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Hypertrophic growth in cardiac myocytes is mediated by Myc through a Cyclin D2-dependent pathway

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c-Myc (Myc) is highly expressed in developing embryos where it regulates body size by controlling proliferation but not cell size. However, Myc is also induced in many postmitotic tissues, including adult myocardium, in response to stress where the predominant form of growth is an increase in cell size (hypertrophy) and not number. The function of Myc induction in this setting is unproven. Therefore, to explore Myc's role in hypertrophic growth, we created mice where Myc can be inducibly inactivated, specifically in adult myocardium. Myc-deficient hearts demonstrated attenuated stress-induced hypertrophic growth, secondary to a reduction in cell growth of individual myocytes. To explore the dependence of Myc-induced cell growth on CycD2, we created bigenic mice where Myc can be selectively activated in CycD2-null adult myocardium. Myc-dependent hypertrophic growth and cell cycle reentry is blocked in CycD2-deficient hearts. However, in contrast to Myc-induced DNA synthesis, hypertrophic growth is independent of CycD2-induced Cdk2 activity. These data suggest that Myc is required for a normal hypertrophic response and that its growth-promoting effects are also mediated through a CycD2-dependent pathway.

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Introduction

During development, cell cycle progression is normally tightly coupled to the accumulation of cell mass (cell growth) (Neufeld and Edgar, 1998); however, in some adult postmitotic tissues, cell growth can become uncoupled from proliferation resulting in hypertrophic growth (Dorn and Force, 2005). The molecular mechanisms that regulate hypertrophy and the means whereby proliferation and cell growth are normally coupled are poorly understood, but the fact that they are coupled during development suggests common regulatory mechanisms. One molecule that has been implicated in mediating both forms of growth in many tissues, including the heart, is c-Myc (Myc).

Myc is highly expressed in fetal, proliferating cardiac myocytes. However, soon after birth the myocytes cease to divide corresponding with the downregulation of Myc. Transgenic mice that overexpress Myc in the fetal myocardium develop ventricular enlargement secondary to myocyte hyperplasia, suggesting that Myc is sufficient to induce proliferative growth in the heart as well (Jackson *et al*, 1990). Although Myc is not expressed in the adult heart under normal physiological conditions, it is upregulated rapidly in response to virtually all hypertrophic stimuli (Izumo *et al*, 1988) but the growth response is limited to hypertrophy and not hyperplasia (Soonpaa and Field, 1997). The importance of Myc in mediating this hypertrophic growth in postmitotic myocytes is controversial but several lines of evidence support the concept that Myc can mediate cellular growth in the absence of cell division. In *Drosophila*, decreased expression of dMyc, the ortholog of mammalian Myc, in wing imaginal disc cells reduced cell proliferation and cell size (Johnston *et al*, 1999). In contrast, dMyc overexpression resulted in increased cell size without affecting cell division. In mammalian cells, deleting Myc in B cells and hepatocytes reduced cell size (Iritani and Eisenman, 1999; Baena *et al*, 2005). Conversely, previous studies from our lab demonstrated that activation of Myc specifically in adult myocardium was sufficient to induce hypertrophic growth and this growth was accompanied by cell cycle reentry (Xiao *et al*, 2001). Similarly, overexpression of Myc in B lymphocytes both *in vitro* (Schuhmacher *et al*, 1999) and *in vivo* (Iritani and Eisenman, 1999) is also associated with an increase in cell size, independent of cell cycle progression.

The mechanisms whereby Myc regulates hypertrophic growth are less clear but it is interesting to note that genes responsible for Myc's ability to promote cell cycling have also been implicated in regulating cell size in certain contexts. Myc activation in the heart is accompanied by the upregulation of Cyclin D2 (CycD2) and cyclin-dependent kinase (Cdk) -2 and -4 activities (Xiao *et al*, 2001). The link between this Myc-induced Cdk activity and Myc-induced proliferation has been well established (Amati *et al*, 1998). Myc stimulated Cdk2 kinase activity, which is critical for cell cycle progres-

sion (Amati *et al*, 1998), occurs in large part by antagonizing the association of CdkIs, p27 and p21, with Cdk2 (Bouchard *et al*, 1999; Perez-Roger *et al*, 1999). Upregulation of Myc target genes Cdk4 and CycD2 (Coller *et al*, 2000) leads to the rapid sequestration of p21 and p27, liberating Cdk2. Consistent with this model, cells from CycD2$^{-/-}$ mice, unlike wild-type controls, do not reenter the cell cycle in response to Myc (Bouchard *et al*, 1999). The role of CycD2 in mediating Myc-induced hypertrophic growth was not addressed in these studies.

Interestingly, CycD and Cdk4 have also been implicated in regulating cell size *in Drosophila* but, similar to Myc, the effect of CycD–Cdk4 overexpression varied according to cell type (Datar *et al*, 2000). In undifferentiated proliferating cells, CycD/Cdk4 overexpression caused accelerated cell division (hyperplasia) without affecting cell size (Datar *et al*, 2000). However, in differentiated cells, CycD/Cdk4 caused cell enlargement (hypertrophy), potentially through Rb-independent pathways. More recent studies have suggested that CycD/Cdk4 stimulates cell growth through regulation of mitochondrial activity (Frei *et al*, 2005). In mammals, numerous studies have implicated CycD/Cdk4 in regulating cardiac hypertrophy; however, this connection is, so far, based primarily on cell culture experiments. Hypertrophic signals upregulate CycD2 expression and CycD-dependent kinase activity in the cardiac myocytes (Li *et al*, 1998; Busk *et al*, 2002). Although forced expression of CycD2 in cardiac myocytes provoked cell division and not hypertrophy (Busk *et al*, 2005; Pasumarthi *et al*, 2005), CycD2 was overexpressed at a developmental time point where the myocytes were still capable of reentering the cell cycle. Overexpression of CycD2 in adult, postmitotic cardiac myocytes has not been reported. However, inhibiting G1-Cyc/Cdk activity in adult myocytes blocks hypertrophic growth (Nozato *et al*, 2001).

To determine the role of Myc in regulating hypertrophic growth specifically in adult, postmitotic myocardium and whether its growth effects are mediated through CycD2-dependent pathways in the heart, we developed inducible, cardiac-restricted Myc-deficient mice and a model of inducible, myocardial-specific Myc expression in a CycD2-null background. Deletion of Myc attenuated hypertrophic growth in response to both hemodynamic and pharmacologic hypertrophic stimuli and resulted in an increase in cardiac apoptosis. Myc-induced hypertrophic growth was dependent on the presence of CycD2 similar to what has been reported for Myc-induced cell cycle reentry, but was independent of Cdk2 activity.

Results

Creation of inducible, cardiac-specific Myc-deficient mice

The role of Myc in cell cycle control and cellular proliferation has been well studied and Myc has been shown to be critical for the regulation of mammalian body size during development by controlling cell number (Trumpp *et al*, 2001). However, Myc is also expressed in many adult tissues in pathological conditions, including cardiac hypertrophy, where cell growth but not cell division occurs. Myc's role in regulating normal physiology in these adult tissues remains unknown. To explore the role of Myc in cardiac hypertrophy *in vivo,* we created inducible Myc-deficient mice

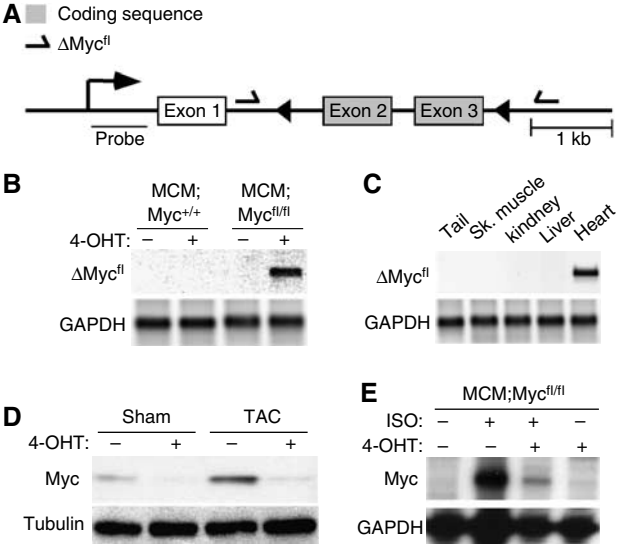

Figure 1 Creation of inducible cardiac-specific Myc-deficient mice. To create inducible, cardiac-restricted Myc-deficient mice, we bred mice with a floxed Myc allele to mice expressing a tamoxifen-inducible Cre only in the heart. (**A**) Schematic diagram depicting the floxed Myc allele (⇀ = ΔMycfl primers; ◀ = LoxP sites). (**B, C**) PCR utilizing ΔMycfl primers was performed on genomic DNA from the indicated tissues and genotypes. (**D**) Myc protein expression in ventricular lysates from vehicle- and 4-OHT-treated MCM;Myc$^{fl/fl}$ mice after Sham or TAC surgery was determined by Western blotting. (**E**) RPA analysis on total ventricular RNA from MCM;Myc$^{fl/fl}$ mice after ISO stimulation.

where Myc can be specifically deleted in adult myocardium with a tamoxifen-regulated Cre, MerCreMer (MCM). When Cre is activated in these mice (MCM;Myc$^{fl/fl}$) with 4-hydroxytamoxifen (4-OHT), the resulting recombination excises Myc coding exons 2 and 3 (Figure 1A). This deletion can be identified with ΔMycfl primers, which give a ~600 bp product when recombination has occurred (de Alboran *et al*, 2001). PCR performed on total ventricular DNA from control (MCM;Myc$^{+/+}$) or Myc-null (MCM;Myc$^{fl/fl}$) mice with ΔMycfl primers demonstrated no spontaneous recombination in the absence of ligand. However, 4-OHT treatment resulted in recombination in the ventricles of MCM;Myc$^{fl/fl}$ mice (Figure 1B). This recombination only occurred in the hearts of the 4-OHT-treated, MCM;Myc$^{fl/fl}$ mice and was not seen in other tissues (Figure 1C). As Myc is expressed at very low levels in adult myocardium at baseline, its expression was induced by subjecting mice to hemodynamic or pharmacological hypertrophic stimuli *in vivo*. MCM;Myc$^{fl/fl}$ mice with or without 4-OHT treatment underwent Sham or transverse aortic constriction (TAC) operation to induce cardiac hypertrophy. TAC induced a 9.7-fold increase in Myc protein expression in vehicle-treated MCM;Myc$^{fl/fl}$ mice when compared to Sham-operated mice. Pretreatment of MCM:Myc$^{fl/fl}$ mice with 4-OHT reduced the expected increase in Myc protein after hemodynamic load by 66.5% (9.67 ± 0.44- versus 3.24 ± 2.11-fold, $P < 0.005$; Figure 1D). This degree of recombination is consistent with previous reports using the MCM mice (Sohal *et al*, 2001) and likely reflects in part, that the heart consists of a number of different cell types although the majority of the protein arises from authentic cardiac myocytes. To confirm this finding, we analyzed Myc expression in total ventricular RNA prepared from treated

or untreated MCM;Myc$^{fl/fl}$ hearts after stimulation with a pharmacologic hypertrophic agonist, isoproterenol (ISO). Myc expression was dramatically attenuated in response to ISO stimulation in 4-OHT treated MCM:Myc$^{fl/fl}$ hearts *in vivo* (Figure 1E).

Cardiac hypertrophy is attenuated in Myc-deficient mice after hemodynamic stress

MCM;Myc$^{fl/fl}$ mice were born with expected Mendelian numbers and appeared phenotypically normal. Baseline left ventricular (LV) size, function and histology were normal in MCM;Myc$^{fl/fl}$ mice after 4-OHT treatment to delete Myc (Figure 2A and Supplementary Table 1). To determine the role of Myc in hypertrophic growth, we subjected adult mice to a hemodynamic stress induced by TAC surgery and measured the increase in heart weight normalized to body weight (HW/BW) after 2 weeks. Vehicle-treated MCM;Myc$^{fl/fl}$ mice demonstrated the expected enhanced LV wall thickness (Figure 2A) and concentric hypertrophy after TAC, while 4-OHT-treated MCM;Myc$^{fl/fl}$ in the absence of Myc did not. When compared to Sham-operated animals, MCM:Myc$^{+/+}$ (4.74 ± 0.01 versus 7.62 ± 0.98 mg/g, $P<0.001$; Figure 2B) or vehicle-treated MCM;Myc$^{fl/fl}$ (5.05 ± 0.16 versus 7.42 ± 0.24 mg/g, $P<0.001$; Figure 2B) with intact Myc expression demonstrated the expected increase in HW/BW ratio after TAC. In contrast, the hypertrophic response in Myc-deficient mice was attenuated. Although 4-OHT-treated MCM;Myc$^{fl/fl}$ mice tolerated TAC hemodynamically over the period studied and did not develop left ventricular dysfunction (Supplementary Table 1), they developed less hypertrophy (4.85 ± 0.17 versus 6.01 ± 0.29 mg/g, $P<0.01$). While TAC induced a 46.9% increase in heart weight in vehicle-treated MCM;Myc$^{fl/fl}$ mice compared to Sham-operated mice, heart weight only increased 23.9% in 4-OHT-treated MCM:Myc$^{fl/fl}$ mice after TAC ($P<0.001$).

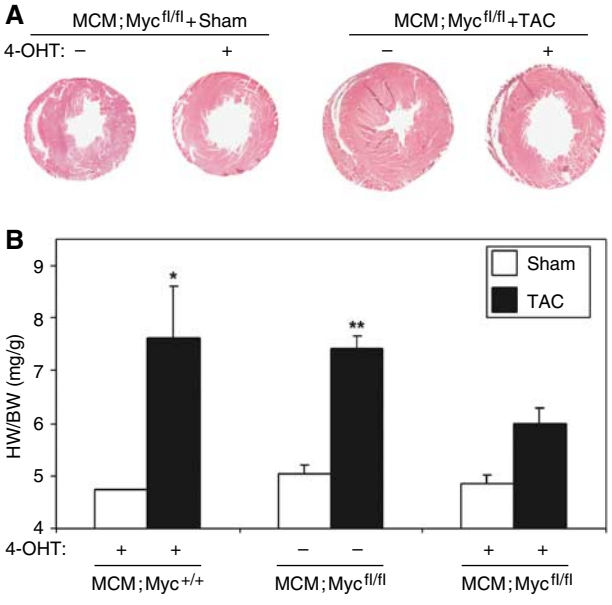

Figure 2 Myc-null mice decrease cardiac mass and cardiac myocyte size. MCM;Myc$^{+/+}$ and MCM;Myc$^{fl/fl}$ mice were treated with vehicle or 4-OHT for 5 days to induce recombination and then subjected to Sham or TAC surgery and followed for 2 weeks. (**A**) H&E-stained, perfusion-fixed MCM;Myc$^{fl/fl}$ hearts demonstrating development of concentric LV hypertrophy in vehicle-treated MCM;Myc$^{fl/fl}$ mice. (**B**) To determine if myocardial hypertrophy had occurred, heart weights (mg) normalized to body weight (g) were analyzed. *$P<0.05$ for 4-OHT-treated MCM;Myc$^{+/+}$ mice after TAC versus MCM;Myc$^{+/+}$ Sham animals. **$P<0.05$ for vehicle-treated MCM;Myc$^{fl/fl}$ mice + TAC versus Sham animals or 4-OHT-treated MCM;Myc$^{fl/fl}$ mice + TAC ($n=6$ in each group).

To confirm that the reduced HW/BW ratio in Myc-deficient hearts after pressure-overload represented a reduction in cell growth, we measured cardiac myocyte fiber width on wheat germ agglutinin-stained myocardial sections in these animals (Figure 3A). Cardiac myocyte width was 45% greater in control vehicle-treated MCM;Myc$^{fl/fl}$ subjected to TAC compared with 4-OHT-treated MCM;Myc$^{fl/fl}$ mice (17.58 ± 0.33 versus 12.08 ± 0.28 μm, $P<0.01$; Figure 3B). Consistent with this reduction in cardiac hypertrophy, Myc-deficient mice also showed attenuated upregulation of hypertrophic marker genes such as atrial natriuretic factor (ANF) and β-myosin heavy chain (βMHC) mRNA compared with untreated MCM:Myc$^{fl/fl}$ mice after TAC (Figure 3C).

Interestingly, there was a significant increase in interstitial fibrosis as measured by picrosirius red staining of ventricular sections from 4-OHT treated MCM;Myc$^{fl/fl}$ mice subjected to Sham versus TAC (1.00 ± 0.04 versus 1.48 ± 0.09-fold, $P<0.01$; Figure 4A and B). To determine if this was in response to cell loss, we assessed apoptosis by TdT-mediated dUTP-biotin nick-end labeling (TUNEL) staining. Apoptotic cardiomyocytes were rarely detected in MCM;Myc$^{fl/fl}$ mice with or without 4-OHT treatment mice that underwent sham operation. However, apoptosis increased 12-fold in 4-OHT-treated MCM;Myc$^{fl/fl}$ mice subjected to TAC compared to untreated Sham animals ($P<0.001$; Figure 4C), suggesting Myc was also necessary for cardiac myocyte survival with hypertrophic stimuli.

Myc-deficient mice display reduced cardiac growth and increased apoptosis with chronic isoproterenol infusion

To determine if this attenuated hypertrophy and fibrosis was a general response to hypertrophic stimuli, we treated MCM:Myc$^{+/+}$ or MCM;Myc$^{fl/fl}$ mice with 4-OHT for 5 days and then subjected the mice to an infusion of ISO or vehicle for 1 week. ISO stimulation resulted in a 30.4% increase in HW/BW ratio in 4-OHT-treated MCM:Myc$^{+/+}$ mice compared to only a 17.4% increase in ISO-treated, Myc-deficient MCM;Myc$^{fl/fl}$ mice ($P<0.05$; Figure 5A). ISO stimulation increased heart-to-body weight ratio in 4-OHT-treated control MCM:Myc$^{+/+}$ mice (5.17 ± 0.12 versus 6.74 ± 0.40 mg/g, $P<0.01$; Figure 5A). In contrast, MCM;Myc$^{fl/fl}$ mice demonstrated no significant increase (4.83 ± 0.62 versus 5.67 ± 0.33 mg/g, $P=$NS). This difference in HW/BW ratio was paralleled by a 19.4% increase in cardiac myocyte fiber width in ISO-stimulated MCM:Myc$^{+/+}$ mice when compared to fiber width in vehicle-treated MCM:Myc$^{+/+}$ or MCM;Myc$^{fl/fl}$ hearts (13.79 ± 0.56 versus 11.55 ± 0.30 or 11.04 ± 0.14 μm, $P<0.01$; Figure 5B). There was no significant increase in cardiac myocyte fiber width in ISO-treated MCM;Myc$^{fl/fl}$ hearts compared to vehicle-treated MCM;Myc$^{fl/fl}$ hearts, suggesting the reduced HW/BW was a result of attenuated myocyte hypertrophy. Similar to the results in the TAC model, ISO treatment in MCM;Myc$^{fl/fl}$ mice was also accompanied by increased interstitial fibrosis as seen on H&E-stained myocardial sections (Figure 5C-b). To further explore the cause for this, we quantified TUNEL +ve nuclei

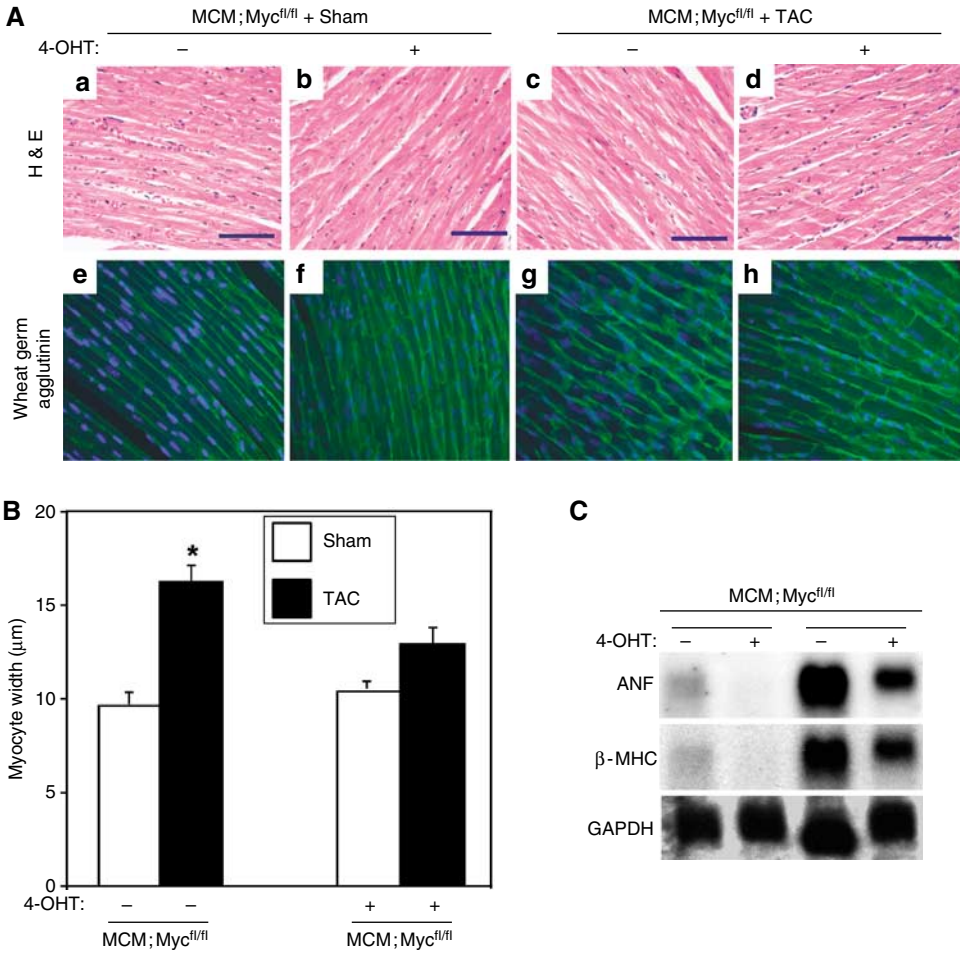

Figure 3 Myc-null mice decrease cardiac mass and cardiac myocyte size. MCM;Myc$^{fl/fl}$ mice with 5 days of 4-OHT or vehicle treatment underwent Sham or TAC surgery. Hearts were harvested after 2 weeks and histological analysis was performed. (**A**) H&E (**a–d**) and wheat germ agglutinin (**e–h**)-stained myocardial sections from Sham-operated MCM;Myc$^{fl/fl}$ mice treated with vehicle (a and e) or 4-OHT (b, f) versus TAC-operated MCM;Myc$^{fl/fl}$ treated with vehicle (c, g) or 4-OHT (d, h). Scale bar = 70 μm. (**B**) Fiber width of wheat germ agglutinin-stained hearts from Sham and TAC mice were quantified ($n = 4$ in each group). *$P < 0.01$ for MCM;Myc$^{fl/fl}$ with vehicle + TAC versus MCM;Myc$^{fl/fl}$ with 4-OHT + TAC. (**C**) Total ventricular RNA (10 μg) from 4-OHT-treated mice with indicated genotypes was probed with specific ^{32}P-labelled probes for atrial natriuretic factor (ANF), β-myosin heavy chain (βMHC), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

on sections of ventricular tissue. As shown, ISO-treated MCM;Myc$^{fl/fl}$ ventricles displayed a 6.3-fold increase in TUNEL +ve nuclei ($P < 0.01$; Figure 5D), suggesting that Myc was also necessary for cardiac myocyte survival with hypertrophic stimuli.

Inhibition of Myc in vitro blocks ET-1-stimulated hypertrophic growth and CycD2 upregulation

To explore the mechanisms underlying the growth effects of Myc in cardiac myocytes, we utilized an *in vitro* model of hypertrophic growth by stimulating neonatal rat ventricular myocytes (NRVMs) with endothelin-1 (ET-1). NRVMs were serum starved for 48 h and then exposed to ET-1, which upregulates Myc expression (Figure 6A) and induces hypertrophic growth (Figure 6Ba versus b) without increasing cell number (data not shown). To confirm our *in vivo* results, we blocked ET-1-induced Myc expression with an adenovirus overexpressing an siRNA to Myc (Ad-siMyc). Overexpressing a control protein (Ad-LacZ) or scrambled siRNA sequence (data not shown) had no effect on Myc induction.

Myc is well known to regulate the expression of a number of G1 cyclins and Cdk activities. As these proteins are also regulated in cardiac myocytes after hypertrophic agonists, we examined the expression of a panel of cell cycle activators (Figure 6A). Although stimulation of NRVMs with ET-1 upregulated CycD2 leading to increased Cdk2 and 4 activity, these changes were blocked by inhibiting Myc expression (Figure 6A). Inhibiting ET-1-induced Myc expression also prevented the expected increase in myocyte size (Figure 6Bb versus d) presumably though the inhibition of Myc-dependent protein synthesis seen in control myocytes after ET-1 stimulation (1.00 ± 0.05 versus 1.54 ± 0.18, $P < 0.01$; Figure 6C). Although ET-1 induced both ANF and β-MHC, the expression of these hypertrophic markers was attenuated by inhibiting Myc expression, similar to the *in vivo* results (Figure 6D). It is well known that Cdk2 and 4 activity cooperate for Myc-induced cell cycle progression but the role of these proteins in mediating Myc-induced hypertrophic growth is unknown.

CycD2 is necessary for Myc-induced cardiac hypertrophy

Our previous studies had demonstrated that cardiac-specific activation of Myc in adult α-MHC-MycER mice with 4-OHT

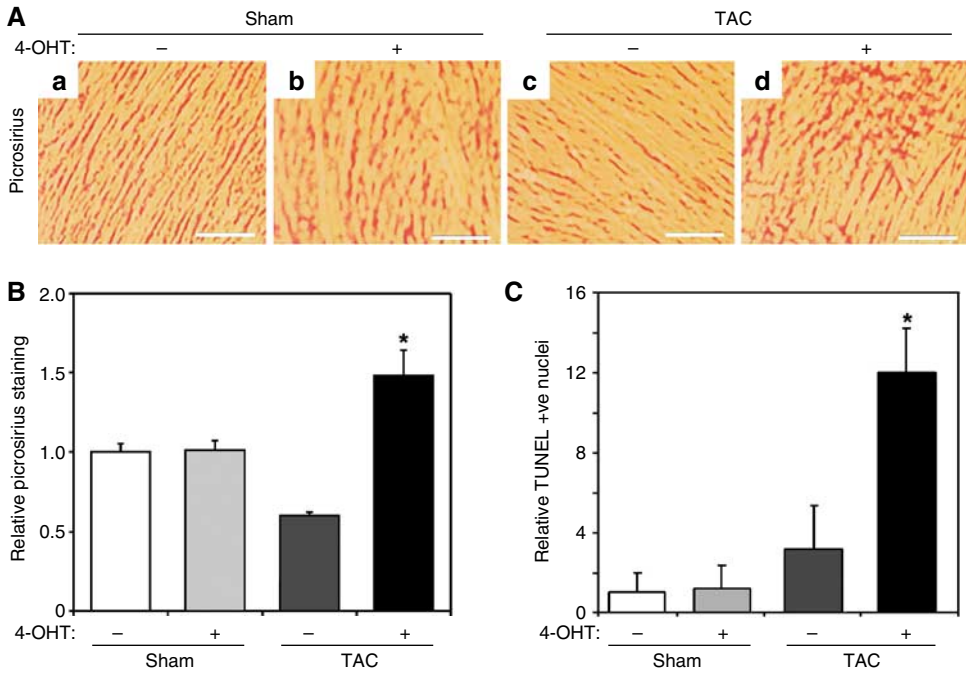

Figure 4 Myc-null hearts demonstrate increased fibrosis and apoptosis after TAC. (**A**) Representative picrosirius-stained myocardial sections from vehicle- or 4-OHT-treated MCM;$Myc^{fl/fl}$ mice subjected to Sham or TAC surgery. Scale bar = 70 μm. (**B**) The amount of ventricular fibrosis was quantified and displayed relative to vehicle-treated MCM;$Myc^{fl/fl}$ + Sham mice ($n = 4$ in each group). *$P < 0.01$ for 4-OHT-treated MCM;$Myc^{fl/fl}$ + TAC versus 4-OHT-treated MCM;$Myc^{fl/fl}$ + Sham or vehicle-treated MCM;$Myc^{fl/fl}$ + TAC. (**C**) Results of TUNEL staining were quantified and the numbers of TUNEL − + ve nuclei expressed relative to vehicle-treated MCM:$Myc^{fl/fl}$ + Sham mice. *$P < 0.01$ for 4-OHT-treated MCM;$Myc^{fl/fl}$ + TAC versus 4-OHT-treated MCM;$Myc^{fl/fl}$ + Sham or vehicle-treated MCM;$Myc^{fl/fl}$ + TAC.

could stimulate both cardiac myocyte hypertrophy and cell cycle reentry but not proliferation when compared to similarly treated nontransgenic (NTg) littermates (Xiao *et al*, 2001). To determine if Myc-induced hypertrophic growth was CycD2 dependent, as has been described for Myc-induced cell cycle entry (Bouchard *et al*, 1999; Perez-Roger *et al*, 1999), we created MycER mice deficient for CycD2. Although Myc activation induced CycD2 expression and Cdk2 and 4 activity in MycER myocardium, no upregulation was seen in similarly treated CycD2-null mice (Figure 7A). Treatment of MycER;$CycD2^{+/+}$ mice with 4-OHT for 1 week resulted in a 26.6% increase in HW/BW ratio (4.36 ± 0.17 versus 5.52 ± 0.19, $P < 0.01$; Figure 7B). However, activation of Myc in CycD2-null mice did not result in a significant change in cardiac mass (4.51 ± 0.11 versus 4.53 ± 0.19, P = NS; Figure 7B). Likewise, the 85-fold increase in BrdU-positive nuclei in MycER;$CycD2^{+/+}$ seen after 4-OHT activation of Myc (0.03 ± 0.03 versus 2.54 ± 0.8; $P < 0.01$) was abolished in MycER;$CycD2^{-/-}$ mice (0.04 ± 0.03 versus 0.35 ± 0.11, P = n.s.; Figure 7C and D).

Myc-induced cell cycle reentry but not hypertrophic growth is Cdk2 depend-

To clarify if the lack of hypertrophic growth in MycER; $CycD2^{-/-}$ mice was due to a lack of CycD2 and potentially Cdk4 activity versus the inability to upregulate Cdk2 activity in these hearts, we examined the role of Cdk2 and Cdk4 in Myc-induced hypertrophic growth and protein synthesis *in vitro*. NRVMs infected with an adenovirus overexpressing Myc (AdMyc) increased Cdk2 activity and to a lesser extent Cdk4, similar to its effects *in vivo* (Figure 8A). To block Cdk activity, we coinfected AdMyc cultures with a dominant-negative Cdk2 (Ad-dnCdk2) or Cdk4 (Ad-dnCdk4) virus. These vectors specifically blocked Myc-induced activation Cdk2 or 4 activity (Figure 8A). The effect of these interventions on cardiac myocyte cell size was assessed with forward scatter by flow cytometry. Forced expression of Myc increased NRVM forward scatter from 420.5 ± 103.3 to 474.0 ± 119.8 indicating larger NRVMs. Although inhibition of Cdk2 activity had no effect on Myc-induced hypertrophic growth (470.2 ± 120.7), blocking Cdk4 activity attenuated Myc-induced hypertrophy (439.5 ± 97.7). Similarly, overexpression of Myc induced protein synthesis by 47% as measured by [^{3}H]phenylalanine incorporation, which was not affected by inhibiting Cdk2 activity but was prevented by blocking Cdk4 activity (Figure 8C). In contrast, both Ad-dnCdk2 and Ad-dnCdk4 inhibited the Myc-induced 6.2-fold increase in DNA synthesis (6.23 ± 1.23 versus 1.18 ± 0.4 or 3.64 ± 0.33-fold, $P < 0.01$; Figure 8D), suggesting that while Myc-dependent reactivation of DNA synthesis in cardiac myocytes requires both Cdk2 and 4, Myc-stimulated cell growth is Cdk2 independent.

Discussion

We have previously shown that overexpression of Myc is sufficient to induce cardiac hypertrophy in adult myocardium (Xiao *et al*, 2001). However, whether Myc is also necessary for cell growth and the mechanisms whereby Myc might induce cardiomyocyte hypertrophy remained unknown. In the present study, we investigated Myc's role in cardiac hypertrophy by inactivating Myc both *in vivo* and *in vitro*. The results demonstrate that an Myc–Cyclin D2-dependent

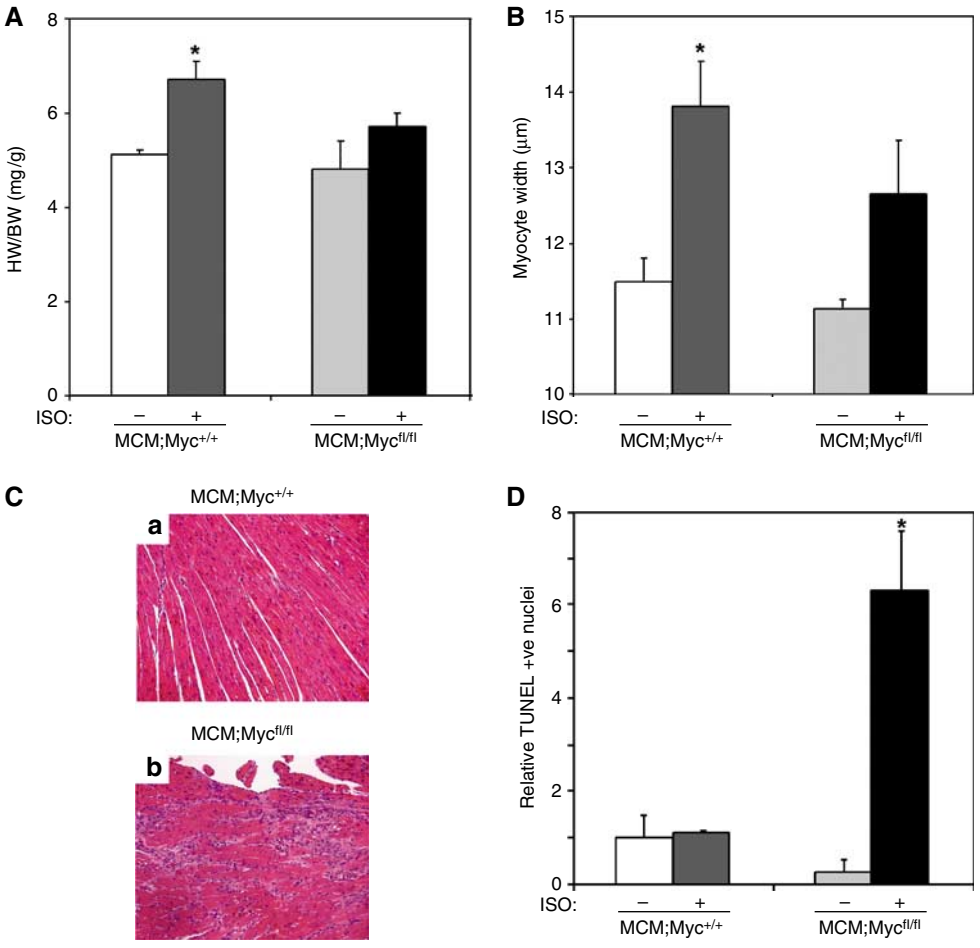

Figure 5 Hypertrophy is attenuated in ISO-stimulated, Myc-deficient hearts. To pharmacologically induce hypertrophy, MCM;Myc$^{+/+}$ and MCM;Myc$^{fl/fl}$ mice were stimulated with ISO for 1 week after 5 days of 4-OHT treatment. (**A**) To assess the hypertrophic response, HW/BW ratios were analyzed. *$P<0.01$ for MCM;Myc$^{+/+}$ + ISO versus MCM;Myc$^{+/+}$ + vehicle and $P<0.05$ versus MCM;Myc$^{fl/fl}$ + ISO ($n=5$ per group). (**B**) To assess cardiac myocyte hypertrophy, cross-sectional myocyte fiber width in hearts were quantified. *$P<0.01$ for ISO-stimulated MCM:Myc$^{+/+}$ versus vehicle-treated MCM:Myc$^{+/+}$ or MCM;Myc$^{fl/fl}$ hearts. (**C**) H&E-stained myocardial sections from ISO-stimulated MCM:Myc$^{+/+}$ (Ca) or MCM;Myc$^{fl/fl}$ (Cb) mice. (**D**) TUNEL + ve cardiac nuclei were quantified and the results expressed relative to vehicle-treated MCM:Myc$^{+/+}$ mice. *$P<0.05$ for ISO-stimulated MCM;Myc$^{fl/fl}$ versus vehicle-treated MCM;Myc$^{fl/fl}$ or ISO-stimulated MCM:Myc$^{+/+}$ hearts.

pathway is required for hypertrophic growth in cardiac myocytes. Deleting Myc in adult myocardium attenuated but did not block cardiac hypertrophy completely. This reduction in cardiac myocyte size was accompanied by a reduction in fetal gene markers normally upregulated in hypertrophic hearts, suggesting that the entire hypertrophic phenotype, not just growth, was suppressed. In contrast, inhibition of Myc in cultured cardiac myocytes completely abolished the hypertrophic response induced by ET-1. One explanation for this divergent response may simply be related to simple technical differences between the two models. ET-1 predominantly stimulates G-protein-coupled, endothelin-A receptors in cardiac myocytes, which activate a downstream signaling cascade mediated by the G(q) heterotrimeric G proteins (Sugden and Clerk, 2005). In contrast, TAC initiates a host of direct and indirect autocrine and paracrine neurohumoral factors, which have been implicated in mediating cardiac growth (Dorn and Force, 2005). It is also possible that the relative role of Myc in the various forms of cardiac hypertrophy explored here varies. Additionally, the time course of the two models differed greatly. The *in vitro* experiments determined the relative importance of Myc to protein synthesis at an early time point when Myc is highly expressed. In contrast, the *in vivo* studies compared hypertrophic responses after 1 and 2 weeks of hemodynamic stress. It is possible that Myc is more important to the initial hypertrophic response and that later redundant mechanisms are able to at least partially compensate. This may explain why the observed attenuation of hypertrophy was greater in the shorter, ISO-stimulated model.

The finding that Myc was important for myocyte survival in hypertrophy was unexpected. Hypertrophic agonists led to a small, but significant increase in apoptosis in Myc-null hearts that resulted in increased interstitial fibrosis. One of the paradoxes facing investigators studying Myc function is the observation that both Myc over- and underexpression results in apoptosis. Of the two, Myc-induced apoptosis has been studied in much more detail (Pelengaris *et al*, 2002). Ectopic expression of Myc sensitizes cells to a wide range of apoptotic stimuli including tumor necrosis factor-alpha (TNFα) (Klefstrom *et al*, 1994) by inducing cytochrome *c* (Morrish *et al*, 2003) release from the mitochondrial inter-

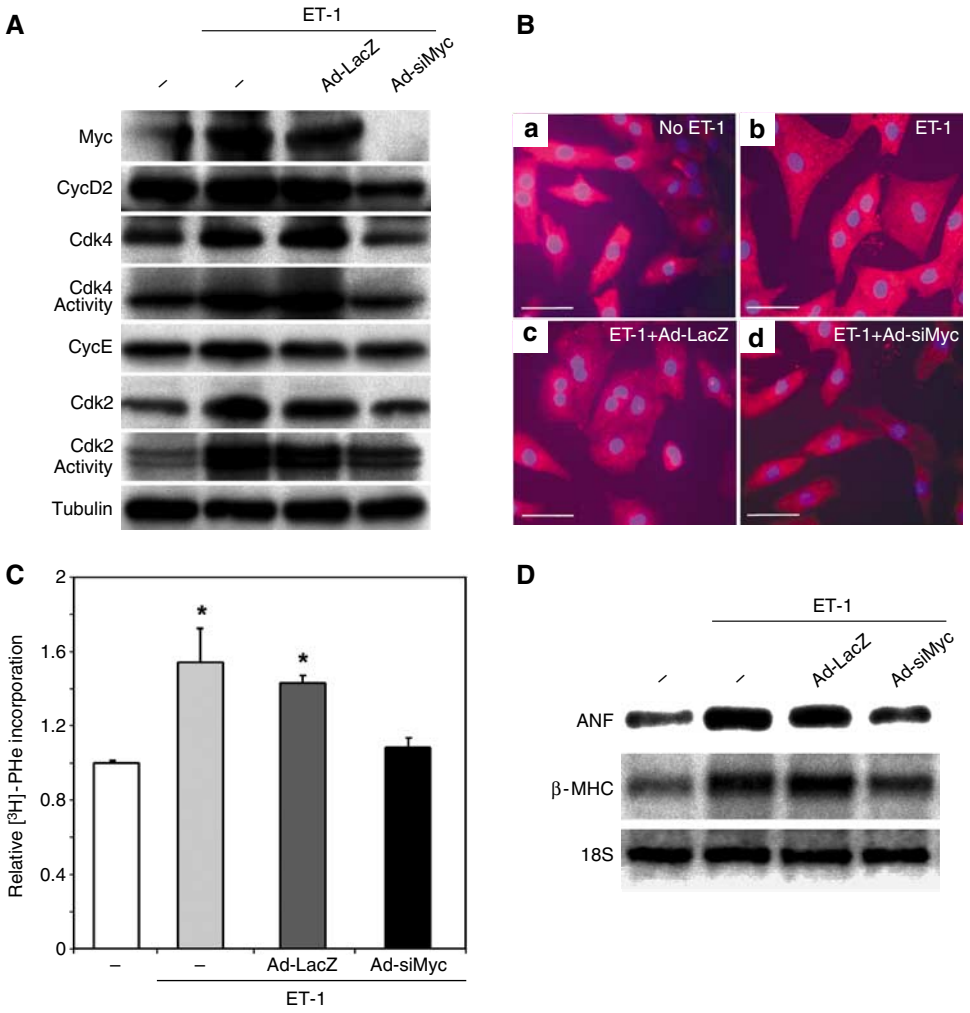

Figure 6 Inhibition of Myc blocks ET-1-dependent hypertrophy *in vitro*. (**A**) NRVMs were infected with no virus (−), AdLacZ or AdsiMyc. Myocytes were stimulated with 100 nM ET-1 for 8 h and protein lysates probed for the indicated proteins or Cdk kinase activities determined and autoradiographs shown. (**B**) To assess the role of Myc on cardiac myocyte cell size, NRVMs were not infected with virus (a, b) or infected with Ad-LacZ (c) or Ad-siMyc (d). NRVMs (b–d) were stimulated with ET-1 for 40 h and then fixed and immunostained with MF20 (red) and DAPI (blue). Scale bar = 50 μm. (**C**) To determine relative protein synthesis, NRVMs were incubated with or without ET-1 for 16 h in the presence of no virus (−), Ad-LacZ or Ad-siMyc and [^{3}H]Phenylalanine incorporation was determined. Results were normalized to uninfected, vehicle-treated cells. *$P<0.01$ for ET-1-stimulated, uninfected or Ad-LacZ-infected NRVMs versus unstimulated or ET-1-stimulated Ad-siMyc cultures. Each experiment was repeated three times. (**D**) Total NRVM RNA (2 μg) from the indicated treatments was probed with specific Dig-labeled probes for atrial natriuretic factor (ANF), β-myosin heavy chain (βMHC) and ribosomal 18S RNA.

membrane space into the cytosol where it can promote apoptosis (Iaccarino *et al*, 2003). The link between Myc deficiency and apoptosis is less clear, but may also be mediated through a mitochondrial pathway. A large proportion of the nuclear-regulated mitochondrial genes are direct Myc targets (Morrish *et al*, 2003), and are induced in cells upon growth stimulation. Ensuring that the cell maintains the correct stoichiometry of respiratory complex subunits and their correct assembly in the mitochondria is critical for adapting to the increased metabolic demands associated with cell growth (Poyton and McEwen, 1996). Disruption of this balance can have detrimental effects, and examples exist of mitochondrial dysfunction that result from both reduced (Schapira and Cock, 1999) or increased expression of genes involved in mitochondrial function, such as adenine nucleotide translocase-1 (Bauer *et al*, 1999) and the mitochondrial hinge protein (Okazaki *et al*, 1998). Therefore, Myc underexpression could lead to mitochondrial dysfunction and apoptosis by deregulating genes involved in mitochondrial function.

A major finding of this study is that a CycD2-dependent pathway mediates Myc-induced hypertrophic growth, although the end effectors remain unclear. It is generally accepted that Cdk2 kinase activation is an essential step in Myc-induced G1-exit and that induction of CycD2 is a critical preliminary step in this process, as the newly formed CycD/Cdk4 complexes sequester Cdk inhibitors p27 and p21 (Bouchard *et al*, 1999; Perez-Roger *et al*, 1999). We found that both Myc-induced cardiac cell cycle entry and cellular growth was blocked in CycD2-null mice. However, in contrast to its effects on cell cycle progression, Myc-induced hypertrophic growth was independent of Cdk2 activity. These results are consistent with the preferential role of Cdk2 in cardiac hyperplasia not hypertrophy, shown by forced expression *in vivo* (Liao *et al*, 2001). Given that Cdk2 is critical for Myc's proliferative effects, whether CycD2 leads to

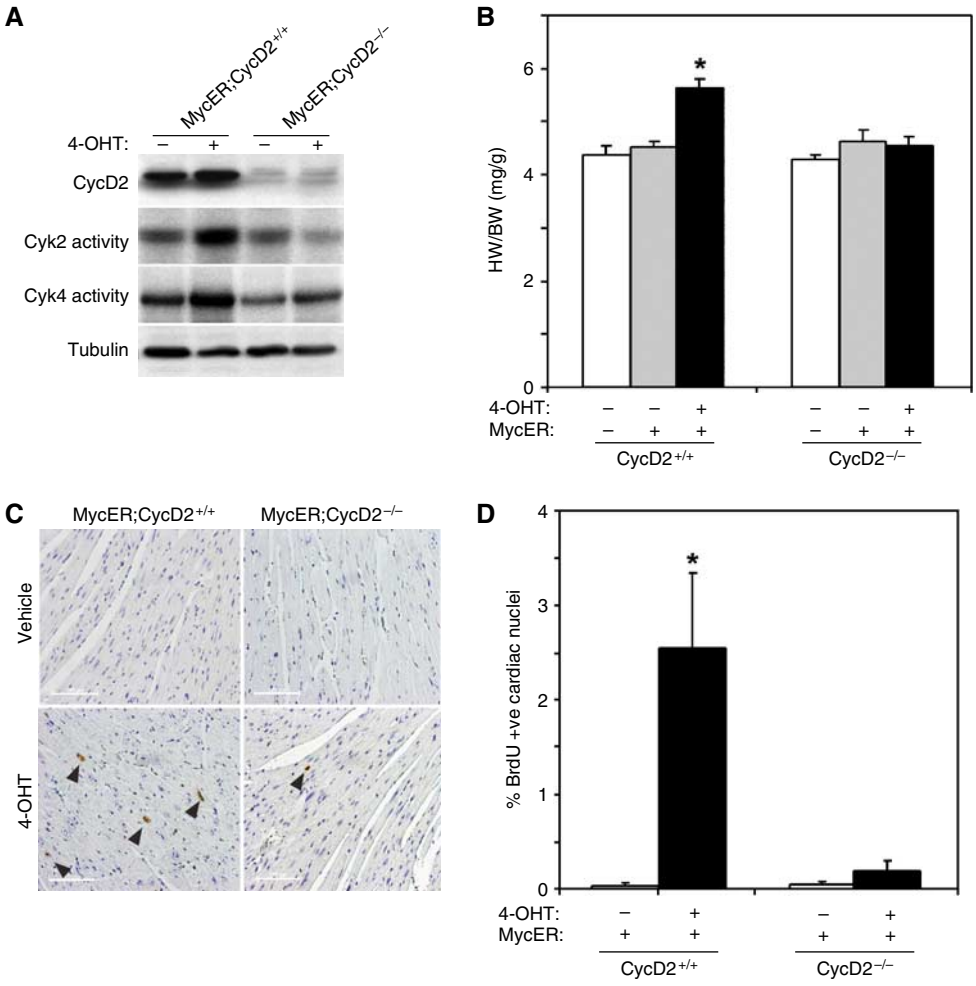

Figure 7 Cyclin D2 is necessary for Myc-induced cell cycle re-entry and hypertrophic growth in the heart. To determine the relative importance of Cyclin D2 to Myc's ability to induce cell cycle re-entry and/or hypertrophic growth in the heart, bigenic MycER;CycD2$^{-/-}$ mice were created. (**A**) Expression of CycD2 determined by Western blotting and Myc-induced Cdk kinase activity in 4-OHT-treated MycER;CycD2$^{+/+}$ versus MycER;CycD2$^{-/-}$ hearts is shown. (**B**) The ratio of HW/BW after 7 days of Myc activation in control (MycER;CycD2$^{+/+}$) or CycD2-null mice (MycERCycD2$^{-/-}$) was determined. *$P<0.01$ for 4-OHT-treated MycER;CycD2$^{+/+}$ versus MycERCycD2$^{-/-}$ hearts. (**C, D**) Myocardial sections from 4-OHT-treated MycER;CycD2$^{+/+}$ or MycER;CycD2$^{-/-}$ were stained for BrdU incorporation (arrowheads identify BrdU + ve cardiac nuclei). The percentage of BrdU + ve nuclei was quantified ($n=5$ per group). *$P<0.05$ for 4-OHT-treated MycER;CycD2$^{+/+}$ versus MycERCycD2$^{-/-}$ hearts. Scale bar = 100 μm.

proliferation or hypertrophy might be dependent on whether excess Cdk inhibitors are present. Thus, the differential growth effect of CycD2 in proliferative versus postmitotic cells may be attributable to a developmental increase in Cdk inhibitors, as is seen in the postnatal heart (Poolman *et al*, 1998). This report represents the first evidence that Myc-dependent hypertrophic growth is also CycD2 dependent and that the pathways controlling cell cycle progression and cellular growth diverge at CycD.

Several lines of evidence suggest that G1 cyclins and Cdks might be critical for the control of cell growth. In *Drosophila*, CycD/Cdk4 stimulates and controls cell growth in postmitotic cells (Datar *et al*, 2000). This CycD/Cdk4-induced cell growth was dependent on a gene encoding the mitochondrial ribosomal protein, mRpL12 (Frei *et al*, 2005). In the absence of mRpL12, cells demonstrated reduced growth and mitochondrial activity suggesting that CycD/Cdk4 controls cell growth via a mitochondrial-dependent pathway. A number of reports have implicated CycD/Cdk4 in regulating cardiac hypertrophy in mammalian cells as well, although the downstream effectors have not been identified (Busk *et al*, 2002; Tamamori-Adachi *et al*, 2002). Likewise, several reports have documented that inhibiting G1-Cyclin/Cdk activity in adult, postmitotic cardiac myocytes can attenuate hypertrophic growth (Tamamori *et al*, 1998; Nozato *et al*, 2001). We recently demonstrated that cardiac hypertrophy is accompanied by increased RNA polymerase (pol) III transcription, which is related to changes in both the activity and level of the RNA pol III-specific transcription factor TFIIIB (Goodfellow *et al*, 2006). Myc can potentiate TFIIIB activity directly by binding and activating transcription (Gomez-Roman *et al*, 2003) or potentially indirectly, by removing the inhibiting effects of the retinoblastoma gene product (Rb). Hypophosphorylated, active Rb binds TFIIIB and prevents its interactions with TFIIIC or RNA pol III (Larminie *et al*, 1997). Myc-induced Cdk activity could potentially remove this inhibitory effect. Given the fundamental role Myc plays in regulating cell growth, it will be critical in future studies to determine the role and mechanisms whereby CycD/Cdk4 might induce cardiac hypertrophy.

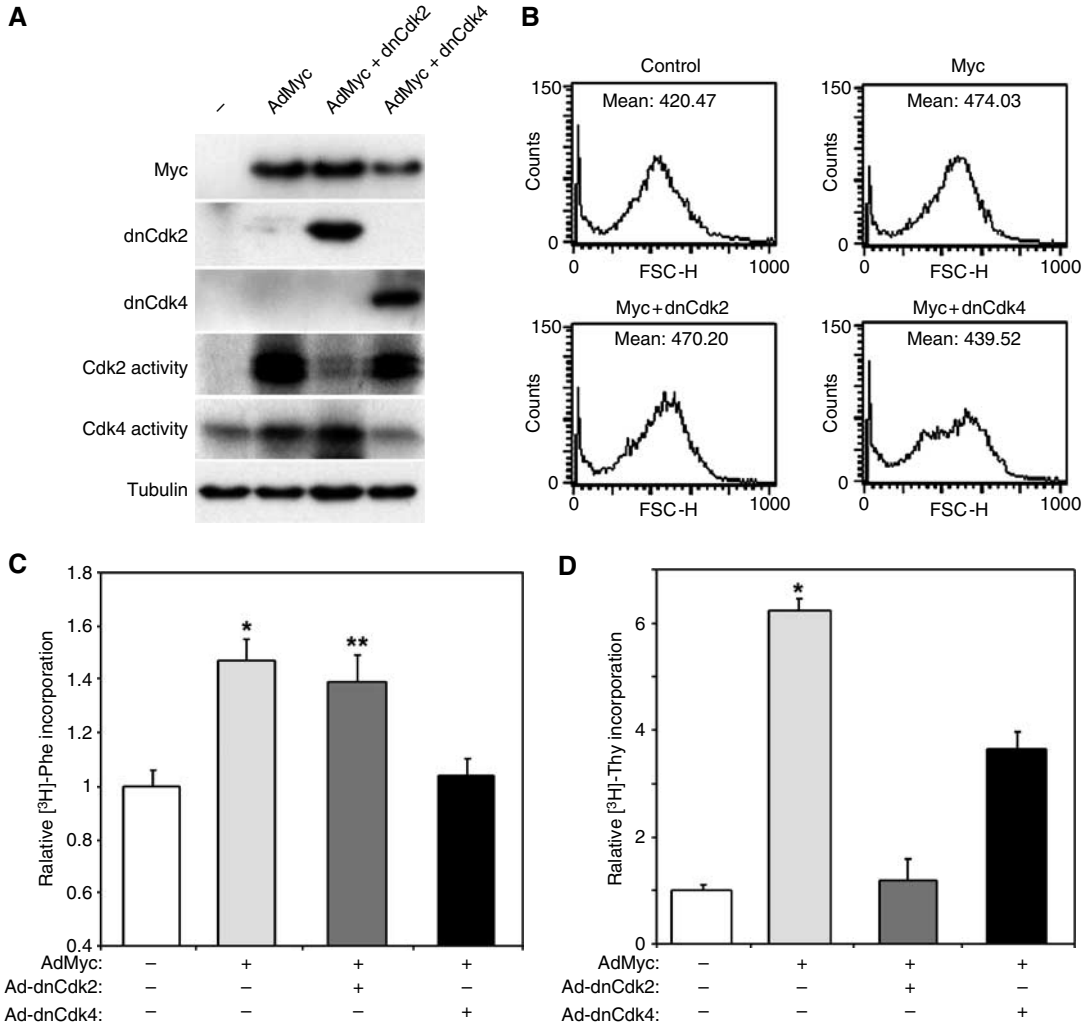

Figure 8 Myc-induced DNA synthesis but not hypertrophy requires Cdk2 activity. To explore the dependence of Myc's growth effects on Cdk2 and 4, we infected NRVM with no virus (−) or AdMyc with or with adenoviruses expressing dominant forms of Cdk2 and Cdk4. (**A**) Expression of infected proteins was confirmed by Western blotting on lysates prepared from NVRMs infected by the indicated vectors. The ability of the dominant negative constructs to specifically inhibit Myc-induced Cdk kinase activity was determined on parallel lysates. (**B**) To assess cardiac myocyte cell size, NRVMs were analyzed by flow cytometry and forward scatter (FSC) was measured. Histograms of FSC in a representative experiment of NRVMs infected by the indicated viruses are shown. The mean FSC is shown for each condition. (**C**) Relative protein synthesis was determined by [^{3}H]Phenylalanine incorporation of myocytes infected with the indicated adenoviral vectors. Results are presented relative to uninfected cultures. *$P<0.001$ AdMyc versus uninfected cultures or AdMyc + dnCdk4. **$P<0.01$ for AdMyc + dnCdk2 versus uninfected cultures or AdMyc + dnCdk4. (**D**) Relative DNA synthesis was determined by [^{3}H]thymidine incorporation of NRVMs infected with the indicated adenoviral vectors. *$P<0.0001$ for AdMyc versus uninfected cultures, $P<0.001$ versus AdMyc + dnCdk2 and $P<0.05$ versus AdMyc + Ad-dnCdk4.

Materials and methods

Transgenic mice and animal studies

The inducible α-myosin heavy chain (α-MHC)-MycER transgenic mice have been described (Xiao *et al*, 2001). Myc-floxed mice (Myc$^{fl/fl}$) were provided by Dr F Alt and genotyped as described (de Alboran *et al*, 2001). The tamoxifen-inducible MerCreMer (MCM) mice were generated by Dr J Molkentin under the control of the α-MHC promoter (Sohal *et al*, 2001). To activate Myc or Cre in these transgenic mice, 1 mg of 4-OHT (Sigma) dispersed in peanut oil by sonication was injected intraperitoneally daily. Control littermates were injected with peanut oil alone. To induce excisional recombination of Myc, mice were treated with 4-OHT for 5 days. To identify recombination, PCR conditions were chosen for ΔMycfl primers that do not amplify the >2.5 kb fragment in wild-type mice and no product is obtained. CycD2-deficient mice were a kind gift from Dr P Sicinski (Sicinski *et al*, 1996). All mice were maintained on a C57 (MycER, CycD2) or FVB (MCM;Myc$^{fl/fl}$) background. Animals were handled in accordance with institutional guidelines.

For TAC, a fixed pressure overload was obtained by surgically constricting the transverse aorta, as has been previously described (Rockman *et al*, 1994). Age-matched Sham-operated animals underwent the identical surgical procedure, except that the aortic constriction was not placed. For the ISO infusion model, 7-day osmotic minipumps (Alzet, model 2001) loaded with 0.2 ml of ISO (28 μg/ml per 25 g body weight) were implanted into the subcutaneous space of 10-week-old mice via a small intrascapular incision.

Cell culture and adenovirus preparation

NRVM were prepared as previously described (MacLellan *et al*, 2000). For all cell culture experiments, NRVM were serum-starved in serum-free DMEM for 36 h before use. Construction of AdMyc (Mitchell and El-Deiry, 1999), dnCdk2 and dnCdk4 (Ferguson *et al*, 2000) has been previously described. Viruses were propagated and titered according to established protocols (MacLellan *et al*, 2000).

To create an siRNA adenovirus to Myc, a shuttle plasmid was constructed by inserting a 21-mer RNA oligonucleotide directed

against the rat and mouse Myc sequence (5′-aagaggcggacacac aacgtc-3′) into the unique *Apa*I and *Hin*dIII sites of pDC.silencer(U6), which was kindly supplied by Dr Abdellatif (Yue *et al*, 2004). Adenoviruses were propagated and titered according to published protocols (MacLellan *et al*, 2000). For cell culture experiments, NRVM were infected with adenoviruses 36 h before treatment.

Protein analysis

Western blots were performed on protein extracts from whole ventricles, according to established protocols (MacLellan *et al*, 2000). Antibodies were obtained from Santa Cruz Biotechnology, Inc. unless otherwise noted. Protein expression was visualized using horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents (Amersham Biosciences). Immune complex kinase assays for Cdk2 and Cdk4 activity were performed on 500 μg of ventricular lysates (Li *et al*, 1998). The immunocomplexed pellet was incubated in 30 μl kinase buffer with 1 μg of Rb (Santa Cruz Biotech.) or histone H1 (Upstate Biotech, Inc.) substrate, 5 μCi of [γ-^{32}P]ATP, 1 mM DTT and 5 μM ATP and then electrophoresed through 10% acrylamide gels.

Histology and morphometric analysis

Hearts were fixed overnight in 4% paraformaldehyde buffered with PBS and routinely processed. BrdU labeling was achieved by injecting 50 mg of BrdU per gram of body weight intraperitoneally. To identify DNA synthesis, paraffin-embedded sections were probed with antibodies against BrdU (Zymed). Antibody against sarcomeric myosin heavy chains (MF20; Developmental Hybridoma Studies Bank) and diamidinophenolindole (DAPI) were used according to the manufacturer's instructions. Apoptosis was determined using ApopTag fluorescein *in situ* apoptosis detection kit (Chemicon) or TACS^{TM} 2 TdT blue label *in situ* apoptosis detection kit (Trevigen). Apoptotic cells were detected with fluorescein conjugate or visualized with an enzymatic reaction using the TUNEL method. The apoptosis rates were determined by examining >2500 cardiac nuclei per heart sections. Secondary antibodies were purchased from Molecular Probes. Myocyte fiber widths were measured on perfusion-fixed myocardial sections using a computerized morphometric system (SigmaScan, Systat Inc.). All myocytes were measured at the same magnification with the observer blinded to the genotype of the animals.

Northern blot analysis

Total RNA was isolated from ventricles using RNA STAT 60 Kit (Tel-Test, Inc.). Northerns blots were performed according to established protocols using radioactive (MacLellan *et al*, 2000) or digoxigenin (DIG)-labeled probes (MacLellan *et al*, 2000). The oligonucleotide and cDNA probes used have been reported (Ross *et al*, 1998).

Measurement of myocyte cell number, cell size, DNA and protein synthesis

To determine myocyte cell number, DNA and protein synthesis, serum-starved myocytes were infected with the indicated adenoviral vectors (50 PFU/myocyte). To determine cell number, myocytes were trypsinized and total cell counts determined using a Coulter Counter (Becton-Dickinson). To estimate protein synthesis, myocytes were labeled with 5 μCi/ml of [^{3}H]phenylalanine (Amersham Corp.) for 4 h after which cell precipitates were solubilized and counted. To determine the relative DNA synthesis, myocytes were cultured in media containing 5 μCi/ml of [^{3}H]thymidine (ICN) for 6 h and thymidine incorporation quantified. Flow cytometry to assess cell size was performed at the UCLA Flow Cytometry Laboratory based on forward scatter as previously described (Gylys *et al*, 2004). Aliquots of 10^5 cells in 500 μl 1 × PBS were dispensed into small conical tubes (Falcon). At least 10 000 events were collected before analysis. All samples were analyzed using a Becton Dickinson FACScan analytic flow cytometer (Becton Dickinson) with FCS Express software (DeNovo).

Statistical analysis

All data are presented as mean ± s.e.m. except results of forward scatter, which are presented as mean ± s.d. Results were compared by analysis of variance and Fisher's PLSD tests, using significance at a P-value <0.05.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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