Deregulation of the Transcriptional Repressor E2F6 in Myocardium Leads to Gene Activation and Dilated Cardiomyopathy

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ABSTRACT

The E2F family of transcription factors regulate cellular growth, death and differentiation, but their role in cardiac biology remains to be fully explored. We hypothesized that the balance of the E2F pathway would determine cardiac development and function. We provide evidence for this via modulation of the E2F6 repressor, in a transgenic (Tg) mouse model. Targeted expression of E2F6 in the heart led to dilated cardiomyopathy (DCM) and death. Microarray analysis revealed that E2F responsive pathways were activated in Tg mice. Furthermore, we found that E2F6 and YY1 (E2F-co-factor) were translocated to the nucleus in Tg mice, providing a potential mechanism for the observed transcriptional activation. We also observed a marked decrease of Connexin43 protein in the myocardium, and reduced atrial conductivity in Tg mice which may lead to reduced cardiac function. The data demonstrates a novel role for E2F pathway outside of cell cycle control in the heart.

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APS: Ammonium persulphate

cDNA: Complementary DNA

dNTP: Deoxynucleotide-triphosphate

DEPC: Diethylpyrocarbonate

DCM: Dilated Cardiomyopathy

DNA: Deoxyribonucleic acid

ECG: Electrocardiogram

EDTA: Ethylene-diamine-tetra-acetic acide

EtOH: Ethanol

EtBR: Ethidium Bromine

g: Gravitational force.

HCL: Hydrochloride

HEPES: 4-2-hydroxyethyl-1-piperazineethanesulfonic acid

Hrs: Hours

Kb: Kilobases

kDa: Kilo-Daltons

min: Minutes

$MgCl_2$: Magnesium Chloride

mRNA: Messenger RNA

N: Normal

NaCl: Sodium chloride

PAGE: Polyacrylamide Gel Electrophoresis

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PMSF: Phenyl methyl sulfonyl fluoride

RNA: Ribonucleic acide

rpm: revolutions per minute

RT-PCR: Real Time PCR

Sec: Seconds

SDS: Sodium dodecyl sulphate

SSII: Superscript II Reverse Transcriptase

TAE: 40mM Tris Acetate buffer with 1mM EDTA

TBST: Tris base buffer with Tween 20

TEMED: N,N,N',N'-Tetramethylethylenediamine

CHAPTER 1: INTRODUCTION

Proper cardiac development requires the tight and coordinated expression of genes controlling both the cell cycle and differentiation. During embryonic development mammalian cardiomyocytes divide rapidly, but as cardiac precursor cells begin to differentiate they lose their proliferative capacity resulting in only ~45% of cardiomyocytes synthesizing DNA (Pasumarthi and Field, 2002). Several days after birth cardiomyocytes undergo a final round of DNA synthesis without cell division termed acytokinetic mitosis, resulting in the binucleation of many cardiomyocytes. Historically, mammalian cardiomyocytes are believed to then permanently withdraw from the cell cycle, thus all postnatal cardiac growth depends on physiological hypertrophy. As the heart ages and is exposed to stressors and damage this triggers abnormal or pathological cardiac hypertrophy, often leading to heart failure. This abnormal growth is associated with a reactivation of the cardiac fetal gene program as well the expression of genes involved in cell cycle re-entry (Reviewed in Frey and Olson, 2003 and Li *et al*, 1998).

In the past decade it has been demonstrated that Zebrafish have the capacity to regenerate large sections of the myocardium following injury (Poss *et al*, 2002) (Lien *et al*, 2006). This phenomenon has been attributed to the dedifferentiation of cardiomyocytes which renders them capable of cell cycle re-entry and division (Jopling *et al*, 2010). Similarly, fifteen percent of the neonatal mouse heart was regenerated by cardiomyocyte cell division, but this capacity was lost seven days after birth which corresponds to the approximate timing of cell cycle withdrawal (Porrello *et al*, 2011). Although such a dramatic potential has yet to be demonstrated in the adult mammalian heart, it has become evident that the human heart has a 1% renewal rate per year, and in

the adult heart approximately 45% of cardiomyocytes are generated after birth (Bergman *et al*, 2009). Thus it appears that regulation of the cell cycle may be key to embryonic and postnatal cardiac development, as well as potential therapeutic intervention in heart disease.

1.1 The Cell Cycle:

The cell cycle is a complex and tightly regulated process which exists in five distinct phases and has numerous checkpoints to inhibit abnormal cell cycle entry and proliferation. The cell cycle begins in Gap 1(G1) in which cells grow and prepare to enter the cell cycle. Cells which remain in G1 for extended periods of time can exit the cell cycle and are said to be in a quiescent stage termed G0. They may re-enter the cell cycle or express genes necessary for terminal differentiation and become senescent. When the appropriate growth signals are received cells can pass through a restriction point in G1, after which they are committed to the cell cycle. The next stage is the synthesis (S) phase in which DNA is duplicated, followed by a second preparatory phase Gap 2 (G2). Cells then enter mitosis (M) during which chromatin is condensed and assembled at the mitotic plate. DNA and cellular components are separated into two daughter cells which divide in a process called cytokinesis.

Cell cycle regulation is managed in large by cyclin dependant protein kinases (CDKs) which, in association with cyclins, phosphorylate a wide range of proteins leading to the appropriate expression of genes for coordinated induction and passage through the cycle. Cyclin type and level of expression fluctuates throughout the cycle and different cyclins positively regulate CDKs, which remain at stable levels. Cyclin D

associates with CDK4 and CDK6 during G1, which is important for passage through the G1/S restriction point. Cyclins E or A associate with CDK2 to create the S phase promoting factor (SPF). Cyclin B or A associates with CDK1 to promote mitosis by creating the M-phase promoting factor (MPF). While the embryonic heart contains large amounts of cyclins and CDKs, they are down-regulated in quiescent adult cardiomyocytes (Reviewed in Ahuga *et al*, 2007).

CDK inhibitors (CDKIs) negatively regulate CDKs by competing with cyclins for binding, thereby blocking cell cycle progression. They exist in two groups: Cip/Kip (p21, p27, and p57) which broadly inhibit both CDK4/6 and CDK1/2 and Ink4 (p15, p16, p18, and p19) which selectively inhibit CDK4/6 (Sherr *et al*, 1994) (Sherr *et al*, 1995). In contrast to cyclins, Cip members are highly expressed in adult cardiomyocytes which have permanently withdrawn from the cell cycle (Lim *et al*, 1997).

Together cyclins and CDKIs respond to signalling pathways and mitogens to activate or inactivate the appropriate CDK complexes throughout the cell cycle. This process appears to be crucial to cardiac development. Deletion of CDK2 and CDK4 is embryonic lethal due to cardiac defects (Berthet *et al*, 2006) and hyperplasia is observed in mice which over express cyclin D (Soonpa *et al*, 1997) and CDK2 (Liao *et al*, 2001). These defects have been attributed to hypophosphorylation of the pocket protein pRb and consequently activation of the E2F pathway. In fact, the pocket protein and E2F families are amongst the most widely studied targets of CDK complexes. This is because the E2F/Pocket protein families play a pivotal role in cell cycle control by regulating the expression and repression of genes involved in cellular proliferation, differentiation, and death (Ishida *et al*, 2001)(Muller *et al*, 2008)(Ren *et al*, 2002).

1. 2 E2F Family Member Structure

The E2F family consists of nine transcription factors: E2F1, E2F2, E2F3a, E2F3b, E2F4, E2F5, E2F6, E2F7, and E2F8 (Figure 1). Each member shares a DNA binding domain in common consisting of a winged helix motif. In order to form functional DNA binding complexes E2Fs 1-6 also share a dimerization domain containing a leucine zipper which allows them to heterodimerize with Differentiation Proteins DP1 and DP2 (Tao et al, 1997)(Helin *et al*, 1993). DPs also share regions of homology to the E2F family in their DNA binding and dimerization domains. E2Fs 7 and 8 lack a dimerization domain and instead have two DNA binding domains, and form homo and/or heterodimers with one another (DiStefano *et al*, 2003) (Baidehi *et al*, 2005).

E2F 1-5 also share homology at their carboxyl-termini termed a transactivation domain, which is thought to recruit basal transcriptional machinery and promote gene transcription (Johnson *et al*, 1998). Embedded within this domain is a pocket protein binding domain, which when bound by pocket proteins inhibits transactivation and forms a repression complex (Flemington *et al*, 1993). Additionally, E2F 1-3 share regions of homology in their amino-termini including a domain which binds cyclin A, which promotes their phosphorylation (Kitagawa *et al*, 1995) (Krek *et al*, 1994), and a nuclear localization signal (Takahashi *et al*, 2000) (Mariconti *et al*, 2002). E2F4 and E2F5 instead share nuclear export signals embedded within their DNA binding domains, while E2F6-8 lack any recognizable nuclear import or export signals. E2F6 contains a region at its carboxyl-terminus termed a “repression” domain which has been demonstrated to interact with a variety of different proteins which form gene repression complexes (Trimarchi *et al*, 2001) (Atwool *et al*, 2005).

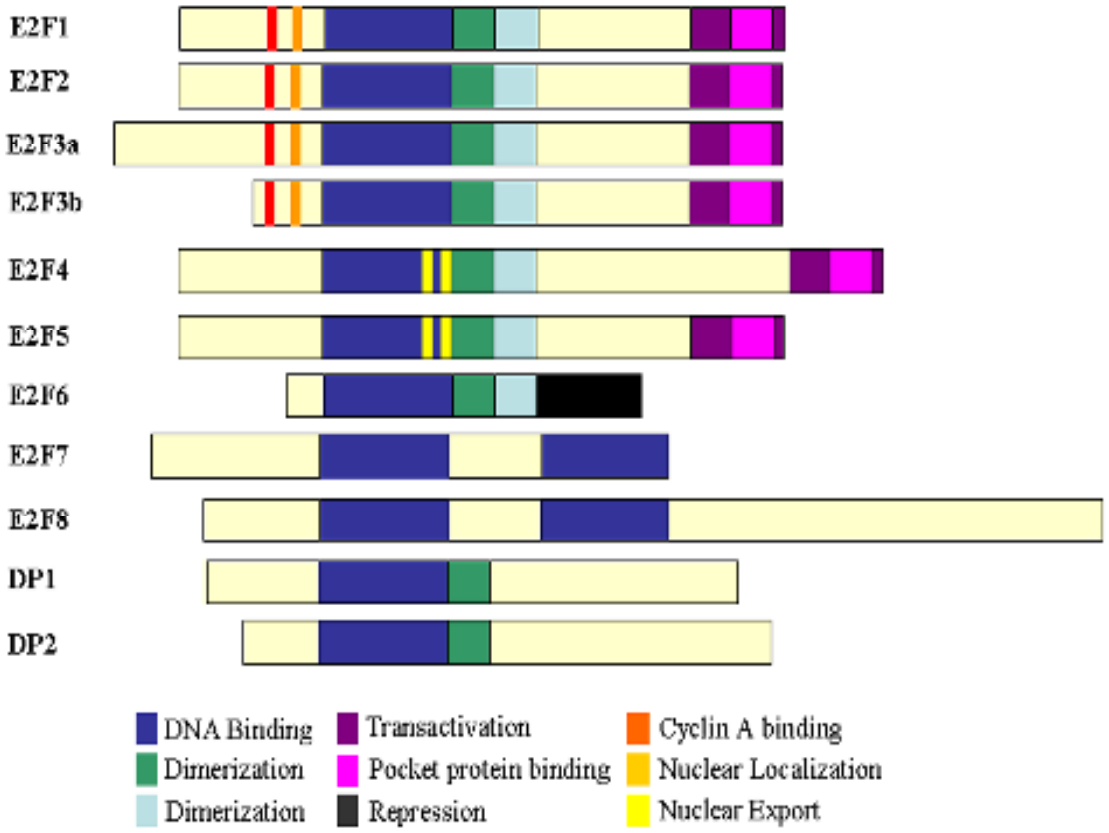


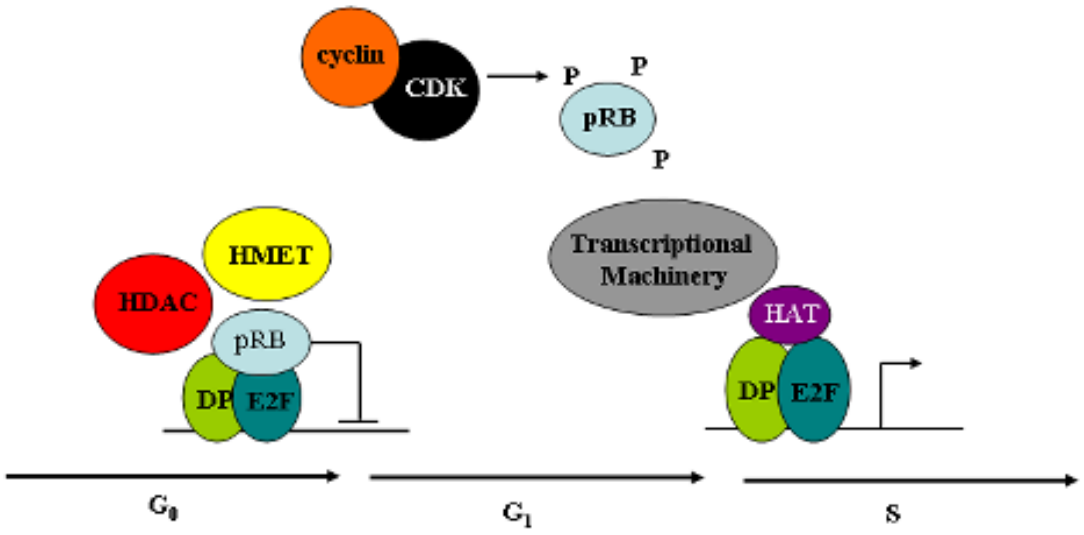
Figure 1: *Schematic of the E2F and DP Family Members*. All members share a DNA binding domain and E2F1-6 dimerize with DPs through their dimerization domain in order to form functional DNA binding heterodimers. E2F7 and E2F8 do not bind to DPs and instead have two DNA binding domains. E2F1-5 share a C-terminal domain that is responsible for transcriptional transactivation which also contains a pocket protein binding domain. E2F1-3 share a Cyclin A binding motif as well as a nuclear localization signal in their amino-terminal while E2F4 and E2F5 share nuclear export signals embedded in the DNA binding domain. The C-terminus of E2F6 contains a domain important for the recruitment of polycomb proteins for gene repression.

1.3 E2F/Pocket Protein Pathway

Underscoring the importance of the E2F-Rb pathway is its evolutionary conservation in *D. Melanogaster* (Ohtani *et al*, 1994), *C. Elegans* (Ceol *et al*, 2001), and *A. Thaliana* (Ramirrez *et al*, 1999). In more primitive animals there exists only a single E2F activator and repressor, and one pocket protein: pRb. The mammalian pocket proteins include pRb, p130, and p107 which interact with the "classical" E2Fs 1-5. E2Fs 1-3 interact primarily with pRb (Lees *et al*, 1993), E2F5 with p130 and p107, and E2F4 interacts with all members (Moberg *et al*, 1996). Although the mammalian E2F/pocket protein family is much more diverse, E2F1-5 are regulated by pocket proteins in a fashion which is similar to that of invertebrates (Figure 2A).

During G_0 and early G_1 stages of the cell cycle hypophosphorylated pocket proteins bind to E2Fs masking the transactivation domain at their C-terminus, thus leading to transcriptional repression (Ginsberg *et al*, 1994) (Figure 2A). Pocket proteins can recruit a number of histone modifying enzymes including histone deacetylases (HDACs), and heterochromatin proteins (HP1) which recruit histone methyltransferases (HMETs) in order to form heterochromatin and maintain gene repression (Brehm *et al*, 1998) (Ferreira *et al*, 1998). As cells receive growth signals CDK4 (in association with cyclin D) phosphorylates pocket proteins causing them to become hyperphosphorylated and release the E2Fs. Subsequently, E2Fs can recruit chromatin modifying enzymes such as the histone acetyl transferases (HAT) p300 and Tip60 in order to relax chromatin architecture and activate gene expression (Marzio *et al*, 2000)(Taubert *et al*, 2004).

A


cyclin
CDK
P
P
pRB
P
HMET
HDAC
pRB
DP
E2F
Transcriptional Machinery
HAT
DP
E2F
G0
G1
S

B

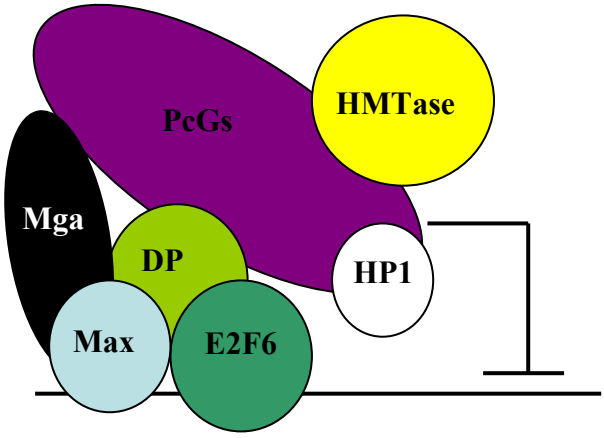

PcGs
HMTase
Mga
DP
HP1
Max
E2F6

Figure 2: *E2F/Pocket Protein Family Function and Regulation*. (**A**) During G0 E2Fs' transactivation domains are masked by pocket proteins, resulting in E2F responsive gene repression. Pocket proteins can recruit histone deacetylases (HDACs) and histone methyltransferases (HMETs) to actively repress gene activity by chromatin remodelling. As cells transition into G1/S phase, pocket proteins become phosphorylated by cyclin/CDK complexes freeing E2F/DP complexes. Histone acetyl transferases (HATs) are recruited to open chromatin architecture and transcriptional machinery activates the E2F responsive genes necessary for cell cycle progression. (**B**) E2F6 is not regulated by pocket proteins. It represses gene activity by recruiting polycomb group proteins (PcGs) and heterochromatin protein 1(HP1) which recruits HMETs forming a large repression complex. This complex modifies chromatin leading to gene silencing. E2F6 may also interact with other transcription factors such as Max and Mga.

1.4 E2F Family Member Function

E2F family members are generally grouped in two categories based on their capacity to drive quiescent cells into S phase and their presence at E2F responsive promoters during the cell cycle. E2F1-3a are described as activators of transcription while E2Fs 3b-8 are gene repressors. The repressors are usually subdivided into those which interact with pocket proteins (E2F3b-5) and those whose mechanism of repression is pocket protein independent (E2F6-8). These categories appear to be over simplistic as increasing evidence suggests that repressor E2Fs can activate and activator E2Fs can repress transcription (discussed further in next section). Further complicating the understanding of individual E2Fs is their large family size. When an individual member is deleted other members have the capacity to compensate highlighting their redundancies (Giangrande *et al*, 2004). Furthermore, over expression and/or knockout (KO) of individual E2Fs shifts the balance of activator: repressor E2Fs as well as the availability of DP and pocket proteins. Thus these types of studies must be interpreted with caution. Granting all this, knockout and double knockout (DKO) mouse models have aided in determining some of the tissue and temporal specific functions of particular E2Fs, and have contributed to our overall understanding of the importance of proper regulation of this pathway.

1.4.1 Activating E2Fs

E2F1-3a represent the activator E2Fs which appear to have both overlapping and specific functions. When over-expressed each member induced activation of E2F responsive genes and had the capacity to cause cell cycle re-entry in quiescent cells (Lees *et al*, 1993) (Lukas *et al*, 1996). Deletion of individual activators did not affect fibroblast

proliferative capacity, but deletion of all three inhibited proliferation suggesting a level of functional redundancy between members (Wu *et al*, 2003). In spite of this, their temporal and tissue specific expression points towards non-redundant roles as well. E2F1 and 2 are up-regulated at the G1/S boundary of the cell cycle (Johnson *et al*, 1994) while E2F3a is expressed very highly during mid G1 and remains stable through S phase (Leone *et al*, 1998). In addition, E2F1 is the only activator expressed in the brain after early embryonic development (Helin *et al*, 1993), and while E2F1 and 3 are expressed in the heart, E2F2 is not (Slansky *et al*, 1996).

E2F1 was the first family member identified and its function is the best described. $E2F1^{-/-}$ mice are viable and isolated fibroblasts have a normal proliferative capacity, but adult mice have a higher incidence of tumour development (Field *et al*, 1996) (Yamasaki *et al*, 1996) (Cloud *et al*, 2002). In support of its role in cancer elevated levels of E2F1 have been found in breast (Han *et al*, 2003), ovarian (Reimer *et al*, 2006), and gastric (Xiao *et al*, 2007) cancers. Furthermore, mutations of its inhibitor pRb have been implicated in dozens of cancers, and DKO of pRb and E2F1 can reverse tumour incidence (Yamasaki *et al*, 1998).

In addition to cancer, E2F1 has been demonstrated to participate in both p53 dependant and independent apoptotic pathways. E2F1 can directly activate the expression of the CDKI: $p14^{ARF}$, which in turn inhibits MDM2 (an inhibitor of p53), resulting in the stabilization of p53 and activation of apoptotic pathways (Haupt *et al*, 1997) (Hiebert *et al*, 1995). It has also been suggested that E2F1 can directly interact with p53 through its cyclin-A binding domain (Hseih *et al*, 2002). Thus E2F1's potential to activate p53 would depend on competitive binding between cyclin A and p53 (Rogers *et al*, 1996).

Like E2F1 null mice, E2F2$^{-/-}$ mice are also viable and isolated fibroblasts proliferate at a normal rate (Murga *et al*, 2001). Adult E2F2$^{-/-}$ mice are more susceptible to infections and develop late-onset autoimmune disease. Although E2F2 is usually described as a driver of cellular proliferation, its ablation resulted in hyper proliferation in T-lymphocytes suggestive of an additional function in repressing T-cell proliferation.

E2F3 has also been widely researched and its ablation is the only individual E2F knockout which appears to affect embryonic viability. E2F3 KO leads to partial embryonic lethality which has been attributed to defects in proliferation (Humbert *et al*, 2000). The lethality of E2F3$^{-/-}$ appears to be dependent on mouse strain. Pure strain 129/Sv mice have 100% embryonic lethality while in a mixed background (C57BL/6 x 129/Sv) 30% die in utero, 45% die within 24hrs after birth, and 25% survive to adulthood, 85% of which eventually die of congestive heart failure that is preceded by dilated cardiomyopathy (Cloud *et al*, 2002).

The major time points of E2F3$^{-/-}$ death correlate to major cardiac proliferative events in embryonic development as well as during the final round of the cardiac cell cycle following birth (King *et al*, 2008). This hinted at a potential cardiac proliferation defect. Upon histological analysis of E2F3 KO mice, several cardiac defects were discovered including hypoplastic septal walls and defects in septal development. A decrease in the proliferative index (measured by BRDU incorporation) in the embryonic heart of KO mice was also observed. Thus it appears that E2F3 is necessary for cardiomyocyte proliferation during embryonic development. Additionally, autopsy of KO mice which survived into adulthood revealed dilated hearts and atrial thrombi (King *et al*, 2008). The late-onset dilated cardiomyopathy is potentially due to the progressive

accumulation of stress on the heart due to defects in sarcomere organization which were observed. Thus it is evident that E2F3 is crucial for both embryonic and postnatal cardiac development and function.

Despite the array of studies depicting specific roles for individual activators, a group provided evidence suggesting that like their invertebrate counterparts, mammals too can survive with a single activator E2F (Tsai *et al*, 2008). The authors created different combinations of compound knockout mice in order to more closely evaluate the individual and redundant roles of activator E2Fs. Their studies confirmed many previously published results with respect to adult phenotypes of KO of individual E2F members, such as increased incidence of tumour development in $E2F1^{-/-}$ mice. They also took a closer look at E2F3 by creating separate E2F3a and E2F3b KOs. Although mice with deletion of E2F1; E2F2; E2F3b were viable, mice with a deletion of E2F1; E2F2; E2F3a mice were not, thereby suggesting that the crucial role of E2F3 in embryonic development can be attributed to E2F3a. Most interestingly, when E2F3b or E2F1 was expressed from the same genetic locus as the deleted E2F3a, mice were rescued from the phenotype of $E2F3a^{-/-}$ (Tsai *et al*, 2008). This suggests that the specificity of activating E2Fs has more to do with genetic context which controls spatial and temporal expression than a specific function of an individual family member.

1.4.2 Repressor E2Fs

A) E2F4 and E2F5

E2F4 and 5 represent the class of E2F repressors which are governed by pocket proteins. Both members are nuclear localized during early stages of the cell cycle at

which time they are thought necessary to repress genes involved in cell cycle progression. They become exported from the nucleus as the cell cycle advances and genes required for progression should be expressed (Lindeman *et al*, 1998) (Verona *et al*, 1997). E2F4 is constitutively expressed throughout the cell cycle, while E2F5 is expressed more highly during mid-G1 (Moberg *et al*, 1996). Over expression of E2F4 and 5 revealed they did not have the same capacity as activator E2Fs to drive cell cycle re-entry in quiescent cells. Instead, they tend to act as repressors of E2F responsive genes (Trimarchi *et al*, 2001). This repressor effect may be tissue specific as other groups found that E2F4 over expression drove neonatal cardiomyocytes into S phase (Ebelt *et al*, 2005) and promoted cellular proliferation during foetal erythropoiesis (Kinross *et al*, 2006).

Knockout mouse models of E2F4 and 5 are in agreement with early studies depicting them as negative regulators of the cell cycle which are important for terminal differentiation. Mouse fibroblasts lacking E2F4 and E2F5 are unable to exit the cell cycle (Bruce *et al*, 2000) and DKO mice die late in embryogenesis due to defects in differentiation (Gaubatz *et al*, 2000). Adult $E2F4^{-/-}$ mice display defects in differentiation of erythrocytes as well as gut epithelial cells, and die early due to increased susceptibility to infections (Humbert *et al*, 2004)(Rempel *et al*, 2000). $E2F5^{-/-}$ mice develop a non-lethal hydrocephalus due to excess cerebrospinal fluid production which has been attributed to a defect in choroid plexus differentiation (Lindeman *et al*, 1998). Like E2F1, E2F5 also appears to play a role in the development of cancer (Kothandaraman *et al*, 2010) (Polanawska *et al*, 2000).

B) E2F6:

E2F6 was the first E2F family member identified which lacked a C-terminal activation domain (Gaubatz *et al*, 1998). When over-expressed, E2F6 was a potent repressor of E2F responsive gene expression and was capable of inhibiting cell cycle progression and re-entry in quiescent cells (Gaubatz *et al*, 1998) (Cartwright *et al*, 1998). The truncated C-terminus of E2F6 alluded to the fact that its mechanisms of regulation and manner of repression are independent of the classical pocket protein pathway (Figure 2). Although not regulated in the same manner as other E2Fs, E2F6 expression does fluctuate throughout the cell cycle. Its expression is up-regulated at both the mRNA and protein level during the G1/S phase boundary. Our lab has shown that the E2F6 promoter contains two E2F binding elements which when bound by other E2Fs activates E2F6 expression (Lyons *et al*, 2006). This implies that when activating E2Fs are expressed early in the cell cycle they up-regulate E2F6 expression to ensure no improper passage through the G1/S checkpoint. In support of this Chromatin Immunoprecipitation (ChIP) studies demonstrated that E2F6 is present at E2F responsive gene promoters during G1 and S phase but not other phases of the cell cycle (Takahashi *et al*, 2000).

E2F6's capacity to repress gene expression appears to involve the recruitment of polycomb group proteins (PcGs) (Figure 2B). PcGs include a wide variety of chromatin modifying enzymes found in two distinct types of complexes which are epigenetic gene silencers during development (Reviewed in Sparmann, 2006). The PRC2 complex is important for the definition and trimethylation of histones to be repressed at histone 3 lysine 27 (H3K27). This marker recruits the PRC1 complex for long term gene silencing marked by trimethylation at histone 3 lysine 9 (H3K9). E2F6 has been identified in

complexes containing proteins from both types of complexes including Bmi1, Ring and YY1 binding protein (RYBP), EPC1, and EZH2 (Trimarchi *et al*, 2001)(Atwool *et al*, 2005).

Complimenting E2F6's interaction with PcG proteins, deletion of E2F6 has a similar phenotype to the deletion of the PcG protein Bmi1. This includes posterior homeotic transformations of the axial skeleton (Storre *et al*, 2002). Interestingly, embryonic fibroblasts from these mice did not display any defect in proliferative potential. The authors have attributed this to functional compensation by E2F4. A similar effect was previously demonstrated by a ChIP experiment in which E2F4 replaced E2F6 at E2F responsive promoters in E2F6 KOs (Giangrande *et al*, 2004).

E2F6 has also been described in a unique complex in HeLa cells capable of binding to E2F sites during G_0 (Ogawa *et al*, 2002). This E2F6 complex includes DP1, HMET, HP1, various PcGs as well as the transcription factors Max and Mga (Figure 2B). This complex was capable of binding and repressing E2F as well as Max and Mga responsive genes (E-box and T-box sites), thereby implicating E2F6 in a complex which could be important for the repression of an expansive array of genes during cell cycle arrest. Although this study differs from other studies suggesting only E2F4 is expressed and bound to promoters during G0 (Takahashi *et al*, 2000), the authors have attributed the differences to the use of different cell types and mechanisms of cell cycle arrest. This appears plausible since ChIP studies done by Xu and colleagues demonstrated that E2F1, E2F4, and E2F6 were present at the same promoters in various cell types, yet E2F6 may also have a specific function in Ntera2 cells, in which it bound to a novel set of gene promoters (Xu *et al*, 2007). These studies suggest that E2Fs can share interchangeable

roles, yet in specific cells and settings E2F6 (as well as other E2Fs) could play some unique roles in gene expression. These special roles likely involve interactions with other transcription factors and chromatin modifying enzymes.

C) E2F7 & E2F8

E2F7 and 8 represent the last group of E2Fs which have been identified. Like E2F6 they are not regulated by pocket proteins and unlike all other E2Fs they do not dimerize with DPs. They appear to be strong repressors of E2F activity which are capable of inhibiting cellular proliferation when over expressed (Christensen *et al*, 2005) (Li *et al*, 2007). The two proteins appear to have overlapping roles in development since individual KO mice develop normally, but DKO mice die very early during embryonic development due to apoptosis in various cell lineages (Moon and Dyson, 2008). Their redundancy is also supported by their capacity to form heterodimers which repress gene expression.

Moon and Dyson review a feedback loop, similar to what we described for E2F6 (Lyons *et al*, 2006), in which E2F1 expression activates the transcription of E2F7 and 8 which in turn represses E2F responsive genes including E2F1 itself (Moon and Dyson, 2008). This loop appears to be vital since the lethal apoptosis in E2F7/8 DKO mice has been attributed to elevated levels of both E2F1 and p53 (Li *et al*, 2007). Interestingly, the KO mice also exhibit severe dilation of blood vessels which is not yet understood, but may represent a specific function for these outlier E2Fs.

Counter-intuitive to its role as a repressor of proliferation and E2F1 mediated apoptosis, E2F8 is up-regulated in human hepatocarcinoma (HCC) specimens (Deng *et al*, 2010). Its knockdown is sufficient to inhibit colony formation of HCC derived cell

lines and decrease tumorogenicity *in vivo*. The authors found that E2F8 over expression caused an increase in DNA synthesis and cyclin D1 expression, which has been previously linked to HCC (Tashiro *et al*, 2007), while knockdown had the opposite effect. The authors have attributed the unexpected up-regulation of cyclin D1 (an E2F target) to excess E2F8 outcompeting E2F1 which would normally bind in association with pRb leading to gene repression (Deng *et al*, 2010).

This study is a perfect example of how E2Fs cannot be restricted to activators and repressors of gene expression and cellular proliferation. Instead, the outcome of E2F activity will depend on the regulation of a delicate balance in E2F activity. Although not all E2F members are necessary for viability the largeness of the family not only safeguards against mutation but also allows the tight regulation of development and function in complex mammalian tissues and organisms. E2F activity will be dictated by a variety of different factors including: genetic context, sub-cellular localization, spatial-temporal specific expression, and cell cycle stage/signals.

1.5 E2Fs in Cardiac Growth and Pathology:

Proper cardiac development requires a very tight regulation of the cell cycle. Thus it is likely that the E2F pathway will play a pivotal role in cardiac development and pathology as demonstrated in the E2F3 KO studies described earlier. In fact, many of the E2F/pocket protein family members appear to play important roles in cardiac growth and disease (Summarized in Table 1) (Reviewed in Rueger and Tuana, 2011).

Table 1: E2F/Pocket Protein Members Function in the Heart. Knockout mouse models of E2F and pocket proteins. * Cardiac restricted deletion.

Gene	Phenotype	Reference
E2F1	Cardiac Apoptosis	Field *et al* 1996; Yamasaki *et al*, 1996.
E2F3	Partial penetrant embryonic lethality due to cardiac developmental defects. Adults develop congestive heart failure.	Humbert *et al*; 2000a; Cloud *et al*, 2002;King *et al*, 2008
E2F1;E2F2;E2F3a	Partial embryonic lethal, perinatal lethal. Reduction in white adipose tissue deposits.	Tsai *et al*, 2008
E2F1;E2F2;E2F3b	Viable, but have reduced body weight.	Tsai *et al*, 2008
E2F7;E2F8	Embryonic lethal. Excess apoptosis (due to increased E2F1 and p53) and blood vessel dilation.	Li *et al*, 2008
Rb*; p130	Cardiac hyperplasia.	Maclellan *et al*, 2005
Rb*;p107	Embryonic lethal. Increased proliferation in central nervous system, blood vessel endothelial cells, and heart defects (double-outlet right ventricle).	Berman *et al*, 2009

E2F1 was the first of the E2F family which was described to play a role in cardiomyocyte cell cycle control. When neonatal cardiomyocytes were transfected with E2F1 they displayed a marked increase in DNA synthesis accompanied by a high rate of apoptosis, thereby demonstrating the capacity of the E2F pathway to control cardiomyocyte proliferation and death (Kirshenbaum *et al*, 1996) (Agah *et al*, 1997). Neonatal cardiomyocytes still retain some proliferative capacity and differ greatly from post-mitotic adult cardiomyocytes, thus adult rat ventricular myocytes were transfected with E2F1. This also induced DNA synthesis, but to a lesser extent (19% vs. 47%) which again was accompanied by apoptosis (Agah *et al*, 1997). The effects of ectopic expression of E2F1 were also explored *in vivo* by adenoviral injection of E2F1 into the myocardium of adult mice. Similar to the cardiomyocytes, an increase in DNA synthesis

was observed and cardiomyocytes accumulated in G2/M, but none were capable of overcoming the G2/M checkpoint and proliferating. Since E2F1 interacts with the p53 pathway, Agah and colleagues transfected E2F1 into the myocardium of p53 null mice. Surprisingly, this did not alleviate the rate of apoptosis indicating that E2F1 can induce cell death in a p53 independent pathway.

In addition to the p53 pathway of apoptosis the mitochondria plays an important role in programmed cell death. During hypoxic injury the mitochondrial permeability pore opens causing a loss of membrane potential and cytotoxic protein release. This activates apoptotic pathways leading to ventricular myocyte death (Reviewed in Wang, 2009). It has previously been demonstrated that the mitochondrial death protein Bnip3 plays a role as a sensor of oxidative stress during myocardial infarction (MI) (Kubli *et al*, 2008). Furthermore the induced expression of Bnip3 leads to ventricular myocyte death (Regula *et al*, 2002). Yurkova showed that ectopic E2F1 directly activates the transcription of this death factor *Bnip3* (Yurkova *et al*, 2008). Protein levels of Bnip3 were not confirmed, but two inhibitors of Bnip rescued cells from the apoptosis incurred by E2F1 in cardiomyocytes. Thus it appears that in addition to interacting with p53 (in cardiomyocytes) E2F1 can regulate apoptosis by controlling the levels of hypoxia inducible pro-apoptotic factors.

In addition to regulating apoptosis in cardiomyocytes, it appears that the E2F family also plays a central role in regulating cell growth- a key aspect of cardiomyopathic heart failure. Upon hypertrophic stimulation with phenylephrine E2Fs 1-4 and 6 became up-regulated in neonatal cardiomyocytes (Vara *et al*, 2003) (Movassagh *et al*, 2005). The importance of the E2F pathway in cardiac hypertrophy was highlighted by inhibiting the

pathway with specific inhibitors for E2F/DP heterodimerization. This resulted in a decrease in the intensity of hypertrophy, as well as blocked the expression of hypertrophic markers ANP and BNP (Vara *et al*, 2003).

The capacity of E2Fs to induce cell cycle re-entry in the heart was evaluated and, indeed, over expression of E2Fs1-4 induced S phase in neonatal cardiomyocytes and induced transcription of cyclin A and E (Ebelt *et al*, 2004). In this study only E2F2 and E2F4 induced S phase without also causing apoptosis, and only E2F2 over-expression resulted in mitosis. In order to determine if this was relevant in the adult heart Ebelt and colleagues stably over expressed E2F2 and E2F4 in the adult mouse myocardium by adenoviral infection (Ebelt *et al*, 2008). Similar to what was observed in the neonatal cardiomyocytes, over-expression of both E2F2 and 4 resulted in the re-entry of cardiomyocytes into S phase and cardiomyocyte hypertrophy. More importantly, expression of E2F2 also resulted in a modest increase in the number of mitotic adult cardiomyocytes. This is especially interesting since E2F2 is not normally expressed in the heart (Dirlam *et al*, 2007). Perhaps a lack of E2F2 hints at a protective mechanism against excess proliferation in the heart in order to maintain postnatal cardiac function. It also points towards a therapy to stimulate cardiac regeneration post MI.

Recently van Amerongen and colleagues demonstrated a specific function for E2F4 during cardiomyocyte mitosis (van Amerongen *et al*, 2010). In this study E2F4 over expression did not induce cell cycle entry in cardiomyocytes. Although this differs from earlier work in which Ebelt found that E2F4 over expression induces DNA synthesis, the manner in which cardiomyocytes were isolated was different in each study (Ebelt *et al*, 2005). In Ebelt's study cardiomyocytes were isolated from 0 day old rats,

while in van Amerongen's study cardiomyocytes were isolated from 3 day old rat hearts. Considering that cardiomyocytes permanently exit the cell cycle within a few days of birth the 3 day old hearts may have lost some proliferative potential. In this study both the expression and nuclear localization of E2F4 were correlated to cardiac development and the proliferative potential of cardiomyocytes, supporting its role in normal cardiac growth and development (van Amerongen *et al*, 2010). Unexpectedly, the authors found that E2F4 co-localized with kinetochores in cardiomyocytes, and when knocked down by siRNA restricted mitosis. This suggests a potential novel role for E2F4 in cell cycle regulation outside of transcriptional regulation. The relevance of these results in the adult myocardium is unknown as this was tested in postnatal cardiomyocytes which were artificially stimulated to proliferate (using FGF1 and a p38 inhibitor), but may be a useful tool in cardiac regeneration studies.

In addition to a balance in levels of individual E2F family members, appropriate regulation of the E2F pathway by pocket proteins is crucial for normal cardiac development. Although cardiac specific deletion of individual pocket proteins does not lead to a specific cardiac defect, compound knockouts tell a different story. Mice which are null for pRb are not viable thus cardiac restricted KO of pRb in conjunction with p130 or p107 knockouts have been utilized. Ablation of p130 and cardiac pRb led to a threefold increase in heart weight: body weight ratio due to abnormal hyperplasia (MacLellan *et al*, 2005). In this model an increase in Myc, E2F1, and G1 CDK activity was observed, indicating that indeed the E2F pathway and cell cycle had been activated.

The importance of p107 in the heart was highlighted in another study in which DKO of pRb and p107 led to cardiac defects and embryonic lethality (Berman *et al,*

2009). Mice lacking the two proteins developed a double outlet right ventricle (pulmonary artery and aorta exit from the right ventricle) and many embryos also displayed thinner myocardium, dilated atria, and septal defects. Thus it appears that all three pocket proteins play an important role in cardiac development although much like the E2Fs they have the capacity to compensate for each other's loss.

1.6 Clinical Relevance:

Accumulating evidence suggests that appropriate regulation of the E2F/pocket protein pathway is crucial to normal cardiac development. Highlighting this is the developmental regulation of individual E2F and pocket protein members within the heart (Ahuga *et al*, 2007) (Movassagh *et al*, 2006) (van Amerengon *et al*, 2010). Recently, a direct link between E2Fs and congestive heart failure (CHF) was demonstrated in humans. In this study patients with CHF displayed up-regulated levels of E2F1, pRb, p107, and p130 in comparison to control patients (Wohlschlaeger *et al*, 2010). A positive correlation between pRb and p130 with cardiomyocyte diameter was also described, thereby suggestive of their role in cardiomyocyte hypertrophy. Following unloading by left ventricular assistance device a significant decrease in expression of E2F1 and pocket proteins was observed, indicating that ventricular unloading can reverse the process. Thus it appears that similar to studies in cultured cardiomyocytes and mouse models, the E2F/pocket protein pathway is a pivotal player in human cardiac pathology.

The idea of transcriptional networks governing cardiac development and disease is an evolving concept. Transcription of the cardiac fetal gene program during the cardiac remodelling process has been well described (reviewed in Rajabi *et al*, 2007). During this

process fetal forms of genes such as myosin heavy chain and actin are expressed and the expression of adult forms of particular genes such as the cardiac calcium pump SERCA is reduced. Interestingly, this pathway can be activated by calcium deregulation. For example, excess calcium in the cell activates the calcium binding protein calmodulin which in turn activates calmodulin dependent kinase (CAMK). CAMK can enter the nucleus and phosphorylate HDACs which leads to chromatin relaxation and allows transcription factors such as Myc and GATA4 to transcribe fetal genes (reviewed in Bers, 2008). Thus the cardiomyocyte has the capacity to monitor the cellular environment and respond to fluctuations potentially caused by cardiac dysfunction. Although meant to correct defects, the prolonged effects of this process will eventually become more detrimental to heart function then beneficial, often leading to heart failure. Thus it appears that hypertrophy is governed by transcriptional networks including the induction of the fetal gene program as well as the E2F/Rb pathway which controls the induction of the cell cycle- associated with cardiac hypertrophy.

1.7 MicroRNAs in the Heart

Further complicating the control of gene expression is the relatively recent identification of a group of small non-coding RNAs which regulates gene expression post-transcriptionally (reviewed in Boyd, 2008).These microRNAs are very short RNAs which can bind to short complimentary regions of mRNA, thereby targeting their destruction by the DICER complex.

MicroRNAs are known to have tissue specific expression and thus can be important in regulating specific biological processes. It has become evident that these

tiny messengers play an incredibly important role in cardiac development and disease (reviewed in Zhang *et al*, 2008). Several microRNAs in particular have been identified as crucial, including miR-1 which plays a large role in the balance of cardiomyocyte differentiation vs. proliferation. Under conditions of over-expression, miR-195 and miR-208 lead to cardiac hypertrophy (van Rooij *et al*, 2006) (van Rooij *et al*, 2007), and others have been linked to cardiac arrhythmias including miR-133 (Xiao *et al*, 2007). Thus the expression of these small but mighty players must be kept in mind when exploring cardiac gene expression.

1.8 Dilated Cardiomyopathy

Dilated cardiomyopathy is the leading cardiomyopathy in North America. Unlike hypertrophy, the causes and pathways involved in the disease are less well defined. Dilated Cardiomyopathy (DCM) is characterized by enlargement of the left or both ventricular chambers. This is often accompanied by a thinning of the myocardium and may also result in a hypertrophic response. This leaves the heart weakened and inhibits its capacity to function properly as a pump, leading to a dangerous cycle of blood pooling and myocardial stretching. This eventually can lead to heart failure, arrhythmias, and sudden death.

Although many cases of DCM are idiopathic (unknown cause) a variety of factors have been linked to it, including but not limited to: alcohol consumption, toxic metals (lead, mercury), and viral infections causing myocardial inflammation. Microarray studies have uncovered that genes involved in inflammation and immunity are down-regulated in patients with DCM (Barth *et al*, 2006). In addition to these factors a large

portion of DCM cases (~30%) are associated with mutations found in genes involved in force generation and transmission (reviewed in Luk *et al*, 2009).

The sarcomere is the basic contractile unit in the cardiomyocyte. Myosin heads compose the thick filament which uses ATP to move along the thin filament composed of actin. The end result of which is contraction. Myosin and actin as well as various other proteins including the troponin-tropomyosin complex, which regulates myosin-actin interactions, dictate force generation. Scaffolding proteins in the cytoskeleton and sarcolemma such as desmin, vinculin, titin, dystrophin, and sarcoglycan determine force transmission. Point mutations, generally causing loss of function, have been found in all the above mentioned genes in patients with dilated cardiomyopathy (reviewed in Chang *et al*, 2005). Presumably the accumulated stress on the heart from ineffective force generation and transmission leads to cardiac remodelling and chamber dilation.

Numerous mutations in nuclear Lamin A and C have also been identified in cases of DCM. A loss of Lamin A or nuclear emerin is known to cause Emery-Dreifuss muscular dystrophy (EMD1) associated with cardiac conduction defects and atrio-ventricular (AV) block in addition to DCM. In a model of EMD1 an increase in both MyoD and E2F/Rb responsive genes was observed resulting in a defect in myogenic differentiation (Melcon *et al*, 2006). This was associated with an increase in hyperphosphorylated pRb. It has previously been demonstrated that the correct distribution of Lamin A/C is necessary for docking of hypo-phosphorylated pRb (Markiewicz *et al*, 2002). Thus it appears that structural and scaffolding proteins can induce changes in gene expression through their interactions with the E2F pathway, in this particular case leading to DCM.

As previously mentioned, E2F3 null mice which survive into adulthood develop late onset dilated cardiomyopathy with early defects in sarcomere organization (King *et al*, 2008). These defects could presumably cause defective force transmission over time leading to the observed DCM phenotype. Similar to the mouse model of EMD1, E2F3B$^{-/-}$ myocytes also show defects in muscle differentiation (Asp *et al*, 2009). Furthermore, E2F3B has been shown to be the major E2F family member which interacts with pRb in quiescent cell types (Asp *et al*, 2009). This suggests that there may be a link between E2F3B/pRb regulation and dilated cardiomyopathy induced by defects in cardiomyocyte differentiation and development.

1.9 Statement of the Problem:

The E2F pathway regulates cardiomyocyte growth, differentiation, and death, but the exact mechanism of such pathways has yet to be described in cardiac biology. In fact, the majority of studies on E2F in cardiomyocytes have been performed in cell culture, while those done *in vivo* focussed on pocket protein pathways and have led to drastic phenotypes, which cannot be properly evaluated. We have taken a different approach by changing the fine tuning of the E2F pathway in the heart using a unique member of the E2F family: the E2F6 transcriptional repressor. We hypothesize that *regulated levels of E2F6 in the heart would be necessary for proper heart development and function.* In support of this we found that E2F6 is expressed in cardiomyocytes and in the heart throughout murine development (Westendorp *et al*, 2008). Thus in order to test our hypothesis, we deregulated levels of E2F6 in the heart using a transgenic mouse model. The transgene was placed under the control of an αMHC promoter to ensure cardiac

specific expression and was tagged with a 6-myc repeat to track transgene expression. Intriguingly, the over expression of E2F6 led quickly to the development of Dilated Cardiomyopathy (DCM) in mice as young as 6 weeks old, as well as decreased survival rates of Tg mice (Westendorp *et al*, 2008)(Rueger *et al*, 2009)**.** The de-regulation of the E2F pathway caused a phenotype unique from previous studies suggesting that E2F6 is a novel and important regulator of cardiac development and disease.

My primary objective is to use our animal model in order to elucidate the role of the E2F pathway in the heart by altering the activity of E2F6 and evaluating how it might impact gene expression and cause the observed DCM phenotype. In doing this I will test our hypothesis that a shift in the balancing act of E2F activity will lead to changes in the normal functioning of the heart and in this case cause the disease state of dilated cardiomyopathy. In order to address this, I will focus on three main aspects of E2F6 in the heart including how it modulates: (1) the E2F pathway by exploring the expression and sub-cellular localization of other E2Fs and interacting proteins (2) E2F responsive gene activity, and (3) the development of dilated cardiomyopathy.

CHAPTER 2: MATERIALS AND METHODS

2.1 Transgenic Mice:

a) Mouse line: Previously established Tg mouse lines (Westendorp *et al*, 2008) were used and bred with B6C3F1 mice. All animals were handled according to protocols approved by the Institutional Animal Care Committee.

b) Genotyping: Genomic DNA was extracted from ear clips using Red Extract Tissue (Sigma). Transgenic mice were identified by PCR using a forward primer located in exon 6 (ELFT) and reverse primer located in exon 7 (MHCR). (Primer sequences in Table 2) This resulted in a ~650bp difference between endogenous and 6myc-tagged E2F6 (no introns) PCR products, which were visualized by EtBR staining on a 1% agarose gel. Genotyping was routinely verified at the protein level by western blot using anti-myc to detect 6myc-tagged E2F6.

Table 2: *Primer Sequences Used in this Study*. Primers used for Quantitative Real Time PCR and genotyping. (F) Forward primer, (R) Reverse primer.

Primer	Targeted Gene	5'-3' Sequence
18S (F)	18S rRNA	AATACATGCCGACGCGCCCTGACC
18S (R)	18S rRNA	AGTGGGTAATTTGCGCGCCT
ATF3 (F)	Activating Transcription Factor 3	CAGGCAGAAGTGTCTACCTTGAT
ATF3 (R)	Activating Transcription Factor 3	GGATACCCTATGCACAGTCATTC
ATF4 (F)	Activating Transcription Factor 4	ATGTGTAAAGGAGGAAGACACTCC
ATF4 (R)	Activating Transcription Factor 4	TAGCTGTCAAACTAACTCCAGGTG
CTGF (F)	Connective Tissue Growth Factor	GGACACCTAAAATCGCCAAG
CTGF (R)	Connective Tissue Growth Factor	GTAATGGCAGGCACAGGTCT
Cx 43 (F)	Connexin 43	GAACACGGCAAGGTGAAGAT
Cx 43 (R)	Connexin 43	GACGTGAGAGGAAGCAGTCC
E2F1 (F)	E2F1	AGT CCC TTT GTA CCA GTA CTC CAG
E2F1 (R)	E2F1	GAG GGA ACA GAA CTG TTA GGA AAC
E2F2 (F)	E2F2	GTAGGCAGGGAACTATTTGAAGAC
E2F2 (R)	E2F2	GGATATCCTGGTAGGTCACATAGG
E2F3A (F)	E2F3A	CCGTATCCCTTCATTCATTGTC
E2F3A (R)	E2F3A	CGA ACCCTCTCTCTCTCTTTTCTT
E2F3B (F)	E2F3B	ATGCCC ACAGCAGCAGGCAAAGC
E2F3B (R)	E2F3B	GAGCTGAATGAACTTCTTGGTGAG
E2F4 (F)	E2F4	CCTACCAGTCACTATGAAGGAGGT
E2F4 (R)	E2F4	CAGACTCCTAGCTAGACACAGCAA
E2F5 (F)	E2F5	AGAGTCCAGTTCATCTAAGCCAGT
E2F5 (R)	E2F5	GAAGATACTTCTGCAGCTGGTGT
ELF-T (F)	E2F6 (genotyping)	ATCACAGTACATATTAGGAGCACC
Gins2 (F)	Gins Complex 2	CAAGCTTCGAGTGTCTGCTG
Gins2 (R)	Gins Complex 2	GGCCTCTGGCTAGAAGTCCT
Hp1 (F)	Heterochromatin Protein 1α	TAC TTC TGT GCT AGC CTC CCT ACT
Hp1 (R)	Heterochromatin Protein 1α	AAG TAG CTC AGT GAG GGC TAA AAA
Kcne1 (F)	Potassium Rectifying Channel Subunit	CCTGCCATAGTCTTTATTGTAGGG

Kcne1 (R)	Potassium Rectifying Channel Subunit	TGTCTCCCATCAGAACTTAGTCAC
Mga (F)	Max Gene Associated	GCACCTTCACTGCTTTCCTC
Mga (R)	Max Gene Associated	AGCTGCAACAAGGCAAAACT
MMHC (R)	E2F6 (genotyping)	CTACCAGTCTACAATAGAGC
miR-122	miR-122 (Taqman probe)	GAGTGTGACAATGGTGTTT
miR- 124	miR-124 (Taqman probe)	TAAGGCACGCGGTGAAT
miR-206	miR-206 (Taqman probe)	TGGAATGTAAGGAAGTGTGT
Myl7 (F)	Myosin Light Chain 7	CAAGTTCTCTCCTGCTGAGGTAG
Myl7 (R)	Myosin Light Chain 7	GGCACAGAGTTTATTGAGGTGAC
Ned4 (F)	E3 Protein Ubiquitin Ligase	CTGATACCACAGGATCTCATCAAG
Ned4 (R)	E3 Protein Ubiquitin Ligase	GTGCCAGTGACAAACTGAAGTAAG
PDZ/Lim (F)	PDZ and Lim Domain containing 5	GCATGTGTCCTGTTTTGTGTG
PDZ/Lim (R)	PDZ and Lim Domain containing 5	ACATGTCACCAGCCTCTATGG
RCF4 (F)	Replication Factor 4c	CTGTATCAGGAAGTCGTTCAGATG
RCF4 (R)	Replication Factor 4c	GAACATCTAGAAGTGAGGGGTTCA
Ren (F)	Renin Binding Protein	TGAACCAAGAGGGAAAGGTG
Ren (R)	Renin Binding Protein	TTTCGAGCCTTCATGGGTAG
RPA2 (F)	Replication Factor 2	GGTCACTATTGTGGGGATAATCAG
RPA2 (R)	Replication Factor 2	GAACTCATTCATGTCTTCCAGAGG
SERCA (F)	Sarcoplasmic Recticulum Calcium ATPase	CTGTGGAGACCCTTGGTTGT
SERCA (R)	Sarcoplasmic Recticulum Calcium ATPase	CAGAGCACAGATGGTGGCTA
Sln (F)	Sarcolipin	TGAAGACAAGCCTTGGTGTG
Sln (R)	Sarcolipin	TGGCCCCTCAGTATTGGTAG
sno-202	Small nucleolar RNA-202 (Taqman probe)	GCUGUACUGACUUGAUGAAAGUACUUUU GAAC CCUUUUCCAUCUGAUG
Tnt2 (F)	Troponin T 2 (cardiac)	AAGATGCTGAAGAAGGTCCAGTAG
Tnt2 (R)	Troponin T 2 (cardiac)	TCGATCAGAGTCTGTAGCTCATTC
UNG (F)	Uracil DNA Glycosylase	GCTTCAGTGTCCAAAGACCAGTT
UNG (R)	Uracil DNA Glycosylase	AATGACACTGCCCTTCTTCTGAG

2.2. Microarray:

a) RNA Isolation and Preparation: RNA was previously isolated by Dr. Bart Westendorp from 3 Tg and 3 Wt hearts 7 days after birth using RNeasy Fibrous Tissue mini kit (Qiagen). RNA was processed at StemCore Laboratories in Ottawa, Ontario using the Affymetrix Gene Chip 430.2 mouse array.

b) Analysis: Results were analyzed using Flexarray software (Blazejczyk *et al*, 2007). Four normalization techniques (GCRMA, RMA, MAS5, and PLIER) were employed and statistically analyzed using SAM, 2 Sample Bayes T, and LPE algorithms. Fold change thresholds were set at >1.5 or < 0.75 accompanied by a p-value of <0.05. A gene list was compiled for each normalization method which was most representative of the three statistical methods (Found on Accompanying CD). The four lists were compared and genes which met the criteria in three or more lists were considered.

c) Pathway analysis: PANTHER gene expression tools were used to identify biological processes and molecular functions of proteins which were significantly deregulated in our microarray (Thomas *et al*, 2003). Up and down-regulated genes were analyzed separately in comparison to the NCBI mouse gene database. Results were corrected by the Boneferroni correction for multiple hypothesis testing. Biological processes and molecular functions significantly enriched in Tg animals with a p-value of <0.05 were considered.

2.3 microRNA array:

a) microRNA preparation: Total RNA was isolated from 3 Wt and 3Tg mouse heart tissues 7 or 37 days after birth using the mirVANA kit (Ambion) as per the manufacturer's protocol. Samples were processed at the Centre for Applied Genomics at the Sick Kids Microarray Facility in Toronto, Ontario using Illumina mouse miRNA 380 chip.

b) Analysis: Results for pups and young adults were analyzed separately using Flexarray software (Blazejczyk *et al*, 2007). Only one normalization algorithm was available for the Illumina array, thus it was used and five statistical analyses were employed: SAM, LPE, Cyber T, EB, and 2S to create five gene lists. Fold change thresholds were >1.5 or < 0.75 and a p-value of <0.05. A microRNA gene was considered if it met the criteria in 3 or more of the 5 lists.

2.4 Real Time Quantitative Reverse Transcription –PCR:

a) RNA Preparation: Total RNA was isolated from 7 or 37 day old mouse heart tissue using the mirVANA (Ambion) kit as per the manufacturers' protocol.

b) mRNA: 2ug of RNA and oligoDT primers (Invitrogen) were used to synthesize cDNA using SSII reverse transcriptase (Invitrogen) as per the manufacturer's protocol. A 1:50 dilution of the cDNA was used as a template in real-time QPCR using Fast Start SYBR Green with Rox (Roche) in a BioRad Thermo Cycler in a volume of 50µL. Reaction conditions were: 95^{o}C 10 min, (95^{o}C 15s, 60^{o}C 30s, 72^{o}C 45s, data collection) x 40, followed by a melting curve analysis. Samples were routinely run on 1.5% agarose gel to ensure only one product per QPCR reaction. (Primer sequences are listed in Table 2).

c) microRNA: Taqman microRNA assays (Applied Biosystems) were used to amplify microRNA. Approximately 30ng of total RNA was used per reaction as per the manufacturer's protocol. A 1:15 dilution of the cDNA was used in real time QPCR as recommended by the manufacturer in a BioRad thermo cycler. (Taqman probe sequences listed in Table 2)

d) Statistical Analysis: At least three trials were performed for each gene of interest to ensure reproducibility of results using 3-7 Wt and Tg hearts. Threshold values (Ct) were recorded and the fold induction of genes of interest were calculated for each Wt and Tg sample compared to a control gene (18S for mRNA and sno-202 for microRNA) using the ΔCt method. Student's T-tests were performed using Graph Pad.

2.5 Tissue Fractionation:

a) Tissue Collection and Fractionation: Hearts were collected from mice euthanized by CO_2 and rinsed in 1X PBS buffer on ice. Qiagen's Q-Proteome cell compartment kit was used to separate cytosolic and nuclear fractions according to the manufacturer's protocol.

b) Histone Isolation: In order to enrich histone proteins the following protocol was modified from a tissue subcellular fractionation protocol (Cox and Emil, 2006). Hearts were minced with scissors and rinsed twice in 1X PBS and once in 250 STM buffer (250mM sucrose, 50mM Tris-HCl pH 7.4, 5mM $MgCl_2$, 1% protease inhibitor cocktail (Roche), and 5mM sodium butyrate to inhibit histone deacetylases). Hearts were homogenized with a dounce homogenizer in 250 STM buffer. The lysate was centrifuged at 1000g at 4^{o}C for 10 min to pellet nuclei. Pelleted nuclei were resuspended in 0.2N HCl to solubilise histones, vortexed to ensure resuspension, and left rotating overnight at 4^{o}C.

Samples were centrifuged at 6500g for 10 min at 4^{o}C to pellet insoluble nuclear debris, and the supernatant was saved as the histone enriched fraction.

c) Protein Measurements: The protein concentrations of cytosolic and nuclear fractions were determined using the BCA protein assay kit (Thermo Scientific) as per the manufacturer's protocol. The protein concentration of histones was determined using the Bradford method due to their acidic nature.

2.6 Immunoblotting:

Hearts were collected from mice euthanized by CO_2 and rinsed in 1X PBS buffer on ice. Tissues were homogenized using an electric rotor in RIPA buffer (0.25% deoxycholate, 1 mM EDTA, 50 mM Tris base, 1% Nodidet P-40, 100 mM sodium chloride, and 1% complete mini EDTA free protease inhibitor cocktail (Roche)). Protein concentrations were determined using BCA protein assay kit (Thermo Scientific) as per the manufacturer's recommendations. Isolated protein or fractions (see Tissue Fractionation) were run on a denaturing 10% SDS-PAGE gel and histones were run on a 15% SDS-PAGE gels. Gels with fractionated samples were stained with Coomassie Blue (50% methanol, 10% acetic acid, and 0.05% coomassie blue) for 1 hour at room temperature and destained in 5% Methanol and 7% acetic acid for 2 hours at room temperature.

For immunoblotting gels were transferred overnight in buffer (25mM Tris, 190mM Glycine, 20% methanol) to a PVDF membrane (Bio-Rad). All membranes were blocked in TBST (1M Tris, 290 mM NaCl, 0.1% TWEEN pH 7.2) with 5% milk for 1hr at room temperature and incubated with primary antibodies (listed in Table 3) in TBST

with 5% milk for 1 hour at room temperature or at 4^{o}C overnight. Membranes were washed 3 times for 10min in TBST before adding the appropriate horseradish peroxidase labelled secondary antibody (Jackson) in TBST with 5% milk. Membranes were washed again 3 times for 10min in TBST and the conversion of ECL substrate (Roche) was detected on film. Bands were quantified by densitometry using Alpha Ease (Alpha Innotech). Membranes were stripped (25mM glycine, 1% SDS pH 2.5) and reprobed with different antibodies.

Table 3: *Antibodies Used in This Study*. Primary antibodies used for western blot.

Target Protein	**Catalogue Number**	**Company/Source**
Acetyl-Histone H3K9	9761	Cell Signalling
Acetyl-Histone H3K18	9675	Cell Signalling
Bmi1	sc-10745	Santa Cruz
Connexin 43	ab11370	Abcam
Connexin 40	AB1726	Millipore
DP1	MA1-21251	Affinity Bioreagents
DP2	11500-1-AP	Affinity Bioreagents
E2F1	sc-193	Santa Cruz
E2F3	sc-878	Santa Cruz
E2F6	sc-8366	Santa Cruz
Fibrillarin	sc-25397	Santa Cruz
GAPDH	RGM2	Advanced Immunochemicals
Histone H3	ab1791	Abcam
HP1 alpha	C7F11	Cell Signalling
Lamin A/C	sc-6215	Santa Cruz
c-Myc clone 9E10		(in house- mouse ascites)
p42/44 (ERK)	9102	Cell Signalling
Phospho-p42/44 (ERK)	9101	Cell Signalling
Tri-methyl histoneH3K9	9754	Cell Signalling
Tri-methyl histone H3K27	9733	Cell Signalling
γ-Tubulin	T6557	Sigma
Vinculin	V-4505	Sigma
YY1	H00007528-D01P	Abnova

2.7 Immunohistochemistry:

Hearts were isolated from mice euthanized by CO_2, rinsed in 1X PBS, and fixed in 10% paraformaldehyde overnight. Hearts were embedded in paraffin and 5µm sections were embedded on slides. The slides were placed in an organic solvent (xylene) to deparaffinise and subsequently rinsed in a descending ethanol gradient (100%, 95%, 70%) followed by ddH_2O to hydrate the tissue. Antigen retrieval was performed using 95^oC sodium citrate (10mM sodium citrate, 0.1%Tween, pH 6.0) for 10min. Samples were rinsed in 1X PBS, treated with 0.1% H_2O_2 1X PBS for 15 min, and rinsed twice in 1X PBS for 5min. Blocking was performed using 5% bovine serum albumin (Sigma) and 0.3% Triton X-100 for 30 min. Primary antibody to Cx43 (ab11370) was added overnight at 4^oC in TBST containing 5% BSA and 0.3% Triton X-100. Slides were rinsed 3 times in 1X PBS for 5 min prior to the addition of Goat- Anti-Rabbit HRP (Jackson) in TBST containing 5% BSA and 0.3% Triton X-100 for 1hr at room temperature. The slides were rinsed three times in 1X PBS for 5 min and immersed in DAB prepared according to the manufacturer's protocol (Sigma) for 20min. The sections were rinsed in dH_2O and counterstained in filtered Haematoxylin for 5min. The slides were dipped in 100% EtOH (~20 times) to dehydrate the sections and then in xylene (~30 times). Permount (Fisher) was used to mount cover slips and slides were observed using an analog light microscope.

2.8 Electrocardiography

Mice aged 6-8 weeks were lightly anaesthetized using isoflurane. Twelve lead electrocardiograms (ECG) were measured using surface electrodes attached to the limbs, amplified by the bol amplifier (EMKA technologies), and digitally recorded using the IOX2 version 2.4.2.6 (EMKA technologies). Readings from lead II were analyzed using ECG Auto version 2.5.1.18 (EMKA technologies) to obtain interval measurements. Student's T-tests were performed using Graph Pad.

CHAPTER 3: RESULTS

3.1. E2F6 Serves to Activate Transcription of E2F Responsive Genes in the Myocardium

Transcriptional networks are vital regulators of cardiac development and disease. Activation of the foetal gene program and genes involved in cell cycle progression has been noted as key in cardiac remodelling and cardiomyopathy, of which the E2F family plays a fundamental. E2F6 represents a unique modulator of E2F responsive genes, thus the transcript profile of Tg mice was evaluated by microarray. Thereby a basis for the E2F pathway and E2F6's role in the myocardium could be deduced as well as linked to the dilated cardiomyopathy phenotype observed. Seven day pups were used as opposed o adults in order to avoid any noise caused by the disease process.

In order to evaluate transcript levels with a microarray, the expression of genes of interest are normalized to control probesets and fold changes in expression are calculated. Gene ontology (GO) is employed to statistically determine if genes which are deregulated belong to similar biological pathways or processes. The general threshold values for calculating significant fold changes in gene expression are >2 or <0.5 and a p-value <0.05. When these restrictions were applied to our microarray 86 up-regulated and 104 down-regulated genes were identified. GO analysis revealed that the down-regulated genes were significantly enriched in one biological process: muscle contraction (p-value <0.05), while the up-regulated genes were not significantly enriched in any biological processes or molecular functions. Since the design of microarray analysis (see materials and methods) was already quite stringent and a much smaller change in gene activity could have a biological effect we decreased the stringency of analysis to include genes

which have a fold change of >1.5 or <0.75. (Data sets for each normalization method can be found in the accompanying CD) Although this resulted in approximately the same number of down-regulated genes (gene symbols listed in Appendix 1) the number of up-regulated genes increased dramatically to 302 (gene symbols listed in Appendix 2). Gene ontology analysis of the up-regulated genes revealed a number of biological process and molecular functions which were significantly enriched in our array; including some of the classical E2F pathways such as DNA replication and cell cycle control (Table 4). Genes involved in protein modification, trafficking, and metabolism were also significantly enriched. Results of the down-regulated gene ontology analysis included an enrichment of genes involved in muscle contraction and cell structure and motility (Table 5).

Table 4: *Gene Ontology of Up-regulated Genes in Tg Hearts.* GO Terms associated with genes which were predicted by microarray analysis to be up-regulated by at least 1.5 fold. * p-value <0.05, **p-value<0.01, ***p-value<0.001, ****p-value<0.0001, *****p-value<0.00001

	Gene Ontology Term	# of Genes
Biological Process	DNA Metabolism	19*****
	DNA Replication	13*****
	Cell Cycle	30****
	Protein Modification	32**
	Intracellular Protein Traffic	25*
	DNA Repair	9*
	Muscle Development	8*
	Transport	28*
	Protein Metabolism and Modification	58*
	Cell Structure and Motility	23*
Molecular Function	Transferase	30****
	Cytoskeletal Protein	23*
	Actin Binding Cytoskeletal Protein	15*
	Oxidoreductase	18*
	Hydrolase	19*
	Transporter	17*

Table 5: *Gene Ontology of Down-regulated Genes in Tg Hearts.* GO Terms associated with genes which were which were predicted by microarray analysis to be down-regulated by at least 1.5 fold. * p-value <0.05, **p-value<0.01

	Gene Ontology Term	# of Genes
Biological Process	Muscle contraction	6*
	Cell structure and motility	14*
Molecular	Cytoskeletal protein	14**
	Actin binding cytoskeletal protein	10**

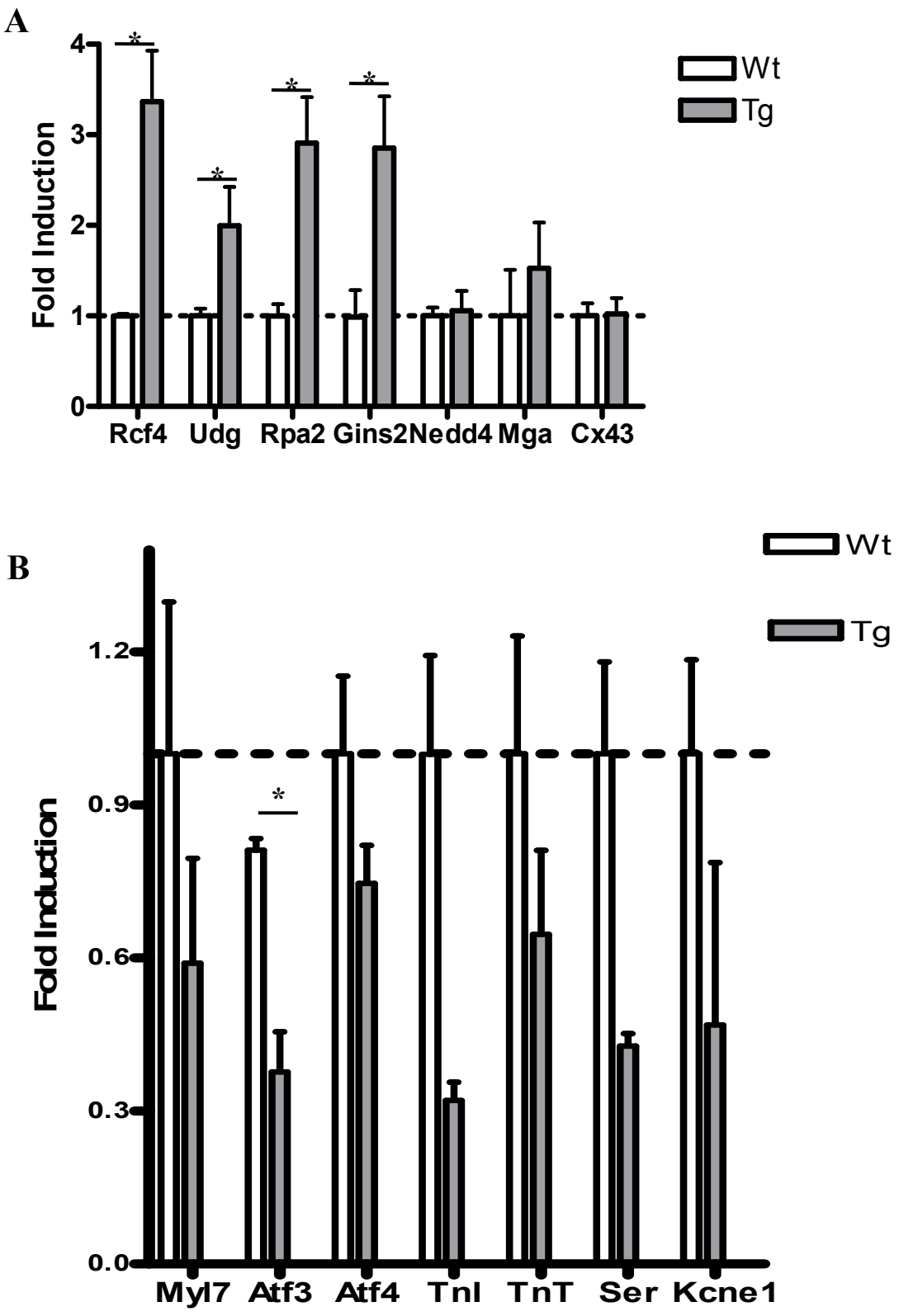
The up-regulation of several genes involved in DNA replication and repair including: Replication factor 4 (Rcf4), Uracil DNA glycosylase, (Ung), Replication factor A2 (RPA2), and Gins Complex 2 (Gins2) was validated by QPCR (Figure 3A). Of particular interest, RPA2 is a verified E2F target (Xu *et al* 2007). Also, the transcription factor Max gene associated (Mga), which interacts with E2F6 in a G0 complex (Ogawa *et al*, 2002), appeared to be up-regulated, but was not statistically significant (Figure 3A).

As expected in any microarray, several genes analyzed by Q-PCR did not exhibit the expected fold change predicted by microarray. For example, the ubiquitin ligase Nedd4 was predicted to be up-regulated in Tg mice, but had an insignificant fold change of 0.97 (Figure 3A). Also, various genes had a fold change close to what was predicted by microarray, but were not associated with a significant p-value including: the contractile protein myosin light chain 7 (Myl7), Troponin T (Tnt2), and the potassium rectifying channel (Kcne1).

Many of the non-significant results hover around the less stringent microarray cut off of >1.5 or <0.75 fold change which may be too small of a change to be accurately

assessed by Q-PCR without a larger sample size. Interestingly, although several other genes involved in regulating muscle contraction were down-regulated, the transcript levels of gap junction protein connexin43 (Cx43), which were predicted to be up-regulated in Tg hearts, did not fluctuate (Figure 3A).

The only down-regulated gene which was significantly changed in Tg mice was Activating transcription factor 3 (ATF3) (Figure 3B) which, interestingly, is a signature of gene expression in human dilated cardiomyopathy (Barth *et al*, 2003). Thus it appears that various indicators of dilated cardiomyopathy are present at an early stage of development before any gross cardiac phenotype is observed.


A
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3
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Rcf4
Udg
Rpa2
Gins2
Nedd4
Mga
Cx43
B
Wt
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Fold Induction
1.2
0.9
0.6
0.3
0.0
*
Myl7
Atf3
Atf4
Tnl
TnT
Ser
Kcne1

Figure 3. *Analysis of Genes Involved in Cell Cycle and Contraction in Tg Hearts*. Real Time PCR of various genes which were predicted to be up-regulated (**A**) or down-regulated (**B**) by microarray analysis. Bars represent the average fold induction (+/-SEM) of 4-7 hearts compared to control gene 18S. * $P<0.05$. ** $P<0.01$, *** $P<0.001$

3.2 E2F6 Does Not Impact Global Chromatin Remodelling

Gene expression is largely controlled by chromatin architecture. The methylation and deacetylation of histones are associated with the formation of heterochromatin and gene repression, while histone acetylation is associated with the relaxation of chromatin structure and gene expression. The E2F and pocket protein families interact with numerous histone modifying enzymes, thus enabling them to modulate gene expression by remodelling chromatin. E2F6 has been described in Polycomb complexes which repress gene expression by histone tri-methylation, thus we explored histone modifications in order to evaluate global chromatin architecture in E2F6 Tg hearts.

Previous analysis of Tg hearts by immunohistochemistry suggested that Tg cardiomyocyte nuclei were larger and irregular. Images taken with an electron microscope also suggested that there could potentially be a difference in chromatin architecture in Tg nuclei (Westendorp *et al*,2008)(Appendix 3). We hypothesized that excess E2F6 might sequester chromatin modifying enzymes resulting in a net loss of heterochromatin, leading to the gene activation observed by microarray analysis. Thus we expected to find a decrease in markers of heterochromatin formation. The expression of heterochromatin protein HP1α, which recruits HMET and is a marker of heterochromatin formation, was first evaluated. Contrary to what was expected, HP1α was up-regulated at both the mRNA and protein levels (Figure 4A/B).

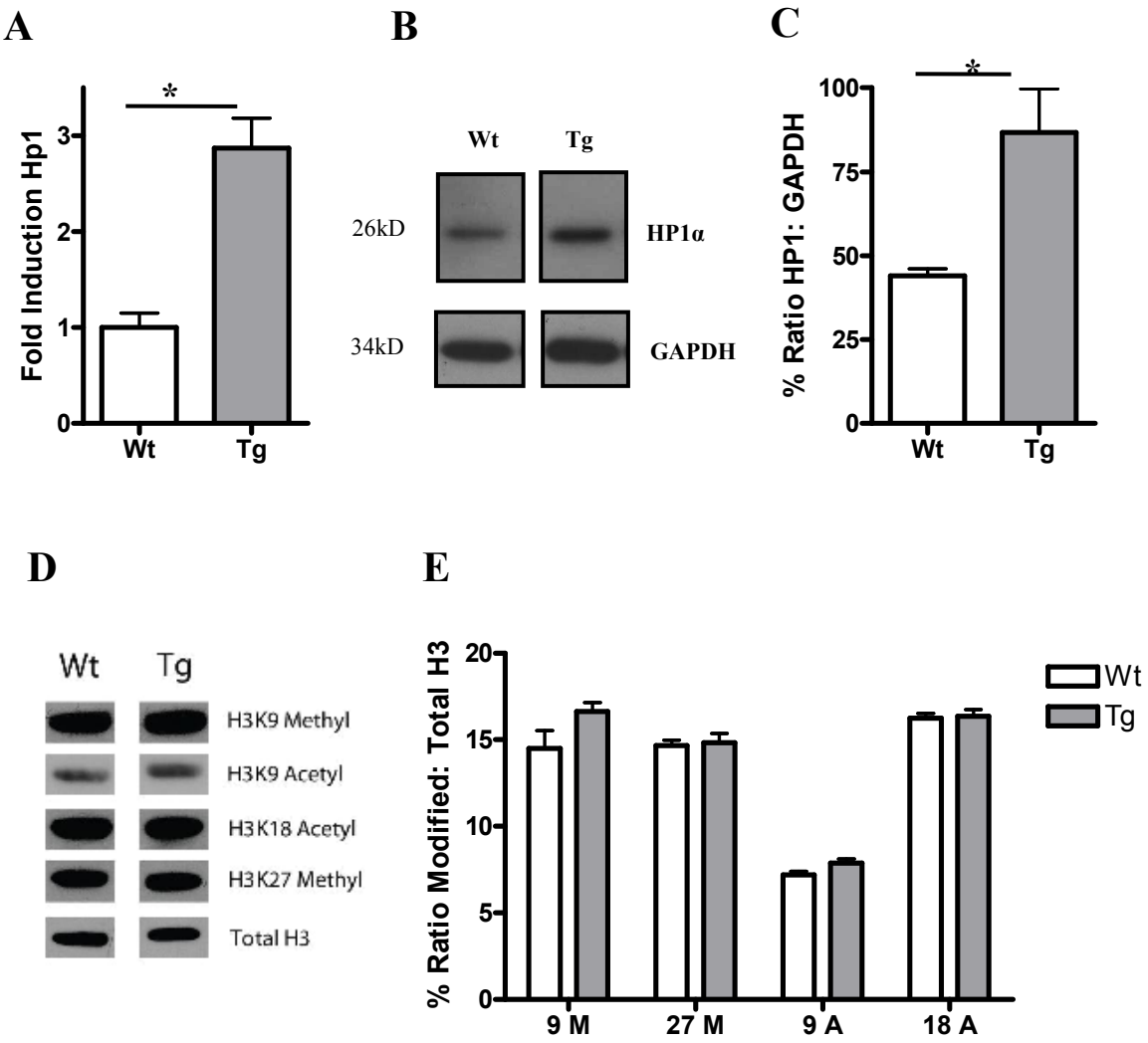


Figure 4: *Chromatin Modifications in Tg Hearts*. Data represents the evaluation of Hp1α by QPCR (**A**), and western blot of total cardiac protein examined with anti- Hp1α (**B**). Percent Ratio of HP1α: GAPDH quantified by densitometry (**C**). Analysis of various histone modifications by western blot (**D**). Percent Ratio of methylated (M) or acetylated (A) lysines on histone 3 : total histone 3 (H) quantified by densitometry (**E**). Sample means are presented +/- SEM. n= 3. *p-value <0.05

Western blot of several histone methylation and acetylation modifications were performed to detect global changes to histones which could impact chromatin status and transcriptional activity (Figure 4C). Tri-methylation of lysine 9 and 27 of histone three (H3K9/27), markers of gene silencing by the Polycomb PRC1 and PRC2 complexes respectively, and histone three acetylation at lysine 9 and 18 (H3K9/18), markers of gene activation, were evaluated. A modest increase in histone acetylation at H3K9 was observed, but results were insignificant (p-value 0.07). Furthermore, no significant changes in the acetylation of H3K18 or the tri-methylation of H3K9/27 were observed. Thus the changes in Tg gene expression cannot be attributed to a global phenomenon concerning chromatin architecture.

3.3 Connexin43 Down-Regulation in Tg Hearts and Changes in Electrocardiogram

In order to coordinate contraction of cardiac chambers cardiomyocytes employ numerous ion channels to control electrical conductance. Deregulation of which can lead to arrhythmias and reduced contractile function- a characteristic of dilated cardiomyopathy. In fact, changes in the protein composition of gap junctions and a down-regulation of connexin43 are hallmarks of cardiac dysfunction and failure (Severs *et al*, 2008). Interestingly, although other genes involved in regulating contraction were down-regulated, connexin43 was predicted to be up-regulated, and upon further analysis we discovered transcript levels did not significantly change (Figure 3A).

Analysis of Wt and Tg cardiac protein with anti-Cx43 resulted in the detection of a 43kDa band (Figure 5A/B), which is the predicted molecular weight of the protein, as well as two additional higher molecular weight bands (~45/47kDa) in adult samples

(Figure 5A). These less mobile bands likely represent phosphorylated isoforms of the protein which have previously been described to run at approximately 44-46kDa (Laird *et al*, 1995). In fact, Connexin43 is known to be highly phosphorylated and ubiquitinated during turnover, which can affect its mobility on a gel (reviewed in Barthoud *et al*, 2004). It is curious that these higher molecular weight bands are not detected at this level of exposure in samples of pup myocardium (Figure 5B). This could potentially reflect different levels of Cx43 protein stability/turnover at different stages of development. In order to validate that these bands are in fact phosphorylated forms of the Cx43 protein, phospho-specific antibodies could be utilized. This might also be useful in detecting if particular pathways or events target Connexin 43 throughout postnatal development, or in Tg hearts.

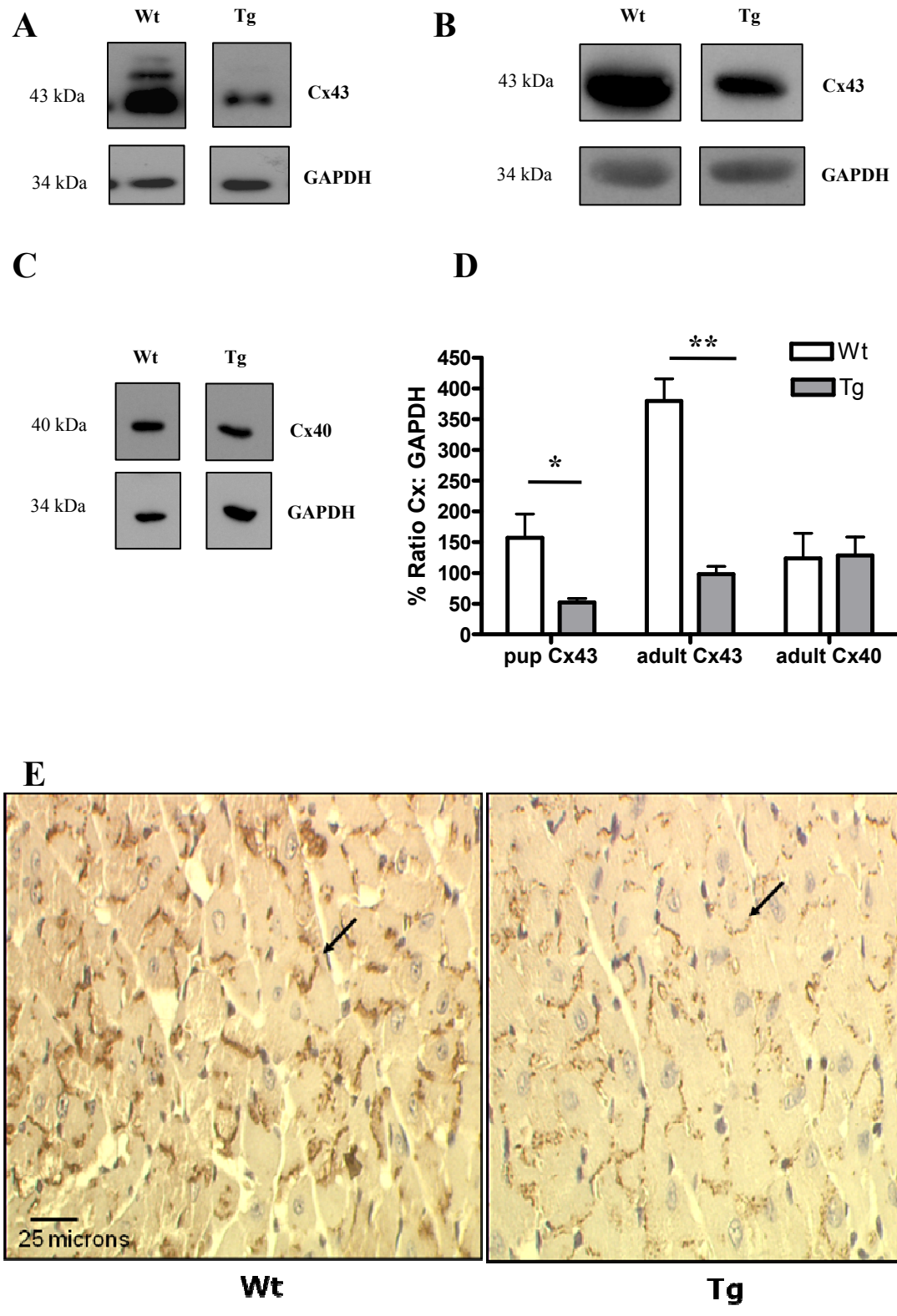
Quantification of the 43kDa band detected by anti-Cx43 revealed that it is significantly down-regulated in both pup and adult Tg myocardium (Figure 5C). Since mRNA levels did not fluctuate, this down-regulation occurs post-transcriptionally in Tg hearts. The down-regulation of Cx43 was confirmed by Immunohistochemical analysis with anti-Cx43, which revealed much less staining in Tg hearts (Figure 5E). Interestingly, Cx43 still appeared to be targeted between adjacent cardiomyocytes (indicated by arrows), which would presumably represent gap junctions. Co-immunofluorescence of Connexin 43 and cell adhesion molecule N-cadherin was performed in an attempt to determine if Cx43 could be found at sites of focal adhesion between cardiomyocytes. Unfortunately under the conditions used N-cadherin had high background and stained the vast majority of myocardial tissue, but did not co-localize with Connexin 43 (data not shown). Thus it did not serve as a useful control for this study. Further analysis is

required in order to validate the localization of Connexin43 at gap junctions in Wt and Tg myocardium.

Connexin43 is crucial to both cardiac development and conductivity (Reaume *et al*, 1995) (Gustein *et al*, 2001). Thus electrocardiograms (ECG) were performed in order to evaluate if there were any changes in the electrical activity of Tg hearts. In a normal electrocardiogram (Figure 6A) the PR interval represents the depolarization of the atrial chambers, the QRS complex represents the rapid depolarization of ventricles, and the T-wave represents the repolarisation of the heart following contraction.

Not surprisingly, we found that the electrical profile of Tg mice was quite different from Wts (Figure 6B). The most striking difference between Wt and Tg ECG readings was a 24% increase in PR interval length (Table 6). This measurement gives an indication of atrial conductivity, which appears to be dampened in Tg hearts. In light of this we also evaluated the other major atrial connexin: Cx40. To our surprise there was no compensatory change in expression of Cx40 between Wt and Tgs (Figure 5C).

In addition to atrial changes, the R amplitude was significantly increased in Tg hearts (Figure 6B/Table 6) which could indicate an increase in sodium channel activation in Tg hearts. The ST segment was also depressed (Figure 6B), which is a hallmark of cardiac failure.


A
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43 kDa
Cx43
34 kDa
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B
Wt
Tg
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Cx43
34 kDa
GAPDH
C
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Tg
40 kDa
Cx40
34 kDa
GAPDH
D
% Ratio Cx: GAPDH
450
400
350
300
250
200
150
100
50
0
*
**
Wt
Tg
pup Cx43
adult Cx43
adult Cx40
E
25 microns
Wt
Tg

Figure 5: *Connexin43 is Down Regulated in the Myocardium of Tg Mice*. Data represents western blots of Wt and Tg adult (**A/C**) and pup (**B**) cardiac protein examined with anti-Cx43 (**A/B**) and anti-Cx40 (**C**). Percent ratio of Connexin: GAPDH quantified by densitometry (**D**). Results are presented as means +/- SEM. n=3. Representative image of immunohistochemical analysis of Cx43 (brown) in adult myocardium of Wt and Tg mice counterstained with Haematoxylin (purple) (**E**). Arrows indicate connexin43 located between adjacent cardiomyocytes. * p-value <0.05. **p-value<0.01

A

B

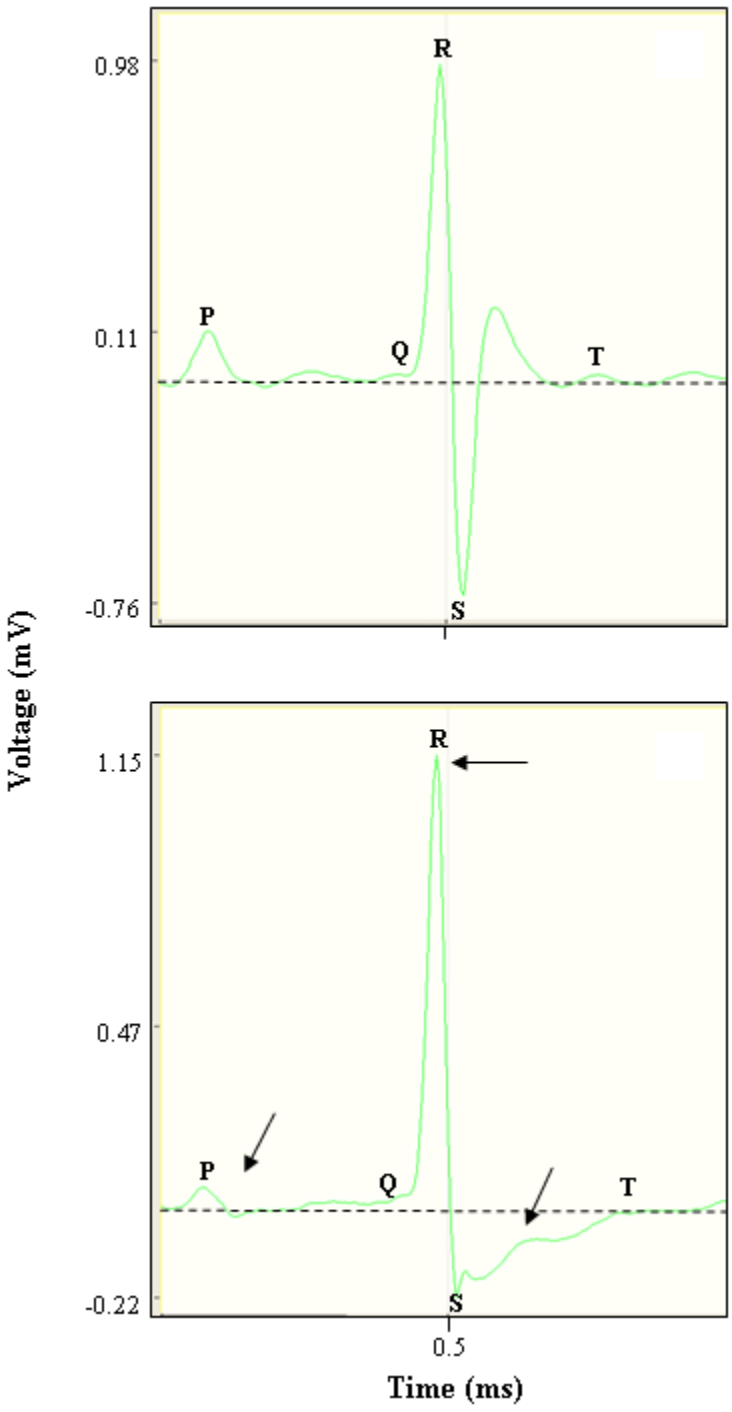


Figure 6: *Changes in Electrical Profile of Tg Hearts*. Representative electrocardiogram recordings from lead II in Wt (**A**) and Tg (**B**) mice. Voltage (mV) is indicated on the Y-axis and time (ms) is indicated on the x-axis. The dashed line indicates the baseline reading ~0mV. Arrows indicate major differences between Wt and Tg recordings.

Table 6: *Analysis of the Electrocardiogram of Tg Mice*. Results are presented as means +/- SEM. n= 7-9. RR: RR interval, PR: PR interval, QRS: QRS complex, QT: QT interval, R amp: R wave amplitude, Ste: ST segment elevation, HR: heart rate.

		Wt	**Tg**	**p value**
RR	(ms)	127.1 ± 1.7	134.7 ± 5.0	
PR	(ms)	33.5 ± 1.4	41.6 ± 2.9	0.024
QRS	(ms)	13.9 ± 0.5	14.6 ± 0.7	
QT	(ms)	45.6 ± 1.7	48.1 ± 2.9	
R amp	(mV)	0.656 ± 0.087	0.993 ± 0.077	0.014
STe	(mV)	0.172 ± 0.021	0.0425 ± 0.046	0.0513
HR	(bpm)	472.6 ± 6.1	452.0 ± 16.6	

3.4 MicroRNA Analysis in Hearts of Tg Mice

MicroRNAs have been recognized as fundamental post-transcriptional regulators in cardiac biology and disease. In fact, deregulation of numerous microRNAs has been demonstrated to inflict cardiac remodelling and produce arrhythmias. Analysis of connexin43 indicates that it is down-regulated post-transcriptionally in Tg hearts, thus we also performed a microRNA array of Wt and Tg hearts. Seven days after birth only a few microRNAs were significantly changed in Tg mouse hearts (Table 7), but after ~5 weeks a larger number were deregulated (Table 8). The increase in young adult hearts could perhaps reflect the development of the DCM phenotype, suggesting that these micro-RNAs may be important in the development of dilated cardiomyopathy or the cardiac remodelling process.

Table 7: *Deregulated microRNAs in Tg Pup Hearts*. Genes were predicted by microarray analysis to be either up-regulated by >1.5 (Up) or down-regulated by <0.75 (Down) fold change. Gene symbols of potential targets which were deregulated in microarray analysis are listed.

Micro RNA	Up/Down	Potential Targets
mmu-miR-763	Up	Tnnt2, CTGF, Ank1, Pde4b, Rif1, Thrap3
mmu-miR-465a-3p	Up	Casc3, Dlc1, Flot1, Pde4b, Rif1,
mmu-miR-122	Down	Dst, Cx43, Immt, Tpp2, Vcl, Zranb3
mmu-miR-19b	Down	Acta2, Dst, Immt, Oxct1, Ppp1r12a, Zranb3
mmu-miR-7a	Down	Cx43,Cxcr4, Dst, Vcl, Zranb3

Table 8: *Deregulated microRNAs in Tg Adult Hearts*. Genes were predicted by microarray analysis to be either up-regulated by >2 (Up) or down-regulated by <0.5 (Down) fold change. Gene symbols of experimentally proven targets are listed.

Micro RNA	Up/Down	Targets
mmu-miR-106b	Up	p21 (Ivonaska *et al*, 2008)
mmu-miR-1196	Up	
mmu-miR-122	Up	Cyclin G1 (Gramantieri *et al*,2007) , Bckdk, Ndrg3, AldoA (Elmen *et al*, 2008)
mmu-miR-124	Up	MAPK14, Mtpn (Krek *et al*, 2005) many genes involved in G1/S transition (Lim *et al*, 2005)
mmu-miR-138	Up	
mmu-miR-196a	Up	HOXB8, (Yetka *et al*, 2004)
mmu-miR-196b	Up	HOXB8, HOXA7, HOXC8 (Yetka *et al*, 2004)
mmu-miR-201	Up	
mmu-miR-206	Up	Cx43, Pola1, Utrn, Fstl (Kim *et al*, 2006) Up-regulated post MI (Shan *et al*, 2009)
mmu-miR-296-3p	Up	
mmu-miR-296-5p	Up	
mmu-miR-298	Up	
mmu-miR-344	Up	
mmu-miR-346	Up	
mmu-miR-154	Down	Up-regulated in cardiac hypertrophy (van Rooij *et al*, 2006)
mmu-miR-18a*	Down	
mmu-miR-467a	Down	
mmu-miR-467e	Down	
mmu-miR-669a	Down	
mmu-miR-669e	Down	
mmu-miR-669f	Down	

Significantly changed microRNAs were put through a target gene prediction program and compared to microarray data to elucidate any changes in mRNA levels which could be accounted for by a change in microRNA (Table 7). It appears that several down-regulated genes could be regulated by various microRNAs including Tnnt2 by mmu-miR763. Unfortunately there were no Taqman probes available for the majority of microRNAs which were de-regulated in pups and attempts at Northern blot were largely unsuccessful, so we focussed on a few major microRNAs in adult mice which had available probes. Many of these microRNAs have described targets and are involved in identified pathways (Table 8).Of particular interest, miR-206 was up-regulated in E2F6 Tg mouse hearts. This microRNA targets the degradation of Connexin 43 (Kim *et al*, 2006), which is down-regulated post-transcriptionally in Tg hearts. Q-PCR was utilized to confirm its up-regulation as well as that of miR-124 and miR-122 in comparison to the control gene small nucleolar RNA 202 (sno-202) (Figure 7). Interestingly, the down-regulation of miR-122 was also confirmed in pup hearts, indicating that there may be a switch in its regulation between one and five weeks. It should be noted that the results of miR-122 were not statistically significant, and a larger sample size would be necessary to evaluate the results more accurately.

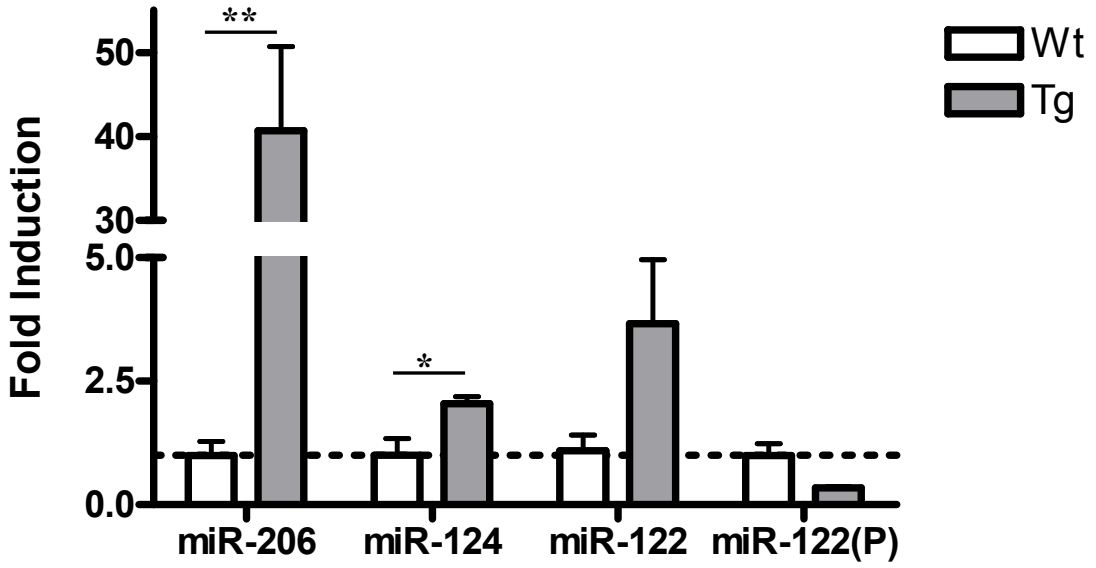


Figure 7: *Non-Cardiac MicroRNAs are Up Regulated in Hearts of Tg Mice***.** Data represents fold induction of expression of microRNAs determined by Quantitative Real Time PCR compared to control gene sno-202. Results are presented as the mean of 3-5 mouse hearts +/- SEM. (P) Represents result of experiment performed using microRNA collected from p-7 pup. * p-value <0.05, ** p-value<0.01.

3.5 The ERK (p42/p44) Pathway is Activated in Tg Mice

Although mir-206 could target Cx43 mRNA for degradation in Tg hearts, it does not appear to be up-regulated in pups (data not shown) at which point in time there is a decrease in protein levels. (Figure 5B). The gap between Wt and Tg Cx43 expression level seems to increase between 7d and 6 weeks, of which the elevated levels of miR-206 at 5 weeks could be a factor. MiR-206 expression cannot alone explain the overall decrease in Cx43 protein. Other mechanisms of post-transcriptional down-regulation could include: a decrease in stability, an increase in degradation, or an inhibition of the translation of Connexin43. Cx43 has multiple phosphorylation sites which are known to affect its turnover. In particular, the mitogen activated protein kinase (MAPK) phosphorylation cascade has been demonstrated to play a role in turnover as well as translation inhibition (reviewed in Rose *et al*, 2010) (Kalma *et al*, 2004). We evaluated the activation of the MAPK pathway by the examination of the downstream protein kinases in this cascade: ERK (p42/44). The ERK kinases are activated by phosphorylation, thus their activity in Wt and Tg hearts was evaluated by immunoblotting with anti-p42/44 and anti-phospho p42/44 (Figure 8A). The ratio of phosphorylated: total p42 and p44 indicated that the ERK pathway was activated in Tg hearts (Figure 8B). More specifically, p42 (ERK2) is activated (phosphorylated), while p44 (ERK1) is not. It should be noted that the amount of ERK detected in Wt samples was not much higher than background, which could affect the accuracy of quantitation. Thus future experiments should involve loading a larger amount of protein in order to ensure accuracy.

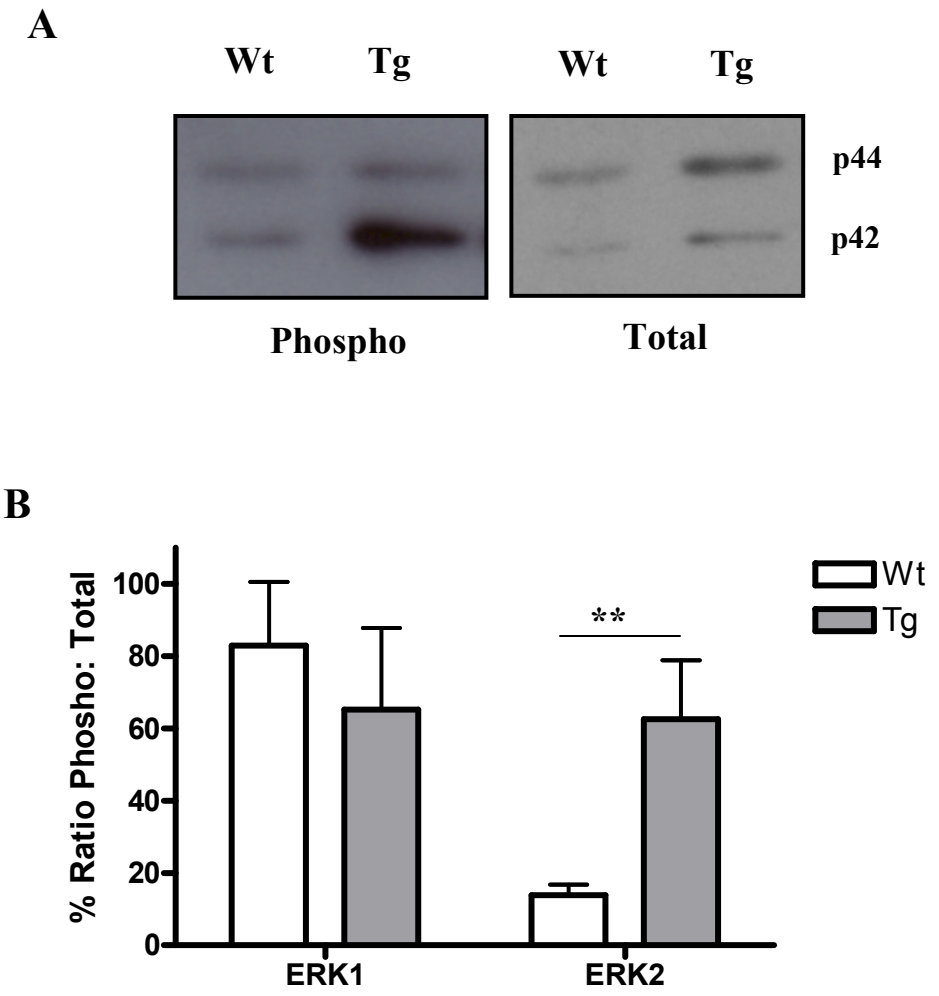


Figure 8: *ERK (p42/44) Pathway is Activated in Tg Hearts.* The data represents western blots examined with anti-p42/44 antibodies in Wt and Tg hearts (**A**). Percent ratio of phosphorylated: total p42 and p44 quantified by densitometry (**B**). Results represent mean values +/- SEM. n= 3 ** p-value < 0.01.

As mentioned, other possibilities for the observed post-transcriptional decrease in Cx43 could include decreased stability or more rapid rate of turnover. In addition to being linked to phosphorylation, Cx43 degradation is correlated to ubiquitination by the ubiquitin ligase Nedd4 (reviewed in Leithe and Rivedal, 2007). As mentioned, Nedd4 was predicted by microarray analysis to be up-regulated in Tg hearts, but its transcript levels did not change (Figure 3A). Furthermore, initial protein analysis evaluated by western blot suggests no change in Nedd4 expression (data not shown), but this does not assess whether any changes in its activity have occurred.

3.6 E2F6 Modulates the Expression of Specific E2F Family Members

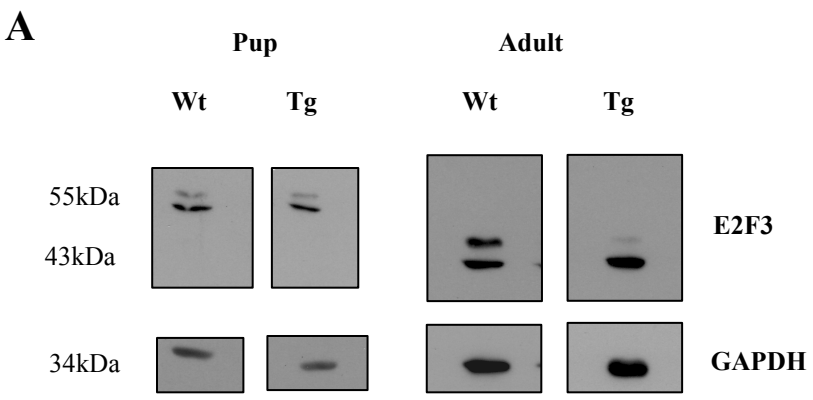
E2F6 is a potent modulator of the E2F pathway which represses gene activity. It appears that E2F responsive genes are activated in E2F6-Tg hearts (Table 4/Figure 3A), thus it is likely that these changes could be associated with changes in other E2F/DP family members. In order to address this they were examined by QPCR and western blot.

Of particular interest were the changes in E2F3 protein observed in Tg hearts. Western blotting using anti-E2F3 resulted in the detection of multiple bands (Figure 9A/C). In order to verify which bands were specific they were compared to a control cell line K962 (Figure 9B) provided by the antibody source (Santa Cruz), as well as the predicted molecular weights of the two E2F3 proteins which would result in a 10kDa size difference. Through this comparison it was deduced that the major bands detected represent E2F3A (~55kDa), E2F3B (~43kDa) and a non-specific band which is detected just below E2F3B (Figure 9A). In pup hearts E2F3A is represented by two bands and is easily detectable while only minor levels of E2F3B were observed. Statistical analysis

revealed no significant changes between Wt and Tg mice (Figure 9C). In adult hearts E2F3A was undetectable and E2F3B was down-regulated by approximately 55% (Figure 9C).

Unfortunately, other E2F proteins could not be properly evaluated by western blot due to the poor quality of antibodies available, although an attempt has been made in the case of E2F1 (discussed in next section). Instead, expression was evaluated at the mRNA level by Real Time QPCR (Figure 10). E2F1 was significantly up-regulated by ~18fold and E2F3A and B were up-regulated by approximately 2-3 fold in Tg mice compared to 18S. Both E2F4 and E2F5 expression did not fluctuate in Tg hearts.

Since E2Fs require DP dimerization in order to bind to gene promoters we also evaluated the levels of DP1 and DP2 by western blot (Figure 11). DP1 protein was up-regulated by 92% in Tg mice whereas DP2 levels were not significantly altered in Tg mice (Figure 11/12C).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Tg
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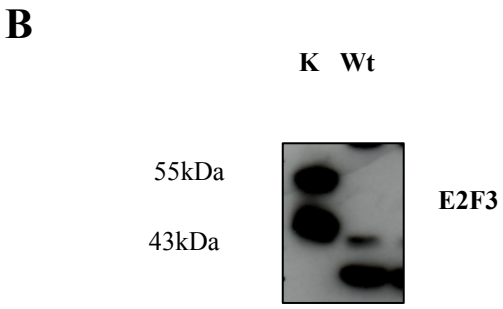
43kDa
E2F3
34kDa
GAPDH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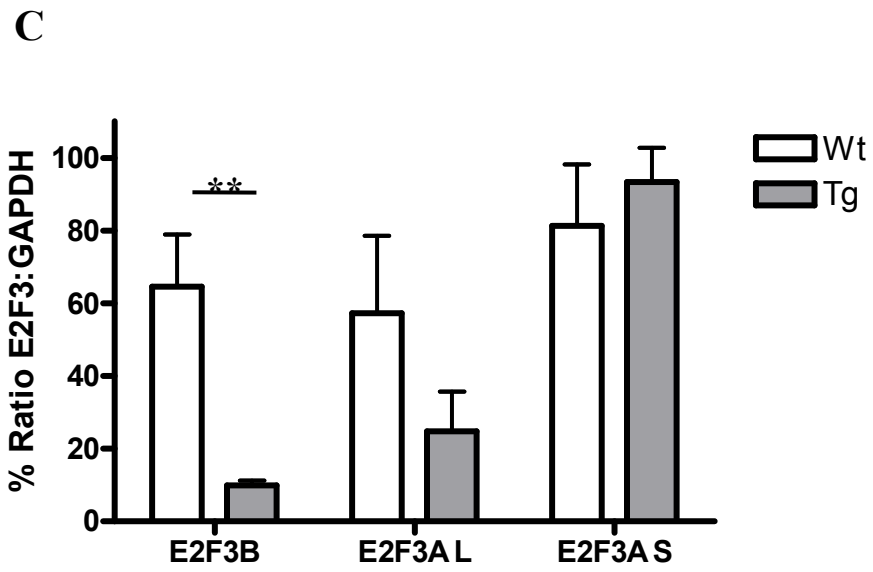
55kDa
43kDa
E2F3


C
% Ratio E2F3:GAPDH
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80
60
40
20
0
**
E2F3B
E2F3A L
E2F3A S
Wt
Tg

Figure 9: *E2F6 Modulates E2F3 Protein Expression*. Data represents western blots of protein from Wt and Tg hearts (**A**) and control cell line K962 (K) and Wt heart (**B**) examined with anti-E2F3. Molecular weights are indicated on the left. Percent ratio of E2F3: GAPDH quantified by densitometry (**C**). E2F3A S and L represent smaller and larger molecular weight bands in pup hearts. Results are presented as means (+/- SEM). n= 4-5. ** p-value <0.01.

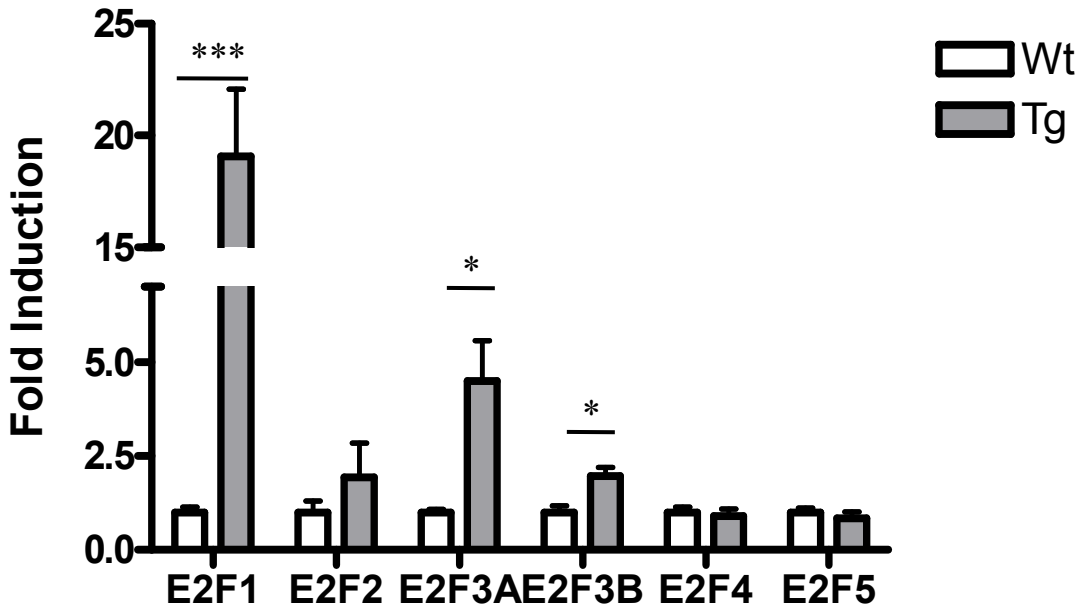


Figure 10: *E2F6 Modulates Transcript Levels of Specific E2F Family Members*. Data represents QPCR results for various E2F family members. Results are presented as the average fold induction (+/- SEM) compared to control gene 18S. n=5-7.

*p-value <0.05, ***p-value<0.001.

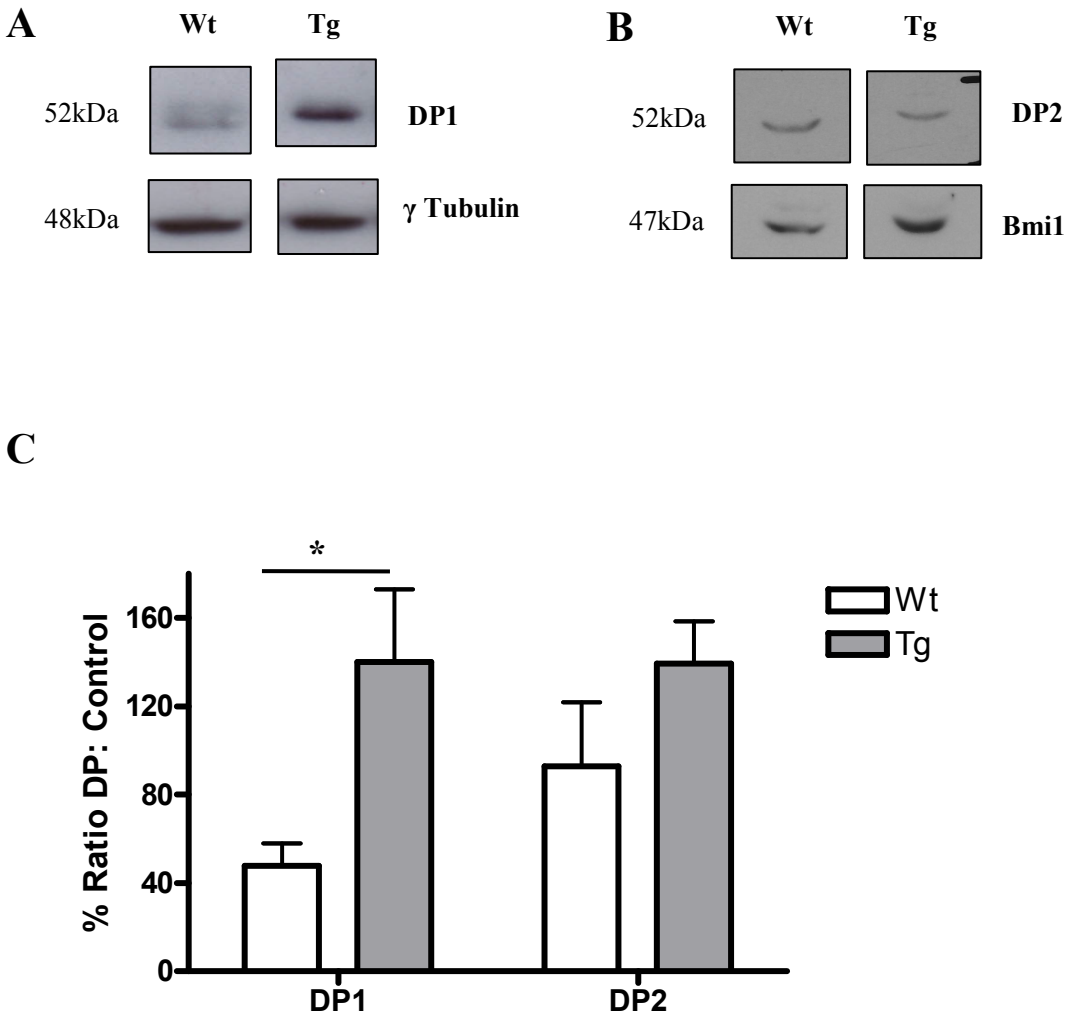


Figure 11: *E2F6 Modulates DP1 but not DP2 Protein Expression*. Data represents western blots of Wt and Tg heart protein investigated with anti-DP1 (**A**) and anti-DP2 (**B**). Molecular weights are indicated on the left. Percent ratio of DP: control protein quantified by densitometry (**C**). Nuclear enriched fractions were used for immunoblotting (B). Results represent means +/- SEM. n= 3. * p-value <0.05

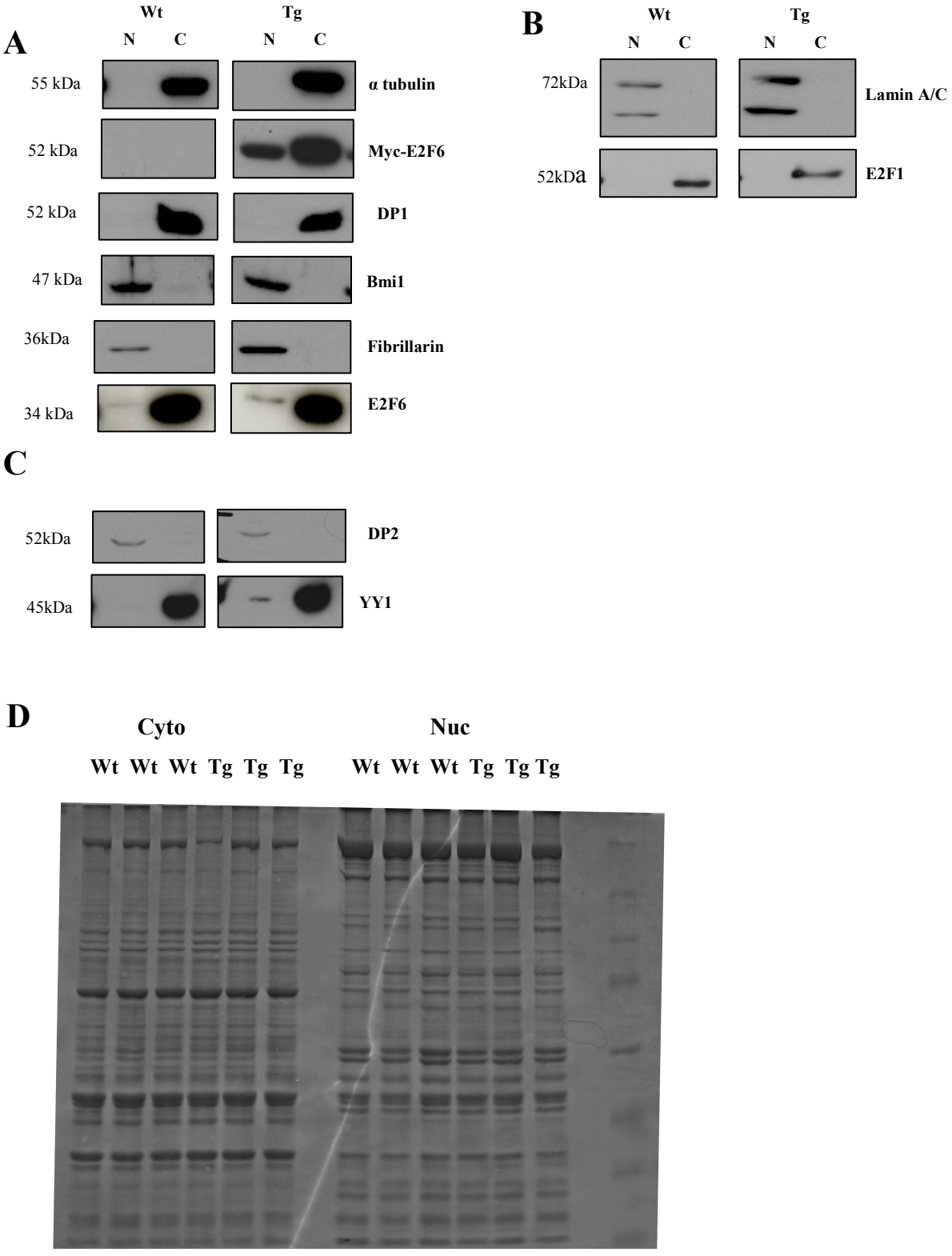
3.7 Subcellular Fractionation of Hearts

In addition to the level of expression, the regulation of various E2F/DP family members is also dictated by their subcellular localization. Furthermore, the localization of E2F family members remains to be explored in the heart. In order to determine where these proteins are localized in the myocardium, and if E2F6 over expression could cause any changes in location, subcellular fractionation was employed (Figure 12). Separation of nuclear and cytosolic fractions was verified by the localization of nuclear markers Lamin A/C (nuclear membrane) and Fibrillarin (nucleolar) solely in nuclear fractions, and cytosolic marker α-Tubulin in cytosolic fractions. Interestingly, both Lamin A/C and fibrillarin appeared to be up-regulated (Figure 12 A/B) in Tg nuclear fractions. This was surprising since both are generally used as nuclear markers and loading controls. As mentioned, we had previously found indications that Tg cardiomyocyte nuclei were large and irregularly shaped (Westendorp *et al*, in prep) (Appendix 3). Thus the up-regulation of Lamin A/C could potentially reflect an overall increase in nuclear membrane (due to the increase in overall size and an increase in the number of invaginations) in Tg cardiomyocytes. In another study the levels of fibrillarin in HeLa cells affected overall nuclear morphology (Amin *et al*, 2007). Thus it is possible that the deregulation of these proteins in E2F6-Tg mice could potentially be part of the cause or a consequence of the irregular nuclear morphology observed. This would require further validation including a statistical analysis of nuclear size/shape.

In order to ensure equal nuclear loading the same fractionation samples were run on a separate gel and stained with Coomassie Blue (Figure 12D). The total protein in each lane was quantified by densitometry and equal loading in both nuclear and cytosolic

fractions in Wts and Tgs was validated (Figure 12D/E). The total protein in each lane was then utilized as a reference to calculate the expression of proteins of interest in a particular sample (Figure 12F/G).

Immunoblotting with anti-E2F1 detected a ~52 kDa band in the cytoloic fraction which was not significantly changed in Tg samples (Fig 12B/F). Although larger than the predicted molecular weight, E2F1 is described to be detected at ~50-60kDa when phosphorylated. Although E2F1 transcript levels were up-regulated it is not surprising that the protein was not up-regulated to a similar degree since this would be expected to cause an increase in E2F6-Tg apoptosis which was not the case (Westendorp *et al*, 2008).


A
Wt
Tg
N
C
N
C
55 kDa
α tubulin
52 kDa
Myc-E2F6
52 kDa
DP1
47 kDa
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36kDa
Fibrillarin
34 kDa
E2F6
B
Wt
Tg
N
C
N
C
72kDa
Lamin A/C
52kDa
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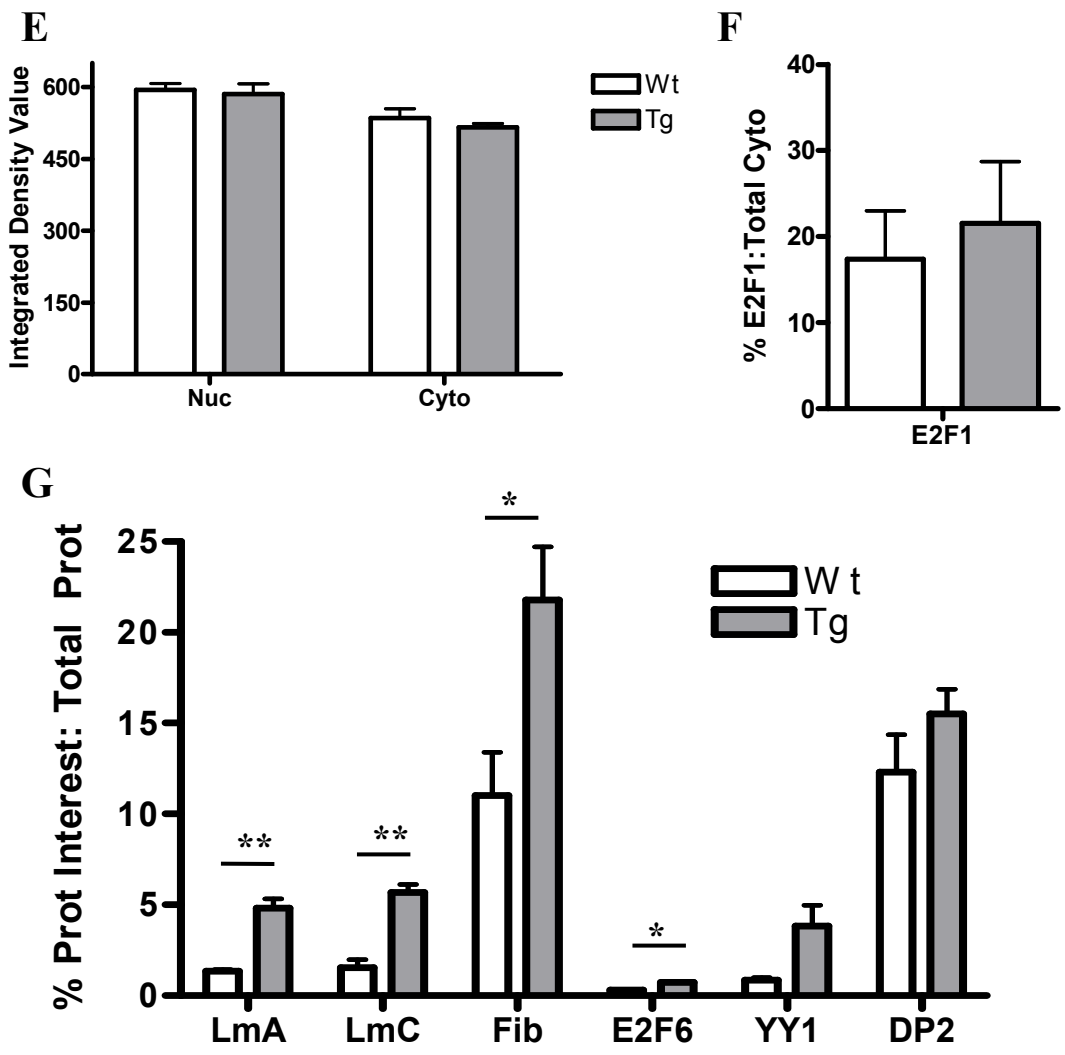

Figure 12: *Subcellular Fractionation of Hearts*. Representative western blots of nuclear (N) and cytosolic (C) enriched fractions of Wt and Tg hearts on sister blots (**A/B/C**). Antibodies used are indicated on the right and molecular weights are indicated on the left. Grayscale image of nuclear and cytosolic fractions stained with coomasie blue (**D**). Total protein quantified by densitometry (**E**). Percent ratio of E2F1: total cytosolic protein quantified by densitometry (**F**). Percent ratio of nuclear proteins of interest: total nuclear protein quantified by densitometry (**G**). Results are presented as means (+/-SEM). n=3. * p-value <0.05, ** p-value <0.01.

Immunoblotting with anti-E2F6 revealed that endogenous E2F6 (~34kDa) was localized primarily in the cytosol and to a much lesser extent in the nucleus (Figure 12A). Similarly, myc-tagged E2F6 (~52kDa), evaluated with anti-myc, was also primarily located in the cytosol and a smaller amount was detected in the nucleus of Tg cardiomyocytes. A ratio of nuclear: cytosolic localization of protein was calculated in order to determine if there was any shift in localization of proteins (nuclear and cytosolic values were normalized to total protein). Interestingly, the ratio of nuclear: cytosolic endogenous and myc-tagged E2F6 were ~2 and ~43 fold higher respectively in Tg hearts (Figure 13). A similar observation was made for polycomb and E2F interacting transcription factor Ying Yang 1 (YY1), which was localized primarily in the cytosol of Wt hearts (Figure 12C) and displayed a 5.4 fold increase in nuclear: cytosolic localization in Tg hearts (Figure 13).

Interestingly, although DP1 levels were increased in Tg hearts (Figure 11 and 12A), they were localized primarily in the cytosol without a significant shift into the nucleus (Figure 12A). In contrast, DP2 levels were not significantly altered in Tg hearts, and were present primarily in nuclear fractions (Figure 12C).

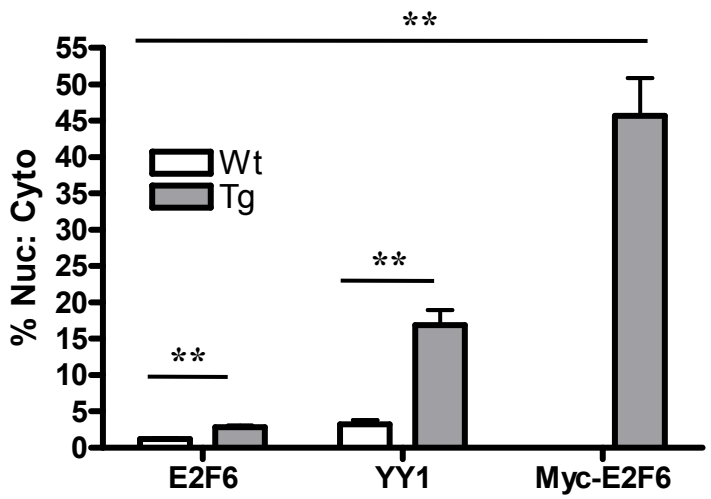

Figure 13: *E2F6 and YY1 are Translocated to the Nucleus in Tg Hearts*. Percent ratio of nuclear: cytosolic localization of proteins in Wt and Tg hearts quantified by densitometry. Values were normalized against total protein. Results are presented as means (+/- SEM). n=3. ** p-value <0.01

CHAPTER 4: DISCUSSION

4.1 E2F6 Can Behave as a Transcriptional Activator

E2F6 has previously been described as a transcriptional repressor which inhibits cell cycle progression. Surprisingly, microarray analysis of E2F6-Tg mouse hearts uncovered an overall net increase in gene transcription. Even more surprising was the discovery that many of the activated genes were part of classical E2F pathways. Quantitative RT-QPCR revealed that previously described E2F targets: Replication factor 4c and uracil DNA glycosylase were up-regulated in Tg hearts. Another E2F target, HP1α, was also up-regulated at both the mRNA and protein levels. Furthermore we previously demonstrated that its promoter was occupied by myc-tagged E2F6 in Chromatin Immunoprecipitation studies (Westendorp *et al*, 2008). Thus it appears that over-expressed E2F6 has the capacity to directly activate the transcription of E2F target genes. This is quite a novel find, since others have previously reported that E2F6 behaves as a potent repressor of E2F responsive genes (Cartwright *et al*, 1998) (Livingston *et al*, 1998). In fact, these groups found that over-expressed E2F6 inhibited cell cycle re-entry in growth arrested cells.

Mammalian cardiomyocytes naturally become arrested shortly after birth. Postnatal cardiac growth is restricted to hypertrophy which is associated with activation of the E2F pathway and induction of genes involved in G1/S phase. In our E2F6-Tg model although the heart is under stress no hypertrophic response was observed. We also did not observe any changes in cardiomyocyte number or size and no induction of S phase (Westendorp *et al*, 2008) as inflicted by over expression of other E2Fs. Thus although over expression of E2F6 has led to an activation of E2F responsive genes there

is something unique about our model that has inhibited the normal cardiac adaptation of cell cycle re-entry and hypertrophic growth. Thus similarly to those studies done by Cartwright and Livingston, over-expression of E2F6 in Tg mouse hearts has led to the inhibition of cell cycle re-entry in quiescent cells.

Even considering this our study differs from previous studies in a very key aspect: E2F6 is not behaving as an inhibitor of E2F responsive genes, but instead appears to be activating them. Originally we hypothesized that perhaps an excess of E2F6 could sequester the important chromatin modifying enzymes it interacts with, leading to an impairment of heterochromatin formation. In fact, routine immunochemical staining and electron microscopy suggested that Tg mice had large irregularly shaped nuclei, with a possible loss of heterochromatin (Westendorp *et al*, 2008) (Supplemental Figure 1). Upon further analysis it appears that over expression of E2F6 did not significantly alter the tri-methylation status of chromatin at histone 3 lysine 9 or 27, suggesting that there were no changes in global heterochromatin formation. This was quite surprising since numerous studies have shown that E2F6 interacts with members of Polycomb groups PCR1 and PRC2 which are responsible for the initiation and maintenance of long term gene silencing by histone tri-methylation respectively (Trimarchi *et al*, 2001)(Atwool *et al*, 2005). This is even more surprising since deletion of E2F6 causes a phenotype which is complimentary to the deletion of Polycomb protein Bmi1 (Storre *et al*, 2002). Thus, despite an array of studies showing that E2F6 interacts with a large handful of proteins involved in gene silencing, E2F6 expression is not correlated to histone tri-methylation or gene repression in our cardiac mouse model. In fact, we did observe a modest increase in histone acetylation at H3K9. Although this result was not significant (p-value 0.07), it

supports microarray data suggesting that E2F6 over expression leads to a net increase in gene transcription. A ChIP study done by Xu and Oberley also found that E2F6 DNA binding is not strongly associated with histone methylation, and furthermore that 60% of promoter regions occupied by E2F6 represented genes which were actively transcribed (Xu and Oberley, 2007). Thus our studies as well as those done by Xu suggest that the view of E2F6 as a transcriptional repressor needs to be re-evaluated. The role of E2F6 likely depends on complex interactions with other proteins and signalling pathways which respond to the environment in order to create cell/tissue specific gene expression profiles.

4.2 Unique MicroRNAs are Expressed in E2F6- Induced Dilated Cardiomyopathy

Young adult Tg mice demonstrated a unique microRNA profile. This appears to be associated with the disease process- since few changes were observed at 7 days, at which point a cardiac phenotype is not observed. Interestingly, several of the microRNAs which were up-regulated have been described as non-cardiac tissue specific microRNAs. Of particular interest was the observed up-regulation of miR-206 in Tg hearts, which was previously described as a muscle specific microRNA (Kim *et al*, 2006). In this study Northern blot analysis of 5ug of total RNA was performed which could easily miss the small amounts of the microRNA easily detectable in the more sensitive Taqman assay we used. Another group found that miR-206 was up-regulated in the heart of a rat model of MI (Shan *et al*, 2009), suggesting that perhaps miR-206 is expressed under conditions of stress in the myocardium. Of particular interest, miR-206 was demonstrated to be regulated by YY1 in the liver (Song and Wang, 2009). Since YY1 is more nuclear

localized and presumably activated in Tg mice this could point towards a potential mechanism for mir-206 up-regulation in Tg hearts.

In addition to the expression of a muscle specific microRNA, miR-122, which is enriched in the liver (reviewed in Girard *et al*, 2008), was also deregulated in Tg hearts. This microRNA is an important regulator of lipid metabolism, which although is not as important in the young heart, becomes increasingly important as the heart ages and lipids becomes the major source of energy. Surprisingly we observed an inverse relationship of miR-122 expression in Tg hearts, which was down-regulated in pups but increased in adult hearts. This may reflect the reversion to the foetal gene program observed in Tg hearts (Westendorp *et al*, 2008) which is also associated with a transition in energy sources.

MiR-124, which is enriched in the brain (Lim *et al*, 2005), was also up-regulated in adult Tg hearts. This microRNA was demonstrated to target a number of genes involved in the G1/S transition (Lim *et al*, 2005). Additionally, it is predicted by the program Target Scan to target the 3'UTR of E2F6. Thus, perhaps its up-regulation reflects a compensatory response to regulate excess E2F6 and the up-regulated G1/S targets in Tg hearts.

Finally, we also observed a down-regulation of miR-154, which when over expressed can inflict cardiac hypertrophy (van Rooij *et al*, 2006). Although this target remains to be verified by real time QPCR it could be involved in the inhibition of a hypertrophic response in Tg mice. A variety of other microRNAs were also significantly changed in young adult Tg mice which could be important in the development of DCM which has yet to be well explored.

4.3 E2F6 is Localized in the Cytosol of Adult Myocardium

Contrary to what might be expected for a transcription factor, we found that E2F6 is primarily localized in the cytoplasm in adult myocardial tissue. Previous studies have demonstrated that E2F6 is localized in the nucleus of U2OS cells (Cartwright *et al*, 1998). These discrepancies could be explained by the fact that cardiomyocytes are quiescent cells as opposed to an actively dividing cancer-derived cell line such as U2OS. The E2F family is highly regulated throughout the cell cycle and different members play unique roles in different phases, thus the way E2F6 behaves in a cycling cell might be quite different from its behaviour in an arrested cardiomyocyte. In support of this idea, Ogawa and colleagues found that E2F6 can interact in a unique complex in HeLa cells which binds to promoters during G0 (Ogawa *et al*, 2002) while others found that E2F6 only binds to promoters during G1/S phases of the cell cycle. Thus, just as E2F6 can behave differently in various cell types and under different culture conditions, it appears that E2F6 may also have unique capacities in the adult myocardium.

E2F6 has no recognizable nuclear localization signal and it might be expected that it is shuttled in with other proteins. This is similar to E2F4 and 5 which depend on pocket proteins for appropriate localization during the cell cycle (Gaubatz *et al*, 2001). Since E2F6 does not have a pocket protein binding domain its shuttling likely depends on interactions with other proteins.

4.4 E2F6 and YY1 are Translocated to the Nucleus in Tg Hearts

Although endogenous E2F6 resides primarily in the cytosol of Wt adult myocardium both endogenous and Myc-tagged E2F6 were observed at a higher ratio in the nucleus of Tg hearts. This supports our model that E2F6 directly activates gene expression in Tg mice, since it would have to be present in the nucleus. Since E2F6 lacks the conserved transactivation domain one can presume that it likely interacts with other factors in order to activate gene transcription. Thus we also evaluated the sub-cellular localization of other E2Fs and interacting proteins.

In addition to E2F6, DP1 was also found primarily in the cytoplasm and to a much lesser extent in the nucleus. This appears to be in agreement with other studies performed in cell culture in which DP1 is only translocated to the nucleus under conditions of excess "activator" E2Fs such as E2F1 (Datta *et al*, 2002)(Zhang *et al*, 2010). Thus it appears that in quiescent adult myocardium both E2F6 and DP1 are localized in the cytoplasm, presumably not required for transcriptional regulation of cells which are not cycling. In contrast, DP2 was found primarily in the nucleus of Wt and Tg mice, consistent with other studies (Magae *et al*, 1996). Perhaps DP2 is the major DP protein which dimerizes with E2Fs in quiescent cardiomyocytes (presumably for gene silencing) while DP1 only enters the nucleus in order to aid in gene activation during the cell cycle.

Interestingly, the YY1 transcription factor also displayed a shift from cytoplasmic to nuclear localization. Although it has not been demonstrated to interact with E2F6 directly it does interact with Ring and YY1 binding protein (RYBP), which does interact directly with E2F6 (Trimarchi *et al*, 2001), and it is by this mechanism which it interacts

with E2F2 and 3 (Schlisio *et al*, 2002). YY1 has been demonstrated to co-regulate E2F responsive genes including cdc6 and Hp1α (Schlisio *et al*, 2002) (Giangrande *et al*, 2004) which are up-regulated in Tg hearts, and whose promoters are occupied by myc-E2F6. It also interacts with pocket protein pRb (Petkova *et al*, 2001) which regulates its activity and may be involved in its transport to the nucleus at the G1/S phase checkpoint (Palko *et al*, 2004). Finally, YY1 is an important gene regulator in the heart which protects it from pathological hypertrophy (Sucharov *et al*, 2008). Thus it is possible that YY1 in association with pocket proteins and/or other factors could aid in the transport of E2F6 to the nucleus and/or in the regulation of gene expression and hypertrophy in Tg hearts. Further experimentation including IP and ChIP could be performed to evaluate these ideas.

4.5 E2f6 Modulates E2F3B Activity in Tg Hearts

Analysis of the E2F family revealed no great changes in protein expression with the exception of E2F3B. In adult mice the protein was reduced by approximately 55%. Surprisingly E2F3A/B mRNA was up-regulated. A similar observation was made for E2F1 which exhibited an 18 fold increase in mRNA (Westendorp *et al*, in prep) yet does not appear to have such a large increase in protein (to be confirmed). Thus it is apparent that they are down-regulated post-transcriptionally. This result does not come as a surprise since over expression of E2F1/3 would be expected to cause a re-activation of the cell cycle and hypertrophy. In fact, no increase in cell size or number, and no difference in the incorporation of BRDU was observed in Tg cardiomyocytes (Westendorp *et al*, 2008). Furthermore, an increase in E2F1 protein would also be

expected to cause an increase in the number of apoptotic cardiomyocytes, which is also not the case (Westendorp *et al*, 2008). It seems likely that the increases in E2F transcript levels are due to E2F6's activation of the E2F pathway, since they are E2F-responsive genes.

E2F1 and E2F3 turnover has been associated with the expression of p19ARF (Martelli *et al*, 2001). P19 is an E2F responsive gene, in particular it appears to be negatively controlled by E2F3B (Danielian *et al*, 2008). It is possible that the increase in E2F6 has led to the impairment of E2F3B mediated regulation, thereby initiating transcription of p19 and consequently the degradation of excess E2F1/3.

An alternative mechanism for the observed post-transcriptional loss of E2F could involve targeting to the ubiquitin proteasome pathway which has been demonstrated to play a role in E2F turnover (Hateboer *et al*, 2010). In support of this, gene ontology analysis of the microarray did point toward an increase in genes involved in protein modification and metabolism. Further analysis would be necessary to confirm either of these hypotheses.

Regardless of the mechanism, a loss of E2F3B may be a critical key in understanding the development of DCM in our Tg mice. As previously mentioned, deletion of E2F3 caused partial embryonic lethality and surviving mice develop late onset dilated cardiomyopathy (King *et al*, 2008). In this study both E2F3A and B were knocked out, but the early proliferation defect was largely attributed to a loss of E2F3A. It seems unlikely that later defects (such as DCM) could be wholly attributed to a loss of E2F3A, since at this time it is not expressed in the adult myocardium (reviewed in Ahuga *et al*, 2007), whereas E2F3B is. Furthermore, E2F3B has been described as a key player in

muscle differentiation and development (Asp *et al*, 2009) and may be a more likely perpetrator than E2F3A in the DCM phenotype observed.

King and colleagues also observed defects in sarcomere organization by electron microscopy which could potentially over time lead to late onset DCM (King *et al*, 2008). This correlates with dozens of studies linking mutations in genes involved in sarcomere and cytoskeletal organization to DCM. We also observed some defects in sarcomere organization in E2F6-Tg mice (Westendorp *et al*, 2008), but our mice develop a severe DCM phenotype at a much earlier age (2-6 months) than E2F3 KO mice (18 months). Thus other factors must be involved in the early-onset DCM observed in E2F6-Tg mice.

4.6 E2F6 May Cause DCM by Changes in Cx43 and Electrical Conduction

With respect to the cause of the DCM developed in Tg mice, one of the players identified in our microarray was connexin43. This protein is one of the major types of connexin which forms gap junctions in the electrical conduction system in both atria and ventricles. Since mRNA levels did not fluctuate, and Cx43 protein down-regulated throughout pot-natal development, it is apparent that the down-regulation has occurred post-transcriptionally. MicroRNA 206, which is up-regulated in Tg hearts, is known to target Cx43 in muscle tissue during differentiation (Kim *et al*, 2006). Preliminary results suggest that the up-regulation of miR-206 does not occur in pups but only at a later stage, thus other factor must be involved in the loss of Cx43 in young pups. This agrees with results demonstrating that Cx43 down-regulation is more prominent in adult Tg hearts at which point additional factors including elevated levels of miR-206 and heart failure are

in effect. In support of this others found that miR-206 is up-regulated post MI and during heart failure, suggesting it may be correlated to cardiac disease.

Kalma and colleagues demonstrated that Cx43 translation was inhibited by luteinizing hormone in oocytes (Kalma *et al*, 2004). The pathway for down-regulation appeared to involve the MAPK phosphorylation cascade resulting in the activation of the ERK protein kinases. Interestingly the MAPK pathway, including ERK, is crucial for cardiac development and plays an important role in the cardiac hypertrophic response (reviewed in Rose *et al*, 2010). In addition, activation of the MAPK pathway in diabetic mice was demonstrated to activate the expression of miR-206 in the heart (Shan *et al*, 2010). It is plausible that the activated ERK in Tg mice has led to both the inhibition of Connexin 43 translation as well as the transcription of miR-206, leading to a significant loss of Connexin43.

Another possibility for the down-regulation of Cx43 in Tg mice is an increase in turnover. Cx43 is an unstable protein with a short half-life (reviewed in Saffitz *et al*, 2000) and its degradation has been linked to both phosphorylation and ubiquitination events (reviewed in Berthoud *et al*, 2004). It is highly likely that this could play a role in the observed post-transcriptional down-regulation of Cx43. As mentioned, E2F1 and 3 are also post-transcriptionally down-regulated in Tg hearts and could be potentially targeted by similar ubiquitin-proteasomal pathways as Cx43, which may be up-regulated in our Tg mice.

The electrocardiogram of E2F6-Tg mice is significantly altered, and it appears that Tg mice exhibit dampened atrial conductivity. It seems highly plausible that the loss of a connexin could play a major role in this observation. In fact, the PR interval in E2F6-

Tg mice is comparable to those in Cx40 KO mice which have both AV and bundle branch blocks (Simon *et al*, 1998). Interestingly, Cx40 protein levels did not change in Tg mice suggesting there was no compensatory response to the loss of Cx43.

The effect of Cx43 on the atria is under debate. $Cx43^{+/-}$ mice have normal atrial conductivity, but altered ventricular activity (Thomas *et al*, 1998). In contrast, isolated atrial cardiomyocytes have greatly reduced conductivity in response to a loss of Cx43 while a loss of Cx40 had only minor effects (Beauchamp *et al*, 2006). Another group found that both Cx40 and 43 are found in equal abundance and play similar contributions to gap junction voltage gating in atrial cardiomyocytes (Lin *et al*, 2010). The reason for these discrepancies may lie in the availability of other connexin proteins. Although levels of Cx40 and Cx45 remained stable in $Cx43^{+/-}$ mice (Thomas *et al*, 1998), it is possible that the composition of gap junctions were altered in order to compensate for loss, as observed in many cases of heart failure.

A decrease in Cx43 is a hallmark of cardiac failure including that caused by DCM (reviewed in Severs *et al*, 2008). Connexin43 has also been demonstrated to be down-regulated at intercalated disks by approximately 3-fold in a desmin model of DCM (Gard *et al*, 2005). Furthermore, a cardiac restricted KO of Cx43 led to reduced conduction velocities and sudden death by 2 months of age in 100% of mice (Gutstein *et al*, 2001), which is quite similar to the age in which our Tg mice die. Connexin43 is down-regulated in our Tg mice from a very young age, before any cardiac remodelling has occurred, suggesting two things (1) The E2F pathway could play a role in Cx43 translation and/or degradation (2) the accumulation of stress due to a lack of Cx43 beginning at a very

young age could lead to a compensatory cardiac remodelling response and eventually to dilated cardiomyopathy and death.

4.7 A Model for E2F6 as a Transcriptional Activator

Normally Rb is hypo-phosphorylated in cells in G_0 (such as adult cardiomyocytes) and thus capable of binding to activating E2Fs such as E2F1 and E2F3 masking their ability to transactivate gene expression. It also recruits chromatin modifying enzymes leading to gene repression (Figure 14A). We have demonstrated that E2F6 remains mainly cytosolic in cardiomyocytes and thereby unable to bind DNA (Figure 12). In Tg mice excess E2F6 and myc-tagged E2F6 are translocated to the nucleus, and can out-compete other E2Fs. E2F6 may cooperate with other transcription factors such as YY1, which is also translocated to the nucleus in Tg mice (as well as perhaps Max, and Mga) in order to recruit the basal transcriptional machinery leading to gene activation (Figure 14B).

Since E2F6 can associate with other transcription factors- it could potentially regulate the expression of a large variety of genes. Furthermore, it has become evident over the past several years that E2Fs do not only target genes with the E2F consensus site (Xu *et al*, 2007). In fact, only a very small percentage of promoter sequences occupied by E2Fs contain the consensus sequence (Bieda *et al*, 2006). Instead, the only obvious pattern of E2F binding appears to be within 2kB of transcription start sites and nearby other transcription factor biding sites. Thus it seems likely that E2Fs, including E2F6, could coordinate the expression of a vast variety of genes in a tissue specific manner dependant on the availability of other proteins.

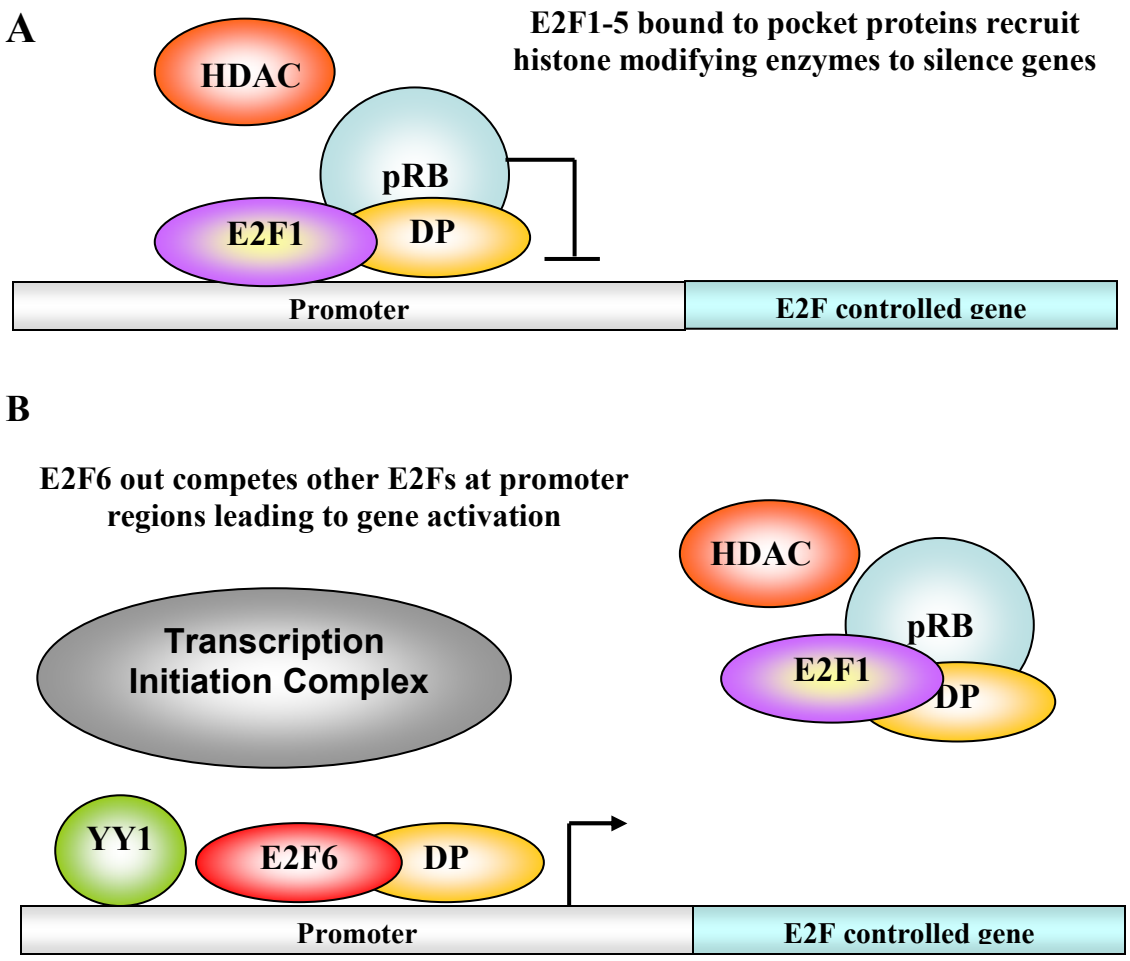

Figure 14: *A Model for How E2F6 Activates Genes in the Heart*. In normal quiescent cardiomyocytes pocket proteins bind to E2F/DP complexes leading to gene repression. Pocket proteins may also recruit histone modifying enzymes for active gene silencing (**A**). In Tg mice excess E2F6 is translocated to the nucleus where it can outcompete other E2F/DP/RB complexes leading to either a leak in transcriptional repression causing gene activation, or E2F6 may recruit other transcription factors such as YY1 which are capable of initiate transcription (**B**).

Conclusions:

This study demonstrates that appropriately regulated levels of E2F in the heart are necessary for proper cardiac development and function. Transgenic mouse hearts exhibit chamber dilation and thinning of the myocardium, leading to severe cardio respiratory distress and death at a very young age. Our data points toward de-regulation of genes and proteins involved in regulating muscle contraction to the progression of the disease. Of particular importance, we detected a profound decrease of Connexin43 in Tg mouse hearts beginning at a very young age. This loss could lead to the impairment of appropriate coordination of cardiac contraction, the accumulated stress of which could lead to cardiac remodelling and DCM. Additionally, we observed a marked decrease in E2F3B which has been previously linked to defects in muscle differentiation and late-onset dilated cardiomyopathy.

In addition to demonstrating an unprecedented role for the E2F pathway in the heart, our study also paints a unique role for E2F6 that does not involve gene repression. Unlike the potent repressor normally depicted in literature, when E2F6 was over-expressed in the murine heart it led to a small net activation in transcription with a specific increase in the classical E2F responsive pathways. We did not observe any significant changes in chromatin architecture despite E2F6's capacity to bind to a variety of chromatin modifying enzymes. A modest increase in H3K9 acetylation was observed which may reflect the net increase in transcription, but no changes in DNA methylation status were detected.

We also found that in the adult myocardium endogenous E2F6 is primarily localized in the cytosol with a very small amount present in the nucleus. The myc-tagged

E2F6 we over-expressed was also highly expressed in the cytosol with a higher ratio in the nucleus than endogenous E2F6. It appears that the excess E2F6 in the nucleus activates transcription and this likely depends on interactions with other transcription factors which are present in the nucleus such as perhaps YY1.

The data suggests that the view of E2F6 as a transcriptional repressor needs to be re-evaluated. The role of E2F likely depends on complex interactions with a variety of different proteins and signalling pathways which leads to a unique role in the adult myocardium. Furthermore, this study provides evidence that the E2F pathway has a role in the heart outside of cell cycle control. Modulation of the E2F pathway led to an impairment in ventricular function and cardiac conduction thereby providing evidence that a strict regulation of the E2F pathway is critical for normal cardiac development and function.

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Appendixes:

Appendix 1: *Down regulated gene list*. Symbols of genes which were predicted to be down-regulated by at least 0.75 fold with a p-value <0.05 by microarray analysis. Non-annotated genes and those which appeared more than once were not included.

1500015O10Rik	Jun
2310040G07Rik	LOC676300
4933439C20Rik	Mlana
4933439C20Rik	Myl4
4933439C20Rik	Myl7
6330564D18Rik	Otud1
6530404N21Rik	Pde4b
6720477C19Rik	Pdk4
9530006C21Rik	Pim1
Acot2	Pisd
Angptl4	Pkp2
Atf3	Pnpt1
Atp2a2	Rabgap1
Atp5a1	Rn18s
Baiap2l1	Sfrp2
Bxdc1	Sln
C78692	Socs2
Cap1	Sod1
Cd24a	Sorbs1
Cdca3	Stard10
Ctgf	Thrap3
Dkk3	Tm2d2
EG665189	Tnni1
Egr1	Tnxb
Fmod	Trp53inp2
Fmod	Ttc27
Fos	Ush1c
H2-K1	
Hba-a1 /// Hba-a2	
Hist1h4	
Hmgcs2	
Hmgcs2	
Ier3	
Jun	

Appendix 2: *Up-regulated Gene List.* Symbols of genes which were predicted to be up-regulated by at least 1.5 fold with a p-value <0.05 by microarray analysis. Non-annotated genes and those which appeared more than once were not included.

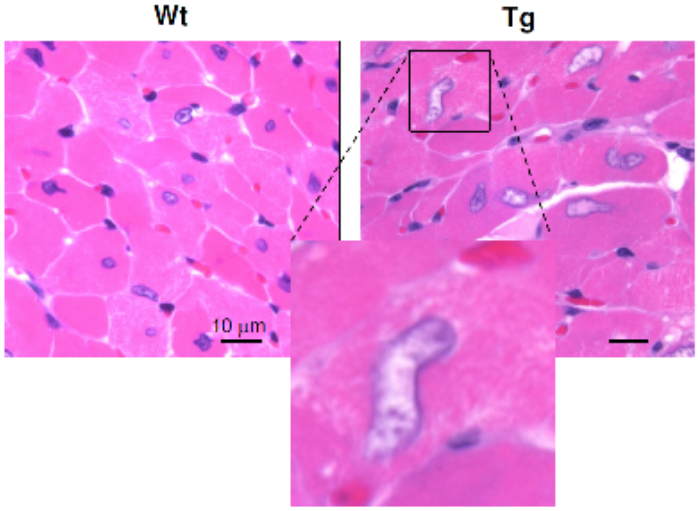
Klf9	Ahcyl1	D7Wsu130e	Hccs	Msrb3
Map3k5	Ak3	Dcbld2	Hfe	Mthfd1
Opa1	Aplp2	Dmd	Hipk3	Mtif2
Rab12	Arhgap5	Dnaja3	Hk1	Mtpn
Ranbp9	Arl6	Dnajc9	Hnrnpc	Myh6
1110001A07Rik	Armc1	Dnm1l	Hsd17b11	Myl1
1110031B06Rik	Asb8	Dpyd	Hsp90aa1	Myl9
1110032E23Rik	Atp1a2	Dsn1	Hspa4l	Myo1c
1110054O05Rik	Atp1b1	Dsp	Ifrd2	Nat11
1500003O03Rik	Atp6v0e2	Dst	Ift122	Ncam1
1500003O03Rik	Atp6v1b2	Dusp7	Immt	Ncapg2
1700029F09Rik	Atpaf1	Efr3a	Itgb1	Ncl
1700040L02Rik	AU021838	EG277333	Ivd	Ndufa12l
1700081L11Rik	B2m	Eid1	Kif3b	Nedd4
1700113I22Rik	B3gnt2	Eif2s3x	Kif5b	Nid2
2310007F21Rik	Bach2	Eif4a1	Kpna1	Nudt19
2410004L22Rik	BC022623	Ets1	Kpna3	Obfc2a
2410127L17Rik	Bckdhb	Etv1	Lama4	Ociad2
2410129H14Rik	Bclaf1	Exosc8	Lamc2	Osgepl1
2610030H06Rik	Blm	F730047E07Rik	Laptm4b	Oxct1
2610036L11Rik	Brp44l	Fblim1	Larp4	Papss2
2810407C02Rik	Bzw1	Fen1	Lin54	Pcmtd1
4632404H22Rik	Cacnb2	Fgf1	Lin7c	Pdk2
4632434I11Rik	Capzb	Fgl2	LOC100047837	Pdk3
4833420G17Rik	Ccdc5	Frag1	LOC100048021	Pdlim5
4930452B06Rik	Ccdc88a	G3bp2	Lrrc2	Pdss1
5730410I19Rik	Ccng1	Gapdh	Lsm3	Pdxp
5730427N09Rik	Cenpj	Gdi2	Ly6e	Phlda1
5730536A07Rik	Chek1	Gfer	Lyrm5	Pkia
6430514L14Rik	Ciapin1	Gins1	Mad2l1	Plcb4
6820431F20Rik	Cks1b	Gins2	Maob	Pop4
A130040M12Rik	Clic5	Gja1	Mat2b	Ppap2b
Aaas	Cops7a	Glb1	Mbnl2	Ppil5
Abcb7	Coro6	Glo1	Mcm5	Ppp1r12a
Abcc9	Cox18	Glrx	Mcm7	Ppp1r14c
Abce1	Cpd	Gmcl1	Med14	Ppp1r3c
Abhd10	Cpeb4	Gmnn	Mest	Prkab2
Abhd5	Crk	Got1	Metapl1	Prnd
Acat2	Ctnnb1	Got2	Mga	Pten
Acox1	Cxcr4	Grhpr	Mlstd2	Ptp4a1
Acp1	Cyb5r3	Gspt1	Mpdz	Ptrf
Acta1	Cyfip2	Gstz1	Mrps23	Pttg1
Acta2	D030056L22Rik	H2-D1	Msh6	Pum2
Adam10	D430042O09Rik	Hat1	Msn	Pygl

Appendix 2 Continued:

Rab18
Rab7
Rad23a
Rfc3
Rfc4
Rgs5
Rgs5
Rnaseh2a
Rnaseh2c
Rpa1
Rpa2
Rpp30
Rrm1
Rsrc1
Sc4mol
Scd2
Sdc2
Senp2
Serinc3
Sfrp1
Sfrs1
Sh3kbp1
Shmt1
Slc16a1
Slc20a2
Slc25a10
Slc25a13
Slc2a1
Slc31a1
Slc37a2
Slc6a6
Slc9a3r1
Slk
Slmap
Smyd1
Spc24
Srd5a2l2
St3gal5
St3gal6
Stat1
Stip1
Syncrip
Sypl
Tesc
Tgm2
Tm6sf1
Tmed2
Tmem32
Tmem38a
Tmem41b
Tmem48
Tnfaip8
Tnfrsf12a
Topbp1
Tpd52l1
Tpp2
Trp53
Tsc22d3
Tspan7
Ttc30b
Ttc5
Ttrap
Tubgcp2
Txndc14
Ube2d3
Ublcp1
Uchl5
Ung
Usp8
Usp9x
Vamp3
Vcl
Vrk1
Wdr51a
Yipf4
Ythdf3
Zfp386
Zfp650
Zranb3
Zwilch
Zwint

Appendix 3: *Supplemental images of cardiomyocyte nuclei.* Images represent transverse sections of Wt and Tg hearts stained with haematoxylin and eosin (**A**) taken with an analog microscope or examined by electron microscope (**B**). (Westendorp *et al*, 2008)

A



B