MOLECULAR AND CELLULAR BIOLOGY, Sept. 2004, p. 7491–7502 Vol. 24, No. 17
0270-7306/04/$08.00+0 DOI: 10.1128/MCB.24.17.7491–7502.2004


# Decoding Hematopoietic Specificity in the Helix-Loop-Helix Domain of the Transcription Factor SCL/Tal-1

Thorsten M. Schlaeger,[1]† Anna Schuh,[2] Simon Flitter,[3] Andreas Fisher,[1]‡ Hanna Mikkola,[1] Stuart H. Orkin,[1] Paresh Vyas,[2] and Catherine Porcher[3]*

*Department of Hematology/Oncology, Children's Hospital and Dana Farber Cancer Institute, Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts,[1] and MRC Molecular Haematology Unit[3] and Department of Haematology,[2] Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom*

Received 2 February 2004/Returned for modification 26 March 2004/Accepted 28 May 2004

**The helix-loop-helix (HLH) domain is employed by many transcription factors that control cell fate choice in multiple developmental settings. Previously, we demonstrated that the HLH domain of the class II basic HLH (bHLH) protein SCL/Tal-1 is critical for hematopoietic specification. We have now identified residues in this domain that are essential for restoring hematopoietic development to SCL$^{-/-}$ embryonic stem cells and sufficient to convert a muscle-specific HLH domain to one able to rescue hematopoiesis. Most of these critical residues are distributed in the loop of SCL, with one in helix 2. This is in contrast to the case for MyoD, the prototype of class II bHLH proteins, where the loop seems to serve mainly as a linker between the two helices. Among the identified residues, some promote heterodimerization with the bHLH partners of SCL (E12/E47), while others, unimportant for this property, are still crucial for the biological function of SCL. Importantly, the residue in helix 2 specifically promotes interaction with a known partner of SCL, the LIM-only protein LMO2, a finding that strengthens genetic evidence that these proteins interact. Our data highlight the functional complexity of bHLH proteins, provide mechanistic insight into SCL function, and strongly support the existence of an active SCL/LMO2-containing multiprotein complex in early hematopoietic cells.**

Proper development requires integration of signaling pathways and transcriptional regulatory networks. Transcriptional regulators of the basic helix-loop-helix (bHLH) family are important in diverse settings. The shared bHLH motif mediates interaction with DNA through the basic (b) domain and promotes homo- or heterodimerization through the HLH domain. bHLH proteins have been assigned to seven classes (28). Class I includes the ubiquitously expressed E proteins, such as E12/E47, HEB, and E2-2 (28). Class II includes tissue-specific bHLH proteins, e.g., MyoD (33), NeuroD (22), and SCL/Tal-1 (hereafter called SCL; see below). Class V members (Id proteins) lack the basic domain and act as dominant-negative regulators of class I and class II proteins (4).

SCL was discovered through its involvement in T-cell acute lymphoblastic leukemia (T-ALL) (for a review, see reference 21). During normal development, SCL is first expressed in the presumptive hemangioblast and is believed to specify hematopoietic fate within this specialized mesoderm (11–13, 24, 42). SCL is required for the emergence of embryonic erythroid cells as well as for the development of all adult blood lineages. Although it is essential for generation of hematopoietic stem cells, inactivation of SCL in the adult does not ostensibly impair stem cell maintenance or function. In contrast, SCL is required for maturation of erythroid and megakaryocytic lineages (14, 31, 38).

SCL forms heterodimers with E proteins that bind to E-box DNA motifs (28). SCL is also involved in protein complex formation. It interacts in vitro with the LIM-only protein LMO2. Like SCL, LMO2 has been implicated in development of T-ALL and is essential for normal blood cell differentiation (40). There is evidence for both physical and functional interactions between bHLH heterodimers, LIM domain proteins (such as LMO2), and the LIM domain-binding protein Ldb1 (NLI, Chip) (1, 19). The existence of such a multimeric complex was first observed in vitro in erythroid cells, where LMO2, a non-DNA-binding protein, acts as a bridging molecule to assemble a complex containing SCL, E2A, Ldb1, and the hematopoietic-specific zinc finger protein GATA-1 (46). This multiprotein complex (with or without GATA-1) is able to form on the c-*kit* promoter, modulating its activity in transiently transfected cells (20, 44). Moreover, a similar complex is required in vivo for proneural patterning in *Drosophila* (41), and bHLH dimers, LIM-HD proteins, and the adaptor protein NLI cooperate to synchronize neuronal subtype specification and neurogenesis (23). Finally, SCL also interacts with the coregulators P/CAF, p300, and mSin3A (16–18).

To understand how SCL acts in hematopoiesis, we previously performed a structure-function analysis employing in vitro differentiation of gene-targeted SCL$^{-/-}$ embryonic stem (ES) cells. Two unexpected findings emerged from these studies (38). First, the SCL bHLH region is the only domain that is essential for activity in hematopoietic development. Second, the SCL DNA-binding activity is dispensable for specification

* Corresponding author. Mailing address: MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, United Kingdom. Phone: 44 (0)1865 222 309. Fax: 44 (0)1865 222 500. E-mail: catherine.porcher@imm.ox.ac.uk.

† Present address: Cell and Molecular Technologies, Phillipsburg, NJ 08865.

‡ Present address: Institute of Physiological Chemistry I, Theodor Boveri Institute, University of Würzburg, Würzburg, Germany.

of hematopoiesis, although it is required for full erythroid and megakaryocyte differentiation. These data focused attention on the HLH region as the critical determinant of biological activity with respect to hematopoietic specification.

At least two possibilities might explain the requirement for an HLH domain. First, expression of any class II or V HLH protein in mesoderm may be sufficient to specify hematopoiesis. This hypothesis would be consistent with models in which SCL functions to sequester E proteins (15). Alternatively, hematopoietic development might rely on specific residues within the SCL HLH region, as would be expected if SCL was engaged in crucial protein-protein interactions. To address this, we have performed a detailed mutational analysis of the SCL HLH domain and show that specific residues required for hematopoietic specification reside in this domain. Through loss-of-function studies, we have identified multiple critical amino acids in the SCL helix 2 and loop. Importantly, introduction of these residues is sufficient to convert the muscle-specific HLH domain of MyoD into one capable of rescuing hematopoiesis from $SCL^{-/-}$ ES cells. Lastly, we show that one of the identified residues promotes interaction with LMO2, thereby validating the strategy, strengthening inferences from genetic findings that an interaction between SCL and LMO2 is required at the earliest stages of hematopoietic development, and providing mechanistic insight into SCL function.

## MATERIALS AND METHODS

**Plasmid constructs. (i) Rescue assay.** PCR fragments containing wild-type (wt) or chimeric cDNAs were subcloned into the murine stem cell virus (MSCV) retroviral vector (38). *NSCL1* (25) and *MyoD* cDNAs were kindly provided by Trang Hoang and Sergei Tevosian, respectively. The Id3 HLH domain was amplified from an expressed sequence tag (gi:12833084).

**(ii) Mammalian two-hybrid system.** SCL-, LMO2-, and Ldb1-coding sequences were PCR cloned into pVP16 and pM (Clontech) to produce VP16 activation domain (VAD) and Gal4 DNA-binding domain (GBD) fusion proteins, respectively. Sequences encoding the bHLH domain of human E47 (amino acids 526 to 614) were similarly cloned into pM.

**(iii) Coimmunoprecipitations.** Flag-SCL was generated by introduction of an oligonucleotide encoding an N-terminal Flag tag between the BglII and NotI sites of pMSCV-SCL (38). Mutant forms of SCL cDNA were subsequently introduced into this vector.

**(iv) pMSCV-Ldb1 and pMSCV-LMO2.** The complete open reading frames of mouse Ldb1 and LMO2 (43) were PCR cloned into MSCV; pcDNA-E47 (36) was kindly provided by Xiao-Hong Sun.

All constructs generated by PCR were verified by sequencing. Details of cloning and mutagenesis strategies are available upon request.

**Retroviral infection of $SCL^{-/-}$ ES cells.** Transient transfection of the packaging cell line BOSC23 and infection of $SCL^{-/-}$ ES cells were performed as described previously (39).

**In vitro ES cell hematopoietic differentiation.** wt or rescued ES cells were subjected to the first step of in vitro hematopoietic differentiation to form embryoid bodies (EBs) (39). Percentages of red EBs were reported after 8 days of differentiation. At least 200 EBs were scored. Primary replatings were repeated three to six times for each type of rescued ES cells. To generate primitive erythroid colonies, day 6 EBs were disaggregated and the cells were replated in methylcellulose in presence of erythropoietin (2 U/ml). Colonies were scored at day 4, and the morphology of the cells was checked by May-Grunwald-Giemsa staining.

**Coimmunoprecipitations and Western blot analyses. (i) Immunoprecipitation from in vitro-translated products.** PCR fragments containing a T7 promoter sequence at their 5′ end were generated from Flag-tagged SCL (wt and mutant) constructs and E12 cDNA by using the Expand long-template polymerase (Roche). Equal amounts of SCL (wt or mutant) and E12 PCR products were mixed and used as templates for coupled in vitro transcription-translation with the TNT T7 Quick for PCR DNA (Promega). After the efficiency of the in vitro transcription-translation procedure was checked by Western blot analysis, heterodimers were immunoprecipitated with anti-Flag antibodies covalently attached to agarose beads (M2 affinity gel; Sigma) according to the manufacturer's instructions. Western blot analyses were performed as described previously (38). Anti-SCL and anti-E2A.E12 antibodies were used for detection. Secondary antibodies were anti-rabbit antibody–horseradish peroxidase. Detection was performed with an ECL kit (Amersham/Pharmacia).

**(ii) Immunoprecipitation from HEK293T cells.** Cells were transiently transfected with 5 μg each of pMSCV-SCL (wt and mutant, Flag tagged), pMSCV-LMO2, and pMSCV-Ldb1 and 3 μg of pcDNA3-E47 by using the Fugene 6 reagent (Roche). At 48 h posttransfection, cells were harvested by scraping and washed with phosphate-buffered saline. Total protein lysates were prepared by extraction for 30 min at 4°C with continuous agitation in 500 μl of lysis buffer (50 mM Tris HCl [pH 8.0], 10% glycerol, 0.7% NP-40, 0.1 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1 mM $NaVO_4$, 0.7 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, proteinase inhibitor cocktail [Roche]). The lysates were spun for 5 min at 20,000 × *g*; 2% of the supernatant were used per lane for Western blot analysis, and 90% was immunoprecipitated with 40 μl of M2 anti-Flag antibody bound to agarose (Sigma). Binding was carried out for 3 h at 4°C. The agarose was washed four times with TBS (20 mM Tris HCl [pH 7.5], 150 mM NaCl, 0.7 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, protease inhibitor cocktail) before elution with sodium dodecyl sulfate gel loading buffer, and 20% of the precipitated proteins were loaded per lane and subjected to Western blot analysis with anti-SCL, -LMO2, -E47, and -Ldb1 antibodies. Detection was performed as described above.

**(iii) Antibodies.** The C-terminal SCL antiserum and the LMO2 and Ldb1 antisera have been described previously (38, 43); the E47 (N-649), E2A.E12 (V-18), and HEB (A-20) antibodies were obtained from Santa Cruz; and anti-Flag M2 antibodies were obtained from Sigma.

**Semiquantitative RT-PCR.** RNA was isolated from day 6 EBs and primitive erythroid colonies, and reverse transcription-PCR (RT-PCR) was performed as described previously (38). The sequences of the oligonucleotides used in the PCRs have been reported previously (39). Aliquots were analyzed on a 4% polyacrylamide gel after cycles 16 to 22 for amplification of βH1 sequences and after cycles 18 to 24 for amplification of SCL and hypoxanthine phosphoribosyltransferase sequences. Quantitation of the PCR products was carried out with a PhosphorImager (Storm; Molecular Dynamics).

**Mammalian three-hybrid assay.** VAD and GBD vectors were obtained from Clontech's mammalian two-hybrid kit. The Gal4-responsive pFR-luc (Stratagene) was used as a reporter gene. HEK293 cells were transiently transfected with 100 ng of the luciferase reporter gene construct, 10 ng of a cytomegalovirus beta-galactosidase (Clontech), and the indicated combinations of test vectors (500 ng per GDB and VAD fusion protein vector and 250 ng per E47 expression vector) in six-well plates by using the Fugene 6 reagent. Total amounts of DNA were kept constant through addition of pMSCV. Luciferase and beta-galactosidase activities were determined in total cell lysates at 48 h posttransfection by using a dual-light kit (Tropix). Each combination of constructs was tested 3 to 14 times.

**Biotin-streptavidin pull-down experiments.** The bacterial BirA ligase (8) was stably transfected into murine erythroleukemia (MEL) cells (clone 585). A clone expressing high levels of the BirA mRNA was selected by Northern blotting (A. Schuh, J. Strouboulis, P. Vyas, and C. Porcher, unpublished data) and subsequently cotransfected with wt SCL or mutant H2(F-G) cDNA harboring in its 5′ end a 23-amino-acid biotinylation tag (bio-tag) (8). Clones that expressed biotinylated SCL (bio-SCL) (wt or mutant) to levels similar to that of endogenous SCL in untransfected cells were selected for further analysis (Schuh et al., unpublished data). Expression of the ligase and bio-tagged SCL cDNAs was driven by the EFI-α promoter. Nuclear extracts were prepared from 3 liters of the selected MEL cell clones by salt extraction (9). For pull-down experiments, paramagnetic streptavidin beads (Dynabeads M-280; Dynal) were blocked by washing three times in 1× TBS with 200 μg of ovalbumin (Sigma-Aldrich) per ml. Five milligrams of nuclear extracts was incubated in TBS–0.3% NP-40 with 100 μl of beads at 4°C overnight on a rotating wheel. After six washes in 250 mM NaCl–TBS–NP-40, the beads were boiled for 5 min in 30 μl of Laemmli sample buffer, and 1 μl was used for Western blotting. For Western blot analysis, nuclear extracts and pull-down products were resolved on a 4 to 12% bis-Tris NuPage gel in MOPS (morpholinepropanesulfonic acid) buffer (Invitrogen) and blotted on nitrocellulose membrane under standard conditions. Anti-E2A.E12, anti-HEB, anti-SCL, or anti-LMO2 antibodies were used, and detection was performed as described above.

## RESULTS

To identify residues crucial for SCL function in hematopoietic development, portions of the SCL HLH region were re-

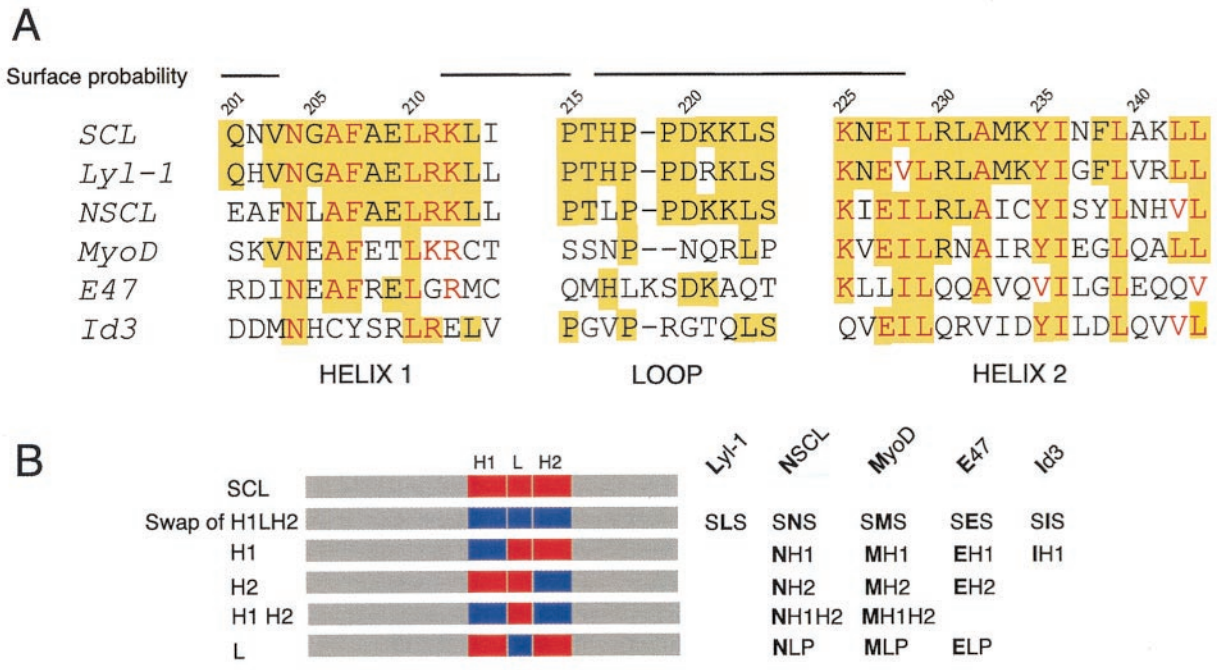


FIG. 1. bHLH proteins used in the study to generate chimeric mutants. (A) Amino acid sequence alignment of the HLH regions of the proteins used in the study. The residues highlighted in yellow are identical to those of SCL. The residues in red are residues conserved in the vast majority of HLH proteins and therefore presumed to be structurally important for folding, dimerization, or DNA binding. Prediction of surface probability of the SCL HLH region was achieved by measuring the solvent-accessible surface area of individual residues (MacVector; Accelrys). (B) Left, schematic representation of the chimeric proteins analyzed in this study. In red are the domains of SCL that have not been substituted, and in blue are the swapped domains. H1, helix 1; H2, helix 2; L, loop. Right, the chimeric proteins were named according to the type of substitution involved and the nature of the HLH protein providing the swapped domain.

placed by the corresponding segments of other HLH proteins. The amino acid sequences of various HLH proteins are shown in Fig. 1A. Lyl-1 is a hematopoiesis-specific bHLH protein that is highly homologous to SCL (30). The other, nonhematopoietic HLH proteins show less similarity to the SCL HLH domain and include NSCL, a neuronal protein (25); MyoD; E47; and Id3 (26). Schematic representations of the chimeric proteins tested are shown Fig. 1B. Point mutations were also introduced into the SCL HLH domain (see below). All of the variants we examined contain an unmodified SCL basic domain.

EBs generated from $SCL^{-/-}$ ES cells entirely lack hematopoietic progenitors. Retroviral transfer of wt SCL cDNA fully rescues hematopoietic development (39). We have taken advantage of this stringent rescue assay to test the hematopoietic potential of the SCL variants. Below are outlined three key aspects of this assay.

First, expression of all the mutants was verified after transfection into producer cells by Western blot analysis. All of the variants were expressed at similar levels, as represented in Fig. 2A, where expression of selected mutant proteins is shown.

Second, the mutant proteins were expressed in $SCL^{-/-}$ ES cells following retroviral transfer. Transduced ES cells were differentiated into EBs. The proportion of red EBs (sites of primitive erythropoietic activity) reflects the level of rescue of hematopoietic development by the mutant proteins. When a domain of SCL containing residues critical for its function was replaced by a domain lacking these specific residues, the protein was less active or inactive in forming hemoglobinized EBs. Examples of EBs derived from wt or rescued ES cells are shown in Fig. 2B. Approximately 75% of wt EBs are red. Fifteen to 30% (average ± standard deviation, 21.3% ± 9.3%) of EBs derived from $SCL^{-/-}$ ES cells rescued with wt SCL cDNA ($SCL^{-/-}$/SCL ES cells) show hematopoietic activity. Examples of EBs representing rescued $SCL^{-/-}$ ES cells containing mutant constructs are also shown (from 15 to 30% to no rescue). This assay was used as an initial screen to assess the function of a large series of mutant proteins. The extent of rescue by specific mutants was investigated further.

Third, as heterodimerization of SCL with its bHLH partners, E12 or E47, is a prerequisite for biological activity (38), an inability to heterodimerize constitutes a trivial reason for inactivity in the rescue system. Therefore, the heterodimerization capacities of the mutants were tested by coimmunoprecipitation experiments, when appropriate. E12 and Flag-tagged SCL (wt or mutant) were in vitro transcribed and translated. Complexes were immunoprecipitated with anti-Flag antibodies, and the presence of E12 was analyzed by Western blotting. Figure 2C shows examples of immunoprecipitated products.

**The SCL HLH region contains key residues for specification of the hematopoietic program.** To determine whether the SCL HLH region contains specific residues that are functional in hematopoietic development, we first replaced the entire SCL HLH domain with the corresponding domains of the proteins listed in Fig. 1A. Lyl-1 HLH sequences were able to substitute

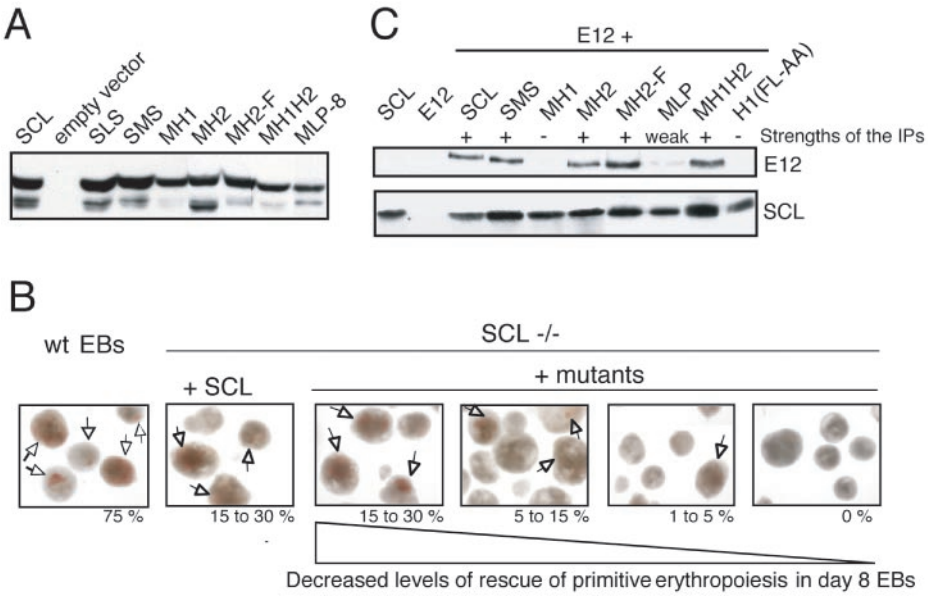


FIG. 2. Biological and biochemical assays used to test the SCL mutants. (A) BOSC23 cells were transfected with MSCV-SCL (wt or mutant forms [discussed in the text and shown in Fig. 1 and 3]). Cell lysates were then subjected to Western blot analysis to test for expression of the SCL constructs, using anti-SCL antibodies. (B) EBs obtained after in vitro differentiation of wt ES cells or $SCL^{-/-}$ ES cells rescued with wt SCL cDNA (+ SCL) or mutant forms of SCL (+ mutants). Red EBs (arrows) reflect primitive hematopoietic activity. Representative fields of EB cultures exhibiting various levels of rescue are shown. Numbers indicate the percentages of red EBs. (C) Coimmunoprecipitations. In vitro-translated E12 and SCL (wt or mutant, Flag tagged in the N terminus), as indicated at the top, were immunoprecipitated (IP) with anti-Flag antibodies and subjected to Western blot analysis with anti-E2A.E12 antibodies (top panel) and anti-SCL antibodies (bottom panel).

functionally for SCL HLH sequences, as the chimeric protein, SLS, had activity equal to that of wt SCL (Fig. 3A). Among the seven differences observed between the SCL and Lyl-1 HLH regions (Fig. 1A), only two are nonconservative (N202 versus histidine in helix 1 and N237 versus glycine in helix 2), suggesting that these two asparagine residues are not crucial for SCL activity. The other swap mutants failed to rescue hematopoiesis (Fig. 3A, constructs SNS, SMS, SES, and SIS), while still being competent to dimerize with E12 (except for SES, which showed weak heterodimerization ability).

Thus, initiation of the hematopoietic program requires specific residues within an HLH domain beyond those necessary for dimerization with the E2A proteins. To characterize these residues, we studied individual portions of the SCL HLH domain.

**Helix 1 lacks hematopoiesis-specific residues.** We first replaced helix 1 of SCL with the corresponding domains of NSCL, MyoD, E47, and Id3 (Fig. 3B, constructs NH1, MH1, EH1, and Id1). Interestingly, the NSCL helix 1 fully substituted for SCL helix 1 (Fig. 3B, construct NH1). In contrast, helix 1 of MyoD, E47, and Id3 did not coimmunoprecipitate with E12 and hence failed to substitute functionally for SCL helix 1 (Fig. 3B, MH1, EH1, and IH1).

We then introduced point mutants into SCL helix 1 to identify specific residues. As Lyl-1 and NSCL helix 1 rescue hematopoiesis, we individually mutated residues that are conserved between SCL, Lyl-1, and NSCL helix 1 sequences (apart from residues conserved between all bHLH proteins [Fig. 1A]): A208, E209, and L213 [constructs H1(A-K), H1(E-T), and H1(L-C)]. We also introduced random mutations into SCL helix 1 [constructs H1(Q-G), H1(R-A), and H1(I-C)]. None of these changes significantly affected SCL activity (data not shown). As a control, we mutated two residues (F207 and L210) previously shown to be essential for heterodimerization [construct H1(FL-AA)] (38). This totally abolished functional activity (data not shown). Taken together, these results indicate that rescue by NH1 and SLS is not due to the presence of specific amino acids important for SCL function in NSCL and Lyl-1 helix 1 but is possible because these two swap mutants are able to heterodimerize and because SCL helix 1 lacks hematopoiesis-specific residues.

**The phenylalanine residue in SCL helix 2 (F238) is crucial for function.** None of the chimeric swap proteins bearing substitutions of helix 2 (Fig. 3C, constructs NH2, MH2, and EH2) restored hematopoiesis. To pinpoint residues essential for SCL activity, selected amino acids in helix 2 were mutated. They included all nonconserved residues except N237, which was already identified as nonessential for SCL activity (see above). Only two variants out of eight tested failed to rescue SCL activity (Fig. 3C and data not shown). One corresponded to mutation of a conserved hydrophobic amino acid [Y235, H2(Y-A)] and failed to dimerize. The other mutation changed F238 to a glycine [H2(F-G)] and abolished rescue activity but retained heterodimerization capacity. As this residue is not present in NSCL and MyoD helix 2, we next tested whether introduction of a phenylalanine residue at the corresponding positions of constructs NH2 and MH2 would be sufficient to confer hematopoietic potential. Remarkably, the resulting mutants (NH2-F and MH2-F) restored hematopoietic activity (Fig. 3C). We then compared the amino acid sequences of SCL, Lyl-1, NSCL, and MyoD helix 2. The only common residue (apart from the conserved residues shown in Fig. 1A) that

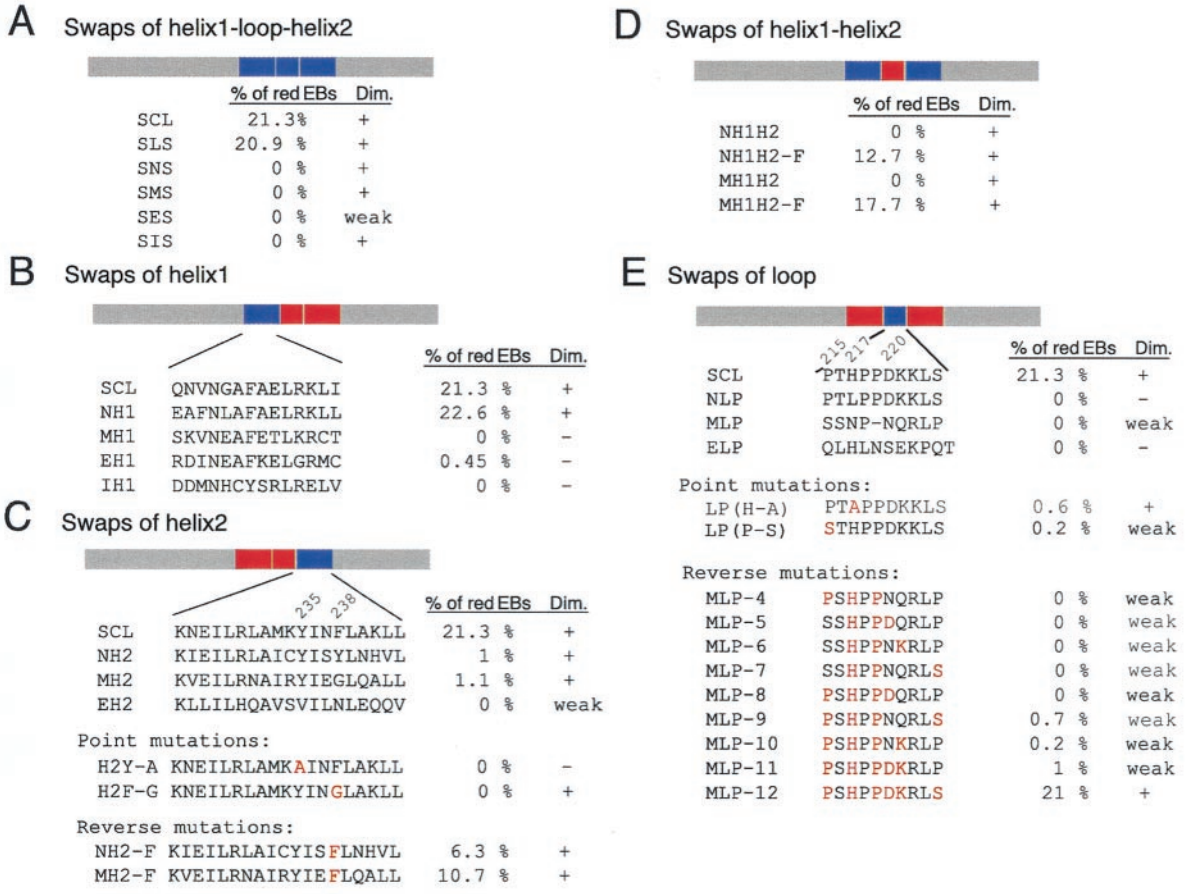


FIG. 3. Summary of the results of in vitro hematopoietic differentiation assays (percentages of red EBs) and coimmunoprecipitation experiments (Dim.). Results obtained with informative chimeric and mutant proteins bearing various substitutions within the SCL HLH domain are shown. At the top of each panel is a schematic representation of the chimeric proteins, as set out in Fig. 1B. In red are shown point mutations. Examples of the various amounts of immunoprecipitated products are illustrated in Fig. 2C.

showed some functional activity is R230 [H2(R-A)] (data not shown). When this residue was mutated, there was a 4.4-fold reduction in hematopoietic rescue, suggesting that it might also play a role in SCL function. In conclusion, F238 is critical for SCL activity, and its introduction into two nonhematopoietic helix 2 domains enabled them to activate hematopoietic development in ES cells.

We then modified both SCL helices in the same construct by replacing them with corresponding sequences of NSCL or MyoD (Fig. 3D, constructs NH1H2 and MH1H2, respectively). Neither of the two mutants had detectable activity despite being able to heterodimerize. However, introduction of the crucial phenylalanine residue F238 in helix 2 of these mutants conferred significant rescue activity (Fig. 3D, constructs NH1H2-F and MH1H2-F). Sequence comparison between SCL helix 1 and MyoD helix 1 shows no homology except for the conserved residues shown in Fig. 1A. Taken together, these findings confirm the absence of key residues in SCL helix 1 and the crucial role played by F238 in helix 2.

**Numerous residues in the SCL loop are biologically important.** To complete the structure-function study, we altered specific residues in the SCL loop. We replaced it with that of NSCL, MyoD, and E47 to generate variants NLP, MLP, and ELP (Fig. 3E). All of these chimeric proteins were impaired in their heterodimerization capacity and inactive in the rescue assay. This included the NSCL chimera (NLP), which differs from SCL at only one residue in the loop (leucine versus H217). This prompted us to specifically mutate H217 in the loop of SCL [mutant LP(H-A)]. This change markedly impaired function (Fig. 3E), confirming the importance of H217. In contrast to the case for the NLP mutant, heterodimerization was retained. It is very likely that this difference reflects the nature of the amino acid replacing H217 (alanine versus leucine). Another point mutation, the change of P215 to a serine, affected heterodimerization and nearly abolished biological activity [Fig. 3E, LP(P-S)]. As proline residues usually bend or even break α-helices, the lack of heterodimerization observed with variant LP(P-S) suggests that P215 might have a specific structural role. Alternatively, P215 could be involved in heterodimerization by directly interacting with the E proteins. Other mutations only partially affected SCL activity (data not shown). Interestingly, mutation of K221 and K222 [mutant LP(KK-RR)], two residues recently described as being acetylated by P/CAF and important for SCL DNA-binding activity and function in MEL cell terminal differentiation (17), did not

appreciably affect primitive or definitive erythropoiesis (data not shown).

From the point mutation analysis, we concluded that two residues are essential for SCL activity: P215, for structural reasons or for directly promoting heterodimerization, and H217, for reasons that remain to be determined.

We next sought to confer hematopoietic activity to the MyoD loop in the context of the otherwise intact SCL molecule. Interestingly, this approach led to the identification of three additional important residues in the loop of SCL which appeared to be necessary for heterodimerization. We first inserted an extra proline in the MyoD loop sequence (to match the size of the SCL loop) and replaced residues at positions 1 and 3 by the corresponding residues defined as important for SCL activity (H217 and P215). This was insufficient to produce functional swap proteins (Fig. 3E [mutants MLP-1 to MLP-4] and data not shown). Replacement of a total of six residues in the MyoD loop (Fig. 3E, mutant MLP-12) was necessary to produce a chimeric protein that was able to heterodimerize and to rescue hematopoiesis from $SCL^{-/-}$ ES cells to extents similar to that observed with wt SCL. Therefore, in addition to P215 and H217, D220, K221, and S224, together, were also important determinants of biological activity. Of the four residues we did not change in the MyoD loop, two are identical to the residues found in the SCL sequence (P218 and L223), and two are conservative changes (serine in place of T216 and arginine in place of K222). The impaired heterodimerization observed with mutants MLP-4 to MLP-11 precluded any assumption about the possible functional roles of these residues. From these findings, we conclude that the majority of the loop residues are necessary to specify dimerization of SCL and that at least one residue (H217) is essential for its functional activity.

**Modification of critical loop and helix 2 residues confers hematopoietic activity to the MyoD HLH domain.** To validate these conclusions, we introduced the residues defined above as critical for SCL activity into the HLH region of MyoD. Our goal was to convert this muscle-specific domain into one capable of rescuing hematopoiesis when introduced into $SCL^{-/-}$ ES cells. A chimeric protein consisting of SCL N-terminal and basic DNA-binding domains followed by MyoD helix 1, the MyoD loop bearing the changes described for mutant MLP-12, MyoD helix 2 with a phenylalanine in place of a glycine, and the SCL C-terminal region was engineered (Fig. 4A, mutant mMyoD). This chimeric protein heterodimerized with E12 (Fig. 4B) and rescued primitive erythropoiesis from $SCL^{-/-}$ ES cells similarly to wt SCL (Fig. 4A).

To assess hematopoietic rescue more precisely, we analyzed primitive erythroid colonies following replating of EB cells into erythropoietin-containing methylcellulose. $SCL^{-/-}$/mMyoD EBs generated primitive erythroid colonies, but to a lesser extent than $SCL^{-/-}$/SCL EBs (Fig. 4C). As expected, no primitive colonies were derived from EBs rescued with SMS. The quality of erythroid maturation was assessed by morphological examination of pure colonies. Complete maturation was observed in both types of rescued colonies (Fig. 4C). To document further the extent of rescue, we quantitated the level of βH1 globin expression in day 6 EBs and primitive erythroid cells by semiquantitative RT-PCR (Fig. 4D, bottom panels). We did not detect noticeable differences in the levels of βH1 globin expression in cells rescued with mMyoD compared to cells rescued with wt SCL. Moreover, these levels were similar to levels of expression detected in wt cells. Finally, levels of expression of the cDNAs introduced into $SCL^{-/-}$ ES cells (wt SCL or mMyoD) (Fig. 4D, top panels) were similar.

In conclusion, we have confirmed the importance of the residues defined in the loss-of-function studies by introducing them into MyoD and switching a muscle-specific HLH domain to a hematopoiesis-specific HLH domain.

**The phenylalanine residue in SCL helix 2 (F238) promotes binding of LMO2.** Like SCL, the LIM-only protein LMO2 is a key regulator of hematopoietic development. LMO2 has been shown to interact in vitro with SCL to form an erythroid-specific multiprotein complex (see the introduction). Using our extensive mutant panel, we characterized the relationship between SCL and LMO2. We employed a mammalian three-hybrid system whereby SCL (wt or mutant), E47, and LMO2 were cotransfected into 293T cells together with a luciferase reporter gene under the control of Gal4 binding sites. Two of the proteins were fused to either GDB (g in designations) or VAD (v in designations). In Fig. 5A, we show that E47 promotes activation of the reporter gene when cotransfected E47 is recruited to the promoter through interaction with gSCL. As expected, this activation is abolished when heterodimerization-deficient mutants [SCL H1(FL-AA) and SCL H2(Y-A)] are used in the assay. Upon transfection of vLMO2 fusion protein with gSCL and E47, activation of the reporter gene is enhanced 18-fold compared to results obtained with gSCL and E47 alone. No activation was observed when VAD or VAD-GATA-1 was cotransfected with gSCL:E47 (data not shown), indicating that in presence of gSCL:E47:vLMO2, enhancement of the reporter gene activity is due to binding of LMO2 to the heterodimer and, consequently, recruitment of the VAD. In contrast, vLMO2 is not recruited to the dimerization-deficient SCL mutants, suggesting that LMO2 interacts principally with SCL:E47 heterodimer.

As SCL has both DNA-binding-dependent and -independent activities (38), we determined whether an intact SCL basic domain is required for interaction with LMO2. We used two mutants of SCL in which the basic domain was inactivated as a result of point mutations (SCL-RER) or a deletion (SCL-ΔB) (38). In both instances, activation of the reporter gene is preserved (Fig. 5B), indicating that the DNA-binding domain of SCL is not required for binding of LMO2 in this context.

Next, selected chimeric proteins were analyzed for their ability to interact with E47 and LMO2 in the three-hybrid assay. First, they were tested for heterodimerization. vSCL and mutants thereof were cotransfected with the E47 bHLH domain fused to the GDB (Fig. 6A). In this assay, SMS and NH1 showed weaker interaction with E47 compared to wt SCL, and MLP failed to show any interaction with the E47 bHLH domain. In contrast, MH1H2 bound E47 at least as well as wt SCL, and reintroduction of the critical phenylalanine residue into mutant MH1H2 did not affect heterodimerization.

We then investigated whether these swap and point mutations affected binding to LMO2 (Fig. 6B). We cotransfected vLMO2 with gE47 and SCL (wt and mutants). Fusion of LMO2 to VAD enabled us to monitor binding of LMO2 to the heterodimer. As expected, vLMO2 strongly activated reporter gene expression in the presence of gE47 and SCL. No activa-

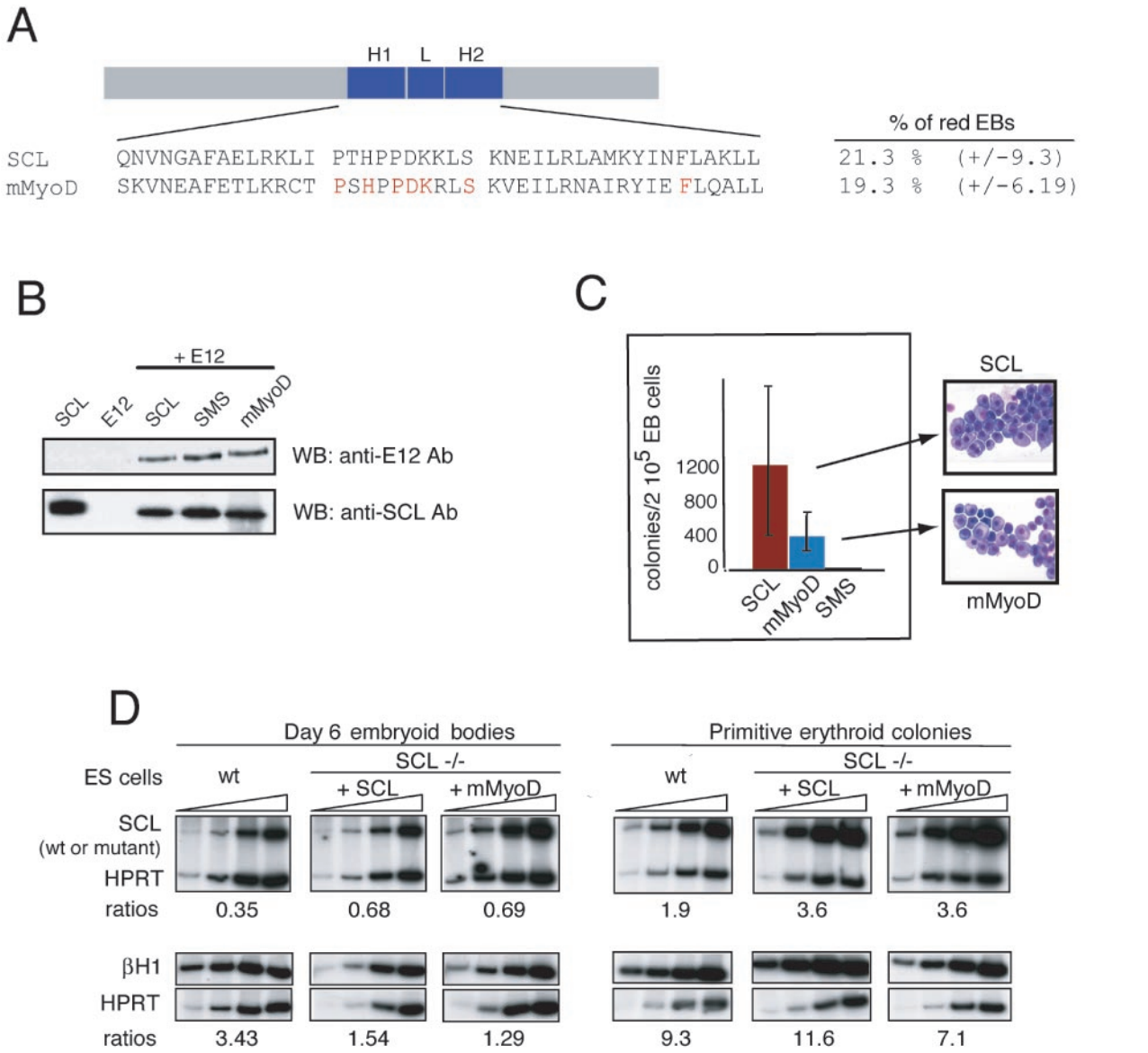


FIG. 4. mMyoD rescues hematopoietic development from SCL$^{-/-}$ ES cells. (A) Left, amino acid sequences of the HLH regions of wt SCL and of the chimeric protein containing the modified MyoD HLH region (mMyoD). Residues of SCL introduced into the MyoD HLH region are shown in red. Right, results of the in vitro differentiation assays. Standard deviations are shown in parentheses. (B) Coimmunoprecipitation experiments. For details, see the legend to Fig. 2C. WB, Western blotting; Ab, antibody. (C) Colony assays. Left, number of primitive erythroid colonies derived from $2 \times 10^5$ EB cells rescued with wt SCL, mMyoD, and SMS. Error bars indicate standard deviations. Right, morphology of cells present in colonies derived from ES cells rescued with wt SCL and mMyoD after May-Grunwald-Giemsa staining. (D) Analysis of RNA expression. RT-PCR was performed with RNAs isolated from day 6 EBs (left panels) or primitive erythroid colonies (right panels) derived from wt ES cells or SCL$^{-/-}$ ES cells rescued with wt SCL (+SCL) or the modified MyoD construct (+mMyoD). Triangles represent increasing PCR cycles. Ratios represent the amount of products amplified from SCL (top panels) or βH1 (bottom panels) sequences relative to the amount of products derived from hypoxanthine phosphoribosyltransferase (HPRT) sequences.

tion was observed with constructs containing nonhematopoietic HLH domains (SMS and SIS). Interestingly, although MLP was not able to heterodimerize (Fig. 6A, gE47 plus vMLP), vLMO2 did promote activation when cotransfected with gE47 and MLP. As LMO2 binds principally to heterodimers (Fig. 5A), our finding suggests that, in this setting, vLMO2 is able to stabilize an otherwise unstable bHLH heterodimer (see below). Replacement of SCL helix 1 by NSCL helix 1 (construct NH1) did not impair binding of LMO2. Finally, and importantly, introduction of a phenylalanine residue into the MyoD helix 1/helix 2 swap mutant strongly increased recruitment of LMO2 to the heterodimer (compare gE47 plus MH1H2 plus vLMO2 and gE47 plus MH1H2-F plus vLMO2 in Fig. 6B). In contrast, dimerization with E47 was not affected by this mutation (Fig. 6A). From these results, we concluded that helix 2 F238 contributes to the hematopoietic activity of SCL by specifically promoting interaction with LMO2.

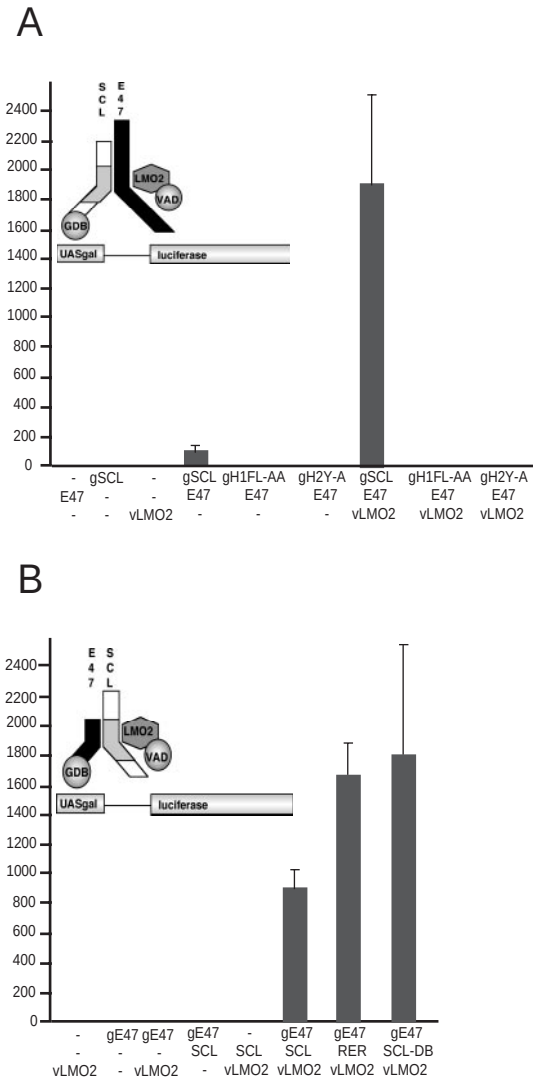


FIG. 5. LMO2 interaction with the SCL/E2A heterodimer does not require the SCL DNA-binding domain. In a mammalian three-hybrid assay, 293T cells were transfected with various combinations of expression constructs for E47, SCL (wt or mutant), and LMO2 sequences, together with a Gal4-responsive luciferase reporter construct. Where indicated, proteins were expressed as VAD (v) or GBD (g) fusion proteins. Levels of expression of the reporter gene are shown as arbitrary units. Error bars indicate standard deviations.

To test whether wt LMO2 was able to stabilize heterodimers, we cotransfected LMO2 with vSCL (wt or mutant) and gE47 (Fig. 6C). We show that LMO2 can enhance both stable (gE47:vSCL) and unstable (gE47:vMH1) dimers but not those dimers containing only nonhematopoietic HLH domains to which LMO2 cannot bind (gE47:vSIS and gE47:vSMS) (Fig. 6C).

Because the mammalian three-hybrid assays do not readily distinguish transient from durable interactions and because VAD or Gal4 domains could modify the conformation and function of the proteins they are fused to, we confirmed these results by coimmunoprecipitation experiments with full-length proteins. 293T cells were cotransfected either with Flag-SCL (wt and mutant) and E47 (Fig. 6D, left panels) or with Flag-SCL (wt and mutant), E47, LMO2, and the LIM-domain binding protein Ldb1 (Fig. 6D, right panels). Coimmunoprecipitation experiments confirmed the heterodimerization data (compare results in Fig. 6A and D, left panels). They also confirmed the enhancement of the heterodimerization ability of unstable heterodimers (MLP:E47 and NH1:E47) in the presence of LMO2 (Fig. 6D, compare the E47 Western blots in the left and right panels) and the role of F238 for efficient recruitment of LMO2 to MH1H2 (Fig. 6D, right panels). Moreover, the pattern of immunoprecipitation of Ldb1 is comparable to that of LMO2. This was expected, as Ldb1 does not bind to SCL directly but is recruited through LMO2 (data not shown). Total protein levels of LMO2 and Ldb1, but not E47, were consistently found to correlate well with the extent of coprecipitation. This could suggest that E47 protein stability is independent of its interaction with SCL, whereas unbound LMO2/Ldb1 is unstable.

To validate these findings with hematopoietic cells, we recovered SCL-containing protein complexes from MEL cells transfected with either wt SCL or the F238 mutant [H2(F-G)] and assayed for the presence of endogenous E proteins and LMO2 in the precipitates. Polypeptides generated from SCL and H2(F-G) cDNAs were tagged by in vivo biotinylation [to generate wt bio-SCL and bio-H2(F-G)], thereby permitting streptavidin affinity purification of SCL-containing complexes. The presence of the proteins of interest was analyzed by Western blotting. Figure 7 shows pull-downs of biotinylated SCL from cells expressing wt bio-SCL and from cells expressing the bio-H2(F-G) mutant. Most of the biotinylated SCL (wt and mutant) bound to the streptavidin beads, as little or no biotinylated SCL is present in the unbound fractions. The F238 mutation fails to affect binding of the E proteins, as both HEB and E12/E47 were pulled down with bio-H2(F-G) to the same extent as with wt bio-SCL. Interestingly, while LMO2 binds very efficiently to wt bio-SCL, some LMO2 is pulled down with bio-H2(F-G), but enrichment is not as complete as with wt bio-SCL, as there is a substantial amount of unbound LMO2. Taken together, these findings confirm the results presented above: F238 does not mediate interaction of SCL with the E proteins but promotes association of LMO2 with the SCL/E-protein heterodimer.

## DISCUSSION

bHLH proteins consist of conserved modules sharing amino acids necessary for proper folding of monomers, dimerization with bHLH partners, and DNA binding. Tissue-specific bHLH proteins exert discrete functions that depend on the nature of the multiprotein complexes they are part of. To mediate interactions with components of these protein complexes, functionally important residues must coexist with conserved structural residues in bHLH proteins.

Here, we sought to define residues within the HLH domain of SCL that are necessary to specify hematopoiesis, as a prelude to studying formation of tissue-specific multiprotein complexes. Our approach has several strengths. First, we have used an ES cell differentiation assay that allows functional studies in the appropriate cellular context, therefore permitting biologically relevant conclusions to be drawn. Second, conversion of a

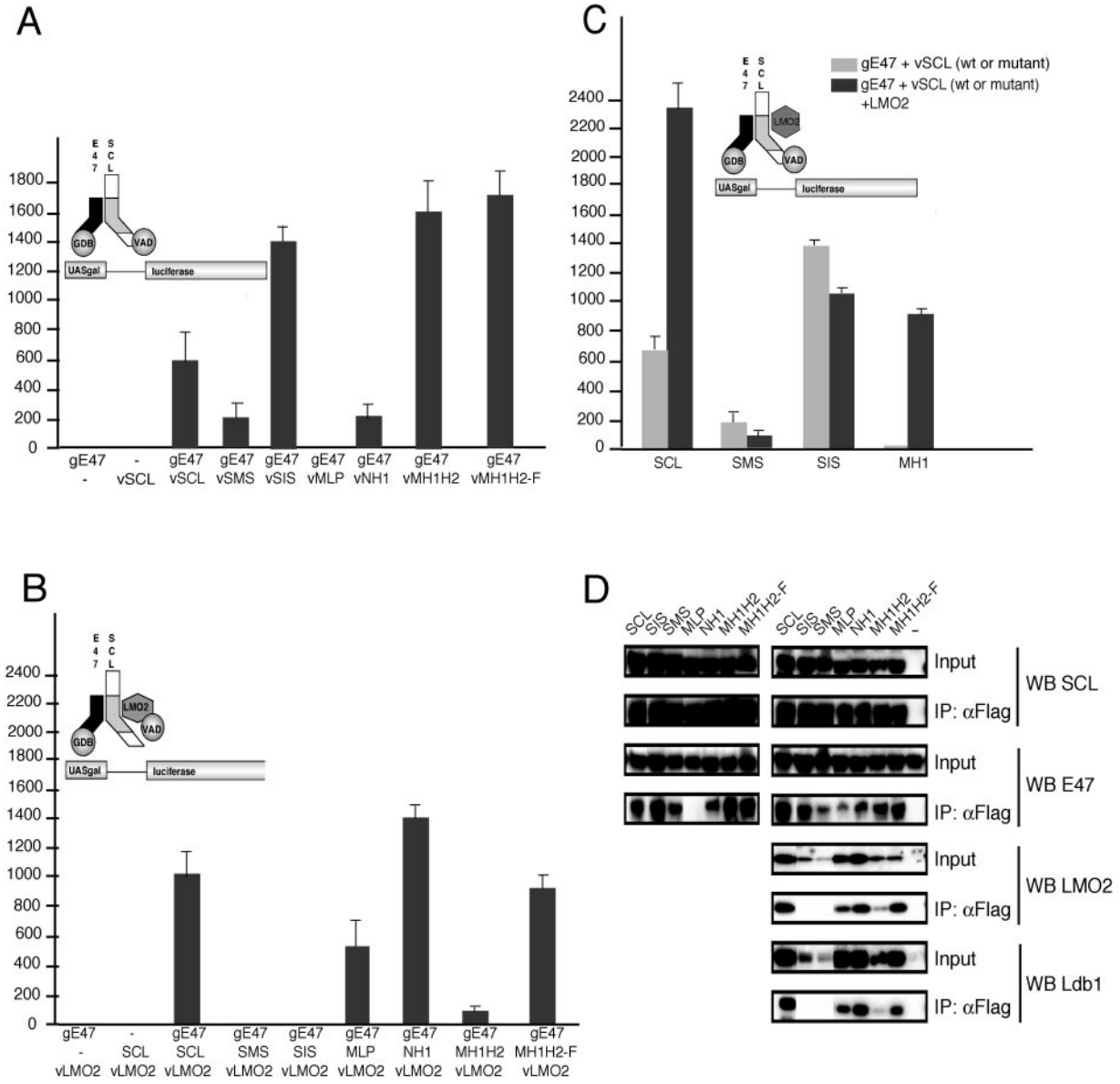


FIG. 6. F238 in SCL helix 2 promotes interaction with LMO2 in heterologous cells. (A to C) Mammalian three-hybrid assays, as described for Fig. 5. In panel C, the nature of the SCL protein (wt or mutant) is indicated on the *x* axis and light and dark grey bars represent transfection of gE47:vSCL (wt or mutant) and gE47:vSCL:LMO2 (wt or mutant), respectively. Error bars indicate standard deviations. (D) Coimmunoprecipitations. Flag-tagged SCL constructs (wt and mutant, as indicated at the top) were cotransfected into 293T cells with E47 (left panels) or with E47, LMO2, and Ldb1 (right panels). After immunoprecipitation (IP) with anti-Flag antibodies, products were analyzed by Western blotting (WB) with the antibodies indicated on the left.

muscle-specific HLH domain into one able to rescue hematopoiesis from $SCL^{-/-}$ ES cells affords a stringent test of those residues sufficient to initiate hematopoiesis. Finally, our demonstration that one of the identified residues promotes interaction with a previously recognized interacting protein, LMO2, validates our strategy, provides mechanistic insight into SCL function, and confirms the biological relevance of a multiprotein SCL-LMO2-containing complex.

**The determinants crucial for SCL function are located in the loop and helix 2.** We defined seven residues (six in the loop and one in helix 2) as the minimal requirements for activity of a bHLH protein in hematopoiesis. These residues serve different roles in SCL function. Two, H217 in the loop and F238 in helix 2, are not required for heterodimerization and therefore are likely to be involved in mediating protein-protein interactions. P215 in the loop may serve a structural role or be directly involved in heterodimerization. Three other amino acids in the loop (D220, K221, and S224), characterized by gain-of-function studies, were found to be necessary to allow the MyoD loop to heterodimerize in the context of the SCL HLH domain. Surface probability algorithms (MacVector; Accelrys) predicted that these residues lie at the surface of the protein (Fig. 1A), also making them attractive docking sites for partner proteins. Finally, the seventh residue, a proline (P219), was

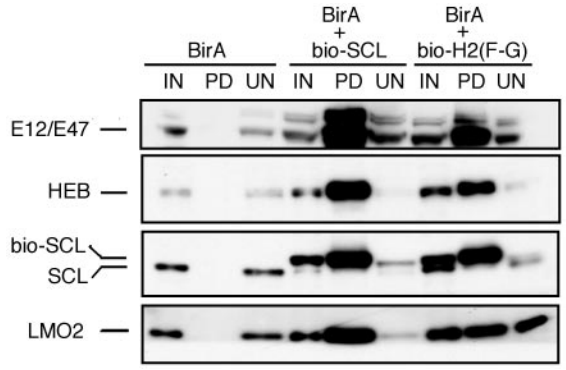


FIG. 7. The SCL-LMO2 interaction in hematopoietic cells requires F238. Nuclear extracts prepared from MEL cells transfected with BirA ligase only or cotransfected with BirA ligase and either bio-SCL or bio-H2(F-G) were incubated with streptavidin beads. Pull-down products were analyzed by Western blotting with anti-E.2AE12 (first panel), anti-HEB (second panel), and anti-SCL C-terminal antibodies (third panel, showing both the endogenous SCL and the biotinylated form of SCL) and LMO2 antiserum (bottom panel). IN, input (equivalent to 1/10 of the amount of nuclear extracts used in the pull-down experiments); PD, pull-down fraction; UN, unbound.

required to confer the appropriate length to the MyoD loop. Together, these findings suggest that the loop of SCL may play an important functional role. Further analysis will be required to fully address the function of the loop residues.

No specificity could be ascribed to helix 1, probably because of the general conformation of HLH domains: while the loop and helix 2 are exposed to the solvent, helix 1 is a prolongation of the basic domain, located between the DNA and the loop and, therefore, not easily accessible (10, 27). Thus, it is likely that SCL helix 1 does not serve any vital function other than providing residues involved in formation of the four-helix bundle of the heterodimer (38; this study).

Although they are able to confer hematopoietic activity to the MyoD HLH domain, the seven critical residues are probably not the only residues in the SCL HLH region that are necessary for full hematopoietic activity. The bHLH domain of SCL has been entirely conserved from zebra fish to human, suggesting that each amino acid may be functionally important. Moreover, the chimeric protein mMyoD was less active than wt SCL in promoting hematopoiesis (Fig. 4C). Therefore, the residues we have defined represent the minimal crucial determinants that confer hematopoietic specificity to an HLH domain. Additional residues are required to achieve maximal biological function.

**The loop of SCL shares similarities with that of the HLH protein Id1.** A similar structure-function analysis of MyoD was performed previously (6) and showed that the MyoD loop could be replaced by loops of various lengths and sequences without altering function. Therefore, this domain has been considered to act as a spacer but not to confer myogenic specificity (6, 7). These data suggest fundamental functional differences between SCL and MyoD.

While the MyoD loop tolerates important perturbations, the loop of Id1 appears to be less flexible and, in this context, shares similarities with the SCL loop (37). Remarkably, residues required for activity of Id1 are located at the same position as some of those that are crucial for the activity of SCL. The respective residues are a proline at the first position (in the SCL loop, this is also a proline, P215) and a leucine at position 3 (in the SCL loop, this is a histidine, H217). Pesce and Benezra (37) suggest that the geometry imposed by the side chain of the proline imposes a specific three-dimensional structure on the HLH domain and is necessary for its activity. Whether this is applicable to the SCL structure remains to be determined. In conclusion, similarities between Id1 and SCL reinforce the view that SCL may function like Id proteins in cell contexts where its DNA-binding activities are dispensable, for instance, in specifying hematopoietic development (38) and promoting leukemogenesis (34). In these settings, SCL might sequester other bHLH proteins and act as a dominant-negative protein (2, 3). Alternatively, and more likely based on our findings reported here, the SCL heterodimer may function by interacting with other proteins (see below).

**A multimeric protein complex builds around the phenylalanine.** The phenylalanine residue in helix 2 is instrumental for the activity of SCL. Not only does its mutation abolish SCL function, but its introduction into two nonhematopoietic helices confers function in a hematopoietic environment. Because F238 is functionally crucial but does not appear to mediate heterodimer formation, we postulated that it might promote recruitment of a protein partner. Given what is known about SCL hematopoietic partners (see the introduction), we considered the possibility that LMO2 might be recruited through F238. Association between SCL and LMO2 has not been formally demonstrated in early hematopoietic progenitors but has been strongly suggested, as loss-of-function mutations of either protein lead to the same early hematopoietic phenotype in mouse embryos and as these proteins do interact in other cell types and cooperate in development of T-ALL in transgenic mouse models (reviewed in reference 40).

Our analysis of the SCL-LMO2 interaction confirmed earlier reports (45) that LMO2 binds principally the SCL/E-protein heterodimer, and it also showed that the integrity of the SCL DNA-binding domain is not required. Therefore, in early hematopoietic progenitors, SCL and LMO2 may reside in a complex that does not bind DNA or that binds through the DNA-binding domains of other transcription factors (20, 35).

Several lines of evidence indicate that F238 specifically mediates the SCL-LMO2 interaction (Fig. 6 and 7). Although F238 is not directly required for heterodimerization, recruitment of LMO2 may reinforce the interactions between SCL and the E proteins (Fig. 6). A number of observations also suggest that SCL and LMO2 are key to the formation of a critical tissue-specific transcriptional complex that consists, at a minimum, of SCL, E2A, LMO2, Ldb1, and, very likely, a GATA protein (20, 29, 35, 44, 46). This multiprotein complex is likely to have activator function, as fusion of SCL to activator domains (E47 or VP16), but not to the Engrailed transcriptional repressor domain, allows for hematopoietic rescue of $SCL^{-/-}$ ES cells (unpublished data). In summary, our data provide persuasive evidence of a functional interaction between SCL and LMO2, thereby extending prior genetic evidence suggesting a link between these regulators in early hematopoiesis.

It is informative to compare the SCL-LMO2 interaction with that of the myogenic bHLH proteins (MRFs) and their protein

partners (MADS or MEFs). Like SCL, the bHLH domain of the MRFs contains conserved "myogenic" amino acids that are sufficient to confer myogenic potential to a nonmyogenic bHLH domain (7) and that are crucial for functional interaction of the MRFs/E-protein heterodimers with MEFs (5). However, in contrast to SCL, the basic region of the MEFs is necessary for imparting myogenic potential and for interaction of MEFs and MRFs. Moreover, in contrast to LMO2, MEFs contain a DNA-binding domain and an activation domain (32). These observations strongly argue that there are differences in the mechanisms of action of class II bHLH proteins. Our detailed structure-function analysis supports this functional complexity, by showing that SCL shares similarities with HLH-only proteins (Id) rather than just with other class II bHLH proteins.

In conclusion, we have identified residues that confer hematopoietic specificity to the HLH domain in early hematopoietic cells, presumably by promoting protein-protein interactions, as shown for the phenylalanine residue in helix 2. We also demonstrate that the loop of SCL is critical for its activity; elucidating the role of the loop residues will shed light on mechanisms of SCL action. More generally, this study provides a biochemical and genetic platform to elucidate the nature of a hematopoietic stem cell-specific protein complex. Finally, our findings should encourage similar molecular dissections of other HLH regions. Identification of residues residing at conserved crucial positions may reveal unexpected structural or functional links between HLH proteins (not reflected by present classifications) and, consequently, reorient thinking about the functions of these key transcription factors.

## ACKNOWLEDGMENTS

We thank Doug Higgs and Bill Wood for critical reading of the manuscript.

T.M.S. was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG). A.S. is a recipient of a Leukemia Research Fund Clinical Fellowship. P.V. is a Wellcome Trust Senior Clinical Fellow. S.F. and C.P. are funded by the Medical Research Council.

## REFERENCES

1. **Agulnick, A. D., M. Taira, J. J. Breen, T. Tanaka, I. B. Dawid, and H. Westphal.** 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.
2. **Aplan, P. D., C. A. Jones, D. S. Chervinsky, X. F. Zhao, M. Ellsworth, C. Wu, E. A. McGuire, and K. W. Gross.** 1997. An *scl* gene product lacking the transactivation domain induces bony abnormalities and cooperates with LMO1 to generate T-cell malignancies in transgenic mice. EMBO J. **16:** 2408–2419.
3. **Bain, G., I. Engel, M. E. C. Robanus, H. P. te Riele, J. R. Voland, L. L. Sharp, J. Chun, B. Huey, D. Pinkel, and C. Murre.** 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.
4. **Benezra, R., R. L. Davis, M. Lockshon, D. L. Turner, and H. Weintraub.** 1990. The protein Id: a negative regulator of helix-loop-helix DNA binding proteins. Cell **61:**49–59.
5. **Black, B. L., J. D. Molkentin, and E. N. Olson.** 1998. Multiples roles for the MyoD basic region in transmission of transcriptional activation signals and interaction with MEF2. Mol. Cell. Biol. **18:**69–77.
6. **Davis, R. L., P.-F. Cheng, A. B. Lassar, and H. Weintraub.** 1990. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. Cell **60:**733–746.
7. **Davis, R. L., and H. Weintraub.** 1992. Acquisition of myogenic specificity by replacement of three amino acid residues from MyoD into E12. Science **256:**1027–1030.
8. **de Boer, E., P. Rodriguez, E. Bonte, J. Krijgsveld, E. Katsantoni, A. Heck, F. Grosveld, and J. Strouboulis.** 2003. Efficient biotinylation and single-step purification of tagged transcription factors in mammalian cells and transgenic mice. Proc. Natl. Acad. Sci. USA **100:**7480–7485.
9. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
10. **Ellenberger, T., D. Fass, M. Arnaud, and S. C. Harrison.** 1994. Crystal structure of transcription factor E47: E-box recognition by a basic region helix-loop-helix dimer. Genes Dev. **8:**970–980.
11. **Ema, M., P. Faloon, W. J. Zhang, M. Hirashima, T. Reid, W. L. Stanford, S. Orkin, K. Choi, and J. Rossant.** 2003. Combinatorial effects of Flk1 and Tal1 on vascular and hematopoietic development in the mouse. Genes Dev. **17:**380–393.
12. **Gering, M., A. R. F. Rodaway, B. Gottgens, R. K. Patient, and A. R. Green.** 1998. The SCL gene specifies haemangioblast development from early mesoderm. EMBO J. **17:**4029–4045.
13. **Gering, M., Y. Yamada, T. H. Rabbits, and R. K. Patient.** 2003. Lmo2 and Scl/Tal-1 convert non-axial mesoderm into haemangioblasts which differentiate into endothelial cells in the absence of Gata1. Development **130:**6187–6199.
14. **Hall, M. A., D. J. Curtis, D. Metcalf, A. G. Elefanty, K. Sourris, L. Robb, J. R. Gothert, S. M. Jane, and C. G. Begley.** 2003. The critical regulator of embryonic hematopoiesis, SCL, is vital in the adult for megakaryopoiesis, erythropoiesis, and lineage choice in CFU-S12. Proc. Natl. Acad. Sci. USA **100:**992–997.
15. **Hsu, H.-L., I. Wadman, J. T. Tsan, and R. Baer.** 1994. Positive and negative transcriptional control by the TAL1 helix-loop-helix protein. Proc. Natl. Acad. Sci. USA **91:**5947–5951.
16. **Huang, S., and S. J. Brandt.** 2000. mSin3A regulates murine erythroleukemia cell differentiation through association with the TAL1 (or SCL) transcription factor. Mol. Cell. Biol. **20:**2248–2259.
17. **Huang, S., Y. Qiu, Y. Shi, Z. Xu, and S. J. Brandt.** 2000. P/CAF-mediated acetylation regulates the function of the basic helix-loop-helix transcription factor TAL1/SCL. EMBO J. **19:**6792–6803.
18. **Huang, S., Y. Qiu, R. W. Stein, and S. J. Brandt.** 1999. p300 functions as a transcriptional coactivator for the TAL1/SCL oncoprotein. Oncogene **18:** 4958–4967.
19. **Jurata, L. W., D. A. Kenny, and G. N. Gill.** 1996. Nuclear LIM interactor, a rhombotin and LIM homeodomain interaction protein, is expressed in neuronal development. Proc. Natl. Acad. Sci. USA **93:**11693–11698.
20. **Lecuyer, E., S. Herblot, M. Saint-Denis, R. Martin, C. G. Begley, C. Porcher, S. H. Orkin, and T. Hoang.** 2002. The SCL complex regulates c-kit expression in hematopoietic cells through functional interaction with Sp1. Blood **100:**2430–2440.
21. **Lecuyer, E., and T. Hoang.** 2004. SCL: from the origin of hematopoiesis to stem cells and leukemia. Exp. Hematol. **32:**11–24.
22. **Lee, J. E.** 1997. Basic helix-loop-helix genes in neural development. Curr. Opin. Neurobiol. **7:**13–20.
23. **Lee, S. K., and S. L. Pfaff.** 2003. Synchronization of neurogenesis and motor neuron specification by direct coupling of bHLH and homeodomain transcription factors. Neuron **38:**731–745.
24. **Liao, E. C., B. H. Paw, A. C. Oates, S. J. Pratt, J. H. Postlethwait, and L. I. Zon.** 1998. SCL/Tal-1 transcription factor acts downstream of *cloche* to specify hematopoietic and vascular progenitors in zebrafish. Genes Dev. **12:**621–626.
25. **Lipkowitz, S., V. Gobel, M. L. Varterasian, K. Nakahara, K. Tchorz, and I. R. Kirsch.** 1992. A comparative structural characterization of the human NSCL-1 and NSCL-2 genes. Two basic helix-loop-helix genes expressed in the developing nervous system. J. Biol. Chem. **267:**21065–21071.
26. **Lyden, D., A. Z. Young, D. Zagzag, W. Yan, W. Gerald, R. O'Reilly, B. L. Bader, R. O. Hynes, Y. Zhuang, K. Manova, and R. Benezra.** 1999. Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. Nature **401:**670–677.
27. **Ma, P. C. M., M. A. Rould, H. Weintraub, and C. O. Pabo.** 1994. Crystal structure of MyoD bHLH domain-DNA complex: perspectives on DNA recognition and implications for transcriptional activation. Cell **77:**451–459.
28. **Massari, M. E., and C. Murre.** 2000. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. Mol. Cell. Biol. **20:**429–440.
29. **Mead, P. E., A. E. Deconinck, T. L. Huber, S. H. Orkin, and L. I. Zon.** 2001. Primitive erythropoiesis in the Xenopus embryo: the synergistic role of LMO2, SCL and GATA-binding proteins. Development **128:**2301–2308.
30. **Mellentin, J. D., S. D. Smith, and M. L. Cleary.** 1989. lyl-1, a novel gene altered by chromosomal translocation in T cell leukemia, codes for a protein with a helix-loop-helix DNA binding motif. Cell **58:**77–83.
31. **Mikkola, H. K., J. Klintman, H. Yang, H. Hock, T. M. Schlaeger, Y. Fujiwara, and S. H. Orkin.** 2003. Haematopoietic stem cells retain long-term repopulating activity and multipotency in the absence of stem-cell leukaemia SCL/tal-1 gene. Nature **421:**547–551.
32. **Molkentin, J. D., B. L. Black, J. F. Martin, and E. N. Olson.** 1995. Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. Cell **83:**1125–1136.
33. **Molkentin, J. D., and E. N. Olson.** 1996. Defining the regulatory networks for muscle development. Curr. Opin. Genet. Dev. **6:**445–453.
34. **O'Neil, J., M. Billa, S. Oikemus, and M. Kelliher.** 2001. The DNA binding

activity of TAL-1 is not required to induce leukemia/lymphoma in mice. Oncogene **20:**3897–3905.
35. **Ono, Y., N. Fukuhara, and O. Yoshie.** 1998. TAL1 and LIM-only proteins synergistically induce retinaldehyde dehydrogenase 2 expression in T-cell acute lymphoblastic leukemia by acting as cofactors for GATA3. Mol. Cell. Biol. **18:**6939–6950.
36. **Park, S. T., and X. H. Sun.** 1998. The Tal1 oncoprotein inhibits E47-mediated transcription. Mechanism of inhibition. J. Biol. Chem. **273:**7030–7037.
37. **Pesce, S., and R. Benezra.** 1993. The loop region of the helix-loop-helix protein Id1 is critical for its dominant negative activity. Mol. Cell. Biol. **13:**7874–7880.
38. **Porcher, C., E. C. Liao, Y. Fujiwara, L. I. Zon, and S. H. Orkin.** 1999. Specification of hematopoietic and vascular development by the bHLH transcription factor SCL without direct DNA binding. Development **126:**4603–4615.
39. **Porcher, C., W. Swat, K. Rockwell, Y. Fujiwara, F. W. Alt, and S. H. Orkin.** 1996. The T-cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. Cell **86:**47–57.
40. **Rabbitts, T. H.** 1998. LMO T-cell translocation oncogenes typify genes activated by chromosomal translocations that alter transcription and developmental processes. Genes Dev. **12:**2651–2657.
41. **Ramain, P., R. Khechumian, K. Khechumian, N. Arbogast, C. Ackermann, and P. Heitzler.** 2000. Interactions between chip and the achaete/scute-daughterless heterodimers are required for pannier-driven proneural patterning. Mol. Cell **6:**781–790.
42. **Robertson, S. M., M. Kennedy, J. M. Shannon, and G. Keller.** 2000. A transitional stage in the commitment of mesoderm to hematopoiesis requiring the transcription factor SCL/tal-1. Development **127:**2447–2459.
43. **Visvader, J. E., X. Mao, Y. Fujiwara, K. Hahm, and S. H. Orkin.** 1997. The LIM-domain binding protein Ldb1 and its partner LMO2 act as negative regulators of erythroid differentiation. Proc. Natl. Acad. Sci. USA **94:**13707–137112.
44. **Vitelli, L., G. Condorelli, V. Lulli, T. Hoang, L. Luchetti, C. M. Croce, and C. Peschle.** 2000. A pentamer transcriptional complex including tal-1 and retinoblastoma protein downmodulates c-kit expression in normal erythroblasts. Mol. Cell. Biol. **20:**5330–5342.
45. **Wadman, I., J. Li, R. O. Bash, A. Forster, H. Osada, T. H. Rabbitts, and R. Baer.** 1994. Specific in vivo association between the bHLH and LIM proteins implicated in human T cell leukemia. EMBO J. **13:**4831–4839.
46. **Wadman, I. S., H. Osada, G. G. Grutz, A. D. Agulnick, H. Westphal, A. Forster, and T. H. Rabbitts.** 1997. The LIM-only protein Lmo2 is a bridging molecule assembling an erythroid, DNA-binding complex which include TAL1, E47, GATA-1, and Ldb1/NL1 proteins. EMBO J. **16:**3145–3157.