THE NUCLEAR FUNCTION OF THE SPLICE VARIANT AMPLIFIED IN BREAST CANCER 1-Δ4 CONTRIBUTES TO ITS ONCOGENICITY

A Dissertation submitted to the Faculty of the Graduate School of Arts and Sciences of Georgetown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Tumor Biology

By

Christopher Daniel Chien, M.S.

Washington, DC
July 11, 2011
Copyright 2011 by Christopher Daniel Chien
All Rights Reserved
THE NUCLEAR FUNCTION OF THE SPlice VARIANT AMPLeIFIED IN BREAST CANCER 1-Δ4 CONTRIBUTES TO ITS ONCOGENICITY

Christopher Daniel Chien, M.S.

Thesis Advisor: Anna T. Riegel, Ph.D.

ABSTRACT

The oncogene amplified in breast cancer 1 (AIB1) is a nuclear receptor coactivator that plays a major role in the progression of various cancers. We previously identified a splice variant of AIB1 called AIB1-Δ4 that is overexpressed in breast cancer. In this same report AIB1-Δ4 was found to be a more potent coactivator of steroid hormone transcription that full-length AIB1. The underlying mechanism to explain this potent coactivation had yet to be explored. The AIB1-Δ4 protein is a N-terminally truncated isoform of AIB1 and we propose that loss of this N-terminal region is the reason why AIB1-Δ4 is a more potent coactivator. In this study we used mass spectrometry to define the translation initiation of AIB1-Δ4 at Met224 of the full-length AIB1 sequence and have raised an antibody to a peptide representing the acetylated N-terminus. We determined that AIB1-Δ4 is predominantly localized in the cytoplasm, although leptomycin B nuclear export inhibition demonstrates that AIB1-Δ4 can enter and traffic through the nucleus. Our data indicate an import mechanism enhanced by other coactivators such as p300/CBP and AIB1. We report that the endogenously and exogenously expressed AIB1-Δ4 is recruited as efficiently as full-length AIB1 to estrogen-response elements of genes, and it enhances estrogen-dependent transcription more effectively than AIB1. Expression of an N-terminal AIB1 protein fragment, which is lost in the AIB1-Δ4 isoform, potentiates AIB1 as a coactivator. This suggests a model whereby the transcriptional activity of AIB1 is squelched by
a repressive mechanism utilizing the N-terminal domain and that the increased coactivator function of AIB1-Δ4 is due to the loss of this inhibitory domain. We observed that this N-terminal region of AIB1 is a region of negative phosphorylation and possibly a domain of protein-protein interaction. Using Scorpion primer technology, we show that AIB1-Δ4 expression is correlated with metastatic capability of human cancer cell lines. And lastly, we do not see an effect on in vitro proliferation of cells or invasiveness due to expression of AIB1-Δ4 protein.
DEDICATION

I dedicate this thesis and the work in it to the special people who have made this work possible. To my wife Jennifer and daughter Natalie who have kept the moments in between experiments fun and exciting. To Tina Tang and Paul Chien for taking the role of babysitter so that I could pursue my research without worrying about Natalie. To Rosa Chien without whose support I would not be able to do half of the things I have done in my life. To my sister Jenny who made me a stronger person and taught me how to have a sense of humor about my life by doing what a big sister should do. And to all other family and friends for their support and interest in my research.
ACKNOWLEDGMENTS

I would like to acknowledge the people who have contributed to this work. First and foremost to Dr. Anna Riegel who has guided me through this process. Your critical and helpful reviews of my work have brought me to where I am today. To Dr. Anton Wellstein in whose presence I could not help but learn something new about science everyday. Together your pursuit of good science is unparalleled and your optimism about the project helped keep me going when I thought the project was not going to go any further. I have learned so much under your mentorship and I feel like I am prepared to take on any project now.

I would like to thank Dr. Alex Kirilyuk for his mass spectrometry expertise and help with generating and validating the AIB1-Δ4 antibody. Without this work I would not have a paper. To Jordan Li for her help with the Scorpion primer work and to Wentao Zhang for his insightful conversations and for his contribution to this work. I would like to thank Geoffrey Storchan for providing the model system for the ChIP experiments. To all of the AIB1 group members past and present as a whole for creating a safe and instructive environment to present my work. In particular I would like to thank Dr. Annabell Oh for all of her help with everything I have worked on in this lab and to her husband Dr. Ronald Reiter, the father of AIB1-Δ4.

I thank Dr. Christopher Albanese, Dr. Stephen Byers, Dr. Eliot Rosen, and Dr. John Hanover for serving on my committee. Your guidance, discussions, and suggestions for experiments have shaped this project and help bring it to completion.

And finally to all the members of the Riegel and Wellstein labs. Thanks for all of the science and non-science related discussions. It has been a fun ride that neither my stomach or I will forget.
TABLE OF CONTENTS

Title Page
Abstract
Dedication
Acknowledgements
Table of Contents
List of Figures

I. Introduction

A. Nuclear Receptor Signaling 2
B. Nuclear Receptor Coregulators 3
C. p160 Steroid Receptor Coactivator Family 4
D. AIB1 Affects Multiple Signaling Pathways at Different Levels 6
E. AIB1 is an Oncogene that is Amplified in Cancer and is Necessary for Tumorigenesis 7
F. Regulation of AIB1 mRNA, Protein, and Activity
   1. Regulation of AIB1 mRNA 10
   2. Regulation of AIB1 protein levels 10
   3. Phosphorylation status of AIB1 regulates its function 12
   4. Other posttranslational modifications alter AIB1 activity 13
G. A Splice Variant of AIB1, AIB1-Δ4 is a More Potent Coactivator 14
H. AIB1-Δ4 Bridges the Interaction of EGFR and FAK to Promote Cell Migration 15
I. The Role of AIB1-Δ4 in the Cytoplasm vs. the Nucleus 16

vi
II. Materials and Methods

A. Plasmids 18
B. Cell Lines and Transient Transfection 18
C. Identification of N-terminus of AIB1-Δ4 19
D. Western blot (WB) analysis and Immunoprecipitation (IP) 21
E. Nuclear Cytoplasmic Fractionation 22
F. Immunofluorescence and Leptomycin B Treatment 22
G. Chromatin Immunoprecipitation (ChIP) Assays 23
H. Real Time PCR Analysis 25
I. Quantitation of AIB1-Δ4 mRNA levels using Scorpion primer based quantitative RT-PCR 25
J. Luciferase Reporter Assay 26
K. Proteasomal Degradation of AIB1 and AIB1-Δ4 27
L. Lentiviral Transduction of Cell Lines 27
M. Growth Assays 27
N. Scratch Assays 28

III. Results

A. Identification of the N-terminus of AIB1-Δ4 and Generation of AIB1-Δ4 Specific Antibodies 30
1. Isolation of AIB1-Δ4 protein independent of AIB1 protein 30
2. Identification of N-terminus of AIB1-Δ4 31
3. Generation of AIB1-Δ4 specific antibodies 36
B. AIB1-Δ4 Enters the Nucleus and is Recruited to Endogenous Estrogen Regulated Genes

1. Cellular localization of AIB1 and AIB1-Δ4

2. AIB1-Δ4, like AIB1 can enter the nucleus

3. AIB1-Δ4 enters the nucleus possibly through interaction with other NLS containing proteins

4. AIB1-Δ4 is recruited to endogenous estrogen responsive genes like AIB1

5. AIB1-Δ4 increases the expression of estrogen regulated genes

C. AIB1-Δ4 Lacks the Inhibitory Domain Contained in the N-terminus of Full-length AIB1

1. The N-terminus of AIB1 contains an inhibitory domain

2. The AIB1-Δ4 is more resistant to proteasomal degradation than full-length AIB1

3. The N-terminus of AIB1 has potential sites of inhibitory phosphorylation

D. AIB1-Δ4 Expression Correlates With Metastatic Potential

1. Semi-quantitative detection of AIB1-Δ4 protein relative to AIB1 in breast cell lines

2. Quantitative detection of AIB1-Δ4 mRNA independent of AIB1 mRNA using Scorpion primer technology
   a. Scorpion primer design and validation
   b. AIB1-Δ4 correlates with invasiveness in pancreatic cancer cell lines
   c. AIB1-Δ4 correlates with metastatic potential in breast cancer cell lines
The Effects of AIB1-Δ4 Expression on Cell Growth and Migration 70

1. There is no difference on cell growth due to AIB1 or AIB1-Δ4 in AIB1 knockout mouse embryonic fibroblasts 70

2. There is no effect on cell growth due to increased AIB1-Δ4 expression in MCF10DCIS.com cells 72

3. Cell migration of DCIS.com cells is not enhanced by AIB1-Δ4 expression 73

IV. Discussion

A. AIB1-Δ4 is a predominantly cytoplasmic protein but has a role in the nucleus 76

B. The nuclear import mechanism of AIB1-Δ4 may involve binding to other NLS containing proteins 77

C. Translation of AIB1-Δ4 May be Directed by a Eukaryotic Internal Ribosome Entry Site 77

D. Loss of a Regulatory Region in the N-terminus of AIB1 Allows AIB1-Δ4 to be a More Potent Coactivator 78

E. A Signaling Loop Between AIB1/AIB1-Δ4 and Epidermal Growth Factor Receptor Exists. 81

F. The effect of AIB1-Δ4 expression on the phenotype of cells 82

G. Future studies of AIB1-Δ4 83

H. Differences between AIB1 and AIB1-Δ4 in the nucleus and cytoplasm 86

V. Appendix

A. Role of AIB1 in Tamoxifen Resistance 89

VI. References 94
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Model of normal steroid receptor coactivator function</td>
<td>6</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Isolation of AIB1-Δ4</td>
<td>31</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Potential methionines for translation start site of AIB1-Δ4</td>
<td>32</td>
</tr>
<tr>
<td>Figure 4</td>
<td>MS/MS spectra of N-terminal peptide of AIB1-Δ4</td>
<td>33</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Translation initiation of AIB1-Δ4 is possibly dependent on mRNA secondary structure</td>
<td>35</td>
</tr>
<tr>
<td>Figure 6</td>
<td>mRNA and Protein structure of AIB1 and AIB1-Δ4</td>
<td>36</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Characterization of affinity purified AIB1-Δ4 polyclonal antibodies</td>
<td>38</td>
</tr>
<tr>
<td>Figure 8</td>
<td>AIB1-Δ4 is a predominantly cytoplasmic protein</td>
<td>40</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Cellular Localization of AIB1 and AIB1-Δ4</td>
<td>42</td>
</tr>
<tr>
<td>Figure 10</td>
<td>AIB1-Δ4 enters the nucleus</td>
<td>44</td>
</tr>
<tr>
<td>Figure 11</td>
<td>AIB1-Δ4 interacts with AIB1</td>
<td>45</td>
</tr>
<tr>
<td>Figure 12</td>
<td>AIB1 can facilitate the nuclear import of AIB1-Δ4</td>
<td>47</td>
</tr>
<tr>
<td>Figure 13</td>
<td>AIB1-Δ4 is imported into the nucleus by AIB1</td>
<td>49</td>
</tr>
<tr>
<td>Figure 14</td>
<td>AIB1-Δ4 interacts with p300</td>
<td>50</td>
</tr>
<tr>
<td>Figure 15</td>
<td>p300 can facilitate the nuclear import of AIB1-Δ4</td>
<td>51</td>
</tr>
<tr>
<td>Figure 16</td>
<td>AIB1-Δ4 is imported into the nucleus by p300</td>
<td>53</td>
</tr>
<tr>
<td>Figure 17</td>
<td>AIB1 and AIB1-Δ4 are recruited to ERE in endogenous estrogen regulated genes</td>
<td>55</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Endogenous AIB1 and AIB1-Δ4 are recruited to ERE in endogenous estrogen regulated genes</td>
<td>56</td>
</tr>
<tr>
<td>Figure 19</td>
<td>AIB1-Δ4 can increase the expression of estrogen regulated genes</td>
<td>58</td>
</tr>
</tbody>
</table>
The N terminus of AIB1 contains an inhibitory domain that is lost in AIB1-Δ4

The N terminus of AIB1 plays a critical role in proteasomal regulation

The N-terminus of AIB1 harbors potential negative regulatory sites of phosphorylation

AIB1-Δ4 in non-metastatic and metastatic breast cell lines

Validation of AIB1 and AIB1-Δ4 Scorpion primers

AIB1-Δ4 expression is higher in in vivo selected metastatic pancreatic cancer cells

AIB1-Δ4 expression is higher in in vivo selected metastatic breast cancer cells

AIB1 and AIB1-Δ4 do not have differing effects on cell proliferation in AIB1 KO MEFs

AIB1-Δ4 does not have an effect on cell proliferation in MCF10DCIS.com cells

AIB1-Δ4 does not have an effect on cell invasiveness of MCF10DCIS.com cells

Proposed model of AIB1-Δ4 enhanced coactivator function
I. INTRODUCTION
A. Nuclear Receptor Signaling.

The idea of a hormone was first proposed by Bayliss and Starling in 1902 in describing secretions from the pancreas (1). Since then the term hormone has been applied to describe secretions from any endocrine gland. Hormones act at great distances from their tissue of origin with great tissue specificity and have been well characterized through the first half of the 20th century. The prevailing hypothesis at the time generated for the mechanism of hormone action was that direct interaction of hormones with enzymes would induce a conformational change in the enzymes they interacted with to activate them. However, this theory was abandoned due to the unusually high concentrations of hormone needed to obtain effects and that such high levels of hormone would not explain the exquisite tissue specificity of hormones. In 1956 hormones were known to influence the production of proteins such as tyrosine aminotransferase (2). Whether this regulation was due to influences of hormone at the transcriptional or translational level was not clear until it was seen that hormones had no effect on cell free protein translation systems. In addition, the identification of transcriptional inhibitors such as actinomycin D and α-amanitin centered the focus of how hormones acted to transcription.

The major discovery that led to the current paradigm of hormone action was in 1961 from Elwood Jensen and colleagues. In this work radioactively labeled estradiol-17β was tracked in female rats to track the localization of the hormone in the body. Estrogen was found in the reproductive tissues of the rat and in addition they found that it was in a complex with protein in the nucleus of the cells from these tissues (3, 4). This protein turned out to be the estrogen receptor and it was the discovery of the hormone receptor that changed the dogma of hormone action. Hormones were now believed to act through receptors to activate transcription. The location of these receptors stratifies hormones into two major groups, hormones that act through
receptors in the cell membrane and hormones that act through receptors that translocate into the nucleus called nuclear receptors.

Jensen in 1968 went on further to describe a two-step model to explain how estrogen acts through the estrogen receptor to activate transcription and is thought of as a general mechanism for how all nuclear receptors function (5). The Jensen two-step model involved hormone binding to nuclear receptor and subsequent activation of the nuclear receptor complex. Unbound nuclear receptors are found in the cytoplasm and structurally altered upon hormone binding. This change in conformation was confirmed by x-ray crystallography (6, 7). Once bound by hormone, the nuclear receptors in their new conformation can translocate into the nucleus via the nuclear localization signal of the receptor and dimerize to activate gene transcription by directly binding estrogen response elements (ERE) in the genomic DNA. This is the predominant theory of how nuclear receptors exert their action but there are additional levels of complexity to the modulation of gene transcription due to hormone stimulation.

B. Nuclear Receptor Coregulators

Gene transcription in eukaryotes is a complex and highly regulated process. One of the major controls of gene transcription is exerted by the coregulator family of proteins. These include both corepressors, which dampen transcription, and coactivators, which potentiate transcription. The existence of transcriptional coregulators was proposed in studies looking at transcriptional “squelching”. The yeast Gal4 transcription factor was able to negatively regulate the expression of genes without the need to bind to DNA or for the Gal4 target sequence (8). A similar effect was seen with steroid receptors, which are a type of nuclear receptor, when ERα was shown to prevent the activation of progesterone and glucocorticoid receptor on promoters
which did not contain any ERE (9). These data suggest that the steroid receptors competed for a rate limiting pool of factors responsible for the decrease in their transcriptional activation. This work preceded the identification of the numerous factors shown to bind to and regulate the function of steroid receptors, and in particular the estrogen receptor. These factors are known as the steroid receptor coregulators, which can be divided into coactivators that facilitate the enhancement of transcription and corepressors that dampen transcription.

More recent data suggests that these factors are critical for determining response to ligand receptor interactions in studies with selective estrogen receptor modulators such as Tamoxifen. Tamoxifen, a Selective Estrogen Receptor Modulator (SERM) is the standard of care for estrogen receptor positive breast cancer (10). SERMs are classified as selective due to their differential agonist/antagonist effects in a cell or tissue specific context. In the breast, Tamoxifen acts as an antagonist and competes with estrogen for binding to estrogen receptors to downregulate signaling through ERα. In the skeleton and uterus it acts as an agonist. This tissue specific response from the same receptor ligand interaction suggests that there is another mechanism driving the outcome from the same stimulus in different organs. As we know now, this difference is primarily due to the difference in recruitment and levels of the steroid receptor coregulators.

C. p160 Steroid Receptor Coactivator Family

A subgroup of nuclear receptor coactivators has been shown to be critical for the malignant progression of cancer and is known as the p160 Steroid Receptor Coactivators (SRC) (11). Steroid receptor coactivators are named for the fact that they increase the transcriptional activity of ligand bound steroid receptors. This family consists of three members Steroid
Receptor Coactivator-1 (SRC-1, NCOA1), Transcriptional Intermediary Factor 2 (TIF2, GRIP1, NCOA2), and Amplified in Breast Cancer 1 (AIB1, SRC-3, ACTR, p/CIP, RAC3, TRAM-1, NCOA3). The founding member of the p160 SRC family was cloned in the laboratory of Dr. Bert O’Malley in 1995 (12). In this study SRC-1 was shown to associate with both ERα and progesterone receptor in the presence of agonist and this association was inhibited by antagonist. The mouse homolog of TIF2, GRIP1, was cloned in the laboratory of Dr. Michael Stallcup and was identified in yeast two hybrid screens to interact in a ligand dependent fashion with the hormone binding domains of the glucocorticoid (GR), estrogen (ER), and androgen receptors (AR) (13). AIB1 was cloned independently in different labs as p/CIP (14), ACTR (15), RAC3 (16), and TRAM-1 (17) in Dr. Michael Rosenfeld, Dr. Ronald Evans, Dr. J. Don Chen, and Dr. William Chin’s laboratories respectively. They all contain a basic helix loop helix (bHLH), Per Arnt Sim (PAS) A and B domain, nuclear receptor interaction domain, CBP/p300 interaction domain, polyglutamine region, and activation domain which contains a domain with histone acetyltransferase activity in SRC-1 and AIB1 (Figure 6). The bHLH domain is typically a DNA binding domain in transcription factors. This is not the case for the p160 SRC family members as none of the members has been shown to directly bind to DNA. The PAS domain is a region of homology shared with the period circadian (Per), aryl hydrocarbon receptor nuclear translocator (ARNT), and single minded (Sim) proteins. They are thought to be areas of protein protein interaction and in particular of homo/heteromdimerization of PAS domain containing proteins. The nuclear receptor interaction domain facilitates the binding of the SRC with nuclear receptors through the presence of LXXLL motifs while the CBP/p300 interaction domain is the binding site for those proteins. In addition, several proteins have been shown to bind to the SRC family of coactivators (18). The predominant function of these proteins is to bind to activated
nuclear receptors and recruit basal transcription machinery and other molecules involved in chromatin remodeling such as CREB Binding Protein (CBP)/p300 (15), Coactivator-Associated Arginine Methyltransferase-1 (CARM-1) (19), and p300/CBP-associated factor (PCAF) (20) (Figure 1). Their general effect is an enhancement of transcription due to modifications to allow a more relaxed conformation of the chromatin in the area of activated steroid receptor binding to DNA (11).

**Figure 1. Model of normal steroid receptor coactivator function.** The normal function of steroid receptor coactivators (SRC) is bind to ligand bound nuclear receptors (NR) to potentiate transcription. They enhance transcription by recruiting molecules that are involved in chromatin remodeling such as CBP/p300 and CARM-1. These molecules as well as the SRC can acetylate histones (blue ovals) to put the chromatin (black ladder) in a relaxed state so it is accessible to proteins involved in transcription such as RNA polymerase II (POLII).

D. AIB1 Affects Multiple Signaling Pathways at Different Levels

It has been established that AIB1 interacts with various nuclear receptors but it was not until a genetic knockouts of AIB1 that the role of AIB1 in growth factor signaling would be examined (21, 22). Circulating IGF-1 levels in were decreased and overall size of mice was decreased in both models due to loss of AIB1. The Xu et al model also showed effects in reproductive tissues, which manifested as a delay in the onset of puberty, reduced female...
reproductive function, and stunted mammary gland development. The difference in the phenotypes seen may be due to differences in the design of the mouse models as different exons of the AIB1 mouse gene (p/CIP) were deleted. These studies were some of the first indications that AIB1 could play a role in growth factor signaling. Later studies would also show that AIB1 plays a major role in the IGF-1 pathways. IGF-1-stimulated anchorage independent growth of MCF-7 breast cancer cells was reduced with small interfering RNA (siRNA) knockdown of AIB1 in our lab (23). In contrast to AIB1 knockout mice, overexpression of AIB1 in mice caused increased IGF-1 serum levels, IGF-1 receptor tyrosine phosphorylation, and IGF-1 signaling in mammary epithelial cells. Additionally AIB1 levels modulate human epidermal growth factor (HER) signaling pathways. Work from our lab has shown that AIB1 is critical for maintaining phosphorylation of Epidermal Growth Factor Receptor (EGFR) (24) after EGF stimulation. More studies from our lab also showed that AIB1 is also necessary for HER2 induced tumorigenesis as genetic loss of the AIB1 gene prevented the HER2 induced mammary tumor formation (25). Downstream of growth factor receptors, AIB1 has been shown to interact with numerous transcription factors such as E2F1, NF-κB, Smads, STATs, HIF1, p53, and Rb (11). In most cases AIB1 activates signaling from these transcription factors, however AIB1 repressed p53 dependent transcriptional activity (26). In summary, AIB1 has effects on multiple signaling pathways not only at the level of the receptor but also at the level of the transcription factor.

E. AIB1 is an Oncogene that is Amplified in Cancer and is Necessary for Tumorigenesis

Amplified in Breast Cancer 1 has been shown to be gene amplified in breast cancer (27). In this hallmark study AIB1 was identified from microdissected regions of chromosome 20q,
which is a region that is frequently amplified in breast cancer. The level of expression of the AIB1 gene in various ER positive and ER negative breast cancer cell lines as well as ovarian cancer cell lines was also determined by Fluorescent In Situ Hybridization (FISH) in this report. In four of the five ER positive breast cancer cell lines overexpression of the AIB1 gene was found, while no AIB1 gene amplification in the ER negative breast cancer cell lines was detected. The BG-1 ovarian cancer cell line and the breast cancer cell lines BT-474, MCF-7, and ZR75-1 showed AIB1 gene amplification of greater than 20 fold. To confirm that AIB1 gene amplification was not a result of cell culture selection of cell lines, AIB1 gene expression in 105 unstratified breast cancer tissue specimens from tumor biopsies was determined. Ten of the specimens displayed AIB1 gene amplification, however not to the degree as in the cancer cell lines. At the mRNA level AIB1 was found overexpressed in the cell lines with gene amplified AIB1 while the expression of the other p160 SRC family members stayed constant. This overexpression at the transcript level was also true for the tissue specimens tested previously for AIB1 copy number. The highest levels of AIB1 transcript were in the 10 samples that had gene amplification of AIB1. Additionally more than half of the breast cancer samples without gene amplification had a higher expression of AIB1 transcript relative to normal mammary epithelium. Finally, AIB1 was found to be able to coactivate an estrogen inducible reporter and that it interacted with ER in the nuclear receptor interaction domain. These data were the first evidence that AIB1 may play a significant role in carcinogenesis.

In addition to breast cancer, AIB1 is also overexpressed at the mRNA and protein level in various cancers (11, 28, 29). AIB1 is gene amplified or overexpressed at the mRNA or protein level in colorectal (30, 31), endometrial carcinoma (32-34), esophageal squamous cell carcinoma (35, 36), gastric cancer (37), hepatocellular (38, 39), meningioma (40), nasopharyngeal (41), non
small cell lung cancer (42, 43), oral squamous cell carcinoma (44), ovarian cancer (45-47), pancreatic cancer (29), and prostate cancer (48). Its role in tumorigenesis is attributed to its ability to coactivate both steroid hormone and growth factor dependent transcription (23, 24, 28, 49).

AIB1 has been defined as an oncogene based on studies of a mouse mammary tumor virus (MMTV) AIB1 transgenic mouse model (50). In this model overexpression of AIB1 led to mammary gland hypertrophy, hyperplasia, delays in mammary gland involution, and the formation of spontaneous malignant mammary tumors. Tumors in other organs were seen but the majority of cancers observed were in the mammary gland, pituitary gland, and lung. In addition, increases in the activation of the IGF-1 signaling pathway were also detected. AIB1 is not only sufficient but necessary for the generation of tumors. In MMTV-v-Ras and HER2 driven oncogene mouse models, reduction of AIB1 levels led to a decrease in tumorigenesis (25, 51). 7,12-dimethylbenz[a]anthracene (DMBA) chemical carcinogen induced mammary tumor formation was also ablated with loss of AIB1 expression. Clinically, AIB1 expression in breast cancer cases is correlated with high HER2 levels, larger tumor size, higher tumor grade and shorter disease free survival (47, 52, 53). Also, high levels of AIB1 in conjunction with high HER2 levels coincide with reduced disease free survival in patients treated with tamoxifen, suggesting a role for AIB1 in tamoxifen resistance (54). The pre-clinical and clinical role data that support a role for AIB1 in regulating response to tamoxifen is reviewed in the appendix (p. 91).
F. Regulation of AIB1 mRNA, Protein, and Activity

1. Regulation of AIB1 mRNA levels

There are only a few studies that explore the transcriptional regulation of AIB1. Our lab has reported that estrogenic compounds and Transforming Growth Factor-β (TGF-β) can regulate the levels of expression of AIB1 transcript (55). TGF-β, all-trans retinoic acid, Tamoxifen, and ICI 182,780 treatment of MCF-7 cells caused an increase in the mRNA expression of AIB1 while estrogen treatment decreased AIB1 transcript levels. Two other studies found that E2F1 binds to a region in the AIB1 promoter to increase its expression (56, 57) and another showed that Specificity Protein 1 (Sp1) binding also increased AIB1 expression. Regulation of AIB1 gene expression is still relatively understudied as there are large regions of intronic genomic space between exons 1, 2, and 3, which may also contain enhancer/repressor regions that may contribute to control of AIB1 mRNA expression.

2. Regulation of AIB1 protein levels

The proteasomal regulation of AIB1 has been well documented. Canonical proteasomal degradation occurs when a protein is has a poly-ubiquitin modification on lysine residues on the protein, which is recognized by the cap of the 26S proteasome complex. These ubiquitin modifiers are added through an enzyme cascade that involves E1 ubiquitin activating, E2 ubiquitin conjugating, and E3 ubiquitin ligase enzymes. We have previously published that AIB1 interacts with the E3 ubiquitin ligase E6-AP which may facilitate the ubiquitination and degradation of AIB1 (58). SCF^Fbw7alpha was shown to add ubiquitin to lysine 723 and 786, which led to a decrease in AIB1 protein (59). Atypical Protein Kinase C (PKC) phosphorylates AIB1 to prevent its proteasome dependent turnover through inhibition of the interaction of AIB1 with
the C8 subunit of the 20S core proteasome (60). Through interaction with REGγ targets AIB1 for destruction by the 20S core proteasome in a ubiquitin and ATP independent manner (61). The identification of a phospho degron at serine 101 and 102 in the N-terminus of AIB1 regulates AIB1 protein levels as well. Protein phosphatase 1 (PP1) dephosphorylates these residues and prevents the proteasome dependent turnover of the AIB1 protein (62). In addition, casein kinase Iε (CKIε) phosphorylates S102 and promotes the E3 ligase speckle-type POZ protein (SPOP) dependent degradation of AIB1 (63).

Although microRNAs (miR) are a relatively new field, there are a few reports of regulation of AIB1 by miR. The study with the most thorough examination of miR regulation of AIB1 identified miR-17-5p as a regulator of AIB1 mRNA translation (64). A sequence was found in the 3’ untranslated region (UTR) as the target for miR-17-5p binding and this resulted in translation inhibition of the mRNA transcript. The expression of miR-17-5p was low in breast cancer cell lines. Overexpression of this miR decreased the expression of estrogen and E2F1 gene targets, inhibited the growth of estrogen dependent and independent cell lines, and inhibited the IGF-1 stimulated anchorage independent growth due to AIB1 down regulation. The other reports showed that AIB1 protein levels were modulated via the 3’UTR with either overexpression of specific miR (65, 66) or by inhibiting specific miR with an antagomiR (67) though whether these miR regulated AIB1 protein expression by translation inhibition or by degradation of the mRNA transcript of AIB1 was not examined. Undoubtedly there will be more miRs that will be found to regulate the expression of AIB1.
3. Phosphorylation Status of AIB1 regulates its function

One of the predominant controls of the function of AIB1 is through phosphorylation. Several phosphorylation sites have been identified to modulate AIB1 activity. The first published report of phosphorylation regulating AIB1 function showed that mitogen activated protein kinase (MAPK) phosphorylates AIB1 primarily on serine residues and increases its activity after phosphorylation. Later work would highlight the fact that different stimuli could induce the phosphorylation of AIB1. Estrogen, androgen, tumor necrosis factor-α, IκB kinase (IKK), Casein kinase 1δ (CKIδ), p38MAPK, IGF-1, and EGF all increase the phosphorylation of AIB1 (68-73). Another group identified several phosphorylation sites from AIB1 protein isolated from SF9 insect cells subjected to analysis of mass spectrometry (71). Serines at position 505, 543, 857, 860, and 867 and a threonine at position 24 were all identified as sites of phosphorylation. These sites were confirmed in human cells by mutational analysis and generation of site specific phospho AIB1 antibodies and these sites when mutated were able to modulate the coactivator function of AIB1. MAPK, c-Jun N-terminal kinase (JNK), p38MAPK, glycogen synthase kinase-3 (GSK-3), protein kinase A (PKA), and IKK were identified as the kinases responsible for phosphorylations at these sites. In this same study some of the serine phosphorylation mutants showed decreased association with CBP, which is a major contributor to the coactivation function of AIB1 (15). In subsequent work, the E3 ligase SCF^{Fbw7alpha} dependent degradation of AIB1 relied on S505 phosphorylation by GSK-3 to enhance AIB1 activity (59). In addition to these sites a phospho degron in the N-terminus of the AIB1 protein was identified (62). Protein phosphatase 1 (PP1) regulated the phosphorylation at S101 and S102 and dephosphorylation at these residues stabilized the AIB1 protein but hindered the oncogenic functions of AIB1. S102 in particular was shown to be phosphorylated by
CKIε, which is required for SPOP degradation and inhibition of AIB1 oncogenic signaling (63). Our lab has found tyrosine phosphorylation at Y1357 as yet another modification that regulates AIB1 activity (73). This site is phosphorylated by V-abl Abelson murine leukemia viral oncogene homolog 1 (Abl) kinase and loss of phosphorylation at this site in AIB1 led to decrease in interaction with p300 and ERα. All of these data suggest a major role for phosphorylation in the regulation of AIB1 activity.

4. Other posttranslational modifications alter AIB1 activity

We have seen through three other types of posttranslational modification (PTM) of AIB1. Small ubiquitin related modifier (SUMO) is another PTM similar to ubiquitin and both modifications compete for lysine residues in proteins to modify their activity and stability. Through this competition sumoylation can prevent the ubiquitination and eventual proteasomal degradation of a protein or may induce an inactive conformation of the protein. AIB1 was identified to be sumoylated and MAPK phosphorylation of AIB1 was decreased when sumoylation was high and vice versa (70). AIB1 was also found to be acetylated by CBP/p300 (74). In this study AIB1 was determined to be acetylated at lysine 626, 629, and 630. The association of AIB1 with ERα after acetylation was decreased through altering the nuclear receptor interaction domain conformation and thereby altering AIB1 activity. This acetylation is thought to be how the nuclear receptor AIB1 complex is disassembled and how transcription is attenuated. Lastly, methylation of AIB1 was shown in two studies to modulate its activity. The first study identified R1171 as the major site of methylation by CARM1 and this methylation played a critical role in disassembly of the AIB1 transcriptional complex (75). The second study found two areas of methylation with R839 to R961 and R1163 to R1195 (76). Loss of
methylation also led to increases in AIB1 levels and AIB1 activity suggesting that methylation of AIB1 regulates its stability and function.

G. A Splice Variant of AIB1, AIB1-Δ4 is a More Potent Coactivator

We had previously identified a splice variant of AIB1, where exon 3 was spliced from the mature mRNA and the resulting protein named AIB1-Δ3 (77). More recently, an additional 5’ exon, 81,164 bases upstream of the known 5’UTR, was identified. Thus the deleted exon is now exon 4 and we now refer to the splice variant as AIB1-Δ4. We had reported that AIB1-Δ4 mRNA results in an N-terminally truncated isoform of the AIB1 protein that was found to be a more potent coactivator of steroid dependent transcription on a per mole basis when compared with the full-length AIB1 protein. AIB1-Δ4 mRNA expression was elevated in breast tumor tissue relative to normal breast tissue (77). It was also shown to increase the efficacy of estrogenic compounds and the agonist effects of the selective estrogen receptor modulator tamoxifen in breast and endometrial tumor cells (77, 78). To try to elucidate the role of AIB1-Δ4 in vivo we made transgenic mice with human AIB1-Δ4 driven by the CMV promoter (79). We saw significant increases in mammary epithelial cell proliferation, cyclin D1 expression, IGF-I receptor protein expression, and mammary gland mass. Ductal ectasia was observed in the mammary gland at 13 months of age with an increased expression of proliferative markers such as proliferating cell nuclear antigen (PCNA), phospho-histone H3, and Cyclin D1. Recently we have also published that AIB1-Δ4 impacts ERα mediated effects in the breast stroma and epithelium (80). Tetracycline inducible AIB1-Δ4 and tetracycline inducible Conditional Estrogen Receptor Mice (CERM) were generated and compared to CERM mice alone.
Simultaneous AIB1-Δ4 and ERα expression (AIB1-Δ4/CERM) in the mammary gland caused a significant increase in ductal hyperplasia and hyperplastic alveolar nodules. Out of 22 AIB1-Δ4/CERM mice, two developed mammary adenocarcinomas where one was ERα/PR positive and the other ERα/PR negative. In the stroma, AIB1-Δ4 and ERα expression induced significant increases in collagen expression. Estrogen and progesterone target genes were also significantly increased due to AIB1-Δ4 overexpression. These studies suggest that AIB1-Δ4 is an important factor to study in oncogenesis.

**H. AIB1-Δ4 Bridges the Interaction of EGFR and FAK to Promote Cell Migration**

More recently, AIB1-Δ4 was shown to act as a molecular bridge between EGFR and Focal Adhesion Kinase (FAK) in the cytoplasm (81). In this study AIB1-Δ4 and not AIB1 was able to interact with EGFR and FAK at the cell membrane. AIB1-Δ4 interacted with FAK through the N-terminus of FAK and the RID of AIB1-Δ4. AIB1-Δ4 was able to increase the EGF stimulated migration of HeLa cells in a FAK dependent manner. The N-terminus of AIB1-Δ4 was defined as the EGFR interaction domain. p21 activated kinase (PAK1) phosphorylates AIB1-Δ4 at T56, S659, and S676 of the AIB1-Δ4 sequence to facilitate the interaction of AIB1-Δ4 with EGFR and FAK. These sites of phosphorylation were critical to the effect of AIB1-Δ4 on EGF stimulated migration. In addition, it was shown that AIB1-Δ4 overexpression increased the number of lymph node and lung metastasis of MDA-MB-231 metastatic breast cancer cells without affecting the growth rate or tumor growth rate of the cells. Finally, the number of circulating tumor cells was also increased with AIB1-Δ4 overexpression.
I. The Role of AIB1-Δ4 in the Cytoplasm vs. the Nucleus

Since AIB1-Δ4 lacks a nuclear localization sequence (NLS) any function for this protein in cancer to date has been attributed predominantly to its role in the cytoplasm (81). This stands in contrast to our initial identification of AIB1-Δ4 and observation that it is a more potent coactivator of steroid and growth factor transcription, which occurs in the nucleus (77, 78). In our present study we wanted to determine if AIB1-Δ4 has a nuclear function in the regulation of gene transcription and the mechanisms underlying the potent coactivator function of AIB1-Δ4. We now show that AIB1-Δ4 can enter the nucleus by a non-canonical nuclear import mechanism. AIB1-Δ4 is recruited to estrogen response elements of endogenous estrogen-regulated genes and increases their expression. We also determined that the N-terminal region absent from the AIB1-Δ4 protein contains an inhibitory domain. Through the use of Scorpion primer technology, we have created the first quantitative assay for the AIB1-Δ4 transcript and found a correlation between AIB1-Δ4 expression and the metastatic phenotype of human cancer cell lines. These data suggest that the nuclear activities of AIB1-Δ4 can contribute to its function in malignancy.
II. MATERIALS AND METHODS
A. Plasmids

p300-HA and ERα constructs were provided by Dr. Maria L. Avantaggiati (Georgetown University) and from Dr. Pierre Chambon (INSERM, Strasbourg) respectively. AIB1, AIB1-Δ4, FLAG AIB1, and FLAG AIB1-Δ4 were described previously (ref 17,22). A C-terminal FLAG was added to the AIB1-Δ4 cDNA by deletion of the stop codon in AIB1-Δ4 and addition of FLAG peptide sequence by site-directed mutagenesis (Stratagene). HA AIB1 was generated by PCR amplification of the AIB1 sequence from pcDNA3-AIB1 and cloned into phCMV2. The AIB1 N term construct was created by PCR amplification of the ACTR/AIB1 cDNA (184 bp to 777bp) to add a new 5’ NotI site and 3’BglII site. The PCR product was then cloned into p3XFLAG-CMV-10 (Sigma). pCDF1-MCS2-EF1-puro lentiviral vector and pFIV-34N packaging plasmid were provided by Dr. Todd Waldman (Georgetown University). FLAG AIB1 or FLAG AIB1-Δ4 sequence was cloned into pCDF1-MCS2-EF1-puro into the BglII and NotI sites.

B. Cell Lines and Transient Transfection

MDA231-BrM2 were kindly provided by Dr. Joan Massagué (Sloan Kettering Institute), 4175-TR and SCP2-TR cells by Yibin Kang (Princeton University), AIB1 KO/SRC-3+/− mouse embryonic fibroblasts (MEFs) by Dr. Jiangming Xu (Baylor College of Medicine), T47D A1-2 cells by Dr. Steve Nordeen (University of Colorado), and COLO SL and COLO PL cells by Dr. John M. Jessup (Georgetown University). Chinese Hamster Ovary (CHO), COLO 357 were purchased from ATCC. HEK293, HEK293T, COS-7, MCF-7, and MDA-MB-231 were obtained from the Tissue Culture Shared Resource at Georgetown University. The Human mammary epithelial cells (HMEC) were purchased and cultured in commercially supplied medium
HEK293T, COS-7, COLO 357, COLO SL, and COLO PL, MDA-MB-231, MDA231-BrM2 (brain), 4175-TR (lung), SCP2-TR (bone), and AIB1 KO MEFs were grown in Dulbecco modified Eagle medium (DMEM, Invitrogen) with 10% FBS. CHO cells were grown in DMEM F12 (Invitrogen) with 10% FBS. HEK293 cells were grown in phenol red free Iscove’s modified Eagle medium (IMEM, Invitrogen) with 10% Charcoal stripped serum (CCS). T47D A1-2 and MCF-7 cells were grown in phenol red free IMEM+5%CCS. HEK293, HEK293T, CHO, COS-7, and T47D A1-2 cells were transiently transfected with FuGENE 6 (Roche).

C. Identification of N-terminus of AIB1-Δ4

HEK293T cells grown to 80% confluence were transiently transfected with 18 µg C-terminal FLAG AIB1-Δ4 cDNA. 24 hours later whole cell lysates were prepared and subjected to immunoprecipitation using Anti-FLAG M2 affinity gel (Sigma). After washing AIB1-Δ4 protein was recovered by heating the affinity gel to 95°C and the sample was subjected to SDS-PAGE. A band corresponding to AIB1-Δ4 protein was isolated and trypsinized using a conventional in-gel digestion protocol where cysteines were reduced with DTT and alkylated by iodoacetamide. Extracted tryptic peptides were analyzed using the MIDAS-MS based algorithm on an LC-ESI-MS 4000QTRAP instrument (AB SCIEX, Framingham, MA). In silico predicted peptides and corresponding collision energy settings were generated using recommended settings in MRMpilot software (AB SCIEX, Framingham, MA). The list of predicted precursors includes the potential variable modification of methionine oxidation and the fixed modification of cysteine alkylation. A final MS method was created for detection of the tryptic peptides produced from the full-length AIB1 protein. This approach allows detecting only tryptic
peptides that overlaps with the spliced version of AIB1 and was tested on endogenous AIB1 protein isolated by immunoprecipitation followed by SDS-PAGE. In silico predictions for tryptic peptides with methionine as an initial amino acid residue were applied to data for identification of the N-terminus.

Peptides were fractionated using a reverse phase BEH C18 column (1.7 µm, 75 µm x 150 mm, Waters) on a nanoUPLC Acquity system (Waters) using buffer A (2% acetonitrile, 0.1% formic acid) and buffer B (98% acetonitrile, 0.1% formic acid). Analytes were eluted over a 30 min linear gradient of 0-60% of solvent B with 300 nl/min flow rate. The nanoUPLC instrument was coupled to a 4000QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (AB SCIEX, Framingham, MA). Eluted peptides were ionized in positive mode using a fused silica PicoTip emitter (New Objective, Woburn, MA) with a spray voltage of 2300 V, curtain gas of 13, nebulizer gas of 13, interface heater temperature of 180°C, unit resolution for Q1 and Q3 for MRM mode, and at low resolution in enhanced product ion scan. The method for the 4000QTRAP mass spectrometer includes a MIDAS algorithm for dependent Enhanced Product Ion scans (MS/MS), which are triggered when the MRM signal exceeds a threshold (typically 50 counts/s). Precursor ions were dynamically excluded for one minute after two occurrences.

Database search MS/MS fragment ion data were searched against a human database using the Mascot algorithm (Matrix Sciences, London, UK) in ProteinPilot 3.0 software (AB SCIEX, Framingham, MA) with a mass tolerance for precursor ions of 0.05 Da, fragment ion tolerance 0.6 Da, no missed cleavage, carboxymethylation as a fixed modification for cysteines, and oxidation for methionine as variable modifications. In order to match experimental data to the translational start of AIB1-Δ4 the database search was performed using semi-trypsin pattern of fragmentation, where tryptic specificity was assigned at C-terminus and the N-terminus was
allowed to be a non-tryptic cleavage and additional variable modifications such as N-terminal peptide acetylation and de-methionation.

D. Western Blot (WB) Analysis and Immunoprecipitation (IP)

Western blotting was done with the following antibodies: AIB1 (5E11, Cell Signaling), FLAG M2 (Sigma), HA (Cell Signaling), ERα (Ab-10, Neomarkers), ERα (G-20, Santa Cruz), human Actin (Millipore). (i) Interaction of AIB1 with AIB1-Δ4. HEK293T cells were transfected with 6µg of either FLAG AIB1, AIB1-Δ4, or FLAG AIB1 and AIB1-Δ4 together. After washing with cold 1X PBS whole cell lysates were prepared by adding 1% NP-40 lysis buffer containing 1mM NaO₃VO₄ and 1x Complete protease inhibitor tablet (Roche). IP was performed with Anti-FLAG M2 affinity gel as described previously (6) and samples were subjected to SDS-PAGE. (ii) Interaction of AIB1 and AIB1-Δ4 with p300-HA. HEK293T cells were transfected with either FLAG AIB1, FLAG AIB1-Δ4, or p300-HA. Whole cell lysates were prepared as in section (i). Equal amounts of FLAG AIB1 and FLAG AIB1-Δ4 were added to equal amounts of p300-HA lysate. After immunoprecipitation using HA antibody (Cell Signaling) the amounts of FLAG AIB1 or FLAG AIB1-Δ4 were detected with FLAG M2 antibody (Sigma). Densitometry was performed by using Adobe Photoshop 7.0 normalizing AIB1-Δ4 bands to AIB1 bands for both input and IP. (iii) Immunoprecipitation with AIB1-Δ4 antibody. 500 µg of lysate from MCF-7 or HEK293T transfected with C-terminal FLAG tagged AIB1-Δ4 were subjected to IP with 5µg AIB1-Δ4 antibody. AIB1 or AIB1-Δ4 proteins were detected with AIB1 antibody (5E11, Cell Signaling).
E. Nuclear Cytoplasmic Fractionation

Fractionation of lysates was carried out as per the protocol recommended by the NE-PER Nuclear and Cytoplasmic extraction reagents (78833, Pierce). Endogenous or transfected AIB1-Δ4 was detected by either AIB1 (5E11) or FLAGM2 antibody. Controls use for nuclear and cytoplasmic fractions were HDAC1 (#2062, Cell Signaling) and HSP90 (05-594, Upstate).

F. Immunofluorescence and Leptomycin B Treatment

CHO cells were plated on glass coverslips and transfected with 500ng of either FLAG AIB1 or FLAG AIB1-Δ4. 24 hours later cells were fixed with 3.7% paraformaldehyde in PBS for 10 minutes at 25°C. Cells were then washed three times with 1x PBS and permeabilized with 1x PBS containing 0.2% Triton X-100 for 5 minutes 25°C. Cells were then washed with three times with 1xPBS. Coverslips with cells were then blocked for 30 minutes with 1%BSA in PBS. Cells were then incubated with FLAG M2 antibody (1:500, Sigma) and HA antibody (1:500, Abcam ab9110) for 20 minutes. After three 5 minute washes with 1xPBS, cells were incubated with anti-mouse IgG AlexaFluor488 (1:1000, Invitrogen) and anti-rabbit AlexaFluor594 (1:1000, Invitrogen) for 20 minutes. Coverslips were then washed three times with 1x PBS and mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) on to glass slides. 50 nM leptomycin B was added into the culture medium 4 hours before fixation. 200 cells were counted and the percentage of nuclear, nuclear/cytoplasmic, and cytoplasmic stained cells was quantified from 3 different experiments. Nuclear staining was defined as protein specific signal that overlaid with the DAPI signal only. Nuclear/cytoplasmic was defined as protein specific staining that overlaid with the DAPI signal but also showed staining in the cytoplasmic compartment. Cytoplasmic staining was defined as protein specific staining that did
not overlay with the DAPI staining of the nucleus. Imaging of stained CHO cells was performed on a Nikon E600 Fluorescence Digital Microscope System and analyzed with Nuance multi-spectral imaging system and software (Cambridge Research & Instrumentation).

$2 \times 10^6$ AIB1 KO MEFs were transiently transfected with 4 µg FLAG AIB1-Δ4 alone and either 2, 4, or 6 µg of p300-HA or AIB1 using the MEF 2 Nucleofector kit (Amaxa, Lonza) and plated on glass coverslips after transfection. 24 hours later cells were fixed, permeabilized, stained, mounted, and quantified as CHO cells in experimental methods. p300 (1:500, Abcam) and anti-rabbit AlexaFluor594 (1:1000, Invitrogen) were used to stain for p300-HA. Stained AIB1 KO MEFs were analyzed on Olympus Fluoview-FV300 Laser Scanning Confocal System in the Microscopy and Imaging Shared Resource at Georgetown.

**G. Chromatin Immunoprecipitation Assays (ChIP)**

HEK293 cells in a 10 cm dish were transfected with 5µg ERα and either 6µg FLAG AIB1 or 3 µg FLAG AIB1-Δ4 in phenol red free IMEM+10%CCS. 24 hours later cells were treated with Estrogen (E2) for 0, 15, 30, 45, or 60 minutes. Cells were fixed with formaldehyde fixation solution (3.7% formaldehyde, 100 mM NaCl, 50 mM Tris/HCl pH 8.0, 1 mM EDTA, 0.5 mM EGTA) for 10 minutes at 37°C and stopped with 0.125 M Glycine in 1x PBS for 5 minutes at 25°C. Cells were washed three times 1xPBS and resuspended in SDS lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 1% SDS). Cells were sonicated and resuspended in ChIP dilution buffer (20 mM Tris pH8.0, 2 mM EDTA pH 8.0, 150mM NaCl, 1% Triton-X-100) and pre-cleared with 30 µl of protein G agarose/salmon sperm DNA (Millipore) for 1 hour. 500 µg of total protein was immunoprecipitated with 2 µl FLAG M2 antibody (Sigma) 16 hours and immunoprecipitated with 30 µl protein G agarose/salmon sperm DNA for 2 hours. Agarose was
washed with once with low salt buffer (20 mM Tris pH 8.0, 2 mM EDTA pH 8.0, NaCl 150 mM, 0.1% SDS, 1% Triton-X-100), twice with high salt buffer (20 mM Tris pH 8.0, 2 mM EDTA pH 8.0, 500 mM NaCl, 0.1% SDS, 1% Triton-X-100), once with LiCl salt buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0, 250 mM LiCl, 1% Na deoxycholate, 1% NP-40), and twice with TE buffer (10 mM Tris pH 8.0, 1 mM EDTA, pH 8.0). Samples were eluted with elution buffer (1%SDS, 0.1M NaHCO₃) for 15 minutes on rotator and 10 minutes on vortexer. Crosslinks were removed with 200mM NaCl for 6 hours at 65°C and proteins digested with 1 µg proteinase K for 1 hour at 45°C. DNA was purified using GENECLEAN Turbo kit (Q-Biogene). Samples were analyzed by real time PCR to examine the ERE recruitment of FLAG AIB1 or FLAG AIB1-Δ4 with the following primers: pS2 ERE s: 5’GGCCATCTCTCAGTATGAATCACTTC, pS2 ERE as: 5’-GGCAGGCTCTGCTTAAAGAGCG-3’, hC3 ERE s: 5’-GTTCCTCCCTCCTGTTC-3’, hC3 ERE as: 5’-GAGAAAGGTCTGTGGTCACCAG-3’, HER2 ERE s: 5’-GAGAAAGGTCTGTGGTCACCAG-3’, HER2 ERE as: 5’-GAGAAAGGTCTGTGGTCACCAG-3’. Cycling conditions for real time PCR using iCycler were 95°C 3 minutes followed by 40 cycles of 95°C 20 sec, 57°C 30 sec, 72°C 40 sec for hC3 ERE and pS2 ERE. For HER2 ERE cycling conditions were 95°C 3 minutes followed by 40 cycles of 95°C 20 sec, 65°C 30 sec, 72°C 40 sec. The percentage of the input for each time point is plotted on the graphs and normalized time 0 for each transfection.

For ChIP assays with endogenous AIB1-Δ4, MCF-7 cells were plated in 15 cm dishes in phenol red free IMEM+5%CCS. Fresh IMEM+5%CCS was added for three days. Cells were treated as the HEK293 cells except they were subjected to immunoprecipitation with either 4 µg affinity purified AIB1-Δ4 antibodies (Abgent), 2µg AIB1 (C-20, Santa Cruz), or 2µg ER
antibody (HC-20, Santa Cruz). After immunoprecipitation the ChIP procedure for HEK293 cells was then followed. All primers were synthesized by Integrated DNA Technologies (IDT).

H. Real Time PCR Analysis

HEK293 cells were transfected with FLAG AIB1 or FLAG AIB1-Δ4 at levels that give equal amounts of transfected protein in IMEM +10% CCS. 16 hours later cells were stimulated with E2 for 0, 4, 8, and 24 hours. Total RNA was harvested using RNeasy mini kit (Qiagen) and reverse transcribed with iScript cDNA synthesis kit (Bio-Rad) using 1 µg of total RNA. Samples were analyzed by real time PCR (iCycler, Bio-Rad) using the following conditions: 95°C 3 min, 40 cycles of 95°C 20 sec 56°C 30 sec 72°C 40 sec. Primer sequences used were: pS2 s: 5’-CCCCGTGAAAGACAGAATTGT-3’, pS2 as: 5’-GGTGTCGTCGAAACAGCAG-3’, hC3 s: 5’-CTGTCCACCGACTTCCAGG-3’, hC3 as: 5’-CCCTTTTCTGACTTGAACCTCCC, HER2 s: 5’-AAAGGCCCAAGACTCTCTCC, HER2 as: 5’-CAAGTACTCGGGGTCTCCA-3’, human actin s: 5’-CCTGGGCCACCCAGCAAACT-3’, human actin as: 5’-GCCGATCCACACGGAGTACT-3’. All primers were synthesized by IDT. Expression level for each gene is normalized to actin expression and multiplied by either 1000 or 100,000 to obtain whole value numbers.

I. Quantitation of AIB1-Δ4 mRNA Levels Using Scorpion Primer Based Quantitative Real Time PCR

A total of 2 x 10^6 cells were plated for each cell line. 24 hours later total RNA was extracted with RNeasy mini kit (Qiagen) and reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad) using 1µg of total RNA. Real-time PCR was performed using IQ SYBR Green
Supermix (Bio-Rad) with AIB1\Delta 4-Scorpion primer and human actin primers. Cycling conditions for the AIB1-\Delta 4-Scorpion primer consist of an initial denaturing step at 94°C (2 min), and 50 cycles (20 seconds at 94°C, 15 seconds at 55.5°C and 20 seconds at 72°C). Unlike SYBR green real time PCR analysis where data is collected during the extension step, data for the Scorpion primer reactions were collected during the 55.5°C annealing step (iCycler; Bio-Rad). Cycling conditions for the human actin primers include a denaturing step at 94°C (2 min), and 45 cycles (20 seconds at 94°C, 30 seconds at 58°C and 40 seconds at 72°C). The AIB1-\Delta 4 Scorpion primer was custom designed and purchased from Sigma-Aldrich. Primer sequences for the AIB1-\Delta 4 Scorpion reaction: 5’FAMCCCGCGCTTGGAAATAGTTTTTCCCTTGTCCGCGGGBHQ1 HEGCGCAAATTGCCATGTGATAC. AIB1-\Delta reverse primer: 5’-CCATCCAATGCCTGAA GTAA-3’. The expression level of AIB1-\Delta 4 is normalized to actin expression levels and multiplied by either 10,000 or 100,000 to obtain whole number values.

**J. Luciferase Reporter Assay**

25,000 COS-7 cells per well in a 24 well dish were transfected in DMEM without serum with 100 ng MMTV luciferase, 25 ng Progesterone receptor (PR), 5 ng Thymidine Kinase (TK) Renilla luciferase, and either 500 ng pcDNA3, 500 ng FLAG AIB1, 500 ng FLAG N term, or 125, 500, and 750 ng of FLAG N term with 500 ng FLAG AIB1. 24 hours later cells were treated with 10 nM R5020 or an equivalent volume of ethanol. 24 hours after stimulation cells were lysed and luciferase values were measured using the Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase values were normalized to Renilla luciferase values and averaged for each transfection condition plated in triplicate.
K. Proteasomal Degradation of AIB1 and AIB1-Δ4

HEK293T cells were transfected with either FLAG AIB1 or FLAG AIB1-Δ4 at 50% confluence. 24 hours later cells were treated with either ethanol or 10µM MG132. 16 hours later cells were at high confluence and cell lysates harvested and the amount of transfected FLAG AIB1 or FLAG AIB1-Δ4 was detected by Western blot with FLAG M2 antibody.

L. Lentiviral Transduction of Cell Lines

pCDF1 FLAG AIB1 and pCDF1 AIB1-Δ4 were prepared as per protocol (Systems Biosciences). Titering of lentivirus was done in HEK293T cells. Briefly 300,000 HEK293T cells were infected in 6 well dishes 24 hours after plating. Dilutions of the virus (1:100-1:1,000,000) were made in DMEM+10% FBS with 8mg/ml polybrene. After 24 hours media containing 5µg/ml puromycin was added to the cells and changed every 2-3 days until colonies were visible by eye. Colonies were washed 2 times in PBS and then stained with crystal violet solution for 10 minutes (0.5% crystal violet in 25% methanol). Colonies were then washed until no color was washing off in the washes. Plates were then dried at room temperature. The number of colonies was counted in each well. Viral titer was calculated to determine the infectious units per ml of virus containing media with the following formula:

Viral titer= (# colonies/volume stock virus added to cells)* dilution factor.

M. Growth Assays

For transduced AIB1 KO MEFs 1250 cells were plated in opaque 96 well plates and the protocol for Cell Titer glo (Promega) luminescent cell proliferation assay was followed. For
transduced MCF10DCIS.com cells 500 cells were plated in opaque 96 well plates and standard Cell Titer glo protocol followed. For both assays cells were assayed on day 2, 4, and 6.

N. Scratch Assay

Scratch assays were performed in 6 well dishes with MCF10DCIS.com cells plated in cell culture inserts (Ibidi) to make the scratched/wound area. After the formation of a monolayer in the tissue culture inserts, the cell culture insert was removed by sterile forceps and the images were taken on the Nikon Eclipse TE-300 Spinning Disk Timelapse Microscope System. Images were taken every hour for 24 hours. Data analysis was done at 0 and 12 hours and the scratch area measure with ImageJ software.
III. Results
A. Identification of the N-terminus of AIB1-Δ4 and Generation of AIB1-Δ4 Specific Antibodies

1. Isolation of AIB1-Δ4 protein independent of AIB1 protein

To date there is no antibody to specifically detect the AIB1-Δ4 isoform without detecting the full length AIB1 protein. The lack of this reagent makes it difficult to determine the level of expression of the AIB1-Δ4 protein in assays that do not distinguish the two isoforms of the protein by size. Based on the migration of the AIB1-Δ4 protein on an SDS-PAGE gel, the translation start site was predicted to be at the methionine at position 199 of the full-length protein (77). However, the translation start site of AIB1-Δ4 was not identified experimentally. We employed mass spectrometry analysis, which allows for identification of peptides in small quantities, to determine the translation start site of AIB1-Δ4. The first step in this process involved isolating AIB1-Δ4 protein independent of full-length AIB1 protein in relatively large quantities. To accomplish this task, we transfected HEK293T cells with C-terminal FLAG tagged AIB1-Δ4 construct and immunoprecipitated lysates from these cells with FLAG antibody agarose conjugate and the levels of AIB1 and AIB1-Δ4 were detected before and after immunoprecipitation. We were able to immunoprecipitate only the C-terminally FLAG tagged AIB1-Δ4 from the expression construct (Figure 2). A pan AIB1 antibody that recognizes both full-length AIB1 and AIB1-Δ4, detected the presence of AIB1 and AIB1-Δ4 in the cell lysates before IP (Figure 2, lane 1). After immunoprecipitation with FLAG antibody, we only detect full-length AIB1 in the supernatant from the IP (Figure 2, lane 2). In the recovered protein sample from the IP agarose beads we see an enrichment of the AIB1-Δ4 and no full-length AIB1
2. Identification of N-terminus of AIB1-Δ4

The AIB1-Δ4 protein migrates at approximately 130 kDa. Translation from methionines at positions 199, 201, 217, 224, 235, 236, 246, and 289 of the full-length AIB1 protein sequence will give a predicted molecular mass of 130 kDa (Figure 3a). To determine which of these methionines is the translation start site of AIB1-Δ4 we subjected the AIB1-Δ4 proteins to mass spectrometric analysis. Following the immunoprecipitation procedure outlined in Figure 2, we separated the immunoprecipitated proteins by gel electrophoresis, isolated a gel slice containing the AIB1-Δ4 protein from a Coomassie stained gel, and subjected it to digestion with trypsin. These tryptic fragments were then analyzed by mass spectrometry. Using an MS method file used to study full-length AIB1, we confirmed that an AIB1 related protein was identified in the tryptic fragments from the gel slice containing AIB1-Δ4. The most N-terminal fragment identified in this sample was $\text{AM}_{235}\text{M}_{236}\text{EEGEDLQSCM}_{246}\text{ICVAR}$ (underlined in Figure 3b). This eliminated the possibility that translation was initiated from $\text{M}_{235-236}$, $\text{M}_{246}$, or $\text{M}_{289}$. From previous studies mass spectrometry studies on AIB1 (73), we have seen that the tryptic peptide
This ruled out translation initiation of AIB1-Δ4 from M$_{199}$ or M$_{201}$.

Of the eight methionines that could be the translation start site of AIB1-Δ4, only M$_{217}$ and M$_{224}$ remained as possible translation start sites. A new method file was created to analyze the MS/MS spectra from the AIB1-Δ4 tryptic fragments, which included in silico predictions for tryptic peptides with methionine as the first residue and cotranslational modifications such as N-terminal acetylation. Information for N-terminal acetylation since 80% of all proteins have been described to have an acetyl moiety added to the N-terminus (82, 83). After analysis with the new method file we only detected one predicted peptide M$_{224}$QCFALSQPR that retained an initiator methionine. This peptide contained N-terminal acetylation and was found with the M$_{224}$ residue in the non-oxidized (m/z 640.3) and oxidized states (m/z 648.3) (Figure 4).
In addition to using mass spectrometry we also tried to confirm N-terminus of AIB1-Δ4 by mutating the translation initiation site by single nucleotide polymorphism. Using site-directed mutagenesis we mutated the adenine base in the ATG codon, which encodes the M224 amino acid, to a guanine in the AIB1-Δ4 cDNA. This changes the sequence from a Methionine to a Valine and therefore prevents translation initiation at this position in the mRNA sequence of AIB1-Δ4. We mutated the codons that encode the methionines at position M199 and M235 to serve as negative controls. We transfected these constructs along with wild type (wt) AIB1-Δ4 cDNA to determine if the expression of AIB1-Δ4 protein would be prevented by mutating the start site at M224 (Figure 5a). After detecting with a pan AIB1 antibody, we see that mutations at all sites led to an increase in the amount of expression of an AIB1-related protein that migrates at

![MS/MS spectra of N-terminal peptide of AIB1-Δ4.](image)

**Figure 4.** MS/MS spectra of N-terminal peptide of AIB1-Δ4. Shown above are the annotated fragmentation spectra of the MQCFALSQPR peptide. As a result of cotranslational modification, this tryptic peptide contains acetylation at the initial methionine and is in the double charge state. The left spectrum shows collision induced dissociation (CID) fragmentation of the peptide with a non-oxidized methionine and m/z ratio of 640.3. The right spectrum depicts fragmentation of the peptide with mass shifted m/z ratio of 648.3 caused by oxidation of the initial methionine. Stars indicate the position of parental ions in the MS/MS spectra.
the same size as AIB1-Δ4 as compared to wt AIB1-Δ4 cDNA transfection. In fact we get the least expression with wt cDNA transfection. This suggests that translation initiation of AIB1-Δ4 may occur via an internal ribosomal entry site (IRES) and mutating the mRNA sequence alters the secondary structure of the mRNA and allows greater expression from other methionines in the mRNA. We know that translation from 8 different methionines in the full-length AIB1 sequence can give rise to a protein of molecular mass of approximately 130kDa so after mutation of a single methionine codon translation may be initiated at another methionine in the sequence (Figure 5b). Indeed if we look at the Kozak sequence for the 8 methionines in comparison to the full-length AIB1 Kozak sequence, we see that M\textsubscript{236} has the most prevalent Kozak sequence which is after where all the mutations have been made (Figure 5c). It is possible that disrupting the native mRNA structure of the AIB1-Δ4 transcript that translation is now freely able to occur at M\textsubscript{236}. 
The AIB1 and AIB1-Δ4 mRNA and protein are depicted in Figure 6. Full-length AIB1 contains 23 exons, which code for a 155 kDa protein of 1424 amino acids. Translation of AIB1 is initiated in exon 3 and continues to the stop codon in exon 23. The alternatively spliced AIB1-Δ4 lacks exon 4 and initiates translation in exon 7 at M_{224} of the full-length AIB1 sequence, but retains the same stop codon as full-length AIB1. Thus, AIB1-Δ4 is a protein of 1201 amino acids and molecular mass of 130 kDa.
3. Generation of AIB1-Δ4 specific antibodies

With knowledge of the translation initiation site of AIB1-Δ4 and the fact that the majority of the AIB1-Δ4 is N-terminally acetylated, we attempted to generate an antibody against the unique N-terminal acetylated AIB1-Δ4 peptide. This peptide would not exist in the full-length AIB1 sequence as N-terminal acetylation is not possible in the middle of a polypeptide chain. We contracted the polyclonal antibody generation to Abgent who immunized rabbits against the N-terminal acetylated peptide MQCFALSQPRK and monitored the generation of antibodies in serum by ELISA. After purification by positive selection with acetylated AIB1-Δ4 peptide and
negative selection with a non-acetylated AIB1-Δ4 peptide (Figure 7a), affinity purified antibodies were characterized for their ability to work in direct Western blotting and immunoprecipitation, and immunofluorescence. These polyclonal antibodies did not work in direct Western blotting or immunofluorescence, but did work for immunoprecipitation. We tested the ability of the antibodies to immunoprecipitate the transfected AIB1-Δ4 cDNA construct that we used to determine the N-terminus of AIB1-Δ4. We transfected HEK293T cells with AIB1-Δ4 FLAG and immunoprecipitated with the AIB1-Δ4 affinity purified antibodies (Figure 7b). We used a pan AIB1 antibody to determine the specificity of the AIB1-Δ4 antibodies. After immunoprecipitation with antibodies specifically selected to bind to the N-acetylated AIB1-Δ4 peptide, we saw that antibodies that detected both acetylated and non-acetylated peptides were able to immunoprecipitate both AIB1 and AIB1-Δ4 and such antibodies from rabbit#1 showed more affinity for the full-length AIB1 as opposed to rabbit#2 which had more affinity for AIB1-Δ4 (Figure 7b lanes 4 and 6). Antibodies that only bound to acetylated AIB1-Δ4 and did not bind to non-acetylated AIB1-Δ4 peptide specifically immunoprecipitated AIB1-Δ4 protein (Figure 7b lane 5). Antibodies purified from rabbit #2 were more specific for the AIB1-Δ4 protein as the seen in comparison of lanes 3 and 5, so all following experiments with affinity purified AIB1-Δ4 antibodies were performed with antibodies from rabbit #2.

To test if these antibodies were able to detect the endogenous AIB1-Δ4 we used MCF-7 cells, which have high levels of expression of AIB1-Δ4, for immunoprecipitation (Figure 7c). MCF-7 cells were immunoprecipitated with AIB1-Δ4 antibodies and subjected to Western blotting with pan AIB1 antibody. We found that these antibodies specifically detected only the AIB1-Δ4 protein independent of AIB1 (Figure 7c lane 2). To determine if the band we see is N-
acetylated AIB1-Δ4, we performed peptide competition experiments with either the N-acetylated or the non-acetylated immunizing peptide (Figure 7c, lanes 3 and 4). N-acetylated peptides added during immunoprecipitation with the AIB1-Δ4 antibodies competed for binding of the antibody and the presence of an AIB1-Δ4 while non-acetylated peptides had no effect on the ability of the antibody to immunoprecipitate AIB1-Δ4.

**Figure 7.** Characterization of affinity purified AIB1-Δ4 polyclonal antibodies. 

a) Purification scheme of affinity purified AIB1-Δ4 antibodies. Serum from rabbits immunized with N-acetylated MQCFALSQPRK peptide was positively selected on a N-acetylated AIB1-Δ4 peptide column. Antibodies eluted from this column were subjected to negative selection with a non-acetylated AIB1-Δ4 peptide column. The antibodies that bound the first column and passed through the second column were used as affinity purified AIB1-Δ4 antibodies. b) HEK293T cells were transfected with AIB1-Δ4 FLAG cDNA construct. 24 hours later cell lysates were harvested and used for immunoprecipitation with either FLAG antibody, antibodies selected to only recognize the N-terminal acetylated peptide of AIB1-Δ4, or antibodies that recognized both the N-terminal acetylated and non-acetylated AIB1/AIB1-Δ4 peptide. c) MCF-7 cell lysates were immunoprecipitated with affinity purified AIB1-Δ4 antibodies and competed with either N-acetylated or non-acetylated immunizing peptides. The immunoprecipitates were blotted with pan AIB1 antibody that detects both isoforms of AIB1.
B. AIB1-Δ4 Enters the Nucleus and is Recruited to Endogenous Estrogen Regulated Genes

1. Cellular localization of AIB1 and AIB1-Δ4

Our initial observation of the AIB1-Δ4 protein was that AIB1-Δ4 was a more potent coactivator of steroid receptor and growth factor dependent transcription than the full-length AIB1 (77). To investigate if the difference in coactivator activity of AIB1 and AIB1-Δ4 is due to difference in localization of the two isoforms, we looked at the distribution of AIB1 and AIB1-Δ4 throughout the cell. We took MCF-7 cells, which express endogenous AIB1-Δ4, and extracted nuclear and cytoplasmic proteins (Figure 8a). After fractionation of MCF-7 cell lysates we see that the majority of AIB1-Δ4 resides in the cytoplasm with a small proportion of AIB1-Δ4 in the nucleus (68% cytoplasmic vs. 32% nuclear). In comparison, AIB1 has equal distribution between the nuclear and cytoplasmic compartments (50% cytoplasmic vs. 50% nuclear). Although AIB1 is thought of primarily as a nuclear protein, this finding corroborates previous data that suggested that most of AIB1 is not tightly associated with non-extractable structures in the nucleus and is a readily extractable protein (84, 85) in cellular fractionation experiments.

With the generation of polyclonal antibodies that detect AIB1-Δ4 protein independent of AIB1 we hoped to be able to distinguish the localization of the endogenous AIB1-Δ4 protein using these antibodies in immunofluorescence. Unfortunately, these antibodies do not work well in immunofluorescence so we instead created a FLAG tagged cDNA construct of AIB1-Δ4 in order to specifically detect the cellular distribution of AIB1-Δ4. We also created a FLAG AIB1 cDNA construct so that we could determine the cellular distribution of AIB1 independent of AIB1-Δ4 as well. We saw that FLAG AIB1-Δ4 when transfected into HEK293T cells
recapitulates the cellular distribution of the endogenous AIB1-Δ4 protein in cell fractionation experiments (Figure 8b). After fractionation we found that the majority of the AIB1-Δ4 is in the cytoplasm with 77% of the protein in the cytoplasm vs. 23% in the nucleus.

To get around the issue of AIB1 being easily extracted from the nucleus in cell fractionation experiments, we used immunofluorescence to determine the localization of both isoforms of the AIB1 protein. In immunofluorescence proteins are fixed by paraformaldehyde fixation, which creates covalent chemical bonds between proteins and stabilizes the protein-protein interactions and may prevent AIB1 from exiting the nucleus as in fractionation experiments. We transfected FLAG AIB1 and FLAG AIB1-Δ4 constructs into Chinese Hamster Ovary (CHO) cells. After transfection with FLAG AIB1 (Figure 8; top panel of images), we saw two major distribution patterns of AIB1 in the cell. The first was a nuclear pattern of staining for AIB1 (green) which overlaid completely with the nuclear DNA stain 4',6-diamidino-2-phenylindole (DAPI, blue) (Figure 9; highlighted by red arrowhead in low and red bar in high

Figure 8. AIB1-Δ4 is a predominantly cytoplasmic protein. a) Nuclear and cytoplasmic fractions were isolated from MCF-7 cell lysates and subjected to Western blotting with pan AIB1 antibody that recognizes both isoforms of AIB1. HSP90 and HDAC1 blotting was performed as controls for cytoplasmic and nuclear proteins. b) HEK293T cells were transfected with FLAG AIB1-Δ4 and nuclear and cytoplasmic cell fractionation fractions were isolated and analyzed as in a. Densitometry for AIB1-Δ4 was performed using Adobe Photoshop.
magnification images). The second pattern of staining for AIB1 was a nuclear/cytoplasmic distribution of staining, which overlaid with the DAPI area and the cytoplasm outside of the DAPI stained area (Figure 9; highlighted by yellow arrowhead in low and yellow bar in high magnification images). For AIB1-Δ4, we observed three distinct staining patterns (Figure 9; bottom panels). The two predominant staining patterns that we observed for AIB1-Δ4 were a nuclear/cytoplasmic pattern (Figure 9; highlighted by yellow arrowhead in low and yellow bar in high magnification images) and a cytoplasmic pattern of staining, which did not overlay with DAPI staining (Figure 9; highlighted by blue arrowhead in low and blue bar in high magnification images).

We then quantified the steady state staining patterns for both AIB1 and AIB1-Δ4. AIB1 showed nuclear and nuclear/cytoplasmic staining in 75% and 25% of cells respectively. In contrast, the percentage of cells with nuclear, nuclear/cytoplasmic, and cytoplasmic only staining for AIB1-Δ4 was 1.5%, 41.3%, and 57.2% of cells. These data show that AIB1 is a predominantly nuclear protein as shown by other groups and the nuclear/cytoplasmic distribution seen in the cellular fractionation experiments likely a result of the loose association of AIB1 to nuclear proteins. In contrast, AIB1-Δ4 is localized primarily in the cytoplasm at steady state. This is not surprising since three groups have previously identified the nuclear localization sequence (NLS) of AIB1 (84, 86, 87). The bipartite NLS resides in amino acids 16-19 and 35-38 of AIB1. Since we have identified AIB1-Δ4 to initiate translation at amino acid 224 of the full-length AIB1 sequence, AIB1-Δ4 has lost the bipartite NLS. Based on this information we would predict that AIB1-Δ4 would be localized in the cytoplasm of the cell. We confirm this prediction.
with our data, however, it is still curious that AIB1-ΔΔ₄ is a more potent coactivator of nuclear hormone receptors than AIB1.

**Figure 9. Cellular Localization of AIB1 and AIB1-Δ4.** CHO cells were grown in DMEM F12+10%FBS and transfected with either FLAG AIB1 or FLAG AIB1-Δ4 and the cellular distribution of staining for the isoforms was determined by indirect immunofluorescence staining with FLAG antibody. FLAG staining is shown in the left panels (green), DAPI staining DNA in the nucleus is shown in the middle panels (blue), and merge images in the right panels. Typical nuclear, nuclear/cytoplasmic, and cytoplasmic staining is shown in the smaller panels below the larger images. Red, yellow, and blue arrowheads note the nuclear, nuclear/cytoplasmic, and cytoplasmic stained cells shown in the insets below the large field images. The percentage of nuclear, nuclear/cytoplasmic, and cytoplasmic staining cells was quantified by counting 200 cells per condition and the data are a summary of three independent experiments.
2. AIB1-Δ4, like AIB1 can enter the nucleus

Since we observed that the localization of AIB1-Δ4 is predominantly cytoplasmic, we wanted to determine if AIB1-Δ4 could enter the nucleus like AIB1. AIB1 and AIB1-Δ4 both contain a nuclear export sequence (NES) in the C-terminal half of the protein (Figure 6). If AIB1-Δ4 is able to enter the nucleus, blockade of nuclear export should increase the amount of AIB1-Δ4 staining the nuclear compartment of the cell. To block nuclear export we used leptomycin B. Leptomycin B is a compound isolated from Streptomyces bacteria found to be an anti-fungal agent and inhibits export of proteins with a nuclear export sequence (NES). CRM1/XPO1 (Chromosome region maintenance 1/exportin 1) binds to proteins with a NES to transport them out of the nucleus (88) and leptomycin B binds directly to CRM1 to inhibit its interaction with these proteins.

We transfected CHO cells with FLAG AIB1-Δ4 and treated the cells with either carrier or 50 nM leptomycin B for 4 hours before fixing and analyzing the cells via immunofluorescence (Figure 10). We observed a mainly cytoplasmic distribution of AIB1-Δ4 in cells after treatment with carrier (Figure 10; top panels). After treatment with leptomycin B, the major staining pattern of AIB1-Δ4 is the nuclear/cytoplasmic phenotype (Figure 10; bottom panels). CHO cells transfected with AIB1-Δ4 stained the nuclear, nuclear/cytoplasmic, and cytoplasmic compartments in 18%, 60%, and 22% of the cells respectively after treatment. We detected an increase in the percentage of cells staining in the nuclear and nuclear/cytoplasmic compartments with a decrease in the percentage of cells staining only the cytoplasm. The increase in the percentage of cells that stained in the nucleus after nuclear export blockade for AIB1-Δ4 indicates that AIB1-Δ4 can indeed enter the nucleus.
Figure 10. AIB1-Δ4 enters the nucleus. CHO cells were transfected as in Figure 8 except 24 hours after transfection cells were treated with carrier (ethanol) or 50 nM leptomycin B for 4 hours. AIB1-Δ4 transfected cells stained primarily in the cytoplasm without treatment and in the nuclear/cytoplasmic compartments after blockade of nuclear export. The compartment percentage was determined by counting 200 cells per condition in three independent experiments.

3. AIB1-Δ4 enters the nucleus possibly through interaction with other NLS containing proteins

Since we determined that AIB1-Δ4 can enter the nucleus, we tried to identify possible mechanisms for how AIB1-Δ4 can be imported into the nucleus without a canonical NLS. Proteins involved in signaling, such as β-catenin, ERK MAP kinase, ERK2, and SMAD2 are imported into the nucleus in a NLS independent manner (89-93). A potential mechanism of nuclear import is interaction of AIB1-Δ4 with another protein that contains a NLS. It was shown that the PAS B domain was sufficient for heterodimerization and homodimerization of p160 SRC family members (94). Since both AIB1 and AIB1-Δ4 both contain the PAS B domain, it is possible that AIB1-Δ4 may bind to AIB1 and be imported into the nucleus with AIB1. To
Figure 11. AIB1-Δ4 interacts with AIB1. HEK293T cells were grown in DMEM+10%FBS and transfected with either FLAG AIB1, AIB1-Δ4, or FLAG AIB1 and AIB1-Δ4 together. Lysates from these cells were then immunoprecipitated using FLAG antibody to pulldown FLAG AIB1 and any interacting proteins. Low (top panel) and high exposures (bottom panel) are shown for AIB1 Western blot.

To determine if AIB1-Δ4 and AIB1 interact, we transfected HEK 293T cells with FLAG AIB1 and AIB1-Δ4 and immunoprecipitated for FLAG AIB1 (Figure 11). Immunoprecipitation of lysates from cells transfected with FLAG AIB1 and AIB1-Δ4 and Western blotted with an AIB1 antibody that recognizes both isoforms showed that AIB1-Δ4 immunoprecipitates with AIB1 (Figure 11, lane 6, top panel). Interestingly, there are detectable amounts of endogenous AIB1-Δ4 protein in HEK293T cells and immunoprecipitation with FLAG AIB1 is able to pull down endogenous AIB1-Δ4 protein as well (Figure 11, lane 4, bottom panel). When no FLAG AIB1 is present, we do not detect any AIB1 or AIB1-Δ4 after immunoprecipitation with FLAG antibody (Figure 11, lane 5, top and bottom panels). Therefore we see that AIB1-Δ4 is able to interact with AIB1.

After identifying that AIB1-Δ4 can interact with AIB1, we hypothesized that AIB1 might be able to facilitate the nuclear import of AIB1-Δ4 by allowing AIB1-Δ4 to “piggyback” with AIB1 into the nucleus. To test this hypothesis we transfected CHO cells with FLAG AIB1-Δ4 and examined the distribution of AIB1-Δ4 after co-transfection with HA AIB1 (Figure 12).
see that CHO cells transfected with FLAG AIB1-Δ4 alone had a similar distribution of AIB1-Δ4 as before with 67±3.6% and 33±3.6% of cells staining in the cytoplasm and nuclear/cytoplasmic compartments of the cell respectively. Co-transfection of HA AIB1 with FLAG AIB1-Δ4 shifted the staining pattern for AIB1-Δ4 to 2.7±1.5%, 63.3±1.5%, and 34±1% in the nuclear, nuclear/cytoplasmic, and cytoplasmic compartments. There was a statistically significant increase in the nuclear/cytoplasmic staining and decrease in cytoplasmic staining in cells transfected with both AIB1 and AIB1-Δ4 as compared to cells transfected with AIB1-Δ4 alone. This data suggests that AIB1 can facilitate the nuclear import of AIB1-Δ4 possibly through interaction with AIB1 or complexes involving AIB1.
We see that this effect is not an effect related only to CHO cells as we have transfected AIB1 knockout mouse embryonic fibroblasts (KO MEFs) with FLAG AIB1-Δ4 with increasing amounts of AIB1 protein (Figure 13). We observed an increase the nuclear staining pattern of AIB1-Δ4 after transfection with increasing amounts of AIB1 and the increase in nuclear AIB1-Δ4 staining was dose dependent. The pattern of staining of AIB1-Δ4 that was cytoplasmic,
nuclear/cytoplasmic, or nuclear was 86±1%, 14±1%, and 0% respectively and shifted to 70.3±3.5%, 29.3±4%, and 0.3±0.6% with the highest level of full-length AIB1 co-transfection. We believe that this may be an underestimate since in this experiment we do not know how many of the AIB1-Δ4 transfected cells were also transfected with AIB1 as well. However, the increase in the number of nuclear/cytoplasmic and decrease in cytoplasmic stained cells after transfection with increasing amounts of AIB1 is statistically significant. Together these data suggest that interaction of AIB1 with AIB1-Δ4 localizes AIB1-Δ4 to the nucleus.
Figure 13. AIB1-Δ4 is imported into the nucleus by AIB1. AIB1 KO MEFs were transfected by electroporation with 4 μg FLAG AIB1-Δ4 alone or with 2, 4, or 6 μg of untagged AIB1 and plated on glass cover slips. Cells were fixed and permeabilized 24 hours after plating and stained for FLAG AIB1-Δ4 and nuclei stained with DAPI. Cells were then analyzed by confocal microscopy and some representative nuclear/cytoplasmic and cytoplasmic stain were analyzed by o

*=p<0.05, ***=p<0.0
We wanted to test if AIB1-Δ4 could also bind to other NLS-containing proteins to be imported into the nucleus. The interaction of AIB1 with other transcriptionally active proteins is well characterized. One of these proteins is p300/CBP (15) and we show that AIB1-Δ4 also interacts with p300 by immunoprecipitation (Figure 14). HEK293T cells were transfected with either p300-HA, FLAG AIB1, or FLAG AIB1-Δ4 and cell lysates were harvested. After assaying the lysates for protein content, equal amounts of either FLAG AIB1 or FLAG AIB1-Δ4 were added to p300-HA cell lysate. The amount of FLAG AIB1 and FLAG AIB1-Δ4 proteins were detected after immunoprecipitation with p300-HA antibody. We found that both AIB1 and AIB1-Δ4 associated with p300. Interestingly, with equal amounts of FLAG AIB1 or FLAG AIB1-Δ4 in each immunoprecipitation we found more AIB1-Δ4 associated with p300. This may suggest that AIB1-Δ4 is in active transcriptional complexes since p300 is typically found in active transcriptional complexes.

**Figure 14. AIB1-Δ4 interacts with p300.** HEK293T cells were transfected with either p300-HA, FLAG AIB1, or FLAG AIB1-Δ4. Equal amounts of FLAG AIB1 or FLAG AIB1-Δ4 lysate were incubated with equal amounts of p300-HA cell lysate. After immunoprecipitation with HA antibody to pull down p300 and associated proteins, a FLAG Western blot was performed to determine how much AIB1 and AIB1-Δ4 immunoprecipitated with p300. Densitometry was performed with Adobe Photoshop.

To ascertain if AIB1-Δ4 was similarly more nuclear after co-transfection with another protein with a NLS, we transfected CHO cells with FLAG AIB1-Δ4 and p300-HA and...
Figure 15. **p300 can facilitate the nuclear import of AIB1-Δ4.** CHO cells were transfected with FLAG AIB1-Δ4 alone (top panels) or with an equal amount of p300-HA (bottom panels) and plated on glass cover slips in DMEM F12+10% FBS. Cells were fixed and permeabilized 24 hours after plating and stained for FLAG AIB1-Δ4 (green), p300-HA (red) and nuclei (blue) with DAPI. Cells were analyzed by indirect immunofluorescence. The number of nuclear, nuclear/cytoplasmic, and cytoplasmic staining cells was quantified for three experiments as in Fig 9. Data were analyzed by t-test. **=p<0.01 when compared to AIB1-Δ4 transfection alone.

AIB1-Δ4 alone showed staining in the nuclear/cytoplasmic and cytoplasmic cell compartments 32.3±1.5% and 67.7±1.5% respectively, which shifted to 56±5.3% and 44±5.3% with co-transfection with p300.
We also transfected AIB1-Δ4 with p300-HA into the AIB1 KO MEFs (Figure 16). Again, we saw that the nuclear staining pattern of AIB1-Δ4 was increased with increased expression of p300-HA and it was not cell line dependent. The pattern of staining of AIB1-Δ4 that was cytoplasmic, nuclear/cytoplasmic, or nuclear was 86±1%, 14±1%, and 0% respectively and shifted to 23.7±2.5%, 63±4.6%, and 13.3±2.3%. Again we observed that the accumulation of nuclear AIB1-Δ4 was dependent on the level of expression of p300-HA in a dose dependent fashion. These data together suggest that AIB1-Δ4 may interact with other NLS containing proteins to traffic into the nucleus.
Figure 16. AIB1-Δ4 is imported into the nucleus by p300. AIB1 KO MEFs were transfected by electroporation with 4 µg AIB1-Δ4 alone or with 2, 4, or 6 µg of p300-HA and plated on glass cover slips in DMEM+10% FBS. Cells were fixed and permeabilized 24 hours after plating and stained for DAPI, FLAG, and HA containing proteins. Cells were then analyzed by confocal microscopy. The number of nuclear, nuclear/cytoplasmic, and cytoplasmic cells were analyzed by three experiments were analyzed by one way ANOVA with Tukey’s multiple comparison test. When compared to AIB1-Δ4 alone. ***p<0.001.
4. AIB1-Δ4 is recruited to endogenous estrogen responsive genes like AIB1

AIB1-Δ4 like AIB1 is imported into the nucleus, but we have yet to see if AIB1-Δ4 is found at active sites of transcription. Since AIB1 and AIB1-Δ4 have been shown to coactivate estrogen-induced transcription (77), we wanted to determine if AIB1-Δ4 is recruited to estrogen response elements (ERE) in estrogen responsive genes. Trefoil Factor 1 (TFF1/pS2), human complement 3 (hC3), and human epidermal growth factor receptor 2 (HER2) are genes with ERE that are regulated by estrogen (95-97). We transfected HEK293 cells with ERα and either FLAG AIB1 or FLAG AIB1-Δ4 and performed chromatin immunoprecipitation analysis (ChIP) to look at the recruitment of the two isoforms to ERE in the endogenous pS2, hC3, and HER2 genes after 15, 30, 45, and 60 minutes of estrogen stimulation (Figure 17). Maximal recruitment of FLAG AIB1 occurred at 15 minutes to 8.7%, 4.3%, and 5.0% of the EREs in the pS2, hC3, and HER2 genes respectively. In contrast, maximal recruitment of FLAG AIB1-Δ4 happened at 30 minutes to 3.5%, 4.2%, and 3.1% of the EREs in the pS2, hC3, and HER2 genes respectively. This indicates the both AIB1 and AIB1-Δ4 are recruited to the EREs in endogenous genes confirming that AIB1-Δ4 is found in the nucleus and found at active sites of transcription. Interestingly, the time to maximal recruitment of AIB1-Δ4 recruitment is delayed relative to AIB1, which is possibly due to inefficient or altered nuclear transport because of lack of a canonical NLS. The corresponding protein levels are shown by Western blot for all the different times of estrogen stimulation.
To confirm that this effect of nuclear recruitment of AIB1-Δ4 to gene elements was not an artifact of the overexpression system, we utilized the affinity purified AIB1-Δ4 antibodies to determine if endogenous AIB1-Δ4 is recruited to the same EREs in pS2, hC3, and HER2 in MCF-7 cells. We compared the ChIP with AIB1-Δ4 antibodies to a pan AIB1 antibody that detects both AIB1 and AIB1-Δ4 as a control since the amount of AIB1 protein is significantly greater than the level of expression of AIB1-Δ4 (Figure 7c, input lane) and the majority of the signal from the ChIP with this antibody will be due to full-length AIB1. After ChIP we see that AIB1 and AIB1-Δ4 are recruited to ERE in endogenous estrogen regulated genes. HEK293 cells grown in phenol red free IMEM+10% charcoal stripped serum were transfected with ERα and either FLAG AIB1 or FLAG AIB1-Δ4. 24 hours later cells were stimulated with estrogen and harvested at 0, 15, 30, 45, and 60 minutes after estrogen using a FLAG for each ERE independent of transfection for Figure 17.
both AIB1 and AIB1-Δ4 are recruited to the three estrogen responsive genes and the same delay in the recruitment of AIB1-Δ4 relative to AIB1 for pS2 and HER2. Maximal recruitment occurred at 15 minutes for full-length AIB1 for pS2 and HER2 while peak recruitment for AIB1-Δ4 was delayed to either 30 minutes or 45 minutes for pS2 and HER2 respectively.

**Figure 18.** Endogenous AIB1 and AIB1-Δ4 are recruited to ERE in endogenous estrogen regulated genes. MCF-7 cells grown in phenol red free IMEM+10% charcoal stripped serum for three days to remove estrogens. 24 hours later cells were stimulated with 10 nM estrogen and harvested at 0, 15, 30, 45, and 60 minutes after estrogen stimulation. The AIB1s and AIB1-Δ4 subjected to immunoprecipitation ChIP analysis using either pan-AIB1 or AIB1-Δ4 antibodies. The percentage of the input recovered after immunoprecipitation for each ERE in pS2, hC3, or HER2 was determined. Data is representative of two independent experiments.
5. AIB1-Δ4 increases the expression of estrogen regulated genes

We next wanted to confirm that the recruitment of AIB1-Δ4 to these EREs in endogenous estrogen responsive genes was correlated with an increase in expression of these genes. To determine if AIB1-Δ4 expression leads to an increase in expression of these genes we transfected HEK293 cells with ERα and either FLAG AIB1 or FLAG AIB1-Δ4 and observed the transcript levels of pS2, hC3, and HER2 genes at 4, 8, and 24 hours (Figure 19). In our previous studies (77, 78) we have noted an increase of coactivation of steroid hormone and growth factor induced signaling after expression of AIB1-Δ4 in cell lines so we would predict that AIB1-Δ4 recruitment to the ERE in these genes would lead to an increase in their expression. Indeed we observed an increase in the expression of these estrogen responsive genes after transfection with AIB1-Δ4. For AIB1 transfected cells we detected maximal gene expression at 24 hours for all three genes. We found maximal gene expression in AIB1-Δ4 transfected cells at 8 hours for HER2 and at 24 hours for pS2 and hC3. Significant differences in gene expression were only observed in the AIB1-Δ4 transfected cells suggesting that AIB1-Δ4 is better able to stimulate expression of estrogen responsive genes than full-length AIB1. This is quite interesting since we the majority of the AIB1-Δ4 protein in the cytoplasm (Figure 8b).
C. AIB1-Δ4 Lacks the Inhibitory Domain Contained in the N-terminus of Full-length AIB1

1. The N-terminus of AIB1 contains an inhibitory domain

Based on our previous data we have seen that AIB1-Δ4 is a more potent coactivator of transcription than full-length AIB1 on a per mole basis (77). This is interesting since we find at steady state levels that the amount of AIB1 in the nucleus is far greater than the amount of AIB1-Δ4 (Figure 8a). How this little amount of AIB1-Δ4 is able to coactivate transcription when the majority of it is in the cytoplasm was a question we wanted to explore further. One possible
explanation is that the N-terminus of the full-length protein contains an inhibitory domain. This region is lost from AIB1-Δ4 and allows AIB1-Δ4 to be a potent coactivator. AIB1 function on the other hand would be inhibited through the N-terminus.

To test this hypothesis we utilized a reporter assay, which is a readout of AIB1 coactivator function and tested if overexpression of this N-terminal fragment (AIB1 N term) could relieve the repression on the full-length AIB1 protein (Figure 20a). This fragment contains the bHLH and PAS A domains not present in the AIB1-Δ4 protein and should have no coactivator function since it lacks the C-terminal activation domain responsible for binding to nuclear receptors and molecules that affect transcription such as p300/CBP and CARM1. We transfected COS-7 cells with a mouse mammary tumor virus (MMTV) luciferase, progesterone receptor (PR), and FLAG AIB1 with increasing amounts of AIB1 N term or without co-transfection (Figure 20b). We chose a progesterone inducible system since in previous studies it showed the greatest effect of coactivation (73, 77). As expected, AIB1 is able to coactivate transcription of the luciferase reporter relative to empty vector transfected cells (AIB1 0 vs. 500). Co-transfection of AIB1 with AIB1 N term significantly increased the amount of transcription from the luciferase reporter. This effect was increased with the amount of AIB1 N term co-transfected with AIB1 (AIB1 co-transfection with AIB1 N term 125, 500, 750). A relief of repression on endogenous AIB1 coactivator activity due to co-expression of the AIB1 N term construct alone was also apparent (AIB1 N term 0 vs. 500). The levels of AIB1 and AIB1 N term protein are shown by Western blot and there is no increase in the AIB1 protein with co-transfection of higher levels of AIB1 N term (Fig. 20c). This data suggests a suppressor role of the N-terminal region of AIB1 in the regulation of the coactivator function of AIB1.
The N terminus of AIB1 contains an inhibitory domain that is lost in AIB1-Δ4. a) Proposed mechanism of repression of the full-length AIB1 protein. An inhibitor that binds to the N terminus of AIB1 ordinarily represses its coactivator function. Overexpression of an N-terminal fragment of AIB1 lost in AIB1-Δ4 may bind to the squelching factor, which normally regulates the coactivator function of AIB1 thereby relieving repression on the full-length AIB1 protein. b) COS-7 cells were transfected with human progesterone receptor B (25 ng), MMTV luciferase (100 ng), and either pcDNA3 or FLAG AIB1 (500 ng) with increasing amounts of FLAG AIB1 N term (125, 500, and 750 ng). 24 h later, cells were treated with 10 nM R5020 for 24 h before reporter activity was determined. Luciferase values were normalized to thymidine kinase Renilla (10 ng) reporter activity. The assay was plated in triplicate, and a representative graph is shown from three separate experiments. Data were analyzed by one-way ANOVA with Tukey’s multiple comparison post-test. **, p<0.01; ***, p<0.001 when compared with FLAG AIB1 transfection alone. c) The relative amount of FLAG proteins in the COS-7 cells is shown by Western blot.

![Graph and Western blot image]
2. The AIB1-Δ4 is more resistant to proteasomal degradation than full-length AIB1

The proteasomal regulation of full-length AIB1 levels has been studied extensively (28, 58-63). Based this work there appears to be two major sites of regulation of the AIB1 protein. Work from our lab has suggested that a site of regulation of the full-length AIB1 protein resides in the C-terminus of the protein (58). While work from others has shown that there is a region in the N-terminus that regulates the protein levels of AIB1, which is a regulated by phosphorylation and they term a phospho-degron (62). Based on this information we wanted to determine if the AIB1-Δ4 protein is differentially regulated from the full-length AIB1 protein. We measured the stability of the AIB1-Δ4 relative to the full-length AIB1 protein by subjecting the both isoforms of the protein to conditions that favor the degradation of the full-length protein. Our group has published that in response to growth of cells at high density or withdrawal of serum leads to a dramatic decrease in the protein expression of full-length AIB1 and this is mediated through the proteasome (58, 63). To observe the regulation of the two isoforms of the protein we transfected HEK293 cells with either FLAG AIB1 or FLAG AIB1-Δ4, plated the cells at high density, and treated cells with carrier or MG132, which is a specific reversible inhibitor of the proteasome (Figure 21). We saw that AIB1 protein is degraded under high confluence conditions and the protein expression can be rescued by treatment with MG132 (lanes 3 vs. 4). In contrast, the levels of AIB1-Δ4 protein were much higher than full-length AIB1 and addition of MG132 did not increase the level of expression of AIB1-Δ4 (lanes 5 vs. 6). These data suggest that there is a level of regulation on AIB1 that is lost in the AIB1-Δ4 protein and that the N-terminus plays a critical role in the proteasomal regulation of the AIB1 protein.
3. The N-terminus of AIB1 has potential sites of inhibitory phosphorylation

Prior to the identification of the phosphor-degron in the N-terminus we attempted to determine if there were potential sites of phosphorylation in the N-terminus that might be negative sites of regulation due to phosphorylation. To achieve this we used the FLAG AIB1 N term fragment which contains the bHLH and PAS A domains used in the inhibitor competition assay (Figure 20). We postulated that conditions that favor degradation of the full-length AIB1 protein would be suitable for detecting phosphorylations in the N-terminus that could be negative sites of regulation. We transfected HEK293T cells with FLAG AIB1 N term and either subjected the cells to growth medium with or without serum. These conditions had been shown to favor the degradation of the full-length protein (58). After growth under these conditions the fragment was immunoprecipitated from whole cell lysates and subjected to mass spectrometric analysis to identify phosphorylation sites (Figure 22). We identified numerous phosphorylation sites and interestingly the serum withdrawal conditions led to more sites of phosphorylation than in the cells grown in serum containing media. The strongest peak in the serum withdrawal

![Figure 21](image-url)
Figure 22. The N-terminus of AIB1 harbors potential negative regulatory sites of phosphorylation. HEK293T cells were grown in DMEM+10%FBS and transfected with either FLAG AIB1 N term. 24 hours later cells were grown in media with or without serum. Whole cell lysates were harvested and immunoprecipitated with FLAG M2 antibody. After SDS-PAGE, a band corresponding to the FLAG AIB1 N term fragment was isolated and subjected to analysis by mass spectrometry. This site is in the phospho-degron and has been shown to be modulated by both protein phosphatase 1 (PP1) and speckle-type POZ protein (SPOP) (62, 63).
D. AIB1-Δ4 Expression Correlates with Metastatic Potential

1. Semi-quantitative detection of AIB1-Δ4 protein relative to AIB1 in breast cell lines

As we have previously reported, the expression of AIB1-Δ4 is higher in breast cancer cell lines relative to normal or immortalized breast cells (77). Based on the recent publication that AIB1-Δ4 increased the metastatic potential of the metastatic breast cancer cell line MDA-MB-231 through mediating the interaction of FAK and EGFR (81), we decided to look at a panel of cells that would represent a spectrum of invasive and non-invasive breast cells (Figure 23). MCF10A are immortalized breast cells and are not tumorigenic. MCF10DCIS.com are a sub line derived from a MCF10AT cells stably transfected with activated T24 c-Ha-Ras that are tumorigenic but form ductal carcinoma in situ (DCIS) lesions with a comedo type pathology (98). T47D and MCF-7 cells are estrogen positive ductal carcinoma cell lines that are generally thought of as less invasive (99). MDA-MB-231 cells are estrogen receptor negative breast cancer cells that are highly metastatic (100-102). With this panel of cell lines we wanted to observe any correlation of AIB1-Δ4 expression with metastatic phenotype. We probed whole lysates from these cell lines by Western blot with a pan AIB1 antibody to determine the relative levels of full-length AIB1 protein and AIB1-Δ4 protein. We see that the expression of AIB1-Δ4 is higher in tumor forming cells and in more metastatic cells. The non-tumorigenic MCF10A and DCIS forming MCF10DCIS.com cells show no AIB1-Δ4 expression. The less metastatic T47D cells show lower levels of AIB1-Δ4 relative to the highly metastatic MDA-MB-231 cells. MCF-7 cells have the highest levels of AIB1 and AIB1-Δ4, however this due to the fact that MCF-7 cells have amplification of the AIB1 gene (27). Overall we see that AIB1-Δ4 correlates with metastatic potential.
2. Quantitative detection of AIB1-Δ4 mRNA independent of AIB1 mRNA using Scorpion primer technology

a. Scorpion primer design and validation

Detection of proteins by Western blotting is semi-quantitative and even with the use of the affinity purified AIB1-Δ4 antibodies we would still only be able to compare the relative expression levels of AIB1-Δ4 between various cancer cell lines or tissue samples. We set out to develop a quantitative and high throughput methodology to determine the relative expression of AIB1-Δ4. Real time PCR allows for quantitative and high throughput detection of AIB1-Δ4 mRNA. To distinguish the full-length AIB1 from the spliced AIB1-Δ4 mRNA we took advantage of the unique sequence that arises in the AIB1-Δ4 mRNA due to the splicing of exon 3 to exon 5 and the loss of exon 4 from the AIB1-Δ4 mRNA. We have tried to use both Taqman

![Figure 23. AIB1-Δ4 in non-metastatic and metastatic breast cell lines. Non-tumorigenic MCF10A, DCIS lesion forming MCF10DCIS.com, T47D and MCF-7 less invasive, and MDA-MB-231 highly metastatic cells were analyzed by Western blot for expression of AIB1-Δ4 protein. Cells were grown in their respective media as described in the methods and kept at subconfluence before cell lysate harvest.](image-url)
and SYBR green based primers to distinguish between the two splice variants with no success (data not shown). Our final strategy involved the use of Scorpion based primers to specifically detect the AIB1-Δ4 mRNA independent of the AIB1 mRNA. Scorpion primers have been used to distinguish single nucleotide polymorphisms as well as for identifying splice variants of gene products (103, 104). The Scorpion primer reaction consists of a forward primer coupled to a stem loop structure, whose loop is a probe sequence, and a reverse primer. Extension from the forward primer creates a target sequence to which the probe is complimentary. The thermodynamics of the stem loop sequence is such that if an appropriate target is generated during cycling of the reaction that the forward primer, the probe target interaction is favored over the stem loop conformation. If no appropriate target is generated then the stem loop conformation is favored over and non-specific probe interaction. The reaction for the AIB1-Δ4 Scorpion reaction binding to either full-length AIB1 or AIB1-Δ4 mRNA is depicted in Figure 24a. For the AIB1-Δ4 Scorpion primer the forward primer sequence is in exon 3 and the probe target sequence is the unique sequence derived from the splicing of exon 3 to exon 5. For the AIB1 Scorpion primer the forward primer sequence is in exon 3 with a probe target sequence in exon 4, which is not contained in the AIB1-Δ4 transcript. We tested both AIB1 and AIB1-Δ4 Scorpion primers against plasmids that contain cDNA for either AIB1 or AIB1-Δ4 (Figure 24b and c). With the AIB1 Scorpion primer we only detect an increase in fluorescence by real time PCR when the template is AIB1 cDNA and not AIB1-Δ4 cDNA (Figure 24b). For the AIB1-Δ4 Scorpion primer fluorescence is only detected when AIB1-Δ4 cDNA is the template and not AIB1 cDNA (Figure 24c). We now have developed a quantitative and high throughput method to detect AIB1 and AIB1-Δ4 mRNA independently.
Figure 24. Validation of AIB1 and AIB1-Δ4 Scorpion primers. a) Scorpion primers were designed to specifically recognize AIB1 or AIB1-Δ4 mRNA and consist of forward primer (black half arrow), blocker (blue jagged line), quencher (purple octagon), probe (red - exon3, light blue - exon 5), stem (black attached lines), and reporter (green ball). The stem region was used to specifically recognize AIB1 or AIB1-Δ4 mRNA and consist of forward primer (black half arrow), blocker (blue jagged line), quencher (purple octagon), probe (red - exon3, light blue - exon 5), stem (black attached lines), and reporter (green ball). The stem region will only be dissociated when a target sequence is created during the process of PCR. For AIB1-Δ4 Scorpion primers the unique splice junction of exon 3 and exon 5 was used as a probe target sequence. When AIB1-Δ4 Scorpion primers use AIB1 mRNA as a template, no appropriate target is generated so the preferred conformation is to remain in the stem loop with the reporter quenched. When AIB1-Δ4 transcript is used as a template, an appropriate target is generated and the probe can then bind its target sequence allowing the reporter to fluoresce. AIB1 Scorpion primers had a probe sequence complementary to exon 4 not contained in AIB1-Δ4 transcripts.

b) AIB1 Scorpion primer reaction with AIB1 (open circle) and AIB1-Δ4 (closed triangle) cDNA as templates. c) AIB1-Δ4 Scorpion primer reaction with AIB1 (open circle) and AIB1-Δ4 (closed triangle) cDNA as templates.
b. AIB1-Δ4 correlates with invasiveness in pancreatic cancer cell lines

With the data that AIB1-Δ4 protein levels correlate with metastatic phenotype (Figure 23) and the development of AIB1 and AIB1-Δ4 specific primers, we wanted to determine if the AIB1-Δ4 mRNA levels also correlated with invasiveness. The pancreatic cancer cell line COLO357 has a well characterized model of orthotopic mouse model of metastasis (105). In this model a fast growing variant of the COLO357 human pancreatic cancer cells were injected to the pancreas or spleen of nude mice. Liver metastases were harvested, cultured and re-implanted to either the pancreas or spleen again. After three in vivo selections the cells the obtained COLOPL (pancreas to liver) and COLOS (spleen to liver) cell lines and compared the growth potential in vitro and in vivo relative to the COLO357 cell line. The metastatic variants had a shorter doubling time and had a decreased time to tumor formation than the parental COLO357 cells suggesting that they were more aggressive and invasive. We decided to determine the ratio of AIB1-Δ4 mRNA to AIB1 mRNA in these three cell lines to determine if AIB1-Δ4 correlated with the metastatic phenotype of these cells (Figure 25). After normalizing the expression of each transcript to actin we see that the ratio of AIB1-Δ4 transcript to AIB1 transcript is higher in the two metastatic variants of the COLO357 cells indicating a correlation of AIB1-Δ4 expression with metastatic capability.
c. AIB1-Δ4 correlates with metastatic potential in breast cancer cell lines

Since we saw the correlation of AIB1-Δ4 expression in the pancreatic cancer cell lines we wanted to confirm if AIB1-Δ4 correlates with metastatic potential in breast cancer cell lines. MDA-MB-231 breast cancer adenocarcinoma cells have been shown to be highly metastatic (106). More recently MDA-MB-231 cells have been in vivo selected to home to either the bone, brain, or lung (100-102). These three sub lines of MDA-MB-231 have been injected into the arterial circulation and bone (SCP2-TR), brain (MDA231-BrM2), and lung (4175-TR) homing variants were harvested and characterized. We used these cell lines as well as the parental cell line MDA-MB-231 and compared their relative AIB1-Δ4 mRNA expression (Figure 26). We also determined the AIB1-Δ4/AIB1 mRNA ratio in HMEC cells as a comparison to a non-tumorigenic non-immortalized cell line. We see that HMEC have expression of AIB1-Δ4 mRNA and that they have lowest AIB1-Δ4/AIB1 ratio compared to the parental and tissue
specific MDA-MB-231 cells correlating AIB1-Δ4 expression with malignancy. Of all the MDA-MB-231 variants the parental cells have the lowest AIB1-Δ4/AIB1 mRNA ratio. All of the in vivo selected tissue specific variants have a higher AIB1-Δ4/AIB1 ratio than the parental MDA-MB-231 cells suggesting that these AIB1-Δ4 expression is correlated with metastatic potential.

**E. The Effects of AIB1-Δ4 Expression on Cell Growth and Migration**

1. There is no difference on cell growth due to AIB1 or AIB1-Δ4 in AIB1 knockout mouse embryonic fibroblasts

   Since we see that cells with higher levels of AIB1-Δ4 expression have higher levels of coactivation and metastatic potential, we were curious to see if AIB1 and AIB1-Δ4 have differing effects on the phenotype of cells. To test the differing roles of AIB1 and AIB1-Δ4 on

---

**Figure 26. AIB1-Δ4 expression is higher in in vivo selected metastatic breast cancer cells.** Scorpion primers were used to quantitate the amount of AIB1 and AIB1-Δ4 transcript from the RNA of HMEC, parental MDA-MB-231 breast cancer cells, and three tissue-specific metastatic variants of MDA-MB-231 cells. The Ct values were normalized to actin expression as a control. The ratio of AIB1-Δ4 to AIB1 is shown. Data were analyzed by one-way ANOVA with Bonferroni post-test. *=p<0.05, **=*p<0.001 when compared with HMEC.

- HMEC
- MDA-MB-231
- MDA231-Bm2
- SCP2-TR
- 4176-TR

![Graph showing AIB1-Δ4 expression ratio](image-url)
cells we used AIB1 knockout mouse embryonic fibroblasts (AIB1 KO MEFs). We chose the AIB1 KO MEFs to determine the role of AIB1-Δ4 independent of AIB1 and vice versa. FLAG AIB1 and FLAG AIB1-Δ4 were lentivirally transduced into cells at different multiplicities of infection (MOI) (Figure 27a). We see with higher MOI we get higher expression of AIB1 and AIB1-Δ4. The steady state levels of AIB1-Δ4 are higher than that of AIB1 so we chose the AIB1 KO MEFs transduced with FLAG AIB1 at an MOI of 10 and the AIB1 KO MEFs transduced with FLAG AIB1-Δ4 at an MOI of 2 for further analysis. We decided to test cell proliferation as a phenotype that may be altered in the presence of either AIB1 or AIB1-Δ4 (Figure 27b). We assayed cells for cell proliferation on days 2, 4, and 6 after plating the same number of cells in the initial culture and we observed no significant difference except on day 4 or cell growth. Overall it does not appear that AIB1 or AIB1-Δ4 independently have differing effects on cell proliferation.
2. There is no effect on cell growth due to increased AIB1-Δ4 expression in MCF10DCIS.com cells

Since recent data suggested that AIB1-Δ4 is associated with metastatic phenotype in breast cancer cells, we wanted to determine if expressing AIB1-Δ4 in a model of ductal carcinoma in situ (DCIS) would lead to a more invasive phenotype of cells. We used the MCF10DCIS.com cells, which have been shown to form DCIS lesions of a comedo pathology in
We introduced AIB1-Δ4 expression into the MCF10DCIS.com cells by lentiviral transduction (Figure 28a). We wanted to determine if there was any effect on cell proliferation due to increased expression of AIB1-Δ4. Compared to empty lentiviral vector infected cells we do not see any significant difference in cell proliferation at days 4 and 6. These data suggest that there is no effect on cell proliferation due to increased expression of AIB1-Δ4.

3. Cell migration of DCIS.com cells is not enhanced by AIB1-Δ4 expression

Since we observed no difference in cell proliferation and previous data suggest that breast cancer cells can be made more invasive with increased AIB1-Δ4 expression (81), we wanted to determine if there was any effect of increased AIB1-Δ4 expression on the invasiveness of the DCIS forming MCF10DCIS.com cells. We tested the lentivirally transduced MCF10DCIS.com cells in an in vitro scratch assay (Figure 29). We allowed these cells to create a monolayer and created a scratch in the middle of the monolayer. We then measured the wound at time 0 and at
**Figure 29.** **AIB1-Δ4 does not have an effect on cell invasiveness of MCF10DCIS.com cells.**  

a) MCF10DCIS.com cells were lentivirally transduced with AIB1-Δ4 at a MOI of 10. Cells were allowed to form a monolayer prior to making a scratch in the middle of the monolayer. Representative pictures are shown at 0 and 12 hours after scratching the monolayer. b) The percentage of wound closure was determined. No significant difference in the ability of the AIB1-Δ4 overexpressing cells to migrate into the scratch area relative to the MCF10DCIS.com cells infected with empty lentiviral vector. These data show that AIB1-Δ4 expression cannot enhance the metastatic potential of MCF10DCIS.com cells in vitro.
IV. DISCUSSION
A. AIB1-Δ4 is a Predominantly Cytoplasmic Protein But has a Role in the Nucleus

In this study we show that AIB1-Δ4 cycles through the nucleus and has a nuclear function. In accordance with previous data that the NLS is contained in the N terminus of AIB1 (84, 86, 87), we found that the majority of AIB1-Δ4 which lacks an NLS is predominantly in the cytoplasm at steady state levels. This is in contrast to the localization of AIB1, which resides mostly in the nucleus. This is interesting since previous data showed that AIB1-Δ4 was a more potent coactivator of steroid and growth factor dependent transcription than AIB1 on a per mole basis (77, 78). Coactivation occurs in the nucleus and we wondered how the mainly cytoplasmic AIB1-Δ4 had such potent effects on transcription. To try to explore the potent coactivation of AIB1-Δ4 the first question to address was if AIB1-Δ4 cycles through the nucleus. We saw that both endogenous and transfected AIB1-Δ4 was recruited as efficiently as AIB1 to ERE in estrogen-regulated genes in the nucleus despite significantly lower steady-state nuclear levels of AIB1-Δ4. We also showed that AIB1-Δ4 can be enriched in the nucleus after blockade of nuclear export suggesting it was indeed being imported into the nucleus. The next question to address was whether AIB1-Δ4 had a functional role in the nucleus. We saw that AIB1-Δ4 was able to significantly increase the expression of estrogen regulated genes despite significantly lower nuclear levels of AIB1-Δ4. These data together suggest that AIB1-Δ4 not only cycles through the nucleus but has a role in gene control in the nucleus.
B. The Nuclear Import Mechanism of AIB1-Δ4 May Involve Binding to other NLS Containing Proteins

The mainly cytoplasmic distribution of AIB1-Δ4 is potentially due to either an inefficient nuclear import mechanism or through a rapid nuclear export mechanism. We argue for the former scenario given that AIB1-Δ4 lacks the N-terminal NLS and there is no alteration in the nuclear export sequence of AIB1-Δ4. It is known that molecules larger than 40 kDa have to be actively transported through the nuclear pore complex through interaction of the NLS with nuclear importins (107). Another potential mechanism for nuclear import termed piggybacking was demonstrated for various proteins such as eIF4E, IκBα, and Cdk2, and BRCA1 (108-113). BRCA1 has a naturally occurring splice variant that lacks an NLS and it is able to localize to the nucleus through interaction with another protein BARD1, which contains a canonical NLS. We believe that AIB1-Δ4 is able to similarly piggyback on AIB1, p300/CBP, and/or other NLS containing proteins to enter the nucleus. We believe this mechanism is an inefficient process and that this accounts for the largely cytoplasmic distribution of AIB1-Δ4 in the cell. Also consistent with this theory is the difference in the kinetics of recruitment of AIB1 and AIB1-Δ4. We saw a delay in the recruitment of AIB1-Δ4 to the ERE relative to AIB1 suggesting that the mechanism of nuclear import of AIB1-Δ4 is less efficient than that of AIB1.

C. Translation of AIB1-Δ4 May be Directed by a Eukaryotic Internal Ribosome Entry Site

We propose that the AIB1-Δ4 mRNA contains a eukaryotic internal ribosomal entry sequence (IRES). The presence of eukaryotic IRES sequences has been described (114). Although still controversial what the structure may be for constituting a cellular IRES, in general
they seem to be less efficient than their viral counterparts. Expression of the AIB1-Δ4 cDNA leads to low levels of AIB1-Δ4 protein and by altering the secondary structure of the mRNA by site directed mutagenesis we find that expression of an AIB1-like protein the size of AIB1-Δ4 increases (Figure 5a). We believe that the secondary structure of the AIB1-Δ4 mRNA leads to a cap independent translation of the AIB1-Δ4 protein. Disruption of this structure allows for translation to be initiated at another methionine since translation from different methionines can give rise to AIB1 related proteins with a predicted molecular mass the size of AIB1-Δ4.

D. Loss of a Regulatory Region in the N-terminus of AIB1 Allows AIB1-Δ4 to be a More Potent Coactivator

The fact that we saw more potent coactivation with AIB1-Δ4 despite having much more AIB1 in the nucleus than AIB1-Δ4 suggests that there is a regulation of AIB1 coactivator activity that does not exist for AIB1-Δ4. Since we observed that the levels of AIB1-Δ4 interacting with p300 was higher than the levels of AIB1 associated with p300, and p300 is generally found in active transcriptional complexes, we believe that AIB1-Δ4 is highly recruited to sites of active transcription. The increased coactivator activity of AIB1-Δ4 was confirmed by a higher increase in endogenous estrogen regulated gene expression in cells transfected with AIB1-Δ4. This data suggests that the large amount of AIB1 that resides in the nucleus is not in active transcriptional complexes and the reason why AIB1-Δ4 is a more potent coactivator than AIB1 can be explained by the presence of an inhibitory domain in the N-terminal 223 amino acids of AIB1. We conjectured that this N terminal fragment containing the bHLH and PAS A domains would be able to bind N terminal repressors of AIB1. We found that the N-terminal fragment containing
the bHLH and PAS A domains of AIB1 when co-transfected with AIB1 is able to relieve repression of the coactivator function of the AIB1 protein. This effect was dose dependent and the more AIB1 N term fragment added to the cells the less repression there was on the AIB1 protein. Interestingly the fragment when transfected by itself showed an increase in luciferase activity which is probably not due to an inherent coactivator function of this fragment since most of the transcriptional activity of the AIB1 and AIB1-Δ4 proteins reside in their recruitment of p300/CBP (15). This increase in transcription is most likely due to a relief of repression of the endogenous AIB1 in the COS-7 cells since we are able to detect endogenous AIB1 protein by Western blot (77). Previous studies from our group and others have suggested that the N-terminal region containing the bHLH and PAS A domains contain an inhibitory domain that represses activity in both the nucleus and cytoplasm (15, 77, 78, 81, 86). Our studies and those from Chen et al have seen that loss of the N-terminal region leads to potent coactivation of nuclear hormone receptor mediated transcription. Expression of AIB1-Δ4 (loss of amino acids 1-223 of AIB1) or ACTR38 (loss of amino acids 1-447 of AIB1) leads to potent coactivation of nuclear receptor dependent transcription from estrogen, progesterone, retinoic acid, thyroid, glucocorticoid, vitamin D, and retinoid X receptors. Data from Li et al showed expression of AIB1 constructs containing mutations in the either or both NLS (NLS amino acids 16-19 and 35-38 of AIB1) or with the bHLH domain deleted (amino acids 16-88 of AIB1) had no coactivator activity presumably because of lack of import into the nucleus. The other possibility is that they still retained amino acids 88 through 224, which would support the evidence that there is an inhibitory domain in this region. Loss of these amino acids in AIB1-Δ4 and ACTR38 allows these proteins to be potent coactivators.
Given that we have found that the expression of AIB1-Δ4 at the protein level is higher in breast and pancreatic cells (29, 77), it was interesting to observe if AIB1-Δ4 is regulated by cells at the protein level. We have previously shown that AIB1 protein levels are greatly reduced in response to growth of cells at high confluence and the removal of growth factors (58). Interestingly we now find that AIB1-Δ4 isoform is not regulated in the same fashion as the AIB1 protein. AIB1-Δ4 is resistant to proteasomal degradation induced by high confluence. This is probably due to loss of a site of regulation that resides in the N-terminal 223 amino acids. The proteasomal regulation of AIB1 has been well characterized (58, 61-63). E6-AP and REGγ were shown to bind in the C-terminal region contained in both AIB1 and AIB1-Δ4 and therefore are not candidate sites for the difference we see in the stability of the AIB1-Δ4 protein. Regulation of a phospho-degron at S102 by protein phosphatase 1 (PP1) was shown to be important for regulating the activity of AIB1 and the stability of the protein. PP1 stabilized the AIB1 protein but inhibited its coactivator function and cell proliferative ability. The S102 site is also a site of regulation by the ubiquitin ligase SPOP (63). This site is lost in AIB1-Δ4 and could explain the high levels of the AIB1-Δ4 protein.

We tried also to identify possible phosphorylation sites or regions of protein-protein interaction that may negatively regulate the function of the AIB1 protein and not have any effect on the AIB1-Δ4 protein. We also identified that S102 was a major site of phosphorylation in cells grown under conditions that favor the degradation of full-length AIB1 protein. More work needs to be done to further validate and determine mechanisms of regulation at the phosphorylation sites that we identified through mutational analysis and in silico kinase/phosphatase screening.
**E. A Signaling Loop Between AIB1/AIB1-Δ4 and Epidermal Growth Factor Receptor Exists**

Both AIB1 and AIB1-Δ4 have been shown to have effects in the Epidermal Growth Factor (EGF) signaling. Data from our lab has shown that loss of both AIB1 and AIB1-Δ4 protein together can lead to a decrease in EGF receptor (EGFR) phosphorylation (24). Long et al have shown that AIB1-Δ4 acts as a bridging molecule between EGFR and Focal Adhesion Kinase (FAK) and this interaction facilitates the motility and metastatic capability of MDA-MB-231 breast cancer cells (81). They also show that AIB1-Δ4 is phosphorylated by p21 activated kinase (PAK1), which increases the association of AIB1-Δ4 with EGFR and FAK. Intriguingly in this study the N-terminal region of AIB1 inhibited the interaction of AIB1 with Focal Adhesion Kinase (FAK) and therefore was unable to stimulate the EGF induced migration of cancer cells, suggesting that even in the cytoplasm the N-terminal region of AIB1 was repressive. Expression of a NLS mutant of AIB1, which resides predominantly in the cytoplasm showed much weaker interaction with FAK in cells. Another recent publication from Cai et al (43) show in non small cell lung cancer (NSCLC) that AIB1 expression is correlated with poor prognosis in lung cancer patients and knockdown of AIB1 in NSCLC cell line resistant to gefitinib treatment restored sensitivity to EGFR inhibition by gefitinib. Taken together these data indicate that there is a signaling loop existing between AIB1-Δ4 and EGFR where EGFR can affect AIB1-Δ4 through PAK1 activation and AIB1 can affect EGFR signaling as well. It is unclear if the siRNA used to target AIB1 in the gefitinib study also targeted AIB1-Δ4 as well, so the effects of AIB1-Δ4 on EGFR signaling are not currently known. Overall the contribution of
nuclear versus cytoplasmic function of AIB1-Δ4 to steroid and growth factor signaling and metastatic spread of tumors needs to be further explored.

F. The Effect of AIB1-Δ4 Expression on the Phenotype of Cells

A major question that arises from these studies on AIB1-Δ4 is whether there are distinct biological functions for this isoform. To try to elucidate the possible distinct functions of full-length AIB1 and AIB1-Δ4 we utilized mouse embryonic fibroblasts derived from AIB1 KO mice. Expressing either AIB1 or AIB1-Δ4 in these cells would allow us to examine the function of one isoform independent of the other. After lentivirally transducing AIB1 KO MEFs we found that there was no significant difference in the cell proliferative ability of AIB1 vs. AIB1-Δ4. These studies are far from exhaustive and it is possible that the phenotype is more nuanced and the response to certain growth factors or challenges would help distinguish the distinct functions of the two isoforms. The other possibility is that these cells may no longer be dependent on AIB1 for growth. These cells have been immortalized and may have already compensated for the loss of AIB1 by upregulating other p160 SRC family members to maintain growth. More experiments with specific stimuli (ie growth factor or hormone) may be required to bring out the differences between AIB1 and AIB1-Δ4 and their contribution to cellular phenotypes.

A previous report showed that overexpression of AIB1-Δ4 increased the invasiveness of the metastatic breast cancer cell line MDA-MB-231 (81). We wondered if we were able to translate the increased metastatic potential to another breast cancer cell line. MCF10DCIS.com cells form ductal carcinoma in situ tumors in nude mice and we expressed AIB1-Δ4 in these cells
to determine if their invasiveness increased. In contrast to the other study we did not see a difference in an in vitro assay of cell invasion. This may be due to the fact that the phenotype is driven by EGF. The previous study showed in vitro studies using transwell migration assays in which cells were migrating in response to an EGF gradient. In our study we were just observing the migratory ability in media with 10% serum. Our cells were not induced with a particular stimulus, which suggests that the metastatic phenotype due to AIB1-Δ4 expression is primarily driven through EGF. Another possible explanation is that epithelial stromal interactions are required for the migration of cells due to AIB1-Δ4. Our recent publication describing that AIB1-Δ4 in conjunction with ERα expression is able to stimulate stromal and epithelial changes suggests that AIB1-Δ4 affects epithelial stromal interactions (80). Interestingly in this study AIB1 did not have a statistically significant increase in collagen in the stroma when coexpressed with ERα. We also saw that as in the AIB1 KO MEFs that there was no difference in proliferation rate in cells transduced with empty lentiviral vector vs. those that were infected with AIB1-Δ4 virus. This is interesting given that in the CMV AIB1-Δ4 mouse there were signs of increased proliferation in the mammary gland (79). It is possible that epithelial stromal interactions are required for the cell proliferation of breast derived epithelial cells.

**G. Future Studies of AIB1-Δ4**

We plan on trying to identify what proteins may interact with the N-terminus of AIB1. These proteins may be kinases, phosphatases, or other posttranslational modifying proteins. Proteins that immunoprecipitate with the AIB1 N term fragment would be interesting to look at as candidate repressor proteins. After validation for binding to the AIB1 N term we would see
which ones interact with AIB1 and not AIB1-Δ4 and assess these proteins for their effect on the coactivator function of AIB1 and AIB1-Δ4. These interactions may be difficult to detect because the interaction of kinases or phosphatases that are responsible for the regulation of the N-terminus may be brief at best. Kinase substrate interactions have been suggested to be transient (115) and may make it harder to detect interaction with our AIB1 N term fragment.

We believe that the next important studies to pursue are in dissecting out the distinct functions of AIB1-Δ4 from AIB1. To achieve this goal we have generated affinity purified AIB1-Δ4 antibodies and AIB1-Δ4 Scorpion primers. We are interested in using the antibodies to look at clinical samples to determine in what cells and what diseases AIB1-Δ4 is expressed. We are currently optimizing the use of these antibodies for immunofluorescence and immunohistochemistry to probe different cancer tissue samples. With the AIB1-Δ4 Scorpion primer we took advantage of the unique splice junction sequence that exists in the AIB1-Δ4 transcript to develop a new technique to specifically measure the amounts of AIB1-Δ4 mRNA independent of AIB1 transcript. By utilizing Scorpion primer technology we see higher expression of AIB1-Δ4 in cancer cell lines relative to normal cell lines and higher expression in more metastatic cancer cell lines.

Most studies published on AIB1 do not account for the distinction of the role of AIB1 independent of AIB1-Δ4 due to the lack of tools to specifically detect AIB1-Δ4 protein or mRNA. In fact, most reports on the role AIB1 in disease do not make the distinction between the contribution of AIB1 or AIB1-Δ4 to the phenotype. Most studies that analyze mRNA expression of AIB1 utilize primers that will detect both AIB1 and AIB1-Δ4. We believe that it will be important to separate the contribution to phenotypes attributed to AIB1-Δ4 independent
of AIB1 and vice versa. With the development of the AIB1-Δ4 Scorpion primer and affinity purified AIB1-Δ4 antibodies we believe that this is an important step forward in the initiation of this process.

The role of AIB1-Δ4 in tumorigenesis compared to its normal function needs to be determined. Proteins overexpressed in cancer initially have a normal function and in accordance with this we see a low level of expression of AIB1-Δ4 in normal breast cells. We see that the levels of AIB1 protein are highly regulated by the proteasome in conditions where growth is not favored. The AIB1 protein is rapidly degraded whereas the AIB1-Δ4 protein is stable. In normal cells grown in an environment not favoring growth the cell may degrade AIB1 protein. Once the cell receives conditions that favor growth, AIB1-Δ4 may serve as a reservoir of transcriptionally active protein to restart pro-growth signaling while AIB1 protein is being synthesized. A cancer cell may take advantage of AIB1-Δ4 protein to continue to grow in conditions not favoring growth. Solid tumor cancer cells initially grow in a single mass and these conditions do not favor growth of normal cells. Cancer cells have to overcome inhibition of growth due to contact inhibition. AIB1-Δ4 can survive these conditions since the protein is stable in high confluent conditions. The inside of the tumor before it becomes vascularized is a nutrient and growth factor deprived area. Since we have seen that AIB1 levels are highly degraded under serum deprived conditions and AIB1-Δ4 is resistant to this degradation, the overexpression of AIB1-Δ4 in cancer cells may also allow the cells to survive nutrient and growth factor deprived conditions by increasing expression of growth factor receptors such as HER2 and through potentiation of signals through growth factors such as EGFR. Cancer cells may also upregulate the expression of the AIB1-Δ4 through the alteration of splicing, since splicing has been shown to be different
in cancer cells versus normal cells (116). The regulation of splicing to favor the production of the AIB1-Δ4 would be interesting to explore in cell lines with high levels of AIB1-Δ4 mRNA.

H. Differences Between AIB1 and AIB1-Δ4 in the Nucleus and Cytoplasm

AIB1 and AIB1-Δ4 are differentially regulated in the nucleus and have distinct roles in the cytoplasm (Figure 31). In the cytoplasm AIB1-Δ4 has been shown to act as a scaffold tying together EGFR and FAK signaling pathways to mediated metastatic potential. AIB1 in the cytoplasm is not capable of facilitating this interaction. AIB1 contains a NLS sequence and is imported into the nucleus through the canonical nuclear import pathway. AIB1-Δ4 on the other hand is imported in an inefficient fashion and leads to the predominantly cytoplasmic localization of the protein. A potential mechanism of nuclear import is through the interaction with other NLS containing proteins to “piggyback” into the nucleus and this leads to a delay in recruitment into the nucleus relative to AIB1 protein. In the nucleus AIB1 and AIB1-Δ4 both serve as coactivators. AIB1-Δ4 acts as a potent coactivator relative to AIB1 because of the lack of an inhibitory domain contained in the N-terminus of AIB1. This explains why we see less coactivation with AIB1 protein when there is clearly more nuclear AIB1 than nuclear AIB1-Δ4. There is some protein-protein interaction or posttranslational modification that occurs on the N-terminus of AIB1 that dampens its coactivator function. This same region also determines the proteasomal regulation of AIB1, which is lost in AIB1-Δ4 and leads to AIB1-Δ4 to be a more stable protein.
Figure 30. Proposed model of AIB1-Δ4 enhanced coactivator function. AIB1-Δ4 in the cytoplasm bridges the interaction of EGFR and FAK. AIB1-Δ4 is trafficked into the nucleus by an inefficient mechanism involving binding to other NLS proteins while AIB1 is imported into the nucleus through a canonical NLS mechanism. AIB1-Δ4 is resistant to proteasomal degradation due to loss of the N-terminal inhibitory domain. AIB1 is rapidly degraded through a mechanism involving its N-terminus. In the nucleus AIB1-Δ4 may be a more potent coactivator because it is not bound by an inhibiting protein or modified in the N-terminal region deleted in the AIB1-Δ4 protein.
V. APPENDIX
A. Role of AIB1 in Tamoxifen Resistance

The most highly studied coactivator in relation to modulating the response to SERMs and in particular tamoxifen is the Amplified in Breast Cancer 1 (AIB1) protein. AIB1 plays an important role in the response to SERMs in breast cancer and there is both pre-clinical and clinical data to support this claim. Most studies have focused on the co-expression of AIB1 with HER2. The earliest indication that AIB1 and HER2 might have a role in tamoxifen resistance is from in vitro experiments from Dr. C. Kent Osborne’s lab (117). They created the MCF-7/HER2-18 breast cancer cell sub-line that expressed HER2 receptor at 45 times the level expressed in the parental MCF-7 cell line. These parental and MCF-7/HER2-18 cells were allowed to form tumors in ovariectomized mice supplemented with a slow release estrogen pellet. When the tumors reached 500 mm$^2$ the estrogen pellet was removed and the animals were treated with tamoxifen. Strikingly, the MCF-7/HER2-18 tumors grew in response to tamoxifen treatment while the parental MCF-7 tumors did not grow. This implicated HER2 in acquisition of tamoxifen resistance but not necessarily AIB1. However MCF-7 cells also have greater than 20 fold expression in the AIB1 gene and have high levels of AIB1 mRNA (27) suggesting that there may be a role for AIB1 in tamoxifen resistance. Later data would show that in tamoxifen resistant HER2 overexpressing BT474 breast cancer cells depleted of AIB1 became sensitized to the growth inhibitory effects of tamoxifen compared to the parental cells (118) indicating a role for AIB1 in modulating the response to tamoxifen. In addition, overexpression of an isoform of AIB1 in breast cancer and endometrial carcinoma cells in vitro increased the agonist properties of tamoxifen (78).

Confirmation of the cross talk between AIB1 and HER2 in the development of tamoxifen resistance came in two papers. The first paper described a correlation between AIB1 and HER2
and worse outcomes after treatment with tamoxifen (54). In this paper they measured the levels of AIB1 and HER2 in samples from breast cancer patients who either did or did not receive adjuvant tamoxifen by Western blot. Patients with high levels of AIB1 irrespective of HER2 expression who were treated with tamoxifen had a worse disease free survival (DFS). However, the most significant finding from this paper was that patients who had high AIB1 and high HER2 expression treated with tamoxifen had worse DFS than all other patients indicating that a potential acquisition of tamoxifen resistance developed in these patients. Interestingly also in this study they found that patients with high levels of AIB1 irrespective of HER2 expression who were not treated with tamoxifen had longer disease free survival than those patients with low levels of AIB1. This is not the only evidence of high levels of AIB1 being a good prognostic factor in breast cancer. More recent data have shown that tamoxifen treated ER positive patients with high levels of AIB1 have reduced recurrence free survival and overall survival compared to patients who were not treated with tamoxifen (119). These data stand a bit in opposition to the previous data but may be explained by the difference in patient populations and comparisons of treatment groups. In the first study the percentage of HER2 positive patients was greater than in the latter study. Also the comparison in the first paper of DFS was within a particular treatment group (tamoxifen treated) and in the following study was in between treatment groups (not treated with tamoxifen vs. tamoxifen treated) so the results are not directly comparable and not necessarily opposing.

These studies were followed up with in vitro work to explore the possibility of crosstalk between the estrogen and growth factor signaling pathways (120). They made use of the tamoxifen resistance MCF-7/HER2-18 breast cancer cell line described previously (117) to show that several molecules downstream of the human epidermal growth factor receptors (Epidermal...
Growth Factor Receptor or EGFR and HER2) and molecules associated with estrogen signaling were activated/phosphorylated upon stimulation with growth factors (Epidermal Growth Factor or EGF and Heregulin or HRG) or estrogenic compounds (estrogen or tamoxifen). They showed that the activation of these same molecules and other signaling molecules were reduced when MCF-7/HER2-18 cells were treated with Gefitinib, a small molecule inhibitor targeted specifically for the tyrosine kinase domain of EGFR. They also showed that AIB1 is recruited after treatment with tamoxifen in the tamoxifen resistant MCF-7/HER2-18 cells but not in tamoxifen sensitive parental MCF-7 cells to ERE in the pS2 promoter indicating that the switch of tamoxifen from an antagonist to an agonist is in part due to the recruitment of AIB1 to estrogen responsive genes. They went on to validate in a pre-clinical in vivo model that the growth response to tamoxifen of tumors derived from tamoxifen resistant MCF-7/HER2-18 cells could be completely abrogated by treatment with gefitinib. Interestingly in this same experiment estrogen and gefitinib treated tumors grew just as well as tamoxifen treated MCF-7/HER2-18 cells which the authors suggested that growth due to estrogen stimulation was not completely dependent on human epidermal growth factor (HER) signaling, but that the growth stimulation due to tamoxifen was dependent on HER signaling. They also saw that the activation/phosphorylation of AIB1 was increased by HRG, estrogen, and tamoxifen in the MCF-7/HER2-18 cells relative to the parental MCF-7 cells and this activation was reversed by Gefitinib treatment. They observed the same phosphorylation of AIB1 in extracts from the tumors in the in vivo experiment as well. These data suggest a role for AIB1 in the regulation of tamoxifen response through a mechanism that involves growth factor signaling activation of AIB1. Consistent with this correlation is that the development of hormone independence and SERM resistance in breast tumors is widely associated with increases in growth factor signaling
through receptor tyrosine kinases such as EGFR, HER2 and HER3 (120, 121). In addition, AIB1 has also been shown to potentiate HER family signaling in vitro and in vivo (24, 25, 77). Thus it is perhaps not surprising that high levels of AIB1 coupled with high EGFR or HER2 are associated with increased relapse after tamoxifen therapy (54, 122, 123).

In terms of mechanism of AIB1 and HER2 induced tamoxifen resistance, one possibility is that the increases in HER2 and AIB1 lead directly to enhancement of agonist properties of tamoxifen in breast cancer cells (124). Alternatively the unliganded ERα activated by HER kinase pathways (such as MAPK) (120) or other kinase pathways that activate AIB1 (71) could be capable of activating a mitogenic AP-1 dependent gene set (125) that possibly bypasses tamoxifen repressive effects. Of note is that AIB1 can also coactivate AP-1 dependent genes and may be involved directly in potentiating the ER signaling at AP-1 dependent promoters (73, 77).

In a tamoxifen resistant cell line derived from MCF-7 cells (Tam-R) (126) the total levels of HER2 were increased and the total levels of PAX2 were decreased. Tamoxifen treatment of Tam-R cells led to the recruitment of AIB1 and no recruitment of PAX2 to the ERE in an intron of HER2 and in tamoxifen sensitive parental MCF-7 cells the opposite was observed. This suggests that AIB1 regulation of HER2 levels is critical in the modulation in the SERM activity of tamoxifen.

Tamoxifen also induces the expression of AIB1 in vitro (55, 127) suggesting a positive feedback loop that would increase AIB1 mediated signaling in tumors. Consistent with this, tamoxifen induces AIB1 in breast tumors of patients even at low doses (128) suggesting that this positive feedback is occurs also in vivo. Overall, the in vitro and clinical data support a
sequential powerful positive feedback model of anti-estrogen resistance whereby tamoxifen treatment initially leads to an increase in AIB1 that potentiates the agonist properties of tamoxifen, as well as any estrogen-liganded ER. This leads to increased levels of HER2 and subsequent stimulation of kinases cascades. HER family signaling is also potentiated directly by the high AIB1 levels. The signaling cascades can further activate AIB1 and the unliganded ER leading to hormone independent stimulation of proliferation and endocrine resistance. Also of note is that the mechanisms of resistance to aromatase inhibitors reported to date seem to also involve increases in coactivator activity and cross talk of HER2 and AIB1 (129-131).
VI. REFERENCES


