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14. ABSTRACT
A prognostic feature of many human cancers is a high mitotic index and the inability to maintain a terminal cell cycle arrest (TCCA). The *Rb* gene product retinoblastoma protein has been implicated in the maintenance of a terminal cell cycle arrest. Likewise the inactivation of retinoblastoma gene (*Rb*) is observed in several human cancers including those of the breast. However, in contrast to our knowledge of how pRb regulates proliferation in a cycling population, little is known how it maintains a permanent cell cycle arrest. The proposed study was aimed at elucidating the molecular mechanism by which pRb accomplishes this task and plays the role of tumor suppressor of tumor formation. Our working hypothesis was that pRb in cooperation with basic helix loop helix (bHLH) protein MyoD participates in the transcriptional repression of one or more immediate early genes required for the induction of cyclin D1. And this event ultimately prevents the re-entry into the cell cycle, thus maintaining a terminal cell cycle arrest. To test this hypothesis myogenic differentiation has been used as model, because it represents a differentiation system in which pRb has been implicated in a terminal cell cycle arrest both *in vitro* and *in vivo*. In this study I have established that, among various immediate early genes only the induction of Fra-1 is blocked in pRb and myoD dependent manner leading to cyclin D1 control and is independent of the cell cycle inhibitory protein p16. Whereas an ectopic expression of Fra-1 by adenovirus-vector which leads to induction of cyclin D1 in identical settings confirmed a causal relationship between Fra-1 expression and cyclin D1. Further a biochemical analysis of the Fra-1 gene regulation using wild type promoter reporter (LUC) and a mutant for MyoD binding constructs confirmed the loss of MyoD binding to the promoter renders its ability to inhibit the Fra-1 induction. Consistent with this result a Chromatin Immunoprecipitation (ChIP) assay confirmed that MyoD indeed directly binds to the Fra-1 promoter in terminal cell cycle arrest. Interestingly a MyoD mutant (MyoD-RRR) incapable for muscle differentiation maintained the terminal cell cycle arrest thus confirmed that the role of pRb and MyoD in TCCA is separable from its role in skeletal muscle differentiation. These results and reagents provide the basis to discover the detailed mechanism how pRb may be participating with other bHLH proteins in maintaining a terminal cell cycle arrest.

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Introduction:

A characteristic feature of most cancers is an increase in the percentage of proliferating cells, often referred as mitotic index. Upon differentiation most cells in the body enter an irreversible terminal cell cycle arrest. Failure to maintain this growth-arrested state is thought to significantly contribute in the development of most forms of human cancer including those of the breast. The retinoblastoma protein (pRb) has been shown to participate in the maintenance of a terminal cell cycle arrest (Novitch, Mulligan et al. 1996), however, the mechanisms by which it accomplishes this task are not understood. This is in contrast to the role of pRb in controlling proliferation in a cycling population, which is well characterized. The purpose of these studies described here are to elucidate the molecular mechanism by which pRb maintains a terminal cell cycle arrest. Initial focus is placed on the study on skeletal muscle differentiation since both *in vitro* and *in vivo* studies have clearly demonstrated a role for pRb in maintaining an arrested state following terminal differentiation of this tissue type (13, 19). Information gained from this analysis will then be applied to study of mammary gland differentiation. Since the inactivation of retinoblastoma gene (*Rb*) is a common event in development of several human cancers including those of the breast. Therefore, the above studies likely have a direct bearing of how loss of *Rb* contributes to the development of cancer.

Body:

Task 1: To establish various experimental systems to study terminal growth arrest

Initially the genuine mouse myoblast line (C2C12) was utilized as an experimental tool, as these cells express both endogenous MyoD and pRb. Also, they respond differently to their cell culture conditions and can achieve either an irreversible growth arrest or a state of quiescence (reversible). To establish the condition for the irreversible growth arrest or quiescence, a confluent culture of C2C12 cells were grown for extended period in 1% horse serum (for differentiation) or 0.5% FBS (for quiescence) containing media respectively. Cells were growth arrested for at least 72 hours in either of above media. Both differentiated or quiescence cells were serum stimulated with 20%FBS containing media to induce proliferation. The S-phase induction of re-stimulated cells was analyzed for by BrdU incorporation. Cells grown in condition known to cause differentiation (1%HS media) as described above did not show any S-phase induction whereas the cells grown to quiescence (0.5% FBS media) clearly incorporated BrdU upon serum re-stimulation indicating a S-phase induction (data not shown). These observations suggest a success of culture conditions established to study an irreversible or reversible cell cycle arrest.

Next, I extended these observations to littermate matched $Rb^{+/+}$ and $Rb^{-/-}$ 3T3 mouse fibroblasts. These cells, with help of ectopic expression of a retrovirus transduced MyoD can be converted to myoblasts. Use of this experimental system affords the ability to simultaneously assess the contribution of MyoD and pRb to a terminal cell cycle arrest. Initial efforts were placed on establishing the conditions for successful retroviral transduction to the 3T3 fibroblast cells, utilizing a virus encoding MyoD. The experimental conditions were established such that *Rb*-positive fibroblasts transduced with a MyoD-encoding retrovirus cultured under conditions known to induce myogenesis (1% horse serum containing medium for 72 hours) would not re-enter the cell cycle, but the same cells when infected with an 'empty' retrovirus vector do re-enter S phase upon restimulation. I found that indeed pRb and MyoD cooperate to prevent cell cycle re-entry of differentiated myoblasts following serum restimulation (data not shown). We feel that these observations may extend to other tissues, e.g., pRb may cooperate with other 'MyoD-like' basic helix loop helix (bHLH) factor/s to bring about a terminal cells cycle arrest in the breast and may be in other tissues.

Task 2: To perform analysis of immediate early and delayed early gene expression

Our working hypothesis is that the lack of cell cycle re-entry in pRb- and MyoD-positive myoblasts (following restimulation) is achieved by inhibiting the induction of one or more immediate early genes, which prevent the induction of cyclin D1, an event required for cell cycle re-entry. First we used the genuine mouse myoblast C2C12 cells to establish our hypothesis of a correlation between expression of immediate early gene/s and cyclin D1. As described earlier, C2C12 cells were grown under conditions known to cause irreversible (differentiation) or reversible arrest (quiescence) and then challenged with the addition of 20% FBS containing growth media. The expression of several immediate early genes was assessed by immunofuorescence and only Fra-1 was not induced in terminally differentiated myoblasts in direct correlation of lack in cyclin D1 induction (see figure 1 in appendix). Whereas restimulated quiescent myoblasts show a clear induction of Fra-1 and cyclin D1. This lack of induction of Fra-1 suggests the possibility that among various immediate early gene the Fra-1 may be the target of pRb and MyoD in their role of controlling the cyclin D1 in maintaining a terminal cell cycle arrest.

In order to further validate our working hypothesis of cooperative role of pRb and MyoD in blocking the induction of Fra-1 leading to inhibition of cyclin D1 induction. The *Rb* positive or negative 3T3 fibroblasts turned myoblasts (after transduction with MyoD or empty retrovirus control and differentiation) were restimulated with 20% FBS containing media. These myoblasts were harvested at various time points and the expression of several immediate early genes was carried out by Western blot analysis. The *Rb* positive fibroblasts transduced with MyoD did not show the induction of Fra-1 and cyclin D1 upon serum re-stimulation, whereas other immediate early genes, e.g., c-Fos, Fra-2 or c-Jun, showed a clear and significant induction. Whereas in vector transduced *Rb* +/+ cells the Fra-1, cyclin D1 and other immediate early genes were induced similar to *Rb*-deficient myoblasts (see figure. 2 in appendix). It is worth mentioning here that to analyze the expression of c-Fos protein in *Rb*—MyoD dependent manner the cell lysates from early time points were used for Western blot. Also the expression of MyoD in both *Rb* +/+ and *Rb* -/- cell lysate is found to be at comparable level as seen by Western blot. These observations suggest that only Fra-1 among other immediate or delayed early genes shows a lack of induction upon serum stimulation in a pRb- and MyoD- dependent manner. These results, together with our analyses of C2C12 myoblasts (see above), further support the hypothesis that cooperative function of MyoD and pRb specifically inhibit the induction of immediate early gene Fra-1, which in turn prevents the induction of the essential cell cycle protein cyclin D1, thus allowing the maintenance of a terminal cell cycle arrest.

One of my working hypotheses is that the role of pRb in maintaining a terminal cell cycle arrest is distinct from its participation in cell cycle arrest at mid G1. Consistent with this notion others have recently separated these two functions of pRb genetically (11). Further, we hypothesize that an ultimate target of pRb during a terminal cell cycle arrest is cyclin D1. This is in contrast to the mid G1 arrest brought about by pRb where cyclin D1 expression is not affected. We have confirmed the above hypothesis using *Rb*+/+ fibroblasts transduced with retrovirus encoding the cdk inhibitor p16, retrovirus-vector or retrovirus-MyoD. The transduced fibroblasts were treated with differentiation conditions for 72 hours and followed by re-stimulation with 20% fetal bovine serum containing media. Cells expressing either p16 or MyoD failed to re-enter the cell cycle as determined by FACS analysis of BrdU incorporated cells (data not shown), whereas vector infected cells, as expected, progressed into S phase. Importantly, in the cells infected with the p16-encoding virus or vector control, both Fra-1 and cyclin D1 proteins were induced following re-stimulation. By contrast, only in MyoD expressing cells the Fra-1 and cyclin D1 was not induced as observed in Western blots (see figure 3 in appendix). These

observations suggest that the pRb target cyclin D1 during a terminal cell cycle arrest and its functional role in terminal cell cycle arrest and a mid G1 arrest are separable.

In order to further support the above observation, these genetically defined cells (*Rb*+/+ or *Rb*-/- 3T3) when treated to the conditions known to cause differentiation and restimulated (without being transduced with either MyoD or retrovirus vector), shows a clear induction of Fra-1 and cyclin D1. Importantly the temporal expression of Fra-1 is prior to cyclin D1 expression (see figure 4 in appendix), which is consistent with our hypothesis that Fra-1 can be a participant in the induction of cyclin D1. Also the earlier induction of Fra-1 expression indicates that Fra-1 is more proximal to the affect of MyoD and pRb during a terminal cell cycle arrest.

With regard to the experiments outlined above it is noteworthy that while several immediate early genes have been implicated in the induction of cyclin D1 (3,5,11,19) only one implicates Fra-1 (1). Thus, I sought to causally connect Fra-1 expression with cyclin D1 induction using the myoblasts system. As we know from reports of previous other investigators that the Fra-1 protein does not have a Transactivation Domain (TAD) to be able to function alone (4,16,18). And it is been shown that Fra-1 protein to be functionally active needs to heterodimerize with c-Jun family proteins and cannot homodimerize with any other c-Fos family protein (6,7,11,15). We also know that c-Fos and c-Jun family proteins are partners in AP-1 complex formation to participate in various cell cycle regulatory activities (2,6,9). Since Fra-1 but none of the other immediate early genes show any inhibition in *Rb*-MyoD dependent manner upon serum stimulation, therefore a presence of ectopic Fra-1 at time of serum restimulation may be able to cause the cell cycle re-entery and annul the effect of *Rb* and MyoD positive cells to undergo terminal cell cycle arrest. In order to achieve this, I have constructed an adenovirus capable of directing the expression of HA-tagged mouse Fra-1 using a slight modified protocol from He TC and colleagues (8). Considering the advantage of adenovirus vectors, which can successfully transduce and ectopically express the protein of interest in various differentiated cell thus can probably work in the differentiated myoblasts as well. Importantly, it circumvents the limitations of retrovirus mediated expression, which occurs only in dividing cells, as well as the continued expression of Fra-1 which may even prevent the cells to enter the terminal cell cycle arrest. Therefore the adenovirus vector mediated ectopic expression of Fra-1 was carried out in the C2C12 myoblasts and MyoD positive *Rb*+/+ cells after differentiation as described earlier. Cells were infected with Adeno-Fra-1 or vector control (at MOI of 100pfu/cell) and continued to grow in differentiation media for another 36 hours before serum restimulation. The protein expression for cyclin D1 and Fra-1 of cell lysates at time zero and eight hours after serum stimulation was analysed. Here the ectopic Fra-1, but not vector control in both C2C12 myoblasts and *Rb*+/+ myoblasts showed the induction of cyclin D1 upon serum restimulation (see figure 5 in appendix). Further provides the evidence that presence of ectopic Fra-1 can over-ride the inhibition of cyclin D1 induction even in MyoD and pRb positive cells. Moreover, this result establishes a causal link between Fra-1 expressions and cyclin D1 induction, thereby supporting the hypothesis that it is the block to Fra-1 induction, which leads to inhibition of cyclin D1 expression in terminally arrested cells.

Earlier reports by Sartorelli V et al suggested that acetylation of MyoD directed by PCAF is necessary for the execution of muscle program (14). Since my model for terminal cell cycle arrest is with ectopic expression of MyoD in defined genetic background of *Rb* and differentiation to myoblast. Therefore, I consider it will be informative to check the functional significance of acetylation negative mutant of MyoD in terminal cell cycle arrest. In order to analyze this I have generated lysine residue mutant of MyoD by site directed mutagenesis (MyoD-RRR mutant as published by Sartorelli V et al). I have compared the wild type and acetylation negative mutant of MyoD in *Rb* +/+ and -/- genetic background fibroblasts. These ectopic MyoD or RRR-mutant of

MyoD expressed in fibroblasts and treated to conditions establish for differentiation and serum restimulation to assay the expression of Fra-1 and cyclin D1. Both wild type and MyoD-RRR mutant were able to inhibit the induction of Fra-1 and cyclin D1 in Rb+/+ cells to similar extent (see figure 6 in appendix). This result is in agreement with our previous double knock out study of Rb and Nras, which rescues the myogenic differentiation defects but not the defect in terminal cell cycle arrest and further supporting that myogenic differentiation and terminal cell cycle arrest are separable function of pRb and MyoD.

Task 3: To characterize and elucidate the molecular involvement of retinoblastoma protein in terminal growth arrest

Our preliminary data with defined genetic background myoblast suggests that the Fra-1 gene is a primary target for pRb and MyoD during a terminal cell cycle arrest. A major goal in this line of research is to determine how, mechanistically, pRb and MyoD cooperate to prevent the induction of Fra-1 following restimulation of myoblasts cultured under differentiation conditions. We suspect that somehow pRb and MyoD actions converge upon the Fra-1 promoter to achieve such sustained silenced mode. Numerous investigators have studied the regulation of the Fra-1 gene. Regulation of this gene is complex, as both the 5' flanking sequence and intron-1 have been implicated in its regulation (15). Thus, I have generated a Fra-1 promoter fused with luciferase reporter construct where both 5'UTR and intron-1 were cloned upstream to the reporter gene as closely identical to the physiological promoter and designated it as wild type promoter. I first confirmed the functional viability of such an in-frame (fused to luciferase) wild type Fra-1 promoter reporter by transient transfection of dividing C2C12 myoblast cells and found it responds faithfully to promoter activation as compared to basal activity from empty vector control. Thus a functionally active Fra-1 promoter reporter (Luciferase) can effectively be used as a tool to further analyze the Fra-1 transcription regulation as primary target of pRb and MyoD in a terminally arrested stage.

Considering the functional integrity of wild type promoter, I have decided to generate various deletion mutants from this full-length promoter of Fra-1 (see figure 7 in appendix). The idea behind generating such deletion mutant was to see if it provides evidence of some conserved responsive elements in different regions of Fra-1 promoter and which may be regulated by the presence of Rb-MyoD in maintaining terminal cell cycle arrest. Another goal of transient transfection of wild type and various deletion mutants of promoter reporter constructs was to find out whether this extra chromosomal constructs responds identical to the one seen from the endogenous promoter activation reflecting the induction of Fra-1 protein expression. To accomplish this task, C2C12 myoblasts were transfected either with full-length wild type or various deletion mutants of promoter reporter construct. The transfected C2C12 cells grown in previously established differentiation (DMEM plus 1% horse serum) or quiescence (DMEM plus 0.5%FBS) conditions for the same duration and re-stimulated with 20% fetal bovine serum containing media. The differentiated promoter constructs did not show any significant induction in luciferase activity, in contrast to quiescence treated showed a clear and significant induction upon serum stimulation. (See figure 8 in appendix). Interestingly, this positive induction in quiescence treated cells and lack of induction in differentiation treated cells was similar for various deletion mutants as well but all the deletion mutants had nearly 10 fold lower activation than that of the full-length promoter (see figure 9 in appendix and data not shown). As these deletion mutants show the loss of functional integrity as compare to full-length wild type promoter by transient transfection assay suggesting that promoter designated as wild type is actually the Fra-1 minimal promoter. Further the difference in promoter activity indicates that maybe the full activation of Fra-1 promoter involves multiple regions of the minimal promoter. Also these results by transient

transfection indicate that an extra chromosomal Fra-1 promoter reporter constructs faithfully reflect the functional outcomes to growth conditions of host cells endogenous promoter.

As we reported last year the possible technical constrains for Fra-1 promoter reporter analysis in defined genetic background fibroblasts (*Rb* +/+ and *Rb* -/-), which is due to their poor transfection efficiency. Therefore the wild type promoter reporter construct was co-transfected with retroviral vector for stable integration into litter matched *Rb* +/+ and *Rb* -/- 3T3 cells. Both, the clonal lines or the pooled population for full-length promoter have been antibiotic selected and the functional integrity has been verified by assaying the luciferase activity. Mainly the cell line from a pooled population of Fra-1 promoter reporter was used for subsequent biochemical analysis of Fra-1 promoter regulation. The stably integrated and functionally active Fra-1 promoter reporter line helps to circumvent the technical constrains of poor transient transfection and paves the way to carry out promoter regulation analysis in pRb and MyoD dependent manner.

Continuing our effort to establish the cooperative function of pRb and MyoD in regulation of Fra-1 promoter, the above described stable lines was Fra-1 full-length promoter reporter was transduced with MyoD or empty retrovirus vector. These transduced cells were subjected to differentiation conditions and challenged with serum stimulation for a growth induction as described earlier. The cells were harvested at various time points after serum stimulation and promoter activity was analysed by Luciferase activation. The pRb +/+ cells expressing MyoD did not show any induction of Fra-1 promoter (see figure 10 in appendix), whereas the empty vector infected cells showed a clear up-regulation in Luciferase activity. In contrast the Rb-/- cells transduced either with MyoD or vector retrovirus showed a similar level of promoter activation at various time points. Indeed the promoter reporter activity is in full agreement with the Fra-1 and Cyclin D1 protein expression, thus further confirming a clear cooperative function between pRb and MyoD in regulation of Fra-1 promoter.

The above observations of Fra-1 promoter reporter activation and inhibition of induction further support our hypothesis that the MyoD may be directly binding to the Fra-1 promoter. In order to validate our hypothesis that direct binding of MyoD is crucial event in terminal cell cycle arrest a sequence analysis of the Fra-1 promoter was carried out. Using Bayesian block alignment phylogenetic analysis (see figure 11 in appendix) both for 5'upstream and intron-1 sequences from mouse, human and rat revealed a pair of conserved E-box elements in intron-1 region. Interestingly the E-box elements are in close proximity of three conserved AP-1 elements, which have already been reported as the key regulator of Fra-1 expression (4). Such high sequence conservation encouraged us to investigate the possibility of direct binding of MyoD to Fra-1 promoter. To confirm the likely binding of MyoD to E-boxes in Fra-1 promoter, a Chromatin immunoprecipitation (ChIP) assay was carried out. First, the genuine mouse myoblast C2C12 cells were used to determine if MyoD is actually binding to the Fra-1 promoter. Here these cells were treated with conditions known to cause differentiation as described earlier and serum stimulated. Cells were cross-linked with 1% Formaldehyde, collected and washed and then subjected to sonication Aim was to generate chromatin fragments in range of 500 to 1000 base pairs in length. Cleared lysate was subjected to immuno-precipitation using the antibody against MyoD (M318, Santa Cruz Biotech, USA) and co-precipitated chromatin released by reversing the cross linking at 65^0C over night and DNA was purified by Qiagen columns for PCR amplification. One microliter of each sample was used as template for PCR amplification of Fra-1 promoter fragment from the intron-1 region with conserved MyoD binding sites. In addition, primer pairs predicated to amplify DNA fragment 3000 base pair downstream to first exon used as negative control. Also a primer pair for the known MyoD responsive promoter of late differentiation marker gene the muscle creatine kinase (MCK) was used as positive control. As predicted DNA template generated through ChIP from both the differentiated and stimulated C2C12 cells clearly showed a PCR amplification of

Fra-1 promoter fragment (see figure 12 in appendix), thus supporting our hypothesis that MyoD is directly binding to Fra-1 promoter both during differentiation and continued to be present even after stimulation.

Next, to further demonstrate that the pRb and MyoD play a cooperative function in inhibition of induction of Fra-1 promoter in maintaining terminal cell cycle arrest, the defined genetic background (*Rb*+/+ and/or *Rb*-/- 3T3 cells) myoblasts with ectopic MyoD were used for ChIP analysis. As described earlier the *Rb*+/+ 3T3 cells were transduced with MyoD or empty vector and grown under conditions known to cause their differentiation to myoblast. Cells were harvested before and after serum stimulation for ChIP assay identically as described above for genuine myoblast (C2C12 cells). The DNA templates generated from ChIP assay with anti-MyoD antibody were used for PCR amplification of Fra-1 promoter region. The ethidium bromide staining of 1% agrose gel resolved PCR products clearly shows the presence of Fra-1 promoter specific fragment in MyoD transduced *Rb*+/+ cells whereas no bands were visible in vector infected cells. The PCR amplification for fragment of MCK promoter region was used as positive control, a late marker of skeletal muscle differentiation and to which MyoD is known to directly bind during differentiation. A non-specific DNA fragment for HSC70 gene and sequences 3000 base pairs downstream to MyoD binding site in Fra-1 promoter were used as negative control for anti-MyoD antibody, which clearly scored negative for PCR amplification (see figure 13 in appendix). These observations with defined genetic background myoblasts strongly support our hypothesis that pRb somehow regulate the MyoD to converge upon Fra-1 promoter to maintain a terminal cell cycle arrest.

In order to further support our hypothesis that pRb and MyoD actions somehow converge upon Fra-1 promoter and direct binding of MyoD is crucial event in terminal cell cycle arrest, a mutational analysis of the MyoD binding site (E-box elements) in Fra-1 promoter was carried out. As evident from the Bayesian block alignment analysis of the Fra-1 promoter the E-box elements (Myo D responsive elements) as well as AP-1 binding sites (growth stimulus responsive elements) were localized in stretch of conserved sequence. Thus, we generated three different point mutations of putative E-box elements present in intron-1 region of Fra-1 promoter reporter. The mutations were made by site directed mutagenesis, out of which one E-box mutant is from non-conserved sequences (E-box3) and two E-box elements from the conserved regions (E-box1 and E-Box2) changed to a non-functional E-box element. The idea behind generating such point mutation was to see if the loss of E-box element in Fra-1 promoter directly reflects to loss of inhibition of induction in presence of both pRb and MyoD, which is otherwise seen in direct correlation. To establish the above hypothesis, a pooled population of wild type promoter and three of the E-box mutant (E-box1, E-box2 and E-box3 mutants) stable lines in pRb+/+ cells were transduced with retrovirus expressing MyoD or vector. As described earlier, cells were treated with the conditions known to cause differentiation and serum stimulated to check the Fra-1 promoter activation at various time points by luciferase reporter assay. The wild type promoter and E-box3 (distal from conserved AP-1 sites) showed a significant inhibition of induction in presence of MyoD and activation in vector transduced cells (see figure 14 in appendix). However, the E-box1 and E-box2 mutant (proximal to conserved AP-1 sites) showed similar level of activation upon serum restimulation in both MyoD or vector infected cells at various time points. This result further supports that MyoD may be directly binding to the E-box element in Fra-1 promoter in order to inhibit the induction. Moreover the loss of inhibition in E-box mutated promoter (proximal to AP-1 site) also indicates that either MyoD is somehow involved in chromatin remodeling of Fra-1 promoter or helping some other transcriptional regulators to converge at promoter.

Together, these observations of cooperative role played by pRb and MyoD in maintaining a terminal cell cycle arrest, it becomes utmost important to access the physiological relevance of pRb and MyoD function. In order to analyze this, the ongoing experiments are to carry out an *in-situ* hybridization on skeletal muscles sections generated from defined genetic background (Rb+/+ or -/-) E14.5 day mouse embryo. As we know from our

earlier observations in the lab that the *Rb*-/- mouse embryos die at E13.5-E14.5 days and show defects in skeletal muscles differentiation and aberrant cell proliferation, which is not rescued by the loss of N-ras. Thus indicating a separable function of *Rb* in differentiation and terminal cell cycle arrest. Therefore we decided to check the levels of cyclin D1 and Fra-1 messages in those proliferating skeletal muscle cells with large endoreduplicating nuclei using riboprobe against either of these protein messages. We have already generated the riboprobe for mouse cyclin D1 and Fra-1 and confirmed the specificity of probes. The presence of cyclin D1 and Fra-1 mRNA has already been established in different embryonic regions during embryogenesis. A standardization of *in-situ* hybridization conditions is already being carried out using embryonic sections from wild type mouse E14.5 embryos. Sections from brain and eye lens regions, which are reported positive for Fra-1 and cyclin D1 expression respectively were used as positive control. Right now I am working to generate longitudinal sections of skeletal muscles from various genotype background to complete this analysis. We hope this *in-situ* hybridization study will provide evidence of Fra-1 and cyclin D1 regulation and further strengthen our hypothesis of the cooperative role played by pRb and MyoD in maintaining the terminal cell cycle arrest in a physiological relevant manner.

These research accomplishments strongly support the hypothesis being tested in this proposal. Further, the established cell culture conditions along with inclusion of various other analytical tools assisted us to propose how mechanistically pRb and MyoD may be cooperating to maintain a terminal cell cycle arrest. Further, they form a working foundation on which to test the possibility that a similar mechanism is employed by other cell types, e.g. mammary epithelial cells, to maintain an arrested state— also a goal of future research.

This line of investigation provides an excellent training in basic molecular biological techniques pertaining to cell cycle and differentiation— two key aspects to the study of breast cancer. It also taught me how to carefully design an experiment to test a hypothesis.

Key research accomplishments:

- Cell culture conditions established for the defined genetic system generated from *Rb*+/+ and *Rb*-/- mouse fibroblast allowing the study of a terminal cell cycle arrest
- Condition for efficient retroviral infection for MyoD and p16 to the defined genetic system of mouse fibroblasts has been established
- Established that terminal cell cycle arrest is distinct from the well characterized mid G1 arrest brought about by the cdk inhibitor p16
- Determined that the Fra-1 induction precedes the cyclin D1 induction in my defined genetic system of *Rb*+/+ and *Rb*-/- mouse fibroblasts, suggesting that Fra-1 participates in the induction of cyclin D1 and that Fra-1 gene regulation is likely the target of pRb and MyoD action in maintenance of a terminal cell cycle arrest.
- Determined Fra-1 and not any other immediate early genes is the target of pRb and MyoD action during a terminal cell cycle arrest, suggesting some degree of specificity in the mode of action of pRb and MyoD in maintaining a terminal cell cycle arrest.
- Constructed an Adenovirus and Retrovirus vector for ectopic expression of Fra-1 to establish a causal relationship between Fra-1 and cyclin D1 expression.
- Established a causal relationship between Fra-1 and cyclin D1 by ectopic expression of Fra-1 using Adeno-Fra-1 vector.
- Determined that the activity of a Fra-1 promoter reporter construct faithfully recapitulates the expression of the endogenous gene during restimulation of quiescent and differentiated myoblasts.

- Established the Fra-1 promoter reporter stable clonal lines in pRb defined genetic background 3T3 fibroblast.
- Determined that the Fra-1 promoter reporter line of *Rb*+/+ cells show a clear inhibition of induction in presence of MyoD.
- Determined the presence of MyoD on Fra-1 promoter by ChIP analysis of genuine myoblast defined genetic background Myoblast both after differentiation and stimulation.
- Generated E-box mutation carrying clonal line in defined genetic background and determined the loss of one of the MyoD binding loses the property on inhibition of induction.
- Generated E14.5 day old mouse embryo for in situ hybridization analysis for Fra-1 and cyclin D1 in Rb-/- and Rb+/+ state.

Reportable outcomes:

- Fra-1 promoter reporter constructs: full length and various deletion mutants of Fra-1 promoter reporter cloned upstream to Luciferase gene
- Rat Fra-1 coding gene is cloned in to pBabe-puro retrovirus expression vector
- Cell lines: Fra-1 promoter reporter constructs stably transfected to cell lines of Rb+/+ and Rb-/- mouse fibroblast and clonal lines are created
- Murine Fra-1 coding gene with HA-tag is cloned into adenovirus vector and used to generate Adeno-Fra-1_HA virus.
- Ectopic expression of Fra-1 in differentiated C2C12 cells can induce the cyclin D1 expression upon serum restimulation.
- Chromatin immunoprecipitation (ChIP) analysis: localize MyoD on the E-box elements in Fra-1 promoter both in genuine myoblast (C2C12) and MyoD transduced pRb+/+ 3T3 fibroblast under differentiation and stimulation condition.
- Site directed mutagenesis of conserved E-box element in Fra-1 promoter: established a clonal line for the mutants.
- Lack of inhibition of induction in one of the E-box mutant indicating a direct binding of MyoD on Fra-1 promoter.

Conclusions:

One of the characteristic features of cancer is the inability to maintain a terminal cell cycle arrest. Various human cancers including those of the breast has been reported for an inactivated *Rb*. Moreover the pRb protein has been implicated in maintaining the terminal cell cycle arrest, though the mechanism by which it accomplishes this is not known in contrast to a well-understood role in regulation of proliferation in cycling cells. Our studies supported by this fellowship to date have been directed towards developing a system to study mechanistically how pRb participates in maintaining a terminal cell cycle arrest. My data suggest that pRb maintains a terminal cell cycle arrest by specifically blocking the expression of immediate early gene Fra-1, which in turn is responsible for the lack of induction of cyclin D1—otherwise an essential event required for re-entry of cells into the cell cycle. I have provided evidence that this mode of action of pRb is distinct from its well-characterized ability to mediate a mid G1 arrest. Importantly, I have confirmed that an ectopic expression of Fra-1 can override the pRb and MyoD dependent inhibition of cyclin D1 induction. Further to support my hypothesis I have developed a Fra-1 promoter reporter that behaves in a manner similar to the endogenous gene and successfully recapitulate the expression patterns of endogenous gene. In order to provide more convincing evidence, I have used the Chromatin Immunoprecipitation (ChIP) assay for defined genetic background

fibroblast with ectopic MyoD and genuine mouse myoblast C2C12 cells, which gave confirmed that MyoD directly binding to Fra-1 promoter. It also indicated the direct binding of MyoD may be a critical factor in likely mechanism of terminal cell cycle arrest. The results from stably integrated wild type Fra-1 promoter reporter and the E-box mutant cell lines further confirmed the direct binding of MyoD on Fra-1 promoter for its regulation where the mutant dissimilar to wild type promoter reporter lost the inhibition of induction. Specifically, it strongly suggests that pRb and MyoD cooperate to maintain the Fra-1 in a silenced state following restimulation of differentiated myoblasts. The important observation is likely to be generated from *in-situ* hybridization of mouse embryo, which is ongoing and will support our hypothesis with the physiological significance of pRb and MyoD roles in terminal cell cycle arrest. Significantly the results obtained so far, provide a framework on which the study of a terminal cell cycle arrest can be extended to other cell types. Also it will help to find out the likely mechanism of pRb action in the suppression of tumor formation.

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1. Appendix:

Figure Legends:

Figure 1. Fra-1 is the only immediate early or delayed early gene getting specifically inhibited from induction in genuine mouse myoblast line C2C12 after serum stimulation of these differentiated myobalsts. (A-H) C2C12 cells incubated under conditions that induce myogenesis (1%HS) (A-D) or cause quiescence (E-H). Differentiated and quiescence cells were stimulated with 20%FBS containing media for 4 hours (B, D, F and H). Differentiated myoblast that had not (A) and had (B) been restimulated were then fixed, permiablized and stained with antibody to Fra-1 (rhodamine, Red) or myosin heavey chain (MHC; marker of muscle differentiation; FITC, green) and counterstained with DAPI to visualize nuclei (C and D). C2C12 rendered quiescent without (E) or with (F) restimulation were stained with same antibody to Fra-1 (rhodamine) or counterstained with DAPI (G and H).
Similarly (I-P) was analyzed as in A-H, except antibody to c-Fos was used and C2C12 cells were stimulated for 2 hours.

Figure 2. Fra-1 and cyclin D1 proteins are not induced following restimulation of *Rb*+/+ 3T3 mouse fibroblasts with MyoD after getting differentiated (1% Horse serum containing media) and Fra-1 is the only immediate early or delayed early gene getting specifically inhibited from induction as shown in western blot, but shows a clear induction in cells infected with vector or in the *Rb* -/- fibroblasts with MyoD. These differentiated myoblasts were stimulated for 8 hours in presence of 20% fetal bovine serum containing media. Cells were harvested in lysis buffer with protease inhibitors (50 mM Tris-Cl pH 8.0, 200 mM NaCl, 1% NP-40, 0.25% Na deoxycholate, 25 mM NaF, 1 mM EDTA, 1 mM Na3VO4, 250 μM phenylmethylsulfonyl fluoride, 10 μg of Leupeptin per ml and 10 μg of aprotenin per ml). Equal amount of whole cell lysates were loaded in 10% SDS-polyacrylamide gel and transferred to Polyvinylidine difluoride membrane, probed with Fra-1 (N-17, SantaCruz Biotech) or cyclin D1 (AB-3, Neomarker) antibody, c-Fos, c-Jun, Fra-2 and MyoD antibody (SantaCruz Biotech Inc., USA).

Figure 3. The temporal expression of cyclin D1 and Fra-1 in Rb+/+ cells after treating to differentiation conditions followed by serum stimulation shows that Fra-1 expression preceeds the cyclin D1 thus more likely be influencing the cyclin D1 regulation and proximal to MyoD effects.

Figure 4. Ectopic expression of p16 has no effect on the induction of cyclin D1 or Fra-1 proteins in differentiated (1% Horse serum containing media) *Rb*+/+ 3T3 mouse fibroblasts upon serum stimulation. These differentiated myoblasts were stimulated for 4 hours with 20% fetal bovine serum containing media. Cell lysates were prepared as described and equal amount of whole cell lysates were loaded in 12% SDS-polyacrylamide gel and transferred to Polyvinylidine difluoride membrane, western blot for p16 was carried out using ZJ-11 mouse monoclonal antibody.

Figure 5. A mutant of MyoD (MyoD RRR) lacking the function of skeletal muscle differentiation can still maintain a terminal cell cycle arrest by inhibiting the induction of cyclun D1 and Fra-1 same as wild type MyoD. Also the FLAG-MyoD generated for future experiments behaves same as wild type MyoD in inhbiting the induction of Fra-1 and cyclin D1

Figure 6. An ectopic expression of Fra-1 using Adeno-Fra-1_HA virus in C2C12 cells after differentiation shows a clear induction of cyclin D1 in response to serum restimulation (compare lanes 4 and 6) but not in the cells without any ectopic Fra-1 (compare lanes 3 and 5). Differntiated C2C12 cells express similar levels of ectopic Fra-1 at 48 hours and 72 hours post infection (compare lanes 1 and 2).

Figure 7. Schematic diagram of Fra-1 promoter reporter construct and its deletion mutants generated from the full length Fra-1 promoter region.

Figure 8. Fra-1 promoter reporter construct (designated as close to wild type promoter) is transfected into C2C12 myoblast cells followed by treatment to conditions causing differentiation (1% Horse serum containing media) or irreversible quiescence (0.5% Fetal bovine serum containing media) before stimulation with 20% fetal bovine serum containing media at various time points. Relative luciferase reporter activation was analysed for Fra-1 promoter activity.

Figure 9. Various deletion mutants of Fra-1 promoter reporter construct containing part of Fra-1 promoter was transfected to C2C12 myoblast cells followed by treatment to conditions causing differentiation (1% Horse serum containing media) or quiescence (0.5% Fetal bovine serum containing media) after stimulation with 20% fetal bovine serum containing media at various time points (Two different mutant with largest part of promoter). Relative luciferase reporter activation was analysed for Fra-1 promoter activity.

Figure 10. Fra-1 promoter reporter construct (designated as close to wild type promoter) stably integrated into Rb+/+ or Rb-/- fibroblasts. A pooled population of integrated stable line transduced with MyoD expressing retrovirus followed by treatment to conditions causing differentiation (1% Horse serum containing media). These myoblasts were stimulated with 20% fetal bovine serum containing media. Relative luciferase reporter activation was analysed at various time points after re-stimulation for Fra-1 promoter activity.

Figure 11. Bayesian block phylogenetic alignment from Intron-1 region of human and mouse Fra-1 promoter sequences to check the conserved regions across species. This helped us to design the primers for ChIP assay and generating E-box mutant of the Fra-1 promoter to assist in understanding the possible mechanism of pRb and MyoD mediated regulation of Fra-1 promoter.

Figure 12. Chormatin Immunoprecipitation (ChIP) assay for differentiated genuine mouse myoblast cells using MyoD antibody (SantaCruz Biotech, SC-760) to localize MyoD on Fra-1 promoter. The primer pair for known MyoD target of mouse creatin kinase (MCK) promoter was used as positive control and primers designed for 3000 base pair down stream of exon-II and a non-specific gene HSC-70 were used as negative control for MyoD binding.

Figure 13. Chormatin Immunoprecipitation (ChIP) assay for differentiated Rb+/+ mouse fibroblasts turned myobalsts using MyoD antibody (SanatCruz Biotech, SC-760) to localize MyoD on Fra-1 promoter. The primer pair for known MyoD target of mouse creatin kinase (MCK) promoter was used as positive control and primers designed for 3000 base pair down stream of exon-II and a non-specific gene HSC-70 were used as negative control for MyoD binding.

Figure 14. Fra-1 promoter reporter constructs (designated as wild type, E-box1, E-box2 and E-box3 mutants) stably integrated into Rb+/+ fibroblasts. A pooled population of integrated stable line transduced with MyoD expressing retrovirus followed by treatment to conditions causing differentiation (1% Horse serum containing media). These myoblasts were stimulated with 20% fetal bovine serum containing media. Relative luciferase reporter activation was analysed at time point after re-stimulation for Fra-1 promoter activity.

Immediate early gene Fra-1 is inhibited from induction in differentiated genuine mouse myoblast upon serum stimulation

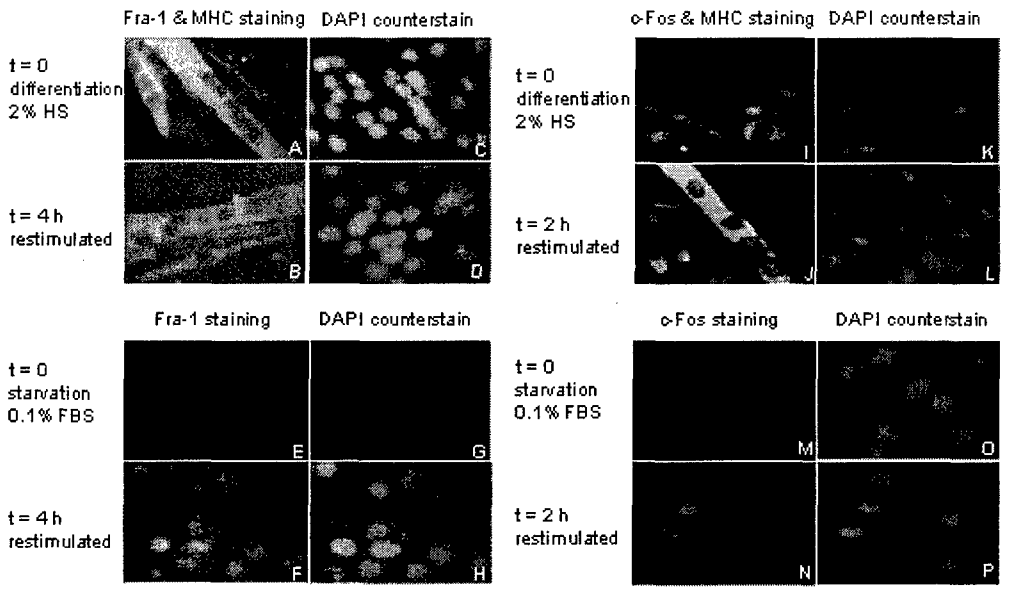


Figure 1

Fra-1 is the only immediate early gene being inhibited from the induction upon serum stimulation in Rb+/+ fibroblasts

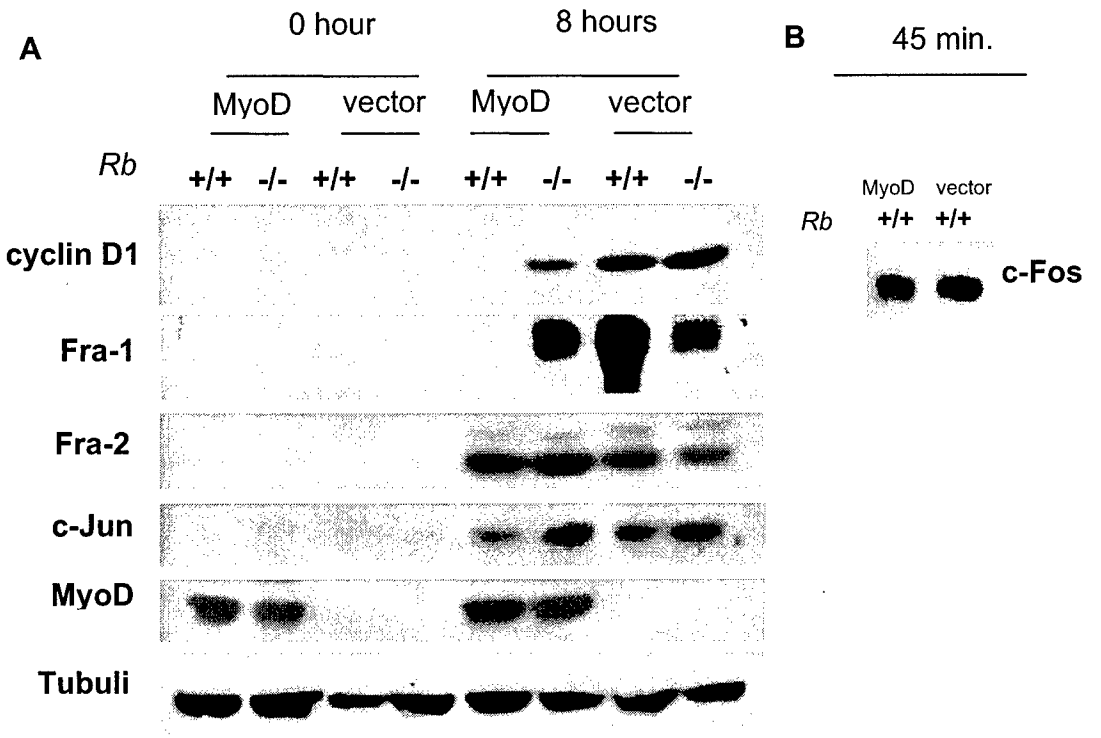


Figure 2

Terminal cell cycle arrest mediated by the cooperation of pRb and MyoD is distinct from mid G1 arrest that mediated by p16

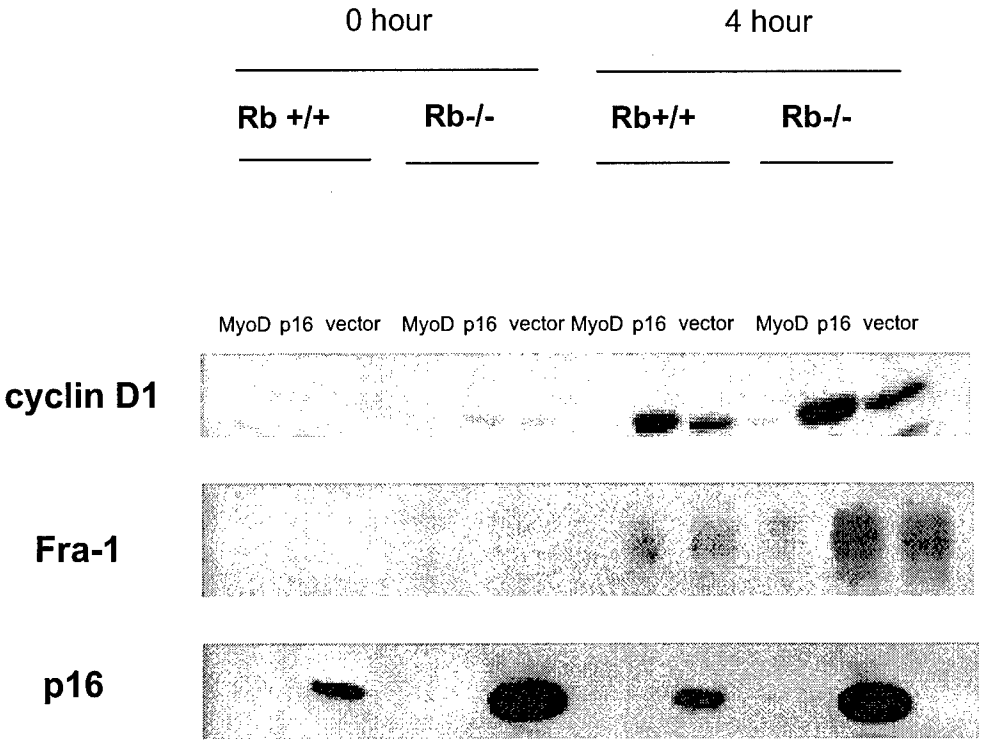


Figure 3

Temporal expression pattern of Fra-1 and cyclin D1 is consistent with the notion that Fra-1 participates in the induction of cyclin D1

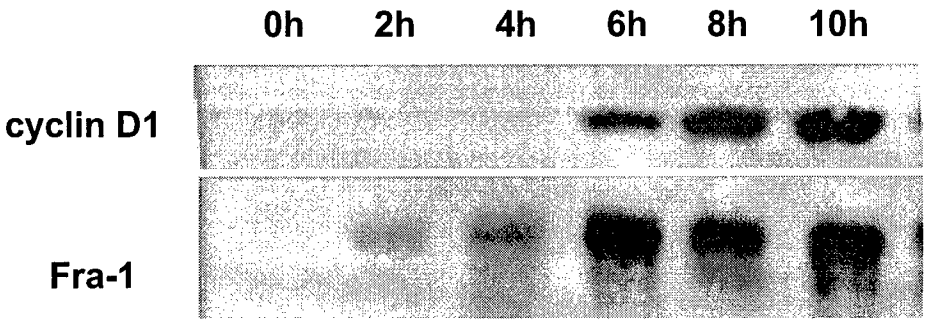


Figure 4

Comparison of RRR mutant of MyoD and FLAG-tag MyoD with wild type MyoD in maintaining a terminal cell cycle arrest

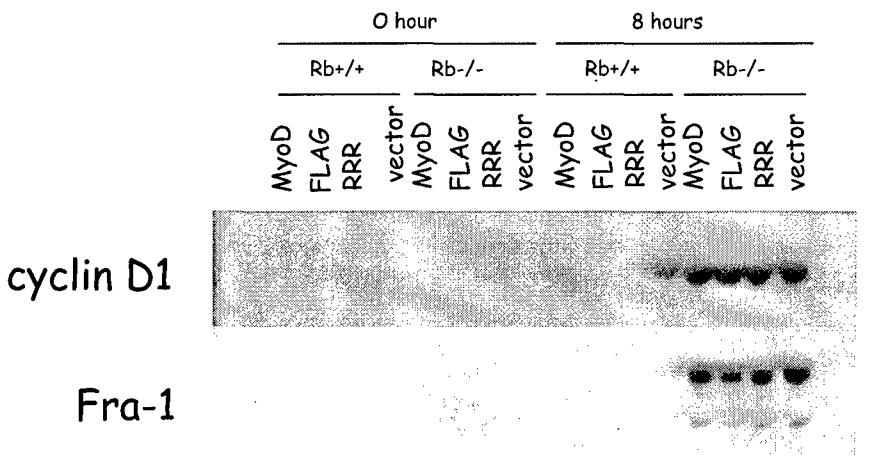


Figure 5

Ectopic expression of Fra-1 in differentiated C2C12 cells using Adeno-Fra-1_HA virus

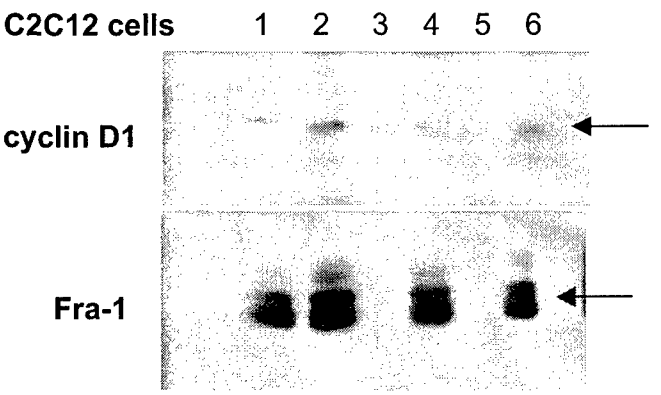


Figure 6

Schematic diagram of Fra-1 promoter reporter constructs

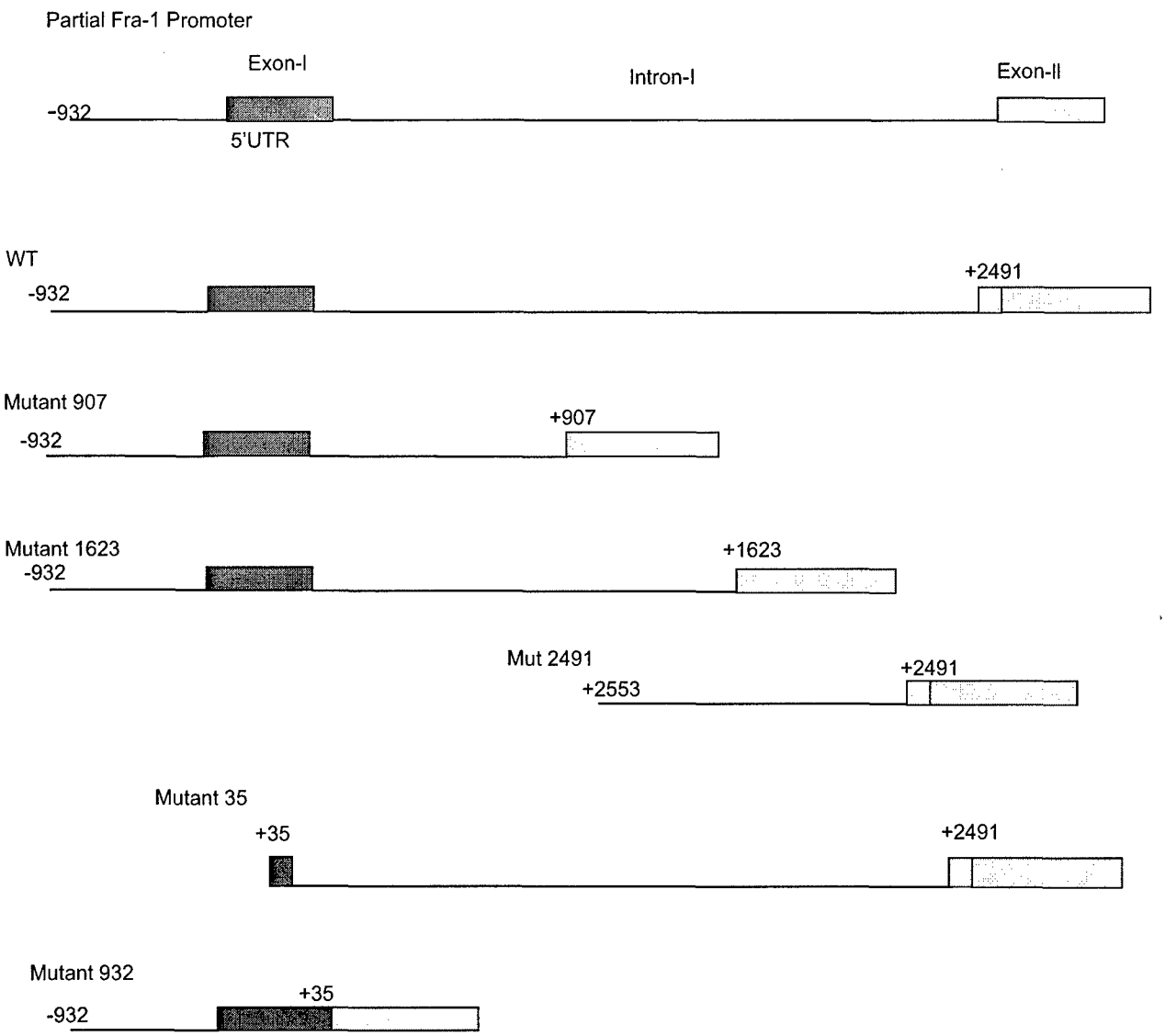


Figure 7

A wild type Fra-1 promoter reporter construct faithfully Recapitulates the expression of the endogenous Fra-1 gene

Fra-1 promoter reporter (WT) in C2C12 cells with 1% Horse Serum or 0.5% Fetal Bovine Serum followed by serum stimulation

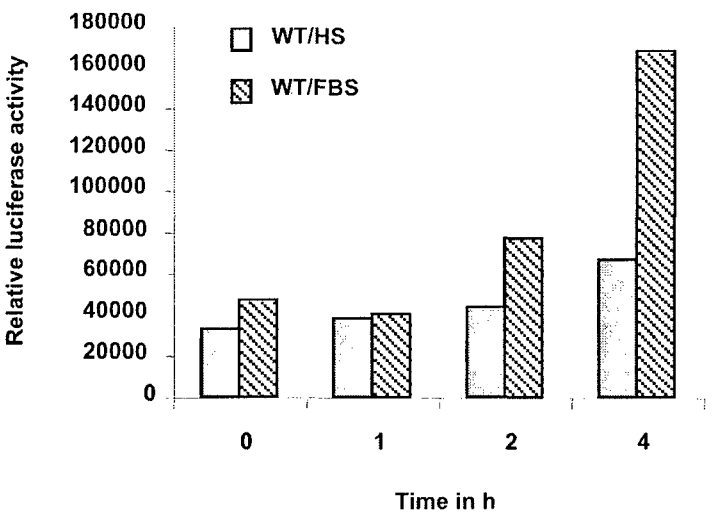
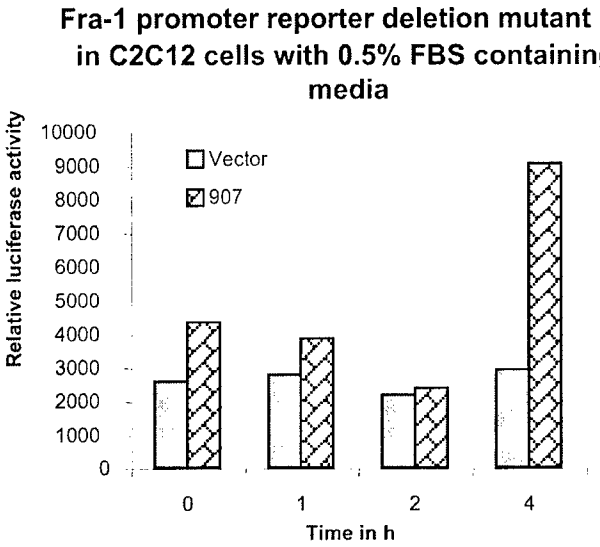
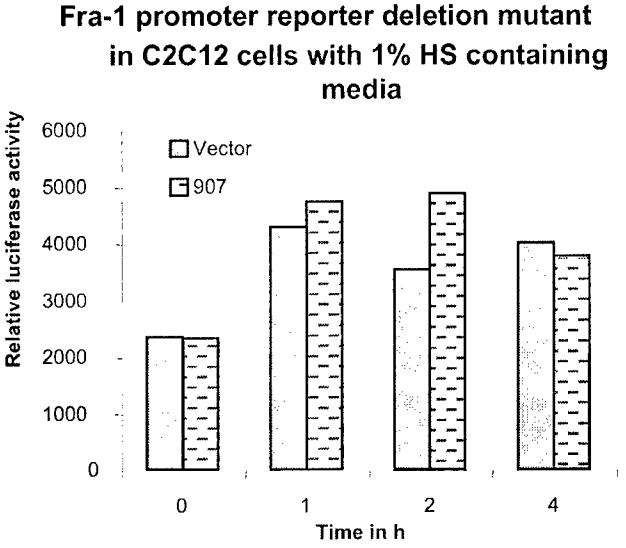
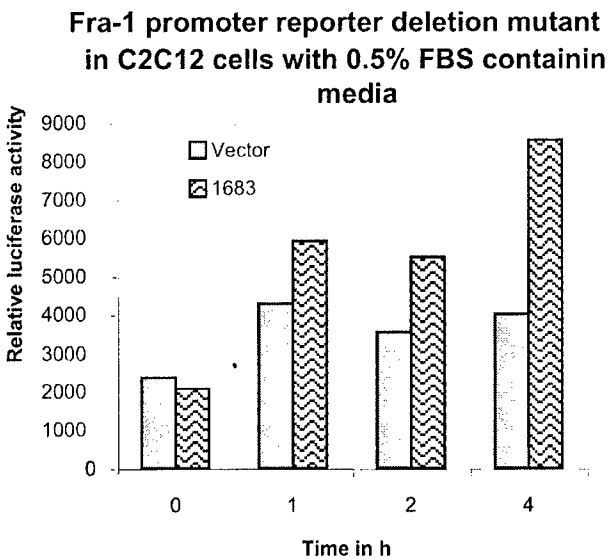


Figure 8

Deletion mutants of Fra-1 promoter reporter construct showing activation of Fra-1 gene







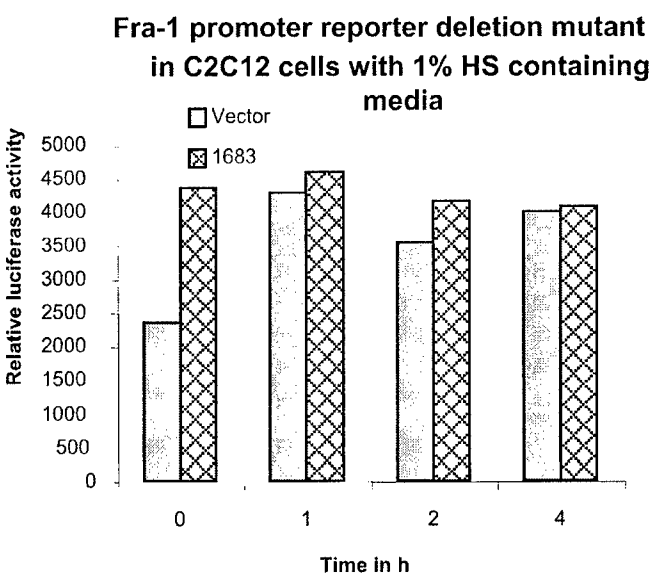


Figure 9

Stably integrated lines of full-length Fra-1 promoter reporter showing a Rb/MyoD dependent inhibtion of induction upon serum re-stimulation

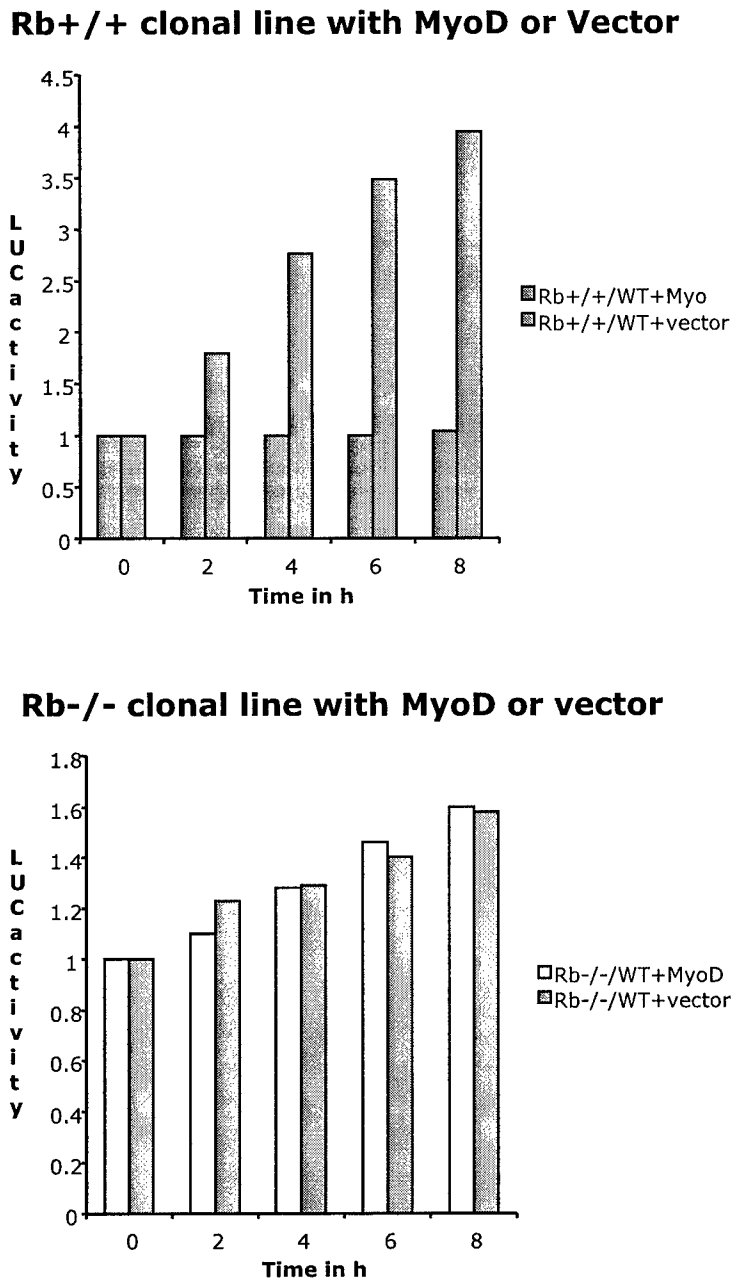


Figure 10

Site directed mutagenesis for the conserved E-box element localized by Bayesian block alignment of intron-1 region between mouse and human Fra-1 promoter

Sequence homology from Intron-1 region of Fra-1 promoter

agaattcttagcagcctgtccgaggctgtccgtgtgttgctctggttgtccgtgtccct
tatccggtcaagtcctcatctctttgtgcgcagtatagagcccatgggccccaggcagt
gttccgaggggttcctggagaccacgaagtgttgggatgtgcgcggggt acctgcccg
ccacactcgcgctccacattctcggcacccgacgtctctcactgctggataggggcact
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gaaggagc gcgtccgtttcgccgagtcacagcgggccgagtca tg ggctgagtcacc
ctgg tg ccctcccttcctgg cccaaacggcccctaaggaccgacgacctgggagcga
gagatgcccctggcagtgcttctagcccag acgggggtcact agatgctgggtccccc
agtattgggctggggacatagctgtcca acttgcctaagcatgtgaggt cttc tgg
ctggaggggccccacatccttagctca agagcttgaacc agttttc ctcccaga cg

the E boxes and AP-1 sites are all in conserved

E box CANNTG

AP-1 site CGGGTC TGGGTC

Figure 11

PCR amplification of Fra-1 promoter fragment from DNA template generated by Chromatin Immunoprecipitation (ChIP) using MyoD specific antibody in C2C12 myoblasts

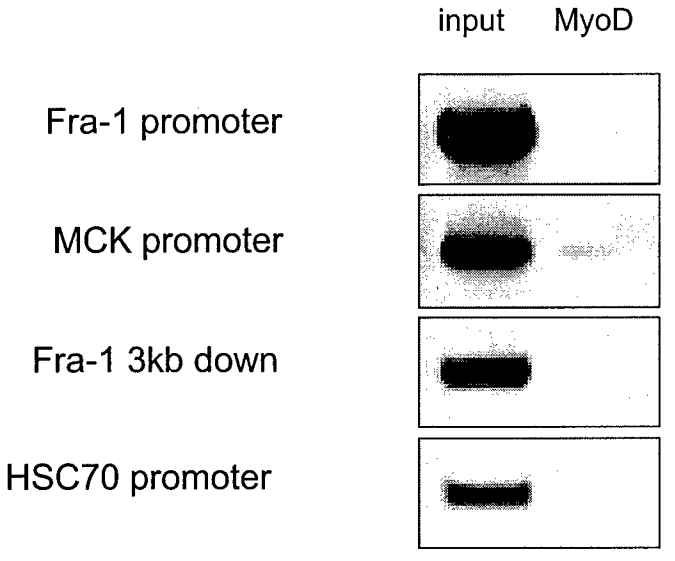


Figure 12

PCR amplification of Fra-1 promoter fragment in Rb+/+ myoblasts with DNA template generated from Chromatin Immunoprecipitation (ChIP) using MyoD specific antibody

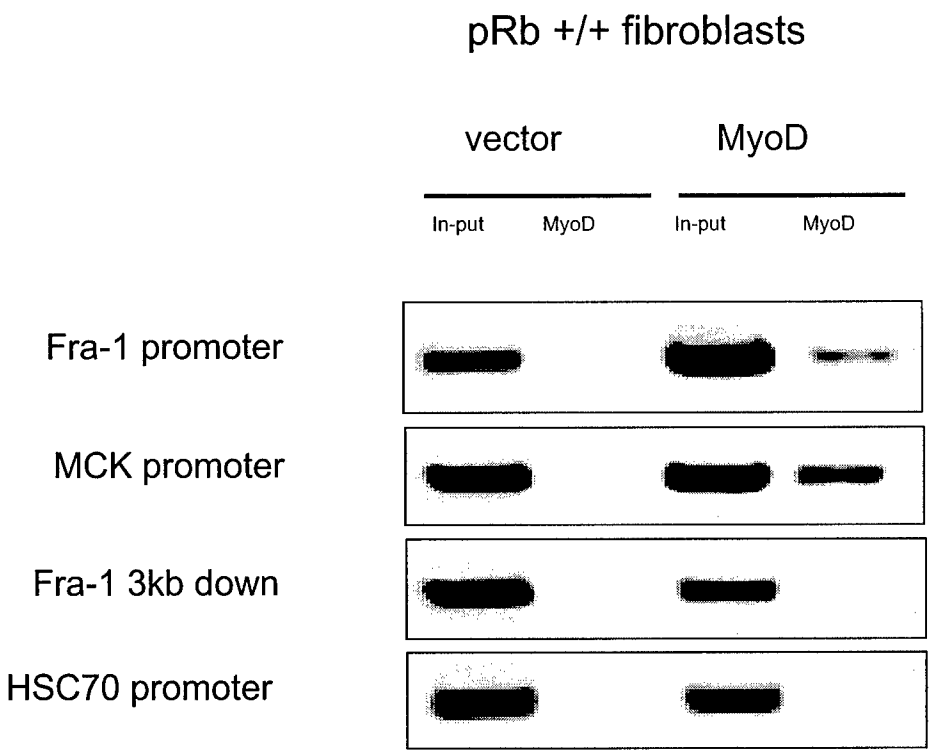
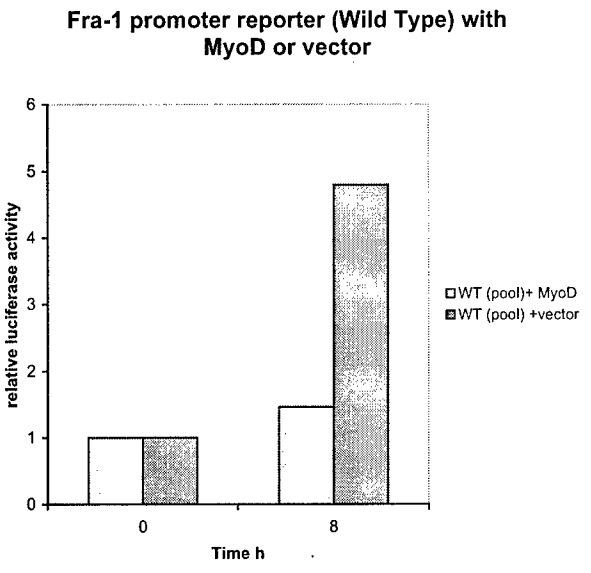
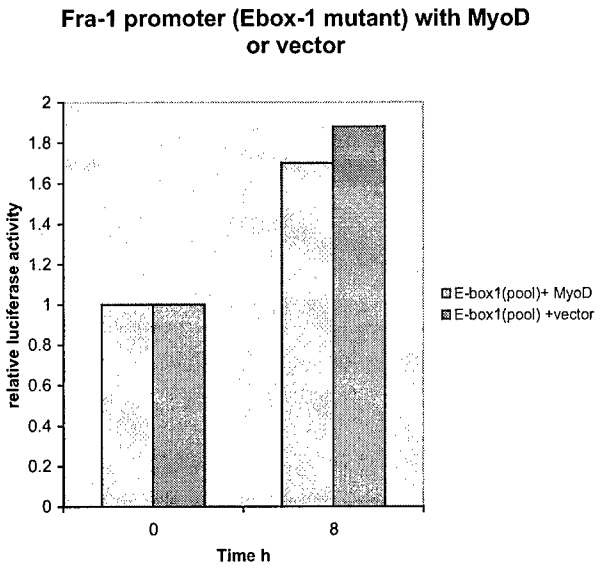
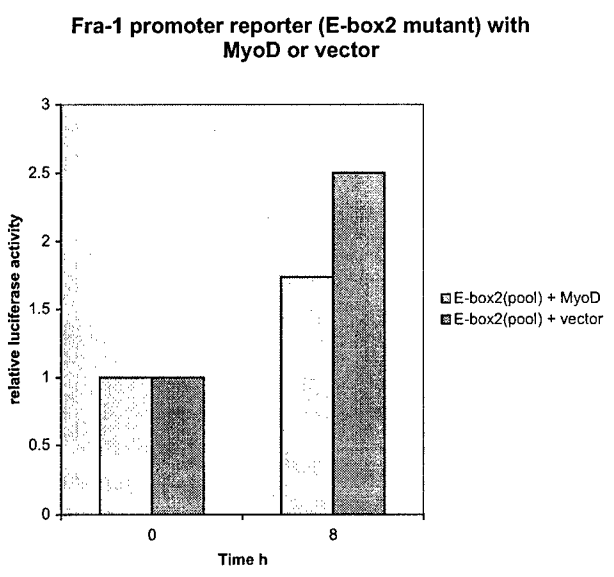


Figure 13

Fra-1 promoter reporter analysis of Rb+/+ stable lines of wild type and E-box mutant myoblasts before and after serum stimulation







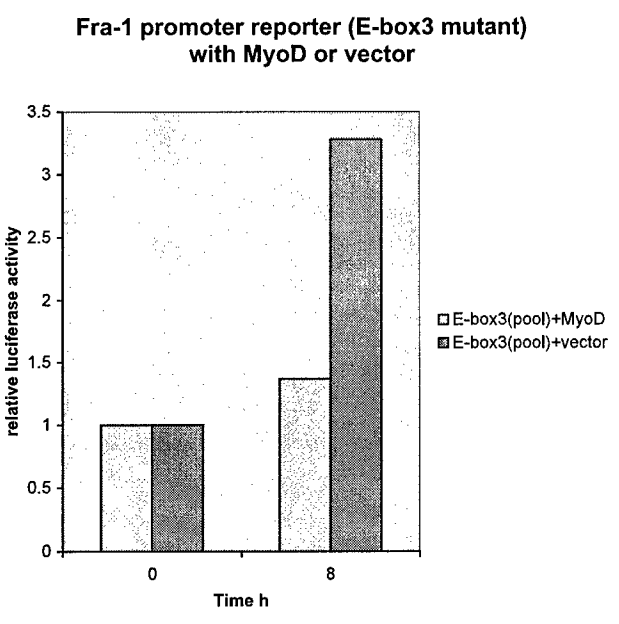


Figure 14