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Myc/Max/Mad in Invertebrates: The Evolution of the Max Network

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Abstract The Myc proto-oncogenes, their binding partner Max and their antagonists from the Mad family of transcriptional repressors have been extensively analysed in vertebrates. However, members of this network are found in all animals examined so far. Several recent studies have addressed the physiological function of these proteins in invertebrate model organisms, in particular *Drosophila melanogaster*. This review describes the structure of invertebrate Myc/Max/Mad genes and it discusses their regulation and physiological functions, with special emphasis on their essential role in the control of cellular growth and proliferation.

Abbreviations

BDGP	Berkeley Drosophila Genome Project; http://www.fruitfly.org/
BHLHLZ	Basic-helix 1-loop-helix 2 leucine zipper
EST	Expressed sequence tag
SID	mSin3 interaction domain
Wg	Wingless
Dpp	Decapentaplegic
PI3K	Phosphatidylinositol-3-OH kinase
PIP3	Phosphatidylinositol 3,4,5-triphosphate
TOR	Target of rapamycin
TSC1/2	"Tuberous sclerosis" tumour suppressor gene 1/2

1
Identification of *myc/max*-Related Genes in Invertebrates

The importance of *myc* genes in normal development and disease has been amply documented (Oster et al. 2002). Myc activity has been shown to be required for normal proliferation and growth (Oster et al. 2002); conversely, deregulated activation of Myc contributes to cellular transformation, immortalization and genome instability, and appears to promote growth, cell cycle progression, apoptosis and angiogenesis (Oster et al. 2002). All of these effects are associated with Myc's ability to regulate the expression of a number of target genes, whereby Myc can act as an activator on some targets and as a repressor on others. The mechanism of transcriptional repression by Myc has been recently reviewed and it will not be further addressed here (Wanzel et al. 2003; D. Kleine-Kohlbrecher et al., this volume). Transcriptional activation by Myc is mediated by heterodimers between Myc and Max which bind to specific DNA sequences called E-boxes. These E-boxes can also be bound by heterodimers of Max with Mad proteins, which results in repression of the corresponding genes. Thus, a model has emerged where Max is located at the centre of a network of transcriptional activators and repressors. Since Max levels appear to be fairly constant, it is the relative levels of Myc and Mad proteins which determines the transcriptional status of E-box-containing target genes. The analysis of this network is complicated by a high degree of functional redundancy; mice and humans, where the Max network has been most extensively studied, contain only one *max* gene, but at least 3 partially redundant *myc* genes (c-*myc*, N-*myc*, L-*myc*, plus additional genes derived from processed transcripts) and 5 *mad*-like genes (*mad1*, *mxi1*, *mad3*, *mad4*, *mnt*). To complicate matters further, targeted disruption of either c-*myc* or N-*myc* results in lethality during mid-embryogenesis (Charron et al. 1992; Davis et al. 1993; Sawai et al. 1993).

To circumvent these problems, different approaches were undertaken to identify the Max network in simpler and genetically tractable organisms. Low stringency hybridization approaches led to the cloning of Myc in the sea star *Asterias vulgaris* (Walker et al. 1992), but failed to molecularly identify any *myc* genes in protostomes (see e.g. Shilo and Weinberg 1981; Bishop 1983; Madhavan et al. 1985; Sarid et al. 1987; Blackwood and Eisenman 1991). Instead, the single *Drosophila* Myc orthologue, termed dMyc, was found in yeast two-hybrid screens of a *Drosophila* library where vertebrate Max was used as the bait (Gallant et al. 1996; Schreiber-Agus et al. 1997). *Drosophila* Max (dMax) was cloned in a subsequent yeast two-hybrid screen with dMyc as the bait (Gallant et al. 1996), and the single *Drosophila* Mad/Mnt orthologue (dMnt) was found in yet another yeast two-hybrid screen with dMax as the bait (L. Loo

et al., manuscript submitted), and independently by in silico screens of the published *Drosophila* genome sequence (Peyrefitte et al. 2001). The availability of full-genome sequences also allowed the identification of Max network components in *Caenorhabditis elegans* (Yuan et al. 1998), *Anopheles gambiae* (Holt et al. 2002; P. Gallant, unpublished observation) and *Ciona intestinalis* (Dehal et al. 2002; P. Gallant, unpublished observation). In contrast to the situation in metazoans, no *myc*, *max* or *mad* genes are found in fungi or in plants. Two proteins in *Arabidopsis thaliana* called ATmyc1 (Urao et al. 1996) and ATmyc2 (Abe et al. 2003) share sequence similarity with the Myc C-terminus, the BHLHLZ domain (basic-helix 1-loop-helix 2-leucine zipper), but lack the N-terminal hallmarks of animal Myc proteins (Myc Box 1, Myc Box 2; see Sect. 2.2) and therefore probably do not correspond to true Myc orthologs.

2
Analysis of *myc*, *max*, *mad* Sequences in Invertebrates

The last common ancestor of insects, nematodes and chordates lived almost 1 billion years ago (Hedges 2002). Any motif that is conserved between orthologous proteins from these different groups is likely to be of functional significance. In the following sections, such evolutionary sequence conservation is discussed for Max network components from different invertebrates and one representative vertebrate, human (for an extensive comparison of vertebrate Myc proteins, see Miyamoto and Freire 2000; Johansson et al. 2001).

2.1
Max

All analysed species encode one Max orthologue, with the exception of *C. elegans*, which contains two *max*-like genes (*mxl-1* and *mxl-3*). As Max needs to interact with Myc and Mad proteins and possibly additional transcription factors such as Mga, TEF-1 and α-Pal (Hurlin et al. 1999; Gupta et al. 1997; Shors et al. 1998), it is not surprising that it is evolutionarily the most conserved component of the network (Atchley and Fitch 1995). The conservation is particularly high in the BHLHLZ domain, which is involved in protein:protein interactions and DNA binding (Fig. 1a, b). Fig. 1a also indicates the positions of exon–exon junctions with respect to the coding sequence; these junctions have been predicted based on comparisons between complementary (c)DNAs and published genomic sequences (Adams et al. 2000; Lander et al. 2001; Venter et al. 2001; Holt et al. 2002). The predicted human gene structure is identical to the published structure of the chicken gene (Sollenberger et al. 1994), sug-

a

```
                                                      "9 amino acids"
Ciona        1                                                                  KRS
Human        1                    MSDNDDIEVESD EEQPRFQSA............... ADKRA
Drosophila   1                 MSMSDDDRDIDIESD EDGDSDTGLGSSRHTNTANFTQ.. AEKRA
Anopheles    1                   MSDDDRDIDIESD DGDDSDNTKSQSRSGAGSHLYSQV AEKRA
Ce Mxl-3     1 MSAIVEDMYLLSSSMKMEKQFRKRHHSDSSD DDSSSPKSASPSMD.......... DDRRA
Ce Mxl-1     1                    MSDMSDLEDDQT GHCGSGEHSGPFDPKRHA...... ...RE
consensus    1                      d rdie esd ee  s  s   s             adkRa

             basic region  helix 1       loop        helix 2
                                                                 (*)
Ciona        4 HHNALERKRRDHIKESFNGLRDSVPSLQGEK ..TSRAQILNKATEYIQDVSRKNNADRQD
Human       27 HHNALERKRRDHIKDSFHSLRDSVPSLQGEK ..ASRAQILDKATEYIQYMRRKNHTHQQD
Drosophila  43 HHNALERRRRDHIKESFTNLREAVPTLKGEK ..ASRAQILKKTTECIQTMRRKISENQKD
Anopheles   43 HHNALERKRRDHIKDSFTSLRDSVPSLQGEK ..ASRAQILKKAAEYIQFMRRKNNSHQQD
Ce Mxl-3    51 HHNELERRRRDHIKDHFTILKDAIPLLDGEK ..SSRALILKRAVEFIHVMQTKLSSQGKA
Ce Mxl-1    33 QHNALERRRRDNIKDMYTSLREVVPDANGER VQASRAVILKKAIESIEKGQSDSATLSVD
consensus   61 hHNaLERkRRDhIKdsftsLrdsvPslqGEk  aSRAqILkkatEyIq mrrkn t qqd

               leucine zipper
                  *      *      *
Ciona       62 IDELKKQNNQLEMR IRALE
Human       85 IDDLKRQNALLEQQ VRALEKARSSAQ.....LQTNYPSSDNSLYTNAKGSTISAFDGGSD
Drosophila 101 IEEIKRQNNIIAKQ IQALESSNGD..........QFSEFLSDEEVGSEEADDEDLDQDFS
Anopheles  101 IDDLRRQNNMLESQ IRHLEQARASGN.....F.VDGADLGLAFQDGSRGSDSSDIDEVPT
Ce Mxl-3   109 IEDLTRKNELLEER LLERESSGSPSSSRLPALAVSSSQMQLTMPIIPQMQNIAQLSQYPQ
Ce Mxl-1    93 VAEQESKNAKLREE IARLKAKKDPSSSQSIIQ
consensus  121 iddlkrqNnlle q irale  r                                   d

Human      140 SS SESEPEEPQSRKKLRMEAS
Drosophila 151 RR NKKMKTFHA
Anopheles  155 NN NNGLGARRNKKMKTNANY
Ce Mxl-3   169 QA NIIAQSTNPAQLDGLIALNNDAILALLGSFQSVSPSLLDSAPGTPPSGFYPCAFSPVD
consensus  181    n

Ce Mxl-3   229 QQMAVKI    37% / 53%
```

b

```
                    Identity with human Max
           (overall / basic-helix-loop-helix-zipper)
Human                     100% / 100%
Ciona                       -  / 75%
Drosophila                 49% / 66%
Anopheles                  56% / 85%
Ce Mxl-3                   37% / 53%
Ce Mxl-1                   41% / 46%
```

gesting that it reflects a generic vertebrate *max* gene structure. Interestingly, the *max* gene structure is identical in insects (but not in worms; Fig. 1). This evolutionary conservation is particularly intriguing in light of the existence of alternatively spliced *max* messenger (m)RNAs in vertebrates: coding exons 2 (labelled "9 amino acids" in Fig. 1a) and 3 (coding for the "basic-helix 1-loop" domain) are facultatively included in mature *max* mRNAs, as is an exon between the last two indicated coding exons (this facultative exon is not shown

Fig. 1a, b Comparison of Max proteins from different species. **a** amino acid alignment. Shown *above* the sequence are the functional elements of Max; *asterisks* denote hydropic amino acids constituting the "leucine zipper". *Red vertical bars* show the positions of exon–exon junctions (except for *Ciona* Max). Full-length proteins are shown, except for *Ciona* Max where only the predicted BHLHLZ region is depicted. **b** Percentage identity of Max proteins with human Max, indicated for the full-length protein and for the BHLHLZ region only. Species shown are: *Ciona intestinalis* (sea squirt); *Drosophila melanogaster* (fruit fly); *Anopheles gambiae* (mosquito); *Caenorhabditis elegans* (nematode worm). Sources of unpublished sequences: *Anopheles gambiae*—accession number BX049732 (EST); *Caenorhabditis elegans* Mxl-3—accession number NP_510223 (protein); *Ciona intestinalis*—genomic scaffold 50, co-ordinates 2920 to 3318 (best match in a TBLASTN search with dMax)

in Fig. 1a) (Blackwood and Eisenman 1991; Makela et al. 1992; King et al. 1993; Vastrik et al. 1993; Koskinen et al. 1994; Tonissen and Krieg 1994; Arsura et al. 1995; FitzGerald et al. 1999). In insects only one mature *max* mRNA has been characterized (Gallant et al. 1996) and one more alternatively spliced EST has been reported (BDGP), but this conservation in gene structure indicates the possible existence of different additional splice isoforms. Furthermore, it suggests that such alternative forms of Max protein might play an essential role in vivo, even though their importance has not been demonstrated so far.

2.2
Myc

Vertebrates contain multiple *myc* genes (see above). They share a three-exon structure, whereby the major translation initiation codon is located at the beginning of the second exon and the open reading frame extends into exon 3 (Spencer and Groudine 1991); a few *myc* genes that deviate from this pattern and are encoded on a single exon probably derive from processed transcripts (e.g. human L2-*myc*). The same three-exon structure has also been found for *Drosophila myc* (P. Gallant, unpublished; however, the existence of additional non-coding exons 3′ of exon 3 has not been rigorously excluded), and the junction between exons 2 and 3 is located at the same codon as in vertebrate *myc* genes (Fig. 2c). This junction is also conserved in the single *myc* gene of *C. intestinalis* (as indicated by a comparison of EST and genomic sequences—P. Gallant, unpublished; Fig. 2c), and presumably also in the *Anopheles gambiae myc* (exon prediction based on the sequence similarity of conceptual translations of genomic DNA with Myc proteins from other species—see Fig. 2c; P. Gallant, unpublished). No *myc* gene has been found in the *C. elegans* genome (Yuan et al. 1998).

a. Myc-Box I

```
Human L-        17 EDFYRSTAPSEDIWKKFELVP
Human N-        29 FGGPDSTPPGEDIWKKFELLP
Human c-        37 QSELQPPAPSEDIWKKFELLP
Ciona           34 SSPTYGACLSEEIWKKFELLP
Asterias        29 SSTLTPPTPSEDIWKKFELYP
Urchin          27 AASPNSTTPSEDIWKKFDDME
Drosophila      42 QSDLEKIEDMESVFQDYDLEE
Anopheles        7 HWDLIKMEPMDDADTNELGML
consensus                  psediwkkfelvp
```

b. Myc-Box II

```
Human L-        89 IIRRDCMWSGFSARER
Human N-       102 VILQDCMWSGFSAREK
Human c-       128 IIIQDCMWSGFSAAAK
Ciona          129 KLIKDCMWNGIGHKPH
Asterias       112 ALIQDCMWSSIIAEER
Urchin         136 FLIQDCMWSAIQAEER
Drosophila      68 IRNIDCMWPAMSSCLT
Anopheles      118 QIRHDCMWAGMCADQS
consensus          vii DCMWsgisa er
```

c. Acidic region

```
Human L-       159 SESPSDS .........ENEEIDVVTV.EKR
Human N-       249 EDTLSDS DDEDDEEEDEEEEIDVVTV.EKR
Human c-       246 PTTSSDS ....EEEQEDEEEIDVVSV.EKR
Ciona          202 LETTSDS ..........DEEIDVVTV.DKA
Asterias       193 TNTPSDS ..........EEEIDVVTV.EKK
Urchin         205 STTPSDS ..........EEEIDVVTV.EKK
Drosophila     403 LETPSDS ..........DEEIDVVSYTDKK
Anopheles      891 VQTPSDS ..........DEEIDVVSIGDKN
consensus            tpSDS           eEEIDVVtv eKr
```

Fig. 2a–c Partial sequence alignments of Myc proteins from different species. Conventions are as for Fig. 1. Species shown are: urchin—*Strongylocentrotus purpuratus* (purple urchin); *Asterias vulgaris* (sea star); others are described in the legend to Fig. 1. Sources of unpublished sequences: *Ciona*—gene name ci0100150934; the BHLHLZ domain of *Anopheles* Myc was identified in a TBLASTN search of the *Anopheles* genome with dMyc as the query; the position of the exon boundaries was predicted based on the position of splice junctions, the amino acid homology at the ends of both exons, and the length of the predicted intron *(Anopheles:* 8,163 bp; *Drosophila:* 8,152 bp for the corresponding intron)

At the amino acid sequence level, Myc proteins are moderately conserved throughout evolution; for example, dMyc and human c-Myc are only 26% identical over the whole sequence (Gallant et al. 1996). However, interspersed in oceans of divergence lie islands of high sequence conservation that correspond to functionally important motifs. Best known are the N-terminally

d. Basic region – helix 1 – loop - helix 2 - leucine zipper

```
                        basic region    helix 1       loop     helix 2
                                                                          *       *
Human L-Myc   282     KRKNHNFLERKRRNDLRSRFLALRDQVPTLASCSKAPKVVILSKALEYLQALVGAEKRMA
Human N-Myc   374     RRRNHNILERQRRNDLRSSFLTLRDHVPELVKNEKAAKVVILKKATEYVHSLQAEEHQLL
Human c-Myc   355     KRRTHNVLERQRRNELKRSFFALRDQIPELENNEKAPKVVILKKATAYILSVQAEEQKLI
Ciona         512     IRVAHNVLERQRREGLRTSFHTLRKCVPELAEQERTPKVTILKKARDYSFQLQQEHAQLQ
Urchin        350     RRANHNVLERRRREDLRTSFFKLRDQVPELASQERAAKIVILKKATDYVHHLHADEESHT
Asterias     (322)    KRACHNVLERQRREDLRTSFLLLRDEVPELGTCDRAAKVVILKKATDYVSSLRDREETLR
Anopheles   (1086)    KRNLHNNLERQRRIGLKNLFEELKRQIPQLRDKERAPKVNILREAAALCTRLNQEAEQVN
Drosophila    626     KRNQHNDMERQRRIGLKNLFEALKKQIPTIRDKERAPKVNILREAAKLCIQLTQEEKELS
consensus             kR  HN lERqRR dLrs F  Lrd vP l   era KvvILrkA eyl  l  ee  l

                      leucine zipper
                         *          *      *
Human L-      342     TEKRQL....RCRQQQLQKRIAYL
Human N-      434     LEKEKL....QARQQQLLKKIEHA
Human c-      415     SEEDLL....RKRREQLKHKLEQL
Ciona         572     SEKARL....QQRQLALQQRLRSL
Urchin        410     RTHNAL....KRRHHALLLRLRQL
Asterias     (382)    MDMATE....KNRNLQLRRRLEAL
Anopheles   (1146)    ELRQRQ.MKLYERVRYLRASIHSQ
Drosophila    686     MQRQLLSLQLKQRQDTLASYQMEL
consensus              er  l      R  L  ri  l
```

e. Identity with human c-Myc (BHLHZ)

Human L-	48%
Human N-	59%
Human c-	100%
Ciona intestinalis	48%
Strongylocentrotus purpuratus (purple urchin)	49%
Asterias vulgaris (sea star)	50%
Drosophila melanogaster	41%
Anopheles gambiae	44%

Fig. 2d, e (continued)

located "Myc Box 2", which is part of the transcriptional regulation domain and important for the biological activities of Myc (Fig. 2b; Amati et al. 2001), and the C-terminal BHLHLZ domain, which mediates DNA binding and heterodimerization (Fig. 2d, e; Amati et al. 2001); the presence of these two motifs is a hallmark of all Myc proteins. A second N-terminal motif, known as "Myc Box 1", is also part of the transactivation domain and highly conserved in deuterostome Myc proteins, but much less so in the insect proteins (Fig. 2a). While these motifs have been extensively characterized in vertebrate Myc, considerably less is known about a highly conserved "acidic domain" located in the centre of the protein (Fig. 2c). The corresponding region in the v-Myc oncoprotein is specifically required for the transformation of adult chicken bone marrow cells and peripheral blood macrophages, whereas it is dispensable for the transformation of embryonic chicken cells or quail peripheral blood macrophages (Heaney et al. 1986; Biegalke et al. 1987). The high degree of evolutionary conservation suggests a much broader and more important role for this domain that needs to be defined. Evolutionary constraints on the

nucleotide sequence coding for this motif may also explain why the position of the junction between exons 2 and 3 has been conserved in *myc* genes (Fig. 2c).

2.3 Mad/Mnt

In humans and mice, the *mad* family is represented by five genes: *mad1*, *mad3*, *mad4*, *mxi1* and *mnt*. Two family members have been identified in the genome of *C. intestinalis* (P. Gallant, unpublished), whereas *Drosophila* and *Caenorhabditis* only encode one such gene each (*dmnt* and *mdl-1*, respectively; Peyrefitte et al. 2001, Yuan et al. 1998); the same appears to be true for *Anopheles* as well (P. Gallant, unpublished). Thus, early in chordate development a gene duplication involving *mad* seems to have taken place.

Figure 3a shows partial amino acid alignments of the Mad family proteins. The *Ciona* and *Anopheles* proteins are derived from conceptual translations of genomic DNA, and no EST evidence has been published yet; hence only their BHLHLZ region is shown, as the remainder of the protein cannot be predicted with high confidence. Like all members of the Max network, Mad/Mnt proteins are characterized by a BHLHLZ domain. In addition, they contain a region that mediates interaction with the transcriptional corepressor Sin3 known as "Sin3 interaction domain" or SID (Ayer et al. 1995; Eilers et al. 1999; Schreiber-Agus et al. 1995). Based on a comparison of the BHLHLZ regions, the dipteran Mad proteins are most closely related to vertebrate Mnt; the same appears to be true for the worm orthologue (Fig. 3b).

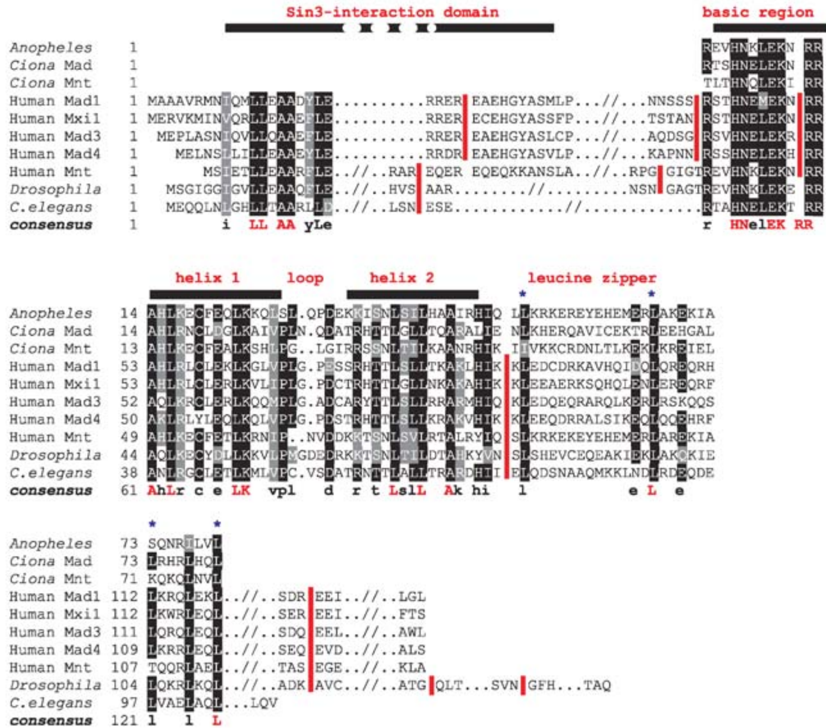
The structure of *mad* genes is less conserved than that of *myc* or *max*. However, in all genes the SID is encoded on a different exon than the BHLHLZ. This opens the possibility for alternative splicing to generate proteins that are able to bind DNA and Max, but lack the interaction with transcriptional corepressors; the resulting proteins could potentially differ radically in their transcriptional properties from SID-containing isoforms. Such alternatively spliced forms have indeed been reported to be produced from the murine *mxi1* locus (Schreiber-Agus et al. 1995) and from the *dmnt* gene (L. Loo et al., manuscript submitted).

3 Function of the Max Network in Invertebrates

3.1 Drosophila Myc

In invertebrates, the function of Max network components has predominantly been addressed in *Drosophila*. The *dmyc* gene has long been known to the

a. Alignment of Mad / Mnt proteins



b. Tree of bhlhz regions of Mad/ Mnt proteins

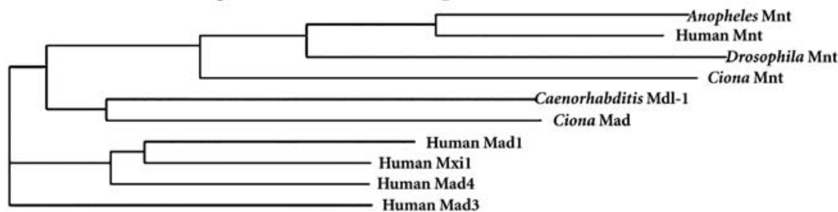


Fig. 3a, b Comparison of Mad/Mnt proteins from different species. **a** Partial amino acid alignment; conventions are as in Fig. 1. **b** Phylogenetic tree of BHLHLZ domains of different Mad/Mnt proteins constructed using CLUSTALW. Species are the same as in Fig. 1. Sources of unpublished sequences: *Anopheles* Mnt—accession number EAA07540 (protein); *Ciona* Mad—gene name ci0100137424; *Ciona* Mnt—gene name ci0100131159

fly-research community under the name of *diminutive* (*dm*), although the identity of *dm* with *dmyc* was only recently recognized (Bridges 1935; Gallant et al. 1996; Schreiber-Agus et al. 1997). While *dmyc* is an essential gene, several hypomorphic viable *dmyc* alleles have been described; flies carrying such mutations are characterized by a number of traits, including reduced body size, slender bristles, a delay in development and female sterility (Bridges 1935; Johnston et al. 1999). The cellular cause for the female sterility is currently unknown, but one of the contributing factors presumably is a defect in the migration and differentiation of somatic follicle cells, in particular of the border cells (J. Maines, personal communication; King 1957; King and Vanoucek 1960). In contrast, the other defects reflect dMyc's role in the control of cellular growth and proliferation: a reduction in *dmyc* activity reduces cellular size and increases the fraction of cells in G1 phase of the cycle (Johnston et al. 1999; T. Hulf and P. Gallant, unpublished data), whereas overexpression of dMyc promotes entry into S-phase and increases cellular size and the rate of mass increase (growth) in clones of cells (Johnston et al. 1999). In contrast to vertebrates, the forced expression of dMyc in flies does not accelerate cell division rates, since the G2–M transition is independent of dMyc activity in flies and becomes rate-limiting under conditions of dMyc overexpression where the duration of G1 phase is greatly reduce (Johnston et al. 1999). In endoreplicating cells that lack M-phases, however, forced expression of dMyc induces additional S-phases and results in hyperploidy (Britton et al. 2002; S. Pierce et al., submitted; J. Maines et al., submitted). These effects on growth are presumably mediated by the transcriptional regulation of a similar set of target genes as has been proposed for vertebrate Myc, including many genes involved in ribosome function and nucleolar biogenesis (Zaffran et al. 1998; Orian et al. 2003). In addition, overexpressed dMyc has been reported to control several cell-cycle regulators at the transcriptional level (Orian et al. 2003), as well as the important regulator of the G1–S transition, cyclin E, at the post-translational level (Prober and Edgar 2000). However, the involvement of these different putative dMyc targets in dMyc-controlled processes has not been addressed genetically.

These initial studies demonstrate a central role for dMyc in the control of growth. What then controls *dmyc* activity itself? So far, three signalling pathways have been implied in this process. The Wnt-family member Wingless was proposed to repress *dmyc* transcription in the presumptive wing margin (Johnston et al. 1999), and Dpp signalling positively regulates dMyc protein levels in the wing imaginal disc (C. Martin-Caballeros, cited in Prober and Edgar 2002). An interesting connection was also made between dMyc and Ras: Activated Ras itself promotes cellular growth, and this effect is mediated in part by an activation of the Raf-MAPK (mitogen-activated protein kinase)

module, which results in the accumulation of dMyc protein (Prober and Edgar 2000, 2002). By analogy with the situation in vertebrates, it was speculated that this effect is based on the stabilization of dMyc protein (Sears et al. 1999; Prober and Edgar 2000). A similar process might also occur during normal development, as cells lacking Ras also may have reduced dMyc protein levels (Prober and Edgar 2002). These observations suggest that receptor-tyrosine kinases controlling Ras might also be implied in the regulation of *dmyc*.

Ectopically expressed activated Ras also affects growth by stimulating PI3K activity, but PI3K and dMyc reside on parallel growth-regulatory pathways; forced expression of PI3K does not affect dMyc protein levels, and conversely, forced dMyc expression does not alter the levels of PIP3, the product of PI3K enzymatic activity (Britton et al. 2002; Prober and Edgar 2002). The difference between dMyc and PI3K is illustrated by their different response to environmental conditions. During normal development, PI3K is controlled by nutrient availability, via the activity of the insulin-receptor, and starvation leads to down-regulation of PI3K activity (Britton et al. 2002). If this down-regulation is prevented by constitutive expression of PI3K, larvae become hyper-sensitive to starvation. In contrast, larvae constitutively expressing dMyc survive starvation as well as wild-type larvae, consistent with the idea that nutrient and insulin signalling does not feed into *dmyc* (Britton et al. 2002). The growth-relevant targets downstream of dMyc and PI3K also seem to be different, as co-expressed PI3K and dMyc strongly synergize in the promotion of cellular growth (L. Johnston and P. Gallant, unpublished observations).

While these studies have directly addressed the regulation of *dmyc* protein and mRNA levels, forced dMyc expression has also been shown to overcome proliferation defects caused by genetic lesions in other pathways. Interference with the activity of the Tor kinase (Schmelzle and Hall 2000), either by expression of dominant-negative or wild-type forms of Tor in the wing (both of which function in a dominant-negative fashion), or by overexpression of the tumour suppressors TSC1 and TSC2 in the eye, inhibits growth and reduces organ size; these defects can be reversed by co-expression with dMyc (Tapon et al. 2001; Hennig and Neufeld 2002). Ectopic expression of different transcription factors in the eye primordium interferes with the normal development of the head capsule and results in a striking reduction in head size; this defect can be partially rescued by co-expression with dMyc (Jiao et al. 2001). Finally, certain combinations of mutations in the Pax gene *prd* with partial genomic rescue constructs allow the development of adult male flies that are characterized by small accessory glands; this size defect is rescued by ectopic expression of dMyc (Xue and Noll 2002). These examples further illustrate the ability of dMyc to promote growth and proliferation in different situations. However, additional work is required to determine to what extent

and at which level dTOR or Prd, for example, control *dmyc* activity during normal development.

The examples described above indicate that two principal biological activities of Myc proteins have been conserved between flies and vertebrates: the control of growth and proliferation (Elend and Eilers 1999). Indeed, fly and vertebrate Myc proteins are very similar in their molecular function and they can substitute for each other in different assays: When expressed together with dMax in human 293 cells, dMyc activates the expression of a c-Myc responsive reporter construct (Gallant et al. 1996); upon co-expression with human RasV12 dMyc is able to transform rat embryo fibroblasts (Schreiber-Agus et al. 1997); the proliferation defect of mouse embryo fibroblasts that are mutant for *c-myc* is partially rescued by ectopic expression of dMyc (Trumpp et al. 2001). Conversely, different forms of human c-Myc are able to partially rescue the lethality of strong *dmyc* alleles in flies (C. Benassayag et al., personal communication). In light of these observations, it is likely that dMyc and human c-Myc fulfill the same molecular tasks, and notably that they control the expression of their target genes in similar ways, by recruiting similar types of transcriptional co-factors as have been described in the vertebrate system, e.g. TRRAP, SNF5, Tip48, Tip49, BAF53, p300/CBP—all of which are also present in the fly genome (McMahon et al. 1998; Cheng et al. 1999; Wood et al. 2000; Park et al. 2002; Vervoorts et al. 2003; Adams et al. 2000).

3.2 Mad and Max

The other components of the Max network have not been extensively studied in flies. No mutations are known for *dmax*, but a null mutation in *dmnt* has recently been identified (L. Loo et al., manuscript submitted). An initial characterization suggests that overexpression of dMnt inhibits growth and proliferation, and a mutation in *dmnt* has the opposite effect, consistent with the expected properties of an antagonist of dMyc (L. Loo et al., manuscript submitted).

In contrast to flies, *C. elegans* contains two *max* genes (*mxl-1* and *mxl-3*) and one *mad* gene (*mdl-1*), but no *myc* (Yuan et al. 1998). Little is known about the normal function of these genes. Overexpression of dominant-negative forms of Mdl-1 or Mxl-1 (lacking the basic region) or RNA interference with *mxl-1* or *mxl-3* produces no discernible phenotype (Yuan et al. 1998; Maeda et al. 2001; Kamath et al. 2003), whereas RNA interference with *mdl-1* slightly reduces longevity in *daf-2* mutant worms (Murphy et al. 2003). Interestingly, *mdl-1* expression is also negatively regulated by the insulin receptor *daf-2* (Murphy et al. 2003), mutations of which extend lifespan in worms, raising

the possibility that Mdl-1 might also contribute to the regulation of lifespan in worms.

Although these experiments do not reveal any involvement in the control of proliferation and growth, Mdl-1 and Mxl-1 do show Mad- and Max-like properties when assayed in a heterologous system. Mdl-1 (and to a lesser extent Mxl-1) is able to interfere with the co-transformation of rat embryo fibroblasts by activated mammalian Ras and c-Myc. The interference by Mdl-1 depends on SID in Mdl-1, suggesting that Mdl-1 functions like other Mad proteins by recruiting the Sin3-corepressor complex and repressing transcription (Yuan et al. 1998). This result—as well as the sequence similarity—indicates that the (rudimentary) Max network in worms might fulfill similar functions to the vertebrate network. On the other hand, the Max network in worms shows several unique features not found in other metazoans—the absence of a *myc* gene, the existence of two *max*-like genes, the unique genomic structure of the *max* genes and the inability of Mxl-1 to homodimerize (Yuan et al. 1998).

As the phylogenetic relationship between nematodes, arthropods and chordates is still under debate (Hedges 2002), two main hypotheses can be invoked to explain these peculiarities in worms. The first is that worms contain an ancestral form of the Max network; hence, activities executed by Mad:Max complexes are the primary duty of the network, and Myc-like genes have been added later in evolution. The alternative is that *C. elegans* contains a derived Max network that differs in several aspects from an ancestral Max network. As Myc is essential in flies (Bourbon et al. 2002) whereas Mad/Mnt is not (L. Loo et al., manuscript submitted)—suggesting that Myc function is more important for survival—we favour the latter possibility.

4 Speculations and Conclusions

The availability of complete genome sequences enables biologists for the first time to make (reasonably accurate) predictions about the presence *and* absence of certain gene functions in many different species. Based on such information, we can state that components of the Max network exist in all analysed animals, but neither in unicellular organisms nor in plants, suggesting that this network originated early during the evolution of animals. The principal function of the Max network resides in the control of growth and proliferation. These processes are essential for all living cells, and accordingly Myc activity is required for the proliferation of many cells. However, the Max network is not absolutely required in all cell types and it might not be an integral part of the basic cell-cycle machinery or growth apparatus in animals, as

indicated by the existence of several vertebrate cell lines that lack core components of the Max network—either Myc (Miyazaki et al. 1995; Mateyak et al. 1997) or Max (Hopewell and Ziff 1995). Rather, it appears that Max network components might relay signals that are typical for multicellular organisms (e.g. patterning signals involved in cell–cell communication) down to the core cell-cycle and growth machinery. The Max network affects the activity of this machinery by modulating, or fine-tuning, the expression of many of its core components (Eisenman 2001). In contrast, Max network components might not be involved in the transmission of nutrient signals (at least in simpler animals), a function that is not specific to metazoans but of equal relevance for unicellular organisms.

The evolutionary conservation and, by inference, the central importance of the Max network is dramatically illustrated by the partial functional interchangeability of Myc proteins from flies and mammals, which further implies that not only core components of the Max networks are conserved (Max, Myc, Mad) but also associated factors that interact with these core components. This high degree of conservation opens new possibilities for the experimental dissection of the Max network, based on one hand on a functional analysis in appropriate model organisms (such as flies) and on the other hand on a bioinformatic analysis of the components making up the Max network. A sequence comparison of components from widely divergent species (in particular flies and mammals) reveals several highly conserved features that did not stand out when only mammals were included in the analysis. Of particular note are the gene structure of Max, which hints at the potential relevance of alternative Max isoforms, and the acidic domain located in the centre of the Myc protein. Clearly, despite intensive research over the last 20 years, the Max network still holds many secrets that will keep biologists busy for some time to come.

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