A Myc–Groucho complex integrates EGF and Notch signaling to regulate neural development

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Integration of patterning cues via transcriptional networks to coordinate gene expression is critical during morphogenesis and misregulated in cancer. Using DNA adenine methyltransferase (Dam)ID chromatin profiling, we identified a protein–protein interaction between the Drosophila Myc oncogene and the Groucho corepressor that regulates a subset of direct dMyc targets. Most of these shared targets affect fate or mitosis particularly during neurogenesis, suggesting that the dMyc–Groucho complex may coordinate fate acquisition with mitotic capacity during development. We find an antagonistic relationship between dMyc and Groucho that mimics the antagonistic interactions found for EGF and Notch signaling: dMyc is required to specify neuronal fate and enhance neuroblast mitosis, whereas Groucho is required to maintain epithelial fate and inhibit mitosis. Our results suggest that the dMyc–Groucho complex defines a previously undescribed mechanism of Myc function and may serve as the transcriptional unit that integrates EGF and Notch inputs to regulate early neuronal development.

T
he Myc family of oncoproteins is intimately involved in the genesis of cancer (1). Myc proteins function within the context of a highly conserved basic helix–loop–helix (bHLH) Myc/Max/Mxd(Mad-Mnt) transcriptional network that is essential for normal development (2). Loss-of-function mammalian Myc mutants exhibit embryonic lethality probably because of Myc’s role in organogenesis (1, 2). Myc proteins dimerize with Max, and the resulting Myc-Max heterodimers bind to CACGTG (E-box) sequences, where they are associated with gene activation. Max can also heterodimerize with Myc antagonist proteins belonging to the Mxd family. Max-Mxd heterodimers bind to the same E-box sequences; however, this binding results in repression of many Myc-Max target genes. The transcriptional antagonism between Myc and Mxd proteins is well established biologically in the regulation of cell size and cellular growth (1, 2).

The Myc/Max/Mxd network is highly conserved and, in Drosophila, is represented by single dMyc, dMax, and dMnt genes (3, 4). Similar to the vertebrate Myc genes, dmyc is an essential gene involved in cell growth, affecting endoreplication, regulation of cell size, cell competition, and apoptosis (reviewed in ref. 5), whereas dmnt is a nonessential gene that is associated with differentiation, where it functions to limit cell growth (4).

A major challenge in understanding Myc function has been to identify the number and nature of the direct targets that it regulates (6). To this end, we previously used a microarray-based genomic chromatin profiling method termed DNA adenine methyltransferase (Dam)ID to identify the direct binding sites of the Drosophila Myc network (7). DamID, similar to the ChIP-chip chromatin profiling technique, is a powerful tool that allows systematic and global identification of in vivo direct targets of transcriptional networks (8).

We have also used the DamID approach to map the direct binding sites of the bHLH repressor Hairy and its associated cofactors Sir2, CtBP, and Groucho (9). Strikingly, a comparison of the two networks revealed a group of dMyc target genes that overlaps with targets recruiting the Groucho corepressor. Groucho (Gro) and its mammalian orthologs, collectively called transducin-like Enhancer of split (TLE) (TLE1–4), are developmentally regulated corepressors. Groucho was the first cofactor shown to be required for Hairy-mediated repression and was subsequently shown to mediate repression through several other classes of DNA-binding transcriptional regulators, including Engrailed, Dorsal, Tcf, and Runt (10).

Here we show that dMyc and Gro antagonistically coregulate a subset of cell fate and mitotic targets, defining a previously undescribed mechanism of dMyc function. Consistent with this, our phenotypic analyses show that dMyc and Gro are required for neuronal fate and mitosis and phenecopy EGF and Notch signaling, respectively. We also demonstrate a genetic link between dMyc, Gro, and the EGF/Notch pathways and propose that dMyc and Gro integrate EGF/Notch signaling during neuroectoderm development.

Results

dMyc and Gro Share Many Direct Targets. We identified 37 transcriptional direct targets shared between dMyc and Gro in Kc cells [Fig. 1 A; supporting information (SI) Table 2; chance probability of overlap is 3 × 10 e-14 (hypergeometric distribution); SI Fig. 5A]. These dMyc-Gro shared targets are not bound by other network proteins (dMax, dMnt, Hairy, dSir2, or dCtBP). Consistent with this, we find that the Myc-Max canonical binding consensus CACGTG (E-box) and its derivatives are absent from most (75%) of these dMyc-Gro target gene promoters. Functional classification of the dMyc-Gro targets show that 37% (14/38) of these genes play roles in mitosis. In addition, these shared targets also include genes encoding fate-determining transcription factors such as ventral nerve defective (ynd); intermediate nerve defective (ind); dMyc’s heterodimerization partner, dMax; and other genes involved in early neurogenesis (Fig. 1B; SI Table 2; data not shown). We hypothesized that dMyc and Gro coregulate this subset of transcriptional targets to determine mitotic potential and cell fate during development.

dMyc and Gro Antagonistically Regulate the Expression of Their Shared Targets. Gro/TLE proteins are corepressors that are recruited by dedicated repressors like Hairy/E(spl) (HES) proteins

Abbreviations: bHLH, basis helix–loop–helix; bHLHZip, bHLH leucine zipper motif; Dam, DNA adenine methyltransferase; dm, diminutive; IVT, in vitro translation; LOF, loss-of-function; PNS, peripheral nervous system; UAS, upstream activating sequences.

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Gene expression at both the protein and transcriptional levels (i.e., expression). Reduction of dMyc levels resulted in reduced target gene expression, whereas in model (i) dMyc would be expected to increase target gene expression, or (ii) dMyc and Gro targets. Western blot analyses indicating that dMyc/Gro target gene expression (a shared Myc-Gro target whose expression overlaps with that of Gro) is direct, but not indirect, because this effect was also observed with classical Myc target gene (fibrillarin; Fig. 1F). We also find that, although both dMyc and Gro are expressed in proliferating Kc and S2 cells, the dMyc antagonist dMnt is not detected under these conditions, and RNAi to dMnt had no effect on expression of these targets (data not shown). Thus, our data support the second model; dMyc is positively required for target gene expression, whereas Gro inhibits target expression independent of dMnt.

**dMyc-Gro Antagonistic Interactions Regulate Fate and Mitosis in the Developing Nervous System and Phenocopy EGF/Notch Antagonistic Signaling.** Gro is a downstream transducer of several signaling pathways and was placed at the crossroads of the Notch and EGF signaling pathways during patterning of the Drosophila nervous system, where EGF-induced site-specific phosphorylation of Gro attenuates its repression activity (13–15). During embryonic stage 9, the CNS matures in three bilaterally symmetrical longitudinal rows of neuroblasts (16), with the homeobox transcription factors, Vnd, Ind, and Msh, specifying the medial (ventral), intermediate, and lateral rows, respectively (Fig. 24; ref. 17). EGF regulates the expression of both Vnd and Ind and is thus required for the formation of the ventral and intermediate rows (13, 17). Interestingly, we find that both Vnd and Ind are among the 38 dMyc-Gro shared targets we identified (SI Table 2), and that Gro and dMyc, but not dMnt, are expressed in neuroblasts of stage 9 embryos (Fig. 2B–C’; data not shown). Because dMyc-Gro targets are associated with both neuroblast fate and mitosis, we hypothesized that EGF and Notch coregulate cell fate and mitosis within the developing neuroectoderm via dMyc-Gro antagonism. We compared Vnd expression (a shared Myc-Gro target whose expression overlaps with that of Gro) to that of Gro. Since Gro is not required for establishment of S1 neuroblasts, the overall number of neuroblasts, and mitotic activity in wild-type embryos to gro+7 loss-of-function (LOF) mutants (in which the maternal contribution of Gro is removed), Egfr2, or Notch55e11 (note that dMyc LOF embryos cannot be generated (18); Fig. 2 D and D’, G–F, J, and M–O]). We also evaluated these parameters in embryos overexpressing either dMyc or Gro using the conditional Gal4/+ upstream activating sequence (UAS) expression system (Fig. 2E, F, K, and L). Vnd expression is stronger and expanded in both Notch and gro LOF embryos, as well as in embryos overexpressing dMyc (Fig. 2I, G, and E, respectively) when compared with wild-type embryos (Fig. 2D). These mutants also show neuroblast hyperplasia (Fig. 2F, G, G’, H, and E’, respectively; Fig. 2P) and elevated mitotic activity (Fig. 2O, M, and K, respectively; Fig. 2Q). Furthermore, Egfr LOF or Gro overexpressing embryos show reduced Vnd expression (Fig. 2H and F), neuronal hypoplasia (Fig. 2H’, F’, and P), and reduced mitotic activity (Fig. 2N, L, and Q), consistent with the molecular nature of the dMyc-Gro common targets.

Another patterning/fate determination process governed by EGF and Notch signaling is the specification of mesothoracic sensory bristles in the peripheral nervous system (PNS) (13–15). Similar to our findings during neuroblast development, we find that loss of Gro or dMyc overexpression phenocopies activation of the EGF pathway, whereas loss of dMyc expression or overexpression of Gro phenocopies activated Notch signaling (SI Fig. 7). Importantly, cooverexpression of dMyc along with Gro results in a dose-dependent partial rescue of the Gro phenotype (SI Fig. 7 and SI Text).

**Fig. 1.** Regulation of dMyc and Gro shared targets. (A) Venn diagram of dMyc/dMax/dMnt and Gro targets (see SI Text). (B) Functional distribution of the 37 dMyc and Gro shared targets identified by DamID (plus one target identified experimentally; SI Table 2). (C) dMyc is required for expression of dMyc/Gro targets. Western blot analysis indicating that dMyc/Gro target gene expression is reduced in S2 cells treated with dMyc RNAi (dMyc-i). (Kr, Kruppel; γTub, γTubulin; Vnd, ventral nerve defective; cyclin B, cyclin 8; fibrillar, fibrillarin. (D) and E) dMyc is required for dMyc/Gro target genes transcription. (D) Western blot analysis of dMyc and Gro in untreated S2 cells or in cells treated with either dMyc or control (GFP) RNAi. (E) Northern blot analysis of Nop60B expression using the same extracts depicted in D. (F) Decline in target gene expression because of reduced dMyc levels can be partially restored by the simultaneous reduction of Gro levels. Western blot analysis of S2 cells treated with RNAi to the indicated gene(s) and assessed for the level of dMyc, Gro, or the shared target proteins Nop60B, CycA, Cyclin B, γTubulin, Barren (Barr), and the direct Myc-Max target, fibrillarin. Relative quantification of the proteins is given below the lanes with the first 0 μg of dMyc-i RNAi lane (wild type) being set to 100 (see Materials and Methods).
dMyc Interacts Genetically with EGF Receptor and Notch During Neurogenesis. The genetic interaction between dMyc and Gro fits well with previously reported genetic interactions between Notch-Gro and EGF-Gro ([15]). We find that dMyc also interacts genetically with EGF and Notch, leading to disruptions in neurogenesis. We tested for interactions between an EGF receptor mutant (Egfr; ref. 19) and two dMyc mutants (dm1 and dm2; refs. 4 and 18) and observed a dose-sensitive genetic interaction in which a reduced number of transheterozygous progeny survive (i.e., synthetic lethality; Table 1). Embryos from mothers heterozygous for either Egfr or dMyc alone (or hemizygous for dm* in males; dm*/Y) are viable. In contrast, simultaneously reducing the dose of both dMyc and Egfr (dm*/+/; Egfr*/+; Egfr*/+ or dm*/Y; Egfr*/+ or dm*/Y) results in inappropriate development and subsequent increased embryo lethality. Analysis of dm1/Egfr2 heterozygous mutant embryos using the panneuronal marker 22C10 revealed severe patterned phenotypes and aberrant neurogenesis due in part to improper development of neuroblasts (Fig. 3).

Because Gro is a downstream transducer of the Notch pathway during neurogenesis ([14, 15]), our results suggest that an antagonistic relationship will exist between dMyc and Notch during neurogenesis. Because dMyc and Notch are located in close proximity on the X chromosome, we tested for dominant genetic interaction between a LOF mutant in Notch (N55e11; ref. 20) and dm1. Although homozygous N55e11 is lethal, heterozygous N55e11 females are viable (Table 1) and display a greatly increased number of mesothoracic bristles (SI Fig. 7J). We hypothesize that, if dMyc antagonizes Notch and is required to promote neuronal fate, partial loss of dMyc will result in suppression of the ectopic bristle formation observed in N55e11 heterozygous females. Indeed, dm1 exhibits a dominant genetic interaction with N55e11: ectopic bristle formation is partially rescued (>50%) in doubly heterozygous N55e11/dm1 females (Table 1; SI Fig. 7L). Thus, our genetic results suggest that EGF and Notch are the upstream regulators of dMyc-Gro function: dMyc is required for establishing the neuroblast fate and mitosis in the developing CNS/neuroectoderm and Gro is a direct target of dMyc that works together to establish neuronal number and maintenance.

Table 1. dMyc(dm) exhibits genetic interaction with Egfr and N

<table>
<thead>
<tr>
<th>Female</th>
<th>Male</th>
<th>N</th>
<th>Percent flies with ectopic bristles*</th>
<th>Percent lethality†</th>
</tr>
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<tbody>
<tr>
<td>+/+</td>
<td>+/+</td>
<td>2,969</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>dm1/+, Egfr2/+</td>
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<td>2,575</td>
<td>3</td>
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</tr>
<tr>
<td>dm1/+, Egfr2/+</td>
<td>+/+</td>
<td>2,028</td>
<td>17</td>
<td></td>
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</tbody>
</table>

*dm1 suppresses ectopic bristle formation associated with N55e11.
†dm1/Egfr doubly heterozygous embryos exhibit synthetic lethality.
dMyc and Gro associate with each other in the immunoprecipitated complex. We find that Gro binds to two regions within the dMyc protein: domains within dMyc and Gro that are required for their interaction (Fig. 4). To test this, we immunoprecipitated endogenous dMyc or Gro from 3.5- to 4.5-h neuron stage embryos and tested for the presence of the Gro dominant-negative dMyc or Gro from 3.5- to 4.5-h neuron stage embryos and tested for the presence of the Gro dominant-negative dMyc–Gro direct target whose expression overlaps with and is required for S1 neuroblasts (D and E), or the mitosis marker phosphohistone3 (pH3; green) (F and G). Hemisegments are indicated by a white rectangle. All other abbreviations are as in Fig. 2. Anterior is to the left.

Discussion
Identification of a Gro–dMyc Interaction Outside the Canonical dMyc/dMax/dMnt Network. Myc proteins are required for both cell growth/size and cell proliferation. The model in which Myc functions are mediated by heterodimerization with Max and antagonized by Mxd (Mad/Mnt) proteins has been well established (1, 2). However, recent studies suggest that a set of interactions outside the canonical Myc/Max/Mxd network also regulate some of Myc’s functions (2, 7). Interestingly, our studies point to a subset of dMyc direct targets that are not shared by either dMax or dMnt (Fig. 1A; ref. 7). Furthermore, dMnt-Dam and dMax-Dam were not recruited to these dMyc targets even in experiments where the Dam protein was coexpressed in the presence of high levels of dMax or dMyc, respectively (A.O. and R.N.E., unpublished data), suggesting that previously uncharacterized mechanisms may mediate Myc’s recruitment to DNA, and proteins other than dMnt may antagonize its transcriptional activity on this set of targets. Here we report the identification of Gro as the first component in a pathway that antagonizes dMyc function independent of dMnt and operates during Drosophila neurogenesis.

Gro Antagonizes dMyc Function. Transcriptionally, we find that dMyc is positively required for the expression of dMyc-Gro targets, activity that is antagonized by Gro. Importantly, dMyc is not a Gro target, and reducing Gro levels does not affect dMyc protein levels (Fig. 1D; ref. 9). Furthermore, Gro antagonism is limited only to the dMyc-Gro subset of shared targets and does not involve dMnt: there is no overlap between genes bound by dMnt or Gro, dMnt is not expressed in cells where the dMyc–Gro interaction is observed, RNAi to dMnt does not affect Myc-Gro shared target expression (not shown), and overexpression of dMnt does affect PNS development (SI Fig. 6).

Although we cannot exclude the possibility that dMyc-Gro targets are coregulated by individual dMyc and Gro complexes, our results suggest that dMyc and Gro are part of a single larger protein complex. First, the observation that RNAi to dMyc results in reduction of target expression and is restored by coregulating Gro suggests that other activators coregulate shared target expression along with dMyc. Second, our biochemical purification, binding data, and DamID Southern analyses support the idea that both
proteins physically interact with one another yet associate with DNA through distinct binding sites. Third, Gro does not bind directly to DNA but must be recruited to targets by sequence-specific DNA-binding transcription factors. Fourth, most of the dMyc-Gro targets lack E-box sequences associated with canonical Myc network targets, suggesting that dMyc and Gro may be recruited to shared targets via a novel mechanism or by other protein(s) yet to be identified. Candidates for recruiting Gro may be the E(spl) proteins that convey the Notch signal, antagonize the EGF pathway, interact with Gro, and exhibit similar phenotypes (14). Thus, the identification of the entire dMyc–Gro complex and its regulation will be an important next step.

Biological Role of dMyc-Gro During Neuroectoderm Development. Gro’s role as a downstream transducer of Notch signaling during neurogenesis is well documented (15), and mounting evidence supports Myc as a key player in progenitor cell proliferation (23, 24). Here we have identified a previously undescribed role for dMyc, together with Gro, during Drosophila early neuronal development. dMyc and Gro are required to directly regulate key fate controlling genes such as the homeodomain proteins vnd and ind that are downstream targets of EGF signaling (SI Table 2; Fig. 3). Because Vnd was identified as a regulator of the proneural gene complex (25, 26), the differential regulation of Vnd by dMyc and Gro implicates them as antagonistic regulators upstream of proneural genes. Thus, we propose that dMyc is transiently required within the neuroectoderm, where it promotes specific fate acquisition and allows mitotic expansion of committed neuronal cells.

Phenotypically, we observe that, similar to EGF, dMyc promotes neurogenesis both in the PNS and CNS, whereas Gro and Notch inhibit neuroblast formation and mitosis (Figs. 3 and 4; SI Fig. 6). This is a different role than that previously ascribed to dMyc, because it is usually associated with regulation of cell size and organismal growth (5), functions that are antagonized by dMnt.
Consistent with this, a recent study identified EGF-induced phosphorylation of e-Myc, Max, and TLE proteins in mammalian cells (27). The antagonistic relationship of Myc/EGF to Gro/Notch is likely to be highly dependent on the developmental context and the specific progenitor niche (28). For example, in cellular contexts in which Notch promotes proliferation, such as during the development of T cells in acute leukemia, Myc is a direct target of mutated Notch1 and is required for T cell proliferation and development (29). Our findings also fit well with observations that N-Myc is required during mouse progenitor development, and that the fly tumor suppressor Brat regulates dMyc levels posttranscriptionally in larval neuroblasts resulting in a “tumorous” phenotype (30, 31).

A Proposed Pathway for Transducing Signaling Cues into Transcriptional Outputs. Taken together, the snapshot provided by our DamID data leads us to suggest a model in which changes in neuronal progenitor fate and mitosis are determined by the balance between EGF and Notch signaling that is likely transcriptionally mediated by the dMyc–Gro complex. During epithelial develop-

Plasmids and Constructs. A detailed list of constructs used in this study is provided in SI Text.

Binding Assays. In vitro binding experiments were performed by using GST-pulldown assays similar to those described (4, 34, 35).

Immunoprecipitations and Immunofluorescence. Embryos (3.5–4.5 h) or Kc cells were lysed on ice for 15 min in buffer L (1× PBS/1% BSA/0.5% Nonidet P-40) with 10 mM EDTA and protease and phosphatase inhibitors, and then sonicated, followed by centrifugation at 4°C. Protein (750 μg) was used for each immunoprecipitation with the indicated antibody and then subjected to SDS–PAGE and Western blot analysis. Embryos and cells were immunostained as described (ref. 35; see also SI Text).

Drosophila Cell Culture and RNAi Experiments. S2 cells were maintained at 25°C Schneider’s media (Invitrogen, Carlsbad, CA), 10% FBS/10 mM glutamine. RNAi to Drosophila cells was performed by using the MegaScript RNAi Kit (Ambion, Austin, TX). One hundred fifty micrograms of cell extract per lane was separated via SDS–PAGE.