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PRINCIPAL INVESTIGATOR: Daitoku Sakamuro, Ph.D.

CONTRACTING ORGANIZATION: Purdue University
West Lafayette, Indiana 47907-1063

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Daitoku Sakamuro, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Purdue University
West Lafayette, Indiana 47907-1063

E-mail: daitoku@pharmacy.purdue.edu

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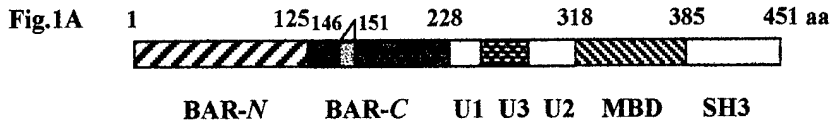
A. INTRODUCTION:

Cancer is a disease of aberrant cell growth. The c-Myc oncogene is commonly activated in tumors, including prostate cancers, and its over expression has been linked to both cell cycle progression and the induction of apoptosis; however the ambiguous role of this transcription factor has yet to be elucidated. The c-Myc function lies between two signaling networks that target its *C*-terminal DNA binding domain as well as its *N*-termini transcriptional activation domain. Bin1 (**B**ridge **in**tegrator-**1**) is a Myc-interacting protein that associates with the *N*-terminus of the Myc oncoprotein and inhibits malignant transformation induced by c-Myc and adenovirus E1A[1]. However, deletion mutants of Bin1 lacking the Myc-binding domain (MBD) were still capable of inhibiting cellular transformation of oncogenic Ras mediated by adenovirus E1A, thus indicating that Bin1 can inhibit malignant cell growth in a Myc-independent manner[2] (see Figure 1A & 1B).



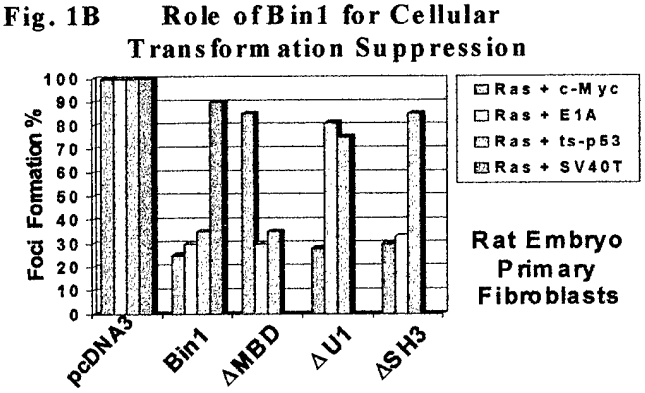


Figure-1. Structure and domain mapping of Bin1[1,2] A) Schematic diagram of Bin1 structure. BAR region shows structural homology among **B**in1, **A**mphiphysin (neuronal vesicle protein), and **R**vs167 (yeast cell cycle inhibitor). U1, U2, and U3 regions are **u**nique to Bin1. MBD is **M**yc-**B**inding **D**omain. SH3 is Src Homology-3 domain. Δ4 is a deletion of six amino acids (146-151aa) (see below). **B)** Transformation suppression by Bin1. Rat primary embryo fibroblast (REF) cells were transformed by H-Ras^{G12V} with various nuclear oncogenes[19,20], such as c-myc, adenoviral E1A, or oncogenic mutant p53. Foci were scored ~3 weeks later by methanol fixation and Giemsa stain.

Bin1 inhibits c-Myc-associated oncogenic activity by activating apoptosis[3], but how Bin1 inhibits E1A-dependent transformation is largely unknown. Unlike c-Myc/Bin1 association, we did not observe any binding activity of E1A to Bin1 in our biochemical conditions[9]. Therefore, we hypothesized that Bin1 is involved in the downstream pathways regulated by E1A.

E1A-dependent signals: E1A physically interacts with several cellular proteins that play key roles in cell proliferation and apoptosis, such as Rb retinoblastoma tumor suppressor [30-36] and p300/CBP transcriptional co-activator[37-42] (see Figure 2). Inactivation of the Rb protein by E1A is dependent on the physical interaction between the Rb's AB pocket domain and the E1A's Conserved Region-2 (CR2 region). The Rb inactivation by E1A is crucial for sustaining cell proliferation induced by E1A, at least in rodent fibroblasts, because it releases E2F1 transcription factor that is sufficient to promote cell cycle in quiescent cells[18], but under certain conditions, E2F1 induces apoptosis in a manner dependent p53 [17] and p73 [21-24]. Similarly, c-Abl tyrosine kinase induces p73-dependent apoptosis when Rb is deficient [25-29]. Furthermore, the E1A/Rb interaction stabilizes p53 tumor suppressor protein by a p14ARF-dependent sequestration of MDM2 protein in cells [43-51].

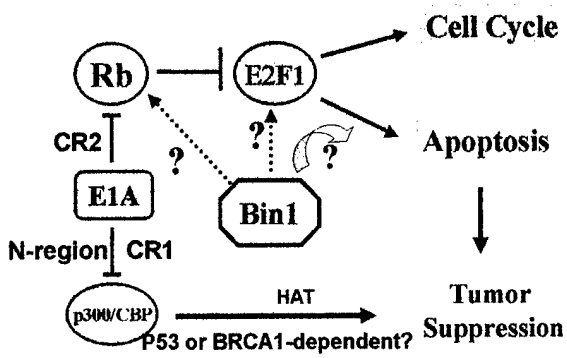


Figure 2. E1A-dependent Signal Pathways

We have developed research initiated by our initial observation as to the E1A/H-Ras co-transformation suppression by Bin1. Our preliminary studies have demonstrated that Bin1 is physically and functionally involved in the Rb/E2F1-dependent signaling pathways (see Task-1 and Task-2).

Bin1 gene expression and structure in cancer: The gene for Bin1 consists of 16 exons that lie at a region that is deleted approximately 42% of metastatic prostate cancers.[2] Bin1 ubiquitously expressed in both normal cells and tissues, with the highest levels in skeletal muscle and brain tissues, but is poorly detectable in almost 50% of carcinoma cell lines and primary breast tumors. Lack of endogenous expression in tumor cells is functionally meaningful because ectopic Bin1 expression inhibits tumor cell growth. Thus, Bin1 displays features as a tumor suppressor [5-8, 14].

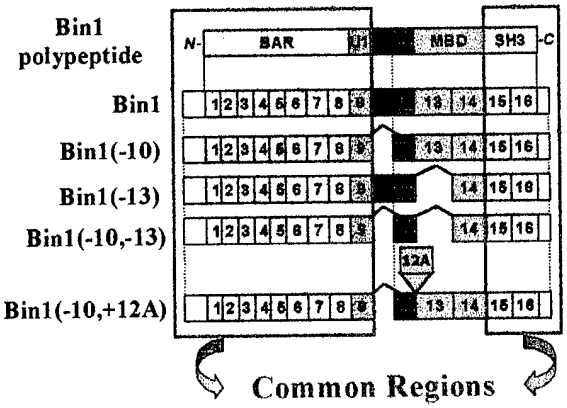


Figure 3. Non-neuronal Bin1 isoforms

Amphiphysin II, a brain-specific isoform of Bin1, contains brain-specific exons 12A, 12B, 12C and 12D that lacks tumor suppressor activity.[12, 15, 16] The exon 12A-containing Bin1 messenger RNA, Bin1(+12A) mRNA was identified in DU145 and PC3 androgen-independent prostate cancer cell lines and metastatic melanomas, suggesting that this exon could act as a signal for attenuating tumor suppression function of Bin1 in these cancers. Consistently, primary rat embryo fibroblast transformation assays indicated that the presence of exon 12A relieved the ability of Bin1 to inhibit focus formation of both c-myc co-transfected with oncogenic Ras and adenovirus EIA co-transfected with activated Ras.[4] However, there was no direct evidence to indicates the effect of Bin1(+12A) on the growth of androgen-dependent prostate cancer cell line LNCaP. In this progress report, we have addressed whether aberrant splicing of exon 12A leads to a loss of tumor suppressor activity of Bin1 in LNCaP (see Task-3).

Here is discussed the progress that has been made since February 2002 to determining the growth inhibitory effects of Bin1 and Bin1(+12A) in the context of the Rb/E2F1-dependent signal pathways in prostate cancer cell lines. Approaches first have been centralized to the initial confirmation of the physical involvement of Bin1 in the Rb/E2F1-dependent mechanisms and Bin1-dependent growth suppressive activities in the androgen-dependent LNCaP prostate carcinoma cell line. We also addressed whether Bin1(+12A) is involved in the sensitivity to androgen. We will elucidate the effects of Bin1 (+12A) and/or any involvement of p73 in these phenomena.

B: APPROVED STATEMENT OF WORK:

Task 1: ***To examine the physical and functional interaction of Bin1/E2F1 in vitro and in vivo (Months 1 – 18)***

1) GST-fusion protein-pull down assay in prostate cancer cell lysates (Months 1 – 6)

   a) Test the effect of E1A, oncogenic Ras, and Cyclin D1 on the Bin1/E2F1 interaction in LNCaP cell lysate (Months 1 – 6).
   b) Test the effect of androgen withdrawal on Bin1/E2F1 interaction (Months 1 – 6).

c) Examine if androgen-independent prostate cancer-associated mutant Bin1 binds E2F1 in DU145 cell lysate (Months 1 – 3).
d) Test if the Marked Box of E2F1 is sufficient for the interaction with Bin1 (Months 3 – 6).
e) Test if the BAR-N and/or the SH3 domain of Bin1 is sufficient for the interaction with E2F1 (Months 3 – 6).

2) Impact of Bin1 on E2F1-dependent transcriptional activation: Luciferase reporter assay (Months 6 – 12)

a) Examine if up-stream signals, such as oncogenic Ras, E1A, or Cyclin D1 that disrupts an Rb/E2F1 protein interaction, negatively regulates Bin1/E2F1 association in LNCaP cells (Months 6 –12).
b) Test if attenuation of Bin1 stimulates E2F1-dependent gene expression in LNCaP cells (Months 9 – 12).

3) Immunoprecipitation and Western blotting of the Bin1/E2F1 complex in LNCaP cells (Months 12 – 18)

a) Transient transfection of both Bin1 and E2F1 expression constructs into LNCaP cells and prepare cell lysates to detect a Bin1/E2F1 complex (Months 12 – 15).
b) Once the condition of transient transfection experiment is optimized to detect a Bin1/E2F1 complex in LNCaP cells, co-transfect the E1A, oncogenic Ras, or Cyclin D1 plasmids to see if they disrupts the Bin1/E2F1 association in LNCaP cells (Months 12 – 15).
c) Demonstrate a natural Bin1/E2F1 protein complex in plain LNCaP cells and also E1A-expressing LNCaP (Months 15 – 18).
d) If it does not work, put LNCaP cells in a pro-apoptotic stressful culture condition, then harvest cell lysates to see if Bin1 interacts with E2F1 (Months 15 – 18).

Task 2: ***To define the role of Bin1 for E2F1-dependent biological functions (Months 6 – 30)***

a) Develop a series of retrovirus plasmids required for expressing antisense Bin1, dominant negative Bin1, and an androgen-independent prostate cancer-related mutant Bin1 (Months 6 – 12)
b) Prepare replication-incompetent amphotropic retrovirus of the gene of interest, and infect them into E2F1-inducible LNCaP cells and E1A-expressing, E2F1-inducible LNCaP cells (Months 12 – 18).
c) Ascertain the expression of each protein in cells by western blot analysis (Months 12 – 18).
d) Perform analysis of cell morphology, growth curve, flow cytometry, chromatin condensation, and DNA ladder/TUNEL assay to see the effect of Bin1 attenuation on E2F1-dependent cell cycle progression, cellular senescence, and/or apoptosis in LNCaP cells and E1A-expressing LNCaP cells (Months 18 – 30).

Task 3: ***To test the impact of the Bin1/E2F1 association on androgen-sensitivity and chemo-resistance of prostate cancer cells (Months 30 – 36)***

a) Characterize the growth of LNCaP cells expressing Bin1(+12A) with or without E2F1 induction and evaluate the requirement of androgen (Months 30 – 36)

C. PROGRESS REPORTS:

Task 1: ***To examine the physical and functional interaction of Bin1/E2F1 in vitro and in vivo (Months 1 – 18).***

Bin1 interacts with both Rb and E2F1 in LNCaP prostate cancer cell lysate: We previously observed the physical interaction between E2F1 and Bin1 *in vitro* and thus hypothesized that Bin1 directly associates with E2F1 in prostate cancer cells and mediates E2F1-dependent apoptosis and/or cellular senescence in prostate cancers. According to Task-1-(1), we first performed GST-pull down experiments to examine whether E2F1 physically interacts with Bin1 in LNCaP androgen-dependent prostate cancer cell lysate. Since LNCaP cells express endogenous Bin1 protein, we used GST-E2F1 fusion protein as bait. As reported, E2F1 associates with DP1 to constitute a hetero-dimmer transcriptional protein complex. Hypo-phosphorylated Rb retinoblastoma protein physically binds to E2F1's transactivation domain and inhibits the E2F1-dependent transcriptional activation. Thus, besides GST alone, we included GST-Rb (ABC pocket region) that includes the three Rb pocket-domains-A, -B, and -C and GST-DP1 as additional negative controls for Bin1-binding. As Bin1 protein-expression negative control, we used cell lysates of MCF7 breast cancer cell line that does not express endogenous Bin1 message.

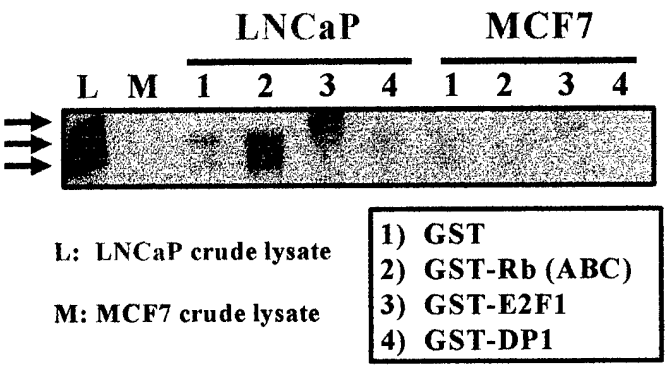


Figure 4. Western Blot Analysis of Bin1 Interaction with Rb, E2F1 and DP1 in LNCaP Cell Lysate

As shown in Figure 4, we observed that LNCaP cell line expresses three different sizes of Bin1 protein isoforms and, interestingly, only the longest Bin1 isoform interacts with E2F1 and the other ones interact with Rb (ABC). Neither one interacts with DP1. These data clearly indicate that Bin1 is physically involved in the Rb/E2F1-dependent cell growth regulation machinery in LNCaP prostate cancer cells.

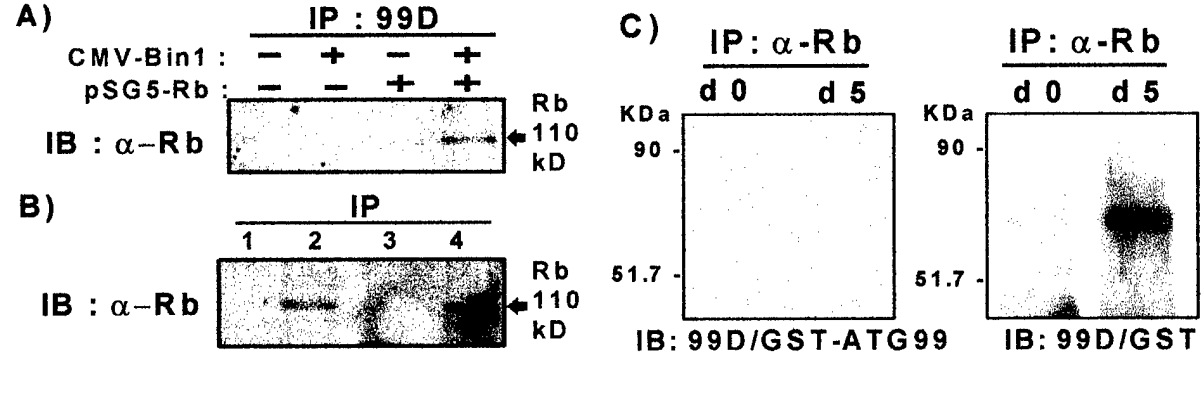
Although GST-fusion protein pull down assay is a very powerful method to study protein-protein physical interaction in cell lysate, the physiological relevance of such protein-protein association need to be evaluated through more physiological approach, such as immunoprecipitation. Thus, before investigating the effect of oncogenic stimuli generated by Ras, Cyclin D1, and/or adenovirus E1A that all could disrupt Rb-E2F1 association, on the Bin1-Rb and Bin1-E2F1 interaction, we next performed transient transfection experiments of both Bin1 and Rb (or E2F1) expression vectors into COS cells, followed by immunoprecipitation and Western blot analysis (Task-1-(3)).

Bin1-E2F1 association was hardly detectable in vivo: We clearly detected the Bin1-E2F1 protein physical interaction in both *in vitro* (previous preliminary data in our original grant proposal) and in cell lysate (see above, Figure 4). However, it has been difficult to confirm the Bin1-E2F1 association *in vivo* in our biochemical conditions (data not shown). Perhaps it was because the Bin1/E2F1 protein complex turned over very rapidly in mammalian cells. Or, it may be also possible that cells possessing the Bin1/E2F1 protein complex immediately undergo apoptosis (see below). In this regard, only a small fraction of either Bin1 protein or E2F1 protein (or both) might be necessary and sufficient for promoting apoptosis.

Bin1 physically interacts with Rb in vivo[9]: Transient co-transfection of Bin1 and Rb expression plasmids into COS-1 cells shows that full-length Bin1 interacts with Rb protein in cells (Figure 5A). To test the immuno-specificity of the Bin1/Rb complex, we extensively pre-cleaned anti-Bin1 monoclonal antiserum 99D [13] with excess amount of GST alone or GST-ATG99 polypeptide that encodes an epitope for the 99D [1,13]. We confirmed that Bin1 specifically interacts with Rb in COS-1 cells (Figure 5B).

However, it should be noted that protein-protein association observed by transient co-transfection of expression plasmids in COS-1 cells may be due to extremely high level of expression of each protein in cells and thus it may not be physiologically relevant. Therefore, we next used C2C12 murine myoblasts in which we know both Bin1 protein and Rb protein are naturally expressed. In this experiment, we did not transfect any expression plasmid DNA. We observed a strong Bin1-Rb protein association only in differentiated C2C12 cell lysate (day 5) but not in undifferentiated lysate (day 0) (Figure 5C). In differentiated C2C12, Rb protein should be hypophosphorylated. Thus, we conclude that Bin1 physically associated with hypo-phosphorylated Rb protein that interacts with and inhibits E2F1.

Bin1 spliced isoforms have differential Rb-binding affinity[9]: Since there seems to be differential affinity of different Bin1 isoforms to Rb interaction (see Figure 4), we next examined what spliced isoforms of Bin1 preferentially interact with Rb. As shown in Figure 5D, when Bin1 includes the exon 10, the binding affinity to GST-Rb(ABC) polypeptide was markedly increased. Moreover, we noticed that removal of exon13 that encodes Myc-binding domain increases the Bin1/Rb binding capability *in vitro*, indicating that different Bin1 splice isoforms may choose their preferable binding partners, such as Rb, c-Myc, or E2F1 by including (or excluding) exon 10 and/or exon 13 during normal cell proliferation or differentiation.



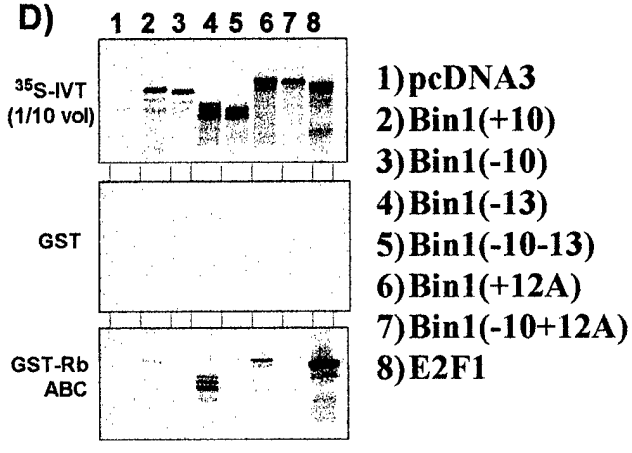


Figure-5. Physical interaction between Bin1 and Rb *in vivo* and *in vitro*. A) Twenty μg of CMV-Bin1 (which includes exon 10) and pSG5-Rb (a gift from Dr.W. Kaelin, Dana-Farber Cancer Inst.,Boston)[35] were transfected into COS-1 cells by DEAE-dextran method[52]. Each lysate was subjected to immunoprecipitation (IP) and immunoblot (IB) analysis with 99D anti-Bin1 and anti-Rb antibodies (Cell Signaling), respectively. **B)** The 99D anti-Bin1 monoclonal antiserum was pre-cleaned by GST peptide alone or GST-ATG99 peptide (encoding murine Bin1 fragment that encodes a 99D epitope). **(1)** mouse IgG, **(2)** 99D, **(3)** 99D pre-incubated with GST-ATG99, and **(4)** 99D pre-incubated with GST alone. **C)** IP/IB of C2C12 cell lysate. **d0**: undifferentiated, **d5**: differentiated. **D)** *in vitro* GST-pull down assay. Ten μL of [^{35}S-Met]-labeled *In Vitro* Translated (IVT) product (Promega) of Bin1 polypeptides was incubated with 5 μg of GST alone or GST-Rb(ABC) protein (a gift from Dr.E.Liu, NCI/NIH)[53] in NP40 binding buffer for 1 hour at 4 °C. Unbound IVT product was washed out four times at 4 °C.

***Domain mapping of Bin1 and Rb proteins required for the interaction*:** Domain mapping experiments show that both BAR/U1/U3 region and the SH3 domain of Bin1 are sufficient for the Rb-interaction (Figure 6A). Except the U3 region (almost encoded by exon 10), these regions are common among the all non-neuronal Bin1 spliced isoforms (Figure 3). In contrast, we found that Rb-C pocket region was sufficient to interact with Bin1 *in vitro* (Figure 6B). The binding efficiency of Bin1 to the Rb-C pocket was weaker than that of E2F1 to the Rb-AB pocket. This is probably due to different nature of the Rb requirement for E2F1 and Bin1, or the Rb-C pocket by itself may not be sufficient to support the Rb/Bin1 interaction. As shown in Figure 6A, there are two separate regions of Bin1 participating in the Rb interaction; the BAR-*C* region and the SH3 domain of Bin1. Thus, a second interaction between the Bin1-SH3 domain and Rb's amino-terminal proline-rich region may be required to stabilize the full-length Bin1/Rb association.

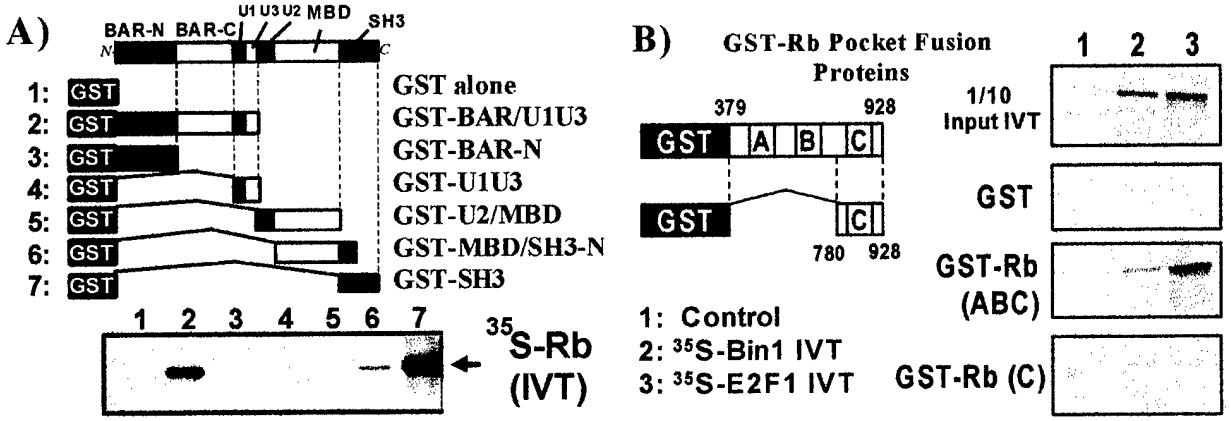


Figure-6. Domain mapping of Bin1/Rb interaction and Bin1/c-Abl interaction. GST-pull down experiments using [^{35}S-Met]-IVT products. 10 uL of ^{35}S-Methionine labeled IVT product was incubated with 2 ug each GST-fusion polypeptide.

Conclusion and Future Directions of Task-1: Taken all together, we conclude that Bin1 is physically involved in the Rb/E2F1-dependent cell growth control machinery. We will determine whether Bin1 association with Rb is disrupted when oncogenic Ras, Neu, and Cyclin D1 are overexpressed in LNCaP cells. We will also determine whether Bin1 association with Rb affects the phosphorylation status of Rb by cyclin-dependent kinase-4/6/Cyclin D/E complex.

Task 2. To define the role of Bin1 for the E2F1-dependent biological functions (Months 6-30):

Bin1 is required for E2F1-dependent apoptosis: Like c-Myc, E2F1 enhances cell cycle progression in quiescent cells, but if survival signals (in serum) are depleted, it enhances apoptosis in both p53-dependent manner and p53-independent manner. Recent reports have identified p73, one of the p53 protein family members, as a crucial target of E2F1 to mediate apoptosis. However, E2F1-dependent apoptosis does not naturally occur in normal cells because serum deprivation immediately dephosphorylates the Rb protein and such Rb physically binds to and inhibits E2F1-dependent transactivation. Thus, as long as the Rb/E2F1 interaction is properly controlled, serum deprivation induces cell cycle arrest by Rb, but not apoptosis by E2F1. As described above, Bin1 physically interacts with hypophosphorylated Rb (Figure 5C), and forced expression of ectopic Bin1 induces apoptosis selectively in cancer cells, including LNCaP prostate cancer cells (see below). These data prompted us to examine whether Bin1 is functionally required for E2F1-dependent apoptosis.

We have developed a zinc-inducible E2F1-expression system in Rat1 fibroblast cell line. Rat1 cell line expresses endogenous Bin1 protein (D.S, unpublished observation) and is sensitive to

apoptosis induced by E2F1. We isolated Rat1-clone #1 line that showed a highest level of ectopic E2F1 induction by zinc treatment (Figure 7A). Next, we examined E2F1-dependent transactivation activity by using two E2F1-responsive luciferase reporters; adenovirus E2A-promoter and c-myc-promoter as described in our original proposal. We observed approximately 50 times and 7 times hold induction of luciferase activity by the zinc-dependent induction of E2F1 protein expression, respectively. Thus, we conclude that the zinc-induced ectopic E2F1 is functional in Rat1 cells.

Next, we stably infected into Rat1-clone#1 cells (G418-resistant) a retrovirus pBabe-Bin1Δ4 (Puromycin-resistant) that expresses a dominant negative inhibitor of Bin1. When the cells resistant to both G418 and Puromycin were incubated with zinc (100 μM) in 0.5% FBS for 24 hours, apoptotic cell death was induced, but attenuated in the Bin1Δ4 retrovirus-infected cells (see Figure 7B). As reported before, Bin1Δ4 attenuates c-Myc-dependent apoptosis in Rat1 cells[3] and importantly, a recent paper demonstrates that c-Myc requires E2F1 to induce apoptosis in primary mouse embryo fibroblast cells[54]. Taken together, we conclude that Bin1 is a proapoptotic effector downstream of (or parallel to) E2F1.

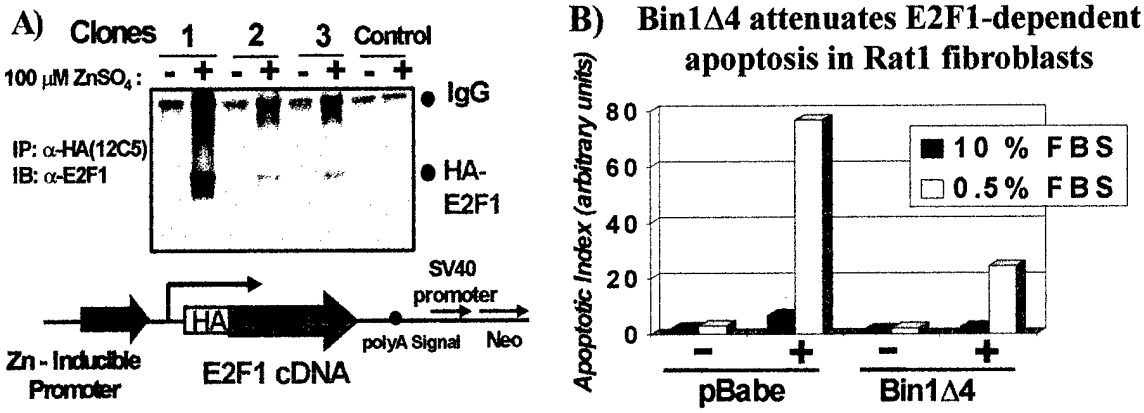


Figure 7. Bin1 activity is required for E2F1-induced apoptosis. A) Establishment of an E2F1-inducible Rat1 fibroblast cell line. CB6MT-HA-E2F1, a Zinc-inducible E2F1-expression plasmid vector includes a Metallothionein-derived, Zinc-inducible promoter (a gift of Dr.F.Rauscher III, Wistar Inst., Philadelphia) and an HA-tagged E2F1 cDNA (a gift of Dr. W.Kaelin, Dana-Farber Cancer Inst., Boston). Induced HA-E2F1 protein in Rat1 cells was detected by IP (anti-HA) followed by IB (anti-E2F1). **B)** Requirement of Bin1 for E2F1-dependent apoptosis. Retrovirus Bin1Δ4 was infected into the Rat1-clone#1 cell line. After incubating the cells in culture medium including 0.5% FBS wirh (+) or without (-) 100 μM Zinc for 24 hours, cells were stained by DAPI and chromatin condensed cells were counted under fluorescence microscope as described previously[8-10].

Synergistic Effect of Bin1/E2F1 but not Bin1/c-Myc on LNCaP cell death[10]: Since Bin1 is required for apoptosis induced by c-Myc[3] and E2F1 (Figure 7), we next examined whether either E2F1 or c-Myc or both is apoptotic to LNCaP cells and whether these transcription factors synergistically increase the apoptosis induced by Bin1. As shown in Figure 8, we observed that both E2F1 and c-Myc show similar toxicity in LNCaP cells.

Interestingly, when Bin1 was co-transfected with E2F1, the moderate level of toxicity of Bin1 was markedly increased. This may be simply because two toxic proteins are expressed simultaneously and thus combined toxicities from two apoptotic proteins killed LNCaP cells more effectively. However, when Bin1 was co-transfected with c-Myc, we did not see the similar synergistic effect. Transfected c-Myc vector was functional because it showed similar synergistic toxicity when co-transfected with E2F1 (Figure 8B). Thus, it is hypothesized that Bin1 protein (or c-Myc protein) is destabilized when c-Myc (or Bin1) is overexpressed in LNCaP cells; otherwise overexpressed c-Myc may cancel Bin1's apoptotic function. We will test these possibilities by transfecting Bin1(-13) that

does not interact with c-Myc but still toxic to LNCaP. In either case, we conclude that Bin1 and E2F1 cooperatively activate apoptosis in LNCaP cells.

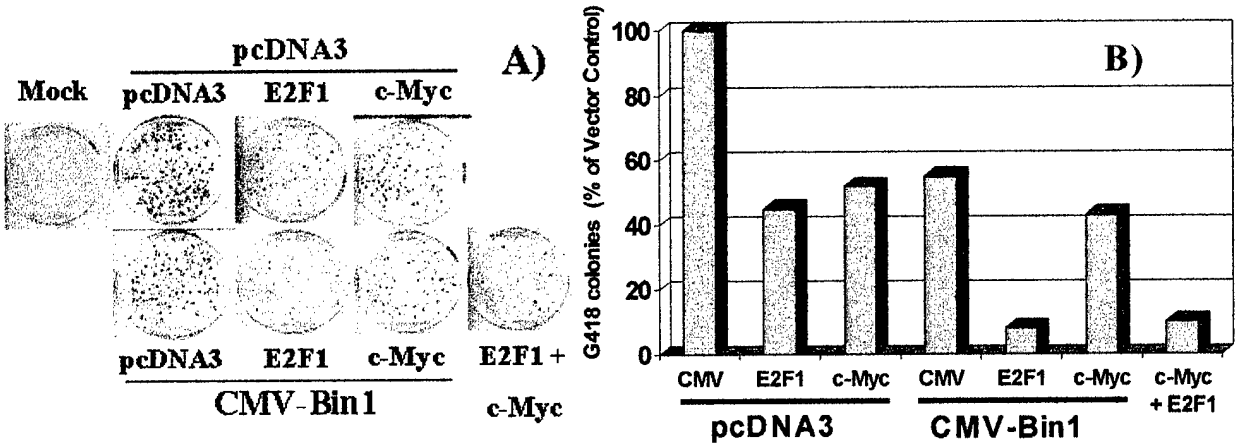


Figure 8. LNCaP prostate carcinoma cell colony formation suppression assay by Bin1, E2F1, and c-Myc. 1 μg of pcDNA3 or CMV-Bin1 was co-transfected with 1 μg of pcDNA3, CMV-E2F1, or CMV-Myc by Fugene-6 transfection reagent into 5 x 10^5 LNCaP cells in a 6-cm dish. Data represent the results from two independent experiments

Conclusion and Future Directions of Task-2: We found that 1) Bin1 mediates E2F1-induced apoptosis in Rat1 cells and 2) both Bin1 and E2F1 are toxic to LNCaP prostate cancer cells. As reported by Elliott *et al*[11], Bin1 induces apoptosis independent of caspase and p53. Since E2F1-induced apoptosis is dependent on p53 and p73 and caspase-cascades, our observation suggests that the Bin1-E2F1 apoptosis may be dependent on p73 or totally new mechanism. Thus, we will next determine whether Bin1-induced apoptosis is attenuated by dominant negative mutant of p73 in LNCaP cells. We will also determine whether p73-induced apoptosis is blocked by antisense-Bin1 in LNCaP cells that express endogenous Bin1. Our recent preliminary data show that LNCaP cell line is sensitive to p73 beta isoform (data not shown).

Task 3: ***To test the impact of the Bin1/E2F1 association on androgen-sensitivity and chemo-resistance of prostate cancer cells (Months 30 – 36)***

In androgen-independent prostate cancer cell lines DU145 and PC3, but not androgen-dependent prostate cancer cell line LNCaP, an aberrant spliced isoform of Bin1, Bin1(+12A) that incorporates brain-specific exon 12A was detected. In rat embryo primary fibroblast transformation assay, Bin1(+12A) did not exert any growth suppression activity. This observation prompted us to hypothesize that Bin1(+12A) could act as a dominant negative inhibitor to Bin1 and thus promote prostate cancer cell proliferation.

G418 resistant colonies were isolated and screened for the presence of the Bin1(+12A) protein product using the mouse monoclonal anti-Bin1(+12A). From screening twelve clones, two independent clones were shown to contain the +12A exon (clone #6 and #8) as evident through Western analysis (Figure 9).

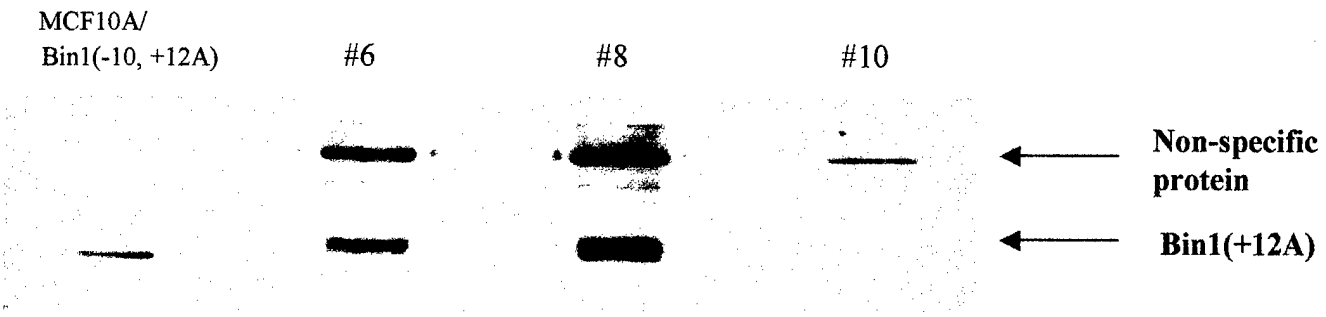


Figure 9. Screening of Bin1 (+12A) in LNCaP G418-resistant clones.

Bin1(+12A) is not toxic to LNCaP but moderately inhibits cell proliferation[10]: To examine if splicing of exon 12A in Bin1 causes an increase or decrease in the growth rate of LNCaP cells that endogenously express Bin1, a growth curve was generated. Results indicated that aberrant splicing does not increase the growth rate of LNCaP cells. LNCaP cell lines overexpressing Bin1(+12A) clone #6 and clone #8 show moderate growth suppressive activity (Figure 10).

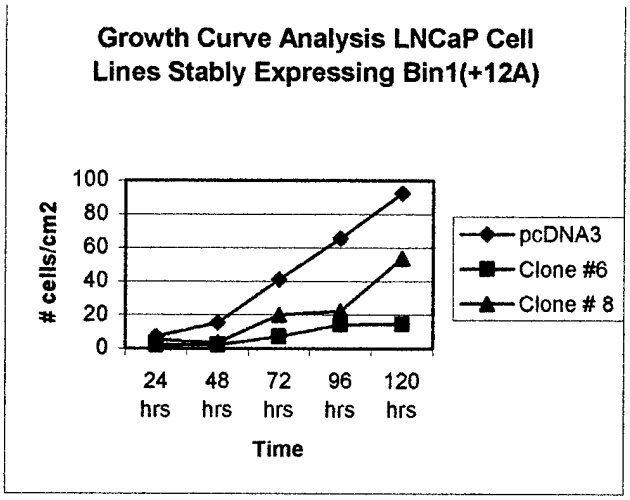
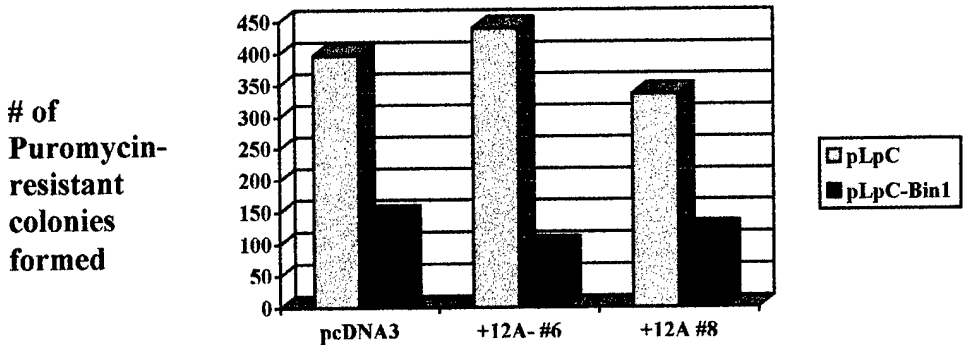


Figure 10. Growth Curve Analysis of LNCaP stably expressing Bin1(+12A)

Bin1(+12A) is not a dominant negative inhibitor of Bin1 in LNCaP cell line[10]: A colony formation assay was performed to determine whether growth suppression by ectopic Bin1 transfection was attenuated in LNCaP stably expressing Bin1(+12A). Based on the reduced number of Puromycin-resistant colonies in clones #6 and 8 of LNCaP cells being comparable to that of the control LNCaP cell line when transfected with a Puromycin-resistant Bin1 expression vector, it appears that Bin1(+12A) does not act as a dominant negative inhibitor to Bin1 in LNCaP cells[10] (Figure 11).

Therefore, another approach to generating a Bin1 inhibitor is in its initial trials. The utilization of antisense (AS)-Bin1 will be used to inhibit endogenous Bin1 activity. A plasmid transfection of AS-Bin1 will be performed, and G418-resistant colonies will be isolated and screened through Western Analysis. The absence of Bin1 expression through Western blot analysis will indicate that this antisense Bin1 is in fact inhibiting endogenous Bin1 in LNCaP cells.



# of G418 Resistant colonies formed

	pcDNA3	+12A- clone#6	+12A clone#8
pLpC	398	440	336
pLpC-Bin1	142	93	118

Figure 11. Bin1-mediated apoptosis is not attenuated by the co-existence of Bin1(+12A) in LNCaP cells

Splicing of exon 12A is not a signal for androgen independence of prostate cancer cells[10]: The brain-specific Bin1(+12A) message was detected in androgen independent cell lines, DU145 and PC3 metastatic prostate cancer.[4] Being that LNCaP cells are androgen dependent, we hypothesized that the presence of this spliced isoforms could act as a signaling target to convert this androgen dependent cell line into one of its androgen independent tissue family members. LNCaP stably expressing Bin1(+12A) clone #6 and clone #8 were subjected to androgen-depleted conditions in 10% charcoal-dextran treated (CDT)-FBS (Fetal Bovine Serum) media, which strips the media of its androgen components, for five days. The cells were visualized under a light microscope (Figure 12). DU145 androgen-resistant prostate cancer cell line was included as control. Our results clearly indicate that the aberrant splicing of exon 12A does not contribute to the survival of LNCaP in androgen-depleted conditions.

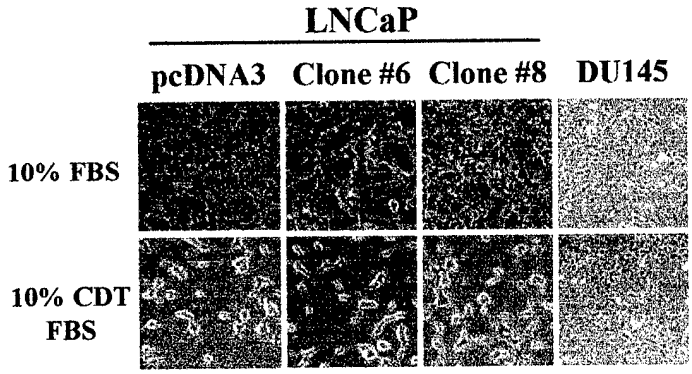


Figure 12 Bin1(+12A) Expressing LNCaP Cells Are Still Androgen-Sensitive

Conclusion and Future Directions of Task-3: We found that 1) androgen-resistant prostate cancer-associated Bin1 aberrant sliced isoform, called Bin1(+12A), is a moderate growth suppressor in LNCaP cell line, 2) Bin1(+12A) does not interfere with apoptotic property of Bin1 in LNCaP cell line, and 3) Bin1(+12A) does not convert androgen-dependent phenotype of LNCaP cell line to androgen-resistant one. Our data clearly demonstrate that abnormal splicing event of Bin1 is associated with androgen-resistance but is unlikely to play a role to promote such malignant conversion. To determine whether Bin1 tumor suppressor function is involved in chemosensitivity and chemoresistance of prostate cancer cells, we will next generate antisense-Bin1 expressing LNCaP and sense-Bin1 expressing DU145 cell lines and put them in anticancer drug containing culture medium.

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