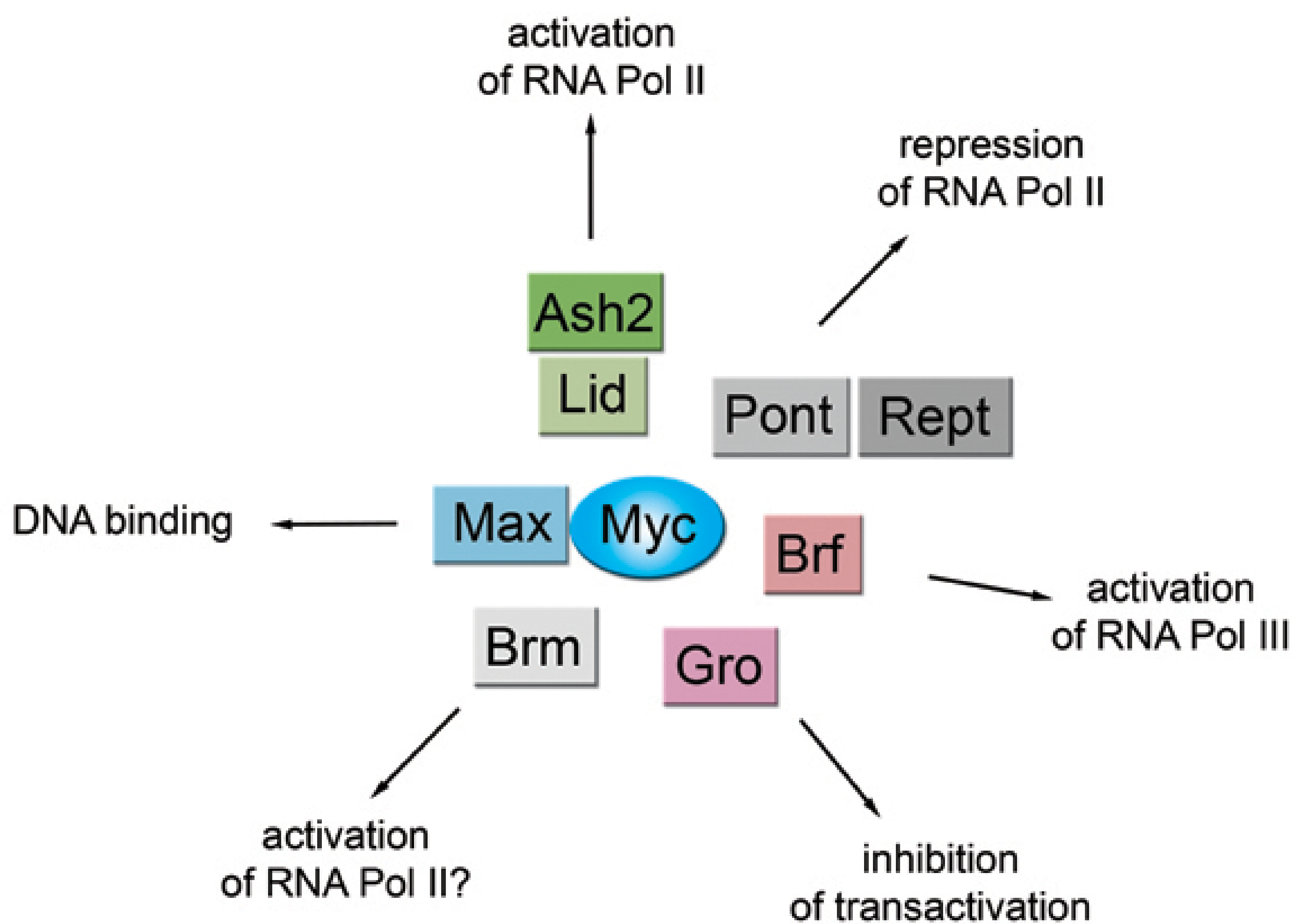

Figure 4