Mammalian Atypical E2Fs Link Endocycle Control to Cancer

DISSERTATION

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By

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Abstract

The endocycle is a developmentally programmed variant cell cycle consisting of successive S (DNA synthesis) and G (Gap) phases without an intervening M phase or cytokinesis. As a consequence of the regulated “decoupling” of DNA replication and mitosis, which are two central processes of the traditional cell division program, endocycling cells acquire highly polyploid genomes after having undergone multiple rounds of whole genome reduplication. Although essential for metazoan development, relatively little is known about the regulation of endocycle or its physiologic role in higher vertebrates such as the mammal.

A substantial body of work in the model organism Drosophila melanogaster has demonstrated an important function for dE2Fs in the control of endocycle. Genetic studies showed that both endocycle initiation and progression is severely disrupted by altering the expression of the fly E2F activator (dE2F1) or repressor (dE2F2). In mammals, the E2F family is comprised of nine structurally related proteins, encoded by eight distinct genes, that can be classified into transcriptional activators (E2f1, E2f2, E2f3a and E2f3b) or repressors (E2f4, E2f5, E2f6, E2f7 and E2f8). The repressor subclass may then be further divided into canonical (E2f4, E2f5 and E2f6) or atypical E2fs (E2f7 and E2f8). Until this study, there has been sparse knowledge regarding the role of E2fs in
the control of mammalian endocycle, or what the physiologic consequences might be of perturbed endocycles in tissues composed of polyploid cells such as the liver.

Now using a combination of novel and established lineage-specific cre mice we identified that two opposing arms of the E2F program, one driven by canonical transcription activation (E2F1, E2F2 and E2F3) and the other by atypical repression (E2F7 and E2F8), converge on the regulation endocycles in vivo. Ablation of canonical activators in the two endocycling tissues of mammals, trophoblast giant cells (TGCs) in the placenta and hepatocytes in the liver, augmented genome ploidy, whereas ablation of atypical repressors diminished ploidy. Surprisingly, the severe reduction of ploidy caused by E2f7/E2f8 deficiency had no apparent adverse impact on placental and hepatic physiology. However, the sole inactivation of E2f8 in hepatocytes, which biases toward a diploid state, resulted in the development of spontaneous hepatocellular carcinoma (HCC). Loss of E2f8 also potentiated the development of carcinogen-induced HCC, suggesting that E2F8 functions to suppress tumor initiation and impede tumor progression. Taken together, the results presented within provide the first in vivo evidence for a direct role of E2Fs in regulating non-traditional cell cycles in mammals and genetically link endocycle control with cancer.
Dedication

This thesis is dedicated to my family, with immense love and gratitude.

This is also dedicated to Dr. Allan Yates, whose kindness changed the course of my life in more ways than one.
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A tremendous thank you to my dissertation committee members and my advisor, for your contributions to my scientific footprints. I would be gracious to have your continued support as I now forge ahead, creating new paths. Also a sincere thank you to IBGP and MSTP directors and administrators for your guidance and support.

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**Fields of Study**

**Major Field:** Integrated Biomedical Science Program
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Chapter 1

General Introduction

More than two decades of experimentation in vitro link E2F activity to cell cycle regulation. The E2Fs are a large family of transcription factors containing one or more highly conserved DNA binding domains (DBDs) that bind target promoters and regulate their cell cycle dependent expression. Global gene expression profiling (ChIP-on-chip) and promoter occupancy (ChIP-sequencing) arrays have confirmed many genes critical for proper cell cycle progression as bona fide E2F targets, thus establishing a direct role for E2Fs in governing cell proliferation.

1.1 Role of E2Fs in Cell Cycle Regulation: The Classic Paradigm

_E2F function is controlled by cyclin-dependent kinases (Cdks) and Rb_

It was discovered approximately two decades ago that infection by DNA tumor viruses caused the excessive proliferation of host cells that was associated with the binding of specific cellular factors to viral gene promoters (Kovesdi et al., 1986; Yee et al., 1987). These factors were eventually deemed E2-associated factors, or E2Fs. Since discovery of the founding E2F family member, _E2f1_ (Helin et al., 1992; Kaelin et al., 1992), eight additional genes in mammals have subsequently been identified (Figure
1.1). The key finding that over-expression of E2F activators could trigger quiescent cells to enter G\textsubscript{1} phase of the cell division cycle (Johnson et al., 1993), independently of serum stimulation, spurred the initial identification of numerous E2F-regulated genes involved in cell proliferation. Thereafter, studies that sought to determine how E2Fs contributed to cell proliferation elucidated an intricate molecular signaling network converging on the post-translational modifications of retinoblastoma (RB) and RB-related proteins by cyclin-Cdk complexes, culminating in the control of E2F transcriptional activity (Figure 1.2). The result was then sequential waves of E2F-dependent transcriptional activation and repression that would guarantee the timely movement of cells through all four phases of the cell cycle: G\textsubscript{1}, S, G\textsubscript{2} and M (Figure 1.3).

**Complexities of E2F function: redundancy, antagonism and feedback**

Several obstacles have precluded a thorough analysis of the role of mammalian E2Fs \textit{in vivo}, with the first being a high degree of functional redundancy among activators and repressors that was initially revealed by the systematic analyses of mice harboring single and compound \textit{E2f} mutations (DeGregori and Johnson, 2006; Li et al., 2008). Recent gene knockin strategies in mice corroborated this by showing that expression of the highly related \textit{E2f3b} isoform, or the more distantly related \textit{E2f1} gene, from the endogenous \textit{E2f3a} locus suppressed majority of the early postnatal developmental phenotypes associated with \textit{E2f3a} inactivation (Tsai et al., 2008).

The second obstacle impeding a complete understanding of E2F functions \textit{in vivo} is the apparent functional antagonism between E2F-mediated transcriptional activation
and repression in the control of normal cell proliferation. Results from early studies in *Drosophila melanogaster*, which contain just one E2F activator and one repressor (dE2F1 and dE2F2, respectively), appeared to validate the relatively straightforward model of E2F function as an activity that either promoted or inhibited progression through the cell cycle. Loss of function mutations in *dE2f1* led to reduced expression of classic E2F-regulated genes, DNA synthesis and larval survival (Duronio et al., 1995; Frolov et al., 2001). Surprisingly, loss of *dE2f2* reversed the block on cell proliferation in *dE2f1* mutant larvae and significantly delayed lethality until mid- or late pupal stages (Frolov et al., 2001). These results demonstrated that the net effect of E2Fs on cell proliferation appeared to depend on the balanced activation and repression of a core set of target genes. However, subsequent microarray analysis of gene expression in S2 cells lacking *dE2f1*, *dE2f2* or *dDP*, which abrogates total E2F activity in flies, revealed surprisingly little overlap in genes regulated by *dE2f1* and *dE2f2* (Dimova et al., 2003). Consistent with this gene expression data, a recent genetic mosaic screen found that the cell cycle defects induced by loss of dE2F1 could be rescued without functionally inactivating *dE2f2* and abrogating dE2F2-mediated repression (Ambrus et al., 2007). Therefore, in addition to regulating a common set of target genes, dE2F1 and dE2F2 also contribute independently to the normal proliferative potential of a cell.

A third reason why studying E2F function remains a challenge is the ability of E2F family members to regulate the expression of one another. This is presumably so to ensure a balanced level of E2F activators and repressors that mediate transition from one phase of the cell cycle to the next. A clear example of such feedback regulation was
revealed by the recent molecular analysis of mouse embryos doubly deficient for E2f7 and E2f8. Consistent with chromatin immunoprecipitation (ChIP) experiments that demonstrated loading of E2F7 and E2F8 to the E2f1 promoter (Di Stefano et al., 2003; Christensen et al., 2005), ablation of E2f7/E2f8 in the mouse embryo resulted in derepression of E2f1, ectopic levels of E2F1 protein and E2F1-dependent apoptosis. On the other hand, the presence of multiple putative E2F binding sites in the promoter regions of E2f7 and E2f8 indicates that their expression may be activated by E2F1. Thus E2F1 function, peaking at the G1/S transition, robustly transactivates the expression of E2f7 and E2f8 in addition to genes essential for S phase progression. As a result, the level of E2F7 and E2F8 proteins accumulate and reach maximal levels in late S phase or near S/G2, which coincides with the timely cessation of DNA replication that in part is due to direct repression of E2f1 (Moon and Dyson, 2008).

**Rb–E2F developmental mouse models support the canonical pathway**

Developmental studies using Rb and Rb–E2F compound mutant mice appear to reinforce the role of E2Fs in cell proliferation and apoptosis that was originally identified in vitro. Homozygous inactivation of Rb in mice leads to embryonic lethality that is accompanied by massive ectopic proliferation and apoptosis in multiple tissues (Jacks et al., 1992; Clarke et al., 1992). The proliferative and apoptotic phenotypes of Rb deficiency were partially rescued by the genetic inactivation of E2f1 (Tsai et al., 1998) or E2f3a, which also extended the viability of Rb knockout embryos to late stages of embryogenesis (Chong et al., 2009). Interestingly, the loss of E2f3b, which encodes an
E2F3 isoform that is constitutively expressed during the cell cycle and in G₀ (Leone et al., 2000), also alleviated a subset of Rb mutant phenotypes. This latter result demonstrates significant functional redundancy for E2F3a and E2F3b during development (Danielian et al., 2008; Tsai et al., 2008). The observation that ablation of repressor E2F4 or E2F5 failed to rescue Rb mutant phenotypes in the mouse embryo is consistent with their role as binding partners of Rb that mediate transcriptional repression (Wenzel et al., 2007). Taken together, the results of these knockout mouse models are generally consistent with a canonical role of E2F1-3 activators as downstream effectors of Rb that positively control cell proliferation and apoptosis.

**E2F target genes and the mechanisms of their regulation**

The specificity of E2F-dependent activation and repression is conferred by the ability of E2F family members to physically interact with a diverse group of transcriptional co-factors. Indeed, pull-down assays in vitro have shown that E2Fs associate with different chromatin remodeling enzymes such as histone deacetylases or histone methyltransferases to modulate the transcriptional landscape (Blais and Dynlacht, 2007). Given that changes in gene expression may reflect both direct and indirect functions of transcription factors, several laboratories have extensively utilized ChIP-based technologies to identify direct E2F-regulated targets (Takahashi et al., 2000; Wells et al., 2000; Jin et al., 2006; Xu et al., 2007). These approaches have identified many novel E2F-responsive genes, consistent with numerous reports that have exposed context-dependent functions in differentiation and development for different E2Fs in vivo.
(McClellan and Slack, 2007). Work from the Farnham laboratory has shown that E2F1 could be recruited to a number of gene promoters that do not contain consensus E2F binding sites (Bieda et al., 2006). The promiscuity in E2F-chromatin associations could be attributed to either the co-recruitment of E2Fs by other transcription factors such as NF-Kβ (Lim et al., 2007), MYC (Leung et al., 2008) and CEBPα (Iakova et al., 2003), or by components of the transcriptional machinery. Although intriguing, these studies are hampered by the fact that most have been performed in cell culture systems. Whether the newly identified transcriptional networks involving E2Fs reflect their true physiological roles in vivo remains to be determined.
Figure 1.1. The Evolution of E2F Gene Families.

(A) The mammalian E2F gene family. Note the classification of E2F3b as an activator because recent developmental studies (Tsai et al., 2008; Danielian et al., 2008) support
redundant functions for the two E2F3 isoforms \textit{in vivo}. Interestingly, the E2F7 family member also has two isoforms, E2F7a and E2F7b. However, unlike the \textit{E2F3} isoforms that are transcribed from distinct promoters, \textit{E2F7a} and \textit{E2F7b} isoforms are produced by alternative splicing of the primary transcript (Di Stefano et al., 2003), resulting in excision of exon 12 from E2F7a. While the E2F7a protein is detectable in all phases of the cell cycle, the level of E2F7b remains minimal in mitosis and early G1 but begins to elevate as cells enter the S phase. Whether these two E2F7 proteins are functionally distinct has not yet been established. DBD, DNA binding domain; LZ, leucine zipper; MB, marked box; RB, pocket binding; NLS, nuclear localization sequence; CycA, cyclin A binding; NES, nuclear export sequence; DP, dimerization partner protein.

\textbf{(B)} Proteins related to the E2Fs are conserved across various species of plants and animals. The efforts to elucidate E2F function in mammals have traditionally been complicated by the existence of a large number of family members with extensive functional overlap. Rb-E2F studies in the elegant model organism \textit{Drosophila melanogaster}, which has one activator (dE2F1) and repressor E2F (dE2F2), have yielded many essential insights into the role of E2F proteins in cell proliferation, differentiation and apoptosis (van den Heuvel and Dyson, 2008). In \textit{Caenorhabditis elegans}, three E2F-like proteins have been described comprising of EFL-1, EFL-2 and EFL-3. It is well established that EFL-1, DPL-1 (DP) and LIN-35 (Rb) are all components of the SynMuv
B pathway that inhibits ectopic vulval development (Ceol and Horvits, 2001; Myers and Greenwald, 2005; Winn et al., 2011). Consistent with emerging cell cycle-independent functions for E2Fs in higher eukaryotes, efl-1 and dpl-1 have recently been shown to be essential for fertility in C. elegans by regulating the expression of developmental rather than cell cycle-related programs independent of lin-35. EFL-1 is most closely related to the mammalian repressor E2F4, but the function of EFL-2 is unknown since its knockdown through RNAi did not result in notable phenotypes in the worm. EFL-3 has recently been identified as the homolog of mammalian E2F7/E2F8 with a conserved role in apoptosis regulation during worm development (Winn et al., 2011; Li et al., 2008). In the model plant system Arabidopsis thaliana, two activators and 4 repressors have been identified (Mariconti et al., 2002). Like mammalian E2F activators, E2Fa/E2Fb interacts with DP (DP) to bind DNA and activate E2F-responsive genes. Studies have shown that E2Fa expression is at a maximum late in G1, acts to induce S phase and endoreplication and that E2Fb plays a similar role (Rossignol et al., 2002; Sozzani et al., 2006). Interestingly, E2Fc has a truncated C-terminal transactivation domain and has been shown in vitro to down-regulate the expression of E2F targets such as CDC6 (Cdc6) (Kosugi et al., 2002; del Pozo et al., 2002). E2Fa-c are all capable of binding the plant RBR (Rb) protein. In contrast, the “atypical” E2Fd-f are considered the plant homologues of mammalian E2F7/E2F8 and participate in the regulation of plant cell growth and endoreplication (Lammens et al., 2009).
Figure 1.2. The Canonical Cdk-Rb-E2F pathway. Uncontrolled cell growth is an invariable characteristic of human cancer (Hanahan and Weinberg, 2000). The proliferation of cancer cells is sustained in the absence of growth factors and is insensitive to growth-inhibitory signals (Hanahan and Weinberg, 2000). Members of the human epidermal growth factor receptor (HER) family of receptor tyrosine kinases (RTK) are deregulated in many types of human cancer (Yarden et al., 2001). Overexpression or mutation of these receptors leads to the constitutive activation of downstream signaling pathways (Hynes et al., 2005).

Upon mitogenic stimulation, intracellular levels of D-type cyclins (D1, D2 and

(continued)
Figure 1.2: continued

D3) increase, resulting first in the formation and subsequent nuclear localization of cyclin D-CDK4 and cyclin D-CDK6 complexes that phosphorylate RB early in G₁ (Harbour et al., 1999). After this initial wave of Rb phosphorylation, levels of mitogen-independent E-type cyclins (E1 and E2) accumulate and associate with CDK2 to further inactivate RB late in G₁ in preparation for progression into S phase (Harbour et al., 1999). Genetic alterations of G₁/S regulators have been extensively documented in human cancers (Malumbres and Barbacid, 2005, 2009). The G₁ cyclins, in particular D1 and E1, and their catalytic partners CDK4 and CDK6 are overexpressed, mutated or their genomic loci amplified in a wide spectrum of tumors. The activity of CDKs is regulated by two families of CDK-inhibitors (CKIs), with the first family comprised of the INK4 proteins (INK4A, INK4B, INK4C and INK4D). The second family consists of the CIP and KIP proteins (p21, p27 and p57). Decreased expression of these negative G₁/S regulators, including RB, in human cancer has been attributed to a number of genetic as well as epigenetic changes (Harbour and Dean, 2000; Malumbres and Barbacid, 2001, 2009). In short, deregulation of factors that control the G₁/S transition, which ultimately relies on the engagement of E2F activity in a textbook view of cell cycle regulation, appears to be a universal theme in the process of neoplastic transformation.
Figure 1.3. Expression and Activity of Mammalian E2F Family Members in the Mitotic Cell Cycle.

(A) In quiescent (G₀) cells, the ubiquitously expressed E2F4/E2F5 associate with pocket proteins and other co-repressors to maintain repression of genes that promote entry (G₁) into the cell cycle. Upon mitogenic stimulation, the sequential phosphorylation of RB mediated by cyclin D-CDK and cyclin E-CDK complexes results in the loss of RB (continued)
Figure 1.3: continued

function and the release of E2F4/E2F5 from RB-E2F repressor complexes. The inactivation of RB also leads to the maximal accumulation of newly synthesized free E2F1-3 late in G1. Together, these events initiate a transcriptional program that drives cells into S phase. This G1/S-specific transcriptome is then attenuated upon completion of S phase in G2 by the action of repressors E2F6-8 thought to function independently of RB and RB-related proteins. In addition to the direct repression of genes involved in DNA replication, the unique E2F repressor arm consisting of E2F7/E2F8, which lack the canonical structural features of the repressors E2F4/E2F5, can also mediate the repression of E2F activators such as E2f1. Heterodimeric or homodimeric E2F7-E2F8 complexes directly bind the E2f1 promoter and repress its expression throughout S phase to restrain excessive activator E2F-dependent transcription. Without this “brake”, E2F1 proteins would persist inappropriately and continue activation of genes encoding components of the transcriptional machinery, resulting in ectopic DNA replication.

(B) Modifications of nuclear histone proteins unfold or compact chromatin to facilitate or impede, respectively, the loading and activation of the transcriptional machinery. E2F family members contribute to the organization of both euchromatin and heterochromatin. During cell proliferation when RB is hyperphosphorylated, E2F activators recruit the basal transcription factor TFIID and other co-activators such as histone acetyltransferases (HATs) p300/CBP, GCN5 and Tip60 to specific gene promoters (Taubert et al., 2004; (continued)
Nicolas et al., 2003; Lang et al., 2001), leading to an open chromatin configuration and transcription initiation. In quiescent or differentiated cells, pocket protein-bound E2F4/E2F5 have been found to associate with a variety of co-repressors such as histone methyltransferases (HMTs) and deacetylases (HDACs), DNA methyltransferase (DNMT1) and C-terminal binding protein (CtBP), leading to chromatin compaction and transcription inhibition (Tyagi et al., 2007; Ogawa et al., 2002; Rayman et al., 2002; Ferreira et al., 1998). The more recently identified repressors E2F6-8 mediate repression of E2F-responsive genes independent of binding to pocket proteins. While E2F6, through interaction with the Polycomb complex (Trimarchi et al., 2001), still requires dimerization with DP to function in transcription repression, E2F7/E2F8 are distinctly unique in that they form homo (E2F7-E2F7, E2F8-E2F8) or heterodimers (E2F7-E2F8) to repress transcription of cell cycle-related genes (Li et al., 2008; Di Stefano et al., 2003; Christensen et al., 2005). The co-repressors that associate with E2F7/E2F8 in vivo are currently unknown and remain to be identified.
1.2 The Endocycle: Variant Cell Cycle with a Purpose

The endocycle is characterized by alternating DNA synthesis (S) and Gap (G) phases without an intervening mitosis or cytokinesis. Endocycling cells are destined to achieve genome polyploidy, some as high as 1000n, with multiple rounds of whole genome reduplication (Lee et al., 2009). The increase in genome ploidy is usually accompanied by a corresponding increase in nuclear size (Varmuza et al., 1988). The conserved utilization of endocycle in organisms ranging from the unicellular protist to eukaryotes as complex as metazoans suggests an evolutionary significance to this process. Indeed, many biological advantages of endocycles have been proposed, including an augmented response to strenuous cellular metabolic demands (Lee et al., 2009); buffering against environmental, metabolic-derived or replication-associated DNA damage (Otto and Whitton, 2000); and dampened sensitivity to apoptotic stimuli such as telomeric attrition and increased p53-p21 checkpoint function (Lilly and Spradling, 1996; Lazzerini Denchi et al., 2006; Mehrotra et al., 2008).

**Mechanisms of endocycle regulation: lessons from Drosophila**

*Drosophila melanogaster* has been used as a model organism to extensively study the endocycle because many tissues in the fly embryo and larva switch from the mitotic cell cycle to endocycle upon receiving developmental cues. For example, somatic follicular epithelial cells in the fly ovary switch to endocycle mid-oogenesis and together with the germline-derived polyploid nurse cells are essential for the development of oocytes and embryogenesis (Lilly and Spradling, 1996). The Notch signaling pathway
has been identified as a key regulator that promotes the mitosis-to-endocycle (M-E) transition (Deng et al., 2001), while Hedgehog signaling has been shown to antagonize the M-E transition (Zhang and Kalderon, 2000). An important intracellular event induced by Notch signaling is the increased expression of fizzy-related protein (fzr, or Cdh1 in mammals), which activates the anaphase promoting complex/cyclosome (APC/C) leading to the ubiquitin-mediated destruction of mitotic cyclins. Other key events in response to Notch activation include downregulation of both the S phase cyclin-dependent kinase (Cdk) inhibitor, dacapo (dap$^{p21/p27}$), to permit DNA replication and the M phase Cdk1 activator, string$^{cd25}$, to inhibit mitosis initiation. These events are viewed as necessary for endocycle onset.

Subsequent to the M-E transition, progression through endocycles is thought to be regulated by a subset of the same factors that control progression through mitotic cell cycles (Diaz-Trivino et al., 2005; Park and Asano, 2008). It has been firmly established that cyclin E expression (and thus cyclin E-Cdk2 activity) must occur in a 'pulsatile' manner, peaking at the G/S transition, to create conditions permissive for endocycle (Follette et al., 1998; Weiss et al., 1998). Drosophila embryos mutant for cyclin E are unable to endoreplicate, but specialized embryonic cells are also blocked from endoreplication if cyclin E is constitutively expressed. To prevent re-replication and limit whole genome reduplication to once per endocycle, cyclin E-Cdk2 induces dissociation of pre-replicative complexes (pre-RCs) that is composed of Orc1, Cdc6 and Cdt1. Additionally, cyclin E-Cdk2 function also prevents inappropriate APC/C activation during endo S phase by mediating inhibitory phosphorylation of fzr/Cdh1 (Arias and
Walter, 2007; Narbonne-Reveau et al., 2008; Zielke et al., 2008). Minimal function of APC/C during S phase ensures high levels of Geminin, a metazoan-specific protein that binds Cdt1 proteins to preclude their re-incorporation into pre-RCs and thereby inhibiting the re-licensing of origins of DNA replication (Shcherbata et al., 2004). Finally, through a kinase independent mechanism, cyclin E has been shown to stimulate the association of minichromosome maintenance (Mcm) proteins, which form the essential replicative DNA helicase, with chromosomes to facilitate DNA synthesis.

Given the key functions of cyclin E that have just been described, it is not surprising that cellular factors governing its expression would also be critically involved in endocycle control. The E2F family in Drosophila, unlike in mammals, is comprised of two genes encoding one activator, dE2F1, and one repressor, dE2F2 (Figure 1.1B). There is also only one DP gene encoding the essential dimerization partner of dE2F1 and dE2F2. The simplicity of this system has yielded significant insight into the role of dE2Fs in endocycle onset and regulation. Numerous target genes activated by dE2F1 are required for G/S transition and DNA synthesis. Among these targets is cyclin E and indeed, genetic or functional ablation of dE2F1 results in drastic reduction in DNA synthesis and endocycle progression in the Drosophila embryo (Duronio and O’Farrell, 1995; Duronio et al., 1995, 1998; Royzman et al., 1997). Conversely, expression of a mutant form of dE2F1 unable to undergo ubiquitin-mediated proteolysis results in constitutive cyclin E expression and consequently, a block in endoreplication in larval salivary glands (Shibutani et al., 2008). Loss of dE2F2, a component of the multi-protein Myb-Muv or dREAM repressor complex, also led to continuous cyclin E expression and
severe developmental defects in tissues that endocycle (Weng et al., 2003). Finally, an important point to note in these loss and gain of function studies is that DNA replication defects were not strictly limited to endocycling tissues in dE2F mutant flies. Thus, these studies also lend support to the notion that mitotic cell cycles and endocycles may share a subset of regulatory factors (e.g. E2Fs) to ensure faithful genome duplication.

**Polyploidy in mammalian cells**

Polyploidy of whole organisms is frequently found in plants, invertebrates and lower vertebrates but is rare in higher vertebrates, where polyploidy of a limited subset of cells is achieved in most developmental contexts by the endocycle or in pathological contexts as part of an adaptive response to stress and disease (Storchova and Pellman, 2004). Polyploidy achieved through developmentally programmed endocycles in mammals is restricted to trophoblast giant cells (TGCs) of the placenta and hepatocytes of the liver. The megakaryocyte also achieves polyploidy but by means of undergoing ‘abortive’ mitosis or endomitosis (Ullah et al., 2009). Polyploidy achieved under conditions of stress, seen for example in cardiomyocytes and vascular smooth muscles in a hypertensive setting (Vliegen et al., 1995; Hixon et al., 2000) and in hepatocytes that have sustained oxidative damage (Gorla et al., 2001; Gupta 2000), has been hypothesized to occur through cell fusion.

The developmentally programmed polyploidization of hepatocytes begins at the time of weaning in rodents, when animals are approximately three weeks of age (Barbason et al., 1974; Dallman et al., 1974). This process continues throughout normal
growth and aging such that up to 90% of hepatocytes in adult mice may be polyploid (Gerhard et al., 1971; Saeter et al., 1988). Similarly, hepatocyte polyploidization in humans begins at a young age, and polyploid hepatocytes accumulate in the liver at a moderate rate until approximately fifty years of age, after which the rate of polyploidization increases significantly (Kudryavtasev et al., 1993). Hepatocytes achieve polyploidy through endocycles, which generate mononuclear cells with high DNA content. Interestingly, hepatocytes also undergo a process called acytokinetic mitosis, in which S and M phases are actually completed but cytosolic partitioning does not occur (Ullah et al., 2009), resulting in the appearance of bi- or multinucleated cells. A mature differentiated hepatocyte could, therefore, have multiple diploid nuclei or a single polyploid nucleus. The molecular mechanisms leading to the inhibition of cytokinesis are thought to be dependent on modifications of processes involved in actin cytoskeleton assembly and microtubule reorganization (Margall-Ducos et al., 2007), but remain in general much less well understood than mechanisms that control endocycles.

**The Cdk-Rb-E2F pathway appears to be involved in regulating mammalian endocycles**

In mammals, a thorough understanding of the mechanisms regulating endocycles has been difficult to achieve due to functional complexity of the cyclin, Cdk and E2F/DP protein families (Malumbres and Barbacid, 2009; Chen et al., 2009a). However, loss of function studies in mice have supported a general involvement of the molecular pathways from *Drosophila* (e.g. Notch and cyclin E) in the control of mammalian endocycles. For instance, the closely related mammalian Cyclin E1 and E2 isoforms are required for TGC
endocycles but in contrast to their fly counterpart, are not strictly required for embryonic mitotic cell cycles (Geng et al., 2003; Parisi et al., 2003). Mice mutant for Cdk2, the kinase partner of Cyclin E, are infertile but viable, suggesting compensation by other Cdk5s (Berthet et al., 2003; Ortega et al., 2003). Consistent with this, pharmacologic inhibition of Cdk1 function in Cdk2 knockout trophoblast stem (TS) cells, unlike in wildtype TS cells, potently suppressed their ability to endoreplicate and differentiate into TGCs (Ullah et al., 2008). An important substrate of cyclin-Cdk complexes is the retinoblastoma (Rb) tumor suppressor protein, which in turn regulates activity of the E2F transcription factors. All mammalian E2Fs, with the exception of E2F7 and E2F8, require association with one of two DP proteins to bind DNA. Interestingly, the functions of Rb and DP1 are both required for proper extraembryonic development (Wu et al. 2003; Kohn et al., 2003), but only DP1 ablation, which was accompanied by a compensatory increase in the level of DP2, resulted in severe disruption of endocycle in TGCs. Mice mutant for Cdh1, which encodes an activating partner of the APC/C E3 ligase that targets mitotic cyclins for degradation, exhibit a block in TGC differentiation (Garcia-Higuera et al., 2008), which also may involve Notch mediated downregulation of the transcription factor Mash-2 (Nakayama et al., 1997). Finally in the murine liver, altered hepatocyte ploidy levels were observed in response to Rb inactivation (Mayhew et al., 2005), E2fl overexpression (Conner et al., 2003) and p27Kip1 accumulation as a consequence of Skp2 ablation (Minamishima et al., 2002). Transgenic mice with hepatocyte-specific overexpression of p21, which blocks S phase entry, have significantly decreased liver
mass due to reduced number of mature hepatocytes (Wu et al., 1996), consistent with a defect in hepatocyte differentiation and polyploidization.
Chapter 2

Role of Mammalian E2F Activators in Endocycle Regulation

2.1 Introduction

Years of experimentation using cell culture systems have demonstrated that the activator subclass of mammalian E2Fs, consisting of E2F1, E2F2 and E2F3 (E2F1-3), promotes G1/S transition of the mitotic cell cycle, and that this function is potently inhibited by their physical association with Rb. In the textbook view of cell cycle progression, the levels and activities of E2F1, E2F2 and E2F3 peak at the G1/S transition, a time when Rb is maximally inactivated through hyperphosphorylation by cyclin D-Cdk4/6 and cyclin E-Cdk2 complexes, to increase the transcription of genes involved in DNA replication. More recent studies in mice, however, have begun to challenge this view by demonstrating that E2F1-3 function is not required for mammalian cell proliferation, and that the so-called ‘activators’ may also have context-dependent repressor function (Chen et al., 2009b; Chong et al., 2009; Wenzel et al., 2011). Nonetheless, E2F-mediated transcriptional activation has been shown to contribute to endocycle control in flies (Lee et al., 2009), but until now its role in mammalian endocycles has not been explicitly evaluated. In this chapter, we utilized an inducible loss of function approach to investigate the role of E2F1-3 in hepatocyte endocycles.
2.2 Results

Genome reduplication in mammalian endocycling cells is temporally regulated during development with major ploidy increases in hepatocytes following weaning at approximately three weeks of age (Barbason et al., 1974). The expression of E2f activators was examined in livers by quantitative real-time polymerase chain reaction (qRT-PCR). This standard assay showed relatively high levels of E2f1 and E2f2 expression in the liver during fetal development (E13.5) and a precipitous decrease following birth (post-natal day 0, P0), whereas expression of E2f3 (E2f3a and E2f3b) only begun to decrease or was stable following weaning of pups (Figure 2.1A). The expression of E2f activators in livers, once diminished, remained attenuated as mice reached old age. Thus, it would appear that total E2F1-3 levels are at a minimum during the time when hepatocytes are actively endocycling.

We next utilized targeted gene inactivation strategies in mice to rigorously evaluate the role of E2F activators in endocycling hepatocytes. To avoid functional compensation among E2F activators (Tsai et al., 2008; Danielian et al., 2008), we obtained murine livers deficient for the entire E2F activator subclass (E2f1-3) by crossing polyIC-inducible Mx-cre mice (Kuhn et al., 1995) with mice carrying conditional alleles of E2f3 (Mx-cre;E2f1”;E2f2”;E2f3”f/f; Mx-123tko). As soon as two weeks after polyIC induced expression of cre, Mx-123tko hepatocytes appeared larger than age-matched controls (Figure 2.1B), and liver weights as well as hepatocyte size increased proportionately (Figure 2.1C). Flow cytometry demonstrated ectopic polyploidization in hepatocytes of Mx-123tko livers (Figure 2.1D), with significant increases in the fraction
of 8n and 16n nuclei populations. This marked increase in genome ploidy was consistent with the enlarged appearance of Mx-123tko hepatocytes (Figure 2.1B). Interestingly, loss of E2f1 alone or in combination with E2f2 resulted in progressive increases in hepatocyte ploidy (Figure 2.1D), suggesting a synergistic role for E2f activators in the control of hepatocyte endocycles. To control for the interferon response, we also analyzed the livers from wild type mice injected with polyIC and could not detect an effect on ploidy (data not shown). Thus our analysis of genome ploidy levels in E2f1-3 deficient hepatocytes demonstrates that mammalian E2F activators have a physiologic role in suppressing endocycles.

2.3 Discussion

Role of E2F activators in TGC endocycles

Parallell studies evaluating the consequences of E2f1-3 ablation in TGCs of the placenta were carried out (M. Ouseph, Figure 2.2). Expression analysis with NanoString technology showed that E2f1 and E2f2 mRNA levels were relatively low in wild type TGCs throughout placental development (from E6.5-E17.5), whereas E2f3 mRNA levels were high prior to E8.5 and following E13.5. Thus similar to the pattern of E2f1-3 expression in the liver, levels of E2f activators appeared to be at a minimum during the time when TGCs are actively endocycling (Cross, 2005). Moreover, consistent with ploidy results obtained in the liver, loss of all E2F activators produced a marked increase in the genome ploidy of TGCs at E9.5 as determined by the Feulgen technique. Analysis
of $E2f1^{-/-};E2f2^{-/-};E2f3^{-/-}$ TGCs at later stages of development was precluded due to embryo lethality at E10.5 (Chong et al., 2009).

**How might E2F activators suppress TGC and hepatocyte endocycles?**

In this chapter, we reveal a novel role of $E2f1-3$ in suppressing mammalian endocycles. TGCs and hepatocytes became aberrantly polyploid with the ablation of $E2f1-3$, suggesting that loss of E2F activators either prematurely precipitated the onset of M-E transition or accelerated the kinetics of G/S oscillations (thereby resulting in more rounds of genome reduplication in the same span of time) subsequent to endocyte onset. It has been reported that a number of E2F1-DP target genes promote G2/M transition and M phase progression (Maqbool et al., 2010; Ishida et al., 2011). Therefore we presumed that a high level of E2F activators would prevent endocycles and conversely, low level of E2F activators would permit endocycles.

Loss of function studies in *Drosophila* (Duronio et al., 1995; Royzman et al., 1997) and now as we show in mice suggest a critical role for E2F mediated transcriptional activation in regulating endocycles. But surprisingly, the mechanisms involved in flies and mice appear, at least on the surface, to be reversed. In flies, loss of $dE2f1$ diminishes the capacity of cells to enter S phase and as a result both the mitotic cycle and endocycles cease, whereas $dE2f1$ overexpression accelerates endocycles and cells gain further ploidy. Presumably, overproduced dE2F1 protein can still be targeted for degradation late in S phase by the E3 ubiquitin ligase Cul4$^{cdt2}$ and thus, total dE2F1 level still oscillates during the cell cycle (Shibutani et al., 2008). Consistent with a
requirement for dE2F1 oscillatory expression that in turn drives periodic transcription of its key target cyclin E, salivary gland cells that express a constitutively stable form of dE2F1 protein arrest in S phase and fail to endocycle (Shibutani et al., 2008). In contrast to flies, even the absence of all three E2F activators in the mouse embryo is insufficient to preclude cells from entering S phase or completing mitotic cell division (Chong et al., 2009). Furthermore, their absence in endocycling TGCs and hepatocytes as we have shown leads to pronounced ploidy gains. Conversely, overexpression of E2F1 in hepatocytes leads to a block in their age-associated polyploidization (Conner et al., 2003). This raises an intriguing paradox of why altering the levels of activating E2Fs in flies and mice yield opposite consequences. One possible explanation for this difference may be the number of E2F family members that each species encodes, contributing to the availability of compensatory mechanisms that operate in flies and mice.

In preparation for the M-E transition, it may be that one or multiple upstream signaling pathways would serve to downregulate E2f1-3 expression in order to cease further mitotic cell division. Consistent with this model, we found that levels of E2f1-3 expression were lowest at a time when TGCs and hepatocytes are actively endocycling. The result that expression of E2f3a and E2f3b differed only mildly between embryonic and post-natal livers and that an increase in TGC-specific E2f3 expression corresponded with a period during which TGCs have stopped endoreplicating may be evidence to suggest that E2F3 has non-endocycle related function in these tissues. Based on these results, we suggest that attenuating E2F1-3 function may be a key event in determining the timing of endocycle onset in mammals. In support of this hypothesis, work from the
Calvi laboratory (Maqbool et al., 2010) recently demonstrated that the expressions of \(dE2f1\) and its obligate binding partner \(dDp\) were significantly decreased in fly endocycling tissues (larval salivary gland and fat body). As a result, many E2F1-DP target genes were also expressed at significantly lower levels in these tissues than in mitotic cycling tissues (larval brain and imaginal disc). The precise upstream signaling events that determine how and when E2F1-3 activity becomes downregulated are currently unknown, but may involve components of the Notch pathway.

A second intriguing point that is raised by our study is how DNA replication during endo S phase might occur in the absence of E2F1-3. It has already been shown that n-Myc was sufficient to stimulate DNA replication in retinal progenitor cells lacking \(E2f1-3\) (Chen et al., 2009b), eliciting the possibility that n-Myc might function in a similar capacity in \(E2f1-3\) deficient TGCs and hepatocytes. Thus, the previous observation that hepatocyte-specific overexpression of \(Myc\) resulted in precocious polyploidy (Conner et al., 2001) might be explained by the potential overlapping actions of Myc and E2F1-3 at the G\(_{1}\)/S transition. Finally, the fact that \(Dp1\) ablation in the mouse, which resulted in lethality by E8.5-9.5, suppressed TGC endocycles (Kohn et al., 2003) might seem at first contradictory to our result that loss of \(E2f1-3\) prompted ectopic endocycles in the same cells. However, because trophoblast stem cell differentiation appeared to be severely compromised by the loss of DP1, it was difficult to ascertain how TGC endocycles might have been truly impacted. Furthermore, the DNA binding activity of E2F4-6 was also presumably abrogated by the loss of \(Dp1\), which would have
confounded the analysis of the relative contribution of activator versus canonical repressors to endocycle control.

In summary, we showed by utilizing targeted gene ablation strategies that mammalian E2F activators suppress endocycles in vivo, and presented data to suggest that decreased expression of E2f1-3 may serve as an important developmental trigger to endocycle onset in TGCs and hepatocytes.

2.4 Materials and methods

Mice and genotyping

The conventional E2f1 and E2f2 knockout mice, E2f3^{fl/fl} mice and Mx-cre transgenic mice were maintained in a mixed background composed of 129SvEv, C57B/L6 and FVB. The E2f1^{-/-} and E2f2^{-/-} mice were generously provided by M. Greenberg and S. Orkin, respectively. The E2f3^{fl/fl} conditional mouse was previously generated by L. Wu in the Leone laboratory. All mice were housed in barrier conditions with an automated 12-hour light/dark cycle and had access to food and water ad libitum. All protocols involving mice in this study were approved by the Institutional Animal Care and Use Committee at The Ohio State University. PCR genotyping was performed using genomic DNA purified from tails following a standard protocol (digestion with proteinase K followed by isopropanol precipitation). Mx-cre:

5’ATGCTTCTGTCCGTTTGCCG, 5’CCTGTTTTGCACGTTCACCG; E2f1ko:

5’AGCCACTGGATATGATTCTTGGAC, 5’AGAAGTCACGCTATGAAACCTCAC, 5’AGTGCCAGCGGGGCTGCTAAAG; E2f2ko:
5’GCCCCTAACACATGCACCCATTGG, 5’CCTGAGCGAGTCGGAGGATGG, 5’ACCAAAGAACGGAGCCGGTTGGCG;
E2f3ko and E2f3f:
5’TGTGAATAATTTTTGGCATGTTTT, 5’CTTATTCTGAGTGTGGACATACCG, 5’AAGGGGAAGGGAAAAATTAATCTGA.

Quantitative RT-PCR

Total liver RNA was extracted from tissues that were snap-frozen in liquid nitrogen using TRIzol® (Invitrogen). Five μg of total RNA from all developmental time points evaluated (E13.5-12mo) was used for reverse transcription at once with Superscript III (Invitrogen) and oligo(dT) in the presence of RNAse inhibitor (Roche). cDNA was then diluted 1:5 prior to being used as template in quantitative RT-PCR. SYBR Green (BioRad) Real-Time amplification was performed using gene-specific primers that spanned exon-exon junctions in the BioRad iCycler. Each sample was tested in triplicate 25μl reactions. Target gene transcripts were quantified relative to housekeeping genes (GAPDH and 16s rRNA) using the ΔCT method, and relative gene expression for each sample is presented as average ± SD. Primers were designed using the publicly available program Primer3 version 0.4.0 (Rozen and Skaletsky, 2000), and optimal annealing temperature conditions were determined by using the gradient run feature on iCycler.

polyIC injection
Six-week-old mice received five intraperitoneal injections of 250μg of polyinosine-polycytidine (Sigma) dissolved in sterile PBS every alternate day. Mice were sacrificed 24 hours after the last injection, and their livers harvested for FACS and microarray analyses. Body weight (BW) and liver weight (LW) information were gathered at time of sacrifice to calculate percent hepatic mass (LW/BW x 100).

**Flow cytometry**

Frozen liver tissue (~100mg) was finely minced with a razor followed by 5-10 minutes of incubation on ice in a small volume (~0.5mL) of hypotonic lysis buffer containing 25mM Tris (pH 7.5), 50mM KCl, 2mM MgCl₂, 1mM EDTA and fresh 1mM PMSF (phenylmethylsulfonyl fluoride). Nuclei were then gently released using a Dounce homogenizer with a tight pestle and washed twice more with the lysis buffer, once with cold sterile PBS, and resuspended in 0.1% PBS-Triton solution containing 50μl of propidium iodide (P.I., 0.5mg/ml, Roche) and 1μl of RNase A (20mg/ml, Invitrogen). A minimum of 30,000 nuclei per liver sample were analyzed for total DNA content using LSR II (BD Biosciences), and cell cycle profiles were generated using FlowJo.

**Hepatocyte quantification**

Images of hematoxylin and eosin (H&E) stained liver sections were captured using either the Eclipse 50i (Nikon) or Axioskop 40 (Zeiss) microscope. For quantification of hepatocytes, cells were manually counted in the Metamorph Imaging 6.1 software from images representing a minimum of five 40x lens objective fields per
sample. A minimum of three samples were evaluated per genotype. Results were reported as an average number ± SD of hepatocytes in control and mutant samples analyzed.
Figure 2.1. Loss of E2f Activators Promotes Hepatocyte Endocycles.

(A) Quantitative RT-PCR expression analysis of E2f activators in wild type pre- (E13.5), neo- (P0) and post-natal (2wk-12mo) livers. 3wk, weaning age; 3wk*, one day post weaning. Pups sacrificed at 3wk and 3wk* for tissue collection were littermates. n, number of independent mice analyzed per developmental time point. Relative gene expression is presented as average ± SD.

(continued)
Figure 2.1: continued

(B) Representative H&E sections showing control and Mx-cre;E2f1+/-;E2f2+/-;E2f3+/- (Mx-123tko) livers from eight-week-old mice. Mice were sacrificed 24 hours after the last polyIC injection.

(C) Top, percent hepatic mass measurements showing significantly enlarged Mx-123tko livers relative to control livers. Bottom, hepatocyte quantification showing significantly decreased number of number of cells per 40x objective lens field in Mx-123tko livers relative to control livers. n, number of independent mice analyzed. Data is presented as average ± SD. Two-tailed Student T-test, ** p≤0.01.

(D) Flow cytometry of control and Mx-123tko liver nuclei from eight-week-old mice demonstrating significant increases in the fraction of Mx-123tko cells with 8n and 16n ploidy levels. Loss of E2f1 alone or E2f1 and E2f2 resulted in dosage-dependent increases in the 8n and 16n ploidy classes. n, number of independent mice analyzed. Data is presented as average ± SD. One way ANOVA test, ** p≤0.01; ***p≤0.001. control in (B), polyIC injected Mx-cre;E2f1+/-;E2f2+/-;E2f3+/- livers; control in (C-D), wild type livers.
Figure 2.2. Loss of $E2f$ Activators Promotes TGC Endocycles.

(A) NanoString expression analysis of $E2f1$, $E2f2$ and $E2f3$ from laser capture microdissected TGCs (PALM MicroLaser) at the different embryonic stages indicated. n,
number of independent wild type OCT embedded placentas from which TGCs were captured; TGCs collected from multiple 8µm H&E sections of each placenta were pooled for total RNA extraction using Arcturus PicoPure RNA Isolation Kit (Applied Biosystems) according to manufacturer’s protocol. Relative gene expression is presented as average ± SD.

(B) Representative E9.5 H&E placenta sections showing control and E2f1−/−;E2f2−/−;E2f3−/− (123tko) TGCs.

(C) Quantification of genome ploidy using the established Feulgen technique in E9.5 control and 123tko TGCs. This analysis demonstrated a significant overall shift of 123tko TGCs towards higher levels of polyploidy. n, number of independent placentas for which TGC ploidy was analyzed. Data is presented as average ± SD. One way ANOVA test, *, p≤0.05; ** p≤0.01; ***p≤0.001.
Chapter 3

Role of Mammalian E2F Repressors in Endocycle Regulation

3.1 Introduction

The E2F family is defined by presence of the highly conserved winged-helix DNA binding domains (Figure 1.1). Unique among the mammalian E2Fs, E2F7 and E2F8 possess two tandem DBDs and do not require heterodimerization with DP1/DP2 to bind target sequences (de Bruin et al., 2003; Maiti et al., 2005). Co-immunoprecipitation (co-IP) experiments in vitro indicate that E2F7 and E2F8 instead form homo- or heterodimers through physical interaction between DBDs. The C-terminal transactivation and Rb binding domains are also lacking in these two E2Fs, which are therefore presumed to mediate transcriptional repression independently of association with pocket proteins. Due to their distinct structural and biochemical properties, E2F7 and E2F8 have fittingly been bestowed the title “atypical” E2F repressors.

Homologues of mammalian atypical E2Fs have been identified in Arabidopsis thaliana (DEL1-3 or E2Fd-f) and most recently in Caenorhabditis elegans (EFL-3; Winn et al., 2011) but are conspicuously lacking in Drosophila. Overexpression and RNAi studies in Arabidopsis have convincingly implicated a role for DEL1 proteins in the regulation of the timing of endocycle onset. Plant cells overexpressing DEL1 do not increase their DNA content and continue mitotic cell division past the developmental
time when endocycle is supposed to be initiated. Conversely, those lacking or with decreased expression of DEL1 display precocious and enhanced endoreplication (Vlieghe et al., 2005). The underlying mechanism for how DEL1 controls timing of plant endocycles was shown to involve direct repression of CCS52A (Cdh1 in mammals), a subunit that is required for activation of the APC/C complex to promote ubiquitin-mediated degradation of mitotic cyclins (Lammens et al., 2008). However, it is not known whether this mechanism is conserved in higher vertebrates. Finally in C. elegans, the newly identified EFL-3, like E2F7 and E2F8 (Li et al., 2008), was reported to be involved in the control of apoptosis during worm development. It would be highly interesting to determine whether EFL3 would also function in the control of endocycles in the worm, which contain highly polyploid tissues such as the hypodermal syncytium (Lozano et al., 2006).

In this chapter, we show by lineage-specific gene ablation strategies that the atypical E2F proteins are cell autonomously required for mammalian endocycles, and that their functions appear to involve the suppression of molecular events in mitosis, karyokinesis and cytokinesis.

3.2 Results

*Loss of E2f8 prevented hepatocyte polyploidization*

Having found that E2F activators suppress endocycles, we wished to explore the role of E2F repressors in endocycle control. Initially, we evaluated the representative canonical repressor E2F4 since quantitative RT-PCR assay showed that it is the most
abundant member of its subclass in hepatocytes (Figure 3.1A). Previous studies had also suggested that E2F4 is involved in maintaining hepatocyte quiescence through interaction with C/EBPα and repression of S phase related genes (Iakova et al., 2003). Flow cytometry analysis of E2f4−/− livers, however, failed to reveal any major alteration in hepatocyte ploidy (Figure 3.1B). We next evaluated the role of E2F7 and E2F8 in hepatocyte endocycles, and began by examining their mRNA levels using quantitative RT-PCR and NanoString assays. In wild type fetal livers, expression of E2f7 was high throughout embryogenesis, decreased rapidly at birth (P0) and remained barely detectable thereafter (Figure 3.2A and 3.2B). The expression of E2f8 was also high in fetal livers and decreased at birth, but in contrast to E2f7, it transiently increased during weaning (Figure 3.2A and 3.2B), coinciding with the time when hepatocytes switch from mitotic cell cycles to endocycles as the main mechanism of organ growth through cellular hypertrophy. The transient but distinct elevation in E2f8 expression suggests its involvement in coordinating the M-E transition.

Genetic ablation of E2f7 and E2f8 causes embryonic lethality at E11.5 (Li et al., 2008). Thus to examine their roles during postnatal liver development we crossed E2f7+/− and E2f8+/− mice with the established Albumin-cre mouse model (Alb-cre; Postic and Magnuson, 2000), which begins to expresses cre in hepatocytes around the time of birth (Weisend et al., 2009). The conditional inactivation of E2f7, E2f8 or both did not significantly alter hepatic mass at any of the ages analyzed (Figure 3.3B). However, evaluation of H&E stained liver sections revealed that the size of hepatocytes and their nuclei in aged Alb-cre;E2f8−/− (Alb-8ko) and Alb-cre;E2f7−/−,E2f8−/− (Alb-78dko) mice were
markedly smaller than in control animals (Figure 3.3A and 3.3C). These differences were detectable by about a week after weaning (data not shown), became more pronounced with age as shown in Figure 3.3A and were compensated by an increase in the total number of Alb-8ko and Alb-78dko hepatocytes (Figure 3.3D). Moreover, loss of E2f8 or E2f7/E2f8 significantly decreased the proportion of bi-nucleated hepatocytes (Figure 3.3E). Strikingly, flow cytometry showed that hepatocytes in Alb-8ko and Alb-78dko livers remained diploid over the entire lifetime of the mouse, whereas hepatocytes in Alb-cre;E2f7f/f (Alb-7ko) livers had only a modest reduction in genome ploidy compared to wild type controls (Figure 3.4). The percentage of Ki67-, cyclin A2, cyclin B1- and P-H3-positive hepatocytes was significantly elevated in 2-month-old Alb-8ko mice and even more so in Alb-78dko mice (Figure 3.5). These data suggest that loss of E2f8 alone was sufficient to stimulate hepatocytes to undergo ectopic mitotic cell divisions and prevent their polyploidization.

Finally, analysis of livers from aged (12-15 months) mice globally deleted for E2f7 (E2f7f/f) or E2f8 (E2f8f/f) yielded ploidy phenotypes identical to livers from conditionally deleted Alb-7ko and Alb-8ko mice (Figure 3.6A and 3.6B). From these results, we conclude that E2F8 and to a lesser extent E2F7, act cell autonomously to promote hepatocyte endocycles.

3.3 Discussion

*E2F8 is the principal organizer of hepatocyte endocycles*
It has been difficult to contextualize a definitive role of E2Fs in regulating mammalian endocycles given the restricted number of polyploid tissues in these complex organisms. In mice, the process of hepatocyte polyploidization begins early during postnatal growth and becomes more extensive with age. We now demonstrate that this process requires the function of E2F8, with just a small degree of contribution from E2F7, given that a failure to accumulate polyploid hepatocytes was evident in murine livers lacking $E2f8$ alone, but was most pronounced in those with concomitant loss of $E2f7$ and $E2f8$. Hepatocytes of all four genotypes (control, Alb-7ko, Alb-8ko and Alb-78dko) examined in this chapter underwent mitotic cell divisions at similar rates near the age of weaning (data not shown), when flow cytometry confirmed that more than 75% of the nuclei were diploid (Figure 3.4). At this time prior to the M-E transition, the liver rapidly expands in size using a ‘proliferative’ mode of growth. Subsequent to weaning and for the remainder of an animal’s life, wild type hepatocytes switch to a ‘hypertrophic’ mode of growth while we showed that $E2f8$- and $E2f7/E2f8$-deficient hepatocytes fail to engage this switch.

Interestingly, the observation that functional livers develop from hepatocytes lacking $E2f8$ or $E2f7/E2f8$ would suggest that E2F8 activity is dispensable for mitotic cycles in hepatic progenitor cells and immature hepatocytes. Moreover, although one could argue that E2F7 may functionally compensate for the loss of E2F8 activity in mitotic cell cycles, this is unlikely given that young hepatocytes deficient for both $E2f7$ and $E2f8$ also proliferate. An alternative interpretation of this observation would be that E2F8 activity potently suppresses mitotic cell cycles, and therefore its expression must be
kept low during the ‘proliferative’ mode of organ growth in the liver. This would suggest that physiologic or developmental signals in the liver during weaning might serve to transiently release the repression of $E2f8$, which then mediates the cell autonomous induction of events (e.g. inhibition of mitosis) necessary for the beginning of polyploidization. The pattern of E2F8 expression and activity would thus be the opposite of what had already been described for the activator E2Fs (Chapter 2), which must be kept low during the ‘hypertrophic’ mode of growth.

One caveat of our liver studies so far is that results were obtained using a non-inducible gene ablation system. The expression of cre, driven by the *Albumin* gene promoter, reportedly begins before weaning age (at around birth) in the liver. However, cre expression has also been detected as early as mid-gestation in the fetal liver (Postic and Magnuson, 2000), suggesting that cre-mediated recombination of conditional alleles has the potential to occur in the bi-potential hepatoblasts, which can give rise to both hepatocytes and cholangiocytes (or mature bile duct epithelial cells). To avoid inadvertent effects caused by loss of $E2f7$ and $E2f8$ in cholangiocytes on hepatocyte physiology, it might be necessary to utilize a system that is more restricted in targeting cre expression.

It would be important to determine whether deletion of $E2f8$, when induced shortly before the scheduled onset of endocycle, might result in the same defect in polyploidization as deletion at or near birth. Moreover, to assess whether E2F8 might function in endocycle progression after M-E transition, its inactivation would need to be induced after the scheduled onset of polyploidization. Finally, the neonatal ablation of
E2f7/E2f8 precludes the analysis of possible endocycle independent functions of atypical E2Fs that may be important for hepatocyte biology (e.g. xenobiotic detoxification) or organ homeostasis in the adult animal. In the near future, information imparted by careful studies involving functional assessments of E2f7/E2f8-deficient livers in the setting of metabolic and physiologic stress may generate novel insights into the molecular basis of hepatic dysfunction.

**E2F7 and E2F8 synergistically regulate TGC endocycles**

TGCs in the placenta are remarkable among all mammalian cell types because they are highly polyploid, with as many as 1000 copies of the genome arranged in a polytene configuration (Varmuza et al., 1988). Parallel studies evaluating the consequences of E2f7/E2f8 ablation in TGCs of the placenta were carried out (M. Ouseph), which revealed many E2f7−/−;E2f8−/− (78dko) TGCs by E10.5 at various stages of mitosis, implying an interruption of normal endocycles. Indeed, the ploidy of 78dko TGCs was dramatically reduced and never exceeded 64n, whereas wild type TGCs with genomes >1000n could be readily detected. Similar to what was observed in hepatocytes, the combined inactivation of E2f7/E2f8 also led to an increase in the levels of G2/M (cyclin A2) and M phase-specific proteins (cyclin B1 and phospho-H3). Confocal microscopy and 3D reconstruction of serially imaged E2f7−/−;E2f8−/− placentas showed a significant number of TGCs containing at least two closely apposed nuclei (S. Singh and S. Raman), suggesting that they had actually undergone karyokinesis. Placentas lacking E2f7, E2f8, or one allele of each (E2f7+/−;E2f8+/−), exhibited TGCs with an intermediate
reduction in ploidy and a proportionate increase in G2/M and M phase related events, indicating a gene dosage effect in their function. We interpret these results to mean that E2F7/E2F8 are not required for the initiation of TGC endocycles per se, since their ablation did not preclude trophoblast stem cells from differentiating into giant cells, as p57Kip2 expression was still maintained (data not shown; Ullah et al., 2008), and 78dko TGCs once formed was able to acquire polyploid genomes (although only up to 64n). Rather, our results suggest that E2F7 and E2F8 may be required to promote successive G/S oscillations in TGC endocycles after M-E transition through synergistic repression of key factors involved in the onset of mitosis and karyokinesis.

Because the expression of multiple trophoblast cell lineage markers was altered in placentas globally deleted for E2f7/E2f8 (Ouseph et al., in review), we could not formally rule out a general disruption of differentiation as a possible cause for the endocycle defects observed in 78dko TGCs. A graduate student in the Leone lab (M. Ouseph) thus used homologous recombination to develop a novel knockin mouse model where the cre open reading frame was inserted directly downstream of the endogenous start codon of the TGC-specific Proliferin gene (Plfcre/+), which allowed us to directly evaluate the consequence of E2f7/E2f8 ablation in TGCs, beginning as early as E6.5. TGC-specific ablation of E2f7/E2f8 (Proliferincre/+ ) remarkably did not result in lethality of mouse embryos (Ouseph et al., in review) but recapitulated virtually all ploidy and molecular abnormalities we detected in TGCs from E2f7−/−;E2f8−/− placentas. An immediate supposition provided by this result is that atypical repressors act cell autonomously to control endocycle progression. Second and perhaps more interestingly, this data would
argue that ‘binucleated’ TGCs with drastically reduced ploidy were sufficient to support placental function. Indeed, E2f7/E2f8-deficient TGCs were still able to produce, albeit at a reduced level, the myriad of cytokines and proteins thought to be critical for a successful pregnancy (Ouseph et al., submitted). What then might the consequences be of interrupted endocycles? It would appear that, at least in the giant cell, once the endocycle machinery has been switched on, numerous mechanisms may be immediately engaged to ensure ‘longevity’ of these cells. As such, they become more resilient to extra- and intracellular stresses and may adapt to functioning under less than ideal conditions, including sub-optimal configurations of their typically highly organized genomes. Given that many studies in the past have relied on gene ablation strategies that target the entire extra-embryonic compartment or the whole conceptus, the full impact of endocycle on TGC function and together their contribution to placental biology remains to be carefully evaluated.

In conclusion, by using targeted gene ablation strategies in mice we showed that the functions of mammalian atypical E2Fs are required to promote TGC and hepatocyte endocycles. Furthermore, loss of E2F7 and E2F8 also significantly reduced the ploidy of megakaryocytes (M. Ouseph, data not shown), which normally undergoes ‘abortive’ mitosis or endomitosis to achieve a genome ploidy level of 64n. Notably, neither loss of E2f4 nor E2f5 in mice appeared to affect polyploidization of TGCs and hepatocytes (Figure 3.1 and data not shown). While we cannot rule out a role for these canonical repressors in endocycle control that might otherwise be exposed in different genetic or
environmental contexts, it would appear that their involvement is not essential. We therefore suggest that $E2f7/E2f8$ evolved to specifically regulate variant cell cycles in metazoans in a manner distinct from the function of canonical E2F repressor proteins as well as their plant counterparts.

3.4 Materials and methods

Mice and genotyping

$E2f7^{0/0}$, $E2f8^{0/0}$ and Alb-cre transgenic mice were maintained in a pure FVB/N genetic background. The $E2f7^{0/0}$ and $E2f8^{0/0}$ conditional mice were previously generated by former members A. de Bruin and E. Li, respectively, of the Leone laboratory (Li et al., 2008). The Alb-cre transgensics were generously provided by E. Knudsen. All mice were housed in barrier conditions with an automated 12-hour light/dark cycle and had access to food and water *ad libitum*. All protocols involving mice in this study were approved by the Institutional Animal Care and Use Committee at The Ohio State University. PCR genotyping was performed using genomic DNA purified from tails following a standard protocol (digestion with proteinase K followed by isopropanol precipitation). Alb-cre: 5′ATGCTTCTGTCCGTTTGCCG, 5′CCTGTTTTGCACGTTCACCG; $E2f7^{ko}$ and $E2f7^f$: 5′AGGCAGCACAACCTTGACACCG, 5′ACTTTTGAGGACAGAAGGTAGGA, 5′CCAAGATGAAGGCCGAGATGCTAC; $E2f8^{ko}$ and $E2f8^f$: 5′TAAAAAGCTTTTGGGTCGGTTAGA, 5′AAGCCAACCTCGATGAATTG, 5′CTCGCATCATCGCTCTGCTAA.
**NanoString nCounter analysis**

The NanoString nCounter system was used to quantitatively assess the expression of all E2f family members in wild type liver samples (although the expression of E2f1-3 was not presented). Approximately 200ng of total RNA from individual liver samples was submitted to the OSUCCC Nucleic Acid Shared Research core facility. The RNA samples were profiled on a custom designed mRNA codeset, which included probes specific for the murine E2f1-8 transcripts, according to manufacturer’s instructions. Ten house keeping genes (Actb, AldoA, B2m, Gapdh, LdhA, Oaz1, Pgk1, Rpl27, Rps13 and Rps20) were also selected to be included in the codeset (de Jonge et al., 2007). The nCounter data was first normalized to the geometric mean of positive control spike counts in each lane of a cartridge, followed by subtracting the minimum negative control spike counts in each lane to remove the background (technical normalization). Finally, expression of each gene in the codeset was normalized with respect to the geometric mean of the ten house keeping genes (biological normalization). The geometric mean of E2f4, E2f5, E2f6, E2f7 and E2f8 from livers (n=2) at the P0 time point was set as reference, and fold-change in gene expression was calculated between P0 and other developmental time points (p<0.05).

**Quantitative RT-PCR**

This procedure was performed exactly as described in Chapter 2.

**Flow cytometry**
This procedure was performed exactly as described in Chapter 2.

**Immunostaining**

For immunohistochemistry (IHC), five μm paraffin sections of liver tissues were incubated with primary antibodies against Ki67 (550609, BD Pharmigen), phospho-Histone 3 (Ser10) (06-570, Millipore), Cyclin A2 (sc-596, Santa Cruz) and Cyclin B1 (sc-752, Santa Cruz). Sections were deparaffinized in xylene and gradually rehydrated in 100%, 95% and 70% ethanol followed by incubation in water. Endogenous peroxidase activity was quenched by incubating rehydrated sections in 3% hydrogen peroxide PBS solution. Antigen retrieval was achieved by heat treatment (near boiling) in Target Retrieval Solution (DAKO) followed by blocking in a PBS solution containing 5% goat serum (GS) and 2% bovine serum albumin (BSA). Primary antibodies were then applied to sections, and all were allowed to incubate overnight at 4°C. Appropriate species-specific biotinylated secondary antibodies were applied to sections for one hour at room temperature the next day, followed by ABC incubation (Vector) and finally development using DAB (Vector). Sections were counterstained with Mayer’s hematoxylin and dehydrated in an ascending alcohol series ending in xylene. For immunofluorescence, five μm paraffin sections of liver tissues were deparaffinized and rehydrated. Antigen retrieval was performed, after which sections were incubated in 10% PBS-Triton for ten minutes, followed by blocking (5% GS/2% BSA in PBS) and incubation with anti-E-Cadherin (ab53033, Abcam) overnight at 4°C. Fluorophore-conjugated secondary were applied to sections the next day for one hour at room temperature in low light conditions.
Sections were counterstained with DAPI solution and stored in the dark at 4°C until visualization or imaging.

Confocal microscopy and nuclei volume analysis

Livers samples were collected from 6-month-old mice of the appropriate genotypes (control and Alb-78dko), fixed in 4% paraformaldehyde in PBS overnight at 4°C and subsequently embedded in OCT. Seventy µm-thick frozen sections were cut, washed with 0.2% PBS-Triton with gentle agitation for 1.5 hours at 4°C, and stained with 1mM DAPI solution in the dark at 4°C overnight. After extensive washing the next day to removal excess unbound DAPI (three consecutive one-hour incubations in PBS at 4°C with gentle agitation), the stained sections were captured onto a glass slide and mounted in a non-xylene based medium and allowed to dry in the dark for at least one hour. Subsequently, stained liver sections were examined and their images captured using confocal laser scanning microscopy (Zeiss LSM 510) with the use of a 63X objective and 0.7X scan zoom (S. Singh and S. Raman). Optical sections with between-plane-plane resolution of 0.40µm and axial resolution of 0.42µm were acquired. Four to five non-overlapping regions per independent liver sample were imaged with a field of view of 207µm x 207µm and depth of 40 ± 2µm, resulting in a stack of 100 images for each region. For 3D reconstruction of hepatocyte nuclei, the image stacks were processed using Insight Toolkit and ITK-SNAP (Yushkevich et al., 2006). Each hepatocyte nucleus was segmented by active contour segmentation using region competition as the stopping criterion (M. Leone; Zhu and Yuille, 1996). Only the volumes of individual hepatocyte
nuclei were measured in cubic units (as a function of the number of pixels in each reconstructed nucleus).

Statistics

Two-tailed Student T-test was used to evaluate differences in hepatocyte nuclei volume. One way ANOVA method was used for data analysis unless otherwise indicated. In the case of multiple comparisons (e.g. more than two groups being considered), Holm’s method was used to adjust resulting p-values. All IHC quantification data were reported as an average percentage ± SD of positive cells from the total cell population in the number (n value indicated in Figures) of control and mutant samples analyzed.
Figure 3.1. Classical Repressor *E2f4* Does Not Contribute to Hepatocyte Polyploidization.

(A) Quantitative RT-PCR (qRT-PCR) expression analysis of canonical repressors *E2f4*-6 in pre- (E13.5), neo- (P0) and post-natal (2wk-12mo) livers. 3wk, weaning age; 3wk*, one day post weaning. Pups sacrificed at 3wk and 3wk* for tissue collection were littermates. n, number of independent mice analyzed per developmental time point. Relative gene expression is presented as average ± SD.

(B) Flow cytometry comparing the nuclear ploidy of livers from *E2f4*+/+ and 3-month-old *E2f4*−/− mice demonstrating no significant differences among any of the ploidy classes.
Figure 3.2. Expression of Atypical Repressor $E2f8$ Transiently Increases in the Liver During Weaning.

(A) NanoString analysis of liver-specific expression of atypical $E2f$ repressors demonstrating a transient peak in post-natal $E2f8$ expression that coincides with the time of weaning. The expression of $E2f7$ declined slightly after birth from embryonic levels and remained low in the young adult liver and through old age. 3wk, weaning age; 3wk*, one day post weaning. Pups sacrificed at 3wk and 3wk* for tissue collection were littermates. n, number of independent mice analyzed per developmental time point (a (continued)
Figure 3.2: continued

subset of the same samples as in Figure 3.2B). Relative gene expression is presented as average ± SD.

(B) Quantitative RT-PCR expression analysis of E2f7 and E2f8 in pre- (E13.5), neo- (P0) and post-natal (2wk-12mo) livers. Although the peak in E2f8 expression is slightly shifted by quantitative RT-PCR (3wk*) compared with NanoString (3wk), the overall pattern of its expression (as well as the pattern of E2f7 expression) coincides significantly between the two methods. 3wk, weaning age; 3wk*, one day post weaning. Pups sacrificed at 3wk and 3wk* for tissue collection were littermates. n, number of independent mice analyzed per developmental time point. Relative gene expression is presented as average ± SD.
Figure 3.3. Loss of E2f8 Resulted in Reductions in Hepatocyte Size, Hepatocyte Nuclear Volume and Rate of Binucleation.

(A) Representative H&E sections of livers from 12-month-old mice demonstrating a
Figure 3.3: continued

decrease in the size of Alb-8ko and Alb-78dko hepatocytes relative to control cells. Note that the size of their nuclei (inset) also appears correspondingly diminished. The Alb-7ko hepatocytes do not appear different than control cells at this age.

(B) Percent hepatic mass in weaning age (3wk), adult (2-6mo) and aging (12mo) livers demonstrating no significant differences in this measurement between any two age groups or genotypes assessed. Percent hepatic mass is liver weight divided by body weight, multiplied by 100. Normal range for a rodent is between 4.5-6%. n, number of independent mice analyzed per indicated genotype. Data is presented as average ± SD.

(C) Left, assessment of hepatocyte nuclei volume in livers from 6-month-old control and Alb-78dko mice. n, total number of nuclei evaluated per genotype. Data is presented as average ± SD. Two-tailed Student T-test, ***p≤0.001. Right, representative confocal images of liver sections with DAPI-stained nuclei (S. Singh and S. Raman). Although multinucleated hepatocytes were clearly present in the control sample (top), only individual nuclei in both control and Alb-78dko samples were selected for the volume analysis (M. Leone).

(D) Quantification of number of hepatocytes per 40x objective lens field. A semi-quantitative assessment of hepatocyte size was inferred by number of cells in a given (continued)
area, with greater numbers representing smaller size and vice versa. At weaning (3wk), already *Alb*-78dko hepatocytes appeared smaller than control hepatocytes; this difference was magnified as mice reached 12mo of age. *Alb*-7ko and *Alb*-8ko hepatocytes had significantly intermediate reductions in size, with *E2f8* having a slightly more dominant effect. n, number of independent mice analyzed per indicated genotype. Data is presented as average ± SD. One way Anova, ** p≤0.01; ***p≤0.001.

(E) Left, quantification of binucleated hepatocytes in livers from different age groups demonstrating a significant decrease in *Alb*-78dko livers relative to control tissues. Loss *E2f8* alone also had a moderate but significant effect at 3wk and 2months. n, number of independent mice analyzed per indicated genotype. Data is presented as average ± SD. One way Anova, ** p≤0.01; ***p≤0.001. Right, immunofluorescence with anti-E-cadherin marking the boundary of hepatocytes counterstained with DAPI. *control, E2f7f/f;E2f8f/f; Alb-7ko, Alb-cre;E2f7f/f; Alb-8ko, Alb-cre;E2f8f/f; Alb-78dko, Alb-cre;E2f7f/f;E2f8f/f.*
Figure 3.4. Activity of Atypical Repressor E2f8 Alone is Sufficient to Support Hepatocyte Polyploidization. Left (top to bottom), flow cytometry of liver nuclei in (continued)
Figure 3.4: continued
young (3wk), adult (2mo) and aging (1yr) livers with the four indicated genotypes. Individual bars in each bracketed ploidy class represent one age group. n, number of independent mice analyzed per indicated genotype. The one way Anova (with Holm’s adjustment) significance test was performed to determine differences in each ploidy classes between the following comparisons: 1) Alb-78dko and control, 2) Alb-8ko and control, and 3) Alb-7ko and control. Results for comparisons 1 and 2 were significant (at least p≤0.01) for all four ploidy classes. Result for comparison 3 was not significant for any of the four ploidy classes. Right (top to bottom), representative FACS profiles of liver nuclei from aging mice (last bar from each bracketed ploidy class). This analysis clearly demonstrated that Alb-78dko and Alb-8ko livers failed to undergo age-dependent polyploidization, consistent with their smaller appearance in H&E (Figure 3.3A). FACS, fluorescence activated cell sorting. control, E2f7floxp;E2f8floxp; Alb-7ko, Alb-cre;E2f7floxp; Alb-8ko, Alb-cre;E2f8floxp; Alb-78dko, Alb-cre;E2f7floxp;E2f8floxp.
Figure 3.5. Loss of Atypical E2f Repressor Function Stimulated Ectopic Mitotic Proliferation in Hepatocytes. Representative immunohistochemistry images (right) and quantification of hepatocyte proliferation (left) in livers from 2-month-old mice. Liver-specific ablation of E2f7 and E2f8 (Alb-78dko) resulted in significant increases in the (continued)
Figure 3.5: continued

number of Ki67, cyclin A2, cyclin B1 and P-H3 positive hepatocytes relative to control tissues. Pattern of cyclin A2 and cyclin B1 staining in Alb-78dko hepatocytes is characteristic of the intracellular distribution of mitotic cyclins (cytoplasmic and nuclear). Loss of E2f8 (Alb-8ko) alone also resulted in a significant increase in P-H3 positive hepatocytes (p≤0.05, images not shown). n, number of independent mice analyzed per genotype. Quantification data presented as average ± SD. One way Anova, * p≤0.05; ** p≤0.01; ***p≤0.001. control, E2f7f/f;E2f8f/f; Alb-7ko, Alb-cre;E2f7f/f; Alb-8ko, Alb-cre;E2f8f/f; Alb-78dko, Alb-cre;E2f7f/f;E2f8f/f.
Figure 3.6. *E2f8* Function in Hepatocyte Endocycles is Cell Autonomous.

(A) Representative H&E liver sections from aged (12-15mo) *control*, *E2f7*⁻/⁻ (7ko) and *E2f8*⁻/⁻ (8ko) mice. Hepatocytes deleted for *E2f8* were dramatically smaller than either *control* or 7ko hepatocytes, consistent with their lack of endocycles.

(B) FACS profiles of liver nuclei suspension from aged (12-15mo) *control*, *E2f7*⁺/⁺ (7ko) and *E2f8*⁺/⁺ (8ko) mice. Livers from 8ko mice were almost exclusively diploid and tetraploid. In contrast, both *control* and 7ko livers have considerable proportions of 8n and 16n hepatocytes. *control*, *E2f7*⁺/⁺;*E2f8*⁺/⁺. FACS, fluorescence activated cell sorting.
Chapter 4

Identification of Endocycle-related Genes Regulated by E2F Atypical Repressors

4.1 Introduction

Consistent with their function as transcriptional repressors, ectopically expressed E2F7 and E2F8 in human embryonic kidney (HEK) 293 cells could be pulled down in complexes containing bona fide co-repressors such as C-terminal binding protein (CtBP) and histone deacetylase (HDAC) proteins (J. Li, unpublished). Unlike E2f7/E2f8 double knockout mouse embryos that die at mid gestation due to widespread apoptosis and hemorrhage (Li et al., 2008), E2f7 and E2f8 single knockout mice are viable and do not exhibit overt developmental phenotypes. It is therefore thought that these two proteins are functionally redundant and regulate an overlapping set of gene targets in vivo. Protein and mRNA expression studies in cultured cells indicate that levels of E2F7/E2F8 during the cell cycle are highest at the S/G2 transition, suggesting that they normally usher cells out of S phase by reducing the expression of genes involved in DNA replication. Chromatin-immunoprecipitation (ChIP) experiments demonstrated that E2F7/E2F8 indeed bind to and repress the promoters of E2F1 and E2F3 (Di Stefano et al., 2003; Christensen et al., 2005), which activate the expression of genes such as dihydrofolate reductase (DHFR) and proliferating cell nuclear antigen (PCNA).

The E2F7a and E2F7b isoforms are produced by alternative splicing of the primary transcript and differ only at the C-termini (Di Stefano et al., 2003), with the
exclusion of exon twelve from E2F7a that encodes a putative, non-LXCXE pocket protein binding motif. It is currently unclear whether these two isoforms are functionally distinct and whether their expression varies among different tissues in the mammal.

In this chapter, we profiled the transcriptomes of livers deficient for $E2f7$, $E2f8$ and $E2f7/E2f8$ from weaning age mice. We show that disruption of the $G_2/M$ transcriptional network induced by loss of E2F7 and E2F8 was the principal underlying cause of endocycle failure in $E2f7/E2f8$ deficient hepatocytes.

4.2 Results and Discussion

Gene expression profiling analysis (Affymetrix Mouse Genome 430 2.0) using RNA purified from livers of weaning age wild type, Alb-7ko, Alb-8ko and Alb-78dko mice showed that there were significantly more upregulated than downregulated genes in mutant samples, consistent with E2F7/E2F8 functioning as transcriptional repressors (Figure 4.1). Changes in expression of a subset of these genes were confirmed by NanoString technology (Figure 4.2A). Supervised clustering methods identified three main classes of differentially expressed genes (Figure 4.1). Class I includes genes differentially expressed in all three mutant genetic groups relative to wild type controls. These genes may represent targets of E2F7 and E2F8 since loss of either member led to their derepression. Class II includes genes differentially expressed in two of the three mutant genetic groups relative to wild type controls. A subset of these may represent targets repressed by either E2F7 or E2F8. Class III includes genes differentially expressed in only one mutant genetic group relative to wild type controls. More
specifically, the subset upregulated in *Alb-78dko* samples may represent targets that are synergistically repressed by E2F7 and E2F8.

We next focused our analyses on differentially expressed genes that have the potential to be involved in endocycle control. These included genes upregulated in both *Alb-8ko* and *Alb-78dko* livers (in Class II), since hepatocytes in these two genetic groups displayed the most pronounced reduction in genome ploidy, as well as genes upregulated in all three mutant cohorts (in Class I), as these could also contribute to the observed polyploidy defects. Close inspection of Class I genes by Ingenuity Pathway Analysis (IPA) revealed a striking bias for functions related to cytokinesis, G2/M progression and various stages of mitosis, such as chromosome condensation, stabilization and segregation (Figure 4.2B). By manual annotation of Class II genes, we also noted a bias for gene products with GTPase-activating (GAP) or GTP exchange factor (GEF) functions, which have the potential to regulate the Rho family of small GTPases that are essential for successful cytokinesis (Normand et al., 2010). A NanoString custom codeset was then used to interrogate the expression of these genes in TGCs isolated from wild type, *E2f7−/−*, *E2f8−/−* and *E2f7−/−;E2f8−/−* placenta by laser capture microdissection (Figure 4.3A). Given the striking functional bias noted above through IPA, we also included additional hand-selected genes with reported G2/M related functions. As shown in Figure 4.2, 66 of the 99 genes analyzed had predominantly increased expression in *E2f7/E2f8* deficient TGCs, suggesting a common mechanism for how E2F7 and E2F8 may regulate endocycles in TGCs and hepatocytes.

Interestingly, a significant portion of common upregulated genes in TGCs and hepatocytes have E2F binding elements on their promoters, raising the possibility that
these may represent direct targets of E2F7/E2F8. To test this hypothesis, we transfected human embryonic kidney cells (HEK 293) with flag-tagged versions of E2F7 and E2F8 plasmids and performed chromatin immunoprecipitation (ChIP) assays with anti-flag antibodies (Figure 4.3B). Flag-specific antibodies, but not control IgG, co-immunoprecipitated Ccna2, Chek1, Dtl and E2f1 promoter sequences containing E2F binding elements, but not irrelevant sequences lacking E2F binding sites (downstream extronic sequences of E2f1 (E2f1ds) and Tubulin (Tub)). The specificity of these ChIP assays was confirmed by parallel experiments with HEK 293 cells expressing mutant versions of E2F7 and E2F8 that lack DNA-binding capacity.

The results of our ChIP assays suggest that E2F7 and E2F8 repress a core group of genes including Ccna2 and E2f1 with prominent involvement in the regulation of G2/M related events. A third candidate gene that we have tested is denticleless homolog or Dtl (also known as Cdt2), which encodes a protein that acts as the substrate recognition factor of the Cul4A-RING ubiquitin ligase complex (Abbas and Dutta, 2009). The function of Dtl appears to be essential for mammalian development, as its targeted disruption resulted in death of mouse embryos due to failure of cell cycle progression at the 4-8-cell stage (Liu et al., 2007). Anti-sense depletion studies in wild type mouse embryos further revealed that cells with reduced Dtl proteins display hallmarks of mitotic catastrophe, including fragmentation of mitotic chromosomes and chromosomal lagging. Moreover, knockdown of human DTL using siRNA in HeLa cells led to a striking phenotype defined by irregular multinucleation, suggesting defects in the mechanisms that control karyokinesis and cytokinesis (Liu et al., 2007). This latter observation indicates that excess Dtl function would result then in an enhancement of mitosis,
karyokinesis and cytokinesis. In summary, the derepression of E2F7/E2F8 regulated genes, such as *Ccna2* and *Dtl* that positively coordinate G2/M, provide a logical basis for the mechanisms underlying endocycle failure in *E2f7/E2f8* deficient cells.

Finally, we performed global gene expression profiling in hepatocytes lacking the three E2F activators (*Mx-123tko*). This experiment revealed that loss of E2F1-3 resulted in the downregulation of a significant number of genes, which strikingly had been upregulated in *E2f7/E2f8* deficient hepatocytes (Figure 4.4A). Moreover, using IPA we again determined that many of these genes also have annotated functions related to G2/M transition and mitosis (Figure 4.4B), while a survey of their promoters revealed that the majority contain just one consensus E2F binding element (Figure 4.4C). This data is consistent with E2F activators and atypical repressors having antagonistic roles in the transcriptional control of a core group of genes that have, based on their functional classification (Figure 4.4B), a strong potential to affect endocycle control. Indeed, this subject of functional antagonism will be thoroughly explored in the next chapter.

### 4.4 Materials and methods

**Global gene expression profiling analysis**

Total RNA was extracted from a minimum of three independent livers per genetic group using TRizol® (Invitrogen) following manufacturer’s instructions. RNA quality was verified using the Agilent 2100 Bioanalyzer. RNA samples were submitted to the OSUCCC Microarray Shared Resource core facility for generation of biotinylated cRNA and hybridization to Mouse Genome 430 2.0 (MOE4302.0) Affymetrix chips. A GeneChip Scanner 3000 (Affymetrix) was used for data acquisition. For each sample,
pre-processing of raw signal intensities from .CEL files was performed using the Robust Multi-chip Average (RMA) method (Irizarry et al., 2003) and consisted of background adjustment, quantile normalization and median polish. BRB-Array Tools 3.8.1 (developed by R. Simon) was subsequently used to perform various class comparisons to determine differentially expressed genes at a significance level of p<0.05 (two-sample T-test with random variance model). For the construction of heatmaps (T. Pecot), the geometric mean of genes from control liver samples after RMA normalization was set as reference. Colors in the heatmap correspond to the ratio between expression of genes in mutant (e.g. \textit{Alb-78dko}) samples and the reference value (blue indicating decreased expression relative to reference, red indicating increased expression relative to reference). Supervised clustering was performed to group genes by their pattern of deregulation in mutant samples relative to control samples, which classified them as belonging to Class I, II or III. Functional annotation of differentially expressed genes was performed using Ingenuity Pathway Analysis (Ingenuity Systems, http://www.ingenuity.com).

\textit{NanoString nCounter analysis}

The NanoString nCounter system was used to profile the transcriptome of TGCs and to validate gene expression changes (as first determined by Affymetrix analysis) in the liver samples. For the analysis of gene expression in TGCs, TGCs were isolated (M. Ouseph and J. Thompson) from 8µm H&E stained sections of OCT (Sakura) embedded frozen placental tissues using laser capture microdissection (PALM MicroLaser system). Total RNA was then extracted using Arcturus PicoPure RNA Isolation Kit (12204-01, Applied Biosystems) according to manufacturer’s protocol. For validation of gene
expression changes in liver samples, the same TRIzol®-isolated RNA was submitted to core facilities for both Affymetrix and NanoString assays. 200ng of total RNA from each TGC or liver sample was profiled on a custom mRNA codeset according to manufacturer’s instructions. The codeset contained approximately 200 genes, 130 of which were selected based on their expression status in the liver and based on their ability to regulate cellular functions involved in S phase, G2/M transition and M phase of the cell cycle. Additionally, ten house keeping genes (Actb, AldoA, B2m, Gapdh, LdhA, Oaz1, Pgk1, Rpl27, Rps13 and Rps20) were also included in the codeset (de Jonge et al., 2007). Technical and biological normalization of nCounter data was performed as previously described in Chapter 2 (T. Pecot). For the construction of heatmap in Figure 4.3A, the geometric mean of genes from control TGCs was set as reference, with the colors (blue or red) corresponding to fold-change in gene expression between control and mutant TGCs at a significance level of p<0.05 (two-sample T-test with random variance model). For the construction of heatmap in Figure 4.2A comparing Affymetrix and NanoString results, colors in both “Array” and “Nano” columns correspond to fold change in expression of genes in mutant (e.g. Alb-78dko) samples relative to control samples. Genes in heatmap were ordered top to bottom starting with one that was most down-regulated in Alb-78dko relative to control samples (blue block with descending gradient), followed by one that had most up-regulated expression in Alb-78dko relative to control samples (red block with descending gradient).

*E2F binding site analysis*

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Promoter sequences (from -3000bp to +2000bp relative to the Transcriptional Start Site) of genes antagonistically regulated by E2F1-3 and E2F7/E2F8 were obtained from the UCSC genome browser (http://genome.ucsc.edu/). E2F consensus binding sites in these sequences were then identified using the publicly available program TFSearch (http://www.cbrc.jp/research/db/TFSEARCH.html). Only sequences with a score of >85 were included in the final analysis.

Chromatin immunoprecipitation (ChIP) assay

For ChIP experiments, the EZ-CHIP™ assay kit (Millipore) was used following manufacturer’s instructions. 70% confluent human embryonic kidney (HEK) 293 cells were transiently transfected using the calcium phosphate transfection method with 25μg of plasmids overexpressing wild type flag-E2F7 and flag-E2F8. Cells were transfected in parallel with plasmids overexpressing mutant flag-E2F7 and flag-E2F8, which harbored mutations in their DNA binding domains that abrogated their ability to associate with DNA. After 48 hours, cells were crosslinked and lysed, and the chromatin sonicated to obtain approximately 200-300bp fragments. Sonicated chromatin was incubated with 2μg of anti-flag (M2, Sigma) or normal mouse IgG (Oncogene) antibodies at 4°C with gentle rotation overnight. The next day antibody-protein-DNA complexes were recovered by addition of 30μl of Salmon Sperm DNA/Protein G agarose slurry (Millipore). Immunoprecipitated DNA fractions were de-crosslinked at 65°C overnight and purified using Qiaquick columns (Qiagen). Quantitative RT-PCR of de-crosslinked immunoprecipitated DNA was performed using Sybr Green (BioRad) in the iCycler machine with primers flanking specific E2F binding sites. Quantitative RT-PCR reactions
were performed in triplicate with 25μl per reaction in the following conditions: 1 cycle at 95°C for 5 minutes, 20-40 cycles of 95°C for 15 seconds, 60-64°C for 30 seconds and 72°C for 30 seconds. Cycle numbers of genes tested were normalized to the threshold cycle numbers of 1% total input material. Relative enrichment presented as average ± SD.
Figure 4.1. Loss of *E2f7* and *E2f8* in Weaning Age Livers Resulted in a Globally Deregulated Transcriptome. Heatmap of approximately 4,500 differentially expressed genes in weaning age (3wk) livers. Class I: genes repressed by E2F7 and E2F8 (labeled),
Figure 4.1: continued

as this class contained genes that were deregulated in all three mutant groups profiled. Class II: genes repressed by either E2F7 or E2F8 (labeled), as this class contained genes that were deregulated in at least two of the three mutant groups profiled (Alb-78dko and Alb-8ko v. control, Alb-78dko and Alb-7ko v. control, and Alb-8ko and Alb-7ko v. control). Class III: genes synergistically repressed by E2F7/E2F8 (labeled), as this class contained genes that were deregulated in only one of three mutant groups profiled (Alb-78dko v. control, Alb-8ko v. control, and Alb-7ko v. control). Minimum three independent livers were profiled per genetic group. Colors in heatmap represent range of ratio (-1.5 to +1.5) between expression of genes in mutant (e.g. Alb-78dko) samples and the reference value, which is set as the geometric mean of genes in control samples after RMA normalization. control, E2f7<sup>fl/fl</sup>; E2f8<sup>fl/fl</sup>; Alb-78dko, Alb-cre; E2f7<sup>fl/fl</sup>; E2f8<sup>fl/fl</sup>; Alb-8ko, Alb-cre; E2f8<sup>fl/fl</sup>; Alb-7ko, Alb-cre; E2f7<sup>fl/fl</sup>.
Figure 4.2. Validation of Affymetrix Gene Expression Profiling and Functional Annotation of Genes Regulated by E2F7 and E2F8 in Weaning Age Livers.

(continued)
Figure 4.2: continued

(A) Combined heatmap representation of gene expression changes as detected by Affymetrix and NanoString assays in weaning age livers. Values for four independent samples profiled per genetic group were averaged. Colors in “Nano” columns represent fold changes (-3.0 to +3.0) in the expression of 130 genes in a custom NanoString mRNA codeset, of approximately half were selected based on their significant expression changes in Affymetrix arrays. The raw signal intensity values from Affymetrix arrays, after RMA normalization, were extracted for these same 130 genes, and fold change in expression between indicated mutant groups and control group was determined. Expression data from NanoString corroborated Affymetrix results, as majority of the 130 genes assayed demonstrated the same direction of expression change in both assays. The NanoString codeset also contained ten housekeeping genes (not represented in the heatmap) and genes with annotated functions relating to the control of DNA replication, G2/M transition and mitosis. The latter genes (comprising approximately half of the 130) were selected based on their potential to be involved in endocycle control and included a number of bona fide E2F target genes.

(B) Top five categories in Molecular and Cellular Functions represented by Class I genes (genes with increased expression in any of the three mutant groups, see Figure 4.1) as revealed by Ingenuity Pathway Analysis. The category ‘Cell Cycle’ (pie chart) contained the most abundant number of genes, many of which had functions relating to cytokinesis, mitosis and G2/M transition.
Figure 4.3. Loss of E2f7 and E2f8 Resulted in the Depression of a Core Set of Target Genes in TGCs and Weaning Age Livers.

(A) Left, heatmap representation of genes deregulated in 78dko, 8ko and 7ko E10.5 TGCs relative to control samples. Total RNA was isolated from E10.5 TGCs with the indicated genotypes that were obtained through laser capture microdissection. Genes shown in heatmap had deregulated expression in weaning age mutant livers and TGCs. Right, G2/M related genes in the mRNA codeset that were deregulated in TGCs. Colors represent expression fold change in mutant samples relative to control. control, E2f7+/+;E2f8+/+; 78dko, E2f7−/−;E2f8−/−; 8ko, E2f8−/−; 7ko, E2f7−/−.

(B) Chromatin immunoprecipitation (ChIP) assays in human embryonic kidney (HEK) 293 cells demonstrating enhanced occupancy of flag-tagged E2F7 (top six panels) and (continued)
Figure 4.3: continued

E2F8 (bottom six panels) on E2F binding sites in the promoter of G2/M (Ccn2, Cdt2 and Chek1) and G1/S (E2f1) genes. Promoter sequences that served as negative controls, E2f1ds (downstream extronic sequence) and Tub (tubulin), showed lack of binding by E2F7 or E2F8 to these irrelevant sites, demonstrating specificity of these assays. F, anti-flag; Ig, immunoglobulin control; w, flag-E2F7 or E2F8 with wild type DNA binding domains (DBD); m, flag-E2F7 or E2F8 with mutant DBD.
Figure 4.4. Functional Antagonism by E2F Activators and Atypical E2F Repressors in the Transcriptional Control of Target Genes with G2/M Related Functions.

(A) Heatmap representation of downregulated genes in *Mx-123tko* livers whose expression were significantly deregulated in *Alb-78dko* livers. Note majority of genes with decreased expression in *Mx-123tko* samples had instead increased expression in *Alb-78dko* samples (genes antagonistically regulated). Colors in heatmap represent range of ratio (-1.5 to +1.5) between expression of genes in mutant samples and the reference (continued)
Figure 4.4: continued

value, which is set as the geometric mean of genes in control samples after RMA normalization. Two sets of control samples are indicated because RNA from \textit{Mx-123tko} samples were analyzed in a separate Affymetrix experiment. \textit{Alb-78dko, Alb-cre;E2f7^{−/−};E2f8^{−/−}}, \textit{Mx-123tko, Mx-cre;E2f1^{−/−};E2f2^{−/−};E2f3^{−/−}}.

\textbf{(B)} Top five Molecular & Cellular Functions of genes antagonistically regulated by E2F1-3 and E2F7/E2F8 as determined by Ingenuity Pathway Analysis. Detailed annotation of the 53 ‘Cell Cycle’ genes revealed a relative enrichment of genes with G2/M and M phase related functions.

\textbf{(C)} Promoters of genes antagonistically regulated by E2F1-3 and E2F7/E2F8 were analyzed for the presence of E2F binding sites. The analyzed region spanned from 3.0kb upstream of the predicted transcriptional start site (TSS) and 2.0kb downstream of TSS. Of the approximately 700 genes regulated oppositely by E2F1-3 and E2F7/E2F8, 580 contained at least one E2F binding site within the promoter region examined. Of the 580 genes, 45\% had just one E2F binding site that might be competitively bound by either E2F activators or repressors at any given time to dictate transcriptional output (i.e. transactivation or active repression).
Table 4.1. List of genes in NanoString mRNA codeset.

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Chapter 5

Canonical E2F Activators and Atypical E2F Repressors Oppositely Regulate Transcriptional Programs Involved in Endocycle Control

5.1 Introduction

Progression through the mitotic cell cycle is thought to require coordinated E2F dependent transcriptional activation and repression. Consistent with this paradigm, overexpression of E2f activators (E2f1-3) in a variety of cell lines induces ectopic proliferation (Johnson et al., 1993; DeGregori et al., 1997) and their combined ablation in mouse embryo fibroblasts (MEFs) elicits a profound cell cycle arrest (Wu et al., 2001). Conversely, overexpression of canonical E2f repressors (E2f4-6) halts cell cycle progression and their combined ablation in MEFs evokes insensitivity to inhibitory mitogenic signals (Gaubatz et al., 2000). While alluringly simple and supported by decades of experimentation in vitro, key aspects of this paradigm have been difficult to validate in the many E2f knockout and knockin mouse models that have been developed in recent years. In fact, studies in mice have now demonstrated that mitotic cell cycles in a variety of cell lineages proceed without E2F1-3 (Chen et al., 2009b; Chong et al., 2009; Wenzel et al., 2011). Moreover, the prediction that mice with mutant alleles of E2f4, E2f5 or E2f6 would harbor phenotypes opposite to that observed in mice lacking E2f1-3 has yet to be substantiated. Here in this chapter we show that E2F activators antagonize the functions of atypical E2F7 and E2F8 repressors in the regulation of endocycles in vivo.
To the best of our knowledge, these findings represent the first demonstration in which distinct arms of the E2F program coordinate gene expression to control fundamental aspects of mammalian cell cycles \textit{in vivo}.

5.2 Results

Based on the opposite ploidy and gene expression changes exhibited by \textit{Mx-123tko} and \textit{Alb-78dko} hepatocytes we entertained the hypothesis that canonical E2F activators may functionally antagonize atypical E2F repressors in the control of endocycles. To rigorously test this possibility we generated and analyzed mice with hepatocytes deficient for E2F7/E2F8 and one of the E2F activators, E2F1 (\textit{E2f1}\textsuperscript{+/−}). The specific ablation of \textit{E2f7} and \textit{E2f8} alleles in hepatocytes was again achieved by crossing conditional \textit{E2f} mice with \textit{Alb-cre} transgenic mice (\textit{Alb-178tko}). We first examined livers from 2-month-old \textit{Alb-178tko} mice and noticed that these tissues had many more binucleated hepatocytes (\textbf{Figure 5.1A}). Unlike the binucleation we have previously described in \textit{E2f7/E2f8} deficient TGCs that is indicative of inappropriate karyokinesis, binucleation in hepatocytes has been proposed as a possible mechanism leading to their polyploidization (Margall-Ducos, 2007). Next, we observed that 2-month-old \textit{Alb-178tko} livers also had fewer Ki67-, P-H3- and cyclin B1-positive hepatocytes relative to \textit{Alb-78dko} livers, even though the percentage of cyclin A2-positive hepatocytes was not reduced (\textbf{Figure 5.1B}). Thus it would appear that loss of \textit{E2f1} suppressed most if not all mitotic defects in hepatocytes caused by \textit{E2f7/E2f8} deficiency. Given this rescue of mitotic phenotypes, we hypothesized that loss of \textit{E2f1} might also restore polyploidy in cells lacking \textit{E2f7/E2f8}. Thus the ploidy of livers from 2- and 6-month-old mice was
evaluated. This analysis showed that while by 2 months of age ploidy had not been sufficiently increased in Alb-178tko hepatocytes relative to Alb-78dko cells (Figure 5.2A), by 6 months of age ploidy at the 4n and 8n levels was significantly increased in Alb-178tko livers (Figure 5.1E). This age-dependent increase was accompanied by a correspondingly significant reduction in the fraction of 2n hepatocytes in Alb-178tko livers (Figure 5.1E). Inspection of H&E sections confirmed an increase in size of Alb-178tko hepatocytes without dramatically altering hepatic mass (Figure 5.1C and 5.1D).

Finally, given that loss of E2F8 alone resulted in the suppression of hepatocyte endocycles, we also generated and analyzed E2f1+/− mice with ablation of E2f8flo/flo in the liver (Alb-18dko). Ploidy analyses of 2- and 6-month-old Alb-18dko livers recapitulated the results that were obtained in Alb-178tko livers (Figure 5.2E). The same pattern of decreased mitotic cell divisions as well as measurement of cell size and hepatic mass was also observed in young Alb-18dko hepatocytes (Figure 5.2B-5.2D). In summary, loss of E2f1 attenuated the ectopic mitotic proliferation of E2f7/E2f8 hepatocytes early on during development, resulting in a rescue of polyploidization that became evident with increasing age. These data suggest that excess E2F1 function was an important underlying cause of the endocycle defects induced by E2f7/E2f8 deficiency, and are consistent with E2F activators and E2F7/E2F8 having antagonistic functions in hepatocyte endocycles.

5.3 Discussion

Parallel studies examining the effects of E2f1 ablation (E2f1−/−) on ploidy levels of E2f7/E2f8 deficient TGC were carried out in placentas (M. Ouseph). TGC-specific
Ablation of E2f7 and E2f8 was achieved using the Ptfcre/+ system. Triple knockout TGCs from E10.5 Ptf-178tko placentas had visibly larger nuclei and significantly more polyploid genomes than TGCs from Ptf-78dko placentas. Moreover, this increase in ploidy was accompanied by a decrease in the number of multinucleated Ptf-178tko TGCs, consistent with a reduction in karyokinesis. These analyses further support our conclusion from the liver studies that the endocycle defects induced by loss of atypical E2F repressors was due to unopposed function of activator E2Fs, which normally have a role in suppressing endocycles (Chapter 2). A recent survey in vitro of E2F1-3 target genes revealed that a subset of these is involved in the positive control of mitosis, including Cyclin A2, Cyclin B1, Cdc25C and Cdc2 (Ishida et al., 2011). Thus, an early decline in E2F activator function, as ‘artificially’ caused by their genetic inactivation, might have been sufficient to induce TGCs and hepatocytes to precociously enter endocycles, an act that would have led to their excess polyploidization.

Several lines of evidence (presented in this and previous chapters) support antagonistic roles for canonical E2F activators and atypical repressors in orchestrating mammalian endocycles. First and most obvious, the genetic inactivation of activators and atypical repressors in the two endocycling cell lineages of mice, TGCs and hepatocytes, resulted in opposite cellular phenotypes, with E2f1-3 loss promoting larger nuclei with increased ploidy (Chapter 2) and E2f7/E2f8 loss promoting smaller nuclei with reduced ploidy (Chapter 3). Second, their inactivation in these cells led to the altered expression of a common core set of target genes, with E2f1-3 loss leading to their downregulation and E2f7/E2f8 loss leading to their upregulation (Chapter 4, Figure 4.4A). Here we consider two potential mechanisms for how these two E2F arms might normally
coordinate gene expression *in vivo*. The first involves the recruitment of E2F activators and repressors to distinct E2F binding elements present on target promoters. The occupancy of either activators or repressors on these promoter elements would thus be predicted to dictate transcriptional output. Interestingly, a survey performed within this study of common genes regulated by E2F1-3 and E2F7/E2F8 revealed that approximately 40% of target promoters contained just a single E2F binding element within 5.0kb distance spanning the transcriptional start site (Figure 4.4C). This raises a second possibility where competitive binding to the same element would rely on different DNA binding affinities of activators and repressors, or on the spatial and/or temporal regulation of their expression and activity.

Finally, although we did not closely examine these promoters for the presence of other transcriptional binding elements, it is possible that co-factors might also have been intimately involved in recruiting E2Fs to these target promoters. This latter scenario would not be surprising given that both E2F activators and repressors have been found to be capable of associating with a rather diverse cohort of transcriptional factors in chromatin-bound complexes. Because we and others (Maqbool et al., 2010) have demonstrated that E2F1-3 expression appears to be significantly diminished in endocycling cells, E2F7/E2F8 mediated repression would thus be expected to dominate and enforce the mitotic block necessary for continuous endocycles after M-E transition. In line with this interpretation, we propose that the antagonism exhibited by E2F activators and atypical repressors would mainly be responsible for coordinating cellular events that enable the M-E switch.
Finally, in this chapter we show that the inactivation of a single E2F activator (E2F1) is sufficient to suppress endocycle defects caused by $E2f7/E2f8$ deficiency. Interestingly, the level of cyclin A2 protein, which had been elevated in $E2f7/E2f8$ deleted TGCs and hepatocytes, was not reduced by the ablation of $E2f1$. This observation would suggest that cyclin A2, given its critical role at the G2/M transition, either is regulated independently of E2F1 at the transcriptional level or perhaps more likely, is predominantly regulated at the post-transcriptional level (e.g. by APC/C). Intriguingly, genetic inactivation of cyclin A2 led to a dramatic increase in the ploidy of hepatocytes. How this might be accomplished will be the subject of next chapter’s investigation.

To summarize, our results in Chapters 2-5 suggest that two arms of the E2F program, an activating arm (E2F1-3) that is dependent on dimerization with DP proteins and is regulated by canonical Cdk-Rb signaling and a second atypical repressor arm ($E2f7/E2f8$) that is both Rb- and DP-independent, converge to antagonistically regulate the endocycle. Interestingly, a third polyploid cell type in mammals is the megakaryocyte, and here again loss of $E2f7/E2f8$ leads to decreased polyploidization (M. Ouseph), although the consequence of concomitant $E2f1$ ablation was not evaluated in this context. Thus, the conserved role of atypical E2Fs in promoting polyploidy in three distinct tissues of the mouse likely reflects a generalized role in mammalian endocycle control that is also critically dependent on balanced E2F activator function.

5.4 Materials and methods

*Mice and genotyping*
E2f7^ff, E2f8^ff and Alb-cre transgenic mice were maintained in a pure FVB/N background. E2f1^- mice were maintained in a mixed background composed of 129SvEv, C57B/L6 and FVB. All mice were housed in barrier conditions with a 12-hour light/dark cycle and had access to food and water ad libitum. All protocols involving mice in this study were approved by the Institutional Animal Care and Use Committee at The Ohio State University. PCR genotyping was performed using genomic DNA purified from tails following a standard protocol (digestion with proteinase K followed by isopropanol precipitation). Sequences for genotyping primers have been provided in Chapters 2 and 3.

Flow cytometry

This procedure was performed exactly as described in Chapter 2.

Immunostaining and hepatocyte quantification

These procedures were performed exactly as described in Chapter 3. All IHC quantification data were reported as an average percentage ± SD of positive cells from the total cell population in the number of control and mutant samples analyzed.
Figure 5.1. Loss of E2f1 Restored Polyploidy in E2f7/E2f8 Deficient Hepatocytes by Partially Reducing Mitotic Division.

(A) Quantification of binucleated hepatocytes in 2-month-old livers (see genotype key in E). Note significant increase in percent binucleated hepatocytes in Alb-178tko livers relative to Alb-78dko livers. There was no significant difference in percent binucleated hepatocytes between control and Alb-178tko livers. Quantification data presented as average ± SD. At least n=3 independent mice analyzed in control and Alb-78dko (continued)
Figure 5.1: continued

genotypes; n=3 independent mice analyzed in *Iko* and *Alb-178tko* genotypes. Two-tailed Student T-test, ** p≤0.01.

(B) Quantification of hepatocyte proliferation in 2-month-old livers (see genotype key in E). Note significant decrease in percent Ki67 and P-H3 positive hepatocytes in *Alb-178tko* livers relative to *Alb-78dko* livers. Quantification data presented as average ± SD. At least n=3 independent mice analyzed in *control* and *Alb-78dko* genotypes; n=3 independent mice analyzed in *Iko* and *Alb-178tko* genotypes. Two-tailed Student T-test, * p≤0.05, *** p≤0.001.

(C) Representative H&E liver sections from 6-month-old mice demonstrating enlarged appearance of *Alb-178tko* hepatocytes relative to *Alb-78dko* hepatocytes.

(D) Measurement of percent hepatic mass in 6-month-old mice demonstrating a significant decrease in hepatic mass (although within normal limits) in *Alb-178tko* livers relative to *Alb-78dko* livers. Hepatocyte quantification also demonstrated a significant reduction in number of cells per 40x field in *Alb-178tko* livers (indicative of increased hepatocyte size) relative to *Alb-78dko* livers. Quantification data presented as average ± SD. At least n=3 independent mice analyzed in *control* and *Alb-78dko* genotypes; n=5 independent mice from *Iko* and n=3 *Alb-178tko* mice analyzed. Two-tailed Student T-test, ** p≤0.01, *** p≤0.001.

(continued)
Figure 5.1: continued

(E) Flow cytometry of liver nuclei in 6-month-old mice. Note significant increase in proportion of 4n nuclei in Alb-178tko livers relative to Alb-78dko livers, and the significant decrease in proportion of 2n nuclei. Loss of E2f1 alone resulted in significant increases in proportion of 8n and 16n nuclei relative to control livers (a small percentage of 32n nuclei was even detected). Quantification data presented as average ± SD. n, number of independent mice analyzed per genotype. One way Anova, *** p≤0.001. control, E2f7<sup>−/+</sup>;E2f8<sup>−/+</sup>; 1ko, E2f1<sup>−/−</sup>; Alb-78dko, Alb-cre;E2f7<sup>−/+</sup>;E2f8<sup>−/+</sup>; Alb-178tko, Alb-cre;E2f1<sup>−/−</sup>;E2f7<sup>−/+</sup>;E2f8<sup>−/+</sup>. 
Figure 5.2. Loss of E2f1 Restored Polyploidy in E2f8 Deficient Hepatocytes.

(A) Flow cytometry demonstrating similar ploidy levels between Alb-78dko and Alb-178tko livers from 2-month-old mice. However, hepatocyte ploidy levels increased in Alb-178tko livers by 6 months of age, whereas ploidy levels remained the same in Alb-78dko livers (compare with Figure 5.1E). This data suggest that Alb-178tko hepatocytes, unlike Alb-78dko hepatocytes, were able to undergo polyploidization over time. Quantification data presented as average ± SD.

(continued)
Figure 5.2: continued

(B) Quantification of hepatocyte proliferation in 2-month-old livers (see genotype key in E). Note significant decrease in percent Ki67 and cyclin A2 positive hepatocytes in Alb-18dko livers relative to Alb-8ko livers. Quantification data presented as average ± SD. At least n=3 independent mice analyzed in control and Alb-8ko genotypes; n=3 independent mice analyzed in Iko and Alb-18dko genotypes. Two-tailed Student T-test, * p≤0.05, *** p≤0.001. Quantification of hepatocyte proliferation in Alb-78dko and Alb-178tko livers is presented again (from Figure 5.1B) for comparison.

(C) Measurement of percent hepatic mass in 6-month-old mice demonstrating no significant difference between Alb-18dko and Alb-8ko livers (see genotype key in E). Hepatocyte quantification demonstrated a significant reduction in number of cells per 40x field in Alb-18dko livers (indicative of increased hepatocyte size) relative to Alb-8ko livers. Quantification data presented as average ± SD. At least n=3 independent mice analyzed in control and Alb-78dko genotypes; n=5 independent mice from Iko and n=3 Alb-178tko mice analyzed. Two-tailed Student T-test, ** p≤0.01. Data for Alb-78dko and Alb-178tko mice are presented again (from Figure 5.1D) for comparison.

(D) Representative H&E liver sections from 6-month-old mice showing similar cell size between control and Alb-18dko hepatocytes.

(E) Flow cytometry of liver nuclei in 6-month-old mice. Note significant increase in
Figure 5.2: continued

proportion of 4n nuclei in Alb-18dko livers relative to Alb-8ko livers, and the significant decrease in proportion of 2n nuclei. Loss of E2f1 alone resulted in significant increases in proportion of 8n and 16n nuclei relative to control livers. Quantification data presented as average ± SD. n, number of independent mice analyzed per genotype. One way Anova, *** p≤0.001. Ploidy data for Alb-78dko and Alb-178tko mice are presented again (from Figure 5.1E) for comparison. control, E2f7f/f;E2f8f/f; 1ko, E2f1−/−; Alb-8ko, Alb-cre;E2f8f/f; Alb-18dko, Alb-cre;E2f1−/−;E2f8f/f; Alb-78dko, Alb-cre;E2f7f/f;E2f8f/f; Alb-178tko, Alb-cre;E2f1−/−;E2f7f/f;E2f8f/f.
Chapter 6

Reinstating G2/M Block by Ablating Cyclin A2

Function Restores Polyploidy of E2f7/E2f8 Deficient Cells

6.1 Introduction

Cyclin A was the first cyclin to be identified and cloned in any organism (Swenson et al., 1986). The mammalian cyclin A family contains just two isoforms, cyclin A1 and cyclin A2 (Nieduszynski et al., 2002), that are encoded by two distinct genes and differ by just one amino acid residue at their N-termini. In mice, expression of cyclin A1 is restricted to the developing testes (Sweeney et al., 1996; Yang et al., 1997) while cyclin A2 is ubiquitously expressed in all proliferating cells (Pines and Hunter, 1990). The targeted inactivation of cyclin A2 results in early lethality of mouse embryos shortly after implantation by approximately E6.5 (Murphy et al., 1997), suggesting that cyclin A2 is essential for the proliferation of mammalian cells. However, recent work from the Sicinski lab has demonstrated a requirement for cyclin A2 function in the proliferation of hematopoietic and embryonic stem cells while in fibroblasts, the loss of A2 was compensated by an upregulation in the levels of cyclin E. This compensatory increase in E-type cyclins permitted already proliferating mouse embryonic fibroblasts to continue cell division, although it was insufficient to induce their re-entry into the cell cycle from a quiescent state (Kalaszczynska et al., 2009).
The expression of cyclin A2 is induced at the G₁/S transition, and the activity of cyclin A2-Cdk2 is believed to be necessary for S phase progression. An key substrate of the cyclin A2-Cdk2 complex that is phosphorylated at the beginning of S phase is Cdh1, a subunit of APC/C that when bound determines target specificity of this E3 ubiquitin ligase complex. The inhibitory phosphorylation of Cdh1 mediated by cyclin A2-Cdk2 has been shown to require an intact cyclin-binding motif in the WD-40 domain of Cdh1 (Sorensen et al., 2001). An important consequence of inhibiting APC/C function during S and G₂ phases is the gradual accumulation of cyclin B1. While cyclin B1 is not required for entry into mitosis (this has been postulated to be a role that is instead fulfilled by cyclin A2), cyclin B1-Cdk1 mediated phosphorylation of numerous substrates is essential for completing and exiting the M phase given that cyclin A2 is rapidly degraded after mitosis begins. Whether cyclin B1 is functionally interchangeable with cyclin A2 in inducing entry into mitosis is currently unclear and has not been determined by knockin approaches in the mouse. However, it is likely that different cyclin partners (e.g. cyclin B1-Cdk2) may confer either different substrate specificities of the associated Cdks or different affinities of the Cdks for a subset or all of the same substrates. Therefore, in addition to being generally regarded as the essential S phase cyclin, a view that incidentally has now been challenged (Kalaszczyńska et al., 2009), cyclin A2 is also required to propel cells into M phase. With emerging studies reporting novel kinase independent functions of mammalian cyclins (e.g. D-type cyclins) that are not restricted to cell cycle regulation (Bienvenu et al., 2010; Jirawatnotai et al., 2011), it remains to be seen whether this might be true for other members of the cyclin family, which is believed
to be comprised of up to 29 structurally related proteins (Malumbres and Barbacid, 2009).

In this chapter, we show that quadruple deficiency in cyclin A1, cyclin A2, E2f7 and E2f8 ‘normalized’ the ploidy of hepatocytes to near wild type levels. Furthermore, ablation of just cyclin A1 and A2 in hepatocytes was sufficient to produce a drastic increase in their ploidy, which was accompanied by a number of molecular and physiologic abnormalities. Beyond a cursory examination of these fascinating phenotypes, we did not explore the precise mechanisms underlying them in the context of our endocycle studies. However, they would undoubtedly serve well as the basis of future investigations on metabolic dysfunctions relating to the hepatobiliary system.

6.2 Results

Thus far our results have demonstrated that the inactivation of E2f7/E2f8 induces ectopic mitoses to occur but without inhibiting DNA replication, as many cell lineages in double knockout placentas, including TGCs, remained competent for BrdU incorporation (Ouseph et al., in review). Similarly, the conditional inactivation of E2f7/E2f8 in the liver led to an increase in the number of hepatocytes expressing the proliferation associated antigen, Ki67 (Chapter 3). Based on these observations, we predicted that re-establishment of a mitotic block, without perturbing S phase progression, would restore genome polyploidy in E2f7/E2f8 deficient cells. Therefore, we evaluated the consequence of inactivating cyclin A2 in E2f7/E2f8 deficient hepatocytes. Cyclin A2 was a logical candidate given that its expression had been significantly increased in E2f7/E2f8 deficient TGCs and hepatocytes. Moreover, their elevated expression was presumably the
underlying cause of the mitotic phenotypes (Sorensen et al., 2001; Lukas et al., 1999) induced by ablation of E2f7/E2f8 in TGCs and hepatocytes. A second important reason that led us to focus on cyclin A2 is the observation that loss of E2f1 was not sufficient to result in a ‘rescue’ of the ectopic cyclin A2 protein levels in E2f7/E2f8 deficient cells. The persistent levels of cyclin A2 in Alb-178tko hepatocytes and Plf-178tko TGCs could therefore explain the result that polyploidy in these triple knockout cells was unable to reach completely wild type levels (Figure 5.1B and 5.1E). This would additionally suggest that cyclin A2 is unlikely to be directly regulated by E2F activators, or that post-transcriptional mechanisms governing cyclin A2 stability may have greater influence over the rate of transcriptional output in dictating the final intracellular levels of A2.

To avoid possible compensation by cyclin A1, we intercrossed cyclin A1<sup>−/−</sup>;cyclin A2<sup>+/−</sup> mice with Alb-cre;E2f7<sup>−/−</sup>;E2f8<sup>−/−</sup> mice to generate offspring with quadruply deficient hepatocytes (Alb-A1278qko). Remarkably, hepatocytes in 3-month-old Alb-A<sup>1278qko</sup> mice had similar ploidy levels, as determined by the Feulgen technique, as hepatocytes in age-matched wild type mice (Figure 6.1B). Interestingly, the loss of cyclin A1 and cyclin A2 (Alb-A<sup>12</sup>) or cyclin A2 alone (Alb-A<sup>2</sup>) resulted in hyperploidy (Figure 6.1B and data not shown) while loss of cyclin A1 alone had no effect on genome size (data not shown). Furthermore, the striking hyperploidy induced by cyclin A2 inactivation was associated with a number of notable abnormalities in the liver (Figure 6.2), but the exact basis for this latter observation remains to be determined. While these molecular abnormalities appeared to be partially corrected in Alb-A<sup>1278qko</sup> livers (H&E, Figure 6.1A), it is unknown at the moment whether hepatocyte physiology might have been altered. In summary, inactivating a core component of the mitotic cell cycle machinery (cyclin A2)
re-established a mitotic block and reinstated higher levels of polyploidy in $E2f7/E2f8$ deficient cells.

6.3 Discussion

Our gene ontology analyses of genes repressed by E2F7 and E2F8 at the key developmental time of M-E switch in hepatocytes revealed a bias towards mitosis related genes. Certainly, a key component among these genes pertinent to the control of endocycle is cyclin A2. Indeed, transcriptional repression of all members of the Arabidopsis CYCLINA2 family by ILP1 (polyploidy1) represents a key event in endoreplication control, with the loss of CYCA2;1 producing an ectopic increase in polyploidy (Yoshizumi et al., 2006). Another A-type cyclin CYCA2;3 is also a critical regulator of ploidy levels in Arabidopsis, whose genome encodes multiple highly redundant cyclin A genes (Imai et al., 2006). Co-expression of CYCA2;3 with the M phase-associated cyclin dependent kinase CDKB1;1 (the equivalent of Cdk1 in mammals) inhibited endocycle onset and triggered excess mitotic divisions in plant cells (Boudolf et al., 2009). Thus, a series of elegant studies in plants have already explored the role of cyclin A2 in endocycle control, but until our study its role in mammalian endocycles has not been directly evaluated, in part due to the early death of cyclin A2 null embryos shortly after implantation.

Parallel studies evaluating how loss of cyclin A family members might impact the ploidy of $E2f7/E2f8$ deficient TGCs were carried out (M. Ouseph). $Cyclin A1^{-/-};cyclin A2^{ff}$ mice were intercrossed with $Plf^{rel+};E2f7^{ff},E2f8^{ff}$ mice to generate embryos and associated placentas with quadruply deficient TGCs ($Plf^{-A^{12}78qko}$). As revealed by
Feulgen analysis, TGCs in E10.5 Plf-A\textsuperscript{12}78\textit{qko} placentas had larger nuclei with higher ploidy levels than TGCs in Plf-78\textit{dko} placentas, with some quadruple knockout cells having reached 256n. However, unlike in the liver, inactivation of the two cyclin A isoforms alone (Plf-A\textsuperscript{12}) did not result in hyperploidy of TGCs when compared with wild type TGCs. This should be considered a landmark experiment because to the best of our knowledge, it is the first time that the inactivation of a core component of the cell cycle machinery with a strong implication in endocycle control has been achieved in a lineage (TGC)-specific manner. It would now be important to re-evaluate, using the Plf\textsuperscript{cre/+} system, the role of other proteins (e.g. cyclin E1/E2 and Cdh1) that previously based on gene inactivation strategies targeting the whole conceptus (placenta and embryo) have been deemed necessary for mammalian endocycles.

Small but fundamental differences in the biology of TGC and hepatocyte cell cycles may explain the disparate effects on their ploidy levels resulting from cyclin A2 inactivation. First, it is known that TGCs exclusively utilize endocycles upon reaching terminal differentiation (Cross, 2005) whereas hepatocytes have been shown to be capable of utilizing additional mechanisms to achieve polyploidy, such as fusion with bone marrow derived cells (Willenbring et al., 2004; Wang et al., 2003). Second, TGCs have rather short life spans when compared with hepatocytes, whose survival (as a whole population of cells that comprise the liver) must endure over the course of an organism’s lifetime. Although the liver is normally considered one of the most quiescent organs in the body, there is still a physiologic level of hepatocyte turnover necessary for maintaining tissue homeostasis (Gupta, 2000). A fraction of hepatocytes in a wild type liver might be expected to express moderate levels of cyclin A2, and these hepatocytes
might represent the small pool of liver parenchymal cells that ‘self-renew’ through mitotic proliferation at any given time. Certainly the loss of A2 function in these specialized cells might be expected to result in continuous DNA replication without mitosis thereby explaining their hyperploidy. But because our analysis demonstrated that almost all Alb-A\textsubscript{12} hepatocytes had ectopic ploidy, it is possible to attribute this to the timing of A2 inactivation achieved by Alb-cre. Using this system, the onset of deletion would have coincided with a time when A2 function was still essential in proliferating hepatocytes in the perinatal period, thereby resulting in a strong mitotic block in most if not all hepatocytes significantly prior to the scheduled onset of the M-E transition. To determine whether A2 inactivation would lead to hyperploidy in fully quiescent and mature hepatocytes, an inducible gene ablation strategy such as SA-cre\textsubscript{ER}\textsuperscript{T2} (Metzger et al., 2002) would have to be utilized. Finally, it could be that Plf\textsuperscript{cre/+} mediated ablation of cyclin A2, starting at approximately E6.5 (data not shown), failed to further augment TGC ploidy because the timing of A2 inactivation coincided with the start of terminal differentiation in TGCs. This terminal differentiation signals the beginning of M-E transition (Cross, 2005) and would naturally have required the complete inhibition of cyclin A2 expression, thus rendering its genetic or ‘artificial’ inactivation rather obsolete.

The additional loss of cyclin A1/A2 from E2f7/E2f8 deficient hepatocytes restored their ploidy to near wild type levels (Figure 6.1B) but without completely correcting all aberrant molecular events, which also included the ectopic accumulation of cyclin B1 proteins (Figure 6.2). The persistent level of cyclin B1 in 3-month-old Alb-A\textsubscript{12}\textsuperscript{78qko} hepatocytes might have been the result of compensatory upregulation due to absence of cyclin A2. Another possibility is that cyclin B1 proteins accumulated over multiple
earlier division cycles due to deregulated APC/C function. The observation that many Alb-A1278qko hepatocytes stained strongly positive for cyclin B1 in the cytoplasm suggests a lack of Cdk1 activation in the nucleus, which would explain how Alb-A1278qko hepatocytes exhibited ectopic polyploidy despite high levels of B1. Interestingly, the Kaldis laboratory recently showed that hepatocyte specific ablation of Cdk1 function, utilizing also the Alb-cre transgenic system, led to a hyperploidy phenotype in the liver reminiscent of that accomplished by cyclin A2 deletion (G. Leone and P. Kaldis, personal communication). On a broader level, these results suggest that mechanisms regulating endocycle, no matter how divergent they may appear, must ultimately converge on the control of the M phase associated Cdk1 function.

6.4 Materials and methods

Mice and genotyping

E2f7fl/fl, E2f8fl/fl and Alb-cre transgenic mice were maintained in a pure FVB/N background. The conditional cyclin A2 and cyclin A1fl/fl mice were maintained in a mixed genetic background composed of 129SvEv, C57B/L6 and FVB. The cyclinA1fl/fl;cyclinA2fl/fl mice were generously provided by D. Wogelmuth and P. Sicinski. All mice were housed in barrier conditions with a 12-hour light/dark cycle and had access to food and water ad libitum. All protocols involving mice in this study were approved by the Institutional Animal Care and Use Committee at The Ohio State University. PCR genotyping was performed using genomic DNA purified from tails following a standard protocol (digestion with proteinase K followed by isopropanol precipitation). cyclinA1ko:

5’CCTTTGGGCGGATTGTGTTT, 5’GGTCCTCCTGTACTGCTCAT,
Ploidy analysis using the Feulgen technique

Five μm paraffin liver sections were deparaffinized in xylene, treated with 1N hydrochloric acid for eight minutes and stained with the Schiff reagent for one hour at room temperature. Aqueous light green solution (1%) was used to counterstain the cytoplasm. Stained tissues were imaged using Axioskop 40 (Zeiss). Nuclear intensity was quantified using the software ImageJ (http://rsbweb.nih.gov/ij/) following standard protocols (Hardie et al., 2002). Hepatocyte ploidy in liver samples from 3-month-old mice (in Figure 6.1A and 6.1B) was expressed relative to diploid-tetraploid (2n-4n) hepatocytes from wild type weaning age (3wk) livers. The average percent (± standard deviation) of hepatocytes classified into different bins, which represent different ploidy levels, was plotted in histogram format. Hepatocyte ploidy is expressed on a per nucleus basis.

Immunostaining and quantification

These procedures were performed exactly as described in Chapter 3. All IHC quantification data were reported as an average percentage ± SD of positive cells from the total cell population in the number of control and mutant samples analyzed.
Figure 6.1. Hepatocytes Quadruply Deficient for *cyclin A1*, *cyclin A2*, *E2f7*, and *E2f8* Exhibited Near Normal Levels of Polyploidy.

(A) Representative H&E and Feulgen stained liver sections from 3-month-old mice with the indicated genotypes. Note as before the appearance of smaller hepatocytes in *Alb-78dko* livers relative to *control* livers. Loss of *cyclin A1* and *cyclin A2* (*Alb-A12*) resulted in significantly enlarged hepatocytes, a subpopulation of which had dysmorphic nuclei containing what appeared to be cytoplasmic intrusions; also note the appearance of apoptotic bodies in these livers. Quadruple ablation of *cyclin A1*, *cyclin A2*, *E2f7* and *E2f8* (continued)
Figure 6.1: continued

*E2f8* (*Alb-A<sup>1278qko</sup>*) function resulted in more normal appearing hepatocytes in the liver.

**(B)** Quantification of hepatocyte ploidy using the Feulgen method in livers of 3-month-old mice. Ploidy levels in *control* and *Alb-78dko* hepatocytes as determined by the Feulgen method corroborate the ploidy data obtained in similarly aged liver samples by flow cytometry. Livers lacking *cyclin A1* and *cyclin A2* had increased proportion of hepatocytes with higher ploidy levels (16n, 32n and 64n) relative to *control* livers. Livers quadruply deficient for *cyclin A1*, *cyclin A2*, *E2f7* and *E2f8* had significantly increased proportion of hepatocytes with more polyploid genomes relative to *Alb-78dko* livers. n, number of independent mice analyzed per genotype. Quantification data presented as average ± SD. One way Anova, * p≤0.05.

**(C)** Measurement of percent hepatic mass and hepatocyte proliferation. Percent hepatic mass of 3-month-old *Alb-A<sup>12</sup>* and *Alb-A<sup>1278qko</sup>* mice was compared with that of 2-month-old *control* and *Alb-78dko* mice. *Alb-A<sup>12</sup>* and *Alb-A<sup>1278qko</sup>* mice had percent hepatic mass that were beyond upper limit of normal range, approaching 6%. Quantification of immunostained liver sections showed a dramatic increase in the percent of Ki67 and P-H3 positive *Alb-A<sup>12</sup>* hepatocytes relative to *control* hepatocytes. The percent of Ki67 positive hepatocytes was similar between *Alb-A<sup>1278qko</sup>* and *Alb-A<sup>12</sup>* livers, while the percent of P-H3 positive hepatocytes was decreased in *Alb-A<sup>1278qko</sup>* relative to *Alb-A<sup>12</sup>* livers. Comparisons were carried out between 3-month-old *Alb-A<sup>12</sup>* and *Alb-A<sup>1278qko</sup>* (continued)
Figure 6.1: continued

livers and 2-month-old control and Alb-78dko livers. n, number of independent mice analyzed per genetic group. Quantification data presented as average ± SD. control, E2f7^{+/−};E2f8^{+/−}; Alb-A^{12}, Alb-cre;cyclinA1^{+/−};cyclinA2^{+/−}; Alb-78dko, Alb-cre;E2f7^{+/−};E2f8^{+/−}; Alb-A^{12}78qko, Alb-cre;cyclinA1^{+/−};cyclinA2^{+/−};E2f7^{+/−};E2f8^{+/−}. 
**Figure 6.2. Loss of cyclin A2 Led to Prominent Molecular and Physiologic Abnormalities in the Liver.** Livers from 3-month-old *control* (not shown), *Alb-cre;cyclin A1* (Alb-A1), *Alb-cre;cyclinA2* (Alb-A2, not shown) and *Alb-cre;cyclinA1*;*cyclinA2* (Alb-A12) were harvested and examined. The *control* and Alb-A1 livers were indistinguishable in their gross and microscopic (H&E) appearance, suggesting cyclin A1 is dispensable for hepatocyte cell cycles consistent with their tissue-restricted expression during development. Note the advanced cirrhotic appearance of Alb-A12 livers grossly, while evaluation of H&E stained sections revealed significantly enlarged hepatocytes (with dramatically enlarged nuclei containing greater DNA content, see Figure 6.1B).

There also appeared to be a significant amount of lymphocytic infiltration accompanied by the presence of apoptotic bodies. H&E sections further demonstrated the presence of highly prominent bile ducts and biliary network structures. Co-immunofluorescence with Ki67 and cytokeratin 8 (CK8) antibodies confirmed many bile duct epithelial cells are proliferating (M. Mair and R. Kladney, not shown). Trichrome staining revealed a (continued)
significant amount of fibrosis in the liver, while cyclin B1 immunohistochemistry showed
abnormal accumulation of cyclin B1 protein in the hepatocytes. P-H3 immunohistochemistry (not shown, quantified in Figure 6.1C) also revealed a number of hepatocytes in abnormal mitoses (e.g. tripolar). The Alb-A2 and Alb-A12 livers were indistinguishable in their gross and microscopic appearance, demonstrating that liver-specific loss of cyclin A2 was the cause of these highly aberrant phenotypes. All images shown are representative of at least n=3 independent mice analyzed per genotype.
Chapter 7

The Atypical Repressor E2f8 Links Endocycle Control to Cancer

7.1 Introduction

The deregulated expression of E2F7 and E2F8 has been observed in several types of human cancer, and this is believed to contribute to the uncontrolled proliferation of malignant cells (Reimer et al., 2006, 2007; Endo-Munoz et al., 2009; Deng et al., 2010; Hazar-Rethinam et al., 2011). Given the general role prescribed for these E2Fs in transcriptional repression of genes related to cell cycle progression (de Bruin et al., 2003; Maiti et al., 2005), it is not difficult to conceptualize how E2F7 and E2F8, by inhibiting cell proliferation, might act as potent tumor suppressors. However, inconsistent with the prediction for an involvement in tumor suppression, E2F repressors as a whole have not been frequently found mutated, deleted or their expression silenced in human cancers (Chen et al., 2009a). Instead, increased copy numbers through amplification (E2F5 in breast cancer) as well as overexpression (E2F8 in primary liver cancer) have been reported (Polanowska et al., 2000; Umemura et al., 2009; Deng et al., 2010). Moreover, efforts over the past decade to construct mouse models investigating the cancer related roles of canonical repressors have produced rather confounding results (Lee et al., 2002; Parisi et al., 2009), while innovative mouse models of the atypical E2F repressors have simply been lacking.
The primary liver cancer hepatocellular carcinoma (HCC) is one of the deadliest cancers globally, with an incidence of approximately 600,000 new cases each year (Villanueva and Llovet, 2011). Most cases are diagnosed at advanced stages and therefore are not amenable to curative treatments such as surgical resection, organ transplantation or radiofrequency ablation (Villanueva and Llovet, 2011). In rodent models it had been noted that HCC displays a “diploid pattern of growth”, meaning that the rapidly proliferating malignant cells were predominantly or even exclusively diploid (Sarafoff et al., 1986; Sargent et al., 1989). Moreover, a direct negative correlation between degree of polyploidization and proliferative capacity was consistently observed in both benign neoplastic nodules (Saeter et al., 1988) and HCC (Schwarze et al., 1991; Gerlyng et al., 1992). Finally, the pattern of HCC growth has additionally been described as ‘non-binucleating’, which is observed during liver regeneration induced by 70% partial hepatectomy (in rodents, complete regeneration occurs within 7-10 days). Therefore, suppression of both binucleation and polyploidization was presumed to confer a proliferative advantage by being energetically less expensive. Additionally, diploid genomes were presumed to be less protected against recessive mutations (less ‘buffering’) and be more prone than polyploid cells to undergo stochastic or mutation-based progression towards malignancy (Schwarze et al., 1984).

A study now has reported deregulated expression of E2F8 in human HCC, a highly interesting finding given the seemingly indisputable connection we (and another independent lab group) had made between E2F8 function and control of hepatocyte polyploidization. Thus an emerging question became whether the cancer related role of
E2F8 in HCC would be mechanistically linked to its physiologic role in endocycle control, a question that we will attempt to address with studies presented in this chapter.

7.2 Results

It has been assumed that polyploidy in mammals, as in plants and flies, is integral to the biological functions of endocycling cells. We thus examined the physiological consequences of reduced hepatocyte ploidy in adult development. Surprisingly, Alb-78dko mice containing livers with diploid hepatocytes survived to old age (up to >20 months) in apparent good health and retained the capacity to regenerate their livers upon chemical (carbon tetrachloride) or physical (partial hepatectomy) injury (Figure 7.1 and data not shown). Histopathology of Alb-78dko male livers, however, revealed a marked incidence (~65%) of spontaneous hepatocellular carcinoma (HCC) by nine months of age (Figure 7.2A). No spontaneous HCC lesions were observed in Alb-78dko female mice or in wild type control male and female mice. In order to ascertain which of the two atypical E2Fs harbors the tumor suppressor function, we subjected wild type, Alb-7ko, Alb-8ko and Alb-78dko male and female mice to an established model of diethylnitrosamine (DEN)-initiated HCC, which induces malignancies within a reasonable experimental time frame (Vesselinovitch and Mihailovich, 1983). Under this experimental regimen and consistent with results from past studies (Naugler et al., 2007), only male Alb-8ko and Alb-78dko mice developed highly advanced HCC, with total tumor mass reaching 2-3 times the normal liver weight by nine months of age (Figure 7.2B, 7.2D and 7.2E). Importantly, the frequency of HCC lesions was also much higher in DEN-treated male Alb-8ko and Alb-78dko mice than in gender- and age-matched wild type controls (Figure
7.2C and 7.2D). Together these results suggest that E2F8 suppresses both the initiation and progression of HCC.

We further evaluated the expression of a selected group of E2F7/E2F8 targets with established oncogenic roles, including Stnn1, Survivin (Birc5), Iqgap1, and cyclin A2 in both tumor and normal adjacent liver tissue. Consistent with the higher proliferative status of tumor cells, the expression of these genes was increased in tumors from DEN-treated wild type mice relative to adjacent normal tissue (Figure 7.3A and 7.3B), and even higher in tumors from DEN-treated Alb-8ko (Figure 7.3A) or Alb-78dko (Figure 7.3B) mice. The ploidy status of tumor samples was also evaluated. Flow cytometry analysis demonstrated that every tumor derived from DEN-treated wild type mice contained predominantly diploid cells, with some tumors containing a small proportion of cells with >4n (Figure 7.3C and 7.3D), suggesting that tumors are more likely to arise from a diploid cell of origin. In contrast, adjacent normal tissue was composed of hepatocytes having a range of ploidy, with cells >4n representing a significant proportion of the cell population (Figure 7.3C and 7.3D). Tumor cells derived from DEN-treated Alb-8ko and Alb-78dko mice were strictly diploid, just like their normal counterparts (Figure 7.3C). Together, these data demonstrate for the first time a tumor suppressor function for E2F8, which coincides with reduced ploidy of tumor precursor cells and elevated expression of genes having oncogenic potential.

7.3 Discussion

Endocycles in mammals are utilized to achieve polyploidy, which is thought to support essential functions in developing placentas and livers of young adults. Here, we
generated the first mouse models to significantly alter ploidy state that can be used to assess how trophoblasts and hepatocytes respond to a variety of external and internal signals. Surprisingly, we show here that TGCs and hepatocytes with severe reductions in ploidy can nonetheless bestow placentas and livers, respectively, with apparently normal physiological function. Unexpectedly, Plf-78dko embryos containing TGCs with abnormally low ploidy (<64n compared to <1054n in wild type embryos) could be carried to term with no deleterious consequence to either placentation or fetal development (Ouseph et al., in review). Moreover, the only adverse outcome related to diminished ploidy in E2f8 deficient livers was development of HCC in male mice.

How E2F8 imparts tumor suppression is up for speculation. Studies in yeast have linked polyploidy to genomic instability by showing that newly formed polyploid cells display a higher rate of chromosome loss (Storchova and Pellman, 2004), but this association remains poorly defined. On the other hand, it has been proposed that polyploidy may buffer against somatic mutations by providing cells with additional copies of functional wild type alleles (Otto and Whitton, 2000). In this study we show that livers furnished exclusively with diploid hepatocytes develop both spontaneous and chemical-induced HCC with much more severity than livers composed of hepatocytes with polyploid genomes. Whether or not diploidy is a strict requirement for cancer initiation is unclear, but our analysis of DEN-treated wild type mice containing hepatocytes with a range of ploidy states suggests that there is a strong bias for cancer to arise from diploid cells. In this view, the role of E2f8 in tumor suppression may be co-dependent on its ability to promote hepatocyte endocycles.
An intriguing question raised now is whether polyploid cells might be capable of initiating tumorigenesis, since an implicit assumption we could have made based on existing studies (Schwarze et al., 1991; Gerlyng et al., 1992) was that tumors in wild type livers arose from diploid and not polyploid progenitors. However, at the time of DEN administration to 20-day-old pups just before weaning, livers in both wild type and mutant (Alb-7ko, Alb-8ko and Alb-78dko) mice were composed of primarily diploid hepatocytes. Thus the pool of cells susceptible to DEN-induced mutagenic change would have been equivalent in livers of all genotype we evaluated. The result that only E2f8 (Alb-8ko and Alb-78dko) deficient but not wild type livers went on to develop highly aggressive HCC would appear to suggest additional anti-tumor functions for E2F8, such as its association with induction of DNA damage repair mechanisms (Zalmas et al., 2008), that might be activated by carcinogen exposure. Another interpretation of our result would also suggest that polyploidization of hepatocytes in wild type livers subsequent to carcinogen treatment provided a certain degree of protection from tumor initiation. However, this latter interpretation makes it difficult to determine whether polyploidization, which is promoted by E2F8, acted alone in suppressing tumor initiation.

Therefore, our previous question of if and how polyploid cells might give rise to cancer is difficult to address but is certainly not without precedence. Recent studies from the Grompe laboratory, in fact, examined the frequency of ‘ploidy reversal’ in vivo resulting from naturally as well as fusion-derived polyploid hepatocytes undergoing mitotic cell divisions (Duncan et al., 2010; Duncan et al., 2009). The result of these elegant studies showed that one-step somatic ‘reductive mitoses’ is accompanied by a rather high incidence of aneuploidy in the newly formed offspring. Furthermore, a novel
cell cycle that has been termed ‘polyploid mitotic cycle’ was recently found to occur in a
*Drosophila* cell type that forms the adult rectal papillae (Fox et al., 2010). These
polyploid mitoses were observed to be highly error-prone, characterized by the
appearance of extended anaphases, chromosome bridges and lagging chromosomes.
Together, these intriguing observations argue that the switch to endocycles during
development is not irreversible, but one deleterious consequence of ploidy reversal is
genomic instability. Therefore, it might be that polyploid cells do not give rise directly to
tumor initiating cells but could give rise to genetically unstable ‘intermediates’ that are
predisposed to further progression towards malignancy. In this context, it would also be
exciting to determine whether loss of E2F8 in already highly polyploid hepatocytes might
possibly lead to increased rate of reductive mitoses. If so, one function of E2F8 after the
M-E transition could be to suppress the ectopic occurrence of reductive mitoses *in vivo*
by repressing mitosis and cytokinesis, suggesting an indirect but important involvement
for this E2F family member in maintaining genomic integrity.

Finally, gene expression analysis of tumors raised an additional possibility for
how *E2f8* loss may lead to development of HCC in mice. Comparison of DEN-treated
wild type and *E2f8* deficient tumors suggests that E2F8 may act in tumor suppression
more directly by participating in the repression of gene products that have oncogenic
potential. By leading to increased expression of genes that promote proliferation (*Stmn1*,
*Survivin*, *Iqgap1* and *Cyclin A2*), the loss of *E2f8* may position hepatocytes with a
proliferative advantage that would facilitate their transformation upon further genetic
alterations. The fact that many more HCC lesions were observed in DEN-treated *E2f8*
deficient livers than wild type livers implies that E2F8 also suppresses tumor initiation. In
summary, genetic, systems-based and biochemical approaches revealed a fundamental role for the most ancient ‘atypical’ arm of the E2F program in directing mammalian endocycles, while exposing unanticipated roles for E2f8 in tissue physiology and cancer.

7.4 Materials and methods

Mice and genotyping

E2f7ff, E2f8ff and Alb-cre transgenic mice were maintained in a pure FVB/N background. All mice were housed in barrier conditions with a 12-hour light/dark cycle and had access to food and water ad libitum. All protocols involving mice in this study were approved by the Institutional Animal Care and Use Committee at The Ohio State University. PCR genotyping was performed using genomic DNA purified from tails following a standard protocol (digestion with proteinase K followed by isopropanol precipitation). Sequences of genotyping primers are provided in Chapter 2. For the tumor studies, all pups were genotyped between 14-17 days of age prior to injection with the carcinogen. Control animals consisted of Alb-cre transgene negative littermates of transgene positive pups.

DEN injections

After genotyping, twenty-day-old pups received a single intraperitoneal injection of diethylnitrosamine (DEN, Sigma) dissolved in sterile PBS at a concentration of 20mg/kg body weight. Pups were weaned by 24 days of age. Mice were sacrificed eight months after injection and their livers were assessed for the development of hepatocellular carcinoma.
**Carbon tetrachloride injection**

Six-month-old mice received a single intraperitoneal injection of 10% solution of carbon tetrachloride (Sigma) dissolved in corn oil. Mice were sacrificed 0 (no injection), 24, 48, 60 and 168 hours post-treatment and their percent hepatic mass (liver weight/total body weight x 100) were determined at each time point. Mice were administered an intraperitoneal injection of BrdU solution to label hepatocytes in S phase of the cell cycle (100µg/g body weight) one hour prior to sacrifice.

**Flow cytometry**

This procedure was performed exactly as previously described, except with tumors that were dissected out from livers of mice at time of sacrifice. Adjacent ‘normal’ tissue is non-tumor tissue that is immediately surrounding the dissected tumors.

**Quantitative RT-PCR**

This procedure was performed as described in previous chapters, with the following statistical considerations. The linear mixed effect model, considering observational dependencies on the same mouse (given some Alb-78dko or Alb-8ko tumors were obtained from the one mouse), was used to evaluate the effect E2f7/E2f8 or E2f8 loss on expression of genes evaluated by quantitative RT-PCR. Multiplicity was adjusted by Holm’s method within each experiment. Total RNA from the same set of control normal and control tumor tissues were reverse transcribed in two separate experiments (once with Alb-8ko tumor RNA and once with Alb-78dko tumor RNA).
**BrdU immunostaining and quantification**

Mice received a single intraperitoneal injection of bromodeoxyuridine (BrdU, Sigma) dissolved in sterile saline at a concentration of 100µg/g body weight one hour prior to sacrifice. Livers were collected for fixation in 10% neutral buffered formalin, and 5µm paraffin embedded-sections were sectioned for BrdU assays. After deparaffinization, anti-BrdU (MO-0744, DAKO) antibodies were applied to tissues overnight 4°C to detect BrdU incorporation. Prior to application of primary antibody overnight, tissues were quenched in 3% hydrogen peroxide for 10 minutes, digested in 2N HCl at 37°C for 1 hour, neutralized with 10mM sodium borate (pH 8.0) for 10 minutes at room temperature, and blocked with 5% GS/2% BSA. Biotionylated secondary antibodies were applied to sections the next day, followed by ABC (Vector) incubation and DAB (Vector) development. Slides were counterstained with Mayer’s hematoxylin. Quantification data were reported as an average percentage ± SD of BrdU positive hepatocytes from the total hepatocyte population in the number (n value indicated in Figure 7.1) of control and mutant samples analyzed.

**Quantification of lesion**

Seven images of H&E stained liver sections from control and mutant mice (only male mice were considered for this analysis) were obtained using the 4x objective lens on Axioskop (Zeiss) light microscope. The number of lesions (including foci of cellular alteration, adenomas and carcinomas) regardless of stage or grade was then manually
scored per 4x image, and an average was obtained for the seven images per mouse. Number of male mice evaluated per genotype is indicated in Figure 7.2C.
Figure 7.1. Loss of E2f7 and E2f8 Did Not Affect Liver Regeneration Upon Chemical Injury Induced by Carbon Tetrachloride Administration.

(A) Quantification of S phase (BrdU) positive hepatocytes at 48 and 60 hours after a single intraperitoneal injection of carbon tetrachloride (CCl₄) to 6-month-old mice, which caused fulminant hepatic necrosis with 12-24 hours (data not shown) followed by regeneration. In this model, shortly after chemical-induced death of tissue, almost all remaining hepatocytes are mobilized to synchronously re-enter the cell cycle and

(continued)
contribute to parenchyma regeneration (Gerhard et al., 1975). Peak DNA synthesis in wild type mice typically occurs between 48 and 60 hours. Red arrows point to BrdU-negative hepatocytes. n, number of independent mice analyzed per genotype. Quantification data presented as average ± SD.

(B) Quantification of M phase (P-H3) positive hepatocytes at 48 and 60 hours after CCl4 injection. n, number of independent mice analyzed per genotype. Quantification data presented as average ± SD. No statistically significant difference was detected in either the number of S or M phase positive hepatocytes between control and Alb-78dko mice at the two time points. Red arrows point to hepatocytes in mitosis.

(C) Measurements of percent hepatic mass at all time points examined after CCl4 injection showing a transient increase in liver weight in both control and Alb-78dko mice after injury. By the end of one week (168 hours) marking the end of time period for liver regeneration in rodents (Schultze et al., 1975), liver weight in both control and Alb-78dko mice returned to near baseline levels. BrdU assay at 168 hours (not shown) demonstrated that almost all hepatocytes had exited S phase. Flow cytometry of regenerated liver tissues at 0 and 168 hours (data not shown) showed similar ploidy profiles in control (polyploid) and mutant (diploid) livers before and after regeneration. n, number of independent mice analyzed per genotype. Quantification data presented as average ± SD. control, E2f7flo;E2f8flo; Alb-78dko, Alb-cre;E2f7flo;E2f8flo.
Figure 7.2. Loss of E2f7/E2f8 or E2f8 Alone Resulted in Enhanced Susceptibility to the Development of Spontaneous and Carcinogen-Induced Hepatocellular Carcinoma (HCC).

(A) Incidence of spontaneous HCC and pre-neoplastic lesions (pre) in 9-month-old control and Alb-78dko mice segregated by sex demonstrating increased development of HCC in males. n, number of independent mice analyzed per genotype.
Figure 7.2: continued

(B) Measurement of percent hepatic mass in 9-month-old male and female mice treated with diethylnitrosamine (DEN). By this age, only Alb-8ko and Alb-78dko male mice developed highly aggressive HCC reflecting a dramatic increase in their liver weight, which was significantly disproportionate to their body weight. Injection of vehicle alone (sterile PBS) did not result in any changes in percent hepatic mass (data not shown). n, number of independent mice analyzed per genotype. Quantification data presented as average ± SD. One way Anova, *** p≤0.001.

(C) Quantification of number of lesions per 10.2 mm² ranging from pre-neoplastic foci to frank carcinomas in livers of DEN-treated 9-month-old males. Alb-8ko and Alb-78dko males had significantly increased number of lesions in a given area of tissue relative to control mice. n, number of independent mice analyzed per genotype. Quantification data presented as average ± SD. One way Anova, *** p≤0.001.

(D) Histopathology of control and Alb-78dko livers from DEN-treated 9-month-old male mice. Compression of normal appearing parenchyma by tumor tissue is evident. N, adjacent normal parenchyma; T, HCC.

(E) Representative gross appearance of livers in 9-month-old male mice with the indicated genotypes. control, E2f7⁺⁺;E2f8⁺⁺; Alb-7ko, Alb-cre;E2f7⁺⁺; Alb-8ko, Alb-cre;E2f8⁺⁺; Alb-78dko, Alb-cre;E2f7⁺⁺;E2f8⁺⁺.
Figure 7.3. Quantitative RT-PCR Analysis of Gene Expression and Ploidy Assessment of E2f7/E2f8 and E2f8 Deficient Tumor Tissues.

(A-B) Boxplot representation of quantitative RT-PCR expression analysis of oncogenes in tumors dissected from livers of 9-month-old DEN-treated Alb-8ko (gray), Alb-78dko (red) and control (white) mice. Gene expression in non-cancer liver tissues (‘Adj. nor.’)
Figure 7.3: continued

from DEN-treated control mice was also measured and used to normalize expression in all tumor tissues. n, number of independent tumors analyzed per genotype. Linear mixed effect model, ** p≤0.01, *** p≤0.001 (knockout tumor expression significantly different than expression in control normal tissue), ^ p≤0.001 (knockout tumor expression significantly different than expression in control tumor tissue).

(C) Top, FACS analysis of individual tumors with diploid profiles (black, T1-4) and one representative polyploid adjacent normal tissue (white, N) from DEN-treated control mice. Bottom, FACS analysis of individual tumors from DEN-treated Alb-8ko (gray, T1-6) and Alb-78dko (red, T1-3) mice. FACS, fluorescence activated cell sorting.

(D) Flow cytometry of adjacent non-cancer tissues and matching tumors (five total pairs, represented by first five bars in each ploidy class enclosed in bracket) from DEN-treated control mice demonstrating a trend for decreased ploidy in tumor cells. Three additional control tumor samples without paired non-cancer tissues were analyzed for DNA content (represented by the last three bars in each ploidy class enclosed in brackets, right graph only) that had a majority of 4n nuclei.
Chapter 8

Cell Non-Autonomous Regulation of \(p19^{Arf}\) by E2F3:
Connecting Cell Cycle Regulation to Anti-Tumor Immunity

8.1 Introduction

In the mammalian cell cycle, engagement of growth factor receptors (e.g. HER2) by their cognate ligands elicits a series of signaling cascades that converge on the activation of cyclin-dependent kinases (CDK) and phosphorylation of retinoblastoma (RB) (Sherr and Roberts, 2004). This results in the release of E2F factors from RB-E2F complexes and the execution of a transcriptional program that is required for S phase entry (Harbour and Dean, 2000). Components that promote mitogenic signaling, including \(HER2\), \(CCND1\) and \(CDK4\), are often overexpressed, amplified and/or harbor activating mutations in breast cancer (Slamon et al., 1987; Bartkova et al., 1994; An et al., 1999), whereas components that oppose mitogenesis, such as the \(p16^{INK4A}\) CDK-inhibitor or \(RB\), are frequently silenced, chromosomally lost or mutated (Deshpande et al., 2005; Esteller, et al., 2001). In breast cancer cells, these genetic alterations culminate in excessive E2F-dependent gene transcription and are believed to tip the balance towards cellular programs favoring unrestricted cell proliferation, a prerequisite to oncogenic transformation and tumor growth (Hanahan and Weinberg, 2000).
The mammalian E2F family is complex, with eight distinct gene loci encoding structurally-related proteins (DeGregori and Johnson, 2006). Clinical studies have suggested that the E2F3 family member may contribute significantly to the etiology of human malignancies (Foster et al., 2004; Oeggerli et al., 2004; Cooper et al., 2006), including breast cancer (Gauthier et al., 2007). Overexpression of E2F3 is prevalent in HER2-positive and basal-like tumors, two of the most aggressive subtypes of breast cancer with poor clinical outcome (Gauthier et al., 2007). Despite extensive clinical and mouse modeling data linking E2F3 to the control of tumor cell proliferation, direct evidence for a causal role for E2F3 in breast cancer is still lacking. Using transgenic mouse models of breast cancer and an inducible system to conditionally inactivate gene function in the mammary epithelium, we show that E2f3 is critical for HER2 but not MYC-induced mammary tumorigenesis. Intriguingly, this oncogene-specific requirement for E2f3 extends beyond its classic role in cell proliferation to include the suppression of anti-tumor innate and adaptive immune responses. These findings challenge the current dogma and identify a novel role for the CDK-RB-E2F axis in breast cancer that is critical in modulating the tumor microenvironment.

8.2 Results

*Loss of E2f3 delays initiation and causes regression of ErbB2 tumors*

Given the positive relationship between E2F3 overexpression and HER2-positive and basal-like subtypes of breast cancer (Gauthier et al., 2007), we investigated its causal involvement in mammary tumor initiation and progression. To this end, we used two relevant mouse models of breast cancer that overexpress the HER2/ErbB2 (Muller et al.,
and MYC (D’Cruz et al., 2001) oncogenes along with a conditional allele of E2f3 (E2f3\textsuperscript{LoxP}; Wu et al., 2001). The ErbB2 and MYC transgenic models have unique molecular, cellular, and clinical features of breast cancer that can be used to evaluate different aspects of this heterogeneous disease. ErbB2 tumor cells are uniformly undifferentiated with small nuclei that form solid tumors with little stromal contribution, whereas MYC tumor cells are larger and have pleomorphic nuclei that form glandular tumors with abundant stromal contribution (Cardiff, et al., 2000). Because loss of E2f3 in mice results in embryonic lethality (Tsai et al., 2008), we used an inducible bitransgenic cre-expression system (MMTV-rtTA;teto-cre) to conditionally delete E2f3 (E2f3\textsuperscript{LoxP}) in the mammary epithelium of female mice.

Mammary tumor initiation was measured in four cohorts of mice, MMTV-ErbB2;MMTV-rtTA;E2f3\textsuperscript{LoxP/LoxP} (ErbB2-control), MMTV-ErbB2;MMTV-rtTA;teto-cre;E2f3\textsuperscript{LoxP/LoxP} (ErbB2-cre), teto-MYC;MMTV-rtTA;E2f3\textsuperscript{LoxP/LoxP} (MYC-control) and teto-MYC;MMTV-rtTA;teto-cre;E2f3\textsuperscript{LoxP/LoxP} (MYC-cre). Kaplan-Meier graphs presented in Figure 8.1A and 8.1B show that tumor initiation was significantly delayed in ErbB2-cre (p<0.001) but not in MYC-cre cohorts (p=0.971). Southern blot analysis demonstrated that 13% (4 out of 30) of tumors in ErbB2-cre mice and 93% (27 out of 29) of tumors in MYC-cre mice were deleted for E2f3\textsuperscript{LoxP}, suggesting that there was a strong tendency for ErbB2-tumors to develop from E2f3\textsuperscript{LoxP} non-deleted cells (p<0.001; Figure 8.1C and 8.1D). Importantly, ablation of E2f3\textsuperscript{LoxP} did not adversely impact mammary gland development or pregnancy-related functions (data not shown), excluding altered epithelial cell differentiation as a cause for the observed delay in tumor initiation in ErbB2-cre mice. Moreover, tumor onset in ErbB2-cre* mice containing wild type alleles
of $E2f3$ was not delayed ($p=0.939$), ruling out any inadvertent effects of $cre$ expression on tumor initiation/progression (Figure 8.1A and 8.1B). Together, these observations suggest a critical role for $E2f3$ in the genesis of ErbB2-driven mammary tumors.

To determine whether $E2f3$ is required to sustain tumor growth, female mice bearing 1-2 cm$^3$ tumors were treated with doxycycline and then monitored daily. Tumors in ErbB2-control mice doubled in size within the first week of doxycycline treatment and all females had to be sacrificed two weeks later due to excessive tumor burden (Figure 8.1E). In contrast, most tumors in ErbB2-cre females treated with doxycycline stopped growing and by three weeks many tumors had decreased in size and/or disappeared. These observations suggest that E2F3 function is continuously required for ErbB2-driven tumor growth.

Ablation of $E2f3$ elicits innate-adaptive immune and oncogene-stress ($p19$) responses

To explore the cellular and molecular mechanisms underlying tumor regression in ErbB2-cre females, we profiled global gene expression in doxycycline-treated ErbB2-control and ErbB2-cre tumors. Among the ~40,000 transcripts queried by Affymetrix microarrays, 298 genes were found to be differentially expressed between the two groups ($p<0.005$; Figure 8.2A). Quantitative RT-PCR (qRT-PCR) confirmed the majority of expression changes (15/15 upregulated genes and 15/17 downregulated genes; Figure 8.2B and data not shown). Bioinformatics analysis of the 298 genes revealed three important aspects of E2F3 function. First, the expression of classic E2F target genes involved in cell proliferation, such as Cdc6, Dhfr, Pena and Mcm3, was unaffected by the loss of $E2f3$. This was consistent with the continued proliferation of $E2f3$-deleted tumor
cells in ErbB2-initiated lesions, including in pre-neoplasms, adenomas, carcinomas, as well as in regressing tumors (Figure 8.2D and 8.2E, and data not shown). Moreover, there was no increase in apoptosis or apoptosis-related gene expression in E2f3-deleted tumors (Figure 8.2B, 8.2D and 8.2E). We thus conclude that the requirement for E2f3 in ErbB2-mammary tumorigenesis is independent of its role in proliferation and apoptosis.

Second, loss of E2f3 resulted in the enhanced expression of genes encoding proteins with immune-related functions, including Cxcl1, Cxcl14 and Lif (Figure 8.2B and 8.2C). This immune expression signature was consistent with the histopathology observed in E2f3-deleted ErbB2-tumors, which exhibited widespread necrosis, hemorrhage and leukocytic infiltration, resulting in cyst-like tumor masses filled with blood and inflammatory exudates (Figure 8.3A and 8.3B). Immunohistochemistry (IHC) assays revealed that the massive immune cell infiltration consisted of CD3-positive T lymphocytes and F4/80-positive macrophages, but not B220-expressing B lymphocytes (Figure 8.3C). In contrast, loss of E2f3 in MYC-tumors did not result in any observable necrosis, hemorrhage or immune cell infiltration (Figure 8.3).

Third, the E2f3-specific expression signature contained a strong bias for genes involved in stress responses and included the activation of p19ARF, which is known to be induced by oncogene-mediated stress (Lowe and Sherr, 2003; Quelle et al., 1995). Quantitative RT-PCR and Western blot assays showed that p19ARF mRNA and protein levels were markedly elevated in E2f3-deleted ErbB2-cre tumors (Figure 8.4A) and IHC assays showed its distinct nuclear/nucleolar accumulation (Figure 8.4B). Side by side comparison of ErbB2-cre and MYC-cre tumors demonstrated that the induction of p19ARF was specific to ErbB2-cre tumors (Figure 8.4A and 8.4B). Given the known role of
p19^{ARF} in stabilizing p53 protein (Weber et al., 1999), we also measured the levels of p53 in ErbB2-control and ErbB2-cre tumors (Figure 8.4C and 8.4D). IHC and Western blots assays demonstrated a robust accumulation of nuclear p53 protein in E2f3-deleted tumors. This was accompanied by the induction of p53-target genes, including gene products having immune (Lif) and angiogenic (Thbs2) functions (Figure 8.4E). But not all p53-targets were induced by loss of E2f3; targets involved in the execution of apoptosis, such as Killer and Bax, remained unchanged (Figure 8.4E). It would thus appear that ErbB2-oncogenic signaling utilizes E2f3 to suppress a specific effector arm of the p19^{ARF}-p53 axis that has immune-related functions.

**Cell non-autonomous suppression of the p19^{ARF}-p53 axis**

While only four of the thirty-four tumors in the ErbB2-cre cohort were fully deleted for E2f3 (Figure 8.1A), all tumors - deleted and non-deleted - exhibited the hemorrhagic, necrotic and leukocytic-rich histopathology (Figure 8.3A and 8.3B). Similarly, pre-existing ErbB2-cre tumors treated with doxycycline displayed this typical inflammatory histopathology and regressed, irrespective of E2f3^{LoxP} deletion (Figure 8.1E, 8.5A and 8.5B). These observations suggest that inactivation of E2f3 in the surrounding mammary epithelium was sufficient to delay tumor initiation and induce regression in ErbB2-cre females. To investigate the molecular mechanism underlying this remarkable phenomenon, we compared expression of p19^{ARF} in E2f3-deleted and non-deleted ErbB2-cre tumors. Surprisingly, qRT-PCR assays showed a marked induction of p19^{ARF} mRNA levels in both deleted and non-deleted tumors (Figure 8.5C). Moreover, IHC assays demonstrated the nuclear accumulation of p19^{ARF} and p53 proteins
in these tumors, irrespective of the status of $E2f3^{LoxP}$ (Figure 8.5D), thus confirming that their induction is cell non-autonomous.

We tested the possibility that $E2f3$ may also regulate $p19^{ARF}$ expression in MEFs via a cell non-autonomous mechanism. To this end, we co-cultured GFP-labeled $E2f3^{LoxP/LoxP}$ MEFs with non-labeled $E2f3^{LoxP/LoxP}$ MEFs infected with cre- or control-expressing retroviruses and measured $p19^{ARF}$ protein in GFP-positive MEFs (non-deleted) by immunocytochemistry. These experiments showed a marked induction of nucleolar $p19^{ARF}$ in GFP-positive cells cultured with non-labeled $E2f3^{LoxP/LoxP}$ MEFs expressing the cre-retrovirus (Figure 8.5E and 8.5F). As expected, $p19^{ARF}$ expression was elevated in GFP-negative MEFs expressing cre-retroviruses. Thus, contrary to previous results implicating a role of E2F3b in repressing $p19^{ARF}$ (Aslanian et al., 2004) expression, our current findings provide evidence for a cell non-autonomous role of E2F3 in activating the $p19^{ARF}$-p53 axis.

**Suppression of p53 is required for E2F3 oncogenic activity**

The above genetic and biochemical experiments suggest that upon loss of $E2f3$, tumor delay/regression in ErbB2-cre female mice is mediated by $p19^{ARF}$ and p53. To test this possibility we analyzed tumor onset in ErbB2-cre mice that also contained a conditional $p53^{LoxP}$ allele ($MMTV-ErbB2;MMTV-rtTA;teto-cre;E2f3^{LoxP/LoxP};p53^{LoxP/LoxP}$). As illustrated by the Kaplan-Meier graph in Figure 8.6A, tumor onset was significantly accelerated in mammary glands deleted for both $E2f3^{LoxP}$ and $p53^{LoxP}$ ($T_{50}= 206$) relative to glands deleted for only $E2f3^{LoxP}$ ($T_{50}= 285$, $p<0.001$). Ablation of $p53^{LoxP}$ alone marginally accelerated tumorigenesis when compared to the ErbB2-control cohort (data not shown). Importantly, Southern blot analysis of tumor samples showed that $E2f3^{LoxP}$
was efficiently deleted in tumors that were simultaneously deleted for $p53^{\text{LoxP}}$ (Figure 8.6C and data not shown). These findings demonstrate that the bias against deletion of $E2f3^{\text{LoxP}}$ in ErbB2-cre tumors was mitigated by the concomitant inactivation of $p53^{\text{LoxP}}$ (p=0.001 versus p=0.338, respectively). Importantly, ErbB2-cre tumors deleted for both $E2f3^{\text{LoxP}}$ and $p53^{\text{LoxP}}$ failed to exhibit any of the inflammatory histopathologic features associated with the sole inactivation of $E2f3^{\text{LoxP}}$ (Figure 8.6B and 8.6D). In summary, these in vivo results provide compelling genetic evidence for a critical role of E2F3 in the cell non-autonomous control of the p19ARF-p53 axis and regulation of anti-tumor immune responses.

8.3 Discussion

Breast cancer is a heterogeneous disease with immense genetic variability. Clinical data implicate important roles for $CCND1$, $CDK4/6$ and $RB$ in HER2-dependent breast cancer (Slamon et al., 1987; Bartkova et al., 1994; An et al., 1999). Oncogenic involvement of these core cell cycle components in breast malignancies is further supported by results of many elegant mouse models (Yu et al., 2001; Landis et al., 2006; Yu et al., 2006; Lin et al., 2008). RB and E2F transcription factors are believed to represent the ultimate effectors of HER2-mitogenic signaling that ensure proper progression through the cell cycle (Dimova and Dyson, 2005). Our current findings suggest that the $E2F3$ family member is a key downstream component of HER2-signaling (ErbB2 in mouse) that is necessary to initiate and sustain tumor growth, and provide fresh insight into how $E2F3$ overexpression contributes to HER2-driven breast cancer. This
novel role of E2F3 in mammary tumorigenesis is oncogene-specific since MYC-driven
tumors were unaffected by the loss of E2f3.

Expression profiling and biochemical analysis of mammary tumors and their
microenvironment showed that E2F3’s oncogenic function was unrelated to its classic
role in cell cycle control. Instead, its oncogene-specific function was associated with the
expression of genes involved in immune function (e.g. Cxcl1, Cxcl14 and Lif), in cellular
metabolism/stress response (e.g. Pdk1, Cyba and Higdl1a) and in angiogenesis (e.g.
Vegfa, Cd44 and Thbs2). Consistent with this transcriptome, loss of E2f3 in ErbB2-
positive mammary epithelium resulted in the formation of cystic tumors exhibiting
extensive necrosis, hemorrhage and immune cell infiltration. While the exact mechanism
remains to be elucidated, the genetic analysis presented within demonstrates that the
capacity of E2F3 to regulate immune functions is mediated through the control of p19ARF
and p53 expression via a cell non-autonomous mechanism. Targeting E2F3 or E2F3-
regulated signals that control p19ARF and p53 levels in neighboring tumor cells will
provide an attractive platform for therapeutic intervention of HER2-positive breast
cancer. Interestingly, this unexpected function of the p19ARF-p53 axis may not be
restricted to breast cancer since transient restoration of p53 in murine hepatocellular
carcinomas can also trigger tumor clearance via the activation of innate immunity (Xue et
al., 2007). In lymphomas and sarcomas, however, restoration of p53 was associated with
apoptotic death of tumor cells (Ventura et al., 2007; Martins et al., 2006). Thus, it
remains unclear how p53 selectively engages alternative arms of its anti-tumor arsenal in
a tumor-type specific manner.
In summary, we have identified E2F3 as a critical component of the HER2-CDK-RB signaling pathway that links, for the first time, the cell cycle machinery with p53 networks to modify immune responses in the tumor microenvironment. We suggest that activation of E2F3 contributes to HER2-tumor activity by specifically regulating the p19ARF-p53 axis through a cell non-autonomous manner. The fact that loss of E2f3 leads to the execution of immune programs that are tumor-specific, sparing normal development and mammary function, emphasizes the potential therapeutic utility of targeting E2F3 in HER2-positive breast cancer.

8.4 Materials and methods

Mice and genotyping

The MMTV-rtTA and teto-MYC mice were obtained from L. Chodosh; teto-cre from J. Whitsett; MMTV-ErbB2 from B. Muller; p53LoxP from A. Berns; and Rosa26LoxP from P. Soriano. The conditional E2f3 knockout mouse (E2f3LoxP/LoxP) was generated by L. Wu (former post-doc in the Leone lab). Animal maintenance and experiments were carried out in accordance with the animal care guidelines set forth by the Institutional Animal Care and Use Committee at The Ohio State University. All tumor studies were performed with mice bred into FVB genetic background (5th generation). Doxycycline hydrochloride (D9891, Sigma) was dissolved in water and stored at −20°C in the dark until use. Doxycycline was given to the mice in their drinking water (2mg/ml) and changed every week. Mice were monitored for tumor formation by palpation twice a week over the period of one year. Tumor diameter was measured with the Fowler SNAP-
CAL digital caliper from which tumor area was calculated. Genotyping of mice was performed by PCR of genomic DNA isolated from mouse tails.

**Immunostaining and western blot assays**

Mammary tumors, mammary glands with hyperplastic nodules or normal mammary gland tissues were dissected and fixed in 10% neutral buffered formalin or 4% paraformaldehyde (PFA), or directly embedded in OCT (Sakura). Formalin or PFA fixed tissue were embedded in paraffin and cut into 5μm sections, which were stained with H&E for histopathology. Immunohistochemistry or immunofluorescence assays using antibodies specific for Ki67, cleaved caspase 3, CD3, F4/80, p19ARF, p53 or GFP were performed following a standard protocol, which has been described in detail in previous chapters. For Western blot assays, approximately 100mg of mammary tumor tissues were homogenized in RIPA lysis buffer (150mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris pH8.0) with addition of fresh protease inhibitor cocktail (Roche) and phosphatase inhibitors. Lysates were centrifuged for 15 minutes at 12,000 rpm at 4°C to remove cellular debris. Protein concentration was determined using the Bradford assay (BioRad) following manufacturer’s instructions. 50-75ug of total proteins were separated by 10% SDS-PAGE gel electrophoresis, transferred onto PVDF membranes (constant amperage, one hour at 4°C) and blocked with 5% milk-TBST solution prior to antibody incubation overnight at 4°C. The next day HRP-conjugated secondary antibodies, anti-mouse or anti-rabbit IgG (GE Healthcare), were applied to the membranes, which were subsequently developed with ECL (Amersham).
For IHC, the following antibodies were used: Ki67 (Pharmingen 550609), cleaved caspase 3 (Cell Signaling 9661-S), p53 (Visionbiosystems NCL-p53-CM5p), CD3 (DAKO A0452), F4/80 (Invitrogen MF-48004), and p19ARF (Abcam ab80). For western blot assays, the following antibodies were used: E2F3 (Upstate PG37), p53 (Visionbiosystems NCL-p53-CM5p), p19ARF (Abcam ab80) and α-tubulin (Sigma T6199) were used in western blot assays.

**Co-culture of mouse embryonic fibroblasts (MEFs)**

Sub-confluent immortalized \( E2f3^{LoxP/LoxP} \) MEFs were infected with GFP-expressing retroviruses (PINCO-GFP), and GFP-positive MEFs were sorted from non-GFP expressing cells by flow cytometry. In 60mm\(^2\) cell culture dishes, \( 5 \times 10^4 \) GFP-tagged MEFs were co-cultured with \( 5 \times 10^4 \) \( E2f3^{LoxP/LoxP} \) MEFs infected with retroviruses expressing the control \( pBP \) vector (\( E2f3 \) non-deleted) or \( pBP-cre \) (\( E2f3 \)-deleted) for 72 hours in DMEM (Invitrogen) supplemented only with 10\% FBS (Gibco). The co-cultured cells were washed with PBS and then fixed in 4\% PFA *in situ*. Fixed cells were permeabilized with ice-cold 1:1 methanol/acetone mixture for immunocytochemistry using p19ARF- and GFP-specific antibodies.

**Affymetrix profiling and quantitative RT-PCR**

Total RNA was isolated from 4 \( MMTV\text{-ErbB2;MMTV\text{-rtTA};E2f3}^{LoxP/LoxP} \) (control) and 4 \( MMTV\text{-ErbB2;MMTV\text{-rtTA};teto\text{-cre};E2f3}^{LoxP/LoxP} \) (cre) tumors harvested from doxycycline-treated mice for global gene expression profiling (Mouse Genome 430 2.0 Array). \( E2f3^{LoxP} \) deletion was confirmed with both PCR-based genotyping and Southern
blot analyses. 1µg of total RNA per tumor sample from control and cre genetic cohorts (total 8 samples) was submitted to the OSUCCC MicroArray Shared Resource facility. RNA quality was verified using the Agilent 2100 Bioanalyzer. A GeneChip Scanner 3000 (Affymetrix) was used for data acquisition. For each sample, pre-processing of raw signal intensities from .CEL files was performed using the Robust Multi-chip Average (RMA) method (Irizarry et al., 2003) and consisted of background adjustment, quantile normalization and median polish. BRB-Array Tools version 3.6.0 (developed by R. Simon) was subsequently used to perform class comparisons to determine differentially expressed genes at a significance level of p<0.005 (two-sample T-test with random variance model). Unsupervised hierarchical clustering was then performed and a heatmap constructed using Cluster 3.0 and TreeView.

To validate gene expression changes as determined by Affymetrix (as in Figure 8.2B), the same set of RNA samples was used for cDNA synthesis with Superscript III (Invitrogen) and oligodT in the presence of RNase inhibitor (Roche). cDNA was then diluted 1:5 prior to being used as template in quantitative RT-PCR. SYBR Green (BioRad) Real-Time amplification was performed using gene-specific primers than spanned exon-exon junctions in the BioRad iCycler. Each sample was tested in triplicate 25µl reactions. Target gene transcripts were quantified relative to housekeeping genes (GAPDH) using the ΔCT method, and relative gene expression for each sample is presented as average ± SD. Primers were designed using the publicly available program Primer3 version 0.4.0 (Rozen and Skaletsky, 2000), and optimal annealing temperature conditions were determined by using the gradient run feature on iCycler.
Statistical analysis (J. Stephens and S. Fernandez)

Pair-wise Log-Rank tests were used to compare the percentage of tumor free mice among the different genetic groups as shown in the Kaplan–Meier graphs. Repeated measures Analysis of Variance (ANOVA) was used to compare the tumor area between mice of the indicated genotypes in the tumor regression study. Data for sacrificed mice due to large tumor burden (meeting Early Removal Criteria) were conservatively imputed as the last known tumor size (as in Figure 8.1E). Chi-square and Fishers’ Exact tests were used to compare the deletion efficiency of $E2f3^{LoxP}$ and/or $p53^{LoxP}$ as analyzed by Southern blot. P-values were adjusted for multiple comparisons when necessary using the Bonferroni method of adjustment.
Figure 8.1. *E2f3* Promoted Oncogene-Specific Mammary Tumor Initiation. (*This Figure is generated entirely by a former post-doc in the Leone lab, Hui Wang*)

(A) Kaplan-Meier tumor-free curves of *MMTV-ErbB2;MMTV-rtTA;E2f3LoxP/LoxP* (*ErbB2-control*, black) and *MMTV-ErbB2;MMTV-rtTA;teto-cre;E2f3LoxP/LoxP* (*ErbB2-cre*, red) cohorts showed a significant difference in time-to-tumor onset (*p*<0.001). Arrows indicate a total of four mice with *E2f3LoxP*-deleted tumors (see Southern data in Figure 8.1C showing two of four deleted ErbB2-cre tumors). Grey line (*ErbB2-cre*) is tumor-
Figure 8.1: continued

free curve of MMTV-ErbB2;MMTV-rtTA;teto-cre mice; cre expression alone in the presence of intact E2f3 did not impact tumor onset (p=0.939). n, number of mice in each tumor cohort.

(B) Kaplan-Meier curves of teto-MYC;MMTV-rtTA;E2f3<sup>LoxP/LoxP</sup> (MYC-control, black) and teto-MYC;MMTV-rtTA;teto-cre;E2f3<sup>LoxP/LoxP</sup> (MYC-cre, red) cohorts showed no significant difference in time-to-tumor onset (p=0.971). n, number of mice in each tumor cohort.

(C) Southern blot analysis of E2f3<sup>LoxP</sup> deletion in mammary tumors derived from 10 different ErbB2-cre mice (top panel), 8 MYC-cre mice and one MYC-control (wt) mouse (bottom panel). LoxP, E2f3<sup>LoxP</sup> allele; null, E2f3<sup>LoxP</sup>-deleted allele.

(D) Table tabulating the efficiency of cre-mediated deletion of E2f3<sup>LoxP</sup> and Rosa26<sup>LoxP</sup> in ErbB2- and MYC-overexpressing mammary tumors. Hyperplastic lesions and tumors deleted for E2f3<sup>LoxP</sup> or Rosa26<sup>LoxP</sup> were identified and scored by Southern blots or X-Gal staining, respectively. The deletion rate of E2f3<sup>LoxP</sup> in ErbB2-cre tumors was significantly lower than that of Rosa26<sup>LoxP</sup> (13%, <sup>a</sup>p<0.001), where as its deletion rate in MYC-cre tumors was not different than Rosa26<sup>LoxP</sup> (93%, <sup>b</sup>p=0.068). Total # tumors, total number of tumors analyzed; # deleted or % deleted, number or percentage of ErbB2- and MYC-cre tumors showing E2f3<sup>LoxP</sup> or Rosa26<sup>LoxP</sup> deletion.
Figure 8.1: continued

(E) Pre-existing 1-2 cm² tumors in ErbB2-control and ErbB2-cre mice were monitored at the indicated times (number of days) following doxycycline administration via drinking water. Measurements of tumor area for $E2f3^{LoxP}$ non-deleted (grey/red stippled) and deleted (red) ErbB2-cre tumors are shown separately. Note the significant difference in overall tumor area between ErbB2-control (black) and ErbB2-cre (red and grey/red stippled) cohorts, irrespective of $E2f3^{LoxP}$ deletion. Repeated measures ANOVA, * p<0.01, ** p<0.001.
Figure 8.2. Ablation of E2f3 in ErbB2-Induced Mammary Tumors Resulted in the Deregulated Expression of Genes Unrelated to Cell Cycle Control.
Figure 8.2: continued

(Figures 8.2A-C were generated by H.-Z. Chen)

(A) Hierarchical clustering of 298 differentially expressed (p<0.005) genes between tumors derived from doxycycline-treated (+14 days) ErbB2-control and ErbB2-cre (E2f3LoxP-deleted) mice. Four independent tumors were profiled per genetic cohort. Colors in heatmap represent fold change in gene expression.

(B) Quantitative RT-PCR validation of representative deregulated genes in the eight tumor samples profiled using Affymetrix arrays in Figure 8.2A. Gene expression data are shown as fold change in ErbB2-cre tumors relative to one ErbB2-control tumor (one with lowest expression). Fold change in gene expression per tumor or control sample presented as average ± SD. Genes in left column (red) were determined to be significantly upregulated by Affymetrix in ErbB2-cre tumors relative to ErbB2-control tumors. Genes in right column (green) were determined to be significantly downregulated by Affymetrix in ErbB2-cre tumors relative to ErbB2-control tumors.

(C) Gene ontology of the 298 differentially expressed genes in Figure 8.2A.

(D) Immunofluorescent detection of Ki67 and cleaved-caspase 3 in primary mammary tumors. Inserts show high magnification views.

(E) Quantification of Ki67- (% Ki67) and cleaved-caspase 3- (% caspase 3) positive (continued)
Figure 8.2: continued

tumor cells in primary and regressing mammary tumors derived from ErbB2-control and ErbB2-cre mice. $E2f3^{LoxP}$ non-deleted (grey/red stippled) and deleted ErbB2-cre (red) tumors are shown separately. Quantification data presented as average ± SD.
Figure 8.3. Loss of E2f3 Resulted in Tumor Inhibition Associated with Immune Cell Infiltration. *(Figure 8.3C was generated by H.-Z. Chen)*
Figure 8.3: continued

(A) Gross appearance (top panels, arrows point to primary mammary tumors; bottom panels, dissected primary tumors) of ErbB2-control, ErbB2-cre \((E2f3^{LoxP}\)-deleted) and MYC-cre \((E2f3^{LoxP}\)-deleted) tumor mice. Note that primary \(E2f3^{LoxP}\)-deleted ErbB2-cre tumors appear hemorrhagic and cystic.

(B) Representative H&E sections of primary mammary tumors from the three genetic groups shown in Figure 8.3A. Bottom panels are enlarged view of boxed areas from corresponding top panels. Note massive tumor necrosis and hemorrhage with a mixture of leukocytic and lymphocytic infiltrate at periphery of the remaining viable tumor areas in ErbB2-cre tumors. T, remaining viable tumor; N, necrosis; L, leukocytes and/or lymphocytes.

(C) Immunohistochemistry of primary mammary tumors derived from ErbB2-control, ErbB2-cre \((E2f3^{LoxP}\)-deleted) and MYC-cre \((E2f3^{LoxP}\)-deleted) mice using F4/80- and CD3-specific antibodies. Note widespread infiltration of F4/80-positive macrophages and CD3-positive T cells in ErbB2-cre but not MYC-cre tumors.
Figure 8.4. Loss of E2f3 Resulted in the Up-Regulation of p19ARF and p53 in ErbB2-but Not MYC-Induced Mammary Tumors.
(Figures 8.4A, 8.4B and 8.4E were generated by H.-Z. Chen)

(A) Quantitative RT-PCR analysis of p19ARF expression in MYC- (MYC-control, MYC-cre) and ErbB2- (ErbB2-control, ErbB2-cre) overexpressing primary mammary tumors. The E2f3LoxP was deleted as assessed by Southern blot assay in the four ErbB2-cre and four MYC-cre tumors analyzed. Relative expression of p19ARF in ErbB2-cre or MYC-cre tumor samples was normalized to the expression one tumor (having lowest expression) in ErbB2-control or MYC-control samples, respectively. Fold change in gene expression per tumor or control sample presented as average ± SD. Red, tumor samples with E2f3 deletion.

(B) Immunohistochemistry of primary mammary tumors derived from ErbB2-control, ErbB2-cre and MYC-cre mice using p19ARF antibodies. Note a significant increase in nucleolar p19ARF protein levels (consistent with their normal pattern of subcellular localization) in ErbB2-cre but not MYC-cre tumor samples. Western blot analyses using same tumor lysates as in Figure 8.4C also demonstrated an increase in p19ARF levels (data not shown).

(C-D) Western blot and immunohistochemistry analysis of p53 protein levels demonstrating an increase in p53 expression in ErbB2-cre tumors (these are the same four tumors that had high p19ARF expression, as shown in Figure 8.4A). Note that expression of p53 was high in the nuclei of ErbB2-cre tumor cells (Figure 8.4D).
Figure 8.4: continued

(E) Quantitative RT-PCR assay demonstrating an increase in the expression of a subset of p53 target genes in primary mammary tumors overexpressing ErbB2 (same set of samples analyzed for p19ARF expression in Figure 8.4A). The expression of classical p53 target genes with pro-apoptotic functions (killer and bax) was unaffected by the loss of E2f3, while expression of non-traditional p53 target genes (thbs2 and lif) was significantly elevated with the loss of E2f3. Relative expression of p53 target genes in ErbB2-cre tumor samples was normalized to the expression of one tumor (having lowest expression) in ErbB2-control samples. Fold change in gene expression per tumor or control sample presented as average ± SD. Red, tumor samples with E2f3 deletion.
Figure 8.5. A Cell Non-Autonomous Role for E2f3 in Suppressing Anti-Tumor Immune Responses. (Figures 8.5B, 8.5C, 8.5D (p19^ARF), 8.5E and 8.5F were generated by H.-Z. Chen)
Figure 8.5: continued

(A-B) H&E of *ErbB2-control* and *ErbB2-cre* (non-deleted) tumors, and immunohistochemistry using F4/80- and CD3- antibodies on paraffin sections obtained from corresponding tumors. Regressing tumors shown here from *ErbB2-cre* mice are non-deleted for *E2f3<sup>LoxP</sup>. However, like *E2f3<sup>LoxP</sup>-deleted ErbB2-cre* tumors in Figure 8.3A and 8.3B, *E2f3<sup>LoxP</sup>* non-deleted tumors also had large areas of necrosis and hemorrhage accompanied by infiltration of macrophages and T cells. T, remaining viable tumor; N, necrosis; L, lymphocytes.

© Quantitative RT-PCR analysis of *p19<sup>ARF</sup>* expression in tumors derived from *ErbB2-control* and *ErbB2-cre* mice. Both *E2f3<sup>LoxP</sup>*-deleted (red) and non-deleted (grey/red stippled) tumors showed significantly elevated *p19<sup>ARF</sup>* expression levels. Relative expression of *p19<sup>ARF</sup>* in *ErbB2-cre* tumor samples was normalized to the expression of one tumor (having lowest expression) in *ErbB2-control* samples. Fold change in gene expression per tumor or control sample presented as average ± SD.

(D) Immunohistochemistry of tumors derived from *ErbB2-control* and *ErbB2-cre* mice using *p19<sup>ARF</sup>*- and *p53*-specific antibodies. Regressing tumors shown here from *ErbB2-cre* mice are non-deleted for *E2f3<sup>LoxP</sup>* but still demonstrate increases in both *p19<sup>ARF</sup>* and *p53* protein levels (compare with regressing tumors that have *E2f3* deletion in Figure 8.4B and 8.4D).

(continued)
Figure 8.5: continued

(E) Immunofluorescent detection of p19\textsuperscript{ARF} proteins in GFP-positive $E2f3^{LoxP/LoxP}$ MEFs co-cultured with either non-GFP-tagged $E2f3^{LoxP/LoxP}$ MEFs ($pBP$-control, left 2 panels) or $E2f3^{-/-}$ MEFs ($pBP$-cre, right 2 panels). GFP-positive MEFs were detected using GFP antibody. Note in bottom right panel, a GFP-negative $E2f3$-deleted cell stained positive for p19\textsuperscript{ARF}, and next to this cell is a GFP-positive cell with intact $E2f3$ also expressing p19\textsuperscript{ARF}.

(F) Quantification of p19\textsuperscript{ARF}-expressing GFP-positive and -negative cells co-cultured with either non-GFP-tagged $E2f3^{LoxP/LoxP}$ MEFs (black bar) or $E2f3^{-/-}$ MEFs (red bar). Note an increase in the percent of double-positive (GFP+:p19+) cells as a result of the cell non-autonomous induction of p19\textsuperscript{ARF} expression in $E2f3$-containing MEFs by neighboring $E2f3^{-/-}$ MEFs.
Figure 8.6. *E2f3* Supported *ErbB2*-Induced Mammary Tumorigenesis by Suppressing a Novel Anti-Tumor Effector Arm of *p53*. (*Figure 8.6D was generated by H.-Z. Chen*)

(A) Kaplan-Meier tumor-free curves of *MMTV-ErbB2;MMTV-rtTA;E2f3\textsuperscript{LoxP/LoxP} (control, black), *MMTV-ErbB2;MMTV-rtTA;teto-cre;E2f3\textsuperscript{LoxP/LoxP} (cre, red), and *MMTV-
Figure 8.6: continued

ErbB2;MMTV-rtTA;teto-cre;E2f3LoxP/LoxP;p53LoxP/LoxP (cre;p53LoxP/LoxP, yellow) cohorts. Ablation of p53LoxP abrogated the delay in tumorigenesis caused by E2f3LoxP loss (yellow versus red; p<0.001). Tumor onset was not significantly different between the control (black) and cre;p53LoxP/LoxP (yellow) cohorts (yellow versus black; p=0.167).

(B) Gross appearance and histopathology of tumors in control mice (control) and E2f3LoxP/LoxP;p53LoxP/LoxP (cre, yellow cohort) mice with indicated genotypes in Figure 8.6A. Note the absence of necrotic lesions and leukocytic infiltration in tumors derived from the cre group (compare with Figure 8.3B).

(C) Table tabulating the efficiency of cre-mediated deletion of E2f3LoxP and/or p53LoxP in ErbB2 tumors. No difference in p53LoxP deletion percentage was detected between E2f3+/+;p53LoxP/LoxP and E2f3LoxP/LoxP;p53LoxP/LoxP tumors (p=0.952). No difference between E2f3LoxP and p53LoxP deletion percentage was detected in E2f3LoxP/LoxP;p53LoxP/LoxP tumors (p=0.338); note the absence of a bias in the frequency of E2f3LoxP deletion in E2f3LoxP/LoxP;p53LoxP/LoxP tumors (compare with Figure 8.1D).

Total # tumors, total number of tumors analyzed; # deleted or % deleted, number or percentage of ErbB2- tumors showing E2f3LoxP and/or p53LoxP deletion.

(D) Immunohistochemistry of tumors derived from mice with the indicated genotypes using F4/80- and CD3-specific antibodies. Note the absence of F4/80-positive (continued)
macrophages and CD3-positive T cells in tumors derived from $E2f3^{LoxP/LoxP}$;$p53^{LoxP/LoxP}$ (cre, yellow cohort) mice.
References


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