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THE ROLE OF ADA3 OVEREXPRESSION IN PROLIFERATION THROUGH ENHANCING MYC EXPRESSION

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Breast cancer is a heterogeneous disease that is the second leading cause of cancer related deaths in women. Cancer is defined as abnormally heightened proliferation. In order for gene transcription and eventual translation to occur to drive the cell cycle to generate more cells, DNA must be uncoiled from nucleosomes by histone acetylation complexes. One of the key evolutionarily conserved components of these HAT complexes is alteration/deficiency in activation 3 (ADA3). In addition to the role in histone acetylation, this protein also functions as a coactivator for nuclear hormone receptors. Recent findings indicated that nuclear Ada3 correlates with ER+ breast cancers and a favorable survival, while cytoplasmic (overexpression) Ada3 correlates with ErbB2+ and EGFR+ breast cancers and a poor survival.

My thesis work focused on the role of Ada3 knockdown and overexpression on cellular proliferation in human derived cell lines. We showed knockdown of ADA3 in immortal hMECs led to inhibition of cell cycle progression, decrease in cyclin B, c-myc or H3K56 levels, and increase in p27 protein. In contrast, ADA3 overexpression in two immortal hMECs and two ER+ breast cancer cell lines enhanced proliferation. Cell cycle analyses showed increased cyclin B and decreased p27 upon ADA3 overexpression. Decreased p27 levels were due to increased turnover of p27 protein. Furthermore, ADA3 overexpression led to increased mRNA levels of early response genes c-fos, EGR1, and c-myc. Analysis of a large cohort of ER+ breast cancer tissue specimens showed a subset of ER+ tumors express higher levels of ADA3 and these tumors also

express higher levels of c-myc ($p < 0.0001$). Overexpression enhances proliferation through increasing c-myc expression, and expression patterns with ADA3 high/low and c-myc high/low can divide patients into four subgroups with significant differences in molecular biomarkers' status and clinical-pathological parameters. Thus, we suggest ADA3 nuclear overexpression in ER+ breast cancer patients can serve as a biomarker to predict tumor progression. In order to better decipher the role of ADA3 in the murine mammary gland I generated a tamoxifen inducible deletion mouse model for use in future studies.

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ABBREVIATIONS

ADA3	alteration/deficiency in activation 3
ER	estrogen receptor
HAT	histone acetyltransferase
HDAC	histone deacetylase
MEF	mouse embryonic fibroblast
PCR	polymerase chain reaction
Rb	retinoblastoma
GFP	green fluorescent protein
SWI/SNF	Switch/sucrose nonfermentable
Gcn5	general control nonderepressible 5
PCAF	p300/CBP-associated factor
CBP	CREB-binding protein
SAGA	Spt-Ada-Gcn5 acetyltransferase complex
SLIK	SAGA-like complex
CDK	cyclin dependent kinase

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Gene transcription is an integral part of all cellular processes, as proper cellular function depends on its final downstream products, proteins. However, genomic DNA in eukaryotic cells exists in multiple ordered states ranging from the highly compacted chromosome to the transcriptionally accessible double helix. As a result of this compaction, most chromatin is not transcriptionally active and is referred to as heterochromatin. In order for cells to begin the transcriptional process to generate mRNA which is then translated into new proteins, the highly ordered DNA must made more readily accessible to the transcriptional machinery by being converted into the active euchromatin. Therefore it is necessary that the DNA be uncoiled from the nucleosome units composed of an octamer of histones around which it is wrapped (Luger et al., 1997). The histone octamer is composed of two copies of each of the four primary histone proteins: H3, H4, H2A, and H2B. This central set of proteins provides the basic structural unit for the compaction of DNA.

The post-translational modification of these eight core proteins allow the conversion of heterochromatin to euchromatin and thus grant the transcriptional machinery access to the DNA (Strahl & Allis, 2000). The key post-translational modification responsible for this interconversion is acetylation. Without the addition of these C_2H_3O functional groups to the core histones, chromatin would remain compact and inaccessible to DNA binding proteins (Strahl & Allis, 2000). Consequently, the process of histone modification is widely conserved across all eukaryotes, making it of great interest to biologists studying both normal and cancerous cells (Grunstein, 1997). An ever changing state of acetyl group addition and removal exists governed by opposing proteins named histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Kouzarides, 1999). Both of these post-translational modification causing

proteins function in concert with multiple other proteins in complexes, thus forming HAT and HDAC complexes that can work in governing gene transcription. Of greater interest to us are the HAT complexes, as they contain alteration/deficiency in activation 3 (Ada3), one of the primary proteins studied by our lab.

1.1 EVOLUTIONARY CONSERVATION OF ADA3

HAT containing protein complexes usually consist of multiple subunits that are evolutionarily conserved (Lee & Workman, 2007). The HAT protein complex named alteration/deficiency in activation, or ADA complex, was first discovered in yeasts during the 1990s. The ADA complex is composed of the adaptor proteins Ada2 and Ada3 in addition to ADA HAT complex component 1(AHC1), and the core histone acetylase general control nonderepressible 5 (Eberharter et al., 1999). The primary function of the adaptor proteins is to act as binding partners to keep the different subunits of the complexes in close proximity through protein binding motifs. The proteins of the Ada family (Ada1-5) were originally found to be involved in resistance when mutated (Marcus et al, 1994; Marcus et al., 1996). Specifically, the Ada3 protein was initially discovered due to it promoting resistance to toxicity induced by exposure to GAL4-VP16 (Pina, et al., 1993). The ADA complex primarily functions in the regulation of gene transcription in yeasts (Grant, et al, 1997; Grant, et al, 1998). A subset of these ADA complex components (Ada2, Ada3, and Gcn5) functions in two other yeast complexes named the Spt-Ada-Gcn5 acetyltransferase complex and the SAGA-like (SLIK) complex to promote transcription (Grant, et al 1997; Pray-Grant et al, 2002). This is done by recruiting RNA polymerase II to all active gene promoters (Bonnet et al, 2014). The evolutionary importance of these subunits continues into mammalian cells where they form the

homologous Spt3/Taf9/Gcn5 (STAGA) complex, as well as TBP-free TAF complex (TFTC), and Ada2a-containing (ATAC) complex (Lee & Workman, 2007).

Within mammalian cells the HAT containing complexes are much more complex than in the yeast counterparts. Gcn5 was the core HAT in yeasts; however as the complexes proceed up the evolutionary ladder, HATs can work singly or in concert with one another. Such examples of mammalian HATs include p300, Tip60, p300/CBP-associated factor (PCAF), and CREB-binding protein (CBP) (Lee & Workman, 2007; Nagy & Tora, 2007). The Ada3 adapter remains, however the Ada2 adapter has a pair of homologs known as Ada2a and Ada2b with different functions (Kusch, et al, 2003), leading to more complex and varied HAT complexes in higher eukaryotes.

1.2 ADA3 COMPLEXES AND HISTONE ACETYLATION

Histone acetylation leading to changes in chromatin structure is critical for the transcriptional machinery to gain access to DNA. As such, the HAT complexes play a crucial role in this loosening of the highly ordered chromatin. The yeast ADA complex, through Gcn5, controls histone acetylation levels of multiple gene promoters important to the cell (Grant et al, 1998). However without both Ad2 and Ada3, Gcn5 cannot properly acetylate histones to unwind DNA from the nucleosomes (Balasubramanian et al, 2002). These three proteins also form the core of the yeast SAGA complex, which was previously mentioned as an important HAT complex within yeast cells (Balasubramanian et al, 2002). Histone H3 has been shown to be the most favored target for ADA complex acetylation, with modifications added to lysines 9, 14, and 18 (Balasubramanian et al, 2002). Additionally, they found that in order for Gcn5 to acetylate nucleosomes, Ada3 must be present and this process is augmented by the presence of Ada2 (Balasubramanian et al, 2002). In the mammalian STAGA complex, Gcn5, in concert

with Ada3 and Ada2b, has been found to acetylate nucleosomes. However, when Ada2b and Ada3 were absent, acetylation was suboptimal (Gamper et al, 2009). When Ada2a was substituted for Ada2b, the complex was able to form, but acetylation did not occur, thus demonstrating that the pair of Ada2 homologs has separate functions in mammalian cells, just as there are separate functions in *Drosophila* (Gamper et al, 2009). When Ada2a is deleted in *Drosophila* there is no change in the levels of acetylation of H3K9 or H3K14 controlled by Ada2b (Pankotai et al, 2005). When Ada3 is deleted in *Drosophila* embryo viability is greatly decreased (Grau et al, 2008). Building upon this, when Ada3 is deleted in mammalian cells, the acetylation levels of multiple lysine residues are affected. These include H2AK5, H2BK5, H3K9, H3K56, and H4K8 (Mohibi, et al, 2012). Thus when any members of this core complex are downregulated the ability of a cell to properly acetylate nucleosomes to allow the subsequent transcription of DNA is greatly reduced.

Histone acetylation typically occurs concurrently with the remodeling of chromatin in order for proteins that interact with DNA to gain access to it. The HAT complexes work with Switch/sucrose nonfermentable (SWI/SNF) to remodel chromatin by displacing nucleosomes to make DNA more accessible for transcriptional initiation (Collingwood et al, 1999). Chromatin remodeling governed by nucleosome sliding occurs at promoter sites or sites of DNA damage and is controlled by the SWI/SNF complex (Martens & Winston, 2003). Recruitment of this complex is thought to coincide with SAGA mediated histone acetylation to displace nucleosomes for promoter accessibility (Mitra, et al, 2006; Chandy et al, 2006). In yeast, Gcn5 mediated acetylation of the Snf2 subunit controls the interaction of SWI/SNF with histones (Kim et al, 2010). Thus, chromatin remodeling complexes are dependent upon HAT complexes to carry out proper structural changes to allow DNA binding proteins access to initiate transcription.

1.3 ADA3 FUNCTIONS AS A CO-ACTIVATOR FOR NUCLEAR HORMONE RECEPTORS

In general, for nuclear receptors (NRs) to function they bind their respective ligands and translocate to the nucleus to initiate target gene transcription. The level of gene expression is governed by the specific coregulators with which the NR interacts (McKenna & O'Malley, 2002). Coregulators which promote target gene expression are considered co-activators, while ones that impede expression are considered co-repressors. The basic functional structure used by coactivators to interact with NRs is through a leucine (L) rich helical domain commonly called an LXXLL motif, where X in any amino acid that binds the NR ligand binding domain (He & Wilson, 2003). Ada3 is such a protein containing this LXXLL motif, and functions as a coactivator.

The role of Ada3 as a coactivator was first discovered in yeasts expressing exogenous nuclear receptors. The complete yeast ADA complex was found to transactivate the glucocorticoid receptor, and ablation of Ada3 led to greatly reduced transactivation (Henriksson, et al, 1997). It was later found that yeast Ada3 interacts with estrogen receptor alpha (ERα), thyroid hormone receptor alpha (TRα), retinoid X receptor alpha, (RXRα), but not with retinoic acid receptor alpha (RARα) (vom Baur et al, 1998). This led to earlier work performed in our laboratory where it was found that Ada3 serves as a coactivator for ERα and RXRα, leading to increased levels of the respective target genes as well as increased transactivation potential (Zeng et al, 2002; Meng et a;, 2004; Germaniuk-Kurowska et al, 2007). Later work by other researchers found that the LXXLL motifs present within Ada3 do indeed interact with the RAR receptor (Let et al, 2010). This work demonstrates that the coactivator function of Ada3 is very important to nuclear receptor target gene transcription. Of great interest to our lab is the interaction of Ada3 with the signaling of the estrogen receptor.

Estrogen receptor alpha (ERα), a 66kDa protein, is composed of four primary domains and is a member of the steroid hormone receptor family. The amino terminus of ER contains the activation functions (AF) domain AF-1 and it is constitutively active in the absence of ligand. AF-1 is subsequently followed by a DNA binding domain, a hinge/nuclear localization region, and the ligand binding domain/AF-2 region that is only activated when bound by estrogen. The phosphorylation of ER on serine 167 in the AF-1 domain by AKT following growth factor signaling leads to ER activation and dimerization (Osborne et al., 2001). Additionally, phosphorylation of serine 118, another important serine residue for ER activation, is mediated by MAPK signaling during estrogen stimulation (Chen et al., 2002). Two truncation mutants exist that lack the AF-1 domain, ERα-46 and ERα-36, though the role of these in breast cancer is not well characterized (Nilsson et al., 2001). Interestingly, the truncation mutant ERα-36, which lacks the AF-1 domain as well as part of the LBD/AF-2 domain, has primarily been identified as part of the cell membrane in patient samples and implicated in anti-estrogen resistance (Shi et al., 2009). Signaling by ER is directed by the AF domains and can be subdivided into four major types, three dependent on and one independent of estrogen stimulation. The direct classical ligand dependent ER signaling pathway consists of estrogen binding the receptor in the cytoplasm, dimerizing with a second receptor, and translocating to the nucleus to bind estrogen response elements (EREs). Nuclear receptor coactivators function in transcriptional activation by linking receptors, chromatin remodeling, and the transcriptional machinery together (Collingwood et al., 1999.) Active ER dimers in the presence of estrogen recruit histone acetyltransferases and coactivators for chromatin remodeling and transcription of ER target genes such as the progesterone receptor, Cyclin D, c-myc, pS2, and cathepsin D (Lin et al., 2004; Heldring et al., 2007; Thomas & Gustafsson, 2011). One well defined family of NRCs is the p160 family, composed of the steroid receptor coactivators 1-3. SRC-3, a coactivator well

characterized by the O'Malley lab, has been shown to bind ER and histone acetyltransferases in the presence of estrogen stimulation to enhance transcription of ER target genes during normal development as well as during the progression of breast and other cancers (Xu et al., 2009.) Our laboratory has shown through *in vitro* experiments that the protein alteration/deficiency in activation 3 (Ada3) binds to ER regardless of estrogen stimulation. Further examination of Ada3 and ER interactions revealed Ada3 to be a coactivator necessary for ER directed gene transcription (Meng et al., 2004). Successive knockout studies by our lab found that Ada3 is required for histone acetyltransferase (HAT) recruitment to estrogen dependent promoters and estrogen mediated breast cancer cell proliferation (Germaniuk-Kurowska et al., 2007). Recently, our Ada3 coactivator and HAT complex interaction findings have been reproduced in cell free systems by the O'Malley lab (Foulds et al. 2013). Another member of the p160 family, SRC-1, functions as a coactivator in second type of estrogen dependent signaling (Xu et al., 2009.) This form of signaling transpires following ligand induced receptor dimerization after which the ER dimer interacts with general transcription factors that tether it to DNA to induce transcription at sites other than EREs. ER associates with the transcription factors Fos/Jun to facilitate binding to the non-ERE AP-1 sites which are responsible for the expression of genes such as insulin like growth factor 1 (IGF-1) and collagenase (Hall et al., 2001; Kushner et al., 2000). Insulin modulated signaling has been found to synergize with ER signaling when ER causes an upregulation of IGF-1R and its downstream pathway components (Lanzino et al., 2008). The third method of estrogen dependent signaling requires ER to bind estrogen in the cytoplasm to activate second messengers and induce ion channel alterations, nitric oxide synthesis, or phosphorylation of transcription factors that directly bind DNA (Hall, et al., 2001). Lastly, ligand independent ER signaling involves the activation of IGFR or EGFR by their respective growth factors to activate ERK and AKT kinases which in turn

phosphorylate ER. This then activates ER mediated transcription (Thomas & Gustafsson, 2011). Of these four types of ER directed signaling, only non-genomic signaling mediated by phosphorylated transcription factors does not require coactivators, denoting the importance of coactivators in signaling events. The classical ligand dependent ER signaling pathway is potentially the most important to our laboratory due to Ada3 being defined as a coactivator for ER. The myriad of ER signaling methods is complex; however, their overall goal is to activate gene transcription by recruiting the transcriptional machinery to chromatin that was made accessible by HAT complexes.

1.4 ADA3 AND THE CELL CYCLE

It is well known that eukaryotic cells must pass through the G1, S, G2, and M phases of the cell cycle in order to undergo cell division. Progression through these phases is tightly controlled and regulated by many interacting proteins (Schafer, 1998). G1 is the first and longest phase of the cell cycle during which the cell prepares for division by ensuring conditions are favorable for division. During transition out of G1 and into the S phase, cyclin D binds cyclin dependent kinase (Cdk) 2/4 to phosphorylate retinoblastoma (Rb) causing the release of the transcription factor E2F1. E2F1 stimulates the transcription of multiple genes involved in the cell cycle and proliferation, during which it uses GCN5 and TRRAP as cofactors (Lang et al., 2001). Ablation of TRRAP, a protein also known to interact with GCN5, has been shown to alter cell cycle progression (Herceg et al., 2001). One E2F1 target gene, cyclin E, drives the transition from G1 to S phases. Recently, after a detailed analysis using conditional deletion in a MEF system, our lab found that Ada3 regulates progression of the cell cycle from G1 to S phase through a c-myc-Skp2-p27 mechanism (Mohibi et al, 2012). ER mediated signaling stimulates SKP2, an E3 ubiquitin ligase, to transactivate E2F1 to amplify cyclin

E production (Zhou et al., 2014), thus this mechanism could function in cancer cells. Increases in cyclin E induce Rb hyperphosphorylation and subsequently lead to Rb deactivation, allowing the cell to continue through the cell cycle. When Ada3 is deleted the levels of phospho-Rb are decreased (Mohibi et al, 2012). However, Rb phosphorylation status is not the only hurdle in the early stages of cell cycle progression to the S phase. The tumor suppressor p27 inhibits cyclin D and the cyclin D/ Cdk4 complex from phosphorylating Rb until the levels of the cyclin E/Cdk2 complex are high enough to induce ubiqutination of p27, causing degradation. Of note, Ada3 ablation causes an increase in the stability of p27 leading to an inhibition of the cell cycle via the aforementioned pathway, but when p27 is removed the cell cycle progression is partially rescued (Mohibi et al, 2012).

Interestingly, p27 ablation positively affects estradiol mediated ER target gene transcription while overexpression of p27 negatively regulates ER mediated transcription by sequestering ER to the cytoplasm and by interfering with ER/ cyclin D interactions that initiate the cell cycle, thus the levels of p27 influence transcription with respect to ER mediated transcription (Jeon, et al., 2012). During DNA synthesis in the S phase, HAT complexes acetylate nucleosomal histones to modify the chromatin structure to allow transcriptional factors to gain access to synthesize specific genes. Post replication, the G2 phase begins during which the cell prepares for M phase or mitosis, by transcribing mitotic genes that are made accessible by HAT complexes. Our lab recently found that Ada3 interacts with centromere protein B (CENP-B). The Once these proteins are fully synthesized, chromosomes replicated in S phase are equally divided between the daughter cells. However, if Ada3 is deleted from cells chromosomes cannot properly segregate, thus leading to an increase in anaphase bridging (Mohibi et al, 2015). Following the completion of accurate division, the cells return to the G1 phase to await

stimulation by growth factors to begin preparations for further divisions. However, when the cell cycle goes unchecked and cells proliferate out of control cancer can develop.

1.5 ADA3 AND CANCER

While ADA3 must be important in several cancers, our research is particularly focused on breast cancers and therefore we have analyzed role of ADA3 in breast cancers. Breast cancer is a heterogeneous disease that is the second leading cause of cancer related deaths in women. Historically breast cancer was solely classified by histological features and hormone receptor expression (Schnitt, 2010; Ignatiadis & Sotiriou, 2010). Histological classification can be split between invasive and *in situ* with each being subdivided further by growth and structure characteristics (Malhotra et al., 2010). Despite these histological divisions, treatment regimens were mainly generalized based upon the estrogen receptor (ER), progesterone receptor (PR), and HER2 receptor status. In the past 15 years these two main forms of identification have been joined by a third type of stratification: gene expression profiling. Largely due to the work of Perou and colleagues we now know there are six molecularly different subtypes of breast cancer, each with a different gene profile (Perou et. al, 2000). These subtypes include luminal A (50-60%), luminal B (10-20%), HER2+/ER- (10-15%), basal-like (10-20%), claudin low (12-14%), and normal breast-like (5-10%) (Eroles et al, 2012). This division into subtypes has greatly helped to stratify patients for more helpful therapeutic intervention and prediction of potential causes of future disease relapse. Each subtype has intrinsic differences in susceptibility to chemotherapy and anti-endocrine therapy. While basal-like breast cancer may be the most difficult to treat due to a lack of targetable receptors, the most prevalent subtype of breast cancer is luminal A, expressing both the estrogen receptor (ER) and its transcriptional target, the

progesterone receptor (PR), while lacking the HER2 receptor tyrosine kinase. Luminal A tumors are susceptible to anti-estrogen therapy, the accepted standard of treatment for such breast cancers in premenopausal women. Resultantly these drugs are administered to kill tumor cells in addition to surgical removal of the tumor. In some cases anti-estrogens are administered after surgery as an adjuvant to limit potential recurrence if node status, tumor size, stage, and genomic prediction arrays warrant it (Ignatiadis & Sotiriou, 2010). The compounds administered to patients during anti-estrogen therapy function to antagonize growth by competitively inhibiting the binding of estrogen with ER to interfere with signaling pathways and target gene transcription that direct cellular proliferation and cell cycle progression. The luminal B subtype of ER+ breast cancer can be PR- and even HER2+. This second type of luminal tumors have a higher proliferative marker staining (Ki67) than their luminal A counterparts (Ignatiadis & Sotiriou, 2010). Combining the more heterogeneous receptor profile of the luminal B subtype with luminal A demonstrates that approximately 70% of all breast cancers are ER+, thus since Ada3 functions as an ER coactivator it could have an important role in nearly 3/4 of breast cancer.

Previously we demonstrated that ADA3 binds to ER. Through the use of sedimentation gradients and co-immunoprecipitation experiments it was determined that Gcn5, PCAF, and p300 all associate with ER and ADA3. ChIP analysis demonstrated that ADA3 knockdown with shRNA led to decreased Gcn5, p300, and PCAF at active pS2 promoter sites in the ER+ MCF-7 and ZR-75-1 cell lines. Subsequent analysis found that ADA3 knockdown in these ER+ cell lines led to decreased estrogen dependent proliferation both in culture dishes and in Matrigel. Thus demonstrating ADA3 was integral to the transcription of ER target genes and estrogen dependent breast cancer cell proliferation.

Recent work performed by our lab was able to determine the effect of Ada3 expression on the different subtypes of breast cancer. Due to Ada3 being a nuclear protein, localization into the cytoplasm would denote a form of overexpression or mislocalization. Mirza et al found that cytoplasmic/overexpression of Ada3 correlated with poor prognosis and EGFR+/ErbB2+ breast cancer patients; while nuclear Ada3 correlates with a good prognosis and ER+ breast cancer patients (2012).

Recent unpublished data from our laboratory demonstrates nuclear overexpression of ADA3 is seen in a subset of ER+ breast cancers; these tumors also overexpress c-myc, as well as express high levels of the proliferation marker Ki67. Interestingly, c-myc is observed to be amplified in 15% of breast cancers (Xu et al, 2010). When overexpressed, prognosis of patients is decreased and is connected to the appearance of endocrine therapy resistance (Xu et al, 2010; Dang, 2012; Wolfer et al, 2010). ER+ breast cancer cell lines long term deprived of estrogen develop altered proliferative abilities and an altered gene expression profile that correlates with myc mediated endocrine therapy resistance (Miller, T.W. et al, 2011.). During periods of low nutrient availability, therapy resistant cell lines with increased c-myc expression can metabolize glutamine and glucose (Shajahan-Haq, et al, 2014). C-myc was additionally seen to independently predict distant metastases-free survival in endocrine therapy treated luminal A patients who were lymph node positive (Green et al, 2016). Taken together these results suggest the ADA3 complex regulates c-myc mediated proliferation in these breast cancers and may be involved in endocrine therapy resistance.

CHAPTER 2

ADA3 overexpression promotes estrogen receptor-positive breast cancer cell proliferation through c-myc

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2.1 INTRODUCTION

Coordination of cell cycle progression with chromosomal duplication maintains genomic stability; a critical cancer-associated trait (Schvartzman et al, 2010). Deregulated cell cycle components have now also emerged as key biomarkers and therapeutic targets in cancer (Lapenna & Giordano, 2009). Thus, a better understanding of cell cycle machinery and its aberrations in cancer are of fundamental importance in cell and cancer biology. Among cell cycle regulatory pathways, histone acetylation has emerged as a conserved mechanism that is invariably altered in cancer (Kouzarides, 1999)as it plays key roles in chromatin assembly, accessibility to transcription and replication machineries, and genome stability (Peng & Karpen, 2007). Steady-state levels of histone acetylation and its dynamic changes represent a balance between HATs and HDACs (Kouzarides, 2007). Given the therapeutic efficacy of relatively general HDAC inhibitors in cancers (Johnstone, 2002), it is likely that more targeted agents to alter acetylation in cancer cells will provide improved anticancer strategies. Despite these promising prospects, the physiological regulation of histone acetylation in relation to cell cycle progression in epithelial cells, precursors of most human cancers, remains incomplete.

Breast cancer is the single most common malignancy in women (Malhotra et al, 2010). Towards identifying novel regulators of cell cycle in hMECs, we previously used the dominant hMEC-immortalizing HPV16 E6 oncogene and identified ADA3 as a E6-binding protein (Kumar et al, 2002) and showed its coactivator function for p53 (Kumar et al, 2002; Nag et al, 2007), retinoic acid (Zeng et al, 2002), and estrogen receptors (ER) (Meng et al, 2004; Germaniuk-Kurowska et al, 2007) transactivation; others showed its role in androgen receptor (Zhao et al, 2008). We observed in breast cancer

cells ADA3 is in a large complex that include components of the yeast Ada complex (Ada2, Ada3 and GCN5, a HAT) but also other cell cycle- and cancer-associated HATs, p300 and PCAF (Germaniuk-Kurowska et al, 2007). ADA3 is also a component of other HAT complexes such as the Spt3/Taf9/Gcn5 acetyltransferase complex (STAGA) and the Ada2a-containing (ATAC) complex (Spedale et al, 2012). We demonstrated that ADA3 is essential for p300-mediated p53 acetylation (Nag et al, 2007). Together, these studies suggested a potentially important role of ADA3 in breast cancer.

To explore the physiological function of ADA3, we engineered Ada3 fl/fl mice that showed germline homozygous deletion of Ada3 was early embryonic lethal (Mohibi et al, 2012). The most dramatic result of conditional deletion of Ada3 in Ada3 fl/fl MEFs was defects in cell cycle progression, including delayed G1 to S transition, mitotic catastrophe, and defective cytokinesis (Mohibi et al, 2012), suggesting lack of coordination between DNA replication and subsequent cytokinesis, a precursor for accumulation of DNA damage and genomic instability (Blow & Tanaka, 2005). Indeed, Ada3-null MEFs exhibited increased basal levels of DNA damage response, a delay in the repair of γ-irradiation-induced DNA damage; and increased chromosomal aberrations that increased further upon DNA damage (Mirza et al, 2012), suggesting critical roles of histone acetylation in cell cycle-associated transcription, chromatin assembly around newly-synthesized DNA; resolution of stalled replication forks and replication-coupled DNA damage repair (Lukas et al, 2011; Alcasabas et al, 2001). Loss of Ada3 in MEFs was associated with markedly reduced acetylation of core histones and levels of p300 and PCAF (Mohibi et al, 2012). Another study using RNAi knockdown showed the role of Ada3 in G2/M progression (Orpinell et al, 2010). Together, these studies demonstrate an essential role of Ada3 in cell cycle progression in MEFs and in tumor cell lines (Kumar et al, 2002; Nag et al, 2007; Zeng et al, 2002; Meng et al, 2004; Germaniuk-Kurowska et al, 2007; Mohibi et al, 2012; Mirza et al, 2012).

Further studies from our laboratory examined the expression of ADA3 in over 900 breast cancer tissue specimens (Mirza et al, 2013) with known clinicopathological parameters and survival data (Zhao et al, 2012). We showed ADA3 was predominantly nuclear in ER+ breast cancers, confirming our previous studies that ADA3 functions as an ER coactivator (Meng et al, 2004; Germaniuk-Kurowska et al, 2007); whereas ADA3 expression was both nuclear and cytoplasmic in ER- breast cancers and this expression pattern correlated with high ErbB2/EGFR status and predicted poor patient survival (Mirza et al, 2013).

In this study, we first confirmed our previous studies in ADA3fl/fl MEFs in immortal hMECs where ADA3 deletion led to delayed cell cycle progression. Detailed analyses in this system revealed the same Ada3-c-myc-Skp2-p27 axis that controls G1 to S phase progression and partly contributes to cell cycle delay upon Ada3 deletion. Additionally, loss of Ada3 showed dramatic decrease in acetylation of H3K56 that is known to play an important role in cell cycle. Next, we examined the consequence of ADA3 overexpression in immortal hMECs as well as in ER+ breast cancer cell lines. ADA3 overexpression in both immortal hMECs, as well as in cancer cell lines dramatically enhanced cellular proliferation. Cell cycle analyses of ADA3 transfectants showed increased cyclin B, c-myc and decreased p27 levels. ADA3 overexpression led to increase in mRNA levels of early response genes c-fos, EGR1 and c-myc levels. Analysis of a large cohort of ER+ breast cancer tissue specimens showed a subset of ER+ tumors express higher levels of ADA3, c-myc, and Ki67. Comparison of four groups (ADA3 high/c-myc high, ADA3 high/ c-myc low, ADA3 low/c-myc high and ADA3 low/c-myc low) showed significant a difference in cancer molecular subtypes, tumor grade, Nottingham Prognostic Index, and tumor types. Thus, ADA3 expression in ER+ breast cancers can serve as a marker for tumor progression.

2.2 MATERIALS & METHOD

Cells and Media: 76NTERT and 81NTERT, two immortalized human mammary epithelial cell lines, were grown in DFCI-1 medium, as described earlier (Band et al, 1990; Band & Sager 1989). MCF-7 and ZR-75-1 cell lines were grown in alpha-MEM supplemented with 10% fetal calf serum. For estradiol starvation and stimulation experiments, MCF-7 and ZR75-1 cell lines were deprived in phenol red-free MEMA media (ThermoFisher Scientific, Waltham, MA, USA) supplemented with 5% charcoal stripped fetal calf serum (Atlanta Biologicals, Flowery Branch, GA, USA) and stimulated with 1nM β-estradiol (Sigma, St. Louis, MO, USA) for synchronization experiments (Germaniuk-Kurowska et al, 2007).

Antibodies: Generation of anti-hAda3 mouse monoclonal antiserum has been described previously (Mohibi et al., 2012). Antibodies against cyclin D1, cyclin E, ERα, Hsc70, PARP, and β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX); SV-40, p27 and Cyclin B1 from BD Biosciences (San Jose, CA); c-MYC from Abcam, Inc (Cambridge, MA); Ki-67 from Dako (Carpinteria, CA); GAPDH from Cell Signaling (Danvers, MA); and H3 and H3K56 antibodies from Millipore (Billerica, MA).

IHC Analysis: Cut sections of paraffin embedded patient derived xenograft (PDX) ER^+ tumors samples were processed for ADA3, ERα and Ki-67 IHC staining using the method as described previously (Mirza, et al, 2013).

Generation of stable hADA3 shRNA knockdown cells and Ada3 overexpressing cells: The hAda3-specific RNAi sequence used in shRNA constructs is as follows:

GCAATCAGAACAAGCCCTT. The oligonucleotide was cloned into the pSUPER-Retro vector (OligoEngine, Seattle, WA). 76NTERT cells were infected with hAda3 RNAi retroviral supernatants as described previously (Germaniuk-Kurowska, et al., 2007). Virally transduced cells were selected in 0.5 µg/ml puromycin for 3 days, and expression of endogenous hAda3 was assessed in the whole cell lysate using Western blotting using anti-Ada3 monoclonal antibody generated in our laboratory (Mohibi et al., 2012). The overexpressing constructs were generated by cloning the oligonucleotides in the pMSCV-Retro vector. 76NTERT, 81NTERT, MCF7, and ZR75-1 cells were infected with hAda3 overexpression retroviral supernatants as described in the same manner as shRNA infection. Virally transduced cells were selected in 0.5 µg/ml puromycin for 3 days, and expression of endogenous hAda3 was assessed in the whole cell lysate using Western blotting using anti-Ada3 monoclonal antibody generated in our laboratory (Mohibi et al., 2012).

Proliferation assay and cell cycle analysis: To perform proliferation assays 76NTERT and 81NTERT cells were plated at different numbers in 6 well plates in triplicates [5.0 x 10^4(for counting on day 3), 2.5 x 10^4 (for counting on day 5), 1.25 x 10^4 (for counting on day 7) and 0.625 x 10^4 (for counting on day 9] and counted at the indicated time points. For cell cycle analysis, two days after plating 2 X 10^5 cells in 100 mm culture dishes, cells were synchronized by replacing the complete medium (DFCI-1 medium) with D3 media and incubating for 72 h. Synchronized cells were stimulated with complete medium (DFCI-1 medium) for various time points and harvested and stained with propidium iodide (PI) for FACS analysis. MCF7 or ZR75-1 cells were plated 2 x 10^4 cells per well in 6 well plates in triplicates and counted every two days. For estrogen independent growth analysis MCF7 and ZR75-1 cells were deprived of estrogenic compounds in phenol free MEMA supplemented with 5% charcoal dextran stripped FBS

for 72rs, stimulated with 1nM β-estradiol, and counted every two days. C-myc level analysis in MCF7 cells was performed by 72hrs of estrogen deprivation and stimulation with 1nM β-estradiol.

RNA Extraction and Quantitative Real-time PCR: TRIzol reagent (ThermoFisher Scientific, Waltham, MA) was used to isolate total RNA from cells. 2 µg of total RNA was used for reverse transcriptase reaction using SuperScriptTM II reverse transcriptase (Invitrogen). Real-time PCR quantification was performed in triplicates using SYBR Green PCR master mix (Applied Biosystems) and the primers are c-fos F: GGGGCAAGGTGGAACAGTTATC, c-fos R: TAGTTGGTCTGTCTCCGCTTGG, EGR1 F: ACCTGACCGCAGAGTCTTTTCC, EGR1 R: CAGGGAAAAGCGGCCAGTATAG, c-Myc: TCAAGAGGCGAACACACAAC, c-Myc R: GGCCTTTTCATTGTTTTCCA, β-actin F: ATCGTCCACCGCAAATGCTTCTA, β-actin R: AGCCATGCCAATCTCATCTTGTT, pS2 F: CCGAGGCCCAGACAGAGA, pS2 R: ACAGCAGCCCTTATTTGCAC, PR F: CCATGTGGCAGATCCCACAGGAGTT, PR R: TGGAAATTCAACACTCAGTGCC.

Expression levels were normalized against β-actin RNA levels, and the results were calculated by the ΔΔCt method.

Analysis of the p27 Protein Turnover: For analyzing p27 protein half-life in exponentially growing cells, cells were treated with 25 µg/ml of cycloheximide (Sigma, St. Louis, MO) and harvested at the indicated time points. Total cell extracts were prepared, and equivalent amounts were run on SDS-PAGE and analyzed by Western blotting. The intensity of p27 bands was quantified by densitometry, normalized to β-actin using ImageJ software. Percentage of normalized intensities were calculated and then converted to log values at base 2 and plotted on Y axis against time of cycloheximide treatment represented on X axis (Mohibi et al, 2012).

Nuclear and cytoplasmic fractionation: Nuclear and cytoplasmic fractionation was performed using a purchased kit (ThermoFisher Scientific, Waltham, MA). In brief, cells were trypsinized, pelleted, and washed with 1X PBS. The pellet was resuspended in cytoplasmic extraction buffer 1, vortexed, and incubated on ice prior to the addition of cytoplasmic extraction buffer 2. The tube was spun in a microcentrifuge (~16000 x *g*) before removal of the supernatant (cytoplasmic fraction). Nuclear extraction reagent was added and the tub vortexed periodically over the course of 40 minutes before centrifugation and supernatant removal. Equivalent supernatant volumes were run on SDS-PAGE and analyzed by western blotting. PARP and GAPDH antibodies were used to assess nuclear and cytoplasmic extract purities, respectively.

Statistical analysis of ADA3 and c-Myc IHC expression: ADA3 immunohistochemical (IHC) expression status of ADA3 was determined as per our previous publication (nuclear expression =>1 % was considered positive) (Mirza et al, 2013). In addition, the nuclear IHC expression of c-myc was divided into in negative/weak (myc low) and moderate/strong (myc high) groups, nuclear expression of ADA3 as published previously (Mirza et al, 2013; Green et al, 2016). Accordingly, ADA3 and c-myc co-expression phenotypes were defined as follows: ADA3 low/ c-myc low, ADA3 high/ c-myc low, ADA3 low/ c-myc high , and ADA3 high/ c-myc high. Associations of these combinatorial phenotypes with different clinicopathological, as well as molecular markers were performed. The statistical analysis was performed using Statistical Package for Social Sciences SPSS version 21 for Windows (Chicago, IL, USA). A p value of less than 0.05 (two-tailed) was considered significant. Cut-off values for the different biomarkers included in this study were chosen before statistical analysis. Standard cut-offs were

used for established prognostic factors and were the same as for previously published patient series (Rakha et al, 2009).

Karyotypic analysis: 76NTERT vector or ADA3 overexpressing cells were processed for karyotyping as done previously by our laboratory (Mirza et al, 2012).

Invasion and migration analysis: Cells were deprived of estrogen for 72 hrs in phenol red free MEMA media supplemented with 5% charcoal dextran stripped fetal calf serum. $5.0x10^3$ cells in 500µl of deprivation media were added to the Matrigel or migration insert. Two hours later 700µl of medium containing 1nM β-estradiol was added to the well to serve as the chemoattractant. Inserts were subsequently stained using the Hema 3 kit from Fisher (Waltham, MA) and the cells counted. Invasion and migration of ADA3 cells were normalized with respect to vector controls.

Microarray analyses: Microarray preparation was performed as mentioned previously (Zhao, et al, 2010). Analysis of the array was performed using Ingenuity Pathway Analysis software.

2.3 RESULTS

ADA3 is a nuclear protein in immortal hMECs, ER+ breast cancer cell lines, and is overexpressed in some ER+ breast cancer patient derived xenografts (PDX).

Previous work performed by our laboratory showed Ada3 ablation in mouse embryonic fibroblasts (MEFs) led to delay in cell cycle progression and that cytoplasmic ADA3 expression correlated with poor prognosis and poor survival of ER- breast cancers (Mohibi et al, 2012; Mirza et al, 2013). In this study, we focused on nuclear function of ADA3 using normal hMECs, ER+ breast cancer cell lines and ER+ breast cancer patients' derived tissues.

We initially examined immortal hMECs and ER+ breast cancer cell lines for ADA3 expression and found that ADA3 is differentially expressed (Figure 1A). Next, we performed immunofluorescence staining and subcellular fractionation and found that ADA3 is localized in the nucleus (Figure 1B, 1C). We then examined two ER+ patient derived xenograft (PDX) tumors using immunohistochemistry to assess ADA3 expression. PDX tumors were generated by Dr. Welm's laboratory by transplanting a portion of a tumor obtained from a patient directly into an immunocompromised mouse (DeRose et al, 2011). By using these PDX samples, we observed that the ER+ Sample 3 expressed intermediate levels of ADA3, while Sample 11 expressed high levels of ADA3. Based on the known function of ADA3 in proliferation in MEFs, we assessed if high ADA3 expression correlates with Ki67, a well-known marker of proliferation (Urruticoechea et al, 2005). Notably, ADA3 high tumor expressed much higher levels of Ki67 (Figure 2), hinting towards role of ADA3 in ER+ tumor proliferation.

Figure 1. ADA3 is expressed and shows nuclear localization in the nucleus of normal and cancer mammary cell lines.

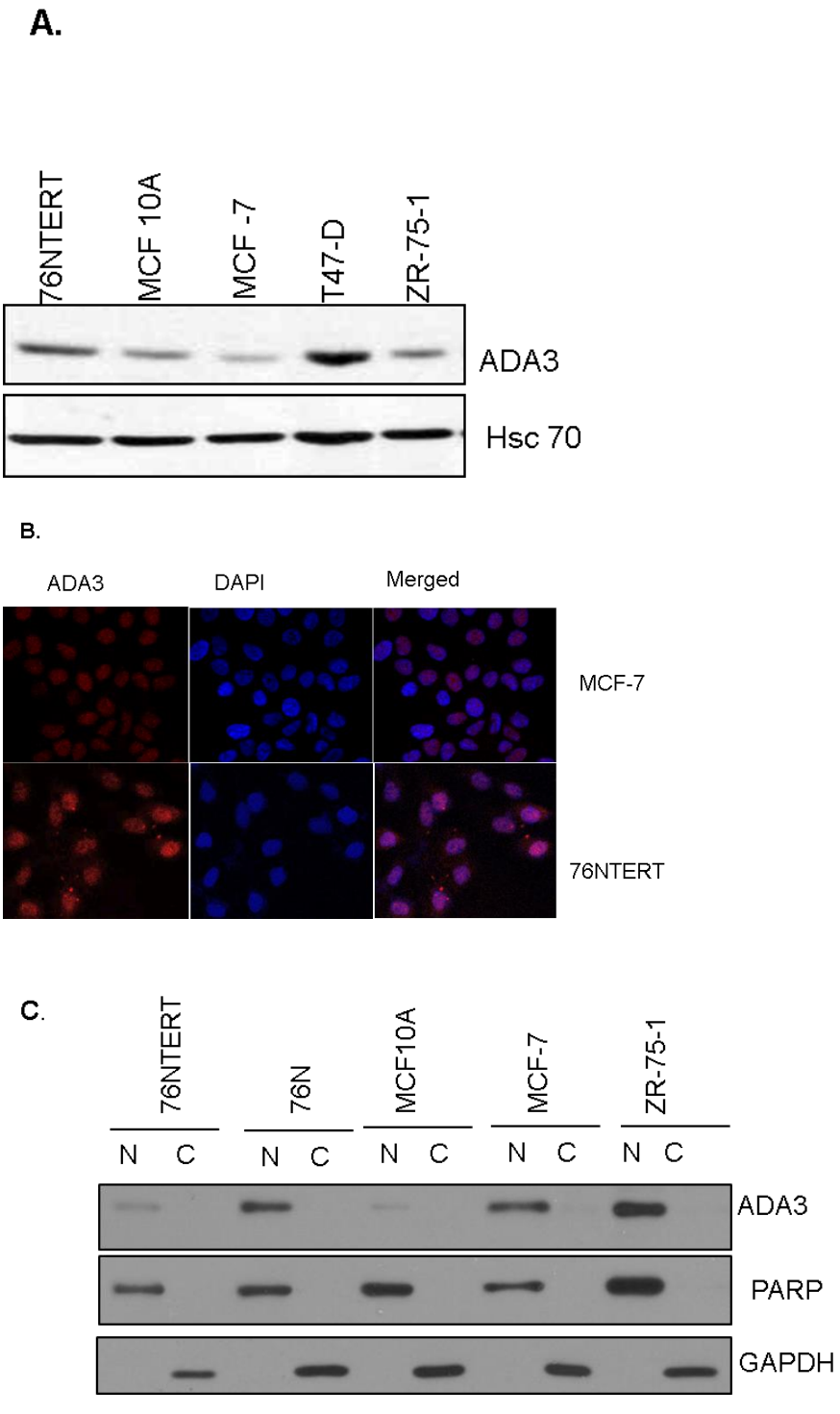


Figure 1. ADA3 is expressed and shows nuclear localization in the nucleus of normal and cancer mammary cell lines. (A) Western blot analysis of ADA3 in immortal hMECs (76NTERT, MCF-10A) and ER+ breast cancer cell lines (MCF-7, T47D, ZR-75-1). **(B)** ADA3 immunofluorescence staining in MCF-7 and 76NTERT cell lines, DAPI was used for nuclear staining. **(C)** Nuclear and cytoplasmic fractions were prepared from normal mammary epithelial cell (76N), immortal mammary epithelial cells (76NTERT and MCF-10A), and ER+ breast cancer cell lines (MCF-7, ZR-75-1). Protein concentration was quantitated and equal amount of protein was separated on SDS-PAGE gel and expression was assessed with anti-ADA3, PARP or GAPDH. PARP was used as nuclear control, and GAPDH was used as cytoplasm control.

Figure 2. High ADA3 expression in patient derived xenografts correlates with elevated Ki67.

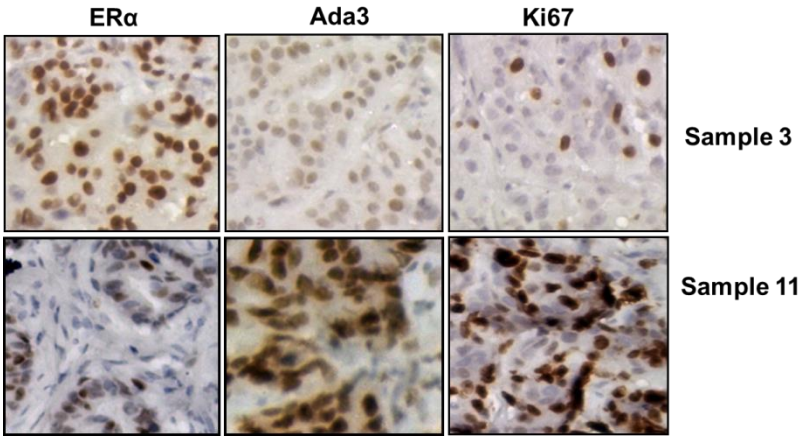


Figure 2: High ADA3 expression in patient derived xenografts correlates with elevated Ki67. Patient-derived xenografts were sectioned and stained with antibodies indicated (brown staining). Blue staining is hematoxylin.

ADA3 knockdown in immortal hMECs delays cell cycle progression.

We previously reported that homozygous deletion of Ada3 in mice led to embryonic lethality (Mohibi et al, 2012). Furthermore, conditional deletion of Ada3 in *Ada3*$^{fl/fl}$ mouse embryonic fibroblasts (MEFs) showed a defect in cell cycle progression, including delayed G1 to S transition, mitotic catastrophe, and defective cytokinesis (Mohibi et al, 2012).

However, no studies have been performed so far to demonstrate such a role of ADA3 in hMECs. Therefore, we first performed experiments to assess if ADA3 regulates cell cycle progression in hMECs. For this purpose, we used control (scrambled) or a well characterized specific ADA3 shRNA (Nag et al, 2007) to knockdown Ada3 in the immortal hMEC cell line, 76NTERT. Next, we synchronized 76NTERT cells by growth factor deprivation for 72 hours (0 time point), released the synchrony by adding medium containing growth factors, and then followed cell cycle progression over various indicated time periods. FACS-based cell cycle analysis of propidium iodide-stained cells showed significant delay in G1 to S progression in Ada3-deleted 76NTERT cells, as compared to control (Figure 3A). Of note, the relative distribution of S phase in Ada3-knockdown hMECs after 16 h of growth factor stimulation was much lower (46 %) as compared to control (64 %) (Figure 3A). These results demonstrate that knockdown of Ada3 leads to delay in G1 to S progression in immortal hMECs, indicating essential role of ADA3 in efficient G1/S progression in hMECs, similar to our previous results in Ada3$^{fl/fl}$ MEFs upon cre-mediated deletion (Mohibi et al, 2012). Knockdown of ADA3 was confirmed by western blotting (Figure 3 B, C).

Next, to examine if ADA3 plays a role in cell cycle progression, Ada3 knockdown or control 76NTERT cells synchronized and released from synchrony as above, were

analyzed for the levels of cell cycle proteins using western blotting. Notably, significant alterations in the levels of cyclin B and E, and an increase in cyclin-dependent kinase inhibitor (CDKI) p27 levels during cell cycle progression were observed in Ada3 shRNA expressing immortal hMECs (Figure 3B).

In consistent with our previous findings, western blotting using antibodies against histone H3 and H3-K56, a significant reduction in acetylation of H3-K56 was observed upon Ada3 knockdown as compared to control (Figure 3C), indicating that ADA3 is essential in maintaining histone acetylation during cell cycle progression. Taken together, we confirmed that ADA3 is essential for cell cycle progression in hMECs.

Figure 3. Knockdown of ADA3 causes a delay in cell cycle progression in immortal hMECs.

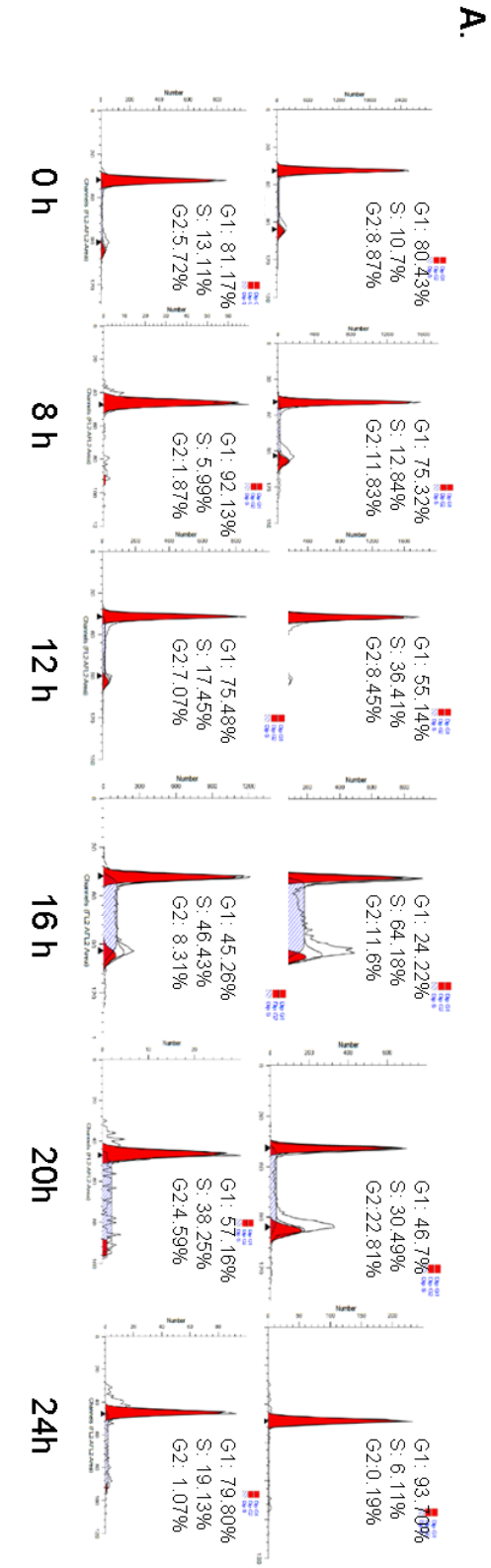
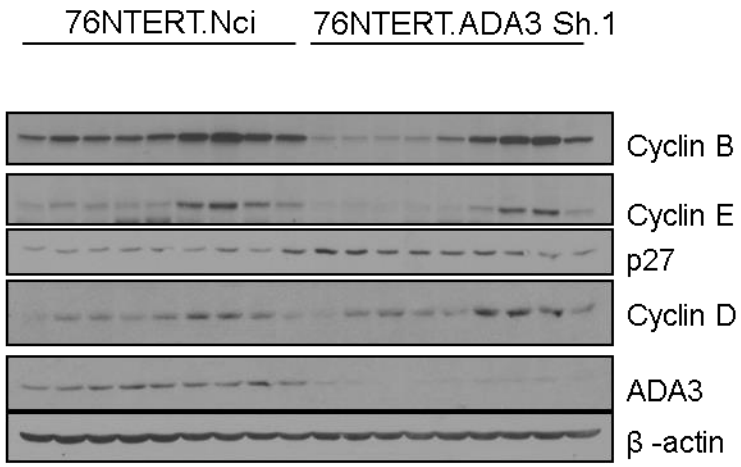


Figure 3. Knockdown of ADA3 causes a delay in cell cycle progression in immortal hMECs.

B.



C.

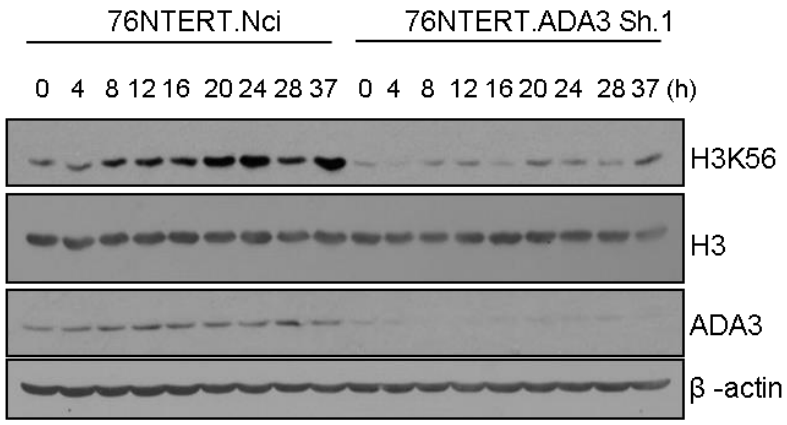


Figure 3. Knockdown of ADA3 causes a delay in cell cycle progression in immortal hMECs.

76NTERT cells expressing scrambled shRNA or Ada3 shRNA were deprived of growth factors in DFCI-3 media for 72hrs, then released from synchrony by adding growth factor containing DFCI-1 medium. Cells were then collected at the indicated time points for various analyses. **(A)** 76NTERT cells expressing scrambled shRNA or Ada3 shRNA were processed for FACS analysis after propidium iodide staining. Cell cycle profile (G1/S/G2/M) at selected time points is shown **(B)** Lysates were immunoblotted with indicated antibodies. β-actin was used as a loading control. **(C)** Western blotting with anti-histone 3 or H3-K56 specific antibodies is shown.

Exogenous overexpression of ADA3 in immortal hMECs enhances proliferation and alters cell cycle regulatory proteins.

Human ADA3 was originally identified in our laboratory as a human papilloma virus (HPV) type 16 E6 interacting protein (Kumar et al, 2002) and by others' as a p53 stabilizing protein (Wang et al, 2001). Initial studies by us and others showed that HPV16 E6 targeted ADA3 for degradation (Kumar et al, 2002; Shamanin et al, 2008), suggesting a tumor suppressor function of ADA3. Furthermore, we reported that ADA3 expression in normal mammary tissues and in ER+ breast cancer tissue is nuclear but ADA3 expression is cytoplasmic in ER- breast cancers (Mirza et al, 2013). In ER- breast cancers, cytoplasmic expression of ADA3 correlated with poor prognosis and poor survival of patients. Based on these published results, and our results shown in Figure 2 where ADA3 overexpression in ER+ breast cancer PDX correlated with high Ki67, we wished to assess if overexpression of nuclear ADA3 in immortal hMECs or in ER+ breast cancer cells have any consequence on proliferation.

For this purpose, we overexpressed ADA3 in two immortal hMECs 76NTERT and 81NTERT and then confirmed ADA3 overexpression using western blotting (Figure 4A). We then performed proliferation experiments by plating equal numbers of vector or ADA3 transfectants on day 0 and then every other day counted cells using a hemocytometer and trypan blue exclusion. Notably, ADA3-overexpressing immortal hMECs exhibited a significantly higher rate of proliferation as compared to control cells (Figure 4B, C). Notably, these cell lines despite of ADA3 overexpression, do not exhibit any chromosomal rearrangements, as seen by karyotype analyses (Figure 4G, E). Vector cells exhibit 19 breaks per 50 diploid metaphases scored, while ADA3 transfectants exhibited 16 breaks per 50 metaphases scored. Next, we analyzed

cell cycle regulatory proteins in vector or ADA3 overexpressing transfectants upon synchronization and then release from synchrony, as described above in Figure 3. Western blotting of lysates at various time periods during cell cycle progression showed clear alterations in the levels and timings of appearance of peak levels of cyclin B protein. Significantly, in contrast to knockdown results, levels of p27 protein was dramatically decreased on cell cycle progression, indicating that decreased levels of p27 may account for hyperproliferation of these cells (Figure 4D). Based on our previous findings in MEFs where knockdown of ADA3 significantly enhanced p27 half-life (Mohibi et al, 2012), we performed half-life experiments of p27 in vector or ADA3 overexpressing hMECs. Transfectants were treated with cycloheximide to block new protein synthesis (0 time point) and then lysates were western blotted at various time points to assess p27 levels. Densitometric quantitation analyses showed turnover of p27 in ADA3 overexpressing cells was much faster than in vector transfectants. Treatment of ADA3 overexpressing cells with the ubiquitin proteasome inhibitor MG132, showed an increase in the level of p27 protein (compare 3 hour lane with or without MG132) (Figure 4E, F) confirming p27 turnover is proteasome-ubiquitin pathway dependent. Analysis of mRNA levels of p27 showed no change (data not shown). Taken together, our published data and results described here show p27 as an important mediator of ADA3 regulated cell proliferation in hMECs.

Consistent with our previous publication of ADA3 regulating c-myc-skp2-p27, we also analyzed c-myc levels in these experiments and observed a significant increase in c-myc levels in ADA3 overexpressing cells, underscoring a positive connection of ADA3 and c-myc levels (Figure 4D).

Figure 4. Exogenous overexpression of ADA3 leads to increased proliferation.

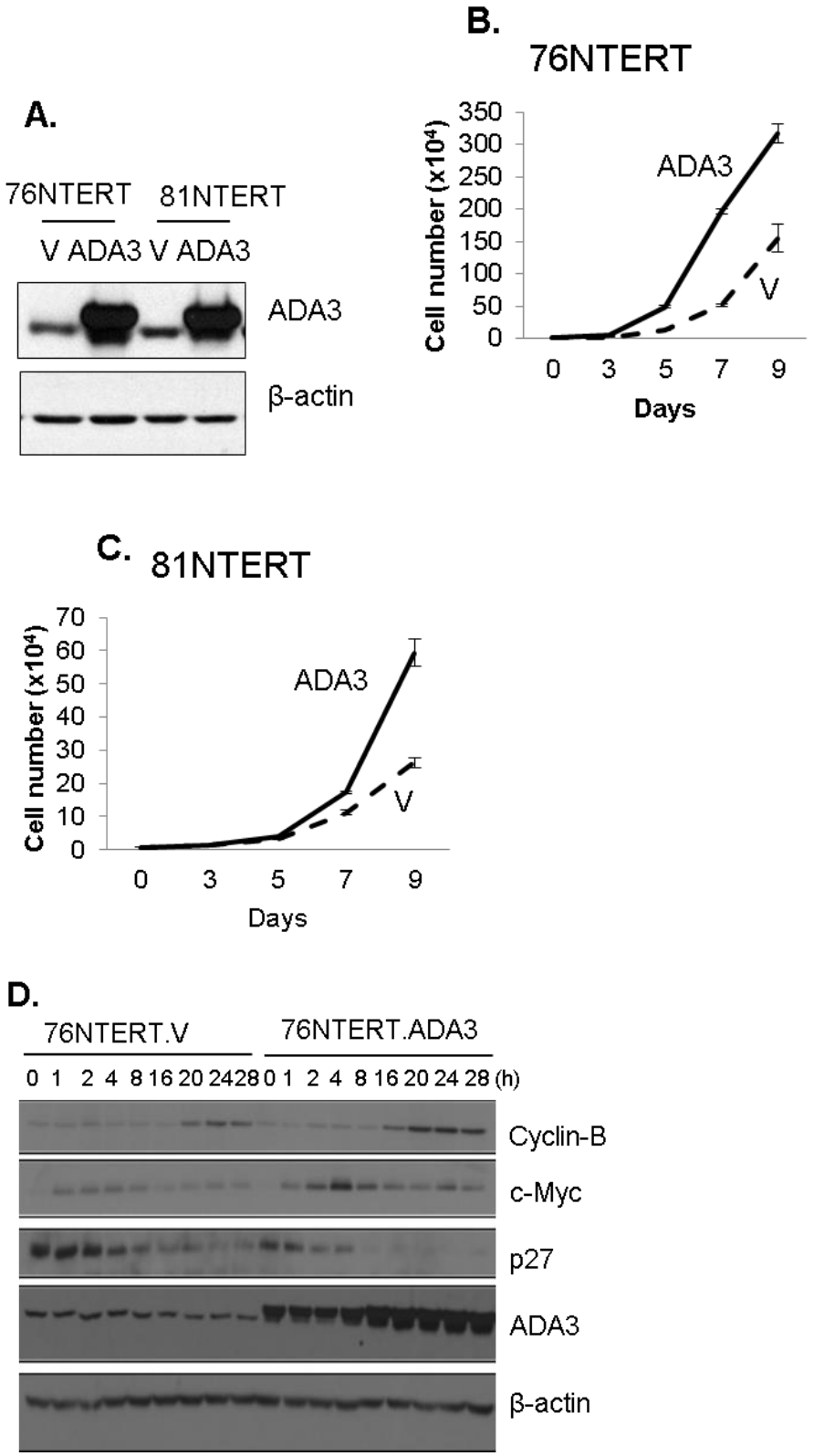
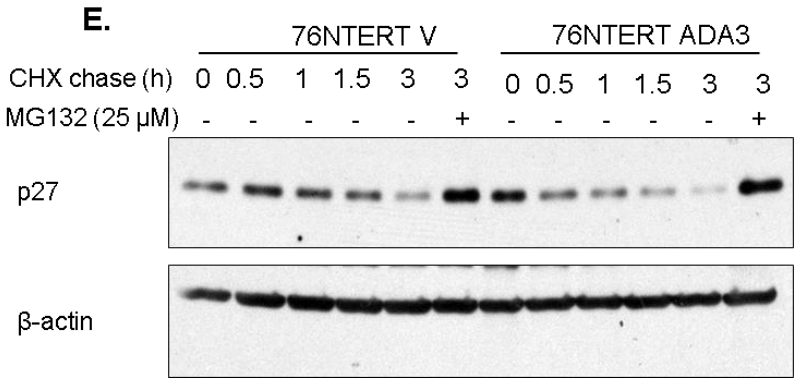
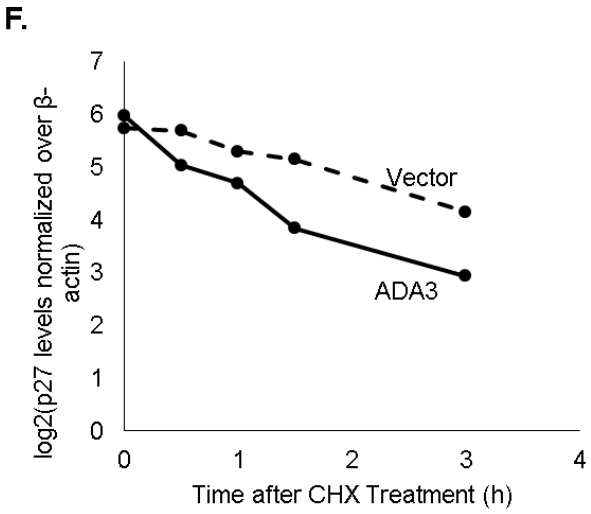


Figure 4. Exogenous overexpression of ADA3 leads to increased proliferation.





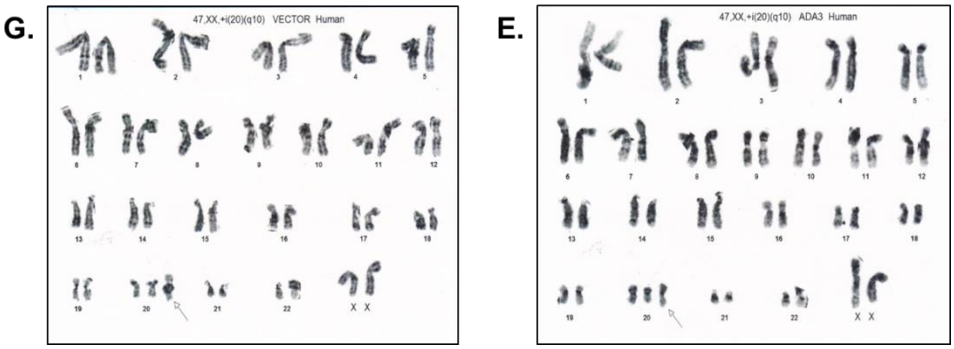


Figure 4. Exogenous overexpression of ADA3 leads to increased proliferation. **(A)** Western blotting of cells overexpressing ADA3. 76NTERT cell line **(B)** or 81NTERT cell line **(C)** expressing an empty vector or ADA3 were plated at a density of 1 x 10^4 and then counted using a hemocytometer on alternate days to measure proliferation. **(D)** western blotting of cell lysates from synchronized (0 time point) or cells released from synchrony (1-28 hours) were immunoblotted with indicated antibodies. β-actin was used as a loading control. **(E)** p27 half-life analysis. 76NTERT cells overexpressing ADA3 were treated with cycloheximide, cells lysates at indicated time points were immunoblotted with anti-p27 antibody. Last lane 3 hour time point of cells treated with MG132 **(F)** The intensity of p27 bands was quantified by densitometry, normalized to β-actin using ImageJ software, and plotted against the time of cycloheximide treatment. **. G)** Representative karyotype of 76NTERT vector cells and **E)** Representative karyotype of 76NTERT ADA3 cells.

Exogenous overexpression of ADA3 in immortal hMECs enhances transcription of early response genes.

Given the change in c-myc protein levels and previous findings that ADA3 as part of STAGA complex enhances c-myc transcription; we analyzed transcription of c-myc along with two other early response genes c-fos and EGR1. For this purpose, vector or ADA3-overexpressing transfectants were growth factor deprived for 72 hrs, and then released from synchrony via the addition of growth factor containing media, followed by analyses of mRNA levels of early response genes by quantitative-PCR (qPCR) analysis at different time points. Significantly, in each case mRNA levels of early response genes, c-fos, EGR1 and c-Myc peaked at 1 hour in both vector and ADA3 transfectants; however levels were much higher in ADA3 transfectants as compared to vector alone. Significantly, the levels of all three early response genes remained higher in ADA3 transfectants in all time points analyzed (Figure 5). These results suggest myc and early response genes are targets of ADA3-mediated proliferation of hMECs.

Figure 5. **ADA3 transcriptionally regulate early response genes.**

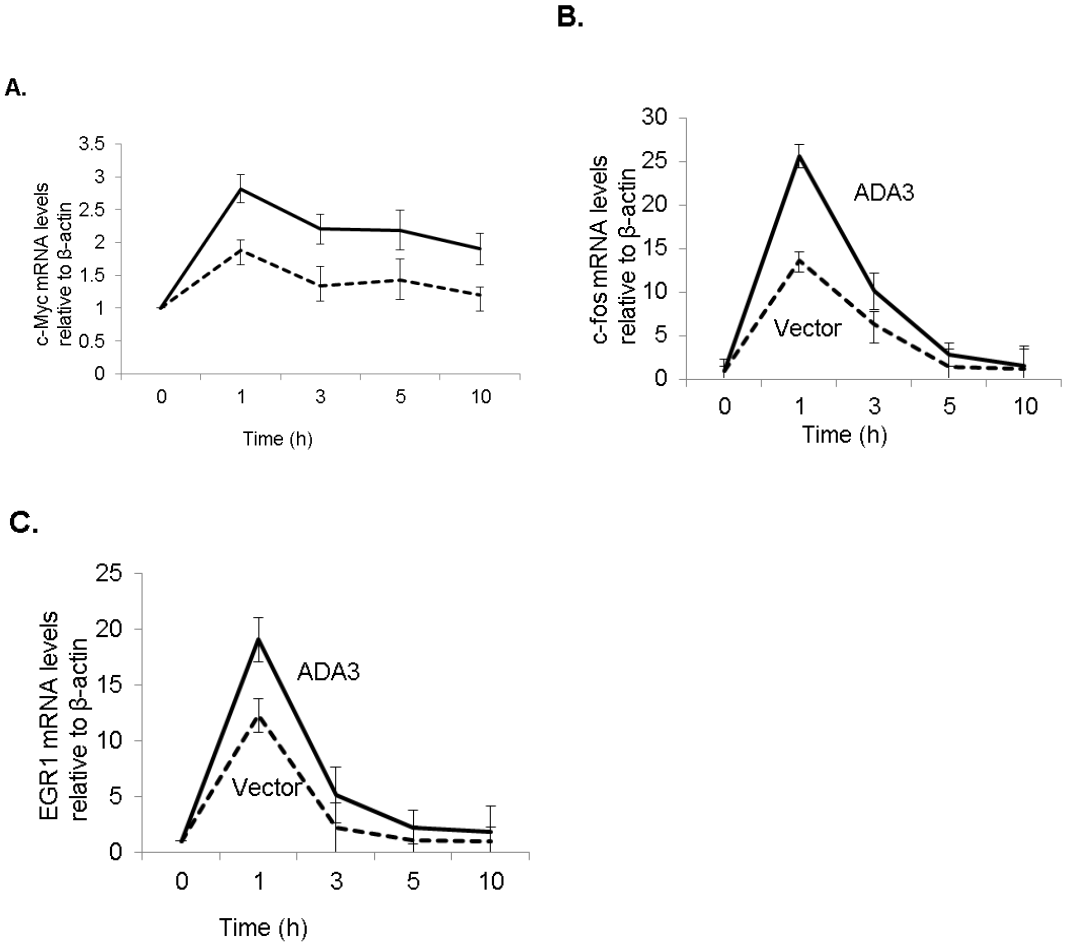


Figure 5. ADA3 transcriptionally regulate early response genes. 76NTERT cells vector or ADA3 overexpressing were deprived of growth factors for 72 hours and then released from synchrony via growth factor stimulation. Cells were collected at the indicated time points and total RNA was extracted for real time PCR analyses. Real time PCR analysis of c-myc **(A)**, c-fos **(B)** and EGR1 **(C)** is shown.

Exogenous overexpression of ADA3 in ER+ breast cancer cell lines enhances proliferation.

Given the role of ADA3 in cell proliferation in hMECs, its overexpression seen in PDX models of ER+ breast cancers, and ADA3 connection with c-myc transcription, we assessed if ADA3 overexpression in ER+ breast cancer cells also influences cell proliferation. For this purpose, we selected two well studied ER+ breast cancer cell lines MCF-7 and ZR-75-1, both of these express similar levels of nuclear ADA3 as seen in immortal hMECs. Overexpression was confirmed using western blotting (Figure 6A). Significantly, similar to ADA3 overexpression in immortal hMECs, overexpression of ADA3 in both ER+ breast cancer cell lines led to hyperproliferation (Figure 6B, C). Next, we examined if ADA3 overexpression induced estrogen independence for proliferation in these cell lines. For this purpose proliferation of both transfectants were analyzed under both estrogen deprivation and estrogen presence. These analyses showed ADA3 overexpression does not alter estrogen dependence of these cells (Figure 7A, B). Taken together, our results demonstrate that ADA3 overexpression induces hyperproliferation in both immortal and ER+ breast cancer cell lines.

Figure 6. Exogenous overexpression of ADA3 leads to increased proliferation of ER+ breast cancer cell lines.

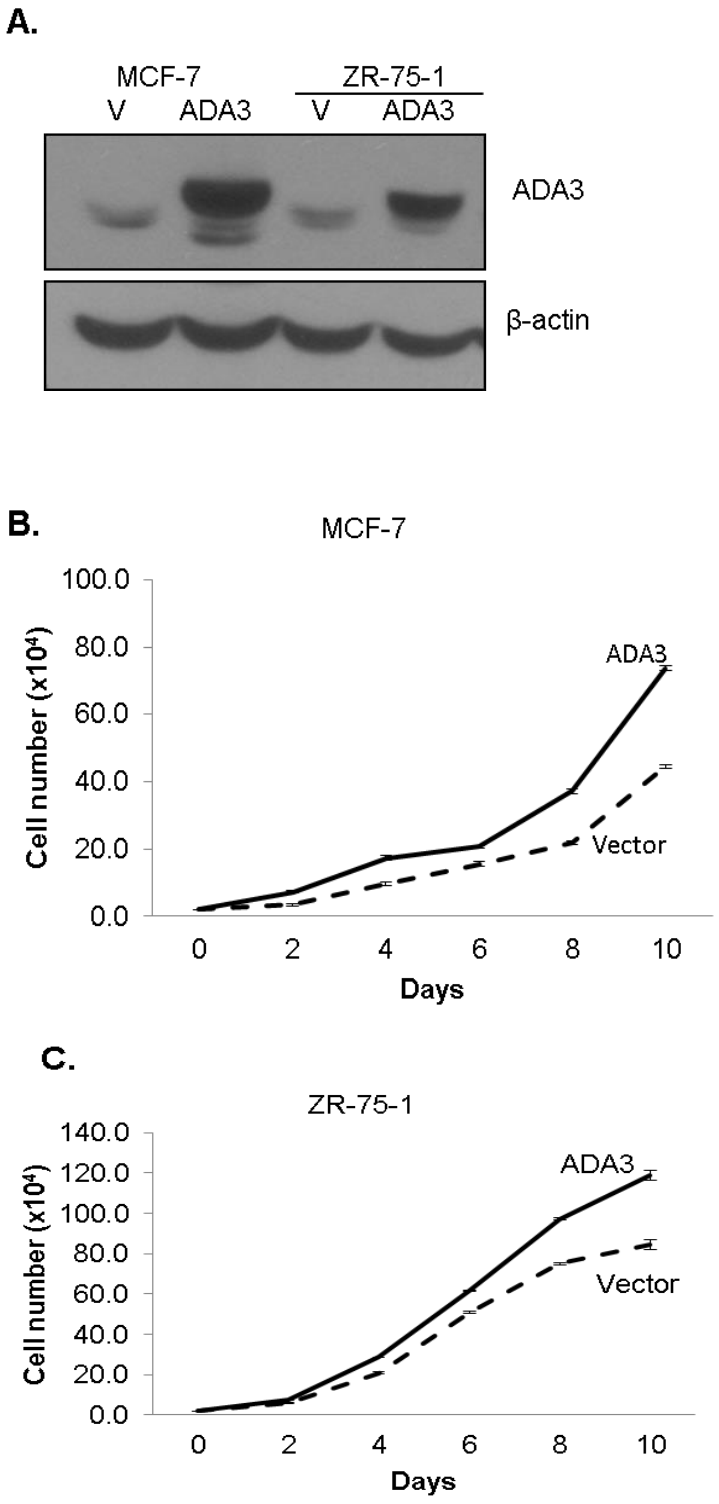


Figure 6. Exogenous overexpression of ADA3 leads to increased proliferation of ER+ breast cancer cell lines. (A) Western blotting of cell lysates from MCF7 and ZR-75-1 expressing vector or ADA3. MCF-7**(B)** or ZR-75-1 **(C)** cell lines expressing an empty vector or ADA3 were plated at a density of $1.0x10^4$ cells and counted on alternate days using a hemocytometer to measure proliferation.

Figure 7. ADA3 overexpression does not induce estrogen independence for proliferation.

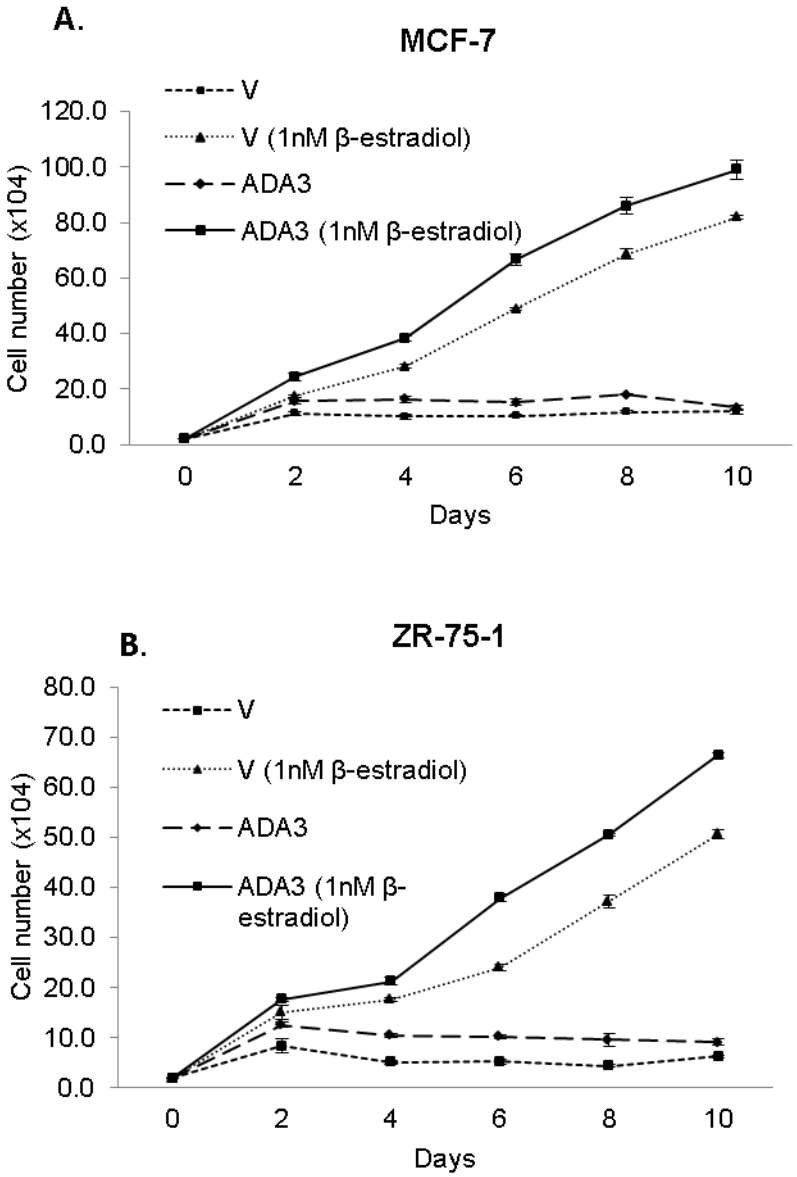


Figure 7. ADA3 overexpression does not induce estrogen independence for proliferation. Indicated cell lines overexpressing vector (V) or ADA3 were deprived of estrogen in phenol red-free MEMA medium supplemented with 5% charcoal dextran stripped fetal calf serum for 72hrs and stimulated with 1nM of β-estradiol. Fresh medium was added on alternate days and total number of cells were determined using a hemocytometer and trypan blue exclusion method.

ADA3 overexpression in immortal hMECs or ER+ breast cancer cell lines does not alter invasion or migration properties of cells.

While hyperproliferation is shown to be an important step in tumor formation, there is ample evidence that hyperproliferation does not always correlate with invasion or migration of tumor cells (Khoshyomn et al, 1999). Given that ADA3 overexpression led to hyperproliferation, we assessed f hyperproliferation provides these cells ability to better invade or migrate. For this purpose standard invasion and migration assays were performed. As shown in Figure 8 (A and B) no significant differences were observed in vector and ADA3 overexpressing ZR-75-1 cells. These results clearly demonstrate the role of ADA3 in proliferation but not in invasion or migration.

Figure 8. ADA3 overexpression has no effect on invasion or migration of cells.

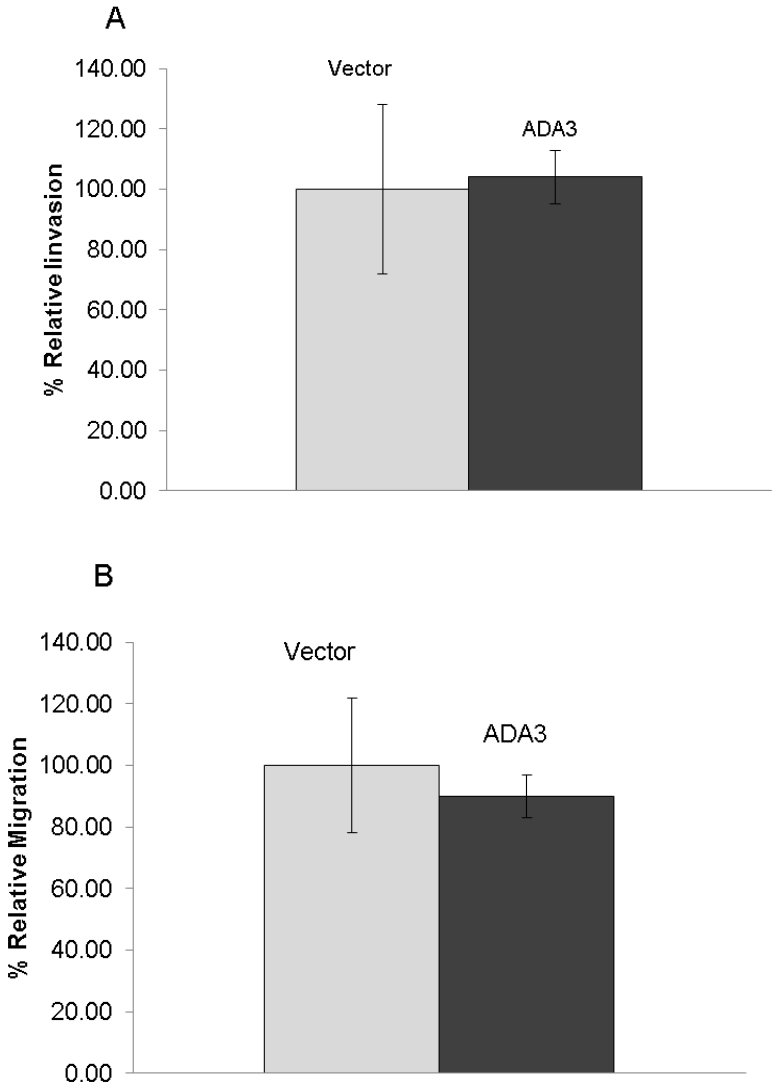


Figure 8. ADA3 overexpression has no effect on invasion or migration of cells. ZR-75-1 , an ER+ breast cancer cell line expressing vector (V) or ADA3 was examined for changes in invasive (A) or migratory (B) potential using the Boyden chamber method.

ADA3 is overexpressed in a subset of ER+ breast cancers, and its overexpression positively correlates with expression of c-myc.

Given the vast body of literature supporting a role of c-myc in breast cancers (Xu et al, 2010; Dang, 2012), amplification of c-myc is seen in approximately 15% of breast cancers (Xu et al, 2010), and its overexpression is associated with poor outcome (Xu et al, 2010; Wolfer et al, 2010; Huang, et al 2014). Our collaborators analyzed a large cohort of breast cancer specimens containing various subtypes and observed that c-myc overexpression was significantly associated with poor prognostic factors, including grade and basal-like breast cancers (Green et al, 2016). C-myc was also observed to be an independent predictor of a shorter distant metastases-free survival in luminal A LN+ tumors treated with endocrine therapy (Green et al, 2016). These patients' tissue specimens were also analyzed for ADA3 protein in our previous study (Mirza et al, 2013). In this study, we used this set of tissue specimens to access if ADA3 and c-myc expression correlated in these tissue specimens.

There were 598 ER+ patient samples where ADA3 expression was examined by IHC; and 796 samples had c-myc data. When combined these two markers, there were 429 patient samples suitable for comparative analysis of ADA3 and c-myc levels. Of these samples, 172 had low levels of both ADA3 and c-myc, 178 were low for ADA3 and high for c-Myc, 69 were high for ADA3 and low for c-myc, and 169 were high for both (Table 1). Notably, these analyses showed a highly significant difference ($p < 0.0001$) among ADA3 high/c-myc high, ADA3 high/ c-myc low, ADA3 low/c-myc high and ADA3 low/c-myc low groups of patients.

Table 1. Significant association between Positive Nuclear expressions of ADA3 and c-myc in unselected breast cancer cases.

Parameter	c-myc Nuclear Expression		Significance	
	Low	High	x^2	p value
ADA3 Nuclear Expression				
Negative/Low	172 (49.1)	178 (50.9)	23.79	< 0.0001
Positive/High	69 (29.0)	169 (71.3)		

Using tissues from the breast cancer specimen cohort we analysed only ER+ samples for high and low ADA3 and c-myc expression. We found that among the four groups there is a statistically significant difference.

Next, we compared these four group ADA3 high/c-myc high, ADA3 high/ c-myc low, ADA3 low/c-myc high and ADA3 low/c-myc low in ER+ and ER- patient samples for phenotypic groups / molecular biomarkers' status and clinical-pathological parameters and found that these 4 groups showed significant difference in tumor grade, Nottingham Prognostic Index and tumor types (Table 2) and cancer molecular subtypes, Ki67, as well as p27 (Table 3). Lastly, when we analyzed nuclear co-expression of ADA3 and c-myc with molecular biomarker status within only ER+ tumors we observed statistically significant correlations in tumor grade, nuclear pleomorphism, Nottingham Prognostic Index, Lymphovascular Invasion, histological tumor type, Ki67, and p27 across the four groups (Table 4).

Taken together, these results show i) ADA3 overexpression is seen in a subset of ER+ patients ii) ADA3 overexpression correlates with c-myc overexpression and iii) ER+ breast cancers can be categorized into 4 groups ADA3 high/c-myc high, ADA3 high/ c-myc low, ADA3 low/c-myc high and ADA3 low/c-myc low iv) these four subgroups show significant differences in their association with biomarkers, particularly c-myc and p27 expression, grades of tumors. Future study will be extended to compare therapy and survival response of these four subtypes of ER+ breast cancer patients.

Table 2. Relationship between nuclear ADA3 and nuclear c-myc co-expression groups and clinic-pathological parameters.

Parameters		ADA3 Nuclear c-myc Nuclear expression phenotypes					
		ADA3^{low} c-myc^{low}	ADA3^{high} c-myc^{low}	ADA3^{low} c-myc^{high}	ADA3^{high} c-myc^{high}	X^2	P
Patient	**≤ 50**	66 (31.7)	24 (11.5)	65 (31.1)	53 (25.5)	1.862	0.602
	> 50	106 (28.0)	45 (11.9)	113 (29.8)	115 (30.3)		
Menopa	**Pre**	66 (30.4)	25 (11.5)	68 (31.3)	58 (26.7)	0.907	0.871
	Post	106 (28.6)	44 (11.9)	110 (29.7)	110 (29.7)		
Tumour	**≤ 2 cm**	96 (28.0)	14 (12.0)	99 (28.9)	107 (31.2)	2.765	0.429
	>2 cm	76 (31.4)	28 (11.6)	77 (31.8)	61 (25.2)		
Tumour	**1**	20 (23.5)	14 (16.6)	17 (20.0)	34 (40.0)	32.961	**<**
	2	47 (25.3)	18 (9.7)	48 (25.8)	73 (39.2)		
grade	**3**	105 (33.3)	37 (11.7)	112 (35.4)	62 (19.6)		**0.001**
	1	5 (22.7)	7 (31.8)	5 (22.7)	5 (22.7)	13.345	
	2	47 (25.1)	21 (11.2)	59 (31.6)	60 (32.1)		
Tubules	**3**	116 (32.7)	35 (9.9)	107 (30.1)	97 (27.3)		**0.037**
	1	1 (14.3)	1 (14.3)	3 (42.9)	2 (28.6)	29.138	**<**
	2	57 (26.0)	31 (14.2)	46 (21.0)	85 (38.8)		
Pleomo	**3**	110 (32.6)	31 (9.2)	122 (36.2)	74 (22.0)		**0.001**
	1	43 (24.6)	23 (13.1)	40 (22.9)	69 (39.4)	23.706	**0.001**
	2	30 (29.1)	9 (8.7)	30 (29.1)	34 (33.0)		
Mitosis	**3**	95 (33.2)	31 (10.8)	101 (35.3)	59 (20.6)		
Axillary	**1**	93 (28.5)	42 (12.9)	90 (27.6)	101 (31.0)	6.617	0.353
	2	57 (28.2)	23 (11.4)	68 (33.7)	54 (26.7)		
nodal	**3**	22 (37.3)	4 (6.8)	20 (33.9)	13 (22.0)		
NPI	**Good**	30 (19.2)	21 (13.5)	39 (4)	66 (25.0)	33.968	**<**
	Moderate	103 (33.0)	40 (12.8)	88 (8.8)	81 (28.2)		
	Poor	39 (33.1)	8 (6.8)	50 (36)	21 (17.8)		**0.001**
Lympho	**Negative**	106 (28.0)	52 (13.8)	103 (27.2)	117 (31.0)	10.624	**0.014**
	Positive	64 (31.4)	16 (7.8)	75 (36.8)	49 (24.0)		
	Invasive Ductal/NST	123 (34.6)	39 (11.0)	120 (33.7)	74 (20.8)		
	Invasive Lobular	13 (22.4)	8 (13.8)	4 (6.9)	33 (56.9)		
Tumour	**Medullary-like**	2 (11.1)	3 (16.7)	9 (50.0)	4 (22.2)		**<0.00**
type	**Excellent**	5 (25.0)	6 (30.0)	5 (25.0)	4 (20.0)	61.858	**1**
	Tubular Mixed	20 (20.6)	11 (11.3)	28 (28.9)	38 (39.2)		
	Mixed NST &	4 (19.0)	2 (9.5)	6 (28.6)	9 (42.9)		
	Mixed NST &other	2 (25.0)	0	3 (37.5)	3 (37.5)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. NST= No Special Type. NPI= Nottingham Prognostic Index. * Include: Invasive mucinous, invasive tubular, invasive cribriform, and invasive papillary

Analysis of all patient specimens from all members of the unselected invasive breast cancer cohort demonstrates a significant correlation between nuclear ADA3 and c-myc levels in the areas of tumor grade, pleomorphism, tubule formation, mitotic scores, NPI, and tumor type across the four groups.

Table 3. Relationship between nuclear ADA3 and nuclear c-myc co-expression combinatorial phenotypic groups with molecular biomarker status.

		ADA3^{low} C Myc^{low}	ADA3^{high} C Myc^{low}	ADA3^{low} C	ADA3^{hig} ^{h}C	Significance	
						X^2	P
ER Status	**Negativ**	59 (39.6)	12 (8.1)	56 (37.6)	22 (14.8)	25.8	< 0.001
	Positiv	113	56 (12.9)	121	144		
PR Status	**Negativ**	78 (36.0)	25 (10.3)	74 (30.6)	56 (23.1)	12.8	0.005
	Positiv	79 (23.7)	43 (12.9)	102	110		
HER2	**Negativ**	6 (54.5)	2 (18.2)	1 (9.1)	2 (18.2)	21.7	0.010
	Positiv	132	55 (11.2)	152	150		
Molecular	**Lumina**	99 (25.4)	49 (12.6)	110	131	25.4	< 0.001
	HER2	34 (40.0)	10 (11.8)	24 (28.2)	17 (20.0)		
Subtype	**Triple**	38 (36.2)	7 (6.7)	43 (41.0)	17 (16.2)	05	
Ki67	**Low**	42 (23.0)	27 (14.8)	40 (21.9)	74 (40.4)	22.0	< 0.001
	High	83 (29.9)	30 (10.8)	101	64 (23.0)		
p27	**Low**	114	33(11.2)	89 (30.3)	58 (19.7)	38.2	< 0.001
	High	40 (16.7)	33 (13.8)	77(32.1)	90 (37.5)		

*HER2 Status was assessed using American Society of Clinical Oncology/College of American Pathologists Guidelines Recommendations for HER2 Testing in Breast Cancer and Equivocal

Analysis within the whole unselected invasive breast cancer series of all patient specimens with respect to receptor status, molecular subtype, Ki67 labeling index, and p27 demonstrated a statistically significant correlation across the four groups.

Table 4. Relationship between nuclear ADA3 and nuclear c-myc co-expression groups with molecular biomarker status within ER+ tumors only.

Parameters		ADA3 Nuclear C Myc Nuclear expression phenotypes					
		$ADA3^{low}$ C Myc^{low}	$ADA3^{high}$ C Myc^{low}	$ADA3^{low}$ C Myc^{high}	$ADA3^{high}$ C Myc^{high}	X^2	P
Patient age	**≤ 50**	35 (26.3)	18 (13.5)	39 (29.3)	41(30.8)	0.469	0.936
	> 50	78 (26.0)	38 (12.7)	82 (27.3)	102 (34.0)		
Menopausal	**Pre**	35 (24.5)	19(13.3)	44 (30.8)	45 (31.5)	1.002	0.801
	Post	78 (26.9)	37 (12.8)	77 (26.6)	98 (33.8)		
Tumour size	**≤ 2 cm**	65 (24.3)	34 (12.7)	74 (27.6)	95 (35.4)	1.968	0.579
	>2 cm	48(29.3)	22 (13.4)	45 (27.4)	49 (29.9)		
Tumour grade	**1**	18 (22.0)	14 (17.1)	17 (20.7)	33 (40.2)	15.477	**0.017**
	2	44 (25.6)	16 (9.3)	44 (25.6)	68 (39.5)		
	3	51 (28.5)	26 (14.5)	59 (33.0)	43 (24.0)		
Tubules	**1**	5 (22.7)	7 (31.8)	5 (22.7)	5 (22.7)	9.813	**0.133**
	2	41 (25.2)	17 (10.4)	51 (31.3)	54 (33.1)		
	3	65 (28.1)	27 (11.7)	60 (26.0)	79 (34.2)		
Pleomorphis	**1**	1 (14.3)	1 (14.3)	3 (42.9)	2 (28.6)	19.460	**0.003**
	2	52 (24.9)	31 (14.8)	42 (20.1)	84 (40.2)		
	3	58 (29.1)	19 (9.5)	71 (35.7)	51 (25.6)		
Mitosis	**1**	40 (24.0)	22 (13.2)	38 (22.8)	67 (40.1)	10.175	**0.117**
	2	23 (25.3)	8 (8.8)	30 (33.0)	30 (33.0)		
	3	48 (30.4)	21 (13.3)	48 (30.4)	41 (25.9)		
Axillary nodal stage	**1**	58 (24.6)	35 (14.8)	61 (25.8)	82 (34.7)	3.652	0.724
	2	43 (26.7	17 (10.6)	50 (31.1)	51 (31.7)		
	3	12 (32.4)	4 (10.8)	10 (27.0)	11 (29.7)		
NPI	**Good**	27 (18.6)	20 (13.8)	37 (25.5)	61 (42.1)	15.643	**0.016**
	Moderate	63 (29.3)	31(14.4)	56(26.0)	65(30.2)		
	Poor	23 (31.5)	5(6.8)	27 (37.0)	18 (24.7)		
Lymphovascular Invasion	**Negative**	75 (26.2)	44 (15.4)	67(23.4)	100 (35.0)	11.090	**0.011**
	Positive	37 (25.3)	12 (8.2)	54 (37.0)	43 (29.5)		
Tumour type	**Invasive**	71 (31.0)	28 (12.2)	71 (31.0)	59 (25.8)	44.333	**<0.001**
	Invasive Lobular	13 (22.4)	8 (13.8)	4 (6.9)	33 (56.9)		
	Medullary-like	0(0)	1(33.3)	2 (66.7)	0 (0)		
	Excellent	5 (26.3)	6 (31.6)	5 (26.3)	3 (15.8)		
	Tubular Mixed	16 (17.4)	11 (12.0)	28 (30.4)	37 (40.2)		
	Mixed NST &	4 (19.0)	2 (9.5)	6 (28.6)	9 (42.9)		
	Mixed NST	2 (28.6)	0	3 (42.9)	2 (28.6)		
Ki67	**Low**	35 (23.0)	25 (14.8)	33 (21.9)	68 (40.4)	11.217	
	High	49 (29.9)	22 (10.8)	38 (36.3)	50 (23.0)		
p27	**Low**	61 (35.5)	21 (12.2)	47 (27.3)	43 (25.0)	18.993	**<0.00**
	High	37 (16.9)	32 (14.6)	67 (30.6)	83 (37.9)		

N= number of cases. c. = cytoplasmic, n. = nuclear expression. NST= No Special Type. NPI= Nottingham Prognostic Index. * Include: Invasive mucinous, invasive tubular, invasive cribriform, and invasive papillary

Analysis of ER+ patient specimens with respect to tumor grade, nuclear pleomorphism, NPI, LVI, histologic tumor type, Ki67 labeling index, and P27 demonstrated a statistically significant correlation across the four groups.

DISCUSSION

Precisely regulated cell cycle progression is essential for embryonic development as well as in adult tissue homeostasis (Kastan & Bartek, 2004). Coordination of cell cycle progression with chromosomal duplication maintains genomic stability; a critical cancer-associated trait (Schvartzman et al, 2010). Deregulated cell cycle components have emerged as key biomarkers and therapeutic targets in cancer (Lapenna & Giordano, 2009). Thus, a better understanding of cell cycle machinery and its aberrations in cancer are of fundamental importance in cell and cancer biology.

We previously identified the ADA complex component ADA3 as an HPV 16 E6 oncoprotein partner as well as a coactivator of cell cycle checkpoint regulator and tumor suppressor p53 (Kumar et al, 2002; Nag et al, 2007). We and others have shown that ADA3 is a target for E6-mediated degradation, suggesting its tumor suppressor function. Several studies have shown that ADA3 is an essentially universal component of a multitude of HAT-based transcriptional regulatory complexes. We previously demonstrated that ADA3 interacts with steroid hormone receptors retinoic acid (Zeng et al, 2002), and estrogen receptors (ER) (Meng et al, 2004; Germaniuk et al, 2007) and functions as a coactivator to enhance their transactivation and function. We also showed that in breast cancer cells ADA3 is in a large complex that include components of the yeast Ada complex (Ada2, Ada3 and GCN5, a HAT) but also other cell cycle- and cancer-associated HATs, p300 and PCAF (Germaniuk et al, 2007). Further analyses from our laboratory demonstrated Ada3 deletion in mice is embryonic lethal and knock out from $Ada3^{fl/fl}$ MEFs led to cell cycle delay. Our previous analyses of a large cohorts of breast cancer tissue specimens, showed while ADA3 is a nuclear protein in normal mammary cells, as well as in ER+ breast cancer cells, it is localized to cytoplasm in ER- breast cancers, where ADA3 expression was correlated with poor survival of the ER- patients.

In this study, we focused our analyses to mammary epithelial cells, which are the precursors of most breast carcinomas and explored the role of ADA3 in human mammary epithelial cells and in ER+ breast cancers. Using immortal (non-tumorigenic) hMEC lines and ADA3 shRNA-mediated knockdown and overexpression, we established a clear role of ADA3 in breast cells. We demonstrated that knockdown of ADA3 in immortal hMECs led to delay in cell cycle progression as seen by accumulation of cells in G1 to S transition. As cell cycle progression is a tightly regulated process and is dependent on sequential and stringently controlled, concerted activation of Cdks and their inhibition by Cdk inhibitors, we analyzed effect of ADA3 knockdown in mammary cells. We observed dramatic changes in regulation of Cyclin E and B, as well as upregulation of p27 protein, consistent with our previous findings in MEFs. In addition, we observed a dramatic decrease in H3K56 protein in immoral hMECs. These results are consistent with a vast body of literature suggesting highly significant role of H3-K56 acetylation in DNA replication and cell cycle (Wurtele et al, 2012; Li et al, 2008; Yu et al, 2012). These results, together with our previous published findings clearly demonstrated a role of ADA3 in hMECs cell cycle progression.

Given that several cell cycle regulatory proteins are upregulated in cancers to help maintain the higher proliferation demand of cancer cells, we analyzed patient derived xenograft tissue from two ER+ breast cancer patients. Surprisingly, we observed in one tumor specimen ADA3 levels were substantially higher than other ER+ tissue specimen, suggesting that ADA3 expression may be different in various ER+ breast cancers. Interestingly, high ADA3 expression in one sample also correlated with high K67 protein, a marker of cell proliferation. These results prompted us to overexpress ADA3 in immortal hMECs and assess if ADA3 overexpression influences cell proliferation.

While ADA3 knockdown/knockout significantly halts cell cycle progression, overexpression of ADA3 significantly induced hyperproliferation. Importantly, hyperproliferation was associated with increased Cyclin B levels and downregulation of p27 protein due to rapid turnover. As our previous results showed ADA3 enhances c-myc transcription (Mohibi et al, 2012), we assessed c-myc levels in these ADA3 overexpressing cells and found that not only c-myc but also two other early response gene, c-fos and EGR1 were significantly high in ADA3 overexpressing cells. C-fos has long been known to be involved in proliferation and EGR1 has also been found to be important for cell proliferation and migration (Angel & Karin, 1991; Mitchell et al, 2004; Min et al, 2008), therefore elevated expression levels of both in ADA3 overexpressing cells is consistent with their role in proliferation. These results are consistent with our previous findings where we defined ADA3-c-myc-skp2-p27 pathway for role of ADA3 in cell cycle regulation in MEFs. We and others have previously reported that Ada3 as part of STAGA complex binds to c-myc enhancer elements (Wang et al, 2001; Shamanin et al, 2008). Notably, another ER coactivator, SRC3 has also been shown to enhance proliferation when it is overexpressed (Louie et al, 2004) suggesting role of ER coactivators in cellular proliferation. However, we and others have not seen presence of SRC3 in ADA3 containing complexes, suggesting different mechanism of action of these two ER coactivators.

Finally, given a vast body of literature linking c-myc expression, as a key regulator of cell proliferation, metabolism, differentiation, and apoptosis and our results that ADA3 regulates c-myc levels, prompted us to analyze ER+ breast cancer specimens where c-myc is shown to be important for breast cancer development and progression and is associated with poor outcomes.

Analyses of a well characterized cohort of breast cancer tissue samples, we report that ER+ tumors can be divided into high and low groups, based on ADA3 protein levels.

Notably, analyses showed a highly significant difference among four groups; ADA3 high/c-myc high, ADA3 high/ c-myc low, ADA3 low/c-myc high and ADA3 low/c-myc low group of patients (Table 1). When combined both ER+ and ER- cases, ADA3 high/c-myc high, ADA3 high/ c-myc low, ADA3 low/c-myc high and ADA3 low/c-myc group of patients showed significant differences in their correlations with various biomarkers (p27 and c-myc), cancer molecular subtypes, pleomorphism, mitosis, tumor grade, Nottingham Prognostic Index and tumor types.

Conclusions

We demonstrate that ADA3 is overexpressed in ER+ breast cancers, its overexpression induces c-myc induction and together expression of both ADA3 and c-myc proteins induced hyperproliferation of cells. ADA3 overexpression together with c-myc can divide patients into four groups with significant differences in phenotypic groups, and molecular biomarkers' status and clinical-pathological parameters.

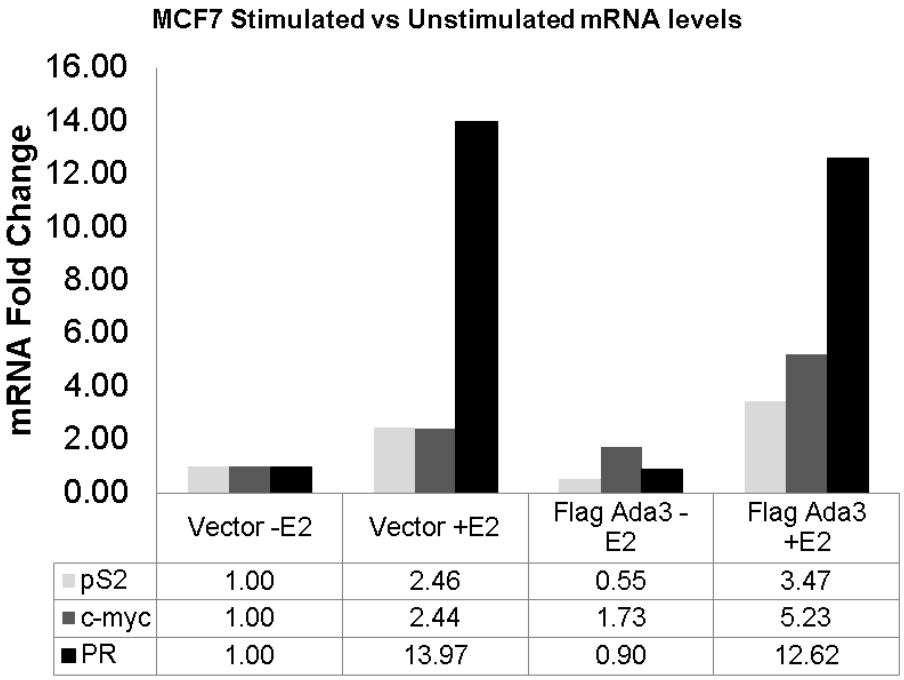
Other experiments performed during my Ph.D. studies

In addition to the above studies that have been compiled into a manuscript, additional experiments were performed to determine ER target gene expression and tamoxifen sensitivity in ER+ breast cancer cell lines. Invasion and migration was measured in the 21PT and 21MT2 ErbB+ breast cancer cell lines. Lastly, microarray analysis of asynchronous 76NTERT vector and ADA3 transfectants was performed to ascertain alterations in basal gene transcription.

Ada3 overexpression leads to increased ER target gene expression

In order to determine if any ER target genes were affected by ADA3 overexpression in MCF-7 cells, we deprived and stimulated cells as in Figure 5A, then collected triplicate samples of mRNA after 24hrs for real time PCR analysis. After normalization with β-actin and untreated vector cells, we found that ADA3 overexpressing cells expressed a greater amount of mRNA for pS2 and c-myc, two well-known ER target genes. However, there was no change in progesterone receptor levels, which could be attributed to experimental error (Figure 9). These data are consistent with that ADA3 functions as a coactivator of ER and enhances the expression of its target genes.

Figure 9. Ada3 overexpression leads to increased ER target gene expression



	Vector -E2	Vector +E2	Flag Ada3 -E2	Flag Ada3 +E2
pS2	1.00	2.46	0.55	3.47
c-myc	1.00	2.44	1.73	5.23
PR	1.00	13.97	0.90	12.62

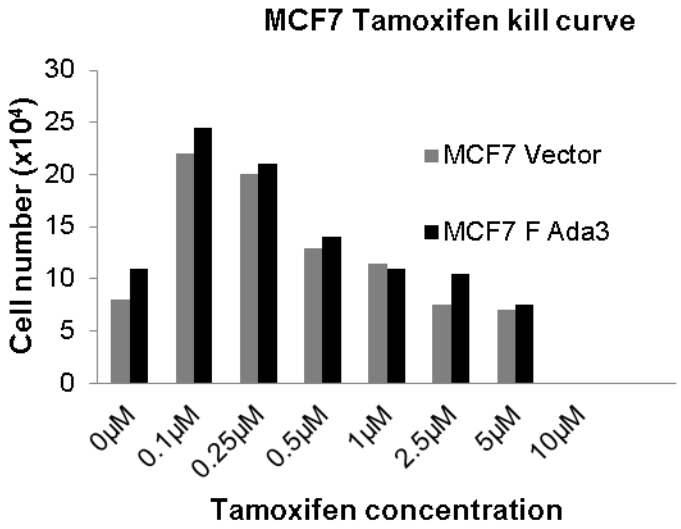
Figure 9. Ada3 overexpression leads to increased ER target gene expression. Following 72hr deprivation and subsequent β-estradiol **stimulation for 24hrs,** MCF7 cells overexpressing ADA3 have increased ER target gene expression levels as compared to vector cells.

ADA3 overexpression does not alter tamoxifen sensitivity

To further examine the effect of ADA3 and consequently c-myc overexpression on ER+ breast cancer cell lines, we treated them with varying doses of 4-hydroxy-tamoxifen (4-OHT), a well-known estrogen antagonist. MCF-7 (Figure 10A) and ZR-75-1 (Figure 10B) transfectants with vector or Ada3 were treated with the indicated doses of tamoxifen for seven days. Following the course of treatment, live cells were counted using trypan blue exclusion dye and a hemocytometer. As expected, at lower doses of tamoxifen, it functions as an estrogen agonist thus enhances proliferation (Smith et al., 1997); however, there was no discernible cell survival difference between vector cells and cells overexpressing ADA3 at higher doses of tamoxifen. Using the more sensitive cell titer glow approach, $1.0x10^4$ ZR-75-1 vector or ADA3 overexpressing cells were treated with concentrations of 4-OHT as indicated in Figure 10 (C-F) for eight days in a 96 well plate. Fluorescence readings were taken every two days and normalized to the initial day of plating. Again, no significant change in tamoxifen sensitivity was observed between vector and ADA3-overexpressing cells. Thus, ADA3-induced overexpression of c-myc is not sufficient to induce tamoxifen resistance.

Figure 10. Ada3 overexpression does not alter tamoxifen sensitivity

A



B

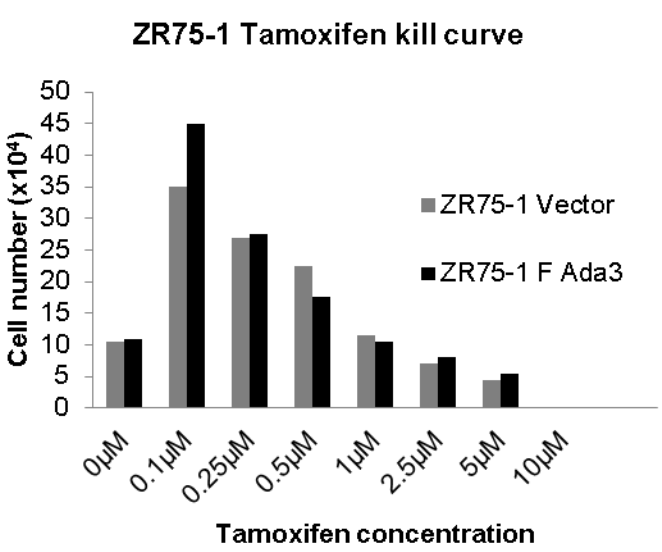


Figure 10. ADA3 overexpression does not alter tamoxifen sensitivity.

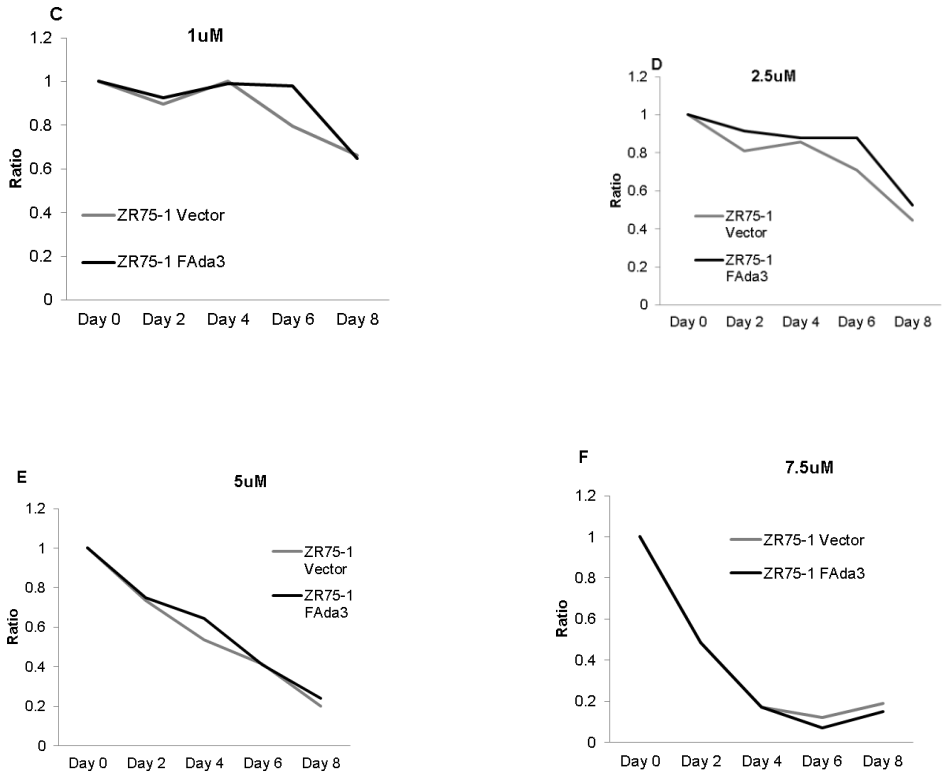


Figure 10. Ada3 overexpression does not alter tamoxifen sensitivity.

(A) MCF7 or **(B)** Zr75-1 cell lines overexpressing Ada3 were continuously treated with 4-hydroxy-tamoxifen (4-OHT). 1.0x10^4 cells were treated with the indicated concentrations of 4-OHT and live cells counted after seven days using a hemocytometer. **(C-F)** ZR75-1 cells overexpressing Ada3 were treated with 4-OHT and quantified using cell titer glow. 1.0x10^3 cells treated with the indicated concentrations of 4-OHT and fluorescence measured every two days were normalized to Day 0.

ADA3 overexpression does not alter tumorigenic phenotypes, invasion and migration in ErbB2+ cell lines.

In addition to proliferation, two other processes known to be altered in cancers are invasion and migration. Invasion is the ability of a cell to pass through the extracellular matrix by cleaving proteins in the way, while migration is when the cell moves by rearranging the cytoskeleton or membrane. Matrigel serves as medium through which cells invade, while migration occurs across a porous membrane. In addition to the two ER+ breast cancer cell lines (MCF-7 and ZR-75-1) we examined in Chapter 2, we took two ErbB2+ (21PT and 21MT2) ADA3 overexpressing transfectants and examined their abilities to invade and migrate. Following the conclusion of the invasion and migration time points, cells were stained, photographed, and counted with ImageJ software. The average values of 4 Ada3 overexpressing inserts were normalized to that of vector inserts. 21PT and 21MT2 cells were plated in serum free MEMA, while the lower chamber was supplemented with 10% serum as a chemo-attractant. 21PT cells underwent both invasion and migration after 24hrs (Figure 11A and 11B), while the 21MT2 cells invaded after 72hrs (Figure 11C) and migrated after 40hrs (Figure 11D). The overexpression of ADA3 did not alter the ability of the cell lines to invade or migrate.

Figure 11. Ada3 overexpression has no effect on invasion and migration in ErbB2+ cell lines.

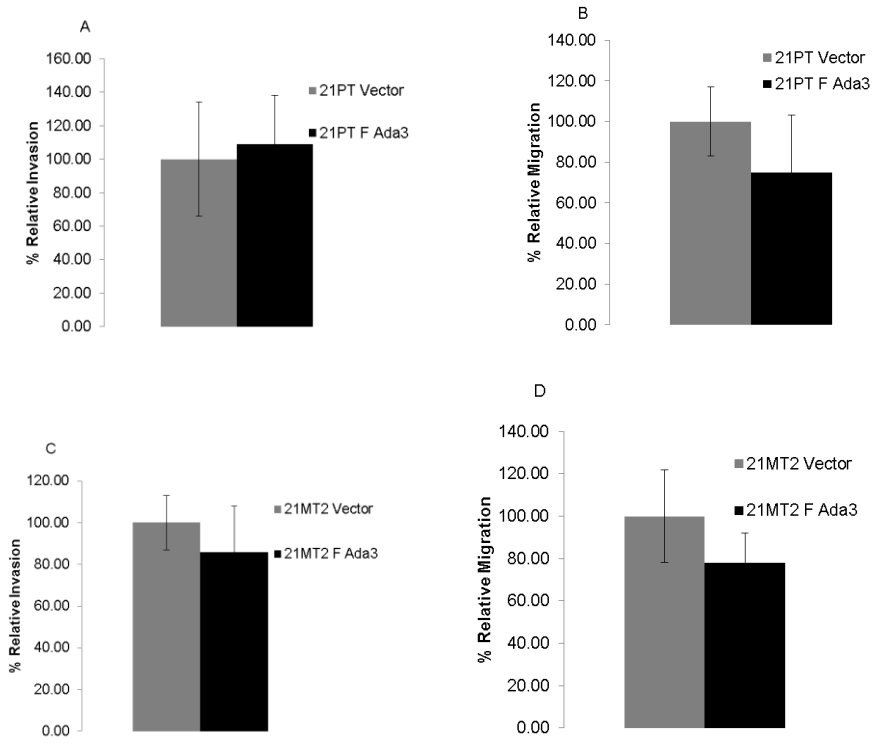


Figure 11. Ada3 overexpression has no effect on invasion and migration. ErbB2+ breast cancer cell lines were examined for changes in invasive and migratory potential. 21MT2 **(A)** and **(B)** cell lines overexpressing Ada3 were assessed using 10% FBS as the chemoattractant. Invasion and migration were normalized with respect to vector controls and are representative of 4 chambers.

Overexpression of Ada3 leads to global gene expression changes

Due to the observed increased in proliferative capability, we performed microarray analysis on 76NTERT vector control cells and Ada3 overexpressing cells. As anticipated, the expression levels of many genes were changed. 582 genes were upregulated and 587 genes were downregulated ≥1.2 fold upon the overexpression of Ada3 in 76NTERT cells. This differential expression is similar to previous work performed in our lab by Mohibi, et al. in 2012. Analysis of the array done using Ingenuity Pathway Analysis showed that the primary biological functions most affected by Ada3 overexpression were related to the cell cycle (379 genes) and DNA replication, recombination, and repair (309 genes) (Table 5). Of note, few of the primary altered biological functions are shared with the observations obtained when Ada3 was deleted (Mohibi et al, 2012). When further examining the well mapped out canonical pathways in IPA, we found that the top affected pathways followed the general trend of being involved with the cell cycle (Table 6).

Table 5. Top biological functions affected in overexpressing Ada3 cells as obtained from Ingenuity Pathway Analysis

Name	p-value	# Molecules
Diseases and Disorders		
Cancer	3.19E-46 - 5.12E-03	2072
Gastrointestinal Disease	4.98E-30 - 3.55E-03	1469
Organismal Injury and Abnormalities	6.56E-19 - 5.26E-03	982
Reproductive System Disease	6.56E-19 - 5.24E-03	870
Hepatic System Disease	7.77E-12 - 3.55E-03	876
Molecular and Cellular Functions		
Cell Cycle	4.20E-23 - 5.12E-03	379
Cellular Assembly and Organization	4.20E-23 - 4.67E-03	244
DNA Replication, Recombination, and Repair	4.20E-23 - 5.26E-03	309
Nucleic Acid Metabolism	7.81E-10 - 2.17E-03	141
Small Molecule Biochemistry	7.81E-10 - 5.11E-03	164
Physiological System Development and Function		
Embryonic Development	5.82E-07 - 3.43E-03	199
Organismal Survival	5.82E-07 - 5.82E-07	42
Reproductive System Development and Function	1.14E-05 - 5.22E-03	119
Connective Tissue Development and Function	2.02E-05 - 4.61E-03	39
Organ Morphology	3.22E-05 - 5.22E-03	73

Table 6: Top canonical pathways affected in overexpressing Ada3 cells as obtained from Ingenuity Pathway Analysis

Name	p-value	Ratio
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	1.41E-12	26/49 (0.531)
Role of BRCA1 in DNA Damage Response	4.59E-10	27/64 (0.422)
Mitotic Roles of Polo-Like Kinase	5.59E-09	26/66 (0.394)
ATM Signaling	2.6E-07	22/59 (0.373)
Cell Cycle Control of Chromosomal Replication	3.24E-07	14/27 (0.519)

Interestingly, the top affected network controls cell death and survival, amino acid metabolism, and post-translational modification (Figure 12A), while the second most affected network influences cellular assembly and organization, cellular function and maintenance, and the cell cycle (Figure 12B). The top five differentially regulated networks are compiled in Figure 12 (A-E). The dysregulated networks are similar when Ada3 is overexpressed in that cell cycle related networks and developmental networks are altered, however the changes are not an exact match.

Figure 12. Network 1: Cell death and survival, amino acid metabolism, post-translational modification

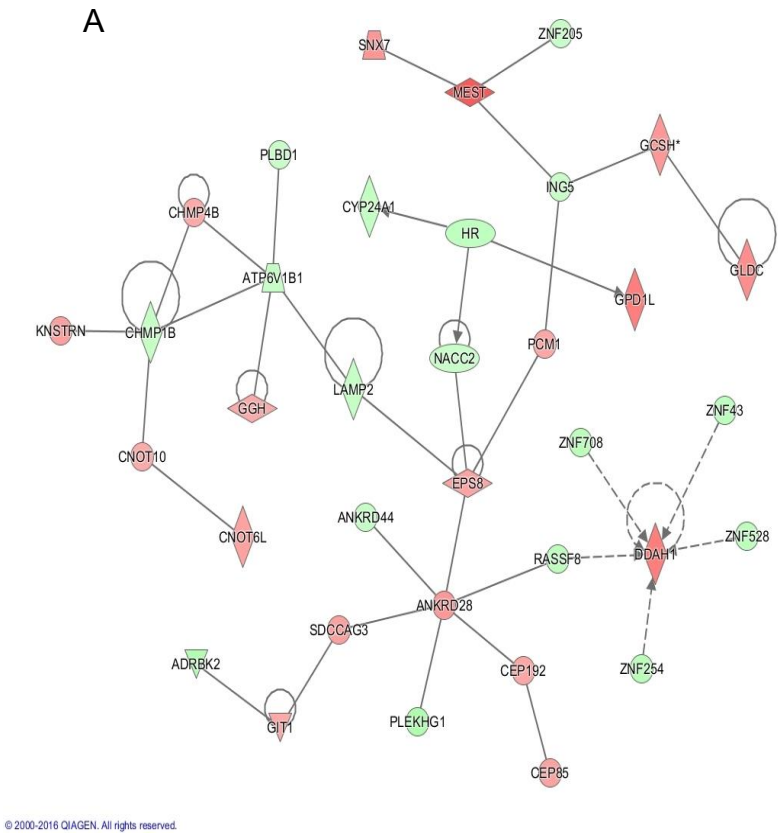


Figure 12. Network 2: Cellular assembly and organization, cellular function and maintenance, cell cycle

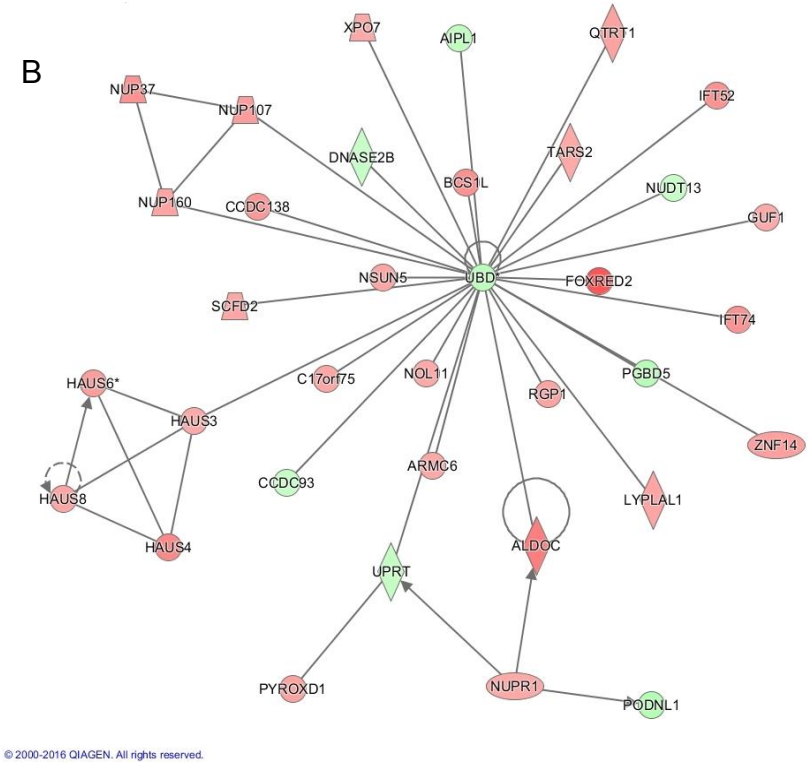


Figure 12. Network 3: Cell-to-cell signaling and interaction, cardiovascular system development and function, tissue development

C

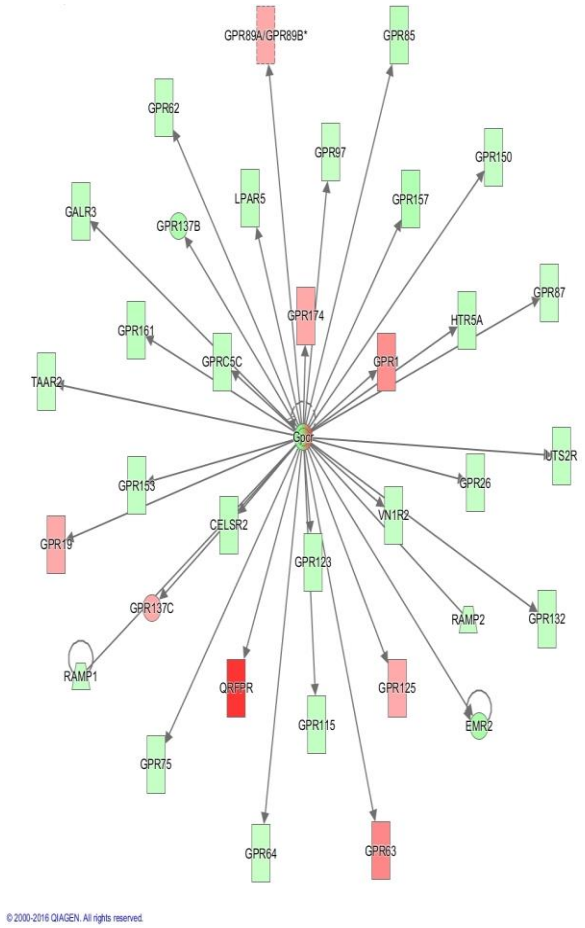


Figure 12. Network 4: Metabolic disease, amino acid metabolism, small molecule biochemistry

D

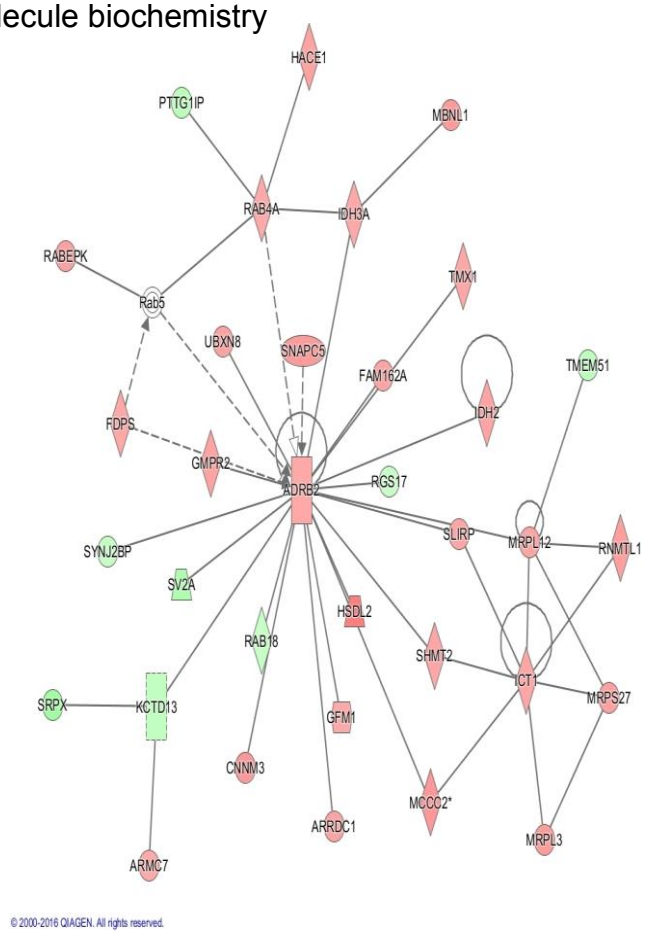


Figure 12. Network 5: Dermatological diseases and conditions, developmental disorders hereditary disorders

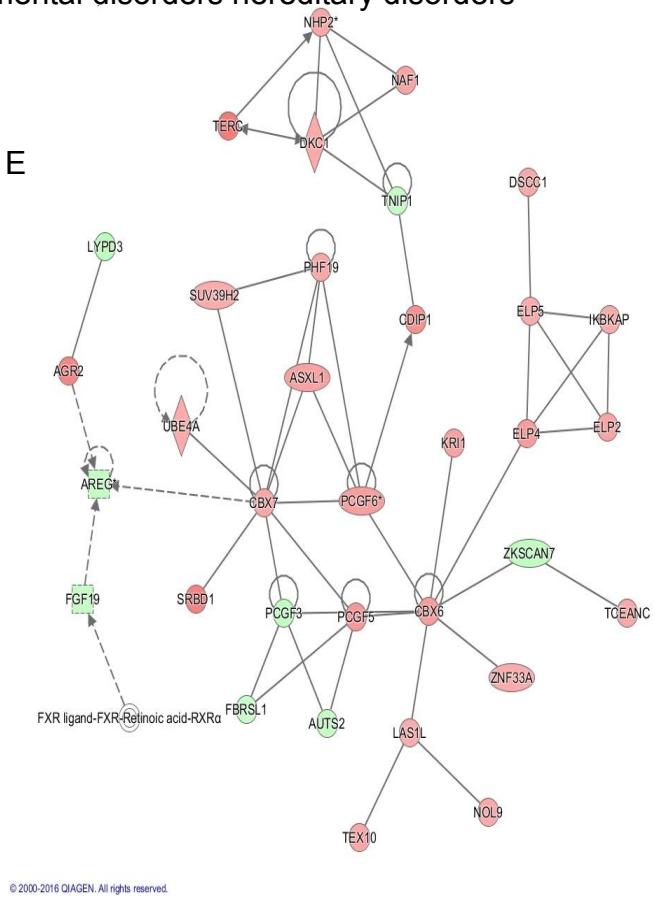


Figure 12. Top 5 cellular networks affected by Ada3 overexpression as obtained from Ingenuity Pathway Analysis. (A-E) The top 5 networks affected upon Ada3 overexpression as obtained from Ingenuity Pathway Analysis. All the genes up or down regulated at least. 1.2 fold were used for this analysis.

CHAPTER 3

GENERATION OF AN ADA3$^{FL/FL}$, GFP, CRE-ERT2 MOUSE MODEL

Note: The data presented within this chapter represents unfinished, ongoing research

3.1 INTRODUCTION

The majority of mammary gland development occurs postnatally after puberty, making it a unique organ system (Macias & Hinck, 2012). In mice, rudimentary ducts are present at birth, which grow into the surrounding tissue post puberty. The ducts give rise to the ductal tree consisting of mammary epithelial cells and the surrounding tissue composed of adipocytes form the stroma containing the mammary fat pad. Together these make up the two primary parts of the mammary gland. During pregnancy and subsequent involution the epithelial cells of the ductal tree go through multiple cycles of increased proliferation followed by apoptosis (Macias & Hinck, 2012). The initial proliferation is stimulated by hormonal changes, leading to the formation of alveolar structures within the ducts that lactate. After lactations ends, the apoptosis begins leading to involution and a return of the gland to the pre-pregnancy state (Macias & Hinck, 2012).

Estrogen receptors are well known as being critical to the development of the mammary gland (Bocchinfuso & Korach, 1997). Previous work from our laboratory found that Ada3 is an important co-activator for the estrogen receptor (Meng, et al, 2004; Germaniuk-Kurowska et al, 2007). Additionally, it was found that Ada3 regulates c-myc transcription and deletion leads to block in cell cycle progression (Mohibi et al, 2012). Our recent work showed that Ada3 is overexpressed in ER+ breast cancers and its overexpression induces an increase in proliferation and consequently increase in c-myc levels through the same c-myc-Skp2-p27 mediated pathway as we observed in MEFs (Griffin et al, submitted). Thus, in order to examine the effects of Ada3 on the development of the mammary gland we bred our floxed Ada3 mice with an inducible Cre-ERT2 model.

3.2 MATERIALS & METHODS

Mice, genotyping, and DNA isolation

The generation of mice containing floxed ADA3 genes ($ADA3^{FL/FL}$) with a GFP reporter has been previously described (Mohibi, et al., 2012). FVB background Cre-ERT2 tamoxifen inducible transgenic mice were ordered from the Jackson Laboratory to maintain the FVB/N background. Genotyping occurred at 2-3 weeks of age when pups were tattooed and a tail piece trimmed for DNA isolation using a Qiagen kit (Valencia, CA). Proteinase K was added to the cell lysis solution and tail pieces were digested overnight at 65˚C. DNA was precipitated with ethanol and reconstituted using DNA hydration buffer.

Breeding strategies

The harem mating strategy was used to generate all mice used in this study, with pups being weaned at 21days of age. Vaginal plugs were monitored to track timed pregnancies. Cre-ERT2 breeding was set so only one parent possessed the Cre gene as per the recommendation of the Jackson Laboratory, as two copies of Cre result in lethality.

Cell line generation

Breeding was set to ensure parental mice were both $ADA3^{FL/+}$ and GFP+, while only the mother was Cre-ERT2+. 13.5 days following verification of a vaginal plug, the embryos were isolated and immortalized following the 3T3 protocol (Todaro & Green, 1963) using Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum. Verification of Cre-ERT2 mediated deletion was performed by treating cell lines

genotyped as ADA3$^{FL/FL}$, GFP+, Cre-ERT2+ or ADA3$^{FL/+}$, GFP+, Cre-ERT2+ with 1μM of tamoxifen (4-OHT) for 72hrs, with media changes occurring daily.

Antibodies: Generation of anti-hAda3 mouse monoclonal antiserum has been described previously (Mohibi et al., 2012). β-actin and GFP antibodies were purchased from Santa Cruz.

3.3 RESULTS

Generation of Cre-ERT2 conditional deletion MEF cell lines

After the isolation of embryos to generate inducible deletion cell lines, DNA was isolated and genotype using the methods and primers described previously in Mohibi, et al, 2012. A total of ten embryos were analyzed. Embryos 3, 7, and 9 exhibited the desired ADA3$^{FL/FL}$, GFP+, Cre-ERT2 genotype, while embryos 2, 4, 5, and 10 exhibited the desired control ADA3$^{FL/+}$, GFP+, Cre-ERT2 genotype (Figure 10A). These lines were immortalized following the well-established 3T3 protocol developed by Todaro & Green in 1963. Following immortalization cells were treated with 1μM 4-OHT for 72hrs to activate Cre-ERT2 to delete at loxP sites and to induce recombination to remove ADA3 and activate the GFP reporter (Figure 10B). Ada3 protein is not expressed in ADA3$^{FL/FL}$ treated with 4-OHT; however protein is expressed in heterozygotes.

Figure 13. Generation of Cre-ERT2 inducible deletion MEF cell lines.

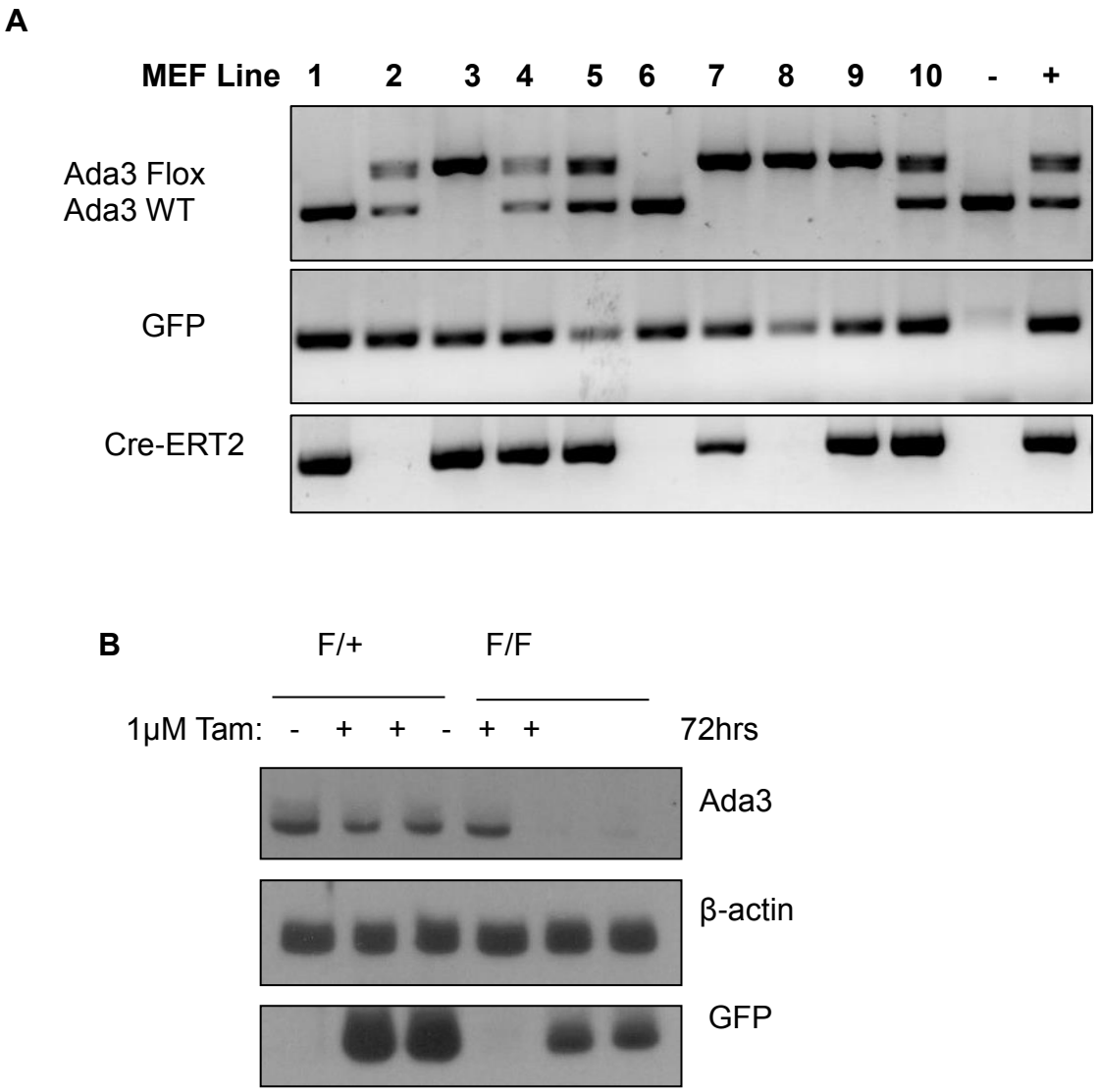


Figure 13. Generation of Cre-ERT2 inducible deletion MEF cell lines. (A) Ten embryos were isolated and genotyped for Ada3, GFP, and Cre-ERT2 in conjunction with negative and positive controls for each gene. **(B)** Immortalized MEFs heterozygous or homozygous for Ada3, and heterozygous for GFP and Cre-ERT2 were treated with 1nM 4-OHT for 72hrs and analyzed for Ada3 deletion.

3.4 DISCUSSION AND FUTURE DIRECTIONS

Recently, the role of Ada3 in cell cycle progression and embryonic development has been determined to be highly important (Mohibi et al, 2012). Deletion leads to embryos not surviving past day E8.5 (Mohibi et al, 2012). Additionally, Ada3 has been found to function as a coactivator for the estrogen receptor and is overexpressed in breast cancer, leading to an increase in the levels of c-myc (Meng et al, 2004; Griffin et al, submitted). The inducible deletion MEF cell lines generated can be used for future analysis of Ada3 mutants generated in the lab because deletion is complete, unlike the mosaic deletion seen when Cre-mediated gene deletion is performed using adenoviruses. Our recent finding that high levels of nuclear Ada3 correlated with high levels of c-myc in ER+ breast cancer patients pushes us towards analyzing the interplay between Ada3 and c-myc in a breast cancer model system. In order to determine the role of Ada3 in breast cancers we will breed our conditional deletion mice with the doxycycline inducible PIK3CA driven mammary tumor model mouse. By transplanting the mammary gland from a Cre-ERT2+ mouse into a non Cre-ERT2 mouse we will be able to study the effects of Ada3 deletion on c-myc mediated tumor progression. We expect that deletion will lead to a decreased tumor burden due to our observations *in vitro*.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Discussion

Recent studies from our laboratory demonstrated the role of Ada3 led to cell cycle delays and that Ada3 functions as a prognostic marker in concert with hormone receptor status provided the rationale to examine the role of Ada3 overexpression in proliferation (Mohibi et al, 2012; Mirza et al, 2013). After examining multiple breast cancer cell lines and tumors grown *in vivo* we were able to ascertain that when compared to immortal mammary cells, cancerous cells expressed a higher amount of Ada3 protein. The increased Ada3 protein level also correlated with increased levels of Ki67, a well-known marker of proliferation, in PDX tumors. Ada3 levels were also higher in ER+ samples as opposed to other subtypes. Consequently, we chose to overexpress Ada3 in immortal mammary epithelial cells and ER+ breast cancer cell lines.

Upon overexpression of Ada3, we found a significant increase in cell proliferation in the immortal mammary epithelial cell lines and ER+ breast cancer cell lines. These results were not wholly unexpected, because recent findings made by our laboratory demonstrated that deletion of Ada3 led to decreased proliferation (Mohibi et al, 2012). The well-known SRC3 coactivator has been shown to enhance proliferation when it is overexpressed (Louie et al, 2004). This protein also promotes ER mediated gene transcription and interacts with the p300 and PCAF histone acetyltransferases (Lahusen et al, 2009). However, observations from our laboratory and from the O'Malley laboratory have not demonstrated that Ada3 and SRC3 function together in the same complex. In an attempt to discern if there were any chromosomal changes linked to overexpressing Ada3, we examined the karyotype of both 76NTERT vector and Ada3 overexpressing cells. The karyotype was not significantly altered in Ada3 overexpressing cells as compared to vector cells. Thus, the observed increased proliferation was not the byproduct of an unstable genome.

We examined cell cycle related genes after both the overexpression and knockdown of Ada3 to determine if cell cycle alterations were the source of the proliferative change. Using FACS analysis we found that increased cell cycle progression from the G1 phase into the S phase was shifted approximately four hours earlier in the overexpressing cells to 16hrs (26.6%) as opposed to 20hrs (24%) in vector cells. Conversely, when Ada3 was knocked down the 12hr time point for scrambled control marked the beginning of the transition of cells into S phase, while that didn't begin until the 16hr time point in KD cells. Overall fewer cells were observed to be entering the cell cycle when Ada3 was ablated, much in the same way that was observed previously (Mohibi et al, 2012). Interestingly, p27 levels were decreased during Ada3 overexpression and c-myc levels were increased. It has been previously documented that c-myc deletion is embryonically lethal and that KD of Ada3 leads to a decrease in c-myc levels (Davis et al, 1993; Mohibi et al, 2012). Similarly to the findings of Mohibi et al, Ada3 knockdown in immortal mammary epithelial cells led to a delay in phase transitions and an increase in p27 levels (2012). Two well-known early cell cycle response genes, c-fos and EGR1, were found to have greatly increased mRNA levels in overexpressing cells as compared to vector. C-fos has long been known to be involved in proliferation and EGR1 has also been found to be important for cell proliferation and migration (Angel & Karin, 1991; Mitchell et al, 2004; Min et al, 2008), therefore expression levels for both being elevated in our overexpressing cells makes sense.

As the mechanism governing cell cycle transition delays in MEFs is mediated by a mechanism containing both p27 and c-myc we examined the levels of these gene products. When overexpressed, the levels of c-myc mRNA and protein are higher than that of vector cells, while the p27 half-life is decreased, again functioning in the opposite way of the knockdown studies performed previously (Mohibi et al, 2012). Just as in previous studies, we observed a decrease in histone acetylation when Ada3 was

knocked down in human derived cell lines; however levels were increased when Ada3 was overexpressed. This increase makes sense in that for cells to become more proliferative there must be an increase in acetylated histones to allow DNA binding proteins access to DNA.

In attempt to build upon previous work from our lab showing Ada3 is an important ER coactivator (Meng et al, 2004; Germaniuk-Kurowska et al 2007) we scrutinized the effect of Ada3 overexpression on estrogen independence in ER+ breast cancer cell lines. After depriving the commonly used MCF7 and ZR75-1 cell lines cells of growth factors other than estrogen and performing proliferation assays, we found no link between the levels of Ada3 expression and estrogen independent growth. The overexpression cells exhibited proliferation similar to that of vector cells when deprived of estrogen. Had this occurred we would have examined the components of the AKT pathway to determine if these were elevated to cause proliferation and growth (Miller et al, 2010). We did find that the mRNA for ER target genes was increased post stimulation which was in line with the previous findings of our lab. Additionally, the overexpressing cells proliferated at a greater rate than the vector cells when stimulated with estrogen, similar to the amount of proliferation seen in complete media so even with limited defined growth factors Ada3 can lead to increased proliferation. Next we treated cell lines with varied doses of tamoxifen to determine if there was a link between Ada3 and resistance as observed with SRC3 (Osborne et al, 2003; Shou et al, 2004; Dihge et al, 2008; Kamakar et al 2010). We found no change in the survival of overexpressing cells when compared to vector cells. This is more than likely due to these coactivators functioning in the same manner, but in separate mutually exclusive complexes. We then desired to ascertain if there was a change in the invasion and migration potential in both ER+ and ErbB2+ breast cancer cell lines overexpressing Ada3 as was observed using an SRC3 variant (Long et al, 2010.) Again, we found no significant difference in these

measurements of oncogenic potential. Due to the increased levels of Ada3 in tumor cell lines as opposed to immortal cell lines and that tumor tissues were observed to contain cytoplasmic Ada3, we examined 2D grown tumor cell lines for changes in localization. We found that during *in vitro* growth Ada3 is a nuclear protein. This may be due to cells grown in culture lacking the specific microenvironment necessary to redistribute Ada3 to the cytoplasm as observed in patient samples. We then examined the global changes caused by Ada3 overexpression in asynchronous 76NTERT cells through microarray analysis. We found that there is a differential change in expression in the levels of multiple genes with some affected pathways akin to what was observed in MEFs. Of the most potential interest are networks involving the regulation of the cell cycle; however the majority of these differentially regulated proteins were not within the standard range of greater than 1.5 fold change in expression levels. Our analysis of these dysregulated pathways was not exhaustive, thus delving deeper into potential alternate mechanisms to the established Ada3-c-myc-Skp2-p27 pathway could be performed in the future.

The observed significant connection between nuclear Ada3 expression levels and nuclear c-myc levels in patient samples can be further examined to ascertain if there is an alteration in tumor burden when Ada3 is deleted in a mammary tumor model exhibiting c-myc amplification. The findings of this study together with previous studies examining the role of Ada3 ablation in the cell cycle, the role of Ada3 in mitosis, and the effect of expression on cancer prognosis help to better emphasize the importance of Ada3 in cell growth and proliferation.

FUTURE DIRECTIONS:

While I have attempted to thoroughly examine the role of Ada3 in proliferation and oncogenesis, there are still several important areas that need to be explored in the future. These include, but are not limited to:

- Examine E2F related transcription factors for alterations in expression level as an alternative or additional pathway to increased proliferation
- Determine if there are novel Ada3 target genes in ER+ cell lines via ChIP
- Examine the role of Ada3 in murine development using the $Ada3^{FL/FL}$, GFP, Cre-ERT2 mice
- Breed the $Ada3^{FL/FL}$, GFP, Cre-ERT2 mice with PIK3CA mice to allow for the deletion ofAda3 in the mammary gland and to observe mice for altered tumor burden
- Examine the role of Ada3 functionally deficient mutants using Cre-ERT2 MEF cell lines

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