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The *Drosophila* Basic Helix-Loop-Helix Protein DIMMED Directly Activates *PHM*, a Gene Encoding a Neuropeptide-Amidating Enzyme▽†

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The basic helix-loop-helix (bHLH) protein DIMMED (DIMM) supports the differentiation of secretory properties in numerous peptidergic cells of *Drosophila melanogaster*. DIMM is coexpressed with diverse amidated neuropeptides and with the amidating enzyme peptidylglycine α-hydroxylating monooxygenase (PHM) in approximately 300 cells of the late embryo. Here we confirm that DIMM has transcription factor activity in transfected HEK 293 cells and that the *PHM* gene is a direct target. The mammalian DIMM orthologue MIST1 also transactivated the *PHM* gene. DIMM activity was dependent on the basic region of the protein and on the sequences of three E-box sites within *PHM*'s first intron; the sites make different contributions to the total activity. These data suggest a model whereby the three E boxes interact cooperatively and independently to produce high *PHM* transcriptional activation. This DIMM-controlled *PHM* regulatory region displayed similar properties in vivo. Spatially, its expression mirrored that of the DIMM protein, and its activity was largely dependent on *dimm*. Further, in vivo expression was highly dependent on the sequences of the same three E boxes. This study supports the hypothesis that DIMM is a master regulator of a peptidergic cell fate in *Drosophila* and provides a detailed transcriptional mechanism of DIMM action on a defined target gene.

Neurons release numerous biologically active transmitters, including neuropeptides, which are derived from larger precursor proteins. Certain neurons termed neurosecretory cells (NSCs) are specialized in neuropeptide production and have greatly amplified secretory capabilities that are akin to those of peripheral endocrine cells. NSCs play essential roles in animal physiology by releasing biologically active peptides that can act at long distances. They represent a distinctive neuronal class because their secretory properties are amplified: such cells are specialized to produce, package, and release large amounts of such signaling molecules. Additionally, NSC properties can be strongly modified in response to changes in environment or internal homeostasis (9). Examining the intracellular regulatory pathways that organize and modulate these specialized properties is therefore critical to understanding NSC physiology.

In vertebrates, several transcription factors have been identified that are required for the proper differentiation of neuroendocrine cells in the brain and periphery, including Mash1, Sim1, Otp, and others (1, 8, 24, 26, 31, 32, 36, 47, 52). In the case of Sim1, this requirement involves early aspects of development prior to terminal differentiation (e.g., cell migration [56]). In *Drosophila melanogaster*, only the basic helix-loop-helix (bHLH) protein DIMMED (DIMM) has so far been implicated in the development of peptidergic cells (2, 17, 20, 21). The *Drosophila* genome contains at least 30 neuropeptide precursor-encoding genes; remarkably, more than 90% of the known or predicted *Drosophila* neuropeptides are amidated (22). In the *Drosophila* central nervous system (CNS), neuropeptides are expressed by cells at all axial levels and individual neuropeptide genes are typically expressed by small subsets among the approximately 10,000 neurons of the larval CNS. DIMM displays a highly regulated pattern of expression in approximately 300 diverse larval CNS neurons. DIMM neurons (and the few DIMM-positive peripheral cells) are chemically heterogeneous but share certain properties: (i) individual DIMM cells contain large amounts of neuropeptides (as indicated by immunocytochemistry), and (ii) DIMM cells all express high amounts of the critical neuropeptide biosynthetic enzyme peptidylglycine α-hydroxylating monooxygenase (PHM), which is required for peptide α-amidation.

The results of previous studies of DIMM have paralleled those for regulatory factors for other transmitters. For example, PET-1 (an ETS domain transcription factor) coordinates the terminal differentiation of the serotonergic phenotype and is precisely expressed by the precursors to the vast majority of serotonergic neurons, but not elsewhere (18). Likewise, the Paired-like homeodomain proteins Phox2a and Phox2b have been implicated as essential determinants of the noradrenergic phenotype (35, 41). However, in contrast to the situations for serotonergic and noradrenergic cell fates, in which all cells sharing the same fate express the same transmitter, the NSC cell fate represents the expression of many different secretory peptides by diverse cells. We estimate that there are at least 31

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different neuropeptide genes in the *Drosophila* genome (51). Thus, NSC cell differentiation must include both cell type-specific features (e.g., neuropeptide selection) and generic features that support the expression, packaging, and release of amidated neuropeptides. Reflecting this fact, we previously concluded that, within single neurons, DIMM operates in two distinct mechanisms: one is termed combinatorial and the other a form of master regulation (3).

In *Drosophila*, the selection of an appropriate neuropeptide gene is controlled by combinations of transcription factors. The compositions of codes are different for different cells, but many include DIMM as a constant element to help drive the selection of different neuropeptide precursors. This DIMM activity represents the first mechanism by which it regulates NSC development. For example, in identified Tv neuroendocrine neurons of the CNS, *dFMRFa* neuropeptide expression depends on DIMM but also on the LIM homeodomain protein Apterous (AP) (6) and the zinc-finger protein SQUEEZE (SQZ) (3, 40). No single member of this three-factor code produces ubiquitous *dFMRFa* expression when broadly misexpressed, and the triple comisexpression of *dimm*, *ap*, and *sqz* is more effective at producing ectopic *dFMRFa*. Thus, different sets of positively acting factors combine with DIMM to drive specific neuropeptide gene expression in different neurons (19, 33, 40).

Independent of combinatorial codes, DIMM exerts a second critical influence, its so-called master regulatory role, on developing NSCs (2). DIMM displays singular control of properties held in common by diverse NSCs and has the ability to impose them onto non-NSCs. For example, DIMM misexpression throughout the entire CNS confers PHM expression (a common NSC property) on all neurons. Other common NSC features controlled by a master regulator like DIMM could involve mechanisms of precursor processing and routing and of dense-core secretory granule generation, trafficking, and accumulation. Therefore, the current conception of NSC cell organization supposes two interlocked regulatory networks (combinatorial codes and single master regulator) operating within single cells.

Previous work has demonstrated that DIMM controls the expression of the neuropeptide biosynthetic enzyme PHM (15, 28). This monooxygenase is most closely related to dopamine-β-hydroxylase (DBH) and is selective and required for secretory peptide C-terminal amidation (25). Loss-of-function genetic studies indicate that DIMM normally controls PHM (21), while gain-of-function studies show that it can confer ectopic PHM expression efficiently on all neurons (2). We used in vitro and in vivo experiments to show that DIMM directly activates *PHM* via three specific E boxes located in the first intron of *PHM*. Furthermore, we extend these results by showing that the same *cis* mechanism operates robustly in vivo and can explain in large part the specific high-level expression of PHM in peptidergic neurons and endocrine cells. Finally, we demonstrate that the mammalian orthologue Mist1 is capable of transactivating *Drosophila* PHM both in cell lines and in transgenic flies. Thus, DIMM and Mist1 share functional as well as sequence attributes, and the regulatory features we describe for specialized secretory cells in *Drosophila* are likely to be broadly applicable across animal phyla.

MATERIALS AND METHODS

Fly stocks. The following fly lines were used in this study: *dimm* mutant allele (*Rev4* and *Rev8*); UAS (upstream activation sequence)-*dimm*-myc (21); UAS-*2X EGFP*, UAS-*mist1*, and *ap*-gal4 lines. *PHM*-gal4, *PHM*-GFP, *PHM*-E1^{M}-gal4, *PHM*-E2^{M}-gal4, *PHM*-E4^{M}-gal4, *PHM*-E24^{M}-gal4, and *PHM*-E124^{M}-gal4 lines were established for this study and are described below. Two plasmids, pPT-gal4 and pStinger (4), were used for gal4 and green fluorescent protein (GFP) constructs, respectively. Some *PHM*-gal4 transgenic flies were generated by a microinjection into w^{1118} embryos, and the other transgenic flies were established commercially by Model Systems Genomics (http://www.biology.duke.edu/model-system/).

Luciferase assay. PHM fragments were subcloned into the pGL3 and *sv40*-pGL3 luciferase (luc) vectors (Promega, Madison, WI). To avoid nonsense-mediated decay, the translation start site of *PHM* was mutated by site-directed mutagenesis. *Drosophila* bHLH proteins (DIMM, DIMM-MB, Daughterless [DA], Atonal [ATO], HLH4C, and NAUTILUS [NAU]), the LIM homeodomain protein APTEROUS (AP), and the mammalian bHLH Mist1 were subcloned into the hemagglutinin (HA)-tagged pCDNA3 vector. *dimm-MB* encodes a DIMM protein with a mutated basic region that is projected to display minimal DNA binding. Three amino acids of the basic region of DIMM were changed to glycines (R164G, E165G, and R165G); the primers used for the site-directed mutagenesis are listed in Table S1 in the supplemental material. For luciferase assays, HEK 293 cells were transiently transfected with 2.1 μg of DNA using Lipofectamine 2000 (Invitrogen, San Diego, CA) and the activity of cell lysates was measured with a Victor-Wallac2 plate reader (Perkin-Elmer, CA). For each experiment, a vector containing the thymidine kinase-*Renilla* luciferase gene (*TK-Rluc*) was cotransfected for normalization, and each transfection was performed at least three times independently.

Site-directed mutagenesis. We performed site-directed mutagenesis following the manufacturer's protocols (Stratagene) using the set of primers listed in Table S1 in the supplemental material. Mutagenesis was performed in the pGEM-TA vector, and sequence variants were then subcloned into the pGL3 vector (both from Promega, Madison, WI). All constructs were confirmed by sequencing.

DNA-protein interaction assays. Electrophoretic mobility shift assays (EMSAs) were performed as previously described (29). A double-stranded DNA probe corresponding to the E1 E box (see below) was radiolabeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and then column purified. In vitro-translated proteins were prepared following the manufacturer's protocols using a TNT kit (Promega, Madison, WI). The binding reaction mixtures contained 5 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, 2% glycerol, 0.5 μg of poly(dI-dC), 3 μl of in vitro-translated protein, and radiolabeled DNA probe (25-μl final volume) and were incubated at room temperature for 30 min. For competition assays, the reaction mixtures included 20 pmol of cold competitor. For supershift assays, the reaction mixtures included 1 μl of antibody and were preincubated for 30 min on ice. After incubation, all reaction mixtures were analyzed by 6% nondenaturing gel electrophoresis, which was run at 200 V at 4°C for 4 h. Signals were detected by autoradiography. The primers are listed in Table S1 in the supplemental material.

Antibodies, immunocytochemistry, and fluorescence imaging. Affinity-purified guinea pig anti-Dimm (1:200) (2), mouse monoclonal anti-GFP 3E6 (1:800; Molecular Probes, Carlsbad CA), rabbit anti-Mist1 (1:250) (29), rabbit anti-FMRFamide (1:1,000) (50), and rabbit anti-GFP (1:500, rabbit polyclonal, catalogue no. AB3080; Chemicon, Temecula, CA) as primary antibodies and Cy3-conjugated or Alex-488-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA) were used for immunocytochemistry. Immunostaining methods were as previously described (21). Images were acquired on an Olympus FV500 confocal laser scanning microscope and manipulated by Adobe Photoshop software to adjust contrast and/or levels.

RESULTS

DIMM activates *PHM* in vitro. Our previous data indicated that *dimm* activity is required for the expression of *PHM*. To test the possibility that such regulation is direct, we tested four genomic regions around the *PHM* locus for enhancer activity by creating *luciferase* fusion constructs and transfecting them into mammalian HEK 293 cells (Fig. 1A). These regions included the complete intergenic domain (249 bp) between *PHM* and its nearest annotated neighbor and the first intron, which includes an unexpectedly large number of E boxes (see below).

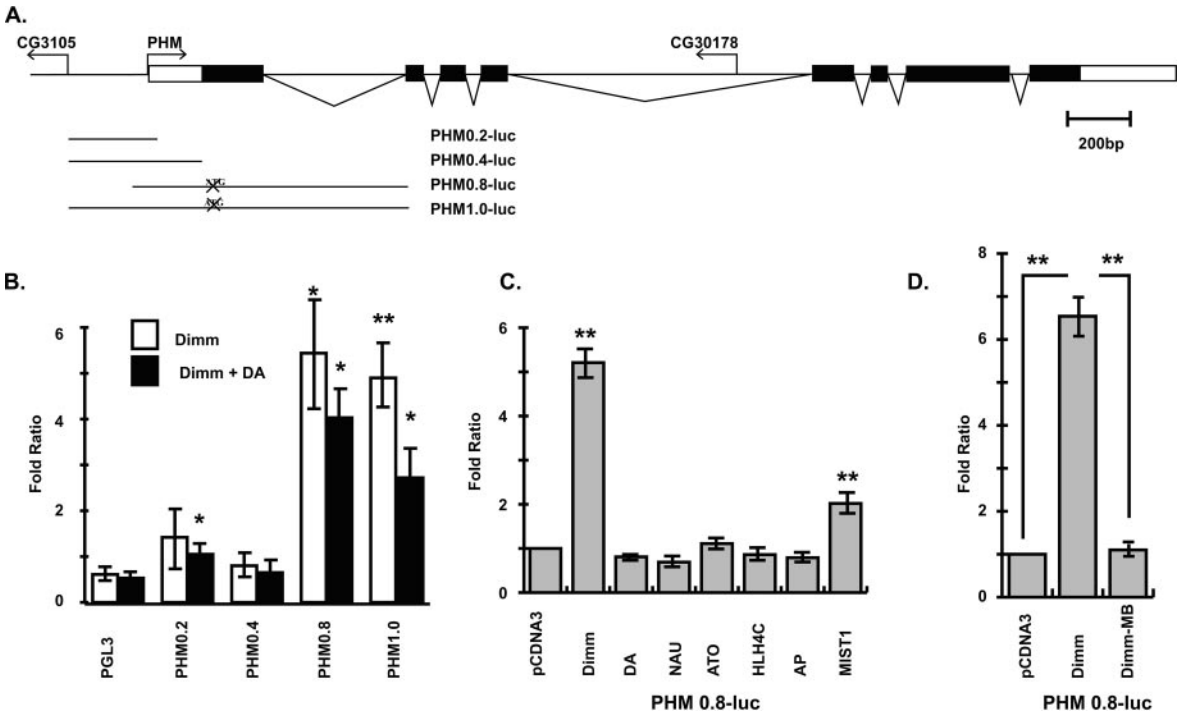

FIG. 1. Transactivation of *PHM* fragments by DIMM in mammalian HEK 293 cells. (A) Schematic diagram of the *PHM* gene and *PHM*-luciferase constructs used for these experiments. When present, the *PHM* ATG site (shown by X) was mutated. (B) The activities of *PHM* promoter constructs with and without cotransfected effectors (DIMM or DIMM and DA). (C) Evaluating the specificity of *PHM* activation by DIMM. NAU, NAUTILUS; ATO, ATONAL; AP, APTEROUS; Mist1, murine Mist1. (D) *PHM* transactivation depends on the presence of a wild-type basic (DNA binding) domain in DIMM. Histograms represent means and standard errors of the means of the results. *, $P < 0.05$; **, $P < 0.01$. *P* values in comparison to the control values were obtained by using Student's *t* test.

The two larger constructs (containing ~0.8 and 1.0 kb of *PHM*, respectively) displayed fivefold or greater increases in luciferase activity when *dimm* was cotransfected. In contrast, the two smaller constructs (~0.2 and 0.4 kb of *PHM*) did not respond to cotransfected *dimm*. We then asked whether cotransfection of *dimm* and the proneural E protein bHLH daughterless gene (*da*) would increase *PHM* transactivation since *da* encodes a putative heteromeric binding partner (10, 11, 13, 14). DA did not interact with DIMM in our previous in vitro biochemical measures (2). While the cotransfection of *dimm* with *da* increased *PHM* transactivation compared to the levels in controls, it was always to a level lower than that of the response to transfection of *dimm* alone (Fig. 1B). Next, we evaluated the specificity of DIMM's transcriptional activity by comparing the response to this *PHM* fragment to that for cotransfection with other transcription factors. All four additional bHLH proteins tested (DA, NAU, ATO, and HLH4C) failed to activate the 0.8-kb *PHM* fragment, as did the LIM-HD protein AP (Fig. 1C). The mammalian protein Mist1 includes a bHLH domain with >90% identity to that of DIMM (29). *Mist1* increased *PHM-luc* activity significantly, although not to the same extent as DIMM (Fig. 1C). Finally, to ask whether DIMM's DNA binding properties were required for its specific transactivating properties, we mutated the basic region within the binding domain of DIMM; we observed little residual activity (Fig. 1D).

In summary, we found that a small *PHM* fragment is transactivated by DIMM following transient transfection in a heterologous cell line. Furthermore, these activities are specific, they are not augmented by cotransfection of a class I (E class) bHLH, and they appear to require DNA binding by DIMM.

Three E boxes within the first intron of *PHM* are necessary for activation by DIMM. Next, we searched for critical *cis*-regulatory elements within the *PHM* genomic fragment by functional assay and by DIMM binding. We found that the ~400-bp first intron of the *PHM* gene, when placed upstream of a heterologous promoter, displayed significant, orientation-independent enhancer activity (Fig. 2B). While both orientations were active, the antisense construct was more so: in response to DIMM transactivation, the *PHM*-E71 construct (antisense) produced an increase similar to that from the wild-type *PHM* promoter; however, the *PHM*-E17 construct (sense) produced approximately half that level of activity. Generally, most bHLH proteins are known to bind a canonical 6-bp sequence, CANNTG, called the E box (7), although noncanonical sites have also been observed (54). Therefore, we searched for and found seven E boxes (putative DIMM binding sites) within this *PHM* intronic fragment; by random estimate, less than two E boxes are expected within this ~400-bp domain. We named them E1 to E7, from 5′ to 3′ within the intron (Fig. 2A). E boxes E1 to E7 display at least some sequence conservation among related *Drosophila* species (see Fig. S1 in the supplemental material).

To examine the functional contributions of these individual E-box elements to transactivation by DIMM, we performed selective site-directed mutagenesis and tested the resulting lev-

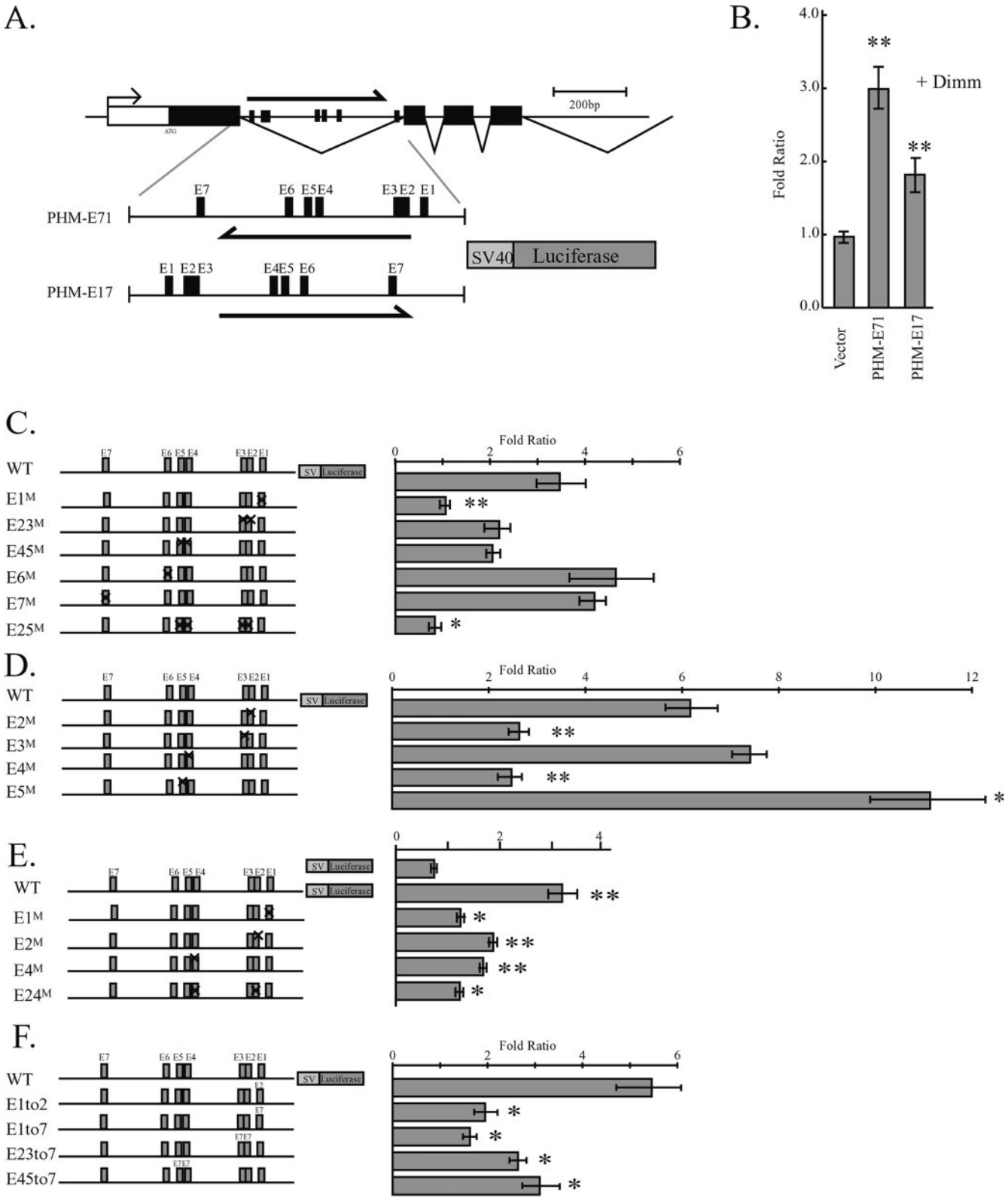

FIG. 2. DIMM transactivates the *PHM* via the subsets of E boxes in its first intron and binds the E1 box directly. (A) A schematic of the first *PHM* intron indicating the positions of seven E boxes, E1 to E7 (see Table 1 for sequences of wild-type and mutated E boxes). Arrows indicate the original orientation of the DNA fragments. (B) Activity of the two constructs and the control vector diagrammed in panel A. In response to DIMM cotransfection, the first intron of *PHM* displays strong enhancer activity when fused to a heterologous simian virus 40 (SV40) promoter. (C to F) Effects on *PHM* transactivation by DIMM following site-directed mutagenesis of different E-box sequences within *PHM's* first intron. A gray box indicates a particular E-box mutation site. Ratios were calculated by dividing values resulting from *dimm* cotransfection by those resulting from no cotransfection. The histogram represents the means and standard errors of the means of the results. *, $P < 0.05$; **, $P < 0.01$. *P* values in comparison to the wild type were obtained by Student's *t* test.

TABLE 1. Wild-type and mutant sequence probes representing the seven E box sites present in the first *PHM* intron[a]

E box(es) and probe	Sequence
E1	
E1 box	5′-cgc tcCATATGga tga-3′
E1^{M} box	5′-cgc tc**T**ATA**CT**ga tga-3′
E1 to 2 box	5′-cgc **c**cCA**GC**TG**c**a tga-3′
E1 to 7 box	5′-cgc **c**cCA**TT**TG**a**a tga-3′
E2 and E3	
E23 box	5′-tcg ccCAGCTG CAGCTGtg cgc-3′
E23^{M} box	5′-tcg cc**T**AGC**CT TAGCCTtg** cgc-3′
E2^{M} box	5′-tcg cc**T**AGC**CT** CAGCTGtg cgc-3′
E3^{M} box	5′-tcg ccCAGCTG **T**AGC**CT**tg cgc-3′
E23 to 7 box	5′-tcg ccCA**TT**TG CA**TT**TG**aa** cgc-3′
E4 and E5	
E45 box	5′-ttt tcCATATGt tCAGTTGta ccc-3′
E45^{M} box	5′-ttt tc**T**ATA**CT**t t**T**AGT**CT**ta ccc-3′
E4^{M} box	5′-ttt tc**T**ATA**CT**t tCAGTTGta ccc-3′
E5^{M} box	5′-ttt tcCATATGt t**T**AGT**CT**ta ccc-3′
E45 to 7 box	5′-tcg **c**cCAT**T**TGt tCA**T**TTG**a**a cgc-3′
E6	
E6 box	5′-cca ccCACATGta agg-3′
E6^{M} box	5′-cca cc**T**ACA**CT**ta agg-3′
E7	
E7 box	5′-act ccCATTTGaa taa-3′
E7^{M} box	5′-act cc**T**ATT**CT**aa taa-3′

[a] The 6-bp E-box sequences are in uppercase and in order as named in column 1. Sequence alterations are shown in bold. Underlines indicate the final extent of sequence matching to the target E-box site (e.g., E1 to 2 box is the 10 bp surrounding E2).

els of enhancer activity in HEK 293 cells. Different E boxes made different contributions to DIMM activity. Sequence alteration of the single E box E1 strongly reduced responsiveness to DIMM. In contrast, sequence alteration to either E6 or E7 had no such effect (Fig. 2C). Likewise, the alteration of either E3 or E5 did not lessen DIMM responsiveness (Fig. 2D), and in fact, the result with the E5 variant was significantly elevated over that with the wild-type sequence control (Fig. 2D). Simultaneously altering four E boxes (E2, E3, E4, and E5) strongly reduced responsiveness to DIMM, to a level equal to that from altering E1 alone (Fig. 2C). Sequence alteration of E2 or of E4 individually resulted in a decrease of approximately 50% (Fig. 2D). Thus, the three E boxes E1, E2, and E4 appeared to make the biggest contributions; we therefore compared their activities directly to the activity of the vector only (Fig. 2E). The single E2 or E4 mutation displayed about half the activity of the wild-type sequence, while the single E1 mutation and the double E2/E4 mutation had even greater effects and displayed only about 20% of that level. All the activities were significantly different from that of the empty vector control. Finally, to determine whether (i) the E-box position or (ii) its specific sequence was the critical determinant of functionality, we tested additional E-box sequence variants (Fig. 2F). Exchanging the sequence of E1 (plus its four adjacent bp [TCCATATGGA]) for that of the nonfunctional E7 (CCCATTTGAA) (Table 1) resulted in the loss of all DIMM responsiveness. Likewise, the exchange of both E2 and E3 with E7 or both E4 and E5 with E7 produced a *PHM*-enhancer fragment with half of its normal

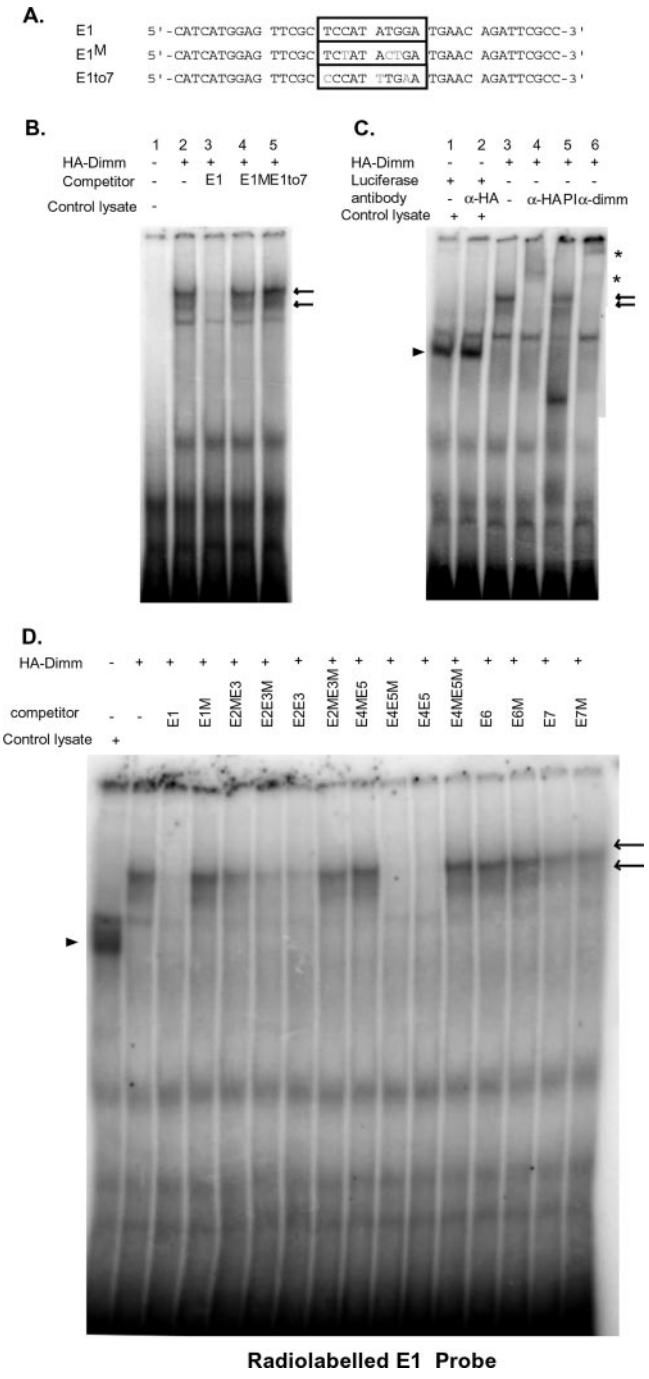

FIG. 3. Direct binding of DIMM to the E1 E box. (A) E1, the sequence of the probe representing the wild-type E1-box region; E1^{M} and E1to7, the sequences of two E1-box variants that were used as competitors for EMSA. The box demarcates the E box and 2 bp on either side. (B) HA-tagged DIMM binds to the radiolabeled E1-box probe directly and specifically. (C) The E1-DIMM complex was supershifted by its specific antibody, and not by control antibodies. Arrows indicate the shifted bands; asterisks indicate supershifted bands. α, anti. (D) The E1 box competes differently with the other E boxes of the first intron of the *PHM* gene. Arrowheads in panels C and D indicate nonspecific bands observed when lysate (control or experimental) was added. These assays were performed twice, with similar results. +, present; −, absent.

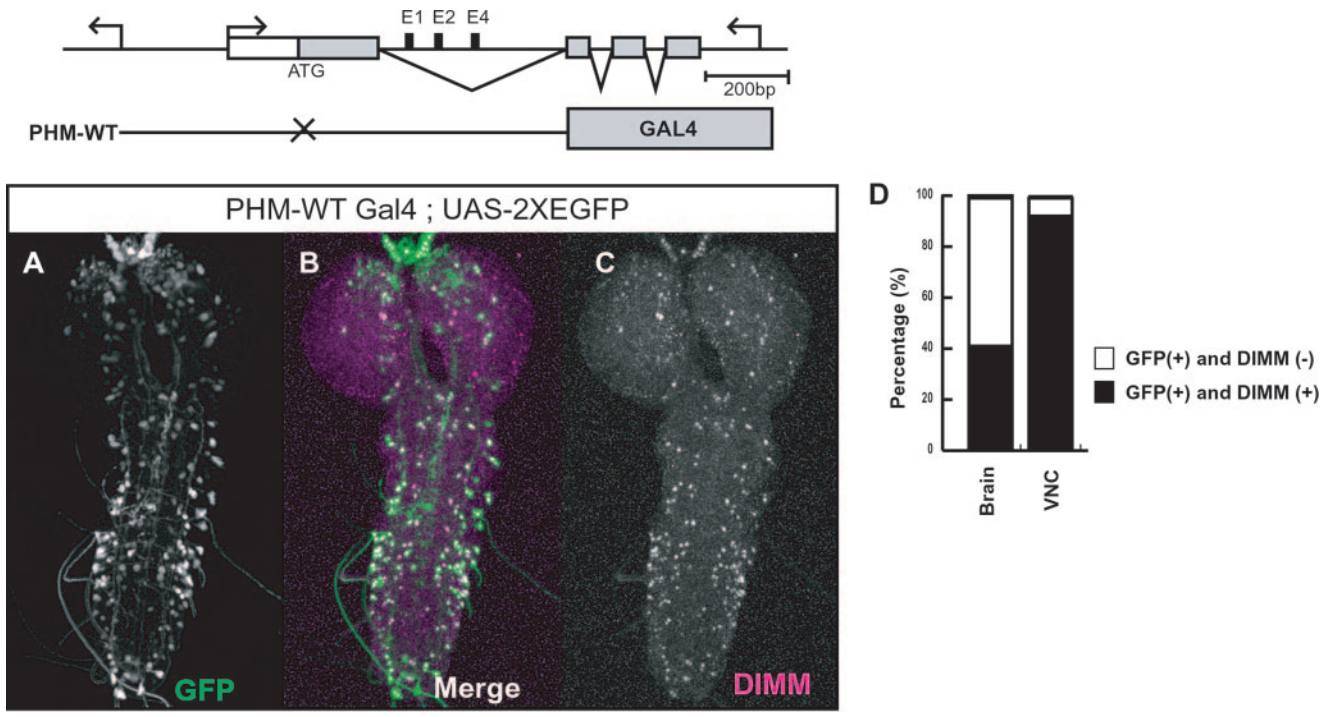

FIG. 4. High coincidence between the expression pattern of the ~1.0-kb *PHM* transgenic reporter and that of DIMM in the third instar larval CNS. The diagram at the top describes the *PHM*-gal4 construct. Arrows indicate the orientation of gene transcription, and the X indicates mutation of the *PHM* AUG sequence. (A to C) Double-staining with anti-DIMM and anti-GFP in the third instar larval brain of *PHM*-WT line. (D) Quantification of the coincidence of patterns in *PHM*-WT flies. About 41% of GFP^+ [GFP(+)] cells in the brain were also $DIMM^+$ ($n = 112$ total cells); more than 90% of those that were $DIMM^+$ in the VNC are GFP^+ ($n = 156$ total cells). The number of $GFP^-/DIMM^+$ cells was typically less than 1% and, for clarity, was not reported in the graphs.

DIMM responsiveness (Fig. 2F), mimicking the effect of the loss of the paired sites.

In summary, the first intron of the *PHM* gene has seven E boxes. Three of these, E1, E2, and E4, proved especially important for the transactivation by DIMM protein in HEK 293 cells, but our experiments indicated that they make different contributions (E1 > E2 = E4). Additional data implicated the specific sequences within each of these E boxes, and not simply their positions, as critical determinants of their specific contributions. Together, these data indicate that the three *cis* regions within the first intron represent critical elements of a putative DIMM response element.

DIMM binds the E1 box of the *PHM* gene in vitro. Next, we examined DIMM's ability to bind the relevant E boxes within the *PHM* intron (Fig. 3A to C). We performed EMSAs to address this question, using an E1-box probe. In vitro-translated DIMM bound the E1 probe specifically: the wild type, but not the sequence-mutated E1 probe, was able to compete such binding efficiently (Fig. 3B, lanes 2 to 4). Likewise, we observed poor competition by a cold E1 box probe in which the E-box sequence had been switched to that of E7 (Fig. 3B, lane 5). Supershift assays using either anti-HA or anti-DIMM antibodies confirmed the presence of DIMM protein (Fig. 3C).

We also examined the degree to which the DIMM-E1 interaction could be competed by the six other *PHM* intronic E-box sequences, E2 to E7. DIMM-E1 interaction was dose dependent (see Fig. S2 in the supplemental material). DIMM binding with E1 was competed efficiently by probes containing both E2 and E3 and also ones containing both E4 and E5 (Fig. 3D). Within the E2/E3 pair, the competition derived primarily but not exclusively from E2; within the E4/E5 pair, all the competing activity derived from E4 (Fig. 3D). We note that E2 and E3 share the same core 6-bp sequence, while the E4 and E5 sequences are different from each other (Table 1). The E6 and E7 oligonucleotides competed poorly with E1 binding and at levels that did not differ when they were tested following mutation of their specific E-box sequences (Fig. 3D). Next, we investigated the sequence requirements within the 10-bp E1 sequence (NNCANNTGNN) in greater detail by using site-directed mutagenesis. We found that specific sequences outside the canonical 6-bp E box (CANNTG) were required for activation by DIMM. Specifically, the nucleotides at the ±1, but not at the ±2 region, could not be altered without loss of DIMM activity. The results also indicated that, within the 6-bp sequence, DIMM prefers TA (and to a lesser extent, GC) to AT or CG for the internal NN sequences (see Fig. S3 in the supplemental material).

In summary, we found that DIMM directly binds E box E1 located within the first intron of *PHM*; this binding was efficiently competed by oligonucleotides corresponding to E2 and E4, but not by those corresponding to E3, E5, E6, or E7. These results further demonstrate differential E-box contributions to transactivation by DIMM, ranking them in an order consistent with the results from the luciferase reporter assays.

A 1-kb enhancer fragment from the first intron of *PHM* recapitulates the expression of the endogenous gene in vivo and is dependent on DIMM. We next asked whether the *PHM* first intronic fragment contains sufficient *cis*-regulatory information to drive a normal *PHM*-like expression in vivo, particularly within NSCs that are strongly DIMM expressing. We established transgenic flies that contain the ~1.0-kb *PHM* fragment as a GAL4 fusion (*PHM*-WT-GAL4) and crossed them into a UAS-GFP reporter background. Gene expression was studied by immunocytochemistry in the third instar larval brain. We found that *PHM*-WT-GAL4 was expressed normally throughout the brain (Fig. 4). GFP-expressing cells were predominantly DIMM positive (Fig. 4B). Forty-one percent of GFP^+ cells in the brain and 91% of GFP^+ cells in the ventral nerve cord (VNC), respectively, were colocalized with DIMM (Fig. 4D). Notably, most DIMM-positive cells were strongly GFP positive (in one representative specimen, we counted 189 GFP-positive cells among the 192 DIMM-positive cells [98.4%] throughout the entire CNS). The DIMM-negative, GFP-positive brain cells were primarily Kenyon cells of the larval mushroom bodies (MBs). PHM is found in moderate levels in both larval and adult MBs (49).

Next, we wished to determine whether DIMM protein regulates the expression of the *PHM* transgene as it does the endogenous PHM locus, and so performed *dimm* loss-of-function analyses. The strongest *dimm* loss-of-function alleles display lethality in the early larval stages (21): in the second instar larval CNS, we observed a strong correspondence between DIMM protein expression and *PHM*-GAL4 transgene expression (Fig. 5A to C). This activity was greatly reduced in a strong *dimm* mutant background (Fig. 5D to F). Some residual GFP activity was detectable in a few neurons in mutant tissues; several of these cells were DIMM positive, likely reflecting that, even in the severe *dimm* hypomorphic background, a small number of DIMM-positive neurons remain. *PHM* transgene activity in Kenyon cells was not apparent at that age and so could not be scored in the *dimm* loss-of-function state. Together these data indicate that the DIMM-responsive ~1.0-kb *PHM* fragment also drives patterned gene expression in vivo. The reporter activities are largely coincident with the expression of DIMM and are largely dependent upon normal DIMM expression.

The *PHM* transgene also responds strongly to DIMM overexpression in vivo. To analyze the effects of DIMM gain-of-function in vivo, we constructed *PHM*-GFP transgenic reporter lines using the same ~1-kb *PHM* fragment. In the larval CNS, we again found high-level GFP expression largely coincident with and dependent on DIMM expression (see Fig. S4 in the supplemental material). Second, we found that within the CNS, misexpressed DIMM protein produced strong, ectopic *PHM* transgene expression (Fig. 6). In the VNC, AP is expressed in three cell groups: (i) the four-cell Tv cluster (Tv, Tva, Tvb, and Tvc), (ii) the ventral AP pair (vAP), and (iii) the dorsal single Aplet cell (dAP) (2, 40). Within the Tv cluster, DIMM is normally found in two cells, Tv and Tvb, but not in Tva or Tvc (the latter are here called Tv3 and Tv4 due to lack of identifying markers [Fig. 6]). Likewise, DIMM is normally found in the dAP, but not in vAP, cells. Using an *ap*-GAL4 driver, we found that *PHM*-GFP expression was responsive to DIMM overexpression in vivo throughout all four neurons of the Tv cluster (Fig. 6D) and in both vAP neurons (data not shown). This is consistent with the results of previous studies demonstrating that DIMM misexpression throughout all these AP neurons directs ectopic PHM protein in them all (2, 16). Notably, the mammalian homologue, MIST1, also showed an ability to activate the *PHM*-GFP reporter in vivo throughout all four cells of the Tv cluster (Fig. 6E and F) and in the vAP cells (data not shown).

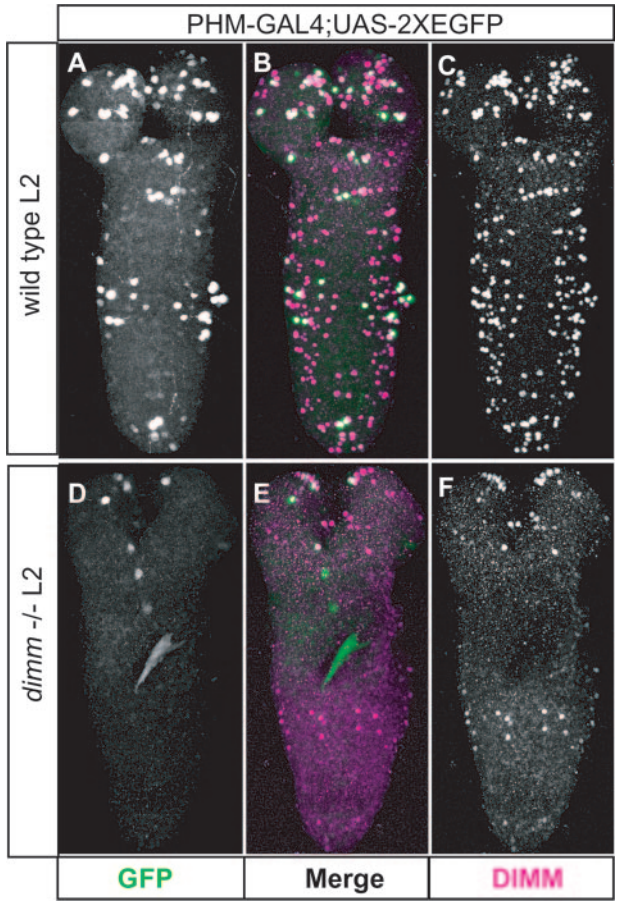

FIG. 5. The activity of the ~1.0-kb transgenic *PHM* reporter largely depends on DIMM. *PHM*-GAL4 expression in the second instar larval CNS is much stronger in *dimm* heterozygotes (*Rev8*/+) (A to C) than in *dimm* transheterozygotes (*Rev8*/*Rev4*) (D to F). At least 10 samples were tested per genotype. Note that the *PHM*-GAL4 activity is not as prevalent in these second instar tissues as in the older tissues displayed in Fig. 4.

In summary, we found that in vivo, dimm regulated the activity of an ~1-kb *PHM* regulatory fragment in a manner consistent with its regulation of the endogenous PHM protein. This further validates this ~1-kb fragment as a primary response element mediating the transcriptional control of PHM by *DIMM*. Additionally, the mammalian orthologue *Mist1* also activated this same *PHM* fragment in vivo, supporting the hypothesis that these related factors share functional properties.

Specific E-box sequences within the first *PHM* intron are essential for the activity of the *PHM* transgene. We further asked which *cis*-regulatory sequences within this ~1-kb *PHM* fragment contribute to its activity as a transgene in vivo. Based

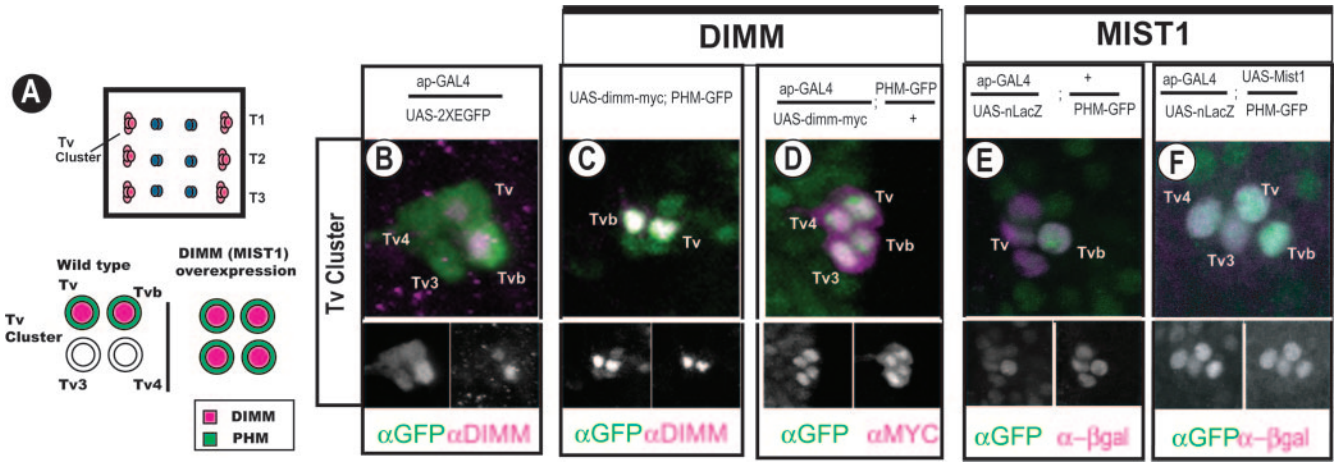

FIG. 6. Misexpressed DIMM drives ectopic expression of the *PHM*-GFP reporter in vivo. (A) Top diagram indicates the positions of two of the three normal *ap* cell groups present in three thoracic segments of the larval VNC: the four-cell Tv cluster (red circles) and the pair of vAP neurons (blue circles). Two of the Tv cluster neurons in the larval stage (called Tv and Tvb) are peptidergic, and both express DIMM and PHM (3, 44). The bottom diagram schematizes the expression of DIMM and PHM in the Tv cluster in the wild type (left) and following *DIMM* misexpression driven by *ap*-GAL4 (right). (B) *ap*-gal4 reporter (UAS-*2X EGFP* line) is expressed in all four cells of the Tv cluster. (C) Without *ap*-GAL4 activity, *PHM*-GFP displays normal restriction in the DIMM$^+$ Tv and Tvb cells and not in the Tv3 or Tv4 cells and is not found in vAPs (not shown). (D) When DIMM is misexpressed in all *ap* neurons, *PHM*-GFP is seen in all four Tv cluster neurons and in the two vAP neurons (data not shown). Anti-MYC recognizes the epitope-tagged DIMM (UAS-*dimm-myc*). (E and F) Misexpressed MIST1, the mammalian homologue of DIMM, also drives *PHM*-GFP throughout the four-cell Tv cluster. (E) An example is shown without the UAS-*Mist1* gene. (F) When the UAS-*Mist1* gene was included, GFP was found to be elevated in all four Tv cells. The results shown are representative for at least five specimens studied per experiment. α, anti. In designations of genotypes, + indicates the wild type.

on the functional analysis in HEK 293 cells, three E boxes (E1, E2, and E4) are required for activation by DIMM. We therefore asked whether these specific *cis*-regulatory sequences also contribute to the activity of this *PHM* fragment in vivo. In the third instar larval brain, all activity was lost in flies bearing a GAL4 fusion of the 1-kb *PHM* fragment in which E1, E2, and E4 E boxes were mutated ($n = 10$; Fig. 7A to C). We note that activity was lost equally from the DIMM-positive and DIMM-negative (primarily MB) cells.

To investigate contributions by individual E boxes or subsets of them, we also established four different *PHM*-gal4 lines harboring either single ($E1^M$, $E2^M$, and $E4^M$) or double ($E24^M$) E-box mutations. Analysis of the single E-box mutations revealed that reporter activity was very low in each, with, typically, a few weakly expressing cells at most (Fig. 7). The reporter activity in the double-mutant line was likewise very low. These results suggest that all three E boxes (E1, E2, and E4) are required for the normal activities of this *PHM* fragment in vivo and that the contribution of these E boxes is crucial.

DISCUSSION

The experiments reported here address the mechanisms underlying DIMM's regulatory functions within peptidergic NSCs in *Drosophila*. We have shown that the tissue-restricted bHLH factor DIMM works as a transcriptional activator and that *PHM* is a direct DIMM target both in heterologous mammalian cells and in vivo. We have also resolved the enhancer activity of *PHM* to a region that contains its first intron and further shown that three tandem E boxes within that intron are essential for full DIMM activation.

DIMM is a transcription factor that regulates *PHM* directly. Class I bHLH factors (e.g., E12, E47, and HEB) are widely expressed, while class II bHLH proteins (e.g., MyoD, myogenin, Mash1, and NeuroD) exhibit tissue-restricted expression profiles. Class I and II bHLH proteins function as heterodimer complexes to regulate the expression of target genes by binding to E-box (CANNTG) DNA elements (16, 30). Some bHLH proteins also form homodimers; for example, HAIRY is thought to form homodimers exclusively (39, 46). Likewise, the *Drosophila* mesoderm regulator TWIST forms heterodimers with DA but also forms homodimers: these different TWIST molecular pairs produce different transcriptional readouts in vivo (12).

At present, we favor the hypothesis that the master regulatory functions of DIMM (such as its control of *PHM*) represent actions as a homomeric dimer or oligomer. Four lines of evidence support this conclusion. (i) Cotransfection of *PHM*-luc with *dimm* was sufficient to transactivate *PHM* in heterologous (mammalian) cells, (ii) whereas cotransfection with *dimm* and *da* produced a less robust transactivation (Fig. 2). (iii) In vitro, DIMM did not bind DA, but did bind itself efficiently when tested as a GST fusion protein or via coimmunoprecipitation (2). (iv) In the absence of a class I bHLH, DIMM was nevertheless able to bind to the *PHM* E1 probe in vitro, presumably as a homodimer (Fig. 2). It may be significant that the three E-box sequences indicated by functional analysis display palindromic core sequences, which is consistent with binding by a homomeric bHLH dimer. We also note that the DIMM mammalian sequence orthologue (called Mist1) forms heterodimers with class I bHLH factors under certain conditions but also forms homodimers to directly regulate the fate of developing acinar cells of the pancreas (29, 45, 56).

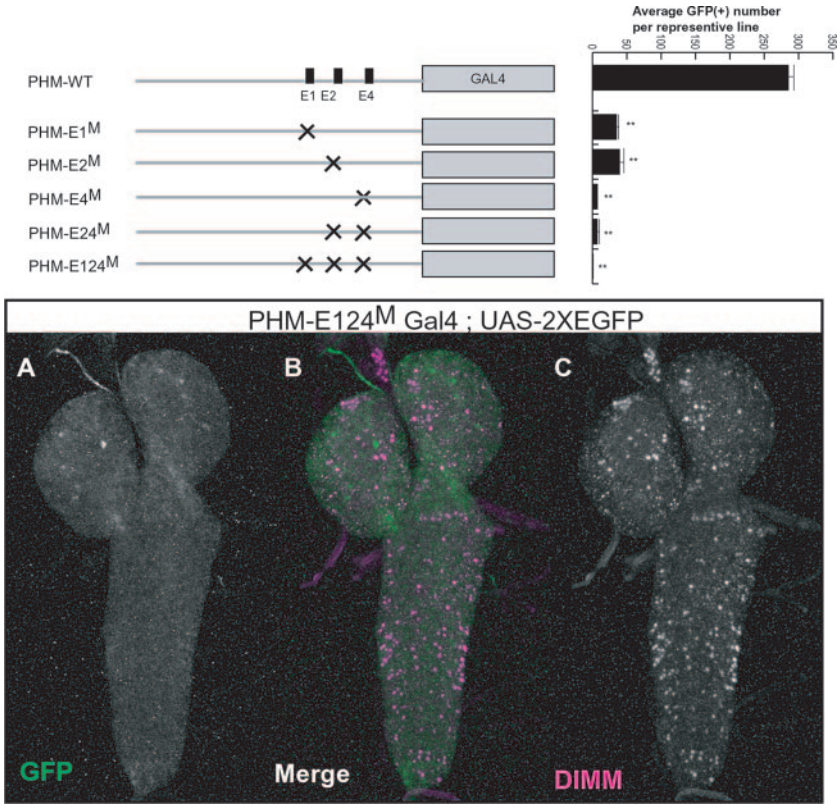

FIG. 7. The three E boxes E1, E2, and E4 are essential for *PHM*-GFP activity in vivo. The in vivo activities of three transgenic *PHM*-gal4 lines were evaluated with UAS-*2X EGFP* in the CNS of third instar larvae. Xs in diagrams on the left indicate positions of site-directed alterations in the ~1.0-kb *PHM* genomic fragment; all constructs altered the *PHM* AUG to prevent initiation at that site. *PHM*-WT was otherwise wild type in sequence. *PHM*-E1^M, *PHM*-E2^M, *PHM*-E4^M, *PHM*-E24^M, and *PHM*-E124^M harbor alterations of specific E-box sequences. Each altered *PHM* transgene displayed significant reductions of promoter activity, as indicated by the results shown in the histogram on the right for the average number of GFP$^+$ neurons ($n = 5$) observed in a representative line for each construct. Error bars show the standard errors of the means. **, $P < 0.01$. For each construct, there was good consensus in the results for independent transgenic lines: *PHM*-WT (5 lines), *PHM*-E1^M (8 lines) *PHM*-E2^M (3 lines), *PHM*-E4^M (3 lines), *PHM*-E24^M (3 lines), and *PHM*-E124^M (10 lines).

The significance of *PHM* as a direct regulatory target of the prosecretory factor DIMM. In the context of establishing transmitter phenotypes, there is special significance to having established direct transcriptional regulation of *PHM* by DIMM. DIMM and PHM, among those neurons that display high levels of neuropeptide expression, display highly congruent patterns of expression within the nervous system (2, 21). PHM is a monooxygenase that is required for neuropeptide amidation and whose closest homologue is the hydroxylase DBH. Phox2a and Phox2b are critical regulators of the noradrenergic phenotype and have been shown to directly regulate *DBH* transcription (27, 48, 53, 55). Likewise, PET-1, which is a critical regulator of the serotonergic phenotype, directly regulates the transcription of *tryptophan hydroxylase,* which encodes the rate-limiting enzyme for serotonin biosynthesis (18). In each case cited, genes encoding biosynthetic hydroxylases appear to be critical points of regulation. Thus, direct transcriptional control of such enzymes by highly dedicated developmental regulators represents a mechanistic parallel between aminergic and peptidergic transmitter cell fates.

DIMM binding sites on *PHM*. We propose that the high level of *PHM* expression found in *Drosophila* NSCs results from direct control by DIMM acting via specific *PHM cis*-regulatory elements (Fig. 8A). The full activity of Class 2 bHLH proteins often requires interactions with multiple tandem E boxes (37), although single E boxes are sometimes sufficient (54). In the current study, we identified a cluster of seven E boxes in the enhancer region of *PHM*, of which at least three (E1, E2, and E4) are critical. bHLH proteins exhibit specificity as to which E-box sequences they utilize (44). The loss of these three motifs in *PHM* resulted in a substantial reduction in luciferase reporter assays and transgenic flies, indicating that there is an essential requirement of multiple E boxes for *PHM* activation by DIMM. We observed small differences in the activities of mutated *PHM* regulatory regions when tested in vitro versus in vivo. Focusing on the in vivo results, we propose a simple model of synergistic or cooperative interactions between DIMM homodimers bound to E1, E2, and E4 (Fig. 8A).

Mutation of the E3 and E5 E boxes produced elevated *PHM*-luc levels upon *dimm* cotransfections. The simplest explanation is that these E boxes bind one or more bHLH proteins that can repress *PHM* expression. The spacing of these potentially "inhibitory" E boxes, approximately one-half turn away from the "activating" E2 and E4 boxes, respectively, may be significant in effecting such negative regulation. Interest-

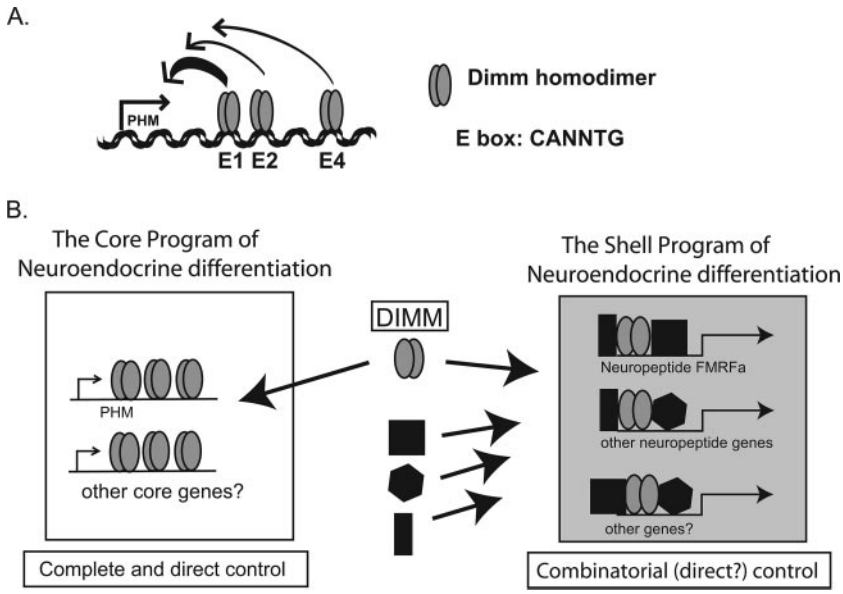

FIG. 8. A proposed mechanism and model for DIMM activity. (A) DIMM homodimers activate *PHM* directly via three specific E boxes (E1, E2, and E4). Presumed coactivators present in HEK 293 cells and in vivo are not shown. In vitro analysis showed that the three E boxes contribute differentially to the total amount of transactivation: the E1 box is absolutely required for in vitro activity, while E2 and E4 have only partial activity. To display full activity, the DIMM-bound E1 box must interact with E2-bound DIMM and E4-bound DIMM independently and cooperatively. However, in vivo, all three E boxes contribute equally to the activity. (B) DIMM plays two distinct roles in the regulation of neuroendocrine cell fate. (i) The left panel shows a "core program" of neuroendocrine differentiation that defines that set of genes that is utilized by all cells displaying an amidated peptide secretory cell fate. It is directly and completely controlled by DIMM. The core program consists of PHM and other as-yet-unidentified gene products. (ii) The right panel shows a "shell program" of neuroendocrine differentiation that defines that set of genes which varies among neuroendocrine cell types and which are controlled in combinatorial fashion. DIMM contributes partial control in concert with diverse other transcription factors which are symbolized as different shapes. Genes in the shell program include neuropeptide-encoding genes, as well as other as-yet-unidentified ones.

ingly, Mist1 functions as a transcriptional repressor when bound as an E47 heterodimer to troponin E boxes (29). DA cotransfection reduced the DIMM transactivation of *PHM*: one possible explanation for this effect is that DA may bind to one or more of the "inhibitory" *PHM* E boxes. Whether and how other factors modulate the level of DIMM's activation by binding *PHM* E3 and/or E5 in vivo will require additional studies.

***PHM* regulatory elements tested in vivo.** We extended the scope of our analysis by considering the behavior of the ~1-kb *PHM* fragment in vivo. Importantly, DIMM regulation of *PHM* in *Drosophila* cells paralleled its activity in HEK 293 cells. Although *PHM* regulation in vivo is complex and subject to multiple influences, these data suggest that much of PHM expression in *Drosophila* NSCs can be ascribed to DIMM direct regulation via the ~1-kb *PHM* fragment that includes its first intron. Significantly, most DIMM-positive cells were *PHM*-GAL4-positive cells, suggesting a strong correlation between DIMM and the same 1-kb *PHM* fragment that displayed transactivation in vitro. In addition, the promoter activity that could be assayed in second instar larvae (before *dimm* mutant animals die) was essentially all DIMM dependent: the few resilient GFP-positive cells that remained in *dimm* mutants were those that also retained DIMM protein—the strongest *dimm* alleles are not protein nulls. As found in HEK 293 cells, specific E boxes appeared to be especially important in vivo as well. The same three *PHM* E boxes that were implicated from in vitro experiments (E1, E2, and E4) were again implicated as critical for supporting *PHM* regulatory activity in vivo. This work therefore defines the *PHM* enhancer region and the constituent *cis*-regulatory sequences therein that confer sensitivity to DIMM. The data also indicate additional complexity of *PHM* regulation. The reliable incidence of GFP-positive/DIMM-negative neurons (especially some or all MB cells in the brain lobes) indicated that other factors besides DIMM may regulate *PHM* expression in other domains of the CNS. Interestingly, the loss of *PHM* transgene expression by MB neurons in flies bearing mutated *PHM* E boxes suggests that the regulatory factor(s) driving *PHM* in MBs may also be a bHLH protein(s).

What is the precise role of DIMM in the differentiation of NSC? In mammals, several transcription factors have been implicated in directing the differentiation of neuroendocrine lineages (1, 8, 24, 26, 31, 32, 36, 47, 52). Based on information so far available, DIMM does not resemble any known mammalian regulator of neuroendocrine development. For example, while DIMM is activated postmitotically in NSCs, Mash1 appears required for the generation of pulmonary neuroendocrine cells (8). Like *dimm*, *Sim1* and *Otp* are thought to act in parallel as determinants of cell fate in hypothalamic neuroendocrine cells and are needed for cell generation or survival (1, 32, 52). Recently, *Sim1* mutant cells were shown to reach their final cell division with normal display of their molecular phenotype but subsequently fail to differentiate as a function of altered cell migration (56). These factors, therefore, appear to

act early and prior to the terminal differentiation of the peptidergic cells.

Based on several observations of normal expression and from genetic analyses, we consider DIMM to be the major regulator of terminal cell differentiation in *Drosophila* NSCs. Our previous work proposed two, nonexclusive mechanisms to explain *dimm* actions. One is a model of transcription factor combinatorial regulation to drive specific neuropeptide expression (e.g., *dFMRFa* and *NPLP1*) (2, 5) in different neurons. In this scenario, DIMM acts combinatorially with several locally expressed transcription factors to drive neuropeptide gene expression. In no case, however, has it been possible to detail the precise identities of any particular combination regulating one specific neuropeptide gene. DIMM also acts independently to regulate the transcription of genes that control common NSC traits. *dimm* is a "master regulator," as its overexpression confers high-level PHM expression in all neurons of the CNS (2). In this dual manner, we speculate that *dimm* contributes both to specific cellular properties of diverse NSCs (e.g., neuropeptide gene selection—a "shell program" of neuroendocrine differentiation) and to more general ones involving generic NSC properties (e.g., PHM expression—the "core program" of neuroendocrine differentiation) (Fig. 8B). The present results in vitro and in vivo strongly support the second model of *dimm* actions: we have shown that the control of PHM protein expression derives from the very strong, direct regulation of *PHM* transcription. Given its potent transcriptional activity, we suspect that DIMM promotes a general NSC phenotype by also activating target genes besides *PHM*, but this supposition will require further analysis.

The relationship of DIMM and the mammalian bHLH Mist1. Among mammalian bHLH proteins, Mist1 is the most similar to DIMM (34). At the sequence level, the bHLH regions of DIMM and Mist1 exhibit greater than 90% sequence identity. Both proteins are enriched in cells that are specialized for secretion, and genetic analyses suggest that both *dimm* and *Mist1* are required for the proper expression of secretory properties by the cells that express them (21, 42, 43, 44a). Also, we showed that Mist1 can transactivate *Drosophila PHM* in HEK 293 cells with 50% of the activity displayed by DIMM and was able to modestly activate the *PHM*-GFP reporter in vivo. Notably, no other transcription factors that we tested (including other, closely related *Drosophila* bHLHs) displayed any effect on *PHM* transcription in vitro.

Even within *Drosophila* tissues, *dimm* is not the sole regulator of all "peptidergic" NSC fates. MB neurons express PHM (49) and specific neuropeptides (as studied in other insects [38]), yet they do not contain DIMM. In addition, other known peptidergic neurons, like the small LNv neurons expressing PDF (49) or the proctolin-containing neurons (D. Park and P. H. Taghert, unpublished data) do not contain DIMM. Thus, the fly nervous and endocrine systems appear divisible into different peptidergic cell domains, as defined by different developmental transcriptional regulators. In *Drosophila*, the *dimm* expression domain is large and diverse, containing many different kinds of NSCs, but there must be at least one more domain whose regulatory transcription factors as yet await identification.

Our studies of DIMM transcriptional activities suggest the hypothesis that there exists a core regulatory program underlying the differentiation and organization of NSCs. Many proteins are enriched in neuroendocrine tissues, and some are coordinately regulated under different physiological states (23). These differentiated gene batteries result from the activity of complex regulatory circuits whose identities and details are largely unknown. Which of these many proteins are primary targets of neuroendocrine cell-type transcriptional control (like DIMM) and which are derivative targets? We anticipate that further analysis of the core gene expression program driven by DIMM will help to explain the organization and evolution of NSCs and other neuroendocrine cell types. DIMM protein persists in neurons through adulthood (D. Park and P. H. Taghert, unpublished observations); studies of DIMM may therefore also help to address mechanisms of peptidergic cell physiology and plasticity.

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