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Ets1 Regulation of the Small Heat Shock Protein alphaB-Crystallin and Involvement in Breast Cancer Cell Transformation

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ABSTRACT

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The small heat shock protein αB-crystallin is expressed primarily in lens and muscle tissue, but it is also found in lung, kidney and many cancers. Regulators of αB-crystallin have been identified almost exclusively using mouse muscle and lens specific models. It has been well documented that αB-crystallin is expressed in multiple cancers and that its expression correlates with poor prognosis. More specifically, it is commonly expressed in the poor prognosis basal-like breast tumor subtype and its expression correlates with resistance to neo-adjuvant chemotherapy and an invasive phenotype in breast cancer. This study worked to develop a better understanding of the transcriptional regulators of αB-crystallin in breast cancer. A bioinformatics analysis of the human αB-crystallin promoter revealed a putative binding site for the Ets1 transcription factor. Ets1 is the founding member of the ETS-family of transcription factors, and its expression in breast tumors correlates with poor prognosis. Furthermore, Ets1 expression correlates with the basal-like phenotype in breast cancer cell lines making the potential regulation of αB-crystallin by Ets1 a plausible hypothesis. Here we show using a human αB-crystallin reporter system, gel shift analysis, chromatin immunoprecipitation and siRNA

technology that Ets1 regulates human αB-crystallin gene expression in breast cancer cell lines. Furthermore, we probed the role of Ets1 in regulating migration, invasion, proliferation and anchorage-independent growth in breast cancer cell lines. Our results provide evidence for Ets1 regulation of αB-crystallin and suggest a highly context dependent role for Ets1 in regulating a motile or transformed phenotype in breast cancer cell lines.

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List of Abbreviations

AAA+	ATPase associated with diverse cellular activities
αB-crystallin	alphaB-crystallin
αBE	alphaB-crystallin enhancer
ADP	adenosine di-phosphate
AIF	apoptosis inducing factor
Akt	serine/threonine specific protein kinase
ANOVA	analysis of variants
AP1	activator protein 1
Apaf-1	apoptotic protease-activating factor-1
ATP	adenosine tri-phosphate
BCCL	breast cancer cell line
bFGF	basic fibroblast growth factor
B-Raf	v-raf murine sarcoma viral oncogene homolog B1
BSA	bovine serum albumin
BCA	bicinchoninic acid
BCR-ABL	breakpoint cluster region Abelson
BRCA1	breast cancer 1, early onset, included
CAT	chloramphenicol acetyltransferase
CBP/p300	creb binding protein
CDC37	cell division cycle 37
CDK	cyclin dependent kinase

cDNA	complementary deoxyribonucleic acid
CHIP	carboxyl terminus of Hsc70-interacting protein
ChIP	chromatin immunoprecipitation
Clp	caseinolytic protease
CRYAB	αB-crystallin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EBS	Ets binding site
ECL	electrochemiluminessence
EDTA	ethylene-diamine-tetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
eIF2α	eukaryotic initiation factor alpha
Elk	ETS-domain protein (SRF accessory protein 1)
EMSA	electrophoretic mobility shift assay
EMT	epithelial to mesenchymal transition
ER	estrogen receptor
ERα	estrogen receptor alpha
ERK	extracellular-signal regulated kinase
Ets	erythroblastosis virus E26 transformation specific sequenc
Ets1	erythroblastosis virus E26 oncogene homolog 1
ESX/ESE1	epithelium specific Ets

FDA	food and drug association
FLT-3	FMS-like tyrosine kinase 3
GABP	growth-associated binding protein
GATA-1	globin transcription factor 1
GKLF	gut-enriched Kruppel-like factor
HER2	human epidermal growth factor receptor 2/ERB-B2
HIF1α	hypoxia inducible factor 1 alpha
HIP	Hsc70 interacting protein
HIV	human immunodeficiency virus
HNF3α	hepatocyte nuclear factor 3 alpha
HOP	Hsp organizing protein
HRP	horse-radish peroxidase
HSE	heat shock element
HSF	heat shock factor
Hsp	heat shock protein
HspB1	heat shock protein B1, heat shock protein 27-1
HspB2	heat shock protein B2, heat shock protein 27-2, MKBP
Hsp40	heat shock proein 40kDa, DnaJ
Hsp60	heat shock protein 60kDa, GroEL
Hsp70	heat shock protein 70kDa, GrpE, DnaK
Hsp90	heat shock protein 90kDa
Hsp100	heat shock protein 100kDa
IGFβ	insuling-like growth factor beta

IGF-1R	insulin-like growth factor 1 receptor
IHC	immunohistochemisty
JNK	Jun N-terminal kinase (SAPK, stress activated protein kinase)
KLF4	Kruppel-like factor 4
LIV-1	solute carrier family 39 (zinc transporter), member 6
LSR	lens specific region
LTR	long terminal repeat
MAPK	mitogen-activated protein kinase
MEK	MAP kinase
MET	hepatocyte growth factor receptor
MMP2	matrix metalloproteinase 2, gelatinase A
MMP3	matrix metalloproteinase 3, stromelysin
MMP9	matrix metalloproteinase 9, gelatinase B
MRF	muscle regulatory factor
mRNA	messenger ribonucleic acid
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt
Myo-D	myogenic differentiation 1
NEB	New England Biolabs
NFκB	nuclear factor-kappa B
p38	stress activated protein kinase 38kDa (MAPK14)
PAI-1	plasminogen activator 1
Pax-6	paired box 6
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PNT	pointed domain
$pp60^{src}$	proto-oncogene tyrosine-protein kinase SRC
PR	progesterone receptor
pRb	retinoblastoma protein
PSG	penicillin-streptomycin-glutamate
PTHrP	parathyroid hormone-related protein
RAR/RXR	retinoic acid receptor/retinoid X receptors
RET	rearranged during transfection proto-oncogene
RIPA	radio immunoprecipitation assay
RNA	ribonucleic acid
RNase	ribonuclease
RT-qPCR	real-time quantitative polymerase chain reaction
SDM	site-directed mutagenesis
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	short-hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
Sox	sex determining region Y-related high mobility group box
SP1	specificity protein 1
SRF	serum response factor
TATA	Goldberg-Hogness box
TFCP2L1	transcription factor CP2-like 1
TBS/T	tris-buffered saline tween-20

TMA	tissue microarray
TNBC	triple-negative breast carcinoma
TP53	tumor protein 53
TSS	transcriptional start site
uPA	urokinase plasminogen activator
USF	upstream stimulating factor
VEGF	vascular endothelial growth factor

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1 INTRODUCTION

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I. The War on Cancer

Approximately 100 years ago a group of scientists and physicians gathered together "to further investigate and spread the knowledge of cancer" by establishing the American Association for Cancer Research. Thirty years after that, the Franklin D. Roosevelt administration established the National Cancer Institute "to promote research in the cause, prevention, and methods of diagnosis and treatment of cancer, to provide better facilities for the diagnosis and treatment of cancer…" These groups made advances early on in the knowledge of cancer biology, however the major advances have come in the years since President Nixon declared war on cancer in his 1971 state of the union address:

> *"I will also ask for an appropriation of an extra $100 million to launch an intensive campaign to find a cure for cancer, and I will ask later for whatever additional funds can effectively be used. The time has come in America when the same kind of concentrated effort that split the atom and took man to the moon should be turned toward conquering this dread disease. Let us make a total national commitment to achieve this goal. America has long been the wealthiest nation in the world. Now it is time we became the healthiest nation in the world."*
>
> President Richard Nixon - 1971 State of the Union Address

This statement led to the drafting and passing of the National Cancer Act in December of 1971, in which Congress recognized that an increasing incidence in cancer concerned Americans, that new research may advance cancer therapies, and that a national effort against cancer is needed to bring about the most effective research. Science has certainly made advances since then, and by the 1990's cancer deaths leveled off and began to decrease in the United States. Currently, the circle of knowledge we have on the biology of cancer is significantly larger than it was 25 or even 10 years ago,

and even as that circle continues to grow, so does the area of the unknown just beyond the grasp of what we can answer with that knowledge.

In 2000, Douglas Hanahan and Robert Weinberg wrote a review that summarized the wide breadth of knowledge gained over the years by cancer biology research to a set of "rules that govern the transformation of normal human cells into malignant cancers" (Hanahan and Weinberg, 2000). These rules, known as the "Hallmarks of Cancer", are: 1) Self-sufficiency in growth signals 2) Insensitivity to anti-growth signals 3) Evading apoptosis 4) Limitless replicative potential 5) Sustained angiogenesis 6) Tissue invasion and metastasis. Collectively, these hallmarks are all the necessary changes that a normal cell needs to undergo to become a cancer cell.

II. Breast Cancer

A. Breast cancer incidence

The number of new breast cancer cases worldwide in 2002 alone was 1.15 million women, and nearly 1 of every 4 women diagnosed with cancer will have breast cancer, making it the most common cancer in women worldwide (Parkin et al., 2005). With such a high incidence, it is not surprising that breast cancer is the leading cause of death in women with cancer, and fifth overall. Breast cancer incidence rates in more developed and affluent regions such as North America, Western Europe, and Australia range from 85-100 per 100,000 women, while incidence rates in less developed, poorer areas such as China and most of Africa range from 17-23 per 100,000 women. Although breast cancer incidence rates are roughly 3 times higher in more affluent regions, the breast cancer

mortality rates globally differ less dramatically indicating that there is a disconnect in effectiveness or availability of breast cancer treatments in these regions (Parkin and Fernandez, 2006). One could speculate as to why there are such dramatic differences worldwide in breast cancer incidence rates, but clearly the lifestyle of women in affluent, developed countries along with an increased awareness and early detection all play a major role. When looking at lifestyle factors that could account for the higher incidence rates in North America and Western Europe, convincing evidence exists that early menarche, late menopause, late first pregnancy or not having children, and lack of breast feeding all increase the risk for developing breast cancer (McPherson et al., 2000). An additional risk factor for increasing chances of developing breast cancer is alcohol intake, as increased alcohol intake results in increased risk. Meta-analysis shows up to a 10 percent increased risk with 10 grams of ethanol per day or a 5 percent increased risk with 5 drinks per week (Hamajima et al., 2002; Smith-Warner et al., 1998). Other factors that likely have an impact on developing breast cancer include increase in body fat (increasing risk) in adults and increase in physical activity (decreasing risk) (Adams et al., 2006; World Cancer Research Fund and American Institute for Cancer Research, 2007).

While lifestyle factors have a significant role, genetics cannot be overlooked. It is estimated that between 4 and 9 percent of breast cancer is hereditary, usually involving mutation in BRCA1 or BRCA2 (Anderson and Badzioch, 1993; Blackwood and Weber, 1998). Even with higher incidence rates resulting from a variety of lifestyle factors, early diagnosis and the better treatment available in higher-income countries account for a

higher average 5-year survival rate compared to middle- and low-income countries: approximately 73 percent compared to 57 percent (WCRF/AICR, 2007; World Cancer Research Fund and American Institute for Cancer Research, 2007).

B. Progress in the knowledge of the biology and treatment of breast cancer

Early detection, mammographic screening and improved treatment over the last 40 or so years have worked to decrease the breast cancer mortality rate among women in affluent countries (Jatoi and Miller, 2003). Mortality rates peaked in affluent countries such as the United States, Canada, the Netherlands, and the United Kingdom during the mid 1980's and continue to decline today. About 10 years after implementing mammographic screening there is an approximate 20 percent decrease in mortality rates (Blanks et al., 2000; Jatoi and Miller, 2003). Reductions in mortality not accounted for by screening alone suggest advancement in treatment and improved management. Some of the significant advancements in breast cancer treatment include the discovery and use of tamoxifen (1978), taxol (1992), herceptin (1998), as well as the Women's Health Initiative's recommendation to stop the use of some types of hormone replacement therapy in post-menopausal women. Even with such advancements, progress in all areas of breast cancer research is necessary. For example, even though breast cancer incidence rates in white women in North America are higher than those of other ethnic groups, mortality rates are highest in black women (Demicheli et al., 2007; McClintock et al., 2005).

C. Molecular portraits and advances in the treatment of breast cancer

Doctors and researchers have known for some time that not all breast carcinomas are the same and that some tumors respond to a specific treatment while others do not. Recent advances describe the phenotypic diversity that exists within breast cancer, and can predict clinical outcome of these diverse classes of tumors. The first leap forward in describing the variety observed within breast cancer came in 2000 when the Stanford group of Perou, Sorlie, Brown, Botstein and colleagues hypothesized "that the phenotypic diversity of breast tumours might be accompanied by a corresponding diversity in gene expression patterns" (Perou et al., 2000). Using cDNA microarrays, mRNA expression levels were studied in normal or malignant human breast tumors from 42 patients. Clustering of gene expression patterns in these tumors led the authors to propose four groups of breast tumors: ER positive/luminal-like, basal-like, HER2 positive, and normal breast. In the discussion of their work, it was suggested that these groups will need to be treated as distinct diseases and that future studies are needed to further classify breast tumor phenotypes (Perou et al., 2000). Indeed, studies continued by this group and others have further classified breast tumor diversity as well as the clinical outcome of each subtype. In 2001 Sorlie, Perou and colleagues again collaborated to study gene expression patterns from 78 tumors along with the clinical outcome of the patients from which the tumors were removed. They confirmed their initial four groups of breast cancer and expanded the ER positive/luminal group into three subtypes (luminal A, B, or C) for a total of six subtypes. Additionally, they linked the gene expression patterns of the breast tumors with patient overall and disease-free survival. Survival analyses of the 6 subtypes revealed that the basal-like and HER2 positive subtypes had the poorest

overall survival, with basal-like tumors and HER2 positive tumors having approximately 30% survival after 5 years (Figure 1.1). Meanwhile the luminal A had the best survival with approximately 90% survival after 5 years suggesting that each subtype could indeed be thought of as distinct cancer (Sorlie et al., 2001). Continuing studies confirmed the observation of independent breast tumor subtypes with significant differences in clinical outcome within an additional 115 malignant tumors and refined the classification into 5 distinct subtypes: basal-like, HER2-overexpressing, luminal A, luminal B, and normal breast tissue-like (Sorlie et al., 2003).

The next step in using the power of gene expression profiling to study the biology of breast cancer came when the Dutch group of van't veer, Dai, van de Vijver and colleagues published their results on providing a way to determine which patients would benefit from adjuvant therapy. Again, using mRNA isolated from 98 breast tumors from 117 lymph node-negative patients along with the clinical data, they were able to identify 70 genes out of 25,000 that could be used to predict clinical outcome as 'poor prognosis' (cancer that was likely to metastasize) or 'good prognosis' (cancer that was unlikely to metastasize) (van 't Veer et al., 2002). Soon afterwards this group expanded their studies to include 295 patients from the Netherlands Cancer Institute all under the age 55 with stage I or stage II tumors; including both lymph node-negative and node-positive tumors. This new study concluded that their 70-gene prognosis profile could more accurately predict (90% v 86%) whether a tumor would metastasize (poor prognosis vs. good prognosis) than the standard clinical and histological methods available (van de Vijver et al., 2002). Additionally, it was discovered that the ability to develop metastasis was an

	Breast cancer Subtypes	5-year survival	Treatments
ER-positive subtypes	Luminal A	~90%	Tamoxifen, Chemotherapy, Aromotase-Inhibitors
	Luminal B	~45%	
	Normal-like	~50%	
ER-negative subtypes	HER2-positive	~30%	Herceptin, Chemotherapy
	Basal-like	~30%	Chemotherapy

Figure 1.1 Breast cancer subtypes, their 5-year survival rates and available therapies.

The five expression based tumor subtypes and approximate survival rates were determined from the classification and Kaplan-Meier curves published by Sorlie et al., 2001 PNAS.

"early and inherent" property of breast cancers and that the poor prognosis signature was preserved throughout the metastatic process (Weigelt et al., 2003; Weigelt et al., 2005), suggesting that more deadly tumors could be identified early in the diagnostic process. With this new information, doctors can more accurately predict the patients with poor clinical outcome and therefore identify the patients that will likely have metastasis and benefit the most from adjuvant therapies. This 70-gene prognosis profile will open the door to patient-specific therapies that will more precisely treat patients and reduce the adverse side effects and costs of ineffective or unnecessary therapies. In fact, in February 2007 the FDA approved the MammaPrint® from the Amsterdam-based company Agendia™. MammaPrint® is a test using this exact 70-gene profile that can help predict whether or not the breast tumor will metastasize within 5-10 years in women under the age of 61, and therefore identify patients that would benefit from adjuvant therapy. In November 2007, TIME Magazine referred to MammaPrint® as "Cancer's Crystal Ball" and named it one of the top 5 innovations in the health sector for the year 2007 (TIME, 2007).

D. The basal-like breast cancer phenotype

Of particular interest to our studies is the breast tumor subtype with the worst clinical outcome, the basal-like subtype. Unlike the ER-positive luminal A or B subtypes and the HER2 positive subtype, which respond well to some of today's conventional therapies, the basal-like subtype tumors are generally ER, PR and HER2 negative, and therefore lack a molecular target or a precise treatment regimen. The ER-positive luminal A group is generally characterized by expression of ERα, GATA binding protein 3, X-

box binding protein 1, trefoil factor 3, HNF-3α, and LIV-1; while the luminal B and C groups tend to have lower expression of the luminal A subtype and the luminal C subtype is distinguished from the A and B groups by high expression of a set of genes with unknown function (Perou et al., 2000; Sorlie et al., 2001). Like the basal-like subtype, the HER2-positive group is associated with poor clinical outcome as well as high expression of HER2 and several genes linked to its amplicon at chromosome 17q22-24. Genes associated with the basal-like subtype include high expression of keratins 5 and 17, laminin, and fatty acid binding protein 7, with low expression of ER, PR, and HER2 (triple-negative) (Perou et al., 2000; Sorlie et al., 2001). Thorough study has been given to the basal-like subtype as it is associated with worse disease-free and overall survival.

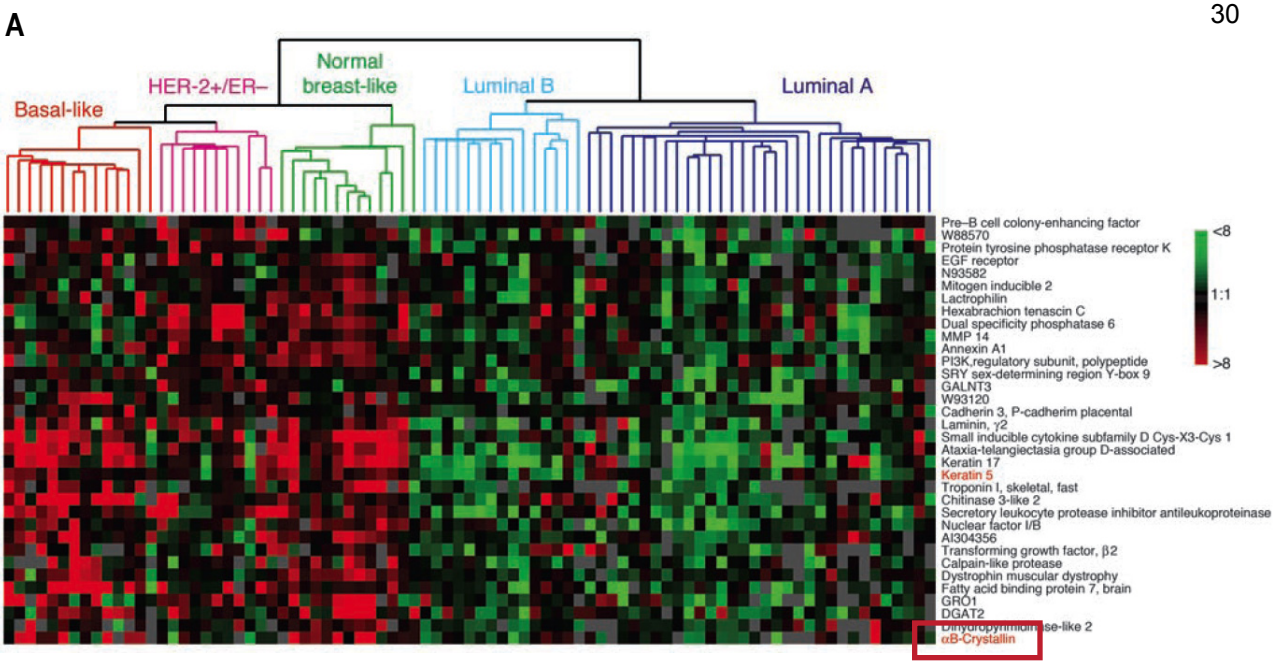
Although the basal-like carcinomas have poorer disease-free and overall survival they do respond well to initial chemotherapy. This has been referred to as the "Triple Negative Paradox" (Carey et al., 2007). For example, in the Carey *et al.* study, clinical response to chemotherapy was 85% for the basal-like subtype and 47% for the luminal subtypes, while patients with basal-like tumors had worse disease-free survival ($P = 0.04$) and overall survival ($P = 0.02$). In another study of 1,118 patients receiving neo-adjuvant therapy, patients with triple-negative status had significantly higher pathological complete response rates compared to patients with non-triple-negative status (22% v 11%) (Liedtke et al., 2008). However, the triple-negative patients had decreased 3-year progression-free survival rates and 3-year overall-survival rates. These studies only highlight the importance of developing a molecular target or a more precise therapy to target those triple-negative tumors that don't respond to traditional chemotherapy.

In addition to being triple-negative, basal-like tumors are associated with TP53 mutations (Sorlie et al., 2001), and expression of the small heat shock protein αB-crystallin (Moyano et al., 2006; Perou et al., 2000). Interestingly, the sporadic basal-like breast tumors do not have BRCA1 mutations; however, women with BRCA1 mutations develop tumors with basal-like features. It is well documented using both immunohistochemistry and genetic profiling that breast tumors with mutated BRCA1 display the basal-like phenotype (Foulkes et al., 2004; Lakhani et al., 2005; Sorlie et al., 2003). Correspondingly, BRCA1 and the basal-like phenotype have been the subject of multiple reviews (Tischkowitz and Foulkes, 2006; Turner and Reis-Filho, 2006; Turner et al., 2007; Yehiely et al., 2006). Two explanations have been suggested for the correlation of BRCA1 tumors and the basal-like phenotype (Turner and Reis-Filho, 2006). One suggestion is that the resilience of the basal-like phenotype allows cell survival following the loss of BRCA1 function, the other view suggests that the BRCA1 mutation generates a cellular environment that results in a transition to the basal-like phenotype. In any case, it is clear that a better understanding of BRCA1 mutation and the basal-like phenotype, as well as other genes associated with the phenotype, is needed for the development of novel therapies targeting this difficult to treat cancer.

E. αB-crystallin and basal-like breast tumors

As previously mentioned, another gene observed to cluster with the basal-like phenotype is the small heat shock protein αB-crystallin. Among the many functions of αB-crystallin, it is capable of acting as a molecular chaperone that can prevent protein

aggregation and apoptosis induced by multiple stimuli, at least in part through its ability to prevent activation of caspase-3 (Alge et al., 2002; Kamradt et al., 2001; Kamradt et al., 2002; Kamradt et al., 2005; Liu et al., 2004a; Liu et al., 2004b; Morrison et al., 2003; Morrison et al., 2004; Nagaraj et al., 2005; Somasundaram and Bhat, 2000; Yaung et al., 2007). Our lab has shown using immunohistochemistry of a tissue microarray including invasive breast tumors that αB-crystallin is expressed in 39 of the 361 (11%) tumors, and 19 of the 40 (45%) basal-like tumors, while only 17 of the 288 (6%) non-basal-like tumors indicating that αB-crystallin is more commonly expressed in the basal-like breast cancer subtype. Furthermore, expression of αB-crystallin correlated with poor prognosis in breast cancer patients independent of lymph node status, tumor grade or size, and ER or HER2 expression (Figure 1.2) (Moyano et al., 2006). We also showed that αB-crystallin was capable of inducing neoplastic-like changes *in vitro* through a MEK signaling dependent mechanism in the immortalized, but not transformed mammary epithelial cell line MCF-10A. When injected into nude mice, αB-crystallin overexpressing MCF-10A cells developed into tumors with an immunohistochemical profile similar to that of the basal-like phenotype. Furthermore, a recent study showed that strong αB-crystallin staining was specific to basal-like breast carcinomas and αB-crystallin staining was observed in 86% of the metaplastic breast carcinomas suggesting that αB-crystallin may provide a means to histologically identify a subset of basal-like breast tumors (Sitterding et al., 2008). Perhaps more significant, recent results in our lab suggest αB-crystallin may confer resistance to neo-adjuvant chemotherapy in patients with breast cancer (Ivanov et al., 2007). In 112 breast cancer patients, αB-crystallin expression correlated with ER-negative and ER/PR/HER2-negative tumors, confirming



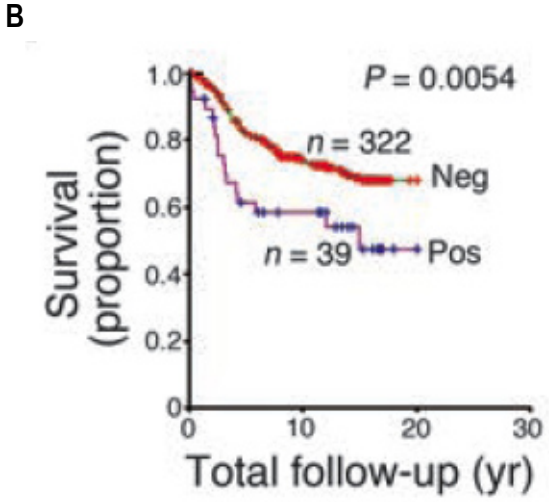


Figure 1.2 αB-crystallin,is expressed in the basal-like subtype of breast cancer and independently predicts shorter survival.

(A) View of the genetic profile of the basal-like breast cancer subtype from as published in Moyano et al., 2006 (red, indicates above average gene expression; green, indicates below average gene expression). (B) Kaplan-Meier survival analysis of breast cancer survival based on positive or negative αB-crystallin,expression in invasive breast carcinomas from Moyano et al., 2006.

the association between αB-crystallin expression and triple negative status. In addition, αB-crystallin positive tumors had a poorer overall response to chemotherapy (21% vs. 59%) compared to αB-crystallin negative tumors. It remains to be seen whether αB-crystallin could be targeted as a therapy against basal-like breast cancer. This approach requires future studies on the mechanisms by which αB-crystallin confers resistance to chemotherapy, as well as identifying the mechanisms that regulate αB-crystallin expression in breast tumors.

III. The Role of Heat Shock Proteins in Tumor Formation

Heat shock proteins perform a wide variety of tasks in cells as they work towards their overall goal to help maintain homeostasis in a cell at any given time. It is no surprise then to observe increased expression of heat shock proteins following and during stressful cellular events. In order for a cell to develop into a tumor, it progresses through stages acquiring each of hallmarks of cancer needed to become a malignant carcinoma. In the particular case of tumor formation, the function of heat shock proteins may eventually bring harm the cell's host by enabling normal cells safe passage to a tumorigenic, malignant and lethal state (Figure 1.3). Below is a brief review of how heat shock proteins can play a role in tumorigenesis.

A. Large heat shock proteins

1. Hsp100

In yeast, Hsp100's are not essential for survival and do not affect growth at normal temperatures. However, they are important in thermotolerance and response to

Self-sufficiency in growth signals
- sustained MEK signaling (αB-crystallin, Hsp70, Hsp90)
- HER2 stabilization (Hsp90)

Insensitivity to anti-growth signals
- CDK4/6 stabilization (Hsp90)

Evading apoptosis
- preventing apoptosome formation (Hsp27, 70, 90)
- preventing caspase-3 activation (αB-crystallin, Hsp70)
- preventing release and nuclear import or AIF (Hsp70)

Sustained angiogenesis
- HIF-1α chaperoning (Hsp90)

Limitless replicative potential
- promoting telomerase DNA binding and extension (Hsp90)

Tissue invasion and metastasis
- association with actin nucleation components (αB-crystallin)
- MMP2 activation (Hsp90)

Figure 1.3 The role of chaperones in tumorigenesis.

Molecular chaperone function can be implicated in establishing each of the hallmarks of cancer as proposed by Hanahan and Weinberg, 2000. Each hallmark is listed and a specific example of how a molecular chaperone helps establish each hallmark.

other stresses (Lindquist and Kim, 1996; Sanchez and Lindquist, 1990). The Hsp100 chaperones form larger hexameric ring structures and have been implicated in disaggregating protein clusters and lead them either to degradation or refolding depending on the partner proteins, which is why they are also referred to as ATPases associated with diverse cellular activities (AAA+ ATPases) (Horwich et al., 1999). For example, on one hand, the bacterial Hsp104 homologue ClpB has no known protease partners and serves more of a quality control role; however, it can work with the DnaK/Hsp70 chaperone to refold disaggregated proteins. On the other hand, ClpA readily forms complexes with the proteasome-like protease ClpP leading to degradation of the protein aggregate (Horwich, 2004; Weibezahn et al., 2004). Interestingly, the human Hsp100 orthologs, hClpP and hClpX, are mitochondrial proteases (Kang et al., 2002); however the yeast Hsp104 introduced into mammalian cells is capable of disaggregating proteins (Mosser et al., 2004).

2. Hsp90

Hsp90 does not affect growth in *E.coli*, however it is necessary for survival in all eukaryotes (Borkovich et al., 1989). Hsp90 acts as a key molecular chaperone in protein homeostasis through its ability to hold nearly mature proteins while helping them achieve their functional state thus influencing everything from signal transduction to evolution (Rutherford, 2003). Nucleotide exchange and ATP hydrolysis drive the chaperone ability of Hsp90 as it cycles between binding, chaperoning and then releasing client proteins (Pearl and Prodromou, 2006). Some of the identified Hsp90 client proteins include hormone receptors, such as glucocorticoid, estrogen and androgen receptors, which

require Hsp90 to mature and signal properly (Neckers, 2002b). Additional client proteins consist of protein kinases (FLT-3, BCR-ABL, MET, RET, $pp60^{src}$, eIF-2a, casein kinase II, and cdc37), B-raf, EGFR, HIF-1α, calmodulin, actin and tubulin (Neckers, 2007). Given its role in chaperoning multiple signaling pathways, Hsp90 can have a significant role in tumor development. Indeed, Hsp90 chaperoning has been implicated in each of the six "hallmarks of cancer". The following is a sampling of how the molecular chaperone ability of Hsp90 enables transformation to a malignant cell: evading apoptosis (IGF-1R, AKT), growth factor independent proliferation (HER2, MET), metastasis (MMP, urokinase), angiogenesis, (HIF-1α, MET), unchecked cell-cycling (CDK4, cyclinD), and continued replicative potential due to chaperoning of telomerase (Neckers, 2007). With such a prevalent role in tumorigenesis, targeting Hsp90 function is a potential therapy against tumors 'addicted' to Hsp90. In fact, geldanamycin and its derivatives (17-AG, 17-AAG, and 17-DMAG) inhibit Hsp90 by binding to its nucleotide binding pocket and preventing the chaperone from its cycling, which leads to its eventual degradation (Neckers, 2006; Whitesell et al., 1994). These drugs are the topic of many reviews and their use has given promising results in a variety of cancer models including clinical trials (Bagatell et al., 2007; Blagosklonny et al., 2001; Chiosis and Neckers, 2006; Isaacs et al., 2003; Kasibhatla et al., 2007; Koga et al., 2007; Neckers, 2000, 2002a, b, 2006; Neckers et al., 2007; Neckers et al., 1999; Neckers and Neckers, 2002, 2005; Schumacher et al., 2007; Solit et al., 2007; Weigel et al., 2007; Williams et al., 2007; Workman et al., 2007; Xu and Neckers, 2007).

3. Hsp70

The Hsp70 family consists of 12 Hsp70 chaperones in *H.sapiens* and is one of the most studied of the chaperone families. Hsp70 acts primarily as a molecular chaperone by refolding and stabilizing denatured or nascent proteins. Refolding occurs over a cycle as Hsp70 binds ATP, hydrolyzes ATP to ADP, binds substrate, refolds and releases substrate and nucleotide, and then starts over (Mayer and Bukau, 2005; Weibezahn et al., 2005). The chaperone's cycle is regulated by Hsp40 (DnaJ/Hdj) and other co-chaperones such as HIP, HOP, CHIP and Bag proteins (GrpE) (Bimston et al., 1998; Connell et al., 2001; Gassler et al., 2001; Laufen et al., 1999; Liberek et al., 1991; Mayer and Bukau, 2005; Nollen et al., 2001; Takayama et al., 1997). Hsp40 itself appears to have some chaperone ability (referred to as a 'holder'), but most study has been on its ability to positively regulate Hsp70 by accelerating the rate of ATP hydrolysis on ATP-bound Hsp70 (Laufen et al., 1999; Liberek et al., 1991). ATP-bound Hsp70 has low affinity for substrates and therefore exchanges substrates too quickly to effectively chaperone, so the function of Hsp40 is to maintain Hsp70 in the ADP bound state, which favors high affinity binding of substrate and promotes cycling between 'holding' and 'folding'. The exact role of the co-chaperone Bag proteins is somewhat controversial, but studies do agree that Bag proteins increase the rate of nucleotide exchange on Hsp70, which may help either increase folding by cycling faster or prevent folding by cycling too fast (Bimston et al., 1998; Brehmer et al., 2001; Gassler et al., 2001; Nollen et al., 2001; Takayama et al., 1997). The other co-chaperones may serve to drive specific roles of the Hsp70 chaperone. For example, HOP is known to help regulate the interactions between the Hsp90 and Hsp70 chaperone machines and specifically functions in the maturation of

the progesterone receptor complex (Smith et al., 1993). HIP can stimulate the activation of the glucocorticoid receptor (Picard et al., 1990), and CHIP has an E3-ubiquitin ligase activity that promotes degradation of Hsp70/Hsp90 substrates by the proteasome (Connell et al., 2001).

Hsp70 chaperone proteins contribute to tumor formation in several ways, one of which is preventing cells from entering a quiescent state through its preferential interaction with the non-phosphorylated form of the retinoblastoma protein (pRb), which prevents pRB from phosphorylation (Inoue et al., 1995). Extracellular Hsp70 can also work towards a malignant cell's self-sufficiency from growth signals by altering EGFR transactivation in the A431 carcinoma cell line (Evdonin et al., 2006).

Evading apoptosis is another one of the hallmarks of cancer that Hsp70 can help a cell acquire. For example, overexpression of Hsp70 can prevent heat shock or ceramide-induced apoptosis most likely through the prevention of the processing of procaspases 3 and 9 without altering the activity of previously cleaved caspase 3 (Mosser et al., 1997). Furthermore, Hsp70 may prevent caspase activation through its ability to prevent cytochrome *c* and apoptosis inducing factor (AIF) release from the mitochondria as well as working to maintain mitochondrial membrane potential following stress (Creagh et al., 2000; Mosser et al., 2000; Ravagnan et al., 2001). Additionally, Hsp70 is able to bind to apoptotic protease-activating factor-1 (Apaf-1) preventing formation of the apoptosome and downstream activation of procaspase 9 (Beere et al., 2000). Preventing the activation of the JNK and p38 stress-activated signaling pathways is an alternative way Hsp70 helps

cells evade apoptosis. Briefly, Hsp70 is thought to prevent the inactivation, and potentially increases the activity, of JNK phosphatase resulting in a deactivation of JNK signaling (Palacios et al., 2001; Yaglom et al., 1999).

B. Small heat shock proteins

Compared to the large heat shock proteins Hsp90 and Hsp70, relatively few studies have characterized the function of the small heat shock family of molecular chaperones. Although almost dispensable in the yeast heat shock response, they play a significant role in thermotolerance in mammals (Jakob et al., 1993; Kurtz et al., 1986). In mammals, the sHSPs consist of 10 members which all share a common C-terminal α-crystallin domain. The earliest studies of these crystallin proteins attributed the transparency of the lens to their over abundance in that tissue (Taylor and Benjamin, 2005). However, we now know that the sHSPs are not exclusively expressed in the lens and have a wide range of functions and specializations. sHSPs form oligomeric structures ranging from 10-50 units with chaperone-like abilities enabling them to interact with non-native proteins preventing their aggregation and with the capacity to help restore protein function in a nucleotide-independent manner (Taylor and Benjamin, 2005).

1. HSPB1/Hsp27-1

The specific role of HSPB1/Hsp27-1 in tumor formation depends primarily on the type of cancer and model system studied. For example, overexpression of Hsp27-1 in rat colon carcinoma cells makes them more resistant to apoptosis *in vitro* and when injected

into rats they display enhanced tumorigenicity (Garrido et al., 1998). The mechanism of Hsp27-1 induced resistance to apoptosis, including apoptosis induced by chemotherapeutic agents, likely comes from its ability to prevent activation of procaspases 9 and 3 (Garrido et al., 1999) or its ability to prevent the formation of the apotosome by either binding released cytochrome *c* (Bruey et al., 2000) or preventing the release of cytochrome *c* from mitochondria (Paul et al., 2002).

Hsp27-1 can also help a cell during the metastasis and invasion process. In an *in vitro* breast cancer model, high expressing Hsp27-1 clones of the human MDA-MB-231 cell line displayed increased invasion, adhesion, and anchorage-independent growth, but decreased motility (Lemieux et al., 1997). *In vivo,* injecting the Hsp27-1 overexpressing cells into nude mice resulted in an increased number of lung metastases compared to control cells, and decreasing the level of Hsp27-1 via anti-sense showed the opposite results of the overexpression experiments. Although increased Hsp27-1 expression can prevent apoptosis and increase metastasis, clinical studies indicate some uncertainty of the role of Hsp27-1 in women with ovarian or breast cancer. For example, decreased Hsp27-1 levels correlated with decreased survival in women with ovarian cancer, and that in a subset of breast cancer patients Hsp27-1 levels had no correlation to disease-free survival (Geisler et al., 2004; Oesterreich et al., 1996). The role of Hsp27-1 in prostate cancer is somewhat more clear in that Hsp27-1 plays a protective role both *in vivo* and *in vitro* where it inhibits apoptosis by preventing caspase-3 activation (Aloy et al., 2008; Rocchi et al., 2006; Rocchi et al., 2004). The diversity of Hsp27-1 roles in cancer

indicates that more studies are needed to elucidate the functions of Hsp27-1 in tumor biology.

2. HSPB5/αB-crystallin

Another member of the small heat shock protein family with a role in tumorigenesis is HSPB5/CRYAB/αB-crystallin. Specifically, αB-crystallin is recognized to be important for protection from apoptosis induced by multiple stressors in a variety of tissues (Alge et al., 2002; Kamradt et al., 2001; Kamradt et al., 2002; Kamradt et al., 2005; Liu et al., 2004a; Liu et al., 2004b; Morrison et al., 2003; Morrison et al., 2004; Nagaraj et al., 2005; Somasundaram and Bhat, 2000; Yaung et al., 2007). Its role in preventing apoptosis ranges from preventing caspase-3 activation (Kamradt et al., 2001; Kamradt et al., 2002; Kamradt et al., 2005; Mao et al., 2004) to binding p53 and potentially preventing its translocation to the mitochondria (Liu et al., 2007a). In rabbit lens epithelial cells, mouse αB-crystallin can prevent calcimycin induced apoptosis by preventing Ras activation, therefore preventing activation of Raf, MEK, ERK which is responsible for induction of apoptosis through upregulation of p53, release of cytochrome *c* and downstream activation of caspase-3 (Li et al., 2005).

In addition to αB-crystallin's clear role as an anti-apoptotic molecular chaperone, a recent study revealed stable αB-crystallin expression is capable of enabling neoplastic-like changes in the mammary epithelial cell line MCF-10A, and these neo-plastic changes can be prevented by αB-crystallin directed RNA interference (Moyano et al., 2006). Additionally, this study showed other indicators of αB-crystallin's potential oncoprotein

status, such as EGF-free and anchorage-independent growth, increased migration, decreased apoptosis, and increased tumor volume in nude mice. As opposed to Hsp27-1 the clinical studies on αB-crystallin are more clear, αB-crystallin expression in breast cancer as well as head and neck cancer correlates with poor clinical outcome (Chin et al., 2005; Moyano et al., 2006). Furthermore, αB-crystallin expression was found to be higher in breast carcinomas with lymph node metastasis (Chelouche-Lev et al., 2004), strong αB-crystallin expression was recently identified as a novel marker of invasive basal-like breast tumors (Sitterding et al., 2008), and finally, αB-crystallin expression in breast cancer appears to confer resistance to neo-adjuvant chemotherapy (Ivanov et al., 2007).

IV. Transcriptional Regulation of αB-crystallin

A. The CRYAB/HSPB2 shared promoter

The human αB-crystallin gene is approximately 3.2kb, composed of 3 exons, 2 introns and exists on chromosome 11 region q22.3 in a divergent head-to-head arrangement with another small heat shock protein HSPB2 (Figure 1.4) (Dubin et al., 1990). The bidirectional gene arrangement of less than 1,000 base pairs observed between the transcriptional start sites (TSS) of CRYAB and HSPB2 accounts for approximately 11 percent of the genes in the human genome (Trinklein et al., 2004). While most genes with bidirectional arrangement co-express, αB-crystallin and HSPB2 usually do not, and have very recently been described to co-express in only one instance by GR (Swamynathan and Piatigorsky, 2007). The most likely cause of this bidirectional arrangement was from a gene duplication event resulting from an inversion that produced

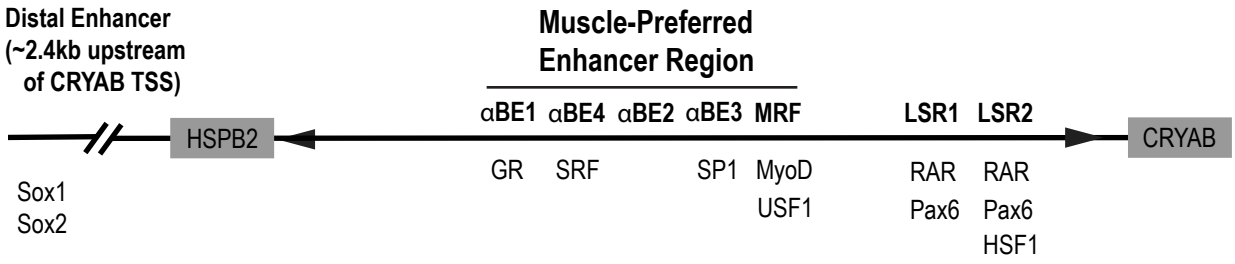


Figure 1.4 Published functional regulators of murine αB-crystallin.

Schematic representation of the murine αB-crystallin,/HSPB2 shared promoter. The shared promoter is approximately 1kb in both humans and mice and above is a summary of published regions (indicated above line) within the promoter that have been shown to have transcription factors that bind to and regulate αB-crystallin, gene expression (indicated below line) in a murine model. ► Indicates TSS for each gene, and gray box indicates location of gene.

two copies of the same gene in this head-to-head arrangement. Slowly during the course of evolution, the gene diverged into the two highly related genes we see today, αB-crystallin and HSPB2. The inversion event occurred early as the head-to-head arrangement is preserved in all the vertebrae orthologs studied thus far, with the intergenic region ranging from 866bp in mice to 1.6kb in duck (Doerwald et al., 2004). Initial analysis of αB-crystallin gene expression by Northern blot analysis in mouse and rat revealed two different sized transcripts depending upon the tissue expressing αB-crystallin (Dubin et al., 1989; Iwaki et al., 1990). A prevalent 800-900 base mRNA transcript is observed in lens, heart and skeletal muscle, and kidney, while a longer 1100-1400 base transcript was observed in brain and lung tissues, although this tissue specificity does not appear to be absolute (Frederikse et al., 1994).

B. Transcriptional regulation of αB-crystallin by stress

One of the first studies researching the regulation of αB-crystallin identified it as a *bone fide* small heat shock protein by observing the induction of αB-crystallin following heat shock or heavy metal exposure (Klemenz et al., 1991). This study also observed translocation of αB-crystallin from the cytoplasm to the nucleus following stress and identified the proximal heat shock factor consensus site just upstream of the TATA box. Multiple studies confirm stress regulation of αB-crystallin in various cell lines (Iwaki et al., 1993; Kato et al., 1993a; Kato et al., 1993b) and it appears that its regulation is primarily through heat shock factor 1 and the proximal HSE, but is not limited to that mechanism. A study involving cadmium or hypertonic stress in astrocytes, found transcriptional upregulation of αB-crystallin following heat shock, cadmium

exposure and hypertonic stress (Head et al., 1996). However, they did not observe upregulation of Hsp27-1, a known HSF1 target gene, following hypertonic stress suggesting an HSF1-independent mechanism of αB-crystallin upregulation. Using gel shift analysis, they observed binding by HSF1 to the αB-crystallin proximal and distal heat shock elements following cadmium stress, but not following hypertonic stress. Furthermore, they observed binding of an unidentified, non-AP1-like factor to the AP1 consensus binding site within the αB-crystallin promoter, providing evidence for hypertonic stress activation of αB-crystallin transcription independent of HSF1 and AP1. It has also been reported that αB-crystallin is regulated by heat shock factor during development (Somasundaram and Bhat, 2000). More specifically, it appears that HSF1 may regulate αB-crystallin expression in neo-natal rats lens as whole cell extracts from the neo-natal rat cells results in binding to and shifting of an oligonucleotide containing the HSE from the αB-crystallin promoter. This shift is observed only in extracts from the lens cells and not brain, liver or heart extracts. Interestingly however it was later revealed that HSF4 was responsible for post-natal regulation of αB-crystallin in rat lens (Somasundaram and Bhat, 2000, 2004).

C. LSR1/LSR2

The transcriptional regulation of αB-crystallin has been well documented in the mouse using the mouse αB-crystallin promoter and 'mini-gene' used for *in vitro* and mouse transgenic experiments (Dubin et al., 1990; Dubin et al., 1991; Dubin et al., 1989; Frederikse et al., 1994; Gopal-Srivastava et al., 1996, 1998; Gopal-Srivastava et al., 1995; Gopal-Srivastava et al., 2000; Gopal-Srivastava and Piatigorsky, 1993, 1994;

Haynes et al., 1996; Haynes et al., 1995; Haynes et al., 1997; Li et al., 2007; Sax and Piatigorsky, 1994; Swamynathan and Piatigorsky, 2002, 2007). Northern blot analysis of αB-crystallin RNA expression in one to two week-old mice indicated strong αB-crystallin gene expression in lens, heart and skeletal muscle, and weak expression in lung, kidney, and brain (brain only at ~2 months). *In vitro* experiments with primary embryonic chick lens cells revealed that the region between -222 and -68 was required for efficient αB-crystallin promoter activity (Dubin et al., 1989). Transgenic mice generated to include the αB-crystallin mouse promoter from -164 to +44 fused to bacterial chloramphenicol transferase (CAT) showed CAT activity exclusively in lens tissue, while transgenic mice with the -426 to +44 mouse αB-crystallin promoter showed activity in lens, skeletal and heart muscle. Transient transfection experiments in mouse lens cells with the -164 to +44 CAT reporter and mutated versions of the reporter revealed that the region between -147 to -118 are most important for promoter activity. Finally, DNase I protection assays indicated protection of the -147 to -118 region in nuclear extracts from lens cells but not myotubes (Gopal-Srivastava and Piatigorsky, 1994). The region from -147 to -118 was later termed lens-specific region 1 (LSR1), and continuing studies of the mouse αB-crystallin promoter in transgenic mice and multiple *in vitro* experiments revealed another less robust lens-specific region called LSR2, -78 to -46 (Figure 1.4). The -115 to +44 CAT transgenic mice showed CAT activity in lens tissue only, however at levels nearly 30 times less than that of the -164 to +44 CAT transgenic mice. Mutation to LSR1 or LSR2 significantly decreased CAT activity in transient transfection experiments in lens cells. Again DNase I protection assays displayed protection at both LSR1 and LSR2 from lens nuclear extracts and purified Pax-

6 (Gopal-Srivastava et al., 1996). Furthermore, gel shift experiments, site-directed mutagenesis and transient transfection reporter experiments provided further evidence for the Pax-6 specific role in regulating the αB-crystallin promoter in lens. A similar set of experiments by the same group indicates that retinoic acid can also regulate αB-crystallin expression in lens cells. Using DNase I protection, gel-shift, site-directed mutagenesis, and transient trasfection assays they showed that retinoic acid receptor/retinoid X receptor binds and regulates LSR1 and LSR2 in mouse lens cells (Gopal-Srivastava et al., 1998). A series of transgenic mice with 5' truncations of the mouse αB-crystallin promoter from -426/-339/-257/-164 fused to CAT revealed the lens epithelial, as well as corneal epithelial, cell-specific nature of the promoter activity of the -164 to +44 region (Gopal-Srivastava et al., 2000).

D. The αB-crystallin/HSPB2 enhancer region

Even though αB-crystallin expression is strongest in the lens, its expression is also found in the skeletal and heart muscle, kidney, lung and brain (Bhat et al., 1991; Bhat and Nagineni, 1989; Dubin et al., 1989). A transgenic mouse expressing the αB-crystallin promoter from -661 to +44 fused to CAT revealed CAT activity primarily in lens and skeletal muscle, and a series of 5' truncations of the αB-crystallin promoter fused to CAT transfected into both lens cells and muscle cells showed muscle cell specific activity. Additionally, nuclear extracts from myoblasts bound multiple areas within a region called the muscle-preferred enhancer between -427 and -259 (Dubin et al., 1991). DNase I footprinting of the muscle-preferred enhancer with nuclear extracts from myotubes, lens epithelial cells, and fibroblasts showed that nuclear exptracts from

all three cell lines footprint to some degree at the same three areas (called αBE1, αBE2, αBE3) within the enhancer, and that only the myotube nuclear extracts protected a fourth site (called MRF) (see Figure 1.4). Gel-shift analysis of the MRF region using nuclear extracts from myotubes showed binding of a factor that is at least antigenically related to MyoD and myogenin. Mutation analysis of the MRF region provides further evidence for the muscle specific nature of the MRF binding site and co-transfection of various αB-crystallin reporters with MyoD displayed transactivation of the reporter in the presence of MyoD dependent upon an E-box within the MRF region (Gopal-Srivastava and Piatigorsky, 1993). Further studies of the mouse αB-crystallin promoter using transgenic mice confirmed αB-crystallin promoter activity in heart tissue and specifically within cardiac myocytes (Gopal-Srivastava et al., 1995). Footprint analysis using heart nuclear extract displayed protection of the previously identified αBE1, αBE2, αBE3, and MRF site as well as a new, cardiac-specific site αBE4. Gel-shift analysis using heart nuclear extract discovered binding of a cardiac factor related to serum response factor (SRF) to αBE4 and a factor similar to USF binding the E-box within the MRF (Gopal-Srivastava et al., 1995). Recently, a study identified interaction and transactivation by glucocorticoid receptor at αBE1 and SP1 at αBE3 (Swamynathan and Piatigorsky, 2007). The αBE3/SP1 interaction transactivates αB-crystallin preferentially and the αBE1/GR interaction transactivates both αB-crystallin and HSPB2 equally, providing the first and only example of regulation of the CRYAB/HSPB2 enhancer by a shared element.

A possible explanation for the antiregulated nature of the shared CRYAB/HSPB2 shared promoter came in 2002 when Swamynathan and Piatigorsky discovered the

orientation-dependent nature of the intergenic muscle-preferred enhancer region (Swamynathan and Piatigorsky, 2002). Truncation of the HSPB2 promoter showed that the muscle preferred enhancer only confers a two-fold activation in myocytes compared to an approximate 30 fold activation towards αB-crystallin. Furthermore, a dual reporter construct composed of renilla (representing CRYAB) and firefly (representing HSPB2) luciferases with the CRYAB/HSPB2 intergenic region between them displays activity almost exclusively toward renilla/CRYAB.

E. Additional regulation of αB-crystallin expression

An interesting study using a mouse model similar to the Piatigorsky studies, observed a well conserved genetic region 130bp downstream of the HSPB2 gene and approximately 2.4kb upstream of the αB-crystallin TSS (Figure 1.4) (Ijichi et al., 2004). Further study of this conserved area identified a new distal enhancer region that drives expression of αB-crystallin in mouse lens cells and is dependent upon binding of Sox1 and Sox2, indicating regulation of αB-crystallin from a distal enhancer region.

F. Expression of αB-crystallin in cancer

Many cancers have been found to express αB-crystallin, including prostate cancer, gliomas, renal cell carcinomas, and oral squamous cell carcinomas (Aoyama et al., 1993; Chelouche-Lev et al., 2004; Chin et al., 2005; Liu et al., 2007b; Moyano et al., 2006; Sitterding et al., 2008). In addition, αB-crystallin expression correlates with poor prognosis in breast cancer, and head and neck cancers (Chin et al., 2005; Moyano et al., 2006). Conversely, anaplastic thyroid carcinomas have decreased expression of αB-

crystallin by immunoblot and RT-PCR analysis when compared to benign goiters, which maybe due to downregulation of the transcription factor TFCP2L1 in that cancer (Mineva et al., 2005), although regulation of αB-crystallin expression by TFCP2L1 was not tested. To date, mechanisms of αB-crystallin gene regulation in cancer have not been well studied.

V. The Biology of Ets1

A. Introduction to Ets1

Ets1 is the founding member of the ETS-domain of transcription factors. Its name comes from the avian erythroblastosis virus E26, from which the viral-*ets* (E Twenty-Six or E26 Transformation Specific) oncogene was first isolated and described (Nunn et al., 1983). Later it was discovered that the *v-ets* sequence existed in human DNA on chromosomes 11 and 21; the human cellular homologue, *c-ets*, genes were named Ets1 and Ets2 (Watson et al., 1985). To date, approximately 30 transcription factors have been found to contain an ETS domain and therefore are considered to be part of the winged helix-loop-helix ETS family of transcription factors (Sharrocks, 2001). The ETS-domain is defined as the DNA binding domain of Ets1 (Karim et al., 1990) an approximately 85 amino acid region of Ets1 that recognizes the core consensus sequence of 5'-GGA$^{A}/_{T}$-3' with variation within the flanking sequences depending on the ETS protein (Nye et al., 1992; Sementchenko and Watson, 2000). Another domain present in Ets1 and other ETS-domain proteins is the Pointed (PNT) domain, which was named after the Ets1-related protein Pointed-P2 found in *Drosophila melanogaster* (Klambt, 1993). The PNT domain is involved in ETS protein homo-oligomerization, heterodimerization, and

transcriptional repression (Baker et al., 2001; Fenrick et al., 1999; Kim et al., 2001). Ets1 transcriptional activity can be regulated by Ras-responsive phosphorylation at threonine-38 near the N-terminus (Yang et al., 1996) or a C-terminal cluster of acidic residues (Gegonne et al., 1992). The exon VII domain contains a Ca^{++}-responsive phosphorylation site that can regulate autoinhibition/DNA binding activity of Ets1 without affecting its localization (Cowley and Graves, 2000; Rabault and Ghysdael, 1994). A schematic of the Ets1 protein with its domains and proteins that interact with Ets1 and regulate its activity can be seen in Figure 1.5, with further discussion in the next section.

B. Regulation of Ets1 by protein-protein interactions

In addition to Ets1's transcriptional activity being regulated by phosphorylation within specific domains, Ets1 also may be regulated by interactions with DNA, other transcription factors or even homodimers (Li et al., 2000a). As a transcription factor, DNA binding is of primary importance and regulation of DNA binding therefore dictates its potential transcriptional activity. Ets1 forms a ternary complex with another transcription factor Pax-5 that stabilizes the DNA binding of Ets1 (Fitzsimmons et al., 1996), and it has been shown that the Pax transcription factors bind DNA at Pax-Ets composite sites and recruit Ets1 to the promoter to activate transcription (Wheat et al., 1999). In a slightly different way, Ets1 interaction with both CBF and DNA increases the affinity of Ets1 to DNA resulting in decreased disassociation of the complex from DNA and synergistic activation of the target gene T-cell receptor β (Wotton et al., 1994). In another protein interaction dependent on DNA binding, Ets1 interacts with NFκB to

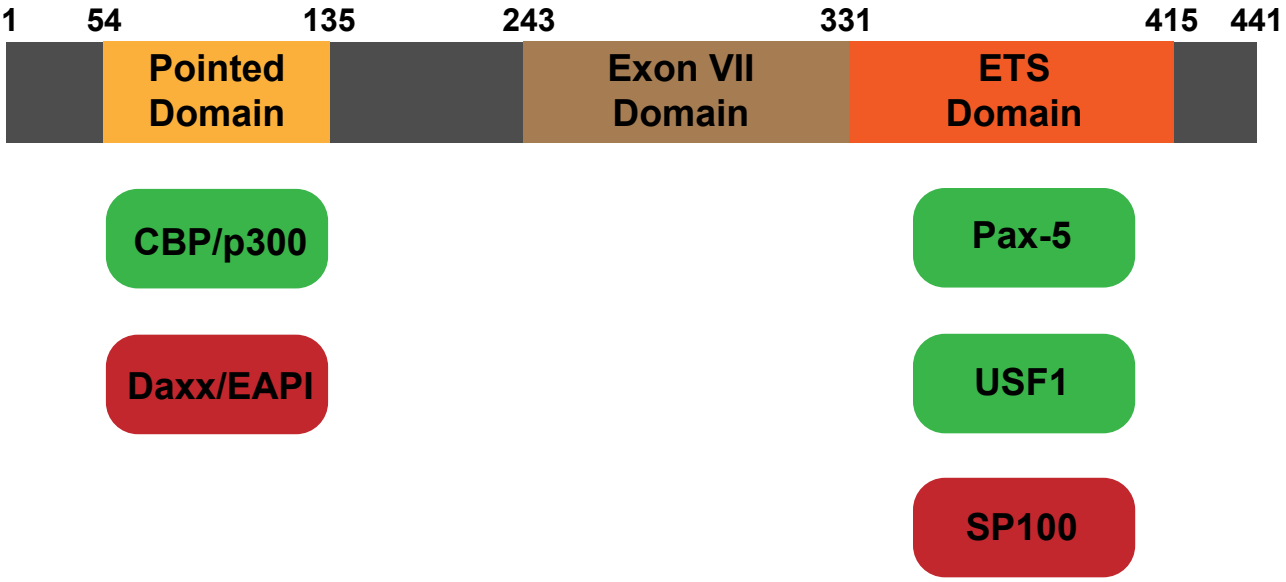


Figure 1.5 Domains of the Ets1 transcription factor and some proteins that interact with Ets1.

Proteins that interact with Ets1 as discussed in text and domain within Ets1 documented to physically interact. In red are transcription factors described to repress Ets1 activty and in green are transcription factors described to activate Ets1 activity. CBP/p300, CREB binding protein/p300; Daxx/EAPI, Ets1-associated protein; Pax-5, paired box containing gene 5; USF1, upstream stimulating factor 1; SP100, speckled 100kDa.

synergistically transactivate the enhancer of human immunodeficiency virus types I and II in activated human T-cells (Bassuk et al., 1997). Synergistic transactivation of the enhancer of HIV-I LTR also was observed with Ets1 and USF1. In this case, Ets1 and USF1 interaction is independent of DNA binding and can synergistically increase their DNA binding and transactivation in a manner dependent upon protein-protein interaction (Sieweke et al., 1998). A transcriptional co-factor that interacts with and regulates Ets1 activity is CBP/p300. Multiple studies have observed DNA-free interactions between Ets1 and CBP/p300, in part regulated by Ras/MAPK signaling, that mediate the transactivation potential of Ets1 (Foulds et al., 2004; Jayaraman et al., 1999; Yang et al., 1998). The cases mentioned thus far are examples of how protein-protein interactions result in activation or enhancement of Ets1 transactivation, but some interactions can cause a transcriptional repression. Examples of this occur by interaction of the Daxx protein EAP with Ets1 and the repression of MMP1 and Bcl2 expression (Li et al., 2000b) or interaction of SP100 with Ets1 and repression of MMP1 and uPA and other targets involved in migration and invasion (Yordy et al., 2004; Yordy et al., 2005). Functionally, overexpression of SP100 inhibits migration and invasion of breast cancer and primary epithelial cell lines. Finally, Ets1 has been shown to homodimerize in the presence of palindromic Ets binding sites blocking the autoinhibitory mechanism and enhancing transactivation of the MMP3 promoter (Baillat et al., 2002; Baillat et al., 2006). A list of Ets1 interacting proteins can be found in Table 1.1 and a schematic of the Ets1 domains along with some interacting proteins can be seen in Figure 1.5.

C. Ets1 and breast cancer

Ets1 was first identified through its homology to the viral E26 transformation specific sequence and it is therefore not surprising to find Ets1 expression in multiple cancers including: brain, breast, cervix, colon, esophagus, liver, lung, lymphoid tissue, ovary, pancreas, and thryoid (Dittmer, 2003). Of the known target genes for Ets1, many are associated with the hallmarks of cancer; for example: p53, NFκB, uPA, MMP3, MMP9, MET/HGFR, and PTHrP. More specifically related to breast cancer, Ets1 mRNA and protein was found to be upregulated along with MMP1 and MMP9 in the both invasive and pre-invasive breast tumor tissues (Barrett et al., 2002; Behrens et al., 2001a; Behrens et al., 2001b) suggesting Ets1 may have a role in tumor invasion. It has also been suggested that Ets1 may have a role in breast cancer angiogenesis (Oda et al., 1999; Span et al., 2002). A study of 123 primary breast tumor samples discovered that Ets1 mRNA expression levels significantly correlated with VEGF and PAI-1 mRNA levels. Furthermore, with a median follow-up of approximately 5 years, this study showed that Ets1 expression is a strong, independent predictor of poor prognosis in breast cancer (Span et al., 2002). Additional studies have found that Ets1 levels were higher in malignant breast tumors compared to surrounding tissue (Buggy et al., 2004; Katayama et al., 2005). In *in vitro* systems, Ets1 expression in breast cancer cell lines is well documented (Barrett et al., 2002) and a recent study of breast cancer cell lines identified Ets1 as one of the genes associated with the basal-like breast cancer cell types (Charafe-Jauffret et al., 2006).

Protein	Target gene or genes
AML-1	TCRalplha/beta, osteopontin, GM-CSF
AR	UNQ9419, CCNG2, PRAME
ATF-2	TCRalpha
AP-1 (cJun/cFos)	GM-CSF, TIMP-1, MMP-1, CD226
CBP/p300	MMP-3
ERK1/2	uPA, MMP-3, prolactin
Ets1	MMP-3
ER	artificial promoter
GATA-3	IL-5
GFI-1	Bax
HIF-2alpha	Flk-1
HTLV-1	PTHrP, IL-5
LEF-1	TCRalpha
cMyb	alpha4 integrin, MIM-1
NFAT	HIV-LTR
NFkappaB	GM-CSF, HIV-LTR
Pax-5	MB-1
Pit-1	Prolactin
PKCalpha	PTHrP
mutant p53	MDR-1
wildtype p53	MDM2, Bax
Ras	uPA, MMP-3, prolactin
Smad3/4	PTHrP
SPBP	cMyc, MMP-3
SP-1	PTHrP, MRG-1, HTLV-1 LTR, FasL, TN-C, PDGFa
SP100	MMP3
Stat-5	GAS/Ets elements
TFE-3	Immunoglobulin u heavy-chain
huUBC9	synthetic promoter
USF-1	HIV-LTR
VDR	artificial promoter

Proteins that repress Ets1 transactivation

Protein	Target gene or genes
CaMKII	GM-CSF
Daxx	MMP-1, Bcl2, VEGF
EAPII	MMP-1
MafB	transferrin receptor, parphobilinogen deaminase
ZEB	alpha4 integrin

Table 1.1 Proteins that have been identified to coregulate Ets1 activity.

D. Ets1 involvement in tumor invasion and metastasis

Of the hallmarks of cancer, Ets1 has been implicated in regulating expression of multiple genes involved in cellular transformation. Of particular interest here is Ets1 regulation of two gelatinases involved in tumor invasion and migration, MMP2 and MMP9. MMP2 and MMP9 are matrix metalloproteinases that belong to a family of approximately 20 proteins responsible for degradation of the extracellular matrix and basement membranes (Curran and Murray, 2000). Generally, MMPs consist of signal peptide, propeptide, catalytic domain containing a well-conserved zinc binding site and a hemopexin region (Woessner, 1998). The proposed role of MMPs in tumor invasion is that they allow a malignant cell to leave the primary tumor, migrate through surrounding tissue and eventually settle in a new environment, a process that crosses many physical barriers. Some evidence for the proposed role of MMPs in tumor invasion is the high level of MMP expression in invasive cancer (Basset et al., 1997; Johnsen et al., 1998). An example of the importance of MMP2 in tumor progression is suggested by the MMP2 knockout mouse, which show a marked decrease in tumor progression and angiogenesis (Itoh et al., 1998). MMP3 enhances tumorgenicity and promotes epithelial to mesenchymal transition *in vitro* and MMP3 transgenic mice develop malignant mammary lesions (Sternlicht et al., 1999). Furthermore, it has been shown that patients with breast cancer show elevated serum levels of both MMP2 and MMP9 (Somiari et al., 2006a; Somiari et al., 2006b). Finally, a rat model indicates that MMP2, MMP3, and MMP9 may play role in breast cancer metastasis to the brain (Mendes et al., 2005). As the role of MMPs in tumor progression becomes more clear, additional studies should look into additional factors that control the expression of MMPs in cancer.

The Ets family of transcription factors are well documented regulators of MMPs (Sementchenko and Watson, 2000). Ets1 in particular regulates expression of MMP1, MMP2, MMP3, and MMP9 in a variety of cell types (Jiang et al., 2001; Oda et al., 1999; Sato et al., 2000). Therefore it appears that the specific environment and cell type dictates whether or not Ets1 regulates a given MMP or not.

I. Bioinformatics Analysis

To identify putative binding sites for known transcription factors within the human αB-crystallin promoter, the putative promoter region from human αB-crystallin (identified as the region between the transcriptional start sites of HSPB2 and CRYAB) was queried for DNA motifs within the TRANSFAC library using the web-based software MOTIF-search (http://motif.genome.jp/). The cut-off score used to search for motifs of published regulatory regions was 90, therefore confirming the validity of MOTIF search software. The cut-off score used to search for putative sites that could be related to breast cancer was 80.

II. Cloning and Plasmid Constructions

A. Cloning of human αB-crystallin promoter

The human αB-crystallin promoter and nested deletion reporters were generated as follows. Using PCR methods, a SacI endonuclease site was generated -1081 base pairs upstream of the αB-crystallin start site (this plasmid is further referred to as the full-length reporter) and a BglII endonuclease site was generated at the start ATG of CRYAB with MCF-10A genomic DNA as template. An approximately 1100bp fragment was generated by PCR using KOD Hot Start DNA polymerase (Novagen; Madison, WI; Cat#71086-EA) following the manufacturer's protocol and was cloned into the pGL3-Basic reporter (Promega; Madison, WI) in front of the luciferase reporter resulting in the full-length human αB-crystallin promoter reporter. All of the human αB-crystallin

promoter truncations were generated by PCR methods with primers containing SacI endonuclease sites specific to each truncation generated (See table below for primers).

B. Site-directed mutagenesis of the human αB-crystallin promoter

Site directed mutatgenesis was used to generate the mutant forms of the putative EBS. The QuickChange® Site-Directed Mutatgenesis Kit (Stratagene; La Jolla, CA; Cat# 200519) was used according to the manufacturer's protocol with the full-length human αB-crystallin promoter in pGL3-Basic as template (See table below for primers).

Name	Sequence	Size	Restriction Site added
Full-length 5'	5'-CGAGCTCCATGGCTGCAGATGCAGC-3'	25bp	SacI
Full-length 3'	5'-GGAAGATCTCATGGTGGCTAGGTGAGTGTGGGG-3'	33bp	BglII
-908 5'	5'-CGAGCTCTGAGAGCAACGAGGGTGTGACC-3'	29bp	SacI
-638 5'	5'-CGAGCTCAGCCACATAGAACGAAAGATGCC-3'	30bp	SacI
-516 5'	5'-CGAGCTCTGGTGCTGACATGTTGACC-3'	26bp	SacI
-356 5'	5'-CGAGCTCACACTACGCCGGCTCCCATC-3'	27bp	SacI
-292 5'	5'-CGAGCTCCAGCTTCAGAGAACAGGGG-3'	26bp	SacI
-121 5'	5'-CGAGCTCCATGAACTGCTGGTGAGC-3'	25bp	SacI
SDM 5'	5'-CCTGGGGCTCAGCCTAAAAAGATTTTAGTCCC-3'	32bp	-
SDM 3'	5'-GGGACTAAAATGTTTTTAGGCTGAGCCCCAGG-3'	32bp	-

C. Cloning of human Ets1

The pcDNA3.1-Ets1 construct was generously provided by M. Zhou, M.D., from the Department of Hematology/Oncology at Emory University, Atlanta, Georgia. pcDNA3.1-Ets1 was used for luciferase reporter assays, but was sub-cloned into pLXSN and pBABEpuro for generation of stable cell pools. Ets1 was subcloned into pLXSN and pBABEpuro via blunt end ligations. Briefly, Ets1 was removed from pcDNA3.1-Ets1 via NheI (NEB; Beverly, MA; Cat# R0131), HindIII (NEB; Cat# R0104) double digestion, T4 DNA polymerase (NEB; Cat# M0203) treated, and ligated (Fast-Link™ DNA

Ligation Kit, Epicentre Biotechnologies; Cat# LK6201H) into EcoRI (NEB; Cat# R0101) digested, T4 DNA polymerase treated pLXSN or pBABEpuro at 16°C overnight. DNA was transformed into DH3α, plated on LB agar plates containing 100 μg/ml ampicillin. Colonies were screened for Ets1 insert by PCR amplification and positive colonies were grown up and sequenced for orientation and sequence correctness.

D. The MMP9 promoter reporter

The pGL3-MMP9 luciferase reporter containing -573 to +30 of the MMP9 promoter was provided by M. Sharon Stack of Northwestern University (Currently at the University of Missouri, St. Louis, Missouri).

III. Cell Culture

A. General cell culture

BT-20, T47D, MDA-MB-231, MCF-7, MDA-MB-468 and MCF-10A cell lines were purchased from American Type Culture Collection and cultured in recommended media. Briefly, BT-20 cells were cultured in MEM with Earl's salts plus L-glutamine (Gibco® Invitrogen Corp.; Cat# 11095-080) supplemented with 10% fetal bovine serum (Gibco® Invitrogen Corp.; Cat# 16140-071), 1X nonessential amino acids (Cellgro® Mediatech Inc.; Herndon, VA; Cat# 25-020-CI), 1 mM Sodium Pyruvate (Cellgro® Mediatech Inc.; Cat# 25-000-CI), 1.5 g/L Sodium Bicarbonate (Cellgro® Mediatech Inc.; Cat# 25-035-CI) and 1X Penicillin-Streptomycin-Glutamate (Gibco® Invitrogen Corp.; Cat# 10378-016); MDA-MB-468 cell lines were cultured in DMEM (Gibco® Invitrogen Corp.; Cat# 11965-092) supplemented with 10% FBS and 1X PSG; MDA-MB-231 and

MCF-7 cells were cultured in MEM with Earl's salts plus L-glutamine (Gibco® Invitrogen Corp.; Cat# 11095-080) supplemented with 10% FBS, 1X nonessential amino acids, 10 mM Hepes buffer (Cellgro® Mediatech Inc.; Cat# 25-060-CI) and 1X PSG; T47D cells were cultured in RPMI-1640 with 2mM L-glutatmine (Gibco® Invitrogen Corp.; Cat#11875-093) supplemented with 10 mM Hepes buffer, 1 mM Sodium Pyruvate, 4.5 g/L glucose, 1.5 g/L Sodium Bicarbonate and 10% FBS; and MCF-10A cells were cultured in DMEM/F12 (Gibco® Invitrogen Corp.; Cat# 11320-033) supplemented with 5% horse serum (Gibco® Invitrogen Corp.; Cat# 26050-088), 20 ng/μl of EGF (Sigma-Alderich; Cat# E9644), 0.5 mg/ml hydrocortisone (Sigma-Alderich; Cat# H0888), 100 ng/μl cholera toxin (Sigma-Alderich; Cat# C8052), 10 μg/ml insulin (Sigma-Alderich; Cat# I1882), and 1X PSG. The MDA-MB-435 cells were a gift from Janet E Price and cultured in MEM with Earl's salts plus L-glutamine supplemented with 5% FBS, 2x MEM vitamins (Cellgro® Mediatech Inc.; Cat# 25-020-CI), 10 mM Sodium Pyruvate (Cellgro® Mediatech Inc.; Cat# 25-000-CI), 1X MEM non-essential amino acids and 1X PSG.

B. Retroviral infection and generation of stable pools and cell lines

To generate retrovirus, two million Phi-NX cells (from ATTC) were plated in T25 filter-top the day before transfection. Cells were transfected using standard calcium-phosphate methods with 6 μg endotoxin free DNA of appropriate pLXSN vector, 12 hours post-transfection cells were washed with PBS and media was replaced. 24 hours post-transfection, cells were placed in 32°C incubator for viral production and 48 hours post-transfection viral supernatant media was harvested, passed through a 0.45 μm filter

and added 8 μg/ml polybrene. Media-containing virus was overlaid onto the target cells for infection. Approximately 48 hours post-infection, the cells infected with pLXSN generated virus were put under G418 selection at 1 mg/ml (MDA-MD-231) or 250 μg/ml (MCF-10A). After 10 days of selection, the cell pools were assayed for Ets1 protein levels.

MCF-10A Ets1 cell lines were established from pools generated using retrovirus infection as described above with the addition of the pBABE-puro vector. After infection as described above, target cells under went selection with 2 μg/ml of puromycin for 4 days. Cell lines were generated by serial dilution of cells from MCF-10A stable pools in 96-well plates and single cell lines were grown up and assayed for Ets1 expression.

IV. Transient Transfection and Dual Luciferase Reporter Assay

Luciferase activity of the αB-crystallin promoter reporter was assayed in MDA-MB-231 and MCF-10A cell lines growing in 24 well plates transiently transfected with pcDNA3-Ets-1 and the pGL3-αB-crystallin promoter reporter using the Dual Luciferase Reporter Assay System (Promega; Madison, WI; Cat# E1960). Ets1 and the appropriate reporter were transfected using Lipofectamine2000™ (Invitrogen Corp.; Carlsbad, CA; Cat# 11668-027) following manufacturer's protocol. For the MCF-10A experiments, 700 ng of pcDNA3 plasmid, 100 ng of pGL3 plasmid, 1 ng of pRL-TK plasmid, and 2 μl of Lipofectamine2000™ were used per well and 48 hours after transfection, each well was prepared for reporter activity according the manufacturer's recommended protocol. For

MDA-MB-231 experiments, 25 μM of siRNA, 300 ng of pGL3 reporter, 3 ng of pRL-TK plasmid, and 2 μl of Lipofectamine2000™ were used per well. 72 hours after transfection each well was prepared for reporter activity according to the manufacturer's recommended protocol. Briefly, cells in a 24 well plate were lysed in 100 μl of 1X passive lysis buffer for 20 minutes at room temperature. Lysate was removed and 20 μl was transferred to a 96-well Lumitrac 200 opaque plate. The activity of the pGL3 αB-crystallin promoter constructs was measured using the Clarity™ Luminescence Microplate Reader (BioTek Instruments Inc.; Winooski, VT) for 30 seconds per reporter. Results were expressed as fold induction compared to vector controls and normalized to firefly/renilla luminescence ratio, obtained by transfecting Ets1 vector with control vector in presence of appropriate reporter. Statistical significance was determined by one-way ANOVA using a P-value < 0.05 as significant.

V. Electrophoretic Mobility Shift Assay

To determine the *in vitro* interaction of Ets1 and human αB-crystallin EBS we performed an EMSA using the Pierce LightShift Chemiluminescent EMSA Kit (Pierce; Rockford, IL; Cat# 20148). The human αB-crystallin promoter region containing the putative EBS from -449 to -423 was used as a probe. (Each probe was 5'-biotinylated (Integrated DNA Technologies; Coralville, IA) and its corresponding antisense sequence were duplexed, then 4pmol of labeled probe was added to 10 μg of KNRK nuclear extract (SantaCruz Biotechnologies; Cat# sc-2141) for 20 minutes at RT under the following binding conditions: 1x binding buffer, 50 ng/μl poly dIdC, 5% glycerol, 0.1% NP-40, 50 mM NaCl2, 1 mM DTT, 1 mM EDTA, 1 mg/ml BSA. For the competition

experiments 4 pmol of 5'-biotinylated probe was incubated as before in the presence of 20 fmol of un-biotinylated probe. For antibody abrogation experiments, 5 μg of Ets1 antibody (SantaCruz Biotechnologies; Cat# sc-111) was incubated in the binding reaction for 20 minutes at RT before addition of probe, then allowed to incubate for an additional 20 minutes at RT. Protein-DNA complexes were resolved on a 5% native-PAGE gels using 0.5x Tris-Borate EDTA buffer at 100 Volts for approximately 3 hours at 4°C then transferred to a Biodyne B membrane (Pierce; Cat# 77016), cross-linked to nylon membrane, and biotin-labeled DNA was detected following manufacturer's chemiluninescence protocol.

Probe	Sequence		
WT	5'-TCAGCCTAGGAAGATTCCAGTCCCTGC-3'		
Mutant	5'-TCAGCCTAAAAAGATTTTAGTCCCTGC-3'		

VI. Chromatin Immunoprecipitation Analysis

Chromatin immunoprecipitation was performed using Upstate's EZ-ChIP kit according to the manufacturer's recommended protocol (Upstate/Millipore; Billerica, MA; Cat# 17-371). Briefly, approximately $2x10^7$ cells were fixed with 1% formaldehyde for 10 minutes at room temperature, crosslinked chromatin was sonicated using the Diagenode Bioruptor™ 200 for 30 minutes at full power with 30 second on/off cycles. Sheared chromatin was pre-cleared and incubated with 2 μg appropriate antibody at 4°C overnight (Ets1, SantaCruz Biotechnologies; Cat# sc350, or sc111; rabbit IgG, SantaCruz Biotechnologies; Cat# sc-2027). Immunoprecipitated complexes were washed in low salt, high salt, LiCl and TE at 4°C for 5 minutes each before eluting for 15 minutes at room temperature. Crosslinks were reversed for 5 hours at 65°C, then RNase treated for

30 minutes at 37°C, followed by Proteinase K treatment for 1-2 hours at 45°C and DNA was isolated using the QIAGEN QIAquick® PCR Purification Kit (QIAGEN; Cat# 28106) according to manufacturer's protocol. Isolated DNA was used to perform PCR reaction with the following primers for the αB-crystallin promoter: Forward 5'-AGATGGCTGGTGCTGACATGTTGA-3'; Reverse 5'-AATCAGGCCAGCAACTATC TTGGG-3'.

VII. Immunoblotting

Subconfluent cells were collected by trypsinization (Gibco®; Cat#25300-033), pelleted, and lysed in RIPA buffer supplemented with 1x protease inhibitor cocktail (Sigma-Aldrich; St.Louis, MO; Cat#P8340). Total protein concentration was determined by BCA Protein Assay Kit (Pierce; Rockford, IL; Cat# 23225), 20 μg of total protein was loaded on SDS-PAGE gels and transferred to PVDF membranes (Millipore; Burlington, MA; Cat# IPVH00010) using semi-dry transfer apparatus (BioRad Laboratories; Hercules, CA). Membranes were blocked with blocking buffer (5% nonfat milk in TBS with 0.1% Tween 20) overnight at 4°C, then incubated with primary antibody for 2 hours at RT, after washing with TBS/T the membranes were incubated for 1 hour at RT with appropriate secondary HRP-conjugated antibody. Proteins were visualized with the ECL Western Lightning Chemiluminescence kit (PerkinElmer; Boston, MA; Cat# NEL101). Primary antibodies were used at the following dilutions: αB-crystallin, 1:1000 (Stressgen Biotechnologies/Nventa Biopharmaceuticals Corp.; San Diego, CA; Cat# SPA-222); Ets1-1G11, 1:500 (Abcam® Inc.; Cambridge, MA; Cat# ab10936); MKBP, 1:500 (BD Biosciences; San Jose, CA; Cat# 611298) Actin, 1:1000 (Sigma-Alderich; Cat# 4700).

VIII. Cell Viability Assays

Cell viability was assayed using Promega's CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega; Madison, WI; Cat# G3580). Using a 96-well plate, 3,000 cells were plated per well in phenol-red free growth media. Approximately 5-6 hours after plating, media was refreshed with either full growth media or horse-serum and EGF-reduced conditions (1% horse serum, 5 ng/ul EGF) and 20 μl of MTS reagent to each well. The plate was incubated at 37°C in a humidified, 5% CO_2 incubator for 1 hour before the absorbance at 490 nm was recorded using Bio-Rad's Model 680 microplate reader (Bio-Rad Laboratories; Hercules, CA; Cat# 168-1000).

IX. Real-Time quantitative PCR

RT-qPCR experiments were performed using SuperArray's ReactionReady™ First Strand cDNA Synthesis Kit and RT^2 Real-Time™ SYBR Green/ROX qPCR Master Mix (SuperArray Bioscience Corporation; Frederick, MD; Cat# C-01 and PA-012). RNA was isolated from cell lines using Qiagen's RNeasy® Mini Kit (QIAGEN; Cat# 74103) following the manufacturer's protocol. For each First Strand cDNA Synthesis sample, 2 μg of RNA was added to the RT cocktail and the reaction was performed following manufacturer's protocol. For each RT^2 Real-Time™ PCR sample, 1 μl of undiluted cDNA template was used and the reaction was setup according to the manufacturer's protocol using the following SuperArray primer sets (RT^2 PCR Primer

Set for Human Ets1, Cat# PPH01781, Position 514-534; RT^2 PCR Primer Set for Human MMP2, Cat# PPH00151A, Position 2193-2215; RT^2 PCR Primer Set for Human, MMP9 Cat# PPH00152B, Position 2093-2111) on the ABI 7900HT Fast Real-Time PCR System (Applied Biosystems; Foster City, CA)

X. Small Interfering RNA

Dharmacon's ON-TARGETplus SMARTpool (Dharmacon; Cat# L-003887-00-0005; Lafayette, CO) for human ETS1 (Accession #NM_005238) was used to knockdown Ets1 in various assays. siGenome ON-TARGETplus SMARTpool duplex sequences:

Sense1; AUAGAGAGCUACGAUAGUUUU
Anti-sense1; P-AACUAUCGUAGCUCUCUAUUU
Sense2; GAAUGAUGUCUCAAGCAUUU
Antisense2; P-AUGCUGAGACAUCAUUUCUU
Sense3; GUGAAACCAUAUCAAGUUAUU
Anti-sense3; P-UAACUUGAUAUGGUUUCACUU
Sense4; CAGAAUGACUACUUUGCUAUU
Anti-sense4; P-UAGCAAAGUAGUCAUUCUGUU

siRNA was introduced using Oligofectamine™ Reagent (Invitrogen; Carlsbad, CA; Cat# 12252-011) in OPTI-MEM® I Reduced Serum Medium (Invitrogen; Cat# 31985-062) following the manufacturer's protocol with a 25 μM final concentration of siRNA. All transfections were performed in the evening and cells were washed with 1X PBS and replaced with fresh media approximately 16-18 hours later.

XI. Wound Healing Assay

Cells transfected with siRNA in a 6-well plate were allowed to grow to confluence, a scratch was introduced using a P200 tip, an image was taken at three locations along the wound, cells were grown for 18 hours, and another three images were acquired along the wound at the locations of the original images.

XII. Transwell Migration and Invasion Assay

The day before setting up migration and invasion experiments, cells near 50% confluency were serum-starved 12-18 hours. For migration assays, approximately 50,000 cells were plated per chamber, BD Falcon™ Cell Culture Inserts (BD Biosciences; Bedford, MA; Cat# 353097), in a 24 well plate with normal growth media used as attractant. Cells were allowed to migrate for six hours after which they were fixed in 0.5% crystal violet, 40% ethanol and 60% PBS. Four images were taken for each well at 100X and the total number of cells counted. For invasion assays, approximately 30,000 cells were plated per chamber BD BioCoat™ Growth Factor Reduced Matrigel™ Invasion Chamber (BD Biosciences; Cat# 354483) and allowed to invade for 24 hours before being stained, fixed and quantified as done in migration assays. For migration and invasion assays involving siRNA to Ets1 and controls, cells transfected with siRNA were serum starved 24 hours following transfection and plated out as above.

XIII. Soft Agar Assay

Anchorage-independent growth assays were performed in 6-well plates using a 2 mL 0.6% base layer of noble agar in full growth media and 30,000 cells in a 0.3%

suspension of Noble agar (Marine BioProducts Inc.; Delta, British Columbia, Canada; Cat# M-02) in full media. Approximately 500 μl of growth media was replaced per well every three days and images were taken after 5 weeks.

XIV. Immunohistochemistry of Breast Tumor Tissue Microarray

The breast tissue microarrays were purchased from Biomax (US Biomax; Rockville, MD; Cat# BRC961, BRC962;) and blotted for Ets1 and αB-crystallin as follows. The 4 μm sections of breast tumor tissue embedded in paraffin were baked at 60°C for 2 hours and deparaffinized in xylene and rehydrated in 100% ethanol, 95% ethanol, and 75% ethanol. Antigen retrieval was performed in retrieval buffer (DAKO; Carpinteria, CA; Cat#S1700) at 98°C for 40 minutes for Ets1 or by microwaving for 2 minutes at full power and then 18 minutes at 30% power for αB-crystallin. Following 30 minutes of blocking (DAKO; Cat#X0909;), tissue microarrays were stained in Ets1 monoclonal antibody 1:10 (Abcam), Ets1 polyclonal antibody 1:800 (SantaCruz, sc350) or αB-crystallin antibody 1:200 (Stressgen) (diluted in DAKO; Cat#S3022) for 1 hour at room temperature for Ets1 or for 1 hour at 37°C for αB-crystallin. Slides were washed in TBST, blotted with mouse secondary antibody for 30 minutes at room temperature, incubated with DAB for 5 minutes at room temperature (DAKO Cytomation EnVision+® System-HRP (DAB); Cat#K407), counterstained with hematoxylin and dipped 10 times in bluing (Richard-Allen Scientific; Cat#7301). Finally, slides were coverslipped with VectaMount™ (Vector Laboratories Inc.; Burlingame, CA; Cat#H-5000).

I. Regulation of the small heat shock protein αB-crystallin by Ets1

A. Introduction

Using the mouse αB-crystallin promoter, transcriptional regulation of αB-crystallin has been studied extensively in lens and muscle specific systems, and transcription factors have been identified for regulation in these cell types. Further regulation has been described following stress and during development in various systems, however the transcriptional regulation of αB-crystallin in cancer has not been studied. Here we used a bioinformatics approach, along with promoter reporter, electrophoretic gel shift, chromatin immunoprecipitation, and western blot analyses to demonstrate Ets1 regulation of human αB-crystallin gene expression in breast cancer cells.

B. Bioinformatics of the human CRYAB/HSPB2 shared promoter

To begin, we searched for novel putative regulatory sites that could explain the expression of αB-crystallin described in multiple cancers, including breast cancer. αB-crystallin is commonly expressed in basal-like breast cancer where its expression predicts poor survival and resistance to neo-adjuvant chemotherapy in breast cancer (Ivanov et al., 2007; Moyano et al., 2006; Perou et al., 2000; Sitterding et al., 2008). Using the web-based software MOTIF-search to analyze the CRYAB/HSBP2 shared promoter, we identified many previously discovered binding sites as well as a putative Ets1 binding site

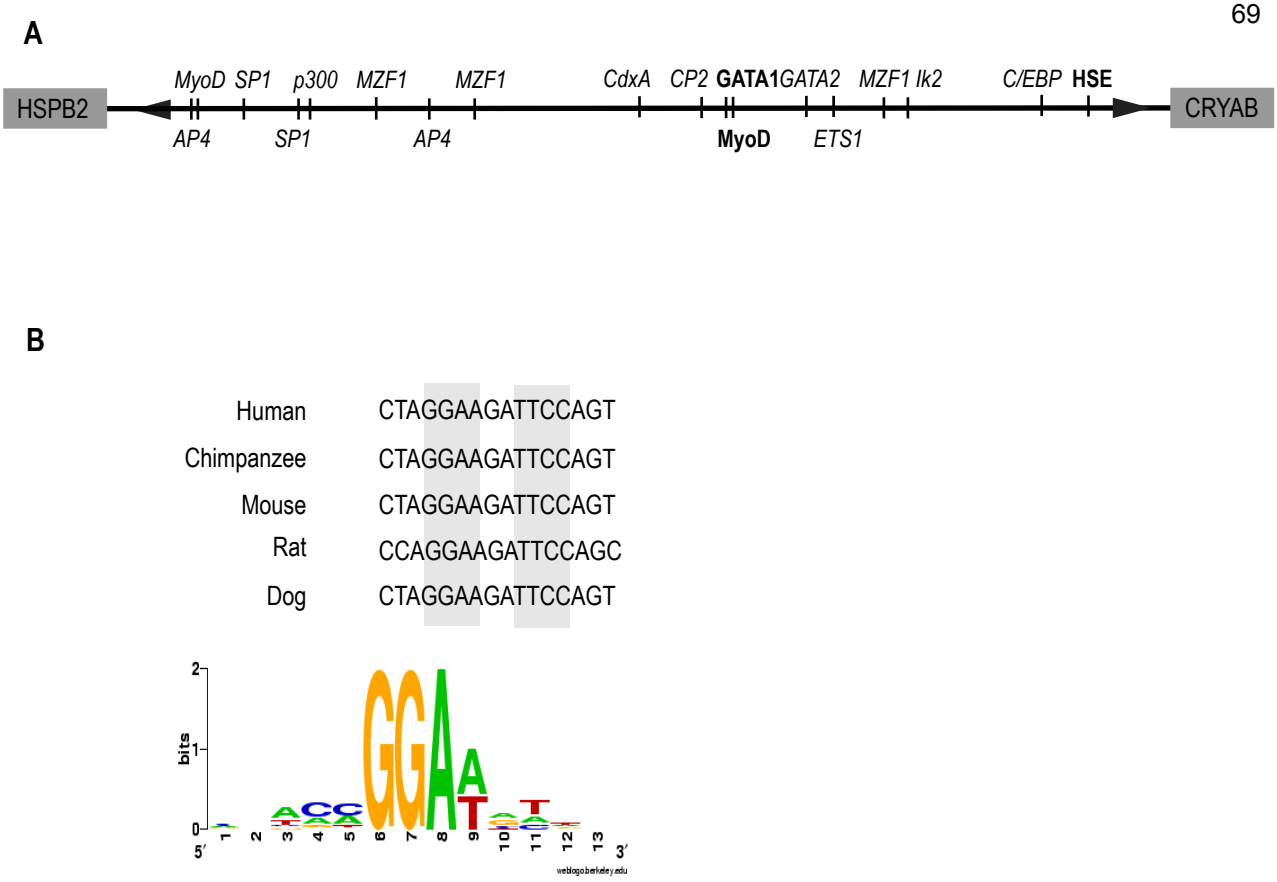


Figure 3.1 Schematic representation of shared αB-crystallin/HSPB2 gene promoter and conservation of putative Ets1 binding site.

(A) Using the MOTIF-search web-based software (http://motif.genome.jp), we performed a bioinformatic analysis of the human αB-crystallin/HSPB2 shared promoter spanning the region between the start ATG for each gene. The search identified published (**bold**) transcriptional regulators of αB-crystallin, as well as additional putative (*italics*) regulators, including the head-to head Ets binding sites.
(B) Alignment of the putative Ets1 binding site from the human αB-crystallin promoter with the corresponding region from other species reveals its conservation. A graphic representing the TRANSFAC matrix used to identify the putative Ets1 binding site (http://weblogo.berkely.edu) demonstrates how well the putative site matches known Ets1 binding sites.

that is evolutionarily conserved (Figure 3.1). Ets1 is the founding member of the ETS family of transcription factors known to play roles in development, angiogenesis, and invasion (Dittmer, 2003). Furthermore in a study of the molecular characteristics of breast cancer cell lines, Ets1 was recently described as a gene that clusters with the basal-like breast cancer cell lines (Charafe-Jauffret et al., 2006). In order to determine whether Ets1 is a *bona fide* regulator of the αB-crystallin gene in breast cancer we cloned the human αB-crystallin promoter from the basal-like human mammary epithelial cell line MCF-10A into a luciferase based reporter to be used for further analysis.

C. Ets1 regulates transcriptional activity of the human αB-crystallin promoter

To determine if the putative ETS binding site of the αB-crystallin promoter is required for activation of αB-crystallin gene transcription, we performed co-transfection experiments with Ets1 in the human mammary epithelial cell line MCF-10A. We chose the MCF-10A cell line because it was classified as a basal-like breast cancer cell type (Charafe-Jauffret et al., 2006) and it has low endogenous Ets1 levels and inducible αB-crystallin therefore making it an ideal cell line to look for alterations of the human αB-crystallin reporter caused by changes in Ets1 levels. Ets1 and the full-length human αB-crystallin promoter, with and without the putative EBS mutated to a known non-Ets1 binding sequence were transfected in to the cells and assayed for luciferase activity. Upon co-transfection of Ets1 with the full-length wildtype αB-crystallin promoter reporter we observed a 2.5 fold activation of the reporter compared to the vector control (Figure 3.2). A slight, non-significant increase in activity (data not shown) was observed

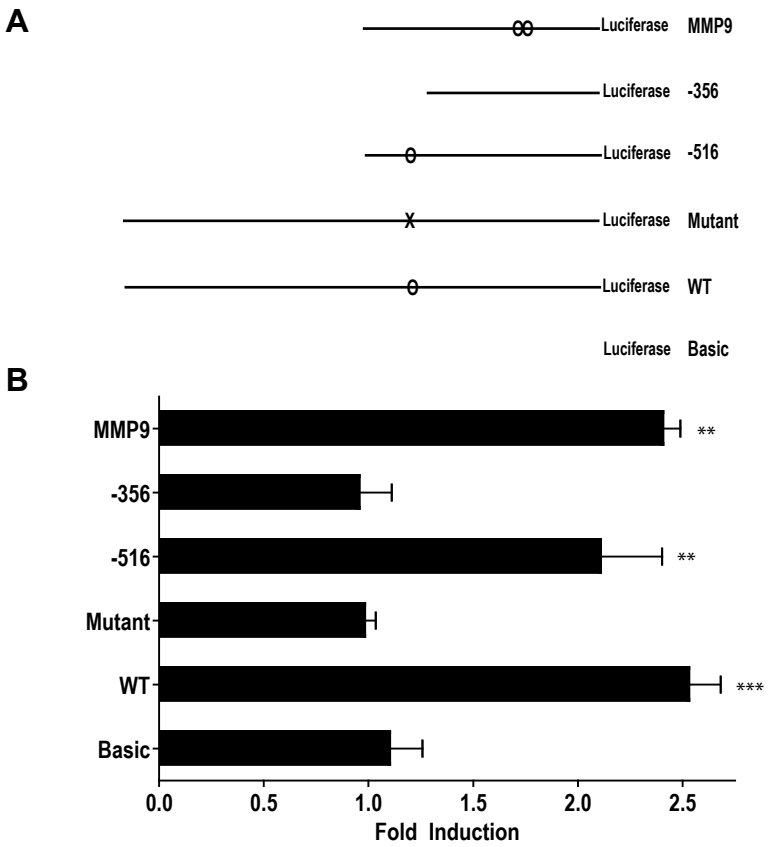


Figure 3.2 Transcriptional activation of the human αB-crystallin promoter by Ets1.

Introducing Ets1 into the MCF-10A mammary epithelial cell line activates the αB-crystallin promoter. (A) Schematic of the human αB-crystallin gene promoter luciferase reporter constructs used. The human αB-crystallin gene promoter and 5' truncations (-516, -356) were cloned from MCF-10A genomic DNA into the pGL3-Basic luciferase plasmid using KOD Hot Start DNA polymerase according to manufacturer's protocol. Site-directed mutagenesis was used according to manufacturer's protocol to generate Ets binding site mutations in the reporter with the following primers: sense, 5'-CCTGGGGCTCAGCCTA AAAAGATTTTAGTCCC-3'; antisense,5'-GGGACTAAAATGTTTTTAGGCTGAGCCCCA GG-3'. All constructs were checked for correctness by sequencing both strands of each reporter. **O** indicates approximate location of wild-type Ets binding site; **X** indicates approximate location of mutated Ets binding site. (B) MCF-10A cells were transiently transfected with 700ug of pcDNA-Ets1 or pcDNA-Empty Vector,100ug of appropriate pGL3 reporter, and 1ng of control renilla reporter using Lipofectamine2000TM. Lysates were assayed for luciferase activity 48 hours later using Promega's Dual Luciferase Reporter Assay system according to manufacturer's instructions on Bio-Tek's Clarity TM Luminescence Microplate Reader. Luciferase activty was normalized to renilla to account for transfection efficiency and activity levels were compared to empty vector. **p<0.01 ***p<0.001

upon co-transfection of Ets1 and the full-length wildtype reporter into the MDA-MB-231 cell line, a cell line with high endogenous Ets1 levels. The degree of Ets1 dependent activation of the wildtype αB-crystallin promoter observed in the MCF-10A cell line was similar to that observed for the MMP9 reporter, a positive control for Ets1 transactivation. However, when the putative EBS was mutated there was no significant Ets1 induced-activation (Figure 3.2). A similar reporter activation pattern was observed in the same system with co-transfection of Ets1 and one of two 5' truncations of the αB-crystallin promoter reporter. The -516 truncation contained the putative Ets1 binding site and the -356 truncation did not. Luciferase activity increased approximately 2.5 fold after cotransfection of Ets1 and the -516 reporter containing the EBS, while no increase in luciferase activity was observed after cotransfection of Ets1 with the -356 reporter (Figure 3.2). Furthermore, expression of another ETS family member, ESX/ESE1 did not result in significant activation of the full length wildtype human αB-crystallin promoter (data not shown).

We also wished to study whether silencing Ets1 altered the activity of the αB-crystallin promoter in another basal-like breast cancer cell line, MDA-MB-231 (Charafe-Jauffret et al., 2006). The MDA-MB-231 cell line has relatively high endogenous levels of both Ets1 and αB-crystallin making it a good cell line to study the consequences of silencing endogenous Ets1 on the human αB-crystallin promoter reporter activity. In the presence of 25 μM Ets1 siRNA, we detected a 5.5 fold decrease in Ets1 levels and observed a 39% reduction in luciferase activity in the αB-crystallin WT promoter reporter and a 41% reduction in the -516 truncation promoter reporter when compared to

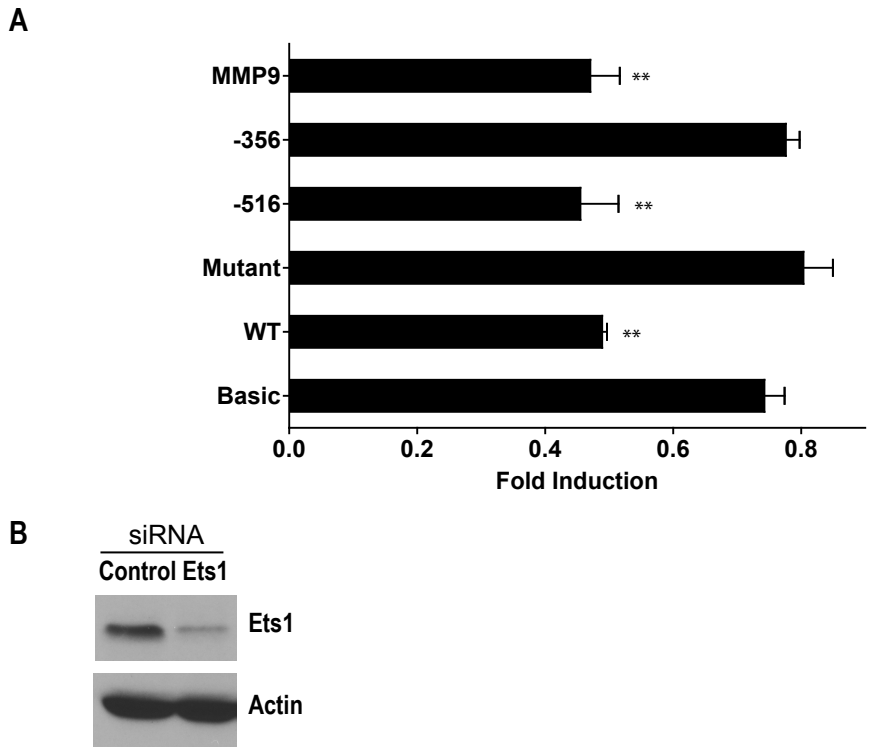


Figure 3.3 Transcriptional regulation of the human αB-crystallin promoter by Ets1.

Silencing of Ets1 decreases activity of human αB-crystallin promoter.
(A) MDA-MB-231 cells were transiently transfected with 300ng of appopriate pGL3 reporter (reporters described in Figure 3.2), 3ng of renilla control reporter and 25uM of siRNA directed to Ets1 using Lipofectamine 2000TM following manufacturer's protocol. Lysates were assayed for luciferase activity 72 hours later using Promega's Dual Luciferase Reporter Assay system according to manufacturer's instructions on Bio-Tek's Clarity TM Luminescence Microplate Reader. Luciferase activty was normalized to renilla to account for transfection efficiency and activity levels were compared to siRNA control. **p<0.01
(B) Western blot of MDA-MB-231 lysates showing silencing of Ets1 72 hours after transient transfection with 25uM siRNA directed to Ets1, 300ng of pGL3 vector, and 3ng of renilla control plasmid using Lipofectamine 2000TM following manufacturer's protocol.

the reporters without a functional ETS binding site (Figure 3.3). We also observed a 40% reduction in MMP9 promoter activity in the presence of 25 μM Ets1 siRNA. Taken together, these results demonstrate that Ets1 expression levels correlate with the activity of the human αB-crystallin gene promoter in two basal-like breast cell lines and that this regulation requires the putative EBS.

D. *In vitro* and *in vivo* binding of Ets1 to the αB-crystallin promoter

After determining that the putative ETS binding site is required for Ets1 induced activation of the human αB-crystallin promoter in basal-like breast cell lines, we studied the *in vitro* requirement of the EBS for Ets1 binding. Electrophoretic gel shift analysis using a probe containing the EBS from the human αB-crystallin promoter showed a shift when incubated in the presence of an Ets1 containing nuclear extract (Figure 3.4). Furthermore, competition experiments with excess unlabeled probe prevented the shift. We also did not observe a shift of a labeled probe with a mutated version of the putative EBS site within a labeled probe. Finally, preincubation of an Ets1 specific antibody in the binding reaction abrogated the shift, indicating that Ets1 is required for the observed shift (Figure 3.4). These results together demonstrate that the wildtype EBS and Ets1 are required to observe a shift of the wildtype EBS containing labeled probe.

In order to determine if Ets1 binds to the putative EBS in the endogenous αB-crystallin promoter, we performed chromatin immunoprecipitation (ChIP) analysis in the MDA-MB-231 cell line. We chose this cell line because, as stated previously, it is a basal-like breast cancer cell line that has high levels of both Ets1 and αB-crystallin.

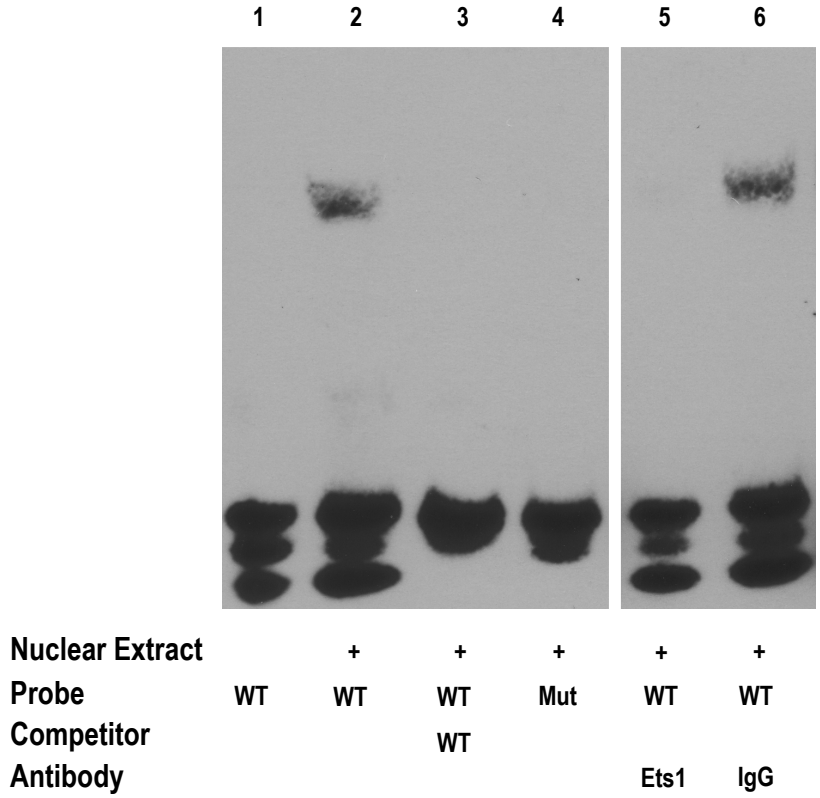


Figure 3.4 Electrophoretic mobility shift assay for Ets1 binding to the putative Ets binding site in the human αB-crystallin promoter.

To determine the *in vitro* interaction of Ets1 and human αB-crystallin EBS we performed an EMSA using the Pierce LightShift Chemiluminescent EMSA Kit (Pierce; Rockford, IL). The human wildtype (WT) αB-crystallin promoter region containing the putative EBS from 5'-TCAGCCTAGGAAGATTCCAGTCCCTGC-3' and its corresponding antisense were duplexed and used as a probe. 4pmol of WT (lane 2) or mutated (lane 4) biotinylated probe 5'-TCAGCCTAAAAAGATTTTAGTCCCTGC-3' was added to 10 µg of nuclear extract for 20 minutes at RT. For the competition experiments (lane 3), 4 pmol of WT biotinylated probe was incubated as before, but in the presence of 20 fmol of un-biotinylated WT probe. For antibody abrogation experiments (lane 5), 5 µg of Ets1 antibody (SantaCruz Biotechnologies; sc-111) was incubated in the binding reaction for 20 minutes at RT before addition of WT probe, then allowed to incubate for an additional 20 minutes at RT. Protein-DNA complexes were resolved on a 5% native-PAGE gels using 0.5x Tris-Borate EDTA buffer at 100 Volts for approximately 3 hours at 4°C then transferred to a Biodyne B membrane, UV cross-linked to the nylon membrane, and biotin-labeled DNA was detected following manufacturer's chemiluninescence protocol.

Approximately $2x10^7$ cells growing under normal growth conditions were formaldehyde-fixed to crosslink DNA to any associated proteins. The crosslinked chromatin was immunoprecipitated with antibodies specific to Ets1 or IgG as a negative control. Using DNA purified from immunoprecipitated complexes as template, PCR was performed using primers specific to the region of the αB-crystallin promoter containing the EBS. The PCR analysis showed a single product for the Ets1 antibody that was 4-fold greater than the product for the IgG antibody control (Figure 3.5). These results indicate that Ets1 binds the ETS binding site with the human αB-crystallin promoter *in vitro* and *in vivo*.

E. Ets1 regulates endogenous αB-crystallin expression in MDA-MB-231 cells

To determine whether Ets1 regulates endogenous αB-crystallin protein levels, we generated stable Ets1 pools in the breast cancer cell line MDA-MB-231 by retroviral infection. Stable Ets1 overexpression resulted in an approximate 2-fold increase in Ets1 protein levels and a 3-fold increase in αB-crystallin protein levels in both pools generated, although only one pool is shown (Figure 3.6). Furthermore, we wished to determine if the opposite was true we assayed whether decreasing endogenous Ets1 results in a decrease in endogenous αB-crystallin levels. Indeed, transfection of 25 μM siRNA specific to Ets1 in the MDA-MB-231 cell line decreased both Ets1 and endogenous αB-crystallin levels (Figure 3.6). Ets1 protein levels decreased 3-fold and αB-crystallin protein levels decreased 8.6-fold compared to non-silencing control siRNA in the parental MDA-MB-231 cell line. Importantly, in both the Ets1 overexpression and Ets1 silencing systems, we did not detect changes in HSPB2 protein levels (Figure 3.6),

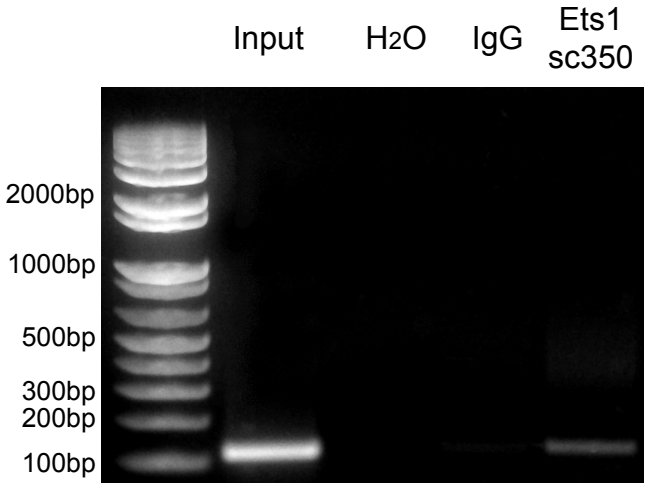


Figure 3.5 Chromatin immunoprecipitation analysis of Ets1 binding to the human αB-crystallin promoter.

Chromatin immunoprecipitation was performed using Upstate's EZ-ChIP kit according to the manufacturer's recommended protocol (Upstate/Millipore; Billerica, MA). Briefly, approximately 2x10^7 cells were fixed with 1% formaldehyde for 10 minutes at RT, crosslinked chromatin was sonicated using the Diagenode Bioruptor 200 for 30 minutes at full power with 30 second on/off cycles. Sheared chromatin was pre-cleared and incubated with 2 µg appropriate antibody at 4°C overnight (Ets1 and Rabbit IgG; SantaCruz Biotechnologies; sc350 andsc-2027). Immunoprecipitated complexes were washed in low salt, high salt, LiCl and TE at 4°C for 5 minutes each before eluting for 15 minutes at RT. Crosslinks were reversed for 5 hours at 65°C, then RNase treated for 30 minutes at 37°C, followed by Proteinase K treatment for 1-2 hours at 45°C and DNA was isolated using the QIAGEN QIAquick PCR Purification Kit according to manufacturer's protocol. Isolated DNA was used to perform PCR reaction with the following primers for the αB-crystallin promoter: Forward 5'-AGATGGCTGGTGCTGACATGTTGA-3'; Reverse 5'-AATCAGGCCAGCAACTATC TTGGG-3'. PCR proucts were resolved on a 2% agarose gel stained with ethidium bromide.

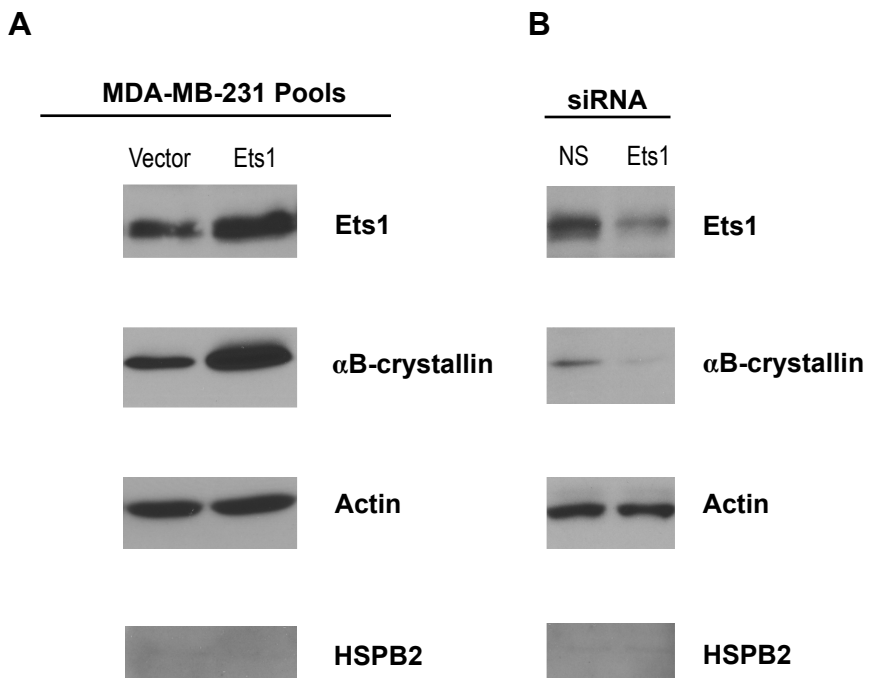


Figure 3.6 Regulation of endogenous αB-crystallin protein levels by Ets1.

(A) We generated retrovirally infected stable pools expressing pBABE-Ets1 or empty pBABE vector in the breast cancer MDA-MB-231cell line. For western blot analysis, subconfluent cells were collected by trypsinization, pelleted, and lysed in RIPA buffer supplemented with 1x protease inhibitor cocktail. Total protein concentration was determined by BCA Protein Assay Kit, 20 µg of total protein was loaded on SDS-PAGE gels and transferred to PVDF membranes using semi-dry transfer. Membranes were blocked with blocking buffer (5% nonfat milk in TBS with 0.1% Tween 20) overnight at 4°C, then incubated with primary antibody for 2 hours at RT, after washing with TBS/T the membranes were incubated for 1 hour at RT with appropriate secondary HRP-conjugated antibody. Proteins were visualized with Pierce's ECL Western Chemiluminescence kit. Primary antibodies were used at the following dilutions: αB-crystallin, 1:1000 (Stressgen Biotechnologies; SPA-222); Ets1, 1:500 (Abcam.; ab10936); HSPB2, 1:500 (BD Biosciences; 611298) Actin, 1:1000 (Sigma-Alderich; # 4700)
(B) For silencing experiments, we transiently transfected parental MDA-MB-231 cells with 25uM of Dharmacon's SMARTpool for Ets1 or a Non-specific (NS) sequencing targeting luciferase using OligofectamineTM according to manufacturer's protocol. Approximately 72 hours following transfection, lysates were collected, processed, and assayed by western blot as described above. Actin was probed as a loading control for both experiments.

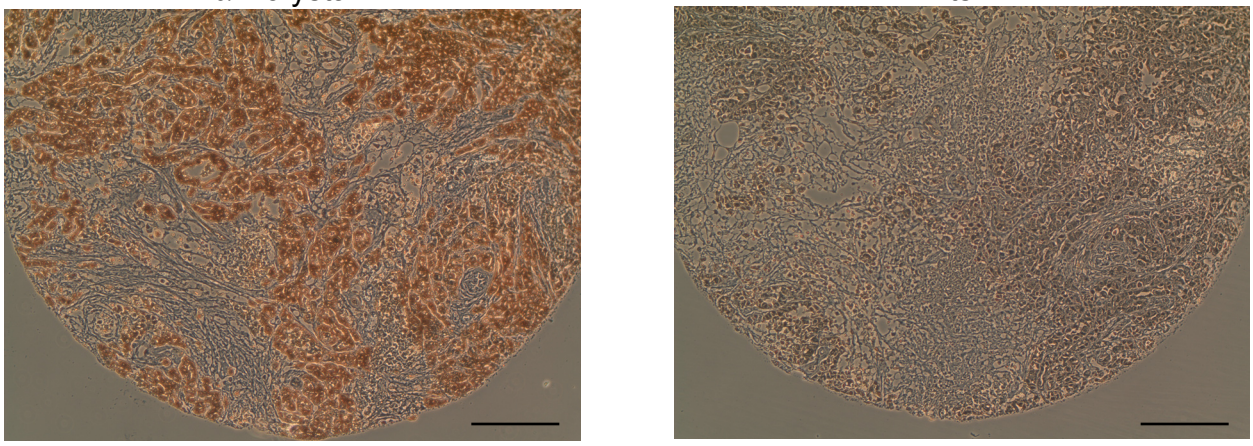
suggesting Ets1 specificity towards αB-crystallin gene expression. Together, these results demonstrate that Ets1 is an endogenous regulator of the human αB-crystallin gene.

F. Immunohistochemistry of Ets1 and αB-crystallin in breast tissue microarray

To test whether the regulatory mechanisms we observed in our *in vitro* and *in vivo* experiments might have relevance in human breast tumors, we obtained breast tissue microarrays and assayed them for both αB-crystallin and Ets1 protein levels by immunohistochemistry. We obtained the tissue microarrays from US Biomax (Rockville, MD). The pathology diagnosis, grade, and type; as well as the immunohistochemistry results for androgen receptor, estrogen receptor, progesterone receptor, and HER2 receptor are known for all the cases. This information allowed us to determine which tumors were triple-negative breast carcinomas (TNBC) and therefore basal-like cases; allowing us to determine if there was correlation between Ets1 and αB-crystallin in basal-like breast tumors.

In previous studies, we observed αB-crystallin staining in 11% of breast tumors and 45% of the basal-like tumors (Moyano et al., 2006), and more recently we observed cytoplasmic αB-crystallin in 81% of basal-like tumors (Sitterding et al., 2008). The results of this study support our previous observations (Moyano et al., 2006), as we observed αB-crystallin staining in 7 (10%) of the tumors and 27% of the triple-negative tumors (Figure 3.7). Of the 7 tumors staining for αB-crystallin, all 7 also had

A



B

Table Summarizing Breast Tissue Microarray

Tissue Type	# of Tissues	% of Tissues	# of TNBC	% of Type
Normal	8	8.3	0	0
Benign	16	16.6	0	0
Malignant	72	75	11	15

Summary of IHC of 72 Malignant Tumors

Antibody	# of Tissues	%	# of TNBC	% of TNBC
αB-Crystallin	7	9.7	3	27
Ets1 (1G11)	1	1.4	0	0
Ets1 (sc-350)	cyto - 69	95.8	cyto - 10	90.9
	nuc - 41	56.9	nuc - 8	72.7

Figure 3.7 Immunohistochemistry of breast tissue microarray.
Staining for Ets1 and αB-crystallin on TMAs with a total of 96 breast tissue cases. (A) Breast tissue microarrays were purchased from US Biomax (Rockville, MD; BRC961 and BRC962) and blotted for Ets1 and αB-crystallin as follows. The 4 μm sections of paraffin embeded tissue were baked at 60°C for 2 hours and deparaffinized in xylene, then rehydrated with graded ethanol. Antigen retrieval was performed in retrieval buffer (DAKO) at 98°C for 40 minutes for Ets1 or by microwaving for 2 minutes at full power and then 18 minutes at 30% power for αB-crystallin. Following 30 minutes of blocking, tissue microarrays were stained in Ets1 polyclonal antibody 1:800 (Santa Cruz; sc-350), or αB-crystallin antibody 1:200 (Stressgen) for 1 hour at RT. Slides were washed in TBST, blotted with secondary antibody for 30 minutes at RT, incubated with DAB for 5 minutes at RT (DAKO Cytomation EnVision+ System-HRP), counterstained with hematoxylin and bluing (Richard-Allen Scientific). Representative images of a tumor staining positive for both αB-crystallin and Ets1 is shown. Line represents 200 μm.
(B) Table summarizing staining from breast tissue microarray, also includes staining for Ets1 with monoclonal antibody 1:10 (Abcam). TNBC, triple-negative breast carcinomas.

cytoplasmic Ets1 staining and 4 (57%) of them stained for nuclear Ets1 with the polyclonal Ets1 antibody. Importantly, staining with the monoclonal Ets1 antibody revealed staining in only one (1.5%) breast tumor within the microarray; this tumor also had the strongest αB-crystallin staining.

A previous study looking at Ets1 staining in breast tissue microarrays found staining in 83% of 78 tumors with no distinction between cytoplasmic or nuclear staining (Buggy et al., 2004). Another study of 149 tumors found cytoplasmic staining in 78% of the tumors and nuclear staining in 46% of the tumors (Mylona et al., 2006). Both of these previous studies used a polyclonal antibody specific to the C-terminus of human Ets1 (Santa Cruz (C-20); sc350). Initial observations in our lab with the Santa Cruz polyclonal antibody suggested a non-specific nature in its binding to antigens; therefore, we stained our tissue microarray with a monoclonal antibody (Abcam, IG11) as well as the Santa Cruz polyclonal antibody. Using the polyclonal Ets1 antibody, 69 (96%) of the 72 tumors stained for cytoplasmic Ets1 and 41 (57%) stained for nuclear Ets1 (Figure 3.7). Looking at Ets1 staining in the TNBCs, ten (91%) of the 11 triple-negative tumors had strong cytoplasmic staining, and 8 (73%) had nuclear staining. The high levels of cytoplasmic staining with the polyclonal antibody suggests non-specific binding of the antibody and therefore we cannot make any significant conclusions from the Ets1 staining data; however, the staining with the monoclonal Ets1 antibody suggests a potential relationship of αB-crytstallin and Ets1 levels in breast tumors.

A. Introduction

Ets1 is a proto-oncogene originally named for its homology to the erythroblastosis virus E26 transformation specific sequence. It is expressed in a variety of cell types and is known to have a significant role in hematopoietic differentiation (Sementchenko and Watson, 2000). In other cell types, such as endothelial cells and epithelial cancer cells, Ets1 encourages an invasive phenotype (Behrens et al., 2001a; Hahne et al., 2005; Rothhammer et al., 2004). Some of the Ets1 target genes thought to be important for a more invasive phenotype are MMP1, MMP2, MMP3, MMP9, uPA and VEGF (Baillat et al., 2006; Behrens et al., 2001a; Behrens et al., 2001b; Delannoy-Courdent et al., 1998; Kitange et al., 1999; Rothhammer et al., 2004). Here we worked to determine the role Ets1 plays in breast cancer cell migration, invasion and transformation. Using siRNA technology, transwell invasion and migration assays, as well as proliferation and anchorage-independent growth assays in various breast cancer cell lines, we determined that Ets1 has a role in transwell invasion, but its exact relationship with migration, invasion, and transformation was unclear and appears to be context dependent.

B. Ets1 protein levels in breast cancer cell lines

To determine Ets1 levels in various breast cancer cell lines (T47D, MCF-7, MDA-MB-468, MDA-MB-231, MCF-10A, BT-20, and MDA-MB-435) lysates were collected and probed for Ets1 by western blot. Figure 3.8 shows that MDA-MB-231 and MDA-MB-435 expressed high levels of Ets1, while MCF-10A had relatively lower levels

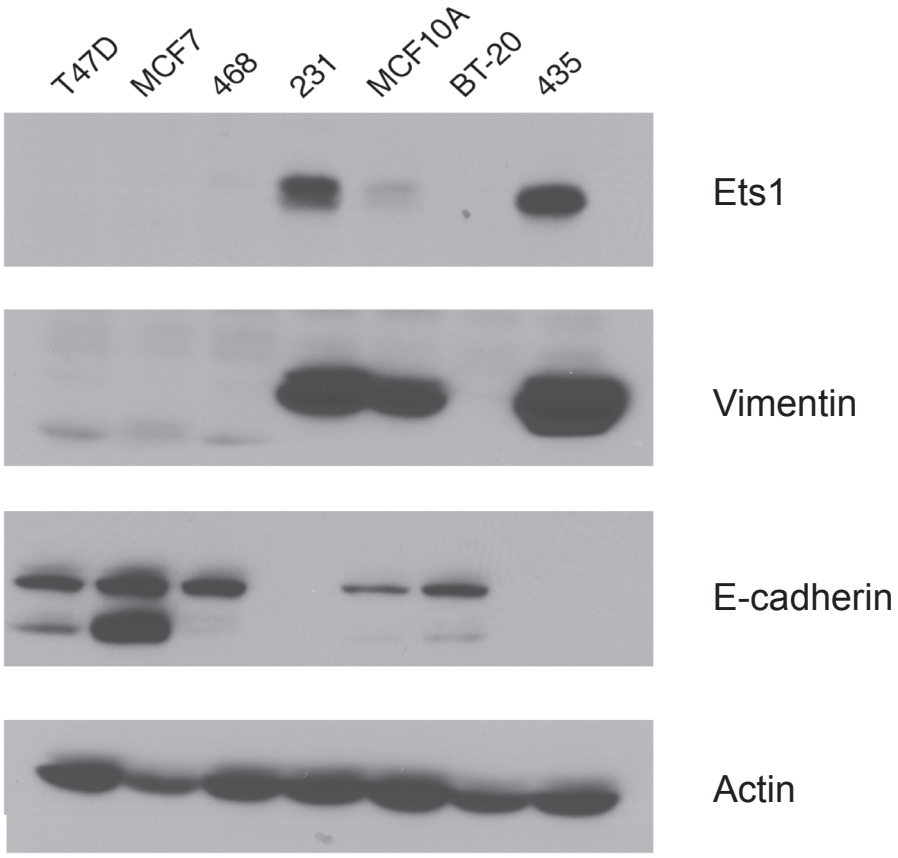


Figure 3.8. Western blot analysis of Ets1 expresssion in various breast cancer cell lines.

For western blot analysis, subconfluent cells were collected by trypsinization, pelleted, and lysed in RIPA buffer supplemented with 1x protease inhibitor cocktail. 20 µg of total protein was loaded on SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked overnight at 4°C, then incubated with primary antibody for 2 hours at RT, washed with TBST, and incubated for 1 hour at RT with secondary antibody. Primary antibodies were used at the following dilutions: Ets1, 1:500; Vimentin, 1:1000; E-cadherin, 1:1000; Actin, 1:1000.
468, MDA-MB-468; 231, MDA-MB-231; 435, MDA-MB-435

and the remaining cell lines had levels that were undetectable by western blotting. Furthermore we checked for increased expression of vimentin and a decreased expression of E-cadherin to provide additional evidence of a more mesenchymal phenotype, which is more migratory and invasive. Indeed, it is known that MDA-MB-231 and MDA-MB-435 cell lines are highly tumorigenic in nude mice (Price et al., 1990), and as these cell lines have the highest levels of Ets1, we hypothesized that Ets1 may have a role in the migratory and invasive phenotypes of these cell lines.

C. Migratory and invasive potential of Ets1 expressing breast cancer cell lines

Before we investigated the role of Ets1 in migration and invasion in breast cancer cell lines, we determined the migratory and invasive phenotypes *in vitro* of the MDA-MB-435, MDA-MB-231, and MCF-10A cell lines to see if Ets1 levels correlated with the migratory and invasive potential in transwell assays. As shown in Figure 3.9, the strongly Ets1 positive cell lines MDA-MB-435 and MDA-MB-231 are 31x and 60x more migratory than the mammary epithelial cell line MCF-10A. Using a transwell migration chamber coated with Matrigel, we assayed the cell lines for their invasive potential. Again, the MDA-MB-435 and MDA-MB-231 cell lines were 6x and 12x more invasive (Figure 3.9) than the MCF-10A cell line. Our results indicate that the strongly Ets1 positive cell lines have both higher migratory and invasive potential than lower Ets1 expressing MCF-10A cell line.

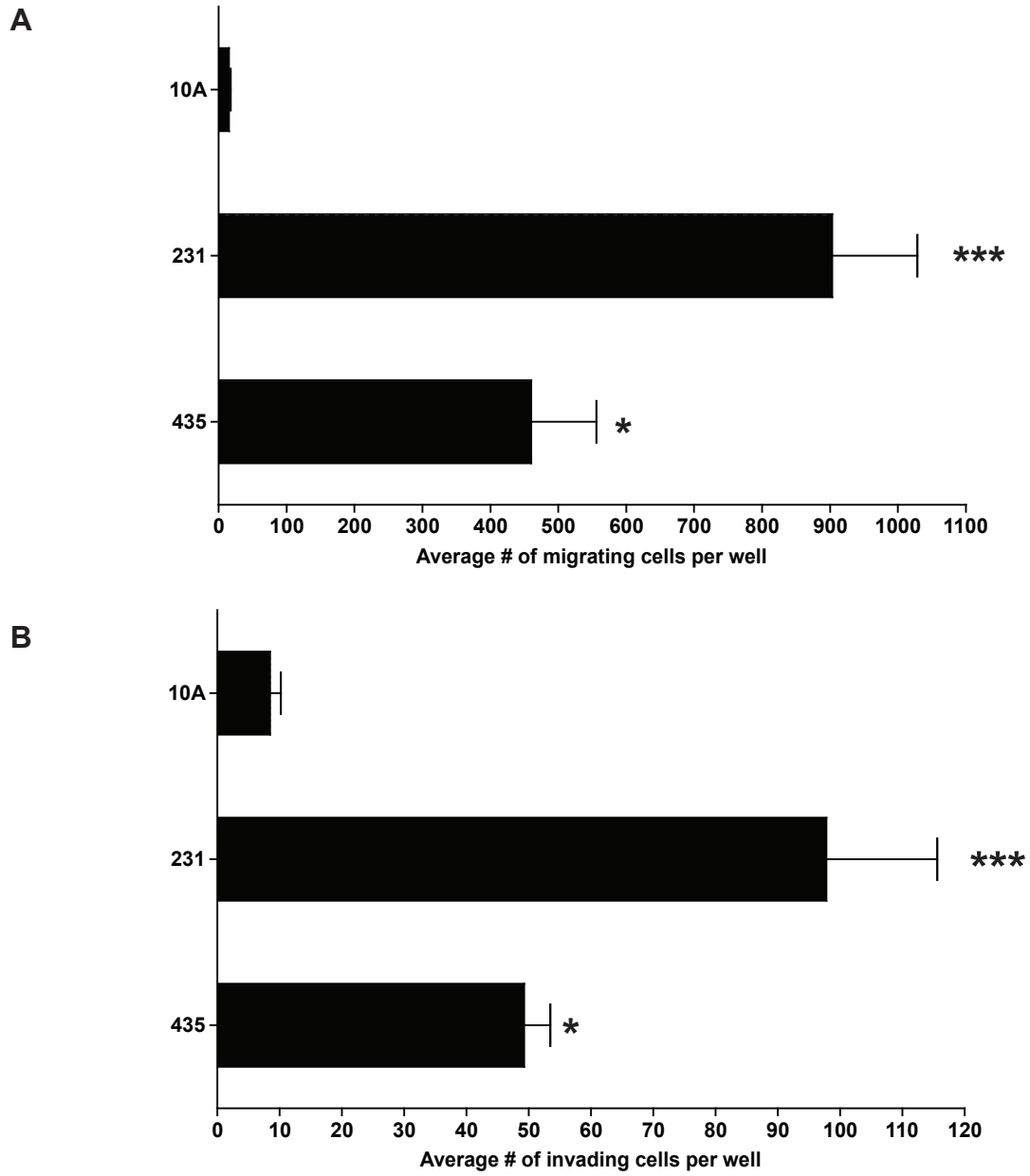


Figure 3.9 Transwell migration and invasion of Ets1 expressing breast cell lines.

(A) For migration assays, 50,000 cells were plated in the migration chamber in duplicate in normal growth media or conditioned media for 435 cells. After 6 hrs the cells were fixed and stained with crystal violet and the number of cells that migragted was counted.
(B) For invasion assays, 30,000 cells were plated in normal growth media or conditioned media for 435 cells. After 24 hours the cells were fixed and stained with crystal violet and the number of cells that invaded through the Matrigel pores was counted. For both the migration and invasion assays, the average number (n=6) of cells counted per well is shown. *P-value <0.05, ***P-value <0.001 compared to 10A cells.
435, MDA-MB-435; 231, MDA-MB-231; 10A, MCF-10A

D. Effect of Ets1 siRNA on migration and invasion in Ets1 expressing breast cancer cell lines

To determine the role Ets1 has on the migratory and invasive potential of the MDA-MB-231, MDA-MB-435, and MCF-10A cell lines, we used siRNA technology to knockdown Ets1 in these cell lines and assayed them for changes in their migratory and invasive phenotypes. First, we determined the ability of the Dharmacon ON-TARGETplus™ SMARTpool® technology to knockdown Ets1 in our cell lines. Using transient transfections of 25 μM of the siRNA Ets1 targeting sequences, we demonstrated a dramatic knockdown of Ets1 protein levels by 49 fold in the MDA-MB-435 cell line and a 3-fold reduction of Ets1 levels in the MDA-MB-231 cell line. Ets1 silencing in the MCF-10A cell line displayed a moderate 1.5-fold knockdown (Figure 3.10). The effect of Ets1 knockdown on migration in the cell lines studies was diverse. We observed no change in transwell migration in the MDA-MB-435 cell line, and a slight, non-significant increase in migration in the MDA-MB-231 cell line, and in the MCF-10A a non-significant 3-fold decrease in transwell migration (Figure 3.11). The knockdown of Ets1 had a surprising effect on the invasive phenotype of the MDA-MB-435 cell line by increasing transwell invasion approximately 2.5-fold. In the MDA-MB-231 and MCF-10A cell lines, we observed an approximate 2.5-fold and 2-fold decrease in transwell invasion, respectively, after silencing Ets1 (Figure 3.11). Our results suggest a cell line specific role of Ets1 silencing on the migratory and invasive potential or that our system is not sensitive enough to distinguish between significant changes and background levels in these cell lines.

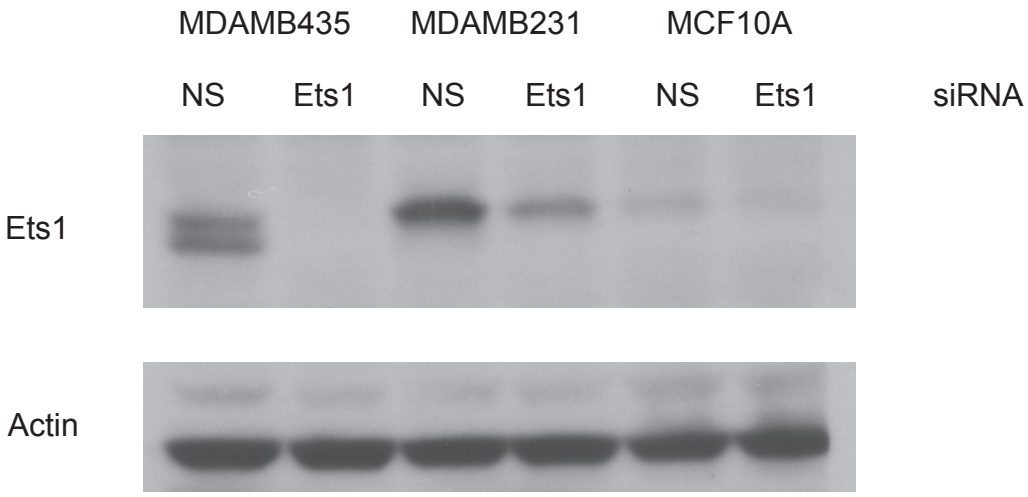


Figure 3.10 Silencing of Ets1 in breast cancer cell lines using siRNA.

For silencing experiments, we transiently transfected parental breast cell lines with 25uM of Dharmacon's SMARTpool for Ets1 or a Non-specific (NS) sequencing targeting luciferase using OligofectamineTM according to manufacturer's protocol. Approximately 72 hours following transfection, lysates were collected, processed, and assayed by western blot. For western blot analysis, subconfluent cells were collected by trypsinization, pelleted, and lysed in RIPA buffer supplemented with 1x protease inhibitor cocktail. Total protein concentration was determined by BCA Protein Assay Kit, 20 µg of total protein was loaded on SDS-PAGE gels and transferred to PVDF membranes using semi-dry transfer. Membranes were blocked with blocking buffer (5% nonfat milk in TBS with 0.1% Tween 20) overnight at 4°C, then incubated with primary antibody for 2 hours at RT, after washing with TBS/T the membranes were incubated for 1 hour at RT with secondary antibody. Proteins were visualized with Pierce's ECL Western Chemiluminescence kit. Primary antibodies were used at the following dilutions: Ets1, 1:500 (Abcam); Actin, 1:1000 (Sigma-Alderich)

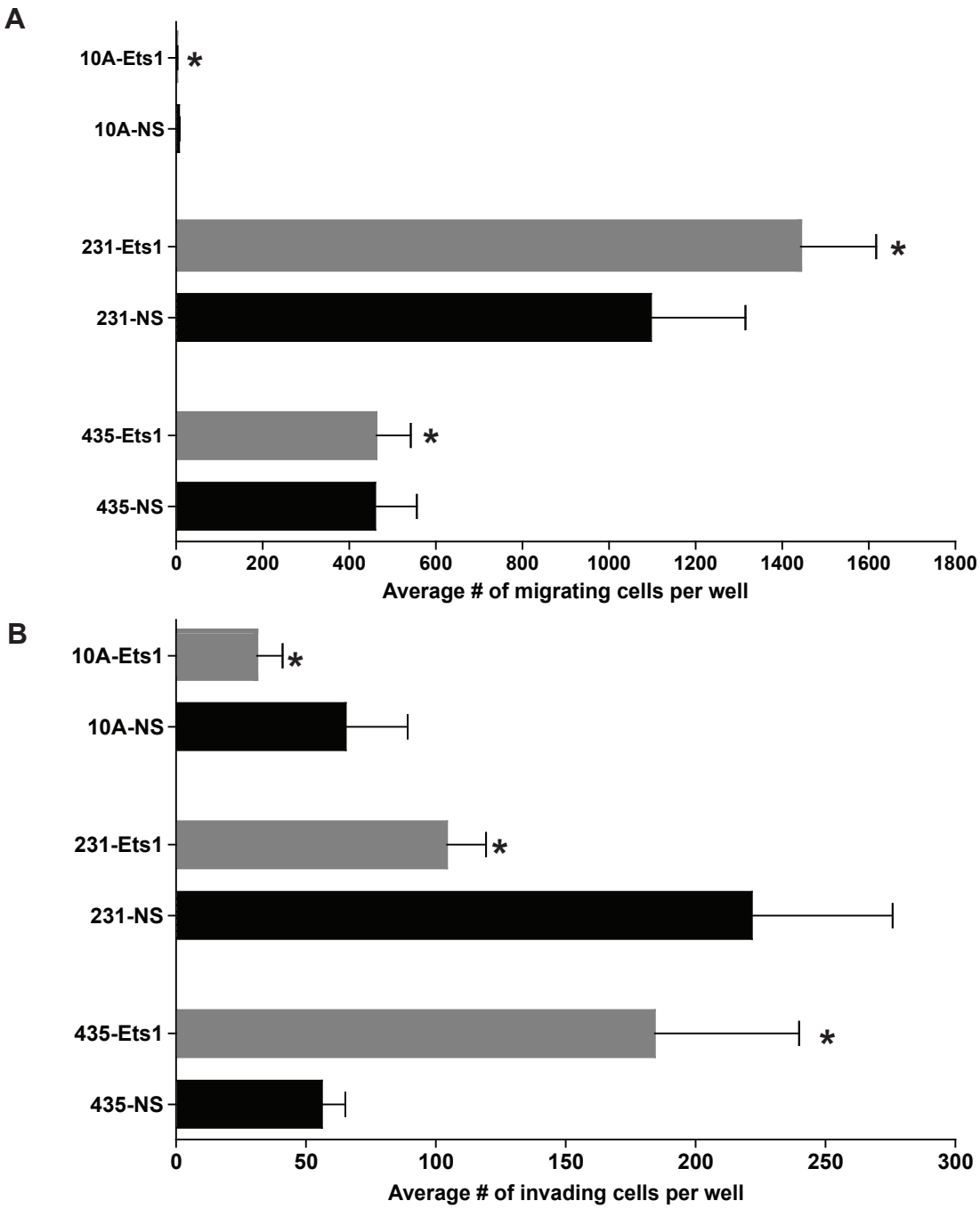


Figure 3.11 Effect of Ets1 siRNA on migration and invasion in breast cell lines.

Transwell migration (A) and invasion (B) assays 72 hours after transfection with 25uM Ets1 or non-specific (NS) siRNA, as described in Fig3.10. The migration and invasion assays were allowed to grow for 6 and 24 hours, respectvely, in normal growth media or conditioned media for 435 cells before fixing and staining, as in Fig3.10. The average (n=6) number of cells counted per well is shown. *P-value>0.05 comparing Ets1 siRNA vs. NS siRNA in each cell line.
435, MDA-MB-435; 231 MDA-MB-231; 10A, MCF-10A; NS siRNA targets luciferase.

E. Ectopic expression of Ets1 promotes invasion in MDA-MB-231 and MCF-10A cell lines

To mimic the overexpression of Ets1 observed in breast cancer, we generated Ets1 overexpressing stable cell pools in the MDA-MB-231 cell line and stable cell clones in the MCF-10A cell line as we were unable to generate stable pools overexpressing Ets1 in MCF-10A cells. Western blot analysis of protein lysates from the MDA-MB-231 pools showed an approximate 2-fold increase in Ets1 levels and these cells displayed a 3.6-fold increase in transwell invasion compared to vector pools (Figure 3.12). In the MDA-MB-231 Ets1 pools, increased Ets1 expression correlated with increased transwell invasion.

In the MCF-10A cell line, both the pLXSN and pBABE retroviral plasmids were used to generate four stable pools for Ets1 overexpression; however, western blot analysis of these pools did not show significant overexpression of Ets1 compared to vector pools (data not shown). We used the MCF-10A pools from both retroviral vectors to generate stable Ets1 clones. As Ets1 mRNA expression has been associated with an EMT-like phenotype (Gilles et al., 1997), we checked Ets1, Vimentin, and E-cadherin protein expression levels in all of the MCF-10A clones (Figure 3.13). Clones #2 and #7 from the pBABE pool and clones #3 and #3A from pLXSN pool express the highest levels of Ets1, and this also appears to correlate with increased Vimentin levels and decreased E-cadherin levels in these clones. Increased Vimentin and decreased E-cadherin are two well known markers for a mesenchymal phenotype of increased migration, invasion, scattering, cell elongation and resistance to anoikis (Lee et al.,

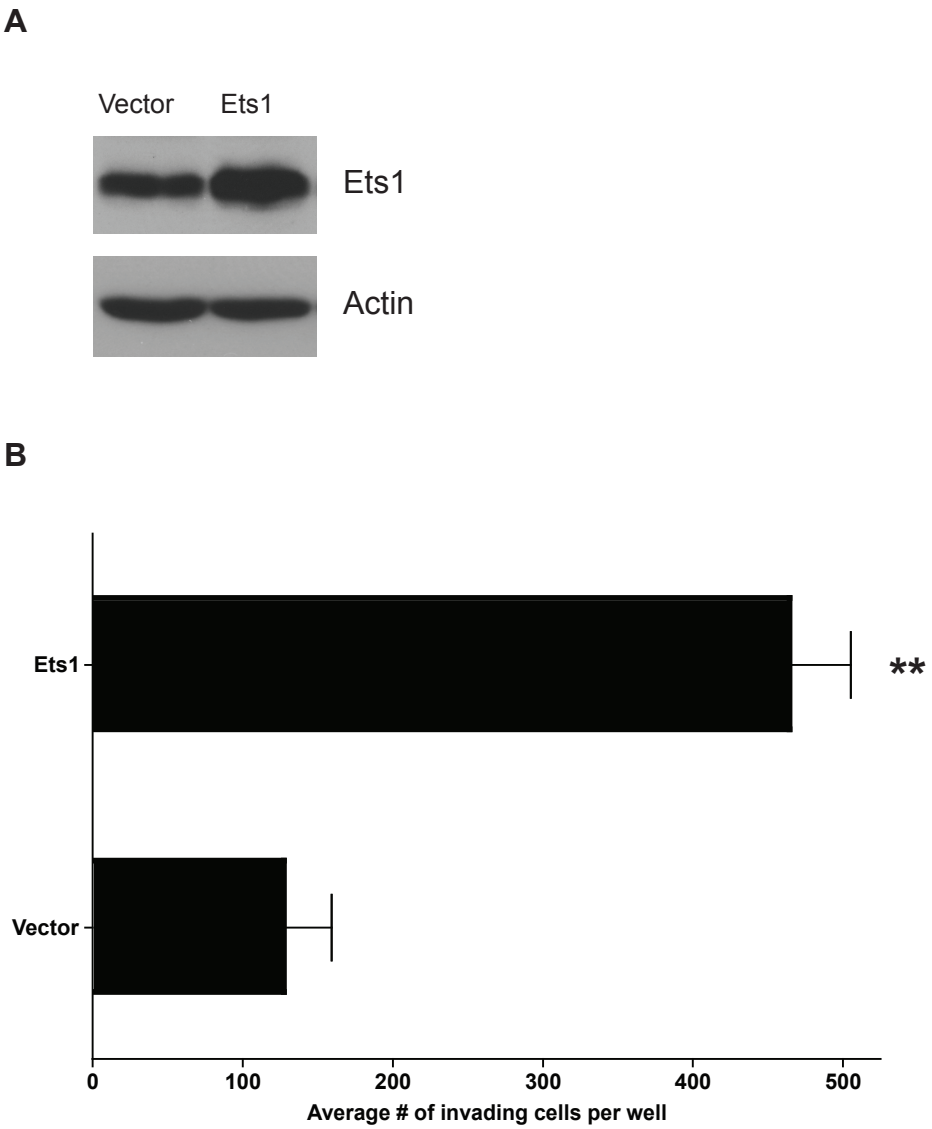


Figure 3.12 Ets1 overexpression increases invasion in MDA-MB-231 pools.

(A) We generated retrovirally infected stable pools expressing pBABE-Ets1 or empty pBABE vector in the breast cancer MDA-MB-231cell line. For western blot analysis, subconfluent cells were collected by trypsinization, pelleted, and lysed in RIPA buffer supplemented with 1x protease inhibitor cocktail. 20 µg of total protein was loaded on SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked overnight at 4°C, then incubated with primary antibody for 2 hours at RT, washed with TBST and incubated for 1 hour at RT with secondary antibody. Primary antibodies were used at the following dilutions: Ets1, 1:500 (Abcam; ab10936); Actin, 1:1000 (Sigma; 4700). (B) Transwell invasion assay of stable pools used above, 30,000 cells were plated per transwell Matrigel-coated chamber and allowed to invade for 24 hours before being fixed, stained and counted. A total of 6 wells were counted and the average number of cells invading per well is shown. ** P-value < 0.01

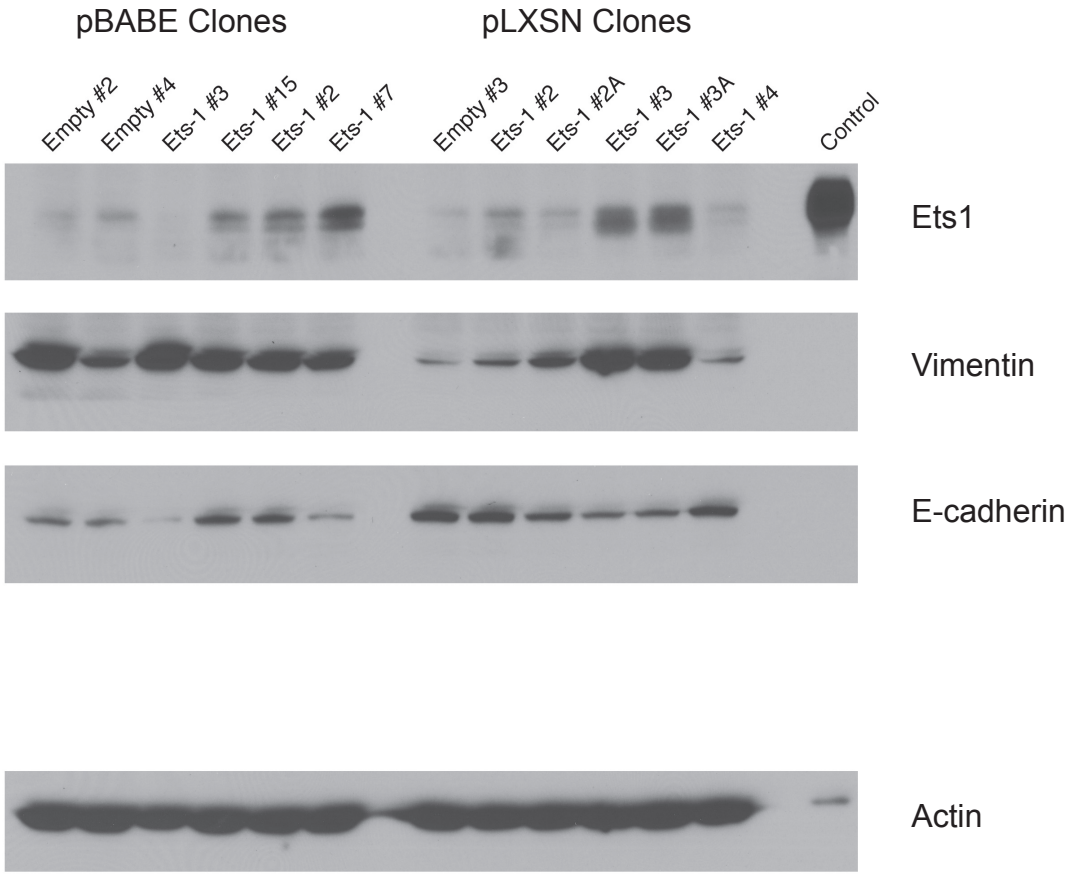


Figure 3.13 Ets1 expression in MCF-10A stable cell clones.

We generated stable MCF-10A clones by serial dilution of retrovirally infected stable cell pools from both pBABE and pLXSN plasmids. For western blot analysis of MCF-10A stable cell clones, subconfluent cells were collected by trypsinization, pelleted, and lysed in RIPA buffer. 20 ug of total protein was loaded on an SDS-PAGE gel, run at 100 Volts, and transferred to PVDF membranes. Membranes were blocked overnight at 4 C, then incubated with primary antibody for 2 hrs at RT, washed with TBST and incubated for 1 hr at RT with secondary antibody. Primary antibodies were used at the following dilutions: Ets1, 1:500; Vimentin, 1:1000; E-cadherin 1:1000, and Actin, 1:1000.

2006); however, in our transwell invasion assays of these clones, we detected varying levels of increased invasion (Figure 3.14). In the stable clones with a detectable increase in Ets1 expression, we observed an increase in invasion, however there does not appear to be a direct correlation with the amount of Ets1 overexpression or increase in Vimentin or decrease in E-cadherin and the observed increase in transwell invasion in the MCF-10A cell line (compare Figure 3.13 and Figure 3.14). These results suggest that there is not a direct relationship between Ets1 protein levels (or even Vimentin and E-cadherin levels) and transwell invasion capacity in the MCF-10A Ets1 clones or the relationship is altered by clonal artifacts generated in our system.

F. Ets1 expression does not influence proliferation and anchorage-independent growth in the MCF-10A cell clones

To further explore the role Ets1 may play in breast cancer, we performed a proliferation assay under growth factor free and reduced serum conditions and an anchorage-independent growth assay. Growing the MCF-10A Ets1 stable clones under EGF-free and reduced serum conditions resulted in no consistent correlations between Ets1 expression and proliferation rates as determined by MTS assays (Figure 3.15). For example, on one hand the pLXSN Ets1 Clone 7 had the high Ets1 protein levels, yet proliferation rates similar to vector controls, suggesting there is no correlation between Ets1 levels and proliferation. However, on the other hand, the pBABEpuro Ets1 Clones 3 and 3A had the highest Ets1 protein levels and a modest growth advantage compared to the vector control.

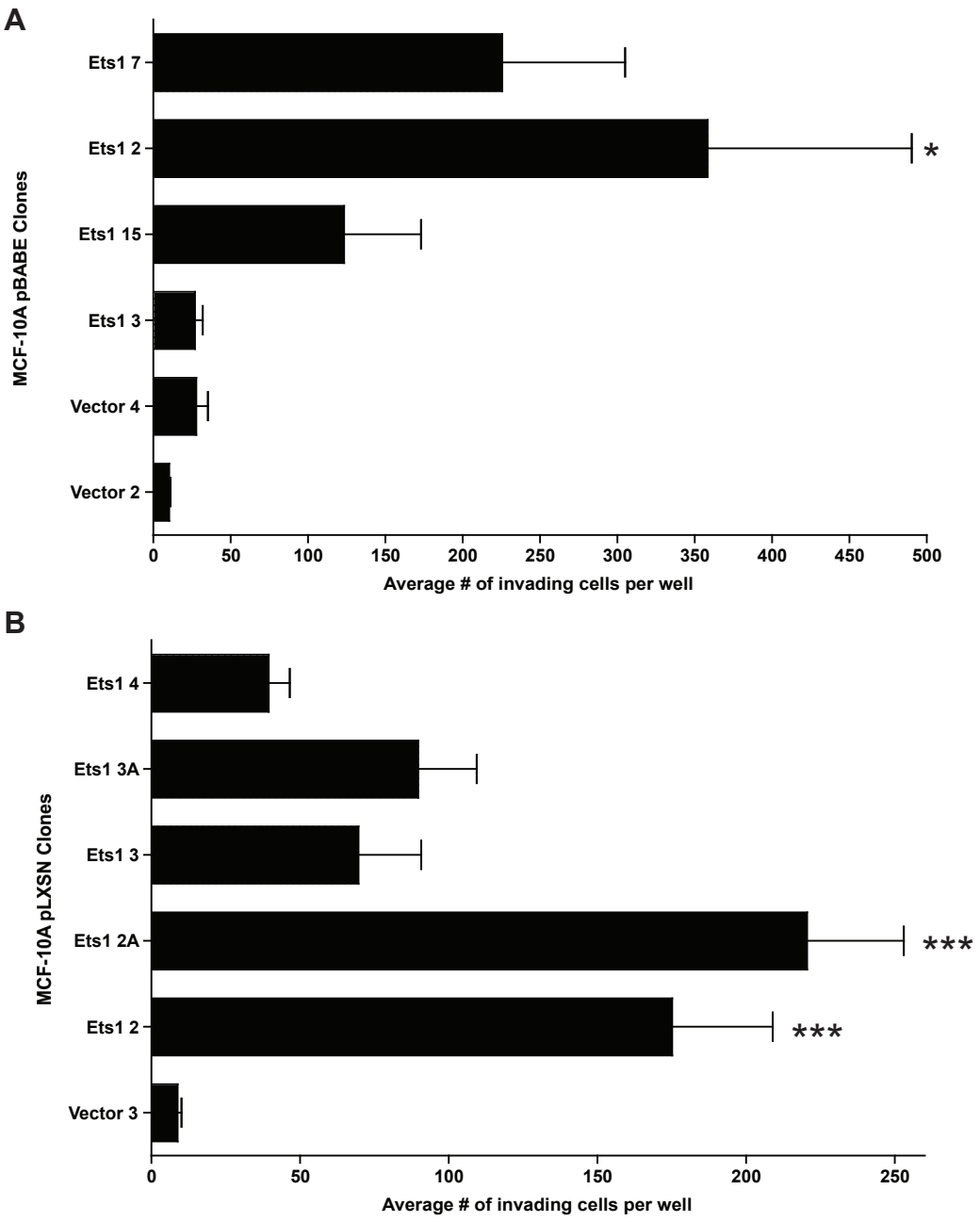


Figure 3.14 Increased transwell invasion in MCF-10A Ets1 expressing clones.

Transwell invasion assay in MCF-10A clones overexrpessing Ets1 from pBABE (A) or pLXSN (B) plasmids. 30,000 cells were plated in the invasion transwell Matrigel coated chamber and allowed to invade in normal growth media for 24 hours before being fixed and stained. A total number of 6 wells were counted from separate experiments and the average number of cells counted per well is shown.
*P-value <0.05 between Vector 2 and Ets1 2; ***P-value <0.001 betweenVector 3 and both Ets1 2 and Ets1 2A.

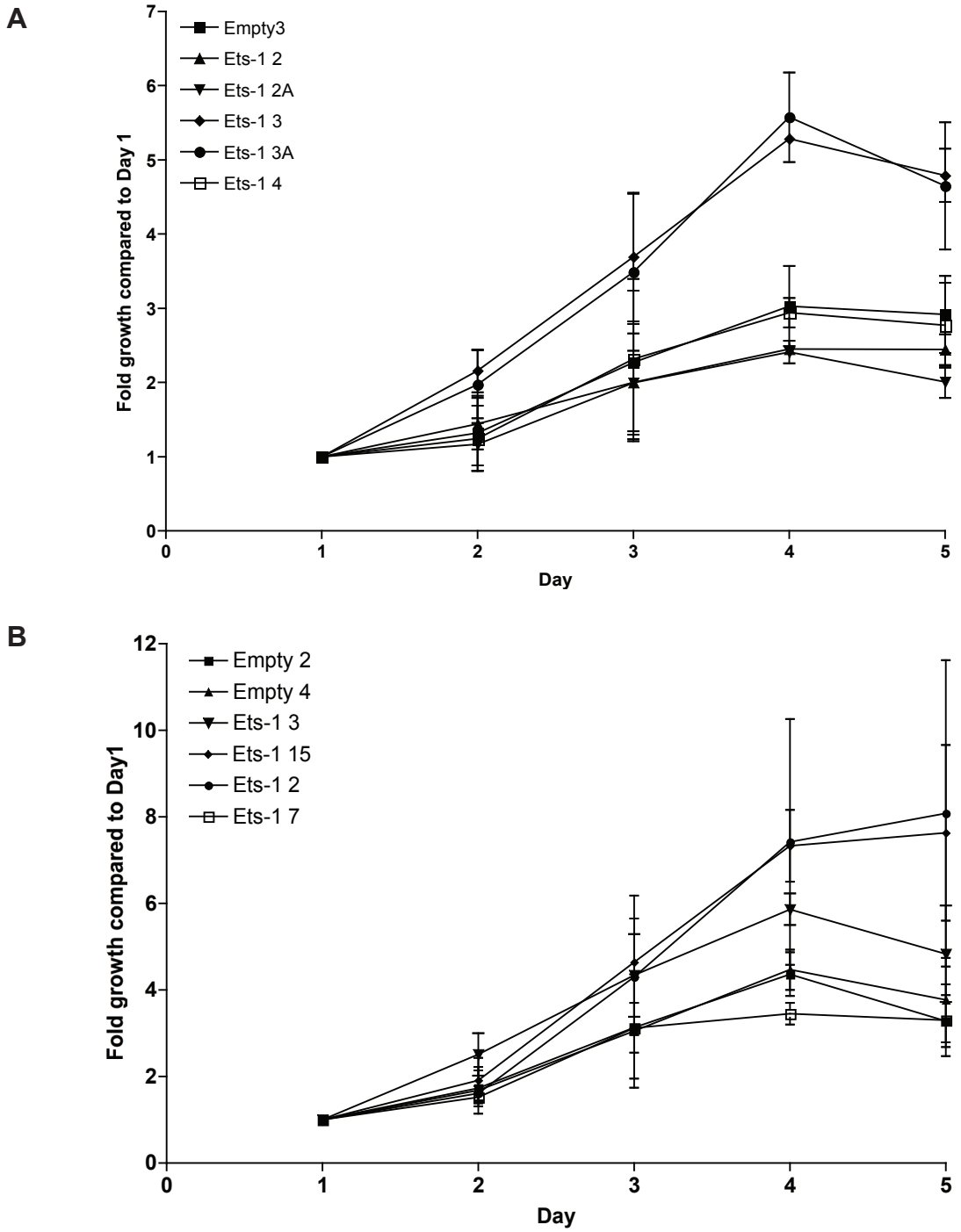


Figure 3.15 Ets1 expression in MCF-10A does not correlate with proliferation.

MTS assay to determine proliferation rates of MCF-10A clones from (A) pLXSN or (B) pBABE expressing Ets1 or vector. 3,000 cells were plated in triplicate in a 96-well plate in normal growth media for 5 hours before switching to 100ul of EGF-free and 1% horse serum, media was refreshed everyday at the time when the reading was taken. 1 hour after adding MTS reagent, absorbance was read at 490nm, and fold growth was compared to the average reading at Day1 (n=3). P-value>0.05 for all data sets.

To determine if any of the MCF-10A Ets1 clones had acquired an anchorage-independent growth phenotype, we performed soft agar assays. Anchorage-independent growth assays with the MCF-10A Ets1 stable clones from the pLXSN vector resulted in no observed differences in growth (Figure 3.16); however, minor growth differences were observed in the clones from the pBABE vector, yet no clone had more colonies than the vector control. These results demonstrate no clear relationship between Ets1 expression and both proliferation and anchorage-independent growth in the MCF-10A cell clones.

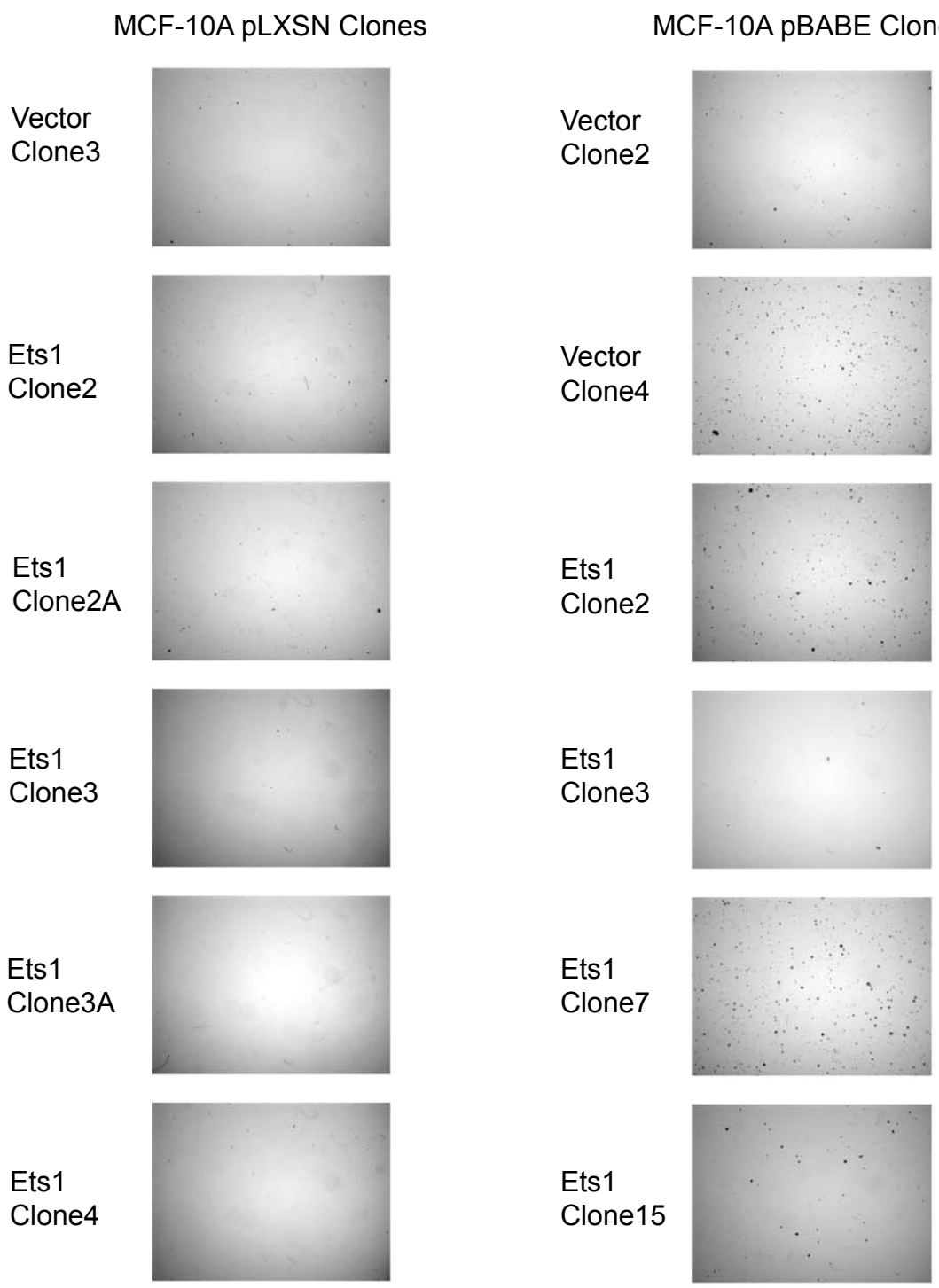


Figure 3.16 Ets1 expression in MCF-10A cell clones does not confer anchorage-independent growth.
Anchorage-independent growth assays were performed in 6-well plates using a 2 mL 0.6% base layer of noble agar in full growth media and 30,000 cells in a 0.3% suspension of Noble agar in full media. Approximately 500 µl of growth media was replaced per well every three days and images were taken after 5 weeks. Representative images shown out of n=3.

CHAPTER 4: DISCUSSION

I. Ets1 Regulation of αB-Crystallin

Of the small heat shock proteins, αB-crystallin is one of the most studied and well characterized. It has established roles in development and preventing apoptosis. Furthermore, αB-crystallin expression in breast cancer is linked to high-grade tumors, metaplastic tumors, poor prognosis, and resistance to neoadjuvant chemotherapy (Chelouche-Lev et al., 2004; Ivanov et al., 2007; Moyano et al., 2006; Sitterding et al., 2008). Recent studies identified ways in which αB-crystallin could promote cancer; including increased MAPK signaling, conferring resistance to apoptosis, and increased invasion (Moyano et al., 2006). Of the studies looking into the regulation of αB-crystallin, few focused on its regulation in cancer. A study of anaplastic thyroid carcinomas, identified different mRNA levels of HSP27-1 and αB-crystallin in thyroid carcinomas (Mineva et al., 2005). However, they showed decreased αB-crystallin levels in the more aggressive tumors compared to the benign goiters and suggested that the lower levels were a result of the lower observed levels of the transcription factor TFCP2L1 in the same tumors as αB-crystallin. The study did not perform any functional assay confirming the regulation of αB-crystallin gene expression by TFCP2L1 in thyroid cell lines. Studies to date on the regulation of αB-crystallin focus on lens and muscle tissues using primarily non-human systems. A general lack of information on the regulation of αB-crystallin in human cancer cell lines, given its relevant roles in cancer, stresses the importance of investigating the transcriptional regulation of αB-crystallin in additional human cellular systems.

Using a bioinformatics approach, we identified Ets1 as a potential regulator of αB-crystallin in breast cancer cell lines. We used a luciferase-based reporter to reveal Ets1 induced activation of the human αB-crystallin promoter, in a manner that is dependent upon the putative ETS binding site. Furthermore, we used electrophoretic gel shift analysis to demonstrate binding of Ets1 to the EBS from the αB-crystallin promoter, but not to a mutated version of the ETS binding site. *In vivo*, we used chromatin immunoprecipitation to verify binding of Ets1 to the αB-crystallin promoter. Importantly in the MDA-MB-231 cell line, we observed that overexpression of Ets1 resulted in increased protein levels of αB-crystallin and that Ets1 silencing resulted in decreased αB-crystallin protein levels. Together these results indicate that Ets1 regulates αB-crystallin gene transcription through an ETS binding site within the αB-crystallin promoter.

To test whether the regulatory mechanisms we observed in our *in vitro* and *in vivo* experiments might have relevance in human breast tumors, we obtained breast cancer tissue microarrays and assessed them for both αB-crystallin and Ets1 protein levels by immunohistochemistry. The results here confirm our previous microarray study (Moyano et al., 2006) in which αB-crystallin was present in about 10% of breast tumors and 27% of the triple-negative tumors. Previous reports found that 83.2% of 137 breast carcinomas showed positive Ets1 staining without distinguishing between cytoplasmic or nuclear localization (Katayama et al., 2005), while a second study, using the same antibody we used, reported 77.9% cytoplasmic and 46.3% nuclear staining in 149 invasive breast carcinomas (Mylona et al., 2006). A third study found Ets1 mRNA in 73% of 179 primary breast carcinomas and 71% of 42 fibroadenomas (Buggy et al.,

2004). In our studies, we observed cytoplasmic Ets1 staining in 96% of 72 breast tumors and nuclear staining in 56% of the breast tumors. We believe that the 96% occurrence of cytoplasmic Ets1 staining in our tissue microarray is due to high levels of non-specific binding with the Ets1 polyclonal antibody. Western blot analysis with this Ets1 polyclonal antibody results in a membrane with multiple bands outside of the expected 50 kDa area for Ets1; further suggesting the non-specific nature of this antibody. Therefore, we cannot make any meaningful conclusions about Ets1 levels and correlation to αB-crystallin staining or the triple-negative status in breast tumors.

The Ets1 binding site in the human αB-crystallin is absolutely conserved in human, chimpanzee, mouse, rat, and dog through evolution (Figure 3.1), suggesting it is a region of importance. Previous studies identified transcription factors involved in the regulation of the mouse αB-crystallin at other regions of the promoter that are well conserved. In the proximal region of the promoter, two lens specific regions were identified and shown to contain RAR/RXR and Pax6 binding sites (Gopal-Srivastava et al., 1996, 1998; Gopal-Srivastava and Piatigorsky, 1994). The proximal region also contains a putative HSE that is most likely responsible for the upregulation of αB-crystallin in response to stress (Tamm et al., 1996); however functional analysis of this HSE, by either HSE mutation or HSF1 binding analyses have been reported in lens models but not cancer model systems (Somasundaram and Bhat, 2000, 2004). An upstream αB-crystallin specific enhancer region also has been identified using the mouse reporter. This enhancer region is in the middle of the HSPB2 and CRYAB genes and contains both lens and muscle specific enhancer elements (Gopal-Srivastava and

Piatigorsky, 1993; Swamynathan and Piatigorsky, 2002). Of the elements identified within the upstream enhancer in the mouse, a region called αBE4 contains the Ets1 binding site studied here as well as a partial HSF binding site. Further study of the αBE4 site in the mouse, suggested binding and regulation by a factor related to serum-response factor (SRF). We did not test for regulation of human αB-crystallin by SRF in our system because our focus was on human breast cancer, and not cardiac muscle, where it plays a role in maturation and development (Du et al., 2003; Wang et al., 2002; Zhang et al., 2001a; Zhang et al., 2001b). Interestingly, another Ets family member Elk1 has been reported to regulate c-fos expression in conjunction with SRF following induction by estradiol in ER-positive MCF-7 cells (Duan et al., 2001). Indeed it is documented that SRF can form a ternary complex with ETS-family members, including Elk1 (Murai and Treisman, 2002) and Elk4 (Cooper et al., 2007; Hassler and Richmond, 2001) to regulate gene expression. While ETS-family members Elk1 and Elk4 are known to bind DNA with SRF and regulate gene transcription, it has been shown that Ets1 and Ets2 are not able to complex with SRF (Watson et al., 1997). Therefore we believe that regulation of αB-crystallin in our system was by Ets1, however we cannot rule out the possible regulation of αB-crystallin through the interaction of SRF with another ETS-family member. However, we also tested for regulation of the αB-crystallin reporter by another ETS-family member ESE1/ESX. ESE1/ESX is the epithelial specific ETS-family member reported to have a role in the transformation of mammary epithelial cells (Prescott et al., 2004; Schedin et al., 2004), however in our studies ESE1/ESX did not significantly activate our luciferase reporter of the human αB-crystallin promoter.

The Ets1 binding site studied within the αB-crystallin promoter exists as a head-to-head orientation of two Ets1 binding sites. This palindromic orientation of two Ets1 binding sites has been documented in the regulation of MMP3/stromelysin-1 (Baillat et al., 2002; Baillat et al., 2006). In that system, two Ets1 monomers formed a homodimer and helped each other maintain a transcriptionally active state by preventing autoinhibition that would lead to down-regulated activity. It is possible that the same situation could exist for the αB-crystallin promoter. Supershift experimental data not reported from the electromobility gel shift assays indicated that Ets1 could bind the promoter as either a monomer or homodimer, but not both as only one supershift band was observed. Therefore, we cannot say conclusively whether Ets1 regulates αB-crystallin as a monomer or homodimer.

As mentioned previously, the Ets1 binding site studied here also overlaps with a partial heat shock element. A complete heat shock element exists as three nGAAn (e.g. 5'-nCCTnnGAAnnCCTn-3') in succession and is bound by a trimer of heat shock transcription factors. However it has been documented that heat shock factor can bind and transactivate from a heat shock element of only two repeats (Kroeger et al., 1993). αB-crystallin is well known as a stress inducible heat shock protein; however, this is most likely from the proximal, complete heat shock element approximately 60 bases upstream from the transcriptional start site. In fact, HSF1 was used as a positive control in our promoter reporter assays, which showed that HSF1 induced activity approximately 3.5 fold more than the vector alone (data not shown), while Ets1 induced an approximate 2.5 fold increase. This suggests that the αB-crystallin promoter is regulated by HSF1

primarily at the proximal HSE and not at the partial HSE overlapping our ETS binding site.

It has been shown that αB-crystallin is commonly expressed in basal-like breast tumors (Moyano et al., 2006; Sitterding et al., 2008) and that Ets1 expression is linked to the basal-like phenotype in breast cancer cell lines (Charafe-Jauffret et al., 2006). Here we provide a possible explanation for the observation of αB-crystallin expression in basal-like breast cancer phenotype by demonstrating Ets1 regulation of αB-crystallin gene expression. Ets1 is known to have roles in cancer progression including increased migration and invasion. αB-crystallin too is known to cause neoplastic-like changes in normal mammary epithelial cell lines, and its expression is linked to resistance to neoadjuvant chemotherapy and an invasive phenotype in breast carcinomas. Future experiments studying the link between these two could offer more insight into the importance of Ets1 regulation of αB-crystallin. As Ets1 and αB-crystallin levels have been linked to poor prognosis in breast cancer and to the basal-like breast cancer subtype, additional studies could lead to more effective therapies for this difficult to treat cancer. This is the first report demonstrating the regulation of the human αB-crystallin gene expression by a transcription factor in a breast cancer model.

II. Future Directions of αB-Crystallin Regulation

This study only begins to look into the regulation of αB-crystallin in humans and human cancer. Although the human and mouse promoters are well conserved, a recent

study of the mouse αB-crystallin promoter confirms how important context is when studying transcriptional regulation (Swamynathan and Piatigorsky, 2007). To date, in the mouse system employed in the Piatigorsky studies, the following transcription factors have been shown to either bind to or transactivate the mouse αB-crystallin promoter: Pax-6, RAR/RXR, SRF, USF1, MyoD/Myogenin, glucocorticoid receptor, and SP1 (Dubin et al., 1991; Gopal-Srivastava et al., 1996, 1998; Gopal-Srivastava et al., 1995; Gopal-Srivastava and Piatigorsky, 1993, 1994; Haynes et al., 1995; Swamynathan and Piatigorsky, 2007). In studies performed by other labs, Sox1 and Sox2 as well as HSF1, 2, 4 have also been implicated in the transcriptional regulation of αB-crystallin (Head et al., 1996; Ijichi et al., 2004; Somasundaram and Bhat, 2000, 2004). In one additional study, the transcription factor TFCP2L1 has been suggested to have a role in regulating αB-crystallin gene expression; however, no functional analysis was reported (Mineva et al., 2005). A bioinformatic analysis performed here indicated many more putative regulatory binding sites for other transcription factors. A few of interest are p53, NFκB, and KLF4.

Briefly, an initial screen of potential regulators of αB-crystallin showed that KLF4 repressed promoter activity and p53 activated promoter activity (data not shown). Others in the lab are investigating the role p53 plays in regulating αB-crystallin expression, and initial unpublished results indicate that αB-crystallin is a target of p53 transactivation. Furthermore, probing published microarray data shows the αB-crystallin mRNA levels increased following p53 and deltaN-p73 expression (Godefroy et al., 2004), indicating potential regulation by the p53/p63/p73 family of transcription factors.

When addressing possible repression by KLF4, microarray data from a KLF-family member, KLF7, knockout mouse displayed upregulation of αB-crystallin in that system (Kajimura et al., 2007), further suggesting negative regulation of αB-crystallin by the KLF family of transcription factors.

NFκB as a potential regulator of αB-crystallin would be of particular interest given the role of NFκB in both tumor formation and as a stress response mediator. Our study only begins to probe into the regulation of αB-crystallin in cancer and opens the door to some of the other transcription factors mentioned here.

Studies have recognized regulation of αB-crystallin by heat shock factors following stress or during development, but these studies have not investigated the regulation of αB-crystallin by HSF1 in cancer. A recent report by Dai *et al* implicates HSF1 as modifier of carcinogenesis (Dai et al., 2007). HSF1 is often considered the master regulator of heat shock protein expression following stress and αB-crystallin is just one of the molecular chaperones transactivated by HSF1 following stress. It would be interesting to study the role of HSF1 in regulating αB-crystallin following stress induced by chemotherapuetic agents, oxygen deprivation, anchorage-independence, serum-free media, cell division, cell migration and cell invasion. Identifying HSF1 regulation of αB-crystallin under any of these conditions could provide further evidence for the significance of αB-crystallin expression in tumor formation or progression and

provide more specific evidence for the role of HSF1 and αB-crystallin in carcinogenesis and chemotherapy resistance.

Ets1 is regulated by protein-protein interactions and the involvement of additional factors in the regulation of αB-crystallin was not studied here. Additional proteins that have been documented to co-regulate gene expression with Ets1 are AP-1, SP1, CBP/p300, NFκB and USF1 (Li et al., 2000a). As the αB-crystallin promoter has putative binding sites for all of the above-mentioned factors, investigating the potential co-regulation by any of those factors would be interesting. Evidence exists for USF1 binding to the mouse αB-crystallin promoter in muscle/lens cells and activated human T-cells (Gopal-Srivastava et al., 1995; Sieweke et al., 1998), suggesting the possibility for co-regulation of αB-crystallin by Ets1 and USF1 in the right cellular environment. Simple co-transfection experiments combining Ets1 with the above mentioned transcription factors along with the αB-crystallin promoter reporter would give an indication of which factors may co-regulate αB-crystallin gene expression.

One final piece of evidence of interest about the regulation of the αB-crystallin promoter comes from study of bi-directional, shared gene promoters. As mentioned previously, αB-crystallin and HSPB2 share a promoter of approximately 1,000 base pairs. A recent study of bidirectional promoters indicated that about 11% of the human genome is composed of genes with bi-directional shared promoters of 1kb or less; furthermore, they identified an over-represented consensus site in these divergent promoters (Collins et al., 2007). The consensus site was for the ETS-related transcription

factor GA-binding protein, and they discovered that ~80% of the 356 shared promoters studied were bound by GABP. Interestingly, GABP binding correlates with bidirectional transactivation in 67% of the luciferase promoter-reporters tested. Most studies of the regulation on the CRYAB/HSPB2 shared promoter focus on the regulation of αB-crystallin gene expression. It also appears that to date, αB-crystallin and HSPB2 are mostly anti-regulated, with only one very recent example of regulation of both HSPB2 and αB-crystallin by the same transcription factor, glucorticoid receptor (Swamynathan and Piatigorsky, 2007). Studies of GABP and the CRYAB/HSPB2 shared promoter could identify cases in which αB-crystallin and HSPB2 are co-regulated, or lead to cases in which GABP binding does not lead to bidirectional transcriptional activity. In any case, these studies would identify novel mechanisms of CRYAB/HSPB2 gene regulation.

III. αB-Crystallin as an Enabler of Tumor Formation

The role of molecular chaperones in tumor formation is often a case of proteins performing their function too well for their given environment. Molecular chaperones as a class of proteins work to protect cells during stressful events and assist the cells in maintaining homeostasis by transporting, folding and preventing aggregation of proteins. During the evolution from a normal cell to a malignant, transformed cell there is a process of selection within that cell's environment. In that sense, cancer can be viewed as an evolutionary and ecological process (Merlo et al., 2006). Evolving from a normal cell to the transformed cell requires acquiring each of the 'hallmarks' of cancer as each hallmark leads to differential reproductive or survival success of that initial cell. It is during the evolution to acquire each hallmark that expression of a particular molecular

chaperone may provide a growth or survival advantage during the stress of the selection process (Figure 1.4). Sometimes, as in the case of Hsp70-2, the transformed cells become dependent on the molecular chaperone to the point where removing it from the cell results in cell death (Daugaard et al., 2005; Nylandsted et al., 2000a; Nylandsted et al., 2000b; Rohde et al., 2005). While the molecular chaperone is not a classical oncogene in the sense that mutation or amplification of the gene results in transformation of the original cell, it does enable the cell to evolve to its transformed status, and has been referred to as non-oncogene addiction (Solimini et al., 2007; Weinstein, 2002).

Targeting a tumor's non-oncogene addiction is a promising therapy as it should only select for the cells actually addicted to the chaperone, and indeed this is the case with the effective anti-cancer drug geldanamycin, which targets and disables Hsp90. Recently, Dai *et al* showed that HSF1 is a potent modifier of the tumorigenic process and that it is required for tumor initiation and maintenance using an assortment of cancer models (Dai et al., 2007). In our lab, we have shown that αB-crystallin protects cells from apoptosis by preventing caspase-3 maturation (Kamradt et al., 2001; Kamradt et al., 2002; Kamradt et al., 2005) and that breast tumors expressing αB-crystallin are commonly basal-like and have poor prognosis, and are resistant to neo-adjuvant chemotherapy (Ivanov et al., 2007; Moyano et al., 2006; Sitterding et al., 2008). These studies suggest that αB-crystallin may act as an enabler of breast tumorigenesis, in particular in the basal-like subset of breast tumors. Additional studies in our lab have shown that overexpression of αB-crystallin in the non-transformed mammary epithelial cell line MCF-10A can induce neoplastic-like changes, including constitutively active

MEK signaling. Interestingly, MEK inhibitors or αB-crystallin shRNA can effectively prevent the neoplastic changes from occurring (Moyano et al., 2006). The effect of αB-crystallin shRNA in particular indicates the enabling ability of αB-crystallin in that system. Furthermore, silencing αB-crystallin expression in the breast cancer cell line MDA-MB-435 makes that cell line more sensitive to apoptosis induced by the potential chemotherapeutic agent TRAIL through a mechanism that prevents activation of caspase-3 (Kamradt et al., 2005). Developing therapies specifically targeting αB-crystallin may make the difficult to treat basal-like breast cancer subtype more treatable (Figure 4.1).

Future therapies targeting αB-crystallin may be more effective if cancer is viewed as a chronic illness and therapies were biological in origin and administered at a lower dose over a long time. This way the negative side-effects of a strong chemotherapy would be avoided while providing a long-term selective process against the enabling ability of αB-crystallin. Traditional chemotherapies provide a short, highly-selective selection process that often effectively cripple a tumor into remission, but may leave a small sub-population that is resistant to the therapy and can eventually re-emerge. But in the presence of constant, minimally-toxic therapy specific to an enabler of the resistance, this sub-population may never re-emerge.

In the case of basal-like breast cancer and αB-crystallin, the ideal treatment would involve delivering αB-crystallin inhibitors or silencing αB-crystallin expression specifically in the basal-like breast cells. For example, the approach of using geldanamycin and its derivatives to target Hsp90 in cancer provides hope that αB-

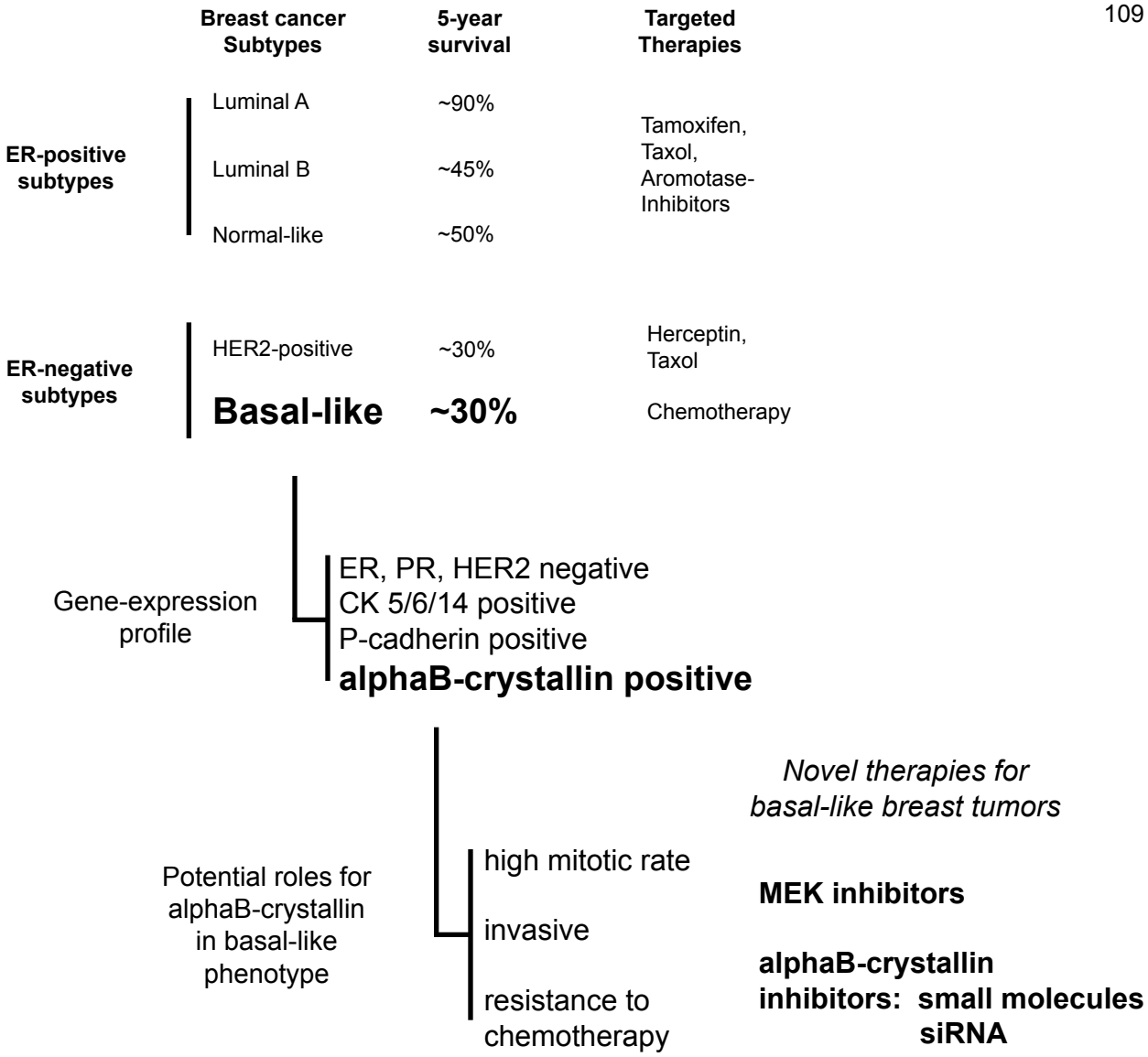


Figure 4.1 Role of alphaB-crystallin in basal-like breast cancer and potential novel therapeutic targets for basal-like breast cancer.

crystallin inhibitors may yield promising results. However, rather than a broad approach of targeting Hsp90 in every cell, which will likely alter the important functions of Hsp90 in non-tumorigenic cells, a more efficient approach is to target Hsp90 specifically in the cancer cells. αB-crystallin expression is common in the triple-negative, basal-like tumors (Sitterding et al., 2008) and can indicate resistance to adjuvant chemotherapy in breast cancer (Ivanov et al., 2007); therefore effective therapies would require identifying the surface markers of the basal-like cells and developing an effective delivery method for the treatment. This approach would allow doctors to target αB-crystallin specifically in the basal-like breast cancer cells. To date, there are no published small molecule inhibitors of αB-crystallin, and currently no cell surface molecules have been identified that are expressed specifically in the basal-like breast carcinomas. siRNA technology today works efficiently *in vitro* to silence αB-crystallin, yet when surface markers are discovered, many hurdles still exist for effective siRNAs *in vivo*. Examples of such hurdles include more effective siRNA design, improved stability of siRNA, and development of an efficient delivery system that targets specific cells, allows siRNA uptake, and yields gene-silencing activity. Retroviral methods of delivery are moving forward with great caution and the realization of this type of therapy may be better achieved through non-viral delivery of siRNAs (Akhtar and Benter, 2007).

IV. Role of Ets1 and Breast Cancer Migration, Invasion, and Transformation

Our results from studying the effects of overexpressing Ets1 in human breast cancer cell lines yielded unexpected results. The initial analysis of breast cancer cell lines showed that the Ets1-expressing breast cancer cell lines also expressed a protein

expression pattern similar to an epithelial to mesenchymal transition, down regulation of E-cadherin, upregulation of vimentin (Figure 3.8). Additionally, we showed that two breast cancer cell lines with high endogenous Ets1 protein levels also have a relatively high migratory and invasive phenotype *in vitro* compared to the low Ets1 expressing MCF-10A. Others have also observed a similar pattern of gene expression in Ets1 expressing breast cancer cell lines (Gilles et al., 1997), and they too were not able to establish a clear relationship between Ets1 expression and invasive potential or expression of three known Ets1 targets MMP1, MMP3 and uPA in multiple breast cancer cell lines. We employed an Ets1 siRNA approach to further elucidate the role Ets1 plays in migration and invasion of breast cancer cell lines and we found that silencing Ets1 did not have a consistent effect on migration and invasion in the three breast cancer cell lines studied. Ets1 silencing had no significant effect on the migration of the highly metastatic MDA-MB-435 cell line, and interestingly also seemed to increase its invasive potential. In the MDA-MB-231 cell line, Ets1 silencing increased the migratory potential, but decreased the invasive phenotype. In the case of the MCF-10A cell line, Ets1 knockdown seemed to decrease the both the migratory and invasive potential, although the results were not statistically significant because of the small total number of migratory and invasive cells. Given the variation of phenotypes from Ets1 siRNA in three different breast cancer cell lines, the data suggest that cellular context has significant control over the phenotype. For example, in the MDA-MB-231 cell line Ets1 siRNA increased migration, but it also increased the proliferation of these cells (data not shown), suggesting that the increase in migration could at least in part be the result of increased proliferation. The results in the MCF-10A cell line seem to fit with what we

would expect, however the number of cells migrating or invading in each assay is so few that the results are not significant. Additionally, we also checked for changes in two gelatinases known to be regulated by Ets1, MMP2 and MMP9. Following Ets1 siRNA, we observed no detectable changes in gelatinase activity of MMP2 and MMP9 in three cell lines assayed by zymography (data not shown). Other than establishing that Ets1 protein expression in the parental breast cell lines tested indicated positive migratory and invasive potential, the mechanisms controlling the mobility potential remain unclear as does the role Ets1 plays in regulating migration and invasion in our system. Others have described Ets1 involvement in migration and invasion and a correlation with uPA expression and activity (Delannoy-Courdent et al., 1998; Kitange et al., 1999; Rothhammer et al., 2004). For example, silencing of Ets1 in bFGF stimulated glioma cells resulted in a decreased invasive phenotype and decreased uPA levels and activity (Delannoy-Courdent et al., 1998; Kitange et al., 1999), while in a mouse mammary epithelial cell system over-expression of a dominant negative Ets1 resulted in decreased transwell migration and invasion that also correlated with decreased uPA expression (Delannoy-Courdent et al., 1998). In a third cell type, silencing of Ets1 in a melanoma cell line resulted in decreased transwell invasion, which was attributed to decreased MMP1, MMP3 and uPA expression (Rothhammer et al., 2004). A possible explanation for the various and cell-specific results observed in our system could be that silencing of Ets1 alone may not be enough to significantly and consistently alter the migration and invasion in our cell lines. With nearly 30 ETS family members, it is possible such redundancy could lead to overlapping regulation of proteins involved in migration and invasion and therefore the absence of only Ets1 results in little change in our system.

Future experiments that could help elucidate the role Ets1 plays in migration and invasion in breast cancer could start with gene profiling in multiple breast cell lines either overexpressing or silencing Ets1 (or any of the ETS family members). Microarray analysis of Ets1 silenced breast cell lines would indicate at a genome-wide scale which genes may be Ets1 targets and knowledge of those genes could help indicate how the cellular context dictates the observed phenotypes. There is an example of a microarray analysis of Ets1 silencing in the MDA-MB-231 cell line (Vetter et al., 2005), which found down-regulation of many genes including MMP1, NQO1, SP100 and MMP9; however, they did not observe any significant changes in morphology or anchorage-independent growth. Additional information from gene-profiling experiments that could help determine the specificity of Ets1 for its target genes could look at the expression of proteins known to interact with Ets1 and regulate its activity. In fact, it appears that Ets1 is an effector of epithelial to mesenchymal transition by being a target in TGFβ-induced EMT, so while Ets1 is not sufficient to induce EMT, it does have a significant role in the process (Shirakihara et al., 2007; Taki et al., 2006). This helps explain the correlation between Ets1 and the EMT phenotype without being able to demonstrate direct regulation of the EMT phenotype by Ets1.

As many cancers express Ets1 and Ets1 expression correlates with poor prognosis (Dittmer, 2003; Span et al., 2002), we wished to determine if Ets1 overexpression could induce a transformed phenotype in the non-transformed, immortalized mammary epithelial cell line MCF-10A. A previous study indicated the Ets1 overexpression could

enhance the already transformed phenotype of the HeLa cell line (Hahne et al., 2005).[114] Furthermore, it is well documented that the epithelial-specific ETS family member ESE1/ESX can induce an EMT transition and a transformed phenotype in the immortalized, but not transformed mammary epithelial cell line MCF-12A (Prescott et al., 2004; Schedin et al., 2004). Our initial attempts to generate stable Ets1 overexpressing pools by retroviral infection and selection failed. In fact, a thorough search of published literature discovered only one stable pool with minimal Ets1 protein overexpression in HeLa cervical cancer-derived cell line (Hahne et al., 2005). The HeLa-Ets1 stable pool in that study had a moderate, yet significant, increase in migration, invasion, and anchorage-independent growth. However, in that study no increase in endogenous MMP1 mRNA expression was detected even though they observed a significant increase of MMP1 promoter activity, suggesting that the Ets1 overexpression was not sufficient to activate endogenous MMP1 expression in their system. As previously mentioned, our system failed to generate Ets1 overexpressing cell pools, which perhaps could be due to the short half-life of Ets1 (Papas et al., 1990) as we could detect no significant changes in proliferation or cell death during the selection process or within the stable pools. In any case, we were able to generate Ets1 overexpressing clones. Our Ets1 overexpressing clones had varying levels of Ets1 with no correlation to the expression levels of E-cadherin or vimentin (Fig 3.14). In fact, the only consistent phenotype we observed in the MCF-10A Ets1 clones was an increase in transwell invasion (Figure 3.15). Even here, there was no direct correlation between Ets1 levels and the invasive phenotype, which could possibly be explained by the harsh selection process required to generate stable cell clones. We additionally determined that the

MCF-10A Ets1 clones had no significant changes in proliferation or anchorage-independent growth. It appears that, at least in the MCF-10A cell line, Ets1 has an apparent role in transwell invasion, but not proliferation or anchorage-independent growth. These data and data from others suggest that Ets1 may be more of an effector of a larger process governing migration, invasion or transformation, and its role within a certain process is dependent upon the cellular context. This appears to be the case with Snail or TGFβ inducing EMT in part through the upregulation of MMP2 and MMP9 by Ets1 (Jorda et al., 2005; Taki et al., 2006). Future studies will likely focus on Ets1 regulation by protein-protein interactions within differing cellular environments in order to elucidate its role in migration, invasion, and transformation.

V. Significance and Speculation

Of the small heat shock proteins, αB-crystallin is one of the most studied and well characterized. It has established roles in development, preventing apoptosis, and significant to our studies here; αB-crystallin expression in breast cancer is linked to high-grade tumors, basal-like breast tumors, metaplastic tumors, poor prognosis, and resistance to neoadjuvant chemotherapy (Chelouche-Lev et al., 2004; Ivanov et al., 2007; Moyano et al., 2006; Sitterding et al., 2008). Recent studies identified ways in which αB-crystallin could promote cancer; including increased MAPK signaling, conferring resistance to apoptosis, and increased invasion (Moyano et al., 2006). When looking into the regulation of αB-crystallin, none have demonstrated regulation of αB-crystallin expression by a transcription factor in a cancer model. Studies to date on the regulation of αB-crystallin focus on lens and muscle tissues using primarily non-human systems. A

general lack of information on the regulation of αB-crystallin in human cancer cell lines stresses the importance of this investigation on the transcriptional regulation of αB-crystallin in human breast cancer. We have demonstrated that Ets1 is a novel regulator of human αB-crystallin gene expression in breast cell lines. This provides a possible explanation for the observation of αB-crystallin expression in basal-like breast cancer phenotype by demonstrating Ets1 regulation of αB-crystallin gene expression. This is especially significant given αB-crystallin's relevant roles in breast cancer and the lack of a specific treatment for the basal-like breast tumors.

As mentioned previously, most basal-like breast tumors initially respond well to conventional therapy, but the basal-like tumors with residual disease have poor prognosis and worse overall survival (Liedtke et al., 2008). It would be interesting if Ets1 and αB-crystallin played a role in the angiogenesis of the residual disease of basal-like tumors. Ets1 is a well-known regulator of VEGF (Hashiya et al., 2004; Mukherjee et al., 2003; Valter et al., 1999), and VEGF is a key player in angiogenesis. VEGF signals in part through MAPK proteins, which αB-crystallin has been shown to help maintain signaling activity. Therefore, Ets1 could regulate both VEGF and αB-crystallin under certain conditions in which both targets work together towards angiogenesis. Under this theory, studying the efficacy of VEGF inhibitors (e.g. Lucentis®/Ranibizumab (antibody), Avastin®/Bevacizumab (antibody), or Sutent®/Sunitinib (small molecule)) in combination with the standard chemotherapy could be a way to more specifically treat basal-like breast cancer. The VEGF inhibitors are currently used for metastatic colon carcinomas and non-small cell lung cancer, and in both cases some resistance to the

VEGF inhibitors has been observed (Baka et al., 2006; Ton and Jayson, 2004). Could αB-crystallin have a role here? Inhibiting VEGF or another RTK usually results in cell death or cell cycle arrest, but αB-crystallin could in theory help a tumor with angiogenesis by preventing apoptosis (this role was recently published) through its interaction with caspase-3 (Dimberg et al., 2008) or by working to maintain kinase signaling downstream of the inhibited RTKs. In both of these scenarios, αB-crystallin could act as an enabler for the tumor allowing it to evolve a new mutation to bypass the therapy/stress. This scenario is not limited to just cancer; Lucentis® and Avantis® both are used for adult macular degeneration to prevent angiogenesis of the retina. In theory, if Ets1 or another ETS-family member is overexpressed in the retina of patients with adult macular degeneration, then there is the potential for expression of αB-crystallin and the development of resistance.

A potential advantage of this scheme would be Ets1 regulation of αB-crystallin and VEGF as a potential therapy in patients following a stroke or heart attack. The stress of ischemia has been shown to activate HSF1 (Higashi et al., 1995) and therefore immediate protection from cell death is most likely through HSF1 and multiple molecular chaperones. However, long term protection and neovascularization could be mediated by this Ets1/VEGF/αB-crystallin scheme; a situation in which the presence of all three proteins working together would be beneficial to the patient.

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