MyoD Can Induce Cell Cycle Arrest but not Muscle Differentiation in the Presence of Dominant Negative SWI/SNF Chromatin Remodeling Enzymes

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running title: Cell cycle arrest is independent of SWI/SNF
Summary

Cell cycle arrest is critical for muscle differentiation and the two processes are closely coordinated but temporally separable. SWI/SNF complexes are ATP dependent chromatin remodeling enzymes that have been shown to be required for muscle differentiation in cell culture and have also been reported to be required for Rb mediated cell cycle arrest. We therefore looked more closely at how SWI/SNF enzymes affect the events that occur during MyoD induced myogenesis, namely, cell cycle regulation and muscle specific gene expression, in cells that inducibly express dominant negative versions of Brahma (BRM) and Brahma related gene 1 (BRG1), the ATPase subunits of two distinct SWI/SNF complexes. Although dominant negative BRM and BRG1 inhibited expression of every muscle specific regulator and structural gene assayed, there was no effect on MyoD-induced activation of cell cycle regulatory proteins and thus cells arrested normally. In particular, in the presence or absence of dominant negative BRM or BRG1, MyoD was able to activate expression of p21, cyclin D3, and Rb, all of which are critical for cell cycle withdrawal in the G1/G0 phase of the cell cycle. These findings suggest that at least one basis for the distinct mechanisms that regulate cessation of cell proliferation and muscle specific gene expression during muscle differentiation is that SWI/SNF mediated chromatin remodeling enzymes are required only for the latter.
**Introduction**

Mammalian SWI/SNF enzymes alter chromatin structure in an ATP-dependent manner and can facilitate the interaction of the transcriptional machinery with nucleosomal DNA in vitro (1-3). In vivo, SWI/SNF enzymes have been implicated in gene regulation by nuclear hormone receptors (4-9), E2F (10), c-MYC (11), c-fos/c-jun heterodimers (12) and of the hsp70 (13,14), interferon-B (15), CD44 (16), and c-fos (17) genes. More recently, SWI/SNF enzymes have been linked to developmentally regulated transcription, including activation of erythroid (18-20), myeloid (21), and muscle-specific (22) gene expression.

Skeletal muscle differentiation involves the activation of skeletal muscle genes by the myogenic proteins (23-25). The myogenic helix loop helix (bHLH) family of transcription factors (MyoD, Myf 5, myogenin, and Mrf 4) heterodimerize with ubiquitously expressed E proteins to bind a consensus E box found in the regulatory regions of many muscle specific genes (26,27). These heterodimers cooperate with members of the myocyte enhancer factor 2 (Mef 2) family to activate myogenesis (28,29). Ectopic expression of the bHLH proteins into non-muscle cells can activate transcription of muscle specific genes (30). Genetic and biochemical evidence indicate that MyoD and Myf 5 establish the myogenic lineage while myogenin promotes terminal differentiation (31-36). MyoD and Myf 5 are expressed in dividing myoblasts which must exit the cell cycle for muscle specific genes to be activated.

Exit from the cell cycle is accomplished by the down-regulation of cyclins except for cyclin D3, which is upregulated during muscle differentiation and contributes to cell cycle withdrawal (37,38). MyoD activity can also promote cell cycle arrest by induction of the cyclin-dependent kinase (CDK) inhibitor, p21, which along with the other members of the CIP1/KIP1 family, p57 and p27, inhibit a wide range of CDK enzymes (39-41). The presence
of p21 or p57 is essential for muscle differentiation during embryonic development in mice (42). Ectopic expression of p21 can activate muscle gene expression even in high concentrations of serum (37).

The activity of cell cycle regulatory proteins is important for coordinating myogenesis with arrest in the G0/G1 phase of the cell cycle, however muscle differentiation and cell cycle arrest are not concomitant events. Introduction of MyoD in transformed and tumor cell lines inhibits growth even when muscle specific genes are not activated (43). Similarly, a MyoD construct in which the basic domain was substituted with the corresponding domain from the E12 transcription factor could inhibit growth but could not activate differentiation (44).

Differentiation of C2C12 myoblasts is an ordered process such that expression of myogenin precedes expression of p21, which is then followed by activation of the muscle structural genes with the subsequent appearance of the contractile phenotype and lastly cell fusion. Induction of p21 correlates with the postmitotic state and failure to reinitiate DNA synthesis upon stimulation with growth factors (45).

Expression of Rb is also upregulated by MyoD and is critical for both activation of muscle specific gene expression and cell cycle withdrawal. Rb activates the Mef 2 factors that are necessary for expression of the muscle specific genes, and it prevents cell cycle progression by repressing the E2F family of transcription factors (46). The activity of RB in turn is modulated by the cyclins and their CDK partners. Recent work has demonstrated that the ATPase chromatin remodeling activity of SWI/SNF is required for Rb mediated repression of cyclin A and possibly cyclin E and arrest in the G1 phase of the cell cycle (47,48).

Activation of myogenesis also involves the reorganization of repressive chromatin structure on previously silent muscle specific loci and requires SWI/SNF enzymes. Expression
of dominant negative versions of the mammalian SWI/SNF ATPase subunits, BRG1 and BRM, abolished the ability of MyoD to activate transcription of two muscle specific genes, myogenin and myosin heavy chain. Inhibition of myogenin gene expression was correlated with suppression of chromatin remodeling in the myogenin promoter region (22). To better understand the role of SWI/SNF chromatin remodeling enzymes during muscle differentiation, we looked at the effects of dominant negative BRG1 and BRM on expression of a number of other skeletal muscle markers and cell cycle regulatory proteins. We found that in the absence of functional SWI/SNF, MyoD can promote normal cell cycle regulation but not the expression of skeletal muscle markers. Though MyoD mediated cell cycle arrest is coordinated with activation of muscle specific genes, the two processes are temporally separable. We show here that at least one basis for the distinct mechanisms that regulate cessation of cell proliferation and muscle specific gene expression is that SWI/SNF mediated chromatin remodeling is required specifically for the induction of muscle specific genes.
Materials and Methods

Cell Culture

Cells were grown as previously described (14). Dominant negative BRG1 and BRM expression were induced by passing cells in media lacking tetracycline and differentiated as described (22).

Protein Extracts and Western Analysis

Isolation of proteins and western blotting were as described (14). Antibodies against the flag epitope, p27, cyclin A, cyclin E (M20), RB (C15), and rel B were from Santa Cruz. The anti-cyclin D1 and D3 antibodies were from Pharmingen/Signal Transduction Laboratories.

Northern Analysis

Total cellular RNA was isolated by Trizol (Invitrogen) as described by the manufacturer. For Northern analysis, 10 to 20 µg of total cellular RNA was electrophoresed on a 1% formaldehyde gel and transferred to Nytran Plus (Schleicher and Schuell). The membranes were baked for 2 hours and prehybridized for at least 2 hours at 42°C in 6X SSC, 50% formamide, 50mM NaPO_4_, pH 6.8, 5X Denhardt's solution, 0.5% SDS, and 100ug/ml salmon sperm DNA (not boiled). The buffer was then changed to 6X SSC, 50% formamide, 50 mM NaPO4, pH 6.8, 1X Denhardt's solution, 0.1% SDS, and 100ug/ml salmon sperm (which was first boiled with a ³²P labeled probe generated by random priming) and membranes were hybridized overnight at 42°C. Probes for myogenin and myosin heavy chain were described (22). A 1.7Kb Xho I restriction fragment was used to detect p21, a 0.7Kb EcoR I restriction
fragment was used to detect myosin light chain, a 0.9Kb Pst I restriction fragment was used to
detect troponin T, and a 700 bp EcoR I-Hind II, restriction fragment was used to detect
GAPDH. Blots were washed sequentially in 2X SSC, 0.1%SDS at room temperature, then in
0.2X SSC, 0.1% SDS at 42°C, and were exposed to a PhosphorImager screen and analyzed
with ImageQuant.

**RT PCR**

Total RNA (1µg) was reversed transcribed with Mo-MuLV reverse transcriptase (Invitrogen)
and a random hexamer (Amersham -Pharmacia) as described (49). 2µl of cDNA was
amplified by PCR with Taq polymerase (Promega) (1 cycle: 95°C/5min, 23 cycles: 95°C/1min,
62°C/30sec, 72°C/30sec). Primers for MyoD, α-skeletal actin, desmin and Mef 2C with the
sizes of the corresponding PCR products were described (49). For p57, a 359 bp PCR product
was amplified using 5'- ATCCAGAC GCAGGAGCCGTCCATCA-3' and 5'-
CCGGCGGCCCAGAACGCGGGATGA-3'. A 500 bp HPRT PCR product was amplified
with 5'-GTTCTTTGCTGACCTGCTGGAT-3' and
5'ATGGCCACAGGAACGACCTGC-3'. For Rb, a 410 bp PCR product was amplified
with 5'-CTTGGCTATTTGGAGAA-3' and 5'- ATCTTCCATCTGTAATACTT-3'. PCR
conditions for Rb were slightly different (1 cycle:95°C/5min, 23 cycles:95°C/1min, 50°C/1min,
72°C/30sec). PCR products were electrophoresed on a 6% nondenaturing polyacrylamide gel.
The gels were dried and exposed to a PhosphorImager screen.
FACs Analysis

Cells were grown and differentiated as described (14,22) and then fixed as described (50).

Incorporation of propidium iodide was determined by flow cytometry.
Results and Discussion

Transcription of muscle specific genes is inhibited by dominant negative BRM and BRG1

Mammalian SWI/SNF complexes contain either the Brm or Brg1 ATPase subunit. When overexpressed in cells, BRM or BRG1 with mutations in the ATPase domains act as dominant negatives and have been shown to inhibit gene activation events that normally require SWI/SNF function. Stable cell lines in which the genes encoding flag tagged dominant negative BRM or BRG1 placed under control of the Tet-VP16 activator were previously described (14). These cells were grown in the presence or absence of tetracycline for 96 hours, then infected with a retrovirus containing the MyoD gene and forced to differentiate in low serum conditions. In the presence of tetracycline, when dominant negative BRM or BRG1 is not expressed, the expression of two muscle specific genes, myosin heavy chain and myogenin, was activated. However, when these cells were differentiated in the absence of tetracycline, dominant negative BRM and BRG1 inhibited activation of these muscle specific markers (22). This indicated that SWI/SNF complexes play an important role in muscle differentiation. To determine how extensively SWI/SNF is required for muscle differentiation, we looked at the effect of dominant negative BRG1 and BRM expression on a number of muscle specific genes. Fig. 1A is a Northern analysis showing muscle specific gene expression in H17 cells, which inducibly express dominant negative hBRM, and B22 cells, which inducibly express dominant negative BRG1. We observed that myosin heavy chain, myosin light chain and troponin T gene expression was induced by MyoD when cells were grown and differentiated in the presence of tetracycline. When cells were grown and differentiated in the absence of tetracycline, dominant negative protein expression inhibited activation of these genes.
Activation of these muscle specific markers was not inhibited when a control Tet-VP16 cell line, which expresses only the Tet-VP16 activator, was grown and differentiated in the absence of tetracycline. Interestingly, expression of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was upregulated during myogenesis but was not affected by dominant negative BRM and BRG1. RT-PCR of two additional muscle specific markers shows that α-skeletal actin and desmin expression is also inhibited by dominant negative BRM and BRG1 (Fig. 1B). The levels of flag tagged dominant negative BRM and BRG1 expression in this experiment were detected by Western analysis (Fig. 1C).

Muscle differentiation involves the activity of the basic helix loop helix family of transcription factors, which heterodimerize with E proteins to bind E boxes on the promoters of muscle specific genes, and the Mef2 family of transcription factors, which bind AT rich sequences on these promoters. We previously showed that myogenin expression is induced by MyoD and inhibited by expression of dominant negative BRM and BRG1 in H17 and B22 cell lines respectively (Fig. 2A and (22)). Fig 2B shows the expression levels of the other bHLH factors and Mef2C. RT-PCR detected Myf 5 and Mrf 4 in C2C12 myotubes but showed that they were not induced by MyoD in our NIH 3T3 fibroblast derived cells. Introduction of MyoD into 10T1/2 fibroblasts also does not induce expression of Myf 5 or Mrf 4 (51). Ectopic expression of MyoD does not activate the endogenous MyoD gene in NIH 3T3 cells (52), therefore, MyoD levels were constant in the presence or absence of dominant negative BRM and BRG1. Although levels of Mef 2C expression in our cell lines is much lower than in C2C12 myoblasts, Mef2C was upregulated during myogenesis in the presence of tetracycline when dominant negative BRM or BRG1 was not expressed and was not activated in the presence of the dominant negative proteins (Fig. 2B). Taken together, these results indicate
that SWI/SNF plays an extensive role in MyoD mediated myogenesis and is critical for the
expression of muscle specific regulatory and structural proteins.

**Cell cycle arrest occurs in the presence of dominant negative BRM and BRG1**

Terminal cell cycle arrest is closely coupled to muscle differentiation and is required for
activation of muscle specific gene expression. To determine if SWI/SNF plays a role in cell
cycle withdrawal, we used FACs analysis to test the ability of cells to cell cycle arrest when
differentiated in the presence or absence of dominant negative BRM or BRG1. DNA content
was measured by propidium iodide staining to determine the number of cells in different phases
of the cell cycle. Dominant negative BRM and BRG1 did not affect the ability of cells
expressing MyoD to arrest in the G1 phase of the cell cycle (Fig. 3). Interestingly, even the
mock differentiated cells, which received no MyoD, arrested regardless of whether functional
SWI/SNF complexes were present. Under the in vitro differentiation protocol, both the
differentiated and mock differentiated cells became confluent in growth medium and then were
subjected to an additional 36 hours in low serum conditions. The results indicate that cell cycle
arrest under such conditions does not require functional SWI/SNF complexes.

These data differ from other reports that have shown that SWI/SNF complexes were
required for pRb mediated cell cycle arrest (47,48). In those studies, proliferating cells
expressing alleles of pRb that could not be phosphorylated, and thus are constitutively active,
did not arrest unless functional BRG1 was present. Similar results were obtained when the
cyclin dependent kinase (CDK) inhibitor p16/ink4a was expressed. In addition, proliferating
cells expressing dominant negative BRG1 did not arrest when treated with cisplatin, a DNA
damaging agent, whereas cisplatin treatment of proliferating cells grown in the presence of
tetracycline (dominant negative off) did undergo cell cycle arrest. Clearly, the experiments presented in Figure 3 differ methodologically from those described in earlier reports. We suggest that while BRG1 and SWI/SNF complexes are required for cell cycle arrest under some conditions, they are not required universally. To further address the role of SWI/SNF complexes in MyoD mediated cell cycle withdrawal and muscle differentiation, we looked more closely at whether dominant negative BRM and BRG1 affect the levels of cell cycle regulatory proteins.

**p21, Cyclin and Rb expression are unaffected by dominant negative BRM and BRG1**

One class of cell cycle regulators, the CDK inhibitors, is generally upregulated during cell differentiation and may play a general role in promoting and maintaining the terminally differentiated phenotype by binding to and inactivating cyclin-CDK complexes (reviewed in (53)). The CIP1/KIP1 family includes p21, p57, and p27, all of which have been implicated in cell cycle withdrawal during muscle differentiation. p21 and p57 mRNAs are both induced during differentiation in C2C12 cells and the presence of one of the two genes is required for myogenesis in mice (42,54,55). We observed that MyoD strongly induced p21 expression and that dominant negative BRM or BRG1 had no effect on its expression (Fig. 4A). RT-PCR showed that p57 mRNA was not expressed in our cells (Fig. 4B). This is consistent with previous reports that showed p57 is not activated by MyoD in 10T1/2 fibroblasts (54). p27 protein levels were similar in mock and MyoD differentiated cells and also were unaffected by expression of dominant negative BRM or BRG1 (Fig. 4C). High levels of p27 have been
associated with density arrest during adipocyte differentiation (56). In our cell lines, both mock and MyoD differentiated cells are confluent and so accumulate similar levels of p27.

Cyclin levels are generally downregulated during differentiation. One exception is cyclin D3, which was previously shown to be transcriptionally upregulated during muscle differentiation and which contributes to irreversible withdrawal from the cell cycle by forming inactive complexes with unphosphorylated Rb, cdk4, p21, and PCNA (38). We therefore looked at whether dominant negative BRM and BRG1 could inhibit induction of cyclin D3 during muscle differentiation by MyoD. Fig. 5A shows that cyclin D3 protein levels were upregulated by MyoD during differentiation in the presence or absence of tetracycline. Cyclin E levels during differentiation can vary depending on cell type and the presence of extracellular factors (reviewed in (57)). In differentiated C2C12 cells, cyclin E was reported both to remain constant and to decline and to associate with p21 to form inactive complexes during differentiation (39,58,59). In our experiments, cyclin E protein levels were higher in MyoD differentiated cells than in mock differentiated or proliferating cells and were unaffected by expression of dominant negative BRM and BRG1 (Fig. 5A). The increase in cyclin E levels may reflect increased protein stability due to the increase in p21 levels. The hypophosphorylated state of Rb in these cells (Fig. 5C) suggests the cyclin E is inactive.

Our results indicate that SWI/SNF complexes are not required for the normal expression of the CIP1/KIP1 CDK inhibitors or cyclins D3 or E under these differentiation conditions. MyoD activates expression of muscle specific markers and cell cycle regulators to coordinate differentiation with cell cycle arrest. However, dominant negative BRM and BRG1 only inhibit activation of the muscle specific genes, suggesting that the induction of differentiation is distinct from the induction of cell cycle arrest. The MyoD mediated induction of p21 and cyclin
D3 may be an explanation for the ability of these cells to arrest when functional SWI/SNF enzymes are compromised.

Cyclin D1, in association with cdk4, plays a key role in progression through the G1 phase of the cell cycle, thus, the levels of cyclin D1 are downregulated during muscle differentiation. Forced expression of cyclin D1 is correlated with phosphorylation of MyoD and inhibition of muscle specific gene activation ((37); reviewed in (57)). Cyclin D1 was present at low to undetectable levels in both mock and MyoD differentiated cells in the presence or absence of dominant negative BRM or BRG1 expression. The levels in these growth arrested cells is contrasted to the higher levels present in proliferating cells (Fig. 5A). Cyclin A is also downregulated when cells exit the cell cycle and its forced expression results in phosphorylation of pRb and in inhibition of muscle differentiation (60). Fig. 5A shows that cyclin A levels were low during growth arrest, even in the presence of the dominant negative proteins. Rb mediated down regulation of cyclin A during cell cycle arrest requires BRG1 (47,48). However, our data demonstrates that in both the mock differentiated as well as the MyoD differentiated cells, cyclin A is downregulated even in the presence of non-functional SWI/SNF enzymes. As discussed above, this suggests that the requirement for SWI/SNF complexes during cell cycle arrest induced by different experimental conditions is not absolute. Instead, the multiple signals received by confluent cells placed under low serum conditions may overcome or bypass the need for SWI/SNF enzymes during exit from the cell cycle. In conclusion, it appears that there are likely to be multiple mechanisms for achieving cell cycle withdrawal, some of which do not require SWI/SNF remodeling enzymes.

Rb has been shown to interact with MyoD and to promote muscle gene activation and cell cycle arrest (61). Rb has also been demonstrated to interact with BRM and BRG1 and has
been implicated in both cell cycle arrest and induction of gene expression by the glucocorticoid receptor (7,47,48,62,63). In C2C12 cells, MyoD upregulates Rb gene expression (64). The phosphorylation state of Rb is also regulated during the cell cycle and during differentiation. Cells in G1 contain mostly the hypo-phosphorylated form while cells approaching S phase accumulate the hyperphosphorylated form of Rb. Cells lacking Rb are competent for induction of early differentiation markers and for cell cycle arrest but fail to induce late muscle specific marker genes (65). We therefore examined Rb mRNA and protein levels in MyoD differentiated cells. Fig. 5B shows that Rb mRNA levels were induced in the MyoD differentiated cells and that dominant negative BRM and BRG1 did not inhibit activation. Fig. 5C shows that the corresponding increase in pRb protein levels and the phosphorylation state of Rb in MyoD differentiated cells also were not affected by dominant negative BRM or BRG1.

Withdrawal from the cell cycle during myogenesis is intricately coordinated with activation of muscle specific gene expression, yet the two events are separable. Here, we demonstrate that functional SWI/SNF chromatin remodeling complexes are required for the induction of muscle specific regulators and structural genes but are not required to induce the expression of a number of cell cycle regulators associated with cell cycle arrest. Thus cells expressing non-functional SWI/SNF complexes can withdraw from the cell cycle upon MyoD mediated muscle differentiation.

Existing data suggests that muscle regulatory factors and cell cycle regulators cooperate to promote muscle differentiation. In most models, MyoD and/or Myf5 stimulate the expression of "early" differentiation markers such as myogenin, MEF2 and P21. This step in the differentiation process promotes cell cycle withdrawal and can occur in the absence of pRb (65). However, additional functions mediated by pRb, including activating the transcriptional
competence of the MEF2 activators, help promote the induction of "late" differentiation markers (46). The involvement of SWI/SNF chromatin remodeling enzymes during MyoD mediated muscle differentiation does not appear specific for either the "early" or "late" differentiation events. Instead, we suggest that the requirement for SWI/SNF complexes is likely traced to its enzymatic properties that enable it to alter chromatin structure. Genes that are off or that require remodeling of chromatin at or near promoter sequences to be upregulated will be dependent on SWI/SNF enzymes for expression during muscle differentiation. In contrast, genes that are upregulated without requiring ATP dependent structural changes in chromatin at or near promoter sequences will be independent of the function of SWI/SNF and related enzymes. According to this hypothesis, we would predict that genes that are off prior to muscle differentiation, such as myogenin, muscle heavy and light chains, troponin T, desmin and α-actin, would require SWI/SNF mediated chromatin remodeling to induce gene expression. In fact, we have previously shown that failure to induce myogenin expression in the absence of functional SWI/SNF complexes correlates with inhibition of chromatin remodeling at the endogenous myogenin promoter (22). Additionally, we would predict that a gene such as Mef2C, which is on in undifferentiated and mock differentiated cells but is upregulated in MyoD differentiated cells, would also require SWI/SNF mediated remodeling of its promoter structure in order for expression to be induced. In contrast, our hypothesis predicts that upregulation of p21, cyclin D3, and pRb does not involve SWI/SNF mediated changes in promoter structure. A schematic model indicating the SWI/SNF dependency of different steps leading to muscle differentiation is presented in Figure 6. Analysis of the endogenous promoter structure of these and other genes, before and after differentiation and in the presence and absence of functional SWI/SNF enzymes, should permit us to directly test our hypothesis.
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Figure Legends

Fig. 1: MyoD-mediated activation of muscle specific gene expression is inhibited by dominant negative BRM and BRG1. Control cell lines (Tet-VP16) or cell lines that express dominant negative BRM (H17) or that express dominant negative BRG1 (B22) were grown in the presence or absence of tetracycline and then cultured in differentiation media in the presence or absence of tetracycline. Cells were either mock differentiated or differentiated by infection with a retrovirus encoding MyoD (65). (A) Northern blot showing the mRNA levels of myosin heavy chain, myosin light chain, troponin T, and GAPDH. Ethidium stained rRNA is shown as a loading control. (B) RT-PCR of α-skeletal actin and desmin mRNA. HPRT levels serve as a loading control. (C) Western blot showing the presence of the flag-tagged dominant negative BRM or BRG1 when Tet-VP16, H17 and B22 cells, respectively, were grown and differentiated in the absence of tetracycline.

Fig. 2: Expression of muscle specific regulators during MyoD mediated muscle differentiation. Tet-VP16, H17, and B22 cell lines were grown and differentiated in the presence or absence of tetracycline. (A) Northern blot showing myogenin mRNA levels and total rRNA. (B) RT-PCR of Mrf 4, Myf 5, MyoD, Mef2C, and HPRT mRNA in each cell line and in C2C12 myotubes. The levels of flag-tagged dominant negative BRM and BRG1 are shown in Fig. 1C.

Fig. 3: FACs analysis of differentiated and proliferating Tet-VP16, H17, and B22 cells. Tet-VP16, H17, and B22 cell lines were either mock differentiated, or differentiated with MyoD in the presence or absence of tetracycline. As a control, each cell line was also cultured in growth media to obtain data for proliferating cells. Data was analyzed by the Modfit software package.
and is shown for MyoD differentiated cells and for proliferating cells. The bar graphs on the right show the percentage of cells in S phase and represent the averages of three to four differentiation experiments. M, mock differentiated; D, differentiated.

Fig. 4: Expression of cyclin dependent kinase inhibitors during MyoD mediated muscle differentiation. Cells were grown and either mock differentiated or differentiated with MyoD in the presence or absence of tetracycline. (A) Northern blot showing p21 mRNA levels with the corresponding ethidium stained rRNA as a loading control. (B) RT-PCR of p57 and HPRT mRNA levels in each cell line and in mouse heart tissue. (C) Western blot of p27 and flag-tagged dominant negative proteins levels. RelB is shown as a loading control.

Fig. 5: Expression of cyclins and Rb during MyoD mediated muscle differentiation. Cells were grown in the presence or absence of tetracycline and either mock differentiated or differentiated with MyoD in the presence or absence of tetracycline. (A) Western blot showing cyclin D3, cyclin E, cyclin D1, and cyclin A protein levels as well as flag protein expression. (B) RT-PCR of Rb mRNA levels. The HPRT mRNA levels for this experiment are shown in Fig. 4B. (C) Western blot showing Rb protein levels in mock and MyoD differentiated cells and in proliferating NIH 3T3 cells.

Fig. 6: Schematic model outlining steps leading to cell cycle arrest and muscle differentiation. The requirement of each step for SWI/SNF enzymes is indicated. The dotted circle around Rb leading to cell cycle arrest in a SWI/SNF dependent manner reflects the possibility that
SWI/SNF dependent, Rb-mediated cell cycle arrest may provide an alternative mechanism for achieving cell cycle arrest during differentiation pathways.
**A**

| Differentiation | Tet-VP16 | H17 | B22 |
|-----------------|---------|-----|-----|
| Tetracycline    | - - + + | - - + + | - - + + |
| Myosin Heavy Chain |
| Myosin Light Chain |
| Troponin T |
| GAPDH |
| 28S rRNA |
| 18S rRNA |

**B**

| Differentiation | Tet-VP16 | H17 | B22 |
|-----------------|---------|-----|-----|
| Tetracycline    | - - + + | - - + + | - - + + |
| α-skeletal actin |
| desmin |
| HPRT |

**C**

| Differentiation | Tet-VP16 | H17 | B22 |
|-----------------|---------|-----|-----|
| Tetracycline    | - - + + | - - + + | - - + + |
| Flag |

---

**Fig. 1**

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Fig. 2

A

|                | Tet-VP16 | H17 | B22 |
|----------------|----------|-----|-----|
| **Differentiation** | - - + +   | - - + +   | - - + +   |
| **Tetracycline**     | + - + -   | + - + -   | + - + -   |

![Myogenin, 28S rRNA, 18S rRNA]

B

|                | Tet-VP16 | H17 | B22 |
|----------------|----------|-----|-----|
| **Differentiation** | - - + +   | - - + +   | - - + +   |
| **Tetracycline**     | + - + -   | + - + -   | + - + -   |

![HPRT, Myf 5, Mrf 4, MyoD, Mef 2C]
Fig. 3

| Cell Type  | M+ | M- | D+ | D- | Prolif. |
|------------|----|----|----|----|---------|
| B22        | ![Histogram](image1) | ![Histogram](image2) | ![Histogram](image3) | ![Histogram](image4) | ![Histogram](image5) |
| H17        | ![Histogram](image6) | ![Histogram](image7) | ![Histogram](image8) | ![Histogram](image9) | ![Histogram](image10) |
| Tet-VP16   | ![Histogram](image11) | ![Histogram](image12) | ![Histogram](image13) | ![Histogram](image14) | ![Histogram](image15) |
**A**

|               | Tet-VP16 | B22 | H17 |
|---------------|----------|-----|-----|
| Differentiation | - - + +  | - - + + | - - + + |
| Tetracycline   | + - + -  | + - + - | + - + - |

**Heart**

|   | p21 | 28S | 18S |
|---|-----|-----|-----|
|   |     |     |     |

**B**

|   | p57 | HPRT |
|---|-----|------|
|   |     |      |

**C**

|   | p27 | rel B | Flag |
|---|-----|-------|------|
|   |     |       |      |
Fig. 5

A

| Differentiation | Tet-VP16 | H17 | B22 |
|-----------------|----------|-----|-----|
| - - + +         | - - + +  | - - + + | - - + - |
| Tetracycline    | + - + -  | + - + - | + - + - |

B

| Differentiation | Tet-VP16 | H17 | B22 |
|-----------------|----------|-----|-----|
| - - + +         | - - + +  | - - + + | - - + + |
| Tetracycline    | + - + -  | + - + - | + - + - |

C

| Differentiation | Tet-VP16 | H17 | B22 | Prolif. cells |
|-----------------|----------|-----|-----|---------------|
| - - + +         | - - + +  | - - + + | - - + + | Rb |
| Tetracycline    | + - + -  | + - + - | + - + - |

Cyclin D3
Cyclin E
Cyclin D1
Cyclin A
Flag
Rel B

Rb
Fig. 6

MyoD

Myogenin
Mef 2

late muscle specific markers

SWI/SNF dependent (?)

Rb

p21

SWI/SNF dependent

SWI/SNF independent

Swi/Snf dependent

Swi/Snf independent

cell cycle arrest

cyclin D3
MyoD can induce cell cycle arrest but not muscle differentiation in the presence of dominant negative SWI/SNF chromatin remodeling enzymes

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