Université de Montréal

ÉTUDE DU MÉCANISME D'ACTION DU FACTEUR bHLH HÉMATOPOÏÉTIQUE SCL

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ÉTUDE DU MÉCANISME D'ACTION DU FACTEUR bHLH HÉMATOPOÏÉTIQUE SCL

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RÉSUMÉ

Un point focal du contrôle de la différenciation cellulaire implique l'activité des facteurs de transcription qui établissent des patrons spécifiques d'expression génique. SCL/TAL-1 est facteur à domaine bHLH jouant un rôle essentiel dans la spécification et la différenciation des cellules hématopoïétiques, dont l'activité aberrante contribue fréquemment au développement de leucémies aigues de cellules T chez l'humain. Malgré l'importance biologique de ce facteur, ses gènes cibles et mécanismes d'action demeurent largement inconnus. Les études présentées dans cette thèse ont permis d'identifier les premiers gènes cibles spécifiquement régulés par SCL dans les cellules progénitrices hématopoïétiques et dans le compartiment érythroïde. L'élucidation des mécanismes d'action de SCL dans la régulation de ces gènes a révélé la nécessité de son association au sein de complexes multi-protéiques, formés de facteurs de transcription hémato-spécifiques et ubiquitaires, démontrant un effet tout-ou-rien durant l'activation transcriptionnelle. Ces études ont identifiées des déterminants moléculaires essentiels à l'assemblage et au fonctionnement des complexes SCL et suggèrent que ces complexes évoluent de manière dynamique au cours de la différenciation afin d'activer des gènes linéage-spécifiques. En plus de révéler les propriétés moléculaires de SCL et de fournir une base solide pour des études futures sur ce régulateur crucial, ces travaux illustrent de nouveaux mécanismes par lesquels les facteurs de transcription créent des codes combinatoires dans l'activation de programmes d'expression génique histo-spécifiques.

MOTS CLÉS

Hématopoïèse, Leucémie, Transcription, Complexes, SCL/TAL-1, GATA, LMO2

ABSTRACT

A focal point in the control of cellular differentiation involves the activity of transcription factors that serve to establish appropriate patterns of gene expression. SCL/TAL-1 is a bHLH domain factor the plays an essential role in the specification and differentiation of hematopoietic cells, while its aberrant activation often contributes to the development of acute T cell leukemias in humans. Despite the biological importance of this factor, its target genes and regulatory functions remain poorly characterized. The studies pressented in this thesis have enabled the identification of the first target genes regulated by SCL in hematopoietic progenitors and cells of the erythroid compartment. Characterization of the mechanistic properties of SCL in the regulation of these genes has revealed the requirement for its association within larger multi-factorial complexes, containing hemato-specific and ubiquitously expressed transcription factors, that exhibit a all-or-none behavior in transcription activation. These studies identify key molecular determinants required for the assembly and function of SCL complexes and further suggest that these complexes evolve dynamically during hematopoiesis to activate lineage specific target genes. In addition to revealing the regulatory properties of SCL and providing a solid framework for future studies on this crucial hematopoietic regulator, this work illustrates the mechanisms utilized by transcription factors in order to create combinatorial codes in the activation of tissue-specific programs of gene expression.

KEYWORDS

Hematopoiesis, Leukemia, Transcription, Complexes, SCL/TAL-1, GATA, LMO2

TABLE DES MATIÉRES

LISTE DES TABLEAUX

LISTE DES FIGURES

LISTE DES ABRÉVIATIONS

AD: Activation Domain

AGM: Aorta-Gonads-Mesonephros

AML-1/CBFβ: Acute Myeloid Leukemia-1/Core Binding Factor β

AS: Antisense

β-gal: β-galactosidase

bHLH: basic Helix-Loop-Helix

BMP-4: Bone Morphogenic Protein-4

BL-CFC: Blast-Colony Forming Cell

BFU-E: Bust-Forming Unit Erythroid

CD: Cluster of Differentiation

CEBP/β: CAAT element binding proteins/β

CFU: Colony Forming Unit

ChIP: Chromatin immunoprecipitation

CLP: Common Lymphoid Progenitor

CMP: Common Myeloid Progenitor

DN: Double Negative for CD4/CD8

DP: Double Positive for CD4/CD8

E: Embryonic/Embryonnaire

EKLF: Erythroid Kruppel Like Factor

EMSA: Electrophoretic Mobility Shift Assay

EPO/EPOR: Erythropoietin/Erythropoietin Receptor

ES cells: Embryonic Stem cells

FCS: Fetal Calf Serum

FDG: Fluoresceine di-β-galacto-pyranoside

FOG: Friend Of GATA

GFP/YFP: Green/Yellow Fluorescence Protein

GMP: Granulocyte Macrophage Progenitor

GPA: Glycophorine A

GST: Glutathione-S-Transferase

HEB: Hela Ebox Binding protein

HSC: Hematopoietic Stem Cell

INF-β: Interferon-β

JAK-2: Janus Kinase-2

Ldb-1: LIM-domain binding protein 1

LIM: Lin-Isl-Mec

LMO: LIM-only

MFI: Mean Fluorescence Intensity

MEP: *Myb-Ets*-transformed progenitors

MyoD: Myogenic Determination Factor

PCAF: P300/CBP associated factor

PolII: ARN polymérase II

RALDH2: Retinaldehyde dehydrogenase 2

Rb: Retinoblastoma

RT-PCR: Reverse transcription-polymerase chain reaction

SAGA: Spt-Ada-Gcn5-Acetyltransferase

SCL/TAL-1: Stem Cell Leukemia/T-cell Acute Leukemia-1

SIL: SCL Interrupting Locus

Sp1: Specificity protein 1

SP: Single Positive for CD4 or CD8

TEL: *translocation-Ets-leukemia*

TPO: Thrombopoietin

uORF: Upstream Open Reading Frame

YS/SV: Yolk Sac/Sac Vitellin

DÉDICACE

À mon épouse, Sonthisa

et mes enfants, Élissya et Kobe.

REMERCIEMENTS

La réalisation d'une telle aventure témoigne de l'encouragement et de l'amour inconditionnel de la part de ma partenaire de vie, Sonthisa. Je la remercie pour les sacrifices qu'elle a faits au cours de ces dernières années et pour le rôle essentiel qu'elle remplit dans ma vie et celle de notre jeune famille. J'aimerais aussi remercier mes enfants, Élissya et Kobe, pour la joie et la dose de réalité qu'ils m'apportent.

À mes parents, Normand et Céline Lécuyer, et aux autres membres de ma famille qui m'ont toujours encouragé et supporté dans la poursuite de mes études et ambitions. Je suis aussi extrêmement reconnaissant envers mes beaux-parents, Salika et Benchamat Mathouchanh, pour leur générosité et l'aide qu'ils nous ont apporté avec les enfants et les recettes magnifiques de ma belle-mère.

J'aimerais remercier le Dr Trang Hoang pour sa supervision, sa passion, sa confiance et son encouragement tout au long de mes études doctorales. Elle m'a permis de m'épanouir durant ces années formatrices. De plus, un gros merci à tous mes collègues du labo qui ont fait de mon séjour avec eux une expérience extraordinaire et qui ont enduré mes blagues redondantes pendant tout ce temps. Je suis aussi reconnaissant envers mes collègues de l'IRCM, du Programme de Biologie Moléculaire et les membres de mon comité de thèse. Enfin, merci aux IRSC, à IRCM et au Programme de Biologie Moléculaire pour le soutien financier qu'ils m'ont accordé durant mes études doctorales.

AVANT-PROPOS ET CONTRIBUTION DES AUTEURS

Cette thèse est subdivisée en six chapitres. Les deux premiers représentent l'introduction et offrent, d'abord, une revue de l'ontogénie du système hématopoïétique et des facteurs qui régulent l'homéostasie de ce système, avec un accent sur les facteurs de transcription (Chapitre 1). Le Chapitre 2 représente un article de revue focalisé sur les fonctions biologiques et moléculaires du facteur SCL/TAL-1, un régulateur essentiel du développement hématopoïétique qui a fait l'objet de nos investigations. Ce manuscrit fut écrit par moi-même sous la supervision du Dr. Hoang et a récemment été publié dans la revue *Experimental Hematology*.

Les trois chapitres suivants (Chapitres 3-5) forment le corps du travail et s'avèrent des articles scientifiques originaux, les deux premiers étant déjà publiés, alors que le chapitre 5 représente un manuscrit récemment soumis pour publication.

L'article présenté au Chapitre 3 a été publié en 2002 dans la revue *Blood* et s'avère un ouvrage à auteurs multiples. La majeure partie du travail expérimental (Figures 3.3-3.8), ainsi que l'écriture du manuscrit, a été réalisé par moi-même sous la supervision du Dr. Hoang. Le Dr. S. Herblot a contribuée les résultats présentés dans les Figures 3.1 et 3.2, alors que M. St-Denis a aidée dans l'optimisation d'essais de retard de mobilité sur gel. R. Martin a participé au clonage initial du promoteur *c-kit*. Enfin, les Dr. C. Porcher, S. Orkin et G. Begley nous ont fournis des outils

importants pour l'accomplissement de ces travaux (vecteurs encodant mutants de SCL et souris SCL^{LacZ}).

Le Chapitre 4 est constitué d'un article récemment publié dans *Molecular and Cellular Biology* et s'avère une étude à contribution égale entre le Dr. R. Lahlil et moi-même. Le Dr. Lahlil a entamé l'analyse du promoteur GPA comme gène cible de SCL et a fourni les résultats des Figures 4.1 (A-D), 4.3, 4.4 (A-C), 4.7 (C-D) et 4.8 (A, D-E). Pour ma part, j'ai généré les résultats des Figures 4.2, 4.4 (D), 4.5, 4.6, 4.7 (A-B), et 4.8 (B-C), et j'ai écrit le manuscrit sous la supervision du Dr. Hoang. Le Dr. S. Herblot a contribuée les résultats de la Figure 4.1 (F-H).

Le Chapitre 5 représente un manuscrit à auteurs multiples récemment soumis pour publication. J'ai générer la majorité des résultats et des réactifs présentés dans cet article, en plus de rédiger le manuscit sous la supervision du Dr. Hoang. S. Larivière a fourni les résultats des Figures 5.6, 5.7 (A-C) et 5.8 (B), alors que le Dr. R. Lahlil a effectué les RT-PCR de la Figure 5.7 (D). Enfin, le Dr. B. Wilkes a généré le model moléculaire de l'hétérodimère SCL/E47 exposé dans la Figure 5.5 (E).

La dernière section (Chapitre 6) contient une discussion élargie des découvertes exposées dans cette thèse, plus particulièrement des mécanismes de régulation génique par SCL et l'importance de son intégration au sein de complexes multifactoriels afin d'activer l'expression de gènes hématopoïétiques.

CHAPITRE 1

INTRODUCTION: SECTION 1

1.1 Hématopoïèse

Chaque jour, l'être humain génère approximativement 10^{12} nouvelles cellules sanguines afin de rencontrer les besoins énergétiques de l'organisme, de répondre aux blessures et de combattre les infections microbiennes [1]. Ce processus complexe, l'hématopoïèse, consiste en la formation d'une douzaine de types cellulaires phénotypiquement et fonctionnellement distinctes à partir de cellules souches hématopoïétiques (HSC) pluripotentes. Les caractéristiques de ce système furent conservées au cours de l'évolution et leur étude expérimentale a permis de définir des paradigmes importants quant aux mécanismes qui régulent la différenciation, la survie, la prolifération et la transformation cellulaire. De plus, la perturbation des fonctions normales de ce système, tel qu'observé chez les patients atteints d'anémies, d'immunodéficiences et de leucémies, entraîne des conséquences désastreuses pour l'organisme, soulignant ainsi l'importance d'en comprendre le fonctionnement normal et pathologique.

1.1.1 Ontogénie du système hématopoïétique

Durant le développement embryonnaire chez les vertébrés, les cellules du système hématopoïétique sont parmi les premiers types cellulaires différentiés à apparaître. Une complexité inhérente à l'étude de ce système vient du fait que les cellules sanguines sont produites par vagues distinctes au cours du développement, tant sur le plan spatial que temporel.

1.1.1.1 Hématopoïèse extraembryonnaire

Le système hématopoïétique dérive du feuillet mésodermal, lequel est formé et segmenté le long de l'axe dorso-ventral de l'embryon durant la gastrulation [2,3]. Chez la souris, les cellules mésodermales dans la partie ventrale de l'embryon contribuent à la formation de structures extraembryonnaires, dont le sac vitellin, l'endroit où apparaîtront les premières cellules hématopoïétiques différenciées (Figure 1.1.A). Parmi les facteurs qui contrôlent la segmentation du mésoderme, certains membres de la famille du TGF-β (transforming growth factor-β), dont TGF-β1 et BMP-4 (bone morphogenic protein-4), jouent un rôle primordial dans la spécification du mésoderme ventral hémogénique [4-6]. Au jour embryonnaire 7 (E7), la composante mésodermale du sac vitellin spécifie la formation de cellules hématopoïtiques et endothéliales dans des structures spécialisées, les îlots sanguins (Figurea 1.1.A-C). Les premières cellules hématopoïétiques ainsi formées sont des cellules érythroïdes nucléées, dites primitives, qui expriment les gènes de globines embryonnaires et assurent un apport approprié d'oxygène à l'embryon lors de l'établissement de la circulation au jour E8.5 [2]. Cette vague initiale d'hématopoïèse primitive se déroule de manière transitoire, les précurseurs primitifs disparaissant rapidement autour du jour E9 [7]. Toutefois, le sac vitellin demeure un organe hémogénique actif jusqu'au jour E12.5, produisant certains précurseurs du système hématopoïétique définitif (Figure 1.1.A).

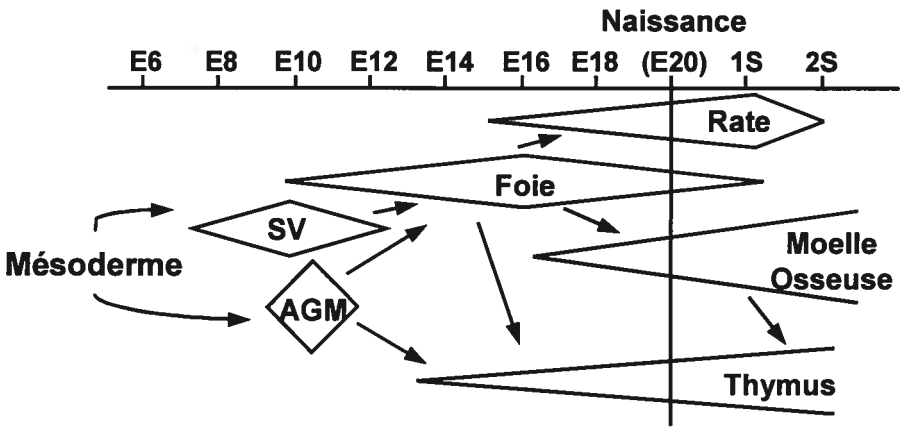
Figure 1.1. Ontogénie du système hématopoïétique.

(A) Les sites hémogéniques sont schématisés selon leur ordre chronologique d'apparition au cours du développement chez la souris. Les évènements de migration des précurseurs et de colonisation de nouveaux sites d'hématopoïèse sont indiqués par des flèches. SV: sac vitellin; AGM: région de l'aorte-gonade-mésonéphros. Adapté de Morrison et al. (1995) *Annu. Rev. Cell Dev. Biol.* 11: 35-71.

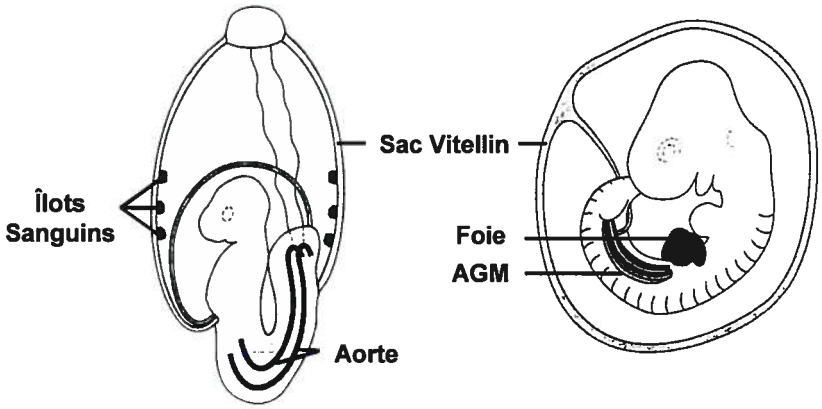
(B) Emplacement des sites hématopoïétiques au cours de l'embryogenèse chez la souris aux jours E8.5/9 (schéma de gauche) et E10.5/11 (schéma de droite). Adapté de Dzierzak et al., (1998) *Immunology today* 19: 228-236.

(C) Représentation schématique de coupes transversales d'un îlot sanguin (gauche) du sac vitellin et de l'aorte dorsale (droite) au jour E10. Dans ces deux sites, une étroite association est observée entre la formation des cellules endothéliales et hématopoïétiques. Adapté de Yoder and Palis (2001) *Hematopoiesis-A developmental approach*, p.180-191 .

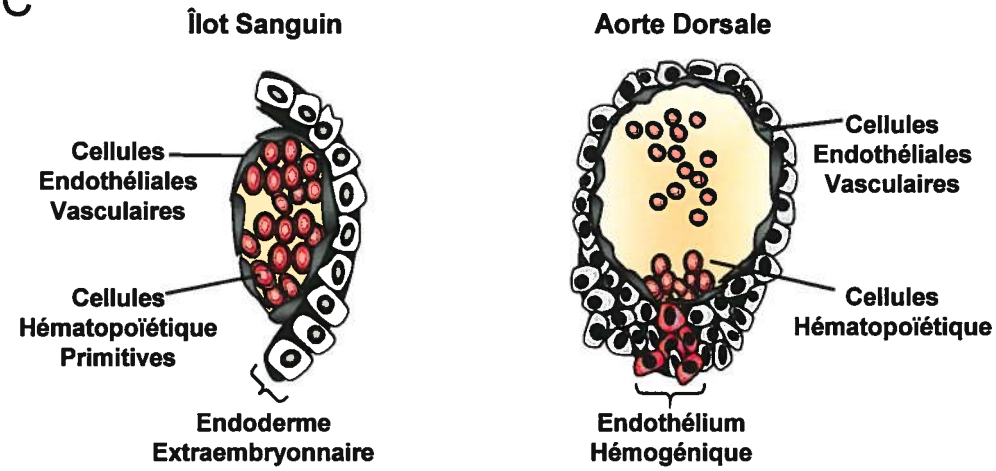
A


Naissance
E6
E8
E10
E12
E14
E16
E18
(E20)
1S
2S
Rate
Foie
SV
Mésoderme
AGM
Moelle
Osseuse
Thymus

B


Sac Vitellin
Îlots
Sanguins
Foie
AGM
Aorte

C


Îlot Sanguin
Aorte Dorsale
Cellules
Endothéliales
Vasculaires
Cellules
Hématopoïétique
Primitives
Endoderme
Extraembryonnaire
Cellules
Endothéliales
Vasculaires
Cellules
Hématopoïétique
Endothélium
Hémogénique

1.1.1.2 Hématopoïèse intraembryonnaire

Outre le sac vitellin, des études plus récentes ont démontrées l'existence d'un second site hémogénique intraembryonnaire, dérivé du mésoderme latéral, dans la région où se développent l'aorte, les gonades et le mésonéphros (AGM) au jour E7.5 (Figure 1.1.A-B) [8]. L'existence de ce site a pour longtemps échappé au regard des expérimentateurs, due à l'absence de foyer hématopoïétique visible, et n'a été révélé que par des études fonctionnelles permettant la détection de progéniteurs et de HSC. Bien que l'AGM ne représente pas un site de maturation terminale des cellules sanguines, il s'agit du premier site où l'on peut détecter la présence de HSC définitives au cours du développement [9-14].

À partir du jour E12, le foie fœtal représente le principal organe hématopoïétique de l'embryon et demeure un site hémogénique important jusqu'à la naissance (Figure 1.1.A). L'hématopoïèse hépatique est caractérisée par la production de cellules sanguines définitives des voies érythroïde, myéloïde et lymphoïde, et résulte d'une colonisation au jour E9.5 dont l'origine cellulaire, intra ou extraembryonnaire, fut longuement disputée. Des études récentes suggèrent que le sac vitellin représente la première source majeure de précurseurs hématopoïétiques qui colonisent le foie [7], assurant une production robuste de cellules définitives à court terme (Figure 1.1.A). Cependant, les HSC responsables de l'établissement à long terme du compartiment hématopoïétique définitif proviendraient plutôt de la région de l'AGM (Figure 1.1.A). Ainsi, des études embryologiques chez le poulet et la grenouille démontrant

que l'hématopoïèse définitive origine d'une source intraembryonnaire homologue à l'AGM viennent appuyer cette hypothèse [15,16]. De plus, des cellules hématopoïétiques bourgeonnant de la portion ventrale de l'aorte ont été observées dans plusieurs espèces (Figure 1.1.C) [16-20]. Chez la souris, ces cellules ont des propriétés de HSC et leur relâchement dans la circulation, autour du jour E10, précède l'apparition de cellules souches dans le foie fœtal [21-23].

Peu après l'établissement de l'hématopoïèse hépatique, une production transitoire de cellules sanguines est observée dans la rate, alors que le thymus devient un site majeur de maturation des cellules lymphoïdes (Figure 1.1.A). Enfin, la dernière transition majeure de sites hémogéniques observée au cours du développement a lieu juste avant la naissance, et implique la colonisation de la moelle osseuse des os par des HSC en provenance du foie fœtal. L'hématopoïèse hépatique s'éteint tôt après la naissance et la moelle osseuse persiste comme site principal d'hématopoïèse définitive tout au long de la vie de l'individu. Ainsi, au cours du développement, l'hématopoïèse est un processus dynamique impliquant la mobilisation de cellules souches et la colonisation de nouveaux sites hémogéniques.

1.1.2 L'hémangioblaste

Une caractéristique intrigante du développement hématopoïétique est le lien intriqué qui existe entre la formation des cellules sanguines et endothéliales vasculaires [24]. Au niveau du sac vitellin (Figure 1.1.C), l'apparition concomitante des cellules

érythroïdes primitives et endothéliales dans les îlots sanguins a mené à l'hypothèse que ces deux types cellulaires dérivent d'un précurseur commun, l'hémangioblaste [25]. Cette association intime est aussi observée dans la région de l'AGM (Figure 1.1.C), où les cellules hématopoïétiques qui émergent de la portion ventrale de l'aorte bourgeonnent à partir de cellules endothéliales sous-jacentes, dites hémogéniques [26-30]. En plus de ces indices histologiques, l'existence d'un précurseur commun aux voies endothéliales et hématopoïétiques est appuyée par plusieurs observations complémentaires. D'abord, ces deux populations cellulaires partagent l'expression de plusieurs marqueurs importants, incluant des protéines de surface cellulaire (flk-1, tie-2, c-Kit, PECAM et CD34) et des facteurs de transcription (SCL/TAL-1, LMO2, GATA-2 et AML-1/RUNX-1) [24,25]. En outre, la fonction de certains de ces gènes est requise dans la formation des deux lignages car l'ablation génique de flk-1, SCL et LMO2 est létale au jour E9 due à une perturbation dramatique de la formation et/ou de la maturation des cellules hématopoïétiques et endothéliales [31-39]. Deuxièmement, l'étude du mutant spontané *cloche* chez le poisson zèbre, caractérisé par des défauts sévères dans la formation des cellules sanguines et endothéliales, a mis en évidence l'existence de déterminants génétiques essentiels pour la formation de l'hémangioblaste [1,40]. Enfin, des études de différenciation in vitro de cellules souches embryonnaires (ES) ont conduit à l'identification du BL-CFC (blast colony-forming cell), un précurseur capable de générer des cellules hématopoïétiques et endothéliales dans des cultures clonales, qui correspond probablement à l'hémangioblaste [41]. Bien que la distinction entre hémangioblaste et endothélium hémogénique demeure ambiguë,

l'ensemble de ces preuves suggère qu'au cours du développement le destin hématopoïétique soit spécifié par des précurseurs qui détiennent également un potentiel endothélial.

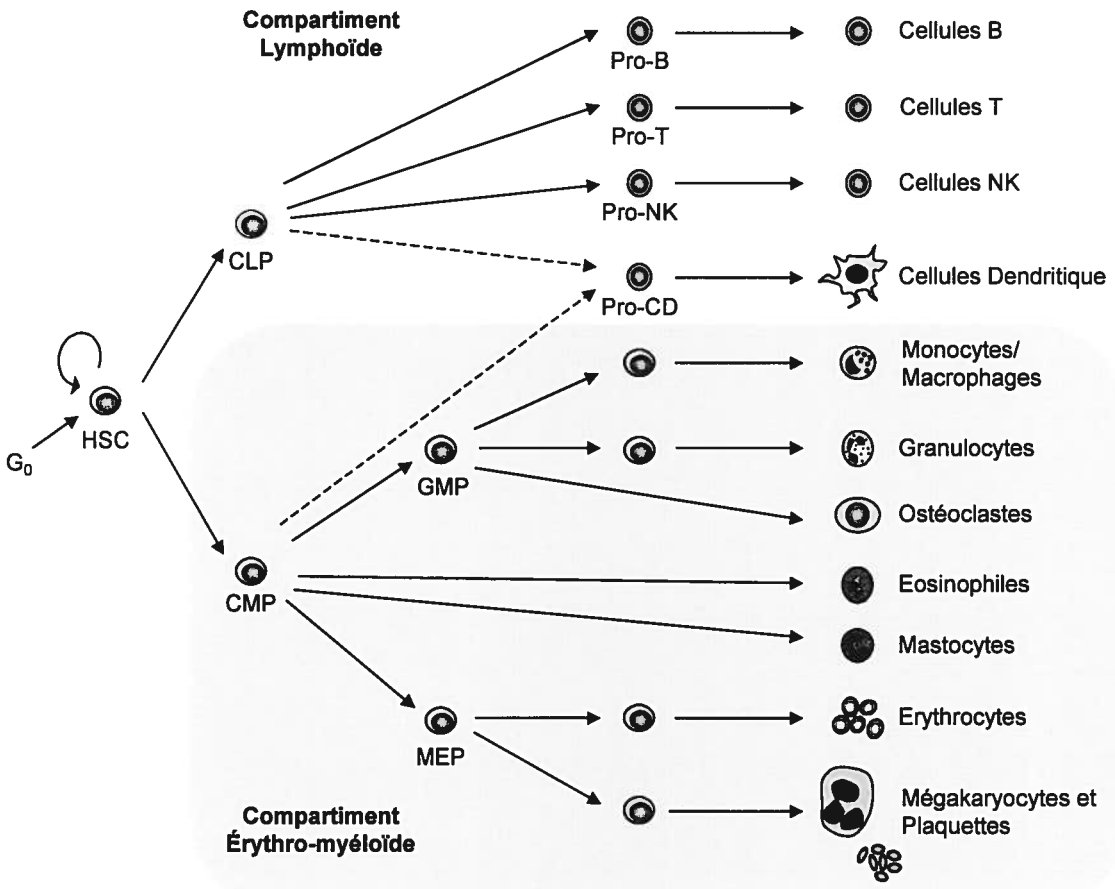
1.1.3 Le système hématopoïétique adulte

Le réseau hématopoïétique définitif démontre une organisation hiérarchique, traditionnellement schématisée sous forme d'un arbre où les cellules souches représentent la racine, alors que les précurseurs différenciés et leur progéniture forment les embranchements (Figure 1.2). L'existence de cellules souches à la base de la hiérarchie hématopoïétique fut d'abord suggérée par des études effectuées il y a une quarantaine d'années par Till, McCulloch et Becker, qui ont démontré que la moelle osseuse contient des cellules clonogéniques capables de former des colonies macroscopiques sur la rate, à composition érythro-myéloïde, après transplantation dans un receveur irradié [42,43]. Ces cellules, désignées CFU-S (colony forming unit-spleen), démontraient des caractéristiques attendues de cellules souches puisqu'elles étaient dotées d'un grand potentiel prolifératif et d'une capacité d'auto-renouvellement après transplantation dans un hôte secondaire [44]. Bien que des études subséquentes ont démontrées que le CFU-S ne représente pas une véritable HSC, mais plutôt un progéniteur commun de la voie érythro-myéloïde [45], son identification a amené l'étude de l'hématopoïèse du niveau descriptif qualitatif vers un niveau quantitatif. D'autre par, ces études nous ont mené à la vision actuelle de la structure du système hématopoïétique adulte.

Figure 1.2. Hiérarchie cellulaire du système hématopoïétique définitif.

Les cellules souches hématopoïétiques (HSC), qui existent majoritairement sous forme quiescentes (G0), peuvent générer l'ensemble des cellules matures du réseau hématopoïétique par un processus qui implique la formation de précurseurs intermédiaires. Par leur capacité d'auto-renouvellement, les HSC assurent un approvisionnement continu en cellules hématopoïétiques à l'organisme. La différenciation s'accompagne d'une restriction graduelle de la capacité prolifération et de la pluripotence. CLP=précurseur commun lymphoïde; CMP=précurseur commun érythro-myéloïde; GMP= précurseur des granulocytes /macrophages; MEP=précurseur érythroïde/mégakaryocytaire. Adapté de Passegué et al. (2003) *Proc Natl Acad Sci U S A.* 100 Suppl 1:11842-11849.

Pluripotence et potentiel prolifératif



Cellules Souches	Précureurs Multipotents	Précureurs Unipotents	Cellules Matures

Les HSC sont définis fonctionnellement par leur capacité à reconstituer à long terme l'ensemble du compartiment hématopoïétique, lymphoïde et myéloïde, après transplantation dans un hôte irradié [44,46]. En plus de leur potentiel de différenciation pan-hématopoïétique, les HSC sont caractérisées par leur propriété d'auto-renouvellement, leur haut potentiel prolifératif et leur capacité à exister sous forme quiescente in vivo [47]. La différenciation des HSC implique d'abord la formation de progéniteurs pluripotents capables de générer l'ensemble des cellules de la voie lymphoïde (CLP, progéniteur commun lymphoïde) et érythro-myéloïde (CMP, progéniteur commun myéloïde), lesquels donnent lieu à des précurseurs bi et unipotents engagés vers des voies spécifiques de différenciation, aboutissant enfin à la formation d'une douzaine de types cellulaires matures fonctionnellement distincts (Figure 1.2). Les divers types de progéniteurs hématopoïétiques ont pu initialement être identifiés et caractérisés grâce au développement d'essais fonctionnels, généralement basés sur la capacité des cellules progénitrices à former des colonies distinctives dans des cultures en milieu semi-solide. Cependant, la caractérisation de marqueurs de surface distinctifs pour des populations précises de cellules hématopoïétiques et l'avènement d'appareils permettant de trier ces cellules à partir de mélanges hétérogènes, ont permis l'isolement de populations enrichies en HSC et en précurseurs selon leurs phénotypes, facilitant ainsi l'analyse de leurs propriétés biologiques [48]. Puisque le potentiel prolifératif des cellules hématopoïétiques décline au cours de la différenciation, à l'exception des lymphocytes B et T, la propriété d'auto-renouvellement des cellules souches est essentielle afin d'assurer un apport constant de cellules hématopoïétiques à l'organisme [44]. Ainsi, la production

de cellules sanguines matures à partir de HSC résulte d'un processus complexe impliquant la formation de précurseurs intermédiaires et s'accompagne d'une restriction graduelle et irréversible du potentiel de différenciation (Figure 1.2) [44].

1.2 Mécanismes de régulation de l'hématopoïèse

Comprendre les mécanismes qui contrôlent l'équilibre entre l'auto-renouvellement et la différenciation est non seulement un thème central dans l'étude de l'organogenèse, mais s'avère aussi un aspect fondamental dans l'étude du cancer où l'auto-renouvellement s'effectue de manière incontrôlée. Les années d'efforts consacrées à la caractérisation du système hématopoïétique en font un modèle extraordinaire pour étudier les mécanismes régulant les choix de destin cellulaire et le maintien d'une réserve de précurseurs non-différenciés. Bien que ces mécanismes demeurent mal compris, l'homéostasie du système hématopoïétique semble être régulée à différents niveaux, impliquant des facteurs extrinsèques retrouvés dans l'environnement et des facteurs intrinsèques aux cellules sanguines.

1.2.1 Régulateurs extrinsèques

1.2.1.1 La niche hématopoïétique

Les cellules hématopoïétiques résident dans des micro-environnements spécialisés (i.e. des niches), où une combinaison d'interactions cellulaires et de facteurs de

croissance module leur développement [49]. L'importance du micro-environnement pour maintenir les cellules souches et les progéniteurs hématopoïétiques dans un état non-différentié est soulignée par le fait que leur potentiel d'auto-renouvellement est rapidement perdu lorsqu'elles sont cultivées in vitro. De plus, une variété d'études portant sur des populations distinctes de cellules souches, soit dans le contexte du système nerveux, des tissus épithéliaux ou des tissus germinaux chez certains organismes invertébrés, ont démontrées que des signaux provenant de cellules spécialisées du micro-environnement jouent un rôle primordial dans l'auto-renouvellement des cellules souches [50]. Outre les cellules sanguines, la niche hématopoïétique est constituée de plusieurs autres types cellulaires incluant des cellules stromales, des ostéoblastes, des fibroblastes et des cellules endothéliales [49,51]. En plus de produire des composantes de la matrice extracellulaire et de sécréter des facteurs de croissance, ces cellules entretiennent des interactions étroites avec les HSC, ayant pour effet de moduler la survie, l'auto-renouvellement et la prolifération de ces dernières [49]. En effet, deux études récentes ont démontré que les ostéoblastes, cellules responsables de la synthèse de la matrice osseuse qui se localisent à la surface interne des os, s'associent étroitement avec les HSC dans la moelle osseuse, et qu'une augmentation du nombre d'ostéoblastes stimule proportionnellement l'expansion des HSC [51-53]. Ainsi, le maintien d'une réserve de HSC non-différenciées est finement régulé via des interactions avec certaines cellules spécialisées du micro-environnement hématopoïétique. Bien qu'une vue d'ensemble des voies de signalisation qui régulent l'auto-renouvellement des HSC demeure incomplète, plusieurs études ont identifié les voies induites par Wnt, SHH

(sonic hedgehog) et Notch, des régulateurs essentiels de la spécification tissulaire chez de nombreux organismes, comme des modulateurs importants de ce processus [52-58].

1.2.1.2 Les cytokines et leurs récepteurs

Les cytokines extra-cellulaires et leurs récepteurs comptent parmi les régulateurs importants de l'hématopoïèse. Certaines cytokines, dont *Steel Factor* (SF), l'interleukine-3 (IL-3) et le facteur de stimulation des granulocytes/macrophages (GM-CSF) agissent au niveau des HSC et des progéniteurs pluripotents, alors que d'autres, comme l'érythropoiétine (EPO), la thrombopoïétine (TPO) et le facteur de stimulation des granulocytes (G-CSF), exercent principalement leurs effets sur des précurseurs unipotents engagés vers une voie particulière de différenciation [59]. Les fonctions biologiques les mieux caractérisées de ces facteurs incluent leur capacité à stimuler la prolifération et la survie des cellules hématopoïétiques qui expriment leurs récepteurs, alors que leur rôle dans l'engagement des HSC et des progéniteurs vers des voies spécifiques de différenciation (i.e. le choix de destin cellulaire) demeure controversé [59]. Deux modèles furent proposés afin d'expliquer le rôle des cytokines dans la différenciation [47,59-61]. Le premier, désigné déterminatif, prétend que les récepteurs de cytokines influencent les choix de destin cellulaire des HSC et des progéniteurs en fournissant des signaux instructifs aux cellules. En contre partie, le modèle stochastique propose que l'engagement d'une cellule souche vers une voie spécifique de différenciation soit déterminé par des facteurs intrinsèques et

que les cytokines n'exercent qu'une fonction de prolifération et de survie afin d'appuyer un programme de différenciation préétabli. Chacun de ces modèles est appuyé par des évidences expérimentales qui laissent place à une certaine interprétation. Prenons l'exemple de l'EPO et de son récepteur (EPOR). Bien que l'ablation des gènes qui encodent ces facteurs cause un blocage de la différenciation des cellules érythroïdes définitives au stade de pro-érythroblaste et la mort au jour E13, les précurseurs unipotents engagés vers la voie érythroïde sont détectables dans ces mutants [62-64], suggérant soit que l'axe EPO/EPOR joue un rôle permissif dans l'expansion du compartiment érythroïde ou que les signaux instructifs émis par l'EPOR sont redondants avec d'autres récepteurs hématopoïétiques lors de l'enclenchement de l'érythropoïèse. L'existence d'une importante redondance fonctionnelle entre l'EPOR et certains récepteurs de cytokines, dont c-mpl (le récepteur de la TPO), est d'abord appuyée par la démonstration que la TPO peut supporter la différenciation érythroïde de cellules $EPOR^{-/-}$ in vitro et que la portion cytoplasmique effectrice de l'EPOR peut être fonctionnellement remplacée par celles de plusieurs récepteurs hétérologues au cours de la différenciation érythroïde [63,65]. De plus, l'ablation du gène encodant la tyrosine kinase JAK2, un effecteur commun de plusieurs récepteurs de cytokines, dont l'EPOR et c-mpl, cause un phénotype érythroïde beaucoup plus sévère que celui observé chez les souris $EPOR^{-/-}$, soit une absence complète de cellules érythroïdes définitives [66,67]. Ces observations suggèrent qu'une combinaison de récepteurs qui utilisent JAK2 comme effecteur pourrait fournir des signaux instructifs afin de consolider la différenciation érythroïde. Ainsi, bien que l'élucidation du rôle exact de ces facteurs dans la

différenciation nécessitera des efforts soutenus, il est clair que le réseau complexe formé par les cytokines et leurs récepteurs joue une fonction centrale dans l'hématopoïèse en favorisant l'expansion et la survie des cellules sanguines. Il est aussi fort probable que les effecteurs finaux des voies de signalisation activées par les récepteurs incluent des facteurs de transcription hémato- ou linéage-spécifiques chargés de consolider la différenciation vers une voie spécifique.

1.2.2 Régulateurs intrinsèques de l'hématopoïèse

Puisque les changements phénotypiques qui accompagnent la différenciation reflètent des variations dans l'expression des gènes, l'étude des mécanismes qui établissent des patrons d'expression génique spécifiques à chacune des voies de différenciation du réseau hématopoïétique représente un point de départ logique pour comprendre les rouages qui contrôlent l'hématopoïèse.

1.2.2.1 La régulation génique

Afin de générer l'incroyable diversité de cellules spécialisées qui composent le corps humain, des groupes spécifiques de gènes, parmi les 35000-50000 gènes qui tapissent le génome, doivent être exprimés au bon moment dans des populations cellulaires précises [68]. Les mécanismes qui assurent cette régulation différentielle sont variés, intimement reliés entre eux et peuvent avoir pour effet de stimuler ou d'inhiber l'expression génique [68,69]. L'activation d'un gène transcrit par l'ARN

polymérase II (PolII) nécessite le recrutement séquentiel et l'assemblage d'énormes complexes protéiques sur des éléments de régulation en *cis* du gène, soit sur le promoteur situé à proximité du site d'initiation de la transcription et sur des séquences activatrices pouvant être localisées à des distances considérables du gène [70-76]. Ce processus est à la base entamé par des facteurs de transcription exprimés dans des populations cellulaires précises et capables de lier l'ADN sur des séquences spécifiques dans les régions régulatrices de leurs gènes cibles. Ces facteurs assurent le recrutement ciblé de complexes de remodelage de la chromatine, afin d'établir une structure chromatinienne propice au recrutement de la machinerie basale de la transcription et de la PolII [72,73,75]. Les facteurs de transcription sont des protéines modulaires, possédant des domaines distinctifs servant à la liaison à l'ADN, à la multimérization sous forme d'homo- et d'hétéro-dimères, ou jouant une fonction trans-effectrice durant l'activation ou la répression transcriptionnelle [45,77]. Une fois que la transcription d'un gène eut été initiée par la PolII, plusieurs étapes additionnelles sont requises avant d'aboutir à l'expression d'une fonction protéique. L'élongation de la transcription, la maturation et le transport des molécules d'ARN, la traduction et le repliement des protéines, et la stabilité des ARN et des protéines représentent des facettes importantes de l'expression génique qui sont susceptibles d'être régulées par des mécanismes histo-spécifiques [68,78,79]. Ce scénario est rendu d'autant plus complexe par l'existence de régulateurs dévoués à la répression transcriptionnelle, tel que les histones déacétylases et les protéines du groupe polycomb [80-82]. Ainsi, l'expression génique implique la coordination d'activités biochimiques complexes qui s'avèrent souvent physiquement et fonctionnellement

inter-reliées [68,73]. Par leur capacité à cibler ces activités biochimiques sur des loci particuliers, les facteurs de transcription jouent un rôle déterminant dans l'établissement et le maintien d'un énorme répertoire de programmes d'expression génique nécessaire à l'élaboration d'un organisme multi-cellulaire complexe.

1.2.2.2 Les facteurs de transcription hématopoïétiques: liaisons intimes avec la transformation leucémique

Curieusement, l'importance de la régulation transcriptionnelle dans l'homéostasie du système hématopoïétique a d'abord été suggérée par l'étude de lésions moléculaires survenant chez des patients atteints de leucémies aiguës. Contrairement aux cellules hématopoïétiques normales, dont la capacité d'auto-renouvellement est finement régulée et décroît durant la différenciation, les leucémies aiguës sont caractérisées par l'accumulation de progéniteurs anormaux bloqués à un stade précis de la différenciation et dans un état d'auto-renouvellement perpétuel [83,84]. L'analyse cytogénique des tumeurs leucémiques a permis d'établir que des réarrangements chromosomiques récurrents sont associés à des sous-types particuliers de leucémies, et que ces anomalies moléculaires jouent un rôle déterminant dans le développement tumoral en perturbant l'expression ou les fonctions normales des gènes retrouvés aux points de jonction des lésions chromosomiques [84,85]. Or, les gènes les plus souvent ciblés par ces réarrangements sont ceux qui encodent des facteurs de transcription, soulignant ainsi l'importance de cette classe de régulateurs dans le contrôle de la différenciation et de l'auto-renouvellement des cellules

hématopoïétiques. De plus, des études subséquentes ont démontré que la majorité des facteurs de transcription identifiés par cette stratégie sont des régulateurs essentiels du développement hématopoïétique normal [84,85]. Ainsi, de manière homologue aux criblages génétiques chez des organismes modèles invertébrés comme la *Drosophile* et *C. elegans*, l'analyse des mutations survenant dans les leucémies humaines s'est avérée une ressource indispensable dans l'identification des gènes et des réseaux moléculaires qui contrôlent l'hématopoïèse normale.

1.2.2.3 La hiérarchie des régulateurs hématopoïétiques

Puisque l'hématopoïèse se déroule par vagues distinctes au cours du développement et implique la génération de plusieurs types cellulaires spécialisés, cette structure laisse présager l'existence d'un réseau complexe de régulateurs transcriptionnels à la base de ces processus. Effectivement, la combinaison d'études moléculaires des syndromes hématopoïétiques, de la régulation de promoteurs linéage-spécifiques (par exemple ceux des gènes de globines et des immunoglobulines), et l'avènement des approches de transgenèse et d'inactivation génique chez la souris, a mené à l'identification d'une panoplie de facteurs de transcription agissant à différents niveaux dans la hiérarchie hématopoïétique (Figure 1.3) [86-88].

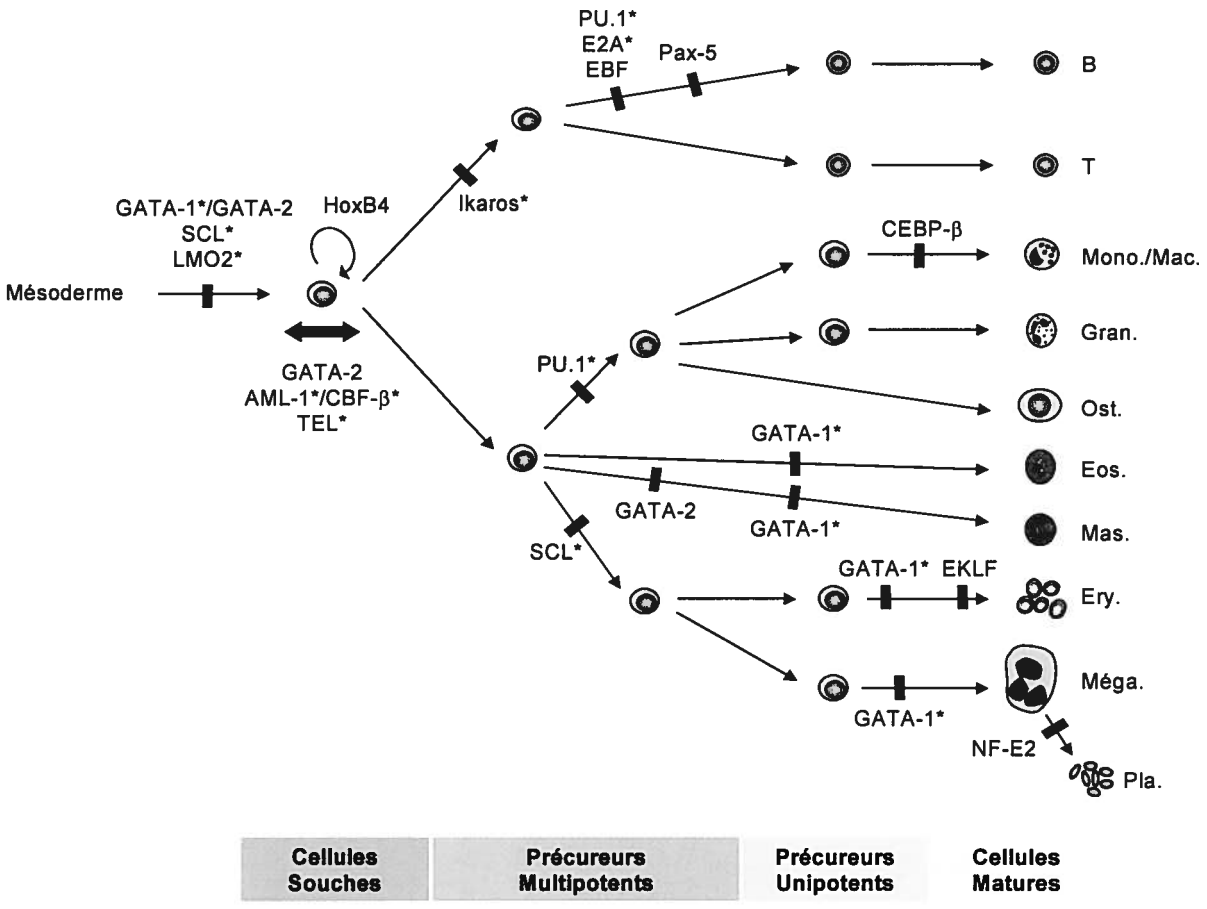


Figure 1.3. Hiérarchie des régulateurs transcriptionnels hématopoïétiques.

Certains facteurs de transcription sont indiqués selon les positions où ils exercent un rôle essentiel au sein du réseau hématopoïétique, tel que révélé par des études d'ablation génique chez la souris (voir le texte pour les détails sur chaque régulateur). Certains régulateurs exercent des fonctions importantes à plusieurs niveaux dans ce réseau. Les astérisques identifient les facteurs impliqués dans la leucémogenèse chez l'humain ou la souris.

1.2.2.3.1 SCL, LMO2 et les facteurs GATA: initiateurs de l'hématopoïèse

Certains facteurs de transcription jouent un rôle primordial dans la spécification du système hématopoïétique à partir de précurseurs mésodermaux. Tel est le cas de SCL et LMO2, des protéines à domaine bHLH et LIM respectivement, dont l'ablation génique cause une absence complète de cellules hématopoïétiques, primitives et définitives, et une mort précoce au jour E8.5 (Figure 1.3) [31,32,34,35,89,90]. Les gènes SCL et LMO2 ont d'abord été identifiés comme des cibles fréquentes de réarrangements chromosomiques chez des patients atteints de leucémies aiguës de type T (T-ALL) et leur potentiel leucémogène a par la suite été confirmé dans des modèles de souris transgéniques (revisé dans [91]). L'importance de LMO2 dans la transformation leucémique humaine a ressurgi récemment, puisque ce gène a été identifié comme une cible d'insertion rétrovirale, menant à un syndrôme lympho-prolifératif de type T-ALL, chez deux jeunes patients immunodéficients qui participaient à une étude clinique basée sur la thérapie génique par l'entremise de vecteurs rétro-viraux [92,93]. Cette découverte a eu pour répercussion de freiner un grand nombre d'études cliniques utilisant des vecteurs semblables et a réitéré le rôle oncogénique de LMO2 dans la leucémogenèse humaine.

Un autre régulateur essentiel de l'hématopoïèse primitive est le membre fondateur de la famille des facteurs GATA, GATA-1, initialement identifié par sa capacité à lier des motifs du même nom dans les régions régulatrices de gènes érythroïdes [94,95]. Chez les vertébrés, la famille GATA est composé d'au moins six membres, dont

GATA-1, -2 et -3 qui remplissent des fonctions importantes au sein du réseau hématopoïétique (discuté dans la référence [96]). Les protéines de cette famille sont caractérisées par la présence d'un domaine distinctif formé de deux doigts de zinc qui modulent la liaison à l'ADN et l'interaction avec des partenaires protéiques. Bien que l'expression de GATA-1 et -2 se chevauche dans les cellules mastocytaires et mégakaryocytaires, seul GATA-1 persiste dans les cellules érythroïdes et éosinophiles en différenciation, alors que GATA-2 est d'avantage enrichi dans les progéniteurs hématopoïétiques multipotents [87]. Contrairement à SCL et LMO2, la perte de fonction de GATA-1 ne cause pas une absence complète de cellules hématopoïétiques, mais conduit plutôt à un blocage de la différenciation des cellules érythroïdes, primitives et définitives, au stade de pro-érythroblaste, causant la mort embryonnaire entre les jours E10.5-11.5 suite à une anémie sévère (Figure 1.3) [97-100]. Comme GATA-1, les souris déficientes pour GATA-2 exhibent une perturbation de l'hématopoïèse primitive et meurent d'une anémie au jour E11 [101]. Cependant, l'absence de GATA-2 n'entraîne pas un blocage de la différenciation érythroïde, mais cause plutôt un phénotype pan-hématopoïétique caractérisé par une réduction globale du nombre de cellules sanguines, suggérant qu'il exerce un rôle dans l'expansion des cellules souches et des progéniteurs hématopoïétiques (Figure 1.3) [101]. Puisque les domaines d'expression de GATA-1 et -2 se chevauchent partiellement et que GATA-2 est hautement sur-exprimé dans le compartiment érythroïde de souris déficientes pour GATA-1, une question importante demeurait: Est-ce que ces facteurs démontrent une redondance fonctionnelle in vivo? Afin d'adresser cette question, Fujiwara et al. ont récemment généré des souris

doublement déficientes pour ces facteurs et ont démontré que leur ablation combinée bloque complètement l'apparition de cellules hématopoïétiques primitives, un phénotype essentiellement identique aux souris $SCL^{-/-}$ et $LMO2^{-/-}$ (Figure 1.3) [102]. Ainsi, en plus d'exercer des fonctions spécialisées non-redondantes au sein de la hiérarchie des cellules sanguines, cette étude démontre l'importance de l'activité combinée des facteurs GATA dans les étapes les plus précoces du développement hématopoïétique. La similitude des phénotypes de perte de fonction de SCL, LMO2 et des facteurs GATA s'explique en partie par le fait que ces protéines interagissent physiquement au sein de complexes multifactoriels (voir section 1.2 ci-dessous). De plus, l'expression ectopique combinée de ces facteurs chez la grenouille et le poisson zèbre conduit à une expansion importante du compartiment hématopoïétique, au détriment de certains tissues d'origine mésordermal [103-105].

1.2.2.3.2 Facteurs requis pour la transition vers différents sites hémogéniques et pour l'expansion des cellules souches

Une autre catégorie importante de régulateurs comprend des facteurs de transcription qui s'avèrent dispensables pour l'hématopoïèse primitive, mais qui remplissent une fonction essentielle dans l'établissement de l'hématopoïèse définitive. Par exemple, l'inactivation des gènes encodant AML1 ou CBFβ, des facteurs à domaine RHD (*Runt homology domain*) qui agissent sous forme de complexes hétéro-dimériques, perturbe complètement le développement des cellules hématopoïétiques définitives causant la mort au jour E12.5 due à une absence d'hématopoïèse hépatique (Figure

1.3) [106-109]. Des études complémentaires ont démontrées que AML1 est un marqueur spécifique des HSC qui émergent de la portion ventrale de l'aorte et que ce facteur est requis pour la formation de cellules souches fonctionnelles [23,27,110]. De plus, les gènes AML1 et CBFβ sont les cibles les plus fréquentes de translocations chromosomiques dans les leucémies aiguës humaines [84]. Dans la majorité des cas, ces translocations mènent à la production de protéines chimériques, contenant une portion des protéines AML1 ou CBFβ fusionnée à des domaines protéiques exogènes, qui démontrent des propriétés moléculaires aberrantes [111,112]. Un autre exemple important est celui du gène TEL (*translocation-Ets-leukemia*), un gène fréquemment transloqué au niveau du locus AML1 dans les leucémies aiguës de type B et qui encode un facteur de transcription de la famille Ets [84]. Wang et al. ont voulu évaluer l'importance du gène TEL pour le développement hématopoïétique en générant des souris chimériques contenant des cellules ES $TEL^{-/-}$, afin de détourner la mortalité précoce observée chez des souris $TEL^{-/-}$ due à des défauts de vascularisation du sac vitellin [113,114]. Or, bien que les cellules TEL déficientes participent à la formation des tissus hématopoïétiques du sac vitellin et du foie fœtal, elles sont incapables de contribuer au compartiment hématopoïétique résidant dans la moelle osseuse (Figure 1.3) [114]. Ces études démontrent l'existence de régulateurs requis spécifiquement pour l'établissement de nouvelles vagues d'hématopoïèse au cours du développement.

1.2.2.3.3 Contrôle de la différenciation des cellules sanguines: l'ajout de nouveaux ingrédients et le recyclage de plusieurs régulateurs

Bien que certains facteurs de transcription, tels que EKLF, NF-E2 et CEBPβ, démontrent des fonctions hautement spécifiques à des points précis du réseau hématopoïétique (Figure 1.3) [115-118], un grand nombre de régulateurs semblent d'avantage exercer des fonctions dans plusieurs embranchements du réseau. Par exemple, Ikaros, une protéine contenant de multiples doigts de zinc initialement identifié comme un régulateur du gène CD3δ et impliqué dans l'activation et la répression transcriptionnelle, est essentiel pour le développement de l'ensemble de cellules lymphoïdes (Figure 1.3) [119,120]. Outre le blocage important du développement lymphoïde observé dans des souris déficientes pour Ikaros, l'inactivation partielle de ce facteur mène à un désordre lymphoprolifératif des cellules T, suggérant qu'il joue des rôles distincts à différents niveaux du compartiment lymphoïde [121]. Un autre exemple concerne le facteur PU.1, une protéine à domaine Ets, dont l'inactivation génique perturbe dramatiquement la formation des monocytes/macrophages, des granulocytes, des ostéoclastes et des lymphocytes B (Figure 1.3) [122-125]. L'étude de PU.1 a ainsi démontré l'existence de déterminants communs aux voies lymphoïdes et myéloïdes, et certains ont même avancé l'hypothèse que PU.1 puisse agir au niveau d'une cellule progénitrice commune à ces deux voies de différenciation [87,122,124]. Similairement, en plus de leur rôle dans l'établissement de l'hématopoïèse primitive, GATA-1 et -2 sont tous les deux requis dans la maturation des mastocytes [101,126,127], alors que GATA-1

est aussi essentiel pour le développement approprié des mégakaryocytes et des éosinophiles (Figure 1.3) [128-130]. Enfin, un exemple encore plus impressionnant est celui de SCL, dont on ignorait largement les rôles spécifiques durant l'hématopoïèse adulte due à la létalité précoce des souris $SCL^{-/-}$ [32,34]. Or, des études récentes, où le gène SCL fut inactivé de manière inductible, ont démontrées qu'il est un déterminant essentiel pour la formation des cellules érythroïdes et mégakaryocytaires chez des souris adultes (Figure1.3) [131,132]. Bien que les exemples exposés ici ne représentent pas un assemblage exhaustif des facteurs de transcription hématopoïétiques, ceux-ci démontrent que le développement des cellules sanguines implique un grand nombre de régulateurs transcriptionnels qui agissent à des stades et dans des populations précises de cellules sanguines, et que plusieurs de ces facteurs sont réutilisés à différents niveaux dans la hiérarchie hématopoïétique.

1.2.2.4 La différenciation hématopoïétique: promiscuité d'expression et régulateurs à effets multiples

Malgré l'identification d'un grand nombre de régulateurs hématopoïétiques, les caractéristiques moléculaires du processus de choix de lignage d'une HSC demeuraient jusqu'à récemment mal définies. La vision traditionnelle de ce processus supposait qu'une cellule souche existait dans un état basal vierge et que l'activation d'un programme de différenciation n'avait lieu qu'à partir du moment ou la cellule s'engageait définitivement vers une voie donnée [133-136]. Cependant, des

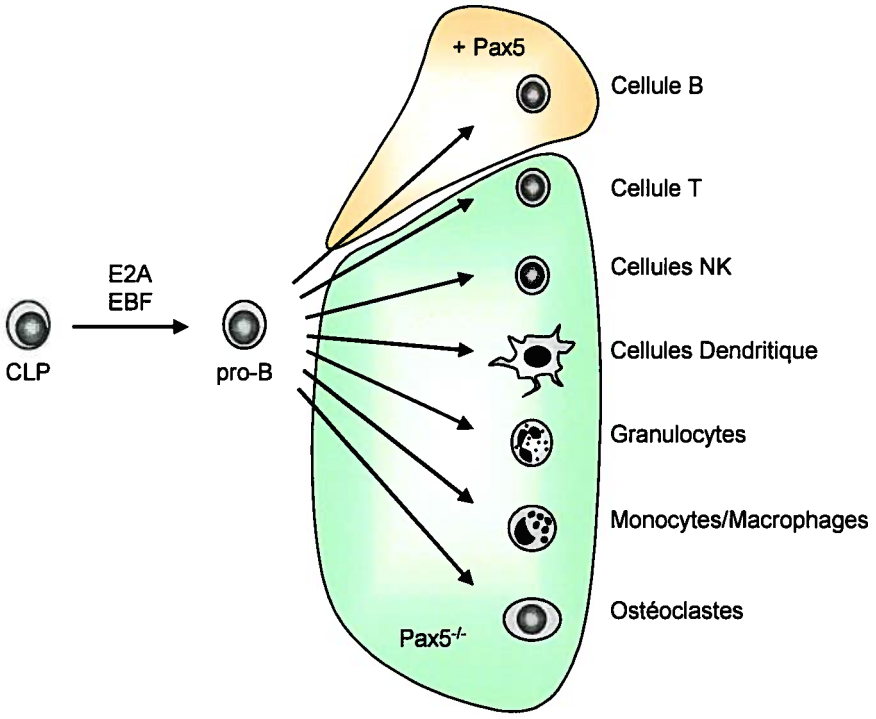
travaux récents, où l'expression génique chez des précurseurs hématopoïétiques (primaires ou transformés) fut analysée de manière clonale, suggèrent que les HSC et progéniteurs pluripotents démontrent une promiscuité surprenante dans leur profils d'expression génique, puisqu'ils co-expriment à de faibles niveaux plusieurs gènes dont l'expression est normalement associée à des cellules hématopoïétiques matures [133,137-141]. Par exemple, certaines cellules HSC et CMP co-expriment la β-globine et la myélopéroxydase, des gènes normalement associés aux voies érythroïdes et granulocytaires, respectivement [140]. Puisque les HSC utilisées dans ces analyses provenaient de mélanges cellulaires hétérogènes et que la fonctionnalité de ces dernières n'a pu être évaluée, il était possible que les cellules qui exprimaient ces marqueurs ne soient pas de véritables HSC. Cependant, une étude récente par Ye et al., qui contourne les faiblesses expérimentales des stratégies précédentes grâce à l'utilisation d'une approche de marquage cellulaire in vivo, démontre que des HSC exprimant le gène lysozyme, un marqueur spécifique de la voie myélo-monocytaire, conservent effectivement leurs propriétés de cellules souches, confirmant la promiscuité d'expression génique des HSC [142]. Puisque cette promiscuité disparaît progressivement au cours de la différenciation et reflète la restriction graduelle du potentiel de différenciation des progéniteurs hématopoïétiques, l'engagement d'une HSC vers une voie de différenciation nécessite non-seulement l'activation accrue d'un programme génique particulier, mais implique aussi une répression des choix alternatifs de différenciation.

Figure 1.4. Propriétés antagonistes des facteurs de transcription.

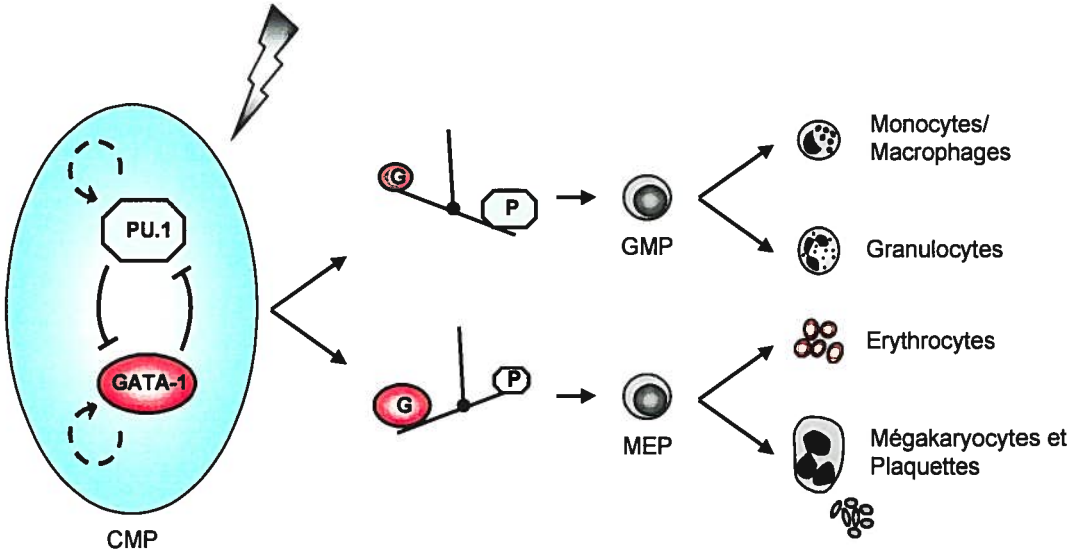
(A) L'enclenchement du processus de différenciation des lymphocytes B nécessite d'abord E2A et EBF, alors que Pax-5 est nécessaire pour restreindre le potentiel de différenciation des cellules pro-B. En absence de Pax-5, les cellules pro-B peuvent se différencier le long de plusieurs voies lympho-myéloïdes. Adapté de Busslinger et al. (2000) *Curr. Opin. Immunol.* 12: 151-158.

(B) L'antagonisme entre GATA-1 et PU.1 peut potentiellement créer un code binaire pour le choix des voies myéloïdes ou érythro-mégakaryocytaires à partir d'un précurseur multipotent. La capacité d'auto-régulation de ces facteurs, combiné à leur effet inhibiteur l'un vis-à-vis de l'autre, en font des régulateurs dominants. L'équilibre entre GATA-1 et PU.1 pourrait vraisemblablement être modulé par des signaux extrinsèques, cependant ceux-ci demeurent inconnus. Adapté Tenen (2003) *Nat. Rev. Cancer* 3: 89-101.

A


+ Pax5
Cellule B
Cellule T
Cellules NK
Cellules Dendritique
Granulocytes
Monocytes/Macrophages
Ostéoclastes
Pax5-/-
E2A
EBF
CLP
pro-B

B


PU.1
GATA-1
CMP
G
P
GMP
MEP
Monocytes/
Macrophages
Granulocytes
Erythrocytes
Mégakaryocytes et
Plaquettes

Un exemple qui illustre parfaitement cette idée concerne l'étude de régulateurs impliqués dans la différenciation des lymphocytes B, incluant E2A et EBF, deux protéines à domaine bHLH, et le facteur à domaine *paired* Pax5 (Figures 1.3 et 1.4.A). Les protéines E2A et EBF jouent un rôle primordial dans l'activation du programme lymphoïde B. En effet, l'ablation génique pour ces facteurs conduit à une absence complète de lymphocytes B (Figure 1.3) [143-145], et leur sur-expression dans des cellules hétérologues induit l'expression de gènes lymphoïde-spécifiques endogènes [146-148]. Or, Pax5 compte parmi les gènes cibles activés par E2A et EBF [149], et ce dernier est requis pour la progression de la différenciation au delà du stade pro-B (Figure 1.3) [150]. Alors que des cellules pro-B normales se différentient exclusivement en lymphocytes B lorsqu'elles sont cultivées in vitro, Nutt et al. ont fait la découverte surprenante que les cellules Pro-B $Pax5^{-/-}$ ne se différencient plus en lymphocytes B, mais elles acquièrent plutôt la capacité de se différentier le long de plusieurs autres voies lympho-myéloïdes (Figure 1.4.A) [151]. Ces observations suggèrent que la différenciation des lymphocytes B nécessite d'abord la présence d'activateurs qui enclenchent le processus (E2A et EBF), suivie de facteurs qui consolident le programme lymphoïde-B (Pax-5) en supprimant les choix alternatifs de différenciation [135,152].

Bien que le mécanisme par lequel Pax-5 restreint le potentiel de différenciation des cellules B demeure floue, des études focalisées sur PU.1 et GATA-1 ont démontré l'importance de l'antagonisme entre les facteurs de transcription dans le contrôle des choix de destin cellulaire. Ces deux facteurs comptent parmi les régulateurs

hématopoïétiques qui détiennent la capacité d'induire un processus de différenciation robuste dans différents contextes cellulaires [135]. Par exemple, la sur-expression de PU.1 dans des progéniteurs hématopoïétiques transformés de poulet, des MEP (*Myb-Ets-transformed progenitors*), qui démontrent un potentiel de différenciation érythro-myéloïde, induit leur différenciation vers la voie myélo-monocytaire tout en inhibant l'expression de marqueurs de cellules progénitrices et mégakaryocytaires [153]. De plus, l'expression ectopique de PU.1 dans des précurseurs érythroïdes bloque leur différenciation et cause une érythroleucémie [154]. Au contraire, l'expression accrue de GATA-1 dans des MEP supprime l'expression de marqueurs myéloïdes et stimule leur différenciation en cellules érythroïdes, mégakaryocytaires et éosinophiles selon un processus dose dépendant [155]. De manière similaire, deux études récentes ont démontré que la sur-expression de GATA-1 dans des fractions de cellules primaires enrichies en HSC, CMP, CLP ou GMP (voir Figure 1.2), perturbe dramatiquement le patron normal de différenciation de ces cellules en favorisant les voies érythroïdes, éosinophiles et mégakaryocytaires [141,156]. Ainsi, PU.1 et GATA-1 agissent comme des facteurs dominants pour stimuler des voies de différenciation complémentaires au sein de la hiérarchie hématopoïétique, une propriété qui découle en partie du fait que ces facteurs s'autorégulent positivement (Figure 1.4.B) [157-159]. De plus, ils démontrent des activités antagonistes, puisque chacun inhibe les voies normalement stimulées par l'autre (Figure 4B). Au niveau moléculaire, cet antagonisme résulte d'une interaction directe entre PU.1 et GATA-1, ayant pour effet de perturber leur liaison à l'ADN ou l'interaction avec des co-facteurs spécifiques [160-162]. Dans le contexte d'un progéniteur pluripotent, ces facteurs existent

probablement dans un état d'équilibre permettant l'activation simultanée de leurs gènes cibles respectifs à faible niveau (Figure 1.4.B), un processus qui pourrait expliquer la promiscuité d'expression génique des cellules progénitrices hématopoïétiques. Lorsque cet état d'équilibre est perturbé, soit par des mécanismes stochastiques ou instructifs, le facteur qui prendra le dessus sera en mesure d'imposer son programme au détriment de l'autre. Ainsi, cette combinaison de propriétés des régulateurs hématopoïétiques, soit la capacité d'activer des gènes spécifiques, d'antagoniser des régulateurs parallèles et d'autoréguler leur propre expression, permet de créer un code binaire robuste afin de moduler les choix de destin cellulaire au sein du réseau hématopoïétique.

1.2.2.5 La combinatoire des facteurs de transcription hématopoïétique

Les exemples illustrés précédemment démontrent l'existence d'un réseau complexe de régulateurs transcriptionnels qui contrôle l'homéostasie du système hématopoïétique. Cependant, l'élucidation des mécanismes d'action de ces facteurs s'avère un défi considérable puisqu'il devient de plus en plus clair que la capacité de ces protéines à réguler l'expression génique nécessite leur intégration au sein de complexes multifactoriels composés d'un amalgame de facteurs ubiquitaires et histo-spécifiques [71,133,163]. L'assemblage des régulateurs sous forme de complexes, combiné à la disposition variable des motifs d'ADN reconnus par ces facteurs dans les régions régulatrices de leurs gènes cibles, fournit les bases moléculaires pour générer un immense répertoire de patrons d'expression génique à partir d'un nombre

limité de régulateurs et représente l'essence de la théorie du code combinatoire des facteurs de transcription [164-166]. Cette structure organisationnelle suggère que des changements dynamiques dans la composition des ces complexes puissent avoir un rôle important à jouer dans la régulation de la différenciation hématopoïétique, tout en offrant une stratégie pour moduler la flexibilité d'un régulateur afin d'activer ou de réprimer la transcription dans différents contextes cellulaire ou en réponse à des signaux particuliers de l'environnement [71,163,167]. Cependant, la caractérisation de complexes d'ordre-supérieur impliqués dans la régulation de gènes hématopoïétiques n'est qu'à ses débuts.

1.2.2.6 SCL comme modèle d'étude de la combinatoire des facteurs de transcription hématopoïétique

Or, le facteur à domaine bHLH SCL représente un modèle fascinant pour l'étude du contrôle combinatoire de la transcription des gènes hématopoïétiques. En effet, il a été démontré que ce facteur s'associe au sein de complexes multifactoriels incluant des régulateurs à expression ubiquitaire (E2A et Ldb-1) et hémato-spécifiques (LMO2 et GATA-1) [168-170]. Tel que mentionné auparavant, ce facteur est essentiel pour la spécification du compartiment hématopoïétique, pour la différenciation des cellules érythroïdes et mégakaryocytaires, et dans le développement des leucémies de type T-ALL. Ces observations suggèrent que les partenaires de SCL doivent sans doute varier selon le contexte cellulaire afin d'assurer ses fonctions de régulateur transcriptionnel. Cependant, malgré

l'importance biologique de ce facteur, ses gènes cibles et mécanismes d'action durant l'hématopoïèse normale et leucémique demeuraient largement inconnus lorsque j'ai entamé mes études doctorales. Ainsi, le but de ma thèse fut de caractériser les mécanismes d'action de ce facteur dans la régulation transcriptionnelle. Au cours de mes recherches, j'ai pu: 1) identifier des gènes cibles régulés par SCL dans le contexte de cellules progénitrices et érythroïdes; 2) élucider le mécanisme d'action de SCL dans la régulation de ces gènes afin de révéler que son association au sein de complexes multi-protéiques est indispensable pour sa fonction; et 3) identifier des déterminants moléculaires essentiels à l'assemblage et au fonctionnement approprié de ces complexes. Les résultats de ces travaux contribuent grandement à notre compréhension des propriétés moléculaires de SCL et fournissent des fondements solides pour des études futures sur ce régulateur central de l'hématopoïèse.

Le chapitre qui suit offre une revue plus approfondie des propriétés biologiques et moléculaires de SCL.

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CHAPITRE 2

INTRODUCTION: SECTION 2

SCL: from the origin of hematopoiesis to stem cells and leukemia

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2.1 Abstract

In the hematopoietic system, lineage commitment and differentiation is controlled by the combinatorial action of transcription factors from diverse families. SCL is a basic helix-loop-helix transcription factor that is an essential regulator at several levels in the hematopoietic hierarchy and whose inappropriate regulation frequently contributes to the development of pediatric T cell acute lymphoblastic leukemia. This review discusses advances that have shed important light on the functions played by SCL during normal hematopoiesis and leukemogenesis, and have revealed an unexpected robustness of hematopoietic stem cell function. Molecular studies have unraveled a mechanism through which gene expression is tightly controlled, as SCL functions within multifactorial complexes that exhibit an all-or-none switch-like behavior in transcription activation, arguing for a quantal process that depends on the concurrent occupation of target loci by all members of the complex. Finally, variations in composition of SCL-containing complexes may ensure flexibility and specificity in the regulation of lineage-specific programs of gene expression, thus providing the molecular basis through which SCL exerts its essential functions at several branch points of the hematopoietic hierarchy.

In eukarytotes, the generation of diverse cell types relies on mechanisms that establish and maintain specific programs of gene expression. Central in this process is the function played by transcription factors, which act in a combinatorial manner to activate or repress specific sets of target genes in the appropriate cellular environment. These themes are particularly relevant in the hematopoietic system, where differentiation and leukemogenesis are controlled by the combined interactions of transcription factors from diverse families [1-6]. Hematopoiesis, which occurs in successive waves during development, is characterized by the production of a dozen distinct cell types through the regulated differentiation of pluripotent hematopoietic stem cells (HSC) and oligo-potent progenitors [7]. The hematopoietic system represents a powerful model to study the mechanisms through which transcription factors control differentiation and lineage commitment decisions [4]. Indeed, gene-targeting studies in mice have identified a series of regulators that function non-redundantly at various stages of hematopoietic cell differentiation and in specific blood cell lineages [1]. Many of the genes that encode crucial hematopoietic transcription factors are also deregulated in human leukemias [8-11], strengthening the view that the appropriate regulation of transcription factor networks is pivotal for maintaining proper tissue homeostasis within the hematopoietic compartment.

A perfect example of this dichotomy is a transcription factor of the basic Helix-Loop-Helix (bHLH) family called SCL/TAL-1/TCL-5, hereafter referred to as SCL. Indeed, gene ablation studies have shown that SCL is an essential regulator at several

levels of the hematopoietic hierarchy [12-17], while it is also the most frequent target of chromosomal rearrangements in children with T-cell acute lymphoblastic leukemia (T-ALL) [18]. As exemplified by SCL, bHLH factors are essential regulators of cell fate determination in organisms ranging from fruit flies to humans [19]. These proteins dimerize through their HLH domain and bind DNA within its major groove on consensus Ebox motifs (CANNTG) via residues in their basic (b) region [20-22]. Like most tissue-specific bHLH factors, the binding of SCL to Ebox motifs requires its heterodimerization with a distinct class of bHLH proteins, designated E-proteins, which includes the products of the E2A (E12 and E47), HEB, and E2-2 genes in vertebrates [23,24]. SCL can either activate or repress transcription, and its ability to regulate gene expression is dependent on its integration within multifactorial complexes containing other essential hematopoietic and ubiquitously expressed regulators. However, the exact mechanisms that account for the range of transcriptional effects that SCL exerts remain to be fully characterized. Here, we review recent progress in our understanding of SCL function at different levels within the hematopoietic system and in T-ALL, and discuss how these studies have revealed novel and unexpected insight into the regulation of hematopoiesis at the cellular and molecular level.

2.2 Developmental expression and function of SCL

Although the SCL gene was originally identified as a recurrent target of chromosomal translocations in T-ALL [25-28], the analysis of its expression pattern

suggested that it might be important for normal hematopoiesis. During embryogenesis, SCL is specifically expressed in hemogenic sites [29-33], as well as endothelial and neural tissues [29,32,34-37], an expression pattern that is conserved from telost fish to mammals [38-40]. Blood cells are specified from mesodermal precursors and are produced through a first wave of primitive hematopoiesis, which begins in yolk sac (YS) blood islands at around embryonic day 7 (E7) and consists mainly of primitive nucleated erythrocytes (Figure 2.1A) [7,41]. This transient wave of primitive hematopoiesis is progressively replaced by a second wave of definitive hematopoiesis, which ensures the production of adult erythroid, myeloid and lymphoid cells. As primitive hematopoiesis declines, cells produced in the YS and an intra-embryonic hemogenic site called the aorta-gonado-mesonephros (AGM) region are thought to colonize the fetal liver around E11 (Figure 2.1A), which becomes the main site of blood production in mid- to late-gestation embryos [42]. Towards the end of gestation, another shift occurs and the bone marrow becomes the predominant site of definitive hematopoiesis and remains so throughout postnatal life. An essential function of SCL for hematopoietic development was first revealed following SCL gene ablation, which results in embryonic lethality at E9.5 due to a complete absence of primitive blood cells [12,13]. Further studies established that $SCL^{-/-}$ embryonic stem (ES) cells are unable to contribute to any hematopoietic lineage in mouse chimeras, demonstrating that SCL is required cell-autonomously for the generation of both primitive and definitive hematopoietic cells (Figure 2.1A) [14,15]. These conclusions are also supported by recent work from Endoh and colleagues, who established an $SCL^{-/-}$ ES cell line in which exogenous SCL

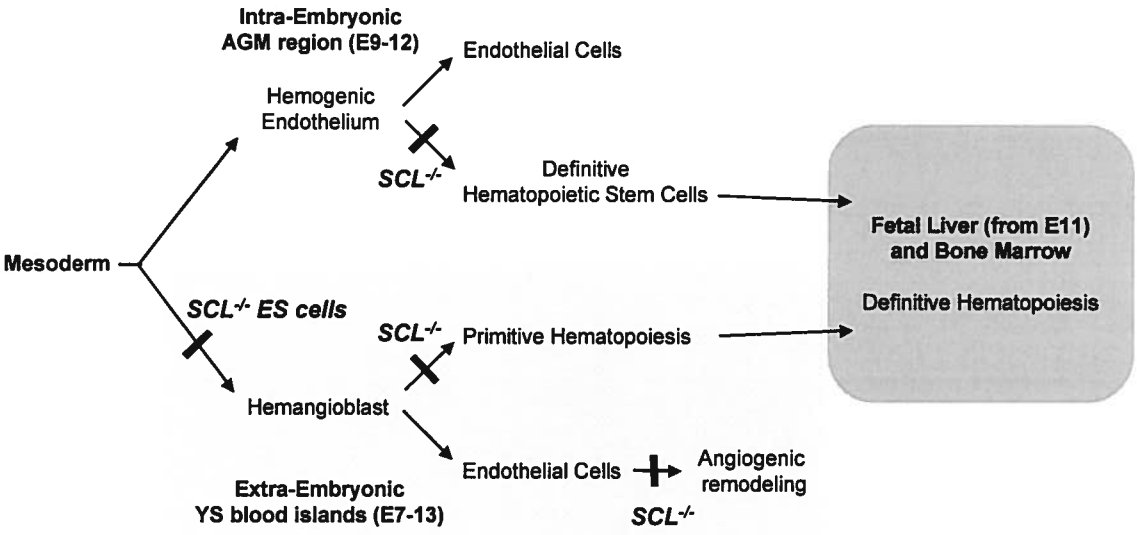
expression could be induced by the addition of tamoxifen to the culture medium [43]. They demonstrated, through in vitro differentiation studies, that specification of both the primitive and definitive hematopoietic compartments requires SCL expression in mesodermal precursors during a critical developmental window of opportunity. Together, these findings identified SCL as one of the earliest acting regulators of HSC specification.

2.3 SCL and the origin of hematopoiesis

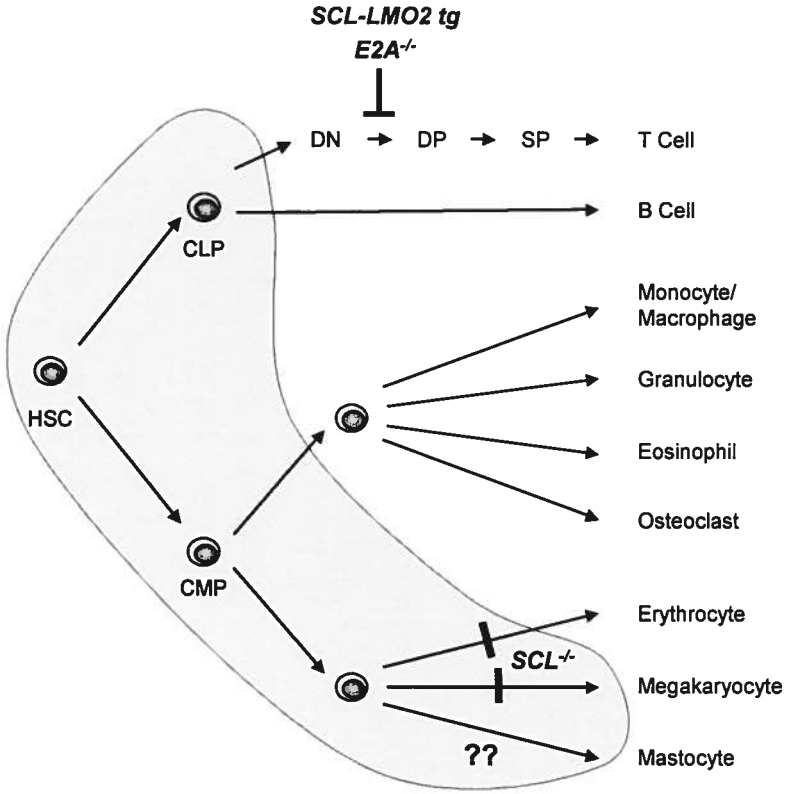
In the YS and AGM, there exists an intimate relationship between the formation of hematopoietic and endothelial cells, which has led to the suggestion that both of these lineages derive from a common precursors, either the hemangioblast or hemogenic endothelium (Figure 2.1A)[44,45]. In addition to histological observations, the existence of the hemangioblast is supported by the finding that hematopoietic and endothelial cells co-express several genes, including CD34, flk-1, PECAM-1, Tie-2, c-kit, GATA-2, LMO2, Runx1 and SCL [44-46], and by the isolation of the blast colony-forming cell (BL-CFC), a precursor identified in differentiating ES cells that can generate both endothelial and hematopoietic progeny in tissue culture [47,48]. Although the characterization of the hemangioblast in its in vivo context represents a daunting challenge, several key studies have implicated SCL as a potential regulator of this elusive precursor. Firstly, in addition to its crucial function in hematopoietic cells, SCL is essential for YS angiogenesis [32,49]. In order to assess whether SCL exerts its functions outside of the hematopoietic

Figure 2.1. Functions played by SCL during ontogeny of the hematopoietic and vascular systems and during adult hematopoiesis. (A) SCL functions during the establishment of the hematopoietic system. Mesodermally derived hematopoietic cells appear in both extra-embryonic (YS blood islands) and intra-embryonic (AGM region) hemogenic sites. In both locations, hematopoietic cells are thought to arise from a precursor (hemangioblast or hemogenic endothelium) that can also generate endothelial cells. Primitive hematopoietic cells, as well as an initial wave of definitive hematopoietic progenitors [33], are seemingly first produced in the YS blood islands, whereas the first detectable adult repopulating HSC are generated in the AGM region (reviewed in [42]). Cells from both of these regions are thought to contribute to the seeding of the fetal liver, the main site of definitive hematopoiesis in the fetus, although this remains a matter of considerable debate. $SCL^{-/-}$ embryos exhibit a complete absence of both primitive and definitive hematopoietic cells (bold lines) [12-15]. Furthermore, hematopoietic-specific rescue of $SCL^{-/-}$ embryos has revealed that SCL is also required for angiogenic remodeling of the primary capillary plexus in the YS [49]. In tissue culture, $SCL^{-/-}$ ES cells are unable to generate BL-CFCs [52,53], the in vitro equivalent to the hemangioblast, although this remains to be clarified in vivo. (B) SCL expression and function during definitive hematopoiesis. Within the definitive hematopoietic hierarchy, SCL is expressed in HSC, multipotent progenitors, as well as cells of the erythroid, megakaryocytic and mastocytic lineages (grey shading). Conditional knockout studies (bold lines) have shown that SCL is essential for the differentiation down the erythroid and megakaryocytic pathways, whereas its requirement in the mast cell lineage remains to be characterized (question marks). Transgenic mice ectopically expressing SCL and LMO2 in the lymphoid lineage exhibit a block in T cell differentiation at the transition of DN to DP cells and develop T cell tumors [68,121,125,127], similar to the phenotype observed in $E2A^{-/-}$ mice [159]. DN, double negative; DP, double positive; SP, single positive cells for CD4/CD8 expression.

A


Intra-Embryonic
AGM region (E9-12)
Endothelial Cells
Hemogenic
Endothelium
SCL-/-
Definitive
Hematopoietic Stem Cells
Fetal Liver (from E11)
and Bone Marrow
Definitive Hematopoiesis
Mesoderm
SCL-/- ES cells
SCL-/-
Primitive Hematopoiesis
Hemangioblast
Endothelial Cells
Angiogenic
remodeling
Extra-Embryonic
YS blood islands (E7-13)
SCL-/-

B


SCL-LMO2 tg
E2A-/-
DN
DP
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T Cell
B Cell
CLP
Monocyte/
Macrophage
Granulocyte
HSC
Eosinophil
Osteoclast
CMP
Erythrocyte
SCL-/-
Megakaryocyte
??
Mastocyte

compartment that might have been missed due to the early lethality of $SCL^{-/-}$ embryos, Visvader and colleagues performed a transgenic rescue of the hematopoietic defects in SCL deficient embryos [32,49]. While hematopoiesis was restored in these mice, angiogenic remodeling of the yolk sac capillary network into larger vessels was deficient resulting in embryonic lethality (Figure 2.1A). These results demonstrated that SCL functions at the interface between the hematopoietic and vascular endothelial lineages, both of which are thought to arise from the hemangioblast [50]. Secondly, recent studies have demonstrated that SCL and flk-1, the receptor for vascular endothelial growth factor (VEGF), are molecular determinants of ES cell derived BL-CFCs and that $SCL^{-/-}$ ES cells are unable to generate these multipotent precursors (Figure 2.1A)[51-54]. Similar to $SCL^{-/-}$ mice, embryos in which the Flk-1 gene is disrupted exhibit a severe defect in the formation of hematopoietic and endothelial cells [55,56], although $Flk\text{-}1^{-/-}$ ES cells are still able to generate these cell types in vitro under particular culture conditions, albeit at a lower frequency than wild type cells [57,58]. The finding that SCL expression is severely reduced in $Flk\text{-}1^{-/-}$ embryos led Ema and colleagues to ask whether enhancing SCL expression in these mice might restore early hematopoietic and endothelial development [59]. To address this question, they generated ES cells in which the SCL cDNA was knocked into the Flk-1 locus, thereby placing SCL expression under control of the Flk-1 gene regulatory elements. These cells were then used to generate mouse chimeras through tetraploid aggregation and were also studied for their in vitro hemangioblastic potential. Although expressing SCL from the Flk-1 locus was insufficient to rescue hematopoietic and endothelial development

in vivo, the authors found that targeting SCL to Flk-1$^+$ cells could clearly enhance their potential to form BL-CFCs and hematopoietic colonies in tissue culture, thereby implicating SCL in hemangioblast specification. In a complementary approach, Martin et *al.* show a genetic interaction between SCL and VEGF, allowing for a partial rescue of the hematopoietic defects associated with a hypomorphic *vegf*^{lo} allele by an SCL transgene (submitted, 2003). These observations are consistent with the view that SCL and VEGF function in the same genetic pathway to favor the hematopoietic fate. Finally, akin to the cell-fate inducing effects seen with other tissue-specific bHLH factors [60-63], ectopic SCL expression in zebrafish embryos can enhance the commitment of mesodermal precursors towards the hematopoietic and endothelial cell fate at the expense of other tissues [38]. Similarly, enforced SCL expression can rescue the formation of hematopoietic and endothelial cells in the spontaneous zebrafish mutant *cloche*, which shows a severe defect in hemangioblast function [39,64,65]. Therefore, SCL is perhaps the best-characterized transcription factor to be involved in the function and specification of the elusive hemangioblast.

2.4 Lineage-specific requirements of SCL

In addition to its crucial role at the earliest stages of hematopoiesis, SCL is also thought to exert important functions in progenitors and in specific lineages of the definitive hematopoietic compartment. In the bone marrow, it is generally accepted that mature cells of each lineage are generated through the progressive differentiation of totipotent HSC into hematopoietic progenitors, which then give rise to precursors

that are committed to specific cell fates. Within this hierarchy, SCL expression follows a differentiation-dependent gradient: it is expressed in HSC and the most primitive progenitors but becomes down regulated as differentiation proceeds into most lineages (Figure 2.1B)[66-70]. However, as an exception to this tendency, SCL remains expressed in cells differentiating towards the erythroid, megakaryocytic and mastocytic pathways [26,34,67,71-76]. Distinct enhancer elements have been identified within the murine SCL locus [77-79], which have been found to direct reporter gene expression to different subregions of the normal SCL expression domain [78,80]. One element found several kilobases downstream of the SCL gene (3'-enhancer) is able to target expression to endothelial cells and primitive hematopoietic progenitors in vivo [81]. In order to asses whether restoring SCL expression in hematopoietic progenitors was sufficient to alleviate the defects observed in $SCL^{-/-}$ embryos, Sanchez and colleagues undertook a genetic rescue approach in which a transgene expressing SCL under control of the 3'-enhancer element was introduced into $SCL^{-/-}$ embryos [81,82]. While this transgene rescued the formation of stem cells, early progenitors and myeloid precursors, the presence of erythroid precursors was barely detectable and these mice died at the same developmental stage as $SCL^{-/-}$ embryos, thus revealing a critical requirement for sustained SCL expression for proper erythropoiesis in vivo (Figure 2.1B). This finding is supported by experiments showing that enforced SCL expression in hematopoietic cell lines and primary bone marrow cells favors erythroid differentiation [76,83-87]. Furthermore, two groups have recently conducted conditional knockout studies of the SCL gene, which bypass the early embryonic

lethality observed in $SCL^{-/-}$ mice and have provided additional evidence that SCL is essential for erythropoiesis and megakaryopoiesis in adult mice (Figure 2.1B)[16,17]. While both studies involved the generation of mice with loxP sites flanking important intronic sequences within the SCL gene (SCL-loxP mice) and their subsequent deletion through the use of an interferon (or PI-PC) inducible Cre-recombinase (Cre), the experimental protocols used to evaluate the effects of SCL gene ablation presented some differences. In the first study, Hall and co-workers directly examined the hematopoietic compartment of SCL-loxP and control mice shortly after inducing Cre-recombinase activity through PI-PC injection [16]. Using this approach, they demonstrated that SCL deletion completely disrupts erythropoiesis and megakaryopoiesis, while myeloid precursors remain unaffected. Interestingly, although primitive progenitors, such as the CFU-S_{12}, were still present following SCL inactivation, their number was reduced by two-fold compared to control samples and they were devoid of red blood cells and megakaryocytes. In the second report, Mikkola and colleagues also observed that direct PI-PC injection into SCL-loxP mice resulted in anemia and thrombocytopenia; however, they proceeded to analyze in more detail the competitive ability of SCL-deleted cells in mouse chimeras [17]. Bone marrow cells from SCL-loxP or control mice were transplanted into wild-type recipients, which were then treated with PI-PC several weeks after transplantation. Similar to the first study, they observed that cells that had undergone SCL deletion were unable to contribute to either the erythroid or megakaryocytic lineage, whereas they still generated hematopoietic progenitors, as well as cells of the myeloid and lymphoid compartments. Whether mast cells, which normally

express SCL, are also affected following SCL gene disruption is unknown (Figure 2.1B). These studies clearly demonstrated the critical function of SCL in the erythroid and megakaryocytic pathways (Figure 2.1B). What about the role played by SCL in the HSC compartment?

2.5 The duality of HSC specification and maintenance, and the robustness of stem cell function

Since SCL is essential for the establishment of the hematopoietic system, it came as a surprise when Mikkola et *al.* found that SCL deleted cells exhibit stem cell activity on serial transplantation, which lead to the conclusion that, once SCL has specified the formation of HCS, its continued expression is dispensable for stem cell function [17]. These observations contrast with previous data suggesting that SCL might regulate the size of the HSC compartment, as transgenic expression of SCL leads to an expansion of Hoechst refractory bone marrow side-population (SP) cells that are endowed with HSC activity [69]. While SCL may indeed be dispensable for HSC homeostasis, other mechanisms might account for the lack of a stem cell defect in SCL deleted bone marrow cells. Firstly, SCL function might be redundant with other hematopoietic bHLH factors such as the closely related Lyl-1 [88]. Alternatively, it is also conceivable that in vivo compensatory mechanisms sustain stem cell function in SCL deficient cells. Indeed, since the maintenance of a stem cell pool is essential for the life-long output of blood cells, its regulation is probably ensured by an extensive network of molecular interactions that would provide a built-in robustness

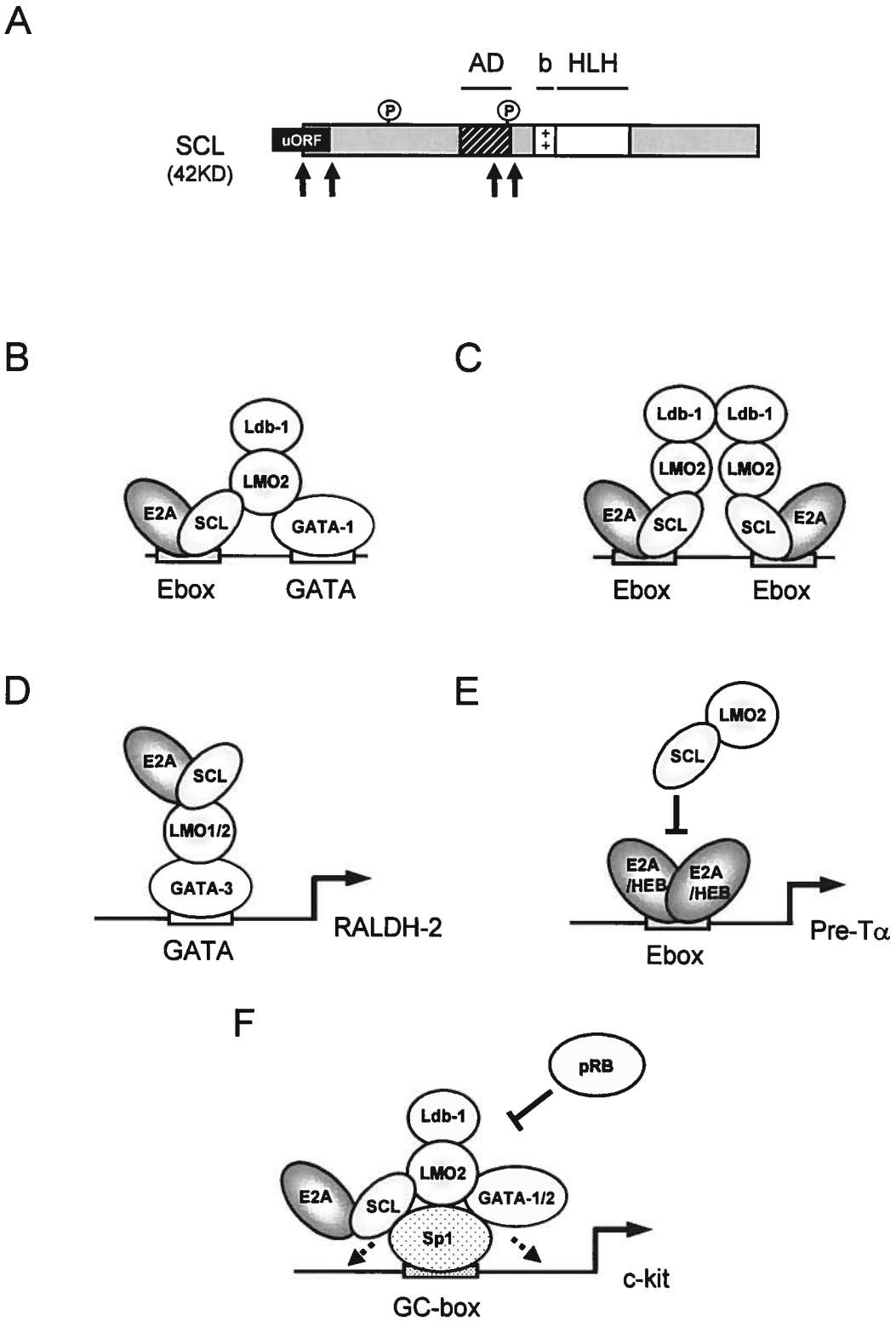
to stem cell function and insulate HSC from single perturbations that may otherwise have catastrophic consequences. Therefore, other genes within this network may adjust their activities in an adaptive response to a total absence of SCL. This type of fail-safe mechanism most likely entails the existence of parallel circuitry, allowing stem cell function to withstand failure of a single circuit. Such parallel circuitry may involve molecules such as STAT-3 [89,90], or developmental genes that include Bmi-1 [91,92] or HoxB4 [93,94]. Interestingly, both Bmi-1 and HoxB4 are involved in the regulation of HSC self-renewal and maintenance, while they are dispensable for stem cell genesis. In comparison, SCL appears to be non-essential for HSC self-renewal but critically controls HSC specification. Therefore, while SCL might be partly dispensable for HSC maintenance, there may exist multiple levels of regulation that could compensate for SCL loss of function within the stem cell compartment. Additional studies will be required in order to address these issues.

2.6 Signaling to SCL

Several lines of evidence suggest that specific signal transduction pathways might modulate SCL function during definitive hematopoiesis. For instance, SCL is required for the survival of hematopoietic progenitors grown in the presence of Steel factor, the ligand for the c-Kit tyrosine kinase receptor, which plays an essential role during definitive hematopoiesis [95,96]. While the requirement of SCL for c-Kit function is partly due to the fact that the c-kit gene is a direct target gene of SCL (Table 2.1)[97], recent evidence suggests that SCL also acts down-stream of c-Kit

signaling pathways to suppress apoptosis in multipotent progenitors (Krosl, Lacombe, Herblot and Hoang, unpublished observations, 2003). Although it is not known whether SCL is a direct target of c-Kit signaling, as has been demonstrated for the bHLH-Zip factor MITF [98], SCL is known to be serine phosphorylated on several residues (Figure 2.2A)[99-105]. In vitro studies have demonstrated that residue S122 can be phosphorylated by the mitogen-activated protein kinase ERK1 and that this modification might modulate the activity of a putative proline-rich transactivation domain within the extreme amino-terminal portion of the full-length SCL protein (Figure 2.2A)[104-107]. SCL can also be phosphorylated on residue S172 by the cAMP-dependent protein kinase (PKA), a modification that has been proposed to affect the binding of SCL to particular Ebox motifs [102,103]. In addition, two independent groups have identified the GTP binding protein DRG as an interacting partner of SCL using the yeast two-hybrid system [108,109]. While the function of DRG remains largely unknown, it has been shown to enhance the transformation of fibroblastic cells induced by c-myc/ras and might be involved in the response of SCL to specific signaling pathways. Finally, although it has long been known that several isoforms of the SCL protein exist in hematopoietic cells [75], Calkhoven and co-workers recently demonstrated that SCL isoform production is controlled by a small upstream open reading frame (uORF) in SCL transcripts (Figure 2.2A), which is responsive to signaling pathways that modulate eukaryotic translation initiation factors (eIFs)[110]. While these isoforms contain different N-terminal portions of the full-length protein, they all retain the bHLH domain, and

Figure 2.2. Schematic representation of the SCL protein and SCL-containing complexes. (A) In addition to the distinctive bHLH region, the SCL protein harbors a putative proline-rich transactivation domain (AD) between positions 117 and 175 [106]. The positively charged basic (b) region is required for DNA-binding, while the HLH motif mediates important protein-protein interactions. The production of different N-terminally truncated isoforms of SCL (arrows) is regulated by an upstream open reading frame (uORF)[110], and SCL can also be phosphorylated (P) on serine residues in response to different signaling pathways [99-105]. (B) A multifactorial complex containing SCL, E2A, LMO2, Ldb-1, and GATA-1, was first identified using a binding site amplification procedure with MEL cell nuclear extracts [146]. Within this complex, LMO2 is thought to act as a bridge between DNA bound SCL/E2A heterodimers and GATA-1, as these factors were found to associate with a composite Ebox-GATA motif. (C) Using a similar approach with extracts of leukemic T cells from LMO2-transgenic mice, a distinct complex was identified that could bind a bipartite Ebox-Ebox motif [148]. In this context, Ldb-1 homodimerization is thought to exert a bridging function between distinct SCL/E2A heterodimers. (D) In T-ALL cell lines, SCL and LMO1/2 have been shown to activate transcription of the Retinaldehyde Dehydrogenase 2 (RALDH2) gene through their recruitment by promoter bound GATA-3 [149]. (E) SCL can also inhibit the transcription of target genes regulated by E2A/HEB homo/heterodimers during lymphoid cell differentiation, such as the pre-Tα gene [168]. (F) A complex containing SCL, E2A, LMO2, Ldb-1, and GATA-1/-2 has been shown to activate the c-kit promoter through functional interaction with promoter bound Sp1 [97]. It remains to be determined whether additional cis elements help to recruit SCL and its partners to the c-kit promoter, as indicated by dotted arrows. The Rb protein is thought to contribute to c-kit promoter repression during erythroid cell differentiation by associating with SCL and its partners [183], although the mechanism of repression remains unclear.


A
AD
b HLH
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P
uORF
SCL
(42KD)
B
Ldb-1
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SCL
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SCL
LMO1/2
GATA-3
GATA
RALDH-2
E
LMO2
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/HEB
Ebox
Pre-Tα
F
pRB
Ldb-1
LMO2
E2A
SCL
GATA-1/2
Sp1
GC-box
c-kit

distinct isoforms were shown to differentially favor differentiation towards the erythroid or the megakaryocytic pathways. Therefore, SCL acts as a crucial regulator at different levels in the hematopoietic hierarchy and might represent a downstream effector of several signaling pathways. Assessing the molecular mechanisms through which SCL regulates gene expression in these different cellular contexts has represented an important challenge. Considerable insight into these processes has emerged through the study of the oncogenic properties of SCL and other transcription factors involved in the development of T cell leukemia.

2.7 Perturbing the equilibrium: SCL in T-ALL

The SCL gene is the most frequent target of chromosomal rearrangements in patients with T-ALL and has long been suspected as a key instigator of T cell leukemogenesis [18,25-28,111-115]. As a consequence of these rearrangements, SCL becomes inappropriately expressed in developing T cells, where it is normally down regulated during differentiation (Figure 2.1B)[68]. SCL gene rearrangements were initially documented in 12-25% of T-ALL patients [113,115-117]; however, more recent studies have demonstrated that ectopic SCL expression can be detected in a considerable proportion of T-ALL samples that lack any detectable SCL locus rearrangements [118,119], reinforcing the notion that SCL activation is a major event in T cell leukemogenesis. Although SCL exhibits weak oncogenicity when expressed in the thymus of transgenic mice [120-123], it efficiently collaborates with other oncogenes such as v-ABL, casein kinase II (CKII), N-Ras, P53, and the LIM only

proteins LMO1 and LMO2 (also known as RBTN1/2 or TTG1/2) to induce aggressive T cell tumors in vivo [68,121-128]. Among these collaborating oncogenes, both LMO1 and LMO2 are also targets of chromosomal rearrangements in T-ALL (reviewed in [6]) and have been shown to specifically associate with SCL in extracts of leukemic cells [129,130]. LMO proteins are comprised of tandem LIM domains, zinc-finger like structures that can mediate specific protein interactions but are seemingly devoid of direct DNA binding capability [131]. The close relationship observed between SCL and LMO proteins in T-ALL seemingly extends to normal hematopoiesis, since both SCL and LMO2 show overlapping expression patterns in early hemogenic sites and in adult hematopoietic lineages [30,74,132-134]. In addition, $LMO2^{-/-}$ mice exhibit a complete absence of hematopoietic cells and defective yolk sac angiogenesis, phenotypes that are highly reminiscent of those observed in $SCL^{-/-}$ mice [135-137]. Therefore, the SCL-LMO interaction is thought to play a central role during normal hematopoietic development and for the generation of T-ALL.

Through their modular structure, LMO proteins are thought to function as adapters allowing the proper assembly of larger protein complexes [131,138,139]. In addition to its association with SCL, LMO2 has been demonstrated to interact with hematopoietic GATA family members (GATA-1, -2, -3) and with the LIM domain binding protein Ldb-1 [140-147]. Insight into the mechanism of leukemogenesis induced by SCL and LMO2 came from the observation that these proteins can associate within a multifactorial complex containing E47, Ldb-1 and GATA-1 in

murine erythroleukemia (MEL) cells (Figure 2.2B)[146]. This complex was revealed through its ability to bind DNA on a composite Ebox-GATA motif and was shown to transactivate an artificial reporter vector driven by multimerized Ebox-GATA sequences. A complex of similar composition, with the exception of GATA-1, was isolated from a leukemic cell line derived from LMO2 transgenic mice and was found to bind an Ebox-Ebox motif through in vitro site selection (Figure 2.2C)[148]. While SCL and LMO2 are normally expressed in the earliest CD4/CD8 double-negative (DN) precursors of the T cell lineage, their expression becomes extinguished prior to the generation of CD4/CD8 double-positive (DP) thymocytes [68]. Therefore, sustained SCL or LMO2 expression, as observed following chromosomal rearrangements in T-ALL or in transgenic mice, might initiate the leukemic process by causing the formation of aberrant protein complexes capable of activating inappropriate gene expression in differentiating T cells. In support of this view, SCL and LMO2 have been shown to induce the expression of the TALLA-1 and RALDH-2 genes in T-ALL cell lines (Table 2.1)[143,149], although the relevance of these target genes for leukemogenesis remains unknown. Surprisingly, rather than requiring an Ebox-Ebox motif, activation of the RALDH-2 gene by SCL and LMO2 occurred through their recruitment to a cryptic intronic promoter by DNA-bound GATA-3 and did not require SCL DNA binding activity (Figure 2.2D)[149]. The identification of additional genes activated by SCL-LMO2 in T-ALL will provide greater insight into the mechanism by which these factors induce aberrant gene expression.

In contrast to inappropriate gene activation, accumulating evidence suggests that SCL can exert its oncogenic effects through yet another mechanism, by inhibiting the normal functions of the E-proteins E2A and HEB (Figure 2.2E), which are important regulators of lymphoid cell differentiation [68,69,127,150,151]. While these factors are expressed in a wide variety of tissues [152-154], their appropriate dosage is essential for the commitment and proper differentiation of B and T lymphoid cells [69,155-158], and E2A has been shown to possess tumor suppressor characteristics in T cells [159-161]. Therefore, through its ability to interact with E-proteins, SCL might interfere with the normal regulatory functions imposed by these factors during T cell differentiation. This view is firstly supported by the fact that $E2A^{-/-}$, $HEB^{-/-}$, and SCL-LMO1/2 transgenic mice all exhibit a strikingly similar defect in T-cell differentiation, characterized by a block at the transition of CD4/CD8 double negative (DN) to double positive (DP) thymocytes [69,121,127,159,160,162-168]. This differentiation blockade precedes the development of T-cell tumors in $E2A^{-/-}$ and SCL-LMO1/2 transgenic mice (Figure 2.1B). Additional studies have suggested that SCL has an antiapoptotic function in T-ALL cell lines that is relieved following the restoration of E2A activity [169,170]. Whether $HEB^{-/-}$ mice also develop tumors is a question that remains to be answered. In addition to genetic evidence, SCL has also been demonstrated to inhibit E2A and HEB mediated transactivation of artificial reporters and of cellular regulatory elements, such as those of the CD4, preT-α and TCR α/δ genes (Table 2.1)[68,127,168,171-175]. This inhibition most likely results from the formation of transcriptionally inactive SCL-E2A and SCL-HEB heterodimers at the expense of E2A and HEB homo/heterodimers, which are

efficient activators of transcription and are essential for T cell differentiation [157,176]. The inhibitory effect of SCL might be due to a DNA binding independent sequestration mechanism similar to that proposed for the Id family of HLH proteins, which lack a DNA interacting basic region [150,177]. In support of this view, transgenic expression of a DNA binding defective mutant of SCL can perturb lymphoid cell differentiation and give rise to T cell tumors in collaboration with CKII [128,151]. Alternatively, since E2A is thought to recruit chromatin remodeling complexes to its target genes in order to facilitate transcription activation [178,179], SCL might exert its inhibitory effects by interfering with this recruitment process or by itself associating with co-factors that hinder transcription activation by E2A. Although this remains to be determined, recent chromatin immunoprecipitation experiments have shown that SCL, E2A and HEB co-occupy the promoter and enhancer elements of the pre-Tα gene, an E2A-HEB target that is inhibited following ectopic SCL expression in lymphoid cells [68,168]. These results suggest that SCL is indeed present on E2A-HEB targets and argues against a simple sequestration model. The identification of several other bHLH factor encoding genes, namely LYL-1, TAL-2 and BHLHB1, as targets of translocations in T-ALL suggests that E-protein inhibition is a recurrent theme in the generation of T-cell leukemias [88,114,119,180,181]. Therefore, the molecular basis through which SCL and LMO proteins contribute to leukemogenesis seems to occur through multiple mechanisms involving both positive and repressive effects on transcription.

Table 2.1. Potential SCL target genes during normal hematopoietic development and in T-ALL.

Hemangioblasts/Endothelial cells	Flk-1 [193,195], otogelin-like [196]
Stem/Progenitor cells	c-kit [97], GATA-1 [191]
Erythroid/Megakaryocytic lineages	GATA-1 [191], GPA, c-kit [183], EKLF [192,194], otogelin-like [196]
T-ALL	TALLA-1 [143], RALDH-2 [149], PreTα [68,168], CD4 [68], TCR α/δ [174]

2.8 Transcription regulation during normal hematopoiesis and the all-or-none behavior of SCL-containing complexes: a case of coincidence detection

While SCL plays important roles at several positions in the hematopoietic hierarchy, target genes directly regulated by SCL in these different hematopoietic compartments remain poorly characterized. Nevertheless, several recent studies have identified potential SCL targets and have revealed important clues as to its normal mechanisms of action (Table 2.1). Among the first direct hematopoietic target genes of SCL to be identified was the c-kit gene [96,97], which encodes an essential tyrosine kinase receptor that is required for the maintenance of HSC and progenitors [95]. The mechanistic link between these two genes was first established through a functional screen in the pluripotent hematopoietic cell line TF-1, in which SCL was

found to be required for the expression and survival mediated by the c-Kit receptor [96]. Molecular analysis revealed that SCL assembles a multifactorial complex containing E47, LMO2, Ldb-1, GATA-1/-2 and Sp1, on the c-kit promoter (Figure 2.2F)[97]. Interestingly, single components have little if any effect on c-kit promoter activity, while co-expression of all partners of the complex leads to synergistic transactivation. This all-or-none switch-like behavior, also described as coincidence detection by Hartwell and colleagues [182], in which the simultaneous occurrence of several events is required in order to activate an output, is a central paradigm in transcription regulation. This property of SCL complexes is dependent on specific protein interactions [97] (Lecuyer and Hoang, unpublished observations, 2003) and allows for tight spatio-temporal control of important target genes, such as c-kit, which may determine whether a given progenitor cell survives, self-renews or differentiates. Through the identification of GATA-2 and Sp1 as functional partners within SCL complexes, this study suggested that hematopoietic progenitor cells might contain variants of the complex first identified in erythroid cell extracts [145,146]. In addition, Vitelli and colleagues demonstrated that the pocket protein pRB can associate with SCL, E12, LMO2 and Ldb-1 in erythroid cells and this pentameric complex was found to inhibit c-kit promoter activity (Figure 2.2F)[183]. Since c-kit is expressed in pluripotent and early erythroid progenitors and becomes progressively down regulated during erythroid cell differentiation, these studies suggest that the proper spatio-temporal regulation of c-kit expression might be achieved through variation in composition of SCL-containing complexes during the differentiation of multipotent progenitors towards the erythroid pathway. While

further investigation is required in order to confirm this hypothesis, recent work characterizing the activation of an erythroid cell target of SCL, the gene encoding the major erythroid membrane glycoprotein glycophorin A (GPA), suggests that distinct SCL-containing complexes show differences in activation efficiency depending on the type of gene that is targeted (Lahlil, Lecuyer, Herblot and Hoang, unpublished observations, 2003). Indeed, the GPA promoter is activated much more strongly by complexes containing GATA-1 compared to those with GATA-2, which is the inverse of what is observed in the context of the c-kit promoter where complexes with GATA-2 are more efficient activators [97]. This functional specificity concurs with the known biological functions of these two GATA factors, as GATA-2 is required for the maintenance of hematopoietic progenitor cells [184,185], while GATA-1 is essential for erythroid cell development [186,187]. Since these GATA family members are also required for the proper development of mast cells and megakaryocytes [185,188,189], lineages in which SCL is suspected to play an important function, it will be interesting to determine whether distinct variants of SCL-containing complexes are required to induce target genes that are specific to these lineages. Consistent with its interaction with LMO2 and SCL in leukemic cells [143,149], GATA-3 might also associate within SCL-containing complexes during normal hematopoiesis, since it is co-expressed with SCL and LMO2 in the AGM region, in the HSC compartment and in early progenitors of the T cell lineage [67,68,134].

2.9 To bind or not to bind DNA

Transcription factors typically bind DNA via a basic domain and recruit the basal transcriptional machinery via their transactivation domains. This linearity has been progressively challenged by the observations that transcription factors work not in isolation but in complex with other factors and can also function as co-activators in the absence of direct DNA binding. As discussed above, SCL is neither a typical bHLH factor nor a co-activator. The DNA binding function of SCL reveals itself to be dispensable or essential, depending on the target gene and cellular context. It was previously shown that SCL DNA binding defective mutants are able to rescue hematopoietic cell specification in $SCL^{-/-}$ ES cells and in the zebrafish mutant *cloche*; however, these mutants were unable to restore the proper differentiation of definitive hematopoietic cells, most notably within the megakaryocytic and erythroid lineages [190]. While DNA binding by SCL was found to be dispensable for c-kit promoter activation [97], it is required for maximal activation of the GPA promoter (Lahlil, Lecuyer, Herblot and Hoang, unpublished observations, 2003). Consistent with the genetic rescue experiments performed by Porcher and colleagues [190], the requirement for SCL DNA binding seems to represent a mechanistic distinction in the activation of progenitor (c-kit) and erythroid cell (GPA) target genes of SCL. Whether these characteristics are broadly applicable to most SCL targets within these hematopoietic compartments remains to be determined. The dispensability of the DNA binding function of SCL in early hematopoietic stem/progenitor cells most likely reflects the fact that SCL can be recruited to target regulatory elements

through specific interactions with partners that provide their own DNA binding function, such as GATA and Sp/XKLF family members [97,149]. In this context, the main purpose of SCL might be to act as a nucleation factor in the assembly of these multifactorial complexes.

Several additional hematopoietic genes have been shown to require Ebox and GATA elements for their appropriate tissue specific expression, which has led to the suggestion that these represent downstream targets of SCL-containing complexes. For instance, a DNAse I hypersensitive (HS1) site upstream of the GATA-1 gene, harbouring an Ebox-GATA motif to which SCL and its partners are able to bind in vitro, has been shown to direct reporter gene expression to erythroid and megakaryocytic cells in transgenic mice (Table 2.1)[191]. Similar motifs identified in regulatory regions of the EKLF and *Flk-1* genes have been shown to direct reporter gene expression to erythroid and endothelial cells, respectively (Table 2.1) [192-195]. However, the direct functional involvement of SCL in the regulation of these genes remains to be demonstrated. Cohen-Kaminsky and co-workers identified an otogelin-like gene as a potential erythroid/endothelial target of SCL through the use of a chromatin immunoselection approach with MEL cell extracts (Table I) [196], although the function and mechanism of regulation of this gene have yet to be characterized. Therefore, despite the critical role played by SCL and its partners during hematopoietic development, there is still much to be deciphered concerning their target genes and molecular mechanisms of action. Nevertheless, these studies suggest that changes in composition of SCL complexes in different hematopoietic

compartments most likely play an important role in generating lineage-specific patterns of gene expression.

2.10 Transcription regulation by SCL in the context of chromatin

Further clues as to the molecular functions played by SCL and its partners during transcription regulation have come from the study of their *Drosophila* orthologues. For example, Chip, the homologue of Ldb-1, both of which are essential for patterning during embryogenesis in flies and mammals [197-199], is thought to regulate gene expression in *Drosophila* by enabling enhancer-promoter communication in developmentally regulated genes [197,200,201]. Chip has been shown to form a complex with Pannier (a GATA family orthrologue), Daughterless (a *Drosophila* E-protein), and bHLH factors encoded by the *achaete* and *scute* (ASC) genes, in which Chip acts as a bridging molecule between its enhancer and promoter bound partners [200]. Similarly, SCL-containing complexes may regulate lineage-specific gene expression by modulating the effects of distally located enhancer elements, a process that could be independent of SCL DNA binding activity. It remains to be determined whether lessons learned from studies in *Drosophila* will provide important missing links in our understanding of the function of SCL-containing complexes.

In addition to its association with other transcription factors, SCL has been shown to interact with coactivator (p300 and P/CAF) and corepressor (mSin3A) proteins

whose functions are linked to histone acetyltransferase or deacetylase activities [202-204]. Huang and colleagues demonstrated that the association of SCL with these cofactors is dynamic during MEL cell differentiation. In parental MEL cells, a portion of SCL proteins, potentially involved in transcriptional repression, is complexed to mSin3A and HDAC1 [203]. As cells are induced to differentiate this association is lost, through a process that seemingly requires acetylation of SCL by the P/CAF coactivator, which is then thought to favor the interaction of SCL with additional cofactors such as p300 [202,204]. Although it remains to be determined what proportion of endogenous SCL proteins is associated with these different cofactors, i.e.: there could be several different types of SCL complexes within the same cell, these results suggest that SCL and its partners might help to direct chromatin remodeling complexes to particular genomic loci during hematopoietic cell differentiation. In doing so, specific SCL complexes could create an epigenetic code ensuring the proper activation or repression of stem/progenitor cell and lineage-specific genes. This type of mechanism might account for the apparent dispensability of sustained SCL expression for the maintenance of HSC and progenitor cells [16,17]. Indeed, if SCL were to specify the hematopoietic cell fate from mesodermal precursors by marking hematopoietic genes with an epigenetic code for activation, its sustained expression might not be required thereafter for the maintenance of the hematopoietic program, as has been observed with transcription factors in other less complex organisms [205,206]. This maintenance function would likely be provided by later acting transcription factors initially specified by SCL, perhaps including such factors as c-myb, RUNX1 and GATA-1, although this remains purely

speculative. Assessing how SCL and its partners regulate gene expression in the context of chromatin will undoubtledly provide us with a clearer view of how these factors control hematopoietic cell fate determination and leukemogenesis.

2.11 Concluding remarks

Altogether, these findings highlight the complexity encountered when studying SCL. At the molecular level, the SCL protein has challenged the prototype of classical tissue-specific bHLH transcription factors that function as heterodimers with ubiquitous bHLH factors. Rather, the study of SCL has lead to the realization that these factors work within large transcription complexes that may exhibit a switch-like behavior in order to control appropriate patterns of gene expression. At the tissue level, SCL acts as a regulator at different critical branch points in the hematopoietic hierarchy. Studying the biological functions of the SCL protein also reveals that each function arises from interactions with other proteins, or with upstream signaling pathways. Furthermore, SCL deletion has unraveled the robustness of hematopoietic stem cell function. It remains to be determined whether cellular responses to fluctuations in SCL function involve positive as well as negative feedback signals that consolidate cell fate. The next challenge will be to integrate SCL function within the global networks that regulate hematopoietic stem cells and their output of mature hematopoietic cells.

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CHAPITRE 3

ARTICLE 1

The SCL-complex regulates c-kit expression in hematopoietic cells through functional interaction with Sp1

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3.1 Abstract

The combinatorial interaction between transcription factors is believed to determine hematopoietic cell fate. SCL/TAL1 is a tissue-specific bHLH factor that plays a central function during hematopoietic development; however, its target genes and molecular mode of action remain to be elucidated. Here we show that SCL and the c-Kit receptor are co-expressed in hematopoietic progenitors at the single cell level, and that SCL induces *c-kit* in chromatin, as ectopic SCL expression in transgenic mice sustains *c-kit* transcription in developing B lymphocytes in which both genes are normally down-regulated. Through transient transfection assays and co-immunoprecipitation of endogenous proteins, we define the role of SCL as a nucleation factor for a multi-factorial complex (SCL-complex) that specifically enhances *c-kit* promoter activity without affecting the activity of myelo-monocytic promoters. This complex, containing hematopoietic-specific (SCL, GATA 1/2) and ubiquitous (E2A/HEB, LMO1/2, Ldb-1) factors, is tethered to DNA via a Sp1 motif, through direct interactions between elements of the SCL-complex and the Sp1 zinc finger protein. Furthermore, we demonstrate by chromatin immunoprecipitation that SCL, E2A, and Sp1 specifically co-occupy the *c-kit* promoter *in vivo*. We therefore conclude that *c-kit* is a direct target of the SCL-complex. Proper activation of the *c-kit* promoter depends on the combinatorial interaction of all members of the complex. Since SCL is down-regulated in maturing cells while its partners remain expressed, our observations suggest that loss of SCL inactivates the SCL-complex, which may be an important event in the differentiation of pluripotent hematopoietic cells.

3.2 Introduction

Members of the bHLH (basic helix-loop-helix) family of transcription factors are crucial regulators of diverse developmental processes such as hematopoiesis, neurogenesis, and myogenesis [1]. This family of proteins includes ubiquitously expressed members (E2A, HEB), tissue-specific factors (SCL/TAL1, MyoD), and non-DNA binding proteins (Id1-4). Tissue-restricted members can regulate gene expression by binding to Ebox DNA sequences (CANNTG) following heterodimerization with ubiquitously expressed E2A gene products (E12 and E47) or HEB. Different dimers exhibit preferential binding to specific E-boxes, and this selectivity is thought to be an important determinant in the spatio-temporal control of gene expression. SCL (stem cell leukemia) is a prototypic tissue-specific bHLH factor normally expressed in pluripotent hematopoietic precursors, vascular endothelial cells, and the central nervous system (reviewed in [2]), and acts as a master regulator of hematopoietic development. Indeed, SCL $^{-/-}$ mice lack all primitive and definitive hematopoietic lineages and precursors [3;4]. Complementary gain of function experiments in zebrafish and Xenopus have demonstrated that SCL plays a role in specifying the formation of hemangioblasts, the common precursors of vascular endothelial and hematopoietic stem cells [5-7].

As for many hematopoietic transcription regulators, the SCL gene was originally identified by virtue of its involvement in a tumor specific translocation [2]. In fact, chromosomal rearrangements causing aberrant activation of SCL are the most

common molecular anomalies associated with childhood acute lymphoblastic leukemia (T-ALL) [2]. Surprisingly, SCL rarely induces leukemia in transgenic mice and requires collaboration with the LIM domain proteins LMO1 and LMO2, whose genes are also translocated in human T-ALL, to induce aggressive T cell tumors [8-11]. This interaction is also relevant to normal development as SCL and LMO2 are co-expressed in normal hematopoietic cells, and the phenotype of $LMO2^{-/-}$ mice is identical to that of $SCL^{-/-}$ mice [12]. LMO proteins are unable to bind to DNA directly and act as bridging molecules within complexes containing SCL, E2A, Ldb-1, and/or GATA-1 in erythroid and immature T cells [13;14]. These complexes were visualized through their ability to bind to *in vitro* selected DNA molecules containing Ebox-GATA or Ebox-Ebox motifs. Variants of these sequences have been found in enhancers of the EKLF and GATA-1 genes and in the intron of an otogelin-like gene [15-17]. It remains to be documented, however, whether SCL and its partners directly regulate these genes. In addition, SCL mutants that are unable to bind to DNA are still highly active *in vivo* in rescuing hematopoietic development in $SCL^{-/-}$ embryonic stem (ES) cells and in inducing leukemia in transgenic mice [18;19]. These data suggest that the molecular mechanism of action of SCL is more complex than initially envisioned and imply the possibility of interactions with novel partners that provide a DNA binding function.

We have previously shown that SCL levels determine *c-kit* gene expression in the TF-1 hematopoietic cell line, suggesting that *c-kit*, which encodes a receptor tyrosine kinase essential for normal hematopoietic development, is a potential downstream

target of SCL [20]. Here, we provide evidence that SCL and *c-kit* are co-expressed in primary hematopoietic precursors and that *in vivo* ectopic expression of SCL increases *c-kit* expression in B cell precursors. In addition, we define a multifactorial complex formed on the *c-kit* promoter containing SCL, E2A, LMO2, Ldb-1, and GATA factors (SCL-complex). A novel partner, the Sp1 zinc finger protein, is also identified. Our data highlight how a key tissue-specific transcription factor can serve to nucleate the assembly of multifactorial complexes containing other tissue-specific or ubiquitously expressed proteins.

3.3 Materials and methods

Transgenic mice

We used the A(5)3SCL line (SCL^{tg}) that expresses the amino-terminal truncated form of human SCL under the control of the ubiquitous SIL promoter [10]. $SCL^{LacZ/w}$ mice have been described previously [21]. Animals were maintained under pathogen free conditions according to institutional animal care guidelines. Mice were genotyped by PCR and Southern blot.

FDG staining, cell sorting and RT-PCR analysis

Bone marrow was extracted from 5-week old wild type (WT), $SCL^{LacZ/W}$, or $SCL^{tg/tg}$ mice. Cells were stained with anti-c-Kit (Pharmingen, Mississauga, ON, Canada) and lineage specific antibodies: B220, Mac1, and TER119. β-galactosidase (β-gal) staining was performed as described previously [21] using Fluorescein di-β-galacto-pyranoside (FDG)(Molecular Probes, Eugene, OR). Immunostaining, sorting of B cell precursors, and RT-PCR were performed as described [11;22]. PCR products were loaded on 1.2% agarose gel, transferred on nylon membrane and hybridized with internal oligonucleotide probes. Oligonucleotide sequences are available upon request.

Plasmid constructs

All promoter segments were cloned into a modified pXPII reporter vector (called pXPIII), in which two E-boxes and two GATA motifs in the proximity of the

multiple cloning site were destroyed. The human *c-kit* promoter (kit-1146) [20] was subcloned into pXPIII using HindIII and BstEII. Deletion mutants were generated as previously described [23]. Point mutations in the Sp1 binding site replaced GGG GCG TGG with GAA GCT TGT. All constructs were verified by sequencing. The pGEX-Sp1 and Gal4-Sp1 were gifts from Dr. D. Kardassis [24], who had originally obtained them from Dr. R. Tjian, University of California, Berkeley. Expression vectors for Ldb-1 [25] and SCL mutants [18] have been described previously. Expression vectors for E47/PAN1 and Gal4-E47, as well as pcDNA-LMO2, were generously provided by Drs. J. Drouin (IRCM, Montréal) and M. Minden (Ontario Cancer Institute, Toronto), respectively. The c-fms and G-CSFR reporter constructs were kindly provided by Dr. D.Tenen (Beth Israel Hospital, Boston).

Cell cultures and transfections

NIH 3T3 and TF-1 cell culture conditions have been described previously [20]. Calcium phosphate was used to transfect NIH 3T3 cells 24 h after plating at 30 000 cells/ml. The amount of reporter was kept at 1.5 μg per well and 100 ng of CMV-βgal was added as an internal control. Total DNA was kept constant at 4.5 μg per well with pGem4. Unless specified otherwise, expression vector doses were 150 ng for SCL, E2A, and GATA factors; and 750 ng for LMO2 and Ldb-1. Luciferase and β-gal activities were assayed after 36 h. For all transfections, results are shown as the mean ± SD of replicate determinations and are representative of (n) independent experiments (see figures).

Electrophoretic mobility shift assays (EMSA)

TF-1 nuclear extracts were prepared as described previously [20]. Binding reactions were allowed to proceed at room temperature for 15 min in 20 mM Hepes [pH 7.5], 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 10 µg BSA, 100 ng poly(dIdC), 50000 CPM of ^{32}P-labeled probes, and 5 µg of nuclear extract. For experiments using *in vitro* synthesized proteins, 2.5 µl of E47 and SCL mutants were placed at 37°C for 30 min before performing the binding reactions. Antibodies and competitor DNA were added to the reaction mixtures before the probes. Samples were resolved by 4% PAGE at 17 mAmps in 0.5×TBE. The oligonucleotides used were Tal-1 consensus [26], kit-GC-box 5'-CGAGGAGGGGCGTGGCCGGCG-3' and reverse, kit-GC-box-mt 5'-CGAGGAGAAGCTTGTCCGGCG-3' and reverse.

Pull-down, immunoprecipitation and ChIP assays

GST and GST-Sp1 were purified from bacteria and coupled to Glutathione Sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ). SCL and SCL deletion mutants, as well as GATA-1, LMO2, Ldb-1, E47, and Luciferase were labeled with ^{35}S-methionine (Promega Corp., Madison, WI). Labeled proteins (10-20 µl) were incubated with 2 µg of immobilized GST fusion proteins in 400 µl of binding buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 1% Nonidet P-40 (NP-40), 5mM dithiothreitol (DTT), 10% glycerol) for 2 h at 4°C with agitation and then centrifuged for 1 min at 3000 r.p.m.. Samples were washed 3 times with binding buffer, resolved by SDS-PAGE, and visualized using phosphor storage plates.

Co-immunoprecipitations were performed for 4 h at 4°C with 1 mg of TF-1 nuclear extract, 3 µg of antibody, and 20 µl of Protein G plus-agarose beads (Calbiochem, San Diego, CA) in 1 ml of IP buffer (20 mM Tris-HCl [pH 8.0], 137 mM NaCl, 1% NP-40, 10% glycerol, 1 mM EDTA, 1mM PMSF). Samples were washed 3 times with IP buffer and subjected to SDS-PAGE. Following transfer on PVDF membranes, proteins were visualized using ECL Plus (Amersham Pharmacia Biotechnology).

For ChIP assays, chromatin cross-linking and extraction was performed as described previously [27;28]using 2×10^7 TF-1 cells/sample. Cells were fixed by adding formaldehyde (1% final) to the cultures for 10 min at room temperature. Formaldehyde was quenched with glycine at a final concentration of 0.125 M, and cells were washed for 15 min each in Triton buffer (10 mM Tris-HCl [pH 8.0], 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100) and NaCl buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl). Cells were resuspended in RIPA buffer (10 mM Tris-HCl [pH 8.0], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% deoxycholate) and sonicated (6 × 10 sec bursts) to make soluble chromatin ranging in size from 500 to 1000 bp. An aliquot of extract was kept for isolation of input DNA, while samples were precleared with Staph A cells (Calbiochem) for 30 min and then incubated overnight at 4°C with antibodies in RIPA buffer. DNA-protein complexes were collected with Staph A cells for 30 min at 4°C and sequentially washed twice with RIPA buffer, LiCl buffer

(10 mM Tris-HCl [pH 8.0], 250 mM LiCl, 1 mM EDTA, 1% NP-40, 1% deoxycholate) and TE. Bound chromatin was eluted at 65°C for 15 min in 300 µl of elution buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% SDS). Samples were diluted by addition of 300 µl of TE and heated overnight at 65°C to reverse cross-links. After RNA and protein digestion, DNA was phenol/chloroform extracted and precipitated using 10 µg tRNA as carrier. PCR reactions were performed for 30 cycles (94°C, 1 min; 62°C, 1 min; 72°C, 20 sec) in 50 µl of PCR buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl, 1.5 mM $MgCl_2$, 5% DMSO, 0.2 mM dNTP, 1 µM of each oligonucleotide, 1.25 U of *Taq* DNA polymerase). PCR products were migrated on a 2% agarose gel, transferred on nylon membranes and hybridized with internal probes. Oligonucleotide sequences are available upon request.

Antibodies

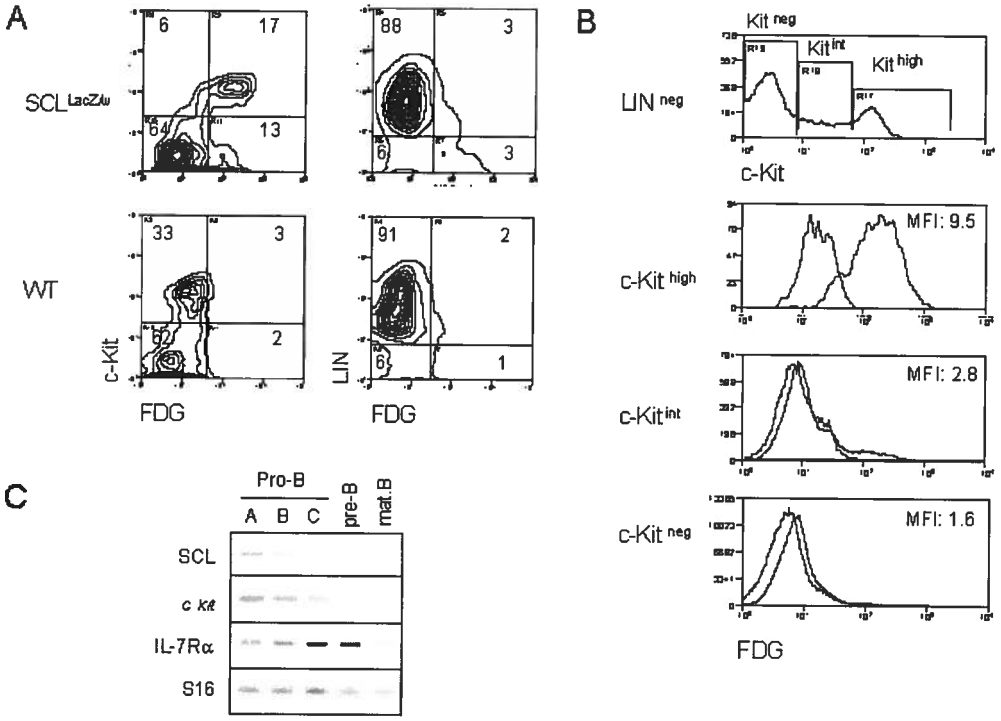
The mouse anti-E2A (YAE) and anti-GATA-2 (CG2-96), rat anti-GATA-1 (N6), rabbit anti-PU.1 (T-21), and goat anti-Sp1 (PEP2) were obtained from Santa Cruz Biotechnology Inc. Rabbit anti-LMO2 and anti-Ldb-1 antisera have been described previously [17]. The mouse monoclonal antibodies against SCL, BTL73 and 2TL136 were generously provided by Dr. D. Mathieu, Institut de Génétique Moléculaire, Montpellier, France.

3.4 Results

Co-linearity of c-Kit receptor and SCL levels

Both the SCL and *c-kit* genes are essential for hematopoietic development, and our previous work demonstrated that SCL is required for *c-kit* expression and function in the hemopoietic cell line TF-1 [20]. To determine whether SCL and the c-Kit receptor are co-expressed at the single cell level in primary hematopoietic cells, we monitored c-Kit surface expression through flow cytometry analysis of bone marrow cells taken from heterozygous $SCL^{lacZ/w}$ mice, in which the *lacZ* gene was knocked into the SCL locus [21]. Cells expressing the *lacZ* gene were revealed through staining with the fluorogenic β-galactodidase (β-gal) substrate FDG. As illustrated in Figure 3.1A, the proportion of c-Kit$^+$ cells in the LIN$^-$ fraction (i.e. negative for Mac-1, B220 and TER119) was in the range of 15-30%. In $SCL^{lacZ/w}$ mice, c-Kit expressing cells were mostly β-gal$^+$, while cells from wild type littermates had only a low background of β-gal activity. The majority (95%) of colony forming cells was found in the c-Kit$^+$/β-gal$^+$ fraction (data not shown). This population included multipotent (CFU-GEMM), erythroid (BFU-E), and granulocyte/macrophage (CFU-GM) precursors (data not shown), shown previously to express c-Kit [29] and SCL [21;30]. Within the LIN$^-$ fraction, c-Kit levels were found, strikingly, to be proportional to the activity of the SCL locus (Figure 3.1B), as shown by the mean fluorescence intensities (MFI) of FDG staining for c-Kit^{high}, c-Kit^{int}, and c-Kit^{neg} populations, which were 9.5, 2.8, and 1.6, respectively. Within the LIN$^+$ fraction, 3% of the cells were β-gal$^+$ (Figure 3.1A),

Figure 3.1. Coexpression of SCL and the c-Kit receptor in hematopoietic precursors. (A) Bone marrow cells from wild type (WT) and *SCL*$^{lacZ/w}$ knock-in mice were stained with lineage markers (B220, Mac-1, TER 119) and the c-Kit antibody, while the SCL locus activity was revealed by β-galactosidase staining with the fluorogenic substrate FDG. Left panels show the c-Kit receptor and FDG fluorescence in the lineage negative population. Right panels show lineage makers and FDG fluorescence. (B) High c-Kit levels correlate with strong FDG staining. Within the lineage negative compartment, FDG fluorescence was analyzed in c-Kit^{high}, c-Kit^{int}, and c-Kit^{neg} populations. The mean fluorescence intensities (MFI) of FDG staining on sorted *SCL*$^{lacZ/wt}$ bone marrow cells are indicated (thick line). FDG staining on WT bone marrow (thin line) was used as a negative control. Dead cells were excluded from analysis by propidium iodide staining. Results shown in A and B are representative of 3 independent experiments. (C) SCL and *c-kit* are co-expressed during B cell differentiation. Bone marrow B cell precursors from wild type mice were purified by flow cytometry [31] and gene expression was investigated by RT-PCR. IL-7Rα levels were determined to confirm the developmental stages of purified B cells and S16 was used as a control for the amount of cDNA.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LIN neg
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MFI: 9.5
c-Kit int
MFI: 2.8
c-Kit neg
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FDG
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and the MFI of FDG staining was 1.5 (data not shown). This staining was mostly due to the presence of erythroid cells (TER119+), and possibly a subset of $B220^+$ cells, as pro-B cells are c-Kit^+. Early B cell precursors expressing the B220 marker were therefore fractionated from wild type mice according to Hardy's protocol [31], and SCL and *c-kit* mRNA levels were assessed by RT-PCR analysis. As shown in Figure 3.1C, the two genes are co-expressed in pro-B cells. Interestingly, both SCL and *c-kit* mRNA levels were down regulated on B cell maturation, while IL-7Rα was up-regulated. Finally, SCL and *c-kit* were absent in mature B cells. In summary, our data indicate that SCL and *c-kit* are co-expressed within the hematopoietic precursor compartment, in multipotent progenitors (CFU-GEMM) and in more committed progenitors (BFU-E, CFU-GM, and pro-B cells). Furthermore, high levels of SCL locus activity correlate with high levels of *c-kit* expression.

Since SCL and *c-kit* are down-regulated at the same stage during B cell development, we assessed whether ectopic SCL expression could induce inappropriate *c-kit* expression in bone marrow derived B cells from homozygous SIL-SCL transgenic mice ($SCL^{tg/tg}$), which express SCL ubiquitously [10]. $B220^+$ cells from wild type and $SCL^{tg/tg}$ mice were isolated by flow cytometry, and *c-kit* expression was assessed by semi-quantitative RT-PCR. As shown in Figure 3.2A, enforced SCL expression induced a 3 to 4-fold increase in *c-kit* mRNA levels, whereas expression of RAG-2 was not affected by the SIL-SCL transgene. In order to identify at which stage of B cell differentiation *c-kit* was being induced, we performed RT-PCR analysis on purified B cell fractions, as described above [31]. As

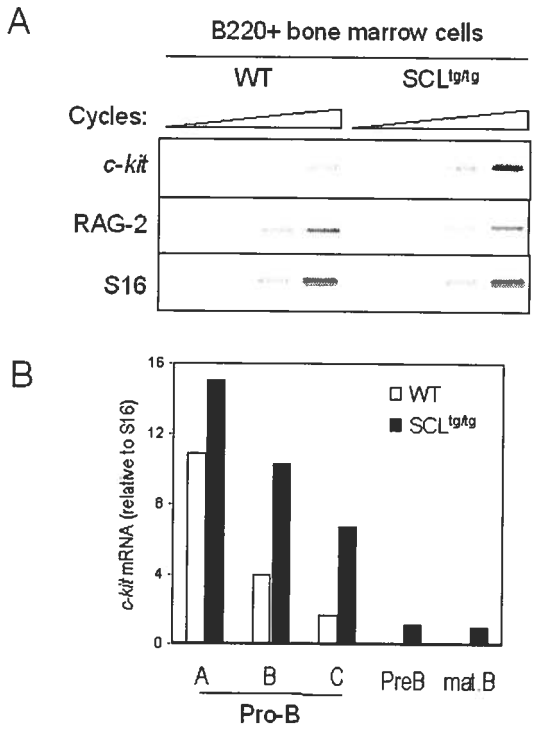


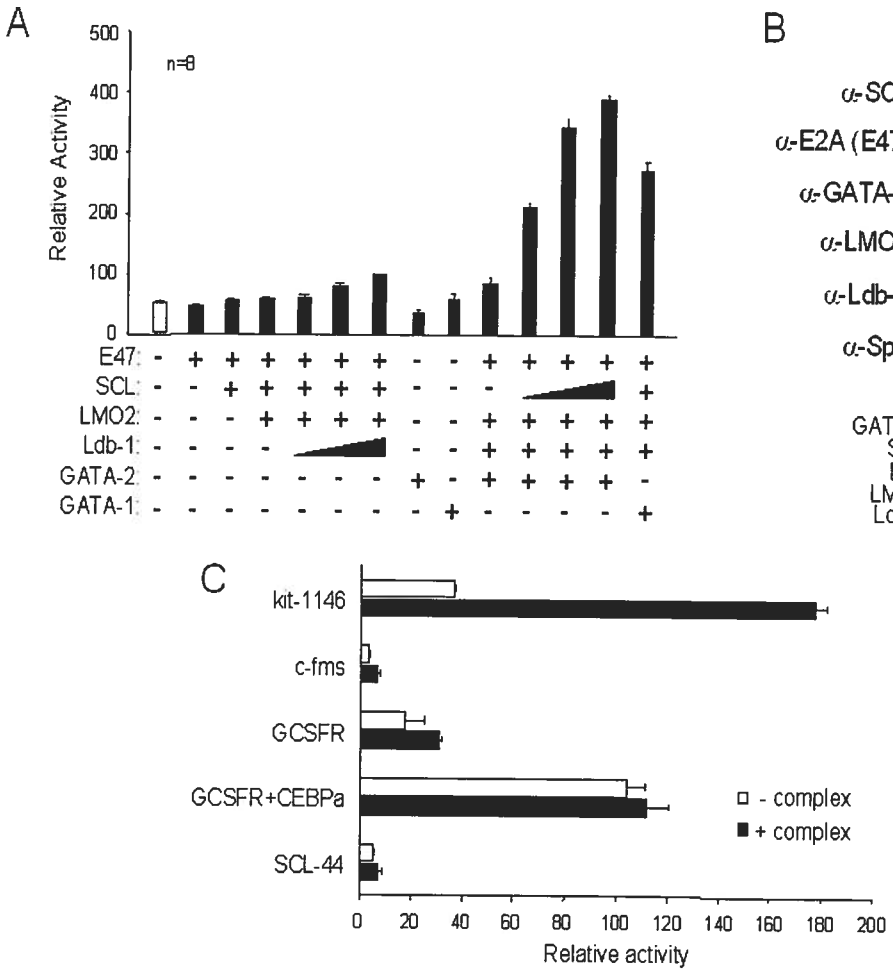
Figure 3.2. In vivo SCL induction of *c-kit* gene expression in developing B cells. (A) Bone marrow derived $B220^{+}$ cells or (B) fractionated B cell precursors from SIL-SCL transgenic mice ($SCL^{tg/tg}$) exhibit increased *c-kit* expression. B lineage cells from WT and $SCL^{tg/tg}$ were purified by flow cytometry [31] and gene expression was assessed by RT-PCR. RAG-2 expression was monitored to confirm the identity of purified B cells and S16 was used as a control for the amount of cDNA. *c-Kit* mRNA levels were quantified using the ImageQuant software (Molecular Dynamics , Sunnyvale, CA) and normalized using S16 signals.

quantified in Figure 3.2B, enforced SCL expression induced a 2 to 3-fold increase of *c-kit* mRNA within late pro-B cells (fractions B and C), in which *c-kit* and SCL are normally down-regulated. Furthermore, *c-kit* expression persisted in pre-B and mature B cells in $SCL^{tg/tg}$ bone marrow but was turned off in wild type mice. The effect of the SIL-SCL transgene was specific to *c-kit*, since the levels of IL-7 receptor α chain mRNA, as well as other B cell specific mRNAs (Pax-5, RAG-2), remained unchanged (data not shown). The increase in *c-kit* mRNA levels does not reflect an immature status of $SCL^{tg/tg}$ B cell progenitors, since we have previously demonstrated that, after an initial block at the B lineage commitment stage, B cell differentiation proceeds normally in $SCL^{tg/tg}$ mice [22]. Taken together, our observations indicate that constitutive SCL expression in the B lineage drives inappropriate *c-kit* expression throughout B cell ontogeny.

The *c-kit* promoter is activated through functional collaboration between SCL, E47, LMO2, Ldb-1, and GATA factors

To directly address whether SCL and its known transcriptional partners regulate *c-kit* expression, we inserted 1146 base pairs of the *c-kit* proximal promoter upstream of the luciferase reporter gene (kit-1146) and optimized a transactivation assay in heterologous cells (NIH 3T3). Expression vectors for SCL and its partners were transfected separately or in combination at several doses ranging from 50 ng to 1500 ng in order to reveal any dose dependent effects on *c-kit* promoter activity (data not shown). Kit-1146 activity was not enhanced upon co-transfection of E47 or GATA

Figure 3.3. Specific activation of the c-kit promoter by a multifactorial complex containing SCL, E47, LMO2, Ldb-1 and GATA factors (SCL complex). (A) NIH 3T3 cells were co-transfected with the kit-1146 reporter and expression vectors encoding SCL (60 to 1500 ng), E47 (150 ng), LMO2 (750 ng), Ldb-1 (60 to 1500 ng), and GATA factors (150 ng). (+) and (-) indicates inclusion or omission of specific expression vectors in the transfection mixtures. Open bar: reporter construct alone. (B) Co-expression of transfected vectors was confirmed by Western blotting. Nuclear extracts of TF-1 cells (12.5 μg), and of NIH 3T3 cells (12.5 μg) transfected with the indicated expression vectors, were immunoblotted using the antibodies shown on the left of each panel. Arrowheads indicate specific bands. The upper band in the Ldb-1 immunoblot corresponds to a cross-hybridizing protein. (C) The SCL-complex does not activate the c-fms, G-CSFR, or minimal SCL (SCL-44) promoters, nor does it affect the activation of the G-CSFR promoter by C/EBP-α (150 ng). Each reporter plasmid was transfected in NIH 3T3 cells in the absence (open bars) or presence (solid bars) of the SCL-complex. For panels A and C, results are shown as luciferase activity relative to the empty pXPIII vector (on average 2000 RLU), represent the average ± SD of replicate determinations and are representative of (n) independent experiments. Luciferase reporter activities were normalized to that of an internal control (CMV-βgal).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SCL:
LMO2:
Ldb-1:
GATA-2:
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TF-1
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α-E2A (E47)
α-GATA-2
α-LMO2
α-Ldb-1
α-Sp1
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GATA-2:
SCL:
E47:
LMO2:
Ldb-1:
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kit-1146
c-fms
GCSFR
GCSFR+CEBPa
SCL-44
- complex
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0 20 40 60 80 100 120 140 160 180 200
Relative activity

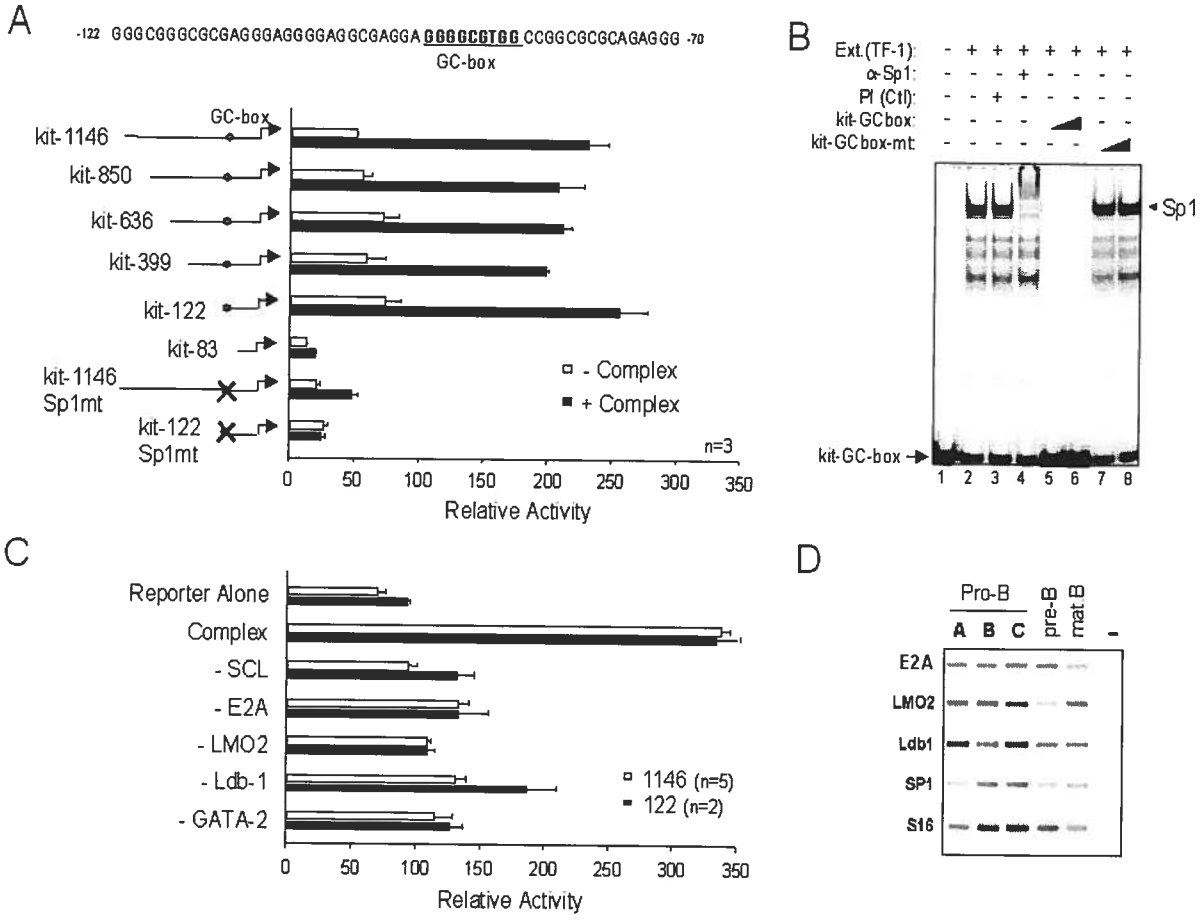
expression vectors (Figure 3.3A) even though E47 homodimers and GATA factors are strong transcriptional activators. Similarly, SCL/E47 did not affect kit-1146 basal activity (Figure 3.3A), a sharp contrast to TAL1 reporters that are activated by E47 homodimers and, to a lesser extent, by SCL/E47 heterodimers [32]. Co-transfection of LMO2 and its co-factor Ldb-1 with SCL/E47 induced a modest dose-dependent increase in luciferase activity (Figure 3.3A). Similarly, the combined effects of GATA-2, E2A, LMO2, and Ldb-1 were modest. In contrast, a high level of synergistic transactivation of the *c-kit* promoter was achieved upon co-transfection of SCL with its partners, and this effect was dose-dependent (Figure 3.3A). Therefore, *c-kit* promoter activation by SCL must rely on the formation of a multifactorial complex (SCL-complex), within which SCL plays a crucial role. In this complex, GATA-1 could substitute for GATA-2, albeit with lower efficiency (Figure 3.3A). Western blotting indicated that all five factors were expressed in the pluripotent c-Kit$^+$ hematopoietic cell line TF-1 but not in untransfected NIH 3T3 fibroblasts (Figure 3B, compare lanes 1 and 7). Transient transfection of expression vectors for SCL and its partners resulted in efficient expression in NIH 3T3 cells (lanes 2-6). Furthermore, the level of expression of each factor was similar whether transfected alone or in combination, indicating that the synergy was not due to cross regulation of transgene expression. Finally, in order to assess whether transcription regulation by the SCL-complex was specific to the *c-kit* promoter, we verified its activity on the promoters of the G-CSFR and c-fms cytokine receptor genes, chosen because of their specificity for myeloid cells that do not express SCL. As above, the SCL-complex activated kit-1146, but did not activate the G-CSFR and c-fms promoters, nor did it

affect transactivation of the G-CSFR promoter by C/EBPα (Figure 3.3C). Furthermore, the empty pXPIII vector included as a negative control was not affected by the SCL-complex. Finally, the SCL promoter (Figure 3.3C) was not activated by the SCL-complex. Together our results demonstrate that SCL and its partners functionally synergize to enhance the activity of the *c-kit* promoter. The results also show that transactivation by the SCL-complex is highly specific for the *c-kit* promoter.

Importance of an Sp1 binding site for transcription activation by the SCL-complex

To identify the *cis* elements through which the SCL-complex activates the *c-kit* promoter, we constructed a series of 5'deletion mutants. Activation of these reporter constructs by the SCL-complex was then assessed in NIH 3T3 cells. As shown in Figure 3.4A, sequences upstream of position –122 could be deleted without affecting promoter activation by the SCL-complex. Further deletion up to position –83 abolished activation, suggesting that a response element involved in recruitment of the SCL-complex was situated between positions –122 and –83 of the promoter. This sequence lacks canonical E-boxes or GATA sites but contains a consensus GC-box that is also conserved in the murine *c-kit* promoter, which is a potential binding site for members of the Sp1 family of zinc finger proteins (Figure 3.4A). To determine which factor associates with this sequence in hematopoietic cells, we performed gel shift assays with TF-1 cell nuclear extracts. The major complex that binds this GC-

Figure 3.4. Role of the Sp1 binding site in *c-kit* promoter activation by the SCL complex. (A) Deletion or point mutation of a GC-box impairs *c-kit* promoter activation by the SCL-complex. NIH 3T3 cells were transfected with mutant *c-kit* reporter constructs in the absence (open bars) or presence (solid bars) of the SCL-complex. Luciferase activities were normalized to that of CMV-βgal. The *c-kit* promoter GC-box and surrounding sequences from position –122 to –70 are indicated. (B) Sp1 is the main factor that binds to the *c-kit* promoter GC-box in TF-1 cells. EMSA were done using ^{32}P-labeled kit-GC-box probe and TF-1 cell nuclear extracts. Supershifting was performed with a pre-immune serum (Ctl) and an anti-Sp1 antibody (lanes 1-4). Competition assays (lanes 5-8) were done using a 10 and 100-fold molar excess of unlabeled kit-GC-box or kit-GC-box-mt double-stranded oligonucleotides. (+) and (-) indicates inclusion or omission of specific ingredients. Arrowhead points to the major Sp1 complex, while arrow points to free probe. (C) All partners of the SCL-complex are required for maximal *c-kit* promoter activation. Expression vectors for SCL, E47, LMO2, Ldb-1, and GATA-2 were co-transfected with the kit-1146 (open bars) or kit-122 (solid bars) reporter plasmids. Where indicated (-), the corresponding vectors were omitted from the transfection mixtures. For A and C, results are shown as luciferase activity relative to the empty pXPIII vector and are representative of (n) independent experiments. (D) Partners of the SCL-complex are expressed in B cell precursors in which SCL induces endogenous c-kit expression. Bone marrow B cell precursors from wild type mice were purified by flow cytometry as in Figure 1C, and expression of SCL partners was assessed by RT-PCR. S16 was used as a control for the amount of cDNA.


A
-122 GGGCGGGCGCGAGGGAGGGGAGGCGAGGA GGGGCGTGG CCGGCGCGCAGAGGG -70
GC-box
GC-box
kit-1146
kit-850
kit-636
kit-399
kit-122
kit-83
kit-1146 Sp1mt
kit-122 Sp1mt
- Complex
+ Complex
n=3
0 50 100 150 200 250 300 350
Relative Activity
B
Ext.(TF-1):
α-Sp1:
PI (Ctl):
kit-GCbox:
kit-GCbox-mt:
Sp1
kit-GC-box
1 2 3 4 5 6 7 8
C
Reporter Alone
Complex
- SCL
- E2A
- LMO2
- Ldb-1
- GATA-2
1146 (n=5)
122 (n=2)
0 50 100 150 200 250 300 350
Relative Activity
D
Pro-B
A B C
pre-B
mat.B
-
E2A
LMO2
Ldb1
SP1
S16

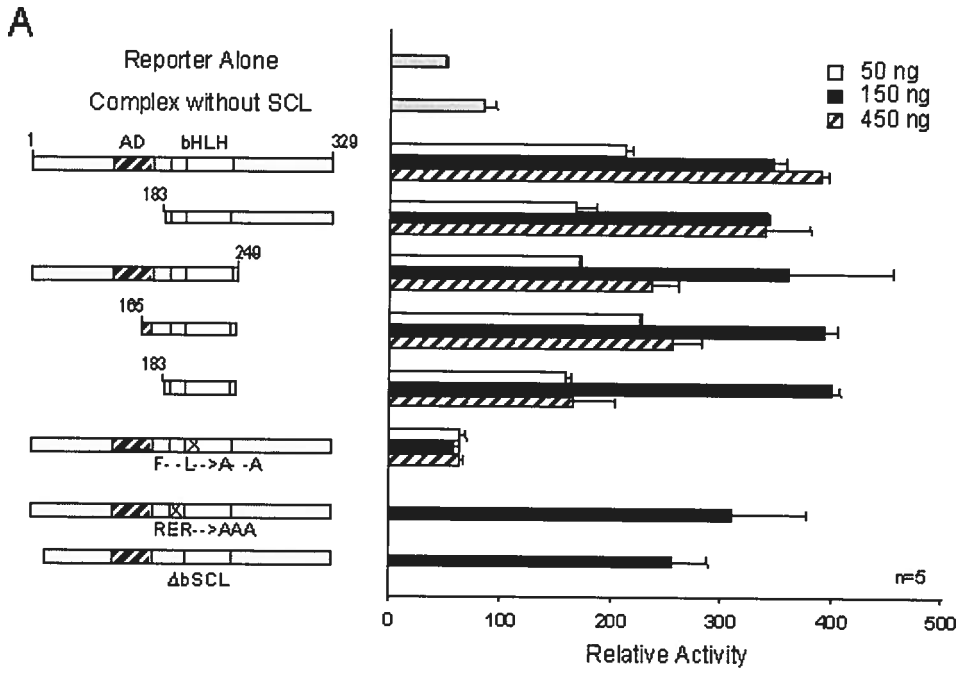
box was supershifted with an anti-Sp1 antibody but not pre-immune serum (Figure 3.4B, lanes 1-4), indicating that it contained Sp1. Binding to the c-kit GC-box probe was efficiently displaced by an unlabeled wild type competitor oligonucleotide (lanes 5-6). In contrast, an oligonucleotide mutated within the GC-box was unable to compete for Sp1 binding (lanes 7-8). These same point mutations were therefore introduced into either the full-length promoter (kit-1146-Sp1mt) or the most proximal promoter deletion mutant that still retained responsiveness to the SCL-complex (kit-122-Sp1mt). Mutating the Sp1 binding site completely abolished *c-kit* promoter activation by the SCL-complex (Figure 3.4A). In light of these results, we confirmed by Western blotting that the Sp1 protein is expressed in NIH 3T3 cells (Figure 3.3C) and that it was not affected by ectopic expression of SCL and its partners, indicating that transcriptional synergy was not due to increased Sp1 expression. Finally, to determine whether transcription activation by all five partners was synergistic or additive, both in the context of kit-1146 and the minimal kit-122, we sequentially omitted each factor from our transfection mixtures. As shown in Figure 3.4C, omission of either of the expression vectors abolished transactivation and reduced luciferase activity at or near basal level. These results demonstrate that all five partners act synergistically to activate both the full length and minimal *c-kit* promoter segments. Together, our observations indicate that the Sp1 binding site is necessary for regulation of the *c-kit* promoter by the SCL-complex, which further suggests that Sp1 can help to recruit these factors to their target genes.

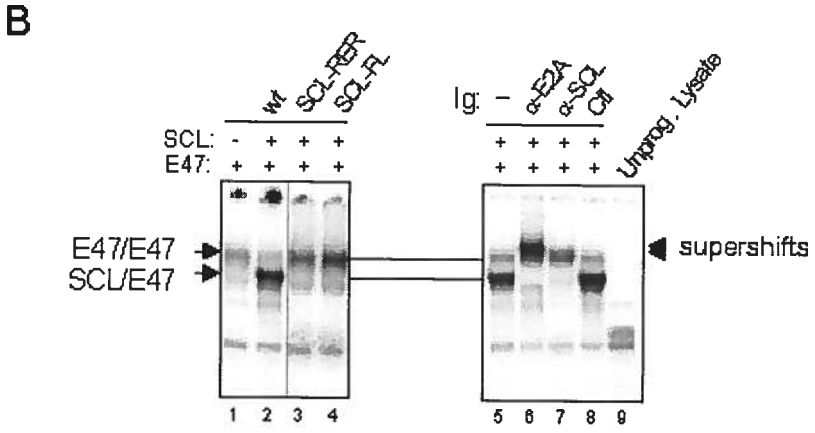
Since the transcriptional activity of the SCL-complex requires recruitment of the LMO2 and Ldb1 cofactors, as well as E2A and GATA transcription factors, we investigated the expression of SCL partners within purified B cell precursors. In addition to E2A, known to be highly expressed in B cells, we observed that LMO2 and Ldb1 mRNAs are present in all bone marrow B cell fractions (Figure 3.4D). The GATA3 transcription factor, highly expressed in T cells, was found to be present at low levels in fractions A to C (data not shown), and could be due to the presence of cells that are not fully committed to the B lineage. Finally, Sp1 mRNA is expressed in the B lineage in which *c-kit* expression is induced by the SCL transgene (Figure 3.4D), suggesting that the induction of *c-kit* by SCL *in vivo* is associated with the presence of appropriate partners within target cells.

Dispensability of SCL DNA binding

Our observations indicate that activation of the *c-kit* minimal promoter by the SCL-complex: 1) occurred in the absence of canonical Ebox binding sites and 2) required multiple partners. We therefore determined which domains of SCL were required to form a functional complex. SCL was previously shown to contain a proline-rich N-terminal transactivation domain lying between amino acids 117 and 175 [33]. In NIH 3T3 cells, this domain could be deleted without affecting SCL transcriptional activity (Figure 3.5A). In fact, SCL could be reduced to its bHLH domain and still remained functionally active. Also, SCL mutants that are unable to bind to DNA due to a deletion (ΔbSCL) or point mutations (SCL-RER) within the basic region (Figure

Figure 3.5. Dispensability of SCL transactivation and DNA binding domains for *c-kit* promoter activation. (A) Mapping of SCL domains required for its function in transcription activation. The kit-1146 reporter was co-transfected in NIH-3T3 cells with complexes containing the indicated SCL point or deletion mutants (50-450 ng) [18]. Numbers correspond to amino acid residues of SCL. bHLH=basic Helix-Loop-Helix (open boxes); AD=Activation Domain (hatched box); F--L→ A--A= point mutations within Helix 1 of the HLH domain that disrupt dimerization with E2A; RER→AAA= point mutations within the basic domain that disrupt DNA binding, n = number of representative experiments. (B) DNA binding properties of SCL mutants. EMSA experiments were performed using *in vitro* synthesized ^{35}S-labeled E2A and SCL mutants, and ^{32}P-labeled TAL1 probe [26]. Where indicated, antibodies were included in the samples before addition of the labeled probes. Arrows point to the binding of E2A homodimers or SCL/E2A heterodimers, and arrowheads indicate the supershifted bands. A monoclonal antibody against c-Myc was used as a control for supershifting (lane 8), while an unprogrammed reticulocyte lysate was included as a negative control for binding (lane 9).


A
Reporter Alone
Complex without SCL
1
AD
bHLH
329
183
249
165
183
F- -L->A- -A
RER-->AAA
ΔbSCL
50 ng
150 ng
450 ng
n=5
0
100
200
300
400
500
Relative Activity


B
wt
SCL-RER
SCL-FL
SCL:
E47:
Ig:
α-E2A
α-SCL
Ctl
Unprog. Lysate
E47/E47
SCL/E47
supershifts
1 2 3 4
5 6 7 8 9

3.5B, lane 3 and data not shown), were still functional (Figure 3.5A). These results are consistent with the fact that *c-kit* promoter activation by the SCL-complex was independent of Ebox binding sites (Figure 3.4A). Finally, point mutations in the HLH domain (SCL-FL) that disrupt heterodimerization with E47 (Figure 3.5B, lane 4) completely abrogate *c-kit* promoter activation by the SCL-complex (Figure 3.5A). Therefore, our observations reveal that the HLH protein interaction motif of SCL is critical for the function of this complex, while the DNA binding and putative transactivation domain are dispensable. These data support the notion that SCL can be recruited to regulatory regions via protein interactions with partners such as Sp1. This mechanism helps to explain why transactivation by the SCL-complex occurs in the absence of E-boxes and why DNA binding by SCL is not strictly required for its function *in vivo* [18;19].

Sp1 physically interacts with multiple elements of the SCL-complex

Since transcriptional synergy may result from direct interactions between transcription factors, we sought to determine, via several approaches, whether Sp1 physically associates with members of the SCL-complex. First, we performed *in vitro* pull-down assays with immobilized GST-Sp1 and *in vitro*-translated, ^{35} S-labeled SCL, GATA-1, LMO2, Ldb-1, and E47. GST columns and *in vitro*-translated Luciferase were used as negative controls for the binding assays. Interestingly, GST-Sp1 columns specifically retained SCL, GATA-1, LMO2 and Ldb-1, but not E47 or luciferase (Figure 3.6A). These interactions were not affected by the presence of

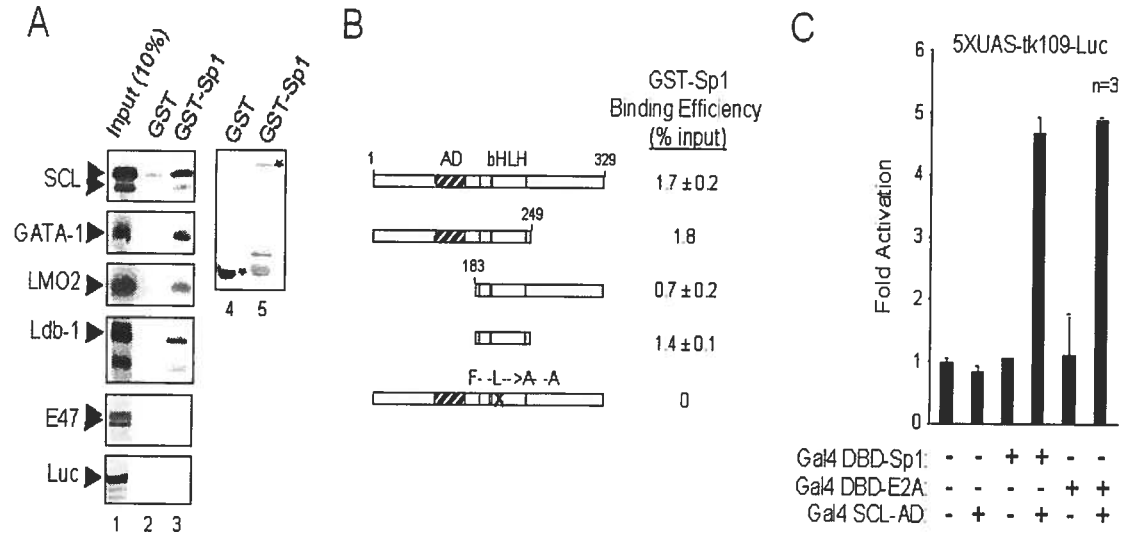


Figure 3.6. Sp1-SCL complex interaction. (A) Sp1 interacts *in vitro* with SCL, GATA-1, LMO2 and Ldb-1. Pull-down assays were performed using immobilized GST and GST-Sp1, and ^{35}S-labeled SCL, GATA-1, LMO2, Ldb-1, E47 and Luciferase. Inputs (lane 1) represent 10% of the amounts used in lanes 2 and 3. Coomasie blue stained GST and GST-Sp1 are shown in lanes 4-5. (B) Sp1 specifically interacts with the bHLH domain of SCL. SCL mutants were labeled with ^{35}S-methionine and used in pull-down assays as described in A. Protein signals were quantified using the ImageQuant software. Binding efficiency (% of input) was calculated by comparison with input samples (10%) after subtraction of background GST signals. (C) *In vivo* interaction between Sp1 and SCL. A two-hybrid assay was performed by transiently transfecting NIH 3T3 cells with the 5XUAS-tk109-Luc reporter (1.5 μg) and Gal4 DBD-Sp1 (10 ng), Gal4 DBD-E2A (10 ng) and Gal4 AD-SCL (1 μg) expression vectors. Results are shown as fold activation over 5×UAS-tk109-Luc transfected alone, are the mean ± SD of replicate determinations and are representative of (n) experiments.

ethidium bromide (200 μg/ml) in the binding reactions, demonstrating that they were direct and not due to bridging by contaminant DNA molecules (data not shown). Quantification of *in vitro* pull-down results indicates that the associations between Sp1 and partners of the SCL-complex were in the range of 1-5% of input proteins. These *in vitro* pull-down results indicate that Sp1 entertains direct physical interactions with four elements of the SCL-complex.

We then sought to identify the domain of SCL that interacts with Sp1 by performing pull-down assays with *in vitro*-translated SCL mutants. Figure 3.6B displays the binding efficiencies of these SCL mutants for GST-Sp1. Deletion of either N-terminal or C-terminal domains of SCL did not affect its association with Sp1, whereas point mutations in the HLH domain (SCL-FL) abrogated this interaction. Furthermore, the bHLH domain of SCL was sufficient for physical interaction with Sp1. These findings are in complete agreement with our transactivation results in which the bHLH domain of SCL was sufficient to form a functional complex that drove *c-kit* promoter activation (Figure 3.5A).

To assess whether SCL and Sp1 interact within transfected cells, we optimized a mammalian two-hybrid assay in which Sp1 or E47 was fused to the Gal4 DNA-binding domain (Gal4 DBD-Sp1 or –E47), while SCL was grafted to the Gal4 activation domain (Gal4 SCL-AD). As shown in Figure 3.6C, Gal4 DBD-Sp1 and Gal4 SCL-AD interacted *in vivo* to produce a 5-fold activation of a reporter construct driven by multimerized upstream activator sequences (5XUAS-tk109-luc). This

interaction was of similar magnitude to that observed with Gal4 DBD-E47 and Gal4 SCL-AD. By contrast, neither of these fusion proteins activated the reporter construct when expressed alone. This result indicates that a physical association between Sp1 and SCL takes place within transfected cells.

Finally, to ascertain whether these interactions occur *in vivo* with endogenous proteins present in *c-kit* expressing hematopoietic cells, we performed co-immunoprecipitations using TF-1 cell nuclear extracts. Antibodies against GATA-1, SCL, Sp1, and E2A efficiently precipitated their respective proteins (Figure 3.7, lanes 4, 6, 10, and 17). Under these conditions, SCL most effectively co-precipitated with anti-Lmo2, anti-Ldb-1, and anti-E2A (lanes 8-9, and data not shown), demonstrating a strong association between SCL and these factors. In addition, the anti-SCL antibody efficiently brought down E2A (lane 16) and to a lesser extent GATA-1 (lane 3). These data demonstrate that previously identified partners of the SCL-complex are indeed associated in pluripotent hematopoietic cells. In addition, antibodies directed against Sp1 consistently co-precipitated GATA-1 and SCL (lanes 2 and 13). Although weak, SCL/Sp1 co-immunoprecipitation was consistently observed, whereas SCL co-precipitation did not occur when either anti-GFP (lane 11) or anti-PU.1 (lane 14) was used as control species matched antisera. Therefore, our data reveal that Sp1 is indeed associated with partners of the SCL-complex *in vivo*, and suggest that Sp1 is a novel component of this complex. The co-immunoprecipitation efficiencies were highest for SCL, LMO2, Ldb-1, and E2A, suggesting that these factors may form a core complex with which other partners

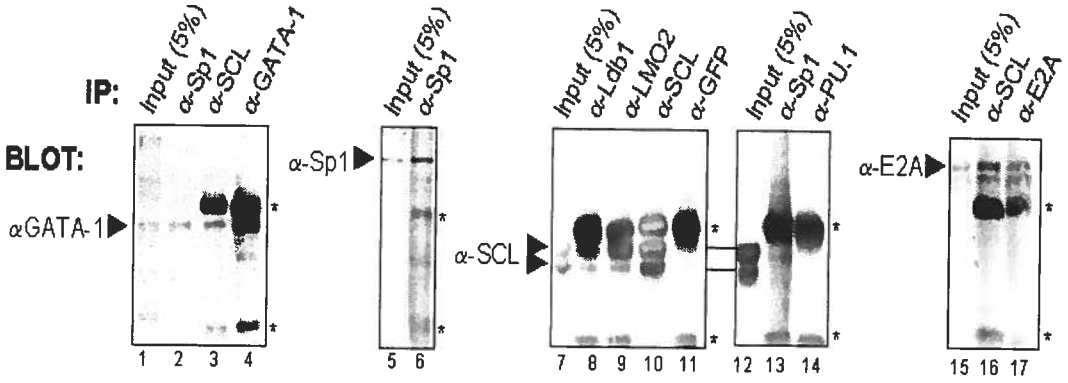


Figure 3.7. Sp1 associates with the SCL complex *in vivo* in hematopoietic cells. Co-immunoprecipitations of endogenous proteins from TF-1 cell nuclear extracts were performed using the antibodies indicated above each panel. Arrowheads point to specific bands that were revealed through Western blotting with the antibodies indicated on the left. Inputs represent 5% (50 μg) of the amount used for each immunoprecipitation (1 mg) and the asterisks indicate immunoglobulin heavy and light chains. Note that GATA-1 is co-immunoprecipitated with α-GATA-1, α-SCL and α-Sp1; Sp1 is immunoprecipitated by α-Sp1; SCL co-precipitates with α-SCL, α-Ldb-1, α-LMO2 and α-Sp1, but not with α-GFP or α-PU.1; while E2A is brought down by α-E2A and α-SCL.

such as GATA-1 and Sp1 can associate. Combined, our results demonstrate that Sp1 directly interacts with partners of the SCL-complex both *in vitro* and *in vivo,* and explain the functional collaboration observed between Sp1 and the SCL-complex.

Sp1, SCL and E2A occupy the *c-kit* promoter *in vivo*

While our results demonstrate that Sp1 and the SCL-complex regulate *c-kit* promoter activity *in vitro*, true proof of principle would have to come from a demonstration that these factors occupy the *c-kit* promoter *in vivo* in hematopoietic cells. To this end, we performed chromatin immunoprecipitation assays using formaldehyde cross-linked TF-1 cells. Chromatin extracts were subjected to immunoprecipitation with anti-SCL, anti-E2A, anti-Sp1, and species matched control antibodies (anti-HA and anti-PU.1). Cross-linking was reversed and DNA fragments that were specifically retained were purified. These samples were then subjected to PCR amplification using specific primers that target different regions in the *c-kit* locus (Figure 3.8). We used serial dilutions of template DNA to ensure that the different samples could be compared within their linear range of amplification. In addition, PCR products were hybridized with ^{32}P-labelled internal oligonucleotide probes to confirm the specificity of the amplified fragments. As shown in Figure 3.8, antibodies to SCL, E2A and Sp1 efficiently recovered a 150 bp fragment of the *c-kit* proximal promoter, encompassing the Sp1 site. In contrast, species-matched control antibodies (anti-PU.1 and anti-HA) were unable to bring down this sequence. Furthermore, all antibodies precipitated negligible amount of DNA located either 2 kb upstream or 13

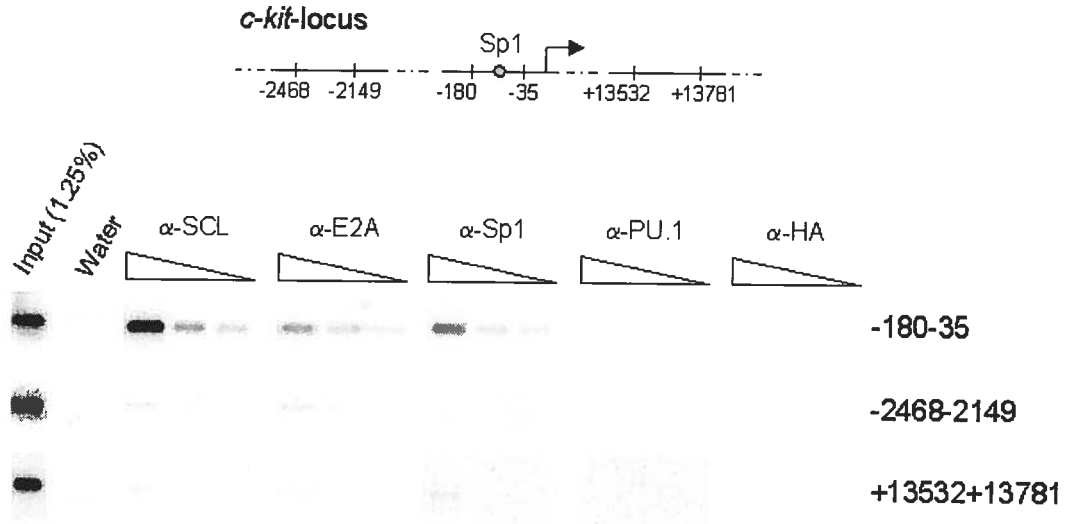


Figure 3.8. Specific association of SCL, E2A and Sp1 with the *c-kit* promoter *in vivo*. Exponentially growing TF-1 cells were fixed in 1% formaldehyde and sonicated. Fragmented chromatin extracts were then subjected to immunoprecipiation with α-SCL, α-E2A, α-Sp1 and isotype matched control antisera (α-PU.1 and α-HA). Precipitated chromatin was heated overnight at 65°C to reverse cross-linking and DNA molecules were purified and subjected to PCR analysis to test for the presence of the *c-kit* promoter (-180 to -35) or the indicated control *c-kit* locus regions (-2468 to -2149 and +13532 to +13781). Input chromatin represents 1.25 % of the amount used in each immunoprecipitation and three fourfold serial dilutions of the immunoprecipitated samples were done prior to amplification. The PCR products were analyzed by agarose gel electrophoresis, transferred onto Biodyne B membrane and hybridized with internal oligonucleotide probes.

kb downstream of the transcription initiation site. These results demonstrate that Sp1, SCL, and E2A directly and selectively associate with the *c-kit* proximal promoter region containing the functional Sp1 binding site in hematopoietic cells.

3.5 Discussion

In the present study, we show that SCL and *c-kit* are co-expressed in primary hematopoietic precursors and that SCL up-regulates *c-kit* expression *in vivo* in developing B-cells. Our study reveals Sp1 as a novel member of the SCL-complex that tethers SCL and its partners to the *c-kit* promoter. Reinforcing this hypothesis is the finding that SCL DNA-binding defective mutants are still functional within the larger protein complex. Finally, we demonstrate that SCL and E2A, essential partners within the SCL-complex, co-occupy the *c-kit* promoter with Sp1 *in vivo* in hematopoietic cells.

Co-linearity of SCL and *c-kit* expression and function

During embryonic development, hematopoiesis occurs in two waves: a first wave of primitive hematopoiesis takes place in the yolk sac, and a second wave of definitive hematopoiesis initiates in the fetal liver and later moves to the bone marrow. In the embryo proper, hemopoietic cells are also found in the AGM (aorta, gonad and mesonephros) region. Within all of these hemogenic sites the expression of *c-kit* and SCL closely overlaps. From E7.5 to E9.5, *c-kit* and SCL are both expressed in yolk sac blood islands, from E9.5 to E11.5 [34;35], in the AGM region and, from E10.5 to E11.5 [34-37], in the fetal liver[34;35;37;38]. We now show that in adult bone marrow cells, the SCL locus is transcriptionally active in cells that express the c-Kit receptor, a population that includes almost all hematopoietic progenitor colony-forming cells.

Our results demonstrate that *c-kit* and SCL remain expressed in committed progenitors, BFU-E, CFU-GM, and pro-B cells, which is consistent with published studies [22;30;39]. Our previous work also indicates that SCL and *c-kit* are co-expressed in early pro-T cells [11]. Finally, both genes are down regulated with terminal differentiation in the granulocyte-macrophage, B and T lymphoid lineages [2]. Thus, the mapping of *c-kit* and SCL expression by a variety of techniques indicates a close temporal association within developing hematopoietic cells.

In mice, severe mutations in the *c-kit* gene (*W* mutants) cause prenatal lethality at 13-15 days of gestation due to the interruption of fetal liver hematopoiesis [40], demonstrating the essential role of the c-Kit tyrosine kinase in definitive hematopoiesis. Similarly, SCL is critical not only for primitive but also for definitive hematopoiesis, as revealed by the study of $SCL^{-/-}$ ES cells *in vitro* and *in vivo* in chimeric mice [41;42]. Taken together, the co-linearity of expression and overlapping biological functions of SCL and *c-kit* suggest that they operate along the same genetic pathway. In order to define SCL function in multipotent cells, we had previously performed a functional screen of TF-1 cells expressing an antisense SCL [20], and found that c-Kit receptor function and expression were defective in these cells. Co-delivery of SCL in the sense orientation rescued *c-kit* gene expression, which suggests that the latter is a potential downstream target of SCL. Here, we demonstrate that ectopic SCL expression in transgenic mice induces sustained *c-kit* transcription *in vivo* in developing B cells, where partners of the SCL-complex are co-expressed. The induction of *c-kit* was monitored in purified B cell fractions,

indicating that increased *c-kit* expression was a direct effect of the SCL transgene on target B cell progenitors rather than an indirect effect on B cell differentiation. Thus, in both $CD34^+$ cells [20] and in $B220^+$ cells (the present study), SCL is shown to induce *c-kit* transcription in chromatin.

c-kit as a target gene of the SCL-complex

The survival and differentiation of hematopoietic cells is critically dependent on the action of hematopoietic growth factors and their receptors. Therefore, genes encoding cytokine receptors represent critical targets for hematopoietic transcription factors. For instance, it has been shown that the major erythroid transcription factor GATA-1 activates the EpoR promoter [43], whereas the myeloid regulator PU.1 regulates promoters of the c-fms and GM-CSFR β-chain genes [44-46]. Despite the critical importance of *c-kit* for the survival of multipotent progenitor cells, the mechanisms controlling *c-kit* gene transcription in hematopoietic cells has not been studied as extensively as that of other cytokine receptor genes. Furthermore, potential binding sites for the SCL-complex were previously identified in other hematopoietic genes, but the functional importance of these sites and their *in vivo* association with SCL and its partners has not yet been demonstrated. In this study, we combine *in vivo* approaches with *in vitro* protein interaction and transactivation assays, and provide direct evidence that the SCL-complex indeed activates the *c-kit* promoter. This activation is selective for *c-kit* promoter sequences, as the SCL-complex has no effect on two myeloid promoters, c-fms and G-CSFR. Finally, we

show via chromatin immunoprecipitation that SCL, E2A, and Sp1 occupy the *c-kit* promoter *in vivo* in TF-1 cells. We conclude therefore that *c-kit* is a direct target of transcription regulation by SCL and its partners. By activating target genes such as *c-kit*, the SCL-complex may ensure the survival of undifferentiated pluripotent hematopoietic cells, a role that has been formerly attributed to the c-Kit receptor [47].

Sp1, a novel member of the SCL-complex

Analysis of the *c-kit* promoter has allowed us to demonstrate novel functional and physical interactions between Sp1 and the SCL pentameric complex. Sp1 is the founding member of a family of zinc finger containing proteins (Sp/XKLF) that bind GC- or GT-boxes [48]. Sp1 is ubiquitously expressed and is involved in chromatin remodeling and maintenance of methylation-free islands. Functionally important Sp1 binding sites have been identified in the regulatory regions of many hematopoietic genes [49-52]. Our observations indicate that Sp1 functionally interacts with the SCL-complex in hematopoietic cells, and co-occupies the *c-kit* promoter with SCL and E2A *in vivo*, highlighting the importance of Sp1 in hematopoietic gene regulation. $Sp1^{-/-}$ embryos die at E11 and show a broad range of abnormalities, and $Sp1^{-/-}$ ES cells fail to contribute to any tissue in mouse chimeras past E9.5 [53]. Although yolk sac and fetal liver hematopoiesis occur in these mice, the early lethality of Sp1 deficient embryos has precluded the study of the hematopoietic compartment. A more detailed analysis of conditional $Sp1^{-/-}$ mutants will be required in order to

clarify additional Sp1 functions during hematopoietic development and in specific blood cell lineages.

Sp1 has a broad binding specificity. Sp1 typically binds to a GC-box consensus motif, (G/T)(A/G)GGGCG(G/T)(A/G)(A/G)(C/T). A high affinity variant of this GC box was defined by *in vitro* CASTing, referred to as s-GC-box, GGGTGGGCGTGGC [54]. Interestingly, the Sp1 binding site found on the proximal c-kit promoter conforms to the high affinity s-GC-box, as A and G are also found at P3 and P4, albeit with lower frequency than G and T. A second type of Sp1 consensus was identified in the myeloid promoters CD14 and CD11b [51;52], (G/T)(A/G)GGC(G/T)(A/G)(A/G)(G/T), identical to two sites found at positions –36 and –15 relative to the major transcription initiation site of the G-CSFR gene [55]. Despite the presence of these two sites, our results indicate the G-CSFR promoter is not activated by the SCL-complex. Two other promoters tested in the present study, the minimal SCL promoter (-44) and the c-fms promoter, do not contain Sp1 binding sites and are not activated by the SCL-complex. Thus, the sequence divergence between the *c-kit* GC-box and the Sp1 binding sites found in the GCSF-R promoter indicate that not all Sp1 sites are competent to recruit SCL and its partners. Our observations suggest that the *c-kit* GC-box may provide a unique molecular environment that allows for the functional interaction between Sp1 and partners of the SCL-complex. It is therefore tempting to speculate that the architecture of the promoter creates unique interfaces for protein interactions.

There seems to exist multiple mechanisms through which SCL regulates gene expression. For example, there is strong evidence in favor of DNA binding independent functions of SCL during early hematopoietic development and induction of leukemia [18;19]. Two potential mechanisms have been proposed. SCL could either be sequestering an inhibitor of hematopoietic development, which is similar to the mechanism proposed for the Id family of HLH factors. Alternatively, since SCL associates into larger complexes with other transcription factors, the DNA binding function of SCL may not be strictly required to activate a subset of its target genes. In support of this second mechanism, SCL and LMO2 have been reported to act as cofactors for GATA-3 to activate the RALDH2 promoter in T-ALL cells [56]. Our results also support this second model and unmask Sp1 as an additional partner that can recruit the SCL-complex to target promoters in a manner that is independent of SCL DNA binding. It remains to be documented whether Sp1 or GATA factors are sufficient to tether the SCL-complex to DNA or, alternatively, whether additional chromatin components contribute to this association, as shown for the IFN-β enhanceosome [57].

Diversification through protein-protein interaction

During the process of differentiation, transcription factor complexes may undergo dynamic changes, through addition or loss of particular components, which modulate their activity or specificity [58]. Since the *c-kit* and SCL genes are transcribed in early progenitors and are progressively extinguished as differentiation proceeds, our study

places SCL among the first events in the hierarchy of transcription factor complexes that regulate hematopoiesis. We have previously shown that SCL genetically interacts with E2A to prevent commitment into the B lineage [22]. The fact that SCL partners are expressed throughout B cell differentiation, suggests that the removal of SCL from this complex is a key event in B lineage determination. In the T lineage, however, SCL *per se* is not sufficient to perturb thymocyte differentiation and requires collaboration with LMO proteins to cause differentiation arrest at the double negative stage [9-11], illustrating the importance of the make-up of transcription complexes in cell fate decisions.

In *in vitro* studies of erythroid differentiation, LMO2/Ldb-1 and SCL/GATA-1 have been shown to exert opposite biological effects, where enforced expression of the former inhibits [59] and of the latter facilitates terminal erythroid maturation [30;60;61]. These seemingly contradictory roles can be reconciled by considering that SCL and GATA-1 are part of dynamically evolving protein complexes during red cell differentiation [62;63]. In early progenitors, SCL, GATA-1/-2 and LMO2 are co-expressed [29;30;64]and collaborate to maintain cells in an undifferentiated state, by activating target genes such as *c-kit*. When the cells receive the proper differentiation signals, induction of modulatory co-factors may inhibit the function of the SCL-complex, allowing SCL and GATA-1 to exert other regulatory functions [18;61]. We believe that the cellular context and the partners that are co-expressed determine the role of SCL as an activator [13;32;56]or repressor [11;32]of transcription. We propose that

dynamic changes in composition of the SCL-complex play an important role in governing cell fate decisions throughout hematopoietic development.

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CHAPITRE 4

ARTICLE 2

SCL assembles a multifactorial complex that determines glycophorin A gene expression

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4.1 Abstract

SCL/TAL1 is a hematopoietic-specific transcription factor of the basic helix-loop-helix (bHLH) family that is essential for erythropoiesis. Here we identify the erythroid-specific gene Glycophorin A (*GPA*) as a target of SCL in primary hematopoietic cells and show that SCL occupies the *GPA* locus in vivo. *GPA* promoter activation is dependent on the assembly of a multifactorial complex containing SCL, as well as ubiquitous (E47, Sp1, Ldb1) and tissue-specific transcription factors (LMO2, GATA-1). In addition, our observations suggest functional specialization within this complex as SCL provides its HLH protein interaction motif, GATA-1 exerts a DNA tethering function through its binding to a critical GATA element in the *GPA* promoter, while E47 requires its N-terminal moiety most likely entailing a transactivation function. Finally, endogenous *GPA* expression is disrupted in hematopoietic cells through the dominant-inhibitory effect of a truncated form of E47 (E47-bHLH) on E-protein activity, of FOG on GATA activity, or when LMO2 or Ldb-1 protein levels are decreased. Together, these observations reveal the functional complementarities of transcription factors within the SCL complex and the essential role of SCL as a nucleation factor within a higher order complex required to activate *GPA* gene expression.

4.2 Introduction

How specific patterns of gene expression are generated represents a fundamental question in understanding cell type specification. There is increasing evidence that this process is controlled by networks of interacting transcription factors and that subtle variations in protein partners may have profound consequences on gene expression programs (56). Among the crucial regulators of cell type specification are transcription factors that contain basic helix-loop-helix (bHLH) domains, such as the hematopoietic master regulator SCL/TAL-1 (6). *SCL* is expressed in hematopoietic stem cells, as well as multipotent, erythroid and megakaryocytic progenitors (7,16,26,47). Loss and gain of function studies in different vertebrate models have shown that *SCL* is essential for the establishment of the hematopoietic system, and that it can specify the hematopoietic cell fate when ectopically expressed (21,34,45,52,53,55). Due to the absence of hematopoietic cells in $SCL^{-/-}$ mice, deciphering its role in the differentiation of particular blood cell lineages has been elusive. Recent conditional knock-out experiments, which bypass the embryonic lethality observed in $SCL^{-/-}$ mice, have demonstrated that *SCL* is essential for erythroid and megakaryocytic differentiation (23,36). In addition to its critical role during normal hematopoiesis, the *SCL* gene is the most frequent target of chromosomal rearrangements in patients with T cell acute lymphoblastic leukemia (T-ALL). This leukemic phenotype is recapitulated in transgenic mice co-expressing SCL and collaborating oncogenes, such as the LIM domain proteins LMO1/2 (6).

Therefore, SCL is an essential regulator at several levels in the hematopoietic hierarchy and its inappropriate regulation leads to severe pathological consequences.

Like other tissue-specific bHLH factors, SCL forms E-box (CANNTG) binding heterodimers with ubiquitous bHLH partners know as E-proteins, which include products of the *E2A* gene (E12 and E47), HEB and E2-2 (27). In erythroid cells, SCL is found in a multifactorial complex (SCL complex) with E47, LMO2, Ldb1, and GATA-1 (68). Although potential binding sites for the SCL complex are found in erythroid genes, such as *GATA-1* and *EKLF* (1,66), functional dissection of the mechanism of action of SCL on erythroid targets remains to be documented. For example, the importance of the N terminal transactivation domain and the basic domain of SCL remains controversial, as they are both dispensable for the genetic rescue of specification of the hematopoietic cell fate (44) and for *c-kit* transcription activation (31), yet DNA binding defective mutants of SCL fail to rescue the maturation of definitive hematopoietic lineages in $SCL^{-/-}$ ES cells (44) and to induce erythroid differentiation in established cell lines (3). In more primitive hematopoietic progenitors, GATA-2 can function within the SCL complex as has been observed in the context of the *c-kit* promoter (31). This study also identified Sp1 as a novel component of the SCL complex, consistent with the importance of Sp1 in hematopoietic gene regulation (57). In leukemic cells, SCL also associates with *E2A* gene products, LMO2 and Ldb1 (22); however, in this cellular context, SCL and LMO1/2 may inhibit the normal functions of E-proteins, which are crucial regulators of lymphoid cell differentiation. Thus, the function of SCL may vary depending on

the cellular context and target genes. In order to clarify our understanding of the functions played by SCL in different hematopoietic lineages, it is crucial to define the mechanisms by which SCL and its partners regulate the expression of candidate target genes in these cellular compartments.

We previously demonstrated that ectopic expression of SCL in TF-1 cells, a bipotent cell line that can be induced to differentiate along the erythroid or monocytic/macrophage lineages, increases cell surface expression of the erythroid marker glycophorin A (GPA) and renders the induction of erythroid differentiation more efficient (26). GPA is one of the most abundant erythrocyte membrane proteins, and its highly glycosylated sialic acid-rich extracellular domain is predominantly responsible for the negative charge of the red cell membrane. Despite the recognition that SCL collaborates with its partners to activate transcription and determine the hematopoietic fate (31,33,68), there is little evidence for the formation of a functional high molecular weight SCL complex on regulatory sequences of physiological target genes. In the present study, we show that the SCL complex determines *GPA* gene expression and that the main function of SCL is to assemble this complex on target gene regulatory elements in order to activate transcription.

4.3 Materials and Methods

DNA Constructs

Expression vectors for SCL and its partners, as well as SCL point and deletion mutants were formerly described (31,44).The murine stem cell virus (MSCV)-neo and MSCV-YFP vectors, expressing SCL or an antisense (AS) RNA of SCL (AS-SCL), were constructed as previously detailed (30). Vectors encoding AS-LMO2 and AS-Ldb1 were generated by subcloning the LMO2 and Ldb1 cDNAs in reverse orientation into the MSCV-neo multiple cloning site. Expression vectors encoding FOG (62), GATA-$1^{V205\rightarrow G}$ (13), and E47-bHLH (46) were generously provided by Drs. Stuart H. Orkin (Harvard Medical School, Boston, MA), John D. Crispino (Ben May Institute for Cancer Research, Chicago, IL) and Jacques Drouin (Institut de Recherches cliniques de Montréal, Montréal, QC), respectively. For retroviral infections, the FOG and E47-bHLH cDNAs were subcloned into the MSCV-neo vector. *GPA* promoter fragments -456, -116, -84 and -79 were PCR amplified from human genomic DNA using forward primers (-456 to -440), (-116 to -100), (-84 to –68) and (-75 to 60), respectively, and the reverse primer (+56 to +40). Amplified fragments were digested with BglII/KpnI and ligated upstream of the luciferase gene in the pXPIII plasmid (31). Nucleotide positions are numbered relative to the transcription initiation site as described by Rahuel and colleagues (48,49). *GPA* promoter point mutations were generated by three step PCR and resulted in the nucleotide substitutions indicated in Figure 3B. Vectors encoding GST-GATA-1, GST-LMO2, and GST-SCL were generated by cloning PCR amplified cDNAs into

the pGex2T plasmid (Amersham Pharmacia Biotech, Piscataway, NJ), while the origin of the GST-Sp1 vector was described previously (31). All vectors were verified by sequencing.

Cells and retroviral infections

TF-1 cells were cultured as described previously (30). NIH 3T3 and BOSC23 cells were maintained in IMDM (GIBCO invitrogen corporation, Burlington, ON) containing 10% fetal calf serum (FCS) (GIBCO invitrogen corporation). For TF-1 cell infections presented in Figure 4.1B-C, high titer viruses encoding SCL or AS-SCL were produced by transient transfection into Bing cells formerly detailed (30), which were irradiated and co-cultured with TF-1 cells for 24 hours in the presence of 8μg/ml of polybrene. Otherwise, the viruses encoding SCL, ΔbSCL, SCL-ΔNt, AS-Ldb1, AS-LMO2, FOG, E47-bHLH, and their MSCV control were produced by transfection of the 293 GPG retroviral packaging cell line (42). The viruses were concentrated by ultra-centrifugation and TF-1 cells were then cultured in presence of concentrated viruses for 24 hours in the presence of 8μg/ml of polybrene. Following infections, the cells were recovered and polyclonal populations were analyzed 1 week after selection in G418 at 1 mg/ml. For infections of primary hematopoietic cells, fetal livers from E14.5 embryos were dissected, disaggregated into single cell suspensions and washed in IMDM containing 10% FCS. The cells were then incubated overnight with control (MSCV-YFP) and AS-SCL expressing (AS-SCL-YFP) retroviruses in the presence of 4 μg/ml polybrene. Following infection, cells were washed and cultured in IMDM containing 10% FCS, 1U/ml Erythropoietin

(EPO), 100ng/ml Steel factor (SF) and 50ng/ml IL-3. After 24h, infected cells were analyzed by FACS and RT-PCR as described below.

FACS analysis

TF-1 cell samples were stained with rat anti-human GPA (YTH 89.1, IgM; Serotec, Oxford, UK) in staining buffer (PBS with 2% FCS), followed by a secondary FITC-conjugated goat anti-rat antibody (CALTAG, Burlingame, CA). Fetal liver cells were labeled with a PE-conjugated antibody directed against murine Ter119 (Pharmingen, Mississauga, ON) in staining buffer. FITC, PE and YFP fluorescence was assessed on stained cells using a FACScalibur apparatus (Becton-Dickinson, San Jose, CA). Dead cells were excluded by adding 1μg/ml of propidium iodide prior to detection.

RT-PCR Analysis

Total RNA from TF-1 or fetal liver cell was prepared as detailed previously (25), and was reverse transcribed using Superscript first strand cDNA synthesis system (GIBCO invitrogen corporation). For PCR amplifications, 2 μl of cDNA sample was added to mixtures containing 1 μM of forward (Fw) and reverse (Rv) primers, 20 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 5% dimethyl sulfoxide [DMSO], 0.2 mM deoxynucleoside triphosphate [dNTP], and 1.25 U *Taq* DNA polymerase. Samples were amplified for 18, 20 and 22 cycles (94°C for 30s, 55°C for 30s and 72°C for 30s), and PCR products were migrated on a 1.5% agarose gel, transferred on nylon membranes, and hybridized with internal (Int) probes. The blots were

exposed to a PhosphoImager screen (Molecular Dynamics, Sunnyvale, CA). hGPA, mGPA, SCL, and Lysozyme mRNA levels were quantified using the Image Quant software (Molecular Dynamics) and expressed as ratio over hS14 or mS16 signals. Oligonucleotides used for amplifications are indicated in Table 1.

Colony assays

Clonal cultures were performed using freshly extracted bone marrow cells from wild type or SIL-SCL transgenic mice (line A(5)3SCL; 2) in 1% methylcellulose, 10% FCS, 200 μg/ml transferrin, 2% bovine serum albumin (BSA), 5ng/ml IL-3, 1 U/ml EPO, 100 ng/ml SF, and 5×10^{-5} M α-monothioglycerol. Colony formation was monitored at appropriate times and day 7 mixed hematopoietic colonies were picked, washed in PBS and subjected to RNA extraction and RT-PCR.

Transfections and nuclear extracts

Transactivation assays were performed essentially as previously described (31). NIH 3T3 cells were transfected using calcium phosphate 24 hours after plating 30 000 cells/well in 12-well culture plates. GPA reporter contructs were kept at 1.5 μg/well, while 100 ng/well of CMV-β-galactosidase (CMV-β-gal) was included in each transfection mixture as an internal control for normalization. Expression vector doses are indicated in figure legends and the total DNA was kept constrant at 4.5 μg/well with pGem4 (Promega, Madison, WI). Luciferase and β-gal activities were measured

36 hours post-transfection. All luciferase values were normalized using β-gal values. Results are shown as the mean ± SD of one experiment performed in triplicate and representative of 3 or more independent experiments (depicted in figures).

TF-1 cell nuclear extracts were prepared as previously described (30). For BOSC23 cell extracts, the cells were first plated at 4.2×10^6 and transfected 24 hours later with the expression vectors for LMO2 and Ldb1 (11.25 μg), as well as GATA-1, E47, and SCL or SCL mutants (2.25 μg). 36 hours after transfection, the cells were harvested, washed twice in cold PBS and subjected to nuclear extraction as indicated above.

Gel shift, pull-down, and chromatin immunoprecipitation (ChIP) assays

Binding reactions were performed at room temperature for 15 min in the presence of 0.5 μg of poly(dI-dC) in 20 mM HEPES(pH7.5), 50 mM KCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 10 μg of BSA, 15 000 cpm of double-stranded probe and 1-20 μg of TF-1 or BOSC23 cell nuclear extract in a total volume of 20 μl. The sequences of the GPA-84 probe and mutant promoter fragments used for competition experiments are indicated in Figure 3B. For antibody supershift assays, 1ug of the following affinity purified antisera were used: goat anti-GATA-1 (M20), mouse anti-E2A (YAE), rabbit anti-E47 (N-649), goat anti-Ldb1/CLIM-2 (N-18), and rabbit anti-Sp1 (PEP-2) antibodies, all from Santa Cruz Biotechnology Inc (Santa-Cruz, CA). The BTL73 mouse anti-SCL antibody was kindly provided by Dr. D. Mathieu (Institut de Génétique Moléculaire, Montpellier, France). As a control, equal

amounts of species matched serum immunoglobulins (Ig) (Sigma, St-Louis, MS) were added to the binding reactions. Protein complexes were resolved by electrophoresis at 150V on 4% polyacrylamide gel in 0.5× TBE at 4°C.

For pull-down assays, GST, GST-SCL, GST-LMO2, GST-GATA-1, and GST-Sp1 were purified from bacteria and coupled to glutathione Sepharose beads (Amersham Pharmacia Biotech). SCL, GATA-1, LMO2, Ldb1, and luciferase were in vitro translated in presence of ^{35}S-methionine using the TnT coupled reticulocyte lysate system (Promega). Labeled proteins (15 µl) were incubated with 2 µg of immobilized GST fusion proteins in 400 µL binding buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 1% Nonidet P-40 [NP-40], 5 mM DTT, 10% glycerol and 200 µg/ml of ethidium bromide) for 2 hours at 4°C with agitation and then centrifuged for 1 minute at 3000 rpm. Samples were washed 3 times with binding buffer, resolved by SDS-PAGE, transferred on PVDF membranes and visualized and quantified using Image Quant (Molecular Dynamics).

Chromatin immunoprecipitation (ChIP) assays were performed essentially as described previously (31,59). Briefly, exponentially growing TF-1 cells were fixed by incubation with formaldehyde (1% final) for 10 minutes at room temperature. After quenching formaldehyde with glycine (0.125 M final), cells were sequentially washed and sonicated to make chromatin extracts ranging in size from 500 to 1000 bp as formerly detailed (31). Protein concentration was determined by Bradford

staining and 500 µg of chromatin extract was incubated overnight at 4°C with specific antisera against SCL and its partners or control immunoglobulins, which are described above. An aliquot of chromatin extract was kept for isolation of input DNA. Samples were then precipitated by addition of Pansorbin cells (Calbiochem, San Diego, CA) for 30 minutes at 4°C. Precipitated chromatin samples were sequentially washed and eluted as previously detailed (31), and incubated overnight at 65°C to reverse cross linking. After RNA and proteins were degraded using 30 µg of RNAse A for 30 minutes at 37°C and 120 µg of proteinase K for 2-3 hours at 37°C, DNA was phenol/chloroform extracted and precipitated with ethanol in the presence of 10 µg of tRNA as carrier. PCR reactions were then performed on precipitated samples as described previously (31). PCR products were migrated on a 1.5% agrose gel, transfered to nylon membranes, and hybridized with internal oligonucleotide probes. Oligonucleotides are indicated in Table 1.

Table 4.1. Oligonucleotides used for RT-PCR and ChIP analysis

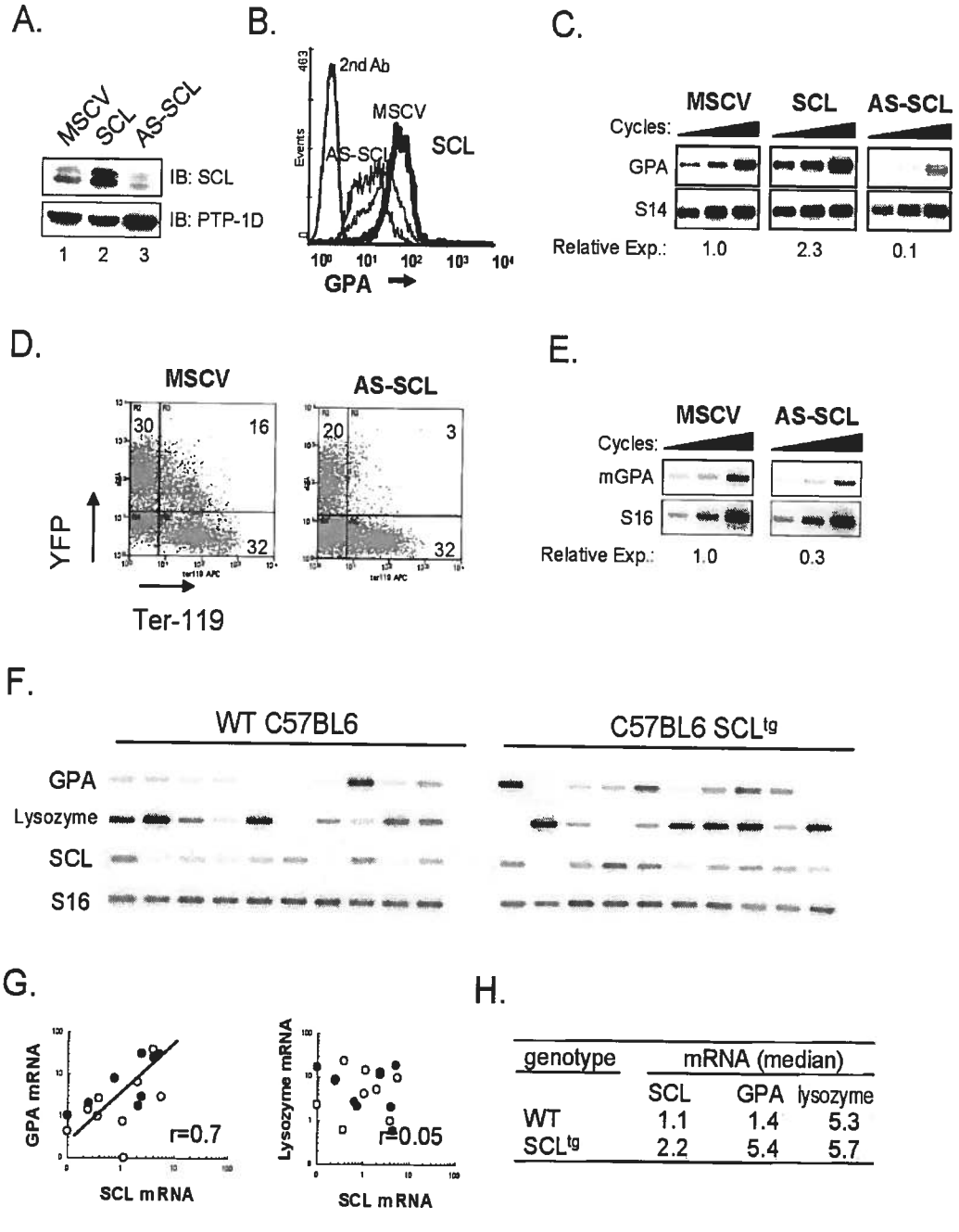
Oligonucleotide	Sequence		
	Forward	Reverse	Internal
Human GPA	ATTGTCAGCAATTGTGAGCATA	TGATCACTTGTCTCTGGATTTT	ATATGCAGCCACTCCTAGAGC
Human S14	GGCAGACCGAGATGATACCTCA	CAGGTCCAGGGGTCTTGGTCC	GACCTGGGTATCACCGCCCT
Murine GpA	ATATGAATTCCTGGGAAGGATGCTTTGC	ATATGGATCCTCCACTGCAAGGAAAGGG	GGAAGTTGCTTTCTTGAATA
Murine S16	AGGAGCGATTTGCTGGTGTGA	GCTACCAGGGCCTTTGAGATG	AAATTTATGCCATCCGACAGTC
Lysozyme	CCAAGGTCTATGAACGTTGTGA	TGCCATCATTACACCAGTATCG	GTCAGCCTGGCCGACTGTAAGTCTC
SCL	ATTGATGTACTTCATGGCAAGG	TCCCCATATGAGATGGAGATTT	TGGAGATTTCTGATGGTCCTCACACCAAA
GPA-ChIP	ATTAGGTACCTCCATGTATCTTTATT	TTAAAGATCTCCTGAGATCATGAGCT	CCCCTGCCTATCAGCTGATGATGGCC
HPRT-ChIP	TGAGGCAAAAATAGAGGCTCA	TCCCAAGACCTTGCACTACC	TGTACAAAACTACAGAGCAG

4.4 Results

GPA expression is dependent on the levels of SCL

We previously demonstrated that over expression of SCL in TF-1 cells increases the expression of *GPA* (26). In order to clarify the mechanism through which SCL regulates gene expression within the erythroid lineage, we performed experiments to address whether *GPA* might be a downstream target of SCL. We first utilized retroviral mediated gene transfer to increase or decrease SCL levels in TF-1 cells. Retroviruses containing an empty MSCV-neo genome, or encoding either SCL or an antisense RNA of SCL (AS-SCL) were generated and used to infect exponentially growing TF-1 cells. Compared to mock infected TF-1 cells (Figure 4.1A, lane 1), SCL protein levels were increased by 2.3-fold in SCL infected cells (lane 2), whereas it was diminished by 5-fold in cells expressing AS-SCL (lane 3). In order to address the question whether *GPA* expression depends on SCL levels, we monitored GPA protein and mRNA expression by flow cytometry and RT-PCR analysis, respectively. Cells over expressing SCL exhibited an increase in cell surface expression of GPA, as shown by labeling with an anti-GPA antibody (Figure 4.1B, thick line). Increased *GPA* expression occurred at the transcriptional level, since GPA mRNA levels were 2-fold higher following ectopic SCL expression (Figure 4.1C). Conversely, decreased SCL protein in AS-SCL infected cells, was associated with a severe 10-fold reduction in GPA protein and mRNA expression compared to mock infected cells (Figure 4.1B and 1C).

Figure 4.1. SCL levels determine *GPA* expression. (A) Immunoblotting with an anti-SCL antibody (upper panel) shows the expression of SCL in TF-1 cells transduced with control retroviruses (MSCV), or viruses encoding SCL or AS-SCL. The blots were stripped and reprobed with an antibody directed against the PTP-1D phosphatase (lower panel) as a loading control. (B) GPA and SCL levels correlate in erythroid cells. The indicated TF-1 transfectants were analyzed by FACS for GPA expression (thick line, SCL transfectant; dotted line, MSCV empty vector; black line: AS-SCL transfectant). Cells labeled with the secondary antibody alone (2^{nd} Ab) were included as negative controls for staining. For simplicity, the 2^{nd} Ab is shown for MSCV control cells only and is representative of 2^{nd} Ab labeling obtained with the SCL and AS-SCL transfectants. (C) GPA mRNA expression in TF-1 cell transfectants was determined by RT-PCR analysis following 18, 20 and 22 cycles of amplification. S14 was used as a normalization control. Amplified fragments were revealed through hybridization with internal oligonucleotide probes. Following normalization with S14, the relative expression of *GPA* was quantified for each transfectant compared to the MSCV control, which was set at 1.0. (D) Expression of an AS-SCL in fetal liver cells decreases of Ter119 labeling. Fetal liver cells were infected with the control YFP and YFP-AS-SCL retroviruses and analyzed by FACS for the expression of YFP and Ter119. (E) Reduced GPA mRNA levels in AS-SCL expressing fetal liver cells. Aliquots of YFP^+ cells infected in (D) were subjected to RT-PCR to measure endogenous *GPA* expression. S16 was used as a control for the amount of cDNA. The relative expression of GPA mRNA was quantified as in (C) following normalization with S16 signals. (E) Increased *GPA* expression in pluripotent colonies from SCL transgenic mice. RT-PCR analysis was performed on pluripotent colonies (CFU-GEMM) derived from WT and SCL transgenic (SCL^{tg}) mice (2). (F) Correlation between GpA and SCL mRNA levels. Data shown in Figure 1E were quantified using the ImageQuant software, and expressed as ratio over S16 controls (○: distribution from WT colonies; ●: SCL^{tg} colonies). The coefficient of correlation between SCL and GPA mRNA levels (r) is shown. There was no correlation with lysozyme mRNA levels (r=0.05). (G) Increased GPA and SCL levels in colony cells from SCLtg mice. Data are the median of the distributions shown in panel F for mRNA expression in individual colonies from bone marrow cells of SCL^{tg} (●) and age-matched control mice (○).



genotype	mRNA (median)		
	SCL	GPA	lysozyme
WT	1.1	1.4	5.3
SCL^{tg}	2.2	5.4	5.7

To determine whether this correlation was also observed in primary hematopoietic cells, we next infected primary E14.5 fetal liver cells with control (MSCV-YFP) and AS-SCL (AS-SCL-YFP) expressing retroviruses, which also encode the fluorogenic protein YFP, allowing for analysis of infected cells in the YFP^+ fraction. It has recently been demonstrated that the Ter119 antibody, which specifically labels cells of the erythroid lineage, recognizes an epitope on mouse GPA (5). Flow cytometric analysis of viable fetal liver cells following gene transfer revealed a dramatic reduction in Ter119 reactivity in the YFP^+ fraction of AS-SCL infected cells compared to control samples, as the percentage of $Ter119^+$ cells drops from 16 % to 3 % (Figure 4.1D). In contrast, the proportion of Ter119 reactive cells in the uninfected (YFP^-) was 32% for both AS-SCL and control MSCV cells. In AS-SCL infected cells, this reduction in Ter119 labeling is concomitant with a 3-fold decrease in GPA mRNA expression as determined by RT-PCR analysis (Figure 4.1E). Together, these results demonstrate a close correlation in SCL and GPA levels in an erythroid progenitor cell line (TF-1) and in primary fetal liver erythrocytes. In both cell types, the level of GPA per cell decreases when SCL levels are lowered, as flow cytometry analysis reveals fluorescence signals at the single cell level.

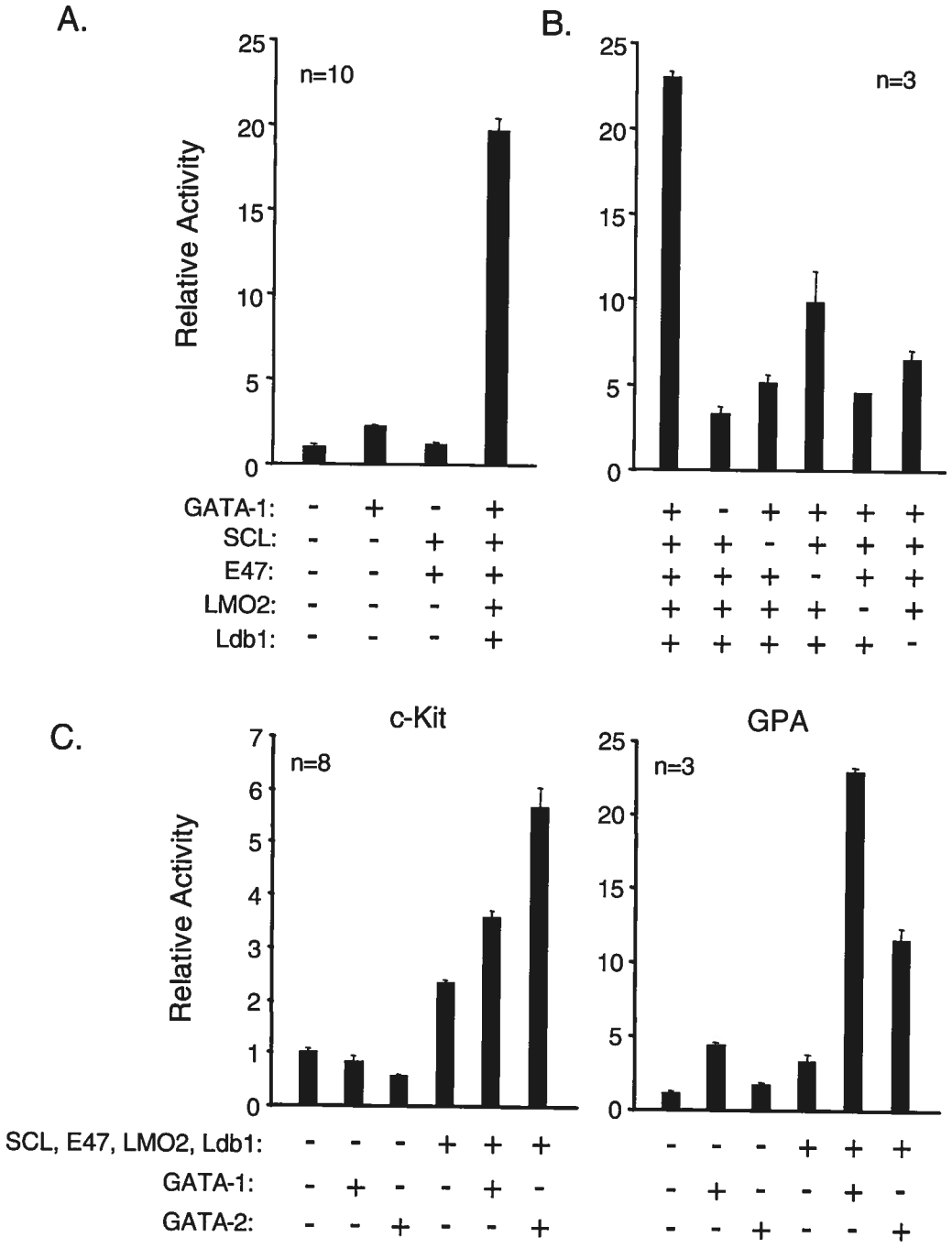
To assess whether *GPA* expression in primary hematopoietic cells increases following SCL gain of function, we next analyzed *SCL* and *GPA* expression in multipotent colonies (CFU-GEMM) derived from bone marrows of wild type or SIL-SCL transgenic mice (SCL^{tg}), which express SCL ubiquitously (2). Total RNA was extracted from individually harvested multipotent colonies and gene expression was

assessed by RT-PCR analysis. Gene expression within single multipotent colonies was normalized on the basis of the control S16 mRNA (Figures 4.1F) and quantified as ratio over S16 (Figure 4.1G). This analysis revealed a linear relationship between the expression levels of SCL and *GPA* in individual colonies from both wild type and SCL^{tg} bone marrows, with a correlation coefficient of 0.7. Furthermore, in colonies from SCL^{tg} mice, which exhibit on average a two-fold increased level of SCL, *GPA* expression was increased four-fold , whereas the myeloid marker lysozyme remained constant (Figure 4.1H). Together, data shown in Figure 1 indicate a close correlation between SCL and GPA levels in the TF1 cell line and in primary hematopoietic cells.

The *GPA* promoter is activated by a complex containing SCL, E47, LMO2, Ldb1 and GATA-1

Increased *GPA* expression in SCL^{tg} colonies might be due to a direct effect of the SCL transgene on *GPA* expression or to an indirect increase in the erythroid content of each colony. To address the question whether *GPA* is a direct target gene of SCL, we performed transactivation assays in heterologous NIH 3T3 cells using a reporter vector in which the *GPA* promoter region, from positions -456 to +56, was inserted in front of the luciferase gene (GPA-456). Using this assay, we previously demonstrated that the regulation of *c-kit* promoter sequences by SCL requires its integration within a multifactorial complex (SCL complex) containing E47, LMO2, Ldb1, and GATA-1/-2 (31). When transfected on its own, the GPA-456 reporter

Figure 4.2. A complex containing SCL, E47, LMO2, Ldb1 and GATA-1 (SCL complex) activates the *GPA* promoter. (A) The *GPA* promoter is synergistically activated by SCL, E47, LMO2, Ldb1 and GATA-1. NIH 3T3 cells were transfected with the GPA-456 reporter (1500 ng) and the indicated expression vectors: SCL, E47 and GATA-1 (150 ng); LMO2 and Ldb1 (750 ng). (B) Each factor of the SCL complex is required for full *GPA* promoter activation. (C) Complexes containing GATA-1 or GATA-2 show varying transactivation efficiency depending on the target promoter. NIH 3T3 cells were transfected with the *c-kit* (left panel) or *GPA* (right panel) reporter constructs and the indicated complexes containing either GATA-1 or GATA-2. For A-C, (+) and (-) indicates inclusion or omission of specific expression vectors. In all transfections, the total amount of transfected DNA was kept constant at 4.5 μg using pGem4. Results are shown as luciferase activity relative to the reporter vector transfected alone and represent the average ± SD of triplicate determinations and are representative of (n) independent experiments. Luciferase reporter activities were normalized to that of an internal control (CMV-β-gal, 100 ng).


A.
B.
C.
n=10
n=3
n=8
n=3
Relative Activity
c-Kit
GPA
GATA-1:
SCL:
E47:
LMO2:
Ldb1:
SCL, E47, LMO2, Ldb1:
GATA-1:
GATA-2:

shows a low background of luciferase expression comparable to the promoter-less reporter pXPIII (data not shown). GATA-1 could activate GPA-456 on its own by 3- to 4-fold (Figure 4.2A), consistent with a previous report on the glycophorin B (*GPB*) promoter, which is highly homologous to the *GPA* promoter (49). In contrast, expression of SCL and E47 had no effect on promoter activity. Strikingly, when we co-expressed SCL with E47, LMO2, Ldb1 and GATA-1, the GPA promoter was synergistically activated by 20- to 25-fold over its basal level (Figure 4.2A and 2B). Omission of either one of the expression vectors from the transfection mixtures severely reduced promoter activation, demonstrating that each partner was required for synergistic transactivation (Figure 4.2B). These results suggest that SCL and its partners regulate *GPA* expression through direct activation of the proximal *GPA* promoter.

Both GATA-1 and GATA-2 can associate in complexes with SCL, although it is not known whether distinct complexes show differences in target gene specificity. GATA-1 is a master regulator of erythroid development (69), while GATA-2 plays a crucial role in maintaining a normal pool of hematopoietic progenitor cells (60). Therefore, we tested whether complexes containing either GATA-1 or GATA-2 showed differences in specificity in activating progenitor or erythroid cell targets of the SCL-complex, i.e. *c-kit* and *GPA*, respectively. In the context of the *c-kit* promoter, we found that activation was more efficient with GATA-2 containing complexes compared to those with GATA-1 (Figure 4.2C, left panel) (31). On the

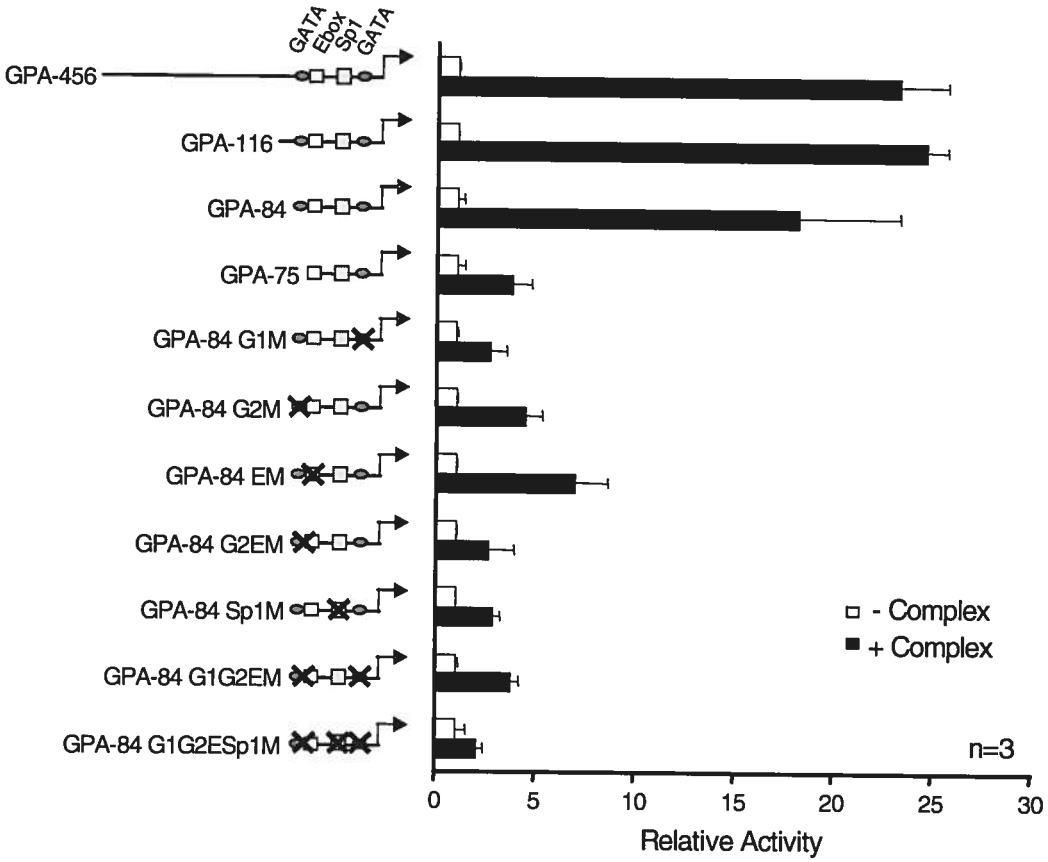
contrary, on the *GPA* promoter, with the same amount of GATA expression vector (150 ng) as in the *c-kit* promoter analysis, we found that GATA-1 containing complexes were much more efficient than GATA-2 complexes (Figure 4.2C, right panel). This functional specificity concurs with the known biological functions of these two GATA factors.

The SCL complex activates the *GPA* promoter through an Ebox motif, two GATA binding sites and a Sp1 biding site

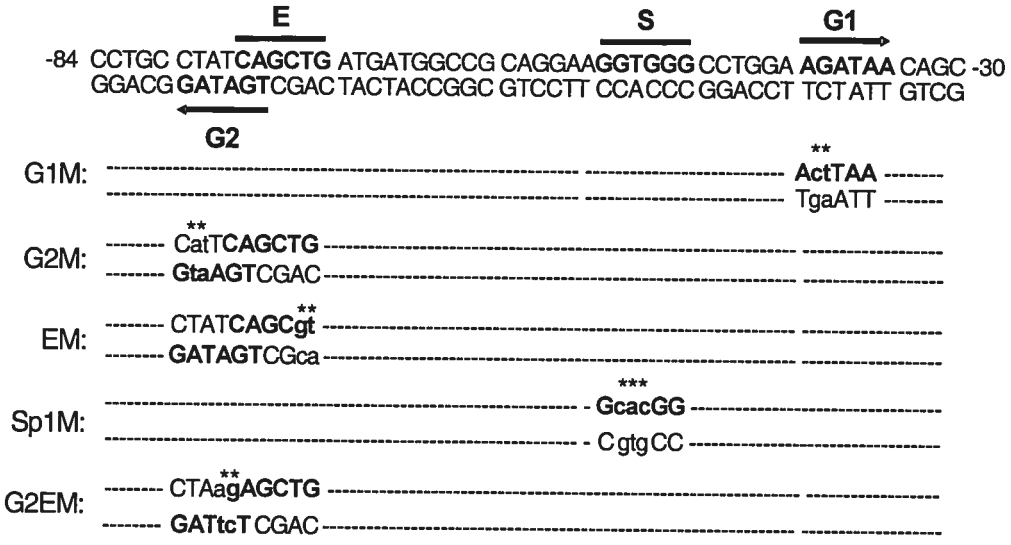
We next sought to identify the *cis* elements that were required to recruit the SCL complex to the *GPA* promoter. We first generated a series of *GPA* promoter 5' deletion mutants in order to identify the minimal promoter sequence that remained maximally activated by the SCL complex. As shown in Figure 4.3A, a promoter segment deleted up to position –84 (GPA-84) was still maximally activated, while further deletion up to position –75 (GPA-75) resulted in a dramatic decrease in activation. Segments of the proximal *GPA* and *GPB* promoters were previously shown to contain sequences necessary for erythroid-specific expression (48,49; data not shown). These studies had highlighted the presence of functionally important GATA motifs at positions –36 (G1) and –74 (G2) and an Sp1 binding site at –48 (Sp1) (Figure 4.3B). An Ebox sequence at position –70 (E) was also previously characterized, although its involvement in erythroid specific expression of the glycophorin promoters remained unclear (10). The E and G2 elements are overlapping between positions –79 and –70 of the promoter, the G2 site being

Figure 4.3. *cis* elements required for *GPA* promoter activation by SCL and its partners. (A) Deletion or point mutations in two GATA sites (G1 at position –36 and G2 at -74), an Sp1 motif (S at position -48) and an Ebox element (E at position -70) impair activation by the SCL-complex. NIH 3T3 cells were transfected with mutant *GPA* reporter constructs in the absence (open bars) or the presence (solid bars) of the SCL complex. Results are shown as luciferase activity relative to each reporter vector transfected alone; luciferase activities were normalized by co-transfection of CMV-β-gal and are representative of (n) independent experiments. The basal promoter activity of the mutant *GPA* reporters was identical to GPA-456 (on average 1000 normalized RLU, which is comparable to the empty pXPIII vector). (B) The *GPA* promoter sequence from positions -84 to -30 is indicated. The G1, G2, S, and E sites are indicated, and the mutations that were introduced into these motifs are designated by asterisks. Note that the G2 and E elements partially overlap on opposite strands.

A.


GATA
EBox
Sp1
GATA
GPA-456
GPA-116
GPA-84
GPA-75
GPA-84 G1M
GPA-84 G2M
GPA-84 EM
GPA-84 G2EM
GPA-84 Sp1M
GPA-84 G1G2EM
GPA-84 G1G2ESp1M
□ - Complex
■ + Complex
n=3
0
5
10
15
20
25
30
Relative Activity

B.


E
S
G1
-84 CCTGC CTATCAGCTG ATGATGGCCG CAGGAAGGTGGG CCTGGA AGATAA CAGC -30
GGACG GATAGTCGAC TACTACCGGC GTCCTT CCACCC GGACCT TCTATT GTCG
G2
G1M:
ActTAA
TgaATT
G2M:
CatTCAGCTG
GtaAGTCGAC
EM:
CTATCAGCgt
GATAGTCGca
Sp1M:
GcacGG
CgtgCC
G2EM:
CTAagAGCTG
GATtcTCGAC

arranged in opposite orientation relative to the *GPA* gene (Figure 4.3B). In the GPA-75 construct, which shows an severe decrease in activation, both of these motifs were affected by the deletion demonstrating that they are important for responsiveness to the SCL complex (Figure 4.3A). In order to address whether the G1, G2, Sp1 and E elements were required for activation by the SCL c1omplex, point mutations were introduced into these motifs as indicated in Figure 4.3B. Promoter activation was greatly reduced when mutations were introduced into each of these elements (G1M, G2M, EM and Sp1M), and was completely abolished when all of the sites were simultaneously mutated (Figure 4.3A). The requirement for an Sp1 binding site for promoter activation by the SCL complex is not unexpected, since we have previously demonstrated that Sp1 helps to recruit the SCL complex to the proximal *c-kit* promoter in hematopoietic progenitor cells (31). These results demonstrate that several *cis* elements collaborate to confer transcription activation by the SCL complex to the *GPA* promoter.

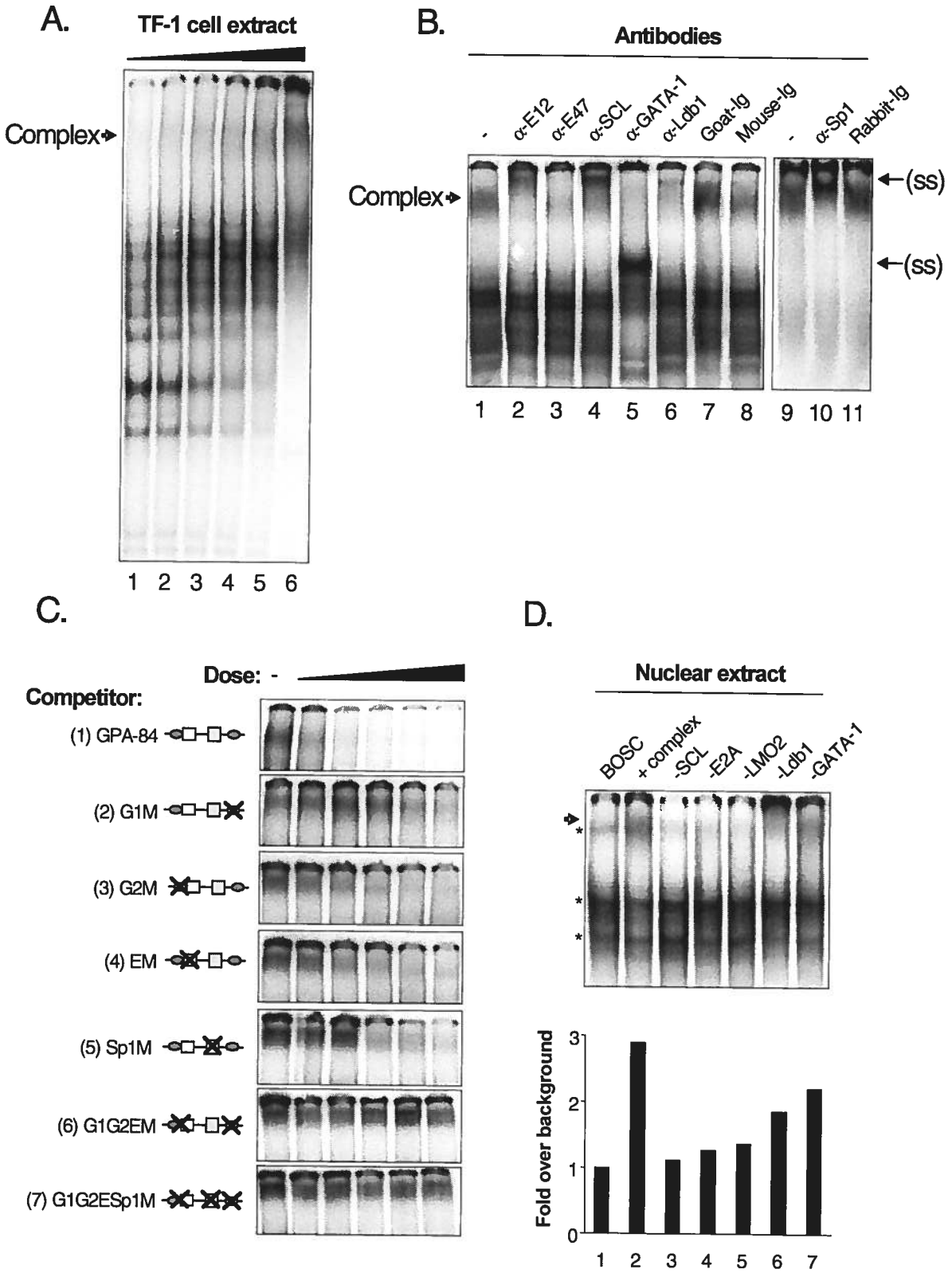
SCL and its partners form a low mobility complex on the *GPA* promoter

We next addressed the question whether the SCL complex assembles on the *GPA* proximal promoter sequence. For this we performed electrophoretic mobility shift assays (EMSA) using the GPA-84 probe, which spans positions –84 to –32 of the *GPA* promoter (Figure 4.3B). We initially performed a titration experiment, in which varying concentrations of TF-1 cell nuclear extracts were incubated with the GPA-84 probe, and found that a very low mobility complex was formed on this probe at

higher concentrations of protein extract (Figure 4.4A, lanes 1-6). This slowly migrating complex was not observed on probes containing the G1 and G2E motifs alone (data not shown). Addition of specific antibodies against SCL, E2A, Ldb1, GATA-1, and Sp1 super-shifted or disrupted the migration of the low mobility complex (Figure 4.4B, lanes 2-6, 10), whereas control immunoglobulins had no effect (lanes 7-8, 11). This demonstrates that the SCL complex can indeed directly associate with the *GPA* promoter in vitro.

To assess the contribution of the G1, G2, Sp1 and E box elements for binding of the SCL complex, we next compared the ability of wild type and mutant *GPA* promoter fragments to compete the binding of the SCL complex to the GPA-84 probe. Competitor fragments were titrated into the binding reactions in amounts ranging from 3- to 300-fold molar excess relative to the ^{32}P-labeled GPA-84 probe. The wild type competitor efficiently displaced the binding of the complex (Figure 4.4C, panel 1). In contrast, competitor fragments mutated in the proximal GATA site (G1M) was 30-fold less efficient (panel 2) while mutations in the G2, E and Sp1 motifs (panels 3-5) were 10-fold less efficient compared to the wild-type competitor. As expected, combined mutations in these *cis* elements completely abolished the ability of the promoter fragments to compete the binding of the SCL complex to the GPA-84 probe (panels 6 and 7). Therefore, in addition to being required for *GPA* promoter activation, the G1, G2, E and Sp1 motifs are also required for direct binding of the SCL complex to the *GPA* promoter.

Figure 4.4. The SCL complex associates with the *GPA* promoter *in vitro*. (A) A low mobility complex from TF-1 cell nuclear extracts forms on the *GPA* promoter. EMSA were performed using the GPA-84 probe (-84 to -30) and increasing amounts of TF-1 cell nuclear extracts (1-20μg). (B) SCL and its partners form a low mobility complex on the GPA–84 promoter sequence. Supershift assays were performed using the antibodies against partners of the SCL complex (lanes 1-6 and 10) or control species matched antiserum (lanes 7-8 and 11). The binding reaction was performed with 10 μg (lanes 1-8) or 20 μg (lanes 9-11) of TF-1 nuclear extracts. (C) Binding of the SCL complex requires the G1, G2, S and E motifs. The indicated competitor fragments were titrated into binding reactions at 3-, 10-, 30-, 100- and 300-fold molar excess compared to the labeled GPA-84 probe. (D) Each partner is required to form a complex on the *GPA* promoter. EMSA were performed with the GPA-84 (lanes 1-7) and nuclear extracts of untransfected BOSC cells (lane 1) or cells transfected with SCL and its partners (lane 2-7). Where indicated (-), particular expression vectors were omitted from the transfection mixtures (lanes 3-7). The asterisks point to complexes formed in control untransfected BOSC23 cells. The arrow heads to the left point to the SCL complex, and the arrows to the right, to the supershifted complexes (ss). The binding intensities of the SCL complexes were quantified using the Image Quant software.


A.
TF-1 cell extract
Complex
1 2 3 4 5 6
B.
Antibodies
- α-E12 α-E47 α-SCL α-GATA-1 α-Ldb1 Goat-Ig Mouse-Ig - α-Sp1 Rabbit-Ig
Complex
←(ss)
←(ss)
1 2 3 4 5 6 7 8 9 10 11
C.
Dose: -
Competitor:
(1) GPA-84
(2) G1M
(3) G2M
(4) EM
(5) Sp1M
(6) G1G2EM
(7) G1G2ESp1M
D.
Nuclear extract
BOSC + complex -SCL -E2A -LMO2 -Ldb1 -GATA-1
Fold over background
0
1
2
3
1 2 3 4 5 6 7

EMSA with TF-1 cell nuclear extracts allowed us to demonstrate that the SCL complex associates with the *GPA* promoter. To assess whether SCL and its partners are necessary and sufficient to form a complex on the *GPA* promoter, we next sought to reconstitute the complex by ectopic expression in heterologous cells. When EMSA were performed with the GPA-84 probe and nuclear extracts of BOSC23 cells transfected with SCL and its partners, we observed the appearance of a low mobility complex that was distinct from the background seen with untransfected BOSC23 extracts (Figure 4.4D, lanes 1-2). In order to determine whether each partner was required for complex formation, we prepared extracts of BOSC23 cells in which the expression vector for each factor was sequentially omitted from the transfection mixtures. We found that subtracting either partner compromised the formation of the complex on the GPA-84 probe (lanes 3-7). Together, these results indicate that all SCL partners are required to form a complex on the *GPA* promoter, consistent with their crucial contribution in promoter transactivation.

Partners of the SCL complex occupy the *GPA* promoter in hematopoietic cells

To address the question of whether SCL and its partners indeed associate with the *GPA* promoter in vivo in hematopoietic cells, we next performed chromatin immunoprecipitation assays with TF-1 cell chromatin extracts (31). Exponentially growing TF-1 cells were treated with formaldehyde and fragmented chromatin was then subjected to immunoprecipitation with antibodies directed against SCL and its partners. Following immunoprecipitation, cross-linking was reversed and associated

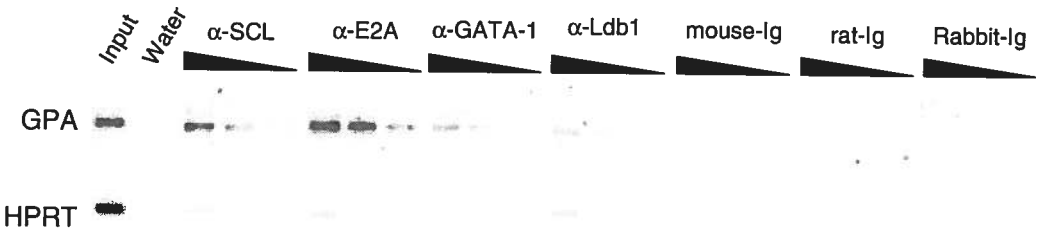


Figure 4.5. Partners of the SCL complex associate with the *GPA* promoter *in vivo*. TF-1 cell chromatin extracts were subjected to immunoprecipiation with α-SCL, α-E2A, α-GATA-1 and α-Ldb1 antibodies and control immunoglogulins. Precipitated chromatin was heated overnight at 65°C to reverse cross-linking and DNA molecules were purified and subjected to PCR analysis to test for the presence of the *GPA* and *HPRT* promoter sequences. Input chromatin represents 1.25 % of the amount used in each immunoprecipitation and three fivefold serial dilutions of the immunoprecipitated samples were used for amplification. Following electrophoresis and transfer, PCR fragments were hybridized with an internal oligonucleotide probe.

DNA fragments were purified, serially diluted and subjected to PCR with primers specific for the *GPA* promoter region, as well as the promoter segment of the ubiquitously expressed HPRT gene as a control. To confirm the specificity of amplification, fragments were hybridized with ^{32}P-labeled internal oligonucleotide probes. We found that antibodies against SCL, E2A, and GATA-1 were able to precipitate the proximal *GPA* promoter 10- to 15-fold more efficiently than the *HPRT* promoter region, whereas control immunoglobulins did not bring down these sequences (Figure 4.5). In contrast, immunoprecipitation with the anti-Ldb1 antibody showed a modest 2-fold enrichment of the *GPA* promoter compared to *HPRT*. Since Ldb1 is a ubiquitously expressed, it is possible that this factor regulates the expression of both tissue-specific and more widely expressed genes. Nevertheless, these results demonstrate that partners of the SCL complex directly and specifically associate with the *GPA* promoter in hematopoietic cells.

Multiple interactions between partners of the complex

Multiprotein complexes formed on DNA are stabilized by protein-protein interactions. To define the network of physical interactions that occur between SCL and its partners, we next performed in vitro pull-down assays. Bacterially produced fusion proteins GST-GATA-1, GST-LMO2, GST-SCL, and GST-Sp1 were immobilized on Sepharose beads and incubated with ^{35}S-methionine labeled SCL, Ldb1, LMO2, and GATA-1. The binding reactions were performed in the presence of a high concentration of ethidium bromide (200 μg/mL) to ensure that contaminant

DNA molecules did not indirectly bridge protein interactions. Columns containing GST alone and reactions with ^{35}S-labeled Luciferase (Figure 4.6, lanes 25-30) were included as negative controls. In addition to confirming previously documented interactions, novel homo- and hetero-typic interactions were observed between partners of the complex. SCL was found to interact efficiently with LMO2, Sp1 and with itself (lanes 4-6 and 17), with binding intensities of 4-6% of input, whereas it did not interact with GATA-1 (lanes 3 and 23). Indeed, the interaction of SCL with LMO2 and Sp1 has been previously documented and SCL has also been shown to interact with itself in the yeast two-hybrid system (31,67,71). We also observed that GATA-1 interacted strongly with LMO2 and Sp1 (lanes 15, 22 and 24, 7-14% of input), while it could also associate with itself more weakly (lane 21; 3% of input), as previously described (14,31,35,43). In addition to its strong interaction with GST-LMO2 (lane 10; 5% of input), we found that Ldb1 could also bind to columns containing GST-GATA-1, GST-SCL, and GST-Sp1 (lanes 9, 11 and 12; 3% of input), albeit with lower efficiency. Finally, we identified a novel high efficiency interaction of LMO2 with itself (lane 16; 8% of input). Together, these results demonstrate that there are multiple direct physical interactions between partners of the SCL complex that may serve to stabilize their association into a higher order complex on the *GPA* promoter.

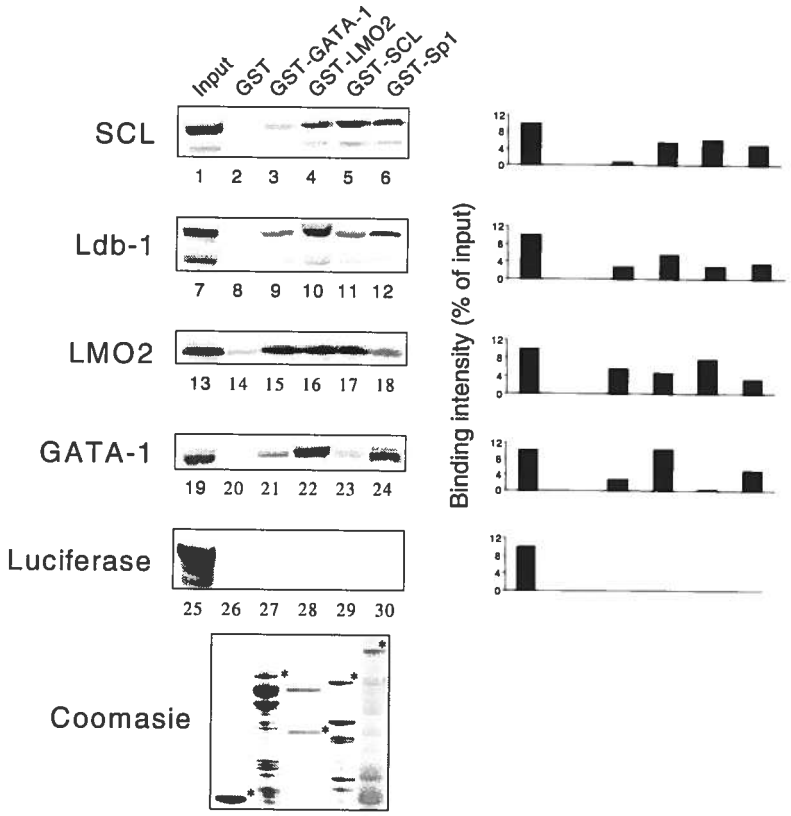
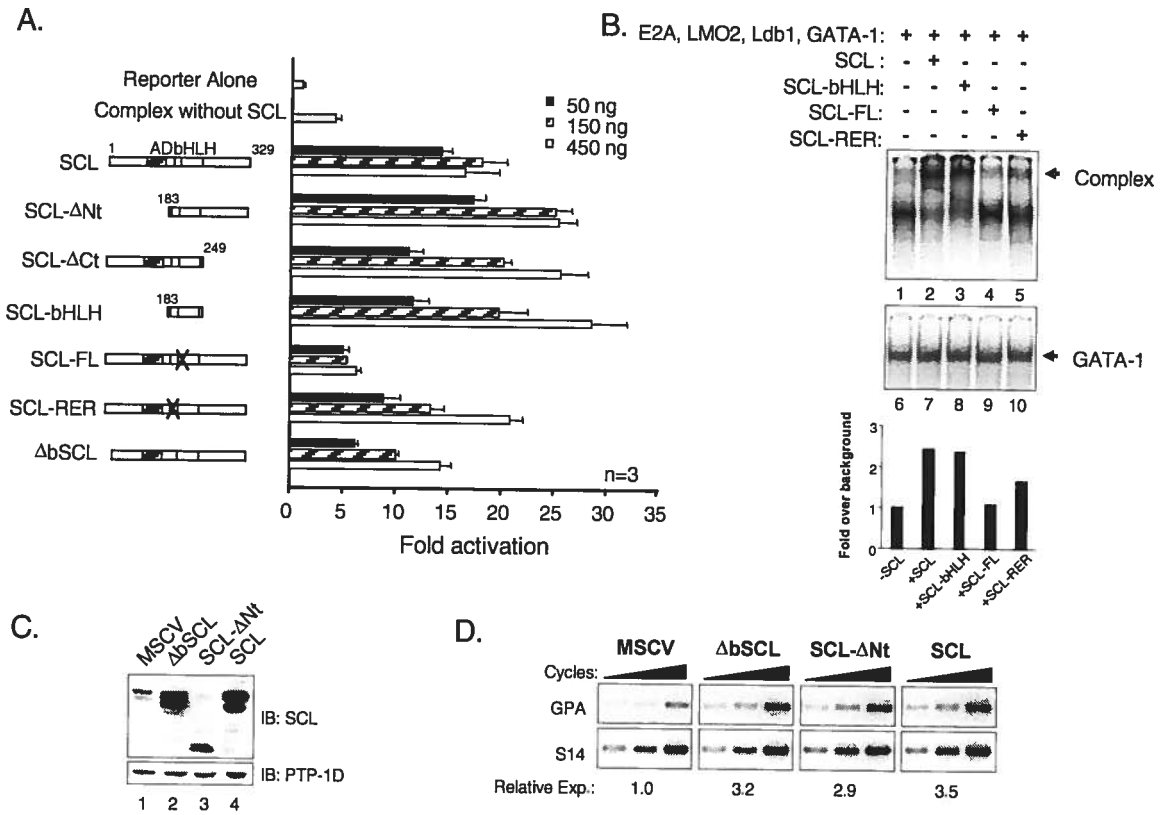


Figure 4.6. Interactions between partners of the SCL complex. Pull-down experiments were performed with immobilized GST, GST-GATA-1, GST-LMO2, GST-SCL and GST-Sp1 as well as ^{35}S-labeled SCL, Ldb1, LMO2, GATA-1 and Luciferase. Protein signals were quantified using the Image Quant software, and the binding efficiency (% of input) was calculated in comparison to input samples (10%) after subtraction of background GST signals. The lower panel shows bacterially expressed GST-fusion proteins, which were resolved by SDS-PAGE and stained with Coomasie blue.

SCL domain requirements for *GPA* gene regulation

Like most transcription factors the SCL protein has a modular structure with a basic DNA binding region, a HLH protein interaction motif, as well as an N-terminal transactivation domain that is absent in shorter SCL isoforms. In order to ascertain which domains of SCL are required for its function, different SCL mutants were tested for their ability to activate the *GPA* promoter in collaboration with the other partners of the complex. As previously observed with the *c-kit* promoter, the bHLH domain of SCL was sufficient for *GPA* promoter activation and a putative N-terminal transactivation domain could be deleted without affecting SCL function in this assay (Figure 4.7A). In addition, the integrity of the HLH domain of SCL was crucial for its function since point mutations in Helix 1 (SCL-FL), which are known to disrupt interactions with E2A and Sp1 (31,44), abolished SCL activity. Since the *GPA* promoter required an Ebox for its full activation by the SCL complex, we tested whether SCL mutants with point mutations (SCL-RER) or a deletion (ΔbSCL) in the basic domain, which render them unable to bind to DNA (31,44), would still be functionally active. These DNA binding defective mutants were less efficient than wild-type SCL at lower doses of expression vector, although they were active at higher doses. These results suggest that DNA binding by SCL is important for maximal *GPA* promoter activation, although it is not essential, which is similar to the intermediate effect on promoter activation that was observed upon mutation of the *GPA* Ebox motif (Figure 4.3A).

Figure 4.7. SCL domain requirements for *GPA* promoter activation and binding. (A) SCL domains required for *GPA* promoter activation. NIH 3T3 cells were co-transfected with the GPA-84 reporter and complexes containing the indicated doses (50-450 ng) of expression vectors encoding SCL point or deletion mutants (44). In all samples, the total amount of transfected DNA was kept constant at 4.5 μg using pGem4. The numbers correspond to amino acid residues of SCL. bHLH = basic Helix-Loop-Helix (open boxes); AD = putative Activation Domain (hatched box); F--L→ A—A = Helix 1 mutations; RER→AAA = basic region mutations, n = number of representative experiments. (B) The bHLH domain of SCL is necessary and sufficient to nucleate a complex on the *GPA* promoter. EMSA were performed with the GPA-84 probe and nuclear extracts of BOSC cells (10μg) transfected with complexes containing wild type or mutant forms of SCL. Arrowhead points to SCL containing complexes. The binding intensities of the complexes were quantified using the Image Quant software. (C) Immunoblotting analysis of extracts of TF-1 cells transduced with control (MSCV) retroviruses, or viruses encoding SCL, ΔbSCL, or SCL-ΔNt. Blots were sequentially hybridized with antibodies indicated on the right of each panel. (D) Induction of endogenous *GPA* expression by SCL mutants. GPA mRNA expression in TF-1 cell transfectants was determined by RT-PCR as described in Figure 1C, following normalization with S16 signals.


A.
Reporter Alone
Complex without SCL
SCL
ADbHLH
1
329
SCL-ΔNt
183
SCL-ΔCt
249
SCL-bHLH
183
SCL-FL
SCL-RER
ΔbSCL
50 ng
150 ng
450 ng
n=3
0 5 10 15 20 25 30 35
Fold activation
B.
E2A, LMO2, Ldb1, GATA-1: + + + + +
SCL : - + - - -
SCL-bHLH: - - + - -
SCL-FL: - - - + -
SCL-RER: - - - - +
Complex
1 2 3 4 5
GATA-1
6 7 8 9 10
Fold over background
-SCL
+SCL
+SCL-bHLH
+SCL-FL
+SCL-RER
C.
MSCV
ΔbSCL
SCL-ΔNt
SCL
IB: SCL
IB: PTP-1D
1 2 3 4
D.
MSCV
ΔbSCL
SCL-ΔNt
SCL
Cycles:
GPA
S14
Relative Exp.: 1.0 3.2 2.9 3.5

We next determined whether the mutants that were tested in our functional assay were able to form complexes on the *GPA* promoter. For this, EMSA were performed with the GPA-84 probe and nuclear extracts of BOSC23 cells expressing different SCL mutants and the other partners of the complex (Figure 4.7B). As predicted from our promoter activation assays, we found that complexes containing full length SCL or only the bHLH domain of SCL were formed efficiently on the *GPA* promoter (Figure 4.7B, lanes 2-3). In contrast, the complex formed with the SCL-FL mutant (lane 4) was comparable to the binding observed when SCL was absent from the extracts (lane 1). Finally, when extracts containing the SCL-RER mutant were tested, we found that complexes were formed with intermediate efficiency (Figure 4.7B, lane 5), being stronger than SCL-FL yet weaker than either wild-type SCL or the bHLH domain of SCL. In control EMSA performed with a probe containing a consensus GATA motif, no variation in GATA DNA binding activity was observed between these samples (lanes 6-10). Together, these findings demonstrate that the integrity of the HLH domain of SCL is crucial for efficient promoter activation and complex formation on *GPA* promoter sequences.

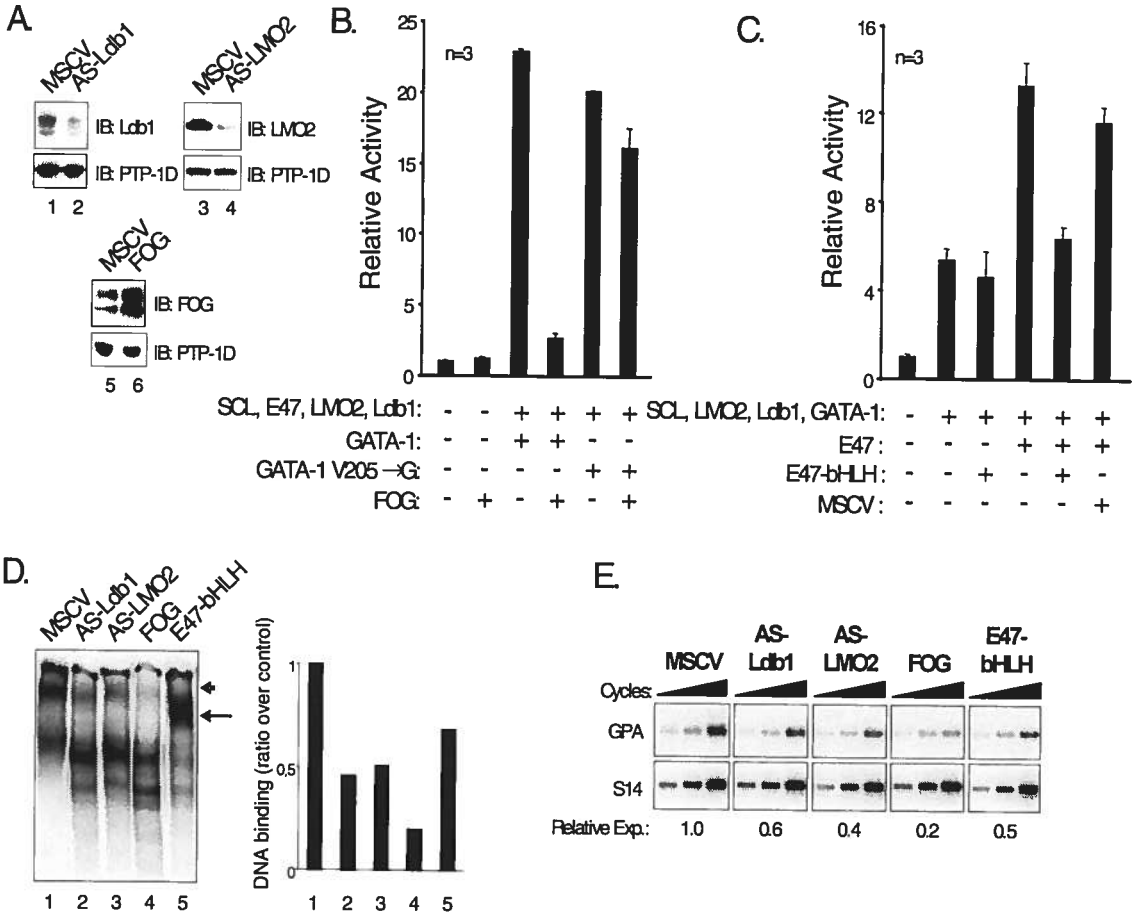
To assess whether the transactivation and DNA-binding domains of SCL were required for the induction of endogenous *GPA* gene expression, we next infected TF-1 cells with viruses encoding SCL, ΔbSCL and SCL-ΔNt, and monitored GPA mRNA expression by RT-PCR analysis. Immunoblotting was first performed to confirm that retroviral mediated gene delivery resulted in efficient over expression of SCL, ΔbSCL and SCL-ΔNt (Figure 4.7C, lanes 2-4), compared to mock infected

cells (lane 1). Interestingly, we found that both ΔbSCL and SCL-ΔNt could induce endogenous *GPA* gene expression at levels that were similar to wild type SCL, from 3- to 3.5-fold (Figure 4.7D). The high levels of expression attained with retroviral infection (Figure 4.7C) may explain why the ΔbSCL is as efficient as wild type SCL, since transactivation assays revealed a difference between the two proteins at low doses but not at high doses (Figure 4.7A). Finally, consistent with transient assays, the integrity of the putative transactivation domain of SCL is dispensable for the induction of endogenous *GPA* gene expression. We therefore conclude that SCL functions mainly as a nucleation factor for a multifactorial complex endowed with a capacity to drive erythroid gene expression.

LMO2, Ldb1, GATA factors and E47 are required for SCL complex assembly on DNA and for *GPA* gene expression in hematopoietic cells.

Transient reporter assays with *GPA* regulatory sequences indicate that a transcriptionally active SCL complex comprises LMO2, Ldb1, E47 and GATA-1. To determine whether the same partners are required for *GPA* expression in chromatin, we next sought to interfere with members of the complex through diverse strategies. For LMO2 and Ldb1, we generated stable TF-1 cells lines exhibiting reduced LMO2 or Ldb1 protein expression through retroviral-mediated delivery of antisense RNA molecules (AS-LMO2 or AS-Ldb1) (Figure 4.8A, lanes 1-4). For GATA factors, our results indicate that both GATA-1 and GATA-2 can contribute to the activity of the SCL complex, although GATA-1 is more active on the *GPA* promoter. TF-1 cells

Figure 4.8. Loss of function of Ldb1 and LMO2, or over expression of FOG or the bHLH domain of E47 leads to a disruption of the SCL complex and to decreased *GPA* activation. (A) Immunoblotting of extracts from control TF-1 cells (MSCV), or cells expressing AS-Ldb1, AS-LMO2 and FOG. Hybridizations were performed with the antibodies indicated on the right of each panel, while detection of PTP-1D served as a loading control. Note the important reduction of Ldb1 and LMO2 expression in AS-Ldb1 and AS-LMO2 cells, and increased FOG expression in FOG infected cells. (B) FOG inhibits *GPA* promoter activation by the SCL complex through specific interaction with GATA-1. Transactivation assays were performed as in Figure 2 using the GPA-84 reporter and mixtures supplemented with the indicated expression vectors (150 ng of GATA-1 or GATA-$1^{V205\rightarrow G}$; 900 ng of FOG). V205→G = point mutation in the N-terminal zinc finger of GATA-1 that disrupts interaction with FOG (13). (C) The bHLH domain of E47 is non-functional and acts as a dominant negative inhibitor of the SCL complex. Transactivation assays were done using the GPA-84 reporter and mixtures containing the indicated expression vectors (150 ng of E47, E47-bHLH or empty MSCV-neo vector). E47-bHLH: bHLH domain of E47 from amino acids 518-610. For B and C, (+) and (-) indicates inclusion or omission of specific expression vectors. For each sample, 100 ng of CMV-β-gal was added as an internal control and the total amount of DNA was kept constant at 4.5μg using pGem4. Results represent the average ± SD of triplicate determinations and are representative of (n) independent experiments. (D) Extracts (10 μg) from TF-1 cells infected with control (MSCV), AS-Ldb1, AS-LMO2, FOG, or E47-bHLH expressing retroviruses were subjected to EMSA with the GPA-84 probe. The arrowhead indicates the usual low mobility SCL complex, whereas the arrow points to a faster migrating complex observed in TF-1 cells expressing the bHLH domain of E47. The binding intensities of the low mobility complexes were quantified using the Image Quant software. (E) Reduced expression of the endogenous *GPA* gene in TF-1 cells expressing AS-Ldb1, AS-LMO2, FOG and E47-bHLH. After normalization with S16 signals, GPA mRNA expression in TF-1 cell infectants was assessed by RT-PCR as described in Figure 1C.


A.
MSCV
AS-Ldb1
IB: Ldb1
IB: PTP-1D
1 2
MSCV
AS-LMO2
IB: LMO2
IB: PTP-1D
3 4
MSCV
FOG
IB: FOG
IB: PTP-1D
5 6
B.
n=3
Relative Activity
SCL, E47, LMO2, Ldb1:
GATA-1:
GATA-1 V205→G:
FOG:
C.
n=3
Relative Activity
SCL, LMO2, Ldb1, GATA-1:
E47:
E47-bHLH:
MSCV:
D.
MSCV
AS-Ldb1
AS-LMO2
FOG
E47-bHLH
1 2 3 4 5
DNA binding (ratio over control)
1 2 3 4 5
E.
MSCV
AS-Ldb1
AS-LMO2
FOG
E47-bHLH
Cycles:
GPA
S14
Relative Exp.: 1.0 0.6 0.4 0.2 0.5

express GATA-1 and -2, and both factors interact with FOG (Friend of GATA) (62), a modulator of GATA activity (20,62). In the context of the *GPA* promoter, our transactivation assay revealed that FOG co-expression drastically reduced *GPA* promoter activation by SCL and its partners (Figure 4.8B). This inhibitory effect of FOG was due to its direct interaction with GATA-1, since the GATA-$1^{V205\rightarrow G}$ mutant, which is deficient for FOG interaction (13), could efficiently replace normal GATA-1 in the SCL complex to activate the *GPA* promoter while conferring resistance to inhibition by FOG (Figure 4.8D). We therefore generated a TF-1 cell line stably expressing FOG, in order to repress both GATA-1 and GATA-2 activities (Figure 4.8A, lanes 5 and 6). Finally, E47 is part of a family of widely expressed proteins that have overlapping functions, comparable to GATA factors. We therefore assessed the activity of a truncated E47 protein, comprising the bHLH domain of E47 (E47-bHLH), within the SCL complex. In sharp contrast with the SCL-bHLH construct, this truncated protein fails to collaborate with other members of the SCL complex in transient assays (Figure 4.8C), indicating that the N-terminal domain of E47 is essential for transcription activation by the SCL complex. Furthermore, this truncated protein was found to be dominant negative over wild type E47 (Figure 4.8C). We therefore stably expressed the truncated E47-bHLH mutant in TF-1 cells.

Since the assembly of a low mobility complex containing SCL and its partners is required for *GPA* activation (Figure 4.4), we first determined the consequences of AS-Ldb1, AS-LMO2, FOG, and E47-bHLH expression on endogenous TF-1 cell complexes by performing gel shift assays with the GPA-84 probe. While a low

mobility complex is revealed in nuclear extracts from parental TF-1 cells (Figure 4.8D, lane 1), decreased LMO2 and Ldb1 protein levels (AS-Ldb1 and AS-LMO2) lead to a reduction in DNA binding by the SCL complex (Figure 4.8D, lanes 1-3). Similarly, FOG over expression severely disrupts complex formation (lane 4), while ectopic expression of E47-bHLH shifts the migration of the DNA bound SCL complex towards higher mobility (lane 5), indicating that this truncated mutant is capable of DNA binding, and that it displaces the endogenous wild type protein from the SCL complex, thus acting as a dominant-negative inhibitor of wild type E47.

Finally, these interventions significantly decreased GPA mRNA expression as assessed by RT-PCR analysis (Figure 4.8E), resulting in a 2-fold reduction when Ldb1 and LMO2 protein levels are decreased, or when E47 binding is displaced by E47-bHLH. Furthermore, there was a 5-fold decrease in GPA mRNA levels when GATA factors are sequestered by FOG. Strikingly, these reductions in GPA mRNA levels closely correlate with the decrease in DNA binding observed by EMSA (compare Figures 4.8D and E). Taken together, these results strengthen the view that LMO2, Ldb1, GATA factors and E proteins (more specifically E47) are important components of SCL-containing complexes and that they are indeed required for *GPA* gene expression in hematopoietic cells.

4.5 Discussion

The present study provides genetic and functional evidence that the erythroid gene *GPA* is a direct target of a multifactorial complex containing SCL, E47, LMO2, Ldb1, GATA-1, and Sp1. Our observations also reveal functional specialization within the complex, as SCL is required as a nucleation factor to assemble the complex on target regulatory elements, while GATA-1 provides a DNA binding function and E47, a potential transactivation function.

SCL in erythropoiesis

The catastrophic consequences of *SCL* gene ablation in mice, which results in early embryonic lethality due to a complete absence of hematopoietic cells (45,52,53,55), has complicated the assessment of the roles that SCL might play in the maturation of particular blood cell lineages. Several lines of evidence point to SCL as an important regulator of erythropoiesis. First, during development SCL is expressed in both primitive and definitive erythroid cells of the yolk sac blood islands and fetal liver (15,29,47). Analysis of hematopoietic precursors has shown that SCL is highly expressed in committed erythroid progenitors (BFU-E and CFU-E/proerythroblasts), whereas it becomes down regulated in terminally differentiated red cells (7,26,29). Therefore, the pattern of SCL expression suggests that it could be involved in the

initial stages of commitment or consolidation of the erythroid cell fate from pluripotent progenitors. Second, enforced SCL expression in hematopoietic cell lines and primary bone marrow cells favors erythroid differentiation (3,17,26,63). Third, the genetic rescue of SCL deficient mice and recent conditional gene targeting experiments have demonstrated that SCL is required for proper erythroid differentiation in vivo (23,36,54). Indeed, Sanchez et al. (54) showed that a transgene driving SCL expression in stem cells was able to rescue early hematopoietic progenitors in *SCL*$^{-/-}$ embryos; however, these mice still exhibited a defect in erythroid differentiation that resulted in embryonic lethality, demonstrating that sustained *SCL* expression is required for erythropoiesis. In recent conditional knockout studies, in which floxed *SCL* alleles were deleted in mice expressing an interferon-inducible Cre recombinase, two groups have demonstrated that *SCL* inactivation leads to a complete block of erythroid and megakaryocytic cell maturation, without seemingly affecting hematopoietic stem cells (23,36). Interestingly, Mikkola et al. (36) observed that a population of Ter119^{+}CD71^{+} cells, representing normal erythroid precursors, disappears following *SCL* inactivation, leading to the accumulation of an abnormal Ter119$^{lo/-}$CD71^{+} population. Since Ter119 recognizes an epitope on murine GPA (5), the observation made by Mikkola et al. provides additional genetic evidence for the importance of SCL in driving *GPA* expression during murine erythropoiesis, shown herein. Therefore, it seems that once SCL has specified the hematopoietic cell fate from uncommitted mesodermal precursors, its sustained expression is not required for stem cell function, but becomes required anew for the generation of red blood cells and megakaryocytes.

Together, these results clearly demonstrate the essential role played by SCL in activating the transcription of erythroid specific genes and driving the erythroid lineage.

Glycophorin genes and *GPA* promoter regulation

The human glycophorins A, B and E are part of a family of erythroid specific membrane glycoproteins, which contribute to the expression of blood group antigens and determine the invasion and growth of parasites, such as the malaria pathogen Plasmodium falciparum (11,12). GPA is thought to form complexes with other erythroid membrane components, such as band 3, ankyrin, and protein 4.2, and their association appears to regulate the mechanical properties of the red cell membrane (8,24). GPA-deficient human red blood cells show decreased sulfate anion transport, due to the association between GPA and band 3, the human erythrocyte anion transporter (9). The *GPA*, *GPB* and *GPE* genes are clustered on chromosome 4q28-q31 and seem to have evolved from successive duplications of the *GPA* gene (40). The *cis* regulatory elements found here to be important for *GPA* promoter activation by the SCL complex are perfectly conserved in the *GPB* and *GPE* promoters (48), suggesting that these genes might also be direct targets of the SCL complex.

Previous studies on glycophorin promoter regulation, mainly focusing on the GPB promoter, demonstrated that these sites are protected from DNase I digestion in the presence of erythroid cell extracts and are required for promoter function (10,48,49). While the authors found that GATA-1 was the main factor binding to the G1 and G2 sites in EMSA, and that the Sp1 motif was important for promoter activity, they did not identify other partners of the SCL complex as potential regulators of the glycophorin genes (49). Using a probe encompassing a longer segment of the *GPA* promoter (GPA-84), we demonstrate the existence of a large protein complex containing SCL and its partners. Within the *GPA* proximal promoter, we show that the G1 motif is the most crucial determinant for binding of the SCL complex, followed by the Sp1, G2 and E elements, and that all these sites are required for optimal binding. We further complement these findings with the demonstration that partners of the SCL complex indeed occupy the *GPA* promoter *in vivo* in hematopoietic cells through chromatin immunoprecipitation. These findings further underscore the importance of Sp1 within the SCL complex, since we previously demonstrated that activation of the *c-kit* promoter by the SCL complex is critically dependent on the presence of a consensus GC-box, and that Sp1 physically interacts with multiple partners of the complex (31).

Involvement of LMO2 and Ldb1 in erythroid gene regulation

The results presented in this study reveal the importance of LMO2 and Ldb1 as essential partners within the SCL complex and establishes their requirement for the

appropriate regulation of an erythroid specific gene, using both transient transactivation assays, chromatin immunoprecipition, and antisense mediated loss of function in TF-1 cells. These findings contrast with a previous study suggesting that LMO2 and Ldb1 are negative regulators of erythropoiesis, as their enforced expression was shown to hinder terminal erythroid differentiation of G1ER cells (65), a GATA-1 deficient cell line blocked at the pro-erythroblast stage of differentiation. While these studies may appear contradictory at face value, previous analyses of the *Drosophila* orthologue of Ldb1, Chip, may help to reconcile these findings. Chip is a widely expressed regulator of several crucial processes including embryonic segmentation (38), neuronal development (50) and dorso-ventral patterning of the *Drosophila* wing (18). During wing morphogenesis, Chip associates within complexes containing the LIM-homeodomain protein Apterous, as well as the *Drosophila* LMO (dLMO) protein, and maintaining the appropriate stoichiometry of these complexes is crucial for proper wing development (18,37,51,64). In this context, both over expression and loss of function mutations of the *Chip* gene lead to the same phenotypic abnormalities in wing morphogenesis (18). Therefore, if the stoichiomoetry of LMO2 and Ldb1 containing complexes is similarly tightly regulated during erythropoiesis, enforced expression of these factors, as performed by Visvader et al. (65), might interfere with endogenous complexes through sequestration mechanisms and lead to the same outcome as loss of function approaches, which were utilized in the present study. In support of this hypothesis, a recent report by Xu and colleagues (70), who identified the *protein 4.2* gene as a erythroid target of SCL and its partners, showed that enforced expression of wild

type or a dominant negative version Ldb1 perturbed activation of the *protein 4.2* gene, consistent with the view that the stoichiometry of these complexes is indeed important for erythroid gene regulation.

In *Drosophila*, Chip was initially identified as an important regulator of enhancer-promoter communication (38), a property that seemingly relies on its ability to self-dimerize and to interact with several families of regulators, including LIM domain and Homeodomain containing proteins (58). Furthermore, it has recently been shown that proper patterning of the *Drosophila* nervous system depends on the ability of Chip to interact physically with Pannier, a *Drosophila* orthologue of GATA-1, and with bHLH factors of the Achaete/Scute complex (50). Our results extend these findings to show that mammalian Ldb1 also directly interacts with GATA family members and bHLH factors. Interestingly, *Ldb1* gene ablation in mice results in severe patterning defects and, among other phenotypes, compromises the development of yolk sac blood islands (39). This hematopoietic phenotype is most likely caused by defects in gene regulation by the SCL complex at the onset of hematopoiesis. We also provide evidence that LMO proteins can self-associate, comparable to the homodimerization of the LIM proteins CRP and MLP (4,19). Therefore, this network of interactions most likely modulates the assembly, targeting and activity of the SCL complex at different levels in the hematopoietic hierarchy.

Functional specialization within the SCL complex

SCL is an important regulator at several positions in the hematopoietic hierarchy. Whether its molecular mode of action varies in different hematopoietic lineages or populations remains ill defined. In our current study, we have found differences in the mechanisms by which SCL regulates the *GPA* gene versus our previous analysis of the *c-kit* gene, which constitute erythroid and stem/progenitor cell targets of SCL, respectively. First, we find that maximal *GPA* promoter activation, at lower concentrations of expression vector, and assembly of the SCL complex on *GPA* promoter sequences requires an Ebox motif and SCL DNA binding activity, although at higher concentrations of SCL, as observed following enforced expression TF-1 cells, SCL DNA binding mutants are active. In contrast, we previously showed that activation of the *c-kit* promoter by the SCL complex was Ebox independent and did not require SCL DNA binding (31). This mechanistic difference is consistent with the observation that SCL DNA binding defective mutants rescue hematopoietic cell commitment in $SCL^{-/-}$ embryonic stem cells, although they are unable to restore the proper maturation of definitive erythroid cells (44). Therefore, the requirement for SCL DNA binding is one characteristic that might delineate SCL function at the onset of hematopoiesis and during erythropoiesis. It is possible that a higher affinity of DNA binding by the SCL complex is required for the proper activation of the erythroid program, which would be provided in part by SCL itself and by other partners of the complex, such as GATA and Sp/XKLF family members. It will be possible to assess whether the necessity of SCL DNA binding is a broad difference

that distinguishes erythroid and stem cell targets of SCL through the identification and molecular characterization of additional SCL target genes.

Unlike SCL, the bHLH domain of E47 is unable to replace the function of the full-length protein during *GPA* promoter activation. Moreover, this truncated protein is dominant-negative over wild type E47 both in transient assays and in chromatin, as it likely competes for DNA binding with endogenous E-proteins. This finding contrasts with a previous study of the POMC promoter, in which E47-bHLH was shown to form a functional tripartite complex with NeuroD and Pitx-1 (46). Besides the C-terminally located bHLH domain, the E47 protein harbors two distinctive activation domains in its N-terminus, designated AD1 and AD2, which are absent in E47-bHLH. These domains are highly conserved in other ubiquitously expressed bHLH factors such as E12, E2-2 and HEB, and we have observed that HEB can functionally replace E47 within the SCL complex (Lecuyer and Hoang, in preperation). Therefore, it is likely that the transactivation domains of E47 are required for the proper function of the SCL complex, although our results cannot exclude the possibility that an unknown function of the N-terminal moiety of E47 might be involved. Interestingly, it has recently been shown that the AD1 domain serves as a recruitment motif for the SAGA histone acetyltransferase complex (32). Since infection of TF-1 cells with E47-bHLH causes an important shift in the mobility of the SCL complex, it is possible that this mutant hinders the recruitment of additional regulatory factors, such as elements of the SAGA complex, to SCL

target genes. Further investigation will be required in order to evaluate this possibility.

Transcription regulation by the SCL complex in different hematopoietic compartments

During differentiation, transcription factor complexes may undergo dynamic changes in composition, a view described as a cocktail party scenario by Sieweke and Graf (56). For example, our observations identified a requirement for Sp1 as a member of the SCL complex in *c-Kit* expressing cells and in erythroid cells (31, the present study), while in T cells there is no evidence for the involvement of Sp1 (41). By evolving in such a manner, the activity and target gene specificity of such multifactorial complexes could be modulated by environmental cues and favor differentiation towards particular cell fates. This type of mechanism would seem energetically cost effective for an organism, as it would bypass the requirement for major dismantling events as a pre-requisite to the commencement and shutdown of different programs of gene expression. In this respect, our finding that FOG can inhibit promoter activation by the SCL complex demonstrates how co-factors can modulate the activity of higher order transcription factor complexes through interactions with specific components. Since the GATA-FOG interaction is essential for erythroid and megakaryocytic cell differentiation (13,61), our findings suggest that during differentiation into theses lineages a proportion of the GATA factor pool

is recruited into FOG containing complexes, thus enabling GATA factors to exert functions that are independent of SCL complexes. Furthermore, our observation that SCL complexes containing GATA-1 or GATA-2 demonstrate preferential activation efficiency for erythroid or stem cell targets, respectively, suggests that SCL containing complexes may evolve dynamically during hematopoiesis to favor the maintenance of pluripotency or to consolidate differentiation towards specific lineages. At the onset of hematopoiesis, when GATA-2 is the predominant GATA family member, SCL complexes would be required for the activation of stem cell targets such as *c-kit* (31), which would favor the maintenance of an undifferentiated phenotype. At this stage, SCL complexes may also start to weakly activate the expression of erythroid targets such as *GPA*, *GATA-1* and *EKLF*, which would help to prime stem/progenitor cells for their eventual commitment towards the erythroid/megakaryocytic pathways. The activity of such a complex would account for the multilineage gene expression priming that is thought to precede the commitment of hematopoietic stem cells into different lineages (28). Since GATA-1 expression increases in progenitors of the erythroid lineage, while that of GATA-2 is down regulated, the replacement of GATA-2 by GATA-1 within the SCL complex might delineate a point at which the activation of erythroid specific genes is engaged more robustly. In the T lineage, however, GATA-3 is preferentially expressed and may substitute for GATA-2 within the SCL complex to drive the expression of T cell specific genes (41). Therefore, subtle variations in composition seem to modulate the specificity of action of SCL containing complexes and may also account for the differential requirement for SCL DNA binding activity in different hematopoietic

compartments. This type of mechanism is most likely a recurrent theme in cell fate determination in many other tissues.

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CHAPITRE 5

ARTICLE 3

The SCL-LMO2 interaction nucleates the assembly of hematopoietic transcription factor complexes through stabilization of LMO2

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5.1 Abstract

The bHLH factor SCL and the LIM-domain protein LMO2 are essential regulators of hematopoiesis and recurrent instigators of pediatric T cell acute lymphoblastic leukemia (T-ALL). The molecular function of these factors is intimately linked to their ability to associate within higher order complexes (SCL complexes); however, the molecular determinants that provide biological specificity in the assembly of these complexes remain poorly defined. By comparing SCL to other tissue-specific bHLH factors, such as the neurogenic factor NSCL-1, we demonstrate that SCL exhibits exquisite functional specificity in hematopoietic gene activation. This specificity is imparted by its exclusive ability to associate with LMO2, an interaction mediated by residues in the Loop and Helix 2 region of SCL. Through the use of SCL mutants that are unable to associate with LMO2, we show that the SCL-LMO2 interaction provides an essential nucleation function for the assembly of active SCL complexes and is required for the stimulation of erythroid differentiation by SCL. We provide further evidence that this interaction stabilizes the expression of the LMO2 protein, thus revealing a novel mechanism ensuring the proper spatio-temporal assembly of multifactorial transcription factor complexes.

5.2 Introduction

Specific programs of gene expression are thought to be generated by networks of interacting transcription factors that provide flexibility and specificity in gene regulation (Tjian and Maniatis, 1994; Grosschedl, 1995). This theme finds particular relevance in the context of the hematopoietic system, where the generation of diverse cellular lineages is controlled by both hematopoietic-specific and broadly expressed transcription factors (Sieweke and Graf, 1998). Indeed, gene ablation studies have identified a panoply of critical regulators that are either essential for the establishment of the hematopoietic system or for the generation of particular lineages (Shivdasani and Orkin, 1996). There are also examples of regulators, such as the bHLH domain transcription factor SCL/TAL-1 (hereafter referred to as SCL), which play important functions at several levels in the hematopoietic hierarchy (Lecuyer and Hoang, 2004).

During mouse embryogenesis, the SCL gene is expressed in all hemogenic sites (Kallianpur *et al.*, 1994; Silver and Palis, 1997; Elefanty *et al.*, 1999; Palis *et al.*, 1999) and gene ablation studies have revealed that SCL is cell-autonomously required for the generation of all hematopoietic cells (Robb *et al.*, 1995; Shivdasani *et al.*, 1995; Porcher *et al.*, 1996; Robb *et al.*, 1996), thus identifying SCL as a master regulator in the establishment of the hematopoietic compartment. This view is supported by the findings that specification of the hematopoietic cell fate requires the expression of SCL in early mesodermal precursors (Endoh *et al.*, 2002), and that

ectopic expression of SCL favors the hematopoietic lineage at the expense of other mesodermal derivatives (Gering *et al.*, 1998; Mead *et al.*, 1998). In addition to its role in early hematopoietic development, SCL is also expressed during adult hematopoiesis in hematopoietic stem cells and multipotent progenitors, as well as cells of the erythroid and megakaryocytic pathways (Brady *et al.*, 1995; Pulford *et al.*, 1995; Hoang *et al.*, 1996; Elefanty *et al.*, 1998; Akashi *et al.*, 2000). Recent conditional knock-out studies, which bypass the early embryonic lethality manifested in traditional $SCL^{-/-}$ mice, have highlighted the requirement for sustained SCL expression in the generation of both the erythroid and megakaryocytic lineages (Mikkola *et al.*, 2003; Hall *et al.*, 2003). The interaction of SCL with multiple partners is thought to provide the molecular basis through which this factor exerts its non-redundant functions at different branch points in the hematopoietic system.

In addition to its essential functions during normal hematopoiesis, the SCL gene is the most frequent target of chromosomal rearrangements in pediatric T cell acute lymphoblastic leukemia (T-ALL) (reviewed in (Begley and Green, 1999)). While SCL has weak oncogenic properties on its own, it efficiently collaborates with other genes that are often rearranged in T-ALL, such as those that encode the LIM-only proteins LMO1/2, to induce aggressive T-cell tumors in transgenic mice (Larson *et al.*, 1996; Aplan *et al.*, 1997; Chervinsky *et al.*, 1999; Herblot *et al.*, 2000). The relationship between SCL and LMO proteins also extends to normal hematopoiesis, since $LMO2^{-/-}$ mice exhibit severe defects in hematopoietic development resembling those found in $SCL^{-/-}$ mice (Warren *et al.*, 1994; Yamada *et al.*, 1998). In fact, SCL

and LMO2 have been shown to physically associate in erythroid and leukemic cell extracts within multifactorial complexes that include the ubiquitous bHLH factors encoded by the E2A gene (E47 and E12), the LIM domain binding protein Ldb-1, and hematopoietic GATA family members (Visvader *et al.*, 1997; Wadman *et al.*, 1997; Grutz *et al.*, 1998; Ono *et al.*, 1998). Since LMO2 is devoid of direct DNA-binding capability, but interacts physically with SCL and GATA proteins, it has been proposed to act as bridging molecule between DNA bound SCL/E2A heterodimers and GATA factors (Wadman *et al.*, 1997). While relatively little is known about the physiological target genes of SCL, recent studies identified the c-kit, glycophorin A (GPA) and *protein 4.2* genes as direct targets of SCL-containing complexes in progenitor and erythroid cells, respectively (Lecuyer *et al.*, 2002; Xu *et al.*, 2003; Lahlil *et al.*, 2004). These studies revealed that the regulation of gene expression by SCL requires its integration within larger complexes that exhibit an all-or-none behavior in transcription activation, which most likely provides a more stringent control over lineage-specific programs of gene expression. Despite these indications, little is known about the molecular determinants that specify critical protein interactions within SCL-containing complexes and to what extent particular interactions modulate the assembly and specificity of these complexes on hematopoietic target genes.

In the present study, we address the question of whether SCL and its partners exhibit specificity or redundancy in hematopoietic gene activation. We demonstrate that the function of SCL cannot be replaced by the closely related bHLH factor NSCL1, and

reveal that this difference in molecular behavior results from the unique ability of SCL to interact with LMO2. On the basis of sequence comparison with NSCL1, we identify residues in the Loop and Helix 2 region of SCL that are required for association with LMO2 and that this interaction provides a nucleation function in the assembly higher order transcription factor complexes that drive hematopoietic gene activation. Furthermore, we provide evidence that the interaction of SCL with LMO2 stabilizes the expression of the LMO2 protein, which is normally rapidly targeted for proteasomal degradation. Therefore, our study reveals novel insight into the mechanisms that establish combinatorial transcription factor codes within the hematopoietic compartment.

5.3 Materials and Methods

Plasmids and mutagenesis

Expression vectors for SCL and its partners, as well as the kit-1146 and GPA-84 reporter plasmids were described previously (Lecuyer *et al.*, 2002; Lahlil *et al.*, 2004). The NSCL1 cDNA was obtained from Dr. J. Drouin (IRCM, Montreal, Canada) and was transferred into MSCV-neo using EcoRI. The HA and FLAG epitopes were added onto the N-termini of NSCL-1 and LMO2, respectively, through ligation of double-stranded oligonucleotides encoding each epitope upstream of the NSCL-1 and LMO2 cDNAs. Vectors encoding GST-LMO2 and GST-E47 were generated by cloning PCR amplified cDNAs into the pGex2T plasmid (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Domain swap mutants between SCL and NSCL1, as well as SCL point mutants, were generated through overlap PCR with the Vent polymerase (New England Biolabs, Mississauga, ON, Canada). PCR fragments were inserted into pGem4 (Promega, Madison, WI, USA) using BamHI/EcoRI and were subcloned into the BglII/EcoRI sites of MSCV-puro or MSCV-neo. All PCR amplified fragments were verified by sequencing and the sequences of oligonucleotides used for mutagenesis are available upon request.

Cell culture, transient transfections and protein extracts

The growth conditions of NIH 3T3, 293 and TF-1 cells were described previously (Krosl *et al.*, 1998; Lahlil *et al.*, 2004). For TF-1 cell infections, cells were incubated overnight with control retroviruses (MSCV-neo) or viruses encoding SCL and SCL-

M13. The cells were then recovered and polyclonal populations were analyzed 1 week after selection in G418 at 1.2 mg/ml. TF-1 infectants (1.5×10^5 cells/ml) were stimulated to differentiate towards the erythroid pathway by adding 1 U/ml of Erythropoietin (EPO) to the culture medium in the absence of selection (Hoang *et al.*, 1996). Samples of cells were then taken at different days following the induction of differentiation for benzidine staining and RT-PCR analysis.

Transient transactivation assays were performed using calcium phosphate precipitation on NIH 3T3 cells seeded in 12-well plates (3×10^4 cells/well) as previously described (Lecuyer *et al.*, 2002). The GPA and c-kit reporter constructs were kept at 1.5 μg/well, while the total amount of DNA was kept constant at 4.5 μg/well using pGem4 (Promega). For normalization, 100 ng of CMV-β-galactosidase (CMV-β-gal) was included in each sample. Expression vector doses used in specific experiments are indicated in figure legends. Luciferase activities were normalized to β-gal values and each experiment was performed in triplicate and was representative of 3 or more independent experiments (see figure legends).

TF-1 and 293 cell nuclear extracts were prepared as documented previously (Krosl *et al.*, 1998; Lahlil *et al.*, 2004). For 293 cell extracts used in immunoprecipitation and electrophoretic mobility shift assays, 4.2×10^6 cells were seeded in 100 mm plates and transfected with combinations of expression vectors (5 μg of each) for SCL, HA-NSCL1, FLAG-LMO2, E47, LMO2, Ldb-1, GATA-1, MSCL-GFP, or different SCL mutants. The total amount of DNA was kept at 25-30 μg using pGem4. For studies

of LMO2 protein stabilization in Fig. 5.8B and 8E, 7×10^5 293 cells were seeded in 6-well plates and transfected 24 h later with 1μg of the F-LMO2-IRES-GFP, E47, SCL or SCL-M13 expression vectors as indicated in figure legends. The total amount of DNA was kept at 5 μg with pGem4. After 36 h, the cells were incubated with proteasomal inhibitors (MG132 or Lactacystine) or cycloheximide for the indicated time periods, washed twice with cold PBS and instantaneously frozen on liquid nitrogen. To obtain total cellular protein extracts, the cells were then partially thawed at 4°C and lysed for 20 min at 4°C with 400 μL of radioimmunoprecipitation assay buffer (10 mM Tris-HCl [pH 8.0], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% deoxycholate) supplemented with protease inhibitors.

Gel shift, pull-down, and immunoprecipitation (IP) assays

For Gelshift assays, each binding reaction was performed using 10 μg of TF-1 or 293 cell nuclear extract in 20 mM HEPES [pH7.5], 50 mM KCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, 10 μg BSA, 0.5 μg dI-dC and the TAL-1 (50 000 cpm) or GPA-84 (10 000 cpm) probes in a final volume of 20 μL. After 15 min at room temperature, protein-DNA complexes were resolved at 150 V on a 4% polyacrylamide (PAGE) gel in 0.5× TBE at 4°C for 4 h.

For pull-down experiments, bacterially expressed GST, GST-LMO2 and GST-E47 were purified and coupled to glutathione sepharose beads (Amersham Pharmacia Biotech, Piscataway, NJ, USA). SCL and NSCL1 were ^{35}S-methionine labeled with the TnT coupled reticulocyte lysate system (Promega), and 15 μL of lysate was

incubated with 3 μg of immobilized GST fusion proteins in 400 μL binding buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 1% NP-40, 5 mM DTT, 10% glycerol) for 2 hours at 4°C with agitation. Samples were then washed 3 times with binding buffer, resolved by SDS-PAGE, transferred to PVDF membranes and visualized using a PhosphorImager apparatus (Molecular Dynamics, Sunnyvale, CA, USA).

For IPs, 10-60μg of 293 cell nuclear extract was incubated overnight at 4°C with 3 μg of antibody in 1 mL of buffer (20 mM Tris-HCl [pH 8.0], 137 mM NaCl, 1% NP-40, 10% glycerol, 1 mM EDTA). Protein complexes were precipitated by adding appropriately conjugated Pansorbin cells (Calbiochem, San Diego, CA, USA) for 30 min at 4°C, washed 3 times with 1 mL of buffer and subjected to SDS-PAGE. Following transfer on PVDF membranes, proteins were visualized by immunoblotting using ECL plus (Amersham Pharmacia Biotech). The following antibodies were used for IP and westernblotting experiments. The mouse anti-E2A (YAE), rat anti-GATA-1 (N6), rabbit anti-GFP (FL) and goat anti-Ldb-1/CLIM-2 (N-18) were all from Santa Cruz Biotechnology Inc (Santa-Cruz, CA, USA). The BTL-73 and 2TL-136 mouse anti-SCL antisera were kindly provided by Dr. D. Mathieu (Institut de Génétique Moléculaire, Montpellier, France). The mouse anti-HA and anti-FLAG antibodies were obtained from Covance (Richmond, CA, USA) and Stratagene (La Jolla, CA, USA), respectively.

Structural modeling

All calculations were performed using the software package SYBYL (Tripos ass., St-Louis, MO, USA) on a silicon graphics indigo^{2}extreme workstation. The Tropois force field was used for energy calculations, and a dielectric constant of 1 was used. The structure of the E47 homodimer/DNA complex (Ellenberger *et al.*, 1994) was obtained from the Brookhaven protein database and was subjected to 1000 steps of conjugate gradient minimization. One of the E47 moieties was transformed into human SCL using homology modeling techniques. After the structure of the DNA bound SCL/E47 heterodimer was obtained, the side chains of the mutated amino acids were packed using the scan subroutine in SYBYL. In this procedure the backbone dihedryl angles are held fixed while the side chains of the individual amino acids are rotated one at a time until a sterically acceptable conformation was obtained. This structure was again minimized, at which time no major conformational changes were observed.

RT-PCR analysis

Total RNA was extracted from TF-1 or 293 cells as previously described (Herblot *et al.*, 2000). To eliminate contaminant DNA molecules, 500 ng of each sample was then subjected to digestion with EcoRI and DNAseI in REact3 buffer (GIBCO invitrogen corporation, Burlingtion, ON, Canada). After the nucleases were heat inactivated for 10 min at 65°C, the samples were subjected to reverse transcription using the Superscript first strand cDNA synthesis system (GIBCO invitrogen corporation, Burlingtion, ON, Canada). For PCR reactions, mixtures containing 1 µl of cDNA, 1 µM of forward (Fw) and reverse (Rv) primers, 20 mM Tris-HCl [pH

8.4], 50 mM KCl, 1.5 mM $MgCl_2$, 5% DMSO, 0.2 mM dNTPs, and 1.25 U of *Taq* DNA polymerase, were subjected to 30 cycles of amplification (94°C for 1 min, 60°C for 1 min and 72°C for 30 sec). PCR fragments were resolved on 1.3% agarose gels, transferred to nylon membranes, hybridized with ^{32}P-labelled internal (Int) oligonucleotide probes, and revealed using PhophorImager (Molecular Dynamics). The following oligonucleotides were used for amplifications and hybridizations: RLIM-Fw: CCTGGCTCAGTTTTTCCTCTTA; RLIM-Rv: CTCTGTTACCAGAAGCTAAGAC; RLIM-Int: GTGTTTGCATTACAGAATA; LMO2-Fw: TACTTCCTGAAAGCCATCGACC; LMO2-Rv: GATCCCATTGATCTTGGTCCAC; LMO2-Int: AGGAGAGACTATCTCAGGCTTT; GPA-Fw: ATTGTCAGCAATTGTGAGCATA; GPA-Rv: TGATCACTTGTCTCTGGATTTT; GPA-Int: ATATGCAGCCACTCCTAGAGC; S14-Fw: GGCAGACCGAGATGAATCCTCA; S14-Rv: CAGGTCCAGGGGTCTTGGTCC; S14-Int: GAGCTGGGTATCACCGCCCT.

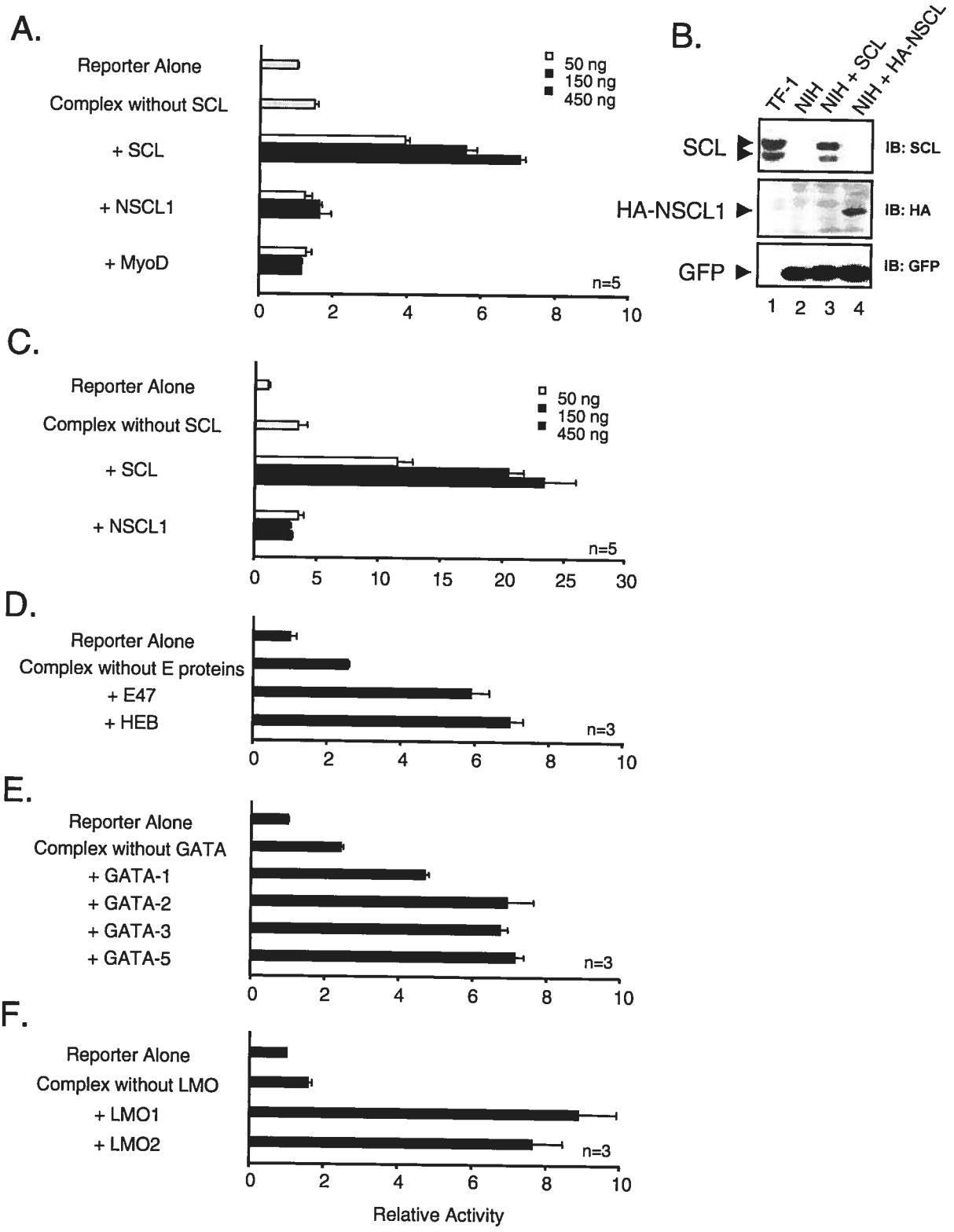
5.4 Results

SCL provides biological specificity in hematopoietic gene activation and assembly of larger transcription factor complexes

SCL is a non-redundant initiator of the hematopoietic system during development and of adult erythropoiesis, but the molecular basis for its biological specificity remains poorly understood. In contrast to bHLH factors like MyoD and E2A that often exhibit strong transcriptional effects when expressed on their own (Massari and Murre, 2000), the activity of SCL is dependent on its integration within higher order transcription factor complexes (Wadman *et al.*, 1997; Lecuyer *et al.*, 2002; Xu *et al.*, 2003; Lahlil *et al.*, 2004), suggesting that protein-protein interactions may be important determinants of this biological specificity. To address this question, we first sought to assess whether SCL and its partners exhibit functional specificity or redundancy during hematopoietic gene activation. We performed transient transactivation assays using a reporter construct driven by the promoter of the c-kit gene (kit-1146), which was previously identified as a progenitor cell target of SCL (Lecuyer *et al.*, 2002). Since full activation of the c-kit promoter requires the presence of each component of the SCL complex (Lecuyer *et al.*, 2002), we utilized this assay to evaluate whether SCL and its partners could be functionally replaced by members of their respective families during c-kit promoter activation. While kit-1146 activity was only weakly increased upon co-transfection of expression vectors for E47, LMO2, Ldb-1 and GATA-2 (Figure 5.1A), efficient promoter activation

occurred in a dose dependent manner upon further inclusion of SCL in the transfection samples. In contrast to SCL, the myogenic bHLH factor MyoD, which shares only 39 % identity with SCL in the HLH domain, was unable to activate the c-kit promoter when co-expressed with E47, LMO2, Ldb-1 and GATA-2 (Figure 5.1A), although this MyoD expression vector mediated efficient activation of cardiac specific promoter sequences (data not shown). Surprisingly, the neurogenic bHLH factor NSCL1/nhlh1, which was originally identified by virtue of its homology to SCL and shows 65 % identity to SCL in the HLH domain, was also inactive. Appropriate expression of SCL and NSCL1 in transfected cells was confirmed by immunoblotting (Figure 5.1B). This functional specificity uncovered for SCL was also observed during the activation of an erythroid target of the SCL complex, the glycophorin A gene promoter (Figure 5.1C) (Lahlil *et al.*, 2004). In contrast to SCL, we found that other partners of the complex were functionally redundant with members of their respective families. Indeed, the ubiquitously expressed bHLH factor E47 could be replaced by HEB (Figure 5.1D), while both hematopoietic (GATA-1, -2, and -3) and cardiac (GATA-5) GATA family members were functional in c-kit promoter activation (Figure 5.1D). Finally, we also observed that the LIM-only proteins LMO1 and LMO2, both of which are targets of chromosomal rearrangements in T-ALL, could participate in synergistic promoter transactivation (Figure 5.1E). Therefore, SCL exhibits exquisite functional specificity in the activation of hematopoietic promoter sequences in conjunction with its partners, which is consistent with the critical non-redundant role played by this factor during hematopoiesis.

Fig. 5.1. Functional specificity for SCL in hematopoietic gene activation by the SCL complex. (A) SCL is non redundant with other tissue-specific bHLH factors during synergistic activation of the c-kit promoter. The kit-1146 reporter (1500 ng) was transfected in NIH 3T3 cells with expression vectors for E47 (150 ng), LMO2 (750 ng), Ldb-1 (750 ng) and GATA-2 (150 ng), and the indicated amounts of SCL, NSCL1 and MyoD vectors (50-450 ng). (B) Nuclear extracts of TF-1 cells (10 μg), or NIH 3T3 cells (10 μg) expressing SCL or HA-NSCL1, were analyzed by immunoblotting with the indicated antibodies. Expression of GFP in transfected cells was monitored as a control for equal loading. Arrowheads point to specific bands revealed by immunoblotting with the antibodies indicated to the right of each panel. (C) SCL specificity is also observed during activation of the erythroid glycophorin A (GPA) promoter. Transactivations were done as in (A) using the GPA-84 reporter (1500 ng) and complexes containing GATA-1 (150 ng). (D-F) Other partners of the SCL complex demonstrate functional redundancy. Transactivation assays were carried out as in (A), except that E47 was replaced by HEB (D), GATA-2 was sustituted by GATA-1, GATA-3 or GATA-5 (E), and LMO2 was replaced by LMO1 (F). For sections A and C-E, results are expressed as fold activation relative to the reporter vector transfected alone, represent the average ± SD of triplicate determinations and are representative of (n) independent experiments. In all samples the amount of total DNA was kept constant at 4.5 μg and 100 ng of the CMV-βgal vector was included to normalize luciferase values.


A.
Reporter Alone
Complex without SCL
+ SCL
+ NSCL1
+ MyoD
50 ng
150 ng
450 ng
n=5
0 2 4 6 8 10
B.
TF-1
NIH
NIH + SCL
NIH + HA-NSCL
SCL
HA-NSCL1
GFP
IB: SCL
IB: HA
IB: GFP
1 2 3 4
C.
Reporter Alone
Complex without SCL
+ SCL
+ NSCL1
50 ng
150 ng
450 ng
n=5
0 5 10 15 20 25 30
D.
Reporter Alone
Complex without E proteins
+ E47
+ HEB
n=3
0 2 4 6 8 10
E.
Reporter Alone
Complex without GATA
+ GATA-1
+ GATA-2
+ GATA-3
+ GATA-5
n=3
0 2 4 6 8 10
F.
Reporter Alone
Complex without LMO
+ LMO1
+ LMO2
n=3
0 2 4 6 8 10
Relative Activity

To determine whether the specificity uncovered for SCL in promoter transactivation is reflected in its ability to nucleate the formation higher order complexes on DNA, we next performed gel shift assays with a probe derived from the proximal GPA promoter (GPA-84). It was previously shown that binding of the SCL complex to the GPA-84 probe requires each partner of the complex and occurs through two GATA motifs, an Ebox element and an Sp1 binding site (Figure 5.2A), all of which are required for maximal GPA promoter activation (Lahlil *et al.*, 2004). Therefore, the GPA-84 probe was incubated with nuclear extracts of TF-1 cells, a hematopoietic cell line that expresses SCL and its partners endogenously, or of heterologous 293 cells ectopically expressing SCL or NSCL1 in combination with the other components of the complex. With TF-1 cell extracts, a very low mobility complex was formed on the GPA-84 probe (Figure 5.2A, lane 2), which was previously shown to contain SCL and its partners in supershift experiments with specific antisera (Lahlil *et al.*, 2004). This complex was reconstituted upon transfection of expression vectors for SCL, E47, LMO2, Ldb-1, and GATA-1 (lane 5), while it was not observed with extracts of untransfected 293 cells (lane 3). Furthermore, omission of the SCL plasmid from the transfection mixtures abrogated the assembly of the complex (lanes 4). Finally, NSCL1 was unable to substitute for SCL in nucleating the formation of the complex on the GPA-84 probe (lane 6) in agreement with our functional experiments. Immunoblotting confirmed the expression of SCL and its partners, as well as NSCL1, in 293 cell extracts (Figure 5.2B, lanes 1-4). These results, therefore, suggest that SCL contains key determinants that are required for the assembly of hematopoietic specific transcription factor complexes on DNA.

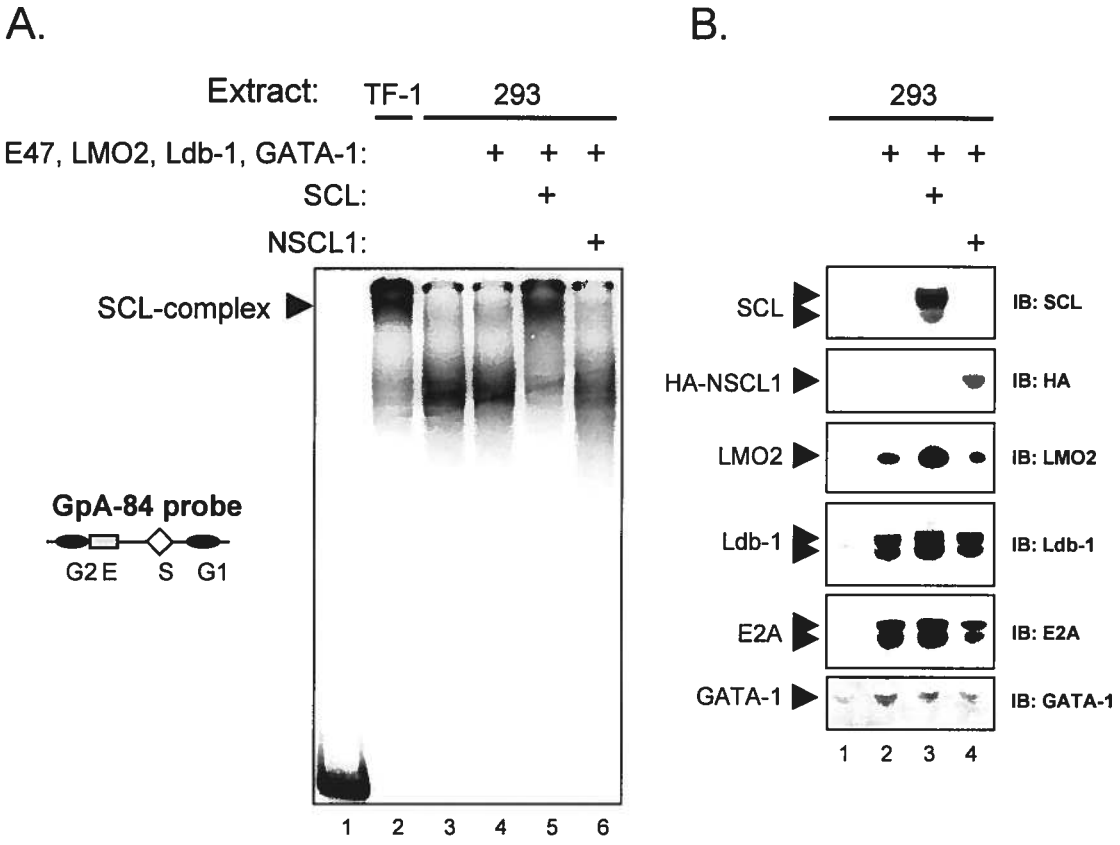


Fig. 5.2. SCL demonstrates specificity in assembling the SCL complex on DNA. (A) EMSA were performed using the GPA-84 probe, which covers positions -84 to -30 of the human GPA promoter (Lahlil *et al.*, 2004), and nuclear extracts (10 μg) of TF-1 cells (lane2) or 293 cells transfected with the indicated expression vectors (lanes 3-6). The arrowhead points to the low mobility complex containing SCL and its partners. The asterisks identify complexes formed in control untransfected 293 cells. (B) The 293 cell nuclear extracts (10 μg) utilized in (A) were subjected to immunoblotting with the indicated antibodies to confirm the appropriate expression of SCL and its partners.

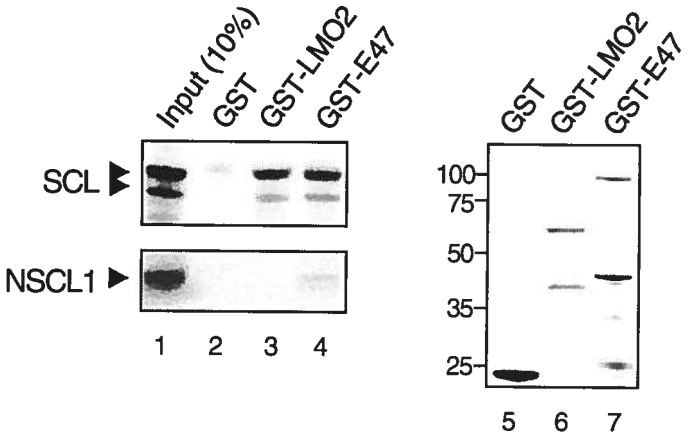
SCL biological specificity results from its specific interaction with LMO2

Within SCL containing complexes, SCL principally interacts with the ubiquitously expressed bHLH factor E47 (or other members of the E-protein family), as well as the hematopoietic cell enriched LIM-only protein LMO2. In order to assess whether these interactions are specific to SCL, we first performed pull-down assays in which columns containing GST, GST-LMO2 or GST-E47 (Figure 5.3A, lanes 5-7) were incubated with ^{35}S-methionine labeled SCL and NSCL1. We found that only SCL, but not NSCL1, was retained on columns containing GST-LMO2 (lane 3), whereas neither factor could bind to control columns containing GST alone (lane 2). Similarly, MyoD was unable to bind to GST-LMO2 columns (data not shown). In contrast, both SCL and NSCL1 bound to immobilized GST-E47 (lane 4), consistent with the view that E47 is a ubiquitous partner of tissue-specific bHLH proteins.

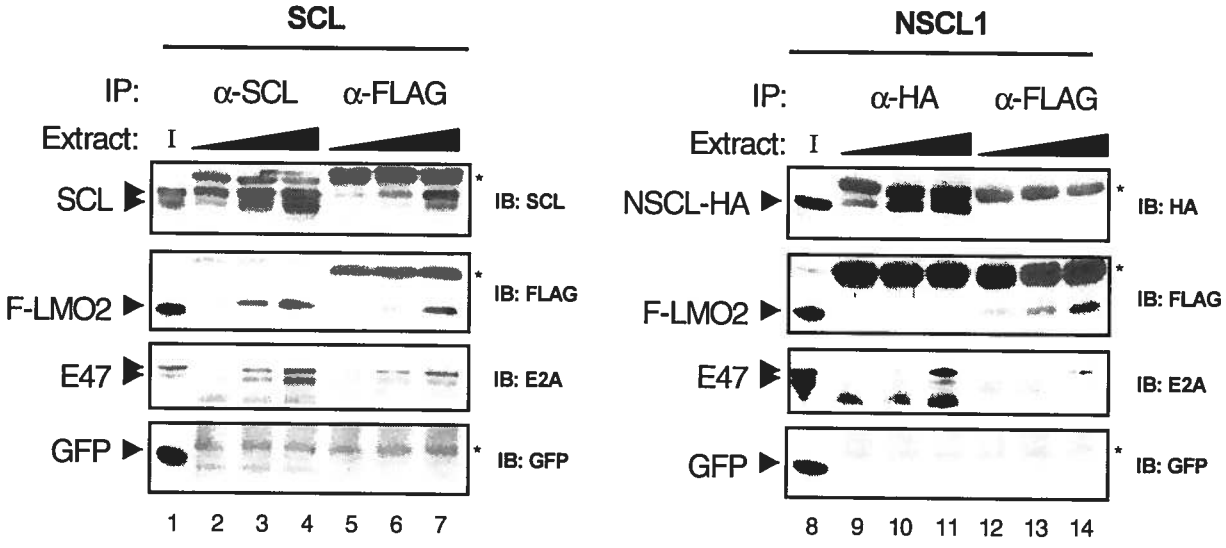
In order to ascertain whether the SCL-LMO2 interaction demonstrated specificity in vivo, we next performed co-immunoprecipitation assays. Nuclear extracts were prepared from 293 cells expressing FLAG-LMO2 (F-LMO2), E47, Ldb-1, GFP, and SCL or HA-NSCL1. Increasing amounts of nuclear extract were then subjected to immunoprecipitation using anti-SCL, anti-FLAG or anti-HA antibodies. In SCL expressing extracts (Figure 5.3B, lanes 1-7), immunoprecipitation with both the anti-SCL and anti-FLAG antibodies efficiently precipitated SCL, F-LMO2 and E47 (lanes 2-7), suggesting that these factors physically associate in transfected cells.

Fig. 5.3. SCL specifically interacts with LMO2. (A) While both SCL and NSCL1 interact with GST-E47, only SCL interacts with GST-LMO2 in vitro. Pulldown experiments were performed with immobilized GST, GST-LMO2 and GST-E47 and ^{35}S-labeled SCL and NSCL1. Input samples (lane 1) represent 10% of the amount used for the binding reactions (lanes 2-4). Coomasie blue stained GST, GST-LMO2 and GST-E47 are shown in lanes 5-7. (B) SCL specifically interacts with LMO2 in vivo. Immunoprecipitation assays were performed with extracts of 293 cells (10, 30 and 60 μg) transfected with expression vectors for E47, F-LMO2, Ldb-1, GFP, and SCL (lanes 1-7) or HA-NSCL1 (lanes 8-14), and with the indicated antibodies. Lanes 1 and 8 represent input (I) samples with 10 μg of nuclear extract. Arrowheads point to specific bands that were revealed following immunoblotting with the antibodies indicated to the right of each panel. Asterisks indicate immunoglobulin light and heavy chains. Note that SCL associates with LMO2 and E47, whereas NSCL1 only associates with E47. In addition, efficient co-precipitation of E47 with LMO2 requires the presence of SCL as a bridging factor.

A.


Input (10%)
GST
GST-LMO2
GST-E47
SCL
NSCL1
1 2 3 4
GST
GST-LMO2
GST-E47
100
75
50
35
25
5 6 7

B.


SCL
IP: α-SCL α-FLAG
Extract: I
SCL
F-LMO2
E47
GFP
IB: SCL
IB: FLAG
IB: E2A
IB: GFP
1 2 3 4 5 6 7
NSCL1
IP: α-HA α-FLAG
Extract: I
NSCL-HA
F-LMO2
E47
GFP
IB: HA
IB: FLAG
IB: E2A
IB: GFP
8 9 10 11 12 13 14

In contrast, when SCL was substituted by HA-NSCL1, the anti-HA antibody was found to precipitate HA-NSCL1 and E47, but was unable to co-precipitate F-LMO2 (lanes 9-11). In agreement, the anti-FLAG antibody precipitated F-LMO2, but did not bring down HA-NSCL1 or E47 (lanes 12-14). Finally, GFP, which was included as a negative control, was not co-precipitated with any of the antibodies. Therefore, since the association of LMO2 and E47 requires the presence of SCL, these data support the view that SCL functions as a bridging factor that can simultaneously interact with both of these partners. Furthermore, while both SCL and NSCL1 are able to associate with the ubiquitous bHLH factor E47, interaction with the hematopoietic-specific co-factor LMO2 is restricted to SCL.

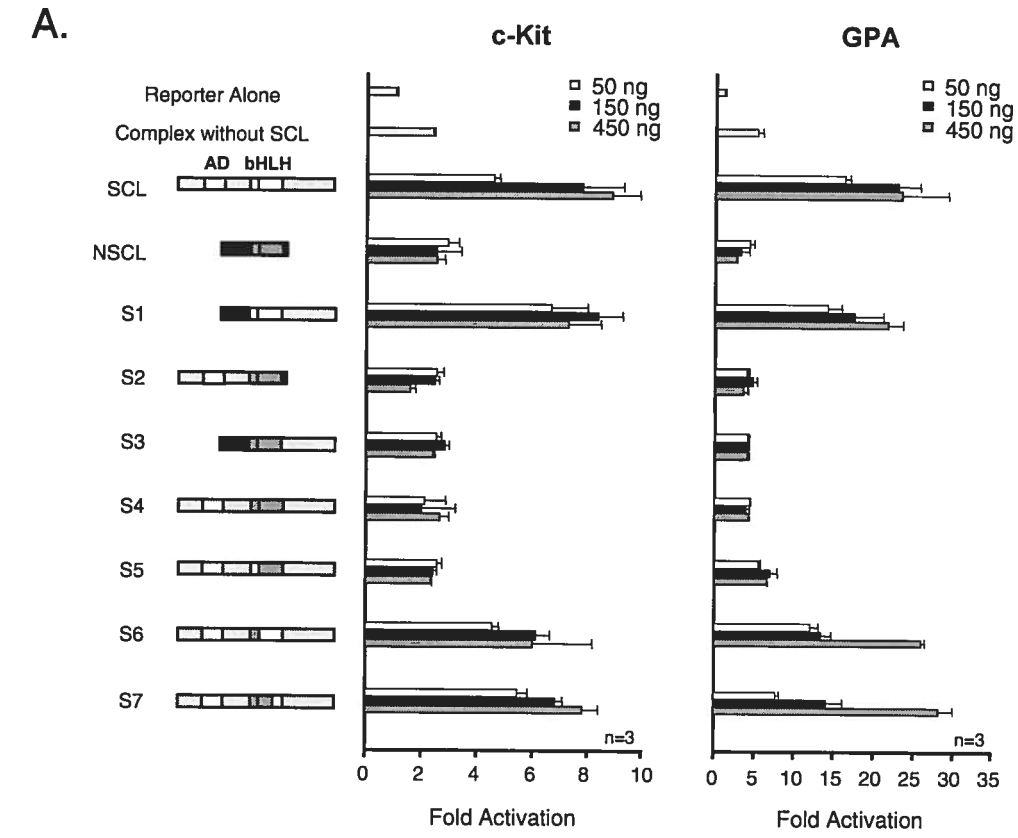
SCL functional specificity and interaction with LMO2 are imparted by residues in the Loop and Helix 2 of the HLH domain

We next sought to identify what portion of the SCL protein accounts for its functional specificity in hematopoietic gene activation. We constructed a series of domain swap mutants between SCL and NSCL1 and tested their ability to activate the c-kit and GPA promoters in collaboration with other partners of the complex. While SCL contains a putative proline-rich activation domain in a region N-terminal to the bHLH domain (Sanchez-Garcia and Rabbitts, 1994), this portion of the SCL protein could be replaced by the N-terminus of NSCL1 without affecting promoter transactivation and was unable to confer activation when fused to the bHLH region and C-terminus of NSCL1 (Figure 5.4A, mutants S1 and S2). Residues found C-

terminal to the bHLH domain of SCL were also unable to confer activation to NSCL1 (mutant S3). However, replacement of the bHLH domain of SCL by that of NSCL1 (mutant S4) led to a complete loss of function, as luciferase values fell to the background levels observed in the absence of SCL. While reintroducing the basic region of SCL in the S4 mutant did not restore SCL function (mutant S5), reinsertion of the HLH domain of SCL (mutant S6) or of the Loop and Helix 2 region (mutant S7), completely restored SCL activity. The expression of all of these mutants was confirmed by western blotting with antibodies directed against C-terminal (BTL-73) or N-terminal (2TL136) portions of the SCL protein (Figure 5.4B). Thus, critical determinants of SCL specificity lie within the Loop and Helix 2 of the HLH domain of SCL, while other portions of the SCL protein, including the DNA-interacting basic region and putative transactivation domain, are unnecessary or redundant for hematopoietic gene activation.

In order to determine whether the Loop and Helix 2 of the SCL protein also specify physical interaction with LMO2, we next performed co-immunoprecipitation assays using extracts of 293 cells expressing different SCL mutants (S4 to S7) in combination with F-LMO2, Ldb-1, E47 and GFP (Figure 5.4C). As revealed by an anti-SCL immunoprecipitation, the transcriptionally inactive S4 and S5 mutants were unable to associate with F-LMO2, while they still interacted with E47 (Figure 5.4C, lane 2 and 4). In contrast, the functional S6 and S7 mutants efficiently interacted with both F-LMO2 and E47 (lanes 6 and 8). Therefore, the same region that specifies SCL function in hematopoietic gene regulation confers physical association with

Fig. 5.4. SCL biological specificity and interaction with LMO2 is conferred by determinants in the HLH domain of SCL. (A) The Loop and Helix 2 region determines SCL functional specificity in hematopoietic gene activation. Transactivation assays were performed as in Fig.1 with either the kit-1146 or GPA-84 reporters (Lahlil *et al.*, 2004), and mixtures containing expression vectors for partners of the complex and varying doses (50-450 ng) of vector encoding SCL, NSCL1 or the indicated domain swap mutants (S1-S7). Portions of the SCL protein are colored dark grey, while those of NSCL1 are in black. The bHLH domains of SCL and NSCL1 are represented as open or hashed boxes, respectively; while the putative transactivation domain (AD) of SCL is light grey. Results are shown as luciferase activity relative to each reporter vector transfected alone. Luciferase values were normalized by co-transfection of CMV-βgal and are representative of (n) independent experiments. (B) Appropriate expression of the SCL/NSCL1 swap mutants (S1-S7) in extracts of transiently transfected NIH 3T3 cells was confirmed by immunoblotting with antibodies directed against the N-terminal (2TL136) or C-terminal (BTL-73) portions of the SCL protein. (C) The Loop and Helix 2 of SCL specifies its physical interaction with LMO2. Immunoprecipitations were carried out as in Fig.3B using the anti-SCL antibody and extracts of cells expressing the indicated SCL swap mutants (S4-S7), as well as E47, F-LMO2, Ldb-1 and GFP. Lanes 1, 3, 5 and 7 represent input samples with 10 μg of nuclear extract; while immunoprecipitations were performed with 60 μg of extract. Arrowheads indicate specific bands revealed by immunoblotting and asterisks indicate immunoglobulin light and heavy chains. Note that the Loop and Helix 2 of SCL is required for co-precipitation of LMO2.


A.
c-Kit
GPA
Reporter Alone
Complex without SCL
AD bHLH
SCL
NSCL
S1
S2
S3
S4
S5
S6
S7
50 ng
150 ng
450 ng
n=3
0 2 4 6 8 10
0 5 10 15 20 25 30 35
Fold Activation
B.
2TL136
BTL73
SCL
- SCL S1 S3 S2 S4 S5 S6 S7
α-SCL (BTL73)
α-SCL (2TL136)
1 2 3 4 5
6 7 8 9
C.
Extract: S4 S5 S6 S7
IP: - + - + - + - +
F-LMO2
SCL
E2A
GFP
IB: FLAG
IB: SCL
IB: E2A
IB: GFP
1 2 3 4 5 6 7 8

LMO2, demonstrating that the SCL-LMO2 interaction is an essential determinant of SCL biological specificity, which is consistent with the crucial functions concomitantly played by both of these factors during hematopoietic development and leukemogenesis.

Alignment of the HLH region of SCL orthologues from humans to fruit flies revealed a high degree of conservation between vertebrate SCL proteins, whereas *Drosophila* SCL (DroSCL) presented a greater number of divergent amino acids (Figure 5.5A). Despite these differences, DroSCL was able to partially substitute for human SCL in GPA and c-kit promoter activation (Figure 5.5A; data not shown). Sequence alignment with the HLH domain of mammalian NSCL1 proteins revealed an even greater number of differences compared to mammalian SCL factors, some of which overlapped with diverging residues in DroSCL (Figure 5.5A). To pinpoint with greater detail the residues that ensure SCL specificity, we systematically mutagenized patches of divergent residues throughout the HLH domain of SCL and replaced them by those found at the corresponding positions in NSCL1 (Figure 5.5B). We reasoned that this strategy would allow us to conserve the general structure of the HLH domain. To our surprise, none of the mutations in single patches of residues (mutants M1-M7) led to SCL loss of function, nor did combinations of mutations in the Helix 2 of SCL (mutants M8-M11) (Figure 5.5B). Rather, loss of SCL transcriptional activity and interaction with LMO2 required the combined mutation of residues throughout the Loop and Helix 2 (mutant M13) (Figure 5B, 5C and 5D, lanes 15-17). This region harbors nine divergent residues

Fig. 5.5. The SCL-LMO2 interaction is specified by multiple residues in the Loop and Helix 2 of SCL. (A) Amino acid sequence alignment of the HLH region of SCL orthologues from humans to fruit flies, as well as those of human and mouse NSCL1 proteins. (+) and (-) indicate the functionality of the different proteins in transactivating the c-kit and GPA promoters in collaboration with other partners of the SCL complex. ND indicates SCL and NSCL1 orthologues for which the activity was not determined in our system. (B) Schematic representation of SCL mutants (M1-M13) generated by systematically converting residues of the HLH region of SCL into those found at the corresponding position in NSCL1. (+) and (-) indicate the functionality of each SCL mutant in synergistic activation of the c-kit and GPA promoters. Asterisks indicate the positions of the H217, N226, and K234 residues, which are evolutionarily conserved in all SCL orthologues andp are required for maximal hematopoietic gene activation. (C) Complete SCL inactivation requires full conversion of the Loop and Helix 2 region, while the combined mutation of H217, N226 and K234 leads to partial inactivation of SCL. Transactivation assays were performed with the GPA-84 reporter and the indicated expression vectors as described in Fig.1. The SCL and SCL-M12 expression vectors were titrated at doses ranging from 1.5 to 150 ng, while the SCL-M13 vector was used at higher doses ranging from 50 to 450 ng. Results are representative of 3 independent experiments. (D) The interaction efficiency of SCL-M12 and SCL-M13 with LMO2 perfectly correlates with their transcriptional activity. Immunoprecipitation assays were performed with the indicated antibodies and 293 cell extracts expressing SCL, SCL-M12 or SCL-M13, as well as E47, F-LMO2, Ldb-1 and GFP. For SCL and SCL-M12, immunoprecipitations were carried out using 10, 30 and 60 μg of extract, whereas only the higher dose (60 μg) of extract was used for SCL-M13. Lanes 1, 8 and 15 represent input (I) samples with 10 μg of nuclear extract. Arrowheads point to specific bands revealed by immunoblotting and asterisks indicate immunoglobulin light and heavy chains. Note that SCL-M12 exhibits a strong ≈10-fold reduction in LMO2 interaction, while SCL-M13 is completely deficient for LMO2 association. (E) Structural model of the SCL/E47 heterodimer bound to DNA, based on the crystal structure of the E47/E47 homodimer (Ellenberger *et al.*, 1994). DNA is shown in grey, E47 in red and SCL green. Residues in the Loop and Helix 2 of SCL that are divergent in NSCL1 are represented in a space-fill configuration. H217, N226, and K234 are colored in cyan, while the other divergent residues are in yellow. Note that H217, N226, and K234 are positioned on the same interface of the SCL protein on the opposite side of the surface that contacts E47.

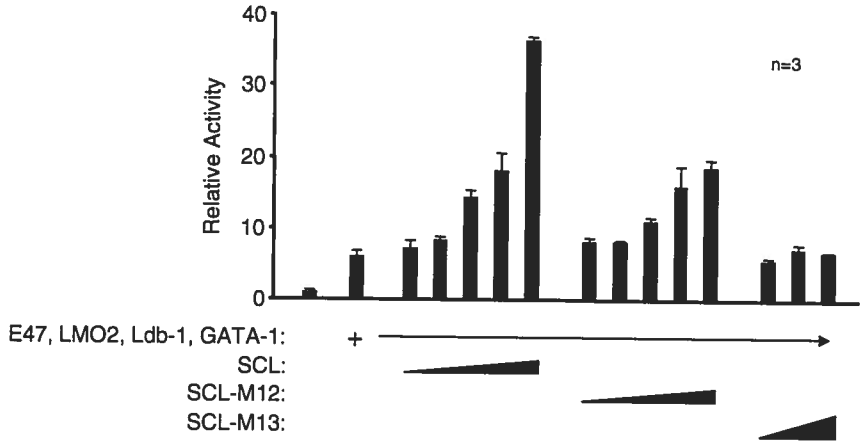
A.

	HELIX 1 / LOOP / HELIX 2	c-kit and GPA activation
H.sapiens SCL	QQNVNGAFAELRKLIPTHPPDKKLSKNEILRLAMKYINFLAKL	+
M.musculus SCL	.....	+
D.rerio SCL	.....	ND
G.gallus SCL	.....	ND
X.laevis SCL	.....	ND
A.mexicanum SCL	.....	ND
T.rubripes SCL	.....S..SN.	ND
D.melanogaster SCL	HE..S.....NVV.....S.I...KL.TGI	+/-
H.sapiens NSCL	VEAF.L.....L..L.....I.....IC..SY.NHV	ND
M.musculus NSCL	VEAF.L.....L..L.....I.....IC..SY.NHV	-

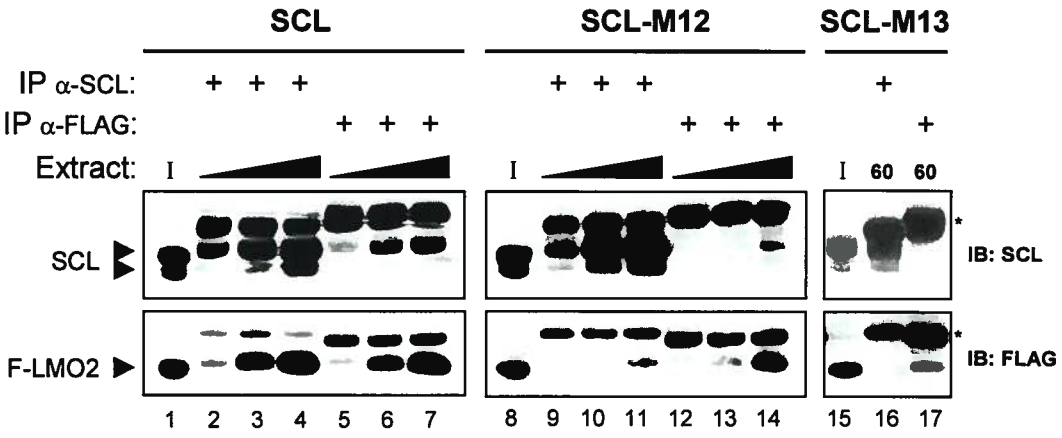
B.

H.sapiens SCL	QQNVNGAFAELRKLIPTHPPDKKLSKNEILRLAMKYINFLAKL	
M1	VEA.....	+
M2	...F.L.....	+
M3	.....L.....	+
M4	.....I.....	+
M5	.....IC.....	+
M6	.....SY....	+
M7	.....NHV	+
M8	.....I.....IC.....	+
M9	.....IC..SY....	+
M10	.....IC..SY.NHV	+
M11	.....I.....IC..SY.NHV	+
M12	.....L.....I.....C.....	+/-
M13	.....L.....I.....IC..SY.NHV	-
	* * *	

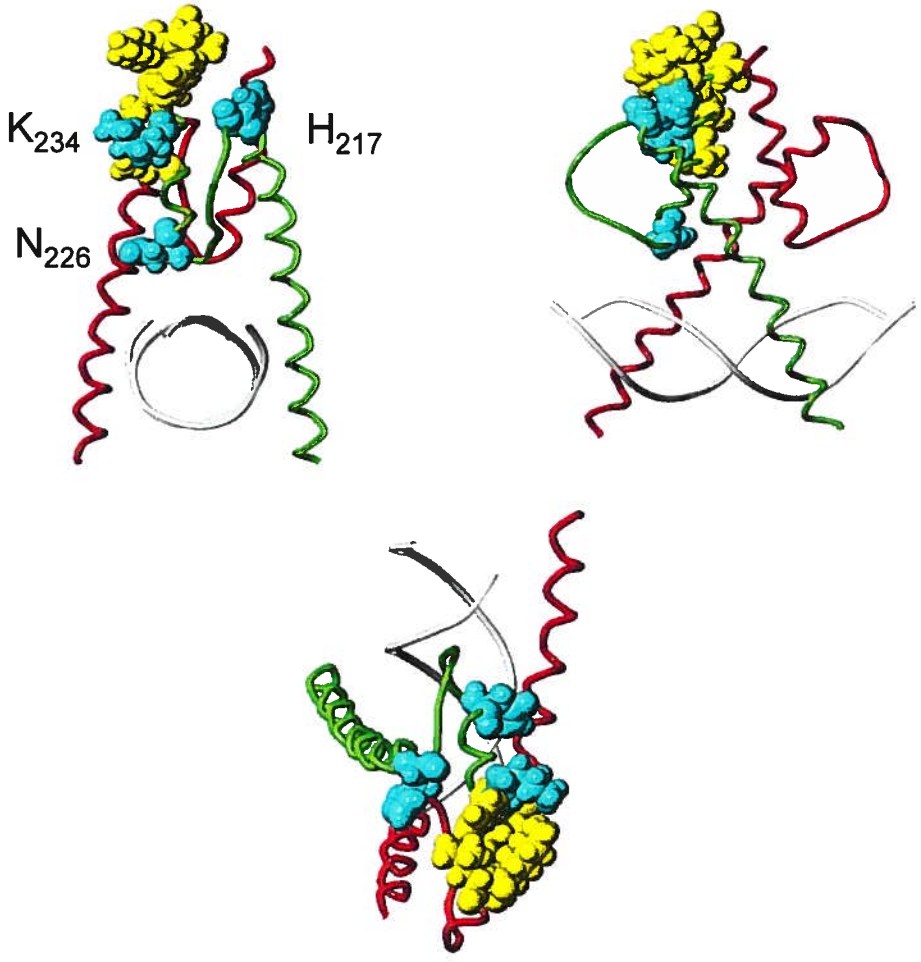
C.



D.


SCL
SCL-M12
SCL-M13
IP α-SCL:
IP α-FLAG:
Extract:
I
60
60
SCL
F-LMO2
IB: SCL
IB: FLAG
1 2 3 4 5 6 7
8 9 10 11 12 13 14
15 16 17

E.


K234
H217
N226

between SCL and NSCL1, six of which are also divergent when comparing human SCL to DroSCL (Figure 5.5A). This led us to speculate that the three remaining residues (H217, N226 and K234), which are conserved in DroSCL but divergent in NSCL1, might be important determinants of SCL functional specificity. Indeed, mutation of these residues (mutant M12) considerably reduced SCL transcriptional activity (Figure 5C) and a 10-fold reduction in LMO2 interaction (Figure 5.5D, lanes 8-14) when compared to wild-type SCL (lanes 1-7). All three of these mutations, H217L, N226I, and K234C, represent non-conservative changes, as the first two polar residues are converted to hydrophobic amino acids, while the third substitution represents a change from a charged to a polar residue. The fact that these amino acids are conserved in organisms ranging from humans to fruit flies suggests that they are important determinants of SCL function. However, since DroSCL is only partially active, additional residues among the 6 that are commonly divergent in DroSCL and NSCL1 are also important for SCL specificity. These results lead us to conclude that the SCL-LMO2 interaction interface is complex and requires several determinants found throughout the Loop and Helix 2 region of SCL.

While the molecular structure of the SCL/E47 heterodimer has yet to be solved, the crystal structure of the E47/E47 homodimer bound to DNA has been previously reported (Ellenberger *et al.*, 1994). In order to understand the structural positioning of the loop and helix 2 residues of SCL that are required for the LMO2 interaction, we conducted molecular modeling of the SCL/E47 heterodimer, using the crystallographic coordinates of the E47/E47 homodimer as a template (Ellenberger *et*

al., 1994). The derived structural model, shown in Figure 5.5E, suggests that most of these residues (represented in spacefill configuration) lie on a single interface that is oriented away from the interaction interface of SCL with E47, or of SCL with DNA. Therefore, these amino acids define a specific docking interface through which LMO2 molecules are recruited to SCL/E47 heterodimers. This type or arrangement thus enables the HLH domain of SCL to accommodate multiple protein interactions at the same time, which is most likely an essential attribute of regulators that function within larger multifactorial complexes.

The SCL-LMO2 interaction nucleates the assembly of higher order complexes

To determine whether the SCL-LMO2 interaction is required for the assembly of the SCL complex on DNA, we next performed EMSA with extracts of 293 cells expressing different SCL mutants in combination with the other partners of the complex. In order to distinguish between the capacities of these mutants to bind DNA as heterodimers with E47 versus their integration into higher order complexes, we performed EMSA with both the TAL-1 and GPA-84 probes. The TAL-1 probe contains an Ebox motif that was identified as a high affinity binding site for SCL/E47 heterodimers (Hsu *et al.*, 1994). As shown in Figure 5.6, the formation of a low mobility complex on the GPA-84 probe was observed with extracts containing SCL and the functional SCL-S7 mutant (lanes 2-3), but was not seen in the presence of the SCL-M12 and -M13 (lanes 4-5), which are deficient for LMO2 interaction. In contrast to the GPA-84 probe, when EMSA were performed with TAL-1, the

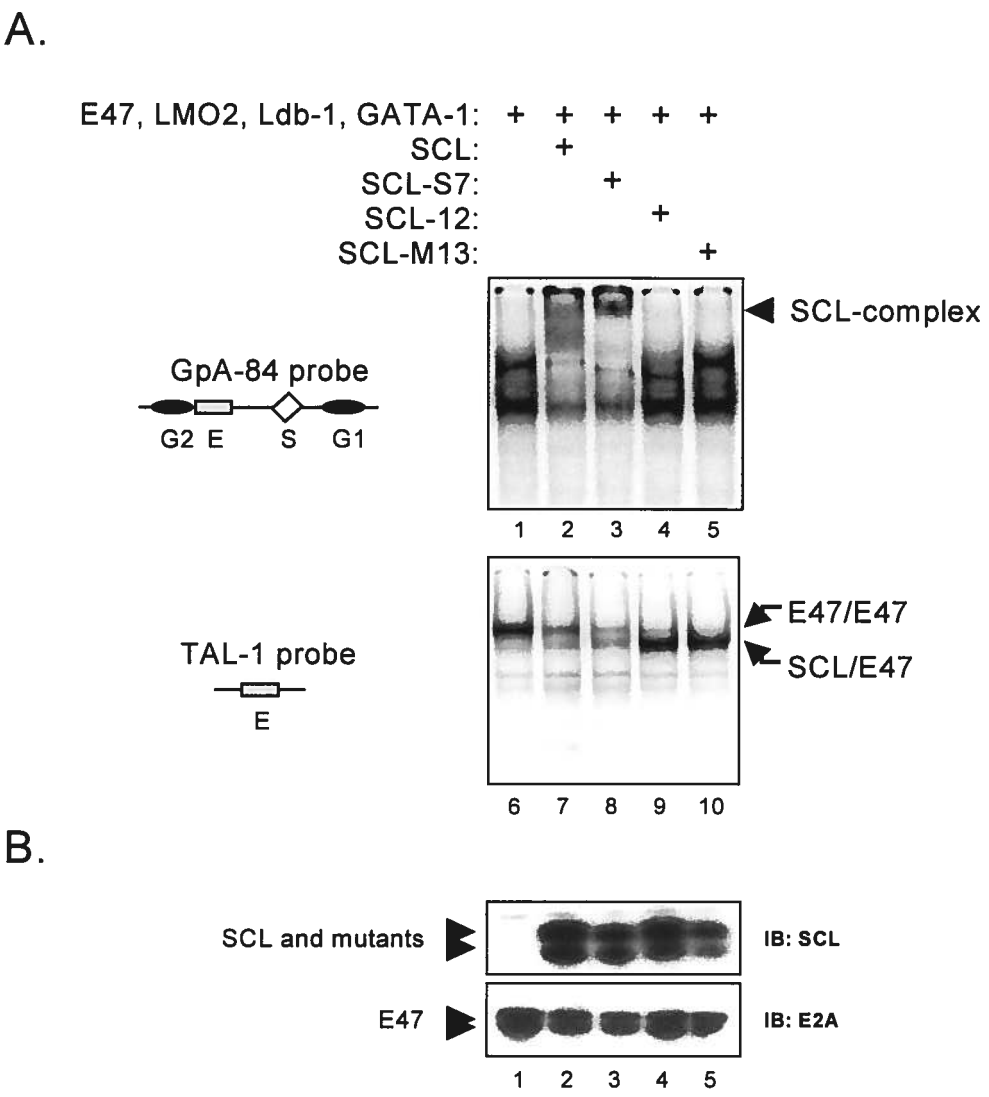


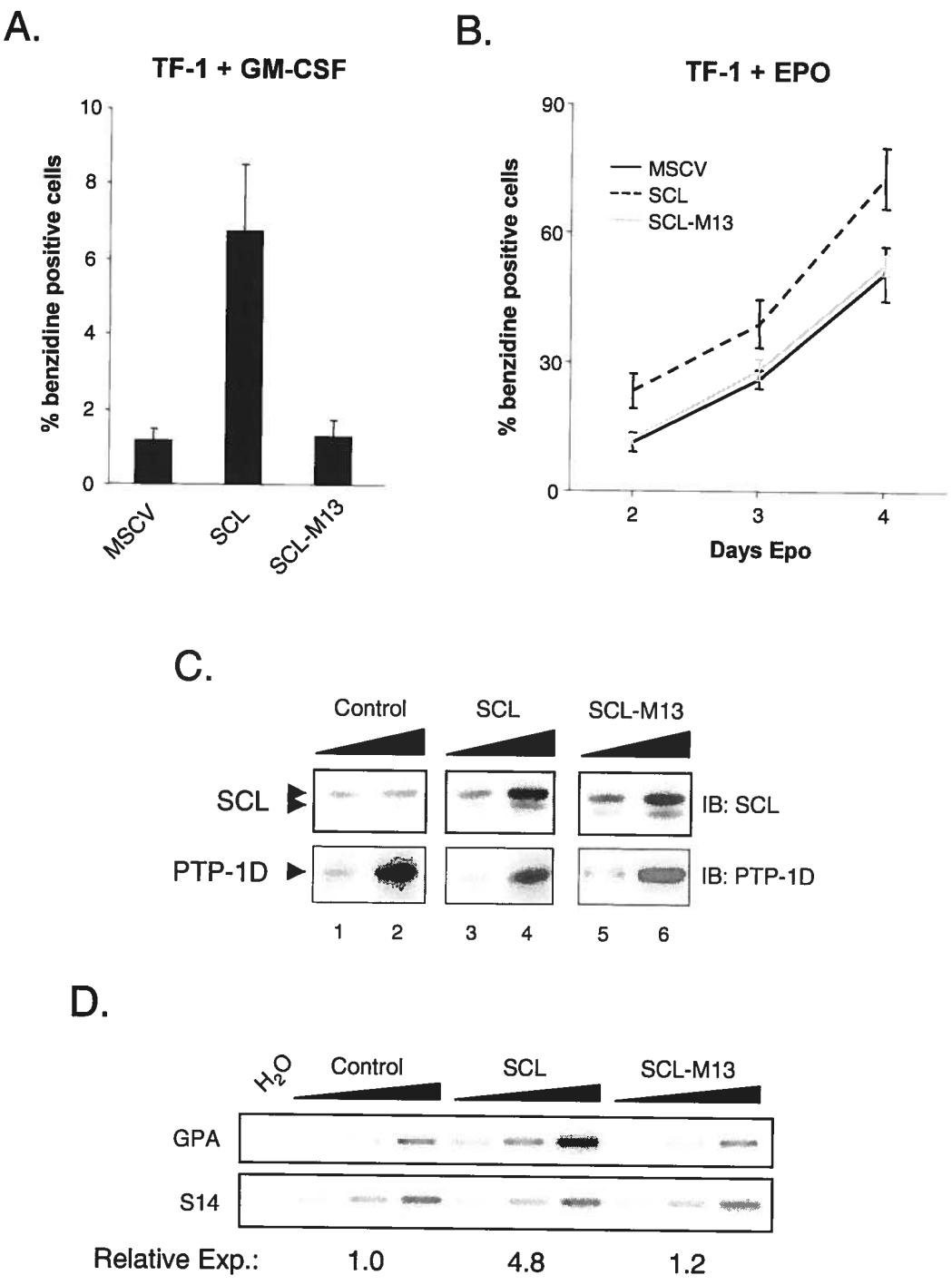
Fig. 5.6. The SCL-LMO2 interaction nucleates the assembly of higher order SCL-containing complexes. (A) EMSA were performed using the GPA-84 (lanes 1-5) and TAL-1 (lanes 6-10) probes, and nuclear extracts (10 μg) of 293 cells transfected with the expression vectors indicated above the panels. The arrowhead points to the low mobility complex containing SCL and its partners, while arrows denote E47/E47 and SCL/E47 dimers. (B) The expression of SCL mutants and E47 in 293 cell extracts was verified by immunoblotting (lanes 1-5).

formation of SCL/E47 heterodimers was much more efficient in extracts expressing SCL-M12 and -M13 (lanes 9-10), compared to SCL and SCL-S7 (lanes 7-8). In fact, most of the E47/E47 homodimers that are observed in the absence of SCL (lane 6) were converted to SCL/E47 heterodimers in extracts with SCL-M12 and -M13 (lanes 9-10). The appropriate expression of SCL mutants and E47 in these nuclear extracts was verified by immunoblotting (lanes 11-15). These results demonstrate that SCL mutants that are unable to associate with LMO2 accumulate as heterodimers with E47, suggesting that the integrity of the SCL-LMO2 interaction is essential to nucleate the assembly of higher order SCL complexes.

The SCL-LMO2 interaction is required for the stimulation of erythroid differentiation by SCL and for endogenous GPA gene activation

SCL has previously been shown to stimulate erythroid differentiation following its enforced expression in hematopoietic cell lines, such as murine erythroleukemia (MEL) and TF-1 cells (Aplan *et al.*, 1992; Hoang *et al.*, 1996). In order to assess whether this biological effect of SCL depends on its ability to interact with LMO2, we utilized retroviral mediated gene transfer to over express SCL or SCL-M13 in TF-1 cells and then assayed for erythroid differentiation. As a control, cells were also infected with retroviruses harboring an empty MSCV-neo genome. Western blotting confirmed that the SCL transgenes were expressed at a three-fold higher level as compared to the endogenome SCL protein (Figure 5.7C). When cultured in the presence of the cytokine GM-CSF, TF-1 cells remain in a proliferative

Fig. 5.7. The SCL-LMO2 interaction is required for the stimulation of erythroid differentiation and for the activation of the endogenous GPA gene by SCL. (A-B) TF-1 cells were infected with control retroviruses (MSCV) or viruses encoding wild-type SCL or SCL-M13. Following selection, the cells were either grown in the presence of (A) 5 ng/mL of GM-CSF or (B) 1U/mL of EPO for the indicated number of days, and stained with benzidine to determine the extent of spontaneous or induced erythroid differentiation. (C) Immunoblotting with an anti-SCL antibody was performed with nuclear extracts of transduced TF-1 cells to verify appropriate over expression of SCL and SCL-M13. The blots stripped and re-hybridized with an antibody directed against the PTP-1D phosphatase as a loading control. (D) GPA mRNA expression in transduced TF-1 cells was assessed by RT-PCR analysis as described in (Lahlil *et al.*, 2004). S14 sequences were amplified as a control for normalization. Amplified fragments were revealed through hybridization with internal oligonucleotide probes. Following normalization, the relative expression (Relative Exp.) of GPA for each cellular sample was quantified compared to that of the MSCV control, which was set at 1.0.


A.
TF-1 + GM-CSF
% benzidine positive cells
10
8
6
4
2
0
MSCV
SCL
SCL-M13
B.
TF-1 + EPO
% benzidine positive cells
90
60
30
0
MSCV
SCL
SCL-M13
2
3
4
Days Epo
C.
Control
SCL
SCL-M13
SCL
PTP-1D
IB: SCL
IB: PTP-1D
1 2 3 4 5 6
D.
H2O
Control
SCL
SCL-M13
GPA
S14
Relative Exp.:
1.0
4.8
1.2

undifferentiated state, and only a weak fraction (≈ 1%) of these cells spontaneously differentiate towards the erythroid pathway and express hemoglobin as revealed by benzidine staining (Figure 5.7A). However, in cells expressing elevated levels of wild-type SCL (Figure 5.7C, compare lanes 1-2 with 3-4), spontaneous erythroid differentiation was stimulated by 6-fold (Figure 5.7A), while this effect was not observed in cells over expressing the SCL-M13 mutant. Similarly, when these cells were grown in the presence of erythropoietin (EPO), a cytokine that induces erythroid differentiation of TF-1 cells, SCL-transduced cells differentiated more efficiently, while the erythroid output of SCL-M13 expressing cells was comparable to control cells (Figure 5.7B). This stimulatory effect of wild-type SCL on erythroid differentiation was accompanied by an induction of the endogenous GPA gene, as GPA mRNA levels were increased by 4.8-fold as determined by semi-quantitative RT-PCR analysis (Figure 5.7D). In contrast, expression of the GPA gene was not increased in SCL-M13 transduced cells. Therefore, these results demonstrate that the stimulatory effect of SCL on erythroid differentiation and its capacity to activate erythroid target genes, such as GPA, in their endogenous chromatin context requires direct interaction with LMO2.

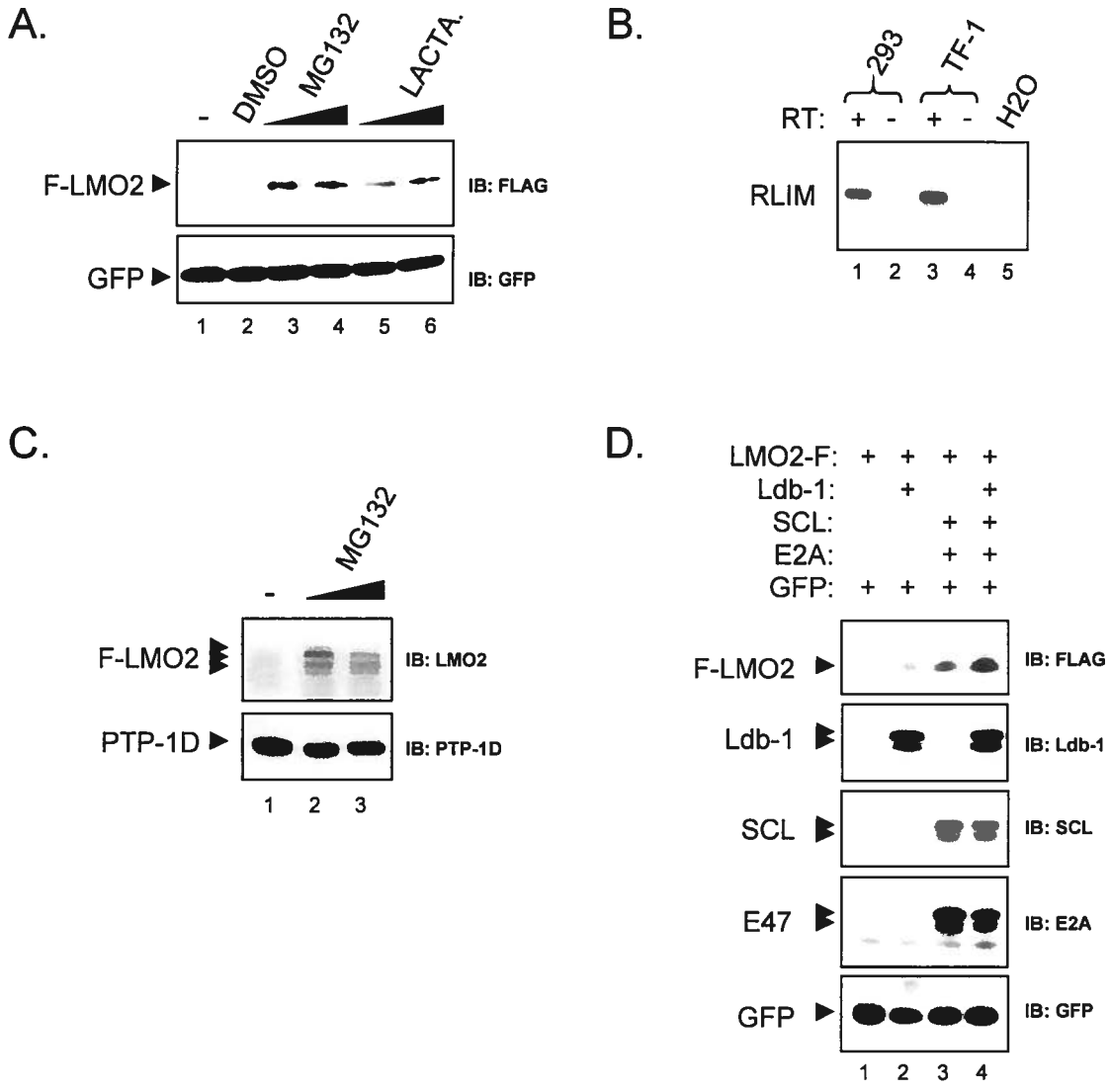
The association of LMO2 with SCL enhances LMO2 protein levels

In recent studies Ldb-1 and LMO2 have been shown to be targeted for proteasomal degradation by the ring-finger ubiquitin protein ligase RLIM/Rnf12 (Ostendorff *et al.*, 2002; Hiratani *et al.*, 2003). Intriguingly, in our experiments we observed that

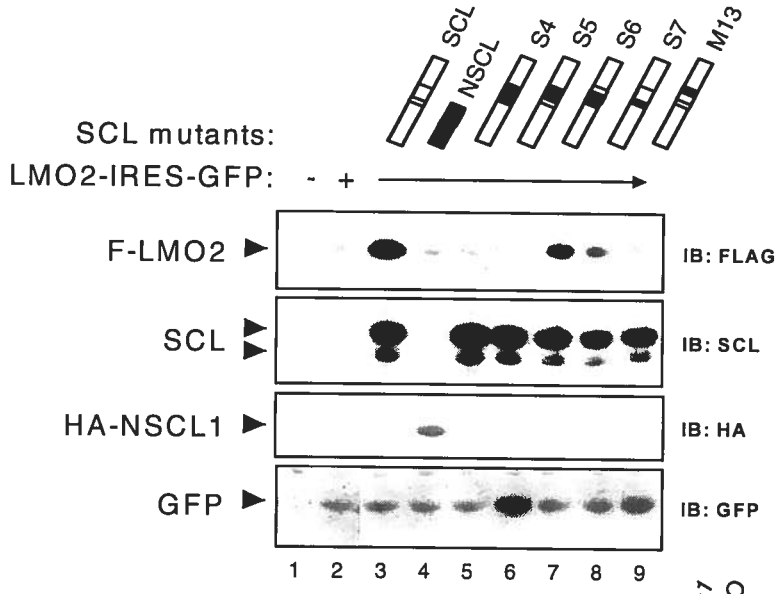
LMO2 was barely detectable when expressed on its own in heterologous 293 cells (Figure 5.8A, lane 1). Since we found that these cells efficiently express RLIM as assessed by RT-PCR analysis (Figure 5.8B, lane 1), we next sought to assess whether LMO2 might be targeted for proteasomal degradation. Therefore, we transfected 293 cells with the MSCV-LMO2-IRES-GFP vector, which produces a bicistronic mRNA molecule that encodes both the LMO2 and GFP proteins, and treated the cells with two proteasome inhibitors, MG132 and Lactacystine. As shown in Figure 5.8A, LMO2 expression was strikingly increased following treatment with both MG132 and Lactacystine (lanes 3-6), but not with DMSO alone (lane 2), while the expression of GFP from the same mRNA remained constant in all of the samples. Since we also found that TF-1 hematopoietic cells strongly express the RLIM ubiquitin ligase (Figure 5.8B, lane 3), we next sought to assess whether endogenous LMO2 protein levels in TF-1 cells are regulated by the proteasome. As shown in Figure 5.8C, while LMO2 protein levels are quite low in control TF-1 cells (lane 1), LMO2 expression increases substantially following treatment with MG132 (lanes 2-3). Together, these results suggest that LMO2 is targeted for proteasomal degradation in both heterologous and hematopoietic cells.

Since LMO2 is essential for the function of SCL-containing complexes, we next asked the question whether efficient LMO2 expression might be dependent on the presence of its interacting partners. This indeed seemed to be the case, as LMO2 protein levels in 293 cells became detectable upon co-expression of either Ldb-1 or SCL/E47 (Figure 5.8D, lanes 1-3), and was further augmented in the presence of

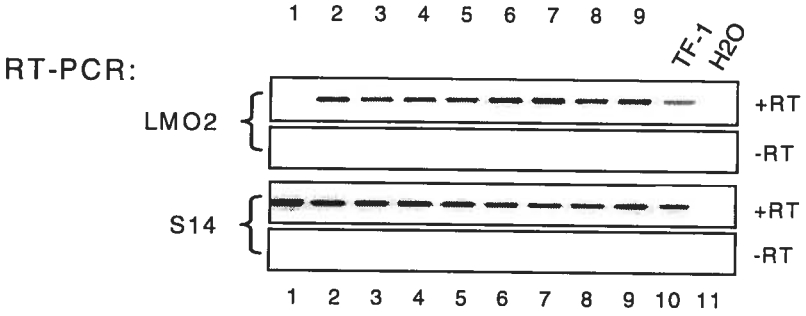
Fig. 5.8. LMO2 protein expression is stabilized through its association with SCL. (A) LMO2 is targeted for proteasomal degredation. 293 cells were transfected with the MSCV-LMO2-IRES-GFP expression vector. After 36 h, the cells were treated for 1 h with MG132 (12.5 and 25 μM) and Lactacystine (10 and 20 μM) and total cell lysates were analyzed by immunoblotting with the anti-FLAG and anti-GFP antibodies. Since MG132 is stored in dimethyl sulfoxyde (DMSO), a control DMSO treated sample was also prepared. (B) The E3 ubiquitin ligase RLIM is expressed in 293 and TF-1 cells. RT-PCR analysis was performed with mRNA from parental 293 and TF-1 cells. PCR products were transferred on membranes and hybridized with internal oligonucleotide probes. (C) Treatment of hematopoietic TF-1 cells for 1h with MG132 (25 μM) leads to an increase in endogenous LMO2 protein levels. TF-1 cell nuclear extracts were first immunoblotted with an anti-LMO2 antibody, while detection of the PTP-1D phosphatase served as a loading control. (D) Efficient detection of the LMO2 protein requires the presence of its interacting partners. 293 cells were transfected with different combinations of expression vectors for F-LMO2, Ldb-1, SCL, E47, and GFP, as indicated above the panels. After 36 hours, nuclear extracts were prepared and analyzed by immunoblotting with the antibodies indicated on the right of each panel. Note that F-LMO2 expression is barely detectable when expressed on its own (lane 1), but becomes efficiently expressed in the presence of Ldb-1, SCL/E47 or when all of the partners are co-expressed (lanes 2-4). (E) The apparent stabilization of LMO2 expression by SCL depends on their direct physical interaction. 293 cells were transfected with the MSCV-LMO2-IRES-GFP, as well as expression vectors for SCL, NSCL1 or different SCL mutants in combination with E47. The expression of F-LMO2 is only efficiently detected in extracts with LMO2-inteacting SCL molecules. (F) LMO2 mRNA levels are comparable in transfected 293 cells. A portion of cellular samples analyzed in (E) were subjected to RT-PCR analysis. TF-1 cells were included as a positive control for LMO2 amplification, and amplification of S14 served as a control for the amount of cDNA. No amplification was observed with mRNA samples processed without reverse transcriptase or in H_2O control PCR reactions. (G) The LMO2 protein is stabilized in the presence of SCL, but not SCL-M13. The MSCV-LMO2-IRES-GFP vector was transiently transfected in 293 cells in the presence of E47 and SCL or SCL-M13. After 36 h, the cells were treated with cycloheximide (100 μg/ml) for the indicated time periods and total cell lysates were analyzed by Western blotting. For (A-C and E-F), arrowheads point to specific bands revealed by immunoblotting with the antibodies indicated to the right of each panel.


A.
-
DMSO
MG132
LACTA.
F-LMO2
IB: FLAG
GFP
IB: GFP
1 2 3 4 5 6
B.
293
TF-1
RT: + - + -
H2O
RLIM
1 2 3 4 5
C.
-
MG132
F-LMO2
IB: LMO2
PTP-1D
IB: PTP-1D
1 2 3
D.
LMO2-F: + + + +
Ldb-1: + +
SCL: + +
E2A: + +
GFP: + + + +
F-LMO2
IB: FLAG
Ldb-1
IB: Ldb-1
SCL
IB: SCL
E47
IB: E2A
GFP
IB: GFP
1 2 3 4

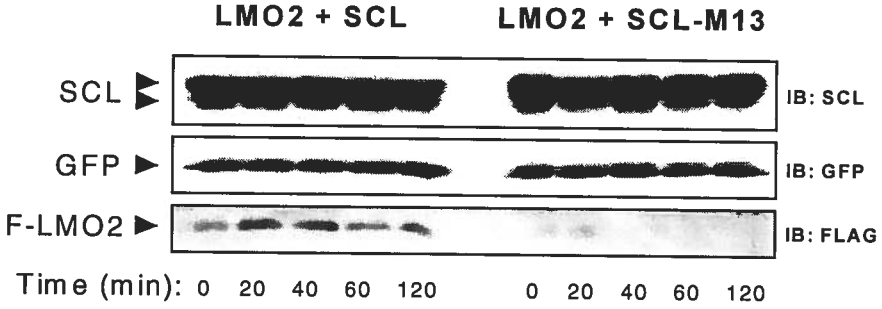
E.

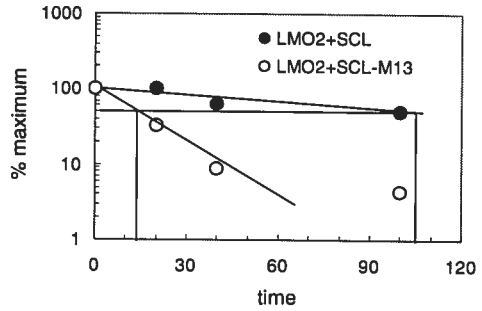

SCL
NSCL
S4
S5
S6
S7
M13
SCL mutants:
LMO2-IRES-GFP: - +
F-LMO2
IB: FLAG
SCL
IB: SCL
HA-NSCL1
IB: HA
GFP
IB: GFP
1 2 3 4 5 6 7 8 9

F.


RT-PCR:
TF-1
H2O
LMO2
+RT
-RT
S14
+RT
-RT
1 2 3 4 5 6 7 8 9 10 11

G.


LMO2 + SCL
LMO2 + SCL-M13
SCL
IB: SCL
GFP
IB: GFP
F-LMO2
IB: FLAG
Time (min): 0 20 40 60 120 0 20 40 60 120


1000
100
10
1
% maximum
LMO2+SCL
LMO2+SCL-M13
0 30 60 90 120
time

all three partners (lane 4). In contrast, GFP expression from an independent vector was constant in each of these samples. Thus, these results suggested that the association of LMO2 with its interacting partners counteracts its degradation. Having identified the determinants of the SCL-LMO2 interaction, we then considered whether the direct physical association of these factors is required for the apparent stabilization of LMO2 expression. Therefore, LMO2 was co-expressed with SCL, NSCL1, or different SCL mutants, in the absence of Ldb-1 so that LMO2 expression would be dependent on the presence of SCL as an interacting partner. As shown in Figure 5.8E, LMO2 was barely detectable when expressed on its own (lane 2), or when co-expressed with NSCL1 or SCL mutants that are unable to interact with LMO2 (lanes 4-6, 9). In contrast, LMO2 was efficiently expressed when in the presence of SCL (lane 3), as well as the functional S6 and S7 mutants (lane 7-8). GFP protein levels, expressed from the same mRNA molecules that encode LMO2, were comparable in all of the samples. Furthermore, when RT-PCR analysis was performed on the same cellular samples as in Figure 5.8E, LMO2 mRNA levels were found to be equivalent in all of the samples (Figure 5.8F), confirming that the effects of SCL are post-translation and require direct interaction with the LMO2 protein. To more precisely determine whether the stability of LMO2 was influenced by SCL, we next measured the half-life of LMO2 in the presence of SCL or SCL-M13 after shutting down protein synthesis using cycloheximide (Figure 5.8G). In cells transfected with the MSCV-LMO2-IRES-GFP, SCL and E47 vectors, the expression levels of SCL, GFP and LMO2 were quite stable throughout the time course of cycloheximide treatement (from 0 to 120 minutes). In contrast, when SCL was

replaced by the SCL-M13 mutant, which is deficient for interaction with LMO2, the basal level of LMO2 was much lower and LMO2 expression became undetectable 40 minutes after cycloheximide treatment, suggesting that interaction with SCL stabilizes the expression of the LMO2 protein. The half-life of LMO2 was calculated to be approximately 14 minutes in the absence of SCL (Figure 5.8G). Therefore, in addition to playing a crucial role in nucleating the assembly of a higher order complexes required for hematopoietic gene regulation, these results suggest that the direct interaction of SCL with LMO2 serves to stabilize LMO2 protein expression by slowing its rapid turnover, which may represent an important mechanism ensuring the appropriate spatio-temporal assembly of SCL-LMO2 nucleated complexes.

5.5 Discussion

Differentiation of hematopoietic cells is controlled by transcription factors that work in concert to establish and maintain specific programs of gene expression (Sieweke and Graf, 1998). By functioning in a combinatorial manner, these factors are believed to achieve greater specificity in regulating appropriate sets of target genes. In the present study, we define the molecular determinants that control the assembly of the SCL complex, as transcription activation requires that SCL associates with specific partners within higher order transcription complexes (Ono *et al.*, 1998; Vitelli *et al.*, 2000; Lecuyer *et al.*, 2002; Lahlil *et al.*, 2004). Here we show that SCL exhibits exquisite functional specificity within this complex, due to its exclusive interaction with the LIM-only protein LMO2. SCL interacts with LMO2 through multiple residues in the Loop and Helix 2 of SCL, and this interaction is essential for the nucleation of larger transcription complexes on target regulatory elements, required for activation of SCL target genes and for the induction of erythroid differentiation. Finally, we demonstrate that this interaction enhances LMO2 protein expression, thus adding an unforeseen post-translational control level that ensures the appropriate spatio-temporal assembly of SCL complexes during hematopoiesis.

The SCL-LMO2 interaction and the combinatorial control of hematopoietic gene expression

The biological functions of the SCL and LMO2 genes are intimately intertwined during normal hematopoiesis and leukemogenesis. First, both genes were initially

identified by virtue of their involvement in recurrent chromosomal rearrangements in childhood T-ALL and their ectopic co-expression in transgenic mouse models recapitulates the leukemic process observed in human T-ALL patients (Larson *et al.*, 1996; Aplan *et al.*, 1997; Chervinsky *et al.*, 1999; Begley and Green, 1999; Herblot *et al.*, 2000). Second, during development, the expression of both genes is first observed in subsets of embryonic and extraembryonic mesodermal cells that give rise to hematopoietic and endothelial tissues in which their expression persists (Silver and Palis, 1997; Elefanty *et al.*, 1999; Manaia *et al.*, 2000; Yamada *et al.*, 2000). In addition, functional studies indicate that $SCL^{-/-}$ and $LMO2^{-/-}$ mice die at mid-gestation (E9-10) due to severe anemia and ES cells that are deficient for either gene fail to contribute to any hematopoietic tissue in adult mouse chimeras (Warren *et al.*, 1994; Robb *et al.*, 1995; Shivdasani *et al.*, 1995; Porcher *et al.*, 1996; Robb *et al.*, 1996; Yamada *et al.*, 1998). The similarities in the biological functions of SCL and LMO2, as well as their ability to synergistically induce the hematopoietic cell fate following their ectopic co-expression in *Xenopus* embryos (Mead *et al.*, 2001), suggests that these proteins function within the same complexes to establish and maintain the homeostasis of the hematopoietic system. Here we provide molecular evidence that the direct interaction of SCL and LMO2 is essential for transcriptional activation of several target genes, explaining how both factors closely interconnect during hematopoietic development.

The critical importance of LMO2 in human leukemogenesis has recently been reiterated by the finding that retroviral insertions in the proximity of the LMO2 gene

resulted in the development of a T-ALL type lympho-proliferative syndrome in two immuno-deficient patients undergoing gene therapy based on retroviral vectors (Hacein-Bey-Abina *et al.*, 2003a; Hacein-Bey-Abina *et al.*, 2003b). Having identified the molecular determinants that specify the association of SCL with LMO2 will allow the development of genetic strategies to clarify which biological functions assigned to these factors require their interaction in vivo and which processes are regulated by each factor independently.

Our findings suggest that the main function of SCL and LMO2 is to nucleate the assembly of higher order transcription factor complexes, thus establishing a combinatorial code for the activation of hematopoietic target genes. The discovery that SCL biological specificity is conferred by residues in the Loop and Helix 2 region of its HLH domain contrasts with previous observations made with the myogenic master regulator MyoD, whose capacity to induce myogenesis in fibroblastic cells is determined by residues located within and in proximity to the basic region (Davis *et al.*, 1990; Davis and Weintraub, 1992). It remains unclear whether these MyoD basic region residues create specificity in DNA target site selection or whether they enable MyoD to interact with key regulatory co-factors required for myogenic conversion. Nevertheless, these characteristics suggest that MyoD and SCL function quite differently at the molecular level, since the integrity of the basic region of SCL is dispensable or redundant for many of its molecular and biological functions (Porcher *et al.*, 1999; O'Neil *et al.*, 2001; Lecuyer *et al.*, 2002; Lahlil *et al.*, 2004; current study). While it has long been known that the myogenic

potential of MyoD depends on the cellular environment and regulators with which it is co-expressed (Schafer *et al.*, 1990), our current study of SCL-containing complexes establishes the view that tissue-specific bHLH factors can execute a nucleation function within higher order transcription factor complexes. This function of SCL differs from that played by the ubiquitous bHLH factor E47 within these complexes, since we recently showed that a truncated form of E47 reduced to its bHLH domain, which lacks highly conserved transactivation domains, is non-functional within the SCL complex (Lahlil *et al.*, 2004), suggesting that E47 provides a transactivation function to the complex. In contrast, on several target genes, GATA family members provide an essential DNA binding function to SCL-containing complexes (Ono *et al.*, 1998; Lahlil *et al.*, 2004), and can contribute to creating specificity in target gene selection (Lahlil *et al.*, 2004). Therefore, functional specialization occurs within the SCL complexes and the composition of these complexes can vary depending on the cellular context and target genes. Nonetheless, a recurrent function of the SCL-LMO2 interaction is to provide the glue that allows the appropriate assembly of these complexes. Since cell fate determination is most likely regulated by competing transcription factor codes, favoring specific protein interactions probably tips the balance towards particular differentiation pathways. One can envision that developmental cues that promote the SCL-LMO2 interaction and the nucleation of larger SCL complexes, either in the context of mesodermal or hematopoietic precursor cells, would enable the selection of the hematopoietic cell fate at the expense of other tissues or would promote differentiation towards particular blood cell lineages. It is likely that different combinations of bHLH-LIM

factor interactions are utilized as a general strategy to control differentiation in many tissues. For instance, NSCL1 might interact with distinct LMO family members during neuronal development (Bao *et al.*, 2000; Manetopoulos *et al.*, 2003), while the LIM-only proteins of the CRP family have been shown to enhance the myogenic activity of MyoD and to function as adaptor proteins in multifactorial complexes that potently activate smooth muscle cell gene expression and differentiation (Kong *et al.*, 1997; Chang *et al.*, 2003). Therefore, it is alluring to speculate that interactions between bHLH and LIM protein family members have evolved to create codes for specific cell fates by enabling the formation of tissue-specific transcription factor complexes.

A striking characteristic of SCL containing complexes is that they demonstrate a switch-like behavior in transcription regulation, since the co-expression of all partners is required in order to observe a substantial transcriptional output ((Lecuyer *et al.*, 2002; Lahlil *et al.*, 2004); present study). This property, described as "coincidence detection" by Hartwell and colleagues (Hartwell *et al.*, 1999), most likely ensures the appropriate spatio-temporal expression of SCL target genes and is reminiscent of previously described enhanceome complexes that control the expression of the interferon-β (INF-β) and T-cell receptor α (TCR-α) genes (Grosschedl, 1995; Merika and Thanos, 2001). Appropriate activation of both of these genes requires the cooperative assembly of higher order complexes, containing tissue-specific and signal induced transcription factors, on enhancer sequences that harbor precise helical arrangements of transcription factor binding sites (Giese *et al.*,

1995; Thanos and Maniatis, 1995; Kim and Maniatis, 1997; Mayall *et al.*, 1997). The function of these complexes also relies on architectural proteins, such as HMG I(Y) and LEF-1, which possess DNA bending activities that are essential for enhanceosome assembly, and on interactions with chromatin remodeling complexes and the basal transcriptional machinery. By analogy to the INF-β and TCR-α enhanceosomes, it is possible that SCL and its partners might represent central components of hematopoietic-cell specific enhanceosome complexes. While it remains to be determined whether additional components regulate the assembly of these complexes on DNA, our data demonstrate how critical protein interactions can nucleate larger complexes, thus providing a binary switch in transcription activation.

Protein stabilization as a mechanism to ensure specificity in transcription factor assemblages

Over the past few years, ubiquitination and proteasomal mediated degradation of transcription factors has emerged as a major mechanism in the control of gene transcription (Thomas and Tyers, 2000; Conaway *et al.*, 2002; Lipford and Deshaies, 2003). In some instances degradation serves to limit the activity of specific transcription factors, while it has also been shown that the high potency of several transcriptional activators is directly correlated with their rate of turnover (Molinari *et al.*, 1999; Thomas and Tyers, 2000; Salghetti *et al.*, 2000; Lipford and Deshaies, 2003). The underlying instability of LMO proteins most likely serves as a mechanism to prevent the assembly higher order complexes in the absence of

appropriate interacting partners, thus providing an efficient strategy for the establishment of specific transcription factor codes. Interestingly, the RING-finger protein RLIM, originally identified as a co-repressor of LIM-homeodomain (LIM-HD) transcription factors (Bach *et al.*, 1999), was recently shown to be a E3-ubiquitin ligase that can ubiquitinate and target Ldb-1 and LMO2 for proteasomal degradation (Ostendorff *et al.*, 2002). This process was proposed as a mechanism to allow the exchange of co-factors associated with DNA-bound LIM-HD proteins (Ostendorff *et al.*, 2002). Ldb-1 is a general co-factor of LIM domain proteins that is essential for patterning in diverse species (Morcillo *et al.*, 1997; Fernandez-Funez *et al.*, 1998; Mukhopadhyay *et al.*, 2003), and it has recently been shown that RLIM mediated degradation of Ldb-1 ensures the proper stoichiometry of Ldb-1 containing complexes during development (Hiratani *et al.*, 2003). Furthermore, work in Drosophila has revealed that LIM-HD and LMO proteins compete for association with the drosophila orthologue of Ldb-1 (Chip) in order to ensure their stability in vivo (Milan and Cohen, 1999; Milan and Cohen, 2000; Weihe *et al.*, 2001). Our finding that efficient LMO2 expression requires the simultaneous presence of either Ldb-1 or SCL, which belong to two distinct protein families, suggests that protein stabilization by interacting co-factors might be a major mode of regulation of LIM domain proteins. Thus, the degradation of LMO2 most likely represents an important control point in the assembly of SCL-containing complexes that may enable a more finely tuned regulation of specific programs of gene expression within the hematopoietic hierarchy. It remains to be elucidated whether protein stabilization

plays an important role in governing the cell fate determining properties of SCL-LMO2 containing complexes during the establishment of the hematopoietic system.

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CHAPITRE 6

DICUSSION

L'élaboration du répertoire cellulaire qui compose un organisme eucaryote complexe est accomplie en grande partie par des réseaux de régulateurs transcriptionnels qui établissent des patrons spécifiques d'expression génique [1,2]. Il est généralement accepté que l'ensemble des interactions homo- ou hétéro-typiques qu'entretiennent ces facteurs entre eux, combiné à l'agencement modulaire des éléments de régulation en *cis* retrouvés dans leurs gènes cibles, permet de diversifier les réponses transcriptionnelles qu'ils orchestrent et représente la source déterminante qui assure la variabilité phénotypique [3,4]. Par sa structure hiérarchique bien caractérisée, qui donne lieu à une douzaine de types cellulaires spécialisés, le système hématopoïétique représente un excellent modèle pour étudier la combinatoire des facteurs de transcription. En effet, un nombre important de régulateurs transcriptionnels ont été identifiés au sein de ce système dont certains, comme le facteur à domaine bHLH SCL, remplissent des fonctions importantes dans plusieurs embranchements du réseau hématopoïétique en agissant au sein de complexes multifactoriels. Ainsi, l'hypothèse à la base de cette thèse était que l'étude des mécanismes de régulation transcriptionnelle de SCL et de ses partenaires fournirait un paradigme pour comprendre les rouages du contrôle combinatoire de la transcription histo-spécifique, un thème dont la portée s'étend à de nombreux systèmes.

6.1 Fonctions biologiques de SCL, un "maître" régulateur de l'hématopoïèse

Bien que le gène SCL fut initialement identifié comme une cible récurrente de réarrangements chromosomiques chez des patients atteints de T-ALL [5], la

réalisation qu'il encode un régulateur central du développement hématopoïétique a d'abord été révélée par l'observation que des souris déficientes pour SCL démontent une absence complète de cellules hématopoïétiques [6,7]. Ces évidences permettaient de placer SCL au tout début de la hiérarchie des modulateurs du système sanguin et suggéraient qu'il représente un 'maître' régulateur dans la spécification du compartiment hématopoïétique [5,8]. Cette propriété de spécification tissulaire est partagée par de nombreux facteurs bHLH dans différentes espèces et dans des processus d'organogenèse variés [9,10], tel qu'illustré par l'étude d'un des membres fondateur de cette famille de protéines, le facteur myogénique MyoD, initialement découvert grâce à sa capacité à convertir des cellules fibroblastiques en myoblastes [11]. Plusieurs membres de cette famille détiennent la capacité d'enclencher un programme ectopique de différenciation suite à leur sur-expression [12-21], suggérant que les facteurs bHLH représentent des instigateurs essentiels des choix de destin cellulaire dans de nombreux tissus. Cependant, bien qu'ils soient des 'maîtres' régulateurs, il est clair que ces facteurs n'agissent pas seuls, mais ils requièrent plutôt la présence de co-facteurs spécifiques afin d'exécuter leurs fonctions de spécification cellulaire [22,23].

Outre son importance dans la l'établissement du système hématopoïétique durant l'embryogenèse, l'expression de SCL est maintenue au sein du compartiment hématopoïétique définitif dans les HSC, les précurseurs pluripotents, et durant la différenciation des cellules érythroïdes et mégakaryocytaires [5,24]. Des études récentes d'ablation génique inductible ont établi que l'expression soutenue de SCL

est essentielle pour la différenciation vers les voies érythroïdes et mégakaryocytaires [25,26], cependant son rôle dans les cellules souches reste controversé. En effet, Mikkola et al. ont démontré que des cellules déficientes pour SCL conservent leurs propriétés de cellule souche après transplantation in vivo, suggérant qu'une fois qu'il a spécifié la formation des HSC, l'expression de SCL n'est pas requise pour l'homéostasie de cette population cellulaire [26]. Bien qu'il soit possible que SCL n'exerce pas de fonction importante au niveau des HSC, il est aussi concevable qu'il démontre une redondance fonctionnelle avec d'autres facteurs bHLH hématopoïétiques hautement apparentés comme Lyl-1, tel qu'observé pour certains facteurs bHLH myogéniques [27,28], ou qu'il y existe des mécanismes compensatoires in vivo pour protéger les HSC d'une perturbation dans un réseau particulier de régulation. Malgré ces incertitudes, il est clair que SCL est un régulateur crucial à plusieurs niveaux durant l'hématopoïèse, soit dans la spécification des HSC et la differentiation des cellules érythroïdes et mégakaryocytaires. Toutefois, les gènes cibles régulés par SCL dans ces différents compartiments cellulaires et ses mécanismes d'action au niveau moléculaire demeuraient énigmatiques au moment où j'ai entamé mes études doctorales. Or, par notre capacité à identifier des gènes cibles de SCL spécifiques aux cellules progénitrices ou érythroïdes, nous avons été en mesure de développer des modèles afin de caractériser les propriétés moléculaires de SCL dans ces deux contextes cellulaires.

6.2 Une cible de SCL dans les HSC et les cellules progénitrices: le gène c-kit

Afin de d'évaluer la fonction de SCL dans un modèle de cellules hématopoïétiques pluripotentes, notre laboratoire a initialement entrepris une approche de criblage fonctionnel à l'aide de cellules TF-1, une lignée hématopoïétique oligopotente dont la survie en culture nécessite la présence de cytokines et dont la différenciation peut être induite vers différentes voies du réseau hématopoïétique [29,30]. Or, Krosl et al. ont observé que l'expression d'ARN antisense dirigé contre SCL dans les cellules TF-1 menait à une perturbation spécifique de l'expression et de la fonction de survie du récepteur à domaine tyrosine kinase c-Kit [30], suggérant que le gène c-kit soit une cible potentielle de SCL. Ces deux gènes sont exprimés dans l'ensemble des sites hémogéniques au cours du développement [31,32], et nous avons démontré qu'ils sont co-exprimés dans des fractions cellulaires de moelle osseuse enrichies en précurseurs hématopoïétiques [33]. De plus, certaines mutations du gène c-kit, tel qu'observé chez les souris mutantes *Whitespotting* (*W*), perturbent dramatiquement l'hématopoïèse définitive en causant une réduction des progéniteurs hématopoïétiques [34]. Par une combinaison d'approches complémentaires, j'ai pu démontrer que SCL est effectivement un régulateur direct du promoteur proximal du gène c-kit [33]. Or, l'activation du promoteur c-kit par SCL nécessite la formation d'un complexe multifactoriel (complexe SCL), incluant des partenaires ubiquitaires (E47, Ldb-1 et Sp1) et hémato-spécifiques (LMO2, GATA-2), dont le recrutement au niveau du promoteur c-kit s'effectue via un site de liaison pour le facteur Sp1. Bien qu'un complexe similaire ait été caractérisé auparavant par une approche de

CASTing avec des extraits de cellules érythroïdes [35], cette étude représentait la première démonstration de l'effet activateur du complexe SCL sur un promoteur cellulaire [33]. Puisque SCL joue un rôle central au sein de ce complexe et que son expression ectopique in vivo conduit à l'induction du gène c-kit endogène [33], nous avons proposé l'hypothèse qu'une des fonctions de SCL soit de nucléer la formation de complexes multifactoriels requis pour l'activation de gènes hématopoïétiques. En activant des gènes cibles comme c-kit dans les HSC et les progéniteurs hématopoïétiques, il est possible qu'une des fonctions du complexe SCL dans ces compartiments cellulaires soit d'assurer la survie ou le maintien d'une réserve de cellules pluripotentes.

6.3 Les gènes cibles érythroïdes de SCL: le cas de la glycophorine A (GPA)

Alors que le promoteur c-kit offrait un modèle intéressant pour l'étude des mécanismes d'action de SCL dans le contexte d'un gène cible de cellules progénitrices, les gènes régulés par SCL dans le compartiment érythroïde demeuraient ambigus. Bien que des sites potentiels de liaison pour le complexe SCL furent identifiés dans des régions régulatrices des gènes GATA-1 et EKLF [36,37], qui encodent des facteurs de transcription érythroïdes, la démonstration que SCL active directement ces gènes n'a jusqu'à maintenant pas été fournie. Or, dans une étude précédente du laboratoire, il avait été démontré que l'expression ectopique de SCL dans les cellules TF-1 cause une augmentation de l'expression du marqueur érythrocytaire GPA tout en rehaussant la différenciation vers la voie érythroïde [29],

ce qui nous a poussé à investiguer la possibilité que le gène GPA soit une cible érythroïde de SCL. Ce gène fait partie d'un regroupement de gènes hautement homologues sur le chromosome 4q28-q31, incluant les gènes GPB et GPE, dont la région promotrice est hautement conservée, alors que la séquence codante est fortement divergente. Ces gènes semblent avoir évolué suite à des événements de duplication du gène GPA et ils encodent tous des glycoprotéines membranaires spécifiques aux cellules érythroïdes [38]. La GPA s'avère une des protéines les plus abondantes de la membrane cytoplasmique des cellules érythroïdes, dont la composition du domaine extracellulaire riche en acide sialique confère une charge globale négative à la membrane des globules rouges, ayant pour effet de minimiser leur agrégation dans la circulation [39,40]. Par des études de gain et de perte de fonction de SCL dans la lignée TF-1 et dans des cellules primaires hématopoïétiques, nous avons démontré qu'il y existe une corrélation parfaite entre les niveaux d'expression de SCL et du gène GPA endogène [41]. De plus, de manière semblable à l'étude du promoteur c-kit, nous avons établi que le promoteur GPA est directement activé par un complexe multiprotéique incluant SCL, E47, LMO2, Ldb-1, GATA-1 et Sp1, qui s'associe avec le promoteur GPA via une combinaison d'éléments en *cis* comprenant deux motifs consensus GATA, une boîte-E et un site de liaison pour Sp1 [41]. Or, l'agencement de ces motifs d'ADN est parfaitement conservé dans les promoteurs des gènes GPB et GPE, suggérant que ces derniers comptent aussi parmi les cibles du complexe SCL dans les cellules érythroïdes. En plus des gènes glycophorines, une étude récente par Xu et al. a permis d'identifier le gène encodant la protéine 4.2, une autre composante structurale essentielle de la

membrane des globules rouges, comme une cible additionnelle du complexe SCL [42]. GPA et la protéine 4.2 font partie de macrocomplexes protéiques membranaires, qui incluent le transporteur d'anions Band 3 et la protéine ankyrine, dont l'intégrité est cruciale pour maintenir la structure membranaire caractéristique des globules rouges et pour le transport ionique [43-45]. Au fait, des mutations qui perturbent la fonction ou l'association de ces facteurs conduisent au développement de désordres hématologiques importants chez l'humain [46-49]. Ainsi, il est intrigant de constater que plusieurs des gènes cibles érythroïdes de SCL encodent des protéines qui exercent des fonctions hautement spécialisées dans ce compartiment cellulaire. Combinés à notre caractérisation du promoteur c-kit, ces études suggèrent que SCL démontre une grande versatilité dans les types de gènes qu'il active, une propriété intimement reliée à son intégration au sein de complexes multifactoriels, ce qui nous amène à discuter des mécanismes qui permettent cette flexibilité moléculaire.

6.4 La dynamique des complexes SCL

Sieweke et Graf ont précédemment proposé que la composition des complexes de régulateurs transcriptionnels évolue de manière dynamique au cours de la différenciation des cellules hématopoïétiques, afin de moduler leur spécificité d'action dans l'élaboration de nouveaux programmes d'expression génique [50]. Une telle stratégie permettrait à la cellule de rentabiliser son énergie en modulant l'activité de complexes transcriptionnels préexistants, via l'ajout ou la soustraction

de sous-unités particulières, évitant ainsi la nécessité de synthétiser ou de démanteler des réseaux entiers de régulateurs. Or, les résultats présentés dans cette thèse, concernant la dissection des mécanismes d'action des complexes SCL sur différents gènes cibles lignage-spécifiques, renforcent cette vision du comportement dynamique des complexes transcriptionnels au cours de la différenciation.

D'abord, nous avons constaté que l'efficacité d'activation des promoteurs c-kit et GPA par SCL et ses partenaires varie dépendant du facteur GATA retrouvé au sein du complexe et que cette spécificité corrèle parfaitement avec les patrons d'expression et les propriétés biologiques connues des facteurs GATA. Ainsi, le promoteur c-kit est plus efficacement activé par des complexes contenant GATA-2, un régulateur crucial au niveau des progéniteurs hématopoïétiques [51], alors que l'activation du promoteur GPA est beaucoup plus robuste avec des complexes contenant GATA-1, un 'maître' régulateur de la voie érythroïde [52]. Cette corrélation surprenante suggère que l'association avec différents facteurs GATA module la spécificité d'action des complexes SCL vis-à-vis des catégories spécifiques de gènes cibles. Bien que les mécanismes responsables de l'activité différentielle des facteurs GATA demeurent spéculatifs, ils pourraient impliquer des différences dans leur efficacité de liaison à l'ADN ou dans leur capacité à interagir avec des partenaires protéiques spécifiquement recrutés sur les promoteurs c-kit et GPA. Dans le contexte du promoteur GPA, où des motifs GATA sont clairement requis pour l'activation par le complexe SCL, il se pourrait que GATA-1 reconnaisse ces motifs avec plus forte affinité que GATA-2, assurant un recrutement plus

efficace du complexe SCL sur ce promoteur. En effet, des différences dans la spécificité de liaison à l'ADN des facteurs GATA, principalement influencées par les nucléotides au pourtour de la séquence centrale 'GATA', ont déjà été documentées [53,54]. En contraste, dans le contexte du promoteur c-kit, où le recrutement du complexe SCL à l'ADN implique principalement un site de liaison pour le facteur Sp1 (boîte-GC) et s'avère indépendant de motifs GATA consensus, il est possible que l'efficacité accrue de GATA-2 résulte d'une plus forte affinité d'interaction avec Sp1 ou d'une capacité à lier l'ADN sur des motifs non-consensus présent dans ce promoteur. En effet, l'existence de déterminants additionnels nécessaire au recrutement du complexe SCL sur le promoteur c-kit est suggéré par le fait que la boîte-GC du promoteur ne permet pas à elle seule de conférer la pleine activation par SCL et ses partenaires (Lécuyer et Hoang, résultats non publiés). À l'avenir, il sera nécessaire de disséquer plus finement le promoteur c-kit minimal afin de déterminer si des sites de liaison non-consensus pour les partenaires connues du complexe ou des motifs pour de nouveaux partenaires insoupçonnés contribuent au recrutement du complexe SCL.

Ainsi, l'image qui se dessine concernant l'évolution du complexe SCL au cours de la différenciation suggère que GATA-2 prédomine au sein de ces complexes dans les cellules souches et progénitrices et devient progressivement remplacé par GATA-1 lorsque ces cellules s'engagent vers la voie érythroïde (Figure 6.1). De plus, des études récentes suggèrent que cette transition des facteurs GATA implique directement l'activité du complexe SCL et, de manière homologue aux voies

enzymatiques traditionnelles, fait appel à des boucles d'auto-activation et de rétro-inhibition. D'abord, Vyas et al. ont illustré que le gène GATA-1 représente fort probablement une cible directe du complexe SCL, suggérant que des complexes plus primitifs contenant GATA-2 seraient requis pour activer l'expression de ce gène [37]. Ainsi, en initiant l'expression de gènes cibles comme GATA-1 dans les progéniteurs hématopoïétiques, le complexe SCL pourrait munir une cellule pluripotente de l'option de se différentier vers la voie érythroïde, une vision appuyée par l'observation que plusieurs gènes érythroïdes sont exprimés à faibles niveaux dans les cellules souches et les progéniteurs hématopoïétiques [55,56]. Or, une fois que l'expression de GATA-1 ait été initié par le complexe SCL, un enclenchement plus robuste du programme érythroïde pourrait être favorisé grâce à la capacité de GATA-1 à auto-activer l'expression de son propre gène en *trans* (Figure 6.1)[57]. À l'inverse, Grass et al. ont récemment démontré que GATA-1 exerce un effet répresseur sur l'expression GATA-2, suggérant l'existence d'un mécanisme de rétro-inhibition qui pourrait servir à consolider la différenciation vers la voie érythroïde de manière irréversible (Figure 6.1)[58]. En effet, il est connu depuis longtemps que des cellules déficientes pour GATA-1 exhibent une forte surexpression de GATA-2 [59], suggérant soit que des mécanismes compensatoires causent une expression accrue de GATA-2 pour palier à une absence complète de GATA-1 ou que GATA-1 soit requis pour inhiber, directement ou indirecteemnt, l'expression de GATA-2 durant la différenciation érythroïde. Or, les données de Grass et al. favorisent cette seconde

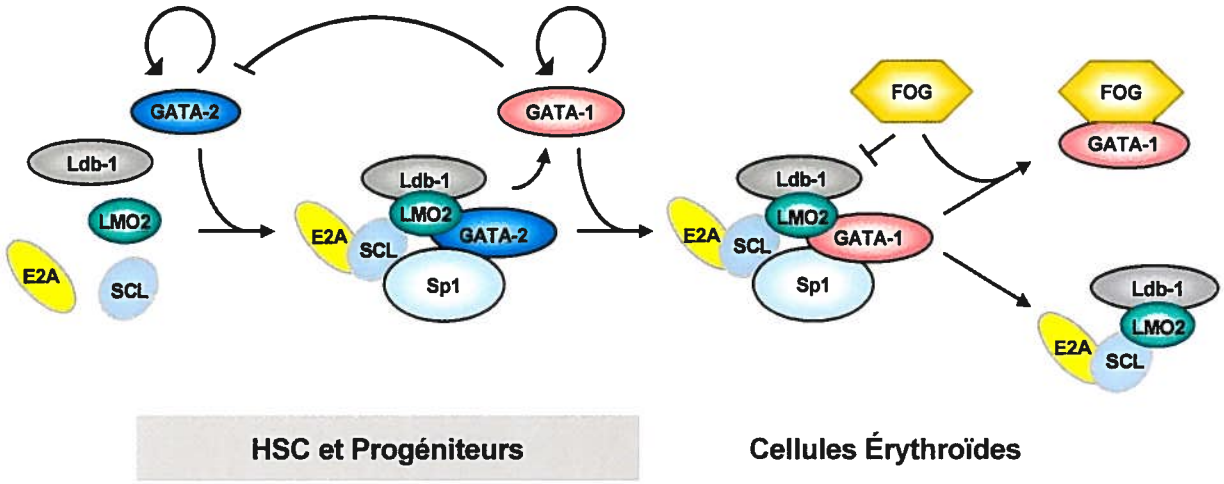

Figure 6.1. L'évolution dynamique des complexes SCL au cours de la différenciation des cellules hématopoïétiques. Dans les cellules souches et les progéniteurs hématopoïétiques, des complexes contenant GATA-2 activent l'expression de gènes comme c-kit qui favorisent le maintien d'une réserve de cellules pluripotentes. De plus, par un processus stochastic ou en réponse à des signaux de l'environnement, ces complexes stimulent l'expression à faible niveau de gènes lignages-spécifiques, tel que GATA-1, menant au remplacement progressif de GATA-2 par GATA-1 au sein du complexe SCL. Cette transition des facteurs GATA pourrait représenter un point critique permettant de modifier la spécificité d'action du complexe SCL et d'enclencher le programme érythroïde de manière irréversible. Par leur capacité à activer leur propre expression en *trans*, les facteurs GATA pourrait créer un code binaire robuste afin de moduler les choix de destin cellulaire au sein du réseau hématopoïétique. Enfin, au cours de la différenciation érythroïde, l'expression de FOG antagonise l'activité du complexe SCL via une séquestration de GATA-1, permettant à nouveau de moduler la spécificité d'action de ces complexes.

interprétation, puisqu'ils illustrent habilement, au moyen d'une stratégie d'immunoprécipitation de la chromatine à l'échelle du locus GATA-2 entier, que GATA-1 est directement impliqué dans l'inhibition du gène GATA-2 via sa liaison sur un élément de régulation situé à 3 kilobases en aval de ce gène, un élément qui permet normalement à GATA-2 d'auto-activer sa propre expression (Figure 6.1)[58]. De plus, des études non-publiés présentées par le Dr. Emery Bresnick lors du congrès annuel des facteurs GATA à l'IRCM à l'été 2003, suggèrent que cette inhibition requière l'interaction de GATA-1 avec son co-facteur FOG-1 (*Friend of GATA-1*), bien que le mécanisme exact par lequel GATA-1 et FOG-1 réprime l'expression du gène GATA-2 demeure floue. FOG-1 est une protéine à multiple doigt de zinc, initialement identifié par sa capacité à interagir avec le doigt de zinc N-terminal de GATA-1 [60], qui ne lie pas l'ADN mais détient la capacité d'inhiber ou de potentialiser l'activation transcriptionnelle par les facteurs GATA dépendent du contexte cellulaire et des gènes cibles [60-62]. L'ablation du gène FOG-1 résulte en un blocage de la différenciation érythroïde au stade de proérythroblase et perturbe complètement le développement de la voie mégakaryocytaire [63]. De plus, des études élégantes effectuées par le groupe du Dr. Orkin, qui ont caractérisé les résidus spécifiques requis pour l'interaction de FOG-1 avec les facteurs GATA, ont permis de démontrer que l'intégrité de ces interactions est essentielle durant la différenciation des cellules éryhroïdes et mégakaryocytaires chez la souris et l'humain [64-66]. Or, dans notre étude du promoteur GPA, nous avons cherché à déterminer si FOG-1 module l'activité du complexe SCL dans le contexte d'un gène cible éryhroïde, étant donné l'importance de GATA-1 au sein de ce complexe. Nous

avons constaté que l'expression de FOG-1 inhibe fortement l'activation du promoteur GPA par le complexe SCL, à la fois dans des essais de transactivation transitoires et dans le contexte du gène GPA endogène dans les cellules TF-1, un effet qui dépend de l'interaction directe de FOG-1 avec GATA-1 [41]. Ces données suggèrent qu'au cours de la différenciation érythroïde, une des fonctions de FOG-1 pourrait être d'inhiber l'activité du complexe SCL via la séquestration de GATA-1, permettant du même coup à GATA-1 d'exécuter des fonctions indépendantes du complexe SCL au sein de la voie érythroïde (Figure 6.1). Ainsi, en plus de la spécificité différentielle conférée au complexe SCL par différents facteurs GATA, nos études démontrent comment l'activité de ce complexe peut être modulée par des co-facteurs comme FOG-1 qui interagissent avec des sous-unités spécifiques du complexe.

Un autre point de divergence dans les mécanismes d'action des complexes SCL découlant de l'étude comparative des promoteurs c-kit et GPA concerne la nécessité de la fonction de liaison à l'ADN de SCL. La vision traditionnelle des facteurs bHLH histo-spécifiques suggère qu'ils activent la transcription sous forme d'hétérodimères avec des partenaires bHLH ubiquitaires (protéines E) suite à leur liaison à l'ADN sur des boîtes-E retrouvées dans les régions régulatrices de leurs gènes cibles [10]. La dimérisation des facteurs bHLH est assurée par des résidus hautement conservés du domaine HLH, alors que leur liaison à l'ADN nécessite une région riche en résidus basiques (b) située du côté N-terminal du HLH. Bien que l'activité biologique de ces facteurs nécessite généralement l'intégrité de leur

domaine basique [67-69], des études récentes suggèrent que certains facteurs bHLH exercent des fonctions indépendantes de leur capacité à lier l'ADN [21,70]. En effet, Porcher et al. ont démontré que des mutants de liaison à l'ADN de SCL restaurent le développement hématopoïétique suite à leur expression dans des cellules ES $SCL^{-/-}$ ou chez le mutant du poisson zèbre *cloche* [70]. Cependant, les cellules qui expriment ces mutants démontrent des défauts de différenciation terminale vers les voies érythroïdes et mégakaryocytaires, suggérant que la propriété de liaison à l'ADN de SCL soit requise pour ses fonctions biologiques reliées à la maturation des cellules hématopoïétiques [70]. Or, nos études des promoteurs c-kit et GPA ont révélé des corrélations semblables, puisque des mutants du domaine basique de SCL sont pleinement actifs dans l'activation du promoteur c-kit, mais sont moins efficaces à faibles doses dans la transactivation et la nucléation du complexe SCL sur les séquences du promoteur GPA [33,41]. De plus, nous avons constaté que la pleine activation du promoteur GPA par SCL et ses partenaires nécessite l'intégrité d'une boîte-E [41], tel qu'observé dans le contexte du promoteur du gène encodant la protéine 4.2 [42], un autre gène cible érythroïde du complexe SCL. Ainsi, la nécessité de la propriété de liaison à l'ADN de SCL semble représenter un point de divergence dans les mécanismes d'activation de ses gènes cibles spécifiques aux cellules progénitrices ou érythroïdes. L'identification et la caractérisation moléculaire de gènes cibles additionnels de SCL dans ces deux compartiments cellulaires permettra d'évaluer si ces différences s'appliquent plus globalement.

Bien que nous ayons davantage concentré nos efforts sur la caractérisation des mécanismes par lesquels les complexes SCL reconnaissent différents promoteurs hématopoïétiques, une question importante qui n'a pas encore été pleinement adressé concerne comment ces complexes créent un environnement propice à l'activation transcriptionnelle. Plusieurs des partenaires du complexe, incluant SCL, GATA-1 et E47 contiennent des domaines putatifs de transactivation, qui pourraient servir à recruter des complexes de remodelage de la chromatine, de co-activateurs ou certains éléments de la machinerie basale [71-74]. Grâce à l'utilisation de mutants de délétion dans nos essais de transactivation, nous avons constaté que parmi ces facteurs, seulement E47 nécessite la présence de ses domaines de transactivation afin de stimuler l'activité des promoteurs c-kit et GPA (33,41; Lécuyer et Hoang, résultats non publiés). De plus, nous avons démontré qu'un mutant de E47 contenant seulement le domaine bHLH (E47-bHLH) exerce un effet dominant négatif sur l'activité du complexe SCL, à la fois dans des essais de transactivation transitoires et dans le contexte de la chromatine dans les cellules TF-1 [41]. Cet effet semble découler de la capacité de E47-bHLH à déplacer la protéine E47 pleine longueur du complexe SCL endogène dans les cellules TF-1, causant une perturbation importante de la composition du complexe SCL, tel qu'évalué par des essais de retardement sur gel avec la séquence du promoteur GPA [41]. Or, il a précédemment été démontré que le domaine d'activation AD1 de E47 interagit avec le complexe multiprotéique SAGA (Spt-Ada-Gcn5-Acetyltransferase) [73], un complexe impliqué dans l'acétylation des histones grâce à sa sous-unité catalytique Gcn5 [75]. Par sa capacité à acétyler les histones associées aux promoteurs où il est recruté, le complexe SAGA

crée une interface de reconnaissance pour des protéines contenant des Bromodomaines, incluant Gcn5 lui-même et la sous-unité Swi2/Snf2 du complexe SWI/SNF [76,77]. Ainsi, une fois qu'il a été recruté sur un locus particulier, le complexe SAGA crée une marque épigénétique qui stabilise sa propre association avec la chromatine et permet le recrutement de complexes enzymatiques additionnels, tel que le complexe de remodelage ATP-dépendent SWI/SNF [76,77]. D'ailleurs, des études récentes ont suggéré que le recrutement du complexe SAGA représente une étape importante dans la séquence des évènements qui mènent à l'activation soutenue de plusieurs gènes [78,79]. Ainsi, la perturbation de la fonction et de la mobilité du complexe SCL observé en présence du mutant E47-bHLH pourrait résulter d'une incapacité de ce mutant à interagir avec les composantes du complexe SAGA, bien que nos données ne permettent pas d'exclure la possibilité qu'une autre fonction de E47 soit altérée avec ce mutant. Si SCL et ses partenaires recrutent effectivement le complexe SAGA sur leurs gènes cibles, cela pourrait permettre d'établir une marque épigénétique susceptible d'être perpétuée même en l'absence de SCL, ce qui pourrait expliquer pourquoi l'expression soutenue de SCL n'est pas requise pour l'homéostasie des HSC, bien qu'elle soit essentielle pour la spécification de cette population cellulaire [25,26]. Une caractérisation biochimique approfondie des complexes SCL dans différents compartiments cellulaires fournira des détails importants sur leur composition, la stœchiométrie de leurs composantes et les activités enzymatiques qui y sont associés. En effet, nos données préliminaires de fractionnement d'extraits nucléaires de cellules TF-1 par chromatographie à filtration sur gel démontrent que SCL, E2A et GATA-1 co-éluent dans des fractions à très haut

poids moléculaire, suggérant qu'ils soient associés avec des partenaires additionnels dont on ignore encore l'identité (Lécuyer et Hoang, résultats non publiés). Ensemble, nos résultats suggèrent que la capacité des facteurs de transcription à fonctionner au sein de complexes multifactoriels diversifie leur répertoire de mécanismes d'action et leur permet d'exercer des fonctions spécialisées dans différents contextes cellulaires ou sur différents types de gènes cibles.

6.5 L'interaction SCL-LMO2 : un point de nucléation du complexe SCL

Une caractéristique intrigante du complexe SCL durant l'activation transcriptionnelle est la nécessité de la présence simultanée de l'ensemble des partenaires du complexe, dont la conséquence biologique devient 'tout ou rien' [33,41]. Cette propriété assure sans doute une régulation plus serrée des gènes cibles de SCL et nous rappelle l'exemple des complexes de type 'enhancéosome', dont les mieux caractérisés incluent ceux qui contrôlent l'expression des gènes encodant l'interféron-β et le récepteur α des cellules T [80,81]. En effet, l'activation de ces deux gènes nécessite la présence simultanée d'une combinaison de facteurs histo-spécifiques et inductibles sur des éléments en *cis* précisément agencés, afin de permettre l'activation synergique de la transcription en réponse à des signaux spécifiques de l'environnement [82-84]. Afin d'identifier les déterminants moléculaires qui permettent l'assemblage du complexe SCL, nous avons cherché à évaluer la spécificité fonctionnelle des partenaires du complexe en les substituant par d'autres membres de leurs familles respectives. Cette approche nous a permis de révéler que

SCL joue un rôle non-redondant au sein du complexe, puisque sa fonction ne peut être remplacée par d'autres facteurs bHLH histo-spécifiques, tels que MyoD ou NSCL-1 (Voir Chapitre 5). Nous avons par la suite établi que la spécificité fonctionnelle de SCL découle de sa capacité à interagir spécifiquement avec LMO2, une interaction qui fournit un point de nucléation pour la formation du complexe. De plus, basée sur la comparaison du domaine HLH de SCL et NSCL-1, nous avons été en mesure d'identifier des résidues spécifiques de la boucle et de l'hélice 2 du HLH de SCL qui lui confère sa capacité d'interaction avec LMO2, et ainsi, sa spécificité hématopoïétique. Or, l'importance cruciale de cette interaction pour les fonctions moléculaires de ces facteurs explique pourquoi leurs fonctions biologiques sont aussi intimement inter-reliées. En effet, les phénotypes des souris $SCL^{-/-}$ et $LMO2^{-/-}$ sont essentiellement identiques, démontrant une absence complète de cellules hématopoïétiques [6,7,85]. De plus, les gènes SCL et LMO2 représentent des cibles récurrentes de réarrangements chromosomiques dans les cas de leucémies T-ALL chez l'humain, et leur co-expression dans des modèles de souris transgéniques récapitule ce phénotype leucémique [86-89]. Ainsi, ayant identifié les déterminants moléculaires qui spécifient l'interaction entre SCL et LMO2, il sera maintenant possible de développer des approches génétiques (insertions géniques et transgenèse) afin de clarifier la nécessité de leur interaction directe pour la spécification du compartiment hématopoïétique, la différenciation des cellules du réseau définitif, et le développement de leucémies T-ALL.

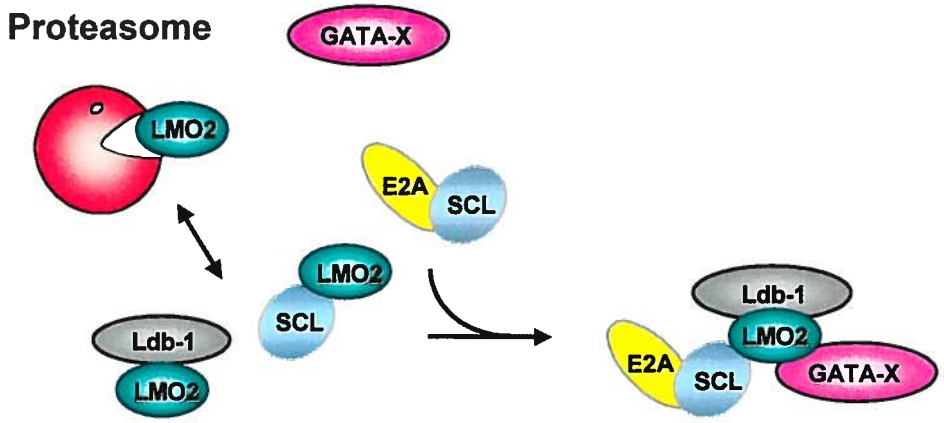

Figure 6.2. La stabilité protéique comme mécanisme de contrôle du complexe SCL. Puisque LMO2 joue un rôle essentiel pour permettre l'assemblage du complexe SCL, la modulation de sa stabilité, dépendant de la présence de SCL ou de Ldb-1, offre un mécanisme puissant pour contrôler la nucléation des complexes transcriptionnels au moment et dans un contexte cellulaire approprié.

6.6 La stabilité protéique: un nouveau mécanisme de régulation du complexe SCL

Au cours des dernières années, de nombreuses études ont souligné l'importance de l'ubiquitylation couplé à la dégradation protéolytique comme un point de contrôle essentiel de l'activité des facteurs de transcription, dont le mécanisme varie selon les circonstances [90-92]. Dans certains cas, la voie ubiquitine-protéasome peut directement limiter l'activité des facteurs de transcription en modulant leur abondance, tel qu'observé dans le cas de p53 et β-caténine [92]. Cependant, il semble y exister un lien encore plus intrigant entre la dégradation et la transcription, puisqu'il a récemment été établi que la vitesse de dégradation de plusieurs régulateurs transcriptionnels corrèle proportionnellement avec la puissance de leur domaine de transactivation [90,93,94]. De plus, il a été démontré que certaines composantes de la voie ubiquitine-protéasome s'associent avec le complexe ARN polymérase II (PolII) et sont recrutés sur les régions régulatrices de gènes activement transcrits [90,95-98]. Ensemble, ces observations ont mené à l'élaboration d'un modèle, le modèle 'suicide', suggérant que certains activateurs soient ciblés pour la dégradation par la PolII au cours de l'activation de la transcription [90,97]. Ce mécanisme permettrait de limiter l'activation continue d'un gène par une même molécule d'activateur, nécessitant ainsi la présence d'activateurs nouvellement synthétisés, et assurerait une plus grande flexibilité dans le contrôle de la transcription en réponse aux signaux de l'environnement.

Or, en plus de la nécessité de l'interaction SCL-LMO2 pour la nucléation et l'activité transcriptionnelle du complexe SCL, nos études ont dévoilé que l'association de ces facteurs empêche la dégradation de la protéine LMO2 (Chapitre 5). Étant donné le rôle crucial de LMO2 dans l'assemblage des complexes SCL, la modulation de sa stabilité représente un mécanisme efficace pour contrôler où et quand ces complexes seront formés (Figure 6.2). Il a récemment été démontré que la protéine RLIM, un facteur à domaine RING initialement identifié comme un co-facteur transcriptionnel des protéines LIM-hémoédomaine [99], agit comme une ubiquitin ligase capable de cibler l'ubiquitylation et la dégradation de Ldb-1 et LMO2 [100]. Or, puisque nous avons confirmé que le gène RLIM est exprimé dans nos modèles cellulaires, ce facteur représente le candidat le plus probable comme enzyme responsable du ciblage de LMO2 pour la dégradation protéolytique. Au fait, des études récentes chez le Xenope, qui ont démontré que RLIM régule la stoechiométrie des complexes contenant Ldb-1 en ciblant ce dernier pour la dégradation [101], suggèrent que RLIM puisse jouer un rôle semblable dans le contexte des complexes SCL. À l'avenir, il sera important d'identifier les déterminants en *cis* et en *trans* responsables de la dégradation de LMO2, afin d'éclaircir le mécanisme par lequel son association avec SCL ou Ldb-1 empêche sa dégradation. Il est fort probable que l'interaction de LMO2 avec SCL ou Ldb-1 masque les déterminants (les dégrons) reconnus par les composantes du système ubiquitine-protéasome. Une situation semblable a précédemment été observée avec les protéines à homéodomaine MATα2 et MATa1, deux régulateurs essentiels du développement chez la levure, dont l'hétérodimérisation masque leurs dégrons et empêche leur destruction par le

protéasome [102,103]. L'identification des dégrons dans LMO2 permettra de développer des stratégies afin d'évaluer si la stabilisation protéique représente effectivement un point de contrôle essentiel de l'activité des complexes SCL durant la spécification du compartiment hématopoïétique et la leucémogenèse. De plus, l'instabilité inhérente de LMO2 pourrait être exploitée afin de développer des agents thérapeutiques dans le traitement des leucémies aiguës de type T.

6.7 Conclusions

En résumé, les études présentées dans cette thèse ont permis d'identifier les premiers gènes cibles spécifiquement régulés par SCL dans les cellules hématopoïétiques progénitrices et érythroïdes, et d'élucider les propriétés moléculaires de ce maître régulateur du développement sanguin. Nos résultats nous ont poussé a défier la vision traditionnelle des facteurs bHLH histo-spécifiques agissant sous forme d'hétérodimères avec des partenaires bHLH ubiquitaires, puisque nous démontrons que la spécificité hématopoïétique de SCL découle de sa capacité a nucléer la formation de complexes d'ordre supérieur sur les régions régulatrices de ses gènes cibles. De plus, nos études suggèrent que l'intégration des facteurs de transcription au sein de complexes multifactoriels offre un moyen efficace pour créer un code binaire durant l'activation de la transcription, assurant ainsi un contôle stringent dans l'établissement de programmes d'expression génique distincts. Puisqu'il est de plus en plus clair que les fonctions variées d'une cellule sont coordonnées par des réseaux de régulateurs organisés sous forme de modules, nous proposons que SCL et ses

partenaires représentent les composantes centrales de réseaux moléculaires impliqués dans la régulation de la différenciation des cellules hématopoïétiques. Un défi important à l'avenir sera d'identifier des composantes additionnelles de ces réseaux et de déterminer comment ceux-ci communiquent avec d'autres fonctions modulaires au sein de la cellule.

6.8 Références

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ANNEXES