Mutational analysis of a *Drosophila* neuroblast enhancer governing *nubbin* expression during CNS development

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Summary
While developmental studies of *Drosophila* neural stem cell lineages have identified transcription factors (TFs) important to cell identity decisions, currently only an incomplete understanding exists of the cis-regulatory elements that control the dynamic expression of these TFs. Our previous studies have identified multiple enhancers that regulate the POU-domain TF paralogs *nubbin* and *pdm-2* genes. Evolutionary comparative analysis of these enhancers reveals that they each contain multiple conserved sequence blocks (CSBs) that span TF DNA-binding sites for known regulators of neuroblast (NB) gene expression in addition to novel sequences. This study functionally analyzes the conserved DNA sequence elements within a NB enhancer located within the *nubbin* gene and highlights a high level of complexity underlying enhancer structure. Mutational analysis has revealed CSBs that are important for enhancer activation and silencing in the developing CNS. We have also observed that adjusting the number and relative positions of the TF binding sites within these CSBs alters enhancer function.

KEYWORDS
CNS, *Drosophila*, enhancers, transcriptional regulation

1 | INTRODUCTION

Enhancers consist of cis-acting DNA elements that control the spatial and temporal aspects of gene expression (reviewed by Epstein, 2009). Although previous studies have shown that enhancers contain clusters of transcription factor (TF) binding sites within blocks of conserved sequences (Berman et al., 2004; Brody et al., 2012; Davidson & Erwin, 2006; Swanson, Evans, & Barolo, 2010), it is not yet well understood just how the distribution of these sites provides a basis for combinatorial logic of enhancer function. One entry point into deciphering the rules that govern an enhancer’s ability to direct gene expression is to manipulate the internal organization of enhancers, that is, position, frequency, and/or order of functionally relevant sequences and examine the effects on cis-regulatory behavior. For example, altering the TF binding sites and other sequences within the sparkling enhancer of shaven (dPax), a gene that encodes a key regulator of photoreceptor fate specification in the developing *Drosophila* retina, switched the specificity of the enhancer from cone to rod photoreceptors (Swanson et al., 2010). A second study has shown that an enhancer of the *Drosophila* Suppressor of Hairless gene is composed of overlapping elements termed submodules that can function independently to activate enhancer activity (Liu & Posakony, 2014). In addition, a recent study of a notochord enhancer structure in *Ciona* points to the importance of TF binding site affinity and arrangement in conferring tissue specificity on enhancer function (Farley, Olson, Zhang, Rokhsar, & Levine, 2016; reviewed by Barolo, 2016; Crocker, Noon, & Stern, 2016). The presence of repeat sequence motifs in these enhancers, and their conserved positioning, points to the necessity of considering binding site position and TF avidity (Levo & Segal, 2014; Sayal, Dresch, Pushel, Taylor, & Arnosti, 2016).

*nubbin* (*nub*) and its closely linked paralog *pdm-2* encode POU homeodomain TFs known for their role in neurogenesis (Billin, Cockerill, & Poole, 1991; Dick, Yang, Yeo, & Chia, 1991; Lloyd & Sakonju, 1991). During embryonic CNS development, the sequential NB expression of the TFs Hunchback—Krüppel—Nubbin and Pdm-

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2. Castor—Grainyhead coordinates specification of neuronal temporal identity (reviewed by Brody & Odenwald, 2002, 2005; Chai, Liu, Chia, & Cai, 2013; Kuzin et al., 2012; Syed, Mark, & Doe, 2017). Loss of any of these TFs, including nubbin and pdm-2, triggers abnormal CNS development and consequently embryonic lethality (Grosskortenhaus, Pearson, Marusich, & Doe, 2005; Kohwi & Doe, 2013; Tran & Doe, 2008; Yang, Yeo, Dick, & Chia, 1993; Yeo et al., 1995). The pdm genes are expressed in overlapping but non-identical patterns within intermediate neuroblast sublineages. Together, Hb and Cas silence pdm expression in early and late forming sublineages, respectively, thereby limiting pdm expression to intermediate sublineages (reviewed by Brody & Odenwald, 2002; Syed et al., 2017). One of the major questions concerning the sequential expression of these TFs is the regulatory basis of their temporal gene expression. Experimental results suggest that the network is regulated by repression of the TFs. This model has been further elaborated to include both activation and feedback repression to achieve temporal gene expression (Nakajima, 2010).

The aim of this study is to understand the regulation of nubbin NB expression in terms of its conserved cis-regulatory sequences. Previous work with a 3.2 Kb fragment that includes a NB enhancer showed that loss of cas function, acting through Cas target sequences, resulted in ectopic activation of pdm expression during embryonic lineage development (Kambadur et al., 1998). A subsequent study identified and delimited the NB enhancers within the pdm locus (Ross, Kuzin, Brody, & Odenwald, 2015). One of these enhancers lies within a nub intron, denoted as nub-46. Phylogenetic footprinting of the nub-46 enhancer reveals that it contains multiple conserved sequence blocks (CSBs). This study describes the functional characterization of the nub-46 NB enhancer in terms of its conserved sequences. Truncation analysis of the CSB cluster was used to delimit core elements that are required for embryonic enhancer expression. We examined the roles of each of the nub-46 CSBs using deletions and sequence rearrangement to resolve sequences that are required for temporal and spatial regulation. Within the core element, we identified two consensus Cas binding sites. Our functional analysis has revealed (a) that the Cas binding sites in nub-46 are the targets of Cas mediated repression; (b) that CSBs containing repeat sequences are not required for enhancer activation; and (c) that novel non-repeated conserved sequences are essential for enhancer activity.

2. MATERIALS AND METHODS

2.1 Comparative genomics

The phylogenetic comparative analysis of the nub-46 enhancer was performed using the EvoPrinterHD program (http://evoprinter.nih.gov/), a program providing alignment of 12 sequenced Drosophila genomes (Odenwald, Rasband, Kuzin, & Brody, 2005; Yavatkar et al., 2008). Instructions for using the EvoPrinterHD comparative tool are provided on the EvoPrinter website.

2.2 Enhancer-reporter transgene vector

A modified pCa4B vector was employed in these studies (Brody et al., 2012). The pCa4B vector was modified to include the following features from the pHStinger vector (Barolo, Castro, & Posakony, 2004): the pHStinger polylinker (replacing the pCa4B polylinker), a minimal Heat shock protein 70 (Hsp70) promoter driving a GFP or RFP reporter gene, and gypsy chromatin insulators to block influence of flanking enhancers that would otherwise modify reporter expression via enhancer trap effects. The vector also contains bacterial attachment (attB) sites for its targeted chromosomal insertion (Groth & Calos, 2004). The site-specific integration vector was selected to ensure that all of the enhancer-reporter constructs were inserted in the same chromosomal environment. In addition to the gypsy chromatin insulators, the nonrandom integration afforded by the PhiC31 integration further reduces integration variability on enhancer function. Integration of the pCa4B vector is facilitated by a serine integrase, phage PhiC31, which mediates recombination between vector attB sites and genomic attP sites (Groth & Calos, 2004).

2.3 Transgene constructs

nub-46 enhancer DNA fragment was cloned from wild-type genomic DNA using standard PCR method (nub-46 5’-primer is TATTAGGCAACTGTCCTCTGCC and nub-46 3’-primer is ACTGAA-CAGGGTAGCTATTCCGG). PCR products were analyzed using gel electrophoresis and were purified by a Qiagen QIAquick Gel Extraction Kit. Purified PCR products were inserted into the Invitrogen pCRII-TOPO TA vectors. For CSB deletions and rearrangements, we employed the Invitrogen GeneArt Gene Service to generate mutated nub-46 enhancers. Verified sequences were inserted into the modified pCa4B vector (details are available upon request).

2.4 Generation of transgenic fly lines

Transgenes were injected into either VK1 (insertion site on chromosome 2R, 59D3) or attP2 (insertion site on chromosome 3L, 68A4) embryos by Rainbow Transgenic Flies, and at least two independent transformant lines for each construct were generated. Standard genetic crosses were performed to generate homozygous transgenic fly lines. Fly lines are maintained at 18°C using standard husbandry procedures (Ashburner, 1989).

2.5 In situ hybridization and immunohistochemistry

Embryo collections and fixations of at least two independent lines per construct were performed according to procedures previously described (Tomancak et al., 2002). For in situ hybridizations, mRNA probes were generated from a PCR amplified GFP ORF. Roche DIG RNA Labeling Mix protocol was used, and staining was visualized using anti-DIG Fab fragments coupled to alkaline phosphatase (1:2,000, Roche). Whole-mount or filleted embryos were photographed using a Nikon Optiphot microscope (10X objective lens). Embryo developmental stages were determined based on morphological features previously described (Campos-Ortega, 2004) Immunolabeling experiments used anti-Cas rabbit antibodies (1:500) and anti-s-Cas mouse antibodies (1:500) purchased from Millipore.
GFP chicken antibodies (1:500, Chemicon). Secondary antibodies included anti-chicken Alexa 488 (1:1,000, Invitrogen), anti-rabbit Alexa 633 (1:1,000, Invitrogen). After immunolabeling, embryos were examined for GFP and Cas expression via serial optical sections that were photographed at 1 μm intervals using a Zeiss LSM 510 confocal microscope. Detailed protocols are available upon request.
RESULTS AND DISCUSSION

3.1 nub-46 sequence conservation and embryonic cis-regulatory dynamics

Our previous enhancer-reporter transgene survey identified an enhancer (denoted as nub-46) that recapitulated nub expression during embryonic cephalic lobe and VNC NB lineage development (Ross et al., 2015). As an initial step to functionally characterize the nub-46 enhancer, we identified its conserved sequence blocks by comparative evolutionary analysis using 12 Drosophila species, including D. melanogaster, D. simulans, D. sechellia, D. yakuba, D. erecta, D. ananassae, D. persimilis, D. pseudoobscura, D. willistoni, D. virilis, D. mojavensis, and D. grimshawi. Our analysis revealed that nub-46 is made up of 11 CSBs (Figure 1a). While many of its conserved elements are novel, we identified a CSB, denoted as “C” (Figure 1a), containing two adjacent 9-mer sequences (TAAAAATTG and CATAAAAAA) that correspond to the DNA-binding site motifs for Cas (Kambadur et al., 1998).

The nub-46 enhancer-reporter transgene expression is dynamic during embryonic CNS development. We observed transient nub-46 activation at the cellular blastoderm stage (data not shown), followed by progressive NB reactivation during embryonic neurogenesis (Figure 1b–d). At stage 9, nub-46 regulates transgene reporter expression in several NBs per ventral cord hemisegment, and enhancer activity is detected in a subset of cephalic lobe neural lineages (Figure 1b). Later in CNS development enhancer/reporter expression is detected in additional cephalic lobe and ventral cord NB lineages (Figure 1c,d). After embryonic stage 13, nub-46 cis-regulatory activity is downregulated in both the brain and ventral cord (data not shown).

3.2 Identification of the nub-46 core enhancer

To delimit the boundaries of the nub-46 enhancer, we generated both 5' and 3' deletions of the full nub-46 enhancer CSB cluster (Ross et al., 2015) and examined the in vivo cis-regulatory activity of these truncated fragments via enhancer-reporter transgenes. This analysis revealed that the centrally located CSBs (Figure 1a, "C" through "I")
were sufficient for embryonic CNS expression (see Figure 1f). However, compared to the full-length enhancer, we observed a reduced enhancer/reporter activity for the core that contains elements "C" through "I" (compare Figure 1e,f). These findings demonstrate that the core fragment consists of activator and repressor sequences required for its wild-type spatial and temporal regulatory dynamics.

3.3 | Castor regulates the nub-46 enhancer during embryonic neurogenesis

Given that Cas is a negative regulator of pdm gene expression in embryonic NBs, we predicted that the putative Cas DNA-binding motifs within nub-46 are required to deactivate enhancer activity. Expression of nub-46 enhancer activity partially overlaps endogenous Cas protein expression in stage 13 embryos (Figure 2a). To determine whether the putative Cas binding-motifs function as Cas binding sites, we examined the regulatory activity of a nub-46 deletion that lacks a 40 bp conserved region containing the two Cas motifs (Figure 2b). Deletion of the Cas DNA-binding sites triggers ectopic enhancer activity in the cephalic lobes during stage 13 (Figure 2b), suggesting that the "C" CB functions as a repressor element during cephalic lobe development. Interestingly, we did not observe significant ectopic enhancer activity in the developing VNC. Therefore, removal of the nub-46 "C" CB does not completely account for the repressive action of Cas on the nub-46 enhancer, especially in the VNC, and other direct or indirect effects of Cas action on the nub should be considered.

3.4 | Deletion analysis of the core nub-46 CSBs

While the "C" element may contain repressor DNA-binding sites, it remained unknown how the nub-46 enhancer is activated in the embryonic CNS. To address this question, we further examined the effects of
internal deletions within the nub-46 enhancer. Each of the 10 remaining CSBs illustrated in Figure 1 were individually removed. Enhancers with these individual deletions were tested in two independent transgenic lines. The wild type control enhancer activity was tested under the same conditions and at the same time as the deletion mutants. We observed that nub-46 variants lacking either the “B” (AGAACGCAAT) element or “E” (CTACCTGAG) element displayed only a modest reduction in enhancer activity compared to the wild-type (Figure 3). Surprisingly, we found that singular removal of other CSBs had only subtle effects on enhancer activity during embryonic NB lineage development (data not shown), suggesting that these CSBs may be either required at later time points or are functionally redundant.

3.5 | nub-46 enhancer contains multiple activator sequences within its conserved core elements

Given that other cis-regulatory enhancers contain a combination of repeat and unique sequence elements, we hypothesized that nub-46 activation may result from a complex set of multiple inputs. Indeed, self-alignment of conserved sequences within nub-46 revealed that the enhancer is made up of 11 distinct repeat and palindromic elements (Figure 4a). Upon closer inspection, we found that seven of the 10 repeat elements are located within the “C” element, and that many of these repeats were also found in CSBs “D,” “H,” and “I” of the enhancer core (Figure 4a).

We next assessed whether the repeat elements within the core are required for enhancer activation. Loss of the “C” element does not significantly affect onset of enhancer-reporter expression during embryonic VNC development (Figure 4c). Among the six repeats identified within the “C” element, nearly all are present in the “D,” “H,” and “I” elements (Figure 4a), and we speculated that these may compensate for the loss of repeats in the nub-46 [C] mutant. To test this hypothesis, we truncated the core to exclude the “C” element (denoted as the [C] in Figure 4c) and then further removed all elements containing repeats (“D,” “H,” and “I” elements) from the core enhancer (referred to as [CDHI] in Figure 4d). Surprisingly, removal of these CSBs had little or no effect on enhancer activity (Figure 4d). One possible explanation for the lack of any significant effect of element “C” (and other elements containing repeat sequences) on enhancer activation is that activator sequences are located within elements lacking repeats (elements “E,” “F,” and “G”). To investigate whether the “E” (CTACCTGAG), “F” (GGGGTGCAATACCAGC), and “G” (TACCGTA) elements are required for enhancer activation, we removed all three elements from the enhancer [CEF] and observed that deletion of these resulted in complete loss of reporter activity, suggesting that “E,” “F,” and “G,” containing only unique sequences, are required to activate reporter expression (Figure 4e).

To determine whether a subset of these elements is necessary for enhancer function, we tested the effect of different combinations of internal deletions on cis-regulatory activity during embryonic neurogenesis. While removal of either the “E,” “F,” or “G” elements had little or no effect on enhancer function (Figure 5b–d), only the combined loss of “E” and

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**FIGURE 5** Deletion analysis of the nub-46 enhancer core reveals functional overlap among individual conserved sequence blocks. Shown are enhancer CSB transgene reporter mRNA expression patterns in whole-mount stage 11 embryos (ventral and dorsal views; anterior up). Each of the constructs lacked CSB “C,” containing repressive elements described in Figure 2: (a) nub-46 enhancer construct containing CSBs “D,” “E,” “F,” “G,” “H,” and “I” (see Figure 1a for sequence); (b) element minus “E”; (c) element minus “F”; (d) element minus “G”; (e) element minus “E” and “F”; (f) element minus “E” and “G”; (g) element minus “F” and “G”; and (h) element minus “E,” “F,” and “G.” Only loss of both “E” and “F” compromised core activity, while higher enhancer activity was obtained with loss of “F” and “G.”
synthesize core enhancers that contain three copies of either
non-repeat elements. We also examined construct expression during multi-
ple stages of CNS development (Figure 6). When we replaced the “F”
and “G” elements with “E” elements (Figure 6b), increasing the number
of “E” elements to three, higher enhancer activity were observed
within subsets of NBs compared to the wild-type during stage 11 (Fig-
ure 6a,b). However, by stage 13, we observed higher levels of
enhancer activity throughout the CNS (Figure 6b’). Notably, we also
observed ectopic expression within putative PNS lineages during
stage 14 (Figure 6b”). It should be noted that additional co-localization
experiments using cell lineage markers would be needed to substanti-
ate the ectopic expression. Increasing the number of “F” elements also
altered core enhancer activity, but the effect was limited to a subset
of lateral VNC NBs and dorso-anterior cephalic lobe cells during early
stage 12. These differences were not apparent at stage 13 and stage
14 (Figure 6c). Increasing the number of “G” elements resulted in
diminished expression at all three stages examined (Figure 6d).

FIGURE 6  Enhancer function is altered by adding multiple CSBs
that lack repeat sequences. Shown are enhancer CSB transgene
reporter mRNA expression patterns in whole-mount stage 11, 13,
and 14 embryos (ventral and dorsal views; anterior up). The
enhancer constructs lack CSB “C,” which contained repressive
elements described in Figure 2, but were engineered to contain
triple occurrence of non-repeat elements “E,” “F,” and “G.” (a) nub-46
enhancer construct containing CSBs “D,” “E,” “F,” “G,” “H,” and “I”;
(b–d) three copies of the “E” CSB were substituted for the “F” and
“G” CSBs; (c) three copies of the “F” CSB were substituted the “E”
and “G” CSBs; (d) three copies of the “G” CSB were substituted for
the “E” and “F” CSBs (see Figure 1a for sequences)

“F” compromised core activity (Figure 5e). Notably, however, we iden-
tified increased enhancer activity with loss of “F” and “G” (Figure 5g),
whereas loss of all three non-repeat elements disrupted enhancer
function (Figure 5h). Individual deletion of non-repeat CSBs exhibited
minor reduction in enhancer activity within brain lineages (see
Figure 5 panels C, D, and G).

Given that all three elements lacking repeat sequences are essen-
tial for enhancer function, we next asked whether enhancer function
is modified by the multiplicity of these sequences. To explore this, we
synthesized core enhancers that contain three copies of either
element, substituting each into the positions of the other two non-
repeat elements. We also examined expression during multi-
ple stages of CNS development (Figure 6). When we replaced the “F”
and “G” elements with “E” elements (Figure 6b), increasing the number
of “E” elements to three, higher enhancer activity were observed
within subsets of NBs compared to the wild-type during stage 11 (Fig-
ure 6a,b). However, by stage 13, we observed higher levels of
enhancer activity throughout the CNS (Figure 6b’). Notably, we also
observed ectopic expression within putative PNS lineages during
stage 14 (Figure 6b”). It should be noted that additional co-localization
experiments using cell lineage markers would be needed to substanti-
ate the ectopic expression. Increasing the number of “F” elements also
altered core enhancer activity, but the effect was limited to a subset
of lateral VNC NBs and dorso-anterior cephalic lobe cells during early
stage 12. These differences were not apparent at stage 13 and stage
14 (Figure 6c). Increasing the number of “G” elements resulted in
diminished expression at all three stages examined (Figure 6d).

4 | SUMMARY

The principal findings of this study are the identification of a core
sequence within the nub-46 NB enhancer that is sufficient to recapitu-
late the embryonic expression pattern of nubb in and that novel non-
repeated conserved sequences are required for enhancer activity. Our
study has delimited the target of Cas repression to a CSB containing
two adjacent 9-mer sequences corresponding to the TF DNA-binding
motif for Cas in CSB “C.” Nevertheless, the possibility still exists that
Cas is not the only repressor of nub-46 during embryonic CNS
development.

We have also localized activator CSBs that contain uniquely
represented sequences within the enhancer, suggesting that the
enhancer may be regulated by as yet uncharacterized TF activators
that play a role in the temporal regulation of nubb in. Our data suggests
that multiple copies of either “E” or “F” can function as an activator
within the enhancer core. While previous studies have suggested that
clusters of repeat regulatory sequences are an important aspect of
enhancer regulation (Brody et al., 2012; Gotea et al., 2010; Lifanov,
Makeev, Nazina, & Papatsenko, 2003; reviewed by Taher, 2013), this
study points to unique non-repeated motifs as targets of transcrip-
tional activators. While our initial observations revealed altered
expression outside the spatial/temporal boundaries of nub-46 activity,
further experiments using cell-type specific markers are needed to
confirm this ectopic expression.

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