Independent roles of the *dachshund* and *eyes absent* genes in BMP signaling, axon pathfinding and neuronal specification

Irene Miguel-Aliaga*, Douglas W. Allan† and Stefan Thor*,‡

Department of Neurobiology, Harvard Medical School, 220 Longwood Avenue, Boston, MA 02115, USA
*Present address: Division of Biology-IFM, Linkoping University, Campus Valla, 581 83 Linkoping, Sweden
†Present address: Department of Neurology, Enders 211, The Children's Hospital, 320 Longwood Avenue, Boston, MA 02115, USA
‡Author for correspondence (e-mail: steth@ifm.liu.se)

Accepted 15 September 2004

Development 131, 5837-5848
Published by The Company of Biologists 2004
doi:10.1242/dev.01447

Summary

In the *Drosophila* nerve cord, a subset of neurons expresses the neuropeptide FMRFamide related (Fmrf). *Fmrf* expression is controlled by a combinatorial code of intrinsic factors and an extrinsic BMP signal. However, this previously identified code does not fully explain the regulation of *Fmrf*. We have found that the Dachshund (Dac) and Eyes Absent (Eya) transcription co-factors participate in this combinatorial code. Previous studies have revealed an intimate link between Dac and Eya during eye development. Here, by analyzing their function in neurons with multiple phenotypic markers, we demonstrate that they play independent roles in neuronal specification, even within single cells. *dac* is required for high-level *Fmrf* expression, and acts potently together with *apterous* and BMP signaling to trigger *Fmrf* expression ectopically, even in motoneurons. By contrast, *eya* regulates *Fmrf* expression by controlling both axon pathfinding and BMP signaling, but cannot trigger Fmrf ectopically. Thus, we show that *dac* and *eya* perform entirely different functions in a single cell type to ultimately regulate a single phenotypic outcome.

Key words: *Drosophila*, *dachshund*, *eyes absent*, BMP signaling, Combinatorial code, FMRFamide, *FMRFa*

Introduction

During development of the nervous system, vast numbers of different neuronal subtypes are generated. Remarkable progress has been made in our understanding of many aspects of nervous system development, including the establishment of neural competence, patterning along the anteroposterior and dorsoventral axes, progenitor (neuroblast) specification and the progression of neuroblasts to postmitotic neurons (Altmann and Brivanlou, 2001; Edlund and Jessell, 1999; Skeath and Thor, 2003). These studies have revealed that neurons do not appear to be specified by the action of any one regulatory gene alone, but rather by the sequential and combinatorial action of many regulators and their unique interplay with key signaling pathways (Briscoe and Ericson, 2001; Shirasaki and Pfaff, 2002). However, once postmitotic neurons are born, it is less clear how the full repertoire of terminal differentiation genes is regulated. How complex are combinatorial codes in postmitotic neurons, how many regulators are required and how do individual regulators contribute to a final and unique neuronal identity?

One particularly well-documented example of a network of regulatory genes controlling organ development is that controlling eye formation in *Drosophila*. Genetic analysis of *Drosophila* eye formation has identified a conserved core group of transcriptional regulators collectively known as the retinal determination network (RDN). This network comprises a hierarchical genetic cascade, wherein *twin of eyeless* (*toy*) activates *eyeless* (*ey*) (Czerny et al., 1999), *ey* in turn activates both *eyes absent* (*eya*) and *sine oculis* (*so*) (Halder et al., 1998; Niimi et al., 1999), and *eya* and *so* activate *dacshund* (*dac*) expression (Chen et al., 1997; Pignoni et al., 1997). Extensive reciprocal positive feedback loops between these genes ensure robust gene expression and potency of the entire network (Chen et al., 1997; Czerny et al., 1999; Halder et al., 1995; Pignoni et al., 1997; Shen and Mardon, 1997). A complex of Eya, So and Dac is generally believed to be central to RDN function, and their coexpression and functional synergism are conserved in numerous vertebrate tissues (Chen et al., 1997; Heanue et al., 1999; Ikeda et al., 2002; Li et al., 2003; Pignoni et al., 1997; Xu et al., 1999). So and the homologous vertebrate Six family are transcription factors characterized by a homeodomain and the conserved Six domain (Kawakami et al., 2000). Eya and the vertebrate Eya family are nuclear co-factors with no known DNA-binding motifs (Bui et al., 2000; Ikeda et al., 2002; Ohto et al., 1999; Silver et al., 2003). Recent studies revealed that Eya proteins have an intrinsic phosphatase activity critical for both their transcriptional activity and in-vivo function (Li et al., 2003; Rayapureddi et al., 2003; Tootle et al., 2003). Dac and vertebrate Dach1-2 have two conserved Dachshund domains, one of which may mediate DNA binding directly (Ikeda et al., 2002). Binding studies have shown direct physical interaction between invertebrate and vertebrate Eya and Six family members (Heanue et al., 1999; Li et al., 2003; Pignoni et al., 1997; Silver et al., 2003). The functional relevance of this interaction has been well demonstrated by mutant analysis (Li et al., 2003; Pignoni et al., 1997) and by their strong phenotypic and transcriptional synergy (Bui et al., 2000; Heanue et al., 1999; Ikeda et al., 2002; Li et al., 2003;

Pignoni et al., 1997; Silver et al., 2003). Direct physical interaction between Dac/Dach and Eya has been observed in several (Chen et al., 1997; Heanue et al., 1999; Li et al., 2003), but not all (Ikeda et al., 2002; Silver et al., 2003), studies.

In spite of these elaborate hierarchical and reciprocal relationships between RDN genes in the eye, evidence suggests that their specific function in photoreceptor neurons may not be identical: *eya* mutant clones appear to have a more dramatic effect on the differentiation of photoreceptor cells than do *dac* mutant clones (Mardon et al., 1994; Pignoni et al., 1997). Furthermore, RDN genes have remarkably divergent expression patterns elsewhere in the *Drosophila* embryo (Bonini et al., 1998; Kammermeier et al., 2001; Kumar and Moses, 2001; Mardon et al., 1994). For example, *toy*, *ey* and *dac* are coexpressed in the developing mushroom bodies of the *Drosophila* central nervous system, but *eya* and *so* are absent (Kurusu et al., 2000; Martini et al., 2000; Noveen et al., 2000). In addition, there appears to be no regulatory relationship between *toy*, *ey* or *dac* in the mushroom bodies (Kurusu et al., 2000). Given the partially overlapping expression patterns of RDN genes in the vertebrate central nervous system (Caubit et al., 1999; Davis et al., 1999; Xu et al., 1997) it will be important to determine the roles that these genes play, independently and possibly combinatorially, in neuronal development.

In the *Drosophila* ventral nerve cord (VNC), a small subset of neurons expresses the LIM homeodomain gene *apterous* (*ap*) (Lundgren et al., 1995). These neurons can be subdivided, based upon differential neuropeptide expression and axon pathfinding (Fig. 1). *ap* itself is an important regulator of these diverse properties (Benveniste et al., 1998; Lundgren et al., 1995) and thus must be acting combinatorially with other regulators. We previously found that *ap* acts with the *squeeze* (*sqz*) zinc finger gene and the BMP pathway to activate expression of the neuropeptide gene *FMRFamide-related* (*Fmrf*) in one subset of *ap* neurons, the Tv neurons (Allan et al., 2003). Reconstitution of this combinatorial code in other peptidergic neurons triggered ectopic Fmrf expression in a subset of them. However, because only a fraction of peptidergic neurons are 'responsive', additional factors probably contribute to Fmrf expression. Here, we find that *dac* and *eya* are expressed in *ap* neurons and play critical roles in Fmrf regulation and *ap*-axon pathfinding. In *dac* and *eya* mutants, *ap* neurons are generated in normal positions and numbers, thus allowing us to address the specific role that each gene plays during neuronal differentiation with single cell resolution. In the VNC, Dac expression is restricted to a subset of interneurons and peptidergic neurons, with no expression observed in motoneurons or glia. Eya shows an early phase of expression in subsets of VNC cells, but rapidly becomes restricted to a subset of *ap* neurons. Expression and mutant analyses show that both Dac and Eya are present in the Fmrf-expressing Tv cells and that both are essential for proper Fmrf expression. However, mutant and misexpression analyses indicate that Dac and Eya have very different functions within *ap* neurons. *dac* has a weak effect on Fmrf expression but, when misexpressed together with *ap*, it potently triggers ectopic Fmrf expression in many peptidergic neurons and motoneurons. This ectopic Fmrf expression is dependent upon BMP signaling, indicating that *dac* acts as a potent member of an *ap*/*sqz*/BMP/*dac* combinatorial code that activates Fmrf expression in postmitotic neurons. By contrast to the weak effect of *dac* mutation, Fmrf expression is almost entirely lost in *eya* mutants. However, *eya* does not act combinatorially with *ap* and BMP signaling to trigger ectopic Fmrf expression. Instead, *eya* appears to play a dual role in Tv neurons, controlling both axon pathfinding and BMP signaling. Thus, our data show that despite being coexpressed in a single identified neuron, *dac* and *eya* perform entirely different functions with a common phenotypic outcome: the activation of Fmrf expression.

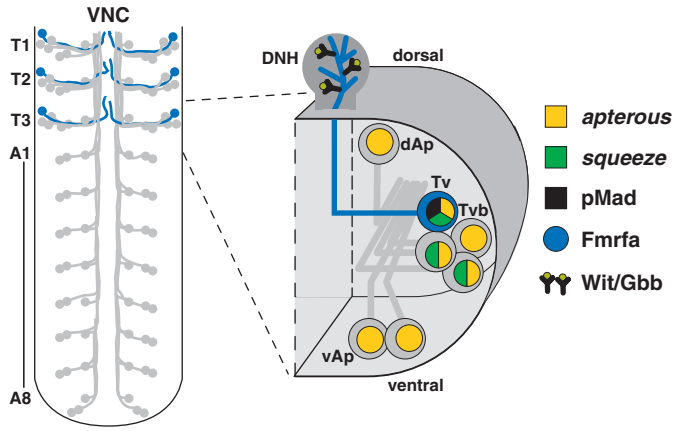

Fig. 1. In the developing *Drosophila* VNC, a small group of 90 neurons express the *ap* gene. Most *ap*-neurons extend axons in a common fascicle running the length of the VNC. The Tv neurons are unique; they innervate the DNH and express the Fmrf neuropeptide. Specification of Tv neurons is dependent upon a combinatorial code of *ap*, *sqz* and a target-derived BMP signal mediated by the Gbb ligand and the Wit receptor (Allan et al., 2003; Marques et al., 2003).

Materials and methods

Fly stocks

The following strains were used in this study: dac^{3}, dac^{4}, dac^{P} (also known as dac^{rK364}) (Mardon et al., 1994), dac^{p7d23} (referred to as dac^{GAL4}) (Heanue et al., 1999), *UAS-dac* (Shen and Mardon, 1997), so^{7} (Cheyette et al., 1994), $eya^{Cli\text{-}IID}$ (Pignoni et al., 1997), *UAS-eya.B.II* (expressing embryonic *eya* transcript; referred to as *UAS-eya*), *Df(2L)eya*^{10} (referred to as eya^{10}) (Bonini et al., 1998), ap^{P44}, ap^{rK568} (referred to as ap^{lacZ}), ap^{md544} (referred to as ap^{GAL4}), *UAS-ap*, wit^{A12}, wit^{B11}, *UAS-tkv*^{A}, *UAS-sax*^{A}, *UAS-gbb*, *UAS-myc-EGFP*^{F}, sqz^{Df}, sqz^{ie}, *UAS-sqz* (Allan et al., 2003), *Fmrf-lacZ#WF3-T2* (Schneider et al., 1993a), *elav-GAL4* (Luo et al., 1994), $elav^{GAL4}$ (Lin and Goodman, 1994), *UAS-nls-myc-EGFP* (Callahan et al., 1998), *apC-tau-lacZ#2.1* (Lundgren et al., 1995), *c929-GAL4* (Hewes et al., 2003), *HB9-GAL4* (Broiher and Skeath, 2002), $repo^{GAL4}$ (Sepp et al., 2001). Mutants were kept over *CyO,Act-GFP* or *TM3,Ser,Act-GFP* balancer chromosomes. w^{1118} was often used as wild type. All crosses were maintained at 25°C.

Immunohistochemistry

Antibodies used were: α-c-Myc mAb 9E10 (1:30), concentrated α-β-gal mAb 40-1a (1:20), α-Dac mAb dac2-3 (1:25), α-Eya mAb 10H6 (1:250) (all from Developmental Studies Hybridoma Bank); rabbit α-proFmrf (1:2000) (Chin et al., 1990), rabbit α-β-gal (1:5000, ICN-Cappel), rabbit α-pMad (1:2000) (Tanimoto et al., 2000), rabbit α-Glutactin (1:300) (Olson et al., 1990), rabbit α-GFP (1:500, Molecular Probes). Immunolabeling was carried out as previously described (Allan et al., 2003).

Analysis of enhancer trap lines

Expression analysis of the 577 second-chromosome lethal lines identified by the BDGP project (Spradling et al., 1999) was carried out using X-gal and anti-β-gal staining. Of these lines, several showed restricted patterns of expression in the VNC. One of them was a *lacZ* insertion in *dac*, referred to as dac^{P}.

Confocal imaging and data acquisition

A Zeiss LSM 510 confocal microscope was used to collect data for all images; confocal stacks were merged using LSM 510 software. Where immunolabeling was compared for levels of expression, wild-type and mutant tissue was stained and analyzed on the same slide. Images were ~2 μm thick (Fig. 2B,C; Fig. 3E-L; Fig. 4I-L,R,S; Fig. 5A-L; Fig. 6G; insets in Fig. 6) or 5 μm thick (Fig. 3B-C″; Fig. 4E-H′,O-Q; Fig. 6H). Images of the entire VNC (Fig. 2A; Fig. 3D; Fig. 4A-D,M,N; Fig. 6A-F,I-L) consisted of 1.2 μm-thick steps through the entire VNC (30-40 μm), which were merged to obtain the final image. The intensity index used to quantify Fmrf expression levels in *dac* mutants and rescues (Fig. 4T) was obtained as previously described (Hewes et al., 2003). Statistical analysis was performed using Microsoft Excel. Where appropriate, images were false colored to help color-blind readers.

Results

The *Drosophila* embryonic/larval VNC contains ~10,000 cells (Schmid et al., 1999). A small subset of these cells (~150) are peptidergic, as defined by expression of high levels of neuropeptide-processing enzymes and one or several of the ~30 identified neuropeptides (Hewes et al., 2003; Nassel, 1996; Taghert, 1999). The neuropeptide gene *FMRFamide-related* (*Fmrf*) is expressed in a small subset of embryonic/larval peptidergic VNC neurons, the six Tv cells located bilaterally in the three thoracic segments (blue cells in Fig. 1) (Schneider et al., 1993b). In each thoracic hemisegment, the Tv cell is one of a cluster of four lateral cells that express the LIM homeodomain gene *apterous* (*ap*) (Benveniste et al., 1998). *ap* is also expressed by three additional neurons per hemisegment throughout the VNC, the single dorsal *ap* (dAp) cell and the doublet ventral *ap* (vAp) cells (Fig. 1) (Lundgren et al., 1995). The Tv neurons are unique among the *ap*-neurons by virtue of their expression of Fmrf and their axonal trajectory; the majority of *ap* cells extend their axons within an ipsilateral longitudinal fascicle, whereas the Tv axons project to the midline, exit the VNC dorsally and innervate the endocrine dorsal neurohemal organs (DNH) (Gorczyca et al., 1994; Nassel et al., 1988). The restricted Fmrf expression and unique axonal trajectory of the Tv cell together provide highly specific terminal differentiation markers with which to ask basic questions concerning cell specification in the central nervous system (Fig. 1).

Peptidergic neurons can be subdivided into two groups

Previous studies had identified several genes acting to specify Tv cell identity. *ap* and the Krüppel-type zinc finger gene *squeeze* (*sqz*) act together to make the Tv cell competent to express Fmrf (Allan et al., 2003). However, Fmrf expression is not triggered until a target-derived retrograde signal, mediated by the BMP ligand Glass bottom boat (Gbb) and the type-II BMP receptor Wishful thinking (Wit), activates the BMP pathway within the Tv cell (Allan et al., 2003; Marques et al., 2003). Additionally, the bHLH gene *dimmed* (*dimm*), which specifies generic aspects of peptidergic cellular identity, is also required for wild-type levels of Fmrf expression (Hewes et al., 2003). Pan-neuronal misexpression of *ap* and *sqz* can trigger ectopic Fmrf expression, but only in a subset of peptidergic neurons: the Va and dMP2 neurons (previously described as Vap neurons) (Allan et al., 2003). All these cells have active BMP signaling, as detected by immunoreactivity to the phosphorylated receptor-Smad protein Mothers against dpp (pMad; Mad – FlyBase). From these studies, we proposed a simple model wherein an *ap/sqz*/BMP combinatorial code would be sufficient to activate Fmrf in all peptidergic neurons (Allan et al., 2003).

To test this hypothesis, we examined immunoreactivity to pMad in the majority of peptidergic neurons, using the *c929-GAL4* line (Hewes et al., 2003) (Fig. 2A-C). Certain peptidergic cells, such as the corazonin cells (Fig. 2B), showed no evidence of BMP activity. However, in addition to the Tv, Va and dMP2 peptidergic neurons (Allan et al., 2003; Miguel-Aliaga and Thor, 2004), we found that a number of peptidergic cells stained for pMad, but were refractory to *ap*/*sqz* misexpression. These include a lateral cluster of peptidergic cells in abdominal segments, here referred to as Plc (peptidergic lateral cluster; Fig. 2C). This indicates that pMad-positive peptidergic cells in the *Drosophila* VNC can be subdivided into two subclasses: those that respond to *ap*/*sqz* by triggering Fmrf expression, and those that are refractory. Thus, other factors besides *ap*, *sqz*, *dimm* and the BMP pathway are probably necessary for proper Fmrf expression (Fig. 2D).

Dachshund and Eyes Absent are expressed in 'responsive' peptidergic neurons

To understand why only a subset of peptidergic cells trigger Fmrf in response to the *ap*/*sqz*/BMP code, we attempted to identify additional genes expressed in subsets of peptidergic cells, including the Tv cells. To this end, we analyzed the expression of a number of enhancer trap lines (see Materials and methods). We found that P-element transposon insertions (*lacZ* or *GAL4*) in the *dac* gene revealed *dac* expression in a large population of interneurons, with no evidence of expression in either glia ($repo^{GAL4}$) or motoneurons (pMad; Fig. 3A-C″). Importantly, however, we observed *dac* expression in a lateral group of cells in the three thoracic segments (Fig. 3A). Using antibodies to Dac, and the *Fmrf-lacZ* and ap^{GAL4} reporter lines, we found that Dac was expressed in all four *ap*-cluster cells at stage 15 (not shown). However, from stage 16 onward, Dac expression was restricted to three of the four cells in the *ap*-cluster (Fig. 3E). In order to identify which *ap*-cluster cells expressed Dac, we co-labeled for *c929-GAL4* (restricted to the peptidergic Tv, Tvb of the *ap*-cluster and dAp cells) (Hewes et al., 2003) and *Fmrf-lacZ* (to distinguish the Tv cell) (Fig. 3G). We found that Dac was absent from the Tvb and dAp cells (*c929-GAL4*-positive, *Fmrf-lacZ*-negative, Fig. 3G), and thus was selectively expressed in the Tv, Tva and Tvc cells. Dac expression was initiated postmitotically in *ap*-neurons, but it was rapidly activated by stage 15 as *ap*-neurons emerged (not shown). We found that Dac expression, as visualized by Dac, dac^{P} (a *lacZ* insertion in *dac*) or dac^{GAL4}, was initiated postmitotically in the majority of neurons, a notion that is substantiated by the onset of expression in *ap*-neurons, and by the expression of Dac in the pCC interneuron but not in its sibling, the aCC motoneuron (Fig. 3C, arrow).

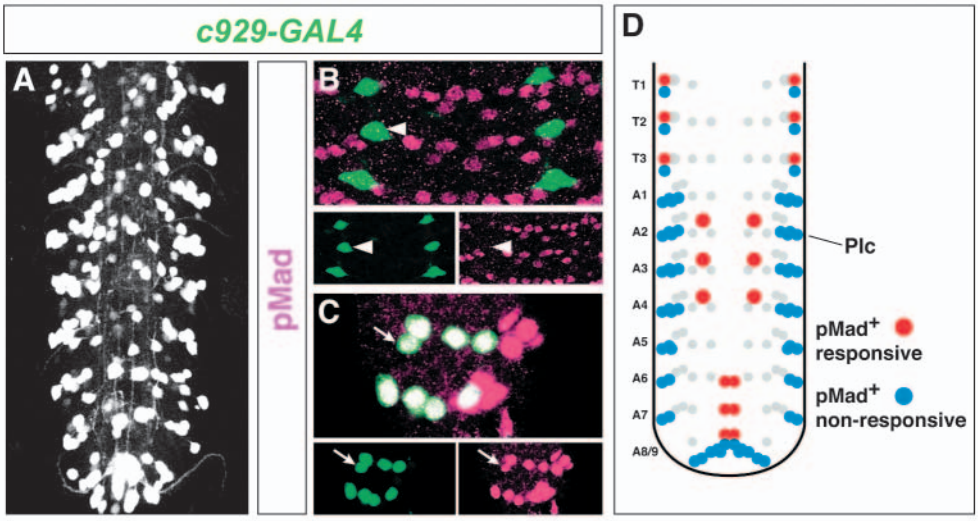

Fig. 2. The BMP pathway is active in many peptidergic cells, but only a subset is responsive to the *ap/sqz Fmrf* code. (A) The *Drosophila* VNC contains ~150 peptidergic neurons, as revealed by c*929-GAL4/UAS-nls-myc-EGFP* expression. (B,C) pMad immunoreactivity in *c929-GAL4*+ neurons reveals that some peptidergic cells, such as Corazonin neurons, do not have active BMP signaling (arrowhead in B), whereas others, such as the peptidergic lateral cluster (Plc) do (arrow in C). (D) Based upon their responsiveness to *sqz/ap* co-misexpression (Allan et al., 2003), the pMad+, peptidergic cells of the VNC can be subdivided into a responsive and a non-responsive 'compartment'.

Next, we examined whether pMad and Dac expression coincided in peptidergic cells, utilizing the *c929-GAL4* reporter to identify VNC peptidergic neurons. pMad/Dac coexpression was restricted to a small subset of peptidergic neurons: the Va and dMP2 cells (Fig. 3C″,I-J), as well as a posterior cluster (Pc) of peptidergic neurons (not shown), all of which exit the VNC. In contrast, neither Dac nor pMad were expressed in several other peptidergic neurons, such as the Crz neurons (Fig. 3K) or the Tvb or dAp cells (Fig. 3G). In the clusters of lateral abdominal peptidergic cells (Fig. 2A,D; Fig. 3L), Dac and pMad expression was mutually exclusive; the pMad+ Plc cells did not express Dac (Fig. 2C, arrow; Fig. 3L, arrowhead) while Dac was expressed in two neighboring pMad-negative peptidergic cells, herein referred to as the ventral intermediate (Vi) neurons (Fig. 3L, arrow).

dac encodes a transcriptional co-factor that plays key roles during *Drosophila* imaginal disc development (Mardon et al., 1994). In the developing eye, *dac* function within the retinal determination gene network is intimately linked to that of the homeobox gene *sine oculis* (*so*) and the transcriptional co-factor *eyes absent* (*eya*) (Hsiao et al., 2001). We analyzed the expression of so^{lacZ} (so^{7}) and *eya* (anti-Eya). As previously described, there is an early phase of both so^{lacZ} and Eya expression in subsets of VNC cells between stages 13 and 15 (Kumar and Moses, 2001) (not shown). *ap*-neurons could first be discriminated at stage 15. Expression of so^{lacZ} was not observed in an *ap*-cluster at any stage (not shown). As the lineage generating *ap*-neurons is unknown, we could not determine whether so^{lacZ} was expressed in the *ap*-neuron precursors. By contrast, Eya expression was observed within a subset of *ap*-neurons, the four *ap*-cluster cells and the dAp cells, even as they first emerged (Fig. 3D,F,H). Remarkably, by stage 16, the expression of Eya within the VNC was entirely restricted to these *ap*-neurons (Fig. 3D).

Dac and Eya were expressed in partially overlapping subsets of *ap*-neurons. The Tv, Tva and Tvc cells expressed both Dac and Eya. However, in the Tvb and dAp cells, Eya was expressed without Dac. With respect to the ability of *ap*/*sqz*/BMP to trigger Fmrf expression ectopically in the VNC peptidergic compartment, we found that all 'responsive' peptidergic cells (the dAp, Va, dMP2 cells) expressed either Dac or Eya, whereas 'non-responsive' peptidergic cells (such as the Plc and Crz cells) did not (Fig. 3M). pMad staining, indicative of active BMP signaling, also contributes to the definition of the responsive/non-responsive peptidergic compartments. pMad was evident in the responsive Va and dMP2 cells, which expressed Dac and responded to *ap*/*sqz* alone. pMad was absent from the responsive dAp cells, which expressed Eya and responded to *ap*/*sqz* only when co-misexpressed with BMP signaling. In the non-responsive population, certain cells (such as the Plc cells) had pMad but did not express Dac or Eya, while others (such as the Crz cells) had neither Dac/Eya nor pMad. The expression of these markers within the VNC peptidergic compartment is summarized in Fig. 3M.

Dachshund and Eyes Absent are important for FMRFamide-related expression but play different roles in *ap*-neurons

To test whether *dac* and *eya* play any roles in the specification of Fmrf-Tv neurons, we analyzed mutants for each gene. In *dac* mutants (Fig. 5C) we found that *ap*-cluster cells were generated and that Tv neurons showed normal innervation of the DNH and pMad staining (Fig. 4F,J). However, there was a small but numerically significant loss of Fmrf expression (97% in wild type compared with 94% in *dac* mutants; $P<0.05$) (Fig. 4A,B). Moreover, quantification of their Fmrf expression levels revealed that Fmrf expression was consistently weaker in *dac* mutants compared with that of wild type ($P<0.0001$) (Fig. 4T). Upon rescue of *dac* mutants, by re-introduction of *UAS-dac* from ap^{GAL4}, we observed a clear upregulation of Fmrf expression above wild-type levels ($P<0.0001$ compared with

control) (Fig. 4T). This supports a cell-autonomous role for *dac* in controlling high-level expression of Fmrf in Tv neurons.

By contrast, in *eya* mutants (Fig. 5J), Fmrf expression was severely reduced, with only 32% of Tv cells expressing Fmrf compared with 97% in wild type ($P<0.0001$) (Fig. 4A,C). We had routinely used ap^{GAL4} as a marker for *ap*-neurons and, although ap^{GAL4} is a strong *ap* allele, we had not seen evidence of genetic interactions between *ap* and either *sqz*, *dac* or BMP signaling (Allan et al., 2003) (not shown). However, upon comparing Fmrf expression in *eya* mutants in the presence or absence of ap^{GAL4}, we found that this *ap* allele enhanced the *eya* phenotype; Fmrf was expressed in only 6% of Tv neurons in an *eya* null, *ap* heterozygous background, compared with 32% for an *eya* null, *ap* wild-type background. This genetic interaction did not result from regulation of *ap* by *eya*, or vice versa, because *ap* expression was normal in *eya* mutant *ap*-cluster cells, and vice versa (Fig. 4I,K; Fig. 5G,H,J). *eya* mutants also displayed a severe pathfinding phenotype with a

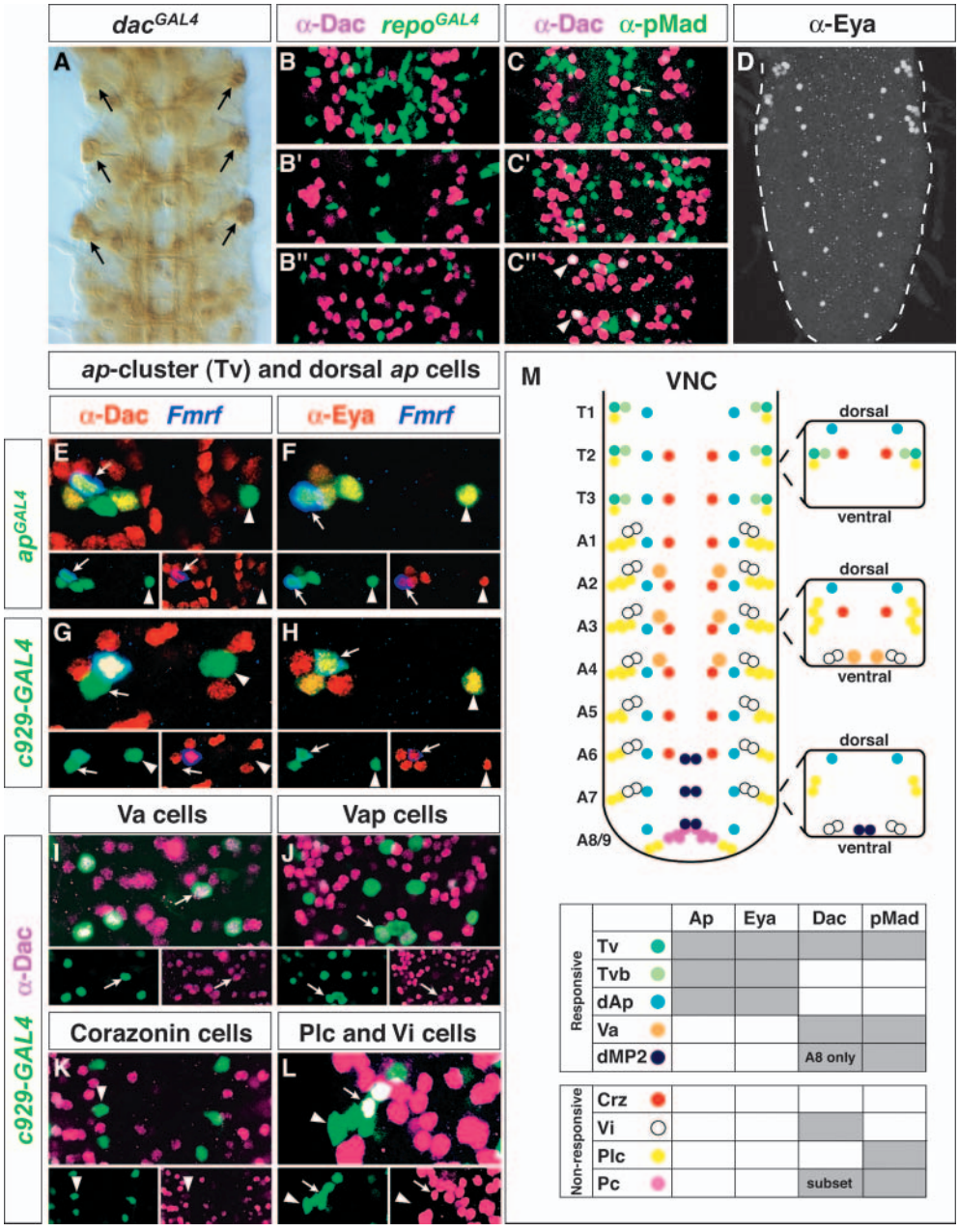

Fig. 3. Dachshund and Eyes Absent are expressed in subsets of peptidergic neurons. (A) Expression of *dac* (*dac*^{GAL4}/*UAS-myc-EGFP*^{F}) is observed in subsets of neurons in the embryonic VNC, including a lateral cluster of cells in the three thoracic segments (arrows). (B-L) Expression of Dac and Eya in late-stage-17 embryos. (B,B′,B″) Expression of Dac and the glia-specific marker *repo* (*repo*^{GAL4}/*UAS-nls-myc-EGFP*) reveals that Dac is not expressed in glia. (C,C′,C″) Expression of Dac and pMad reveals that Dac is not expressed in motoneurons. Expression of Dac is evident in the pCC interneuron (arrow in C) and in the Va peptidergic cells (arrowheads in C″). (D) From embryonic stage 16, expression of Eya is observed in a highly restricted set of cells in the VNC. (E) Expression of Dac (red), *Fmrf* (blue) and *ap* (*ap*^{GAL4}/*UAS-nls-myc-EGFP*; green) shows that Dac is expressed in three of the four *ap*-cluster cells, including the Tv cell (arrow), but is absent from the dAp cell (arrowhead). (F) Expression of Eya (red), Fmrf (blue) and *ap* (*ap*^{GAL4}/*UAS-nls-myc-EGFP*; green) shows that Eya is expressed in all four *ap*-cluster cells, including the Tv cell (arrow) and in the dAp cell (arrowhead). (G,H) *c929-GAL4* and Fmrf-lacZ co-labeling discriminates the Tv cell (*c929-GAL4*+, *Fmrf-lacZ*+) and the Tvb and the dAp cells (*c929-GAL4*+, *Fmrf-lacZ*-negative). (G) Expression of Dac (red), Fmrf (blue) and *c929-GAL4/UAS-nls-myc-EGFP* (green) shows that Dac is not expressed in the Tvb neuron (arrow) or the dAp cell (arrowhead). (H) Expression of Eya (red), Fmrf (blue) and *c929-GAL4/UAS-nls-myc-EGFP* (green) shows that Eya is expressed in both the Tvb neuron (arrow) and dAp cell (arrowhead). (I-L) Expression of Dac and *c929-GAL4/UAS-nls-myc-EGFP* shows that Dac is expressed in Va neurons (arrow in I) and posterior dMP2 neurons (arrow in J), is absent from corazonin neurons (arrowhead in K), present in the Vi neurons (arrow in L) and absent from the Plc neurons (arrowhead in L). The dMP2 neurons were previously described as Vap neurons (Allan et al., 2003). However, subsequent work has revealed that Vap neurons are, in fact, the well-characterized dMP2 neurons (I.M-A. and S.T., unpublished). (M) Summary of the expression of Ap, Dac, Eya and pMad within peptidergic neurons of the stage-17 embryonic and larval *Drosophila* VNC. Note that either Dac or Eya are expressed in all peptidergic neurons that express Fmrf in response to the *ap/sqz*/BMP code.

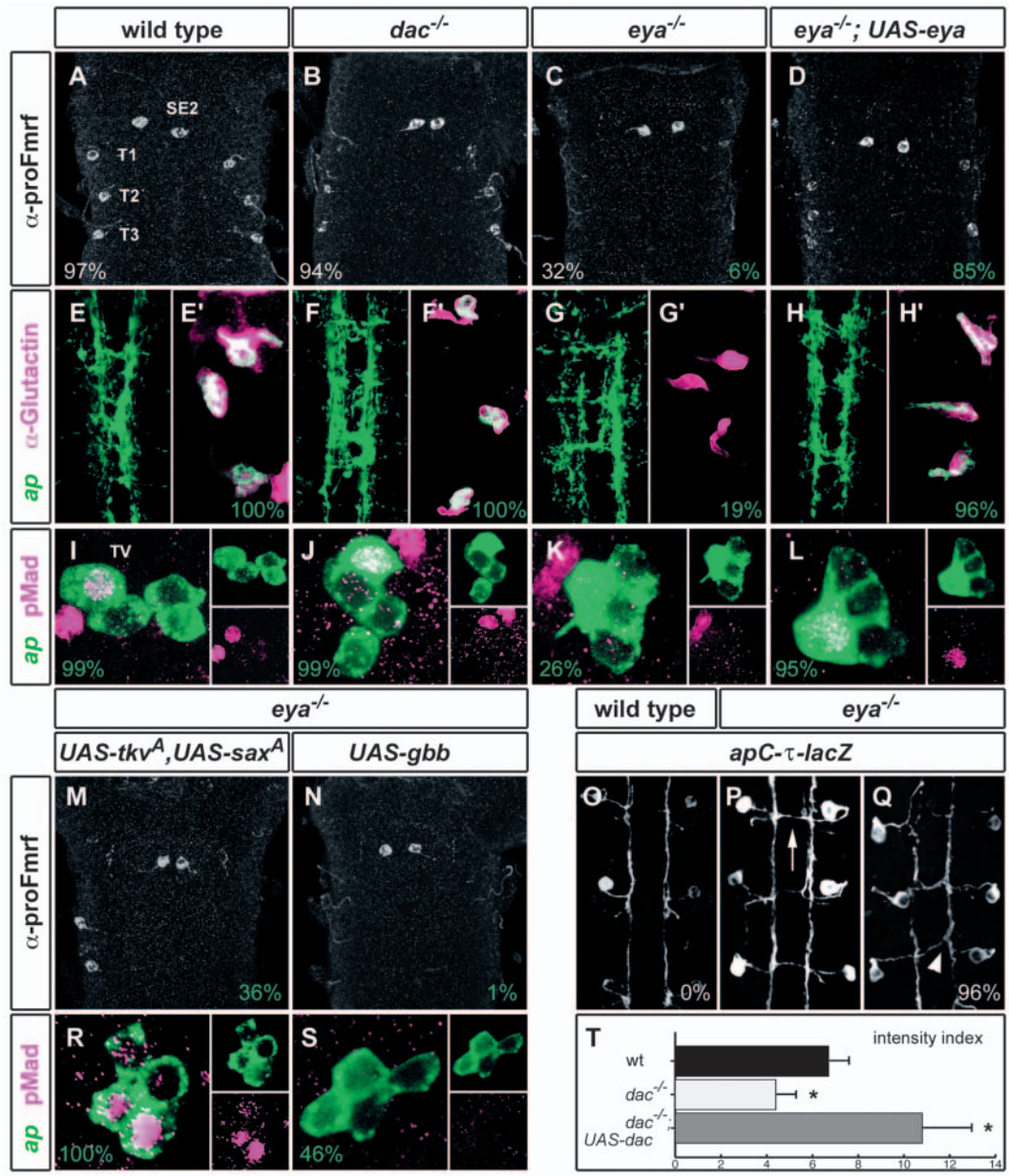

Fig. 4. *dachshund* and *eyes absent* are both important for Fmrf expression but play different roles. Expression of proFmrf (A-D,M,N), morphology of *ap*-axons at the thoracic midline (E-H), innervation of the DNH using ap^{GAL4}*; UAS-myc-*$EGFP^{F}$ (green) and anti-Glutactin to visualize the DNH (red) (E′-H′), and pMad expression in the *ap*-cluster (I-L,R,S) in late-stage-17 embryos. In the wild type (A), proFmrf expression is readily observed in the six lateral Tv neurons in thoracic segments T1 to T3 and in the two anterior, medial SE2 neurons. (A,E,E′,I) In controls (ap^{GAL4}/+*; UAS-myc-*$EGFP^{F}$/+), *ap*-neurons project close to the midline (E) and innervate the DNH (E′), and pMad staining is evident in the Tv cell of the *ap*-cluster (I). (B,F,F′,J) In *dac* mutants (dac^{3}/dac^{4}), the expression of proFmrf is weak and partly lost in Tv cells (B). However, in *dac* mutants (ap^{GAL4}*,* dac^{4}/dac^{3}*; UAS-myc-*$EGFP^{F}$/+), there is entirely wild-type midline and DNH innervation (F,F′), and pMad staining of Tv cells (J). (C,G,G′,K) In *eya* mutants ($eya^{Cli\text{-}IID}/eya^{10}$) proFmrf expression is detected in only 32% of Tv neurons, and this is reduced to 6% by removing one copy of *ap* (C). In *eya* mutants (ap^{GAL4}*,* $eya^{Cli\text{-}IID}/eya^{10}$; *UAS-myc-*$EGFP^{F}$/+), TV axonal projections reach the midline (G) but fail to innervate the DNH (G′, only 19% of DNH). Only 26% of Tv neurons express pMad (K). (D,H,H′,L) Cell-autonomous reintroduction of *eya* (ap^{GAL4}*,* $eya^{Cli\text{-}IID}/eya^{10}$; *UAS-myc-*$EGFP^{F}$*/UAS-eya*) rescues proFmrf (D), DNH innervation (H′) and Tv pMad expression (L). (M,R) Direct activation of the BMP pathway in *eya* mutants (ap^{GAL4}*,* $eya^{Cli\text{-}IID}/eya^{10}$*,* $UAS\text{-}tkv^{A}$*,* $UAS\text{-}sax^{A}$*; UAS-myc-*$EGFP^{F}$/+) only partly restores proFmrf (M), although pMad is expressed in most *ap*-cluster neurons and is rescued to 100% in Tv cells (R). (N,S) Providing *gbb* cell-autonomously in *eya* mutants (ap^{GAL4}*,* $eya^{Cli\text{-}IID}/eya^{10}$*, UAS-gbb; UAS-myc-*$EGFP^{F}$/+) fails to restore either proFmrf (N) or pMad (S). (O,P,Q) Expression of *τ-lacZ* reveals abdominal *ap*-axon projections in the stage 16-17 embryo. In the control (*apC-τ-lacZ*), dAp and vAp neurons project axons within the ipsilateral *ap*-fascicle and do not cross the midline (O). In two different *eya* mutant VNCs ($eya^{Cli\text{-}IID}/eya^{10}$*; apC-τ-lacZ*), the dAp axons frequently (96%) cross the midline (P, arrow, Q). However, they join the contralateral *ap* fascicle and appear to project anteriorly, like wild-type dAp axons (Q, arrowhead). (T) Relative proFmrf staining intensity in wild type, *dac* mutant (dac^{3}/dac^{4}) and *dac* rescue (ap^{GAL4}*,* dac^{4}/dac^{3}*; UAS-dac*/+) late-stage-17 Tv neurons. *dac* mutants have reduced proFmrf expression and the *dac* rescue shows increased intensity, probably due to overexpression of *dac*. Percentages presented in white were obtained in a wild-type *ap* background, whereas those presented in green correspond to an *ap* heterozygous (ap^{GAL4}/+) background.

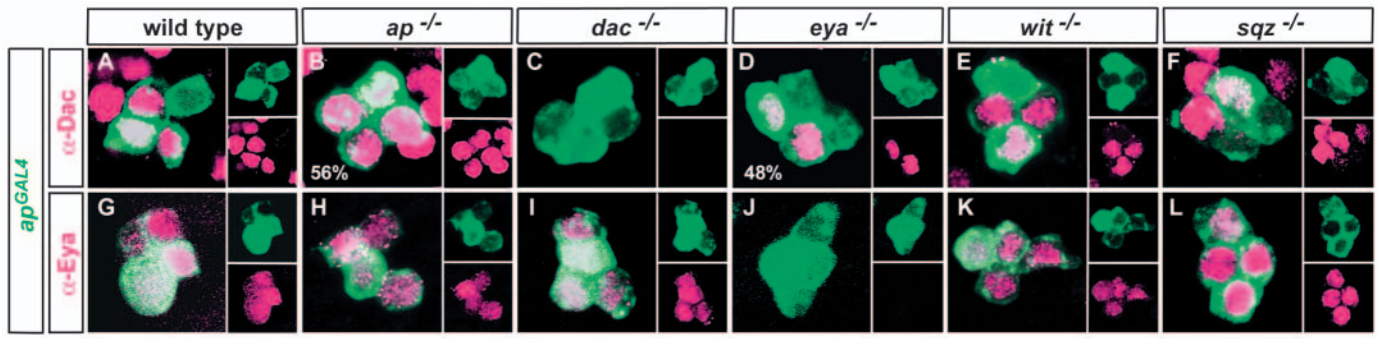

Fig. 5. Expression of *apterous*, Dachshund and Eyes Absent in mutant backgrounds. Expression of *ap* (A-L) Dac (A-F) and Eya (G-L) in control and mutant late-stage-17 *ap*-clusters. In controls, expression of Dac is evident in three *ap*-cluster cells (A), being absent from the Tvb cell (see Fig. 3E,G). In *ap* mutants (ap^{GAL4}/ap^{P44}; *UAS-nls-myc-EGFP*^{F}/+), Dac is often de-repressed in the Tvb cell (B). In *eya* mutants (ap^{GAL4}, $eya^{Cli\text{-}IID}/eya^{10}$; *UAS-myc-EGFP*^{F}/+), Dac is often lost from one cell (D), but not from the Tv cell (identified as the highest EGFP-expressing cell). In *wit* mutants (ap^{GAL4}/+; wit^{A12}, *UAS-myc-EGFP*$^{F}/wit^{B11}$), Dac expression is normal (E). In *sqz* mutants (ap^{GAL4}/+; sqz^{Df}, *UAS-myc-EGFP*$^{F}/sqz^{ie}$), expression of Dac is typically lost from one additional cell per *ap*-cluster (F), but not from the Tv cell (again, identified as the highest EGFP-expressing cell). Eya expression is not affected in any of the mutant backgrounds (G-L). As expected, expression of Dac and Eya is absent in *dac* (C; dac^{4}, ap^{GAL4}/dac^{3}; *UAS-myc-EGFP*^{F}/+) and *eya* (J; ap^{GAL4}, $eya^{Cli\text{-}IID}/eya^{10}$; *UAS-myc-EGFP*^{F}/+) mutants, respectively.

nearly complete failure of DNH innnervation: 19% DNH innervation in *eya* mutants compared with 100% in controls (Fig. 4E′,G′). As predicted, this failure to reach the DNH in *eya* mutants resulted in the nearly complete loss of pMad in the *ap*-cluster (26% pMad staining of Tv cells compared with 99% in controls; $P<0.0001$) (Fig. 4I,K). To analyze axon pathfinding in *eya* mutants without altering *ap* gene dosage, we used the *ap* enhancer construct *apC-τ-lacZ* (Lundgren et al., 1995) instead of ap^{GAL4}. Unfortunately, unlike the membrane-targeted *UAS-myc-EGFP*^{F}, the *τ-lacZ* reporter did not reproducibly reveal the Tv axon terminals in the DNH. Thus, we could not address DNH innervation in *eya* mutants without using ap^{GAL4}. However, since *ap* is not important for Tv pathfinding (Allan et al., 2003; Benveniste et al., 1998), it is unlikely that the severe Tv-axon pathfinding observed in *eya* was exacerbated by the removal of one copy of *ap*. We did find a remarkably strong ectopic midline crossing of dAp axons in *eya* mutants using *τ-lacZ*, most evident in abdominal segments: 96% of segments showed at least one dAp axon crossing the midline in *eya* mutants, compared with 0% in controls (n=24 segments; Fig. 4O-Q). This demonstrates that *eya* is critical for axon pathfinding even in the presence of wild-type *ap*. The ventral pair of *ap*-neurons (vAp) did not express *eya* and did not show any apparent defects in pathfinding (Fig. 4O-Q).

In the embryo, Eya is expressed in certain regions of the lateral mesoderm and in dorsal, anterior structures, and has been shown to be important for embryonic head morphogenesis (Bonini et al., 1998). In spite of these other roles for *eya* during embryogenesis, we found that reintroducing *UAS-eya* from ap^{GAL4} in *eya* mutants rescued DNH innervation to 96% (Fig. 4G′,H′), rescued pMad staining of the Tv cell to 95% (Fig. 4K,L), and rescued Fmrf to 85% (Fig. 4C,D; all $P<0.0001$, compared with *eya* mutants). These data support a cell-autonomous role for *eya* in controlling Tv-axon pathfinding and Fmrf expression.

In summary, *dac* and *eya* act cell-autonomously to regulate crucial, yet different, aspects of Tv cell differentiation. *dac* is important for high-level Fmrf expression but does not affect pathfinding. *eya* regulates axon pathfinding of a subset of *ap*-neurons, including the Tv and dAp cells. We also observed a genetic interaction between *eya* and *ap* with respect to Fmrf expression. Given that *ap* regulates *Fmrf* gene expression directly by binding to its enhancer (Benveniste et al., 1998), the genetic interaction observed between *eya* and *ap* suggests a direct regulation of *Fmrf* gene expression by *eya*.

In addition to pathfinding, Eyes Absent controls BMP signaling

These *eya* mutant results did not discriminate between an effect for *eya* directly on Fmrf, or indirectly on Fmrf via its control of Tv-axon pathfinding to the DNH. Fmrf expression in the Tv neurons is crucially dependent on a target-derived BMP signal mediated by the BMP ligand Gbb, which is accessed by Tv axons at the DNH (Allan et al., 2003; Marques et al., 2003). Fmrf expression is lost when Tv-axon pathfinding is disrupted by *UAS-robo* misexpression (ap^{GAL4}/*UAS-robo*), forcing Tv axons to avoid the midline and DNH. However, Fmrf expression can be efficiently restored in these misguided Tv neurons by providing the Gbb ligand cell-autonomously (ap^{GAL4}/*UAS-robo, UAS-gbb*) (Allan et al., 2003). The severe pathfinding defects observed in *eya* mutants raised the possibility that loss of Fmrf solely reflected a loss of DNH innervation and access to Gbb. Is the loss of Fmrf in *eya* mutants secondary to these axon-pathfinding defects, or does *eya* regulate other aspects of Tv cell differentiation?

To resolve this issue, we tested whether Fmrf expression could be restored in *eya* mutants by providing *gbb* cell-autonomously. Even though *UAS-gbb* rescues *gbb* mutants and misguided Tv neurons (Allan et al., 2003), *UAS-gbb* failed to rescue Fmrf expression in *eya* mutants (Fig. 4N). Surprisingly, we also noted only a partial rescue of pMad staining in Tv neurons; 46% pMad in *gbb*-rescued *eya* mutants compared with 26% in *eya* mutants and 98% pMad in *gbb*-rescued *gbb* mutants ($P<0.0001$) (Fig. 4S) (Allan et al., 2003). This suggested that two aspects of the competence to respond to BMP signaling were affected in *eya* mutants. First, the inability of *gbb* to rescue pMad activation reflects the functional absence

of a component of the BMP signaling pathway upstream of pMad in *eya* mutants. This component may be the BMP type-II receptor Wit, which mediates BMP retrograde signaling in Tv neurons. Unfortunately, the Wit antibody is not sufficiently sensitive to test this hypothesis directly. Second, the complete failure to rescue of Fmrf expression with *gbb*, in spite of its partial rescue of pMad, suggested that a downstream component of the BMP signaling pathway that leads to Fmrf expression was additionally affected in *eya* mutants. Our observations in *eya* mutants, that remaining pMad-positive Tv neurons were frequently Fmrf-negative, is consistent with this hypothesis (26% were positive for pMad staining, whereas only 6% expressed Fmrf; $P<0.0001$). To test this idea directly, we bypassed the Wit receptor by driving activated BMP type I receptors from ap^{GAL4} in an *eya* mutant background. In spite of a full rescue of pMad in Tv cells (100% compared with 26% in *eya* mutants, $P<0.0001$) (Fig. 4K,R), Fmrf expression was only poorly rescued to 36%, compared with 6% in *eya* mutants ($P<0.0001$) (Fig. 4M). This contrasts with the ability of these activated type I receptors to rescue *gbb* and *wit* mutants fully (Allan et al., 2003), and indicates that *eya* controls a component of the pathway downstream of pMad that is essential for activating Fmrf expression. This component may be Eya itself or some other unknown regulatory factor that directly controls Fmrf expression.

In summary, *eya* plays multiple roles in the Tv neuron. *eya* is necessary for Tv innervation of the DNH, as well as normal pathfinding of dAp neurons along the *ap*-fascicle. In addition, *eya* is required in Tv neurons for the activation of pMad in response to *gbb*, as well as for the activation of Fmrf expression following pMad nuclear accumulation.

Dachshund, but not Eyes Absent, is in part regulated by other genes specifying FMRFamide-related cell fate

We next addressed whether the genes controlling Fmrf expression regulate one another. As shown above, there was no effect on ap^{GAL4} reporter activity or *ap* cell numbers in either *dac* or *eya* mutants (Fig. 5A,C,D). Additionally, *dac* did not regulate Eya (Fig. 5I). However, we did note a partial loss of Dac expression in one *ap*-cluster cell in *eya* mutants (Fig. 5D). This cell was probably the Tva or Tvc cell, because Dac was never lost in the Tv cell, identified as the cell with highest ap^{GAL4} activity (Fig. 5D; note pMad staining in cell of highest ap^{GAL4} activity in Fig. 4I,J,L). We found no evidence that the late (stage-17) activation of the BMP pathway was important for the maintenance of either Dac or Eya expression (Fig. 5E,K). In *sqz* mutants, Eya expression was evident within every *ap*-cluster cell (Fig. 5L), including the extra *ap* cells that we typically observed in *sqz* mutants (Allan et al., 2003) (not shown). However, we did observe a partial loss of Dac in *sqz* mutants; it was typically lost from one *ap*-cluster cell (Fig. 5F). In independent studies, we have found that *sqz* regulates the identity and number of *ap*-cluster cells through an interaction with the Notch pathway, resulting in the generation of additional Tvb cells within each *ap*-cluster in *sqz* mutants (D.W.A. and S.T., unpublished). Dac is not normally expressed in the Tvb cell, so we propose that the loss of Dac in one extra cell per *ap*-cluster in *sqz* mutants is due to the generation of an extra Tvb cell, rather than the result of a direct effect of *sqz* on Dac expression. Given these early effects of *sqz* function on *ap*-cluster cell identity via the Notch pathway, we did not examine *sqz* expression in either Dac or Eya mutants, which are expressed exclusively postmitotically and were not found to modulate the number of *ap* cells generated.

Finally, we observed that in *ap* mutants, Dac expression was often maintained in the Tvb neuron (56%), indicating that *ap* normally contributes to the repression of *dac* in Tvb neurons. Because *ap* does not normally prevent Dac expression in the other neurons of the *ap* cluster, additional factors must make the *ap*-mediated repression of Dac context-dependent.

Dachshund, but not Eyes Absent, acts in a combinatorial code to trigger ectopic FMRFamide-related expression

The expression patterns of Dac and Eya, together with their roles in Fmrf regulation, suggested that they are the missing factors in pMad-positive, peptidergic cells that are non-responsive to the *ap/sqz*/BMP combinatorial code. To test this notion we addressed the sufficiency of *dac* and *eya* to activate Fmrf expression ectopically, either alone, in combination with one another, or together with the previously identified Fmrf regulators. This was tested in peptidergic cells (*c929-GAL4*), in *ap*-neurons (ap^{GAL4}) and in all postmitotic neurons ($elav^{GAL4}$).

First, we examined the effects of *UAS-eya* misexpression. UAS-*eya* failed to trigger ectopic Fmrf expression when driven from any *GAL4* driver, in spite of its ability to rescue *eya* mutants and its robust expression in our misexpression conditions (verified by anti-Eya). This held true whether *eya* was misexpressed alone or in combination with either *dac*, *ap* or *sqz*, using any of the three *GAL4*-drivers (n=8 VNCs; not shown). *eya* mutant analysis indicated that *eya* was necessary for competence of the Tv neuron to respond to the Gbb ligand. To address whether *eya* is sufficient to confer Gbb-responsiveness on other neurons, we misexpressed *UAS-eya* in combination with *UAS-gbb* and either *dac* or *ap* [$elav^{GAL4}$/*UAS-gbb; UAS-eya* (*UAS-dac* or *UAS–ap*)]. However, we did not observe any ectopic pMad staining or any ectopic Fmrf expression (n=6 VNCs; not shown). Thus, although *eya* is critical for wild-type Fmrf expression and Gbb responsiveness in Tv cells, it is neither sufficient to activate Fmrf nor sufficient to promote pMad phosphorylation in response to Gbb outside *ap*-neurons.

Misexpression of *UAS-dac* alone in peptidergic cells using *c929-GAL4* triggered little or no ectopic Fmrf expression (Fig. 6A). By contrast, *UAS-dac*/*UAS-ap* co-misexpression within peptidergic cells triggered ectopic Fmrf expression, even within the pMad-positive 'non-responsive' peptidergic cells, such as the peptidergic lateral cluster (Plc) cells (Fig. 6B). We found that this ectopic Fmrf expression was dependent upon BMP signaling, because *UAS-dac*/*UAS-ap* co-misexpression in a *wit* mutant background failed to trigger ectopic Fmrf (Fig. 6C). Thus, *dac* and *ap* co-expression is sufficient to trigger Fmrf expression within pMad+ peptidergic cells. We did not observe ectopic Fmrf activation within the pMad-negative population of peptidergic cells, such as the Crz or dAp cells. However, co-misexpression of *UAS-dac*/*UAS-ap* together with ectopic BMP signaling using $UAS\text{-}tkv^{A}$, $UAS\text{-}sax^{A}$ triggered ectopic expression of Fmrf in these normally pMad-negative peptidergic cells: the Crz, Tvb and dAp cells (Fig. 6D). Thus, *dac* can act with *ap* and BMP signaling to trigger ectopic Fmrf expression in the majority of VNC peptidergic neurons.

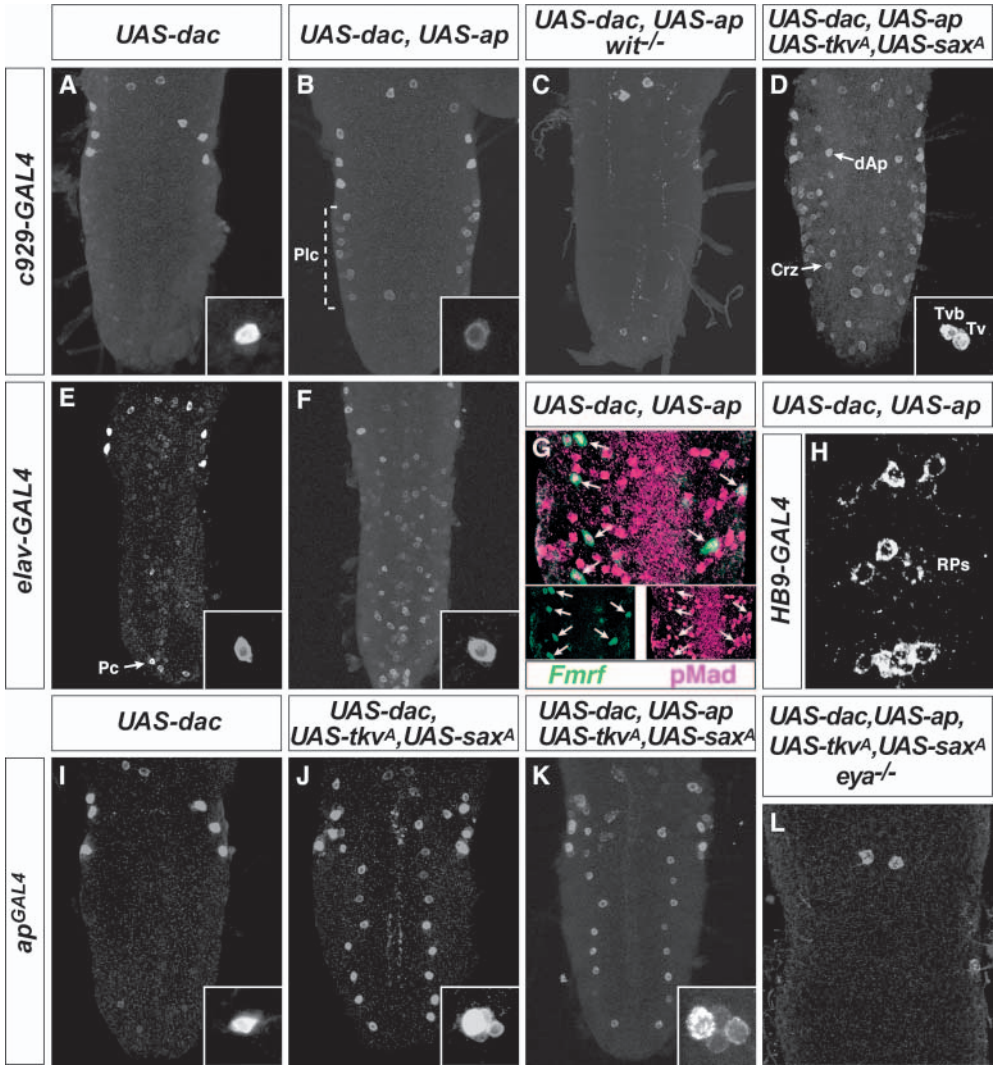

Fig. 6. *dachshund* acts strongly to activate Fmrf expression in combination with *ap* and BMP signaling. Small inset panels show close-ups of Fmrf expression in the *ap*-cluster. (A-D) Misexpression within the peptidergic compartment using *c929-GAL4*. Misexpression of *dac* alone does not trigger ectopic *Fmrf-lacZ* (A), but co-misexpression of both *dac* and *ap* triggers ectopic *Fmrf-lacZ* in the Plc cells (B). Both endogenous and ectopic *Fmrf-lacZ* expression is dependent upon BMP signaling, as only SE2 cells express Fmrf in *wit* mutants (C; *c929-GAL4, Fmrf-lacZ/UAS-ap; wit^{A12}, UAS-dac/wit^{B11}*). Misexpression of *dac* and *ap* together with BMP activation triggers extensive ectopic *Fmrf-lacZ* expression (D; *c929-GAL4, Fmrf-lacZ/UAS-tkv^{A}, UAS-sax^{A}; UAS-ap, UAS-dac/+*). dAp, Crz and Tvb cells (inset) all express Fmrf (D). (E-G) Misexpression within all postmitotic neurons using *elav^{GAL4}*. Misexpression of *dac* alone triggers *Fmrf-lacZ* expression in a small subset of posterior cells (E), but co-misexpression of both *dac* and *ap* triggers extensive ectopic *Fmrf-lacZ* expression (F). Staining for *Fmrf-lacZ* (green) and pMad (magenta) reveals that ectopic *Fmrf-lacZ* cells are all pMad-positive (G). Misexpression of *dac* and *ap* in RP motor neurons using *HB9-GAL4* triggers ectopic proFmrf expression (H). (I-L) Misexpression in *ap*-neurons. Misexpression of *dac* alone does not trigger ectopic *Fmrf-lacZ* (I), but together with BMP activation (J) and *ap* (K), all *ap*-neurons, except the vAp neurons, are triggered to express *Fmrf-lacZ*. (L) Both ectopic and endogenous Fmrf expression is dependent upon *eya* (L; *ap^{GAL4}, eya$^{Cli\text{-}IID}$/eya^{10}, UAS-tkv^{A}, UAS-sax^{A}; UAS-ap, UAS-dac/+*).

Given its potency to trigger Fmrf in peptidergic neurons, we wished to assess the sufficiency of this 'code' to drive Fmrf expression beyond the peptidergic cell population. Pan-neuronal misexpression of *UAS-dac*, using *elav^{GAL4}*, triggered ectopic Fmrf expression that was limited to Pc peptidergic cells (Fig. 6E). By contrast, pan-neuronal co-misexpression of both *UAS-ap* and *UAS-dac* triggered extensive ectopic Fmrf expression (Fig. 6F). Most, if not all, of the neurons that ectopically expressed Fmrf were pMad-positive (Fig. 6G). Thus, *ap/dac* co-misexpression is capable of inducing Fmrf expression in motoneurons. Using *HB9-GAL4*, which is expressed in the majority of motoneurons (Broiher and Skeath, 2002), we found that Fmrf expression could indeed be triggered in defined motoneurons, such as the RP1 and RP4 cells (Fig. 6H). We were unable to test the potency of *dac*/*ap*/BMP in all neurons, due to lethality when activating the BMP pathway ectopically throughout the VNC (Allan et al., 2003).

We next tested the sufficiency of *UAS-dac* to activate Fmrf within *ap*-neurons. As expected, *UAS-dac* alone had no effect in *ap*-neurons (Fig. 6I). As *ap^{GAL4}* is an allele of *ap*, we co-misexpressed *UAS-dac* and *UAS-ap* to test whether a higher level of *ap* expression might work, but again saw no effect (not shown). As the only pMad+ *ap*-neuron is the Tv cell, we activated the BMP pathway ectopically together with *UAS-dac* alone, or together with *UAS-ap*. This led to ectopic expression of Fmrf in the majority of *ap*-neurons, including the four *ap*-cluster cells (Fig. 6J,K). This strong effect of ectopic *dac*/BMP within *ap*-neurons allowed us to address whether *eya* is crucial for this ectopic Fmrf expression in all *ap* neurons, as it is for wild-type Fmrf expression. We misexpressed the same four transgenes in an *eya* mutant background and found that removing *eya* from *ap*-neurons led to loss of both ectopic and endogenous Fmrf expression (Fig. 6L). Since both Dac and pMad expression were clearly observed ectopically in all *ap*-neurons, failure to trigger Fmrf in this case was not due to a failure to drive the transgenes at sufficient levels (not shown). Fmrf expression was also absent from Tv neurons, indicating that the *eya* mutant phenotype cannot be rescued by the addition of other Fmrf regulators. Given these results, we analyzed Eya expression when *UAS-dac* and *UAS-ap* were

misexpressed pan-neuronally from *elav*^{GAL4}. In spite of the extensive ectopic Fmrf expression, Eya expression itself was unaltered from wild type (not shown).

In summary, although *eya* was critical for endogenous Fmrf expression, it was not sufficient to activate Fmrf ectopically in any tested scenario, whether alone or combinatorially. By contrast, *dac* was a potent activator of Fmrf expression, particularly in combination with *ap* in many postmitotic neurons, including motoneurons. *dac*/*ap*-mediated ectopic expression was entirely dependent upon BMP signaling (in all neurons) and also upon *eya* in the neurons that normally express Eya.

Discussion

The retinal determination network in central nervous system development

Phenotypic and transcriptional synergy between So, Dac and Eya during development and in vitro has been well documented (Chen et al., 1997; Ikeda et al., 2002). By contrast, our results indicate that these genes can act independently in the embryonic nervous system to specify neuronal identity. This is the case even when they are coexpressed in the same neuron; while we found no evidence of *so* expression in the *ap*-cluster, *dac* and *eya* functioned together with the previously identified *ap*/*sqz*/BMP combinatorial code to activate *Fmrf* expression in Tv neurons. However, *eya* controlled additional aspects of Tv neuronal identity, such as axon pathfinding and the ability to respond to a BMP signal (Fig. 7). Furthermore, the expression of Dac, but not Eya, So or Ap, in a large number of interneurons suggested that Dac has additional, independent functions in postmitotic neurons.

The molecular mechanisms underlying transcriptional synergy between So (Six), Eya and Dac (Dach) have proven to be quite complex. In most cases examined, So/Six binds DNA and Dac/Dach and Eya regulate its activity (Li et al., 2003; Silver et al., 2003). These biochemical models would not appear to explain our observations fully. In our studies, Dac appeared to act as a potent activator of Fmrf expression but to rely on Eya for activating Fmrf expression only within *ap*-neurons; when *dac* and *ap* were co-misexpressed in all neurons there was widespread ectopic Fmrf expression without any ectopic Eya expression. Why Eya is required in the *ap*-neurons for both endogenous and ectopic Fmrf expression, but not for ectopic Fmrf expression outside *ap*-neurons, is currently unclear.

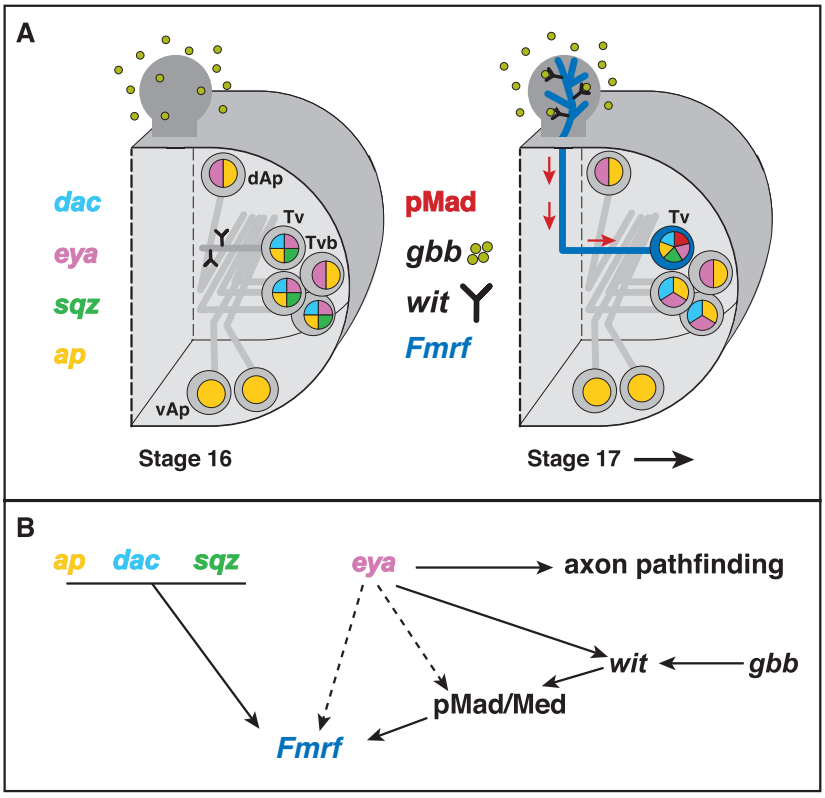

Fig. 7. Summary of Tv cell specification. (A) At stage 16, when *ap*-neurons appear, *dac*, *eya* and *sqz* are expressed in different subsets of *ap*-neurons. At stage 17, BMP activation via the Gbb BMP ligand and the Wit BMP receptor leads to nuclear translocation of pMad and subsequent *Fmrf* activation. (B) In the Tv neuron, our genetic analyses support a differential role for *eya* and *dac*. *eya* plays multiple roles, regulating axon pathfinding, competence to respond to Gbb, and *Fmrf* expression in response to activated BMP signaling. *dac*, by contrast, regulates only *Fmrf*. Although the exact target of *eya* action downstream of pMad is not known, the dashed arrows suggest two possibilities: *eya* may regulate the pMad/Medea (Med) complex and/or the expression of *Fmrf* directly.

Apterous, Eyes Absent and axon pathfinding

Our findings illustrate the fact that regulators acting within a postmitotic neuron can act together in a combinatorial fashion to specify one aspect of neuronal identity (Fmrf expression, in this case). However, some of these regulators can simultaneously function in combinatorial sub-codes to control other aspects of neuronal identity; the additional roles of *ap* and *eya* in Tv axon pathfinding may be one such example. In abdominal hemisegments, Ap is expressed in the two vAp and the single dAp neurons. Normally, the axons of these neurons join a common ipsilateral longitudinal fascicle running the length of the VNC. Previous studies have revealed that *ap* is important for proper *ap*-axon fasciculation as well as for their avoidance of the midline (Lundgren et al., 1995). Eya is not expressed in vAp neurons, and our results indicated that it specifically controls dAp pathfinding. The *eya* mutant phenotype only partially phenocopies the *ap* phenotype, since *eya* affects midline crossing but not fasciculation; once dAp neurons have aberrantly crossed the midline they join the contralateral *ap*-fascicle. Neither the *ap* nor the *eya* mutant phenotypes are due to any apparent crossregulation between these two genes. Surprisingly, our findings indicated that different genetic mechanisms underlie the indistinguishable, *ap*-dependent axon pathfinding of dAp and vAp neurons; dAp axons crucially depend upon *eya* to avoid crossing the midline, whereas vAp axons neither express *eya* nor depend upon it.

An instructive and additive code for *Fmrf* expression

Together with previous findings (Allan et al., 2003; Benveniste et al., 1998; Hewes et al., 2003; Marques et al., 2003) our results indicate that *Fmrf* expression is triggered by the combinatorial action of *ap*, *sqz*, *dimm*, *dac*, *eya* and *BMP* signaling. However, with the exception of *BMP* signaling, none of these factors are absolutely necessary for endogenous *Fmrf* expression – in all mutants, expression of Fmrf is not lost from all Tv neurons. Similarly, although misexpression of a partial code can lead to ectopic *Fmrf* expression, its expression levels are consistently weaker than those seen in Tv neurons. Thus, it appears that a partial code is sufficient for some level of Fmrf expression: the ectopic expression of *Fmrf* in BMP-positive RP neurons – cells that do not express *sqz*, *eya* or *dimm* – in response to *dac* and *ap* is one such example. However, the complete code (*ap*/*sqz*/*dimm*/*dac*/*eya*/*BMP*) appears to be necessary for wild-type (high) levels of expression, as seen in the Tv neurons. It is possible that the simultaneous misexpression of all these factors would lead to robust ectopic *Fmrf* expression in all neurons. Due to obvious technical limitations, we have not been able to test this idea.

Eyes Absent: a pivotal integrator of multiple signal transduction networks?

Multiple signal transduction inputs/outputs appear to revolve around Eya. First, phosphorylation of Eya by the Ras/MAPK pathway has been found to regulate Eya activity and synergy with So (Hsiao et al., 2001; Silver et al., 2003). Second, the transcriptional activity of Eya itself depends upon an intrinsic tyrosine phosphatase activity (Li et al., 2003) that is also required for ectopic eye induction in *Drosophila* (Rayapureddi et al., 2003; Tootle et al., 2003). The target(s) of Eya phosphatase activity are currently unknown. Third, we find that Eya regulates the BMP pathway in Tv neurons and pMad cannot be reactivated in *eya* mutants even by cell-autonomous introduction of the BMP ligand Gbb. A probable explanation for this result is that *eya* regulates the expression or activity of the BMP type receptors Wit, Tkv or Sax. When the BMP pathway is dominantly activated by the use of activated type I receptors, nuclear pMad is restored. However, this still does not reactivate Fmrf expression, indicating that Eya additionally plays important roles downstream of pMad activation. One interpretation of these findings is that Eya acts directly on the *Fmrf* gene. However, it is also tempting to speculate that Eya may act to modulate pMad activity directly. There are several reasons for this proposal. It is known that several other kinase pathways, such as MAPK, can phosphorylate Smad proteins on residues other than those phosphorylated by TGFβ/BMP type I receptors (Derynck and Zhang, 2003). The in-vivo roles of such modifications are unclear, but in-vitro evidence points to both repression and activation of Smad activity (Brown et al., 1999; Engel et al., 1999; Kretzschmar et al., 1999). Nevertheless, given its nuclear localization and phosphatase activity, it is possible that Eya acts to de-phosphorylate inhibitory residues in pMad. A regulatory circuitry between MAPK (and other kinases), Eya and the TGFβ/BMP pathway is an intriguing possibility. Moreover, recent studies reveal that vertebrate orthologs of Dac can physically interact with the Smad complex, thereby affecting TGF-β signaling (Kida et al., 2004; Wu et al., 2003). Together with these previous findings, our results point to a model wherein Eya and Dac play central roles in integrating input from, and controlling the activity of, multiple signal transduction networks. Determination of the precise mechanisms by which Eya and Dac orchestrate these events should enhance our understanding of how both intrinsic and extrinsic signals intersect to affect cellular differentiation.

We thank J.B. Thomas for reading the manuscript critically. We thank J. B. Skeath, H. T. Broiher, P. H. Taghert, G. Mardon, F. Pignoni, N. Bonini, U. Gaul, D. van Meyel, L. Fessler, the Bloomington Stock Center and the Developmental Studies Hybridoma Center for sharing fly lines and antibodies. Confocal imaging was performed at the Harvard Center for Neuro-degeneration and Repair. This work was supported by grants from the Freudenberger Scholarship Fund at Harvard Medical School (S.T.).

References

Allan, D. W., Pierre, S. E., Miguel-Aliaga, I. and Thor, S. (2003). Specification of neuropeptide cell identity by the integration of retrograde BMP signaling and a combinatorial transcription factor code. *Cell* **113**, 73-86.

Altmann, C. R. and Brivanlou, A. H. (2001). Neural patterning in the vertebrate embryo. *Int. Rev. Cytol.* **203**, 447-482.

Benveniste, R. J., Thor, S., Thomas, J. B. and Taghert, P. H. (1998). Cell type-specific regulation of the Drosophila FMRF-NH2 neuropeptide gene by Apterous, a LIM homeodomain transcription factor. *Development* **125**, 4757-4765.

Bonini, N. M., Leiserson, W. M. and Benzer, S. (1998). Multiple roles of the eyes absent gene in Drosophila. *Dev. Biol.* **196**, 42-57.

Briscoe, J. and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. *Curr. Opin. Neurobiol.* **11**, 43-49.

Broihier, H. T. and Skeath, J. B. (2002). Homeodomain protein Extra-extra, the drosophila Hb9 homolog directs neuronal fate via cross-repressive and cell non-autonomous mechanisms. *Neuron* **35**, 39-50.

Brown, J. D., DiChiara, M. R., Anderson, K. R., Gimbrone, M. A., Jr and Topper, J. N. (1999). MEKK-1, a component of the stress (stress-activated protein kinase/c-Jun N-terminal kinase) pathway, can selectively activate Smad2-mediated transcriptional activation in endothelial cells. *J. Biol. Chem.* **274**, 8797-8805.

Bui, Q. T., Zimmerman, J. E., Liu, H. and Bonini, N. M. (2000). Molecular analysis of Drosophila eyes absent mutants reveals features of the conserved Eya domain. *Genetics* **155**, 709-720.

Callahan, C. A., Yoshikawa, S. and Thomas, J. B. (1998). Tracing axons. *Curr. Opin. Neurobiol.* **8**, 582-586.

Caubit, X., Thangarajah, R., Theil, T., Wirth, J., Nothwang, H. G., Ruther, U. and Krauss, S. (1999). Mouse Dac, a novel nuclear factor with homology to Drosophila dachshund shows a dynamic expression in the neural crest, the eye, the neocortex, and the limb bud. *Dev. Dyn.* **214**, 66-80.

Chen, R., Amoui, M., Zhang, Z. and Mardon, G. (1997). Dachshund and eyes absent proteins form a complex and function synergistically to induce ectopic eye development in Drosophila. *Cell* **91**, 893-903.

Cheyette, B. N., Green, P. J., Martin, K., Garren, H., Hartenstein, V. and Zipursky, S. L. (1994). The Drosophila sine oculis locus encodes a homeodomain-containing protein required for the development of the entire visual system. *Neuron* **12**, 977-996.

Chin, A. C., Reynolds, E. R. and Scheller, R. H. (1990). Organization and expression of the Drosophila FMRFamide-related prohormone gene. *DNA Cell Biol.* **9**, 263-271.

Czerny, T., Halder, G., Kloter, U., Souabni, A., Gehring, W. J. and Busslinger, M. (1999). twin of eyeless, a second Pax-6 gene of Drosophila, acts upstream of eyeless in the control of eye development. *Mol. Cell* **3**, 297-307.

Davis, R. J., Shen, W., Heanue, T. A. and Mardon, G. (1999). Mouse Dach, a homologue of Drosophila dachshund, is expressed in the developing retina, brain and limbs. *Dev. Genes Evol.* **209**, 526-536.

Derynck, R. and Zhang, Y. E. (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**, 577-584.

Edlund, T. and Jessell, T. M. (1999). Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. *Cell* **96**, 211-224.

Engel, M. E., McDonnell, M. A., Law, B. K. and Moses, H. L. (1999).

Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. *J. Biol. Chem.* **274**, 37413-37420.

Gorczyca, M. G., Phillis, R. W. and Budnik, V. (1994). The role of tinman, a mesodermal cell fate gene, in axon pathfinding during the development of the transverse nerve in Drosophila. *Development* **120**, 2143-2152.

Halder, G., Callaerts, P. and Gehring, W. J. (1995). Induction of ectopic eyes by targeted expression of the eyeless gene in Drosophila. *Science* **267**, 1788-1792.

Halder, G., Callaerts, P., Flister, S., Walldorf, U., Kloter, U. and Gehring, W. J. (1998). Eyeless initiates the expression of both sine oculis and eyes absent during Drosophila compound eye development. *Development* **125**, 2181-2191.

Heanue, T. A., Reshef, R., Davis, R. J., Mardon, G., Oliver, G., Tomarev, S., Lassar, A. B. and Tabin, C. J. (1999). Synergistic regulation of vertebrate muscle development by Dach2, Eya2, and Six1, homologs of genes required for Drosophila eye formation. *Genes Dev.* **13**, 3231-3243.

Hewes, R. S., Park, D., Gauthier, S. A., Schafer, A. M. and Taghert, P. H. (2003). The bHLH protein Dimmed controls neuroendocrine cell differentiation in Drosophila. *Development* **130**, 1771-1781.

Hsiao, F. C., Williams, A., Davies, E. L. and Rebay, I. (2001). Eyes absent mediates cross-talk between retinal determination genes and the receptor tyrosine kinase signaling pathway. *Dev. Cell* **1**, 51-61.

Ikeda, K., Watanabe, Y., Ohto, H. and Kawakami, K. (2002). Molecular interaction and synergistic activation of a promoter by Six, Eya, and Dach proteins mediated through CREB binding protein. *Mol. Cell. Biol.* **22**, 6759-6766.

Kammermeier, L., Leemans, R., Hirth, F., Flister, S., Wenger, U., Walldorf, U., Gehring, W. J. and Reichert, H. (2001). Differential expression and function of the Drosophila Pax6 genes eyeless and twin of eyeless in embryonic central nervous system development. *Mech. Dev.* **103**, 71-78.

Kawakami, K., Sato, S., Ozaki, H. and Ikeda, K. (2000). Six family genes – structure and function as transcription factors and their roles in development. *BioEssays* **22**, 616-626.

Kida, Y., Maeda, Y., Shiraishi, T., Suzuki, T. and Ogura, T. (2004). Chick Dach1 interacts with the Smad complex and Sin3a to control AER formation and limb development along the proximodistal axis. *Development* **131**, 4179-4187.

Kretzschmar, M., Doody, J., Timokhina, I. and Massague, J. (1999). A mechanism of repression of TGFbeta/Smad signaling by oncogenic Ras. *Genes Dev.* **13**, 804-816.

Kumar, J. P. and Moses, K. (2001). Expression of evolutionarily conserved eye specification genes during Drosophila embryogenesis. *Dev. Genes Evol.* **211**, 406-414.

Kurusu, M., Nagao, T., Walldorf, U., Flister, S., Gehring, W. J. and Furukubo-Tokunaga, K. (2000). Genetic control of development of the mushroom bodies, the associative learning centers in the Drosophila brain, by the eyeless, twin of eyeless, and Dachshund genes. *Proc. Natl. Acad. Sci. USA* **97**, 2140-2144.

Li, X., Oghi, K. A., Zhang, J., Krones, A., Bush, K. T., Glass, C. K., Nigam, S. K., Aggarwal, A. K., Maas, R., Rose, D. W. et al. (2003). Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. *Nature* **426**, 247-254.

Lin, D. M. and Goodman, C. S. (1994). Ectopic and increased expression of Fasciclin II alters motorneuron growth cone guidance. *Neuron* **13**, 507-523.

Lundgren, S. E., Callahan, C. A., Thor, S. and Thomas, J. B. (1995). Control of neuronal pathway selection by the *Drosophila* LIM homeodomain gene *apterous*. *Development* **121**, 1769-1773.

Luo, L., Liao, Y. J., Jan, L. Y. and Jan, Y. N. (1994). Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. *Genes Dev.* **8**, 1787-1802.

Mardon, G., Solomon, N. M. and Rubin, G. M. (1994). dachshund encodes a nuclear protein required for normal eye and leg development in Drosophila. *Development* **120**, 3473-3486.

Marques, G., Haerry, T. E., Crotty, M. L., Xue, M., Zhang, B. and O'Connor, M. B. (2003). Retrograde Gbb signaling through the Bmp type 2 receptor wishful thinking regulates systemic FMRFa expression in Drosophila. *Development* **130**, 5457-5470.

Martini, S. R., Roman, G., Meuser, S., Mardon, G. and Davis, R. L. (2000). The retinal determination gene, dachshund, is required for mushroom body cell differentiation. *Development* **127**, 2663-2672.

Miguel-Aliaga, I. and Thor, S. (2004). Segment-specific prevention of pioneer neuron apoptosis by cell-autonomous, postmitotic Hox gene activity. *Development* (in press).

Nassel, D. R. (1996). Neuropeptides, amines and amino acids in an elementary insect ganglion: functional and chemical anatomy of the unfused abdominal ganglion. *Prog. Neurobiol.* **48**, 325-420.

Nassel, D. R., Ohlsson, L. G. and Cantera, R. (1988). Metamorphosis of identified neurons innervating thoracic neurohemal organs in the blowfly: transformation of cholecystokininlike immunoreactive neurons. *J. Comp. Neurol.* **267**, 343-356.

Niimi, T., Seimiya, M., Kloter, U., Flister, S. and Gehring, W. J. (1999). Direct regulatory interaction of the eyeless protein with an eye-specific enhancer in the sine oculis gene during eye induction in Drosophila. *Development* **126**, 2253-2260.

Noveen, A., Daniel, A. and Hartenstein, V. (2000). Early development of the Drosophila mushroom body: the roles of eyeless and dachshund. *Development* **127**, 3475-3488.

Ohto, H., Kamada, S., Tago, K., Tominaga, S. I., Ozaki, H., Sato, S. and Kawakami, K. (1999). Cooperation of six and eya in activation of their target genes through nuclear translocation of Eya. *Mol. Cell. Biol.* **19**, 6815-6824.

Olson, P. F., Fessler, L. I., Nelson, R. E., Sterne, R. E., Campbell, A. G. and Fessler, J. H. (1990). Glutactin, a novel Drosophila basement membrane-related glycoprotein with sequence similarity to serine esterases. *EMBO J.* **9**, 1219-1227.

Pignoni, F., Hu, B., Zavitz, K. H., Xiao, J., Garrity, P. A. and Zipursky, S. L. (1997). The eye-specification proteins So and Eya form a complex and regulate multiple steps in Drosophila eye development. *Cell* **91**, 881-891.

Rayapureddi, J. P., Kattamuri, C., Steinmetz, B. D., Frankfort, B. J., Ostrin, E. J., Mardon, G. and Hegde, R. S. (2003). Eyes absent represents a class of protein tyrosine phosphatases. *Nature* **426**, 295-298.

Schmid, A., Chiba, A. and Doe, C. Q. (1999). Clonal analysis of Drosophila embryonic neuroblasts: neural cell types, axon projections and muscle targets. *Development* **126**, 4653-4689.

Schneider, L. E., Roberts, M. S. and Taghert, P. H. (1993a). Cell type-specific transcriptional regulation of the Drosophila FMRFamide neuropeptide gene. *Neuron* **10**, 279-291.

Schneider, L. E., Sun, E. T., Garland, D. J. and Taghert, P. H. (1993b). An immunocytochemical study of the FMRFamide neuropeptide gene products in Drosophila. *J. Comp. Neurol.* **337**, 446-460.

Sepp, K. J., Schulte, J. and Auld, V. J. (2001). Peripheral glia direct axon guidance across the CNS/PNS transition zone. *Dev. Biol.* **238**, 47-63.

Shen, W. and Mardon, G. (1997). Ectopic eye development in Drosophila induced by directed dachshund expression. *Development* **124**, 45-52.

Shirasaki, R. and Pfaff, S. L. (2002). Transcriptional codes and the control of neuronal identity. *Annu. Rev. Neurosci.* **25**, 251-281.

Silver, S. J., Davies, E. L., Doyon, L. and Rebay, I. (2003). Functional dissection of eyes absent reveals new modes of regulation within the retinal determination gene network. *Mol. Cell. Biol.* **23**, 5989-5999.

Skeath, J. B. and Thor, S. (2003). Genetic control of Drosophila nerve cord development. *Curr. Opin. Neurobiol.* **13**, 8-15.

Spradling, A. C., Stern, D., Beaton, A., Rhem, E. J., Laverty, T., Mozden, N., Misra, S. and Rubin, G. M. (1999). The berkeley drosophila genome project gene disruption project. Single P-element insertions mutating 25% of vital drosophila genes. *Genetics* **153**, 135-177.

Taghert, P. H. (1999). FMRFamide neuropeptides and neuropeptide-associated enzymes in Drosophila. *Microsc. Res. Tech.* **45**, 80-95.

Tanimoto, H., Itoh, S., ten Dijke, P. and Tabata, T. (2000). Hedgehog creates a gradient of DPP activity in Drosophila wing imaginal discs. *Mol. Cell* **5**, 59-71.

Tootle, T. L., Silver, S. J., Davies, E. L., Newman, V., Latek, R. R., Mills, I. A., Selengut, J. D., Parlikar, B. E. and Rebay, I. (2003). The transcription factor Eyes absent is a protein tyrosine phosphatase. *Nature* **426**, 299-302.

Wu, K., Yang, Y., Wang, C., Davoli, M. A., D'Amico, M., Li, A., Cveklova, K., Kozmik, Z., Lisanti, M. P., Russell, R. G. et al. (2003). DACH1 inhibits transforming growth factor-beta signaling through binding Smad4. *J. Biol. Chem.* **278**, 51673-51684.

Xu, P. X., Cheng, J., Epstein, J. A. and Maas, R. L. (1997). Mouse Eya genes are expressed during limb tendon development and encode a transcriptional activation function. *Proc. Natl. Acad. Sci. USA* **94**, 11974-11979.

Xu, P. X., Adams, J., Peters, H., Brown, M. C., Heaney, S. and Maas, R. (1999). Eya1-deficient mice lack ears and kidneys and show abnormal apoptosis of organ primordia. *Nat. Genet.* **23**, 113-117.