Mes2, a MADF-Containing Transcription Factor Essential for Drosophila Development

Gregor Zimmermann, Eileen E. Furlong,† Kaye Suyama, and Matthew P. Scott*

The development of the Drosophila mesoderm is initiated by the basic helix-loop-helix transcription factor twist. We identified a gene encoding a putative transcription factor, mes2, in a screen for essential mesoderm-expressed genes that function downstream of twist. Mes2 protein belongs to a family of 48 Drosophila proteins containing MADF domains. MADF domains exist in worms, flies, and fish. Mes2 is a nuclear protein first produced in trunk and head mesoderm during late gastrulation. At later embryonic stages, mes2 is expressed in glia of the central and peripheral nervous systems, and in tissues derived from the head mesoderm. We have identified a null mutation of mes2 that leads to developmental arrest in first instar larvae. Increased production of Mes2 in multiple embryonic and larval tissues almost always causes lethality. The ubiquitous or epidermal misexpression of mes2 in the embryo causes a dramatic loss of epidermal integrity resulting in the failure of dorsal closure. Our data show that the precise regulation of mes2 expression is critical for normal development in Drosophila and implicate Mes2 in the regulation of essential target genes. Developmental Dynamics 235:3387–3395, 2006. © 2006 Wiley-Liss, Inc.

Key words: muscle; glia; Drosophila; development; MADF; transcription

Accepted 30 August 2006

INTRODUCTION

Myogenic programs regulate the formation of a wide array of highly specialized muscles with diverse functions and morphologies. In Drosophila, the interplay of transcriptional hierarchies as well as signaling networks specifies how the early undifferentiated mesoderm gives rise to a highly stereotyped system of muscle fibers in larvae and adults (reviewed in Frasch, 1999; Baylies and Michelson, 2001; Furlong, 2004). Work over the past 2 decades has led to the identification of several key transcriptional regulators involved in mesoderm specification and differentiation (Boulay et al., 1987; Thisse et al., 1987; Azpiazu and Frasch, 1993; Taylor et al., 1995). This work established that twist, an evolutionarily conserved basic helix–loop–helix transcription factor (reviewed in Castanon and Baylies, 2002), constitutes the top of a genetic hierarchy that specifies the formation of the early mesoderm. The target genes of Twist include tin, sna, and Dmef2, all of which encode critical transcription factors that regulate gene expression in the mesoderm and neuroectoderm. Dmef2 is required for the differentiation of all muscle types, whereas tin (in conjunction with bap) has a more specific function in the development of the visceral and cardiac muscles. It is clear that many genes that comprise the myogenic program in Drosophila have yet to be studied in detail.

Previous work in our laboratory has investigated the transcriptional control of mesoderm development by identifying novel regulators that function downstream of twist (Furlong et al., 2001; Weiss et al., 2001). These regulators often play roles in tissues other than mesoderm (Weiss et al., 2001). To further elucidate the control of myogenesis, our laboratory carried out a microarray-based characterization of mutants that lack twist expression during early embryogenesis or...
that have twist overexpression (Furlong et al., 2001). This work implicated hundreds of genes in the myogenic program, as have other valuable studies using related methods (Estrada et al., 2006; Sandmann et al., 2006). The work described in this study extends the analysis by testing 26 of the newly identified genes for essential embryonic functions and doing an extensive analysis of one of them, mes2.

RESULTS

Previous work in the laboratory compared the mRNA expression profile of twist loss-of-function embryos to that of wild-type embryos and of embryos that express twist ubiquitously (Furlong et al., 2001). This work led to the identification of hundreds of genes newly linked to mesoderm development. The developmental role of the majority of these genes has not been examined. To evaluate whether these genes are essential for embryonic development, we verified their expression in the mesoderm by in situ hybridization and performed an RNAi-based screen of 26 genes. The 26 genes were selected based on their expression in the embryonic mesoderm, their unknown developmental function, and, in several cases, the presence of protein domains commonly found in signaling proteins. Stage 3 embryos (1 hr after egg lay) were injected with dsRNA probes directed against the coding region of the selected genes and ranging from 500 to 800 bp in length. Embryonic survival was assayed by determining the percentage of embryos that produced motile first-instar larvae able to hatch from the vitelline membrane (Fig. 1). Control-injected embryos show a 60–80% survival rate. Of the targeted genes, only three resulted in significantly lower hatching rates: CG3983, CG8965, and CG11100/mes2. The SD05368 expressed sequence tag does not have a corresponding annotated name.

**Fig. 1.** RNAi screen for genes expressed in the embryonic mesoderm. The embryonic requirement for 26 candidate genes was tested by injecting dsRNA constructs directed against the coding sequence of the indicated genes into stage 3 embryos. Control-injected embryos show a 60–80% hatching rate. Of the targeted genes, only three resulted in significantly lower hatching rates: CG3983, CG8965, CG11100/mes2. The SD05368 expressed sequence tag does not have a corresponding annotated name.

**Mes2 Protein Is a Member of the MADF Family**

The mes2 gene is predicted to encode a 437 amino acid protein. Conserved-domain searches of the public database (Marchler-Bauer and Bryant, 2004) revealed the presence of a single MADF domain (Myb/SANT-like domain in Adf-1: SMART accession no. 00595) within the N-terminal portion of Mes2 (amino acids 62–149). This domain was originally identified in Adf-1, a transcriptional activator of alcohol dehydrogenase (England et al., 1992). The MADF domain shows weak similarity to Myb domains. MADF domains of two Drosophila proteins, Adf-1 and Dip3, can bind DNA directly (Cutler et al., 1998; Bhaskar and Courey, 2002). The Drosophila genome contains 48 predicted proteins that contain at least one MADF domain, including several known or predicted transcriptional regulators such as Adf-1, Dip3, Stonewall, and Regular (England et al., 1992; Cutler et al., 1998; Claridge-Chang et al., 2001; Bhaskar and Courey, 2002). In most cases, the domain occurs within the N-terminal portion of the protein.

An alignment of the MADF domains of Mes2, Dip3, and Adf-1 as well as those of two predicted vertebrate proteins reveals the presence of several highly conserved aromatic residues (Fig. 2). An alignment of all Drosophila MADF domains reveals the following consensus motif: [LFI][ILV][2X][VYI]-[5X][LI][YW][DEN][5X][SF][9X]-[WYF][2][ILV][14-18X][WF][KR][X-][MLI][R][2X][YF]. The three aromatic amino acids at positions 12, 29, and 47 (shown in bold) are especially well conserved and form the basis of the
similarity to the mammalian Myb domain (England et al., 1992). MADF domains have been identified in diverse organisms ranging from worms to fish, although oddly, no such domain has yet been identified in mammalian genomes. Thus, this is a rare case of a substantial family of genes in multiple branches of the animal kingdom that has never been found in mouse or human. The regions of Mes2 outside of the MADF domain show little or no sequence similarity to other proteins. The closest relative of mes2 is another Drosophila gene (CG12768) that occurs ~22-kb upstream of the mes2 locus. This is the only protein that shows any similarity to Mes2 outside of the MADF domain. This additional sequence similarity is confined to a 38 amino acid region in the C-terminus of the proteins. In contrast to mes2, we were unable to detect any CG12768 expression in the embryonic mesoderm (data not shown).

To evaluate the expression patterns and the localization of the Mes2 protein during development, we prepared a polyclonal antibody against the full-length protein. Antibody staining of wild-type Drosophila embryos revealed that Mes2 is a nuclear protein first expressed in the trunk and head mesoderm during late gastrulation (Fig. 3A). This antibody specifically recognizes Mes2, as demonstrated by the absence of staining in Drosophila embryos that do not express the gene (Fig. 4E). Mes2 colocalizes with the mesoderm-specific DMef2 protein within cells of the trunk mesoderm (Fig. 3D–F). Expression of mes2 is restricted to the mesoderm until embryonic stage 10. The timing of mes2 expression in the early mesoderm correlates well with that of the twist target genes Dme2 and tin (data not shown). Our previous microarray analysis (Furlong et al., 2001) revealed that the mes2 transcript is depleted in twist mutant embryos. The ratio of mes2 transcript in these embryos (5–6 hr after egg lay) when compared with stage-matched wild-type embryos was 0.45 (P value of 0.0007). We also see very little Mes2 staining in early (stage 6–10) twist mutant embryos (not shown), which is not surprising given the loss of mesoderm in these mutants. The microarray data also showed enhanced levels of mes2 transcript in early (3–4 hr after egg lay) Toll065 mutant embryos that overexpress twist, with a mutant to wild-type ratio of 3.4 (P value of 0.0003). These data indicate that mes2 is a target gene of twist. We have tested this further by misexpressing twist in the wing disk using the 71B and MS1096 Gal4 drivers. Despite substantial Twist protein production, ectopic production of Mes2 protein is not observed (data not shown). These results suggest that Twist requires additional factors to activate mes2 expression or that repressing influences in the wing disc block Twist from activating mes2.

Beginning at stage 11, the expression pattern of mes2 expands to include neurogenic ectoderm (Fig. 3B). As embryogenesis proceeds, the expression of mes2 declines in muscle tissues derived from the trunk mesoderm (Fig. 3C,M), while expression in the neuronal ectoderm becomes restricted to glia (Fig. 3G–I,M). Costaining with the anti-Repo antibody demonstrates that mes2 is expressed in all glial populations of the CNS and peripheral nervous systems (Fig. 3G–I). At these late embryonic stages, Mes2 is also expressed in tissues derived from the head mesoderm, such as the fat body, lymph glands, pericardial nephrocytes, and hemocytes (Fig. 3C).

Staining larval tissues with the Mes2 antibody demonstrates expression in the same cell types that produce Mes2 in the embryo, such as myoblasts, which stain for Mes2 in the wing disc (Fig. 3J–L), and CNS glia (not shown). The dynamic expression pattern of Mes2 in two Drosophila germ layers suggests a regulatory function in diverse embryonic and larval tissues.

**mes2 Protein Is Essential for Larval Development**

To examine the role of mes2 during development, we obtained a fly line carrying a lethal P-element insertion (KG02901) within the mes2 genomic locus. mes2\(^{KG02901}\) animals were able to complete embryogenesis and hatch out of the vitelline membrane, but homozygotes die during larval stages. The movement of these first instar (L1) larvae is sluggish, although the animals do respond to touch (data not shown). The growth of the mutant larvae is severely impaired when compared with their heterozygous siblings. The mutant animals arrest during the L1 stage and die during the transition to the second instar (L2) larval stage. A small proportion of mutant larvae survive up to 8 days after egg laying, but even at this late time point, all of the animals remain in the L1 stage. We have never observed a live L2 mutant larva. Many larvae display incomplete ec dysis, a failure to shed the L1 cuticle completely. This developmental arrest was not rescued by feeding ecdysone to the larvae (data not shown), indicating that the inability to complete the transition to L2 is not due to a deficiency in the production of molting hormone. These data show that mes2 is essential for larval development and progression to the L2 stage.

Inverse polymerase chain reaction (PCR) sequencing (see Experimental Procedures section) revealed that the KG02901 insertion is associated with a small deletion that removes a portion of the first exon (including the
start codon) and all of the second exon of mes2 (Fig. 4A). The second exon encodes the entire region of the MADF domain. Homozygous mutant embryos have no detectable mes2 transcript (Fig. 4C) or Mes2 protein (Fig. 4E), confirming that mes2<sup>KG02901</sup> represents a null allele of mes2. The mutation is not complemented by the Df(3L)ED5017 deficiency, demonstrating that the lethal phenotype maps to the region that contains the P-element insertion.

To conclusively demonstrate that the lethal phenotype observed in these animals is due to the disruption of the mes2 locus, we constructed a genomic DNA rescue plasmid for this gene.

**Fig. 3.** Mes2 expression in embryonic and larval tissues. A–M: Mes2 protein (shown in red) is detected in wild-type embryos (A–I,M) or larval wing disk (J–L) using a polyclonal anti-Mes2 antibody followed by an Alexa-conjugated secondary antibody. A: Mes2 localizes to nuclei and is first detected in stage 6 embryos during gastrulation. D–F: Double-labeling for Mes2 (red) and the mesoderm marker DMef2 (green) reveals colocalization in the trunk mesoderm of stage 9 embryos. (D, Mes2; E, DMef2; F, merge). B: At embryonic stage 11, Mes2 expression expands to include the neurogenic ectoderm and expression in the trunk mesoderm starts declining. G–I: By stage 16, Mes2 (red) expression in the nervous system is restricted to glial populations and colocalizes with the glial-specific marker Repo (green; G, Mes2; H, Repo; I, merge). C: At these late embryonic stages Mes2 is also expressed in head mesoderm-derived tissues such as the lymph gland, pericardial nephrocytes, and the fat body. M: By embryonic stage 16, Mes2 (red) is no longer expressed in muscle tissues derived from the trunk mesoderm that express DMef2 (green). J–L: During larval stages, Mes2 is expressed in wing-disk myoblasts (J, Mes2; K, DMef2; L, merge) and in central nervous system glia (not shown).

**Fig. 4.** The KG02901 insertion leads to a complete disruption of mes2 expression. A: Graphic representation of the mes2 locus (green, exons; dark green, translated regions; light green, untranslated regions; black bars, introns). The KG02901 P-element insertion (red) deletes a portion of the mes2 coding region. B,C: In situ hybridization against mes2 insertion (red) deletes a portion of the mes2 coding region. B,C: In situ hybridization against mes2 insertion (red) deletes a portion of the mes2 coding region. D: Heterozygous embryos are shown for comparison (red, Mes2; blue, DMef2; green, β-galactosidase carried on balancer chromosome).
**Table 1. Misexpression of mes2 Leads to Lethality**

<table>
<thead>
<tr>
<th>Gal4 Strain</th>
<th>Phenotype (25°C):</th>
<th>Phenotype (18°C):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesoderm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>twist2XPE-gal4</td>
<td>Embryonic/Larval (L1) lethal</td>
<td>Viable</td>
</tr>
<tr>
<td>twist-gal4; 24B-gal4</td>
<td>Embryonic/Larval (L1) lethal</td>
<td>Embryonic/Larval (L1) lethal</td>
</tr>
<tr>
<td>twist-gal4; Dmef2-gal4</td>
<td>Larval (L1) lethal</td>
<td>Viable</td>
</tr>
<tr>
<td>Glia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gcm-gal4</td>
<td>Larval (L2) lethal</td>
<td>Viable</td>
</tr>
<tr>
<td>repo-gal4</td>
<td>Lethal</td>
<td>N/A</td>
</tr>
<tr>
<td>tub-gal4</td>
<td>Embryonic lethal</td>
<td>N/A</td>
</tr>
<tr>
<td>Ubiquitous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ubi-gal4</td>
<td>Lethal</td>
<td>lethal</td>
</tr>
<tr>
<td>NGT4; NGT40</td>
<td>Lethal</td>
<td>N/A</td>
</tr>
<tr>
<td>da-gal4</td>
<td>Embryonic lethal</td>
<td>N/A</td>
</tr>
<tr>
<td>Epithelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>prd-gal4</td>
<td>Lethal</td>
<td>N/A</td>
</tr>
<tr>
<td>69B-gal4</td>
<td>Embryonic lethal</td>
<td>N/A</td>
</tr>
<tr>
<td>MS1096</td>
<td>Pupal lethal</td>
<td>N/A</td>
</tr>
<tr>
<td>71B-gal4</td>
<td>Larval/Pupal lethal</td>
<td>Larval/Pupal lethal</td>
</tr>
</tbody>
</table>

*ila melanogaster* to that in *Drosophila pseudoobscura* revealed two regions with high conservation in the noncoding portions of the gene (Fig. 5A). One region is located ~2 kb upstream of the first exon and the other occurs within the large second intron. The conservation of these regions suggests that they may contain regulatory sequences that control the expression of mes2. Based on this information, we generated a rescue construct containing a 12-kb genomic fragment that encompasses all putative regulatory sequences. This fragment includes the entire coding region of the mes2 sequences that control the expression of mes2. Based on this information, we generated a rescue construct containing a 12-kb genomic fragment that encompasses all putative regulatory sequences. This fragment includes the entire coding region of the mes2, as well as upstream (3.5-kb) and downstream (1.5-kb) regions (Fig. 5A). Introducing the rescue construct into a mes2 mutant background restores the expression of mes2 in a wild-type pattern and fully rescues the lethal phenotype produced by this mutation, resulting in viable adult flies with no phenotypic defects (Fig. 5B vs. wild-type in 5C). Our data demonstrate that the phenotype associated with the KG02901 insertion is indeed caused by the disruption of the mes2 locus.

The survival of zygotic mutant animals up to the L1 larval stage differs from the dramatic embryonic lethality we observed in response to dsRNA injections (Fig. 1). To address the possibility that the genetically deposited mes2 RNA we detect by in situ hybridization (not shown) is able to rescue embryonic lethality, we generated mes2 mutant germline clones (Fig. 6). *mes2* is proximal to the available FRT insertion sites that are usually used to generate clones on the left arm of chromosome three, making it necessary to recombine chromosomes another way. We devised a strategy to eliminate the genomic rescue construct from the germline of adult flies that are homozygous for the *mes2* mutation (see Experimental Procedures section). To this end, we modified the approach of Luschnig et al. (2004) in which germline clones are detected by the absence of a maternally deposited nuclear green fluorescent protein (GFP; Fig. 6A). The disruption of the *mes2* transcript in germline clones was confirmed by carrying out reverse transcription followed by PCR (RT-PCR) with *mes2*-specific primers with RNA from 1- to 2-hr-old embryos (Fig. 6B). The full maternal-zygotic mutants survive into the L1 larval stage, reproducing the phenotype of the zygotic mutant. These results indicate that maternally deposited *mes2* transcript does not mask an embryonic requirement for the gene.

During larval stages, the Mes2 protein is strongly expressed in glia. To examine a requirement for Mes2 in the development of these cells, we adapted the MARCM technique (mosaic analysis with a repressible marker; Lee and Luo, 1999) to create mitotic clones that are homozygous for the null mutation. Mitotic recombination on the second chromosome creates YFP-positive mes2-mutant glia by removing the mes2 genomic rescue construct (see Experimental Procedures section). The overall morphology of glia that lack mes2 is normal, and the cells display no obvious developmental defects (Fig. 7B, compared with wild-type in 7A). These results suggest either that Mes2 protein is not essential for glia development or that the requirement is non–cell autonomous. Alternatively, the *mes2* mutation may disrupt an essential glia function without affecting the gross morphology of these cells. This possibility could explain the larval death caused by loss of the gene.

**Mis- and Overexpression of mes2 Is Highly Damaging**

To evaluate the effect of *mes2* misexpression on embryonic development, we produced transgenic animals that carry an inducible UAST-mes2 construct. We tested many fly strains that produce Gal4 in numerous tissues and patterns (Table 1). We found that increased production of Mes2 in various tissues disrupts normal development and leads to a lethal phenotype (Table 1). We have overexpressed *mes2* in cells that express the endogenous protein using glia-specific (repo-gal4 and gcm-gal4) and mesoderm-specific (twist-gal4, Dmef2-gal4, and 24B-gal4) Gal4 drivers. The morphology of the tissues that overexpress the protein was not disrupted, but in all
The mes2KG02901 allele is completely rescued by a mes2 genomic rescue construct. A: VISTA alignment of the mes2 regions of Drosophila melanogaster and D. pseudoobscura. Regions of high sequence similarity are indicated in pink and include two domains outside of the coding region. Red arrows indicate the limits of the genomic fragment that completely rescues mes2 mutant animals. B,C: Rescued mes2KG02901 flies of the genotype w; rescue; mes2KG02901 (B) are indistinguishable from wild-type CantonS flies (C).
cases, heightened levels of Mes2 resulted in lethality.

The ubiquitous production of Mes2 in embryos results in embryonic lethality and causes gross developmental abnormalities. The most striking phenotype was the failure of dorsal closure. Dorsal closure was also disrupted in embryos that specifically misexpress mes2 in the epidermis (Fig. 8C,D). These embryos appear morphologically wild-type up to stage 11. By stage 13, however, the organization of the epidermis is severely disrupted, especially along the leading edge that contacts cells of the amnioserosa. At the time when dorsal closure normally takes place, the cells of the leading edge do not display the normal elongated morphology and the integrity of the epidermis is disrupted, leading to “tears” in the epidermal sheath (Fig. 8C,D). mes2 misexpression also disrupts the development of the wing-disc epidermis (Fig. 8). Most MS1096-gal4; UAST-mes2 animals die during pupal stages, with very few escapers. Those animals that do survive have severely deformed wing and haltere tissues that form a necrotic mass. Even when the MS1096-gal4; UAST-mes2 animals are raised at 18°C to reduce Gal4 function and, therefore, the level of Mes2, the surviving adults display severely disrupted wing tissues (Fig. 8F). In all cases tested, the exogenous Mes2 protein displays a normal subcellular localization within the nucleus (Fig. 8C).

DISCUSSION

A Large Gene Family in Worms, Insects, and Fish

Mes2 belongs to a large family of predicted transcription factors in Drosophila that contain at least one MADF domain. The MADF domain has been identified in a variety of organisms, including worms, insects (Apis mellifera, honey bee, and Anopheles gambiae, mosquito), and fish. At this time, no clear examples of this domain have been identified in mammalian genomes. The very weak sequence similarities that do exist do not include some of the residues that are conserved among all the family members in other organisms, so they are probably unrelated or so distant as to be impossible to assess. Given that two complete and other partial mammalian genomes lack any MADF domains, the pattern of evolutionary conservation (and lack of it) is unique.

Based on its expression in the early mesoderm we expect that mes2 functions downstream of twist to regulate gene expression in the mesoderm. It was previously shown that another MADF domain protein, DIP3, modulates the synergistic transcriptional activation by Twist and Dorsal of a firefly luciferase reporter construct with enhancer regions that contain binding sites for both of these transcription factors (Bhaskar and Courey, 2002). These results may reflect a function of DIP3 in early mesoderm development. In addition, the circadian periodicity gene regular, which encodes a MADF and zinc finger-containing transcription factor, is strongly expressed in embryonic mesoderm (Scully et al., 2002). Further characterization of the MADF family may thus reveal interactions among multiple MADF-containing proteins in the specification of the mesoderm as well as other tissues.

Essential Functions of mes2

Although mes2 is an essential gene, it does not appear to regulate the embryonic patterning of the tissues in which it is expressed. Both zygotic and maternal-zygotic null mutants are able to complete embryogenesis and display no disruption of embryonic muscle structures, glia, or the lymph gland. Our results also demonstrate that mes2 is not required in a cell-autonomous manner for specification, development, or morphology of glia during larval development. mes2 could play an essential role in the development of other cell types, such as the fat body, or carry out a function in glia that can be provided by surrounding cells and, therefore, would not be detected by our clonal analysis. Alternatively, mes2 may regulate critical cellular processes without affecting glia morphogenesis.

Our data suggest that levels of Mes2 must be precisely regulated during Drosophila development. Too much or too little is fatal. We have shown that a null mutation of the gene results in developmental arrest during the first-instar larval stage and the animals die during the first molt. In addition, depletion of mes2 from hemocyte-derived S2R+ cells by RNAi disrupts cell growth and viability (Boutros et al., 2004). We have obtained similar results in our laboratory using any of three non-overlapping dsRNA probes directed against the coding region of mes2 (data not shown). We observed the most dramatic phenotypes when we used an inducible mes2 transgene to elevate the levels of Mes2 protein. The increased production of Mes2 in cell types that express the endogenous gene (mesoderm and glia) results in lethality. It seems likely that high levels of Mes2 disrupt normal development due to misregulation of mes2 target genes. Such an effect would be detrimental if either the timing or the level of target-gene regulation is critical in a given cell type. When a twist-gal4 driver is used to increase mes2 expression in the mesoderm, for example, the lethal phenotype may be caused by unusually high levels of Mes2 in the mesoderm, or to the perdurance of the protein beyond the embryonic stages during which it is normally expressed in this tissue.

The misexpression of mes2 in epidermal cells that do not normally express the gene results in severe disruptions of epithelial tissues and a failure of dorsal closure. Dorsal closure is the developmental process whereby the two lateral epithelia of the embryo migrate dorsally across the amnioserosa to fuse along the dorsal midline and “seal” the embryo. Mes2 misexpression in these epithelial cells results in “tears” within the epithelial sheath, particularly along the leading edge that contacts the amnioserosa. Mathematical models for dorsal closure (Rouset et al., 2003) have proposed that the lateral epidermis contributes a resistive force, opposing dorsal migration. Such forces place a strain upon the tissue and are likely to cause tearing of the epithelial sheath in situations that weaken epidermal integrity, possibly by disrupting cell–cell interactions.

Two other types of effect may account for the potent toxicity of mes2 over- and misexpression. Excess
Mes2 protein may have a dominant-negative effect by sequestering essential transcriptional cofactors in nonfunctional complexes. This finding would produce phenotypes that are more severe than the loss of mes2 if the putative cofactors have additional functions besides mediating the Mes2 signal. It is also possible that elevated levels of mes2 may misregulate the expression of genes besides the normal Mes2 targets. For instance, Mes2 may have a low affinity for target genes of other MADF-containing transcriptional regulators. In this case, increasing the levels of Mes2 in a cell may result in the misregulation of these genes. Previous studies of two other MADF-containing Drosophila proteins also describe the need for tight gene regulation. naylot, a hypomorphic allele of alpha1, disrupts olfactory memory in adults (DeZazzo et al., 2000). A null mutation of this gene causes larval lethality. Leaky expression from a heat shock promoter-alpha1 transgene can rescue the naylot memory defect, whereas more robust production of Alpha1 from UAS-alpha1 transgenes results in lethality with most Gal4 drivers tested. Similarly, acute overexpression of stone-wall, a gene that encodes a MADF-containing transcription factor essential in germ cell development, causes larval lethality (Clark and McKearin, 1996). All these data suggest that the levels of MADF-containing proteins must be kept within a narrow range during Drosophila development.

### EXPERIMENTAL PROCEDURES

#### RNAi Screen

dsRNA was prepared from PCR products (500–800 bp) flanked by T3 RNA polymerase sequences by in vitro transcription with T3 RNA polymerase (Roche). A total of 100–150 embryos (0–1 hr) were injected with 2 μM dsRNA. Survival was assayed after 24 hr by counting the number of larvae that hatched from the vitelline membrane.

#### Antibody Staining and In Situ Hybridization

Antibody staining with anti-beta-gal (Rockland, 1:1,000 dilution), anti-Repo (kindly provided by Chris Doe, 1:10 dilution), anti-DMEF2 (kindly provided by Hanh Nguyen, 1:1,000 dilution), and anti-Mes2 (1:1,000 dilution) was performed using standard techniques (Sullivan, Ashburner, and Hawley). Staining was visualized with Alexa568- and Alexa488-conjugated secondary antibodies (Molecular Probes, 1:500 dilution). To produce antibodies against Mes2 the entire open-reading frame was PCR-amplified and cloned into the PATH10 Trpe fusion vector. The TrpE-Mes2 fusion protein was expressed in BL21 bacteria and sodium dodecyl sulfate-gel purified from inclusion bodies. Gel slices were injected into rabbits and rats. The mes2 transcript was detected in 0–to-1 hr-old embryos by whole-mount in situ hybridization with a full-length antisense probe using an anti-digoxigenin alkaline phosphatase probe and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Roche) using standard techniques.

#### Fly Strains

Flies carrying the KG02901 insertion in the mes2 locus and the UAS-2xEYFP transgene were obtained from the Bloomington stock center. UAS-mes2 flies were generated by cloning the entire region of the SD09884 EST into the pUAST vector and by transforming the construct into embryos. Flies carrying the genomic mes2 rescue construct were generated by transforming embryos with a pCasper4 transformation vector containing the genomic region of mes2 (see Fig. 6 legend and below for details).

Flies that carry the maternal ubi-GFP transgenes were kindly provided by Stefan Luschnig. Flies carrying the tub-gal80 transgene were kindly provided Liqun Luo. For our overexpression experiments, we used the lines: tub-gal4, ubi-gal4, NGT4-NGT4-gal4, da-gal4, gcm-gal4, repo-gal4, 69B-gal4, twist-and-24B-gal4, twist-2XE-gal4, prd-gal4, MS1096-gal4, 71B-gal4, gmr-gal4.

#### Mes2 Mutant Clones

mes2KG02901 mutant germline clones were produced by females of the following genotype: hs-flp hs-flp; FRT(G13), ubi-nls-gfp, mes2 genomic rescue construct/ FRT(G13); mes2KG02901/mes2KG02901. Flip-induced recombination on the second chromosome results in the loss of the genomic rescue construct and of the maternally deposited GFP. Full maternal-zygotic mutant embryos were produced by crossing these females to heterozygous mutant males. mes2KG02901 mutant glial clones were produced in larvae of the following genotype: hs-flp hs-flp; FRT(G13), tub-gal80, mes2 genomic rescue construct/ FRT(G13); repo-gal4, mes2KG02901/ UAS-yfp, mes2KG02901. Flip-induced recombination on the second chromosome results in the loss of tub-gal80 as well as the mes2 genomic rescue construct. Loss of tub-gal80 results in YFP-positive mitotic clones. Control clones were generated in larvae with the same genotype as above, but lacking the mes2KG02901 mutation.

#### Analysis of Larval Lethality

Heterozygous mes2 mutant adults balanced with TM3, P[w+, act-GFP] were allowed to lay eggs onto molasses/plates supplemented with baker’s yeast paste for 1 hr at 25°C. Twenty-four hours after egg lay, homozygous and heterozygous L1 larvae were picked based on the absence or presence of the act-GFP marker, respectively. Larvae were staged based on the morphology of the trachea and of the mouth hooks. L1 larvae were kept on a molasses plate supplemented with baker’s yeast paste with or without added 20-hydroxyecdysone (20E, 8 μg/g) at 25°C.

#### Molecular Biology

Inverse PCR analysis was performed as described by the Berkeley Drosophila Genome Project (http://www.fruitfly.org/DGC/index.html). RT-PCR was performed with the One-Shot RT-PCR kit (Invitrogen). The mes2 genomic rescue construct was generated by cutting a 12-kb HpaI/Kpn1 fragment that contains the entire coding sequence out of the BAC RPCI-48E5 (Children’s Hospital Oakland Research Institute) and cloning it into a pCasper4 transformation vector.
ACKNOWLEDGMENTS

G.Z. was an Associate and M.P.S. is an Investigator of the Howard Hughes Medical Institute.

REFERENCES


Tsai RY, McKay RD. 2002. A nucleolar mechanism controlling cell proliferation in stem cells and cancer cells. Genes Dev 16:2991–3003.