

The Role of Cdx in Primitive Hematopoiesis

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Authorization

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Abstract

Functional overlap and peri-implantation lethality have made the study of the Cdx family of transcription factors a challenging pursuit. Our research group has generated a conditional genetic knockout model that renders the murine embryo effectively Cdx null, allowing us to explore unknown developmental roles of Cdx. Previously uncharacterized hematopoietic defects are evident in these embryos, and with the use of ChIP-seq technology, a number of hematopoietic genes were identified as putative Cdx targets, including *Scl*, *Lyl1*, *Lmo2*, and *Meis1*. In addition, the chromatin remodeling protein Brg1 was identified from a SILAC-MS screen of potential Cdx2 interactors, and was studied in the context of direct interaction with Cdx2. My studies suggest a critical role for Cdx in primitive hematopoiesis in mice, and indicate that Cdx2 may recruit Brg1 to specific targets to induce transcription of these genes.

L'étude des facteurs de transcription Cdx est difficile principalement à cause de la létalité péri-implantation de la perte de Cdx, ainsi que ces facteurs partagent plusieurs fonctions. Notre groupe a généré un modèle de KO génétique conditionnel qui rend l'embryon murin efficacement Cdx nul, ce qui nous permet d'explorer les rôles de développement inconnus de Cdx. Précisément, on a vu des défauts hématopoïétiques dans ces embryons, et avec l'utilisation de la technologie « ChIP-seq », un certain nombre de gènes hématopoïétiques ont été identifiés comme gènes cibles de Cdx putatifs, dont *Scl*, *Lyl1*, *Lmo2*, et *Meis1*. En outre, on a identifié Brg1, une protéine de

remodelage de la chromatine, à partir d'un écran SILAC-MS de interacteurs Cdx2 potentiels. Dans ce regard, on a étudié interaction directe entre Cdx2 et Brg1. Mes études suggèrent un rôle critique pour Cdx dans l'hématopoïèse primitive chez la souris, et indiquent que Cdx2 peut recruter Brg1 à des cibles spécifiques pour induire la transcription de ces gènes.

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Abbreviations

α MEM	Alpha Modification of Eagle's Medium
ADP	Adenosine diphosphate
AGM	Aorta-gonad-mesonephros
AML	Acute myeloid leukemia
AP	Anterior-posterior
ATP	Adenosine triphosphate
β gal	β -galactosidase
BAF	Brg1-associated factor
BFU-E	Burst-Forming Unit-Erythroid
Brg1	Brahma-related gene 1
Brm	Brahma
BSA	Bovine serum albumin
cDNA	Complementary DNA
CDRE	Cdx response element
Cdx	Caudal-related homeobox
CFU-GEMM	Colony-Forming Unit-Granulocyte, Erythroid, Macrophage, Megakaryocyte
CFU-GM	Colony-Forming Unit-Granulocyte, Macrophage
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation combined with deep sequencing
Cmyb	Myoblastosis proto-oncogene

CO ₂	Carbon dioxide
CPRG	Chlorophenol Red-β-D-Galactopyranoside
Cre	Causes recombination enzyme
DAB	Diamino-benzidine
DAPI	4',6-diamidino-2-phenylindole
DBS	Donor bovine serum
DepC	Diethylpyrocarbonate
dHSCs	Definitive hematopoietic stem cells
Dll1	Delta-like ligand 1
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
E	Embryonic day
EDTA	Ethylenediaminetetraacetic acid
Elf1	E74-like factor 1
EMP	Erythroid-myeloid progenitor
EMSA	Electrophoretic mobility shift assay
ER ^T	Estrogen receptor, responsive to tamoxifen
ESC	Embryonic stem cell
Etv	ETS variant
FBS	Fetal bovine serum
Fli1	Friend leukemia integration 1
Flk1	Fetal liver kinase 1

Flt1	FMS related tyrosine kinase 1
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gata1	GATA binding protein 1
Gata2	GATA binding protein 2
GFP	Green fluorescent protein
α -globin	Hemoglobin α
β -globin	Hemoglobin β
ζ -globin	Embryonic hemoglobin α
$\epsilon\gamma$ -globin	Embryonic hemoglobin β
GST	Glutathione S-transferase
H3K4Me3	Histone 3 lysine 4 trimethylation
HCl	Hydrochloric acid
HEK293	Human embryonic kidney 293 cells
HEPES	Hydroxyethyl piperazineethanesulfonic acid
HI-DBS	Heat-inactivated donor bovine serum
HI-FBS	Heat-inactivated fetal bovine serum
Hox	Homeobox gene
Hsp	Heat shock protein
IgG	Immunoglobulin G
IMDM	Iscove's Modified Dulbecco's Medium
IP	Immunoprecipitation
ISH	<i>In situ</i> hybridization

Kb	Kilobase
Lef1	Lymphoid enhancer binding factor 1
LiCl	Lithium chloride
Lmo2	LIM domain only 2
loxP	Locus of crossover in P1
Lyl1	Lymphoblastic leukemia derived sequence 1
Meis1	Myeloid ecotropic viral integration site 1
mESC	Murine embryonic stem cell
Mll	Mixed-lineage leukemia
MO	Morpholino
mRNA	Messenger ribonucleic acid
MS	Mass spectrophotometry
MyoD	Myogenin D
P19	Murine embryonic carcinoma cell line
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
Pbx	Pre-B-cell leukemia homeobox
PCR	Polymerase chain reaction
PECAM1	Platelet/endothelial cell adhesion molecule 1
PFA	Paraformaldehyde
Pu.1	Purine-rich box 1
RNA	Ribonucleic acid

RT-PCR	Reverse-transcriptase polymerase chain reaction
Runx1	Runt-related transcription factor 1
Sall	Spalt-like transcription factor
Scl	Stem cell leukemia
SDS	Sodium dodecylsulfate
shRNA	Short hairpin RNA
SILAC	Stable isotope labelling with amino acids in cell culture
SWI/SNF	Switch/sucrose-non-fermentable
T-ALL	T-cell acute lymphoblastic leukemia
TBE	Tris, Boric Acid, EDTA buffer
Tbx6	T-box 6
TE	Tris/EDTA
TESS	Transcription element search software
TFSearch	Transcription factor search software
Tie2	Tunica intima endothelial kinase 2
TNF α	Tumour necrosis factor A
TSS	Transcriptional start site
VEGFA	Vascular endothelial growth factor A
Wnt	Wingless-related

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Chapter 1

Introduction

1.1 Introduction

Hematopoiesis is the formation of the cellular components of blood, involving many processes that have been conserved throughout vertebrate evolution. Development of the hematopoietic system involves the coordinated effects of several transcription factors, and the Caudal-related homeobox (Cdx) family of transcription factors has recently been implicated in these processes. Moreover, Cdx members, in particular Cdx2, display aberrant expression patterns in certain blood cancers. By studying the earliest events of hematopoiesis, we hope to gain insight into normal developmental processes, and better understand adult blood disorders.

1.2 Hematopoiesis

During murine development, the process of gastrulation generates three distinct germ layers: ectoderm, mesoderm, and endoderm. Each of these germ layers gives rise to unique structures and tissues of the developing embryo. Ectoderm is the outermost layer of the organism, and the source of the epidermis, sensory organs, and nervous tissues. Endoderm gives rise to the gastrointestinal tract, mouth, lungs, and associated organs. Mesoderm lies between the ectoderm and endoderm, and differentiates into muscles, bones, connective tissue, gonads, and blood. All three layers contribute to extraembryonic tissues, with mesoderm contributing to the yolk sac - the first organ involved in murine hematopoiesis.

Vertebrate blood development involves two waves, termed primitive hematopoiesis and definitive hematopoiesis, the latter of which gradually replaces the primitive system and eventually gives rise to adult hematopoiesis (Galloway and Zon, 2003). In mice, the primitive wave occurs in the yolk sac, giving rise to immature erythrocytes (red blood cells) that supply oxygen to the developing embryo as it undergoes rapid growth (Orkin and Zon, 2008). Primitive erythroid precursors lack pluripotency and renewal capabilities, necessitating their replacement by the definitive wave of hematopoiesis for later developmental events and adult-type blood cell maintenance (Jagannathan-Bogdan and Zon, 2013).

In mice, embryonic hematopoiesis proceeds through sequential steps and different anatomical locations, starting in the yolk sac, then the aorta-gonad mesonephros (AGM), fetal liver, and finally bone marrow in the adult (Figure 1.1; Jagannathan-Bogdan and Zon, 2013). The first stage of blood and blood vessel development occurs in the extraembryonic yolk sac, where blood islands form at approximately E (embryonic day) 7.0-7.5 and generate a belt-like structure around the yolk sac that is composed of hemangioblasts (Haar and Ackerman, 1971). Hemangioblasts are common endothelial and hematopoietic precursors that have the ability to give rise to both primitive erythroblasts and vascular endothelium (Jagannathan-Bogdan and Zon, 2013). Blood and blood vessel development occur concurrently, and erythrocytes communicate with endothelial cells to ensure the maturation of both cell types (Auerbach et al., 1996).

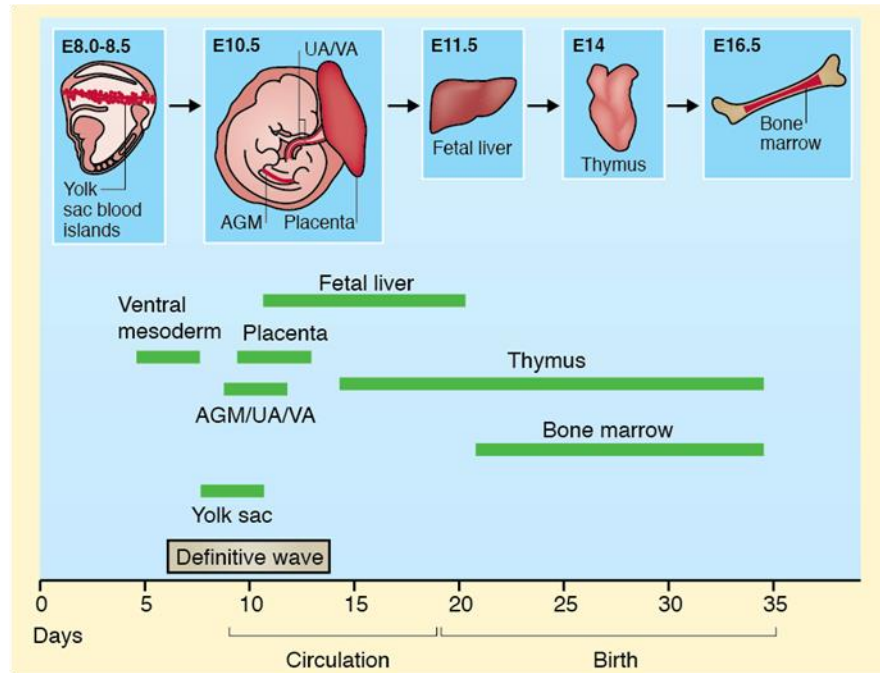


Figure 1.1: Murine hematopoietic development.

Blood islands represent the first hematopoietic precursors, derived from ventral mesoderm. Upon the introduction of circulation, hematopoietic cells colonize other tissues, and at E10.5 the aorta-gonad-mesonephros (AGM), placenta, umbilical artery (UA) and vitelline artery (VA) initiate the generation of definitive hematopoiesis. These cells colonize the fetal liver, thymus, and bone marrow. (Jagannathan-Bogdan and Zon, 2013, reproduced with permission from the Development publishing group)

Hemangioblasts are defined as a subset of mesodermal cells that co-express *Brachyury* (T, Bry) and Fetal liver kinase 1 (Flk1/Kdr/Vegfr2/ CD309), and serve as progenitors for both hematopoietic and vascular lineages (Fehling et al., 2003). Using *Brachyury* and *Flk1* as markers, hemangioblast development was found to be initiated in the mid-streak stage (E6.5), with peak T/Flk1 expression at late streak/neural plate stages (E7), followed by a decline at the head fold stage (E7.5) (Huber et al., 2004). Dissection of E7.5 embryos allowed for the spatial identification of hemangioblasts, which primarily localize to the posterior primitive streak and yolk sac regions of the developing embryo (Figure 1.2; (Medvinsky et al., 2011). The Huber group also determined that, prior to migration to the yolk sac, hemangioblasts are already segregated into distinct hematopoietic, endothelial, and vascular smooth muscle lineages (Figure 1.2 B). Although hemangioblasts were generally believed to be bi-potent progenitors, these data indicated that the original cells were tri-potent and likely derived from the posterior primitive streak region (Huber et al., 2004; Medvinsky et al., 2011).

The primary function of primitive hematopoiesis is to generate primitive erythrocytes that express embryonic globin proteins (embryonic alpha ζ -globin, embryonic beta $\epsilon\gamma$ -globin, embryonic beta globin β H1) in order to supply the developing embryo with oxygen (Orkin and Zon, 2008). In addition to erythrocytes, megakaryocytes are also derived from yolk sac hematopoietic precursors, albeit in lesser numbers (Palis et al., 1999). These cells begin to circulate between E8.0 and E8.5 upon the initiation of the embryonic heartbeat, and by E9.5 the majority of circulating blood cells are large nucleated erythroblasts that have been generated in the yolk sac (Palis et al., 1999). A

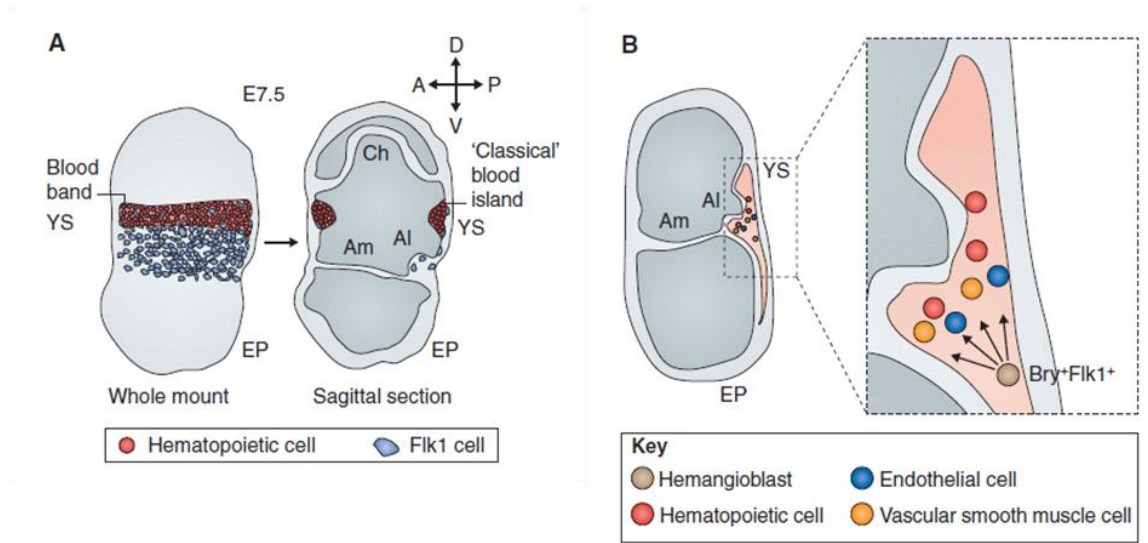


Figure 1.2: Early yolk sac hematopoiesis.

A) Left - Schematic representation of an E7.5 murine embryo, displaying hematopoietic cells (red) in the yolk sac comprising a belt-like structure that encircles the extraembryonic portion of the embryo. Flk1⁺ mesodermal and endothelial cells are shown in blue. Right - A sagittal section through the E7.5 embryo, displaying the classical view of blood islands

B) Left - A sagittal section through the E7.5 embryo, displaying segregated hematopoietic, endothelial, and vascular smooth muscle cells derived from the Brachyury⁺Flk1⁺ hemangioblast. This segregation is thought to occur before hematopoietic cells migrate to the yolk sac (magnified insert). YS - yolk sac; Al - allantois; Am - amnion; Ch - chorion; EP - embryo proper. (Medvinsky et al., 2011, reproduced with permission from the Development publishing group)

wave of definitive erythropoiesis, marked by the expression of adult-type alpha- ($\alpha 1$, $\alpha 2$) and beta- (β^{maj} , β^{min}) globins, begins at approximately E11.5. Primitive lineages are gradually replaced by definitive hematopoietic stem cells (dHSCs) that go on to supply the body with all blood types throughout adulthood (Sugiyama and Tsuji, 2006).

In mice, a transient wave of pseudo-definitive hematopoiesis is thought to be initiated in the blood islands, producing cells termed erythroid-myeloid progenitors (EMPs).

Strictly speaking, definitive hematopoiesis first occurs in the AGM region of the embryo proper between E9.0 and 10.5, and gives rise to dHSCs which are capable of differentiating into all hematopoietic lineages (Cumano and Godin, 2007). Considerable debate exists about the possibility of yolk sac cells giving rise to dHSCs from E9.5 onwards, but it is generally accepted that the AGM region is the primary source of dHSCs in mice (Jagannathan-Bogdan and Zon, 2013). Recent studies have further characterized a tissue found in the AGM known as hemogenic endothelium that consists of endothelial cells that give rise to dHSCs (Tavian et al., 1996). These cells are thought to either bud off from the ventral wall of the aorta (Kissa and Herbomel, 2010) or to be derived from sub-aortic mesenchymal cells that migrate through the wall of the dorsal aorta (Bertrand et al., 2005). Definitive HSCs subsequently populate other hematopoietic organs where they mature into adult-type erythroid, myeloid, and lymphoid lineages (Figure 1.3) (Orkin and Zon, 2008; Medvinsky et al., 2011).

The placenta is another hematopoietic organ that contributes to the exchange of gases and nutrition between the mother and the developing conceptus (Mikkola et al., 2005).

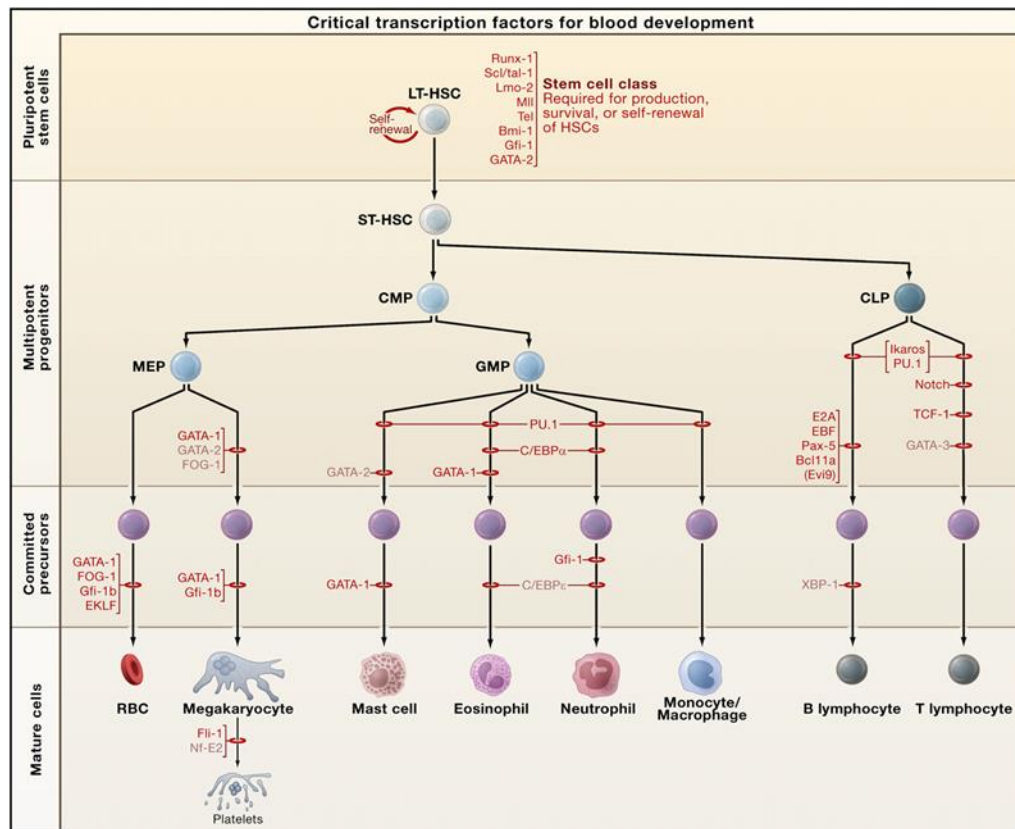


Figure 1.3: Transcription factors involved in hematopoiesis.

Several transcription factors play critical roles during hematopoiesis. Red bars represent the stage at which the knockout of a given gene blocks hematopoietic development. All factors depicted in bold have been associated with human and/or murine hematopoietic oncogenesis. LT-HSC - long-term hematopoietic stem cell; ST-HSC - short-term hematopoietic stem cell; CMP - common myeloid progenitor; CLP - common lymphoid progenitor; MEP - megakaryocyte/erythroid progenitor; GMP - granulocyte/macrophage progenitor; RBCs - red blood cells. (Orkin and Zon, 2008, reproduced with permission from the Cell publishing group)

This organ is a highly vascularized structure that forms after the embryonic allantois fuses to the chorion (Figure 1.2); a structure that integrates into the maternal endometrium (Downs, 2002). The chorion itself, when harvested before fusion to the allantois, can also generate hematopoietic cells in culture (Zeigler et al., 2006). Definitive HSCs are present in the placenta as early as E10.5, but the source of these cells remains unknown (Gekas et al., 2005). Limited data suggests that dHSCs may also form autonomously in the placenta (Rhodes et al., 2008).

1.3 Genetic Control of Hematopoiesis

Several transcription factors are known to play critical roles in the specification, maintenance, and differentiation of hematopoietic stem cells (Figure 1.3). Formation of the hemangioblast from mesodermal tissue, the first critical stage of hematopoietic development, is thought to depend on a number of proteins that control the expression of downstream transcription factors and effectors. Critical transcription factors include stem cell leukemia (Scl/Tal1), LIM-only domain 2 (Lmo2/Rbtn2), Gata1 and Gata2, friend leukemia integration 1 (Fli1), and ETS variant 2 (Etv2/Etsrp). Of particular interest are Scl and Lmo2, each of which is independently required for both primitive and definitive hematopoiesis (Kim and Bresnick, 2007). The activity of these proteins within the hemangioblast is thought to specify hematopoietic, rather than vascular, fate (Kim and Bresnick, 2007).

The *Scl* gene encodes a basic helix-loop-helix transcription factor that is considered to be a master regulator of hematopoietic development (Porcher et al., 1996), and is capable of recruiting mesodermal cells to hematopoietic fates (Ismailoglu et al., 2008). *Scl* directly binds DNA at the canonical “E-box” motif CANNTG, and several direct target genes have been identified, including *Gata1*, *Runx1*, and α -globin (Vyas et al., 1999; Anguita et al., 2004; Landry et al., 2008). In mice, *Scl* is expressed in both intra-embryonic and extra-embryonic mesoderm at E7.5, in blood islands at E7.5-8.5, and in various areas of the E9.5 embryo including the yolk sac, neural tube, and developing liver (Kallianpur et al., 1994). *Scl*^{-/-} embryos do not undergo primitive hematopoiesis, and die at approximately E9.5 (Shivdasani et al., 1995). The development of embryonic dHSCs depends on *Scl*, but this protein is also specifically associated with the differentiation of erythroid and megakaryocyte lineages (Hall et al., 2003). *Scl* and its closely related paralog lymphoblastic leukemia derived sequence 1 (*Lyl1*) are also essential to the survival of adult dHSCs, underlining the importance of these factors in hematopoietic homeostasis (Souroullas et al., 2009). Although closely related to *Scl*, *Lyl1* is predominantly expressed at later developmental stages, and is unable to rescue the early hematopoietic defects observed in *Scl*^{-/-} embryos, indicative of functional specificity (Chan et al., 2007).

Scl is known to act in concert with other critical hematopoietic proteins, including *Gata1*, *Gata2*, and *Lmo2*, in both primitive and definitive hematopoiesis (Pimanda et al., 2007). *Gata1* is essential for erythroid development (Fujiwara et al., 1996), while *Gata2* is required for the maintenance of hematopoietic progenitor cells (Tsai et al., 1994).

Considerable cross-regulation exists among these proteins, and the hematopoietic phenotypes of all deletion mutants are similar, although Scl deletion is more critical at early developmental stages (Wozniak et al., 2008). Consistent with this, concurrent binding of Scl/Gata complexes to E-box/GATA motifs regulates many erythroid-specific genes (Vyas et al., 1999) and, interestingly, *Scl*^{-/-} cells exhibit significant rescue via a mutant version of Scl that is unable to bind DNA, indicating that its binding to E-box elements is dispensable for regulation of at least some target genes (Porcher et al., 1999).

In wild type mice, Lmo2 expression was originally detected in the fetal liver, adult spleen, thymus, and B cells (Foroni et al., 1992). Subsequent knockout studies demonstrated that Lmo2 plays critical roles in primitive hematopoiesis, and *Lmo2*^{-/-} embryos die at E10.5 due to a failure of yolk sac erythropoiesis (Warren et al., 1994). In addition to the erythropoietic defects observed in *Lmo2*^{-/-} embryos, anterior-posterior (AP) development is stunted, and distension of the pericardial sac is evident (Warren et al., 1994). Lmo2 does not directly bind DNA, but rather acts as a scaffold molecule that facilitates protein-protein interactions, enabling the assembly of complexes that typically include Scl and Gata members (Bach, 2000). In zebrafish, concurrent overexpression of Lmo2 and Scl results in the expansion of endothelial and hematopoietic lineages, indicating that these proteins can specify mesoderm to a hemangioblast fate (Mead et al., 2001; Gering et al., 2003).

Gata1 and purine box 1 (Pu.1/Sfpi1) are two additional critical transcription factors involved in primitive hematopoiesis, and Gata1 expression is the first marker of primitive erythroid cells (Pevny et al., 1991). Gata1 is also a master regulator of erythrocyte development, and *Gata1*^{-/-} embryos fail to develop erythrocytes, resulting in death at E11.5 (Fujiwara et al., 1996). Both Gata1 and Gata2 are expressed in the yolk sac starting at E7.5, and *Gata2* deletion also results in embryonic death at E11.5 (Tsai et al., 1994). Unlike Gata2, however, Gata1 both promotes erythroid fate and acts to inhibit myeloid-specific cell (macrophages and granulocytes) formation, possibly by directly competing for Pu.1 binding to target genes (Cantor et al., 2002). Conversely, Pu.1 leads to the formation of myeloid cells, and inhibits Gata1 expression (Scott et al., 1994). *Pu.1*^{-/-} mutants are viable but adult mice lack monocytes, macrophages, and neutrophils (Anderson et al., 1998).

A number of transcription factors also exhibit critical effects in definitive hematopoiesis. A member of the runt family of proteins, Runx1 (AML/Cbfa2) is one such factor, and Runx1 mutants display a lack of definitive erythroid, myeloid, and lymphoid cells (Wang et al., 1996a). This phenotype is due to the requirement of Runx1 within dHSCs in the AGM region of mice (Orkin, 2000). Although Runx1 expression is a hallmark of definitive hematopoiesis, recent evidence also suggests a role in primitive hematopoiesis. Indeed, Runx1 is detected in the allantois prior to fusion with the chorion (Corbel et al., 2007), and LacZ tracing indicates that Runx1 is expressed in the yolk sac at E7.5. These data indicate that Runx1 plays a role in primitive hematopoiesis, and/or supports the hypothesis that the yolk sac has the potential to generate dHSCs (Samokhvalov et al.,

2007). Interestingly, Runx1 is required for blood formation from hemogenic endothelium, but not from yolk sac hemangioblasts (North et al., 2002). Deletion of Runx1 results in decreased expression of *Cmyb*, a protein that is also thought to play role in primitive hematopoiesis (Moriyama et al., 2010). *Cmyb*^{-/-} embryos die at approximately E15.0 due to severe anemia, implicating a critical role for its function in definitive hematopoiesis (Mucenski et al., 1991).

Myeloid ecotropic viral integration site 1 (Meis1) is another transcription factor that has been found to regulate definitive hematopoiesis, with expression evident in the AGM and fetal liver (Azcoitia et al., 2005). *Meis1*^{-/-} embryos die between E11.5-14.5 due to defects in the generation of dHSCs, combined with severe hemorrhaging within the embryo proper (Azcoitia et al., 2005). *Meis1*^{-/-} embryos also exhibit impaired expression of Runx1⁺ cells, although *Runx1* is not thought to be a direct Meis1 target gene (Azcoitia et al., 2005). A recent study in zebrafish indicates a role for Meis1 in the establishment of mesoderm that gives rise to the hemangioblast (Amali et al., 2013), but this effect appears to be species-specific.

Hox proteins are a family of homeobox transcription factors involved in hematopoiesis, and a subset of these proteins require interaction with Meis and/or Pre-B-cell leukemia homeobox (Pbx) homeodomain members for proper function (Mann and Affolter, 1998). Due to the redundancy between certain members of the 39 murine Hox proteins, loss-of-function studies involving individual *Hox* genes do not always exhibit phenotypic changes (Magli et al., 1997). For example, gain-of-function studies have shown that

HoxB4 is a potent enhancer of HSC expansion (Antonchuk et al., 2002), while *HoxB4*^{-/-} embryos exhibit only slight hematopoietic defects (Brun et al., 2004). Similarly, aberrant hematopoietic effects are observed in gain-of-function studies involving HoxA9 (Kroon et al., 1998), HoxA10 (Thorsteinsdottir et al., 1997), and HoxB3 (Sauvageau et al., 1997). The Meis family, in contrast, has only three members, which could explain the severity of developmental defects associated with *Meis1* mutant mice compared to *Hox* mutants. In this regard, Meis1 and Meis2 have recently been shown to strongly induce the formation of hematopoietic cells from embryonic stem cells (Cai et al., 2012), but further study of Meis2 and Meis3 are required to elucidate their functions in hematopoiesis.

1.4 Vasculogenesis

Concurrent with hematopoietic development, vascular networks are generated in the murine yolk sac and embryo proper to facilitate the transfer of oxygen and nutrients to the developing embryo. Hemangioblasts give rise to hematopoietic cells, but also generate primitive vascular cells known as angioblasts. The initial step in blood vessel development, termed vasculogenesis, involves the coalescence of yolk sac angioblasts into simple tube-like structures that interconnect to form the vascular plexus (Risau and Flamme, 1995). Angioblasts gradually branch out from the blood islands to cover the entire surface of the yolk sac (Figure 1.4 A and B; Jones et al., 2006). Subsequent vascular remodelling then occurs, generating the tree-like structure displayed in Figure

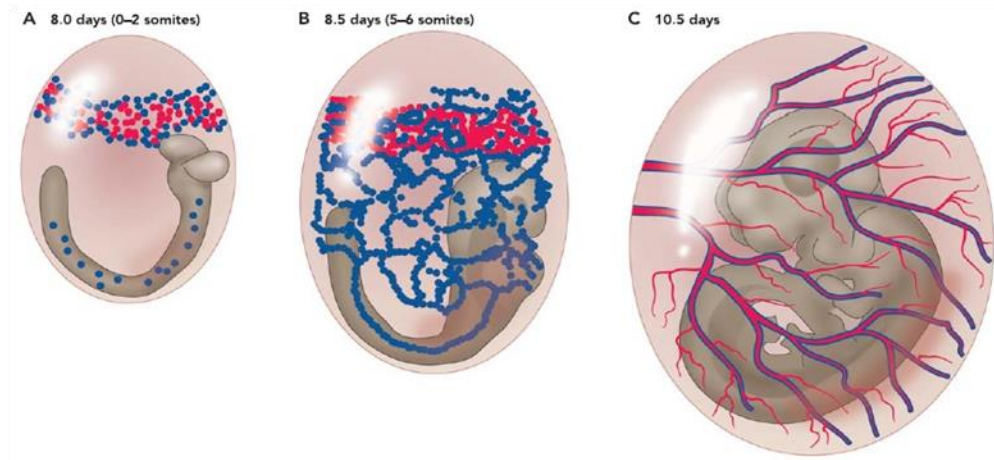


Figure 1.4: Murine extraembryonic vascular development.

Embryonic vascular development occurs in both the yolk sac and embryo proper.

A) Blood islands contain both hematopoietic (red) and endothelial (blue) cells, while the embryo proper only develops endothelial cells

B) Yolk sac cells organize to form the vascular plexus

C) Upon the introduction of blood flow, vessels remodel into a tree-like architecture.
(Jones et al., 2006, reproduced with permission from the Physiology publishing group)

1.4 C. Vasculogenesis is the process of *de novo* generation of endothelial structures, while angiogenesis describes the remodelling of existing structures into higher order architecture.

Intra-embryonic vessels develop concurrently with the extra-embryonic vascular plexus, and interconnect with the yolk sac via the dorsal aorta and cardinal vein (Jones et al., 2006). Upon the formation of a beating heart at E8.0-8.5, blood flow introduces fluid flow and shear stresses into the vessels, contributing to the remodelling of the endothelial network (Lucitti et al., 2007). Due to the high number of erythrocytes and immaturity of the vasculature at early stages, large-scale movement of yolk-sac derived erythroid cells is only apparent from E10.0 onwards (McGrath et al., 2003). The hypoxic environment of the primitive vascular system also plays a critical role in the signalling of angiogenic events via vascular endothelial growth factor (VEGFA/VEGF) (Kuwabara et al., 1995; Mazure et al., 1996), and shear stress impacts the transcription of endothelial-specific transcriptional networks (Dusserre et al., 2004).

1.5 Genetic Control of Vasculogenesis

Genetic factors, hypoxia, and fluid stresses play many roles in the development of yolk sac vasculature, but interplay between these phenomena confounds the understanding of yolk sac development. Without the introduction of fluid flow into developing endothelial vessels, the vascular plexus forms but fails to mature (Wakimoto et al., 2000), clearly demonstrating that genetic factors alone cannot account for the

development of yolk sac vasculature. Interestingly, there are currently no known genes that are expressed in developing endothelial cells that are not also expressed in some hematopoietic cells; conversely, several genes are uniquely expressed in hematopoietic cells that are absent in the endothelium (Medvinsky et al., 2011). As one example of the former, Flk1 is essential for both hematopoietic and vascular development. *Flk1*^{-/-} mice die between E8.5 and E9.5 (Shalaby et al., 1995), and lack blood island formation and all vascular structures, presumably due to the improper formation of hemangioblasts at early stages, or improper migration of mesodermal cells into the yolk sac (Shalaby et al., 1997).

Zebrafish studies indicate that Scl has the ability to induce both hematopoietic and vascular lineages, depending on temporal expression and specific regulatory networks that are unique to each cellular environment (Dooley et al., 2005). Scl and Lyl1 are both expressed in murine endothelial cells, and while Scl plays critical roles in vascular maturation (Visvader et al., 1998), *Lyl1*^{-/-} mice are viable and fertile (Capron et al., 2006), indicating that Lyl1 does not play a role in vasculogenesis, or that Scl compensates in *Lyl1*^{-/-} mutants. When *Scl*^{-/-} embryos were rescued by a transgenic approach that put *Scl* under the control of the hematopoietic-specific *Gata1* promoter, many hematopoietic defects were rescued, but vascular remodelling failed to generate mature vessel architecture (Visvader et al., 1998). These data demonstrate a requirement of Scl in murine angiogenic events, in addition to the known function of Scl in hemangioblasts (Visvader et al., 1998).

VEGFA is a crucial factor involved in vascular development, and expression of this protein is evident from the time of blood island formation through later stages of blood vessel remodelling (reviewed by (Haigh, 2008). Flk1 and Flt1 (VEGFR1) are VEGFA receptor tyrosine kinases (Patan, 2000). As previously mentioned, Flk1 is a marker of hemangioblasts, and similar to *Flk1*^{-/-} embryos, *VEGFA*^{-/-} embryos die between E8.5 and E9.5 due to defects in hemangioblast maturation (Carmeliet et al., 1996). *VEGFA*^{+/-} mice also die at midgestation, highlighting the critical role that VEGFA and Flk1/Flt1 interaction plays in normal development (Ferrara et al., 1996).

Gata1^{-/-}, *Gata2*^{-/-} and *Lmo2*^{-/-} embryos all have defects in primitive erythropoiesis and die from severe anemia at approximately E10-11.5, but none of these mutants display early vascular defects (Tsai et al., 1994; Warren et al., 1994; Fujiwara et al., 1996). *Lmo2* is expressed in vascular endothelium, but again plays a role in vascular remodelling, rather than *de novo* generation of yolk sac vasculature (Yamada et al., 2000). *Meis1*^{-/-} embryos also exhibit vascular defects, leading to extensive hemorrhaging and embryonic death between E11.5 and E14.5 (Azcoitia et al., 2005).

Several members of the Hox family have been implicated in the development of vasculature and subsequent remodelling (reviewed by (Cantile et al., 2008). Hox members are involved in the regulation of both *Flk1* and *Flt1* expression (Wu et al., 2003; Coultas et al., 2005), while HoxD3 plays important roles in angiogenesis (Uyeno et al., 2001; Boudreau and Varner, 2004). *HoxA3*^{-/-} mice exhibit abnormal or absent arterial formation and subsequent vascular defects (Chisaka and Kameda, 2005), while *Hoxa13*^{-/-}

mice die at E11.5-15.5 due to improper development of umbilical vasculature (Warot et al., 1997). Hox members can also induce vascular sprouting in chick chorio-allantoic membranes (Myers et al., 2000). A recent study in zebrafish placed Hoxd4a near the top of a hierarchy that controls the expression of *meis1.1* and *scl* (Amali et al., 2013), and demonstrated a critical role for Hoxd4a in vasculogenesis, hematopoiesis, and angiogenesis.

1.6 Cdx proteins

Homeodomain proteins are transcription factors that play critical roles in embryonic development and are involved in various processes in the adult. The homeobox gene *caudal* was originally identified in *Drosophila*, and encodes a transcription factor that binds DNA through an evolutionarily conserved α -helix domain (Mlodzik et al., 1985). The study of *caudal* mutants gave insights into the roles that this gene plays in AP patterning and posterior specification in *Drosophila* (Macdonald and Struhl, 1986). Homologues of *caudal* were subsequently identified in zebrafish (*cdx1a*, *cdx1b* and *cdx4*), mice (*Cdx1*, *Cdx2*, and *Cdx4*), and humans (*CDX1*, *CDX2*, and *CDX4*), among other species. Vertebrate *Cdx* genes are thought to have arisen from a *ProtoHox* gene cluster that also gave rise to the *Hox* genes (Ferrier and Holland, 2001), and are considered master regulators of *Hox* gene expression.

The expression patterns of murine Cdx members vary both spatially and temporally during development, but exhibit significant overlap in the post-implantation embryo

(Figure 1.5). Cdx1 transcripts are first detected at E7.5 in the caudal-most portion of the primitive streak, and persist in the somites, forelimb buds, and tail bud until E11.5 (Meyer and Gruss, 1993). Cdx2 transcripts are detected in the caudal embryo from E7.5 through E12.5, but also play earlier roles in the extra-embryonic trophoderm, and persist in extraembryonic tissues through E12.5 (Beck et al., 1995). Cdx4 is expressed from E7.5 through E10.5, beginning in the caudal primitive streak and remaining confined to the most caudal portions of the tail bud at later stages. Cdx1 and Cdx2 (but not Cdx4) are also involved in the development and maintenance of the adult intestinal epithelium (Reviewed (Guo et al., 2004). Notably, all Cdx genes are co-expressed in the posterior primitive streak at E7.5 (Gamer and Wright, 1993; Meyer and Gruss, 1993; Beck et al., 1995), the site and developmental stage at which most hemangioblasts are detected (Huber et al., 2004).

Cdx proteins share extensive sequence similarity in their DNA-binding homeodomains; however, there is little conservation between their remaining sequences (Figure 1.6). In mice and *Drosophila*, a canonical Cdx response element (CDRE) has been identified, comprised of the sequence TTTATG, which is recognized by all Cdx members (Dearolf et al., 1989). Although canonical CDRE sequences are associated with Cdx binding, variations of this sequence also impact protein-DNA interaction (Margalit et al., 1993). Cdx proteins are generally known as transcriptional activators, although evidence does exist to suggest repressive roles in some instances (Chun et al., 2007; Wang et al., 2010; Faber et al., 2013).

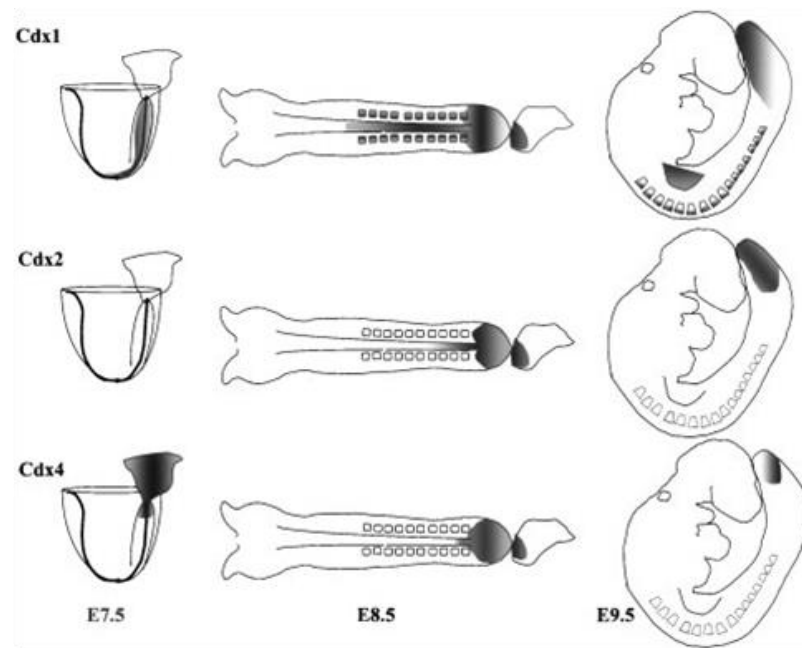


Figure 1.5: Expression profile of Cdx members in E7.5–E9.5 murine embryos.

Cdx members are expressed in the developing embryo proper at different stages and intensities. Allantoic expression is displayed at E7.5 and E8.5. Expression level is denoted by grey shading for each Cdx gene. (Lohnes 2003, reproduced with permission from the BioEssays publishing group).

1.7 Cdx loss of function studies

Mouse, frog, and zebrafish loss of function models have proven useful in the study of vertebrate Cdx function. *Cdx1*^{-/-} mutant mice are viable, but exhibit homeotic transformations of the cervical vertebrae due to disruptions in vertebral patterning, while *Cdx1*^{+/-} mice develop normally (Subramanian et al., 1995). Homozygous deletion of *Cdx2* results in peri-implantation lethality in mice due to critical roles for Cdx2 in the trophectoderm (Chawengsaksophak et al., 1997). *Cdx2* heterozygous mutants display some similarity to *Cdx1*^{-/-} mutants, exhibiting homeotic vertebral transformations of posterior cervical and upper thoracic vertebrae (Chawengsaksophak et al., 1997). This observation correlates with the later onset of Cdx2 expression compared to Cdx1 (Lohnes, 2003). In addition to these vertebral defects, adult *Cdx2*^{+/-} mutants also exhibit intestinal lesions, consistent with the role of Cdx2 in intestinal homeostasis (Chawengsaksophak et al., 1997).

To circumvent the embryonic lethality of *Cdx2*^{-/-} mutants, tetraploid aggregation was employed to study post-implantation *Cdx2* mutants (Chawengsaksophak et al., 2004). Impaired development of the allantois was reported in these mutants, along with defects in yolk sac vascular remodelling, truncation of the embryo at the level of the forelimb bud, and distension of the pericardium (Chawengsaksophak et al., 2004). In recent years a conditional *Cdx2* floxed model has been developed to further study the effect of *Cdx2* deletion. Cre/*loxP*-mediated inactivation of *Cdx2* at post-implantation stages lead to death *in utero* at approximately E11.5, likely due to improper chorio-allantoic fusion or cardiac insufficiency (Chawengsaksophak et al., 2004; Savory et al.,

2009a), consistent with the tetraploid aggregation study (Chawengsaksophak et al., 2004). Novel non-Hox Cdx target genes were also identified, including *Brachyury*, *Wnt3a*, and *Cyp26a1* (Savory et al., 2009a).

Cdx4^{-/-} mutants display no overt phenotypic changes, indicating that Cdx1 and Cdx2 may exhibit functional overlap with Cdx4 (van Nes et al., 2006). Consistent with this, *Cdx4* deletion exacerbates the defects observed in *Cdx1* and *Cdx2* mutants (van Nes et al., 2006). In this regard, several studies have characterized compound Cdx mutants. *Cdx1*^{+/-} *Cdx2*^{+/-} and *Cdx1*^{-/-} *Cdx2*^{+/-} mutants display progressively more severe vertebral defects and truncation of the tail (van den Akker et al., 2002). *Cdx2*^{+/-} *Cdx4*^{-/0} mutants exhibit diminished presomitic mesoderm tissue, impaired chorio-allantoic fusion, and subsequent placental disruption, resulting in embryonic lethality at approximately E10.5 (van Nes et al., 2006). These results indicate overlapping roles for Cdx members in mesodermal tissues (van Nes et al., 2006). This functional overlap was also addressed by gene-swap studies, in which *Cdx2* was knocked into the *Cdx1* genetic locus; such mice exhibit normal vertebral patterning, suggesting that Cdx members converge on common target genes involved in axial morphogenesis (Savory et al., 2009b).

The conditional *Cdx2* knockout model makes use of the Cre/*loxP* system in which *Cdx2* exon two is flanked by *loxP* sites. Loss of this domain was predicted to result in a functionally null protein (Figure 1.7). The Cre recombinase protein used for these studies is fused to a modified estrogen receptor ligand binding domain that responds exclusively to tamoxifen (ER^T), and is sequestered in the cytoplasm by Hsp90 in the

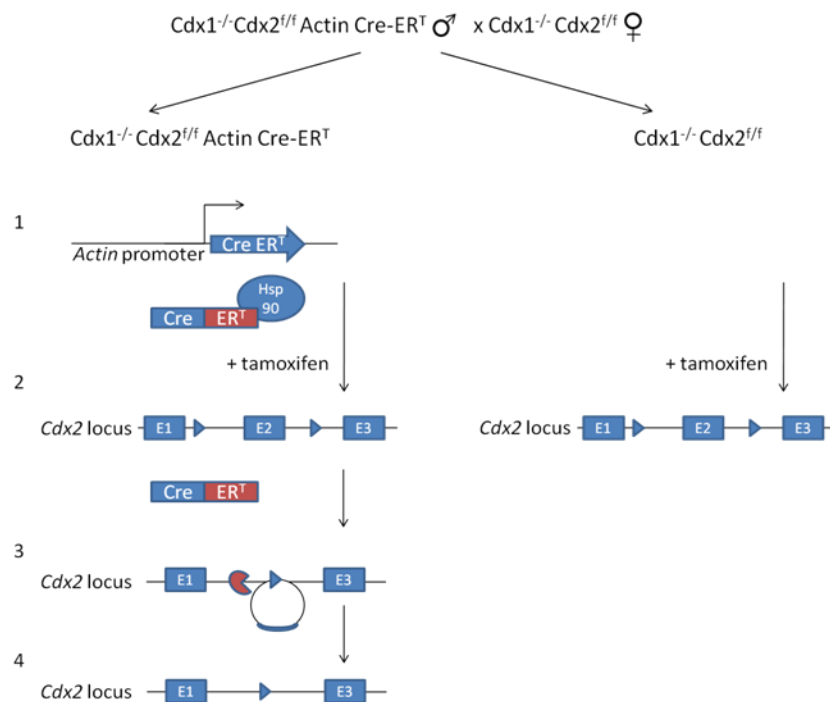


Figure 1.7: Generation of conditional *Cdx1*^{-/-} *Cdx2*^{-/-} knockout.

A *Cdx1*^{-/-} *Cdx2*^{fl/fl} Actin Cre-ER^T male is bred with a *Cdx1*^{-/-} *Cdx2*^{fl/fl} female to produce *Cdx1*^{-/-} *Cdx2*^{fl/fl} offspring with or without *Actin Cre* in a predicted 1:1 ratio. 1) *Cre* is transcribed under the control of the *Actin* promoter. The Cre protein is fused to a modified estrogen receptor ligand binding domain (ER^T) that responds to tamoxifen. In the absence of tamoxifen, association with Hsp90 results in cytoplasmic sequestration of the chimeric protein. 2-4) Exon 2 of *Cdx2* is flanked by two *loxP* sites. Pregnant females are given 2 mg tamoxifen at the desired stage, resulting in the Cre-mediated recombination and excision of *Cdx2* exon 2.

absence of ligand. Tamoxifen administration results in the release of the Hsp90 protein and subsequent translocation of the Cre-ER^T fusion protein to the nucleus, where it excises the *Cdx2* sequences within the *loxP* sites. We have used a Cre transgene driven by the ubiquitously expressed *β-Actin* promoter, thereby targeting all cells of the developing embryo. We have also generated *Cdx1*^{-/-}*Cdx2*^{-/-} embryos, by crossing the *Cdx2* conditional allele with the *Cdx1*^{-/-} mutant (Savory et al., 2011a). These *Cdx1*^{-/-}*Cdx2*^{-/-} embryos lack *Cdx4* expression, likely due to the fact that *Cdx4* is a direct *Cdx2* target gene (Savory et al., 2011b), and are therefore effectively Cdx null. *Cdx1*^{-/-}*Cdx2*^{-/-} embryos exhibit numerous abnormalities observed in previous Cdx mutant models, including chorio-allantoic defects, distended pericardium, and axial truncation caudal to the presumptive forelimb bud, along with novel defects including improper closure of the neural tube, likely due to disrupted planar cell polarity (Savory et al., 2011a). Defects are also evident in the yolk sacs of *Cdx1*^{-/-}*Cdx2*^{-/-} embryos from E8.5 onwards, including disruption of blood formation and endothelial development, prompting the current study.

1.8 Cdx proteins in hematopoiesis

Mutations of *Cdx1* and *Cdx4* have little effect on yolk sac vasculogenesis or hematopoietic development, likely due to functional overlap among Cdx family members (van den Akker et al., 2002; van Nes et al., 2006; Savory et al., 2011a), while *Cdx2* mutants display abnormal yolk sac vasculature and circulation at E8.5

(Chawengsaksophak et al., 2004). In mice, *Cdx2* and *Cdx4* are co-expressed in the allantois (van Nes et al., 2006), and *Cdx4* has been implicated in placental formation by examining *Cdx4* deletion in a *Cdx2*^{+/-} background, strengthening existing evidence that *Cdx* members impact chorio-allantoic fusion events (van Nes et al., 2006). *Cdx4*^{-/-} mutants exhibit a diminished number of erythroid colonies derived from yolk sac tissue (Wang et al., 2008), but these defects are not significant enough to affect the development of *Cdx4*^{-/-} mice (van Nes et al., 2006).

Zebrafish studies have proven useful to gain insights into vertebrate hematopoiesis, frequently utilizing morpholino (MO) knockdown of specific mRNA transcripts or mutant genetic strains. In this context, *cdx1*^{MO} morphants display no overt morphological defects, and hematopoietic events appear to proceed normally (Davidson and Zon, 2006). In contrast, *cdx4*^{-/-} (analogous to murine *Cdx2*^{-/-}) mutants exhibit severe anemia, and decreased expression of *scl*, *lmo2*, and *gata1*. Erythropoiesis can be rescued by overexpression of a subset of Hox proteins, but not *Scl*, indicating that *Scl* is dependent on *Cdx4* to specify hematopoietic fate (Davidson et al., 2003).

Analysis of compound *cdx1a*^{MO}*cdx4*^{-/-} zebrafish mutants revealed a complete lack of both primitive and definitive hematopoiesis (Davidson and Zon, 2006). The expression of several *hox* genes were perturbed in these mutants, while the transcript levels of *scl* were downregulated only in hematopoietic precursors, remaining unaltered in adjacent angioblasts (Davidson and Zon, 2006). *Runx1a* transcripts were also significantly diminished in *cdx1a*^{MO}*cdx4*^{-/-} embryos, consistent with disruption of definitive

hematopoiesis (Davidson and Zon, 2006). Overexpression of *hoxa9a* was sufficient to rescue the formation of both primitive and definitive hematopoietic lineages, but rescue was incomplete, indicating that additional pathways were disrupted upon the loss of *cdx* (Davidson and Zon, 2006). *Cdx1*^{-/-}*Cdx4*^{-/0} mutant mice were found to be viable and fertile, with no reported defects in hematopoiesis (van Nes et al., 2006). Similarly, *Cdx2*^{+/-}/*Cdx4*^{-/0} mutants displayed normal vascular and hematopoietic development until E9.5, but died shortly thereafter, likely due to chorio-allantoic or placental defects (van Nes et al., 2006).

Murine embryonic stem cell (mESCs) models have also been used to examine the role of Cdx in hematopoietic events. Deletion of *Cdx1* or *Cdx4* in mESCs resulted in modest hematopoietic defects, while *Cdx2* knockout resulted in a more severe phenotype (Wang et al., 2008). A microarray analysis of *Cdx2*^{-/-} mESCs revealed a significant reduction of *Hox* transcripts, along with decreased transcript levels of *Scl*, *Gata1*, *βH1 globin* (markers of primitive hematopoiesis), and *Runx1* (a marker of definitive hematopoiesis) (Wang et al., 2008). Notably, all three markers of primitive hematopoiesis (*Scl*, *Gata1*, *βH1 globin*) identified in the Wang study are signatures of blood islands at E7.5-7.75 (Onodera et al., 1997; Silver and Palis, 1997; Belaoussoff et al., 1998). Contrary to the completely bloodless phenotype observed in *cdx1a*^{MO}*cdx4*^{-/-} zebrafish, knockdown of all *Cdx* members in mESCs resulted in a significant but incomplete failure of hematopoietic differentiation (Wang et al., 2008). In mESCs, *Cdx4* overexpression was also found to expand the populations of erythrocytes,

megakaryocytes, granulocytes, and macrophages, indicating that this Cdx member has pro-hematopoietic effects (Wang et al., 2005).

1.9 Blood Cancers

In addition to developmental hematopoiesis, Cdx members also play a role in adult leukemias. Cdx1 and Cdx4 are detected at very low levels in adult bone marrow, while Cdx2 is completely absent (Chase et al., 1999; Koo et al., 2010). In contrast, CDX2 expression is evident in most cases of acute myeloid leukemia (AML) (Scholl et al., 2007), and recent studies also indicate a role for CDX2 in T-cell acute lymphoblastic leukemia (T-ALL) (Riedt et al., 2009). CDX2 translocations are associated with AML in humans (Riedt et al., 2009), while very little data exists to support a link between CDX1 or CDX4 and leukemogenesis.

In support of Cdx2 inducing transformation of adult blood cells, overexpression of Cdx2 in murine progenitor cells effectively induces AML (Rawat et al., 2004; Scholl et al., 2007). Interestingly, overexpression of Cdx4 in murine bone marrow also gives rise to leukemia (Bansal et al., 2006), indicating that a similar role may yet be identified in human patients. Aberrant *Hox* gene expression is also observed in several types of human leukemias (Frohling et al., 2007; Scholl et al., 2007). However, HOX gene expression does not correlate with aberrant CDX2 expression in T-ALL (Lengerke and Daley, 2012), indicating that CDX2 may impact pathways unrelated to HOX in leukemogenesis.

Many of the transcription factors involved in hematopoiesis were originally identified in human leukemias, and their misexpression, or altered function, often results in malignancies in murine models (McGuire et al., 1989; Brown et al., 1990; Miyoshi et al., 1991; Ziemer-van der Poel et al., 1991). For example, translocations of *SCL*, *LMO2*, *RUNX1*, *MLL*, or *ETV6* are evident in almost all cases of human leukemia (Orkin and Zon, 2008), and *SCL* and *LYL1* were originally identified via their chromosomal translocations in T-ALL (Cleary et al., 1988; Begley and Green, 1999). Subsequent studies have revealed overexpression of *SCL* in a significant proportion of T-ALL cases that lack *SCL* chromosomal translocation, further suggesting that aberrant *SCL* activation is critical to the development of T-ALL (Ferrando et al., 2002). Similarly, *LMO2* was originally identified by examining chromosomal translocations in T-ALL (Royer-Pokora et al., 1995), and the close relationship between *SCL* and *LMO2* in embryonic hematopoiesis appears to coincide with the development of T-ALL.

In both humans and mice, *Meis1* overexpression has been linked to the induction of AML and most types of acute lymphoblastic leukemia (ALL) (Moskow et al., 1995; Afonja et al., 2000; Imamura et al., 2002). Interestingly, the leukemia-inducing effect of *Cdx4* overexpression in murine models is exacerbated by concurrent overexpression of *Meis1a* (Bansal et al., 2006). However, a long period of latency existed before *Cdx4* overexpression induced AML in this study (Bansal et al., 2006). This result may reflect aberrant overexpression of Hox genes (driven by *Cdx4*) acting in concert with *Meis1a* to promote AML development.

1.10 Identification of novel hematopoietic genes possibly controlled by Cdx

To better understand the functions of Cdx members, and the networks in which they elicit their effects, it is important to identify specific genes that are transcribed under the control of Cdx members. An experiment was undertaken by members of our research group to identify novel genetic loci bound by Cdx2. This was accomplished by performing a chromatin immunoprecipitation (ChIP) study combined with deep sequencing (ChIP-seq). Using wild type E8.5 CD1 embryos, immunoprecipitation was performed with a custom-designed antibody against Cdx2 (Savory et al., 2009b), using immunoglobulin G (IgG) as a baseline control for non-specific DNA binding. Chromatin that was bound by each antibody was subsequently subjected to deep sequencing to map the binding of Cdx2 to unique regions of DNA, which were then analyzed using statistical algorithms. Several hematopoietic genes of interest were highlighted in this experiment, including *Scl*, *Lmo2*, *Meis1*, and *Lyl1*. Each of these genes was studied using different assays, with particular emphasis being placed on the *Scl* gene.

1.11 Cdx2 Interacts with the SWI/SNF Complex

Cdx proteins are believed to recruit co-regulators to target loci, thereby facilitating transcriptional machinery to access DNA and transcribe target genes. However, the

nature of such Cdx co-regulators, and the mechanisms through which they elicit their functions, are not known. To this end, members of our research group used stable isotope labelling with amino acids in cell culture (SILAC), combined with mass spectrophotometry (MS) to identify putative Cdx2 interactors. The SILAC-MS experiment was undertaken in human embryonic kidney (HEK) 293 cells due to the high level of CDX2 protein expression in these cells. By utilizing a cell line that expressed native CDX2 at high levels, immunoprecipitation was easily achieved, and any proteins or complexes that normally associated with CDX2 were pulled down for identification via MS.

Proteins that were recovered in this analysis included members of the switch/sucrose non-fermentable (SWI/SNF) chromatin remodelling complex. The SWI/SNF complex acts to remodel chromatin structure via conformational or positional changes of nucleosomes, to allow transcriptional machinery access to target genes (Narlikar et al., 2002). Transcriptional regulators can recruit such chromatin remodelling complexes to target loci, inducing an altered chromatin structure, thus conferring temporal and spatial specificity to the transcription of target genes (Kadam and Emerson, 2003).

The SWI/SNF complex was originally identified in yeast (Neigeborn and Carlson, 1984; Stern et al., 1984), and homologs have since been identified in all eukaryotes, highlighting the importance of these complexes throughout evolution. The mammalian SWI/SNF family are ATP-dependent chromatin remodelling complexes (Wang et al., 1996c) that consist of several core members, including the requisite ATPase hub proteins Brahma (Brm/SMARCA2) or Brahma-related gene 1 (Brg1/Baf190/SMARCA4),

and Brg-associated factors (Baf) 45, 47, 53, 57, 60, 155, 170, 180, 200, and 250 (Figure 1.8) (Tang et al., 2010). Brg1/Brm, Baf47, Baf155, and Baf170 are considered core members of the SWI/SNF complex, based on *in vitro* studies demonstrating that the remodelling activity of these few combined factors is equal to that of the complete complex (Phelan et al., 1999). Consistent with the importance of these core members, murine knockout of *Brg1* (Bultman et al., 2000), *Baf47* (Klochender-Yeivin et al., 2000), or *Baf155* (Kim et al., 2001) all result in an early lethal phenotype.

Specific conformations of SWI/SNF complexes are based around either Brm or Brg1 as the central catalytic subunit, but each hub protein is also utilized in unique contexts (Wang et al., 1996b). Peripheral members contribute to sub-complexes in developmental-stage and tissue-specific contexts (Tang et al., 2010). Based on the SILAC-MS data, along with existing reports of Cdx2-Brg1 interactions (Yamamichi et al., 2009; Wang et al., 2010), Brg1 became the focus of my studies. *In vitro* assays indicate that Cdx2 directly binds several members of the SWI/SNF complex, including Baf47, Brm, and Brg1 (Yamamichi et al., 2009). The Yamamichi group also demonstrated the effective chromatin immunoprecipitation of both Cdx2 and Brg1 to a region of the *Villin* promoter that contains two canonical CDREs. Consistent with this, recent work also indicates that Cdx2 and Brg1 co-regulate the transcription of *Oct4* at very early stages of mouse development, and co-localize to the *Oct4* locus in ChIP experiments (Wang et al., 2010).

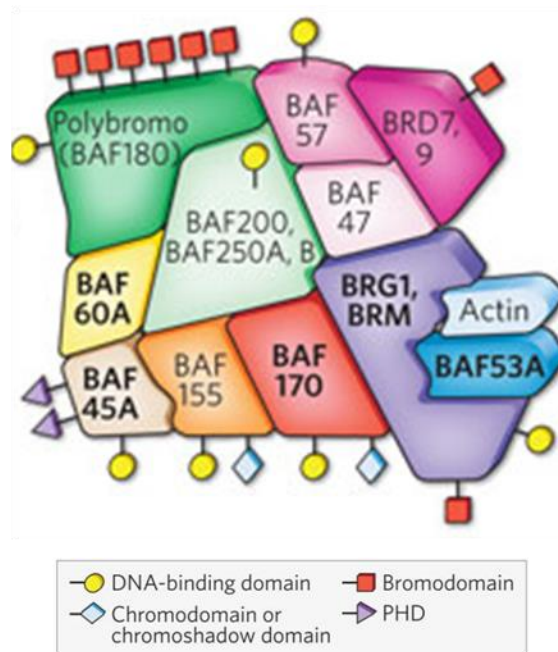


Figure 1.8: Schematic representation of the SWI/SNF complex.

The SWI/SNF family of chromatin-remodelling complexes are composed of multiple subunits, several of which have multiple isoforms (i.e. Brg1-associated factor [BAF] 200A, B). Core members are depicted in boldface font. (Ho and Crabtree, 2010, reproduced with permission from the Nature publishing group)

1.12 Brg1 loss of function studies

Brg1^{-/-} embryos die at the peri-implantation stage of development, demonstrating the importance of this protein during development, and necessitating the conditional deletion of this gene for post-implantation studies (Bultman et al., 2000). Although structurally similar (approximately 75% sequence similarity) to the Brg1 protein, *Brm*^{-/-} mice develop relatively normally, and Brg1 expression is upregulated in *Brm*^{-/-} mice, suggesting that functional compensation by Brg1 can overcome the loss of Brm (Reyes et al., 1998). Based on this finding, and the fact that Brm cannot compensate for the loss of Brg1 protein in a *Brg1*^{-/-} background, Brg1 appears to play the more critical role during development (Griffin et al., 2008).

Conditional knockout models have given insights into many developmental processes that are impacted by Brg1, including cardiac, neuronal, and vascular development, along with primitive erythropoiesis (Wu et al., 2007; Griffin et al., 2008; Hang et al., 2010; Griffin et al., 2011). Using a *Tie2-Cre*, which is expressed in yolk sac blood islands and endothelial cells (Schnurch and Risau, 1993), excision of *Brg1* was achieved in hematopoietic and vascular lineages. In this system, embryos died between E10.5 and E11.0, and although primitive hematopoiesis appeared normal at E8.5, significant disruptions in primitive erythrocyte development were observed at E9.5 (Griffin et al., 2008). Brg1 was found to be recruited to embryonic α - and β -globin gene loci, and severely diminished embryonic hemoglobin synthesis was observed in *Brg1* mutants at E9.5 (Griffin et al., 2008). Erythropoietic defects were followed by apoptosis of cells normally expressing the embryonic globin proteins, which contributed to the death of

these mid-gestation embryos (Griffin et al., 2008). *Brm* deletion in the conditional *Brg1*^{-/-} background produced no observable exacerbation of the *Brg1*^{-/-} phenotype, indicating that Brm does not play a role in hematopoietic or vascular development (Griffin et al., 2008). A previous study also reported that a Brg1 mutant lacking chromatin remodelling activity was unable to transcribe the adult-type beta-globin genes (β^{maj} , β^{min}), resulting in embryonic death due to failure of definitive erythropoiesis (Bultman et al., 2005). This study also demonstrated that Brm is not expressed in erythroid lineages, and was therefore unable to functionally compensate for the loss of Brg1 (Bultman et al., 2005). Vasculogenesis was also found to be perturbed in *Brg1*^{-/-} embryos, again utilizing a conditional *Brg1* knockout in hematopoietic and vascular lineages (Griffin et al., 2008). Only yolk sac angiogenesis was disrupted, since the vascular plexus appeared to develop normally, and intra-embryonic vasculature was unaffected (Griffin et al., 2008). Baf155 is also required for vascular remodelling to occur, but appears to be dispensable at early stages of vasculogenesis (Han et al., 2008). Together, these data suggest that different members of the SWI/SNF complex are required for the proper development of the murine yolk sac, but little data exists to comprehensively understand how these proteins exert their effects in these processes.

1.13 Rationale

Cdx members play critical roles in zebrafish hematopoiesis, and are thought to exert their effects in part through *hox* gene expression (Davidson and Zon, 2006). However,

not all defects associated with hematopoiesis in *cdx* mutant zebrafish can be attributed to the effect of *hox* proteins, indicating one or more novel pathways remain unknown. Defects in murine hematopoiesis have also been discovered in *Cdx* mutant ESCs, although no mouse model to date has addressed this *in vivo*. By conditionally deleting *Cdx2* in a *Cdx1*^{-/-} background, we were able to circumvent the functional overlap of *Cdx* members that has clouded previous studies of the *Cdx* family. In the context of my studies, *Cdx1*^{-/-}*Cdx2*^{-/-} embryos lack structures caudal to the forelimb bud (Savory et al., 2011a), presumably including the AGM region, and display defects in hematopoietic and vascular development by E8.5. Based on this phenotype, my work primarily focussed on primitive hematopoietic events.

Protein-protein interactions that facilitate the transcription of *Cdx* target genes have remained poorly understood. Through the use of SILAC-MS technology, we have recently identified the Brg1 component of the SWI/SNF chromatin remodelling complex as a putative interactor with *Cdx2*. Examination of physical interactions, along with functional consequences of these interactions, will expand our understanding of *Cdx* biology. Phenotypic similarities between *Brg1*^{-/-} and *Cdx1*^{-/-}*Cdx2*^{-/-} mutants indicate that *Cdx*-Brg1 interactions may impact the transcription of genes critical to hematopoiesis.

1.14 Hypothesis

Cdx2 affects the transcription of critical hematopoietic genes and regulates these events in part through interaction with the chromatin remodelling protein Brg1.

1.15 Objectives

- 1) Analyze the hematopoietic defects observed in *Cdx1*^{-/-}*Cdx2*^{-/-} murine embryos.
- 2) Determine if hematopoietic targets identified in the ChIP-seq experiment are regulated by Cdx2.
- 3) Confirm the MS data indicating that Cdx2 interacts with Brg1.
- 4) Identify the site of interaction between Cdx2 and Brg1, and assess the functional consequences of this interaction.

Chapter 2

Materials and Methods

2.1 Mice

Timed matings were set up overnight; E0.5 was considered to be noon of the day that vaginal plugs were detected. Pregnant females were sacrificed by cervical dislocation at various time points, and embryos were harvested in sterile phosphate-buffered saline (PBS). For the isolation of RNA, embryos were collected in sterile diethylpyrocarbonate (DepC, Calbiochem®)-treated PBS and stored in TRIzol® reagent (Ambion Life Technologies). Wild-type CD1 mice were obtained from Charles River.

Cdx1^{-/-}*Cdx2*^{-/-} embryos were generated as previously reported (Savory et al., 2011a) by pairing *Cdx1*^{-/-}*Cdx2*^{fl/fl} females with *Cdx1*^{-/-}*Cdx2*^{fl/fl} *Actin*^{CreERT} males (Figure 1.7). Timed matings were set up as above, with animals maintained in a mixed genetic background. Two mg of tamoxifen (Sigma Aldrich; dissolved in 5% ethanol in corn oil) were administered to pregnant dams at E5.5 to inactivate *Cdx2*. *Cdx1*^{-/-}*Cdx2*^{fl/fl} embryos were considered controls, unless otherwise noted, as *Cdx1*^{-/-} mutants exhibit no yolk sac defects or perturbation in expression of relevant target genes.

2.2 Gross Anatomy of Embryos

Embryos were dissected in sterile PBS and photographed using a Leica DFC320 camera.

2.3 PECAM1 Staining

Embryos were harvested in sterile PBS with yolk sacs attached, rinsed once in PBS, and fixed in 4% paraformaldehyde (PFA, BioShop® Canada)-DepC PBS for 1 hour at room temperature, rinsed three times with PBS, and incubated overnight in blocking solution (2% milk, 0.02% Triton-X 100, 1% BSA [BioShop® Canada]) at 4 °C. Samples were rinsed in blocking solution, and incubated in 1:50 αPECAM1 (CD31, Developmental Studies Hybridoma Bank) overnight at 4 °C. Samples were washed five times for one hour in blocking solution, incubated overnight in 1:2000 mouse-α-Armenian Hamster-TR (Santa Cruz Biotechnology) at 4 °C, and washed five times for one hour with blocking solution. Samples were then washed for 20 minutes in PBS-BSA (0.02% Triton-X 100, 1% BSA), and fixed for 5 minutes in 4% PFA-DepC PBS. Yolk sacs were removed and flat-mounted using Dako Fluorescent Mounting Media (Dako, Inc.). Images were acquired using Zeiss Axio Observer D1, and analyzed using Zeiss AxioVision 4.8.2 software.

2.4 Diamino-benzidine Staining

Embryos were harvested in sterile PBS with yolk sacs attached, rinsed once with PBS, and fixed in 4% PFA-DepC PBS for 1 hour at room temperature, rinsed three times with PBS, and permeabilized in 3% Triton-X-100 for 15 minutes. Embryos were rinsed three times with PBS, then incubated in 4 mL diamino-benzidine (DAB) staining solution (0.1 M Tris Base pH 7.6, 1 mM DAB). Eighty µL 0.5% H₂O₂ were added and embryos were incubated for 10 minutes in the dark. Samples were washed five times with sterile

water, once with 50% ethanol/H₂O, twice with 100% ethanol, then twice with 100% Xylene. Yolk sacs were removed from embryos proper, and flat-mounted using Permunt mounting media (Fisher Scientific). Images were acquired using a Zeiss Axiophot camera and adjusted with Northern Eclipse software.

2.5 Hematopoietic Colony Formation Assays

Colony forming units were assessed using MethoCult (Stem Cell Technologies) Methylcellulose-Based Media with Recombinant Cytokines and EPO for Mouse Cells. Day E8.5 *Cdx1*^{-/-}*Cdx2*^{-/-} mutants and *Cdx1*^{-/-}*Cdx2*^{fl/fl} littermate controls were dissected in sterile PBS, and yolk sacs were removed and washed once in sterile PBS. Each yolk sac was incubated for 30 minutes in 0.1% collagenase/dispase, 20% HI-FBS, in sterile PBS. Cell suspensions were passed through a 26 gauge needle (BD Bioscience), followed by a 30 gauge needle (BD Bioscience) to generate single cell suspensions. Cells were pelleted by centrifugation, and resuspended in 300 µL Iscove's Modified Dulbecco Medium (IMDM) containing 2% FBS and 1% penicillin/streptomycin, which was added to 2.7 mL MethoCult media and mixed by vortexing. Suspensions were plated in 1.5 mL duplicates using 18 gauge blunt end needles (BD Bioscience), and incubated in humidified plates in 5% CO₂ at 37°C. Colonies were assessed 8 days after plating.

For assays involving direct administration of tamoxifen in the MethoCult media, *Cdx1*^{-/-}*Cdx2*^{fl/fl}*Actin*^{CreERT/CreERT} males were paired with *Cdx1*^{-/-}*Cdx2*^{fl/fl} females to exclusively generate *Cdx1*^{-/-}*Cdx2*^{fl/fl} embryos, and yolk sacs were harvested at E8.5. Single cell

suspensions were generated, and pooled together before being pelleted and resuspended in IMDM. Cell suspensions were divided into 300 μ L aliquots and added to 2.7 mL MethoCult media, followed by the addition of vehicle or tamoxifen to a final concentration of 1 μ M. Suspensions were then plated as above.

Colony morphology was used to distinguish between Burst-Forming Unit-Erythroid (BFU-E), Colony-Forming Unit-Granulocyte, Macrophage (CFU-GM), and Colony-Forming Unit-Granulocyte, Erythroid, Macrophage, Megakaryocyte (CFU-GEMM) (See Appendix 1). Distinct colonies were deemed to be growing at least one colony-diameter away from the next group of cells (i.e. two colonies growing side by side were considered to be one single colony forming unit).

2.6 Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR)

Samples from tissue culture cells and yolk sacs were collected in Trizol[®] reagent, and complementary DNA (cDNA) was generated using standard procedures. The cDNA was subsequently amplified by PCR, using *GAPDH* as an input control for murine studies and *ACTIN* for human studies. GoTaq[®] Green PCR master mix (Promega) was used for all PCR reactions. Oligonucleotide sequences for murine qPCR and semi-quantitative RT-PCR are presented in Appendix 2.

2.7 Cdx Genome Occupancy

To identify potential Cdx2 target genes, a ChIP-seq experiment was conducted using E8.5 wild type CD1 embryos. Briefly, 30 embryos were fixed and pooled for chromatin shearing, and immunoprecipitated with either Cdx2 (Savory et al., 2009b) or control IgG (Santa Cruz Biotechnology) antibodies bound to pre-blocked A/G agarose beads (Santa Cruz Biotechnology). Samples were washed, de-crosslinked, and DNA was sent to UCSC for sequencing and statistical analyses comparing Cdx2 immunoprecipitation to control IgG. I analyzed peaks of interest from these data using the UCSC Genome Browser (<http://genome.ucsc.edu/cgi-bin/hgGateway>, July 2007 Assembly, NCBI37/mm9).

2.7.1 ChIP

E8.5 embryos were collected in sterile PBS, rinsed once with PBS, and fixed for 5 minutes in 1% formaldehyde (BioShop® Canada) in PBS at room temperature. Fixation was halted by the addition of 0.125 M glycine for 5 minutes and embryos were rinsed twice with PBS, then snap-frozen for storage at -80 °C. Five µg of each antibody of interest (Cdx2, Savory et al., 2009b; Brg1, Santa Cruz Biotechnology; IgG, Santa Cruz Biotechnology) were incubated with pre-blocked A/G agarose beads (Santa Cruz Biotechnology), then washed three times with PBS/BSA (5 mg/mL BSA, Protease Inhibitor Cocktail), and resuspended in a final volume of 100 µL PBS/BSA. Thirty embryos were used for each immunoprecipitation; embryos were thawed on ice and resuspended in 1 mL RIPA buffer (1% Triton-X-100, 1 mM EDTA, 150 mM NaCl, 20 mM

Tris-HCl pH 8.1, Protease Inhibitor Cocktail). Samples were sheared via sonication for 2 minutes (Branson Sonifier 450; duty cycle 3.0, 30% output), and pooled together. Five percent of the sample was removed as an input control, and the remaining lysate was divided for each immunoprecipitation. Inputs were incubated with antibodies overnight with rocking at 4 °C. The following day, samples were washed five times with ChIP wash buffer (500 mM LiCl, 1% NP-40, 1% sodium deoxycholate, 100 mM Tris pH 7.5), then once with TE buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA). Crosslinking was reversed in 200 µL IP elution buffer (1% SDS, 0.1 M NaHCO₃) at 65 °C overnight. DNA was purified using a Qiagen PCR purification kit, eluted in 50 µL Qiagen Elution Buffer, and amplified by PCR using GoTaq® Green PCR master mix (Promega). Oligonucleotides for ChIP studies are listed in Appendix 3.

2.7.2 Sequence Analyses

DNA sequences upstream of the transcriptional start site (TSS) of putative target genes were scanned using Transcription Element Search System (TESS; <http://www.cbil.upenn.edu/cgi-bin/tess/tess>) or TFSEARCH Software, v1.3 (<http://www.cbrc.jp/research/db/TFSEARCH.html>) to identify canonical Cdx response elements (CDREs).

2.8 In Vitro Association Assays

Proteins were radiolabelled *in vitro* with ^{35}S -methionine (Perkin Elmer) using the TnT[®] T7 Coupled Reticulocyte Lysate System (Promega) following the manufacturer's instructions, using 20 μCi of ^{35}S -methionine.

Cdx2 and Brg1 GST-fusion proteins were generated by standard procedures via PCR using high-fidelity taq polymerase (Hi-Fi taq; Invitrogen) and murine cDNA from existing constructs. Amplicons were isolated using Qiagen PCR purification kits, and ligated via T/A cloning into the pDRIVE subcloning vector (Qiagen). A deletion mutation of Cdx2 was generated by two-step PCR, removing amino acids 60 through 80. The symbol (|) indicates the gap in sequence between nucleotides encoding amino acids 60 through 80 (Appendix 4).

Site directed mutagenesis was carried out using the QuikChange II XL Site Directed Mutagenesis Kit (Agilent Technologies) to alter amino acids 72-78 in the native Cdx2 sequence (amino acids PLREDWN converted to GVGAAGA, referred to as Cdx2Mut). Oligonucleotides used for subcloning GST-fusion vectors are presented in Appendix 4.

All plasmids were confirmed by sequencing and inserts subcloned into the pGEX4T1 (GE Healthcare) vector. Constructs were transformed into the BL21 *E. coli* cell line. Upon induction of BL21 cultures with 0.5 mM IPTG, cells were grown to exponential phase and pelleted, then resuspended in PBS Mix (1 mM DTT/0.02 % Triton X-100 [BioShop[®] Canada]/Protease Inhibitor Cocktail Set III EDTA-free [Calbiochem] in sterile PBS). Cells were lysed by sonication for 2 minutes (Branson Sonifier 450; duty cycle 3.0, 30%), cell

debris was pelleted by centrifugation and the remaining supernatant was incubated with glutathione agarose beads (Invitrogen), rocking overnight at 4 °C. Beads were washed several times with PBS, and Cdx2 concentration assessed by Coomassie Brilliant Blue staining (Bio-Rad Laboratories).

Radiolabelled proteins were incubated with 5 µg of each GST-fusion protein/bead mix in TNEN buffer (50mM Tris HCl pH 7.3/500mM NaCl/0.1% NP-40/5mM EDTA/Protease Inhibitor Cocktail). Beads were washed three times in TNEN buffer, denatured by boiling for 5 minutes in SDS loading buffer (4 µL of 50% glycerol, 2 µL DTT, 2 µL 10X SDS loading dye), and resolved on SDS-PAGE gels with 5% input of each radiolabelled protein. Gels were fixed, enhanced with Amersham™ Amplify solution™ (GE Healthcare), dehydrated, and exposed to BioMax MS Film (Kodak) for visualization.

2.9 Electrophoretic Mobility Shift Assay (EMSA)

Double-stranded oligonucleotides were generated for the potential CDREs (identified by TESS) located in the proximal *Scf* promoter. One hundred ng of each forward oligonucleotide were end-labeled with [γ -³²P] ATP (Perkin Elmer), using T4 polynucleotide kinase (Invitrogen), and purified over a Sephadex G-50 (Sigma Aldrich) column to remove any unincorporated radioactive label. The eluate was mixed with 200 ng of the complementary oligonucleotide, boiled for 5 minutes, and left to cool before use. Incorporation of ³²P was normalized to 50000 counts per minute for each reaction. GST-Cdx2 fusion proteins were incubated with 5.6 µL of binding buffer for 30 minutes

on ice prior to incubation with probes for 30 minutes at room temperature. Samples were resolved on 10% non-denaturing PAGE gels at room temperature in TBE buffer (0.022 M Tris base, 0.022 M boric acid, 0.0005 M EDTA). Gels were dried and exposed to BioMax MS Film (Kodak) for visualization. Oligonucleotides for EMSA probes are presented in Appendix 5.

2.10 Cell Lines

Murine P19 embryonic carcinoma cells were maintained in Alpha Modification of Eagle's Medium (α MEM; Multicell, Wisent), supplemented with 7.5% heat-inactivated donor bovine serum (HI-DBS), 2.5% heat-inactivated fetal bovine serum (HI-FBS) and penicillin/streptomycin. Cells were grown in 5% CO₂ at 37°C. For routine maintenance, cells were passed at a 1:10 ratio every other day.

Human embryonic kidney (HEK293) cells were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM; Multicell, Wisent), supplemented with 10% HI-FBS and penicillin/streptomycin. Cells were grown in 5% CO₂ at 37°C. For routine maintenance, cells were passed at a 1:6 ratio every other day. For shRNA studies, HEK293 cells were maintained in standard media, supplemented with 3 μ g/mL puromycin.

2.11 Transfection Assays

For shRNA knockdown and luciferase-based expression studies, plasmid DNA was transfected into HEK293 or P19 cells using calcium phosphate. DNA was diluted in sterile water, combined with 0.17 M CaCl_2 (BioShop® Canada), mixed with hydroxyethyl piperazineethanesulfonic acid (HEPES)-buffered saline (HBS, Fisher Scientific), and added drop-wise to cells. Growth media were replenished 24 hours later, and cultured for an additional 24 hours before harvest. For nuclear localization immunohistochemistry, P19 cells were transfected using Attractene Transfection Reagent (Qiagen), and cells were harvested 24 hours after transfection.

2.12 Luciferase Reporter Assays

2.12.1 Luciferase Reporter Constructs

Two 1kb regions of the *Scf* promoter (“*Scf* proximal” and “*Scf* distal”) were amplified by PCR from genomic DNA and ligated into pDrive (Qiagen) to encompass the 2kb region immediately upstream of the *Scf* TSS. Following validation by sequencing, this insert was subcloned into the pXP2 luciferase reporter vector using restriction sites incorporated into the oligonucleotide primers (*Scf* proximal utilizing *NotI* and *BglII* sites, *Scf* distal utilizing *XhoI* and *NotI* sites). Similarly, a 1.6 kb region of the *Meis1* promoter immediately upstream of the TSS was amplified and ligated into pDRIVE. Following validation by sequencing, this insert was subcloned into the pXP2 luciferase reporter vector. Oligonucleotides for subcloning are listed in Appendix 6.

2.12.2 Luciferase Reporter Assays

Luciferase reporter assays were performed in triplicate from P19 cells cultured in 6-well plates. Each well was transfected with 0.5 µg of Cdx2 expression vector (wild type or Cdx2Mut, where indicated) or empty vector, 1 µg of reporter vector, 0.3 µg of GFP (for verification of transfection), and 0.3 µg of a β-galactosidase expression vector. At time of harvest, cells were rinsed with sterile PBS, lysed in 150µL of Luciferase lysis buffer (Promega), frozen at -80°C, thawed, and cellular lysates collected. Luciferase activity was analyzed using the Promega Luciferase Assay System following the manufacturer's instructions, using an LMax II microplate reader and SOFTmax PRO Software Version 4.7 (Molecular Devices). β-galactosidase activity was quantified using CPRG substrate, and measured for absorption at 585 nm on a Spectramax M2 microplate reader using SOFTmax PRO Software Version 4.7. β-galactosidase activity was used to normalize transfection efficiency.

2.13 FLAG-tagged Brg1-B2 Interaction Assays

The FLAG-Brg1-B2 construct was transfected into HEK293 cells as above (using 10 µg DNA per 10 cm plate). Immunoprecipitations were carried out using 5 µg of IgG (Santa Cruz Biotechnology) or Cdx2 (Savory et al., 2009b) antibodies bound to protein A/G beads. Cell lysates were incubated with bead/antibody slurries overnight at 4°C, washed several times with RIPA buffer (1% Triton-X-100, 1 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.1, Protease Inhibitor Cocktail), and resuspended in 200 µL 2% CHAPS (Sigma

Aldrich), 8M Urea (EMD Chemicals Inc.), with Protease Inhibitor Cocktail (Calbiochem). Western blots were run to assess co-precipitation.

To identify the region of interaction between Brg1-B2 and Cdx2, GST-Cdx2 fragments were bound to glutathione agarose beads and incubated with cell lysates overnight at 4 °C, washed several times with RIPA buffer, and resuspended in 200 µL 2% CHAPS/8M Urea with Protease Inhibitor Cocktail (Calbiochem). Western blots were used to assess the ability of each truncation protein to immunoprecipitate FLAG-Brg1-B2.

2.14 Subcellular Localization

P19 cells were transfected as above with plasmids containing wild type Cdx2 or Cdx2Mut. 24 hours post-transfection, cells were washed with PBS, fixed in 4% PFA for 10 minutes, permeabilized with 0.3% Triton-X-100 in PBS, and blocked for one hour with 1% goat serum (Millipore) in PBS. Cells were incubated with anti-Brg1 antibody (laboratory; unpublished) overnight at 4 °C in blocking solution at a 1:1000 dilution, washed with PBS, and incubated for 1 hour at room temperature in 1:1000 goat-anti-rabbit Alexa Fluor® 594 (Invitrogen). Cells were washed with PBS, and incubated with 1:1000 anti-Cdx2 primary antibody (Savory et al., 2009b) overnight at 4 °C in blocking solution, washed again with PBS, and incubated with 1:1000 goat-anti-rabbit Alexa Fluor® 488. Finally, cells were washed with PBS and incubated with 1:100 DAPI stain (Invitrogen), washed again with PBS, and mounted using Dako Fluorescent Mounting

Media (Dako, Inc.). Images were acquired using a Zeiss LSM 510 META confocal microscope, and analyzed with Zeiss ZEN 2009 software.

2.15 shRNA Knockdown of CDX2 and BRG1

Lentiviral shRNA targeting human *CDX2* or *BRG1* was obtained from Thermo Scientific Life Science Research. Constructs were transfected into HEK293 cells as above using non-specific shRNA as a negative control. Cells were selected in puromycin (3 µg/mL) for 7 days and individual colonies isolated, expanded, and assessed for CDX2 or BRG1 expression via Western blot and semi-quantitative RT-PCR. Rescue experiments were performed by transfecting knock-down clones with expression vectors encoding Brg1, wild type Cdx2, or Cdx2Mut (5 µg per 10 cm plate). Mature antisense sequences against mRNA transcripts are listed in Appendix 7. Oligonucleotides for RT-PCR analyses are presented in Appendix 8.

2. 15.1 Western Blot Analyses

HEK293 were grown to confluence, rinsed twice with PBS and resuspended in 200 µL 2% CHAPS (Sigma Aldrich), 8M Urea (EMD Chemicals Inc.) and Protease Inhibitor Cocktail (Calbiochem). Samples were sonicated on ice for 30 seconds (Branson Sonifier 450; duty cycle 1.0, 10% output). Protein concentration was normalized via Bradford assays and samples were resolved on 10% SDS-PAGE gels and transferred to Immobilon-P (Millipore) membranes by standard techniques. Membranes were activated with 100%

methanol (Fisher Scientific), blocked with 5% milk powder in PBST (0.1% Tween20, Fisher Scientific, in PBS), and incubated overnight at 4°C with 1:1000 primary antibody (Cdx2, Savory et al., 2009b; Cyclophilin, AbCam; Brg1, Santa Cruz Biotechnology; FLAG, Sigma Aldrich). Membranes were washed with PBST, incubated with 1:25000 HRP-conjugated secondary antibodies (goat-anti-rabbit and goat-anti-mouse HRP, Santa Cruz Biotechnology) for 1 hour at room temperature, and washed again with PBST. Following a final wash in PBS, HRP reactivity was revealed using Luminata Forte ECL substrate (Millipore), and blots were developed on Bioflex® Econo film (Clonex).

2.16 Statistical Analyses

All statistical analyses for CFU assays, semi-quantitative and quantitative RT-PCR, and luciferase reporter assays utilized the student's t-test (paired, two-tailed).

Chapter 3

Results

3.1 *Cdx1*^{-/-}*Cdx2*^{-/-} mutants exhibit gross anatomical defects

Cdx proteins have been shown to play critical roles in zebrafish hematopoiesis (Davidson and Zon, 2006), but their role in murine hematopoietic development is poorly understood. Using the Cre/*loxP* system, we were able to circumvent the requirement for Cdx2 in early implantation events (Chawengsaksophak et al., 1997), to study the effect of Cdx deletion in the post-implantation embryo. The breeding protocol presented in Figure 1.7 was used to generate *Cdx1*^{-/-}*Cdx2*^{-/-} mutants and *Cdx1*^{-/-}*Cdx2*^{fl/fl} littermate controls, utilizing a tamoxifen-inducible Cre transgene to effect deletion at E5.5, as previously described (Savory et al., 2009a). Excision of *Cdx2* in the *Cdx1*^{-/-} background resulted in loss of Cdx4 expression, effectively generating Cdx null mutants (Savory et al., 2011b). At E8.5, *Cdx1*^{-/-}*Cdx2*^{-/-} embryos appeared smaller than those of controls, exhibiting a broader and shortened caudal region (Figure 3.1 A and D), likely related to defects in planar cell polarity, as previously described (Savory et al., 2011a). The anterior regions of *Cdx1*^{-/-}*Cdx2*^{-/-} embryos were similar to controls, and yolk sac morphology appeared to be unaltered (Figure 3.1 B and C). Blood was not easily observed at this stage, but diamino-benzidine (DAB) staining indicated that hemoglobin-expressing cells were significantly perturbed in *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs (Figure 3.2 A and B).

Before death at E11.5, distinct differences were observed between control and *Cdx1*^{-/-}*Cdx2*^{-/-} embryos at E9.5 and E10.5, the most notable of which was the complete lack of posterior tissue beyond the forelimb bud (Figure 3.1 E, H, I and L). Anterior portions of *Cdx1*^{-/-}*Cdx2*^{-/-} embryos were significantly smaller than controls, and the pericardial sacs were distended, with apparent abnormalities in heart looping (Figure 3.1 I and L).

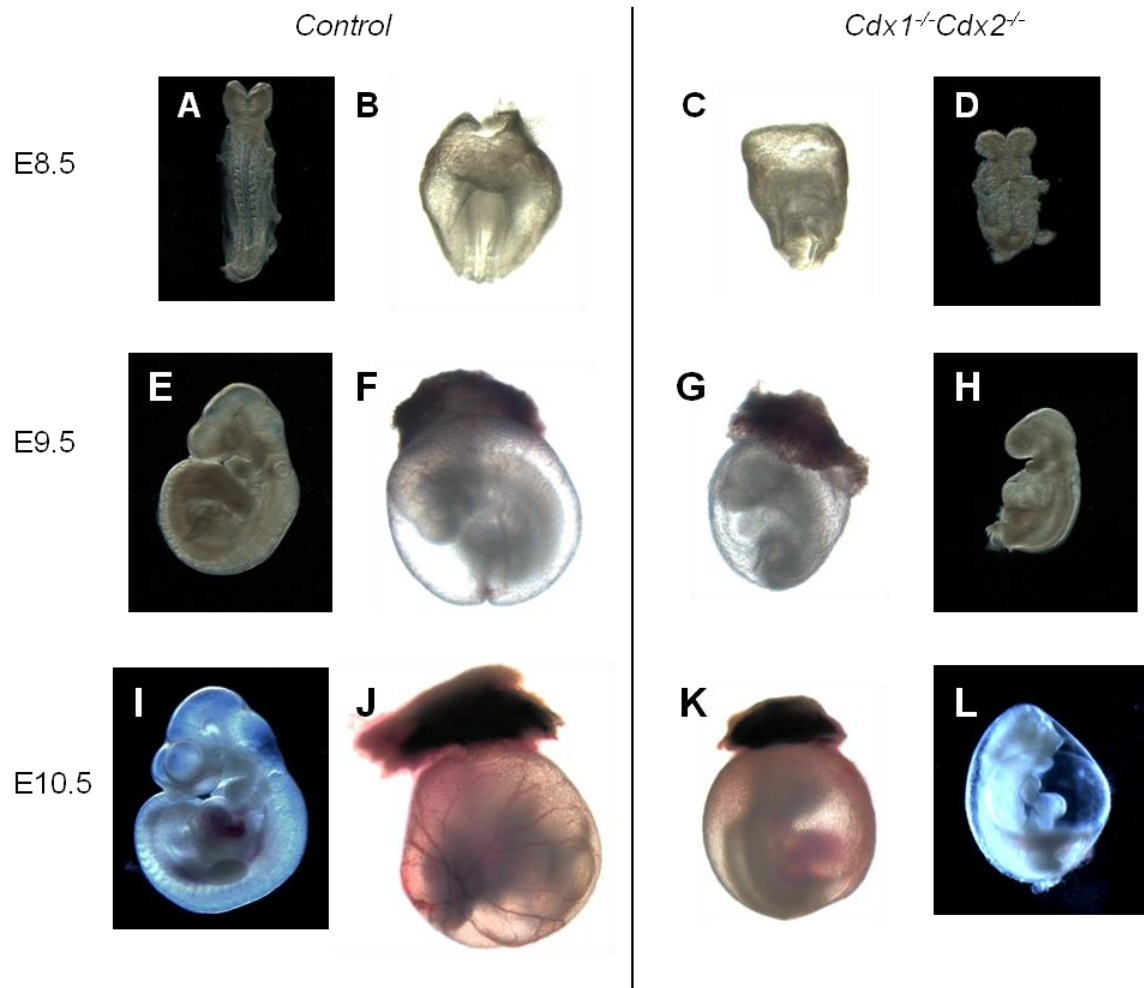


Figure 3.1: Gross anatomy Cdx null mutant embryos.

Cdx1^{-/-}*Cdx2*^{fl/fl} (control) embryos were compared to *Cdx1*^{-/-}*Cdx2*^{-/-} mutants at E8.5 (A,B,C,D) E9.5 (E,F,G,H) and E10.5 (I,J,K,L). Embryos are displayed before and after the removal of the yolk sac. E8.5 mutants appear shorter and broader than controls. E9.5 and E10.5 mutants are smaller than controls, are truncated beneath the forelimb bud, and display disrupted heart formation. E9.5 and E10.5 mutant yolk sacs also lack red blood cells and vascular networks.

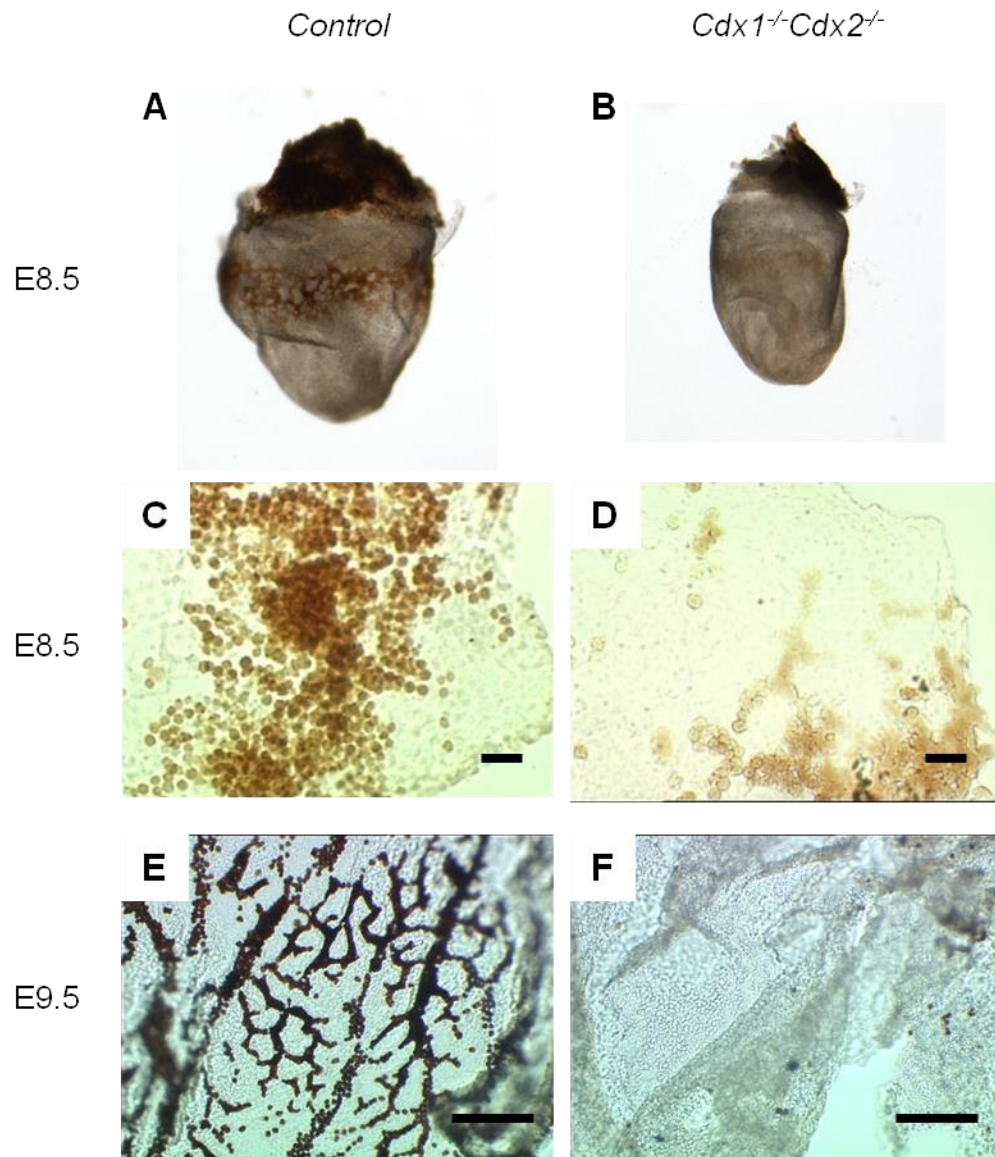


Figure 3.2: Cdx mutant yolk sacs are deficient in hemoglobin-expressing cells.

Cdx1^{-/-}*Cdx2*^{fl/fl} (control) embryos compared to *Cdx1*^{-/-}*Cdx2*^{-/-} mutants at E8.5 (**A,B,C,D**) and E9.5 (**E,F**). Diamino-benzidine (DAB) stained E8.5 embryos before the removal of the yolk sac (**A,B**) and flat-mounted yolk sacs at E8.5 (**C,D**) and E9.5 (**E,F**). Scale bars represent 100 μ m.

Although the hearts of *Cdx1*^{-/-}*Cdx2*^{-/-} embryos did beat, normal fluid flow was possibly disrupted due to the perturbed architecture of the heart tube (Figure 3.1 L). DAB staining at these stages indicated a significant disruption in hematopoiesis, as red blood cells were almost completely absent from *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs (Figure 3.2 C, D, E and F; Figure 3.3). Vascular branching also appeared to be disrupted in *Cdx1*^{-/-}*Cdx2*^{-/-} mutants at E9.5 and E10.5 (Figure 3.1 F, G, J and K).

3.2 Cdx deletion disrupts yolk sac vasculature

To examine the vascular networks of E9.5 and E10.5 yolk sacs in more detail, immunohistochemistry was performed for platelet/endothelial cell adhesion molecule 1 (PECAM1/CD31), a marker of platelets and endothelial cells (Baldwin et al., 1994). Compared to control yolk sacs, only a minor degree of vascular remodelling was apparent in E9.5 *Cdx1*^{-/-}*Cdx2*^{-/-} embryos (Figure 3.4 A and B). The small regions of PECAM1 staining in E10.5 mutants did not make connections with neighbouring structures, and did not appear to label tube-like architecture as seen in control embryos (Figure 3.4 C and D), suggesting a failure in vascular remodelling. DAB staining highlighted a belt of a few hemoglobin-expressing cells in the yolk sacs of *Cdx1*^{-/-}*Cdx2*^{-/-} embryos, indicating that blood islands had likely organized into a primitive vascular plexus, albeit much less pronounced than controls (Figure 3.2 B).

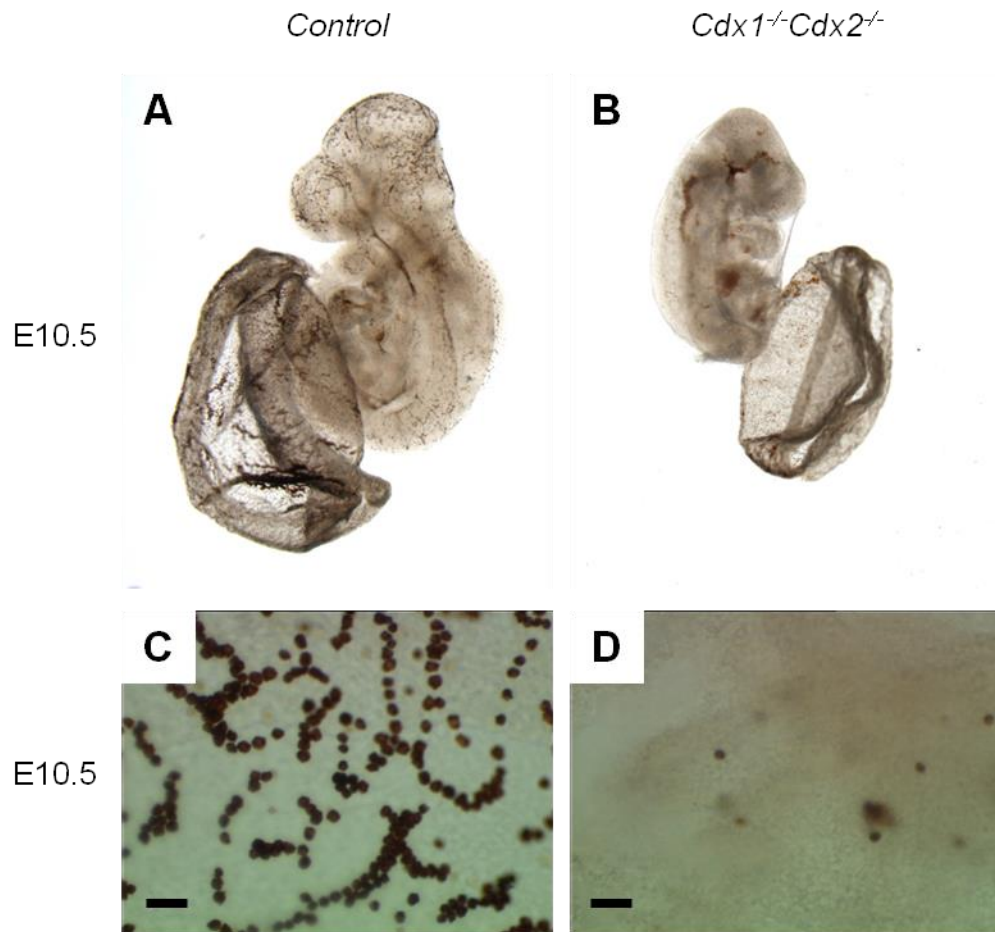


Figure 3.3: Cdx mutants are deficient in hemoglobin-expressing cells.

Cdx1^{-/-}Cdx2^{fl/fl} (control) embryos compared to *Cdx1^{-/-}Cdx2^{-/-}* mutants at E10.5. Diaminobenzidine (DAB) stained E10.5 embryos before the removal of the yolk sac (**A,B**) and flat-mounted yolk sacs (**C,D**). Scale bars represent 100 μm .

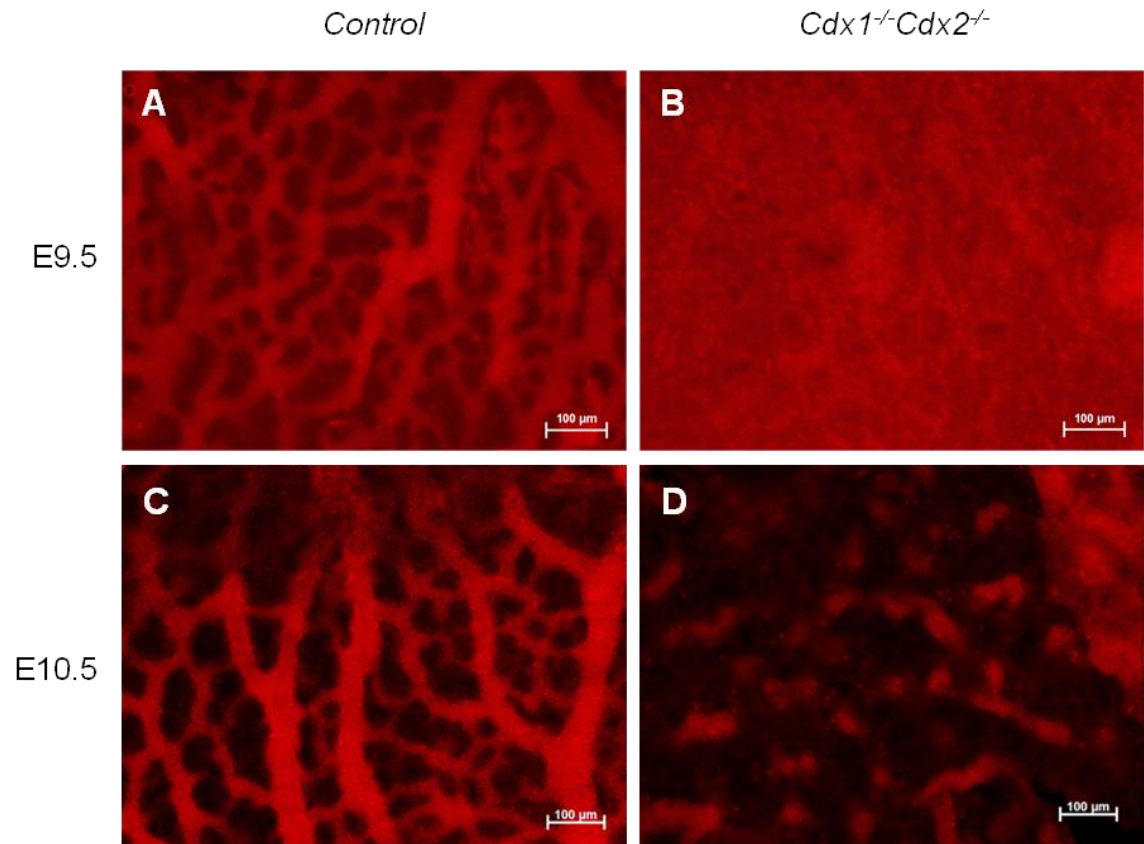


Figure 3.4: Cdx mutant yolk sacs exhibit aberrant vascular formation.

Immunohistochemistry of *Cdx1*^{-/-}*Cdx2*^{fl/fl} (control) and *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs for PECAM1 at E9.5 (**A,C**) and E10.5 (**B,D**). Scale bars represent 100 μm.

3.3 Cdx deletion impacts primitive erythropoiesis

Many genes involved in vascular development also play roles in hematopoiesis.

Hematopoietic events precede vasculogenesis and angiogenesis in the yolk sac, and several assays are available to study these processes. DAB staining of *Cdx1*^{-/-}*Cdx2*^{-/-} mutants suggested that erythropoietic events were disrupted in these embryos, which was further evidenced by flat mounting of DAB-stained E8.5 yolk sacs (Figure 3.2 C and D). Control E10.5 samples displayed DAB staining in the yolk sac, along with extensive staining in the vascular networks of the embryo proper (Figure 3.3), while *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs displayed staining in very few cells, and diminished staining within the embryo proper (Figure 3.3).

3.4 Cdx deletion affects primitive hematopoiesis

To assess the ability of precursor cells within *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs to give rise to hematopoietic lineages, colony forming assays were performed. Following *Cdx2* deletion at E5.5, E8.5 yolk sacs were dissociated into single cell suspensions, and plated in MethoCult Media containing cytokines and erythropoietin. Eight days after plating, colonies were counted and scored as Burst-Forming Unit-Erythroid (BFU-E), Colony-Forming Unit-Granulocyte, Macrophage (CFU-GM), or Colony-Forming Unit-Granulocyte, Erythroid, Macrophage, Megakaryocyte (CFU-GEMM) (Appendix 1). Colony counts were assessed in quadruplicate. E8.5 *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs exhibited a significant reduction

in the overall number of colonies, with all lineages being significantly reduced (Figure 3.5).

To further examine the effect of *Cdx* deletion on hematopoiesis, *Cdx1*^{-/-}*Cdx2*^{fl/fl} embryos were harvested at E8.5, yolk sacs were processed to generate single cell suspensions, and cells were incubated with vehicle or 1μM tamoxifen contained within the media. Colonies were scored 8 days post-plating, and data indicated a small but significant decrease in colony formation in each of the three hematopoietic lineages (Figure 3.6). This experiment suggests that *Cdx2* likely plays a role in yolk sac hematopoiesis after hematopoiesis has been initiated in blood islands.

3.5 Expression of genes involved in hematopoiesis are affected by *Cdx* deletion

To further examine the impact of *Cdx* loss on hematopoietic development in the yolk sac, quantitative PCR (qPCR) was performed at E9.5 on a subset of genes critical to hematopoiesis. Three independent control and *Cdx1*^{-/-}*Cdx2*^{-/-} mutant yolk sacs were used in these trials.

Two genes encoding embryonic hemoglobin proteins (ζ-globin, εγ-globin) were found to be significantly diminished in *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs (Figure 3.7 B), supporting the results of DAB staining. A significant decrease in expression was also observed for *Scl*, a master regulator of hematopoiesis, and the *Scl* target gene *Runx1* (Figure 3.7). These four genes were subsequently examined in E8.5 control and *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs. At

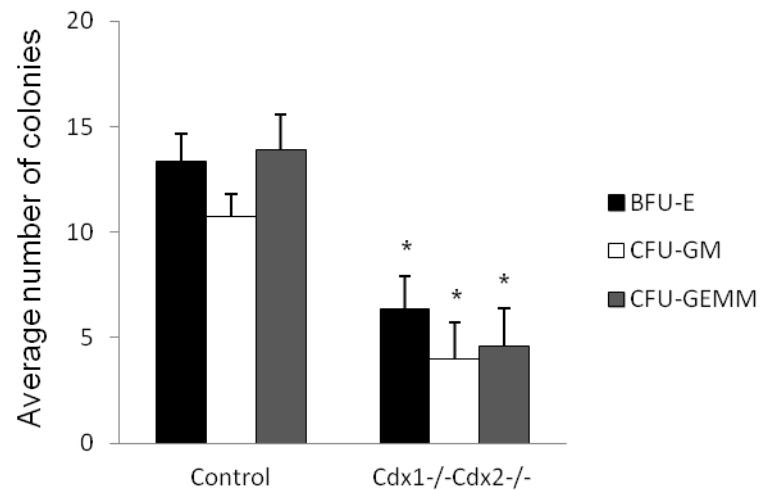


Figure 3.5: Cdx deletion impacts hematopoietic colony formation.

Hematopoietic colony formation from E8.5 $Cdx1^{-/-}Cdx2^{fl/fl}$ (control) and $Cdx1^{-/-}Cdx2^{-/-}$ yolk sacs following Cdx2 excision *in utero* at E5.5. n = 4, *P<0.05 by student's t-test (paired, two-tailed) compared to controls.

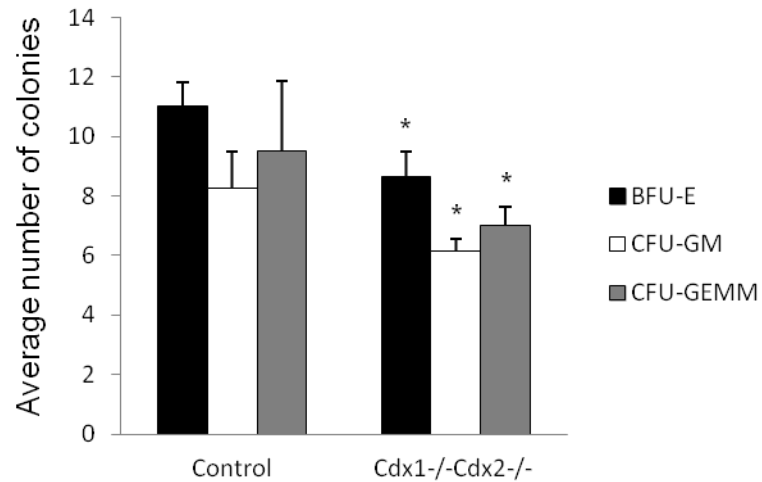


Figure 3.6: Cdx impacts hematopoietic colony formation in a temporal manner.

Hematopoietic colony formation was compared between E8.5 *Cdx1*^{-/-}*Cdx2*^{fl/fl} (control) and *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs. *Cdx2* was excised *in vitro* via tamoxifen administration at the time of plating (E8.5), after single cell suspensions were generated from individual yolk sacs. n = 4, *P<0.05 by student's t-test (paired, two-tailed) compared to controls

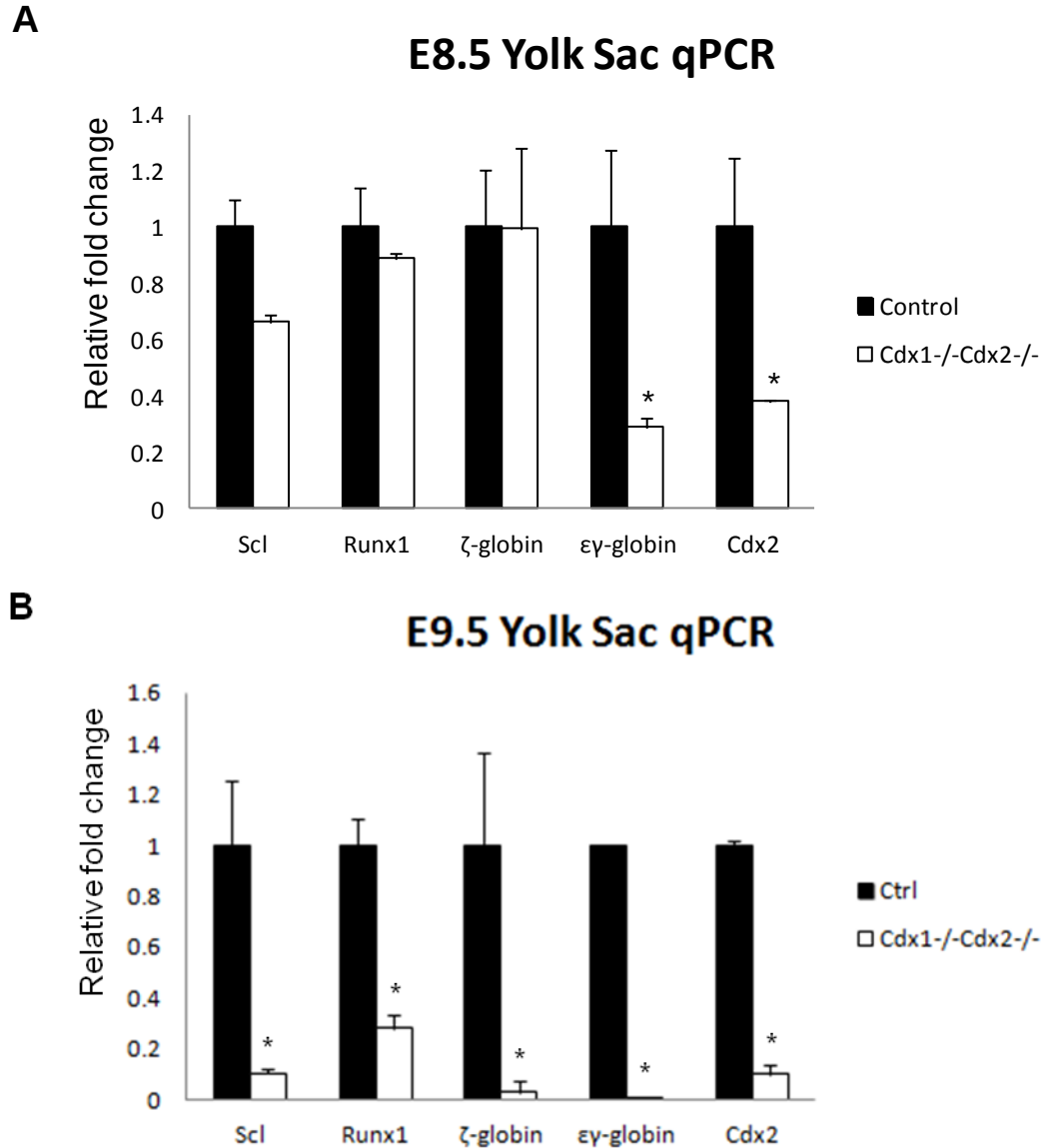


Figure 3.7: Cdx impacts expression of genes involved in hematopoiesis.

Expression of *Scl*, *Runx1*, *ζ-globin*, *εγ-globin*, and *Cdx2* was compared between *Cdx1*^{-/-}*Cdx2*^{fl/fl} (Ctrl) and *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs at E8.5 (A) and E9.5 (B). qPCR was performed using GAPDH as a housekeeping control for normalization. Fold change was calculated relative to control samples. n = 3, *P<0.05 by student's t-test (paired, two-tailed) compared to controls.

this time point, $\epsilon\gamma$ -globin and *Scl* transcripts were significantly downregulated, albeit to a lesser extent than observed at E9.5, while ζ -globin and *Runx1* transcripts were relatively unaffected (Figure 3.7 A). These data indicate that hematopoietic defects in *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs manifest a more pronounced phenotype after hematopoiesis has been initiated in the yolk sac.

3.6 Identification of novel Cdx2 target genes involved in hematopoiesis

Using ChIP-seq, several known Cdx target genes were identified, along with a number of novel target loci including several genes involved in hematopoiesis. Four such hematopoietic genes scored the maximum of 3100 in these analyses: *Scl*, *Lyl1*, *Lmo2*, and *Meis1* (Table 3.1), representing enrichment comparable to values observed for known Cdx target genes. Low false discovery rates further supported their occupancy by Cdx2.

Examination of the ChIP-seq data using the UCSC Genome Browser revealed peaks of putative Cdx2 binding to each of these genes, as well as bona fide Cdx target genes (*Brachyury*, *Dll1*, *Lef1*; Figure 3.8). DNA sequences beneath the peaks were scanned for canonical Cdx response elements (CDREs) using TESS and TFSEARCH softwares, revealing clusters of CDREs beneath each peak (red asterisks in Figure 3.8).

Gene Name	Peak Score	Distance to TSS	Fold Enrichment	FDR (%)
<i>Brachyury (T)</i>	3100	-1457	16.09	6.83
<i>Dll1</i>	845.77	99	8.5	5.08
<i>Lef1</i>	1503.66	-3407	8.91	5.28
<i>Scl</i>	3100	-2018	22.59	6.83
<i>Lyl1</i>	3100	5229	22.09	6.83
<i>Lmo2</i>	1153.8	-125	6.95	4.86
<i>Meis1</i>	3100	-759	7.65	5.11

Table 3.1: ChIP-seq identification of Cdx2 target genes.

ChIP-seq analysis for Cdx2 genome distribution identified several known Cdx2 target genes (*Brachyury*, *Dll1*, *Lef1*), along with novel hematopoietic genes of interest (*Scl*, *Lyl1*, *Lmo2*, *Meis1*). Negative values in the Distance to Transcriptional Start Site (TSS) indicate regions of binding upstream of the genes indicated. Fold enrichment is relative to IgG antibody immunoprecipitation. False discovery rates (FDR) are indicated for all genes.

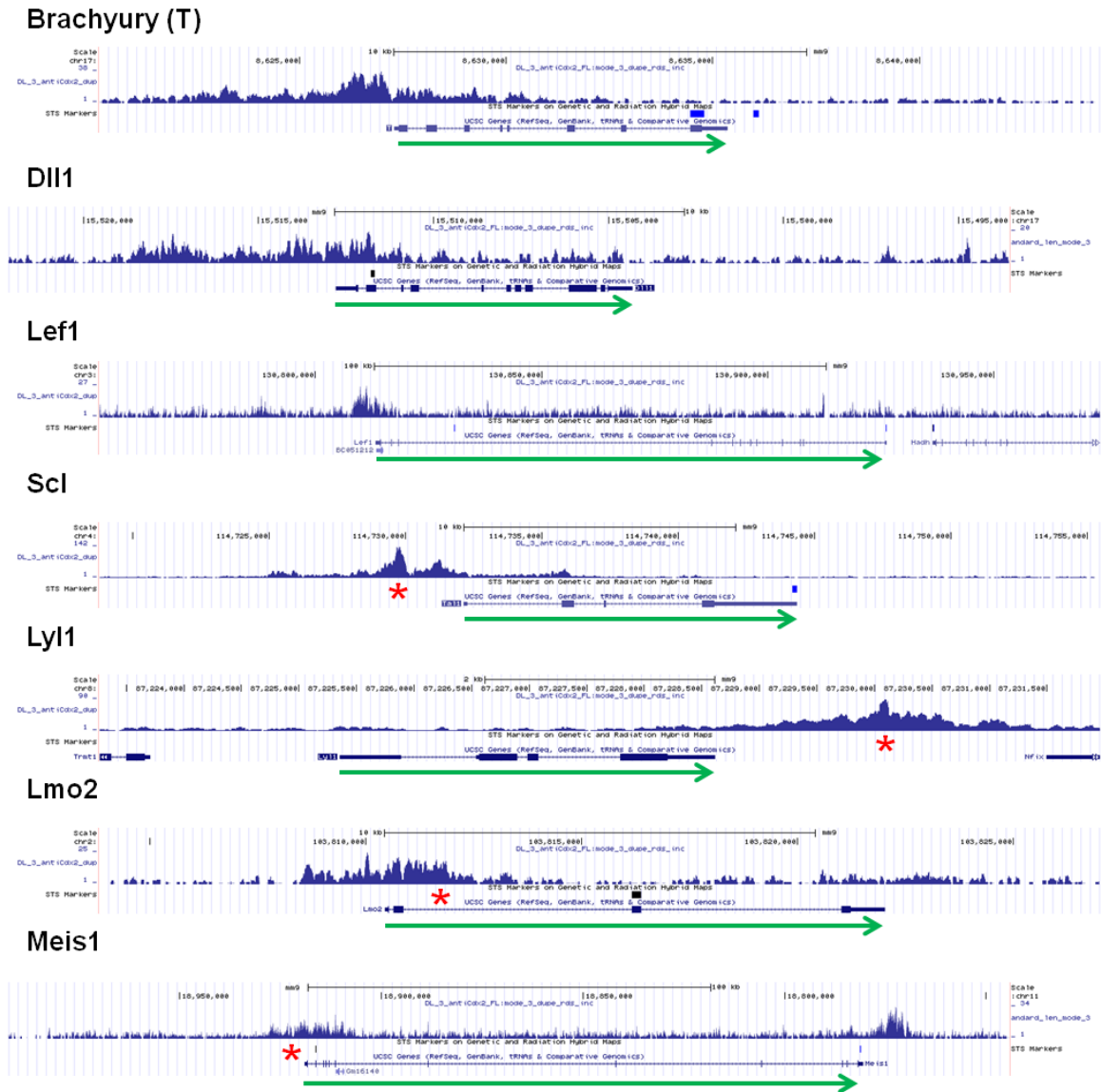


Figure 3.8: ChIP-seq peak distributions.

Analysis of the ChIP-seq data using the UCSC Genome Browser showing peaks relative to genes of interest. Green arrows below the peaks indicate transcripts. Red asterisks mark canonical Cdx response elements (CDREs) identified by TESS or TFSEARCH under the hematopoietic genes of interest. Scale bars (kilobase pairs) unique to each gene are displayed above each peak map.

3.7 Directed ChIP Experiments

Using the ChIP-seq peaks and putative CDREs as landmarks, DNA intervals were assessed by directed ChIP assays. A region of DNA located immediately upstream of the *Brachyury* transcriptional start site (TSS), a known Cdx target gene (Savory et al., 2009a), was used as a positive control, while a region of DNA located in *Wnt3a exon 4* was used as an off-target negative control (Figure 3.9). *Brachyury* displayed strong pulldown with Cdx2, as did a region approximately 1.5 kb upstream of the *Scf* TSS. Interaction was also seen in a region of the *Meis1* promoter located approximately 1.2 kb upstream of the TSS, a region 1.5 kb downstream of the *Lmo2* TSS, and a region located 1.5 kb downstream of the *Lyl1* gene (Figure 3.9). *Wnt3a exon 4* displayed minimal interaction, as did pre-immune IgG.

3.8 Cdx2 Impacts the Expression of Several Hematopoietic Genes

To further examine Cdx-dependent regulation of these candidate target genes, RT-PCR was carried out on E8.5 and E9.5 yolk sacs from control and *Cdx1*^{-/-}*Cdx2*^{-/-} embryos. *GAPDH* was used as a housekeeping gene to normalize data, and *Cdx1*^{-/-}*Cdx2*^{fl/fl} controls were set at a baseline of 1.0. Three independent control and *Cdx1*^{-/-}*Cdx2*^{-/-} mutant yolk sacs were used in these trials. qPCR data already indicated a significant decrease in *Scf* transcripts at E8.5 and E9.5 (Figure 3.7).

Expression of two genes known to be upstream of *Scf* in the development of hematopoietic and vascular precursors, *Elf1* and *Flk1*, were also examined. *Flk1*^{-/-} mice

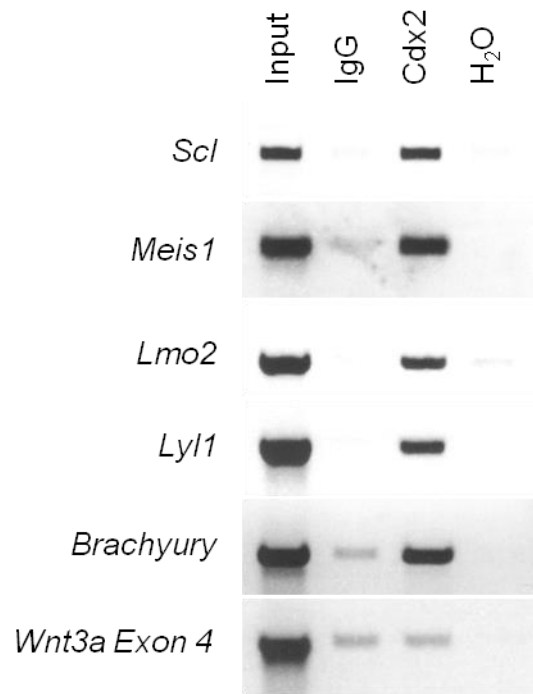


Figure 3.9: ChIP analysis of Cdx2 occupancy of hematopoietic target gene loci.

ChIP was performed on chromatin derived from 30 wild type embryos at E8.5, using a Cdx2 antibody and rabbit IgG as a control for nonspecific binding. Regions encompassing the putative CDRE of *Scl*, *Meis1*, *Lmo2*, and *Lyl1* loci were amplified by PCR. *Brachyury* was used as a positive control, and *Wnt3a Exon 4* was used as a negative control.

die between E8.5 and E9.5 due to the lack of blood island formation, and subsequent lack of vascular structures, due to defects in mesodermal migration into the yolk sac. Elf1 (E74-like factor 1) is a protein that regulates the expression of both *Scf* (Gottgens et al., 2004) and *Lmo2* (Landry et al., 2005). Both *Elf1* and *Flk1* transcripts were found to be unaltered at both E8.5 and E9.5 (Figure 3.10), indicating that these genes are not impacted in *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs.

Expression of *Lyl1*, *Lmo2*, and *Meis1* were also assessed at E8.5 and E9.5. Perturbations were not evident in *Lyl1* transcript levels at E8.5, but did exhibit a slight reduction at E9.5 (Figure 3.10 A and B). Likewise, *Lmo2* levels were only significantly altered at E9.5, while *Meis1* transcripts appear to be elevated above those of controls at E8.5, but drop considerably by E9.5 (Figure 3.10 A and B). *Gata1* and *Pu.1* were not recovered in ChIP-seq analyses, indicating that these factors are not direct *Cdx2* target genes. However, *Gata1* is the first marker of primitive erythropoiesis (Pevny et al., 1991), while *Pu.1* is associated with myeloid fates (Scott et al., 1994), and therefore their expression was surveyed. Both genes exhibited diminished transcript levels in E8.5 *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs, with *Gata1* showing a significant reduction (Figure 3.10 A). Together, these data indicated that the transcription of several hematopoietic genes is altered at E8.5 - E9.5 in *Cdx* mutants.

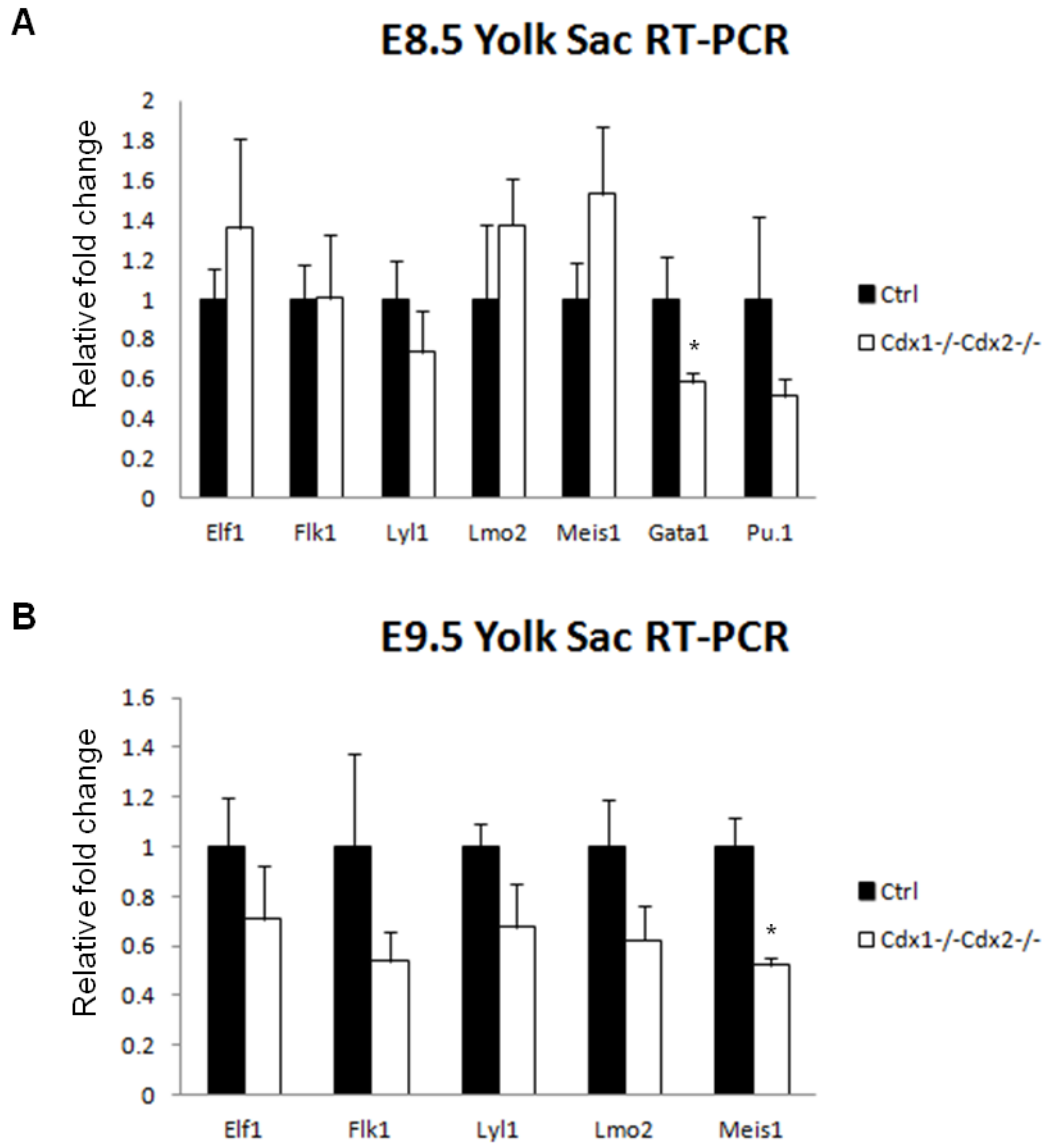


Figure 3.10: Cdx impacts expression of hematopoietic genes in yolk sac.

Expression levels of *Elf1*, *Flk1*, *Lyl1*, *Lmo2*, *Meis1*, *Gata1*, and *Pu.1* were compared between *Cdx1*^{-/-}*Cdx2*^{fl/fl} (Ctrl) and *Cdx1*^{-/-}*Cdx2*^{-/-} at E8.5 (**A**) and E9.5 (**B**). qPCR was performed using GAPDH as a housekeeping control for normalization. Band intensities were calculated for each PCR product using ImageJ software, and fold change was calculated relative to control samples. n = 3, *P<0.05 by student's t-test (paired, two-tailed) compared to controls.

3.9 *Scl* is a putative *Cdx* target gene

Scl is a master regulator of hematopoietic development, is important for vascular remodelling (Visvader et al., 1998), and plays critical roles in the development of the hematopoietic system at early stages of development (Robb et al., 1995). *Scl*^{-/-} embryos exhibit many of the defects observed in *Cdx1*^{-/-}*Cdx2*^{-/-} mutants, such as a lack of visible blood, vascular remodelling defects, and distended pericardium (Shivdasani et al., 1995).

ChIP and expression analyses (Figures 3.7, 3.9) suggested that *Cdx* may directly regulate *Scl* expression. To further examine this, a ChIP experiment was undertaken comparing E8.5 wild type and *Cdx1*^{-/-}*Cdx2*^{-/-} mutant embryos. In addition to *Cdx2*, H3K4Me3 (a transcriptional activation marker) was also used in chromatin pulldowns. In control embryos, the *Scl* promoter showed interaction with *Cdx2*, as well as with H3K4Me3, consistent with *Cdx*-dependent transcriptional activation (Figure 3.11). A region upstream of the *Cdx1* TSS was used as a positive control for *Cdx2* (Mutoh et al., 2009).

Examination of the *Cdx1*^{-/-}*Cdx2*^{-/-} mutant embryo samples indicated that both *Scl* and *Cdx1* loci displayed noticeably diminished occupation by *Cdx2*, accompanied by a decrease in H3K4Me3 occupation (Figure 3.11). Together, these data indicate that *Cdx2* occupies the region of DNA upstream of the *Scl* TSS, and that binding correlates with histone marks associated with transcriptional activation, consistent with the hypothesis that *Scl* is a direct *Cdx2* target gene.

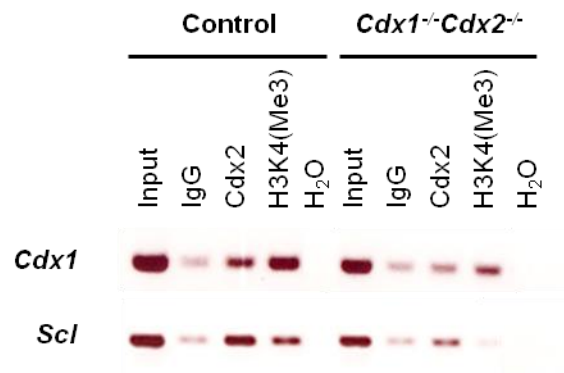


Figure 3.11: Cdx deletion impacts histone methylation at the *Scl* locus *in vivo*.

ChIP was performed on chromatin derived from E8.5 wild type (control) and *Cdx1*^{-/-}*Cdx2*^{-/-} embryos. Cdx2 and Histone 3 Lysine 4 trimethylation (H3K4Me3) antibodies were used for immunoprecipitation, and rabbit IgG was used as a control for nonspecific binding. *Cdx1* was used as a positive control for pulldowns.

The Transcription Element Search System (TESS) revealed three canonical CDREs under the *Scf* ChIP-seq peak (Figure 3.12 A, black diamonds 1, 4, 5). Scanning of this region using TFSEARCH software identified two more potential CDREs (Figure 3.12 A, black diamonds 2, 3). This cluster of CDREs located approximately 1.5 kilobase pairs upstream of the *Scf* TSS correlated with the ChIP-seq peak and is in proximity to the promoter region of the *Scf* gene.

To assess the ability of Cdx2 to bind to these CDREs, an electrophoretic mobility shift assay (EMSA) was carried out. Increasing concentrations of Cdx2 protein were used to assess the ability of Cdx2 to bind oligonucleotides harbouring putative CDRE sequences identified in the *Scf* promoter (black diamonds 1, 4, 5; Figure 3.12 A). Preliminary data indicated that Cdx2 bound to three CDREs (Appendix 9). Although this experiment bears repeating, the direct binding of Cdx2 to each CDRE *in vitro* is consistent with the hypothesis that Cdx2 directly impacts the transcription of *Scf*.

To study the ability of Cdx2 to induce expression of the *Scf* protein, a 2 kb fragment of DNA immediately upstream of the *Scf* TSS, encompassing the CDREs, was cloned into the promoterless pXP2 luciferase reporter vector (Figure 3.12 B). P19 cells were used in this luciferase reporter assay, since Cdx2 is not expressed in this cell line (Appendix 10), allowing for the study of the functional impact of Cdx2 on reporter activity. The *Scf* reporter plasmid was transiently transfected into P19 cells, with and without co-transfection of a Cdx2 expression vector, and luciferase activity was assessed; luciferase reporter activity was unaffected by Cdx2 in these cells (Figure 3.12 C). Since

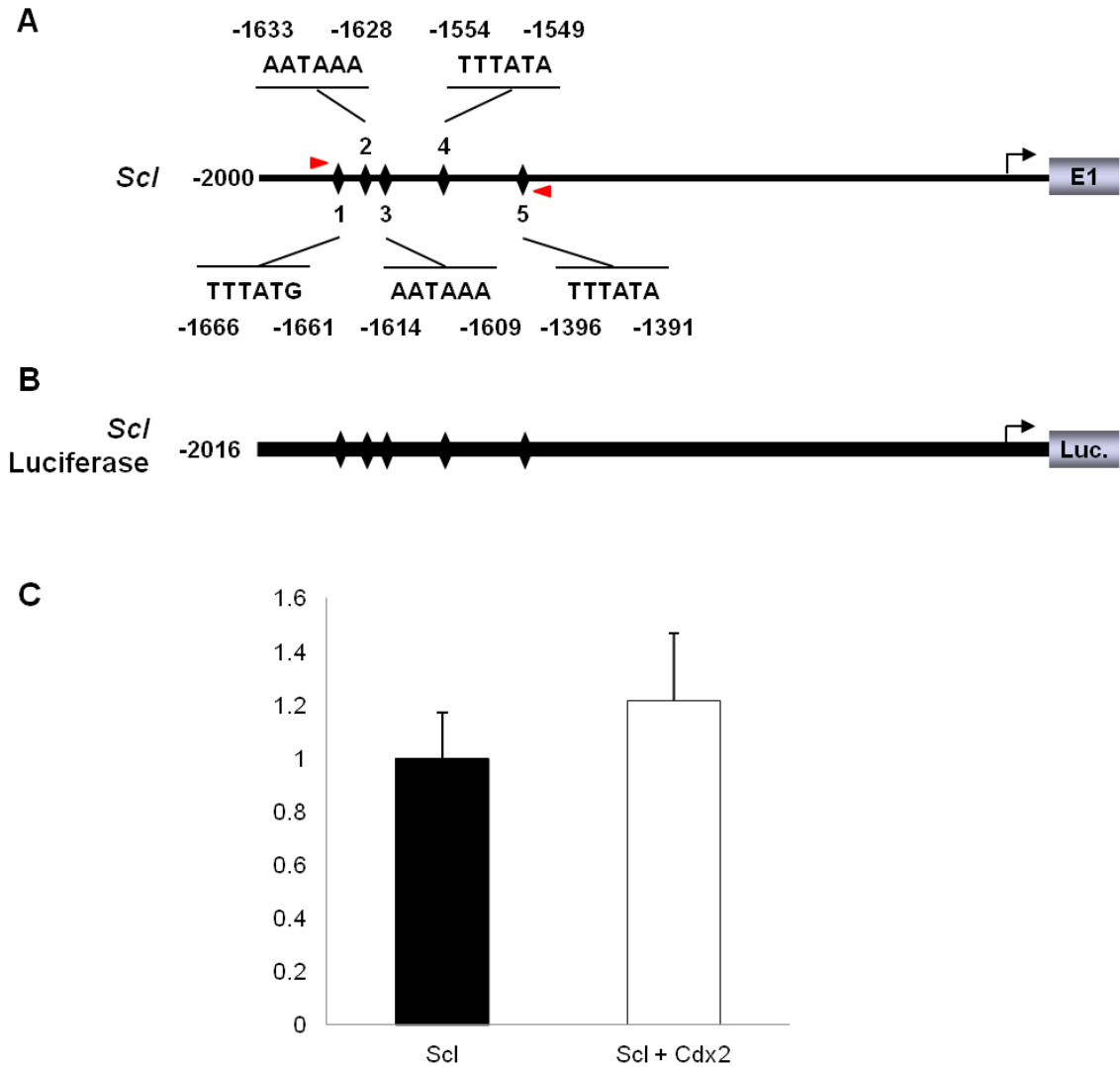


Figure 3.12: Cdx regulation of the *Scf* promoter

A) Five putative CDREs (black diamonds) were identified in the *Scf* proximal promoter using Transcription Element Search System (TESS) and TFSEARCH softwares. Red triangles represent ChIP primers.

B) Schematic of the *Scf* luciferase construct, cloned into the pXP2 luciferase reporter vector.

C) The *Scf* reporter vector was transfected into P19 cells in the absence or presence of a Cdx2 expression vector. Luciferase reporter activity was measured and normalized using β -galactosidase, which was co-transfected into all culture plates.

transcription factors do not act in isolation, it is possible that P19 cells lack the necessary co-factors that are required for Cdx2-dependent transcription of the *Scf* gene. In addition, three enhancer elements (at -4 kb, +19 kb, and +40 kb relative to the TSS) have been identified that modulate *Scf* transcription *in vivo* (Sanchez et al., 2001; Gottgens et al., 2004; Ogilvy et al., 2007). It is plausible that one or more of these elements contribute to Cdx-dependent transcriptional activity. Based on recent data suggesting that *scf* is a direct Cdx target in zebrafish (Paik et al., 2013), it is likely that this reporter construct will be responsive in an appropriate cellular context.

3.10 *Meis1* is a putative Cdx target gene

Meis1 is important for definitive hematopoiesis and vascular maturation (Azcoitia et al., 2005), both of which occur at later developmental stages than the defects observed in *Cdx1*^{-/-}*Cdx2*^{-/-} embryos. ChIP and ChIP-seq data indicated that Cdx2 bound the promoter region of *Meis1* at E8.5 (Figure 3.8, Figure 3.9), while semi-quantitative RT-PCR revealed that *Cdx1*^{-/-}*Cdx2*^{-/-} mutant yolk sacs displayed a significant reduction in *Meis1* expression at E9.5 (Figure 3.10 B).

Based on the ChIP-seq data, a region of the *Meis1* promoter was scanned for canonical CDREs using TFSEARCH software, revealing a cluster of six putative elements approximately 1.2 kb upstream of the TSS (Figure 3.13 A). Directed ChIP revealed strong interaction with Cdx2 and this region in the *Meis1* promoter (Figure 3.9). To further examine the impact of Cdx2 on the transcription of *Meis1*, a fragment of 1.6 kb

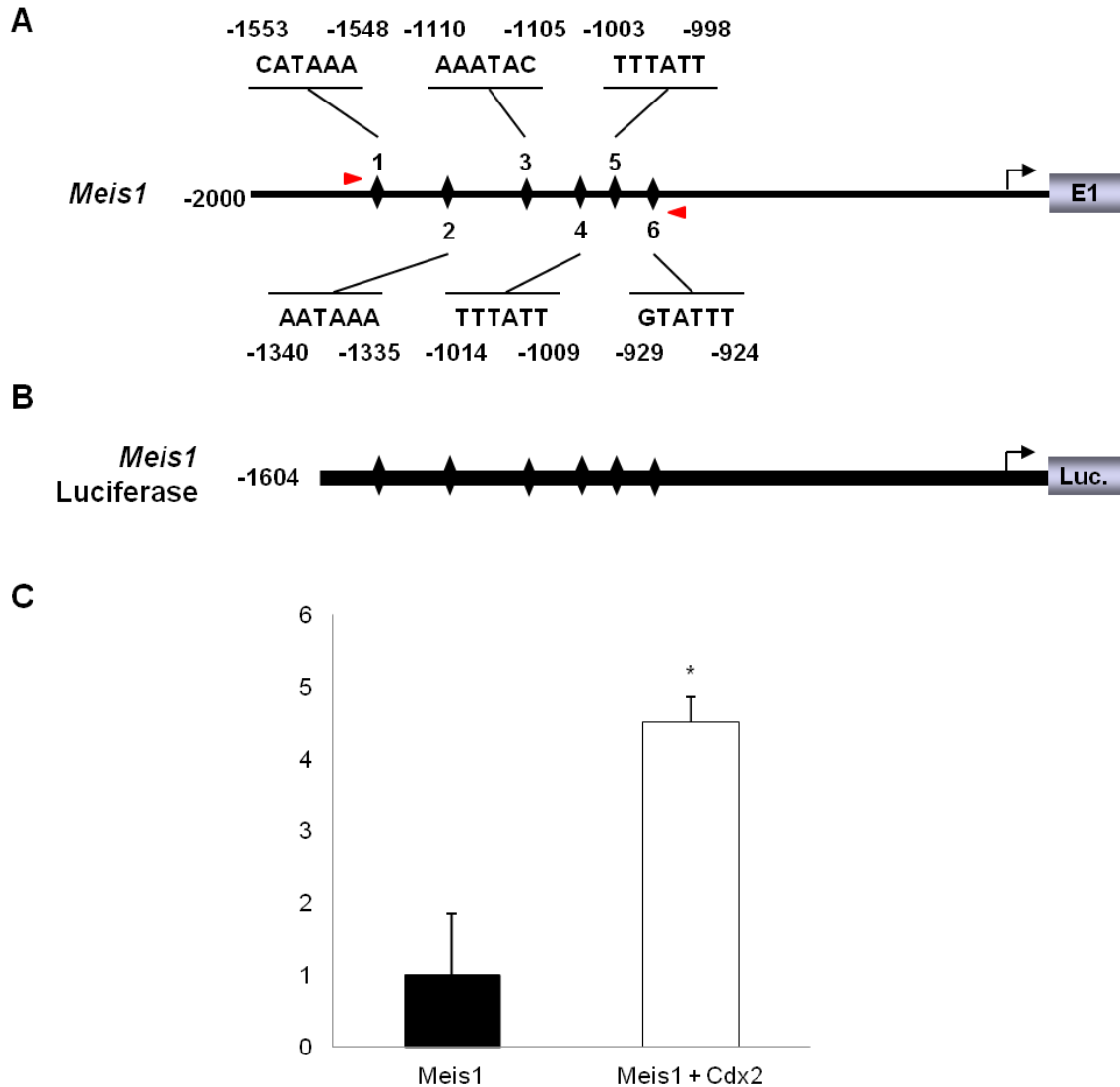


Figure 3.13: Cdx2 regulates the *Meis1* promoter

A) Six putative CDREs (black diamonds) were identified in the *Meis1* proximal promoter using TFSEARCH software. Red triangles represent ChIP primers.

B) Schematic of the *Meis1* luciferase construct, cloned into the pXP2 luciferase reporter vector.

C) The *Meis1* luciferase reporter was transfected into P19 cells, in the absence or presence of Cdx2 expression vector. Luciferase reporter activity was measured and normalized using β -galactosidase, which was co-transfected into all culture plates. Assays were repeated in triplicate. *P<0.05 by student's t-test (paired, two-tailed).

immediately upstream of the *Meis1* TSS was cloned into the promoterless pXP2 luciferase reporter vector (Figure 3.13 B). The luciferase reporter plasmid was transiently transfected into P19 cells in the absence and presence of a Cdx2 expression plasmid, and luciferase activity was assessed. A significant increase in reporter gene expression was observed in this assay, suggesting that Cdx2 positively affects the transcription of the *Meis1* gene (Figure 3.13 C).

3.11 Cdx2-SWI/SNF Interaction

Little is known about the nature of Cdx co-regulator proteins. To this end, we undertook a SILAC-MS experiment to identify Cdx2-interacting proteins using HEK293 cells, which express CDX2 (Appendix 10). This analysis recovered six members of the SWI/SNF chromatin remodelling complex: BRG-1 (BAF190), BAF53, BAF57, BAF155, BAF170, and BAF250 (Table 3.2). The SWI/SNF chromatin remodelling complex alters nucleosome positioning with subsequent impact on transcription factor accessibility (Imbalzano et al., 1994; Schnitzler et al., 1998), consistent with a potential role as a Cdx2 co-regulatory complex. I focussed on Brg1, based on previous evidence that suggested a direct interaction exists between Cdx2 and Brg1 in intestinal tissues (Yamamichi et al., 2009).

3.12 Cdx2 and Brg1 interact *in vivo*

Consistent with MS data, studies from HEK293 cell lysates revealed that Brg1 effectively immunoprecipitated with Cdx2 (Figure 3.14 A). Prior studies have identified a region of

Gene Name	Unique Peptides	Sequence Coverage [%]	Ratio H/L Normalized	log Ratio
CDX2	6	17.6	14.40	3.85
BAF250	16	9.9	7.51	2.91
BAF190 (BRG1)	21	16.5	5.27	2.40
BAF53	2	4.7	4.19	2.07
BAF57	2	6.1	2.78	1.48
BAF155	4	6.2	2.70	1.43
BAF170	2	3.5	2.47	1.30

Table 3.2: CDX2 interacts with the SWI/SNF complex.

SILAC-MS revealed interaction between Cdx2 and multiple members of the SWI/SNF chromatin remodelling complex. The ratio of heavy to light isotope peptides (H/L) indicates relative enrichment of CDX2 interactions.

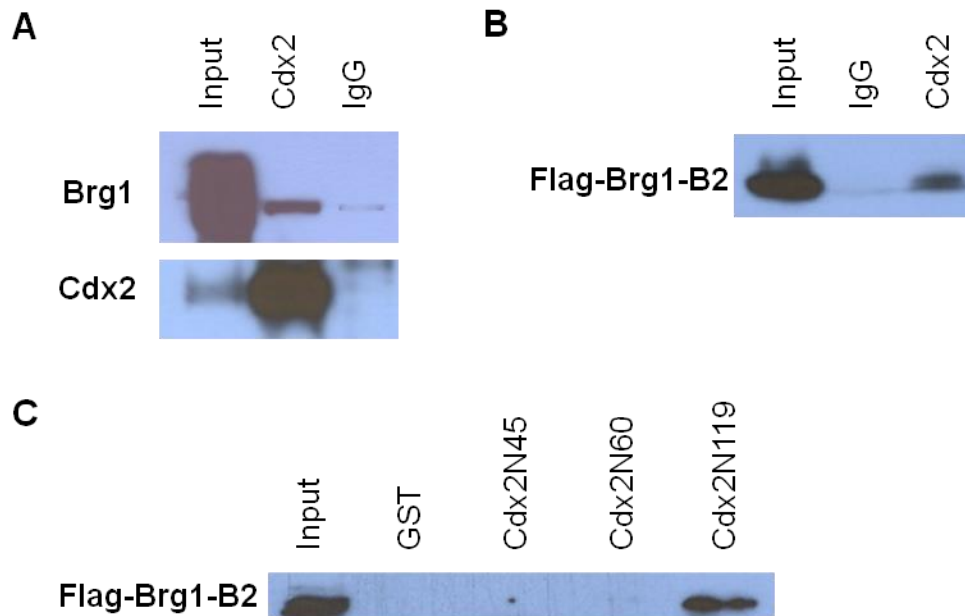


Figure 3.14: Cdx2 and Brg1 co-immunoprecipitate.

A) Co-immunoprecipitation of Cdx2 and Brg1 from HEK293 cell lysate. The Western blot was stripped and re-probed with Cdx2 to illustrate immunoprecipitation. IgG was used as a negative control.

B) A Flag-Brg1-B2 expression vector was transfected into HEK293 cells, and lysates immunoprecipitated with anti-Cdx2 antibody. Brg1-B2 association was assessed by anti-Flag using IgG as a negative control.

C) A Flag-Brg1-B2 expression vector was transfected into HEK293 cells, and lysates incubated with GST-Cdx2 fusion proteins bound to glutathione-agarose beads. Flag-Brg1-B2 interaction was assessed by Western blotting using an anti-Flag antibody. GST was used as a negative control.

Brg1 between amino acids 325 and 611 that interacts with other transcription factors (Mudhasani and Fontes, 2005; Trotter et al., 2008). I therefore assessed the ability of Brg1 amino acids 325 through 526 (termed Brg1-B2) to interact with Cdx2, and found that it displayed strong interaction with Cdx2 in GST-pulldown assays (data not shown). This fragment was then used to develop a FLAG-tagged Brg1-B2 expression vector for co-immunoprecipitation studies, which revealed FLAG-Brg1-B2 interaction with full length Cdx2 and the Cdx2-N119 amino terminal domain (Figure 3.14 B and C). However, smaller N-terminal regions of Cdx2 did not display any interaction (Figure 3.14 C), indicating that Brg1 interacts with Cdx2 between amino acids 60 and 119.

3.13 Cdx members interact with Brg1 *in vitro*

Immunoprecipitation studies from cells do not confirm direct interaction between proteins, as multi-protein complexes can be immunoprecipitated. To determine if the interaction between two proteins is likely direct, *in vitro* assays, such as glutathione S-transferase (GST) pulldowns assays, are a useful tool (Smith and Johnson, 1988; Kaelin et al., 1991). To this end, full length Brg1 protein was generated and radiolabelled with ³⁵S-methionine and assessed for its ability to interact with a GST-Cdx2 fusion protein bound to glutathione agarose beads. Initial assays indicated that full length Brg1 interacted with full length Cdx2 in this system (Figure 3.15 A), corroborating previous data (Yamamichi et al., 2009) and consistent with direct interaction between the two proteins.

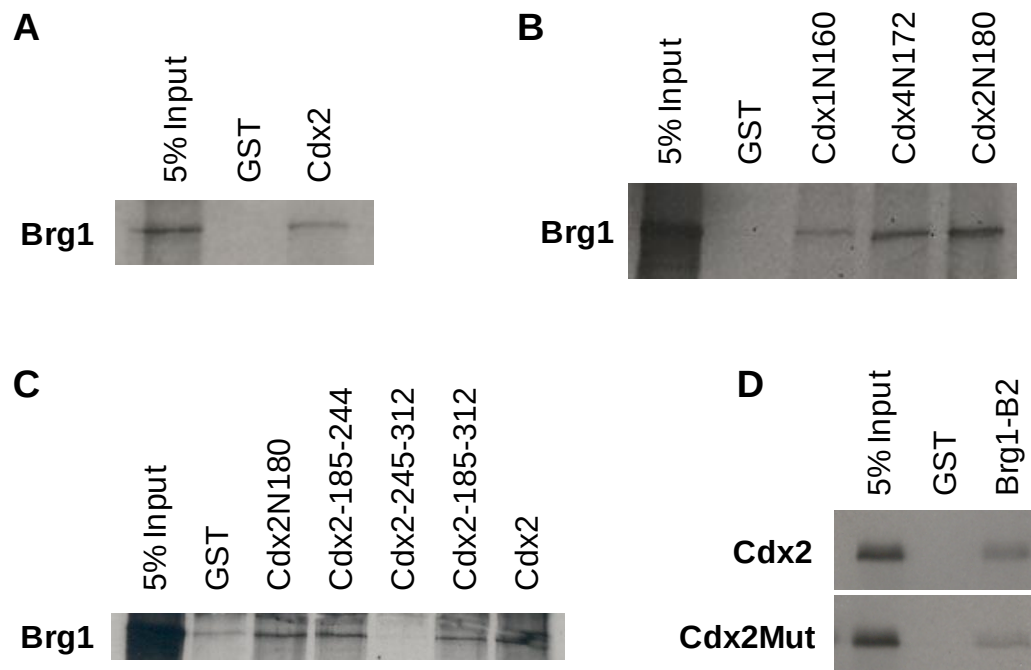


Figure 3.15: Analysis of interaction between Cdx2 and Brg1 *in vitro*.

GST pulldown assays were used to identify the regions necessary for interaction between Cdx2 and Brg1. Cdx2 and Brg1 GST-fusion vectors were bound to glutathione-agarose beads. Full length Brg1, Cdx2, and Cdx2Mut were radiolabelled with ^{35}S -methionine, incubated with beads, and bound protein visualized by autoradiography following resolution by PAGE. GST was used as a negative control.

- A)** Brg1 was assessed for interaction with full length GST-Cdx2.
- B)** Brg1 was assessed for interaction with the N-terminal region of each Cdx member.
- C)** Brg1 was assessed for interaction with the N-terminus, homeodomain, and C-terminus regions of Cdx2, along with full length Cdx2.
- D)** Cdx2 and Cdx2Mut were assessed for interactions with Brg1-B2.

Based on the functional overlap between Cdx members, it was hypothesized that Cdx1 and Cdx4 may also interact with Brg1. Using GST-fusion constructs, the N-terminal regions of all three Cdx members were found to interact with Brg1 in GST-pulldown assays (Figure 3.15 B).

3.14 Brg1 interacts with multiple regions of Cdx2 *in vitro*

To more precisely identify the site of Cdx2-Brg1 interaction, the N-terminal 180 amino acid region of Cdx2 was used to generate a series of GST fusion proteins, outlined in Figure 3.16 A. Re-examination of the homology between Cdx members highlighted a conserved motif within this region (Figure 1.6), spanning from amino acids 72 to 78 in Cdx2 (Figure 3.16 A). Using a GST-pulldown assay, Brg1 was found to interact with a region of Cdx2 between amino acids 60 and 80 encompassing this motif (Appendix 11).

Site directed mutagenesis was used to alter the amino acid 72-78 region in the context of full length Cdx2 (referred to as Cdx2Mut), converting the wild type sequence PLREDWN to GVGAAGA. Cdx2Mut, however, maintained interaction with Brg1 and GST-Brg1-B2 (data not shown and Figure 3.15 D), suggesting that another point of contact existed between Cdx2 and Brg1. To determine the location of the secondary point of contact, the homeodomain (amino acids 185 through 244) and C-terminal (amino acids 245 through 312) regions of Cdx2, along with both the homeodomain and the C-terminal region (amino acids 185 through 312; Figure 3.15 C) were assessed for interaction. Brg1 exhibited association with the Cdx2 homeodomain and the larger

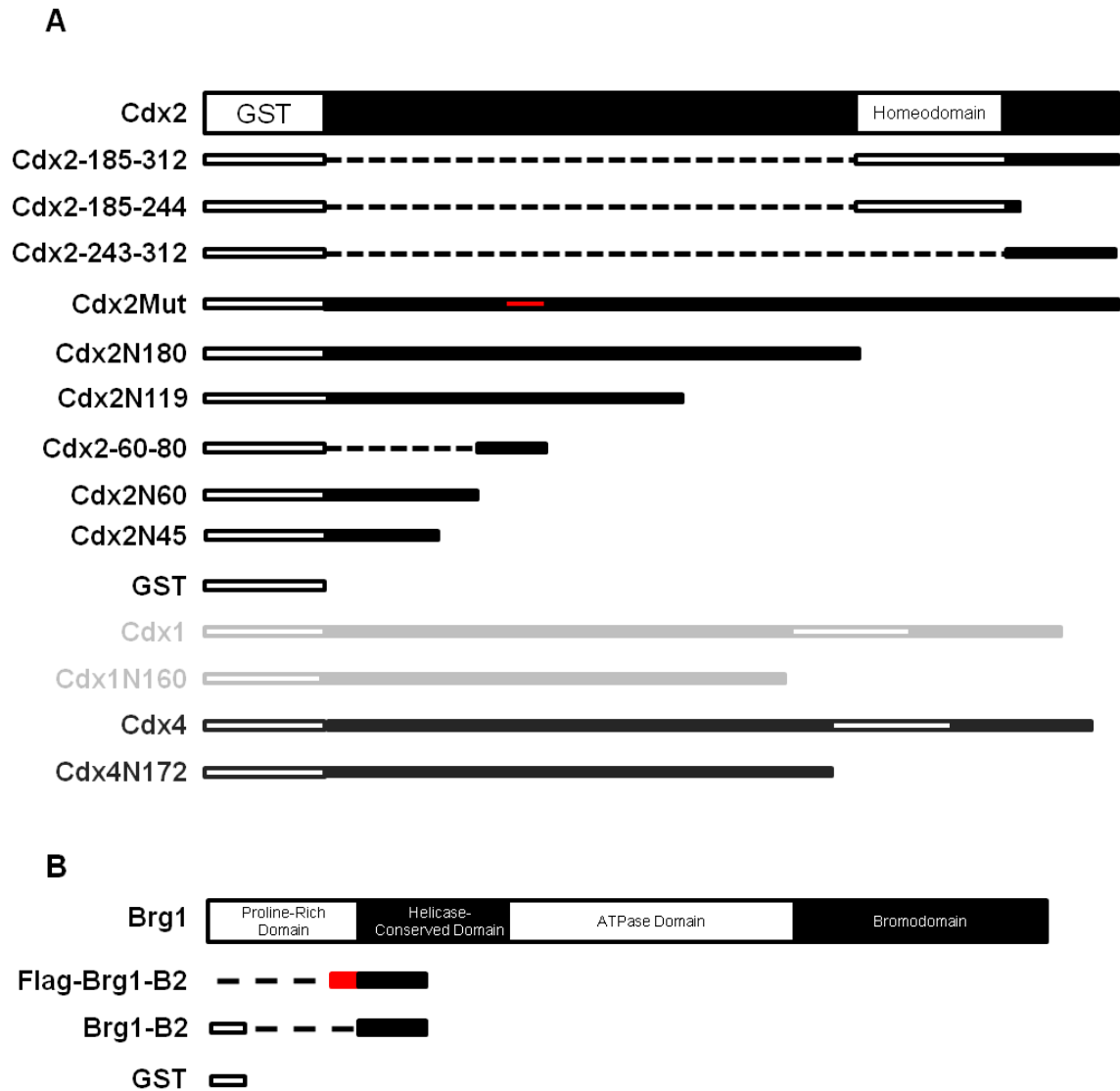


Figure 3.16: Truncation constructs of Cdx2 and Brg1 proteins

A) Schematic representation of GST-Cdx fusion proteins. Red box represents a region of site-directed mutagenesis (Cdx2Mut; amino acids 72-78). Sequences not drawn to scale.

B) Schematic representation of Flag-Brg1-B2 and GST-Brg1-B2 fusion proteins. B2 represents Brg1 amino acids 326 through 527. Red box represents Flag sequence. Sequences not drawn to scale.

homeodomain-C-terminus construct, but failed to interact with the C-terminal region (Figure 3.15 D). These data are consistent with Brg1 interacting with at least two regions of Cdx2 *in vitro*.

3.16 Brg1 and Cdx2 co-localize *in vivo*

To assess the ability of wild type Cdx2 and Cdx2Mut to co-localize with Brg1, P19 cells (which express Brg1; Appendix 10) were transfected with expression vectors encoding Cdx2 and Cdx2Mut proteins. Immunofluorescence revealed that both wild type Cdx2 and Cdx2Mut proteins were nuclear, indicating that the altered amino acid substitutions do not affect nuclear localization (Figure 3.17). Moreover, both wild type Cdx2 and Cdx2Mut appeared to co-localize with Brg1 in this assay (Figure 3.17).

3.17 Brg1 and Cdx2 co-localize on Cdx2 target genes *in vivo*

To determine if Brg1 and Cdx2 co-localized on known Cdx2 target loci, ChIP was performed using wild type E8.5 embryos. *Brachyury* was used as a positive control locus for Cdx2 immunoprecipitation, and *Wnt3a exon 4* was used as a negative control. In addition, a region immediately upstream of the *Myogenin D (MyoD)* locus was used as a positive control for Brg1 immunoprecipitation (Lu et al., 2000). *Brachyury* exhibited occupation by Cdx2, but minimal interaction with Brg1 (Figure 3.18). Similarly, *MyoD* showed clear interaction with Brg1, but not Cdx2 (Figure 3.18). *Wnt3a exon 4* showed

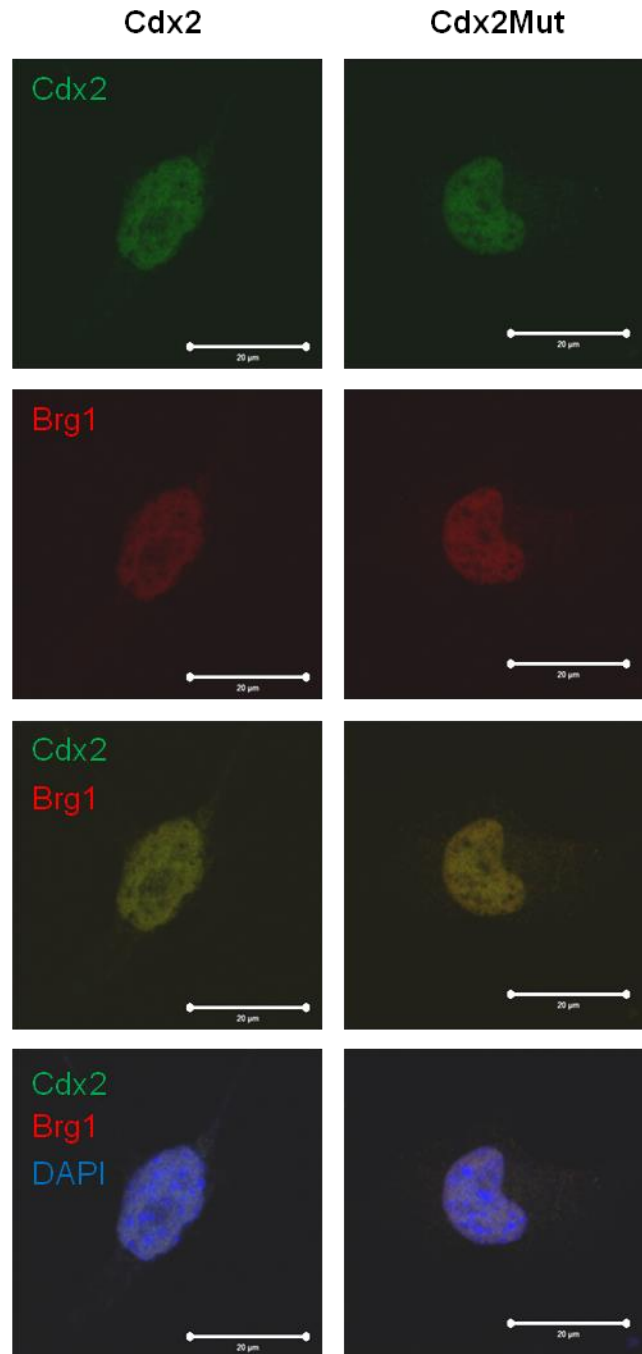


Figure 3.17: Co-localization of Cdx2 and Brg1 in P19 cells.

Immunohistochemistry was carried out on P19 cells transfected with Cdx2 and Cdx2Mut. Signals from antibodies specific to Cdx2 (green) and Brg1 (red) were overlaid to examine co-localization (yellow). DAPI staining was used to reveal nuclei (blue). Scale bars represent 20 µm.

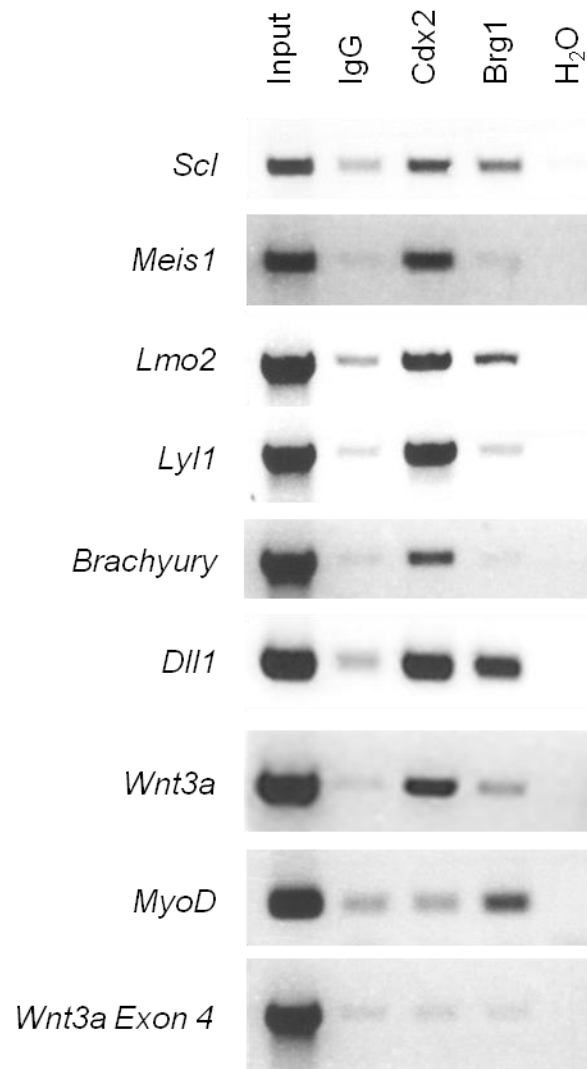


Figure 3.18: Assessment of Cdx2 and Brg1 localization on target genes *in vivo*

ChIP was performed on chromatin derived from E8.5 wild type embryos using Cdx2 and Brg1 antibodies for immunoprecipitation, and rabbit IgG for non-specific binding. Genes are indicated to the left. *Wnt3a Exon 4* was used as a non-specific control locus.

minimal interaction in all conditions, demonstrating the specificity of these interactions (Figure 3.18).

Examination of hematopoietic gene loci also revealed strong interaction with Cdx2 (Figure 3.18), recapitulating the prior ChIP analysis of *Scl*, *Lmo2*, *Meis1* and *Lyl1* loci. *Scl* and *Lmo2* (but not *Meis1* or *Lyl1*) also showed considerable interaction with Brg1 (Figure 3.18). Previous studies have shown that Cdx2 occupies, and regulates, the *Wnt3a* and *Dll1* genes (Savory et al., 2009a; Grainger et al., 2012). In addition to Cdx2 occupation, Brg1 was also found to co-occupy the *Dll1* locus, while *Wnt3a* exhibited a lesser degree of binding, albeit above background (Figure 3.18). Taken together, these observations suggest that Brg1 and Cdx2 co-occupy a subset of target genes.

3.18 Brg1 knockdown results in decreased expression of Cdx2 targets

To assess the importance of Brg1 for Cdx2-dependent transcription, HEK293 cells were transfected with shRNA targeting *BRG1*, and knock-down clones identified via Western blot (Figure 3.19 A) and RT-PCR (Figure 3.19 B). Expression of *TNF α* , a BRG1-dependent gene (Naito et al., 2009), was significantly reduced in BRG1 knock-down cells (Figure 3.19 B), validating these lines. Cdx target genes *DLL1*, *LEF1*, *TBX6* and *WNT3A* were also significantly downregulated by *BRG1* knockdown, while *CDX2* levels remained unchanged (Figure 3.19 B).

Brg1 was reintroduced by transient transfection into the knock-down clones and expression of CDX2 target genes and *TNF α* were assessed. Western blots confirmed the

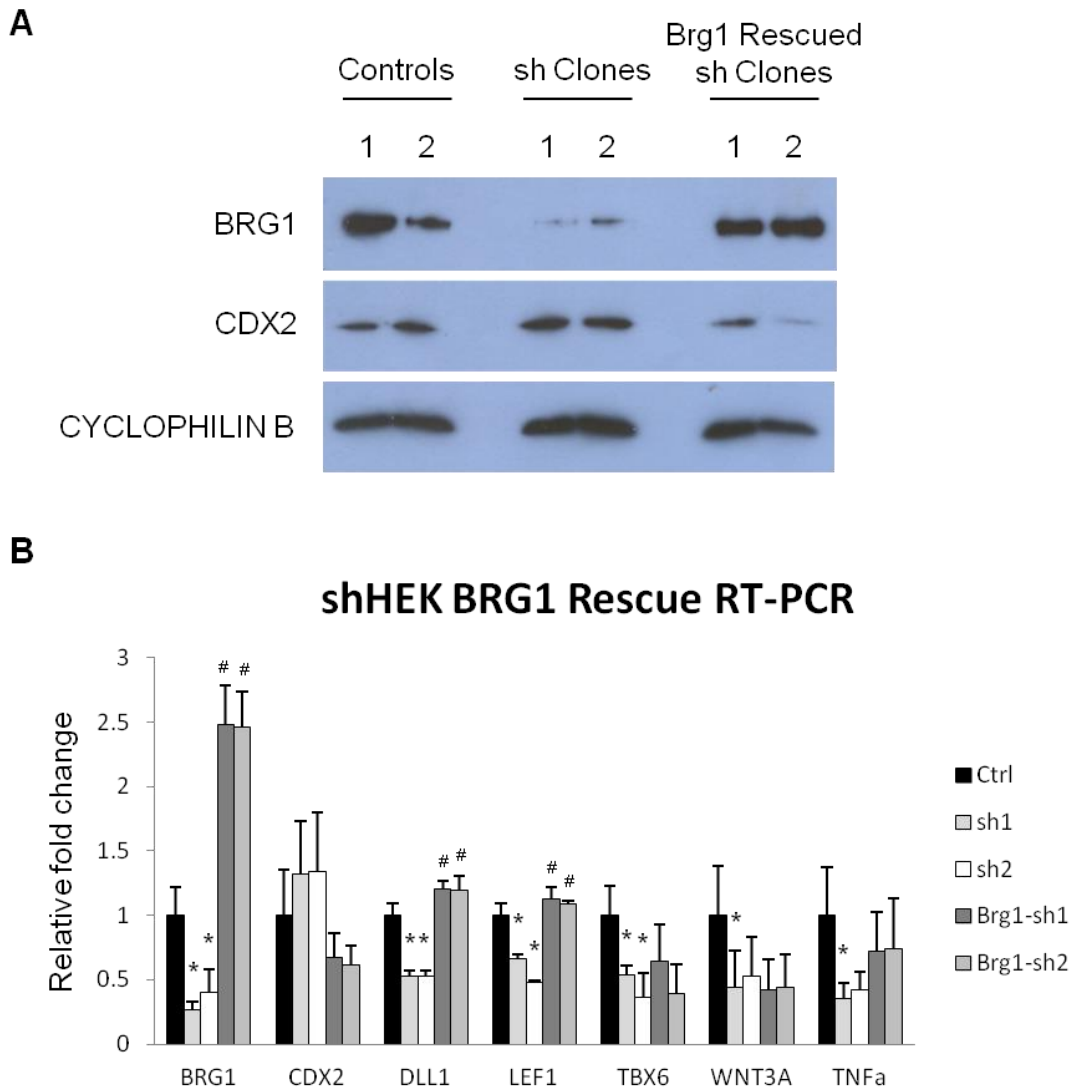


Figure 3.19: Analysis of BRG1 shRNA knockdown in HEK293 cells

A) Western blot analysis of BRG1 and CDX2 in control, two independent BRG1 knockdown lines (sh clones), sh clones re-expressing BRG1. Cyclophilin B was used as a loading control.

B) RT-PCR analysis of gene expression. Expression levels of *BRG1*, *CDX2*, *DLL1*, *LEF1*, *TBX6*, *WNT3A*, and *TNFa* were compared between control (Ctrl), BRG1 knockdown clones (sh1, sh2) and knockdown clones re-expressing BRG1 (Brg1-sh1, Brg1-sh2). ACTIN amplification was used as an input control. Band intensities were assessed using ImageJ, and relative fold expression was calculated compared to control samples. $n = 3$, * $P < 0.05$ by student's t-test comparing sh clones to controls, # $P < 0.05$ by student's t-test comparing Brg1-sh clone to sh clone.

rescue of Brg1 protein levels (Figure 3.19 A). RT-PCR revealed that transcript levels of *DLL1* and *LEF1* were restored to wild type levels upon the reintroduction of Brg1, but rescue was not observed for *TBX6* or *WNT3A* (Figure 3.19 B). *TNF α* levels appeared to partially recover, but these data were not statistically significant (Figure 3.19 B). Interestingly, CDX2 protein and mRNA levels were diminished in the rescue experiment, possibly due to squelching induced by excessive levels of Brg1 (Figure 3.19 A and B).

3.19 Cdx2 Knockdown results in decreased expression of target genes

To further examine regulation of CDX2 target genes in HEK293 cells, *CDX2* shRNA knock-down clones were generated and identified via Western blotting and RT-PCR (Figure 3.20 A). Among target genes examined, *DLL1* exhibited a marked decrease in expression, while only one knock-down clone exhibited decreased *TBX6* transcript levels (Figure 3.20 B). *LEF1* and *WNT3A* transcripts remained relatively unaffected by the loss of *CDX2* (Figure 3.20 B). These results may be due to the fact that *CDX2* transcript levels remained relatively high after shRNA suppression, and protein levels were not diminished to the degree observed in the *BRG1* shRNA experiments.

3.20 Cdx2 and Cdx2Mut differentially impact target gene expression

To examine the impact that Cdx2Mut may have on the transcription of CDX2 target genes, both wild-type Cdx2 and Cdx2Mut were transiently transfected into CDX2 knock-down HEK293 clones. Cdx2 and Cdx2Mut protein levels were assessed by Western

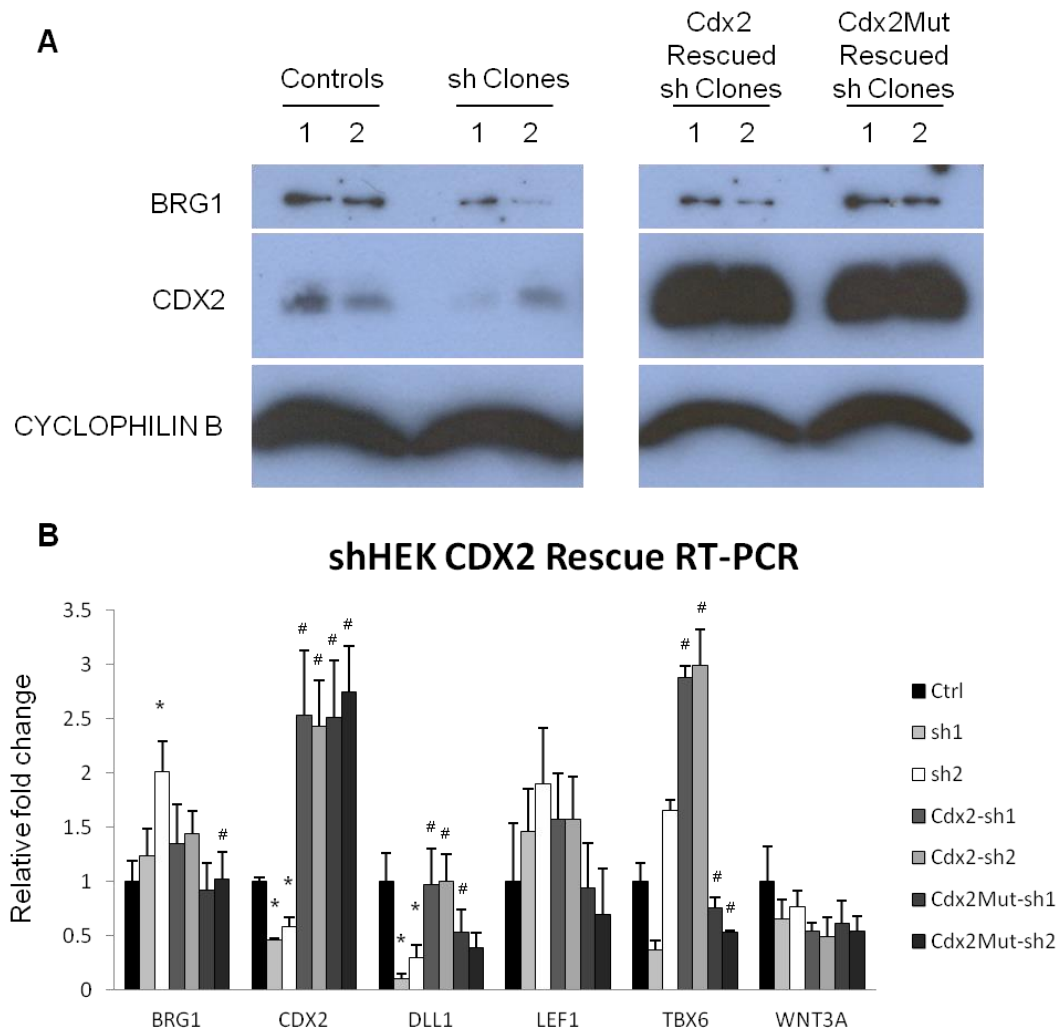


Figure 3.20: Analysis of CDX2 shRNA knockdown in HEK293 cells

A) Western blot analysis of BRG1 and CDX2 protein levels in control, two independent Cdx2 knockdown lines (sh clones), knockdown lines re-expressing Cdx2 (Cdx2-sh), and knockdown lines re-expressing Cdx2Mut (Cdx2Mut-sh). Cyclophilin B was used as a loading control.

B) Expression levels of *BRG1*, *CDX2*, *DLL1*, *LEF1*, *TBX6*, and *WNT3A* were compared between control (ctrl), Cdx2 knockdown lines (sh1, sh2), knockdown lines re-expressing Cdx2 (Cdx2-sh1, Cdx2-sh2), and knockdown lines re-expressing Cdx2Mut (Cdx2Mut-sh1, Cdx2Mut-sh2). RT-PCR was performed using ACTIN as an input control. Band intensities were assessed using ImageJ, and relative fold expression was calculated compared to control samples. $n = 3$, * $P < 0.05$ by student's t-test comparing sh clones to controls, # $P < 0.05$ by student's t-test comparing Cdx2-sh clone or Cdx2Mut-sh clone to original sh clone.

blotting to confirm rescue (Figure 3.20 A). Examination of CDX2 target genes by RT-PCR revealed that *DLL1* transcript levels returned to control levels upon reintroduction of wild type Cdx2, but not Cdx2Mut (Figure 3.20 B). *LEF1* and *WNT3A* remained relatively unaffected by the introduction of either Cdx2; however, *TBX6* transcripts increased upon wild type Cdx2 expression, but less so with Cdx2Mut (Figure 3.20 B). Brg1 showed no difference in protein levels between wild type Cdx2 and Cdx2Mut samples (Figure 3.20 A).

3.21 The Cdx2-Brg1 interaction is functionally relevant to *Dll1* transcription

Several observations suggest that Cdx2 and Brg1 converge on the Cdx2 target gene *Dll1*. CHIP indicated co-localization of both proteins to the *Dll1* promoter while *BRG1* knock down resulted in downregulation of *DLL1* in HEK293 cells. Wild type Cdx2, but not Cdx2Mut, could rescue *DLL1* expression in CDX2-deficient cells, indicating that contact between Cdx2 and Brg1 impacts the transcription of this target gene.

To further examine this relationship, P19 cells were transfected with a Cdx2-responsive reporter derived from the *Dll1* locus, along with wild type Cdx2 or Cdx2Mut, to assess the ability of each protein to impact expression of the *Dll1* reporter. As Brg1 is expressed in P19 cells (Appendix 10), co-transfection of this protein was not necessary. Wild type Cdx2, but not Cdx2Mut, significantly induced the expression of the reporter

(Figure 3.21) indicating that the Cdx2Mut mutation impacted Cdx2 function necessary for regulation of *Dll1* transcription (Figure 3.21).

Regulation of a Cdx2-dependent *Wnt3a* reporter was also examined (Savory et al., 2009a), as Brg1 did not appear to strongly associate with this promoter in ChIP experiments (Figure 3.18). Additionally, *WNT3A* expression was not significantly affected by *BRG1* knock down in HEK293 cells (Figure 3.19 B), and no differences were observed between wild type Cdx2 and Cdx2Mut rescue in CDX2 knock-down cells (Figure 3.20 B). Consistent with these data, both forms of Cdx2 exhibited comparable and significant induction of reporter activity in P19 cells (Figure 3.21). Together, these observations suggest that Brg1 and Cdx2 appear to impact the transcription of a subset of Cdx2 target genes.

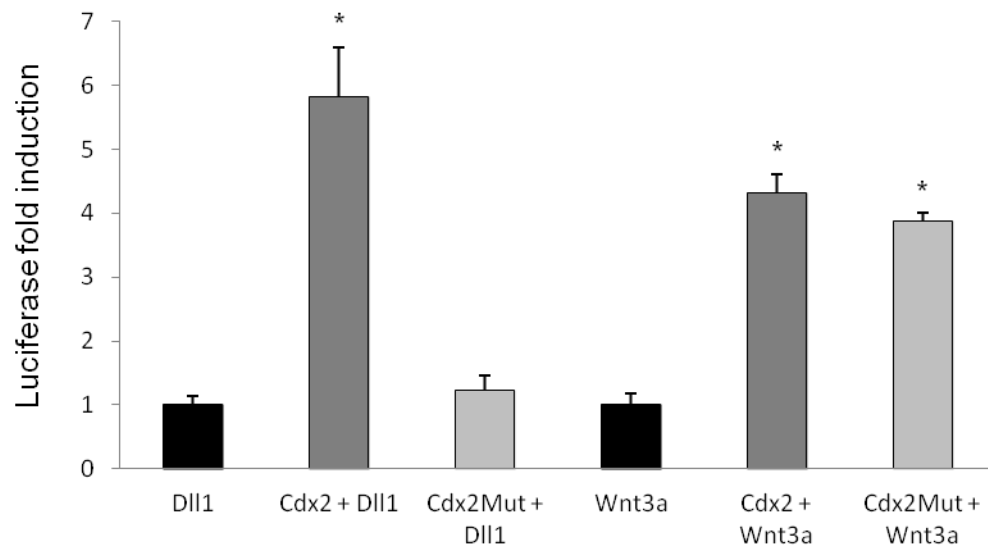


Figure 3.21: Cdx2Mut mutation impacts Dll1 reporter expression.

Dll1 and *Wnt3a* luciferase reporter vectors were transfected into P19 cells, with or without wild type Cdx2 or Cdx2Mut expression vectors. Luciferase activity was assessed and normalized using β -galactosidase, which was co-transfected into all culture plates. Assays were repeated in triplicate. * $P < 0.05$ by student's t-test compared to reporter vectors in the absence of Cdx2 constructs.

Chapter 4

Discussion

My analysis of *Cdx1*^{-/-}*Cdx2*^{-/-} mutants revealed perturbations of primitive hematopoiesis and development of yolk sac vasculature, likely contributing to death at approximately E11.5. A number of novel Cdx target genes were identified that may be contributing to these phenotypes. In addition, Brg1 was identified as a potential Cdx co-factor that impacts the transcription of a subset of Cdx target genes.

4.1 Cdx in hematopoiesis

The complexity of factors involved in hematopoietic and vascular development in the murine embryo makes the analysis of *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs challenging. Several independent events are critical to the development of blood and vasculature, specification of mesodermal derivatives, migration of hemangioblast cells to the yolk sac, formation of the heart and subsequent introduction of fluid force into the developing vasculature, and the maturation of primitive hematopoietic and vascular cells themselves.

In mice, deletion of *Cdx1* or *Cdx4* does not result in gross hematopoietic or vascular defects (Subramanian et al., 1995; Chawengsaksophak et al., 1997; van Nes et al., 2006), although conditional *Cdx2* mutants do exhibit abnormal development of the vascular plexus (Chawengsaksophak et al., 2004). In zebrafish, *cdx4* deletion results in severe anemia (Davidson et al., 2003), and this phenotype is exacerbated with elimination of all primitive and definitive hematopoiesis when *cdx1a* is also knocked down (Davidson and Zon, 2006). Although mESCs have been characterized in the context of complete *Cdx*

knockdown (Wang et al., 2008), our model offers the first characterization of hematopoietic defects in a Cdx murine model that is effectively Cdx null. My studies revealed significant hematopoietic and vascular defects in *Cdx1^{-/-}Cdx2^{-/-}* yolk sacs, along with the identification of potential novel Cdx2 target genes that may impact hematopoiesis.

Primitive hematopoiesis appeared to proceed somewhat normally at early stages in *Cdx1^{-/-}Cdx2^{-/-}* embryos, based on the presence of hemoglobin-positive cells at E8.5. Transcript levels of numerous hematopoietic genes were also relatively unaffected at E8.5, but became significantly altered by E9.5; consistent with this, E9.5 mutants exhibited disrupted hematopoietic and vascular systems in the yolk sac. CFU counts from *Cdx1^{-/-}Cdx2^{-/-}* yolk sacs exhibited a significant reduction in hematopoietic colony formation, but all lineages of normal CFUs developed in this assay, indicating that Cdx2 might impact the development of multiple hematopoietic cell types. Likewise, when *Cdx2* was deleted in yolk sacs after hematopoiesis had been initiated, all CFU lineages formed, albeit at lesser numbers than controls. This observation indicates that Cdx2 plays a role in hematopoiesis after blood islands have formed. These data are also consistent with prior work in mESCs, illustrating that Cdx knockdown does not completely eliminate hematopoietic potential (Wang et al., 2008). In addition, based on the completely bloodless phenotype exhibited in *cdx1a^{MO}cdx4^{-/-}* zebrafish, my studies suggest that differences may exist between zebrafish and mouse hematopoiesis. It remains a possibility, however, that mESC knockdown cells and conditional *Cdx1^{-/-}Cdx2^{-/-}*

mutant mice retain residual Cdx expression, leading to incomplete failure of hematopoiesis.

ChIP-seq and ChIP analyses indicated that *Scf*, *Lmo2*, *Lyl1*, and *Meis1* loci were occupied by Cdx2, suggesting that Cdx2 may be involved in the transcription of these genes. Consistent with this, transcript levels of *Scf* were diminished at E8.5, and decreased further by E9.5. *Lmo2*, *Lyl1*, and *Meis1* transcript levels remained unaffected by Cdx deletion at E8.5, but appeared to decline at E9.5. Although *Gata1* and *Pu.1* were not recovered in the ChIP-seq experiment, transcript levels were studied to better understand the stage at which Cdx deletion impacted hematopoiesis. *Gata1* showed significant downregulation at E8.5, indicating that primitive erythropoiesis was perturbed in Cdx mutants. *Pu.1* transcripts were also diminished, but not to a significant extent. These results are consistent with the observation that *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs exhibit a mildly perturbed phenotype at E8.5, but hematopoiesis is drastically impacted by E9.5.

To further examine specific hematopoietic genes potentially regulated by Cdx, *Scf* and *Meis1* were studied in further detail. Both genes displayed strong peak scores in the ChIP-seq analyses, and directed ChIP experiments verified the validity of these results. A recent ChIP study of the *Meis1* locus identified histone 3 acetylation (a mark of transcriptional activation) at both -2kb and +140 kb regions (Xiang et al., 2013), an observation that correlates closely with the Cdx2 ChIP-seq profile obtained from our data set, indicating that Cdx2 may act to promote transcription of *Meis1*. RT-PCR

analyses also indicated significantly diminished transcript levels of *Scf* and *Meis1* at E9.5, further suggesting that Cdx2 affects the transcription of these genes. Cell-based expression assays indicated that *Scf* was unresponsive to Cdx2, while *Meis1* exhibited significant Cdx2-induced reporter activity, suggesting that Cdx2 directly activates *Meis1* transcription. These differences may reflect the cellular environment of P19 cells, which is unlikely to represent the normal hematopoietic/vascular niche.

A recent study in zebrafish has corroborated *scf* and *lmo2* as direct Cdx target genes (Paik et al., 2013). In this model, a transcriptional circuit involving cross-regulation of Cdx4 and *Sall4*, and co-occupation of these two proteins at target gene loci, was found to be critical to the transition of mesoderm to hematopoietic precursors (Paik et al., 2013). Notably, our ChIP-seq data did not recover *Sall4* (our unpublished data). Reintroduction of *scf* and *lmo2* partially rescued the hematopoietic defects in *cdx4/sall4* deficient zebrafish embryos, but this rescue was incomplete, indicating that other Cdx/*Sall* target genes are likely to contribute to hematopoiesis (Paik et al., 2013).

In zebrafish, a model has been proposed whereby Cdx regulates Hox expression, thereby generating mesoderm that is competent for hematopoietic development, followed by the expression of *Scf* and Gata1 in hematopoietic progenitor cells (de Jong et al., 2010). Another model places Cdx members upstream of Hox proteins, but leaves room for the possibility that Cdx members may also contribute to the expression of *meis1.1* and *scf* (Amali et al., 2013). Based on my studies, it is plausible that Cdx members have an effect at both levels; Hox-dependent mesodermal tissues are

perturbed upon *Cdx* deletion, and the expression of hematopoietic target genes are directly affected by the loss of *Cdx2*. Although two mesodermal markers, *Elf1* and *Flk1*, appeared at normal levels in *Cdx1*^{-/-}*Cdx2*^{-/-} mutants, it remains a possibility that other mesodermal factors are disrupted.

In mice, conditional hematopoietic/vascular knockout of *Scl* bypasses the lethality of the complete *Scl*^{-/-} phenotype, but erythrocytes and megakaryocytes fail to develop, likely due to the requirement of *Scl* expression in these lineages (Hall et al., 2003). If *Cdx* deletion directly impacts the expression of *Scl*, it is plausible that the failure of erythropoiesis in *Cdx1*^{-/-}*Cdx2*^{-/-} embryos is a direct result of this effect. Although *Scl* reporter activity was unaffected by *Cdx2* in P19 cells, further studies in hematopoietic/vascular cell lines may support a regulatory role of *Cdx2* for *Scl* expression.

4.2 Hematopoiesis - Future Directions

My findings have revealed extensive yolk sac hematopoietic and vascular defects in *Cdx1*^{-/-}*Cdx2*^{-/-} embryos, identified potential novel hematopoietic *Cdx* targets, and demonstrated that *Cdx2* directly binds and induces the transcription of at least one such gene, *Meis1*. Despite these insights, a number of questions remain unanswered with respect to the roles of *Cdx* members in hematopoietic development.

The *Actin-Cre* system that was used in my studies generated a wide array of developmental defects, which may have contributed to hematopoietic and vascular

abnormalities in *Cdx1*^{-/-}*Cdx2*^{-/-} embryos. An important experiment to study the effects of Cdx deletion on hematopoietic and vascular events would involve the deletion of *Cdx2* using a hematopoietic/vascular-specific *Cre* transgene alone or in the context of a *Cdx1*^{-/-} background. The *Tie2-Cre* transgenic has been used to selectively delete genes from the hematopoietic/vascular niche (Kisanuki et al., 2001; Schlaeger et al., 2005; Griffin et al., 2008). Tie2 is a receptor tyrosine kinase unique to endothelial cells and their precursors in blood islands, and is activated by Angiopoietin-1 (Angpt1), promoting blood vessel maturation (Maisonpierre et al., 1997). A *Flk1-Cre* transgene would also allow for deletion of *Cdx2* in blood islands, and may further shed light on the roles of Cdx in these cells. By specifically targeting hematopoietic and vascular systems, it may prove possible to study the involvement of Cdx members in these developmental niches.

Using our current *Cdx* knockout system, PECAM1 staining at E8.5 would shed light on the morphology of control versus *Cdx1*^{-/-}*Cdx2*^{-/-} vascular plexuses, and more conclusively identify the stage at which yolk sac vascular development is perturbed. The distinctive belt of DAB staining that highlights the formation of blood islands at E8.5 is indicative of plexus formation in yolk sacs (Figure 3.2 B), but the morphology of these structures remains unknown in *Cdx1*^{-/-}*Cdx2*^{-/-} samples. Little information exists in the literature to characterize the expression profile of Cdx members outside of the embryo proper, and *in situ* hybridizations (ISH) or immunohistochemistry would be useful in the characterization of Cdx localization and temporal expression in the yolk sac.

A complementation assay utilizing the intercross of *Cdx1*^{-/-}*Cdx2*^{+/-} and *Scl*^{+/-} could be used to determine if Cdx2 and Scl act within the same pathway. *Cdx2*^{+/-} and *Cdx1*^{-/-} *Cdx2*^{+/-} mutants display homeotic vertebral defects, but do not exhibit overt hematopoietic abnormalities (Chawengsaksophak et al., 1997; van den Akker et al., 2002). Likewise, *Scl*^{+/-} mice appear phenotypically normal (Shivdasani et al., 1995), so any disruptions of hematopoiesis in *Cdx1*^{-/-}*Cdx2*^{+/-} *Scl*^{+/-} compound mutant embryos would indicate that Scl and Cdx2 act within the same genetic pathway.

Our studies have demonstrated that the proximal *Scl* promoter harbours potential CDREs, and is occupied by Cdx2; a complete EMSA experiment would verify these results. The EMSA should include mutated genetic elements within the CDREs, Cdx2 antibody addition to induce a supershift in the gels, and cold competition in the form of unlabelled *Scl* CDRE oligonucleotides. GST protein alone should also be assessed for the ability of this protein to interact with the CDREs, since a GST-Cdx2 fusion protein was used in my EMSA studies. Further examination of the *Meis1* promoter via EMSA, which appears to be bound by Cdx2 in ChIP and luciferase assays, would further support the theory that Cdx2 activates the transcription of *Meis1*.

Surprisingly, the luciferase reporter assays involving Cdx2 and the promoter regions of *Scl* and *Meis1* yielded markedly different results. *Meis1* promoter activity was induced by Cdx2, while the *Scl* promoter showed no change in activity. This may reflect the cellular environment in which these luciferase assays were performed. P19 cells may not recapitulate the normal environment in which Scl and Meis1 are expressed, and the

differences suggest that the expression of *Scl* may require co-factors that are not necessary for the Cdx2-induced expression of *Meis1*. *Scl* luciferase reporter assays have been examined in Jurkat (Patel et al., 2014) and 416B cells (Smith et al., 2008), which may be better suited to examine Cdx-dependent regulation. *Meis1* promoter activity could also be studied in these contexts. Following these studies, the input or deletion of individual CDREs within the *Scl* and *Meis1* promoter regions could also be examined.

Cdx2-dependent regulation of the *Lyl1* and *Lmo2* promoters was not pursued, although ChIP-seq data and directed ChIP revealed CDREs in an intronic region of *Lmo2*, and downstream of the *Lyl1* gene. Expression of both *Lmo2* and *Lyl1* were diminished at E9.5 in *Cdx1*^{-/-}*Cdx2*^{-/-} yolk sacs, indicating that Cdx members impact the expression of these genes. Future studies, in the form of EMSA and luciferase reporter assays, could shed further light on the role that Cdx2 plays in the transcription of *Lmo2* and *Lyl1*.

Although my studies focussed on Cdx2, it should be noted that Cdx1 and Cdx4 may also play critical roles in hematopoietic and vascular development. As previously reported, Cdx4 expression is lost in *Cdx1*^{-/-}*Cdx2*^{-/-} double mutants, which effectively generated a Cdx null phenotype (Savory et al., 2011b). Published data indicates that *Cdx4*^{-/-} mice exhibit defects in erythroid development (Wang et al., 2008), but these mice survive and exhibit normal fertility (van Nes et al., 2006). Functional overlap between Cdx members likely explains the minor perturbations of normal hematopoiesis in this single knockout model. Consistent with this, *Cdx1*^{-/-}*Cdx2*^{-/-} embryos demonstrated severe hematopoietic defects during development. Additional genetic crosses of *Cdx4* mutants into a *Cdx1*^{-/-}

Cdx2^{fl/fl} background would remove any potential for residual *Cdx4* expression, and further clarify the importance of *Cdx* members during hematopoiesis.

As described in the introduction, many genes that are critical to the development of the hematopoietic system are also misexpressed in murine and human blood cancers. *Scl*, *Lmo2*, *Lyl1*, and *Meis1* are all involved in leukemias (T-ALL and AML), which may be the result of genetic translocations of these genes, or aberrant activation of normally dormant genes in these cells. It is possible that *Cdx2* misexpression in leukemia cells results in the aberrant activation of one or more such hematopoietic gene, thereby initiating a transcriptional cascade that supports the formation of leukemia. Transgenic approaches that activate *Cdx* in the hematopoietic system of adult mice would prove useful in the examination of this hypothesis. Several Cre-transgenic lines have been used to assess the adult hematopoietic system, including *Vav-Cre* in hematopoietic and endothelial cells (Georgiades et al., 2002), *Mx1-Cre* in the induction of T-ALL (Carofino et al., 2013) and *Lck-Cre* in T-cells (Reiss et al., 2010). By analyzing the phenotypes of these animals, the role of *Cdx* in leukemias could be understood in more detail.

4.3 Cdx and SWI/SNF

In an effort to understand how *Cdx* impacts the transcription of target genes, a SILAC-MS experiment was undertaken to identify proteins that interact with *Cdx2*. Following the identification of the SWI/SNF complex as a putative *Cdx2* interactor, the core SWI/SNF protein Brg1 was studied in several contexts. An important role of many

transcription factors is the recruitment of other proteins that allow for the alteration of chromatin structures at target loci. The SWI/SNF complex modifies chromatin in an ATP-dependent manner, thereby altering DNA accessibility (Vignali et al., 2000). Current understanding of Cdx biology suggests a limited role for Cdx members regarding the repression of target genes (Chun et al., 2007; Wang et al., 2010; Faber et al., 2013), which leads to the hypothesis that Cdx proteins normally recruit the SWI/SNF complex to facilitate the transcription of target genes. It also remains a possibility that Cdx may use SWI/SNF recruitment to repress target genes, supported by recent data suggesting that Cdx2 interacts with Brg1 at the *Oct4* locus to repress its transcription (Wang et al., 2010), but this repressive effect may be limited to specific contexts.

Using GST-pulldown assays, it was demonstrated that Brg1 associated with Cdx2 *in vitro*, consistent with direct binding; the N-terminal regions of Cdx1 and Cdx4 also showed binding with Brg1. Based on murine knock-out and knock-in studies, Cdx members exhibit functional overlap (van den Akker et al., 2002; van Nes et al., 2006; Savory et al., 2009b; Savory et al., 2011a; Savory et al., 2011b) and therefore they may all recruit SWI/SNF complexes to target loci in a similar fashion. Although published data suggest that Cdx2 interacts with Brg1 (Yamamichi et al., 2009; Wang et al., 2010), interaction between Cdx1 and Cdx4 with Brg1 has not been demonstrated. In addition, the interaction interface within Cdx2 or Brg1 have not been described; I addressed this by analysis of Brg1-Cdx interaction using a series of Cdx mutations in GST-pulldown assays.

Although interaction assays suggested a distinct N-terminal domain was involved in Brg1 binding, the Cdx2Mut protein (which abrogated this point of association) still bound to Brg1, indicating that additional points of contact exist. Interestingly, however, target gene analyses demonstrated marked differences between Cdx2Mut and wild type Cdx2. In Cdx2-depleted HEK293 cells, Cdx2Mut only marginally increased the expression of the targets *DLL1* and *TBX6*, while wild type Cdx2 significantly increased expression of these genes. Several controls support the validity of these data, including comparable expression of wild type Cdx2 and Cdx2Mut at both the protein and transcript level. Finally, both Cdx2 and Cdx2Mut exhibited similar nuclear localization in P19 cells, and both proteins appeared to co-localize with Brg1.

Based on the observation that knockdown of either BRG1 or CDX2 impacted the expression of *DLL1*, reporter assays were carried out to examine the impact of Cdx2Mut on activity of the *Dll1* promoter. *Wnt3a* was used as a control Cdx2 target gene in these assays, based on data suggesting that knockdown of BRG1 did not significantly impact its expression. These assays revealed that, compared to wild type Cdx2, Cdx2Mut did not induce the expression of *Dll1*, while *Wnt3a* expression was comparably induced by both forms of Cdx2, suggesting that recruitment of Brg1 is not required for the Cdx-dependent expression of *Wnt3a*. In the context of *BRG1* knockdown cell lines, it remains a possibility that any genes impacted by *BRG1* depletion are influenced by other BRG1 target genes, in addition to CDX2-dependent alterations in gene expression.

ChIP analysis revealed that Brg1 and Cdx2 co-occupy the *Dll1*, but not the *Wnt3a*, promoter. Some newly identified candidate Cdx2 target genes, such as *Scf* and *Lmo2*, exhibited a similar pattern of co-occupancy, while others, including *Meis1* and *Lyl1*, were bound by Cdx2 only. These results may reflect temporal differences in the activation of Cdx2 target genes, or that Cdx2 does not recruit Brg1 to these loci. It is interesting to note that *Meis1* and *Lyl1* are bound by Cdx2 at E8.5, but are not normally expressed until later time points (Azcoitia et al., 2005; Chan et al., 2007). This may reflect these loci being poised for activation via Cdx2, and this input may be eliminated by the deletion of Cdx2.

Together with shRNA knockdown studies and reporter assays, current evidence indicates that Cdx2 recruits Brg1 to a subset of Cdx2 target genes. The model presented in Figure 4.1 B outlines a mechanism in which Cdx2 binds CDREs, recruits Brg1 to induce an altered chromatin structure, and allows for increased transcription of the *Dll1* gene. In the absence of Cdx2 (Figure 4.1 A), the chromatin structure impedes the transcriptional machinery and/or other unknown factors from accessing the promoter of *Dll1*, thereby inhibiting transcription. In contrast to this model, Cdx2-induced *Wnt3a* transcription appears to rely on unknown factors. These two models can be extrapolated to other Cdx2 target genes.

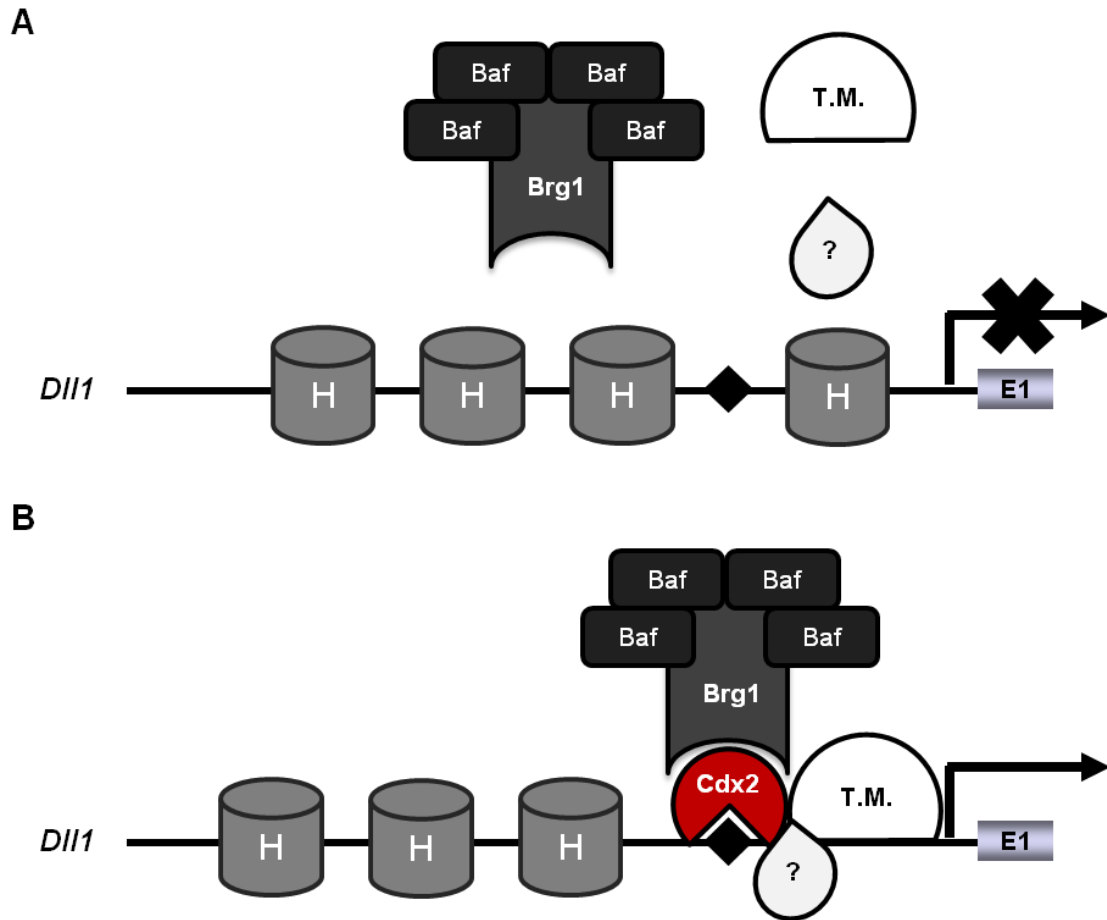


Figure 4.1: Proposed model for Brg1-dependent regulation of Cdx2 target genes

A) In the absence of Cdx2, Brg1 (and the SWI/SNF complex) is not recruited to the promoter region of *Dll1*. Histone (H) distribution prevents the transcriptional machinery (T.M.) and/or unknown factors (?) from accessing the DNA, and the transcription of *Dll1* is inhibited.

B) In the presence of Cdx2, Brg1 (and the SWI/SNF complex) is recruited to the promoter of *Dll1*. Histones are displaced by SWI/SNF remodelling activity, allowing the transcriptional machinery and/or unknown factors to access DNA, and the transcription of *Dll1* is increased.

4.4 Cdx and SWI/SNF - Future Directions

Although it is possible to study the exact amino acid residues within Brg1 that are required for binding Cdx2, it would be technically challenging to identify and delete these regions. Brg1 is a very large protein measuring 190 kilodaltons, and spans 4845 base pairs at the mRNA level. Although the region of Brg1 between amino acids 325 and 526 appeared to interact with Cdx2 in GST-pulldown assays, the possibility remains that other regions within Brg1 also interact with Cdx2. The identification and characterization of these interactions would be laborious, but may prove useful in future studies. For example, if a specific Cdx-interacting domain within Brg1 could be identified and mutated, expression of Cdx target genes could be examined in the context of Brg1 shRNA knockdown/rescue experiments.

Further co-immunoprecipitations were considered to examine the relationship between Brg1 and Cdx2Mut, but confounding issues would limit the usefulness of this assay. In the initial SILAC-MS screen that identified Brg1 as an interactor with Cdx2, several other members of the SWI/SNF complex were also immunoprecipitated (Table 3.2). If GST-pulldown assays were conducted, and Cdx2 directly interacted with another SWI/SNF member (in addition to Brg1), co-immunoprecipitation using Cdx2Mut would likely exhibit indirect interaction with Brg1, even if the principal site of interaction between Cdx2 and Brg1 was disrupted. In addition to these issues, GST-pulldown data must also be considered. If the results of GST-pulldowns are considered an accurate representation of Cdx2-Brg1 interactions *in vivo*, it appears that two unique motifs contribute to contact points between the two proteins (Figure 3.15). Further studies

may identify all of the residues that account for Cdx2-Brg1 interactions, but one of these sites appears to exist in the homeodomain of Cdx2, which is required for DNA binding. Mutating this region would confound our ability to assess functional implications, since alteration of the Cdx2 homeodomain would impact the binding of Cdx2 to CDREs.

To further examine the co-occupancy of target gene loci, an important experiment will be the examination of Brg1 and Cdx2 ChIPs in *Cdx1^{-/-}Cdx2^{-/-}* embryos, and comparing these results to wild type controls. If Cdx2 acts to recruit Brg1 to Cdx target genes, Brg1 interaction should be lost in the *Cdx1^{-/-}Cdx2^{-/-}* background. Consistent with this, preliminary ChIP analyses comparing wild type and *Cdx1^{-/-}Cdx2^{-/-}* embryos indicate that Cdx2 localization at the promoters of *Scf* and *Cdx1* is disrupted in *Cdx1^{-/-}Cdx2^{-/-}* mutants.

To better understand the relationship between Cdx and Brg1, complementation assays could be employed. Preliminary data suggests that all Cdx members interact with Brg1; therefore the intercross of *Brg1^{+/-}* mice with single or compound Cdx mutants would allow for the characterization of the relationship between Brg1 and each Cdx member. These complementation studies would indicate that Cdx proteins and Brg1 work within the same genetic pathways, assuming the phenotypes of Cdx mutant embryos are exacerbated by Brg1 heterozygosity. In support of this, preliminary data suggests that Cdx2 and Brg1 converge on common hematopoietic target genes; namely *Scf* and *Lmo2*. Study of the conditional knockout of *Brg1* in hematopoietic and vascular lineages, as described for *Scf*, would shed further light on the roles that Brg1 and Cdx members play in the development of these niches.

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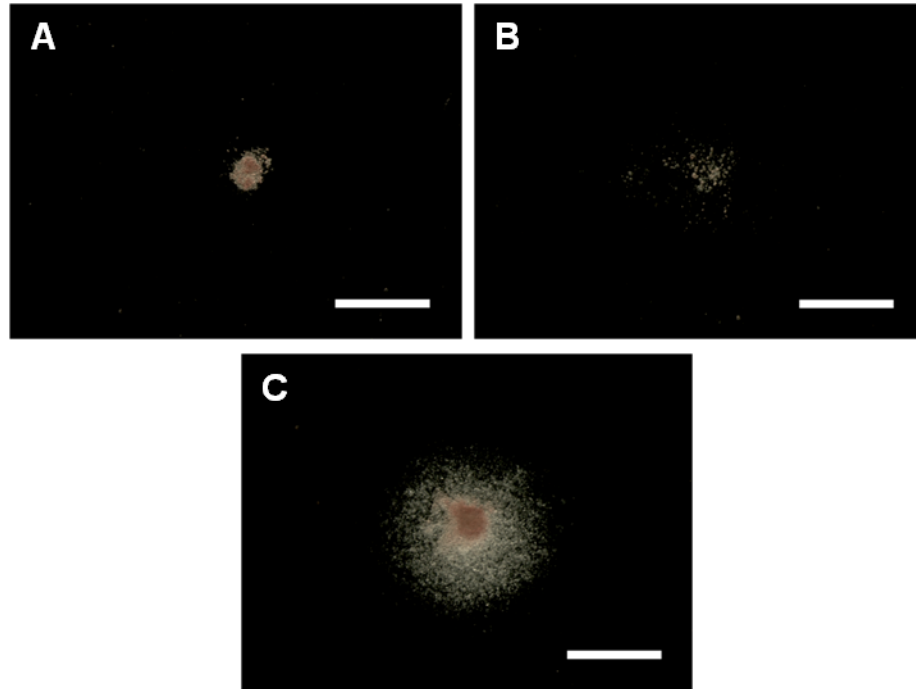
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Appendices



Appendix 1: Hematopoietic colony forming assay qualification.

Single cell suspensions from yolk sacs give rise to three distinct blood progenitor colony types when grown in MethoCult media. (A) Burst-Forming Unit-Erythroid (BFU-E), (B) Colony-Forming Unit-Granulocyte, Macrophage (CFU-GM), (C) Colony-Forming Unit-Granulocyte, Erythroid, Macrophage, Megakaryocyte (CFU-GEMM). Images were acquired using a Leica DFC320 camera, and adjusted using Adobe Photoshop CS4. Scale bars represent 100 μm .

Gene	Forward (5'-3')	Reverse (5'-3')
<i>Cdx2</i>	GTCGATACATCACCATCAGGAGG	TTTCCTCTCCTTGGCTCTGCGG
<i>Elf1</i>	CCCAGAGGAAGCAACCATAGCCA	CCACTACTGGAACCTGGGTTGAAG
<i>Flk1</i>	GGGATGGTCCTTGATCAGAAGAG	CAGACTCCCTGCTTTTACTGGGC
<i>GAPDH</i>	ATCTGACGTGCCGCCTGGAGAA	GTGTAGCCCAAGATGCCCTTCAG
<i>Gata1</i>	AACACTCTGGCGGAGGAATGCCA	TTTCCTCATGGTCAGTGGGCGGT
<i>HbaX</i>	GCGTGTGGATCCGGTCAACTTCA	TTGTCCCAGGCTTCGTGGACCTCA
<i>HbbY</i>	GGAAAAGACCCTCATCAATGGCC	TGGGTCCATGGGTACACAACAAG
<i>Lmo2</i>	GCTGGGACGGAATTGTGCAGG	CCCGCATCGTCATCTCATAGGC
<i>Lyl1</i>	GCGCAGACCAAGCCATAGTGAG	GCGAAGGCGCCGTTAACGTGCT
<i>Meis1</i>	GTCAGCAGCACATGGGCATCAGA	ACTCGGCTGTCCCATACTCACAC
<i>Pu.1</i>	TTGGAGCCTGGGCCAGGTCTT	TTCATGTCGCCGCTGCGCAGCA
<i>Runx1</i>	GCCTCTCTGCAGAACTTCCAGTC	TAGTGCATGCGCGGGTCGGAGAT
<i>Scl</i>	GCCCTCCCCATATGAGATGGAGA	TTCACATTCTGCTGCCGCCATCG

Appendix 2: Oligonucleotides used for murine RT-PCR and qPCR analyses

Gene	Forward (5'-3')	Reverse (5'-3')
<i>Brachyury</i>	TTCAAAGCGTGC GCGGCCCAAT	TCCACTTGAACTCCCCAAGGCTC
<i>Cdx1</i>	GGTAGGTACACAATGCAACTCGGTG	GGGGGTTCCGTCTGTAAGGTAG
<i>Dll1</i>	TTGGCTAGGCCAACATTCAGGC	TTCTCCTCCTCATCCTCCTGT
<i>Lmo2</i>	CCAAAGGCACTGCTAATGACTTGTCGTTTGG	GGTTAGGTTGTGCTGATAGGGAGCCAG
<i>Lyl1</i>	GACGTTTGTTTCGTTGGCATTGACCTCTGC	GCCTTTTTCTGCGTTGACGAGGA
<i>Meis1</i>	CCGCGTTGAGTTTCGTTCTCCAG	GGCACTAACACCACACCCAG
<i>MyoD</i>	GAATCACATGTAATCCACTGGA	ACGCCAACTGCTGGGTGCCA
<i>Scl</i>	GGCCCAGGTGAAAGGAAATCAC	TCATCAGTTGTGGGGGCACATG
<i>Wnt3a</i>	GAGAAGCTGGAGCACATCCAACTTGTACC	CCCTGGATCTCAAGCTCCAAGCACC
<i>Wnt3a Exon 4</i>	GCCATCGCCAGTCACATGCACCTCAAGTGC	CTACTTGCAGGTGTGCACGTCATAGACACG

Appendix 3: Oligonucleotides used to in murine ChIP analyses

Construct	Forward (5'-3')	Reverse (5'-3')	F Cut Site	R Cut Site
Cdx2 Full Length	GGATCCATGTACGTGAGCTACCTT	TCAGAATTCTCACTGGGTGACAGTGGA	BamHI	EcoRI
Cdx2 185-312	GGATCCAAAGACAAATACCGGGTGGTG	TCAGAATTCTCACTGGGTGACAGTGGA	BamHI	EcoRI
Cdx2 185-244	GGATCCAAAGACAAATACCGGGTGGTG	GAATTCCTTGATTTCTCCTTGG	BamHI	EcoRI
Cdx2 243-312	GGATCCATCAAGAAGAAGCAGCAGC	TCAGAATTCTCACTGGGTGACAGTGGA	BamHI	EcoRI
Cdx2 N180	GGATCCATGTACGTGAGCTACCTT	GAATTCCTTGGCTTCTAGGGACTG	BamHI	EcoRI
Cdx2 N119	GGATCCATGTACGTGAGCTACCTT	GAATTCATGCGGGTGATGGTGCGCG	BamHI	EcoRI
Cdx2 60-80 F	GATCCCAGGGCCATCCTGGCCACCGCGTACGGCGCCCTCTCCGCGAGGACTGGAATGGCTACG		BamHI	
Cdx2 60-80 R	AATTCGTAGCCATTCCAGTCTCGCGAGAGGGGCGCCGTACGCGTGGGCCAGGATGGCCCTGGG			EcoRI
Cdx2 N60	GGATCCATGTACGTGAGCTACCTT	GAATTCGGGGACTGAGCGCTGTCC	BamHI	EcoRI
Cdx2 x60-80	TTGGACAGCGCTCAG TACGCACCCGGG	CCCGGGTGCGTA CTGAGCGCTGTCAA		
Cdx2Mut F	ACCGCGTACGGCGCCGGCGTCGGCGCGGCCGGAGCCGGGTACGCACCCGGG			
Cdx2Mut R	CCCGGGTGCGTAGCCGGCTCCGGCCGCGCCGACGCCGGCGCCGTACGCGGT			
Brg1-B2	GGATCCATGCCACCACAAACAGTCCCC	GCGGCCGCTCGCTCCTTCTCAATGCGC	BamHI	NotI

Appendix 4: Oligonucleotides used to generate GST-fusion constructs

Locus	Forward (5'-3')	Reverse (5'-3')
<i>Scl</i> CDRE 1	CTTCAACATTTATGACTGTGG	CCACAGTCATAAATGTTGAAG
<i>Scl</i> CDRE 2	GCAATGTGATTTATAAAGCCTA	TAGGCTTTATAAATCACATTGC
<i>Scl</i> CDRE 3	GGCTGCATTATAAGCATGTGC	GCACATGCTTATAAATGCAGCC

Appendix 5: Oligonucleotides used to generate EMSA probes

Locus	Forward (5'-3')	Reverse (5'-3')	F Cut Site	R Cut Site
<i>Scf proximal</i>	GCGGCCGCGATGCCCTCTCCTAGTGTGTCTGAAGG	AGATCTCTGTAAACAGGGTAAGGGGC	NotI	BglII
<i>Scf distal</i>	CTCGAGGCCTGAATTGCAATCCTTGG	GCGGCCGCGATCCCATTACAGATGGTTCTGAGCC	XhoI	NotI
<i>Meis1</i>	AAGCTTCCGCAAGGTCTGGCTGCTTGGTG	GGTACCCGGCCTCTCTGGCTCCCTCCTACT	HindIII	KpnI

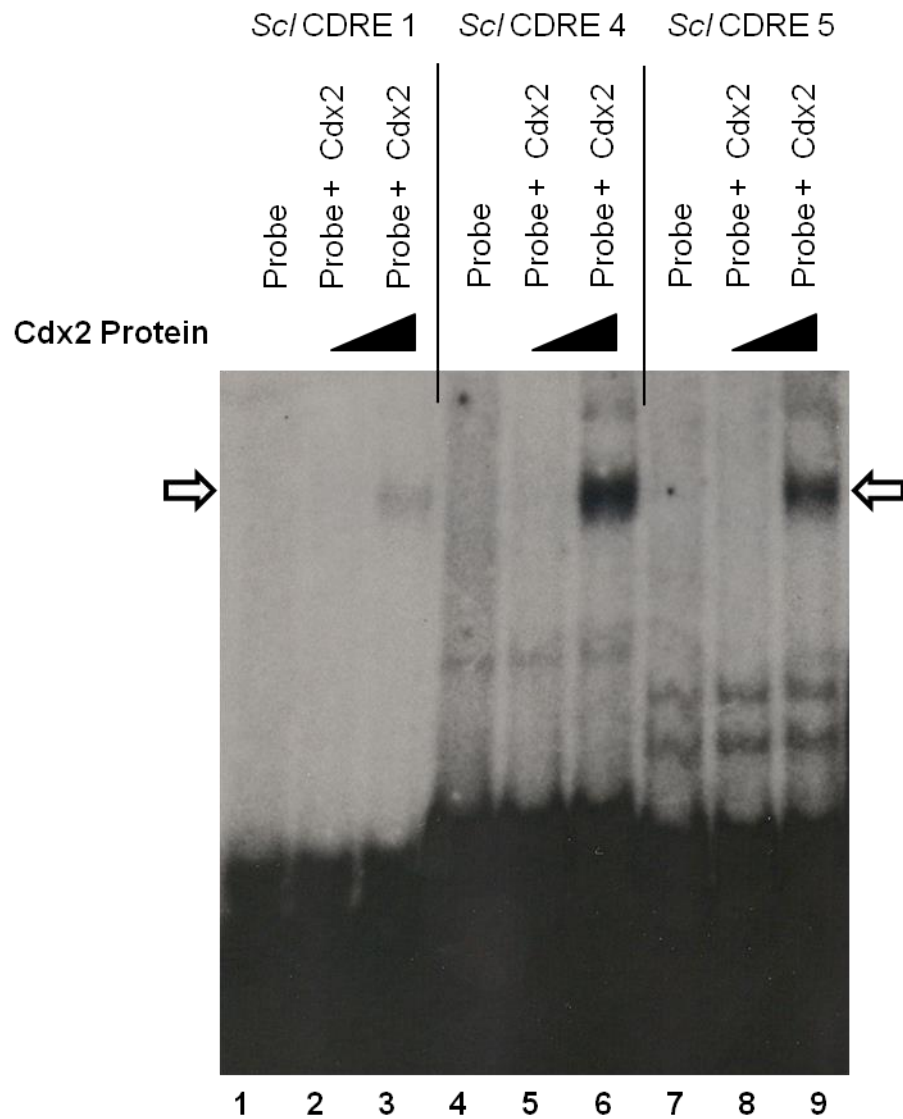
Appendix 6: Oligonucleotides used to generate luciferase constructs

Locus	Annotation	Sequence
<i>BRG1</i>	TRCN0000015552	AAATGATCACAGTGTCTGCCG
<i>CDX2</i>	TRCN0000013685	AAACCCAGGGCTGCCTTGGAAAAG

Appendix 7: Mature shRNA antisense sequences against BRG1 and CDX2

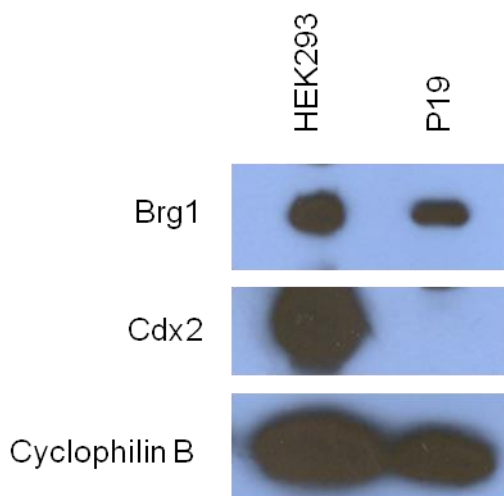
Gene	Forward (5'-3')	Reverse (5'-3')
<i>ACTIN</i>	GCGGGAAATCGTGCGTGACATT	GATGGAGTTGAAGGTAGTTTCGTG
<i>BRG1</i>	GTGCCAGAACGCTCAGACGTTCA	CTCAATCTTCTGCCGTACGCTGG
<i>CDX2</i>	GACAGAGCCAGGCACTGAGGC	CGGAAGCCGGCGCAGCAGTCCC
<i>DLL1</i>	CAAGCGTGACACCAAGTGCCAG	GAAGTTGAACAGCCCGAGTCCG
<i>LEF1</i>	GAGAGCGAATGTCGTTGCTGAGTG	GGCAGCTGTCATTCTTGACCTG
<i>TBX6</i>	CTGCACCCTGCGGCTTCCATG	GGAGAAAGTGCGGGGCAAAGGG
<i>TNFα</i>	TAGATGGGCTCATACCAGGG	CCGTCTCCTACCAGACCAAG
<i>WNT3A</i>	GAACCGCCACAACAACGAGGC	CATGTCTTCACCTCGCAGCTGC

Appendix 8: Oligonucleotides used for human RT-PCR analyses



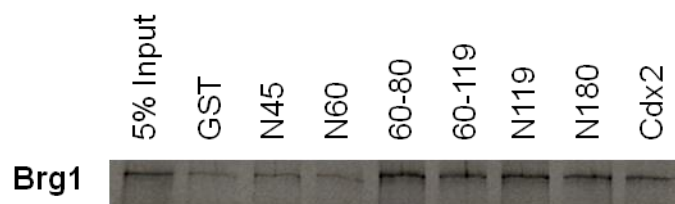
Appendix 9: Examination of Cdx2 binding to the *Scf* promoter *in vitro*.

Cdx2 binding at three putative CDREs within the *Scf* promoter was assessed via EMSA. Oligonucleotide probes corresponding to CDREs 1, 4, and 5, outlined in Figure 3.12 A were incubated with two concentrations of Cdx2 protein (lanes 2, 5, 8 versus lanes 3, 6, 9), or no protein (lanes 1, 4, 7). Arrows indicate binding.



Appendix 10: Expression of Brg1 and Cdx2 proteins in HEK293 and P19 cells.

Brg1 and Cdx2 expression was assessed in HEK293 or P19 cells by Western blot. Cyclophilin B was used as a loading control.



Appendix 11: Analysis of direct interaction between Cdx2 and Brg1 *in vitro*.

GST pulldown assays were used to identify the amino-acid regions required for interaction between Cdx2 and Brg1. Cdx2 sequences were cloned into a GST-fusion vector and proteins bound to glutathione-agarose beads. Full length Brg1 was radiolabelled with ^{35}S -methionine, incubated with beads, and interacting protein visualized by autoradiography following PAGE. GST was used as a negative control.