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

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Summary

In the *Drosophila* nerve cord, a subset of neurons expresses the neuropeptide FMRFamid 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, FMRFamid, 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 *dachshund* (*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 (Ikedà 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.

Materials and methods
Fly stocks
The following strains were used in this study: dacΔ, dacΔc, dacΔp (also known as dacK56Δ) (Mardon et al., 1994), dacΔΔα2 (referred to as dacΔΔα2) (Heanue et al., 1999), UAS-dac (Shen and Mardon, 1997), soΔ (Cheyette et al., 1994), eyaGΔ-β (Pignoni et al., 1997), UAS-eya.B.II (expressing embryonic eya transcript; referred to as UAS-eya), Df(2L)eyaΔ10 (referred to as eyaΔ10) (Bonini et al., 1998), apP4, apK568 (referred to as apK568), apΔα2 (referred to as apΔα2), UAS-ap, apaΔ12, apaΔ11, UAS-ievΔ, UAS-ievΔ (Allan et al., 2003), Fmrf-lacZWF3-T2 (Schneider et al., 1993a), elav-GAL4 (Luo et al., 1994), elavGAL4 (Lin and Goodman, 1994), UAS-nls-EGFP (Callahan et al., 1998), apC-tau-lacZ#2.1 (Lundgren et al., 1995), eyaB11 (Hewes et al., 2001), CyO,Act-GFP or TM3,Ser,Act-GFP (Broicher and Skeath, 2002), repoGAL4 (Sepp et al., 2001). Mutants were kept over CyO,Act-GFP or TM3,SerAct-GFP balancer chromosomes, w1118 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., 1999), rabbit α-β-gal (1:5000, ICN-Cappel), rabbit α-pMad (1:2000) (Tamimoto et al., 2000), rabbit α-Glutamyltyrosine (1:300) (Olson et al., 1990), rabbit α-GFP (1:500, Molecular Probes). Immunolabeling was carried out as previously described (Allan et al., 2003).

![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), which is revealed by the expression of Fmrf in a subset of ap neurons.](image)
Analysis of enhancer trap lines

Expression analysis of the 577 second-chromosomal 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′.

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 peptidergic 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 coronazonin 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 Ple (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 FmrflacZ and apGAL4 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 FmrflacZ (to distinguish the Tv cell) (Fig. 3G). We found that Dac was absent from the Tvb and dAp cells (c929-GAL4-positive, FmrflacZ-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′ (a lacZ insertion in dac) or dacGAL4, 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).
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 (Plc) 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 Tv 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\textsuperscript{lacZ} (so′) and eya (anti-Eya). As previously described, there is an early phase of both so\textsuperscript{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\textsuperscript{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\textsuperscript{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 Tv, dAp 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.

**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 c929-GAL4/UAS-nls-EGFP expression. (B,C) pMad immunoreactivity in c929-GAL4+ neurons reveals that some peptidergic neurons, 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’.

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\textsuperscript{GAL4}, we observed a clear upregulation of FmrF expression above wild-type levels (P<0.0001 compared with...
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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 apGAL4 as a marker for ap-neurons and, although apGAL4 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 apGAL4, 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 (dacGAL4/UAS-myc-EGFP) 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 (repoGAL4/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 (apGAL4/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 (apGAL4/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 FmrflacZ co-labeling discriminates the Tv cell (c929-GAL4+, FmrflacZ+) and the Tvb and the dAp cells (c929-GAL4+, FmrflacZ-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 apGAL4; UAS-myc-EGFPF (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’) In controls (apGAL4/+; UAS-myc-EGFPF/+) 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,E,F) In dac mutants (dac3/dac4), the expression of proFmrf is weak and partly lost in Tv cells (B). However, in dac mutants (apGAL4, dac4/dac3; UAS-myc-EGFPF/+) there is entirely wild-type midline and DNH innervation (F,F’), and pMad staining of Tv cells (J). (C,G,G’) In eya mutants (eya10; eya10), 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 (apGAL4, eya10; eya10; UAS-myc-EGFPF/+) 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,L) Cell-autonomous reintroduction of eya (apGAL4, eya10; UAS-myc-EGFPF/+) rescues proFmrf (D), DNH innervation (H) and pMad expression (L). (M,R) Direct activation of the BMP pathway in eya mutants (apGAL4, eya10; UAS-tkvA, UAS-saxA; UAS-myc-EGFPF/+) 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 (apGAL4, eya10; UAS-gbb; UAS-myc-EGFPF/+) fails to restore either proFmrf (N) or pMad (S). (O,Q) Expression of t-lacZ reveals abdominal ap-axon projections in the stage 16-17 embryo. In the control (apC-t-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 (eya10; eya10; apC-t-lacZ), the dAp axons frequently (96%) cross the midline (P, arrow). 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 (dac3/dac4) and dac rescue (apGAL4, dac3/dac4; 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 (apGAL4/+) background.
Combinatorial regulation of neuropeptide expression

Fig. 5. Expression of aperturon, 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 Tv cell (see Fig. 3E,G). In ap mutants (ap^{Gal4/}; ap^{Pd4:UAS-nsl-myc-EGFP{+/-}}, Dac is often de-repressed in the Tv cell (B). In eya mutants (ap^{Gal4/eya^{ClIId};eya^{10T}:UAS-myc-EGFP{+/-}}, 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^{1L2};UAS-myc-EGFP{+/-}}, Dac expression is normal (E). In sqz mutants (ap^{Gal4/;sqz^{0P};UAS-myc-EGFP{+/-}}, 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^{5};UAS-myc-EGFP{+/-}}) and eya (J; ap^{Gal4/eya^{ClIId};eya^{10T}:UAS-myc-EGFP{+/-}}) mutants, respectively.

nearly complete failure of DNH innervation: 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. 4L). To analyze axon pathfinding in eya mutants without altering ap gene dosage, we used the ap enhancer construct ap^{C-\tau-lacZ} (Lundgren et al., 1995) instead of ap^{Gal4}. Unfortunately, unlike the membrane-targeted UAS-myc-EGFP, the \tau-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 \tau-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 apGAL4 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 contrast with the abilities 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 path-finding 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 apGAL4 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 apGAL4 activity (Fig. 5D; note pMad staining in cell of highest apGAL4 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 contextually, either alone, in combination with one another, or together with the previously identified Fmrf regulators. This was tested in peptidergic cells (c929-GALA), in ap-neurons (apGAL4) and in all postmitotic neurons (elavGAL4).

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 [elavGAL4/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-tkvΔ4. UAS-sax4 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.
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 elavGAL4, triggered ectopic Fmrf-lacZ expression 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; witB11, UAS-dac/witB11). Misexpression of dac and ap together with BMP activation triggers extensive ectopic Fmrf-lacZ expression (D; c929-GAL4, Fmrf-lacZ/UAS-tkvA, UAS-saxA; UAS-ap, UAS-dac/+). dAp, Crz and Tvb cells (inset) all express Fmrf (D). (E-G) Misexpression within all postmitotic neurons using elavGAL4. 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; apGAL4, eyaCLI-IID/eya10, UAS-tkvA, UAS-saxA; UAS-ap, UAS-dac/+).

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 apGAL4 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). 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 \textit{elav\textsuperscript{GAL4}}. In spite of the extensive ectopic Fmrf expression, Eya expression itself was unaltered from wild type (not shown).

In summary, although \textit{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, \textit{dac} was a potent activator of Fmrf expression, particularly in combination with \textit{ap} in many postmitotic neurons, including motoneurons. \textit{dacap}-mediated ectopic expression was entirely dependent upon BMP signaling (in all neurons) and also upon \textit{eya} in the neurons that normally express Eya.

**Discussion**

The retinal determination network in central nervous system development

Phenotypic and transcriptional synergy between \textit{So}, \textit{Dac} and \textit{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 \textit{so} expression in the \textit{ap}-cluster, \textit{dac} and \textit{eya} functioned together with the previously identified \textit{ap/sqz}/BMP combinatorial code to activate \textit{Fmrf} expression in \textit{Tv} neurons. However, \textit{eya} controlled additional aspects of \textit{Tv} neuronal identity, such as axon pathfinding and the ability to respond to a BMP signal (Fig. 7). Furthermore, the expression of \textit{Dac}, but not \textit{Eya}, \textit{So} or \textit{Ap}, in a large number of interneurons suggested that \textit{Dac} has additional, independent functions in postmitotic neurons.

The molecular mechanisms underlying transcriptional synergy between \textit{So} (Six), \textit{Eya} and \textit{Dac} (Dach) have proven to be quite complex. In most cases examined, \textit{So/Six} binds DNA and \textit{Dac/Dach} and \textit{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, \textit{Dac} appeared to act as a potent activator of Fmrf expression but to rely on \textit{Eya} for activating Fmrf expression only within \textit{ap}-neurons; when \textit{dac} and \textit{ap} were co-misexpressed in all neurons there was widespread ectopic Fmrf expression without any ectopic \textit{Eya} expression. Why \textit{Eya} is required in the \textit{ap}-neurons for both endogenous and ectopic Fmrf expression, but not for ectopic Fmrf expression outside \textit{ap}-neurons, is currently unclear.

\textbf{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 \textit{ap} and \textit{eya} in \textit{Tv} axon pathfinding may be one such example. In abdominal hemisegments, \textit{Ap} is expressed in the two \textit{vAp} and the single \textit{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 \textit{ap} is important for proper \textit{ap}-axon fasciculation as well as for their avoidance of the midline (Lundgren et al., 1995). \textit{Eya} is not expressed in \textit{vAp} neurons, and our results indicated that it specifically controls \textit{dAp} pathfinding. The \textit{eya} mutant phenotype only partially phenocopies the \textit{ap} phenotype, since \textit{eya} affects midline crossing but not fasciculation; once \textit{dAp} neurons have aberrantly crossed the midline they join the contralateral \textit{ap}-fascicle. Neither the \textit{ap} nor the \textit{eya} mutant phenotypes are due to any apparent crossregulation between these two genes. Surprisingly, our findings indicated that different genetic mechanisms underlie the indistinguishable, \textit{ap}-dependent axon pathfinding of \textit{dAp} and \textit{vAp} neurons; \textit{dAp} axons crucially depend upon \textit{eya} to avoid crossing the midline, whereas \textit{vAp} axons neither express \textit{eya} nor depend upon it.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{Summary of \textit{Tv} cell specification. (A) At stage 16, when \textit{ap}-neurons appear, \textit{dac}, \textit{eya} and \textit{sqz} are expressed in different subsets of \textit{ap}-neurons. At stage 17, BMP activation via the \textit{Gbb} BMP ligand and the \textit{Wit} BMP receptor leads to nuclear translocation of pMad and subsequent \textit{Fmrf} activation. (B) In the \textit{Tv} neuron, our genetic analyses support a differential role for \textit{eya} and \textit{dac}. \textit{eya} plays multiple roles, regulating axon pathfinding, competence to respond to \textit{Gbb}, and \textit{Fmrf} expression in response to activated BMP signaling. \textit{dac}, by contrast, regulates only \textit{Fmrf}. Although the exact target of \textit{eya} action downstream of pMad is not known, the dashed arrows suggest two possibilities: \textit{eya} may regulate the pMad/Medea (Med) complex and/or the expression of \textit{Fmrf} directly.}
\end{figure}
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/eyal/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.

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### References


Interdependent SMAD and JNK signaling in transforming growth factor-
Gorczyca, M. G., Phillips, R. W. and Budnik, V. (1994). The role of timm-in,
mesodermal cell fate gene, in axon pathfinding during the development of
the transverse nerve in Drosophila. Development 120, 2143-2152.
Acad. Sci. USA 92, 4744-4748.
Eyes absent, regulates the initiation and development of the Drosophila
He, Q., Li, M., Wang, J., Zhang, Y. and Yue, J. (2003). The role of
dysfunction in the dachshund gene in regulating the differentiation of
drosophila. J. Biol. Chem. 278, 51573-51580.
Hengst, R., Park, D., Gauthier, S. A., Schafer, A. M. and Taghert, P. H.
(2000). The bHLH protein Dimmed controls neuroendocrine cell
(2000). The bHLH protein Dimmed controls neuroendocrine cell
Hewes, R. S., Park, D., Gauthier, S. A., Schafer, A. M. and Taghert, P. H.
(2000). The bHLH protein Dimmed controls neuroendocrine cell
bHLH protein Dimmed controls neuroendocrine cell differentiation in
Hsiao, R. C., Fessler, L. I., Nelson, R. E., Sterne, R. E., Campbell, A. G.
membrane-related glycoprotein with sequence similarity to serine esterases.
J. Cell Sci. 95, 113-117.
Pignoni, F., Hu, B., Zavitz, K. H., Xiao, J., Garrity, P. A. and Zipursky,
S. L. (1997). The eye-specific proteins So and Eya form a complex
and regulate multiple steps in Drosophila eye development. Cell 91, 881-
891.
Rayapaddi, J. P., Kattamuri, C., Steinmetz, B. D., Frankfort, B. J.,
Ostrin, E. J., Mardon, G. and Hegde, R. S. (2003). Eyes absent represents
embryonic neuroblasts: neural cell types, axon projections and muscle
targets. Development 126, 4653-4669.
Schneider, L. E., Roberts, M. S. and Taghert, P. H. (1993a). Cell type-
specific transcriptional regulation of the Drosophila FMRFamide
immunocytochemical study of the FMRFamide neuropeptide gene products
Sepp, K. J., Schulte, J. and Auld, V. J. (2001). Peripheral glia directly
direct axon guidance across the CNS/PNS transition zone. Dev. Biol.
238, 47-63.
induced by directed dachshund expression. Development 124, 45-52.
dissection of eyes absent reveals new modes of regulation within the retinal
Spradling, A. C., Stern, D., Benton, A., Rhem, E. J., Laverty, T., Mozden,
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-
5, 59-71.
Tooth, E. L., Silver, S. J., Davies, E. L., Newman, V., Latek, R. R., Mills,
transcription factor Eyes absent is a protein tyrosine phosphatase. Nature
242, 299-302.
Wu, K., Yang, Y., Wang, C., Davoli, M. A., D’Amico, M., Li, A., Cveklova,
inhibits transforming growth factor-beta signaling through binding Smad4.
J. Biol. Chem. 278, 51673-51684.
genes are expressed during limb tendon development and encode a
transcriptional activation function. Proc. Natl. Acad. Sci. USA 94, 11979-
11979.
(1999). Eys-deficient mice lack ears and kidneys and show abnormal