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Competition between LIM-binding domains

Jacqueline M. Matthews[1], Mugdha Bhati, Vanessa J. Craig, Janet E. Deane, Cy Jeffries, Christopher Lee, Amy L. Nancarrow, Daniel P. Ryan and Margaret Sunde

School of Molecular and Microbial Biosciences, The University of Sydney, NSW 2006, Australia

Abstract

LMO (LIM-only) and LIM-HD (LIM-homeodomain) proteins form a family of proteins that is required for myriad developmental processes and which can contribute to diseases such as T-cell leukaemia and breast cancer. The four LMO and 12 LIM-HD proteins in mammals are expressed in a combinatorial manner in many cell types, forming a transcriptional 'LIM code'. The proteins all contain a pair of closely spaced LIM domains near their N-termini that mediate protein–protein interactions, including binding to the ~30-residue LID (LIM interaction domain) of the essential co-factor protein Ldb1 (LIM domain-binding protein 1). In an attempt to understand the molecular mechanisms behind the LIM code, we have determined the molecular basis of binding of LMO and LIM-HD proteins for $Ldb1_{LID}$ through a series of structural, mutagenic and biophysical studies. These studies provide an explanation for why Ldb1 binds the LIM domains of the LMO/LIM-HD family, but not LIM domains from other proteins. The LMO/LIM-HD family exhibit a range of affinities for Ldb1, which influences the formation of specific functional complexes within cells. We have also identified an additional LIM interaction domain in one of the LIM-HD proteins, Isl1. Despite low sequence similarity to $Ldb1_{LID}$, this domain binds another LIM-HD protein, Lhx3, in an identical manner to $Ldb1_{LID}$. Through our and other studies, it is emerging that the multiple layers of competitive binding involving LMO and LIM-HD proteins and their partner proteins contribute significantly to cell fate specification and development.

Introduction

LMO (LIM-only) and LIM-HD (LIM-homeodomain) proteins are closely related protein families that have roles in determining cell fate, and are required for the correct development of many different organs and tissue types (e.g. reviewed in [1]). Both protein families are characterized by two closely spaced LIM domains at or near their N-termini (Figure 1A). LIM domains are a class of zinc finger that ligate two zinc ions, and function as protein-binding domains. The name LIM is derived from the first three proteins in which the motif was found: Lin-11, Isl1 and Mec-3 [2,3]. Whereas LMO proteins contain little additional sequence, LIM-HD proteins also contain a centrally located HD that binds AT-rich sites on DNA and a C-terminal domain, which in most cases is not well characterized. The LIM and HDs are highly conserved, but the C-terminal domains are generally diverse in sequence and are predicted to be largely unstructured. LMO and LIM-HD proteins are also related to LIM-kinase proteins, which resemble LIM-HD proteins but have a kinase domain in place of an HD.

LIM-HD proteins are transcription factors, and LMO proteins, despite having no direct DNA-binding activity, are also involved in transcriptional regulation through association with other transcription factors. Humans have genes for 12 LIM-HD [Lhx1–6, Lhx8 (Lhx8 is sometimes also referred to as Lhx7), Lhx9, Isl1, Isl2, Lmx1a and Lmx1b] and four LMO proteins (LMO1–4), and splice variants

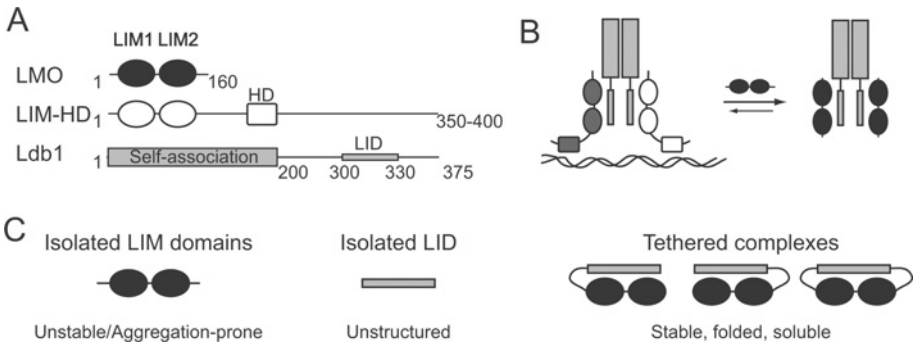
Key words: apterous, competitive binding, LIM domain-binding protein 1 (Ldb1), LIM interaction domain (LID), LIM-homeodomain (LIM-HD), LIM-only (LMO).

Abbreviations used: Ap, apterous; GST, glutathione transferase; Ldb1, LIM domain-binding protein 1; LID, LIM interaction domain; LIM-HD, LIM-homeodomain; LMO, LIM-only.

[1]To whom correspondence should be addressed (email j.matthews@usyd.edu.au).

Figure 1 | LMO, LIM-HD and Ldb1 complexes

(**A**) Schematics of LMO, LIM-HD and Ldb1 proteins. (**B**) Ldb1 contributes to the LIM code by binding multiple LMO/LIM-HD proteins and/or allowing different LMO/LIM-HD proteins to compete for binding. (**C**) Isolated LIM domains from LMO/LIM-HD proteins are prone to aggregation, but are stabilized by tethering to the $Ldb1_{LID}$.



for many of these proteins have been detected. Different combinations of LIM-HD, LMO and co-factor proteins appear to be important in specifying a wide range of different cell types, particularly in the developing central nervous system. These combinatorial expression patterns form what is referred to as a transcriptional 'LIM code' [4,5]. The most important co-factor protein for these proteins is the widely expressed Ldb1 (LIM domain-binding protein 1; also known as CLIM2, NLI and CHIP), which binds the LIM domains of LIM-HD and LMO, but not the closely related LIM-kinase proteins. This interaction is mediated by the 30-residue region known as the LID (LIM interaction domain) [6–8]. LID is highly conserved in Ldb proteins across a wide range of species (reviewed in [9]). Ldb proteins also contain an N-terminal self-association domain, which is required for the biological activity of LIM-HD proteins [10,11]. This self-association domain permits Ldb1 oligomers to form complexes with at least two different target LIM-proteins [8], and this ability to potentially form heterocomplexes is one mechanism by which Ldb1 could contribute to the LIM code (Figure 1B). Another mechanism is the competition of different LIM proteins for the same target in $Ldb1_{LID}$. For example, in *Drosophila* the LIM-HD protein Ap (apterous) up-regulates the expression of dLMO, which then down-regulates the expression of Ap by competing for binding to CHIP (the *Drosophila* homologue of Ldb1; Figure 1B) [12–15]. It is likely that this sort of competition is very common, particularly in the developing central nervous system where LMO, LIM-HD and Ldb genes show complex and dynamic patterns of expression [16]. We wanted to explore the molecular basis of selectivity and competition that contribute to protein complex formation involving LMO, LIM-HD and Ldb proteins.

Generating stable LMO and LIM-HD complexes

The LIM domains from LMO and LIM-HD proteins are difficult to produce in a recombinant fashion; they tend to be insoluble and are prone to aggregation. In order to overcome this problem, we developed a strategy whereby the LIM domains are tethered to $Ldb1_{LID}$ by a flexible linker (Figure 1C) [17]. Tethering the two domains increases the stability of the complexes [18], and this allows the expression and purification of soluble protein complexes. The tethered complexes can be generated in either orientation (LIM–LID or LID–LIM), or as a circularized complex as shown for $LMO4–Ldb1_{LID}$ [18] using a mini-split intein expression system [19,20]. The tethered (intramolecular) complexes form the same structure as intermolecular complexes. This was shown by engineering a protease site into the linker and comparing NMR spectra of tethered with cleaved complexes; $^{1}H,^{15}N$-HSQC spectra of the intra- and intermolecular complexes are identical [7,21,22].

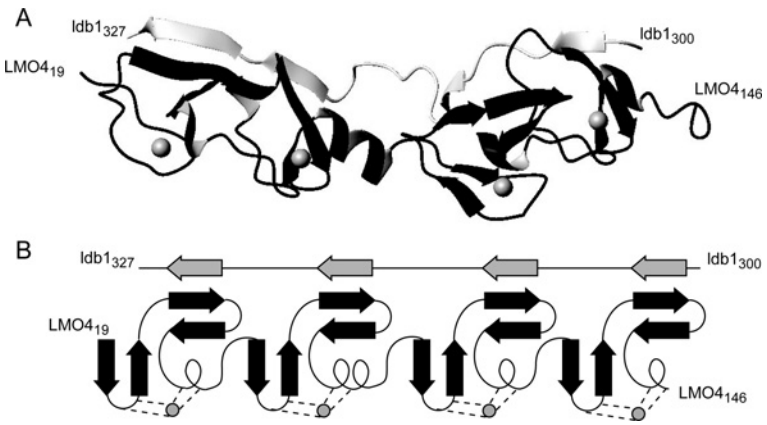
To date we have determined the structures of $Ldb1_{LID}$ in complex with the N-terminal LIM domains of LMO2 and LMO4, and the tandem LIM domains of Lhx3 by NMR methods [21,22], and the structure of $LMO4_{LIM1+2}–Ldb1_{LID}$ by X-ray crystallography [7,18]. The structures of LIM–LID complexes reveal why the tethering strategy works so well; the proteins all bind in a head-to-tail fashion with the N-terminus of one binding domain lying close to (~10 Å) the C-terminus of the other and vice versa (e.g. Figure 2A). We have also used a tethered $LMO2–Ldb1_{LID}$ complex to characterize the assembly of a multiprotein–DNA complex that is essential for normal blood cell development, but which also contributes to T-cell leukaemia [23].

Promiscuity versus specificity of LIM-binding by Ldb1

The structures also reveal why the 30-residue $Ldb1_{LID}$ can bind the LIM domains of up to 16 different LMO and LIM–HD proteins (which have sequence identities of as low as 35% within the LIM domains) but not to the LIM domains from other proteins, such as the LIM kinases, which have closely related LIM domains. $Ldb1_{LID}$ forms an extended structure that binds across both LIM domains; it forms short segments of β-strand that augment β-hairpins in the LIM domains. This tandem β-zipper arrangement (Figure 2B) [24] means that $Ldb1_{LID}$ forms a number of intermolecular

Figure 2 | **The structure of an LMO4–Ldb1$_{LID}$ complex**

(**A**) Ribbon diagram showing Ldb1 in grey and LMO4 in black. The zinc ions in LMO4 are shown as grey spheres (Protein Data Bank accession code 1RUT). (**B**) The tandem β-zipper arrangement of the LMO4–Ldb1$_{LID}$ complex.



backbone–backbone hydrogen bonds that are additionally stabilized by extensive hydrophobic side chain interactions. Both of these types of interactions do not require strong sequence conservation. Specificity of binding appears to be conferred, at least in part, by a small number of salt-bridges and other hydrogen bonds across the interfaces. Standard sequence alignments of the LIM domains from LIM-kinase proteins and LMO/LIM-HD proteins do not reveal any obvious differences between the Ldb1-binding and non-Ldb1-binding LIM domains. However, when the Ldb1-binding residues were identified through analysis of the structures it became evident that a very short hydrophobic-rich stretch of residues that contact Ldb1$_{LID}$ is highly conserved in the N-terminal LIM domains of the LMO/LIM-HD proteins but not the LIM-kinase proteins. Mutation of those residues in LMO4 to the corresponding LIM-kinase residues abolished binding of Ldb1, indicating that those residues contribute to specificity of binding [21].

Competing for binding to Ldb1

In order to understand whether Ldb1$_{LID}$ has different preferences for LMO and LIM-HD proteins, we developed a competition ELISA strategy for measuring binding affinities using versions of LIM–LID complexes with protease sites within the linker. Briefly, cleaved GST (glutathione transferase)–LIM–LID complexes were bound to ELISA plates that had been precoated with anti-GST antibodies. Different concentrations of tagged Ldb1$_{LID}$ were allowed to compete off the cleaved LID peptide and the levels of bound tagged Ldb1$_{LID}$ were measured with standard ELISA methods using antibodies against the tag (usually FLAG) [7,22,26]. Using this approach, we have measured the dissociation constants of binding of Ldb1$_{LID}$ for the LIM domains of LMO4 ($K_d = 10$ nM), LMO2 ($K_d = 20$ nM), Lhx3 ($K_d = 35$ nM) and Isl1 ($K_d = 90$ nM) [22,26]. Thus, for this set of proteins, the LMO proteins bind more effectively to Ldb1 than do the LIM-HD proteins, providing a biophysical mechanism by which LMO proteins can down-regulate the activity of LIM-HD proteins by competing for Ldb proteins.

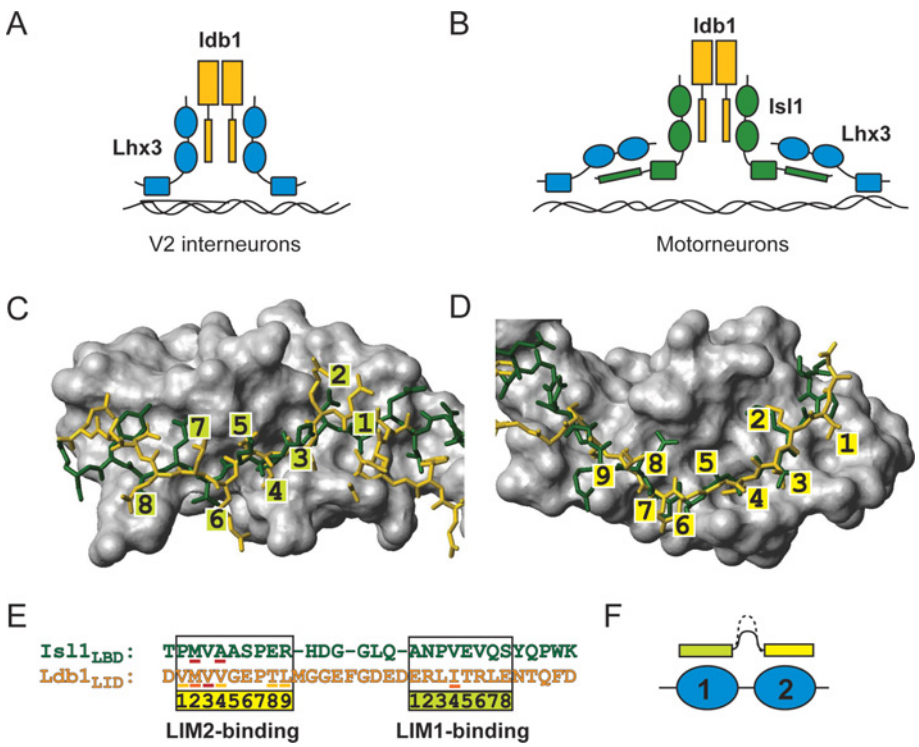
Decoy LIM interaction domains help specify cell type

This competition may extend beyond competition of LIM-containing proteins for Ldb. It has been known for several years that some LIM-HD proteins can interact with each other; Isl1 (and Isl2) can bind Lhx3 in a non-Ldb1-dependent manner [27]. The interaction between Isl1 and Lhx3 is important in the developing ventral spinal cord. Ldb1 and Lhx3 are expressed in and are essential for the development of V2 interneurons, whereas Isl1 is additionally required to specify motor neurons [10]. In V2 interneurons, the LIM domains of Lhx3 bind Ldb1$_{LID}$ to form a binary complex, whereas in motor neurons a ternary complex is formed in which the LIM domains of Isl1 bind Ldb1$_{LID}$, and Lhx3 instead binds the C-terminal region of Isl1 (Figures 3A and 3B) [10].

We recently identified the Lhx3-binding domain on Isl1 (Isl1$_{LBD}$), which, like Ldb1$_{LID}$, is a 30-residue intrinsically unstructured domain [22]. Structures of both Ldb1$_{LID}$ and Isl1$_{LDB}$ bound to Lhx3 indicate that, despite low sequence similarities, both Ldb1 and Isl1 bind Lhx3 in an essentially identical manner (Figures 3C and 3D). The only significant differences in the two structures occur in the regions between the two LIM domains. The angles between the two LIM domains differ in the two complexes, as do the structures of binding peptides where they straddle the LIM domains [22]. A comparison of the structures of the different complexes, combined with a structure-based sequence alignment, and alanine scanning mutagenic screens of the binding domains, reveals that Ldb1$_{LID}$ and Isl1$_{LBD}$ comprise two binding motifs of around nine residues, separated by different length linkers (nine for Ldb1$_{LID}$ and six for Isl1$_{LBD}$; Figure 3E). These results suggest that intrinsically unstructured LMO and LIM-HD interaction domains recognize the tandem

Figure 3 | $Ldb1_{LID}$ and $Isl1_{LBD}$ compete for binding the same site on Lhx3

(**A**) Schematic of the binary complex found in developing V2 interneurons. (**B**) Schematic of the ternary complex formed in post-mitotic motor neurons. (**C** and **D**) Comparison of $Ldb1_{LID}$ (yellow) and $Isl1_{LBD}$ (green) binding to the LIM1 and LIM2 domains of Lhx3 (grey) respectively. Structures (Protein Data Bank accession codes 2JTN and 2RGT) were aligned over the backbone atoms of the relevant LIM domain, and the surface of Lhx3 from the Lhx3-Isl1 structure is shown in grey. Numbers refer to structurally equivalent residue positions in the interaction domains. (**E**) Structure-based sequence alignment of $Ldb1_{LID}$ (yellow) and $Isl1_{LBD}$ (green), structurally equivalent residues are boxed and numbered. Underlined residues were identified as important for binding from mutagenic screens with the relative effect indicated: strongest (red), moderate (orange) and weaker (gold) residues. (**F**) Tandem linear motifs bind tandem LIM domains. Blue represents LIM domains, yellow and light green boxes represent binding motifs from LIM-interaction domains, separated by linkers of different lengths.



LIM domains of their target proteins through tandemly arrayed linear motifs (Figures 3E and 3F). The binding of these linear motifs appears to be directional, i.e. the N-terminal linear motif binds the C-terminal LIM domain, and the C-terminal linear motif binds the N-terminal LIM domain, but not vice versa [7,26]. This modular mechanism of interaction allows pairs of weak-to-moderate affinity 'half-complexes' to form tighter complexes and may have facilitated the fine-tuning of binding affinities that control which LIM domain–interaction domain complexes form in different cells at various stages of development.

Although the Lhx3–$Ldb1_{LID}$ and Lhx3–$Isl1_{LBD}$ complexes look very similar, the latter appears to have significantly lower binding affinity. Indeed, both LIM domain interaction–domain interactions in the ternary motor neuron complex (Lhx3–$Isl1_{LBD}$ and Isl1–$Ldb1_{LID}$; Figure 3B) are weaker than the Lhx3–$Ldb1_{LID}$ interaction in the binary V2 interneuron complex (Figure 3A). Thus the formation of the ternary complex would only be favoured at very high-protein concentrations, but not at the low-protein concentrations expected in the cell. Modelling of the many binding equilibria in this system indicates that the ternary complex should only form through avidity effects if all of the HDs from the LIM-HD proteins bind the same fragment of DNA [22]. This prediction based on binding data is consistent with several different experimental observations.

Thaler et al. [10] used a differentiation assay to show that motor neurons are produced only when ternary complexes that contained functional HDs from both Isl1 and Lhx3 were allowed to form [10]. In line with this, both Isl1 and Lhx3 are required to bind the promoter of *HB9*, the key gene up-regulated by Isl1/Lhx3 in motorneurons; neither protein alone can bind [28]. Other studies have shown that binary Isl1–Ldb1 or Lhx3–Ldb1, and ternary complexes, all appear to recognize different target sites on DNA [29]. Thus the binary and ternary complexes can target different genes to allow the specification of different cell types. There are additional layers of control via competitive binding

throughout motor neuron and V2 interneuron differentiation. For example, the HD-containing protein product of *HB9* acts in synergy with LMO4 to specifically down-regulate the binary Lhx3–Ldb1 complex; HB9 can bind to and compete for Lhx3 target sites on DNA, whereas LMO4 can compete with Lhx3 for binding to Ldb1 [29].

In summary, LMO and LIM-HD proteins and their partners are involved in multiple competitive binding events. By considering structural and biophysical results along with detailed functional studies, we now have a detailed molecular understanding of how the members of this network of proteins interact to control key transcriptional events. However, there is still much to discover about the specific roles and mechanisms of action of this important family of proteins.

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References

1 Bach, I. (2000) The LIM domain: regulation by association. Mech. Dev. **91**, 5–17
2 Freyd, G., Kim, S.K. and Horvitz, H.R. (1990) Novel cysteine-rich motif and homeodomain in the product of the *Caenorhabditis elegans* cell lineage gene lin-11. Nature **344**, 876–879
3 Karlsson, O., Thor, S., Norberg, T., Ohlsson, H. and Edlund, T. (1990) Insulin gene enhancer binding protein Isl-1 is a member of a novel class of proteins containing both a homeo- and a Cys-His domain. Nature **344**, 879–882
4 Gill, G.N. (2003) Decoding the LIM development code. Trans. Am. Clin. Climatol. Assoc. **114**, 179–189
5 Shirasaki, R. and Pfaff, S.L. (2002) Transcriptional codes and the control of neuronal identity. Annu. Rev. Neurosci. **25**, 251–281
6 Agulnick, A.D., Taira, M., Breen, J.J., Tanaka, T., Dawid, I.B. and Westphal, H. (1996) Interactions of the LIM-domain-binding factor Ldb1 with LIM homeodomain proteins. Nature **384**, 270–272
7 Deane, J.E., Ryan, D.P., Sunde, M., Maher, M.J., Guss, J.M., Visvader, J.E. and Matthews, J.M. (2004) Tandem LIM domains provide synergistic binding in the LMO4:Ldb1 complex. EMBO J. **23**, 3589–3598
8 Jurata, L.W. and Gill, G.N. (1997) Functional analysis of the nuclear LIM domain interactor NLI. Mol. Cell. Biol. **17**, 5688–5998
9 Matthews, J.M. and Visvader, J.E. (2003) LIM-domain-binding protein 1: a multifunctional cofactor that interacts with diverse proteins. EMBO Rep. **4**, 1132–1137
10 Thaler, J.P., Lee, S.K., Jurata, L.W., Gill, G.N. and Pfaff, S.L. (2002) LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein–protein interactions. Cell **110**, 237–249
11 van Meyel, D.J., O'Keefe, D.D., Thor, S., Jurata, L.W., Gill, G.N. and Thomas, J.B. (2000) Chip is an essential cofactor for apterous in the regulation of axon guidance in *Drosophila*. Development **127**, 1823–1831
12 Fernandez-Funez, P., Lu, C.H., Rincon-Limas, D.E., Garcia-Bellido, A. and Botas, J. (1998) The relative expression amounts of apterous and its co-factor dLdb/Chip are critical for dorso-ventral compartmentalization in the *Drosophila* wing. EMBO J. **17**, 6846–6853
13 Milan, M. and Cohen, S.M. (1999) Regulation of LIM homeodomain activity *in vivo*: a tetramer of dLDB and apterous confers activity and capacity for regulation by dLMO. Mol. Cell **4**, 267–273
14 Milan, M., Diaz-Benjumea, F.J. and Cohen, S.M. (1998) Beadex encodes an LMO protein that regulates Apterous LIM-homeodomain activity in *Drosophila* wing development: a model for LMO oncogene function. Genes Dev. **12**, 2912–2920
15 Rincon-Limas, D.E., Lu, C.H., Canal, I. and Botas, J. (2000) The level of DLDB/CHIP controls the activity of the LIM homeodomain protein apterous: evidence for a functional tetramer complex *in vivo*. EMBO J. **19**, 2602–2614
16 Bulchand, S., Subramanian, L. and Tole, S. (2003) Dynamic spatiotemporal expression of LIM genes and cofactors in the embryonic and postnatal cerebral cortex. Dev. Dyn. **226**, 460–469
17 Deane, J.E., Sum, E., Mackay, J.P., Lindeman, G.J., Visvader, J.E. and Matthews, J.M. (2001) Design, production and characterization of FLIN2 and FLIN4: the engineering of intramolecular ldb1:LMO complexes. Protein Eng. **14**, 493–499
18 Jeffries, C., Graham, S., Stokes, P.H., Collyer, C.A., Guss, J.M. and Matthews, J.M. (2006) Stabilization of a binary protein complex through intein-mediated circularization. Protein Sci. **15**, 2612–2618
19 Williams, N.K., Liepinsh, E., Watt, S.J., Prosselkov, P., Matthews, J.M., Attard, P., Beck, J.L., Dixon, N.E. and Otting, G. (2005) Stabilization of native protein fold by intein-mediated covalent cyclization. J. Mol. Biol. **346**, 1095–1108
20 Williams, N.K., Prosselkov, P., Liepinsh, E., Line, I., Sharipo, A., Littler, D.R., Curmi, P.M., Otting, G. and Dixon, N.E. (2002) *In vivo* protein cyclization promoted by a circularly permuted *Synechocystis* sp. PCC6803 DnaB mini-intein. J. Biol. Chem. **277**, 7790–7798
21 Deane, J.E., Mackay, J.P., Kwan, A.H., Sum, E.Y., Visvader, J.E. and Matthews, J.M. (2003) Structural basis for the recognition of ldb1 by the N-terminal LIM domains of LMO2 and LMO4. EMBO J. **22**, 2224–2233
22 Bhati, M., Lee, C., Nancarrow, A.L., Lee, M., Craig, V.J., Bach, I., Guss, J.M., Mackay, J.P. and Matthews, J.M. (2008) Implementing the LIM code: the structural basis for cell type-specific assembly of complexes recruited by LIM homeodomain transcription factors. EMBO J. **27**, 2018–2029
23 Ryan, D.P., Duncan, J.L., Lee, C., Kuchel, P.W. and Matthews, J.M. (2008) Assembly of the oncogenic DNA-binding complex LMO2-Ldb1-TAL1-E12. Proteins **70**, 1461–1474
24 Schwarz-Linek, U., Werner, J.M., Pickford, A.R., Gurusiddappa, S., Kim, J.H., Pilka, E.S., Briggs, J.A., Gough, T.S., Hook, M., Campbell, I.D. et al. (2003) Pathogenic bacteria attach to human fibronectin through a tandem β-zipper. Nature **423**, 177–181
25 Reference deleted
26 Ryan, D.P., Sunde, M., Kwan, A.H.-Y., Marianayagam, N.J., Nancarrow, A.L., vanden Hoven, R.N., Thompson, L.S., Baca, M., Mackay, J.P., Visvader, J.E. et al. (2006) Identification of the key LMO2-binding determinants on Ldb1. J. Mol. Biol. **359**, 66–75
27 Jurata, L.W., Pfaff, S.L. and Gill, G.N. (1998) The nuclear LIM domain interactor NLI mediates homo- and heterodimerization of LIM domain transcription factors. J. Biol. Chem. **273**, 3152–3157
28 Lee, S.K. and Pfaff, S.L. (2003) Synchronization of neurogenesis and motor neuron specification by direct coupling of bHLH and homeodomain transcription factors. Neuron **38**, 731–745
29 Lee, S., Lee, B., Joshi, K., Pfaff, S.L., Lee, J.W. and Lee, S.K. (2008) A regulatory network to segregate the identity of neuronal subtypes. Dev. Cell **14**, 877–889

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