Role of Homothorax in region specific regulation of Deformed in embryonic neuroblasts

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\textbf{A B S T R A C T}

The expression and regulation of Hox genes in developing central nervous system (CNS) lack important details like specific cell types where Hox genes are expressed and the transcriptional regulatory players involved in these cells. In this study we have investigated the expression and regulation of Drosophila Hox gene Deformed (Dfd) in specific cell types of embryonic CNS. Using Dfd neural autoregulatory enhancer we find that Dfd autoregulates itself in cells of mandibular neuromere. We have also investigated the role of a Hox cofactor Homothorax (Hth) for its role in regulating Dfd expression in CNS. We find that Hth exhibits a region specific role in controlling the expression of Dfd, but has no direct role in mandibular Dfd neural autoregulatory circuit. Our results also suggest that homeodomain of Hth is not required for regulating Dfd expression in embryonic CNS.

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\section{1. Introduction}

Hox genes are a highly conserved family of homeodomain containing transcription factors which are well known for their role in specification of the anterior–posterior axis (Pearson et al., 2005). A parallel role for Hox genes in central nervous system (CNS) patterning and development is well known yet not completely understood. Therefore, mechanisms underlying their expression and regulation in CNS need to be investigated. Our understanding of the Hox neural regulation and its functional significance needs further investigation for details like specific cell types where Hox genes are expressed, how they are regulated, their functional significance in those cells, and cell type specific molecular players involved therein.

The phenomenon of autoregulation of Hox genes has been suggested as an important mechanism for their sustained expression during development. To this end both neural and non-neural autoregulatory loops have been identified and investigated in Drosophila and vertebrates (Bergson and McGinnis, 1990; Haerry and Gehring, 1996; Kuziora and McGinnis, 1988; Lou et al., 1995; Manzanares et al., 2001; Marty et al., 2001; Muller et al., 1989; Packer et al., 1998; Popperl et al., 1995; Tremml and Bienz, 1992; Yau et al., 2002; Zappavigna et al., 1991).

In Drosophila, Deformed (Dfd), labial (lab) and Ultrabithorax (Ubx) are known to autoregulate their expression during development (Bergson and McGinnis, 1990; Kuziora and McGinnis, 1988; Lou et al., 1995; Marty et al., 2001; Muller et al., 1989; Popperl et al., 1995; Tremml and Bienz, 1992). Amongst these three, Dfd is known to maintain its expression in both embryonic epidermis and CNS through an autoregulatory transcriptional loop. This autoregulation eventually contributes to the development of maxillary and mandibular segments of the body (Bergson and McGinnis, 1990; Kuziora and McGinnis, 1988; Lou et al., 1995; Muller et al., 1989; Pinsonneault et al., 1997; Popperl et al., 1995; Tremml and Bienz, 1992; Zeng et al., 1994). Hox cofactors Extradenticle (Exd) and Homothorax (Hth) have been shown to play a direct role in maintaining the epidermal autoregulation of Dfd in these segments (Bergson and McGinnis, 1990; Joshi et al., 2010; Pinsonneault et al., 1997), but whether they play a similar role in neural autoregulation (Lou et al., 1995) has not been investigated in detail. A role of vertebrate Pbx (Exd homolog) and Meis (Hth homolog)
has been shown in Hox neural autoregulation in vertebrate CNS (Manzanares et al., 2001; Popperl et al., 1995), but cell type specific roles of both Pbx and Meis in neural autoregulation have not been studied.

In case of Dfd mutants, Dfd gene transcription is initiated normally prior to embryonic stage 10. In subsequent stages, Dfd mutants are unable to maintain normal Dfd expression in both epidermis and CNS, suggesting a role for autoregulation in maintenance of Dfd transcription. Previous studies have identified a 3.2 kb intronic enhancer of Dfd responsible for its autoregulation in CNS. This enhancer is referred to as neural autoregulatory enhancer (NAE). A 608 bp fragment of this 3.2 kb successfully recapitulates Dfd neural autoregulation. The expression from this enhancer is first detected at stage 11 (~5 h after egg laying) in mandibular region of CNS (Lou et al., 1995). The activity of NAE is completely abrogated in Dfd mutants, thereby making it a good readout for Dfd neural autoregulation (Lou et al., 1995; Pinsonneault et al., 1997). The identity of the cells where NAE is expressed in CNS and the functional significance of this autoregulation has not been established. While Dfd protein is expressed in both maxillary (Mx) and mandibular (Mn) regions of embryonic CNS, the neural autoregulation has been suggested to be a characteristic of Mn neuronomere only. It is known that in Dfd mutants there is a loss of expression of Dfd in Mn region but a basal level of Dfd transcription is still maintained in Mx segments. This suggests that Dfd expression in Mx region is independent of neural autoregulation (Zeng et al., 1994). Similarly, in maternal and zygotic mutants of Dfd, the expression is still maintained in Mx segments. This suggests that the expression of Dfd in Mn region but a basal level of Dfd transcription is still maintained in Mx segments. This suggests that Dfd expression in Mx region is independent of neural autoregulation (Zeng et al., 1994). Similarly, in maternal and zygotic mutants of Dfd (edf mutant), Dfd levels and NAE3.2-lacZ expression in CNS are lowered but qualitative expression of both are essentially unaffected in Mn neuronomere (Pinsonneault et al., 1997), thereby suggesting that Edx doesn’t play a role in neural autoregulation. A similar role for cofactor Hth has not been checked in embryonic CNS.

In this work, we have investigated the expression and regulation of Dfd in specific cell types of embryonic CNS. Our results show that Dfd is expressed in neural stem cells (also called neuroblasts-Nbs), neurons and in glial cells of both Mx and Mn neuronomeres. We further report the expression of 3.2 kb NAE in all these three cell types of Mn neuronomere, thereby suggesting that Dfd autoregulates itself in Mn cells. We have also addressed the role Hth in Dfd regulation in embryonic CNS. We find that Hth is critically required for Dfd expression in Nbs of Mx neuronomere, while its role in Mn neuronomere is limited only in regulating the expression of Dfd in these cells, and has no function in neural autoregulatory circuit. Our experiments further suggest that homeodomain of Hth is not necessary for Dfd regulation, and HD-less form of Hth is sufficient for Dfd regulation in embryonic Nbs.

2. Results

The current work focuses on identifying specific cell types of CNS where Dfd is expressed and autoregulated; and to understand the role of Hth in regulating Dfd expression in embryonic Nbs.

2.1. Region specific expression analysis of 3.2 kb NAE in embryonic CNS

Dfd autoregulation in embryonic CNS is mediated through a 3.2 kb neural autoregulatory enhancer (NAE3.2) (Lou et al., 1995; Pinsonneault et al., 1997; Zeng et al., 1994). This enhancer primarily expresses in Mn neuronomere and loses its CNS specific expression in Dfd mutants (Lou et al., 1995; Pinsonneault et al., 1997; Zeng et al., 1994), making NAE3.2-lacZ a bona fide readout for neural autoregulation.

We started out by looking at the expression of NAE3.2-lacZ line reported earlier (obtained from McGinnis lab-UCSD, referred to as NAE3.2-lacZ-2P in the text and figures) (Lou et al., 1995; Pinsonneault et al., 1997). A co-staining of β-galactosidase (LacZ), Dfd and Dpn (Nb marker) on embryos of NAE3.2-lacZ-2P showed the expression of LacZ outside Dfd specific region of CNS (Fig. 1A–A’, pink arrow heads, Z-project of multiple slices is shown). Since earlier results had suggested that Dfd maintains its expression through neural autoregulation primarily in Mn neuronomere, we generated and analyzed additional reporter lines and compared their expression to NAE3.2-lacZ-P. These lines were generated by site specific insertion (Bischof et al., 2007) of NAE3.2-lacZ constructs at attP2-68A and attP40-25C6 (and will here on be referred to as NAE3.2-lacZ-68A and 25C).

In case of both lines (NAE3.2-lacZ-68A and 25C), we observed that LacZ reporter expression was confined primarily to Dfd region (Fig. 1B–B’ and C–C’), and very little background was observed outside Dfd region (Z-project of multiple slices is shown in Fig. 1; for complete Z project of Fig. 1B, see Supp. Fig. 1E). It was also observed that the expression of NAE3.2-lacZ-68A and 25C was mainly confined to Mn neuronomere of CNS and there were only very few cells of Mx neuronomere (outside of CNS) which showed LacZ expression compared to NAE3.2-lacZ-P (Fig. 1B–B’ and C–C’). In order to further clarify the region specific expression of NAE3.2-lacZ reporter, a costaining for Dfd, Engrailed and LacZ antibodies was done for both NAE3.2-lacZ-P and NAE3.2-lacZ-68A. It was observed that while the expression of NAE3.2-lacZ-P extended into Mx neuronomere and cells outside Dfd region (Fig. 1D–D’), LacZ expression in NAE3.2-lacZ-68A was confined to Mn neuronomere of the embryo (Fig. 1E–E’).

These results suggest that NAE3.2-lacZ reporter is primarily confined to Mn neuronomere (in both 68A and 25C lines) and thus lines could be used as an accurate readout for neural autoregulation in this region. It further suggests that the expression of NAE3.2-lacZ-P in regions other than Mn neuronomere may not have any functional significance. Thus all subsequent experiments were done with NAE3.2-lacZ-68A reporter line.

2.2. Dfd is expressed and autoregulated in mandibular Nbs, neurons and glial cells

In order to identify the specific cell types where Dfd is expressed and autoregulated in embryonic CNS, a costaining for Dfd protein was done with LacZ, and Nb (Dpn), neuron (Elav) and glial cell (Repo) specific markers. We found that Dfd protein was expressed in all the Nbs (yellow and white arrowhead in Fig. 2A–A’, see Supp. Fig. 1B for two channel merges) and most of the neurons (yellow and white arrowhead in Fig. 2B–B’)) of Mn and Mx neuronomeres of CNS at stage 12 of embryogenesis. We observed that there were few glial cells present at stage 12 of embryogenesis (yellow and white arrowhead in Fig. 2C–C’) and all of them express Dfd at a very low level. More glial cells were observed in later stages of embryonic development (stages 13 and 14) and Dfd expression was found to be more robust in the later stages (stage 13 embryos shown in Supp. Fig. 2D).

To further test if the expression of Dfd in Nbs, neurons and glial cells in Mn neuronomere was maintained through autoregulation, we looked at the expression of NAE3.2-lacZ and Dfd in these cells. Our results showed that LacZ coexpressed with Dfd in Nbs (yellow arrowheads, Fig. 2A–A’); neurons (yellow arrowheads Fig. 2B–B’); and glial cells (yellow arrowheads Fig. 2C–C’). Glial cells showed a very weak expression of LacZ and Dfd (yellow arrowheads Fig. 2C–C’) at stage 12, but the expression of Dfd and LacZ became stronger in subsequent embryonic stages (Supp. Fig. 2D).

These results indicate that Dfd is expressed in all the three cell types of CNS (Nbs neurons and glial cells) in both Mx and Mn neuronomeres. The coexpression of NAE3.2-lacZ in Mn cells further suggests that Dfd expression in these cells is autoregulated, while in Mx cells Dfd is expressed but not autoregulated. Since Nbs are neural progenitor cells (that give rise to all the cells of CNS including neurons and glial cells), we decided to restrict our subsequent analysis to Nbs only.

2.3. Hth shows a region specific effect on Dfd expression in Nbs

Hox genes function with cofactors like Edx and Hth (Pearson et al., 2005), which have been shown to play an important role in non-neural autoregulatory loops for Dfd and labial (Bergson and McGinnis, 1990; Joshi et al., 2010; Kuziora and McGinnis, 1988; Lou et al., 1995; Marty et al., 2001; Popperl et al., 1995; Tremml and Bienz, 1992). While the role of edx has been tested in Dfd neural autoregulation (Pinsonneault et al., 1997), a similar role for Hth has not been investigated.
To this end, we decided to start with \textit{exd} mutant. \textit{exd} homozygous mutants (Peifer and Wieschaus, 1990) showed no significant change in Dfd expression in Nbs, both in Mx and Mn neuromeres (yellow arrowheads Supp. Fig. 2A). This is because Exd is known to be maternally contributed (Peifer and Wieschaus, 1990; Pinsonneault et al., 1997). Since Hth is a known partner of Exd, and plays an important role in its transport into the cell nuclei (Kurant et al., 2001), we next looked at \textit{hth} mutant. We expected that \textit{hth} null mutant will mimic a phenotype similar to \textit{exd} complete loss of function (Kurant et al., 2001; Rieckhof et al., 1997).

Our analysis of \textit{hth} null mutant (\textit{hth}^{2F}) (Rieckhof et al., 1997), showed almost complete absence of epidermal Dfd expression in embryos (data not shown). Interestingly, we observed a region specific effect of \textit{hth} mutation on Dfd expression. We found that Dfd expression was completely missing in Mx Nbs (Fig. 3, Panel B), while the expression in Mn Nbs was dramatically down regulated, but low levels of Dfd could still be observed in these cells (yellow arrow heads, Fig. 3, Panel B). The brightness of the Dfd channel in Fig. 3, Panel B has been increased to highlight the residual expression of Dfd in Nbs of Mn neuromere. Furthermore the expression of
NAE3.2-lacZ was largely unaffected in the Mn Nbs (Fig. 3, Panel-B), suggesting that Dfd autoregulatory transcriptional loop is unaffected in hthP2 mutants. This result is very similar to what was observed in the past for exdmz-mutant embryos (embryos mutant for maternal and zygotic exd). In these mutants only a quantitative decrease in expression of Dfd was observed. The expression of NAE3.2-lacZ showed a slight decrease in Mn neuromere, but the qualitative expression of both Dfd and NAE3.2-lacZ was unaffected in exdmz-mutant (Pinsonneault et al., 1997). These results suggest that Hth plays an important role in regulation of Dfd in Nbs of embryonic CNS. We find that Hth is critical for Dfd expression in Mx Nbs but is important only for maintenance of the levels of Dfd protein in Mn Nbs, and has no role in Dfd neural autoregulation.

2.4. Dfd-Exd-HthFL bind as a trimer on NAE

A general decrease in levels of Dfd was observed in Mn neuromeres in case of both exd (exdmz) (Pinsonneault et al., 1997) and hth mutants (Section 2.3). This suggests that both these factors play an important role in controlling the levels of Dfd in Mn neuromere. Since Mn expression of Dfd is regulated through 3.2 kb NAE, we decided to test the Dfd binding in the presence of Hth and Exd on Hox-Exd composite binding sites present in 3.2 kb NAE. The 3.2 kb NAE enhancer has seven composite Hox-Exd binding sites (with consensus sequence of [T/A]GATNNATNN). We checked all these 7 binding sites for Dfd-Exd-Hth binding by EMSA. Two out of these seven sites are also found in 608 bp NAE which is known to recapitulate the expression of 3.2 kb enhancer (Lou et al., 1995). The binding data for these two sites (sites-1 and 2) has been presented in Fig. 4. We tested the role of Exd and HthFL (Hth Full length) heterodimer along with Dfd for their capacity to bind on sites-1 and 2 by EMSA. We found that while Dfd protein bound to both of the binding sites (Fig. 4, Panel A, lanes 2 and 9); co-purified Exd-HthFL heterodimer didn’t show any significant binding on its own to either of the binding sites (Fig. 4, Panel A, lanes 3 and 10). A Dfd-Exd-HthFL trimer showed cooperative binding on site-1 (Fig. 4, Panel A, lanes 4 to 7) while on site-2 it showed comparatively weaker trimer binding (Fig. 4, Panel A, lanes 11 to 13). The site-1 oligo mutant for Exd binding site alone showed a loss of cooperative binding and only Dfd monomer bound to DNA (Fig. 4, Panel B, lane 13), suggesting that cooperative trimer binding seen on site-1 is due to Exd-HthFL. The oligo mutant for both Hox-Exd binding site showed a complete loss of binding for Dfd monomer as well as for Dfd-Exd-HthFL trimer (Fig. 4, Panel B, lanes 6, 8 and 9). These results show that Dfd forms a cooperative trimer with Exd-HthFL in vitro on NAE.

2.5. Homeodomain-less isoform of Hth is necessary for its role in Dfd regulation in embryonic Nbs

Homeodomain-less (HD-less) isoform of Hth (referred to as HM-Hth) has been shown to be a functionally important isoform in embryonic CNS.
stages of development (Noro et al., 2006). In order to test if Dfd expression (in both Mn and Mx neuromeres) is dependent on full length Hth or HM-Hth, we analyzed embryos which expressed only HM-Hth isoform (hthP2/hth100-1 heteroallelic combination (Noro et al., 2006)). We observed that HM-Hth embryos showed normal expression of Dfd in Nbs of both Mx and Mn neuromeres of embryonic CNS (yellow and white arrowheads, Fig. 3, Panel C).

**Fig. 3.** Region specific role of Hth in regulating Dfd expression in Nbs. Stage 12 embryos of the following genotypes costained with Dfd, Dpn and LacZ are shown, wild type (Panel-A), hthP2 (Panel-B) and HM-Hth expressing embryos (Panel-C, embryos of genotype hthP2/hth100-1 express only the HD-less form of Hth, HM-Hth). hthP2 mutant embryos only show a Mx Nbs specific loss of Dfd expression and a dramatic decrease in level of Dfd in Mn Nbs (yellow arrowheads in Panel B). The NAE3.2-lacZ expression in Mn Nbs is unaffected in hthP2 mutant embryos (yellow arrowhead in Panel B). The brightness of the Dfd channel in Panel B has been increased to clearly show the residual expression of Dfd in Nbs of Mn neurone. Mn and Mx segment boundary is indicated by white dotted line. Yellow arrowheads indicate Mn cells with coexpression of Dfd, Dpn and lacZ, while white arrowheads indicate Mx cells which coexpress Dfd and Dpn only and are lacZ negative. Scale bars shown are for 30 μm.

The fact that Hth is sufficient to carry out its requisite role in both the neuromeres without its HD, suggests that HD of Hth is not necessary for region specific role of Hth in CNS.

### 3. Discussion

Our understanding of expression and regulation of Hox genes in embryonic CNS has been lacking important details like specific cell types where Hox genes are expressed and the regulatory molecular players involved in these cells. In *Drosophila*, Dfd autoregulation has been investigated and established in both epidermal and neural cells. Specific enhancers have been isolated which control Dfd expression in both these tissues (Bergson and McGinnis, 1990; Kuziora and McGinnis, 1988; Lou et al., 1995). Therefore comparison of Dfd expression (in specific cell types) to 3.2 kb **Neuro Autoregulatory Enhancer** (NAE) expression gave us an opportunity to understand the role of Hth in Dfd expression and autoregulation.

#### 3.1 3.2 kb Neuro Autoregulatory Enhancer

Dfd autoregulation happens in Mn neuromere of CNS through a 3.2 kb NAE, which critically depends on Dfd protein for its expression in cells of CNS (Lou et al., 1995; Zeng et al., 1994). Our analysis of the original NAE3.2-lacZ-P line (obtained from McGinnis lab) showed us a
strong expression of LacZ in Nbs and neurons within (Fig. 1 Panel A, yellow and white arrowheads) and outside the region of Dfd expression (Fig. 1 Panel A, pink arrowheads). Since earlier results had suggested that Dfd maintains its expression through neural autoregulation primarily in Mn neuromere our analysis of NAE3.2-lacZ-P line led us to generate and analyze new reporter lines for 3.2 kb NAE by site specific insertion (Bischof et al., 2007). In comparison to NAE3.2-lacZ-P line both NAE3.2-lacZ-68A and 25C lines showed us expression only in Dfd specific region of embryonic CNS. The specificity of NAE3.2-lacZ-68A expression within Dfd region was further established by a costaining of Dfd, LacZ and Engrailed (Fig. 1 Panel E). These results suggested that 3.2 kb NAE shows a very specific expression mainly confined to Mn neuromere of embryonic CNS, with a very minor expression in maxillary region. These results reconfirmed that Dfd neural autoregulation happens mainly in Mn neuromere of embryonic CNS through 3.2 kb NAE. Based on these results we suggest that the expression of NAE3.2-lacZ-P outside Mn neuromere may not be functionally significant.

3.2. Dfd autoregulates itself in mandibular Nbs neurons and glial cells

Using antibodies to cell type specific markers, we found that Dfd is expressed in embryonic Nbs, neurons and glial cells (Fig. 1). Since NAE3.2-lacZ is an established read out of Dfd neural autoregulation in Mn neuromere, the coexpression of Dfd and NAE3.2-lacZ in Nbs, neurons and glial cells suggest that Dfd autoregulates itself through NAE3.2 in these cells. Our analysis of Mn neurons indicated that there were few neurons which were Dfd+/Elav+/lacZ−, we speculate that LacZ expression in these cells was below our detection limit.

The expression of Dfd in glial cells was analyzed in stages 12 (Fig. 2 Panel C) and 13 (Supp Fig. 2D). We found few glial cells at early stage 12 (Fig. 2 Panel C), this probably was because majority of glial cells were yet to be born. Both NAE3.2-lacZ and Dfd also showed a very weak expression in glial cells initially. As the development progress we find more number of glial cells, and expression of Dfd and NAE3.2-lacZ becomes stronger and consistent in these cells (Stage 13 embryo, 195 R. Kumar et al. / Mechanisms of Development 138 (2015) 190–197
Supp Fig. 2D). As expected Mx glial cells expressed Dfd but not LacZ (white arrowheads in Fig. 1, Panel C).

Since Nbs, neurons and glial cells in Mn neuromere do not autoregulate Dfd expression, it will be of future interest to investigate if these cells sustain Dfd expression later in development.

3.3. Segment specific role of Hth in Dfd regulation

Exd is known to play a role in Dfd autoregulation in epidermis (Bergson and McGinnis, 1990), but neural autoregulation had been shown to be independent of Exd (Pinsonneault et al., 1997). Dfd expression in maternal-zygotic exd mutant embryos (exdmz-) showed a significant decrease compared to the controls, but more importantly both Dfd and NAE3.2-lacZ showed only a quantitative decrease in expression while the qualitative expression was unaffected (Pinsonneault et al., 1997). Our results with exd1 mutant showed us no significant change in expression of Dfd in embryonic Nbs (Supp. Fig. 2A), this was due to maternal contribution of Exd. On the other hand, in hthP2 mutants, we find that the expression of NAE3.2-lacZ is unaffected in Mn Nbs but the expression of Dfd in these cells is dramatically reduced. This is in addition to complete loss of Dfd expression from Mx Nbs. This data suggests a region specific role of Hth in regulation of Dfd expression in embryonic Nbs. We conclude that Hth is critically required for the expression of Dfd in Nbs of Mx neuromere, whereas its role in Mn Nbs is limited only in regulating the expression levels of Dfd. These observations further suggest that Hth has no role to play in regulation of core neural autoregulatory circuitry. This is very similar to what was observed in the past for exdmz- mutant embryos (Pinsonneault et al., 1997), therefore it further supports the idea that Exd and Hth could be functioning together in Nbs of both Mx and Mn neuromeres. This is relevant since Exd is known to play an important role in nuclear localization of Exd (Kurant et al., 2001; Noro et al., 2006). We speculate that in case of hthP2 mutants, Exd is not be able to localize to nucleus and thus cannot carry out its role in regulating Dfd expression in Mx cells. In Mn neuromere on the other hand Exd-Hth heterodimer may have a role only in maintaining the levels of Dfd expression.

The expression of Dfd in Mn Nbs is autoregulated through NAE3.2. Our results suggest that Exd-Hth heterodimer plays a role in maintenance of the levels of Dfd in these cells. By this argument, it is expected that expression of NAE3.2-lacZ should decrease in Mn Nbs in case of both exdmz- and hthP2 mutants. Interestingly in both these cases NAE3.2-lacZ levels show a very minor decrease, while Dfd protein levels show a dramatic decrease. There can be two explanations for these observations; first one being that the enhancer for maintenance of the levels of Dfd protein lies outside NAE3.2 or NAE3.2 has two modules, one of which is responsible for the autoregulation and other one is important for the maintenance of the Dfd expression levels. The latter line of thinking is further corroborated by a very low expression of LacZ reporter when a 608 bp sub-fragment of 3.2 kb NAE is used to drive LacZ in vivo (Lou et al., 1995). Therefore it is plausible that Dfd-Exd-Hth may play a role in enhancing the mandibular levels of Dfd through one of these 7 composite Hox-Exd binding sites found in NAE.

Furthermore the fact that NAE3.2-lacZ expression is not affected much in exdmz- and hthP2 mutants (as compared to dramatic decrease of Dfd expression) could be attributed to the universal heat shock promoter used in NAE3.2-lacZ construct, which may make the construct less sensitive towards Exd-Hth mediated expression level control (Lorberbaum and Barolo, 2015; Zabidi et al., 2015). Replacing the universal promoter with endogenous Dfd promoter can be done to address this issue.

3.4. Role of HM-Hth in Dfd autoregulation

Our results also show that HD of Hth is not necessary in Dfd regulation in embryonic Nbs. We find that Mx and Mn expressions (as well as Mn autoregulation) of Dfd is unaffected in embryos expressing only HD-less isoform of Hth (HM-Hth; only isoform expressed in hthP2/hth100-1 embryos (Noro et al., 2006)). HM-Hth is suggested to interact with Exd and promotes its nuclear localization normally (Kurant et al., 2001; Noro et al., 2006). Our EMSA results show that HM-Hth along with Exd was able to form a cooperative trimer (Dfd-Exd-HM-Hth) on both sites-1 and 2 (Fig. 4, Panel C). In fact, our results indicate that Exd-HM-Hth-Dfd trimer on DNA is much more cooperative than Dfd-Exd-HthFL on both these sites (compare lanes 4–7 and 11–13 in Panels A and C of Fig. 4, which used the same concentrations of Dfd, HthFL-Exd and HM-Hth-Exd). The results with hthP2/hth100-1 embryos also suggest that HM-Hth may contribute to Mx and Mn expressions of Dfd primarily with the help of Exd protein. Therefore taking into account our in vitro and in vivo results, we like to speculate that HM-Hth has a limited role along with Exd and Dfd in regulating Dfd levels in Mn Nbs, and HM-Hth doesn’t affect Dfd neural autoregulation in these cells. On the other hand we speculate a direct role of Exd-HM-Hth in regulating Dfd expression in Mn neuromere. The role of Exd in Mx Nbs could be tested either by attempting Nb specific RNA interference or by making germline clones of exd.

Since both Exd and HM-Hth are required only for regulating levels of Dfd expression in mandibular Nbs, and neural autoregulation in these cells is independent of their roles, therefore we propose a role for yet to be identified factor(s) in regulating core neural autoregulatory transcriptional loop.

Identification of this/these factor(s) and characterization of their role in Nbs and differentiated neurons of mandibular region will be an interesting direction for future research.

4. Experimental procedure

4.1. Drosophila strains and genetics

Wild type flies used were w1118. Standard methods were used to recombine and balance the chromosome containing mutation and transgenes. The hthP2 mutation and exd1 mutations (Peifer and Wieschaus, 1990; Rieckhof et al., 1997) were balanced over hh-lacZ-marked TM3-Sb balancer and ftz-lacZ marked FM7 balancer to identify homozygous embryos. The hth100-1 mutation (Noro et al., 2006) was balanced over TM3-Sb balancer marked with KrGal4, UAS-GFP transgene. NAE3.2-lacZ transgenic line was made using the phiC31-based integration system (Bischof et al., 2007) and inserted in attP sites at 68A4 and 25C6. The original NAE3.2-lacZ line (generated using classical P-element based transgenic method) is balanced on X chromosome and was obtained from Prof. W. McGinnis (UCSD) and referred to as NAE3.2-lacZ-P everywhere. All the experiments were done with 4-h egg collections which were aged for 6 h at 25 °C.

4.2. Antibodies and immunohistochemistry

Antibody staining was done as previously described (Noro et al., 2006). The following primary antibodies were used: anti-lacZ (Chicken, AbCam-ab9631, 1:2000), anti-Dfr (Rbt, Preabsorbed, 1:500), anti-Dpn (mouse, Preabsorbed, 1:1000) anti-Repo (mouse, DHSB 8D12, 1:100), anti-Elav (rat, DSHB, 7E8A10, 1:100), anti-Exd (mouse, DSHB, 1:5), anti-Hth (guinea pig, 1:500) and anti-En (mouse, DSHB, 4D9, 1:50). Secondary antibodies conjugated to Alexa fluorophores from Molecular Probes were used, dilution used is in parenthesis: Alexa Fluor405 (1:200), Alexa Fluor488 (1:500), Alexa Fluor555 (1:1000), and Alexa Fluor647 (1:500). Embryos were mounted with Vectashield. Z-series images were collected on Zeiss LSM700 and were analyzed by LSM browser and ImageJ (http://rsweb.nih.gov/ij/). Other image analyses were done with Photoshop CS3. All images were acquired at 63× and 0.5 zoom. All the analysis represented in the figure was done on stage 12 embryos. All figures unless specified were single confocal slices of 0.4 μm thickness, Fig. 1 was Z-project of multiple slices. All images have anterior roughly towards the top (or top left corner) and
posterior towards the bottom (or bottom right corner). Scale bars shown are for 30 μm.

4.3. Protein-DNA binding assay

Site-1 and site-2 were examined by EMSA for protein binding. Sequence of all oligos used for making probes are given below, the specific binding site tested is underlined.

Site-1 (wild type).
ctgacatcctaacagttgcgcgccatttgattt
tgCcGcCttacaacctgaggcgcacctgatttt
tgGCaattacgctgGTCGAGGACGAGG.

Site-1HE-m (mutant for Exd binding site).
tgacatcctaacagtgcgccatttgattt
tgGCaattacgctgGTCGAGGACGAGG.

Site-2.
tgggacctctgacagtgcgtgacacata
tcattaattattcagtGTCGAGGACGAGG.

EMSA was carried out as described previously (Joshi et al., 2010).

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