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Journal Title: Journal of Neuroscience Nursing
Volume: Volume 34, Number 7
Publisher: Lippincott, Williams & Wilkins | 2014-02-12, Pages 2538-2543
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1523/JNEUROSCI.4511-13.2014
Permanent URL: https://pid.emory.edu/ark:/25593/s5n3h

Final published version: http://dx.doi.org/10.1523/JNEUROSCI.4511-13.2014

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Accessed February 6, 2019 6:55 PM EST
The Transcription Factors Islet and Lim3 Combinatorially Regulate Ion Channel Gene Expression

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Expression of appropriate ion channels is essential to allow developing neurons to form functional networks. Our previous studies have identified LIM-homeodomain (HD) transcription factors (TFs), expressed by developing neurons, that are specifically able to regulate ion channel gene expression. In this study, we use the technique of DNA adenine methyltransferase identification (DamID) to identify putative gene targets of four such TFs that are differentially expressed in Drosophila motoneurons. Analysis of targets for Islet (Isl), Lim3, Hb9, and Even-skipped (Eve) identifies both ion channel genes and genes predicted to regulate aspects of dendritic and axonal morphology. Significantly, some ion channel genes are bound by more than one TF, consistent with the possibility of combinatorial regulation. One such gene is Shaker (Sh), which encodes a voltage-dependent fast K+ channel (K+1,1). DamID reveals that Sh is bound by both Isl and Lim3. We used body wall muscle as a test tissue because in conditions of low Ca2+, the fast K+ current is carried solely by Sh channels (unlike neurons in which a second fast K+ current, Shal, also contributes). Ectopic expression of isl, but not Lim3, is sufficient to reduce both Sh transcript and Sh current level. By contrast, coexpression of both TFs is additive, resulting in a significantly greater reduction in both Sh transcript and current compared with isl expression alone. These observations provide evidence for combinatorial activity of Isl and Lim3 in regulating ion channel gene expression.

Key words: aCC; central nervous system; Drosophila; muscle; RP3; Shaker

Introduction

The development of embryonic neurons is regulated by factors that are intrinsic or extrinsic to individual cells. It is, however, in the early stages of development, before axonal growth and circuit formation, that intrinsic factors predominate (Spana et al., 1995; Grosskortenhaus et al., 2005; Grosskortenhaus et al., 2006). These factors, which are likely determined by clonal lineage, control aspects of both morphological and functional (electrical) development. Studies on motoneuron specification, from flies to mammals, have shown that early developmental decisions, such as subclass identity, are dictated, at least in part, by a combinatorial code of LIM-HD transcription factors (TFs) (Thor and Thomas, 1997; Landgraf et al., 1999; Dasen et al., 2005; Landgraf and Thor, 2006; Dasen et al., 2008; De Marco García and Jessell, 2008). However, whether early neuron-type specific ion channel gene expression is similarly influenced through combinatorial activity of these same TFs remains to be demonstrated.

Embryonic Drosophila motoneurons express a stereotypic mix of identified TFs, which are evolutionarily conserved with mammals (Thor and Thomas, 1997; Thaler et al., 1999; Moran-Rivard et al., 2001; Esmaeili et al., 2002; Thaler et al., 2002). For example, the RP subgroup of motoneurons (RPs 1, 3–5), which innervate ventral and lateral muscles, express the TFs Isl (also known as Tail-up), Lim3, and Hb9 (also known as Extra-extra). Motoneurons (e.g., aCC) that project dorsally express Eve (Thor and Thomas, 1997; Landgraf et al., 1999; Landgraf and Thor, 2006). The presence or absence of individual TFs, particularly Isl and Eve, is a known determinant for both axonal projection, neurotransmitter phenotype, and neuron type-specific expression of ion channels (Thor and Thomas, 1997; Landgraf et al., 1999; Pym et al., 2006; Wolfram et al., 2012).

Our previous studies used DNA adenine methyltransferase identification (DamID) (van Steensel and Henikoff, 2000) to identify ion channel genes, in particular slowpoke (slo) and Sh, as targets of Eve and Islet, respectively (Pym et al., 2006; Wolfram et al., 2012). Here we now report the complete identified DamID-derived binding sites for Hb9 and Lim3, together with a reassessment of DamID data previously obtained for Eve and Isl, using Flybase release 5.47. Our analysis identifies ion channel genes as...
targets of all four TFs, and shows that some ion channel genes (e.g., Sh) are bound by multiple TFs (in this instance, Isl and Lim3). We further show that the combined action of Isl and Lim3, in regulating Sh expression, is additive. As such, these findings provide first direct experimental evidence to support combinatorial regulation of a specific ion channel gene.

**Materials and Methods**

*Fly stocks.* Flies were maintained under standard conditions. For larval collections, flies were allowed to lay eggs onto grape juice agar plates. GAL4-UAS-shaker-Bgl2 (homozygous viable on the second chromosome) was used to express UAS-Isl (2× UAS-Isl, third chromosome) or UAS-Lim3 (1× UAS-Lim3, second chromosome) in body wall muscle (both kindly supplied by Dr. Stefan Thor). Coexpression was achieved using a generic cross containing both transgenes (2× UAS-is1 and 1× UAS-Lim3). Embryos of either sex were kept at 18°C to avoid embryonic lethality, which occurs with expression of either transgene in muscle at 25°C.

**DamID analysis.** The construction of DamID constructs and transgenic *Drosophila* lines has been previously described (Pyrm et al., 2006; Wolfram et al., 2012). Briefly, the full-length TF-coding sequences were PCR-amplified from an embryonic cDNA library and cloned into pUASTattB-NDam. Preparation of Dam-methylated DNA from stage 17 embryos was performed as previously described (Pyrm et al., 2006) and gene-targets identified (Wolfram et al., 2012) using Flybase release 5.47 and a stringent false discovery rate (FDR) of 0.1%

**Electrophysiology.** Hatched larvae (1–4 h old) were dissected and the CNS removed (Wolfram et al., 2012). Muscles were treated with 1 mg/ml collagenase (Sigma) for 0.5 to 1 min before whole-cell patch recording. Larvae were visualized using a water-immersion lens (total magnification, 600×) combined with DIC optics (BX51WI microscope; Olympus, Optical). Recordings were made from muscle 6 in segments A3–4 using a Multiclamp 700B amplifier controlled by pClamp 10.2 (Molecular Devices). Recordings were sampled at 20 kHz and filtered at 2 kHz. The voltage protocol used a maintained holding potential of −60 mV and a −90 mV prepulse for 200 ms before a 50 ms step to 40 mV. Leak currents were subtracted online (P/4). Recordings were done in at least 4 animals, and at least 8 muscles were recorded from in total for each manipulation. Cell capacitance was determined by integrating the area under the capacity transients evoked by stepping from −60 to −90 mV (checked before and after recordings). External saline (stewart et al., 1994) consisted of (in mM) as follows: 70 NaCl, 5 KCl, 0.1 CaCl2, 20 MgCl2, 5 HEPES, 115 sucrose, 5 trehalose, pH 7.2. The calcium concentration was kept low to prevent activation of Ca2+/H11002. Gluconate, 2 MgCl2

**qRT-PCR.** Muscle tissue was collected from newly hatched larvae. After RNA extraction (Qiagen RNAesy Micro kit), cDNA was synthesized using the Fermentas Reverse Aid H minus First strand cDNA synthesis kit. RNA concentration was matched for control and experimental samples before cDNA synthesis. qRT-PCR was performed on a Roche LightCycler TaqMan Master reaction mix (Roche; 04553286001). The thermal profile used was 10 min at 95°C followed by 45 cycles of 10 s at 95°C, followed by 20 s at 60°C, and finally 10 s at 72°C. Results were analyzed by the ∆Δ C method and are expressed as fold difference relative to control. C values used were the means of duplicates. TaqMan primers and probes (forward and reverse primers in 5′ to 3′ orientation) were as follows: rp49 primers CCACTGCCGATCGATCATGATCAGT and AGCTTGTGCAACTTGCAAGAA and probe GTACGCGCAATTGTCGTTACCGG conjugated with Cy5; Sh primers TAGGTGTAAGAATATTAAATATATCG and TAAACAAGTACCTATTTATTGATTACACT and probe AGACACCTACGGAGGAATTGAAACACGG AG conjugated with FAM. Levels of expressed transcription factor in muscle were also monitored by TaqMan qPCR.

**Statistics.** Statistical significance was calculated using a one-way ANOVA with Tukey’s post hoc test using confidence intervals of p = 0.05 and p ≤ 0.01. All quantitative data shown are mean ± SEM.

**Accession codes.** The DamID-chip data have been deposited under NCBI GEO accession GSE53446.

**Results**

**Target genes include both ion channel and morphology-associated genes**

The LIM-HD transcription factors Isl and Eve control both axon guidance and ion-channel expression in developing neurons (Thor and Thomas, 1997; Landgraf et al., 1999; Pyrm et al., 2006). Together with other motoneuron-expressed TFs, notably Lim3 and Hb9, these TFs have been postulated to act combinatorially to set both structural and functional properties of embryonic neurons (Landgraf and Thor, 2006; Wolfram and Baines, 2013). However, to date, the gene targets of these TFs remain largely unknown and combinatorial activity remains to be definitively shown. We used DamID to describe binding sites for each of these TFs in *Drosophila*. Using an FDR of = 0.1%, we identify 2670 genes (exhibiting one or more peaks of binding within 5 kb of the transcriptional unit) as targets of Isl; 4105 genes for Lim3, 1771 genes for Hb9, and 1039 genes for Eve. Notably, Islet, Lim3, and Hb9 bind a comparatively larger number of gene targets than does Eve. Analysis of targets indicates that these three TFs share a large number of common putative targets. In contrast, overlap with targets of Eve is relatively much smaller. This is predictable and validates our analysis because it reflects the observed overlap in neuron expression of these transcription factors. Lim3, Isl, and Hb9 are found colocalized in many motoneurons, whereas Eve is expressed in relatively few (Landgraf and Thor, 2006).

Consistent with the possibility of combinatorial regulation, many of target genes are bound by more than one TF (Fig. 1; Table 1). Gene ontology analysis reveals that targets include ion channels and also genes associated with both morphology (axonal and dendritic) and synapse formation (Table 1). This latter subset includes beat-1c, Dscam, fra, Fas2, robo, and Sema1a, which have been studied in some detail (Baines et al., 2002; Corty et al., 2009; Mauss et al., 2009; Timofeev et al., 2012). One gene in
Our DamID shows that some genes are potentially bound by a particular, *beat-lc*, has been shown to be downstream of the LIM-HD combinatorial code: regulation in this instance dictating motoneuron synaptic connectivity (Certel and Thor, 2004).

Of the ion channel gene targets identified, there is considerable evidence to show that the voltage-gated K\(^+\) channel genes, *Sh*, *Shal*, *Shab*, the Ca\(^{2+}\)-dependent K\(^+\) channel gene *slo*, and the Ca\(^{2+}\)-channel gene *Ca-α1D* are expressed in motoneurons (Byerly and Leung, 1988; Leung and Byerly, 1991; Martínez-Padrón and Ferrus, 1997; Baines and Bate, 1998; Engel and Wu, 1998; Worrell and Levine, 2008). Synaptic excitation of motoneurons is also cholinergic (Baines et al., 1999); and in this regard, it is significant that our analysis identifies 5 nicotinic receptor-subunits (of a total of 10) (Sattelle et al., 2002). These genes are *nAcRa-30D*, *nAcRa-7E*, *nAcRa-34E*, *nAcRa-96Aa*, and *nAcRβ-96A*. We have previously validated three of the identified ion channel genes, *Sh* and *slo/nAcRa-96Aa*, as targets of *Is1* and *Eve*, respectively (Pym et al., 2006; Wolfram et al., 2012). It should be noted, however, that *slo* is now only identified (using stricter reassessment criteria) when we relax the FDR to \(0.5\%\), indicative that our current target list (derived from an FDR of \(\leq 0.1\%\)) is restricted.

**Combinatorial transcription factors share the same targets**

Our DamID shows that some genes are potentially bound by more than one TF. For example, *Sh* is a validated target of *Isl* (Wolfram et al., 2012) but also a putative target of *Lim3* (Fig. 2; Table 1). Significantly, a mammalian *Isl1*:Lhx3 (Isl/Lim3 homologs) ATTAGTTAATT “dimer” motif (Lee et al., 2008) underlies both Isl and Lim3 binding sites at this locus (Fig. 2). This overlap is consistent with the hypothesis of combinatorial regulation. We tested for this experimentally.

To determine how *Lim3* influences expression of *Sh* and whether its effect is additive to that of *Isl*, we used body wall muscle in which Kf is carried entirely by Sh channels (in low external Ca\(^{2+}\)) (Singh and Wu, 1990). Such analysis would be complicated in neurons because of the expression of an additional Kf encoded by *Shal* (Tsunoda and Salkoff, 1995) and homeostatic mechanisms (Baines et al., 2001), which maintain consistency in action potential firing through adjustment of ionic conductances. By contrast, muscle does not exhibit this type of homeostasis. To determine the amplitude of Kf in muscle whole-cell recordings, we modeled Kf and Ks, which allowed us to computationally determine peak conductances for both (see Materials and Methods). As previously shown, expression of *isl* in muscle is sufficient to reduce both the magnitude of Kf (Sh-dependent) (Fig. 3A–C) and the abundance of *Sh* transcript (Fig. 3D) consistent with transcriptional regulation (Wolfram et al., 2012). Expression of *isl* led to a significant reduction of Kf (0.17 ± 0.02 vs 0.11 ± 0.01 nS, \(p = 0.01\), \(n = 8\), mean ± SE) and transcript (0.91 ± 0.03-fold difference, \(p = 0.02\), \(n = 6\)). By contrast, expression of *Lim3* did not statistically affect either Kf (0.13 ± 0.01 nS, \(p = 0.08\), \(n = 10\)) or *Sh* transcript level (0.94 ± 0.03-fold reduction, \(p = 0.1\); Fig. 2), even though the *Lim3* transgene was expressed at increased levels relative to *isl* (\(~30\)-fold compared with an \(~15\)-fold increase relative to control, no transgenic expression). Coexpression of both *isl* and *Lim3* was, however, suf-
up-regulated by ~8- and ~12-fold, relative to control. It should be noted that DamID shows that \textit{Lim3} is bound by Islet, which likely explains the significantly lower expression level compared with when \textit{Lim3} was overexpressed alone.

Together, our results are consistent with the previous demonstration of a direct gene–dosage relationship between \textit{Sh} transcript and \textit{Sh}-dependent \textit{Kf} expressed in body wall muscle (Haugland and Wu, 1990). Attempts to verify this through higher TF transgene expression, often achieved by raising the temperature to 25°C, was not possible in our experiments because of lethality at this temperature. Thus, we conclude that repression of \textit{Sh} expression by coexpressing both \textit{isl} and \textit{Lim3} is additive, which is both predictive and supportive of combinatorial regulation.

**Discussion**

In contrast to specification of neuron identity, little is known concerning how embryonic neurons regulate ion channel gene expression before formation of neuronal networks. Recent studies, in a range of animals, suggest that members of a defined set of LIM-HD TFs regulate this early phase of expression. Collectively, these studies have led to the proposition that early neuron-type specific properties, including axonal and dendritic morphology, choice of synaptic target and expression of ion channels, are set by a combinatorial activity of TFs (Landgraf and Thor, 2006; Pym et al., 2006; Wolfram et al., 2012). For example, the presence of \textit{Lim3} is seemingly sufficient to subdivide \textit{Drosophila} \textit{isl}-positive motoneurons into the ISNb and ISNd classes (Thor et al., 1999). Although there is evidence to show that ion channels are also regulated by LIM-HD TFs (Wolfram and Baines, 2013), definitive evidence to show combinatorial regulation is lacking. In this study, we show that the activity of two early expressed combinatorial TFs, \textit{Isl} and \textit{Lim3}, is additive such that their combined effect to suppress expression of the \textit{Sh} \textit{K}^+ channel is significantly greater than either alone.

To show that \textit{Sh} expression is regulated by both \textit{Isl} and \textit{Lim3}, we exploited muscle as a model tissue. There are several advantages of using muscle over neurons. First, \textit{Kf} in muscle (in conditions of low external \textit{Ca}^{2+}) is carried solely by \textit{Sh} channels (Singh and Wu, 1990). Second, muscle is isopotential (Jan and Jan, 1976), which significantly minimizes the impact of space clamp problems, which, in neurons, can be particularly problematic for fine-scale analysis of ionic currents. Third, studies in neurons can be complicated by

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**Figure 2.** DamID demonstrates direct binding of \textit{Isl} and \textit{Lim3} to the \textit{Sh} locus. The transcription unit of \textit{Sh} is shown in blue; arrow indicates direction of transcription. \textit{Sh} exons are shown immediately below in brown. Putative binding of each TF to this locus is indicated by the average of normalized log2-transformed ratios from multiple independent DamID experiments. Areas in red (marked by the black boxes) represent a significant binding peak within the dataset (FDR < 0.1%) for both \textit{Isl} and \textit{Lim3}. No binding peaks were detected for Hb9 and Eve. Open red arrow indicates the location of a mammalian Isl1:LhX3 (Isl/Lim3 homologs) ATTAGTAAT “dimer” motif (Lee et al., 2008).

**Figure 3.** \textit{Isl} and \textit{Lim3} act combinatorially to regulate \textit{Sh} expression. \textit{A}, The fast \textit{K}^+ current (\textit{Kf}) in body wall muscle is carried entirely by the \textit{Sh} channel (in conditions of low \textit{Ca}^{2+}). Traces represent membrane current produced under voltage clamp for a step from –90 to 40 mV for 50 ms in control muscle and when \textit{Isl}, \textit{Lim3}, or both are expressed. Recordings are normalized to cell capacitance. \textit{B}, Modelling of the outward \textit{K}^+ conductance allows \textit{Kf} (carried by \textit{Sh}) and \textit{Kslow} (\textit{Ks}) to be differentiated. Green line indicates model fit to capacitance-adjusted data (control muscle); model-derived \textit{Kf} and \textit{Ks} are shown. \textit{C}, Model-derived peak conductances for \textit{Kf} show that expression of \textit{Isl} is sufficient to suppress the \textit{Sh}-dependent \textit{Kf} current, whereas \textit{Lim3} expression has no statistically significant effect. Coexpression of both \textit{Isl} and \textit{Lim3} further reduces \textit{Kf} to a level that is significantly different from \textit{Isl} alone. \textit{D}, \textit{qPCR} shows that coexpression of \textit{Isl} and \textit{Lim3} reduces \textit{Sh} transcript to a level significantly different from \textit{Isl} expression alone. In isolation, only \textit{Isl} expression significantly reduces \textit{Sh} transcript levels, mirroring the effect on \textit{Sh} current. Values are mean ± SEM, *p < 0.05, **p < 0.01.

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**References**

homeostatic mechanisms, which maintain consistency in action potential firing. These mechanisms, not present in muscle, can modify expression of other channels to compensate for any enforced perturbation to membrane excitability that would be caused, in this instance, by manipulation of $Sh$ expression (Baines et al., 2001; Wolfram et al., 2012). Validation of the effects we report here in muscle will, of course, require analysis of the effect of TF expression in central neurons. An attractive possibility, if endogenous levels of individual TFs can be accurately determined, is to use our understanding of Isl and Lim3 to predict levels of Sh-dependent $K^+$ current in individual motoneurons, which can be subsequently confirmed by electrophysiology.

An important question to be addressed is how coexpression of Isl and Lim3 differs from expression of Isl alone in being able to repress Sh expression. The mammalian homologs, Isl1 and Lhx3, have been shown to form a hexameric complex, together with the self-dimerizing cofactor NL1, to promote motoneuron fate (Lee et al., 2012). Although the Lhx3/Isl1 complex and Isl1 or Lhx3 response elements share A/T-rich sequences, each site is unique (Lee et al., 2008). Isl1 binds a DNA motif containing a core TAAT sequence (Yaden et al., 2005; Lee et al., 2008; Mazzoni et al., 2013). TAAT motif sites occur at a very high frequency in the genome; therefore, examining this site alone is not particularly informative. The first report to describe an Isl1 binding site (Boam and Docherty, 1989) identified a CTAATG, which is present at one of the sites of Isl binding at the Sh locus. Lee et al. (2008) reported a predicted site for Lhx3 binding (AATTAGTTAATT) and a hybrid motif (ATTAGCNTAATT), which is bound by Isl1:Lhx3 dimers. We do not observe the Lhx3 (i.e., Lim3) motif but do find a highly similar ATTAGTTAATT “dimer” motif at a site where both Isl and Lim3 bind the Sh locus (coordinates 17950735–17950745; Fig. 2). Our identification of multiple genes for Isl and Lim3 in Drosophila affords significant opportunities to refine the identification of response elements in this model genome. This may allow identification of not only additional gene targets but will also have significant comparative value for identification of mammalian response elements.

In conclusion, sufficient studies have been reported to show that the TFs required for specification of neuron identity, such as Isl, Lim3, Hb9, and Eve, are present in all nervous systems and, moreover, that the developmental mechanisms they regulate are conserved. Our present DamID results, together with previous studies (Pym et al., 2006; Wolfram et al., 2012), provide experimental evidence that these regulatory factors orchestrate significant aspects of both the morphological and electrical development of embryonic neurons. For at least one gene, but likely more, this regulation is likely combinatorial. The use of Drosophila to describe the gene targets and underlying mechanisms through which these TFs operate will make a significant advance to understanding of neural circuit development.