

AD \_\_\_\_\_

Award Number: DAMD17-01-1-0224

TITLE: The Mechanism of Retinoblastoma Protein-Mediated Terminal  
Cell Cycle Arrest

PRINCIPAL INVESTIGATOR: Hasan N. Rajabi, Ph.D.

CONTRACTING ORGANIZATION: Dana-Farber Cancer Center Institute  
Boston, Massachusetts 02115

REPORT DATE: September 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20040413 032

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY</b> (Leave blank)	<b>2. REPORT DATE</b> September 2003	<b>3. REPORT TYPE AND DATES COVERED</b> Annual Summary (1 Sep 2002 - 31 Aug 2003)	
<b>4. TITLE AND SUBTITLE</b> The Mechanism of Retinoblastoma Protein-Mediated Terminal Cell Cycle Arrest		<b>5. FUNDING NUMBERS</b> DAMD17-01-1-0224	
<b>6. AUTHOR(S)</b> Hasan N. Rajabi, Ph.D.			
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Dana-Farber Cancer Institute Boston, Massachusetts 02115  <i>E-Mail:</i> hasan_rajabi@dfci.harvard.duc		<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>			
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited			<b>12b. DISTRIBUTION CODE</b>
<b>13. ABSTRACT (Maximum 200 Words)</b> Inactivation of retinoblastoma gene (Rb) is observed in several human cancers including those of breast cancer is the inability to maintain a terminal cell cycle arrest, whereas the Rb product (pRb) has been implicated in the maintenance of a terminal cell cycle arrest. 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 studies are 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 is that pRb, in cooperation with MyoD, participates in the transcriptional repression of one or more immediate early genes required for the induction of cyclin D1. And this even 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 the past year, I have discovered that: (1) The induction of Fra-1 and not any other immediate early genes is blocked following restimulation of differentiated myoblasts. (2) Ectopic expression of the cell cycle inhibitory protein p16, which bring about a cell cycle arrest distinct from a terminal cell cycle arrest, has no effect on expressions of both Fra-1 and cyclin D1. In an effort to further study the regulation of the Fra-1 gene I have created Fra-1 promoter reporter and its deletion mutants. Also constructed a retrovirus vector for ectopic expression of Fra-1 to establish a causal relationship between Fra-1 and cyclin D1. These results and reagents provide the basis upon which to discover the detailed mechanism by which pRb participates in a terminal cell cycle arrest.			
<b>14. SUBJECT TERMS</b> No Subject Terms Provided.			<b>15. NUMBER OF PAGES</b> 21
			<b>16. PRICE CODE</b>
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited

## Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	9
Conclusions.....	9
References.....	10
Appendices.....	11

## Introduction:

A characteristic feature of most cancers is an increase in the percentage of proliferating cells, often referred to 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 contribute significantly to 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 (12), however, the mechanism 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 (12, 17). Information gained from this analysis will then be applied to study of mammary gland differentiation. Inactivation of the retinoblastoma gene (*Rb*) is a common event in the development of several human cancers including those of breast. These studies thus likely have 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*

Last year we reported the establishment of cell culture conditions that leads to an irreversible growth arrest or a state of quiescence (reversible) in C2C12 myoblasts. Where the reversibility of the arrest was established by monitoring their ability to re-enter the cell cycle upon serum restimulation using BrdU incorporation and fluorescence-activated cell sorting (FACS) analysis. These culture conditions were ultimately improved to a level necessary for subsequent analysis using a defined genetic system employing wild type and litter matched *Rb*-deficient mouse fibroblasts infected with a MyoD-encoding retrovirus. Use of this system has allowed me to assess the dependence of both pRb and MyoD in the terminal cell cycle arrest. Initial efforts were placed on establishing the conditions for successful retroviral infection of these cells utilizing a virus encoding MyoD. Focusing on the same set of variables utilized for the C2C12 cells, the conditions were established such that *Rb*-positive fibroblasts transduced with a MyoD-encoding retrovirus cultured under conditions known to induce myogenesis would not re-enter the cell cycle following serum restimulation, but the same cells infected with an 'empty' retrovirus or *Rb*-deficient cells infected with MyoD or 'empty' retrovirus do re-enter S phase upon restimulation. Here entry into the S phase was analyzed by BrdU incorporation and I established that indeed pRb and MyoD cooperate to prevent cell cycle re-entry following restimulation of differentiated myoblasts (data not shown)

Our working hypothesis is that the lack of induction of Fra-1 following restimulation of pRb- and MyoD-positive myoblasts prevents the induction of cyclin D1, an event required for cell cycle re-entry. To test the above hypothesis both *Rb* positive and negative fibroblast were transduced with retrovirus expressing MyoD or empty vector. These fibroblasts were cultured under conditions known to induce differentiation and a terminal cell cycle arrest ( DMEM plus 1% horse serum containing medium for 72 hours) followed by restimulation with growth medium containing 20% fetal bovine serum. Cells were harvested before and after serum stimulation to check the expression of Fra-1 and cyclin D1 by

Western blot. The *Rb* positive fibroblasts transduced with MyoD did not show the re-induction of Fra-1 and cyclin D1 upon serum stimulation (see figure 1 in appendix) but showed re-induction in those infected with empty vector. In contrast, the *Rb*-deficient fibroblast infected and cultured under identical conditions show a significant induction of Fra-1 and cyclin D1 in both MyoD or vector infected cells upon serum restimulation. These results indicate that the pRb and MyoD cooperate to block the induction of both Fra-1 and cyclin D1 following restimulation of differentiated myoblasts, events that contribute to 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 a mid G1 cell cycle arrest. 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. Specifically, that cyclin D1 is not induced following restimulation of myoblasts cultured under conditions known to bring about their withdrawal from the cell cycle and to promote differentiation. This is in contrast to the mid G1 arrest brought about by pRb where cyclin D1 expression is not affected.

To test the above hypothesis *Rb*<sup>+/+</sup> fibroblasts were infected with retrovirus encoding MyoD, the cdk inhibitor p16 or vector. Cells were then cultured under conditions known to induce differentiation and then subsequently restimulated with fetal bovine serum. Cells expressing MyoD or p16 failed to re-enter the cell cycle as determined by FACS analysis (data not shown). Vector infected cells, as expected, progressed into S phase. Importantly, in cells infected with the p16-encoding virus or vector control cyclin D1 was induced following restimulation. By contrast, cyclin D1 was not induced in MyoD expressing cells (see Figure 2 in appendix). These observations suggest that the function of pRb during a terminal cell cycle arrest and a mid G1 arrest are distinct and that in the former one target of pRb is cyclin D1.

These research accomplishments are vital to the continuation of this project, as we have already established the working conditions with defined genetic system, which gives liberty to incorporate other analytical tools in a more comprehensive ways. Also the basic mechanistic regulation of cell cycle is more or less same in other defined genetic systems including those of breast epithelial cells. Thus the information generated through this analysis can be extrapolated and may be extended in understanding the role of Rb in different type of cancers including those of breast.

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

Our previous observations in the laboratory using C2C12 myoblasts indicated that the restimulation of differentiated cells with growth medium did not result in the re-induction of cyclin D1. Also among several immediate and delayed early genes only Fra-1 was not induced following restimulation, suggesting the possibility that Fra-1 may be the target of pRb and MyoD in their role of maintenance of a terminal cell cycle arrest. Therefore, the issue then became to determine whether the genetically defined myoblast system described above could faithfully recapitulate what we have previously observed with C2C12 myoblasts. Specifically, that Fra-1 is not induced following restimulation of differentiated pRb-positive myoblasts. Also, by using the genetically tractable system afforded me the ability to determine if the cooperation between pRb and MyoD is responsible for the lack of induction of Fra-1 and that this correlates with the lack of induction of cyclin D1. Thus, the genetic analysis of

pRb and MyoD in maintaining a terminal cell cycle arrest may allow me to assess the contribution of these two proteins to this phenomenon in a system that recapitulates what is observed in a bona fide myoblast line, C2C12.

As indicated above though C2C12 myoblast provides a system with Rb and MyoD where they act as partners in its differentiation, it does not establish a direct relationship between Rb and MyoD cooperation rather as only being the factor for causing a terminal cell cycle arrest stage. Since one of our goals is to determine the mechanism by which pRb and MyoD cooperate to maintain a terminal cell cycle arrest, mouse fibroblasts where I control the expression of both pRb and MyoD will be used. Notably, it is well established that ectopic expression of MyoD in fibroblasts results in the creation of myoblasts capable of differentiating to form myotubes. To extend above observation of specificity of Fra-1 inhibition of induction to my genetically tractable system both *Rb* positive and negative fibroblast were transduced with MyoD expressing or empty retrovirus as described earlier. Fibroblasts were cultured in DMEM plus 1% horse serum for 72 hours followed by restimulation with growth medium containing 20% fetal bovine serum. Cells were harvested at various time points including the early ones for c-Fos protein for western blot analysis. The *Rb* positive fibroblasts transduced with MyoD did not show the re-induction of cyclin D1 or Fra-1 upon serum stimulation. Whereas, the same membrane, when analyzed for Fra-2 or c-Jun, by western blot showed a clear and significant induction of these proteins, irrespective of *Rb* and MyoD status (See Figure 3 in appendix). To analyze the expression of c-Fos protein in *Rb*—MyoD dependent manner the cell lysates from early time points were used in western blot. Here too c-Fos protein did not show any inhibition of induction upon serum stimulation. The expression of MyoD in both *Rb* *+/+* and *Rb* *-/-* cell lysate is found to be at comparable level as seen in the other western blot. These observations strongly suggest that none other than Fra-1 is the immediate early or delayed early gene, which shows a lack of induction upon serum stimulation in *Rb* and MyoD dependent manner. These results further support my hypothesis of cooperative function of MyoD and pRb to specifically inhibit the induction of Fra-1, which in turn prevents the induction of the essential cell cycle protein cyclin D1, thus participates in the maintenance of a terminal cell cycle arrest. Moreover, this observation is in clear agreement with our previous results analyzing a genuine myoblasts line, C2C12 by immunofluorescence.

In contrast these genetically defined cells when treated with the conditions known to cause differentiation and restimulation 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 the 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.

To more solidly link Fra-1 expression with the induction of cyclin D1, I have created a retrovirus capable of directing the expression of Fra-1. This retrovirus may be used to ectopically express the Fra-1 in the *Rb* *+/+* and *Rb* *-/-* cells along with the MyoD expression. Here ectopic Fra-1, but not vector control, in *Rb* *+/+* fibroblast undergoing differentiation might allow the induction of cyclin D1 upon serum restimulation. Such a result will provide evidence that Fra-1 participates in the induction of cyclin D1 expression (under circumstances where a terminal cell cycle arrest is not achieved). Though the retrovirus mediated expression of Fra-1 has its own limitation, considering both the need of dividing cells for retrovirus mediated expression of protein as well as continued expression of Fra-1 may even prevent the cells to enter the terminal cell cycle arrest. Therefore an adenovirus vector

mediated Fra-1 expression in differentiated cells may be utilized, where this adenovirus vector can ectopically express the protein of interest in differentiated myoblasts. Together, these approaches will allow me to link Fra-1 to the expression of cyclin D1, thereby supporting my hypothesis that it is the block to Fra-1 induction that is responsible for the corresponding lack of expression of cyclin D1 following restimulation of arrested myoblasts.

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 (3,5,9,15) only one implicates Fra-1 (1). Thus, I sought to causally connect Fra-1 expression with cyclin D1 induction using my myoblasts system. To this end we know that the Fra-1 protein does not have a Transactivation Domain (TAD) to be able to function alone (4,14,16). And it is been shown by various other investigators 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 (6,7,10,14). We also know that c-Fos and c-Jun family proteins are partners in AP-1 complex formation for to participate in various cell cycle regulation activities (2,6,8). Since no other immediate early genes show any inhibition upon serum stimulation in *Rb*-MyoD dependent manner in cells grown in conditions known to cause differentiation. Therefore, an ectopic expression of Fra-1 may be able to prevent the affect of *Rb* positive cells to undergo terminal cell cycle arrest in presence of MyoD, which is seen otherwise and will cause the cell cycle re-entry upon serum restimulation.

### *Task 3:*

*To characterize and elucidate the molecular involvement of retinoblastoma protein in terminal growth arrest*

My preliminary data suggest 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. I suspect that somehow pRb and MyoD action converge upon the Fra-1 promoter.

In order to understand the possible role of pRb and MyoD in preventing the activation of Fra-1, I need to localize the regions in the Fra-1 promoter that is responsible for its lack of induction following restimulation in a pRb- and MyoD -dependent manner. 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 (13). Thus, I created a luciferase promoter reporter construct where both 5'UTR and intron-1 were cloned upstream to the reporter gene as closely identical to the physiological promoter designated as wild type promoter. I have established that the wild type Fra-1 promoter reporter responds faithfully to differentiation and serum stimulation in C2C12 myoblast cells. Considering the functional integrity of wild type promoter I have further generated various deletion mutants from this full-length wild type promoter of Fra-1 (See Figure 5 in appendix). Further, these constructs of wild type and various deletion mutants of Fra-1 promoter will help me to locate the conserved responsive elements across the species in the promoter, which may be regulated by the presence of *Rb*-MyoD in maintaining terminal cell cycle arrest.

My initial emphasis was to analyze the luciferase activation, cloned downstream to Fra-1 promoter sequences for finding out whether this extra chromosomal promoter and its mutants will respond in

similar manner to the one seen from the endogenous promoter. To accomplish this task, C2C12 myoblast cells were transfected either with full-length promoter or various deletion mutants. Transfected cells were grown under conditions known to cause differentiation (DMEM plus 1% horse serum) for 72 hours and restimulated with 20% fetal bovine serum containing media here the full-length promoter of Fra-1 did not show any significant induction of luciferase activity. In contrast to the promoter transfected C2C12 cells when grown in the conditions known to cause reversible proliferation arrest (quiescence) (DMEM plus 0.5%FBS) did show a clear and significant induction upon serum stimulation (See figure 6 in appendix). Interestingly, this lack of induction in differentiation treated and positive induction in quiescence treated cells was similar for various deletion mutants as well (See figure 7 in appendix and data not shown), but all the deletion mutants had nearly 10 fold lower activation then that of the full-length promoter (See figure 8 in appendix and data not shown). These results indicate that may be the activation of Fra-1 promoter involves more than one region of promoter and work in some synchrony but inhibition of induction can be regulated in isolated regions of promoter as well.

The transient transfection approach for Fra-1 promoter reporter constructs has some technical constrains in my system of studies. Considering the step of retrovirus infection expressing MyoD followed by transfection in genetically defined *Rb* +/+ or *Rb* -/- cells to establish *Rb*-MyoD dependence on Fra-1 promoter regulation. Therefore these various promoter reporter constructs have been stably transfected and integrated into defined genetic background of *Rb* +/+ and *Rb* -/- cells derived from wild type and litter matched *Rb*-deficient mouse fibroblasts. The various clonal lines for full-length promoter and all the deletion mutants have already been selected and the functional integrity of these promoter reporter clonal lines has been checked by their luciferase activity status in asynchronous growing population. These clonal lines can be used in future for various related studies to help in understanding the mechanistic part of Fra-1 promoter regulation.

These research accomplishments suggest that the hypothesis being tested in this proposal is correct. Further, they establish cell culture conditions that can be utilized to determine how, mechanistically, pRb and MyoD cooperate to maintain a terminal cell cycle arrest—the goal of the next year of research. 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 excellent training in basic molecular biological techniques pertaining to cell cycle and differentiation— two key aspects to the study of breast cancer. It also teaches one 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 to the defined genetic system of mouse fibroblasts for MyoD has been established
- Conditions for successful transduction and expression of p16 is established in mouse fibroblast
- 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*<sup>+/+</sup> and *Rb*<sup>-/-</sup> 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.
- Created a retrovirus vector for ectopic expression of Fra-1 to establish a causal relationship between Fra-1 and cyclin D1 expression.
- Determined Fra-1 and not other immediate early genes are 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.
- 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.

### 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*<sup>+/+</sup> and *Rb*<sup>-/-</sup> mouse fibroblast and clonal lines are created

### Conclusions:

One of the characteristic features of cancer is the inability to maintain a terminal cell cycle arrest. *Rb* has been reported to be inactivated in various human cancers including those of breast. Also the pRb 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 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 developed a Fra-1 promoter reporter that behaves in a manner similar to the endogenous gene. Use of the promoter reporter construct provides me an inroad into studying the mechanistic underpinnings of the events that participate in a terminal cell cycle arrest. Specifically, it allows me to begin to explore the means by which pRb and MyoD cooperate to maintain the Fra-1 in a silenced state following restimulation of differentiated myoblasts. The deletion mutants of Fra-1 promoter will also be helpful in localizing the likely responsive regions and thus help for future analysis of exact mechanism, including the CHIP assay for the responsive domain/s in the promoter. Significantly the results obtained, 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.

## References:

1. Albanese C, Johnson J, Watanabe G, Eklund N, Vu D, Arnold A, Pestell RG, *J Biol Chem.* **270** (40):23589-97 (1995).
2. Angel P, Karin M., *Biochim Biophys Acta.* **10**;1072 (2-3):129-57 (1991).
3. Bakiri L, Lallemand D, Bossy-Wetzell E, Yaniv M., *EMBO J.* **19** (9):2056-68 (2000).
4. Bergers G, Graninger P, Braselmann S, Wrighton C, Busslinger M., *Mol Cell Biol.* **15**(7):3748-58 (1995).
5. Brown JR, Nigh E, Lee RJ, Ye H, Thompson MA, Saudou F, Pestell RG, Greenberg ME, *Mol Cell Biol.* **18**(9):5609-19 (1998).
6. Cohen DR, Ferreira PC, Gentz R, Franza BR Jr, Curran T, *Genes Dev.* **3** (2):173-84 (1989)
7. Halazonetis TD, Georgopoulos K, Greenberg ME, Leder P, *Cell.* **55** (5):917-24 (1988).
8. Kovary K, Bravo R., *Mol Cell Biol.* **12** (11):5015-23 (1992).
9. Milde-Langosch K, Bamberger AM, Methner C, Rieck G, Loning T, *Int J Cancer.* **87** (4):468-72 (2000).
10. Nakabeppu Y, Ryder K, Nathans D, *Cell.* **55** (5):907-15 (1988).
11. Narita M, Nunez S, Heard E, Narita M, Lin AW, Hearn SA, Spector DL, Hannon GJ, Lowe SW, *Cell.* **113** (6):703-16 (2003).
12. Novitch BG, Mulligan GJ, Jacks T, Lassar AB, *J Cell Biol.* **135** (2):441-56 (1996).
13. Schreiber M, Poirier C, Franchi A, Kurzbauer R, Guenet JL, Carle GF, Wagner EF, *Oncogene.* **15** (10):1171-8 (1997).
14. Suzuki T, Okuno H, Yoshida T, Endo T, Nishina H, Iba H, *Nucleic Acids Res.* **19** (20):5537-42 (1991).
15. Wisdom R, Johnson RS, Moore C, *EMBO J.* **18** (1):188-97 (1999).
16. Wisdom R, Verma IM, *Mol Cell Biol.* **13** (5):2635-43 (1993).
17. Zacksenhaus E, Jiang Z, Chung D, Marth JD, Phillips RA, Gallie BL, *Genes Dev.* **10** (23):3051-64 (1996).

## **Appendix:**

### **Figure Legends**

**Figure 1**

**Figure 2**

**Figure 3**

**Figure 4**

**Figure 5**

**Figure 6**

**Figure 7**

**Figure 8**

## Figure Legends:

**Figure 1.** Fra-1 and cyclin D1 proteins are not induced following restimulation of *Rb*<sup>+/+</sup> 3T3 mouse fibroblasts with MyoD after getting differentiated (1% Horse serum containing media) as shown in western blot, but shows a clear induction in cells infected with vector or in the *Rb*<sup>-/-</sup> 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 Na<sub>3</sub>VO<sub>4</sub>, 250 μM phenylmethylsulfonyl fluoride, 10 μg of Leupeptin per ml and 10 μg of aprotinin per ml). Equal amount of whole cell lysates were loaded in 10% SDS-polyacrylamide gel and transferred to Polyvinylidene difluoride membrane, probed with Fra-1 (N-17, SantaCruz Biotech) or cyclin D1 (AB-3, Neomarker) antibody

**Figure 2.** 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*<sup>+/+</sup> 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 Polyvinylidene difluoride membrane, western blot for p16 was carried out using ZJ-11 mouse monoclonal antibody.

**Figure 3.** Fra-1 is the only immediate early or delayed early gene getting specifically inhibited from induction in *Rb*<sup>+/+</sup> fibroblasts with MyoD after serum stimulation of these differentiated myoblasts. These differentiated myoblasts were stimulated for different time points with 20% fetal bovine serum containing media. Whole cell lysate was prepared as described and equal amount of whole cell lysates were loaded in 10% SDS-polyacrylamide gel and transferred to Polyvinylidene difluoride membrane, western blot was carried out using cyclin D1, Fra-1, c-Fos, c-Jun and Fra-2 specific antibodies.

**Figure 4.** Expression of endogenous Fra-1 and cyclin D1 proteins in genetically defined (*Rb*<sup>+/+</sup> or *-/-*) mouse fibroblast was carried out after treatment with conditions causing differentiation (1% Horse serum containing media) and serum stimulation with 20% fetal bovine serum containing media at various time points.

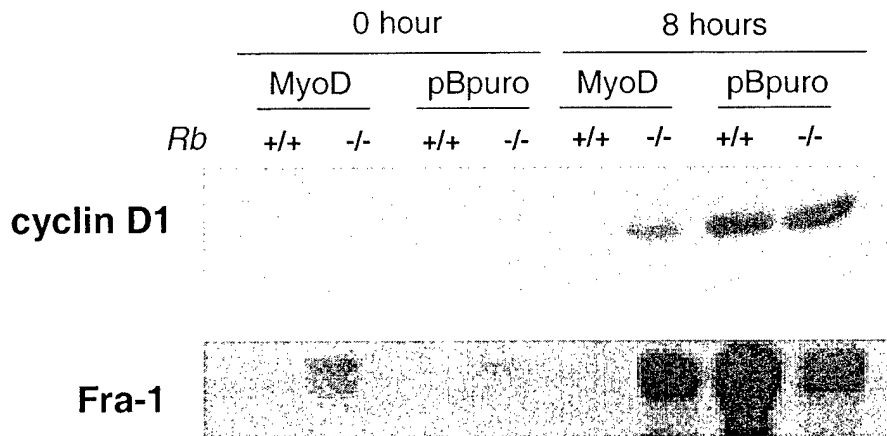
**Figure 5.** Schematic diagram of Fra-1 promoter reporter construct and its deletion mutants generated from the full length Fra-1 promoter region.

**Figure 6.** 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. Luciferase reporter activation was analysed for Fra-1 promoter activity.

**Figure 7.** Deletion mutants of Fra-1 promoter reporter construct containing various regions 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) before stimulation with 20% fetal bovine serum containing media at various time points. Luciferase reporter activation was analysed for Fra-1 promoter activity.

**Figure 8.** Comparison of Fra-1 promoter reporter (WT) with its deletion mutants shows a high activation of full-length promoter compare to deletion mutants upon serum stimulation in both differentiation condition or quiescence condition treated C2C12 myoblast cells.

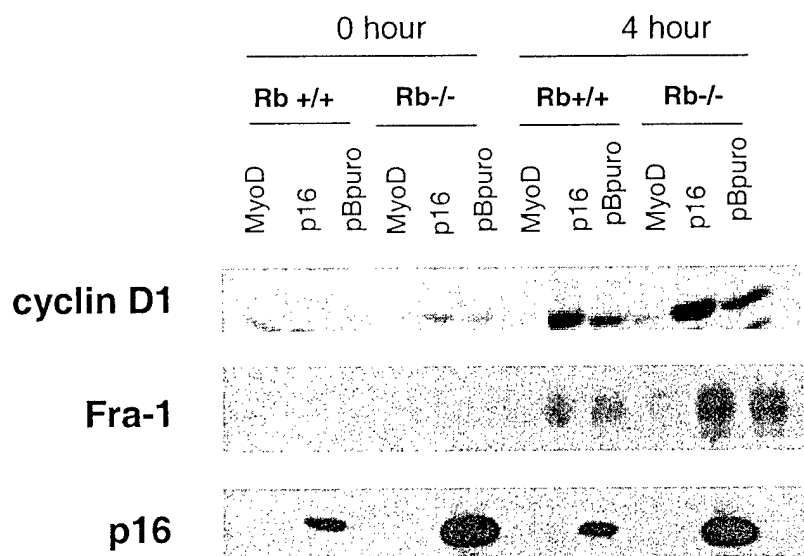
**pRb and MyoD cooperate to prevent the induction of cyclin D1 and Fra-1 following restimulation of differentiated myoblasts**



**BEST AVAILABLE COPY**

**Figure 1**

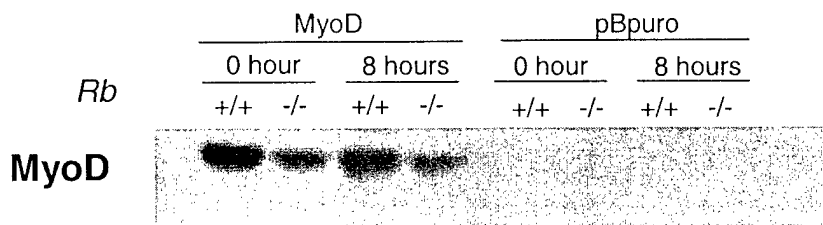
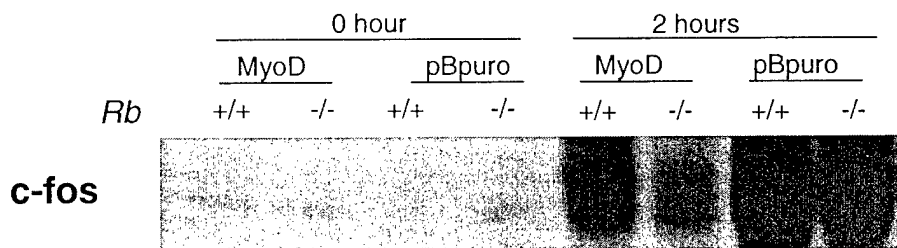
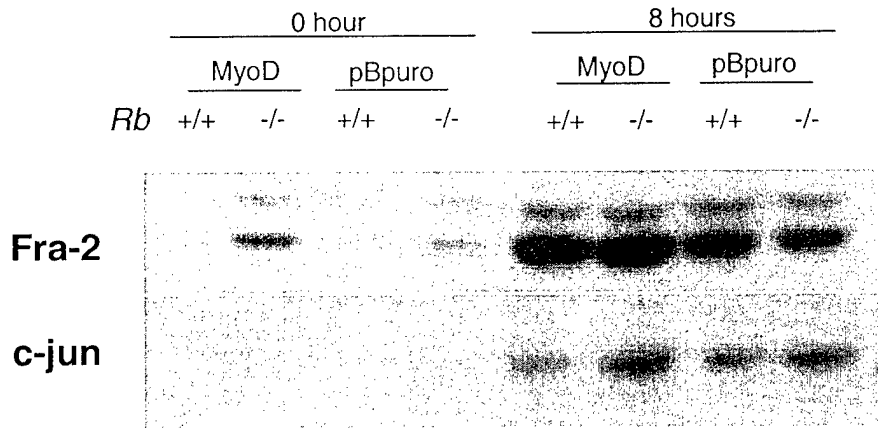
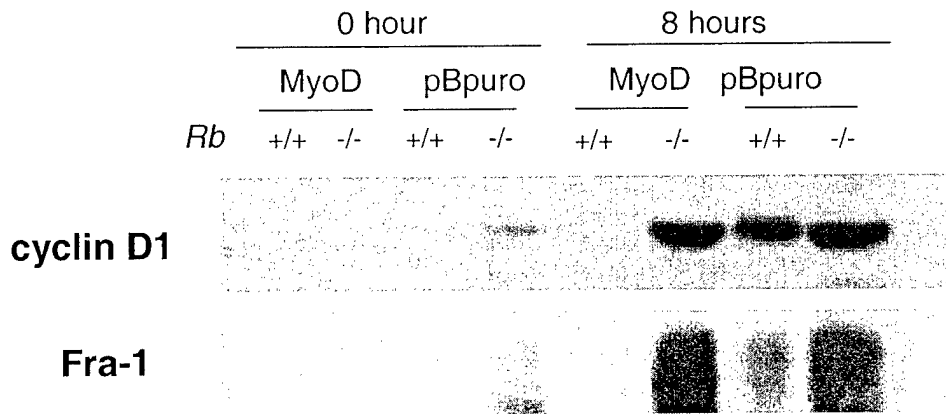
## Terminal cell cycle arrest mediated by the cooperation of pRb and MyoD is distinct from that mediated by p16



BEST AVAILABLE COPY

Figure 2

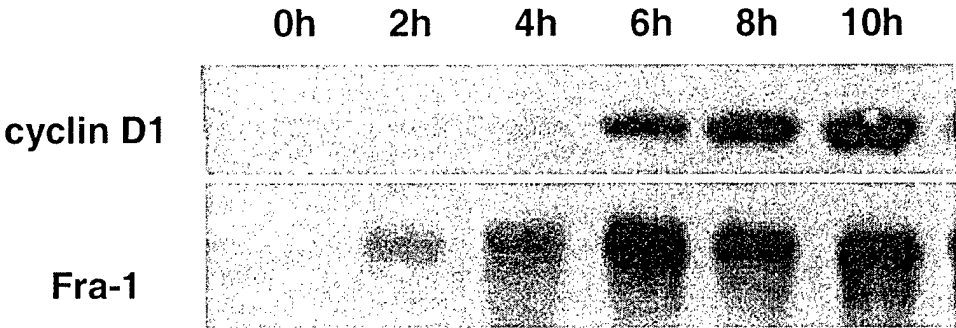
**Fra-1 is the only immediate early gene being inhibited from the induction upon serum stimulation**



**Figure 3**

BEST AVAILABLE COPY

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



BEST AVAILABLE COPY

Figure 4

## Schematic diagram of Fra-1 promoter reporter constructs

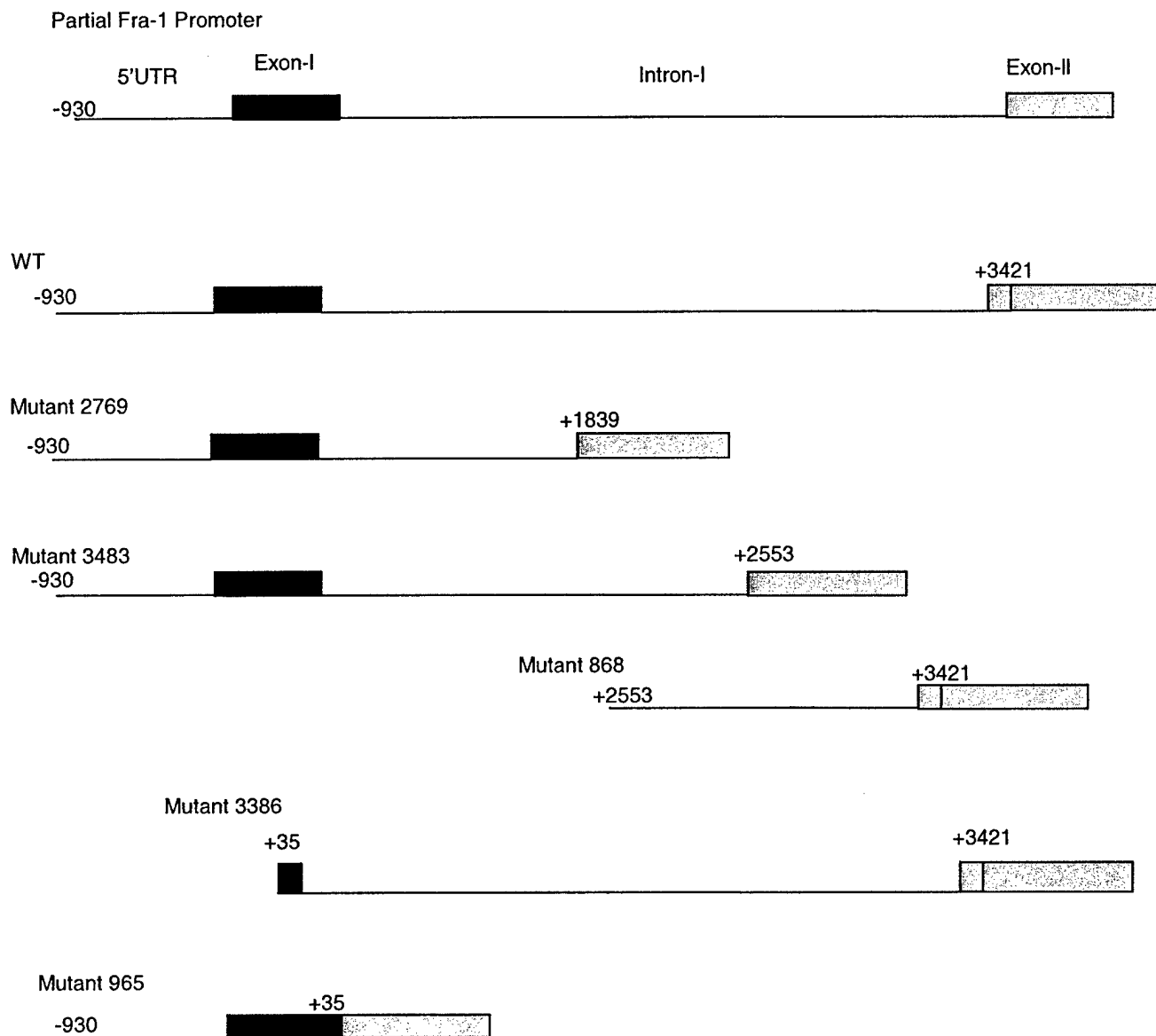


Figure 5

## 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

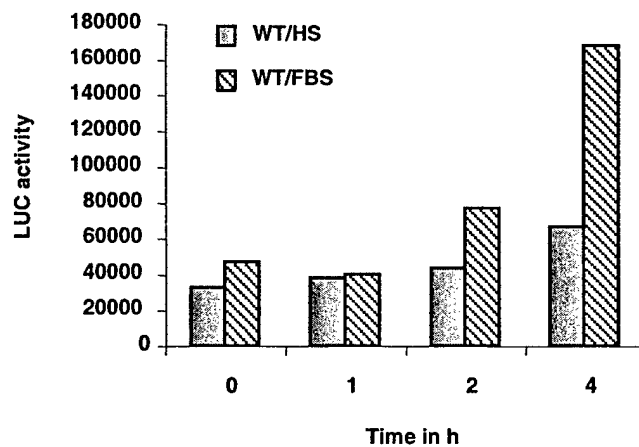
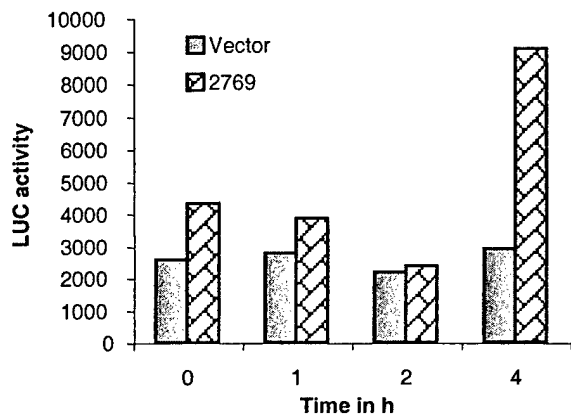


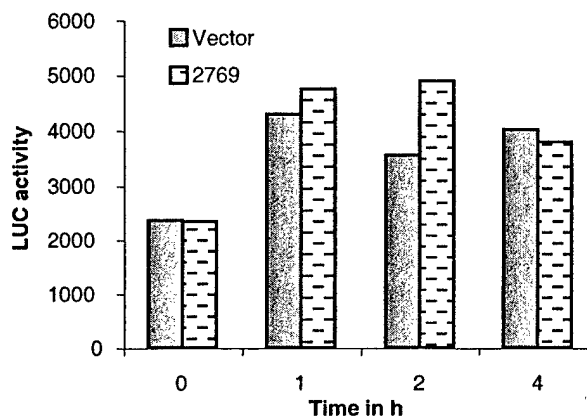
Figure 6

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

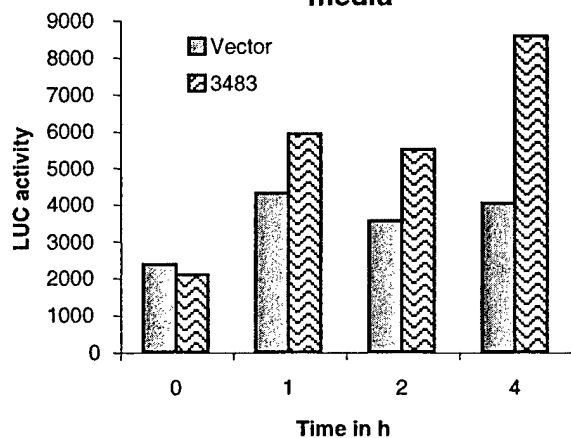
**Fra-1 promoter reporter deletion mutant in C2C12 cells with 0.5% FBS containing media**



**Fra-1 promoter reporter deletion mutant in C2C12 cells with 1% HS containing media**



**Fra-1 promoter reporter deletion mutant in C2C12 cells with 0.5% FBS containing media**



**Fra-1 promoter reporter deletion mutant in C2C12 cells with 1% HS containing media**

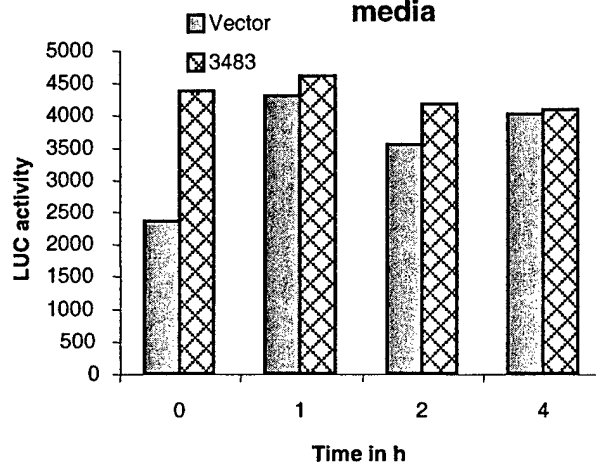
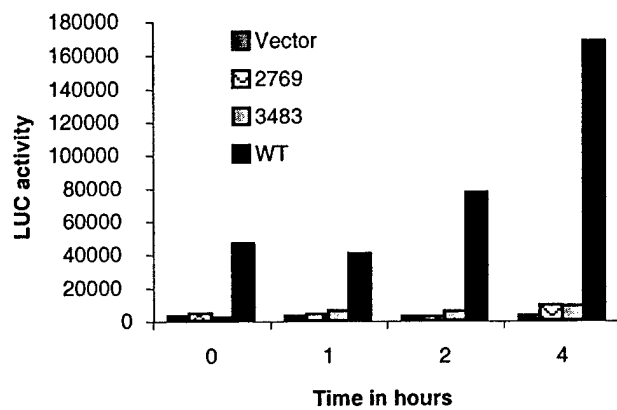


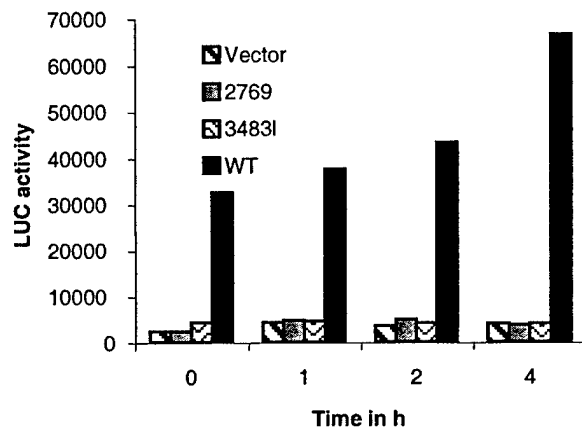
Figure 7

# Full-length Fra-1 promoter reporter showing a higher activation compare to deletion mutants construct upon serum stimulation in C2C12 myoblast cells

### Fra-1 promoter reporter constructs arrested in 0.5% FBS media



### Fra-1 promoter reporter constructs in 1% HS containing media



## Figure 8