CDX2 regulates gene expression through recruitment of BRG1-associated SWI/SNF chromatin remodeling activity.

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AUTHORIZATION

Figure 1.1 (Lohnes 2003). Reproduced with permission from the BioEssays publishing group

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Figure 1.7. (Trotter and Archer 2008) Figure and legends were adapted with permission from Nuclear Receptor Signalling

Figure 1.4. Adapted from Dr. Joanne Savory and Travis Brooke (unpublished data)

Figure 1.5. Adapted from Dr. Joanne Savory (unpublished data)

Figure 3.5. (A) Adapted from Tanya Foley (unpublished data)

Figure 4.1 Adapted and modified from Travis Brooke-Bisschop
ABSTRACT

The packaging of genomic DNA into nucleosomes creates a barrier to transcription which can be relieved through ATP-dependent chromatin remodeling via complexes such as the switch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complex. The SWI/SNF complex remodels chromatin via conformational or positional changes of nucleosomes, thereby altering the access of transcriptional machinery to target genes. The SWI/SNF complex does not possess intrinsic DNA binding ability, and therefore its recruitment to target loci requires interaction with DNA-associated transcription factors. The Cdx family of homeodomain transcription factors (Cdx1, Cdx2 and Cdx4) are essential for a number of developmental programs in the mouse. Cdx1 and Cdx2 also regulate intestinal homeostasis throughout life. Although a number of Cdx target genes have been identified, the basis by which Cdx members impact their transcription is poorly understood. We have found that Cdx members interact with the SWI/SNF complex and make direct contact with Brg1, a catalytic member of SWI/SNF. Both Cdx2 and Brg1 co-occupy a number of Cdx target genes, and both factors are necessary for transcriptional regulation of such targets. Finally, Cdx2 and Brg1 occupancy occurs coincident with chromatin remodeling at certain of these loci. Taken together, our findings suggest that Cdx transcription factors regulate target gene expression, in part, through recruitment of Brg1-associated SWI/SNF chromatin remodeling activity.
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ABBREVIATIONS

αMEM  Alpha Modification of Eagle’s Medium
ADP  Adenosine diphosphate
AP  Anterior-posterior
ATP  Adenosine triphosphate
APC  Adenomatous Polyposis Coli
Axin2  Axin-related protein 2
βgal  β-galactosidase
BAF  Brg1-associated factor
Brg1  Brahma-related gene 1
Brm  Brahma
BSA  Bovine serum albumin
cDNA  Complementary DNA
CDRE  Cdx response element
Cdx  Caudal-related homeobox protein
ChIP  Chromatin immunoprecipitation
ChIP-seq  Chromatin immunoprecipitation combined with deep sequencing
CO₂  Carbon dioxide
CPRG  Chlorophenol Red- β-D-Galactopyranoside
Cre  Causes recombination enzyme
CRISPR  Clustered regularly interspaced short palindromic repeats
Cyp26a1  Cytochrome P450 Family 26 Subfamily A Member 1
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>Cyp3a4</td>
<td>Cytochrome P450 3A4</td>
</tr>
<tr>
<td>DAB</td>
<td>Diamino-benzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DBS</td>
<td>Donor bovine serum</td>
</tr>
<tr>
<td>DepC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>Dll1</td>
<td>Delta-like ligand 1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Elf1</td>
<td>E74-like factor 1</td>
</tr>
<tr>
<td>EMP</td>
<td>Erythroid-myeloid progenitor</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ER^T</td>
<td>Estrogen receptor, responsive to tamoxifen</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>Flt1</td>
<td>FMS related tyrosine kinase 1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>H3K4Me3</td>
<td>Histone 3 lysine 4 trimethylation</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293 cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
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<td>--------------</td>
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<tr>
<td>HEPES</td>
<td>Hydroxyethyl piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HI-DBS</td>
<td>Heat-inactivated donor bovine serum</td>
</tr>
<tr>
<td>HI-FBS</td>
<td>Heat-inactivated fetal bovine serum</td>
</tr>
<tr>
<td>Hox</td>
<td>Homeobox gene</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>Lef1</td>
<td>Lymphoid enhancer binding factor 1</td>
</tr>
<tr>
<td>LiCl</td>
<td>Lithium chloride</td>
</tr>
<tr>
<td>Meis1</td>
<td>Myeloid ecotropic viral integration site 1</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrophotometry</td>
</tr>
<tr>
<td>MUC2</td>
<td>Mucin Glycoprotein 2</td>
</tr>
<tr>
<td>MyoD</td>
<td>Myogenin D</td>
</tr>
<tr>
<td>P19</td>
<td>Murine embryonic carcinoma cell line</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Pbx</td>
<td>Pre-B-cell leukemia homeobox</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>REAA</td>
<td>Restriction enzyme accessibility assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse-transcriptase polymerase chain reaction</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>ScI</td>
<td>Stem cell leukemia</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>SILAC</td>
<td>Stable isotope labelling with amino acids in cell culture</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>Switch/sucrose-non-fermentable</td>
</tr>
<tr>
<td>Tam</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris, Boric Acid, EDTA buffer</td>
</tr>
<tr>
<td>TCF4</td>
<td>Transcription Factor 4</td>
</tr>
<tr>
<td>TE</td>
<td>Tris/EDTA</td>
</tr>
<tr>
<td>TESS</td>
<td>Transcription element search software</td>
</tr>
<tr>
<td>TFSearch</td>
<td>Transcription factor search software</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcriptional start site</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-related</td>
</tr>
<tr>
<td>Wnt3a</td>
<td>Wnt Family Member 3A</td>
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</table>
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CHAPTER 1

INTRODUCTION
The Cdx Family of Transcription Factors

Homeodomain proteins are DNA binding transcription factors that play critical roles in embryonic development as well as various processes in the adult. The homeobox gene caudal (cad) was originally identified in Drosophila, and encodes a 427 amino acid homeodomain transcription factor that binds DNA through an evolutionarily conserved $\alpha$-helix domain (Levine et al. 1985). Cad is required for the specification of the posterior embryo and AP patterning, as well as the patterning of the hindgut and the genital imaginal disk. 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 (Houle, Allan, and Lohnes 2003). The three members of this family are co-expressed in the caudal embryo commencing at mid-gastrulation stages and have been shown to play many important roles in placentation, axis elongation and hematopoiesis (Y. Wang et al. 2008).

The expression patterns of murine Cdx members vary both spatially and temporally during development (Fig 1.1). Cdx1 transcripts are first observed at embryonic day 7.5 (E7.5) in ectodermal and mesodermal cells of the primitive streak, extend to the middle of the prospective hindbrain by E9.5, and persist in the somites, forelimb buds and tail bud until E11.5 (Meyer and Gruss 1993) (Fig 1.1). Cdx2 expression begins at E3.5 in the trophectoderm (Chawengsaksophak et al. 1997) From day 8.5, Cdx2 transcripts are detected in embryonic tissues, including the tail bud and the caudal part of the neural tube (F Beck et al. 1995) (Fig 1.1). At later developmental stages, both Cdx1 and Cdx2 are expressed in the definitive endoderm and subsequently in the intestinal epithelium throughout life (F Beck et al. 1995; Guo, Suh, and Lynch 2004).
The expression of Cdx1 is confined to the crypt cells (Grainger, Hryniuk, and Lohnes 2013), whereas Cdx2 is also expressed in the differentiated progeny that derive from these cells (Houle, Allan, and Lohnes 2003). Furthermore, Cdx2 is more abundant in the rostral colon, while Cdx1 is higher in the caudal end (Fig 1.2). Cdx1 expression is graded along the crypt-villus axis, with higher expression in the crypts. Cdx2 is, however, uniformly expressed along this axis and differentially phosphorylated (F Beck et al. 1995). In addition, Cdx2 has been proposed to have a role in regulating the level of glucagon and insulin in the adult animal (Zhao et al. 2005). 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 (Houle, Allan, and Lohnes 2003). Overall, these observations illustrate that there is an overlapping and dynamic pattern of expression of Cdx members confined largely to the posterior embryo at post-implantation stages and in the adult intestinal epithelium.
Figure 1.1 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).
Figure 1.2. Conserved domains between cdx proteins and cdx expression along the anterior-posterior crypt-villus axis in the small intestine and colon, respectively. Note that Cdx2 expression is unchanged and the gray triangle represents the differential phosphorylation. The hatched areas represent the proliferating zones in the crypts. (Figure and legend adapted with permission from Taylor and Francis) (Guo, Suh, and Lynch 2004).
Cdx proteins possess three highly conserved motifs: an N-terminal signal region necessary for protein processing (Trinh, Jin, and Drucker 1999), a homeodomain DNA binding motif, and a “hexapeptide” sequence N-terminal to the homeodomain (Trinh, Jin, and Drucker 1999). Furthermore, Cdx proteins share extensive homology in these three domains; however, there is little conservation between their remaining sequences (Lohnes 2003).

A Cdx response element (CDRE) has been identified in mice and Drosophila and is comprised of the consensus sequence TTTATG. This sequence is recognized by all Cdx members (Dearolf, Topol, and Parker 1989). Although a consensus CDRE has been demonstrated, and a number of target genes have been identified, the molecular basis by which Cdx proteins regulate transcription remains unclear.

**Functional specificity and redundancy of Cdx members**

Cad is important for the proper development of posterior structures of Drosophila embryos. This function appears to have been conserved in at least two of the mouse Cdx gene products. Cdx1 null mice are viable and fertile. However, these mice exhibit homeotic transformations of the cervical vertebrae due to disruptions in vertebral patterning (Subramanian, Meyer, and Gruss 1995). Certain of these defects are also observed in heterozygotes, however at a lower frequency (Subramanian, Meyer, and Gruss 1995). These vertebral transformations are highly penetrant in the cervical region and to a lesser extend the anterior thoracic region. 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 regulators of Hox gene expression. Hox genes are
expressed from 3’ to 5’, with the earliest genes expression in the primitive streak at pre-somite stages, and more 5’ genes expressed in the posterior part at later time points (Deschamps and van Nes 2005). This is indicative of the spatio-temporal features of Hox gene expression in patterning of the embryonic axis. In the mouse, Cdx1 and Cdx2 are expressed early in a Hox-like manner in the three germ layers (Mallo, Wellik, and Deschamps 2010). Mutations in Cdx1 and Cdx2 result in posterior shift of Hox gene expression (Subramanian, Meyer, and Gruss 1995) which is exaggerated in double mutants (van den Akker et al. 2002). The finding of Cdx response elements in the promoter regions several Hox loci further supports the role of Cdx members in regulating Hox gene expression (F Beck et al. 1995; Subramanian, Meyer, and Gruss 1995). Taken together, these observations suggest that Cdx proteins affect AP patterning at least in part by regulating the Hox genes.

Cdx2 homozygous null embryos fail to implant and do not survive past E3.5, which likely due to a defect in trophectoderm (Chawengsaksophak et al. 1997). Cdx2 heterozygotes are viable and exhibit homeotic transformations exhibiting homeotic vertebral transformations of posterior cervical and upper thoracic vertebrae similar to Cdx1−/− mutants (Chawengsaksophak et al. 1997). This is a reflection of the relative patterns of expression of Cdx1 and Cdx2 during development.

To circumvent the early embryonic lethality of Cdx2 mutants, tetraploid aggregation was employed to study the impact of Cdx2 loss in the embryo proper (Chawengsaksophak et al. 2004). The tetraploid complementation assay is a technique in which cells of two mammalian embryos are combined to form a new embryo (Tam and Rossant 2003). The assay starts with producing a tetraploid cell by fusing an embryo at
two-cell stage. This results in tetraploid cell, which can develop normally until the blastocyst stage, but are then restricted to form the extra-embryonic tissue. These tetraploid cells can be combined with normal diploid embryonic stem cells. The embryo, therefore, will develop exclusively from the diploid ES cells, while the extra-embryonic tissues are exclusively derived from the tetraploid cells (Tam and Rossant 2003). Cdx2 mutant embryo derived from this assay exhibit developmental defects such as impaired development of the allantois, abnormal vasculature in the yolk sac, truncation of the embryo at the level of the forelimb bud, and distension of the pericardium (Chawengsaksophak et al. 1997). Furthermore, novel non-Hox Cdx target genes, including Brachyury, Wnt3a and Cyp26a1, were identified (Savory, Bouchard, et al. 2009).

Several lines of evidence suggest that Cdx4 also has a role in AP patterning and posterior specification. This is supported by studies on the function of Xcad3, the Xenopus homologue of Cdx4 (Faas and Isaacs 2009). Studies have shown that altered Xcad3 function leads to profound defects in the developing animal. For instance, ectopic expression of an active form of Xcad3 inhibits anterior development, and suppression of Xcad3 leads to a dramatic disruption of posterior development (Faas and Isaacs 2009). In the mouse, however, Cdx4 null mutants display no overt phenotypic change suggesting that Cdx1 and Cdx2 may exhibit functional overlap with Cdx4 (van Nes et al. 2006). In this regard, functional overlap has been assessed by studying Cdx compound mutants. Cdx1+/−Cdx2+/− and Cdx1−/−Cdx2+/− mutants display progressively more severe vertebral defects and axial truncation (van den Akker et al. 2002). Cdx2+/−Cdx4−/− mutants exhibit diminished presomitic mesoderm tissue, impaired chorio-allantoic fusion, and placental
disruption, resulting in embryonic lethality at approximately E10.5 (van Nes et al. 2006). Taken together, these observations indicate overlapping roles for Cdx members in mesodermal tissues.

Standard genetic mouse models give a limited view of Cdx function as Cdx2 null embryos die between E3.5 and 5.5 due to failure in implantation (Chawengsaksophak et al. 1997). To circumvent these issues, we have generated a conditional Cdx2 knockout line using the Cre/loxP system in which Cdx2 exon two is flanked by loxP sites in order to generate conditional Cdx2 null allele (Savory, Bouchard, et al. 2009) (Fig 1.3). Loss of this domain is predicted to result in a functionally null protein because exon 2 contains most of the DNA binding domain. The Cre recombinase used for these studies is fused to a modified estrogen receptor ligand-binding domain that responds exclusively to tamoxifen (ER<sup>T</sup>), and is sequestered in the cytoplasm by Hsp90 in the absence of ligand. Tamoxifen administration results in the release of the Hsp90 protein which subsequently allows the Cre- ER<sup>T</sup> fusion protein to translocate into the nucleus and excise the Cdx2 sequence between the loxP sites. Our group used a CreER<sup>T</sup> transgene driven by the ubiquitously expressed β-Actin promoter, thereby targeting all cells of the animal, as well as a CreER<sup>T</sup> transgene driven by the Villin promoter, targeting only cells in the intestinal epithelium (Savory, Bouchard, et al. 2009; Savory, Mansfield, Rijli, et al. 2011; Grainger, Savory, and Lohnes 2010). By crossing the Cdx2 conditional allele with the Cdx<sup>1<sup>−/−</sup></sup> line we also generated Cdx<sup>1<sup>−/−</sup>Cdx2<sup>−/−</sup></sup> embryos (Savory, Mansfield, Rijli, et al. 2011) (Fig 1.3). These mutants lack Cdx4 expression, consistent with the finding that Cdx4 is a direct Cdx target gene (Savory, Mansfield, St. Louis, et al. 2011), and therefore effectively create a Cdx null model.
Cdx1<sup>+/−</sup>Cdx2<sup>+/−</sup> embryos exhibit numerous abnormalities including chorio-allantoic fusion defects, distended pericardium, and axial truncation caudal to the presumptive forelimb bud, along with novel defects including improper closure of the neural tube (Savory, Mansfield, Rijli, et al. 2011). Taken together, these new mutants provide a powerful tool to explore previously uninvestigated roles of Cdx.

Figure 1.3. Generation of conditional Cdx1<sup>+/−</sup>Cdx2<sup>+/−</sup> mutants. A Cdx1<sup>+/−</sup>Cdx2<sup>0/0</sup> Actin Cre-ER<sup>T</sup> male is bred with a Cdx1<sup>+/−</sup>Cdx2<sup>0/0</sup> female to produce Cdx1<sup>+/−</sup>Cdx2<sup>0/0</sup> 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<sup>T</sup>) 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.

**Cdx in the intestine**

The intestinal epithelium is comprised of a single layer of cells that is constantly shed into the lumen, necessitating renewal every four to five days (Yuasa 2003). Cell division driving this renewal is confined to the intestinal crypts, where stem cells both self-renew and provide a population of transiently amplifying (TA) cells. Following several cycles of division, these TA cells subsequently undergo differentiation into the four different intestinal cell types; enterocytes, globlet, and enteroendocrine cells migrate to the luminal surface while Paneth cells, which are produced only in the small intestine, move to the base of the crypts (Yuasa 2003).

Proliferation and differentiation of the intestinal epithelium occurs in an organized manner (Boyd et al. 2010). Several different signaling involved in this process have been identified, including the canonical Wnt pathway. In this pathway, Wnt ligand binds to a membrane Frizzled receptor resulting in inactivation of an APC (Adenomatous polyposis coli)-containing “destructor” complex (Polakis 1999). This leads to stabilization of cytoplasmic β-catenin which subsequently diffuses to the nucleus where it binds to LEF/TCF transcription factors, resulting in increased transcription of Wnt target genes (Polakis 1999). Cdx2 has also been shown to be essential for intestinal differentiation and plays an important role in regulating gene expression in the fetal and adult intestine (Chawengsaksophak et al. 1997). Dysregulation of these processes may lead to many diseases, such as colorectal cancer. Interestingly, work from our group suggests
interaction between Wnt and Cdx pathways, as a number of Wnt target genes and effectors appear to be regulated by Cdx members (Savory, Bouchard, et al. 2009).

Colorectal cancer (CRC) is the third most deadly cancer, resulting in more than 600,000 deaths a year (Siegel, Naishadham, and Jemal 2013; Ferlay et al. 2010). The relevance of Wnt signaling in CRC was discovered over two decades ago with the finding that inactivating mutation of APC is the predominant cause of hereditary (familial adenomatous polyposis, FAP) and sporadic cases of CRC (Gregorieff and Clevers 2005). This loss of APC function results in aberrant activation of the Wnt signaling pathway, leading to hyperproliferation and outgrowth of intestinal crypt cells resulting in the intestinal polyps which are at risk to progress to carcinoma over time (Polakis 2007). Modeling CRC in the mouse showed that mutant mice, such as the multiple intestinal neoplasia (APC\textsuperscript{Min/+}) model, which have a comparable inactivating APC mutation, also exhibit polyps similar to FAP patients (Karim and Huso 2013; Hryniuk et al. 2014). However, polyps in such APC\textsuperscript{Min/+} models are restricted largely to the small intestine and proximal colon, unlike in humans where tumours develop primarily in the distal large intestine and rectum (Hryniuk et al. 2014). In addition, APC\textsuperscript{Min/+} mutant polyps do not typically progress to carcinoma (Halberg et al. 2000).

Several lines of evidence have implicated Cdx members as potential contributors to the CRC phenotype. For instance, loss of Cdx2 is seen in approximately 10-30% of human CRC and correlates with a more aggressive tumor phenotype (Bae et al. 2015). Using a recently generated Cdx2 conditional allele, our group showed that somatic loss of Cdx2 (alone or in a Cdx1 mutant background) increased the frequency and altered the distribution of polyps in a mouse APC\textsuperscript{Min/+} mutant model (Hryniuk et al. 2014).
In addition, Cdx2 deletion leads to a more aggressive tumor phenotype in this model (Hryniuk et al. 2014).

Cdx1 and Cdx2 are differentially expressed in the intestinal epithelium (Fig 1.2). Cdx1 expression is highest in the distal colon, whereas the expression of Cdx2 is maximal in the cecum. Cdx1 appears to be dispensable for the development of the small intestine (Grainger, Savory, and Lohnes 2010). Together with Cdx2, however, Cdx1 may play a role in specification of the colon (Gao, White, and Kaestner 2009). Initial studies have shown that Cdx2 heterozygous mice survive and have metaplastic lesions in the pericecal region and proximal colon (Chawengsaksophak et al. 1997; Felix Beck and Stringer 2010). These lesions exhibit properties of more anterior gastrointestinal tissue (Chawengsaksophak et al. 1997; Felix Beck and Stringer 2010). This suggests that Cdx2 is required for the differentiation of the posterior intestinal epithelium. There is, however, a lack of a gut phenotype in Cdx1 null mutants. This is perhaps explained by the overlapping function of Cdx members in developmental programs such as vertebral patterning (van den Akker et al. 2002), axial elongation (Chawengsaksophak et al. 2004) and neural tube closure (Savory, Mansfield, Rijli, et al. 2011).

To better understand the role of Cdx in the adult intestine, our group used a conditional mutagenesis strategy to delete Cdx2 alone or in a Cdx1 null background in the intestinal epithelium (Grainger, Savory, and Lohnes 2010). Cdx2 deficient mice die within seven days, likely due to intestinal dysfunction. We showed that villus morphology and proliferation, as well as the expression of a number of intestinal makers, were all impacted following the ablation of Cdx2 (Hryniuk et al. 2012). These
observations suggest that Cdx2 plays an important role in the development and homeostasis of the gastrointestinal tract.

**Cdx proteins interact with SWI/SNF complex**

Little is known regarding the mechanisms by which Cdx members regulate transcription, but it is believed that they serve to recruit co-regulators to target genes, and it is the biochemical activity of such co-regulators that impacts transcription of targets. To this end, we used stable isotope labeling 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 expresses 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. This analysis detected significant enrichment of six members of the SWI/SNF chromatin remodeling complex with CDX2, including BRG1 (BAF190), BAF53, BAF57, BAF155, BAF170 and BAF205 (Fig 1.4 A)

BRG1, the ATPase subunit of SWI/SNF, has been shown to interact with a number of transcription factors (Takeuchi et al. 2011; Barker et al. 2001; Lee et al. 2002), suggesting it may serve as an interface between the SWI/SNF complex and CDX2. Co-precipitation of BRG1 with CDX2 was validated by Western blot analysis using BRG1-specific antibodies (Fig 1.4 B). Moreover, both CDX1 and CDX2 associated with BRG1 in E9.5 mouse embryos (Fig 1.4 D).
Previous studies showed that a region of BRG1 between amino acids 325 and 611, termed BRG1-B2, interact with a number of transcription factors (K. Wang et al. 2010; Mudhasani and Fontes 2005), suggesting that it may serve as an interface with CDX2. Consistent with this, our group found interaction between FLAG epitope-tagged BRG1-B2 and endogenous CDX2 in immunoprecipitation assays from HEK293 cells (Fig 1.4 C).

**Figure 1.4. CDX2 interacts with the SWI/SNF complex** (A) 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. (B) Co-immunoprecipitation of CDX2 and BRG1 from HEK293 cells (upper panel). IgG was used as a negative control. (C) A Flag-BRG1-B2, expressed in HEK293 cells, was immunoprecipitated with anti-CDX2 antibody and interaction assessed by anti-Flag. IgG was used as a negative control. (D) Whole cell lysates from
E9.5 mouse embryos were immunoprecipitated with antibodies against Cdx1, Cdx2 or IgG as a negative control and interaction with Brg1 assessed by Western blot. (Adapted from Dr. Joanne Savory and Travis Brooke, unpublished data)

To further examine the association between BRG1 and CDX2, immunofluorescence analysis was carried out in C2BBe1 cells or mouse small intestine. Endogenous BRG1 and CDX2 proteins were found within similar nuclear foci in C2BB2e1 cells, and both proteins also co-localized in murine enterocytes (Fig 1.5). Taken together, these results are consistent with CDX2 and BRG1 forming a complex in vivo.
Figure 1.5. Co-localization of CDX2 and BRG1. Immunohistochemistry was carried out to detect (A) Immunoflorescence analysis of CDX2 (green), BRG1 (red) and merged image in C2bbe1 cells. (B) Innunoflorescence of CDX2 (green), BRG1 (red) and merged image in mouse small intestine. DAPI staining was used to label nuclei (blue). (Adapted from Dr. Joanne Savory, unpublished data)
The SWI/SNF Complex

The packaging of DNA into nucleosomes creates a barrier to transcription by obstructing the binding of transcriptional regulators to sequences within target promoters or enhancers (Trotter and Archer 2008). Alteration of the chromatin structure by ATP-dependent remodeling complexes to alleviate these constraints is important for transcriptional regulation of many eukaryotic genes. A number of chromatin remodeling complexes have been shown to be important in modulating the arrangement and stability of nucleosomes, including the switch/sucrose non-fermentable (SWI/SNF) chromatin remodeling complex (Vignali et al. 2000).

The SWI/SNF family was originally identified in yeast (Khavari et al. 1993), and has been characterized regarding structure, function and enzymatic activity (Trotter and Archer 2008). The Brg1 homologue, Swi2, was first identified in yeast through genetic screening for proteins involved in mating-type switching (SWI) and sucrose non-fermenting (SNF) (Neigeborn and Carlson 1984). These proteins were later found to be able to assemble into a multi-subunit complex, called SWI/SNF. This complex has the ability to alter nucleosomal structure, in an ATP-dependent manner, resulting in an altered conformation of the chromatin (Aoyagi, Trotter, and Archer 2005; Wu et al. 2014; Vignali et al. 2000). The mammalian SWI/SNF family are ATP-dependent chromatin remodeling complexes (Sif 2004) 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 (Fig 1.6) (Takeuchi et al. 2011; Kadoch and Crabtree 2015). Brg1/Brm, Baf47, Baf155, and Baf170 are considered core members of the SWI/SNF
complex based on in vitro studies demonstrating that the remodeling activity of a complex composed of these particular factors is equal to that of the complete complex (Curtis et al. 2011; Trotter and Archer 2008; Bartlett et al. 2011).

The SWI/SNF complex remodels chromatin structure via conformational or positional changes of nucleosomes, altering access of transcriptional machinery to target genes (Narlikar, Fan, and Kingston 2002). Since the SWI/SNF complex does not possess intrinsic DNA binding, its recruitment to relevant target loci is accomplished via interaction with DNA-bound transcription factors (Narlikar, Fan, and Kingston 2002). Human SWI/SNF contains either BRG1 or hBRM (Brahma) as the central ATPase subunit (Fig 1.6), and it is the former which appears to interact with CDX2. As with Cdx2, several observations suggest that Brg1, also known as SMARCA4 (Holik et al. 2014), has a vital role in development. Brg1−/− embryos die at implantation (S. Bultman et al. 2000), while conditional knock-out of Brg1 in hematopoietic cells results in vascular abnormalities observed in yolk sacs (Han et al. 2008).

The Brg1 and hBrm proteins share a high degree of sequence identity (74%) as well as similar biochemical activities (Khavari et al. 1993). These two ATPase subunits, however, play different roles in various cellular processes including proliferation and differentiation (Scott J. Bultman, Gebuhr, and Magnuson 2005; Holik et al. 2014). Beside the catalytic ATPase domain, Brg1 contains a conserved C-terminal bromodomain, AT-hook motif and a less characterized N-terminal region housing conserved domains such as QLQ, HAS, and BRK (Fig 1.7). The bromodomain of Brg1 plays a role in the recognition of acetylated lysines within histone H3 and H4 tails (Chandrasekaran and Thompson 2007). Furthermore, the N-terminus of Brg1 encompasses several regions that
have been proven essential for protein-protein interactions (Trotter and Archer 2008) (1.7). Brg1 is thus composed of a number of domains that may be utilized in the recognition of modified histone and recruit the chromatin remodeling activity of Brg1 to target loci.

Figure 1.6: Schematic of the SWI/SNF complex. The SWI/SNF family of chromatin-remodelling complexes are composed of multiple subunits, several of which have multiple isoforms (e.g. Brg1-associated factor [BAF] 200A, B). Core members are depicted in boldface font (figure and legend adapted with permission from Ho and Crabtree, 2010).
**Role of Brg1 in transcription**

Brg1 has been found in the context of a number of multi-protein complexes (Peterson and Workman 2000). Using variety of methods to identify protein-protein interactions, many studies have shown that Brg1 interact with transcription factors and histone-modifying enzyme complexes (Khavari et al. 1993; K. Wang et al. 2010; Trotter and Archer 2008).

Brg1-containing SWI/SNF complexes are thought to be recruited to specific targets through association with DNA-binding transcription factors, co-regulators and other members of the transcriptional machinery (Trotter and Archer 2008). Although Brg1 contains distinct DNA binding motifs (Fig 1.7), which are believed to have a role in targeting SWI/SNF activity to specific promoters, these motifs do not direct the complex to specific genomic sequences (Trotter and Archer 2008). They instead work in concert with transcription factor activation domains to allow binding and chromatin remodeling (Peterson and Workman 2000).

Numerous studies suggest ATP-dependent chromatin remodeling by Brg1 influences the activity of a variety of transcription factors. For instance, Brg1 was shown to physically interact with, and be recruited by, β-catenin to Tcf target gene promoters, facilitating chromatin remodeling as a prerequisite for transcriptional activation (Barker et al. 2001). Although the role of Brg1 has been widely associated with gene activation, studies suggest the remodeling activity of Brg1 also has critical roles in transcriptional repression through interactions with co-repressors. For example, Brg1 was shown to cooperate with Cdx2 to repress Oct4 expression in the developing trophectoderm to ensure normal development (K. Wang et al. 2010).
Figure 1.7. Domain architecture of BRG1. The BRG1 chromatin remodeling protein contains an evolutionarily conserved ATPase region, as well as domains found within the N- and C-terminus. The conserved domain identification and predictions were performed using the NCBI specialized BLAST conserved domains database (Figure and legends were adapted with permission from Trotter and Archer 2008).

<table>
<thead>
<tr>
<th>Conserved Domain</th>
<th>Putative Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>QLQ</td>
<td>This conserved Gln, Leu, Gln motif is postulated to be involved in mediating protein interactions.</td>
</tr>
<tr>
<td>HSA</td>
<td>This domain is predicted to bind DNA and is often found associated with helicases.</td>
</tr>
<tr>
<td>BRK</td>
<td>The function of this domain is unknown. It is often found associated with helicases and transcription factors.</td>
</tr>
<tr>
<td>DEXHc</td>
<td>Domain associated with DEXH-box helicases. A diverse family of proteins involved in ATP-dependent DNA or RNA unwinding, needed in a variety of cellular processes. This domain contains the ATP-binding region.</td>
</tr>
<tr>
<td>HELICc</td>
<td>This domain is found in a wide variety of helicases and helicase related proteins. Helicases share the ability to unwind nucleic acid duplexes with a distinct directional polarity; they utilize the free energy from nucleoside triphosphate hydrolysis to fuel their translocation along DNA, unwinding the duplex in the process.</td>
</tr>
<tr>
<td>AT-hook</td>
<td>A small DNA-binding protein motif. AT-hook motifs are frequently associated with known functional domains seen in chromatin proteins and in DNA-binding proteins. AT-hook motif is an auxiliary protein motif cooperating with other DNA-binding activities and facilitating changes in the structure of the DNA either as a polypeptide on its own or as part of a multidomain protein.</td>
</tr>
<tr>
<td>Bromo</td>
<td>Bromodomain can interact specifically with acetylated lysine.</td>
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Brg1 in development

Brg1 and Brm are structurally similar (approximately 75% sequence homology); however, Brm<sup>−/−</sup> mice develop relatively normally (Kadam and Emerson 2003).
Interestingly, Brg1 expression is upregulated in Brm−/− mice, suggesting functional compensation by Brg1 can overcome the loss of Brm (Reyes et al. 1998). In contrast to Brm, Brg1−/− embryos die at the peri-implantation stage of development, thus necessitating the conditional deletion of this gene for post-implantation studies (Scott J. Bultman, Gebuhr, and Magnuson 2005). Ex vivo studies suggest that the lethality of Brg1 null embryos might be a result of failure to hatch from their zona pellucidae and fail to implant into the uterus (Scott J. Bultman, Gebuhr, and Magnuson 2005). Furthermore, blastocysts obtained from intercross matings were transferred to pseudopregnant CD1 mice and subsequently dissected between E6.5 and E8.5, but no Brg1 null embryos were recovered (Scott J. Bultman, Gebuhr, and Magnuson 2005). This suggests that the lethality of Brg1 homozygotes is intrinsic to the embryo and not the heterozygous uterine environment. Although the majority of Brg1 heterozygous pups survive they are underrepresented (S. Bultman et al. 2000). As some heterozygotes exhibit exencephaly, such affected Brg1 heterozygous pups likely die at birth and are cannibalized. Furthermore, surviving Brg1 heterozygotes are susceptible to neoplasia (S. Bultman et al. 2000).

To overcome the early embryonic lethality induced by global ablation of Brg1, recent studies utilized conditional knock-out and showed that Brg1 is essential in many developmental processes including hematopoiesis (Griffin et al. 2011), cardiogenesis (Hang et al. 2010), as well as the gastrointestinal tract (Takada et al. 2016). Studies of tissue culture models of differentiation offered more insights into the role of ATP-dependent chromatin remodeling in cellular differentiation and proliferation events. These studies showed that disruption of Brg1 function have an impact on cell cycle
regulation and differentiation in many cellular lineages, including muscle (Hang et al. 2010; Ohkawa et al. 2007), neural (Son and Crabtree 2014), lymphoid (Bossen et al. 2015), and myeloid (Vradii et al. 2006). Furthermore, RNA interference (RNAi)-mediated knockdown of Brg1 in early embryos and ES cells result in down-regulation of self renewal and pluripotency genes, including Oct4, Sox2, Sall14, and Rest, and up-regulation of differentiation genes (Kidder, Palmer, and Knott 2009). This is indicative of loss of self-renewal and pluripotency. Consistent with these observations, Brg1 has been shown to play a role in the maintenance of the intestinal stem cells, and loss of Brg1 impairs stem cell renewal, compromising the long-term renewal capacity of the intestinal epithelium (Holik et al. 2014). A recent study suggests Notch signaling is a key downstream target that mediates these effects of Brg1 on the homeostasis of intestinal stem cells (Takada et al. 2016). The molecular details of the requirement for Brg1 in intestinal stem cell maintenance, however, remain largely unknown.

Taken together, the above observations suggest that Brg1, but not Brm, is indispensable for embryonic development and essential for the development and homeostasis of various organs and cell types.
Rationale

While little is known regarding the means by which Cdx members regulate target gene expression, it is believed that they serve to recruit co-regulators, and it is the biochemical activity of such co-regulators that impacts transcription of such targets. Using immunoprecipitation and mass spectroscopy approaches, we have found that Cdx2 associated with multiple members of the Brg1-containing SWI/SNF complex, suggesting that Cdx2 regulates gene expression, at least in part, through recruitment of Brg1-associated SWI/SNF chromatin remodeling activity. In this study, I will examine the interaction between Brg1 and Cdx2, and the importance of this interaction in regulating gene expression.

Hypothesis

Cdx2 regulates the transcription of its target genes through recruitment of Brg1 and SWI/SNF chromatin remodeling.

Objectives

Aim 1: Identify Cdx2 target genes that are co-occupied by Brg1.
Aim 2: Generate Cdx2 and Brg1 null cell lines using CRISPR-Cas9 gene editing.
Aim 3: Assess the consequence of Cdx2 or Brg1 disruption on Cdx target gene expression and occupancy.
CHAPTER 2

MATERIALS AND METHODS
Cell lines and cell culture

Human embryonic kidney (HEK293) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin at 37 °C with 5% CO2 in air.

CRISPR-Cas9 gene editing

Null cells lines were generated using CRISPR-Cas9 gene editing. Guide oligonucleotides (Appendix D) against the first exon of Cdx2 or Brg1 were cloned into PX459 (Addgene). This plasmid expresses both the guide RNAs and the CAS 9 protein. Targeting plasmids (1 mg) were subsequently transfected into HEK293 cells using the calcium phosphate method (Schenborn and Goiffon 2000) and clones selected by culture in complete media with puromycin (3 mg/ml) for five days. Mutations were assessed by Western blot analysis, and candidates confirmed by sequencing PCR amplicons generated from the targeted interval.

In some experiments, expression vectors encoding Cdx2, Brg1 or an ATPase-dead Brg1 mutant (Khavari et al. 1993) were transfected in wild type or null cell lines by calcium phosphate-mediated transfection.

Western Blot Analyses

HEK293 cells were grown to confluence, rinsed twice with PBS and re-suspended in 200 µL RIPA (1% Triton-X-100, 1 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.1, Protease Inhibitor Cocktail). Samples were incubated on ice for 20 minutes. Protein
concentration was normalized using Bradford assays, and samples were resolved on 10% or 15% 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, Bouchard, et al. 2009); Cyclophilin, AbCam; Brg1, Santa Cruz Biotechnology; FLAG, Sigma Aldrich). Membranes were washed with PBST, incubated with 1:10000 HRP-conjugated secondary antibodies (goat-anti-rabbit or 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 using Odyssey® Fc Imaging System.

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

RNA was isolated from cells using Trizol as per recommendations, and complementary DNA (cDNA) generated using standard procedures. cDNA was subsequently amplified by semi-quantitative RT-PCR with GoTaq or qPCR with SYBR green (Promega) and quantified using Actin as an input control (Appendix D). qPCR was performed using the MX3005P (Agilent Technologies) and results were analyzed using the 2^(-ΔΔCt) method (Schefe et al., 2006). For specificity, the dissociation curve was considered for each amplicon. RT-PCR was performed over a series of cycles and samples within the linear range used for analysis.

**Cdx:Brg1 Interaction Assays**
Sequences encoding Brg1-B2 (amino acids 325-611) were amplified by PCR and ligated in frame in pCEP4-FLAG. This expression vector was transfected into HEK293 cells (10 µg DNA per 10 cm plate), and protein from cell lysates immunoprecipitated using 5 µg of either IgG (Santa Cruz Biotechnology) or Cdx2 (Savory, Bouchard, et al. 2009) antibodies bound to protein A/G and immunoprecipitation assessed by Western blot analysis. To identify the region of interaction between Brg1-B2 and Cdx2, GST-Cdx2 fusion proteins were bound to glutathione agarose beads and incubated with lysates from transfected cells overnight at 4°C and interaction assessed following anti-FLAG immunoprecipitation by Western blot.

For in vitro interaction assays, Cdx2 GST-fusion proteins were generated as described (Pilon et al. 2006). Brg1 proteins were radiolabelled in vitro with 35S-methionine using the TnT T7 Coupled Reticulocyte Lysate System (Promega) and incubated with 5 µg of each GST-fusion protein 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), resolved on SDS-PAGE gels and interactions revealed by autoradiography with BioMax MS Film (Kodak).

**Chromatin Immunoprecipitation (ChIP)**

Formaldehyde was added drop-wise directly to the media of 10 cm tissue culture plates to a final concentration of 1% and rotate gently at room temperature for 10 minutes. Fixation was halted by the addition of 0.125 M glycine for 5 minutes and cells were rinsed twice with PBS. Cells were then collected using a cell scraper and
transferred to 15 ml tube. Samples were centrifuged for 10 minutes at 300xG at 4°C. Pellet was then re-suspended in 3 ml of RIPA buffer (1% Triton-X-100, 1 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.1, Protease Inhibitor Cocktail) and incubated on ice for 20 minutes. Samples were sheared via sonication for 6 minutes (Branson Sonifier 450; duty cycle 3.0, 30% output), and the sonicated chromatin pre-cleared by incubating with 50 ml Protein A/G beads (per sample) for 1 hr. Five percent of the sample was removed as an input control, and the remaining lysate was divided for each immunoprecipitation. Samples were then incubated with 5 mg antibodies with rocking at 4°C. After 1 hour, 50 ml of A/G beads was added to each sample and incubated with antibodies overnight with rocking at 4°C. Samples were then washed two times with each of TSE I (0.1% SDS, 1% TX-100, 2 mM EDTA, 20 mM Tris.HCl pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% TX-100, 2 mM EDTA, 20 mM Tris.HCl pH 8.1, 500 mM NaCl), and LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris.HCl pH 8.1). Crosslinking was reversed in 200 µL IP elution buffer (1% SDS, 0.1 M NaHCO₃) at 65°C overnight, DNA purified using a Qiagen PCR purification kit, and amplified by PCR using GoTaq Green PCR master mix (Promega) (Taneyhill and Adams 2008).

**Restriction Enzyme Accessibility assay**

Cells (approximately 8x10⁶ cells) were rinsed with cold PBS and detached from 10 cm plates by scraping cells into 1 ml cold PBS. Cells were then pelleted by centrifugation at 8000 rpm for 3 min at 4°C. One milliliter of tissue culture cell (TCC) lysis buffer (10 mM PIPES pH 8, 85 mM KCl, 1 mM CaCl₂, 5% sucrose, 0.5% NP-40, plus
protease inhibitors) was added and the suspension transferred to a pre-chilled 10-ml Dounce tissue homogenizer and incubated on ice for 2 minutes. In order to isolate the nuclei, cells were lysed by using 10 complete strokes of the Dounce pestle (tight pestle) and centrifuged. Isolated nuclei were digested with Xhol I (0.5units/nuclei). To assess DNA cleavage, ligation mediated PCR was used (oligonucleotides in appendix). One microgram of digested DNA was ligated to 2.5µL (100mM) adaptor using the Takara ligation kit and PCR performed with GoTaq® qPCR Master Mix, under the following conditions: 95°C for 10 mins, 61°C for 1 min, 72°C for 1 min, 95°C for 1 min, followed by 30 seconds at 55°C and 30 seconds at 95°C. Products were resolved by gel electrophoresis and quantified by densitometric scanning (Ohkawa et al. 2012).

**Cdx-Brg1 Complementation Analysis**

Control, Cdx1^{-/-}Cdx2^{+/F}:Villin-Cre ER\textsuperscript{T} and Cdx1^{-/-}Cdx2^{+/F}Brg1^{+/F}:Villin-Cre ER\textsuperscript{T} animals were treated with 2mg of tamoxifen by oral gavage at 2 months of age. Animals were maintained according to guidelines established by the Canadian Council on Animal Care. Intestines were dissected from the animals 48 hours after treatment, flushed with phosphate buffered saline (PBS), and Swiss rolls prepared as described previously (Moolenbeek and Ruitenberg 1981). Samples were sectioned at 4 microns and stained with Alcian Blue, Churukian Silver or Phloxine-tartrazine. Slides were mounted using Permount (Fisher) and imaged using a Zeiss Axioscope microscope (Zeiss).

**Statistical Analyses**
Statistical analysis and graphs were generated using Graphpad Prism software. Statistical analyses for ChIP, semi-quantitative and quantitative RT-PCR, and luciferase reporter assays utilized student’s t-test (paired, two-tailed).
CHAPTER 3

RESULTS
**Brg1 and Cdx2 co-occupy Cdx2 target genes**

Previously, our group generated a Cdx2 chromatin immunoprecipitation sequencing (ChIP-Seq) dataset using embryonic day 8.5 (E8.5) embryos (Lohnes laboratory, unpublished). By aligning this with a published Brg1 ChIP-seq dataset acquired from embryonic stem cells differentiating toward cardiomyocytes (Alexander et al. 2015), we were able to identify Cdx2 target genes which may also be co-occupied by Brg1 (Appendix A). ChIP was then used to investigate the occupancy of known or potential Cdx2 targets by Brg1 or Cdx2 in HEK293 cells.

We first examined occupancy of the promoters of potential and known CDX2 target genes: DLL1, SCL, CYP26A1, AXIN2, LEF1 and WNT3A (Fig 3.1 A). The results from ChIP experiments suggested that both CDX2 and BRG1 bind to the promoters of DLL1, AXIN2, CYP26A1, WNT3A, SCL, and LEF1. (Fig 3.1 B). CYP3A4 served as a negative control for CDX2 (Medina et al. 2005).
Figure 3.1. Co-occupancy of CDX2 and BRG1 on CDX2 target genes. (A) Schematic diagram of the DLL1, CYP26A1, SCL, LEF1, WNT3A, AXIN2 promoters showing previously identified (DLL1 CYP26A1, WNT3A) or putative (SCL, LEF1) CDX binding sites. (B) ChIP analysis directed at the CDRE regions showing fold enrichment for BRG1 or CDX2 occupancy on the noted promoters in HEK293 cells. * p<0.05 by Student’s t test.
Loss of Cdx2 or Brg1 impacts Cdx target gene expression

In order to assess the consequences of CDX2 or BRG1 disruption on CDX2 target gene occupancy and expression, we first generated knock-out cell lines using RNA-guided nuclease from microbial clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9. CRISPR-Cas9 can be used to cleave virtually any genomic location of choice directed by a 20 nucleotide targeting sequence within its guide RNA (Ran et al. 2013). Subsequent imperfect repair can lead to insertion or deletion mutations, resulting in frame shifts. To ensure that the entire protein is likely to be disrupted, single guided RNAs (sgRNA) were designed to target the first exon of either BRG1 or CDX2. A number of clones were subsequently identified which no longer expressed BRG1 or CDX2 (Fig 3.2 A), and confirmed as due to frame shift mutations elicited by CRISPR-Cas9 targeting (not shown).

We next compared the expression of CDX2 target genes between wild-type, BRG1 and CDX2 null HEK293 cell lines. The relative expression of AXIN2, DLL1, SCL1, WNT3A, MEIS1 and CYP26A1 was decreased significantly in CDX2-null cells relative to controls, while expression of LEF1, AXIN2, DLL1, SCL1, MEIS1, and TCF4 was attenuated in BRG1-null cells (Fig 3.2 B). Thus, expression of a subset of CDX target genes is dependent on both BRG1 and CDX2, while others are independent of either Brg1 (WNT3A) or Cdx2 (LEF1, TCF4), at least in HEK293 cells.

Transfection of a CDX2 expression vector into CDX2-null cells (Fig 3.2 C) resulted in a restoration of expression of genes attenuated by CDX2 loss (Fig 3.2 D), confirming the specificity of the CRISPR-generated mutation. A similar rescue was also
seen upon transfection of a BRG1 expression vector into the cognate null cell line (Fig 3.2 C, E). However, comparable restoration of expression was not seen using a BRG1 cDNA encoding a catalytically-inert ATPase domain mutant (K798R) (Khavari et al. 1993) (Fig 3.2 C, E). As the ATPase activity of BRG1 is essential for the chromatin remodelling activity of SWI/SNF complex (Aoyagi, Trotter, and Archer 2005; Vignali et al. 2000; Wu et al. 2014; Ohkawa et al. 2007), these observations suggest that expression of a subset of CDX target genes depends on BRG1-mediated SWI/SNF remodeling.

The above observations suggest that CDX2 recruits BRG1, which may impact expression at CDX target loci through local alterations in chromatin structure. Consistent with this model, ChIP analysis revealed loss of BRG1 occupancy on a number of target genes in CDX2 null HEK293T (Fig 3.3). Interestingly, the loss of BRG1 also resulted in the loss of CDX2 binding. This result suggests that BRG1 is also required for the binding of CDX2 at its target loci.
Figure 3.2. BRG1 is required for the expression of CDX2 target genes. (A) Characterization of BRG1 and CDX2 null HEK293 cells by Western blot analysis. (B) Relative expression of CDX2 target genes in wild type, CDX2 and BRG1-null cell lines. (C) Western blot analysis of HEK293, BRG1 and CDX2 null cells, or null cells following transfection with vectors encoding wild-type BRG1, BRG1K798R (BRG1 mut), or CDX2. (D) Expression of CDX2 target genes in wild type, CDX2 null cells and cells transfected with a CDX2 expression vector (Rescue). (E) Expression of CDX2 target genes in wild type, BRG1-null cells, null cells transfected with a wild-type BRG1 expression vector (Rescue) or null cells transfected with an ATPase-dead BRG1 expression vector (ATPase mutant). * p<0.05 by Student’s t test.
Figure 3.3. Chromatin occupancy in CDX2 and BRG1 null cells. ChIP analysis for CDX2 from control and BRG1-null cells on DLL1, WNT3A, LEF1, SCL, CYP26A1 and AXIN2 promoters. * p<0.05 by Student’s t test.
**Cdx2 and Brg1 modify chromatin at the DLL1 locus**

SWI/SNF complexes remodel chromatin structure via conformational or positional changes of nucleosomes. Sequences that have been altered in such a manner such that they are in a more open chromatin conformation are typically more sensitive to restriction enzyme cleavage. Conversely, sequences that are resistant to nuclease cleavage are considered to be in a closed conformation, and are less accessible. Restriction enzyme accessibility assays (REAA) utilize ligation-mediated polymerase chain reaction to assess chromatin accessibility to restriction enzyme cleavage at specific DNA sequences of interest.

We used REAA assays taking advantage of a fortuitous XhoI site and found that the loss of either BRG1 or CDX2 had a comparable impact on chromatin accessibility at the CDX target gene *DLL1* in HEK293 cells (**Fig 3.4 A, B**). Similar alterations in accessibility were also seen in Cdx1-Cdx2 compound null murine intestinal epithelial cells, where *Dll1* has been shown to be a Cdx target gene (Grainger et al. 2012), as well as in Brg1 conditional null intestinal epithelium (**Fig 3.4 C**) in agreement with co-regulation between Cdx2 and Brg1 in vivo.

Reintroduction of CDX2 or BRG1 in the cognate null HEK293 cells restored the kinetics of DNA accessibility at the *DLL1* locus (**Fig 3.4 D, E**) while reintroduction of K798R mutant Brg1 did not (**Fig 3.4 E**). Taken together, these observations suggest that CDX2 recruits BRG1 and associated SWI/SNF remodeling activity to regulate expression of at least a subset of CDX target genes.
Figure 3.4. BRG1 and CDX2 impact target gene chromatin accessibility. (A) Schematic representation of CDREs in the Dll1 proximal promoter (Grainger et al 2012). (B) Relative accessibility of chromatin at the Dll1 locus is reduced in the absence of BRG1 or CDX2 in HEK293 cells, or in Cdx1-Cdx2 compound null mutant intestinal epithelial cells (C), as assessed by REAA. (D) Chromatin accessibility in the Dll1 promoter is rescued by reintroduction of wild type CDX2. (E) Wild type BRG1, but not a BRG1 ATPase-deficient mutant (BRG1 mut) rescues chromatin accessibility in BRG1 null cells. * p<0.05 by Student’s t test.
Cdx and Brg1 interact in vivo in goblet cell differentiation.

Cdx and Brg1 both impact differentiation of the intestine and are required for maintenance of a normal intestinal stem cell niche (Holik et al. 2014; Simmini et al. 2014). To determine if Cdx and Brg1 interacted genetically in this program, we quantified intestinal goblet cells, comparing Cdx1+/Cdx2+/+ (control), Cdx1−/−Cdx2+/−, and Cdx1−/−Cdx2+/−/Brg1−/− offspring. While there was no difference in the number of goblet cells per villus between genotypes (Fig 3.5 B and C), there was, however, a significant increase in goblet cell size in Cdx1−/−Cdx2+/−/Brg1−/− animals compared to the other genotypes (Fig 3.5 A and D). This suggests that Cdx2 and Brg1 interact genetically in one of the intestinal differentiation programs.
Figure 3.5. Cdx and Brg1 interact genetically. (A) Alcian blue staining for goblet cells (black arrow) in small intestine of CDX1<sup>-/-</sup>CDX2<sup>+/+</sup> Villin, CDX1<sup>-/-</sup>CDX2<sup>+</sup>/<sup>+</sup>Brg1<sup>+/+</sup>, and CDX1<sup>-/-</sup>CDX2<sup>+/+</sup>Brg1<sup>+-</sup>/ Villin mice (Adapted from Tanya Foley, unpublished data). Quantification of the number of goblet cells in the (B) Crypt region and (C) Villi. (D) Quantification of goblet cell size (μm<sup>2</sup>). * p<0.05 by Student’s t test.
CHAPTER 4

DISCUSSION
Cdx members play critical roles essential to many developmental processes, and also impact intestinal homeostasis and tumorigenesis in the adult (Verzi et al. 2010; Chawengsaksophak et al. 1997; Hryniuk et al. 2014; Young et al. 2009; Savory, Mansfield, Rijli, et al. 2011; Gao, White, and Kaestner 2009; Strumpf et al. 2005; Grainger, Savory, and Lohnes 2010). Little is known, however, regarding the mechanism whereby CDX members regulate target gene expression. In an effort to understand how CDX2 regulates transcription, we utilized a quantitative mass spectrometry approach to identify proteins co-immunoprecipitating with CDX2 from HEK293 cells. This cell line expresses abundant levels of CDX2 and is capable of supporting CDX-dependent transcription. This exercise recovered multiple members of the SWI/SNF chromatin-remodeling complex, including the catalytic subunit BRG1. Additional protein-protein association assays, co-localization analysis, chromatin occupancy and DNA accessibility assays suggest that CDX2 regulate target gene expression, at least in part, through recruitment of SWI/SNF-mediated chromatin remodeling. These findings extend prior work indicative of Brg1-Cdx interactions (K. Wang et al. 2010).

Cdx members interact with Brg1

The SWI/SNF complex is a large multimeric assembly, which is thought to be recruited to specific targets through the association with DNA binding transcription factors or other members of the transcriptional machinery (Trotter and Archer 2008). It has been shown that Brg1 contains distinct DNA binding motifs, which are believed to have a role in targeting SWI/SNF activity to certain promoters (Trotter and Archer 2008). These DNA binding motifs, however, do not direct the complex to specific genomic
sequence (Felsenfeld and Groudiner 2003; Kadam and Emerson 2003). They work in concert with association with transcription factors to allow sequence-specific binding and chromatin remodeling (Peterson and Workman 2000). Our finding that a number of SWI/SNF members, including BRG1, associated with CDX2 in HEK293 cells and in embryos suggests that CDX2 serves to recruit SWI/SNF to specific target genes.

Our SILAC-MS screen from cultured cells showed that BRG1 was significantly enriched in CDX2 immunoprecipitates. Furthermore, \textit{in vitro} GST-pulldown assays revealed that BRG1 interacted with all three CDX members (Appendix A). In addition, using immunofluorescence, we were able to show that CDX2 and BRG1 co-localize both in Caco cells and in intestinal epithelium. Taken together, these findings suggest that CDX transcription factors interact with the SWI/SNF complex through direct association with BRG1.

Consistent with prior studies, we showed that CDX2 interacts with BRG1 through the BRG1-B2 domain (Mudhasani and Fontes 2005; Trotter et al. 2008), although interaction with other regions of BRG1 or additional SWI/SNF subunits cannot be ruled out at present. The promiscuous interaction between all three members of CDX and BRG1 is also consistent with the functional overlap between Cdx members, as evidenced by the interaction between mutant Cdx alleles and the ability of Cdx2 to completely complement Cdx1 function in vertebral patterning (Savory, Pilon, et al. 2009). Moreover, Cdx members appear equivalent in their ability to occupy target genes (Savory, Pilon, et al. 2009; Grainger et al. 2012; Savory, Mansfield, Rijli, et al. 2011), further supporting a common mechanistic basis for their impact on transcription.
Brg1-dependent regulation of Cdx target genes

Cdx members occupy a number of target genes in the developing embryo (Grainger et al. 2012; Savory, Bouchard, et al. 2009) and the intestine (Gao, White, and Kaestner 2009; Grainger, Savory, and Lohnes 2010; San Roman et al. 2015; M. P. Verzi et al. 2011). This occupancy is often predictive of Cdx-dependent expression. Chip-seq analysis from embryonic stem cells undergoing mesodermal differentiation (Alexander et al. 2015) suggest that Brg1 is also resident on a number of these genes in a manner that overlaps known or putative CDRE. Co-occupation of a number of these loci by BRG1 and CDX2 was confirmed by ChIP analysis from HEK293 cells, suggesting that CDX2 serves to recruit SWI/SNF to relevant target loci. Consistent with this model, occupancy of BRG1 at these loci was lost in CDX2 null cells. Interestingly, the occupancy of CDX2 at these target loci was also lost in the absence of BRG1. This suggests that Cdx2 and Brg1 may work in a complex with other factors. Thus, disrupting this interaction, by removing either one of the players, could compromise the function of the complex leading to the loss of occupancy.

A comparison of expression of CDX target genes between wild type and CDX2 or BRG1 null cell lines suggested that BRG1 is essential for normal expression of a number of CDX targets. These results, together with the CDX-dependent recruitment of BRG1, suggest that BRG1 is required for the expression of CDX-dependent genes. A limited number of target genes, however, exhibited co-occupation by CDX2 and BRG1 but were not impacted by loss of one or the other factor. This may be indicative of fortuitous, but non-functional, binding or context-dependent regulation that is not faithfully recapitulated in HEK293 cells. Consistent with this result, prior work examining the
differentiation of intestinal stem cells (ISCs) to Villus cells (VC) showed that Cdx2 occupies hundreds of genomic sites in ISCs (San Roman et al. 2015). This binding, however, correlates poorly with ISC-expressed genes, but is predictive of genes activated in their differentiated progeny (San Roman et al. 2015). This suggests that Brg1 and Cdx2 may co-occupy certain genes in a temporal or context-dependent setting and serves to establish epigenetic landscapes required for later regulation; such a mechanism may not be recapitulated in simple tissue culture models such as used here.

As an ATPase subunit of the SWI/SNF complex, BRG1 utilizes energy from ATP hydrolysis to reposition nucleosomes and modify chromatin structure, thereby facilitating (Wu et al. 2014; Ohkawa et al. 2007; Scott J. Bultman, Gebuhr, and Magnuson 2005) or inhibiting (K. Wang et al. 2010) transcription. We showed that wild type BRG1, but not a catalytically inert mutant, restored the expression of CDX target genes in BRG1 null cells. These data suggests the catalytic activity of BRG1 is required for the expression of CDX target gene. Since ATPase activity is required for the repositioning of nucleosomes, catalytically inert mutant BRG1 compromises the activity of SWI/SNF. Gene expression, therefore, was not rescued with BRG1 mutant.

My data suggests that BRG1 is also required for the binding of CDX2 at certain loci; this binding is important in the transcriptional regulation of these target genes. However, luciferase assay used to access the activity of CDX2 in BRG1 null cells showed an increase in CDX2 activity (Appendix C). This observation is inconsistent with the previous data. I suspect this might be the result of squelching. In the absence of BRG1, CDX2 may not be able to access the genomic DNA, therefore, making more protein available for the luciferase promoter resulting in an increased reporter activity.
Alternatively, it has been demonstrated that transiently transfected plasmids acquire some degree of chromatinization (Cereghini and Yaniv 1984), but this chromatinization occurs randomly. Perhaps, in the presence of BRG1, the CDX reporter vector is remodeled in such a way that it inhibits transcription, leading to a decrease in activity in wild-type cells.

**Cdx2-dependent chromatin remodeling**

The SWI/SNF complex remodels chromatin structure via conformational or positional changes of nucleosomes (Ho and Crabtree 2010). Enzyme accessibility assays have been used as a tool to measure such activity, and have revealed Brg1-dependent changes in chromatin structure (Ohkawa et al. 2007; de la Serna et al. 2005). Sequences that have been altered and considered to be in an open chromatin conformation are more sensitive to restriction enzyme cleavage. On the other hand, sequences that are resistant to nuclease cleavage are considered to be in a closed conformation, and are less accessible. REAA utilizes ligation-mediated polymerase chain reaction to analyze chromatin structural changes at specific DNA sequences of interest (Ohkawa et al. 2012). Using this assay, we assessed the consequence of BRG1 or CDX2 loss-of-function on chromatin structure at the *Dll1* locus, and found that chromatin accessibility was comparably affected by the loss of either factor. Similar altered chromatin accessibility was also seen in Brg1 or Cdx1-Cdx2 null murine intestinal epithelial cells. Furthermore, consistent with the idea that ATPase activity is required for the function of SWI/SNF, wild type, but not mutant, BRG1 was able to restore the chromatin accessibility in BRG1 null cells.
Cdx and Brg1 interact genetically.

The intestinal epithelium is comprised of a single layer of cells that is constantly shed into the lumen, necessitating renewal every four to five days (Yuasa 2003). Cell division driving this renewal is confined to the intestinal crypts where stem cells both self renew and provide a populations of transiently amplifying (TA) cells (Chawengsaksophak et al. 1997). Stem cells residing in the small intestinal crypts have a distinct differentiation program and gene repertoire compared to stem cells found in the pyloric glands of the stomach. Prior studies have shown that Cdx2 is required to maintain the Lgr5-positive stem cells in the small intestine. In the absence of this transcription factor, these cells lose their intestinal identify and are converted cell-autonomously into pyloric stem cells (Simmini et al. 2014). Furthermore, our group showed that Cdx2 is required to maintain the intestinal stem cell population and their progeny (Hryniuk et al. 2012). Taken together, these observations indicate that Cdx2 plays a critical role in the differentiation program of intestinal stem cells. In addition, Brg1 has also been shown to play a role in the maintenance of the intestinal stem cells. Loss of Brg1 impairs stem cell renewal, compromising the long-term renewal capacity of the intestinal epithelium (Holik et al. 2014). Thus, Cdx and SWI/SNF both impact intestinal differentiation programs required for maintenance of the intestinal stem cell niche.

To determine if Cdx and Brg1 interacted genetically in intestinal differentiation programs, we used differential staining for enterocytes, goblet and enteroendocrine cells, comparing Cdx1<sup>−/−</sup>Cdx2<sup>+/−</sup> (control), Cdx1<sup>−/−</sup>Cdx2<sup>−/−</sup>, and Cdx1<sup>−/−</sup>Cdx2<sup>−/−</sup>Brg1<sup>−/−</sup> offspring. While there was no difference in the number of enterocytes or enteroendocrine or goblet
cell numbers between genotypes, there was a significant increase in goblet cell size in Cdx1⁺/⁻Cdx2⁺⁺/⁻Brg1⁺⁺ animals compared to other genotypes.

The mucus layer coating the gastrointestinal tract, secreted by goblet cells, serves as the first line of defense against physical and chemical insult as well as microbes (Birchenough et al. 2015). Goblet cells synthesize and secrete mucin glycoprotein (MUC2) and other bioactive molecules such as epithelial membrane bound mucins, trefoil factor peptides, resistin-like molecule β, and Fc-γ binding protein (Birchenough et al. 2015). MUC2 is the first human secretory mucin to be characterized and the major protein product of mature goblet cells (Pelaseyed et al. 2014; Kim and Ho 2010). The murine homologue of MUC2 has a similar structure motif as well as cell and tissue type-specific expression (Pelaseyed et al. 2014), and is regulated by Cdx2 (Yamamoto, Bai, and Yuasa 2003). Mice lacking Muc2 have the same number of goblet cells; however, these do not have the typical goblet cell shape (Birchenough et al. 2015). Taken together, this suggests that Cdx2 and Brg1 may impact the transcription of Muc2 leading to the irregular shape seen in the present study. Interestingly, studies have shown that in the absence of Muc2 there is no mucus and bacteria can have direct contact with the epithelium. This could lead to inflammation similar to that found in ulcerative colitis (Birchenough et al. 2015). If the inflammation persists for a long period of time, it could potentially lead to cancer (Birchenough et al. 2015). While speculative, this finding is consistent with a functional interaction between Cdx and Brg1 in vivo.

Future Directions

Our data suggests that ATPase activity of BRG1 is required for the remodeling
activity at certain CDX target genes. However, it was not clear whether this catalytic activity is required for the interaction between BRG1 and CDX2, or the occupancy of BRG1 and CDX2 on CDX target genes. We could address this by carrying out a co-immunoprecipitation with the BRG1 DN. Furthermore, we could introduce BRG1 DN into BRG1 null cells and perform a ChIP experiment to assess the binding of CDX2 at its loci.

In addition to containing catalytic ATPase domain, BRG1 contains a bromodomain (Trotter and Archer 2008), which has a role in many chromatin associated proteins as an acetyl lysine binding motif (Chandrasekaran and Thompson 2007). In fact, in yeast, the bromodomain of Swi2/Snf2, the yeast homologue of BRG1, has been shown to be important for anchoring the SWI/SNF complex to acetylated promoters (Hassan et al. 2002). Furthermore, increasing histone H4-K8 acetylation increases Swi2/Snf2 recruitment (Agalioti, Chen, and Thanos 2002). Future studies, therefore, should examine the bromodomain of BRG1 and whether the SWI/SNF chromatin remodeling activity is involved in the stable recruitment of CDX2 to target loci. Our results showed that loss of BRG1 resulted in the loss of CDX2 binding (Fig 3.3). Perhaps, this could be explained by the disruption in stable recruitment of BRG1. We could further examine this by blocking HDAC activity using TSA in BRG1-null cells. This leads to a global hyperacetylation of histones and consequently result more open chromatin conformation. ChIP assay can then be used to access the binding of CDX2. To further confirm the idea that bromodomain of BRG1 plays a role in CDX2 occupancy, we could transfecct BRG1 mutant lacking the bromodomain (BRG1 Δbromo) (Inayoshi et al. 2006) into Brg1 null cells under the two conditions with and without TSA. ChIP assay will be used to access the occupancy of CDX2 at its target loci. In the condition without TSA, we could examine whether BRG1...
bromodomain is important for the binding of CDX2. On the other hand, under the condition with TSA, we could determine whether both ATPase domain and bromodomain are required for the binding of CDX2. Furthermore, we showed that BRG1-B2, region of Brg1 between amino acids 325 and 611, interacts with CDX2. Thus, future studies should look at the importance of this interface between CDX2 and BRG1 by deleting the B2 region of BRG1 of the activation domain on CDX2. Gene expression analysis and ChIP analysis can be used to access the consequences of deleting the interface between CDX2 and BRG1 on target gene expression and occupancy.

We could further examine the co-occupation and transcriptional regulation of Cdx2 and Brg1 in different models. Perhaps, a better system for assessing the consequence of Cdx2 or Brg1 disruption on Cdx target gene expression and occupancy would be mouse ESCs. ESCs are pluripotent cells isolated from the inner mass of the pre-implantation blastocyst (Jackson et al. 2010). They can differentiate into all the cell lineages of the developing embryo. ESCs can aggregate in culture to form embryoid bodies in a manner similar to the early embryo (Jackson et al. 2010). This culture system therefore provides a useful model to study the relatively inaccessible stages of mammalian development. Using ESCs and embryoid bodies, we could assess the co-occupancy of Brg1 and Cdx2 at different stages of differentiation. We could further look at Brg1 and Cdx2 co-occupancy at different stages of development using mouse embryos. Taking advantage of the Cre/Lox system, we can study the effect of Cdx2 and Brg1 deletion at different stages of embryonic development. Quantitative RT-PCR can be performed at different stages to access the change in gene expression. In addition, ChIP
analysis can be used to determine whether the occupancy of Brg1 and Cdx2 correlates with the change in gene expression.

Recent studies looking at the role of Cdx2 in colorectal cancer has shown that Cdx2 has tumor suppressive properties (Velcich et al. 2002; Bae et al. 2015). Our group showed that loss of Cdx2 or Cdx1-Cdx2 in an APC_{min/+} background resulted in a marked acceleration of lethality and an increase in tumor incidence in the small and large intestine (Hryniuk et al. 2014). As with Cdx2, several observations suggest that Brg1 has tumour suppressive properties. For example, loss of Brg1 protein or Brg1 point mutations has been observed in a number of cancers, including lung primary tumours (S J Bultman et al. 2008; Glaros et al. 2008). Moreover, Brg1 heterozygous mice are predisposed to spontaneous tumor formation, further implicating the involvement of Brg1 in cancer (Kadoch and Crabtree 2015). Furthermore, our gene expression analysis suggests both BRG1 and CDX2 affect the expression of AXIN2, which plays a role in regulating the stability of beta-catenin in the Wnt signaling pathway, and is associated with colorectal cancer (Liu et al. 2000). Further studies, therefore, should examine the effect of knocking-out CDX2 and BRG1 in a colorectal cancer cell line, for instance SW480. With these knock-out models, we can look at the expression of target genes that are involved in colorectal cancer. Furthermore, we could examine the morphology of the knock-out cells versus control cells, as well as assess the behaviour of these mutant cell lines using invasion assays. These analyses may lead to further insight into relevant tumorigenic pathways.
Conclusions

Gene expression is tightly regulated at different levels to ensure appropriate transcriptomes for different developmental stages and cell types (Meier and Brehm 2014). The chromatin state in which a gene is embedded determines its expression level to a large extent (Meier and Brehm 2014). Our findings are consistent with a model wherein CDX members impact the chromatin state, and target gene expression, through recruitment of BRG1 and associated SWI/SNF-dependent chromatin remodeling (Fig 4.1). In this regard, prior work has shown CDX2 occupancy of a number of genes in intestinal progenitor cells. Many of these targets, however, are not perturbed in this progenitor population by loss of CDX2, but were impacted in the differentiated progeny thereof (San Roman et al. 2015). Taken together, these observations suggest that one mechanism of action of CDX2 may be to establish the epigenetic landscape in progenitor cells through SWI/SNF-dependent chromatin remodeling for subsequent transcription in descendant lineages.
Figure 4.1. Proposed Model. (A) In the absence of Cdx2, Brg1 (and the SWI/SNF complex) is not recruited to the promoter region of a target loci. Histone (H) distribution prevents the transcriptional machinery (T.M.) and/or unknown factors (?) from accessing the DNA, thereby inhibiting transcription. (B) Brg1 binds to Cdx2 and resembles the complex. (C) The complex is then recruited to the promoter. Histones are displaced by SWI/SNF remodeling activity, allowing the transcriptional machinery and/or unknown factors to access DNA (Adapted and modified from Travis Brooke-Bisschop)
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Appendix A. Chip-seq analysis of Brg1 and Cdx2. Analysis reveals the enrichment of Brg1 and Cdx2 at transcription start sites of some of Cdx2 target genes. Cdx2 ChIP-seq dataset was acquired from embryonic day 8.5 (E8.5) embryos. Brg1 ChIP-seq dataset acquired from embryonic stem cells differentiating toward cardiomyocytes (Alexander et al. 2015).
Appendix B. Brg1 was assessed for interaction with full length GST-Cdx1, 2 and 4, the N-terminus and C-terminus regions of Cdx members. (Adapted from Dr. Joanne Savory, unpublished data)
Appendix C. Cdx2 activity in Brg1 null cells. A Cdx-reporter vector was transfected into wildtype or Brg1 null HEK cells. Luciferase activity was measured and normalized using β–galactosidase, which was co-transfected into all culture plates. Two Brg1 knock-out cell lines were used with 4 different concentrations (0, 1, 2, 3 μg) of Brg1 expression vector.
Table D: List of Oligonucleotides used for RT-PCR, ChIP, REAA and CRISPR-Cas9.

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