ELUCIDATION OF BRG1-DEPENDENT MECHANISMS THAT GOVERN PLURIPOTENCY GENE EXPRESSION IN EMBRYONIC STEM CELLS AND THE TROPHOBLAST LINEAGE

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Biochemistry and Molecular Biology – Doctor of Philosophy

2015

ABSTRACT

ELUCIDATION OF BRG1-DEPENDENT MECHANISMS THAT GOVERN PLURIPOTENCY GENE EXPRESSION IN EMBRYONIC STEM CELLS AND THE TROPHOBLAST LINEAGE

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Brahma-related gene 1 (BRG1), a chromatin remodeling ATPase, is known to function as a key regulator of gene expression eliciting both activator and repressor functions within different cell types. In pluripotent embryonic stem cells (ESCs), BRG1 is found at key regulatory elements of pluripotency genes and functions as a negative regulator to govern lineage determination. However, the underlying mechanisms by which BRG1 regulates pluripotency genes in ESCs and the trophoblast lineage are largely unknown. To elucidate the BRG1-dependent mechanisms that regulate pluripotency during early embryonic development I used a combination of mouse preimplantation embryos and CDX2-inducible ESCs that transdifferentiate into trophoblast-like cells. The cell line allowed for biochemical experiments to be performed that required large amounts of biological material to uncover mechanisms that could then be verified in the embryo.

In the first experimental study of my dissertation I demonstrated that a series of dynamic transcriptional and epigenetic changes occurred at the *Nanog* and *Oct4/Pou5f1* proximal and distal enhancer regions during trophoblast lineage development. Initially, CDX2 was recruited to *Nanog* and *Oct4* enhancers and colocalized with BRG1. Next, OCT4 and RNA polymerase II (RNAPII) were lost and major changes in chromatin structure occurred. Histone H3 lysine 9 and lysine 14 acetylation (H3K9/14Ac) were significantly reduced and p300 and histone deacetylase 1 (HDAC1) were lost at these genes. These changes were accompanied by an increase in nucleosome occupancy as assayed by chromatin accessibility and total histone H3 chromatin

immunoprecipitation (ChIP) experiments. Lastly, I showed that DNA methylation at these regulatory regions was a final step accompanying *Nanog* and *Oct4* silencing in the trophoblast lineage. The results of these early experiments provided an epigenetic framework for subsequent functional experiments that resolved the role of BRG1 in pluripotency and trophoblast lineage development.

In the second experimental study of my dissertation I examined the biological role of BRG1 in pluripotency gene regulation and trophoblast lineage development. To accomplish this a series of experiments were performed in preimplantation embryos and CDX2-inducible ESCs. First, I demonstrated that BRG1 antagonizes histone H3K9/14 acetylation at the *Nanog* proximal enhancer in both pluripotent ESCs and the trophoblast lineage. To understand how BRG1 regulates H3K9/14 acetylation a series of biochemical experiments were performed. I discovered that BRG1 forms a functional deacetylation complex with histone deacetylase 1 (HDAC1) in ESCs and preimplantation embryos. An important observation obtained from the embryo study was that the interaction of BRG1 with HDAC1 occurred at a higher frequency in the trophoblast lineage than in the inner cell mass (ICM). In agreement with a role in transcriptional repression, inhibition of HDAC1 resulted in an increase in *Nanog* expression in ESCs and failure to repress *Nanog* during trophoblast lineage development. Importantly, disruption of HDAC1 phenocopied BRG1 depleted ESCs and embryos, suggesting that HDAC1 cooperates with BRG1 to govern *Nanog* expression. Lastly, I provide nucleosome-mapping data that supports a dual role for BRG1 in histone deacetylation and chromatin remodeling during early embryonic development. Collectively, the results of these experiments provide novel information on the underlying mechanisms by which BRG1 converges with the pluripotency gene network to modulate pluripotency gene expression and support development of the trophoblast lineage.

I dedicate this thesis to my family: to my wife Lisa, sons David and Nolan, and my parents Daniel and Shirley, for their constant love and support

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor, Dr. Jason Knott. His expertise and guidance proved to be invaluable for me both to learn the skills necessary to conduct my research, and in helping me analyze, interpret, and criticize the results. His excitement and passion for science could be observed every time he stepped into the lab, or when we'd have a scientific discussion. I can only hope to one day be considered as passionate for science as he is due to his valuable lessons and guidance.

I would like to thank my committee members, Dr. Monique Floer, Dr. Bill Henry, Dr. David Arnosti, and Dr. George Smith. Each of them displayed a tremendous capacity to always ask the right questions to get me to think about my research in a new and interesting way.

This work could not have been possible without the collaborative efforts of some of my friends and colleagues in Anthony Hall. Tremendous thanks goes to Zubing, Inchul, Katie, and Kai, as well as to Satyaki for all of your help.

Finally, my most heartfelt appreciation goes to my family for supporting me, loving me, and always being there for me. Lisa, your unyielding selflessness in all matters of our lives helped make this process possible. Your ceaseless help with our children and our lives made working the weird hours and long nights to complete this process more bearable. Thank you to my children, for just being the way you are. Lastly, thanks to my parents for helping to shape the person I have become. In this same manner, I would like thank Steve Watrous whom I basically consider family.

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KEY TO ABBREVIATIONS

ARID	AT-rich interactive domain
ART	Assisted reproductive technologies
ATP	Adenosine Triphosphate
BAFs	Brg1 or Brm-associated factors
BMP	Bone morphogenic protein
BRD	Bromodomain containing protein
BRG1	Brahma-related gene 1
BRM	Brahma
CARM	Co-activator associated arginine methyltransferase
CDX2	Caudal type homeobox 2
ChIP	Chromatin immunoprecipitation
DVL2	Dishevelled segment polarity protein-2
EPI	Epiblast
esBAF	Embryonic-specific Brg-1 associated factor complex
ESCs	Embryonic stem cells
FZD2	Frizzled-2 receptor protein
H3K4Me3	Trimethylation mark on lysine 4 of histone subunit H3
H3K9Ac	Trimethylation mark on lysine 9 of histone subunit H3
H3K14Ac	Acetylation mark on lysine 14 of histone subunit H3
H3K27Me3	Trimethylation mark on lysine 27 of histone subunit H3
HDAC	Histone deacetylase

HOX	Homeobox genes
HSA	Helicase-SANT-associated domain
ICM	Inner cell mass
ISWI	Imitation switching mate type complex
KLF4	Kruppel-like factor 4
LIF	Leukemia inhibitory factor
MAPK	Mitogen-activated protein kinase
MBD3	Methyl-CpG-binding domain protein 3
NANOG	Tir Na Nog homeobox transcription factor
NURD	Nucleosome remodeling deacetylase
OCT4	Octamer-binding transcription factor 4, also known as POU5F1
PB1	Polybromo-1
PBAF	Polybromo-associated factors
PcG	Polycomb group
PE	Primative endoderm
POU5F1	POU-domain class 5 transcription factor, also known as OCT4
QLQ	Glutamine-leucine-glutamine motif
RSC	Remodeling structure of chromatin complex
SnAC	Snf2 ATP coupling domain
SNF	Sucrose non-fermentable
SOX2	Sex determining region Y-box 2
STAT3	Signal transducer and activator of transcription 3

SWI	Switching mate type
SWI/SNF	Switching mate type / sucrose non-fermentable chromatin remodeling complex
TCF3	T-cell factor 3
TE	Trophectoderm
TEAD4	TEA domain family member 4
TFAP2C	Transcription factor AP-2γ
WNT	Wingless-related integration site
YAP1	Yes-associated protein 1
ZGA	Zygotic genome activation

CHAPTER 1 -

INTRODUCTION

Tightly controlled regulation of gene expression is a necessary component of maintaining homeostasis in all organisms. The complexity of the mammalian genome adds to the established paradigm of sequence-specific transcription factors binding to enhancer elements in a spatiotemporal-specific manner by providing a dynamic chromatin landscape that contributes to this regulation (1). It has become well established that chromatin organization can disrupt or prevent transcription factor binding to cis-regulatory elements, however changes in chromatin structure and histone modifications can establish an additional layer of regulation that allows for rapid fluctuations in cellular processes that often are required to occur in a time-sensitive manner (2).

In eukaryotic cells, there are two mechanisms used to alter chromatin structure: post-translational modifications of histones and ATP-dependent chromatin remodeling activity (3). The highly basic N-terminal tails of the core histone proteins are often targets that undergo post-translational modification at various residues, including acetylation, methylation, phosphorylation, and ubiquitination (4). These histone modifications play a crucial role in the recruitment of other proteins including chromatin-remodeling complexes (4, 5). Several chromatin-remodeling complexes have been identified in eukaryotes that utilize the power of ATP-hydrolysis to alter nucleosome positioning (6). The enzymatic ATPase subunits in these complexes are part of the SF2 superfamily of helicase-related proteins and most are members of the Snf2 family of proteins. Some well-known examples of these large (>1 mDa) chromatin-remodeling complexes are the SWI/SNF, RSC, NURF, and INO80 complexes (6-8). As discussed below, BRG1 is one of the two highly conserved ATPase subunits found in the mammalian SWI/SNF complex and has been found to have distinct functional roles from BRM, the other ATPase of the mammalian SWI/SNF, as a transcriptional coregulator (9, 10).

BRG1 ATPase, the SWI/SNF complex, and esBAF chromatin remodeling complex

The SWI/SNF complex is named as such because many of its components were first identified in budding yeast, *Saccharomyces cerevisiae,* by genetic screens and were found to be associated with mating-type switching (SWI) and sucrose non-fermenting (SNF) phenotypes (11-14). Originally defined as transcriptional activators, these SWI/SNF proteins were later found to affect transcription by altering chromatin structure (15). Around the same time that many of the molecular mechanisms employed by yeast for undergoing chromatin remodeling by SWI/SNF were being deciphered, several homologs of SWI/SNF proteins were discovered in other eukaryotes ranging from *Drosophila* to mammals.

The SWI/SNF complex in yeast was found to minimally contain SWI1, SWI2/SNF2, SWI3, SNF5, and SNF6 to be able to elicit changes in chromatin structure *in* vivo, which influenced transcription (16-18). The SWI2/SNF2 subunit was ultimately found to be the catalytic subunit for chromatin remodeling and was shown to possess DNA-dependent ATPase activity (19). The highly conserved nature of the Snf2-helicase domain in other chromatin remodeling ATPase proteins led to the discovery of other eukaryotic homologs. The *Drosophila* gene *brahma* (*brm*) was found to share 55% sequence identity with yeast SNF2 over a region spanning residues 740 to 1413 that contained the DEAD-like helicase domain (20). The bromodomain was also characterized during the discovery of the sequence conservation between these two homologs. Defined as a newly discovered domain, the bromodomain was shown to be highly conserved between *brm* and SNF2 and located near the C-terminus of both proteins, and was so named for its discovery in *brm* (20, 21).

Two homologs to SNF2 were identified in humans, and were shown to act as coactivators

for transcription, interacting with a variety of nuclear receptors including the glucocorticoid receptor, estrogen receptor, and retinoic acid receptor (22-24). Sometimes referred to as hSNF2α and hSNF2β, these proteins are more commonly referred to as hBRM (human Brahma) and hBRG1 (human Brahma-related gene 1), respectively. Corresponding murine homologs were also identified for BRM and BRG1 (22, 25). From the onset of the discovery of these two homologs, it appeared their functions were non-redundant. During the initial discovery and cloning of murine BRG1, the protein was shown to have widespread expression during early embryonic development, but later became restricted to the central and peripheral nervous system (25). It was later shown that *Brg1*$^{-/-}$ mice are embryonic lethal and die during the peri-implantation stage, whereas *Brm* was found to be dispensable (9, 26). Moreover, it was shown that *Brg1* is mutated in multiple human tumor cell lines and associated with various malignancies. (27, 28). The idea that these two mutually exclusive ATPase subunits of the SWI/SNF complex serve different roles is reinforced by the observation that BRG1 is able to bind to zinc finger proteins due to a domain in its N-terminus that is missing in BRM (29).

BRG1 and BRM overall share ~75% sequence homology, and the overall primary structure of all of the SWI/SNF ATPase proteins contain the same combination of highly conserved domains (outlined for BRG1 in Fig. 1-1a). Central to the mechanistic function as an ATPase, BRG1 contains a DEAD-like helicase domain that contains two RecA-like domains (Fig. 1-1b). There is also a conserved region associated with the helicase located closer to the C-terminal bromodomain known as the SnAC (Snf2 ATP coupling) domain. This domain has been shown to be critical for remodeling activity and to act as a histone anchor (30, 31). The bromodomain located near the C-terminus provides an additional mechanism for interactions with histones, as the function of these domains is to bind to acetylated lysine residues (21, 32).

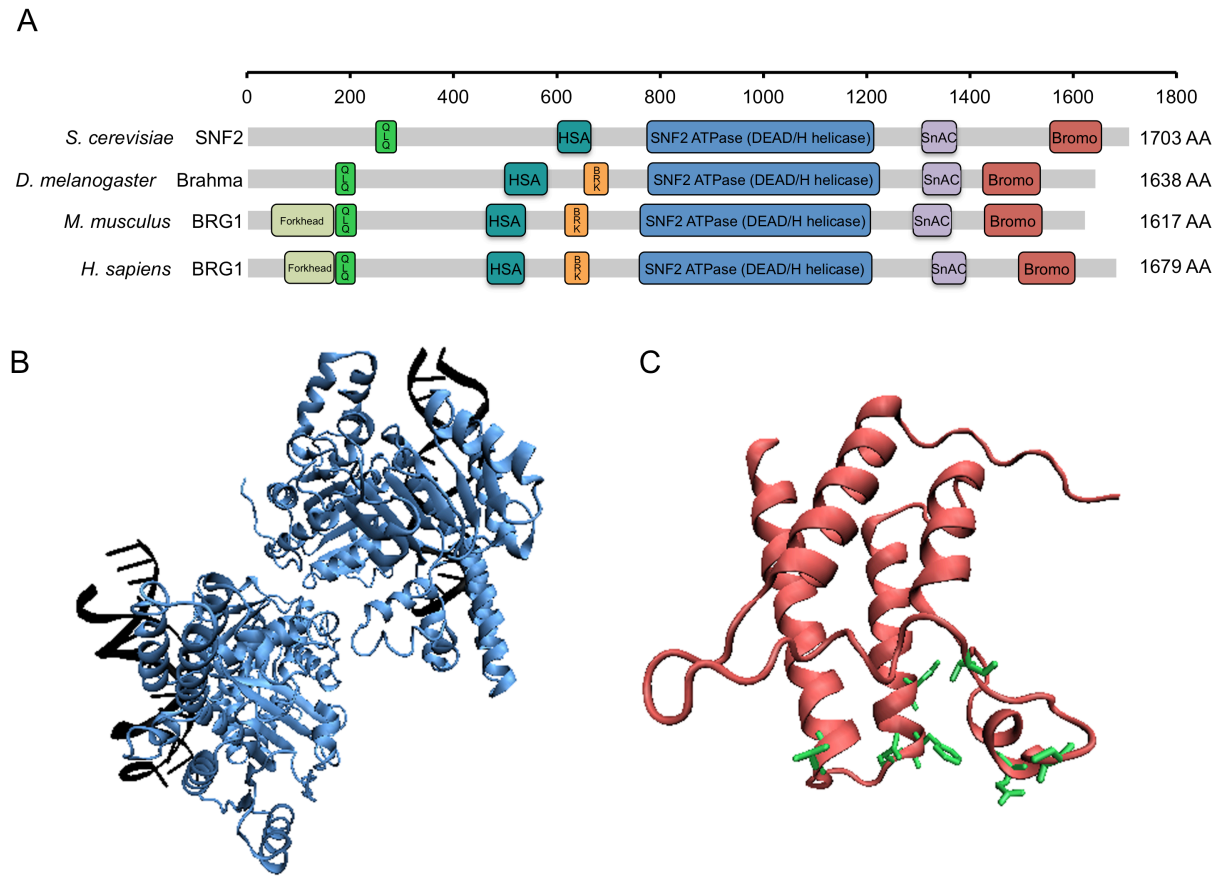

Figure 1-1. Structural aspects of BRG1. (A) Comparison of conserved domains in BRG1 ranging from yeast to humans. (B) Ribbon representation of the structure of the *Sulfolobus solfataricus* SWI2/SNF2 ATPase core from a crystal structure solved to 3 Å bound to DNA. Although this ATPase domain is from a homolog to Rad54, from a structural aspect it is considered representative to the domain present in BRG1. Two RecA-like subdomains are the blue protein lobes making contacts with DNA represented in black. (C) Ribbon representation of the hBRG1 bromodomain from a crystal structure solved to 1.85 Å. The green sidechain residues are sites involved with binding to acetylated lysine.

The bromodomain of BRG1 shows the highest affinity for acetylated H3K14 peptides *in vitro*, but can also bind to acetylated lysine residues of histone H4 peptides (Fig. 1-1c) (33). The N-terminus region of BRG1 contains several domains that are critical for interactions with other proteins. Among these are the QLQ, HSA, and BRK domains (34, 35).

BRG1 has the capacity to remodel chromatin by itself *in vitro*, but must be part of a multi-protein complex to facilitate its chromatin remodeling activity *in vivo* (36). Overall the mammalian complexes containing BRG1 contain a core group of proteins similar to the minimal SWI/SNF chromatin remodeling complexes of yeast, but also contain several distinct proteins. Crabtree and colleagues isolated proteins of the mammalian SWI/SNF complex associated with BRG1 and named the purified proteins as BRG1-associated factors (BAFs) according to their molecular weight (17). A number of the BAFs isolated were found to be homologs of various SWI/SNF yeast proteins. BAF47, also known as INI1 is a homolog of SNF5; BAF155 and BAF170 were identified as homologs of SWI3; and BAF60a was found to be homologous to SWP73, a protein shown to function in the yeast SWI/SNF complex (17, 37, 38). It also became apparent during the initial characterization of BRG1-containing complexes that there was much more complexity and variety of the subunit composition in the mammalian complexes, indicating distinct functional roles in varying cellular contexts. Earlier experiments in HeLa cell nuclear extracts revealed that BRG1 exists in two distinct complexes (39). Crabtree and colleagues also identified two distinct complexes containing BRG1, which were named BAF and PBAF (17, 35, 40). The initial distinction between these two complexes particularly pertained to the presence or absence of some of the larger BAF proteins, specifically BAF250, BAF200, and BAF180. It has since been determined that the subunit BAF250, a homolog of yeast SWI1, is only present in the BAF complex, which is the true mammalian SWI/SNF complex (40, 41). The PBAF

(polybromo BRG1-associated factor) complex is related to the yeast RSC complex and is so named because BAF180, which was thought to be an essential component of PBAF showed 90% sequence homology to the chicken Polybromo protein (PB1)(40). BAF200 (ARID2) was also shown be a factor that specifies the PBAF complex and is essential for the stability of the complex *in vivo* (42).

Ultimately, the ability of BRG1 to interact with DNA is critical for its function as a transcriptional coregulator. The bromodomain would seemingly be a possible way in which BRG1 is recruited to DNA and nucleosomes though its affinity for acetylated lysine residues of histones. Adjacent to the bromodomain there is an AT-hook domain that might facilitate interactions with histone tails (43). However, genetic studies in yeast and flies demonstrated that the bromodomain is dispensable (19, 44). Within the BAF complex, BAF250 plays an important role in recruiting the complex to DNA via its AT-rich interacting domain (ARID). Two isoforms of BAF250 are present in mammals: ARID1a (BAF250a), and ARID1b (BAF250b)(41, 45). ARID1a has been shown to be essential for transcriptional regulation by the BAF complex *in vivo* during early mouse development, and its loss of function is embryonic lethal at day 6.5 (46-48). An interesting facet of the BAF250 isoforms is that much like the mutually exclusive aspect of BRG1 or BRM contained within the BAF complex, either ARID1a or ARID1b is present in the complex but never both at the same time (45). This is a general theme that is observed in the mammalian BAF complexes, and often a particular stoichiometry of subunits is also observed. Therefore, changes in the subunit composition can occur during cellular transitions to provide different functions (49).

Collectively, the overall composition of the mammalian BAF complex is quite heterogeneous. It contains ~12 subunits and its total molecular weight is around 2 MDa. There

are the 5 subunits previously mentioned that are highly conserved with their yeast counterparts: BRG1/BRM, BAF155/BAF170, BAF47, BAF60a, and BAF250a/BAF250b. Actin was also found to be a critical component of the mammalian BAF complex with corresponding actin-related proteins BAF53 and BAF57 (40, 50, 51). This group of subunits seems to define the "core" of the BAF complex to facilitate chromatin remodeling by the central ATPase, therefore providing a nucleosome-remodeling mechanism by which transcription can be regulated. However, several other unique factors can also associate with BAF, and these too can expand its role in regulating gene expression.

The BAF complex has been shown to alter chromatin structure in an ATP-dependent manner just like its yeast counterparts, which can result in altered nuclease sensitivity at its targeted site. BAF can remodel chromatin and then facilitate the transition back to an initial chromatin state by *in vitro* assays (52). This observation suggests that the BAF complex is capable of remodeling chromatin to allow for transcription to take place, but could similarly remodel chromatin back to result in a transcriptionally silent structure. The BAF complex has been shown to associate with transcription factor binding sites to alter the chromatin state to influence transcription, but unlike other chromatin remodelers such as ISWI, it does not make ordered nucleosome structure as would be observed in completely silenced regions of heterochromatin (53, 54). Much of our understanding of the structural aspects of SNF2 chromatin remodeling was not derived from studies of the classical SWI2/SNF2 ATPase or any of its homologs such as BRG1, but by other SWI2/SNF2-related proteins (55-58). All of these chromatin-remodeling ATPases, despite containing the helicase domain, do not demonstrate true helicase activity, but rather ATP-dependent translocation of dsDNA (59). However the structural and functional aspects of chromatin remodeling by SWI/SNF, and specifically the

mammalian BAF generally occur in a similar manner (54, 60). Several mechanistic studies in SWI/SNF remodelers describe a mechanism by which the ATPase subunit is positioned through various interactions of the entire SWI/SNF complex to a position where the DNA and histone interactions are weak (49, 60-63). The translocase activity of the ATPase subunit then creates a loop by ratcheting of a torsion subdomain and tracking subdomains composed within the SNF2 ATPase/translocase domain (49, 54, 60). This loop is then propagated, and the section of DNA not interacting with the histone travels around the histone, ultimately resulting in linker DNA being taken up and being part of the nucleosome, and new-linker DNA formed on the opposite end that was previously part of the nucleosome.

Beyond the core chromatin-remodeling activity of BRG1 in the BAF complex, the protein can associate with numerous transcription factors and histone-modifying proteins that contribute to the coactivator or corepressor function of BRG1. For example BRG1 and several additional members of the BAF complex can interact with the histone methylase CARM1 to activate estrogen receptor target genes (64). Activation of ER-target genes also occurs through the recruitment of BRG1 with histone-acetyltranferse p300, in an estrogen-dependent manner. Conversely estrogen-antagonists can recruit BRG1 to these same target genes in association with the histone deacetylase HDAC1 to repress ER-target genes (65). BRG1 displays coactivator and corepressor functions in the regulation of β-globin in erythroid progenitors. BRG1 is recruited to the proximal promoter of P4.2 in a manner that is dependent on two E-box GATA binding motifs. BRG1 subsequently represses P4.2 transcription by associating with the mSin3a/HDAC2 histone deacetylase complex (66).

Among the cellular contexts where BRG1 has been shown to be particularly important is during early embryonic development and maintaining pluripotency networks in embryonic stem

cells (ESCs) (9, 67, 68). The highly dynamic nature of this developmental signaling pathway with modulation through various epigenetic regulatory mechanisms, including chromatin structure highlights the critical role a chromatin remodeler such as BRG1 might provide to help regulate this pathway (69). As previously mentioned, BRG1, unlike the alternative ATPase BRM, is essential. In mice, *Brg1*$^{-/-}$ embryos have the capacity to form blastocysts but are unable to hatch from the zona pellucida, or form viable inner cell mass or trophectoderm as assayed through *in vitro* outgrowth (9). The effects on embryonic development and the implications of these observations will be further discussed in later sections. Other components of the BAF complex, specifically BAF155, BAF47, and BAF250a/b were also shown to be essential for either embryonic development or in maintaining pluripotency signaling (47, 70-72). As BRG1, BAF47, and BAF155 are core BAF components that are essential for the chromatin remodeling activity of the BAF complex, this suggest that the chromatin remodeling activity of SWI/SNF is essential for early embryonic development and ESC pluripotency.

These observations helped lead to the discovery of an embryonic-specific BAF complex (esBAF) that was initially characterized in mouse ESCs (73). A comparative proteomic analysis was performed on material that was affinity purified using an antibody that recognizes both BRG1 and BRM. Material from ESC nuclear extracts, as well as nuclear extracts from mouse embryonic fibroblasts and neural progenitor cells were all compared. The BAF complex in ESCs contains a unique and lineage-specific composition of BAF subunits, as well as other non-BAF proteins. The esBAF complex exclusively contains BRG1, BAF155, and BAF60a. BRM, BAF170, and BAF60c are all absent from the complex (73). Previously BAF complexes were characterized to contain both BAF155 and BAF170 at an apparent 1:1 ratio (37, 74). It had been proposed that BAF155 and BAF170 form a heterodimer complex that is a critical scaffold

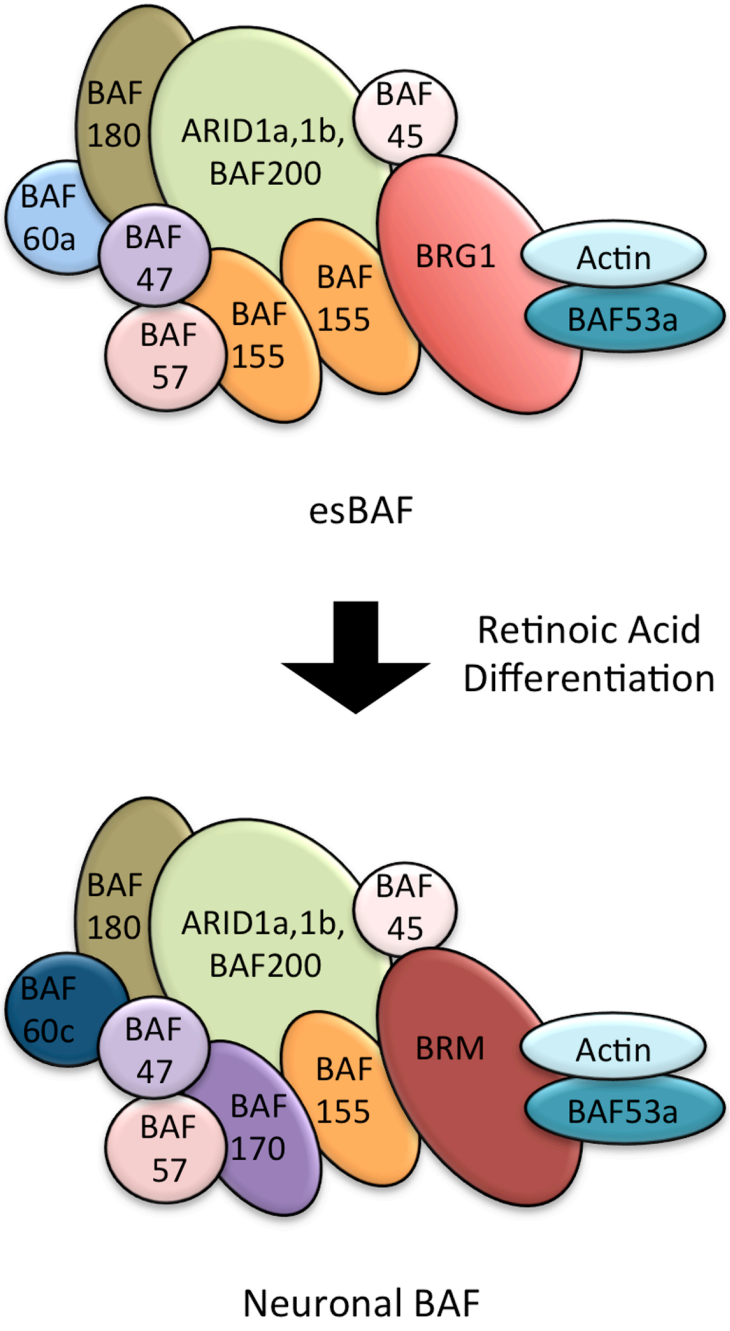

Figure 1-2. Subunit distinction between the esBAF complex and a neuronal BAF complex during differentiation.

for the BAF complex, and the interaction of these proteins, particularly due to domains present on BAF155 regulate the protein levels of BAF57 (74). Discovery of the esBAF complex and its proclivity for BAF155 demonstrates that a homodimer of two BAF155 subunits can form this critical scaffold. Characterization of the subunit composition of the BAF complexes during retinoic acid-induced differentiation of ESCs toward the neuronal lineage revealed a switch from a stoichiometry of two BAF155 subunits present per BAF in ESCs to a 1:1 ratio of BAF155:BAF170 in the neuronal lineage (Fig. 1-2) (73). Additionally a switch to BRM and BAF60c also occurred where these proteins were expressed in high abundance in neuronal cell BAF complex. The manner in which BRG1 and the esBAF complex regulate genes that influence lineage formation in the mouse embryo converges on the same set of transcription factor genes that regulate self-renewal and pluripotency signaling of ESCs. An understanding of these transcriptional networks within both the mouse embryo and in ESCs is critical to understanding how BRG1 participates to regulate their gene expression.

Lineage formation in the mouse blastocyst

The window of preimplantation development in the mammalian embryo culminates in the formation of a blastocyst that is capable of undergoing implantation in the uterine wall. For the mouse embryo, this occurs around 4.5 days after fertilization. The molecular and physiological mechanisms that contribute to a properly formed blastocyst begin prior to fertilization during oogenesis. For the developing mouse embryo a critical step during this overall process is the first cell-fate decision at day 3.5 that results in the formation of two distinct lineages recognized as the inner cell mass (ICM) and the trophectoderm (75).

A vast amount of mRNA and proteins required for transcription are accumulated during

oocyte maturation prior to mitotic arrest at metaphase II. These maternal gene products support the earliest stages of preimplantation embryogenesis (76-78). Development beyond the two-cell stage in the mouse requires the expression of embryo-derived transcripts following a process known as zygotic genome activation (ZGA) (79). A critical element of these earliest developmental processes is symmetrical division from the 1-cell to the 8-cell stage where all of the cells, or blastomeres are considered totipotent, meaning they are capable of contributing to both embryonic and extra-embryonic tissues. This cleavage-stage cell division maintains a constant total volume of cytoplasm and constant size of the entire embryo, while making progressively smaller blastomeres (80). Compaction then begins at the 8-cell stage, which is controlled by factors that increase intercellular adhesion (81-83). Following the 16 and 32-cell stage divisions, the cells in the outside of the embryo become distinct from the inside cells due to a combination of symmetrical and asymmetrical cell divisions (80, 83-85). Lineage-specific transcription factors direct this process, and will be discussed in more detail, but overall it is generally recognized that the outer cells go on to form the trophectoderm lineage while the inner cells form the inner cell mass (ICM).

Tight junction assembly and fluid accumulation during the 16-32 cell stage transitions are important following compaction, and result in cavitation to form the blastocoel (86). Blastocoel formation is a required process to make a functional blastocyst that segregates into its downstream lineages (85). Several families of genes control this process. The Knott lab demonstrated that transcription factor AP-2γ (TFAP2C) acts as an important regulator of many critical genes including aquaporins, Na^+/K^+-ATPases, and tight junction proteins/occludens (87). TFAP2C in recent years has been established as an important transcriptional regulator for trophoblast development and it role in this capacity will be discussed later. The appearance of

the blastocoel around day 3.5 indicates a multi-lineage blastocyst has formed, but the embryo is still not ready for implantation at this stage. Following blastocyst hatching from the zona pellucida, on day 4.5 the ICM further differentiates to form the pluripotent epiblast (EPI) and the primitive endoderm (PE) (88).

A fully developed blastocyst therefore consists of three cell lineages: TE, EPI, and PE. Several transcription factors drive differentiation from the 8-cell stage of the embryo towards these distinct lineages (Fig. 1-3). Caudal type homeobox 2 (CDX2), which is first expressed at the 8-cell stage in the outside polarized blastomeres, specifies the trophectoderm lineage (89). *Cdx2*$^{-/-}$ embryos experience a loss in the epithelial integrity in their TE, and in the expansion of the blastocoel. Moreover, other markers that specify TE showed a decrease in expression in the absence of CDX2, such as Eomesodermin (*Eomes*), a downstream target for CDX2. Transcription factors that maintain the pluripotency of ESCs specify for the ICM lineage (90). These include Octamer-binding transcription factor 4 (OCT4), Nanog homoeobox (NANOG), and Sex determining region Y-box 2 (SOX2), and are expressed in the embryo beginning around the 8-cell stage, but become restricted to the ICM during the morula to blastocyst transition (91-94). Nanog has an expanded role to also specify for EPI (93, 95). GATA-binding factor 6 (GATA6) expression is involved in primitive endoderm (PE) formation (88, 96). GATA6 does not only regulate downstream target genes in PE, but it also negatively regulates genes not associated with the PE lineage (Fig. 1-3) (97). OCT4 and NANOG repress *Cdx2* in the ICM, while conversely CDX2 represses *Oct4* in the TE (89, 98, 99). Similarly NANOG represses *Gata6* in EPI, and GATA6 represses *Nanog* in the PE (100, 101). These transcriptional regulatory loops converge with other regulatory mechanisms to determine lineage formation.

A mechanism delineating compaction and polarity of the developing embryo to these

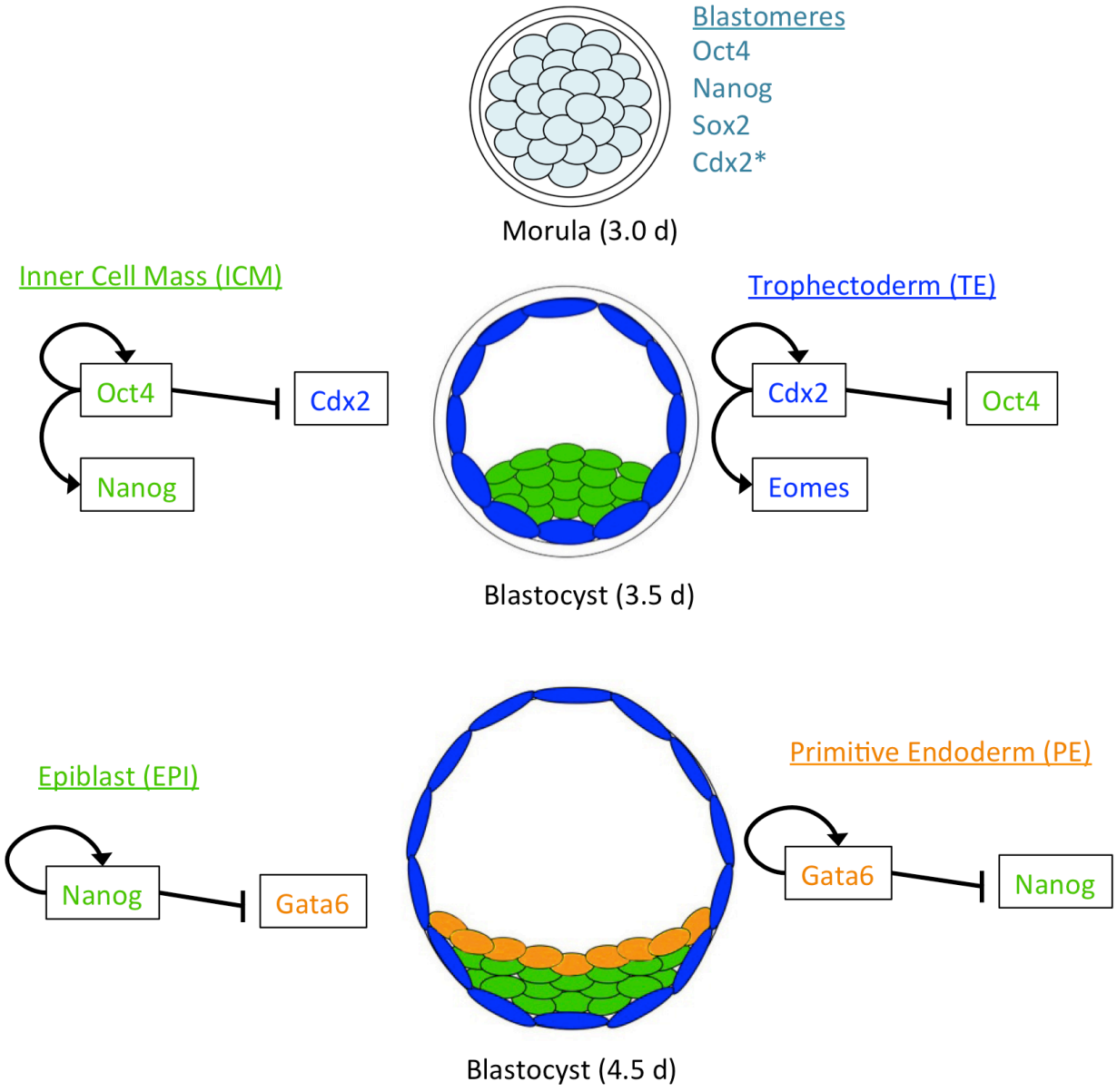

Figure 1-3. The mutually antagonistic transcription network of early lineages in the mouse embryo.

lineage-specific transcription networks occurs through position-dependent HIPPO signaling (102). HIPPO signaling is repressed in outer cells and activated in inside cells by a mechanism dependent on cell polarity (103). The consequence of repressed HIPPO signaling is that YES-associated protein (YAP) is not phosphorylated which results in its localization to the nucleus. Once in the nucleus, YAP is able to cooperate with TEA domain family member 4 (TEAD4), to activate *Cdx2*. We recently reported that TFAP2C also activates *Cdx2* in the TE lineage by two different mechanisms. First as a direct transcriptional activator of *Cdx2* and then through a mechanism that represses HIPPO signaling by acting on of the cell polarity protein PARD6B and the cytoskeleton regulator rho kinase (103). Position dependent HIPPO signaling has also been shown to regulate the expression of SOX2 and to restrict its expression to the ICM and later to the EPI (104).

When taken together, the transcriptional networks of the mouse embryo that confer early lineage formation and cell-fate decisions provide a working blueprint for both the maintenance of pluripotency and possible approaches to differentiate or reprogram cells away from their pluripotent condition.

The pluripotency signaling pathway

There is arguably no better case to make for the role that transcription factors perform in cellular programming than that of the Yamanaka factors for creating induced-pluripotency stem cells (iPSCs). Expression of just four specific factors, namely OCT4, SOX2, Kruppel-like factor 4 (KLF4) and c-Myc, allowed for fibroblasts to be converted back to an embryonic-like state that could give rise to all three germ layers (105). KLF4 is an important reprogramming factor and like OCT4 and SOX2 is able to bind the *Nanog* promoter and to regulate expression of

NANOG during reprogramming (106). Nonetheless these transcription factors do not maintain pluripotency on their own; rather they form the center of a dynamic network that is influenced by various cytokines and their corresponding signal transduction network (107). Furthermore, epigenetic mechanisms play a critical role in pluripotency due to unique aspects of chromatin and histone modifications that ultimately influence downstream targets of the transcription factor network (108).

Mouse ESCs are derived by the expansion of ICM cells from a blastocyst and are characterized by their ability to contribute to all three germ layers and therefore are pluripotent (109). This state of pluripotency is maintained by a transcription factor network consisting of OCT4, SOX2, and NANOG to promote self-renewal and prevent differentiation (110). Both ChIP-on-Chip and ChIP-seq experiments performed on this network established some general themes (111, 112). It was observed that many downstream targets were co-occupied by either two or three of the transcription factors. Additionally these transcription factors have the capacity to both autoregulate their own expression, and to regulate the expression of the other two transcription factors. A good example is NANOG; this transcription factor is considered the most critical factor within this network for establishing pluripotency (95, 113, 114). However the regulatory aspects of NANOG rely on the binding of an OCT4-SOX2 complex to the *Nanog* promoter, similary NANOG has been shown to bind to *Pou5f1* (Oct4 gene) and *Sox2* to positively regulate their expression, composing an autoregulatory loop (90, 115). Autorepression of NANOG is also essential to dictate a fluctuating pattern of its expression, which is critical for maintaining pluripotency (116, 117). NANOG has also been shown to bind to the largest number of downstream targets among the three factors and its association with OCT4 negatively regulates a subset of target genes important for preventing

differentiation (90, 111).

Unique chromatin signatures have been found at many of the pluripotency network target genes (118). Referred to as bivalent domains, chromatin from these regions contains both the activation-associated histone mark of histone H3 lysine 4 trimethylation (H3K4Me3) and the repressive histone mark of trimethylation on lysine 27 of histone H3 (H3K27Me3). This chromatin status suggests that lineage-specific genes are poised for rapid activation, but kept in check by repressive mechanisms. The polycomb-group proteins (PcG) are the repressive machinery involved in this process and are shown to associate with signal transducer and activator of transcription 3 (STAT3), which plays both an activational and repressive role in pluripotency signaling of ESCs (119). ESCs also have a large number of genes marked with histone H3 that has been acetylated at either lysine 9 and/or lysine 14 (H3K9Ac and/or H3K14Ac). H3K14Ac in particular is associated with poised genes where the initiation complex is already loaded to undergo rapid transcription but paused. These acetylation marks can be found within both the promoter and enhancer regions, expanding the classical role of these marks being associated exclusively with active transcription (120).

Various signal transduction pathways are associated with pluripotency network (Fig. 1-4). In mice, leukemia inhibitory factor (LIF) and bone morphogenic protein 4 (BMP4) were shown to block differentiation signaling from the mitogen-activated protein kinase (MAPK) pathway in a manner that utilizes STAT3. (121, 122). LIF/STAT3 signaling is dependent on the interaction of STAT3 with c-MYC to promote pluripotency (119). Interestingly, overexpression of NANOG can overcome the requirement for LIF and can still upregulate *Pou5f1* expression to maintain pluripotency (92). Wnt signaling has also been shown to modulate pluripotency signaling and appears to be conserved between mouse and human ESCs

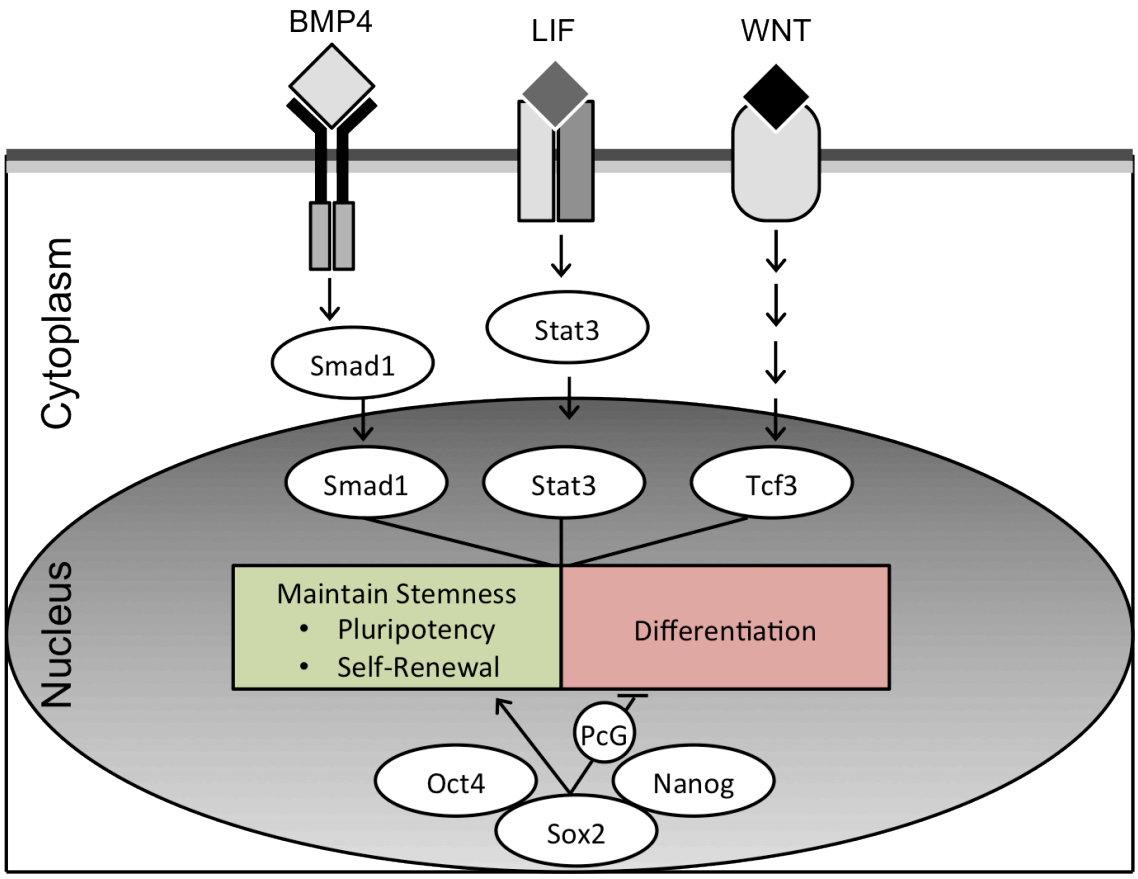

Figure 1-4. The convergence of signal transduction pathways on the pluripotency gene network.

(unlike LIF signaling) (107). While it is still questionable whether Wnt signaling serves to positively or negatively regulate pluripotency signaling versus differentiation, it has been established that Wnt signaling serves to inactivate T-cell factor 3 (TCF3)-mediated repression which is important for maintaining pluirpotency as TCF3 opposes the actions of OCT4 and NANOG at a subset of shared common downstream targets (123, 124).

Lessons from the embryo demonstrate that disruption of lineage-specific transcription factors can have substantial effects on pluripotency signaling and nuclear reprogramming. Either a loss of OCT4 expression or overexpression of CDX2 is capable of differentiating ESCs toward a trophoblast-like state (98, 125). These cells are distinct from true trophoblast stem cells (TSCs) that are derived from the extra-embryonic ectoderm after implantation, and are considered multipotent (89). TS-like cells retain more plasticity characteristic of ESCs due to epigenetic effects more common to the ESCs from which they were derived (126).

Brg1 functions in pluripotency signaling and lineage formation

Genetic ablation of BRG1 using a knockout approach was shown to affect the hatching of mouse blastocysts. Outgrowth experiments demonstrated that while the first lineages appeared morphologically normal, they were ultimately defective and resulted in embryonic lethality (9). The first indication that BRG1 might be associated with pluripotency came from an RNAi screen looking for genes associated with ESC identity (127). This screen identified BRG1, as well as BAF155 and ARID1a, all of which are now recognized to be components of the esBAF complex (73). We and others demonstrated that BRG1 occupies promoters of pluripotency network genes in ESCs and can alter their expression (67, 68). The specific manner as to how BRG1 alters the expression of these pluripotency genes is not well understood. BRG1 is found at genes

associated with differentiation that are silenced in ESCs as well as highly expressed genes associated with pluripotency and self-renewal (67, 68). For the core pluripotency transcription factors *Oct4*, *Nanog*, and *Sox2*, BRG1 initially has a repressive role on their expression, as ablation of BRG1 in ESCs by RNAi methods results in increased expression. However, this expression pattern is a short-term effect and over the course of several days the absence of BRG1 results in decreased expression of *Oct4*, *Nanog*, and *Sox2* and spontaneous differentiation of ESCs (73).

RNAi approaches are adventagous in that they can eliminate both the maternal and embryo-derived supply of transcripts in the preimplantation embryo. RNAi experiments conducted by the Knott lab demonstrated that BRG1 can function as both a coactivator and as a corepressor of pluripotency transcription factor genes in ESCs, but only as a corepressor of these same genes during lineage formation in the mouse embryo (68, 128). This was determined by experiments where gene expression was monitored in BRG1 KD embryos at the blastocyst stage and it was discovered that the transcript levels of *Oct4*, *Nanog*, and *Sox2* had increased. Downregulation of BRG1 had a significant impact on the segregation of OCT4 to the ICM (68, 128). BRG1 depleted embryos confer a similar phenotype at the blastocyst stage as *Cdx2*$^{-/-}$ embryos where OCT4 is expressed in the TE (89, 128). Importantly BRG1 interacts with CDX2 and both proteins occupy the *Oct4* promoter (128). Taken together these observations demonstrate that BRG1 plays an important role in differentiation towards the TE lineage and suggests that the interacting partners of BRG1 or presumably of the esBAF complex can influence whether BRG1 acts as a coactivator or a corepressor.

The unique composition of the esBAF complex in ESCs versus the BAF complexes in somatic cells serves as a possible switch where the function of BRG1 could be altered to act as

either a coactivator or a corepressor, particularly as it relates to switching from supporting pluripotency to inducing differentiation (67, 73, 129). However, esBAF complexes continue to display heterogeneity of their subunit compositions as noted by their ARID domain-containing subunits and the requirement of both an embryo-specific BAF and PBAF to maintain pluripotency (129, 130). Additional bromodomain containing (BRD) subunits, specifically BRD7 and BRD9 have also been shown to be important for regulating pluripotency for both activation and repression, and also display subunit heterogeneity (131, 132). These observations suggest that during differentiation the switching of subunits such as BAF155 or BAF60a to BAF170 or BAF60c, respectively, may serve as one of several cues in a dynamic system of processes that could control the function of BRG1.

BRG1 also converges with LIF/STAT3 signaling to regulate the pluripotency gene network (133). A subset of pluripotency genes which are regulated by LIF and are activated to promote pluripotency are unable to be occupied by STAT3 when BRG1 is absent. This leads to an increase of H3K27me3 histone marks and repression of these target genes by PcG. Withdrawal of LIF alone does not result in an increase in these histone marks, demonstrating that BRG1 opposes the function of PcG while maintaining pluripotency. However, in a way that also supports pluripotency, BRG1 functions through PcG to repress HOX clusters thereby supporting PcG function (133). One facet regarding this type of regulation, which I think the authors might have overlooked is the role of H3K4me3 histone marks in distinguishing the function of BRG1 in the PcG context. The authors mention that the levels of the H3K4me3 histone mark are not affected at target gene sites for STAT3, however there is no mention of the dynamics of the H3K4me3 marks at other genes. The HOX genes were previously shown to be marked by bivalent domains, containing both H3K4me3 and H3K27me3 histone marks, which is associated

with genes that are poised for activation, but repressed in pluripotency signaling (118). Speculatively, this difference could represent another type of cue that refines the expression pattern within pluripotency signaling. This LIF/STAT3/BRG1 regulatory mechanism just described demonstrates the importance of epigenetic markers in regulating pluripotency by associating with H3K27me3 histone marks.

Epigenetic marks also converge with BRG1 in regulation of pluripotency signaling through association of members of the esBAF complex with members of the nucleosome remodeling deacetylase (NURD) complex (134). These two complexes regulate genes that are marked by 5-hydroxy-methylcytosine, providing a method for recruitment to their targets. ChIP-seq analyses showed that occupancy of BRG1 was similar compared to methyl-CpG-binding domain protein 3 (MBD3), a member of NURD and consistently occurred 100-200 bp upstream of MBD3 in an oriented manner with respect to the direction of transcription at a subset of gene promoters. Co-IP experiments further demonstrated interaction between members of esBAF and NURD (73). This finding is important because it illustrates numerous interacting partners for BRG1 that might contribute to modulate its role as a coactivator or a corepressor. It was shown by knocking down either BRG1 or MBD3 that these proteins regulate a common set of genes in an opposing manner. Many of the target genes regulated by this mechanism were shown to be important for pluripotency, including KLF4, but the core pluripotency transcription factors of OCT4, NANOG, and SOX2 were not part of the subset of genes marked by the 5-hydroxy-methylcytosine or affected by the loss of MBD3 (134).

In reviewing a large portion of the known mechanisms through which BRG1 has been shown to affect the expression profile of pluripotency genes, nearly all of the mechanistic information relates to BRG1 acting as a coactivator. Our data suggests that BRG1 may work in

repression of the core pluripotency genes but how it may do this is unclear. As these critical transcription factors influence the establishment of both ESCs and early lineage formation in the pre-implantation embryo, the potential benefits for deciphering the possible repressive mechanisms of BRG1 appear to be vast.

One obvious benefit is increased understanding of how the TE and ICM are established. Understanding the molecular mechanisms involved in lineage formation has implications on reducing early loss of pregnancy and improving methods of assisted reproductive technologies (ART) as these early lineages are crucial for implantation to take place and formation of a functional placenta, which is required for a viable pregnancy. Moreover, new insights could be gained about the formation of pluripotent stem cells, and differentiation of these cells into progenitor cells of various tissue types to improve the understanding of stem cell-based therapies to a number of diseases. Furthermore, there are numerous descriptions about how BRG1 has been recognized as having a role in cancer biology, acting as a tumor suppressor in various types of lung cancer, where as high expression has been found to be associated with metastasis in various types of breast cancer (28, 135, 136). Establishing the molecular mechanisms through which BRG1 regulates pluripotency might be applied to help decipher the molecular mechanisms of cancer stem cells.

I propose that complexity rather than conservation of subunit composition of mammalian BAFs provides a possible mechanism to control distinct functional roles in varying developmental contexts. BAFs and other proteins that associate with the BAF complexes could serve as a context-specific platform to recruit proteins to their site of action through interactions with lineage-specific transcription factors. BAFs, though interactions with other histone binding proteins, have been shown to be recruited to distinct histone modifications (132). I hypothesize

that interactions of BRG1 with corepressors that are recruited via these context-specific epigenetic histone signals serve as a way to potentiate a temporal and lineage-specific repression complex in the early embryo.

In chapter II, I characterize the transcriptional and epigenetic processes that occur during CDX2-induced silencing of *Pou5f1* and *Nanog* as a model system for TE development. Observations from this study helped establish that HDAC1 may be involved in the repression of these genes, acting at the OCT4-SOX2 element where changes in histone H3 K9/K14AC and changes in chromatin structure were also observed. The coordinated timing of these events was also established and was shown to occur before the genes become methylated.

In chapter III, I show that BRG1 interacts with HDAC1 to regulate the expression of NANOG through a process that requires histone deacetylation and chromatin remodeling. I also show that the association of BRG1 with HDAC1 primarily occurs in the TE rather than in the ICM for the developing blastocyst.

In chapter IV, I discuss my major findings on how BRG1 interacts with HDAC1 in a lineage-specific manner to regulate pluripotency gene expression in ESCs and the trophoblast lineage. In addition, I discuss preliminary data that suggest a BRG1-HDAC1 complex modulates Wnt signaling during early embryonic development.

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CHAPTER 2 -

TRANSCRIPTIONAL REPROGRAMMING AND CHROMATIN REMODELING ACCOMPANIES *OCT4* AND *NANOG* SILENCING IN MOUSE TROPHOBLAST LINEAGE[1]

[1]The work described in this chapter was published as the following manuscript: Timothy S. Carey, Inchul Choi, Catherine A. Wilson, Monique Floer, and Jason G. Knott. (2014) Transcriptional reprogramming and chromatin remodeling accompanies Oct4 and Nanog silencing in mouse trophoblast lineage. Stem Cells Dev *23*, 219-229.

Abstract

In mouse blastocysts CDX2 plays a key role in silencing *Oct4* and *Nanog* expression in the trophectoderm (TE) lineage. However, the underlying transcriptional and chromatin-based changes that are associated with CDX2-mediated repression are poorly understood. To address this a Cdx2-inducible mouse embryonic stem (ES) cell line was utilized as a model system. Induction of *Cdx2* expression resulted in a decrease in *Oct4/Nanog* expression, an increase in TE markers, and differentiation into trophoblast-like stem (TS-like) cells within 48 to 120 hours. Consistent with the downregulation of *Oct4* and *Nanog* transcripts, a time-dependent increase in CDX2 binding and a decrease in RNA polymerase II (RNAPII) and OCT4 binding was observed within 48 hours ($P<0.05$). To test whether transcriptionally active epigenetic marks were erased during differentiation, histone H3K9/14 acetylation and two of its epigenetic modifiers were evaluated. Accordingly, a significant decrease in histone H3K9/14 acetylation and loss of p300 and HDAC1 binding at the *Oct4* and *Nanog* regulatory elements was observed by 48 hours. Accompanying these changes there was a significant increase in total histone H3 and a loss of chromatin accessibility at both the *Oct4* and *Nanog* regulatory elements ($P<0.05$), indicative of chromatin remodeling. Lastly, DNA methylation analysis revealed that methylation did not occur at *Oct4* and *Nanog* until 96 to 120 hours after induction of CDX2. In conclusion, our results show that silencing of *Oct4* and *Nanog* is facilitated by sequential changes in transcription factor binding, histone acetylation, chromatin remodeling, and DNA methylation at core regulatory elements.

Introduction

The first cell-fate decision in the mouse preimplantation embryo, inner cell mass (ICM) and trophectoderm (TE) segregation, is mediated by the transcription factors Octamer 3/4 (OCT4), NANOG, and Caudal-like homeobox 2 (CDX2)(1-4). Prior to blastocyst formation OCT4, NANOG, and CDX2 are widely expressed at the 8-16 cell stage, however, during lineage segregation OCT4 and NANOG become restricted to the pluripotent ICM, while CDX2 is confined to the TE epithelium (5). The proper expression of OCT4, NANOG, and CDX2 in blastocysts is required for normal implantation and continued development (2-4).

The current model for segregation of the ICM and TE proposes that CDX2 represses *Oct4* and *Nanog* expression in the TE lineage, whereas OCT4 and NANOG downregulate *Cdx2* expression in the pluripotent ICM (4, 6, 7). In support of this model embryos deficient in *Cdx2* fail to repress *Oct4* and *Nanog* expression in the TE lineage (4, 8, 9). Studies in ES cells showed that forced expression of *Cdx2* or ablation of *Oct4* induces differentiation towards a TE cell-fate via CDX2-OCT4 and CDX2-*Oct4* enhancer interactions (6, 8). Alternatively, in trophoblast stem (TS) cells forced expression of *Oct4* alone or in combination with other reprogramming factors promotes a ES cell-fate through suppression of *Cdx2* and other TS cell regulators (10, 11). Collectively, these findings demonstrate that OCT4, NANOG, and CDX2 participate in a mutually exclusive antagonistic relationship to facilitate the first cell-fate decision in mouse blastocysts.

Previously we demonstrated that CDX2-mediated repression of *Oct4* expression in TE is facilitated by both transcriptional and epigenetic events in the mouse(8). For example, in both preimplantation embryos and Cdx2-inducible ES cells the chromatin remodeling protein Brahma related-gene 1 (BRG1) cooperates with CDX2 to downregulate *Oct4* transcription in the TE

lineage. Interestingly, CDX2/BRG1-dependent repression of *Oct4* expression in the blastocyst TE does not involve DNA methylation (8). In support of this view, during early mouse embryogenesis *Oct4* and *Nanog* do not acquire DNA methylation until after implantation (12). Combined, these data suggest that during blastocyst formation other transcriptional and chromatin-based changes are involved in the repression of *Oct4* and *Nanog* expression in TE.

To further investigate the transcriptional and chromatin-based processes that are associated with *Oct4* and *Nanog* silencing in the emerging TE lineage, we utilized a well-characterized Cdx2-inducible ES cell line that differentiates into TS-like cells (13). Here we report that CDX2-mediated silencing of *Oct4* and *Nanog* expression is associated with a well-orchestrated series of overlapping transcriptional and chromatin-based events at core regulatory elements, *i.e.* the Oct-Sox motif, the proximal promoter regions and the transcriptional start site (TSS). Major transcriptional and chromatin-based changes preceded the onset of DNA methylation, which occurred after *Oct4* and *Nanog* were already downregulated.

Materials and Methods

Embryonic stem (ES) cell culture, differentiation, and trophoblast stem (TS) cell culture

Cdx2-inducible ES cells were provided by Dr. Minoru Ko of the NIA and were cultured as previously described (8, 13-15). In brief, cells were grown on a feeder layer of mitomycin-treated puromycin-resistant mouse embryonic fibroblasts in standard ES cell media, supplemented with 0.2 μg/ml of doxycycline and 1.0 μg/ml of puromycin. Prior to Cdx2 induction cells were switched onto gelatin and cultured with 3.0 μg/ml of puromycin for 3 days. *Cdx2* expression was induced by removal of doxycycline. Leukemia inhibitory factor (LIF) was removed 48 hours after induction. After 96 hours cells were cultured in TS cell medium

containing fibroblast growth factor 4 (FGF4) (8, 16). TS cells were derived as described previously (8, 16). Cdx2 induction was verified by quantitative (q)RT-PCR and by western blot using a Flag antibody (Sigma-Aldrich, St. Louis, MO, USA).

qRT-PCR analysis and western blot

Cells were harvested, flash frozen, and stored at -80°C until isolation. RNA isolation was performed using RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA synthesis was then performed using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR analysis was then performed with TaqMan probes, or gene specific primers using SYBR green detection on a StepOne Plus thermocycler (Applied Biosystems, Foster City, CA, USA). Eukaryotic translation elongation factor 1 alpha 1 (*Eef1a1)* was used as an endogenous control for gene expression analysis. Western blot analysis was performed as previously described (8). In brief, whole cell lysates were size fractionated by SDS-PAGE and transferred to PVDF membrane. Antibodies used to detect the expression of Flag-CDX2, OCT4, NANOG, and β-ACTIN (as a loading control) are listed in Table 2-1 in the appendix. Quantification of the levels of CDX2, OCT4, and NANOG were performed using ImageJ (NIH).

Chromatin-immunoprecipitation (ChIP) analysis

ChIP analysis was performed as previously described (8, 14, 15, 17). In brief, cells were fixed with 1% formaldehyde then flash frozen. Chromatin lysate was prepared by sonication and an extract equivalent to 2 million cells was used for each IP. ChIP was carried out using commercially available antibodies for RNA Polymerase II (RNAPII), acetyl histone H3K9/14, mouse IgG, rabbit IgG, histone H3, OCT4, p300, HDAC1, and Flag. These antibodies are listed

in Table 2-1 in the appendix. The BRG1 anti-serum was obtained from Dr. Anthony Imbalzano (UMASS, Medical School, Worcester, MA). ChIP data were analyzed by quantitative real-time PCR (qPCR) with SYBR green reagents (Applied Biosystems) using the percent input method.

Restriction endonuclease chromatin accessibility assay

Chromatin accessibility assay was adapted from methods developed earlier (18, 19). Five million fresh cells were washed in PBS, pelleted, and lysed for 5 minutes on ice in nuclear lysis buffer containing 10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5% IGEPAL CA-630, 0.15 mM spermine, and 0.5 mM spermidine. Following centrifugation, nuclei were washed in nuclear wash buffer containing 10 mM Tris (pH 7.4), 50 mM NaCl, 10 mM $MgCl_2$, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM β-mercaptoethanol, 0.15 mM spermine, and 0.5 mM spermidine. Nuclei were again pelleted and suspended in restriction enzyme buffer 4 (New England Biolabs, Ipswich, MA, USA). Isolated nuclei were then treated with restriction endonucleases DdeI or MseI (New England Biolabs) specific for single cut sites located within the amplicons for *Oct4* and *Nanog*, respectively. The control intergenic region also contains a cut site for the restriction enzyme MseI, allowing monitoring of an unchangeable chromatin state for MseI digestion, as well as to test for non-specific digestion by treatment with DdeI. The length of digestion as well as specificity of restriction enzymes for their respective amplicons was optimized prior to performing assay (Data not shown). Samples were digested at 37°C for 10 and 40 minutes for DdeI and MseI, respectively. The reaction was stopped by adding SDS and samples were then heated at 65°C for 20 minutes. DNA was then extracted, and compared to uncut genomic samples by qPCR analysis with 25 ng of template DNA. Data are presented as a percentage of the DNA cut by each restriction enzyme (18).

Bisulfite sequencing analysis of DNA methylation

DNA methylation was assessed as previously described (8). Briefly, isolated genomic DNA was bisulfite treated using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). Differentially methylated regions (DMR) were evaluated by performing two successive rounds of PCR using nested primers. PCR products were then TA-cloned into the pCR2.1 TOPO vector (Invitrogen) and colonies were randomly picked for sequencing.

Statistical analysis

Data from qRT-PCR, ChIP, chromatin accessibility assay, and bisulfite sequencing were analyzed using Student's *t*-test to determine statistical differences between time points. A p-value of <0.05 was considered significant.

Results

Experimental model for investigation of Oct4 and Nanog silencing in the TE lineage

To delineate the underlying transcriptional and epigenetic changes that accompany *Oct4* and *Nanog* silencing in the TE lineage, a mouse Cdx2-inducible ES cell line was obtained from Ko and coworkers (13). This cell line is tetracycline repressible and is engineered to express a Flag-CDX2 along with Venus via an IRES element. Previous work from multiple laboratories showed that Cdx2-inducible ES cells can be differentiated into TS-like stem cells that resemble native TS cells in terms of function and potential to contribute to placentas in chimeric embryos (6, 13, 20). Moreover, the inducible ES cell-line used in this study phenotypically resembles TS cells in terms of expression and function (13, 14). A major advantage with using Cdx2-inducible ES cells is that one can monitor transcriptional and epigenetic changes in an identical genetic

background during the differentiation process. The design of our study was based upon work by Ko and coworkers, where global microarray transcriptional profiling and western blot analyses were performed (13). These data provided the framework for the experimental time points that we adopted to monitor silencing of *Oct4* and *Nanog*. Three distinct cellular conditions were chosen: uninduced ES cells, partially differentiated Cdx2 induced cells at 48 hours post-induction, and Cdx2 induced ES cells at 96 hours. For each of these time points cells were collected for qPCR, western blot, ChIP, chromatin remodeling assays, and bisulfite sequencing (Fig. 2-1A). In addition, intermediate collections were obtained at 72 and 120 hours post-induction to monitor the kinetics of DNA methylation.

In preliminary experiments qRT-PCR confirmed that the levels of *Cdx2* transcripts were strongly induced, and the levels of *Oct4* and *Nanog* transcripts progressively decreased at 48 and 96 hours post-induction. *Cdx2* transcripts were increased by ~32 and 93-fold at 48 and 96 hours, respectively, while *Oct4* and *Nanog* were downregulated 3 to 4-fold at 48 hours, but were strongly repressed (> 20-fold) by 96 hours post induction (Fig. 2-1B). Furthermore, western blot analysis confirmed that Flag-CDX2 was strongly expressed at 48 hours and remained constant at 96 hours (Fig. 2-1C). Steady-state protein levels of NANOG and OCT4 seemed to differ slightly, despite the similarities in transcriptional regulation. For example, NANOG was completely depleted by 48 hours post induction, whereas OCT4 was partially reduced, but was then completely lost by 96 hours (Fig. 2-1C and D). Consistent with the increase in CDX2 and decrease in *Oct4* and *Nanog* expression, several TS cell genes such as *Eomes*, *Fgfr2*, and *Elf5* (21, 22) were strongly induced at 48 and 96 hours (Fig. 2-1E). In addition, other markers of TE epithelium such as *Krt18*, *Cldn4*, and *Pard6b* are upregulated in these Cdx2-inducible ES cells (14). These data in combination with previous work (6, 8, 13, 20) demonstrate that Cdx2-

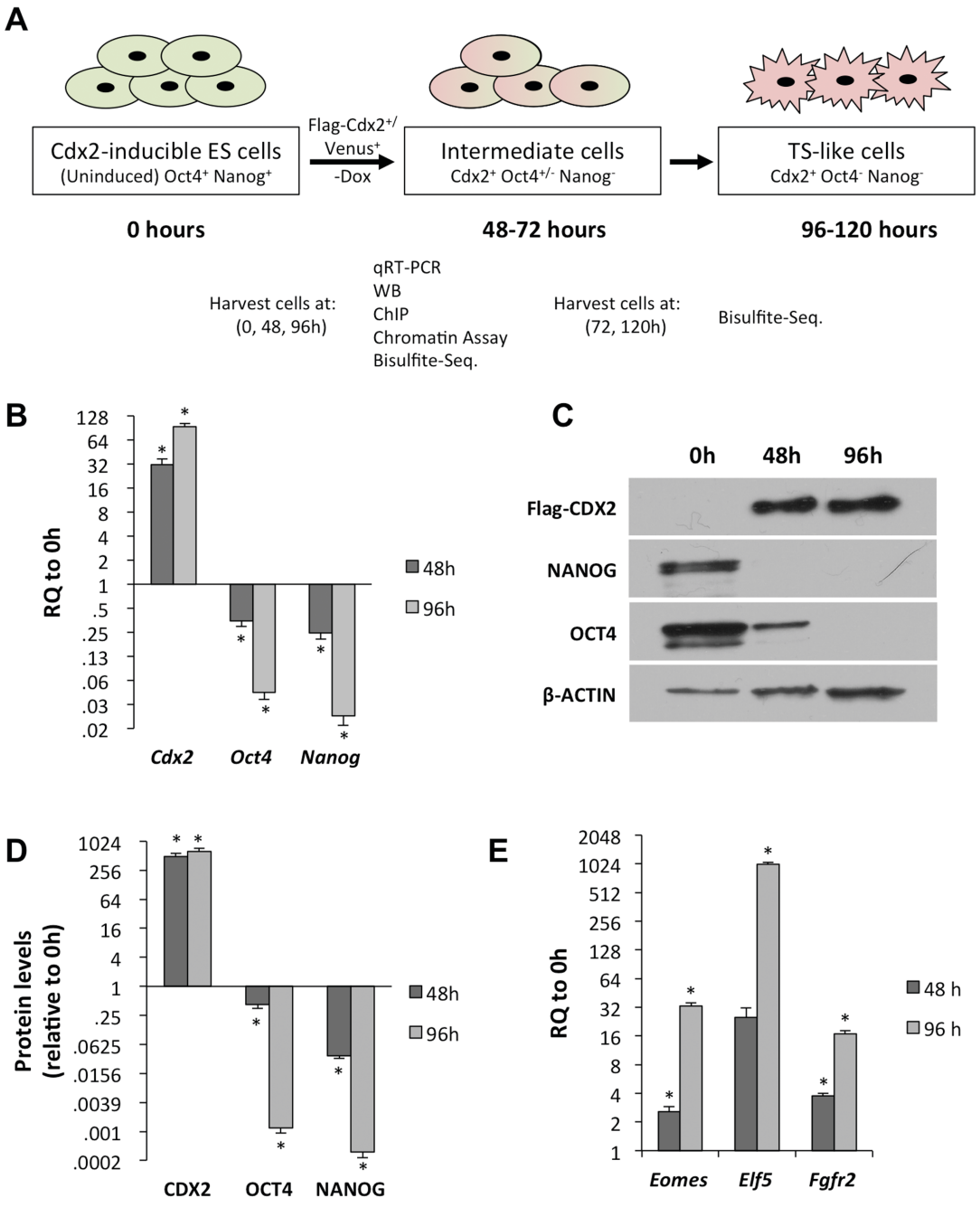

Figure 2-1. Cdx2-inducible ES cell model for *Oct4* and *Nanog* silencing. (A) Schematic describing the developmental time course for sample collection. For each time point samples were subjected to qRT-PCR, western blot, ChIP, chromatin accessibility assays, and bisulfite sequencing. A total of two biological cell replicates were utilized for these experiments. (B) qRT-PCR analysis of *Cdx2*, *Oct4*, and *Nanog* expression at 0, 48, and 96 hours following the

Figure 2-1 (cont'd)

induction of Cdx2; RQ (Relative Quantification). Two technical replicates in duplicate were performed for each biological preparation. (C) Western blot analysis of Flag-CDX2, OCT4, and NANOG expression at 0, 48, and 96 hours following the induction of Cdx2. Samples were normalized to *B*-ACTIN. Two technical replicates were performed for each biological preparation. (D) Quantification of the expression of Flag-CDX2, OCT4, and NANOG protein in Cdx2-inducible ES cells at 48 and 96 hours. The levels of each protein are normalized to *B*-ACTIN and are relative to control uninduced ES cells. (E) qRT-PCR analysis of *Eomes*, *Elf5*, and *Fgfr2* expression at 0, 48, and 96 hours following the induction of Cdx2. Two technical replicates in duplicate were performed for each biological preparation.

inducible ES cells are a suitable cell-based system for investigating the underlying molecular changes that accompany TE development in mice.

Recruitment of CDX2 is associated with loss of OCT4 and RNA polymerase II at Oct4 and Nanog

A critical aspect of transcriptional regulation within the core pluripotency circuitry is autoregulation and activation of other transcription factors within the network. With regard to both *Oct4* and *Nanog*, these genes contain an upstream Oct-Sox cis-regulatory element (23, 24). Previously, we and others showed that CDX2 is recruited to the Oct-Sox binding motif in the *Oct4* promoter within 24 hours of Cdx2 induction (6, 8). Thus, we explored whether this binding was a common occurrence during the repression of both *Oct4* and *Nanog*. To accomplish this we performed a more extensive quantitative ChIP analysis of cells collected at 0, 48 and 96 hours post CDX2 induction. Primers were designed that flanked the Oct-Sox binding motif ~2000 bp upstream of the transcriptional start site (TSS) on *Oct4* and ~180 bp upstream the TSS on *Nanog*. An intergenic region not containing any known cis-elements was used as a negative control. Accordingly, we observed a significant increase in CDX2 binding at the Oct-Sox binding motif at 48 and 96 hours post induction ($p<0.05$). An increase in binding was not observed at the intergenic control region nor in the IgG samples ($p>0.05$; Figuure 2-2A).

To test whether the recruitment of CDX2 was associated with a loss of transcriptional machinery and/or core transcription factors, we utilized qChIP to monitor RNA Polymerase II (RNAPII) and OCT4 occupancy during differentiation. In addition we utilized a second primer set for the *Oct4* proximal promoter/TSS region. The occupancy of RNAPII and OCT4 was

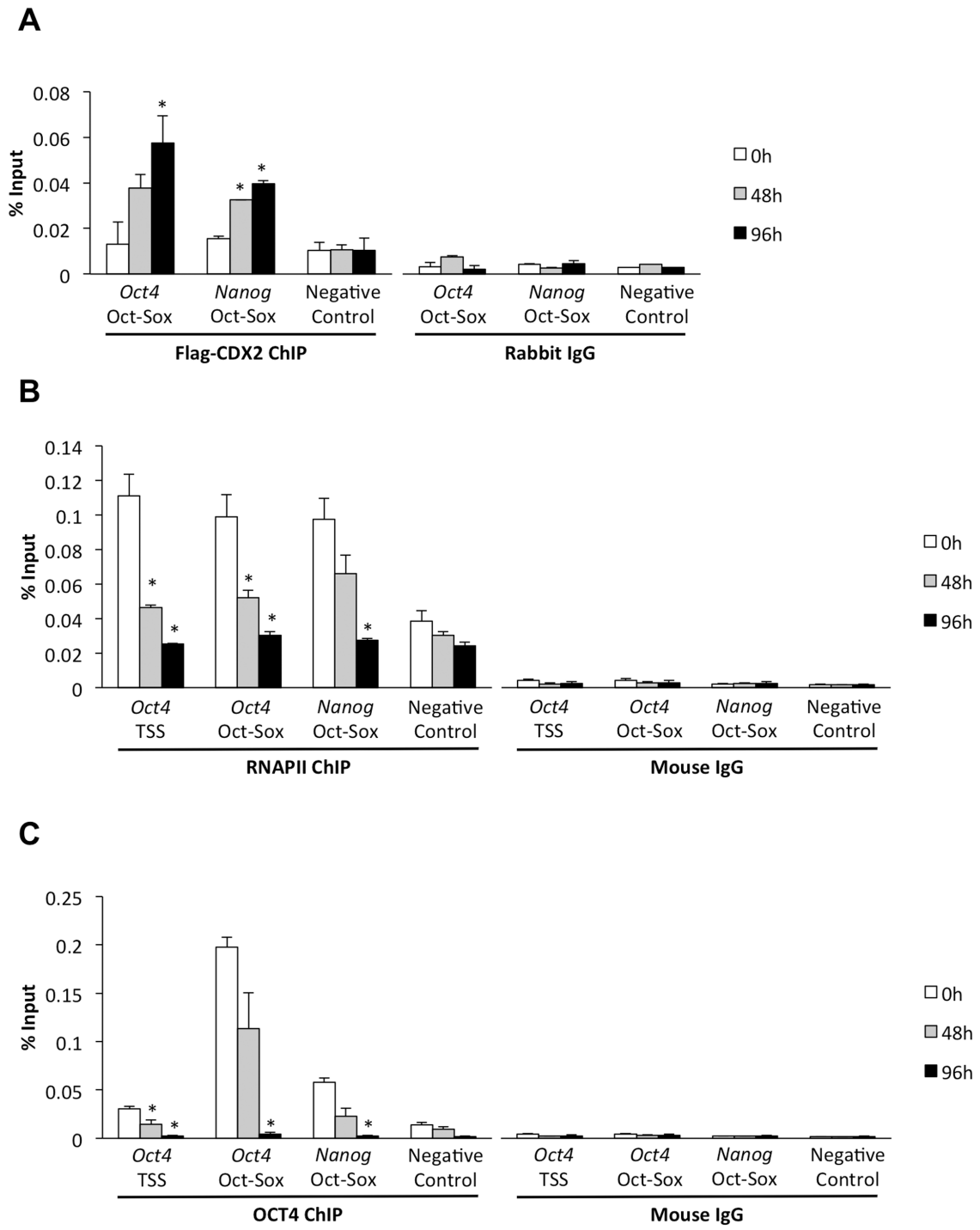

Figure 2-2. Recruitment of CDX2 and loss of RNAPII and OCT4 at *Oct4* and *Nanog*. (A) ChIP analysis of CDX2 recruitment to the Oct-Sox binding motif in *Oct4* and *Nanog* at 0, 48,

Figure 2-2 (cont'd)

and 96 hours. (B) ChIP analysis of RNAPII occupancy at the TSS and Oct-Sox binding motif in *Oct4* and the Oct-Sox binding motif in *Nanog* at 0, 48, and 96 hours. (C) ChIP analysis of OCT4 binding to the Oct-Sox2 binding motif and TSS in *Oct4* and the Oct-Sox binding motif in *Nanog* at 0, 48, and 96 hours. As a negative control a mouse and rabbit non-specific IgG was used. An intergenic region was utilized as a negative control for CDX2, RNAPII, and OCT4 binding. A total of two ChIP replicates were performed for each biological replicate. For each ChIP replicate a total of two PCR reactions were performed in duplicate.

rapidly lost at the *Oct4* and *Nanog* regulatory elements as the cells underwent Cdx2 induced differentiation ($p<0.05$; Fig. 2-2B and C). Interestingly, the recruitment of CDX2 and the loss of RNAPII and OCT4 were tightly correlated with the decrease in *Oct4* and *Nanog* transcripts at these time points (Fig. 2-1 and 2-2). Collectively, these experiments demonstrate that *Oct4* and *Nanog* are bona fide targets of CDX2 and that their transcriptional repression is associated with the loss of OCT4 and RNAPII binding.

Changes in histone acetylation accompany Oct4 and Nanog silencing

To begin to investigate the chromatin-based mechanisms that are associated with *Oct4* and *Nanog* silencing in the emerging trophoblast lineage, histone H3 lysine 9 and 14 (K9/14) acetylation was monitored because it largely associated with active transcription in eukaryotic cells (25). We postulated that loss of H3K9/14 acetylation might be coupled with transcriptional silencing of *Oct4* and *Nanog* in ES cells undergoing differentiation into TS-like cells. Previous studies demonstrated that differences in histone H3 acetylation levels exist between ES cells and TS cells in mice (26, 27), however, temporal changes in histone acetylation at the *Oct4* and *Nanog* regulatory elements were not evaluated during differentiation. To address this qChIP analysis was conducted utilizing primers along the *Oct4* and *Nanog* regulatory elements as described in the previous section. In uninduced ES cells H3K9/14 acetylation was strongly enriched at both the Oct-Sox binding motif and TSS in these genes. However, at 48 and 96 hours after Cdx2 induction the levels of H3K9/14 acetylation were dramatically reduced ($p<0.05$; Fig. 2-3A). Changes in acetylation were not observed at a control intergenic region which exhibited low enrichment of H3K9/14 acetylation throughout differentiation ($p>0.05$; Fig. 2-3A).

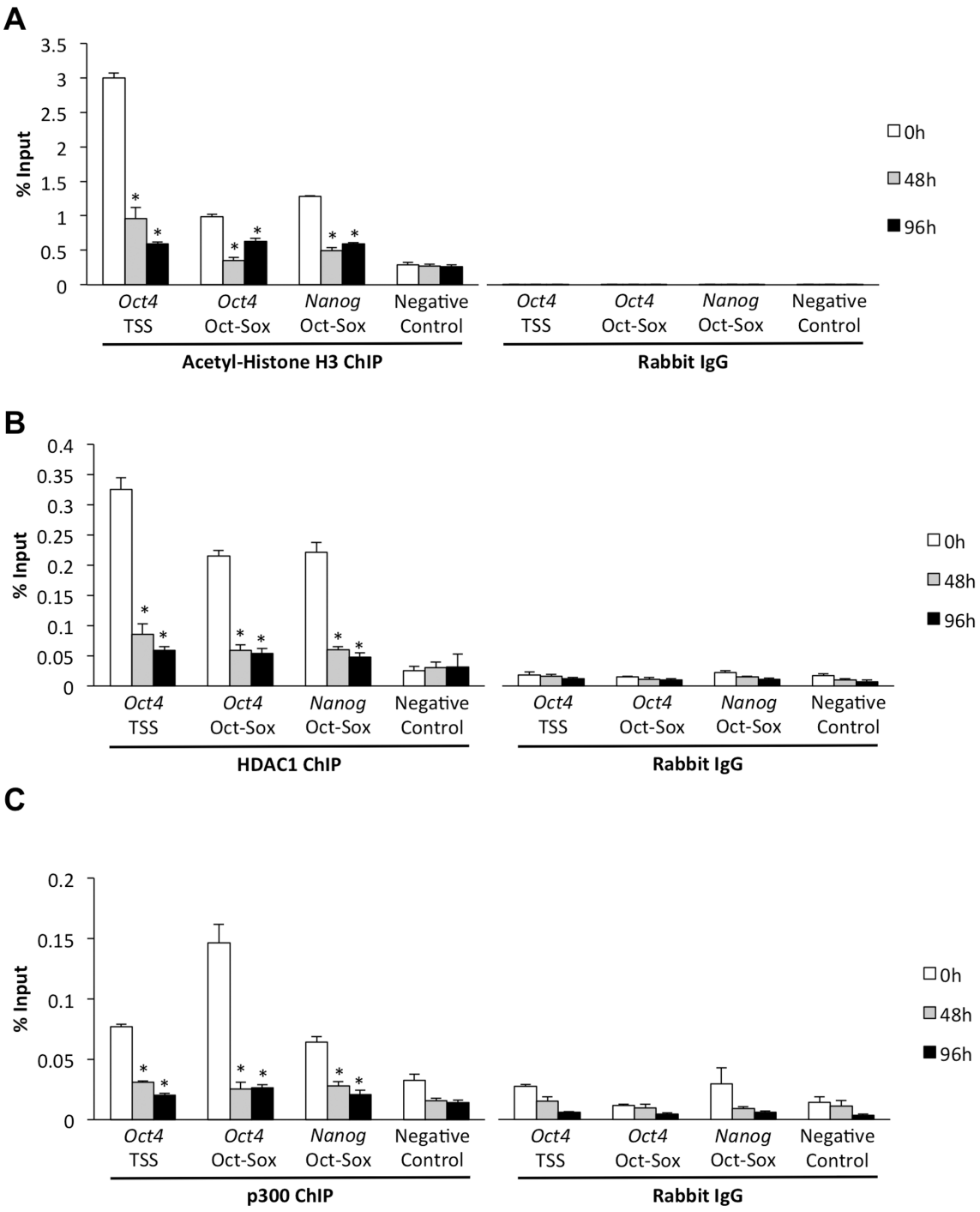

Figure 2-3. Loss of histone H3K9/14 acetylation, HDAC1, and p300 at *Oct4* and *Nanog*. (A) ChIP analysis of histone H3K9/14 acetylation at the Oct-Sox binding motif and TSS in *Oct4* and Oct-Sox binding motif in *Nanog* at 0, 48, and 96 hours. (B) ChIP analysis of HDAC1 occupancy

Figure 2-3 (cont'd)

at the Oct-Sox binding motif and TSS in *Oct4* and the Oct-Sox binding motif in *Nanog* at 0, 48, and 96 hours. (C) ChIP analysis of p300 occupancy at the Oct-Sox2 binding motif and TSS in *Oct4* and the Oct-Sox binding motif in *Nanog* at 0, 48, and 96 hours. As a negative control a rabbit non-specific IgG was used. An intergenic region was utilized as a negative control for H3K9/14 acetylation, and HDAC1 and P300 binding. A total of two ChIP replicates were performed for each biological replicate. For each ChIP replicate two PCR reactions were performed in duplicate.

Two types of epigenetic regulators that are known to either remove or deposit histone acetylation marks include histone deacetylases (HDACs) and histone acetyltransferases (HATs), respectively. HDAC1 and the HAT p300 were previously shown to bind to *Oct4* and *Nanog* regulatory elements in mouse ES cells (28-30). Moreover, in Cdx2-inducible ES cells and other cellular contexts CDX2 can interact with both HDAC1 and p300 (13, 31, 32). Thus, we hypothesized that the recruitment of CDX2 and concomitant decrease in H3K9/14 acetylation was associated with either recruitment and/or loss of HDAC1 and p300 on *Oct4* and *Nanog.* Interestingly, qChIP analysis revealed that HDAC1 and p300 were highly enriched at *Oct4* and *Nanog* in uninduced ES cells, but their binding was rapidly lost during differentiation ($p<0.05$; Fig. 2-3B and C). Collectively, these results suggest that transcriptional repression of *Oct4* and *Nanog* in Cdx2-inducible ES cells is associated with a decrease in histone H3K9/14 acetylation and loss of HDAC1 and p300 occupancy at key regulatory elements.

Changes in chromatin structure at core regulatory regions accompanies Oct4 and Nanog silencing

It is well established that histone acetylation is an active transcription mark and accompanies more open chromatin structure to facilitate binding of transcription factors and core promoter machinery (33-35). Because histone H3K9/14 acetylation was markedly reduced at the Oct-Sox binding motif in *Oct4* and *Nanog* during differentiation, we postulated that chromatin remodeling might accompany gene silencing. To address this we utilized a well-established qPCR-based restriction enzyme chromatin accessibility assay (18). ES cells were collected at 0, 48, and 96 hours after Cdx2 induction and isolated nuclei were exposed to restriction enzymes

specific for a single cut site located proximal to the Oct-Sox2 motif in *Oct4* and *Nanog*, or an intergenic region (no binding site) serving as a negative control (Fig. 2-4A). For both *Oct4* and *Nanog*, restriction enzyme accessibility was the greatest in uninduced ES cells, however, at 48 and 96 hours after Cdx2 induction there was a progressive loss in chromatin accessibility indicative of chromatin remodeling ($p<0.05$; Fig. 2-4B). No changes in chromatin accessibility were observed in the control intergenic region (Fig. 2-4B)

To confirm that the loss of accessibility was due to a change in nucleosome occupancy, histone H3 enrichment was monitored by qChIP at the Oct-Sox binding motif in *Oct4* and *Nanog*; histone H3 ChIP analysis is an excellent method for evaluating and/or confirming nucleosome occupancy at gene promoters (36). In uninduced ES cells the levels of histone H3 were low, however, as differentiation proceeded histone H3 levels dramatically increased ($p<0.05$; Fig. 2-4C). Analysis of *Gapdh*, a constitutively active gene whose expression does not change during differentiation showed that histone H3 levels remained constant ($p>0.05$; Fig. 2-4C). These results demonstrate that transcriptional silencing of *Oct4* and *Nanog* in the developing trophoblast lineage is accompanied with changes in chromatin structure at core enhancers. These findings are consistent with our previous work where we demonstrated that the chromatin remodeling protein brahma-related gene 1 (BRG1) functions as a critical corepressor of *Oct4* and *Nanog* in blastocyst TE (8, 17); reviewed by (37). In support of this ChIP analysis revealed that BRG1 occupies the Oct-Sox binding motif in *Oct4* and *Nanog* in Cdx2-induced ES cells (8) and (See Appendix, Fig. 2-7). Additional functional studies are necessary to establish the chromatin-based mechanism by which BRG1 regulates *Oct4* and *Nanog* silencing in the TE lineage.

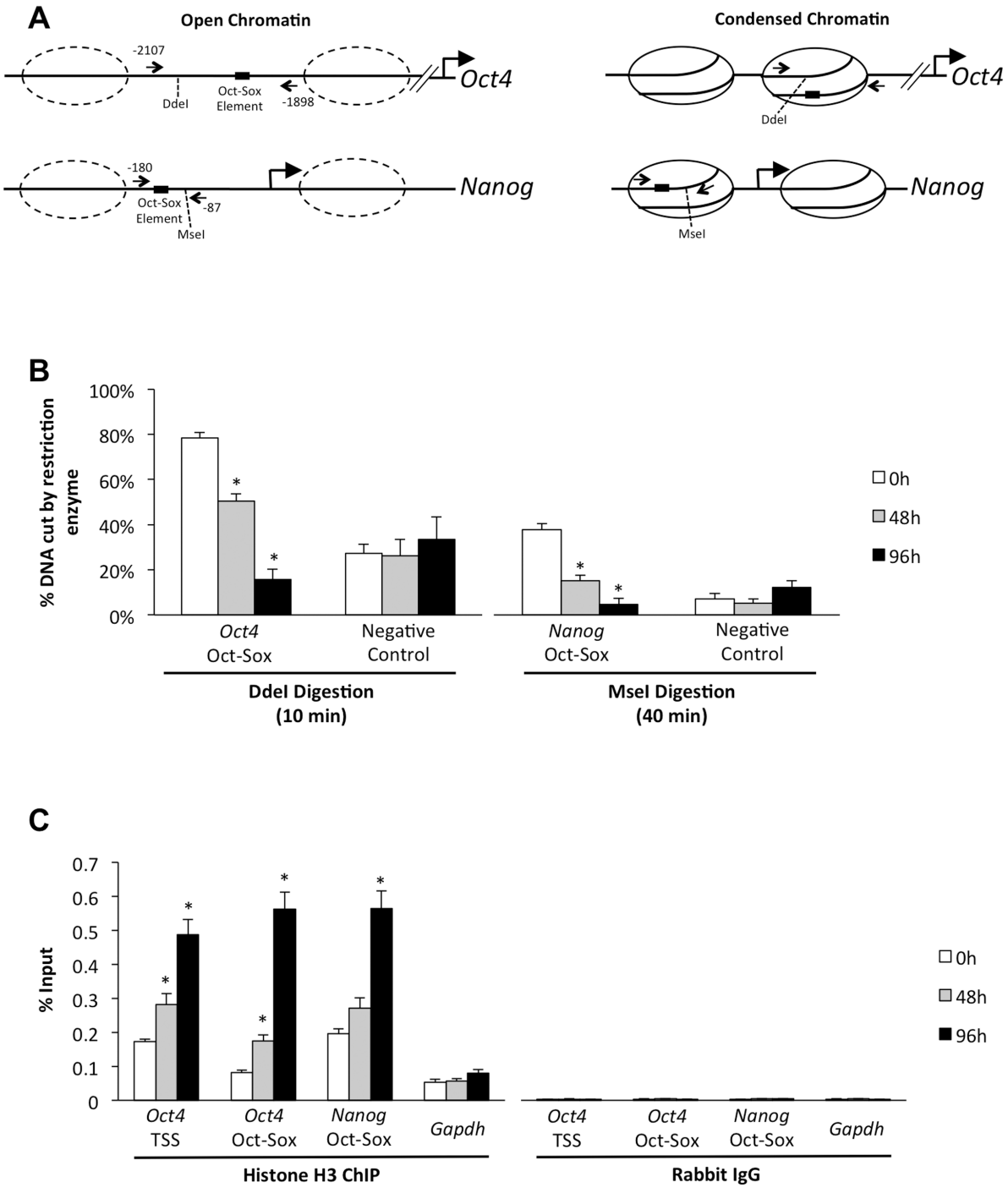

Figure 2-4. Chromatin remodeling at the Oct-Sox binding motif in *Oct4* and *Nanog*. (A) Schematic showing the location of the Oct-Sox binding motif and adjacent restriction enzyme cut site within the forward and reverse primers for *Oct4* and *Nanog*. Shown are the effects of

Figure 2-4 (cont'd)

open and condensed chromatin on the accessibility of DdeI and MseI at *Oct4* and *Nanog*, respectively. (B) qPCR analysis of restriction enzyme accessibility at the Oct-Sox binding motif in *Oct4* and *Nanog* at 0, 48, and 96 hours after induction of Cdx2. As a negative control the accessibility of MseI at an intergenic region was assessed. Data are presented as the percentage of DNA cut by the respective restriction enzyme. Assay was performed on a total of three biological replicates. For each biological replicate two PCR reactions were performed in duplicate. (C) Histone H3 ChIP analysis at the Oct-Sox binding motif in *Oct4* and *Nanog* at 0, 48, and 96 hours after induction of Cdx2. The enrichment of histone H3 on Gapdh was utilized as a control. As a negative control a rabbit non-specific IgG was used. A total of two ChIP replicates were performed for each biological replicate.

CpG methylation of Oct4 and Nanog occurs later during differentiation

DNA methylation, a process known to accompany transcriptional silencing, has also been shown to be important for lineage specific gene expression, genomic imprinting, X-inactivation and is a common theme within mammalian development (38). *Oct4* and *Nanog* have been previously characterized to be hypo and hypermethylated in ES cells and TS cells, respectively (26, 27). Moreover, CpG methylation of *Oct4* and *Nanog* in the TE lineage occurs after blastocyst formation in mice when *Oct4* and *Nanog* are already transcriptionally repressed (8, 12). To examine whether major changes in CpG methylation are associated with *Oct4* and *Nanog* silencing in Cdx2-inducible ES cells, we monitored the timing of DNA methylation at 0, 48, 72, 96, and 120 hours after Cdx2 induction. Bisulfite sequencing was performed at two differentially methylated regions (DMRs) along the *Oct4* gene (Fig. 2-5A). TS cells were used as a positive control. Accordingly, in Cdx2 induced ES cells we observed a time-dependent increase in CpG methylation that occurred after 72 hours. By 120 hours the pattern of CpG methylation resembled native TS cells (Fig. 2-5B and C). This embryonic-like pattern of DNA methylation occurred in a similar manner for a DMR located within the Nanog promoter (See Appendix, Fig. 2-8). Altogether, these data suggest that *Oct4* and *Nanog* silencing occurs in a step-wise fashion with changes in OCT4/RNAPII binding and histone acetylation occurring first followed by major changes in chromatin structure and DNA methylation that transpire during the middle to late stages of silencing.

Discussion

Results presented here provide new insights into fundamental transcriptional and chromatin-based changes that occur during silencing of *Oct4* and *Nanog* in the emerging trophoblast

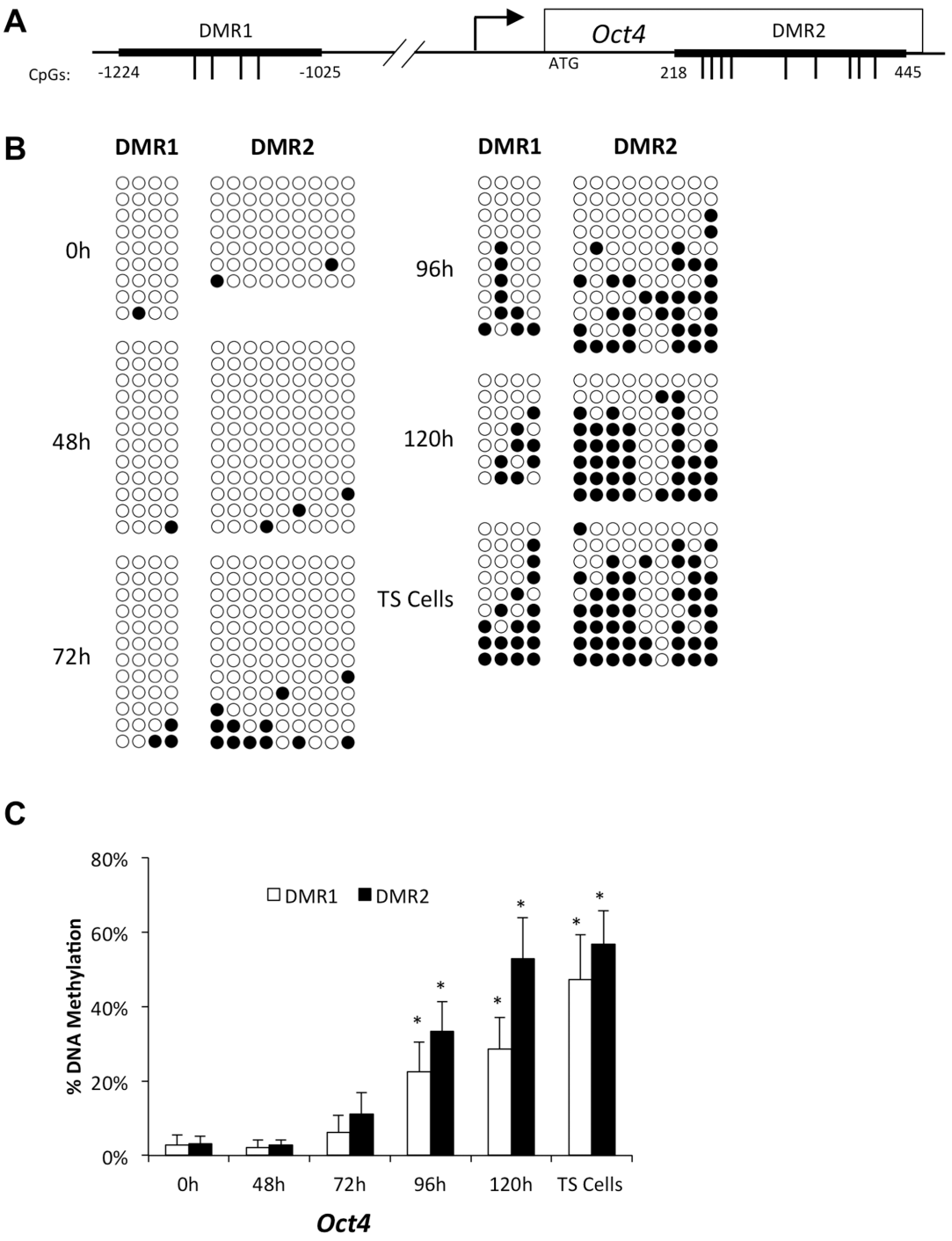

Figure 2-5. Acquisition of DNA methylation at *Oct4* occurs later during differentiation. (A) Schematic showing the location of two DMRs within the *Oct4* proximal promoter and first exon. Base pair numbering corresponds to location of forward and reverse primers used in nested PCR.

Figure 2-5 (cont'd)

Dark vertical lines represent position of CpG sites. Four CpG sites in the DMR1 and nine CpG sites in DMR2 were analyzed by bisulfite sequencing analysis. (B) DNA methylation analysis of *Oct4* DMRs at 0, 48, 72, 96, and 120 hours after induction of Cdx2. Each column represents individual CpG sites and each row corresponds to each colony sequenced. (C) Quantitative analysis of the percent CpG methylation at 0, 48, 72, 96, and 120 hours. As a positive control TS cells were analyzed in parallel. This experiment was performed on two biological preparations.

lineage. To date little is known about the role of chromatin remodeling during early trophoblast development in mice. Our results in ES cells show that following induction of CDX2 (a) the expression of *Oct4* and *Nanog* are rapidly downregulated, (b) CDX2 is recruited and OCT4 and RNAPII are rapidly lost from core regulatory elements, (c) there is a decrease in histone H3K9/14 acetylation and corresponding loss of P300 and HDAC1 binding at core regulatory elements, (d) there is an increase in nucleosome density and loss of chromatin accessibility at core regulatory elements, and (e) the acquisition of DNA methylation occurs later during differentiation after downregulation of *Oct4* and *Nanog* expression. From the data obtained in this study we propose a working model describing a potential mechanism driving the silencing of *Oct4* and *Nanog* in the trophoblast lineage (Fig. 2-6).

In previous work we showed that CDX2-mediated silencing of *Oct4* in mouse blastocysts requires coordinated epigenetic regulation (8). Utilizing a Cdx2-inducible ES cell line as a model system for trophoblast lineage formation we performed a time course analysis to build off of this work. This experimental approach allowed us to monitor differentiation within the same genetic background. From this study and our previous work (8) we established that CDX2 was recruited to the Oct-Sox binding motif in *Oct4* and *Nanog* during the beginning stages of differentiation. Concomitant with the enrichment of CDX2 there was a loss of OCT4 and RNAPII binding. Because OCT4 functions as a key activator of *Oct4* and *Nanog* expression in ES cells (23, 24), it is probable that eviction of OCT4 represents an early event necessary for transcriptional repression of *Oct4* and *Nanog* in the trophoblast lineage.

Accompanying the loss in OCT4 and RNAPII binding was a decrease in histone H3K9/14 acetylation, a well-established epigenetic marker of transcriptional activation. Recent

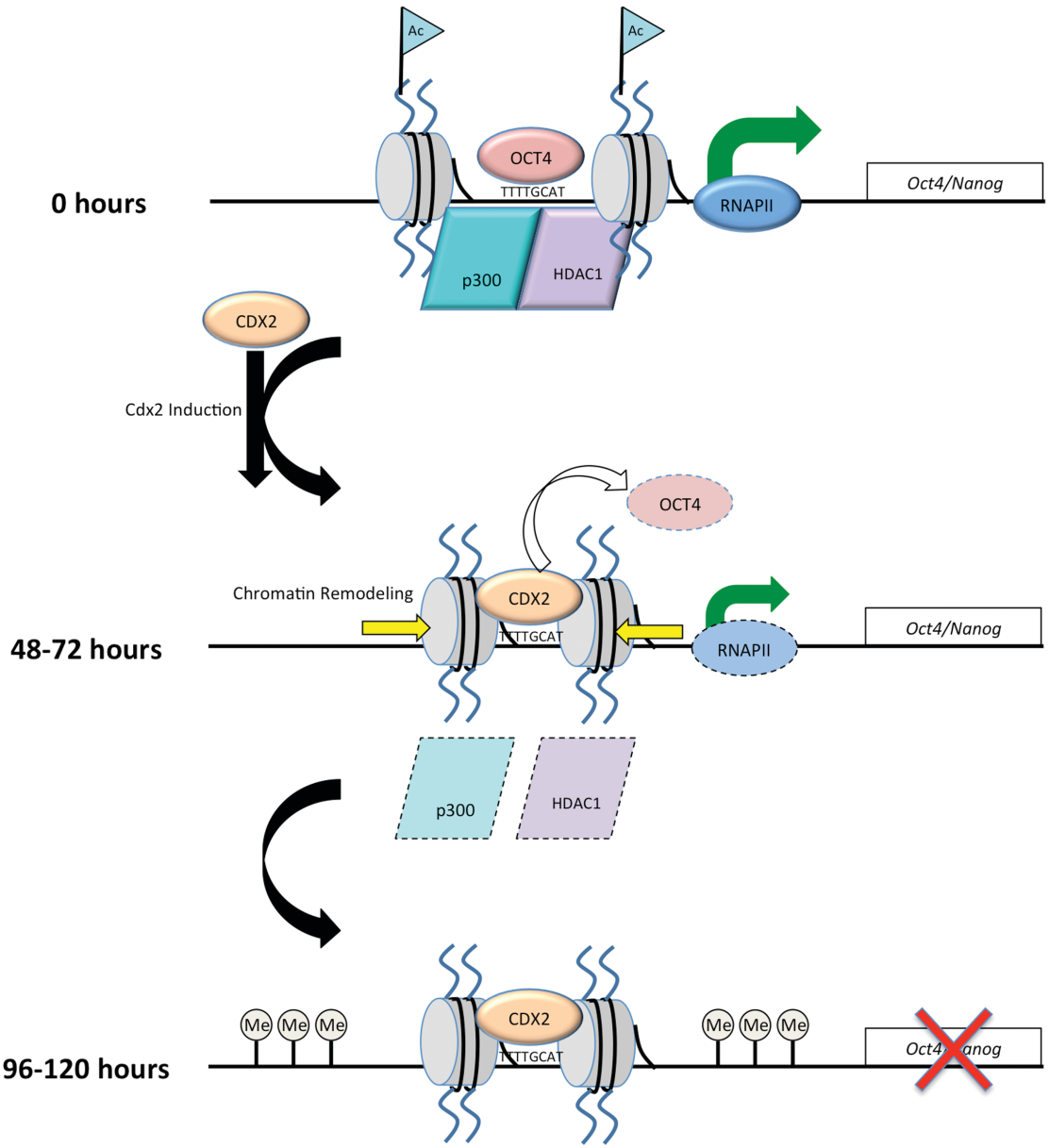

Figure 2-6. Transcriptional and chromatin-based model for silencing of *Oct4* and *Nanog* in the trophoblast lineage. Concomitant with the recruitment of CDX2 the occupancy of OCT4 and RNAPII is lost at the Oct-Sox binding motif and TSS in *Oct4* and *Nanog* during the early stages of transcriptional repression. Loss of histone H3K9/14 acetylation, HDAC, and p300 accompanies transcriptional reprogramming. In parallel with these changes chromatin

Figure 2-6 (cont'd)

remodeling occurs at the Oct-Sox binding motif in *Oct4 and Nanog*. During the later stages of differentiation DNA methylation occurs facilitating transcriptional silencing.

studies showed that HATs and HDACs, modulators of histone H3 acetylation, occupy genes that are highly expressed in ES cells and T lymphocytes (28, 39). These include pluripotency genes such as *Oct4* and *Nanog* in ES cells. Consistent with these findings we observed enrichment of P300 and HDAC1 at *Oct4* and *Nanog* in undifferentiated ES cells. Following the induction of CDX2 the levels of histone H3K9/14 acetylation decreased accompanied by a loss of p300 and HDAC1. An interesting facet that we intend to further explore is the role of HDAC1 in regulation of *Oct4* and *Nanog* in the trophoblast lineage. In this regard, genetic ablation of HDAC1 in ES cells results in increased levels of Nanog and abnormal differentiation (28, 40). Moreover, treatment of human and mouse ES cells with low doses of HDAC inhibitors promotes self-renewal in the absence of growth factors (41). It will be interesting to determine whether HDACs play a central role in fine tuning and/or downregulating *Oct4* and *Nanog* expression in the trophoblast lineage. In this regard, CDX2 forms a complex with HDAC1 in Cdx2-induced ES cells (13) and both CDX2 and HDAC1 colocalize to the Oct-Sox binding motif in *Oct4* and *Nanog* (this study). Thus, it is plausible that CDX2-dependent silencing of *Oct4* and *Nanog* is mediated through HDAC1 and BRG1, while CDX2-dependent activation of trophoblast genes is mediated through interactions with specific coactivators.

A combination of restriction enzyme accessibility and histone H3 ChIP assays established that during differentiation there was an increase in nucleosome density at the Oct-Sox binding motif in *Oct4* and *Nanog*. Previous work in our laboratory demonstrated that the chromatin remodeling factor BRG1 cooperates with CDX2 to silence *Oct4* expression in blastocyst TE (8). The data presented here show that the transcriptional silencing of *Oct4* and *Nanog* in Cdx2-inducible ES cells involves a chromatin remodeling activity that inserts/repositions a nucleosome at the Oct-Sox binding site when OCT4 binding is lost. In

support of this notion You *et al* demonstrated that during retinoic acid (RA)-induced ES cell differentiation there is a loss of OCT4 binding and an increase in nucleosome density at the Oct-Sox binding site in O*ct4* and *Nanog* (42). Since ablation of BRG1 in preimplantation embryos results in the failure to repress *Oct4* and *Nanog* expression in the blastocyst TE (8, 17), we postulate that BRG1 is providing a chromatin remodeling activity that cooperates with CDX2 to silence pluripotency genes in the TE. Ongoing experiments in our laboratory are implementing a combination of RNAi and higher resolution MNase mapping to functionally characterize the requirement of BRG1-containing SWI/SNF complexes in trophoblast development. This will be crucial to decipher whether CDX2/BRG1-dependent repression of *Oct4* and *Nanog* involves chromatin remodeling, or perhaps whether BRG1 acts as a scaffold to recruit additional corepressors to the *Oct4* and *Nanog* promoters.

DNA methylation at CpG sites within the *Oct4* and *Nanog* genes was acquired later within the time course employed in this report. This repressive modification appeared to be a final step during CDX2-mediated differentiation; at 96 and 120 hours the methylation profile was very similar to that observed in native TS cells. This finding is consistent with RA-induced ES cell differentiation where *Oct4* and *Nanog* silencing occurs in a step-wise manner with DNA methylation occurring after chromatin remodeling (42). Interestingly, during early embryogenesis DNA methylation does not occur in the trophoblast lineage until after implantation when *Oct4* and *Nanog* are already downregulated (12). Combined these data highlight the importance of other epigenetic modifications, such as covalent histone modifications and chromatin remodeling, in the specification of the trophoblast lineage during blastocyst formation when *Oct4* and *Nanog* are transcriptionally repressed.

In summary, this study demonstrates that changes in histone H3K9/14 acetylation, chromatin remodeling, and CpG methylation accompany several transcriptional changes during silencing of *Oct4* and *Nanog* in the trophoblast lineage. The timing of these changes, combined with our previous observations in the mouse preimplantation embryo (8, 17), provide novel insights into potential mechanisms that may regulate pluripotency gene silencing in the trophoblast lineage. It will be important to establish whether Brg1- dependent chromatin remodeling at the Oct-Sox binding motif acts as a mechanism to displace OCT4 binding to mediate *Oct4* and *Nanog* repression. A potential broader impact of this research is on understanding how perturbations in the trophoblast specification disrupt implantation and/or post-implantation development. This is especially important for enhancing the efficiency of methods utilized in human assisted reproductive technologies, where a substantial proportion of embryos fail to development into live offspring (43).

Acknowledgements

We are grateful for Ms. Roya Oliai's assistance with the western blot analysis. Furthermore, we thank Dr. Minoru Ko of the NIA for providing us the Cdx2-inducible ES cell line before it became commercially available. We express gratitude to Dr. Anthony Imbalzano for providing us the BRG1 anti-serum for ChIP. This work was supported by NIH grants GM095347 to J.G.K and a MSU gene expression in development and disease (GEDD) fellowship to T.S.C.

APPENDIX

Table 2-1. Antibodies used in this study

Protein	Antibody	Antibody type	Use	Source
H3K9/14Ac	Acetyl-Histone H3 Antibody	Rabbit polyclonal IgG	ChIP	Upstate/Millipore (06-599)
Flag-CDX2	Anti-Flag (M2)	Mouse monoclonal IgG_1	WB	Sigma (F1804)
	Anti-Flag	Rabbit polyclonal IgG	ChIP	Sigma (F7425)
HDAC1	HDAC1 Antibody	Rabbit polyclonal IgG	ChIP	Abcam (ab7028)
Histone H3	Histone H3, C-terminal Ab	Rabbit Serum	ChIP	Active Motif (39163)
NANOG	Nanog Antibody	Rabbit polyclonal IgG	WB	Cosmo Bio (REC-RCAB0002PF)
OCT4	Oct-3/4 Antibody (C-10)	Mouse monoclonal IgG_{2b}	WB, ChIP	Santa Cruz (sc-5279)
p300	p300 Antibody (C-20)	Rabbit polyclonal IgG	ChIP	Santa Cruz (sc-585)
RNAPII	RNAPII Antibody (CTD48H)	Mouse monoclonal IgG_1	ChIP	Upstate/Millipore (05-623)
β-Actin	β-Actin Antibody (AC-15)	Mouse monoclonal IgG_1	WB	Sigma (A5441)

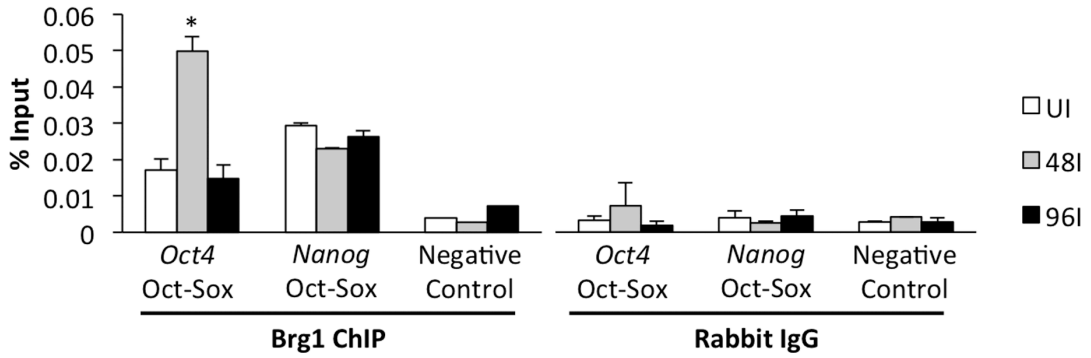

Figure 2-7. BRG1 occupies the Oct-Sox binding motif in *Oct4* and *Nanog* in Cdx2-inducible ES cells differentiating into TS-like cells. ChIP analysis of BRG1 occupancy at the Oct-Sox binding motif in *Oct4* and *Nanog* at 0, 48, and 96 hours. A rabbit non-specific IgG was used as a negative control for the BRG1 antibody. An intergenic region was utilized as a negative control for BRG1 binding. A total of two ChIP replicates were performed for each biological replicate. For each ChIP replicate a total of two PCR reactions were performed in duplicate.

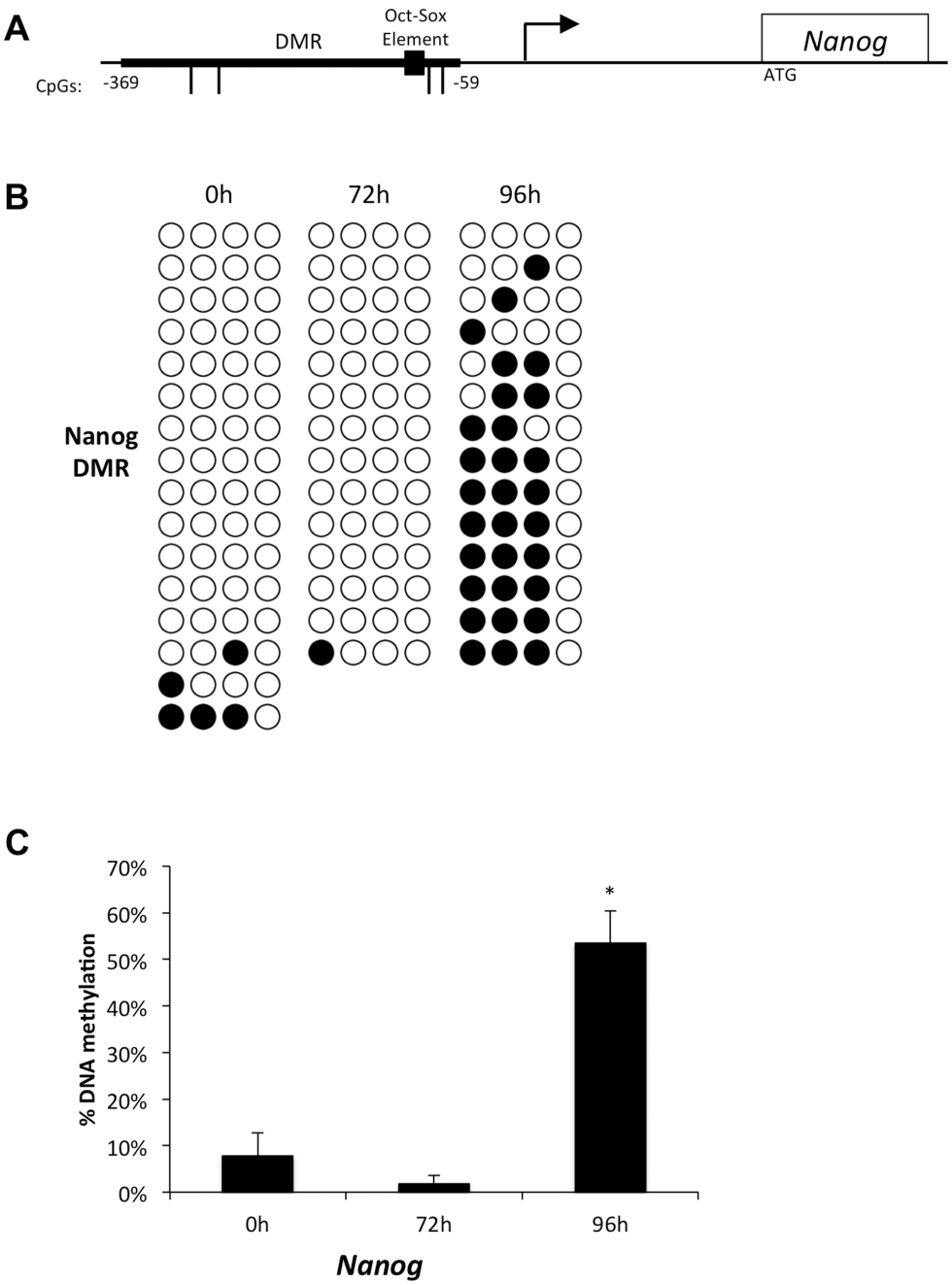

Figure 2-8. DNA methylation analysis along *Nanog* during differentiation. (A) Schematic showing the location of a DMR within the *Nanog* proximal promoter. Four CpG sites located around the Oct-Sox motif were analyzed by bisulfite sequencing. (B) DNA methylation pattern of *Nanog* DMR at 0, 72, and 96 hours after induction of Cdx2. (C) Quantitative analysis of percent CpG methylation for *Nanog* DMR during differentiation.

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CHAPTER 3 -

BRG1 GOVERNS *NANOG* TRANSCRIPTION IN EARLY MOUSE EMBRYOS AND EMBRYONIC STEM CELLS VIA ANTAGONISM OF HISTONE H3 LYSINE 9/14 ACETYLATION[2]

[2]The work described in this chapter was under review for publication at the time of this report, the corresponding author order on that manuscript is as follows: Timothy S. Carey, Zubing Cao, Inchul Choi, Avishek Ganguly, Catherine A. Wilson, Soumen Paul, and Jason G. Knott

Abstract

During mouse preimplantation development the generation of the inner cell mass (ICM) and trophoblast lineages involves upregulation of *Nanog* expression in the ICM and its silencing in the trophoblast. However, the underlying epigenetic mechanisms that differentially regulate *Nanog* in the first cell lineages are poorly understood. Here we report that Brahma related gene-1 (BRG1) cooperates with histone deacetylase 1 (HDAC1) to regulate *Nanog* expression. BRG1 depletion in preimplantation embryos and CDX2-inducible embryonic stem cells (ESCs) revealed that BRG1 is necessary for *Nanog* silencing in the trophoblast lineage. Conversely, in undifferentiated ESCs loss of BRG1 augmented *Nanog* expression. Analysis of histone H3 within the *Nanog* proximal enhancer revealed H3K9/14 acetylation increased in BRG1 depleted embryos and ESCs compared to controls. Biochemical studies demonstrated that HDAC1 was present in BRG1-BAF155 complexes and BRG1-HDAC1 interactions were enriched in the trophoblast lineage. HDAC1 inhibition triggered an increase in H3K9/14 acetylation and a corresponding rise in *Nanog* mRNA and protein, phenocopying BRG1 knockdown embryos and ESCs. Lastly, nucleosome mapping experiments revealed that BRG1 is indispensible for nucleosome remodeling at the *Nanog* enhancer during trophoblast development. In summary, our data suggest that BRG1 governs *Nanog* expression via a dual mechanism involving histone deacetylation and nucleosome remodeling.

Introduction

Cell-fate decisions are crucial for the development of multicellular organisms. In higher animals such as placental mammals, the first cell-fate decision occurs during preimplantation development when the totipotent blastomeres differentiate into the blastocyst inner cell mass

(ICM) and trophoblast lineage (1). The proper development of the blastocyst ICM and trophoblast lineages is critical for embryo implantation, placentation, gastrulation, and full-term development. Abnormal development of the ICM and trophoblast lineages may contribute to pregnancy loss, reproductive disorders, and birth defects.

Early lineage formation in preimplantation embryos is mediated by a combination of transcriptional and epigenetic mechanisms (2, 3). During blastocyst formation the expression of key transcription factors such as octamer-binding transcription factor 4 (OCT4), nanog homeobox (NANOG), and sex determining region Y-box 2 (SOX2) become restricted to the pluripotent ICM, while transcription factor AP-2 gamma (TFAP2C), GATA binding protein 3 (GATA3), and caudal type homeobox 2 (CDX2) are expressed exclusively in the trophoblast lineage (4-10). The spatial and temporal expression of these lineage-specific factors is controlled by position-dependent HIPPO signaling, transcription factor regulatory loops, and chromatin modifications (2, 3, 10-12). For example, the HIPPO signaling pathway differentially regulates lineage-formation via the downregulation of CDX2 expression in the ICM and SOX2 expression in the trophoblast (10, 12). In conjunction with the Hippo pathway, OCT4 and CDX2 negatively regulate one another's expression in the ICM and trophoblast lineage via binding to each other's promoters and cooperating with ERG-associated protein with SET domain (ESET) and brahma related gene 1 (BRG1) to block transcription (13-15). Other epigenetic modifiers, such as embryonic ectoderm development (EED) and lysine (K)-specific demethylase 6B (KDM6B), work in opposition to restrict *Cdx2* and *Gata3* expression to the trophoblast lineage (16). Altogether, these studies demonstrate that ICM and trophoblast lineage development is regulated by overlapping transcriptional and epigenetic mechanisms.

Despite our current understanding of the mechanisms that regulate the spatial and temporal expression of *Oct4*, *Gata3*, and *Cdx2*, less is known about the epigenetic mechanisms that govern *Nanog* expression during the first cell-fate decision in preimplantation embryos. Recent studies in mice revealed that *Nanog* expression is controlled by epigenetic modifications such as tet methylcytosine dioxygenase 1 (Tet1)-dependent 5-hydroxymethylcytosine (17) and coactivator-associated arginine methyltransferase 1 (CARM1)-dependent arginine methylation (18). Previous work in our laboratory demonstrated that *Nanog* expression is upregulated in BRG1 knockdown (KD) blastocysts (19). Furthermore, we and others established that BRG1 occupies the *Nanog* promoter in mouse ESCs (19, 20). Combined, these findings suggest that BRG1 may act as a key regulator of *Nanog* expression during early lineage formation.

Here we report that BRG1 functions as a major regulator of *Nanog* expression during early embryogenesis. In blastocysts BRG1 is required for downregulation of *Nanog* in the trophoblast lineage. Conversely, in pluripotent ESCs BRG1 regulates *Nanog* expression by fine-tuning transcriptional outcome at the *Nanog* locus. We show that this mode of regulation depends on histone deacetylase 1 (HDAC1); in ESCs and preimplantation embryos BRG1 interacts with HDAC1 to antagonize histone H3 lysine 9 and 14 (H3K9/14) acetylation at the *Nanog* proximal enhancer. Disruption of BRG1 and/or HDAC1 augments H3K9/14 acetylation and *Nanog* transcription. Finally, during ESC differentiation into trophoblast-like cells we show that BRG1 is required for nucleosome occupancy at the *Nanog* proximal enhancer and transcriptional start site (TSS). These findings demonstrate that during cell lineage specification in preimplantation embryos and in ESCs BRG1 governs *Nanog* expression via a dual mechanism involving histone deacetylation and nucleosome remodeling.

Materials and Methods

Embryo collection, embryo manipulation, and inhibitor treatment

Mouse embryo collection, *in vitro* culture, and siRNA microinjection were performed as previously described (7, 15, 21). Briefly, embryos were collected from either superovulated CF1 or B6D2/F1 females mated with B6D2/F1 males (Charles River Laboratories, Wilmington, MA, USA). One-cell embryos were microinjected with 5-10 pL of 50-100 μM *Brg1*, *Hdac1*, or non-targeting control siRNA (siGenome SMARTpool; GE Dharmacon, Lafayette, CO, USA). Following injection, embryos were cultured in modified KSOM media (EMD Millipore, Billerica, MA, USA) for 3-4 d. For cell-fate mapping experiments, chimeric embryos were constructed as previously described (7). *GFP* mRNA was coinjected with *Brg1* siRNA to track the fate of BRG1 KD blastomeres. At the 8-cell stage acid Tyrode solution (Sigma, St. Louis, MO) was used to remove the zona pellucida. Two zona pellucida-free embryos were paired in a microwell and cultured until the blastocyst stage. GFP expression was evaluated by epifluorescence on a Nikon ECLIPSE Ti inverted microscope equipped with LED illumination and a FITC filter. In a subset of experiments compacted morulae were cultured in the present of a histone deacetylase inhibitor. A 1M stock solution of sodium butyrate (NaB; Sigma) was prepared in water. NaB was then added to modified KSOM to achieve desired concentration. Embryos were cultured in the presence of the inhibitor for 24 h until blastocyst formation. All animal work in this study was approved by the Institutional Animal Care and Use Committee (IACUC) at Michigan State University and conformed to the institutional guidelines and regulatory standards.

ESC culture, lentivirus transduction, and inhibitor treatment

Cdx2-inducible mouse ESCs (Coriell Institute, Camden, NJ) were cultured as previously described, with slight modifications for lentiviral transduction of shRNA constructs (22). ESCs were initially propagated on mitomicin-treated, puromycin and doxycycline resistant mouse embryonic fibroblasts (MEFs) then switched to gelatinized dishes for growth in feeder-free conditions. Growth media consisted of high glucose DMEM with L-glutamine and sodium pyruvate (Gibco, Life Technologies, Carlsbad, CA) supplemented with 20% FBS (Hyclone™, GE Healthcare Life Sciences, South Logan, UT), 0.1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, 100 U/ml leukemia inhibitory factor (LIF) (EMD-Millipore, Billerica, MA), 1 μg/ml puromycin, and 0.2 μg/ml doxycycline. For lentiviral transduction of shRNA constructs, feeder-free ESCs were passaged and plated in doxycycline and puromycin-free media supplemented with 8 μg/ml Polybrene (Sigma) and 1.25x concentration of lentiviral stock. Eight-hours following viral transduction, doxycycline was returned to the media to prevent expression of the transgene. After 24 h, puromycin was returned to the growth media and during subsequent media changes the level of puromycin was gradually increased to 6 μg/ml by 72 h after transduction. *Cdx2* expression was induced by the removal of doxycycline from the media. Mouse R1 ESCs (ATCC, Manassas, VA) were cultured in growth media and conditions similar to the *Cdx2*-inducible cells, but without supplementation of doxycycline or puromycin. For the histone deacetylase inhibitor experiment, NaB was added to the growth media to the desired concentration from a 1M NaB stock. Cells were grown in the presence of inhibitor for 48 h.

RNAi targeting sequences

Lentiviral pLKO.1 vectors encoding shRNA sequences were used to knockdown BRG1 in ESCs.

The sequences of the hairpins are as follows: *Brg1* shRNA: CCGGCGGCTCAAGAAGGAAGTTGAACTCGAGTTCAACTTCCTTCTTGAGCCGTTTTTG (Open Biosystems TRCN0000071385). Scambled shRNA: CCGGTCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGTTTTTG (Addgene plasmid # 1864). These plasmids were a kind gift from Gerald Crabtree for the *Brg1* shRNA construct and David Sabatini for the scrambled shRNA construct (23, 24). Lentiviral particles were prepared at the University of Michigan Vector Core.

Quantitative reverse transcriptase (qRT)-PCR and western blot analysis

Total RNA was extracted from ESCs using the RNeasy Mini Kit (Qiagen, Valencia, CA) or from pools of 10 embryos using the PicoPure RNA isolation kit (Arcturus, Mountain View, CA). First-strand cDNA synthesis was performed using SuperScript II reverse transcriptase (Life Technologies, Carlsbad,CA). qRT-PCR analysis was performed with TaqMan probes, or gene-specific primers using SYBR green detection on a StepOne Plus thermocycler (Applied Biosystems, Foster City, CA). Data were analyzed by the ΔΔCt method, normalized to *Ubtf* for embryos or *Eef1a1* for ESCs. Western blot analysis was performed as previously described (15, 22). In brief, whole ESC lysates were separated by SDS-PAGE and transferred to PVDF membrane. Antibodies against proteins detected by western blot were BRG1 (Santa Cruz Biotechnology, Dallas, TX; Cat no. sc-10768), M2 FLAG (Sigma; Cat no. F1802), NANOG (Cosmo Bio, Carlsbad, CA; Cat no. RCAB002P-F), β-ACTIN (Sigma; Cat no. A5441), BAF155 (Santa Cruz Biotechnology; Cat no. sc-10756), HDAC1 (Santa Cruz Biotechnology; Cat no. sc-81598), and HDAC2 (Santa Cruz Biotechnology; Cat no. sc-7899). Intensity quantification of BRG1 and NANOG was performed using ImageJ (NIH).

Immunofluorescence, proximity ligation assay (PLA), and confocal microscopy

Immunofluorescent staining of preimplantation embryos was performed as previously described (7). In brief, morula and blastocysts were fixed in 3.7% paraformaldehyde for 20 min, permeabilized, washed, blocked, then incubated overnight in primary antibodies diluted in blocking solution. The following antibodies were used: NANOG (Cosmo Bio; Cat no. RCAB002P-F), CDX2 (Biogenex, Freemont, CA; Cat no. AM392-5M), BRG1 (Santa Cruz Biotechnology; Cat no. sc-10768), HDAC1 (Santa Cruz Biotechnology; Cat no. sc-81598), and acetyl-Histone H3 (EMD Millipore; Cat no. 06-599). After washing, embryos were incubated with secondary antibodies coupled to Alexafluor 488 or Alexafluor 594 (Molecular Probes, Eugene, OR, USA). Embryos were mounted in Vectashield containing DAPI (4',6-diamidino-2-phenylindole). For PLA, the protocol was identical to our immunofluorescence procedure up through the primary antibody incubation step. Washing steps, incubation with PLA probes, ligation, and amplification were performed using the Duolink *in situ* PLA kit according to manufacturer's protocol (Olink Bioscience, Uppsala, Sweden). Imaging was performed on an inverted Olympus IX81 microscope equipped with an Olympus Fluoview 1000 confocal system (Olympus America, Center Valley, PA, USA). Fluorescence intensities were quantified using ImageJ version 1.47 (National Institutes of Health). PLA quantification was performed using Blobfinder v3.2 (Center for Image Analysis, Uppsala University, Uppsala, Sweden).

Chromatin immunoprecipitation (ChIP) assay

Quantitative ChIP in ESCs and micro-scale ChIP coupled with whole genome amplification for mouse blastocysts was performed as previously described (9, 15, 22). ESCs and mouse blastocysts (pool of 30 embryos per replicate) were fixed with 1% formaldehyde, quenched with

glycine, washed in PBS and flash frozen. Sonicated chromatin extracts were incubated with antibodies specific for Histone H3K9/K14 acetylation (EMD-Millipore; Cat no. 06-599) or an isotype control (EMD- Millipore; Cat no. 12-370). Immunocomplexes were washed, eluted, decrosslinked, and purified. Purified ChIP DNA from ESCs was directly ready for analysis. ChIP DNA from mouse blastocysts was amplified using the Genomeplex single cell genome amplification kit (Sigma WGA4). ChIP data were analyzed by real-time quantitative PCR (qPCR) on a StepOne Plus thermocycler with SYBR green reagents (Applied Biosystems) using the percent input method. The following primers were used to analyze the *Nanog* proximal enhancer: 5'-CTGGGTGCCTGGGAGAATAG-3' and 5'-CCAACGGCTCAAGGCGATAG-3'. The intergenic control region was amplified with 5'-TTTTCAGTTCACACATATAAAGCA GA-3' and 5'-TGTTGTTGTTGTTGCTTCACTG-3'.

Co-immunoprecipitation (Co-IP) of nuclear complexes

Nuclear Co-IP was performed using the Nuclear Complex Co-IP Kit according to the manufacturer's protocol with some modifications (Active Motif, Carlsbad, CA). Nuclear extracts were prepared fresh from R1 ESCs. Following a two-step nuclear lysis protocol, extracts were treated with nuclease enzyme included with the kit. Two hundred to 250 µg of nuclear extract was used per immunoprecipitation as quantified by BCA assay (Life Technologies). Dilution of nuclear extract and further washing steps were performed using 1x low stringency Co-IP buffer containing protease inhibitors and 150 mM NaCl. No DTT was added to the Co-IP buffer, contrary to the manufacturer's recommendation of 1 mM DTT. Prepared and diluted nuclear extracts were incubated with BRG1 anti-serum (obtained from Dr. Anthony Imbalzano; UMASS, Medical School, Worcester, MA) or HDAC1 antibody (Abcam, Boston, MA; Cat no.

ab7028) or Rabbit IgG (EMD-Millipore; Cat no. 12-370). Complexes were collected on magnetic protein G Dynabeads (Life Technologies). Beads were washed with Co-IP buffer, and during final wash step, beads were divided for either direct analysis by western blot or for HDAC enzymatic activity assay. For western blot analysis of Co-IP material, proteins were directly eluted from beads with 2x Laemmli Buffer (130 mM Tris-HCl pH 6.8, 4% SDS, 0.02% Bromophenol blue, 100 mM DTT), boiled for 5 min. and loaded onto SDS-PAGE gel.

HDAC activity assay

Enzymatic histone deacetylase activity was assayed using the Fluorescent HDAC Assay Kit (Active Motif). Preparation of standards and control was performed according to manufacturer's protocol. To assay Co-IP material, beads bound with immunoprecipitated complexes were resuspended in 40 µl of HDAC assay buffer. 10 µl of HDAC substrate was added and samples were incubated at 37°C for 1 h. Following this incubation, a magnet was used to collect the beads, and the supernatant was transferred to a 96-well half-volume black microplate (Greiner Bio-One, Monroe, NC). A 50 µl developing solution was then added, following a 10 min incubation, fluorescence was measured using 360 nm excitation and 460 nm emission on a Gemini EM fluorescence microplate reader (Molecular Devices, Sunnyvale, CA).

Nucleosome mapping assay

Micrococcal nuclease digestion conditions and mapping approach were adapted from method previously described (25). Cells were formaldehyde fixed under similar conditions as the preparation of ChIP chromatin. Cells were lysed in a buffer composed of 10 mM Hepes pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.34 M Sucrose, 10% Glycerol, 1 mM DTT, 0.1% Triton X-100,

and 1x protease inhibitor cocktail (Sigma; Cat no. P8340). Following centrifugation, pellets were resuspended in MNase digestion buffer composed of 50 mM Tris–HCl pH 7.4, 25 mM KCl, 4 mM $MgCl_2$, 12.5% Glycerol, and 1 mM $CaCl_2$. Resuspended cells from each experimental group were split into several aliquots and then subjected to various concentrations of MNase (USB, Cleveland, OH, US), or left untreated of nuclease as a genomic control. Samples were then incubated at 37°C for 10 min, and digestion was stopped by addition SDS/EDTA/EGTA buffer. Samples underwent DNA purification, then gel purification on a 1.8% agarose gel for the mononucleosome sized fragment. DNA from the 100 U/ml MNase treatment contained the most mononucleosomal sized DNA and was used for downstream analyses. A tiling primer approach was used to map nucleosome density along a ~ 1kb region centered at the *Nanog* transcriptional start site. Primers were designed to obtain product sizes around 100 bp in length and each primer pair amplified a region located approximately 30 bp away from the adjacent primer pair. Primers sequences will be provided upon request. Relative protection from digestion by MNase was calculated comparing amplification of MNase digested DNA to a undigested control by a ΔCt method. Data were then normalized to the highest protection calculated from a known heterochromatic or repressed region (26).

Statistical analyses

A Student's *t*-test was used to determine statistical significance between control and treatment groups, where appropriate. Analyses of variance (ANOVA) were performed using SAS 9.4 (Cary, NC, USA). *P* values < 0.05 were considered statistically significant unless otherwise stated.

Results

BRG1 regulates Nanog transcription during blastocyst formation

Previously, we demonstrated that BRG1 is required for normal blastocyst development in mice (19). BRG1-depleted blastocysts exhibit defects in the trophoblast lineage and express increased levels of pluripotency genes. However, the precise role of BRG1 in trophoblast lineage specification and *Nanog* regulation was not investigated. To examine the biological role of BRG1 in early lineage formation, we first evaluated the expression and localization of *Nanog* mRNA and protein in BRG1 KD and control blastocysts. To accomplish this we microinjected *Brg1* siRNA or control siRNA into fertilized 1-cell embryos. Using this approach we can obtain a greater than 85% reduction in *Brg1* transcripts (15, 19) and phenocopy *Brg1* null embryos (27). At the blastocyst stage, treated and control embryos were subjected to real-time qPCR and immunofluorescence analysis. To distinguish between the ICM and trophoblast lineage, blastocysts were co-stained with anti-CDX2, a trophoblast-specific marker. These analyses demonstrated that in BRG1 KD blastocysts *Nanog* transcripts were increased ~100% ($P<0.05$; Fig. 3-1A). Immunofluorescence staining showed that in control blastocysts NANOG protein was restricted to cells in the ICM and absent in the CDX2-positive trophoblast cells (Fig. 3-1B). In contrast, in BRG1 KD blastocysts NANOG protein was expressed in both the ICM and trophoblast lineages. These results demonstrate that BRG1 is necessary for downregulation of *Nanog* expression in the emerging trophoblast lineage.

Because loss of BRG1 is associated with increased levels of NANOG, we tested whether BRG1-deficient blastomeres exhibit a preferential commitment towards the ICM lineage. We generated chimeric embryos using blastomeres from 8-cell stage control embryos and GFP labeled (GFP^+) BRG1 KD embryos. Control-control and BRG1 KD-BRG1 KD chimeric

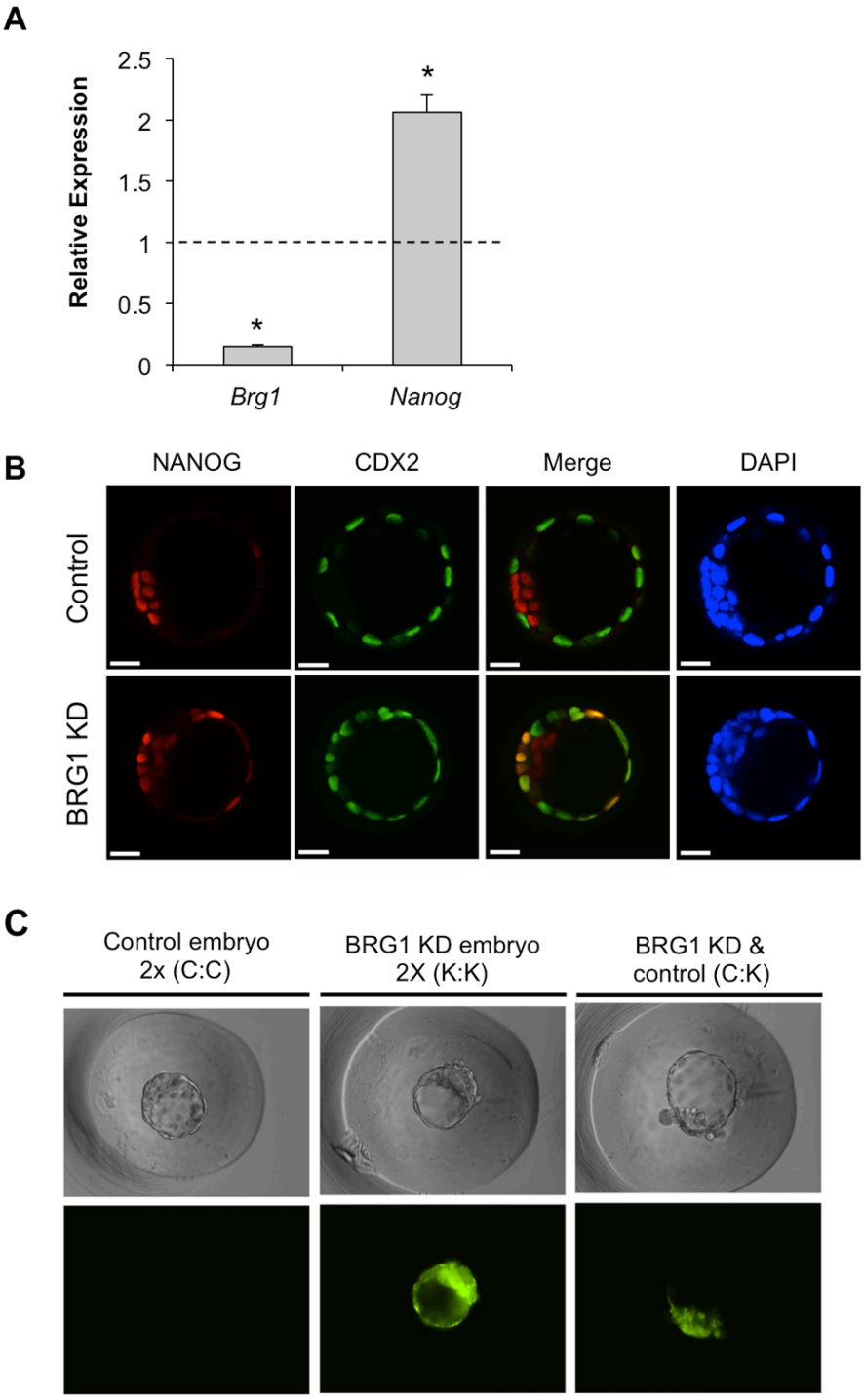

Figure 3-1. BRG1 negatively regulates *Nanog* expression in blastocysts and is required for trophoblast lineage development. (A) qRT-PCR analysis of *Nanog* and *Brg1* transcripts in BRG1 KD blastocysts relative to control blastocysts; error bars represent standard error of the

Figure 3-1 (cont'd)

mean (S.E.M.) from 3 replicates. Dashed line denotes control expression set at 1. Asterisks indicates a significant different between BRG1 KD and control embryos ($p < 0.05$). (B) Confocal immunofluorescence analysis of NANOG expression and localization in BRG1 KD and control blastocysts. Embryos were co-stained with a CDX2 antibody to highlight the ectopic expression of NANOG in the trophoblast lineage. Nuclei were counterstained with DAPI (blue). Scale bar = 20 μm. (C) Lineage tracing experiments using chimeric embryos reveal that GFP positive BRG1 KD blastomeres give rise to the ICM and fail to efficiently contribute to the trophoblast lineage. Top panel shows Hoffman modulation contrast (HMC) and the bottom panel shows GFP fluorescence in the respective groups.

embryos were used as additional controls. These experiments revealed that the vast majority (85%) of control-BRG1 KD blastocysts contained GFP positive cells localized predominantly in the ICM (Fig. 3-1C). Altogether, these results demonstrate that BRG1 negatively regulates *Nanog* expression during blastocyst formation and suggest that BRG1 is important for establishment of the trophoblast lineage.

BRG1 modulates Nanog expression in ESCs and represses Nanog during ESC to trophoblast cell transdifferentiation

To elucidate the underlying molecular mechanisms by which BRG1 regulates *Nanog* expression during trophoblast lineage formation, we utilized a *Cdx2*-inducible ESC model in tandem with preimplantation embryos. *Cdx2*-inducible ESCs differentiate into trophoblast-like cells that resemble native trophoblast stem (TS) cells in terms of gene expression and function (13, 28). Recently, we used this ESC based model to characterize the transcriptional and epigenetic changes associated with pluripotency gene silencing during trophoblast differentiation (22). To determine the biological role of BRG1 in this context, a lentiviral-mediated RNA interference (RNAi) approach was employed to deplete BRG1 (23). Seventy-two hours after *Cdx2*-induction subsequent experiments were performed. An overview of the experimental design can be found in Fig. 3-9 in the appendix. The 72 h time point is based on a previous study were we showed that the majority of transcriptional and epigenetic changes occur between 48 and 96 h after *Cdx2*-induction (22).

In the first set of experiments *Nanog* expression was evaluated in control and *Brg1* KD ESCs with and without *Cdx2*-induction. Real-time qPCR analysis revealed that *Nanog* transcripts were upregulated approximately 50 and 100% in BRG1 KD uninduced and *Cdx2*-

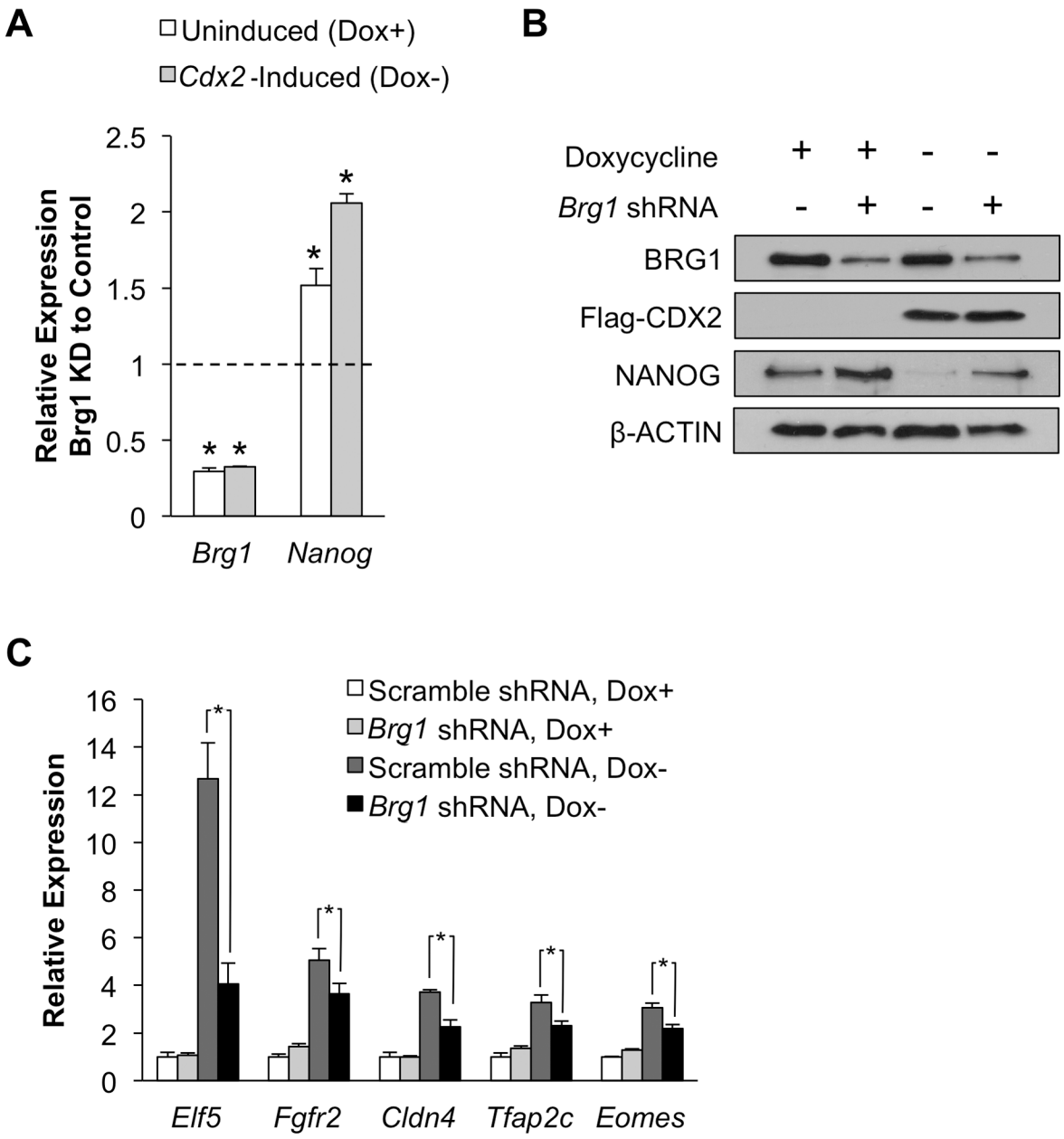

Figure 3-2. BRG1 regulates *Nanog* in ESCs and during ESC to trophoblast cell transdifferentiation. (A) qRT-PCR analysis of *Nanog* and *Brg1* transcripts in BRG1 KD ESCs, relative to a scrambled control in undifferentiated ESCs and *Cdx2*-induced ESCs (72 hours post-induction). Asterisks indicate a significant difference between BRG1 KD versus control cells ($p < 0.05$). (B) Western blot analysis of BRG1, Flag-CDX2, and NANOG in uninduced and *Cdx2*-

Figure 3-2 (cont'd)

induced ESCs (72 hours post-induction) infected with either scrambled or Brg1 shRNA lentivirus. BRG1 protein was reduced by 69% and 65% in uninduced and CDX2-induced ESCs ($P<0.05$). β-ACTIN was used as a loading control. A total of three western blots were used for quantitation. (C) qRT-PCR analysis of trophoblast lineage markers in BRG1 KD ESCs, relative to a scrambled control in *Cdx2*-induced ESCs (72 hours post-induction). Asterisks indicate a significant difference between control and BRG1 KD cells in *Cdx2*-induced groups ($p<0.05$). A total of 3 replicates were performed.

induced ESCs, respectively (P<0.05; Fig. 3-2A). Consistent with this observation, western blot analysis demonstrated that NANOG protein was increased in BRG1 KD uninduced and *Cdx2*-induced ESCs (P<0.05; Fig. 3-2B). To examine the differentiation status of these BRG1 KD *Cdx2*-induced ESCs, several trophoblast and epithelial cell markers were evaluated (Fig. 3-2C). This analysis showed that E74-Like Factor 5 (*Elf5*), fibroblast growth factor receptor 2 (*Fgfr2*), eomesodermin (*Eomes*), claudin 4 (*Cldn4*), and *Tcfap2c* transcripts were expressed at lower levels compared to control *Cdx2*-induced ESCs (P<0.05). Altogether, these results demonstrate that BRG1 functions as a negative regulator of *Nanog* expression and that downregulation of BRG1 in *Cdx2*-induced ESCs impairs trophoblast lineage differentiation. Importantly, the phenotype of BRG1 KD *Cdx2*-induced ESCs closely resembles BRG1 depleted blastocysts, providing a powerful cell-based model to investigate the molecular mechanisms of BRG1-dependent gene regulation during early embryonic development.

BRG1 antagonizes histone H3 acetylation at the Nanog proximal enhancer

Since depletion of BRG1 causes an increase in *Nanog* transcripts, we postulated that BRG1 controls *Nanog* transcription via covalent histone modifications. One such modification is acetylated histone H3 lysine 9 and 14 (AcH3K9/14). In eukaryotic cells AcH3K9/14 is tightly associated with transcriptionally active genes (29, 30), and in ESCs and TS cells, AcH3K9/14 is enriched at regulatory elements of active genes (22, 31-33). To determine whether histone H3 acetylation is altered in BRG1 KD embryos and ESCs, chromatin immunoprecipitation (ChIP) experiments were performed (Fig. 3-3). Real-time qPCR analysis revealed that AcH3K9/14 was significantly increased at the *Nanog* proximal enhancer in BRG1 KD blastocysts and BRG1 KD *Cdx2*-induced ESCs (P<0.05; Fig. 3-3A, B), while AcH3K9/14 was unchanged at an intergenic

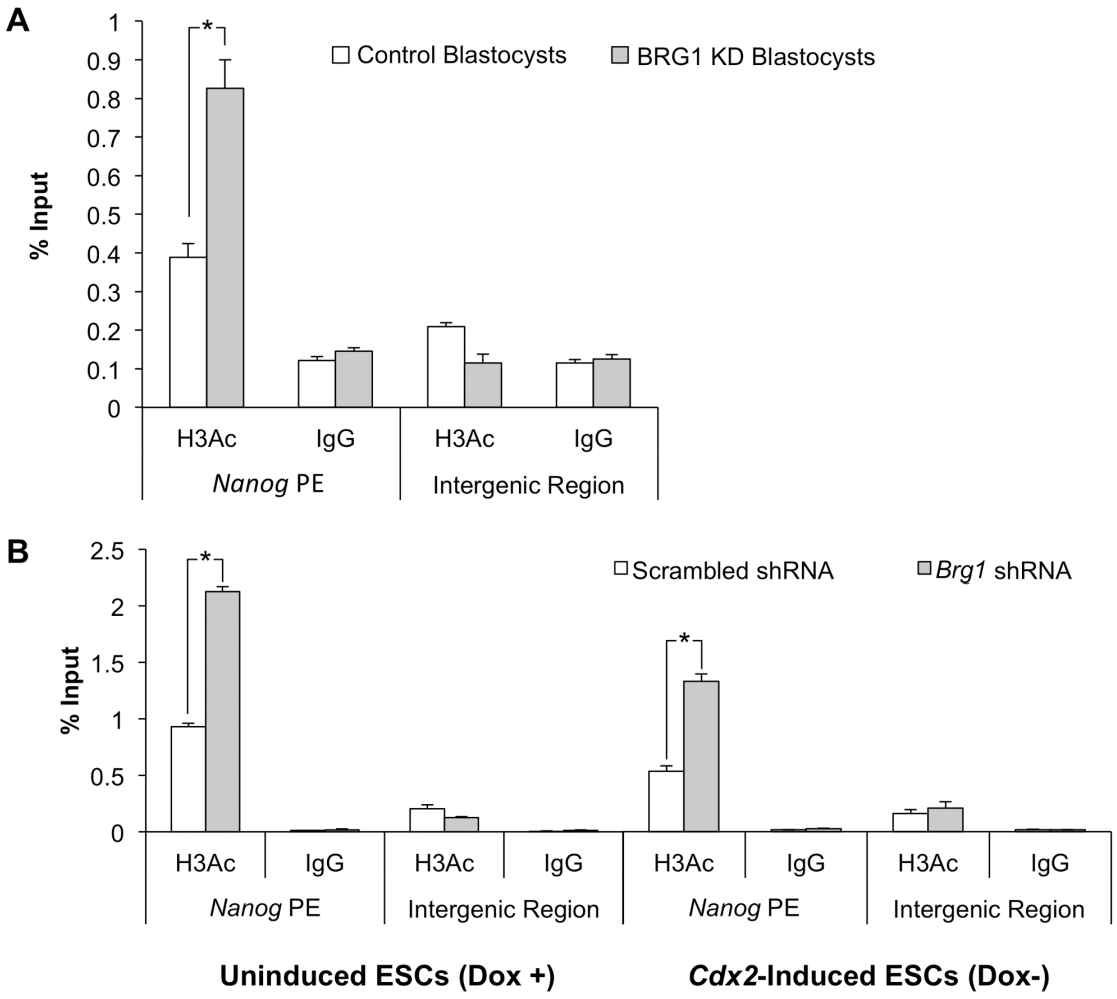

Figure 3-3. BRG1 antagonizes histone H3K9/14 acetylation at the *Nanog* proximal enhancer in blastocysts and ESCs. (A) microChIP analysis of histone H3K9/14 acetylation at the OCT4-SOX2 binding motif (Proximal enhancer; PE) of *Nanog* in control and BRG1 KD blastocysts. Pre-immune rabbit IgG was used as a negative control. An intergenic control region was analyzed for specificity of the acetylation mark. (B) ChIP analyses of histone H3K9/14 acetylation in control and BRG1 KD ESCs. The same promoter regions were analyzed as in the embryo. ChIP was performed with both uninduced and *Cdx2*-induced ESCs (72 hours post-induction) infected with either scrambled or Brg1 shRNA lentivirus. Asterisks indicate a significant difference in enrichment for the acetylation mark between indicated groups ($p<0.05$).

region (P>0.05). Furthermore, in uninduced BRG1 KD ESCs AcH3K9/14 was increased (P<0.05; Fig. 3-3B). These results suggest that BRG1 negatively regulates *Nanog* transcription via antagonizing AcH3K9/14 at its proximal enhancer.

BRG1 and HDAC1 form a functional complex during early embryogenesis

BRG1 can activate and repress gene expression through different mechanisms (34). In some cell-types BRG1 can modulate gene expression independently of its chromatin remodeling activity. For instance, BRG1 can act as a scaffold to recruit various coactivators and corepressors to target genes (35). Recently we showed via ChIP that BRG1 and Histone deacetylase 1 (HDAC1) co-occupy the *Nanog* proximal enhancer in mouse ESCs (22). Hence, we hypothesized that BRG1 antagonizes AcH3K9/14 by interacting with HDAC1. To test this we immunoprecipitated BRG1 from ESC nuclear extracts using a rabbit polyclonal antibody (Fig. 3-4A). To substantiate that BRG1 and HDAC1 interactions were direct and not mediated by DNA bridging, Co-IPs were carried out in ESC extracts treated with a nuclease. We confirmed that BAF155, a major component of BRG1/esBAF complexes in mouse ESCs (23), Co-IPed with BRG1 in these extracts. Western blot analysis revealed that HDAC1, but not HDAC2 was present in these Co-IPs. Conversely, immunoprecipitation of HDAC1 from ESC nuclear extracts pulled out BRG1 and BAF155. The interaction between BRG1 and HDAC1 was found to persist under a variety of Co-IP conditions, including ESC extracts that were not nuclease-treated (data not shown). To test whether BRG1 immunoprecipitates contained an inherent HDAC activity, fluorescent-based HDAC activity assays were carried out in ESC nuclear extracts (Fig. 3-4B). HDAC1 and IgG immunoprecipitates were used as positive and negative controls, respectively. Treatment of the ESC extract or a control HeLa cell extract with trichostatin A abolished the

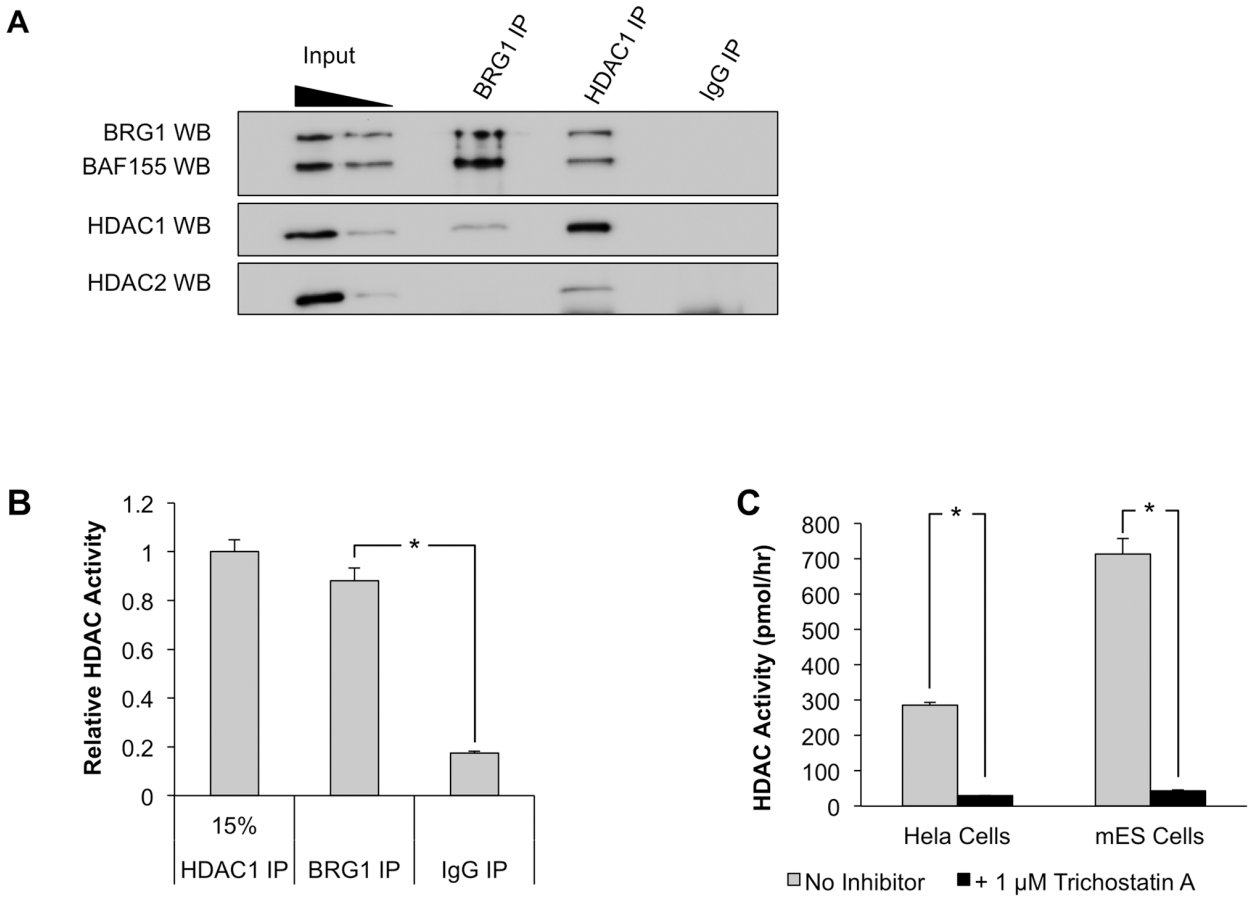

Figure 3-4. BRG1 forms a histone deacetylation complex in ESCs. (A) BRG1 and HDAC1 protein complexes were co-immunoprecipitated from ESC nuclear lysates with antibodies for BRG1, HDAC1 or pre-immune IgG and analyzed by western blot. (B) Histone deacetylase activity of BRG1 immunoprecipitated material was measured by a fluorescence assay. HDAC1 and IgG immunoprecipitates were used as positive and negative controls, respectively. Asterisks indicate a significant difference in deacetylase activity between the indicated groups ($p<0.05$). (C) Treatment of HeLa cell or ESC nuclear extracts with trichostatin A, a potent HDAC inhibitor, abolished the HDAC activity in those extracts.

observed HDAC activity (Fig. 3-4C). Interestingly, BRG1 immunoprecipitates contained an intrinsic HDAC activity that was significantly greater than the IgG control ($P<0.05$), suggesting that BRG1 may antagonize AcH3K9/14 via HDAC1.

To test whether BRG1 interacts with HDAC1 during embryonic development, immunofluorescence and proximity ligation assays (PLAs) were carried out in preimplantation embryos. In the first set of experiments the global expression of BRG1 and HDAC1 were evaluated during the morula-to-blastocyst transition when *Nanog* expression is normally downregulated in the emerging trophoblast lineage (Fig. 3-5). In preliminary experiments the specificity of each antibody was verified in knockdown experiments by microinjecting *Brg1* and *Hdac1* siRNA into zygotes (19) and data not shown. Immunofluorescence analysis revealed that both BRG1 and HDAC1 were broadly expressed in the nuclei of morulae and blastocysts (Fig. 3-5A). During the morula-to-blastocyst transition their overall expression was moderately reduced ($P<0.05$; Fig. 3-5B). Next, PLAs were performed to examine protein-protein interactions between BRG1 and HDAC1. This assay allows the visualization of proteins that are located within 30 nm of each other. To confirm that the observed interactions were specific, control experiments were carried out using BRG1 KD embryos. These experiments demonstrated that BRG1 and HDAC1 interact in morulae and that during blastocyst formation BRG1-HDAC1 interactions are enriched in the trophoblast lineage ($P<0.05$; Fig. 3-5C, D). Collectively, these experiments demonstrate that BRG1 and HDAC1 form a functional complex during early embryonic development that might antagonize AcH3K9/14 at the *Nanog* proximal enhancer to modulate its transcriptional activity.

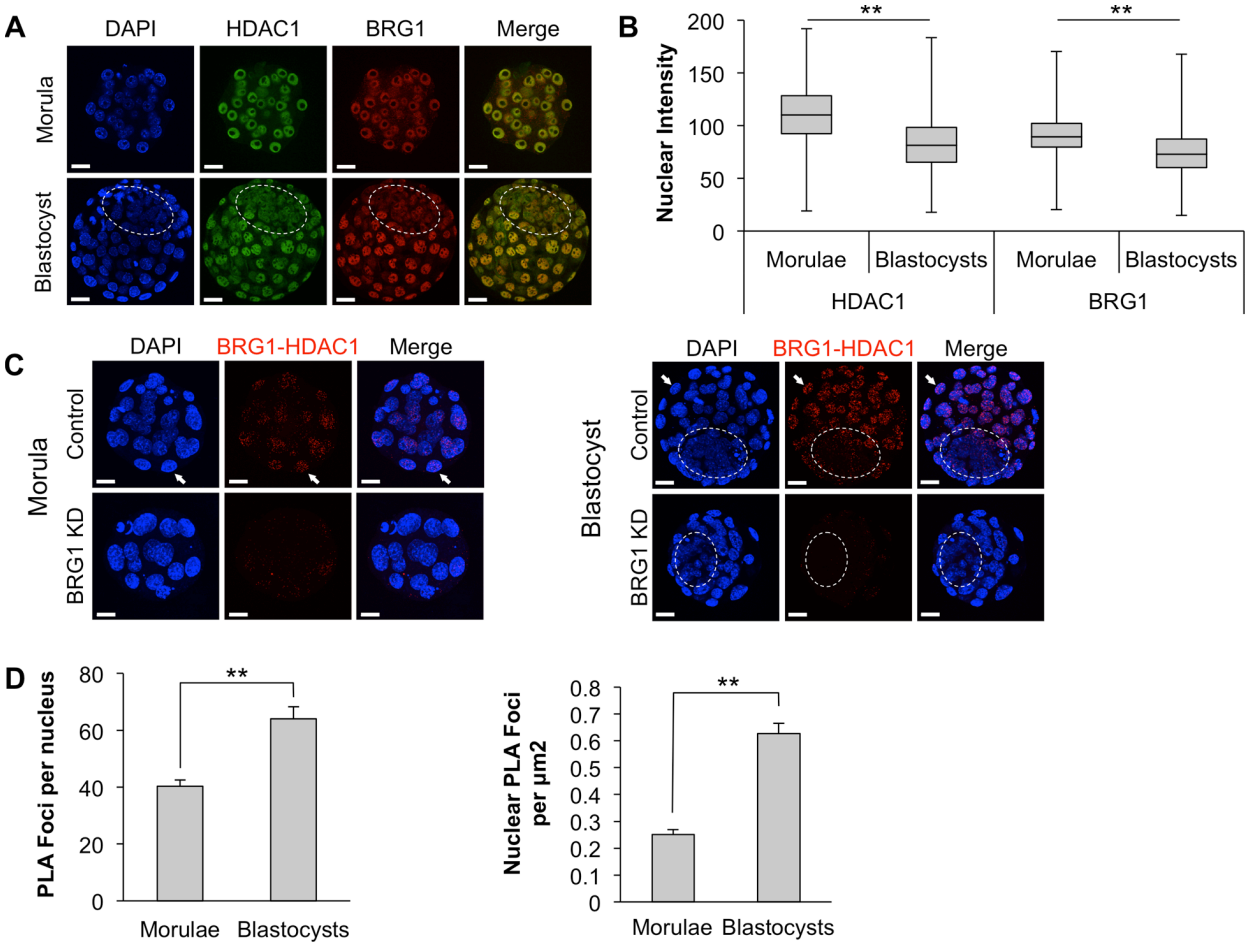

Figure 3-5. BRG1 and HDAC1 interactions are enriched in the trophoblast lineage during the morula to blastocyst transition. (A) Representative z-stack projections of morulae and blastocysts stained with BRG1 and HDAC1 antibodies. Position of the blastocyst ICM is indicated by the dashed circle. Scale bar = 20 µm. (B) Summary of nuclear staining intensity calculated using ImageJ. Box plot represents the distribution of the intensities for all nuclei analyzed in each group. Asterisks indicate a significant difference in the average intensity of the two groups ($p < 0.01$). Centrally located z-sections from 23 morulae and 24 blastocysts were used to calculate the intensities. (C) Specific interactions between BRG1 and HDAC1 in morulae and blastocysts were determined using PLA that detects interacting proteins (< 30nm in

Figure 3-5 (cont'd)

proximity). The red staining is representative of the BRG1-HDAC1 interaction, where a greater number of foci is indicative of increased interactions. BRG1 KD embryos were used as a negative control. White arrows indicate representative nuclei containing BRG1-HDAC1 interactions. Nuclei were counterstained with DAPI. The location of the blastocyst ICM is indicated by the dashed circle. Scale bar = 20 μm. (D) Quantification of PLA foci acquired using Blobfinder software. Centrally located z-sections from 19 morulae and 20 blastocysts, pooled from three-independent PLA experiments were analyzed for quantification of the PLA foci. Nuclei located along the outside edge of morulae and from the trophectoderm of blastocysts were used for the quantitative analysis. Error bars on graph represent the standard error of the mean (S.E.M). Asterisks indicate a significant difference between the comparisons indicated ($p < 0.01$).

A BRG1-HDAC1 complex mediates Nanog repression during early embryonic development

To further explore the potential relationship between BRG1, HDAC1, and *Nanog* transcription, several HDAC1 and/or BRG1 loss of function experiments were performed in ESCs and preimplantation embryos. Previous studies in mouse and human ESCs demonstrated that lower concentrations of sodium butyrate (NaB) promote ES cell pluripotency (36). Furthermore, HDAC1 knockout ESCs exhibit increased levels of *Nanog* mRNA (32). Thus, we hypothesized that HDAC1 cooperates with BRG1 to negatively regulate *Nanog* expression during early embryonic development. In the first set of experiments ESCs and preimplantation embryos were cultured in the presence of increased concentrations of the HDAC inhibitor NaB. ESCs cultured in the presence of 0, 0.125, 0.25, 0.5, 1.25, and 2.5 mM NaB, exhibited a bell-shaped dose-response (Fig. 3-6A). At low (0.125 mM) and high (2.5 mM) concentrations the levels of *Nanog* transcripts were similar to the vehicle, whereas, at intermediate doses (0.5 mM) *Nanog* transcripts were significantly induced ($P<0.05$). Morulae cultured in the presence of 0, 0.125, 0.5 and 2.5mM NaB for 24 h exhibited an increase in histone AcH3K9/14 and a dose dependent rise in *Nanog* transcripts compared to vehicle treated control embryos ($P<0.05$; Fig. 3-6A-C). Similar to the phenotype of BRG1 KD blastocysts (15, 19), embryos cultured in the presence of NaB ectopically expressed NANOG in the trophoblast lineage (Fig. 3-6D).

To test whether manipulation of BRG1 and HDAC1 levels exerts an additive or synergistic effect on *Nanog* transcription, BRG1 and/or HDAC1 were downregulated in preimplantation embryos by microinjection of *Brg1* and *Hdac1* siRNA alone or in combination. Manipulated embryos were cultured to the blastocyst stage and *Nanog* transcripts were evaluated. Depletion of BRG1 or HDAC1 alone triggered a 90 and 40% increase in *Nanog* transcripts, respectively (Fig. 3-6E). Moreover, combined depletion of both BRG1 and HDAC1

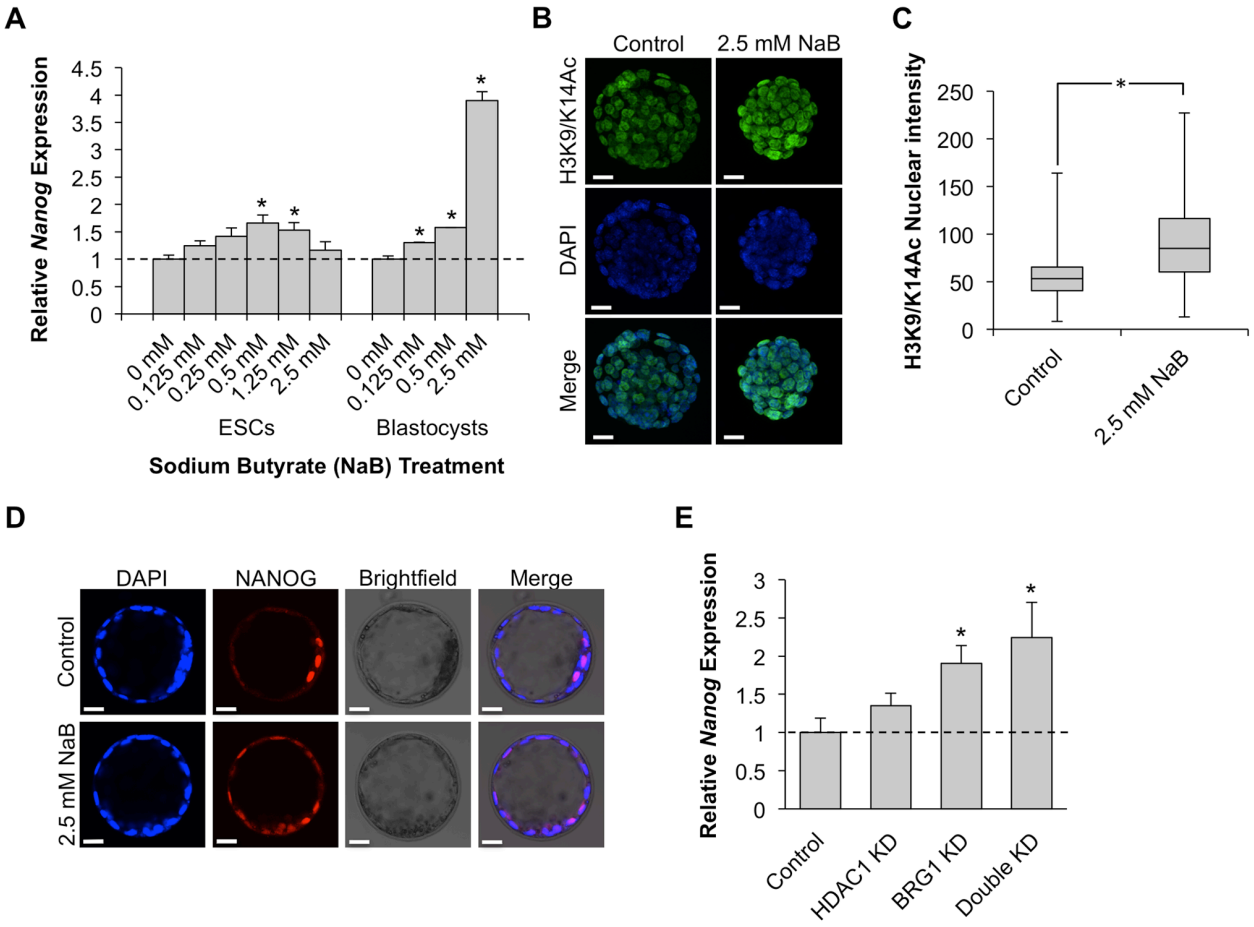

Figure 3-6. HDAC1 cooperates with BRG1 to negatively regulate *Nanog* expression during early embryogenesis. (A) qRT-PCR analysis of *Nanog* transcripts following treatment of ESCs and mouse morulae with various concentrations of NaB. Asterisks indicate doses of NaB that elicited a significant increase in expression versus vehicle-treated samples. Dashed line denotes vehicle-treated control expression set at 1. (B) Representative z-stack projection of immunofluorescent staining for histone H3K9/K14 acetylation (green) in NaB-treated blastocysts. (C) Box-plot summary of nuclear staining intensities of H3K9/K14 acetylation calculated using ImageJ. Asterisk indicates a significant difference between indicated samples ($p < 0.05$). (D) Representative immunfluorescent images of control and treated blastocysts stained for NANOG (red). Nuclei were counterstained with DAPI and transmitted light images

Figure 3-6 (cont'd)

were also collected at the same focal plane to assess embryo morphology. Scale bar = 20 μm. (E) qRT-PCR analysis of *Nanog* transcripts in blastocysts obtained following injection of siRNAs targeting *Hdac1*, *Brg1*, or both *Hdac1* and *Brg1*. Gene expression is shown relative to embryos injected with a non-targeting control, asterisks indicate a significant difference compared to control ($p < 0.05$). Dashed line denotes control expression set at 1.

resulted in a 124% increase in *Nanog* transcripts that was greater than downregulation of BRG1 or HDAC1 alone ($P<0.05$; Fig. 3-6E). All in all, these results indicate that HDAC1 cooperates with BRG1 in an additive manner to modulate *Nanog* expression in ESCs and negatively regulate *Nanog* during trophoblast lineage formation in blastocysts.

BRG1 is required for remodeling nucleosomes at the Nanog proximal enhancer and differentiation of ESCs into trophoblast-like cells

The acquisition of nucleosomes in gene regulatory regions serves as one mechanism for attenuating transcription in eukaryotic cells (37, 38). Recent work in our laboratory established that downregulation of *Oct4* and *Nanog* expression in ESCs is associated with dynamic changes in chromatin structure at core enhancers (22). To test whether BRG1 is required for chromatin remodeling at the *Nanog* proximal promoter, nucleosome mapping experiments were performed. Control and BRG1 KD cells were treated with micrococcal nuclease (MNase) and isolated mononucleosome DNA was subjected to real-time qPCR analysis using overlapping primer sets that span the *Nanog* proximal enhancer and TSS. In addition, chromatin from mouse embryonic fibroblasts (MEFs) was used as a positive control for increased nucleosome occupancy; in this cell-type *Nanog* is epigenetically silenced. In control uninduced ESCs, where *Nanog* is highly expressed, the *Nanog* proximal promoter region was largely devoid of nucleosomes (Fig. 3-7A). Whereas in *Cdx2*-induced ESCs two prominent nucleosomes were established at the *Nanog* proximal enhancer and TSS ($P<0.05$; Fig. 3-7B). Interestingly, in BRG1 KD *Cdx2*-induced ESCs nucleosome remodeling was compromised. Nucleosome occupancy was low and resembled control and BRG1 KD uninduced ESCs ($P>0.05$; Fig. 3-7B). Consistent with these results we found that histone H3 is greatly enriched at the *Nanog* proximal enhancer in *Cdx2*-

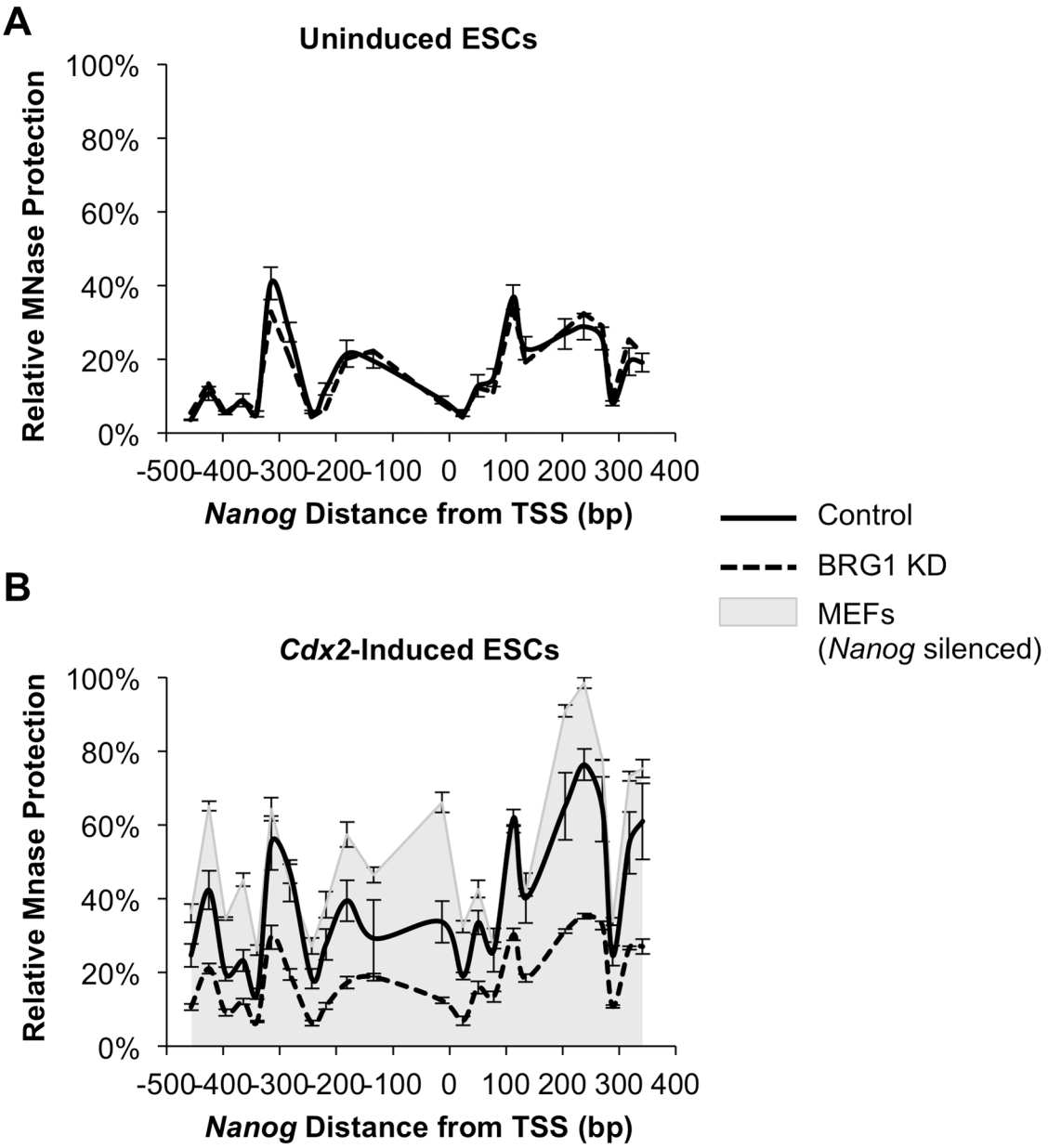

Figure 3-7. BRG1 is required for nucleosome remodeling at the *Nanog* proximal promoter during ESC to trophoblast cell transdifferentiation. (A and B) MNase protection assays were performed on control and BRG1 KD ESCs (uninduced and *Cdx2*-induced) to determine nucleosome occupancy at the *Nanog* proximal promoter. MNase protection was determined by qRT-PCR using 23 primer pairs covering a ~1kb region around the *Nanog* TSS. MEFs were used as a control. Error bars on graph represent the standard error of the mean (S.E.M).

induced ESCs (22). Thus, these results demonstrate that BRG1 is required for nucleosome remodeling at the *Nanog* proximal promoter and suggest that during trophoblast lineage development BRG1 negatively regulates *Nanog* transcription via chromatin remodeling and histone H3K9/14 deacetylation.

Discussion

Previous work from our laboratory and others established that *Oct4*, *Nanog*, and *Sox2* are direct targets of BRG1 in mouse ESCs (15, 19, 20, 22). Notably, these studies demonstrated that BRG1 binding is enriched at key regulatory elements such as enhancers and TSSs within the *Oct4*, *Nanog*, and *Sox2* genes. Results of the present study expand on these observations and provide new insights into the BRG1-dependent mechanisms that govern *Nanog* expression during early embryonic development in mice. We found that during early embryogenesis: (i) BRG1 is required for transcriptional silencing of *Nanog*, (ii) BRG1 regulates *Nanog* expression via interactions with HDAC1 and antagonism of histone AcH3K9/14, and (iii) BRG1-dependent chromatin remodeling activity is required for nucleosome remodeling at the *Nanog* proximal enhancer. Collectively, our data demonstrate that during mouse early embryogenesis a BRG1-HDAC1 complex negatively controls *Nanog* transcription via a combination of histone H3K9/14 deacetylation and nucleosome remodeling. These overlapping modes of regulation are summarized in a model shown in Fig. 3-8.

During preimplantation development proper cell-fate decisions are vital for establishment of the pluripotent ICM and multipotent extraembryonic lineages. NANOG is a key regulator of pluripotency during early development (5, 39). In preimplantation embryos *Nanog* is widely expressed initially and then during the morula-to-blastocyst transition it becomes restricted to the

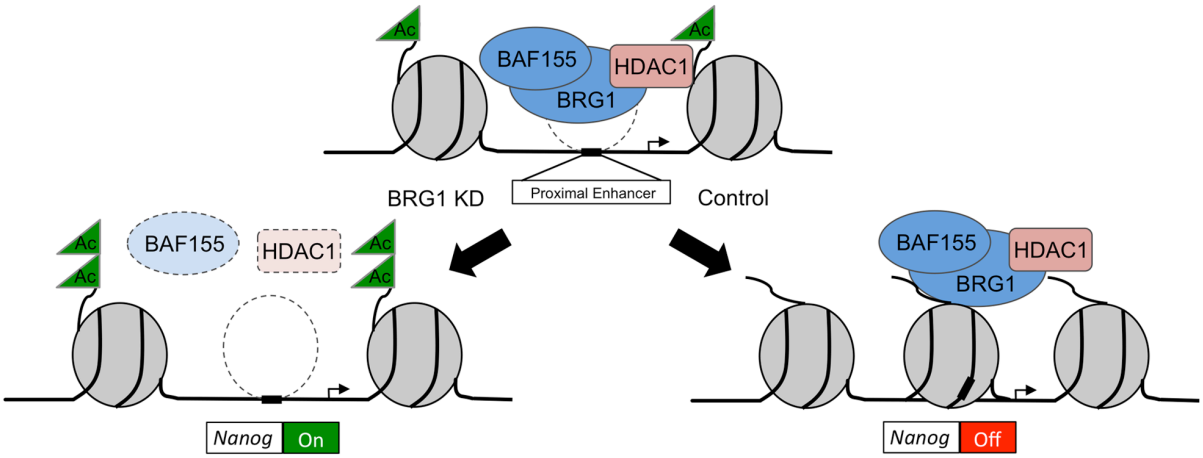

Figure 3-8. Model for BRG1-dependent regulation of *Nanog* transcription. Working model proposing a dual-mechanism on how BRG1 regulates *Nanog* expression during formation of the trophoblast lineage. BRG1 modulates *Nanog* expression in pluripotent cells and the emerging trophoblast lineage via antagonizing H3K9/14 acetylation at the *Nanog* proximal enhancer. BRG1 achieves this by interacting with HDAC1. Upon differentiation, BRG1's chromatin remodeling activity acts in conjunction with histone deacetylation to silence *Nanog* expression in the trophoblast lineage.

ICM, and silenced in the extraembryonic lineages (5, 6). Functional studies in mice demonstrated that NANOG plays a pivotal role in establishment of pluripotency during preimplantation development. Genetic ablation of *Nanog* results in failure to form the embryonic epiblast, an excess in extraembryonic endoderm, and lethality between days 3.5 and 5.5 of development (5). Moreover, *Nanog* deficient ESCs differentiate into extraembryonic lineages, whereas, overexpression of *Nanog* in ESCs promotes self-renewal (5, 39). Thus, *Nanog* is an essential gene required for establishment of pluripotency and its precise regulation is critical for normal embryonic development.

In the current study we show that BRG1-dependent regulation of *Nanog* may be important for trophoblast development in preimplantation embryos and CDX2-inducible ESCs. Proper development of the trophoblast lineage requires downregulation of pluripotency genes (4) and upregulation of trophoblast-specific transcription factors such as TFAP2C, CDX2, ELF5, and EOMES (4, 7, 40). Interestingly, in BRG1-deficient ESCs undergoing trophoblast transdifferentiation there were significantly higher levels of *Nanog* mRNA and protein, and lower levels of *Elf5*, *Tfap2c*, and *Eomes* mRNA. Consistent with this finding, BRG1-deficient blastomeres in chimeric embryos exhibited a bias and preferentially contributed to the ICM. Since NANOG is a known suppressor of the extraembryonic lineages (41-44), we envisage that BRG1-dependent repression of *Nanog* may serve as a mechanism to facilitate trophoblast lineage specification. Ongoing experiments in our laboratory are elucidating the exact role of BRG1-dependent regulation of *Nanog* in early embryonic development.

Although BRG1 was originally identified as an activator of gene expression in yeast (45, 46), work in mammalian cells over the last decade has shown that BRG1 can function as either an activator or repressor of gene transcription (35). Its role as an transcriptional activator or

repressor depends on both the cellular context and which coactivators or corepressors are present (47-49). We showed that BRG1 can differentially regulate *Nanog* transcription in ESCs and trophoblast cells by two mechanisms. The first mode of regulation occurs in ESCs where BRG1 interacts with the corepressor HDAC1 to antagonize histone AcH3K9/14 at the *Nanog* proximal enhancer. Disruption of BRG1 and/or HDAC1 augmented AcH3K9/14 and caused an increase in *Nanog* expression. This type of regulation appears to be important for fine-tuning *Nanog* expression in pluripotent cells where a specific amount of NANOG is important for maintaining an ESC identity (5, 39, 50). The second mechanism operates during formation of the trophoblast lineage when *Nanog* expression is normally silenced. Disruption of BRG1 and/or HDAC1 blocked *Nanog* repression by maintaining an open chromatin structure and sustaining higher levels of AcH3K9/14 at the *Nanog* proximal enhancer. This type of regulation is likely critical for silencing *Nanog* expression in the trophoblast lineage. In future studies it will be exciting to determine whether interactions between HDAC1 and BRG1 stimulate one another's activity to regulate *Nanog* expression. Moreover, it will be interesting to test whether OCT4 and CDX2 are involved in recruiting and/or regulating the activities of BRG1 and HDAC1 at the *Nanog* proximal enhancer. In this regard, we demonstrated that BRG1 can physically interact and/or colocalize with OCT4 and CDX2 at pluripotency gene enhancers (15, 22).

Our most novel and intriguing finding from the current study is the observation that BRG1 can antagonize histone H3K9/14 acetylation to fine-tune and/or negatively regulate pluripotency gene expression in the early embryonic lineages. Consistent with this, a recent study in human ESCs showed that BRG1 can regulate lineage-specific genes via inhibition of histone H3K27 acetylation at their enhancers (51). Interestingly, genome-wide ChIP studies in mouse ESCs and T-lymphocytes unexpectedly revealed that HDACs are enriched at regulatory

elements of highly expressed genes (32, 52). Thus, modulation of histone acetylation levels via a BRG1-HDAC complex may serve as a much larger regulatory mechanism to control transcription of key pluripotency and lineage-specific genes to ensure proper development.

In summary, results reported here demonstrate that BRG1 cooperates with HDAC1 to regulate *Nanog* expression in the early cell lineages. Disruption of BRG1 or HDAC1 activity perturbed *Nanog* expression and blocked embryonic development. Such information may be pertinent to understanding some causes of early embryonic failure in humans. Furthermore, our findings have broader implications in ESCs and induced pluripotency stem (iPS) cells where NANOG plays a crucial role in maintenance of self-renewal and pluripotency, as well as acquisition of pluripotency during nuclear reprogramming. Manipulation of BRG1 and/or HDAC1 expression in some cell-types could serve as a tool to alter cell-identity.

Acknowledgements

This research was supported by a grant from the National Institute of General Medical Sciences to J.G.K. [GM095347] and grants from the National Institute of Child Health and Human Development to S.P. [HD079363, HD062546 and HD075233].

We thank Dr. Gerald Crabtree (Stanford University) for generously providing the *Brg1* shRNA plasmids and Dr. Anthony Imbalzano (UMASS, Medical School) for providing the rabbit BRG1 antiserum. We are grateful for Dr. Monique Floer's (MSU) critical reading of the manuscript.

APPENDIX

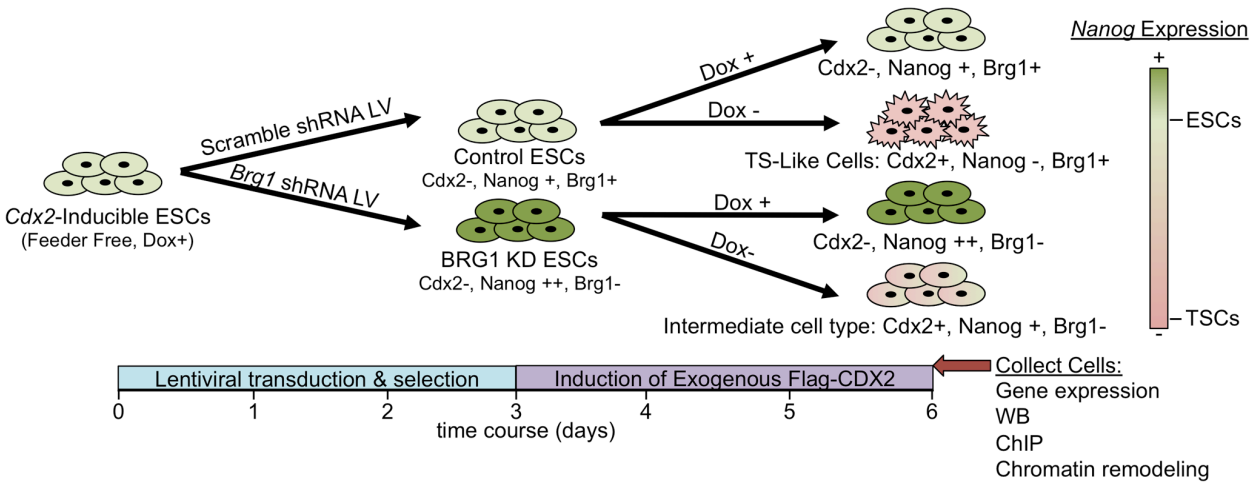

Figure 3-9. Supplemental methods and experiment design for *Brg1* shRNA KD in *Cdx2*-inducable ESCs.

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CHAPTER 4 -

CONCLUSIONS AND FUTURE PERSPECTIVES

BRG1, a chromatin remodeling ATPase, is known to function as a coregulator of gene expression eliciting both coactivator and corepressor functions on the pluripotency gene network. Epigenetic mechanisms such as histone marks are one way in which the coregulator function can switch between these roles by recruitment of coactivators and corepressors that interact with BRG1 and its associated BAF proteins. In this chapter I will describe how my studies on early lineage formation of the mouse embryo has expanded the understanding of how BRG1 regulates the core pluripotency gene network to repress *Pou5f1* and *Nanog* expression during lineage formation. First, I will discuss the importance of our finding that histone H3K9/14 acetylation marks and HDAC1 occupancy are lost during the differentiation of ESCs toward TE lineage. Second, I will discuss our findings that BRG1 associates with HDAC1 to repress *Nanog* by antagonizing H3K9/14 acetylation. Lastly, I will discuss how BRG1 and HDAC1 appear to regulate the Wnt signaling pathway, and describe the implications this has on the pluripotency gene network.

The central role of NANOG within the pluripotency transcriptional network and its regulation by BRG1

The transcriptional regulatory loop of NANOG, which is considered the most important of the core pluripotency transcription factors, directs a distinct gene expression profile in which the mechanistic basis of pluripotency in ESCs can be understood (1, 2). ESCs have the capacity to form any cell type in the body but this pluripotent state requires the stochastic expression of the transcription factor NANOG. Individual cells within a population of ESCs display a heterogeneous expression pattern and are known to fluctuate levels of NANOG expression (3-5). Lineage-specific NANOG expression in the mouse blastocyst is recognized to occur through the

Grb2-MAPK pathway distinguishing EPI from PE (6). This constitutes the second cell-fate decision in the mouse. However as ESCs are derived from ICM, the lineage specificity in this earlier network is established through the mutually exclusive actions of OCT4 and CDX2 to distinguish ICM from TE, respectively (7, 8). NANOG expression was also shown to be upregulated via co-occupancy binding of OCT4-SOX2 on the *Nanog* promoter (9).

Additional mechanisms of how the pluripotency signaling network converge to transcriptionally regulate NANOG expression in ESCs have been discovered and while some of these have been characterized (i.e., P53 and TCF3), the regulation of *Nanog* is not well understood (10). The subtle differences in the processes that regulate *Nanog* expression for maintaining pluripotency in ESCs are predicated on what dictates lineage formation, but it is important to remember that while the expression profile of ESCs matches that of a cell in the EPI, it is actually derived from a cell of the ICM (11). Various signal transduction pathways and epigenetic mechanisms potentiate the pluripotency gene network to establish from ICM lineage cells, a gene expression profile similar to that of the EPI, which can continually divide and has the capacity to form all three germ layers (12).

The BRG1-containing BAF complex is an important coregulator involved with pluripotency gene signaling and lineage formation (13, 14). Importantly, BRG1 has been shown to directly regulate the expression of the core pluripotency transcription factors: NANOG, OCT4, and SOX2. It is for this reason that BRG1 also impacts early lineage formation of the mouse embryo. The established mechanisms though which BRG1 regulates the expression of these core pluripotency factors have mostly been limited to BRG1 acting as a coactivator (15, 16). However it has been established in both ESCs and the mouse blastocyst that BRG1 also negatively coregulates these genes (13, 14). I hypothesized that BRG1, due to interactions with

corepressors via its associated context-specific BAFs, can form a temporal and lineage-specific repression complex that represses pluirpotency genes during trophoblast development.

In Chapter II, I characterized the transcriptional and epigenetic processes that occur during CDX2-induced silencing of *Pou5f1* and *Nanog* in ESCs as a model system for TE development. Induction of *Cdx2* expression results in a decreased *Oct4/Nanog* expression, an increase in TE markers, and differentiation into trophoblast-like stem (TS-like) cells within 48 to 120 h. Consistent with the down-regulation of *Oct4* and *Nanog* transcripts, an increase in CDX2 binding and a decrease in RNA polymerase II (RNAPII) and OCT4 binding was observed within 48 h (Fig. 2-2).

To test whether transcriptionally active epigenetic marks were erased during differentiation, histone H3K9/14 acetylation and two of its epigenetic modifiers were evaluated. A significant decrease in histone H3K9/14 acetylation and loss of p300 and HDAC1 binding at the *Oct4* and *Nanog* regulatory elements was observed by 48 h (Fig. 2-3). Accompanying these changes, there was a significant increase in total histone H3 and a loss of chromatin accessibility at both the *Oct4* and *Nanog* regulatory elements, indicating chromatin remodeling (Fig. 2-4).

Lastly, DNA methylation analysis revealed that methylation did not occur at *Oct4* and *Nanog* until 96 to 120 h after induction of CDX2 (Fig. 2-5 and 2-8). These results showed that silencing of *Oct4* and *Nanog* is facilitated by sequential changes in transcription factor binding, histone acetylation, chromatin remodeling, and DNA methylation at core regulatory elements.

One of the main questions that we asked after performing this study is whether depletion of BRG1 during this differentiation process would influence any of the observed dynamic transcriptional and epigenetic processes. It was also interesting, but somewhat expected that the regulatory aspects of *Oct4* and *Nanog* were so closely related during CDX2-induced silencing.

The protein levels around 48h post-induction are arguably the only variability between the silencing of *Oct4* and *Nanog* that was observed. Of particular interest from this study was that HDAC1 was already present at the *Oct4* and *Nanog* genes in the ESC-condition, but the occupancy of HDAC1 rapidly decreased during induced-differentiation by the overexpression of CDX2. However the presence of both HDACs and HATs at active promoters has become recognized in both ESCs and other cell types at highly active genes (17, 18). This could suggest these genes are needed to be poised for rapid repression. HDAC1 has also been suggested to be critical for ESC differentiation while HDAC2 has not (19).

In the study presented in chapter III, this requirement of HDAC1 over HDAC2 becomes reinforced. Overall we found that BRG1 cooperates with HDAC1 to regulate *Nanog* expression. BRG1 depletion in preimplantation embryos and CDX2-inducible ESCs revealed that BRG1 is necessary for *Nanog* silencing in the trophoblast lineage and in undifferentiated ESCs the loss of BRG1 augmented Nanog expression (Fig. 3-1 and 3-2). Analysis of histone H3 within the *Nanog* proximal enhancer revealed that H3K9/14 acetylation increased in BRG1 depleted embryos and ESCs compared to controls suggesting that BRG1 might be required for deacetylation of occur at these sites (Fig. 3-3). Biochemical studies demonstrated that HDAC1 is found associated with BRG1-BAF155 complexes and BRG1-HDAC1 interactions were found to occur more frequently in the trophoblast lineage (Fig. 3-4 and 3-5). HDAC1 inhibition triggered an increase in H3K9/14 acetylation and a corresponding rise in *Nanog* mRNA and protein, phenocopying BRG1 knockdown embryos and ESCs (Fig. 3-6). Nucleosome mapping experiments revealed that BRG1 is required for changes in chromatin density to occur along the *Nanog* proximal promoter (Fig. 3-7). The results from this study go on to support my overall hypothesis.

An interesting observation is that there is an uncoupling in the manner in which CDX2-

induction in ESCs results in the repression of *Oct4* and *Nanog* when BRG1 is ablated. This is illustrated from comparing the transcriptional profile and protein levels of NANOG reported in Chapter III, to those of OCT4 (previously not shown – See Appendix, Fig. 4-1A and B). This could largely be attributed to different cues that influence the expression of *Oct4* acting through the pluripotency transcription network as well as differences in the context-specific BAFs. Since NANOG was shown to be increased in the TE following BRG1 KD and a similar phenotype is obtained for OCT4 in the mouse embryo, the repressive dynamics occurring at *Oct4* and *Nanog* appear to be more coupled in embryo than in ESCs. It has been shown that the epigenetic properties of ESCs somewhat limit the complete capacity of differentiation to a true TS cell state, supporting the importance of context-specific cues that govern the pluripotency gene network and its coregulation by BRG1 (20). Interestingly ablation of BAF155 in CDX2-induced ESCs results in higher *Oct4* expression illustrating the influence of BAF subunits on these dynamics (See Appendix – Fig. 4-2).

BRG1 and HDAC1 co-occupy several Wnt signaling genes in ESCs

Future studies of how BRG1-HDAC1 coupled repression influences the core pluripotency network are needed, as it appears that these two coregulators are involved in a large number cellular processes (See Appendix Table 4-1). Several reports have supported a collaborative role of these two factors in ESCs. The interactions of the BAF complex with the NURD complex are central to these interactions (16). While initial reports showed that BRG1 interacts with MBD3 to influence genes that impact pluripotency signaling, BRG1 and MBD3 act on a subset of genes that do not affect the levels of the core pluripotency transcription factors. However, a novel complex has been reported that contains alternative members of the NURD

complex, namely MTA1 that is shown to couple the interaction of HDAC1 and HDAC2 to OCT4 and NANOG (21). This complex, referred to as NODE (NANOG OCT4 associated deacetylase), is an ideal candidate to be the HDAC1-containing complex capable of interacting with BRG1via its associated BAF proteins. Because it contains some of the pluripotency transcription factors itself, it might influence the autoregulatory transcriptional network of ESCs. One more important aspect of NODE, is that it does contain substantial levels of MBD3.

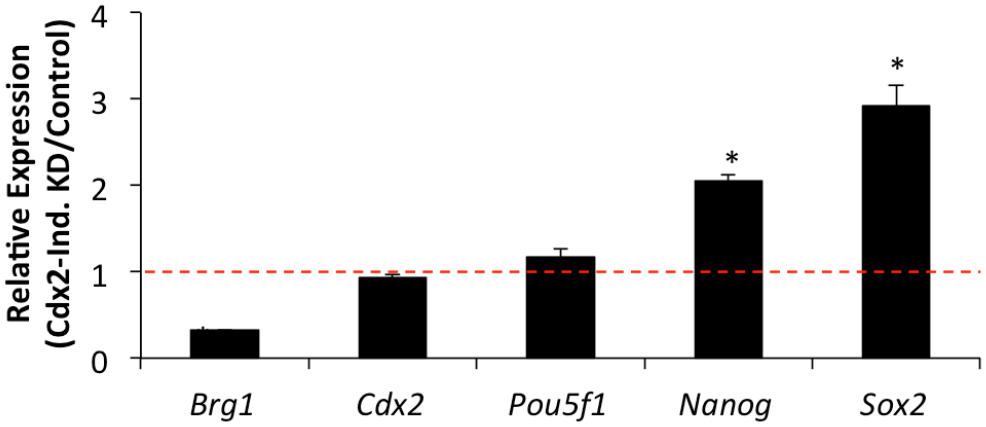
To this end, using published ChIP-on-Chip datasets for BRG1 and HDAC1 in mouse ESCs, I have explored the sets of genes regulated by both of these coregulators (See Appendix – Fig. 4-3 & Table 4-2)(13, 17). The majority of genes bound by BRG1 and HDAC1 were found to not be occupied by Mbd3 suggesting that other genes that might be regulated in a manner similar to *Nanog*. The Database for Annotation, Visualization and Integrated Discovery (DAVID) annotation pathway analysis tool was used to identify possible functions or molecular interactions of the listed genes co-occupied by HDAC1 and BRG1 in ES cells. Several Wnt signaling targets were identified in this subset of genes, a signaling pathway known to impact both pluripotency and embryonic development. Importantly, Wnt signaling has been shown to be associated with blastocyst formation and implantation (22). Moreover, Wnt signaling is considered to be a conserved signaling pathway that establishes pluripotency in both mice and humans (23). Opinion is divided as to whether Wnt signaling serves to positively or negatively regulate pluripotency signaling, alluding to a potential role of a coregulator such as BRG1 in regulating Wnt signaling, and reinforcing that BRG1 might impact the division between pluripotency and differentiation (24, 25). All of these aspects provide justification that BRG1-containing repressive complexes might influence Wnt signaling genes in a manner similar to the regulation of *Nanog*. Preliminary experiments have confirmed that disruption of BRG1 results in

overexpression of some Wnt genes during CDX2 induction (See Appendix – Fig. 4-4).

On a final note, there appears to be a strong connection linking Wnt signaling with pluripotency signaling in the establishment of cancer stem cells (26). Deciphering the regulatory roles of a coregulator such as BRG1 that could influence both of these pathways could serve in the discovery of novel therapeutic approaches in cancer biology.

APPENDIX

A



B

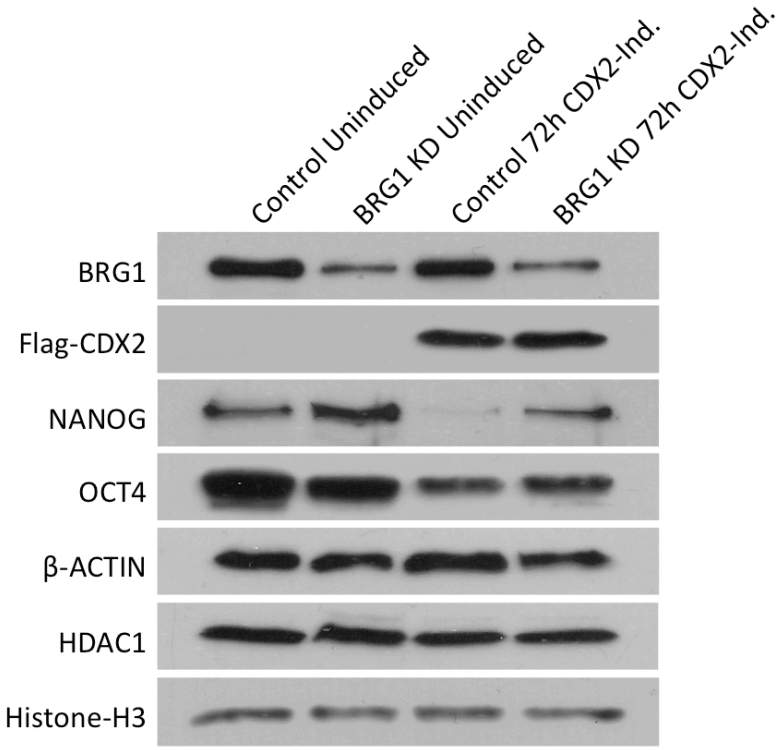


Figure 4-1. Brg1 KD did not increase the expression of *Oct4* during CDX2-induced differentiation. (A) Transcript analysis assessed by qPCR. (B) Western blot analysis.

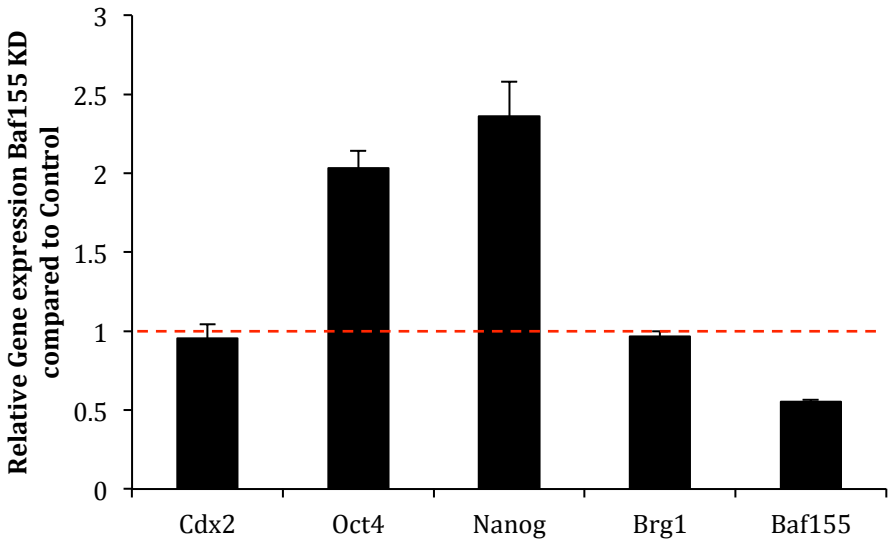


Figure 4-2. BAF155 KD causes *Oct4* transcripts to be higher than control cells following CDX2-induced differentiation of ESCs.

Table 4-1. Biological Function GO of targets occupied by BRG1 and HDAC1 in murine ESCs

Term	Count	%	P-Value
negative regulation of transcription	36	6.383	9.78E-08
negative regulation of gene expression	36	6.383	1.06E-06
stem cell differentiation	7	1.241	5.86E-04
stem cell maintenance	6	1.064	9.20E-04
stem cell development	6	1.064	0.00114
embryonic morphogenesis	25	4.433	0.00142
embryonic development ending in birth or egg hatching	28	4.965	0.00186
positive regulation of gene expression	30	5.319	0.00424
trophectodermal cell differentiation	4	0.709	0.01812

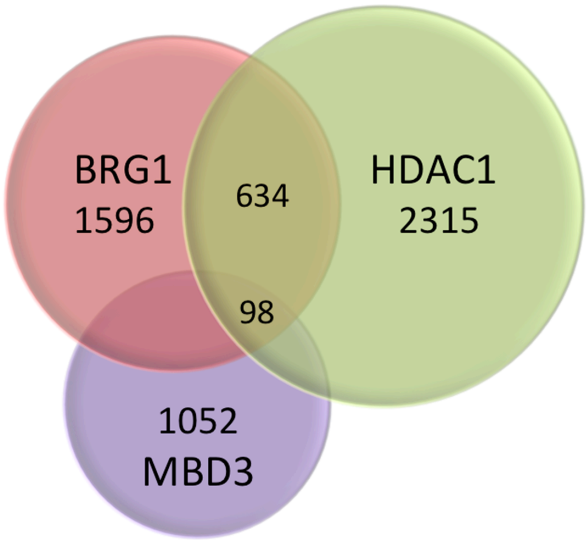


Figure 4-3. Venn diagram of genes occupied by both BRG1 and HDAC1 in mouse ESCs. MBD3 occupancy is also displayed to show that most genes co-occupied by BRG1 and HDAC1 are not occupied by this NURD-complex protein.

Table 4-2. DAVID pathway analysis of targets occupied by BRG1 and HDAC1 in Mouse ESCs.

Term	Count	P-Value	Genes
Adherens junction	8	0.028965703	ACTB, FGFR1, ACTN4, FYN, PVRL3, PVRL2, RHOA, ACTN1
Wnt signaling pathway	12	0.031824211	TRP53, FZD8, SFRP1, SIAH1A, CACYBP, PPP2R5C, RHOA, NFAT5, FZD2, RUVBL1, FZD5, CUL1
Adipocytokine signaling pathway	7	0.046669288	SOCS3, ADIPOR2, PRKAA1, AGRP, IRS1, TRADD, CAMKK2
Prostate cancer	8	0.063486193	TRP53, CCNE1, FGFR1, HSP90B1, HSP90AA1, CREB3L2, PDGFC, GSTP2
RNA degradation	6	0.080129474	CNOT10, RQCD1, LSM4, HSPD1, CNOT4, ENO1

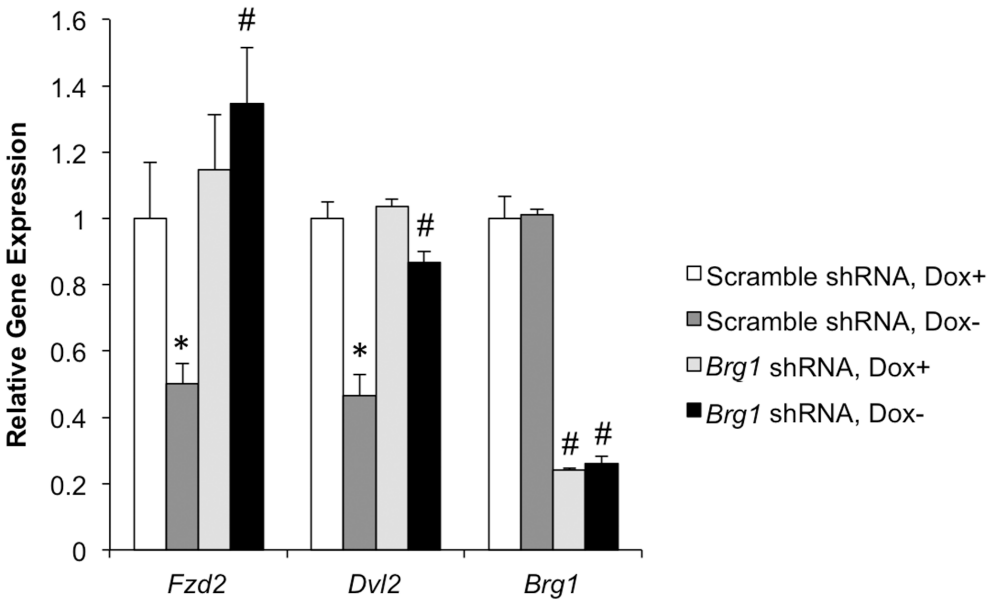


Figure 4-4. Knockdown of BRG1 during CDX2-induced differentiation of ESCs triggers upregulation of a subset of Wnt signaling genes. *Fzd2* encodes for the frizzled-2 receptor protein. *Dvl2* encodes for dishevelled segment polarity protein-2. Error bars represent SEM. * indicates a significant difference of transcript levels between uninduced and induced control cells ($p<0.05$). # indicates a significant difference between transcript levels from control cells and BRG1 KD cells ($p<0.05$).

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