The oncogenic T cell LIM-protein Lmo2 forms part of a DNA-binding complex specifically in immature T cells

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The LIM-only protein LMO2 is expressed aberrantly in acute T-cell leukaemias as a result of the chromosomal translocations t(11;14) (p13;q11) or t(7;11) (q35;p13). In a transgenic model of tumorigenesis by *Lmo2*, T-cell acute leukaemias arise after an asymptomatic phase in which an accumulation of immature CD4[−] CD8[−] double negative thymocytes occurs. Possible molecular mechanisms underlying these effects have been investigated in T cells from *Lmo2* transgenic mice. Isolation of DNA-binding sites by CASTing and band shift assays demonstrates the presence of an oligomeric complex involving Lmo2 which can bind to a bipartite DNA motif comprising two E-box sequences ~10 bp apart, which is distinct from that found in erythroid cells. This complex occurs in T-cell tumours and it is restricted to the immature CD4[−] CD8[−] thymocyte subset in asymptomatic transgenic mice. Thus, ectopic expression of *Lmo2* by transgenesis, or by chromosomal translocations in humans, may result in the aberrant protein interactions causing abnormal regulation of gene expression, resulting in a blockage of T-cell differentiation and providing precursor cells for overt tumour formation.

Keywords: chromosomal translocation/leukaemia/LIM/LMO2/TAL1

Introduction

Chromosomal translocations are found as recurring features in human tumours and have led to the identification of novel oncogenes whose functions in tumour development frequently involve transcriptional regulation (Rabbitts, 1994). One such oncogene is *LMO2* (formerly called *RBTN2* or *TTG2*) which is activated in T-cell acute leukaemias (T-ALLs) by the chromosomal translocations t(11;14) (p13;q11) or t(7;11) (q35;p13) (Boehm *et al.*, 1991a; Royer-Pokora *et al.*, 1991). This gene encodes a nuclear LIM-domain protein which is necessary for embryonic erythropoiesis (Warren *et al.*, 1994) and for adult haematopoiesis (Yamada *et al.*, 1998).

The models of *Lmo2*-driven T-cell tumours have

involved expression of the gene in thymocytes using the promoter/enhancer of the T-cell gene *CD2* (Lang *et al.*, 1988) to drive *Lmo2* expression (Fisch *et al.*, 1992; Larson *et al.*, 1994) or the metallothionein promoter (Neale *et al.*, 1995). A similar situation has been reported for the family member *Lmo1* (Fisch *et al.*, 1992; McGuire *et al.*, 1992). The period before *Lmo2*-induced tumours arise is long, the first ones appearing after ~6 months of age, implying that mutations (in other genes) need to occur before overt disease appears. During this asymptomatic phase, there are marked changes in the thymus T-cell population, principally an increase in immature T cells lacking CD4 and CD8 surface markers (herein referred to as DN thymocytes) (Larson *et al.*, 1995; Neale *et al.*, 1995). The number of T cells in the DN population varies between individual transgenic mice, but increases can be seen in mice soon after birth. Thus it appears that the primary effect of the *Lmo2* transgene is exerted on these immature T cells (Larson *et al.*, 1995), suggesting that this is also the target population in patients with the chromosomal translocation t(11;14) or t(7;11).

The molecular function of the LIM protein Lmo2 in this tumorigenesis is as yet unclear. It is known that in normal erythroid cells, the Lmo2 protein interacts with the basic-helix-loop-helix protein (bHLH) Tal1/Scl (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994) (herein referred to as Tal1). In addition, while *Tal1* transgenic mice (in which the *Tal1* gene is driven by the same *CD2* promoter as used for the *Lmo2* transgene) do not develop leukaemias (Robb *et al.*, 1995b; Larson *et al.*, 1996), nor show any perturbation of T-cell development, *Lmo2*-*Tal1* double transgenic mice exhibit an accelerated tumour development profile compared with *Lmo2*-only mice (Larson *et al.*, 1996). However, in transgenic mice with *Tal1* driven by the *lck* promoter, a proportion of mice developed tumours but there was no reported alteration of surface phenotype of thymocytes prior to tumour induction (Condorelli *et al.*, 1996; Kelliher *et al.*, 1996). Further, double transgenic mice (co-expressing Lmo2 and Tal1) have high levels of thymic DN immature T cells at non-leukaemic stages, even as soon as they are born, showing a marked change in the differentiation pathway resulting in a partial block in the progression to mature CD4⁺ CD8⁺ CD3⁺ T cells (Larson *et al.*, 1996). Thus, the interaction of Lmo2 and Tal1 proteins seems to have a synergistic effect on tumour formation as well as on T-cell differentiation. Such a situation may also occur in some human T-ALLs which acquire both a chromosomal translocation involving *LMO2* and also a chromosomal abnormality involving *TAL1* (Wadman *et al.*, 1994).

Lmo2 is expressed normally in cells of erythroid/myeloid origin (Warren *et al.*, 1994), central nervous system, lung, kidney, liver and spleen, but not in thymus (Feroni *et al.*, 1992). Recent data on the function of Lmo2

have shown that it plays a crucial role in the development of primitive erythroid cells (Warren *et al.*, 1994) and in adult haematopoiesis (Yamada *et al.*, 1998). These functions seem to be mediated through protein–protein interactions involving the LIM-domains of Lmo2, which bind to Tal1 (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994), GATA-1 (Osada *et al.*, 1995) and to the LIM-domain-binding protein Ldb1/Nli (Agulnick *et al.*, 1996; Jurata *et al.*, 1996). Altogether, they form an oligomeric DNA-binding complex in erythroid cells, which also includes the ubiquitously expressed class A bHLH proteins E47/E12, products of the *E2A* gene (Wadman *et al.*, 1997). The bipartite DNA motif recognized by the oligomeric complex is an E-box (CAGGTG) followed 9 bp downstream by a GATA site. Common target genes regulated by this complex might be responsible for the similar phenotype of mice lacking *Lmo2* (Warren *et al.*, 1994), *Tal1* (Robb *et al.*, 1995a; Shivdasani *et al.*, 1995) or *Gata-1* (Pevny *et al.*, 1991). On the other hand, a negative regulatory function of Lmo2 and Ldb1 during erythroid differentiation has been suggested (Visvader *et al.*, 1997), contrary to an activating role of Tal1 and GATA-1 in the same model cell line. In contrast to GATA-1, whose functions seem to be restricted to the erythroid (Pevny *et al.*, 1991) and megakaryocyte lineage (Shivdasani *et al.*, 1997), Tal1 and Lmo2 seem to share a broader range of action in haematopoiesis as null mutations in either of these genes cause failure of adult haematopoiesis (Porcher *et al.*, 1996; Robb *et al.*, 1996; Yamada *et al.*, 1998).

The occurrence of Lmo2 in a complex which contains known DNA-binding proteins (Wadman *et al.*, 1997) suggests that Lmo2 functions in haematopoiesis via its ability to carry out protein contacts. Thus the mode of function may be to facilitate formation of a DNA-binding complex or to participate in a complex which depletes the intracellular concentrations of the DNA-binding protein(s) and thereby affects their normal function. This suggests that the most obvious model for the role of LMO2 in T-ALL is that the protein binds to factors in T cells after the chromosomal translocation, creating an aberrant complex which operates either via binding to DNA or by a sequestration mechanism. This proposal has been investigated utilizing *Lmo2* transgenic mouse T cells to study molecular complexes involving Lmo2. We have detected a DNA-binding complex involving Lmo2 in transgenic tumours and thymi of asymptomatic mice which binds to a bipartite site consisting of two E-boxes separated by an ~10 bp spacer. The complex has some striking similarities to the erythroid-specific Lmo2-containing DNA-binding complex but recruits a second bHLH heterodimer instead a GATA factor into the oligomeric complex. Tal1 can be part of the oligomeric complex. This complex is present in primary tumours from transgenic mice and it seems to be restricted to the immature DN thymocyte subpopulation in asymptomatic transgenics.

Results

Characteristics of a transgenic T-cell line

CD2-Lmo2 transgenic mice develop T-cell tumours from ~6 months of age onwards (Fisch *et al.*, 1992; Larson *et al.*, 1994). We isolated cell lines from these tumours in order to study the biochemical function of Lmo2 protein.

One such line, designated 2114, was made from a transgenic tumour which contains multiple copies of the transgene as judged by filter hybridization of genomic DNA extracted from 2114 cells (Figure 1A, right panel), and lacked expression of CD4 and CD8 surface markers, which is a characteristic feature of immature T cells. The derived cell line 2114 corresponded to this early differentiation stage of thymocytes as fluorescence activated cell sorting (FACS) analysis of 2114 cells (Figure 1C) showed no detectable CD3, CD4 or CD8. But expression of the Thy1.2 T-cell marker as well as CD25 and CD44, which are typical markers for early DN thymocytes (i.e. CD4⁺ CD8⁺) undergoing T-cell receptor (TCR) β -gene rearrangement were detected. The status of TCR β genes in 2114 was examined by filter hybridization with a TCR J β 2 probe (Figure 1A) showing one major rearrangement presumably because one allele of TCR β has undergone correct V–D–J joining. In addition, two bands with weaker hybridization are present, presumably reflecting the breakage within the TCR β probe itself on a second rearranged allele. Labelling of these cells with [³⁵S]cysteine showed that the Lmo2 protein was detectable by immunoprecipitation with anti-Lmo2 antiserum (Figure 1B). The 2114 cells, therefore, appear to originate from a transformed DN immature thymocyte expressing *Lmo2*.

DNA-binding site selection (CASTing) with anti-Lmo2 antiserum

As a direct means to assess if the Lmo2 protein, ectopically expressed in the transgenic T cells, was present in a DNA-binding complex with other proteins, *in vitro* DNA-binding site enrichment (CASTing; Pollock and Treisman, 1990; Wright *et al.*, 1991) was carried out using cellular extracts from 2114 cells and a random pool of oligonucleotides. After seven rounds of CASTing, the final selected oligonucleotide pool was cloned and 57 clones were sequenced.

When these sequences were analysed in detail, it was found that 24 clones (42%) contained an E-box motif (recognition sequence for bHLH proteins) (Figure 2), of which 14 clones had two E-boxes in the same sequence. Of the clones with two E-boxes, nine had a 10 bp spacing between the two E-boxes and other spacings occurred [3 bp (1); 9 bp (1); 11 bp (1) and 12 bp (2)]. The most common E-box was CATCTG or, in the complementary reversed form, CAGATG (63% of all E-box motifs), followed by CAGGTG (24% of all E-box motifs). Unlike the situation found with similar experiments using erythroid extracts (Wadman *et al.*, 1997), no clones with an E-box adjacent to a GATA site were isolated. Interestingly, the most common selected E-box (CAGATG) resembles the preferential binding site for E2A–Tal1 heterodimers (Hsu *et al.*, 1994a) including the flanking regions, suggesting an involvement of Tal1 or a Tal1 homologue in the DNA-binding complex.

Lmo2 is involved in a complex in 2114 cells which recognizes a dual E-box sequence

The involvement of Lmo2 protein in the recognition of the selected binding sites from the CASTing experiments (Figure 2) was assessed further by electrophoretic mobility shift assays (EMSAs) and antibody-mediated supershifts using the same 2114 cellular extracts employed for the CASTing experiments. In these experiments, it was found

Fig. 1. Characterization of the T-cell line 2114. The T-cell line 2114 was established in culture from a thymic tumour which arose in a *CD2-Lmo2* transgenic mouse. (A) Southern hybridization analysis of 2114 genomic DNA. *Hind*III-digested DNA from the cell line 2114 was probed with a *Jβ2* probe (Malissen *et al.*, 1984) and compared with a control liver DNA and lymphoid cell lines [J 558L (B-cell line), BW 5147 (T-cell line)]. The same blot was reprobed with a *Lmo2* probe (right panel) to confirm the presence of the transgenic construct. (B) Immunoprecipitation of Lmo2 from ³⁵S-labelled 2114 cellular extracts using anti-Lmo2 antiserum (Warren *et al.*, 1994) compared with rabbit anti-Lmo2 antiserum blocked by peptide antigen (Lmo2 residues 2–17). (C) Cultured cells from mid-log phase were examined by FACS analysis. Cell surface markers tested for in the upper panel are isotype control with rat IgG_{2a}κ-FITC and IgG_{2a}κ-PE or CD4-FITC/CD8-PE double staining. Lower panel: double staining with anti-CD25-FITC and anti-CD44-PE or single staining with anti-Thy1.2-PE compared with isotype control (dotted line).

that only the sequences with two E-boxes separated by 10 bp (herein called the dual E-box oligo) showed an Lmo2-specific involvement (Figure 3). A number of bands were observed in the EMSAs, shown in Figure 3A using oligonucleotides with either one (E-box/mE-box, designated single E-box oligo) or two E-box motifs (E-box/E-box, designated dual E-box oligo). A low mobility band (herein called band I, Figure 3A, lane 2) appeared in mobility shifts with labelled dual E-box compared with labelled single E-box oligo (Figure 3A, lane 4). This low mobility band I was the only one involving the Lmo2

protein as it is supershifted by addition of anti-Lmo2 antiserum and *Staphylococcus aureus* protein A (Figure 3A, lane 1).

To determine further the nature of this Lmo2-containing low mobility band I, we performed competition assays with non-labelled dual E-box oligo or non-labelled single E-box oligo (Figure 3B). The cold single E-box oligo inhibited the appearance of band II, also seen in the band shift with the labelled single E-box oligo (this band must be due to the binding of a bHLH dimer to the single E-box), but it had no effect on the low mobility band I

A Sequences with a single E-box

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1      tcTCCGCGTCGA CATCTG TTCTCTGTCCcg
2      cgGAC CATCTG GCGTTAGAAGCCACGga
3      cgAA CATCTG TTAAAGTGGCGCCTGCaga
4      cgTGTAAGTGG CATGTG CGTACTATTGga
5      tcACCGGAAGGAGAGACAA CAGGTG TTAcg
6      cgAATCCGTCTGGACAAGC CAGGTG GTga
7      tcTAGTC CAGGTG TCTAGTTAGGAGGTCCg
8      cgCTGGGGGCGTAATAGAC CACGTG TGga
9      cgTATC CAAGTG AAACCTAGCCCTAGTAga
10     cgGC CAAATG TCCATATTTGGGCATGTAgga

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B Sequences with two E-boxes

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1      tcTAAGCGAA CATCTG CCC CATCTG TTCcg
2      tcAC CATCTG TTCGCCAGGAAA CAGCTG cg
3      cgTTAC CATCTG TCGTTAGCTATC CAGGTG TTCAAGTGGATga
4      tc CATCTG CCAGGTTTCAAC CAGGTG TTcg
5      tcA CATCTG GCGGC-CTATCA CAGGTG TGcg
6      cgAA CATCTG TTATG--GATCA CAGGTG Tga
7      cgGA CATCTG TTCTG--TAAAA CAGGTG TTga
8      cgAA CATCTG TTTCA--TGCAA CAGGTG TTga
9      tcCG CATCTG GTGCG--CAAAA CAGATG GCcg
10     tcGC CATCTG TTCAC--TATGA CAGATG GTcg
11     cgA CATCTG GACGT--CCTGG CAGATG GTAgga
12     tcTAC CATCTG CCACG--ACGTC CAGATG Tcg
13     tcAG CATCTG TTCTG--CATAA CAGATG TTcg
14     tc CATCTG TGCGA---ACAA CAGATG Tcg

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C Consensus sequence for E-box dimer

1 st E-box										spacer	2 nd E-box									
-2	-1								+1	+2	9-12bp	-2	-1						+1	+2
50	43	0	100	0	0	0	0	0	7		A	50	64	0	100	0	43	0	0	0
14	43	100	0	0	100	0	0	21	36		C	21	29	100	0	0	21	0	0	7
21	14	0	0	0	0	0	100	21	7		G	14	7	0	0	93	43	0	100	21
14	0	0	0	100	0	100	0	57	50		T	14	0	0	0	7	0	100	0	71
A	M	C	A	T	C	T	G	T	T	cons		A	A	C	A	G	R	T	G	T

Fig. 2. Oligonucleotide sequences obtained by Lmo2-binding site selection (CASTing) using cellular extracts from the 2114 T-cell line. Cellular extracts from 2114 cells were mixed with a random oligonucleotide pool (R76; Pollock and Treisman, 1990) and specific complexes immunoprecipitated with anti-Lmo2 antiserum. After seven rounds of binding and immunoprecipitation, the oligonucleotides were cloned and sequenced. (A) Sequences containing one E-box are aligned by their E-box. (B) Sequences containing two E-boxes. The sequences are aligned by their E-box, and the variation in the spacer (9–12 bp) adjusted by adding bars, with the exception of the first sequence containing a 3 bp spacer. Upper case letters represent the random part of the selected sequence, whereas lower case letters represent the start of constant flanking regions. The E-box motifs are presented in bold and separated by a space from the rest of the sequence. (C) Representation of the consensus sequence as a percentage for nucleotides at each position. The E-boxes and the two flanking positions which had a non-random pattern of nucleotide frequency are shown. The consensus sequence generated from a nucleotide frequency >43% is shown in the last row. In the case of an equal nucleotide frequency, the standard IUPAC code has been used (M = A or C, R = A or G).

except at the very highest concentration of competitor (lane 19) when a small inhibition begins to occur. Conversely, cold competitor dual E-box oligo inhibited the formation of three bands, including the low mobility band I, in the same concentration-dependent way (Figure 3B, lanes 8–13). These results show that the dual E-box site is bound by synergistically interacting bHLH dimers, causing an avidity effect, rather than by independently binding bHLH dimers. Thus, while the intermediate mobility band II seems to be due to a complex binding to a single E-box, not including Lmo2, the low mobility band I is due to an Lmo2-containing complex with two DNA-binding elements each binding to one of the E-boxes.

Lmo2, E47, Tal1 and Ldb1 are part of the complex in 2114 cells binding to the dual E-box motif

The low mobility of the band I which binds to the dual E-box motif must be due to an oligomeric complex involving other proteins in addition to Lmo2, as Lmo2 does not seem to bind to DNA. Indeed, an analogous complex found in erythroid cells which binds to an E-box–GATA motif appears to comprise at least five different proteins (Wadman *et al.*, 1997). Candidates for involvement in the low mobility band I were assessed with antibody-mediated supershifts. Anti-Lmo2 antiserum supershifts the dual E-box oligo-specific band I especially in the presence of *S.aureus* protein A, which binds to the

Fig. 3. Band shift assays using cellular extracts from the 2114 T-cell line. Cellular extracts from the transgenic mouse-derived T-cell line 2114 were used in EMSAs with various oligonucleotides. (A) The labelled oligonucleotides were dual E-box (lanes 1–3; CATCTG–N₁₀–CAGATG) or single E-box (lanes 4–6; CATCTG–N₁₀–CCGATC). Antibody supershifts were performed as indicated with antiserum recognizing Lmo2 (lanes 1 and 4) or Tal1 (lanes 3 and 6). (B) Labelled dual E-box oligo (CATCTG–N₁₀–CAGATG) was competed with unlabelled oligonucleotide corresponding to itself or to the single E-box oligo. The right part of the gel (lanes 8–20) shows competition experiments on mobility shifts of 2114 cellular extracts with the ³²P-labelled dual E-box oligo. Lanes 8, 14 and 20 show the mobility shift without addition of competitor. In lanes 9–13, increasing amounts (5×, 10×, 50×, 100×, 300×) of cold dual E-box oligo were added. In comparison, increasing amounts (5×, 10×, 50×, 100×, 300×) of cold single E-box oligo were added to lanes 15–19. For clarity, only the upper part of the gel is presented in the figure. Diagrams of protein complexes which may cause the particular mobility shifts of interest are indicated as symbols at the side of the gel.

Fig. 4. Antibody supershift of dual E-box oligonucleotide-specific complex. Cell extracts from 2114 cells (lanes 1–14) and EL4 T cells (Lmo2 negative; lanes 16–20) were incubated with ³²P-labelled dual E-box oligo (CATCTG–N₁₀–CAGATG) in the absence or presence of specific antisera (with or without *S.aureus* protein A) as indicated. Left panel: 2114 extract. The mobilities of complexes binding to the dual E-box oligo in the absence of antibody are shown in lane 4. Addition of protein A on its own (lane 11) or the appropriate pre-immune sera (lanes 3, 5, 10 and 12) does not alter the pattern of the bands, with the exception of pre-immune serum of the anti-Tal1 antiserum (lane 5). Mobility shifts in the presence of antisera (plus or minus protein A) are as indicated. Right panel: EL4 cell extract. The right part of the gel (lanes 16–20) shows the mobility shift pattern based on incubating 15 µg of EL4 cellular extracts with ³²P-labelled dual E-box oligo without antibody (lane 7) or with antibody (lanes 16 and 11–20). For clarity, only the upper part of the gel is presented in the figure. Complexes thought to cause the particular mobility shifts of interest are indicated at the side of the gel.

Fc part of the anti-Lmo2 antiserum, whereas the pre-immune serum does not have any influence on the mobility of any band (Figure 4, lanes 1–3). The same supershift behaviour can be produced by a second, independent anti-

Lmo2 antiserum from another rabbit (data not shown). The low mobility complex also contains the recently identified Ldb1/Nli (Agulnick *et al.*, 1996; Jurata *et al.*, 1996) since cellular extracts from 2114 cells were subjected

Fig. 5. EMSA with extract from a primary thymoma from a *CD2-Lmo2* transgenic mouse. Mobility shifts and supershift analysis obtained by incubating 10 µg of nuclear extract, from the primary thymoma 2122 derived from a *CD2-Lmo2* transgenic mouse, with 32 P-labelled dual E-box oligo in the presence of sonicated *E.coli* DNA (lanes 1–10), or mobility shifts of 32 P-labelled dual E-box oligo with 10 µg of nuclear extract from non-transgenic mouse thymus (lanes 12–16) or the 2114 cell line (lanes 17–19).

to band shifts with the dual E-box oligo and supershifted with anti-Ldb1 antiserum with or without the presence of protein A (Figure 4). While the band due to the single E-box-binding complex (band II) was not affected by the anti-Ldb1 serum, that of the low mobility complex (band I) was supershifted.

A second binding partner of Lmo2 is the bHLH protein Tal1 (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994). Thus, Tal1 might also be part of the T-cell-specific dual E-box oligo-binding complex. Therefore, we tested the ability of anti-Tal1 antiserum to alter dual E-box oligo mobility shifts and found that the low mobility band I was supershifted (Figure 3A, lane 3), suggesting the presence of Tal1 protein in this complex. Band III is also affected by the Tal1 antiserum, but this is non-specific as the pre-immune serum also causes a supershift of this band (Figure 4, lanes 5). This unexpected presence of Tal1 in the 2114 cell complex was confirmed by the ability of anti-Tal1 serum (Figure 4, lane 6) or antiserum with *S.aureus* protein A (lane 7) specifically to supershift only the low mobility complex. It was surprising to find Tal1 protein in a T-cell line derived from a *CD2-Lmo2* transgenic thymoma since thymus cells do not express Tal1 (Kallianpur *et al.*, 1994; Pulford *et al.*, 1995). The presence of Tal1 in the 2114 cell line was confirmed by RT-PCR of 2114 cDNA employing several sets of Tal1 primers (data not shown) and sequencing of the RT-PCR products [the largest was a 516 bp fragment spanning position 348, which covers part of Tal1 protein used for antiserum preparation (Hsu *et al.*, 1994a), to 864, which corresponds to the end of bHLH region].

The E-box-GATA binding complex in erythroid cells includes the class A bHLH proteins E47/E12, products of the *E2A* gene (Murre *et al.*, 1989; Sun and Baltimore, 1991; Roberts *et al.*, 1993). Accordingly, the involvement of these molecules in the 2114-specific T-cell complex

was tested using an E2A-specific immune serum. This antiserum was found to reduce the mobility of two specific E-box-binding complexes identified by competition experiments (Figure 3B), namely the low mobility complex containing Lmo2 (band I) and the band due to protein(s) binding to a single E-box [band II, which has the same mobility as the E47 homodimer band in the erythroid cell line MEL (Wadman *et al.*, 1997) and, therefore, is presumably due to the E47 homodimer]. Thus the Lmo2-containing low mobility complex (band I) also contains E47/E12, analogous to the oligomeric E-box-GATA binding complex in erythroid cells (Wadman *et al.*, 1997). This dual E-box-binding complex would therefore appear to have a Tal1-E2A bHLH heterodimer bound to each E-box, with Lmo2 and Ldb1/Nli bridging the two heterodimers.

The Lmo2-containing complex is present in primary T-cell tumours arising in transgenic mice

The representative nature of the 2114 cell line was evaluated by examining primary tumours derived from *CD2-Lmo2* transgenic mice or from *CD2-Lmo2*×*CD2-TAL1* double transgenic mice for the presence of the Lmo2-containing band I complex. Crude nuclear extracts from lymphomas of several transgenic mice were incubated with the 32 P-labelled dual E-box oligo, and mobility shift and supershift patterns were studied. An example is shown in Figure 5 using nuclear extracts of thymus cells from a *CD2-Lmo2*-only mouse (2122) which developed a T-cell lymphoma at 12 months of age. The EMSA pattern was compared with the pattern seen with extract from non-transgenic mouse thymus (2 months of age) and 2114 cells. Gel retardation bands obtained with 2122 extracts were similar to those seen in 2114 (Figure 5). The low mobility band I observed with 2122 nuclear extracts contained Lmo2, Tal1, E2A and Ldb1 proteins as judged

by antibody supershifts (Figure 5, lanes 4–7 and 9 respectively). The specificity of band I for transgenic T cells was confirmed since non-transgenic thymus has no complex that binds to the dual E-box motif (Figure 5, lanes 12–16), consistent with the lack of Lmo2 expression in thymus (Foroni *et al.*, 1992). The complex is also absent in cellular extracts from the non-transgenic T-cell thymoma line EL4 (Figure 4). E2A antibodies did, however, cause in both cases a supershift of band II which corresponds to the binding of an E47 homodimer to a single E-box. This same band II is also evident when EL4 (Figure 4) or 2122 extract was used (Figure 5) and, therefore, can be used as an internal standard.

The dual E-box-binding complex is restricted to the CD4[−] CD8[−] double negative transgenic thymocytes

We have observed a variation in intensity of the Lmo2-containing band I relative to the intensity of the E47 homodimer band II in the panel of transgenic lymphomas tested (data not shown). One difference between the lymphomas tested was the differentiation stage of thymocytes, ranging from CD4[−] CD8[−] DN to CD4⁺ or CD8⁺ single positive (SP) (Larson *et al.*, 1994). Thus, the T-cell differentiation stage might be a key determinant in the ability of Lmo2 to enter the complex which can bind the dual E-box site. This possibility was investigated by analysis of T cells from transgenic mice prior to the development of recognizable disease.

During the early stages of thymus development, the transgenic mouse thymi were found to contain nearly normal numbers of T cells but with an increase in CD4[−] CD8[−] DN thymocytes (Larson *et al.*, 1994), particularly apparent in *Lmo2–Tal1* double transgenics (Larson *et al.*, 1996). This suggested that the DN T-cell precursors might be the target cells in which the oncogenic effect of Lmo2 protein is exerted. Therefore, proteins from thymi of transgenic mice at the age of two months were assessed for the presence of the Lmo2-containing band I. Crude cellular extracts from the whole thymi of these mice, in which the level of DN T cells was assessed by FACS analysis, were subjected to EMSAs using the dual E-box oligo. In the case of a pool of T cells extracted from *Lmo2–Tal1* double transgenic mice (comprising ~22% DN cells in the pool), we observed band I which was supershifted with Lmo2 antiserum (Figure 6A, lanes 1–3). No such complex could readily be seen after EMSA with extracts from a pool of T cells from an *Lmo2*-only transgenic (~6% DN cells) (Figure 6A, lanes 11–13), suggesting that the Lmo2-containing complex was only present in the DN cells of asymptomatic mice.

The presence of band I in CD4[−] CD8[−] DN T cells was verified by purification of this cell population from asymptomatic transgenic mice. A positive selection approach was used to prepare the DN thymocyte subpopulation by taking advantage of the presence of the CD25 (IL2R α chain) surface marker, which is expressed on a subset of DN thymocytes. Two cell populations were compared, one selected with anti-CD25 beads and one selected with anti-CD8 beads. Successful selection was assessed by FACS analysis of the depleted fraction (data not shown). EMSA analysis of the unfractionated thymocytes from the *Lmo2–Tal1* double transgenic mice (com-

prising ~23% CD25⁺ cells) produced a weak band I corresponding to the low mobility complex which could be supershifted with both anti-Lmo2 and anti-Tal1 antisera (Figure 6A, lanes 1–3), whereas the single transgenic T cells (6% DN T cells) did not exhibit this complex. However, when the cell population was enriched for CD25-expressing cells, both single and double transgenic T cells exhibit the oligomeric Lmo2-containing complex (band I; Figure 6A, lanes 4–6 and 14–16). Interestingly, Tal1 was detected in band I of both single and double asymptomatic transgenic mice (according to supershifts with anti-Tal1 antiserum). The CD8⁺ cells [i.e. CD4⁺ CD8⁺ double positive (DP) or CD4[−] CD8⁺ SP thymocytes] do not have this complex in detectable amounts but the single E-box-binding complex (band II) appeared in all samples analysed (acting as an internal standard). We conclude, therefore, that the low mobility oligomeric complex is found predominantly in the immature DN T-cell precursors.

One reason for the observed difference between the two thymocyte subpopulations might be different expression levels of Lmo2, and Tal1 in the case of the double transgenic mice, in the DN compared with DP T cells, but this seems unlikely given the use of the *CD2* promoter cassette to control *Lmo2* and *Tal1* transgene expression. However, we carried out Northern hybridization analysis of RNA prepared from CD25⁺ DN T cells and CD8⁺ DP T cells, which revealed that both RNA populations contain high levels of transgenic *Lmo2* as well as RNA coding for the ubiquitously expressed Lmo2-binding partner Ldb1/Nli (Figure 6C). We cannot exclude differences in expression levels of other unidentified component(s) of the dual E-box-binding complex, which might be restricted to the CD25⁺ DN cells.

In summary, the dual E-box-binding complex which contains Lmo2 is present in asymptomatic thymocytes of the two transgenic mouse lines, either expressing Lmo2 alone or in combination with Tal1 under control of the *CD2* promoter (Larson *et al.*, 1996). It seems to be restricted to the CD25⁺ CD4[−] CD8[−] DN thymocyte subset, which represent early thymocytes undergoing TCR β gene rearrangement.

Discussion

Molecular role of Lmo2 in tumorigenesis of transgenic mice

In human T-ALL, the *LMO2* gene is activated by chromosomal translocations which also involve TCR genes (Boehm *et al.*, 1991a; Royer-Pokora *et al.*, 1991). In transgenic mouse models of Lmo2-induced T-cell tumorigenesis (Fisch *et al.*, 1992; Larson *et al.*, 1994; Neale *et al.*, 1995), there are two features which are outstanding. First, the tumours form after a long latency period (>6 months) during which time secondary mutations in other oncogenes or suppressor genes must occur and are necessary for overt tumour formation (Larson *et al.*, 1994; Neale *et al.*, 1995). Second, there is an impairment of T-cell differentiation manifest in the thymus of transgenic mice as an accumulation of CD4[−] CD8[−] DN immature T cells (Larson *et al.*, 1995; Neale *et al.*, 1995). This effect is particularly evident when mice transgenic for both *Lmo2* and *Tal1* are examined (Larson *et al.*, 1996). This

Fig. 6. The protein complex binding to the dual E-box oligonucleotide is found in CD4⁻ CD8⁻ CD25⁺ transgenic thymocytes. Thymocytes were prepared from pools of 10 thymi from 2-month-old asymptomatic *CD2-Lmo2*-only or *CD2-Lmo2*×*CD2-Tal1* double transgenic mice. Aliquots were treated with anti-CD25 or anti-CD8 magnetic beads to capture cells expressing CD25 or CD8 respectively. FACS analysis was carried out with the unfractionated (unfr) T cells and with the supernatants after capture of the CD25⁺ or CD8⁺ cells, to determine the efficiency of capture. These analyses showed only between 1 and 6% CD25⁺ or CD8⁺ cells remaining in the supernatants after the appropriate bead capture (data not shown). (A) EMSA with an excess of ³²P-labelled dual E-box oligo in the presence of sonicated *E.coli* DNA. Cellular extracts were derived from the same pool of T cells from either *CD2-Lmo2*×*CD2-Tal1* (lanes 1–9) or *CD2-Lmo2* (lanes 11–19) transgenic mice; 10 µg of cellular extracts were used for all lanes. Unfr (lanes 1–3 and 11–13) represents extract from the initial pool of thymocytes, CD25⁺ (lanes 4–6 and 14–16) represents extract from cells captured by the CD25 antibody beads, and CD8⁺ (lanes 7–9 and 17–19) represents extract from cells captured by the CD8 antibody beads. The antisera used to conduct supershifts are indicated at the top of the figure. (B) Northern hybridization analysis of RNA preparations from pools of 10 asymptomatic thymi from non-transgenic, unfractionated thymocytes (unfr) or thymic cells selected with anti-CD25 magnetic beads (CD25⁺) or anti-CD8 magnetic beads (CD8⁺) from *CD2-Lmo2* transgenic mice of the same age. Total RNA has been prepared directly from cells bound to the magnetic beads. After DNase I digestion, 20 µg of total RNA was run on a 1% agarose gel, the gel was blotted to Hybond-N membrane which was hybridized in succession with *Lmo2*, *Ldb1/Nli* and actin probes.

latter observation raised the possibility that a molecular complex between *Lmo2* and *Tal1* was causing an effect in the DN precursor T cells in an analogous way to their interaction in erythroid cells (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1994) where an oligomeric complex has been characterized comprising E2A, GATA-1 and *Ldb1/Nli* in addition to *Lmo2* and *Tal1* (Wadman *et al.*, 1997).

Further investigation of a possible complex in the transgenic T-cell tumours was facilitated by the derivation of tissue culture cell lines from various different tumours; one of these, designated 2114, was found to have a surface marker phenotype typical of the immature T cells expanded in the asymptomatic thymus (i.e. CD4⁻, CD8⁻, CD25⁺, CD44⁺). DNA-binding site selection with anti-*Lmo2* anti-

bodies was used to determine a consensus binding site, and we found that, unlike the site found with analogous experiments with the erythroid *Lmo2*-containing complex, a dual E-box site was present, with two E-box motifs separated by ~10 bp. The complex which binds this motif involves at least the two bHLH proteins, *Tal1* and *E47*, and the LIM-binding protein *Ldb1* in addition to *Lmo2*. There may also be other bHLH protein combinations involved in the oligomeric complex. A model of how this complex might occur is illustrated in Figure 7. *Ldb1/Nli1* may provide binding sites for two *Lmo2* molecules, each of which can bridge to one of the bHLH heterodimers. The potential of *Ldb1/Nli1* as an agent to homo- or heterodimerize LIM-domain-containing proteins has been

Fig. 7. Model of the putative protein complex binding to the dual E-box motif. The hypothetical oligomeric complex binds to the two E-boxes with an optimal spacing of 10 bp. The LIM-only protein Lmo2 may be bridging, together with Ldb1/Nli (Agulnick *et al.*, 1996; Jurata *et al.*, 1996), two bHLH heterodimers into one DNA-binding complex, but the overall stoichiometry of such a complex is unknown. Other proteins could be involved. Additionally, other bHLH protein combinations might be recruited by Ldb1/Nli and Lmo2 into the dual E-box-binding complex. It is also formally possible that the complex may, in physiological circumstances, not bind to DNA to elicit function but may occur as a result of sequestration of components and resultant impediment of function of one or more components.

demonstrated recently (Jurata *et al.*, 1998). No GATA-3 protein was found in this complex, despite the fact that Lmo2 and GATA-3 have been shown to interact (Ono *et al.*, 1997).

Since the dual E-box-binding complex contains proteins with transcriptional activation domains, it may well be that this complex recognizes a new specific set of target gene promoters/enhancers (i.e. those with a dual E-box motif) and causes their transcription. In fact, artificial reporter constructs have been described which fortuitously contain two E-boxes, separated by a 10 bp spacer, and these were activated by co-expression of Lmo2 and Tal1 (Ono *et al.*, 1997). Alternative functions must also be considered, such as sequestration of proteins, hindering their normal function after the ectopic expression of Lmo2 in this transgenic model (and, by analogy, in humans, after chromosomal translocation). For instance, Lmo2 may function in this context to sequester Ldb1 and cause an alteration of its function. *E2A* gene-deficient mice show abnormalities in thymocyte development and develop T-cell lymphoma (Bain *et al.*, 1997; Yan *et al.*, 1997), suggesting that a depletion or inactivation of *E2A* can lead to T-ALL. The inactivation of *E2A* can be achieved by heterodimerization with either Tal1 or Lyl1 (both activated by chromosomal translocations in humans) due to changed binding site specificity (Miyamoto *et al.*, 1996) or repression of activation (Hsu *et al.*, 1994c). Thus it may be that Lmo2 acts via the same mechanism by recruiting *E2A* into a complex with a changed binding site specificity and, therefore, functionally deplete *E2A*. However, there are differences in abnormalities of thymocyte development between *E2A*-deficient mice and *Lmo2* transgenic mice which indicate a different mode of tumorigenesis. The exact gain or loss of function caused by ectopic Lmo2 expression will necessitate models which selectively affect the individual components of the system.

Immature T cells are the target for Lmo2 protein function

The restriction of the dual E-box-binding complex to the CD4⁻ CD8⁻ DN immature thymocyte subset corresponds

well with the observed increase in the DN thymocyte population in the asymptomatic transgenic thymus (Larson *et al.*, 1995, 1996; Neale *et al.*, 1995), suggesting that the pathogenic effect of Lmo2 is confined to these immature T cells because the complex only occurs there. Recognition of the dual E-box oligo motif by the Lmo2-containing oligomeric complex may lead to abnormal regulation of downstream target genes and, therefore, to a partial differentiation block at this T-cell developmental stage. Loss-of-function models must also be considered. In addition, it cannot be ruled out that the immature DN cells normally express *Lmo2* since total thymus cell RNA does have *Lmo2* mRNA as judged by RT-PCR (Boehm *et al.*, 1991b). In this circumstance, the transgenic over-expression of *Lmo2* would provide the molecular basis for the differentiation block as the *Lmo2* gene would, thereby, be constitutively expressed. In this respect, there are similarities to the partial block of erythroid differentiation which has been observed when *Lmo2* is over-expressed in a proerythroblast cell line (Visvader *et al.*, 1997). In our transgenic model of T-cell tumorigenesis, the transgene is necessary but not sufficient for tumours to appear (Larson *et al.*, 1994). During the asymptomatic phase, T-cell differentiation is affected and it is possible that additional chromosomal alterations might happen, particularly since CD25⁺ CD44⁺ are undergoing TCR β gene rearrangements (Fehling and von Boehmer, 1997), and the extended expression of RAG recombinase, due to the impairment of T-cell differentiation, might lead to other gene rearrangements. These and other mutations might cause the overt malignancy to appear.

In the case of the *Lmo2* transgenic model, the RNA levels of *Lmo2* and *Ldb1* are approximately equivalent in both the CD4⁻ CD8⁻ DN and CD4⁺ CD8⁺ DP thymocyte subsets (Figure 6). In addition, Tal1 and E47 proteins are present in both subpopulations as judged by band shift data in double transgenic mice. Therefore, it is surprising that the complex is only detectable in the DN T-cell subset. This suggests that there is an additional unidentified component necessary for the formation of the complex and that this component is only present in the DN and not the DP T cells. An alternative explanation is that post-translational modification of any of the identified components of the complex either enables the formation of the complex in the DN T-cell subset or disables it in later stages of differentiation. Whatever the mode of Lmo2 action, our data suggest that the most likely result of Lmo2 ectopic expression in T cells is an alteration of gene expression within the T cell destined to become an overt tumour.

Materials and methods

Transgenic mice and derivation of cell line 2114

Transgenic mice line 989 (using murine *Lmo2*) and line 2 (using human *TAL1*) and the appropriate cross-breeds have been described before (Larson *et al.*, 1996). The *CD2-Lmo2* transgenic mouse 2114 was sacrificed at 11.5 months and found to have a thymoma with splenomegaly and hepatomegaly. The thymoma was excised and a single cell suspension established by passing it through a 70 μ m cell strainer. After washing with phosphate-buffered saline (PBS), the cells were resuspended in RPMI 1640 + 10% fetal calf serum (FCS) at a density of 10⁷ cells/ml. Cell growth was observed after ~2 weeks. Cells were maintained in this medium in the presence of 10 U/ml of interleukin-7 (IL-7; Genzyme) and 20 U/ml of IL-2 (Boehringer Mannheim).

Antibodies and immunoprecipitation

Rabbit anti-peptide antisera directed against the N-terminus (residues 2–17) of Lmo2 have been described earlier (Warren *et al.*, 1994). The rabbit antisera recognizing Tal1 (residues 1–121; Cheng *et al.*, 1993) and E2A (residues 217–371; Hsu *et al.*, 1994a,b) were kind gifts of Richard Baer. The anti-Ldb1 antiserum, residues 256–270 (Agulnick *et al.*, 1996), was kindly provided by Alan Agulnick.

Antibodies for FACS analysis, directly coupled to either phycoerythrin (PE) or fluorescein isothiocyanate (FITC), were obtained from Pharmingen, San Diego, CA. Antibodies directed against CD2 (RM2-5), CD3- ϵ (145–2C11), CD4 (RM4-5), CD8a (53–6.7), CD25 (7D4), CD44 (1M7), Thy1.2 (53–2.1), TCR $\alpha\beta$ (β -chain specific, H57-597) and TCR $\gamma\delta$ (GL3) were used for characterizing the cell line 2114 and to control the quality of thymocyte subset selection. Biotinylated versions of antibodies directed against CD8a and CD25 have been used for the thymocyte selection.

Immunoprecipitation with anti-Lmo2 antiserum has been performed as described (Valge-Archer *et al.*, 1994).

Preparation of nuclear and cellular extracts

Nuclear extracts for EMSA were prepared as described recently (Wadman *et al.*, 1997). Cellular extracts were prepared by washing the cells twice with PBS/5 mM EDTA and resuspending at a ratio of 150 μ l/5 $\times$ 10⁷ cells in CE solution (10 mM HEPES pH 7.6, 250 mM NaCl, 0.5% NP-40, 5 mM EDTA) with freshly added 1 mM dithiothreitol (DTT) and protease inhibitors [1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml pepstatin A, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin]. The suspension was vortexed vigorously for 30 s and then incubated for 30 min on ice with occasional vortexing. The cell debris was pelleted at 14 000 r.p.m. for 20 min at 4°C in a microfuge and the supernatant (cellular extract) collected. The samples were frozen on dry ice and kept at –70°C. The protein concentration was determined by the Bradford assay (Bio-Rad). Cellular extracts were used either for CASTing experiments or EMSA.

CASTing procedure

The CASTing procedure was performed essentially as described previously (Wadman *et al.*, 1997) with 200 μ g of 2114 cellular extracts per round of CASTing. The immunoprecipitated oligonucleotides were used directly for PCR without further purification. After seven rounds of selection, the final product was amplified for 25 cycles with primers and blunt-end cloned into the *EcoRV* site of phagescript vector. Resulting clones were sequenced and a consensus sequence defined.

EMSA

EMSAs were performed essentially as described (Wadman *et al.*, 1997). One strand of the oligonucleotide used was labelled with ³²P by kinase reaction and annealed to an excess of cold complementary strand. Then 1 μ l (4 nM) of this labelled annealed oligonucleotide was used per reaction together with 5–18 μ g of cellular or nuclear extracts in the presence of 0.8 μ g of sonicated *Escherichia coli* DNA. The samples (10 μ l total volume) were incubated for 30 min at room temperature in loading buffer (20 mM HEPES pH 7.6, 50 mM KCl, 1 mM EDTA, 25% v/v glycerol, 1 mM DTT) and then loaded directly onto a native 4% polyacrylamide gel on top of a 10% cushion. The gel was run for 5 h at 12 mA constant current at 4°C in 0.5 $\times$ TBE (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA), dried and exposed overnight for autoradiography.

For competition assays, both strands of the oligonucleotides were annealed at an equal molar ratio and then simultaneously added together with ³²P-labelled oligonucleotide into the samples at various concentrations, leading to a final excess ranging from 3 to 300 times. For antibody-mediated supershift analysis, 1 μ l of the appropriate pre-immune or immune serum was added to the sample and, where indicated, 1 μ l of *S.aureus* protein A (2 mg/ml) also added. For clarity, only the upper part of the gels are presented in the figures, but all EMSA experiments were carried out with an excess of free probe.

Sequences of oligonucleotides

For EMSA: E-box/E-box (dual E-box oligo), 5'-AGCCGACCATC-TGTTACAGTAAACAGATGGTTCGACA; E-box/mE-box (single E-box oligo), 5'-AGCCGACCATCTGTTCAGGTAACCGATCGTCGACA. For TAL-1 RT-PCR: TAL-N2, 5'-GCCGCGAGGGACTTAAAGGGC; TAL-Or1 (Shivdasani *et al.*, 1995), 5' GTCCTCACACCAAAGTAGTG; TAL-HLHR, 5'-CTTGCCAGGAAATTGATGTA.

Hybridization procedures

For filter hybridization, DNA samples were cleaved with *HindIII* and fractionated on a 0.8% agarose gel prior to transfer (LeFranc *et al.*,

1986) to a Hybond-N nylon membrane. Hybridization of randomly labelled probes (Amersham rediprime manual) was carried out as described (Rabbitts *et al.*, 1993). The *TCR C β* probe was a *HindIII*–*BamHI* fragment from TCR clone 1 (Malissen *et al.*, 1984) and the *Lmo2* probe was a *NotI* fragment of the murine cDNA clone pMR2A12N (Boehm *et al.*, 1991b).

RNA was prepared by the Trizol method (Gibco-BRL). Cells from total thymi of 2-month-old *CD2-Lmo2* transgenic mice (unselected) or C57Bl6/CBA (wild-type) mice were used after passing them through a cell strainer and washing in PBS. For anti-CD25-positive or anti-CD8a-positive selected samples, 8.8 $\times$ 10⁸ thymocytes from 10 *CD2-Lmo2* transgenic mice were selected as described below and the magnetic beads directly incubated and vortexed in 1 ml of Trizol solution. Northern analysis was carried out as described (Rabbitts *et al.*, 1993) with 10 μ g of each RNA per lane. The *Lmo2* probe was the same as used for Southern analysis, the *Ldb1*-specific probe was an *XhoI* fragment of a full-length cDNA of mouse *Ldb1* (Agulnick *et al.*, 1996) (kind gift of Alan Agulnick) cloned into the pEF-BOS vector (Mizushima and Nagata, 1990).

Selection for thymocyte subsets

Ten thymi of 2-month-old mice were pooled and passed through a cell strainer to generate a single cell suspension. Cells were washed once with PBS, resuspended in two times 750 μ l of PBS, transferred into Eppendorf tubes and placed on ice. For FACS analysis of unfractionated cells, 1 $\times$ 10⁷ cells were washed again with PBS/5 mM EDTA, resuspended in 50 μ l of CE solution (see above) and cellular extracts prepared as described above.

The remaining thymus cells (usually ~2–6 $\times$ 10⁸) were used for depletion experiments and were incubated directly with a 1:1 mixture (400 μ l each) of anti-CD4 (L3T4)- and anti-CD8 (Ly2)-coated magnetic Dynabeads (washed before three times with PBS) for 20 min on ice with occasional gentle mixing. The Eppendorf tubes were transferred to a magnetic block and after 2 min the supernatants (non-adherent cells) were collected. The magnetic beads were washed three times with PBS and after the last wash directly resuspended in 30 μ l of CE buffer in order to lyse the cells bound to the beads. The cells of the collected supernatants were pelleted by centrifugation at 2000 r.p.m., 4°C for 8 min. The cell pellet was resuspended in 1 ml of PBS/5 mM EDTA, and an aliquot taken for FACS analysis.

For positive selection with antibodies directed against either CD25 (7D4) or CD8a (Ly2), the remaining thymic cell suspension (after taking the first aliquot for the unfractionated sample, see above) was divided into two halves. The cells were incubated with 30 μ l of the biotinylated antibodies and incubated for 10 min on ice. Then 400 μ l of streptavidin-coated magnetic beads suspension (Dynabeads M280, washed before three times with PBS) was added and the mixture incubated for another 10 min on ice. Washing of the beads and preparing of the samples was performed as described above.

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References

- Agulnick,A.D., Taira,M., Breen,J.J., Tanaka,T., Dawid,I.B. and Westphal,H. (1996) Functional and physical interaction of Ldb1, a novel LIM domain binding factor, with the LIM homeodomain protein Lhx1/Xlim-1. *Nature*, **384**, 270–272.
- Bain,G. *et al.* (1997) E2A deficiency leads to abnormalities in $\alpha\beta$ T-cell development and to rapid development of T-cell lymphomas. *Mol. Cell. Biol.*, **17**, 4782–4791.
- Boehm,T., Foroni,L., Kaneko,Y., Perutz,M.P. and Rabbitts,T.H. (1991a) The rhombotin family of cysteine-rich LIM-domain oncogenes: distinct members are involved in T-cell translocations to human chromosomes 11p15 and 11p13. *Proc. Natl Acad. Sci. USA*, **88**, 4367–4371.
- Boehm,T., Spillantini,M.-G., Sofroniew,M.V., Surani,M.A. and Rabbitts, T.H. (1991b) Developmentally regulated and tissue-specific expression of mRNAs encoding the two alternative forms of the LIM domain oncogene rhombotin: evidence for thymus expression. *Oncogene*, **6**, 695–703.

- Cheng,J.T., Hsu,H.-L., Hwang,L.-Y. and Baer,R. (1993) Products of the *TAL1* oncogene: basic helix-loop-helix proteins phosphorylated at serine residues. *Oncogene*, **8**, 667–683.
- Condorelli,G.L., Facchiano,F., Valtieri,M., Proietti,E., Vitelli,L., Lulli,V., Huebner,K., Peschler,K. and Croce,C.M. (1996) T-cell-directed TAL-1 expression induces T-cell malignancies in transgenic mice. *Cancer Res.*, **56**, 5113–5119.
- Fehling,H.J. and von Boehmer,H. (1997) Early $\alpha\beta$ T cell development in the thymus of normal and genetically altered mice. *Curr. Opin. Immunol.*, **9**, 263–275.
- Fisch,P., Boehm,T., Lavenir,I., Larson,T., Arno,J., Forster,A. and Rabbitts,T.H. (1992) T-cell acute lymphoblastic lymphoma induced in transgenic mice by the RBTN1 and RBTN2 LIM-domain genes. *Oncogene*, **7**, 2389–2397.
- Foroni,L. et al. (1992) The rhombotin gene family encode related LIM-domain proteins whose differing expression suggests multiple roles in mouse development. *J. Mol. Biol.*, **226**, 747–761.
- Hsu,H.-L., Huang,L., Tsan,J.T., Funk,W., Wright,W.E., Hu,J.-S., Kingston,R.E. and Baer,R. (1994a) Preferred sequences for DNA recognition by the TAL1 helix-loop-helix proteins. *Mol. Cell. Biol.*, **14**, 1256–1265.
- Hsu,H.-L., Wadman,I. and Baer,R. (1994b) Formation of *in vivo* complexes between the TAL1 and E2A polypeptides of leukemic T cells. *Proc. Natl Acad. Sci. USA*, **91**, 3181–3185.
- Hsu,H.-L., Wadman,I., Tsan,J.T. and Baer,R. (1994c) Positive and negative transcriptional control by the TAL1 helix-loop-helix protein. *Proc. Natl Acad. Sci. USA*, **91**, 5947–5951.
- Jurata,L.W., Kenny,D.A. and Gill,G.N. (1996) Nuclear LIM interactor, a rhombotin and LIM homeodomain interacting protein, is expressed early in neuronal development. *Proc. Natl Acad. Sci. USA*, **93**, 11693–11698.
- Jurata,L.W., Pfaff,S.L. and Gill,G.N. (1998) The nuclear LIM domain interactor NL1 mediates homo- and heterodimerization of LIM domain transcription factors. *J. Biol. Chem.*, **273**, 3152–3157.
- Kallianpur,A.R., Jordan,J.E. and Brandt,S.J. (1994) The *SCL/TAL-1* gene is expressed in progenitors of both the hematopoietic and vascular systems during embryogenesis. *Blood*, **85**, 1200–1208.
- Kelliher,M.A., Seldin,D.C. and Leder,P. (1996) Tal-1 induces T cell acute lymphoblastic leukemia accelerated by casein kinase II α . *EMBO J.*, **15**, 5160–5166.
- Lang,G., Wotton,D., Owen,M.J., Sewell,W.A., Brown,M.H., Mason,D.Y., Crumpton,M.J. and Kioussis,D. (1988) The structure of the human CD2 gene and its expression in transgenic mice. *EMBO J.*, **7**, 1675–1682.
- Larson,R., Fisch,P., Larson,T., Lavenir,I., Langford,T., King,G. and Rabbitts,T.H. (1994) T cell tumours with disparate phenotypes develop with long latency in mice transgenic for *rbt2*. *Oncogene*, **9**, 3675–3681.
- Larson,R.C., Osada,H., Larson,T.A., Lavenir,I. and Rabbitts,T.H. (1995) The oncogenic LIM protein Rbt2 causes thymic developmental aberrations that precede malignancy in transgenic mice. *Oncogene*, **11**, 853–862.
- Larson,R.C., Lavenir,I., Larson,T.A., Baer,R., Warren,A.J., Wadman,I., Nottage,K. and Rabbitts,T.H. (1996) Protein dimerisation between Lmo2 (Rbt2) and Tal1 alters thymocyte development and potentiates T cell tumorigenesis in transgenic mice. *EMBO J.*, **15**, 1021–1027.
- LeFranc,M.-P., Forster,A., Baer,R., Stinson,M.A. and Rabbitts,T.H. (1986) Diversity and rearrangement of the human T cell rearranging γ genes: nine germ-line variable genes belonging to two subgroups. *Cell*, **45**, 237–246.
- Malissen,M. et al. (1984) Mouse T cell antigen receptor: structure and organization of constant and joining gene segments encoding the β polypeptide. *Cell*, **37**, 1101–1110.
- McGuire,E.A., Rintoul,C.E., Sclar,G.M. and Korsmeyer,S.J. (1992) Thymic overexpression of Ttg-1 in transgenic mice results in T-cell acute lymphoblastic leukemia/lymphoma. *Mol. Cell. Biol.*, **12**, 4186–4196.
- Miyamoto,A., Cui,X., Naumovski,L. and Cleary,M.L. (1996) Helix-loop-helix proteins LYL1 and E2a form heterodimeric complexes with distinctive DNA-binding properties in hematolymphoid cells. *Mol. Cell. Biol.*, **16**, 2394–2401.
- Mizushima,S. and Nagata,S. (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.*, **18**, 5322.
- Murre,C., McCaw,P.S. and Baltimore,D. (1989) A new DNA binding and dimerisation motif in immunoglobulin enhancer binding, daughterless, MyoD and myc proteins. *Cell*, **56**, 777–783.
- Neale,G.A., Reh,J.E. and Goorha,R.M. (1995) Ectopic expression of rhombotin-2 causes selective expansion of the thymus and T-cell tumours in transgenic mice. *Blood*, **86**, 3060–3071.
- Ono,Y., Fukuhara,N. and Yoshie,O. (1997) Transcriptional activity of TAL1 in the T cell acute lymphoblastic leukemia (T-ALL) requires RBTN1 or -2 and induces TALLA1, a highly specific tumor marker of T-ALL. *J. Biol. Chem.*, **272**, 4576–4581.
- Osada,H., Grütz,G., Axelsson,H., Forster,A. and Rabbitts,T.H. (1995) Association of erythroid transcription factors: complexes involving the LIM protein RBTN2 and the zinc-finger protein GATA1. *Proc. Natl Acad. Sci. USA*, **92**, 9585–9589.
- Pevny,L., Simon,M.C., Robertson,E., Klein,W.H., Tsai,S.-F., D'Agati,V., Orkin,S.H. and Costantini,F. (1991) Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature*, **349**, 257–260.
- Pollock,R. and Treisman,R. (1990) A sensitive method for the determination of protein-DNA binding specificities. *Nucleic Acids Res.*, **18**, 6197–6204.
- Porcher,C., Swat,W., Rockwell,K., Fujiwara,Y., Alt,F.W. and Orkin,S.H. (1996) The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell*, **86**, 47–57.
- Pulford,K., Lecoqte,N., Leroy-Viard,E.L., Jones,M., Mathieu-Mahul,D. and Mason,D.Y. (1995) Expression of TAL-1 proteins in human tissues. *Blood*, **85**, 675–684.
- Rabbitts,T.H. (1994) Chromosomal translocations in human cancer. *Nature*, **372**, 143–149.
- Rabbitts,T.H., Forster,A., Larson,R. and Nathan,P. (1993) Fusion of the dominant negative transcription regulator CHOP with a novel gene FUS by translocation t(12;16) in malignant liposarcoma. *Nature Genet.*, **4**, 175–180.
- Robb,L., Lyons,I., Li,R., Hartley,L., Kontgen,F., Harvey,R.P., Metcalf,D. and Begley,C.G. (1995a) Absence of yolk sac hematopoiesis from mice with a targeted disruption of the *scl* gene. *Proc. Natl Acad. Sci. USA*, **92**, 7075–7079.
- Robb,L., Rasko,J.E.J., Bath,M.L., Strasser,A. and Begley,C.G. (1995b) *scl*, a gene frequently activated in human T cell leukaemia, does not induce lymphomas in transgenic mice. *Oncogene*, **10**, 205–209.
- Robb,L., Elwood,N.J., Elefanty,A.G., Kontgen,F., Li,R., Barnett,L.D. and Begley,C.G. (1996) The *scl* gene product is required for the generation of all hematopoietic lineages in the adult mouse. *EMBO J.*, **15**, 4123–4129.
- Roberts,V.J., Steenbergen,R. and Murre,C. (1993) Localization of E2A mRNA expression in developing and adult rat tissues. *Proc. Natl Acad. Sci. USA*, **90**, 7583–7587.
- Royer-Pokora,B., Loos,U. and Ludwig,W.-D. (1991) TTG-2, a new gene encoding a cysteine-rich protein with the LIM motif, is overexpressed in acute T-cell leukaemia with the t(11;14)(p13;q11). *Oncogene*, **6**, 1887–1893.
- Shivdasani,R.A., Mayer,E. and Orkin,S.H. (1995) Absence of blood formation in mice lacking the T-cell leukaemia oncoprotein tal-1/SCL. *Nature*, **373**, 432–434.
- Shivdasani,R.A., Fujiwara,Y., McDevitt,M.A. and Orkin,S.H. (1997) A lineage-selective knockout establishes the critical role of transcription factor GATA-1 in megakaryocyte growth and platelet development. *EMBO J.*, **16**, 3965–3973.
- Sun,X.-H. and Baltimore,D. (1991) An inhibitory domain of E12 transcription factor prevents DNA binding in E12 homodimers but not in E12 heterodimers. *Cell*, **64**, 459–470.
- Valge-Archer,V.E., Osada,H., Warren,A.J., Forster,A., Li,J., Baer,R. and Rabbitts,T.H. (1994) The LIM protein RBTN2 and the bHLH protein TAL1 are present in a complex in erythroid cells. *Proc. Natl Acad. Sci. USA*, **91**, 8617–8621.
- Visvader,J.E., Mao,X., Fujiwara,Y., Hahm,K. and Orkin,S.H. (1997) The LIM-domain binding protein Ldb1 and its partner LMO2 act as negative regulators of erythroid differentiation. *Proc. Natl Acad. Sci. USA*, **94**, 13707–13712.
- Wadman,I., Li,J., Bash,R.O., Forster,A., Osada,H., Rabbitts,T.H. and Baer,R. (1994) Specific *in vivo* association between the bHLH and LIM proteins implicated in human T cell leukaemia. *EMBO J.*, **13**, 4831–4839.
- Wadman,I.A., Osada,H., Grütz,G.G., Agulnick,A.D., Westphal,H., Forster,A. and Rabbitts,T.H. (1997) The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which includes the TAL1, E47, GATA-1 and Ldb1/NLI proteins. *EMBO J.*, **16**, 3145–3157.

- Warren,A.J., Colledge,W.H., Carlton,M.B.L., Evans,M.J., Smith,A.J.H. and Rabbitts,T.H. (1994) The oncogenic cysteine-rich LIM domain protein rbtn2 is essential for erythroid development. *Cell*, **78**, 45–58.
- Wright,W.E., Binder,M. and Funk,W. (1991) Cyclic amplification and selection of targets (CASTing) for the myogenin consensus binding site. *Mol. Cell. Biol.*, **11**, 4104–4110.
- Yamada,Y., Warren,A.W., Dobson,C., Forster,A., Pannell,R. and Rabbitts,T.H. (1998) The T cell leukaemia LIM protein Lmo2 is necessary for adult mouse haematopoiesis. *Proc. Natl Acad. Sci. USA*, **95**, 3890–3895.
- Yan,W., Young,A.Z., Soares,V.C., Kelley,R., Benezra,R. and Zhuang,Y. (1997) High incidence of T-cell tumors in E2A-null mice and E2A/Id1 double-knockout mice. *Mol. Cell. Biol.*, **17**, 7317–7327.

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