

AD _____

Award Number: W81XWH-04-1-0344

TITLE: Targeting of the Nuclear Receptor Coactivator Isoform Delta 3aib1 in Breast Cancer

PRINCIPAL INVESTIGATOR: Christopher Chien

CONTRACTING ORGANIZATION: Georgetown University
Washington, DC 20057

REPORT DATE: July 2007

TYPE OF REPORT: Annual Summary Addendum

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.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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 Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE 01-07-2007		2. REPORT TYPE Annual Summary Addendum		3. DATES COVERED 23 Feb 2007 – 30 Jun 2007	
4. TITLE AND SUBTITLE Targeting of the Nuclear Receptor Coactivator Isoform Delta 3aib1 in Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0344	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Christopher Chien Email: cdc35@georgetown.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Georgetown University Washington, DC 20057				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT AIB1 which stands for "Amplified in Breast Cancer", codes for a protein that is a member of the steroid receptor coactivator (SRC) family. AIB1 is amplified in approximately 5-10% of breast cancers and the mRNA and protein overexpressed in >30% of breast cancers. AIB1 interacts with a superfamily of ligand activated nuclear receptors to potentiate transcriptional activity leading to upregulation of downstream target gene expression. An important finding was that an isoform of AIB1 (Δ 3AIB1) is a significantly more effective coactivator of the estrogen receptor than AIB1 and is highly overexpressed in human breast cancer. Prior work in our lab showed that the downregulation of overall levels of AIB1 plus Δ 3AIB1, using a regulatable AIB1 directed ribozyme, resulted in reduced tumor growth in vivo. Overall, these data indicate a major role for AIB1 and its isoform Δ 3AIB1 in breast cancer development and growth. However the relative roles of AIB1 versus the more highly active Δ 3AIB1 in phenotypic changes in the breast has not been determined. In this investigation, we are developing a method to use siRNA directed at Δ 3AIB1 in order investigate its role in breast cancer and as a possible future therapeutic approach to breast cancer.					
15. SUBJECT TERMS Δ 3AIB1, breast cancer					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	12	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....	page 4
Body.....	page 5
Key Research Accomplishments.....	page 9
Reportable Outcomes.....	page 10
Conclusions.....	page 11
References.....	page 12

INTRODUCTION

Of particular interest to breast cancer was the discovery that an area of chromosome 20q, known to be frequently amplified in breast cancer, harbored the gene for AIB1. AIB1, which stands for " **A**mplified **I**n **B**reast cancer" codes for a protein which is a member of the steroid receptor coactivator (SRC) family. AIB1 is amplified in approximately 5-10% of breast cancers and the mRNA and protein overexpressed in >30% of breast cancers. AIB1 interacts with a superfamily of ligand activated nuclear receptors including the estrogen receptor (ER) and progesterone receptor (PR) to potentiate transcriptional activity leading to upregulation of downstream target gene expression. An important finding was that an isoform of AIB1 ($\Delta 3$ AIB1) is a significantly more effective coactivator of the estrogen receptor than AIB1 and is highly overexpressed in human breast cancer. Prior work in our lab showed that the downregulation of overall levels of AIB1 plus $\Delta 3$ AIB1, using a regulatable AIB1 directed ribozyme, resulted in reduced tumor growth *in vivo*. Overall, these data indicate a major role for AIB1 and its isoform $\Delta 3$ AIB1 in breast cancer development and growth. However the relative roles of AIB1 versus the more highly active $\Delta 3$ AIB1 in phenotypic changes in the breast has not been determined. In this report I will summarize the previous work with a focus on work completed from February 23, 2007 to June 30, 2007.

BODY

Task 1: Design small interfering siRNA molecules that specifically target the nuclear receptor coactivator isoform $\Delta 3AIB1$.

Although we were able to successfully target AIB1 with both siRNA and the short-hairpin RNAs, we were unable to target $\Delta 3AIB1$ specifically without affecting the protein expression of AIB1. The region specifically targeted by various siRNAs and short-hairpin RNAs that we created focused on the splice junction in $\Delta 3AIB1$ of exon 2 with exon 4 (Figure 1). This sequence is absent from AIB1 and is unique to $\Delta 3AIB1$. Successful knockdown of AIB1 is possible by siRNA and short-hairpin RNA as shown in previous reports, however we were unable to specifically decrease the expression of $\Delta 3AIB1$ and always found that some of the full length AIB1 was also targeted (Figure 2). This result is somewhat surprising since dogma indicates that a mismatch of 2 or more nucleotides should not be specific enough for knockdown of expression.

AIB1 transcript

TTCAAG ATG AGTGGATTAGGAGAAAACCTTGGATCCACTGGCCAGTGATTACGAAAACGCAAATT
GCCATGTGATACTCCAGGACAAGGTCTTACCTGCAGTGGTGAAAAACGGAGACGGGAGCAGGAAA
GTAAATATATTGAAGAATTGGCTGAGCTGATATCTGCCAATCTTAGTGATATTGACAATTTCAATGT
CAAACCAGATAAATGTGCGATTTTAAAGGAAAACAGTAAGACAGATACGTCAAATAAAAAGAGCAAG
AAAAACTATTTCCAATGATGATGATGTTCAAAAAGCCGATGTATCTTCTACAGGGCAGGGAGTTAT
TGA TAA AGACTCCTTAGGACCGCTTTTACTTCAGGCATTGGATGGTTTCCTATTTGTGGTGAATCG
AGACGGAAACATTGTATTTGTATCAGAAAATGTACACAATACCTGCAATATAAGCAAGAGGACCT
GGTTAACACAAGTGTTTACAATATCTTACATGAAGAAGACAGAAAGGATTTTCTTAAGAATTTACCA
AAATCTACAGTTAATGGAGTTTCCTGGACAAATGAGACCCAAAGACAAAAAAGCCATACATTTAAT
TGCCGT ATG TTGATGAAAACACC

$\Delta 3AIB1$ transcript

TTCAAG ATG AGTGGATTAGGAGAAAACCTTGGATCCACTGGCCAGTGATTACGAAAACGCAAATT
GCCATGTGATACTCCAGGACAAGGGA~~AAAAACTATTTCCA~~ATGATGATGATGTTCAAAAAGCCGAT
GTATCTTCTACAGGGCAGGGAGTTATTGA TAA AGACTCCTTAGGACCGCTTTTACTTCAGGCATTG
GATGGTTTCCTATTTGTGGTGAATCGAGACGGAAACATTGTATTTGTATCAGAAAATGTACACAAT
ACCTGCAATATAAGCAAGAGGACCTGGTTAACACAAGTGTTTACAATATCTTACATGAAGAAGACA
GAAAGGATTTTCTTAAGAATTTACCAAAATCTACAGTTAATGGAGTTTCCTGGACAAATGAGACCCA
AAGACAAAAAAGCCATACATTTAATTGCCGT ATG TTGATGAAAACACC

Figure 1: AIB1 and $\Delta 3AIB1$ mRNA transcripts. Exon 2 sequence is highlighted in green. Exon 3 sequence is underlined. Exon 4 sequence is highlighted in orange. The translation start site of AIB1 and $\Delta 3AIB1$ are marked by the blue and yellow boxes respectively. The region in $\Delta 3AIB1$ targeted by RNA interference is marked by the dashed box.

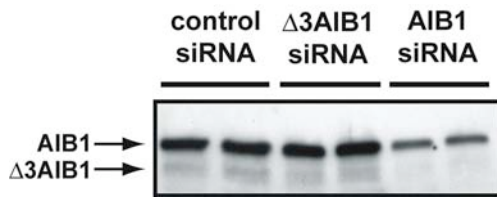


Figure 2: 24 hours before transfection, MCF-7 cells were plated in a 10-cm dish at 50% confluency in IMEM+10% FBS. 60 μ L of 20 μ M siRNA was diluted with 1 mL of IMEM plus 60 μ L of Oligofectamine. After washing, the siRNA-Oligofectamine complex was added to the cells in 5 mL of IMEM and incubated for 4 hours at 37°C. 1.5 mL of IMEM+30% FBS was added to the transfected cells and incubated for 16 to 18 hours at 37°C. Cell lysates were then harvested and subjected to western blot analysis. Control siRNA was a sequence that does not target any mammalian mRNA.

Task 2: To determine if siRNA reduction of cellular levels of AIB1 or Δ 3AIB1 can change the phenotype of breast cancer cell lines.

During ongoing studies of Δ 3AIB1 in breast cancer, we have recently observed that AIB1 is degraded in response to serum withdrawal in several immortalized cell lines. We have further found that the Δ 3AIB1 isoform is resistant to degradation in high cell density conditions (Figure 3). A recent publication from our lab suggests that this is a proteasome-associated effect (Mani et al). It would be interesting to target Δ 3AIB1 by RNA interference developed under Task 1 to see if it is possible to knockdown Δ 3AIB1 protein levels in high cell density conditions. This result may hold important implications for the study we plan to do in this task where we will specifically target the Δ 3AIB1 isoform *in vivo*. It is interesting to note that another construct Δ N19AIB1, which lacks the first 19 amino acids of the N terminus of AIB1 has also been shown to be resistant to proteasomal degradation¹. In this paper they suggest that Δ N19 is resistant to degradation because it lacks a nuclear localization signal and that importation into the nucleus is vital for regulation by the proteasome and for its coactivator function. Δ 3AIB1 may be resistant to degradation because it also lacks the nuclear localization sequence however, we and other groups have shown that Δ 3AIB1 has not lost any coactivator function and in fact is a more potent coactivator than full length AIB1². To further explore this inconsistency, I have examined the localization of Δ 3AIB1 in relation to AIB1 and Δ N19 by immunofluorescence (Figure 4). AIB1 is predominantly nuclear. Δ 3AIB1 and Δ N19 are found primarily in the cytoplasm, but also show staining in the nucleus suggesting there may be either another nuclear localization sequence or other mechanism to transport Δ 3AIB1 and Δ N19 into the nucleus. It is possible that the differential staining of Δ 3AIB1 and AIB1 is due to a difference in how the proteins are trafficked in the cell. I am currently exploring this possibility.

Figure 3: HEK293 cells were plated at 3.8×10^6 cells per 10cm poly d-lysine coated tissue culture plate. Cells were transfected with FLAG tagged AIB1, FLAG tagged $\Delta 3$ AIB1 isoform, or FLAG tagged AIB1 $\Delta 1-19$ 24 hours after plating. Cells were treated with the proteasome inhibitor MG-132 or an equal volume of DMSO overnight 24 hours post transfection. Cell lysates were then prepared in 1% NP-40 lysis buffer and Western blotted for FLAG and actin. Results are representative of two separate experiments.

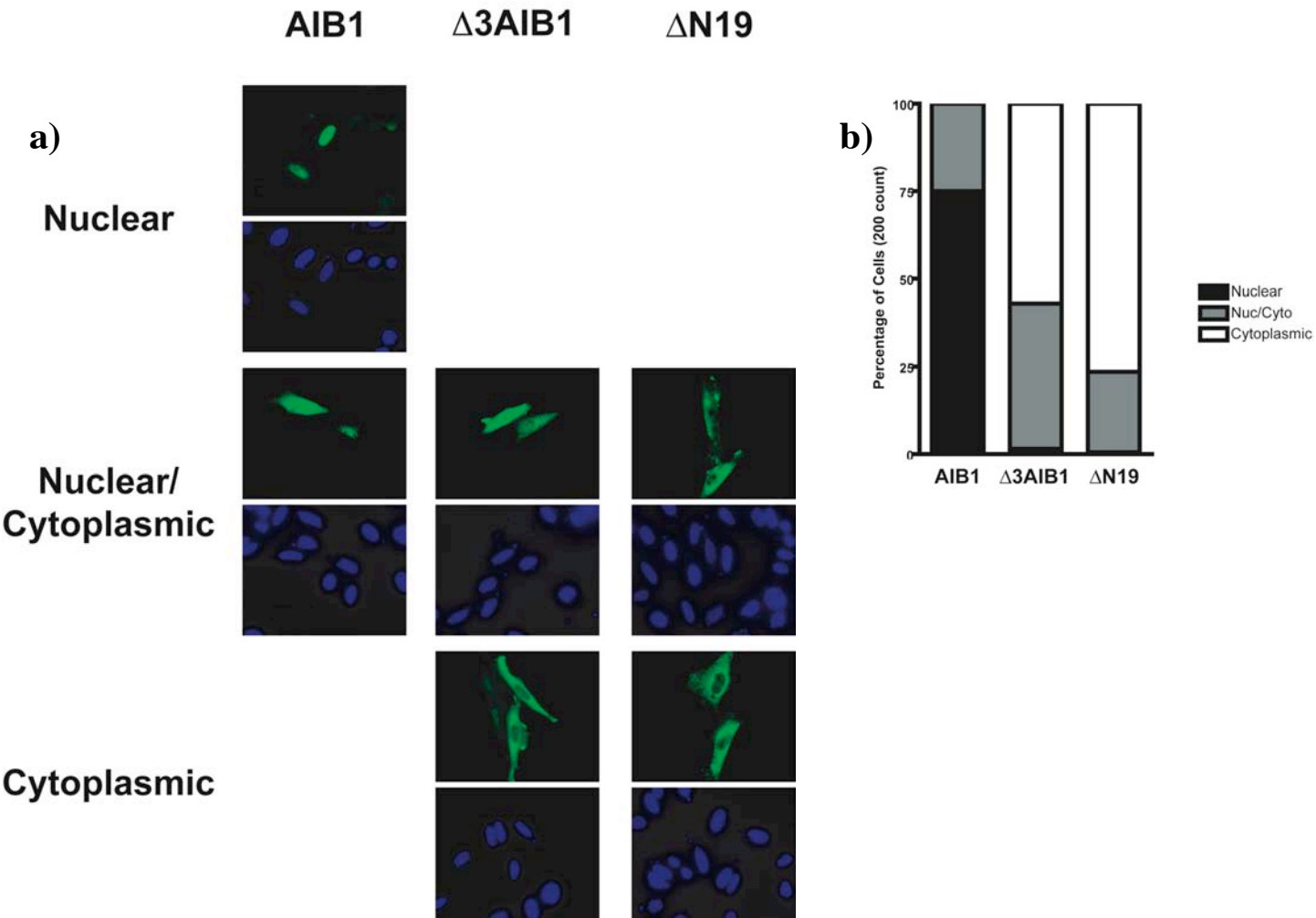
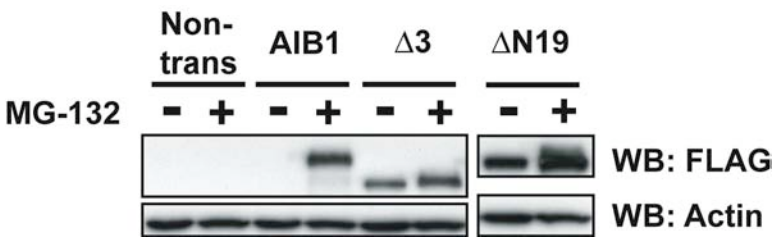


Figure 4: **a)** Chinese Hamster Ovary (CHO) cells were plated on glass coverslips and transfected with FLAG tagged AIB1, FLAG tagged $\Delta 3$ AIB1, or FLAG tagged $\Delta N19$. Cells were fixed in 3.7% paraformaldehyde and permeabilized in PBS+0.2% Triton X100. They were then stained with DAPI, 1:500 diluted FLAG M2 antibody, and 1:1000 AlexaFluor 488 anti-mouse antibody. Cells were then visualized by fluorescence microscopy. **b)** Quantitation of cells that showed staining in the nuclear, cytoplasmic, or nuclear and cytoplasmic compartments of the cell. 200 cells were counted per transfection.

Task 3: To determine if $\Delta 3$ AIB1 siRNA is effective *in vivo*.

We were not able to complete this task since we were unable to develop a siRNA that specifically knocks down $\Delta 3$ AIB1 expression without affecting the levels of AIB1. The recent observation that we made showing that the

$\Delta 3$ AIB1 isoform is resistant to density dependent proteasomal degradation to which the wild-type AIB1 is susceptible is an intriguing and novel finding and may be useful in teasing out $\Delta 3$ AIB1 specific mechanisms. We speculate that resistance to degradation displayed by the $\Delta 3$ AIB1 isoform in a high cell density condition might provide a mechanistic explanation for the finding by Reiter et al that the isoform's ability to promote transcription mediated by the estrogen or progesterone receptors was significantly greater than that of the full-length protein.²

KEY RESEARCH ACCOMPLISHMENTS

Determined that large mismatches in interfering RNAs is still permissible for knockdown of a target sequence.

Defined resistance of $\Delta 3$ AIB1 to proteasomal degradation.

Determined cellular localization of $\Delta 3$ AIB1 relative to AIB1 protein.

REPORTABLE OUTCOMES

“E6AP Mediates Regulated Proteasomal Degradation of the Nuclear Receptor Coactivator Amplified in Breast Cancer 1 in Immortalized Cells.” **Aparna Mani**, Annabell S Oh, Emma T Bowden, Tyler Lahusen, Kevin L Lorick, Allan M Weissman, Richard Schlegel, Anton Wellstein, and Anna T. Riegel, *Cancer Research* 2006 Sep 1;66(17):8680-6.

“Δ3AIB1 Localization Illustrates a Paradigm for P160 Coactivator Function and Regulation.” Annabell S Oh, Chris Chien, Tyler Lahusen, Anton Wellstein, and Anna T. Riegel, *Manuscript in preparation*.

CONCLUSIONS

Although we were unable to specifically target $\Delta 3$ AIB1 by RNA interference, we were able to further define how AIB1 protein is differentially regulated in relation to $\Delta 3$ AIB1. We plan to continue studies on the intriguing finding that the isoform $\Delta 3$ AIB1 is resistant to proteasomal degradation compared to wild-type AIB1. We are currently investigating the coactivator function of $\Delta 3$ AIB1 in relation to $\Delta N19$ to try to resolve the discrepancy that $\Delta 3$ AIB1 is a potent coactivator though it lacks the nuclear localization signal to enter the nucleus. We are also trying to ascertain if there is a difference in how AIB1 and $\Delta 3$ AIB1 are trafficked in a cell.

REFERENCES

1. Li et al. 2007, *Mol Cell Bio.* V27 No.4:1296-1308.
2. Reiter et al. 2001, *J Biol Chem.* V276:39736-41.