E2F6 IS A COMMON REPRESSOR OF MEIOSIS-SPECIFIC GENES

By

SARAH M. KEHOE

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2007

To my dad, who instilled in me his lifelong passion for learning

ACKNOWLEDGMENTS

I would first like to thank my mentor, Dr. Naohiro Terada, for always being optimistic, no matter how confusing science may seem at times. His positive attitude, encouragement, and perseverance will not be forgotten. Dr. Terada's office door being always open for scientific discussions and his willingness to meet regularly with his graduate students despite his busy schedule, exemplifies his dedication. I would also like to thank all of the members of the Terada lab, both past and present, for their support. In particular, I'd like to thank lab manager, Amy Meacham, for her frequent assistance, her reliable smile, and her friendship. I would especially like to thank former graduate student, Amar Singh, for serving as my mentor at the lab bench; he was a wonderful teacher who answered my many questions and helped me to get my feet off the ground.

I am also grateful for my committee members, Dr. Jörg Bungert, Dr. Hideko Kasahara, Dr. Paul Oh, and Dr. Jim Resnick, for their helpful suggestions, feedback, and expertise. I would like to thank our collaborator, Dr. Stefan Gaubatz at the University of Wurzburg for providing invaluable materials and sound advice for my project. Without him, I would not have had the resources necessary to come to the conclusions that I did.

Further, I want to thank my parents, Robert and Patricia, and sister, Karen, for their unconditional love and support. Likewise, I'd like to thank my husband, Joe, for always being there for me at the end of every day. His humor and encouragement have been important to surviving graduate school. Lastly, I'd like to thank my horse, Sundance, for keeping my life balanced and my stress levels at bay. Without being able to escape to the barn and ride my horse on days when the complexity of science seemed too great to ever make headway, I would never have made it this far.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

E2F6 IS A COMMON REPRESSOR OF MEIOSIS-SPECIFIC GENES

By

Sarah M. Kehoe

December 2007

Chair: Naohiro Terada
Major: Medical Sciences--Molecular Cell Biology

Genes whose protein products function specifically during meiosis are strictly repressed in somatic cells. Their aberrant expression may lead to mitotic catastrophe and predispose cells to oncogenic transformation. Although previous studies have demonstrated various mechanisms delineating repression of individual meiosis-specific genes, the existence and identity of a master regulatory protein which coordinately represses multiple meiosis-specific genes in somatic tissues remains to be elucidated. E2F6, a member of the E2F family of transcription factors, was recently shown to play an essential role in the repression of a few meiosis-specific genes including *Stag3* and *Smc1β*. Here we report that E2F6 is required for the repression of another meiosis-specific gene, *Ant4,* in somatic cells.

This discovery prompted us to investigate whether meiosis-specific genes in general, require E2F6 for their repression. We compiled a list of 24 meiosis-specific genes and observed that 19 of them (79.2%) have the core E2F6-binding element (TCCCGC) within 200bp upstream of their transcription initiation sites. This was significantly higher than the frequency found in the promoters of all mouse genes (15.4%) and the mathematical probability of random occurrence (9.3%). Further, using embryonic stem cells, we demonstrate that E2F6 indeed binds to the promoters and represses the transcription of these meiosis-specific genes. These data

suggest that E2F6 serves as a common repressor of meiosis-specific genes. Of interest, E2F6 deficiency alone did not derepress most of these meiosis-specific genes in somatic cells, suggesting that a functional redundancy exists whereby other safeguards are in place to ensure the repression of most meiosis-specific genes in somatic cells.

CHAPTER 1
INTRODUCTION

Transcriptional Control of Gene Expression

With the discovery of the structure of DNA by Watson and Crick in the early 1950s (Watson & Crick 1953) came the understanding that every cell in the body, although diverse, contains the same genetic information as all other cells. Prior to this breakthrough, it was difficult to fathom that two cell types with extreme differences in shape and function, such as cardiomyocytes and neurons, could have been coded from the same genome. In fact, it was speculated that different cell types actually contained different sets of genes due to a selective loss of unnecessary genes as the cells differentiated (Alberts *et al.* 2002). Today, we know that the vast diversity of cell types found in the body is a result of differences in gene expression rather than a consequence of changing the presence of nucleotide sequences in a cell's genome.

Experimental evidence leading to this conclusion involved taking the nucleus from a fully differentiated frog cell and injecting it into a frog egg whose nucleus had been removed (Gurdon 1962). The donor nucleus was able to direct the recipient egg to produce a normal tadpole that contained a full range of differentiated cell types, thereby proving that the genome must be preserved in the differentiated donor cell. Similar experiments done in various species of mammals including: sheep (Campbell *et al*. 1996, Wilmut *et al*. 1997), cattle (Cibelli *et al.* 1998, Kato *et al.* 1998), mice (Wakayama *et al.* 1998), goat (Baguisi et al. 1999), pig (Polejaeva *et al.* 2000), cat (Shin *et al.* 2002), rabbit (Chesne *et al.* 2002), horse (Galli *et al.* 2003), and endangered wolves (Kim *et al.* 2007), have further validated that the genome is upheld in every somatic cell.

Given that the human genome encodes approximately 35,000 genes (Pennisi 2001), specialized cell types have the difficult job of selectively expressing the genes that they need to

support the unique properties of their cell type. If this were not difficult enough, the ability to express the appropriate genes is further complicated by the fact that the eukaryotic genome is packaged into nucleosomes and higher order forms of chromatin which can restrict a gene's accessibility. Fortunately, cells are equipped with the molecular machinery needed to overcome such obstacles. This machinery controls gene expression by operating at various levels using different mechanisms: 1) on a transcriptional level by controlling how often a gene is transcribed to RNA, 2) on an RNA processing level by regulating RNA transcript splicing, 3) on a location basis by selecting which messenger RNA (mRNA) in the nucleus gets transported to the cytoplasm for protein synthesis, 4) on a degradation basis by selectively destabilizing certain mRNA molecules in the cytoplasm, 5) on a translational level by selecting which mRNAs are translated into proteins by ribosomes, and 6) on a post-translational level by affecting protein activity via activation, inactivation, degradation, or compartmentalization (Alberts *et al*. 2002). For the remainder of this dissertation, I will focus on the transcriptional regulation of gene expression.

The General Transcription Machinery

At the heart of transcription is the enzyme, RNA polymerase. RNA polymerase moves along the DNA, unwinding the DNA helix ahead of it to expose a template strand of DNA so that incoming ribonucleotides can complementary base pair and form a growing RNA chain. This RNA chain is then released and depending on the type of RNA, goes on to be processed accordingly. In this study, we focus on mRNA which is transcribed by RNA polymerase II, and after splicing goes on to be translated into protein. In order for the RNA polymerase to transcribe a gene accurately, it must be able to recognize where in the genome to start and end transcription. The starting point of transcription is indicated by a special sequence of nucleotides known as the promoter and when the RNA polymerase comes across this sequence, it binds

tightly to it. The polymerase then begins transcription and continues to produce an RNA transcript until it encounters a termination signal. This end signal then enables the release of the newly made RNA chain and the polymerase from the DNA.

The observation that unlike bacterial RNA polymerase, purified eukaryotic RNA polymerase II could not initiate transcription *in vitro* led to the discovery and purification of additional required factors (Weil *et al.* 1979). These factors are referred to as general transcription factors because they assemble on all promoters used by RNA Polymerase II. Their job is to help the RNA polymerase position itself correctly at the promoter, to assist in pulling apart the DNA strands, and to release the RNA Polymerase from the promoter into elongation mode once transcription has begun. These general transcription factors assemble at the promoter region in a specific order. First, the general transcription factor, TFIID, binds to the TATA box, a short DNA sequence located 25 nucleotides upstream from the transcription start site that is composed primarily of T and A nucleotides. The subunit of TFIID that recognizes the TATA box is called TBP (for TATA-binding protein). TFIID binding to the TATA box causes a distortion in the DNA which brings DNA sequences on both sides of the distortion together to allow for subsequent protein assembly. However, many promoters do not contain TATA boxes. In some large groups of genes, such as housekeeping genes, the TATA box is often absent and the corresponding promoters are referred to as TATA-less promoters. In these promoters, the exact position of the transcription start site and the binding of the general transcription factors is often controlled by an initiator (Inr) nucleotide sequence in the transcription initiation region, or by a downstream promoter element (DPE) which is typically observed 30bp downstream of the transcription start site (Smale 1997). Sequences which have the capacity to function as Inrs or DPEs are less characterized than the TATA-box sequence.

After TFIID binding, other general transcription factors including, TFIIA, TFIIB, TFIIE, TFIIF, and TFIIH along with the RNA polymerase then assemble at the promoter. This cohort of factors at the promoter is termed the transcription initiation complex. After the RNA polymerase is correctly positioned at the promoter, it then gains access to the transcription initiation site with the help of TFIIH's DNA helicase activity. TFIIH then phosphorylates the tail of the RNA polymerase causing a conformational change which releases RNA polymerase from the promoter. RNA polymerase then transcribes along the template DNA strand causing the formation of an elongating RNA transcript (Alberts *et al.* 2002).

Gene Regulatory Proteins

Although the general transcriptional machinery for protein-encoded genes is the same for most eukaryotic promoters, gene regulatory proteins on the other hand are diverse. These are the proteins which regulate gene expression by binding to short stretches of DNA of a defined sequence and in turn, control the accessibility of the general transcriptional machinery to specific promoters. It is estimated that 5-10% of the coding capacity of a mammalian genome is devoted to the synthesis of these regulatory proteins (Alberts *et al*. 2002). This high percentage is not surprising given the vast diversity of cell types found within the body, the constantly changing environmental cues imparted on a cell, and the fact that each gene is regulated by a set of regulatory proteins which are themselves products of genes that are regulated by a whole other set of proteins. Further complexity results from the fact that these regulatory proteins have been shown to regulate transcription at distant sites, often thousands of base pairs away from the promoter. The explanation for this is that DNA can form loops to allow the distantly bound regulatory proteins to come into contact with proteins bound at the promoter. An additional level of complexity comes from the combinatorial nature of transcriptional regulation, where a

regulatory protein's function as either an activator or a repressor is often determined not by the protein itself but by the proteins with which it interacts (Alberts *et al.* 2002).

Although the diversity and complexity of transcriptional regulatory protein networks appears overwhelming, progress has been made to elucidate some of the mechanisms by which regulatory proteins activate and repress transcription. As illustrated in Figure 1-1, regulatory proteins can inhibit transcription via several mechanisms. These include: 1) competing with activator proteins for binding to the same regulatory DNA sequence, 2) binding to the activation domain of an activator protein thereby preventing it from carrying out its activator functions even though it is still capable of binding to DNA, 3) blocking assembly of the transcription initiation complex, 4) recruiting chromatin remodeling complexes or histone modifying enzymes which form more compacted chromatin, and 5) recruiting deacetylases and other histone tail modifying enzymes to add repressive chromatin marks to histones as well as recruiting DNA methyltransferases to methylate DNA and lock a promoter in an inactive state (Alberts *et al.* 2002). Similar mechanisms are used by activators to produce the opposite effect of transcriptional activation. The remainder of this chapter will focus on a specific family of regulatory proteins known as E2Fs and will discuss their involvement in regulating transcription.

The E2F Family of Transcription Factors

E2F transcription factors regulate the expression of genes whose protein products are essential for cell cycle progression, cellular proliferation, DNA repair, and differentiation (Dimova *et al.* 2005, Trimarchi & Lees 2002). E2F was originally identified as a cellular DNA-binding protein that could bind to the sequence, TTTCGCGC, within the adenovirus E2 promoter (Yee *et al.* 1987). Concurrently, La Thangue and coworkers identified DRTF1, a transcription factor which had the same consensus DNA-binding site as E2F during studies of embryonic stem cell differentiation (La Thangue & Rigby 1987). It is now clear that E2F and

DRTF1 are the same factor. In order to bind to DNA, E2F requires a binding partner known as dimerization partner (DP). The E2F and DP proteins heterodimerize at which point E2F becomes functional. Additionally, E2F associates with and is regulated by the retinoblastoma (RB) protein. RB was the first tumor suppressor protein ever identified and it is absent or mutated in at least one-third of all human tumors (Weinberg 1992). In particular, it is known for being mutated in hereditary and sporadic retinoblastoma. The importance of the RB-E2F interaction is demonstrated by the finding that all naturally occurring RB mutants isolated from human tumors lack the ability to bind and negatively regulate E2F.

Currently, there are eight known members of the E2F family (DeGregori & Johnson 2006). E2F1 was the first member to be cloned. It was isolated by three independent groups through E2F1's ability to bind to RB. Subsequently, seven additional members of the E2F family were discovered as a result of their homology to E2F1 or because of their ability to bind to RB. However, some of these E2Fs are unable to bind to RB. Figure 1-2 illustrates the protein binding domains of the E2F family members and shows that only E2Fs 1-5 possess an RB binding domain. This domain allows for E2Fs 1-5 to interact with RB and/or other related pocket proteins. E2Fs 1-5 are also the only E2Fs that have a transcriptional activation domain. However, only E2Fs 1-3a serve as transcriptional activators whereas E2Fs 3b-5 are transcriptional repressors despite the presence of an activation domain. E2Fs 6-8 are also repressors but they are considered to be pocket protein-independent transcriptional repressors. E2Fs 6-8 lack both a transactivation domain and a RB binding domain, but they do contain a highly conserved DNA binding domain. E2Fs 1-6 also contain a dimerization domain for their interaction with the DP proteins, but this domain is absent in E2F7 and E2F8. Generally, the various types of protein domains possessed by individual E2F family members dictate their

specific functions. Below we will describe the attributes of the three main classes of E2Fs: 1) activating E2Fs, 2) pocket protein-dependent repressive E2Fs, and 3) pocket protein-independent repressive E2Fs.

The Activating E2Fs

The activating E2Fs, E2F1-3a, are potent transcriptional activators of E2F-responsive genes. Their key purpose is to activate genes which are essential for cellular proliferation. Briefly, cellular proliferation is regulated by the cell cycle which consists of four phases: G1 (the transition phase to prepare for DNA replication), S (the replication phase), G2 (the transition phase to prepare for cell division), and M (the division phase). Cells which are not cycling are considered to be in a quiescent G0 phase. E2Fs 1-3a activate target genes at the G1/S boundary of the cell cycle, thereby promoting S-phase entry (Schwartz *et al.* 1993). Interestingly, overexpression of any one of these E2Fs is sufficient to induce quiescent cells to re-enter the cell cycle (Johnson *et al*. 1993, Qin *et al*. 1994, Lukas *et al*. 1996). Conversely, reducing the levels of individual activating E2Fs can result in cell-cycle arrest (DeGregori *et al*. 2006). When all three activating E2Fs are mutated, cellular proliferation is completely blocked (Wu *et al.* 2001).

Given their strong ability to alter cellular proliferation, it is not surprising that these E2Fs are tightly regulated and that their expression and activity is restricted to specific windows during the cell cycle. The activating E2Fs exhibit maximum expression levels during late G1 and early S phase (DeGregori *et al.* 2006). During this time, the activity of the E2F protein is also raised and it coincides with a wave of transcription of E2F-responsive genes. The amount of E2F protein activity is largely determined by the interactions between the E2Fs and RB. RB binding to E2Fs 1-3a can inhibit the activation of E2F-responsive genes in one of two ways. First, due to the fact that the RB binding domain is located in the transactivation domain of E2F

proteins (see Figure 1-2); RB binding can physically mask the activation domain. This association prevents the E2F-DP heterodimer from recruiting the general transcription factor, TFIID, thereby inhibiting E2Fs' ability to activate. The other way by which RB blocks E2F activity is through active repression as a consequence of recruiting HDAC, SWI/SNF, SUV39H1, and other chromatin modifying enzymes to the promoters of E2F-responsive genes. RB-mediated repression is relinquished as quiescent cells are stimulated to enter the cell cycle due to phosphorylation of RB by cell cycle-dependent kinases. Upon RB phosphorylation, E2F is released. This sudden abundance of "free E2Fs" results in the activation of E2F-responsive genes during late G1 of the cell cycle (Trimarchi *et al.* 2001). During this time, E2F-responsive promoters are bound by activating E2Fs and this binding is often associated with transcriptionally permissive modifications in local chromatin.

In addition to their roles in promoting cell cycle progression, activating E2Fs are also important during development. This is evidenced by the various E2F mutant mouse models. For instance, *E2f1-/-* mice are viable and fertile but have tissue-specific abnormalities which include: an excess of T cells, exocrine gland dysplasia, and the development of testicular atrophy (Field *et al.* 1996, Yamasaki *et al.* 1996). Also, *E2f1-/-* mice develop various tumors between 8 and 18 months of age. Although E2F1 overexpression in tissue culture cells stimulates cell proliferation and can be oncogenic (Singh *et al.* 1994), the *E2f1-/-* mouse clearly demonstrates that E2F1 can also function as a tumor suppressor. *E2f2* mutant mice display enhanced T lymphocyte proliferation which leads to the development of autoimmunity (Murga *et al.* 2001). *E2f1/E2f2* double-knockout mice exhibit severely impaired hematopoiesis due to defective S phase progression in hematopoietic progenitor populations (Li *et al*. 2003). Combined E2f1 and E2f2 loss also results in polyploidy in the liver, salivary gland, and exocrine pancreas. *E2f3-/-* mice

are shown to die *in utero* and the few that survive to adulthood die prematurely due to congestive heart failure (Cloud *et al*. 2002). It should be noted here that *E2f3-/-* mice are deficient in both *E2f3a* and *E2f3b* isoforms. From the phenotype of *E2f1/E2f3* double-mutant mice (Cloud *et al.* 2002), it is clear that almost all of the developmental and age-related defects arising in the individual *E2f1* or *E2f3* mutant mice are exacerbated by the combined mutation. Interestingly however, these mice did not have an increased incidence of tumor formation, revealing that tumor suppression is a specific property of E2F1 and not E2F3. Overall, in addition to their roles in the induction of proliferation, the activating E2Fs have crucial functions during development.

Pocket Protein-Dependent Repressive E2Fs

Repressing E2Fs that possess an RB binding domain are termed pocket protein-dependent repressive E2Fs. This class of E2Fs consists of E2Fs 3b-5. It has been suggested that their primary roles are to repress growth promoting E2F-responsive genes during G0/early G1 and to induce cell cycle exit and terminal differentiation (DeGregori *et al.* 2006). They diverge considerably in sequence from the activating E2Fs in that they lack most of the sequence that is amino-terminal to the DNA binding domain. Further, they use their RB binding domain to interact with additional RB pocket protein family members. E2F4 interacts with p107, p130, and RB whereas E2F5 interacts mostly with p130. These repressive E2Fs are further distinguished from the activating E2Fs by their expression patterns. Unlike the activating E2Fs, repressive E2Fs are constitutively expressed and can be found in nearly equivalent levels in both quiescent and proliferating cells. In fact, E2F4 is expressed at higher levels than any other E2F and it accounts for at least half of the E2F-pocket protein activity observed *in vivo* (Trimarchi *et al.* 2002). Interestingly, while the protein levels of these E2Fs do not change rapidly, substantial changes in their subcellular location have been observed during the cell cycle.

Unlike the activating E2Fs which are constitutively nuclear, E2Fs 4-5 are predominately cytoplasmic. This difference in location is attributed to the fact that activating E2F proteins have a nuclear localization signal whereas repressive E2Fs have a nuclear export signal. However, when the repressive E2Fs associate with pocket proteins, it is sufficient to induce their nuclear localization. Once in the nucleus, repressive E2F-pocket protein complexes can be found abundantly and this occurs during G0/G1 phases of the cell cycle (Figure 1-3). During this time, these repressive E2F complexes are bound to the promoters of many E2F-responsive genes and their binding is often correlated with repressive chromatin modifications. Then around mid to late G1 in the cell cycle, the repressive E2F complexes are released from the promoters. This release is due to the separation of the repressive E2F from its pocket protein. The timing of the release is often correlated with the upregulation of activating E2Fs which may subsequently replace repressing E2F protein occupancy at E2F-target promoters during S phase. At this point, the repressive E2Fs have been exported back out to the cytoplasm, rendering them inactive (Muller *et al*. 1997).

The role of E2Fs 3b-5 in the repression of E2F-responsive genes was elucidated when these proteins were ablated from cell lines. Mouse embryonic fibroblasts (MEFs) deficient in E2f4 and/or E2f5 have defects in their ability to exit the cell cycle in response to various growth-arrest signals including p16 overexpression and contact inhibition (DeGregori *et al.* 2006). However, these mutant cells are able to respond to growth stimulatory signals and there is no change in their proliferative capacity. Thus, the loss of these repressive E2Fs impairs the repression of E2F-responsive genes and therefore the ability to exit the cell cycle.

In agreement with the phenotype of *E2f4-/-* MEFs, cellular proliferation in *E2f4-/-* mice is also normal. However, *E2f4-/-* mice display defects in terminal differentiation. This is

evidenced by several phenotypic abnormalities in *E2f4-/-* mice: 1) fetal anemia and erythrocyte maturation defects, 2) a lack of mature hematopoietic cell types but an abundance of immature cells, and 3) a thinning of gut epithelium due to a reduction in the density of villi (Rempel *et al*. 2000, Humbert *et al*. 2000). These observations suggest a critical role for E2F4 in controlling the maturation of cells in a number of different tissues. A knockout of the other pocket protein-dependent repressive E2F, *E2f5*, results in mice which are viable but develop hydrocephalus due to an overproduction of cerebral spinal fluid resulting from a choroid plexus defect (Lindeman *et al*. 1998). Thus, E2F4 and E2F5 clearly have individual roles during development. However, the neonatal lethal phenotype of *E2f4/E2f5* double-knockout mice (Gaubatz *et al.* 2000) demonstrates that E2F4 and E2F5 must also have some overlapping functions in biological processes that are essential to survival. Moreover, the individual and combined knockout phenotypes of both mice and cell lines show that these pocket protein-dependent repressive E2Fs are dispensable for cellular proliferation but are required for cell cycle exit and terminal differentiation.

Pocket Protein-Independent Repressive E2Fs

The previously described E2F proteins' roles as transcriptional activators or repressors depended upon whether or not they were bound to a pocket protein. Conversely, pocket protein-independent repressive E2Fs, E2Fs 6-8, lack an RB binding domain (Figure 1-2) and therefore repress E2F-responsive genes through mechanisms which do not require pocket proteins. These E2Fs are localized to the nucleus and their expression fluctuates during the cell cycle. In contrast to pocket protein-dependent repressive E2Fs, E2F6 expression is low in serum-starved, quiescent cells. Instead, E2F6 expression is increased during re-entry into the cell cycle with peak levels in late G1/early S phase. From that point, E2F6 remains expressed throughout the cell cycle. Similarly, E2F7 and E2F8 are also expressed in a cell growth-dependent manner,

accumulating as cells enter the cell cycle and with peak levels found during S phase. This expression pattern suggests that these E2Fs may serve as components of a negative feedback system to counterbalance activating E2Fs during the cell cycle. The finding that E2Fs 6-8 bind to E2F-responsive promoters during S phase agrees with this idea. Additionally, these E2Fs may be involved in defining the distinction between G1/S- and G2/M- regulated transcription. This role was proposed after the observation that E2F6 selectively binds and represses only those E2F-responsive genes that were activated at the G1/S boundary and not the G2/M boundary of the cell cycle (Giagrande *et al*. 2004).

Overexpression studies have further elucidated the roles of pocket-protein independent E2Fs. Ectopic expression of either E2F6 or E2F7 is able to block endogenous E2F-responsive gene transcription as well as block the transactivating activity of ectopic E2F1 (Cartwright *et al.* 1998, Gaubatz *et al.* 1998, Bruin *et al.* 2003). Further, E2F6 overexpression leads to the accumulation of cells in S-phase (Cartwright *et al.* 1998). In agreement with this observation is the prediction that following cellular damage, expression of E2F6 may be upregulated to inhibit E2F-dependent transcription in S-phase and therefore, delay the progression of the cells through S phase until either the damage has been repaired or the cell has been targeted for apoptosis. Additionally, E2F6 overexpression in quiescent cells delays re-entry into the cell cycle (Gaubatz *et al.* 1998). This is consistent with the detection of an E2F6 chromatin-modifying complex at cell cycle-regulated promoters during G0. However, *E2f6-/-* MEFs display no defects in cellular proliferation (Storre *et al.* 2002). It is speculated that proliferation remains normal because many of these cell cycle promoters are also occupied by repressive E2F4 complexes, suggesting that E2F6 is redundant. E2F7 or E2F8 overexpression significantly slows down cellular proliferation in MEFs (Bruin *et al.* 2003, Maiti *et al.* 2005). Ectopic expression of E2F8 has also been shown

to ablate DNA replication of cells in S phase which may reflect the downregulation of E2F-target genes required for DNA synthesis. Taken together, these observations propose that pocket protein-independent repressive E2Fs have overlapping and perhaps synergistic roles during the cell cycle. However, the mechanism by which these pocket protein-independent E2Fs mediate transcriptional repression of E2F-target genes is not known for E2Fs 7-8. Further insights will likely be established with the creation of *E2f7* and *E2f8* knockout mice. Therefore, the remainder of this discussion will focus on the mechanisms of E2F6-mediated repression as there is a substantial amount of experimental evidence on this topic.

One mechanism by which E2F6 mediates transcriptional repression is through its association with polycomb repressive complexes. This association is suggested *in vivo* by the phenotype of *E2f6-/-* mice; posterior homeotic transformations of the axial skeleton which are strikingly similar to those observed in mice deficient in a variety of polycomb proteins (Storre *et al*. 2002). There are two main types of polycomb repressive complexes: 1) PRC2, which is thought to be required at the initiating stage of silencing, and 2) PRC1, which is continuously required for the stable maintenance of repression once the correct gene expression patterns have been established. E2F6 has been shown to associate with members of the PRC1 complex (Trimarchi *et al.* 2001). This E2F6-PRC1 complex includes the PRC1 complex proteins, RING1, mel-18, mph1, Bmi1, and YY1 binding protein (RYBP), and it is presumed that these polycomb proteins associate with their target promoters through binding to E2F6. Recently, another E2F6-polycomb complex (E2F6-G0) was described (Ogawa *et al.* 2002). This complex, containing several novel polycomb proteins including Max and HP1γ, was found to associate with E2F target promoters in G0 but not following re-entry into the cell cycle. This implies a role for E2F6 in the repression of E2F target genes during G0. However, given that E2F6 is

expressed throughout the cell cycle and remains localized to the nucleus, E2F6 most likely forms alternate complexes in proliferating cells. Additional studies have revealed that E2F6 is associated with EPC1 and EZH2, a component of the PRC2 complex, only in proliferating cells (Attwooll *et al.* 2005). Upon overexpression of EPC1, EZH2, and EED, another component of the PRC2 complex, these three proteins will co-immunoprecipitate. Further, this E2F6 proliferation-specific complex was shown to repress reporter activity but a mutation in E2F6 which renders it unable to bind to EPC1 causes a loss of this repression. Interestingly, the PRC2 component EZH2, which is known for its H3K27 methyltransferase activity, has recently been shown to recruit DNA methyltransferases to the promoters of target genes (Vire *et al.* 2006). This recruitment could serve to 'lock in' the silent state initiated by the polycombs through DNA methylation. Overall, E2F6 has been shown to associate with components of both the PRC1 and PRC2 complexes suggesting a role for E2F6 in the establishment and the maintenance of E2F-target gene repression in both quiescent and proliferating cells.

Other mechanisms for E2F6-mediated repression have been studied but not as thoroughly as the E2F6-polycomb interactions. These include: 1) E2F6 blocking activating E2Fs from binding to the promoters of E2F-responsive genes (Oberley *et al*. 2003), 2) E2F6 recruiting DNA methyltransferases to induce CpG hypermethylation and lock target promoters in an inactive state (Pohlers *et al*. 2005), and 3) E2F6 recruiting histone deacetylases and histone methyltransferases which infer repressive chromatin marks on histone tails (Storre *et al*. 2005, Caretti *et al*. 2003, Ogawa *et al*. 2002). For the remainder of this study, we will focus on the E2F family member, E2F6, and E2F6-mediated repression of a select set of target genes.

Summation

The way by which the human body creates so many different cell types from the same genomic sequences is inconceivable. However, we now know that such cellular diversity is at

least made possible through the finite control of gene expression. More specifically, the transcriptional regulation of genes through the binding of transcriptional activators and repressors has proven to be especially important. Therefore, it was the goal of this work to study the transcriptional regulation of gene expression. Considering the complexity of such a task, it is best to simplify the problem into a more manageable solution. Therefore, for the first part of this study, we chose to focus on the transcriptional regulation of an individual gene and a particular transcription factor. The gene we selected to analyze was adenine nucleotide translocase-4 (*Ant4*) because it was recently discovered by our lab (Rodic *et al*. 2005), and prior to this study nothing was known about its transcriptional regulation. We chose to investigate the E2F family of transcription factors, in particular E2F6, for reasons that will become apparent. The biological significance of *Ant4*, along with our findings regarding its transcriptional regulation, is discussed in Chapter 3. For the second part of this study, we examined the transcriptional regulation of 24 additional genes which were similar in expression to *Ant4* because we had reason to believe that they too were regulated by E2F6. More information regarding the transcriptional regulation of these 24 genes is given in Chapter 4. Lastly, in Chapter 5, we present data on the expression of E2F1 and E2F6 in the testis. In summation, the overall goal of this research was to analyze the transcriptional regulation of gene expression in the context of a specific gene/set of genes.

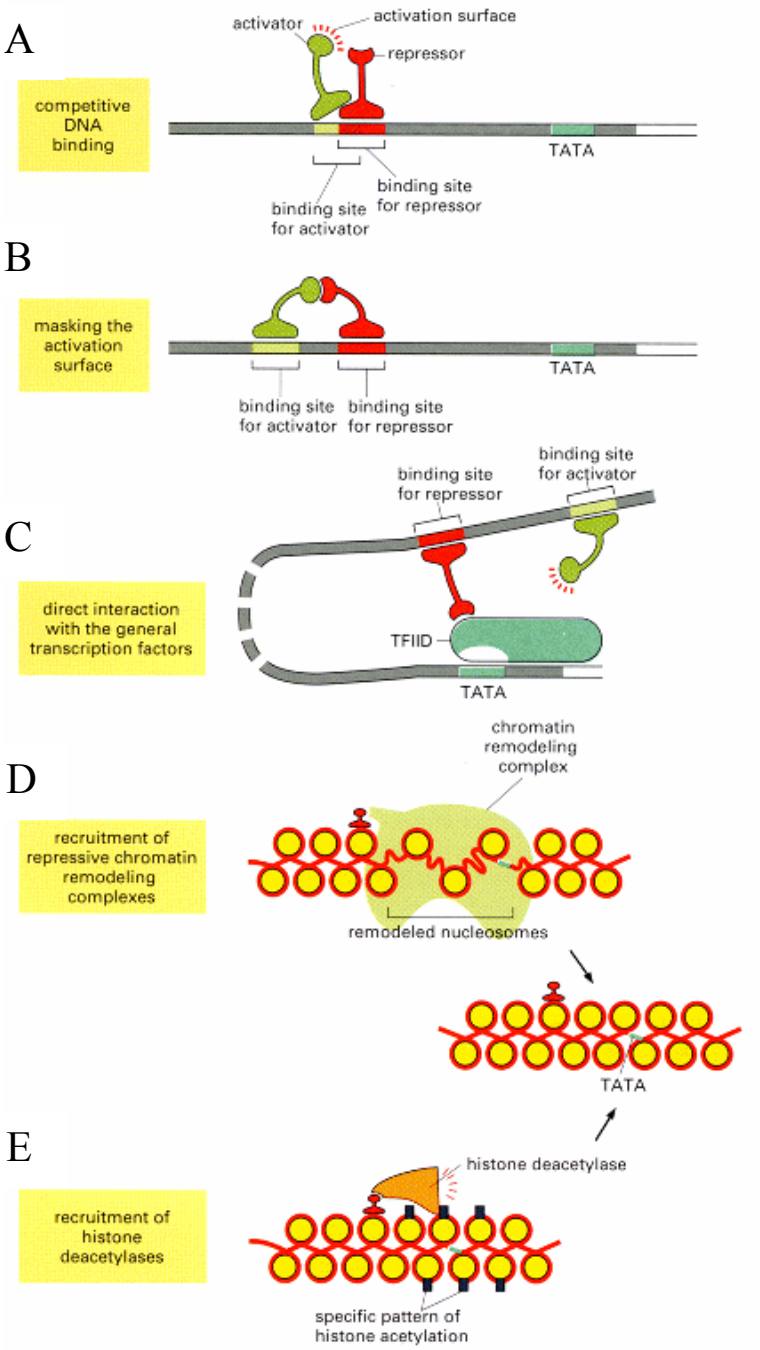


Figure 1-1. Five ways in which regulatory proteins can inhibit transcription. The repressing regulatory proteins can inhibit transcription by: A) competing with activating regulatory proteins for binding to the same DNA sequence, B) blocking the activity of the activating regulatory protein even though it may still bind, C) interacting with the general transcription machinery and blocking it from assembling at the promoter, D**)** recruiting repressive chromatin remodeling complexes, and E) recruiting histone deacetylases and other histone tail and DNA modifying enzymes (from Alberts *et al*. 2002, Figure 7-49 on page 406).

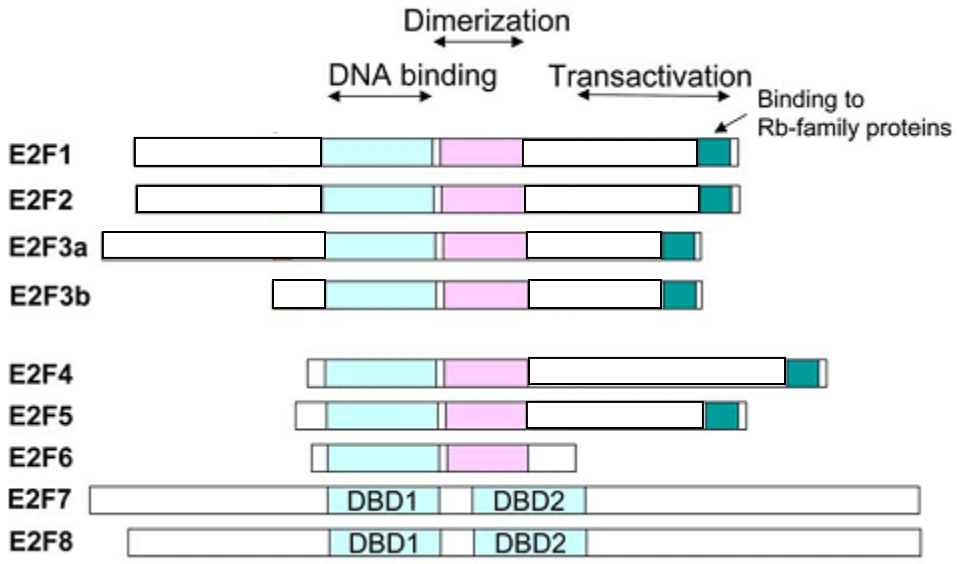


Figure 1-2. The E2F family proteins and their core functional domains. E2Fs 1-6 share domains which mediate DNA binding (light blue) and DP dimerization (pink). E2Fs 7-8 have two of these DNA binding domains (DBD1 and DBD2), but lack a DP dimerization domain. E2Fs 1-5 also have an Rb binding domain (green) within a transactivation domain that only has transactivating activity in E2Fs 1-5 (modified from DeGregori and Johnson 2006, Figure 1 on page 740).

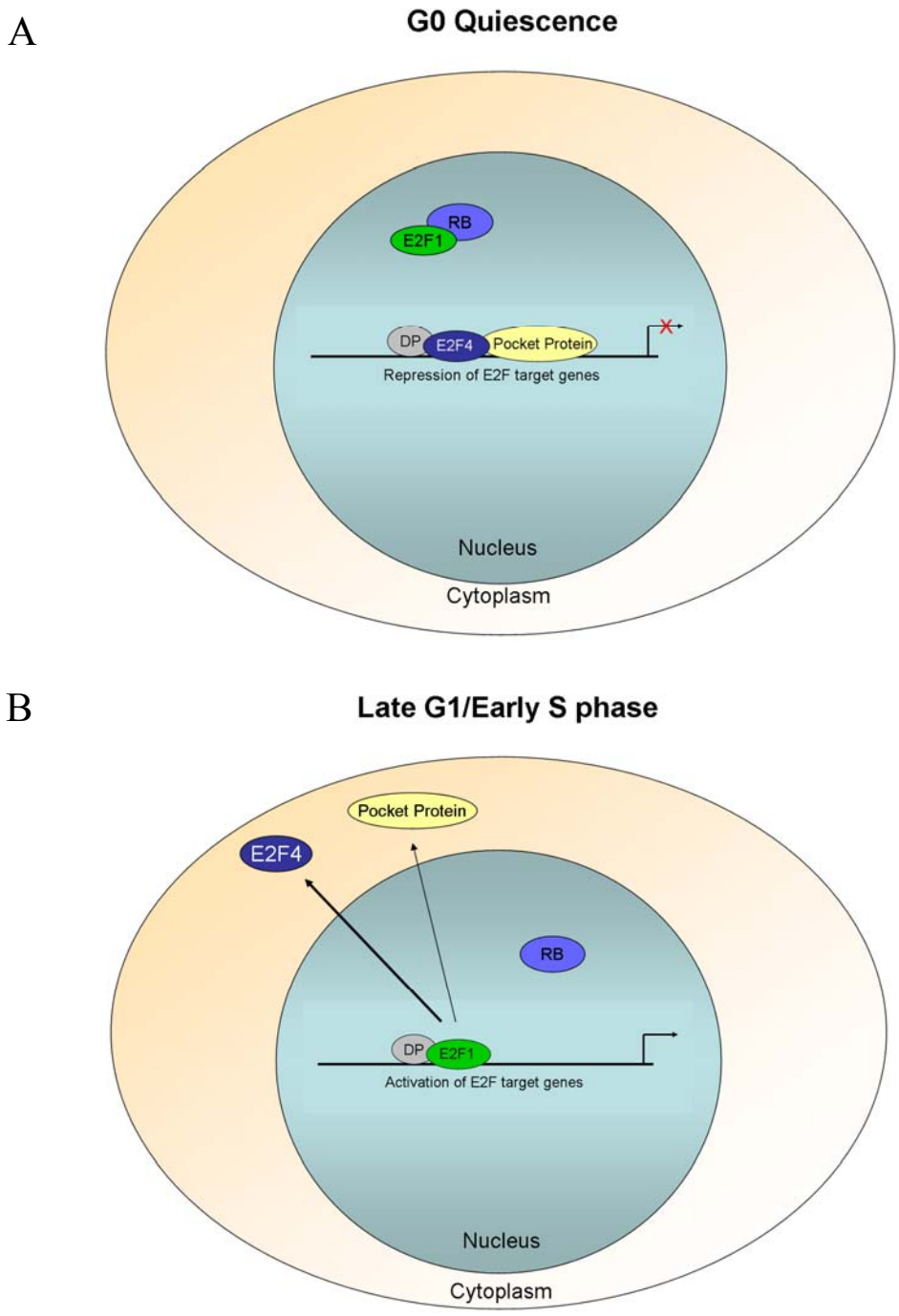


Figure 1-3. Changes in the composition and location of E2F complexes as cells enter the cell cycle. A**)** During G0, complexes containing E2F6 and pocket proteins bind and repress E2F target genes. The function of activating E2Fs is disabled by association with RB. B) As cells enter the cell cycle, the repressive E2F dissociates from its pocket protein causing release from target promoters and export to the cytoplasm. Also, E2F1 is upregulated and released from RB. It can then replace the repressive E2F complexes by binding to and activating E2F target genes.

CHAPTER 2
MATERIALS AND METHODS

Cell Culture and Creation of Stable Cell Lines

The cell lines used in this study were murine WT R1 ES cells (a gift from Dr. A Nagy, Toronto, Canada), HA-E2F6 ES cells, HA-ΔC-E2F6 ES cells, and NIH3T3 cells (American Type Culture Collection, Manassas, VA). Both HA-E2F6 and HA-ΔC-E2F6 ES cell lines were established by stable transfection of linearized HA-E2F6 and HA-ΔC-E2F6 expression vectors (Gaubatz *et al.* 1998) into parental R1 ES cells. Stable clones were subsequently confirmed to express E2F6 by western blotting with anti-HA antibody (Cell Signaling Technology, Danvers, MA), and those with the highest expression were selected and expanded in the presence of Neomycin (G418) (Calbiochem-EMD Biosciences, San Diego, CA). All ES cells were maintained in an undifferentiated state on gelatin-coated dishes in Knock-out™ Dulbecco's Modified Eagle Medium, low glucose (Invitrogen, Carlsbad, CA) containing 10% Knockout™ Serum Replacement (Invitrogen), 1% Fetal Bovine Serum (Atlanta Biologicals, Lawrenceville, GA), 1% Penicillin-Streptomycin-Glutamine (100x) liquid (2mM $_{L}$-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, Invitrogen), 25mM HEPES (Cellgro, Herndon, VA), 300μM monothioglycerol (Sigma-Aldrich, St. Louis, MO), and 1,000 U/ml recombinant mouse LIF (ESGRO) (Millipore, Temecula, CA). NIH3T3 cells were maintained in Dulbecco's Modified Eagle Medium (Invitrogen) containing 10% Fetal Bovine Serum, 1% Penicillin-Streptomycin-Glutamine (100x) liquid, and 25mM HEPES.

Gel Mobility Shift Assay

Gel mobility shift assay was performed as described previously (Gaubatz *et al.* 1998). Briefly, 5μl of reticulocyte lysates with or without *in vitro*-translated proteins (see below) were incubated with 0.1 picomoles of PAGE purified, γ^{32}P-end-labeled, annealed oligonucleotides (5'-

TCAGCGCCCGCTTTCCCGCCAGGGTAAAGCT-3') corresponding to the WT E2F6-binding element (underlined) in the murine *Ant4* promoter. Competition experiments were performed with wild-type and mutant (5'-TCAGCGCCCGCTTTCTTAACAGGGTAAAGCT-3') unlabeled, double-stranded oligonucleotides corresponding to the E2F6 site derived from the *Ant4* promoter, whereby the amount of unlabeled probe relative to the amount of labeled probe was either 20, 50, or 100 fold in excess. For preparation of *in vitro*-translated proteins, one microgram of HA-E2F6 and Myc-DP2 expression vectors (constructed as described previously), were co-translated *in vitro* using a coupled transcription/translation reticulocyte lysate system (Promega, Madison, WI) according to manufacturer's instructions (Gaubatz *et al.* 1998).

Chromatin Immunoprecipitation

R1 ES cells were plated on 100-mm plates at a density of 1 x 10^6 cells per plate and then fixed three days later in 1% formaldehyde for 10 minutes at room temperature. Cross-linking was attenuated by the addition of 125mM glycine. Cells were then collected by scraping with a cell scraper in cold PBS with the following protease inhibitors (PIs): leupeptin, aprotinin, and phenylmethylsulfonyl fluoride. Cells were centrifuged at 1200rpm for 5 minutes at 4°C and then resuspended in cell lysis buffer (5mM PIPES pH 8.0, 85mM KCL, and 0.5% NP-40) plus PIs and incubated for 10 minutes on ice. Nuclei were pelleted at 5000 rpm for 5 minutes at 4°C and then lysed on ice for 10 minutes in nuclei lysis buffer (50mM Tris-Cl pH 8.0, 10mM EDTA, and 1% SDS) plus PIs. Next, chromatin was sheared by sonication for 10 repititions of 10 second bursts with 1 minute rests on ice using a power setting of "4" on a Fisher Scientific Sonicator Dismembrator Model 100. Lysates were centrifuged for 10 minutes and supernatants were collected, diluted, and a sample was kept as Input DNA. The supernatant was then pre-cleared with Protein G agarose (Invitrogen) for twenty minutes and collected again following centrifugation. Approximately 2μg of each antibody; control IgG, (Sigma-Aldrich) and mouse

monoclonal E2f6 (Storre *et al*. 2002), were added to the supernatant and incubated overnight at 4°C. The following day, 60µl of Protein G Agarose-50% slurry was added to the reaction and incubated for 1 hour rocking at 4°C. The agarose complex was then collected, washed seven times with LiCl wash buffer (0.25M LiCl, 0.5% NP-40, 0.5% DOC, 1mM EDTA, and 10mM Tris pH 8.0), and DNA was eluted off with elution buffer (50mM Tris pH 8.0, 1% SDS, and 10mM EDTA). Crosslinking was reversed by incubating the eluted chromatin in 175mM NaCl at 65°C overnight followed by 2 hours of Proteinase K treatment at 55°C to degrade the proteins. DNA was recovered using phenol/chloroform extraction followed by PCR Purification (Qiagen, Valencia, CA). PCR was performed with either semi-quantitative PCR using the Taq DNA polymerase kit (Eppendorf, Westbury, NY) or with real-time quantitative PCR (as described below). The primer sequences used for semi-quantitative PCR analysis of Chromatin Immunoprecipitation (ChIP) are described in Table 2-1. Data from at least three independent experiments were analyzed by semi-quantitative PCR and expressed as means ± standard deviations (SD).

Real-Time Quantitative PCR

The primers and probes used for real-time PCR were designed with Primer Express software and synthesized at Applied Biosystems. Each real-time PCR reaction was performed in a 25µl reaction mixture containing 50ng of cDNA, 10µl of 2X TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), and 1µl of 20X TaqMan Gene Expression Assay Mix (Applied Biosystems) containing the primers and probes. The reaction mixture was incubated for 10 minutes at 95°C and repeated for 40 cycles (denaturing for 15 seconds at 95°C and annealing and extending for 1 minute at 60°C) using Applied Biosystems 7900HT Real-Time PCR System. The TaqMan primers and probes for monitoring transcription levels of *Ant4*, *E2f1, E2f6, Dmc1,* and *Nanog* were assay-on-demand gene expression products labeled with

FAM reporter dye (Applied Biosystems). A mixture of mouse *β-actin* primers with VIC-labeled *β-actin* probe was used as endogenous control (Applied Biosystems, catalog number 4352341E). The primer sequences and probes for quantifying E2f6 enrichment after performing ChIP analysis were custom ordered: *β-actin*: sense primer 5'-CGGAGGCTATTCCTGTACATCTG-3', antisense primer 5'-CGAGATTGAGGAAGAGGATGAAGAG-3', FAM-labeled probe 5'-CCAGCACCCATCGCC-3'; *Ant4*: sense primer 5'-GCTGTTCTCCCAGCATCCT-3', antisense primer 5'-GAGAACTGGAAAACCGCTTCAG-3', FAM-labeled probe 5'-CTTTCCCGCCAGGGTAA-3'; *Dmc1*: sense primer 5'-GGCCCCGCCCATCAA-3', antisense primer 5'-CGCCCTGTCGTCGAACA-3', and FAM-labeled probe 5'-CCGCGGCCCTCATT-3' All samples were tested in triplicate and expressed as means ± standard deviations (SD). Analysis of results was performed using SDS v2.3 software (Applied Biosystems) according to the manufacturer's instructions. The comparative C_T method ($\Delta\Delta C_T$) was used for quantification of gene expression.

Plasmid Construction and Site-Directed Mutagenesis

The *Ant4* promoter region (from -263bp to +25bp) was PCR-amplified from mouse R1 ES cells' genomic DNA with high fidelity LA-Taq™ (Takara, Otsu, Japan) using the following *Ant4* primers: sense primer 5'-AATCACCGGGTTGGTGTAG-3', antisense primer 5'-GCCACACCAACACTCAAGC-3'. The 288bp fragment was excised with Xho1 and HindIII (New England Biolabs, Ipswich, MA) using a QIAquick gel extraction kit (Qiagen). The Takara DNA ligation kit was then used to ligate the fragment of the *Ant4* promoter into a PGL2-basic vector (Promega) containing a luciferase reporter gene. Mutations in the *Ant4*-Luciferase reporter vector at the location of the E2F6-binding element were generated using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) in combination with the following PAGE purified primers: *Ant4*: sense, 5'-

CCCAGCATCCTCAGCGCCCGCGCCAGGGTAAAGCTGAAGCGG -3', antisense, 5'-CCGCTTCAGCTTTACCCTGGCGCGGGCGCTGAGGATGCTGGG -3' (site of mutation is underlined). Reactions consisted of 5µl of 10X reaction buffer, 50ng of vector template, 125ng each primer, 1µl of dNTP mix, and 1µl of pfuTurbo® DNA polymerase up to a final volume of 50µl. Reaction conditions consisted of 1 cycle at 95°C for 30 seconds, and then 18 cycles of 95°C for 30 seconds, 55°C for 1 minute, 68°C for 9 minutes. Template vector was next degraded by the addition of 1µl of DpnI restriction endonuclease and incubated at 37°C for 1 hour. Reactions were transformed into Max Efficiency DH5α chemically competent cells (Invitrogen), analyzed by gel electrophoresis, and sequenced.

Transient Transfection and Reporter Assays

NIH3T3 cells were plated at a density of 1 x 10^5 cells per well in 6-well plates. After 24 hours, FuGENE 6 (Roche) was used to transfect the following according to manufacturer's instructions: 0.5µg *Ant4*-Luciferase reporter vector (wild-type or mutant), 0.1µg pRL-TK-*Renilla* internal control vector (Promega), and 1.0µg of one of the following vectors: HA-E2F6, HA-ΔC-E2F6, HA-E68-E2F6, HA-ΔN-E2F6 (constructed as we previously described) or pCMVTag2 empty control vector (Stratagene) (Gaubatz *et al.* 1998). Twenty-four hours after transfection, the cells were harvested. Firefly and *Renilla* luciferase activities were measured using a dual-luciferase reporter assay system (Promega) according to manufacturer's instructions. The firefly luciferase data for each sample was normalized based on transfection efficiency as measured by *Renilla* luciferase activity. Data from at least three independent experiments were analyzed and expressed as means ± standard deviations (SD). Statistical analysis was performed by Student's *t* test and *P* values of less than 0.05 were considered significant.

Immunoblotting

NIH3T3 cells transiently transfected with one of the following E2F6 expression vectors: HA-E2F6, HA-ΔC-E2F6, HA-E68-E2F6, or HA-ΔN-E2F6 using Fugene 6 (Roche), were lysed in RIPA buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% NP-40, 0.5% Na-Deoxycholate, 0.1% SDS) plus PIs, and then harvested by scraping with a cell scraper. Cells were incubated for 20 minutes on ice and centrifuged. Supernatants were transferred to fresh tubes and total protein was normalized by Lowry assay (Bio-Rad, Hercules, CA). Next, 25μg of total protein was separated on a 12% SDS-PAGE gel and transferred to a nitrocellulose membrane. After blocking with 4% bovine serum albumin (BSA) solution in TBST buffer (50mM Tris-HCl pH 8.0, 100mM NaCl, 0.1% Tween 20) for 1 hour at room temperature, the membrane was incubated overnight with anti-HA primary antibody (1:250 dilution; Cell Signaling Technology). After incubation for one hour with anti-mouse secondary antibody conjugated to horseradish peroxidase (1:5000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), chemiluminescent detection (GE Healthcare, Piscataway, NJ) of horseradish peroxidase activity was performed.

Reverse Transcription-PCR

Total RNA was extracted using an RNAqueous® kit (Ambion, Austin, TX). The cDNA was synthesized using a SuperScript® II first-strand synthesis system with oligo(dT) (Invitrogen) according to manufacturer's instructions. PCR was performed using *Taq* DNA polymerase (Eppendorf) with the primer sequences listed in Table 2-2. For the samples selected to be quantified using real-time PCR, an alternative method of reverse transcription was performed using 1 μg of total RNA and the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to manufacturer's instructions. Primer sequences used in real-time PCR are mentioned above. Regardless of which method of PCR was used, the sense

primers were always designed in different exons than the antisense primers in order to ensure that the PCR product represented the specific mRNA species and not genomic DNA background.

Computational Analysis

Evolutionarily conserved regions (ECRs) of the mouse and human *Ant4* gene and all conserved E2F6 transcription factor binding sites (TFBS) located within the ECRs were identified using the rVista 2.0 software program (Loots & Ovcharenko 2004). Additionally, an interspecies comparison of the *Ant4* promoter sequences containing the E2F6 TFBS was carried out using rVista. All genomic DNA sequences of meiosis-specific genes were obtained using the University of California at Santa Cruz (UCSC) Genome Bioinformatics software (Karolchik *et al*. 2003). A genome-scale DNA pattern matching algorithm was used to identify all core E2F6-binding elements (TCCCGC or GCGGGA depending on the direction of binding) within the proximal promoter regions (-1000bp to +1bp) of all genes in the entire mouse genome (based on 31,113 genes in the database) using Regulatory Sequence Analysis Tools (RSAT) software (Van-Heldin 2003). The statistical relevance of the rate at which core E2F6 binding elements appear in the genome was then examined. We considered DNA sequences with specified lengths of 100bp, 200bp, 500bp, or 1000bp and the probability that a particular subsequence (the E2F6-binding element) appears exactly n times within these sequences was computed under the assumption that each of the four nucleotides (A, G, C, or T) appears randomly and with equal probability (Gentleman & Mullin 1989). This information was used to obtain the probability that a particular subsequence appears *at least* once within a DNA sequence of a given length. Specifically, the individual probabilities corresponding to each possible outcome of a trial must sum to one as a consequence of the three axioms upon which probability theory is based. As such, the probability that a subsequence occurs at least once: $P(n \geq 1)$ is given by Equation 2-1,

where $P(n = 0)$ represents the probability that the subsequence does not appear at all. The expression for $P(n = 0)$ is obtained from Gentleman & Mullin 1989.

$$P(n \geq 1) = 1 - P(n = 0) \quad (2\text{-}1)$$

The present study examines the probability that a bi-directional DNA binding element will appear in a given sequence. Therefore, consideration is required for the case of multiple subsequences, eg. one subsequence corresponding to each binding element. If the subsequences corresponding to each binding element are denoted *A* and *B*, respectively, the probability that *either A* or *B* will occur within a given sequence is denoted as $P(A \cup B)$ and is expressed approximately as in Equation 2-2. This expression also results directly from the axioms of probability. The expression shown in Equation 2-2 is actually an upper bound, as the true value must ideally account for cases where *A* and *B* occur simultaneously.

$$P(A \cup B) = P(A) + P(B) \quad (2\text{-}2)$$

Bisulfite Sequencing and Combined Bisulfite Restriction Analysis (COBRA)

Genomic DNA from WT and *E2f6-/-* MEFs was bisulfite converted using EZ DNA Methylation Kit (Zymo Research, Orange, CA). Approximately 80ng of PCR purified bisulfite converted DNA was used as a template for each PCR analysis. Primers used for *Ant4* COBRA and bisulfite sequencing were: sense primer 5'-TTGTTGTGTATTGATTGAGTATG-3', and antisense primer 5'-AAAAAAAACTAAAAAAC-3'. COBRA was performed by digesting the PCR products with Hha1. After digestion, the COBRA reaction was ran on an 8% PAGE Gel and then stained for 5 minutes with ethidium bromide. For bisulfite sequencing, undigested PCR products were cloned into a pCR-2.1 TOPO cloning vector (Invitrogen) as we have previously described (Rodic *et al*. 2005). Clones were then screened using blue and white screening, and white colonies were subsequently sequenced.

Immunostaining

Testes were harvested from 6-week-old wild-type male mice. All mice have been maintained under standard specific-pathogen-free (SPF) conditions, and the procedures performed on the mice were reviewed and approved by the University of Florida Institutional Animal Care and Use Committee (IACUC). The tissues were then fixed in a mild fixative (10% formalin) overnight with rocking. Following fixation the tissues were dehydrated using an organic solvent (PBS-Citrasol). The tissues were then imbedded in paraffin and sectioned. Deparafinized and rehydrated 5μm tissue sections were stained with goat polyclonal antibodies against mouse E2f6, or IgG control. Slides were blocked for endogenous peroxidase activity and then unmasked in Target Retrieval Solution (Dako, Carpinteria, CA). Antibody was applied at 1:100 for one hour at room temperature prior to identification using the DAB Envision kit (Dako). Slides were counterstained with hematoxylin.

Table 2-1. Primers used for analyzing ChIP experiments with semi-quantitative PCR

Primer name	Oligonucleotide sequence (5' to 3')
Ant4 sense	ACACGTGTTATGGTCACATGC
Ant4 antisense	GCCTTCTTTGAAGACTGCTTCT
Dmc1 sense	CCACCCCCACAACCTGAG
Dmc1 antisense	AGAATTTTCAAGGCGACACC
Nanog sense	TCACACTGACATGAGTGTGG
Nanog antisense	TCTGTGCAGAGCATCTCAGT
Oct-4 sense	ACGCAGAGCCAGCACTTCTC
Oct-4 antisense	CCAGTATTTCAGCCCATGTCC
Smc1β sense	GGAGCAGGGATCCAATTCC
Smc1β antisense	GCCACGACTTGAAATTCTCC
Spo11 sense	AGCTTCATGTCCTCCCTGAA
Spo11 antisense	AGGGCGTCGAAGAACGAG
Stag3 sense	AGGAACGTGGTTAGCACGAG
Stag3 antisense	GGCTGAAGAAAGGTCACCAC
Sycp1 sense	ATCCTCCGACAATTTTGAGC
Sycp1 antisense	ACACCCTCCACACCCTCAC
Tuba3 sense	TTGTTCCATGCTAAGTTGGATGTC
Tuba3 antisense	CCGTTCCTCACCACTGGTCCTCAAC
β-Actin sense	AAATGCTGCACTGTGCGGCG
β-Actin antisense	AGGCAACTTTCGGAACGGCG

Table 2-2. Primers used for semi-quantitative RT-PCR

Primer name	Oligonucleotide sequence (5' to 3')
Ant4 sense	TGGAGCAACATCCTTGTGTG
Ant4 antisense	AGAAATGGGGTTTCCTTTGG
Dmc1 sense	AGAATTTTCAAGGCGACACC
Dmc1 antisense	CCACCCCCACAACCTGAG
E2F6 sense	AAGCTGCAGGCAGAACTCTC
E2F6 antisense	TTCACCCACTCGGGATACTC
Nanog sense	AGGGTCTGCTACTGAGATGCTCTG
Nanog antisense	CAACCACTGGTTTTTCTGCCACCG
Oct-4 sense	TGGAGACTTTGCAGCCTGAG
Oct-4 antisense	TGAATGCATGGGAGAGCCCA
Rec8L sense	AGGGCGTCGAAGAACGAG
Rec8L antisense	AGCTTCATGTCCTCCCTGAA
RecQ1 sense	ACTGGAATCCGTAGCCAGTG
RecQ1 antisense	AGAGCTGGAAGCATTCAACA
RibC2 sense	CCATGGAGGTAGCGATGTCT
RibC2 antisense	GTGTTCCGCATGTCATTGTC
Smc1β sense	GCCACGACTTGAAATTCTCC
Smc1β antisense	GGAGCAGGGATCCAATTCC
Spo11 sense	GTTGGCCATGGTGAAGAGAG
Spo11 antisense	CTCAAGTTGCCAGCAATCAA
Stag3 sense	GGCTGAAGAAAGGTCACCAC
Stag3 antisense	AGGAACGTGGTTAGCACGAG
Sycp1 sense	ACACCCTCCACACCCTCAC
Sycp1 antisense	ATCCTCCGACAATTTTGAGC
Tuba3 sense	GGACCGGATCCGAAAACTGG
Tuba3 antisense	GTCTGGAATTCTGTTAAGTCC
β-Actin sense	TTCCTTCTTGGGTATGGAAT
β-Actin antisense	GAGCAATGATCTTGATCTTC

CHAPTER 3
REPRESSION OF ANT4 GENE EXPRESSION BY E2F6

Motivation

Eukaryotic cells require energy for their survival. The majority of this energy is produced through oxidative phosphorylation, a process whereby ATP is synthesized from ADP and inorganic phosphate. This occurs within the mitochondrial matrix and therefore requires that ADP and ATP be shuttled across both the inner and outer mitochondrial membranes. Although nonspecific porins can mediate this transport across the outer membrane, the inner membrane is highly selective and requires specific carrier proteins for any solutes to get across. The adenine nucleotide translocases (Ants) are a family of solute carriers that facilitate the exchange of ATP for ADP across the inner membrane of the mitochondria (Dahout-Gonzalez *et al.* 2006). In this chapter, the *Ant* isoforms and their critical roles in cellular respiration, as witnessed by defects resulting from their mutations, are discussed. In particular, the regulatory mechanisms governing *Ant* gene expression are emphasized and new results on the transcriptional repression of the *Ant4* isoform are presented.

Background

Adenine nucleotide translocases are the most abundant proteins of the mitochondrial inner membrane and are comprised of approximately 300-320 amino acid residues which form six transmembrane helices. The Ants transport ADP/ATP according to an exchange-diffusion mechanism with a one-to-one stoichiometry, thereby maintaining the adenine nucleotide pool at a constant level within the mitochondrial matrix (Dahout-Gonzalez *et al.* 2006). The Ants are encoded by nuclear genes whereby the ANT proteins are then translocated into the mitochondrial inner membrane (Rehling *et al*. 2004).

Ant genes have been cloned in numerous eukaryotic species including yeast and plants. Mammalian *Ant* genes have been cloned in human, rat, bovine, and most recently mouse. All of these species have multiple *Ant* isoforms of varying tissue specificity. Until recently, it was thought that humans only had three members of the ANT family: *ANT1 (SLC25A4),* which is specific to heart and skeletal muscle, *ANT2 (SLC25A5)*, which is expressed in rapidly growing cells and is inducible, and *ANT3 (SLC25A6)*, which is ubiquitously expressed (Stepien *et al.* 1992, Lunardi *et al.* 1992). In contrast, mice have only two isoforms: *Ant1*, which is expressed in heart, brain, and skeletal muscle and is located on chromosome 8, and *Ant2* which is expressed ubiquitously with the exception of skeletal muscle and is located on the X-chromosome (Levy *et al*. 2000). Mouse Ant2 is the ortholog of human ANT2 and is believed to have the combined functions of both human ANT2 and ANT3 (Ellison *et al.* 1996).

Recently, we and others identified a novel member of the *Ant* family, *Ant4*, in both mouse and humans (Dolce *et al.* 2005, Rodic *et al.* 2005, Kim *et al.* 2007). In humans, the *Ant4* gene is located on chromosome 4 whereas in mice it is located on chromosome 3. In contrast to all other known *Ants* which only have four exons, *Ant4* contains six exons in both mouse and human. The *Ant4* gene is predicted to encode a 320 amino acid protein and a comparison between mouse and human indicates that the protein is mostly conserved, sharing an 88% overall amino acid identity (Figure 3-1A) (Rodic *et al*. 2005). Ant4 also has high amino acid sequence homology with other previously identified mouse Ant proteins (70.1% and 69.1% overall identity to Ant1 and Ant2, respectively) (Figure 3-1B). Closer examination of Ant4's amino acid sequence reveals the presence of a signature sequence common to members of the mitochondrial carrier family, a P-X-[D/E]-X-X-[K/R] motif found three times at ~100 residues apart (Walker 1992). It is noteworthy that Ant4 has a unique extension of amino acids at both the N- terminus and the C-

terminus. From the sequence analysis, it appears that Ant4 shares sufficient homology to be classified as a mitochondrial ADP/ATP transporter but its regions of unique sequence suggest that it may differ in function or tissue-specificity from the rest of the family. Upon further analysis, we did in fact find that *Ant4's* expression pattern is different from all other *Ants* in that its transcripts are detectable only in testis (Brower *et al.* 2007).

Defects resulting from disruption of *Ant* gene expression have been reported. In humans, there is a clinical manifestation associated with ANT1 known as autosomal dominant progressive external opthalmoplegia (adPEO) (Hirano & DiMauro 2001). This adult-onset disorder is characterized by the appearance of external opthalmoplegia, ptosis, and progressive skeletal muscle weakness. Molecularly, adPEO is evidenced by the accumulation of numerous mitochondrial point mutations including many which are found in ANT1: A114P, L98P, A89D, D104G, and V289M substitutions. Deficiency of this ADP/ATP carrier has also been reported in striated muscle of patients affected with myopathy and in patients suffering from Sengers syndrome (Jordens *et al.* 2002). However, in Sengers syndrome, the *ANT1* gene itself was not altered, indicating that the pathology could result from either impairment of the carrier transcription or from impeded import into the mitochondrial membrane.

Studies have also shown an impairment of ADP/ATP carrier function in heart despite the existence of high levels of total carrier proteins. Rather than a defect in total protein levels, this impairment results from a shift in isoform expression. It was shown that an increased amount of ANT1 isoform correlates with a decrease in ANT2 isoform in dilated cardiomyopathy (Dorner *et al.* 2006). Phenotypes similar to those found in humans were observed in *Ant1-/-* mice, where the mice were viable but developed mitochondrial myopathy and severe exercise intolerance along with hypertrophic cardiomyopathy as young adults (Graham *et al*. 1997).

Genetic disruption of *Ant2* in mice presumably results in peri-neonatal lethality but this observation has not yet been published in a scientific paper (www.patentdebate.com/PATAPP/20050091704). ANT2 has been found to be overexpressed in cancer cells (Chevrollier *et al*. 2005). It is established that cancer cells display a glycolytic phenotype resulting from the downregulation of mitochondria-encoded genes and impairment of mitochondrial function. The *ANT2* promoter contains a regulatory element which represses gene expression in response to oxygen. It is hypothesized that ANT2 imports glycolytic ATP into mitochondria in exchange for exporting ADP, a process which would sustain basal mitochondrial activity under the predominant glycolytic status of tumor cell proliferation. There have been no reports regarding *ANT2* or *ANT3* mutations in human.

Defects resulting from disruption of *Ant4* gene expression are evidenced by the *Ant4* knockout mice generated by our lab (Brower *et al.* 2007). We observed that males were sterile and lacked meiotic and post-meiotic cells, thereby establishing that this ADP/ATP mitochondrial translocase is essential for successful progression of male germ cell meiosis. Furthermore, *Ant4*-deficient male mice exhibited increased levels of apoptotic cells within the testis and the majority of these cells were identified as early spermatocytes. Given that *Ant2* is encoded on the X chromosome and the X chromosome is inactivated via meiotic-sex chromosome inactivation (MSCI) (Turner 2007) at the onset of meiosis, we speculate that *Ant4* may have evolved to compensate for the loss of *Ant2* during MSCI. Thus, it is likely that Ant4 functions as the sole mitochondrial ADP/ATP carrier during spermatogenesis.

Although the Ants have been studied fairly extensively on a protein level, very little is known about the regulatory mechanisms governing *Ant* gene expression. The *ANT1* gene is known to contain classic TATA and CCAAT elements within its promoter. Also found within

the promoter is a positive regulatory element known as OXBOX which is bound by proteins present only in muscle cells (Li *et al.* 1990, Chung *et al.* 1992). Muscle-specific binding of transcription factors to OXBOX is thought to account for the induction of *ANT1* expression in muscle. The OXBOX element in the *ANT1* promoter is overlapped by REBOX, a potentially negative regulatory element. Gel shift assays have demonstrated that proteins binding to REBOX are not muscle-specific, suggesting a potential mechanism for mediating *ANT1* repression in non-muscle tissue. However, the mouse *Ant1* gene lacks the OXBOX and REBOX elements identified in the human promoter.

Similar to *ANT1*, a classic TATA box is present in the *ANT2* promoter. *ANT2* also contains three putative Sp1 control elements near its transcription start site. Two of the Sp1 elements, known as the AB box, are located 5' of the TATA box and work synergistically to activate *ANT2* transcription (Li *et al.* 1996). However, activation via the AB box is modulated by three separate repressor regions. One of these is another Sp1 binding element, termed the C box, and is juxtaposed to the transcription start. When the C box is occupied, *ANT2* transcription is decreased. The second repressor region can be found in the distal promoter and is the site for NF1 binding (Barath *et al.* 2004). The third repressor region is located between the AB activation box and the distal repressor region and is also bound by NF1. Similar to *ANT1* and *ANT2*, the *ANT3* gene also has a classical TATA element within its promoter, as well as 16 identified Sp1 elements. *ANT3* has also been shown to be regulated by IL-4 and IFN-γ via STAT-dependent pathways in T cells (Jang & Lee 2003).

In contrast to other *ANTs*, a TATA box is absent in the *ANT4* promoter. We have previously shown that CpG methylation is required for *Ant4* repression (Rodic *et al.* 2005). We have also shown a correlation between methylation and expression whereby in testis, *Ant4* is

highly expressed and hypomethylated while in all of the somatic tissues examined, *Ant4* is repressed and hypermethylated. In particular, we showed *Ant4* derepression in D10 embryoid bodies (EBs) upon addition of the DNA demethylating agent, 5-aza-dC. Likewise, *Ant4* was derepressed in EBs lacking *de novo* DNA methyltransferases (Dnmts). In this study, we have investigated the transcriptional regulation of *Ant4* with the aim of determining how *Ant4* is repressed in all somatic cells. We believe that understanding *Ant4* gene regulation is important given its unique meiosis-specific expression pattern and its essential role in spermatogenesis.

Results

After our recent identification of the novel *Ant4* isoform and further characterization of the *Ant4*-/- mouse, we next examined the promoter region of *Ant4* in search of potential transcription factor binding sites (TFBS). Given that other *Ant* isoforms contain multiple Sp1 TFBS in their promoters which have been previously shown to play a role in their regulation (Li *et al.* 1996), we searched the mouse and rat *Ant4* promoters for Sp1 TFBS. We compared the sequences of mouse and rat *Ant4* (*Slc25a31*) genes and surrounding non-coding regions using rVISTA sequence analysis software (Loots & Ovcharenko 2004). The rVISTA program identified several evolutionary conserved regions (ECRs) of sequence similarity and located conserved Sp1 binding sites within the proximal promoter region (Figure 3-2A). However, when we compared the mouse *Ant4* sequence to human *Ant4*, no conserved Sp1 binding sites were found (Figure 3-2B). We then ran a search for all conserved TFBS between mouse and human *Ant4* and found that there was only a handful of conserved TFBS in the proximal promoter region (data not shown). One of these was an E2F binding site with the core E2F6 sequence, TCCCGC (Figure 3-3A). This potential E2F TFBS was located 36bp upstream of the transcription initiation site and an interspecies sequence comparison indicated that the E2F6 TFBS and surrounding flanking regions were mostly conserved (Figure 3-3B). We chose to

investigate this E2F6 site further due to previous literature implicating E2F6 in the repression of meiosis-specific genes (Storre *et al*. 2005, Pohlers *et al*. 2005).

Next, we investigated the functional significance of the E2F6 TFBS in the murine *Ant4* promoter by analyzing the ability of E2F6 to interact with this site. To address this question, we initially performed a gel retardation assay to determine whether E2F6 would bind *in vitro* to a radiolabeled oligonucleotide encompassing the E2F6 TFBS in the *Ant4* promoter (-54bp to -24bp). In order to prepare E2F6 protein and its binding co-factor DP2, we used a rabbit reticulocyte lysate *in vitro* translation (IVT) system to co-express HA-tagged E2F6 (HA-E2F6) and Myc-tagged DP2 (Myc-DP2). As shown in Figure 3-4, addition of the E2F6/DP2 protein complex to radio-labeled *Ant4* probe generated an additional retarded band. The specificity of this interaction was verified by competition with wild type (wt) and mutant (mut) cold competitors in 20, 50, or 100 fold excess amounts over radiolabeled *Ant4* probe, whereby only the wt competitor was able to efficiently compete for binding to the E2F6 TFBS (filled arrow, Figure 3-4). It should be noted that the reticulocyte lysate used for IVT contains an endogenous protein which interacts with the *Ant4* probe. This interaction is evidenced by the presence of a retarded band when the probe was incubated with control rabbit reticulocyte lysate in the absence of IVT (open arrow, Figure 3-4). However, addition of specific antibodies against HA and Myc into the protein/probe mixture selectively reduced the intensity of the bands indicated by the filled arrow as we previously demonstrated (Gaubatz *et al.* 1998) (data not shown).

Subsequently, we analyzed the occupancy of this E2F6 TFBS *in vivo* using Chromatin immunoprecipitation (ChIP). Here we show that endogenous E2f6 binds to the proximal promoter of *Ant4* but not to *β-actin* in undifferentiated R1 ES cells (Figure 3-5). Both semi-quantitative PCR (Figure 3-5A) and quantitative real-time PCR (Figure 3-5B) analysis of ChIP

resulted in the same conclusion. Collectively, these observations show that *Ant4* contains a conserved E2F6 TFBS that binds E2F6 both *in vitro* and *in vivo*.

To clarify the role that E2F6 plays in the regulation of *Ant4* transcription, we performed luciferase assays using an *Ant4* reporter vector in combination with various E2F6 expression vectors. The *Ant4*-Luciferase reporter contains a 288bp region of the *Ant4* promoter (-263bp to +25bp) which includes the E2F6 TFBS. For transient transfections, we used NIH3T3 cells due to their high transfection efficiency and their apparent absence of endogenous E2f6 expression (data not shown). The activity of the wt *Ant4*-Luciferase reporter significantly decreased upon co-transfection with an HA-E2F6 expression vector (Figure 3-6). However, when the E2F6 TFBS in the *Ant4*-Luciferase reporter was mutated using site-directed mutagenesis, co-transfection with HA-E2F6 failed to repress *Ant4* transcription (Figure 3-6). Similarly, when the wt *Ant4* reporter vector was co-transfected with HA-E2F6 expression vectors in which either the DNA binding domain was mutated (HA-E68-E2F6) or the repression domain was mutated (HA-ΔC-E2F6), reporter activity was not repressed (Figure 3-7A). E2F6 proteins containing a mutation in the N-terminal domain (HA-ΔN-E2F6), which have been shown to retain their repressor activity, had similar repressive effects to that of WT HA-E2F6 (Gaubatz *et al.* 1998). Western blotting confirmed that the E2F6 proteins were expressed at comparable levels when equivalent amounts of DNA were transfected (Figure 3-7B). These reporter assays indicate that the E2F6 protein can repress *Ant4*, providing its DNA binding and repressor domains are intact, and that this repression depends on sequence-specific DNA binding.

To further elucidate the potential role of E2F6 in the repression of the *Ant4* promoter, we next created stable ES cell lines overexpressing either HA-E2F6 or HA-ΔC-E2F6 expression vectors. In contrast to somatic cells, many germ-cell-specific genes including *Ant4* are

considered to be transcriptionally permissive in ES cells and therefore have detectable mRNA transcripts (Rodic *et al*. 2005, Geijsen *et al*. 2004). The CpG island of the *Ant4* gene promoter is hypomethylated in ES cells and a low level of *Ant4* transcription is detectable. Using both semi-quantitative RT-PCR (Figure 3-8A) and quantitative real-time PCR (Figure 3-8B), we show that overexpression of HA-E2F6 but not mutant HA-ΔC-E2F6 is able to reduce *Ant4* transcription relative to parental R1 ES cells. This indicates that increasing the amount of E2F6 protein present in a cell is sufficient to trigger E2F6-mediated repression. Based on this result, it is reasonable to believe that the opposite scenario, in which E2F6 protein has been depleted from a cell, could potentially result in the derepression of *Ant4* transcription. To this end, we examined the expression of *Ant4* in mouse embryonic fibroblast (MEF) cell lines derived from both wild type and *E2f6* null mice. Using semi-quantitative PCR, we demonstrate that *Ant4* is in fact aberrantly expressed in *E2f6-/-* MEFs (Figure 3-9). Taken together, these findings indicate that E2F6 is an essential repressor for the *Ant4* gene in somatic cells.

Given that E2F6 has been shown to be associated with polycomb proteins (discussed in Chapter 1), we analyzed the binding of the polycomb proteins, Eed and EZH2, to R1 ES cells at D0 and D6 of differentiation (Figure 3-10). Interestingly, we observed an increase in binding of Eed and EZH2 to the *Ant4* promoter at D6 but not D0 of ES cell differentiation. The polycomb binding at D6 coincides with *Ant4* repression by D6. However, it is a bit concerning that we still detected acetylated histone 3 (AH3), a marker of active chromatin, on D6 when *Ant4* expression should be silenced. Because the ChIP primers were designed around the E2F6 binding site in the *Ant4* promoter, and both Eed and EZH2 cannot themselves bind to DNA, we speculate that these proteins maybe using E2F6 as a binding platform. Upon binding to E2F6, they would then be able to exert their repressive effects on the *Ant4* promoter.

An alternative mechanism of E2F6-mediated repression of *Ant4* may be through the recruitment of DNA methyltransferases. It was previously shown by our lab that DNA methylation is required for *Ant4* repression during embryonic stem cell differentiation (Rodic *et al*. 2005). We first wanted to confirm that *Ant4* still required DNA methylation to remain repressed in somatic cells and not just initially during ES cell differentiation. Therefore, we treated NIH-3T3 cells with the DNA demethylating agent, 5-Aza-Deoxycytidine (5-AZA-DC) (Figure 3-11). We found that DNA methylation was indeed required for the repression of *Ant4* in somatic cells indicating the continued importance of DNA methylation in *Ant4* repression. Additionally, we examined the effects of the HDAC inihibitor, Trichostatin-A (TSA), on *Ant4* expression. We found that in contrast to 5-AZA-DC, TSA had no affect on *Ant4* expression indicating that histone acetylation was most likely not the mechanism of E2F6-mediated repression. Perhaps *Ant4* repression not being effected by histone acetylation may help to explain how *Ant4* can be repressed at D6 of ES cell differentiation despite the detection of AH3 (Figure 3-10). We next compared the methylation status of *Ant4* in WT and *E2f6-/-* MEFs using both COBRA and Bisulfite Sequencing (Figure 3-12). Interestingly, we observed a reduction in DNA methylation at the *Ant4* promoter in *E2f6-/-* MEFs. However, this decrease, from 96% methylation in WT MEFs to 80% methylation in *E2f6-/-* MEFs was minor. This is in contrast to the high percentage of demethylation found in the promoter region of the germ-cell-specific gene, *Tuba3* (Figure 3-13). Findings regarding *Tuba3* demethylation in *E2f6-/-* MEFs were previously published (Pohlers *et al.* 2005), and the *Tuba3* data presented in Figure 3-13 was generated in collaboration with Emily Smith and Dr. Resnick. Next, we checked for the binding of Dnmt3-containing complexes to the *Ant4* promoter using ChIP assay but the results appeared negative (Figure 3-10A). To further explore the potential involvement of E2F6 in *Ant4*

methylation, we compared our HAE2F6 ES cell line to WT R1 ES cells at D0 and D6 of ES cell differentiation. If E2F6 was repressing *Ant4* by recruiting DNA methyltransferases, then we would expect to observe an increased rate of DNA methylation in those cells overexpressing E2F6. As shown in Figure 3-14, there was no such increase in DNA methylation leading us to believe that DNA methylation is not the primary mechanism of E2F6-mediated repression.

Discussion

In this study, we first demonstrated that the meiosis-specific gene, *Ant4*, contains an evolutionarily conserved E2F6 binding site in its proximal promoter region. This indicates that E2F6-mediated regulation of *Ant4* is likely important both within and between species. The importance of conserving such a binding site is highlighted by the fact that two-thirds of the sequence conserved among mammals is not protein-coding (Adams 2005). Therefore, the noncoding regulatory regions upon which transcription factors bind must play a large part in controlling the expression of protein-coding genes. From this knowledge, it is tempting to speculate that an *in vivo* disruption of the E2F6 binding site located at the *Ant4* promoter could result in: 1) defects in fertility similar to those observed in the *Ant4* knockout mouse, or 2) aberrant expression of *Ant4* in somatic tissues. The data presented here are in support of the latter effect, although both possibilities could be true. Additional studies whereby mice are created from mutations knocked-in to the E2F6 binding site will elucidate if such a binding site is required for *Ant4* expression during meiosis and consequently fertility. For the remainder of this discussion, we will focus on the mechanisms of *Ant4* repression in somatic tissues.

From these experiments, it is clear that there are multiple players involved in the repression of *Ant4* gene expression. The three key players that we found to be involved are E2F6, polycomb proteins, and DNA methylation. Examination of these factors on an individual basis is what led us to the conclusion that they were important to *Ant4* repression. For E2F6,

sufficient evidence came from the combined findings that E2F6 binds to the *Ant4* promoter (Figures 3-4 and 3-5), that overexpression of E2F6 induces *Ant4* repression (Figures 3-6, 3-7, and 3-8), and that *Ant4* derepression occurs in *E2f6-/-* MEFs (Figure 3-9). For polycomb proteins, proof came from the binding of Eed and Ezh2 to the *Ant4* promoter in D6 EBs (Figure 3-10). Based on previous studies in our lab, we already knew that DNA methylation was essential to *Ant4* repression (Rodic *et al.* 2005). These experiments showed that *Ant4* was derepressed in both ES cells following treatment with 5-AZA-DC and in *Dnmt3a-Dnmt3b* double null ES cells. Additionally, we demonstrated the importance of DNA methylation in *Ant4* repression by verifying that *Ant4* was derepressed in somatic cells (NIH-3T3s) after treatment with 5-AZA-DC (Figure 3-11). These findings support a role for E2F6, polycomb proteins, and DNA methylation in the repression of *Ant4*, but fail to explain how these key components coordinate with one another to regulate *Ant4* gene expression.

A relationship between E2F6 and polycomb proteins has been observed in multiple studies (Ogawa *et al*. 2002, Trimarchi *et al*. 2001, Attwooll *et al*. 2005). As discussed in Chapter 1, E2F6 associates with components of both the PRC2 and the PRC1 polycomb repressive complexes. Given that these polycomb proteins do not bind to DNA directly, E2F6 has been suggested to serve as a platform on which polycombs can bind. Binding to E2F6 would then enable these polycomb proteins to exert their repressive effects on DNA. In agreement with this concept is our finding that the PRC2 components, Eed and Ezh2, appear to be associated with the *Ant4* promoter in the same region that E2F6 is bound (Figure 3-10). Further, we demonstrate that only the endogenous E2F6 platform binds at D0 when *Ant4* is expressed, whereas the Eed and Ezh2 polycomb proteins do not bind and assemble until just after *Ant4* is repressed at around D6 of differentiation. This suggests that although a docking platform for the polycomb proteins

may bind to polycomb-target promoters prior to polycomb complex assembly, gene repression will not occur until the full polycomb repressive complex has arrived and assembled on such a platform. Further studies will need to be performed to confirm these findings and to establish when and where the protein-protein interactions involved in such a repressive complex are occurring.

An association between polycomb proteins and DNA methylation has also been reported in literature. Recently, EZH2, a component of the PRC2 complex that is known for its ability to methylate histone H3K27, was shown to physically interact with DNA methyltransferases (Vire *et al*. 2006). This 2006 report was the first time that any evidence suggesting a cross-talk between these two silencing pathways had ever been presented. The study showed that knockdown of Dnmt1, 3a, or 3b led to derepression of EZH2-target genes. These target genes were also found to bind both EZH2 and the DNA methyltransferases. Additionally, EZH2 overexpression increased the CpG methylation at EZH2 target promoters, whereas EZH2 knockdown decreased CpG methylation. Moreover, EZH2 was needed to bring Dnmts to the promoters of EZH2-target genes. Depletion of EZH2 disturbed this recruitment, and enabled the aberrant expression of target genes.

Out of all the possible interactions between the key players involved in *Ant4* repression, we focused our efforts on elucidating the connection between DNA methylation and E2F6. Interestingly, a recent study found that the germ cell-specific gene, *Tuba3*, was derepressed in *E2f6-/-* MEFs (Pohlers *et al.* 2005). This derepression was correlated with a major reduction in DNA methylation at the *Tuba3* promoter, suggesting that E2F6-mediated repression involves the recruitment of Dnmts to target promoters which then locks them in an inactive state. Given that *Ant4* is also a germ cell-specific gene, and that DNA methylation and E2F6 are both essential for

Ant4 repression, we predicted that these key players would behave similarly in mediating the repression of *Ant4*. Surprisingly, *Ant4* methylation in *E2f6-/-* MEFs was only slightly reduced (~16%) (Figure 3-12) in comparison to the massive reduction reported for *Tuba3* methylation (~69%) (Pohlers *et al.* 2005). We have proposed the following theories in an effort to explain why the *Ant4* promoter remains mostly methylated in *E2f6-/-* MEFs.

The first theory to explain why there is only a slight reduction in *Ant4* methylation is that there is heterogeneity among cultured MEF cells where some cells are more differentiated than others. Recent studies have demonstrated that cell populations such as ES cells, which were once believed to be homogeneous, are in fact rather heterogeneous (Singh *et al.* 2007). Additionally, the methylation status of the germ line and pluripotent cell marker, *Oct4*, was shown to be heterogeneous among populations of somatic cells indicating that somatic cells may even exhibit some degree of heterogeneity (Marikawa *et al.* 2005). It is feasible that within the mixture of cultured MEF cells, some cells may be more dependent on E2F6 for DNA methylation than others depending on their differentiation status. However, if this were the case, one would predict that the *Tuba3* promoter would show a similar distribution of methylation among these mixed cell types as *Ant4*. We examined the methylation status of the *Tuba3* promoter in *E2f6-/-* MEFs using COBRA (data not shown), and found that *Tuba3* was indeed less methylated than *Ant4*. This finding eliminates the possibility that variations in culture conditions between our experiments and those from the previous report on *Tuba3* are responsible for this difference in methylation. Thus, it is unlikely that cellular heterogeneity can account for the slightly reduced *Ant4* methylation in *E2f6-/-* MEFs.

An alternative theory for why there is very little demethylation of *Ant4* in *E2f6-/-* MEFs is that in these cells, *Ant4* expression is leaky. The term "leaky" generally refers to genes that

are considered to be specific to one tissue type but then become expressed in another tissue type at much lower levels. Instead of looking at the total percentage of methylation that is occurring on the *Ant4* promoter in *E2f6-/-* MEFs, examining the spatial arrangement of DNA methylation may provide more clues regarding whether *Ant4* expression is indeed "leaky." As shown in Figure 3-12, several of the *Ant4* clones analyzed during bisulfite sequencing are entirely demethylated. Thus, rather than thinking of an 80% overall methylation rate, let us divide the multiple clones of *Ant4* from bisulfite sequencing into two populations: one group which has 0% methylation (3 of the 18 clones) and another group with methylation rates nearly equivalent to that found in WT MEFs (15 out of the 18 clones). Interestingly, we noticed this same "All" OR "None" distribution of methylation for *Tuba3* clones from a previous publication reporting methylation of the *Tuba3* promoter in *E2f6-/-* MEFs (Pohlers *et al.* 2005). We realized that 7 out of 10 of these clones were 0% methylated and 3 out of 10 of the clones had high methylation rates equivalent to WT MEFs. Although the percentage of *Tuba3* clones which were fully demethylated in *E2f6-/-* MEFs was much greater than the percentage demethylated for *Ant4*, the conserved "All" OR "None" pattern implies biological relevance. In collaboration with Dr. Resnick's lab, we further showed that this same "All" OR "None" pattern exists at the *Tuba3* promoter in *E2f6-/-* mouse tail tissue (Figure 3-13) indicating that the heterogeneity of cultured MEF cells is most likely not responsible for these two different populations. Thus, if the difference in methylation between the "All" and "None" populations is not a result of varying degrees of differentiation among MEFs, but instead occurs in a rather homogeneous population of cells, then there must be some other existing phenomenon to explain these findings.

This "All" OR "None" effect may be a result of an incomplete functional redundancy. Although the body has developed backup mechanisms to ensure that processes which are

essential to genome integrity be protected, oftentimes these backup mechanisms are not as efficient as the original. One such process that may exemplify this point is DNA methylation. For successful DNA methylation to occur there needs to be functional DNA methyltransferases (Dnmts) as well as proteins which can recruit these Dnmts to target gene promoters. From the previous publication on *Tuba3* expression in *E2f6-/-* MEFs, it was suggested that E2F6-mediated repression occurred through E2F6 recruitment of Dnmts (Pohlers *et al.* 2005). Previous studies have also shown that E2F family members often compensate for each other when another E2F family member is lost (Kong *et al.* 2007). More specifically, E2F4 has been shown to compensate for E2F6 by repressing E2F6-target genes when E2F6 is absent (Giagrande *et al.* 2004). Further, E2F4 has been found in a complex with Dnmt1 (Macaluso *et al*. 2003).

Therefore, we propose a model whereby under normal conditions, E2F4 and E2F6 may both have roles in recruiting Dnmts to E2F-target genes (such as *Tuba3* and *Ant4*) but that in *E2f6-/-* MEFs, E2F4 must attempt to compensate for the loss of E2F6 by becoming the sole recruiter of Dnmts (Figure 3-15). It should be noted here that many other proteins may be involved in the recruitment of Dnmts to promoters but for the purposes of this discussion, we are specifically focusing on the recruitment of Dnmts to E2F-target genes. Also, here we imply that E2F4 and E2F6 are the only Dnmt recruiters for E2F-target genes, but additional studies are needed to confirm such a theory. However, if this theory holds true, then E2F4-mediated recruitment of Dnmts is serving as a functionally redundant backup for E2F6 in *E2f6-/-* MEFs. We hypothesize that although E2F4 can mostly compensate for the loss of E2F6, it is likely that the efficiency of Dnmt recruitment to target promoters may be lower than under normal conditions. This reduction in efficiency is due to the fact that E2F4 now has double the workload. If E2F4 is successful in recruiting Dnmts to a specific promoter, then the methylation

pattern at that promoter will be nearly identical to WT (Figure 3-15). However, when E2F4 fails to recruit Dnmt to a specific promoter because it is now overwhelmed with having to recruit Dnmts to so many additional promoters, then this promoter will show 0% methylation (Figure 3-15). This theory could explain why in some cells, methylation of *Ant4* and *Tuba3* promoters is completely "missed."

Simply stated, we believe that "leaky" expression of *Ant4* is a direct consequence of incomplete functional redundancy. The extent of leaky expression is determined by the number of times in which E2F4 fails to recruit Dnmts to the *Ant4* promoter in the absence of E2F6 (the clones having 0% methylation). A possible explanation for why a higher percentage of *Tuba3* promoters are demethylated in *E2f6-/-* MEFs could be that *Tuba3* primarily relies more on E2F6 for Dnmt recruitment whereas *Ant4* depends more on E2F4. Therefore, we predict that in an *E2f4-/-* cell line we would see the opposite effect where *Ant4* promoters are more demethylated than *Tuba3* promoters. Additionally, if this functional redundancy model holds true, in a double *E2f4-/- E2f6-/-* cell line we would expect to see a nearly 100% overall demethylation rate for both *Ant4* and *Tuba3* promoters. Taken together, we predict that future experiments such as these will help to further support the theory that *Ant4* expression is leaky in *E2f6-/-* MEFs due to incomplete functional redundancy.

Additional experiments will be necessary to validate that E2F4 and E2F6 are indeed recruiters of Dnmts. We did not present the data here in this study, but attempts were made to demonstrate a physical interaction between Dnmt3 and E2F6. We transiently transfected tagged E2F6, Dnmt3a and Dnmt3b expression vectors in NIH-3T3 cells and then performed co-immunoprecipitation but saw no interaction between E2F6 and Dnmt3. Similarly, we tried to coimmunoprecipitate endogenous Dnmt3 with exogenous HAE2F6 in HAE2F6 ES cell lines but

to no avail. It should be noted here that those studies were preliminary and several repetitions as well as optimization studies would be needed before considering such results as reliable.

Given the possibility that E2F6 and Dnmt3 may not physically interact with each other but could still be part of the same repressor complex, we performed ChIP. As shown in figure 3-10, we were unable to find Dnmt3 association with the *Ant4* promoter region despite the presence of prominent E2F6 binding. However, the possibility that the Dnmt3 antibody quality was insufficient for ChIP assays cannot be ruled out. Additionally, because E2F6 overexpression was unable to enhance DNA methylation at the *Ant4* promoter (Figure 3-14) we cannot draw any conclusions to suggest a relationship between E2F6 and DNA methylation at this time. However, according to our "leaky" expression theory in combination with the previous publication on *Tuba3* (Pohlers *et al.* 2005), E2F6 should recruit Dnmts to E2F-target promoters. The validity of this theory is pending further results which demonstrate an interaction, either direct or indirect, between E2F6 and Dnmts. Moreover, these experiments have attempted to elucidate the regulatory mechanisms governing *Ant4* repression in somatic cells by identifying E2F6 as an essential repressor. In the next chapter, we will further explore the relationship between E2F6 and other genes which are similar to *Ant4*.

A

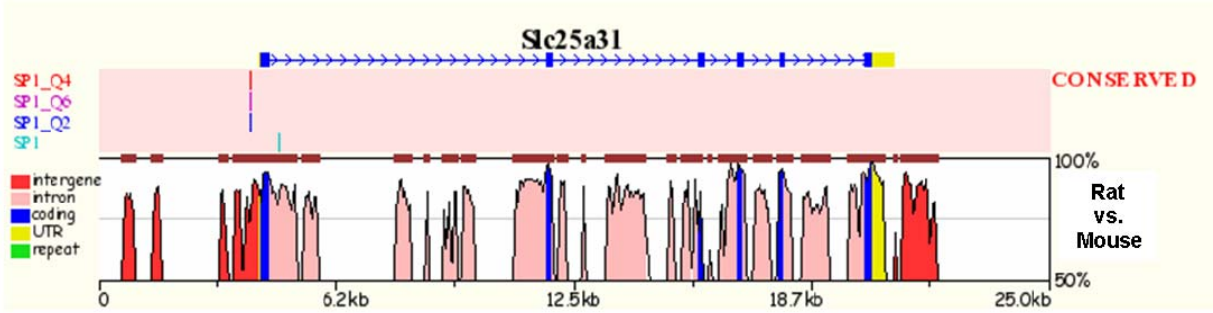
```
hANT4  -MHREPAKKKAEKRLFDASSFGKDLLAGGVAAAVSKTAVAPIERVKLLLQVQASSKQISP
mAnt4  MSNESSKKQSSKKALFDPVSFSKDLLAGGVAAAVSKTTVAPIERVKLLLQVQASSKQISP

hANT4  EARYKGMVDCLVRIPREQGFFSFWRGNLANVIRYFPTQALNFAFKDKYKQLFMSGVNKEK
mAnt4  EARYKGMLDCLVRIPREQGFLSYWRGNLANVIRYFPTQALNFAFKDKYKELFMSGVNKEK

hANT4  QFWRWFLANLASGGAAGATSLCVVYPLDFARTRLGVDIGKGPEERQFKGLGDCIMKIAKS
mAnt4  QFWRWFLANLASGGAAGATSLCVVYPLDFARTRLGVDIGKGPEQRQFTGLGDCIMKIAKS

hANT4  DGIAGLYQGFGVSVQGIIVYRASYFGAYDTVKGLLPKPKKTPFLVSFFIAQVVTTCSGIL
mAnt4  DGLIGLYQGFGVSVQGIIVYRASYFGAYDTVKGLLPKPKETPFLVSFIIAQIVTTCSGIL

hANT4  SYPFDTVRRRMMMQSGEAKRQYKGTLDCFVKIYQHEGISSFFRGAFSNVLRGTGGALVLV
mAnt4  SYPFDTVRRRMMMQSGESDRQYKGTIDCFLKIYRHEGVPAFFRGAFSNILRGTGGALVLV

hANT4  LYDKIKEFFHIDIGGR----
mAnt4  LYDKIKEFLNIDVGGSSSGD
```

B

```
Ant1  ------------MGDQALSFLKDFLAGGIAAAVSKTAVAPIERVKLLLQVQHASKQISA
Ant2  ------------MTDAAVSFAKDFLAGGVAAAISKTAVAPIERVKLLLQVQHASKQITA
Ant4  MSNESSKKQSSKKALFDPVSFSKDLLAGGVAAAVSKTAVAPIERVKLLLQVQASSKQISP

Ant1  EKQYKGIIDCVVRIPKEQGFLSFWRGNLANVIRYFPTQALNFAFKDKYKQIFLGGVDRHK
Ant2  DKQYKGIIDCVVRIPKEQGVLSFWRGNLANVIRYFPTQALNFAFKDKYKQIFLGGVDKRT
Ant4  EARYKGMLDCLVRIPREQGFLSYWRGNLANVIRYFPTQALNFAFKDKYKELFMSGVNKEK

Ant1  QFWRYFAGNLASGGAAGATSLCFVYPLDFARTRLAADVGKGSSQREFNGLGDCLTKIFKS
Ant2  QFWRYFAGNLASGGAAGATSLCFVYPLDFARTRLAADVGKAGAEREFKGLGDCLVKIYKS
Ant4  QFWRWFLANLASGGAAGATSLCVVYPLDFARTRLGVDIGKGPEQRQFTGLGDCIMKIAKS

Ant1  DGLKGLYQGFSVSVQGIIIYRAAYFGVYDTAKGMLPDPKNVHIIVSWMIAQSVTAVAGLV
Ant2  DGIKGLYQGFNVSVQGIIIYRAAYFGIYDTAKGMLPDPKNTHIFISWMIAQSVTAVAGLT
Ant4  DGLIGLYQGFGVSVQGIIVYRASYFGAYDTVKGLLPKPKETPFLVSFIIAQIVTTCSGIL

Ant1  SYPFDTVRRRMMMQSGRKGADIMYTGTLDCWRKIAKDEGANAFFKGAWSNVLRGMGGAFV
Ant2  SYPFDTVRRRMMMQSGRKGTDIMYTGTLDCWRKIARDEGSKAFFKGAWSNVLRGMGGAFV
Ant4  SYPFDTVRRRMMMQSGE--SDRQYKGTIDCFLKIYRHEGVPAFFRGAFSNILRGTGGALV

Ant1  LVLYDEIKKYV----------
Ant2  LVLYDEIKKYT----------
Ant4  LVLYDKIKEFLNIDVGGSSSGD
```

Figure 3-1. *Ant4* encodes a novel isoform of adenine nucleotide translocase. A**)** Deduced amino acid sequence of the mouse *Ant4* gene is aligned with previously identified mouse Ant proteins. B) Deduced amino acid sequence of the mouse *Ant4* gene is aligned with *ANT4* human orthologue (from Rodic *et. al* 2005, Figure 2 on page 1318 with permission).

A



B

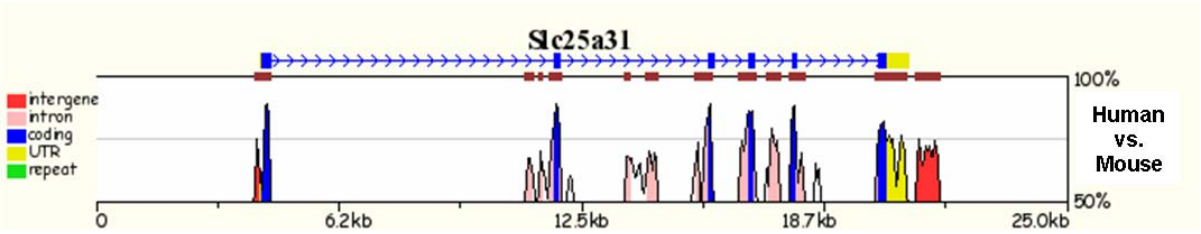


Figure 3-2. The conservation of Sp1 binding sites in the *Ant4* promoter. Evolutionary conserved regions (ECRs) betwen A) rat and mouse and B) human and mouse *Ant4* (*Slc25a31*) genomic regions are indicated as peaks on the graph and are color-coded according to the type of genomic DNA in each ECR. Coding exons are in blue, untranslated regions are in yellow, intronic noncoding ECRs are in pink, and intergenic ECRs are in red. The Y-axis represents the percentage of conservation between the species being compared. Sp1 transcription factor binding sites (TFBS) which are conserved between mouse and rat but not between mouse and human are located within an intergenic ECR and an intron as indicated by vertical tick marks above the graph in A).

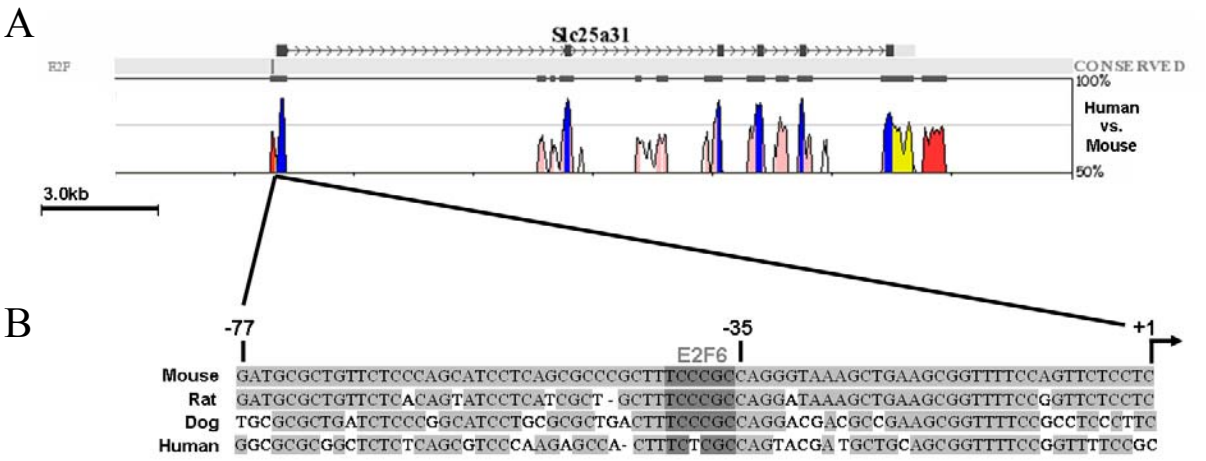


Figure 3-3. The proximal promoter region of the *Ant4* gene has a conserved E2F6 binding site. A) Evolutionary conserved regions (ECRs) of mouse and human *Ant4* (*Slc25a31*) genomic regions are indicated as peaks on the graph and are color-coded according to the type of genomic DNA in each ECR. Coding exons are in blue, untranslated regions are in yellow, intronic noncoding ECRs are in pink, and intergenic ECRs are in red. A scale is shown to indicate the width of the ECRs in kilobases (kb) as measured along the X-axis. The Y-axis represents the percentage of conservation between mouse and human. The position of a single conserved E2F transcription factor binding site (TFBS) with the preferred E2F6 binding sequence (TCCCGC) was found within an intergenic ECR and is indicated by a vertical gray tick mark above the graph. B) The E2F6 TFBS is located at 36 base pairs (bp) upstream of the transcription initiation site. The intergenic ECR containing the site is approximately 149bp in length and is conserved between species. The E2F6 binding site (dark gray) and conserved flanking regions (light gray) are shown for mouse, rat, dog, and human *Ant4*.

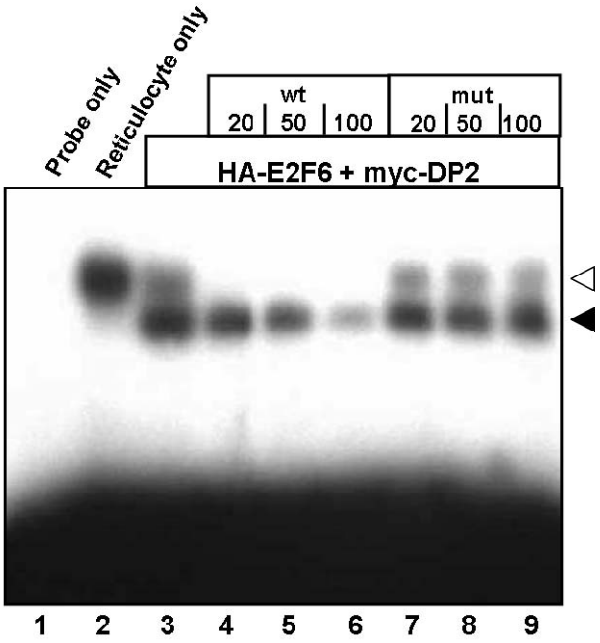


Figure 3-4. Gel mobility shift assay of E2F6 binding to the Ant4 promoter. A labeled oligonucleotide corresponding to the E2F6 TFBS in the mouse Ant4 promoter (lane 1) was incubated with control rabbit reticulocyte lysate alone (lane 2) or in vitro co-translated HA-E2F6 and myc-DP2 proteins (lanes 3-9). Unlabeled Ant4 wild type (wt) (lanes 4-6) or mutated (mut) (lanes 7-9) oligonucleotides were added in excess amounts over the amount of labeled probe as indicated. Filled arrow: position of the specific E2F6/DP2 complex, open arrow: endogenous binding activity in the reticulocyte lysate.

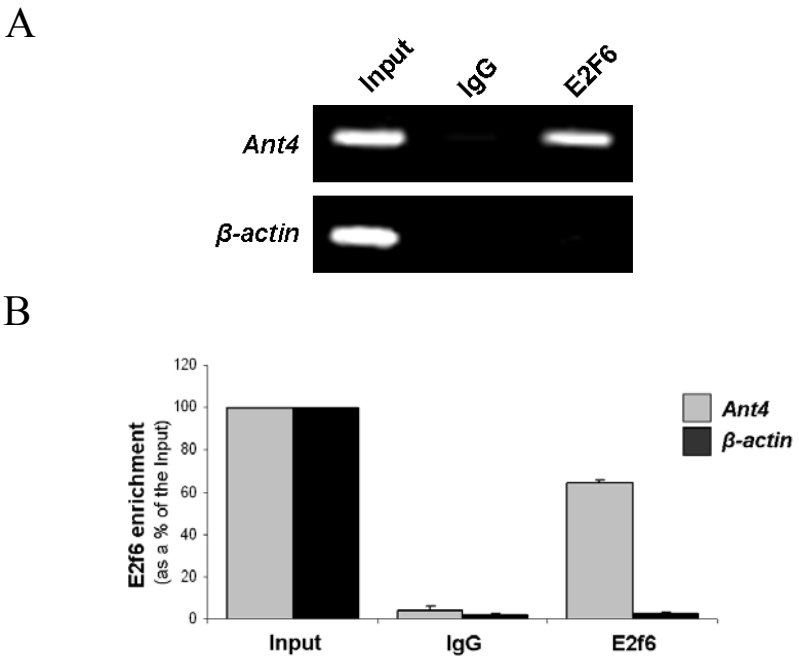


Figure 3-5. The proximal promoter region of Ant4 is bound by endogenous E2f6 in undifferentiated WT R1 ES cells. Chromatin immunoprecipitation (ChIP) was performed using mouse-anti E2f6 antibody. Primers amplifying the Ant4 proximal promoter region containing the E2F6 TFBS and β-actin control primers lacking the E2F6 TFBS were used for both A) semi-quantitative PCR and B) quantitative Real-Time PCR analysis of ChIP. Samples from each primer set were precipitated with IgG to control for nonspecific enrichment. Enrichment of E2f6 binding is expressed as a percentage of the input chromatin. Input samples represent 1% of the starting amount of chromatin and were analyzed to confirm that the different chromatin preparations contain equal amounts of DNA. All samples were tested in triplicate and expressed as means ± standard deviations.

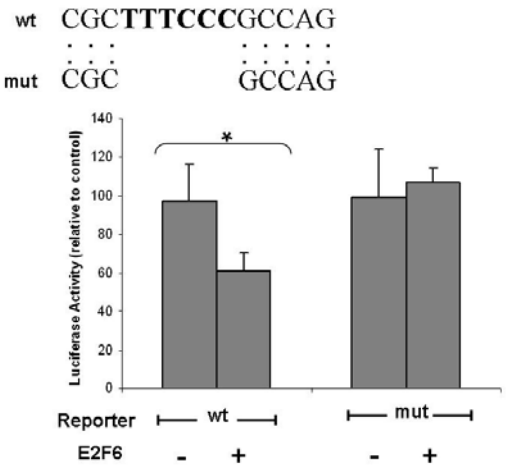


Figure 3-6. E2F6-mediated repression of Ant4 requires an intact transcription factor binding site. Ant4 promoter (-263 to +25bp) luciferase reporter plasmids containing wild type (wt) or mutated (mut) E2F6 TFBS were transiently co-transfected with an empty vector (-) or an E2F6 expression vector (+) into NIH-3T3 cells. Luciferase activity was measured relative to the Renilla internal control vector as described in Materials and Methods Chapter 2. Sequences for both wt and mut E2F6 TFBS reporters are shown above the bar graph. *P<0.05 and data from at least three independent experiments were analyzed and expressed as means ± standard deviations.

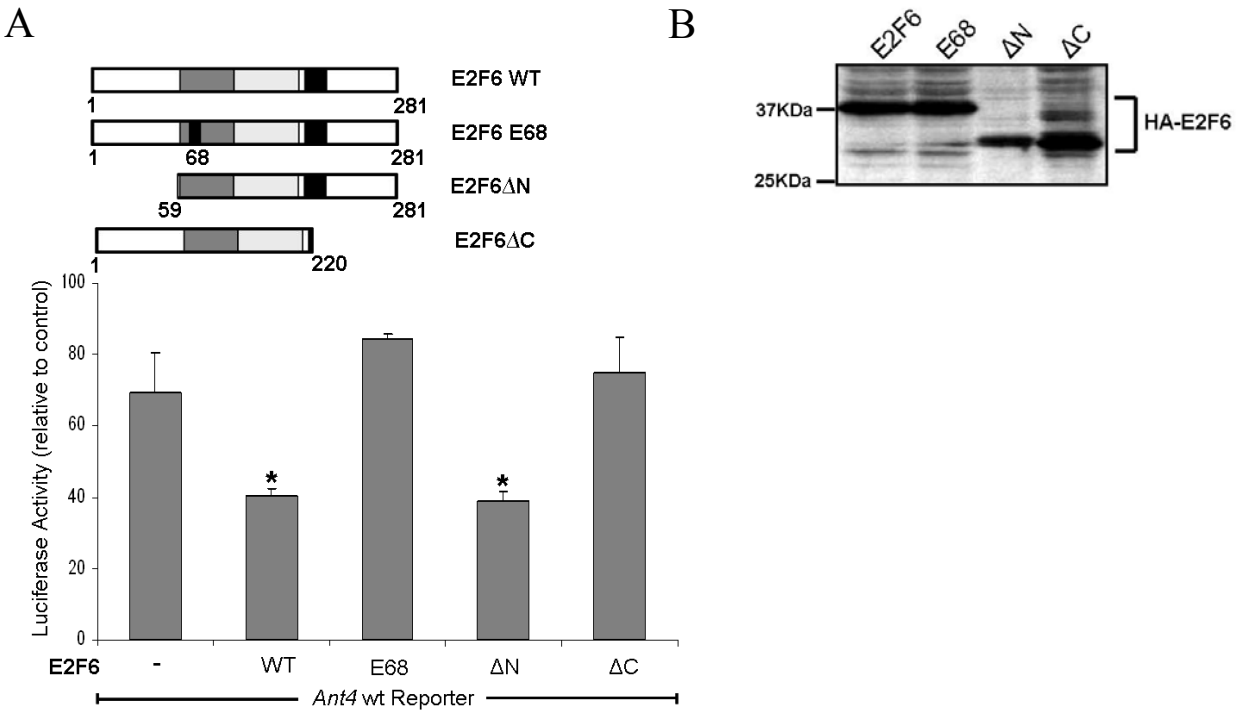


Figure 3-7. E2F6-mediated repression of *Ant4* requires an intact C-terminal and DNA binding domain. A) Luciferase reporter assay in NIH-3T3 cells transiently co-transfected with either empty vector (-), WT E2F6, or mutant (E68, ΔN, or ΔC) HA-E2F6 expression vectors and the *Ant4* wt reporter vector. A schematic representation of the HA-tagged E2F6 vectors is shown above the graph; WT: intact E2F6, E68: a DNA binding domain point mutation, ΔN: an N-terminus deletion mutant, ΔC: a C-terminus deletion mutant, dark gray boxes: DNA binding domains, light gray boxes: dimerization domains, black boxes: marked boxes, and black box inside dark gray box: site of DNA binding domain mutation. B) Western blot analysis using anti-HA antibody, showing that all the E2F6 expression vectors transiently transfected for luciferase reporter assays in A) expressed their respective E2F6 proteins at comparable levels. For A), *P<0.05 and data from at least three independent experiments were analyzed and expressed as means ± standard deviations.

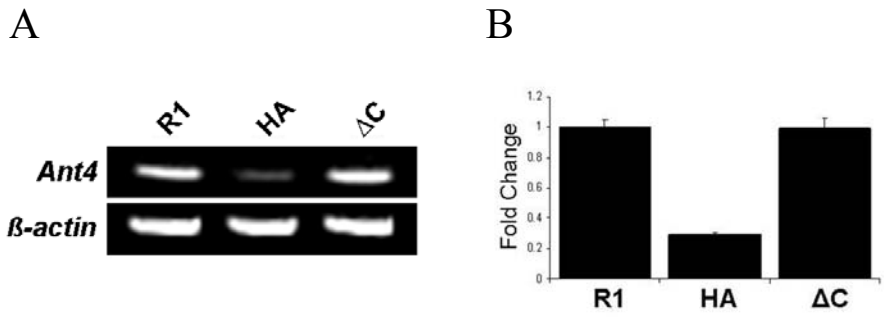


Figure 3-8. *Ant4* transcription is repressed by stable E2F6 overexpression in ES cells. A) Semi-quantitative RT-PCR analysis of *Ant4* and *ß-actin* expression in parental R1, HA-E2F6, and HA-ΔC-E2F6 ES cell lines. B) Quantitative Real-Time PCR analysis of samples shown in A). All samples were tested in triplicate and expressed as means ± standard deviations.

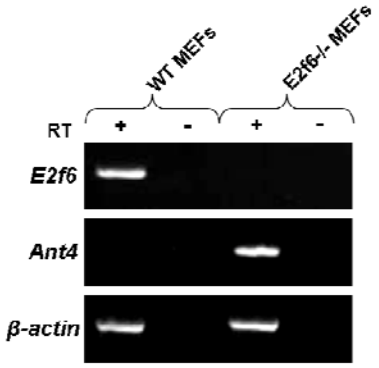


Figure 3-9. *Ant4* transcription is derepressed in *E2f6-/-* MEFs. Semi-quantitative RT-PCR analysis is shown for *E2f6*, *Ant4,* and *β-actin* expression in WT MEFs and *E2f6-/-* MEFs. Reverse transcriptase (RT) minus samples were also amplified to eliminate the possibility of genomic DNA contamination.

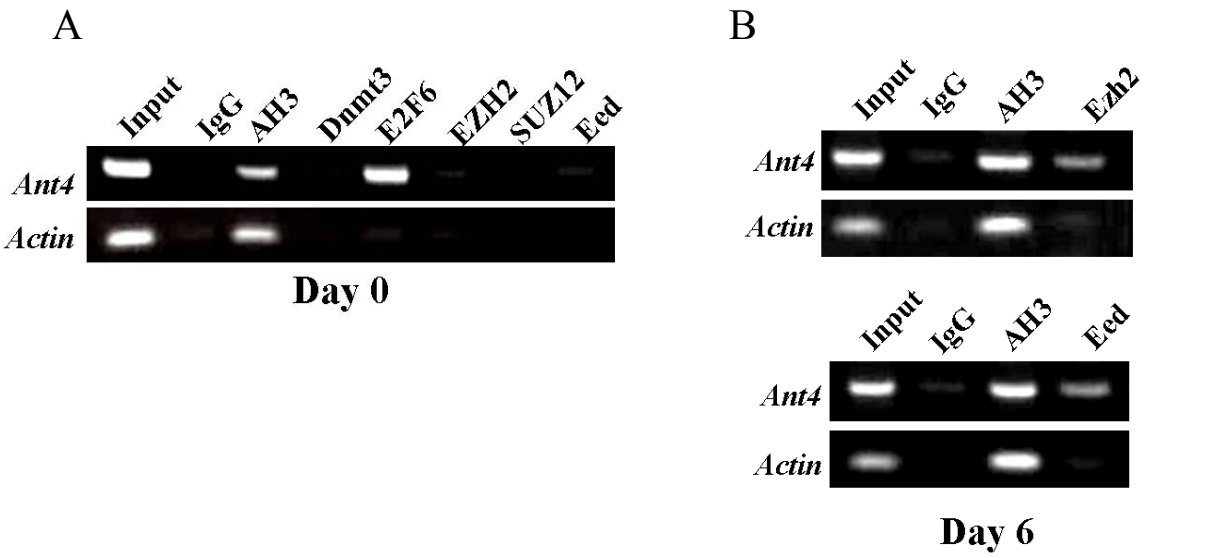


Figure 3-10. The polycomb proteins Eed and Ezh2 bind to the *Ant4* promoter in D6 R1 EBs. Chromatin immunoprecipitation (ChIP) was performed using anti- E2f6, Eed, Ezh2, Dnmt3, Suz12, and acetylated histone H3 (AH3) antibodies. Primers amplifying the *Ant4* proximal promoter region containing the E2F6 TFBS and *β-actin* control primers lacking the E2F6 TFBS were used for semi-quantitative PCR for A) undifferentiated ES cells (D0) and B) differentiated EBs (D6). Samples from each primer set were precipitated with IgG to control for nonspecific enrichment. Input samples represent 1% of the starting amount of chromatin and were analyzed to confirm that the different chromatin preparations contain equal amounts of DNA.

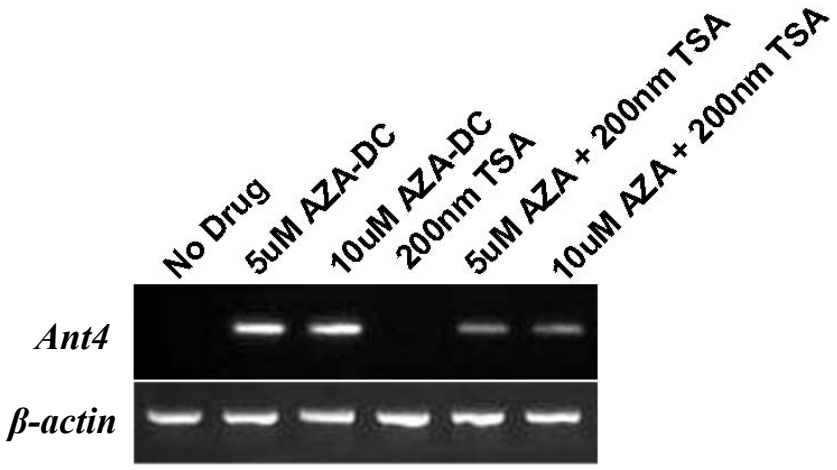


Figure 3-11. *Ant4* is derepressed in NIH-3T3s after treatment with 5-AZA-DC but not TSA. 5μM or 10 μM of AZA-DC were added either alone or in combination with 200nm TSA for 65 hours in NIH-3T3s. RNA was harvested and *Ant4* and *β-actin* primers were used for semi-quantitative RT-PCR.

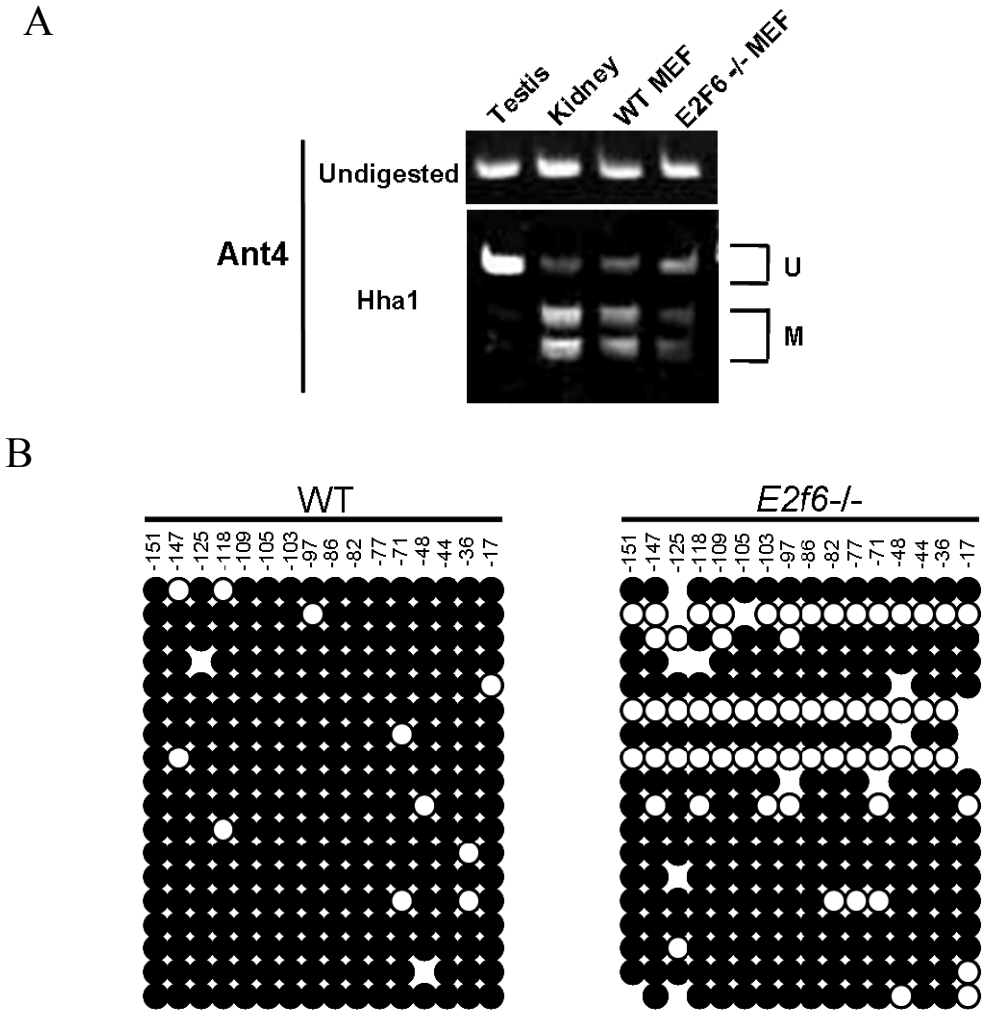


Figure 3-12. CpG methylation at the *Ant4* promoter is partially reduced in *E2f6-/-* MEFs. A) COBRA analysis of the methylation status of *Ant4* in various tissue types. Top band is undigested PCR product prior to COBRA digestion with Hha1. Upon digestion there is three bands, the single band marked as U represents unmethylated DNA; the two bands labeled M represent the methylated DNA. B) Bisulfite Sequencing analysis of the *Ant4* promoter in WT MEFs (left) and *E2f6-/-* MEFs (right) showing a decrease from 96% methylation in WT to 80% methylation in *E2f6-/-*.

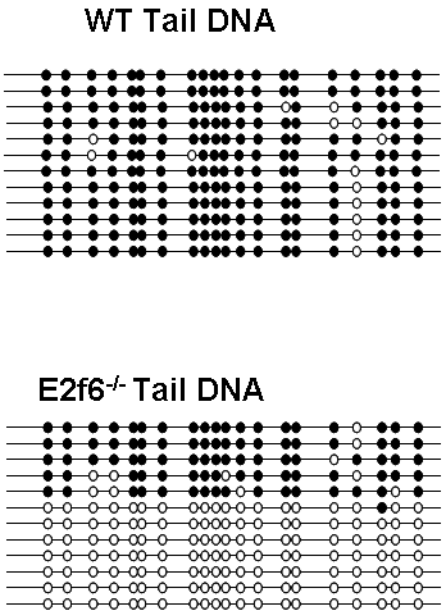


Figure 3-13. *Tuba3* methylation decreases in *E2f6*-/- mouse tail DNA. Bisulfite Sequencing analysis of the *Tuba3* promoter in WT mouse tail (top) and *E2f6*-/- mouse tail (bottom) showing a decrease from 94% methylation in WT to 37.5% methylation in *E2f6*-/- (with permission from Emily Smith and Dr. Resnick).

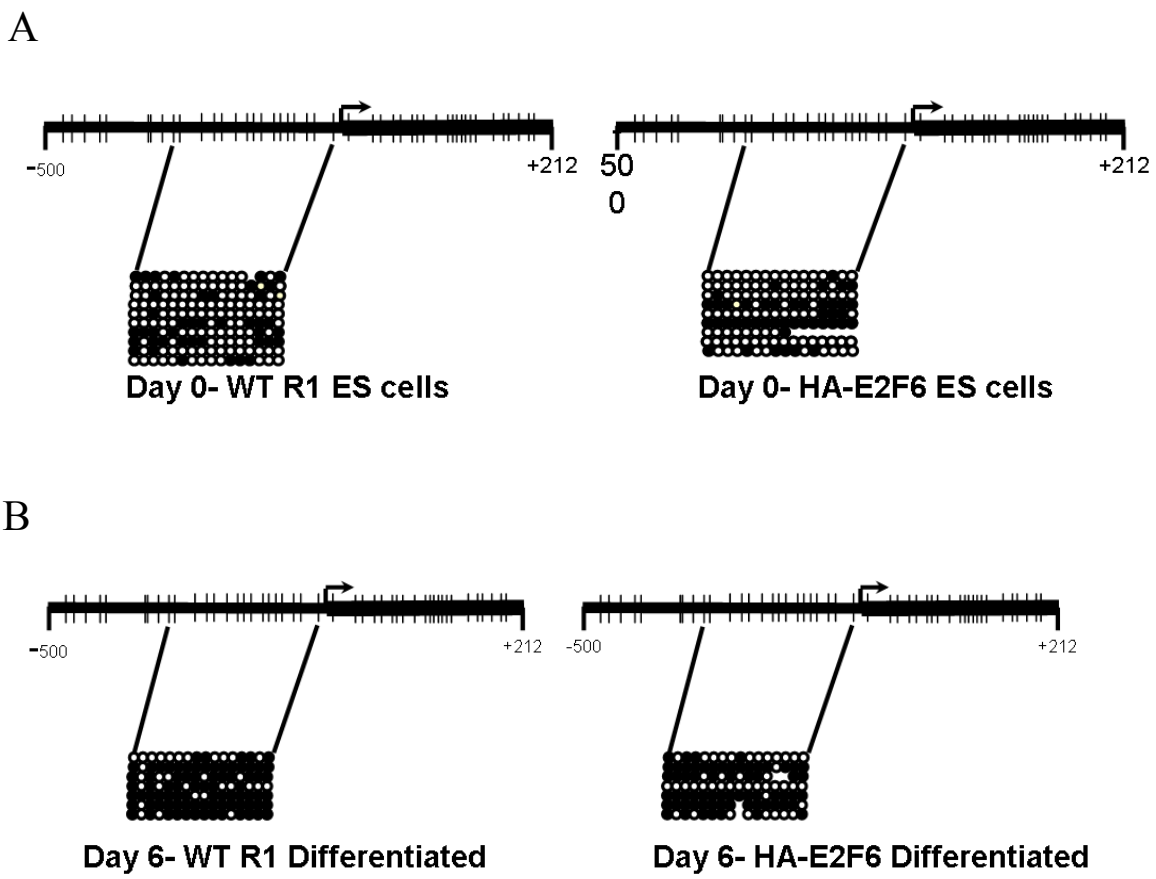


Figure 3-14. E2F6 overexpression does not increase CpG methylation at the *Ant4* promoter. Bisulfite Sequencing analysis comparing the methylation status of the *Ant4* promoter between WT R1 ES cells and HAE2F6 ES cells at A) D0 and B) D6 of ES cell differentiation. The *Ant4* promoter region is shown by a horizontal line with an arrow indicating the transcription initiation site. Each vertical tick mark represents the location of a CpG site with 16 CpG sites residing within the sequenced region.

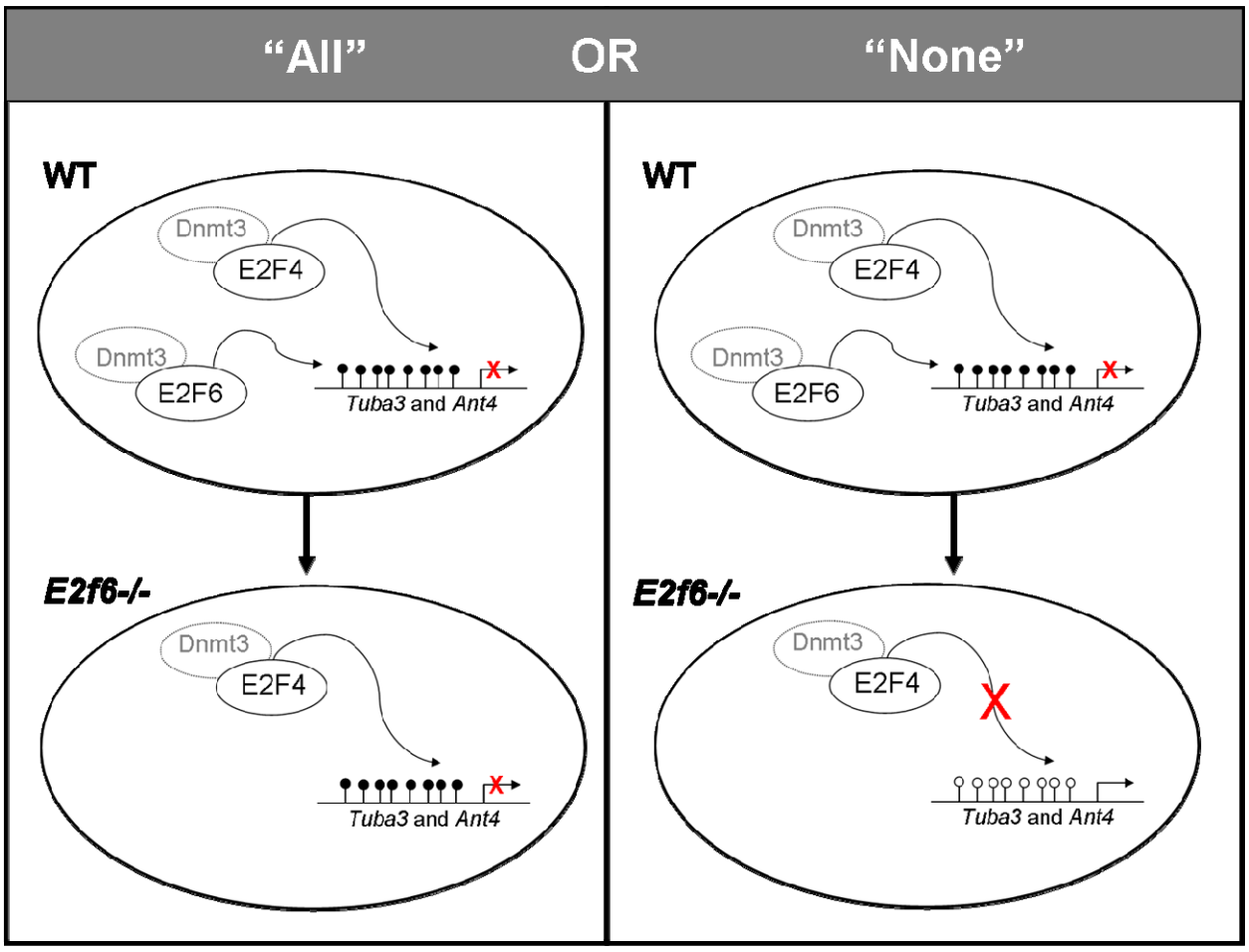


Figure 3-15. The "All" OR "None" model of E2F4 compensation. In WT somatic cell types both E2F4 and E2F6 play a role in the methylation of *Tuba3* and *Ant4* promoters. Then, in *E2f6-/-* tissues one of two things will happen. In the "All" model, E2F4 will compensate for the loss of E2F6 and completely methylate the *Tuba3* and *Ant4* promoters, thereby contributing to transcriptional repression. In the "None" model, E2F4 is unable to compensate for the loss of E2F6 and will not methylate the *Ant4* and *Tuba3* promoters, thereby resulting in "leaky" expression.

CHAPTER 4
REPRESSION OF ADDITIONAL MEIOSIS-SPECIFIC GENES BY E2F6

Motivation

The mechanisms controlling germ-cell-specific gene expression are diverse (Dejong 2006 Eddy 2002, MacLean & Wilkinson 2005). Studies have found these genes to be regulated extrinsically by hormones secreted from the endocrine system, interactively by factors released from neighboring supportive cells in the gonads, and intrinsically by factors affecting transcription, translation, DNA methylation, and histone modifications. Among such germ-cell-specific genes, it is especially important that those genes which are highly expressed and critical during the meiotic phase of gametogenesis be appropriately regulated. Aberrant expression of these genes in somatic cells is presumed to be associated with disruptions in the mitotic cell cycle and may lead to dire consequences such as oncogenic transformation (Sagata 1997, Simpson *et al*. 2005). Many of these critical genes are germ-cell-specific and are therefore repressed in all somatic cells in the body. This chapter focuses on the somatic repression of a group of germ-cell-specific genes which are similar to *Ant4* in that their protein products function specifically during meiosis. Results suggesting the existence of a common transcriptional repressor of these meiosis-specific genes are presented herein.

Background

Much of our current understanding regarding the transcriptional regulation of genes involved in meiosis can be credited to the use of transgenic mouse models. Studies have demonstrated that short proximal promoter regions are sufficient to specify somatic silencing and germ cell activation for meiotic genes such as: *Sycp1*, *CyclinA1, Pgk2, HistoneH1t, Alf,* and *Pdha2* (Bartell *et al*. 1996, Han *et al*. 2004, Iannello *et al*. 1997, Lele & Wolgemuth 2004, Robinson *et al*. 1989, Sage *et al*. 1999). A more detailed analysis of their minimal promoters

revealed DNA regulatory elements harboring transcription factor binding sites. Further, incubation of these sequences with nuclear extracts from either somatic or testicular tissue indicated that nuclear proteins were indeed binding to these elements. Variations in binding between somatic and testicular nuclear extracts imply that these proteins mediate testis-specific expression and somatic cell repression. For instance, *Mos*, a gene found to contain a negative regulatory element in its proximal promoter, is bound by a protein present only in nuclear extracts from somatic cells and not from pachytene spermatocytes (Xu & Cooper 1995). This observation, coupled with the absence of expression of *Mos* in somatic cells, infers that this protein may be serving as a transcriptional repressor. Further studies revealed that this protein was COUP-TF (Lin *et al*. 1999). Additional transcription factors which bind to various germ-cell-specific genes expressed during meiosis are beginning to be uncovered and include Sp1, Sp3, Ctf1, Rfx1, Rfx2, Ctcf, and Bmyb (Bartusel *et al*. 2005, Gebara & McCarrey 1992, Horvath *et al*. 2004, Kim *et al*. 2006, Wilkerson *et al*. 2002). However, the existence and identity of a master regulatory protein or protein family which binds to the proximal promoters and coordinately regulates the expression of multiple meiosis-specific genes uniformly as a group remains to be elucidated.

Recently, the E2F6 transcription factor was shown to be required for the repression of a subset of germ-cell-specific-genes in somatic cells. These six genes, *Tuba3*, *Tuba7*, *XM196054*, *Tex12*, *Stag3*, and *Smc1β*, are aberrantly expressed in *E2f6* null mouse embryonic fibroblasts (MEFs) and/or somatic organs (Pohlers *et al*. 2005, Storre *et al*. 2005). All of these germ-cell-specific genes contain the core E2F6-binding element, TCCCGC, within their proximal promoter regions (Cartwright *et al*. 1998). Although the expression patterns of these genes are similar in that they are all germ-cell-specific, their expression patterns and functions within germ cells are

diverse. Tuba3 and Tuba7 are α-tubulins which heterodimerize with β-tubulins to form microtubules that constitute the primary structural components of mitotic and meiotic spindles (Hecht *et al*. 1988). *Tuba3* and *Tuba7* are encoded by separate genes but have identical protein products which are exclusively expressed in male gonads. More specifically, Tuba3 and Tuba7 are highly expressed in the metaphase spindles of spermatogenic cells undergoing meiosis and in the manchettes of elongated spermatids. Indeed, the *Tuba3* gene initiates significant levels of expression at the onset of meiosis in primary spermatocytes (Wang *et al*. 2005). Little is known about *XM_196054* other than that it is a gene encoding a novel protein of unknown function with testis-specific expression (Storre *et al*. 2005). Tex12 is a male-meiosis-specific component of the synaptonemal complex which is a group of proteins involved in the alignment and pairing of homologous chromosomes during prophase one of meiosis (Hamer *et al*. 2006). Stag3 and Smc1β are also meiosis-specific proteins, but are components of the cohesion complex which is a group of proteins that act as a molecular glue to hold sister chromatids together during prophase one of meiosis (Revenkova & Jessberger 2005). Stag3 is restricted to male meiotic germ cells whereas Smc1β can be found in both male and female meiotic germ cells (Pezzi *et al*. 2000, Remelpva *et al*. 2001).

In the last chapter (Chapter 3), we presented evidence that E2F6 is required for the repression of another germ-cell-specific gene, *Ant4*, in somatic cells. *Ant4* contains the core E2F6-binding element within its proximal promoter region and, similar to Tex12, Stag3, and Smc1β, its protein product is selectively expressed in meiotic cells (Brower *et al*. 2007). This discovery prompted us to investigate whether additional germ-cell-specific genes require E2F6 for their repression. In particular, we focused on a subpopulation of germ-cell-specific genes whose expression is not only high in meiotic cells but is also claimed to be restricted to the

meiotic phase of germ cell development. Here we show that most of the 24 meiosis-specific genes examined in this study have the core E2F6-binding element within 200 basepairs (bp) upstream of their transcription initiation sites. Moreover, using murine embryonic stem (ES) cell culture, we demonstrate that E2F6 indeed plays a broad role in the transcriptional repression of these meiosis-specific genes by binding to their proximal promoter regions.

Results

The discovery that another meiosis-specific gene is regulated by E2F6 prompted us to investigate whether additional meiosis-specific genes require E2F6 for their repression. After an extensive literature search and without bias, we compiled a list of 24 genes which were previously reported to have meiosis-specific expression (Table 4-1). It should be noted here that we excluded genes from the list which continue to have predominant levels of expression post-meiotically. For each of these meiosis-specific genes, we screened a genomic region spanning 1kb upstream of their transcription initiation sites for the presence of E2F6-binding elements (TCCCGC or GCGGGA, depending on the direction of binding) using UCSC Genome Browser (Karolchik *et al*. 2003). As shown in Table 4-1, potential E2F6-binding elements are accumulated within the proximal promoter (~200bp) regions of these meiosis-specific genes. In total, 19 out of the 24 meiosis-specific genes (79.2%) contain at least one E2F6-binding element within 200bp upstream of their transcription initiation sites (white bars, Figure 4-1). The relevance of this finding was examined through the application of a genome-scale DNA pattern matching software tool, known as Regulatory Sequence Analysis Tool (RSAT) (Van-Heldin 2003). The RSAT software was used to identify the E2F6-binding elements within promoter regions (-1000bp to +1bp) of all genes in the entire mouse genome (based on 31,113 genes in the Ensembl database) (gray bars, Figure 4-1). A comparison between the meiosis-specific genes

and all genes in the mouse genome indicates that the proximal promoter regions of these meiosis-specific genes are indeed enriched with E2F6-binding elements.

As an additional measure of enrichment, the probability that an E2F6-binding element would randomly occur within a nucleotide sequence of a specified length was calculated. This calculation was made under the assumption that the four nucleotide base pairs (A, G, C, or T) occur at random and with equal probability at each nucleotide position (Gentleman & Mullin 1989). The resulting value can be considered as the expected rate of random occurrence with which the E2F6-binding element appears throughout a population of actual DNA sequences, and is used here as a reference point for statistical significance (black bars, Figure 4-1). A comparison between this expected rate of random occurrence and the actual frequency of E2F6-binding element appearance indicates that the E2F6-binding element is specifically enriched towards the proximal, but not the distal, promoter regions of all genes (gray bars, Figure 4-1). The rate of occurrence is shown to be appreciably elevated for meiosis-specific genes (white bars, Figure 4-1). The selectivity of the E2F6-binding element to the proximal promoter, a genomic region which is known to harbor binding sites of critical transcriptional regulators, suggests that this E2F6-binding element possesses a high degree of functional significance.

Next, we examined whether E2F6 binds to the E2F6-binding elements found in the proximal promoter regions of meiosis-specific genes. We performed ChIP analysis using undifferentiated ES cells and observed endogenous E2f6 binding to several meiosis-specific genes (Figure 4-2). Figure 4-2A shows E2f6 binding to the promoters of genes which are known to be derepressed in *E2f6 -/-* MEFs. To clarify, *Tuba3* is one of these derepressed genes but its expression is not restricted to meiotic cells and was therefore not included in Table 1. Figure 4-2B suggests that several of the newly identified E2F6-binding elements in the proximal

promoters of meiosis-specific genes (see Table 4-1) are in fact occupied by E2f6. Figure 4-2C verifies the specificity of E2F6 binding as evidenced by the absence of E2f6 binding in both housekeeping and ES cell-pluripotency genes. Figure 4-2D is a quantitative analysis from a sampling of genes in Figures 4-2A, 4-2B, and 4-2C.

Next, we looked to see whether exogenous E2F6 overexpression was capable of repressing meiosis-specific genes. Using HA-E2F6 and HA-ΔC-E2F6 ES cells, we demonstrate that meiosis-specific transcripts are specifically reduced in HA-E2F6 but not in mutant HA-ΔC-E2F6 ES cells relative to parental R1 ES cells (Figure 4-3). This observation brought about the reverse question of whether the absence of E2F6 would result in derepression of additional meiosis-specific genes beyond that of *Stag3*, *Smc1ß*, *Tex12*, and *Ant4*. We examined the expression status of meiosis-specific genes in *E2f6-/-* MEFs for the presence of aberrant gene expression, but found that these additional meiosis-specific genes remain repressed (Figure 4-4). Overall, these observations suggest that E2F6 is not required for somatic cell repression of most meiotic genes and supports the possibility that a functional redundancy exists whereby other safeguards are in place to ensure their repression in the absence of E2F6.

Discussion

During embryonic development, there is a coordinated regulation of gene expression whereby genes whose protein products function in the same physiological processes are concomitantly expressed. Such coordinate regulation is thought to be controlled, at least in part, by the presence of binding sites for the same transcription factors in the promoter regions of these genes. Several studies have revealed common transcription factor binding sites in groups of genes having similar tissue-specific expression patterns or physiological roles (Kel *et al*. 2001, Tronche *et al*. 1997, Wasserman & Fickett 1998). For instance, previous studies have screened DNA sequences from the EMBL and Genbank data banks and found that liver-specific gene

promoters harbor binding sites for the HNF-1 transcription factor 2.5 times more frequently than do other genes (Tronche *et al*. 1997). Likewise, NFAT/AP-1 binding sites are present at rates of 10 times higher in the promoters of immune response genes than in random sequences pulled from EPD and Genbank databases (Kel *et al*. 2001). Further, the promoter regions of genes whose protein products are known to play a role in cell cycle progression were found to have a high frequency of E2F binding site (the traditional TCGCGC site common to E2Fs 1-5 rather than the E2F6-preferred TCCCGC site) occurrence in comparison to the promoters of functionally different genes (Kel *et al*. 2001). In the present study, our examination of the promoter regions of a group of 24 meiosis-specific genes revealed a common E2F6 transcription factor binding site.

Kel et al., defined a set of rules to predict whether a transcription factor binding site could contribute to the shared pattern of gene expression observed in sets of functionally related genes (Kel *et al*. 2001). If a binding site met these criteria, it was termed a "promoter-defining site." Several features of the common E2F6 binding site investigated in this report suggest that it is indeed a "promoter-defining site." First, the site occurs in the meiosis-specific gene promoters at a frequency significantly higher than in random sequences (Figure 4-1). Second, the frequency of E2F6 binding site occurrence significantly differs between the promoters of genes belonging to various functional groups. In this case, the occurrence of E2F6 binding sites in the promoters of the meiosis-specific genes examined in this study was drastically elevated in comparison to the frequency of binding site occurrence in the promoters of all genes in the mouse genome (Figure 4-1). Third, the E2F6 binding site is located in close proximity to the transcription initiation site (Table 4-1). According to Kel et al., in order to be considered a "promoter-defining site," the candidate site should occur within approximately 300bp upstream of the

transcription initiation site (Kel *et al*. 2001). The observation that 19 (79.2%) of the 24 meiosis-specific genes examined here had an E2F6 binding site within 200bp upstream of their transcription initiation sites is a convincing indicator that this E2F6 site should be considered "promoter-defining."

One of the fundamental questions raised by our results pertains to why almost every meiosis-specific gene promoter examined in this study contains an E2F6 binding site which binds E2f6 *in vivo*, yet very few of these genes actually require E2F6 for their repression in somatic cells. This discrepancy does not eliminate the potential role of E2F6 as a broad repressor of meiosis-specific genes, but rather suggests the existence of a functional redundancy whereby even in the absence of E2F6, another repressor can compensate. Previous studies have shown that E2f4 can compensate for the loss of E2f6 by binding to the promoters of G1/S-regulated genes (which under normal circumstances are bound by E2f6) in the absence of E2f6 (Giagrande *et al*. 2004). *E2f4*-knockdown and *E2f6*-null MEFs showed no derepression of these genes individually, but when both E2f4 and E2f6 were simultaneously inhibited using *E2f4*-knockdown-*E2f6*-null MEFs, specific derepression of G1/S-regulated genes was observed. Other studies have shown that E2F4 and E2F6 are concurrently bound to the promoters of numerous genes including: *Cyclin A2*, *Cyclin B2*, *Cdc2*, *Art-27*, *Hp1α*, *Rpab48*, and *Ctip* (Caretti *et al*. 2003, Oberley *et al*. 2003). Interestingly, ChIP analysis of the *Stag3* promoter shows binding of E2f6 and the activating E2f3 but not E2f4 in WT MEFs (Storre *et al*. 2005). Perhaps this lack of redundancy with E2F4 could explain why *Stag3* is aberrantly expressed in *E2f6*-/- MEFs. Conversely, *Tuba3* and *Tuba7* germ-cell-specific genes are also aberrantly expressed in *E2f6*-/- MEFs but do in fact bind E2F4 according to affinity chromatography and gel mobility shift assays (Pohlers *et al*. 2005). This finding implies that the presence or absence of E2F4

binding is not a sole criterion by which to decipher the occurrence of E2F6 functional redundancies. It should be noted that the E2F4 binding status of *Tuba3* and *Tuba7* promoters was not determined in WT MEFs, leaving open the possibility that the discrepancies in binding between these promoters and *Stag3* could be owed to differences in cell type.

It would be naïve to infer that the only transcription factors capable of compensating for the loss of E2F6 are other E2F family members. Transcriptional regulation is a combinatorial process whereby each gene is under the control of a multiplicity of elements, some of which can be dispersed over several kilobases. One example of a redundancy that most likely does not involve other E2F family members may be *Ribc2*. *Ribc2* is one of the many meiosis-specific genes that remain repressed in *E2f6*-/- MEFs (Figure 4-4). This observation is noteworthy given that *Ribc2* and *Smc1β* genes overlap on chromosome 15 and are transcribed in opposite orientations with their promoter regions embedded within each other (Arango *et al*. 2004). These genes are not only transcribed at similar times during meiosis, but they share an E2F6-binding element in their overlapping promoter region which we have shown binds E2f6 *in vivo* (shown as Smc1β, Figure 4-2). There are only 261bp of sequence in between the transcription initiation sites of *Ribc2* and *Smc1β*. Within this narrow region, the shared E2F6 site is located at -175bp to -170bp relative to *Ribc2* and -47bp to -42bp relative to *Smc1β* transcription initiation sites. An explanation regarding why *Smc1β* is derepressed in *E2f6*-/- MEFs but not *Ribc2* could be partially due to a distance effect whereby the proximity of the E2F6-binding element to the transcription initiation site could determine the essentialness of E2F6 binding. Alternatively, DNA elements existing within introns and 3' regions specific to each gene may be accountable for the differential regulation. Although, the mechanisms responsible for this distinction in gene

regulation remain unclear, a redundancy of an E2F family member at the location of this shared E2F6 binding site would most likely not account for such a phenomenon.

The discovery that only a handful of meiosis-specific genes were aberrantly expressed in somatic cells in the absence of E2F6 is in agreement with previous studies. Prior reports have described cDNA microarray experiments with mRNA from WT and *E2f6-/-* MEFs and shown that very few germ-cell-specific genes were upregulated in *E2f6-/-* MEFs (Pohlers *et al*. 2005, Storre *et al*. 2005). Further, our findings are consistent with the mild phenotype observed in *E2f6-/-* mice. Although these mice display homeotic transformations of the axial skeleton, they are viable, fertile, and display no signs of tumor formation (Storre *et al*. 2002). A higher incidence of abnormally regulated meiosis-specific genes would likely result in a more severe phenotype. Evidence supporting this speculation is seen by the aberrant expression of germline genes in cancers whereby expression reflects the acquisition of the silenced gametogenic program in somatic cells (Simpson *et al*. 2005). This acquisition is thought to be one of the driving forces in tumorigenesis. The proteins that become aberrantly expressed as a result of this phenomenon are termed cancer/testis antigens. Two examples of known cancer/testis antigens which have meiosis-specific expression in normal tissues are Spo11 and Sycp1 (Simpson *et al*. 2005, Tureci *et al*. 1998). They are both components of the synaptonemal protein complex but have different functions. Spo11 causes double-stranded-breaks which are thought to initiate recombination events during meiosis (Keeney *et al*. 1997). Sycp1 makes up the transverse filaments of the synaptonemal complex (Pousette *et al*. 1997). It is theorized that aberrant expression of either of these meiosis-specific proteins in mitotic cells leads to abnormal chromosome segregation and aneuploidy, the hallmarks of cancer cells (Simpson *et al*. 2005). The finding that overexpression of Sycp1 in COS cells leads to the formation of synaptonemal

complexes supports this theory (Ollinger *et al*. 2005). In agreement with the absence of tumor formation in the *E2f6-/-* mice; we find here that although E2F6 binds to the promoters of both *Spo11* and *Sycp1* (Figure 4-2), they remain repressed in *E2f6-/-* MEFs (Figure 4-4).

Even though very few meiosis-specific genes examined in this study require E2F6 for their repression in somatic cells, all of the genes analyzed here were at least partially repressed by E2F6 overexpression (Figure 4-3). The obvious question raised by this observation concerns the mechanisms by which E2F6-mediated repression occurs. As discussed in Chapter 1, there are several ways that E2F6 can repress its target genes. Whether E2F6-mediated repression occurs via the same mechanism for all the genes we analyzed or whether each individual gene utilizes a different mode of E2F6-induced repression remains to be determined. In particular, it will be important to distinguish whether those genes which are derepressed in *E2f6-/-* MEFs utilize a different mechanism of E2F6-mediated repression than those which remain repressed. We predict that only those genes which are derepressed in *E2f6-/-* MEFs will have repression mechanisms similar to those found for *Ant4* (discussed in Chapter 3). Further studies analyzing the methylation status as well as polycomb protein occupancy at these meiosis-specific gene promoters are needed before such conclusions can be drawn. Another interesting point is that the repression of *Stag3* and *Smc1ß* can be restored in *E2f6-/-* MEFs reconstituted with E2F6 (Storre *et al*. 2005). This observation brings into question, the role that E2F6 plays in initiating somatic cell repression versus maintaining repression.

Overall, the present study has provided evidence suggesting that E2F6 may play a broad role in the repression of meiosis-specific genes. However, this role is most likely masked *in vivo* by the existence of functional redundancies where other repressive mechanisms are in place to ensure the repression of most meiosis-specific genes in somatic cells. Given the catastrophic

outcome that can result from aberrant expression of meiosis-specific genes (Sagata 1997, Simpson *et al*. 2005); it is not surprising that organisms have developed safeguards to guarantee their repression.

Table 4-1. Locations of E2F6 TFBS (TCCCGC) within upstream promoter regions of meiosis-specific genes relative to their transcription initiation sites

Gene Name	Upstream Promoter Regions in Basepairs (bp)					References
	+1 to -100	-100 to -200	-200 to -300	-300 to -400	-400 to -500	
Ant4 (Slc25a31)	√					(Brower *et al.* 2007)
*Boule**						(Eberhart *et al.* 1996, Xu *et al.* 2001)
Cyclin A1					√	(Ravnick & Wolgemuth 1999, Sweeney *et al.* 1996)
Dmc1 (Lim15h)	√		√			(Habu *et al.* 1996)
*Lamin C2**						(Alsheimer *et al.* 1996)
Meg1 (Calmegin)	√					(Don & Wolgemuth 1992, Watanabe *et al.* 1994)
Mnd1	√					(Petukhova *et al.* 2005, Pezza *et al.* 2006)
Mns1		√				(Furukawa *et al.* 1994)
Mzf6d	√					(Looman *et al.* 2003)
*Prdm9 (Meisetz)**	√					(Hayashi *et al.* 2005)
*Psmc3ip (Hop2)**	√					(Petukhova *et al.* 2005, Pezza *et al.* 2006)
Rec8	√					(Lee *et al.* 2003)
RecQL (isoform beta)	√			√	√	(Wang *et al.* 1998)
Ribc2 (Trib)		√				(Arango *et al.* 2004)
Smc1β	√					(Renekova *et al.* 2001)
Spo11	√					(Shannon *et al.* 1999)
Stag3	√					(Pezzi *et al.* 2000, Prieto *et al.* 2001)
Syce1						(Costa *et al.* 2005)
Syce2 (Cesc1)		√				(Costa *et al.* 2005)
Sycp1 (Scp1)	√					(Meuwissen *et al.* 1992, Yuan et al. 1996)
Sycp2						(Offenberg *et al.* 1998)
Sycp3		√				(Di Carlo *et al.* 2000, Lammers *et al.* 1994)
*Tcte2**		√	√			(Braidotti *et al.* 1997)
Tex12	√					(Hamer *et al.* 2006)

*These genes have an E2F6 TFBS within their 5' UTRs

√ Denotes the presence of an E2F6 TFBS within the upstream region of the corresponding to the column

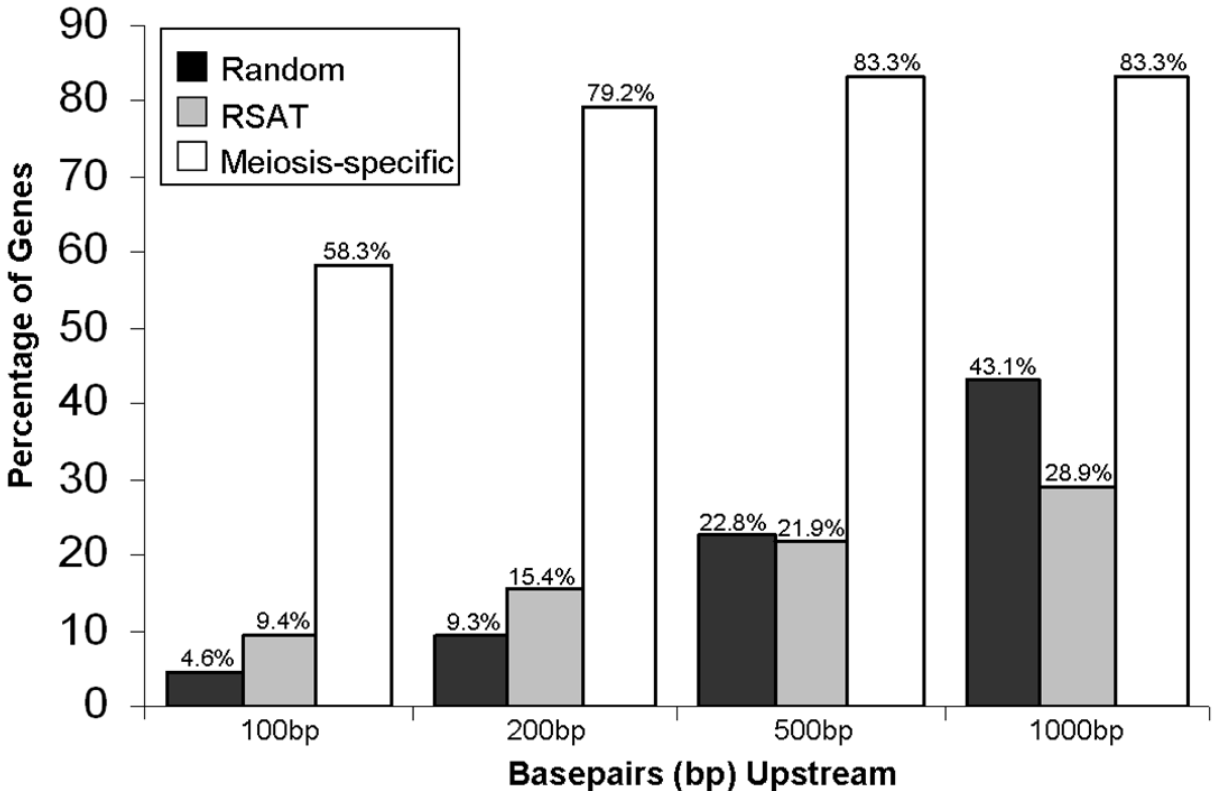


Figure 4-1. Frequency of appearance of E2F6 TFBS within upstream regions of genes relative to their transcription initiation sites. Bar graph indicates the occurrence rate of E2F6 TFBS for the following categories: Random: the probability that an E2F6 TFBS would randomly appear at least once within a sequence of a given length as described in Methods, RSAT: the actual frequency that the E2F6 TFBS occurs at least once within the proximal promoter regions of genes (31,113 genes) in the mouse genome, Meiosis-specific: the actual frequency that the E2F6 TFBS occurs at least once within a population of meiosis-specific genes (those listed in Table 1). The X-axis indicates the location of the E2F6 TFBS to be within 100bp, 200bp, 500bp, or 1000bp upstream of the transcription initiation site; the Y-axis denotes the percentage of genes from each category (see figure legend) that have at least one E2F6 TFBS within a given upstream region.

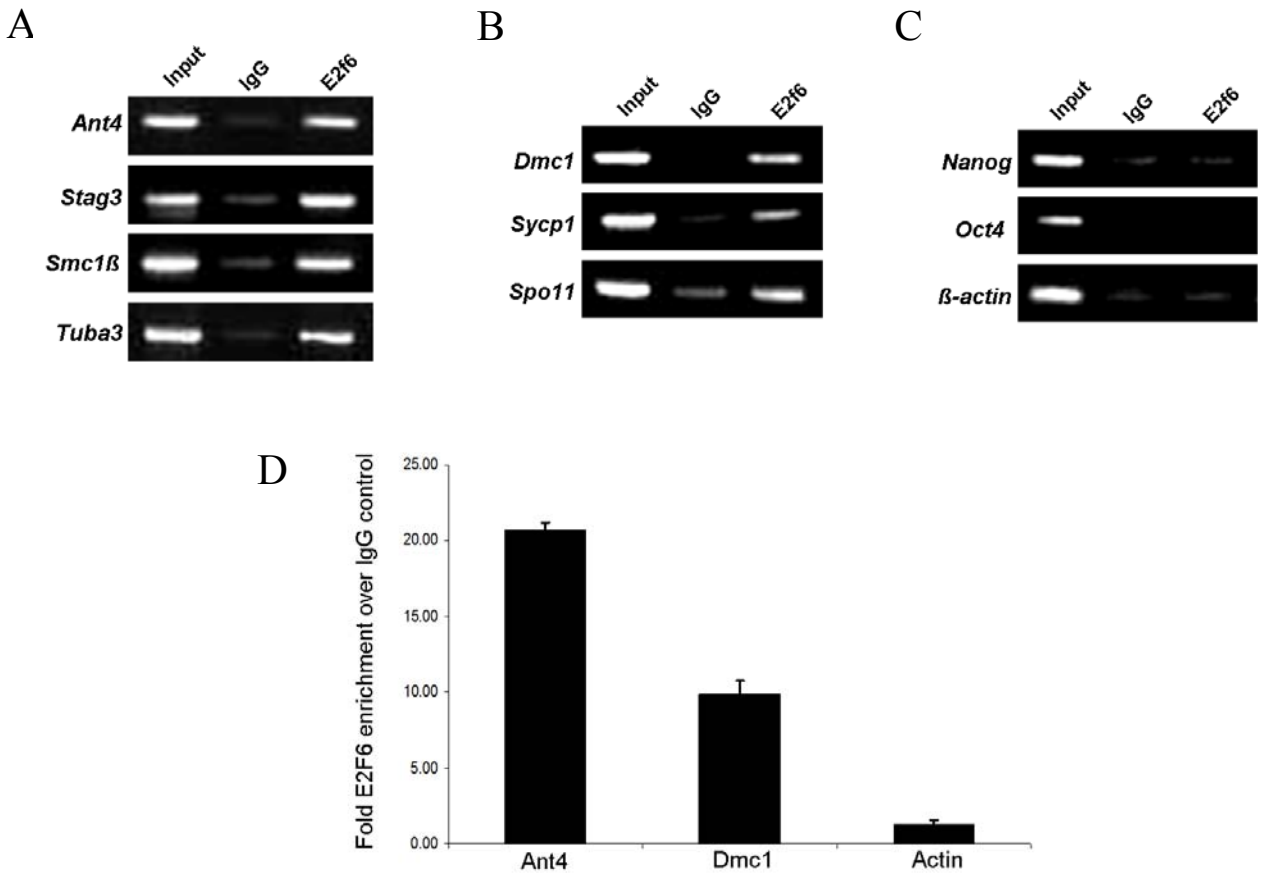


Figure 4-2. E2f6 binds to the promoters of meiosis-specific genes. Chromatin immunoprecipitation (ChIP) of endogenous E2f6 binding activity in WT R1 ES cells. ChIP was performed using mouse-anti-E2f6 antibody and analyzed by semi-quantitative PCR. Input and IgG are shown as controls. E2f6 binding activity on: A) the promoters of meiotic genes which are known to be derepressed in *E2f6-/-* MEFs, B) the promoters of other meiosis-specific genes, and C) the promoters of non-meiotic ES cell pluripotency and *ß-actin* genes. D) Real-time PCR quantification of binding as determined by the ratio of specific ChIP/IgG ChIP relative to input. All samples were tested in triplicate and expressed as means ± standard deviations.

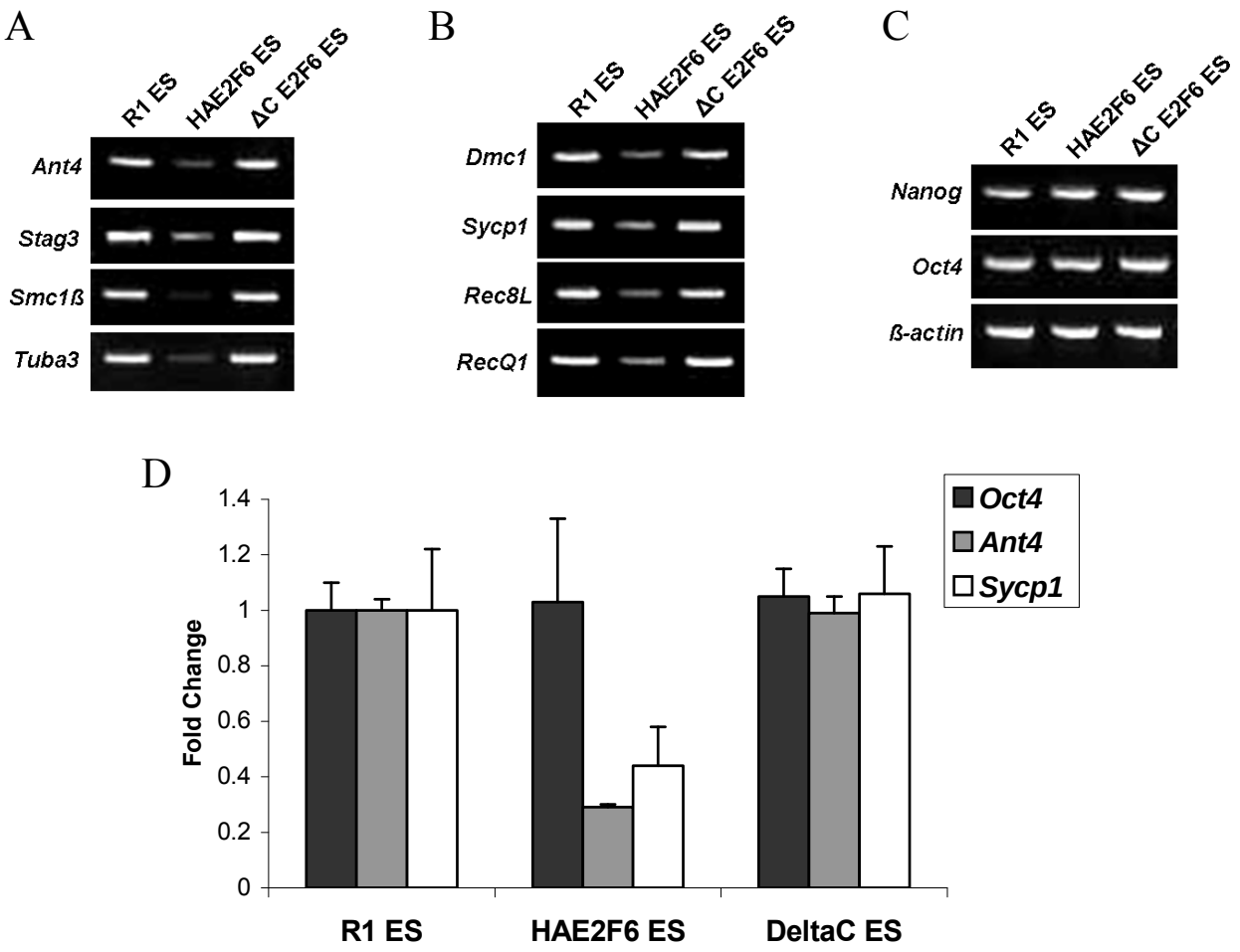


Figure 4-3. Many meiosis-specific genes are repressed by E2F6. Semi-quantitative RT-PCR analysis of meiosis-specific gene expression in R1, HA-E2F6, and HA-ΔC-E2F6 ES cells. A) Genes derepressed in *E2f6-/-* MEFs. B) Other meiosis-specific genes. C) Non-meiotic ES cell pluripotency and *ß-actin* genes. D) Quantitative Real-Time PCR analysis of a representative group of samples from A), B), and C). All samples were tested in triplicate and expressed as means ± standard deviations.

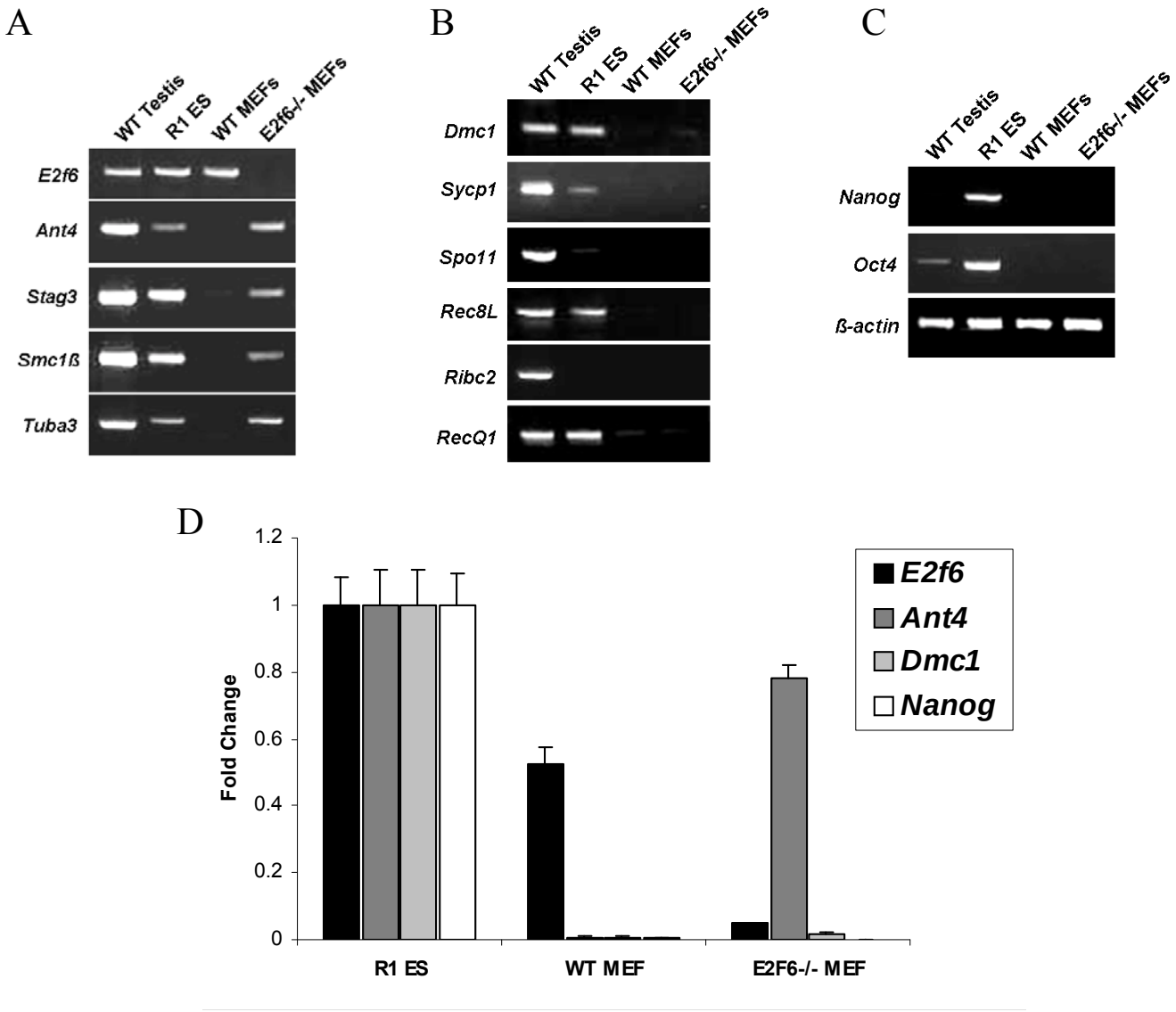


Figure 4-4. Limited meiosis-specific genes are derepressed in *E2f6*-/- MEFs. Semi-quantitative RT-PCR analysis of meiosis-specific gene expression in WT testis from 6-week old mice, R1 ES cells, WT MEFs, and *E2f6*-/- MEFs. A) *E2f6* and genes showing derepression in *E2f6*-/- MEFs. B) Other meiosis-specific genes. C) Non-meiotic ES cell pluripotency genes and *ß-actin* genes. D) Quantitative Real-Time PCR analysis of a representative group of samples from A), B), and C). All samples were tested in triplicate and expressed as means ± standard deviations.

CHAPTER 5
THE ROLE OF E2F6 DURING MALE MEIOSIS AND SPERMATOGENESIS

Motivation

From the data presented in Chapters 3 and 4, we can infer that E2F6 is involved in the repression of several meiosis-specific genes in somatic tissues. One of the remaining questions pertains to whether E2F6 also plays a role in regulating meiosis-specific gene expression in non-somatic tissues. During male germ cell development, there is a rapid transition in the expression of meiosis-specific genes from a repressed state in pre-meiotic spermatogonia, to a highly active state in meiotic spermatocytes, to a restored repressive state in post-meiotic round spermatids. Therefore, we have analyzed the expression pattern of E2F6 in mouse testis with the expectation of finding that, in agreement with E2F6's role as a transcriptional repressor, there would be high E2F6 expression in spermatogonia, followed by low expression in spermatocytes, and then high expression again in spermatids. Surprisingly, the data presented here contradicts our prediction. Such results have convinced us to explore the possibility that during meiosis, E2F6 may instead be serving as a transcriptional activator of meiosis-specific genes. Herein, we discuss what is currently known about meiosis-specific gene activation in male germ cells, as well as speculate how our new findings on E2F6 expression may fit into these already established paradigms.

Background

There are several ways by which meiosis-specific genes may become activated at the onset of meiosis. These include: 1) activation by testis-specific transcription factors, 2) activation by general transcription factors, 3) derepression by removal of repressing factors, and 4) epigenetic alterations in chromatin structure which then promote transcriptional activation. One example of a testis-specific transcription factor that may activate meiotic genes is A-Myb. The murine *A-myb* gene arises from alternatively spliced mRNA and is expressed predominantly

in the testis (Latham *et al.* 1996). *In situ* hybridization analysis of mouse testis shows that *A-myb* expression increases at post-natal day 10, when primary spermatocytes first appear. In the adult, *A-myb* mRNA is highly expressed in a sub-population of spermatogonia and in primary spermatocytes, but is not detectable in spermatids. This expression pattern suggests that *A-myb* may play a role in activating meiosis-specific gene expression. Additionally, in *A-myb-/-* male mice, germ cells enter meiotic prophase and arrest at pachytene, confirming that *A-myb* is required for the proper progression of meiosis (Toscani *et al.* 1997). Another myb family member, B-myb, is not testis-specific but is highly expressed in spermatogonia and early spermatocytes (Latham *et al*. 1996). B-myb expression then decreases by the late pachytene spermatocyte stage. Further studies comparing the differences between protein occupancy at meiosis-specific gene promoters incubated with either testis or somatic tissue nuclear extracts may reveal additional testis-specific activator proteins.

Meiosis-specific genes have also been shown to be activated by general transcription factors which are ubiquitously expressed. For example, the widely expressed Sp1 protein activates transcription of the meiosis-specific gene, *Cyclin A1* (Bartusel *et al.* 2005). *Cyclin A1* contains Sp1 binding sites in its promoter region and it is suggested that Sp1's ability to activate *Cyclin A1* is a result of Sp1 interactions with B-myb. Therefore, it is speculated that Sp1 and B-myb are binding together as an "activation complex" at these Sp1 sites. Mutation of the Sp1 binding sites prevents *Cyclin A1* transcription. Likewise, mutation of the C-terminal domain of B-myb inhibits *Cyclin A1* activation, suggesting that this domain is required for B-myb interaction with Sp1.

Sp1 has also been shown to serve as a transcriptional repressor, but depending on the factors with which it associates, or alternatively, depending upon how it is spliced, Sp1 may

switch between being an activator and being a repressor (Thomas *et al.* 2007). Additionally, Sp1 has been found to associate with E2F family members. When these two proteins associate, they too serve as an "activation complex." Such is the case at the murine thymidine kinase gene promoter (Rotheneder *et al.* 1999). Sp1 also interacts with the p107 pocket protein suggesting that Sp1 may change roles during the cell cycle (Datta *et al.* 1995). Other studies indicate that Sp1 can interact with components of the general transcription machinery including TBP-associated factors (TAFs). The TAFs associate with histone modifying enzymes and chromatin remodeling factors to establish open chromatin during spermatogenesis (Thomas *et al.* 2007).

An alternative way in which meiosis-specific gene transcription may be activated, is through the removal of repressive factors which reside at the promoters of these meiosis-specific genes. For example, polycomb proteins have been shown to repress genes which are involved in promoting differentiation. Interestingly, a recent paper using *Drosophila* as a model organism showed that these polycomb proteins are removed from gene promoters at the onset of meiosis (Chen *et al.* 2005). Five testis-specific TAFs (tTAFs), which associate with the TBP portion of the general transcription factor TFIID and have been shown to be required for meiotic cell cycle progression, are responsible for counteracting these polycomb proteins. Chen and colleagues found that these tTAF proteins are concentrated in a particular subcompartment of the nucleolus. They then become expressed at the initiation of spermatocyte differentiation and persist throughout the remainder of the primary spermatocyte stage, disappearing as cells enter the first meiotic division. This tTAF expression coincides with both the localization of PRC1 components to the nucleolus and the activation of tTAF-target genes. This is in agreement with the finding that polycomb proteins are strongly expressed in all cells of the testis until the onset of meiosis (Ringrose *et al.* 2006).

Further, mutation of tTAFs results in the inability of PRC1 components to localize to the nucleolus, suggesting that tTAFs may be involved in the recruitment of PRC1 components away from target promoters and towards the nucleolus (Chen *et al.* 2005). ChIP analysis revealed that these tTAFs also bound to target promoters just upstream of their transcription start sites, reduced polycomb binding, and promoted local accumulation of H3K4me3, a mark of Trithorax action. Trithorax proteins have been shown to activate transcription by opposing the repressive action of polycomb proteins (Ringrose *et al.* 2006). In tTAF mutant testis, PRC1 components continued to bind to tTAF-dependent target genes. These findings suggest that tTAFs activate germ-cell-specific gene expression by counteracting repression by polycomb proteins. Such transcriptional derepression by sequestration of polycomb proteins has also been observed during HIV-1 infection when the viral Nef protein recruits the PRC2 component, Eed, to the plasma membrane (Witte *et al.* 2004). From these findings, it appears that polycomb proteins may be blocking the expression of meiosis-specific genes. Upon entry into meiosis, polycomb repressive complexes are then disabled through tTAFs and these meiosis-specific genes become expressed. Whether the interactions between tTAFs and polycomb proteins are direct or indirect, remains to be elucidated.

Meiosis-specific gene transcription may also be activated during meiosis as a result of reorganization in chromatin structure towards a conformation that is more accessible to RNA polymerase II. This restructuring often involves a switch from somatic isoforms to unique germ-cell-specific isoforms of various histones and histone modifying enzymes. For example, during male gametogenesis several of the core histones (H2A, H2B, H3, and H4), along with linker histone H1, are partially or completely replaced by testis-specific histone isoforms such as H1t, TH2A, TH2B, and H3t (DeJong *et al.* 2006). These germ-cell-specific histones appear in

spermatogonia, and later in spermatids, but the majority are synthesized and incorporated into chromatin during meiosis. Methylation of somatic histone H1 by the H3K27/H1K26 HMT and polycomb protein, Ezh2, seems to be important for transcriptional repression. However, testis H1 variants have glycine or alanine residues in place of lysine which makes them unlikely to be targeted by Ezh2, and suggests that testis-specific H1 variants may represent a more active chromatin status than their somatic counterparts.

In addition to unique histone isoforms, there are also differences in histone methyltransferases (HMTs) between somatic cells and germ cells. Many of these HMTs have roles in transcriptional repression, but one in particular may aid in the activation of meiosis-specific genes. This meiosis-specific HMT, known as Meisetz (Prdm9), trimethylates histone H3K4. H3K4 trimethylation has been shown to be associated with active chromatin. Meisetz is specifically expressed in early meiotic germ cells in both testis and ovary (Matsui & Hayashi 2007). Interestingly, there is an E2F6 binding site in the proximal promoter region of this HMT (see Table 4-1).

It is suggested that Meisetz may trimethylate H3K4 around a set of genes essential for meiosis, thereby activating their expression. A role for Meisetz in activating meiosis-specific genes is observed in *Meisetz* null mice which have specific abnormalities in meiotic prophase and fail to activate meiosis-specific genes (Hayashi *et al.* 2005). Also, under normal conditions H3K4 trimethylation is upregulated in pachytene spermatocytes, further supporting the involvement of *Meisetz* in transcriptional activation. It should be noted here that although DNA demethylation may be an additional mechanism whereby meiosis-specific genes are derepressed at the onset of meiosis, it was not discussed here because to date, no DNA demethylases have been identified.

Results

After demonstrating that E2F6 indeed plays a role in the repression of meiosis-specific genes, we were curious to see if the expression pattern of E2F6 in the testis could help to explain our findings. As mentioned in the motivation portion of this chapter, we postulated that E2F6 expression would be high in spermatogonia and then suddenly drop off upon entry into meiosis, thereby releasing the repression of meiosis-specific genes. At the completion of meiosis, we predicted that E2F6 expression levels would peak again in order to silence the expression of meiosis-specific genes in post-meiotic cells such as round spermatids and sperm. We also predicted that the activating E2F, E2F1, would increase upon entry into meiosis in order to activate meiosis-specific gene expression.

Surprisingly, when we used real-time PCR to examine the expression patterns of both *E2f1* and *E2f6* in purified spermatogenic cells harvested at various stages of spermatogenesis (cells were a kind gift from JR McCarrey), we found expression patterns which were almost completely opposite to what we had predicted (Figure 5-1). According to these data, *E2f6* expression was highest in those cell types which were undergoing meiosis whereas *E2f1* expression was drastically reduced at this stage. Additionally, both E2f1 and E2f6 expression was low in the somatic population of cells in the testis (sertoli cells). Further confirmation of this expression pattern came from the analysis of E2f6 protein levels in testis from 6 week old mice using immunohistochemistry (Figure 5-2). Staining revealed that E2f6 expression was highest in meiotic cells. The classification of cells as meiotic was determined by cellular morphology and cellular location within the seminiferous tubules. Staining appeared specific when compared to staining using IgG as a negative control. Further immunohistochemistry will be needed to confirm these results.

Discussion

There has only been one published study which describes the expression pattern of E2F family members in the testis (El-Darwish *et al.* 2006). In this report it was found that the E2F1 protein is stage-specific and most abundant in leptotene to early pachytene spermatocytes of mice while strong staining of E2F1 in some cells close to the basal lamina of rat tubules suggest that it may also be expressed in undifferentiated spermatogonia. These findings are in contrast to the expression pattern that we observed for E2F1. Given the inherent nature of high backgrounds during immunohistochemical staining, we are tempted to trust our data more as we have quantitatively shown mRNA expression rather than subjectively characterizing expression. Further, this study showed discrepancies in E2F1 expression patterns between mouse and rat which seems highly unlikely considering the high homology of E2F1 between the two species. Additionally, this study did not examine E2F6 expression in testis. Therefore, for the remainder of this discussion, we will assume that the expression patterns found from our research are correct.

One possible explanation for this unexpected expression pattern is that E2F6 has dual roles as both an activator and a repressor during different stages of the cell cycle. These roles may be determined by differences in the complexes by which E2F6 is bound. For instance, if E2F6 represses transcription by recruiting either Dnmts or polycomb protein complexes to the promoter, then the actual repression activity is coming from the proteins which were recruited by E2F6 rather than from E2F6 itself. Therefore, it is possible that in meiotic cells, E2F6 maybe recruiting an activation complex to meiosis-specific genes rather than a repressive complex. Such an activation complex could consist of components which have already been shown to play a role in meiotic gene activation, such as Myb, Sp1, Meisetz, and tTAFs. Relationships between these proteins and E2Fs have already been suggested but need to be further elucidated.

An explanation for why such a switch in E2F-associated complexes could occur might be that the activation complex is suddenly able to compete with the repressive complex for binding at the onset of meiosis. For instance, if the total level of activation complex was upregulated upon entry into meiosis whereas the repressive complex was downregulated, then this shift in total protein could be responsible for the switch in E2F6-associated complexes. Further, a sudden change in cellular localization of activating and repressing components could also explain the switch in complexes. Interestingly, both a shift in expression patterns and cellular localization was observed for tTAFs and polycomb proteins at the onset of meiosis (Chen *et al.* 2005).

An additional function for the upregulation of E2F6 during meiosis could be that E2F6 is required to repress G1/S genes from becoming activated during this time. Likewise, E2F1 may be downregulated here as it is an activator of genes which promote S phase entry. Therefore, rather than thinking "narrow-mindedly" about only meiosis-specific gene populations, it is plausible that there are a multitude of E2F1- and E2F6-target genes which need to be regulated during meiosis. Indeed, experiments in which E2F1 was conditionally overexpressed in mouse testis resulted in testicular atrophy with seminiferous tubules containing only sertoli cells and clusters of undifferentiated spermatogonia (Agger *et al*. 2005). Likewise, *E2f6-/-* mice displayed a lack of mature spermatocytes (Storre *et al.* 2002). These findings suggest that during meiosis, it is important to keep E2F1 off and E2F6 on in order to ensure proper completion of meiosis by preventing the cell from attempting to initiate a cell cycle program in the middle of meiosis. It is also possible that the role of E2F6, to inhibit G1/S-regulated genes, may not be as important as the role of E2F1, pending that E2F1 is never able to activate these G1/S regulated genes in the

first place. This would explain why the *E2f6-/-* mouse is still fertile but not the mice which overexpress E2F1.

In summary, E2F6 may play a dual role as both an activator and a repressor during meiosis. E2F6-containing activation complexes may be bound to meiosis-specific genes while coexisting repressive E2F6 complexes might be bound to nonmeiotic gene promoters, thereby preventing the disruption of meiosis. Alternatively, E2F6 may only serve as a repressor, and through some unknown mechanism, meiosis-specific gene promoters are protected from the binding of such repressive E2F6 complexes. Future studies using ChIP assays to analyze the binding of E2F6 to both meiosis-specific genes and G1/S target genes during meiosis would clarify this hypothesis.

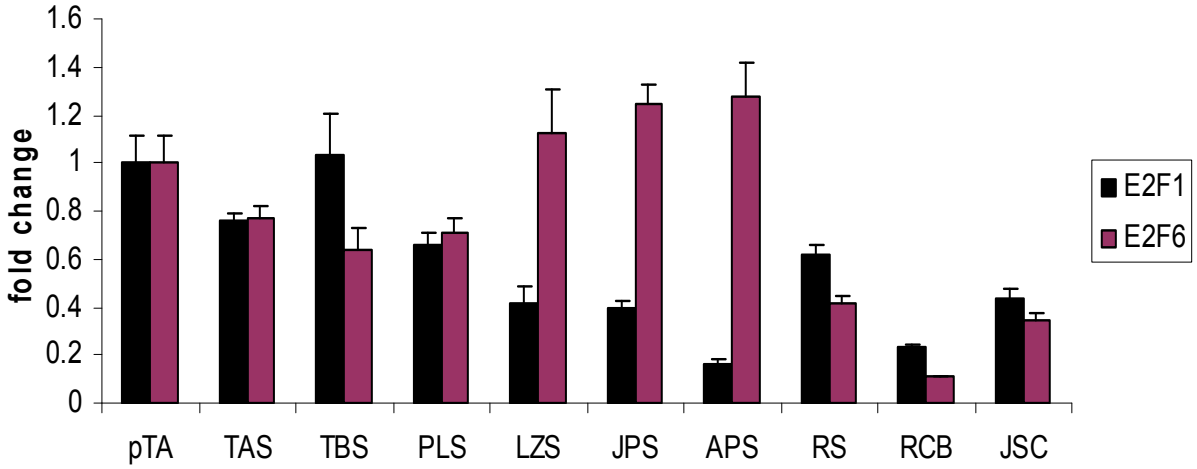


Figure 5-1. *E2f1* and *E2f6* mRNA transcript levels in the testis. Real-time PCR was used to measure *E2f1* (black) and *E2f6* (pink) transcript levels in the following purified spermatogenic cell types: pTA, primitive Type A spermatogonia; TAS, Type A spermatogonia; TBS, Type B spermatogonia; PLS, Pre-leptotene spermatocytes; LZS, Leptotene/Zygotene spermatocytes; JPS, Juvenille pachytene spermatocytes; APS, Adult pachytene spermatocytes; RS, Round Spermatids; RCB, Residual Cytoplasmic Bodies; JSC, Juvenile (D6) Sertoli Cells. The relative transcript levels are shown with the transcript level of pTA for *E2f1* and *E2f6* set as 1. All samples were tested in triplicate and expressed as means ± standard deviations.

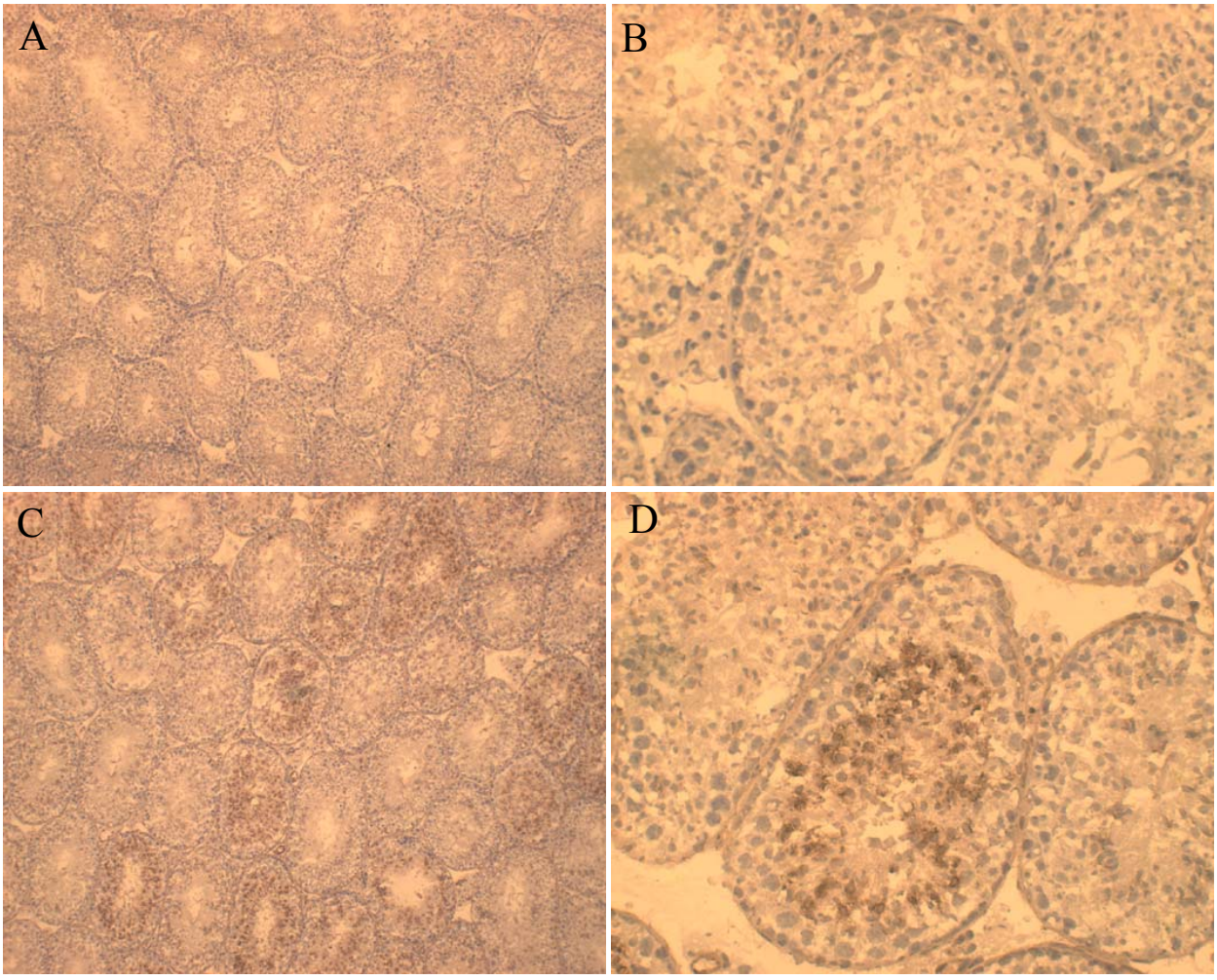


Figure 5-2. Immunohistochemical analysis of E2f6 expression in mouse testis. Formalin-fixed, paraffin-embedded sections of mouse testis from wild type 6-week old mice were incubated with (A) and B)) IgG antibody as a control or (C) and D)) E2f6 antibody. Staining was visualized using DAB (*brown*), and slides were counterstained with hematoxylin. A) and C) images were taken at a 10X magnification and B) and D) were at 40X.

CHAPTER 6
CONCLUSIONS AND SIGNIFICANCE

In summary, the research presented here has demonstrated a role for E2F6 in the repression of meiosis-specific genes in somatic cells. Although many questions remain, we have successfully achieved the original goal of this research: to study the transcriptional regulation of gene expression. We have elucidated the relationship between a particular transcription factor (E2F6) and a specific set of genes (meiosis-specific genes). When transcription factors controlling gene expression are disrupted, dire consequences may result. Therefore, the significance of these findings resides with the common observation that many genes are often aberrantly expressed in cancer cells.

Here we demonstrate that E2F6 depletion results in the expression of a few E2F6-target genes in inappropriate tissues. Such mischievous expression is a classic example of an event which can lead to oncogenic transformation (Simpson *et al.* 2005). Also, the E2F family of transcription factors is highly involved in the regulation of the cell cycle and one of its most common binding partners, retinoblastoma, is a pocket protein which has consistently been demonstrated to play a role in oncogenic transformation (Weinberg 1992). Because E2F6 does not bind to pocket proteins, it is commonly excluded when review papers discuss the role of E2Fs and Rb pocket proteins in cancer. Further, this is to be expected given that there was no indication of tumor formation in the *E2f6-/-* mouse (Storre *et al*. 2002). However, one thing that may be being overlooked is the potential for E2F6 to serve a negative feedback role in keeping the expression of genes which are targets of the activating E2Fs in check. Such an important role for E2F6 may simply be being masked by functional redundancy with E2F4. Although both of these proteins may be involved in negative feedback, their importance is somewhat diminished by the fact that they "share" the job. Therefore, we believe that future experiments which

examine the relationship between E2F4, E2F6, and cancer will provide critical insights regarding the control of E2F-target genes. Taken together, these findings are a small but important contribution to a broader understanding of the body's finite control of gene expression.

LIST OF REFERENCES

Adams MD 2005 Conserved sequences and the evolution of gene regulatory signals. *Current Opinion in Genetics and Development* **15** 625-633.

Agger K, Santoni-Rugiu ES, Holmberg C, Karlstrom O & Helin K 2005 Conditional E2F1 activation in transgenic mice causes testicular atrophy and dysplasia mimicking human CIS. *Oncogene* **24** 780-789.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K & Walter P 2002 Molecular Biology of the Cell, 4th edition. New York: Garland Science.

Alsheimer M & Benavente R 1996 Change of karyoskeleton during mammalian spermatogenesis: expression pattern of nuclear Lamin C2 and its regulation. *Experimental Cell Research* **228** 181-188.

Arango NA, Pearson EJ, Donahoe PK & Teixeira J 2004 Genomic structure and expression analysis of the mouse testis-specific ribbon protein (Trib) gene. *Gene* **343** 221-227.

Attwooll C, Oddi S, Cartwright P, Prosperini E, Agger K, Steensgaard P, Wagener C, Sardet C, Moroni MC & Helin K 2005 A novel repressive E2F6 complex containing the polycomb group protein, EPC1, that interacts with EZH2 in a proliferation-specific manner. *Journal of Biological Chemistry* **280** 1199-1208.

Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW & Echelard Y 1999 Production of goats by somatic cell nuclear transfer. *Nature Biotechnology* **17** 456-461.

Barath P, Poliakova D, Luciakova K & Nelson BD 2004 Identification of NF1 as a silencer protein of the human adenine nucleotide translocase-2 gene. *European Journal of Biochemistry* **271** 1781-1788.

Bartell JG, Davis T, Kremer EJ, Dewey MJ & Kistler WS 1996 Expression of the rat testis-specific Histone H1t gene in transgenic mice. *Journal of Biological Chemistry* **271** 4046-4054.

Bartusel T, Schubert S & Klempnauer K 2005 Regulation of the cyclin D1 and cyclin A1 promoters by B-Myb is mediated by Sp1 binding sites. *Gene* **351** 171-180.

Braidotti G & Barlow DP 1997 Identification of a male meiosis-specific gene, *Tcte2*, which is differentially spliced in species that form sterile hybrids with laboratory mice and deleted in *t* chromosomes showing meiotic drive. *Developmental Biology* **186** 85-99.

Brower JV, Rodic N, Seki T, Jorgensen M, Fliess N, Yachnis AT, McCarrey JR, Oh SP & Terada N. 2007 Evolutionarily conserved mammalian adenine nucleotide translocase 4 is essential for spermatogeneis. *Journal of Biological Chemistry* **282** 29658-29666.

Bruin A, Maiti B, Jakoi L, Timmers C, Buerki R & Leone G. 2003 Identification and characterization of E2F7, a novel mammalian E2F family member capable of blocking cellular proliferation. *Journal of Biological Chemistry* **278** 42041-42049.

Campbell KHS, Ritchie WA, McWhir J & Wilmut I 1996 Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380** 64-66.

Caretti G, Salsi V, Vecchi C, Imbriano C & Mantovani R. 2003 Dynamic recruitment of NF-Y and histone acetyltransferases on cell-cycle promoters. *Journal of Biological Chemistry* **278** 30435-30440.

Cartwright P, Müller H, Wagener C, Holm K & Helin K 1998 E2F-6: a novel member of the E2F family is an inhibitor of E2F-dependent transcription. *Oncogene* **17** 611-623.

Chen X, Hiller M, Sancak Y & Fuller MT 2005 Tissue-specific TAFs counteract polycomb to turn on terminal differentiation. *Science* **310** 869-872.

Chesne P, Adenot PG, Viglietta C, Baratte M, Boulanger L & Renard JP 2002 Cloned rabbits produced by nuclear transfer from adult somatic cells. *Nature Biotechnology* **20** 366-369.

Chevrollier A, Loiseau D, Chabi B, Renier G, Douay O, Malthiery Y & Stepien G 2005 ANT2 isoform required for cancer cell glycolysis. *Journal of Bioenergetics and Biomembranes* **37** 307-317.

Chung AB, Stepien G, Haraguchi Y, Li K & Wallace D 1992 Transcriptional control of nuclear genes for the mitochondrial muscle ADP/ATP translocator and the ATP Synthase β Subunit. *Journal of Biological Chemistry* **267** 21154-21161.

Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce dLn & Robl JM 1998 Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280** 1256-1258.

Cloud JE, Rogers C, Reza TL, Ziebold U, Stone JR, Picard MH, Caron AM, Bronson RT & Lees JA 2002 Mutant mouse models reveal the relative roles of E2F1 and E2F3 in vivo. *Molecular and Cellular Biology* **22** 2663-2672.

Costa Y, Speed R, Ollinger R, Alsheimer M, Semple CA, Gautier P, Maratou K, Novak I, Höög C, Benavente R & Cooke H 2005 Two novel proteins recruited by synaptonemal complex protein 1 (SYCP1) are at the centre of meiosis. *Journal of Cell Science* **118** 2755-2762.

Dahout-Gonzalez C, Nury H, Trezeguet V, Lauquin GJM, Pebay-Paeyroula E & Brandolin G 2006 Molecular, functional, and pathological aspects of the mitochondrial ADP/ATP carrier. *Physiology* **21** 242-249.

Datta PK, Raychaudhuri P & Bagchi S 1995 Association of p107 with Sp1: genetically separable regions of p107 are involved in regulation of E2F- and Sp1- dependent transcription. *Molecular and Cellular Biology* **15** 5444-5452.

Dejong J 2006 Basic mechanisms for the control of germ cell gene expression. *Gene* **366** 39-50.

DeGregori J & Johnson DG 2006 Distinct and overlapping roles for E2F family members in transcription, proliferation, and apoptosis. *Current Molecular Medicine* **6** 739-748.

DiCarlo AD, Travia G & Felici MD 2000 The meiotic specific synaptonemal complex protein SCP3 is expressed by female and male primordial germ cells of the mouse embryo. *International Journal of Developmental Biology* **44** 241-244.

Dimova DK & Dyson NJ 2005 The E2F transcriptional network: old acquaintances with new faces. *Oncogene* **24** 2810-2826.

Dolce V, Scarcia P, Iacopetta D & Palmieri F 2005 A fourth ADP/ATP carrier isoform in man: identification, bacterial expression, functional characterization, and tissue distribution. *FEBS Letters* **579** 633-637.

Don J & Wolgemuth DJ 1992 Identification and characterization of the regulated pattern of expression of a novel mouse gene, *meg1*, during the meiotic cell cycle. *Cell Growth and Differentiation* **3** 495-505.

Dorner A, Giessen S, Gaub R, Grosse Siestrup H, Schwimmbeck PL, Hetzer R, Poller W & Schultheiss HP 2006 An isoform shift in the cardiac adenine nucleotide translocase expression alters the kinetic properties of the carrier in dilated cardiomyopathy. *European Journal of Heart Failure* **8** 81-89.

Eberhart CG, Maines JZ & Wasserman SA 1996 Meiotic cell cycle requirement for a fly homologue of human Deleted in *Azoospermia*. *Nature* **381** 783-785.

Eddy EM 2002 Male germ cell gene expression. *Recent Progress in Hormone Research* **57** 103-128.

El-Darwish KS, Parvinen M & Toppari J 2006 Differential expression of members of the E2F family of transcription factors in rodent testes. *Reproductive Biology and Endocrinology* **4** 63-85.

Ellison JW, Salido EC & Shapiro LJ 1996 Genetic mapping of the adenine nucleotide translocase-2 gene (Ant2) to the mouse proximal X chromosome. *Genomics* **36** 369-371.

Field SJ, Fong-Ying T, Kuo F, Zubiaga AM, Kaelin WG, Livingston DM, Orkin SH & Greenberg ME 1996 E2F-1 functions in mice to promote apoptosis and suppress proliferation. *Cell* **85** 549-561.

Furukawa K, Inagaki H, Naruge T, Tabata S, Tomida T, Yamaguchi A, Yoshikuni M, Nagahami Y & Hotta Y 1994 cDNA cloning and functional characterization of a meiosis-specific protein (MNS1) with apparent nuclear association. *Chromosome Research* **2** 99-113.

Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Ponderato N, Duchi R & Lazzari G 2003 Pregnancy: a cloned horse born to its dam twin. *Nature* **424** 635.

Gaubatz S, Lindeman GJ, Ishida S, Jakoi L, Nevins JR, Livingston DM & Rempel RE 2000 E2F4 and E2F5 play an essential role in pocket protein-mediated G1 control. *Molecular Cell* **6** 729-735.

Gaubatz S, Wood JG & Livingston DM 1998 Unusual proliferation arrest and transcriptional control properties of a newly discovered E2F family member, E2F6. *Proceedings of the National Academy of Sciences* **95** 9190-9195.

Gebara MM & McCarrey JR 1992 Protein-DNA interactions associated with the onset of testis-specific expression of the mammalian Pgk-2 gene. *Molecular and Cellular Biology* **12** 1422-1431.

Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K & Daley GQ 2004 Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* **427** 148-154.

Gentleman JF & Mullin RC 1989 The distribution of the frequency of occurrence of nucleotide subsequences, based on their overlap capability. *Biometrics* **45** 35-52.

Giangrande PH, Zhu W, Schlisio S, Sun X, Mori S, Gaubatz S & Nevins JR 2004 A role for E2F6 in distinguishing G1/S- and G2/M-specific transcription. *Genes and Development* **18** 2941-2951.

Graham BH, Waymire KG, Cottrell B, Trounce IA, MacGregor GR & Wallace DC 1997 A mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in the heart/muscle isoform of the adenine nucleotide translocator. *Nature Genetics* **16** 226-234.

Gurdon JB 1962 Adult frogs derived from the nuclei of single somatic cells. *Developmental Biology* **4** 256-273.

Habu T, Takuyu T, West A, Nishimune Y & Morita T 1996 The mouse and human homologs of DMC1, the yeast meiosis-specific homologous recombination gene, have a common unique form of exon-skipped transcript in meiosis. *Nucleic Acids Research* **24** 470-477.

Hamer G, Gell K, Kouznetsova A, Novak I, Benavente R & Höög C 2006 Characterization of a novel meiosis-specific protein within the central element of the synaptonemal complex. *Journal of Cell Science* **119** 4025-4032.

Han SY, Xie W, Kim SH, Yue L & DeJong J 2004 A short core promoter drives expression of the ALF transcription factor in reproductive tissues of male and female mice. *Biology of Reproduction* **71** 933-941.

Hayashi K, Yoshida K & Matsui Y 2005 A histone H3 methyltransferase controls epigenetic events required for meiotic prophase. *Nature* **438** 374-378.

Hecht NB, Distel RJ, Yelick PC, Tanhauser SM, Driscoll CE, Goldberg E & Tung KSK 1988 Localization of a highly divergent mammalian testicular alpha tubulin that is not detectable in brain. *Molecular and Cellular Biology* **8** 966-1000.

Hirano M & DiMauro S 2001 ANT1, Twinkle, POLG, and TP: new genes open our eyes to opthalmoplegia. *Neurology* **57** 2163-2165.

Horvath GC, Kistler WS & Kistler MK 2004 RFX2 is a potential transcriptional regulatory factor for Histone H1t and other genes expressed during the meiotic phase of spermatogenesis. *Biology of Reproduction* **71** 1551-1559.

Humbert PO, Rogers C, Ganiastsas S, Landsberg RL, Trimarchi JM, Dandapani S, Brugnara C, Erdman S, Schrenzel M, Bronson RT & Lees JA 2000 E2F4 is essential for normal erythrocyte maturation and neonatal viability. *Molecular Cell* **6** 281-291.

Iannello RC, Young J, Sumarsono S, Tymms MJ, Dahl HM, Gould J, Hedger M & Kola I 1997 Regulation of Pdha-2 expression is mediated by proximal promoter sequences and CpG methylation. *Molecular and Cellular Biology* **17** 612-619.

Jang J & Lee C 2003 Mitochondrial adenine nucleotide translocator 3 is regulated by IL-4 and IFN-γ via STAT-dependent pathways. *Cellular Immunology* **226** 11-19.

Johnson DG, Schwarz JK, Cress WD & Nevins JR 1993 Expression of transcription factor E2F1 induces quiescent cells to enter S phase. Nature **365** 349-35.

Jordens EZ, Palmieri L, Huizing M, van den Heuvel LP, Sengers RC, Dorner A, Ruienbee, W, Trijbels FJ, Valsson J, Sigfusson G, Palmieri F & Smeitink JA 2002 Adenine nucleotide translocator 1 deficiency associated with Sengers syndrome. *Annals of Neurology* **52** 95-99.

Karolchik D, Baertsch R, Diekhans M, Furey TS, Hinrichs A, Lu YT, Roskin KM, Schwartz M, Sugnet CE, Thomas DJ, Weber RJ, Haussler D & Kent WJ 2003 The UCSC Genome Browser Database. *Nucleic Acids Research* **31** 51-54.

Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H & Tsunoda Y 1998 Eight calves cloned from somatic cells of a single adult. *Science* **282** 2095-2098.

Keeney S, Giroux CN & Kleckner N 1997 Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family. *Cell* **88** 375-384.

Kel AE, Kel-Margoulis OV, Farnham PJ, Bartley SM, Wingender E & Zhang MQ 2001 Computer-assisted identification of cell cycle-related genes: new targets for E2F transcription factors. *Journal of Molecular Biology* **309** 99-120.

Kim M, Li D, Cui Y, Mueller K, Chears WC & DeJong J 2006 Regulatory factor interactions and somatic silencing of the germ cell-specific ALF gene. *Journal of Biological Chemistry* **281** 34288-34298.

Kim MK, Jang G, Oh HJ, Yuda F, Kim HJ, Hwang WS, Hossein MS, Kim JJ, Shin NS, Kang SK & Lee BC 2007 Endangered wolves cloned from adult somatic cells. *Cloning and Stem Cells* **9** 130-137

Kim YH, Haidl G, Schaefer M, Egner U, Mandal A & Herr JC 2006 Compartmentalization of a unique ADP/ATP carrier protein SFEC (Sperm Flagellar Energy Carrier, AAC4) with glycolytic enzymes in the fibrous sheath of the human sperm flagellar principal piece. *Developmental Biology* **302** 463-476.

Kong L, Chang JT, Bild AH & Nevins JR 2007 Compensation and specificity of function within the E2F family. *Oncogene* **26** 321-327.

Lammers JHM, Offenberg HH, Van Aalderen M, Vink ACG, Dietrich AJJ & Heyting C 1994 The gene encoding a major component of the lateral elements of synaptonemal complexes of the rat is related to X-linked lymphocyte-regulated genes. *Molecular and Celullar Biology* **14** 1137-1146.

Latham KE, Litvin J, Orth JM, Patel B, Mettus R & Reddy EP 1996 Temporal patterns of A-myb and B-myb gene expression during testis development. *Oncogene* **13** 1161-1168.

La Thangue NB & Rigby PW 1987 An adenovirus E1A-like transcription factor regulated during the differentiation of murine embryonal carcinoma stem cells. *Cell* **49** 507-513.

Lee J, Iwai T, Yokota T, & Yamashita M 2003 Temporally and spatially selective loss of Rec8 protein from meiotic chromosomes during mammalian meiosis. *Journal of Cell Science* **116** 2781-2790.

Lele KM & Wolgemuth DJ 2004 Distinct regions of the mouse Cyclin A1 gene, Ccna1, confer male germ-cell-specific expression and enhancer function. *Biology of Reproduction* **71** 1340-1347.

Levy SE, Chen YS, Graham BH & Wallace DC 2000 Expression and sequence analysis of the mouse adenine nucleotide translocase 1 and 2 genes. *Gene* **254** 57-66.

Li FX, Zhu JW, Hogan CJ & DeGregori J 2003 Defective gene expression, S phase progression, and maturation during hematopoiesis in E2F1/E2F2 mutant mice. *Molecular and Cellular Biology* **23** 3607-3622.

Li K, Hodge JA & Wallace DC 1990 OXBOX, a positive transcriptional element of the heart-skeletal muscle ADP/ATP Translocator Gene. *Journal of Biological Chemistry* **265** 20585-20588.

Li R, Hodny Z, Luciakova K, Barath P & Nelson BD 1996 Sp1 activates and inhibits transcription from separate elements in the proximal promoter of the human adenine nucleotide translocase 2 (ANT2) gene. *Journal of Biological Chemistry* **271** 18925-18930.

Lin H, Jurk M, Gulick T & Cooper GM 1999 Identification of COUP-TF as a transcriptional repressor of the c-mos proto-oncogene. *Journal of Biological Chemistry* **274** 36796-36800.

Lindeman GJ, Dagnino L, Gaubatz S, Xu Y, Bronson RT, Warren HB & Livingston DM 1998 A specific, nonproliferative role for E2F-5 in choroid plexus function revealed by gene targeting. *Genes & Development* **12** 1092-1098.

Looman C, Mark C, Abrink M & L Hellman 2003 MZF6D, a novel KRAB zinc-finger gene expressed exclusively in meiotic male germ cells. *DNA and Cell Biology* **22** 489-496.

Loots GG & Ovcharenko I 2004 rVISTA 2.0: evolutionary analysis of transcription factor binding sites. *Nucleic Acids Research* **32** W217-W221.

Lukas J, Petersen BO, Holm K, Bartek J & Helin K 1996 Deregulated expression of E2F family members induces S-phase entry and overcomes p16INK4A-mediated growth suppression. *Molecular and Cellular Biology* **16** 1047-1057.

Lunardi J, Hurko O, Engel WK & Attardi G 1992 The multiple ADP/ATP translocase genes are differentially expressed during human muscle cell development. *Journal of Biological Chemistry* **267** 15267-15270.

Macaluso M, Cinti C, Russo G, Russo A & Giordano A 2003 pRb2/p130-E2F4/5-HDAC1-SUV39H1-DNMT1 multimolecular complexes mediate the transcription of estrogen receptor-α in breast cancer. *Oncogene* **22** 3511-3517.

MacLean II JA & Wilkinson MF 2005 Gene regulation in spermatogenesis. *Current Topics in Developmental Biology* **71** 131-197.

Maiti B, Li J, Bruin A, Gordon F, Timmers C, Opavsky R, Patil K, Tuttle J, Cleghorn W & Leone G 2005 Cloning and characterization of mouse E2F8, a novel mammalian E2F family member capable of blocking cellular proliferation. *Journal of Biological Chemistry* **280** 18211-18220.

Marikawa Y, Fujita T & Alarcon V 2005 Heterogeneous DNA methylation status of the regulatory element of the mouse Oct4 gene in adult somatic cell population. *Cloning and Stem Cells* **7** 8-16.

Matsui Y & Hayashi K 2007 Epigenetic regulation for the induction of meiosis. *Cellular and Molecular Life Sciences* **64** 257-262.

Meuwissen RLJ, Offenberg HH, Dietrich AJJ, Riesewijk A, Van Lersel M & Heyting C 1992 A coiled-coil related protein specific for synapsed regions of meiotic prophase chromosomes *EMBO Journal* **11** 5091-5100.

Muller H, Moroni MC, Vigo E, Petersen BO, Bartek J & Helin K 1997 Induction of S-phase entry by E2F transcription factors depends on their nuclear localization. *Molecular and Cellular Biology* **17** 5508-5520.

Murga M, Fernandez-Capetillo O, Field S, Moreno B, Borlado LR, Fujiwara Y, Balomenos D, Vicario A, Carrera AC, Orkin SH, Greenberg ME & Zubiaga AM 2001 Mutation of E2F2 in mice causes enhanced T lymphocyte proliferation leading to the development of autoimmunity. *Immunity* **15** 959-970.

Oberley MJ, Inman DR, & Farnham PJ 2003 E2F6 negatively regulates BRCA1 in human cancer cells without methylation of histone H3 on lysine 9. *Journal of Biological Chemistry* **278** 42466-42476.

Offenberg HH, Schalk JAC, Meuwissen RLJ, Aalderen MV, Kester HA, Dietrich AJJ & Heyting C 1998 SCP2: a major protein component of the axial elements of synaptonemal complexes of the rat. *Nucleic Acids Research* **26** 2572-2579.

Ogawa H, Ishiguro K, Gaubatz S, Livingston DM, & Nakatani Y 2002 A complex with chromatin modifiers that occupies E2F- and Myc-responsive genes in G_0 cells. *Science* **296** 1132-1136.

Ollinger R, Alsheimer M & Benavente R 2005 Mammalian protein SCP1 forms synaptonemal complex-like structures in the absence of meiotic chromosomes. *Molecular Biology of the Cell* **16** 212-217.

Pennisi E 2001 The human genome. *Science* **291** 1177-1180.

Petukhova GV, Pezza RJ, Vanevski F, Ploquin M, Masson J & Camerini-Otero RD 2005 The Hop2 and Mnd1 proteins act in concert with Rad51 and Dmc1 in meiotic recombination. *Nature Structural & Molecular Biology* **12** 449-453.

Pezza RJ, Petukhova GV, Ghirlando R & Camerini-Otero RD 2006 Molecular activities of meiosis-specific proteins Hop2, Mnd1, and the Hop2-Mnd1 complex. *Journal of Biological Chemistry* **281** 18426-18434.

Pezzi N, Prieto I, Kremer L, Perez-Jurado LA, Valero C, Del-Mazo J, Martinez C & Barbero JL 2000 *STAG3*, a novel gene encoding a protein involved in meiotic chromosome pairing and location of *STAG3*-related genes flanking the Williams-Beuren syndrome deletion. *FASEB Journal* **14** 581-592.

Pohlers M, Truss M, Frede U, Scholz A, Strehle M, Kuban R, Hoffmann B, Morkel M, Birchmeier C & Hagemeier C 2005 A role for E2F6 in the restriction of male-germ-cell-specific gene expression. *Current Biology* **15** 1051-1057.

Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A & Campbell KHS 2000 Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* **407** 86-90.

Pousette A, Leijonhufvud P, Arver S, Kvist U, Pelttari J & Höög C 1997 Presence of synaptonemal complex protein 1 transversal filament-like protein in human primary spermatocytes. *Human Reproduction* **12** 2414-2417.

Prieto I, Suja JA, Pezzi N, Kremer L, Martinez C, Rufas JS & Barbero JL 2001 Mammalian STAG3 is a cohesion specific to sister chromatid arms in meiosis 1. *Nature Cell Biology* **3** 761-766.

Qin XQ, Livingston DM, Kaelin WG & Adams PD 1994 Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proceedings of the National Academy of Sciences* 91 10918-10922.

Ravnik SE & Wolgemuth DJ 1999 Regulation of meiosis during mammalian spermatogenesis: the A-type Cyclins and their associated Cyclin-dependent kinases are differentially expressed in the germ-cell lineage. *Developmental Biology* **207** 408-418.

Rehling P, Brander K & Pfanner N 2004 Mitochondrial import and the twin pore translocase. *Nature Reviews Molecular Cell Biology* **5** 519-530.

Rempel RE, Saenz-Robles MT, Storms R, Morham S, Ishida S, Engel A, Jakoi L, Melhem MF, Pipas JM, Smith C & Nevins JR 2000 Loss of E2F4 activity leads to abnormal development of multiple cellular lineages. *Molecular Cell* **6** 293-306.

Revenkova E, Eupe M, Heyting C, Gross B, & Jessberger R 2001 Novel meiosis-specific isoform of mammalian SMC1. *Molecular and Cellular Biology* **21** 6984-6998.

Revenkova E & Jessberger R 2005 Keeping sister chromatids together: cohesins in meiosis. *Reproduction* **130** 783-790.

Ringrose L 2006 Polycomb, trithorax and the decision to differentiate. *BioEssays* **28** 330-334.

Robinson MO, McCarrey JR & Simon MI 1989 Transcriptional regulatory regions of testis-specific PGK-2 defined in transgenic mice. *Proceedings of the National Academy of Sciences* **86** 8437-8441.

Rodic N, Oka M, Hamazaki T, Murawski MR, Jorgensen M, Maatouk DM, Resnick JL, Li E & Terada N 2005 DNA methylation is required for silencing of *Ant4*, an adenine nucleotide translocase selectively expressed in mouse embryonic stem cells and germ cells. *Stem Cells* **23** 1314-1323.

Rotheneder H, Geymayer S & Haidweger E 1999 Transcription factors of the Sp1 family: interaction with E2F and regulation of the murine thymidine kinase promoter. *Journal of Molecular* Biology **293** 1005-1015.

Sagata N 1997 What does Mos do in oocytes and somatic cells? *Bioessays* **19** 13-21.

Sage J, Martin L, Meuwissen R, Heyting C, Cuzin F & Rassoulzadegan M 1999 Temporal and spatial control of the Sycp1 gene transcription in the mouse meiosis: regulatory elements active in the male are not sufficient for expression in the female gonad. *Mechanisms of Development* **80** 29-39.

Schwartz JK, Devoto SH, Smith EJ, Chellappan SP, Jakoi L, & Nevins JR. 1993 Interactions of the p107 and Rb proteins with E2F during the cell proliferation response. *EMBO Journal* **3** 1013-1020.

Shannon M, Richardson L, Christian A, Handel MA & Thelen MP 1999 Differential gene expression of mammalian *SPO11/TOP6A* homologs during meiosis. *FEBS Letters* **462** 329-334.

Shin T, Kraemer D, Pryor J, Liu L, Rugila J, Howe L, Buck S, Murphy K, Lyons L & Westhusin M 2002 Cell biology: a cat cloned by nuclear transplantation. *Nature* **415** 859.

Simpson AJG, Caballero OL, Jungbluth A, Chen Y & Old LJ 2005 Cancer/testis antigens, gametogenesis and cancer. *Nature Reviews Cancer* **5** 615-625.

Singh AM, Hamazaki T, Hankowski KE & Terada N 2007 A heterogeneous expression pattern for Nanog in embryonic stem cells. *Stem Cells* **25** 2534-2542.

Singh P, Wong SH & Hong W 1994 Overexpression of E2F-1 in rat embryo fibroblasts leads to neoplastic transformation. *EMBO Journal* **13** 3329-3338.

Smale ST 1997 Transcription initiation from TATA-less promoters within eukaryotic protein-coding genes. *Biochimica et Biophysica Acta* **1351** 73-88.

Stepien G, Torroni A, Chung AB, Hodge JA & Wallace DC 1992 Differential expression of adenine nucleotide translocator isoforms in mammalian tissues and during muscle cell differentiation. *Journal of Biological Chemistry* **267** 14592-14597.

Storre J, Elsässer H, Fuchs M, Ullmann D, Livingston DM &Gaubatz S 2002 Homeotic transformations of the axial skeleton that accompany a targeted deletion of *E2f6*. *EMBO Reports* **3** 695-700.

Storre J, Schäfer A, Reichert N, Barbero JL, Hauser S, Eilers M, & Gaubatz S 2005 Silencing of meiotic genes *SMC1β* and *STAG3* in Somatic Cells by E2F6. *Journal of Biological Chemistry* **280** 41380-41386.

Sweeney C, Murphy M, Kubelka M, Ravnik SE, Hawkins CF, Wolgemuth DJ & Carrington M 1996 A distinct cyclin A is expressed in germ cells in the mouse. *Development* **122** 53-64.

Thomas K, Wu J, Sung DY, Thompson W, Powell M, McCarrey J, Gibbs R & Walker W 2007 SP1 transcription factors in male germ cell development and differentiation. *Molecular and Cellular Endocrinology* **270** 1-7.

Toscani A, Mettus RV, Coupland R, Simpkins H, Litvin J, Orth J, Hatton KS & Reddy EP 1997 Arrest of spermatogenesis and defective breast development in mice lacking A-myb. *Nature* **386** 713-717.

Trimarchi JM, Fairchild B, Wen J & Lees JA 2001 The E2F6 transcription factor is a component of the mammalian Bmi1-containing polycomb complex. *Proceedings of the National Academy of Sciences* **98** 1519-1524.

Trimarchi JM & Lees JA 2002 Sibling rivalry in the E2F family. *Nature Reviews Molecular Cell Biology* **3** 11-20.

Tronche F, Ringeisen F, Blumenfeld M, Yaniv M & Pontoglio M 1997 Analysis of the distribution of binding sites for a tissue-specific transcription factor in the vertebrate genome. *Journal of Molecular Biology* **266** 231-245.

Tureci O, Sahin U, Zwick C, Koslowski M, Seitz G & Pfreundschuh M 1998 Identification of a meiosis-specific protein as a member of the class of cancer/testis antigens. *Proceedings of the National Academy of Sciences* **95** 5211-5216.

Turner JM 2007 Meiotic sex chromosome inactivation. *Development* **134** 1823-1831.

Van-Heldin J 2003. Regulatory sequence analysis tools. *Nucleic Acids Research* **31** 3593-3596.

Vire E, Brenner C, Deplu R, Blanchon L, Fraga M, Didelot C, Morey L, Van Eynde A, Bernard D, Vanderwinden J, Bollen M, Esteller M, Di Croce L, Launoit Y & Fuks F 2006 The polycomb group protein EZH2 directly controls DNA methylation. *Nature* **439** 871-874.

Wakayama T, Perry AC, Zuccotti M, Johnson KR & Yanagimachi R 1998 Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394** 369-374.

Walker JE 1992 The mitochondrial transporter family. *Current Opinion in Structural Biology* **2** 519-526.

Wang PJ, Page DC & McCarrey JR 2005 Differential expression of sex-linked and autosomal germ-cell-specific genes during spermatogenesis in the mouse. *Human Molecular Genetics* **14** 2911-2918.

Wang WS, Seki M, Yamoaka T, Seki T, Tada S, Katada T, Fujimoto H & Enomoto T 1998 Cloning of two isoforms of mouse DNA helicase Q1/RecQL cDNA; alpha form is expressed ubiquitously and beta form specifically in the testis. *Biochemical and Biophysical Research Communications* **1443** 198-202.

Wasserman WW & Fickett JW 1998 Identification of regulatory regions which confer muscle-specific gene expression. *Journal of Molecular Biology* **278** 167-181.

Watanabe D, Yamada K, Nishina Y, Tajima Y, Koshimizu U, Nagata A & Nishimune Y 1994 Molecular cloning of a novel Ca^{2+}-binding protein (Calmegin) specifically expressed during male meiotic germ cell development. *Journal of Biological Chemistry* **269** 7744-7749.

Watson JD & Crick FH 1953 Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature* **171** 737-738.

Weil PA, Luse DS, Segall J & Roeder RG 1979 Selective and accurate initiation of transcription at the Ad2 major late promoter in a soluble system dependent on purified RNA polymerase II and DNA. *Cell* **18** 469-484.

Weinberg RA 1992 The retinoblastoma gene and gene product. *Cancer Surveys* **12** 43-57.

Wilkerson DC, Wolfe SA & Grimes SR 2002 H1t/GC-Box and H1t/TE1 element are essential for promoter activity of the testis-specific Histone H1t gene. *Biology of Reproduction* **67** 1157-1164.

Wilmut I, Schnieke AE, McWhir J, Kind AJ & Campbell KHS 1997 Viable offspring derived from fetal and adult mammalian cells. *Nature* **385** 810-813.

Witte V, Laffert B, Rosorius O, Lischka P, Blume K, Galler G, Stilper A, Willbold D, D'Aloja P, Sixt M, Kolanus J, Ott M, Kolanus W, Schuler G & Baur AS 2004 HIV-1 Nef mimics an integrin receptor signal that recruits the polycomb group protein Eed to the plasma membrane. *Molecular Cell* **13** 179-190.

Wu L, Timmers C, Maiti B, Saavedra HI, Sang L, Chong GT, Nuckolls F, Giagrande P, Wright FA, Field SJ, Greenberg ME, Orkin S, Nevins JR, Robinson ML & Leone G 2001 The E2F1-3 transcription factors are essential for cellular proliferation. *Nature* **414** 457-462.

Xu EY, Moore FL & Reijo-Pera RA 2001 A gene family required for human germ cell development evolved from an ancient meiotic gene conserved in metazoans. *Proceedings of the National Academy of Sciences* **98** 7414-7419.

Xu W & Cooper GM 1995 Identification of a candidate c-mos repressor that restricts transcription of germ cell-specific genes. *Molecular and Cellular Biology* **15** 5369-5375.

Yamasaki L, Jacks T, Bronson R, Goillot E, Harlow E & Dyson N 1996 Tumor induction and tissue atrophy in mice lacking E2F-1. *Cell* **85** 537-548.

Yee AS, Reichel R, Kovesdi I & Nevins JR 1987 Promoter interaction of the E1A-inducible factor E2F and its potential role in the formation of a multi-component complex. *EMBO Journal* **6** 2061-2068.

Yuan L, Brundell E & Hoog C 1996 Expression of the meiosis-specific Synaptonemal Complex Protein 1 in a heterologous system results in the formation of large protein structures. *Experimental Cell Research* **229** 272-275.

BIOGRAPHICAL SKETCH

Sarah Michelle Kehoe was born in Washington D.C. in 1980, to Robert and Patricia Williams. She was raised in Fairfax, Virginia and graduated from W.T. Woodson High School in June 1998. Sarah then attended Virginia Polytechnic Institute and State University, where she graduated both "in honors" and summa cum laude with her Bachelor of Science degree in animal and poultry sciences in May 2002. During college, in the laboratory of Dr. Paul Siegel, Sarah conducted poultry genetics research that culminated in the publication of her first-author paper in the August 2002 issue of *Poultry Science*. Sarah was also awarded two summer internships at the National Institutes of Health where she worked at the National Human Genome Research Institute in the laboratory of Dr. Lawrence Brody. In 2003, Sarah enrolled in the Interdisciplinary Program in Biomedical Sciences (IDP) at the University of Florida, College of Medicine and received a Grinter Fellowship upon admission. She began her doctoral study under the guidance of Dr. Naohiro Terada, in the Department of Molecular and Cell Biology. Upon completion of her Ph.D. in December 2007, Sarah plans to pursue a career dedicated to the study of cancer.