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Myc in model organisms: A view from the flyroom

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Abstract

The Myc transcription factor regulates fundamental processes in a cell's life: its growth, division, and survival. Myc is conserved throughout metazoan phyla, and its identification in the fruit fly, *Drosophila melanogaster* has led to new insights in Myc's physiological roles. In this review, we describe recent research on the biology of Myc and its family members in *Drosophila*, paying particular attention to its role in the control of growth during development.

Keywords

Myc; Growth; Cell division; Ribosome biogenesis; Cell competition

1. Introduction

Myc proteins have fascinated biologists for almost 25 years, yet despite the enormous literature that documents their biological functions they still remain mysterious. Myc is the founding member of a family of transcription factors of the basic-helix-loop-helix-leucine zipper (BHLH-LZ) class, charged with directing some of the most basic aspects of a cell's life: its growth, division, and survival. As such, it is not surprising that this family has ancient roots, with members of the Myc "network" – which includes c-, L-, and N-Myc, Myc's binding partner Max, and its functional antagonists, Mad/Mxi/Mnt) – encoded in the genomes of most metazoan phyla [1]. Years of research on c-Myc in mammalian systems have led to a dauntingly diverse range of potential genetic and functional targets. Yet it has remained difficult to evaluate Myc's function in its entirety due to redundancy among family members, tissue specificity, and complex phenotypes.

These problems spurred the search for Myc in genetically tractable animal models, and led to the identification of Myc family members in zebrafish, in the nematode *Caenorhabditis elegans*, and in *Drosophila*. The zebrafish *Danio rerio* genome encodes several transcript variants of Max, as well as homologs of c-Myc, N-Myc, L-Myc, and Mxi/Mnt [2,3]. Interestingly, although *C. elegans* has functional Max and Mnt orthologs, Myc is conspicuously absent from its genome [4]. *Drosophila*, on the other hand, has one homolog each of *myc*, *max*, and *mnt*. The relatively simple genome and the highly developed genetics that *Drosophila* offers have made it the model of choice for study of Myc family members. In fact, the first *myc* mutant of any organism was identified as a spontaneous mutation in *Drosophila* in the 1930s by Eleanor Nichols-Skoog and Calvin Bridges, which they called *diminutive* (*dm*), for its smaller body size [5]. *dm* was identified as a mutation in the *Drosophila myc* (*dmyc*) gene in 1996, and since then more than a dozen mutations of *dmyc* have been characterized [6,7]. In the last several years, work on dMyc has clarified some of the protein's

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more enigmatic functions and also led to the identification of new functional roles. In this review, we focus primarily on work carried out in *Drosophila*, and highlight recent findings regarding the roles dMyc plays during development, their biological importance, and parallels with what is known about Myc in vertebrates.

2. dMyc, dMax, and dMnt: the network in *Drosophila*

The Max protein network – Myc, its antagonists Mad/Mxi/Mnt, and their common binding partner, Max – is conserved in *Drosophila*, but exists in a simple form, with one member of each class. dMyc was the first of the network to be identified, as a binding partner for human Max in two-hybrid screens [6,7]. The isolation of dMyc allowed the subsequent isolation of dMax, and demonstration that as in vertebrates, dMyc and dMax heterodimerize, and bind as a complex to the canonical E-box sequence CACGTG [6]. The last family member to be isolated was dMnt, the sole member of the Mad/Mxi/Mnt cohort of Myc-antagonizing factors [8].

2.1. dMyc

As its physical interaction with Max suggests, dMyc is structurally related to c-Myc and contains its important functional protein domains, including a well-conserved C-terminal BHLH-LZ domain, which mediates DNA-binding and dimerization with Max. The N-terminus of dMyc is substantially longer than that of c-Myc, and less well conserved (for a more detailed account of sequence conservation in dMyc, dMax, and dMnt see ref. [1]). Within the N-terminus of c-Myc are two short motifs that are highly conserved among vertebrate Myc proteins, known as Myc-box I (MBI) and Myc-box II (MBII). MBII is essential for most biological functions of c-Myc [9]. Both MBI and MBII form part of c-Myc's transactivation domain, interact with a variety of cellular proteins, and are responsible for control of the cellular concentration of c-Myc [10]. Not surprisingly, these regions are also hotspots for mutations that transform c-Myc into an oncoprotein. Despite tight conservation among vertebrates, in dMyc the amino acid sequence of this region is only loosely homologous to c-Myc [1]. dMyc has a recognizable MBII, but MBI is not well conserved. Even so, dMyc can functionally substitute for c-Myc in transactivation assays in human cell culture [6], can transform rat embryo fibroblasts when expressed along with human RasV12 [7], and can rescue growth defects of mouse embryo fibroblasts derived from *c-myc* conditional knock-outs [11].

In humans and mice there are three isoforms of c-Myc, translated from distinct start sites, which differ in the length of their N-termini. MycS is the shortest variant and lacks most of the N-terminal transactivation domain, but retains the essential MBII. In *Drosophila*, some variation in *dmyc* transcript length has been reported, but whether the *dmyc* gene has more than one promoter or multiple translation start sites is unknown, and only one protein form of dMyc has been identified [6,12]. However, two c-Myc isoforms – c-Myc2 and MycS – can rescue the lethality of flies carrying a strong *dmyc* allele, allowing their development into fertile adults [12]. It has been hypothesized that the single dMyc protein performs all of the various biological roles that in human cells are carried out by different c-Myc protein isoforms [12], although some controversy exists about their actual physiological role(s) [13].

2.2. dMnt

dMnt was also isolated in a two-hybrid screen, for dMax interacting proteins [8]. While dMnt lacks the proline- and proline/histidine-rich domains characteristic of mouse Mnt, its size, and overall organization is more similar to Mnt than to the Mad/Mxi paralogs [1,8]. In transactivation assays, dMnt/dMax heterodimers bind to, and repress transcription from canonical E-box sequences, and like human Mad proteins, dMnt represses transcription through its association with the corepres-sor *Drosophila* Sin3 [8]. Three Mnt isoforms are synthesized

in *Drosophila*: the full-length dMnt, dMntΔZip, which lacks the leucine zipper, and dMntΔSID, lacking the Sin3-interaction domain [8]. Expression of the full-length dMnt during fly development results in a marked cell size reduction and reduces growth of cell clones. Neither dMntΔZip nor dMntΔSID affect cell size when overexpressed, but do reduce clonal growth, suggesting that interaction with dMax and dSin3 are necessary for dMnt to regulate cell size. These variant dMnts occur naturally and are the result of differential splicing, but their physiological role is currently unknown; additional *dmnt* mutants, which specifically remove individual isoforms will be needed to understand the roles of these dMnt proteins.

Unlike its mouse counterpart, *dmnt* null mutants are viable, thus it is not essential for animal development. This is noteworthy since the mouse Mnt knock-out is lethal shortly after birth, and the absence of strong phenotypes in Mad1, Mad3, Mxi1 knock-out MEFs had been attributed in part to functional overlap between the several Mad/Mxi proteins and Mnt [14]. However, *dmnt* mutants do have growth phenotypes that in general are complementary to *dmyc* mutants (described in detail below). Cells mutant for *dmnt* are larger than normal and consequently, *dmnt* mutant adults are heavier than wildtype flies in overall body weight. Unexpectedly, while *dmnt* adults are healthy, they have a shortened life-span [8]. An intriguing related observation was made in nematodes: the *C. elegans* Mnt/Mad ortholog *mdl1* was identified as a gene that is up-regulated with loss of *daf-2*, which encodes the Insulin/IGF-1 receptor, and down-regulated with *daf-16* RNAi treatment [15]. Daf-2 signaling regulates the activity of Daf-16, a Foxo-family transcription factor, which influences metabolism and lifespan in worms, flies, mice, and humans. The inclusion of a Mad/Mnt homolog in a list of potential Daf-16 targets provides a provocative link between Insulin/IGF signaling and the Myc/Max/Mnt network.

dMnt is expressed in the developing central and peripheral nervous systems coincident with the onset of their differentiation [8]. However, dMnt expression is not strictly limited to differentiating cells; in the salivary glands and fat body, two endoreplicating organs, dMnt is expressed in both replicating and non-replicating cells. Also, dMnt is expressed in the peripodial epithelium encasing imaginal discs at a time when they are proliferating. In some tissues, the expression of dMyc and dMnt proteins is complementary and non-overlapping, but this is not always the case [16].

Indeed, the relationship between dMyc and dMnt is not yet well defined. Whether dMyc and dMnt antagonize one another in the same cell or have opposing roles in separate cells (or both) awaits more detailed study and the generation of *dmyc*, *dmnt* double mutant animals. In general, the various dMnt expression patterns, over-size mutant phenotype, and suppression of growth when overexpressed are certainly consistent with a role in counteracting the growth promoting function of dMyc.

2.3. dMax

Little is known about dMax beyond its roles as a binding partner for dMyc and dMnt. Thus far, no *dmax* mutants have been characterized, but such mutations are expected to eliminate most of the activities of dMyc and dMnt. Along with the *dmyc* and *dmnt* null mutants already available, fly mutants of *dmax* would make possible a thorough genetic epistasis analysis of the Max network. There are some hints, however, that the function of dMax may not be limited to partnering dMyc and dMnt. For example, in experiments designed to map genomic binding by dMyc, dMax, and dMnt, it was found that dMax bound to a large number (365) of genes not bound by dMnt or dMyc [17]. This could be due to binding by dMax homodimers, or, dMax could partner other, unidentified factors. Intriguingly, the unique binding of dMax to these genes did not correlate with the presence of E-boxes, perhaps pointing to the latter possibility [17].

3. The genetics of *dmyc* mutations: dMyc controls growth

The presence of *c-myc*, *N-myc*, and *L-myc* has complicated genetic assessments of Myc's function in mammals, thus the expectation was that a genetic model system, such as *Drosophila* would provide a more straightforward Myc loss of function system. This has been the case, and importantly, has refocused attention on the role of Myc in regulating growth. One of the greatest advantages *Drosophila* provides to Myc biology is the ease with which growth can be studied in a living animal.

All *dmyc* mutations show profound growth defects. Null mutant embryos hatch into larvae at the same time as wild-type animals, but fail to grow and die early in the second larval instar [16]. Hypomorphic alleles are lethal at progressively later stages of development, depending upon their severity [16,18]. In animals bearing the weakest alleles, such as the original dm^1 mutation and $dmyc^{P0}$, development is delayed and yields small flies – the result of smaller cells – with short, thin bristles [19]. Animals carrying the slightly stronger $dmyc^{P1}$ allele also have a significant reduction in cell number. Interestingly, although the hypomorphic mutant flies are smaller than normal they appear normally proportioned (Fig. 1) [19,20]. Many of these same defects are characteristic of flies with growth deficits, and also appear in flies with mutations in genes encoding ribosomal proteins (the *Minute* class of mutations) and other components of ribosome biogenesis.

The existence of a broad *dmyc* allelic series with a range of phenotypes suggests that some developmental processes in the fly require *dmyc* more than others. *dmyc* is expressed in numerous tissues throughout fly development, and its expression occurs in a dynamic pattern (Wu and Johnston, unpublished data) [6,19]. It is expressed in both endoreplicating cells, which oscillate between G1 and S phase but do not divide, and in mitotic tissues [16,19]. Use of the FLP/FRT technique of mitotic recombination to generate somatic *dmyc* mutant cells in an otherwise normal animal demonstrated that in all tissues examined, clones of *dmyc* mutant cells result in pronounced defects in cellular growth. This defect is manifest by a small cell size and by impaired progress through the cell cycle. In endoreplicating larval cells that can normally achieve a DNA content upwards of 2000C, *dmyc* null cells fail to increase in size as development progresses, a defect that is directly related to the number of endocycles a cell undergoes [16]. Since the dramatic cellular growth of these cells is required to sustain the overall growth of a fly larva, the impaired growth of *dmyc* mutant endoreplicating tissue probably accounts for the growth arrest and subsequent death of these null mutant larvae [16]. *dmyc* is also required for growth of polyploid cells within the female germline, and in the diploid imaginal cells that give rise to the adult body structures of the fly [18,19]. Proliferating *dmyc* mutant imaginal cells are markedly reduced in size and spend a disproportionate amount of the cell cycle in the G1 phase; these cells are also smaller in S, G2, and M phases [19]. Conversely, overexpression of dMyc in imaginal cells increases cell size by accelerating cellular growth, and when expressed throughout the animal the size of the fly is increased by nearly 30% (Fig. 1) [19,21]. Collectively, these mutant phenotypes provide solid evidence that dMyc is required in vivo for cellular growth, and that it acts in a dose-sensitive manner.

3.1. Flies and mice: the same but not the same

Both flies and mice carrying *myc* mutations are small in size, but the basis for this effect appears to be different. Like *dmyc* mutant flies, *c-myc* null mice generated with conventional germline "knock-out" techniques die at early stages as small, delayed embryos with a variety of developmental defects [22]. Conditional flox-induced knock-outs of *c-myc* within individual tissues avoids the early lethality and has allowed an analysis of the growth phenotypes with a series of increasingly severe *c-myc* alleles [11,23]. Mice carrying hypomorphic alleles *of c-myc* are smaller in overall body size, and like the fly mutants, stronger *c-myc* alleles result in progressively smaller sizes [11].

A striking difference between flies and mice is that even in the strongest hypomorphic *c-myc* mouse, cell size in several tissues is normal [11]. The small body size of the mutant mice thus appears to be due solely to a dearth of cells. *Drosophila* hypomorphic *dmyc* mutants also have fewer cells, but the cells are significantly smaller than normal [19]. This disparity suggests that there may be inherent differences in the way mice and flies regulate cell (and body) size [11]. However, other observations argue against this generalization. Tissue specific excision of a *c-myc* flox allele to completely inactivate *c-myc* leads to greatly diminished cell size in epidermal keratinocytes and liver hepatocytes [24,25]. The difference in effects on cell size of *c-myc* hypomorphic and null mutations could thus be due to allele strength. Alternatively, the requirement for *c-myc* during mammalian cellular growth may differ with cell type. This is a real possibility since specific inactivation of *c-myc* in a mosaic B-lymphocyte population prevents activation-induced cell growth and proliferation upon mitogen stimulation (although many of the early steps of activation are still induced) [23]; in contrast, a very similar protocol has little effect on cell growth of T-lymphocytes after T-cell receptor activation [11]. Regardless, since growth is a measure of mass accumulation whether scaled by cell number or cell size, the central observation – that both *c-myc* mutant mice and *dmyc* mutant flies have less mass than controls – indicates that Myc is required for animal growth.

4. dMyc and the cell cycle

Given its pivotal role in human cancer and in developmental control of growth, understanding how Myc regulates the cell cycle has historically been of great interest. Like vertebrate Myc, dMyc is required for efficient transit through G1 into S phase, as *dmyc* mutations stall cells in G1, and overexpression of dMyc accelerates G1 [16,18,19]. However, dMyc is not essential for cell cycle progression. This is clearly demonstrated in the female fly germline, where mitotically dividing cytoblast cell clones carrying a strong allele of *dmyc* still undergo all the normal divisions, producing the expected 16 cells of a normal germline cyst [18]. On the other hand, as mentioned above, endoreplicating cells mutant for *dmyc* undergo significantly fewer rounds of S phase [16,18]. The molecular nature of the defect in endoreplication is unclear, as *dmyc* cells still periodically express the G1 Cdk2 regulators Cyclin E and Dacapo, the fly $p27^{cip/kip}$ homolog, and are able to complete the entire endocycle [18]. One possibility is that dMyc regulates the frequency of S phase entry in these cells. Consistent with this idea, overexpression of dMyc increases the rate of endoreplication in cells of the *Drosophila* fat body, and this effect is accompanied by (and dependent upon) oscillating activity of Cyclin E/Cdk2 [16]. Moreover, dMyc expression in endocycling cells can partially reverse a growth arrest imposed by expression of the phosphoinositol-3-kinase (PI3K) adaptor p60, rescuing both endoreplication and cellular growth.

4.1. How does dMyc regulate G1?

Wing imaginal cells undergo canonical mitotic cell cycles in which the rate limiting regulators of G1/S and G2/M are Cyclin E and the Cdc25 phosphatase, String, respectively [26]. Overexpression of dMyc in these cells increases Cyclin E levels and accelerates the G1/S transition [19,27]. Cyclin E mRNA levels are moderately enhanced in response to dMyc expression, but Cyclin E protein is increased disproportionately, suggesting that most of the increase is due to post-transcriptional regulation [27,28]. This post-transcriptional regulation could be mediated by signaling from the small GTPase, Ras, as it is in mammals, since in *ras* mutant cells dMyc expression no longer induces high Cyclin E levels [27]. dMyc also regulates the activity of the E2F/Rb axis. Overexpression of dMyc increases the expression of dE2F targets, such as proliferating cell nuclear antigen (PCNA), ribonucleotide reductase (RNR), and Cyclin E; it also increases expression (both mRNA and protein) of the Rb regulator, Cyclin D, and its obligate cyclin dependent kinase partner, Cdk4 [28]. Cyclin D/Cdk4 is not essential for cell cycle regulation in *Drosophila*, and does not have a strong regulatory role in

G1/S transition [29,30]. However, the complex is required for growth, as animals completely mutant for either *Cyclin D* or *Cdk4* are 25% smaller than wildtype, and their overexpression drives "balanced" growth, where cellular growth rates and cell division rates are in synch (in contrast to dMyc, see below) [21,30]. It appears that G1 cyclins and stimulation of E2F activity are both important effectors of dMyc's ability to promote G1 progression. Nonetheless, dMyc expression cannot bypass a cell cycle arrest in either diploid or polyploid cells. Expression of the vertebrate cyclin dependent kinase inhibitor, p21, which blocks Cyclin E/Cdk2 activity, arrests *Drosophila* endocycling cells, and dMyc expression is not sufficient to overcome that arrest [16]. Likewise, ectopic expression of dMyc is not able to drive arrested diploid cells of the developing wing or eye back into the cell cycle [19].

4.2. G2 regulation is independent of dMyc

When expressed in cell clones in the developing wing, dMyc causes an increase in cell size. This size increase occurs because dMyc accelerates the cellular growth rate (the rate at which a cell accumulates mass), but is not sufficient to speed up their division rate [19]. The failure of dMyc to promote progression through the entire cell cycle was initially perplexing since c-Myc is a potent driver of cell proliferation in cell culture and when de-regulated in cancer. However, the inability of overexpressed dMyc to drive a faster cell cycle is explained at least in part by developmental constraints imposed in vivo. In most (if not all) mitotic *Drosophila* cells the Cdc25 homolog, String (Stg), is not regulated by dMyc, but is under developmentally regulated transcriptional control [19,31]. Thus, even though ectopic dMyc expression drives cells through G1 and into S phase quickly, sufficient levels of Stg for passage through G2 require the appropriate developmental cues. Co-expression of dMyc with Stg does result in a faster cell division rate, matching it to the increased cellular growth rate, and yielding more cells of normal size [19].

Still, there is much that is not understood about how dMyc controls the cell cycle and how its growth promoting activities are linked to cell cycle regulation. For example, whether regulation of Cyclin D and Cdk4 levels by dMyc are important for dMyc's control of growth and the cell cycle is not known. Why, if dMyc overexpression increases the expression of Cyclin D and Cdk4 (which in *Drosophila* promote progression through both G1 and G2 [30]), does not the cell cycle progress faster as a whole? Indeed, in some cases, as in during cell competition (see below), expression of dMyc can increase proliferation rates (Senoo-Matsuda and Johnston, unpublished data). These and other discrepancies imply that dMyc has additional, undefined roles in cell cycle regulation. The fact that dMyc expression can overcome an endoreplicative block due to growth inhibition but not one due to cell cycle arrest suggests that much of dMyc's influence on the cell cycle occurs indirectly, through its role in regulating growth.

5. How does dMyc make cells grow?

The genetic studies in both the fly and the mouse have stressed the importance of Myc's role in regulating growth, yet how Myc does this is still largely unknown. Both genetic experiments in vivo and target identification approaches in vitro have been taken in *Drosophila* to get at this problem. By far, the strongest candidate mechanism for growth regulation by dMyc appears to be through its transcriptional control of key regulators of ribosome biogenesis.

5.1. Identifying dMyc target genes

Several groups have set out to clarify dMyc's mechanism of growth regulation by cataloging dMyc target genes through expression profiling, and both loss- and gain-of-function microarray experiments have been done using cell culture, isolated tissues, or whole animals. A novel approach involving DNA methylation was taken to identify *Drosophila* genes that are direct targets of dMyc, a tactic that was possible because the *Drosophila* genome is otherwise

unmethylated. The DamID technique employs DNA adenine methyltransferase (Dam), fused to a protein of interest, to methylate, and thereby physically mark the protein's binding sites in cultures of *Drosophila* Kc cells, which are derived from wildtype embryos [32]. This technique was used to uncover genomic binding sites of dMyc, dMax, and dMnt [17]. The DamID experiments indicate that when overexpressed, dMyc, dMax, and dMnt bind extensively throughout the genome–to about 15% of loci on arrays that included half of all *Drosophila* coding sequences. This binding occurred with specificity, as genomic regions bound by these proteins were significantly enriched in two motifs, the canonical E-box, and the DNA Replication Element. However, of all genes activated by dMyc, only about 36% were bound by either dMyc or dMnt (either alone or in combination with Max), suggesting that a large number of genes are activated indirectly upon dMyc overexpression [17]. The DamID experiments also indicate that genomic binding by dMyc is very sensitive to dMax levels. Surprisingly, at either low or high dMax levels, a large proportion of dMyc genomic sites (approximately 2/3) were not bound by dMax. It is not yet clear what accounts for dMyc's Max-independent presence, particularly in light of DNA-binding experiments in vitro, where dMyc did not bind to E-box sequences without dMax [6]; however, it is still possible that dMyc can also be recruited to DNA by another, unidentified factor.

In cultured *Drosophila* Schneider's 2 (S2, a cell line derived from embryos) cells, conditional RNA-interference (RNAi) has been used to reduce dMyc activity [33]. Remarkably, especially given the large number of sites bound by dMyc in the genome, under these conditions just 30 genes continuously require dMyc for stable expression. Most of these dMyc-dependent targets encode factors involved in RNA binding, rRNA processing, nucleolar function, and ribosome biogenesis, and include, for example, the nucleolar proteins Fibrillarin and CG1542, which are involved in processing of the 35S primary transcript and of 27S pre-rRNA, respectively [33]. A majority of the targets that are down-regulated by *dmyc* RNAi are induced by overexpression of dMyc in S2 cells, or in experiments using RNA from whole larvae in which dMyc is overexpressed [17,34]. Again, genes involved in ribosome biogenesis dominate the targets increased by dMyc: one-fifth of transcripts up-regulated by dMyc expression encode factors used in ribosome biogenesis [17,33,34]. These targets include RNA Polymerase I- and II-transcribed genes, and many appear to be directly responsive to dMyc activity. For example, RpI135 mRNA, encoding a Pol I subunit, is rapidly increased within 4 h of dMyc expression, as is dTIF-IA, a growth-regulated Pol I-associated factor. This rapid response occurs even before an increase in the synthesis of pre-processed rRNA is detected [34].

5.2. dMyc activity makes more ribosomes

The results of expression profiling experiments are backed-up by genetic manipulations in the developing fly. The increase in expression of pre-rRNA by dMyc overexpression is accompanied by a dramatic increase in nucleolar size, a good indicator of increased ribosome activity [34]. This effect is not observed when other growth regulating factors, such as the PI3K, Dp110, or Cyclin D/Cdk4 are overexpressed (de la Cova & Johnston, unpublished data) [34]. Expression of Fibrillarin is also increased in these larger nucleoli, and the cytoplasm of larval salivary gland cells expressing dMyc are packed with ribosomes and polysomes, with a dense network of rough endoplasmic reticulum (Fig. 2) [34]. By contrast, in *dmyc* mutant larvae, pre-rRNA levels are low compared to controls despite even levels of rDNA, and nucleolar size is reduced [34]. As a whole, the data indicate that modulation of ribosome biogenesis is an important effector of dMyc during normal growth, with dMyc-dependent transcriptional regulation of ribosome biogenesis resulting in greater translational activity within the cell. Similar observations have been made in vertebrate cells, pointing to the control of ribosome biogenesis as a fundamental and conserved part of Myc biology [35,36].

5.3. Transcriptional repression by dMyc

Like c-Myc, dMyc can repress transcription [33,37]. In vertebrates, c-Myc-induced transcriptional repression is not associated with the presence of E-boxes at target genes, and may occur when c-Myc binds and inhibits other transcriptional activators, such as Miz-1 [38]. In *Drosophila*, conditional removal of *dmyc* is not sufficient to activate repressed targets [33]. It is possible that repression of target genes does not continuously require the presence of dMyc, or that other factors act redundantly with dMyc in repression. One dMyc-repressed target, which has been confirmed with experiments in vivo is the *dmyc* locus itself [37]. To identify cellular factors required for repression of the *dmyc* locus, a large-scale genetic screen was conducted using a transposon insertion that, by virtue of its insertion site in the *dmyc* locus, acts as a visible eye-color reporter of *dmyc* transcription. Mutations in *Polycomb* (*Pc*), as well as *Posterior sex combs*, another Pc group gene, derepress *dmyc* transcription. Furthermore, *Pc* is required for dMyc repressive ability because when dMyc is overexpressed in the absence of *Pc*, 73% of dMyc-repressed targets are derepressed [37]. The Pc group complex can mediate long-term “memory” of transcriptional repression and its role in dMyc repression is consistent with the possibility that dMyc is not continuously required for repression of its targets. Why dMyc inhibits expression of itself is unclear, but it is a strategy that could be used to limit growth in some circumstances, or in some cell types.

6. dMyc in development: control of tissue growth and links with pattern formation

One of the biggest mysteries of animal development is how growth is coordinated with pattern formation. By controlling cellular growth and cell proliferation, dMyc also has a major impact on the regulation of animal size. In contrast to the growth-regulating network of Insulin/PI3K and dTOR, *dmyc* expression is regulated by at least two of the major developmental signaling pathways that regulate pattern in *Drosophila*, Wingless (Wg)/Wnt, and Decapentaplegic (Dpp)/BMP/TGF-β. While Insulin/PI3K/dTOR signaling controls growth in response to nutrients, the responsiveness of dMyc to Wg and Dpp, which are also required for growth of many fly organs, suggests a model wherein developmental signals contribute to tissue growth, and ultimately body size and proportion, by regulating dMyc activity. We explore this idea here by describing two specific developmental processes that involve dMyc: a patterned cell cycle arrest, and cell competition.

6.1. Repression of dMyc enforces a developmental growth arrest

Developing wing cells all exit the cell cycle at the end of development, but specific cells at the dorsal-ventral (DV) boundary, a developmental compartment boundary in the wing, arrest more than a day earlier than the rest as part of a neural differentiation program and have been called the zone of non-proliferating cells (ZNC) [39]. A significant fraction of the ZNC arrests in G1, and requires the *Drosophila* Rb family member, Rbf, as cells lacking *rbf*, or overexpressing *Drosophila* E2F1 (dE2F1), fail to arrest [28,39]. Similarly, overexpression of Cyclin E, which is an inhibitor of Rbf activity, is sufficient to prevent the normal G1 arrest [39]. Since dMyc regulates Cyclin E and Cyclin D expression, and the dE2F1 targets PCNA and RNR, dMyc activity must be inhibited in the ZNC to allow unhindered Rbf activity to enforce the cell cycle arrest.

Earlier in development, during the growth phase, *dmyc* is expressed throughout the growing wing, but is later lost in the ZNC through the activity of Wg, which is expressed in cells immediately flanking the DV boundary of the wing [19,39]. Inhibition of dMyc by Wg is necessary to allow Rbf to lock in the cell cycle arrest, and loss of dMyc also ensures that cellular growth is reduced in the arrested cells [19,28]. Wg may inhibit *dmyc* expression by inducing Halfpint (Hfp), a homolog of human FBP interacting repressor (FIR), a pre-mRNA splicing

factor also called PUF60 [40]. In human cells, FIR inhibits *c-myc* transcription and interacts with FBP, a protein that binds an upstream element at the *c-myc* locus [41]. In the fly, *hfp* mutant cells have elevated *dmyc* transcript levels and the ZNC fails to form [40]. Wg signaling thus initiates a program utilizing Hfp and Rbf that restricts *dmyc* expression and ensures that dE2F1 activity remains off. Although, it is currently an open question whether Hfp regulates *dmyc* expression by the same mechanism as human FIR, the ZNC provides an excellent system to examine the relationship between developmental signals, such as Wg and control of dMyc expression.

6.2. A competitive edge: dMyc defines the winners

Recently, work in *Drosophila* has revealed a new aspect of dMyc function: high levels of dMyc provide cells with a competitive edge that allows them to kill nearby cells that have less dMyc. In the fly, cell competition is a process that is operationally defined by the progressive elimination of normally viable (but less “competitive”) cell types. When cell clones overexpressing dMyc are generated in the developing wing, such clones grow faster than the surrounding wildtype cells, which in turn actually grow less than expected and die more frequently [21,42]. The ability of dMyc to induce cell competition is a remarkable property that is not shared by all growth regulators [21].

Competition can be induced whenever neighboring cells differ in levels of dMyc. Wildtype cells, containing endogenous *dmyc*, are only killed when they reside near cells overexpressing dMyc [21,42]. Likewise, although *dmyc* hypomorphic cells are viable when surrounded by each other, when they exist in somatic clones surrounded by wildtype cells, they are eliminated from the wing [19]. Similar competitive outcomes result from other manipulations that allow some cells to have higher dMyc levels than their neighbors. For example, loss of *archipelago* (*ago*), which encodes a *Drosophila* F box protein homologous to human Fbw7, results in elevated dMyc protein levels and a competitive advantage that allows *ago* mutant cells to overtake whole body structures while wildtype cells are eliminated [20]. Also, overexpression of the dMyc antagonist dMnt slows growth of cell clones, and these cells are eventually eliminated [8]. Surprisingly, however, cell competition does not always occur when faster and slower growing cells are neighbors, as overexpression of Cyclin D/Cdk4 or Dp110 does not lead to competition, and cells lacking these regulators in mosaics grow very slowly but are not killed by their wildtype neighbors [21,29,43,44]. Enabling cells with a competitive edge therefore appears be a specific effect of dMyc activity.

How are less competitive cells eliminated from the growing fly? Cells with mutations in receptors for patterning factors, such as Dpp, Wg, or EGF, or those deficient in ribosome biogenesis, such as *Minutes*, a large class of mutations in genes encoding ribosomal proteins, are also subject to competitive elimination [45–48]. Cells lost in competition die by apoptosis, but how the apoptotic program is initiated is not clear [21,42,49]. Two models have been proposed to explain how cell competition occurs. In one model, less competitive cells are deprived of growth factors due to the capture of such factors by their more competitive neighbors [50]. The elimination of cells unable to receive Dpp, Wg, or EGF is certainly consistent with this idea, as is a report that some *Minute* cells are deficient in responding to Dpp [49]. However, the predictions that this model makes do not always hold. For example, dMyc expression neither enhances a cell’s response to signals such as Dpp or Wg, nor alters the response of neighboring cells, as would be predicted from a ligand-capture model (de la Cova, Vargas, and Johnston, unpublished data) [21]. A second model posits that competition is due to secretion of a factor that initiates an apoptotic program in neighboring cells. This model comes from the observation that although physical contact is not necessary for wildtype cells to be killed, they must be in close proximity to dMyc-expressing cells [21]. Thus, dMyc may allow cells to sense each other’s presence, and induce competition via a short-range signal

(Fig. 3) [21]. Interestingly, many of the *Minute* genes, as part of the large number of genes involved in ribosomal biogenesis, are dMyc transcriptional targets. Also, as mentioned above, dMyc expression is influenced by Wg and Dpp [19,21,51]. These observations might imply that all cell competition – competition involving dMyc, a reduction in ribosome biogenesis, or lack of patterning factors – operates by the same mechanism. However, this has not been demonstrated, and the molecular mechanism of cell competition remains to be established.

Cell competition is not an anomaly of flies, as it also occurs in mice. Cells with a mutation in the ribosomal protein L24 are competitively eliminated when wildtype cells are introduced to a mutant mouse blastocyst, a situation reminiscent of the *Minute* mutations of *Drosophila* [52]. Also, somatic inactivation of mouse *c-myc* in B- or T-cell mosaics results in the progressive loss of *c-myc* null lymphocytes [11,23]. Given the deregulation of c-Myc that occurs in many tumors, an exciting possibility that remains to be tested is that a tumor cell population in which c-Myc is activated may out-compete nearby wildtype cells. A Myc-induced competitive advantage, and the ability to kill neighboring cells, could facilitate growth of an incipient tumor. However, there are instances in both mice and flies where differences in Myc levels do not lead to competition. For example, *c-myc* null hepatocytes persist at stable frequencies in mosaic livers [53]. Furthermore, in the developing fly wing, *dmyc* expression is down-regulated in hinge cells as development proceeds, yet these cells are not competed away by nearby cells with higher dMyc levels (Wu & Johnston, unpublished data). Furthermore, post-mitotic cells in the fly seem to be protected from competition. The properties that allow some cells to ignore Myc level differences and others be gravely affected by them is unknown, but should reveal information about how cells perceive themselves as constituents of a growing tissue.

Why does cell competition occur? One hypothesis is that cell competition might contribute to an overall size-control mechanism. A striking observation is that although, for example, dMyc expressing clones in the developing wing proliferate more than wildtype cells, they do not change the final size of the adult wing. Overgrowth is prevented because the competitive elimination of wildtype cells compensates for the increased growth of dMyc-expressing cells. If cells are protected from death, or if dMyc is expressed ubiquitously, thereby preventing competitive interactions, size control is overridden, and a larger wing size results (Fig. 1) [21]. Such a size-controlling role of cell competition is intriguing as, paradoxically, it suggests that the competitive killing of wildtype cells allows dMyc-expressing cells to overtake a structure, and yet also keeps overgrowth of that structure in check. A second attractive theory about cell competition is that it represents a quality control mechanism that removes defective or mispatterned cells [54]. Testing of these ideas has only just begun. Genetic or other conditions that specifically prevent competitive cell death would be remarkable and useful tools for uncovering the normal role of cell competition. dMyc-induced cell competition in *Drosophila* provides a unique model that could lead to the identification of genes that are involved in the earliest steps of cancer progression.

7. Conclusions and perspectives

The simple Myc network, broad allelic series of *dmyc* mutations, and the ease with which growth and cell proliferation can be studied in a living animal has made *Drosophila* an ideal model organism for investigating the biological roles of Myc. Not surprisingly, then, dMyc has been the focus of intense scrutiny in the last several years, yielding several insights as well as some surprises. In this review, we have discussed what has been learned from studies in *Drosophila* and how these findings relate to Myc function in other organisms. Altogether, the data tell us that in all organisms that carry *myc* homologs, the Myc family of interacting proteins is essential for appropriate cellular and animal growth—a huge responsibility for a relatively

small protein network. Myc's role in regulating growth is probably an ancient one, as most of the network is conserved throughout taxa.

Still, several puzzles remain, and as always when research is informative, many additional questions have been raised. For instance, in *Drosophila*, high expression levels of Myc confer an advantage on cells that allows them to compete against and kill cells with less Myc. Why is this aspect of Myc function not shared by growth regulators like PI3K or Cyclin D? What is the molecular mechanism underlying Myc-induced competition, and does it occur in vertebrates? Another question raises an evolutionary issue—why does the nematode *C. elegans* lack *myc*, but retain *max* and *mnt* homologs? The answer to this is unknown, but it is possible that Myc's ability to integrate complex growth regulatory signals is not necessary in *C. elegans*; in contrast to flies, mice, and humans, where growth is "regulative" and allows developmental plasticity, growth in *C. elegans* occurs by proliferation of a fixed developmental lineage of cells [55]. A major gap also remains in our knowledge of how dMyc, dMnt, and dMax function together (or apart) to control growth, and how each contributes to cell cycle regulation. Similarly, we know little of how dMyc expression is controlled during development. In *Drosophila*, developmental signals, such as the Wnt family member Wg modulates dMyc expression in growing tissues [19], and it has been hypothesized that Myc expression, regulated by developmental signals, may provide a link between patterning information and tissue growth [56]. Further study of how the Myc/Max/Mnt network is regulated and functions in vivo is clearly important, and promises to provide insight into many aspects of developmental control of growth and the cell cycle.

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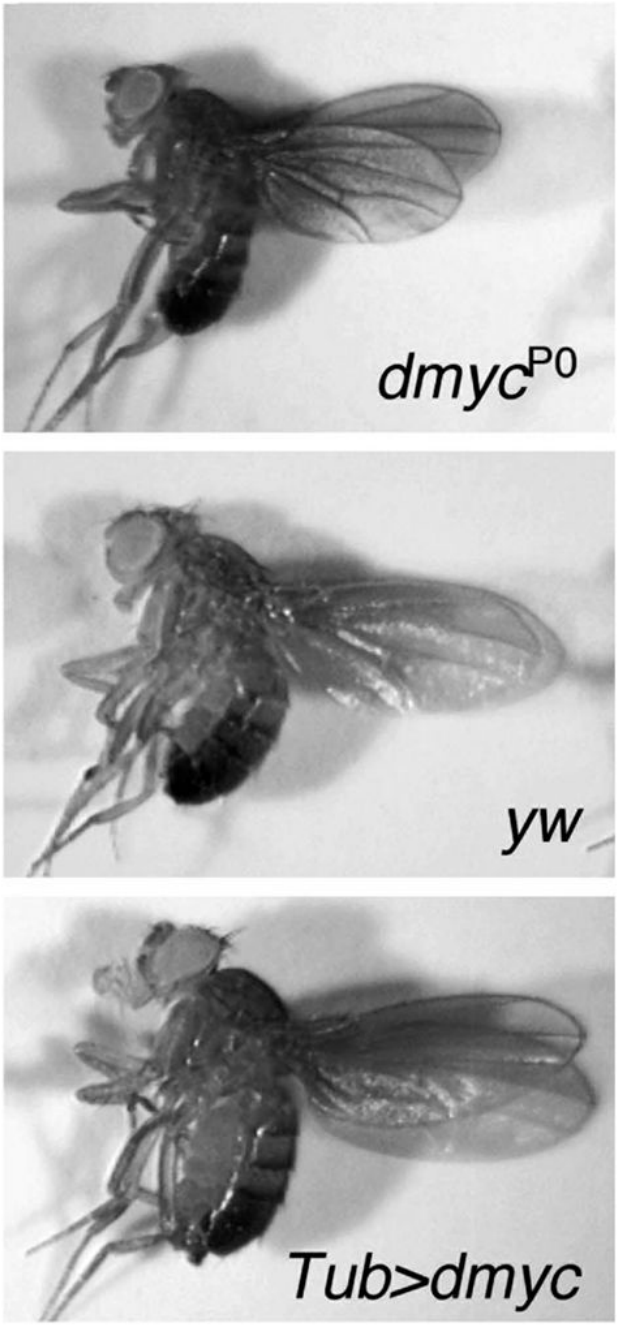

Fig. 1.

dMyc controls animal growth. Flies expressing less *dmyc*, such as a viable *dmyc* hypomorph (*dmyc*^{P0}), are smaller in overall body size than wildtype flies (*yw*), and wing size is reduced approximately 15% (a and b). Conversely, flies with increased *dmyc* expression, from a *Tubulin promoter-dmyc* transgene (*Tub > dmyc*), are approximately 30% larger in overall body size than wildtype flies (b and c).

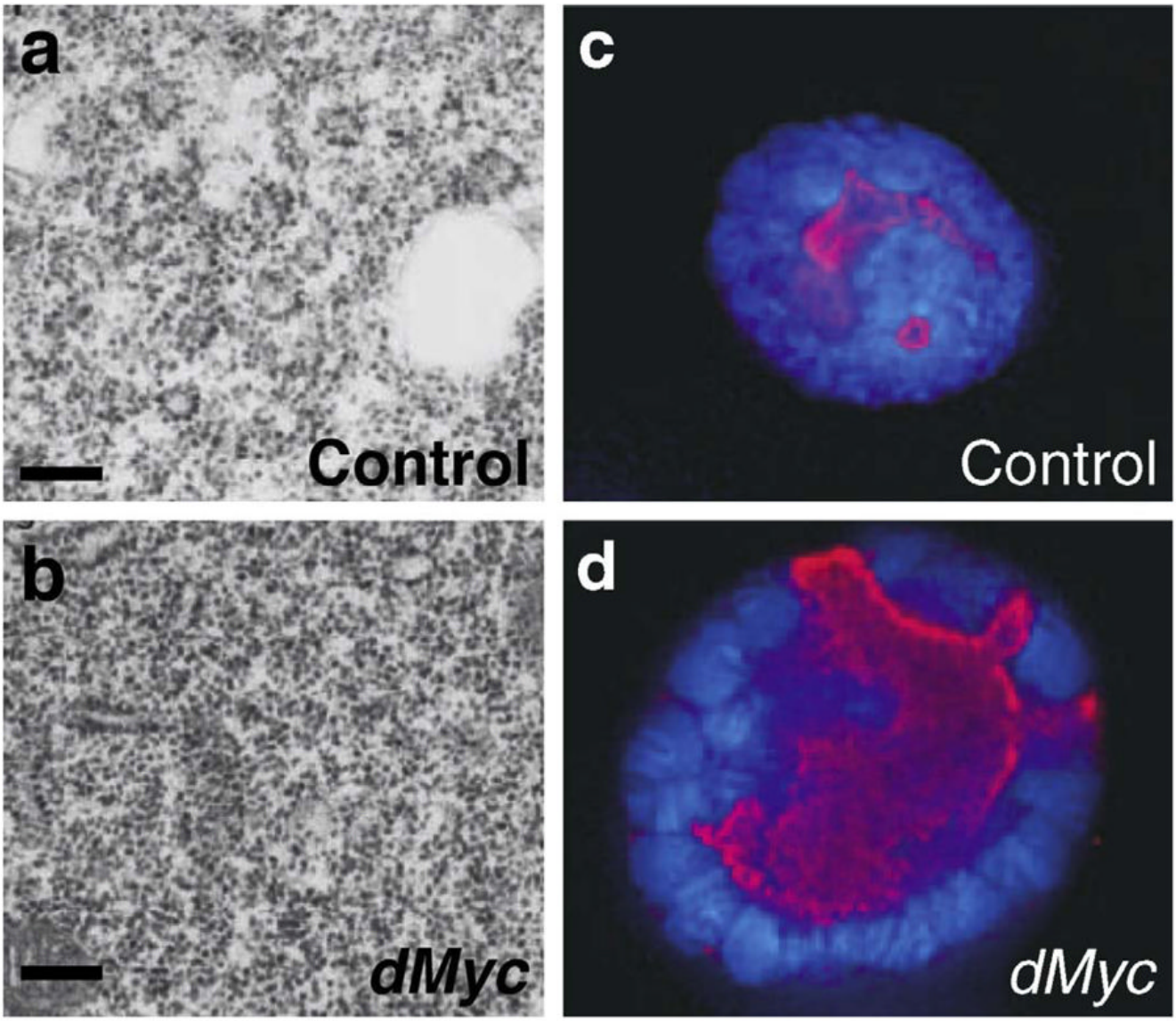

Fig. 2.

dMyc regulates nucleolar size and ribosome biogenesis in vivo. Cells of the fly salivary gland overexpressing dMyc have increased ribosome content (dark dots in a and b), and larger nucleolar size (c and d) as visualized by Fibrillarin (red), and nuclear size (c and d) as seen by DAPI (blue). (Adapted from Grewal, et al. 2005 and used with permission of the authors. See Grewal et al. [34] for more details.)

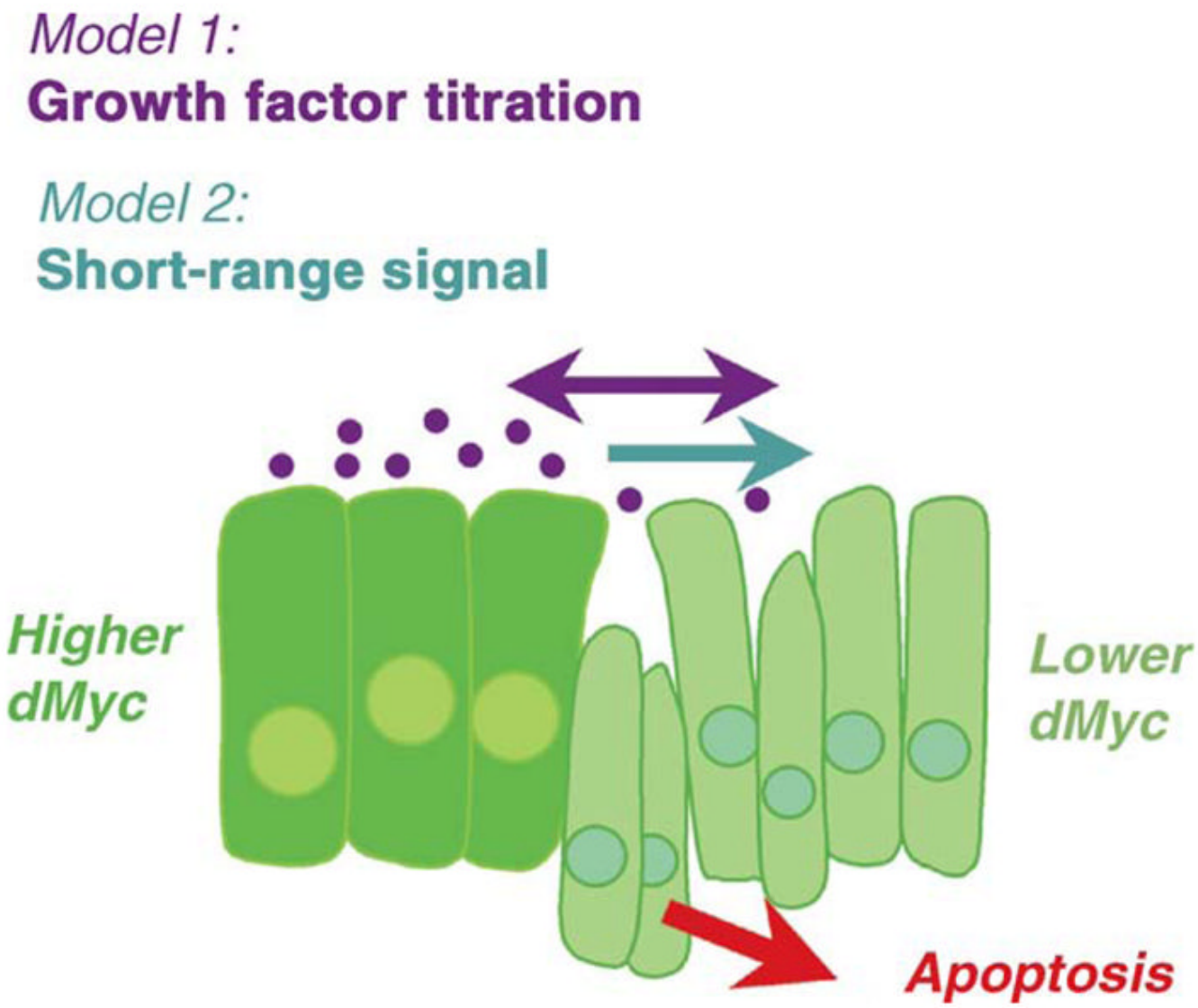

Fig. 3.
dMyc provides growing cells with a competitive advantage. Two models that can explain the competitive advantage of dMyc-expressing cells are: (1) that cells expressing more dMyc deprive their neighbors of growth and/or survival factors and (2) that dMyc expressing cells induce a short-range signal that kills nearby cells that express less dMyc.