To the Graduate Council:

I am submitting herewith a dissertation written by Romaine Ingrid Fernando entitled "BAD is a Central Player in Cell Death and Cell Cycle Regulation in Breast Cancer Cells". I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Jay Wimalasena

Major Professor

We have read this dissertation
and recommend its acceptance:

Roger Carroll

Hwa-Chain Robert Wang

Hildegard Schuller

Ana Kitazono

Accepted for the Council:

Anne Mayhew

Vice Chancellor and the Dean of
Graduate Studies

(Original signatures are on file with official student records.)

# BAD is a Central Player in Cell Death and Cell Cycle Regulation in Breast Cancer Cells

**A Dissertation Presented for the**
**Doctor of Philosophy Degree**
**The University of Tennessee, Knoxville**

**Romaine Ingrid Fernando**
**December 2004**

## DEDICATION

This dissertation is dedicated to my parents, Vivienne Fernando and Marcus Fernando with love.

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# Abstract


**BAD is a Central Player in Cell Death and Cell Cycle Regulation in Breast Cancer Cells**

The estrogen, 17-β estradiol ($E_2$) can stimulate proliferation or induce differentiation or death depending on the cell context. In MCF 7 cells, an estrogen receptor (ER) positive human breast cancer cell line, $E_2$ has been shown to stimulate cell proliferation and may have an anti-apoptotic effect. $E_2$ rapidly activates mitogen activated protein kinases (MAPKs) in mammalian cells in an ER dependent fashion. The mechanisms underlying and the physiological significance of the signaling through MAPK remain to be determined. This signal pathway may represent a potential mechanism by which $E_2$ regulates cell proliferation, growth as well as apoptosis. Recent observation of Raf-1 kinase (which relays signals from cell surface receptor tyrosine kinase to MAPKs) activation by $E_2$ in MCF 7 cells explained a possible link between cell surface and intracellular components of the signaling pathway. PI-3 kinase and its down stream member protein kinase B (PKB/Akt) have been shown to be strongly involved in mitogenic and anti-apoptotic signaling in MCF 7 cells.

Chemical and biological inhibition of PI-3 kinase diminished $E_2$ supported cell survival and concomitant Akt, MAPK (ERK) and its down stream target $p90^{RSK}$ activities. On the other hand, interference of endogenous Ras with a dominant negative construct abolished signal mechanisms involving ERK as well as Akt. These observations suggested that Ras/ERK and PI-3 Kinase pathways could operate as separate entities or cross talk and cooperate to enhance survival or impede apoptosis in MCF 7 cells in the presence of $E_2$.

BCL2 family proteins play critical decision-making roles during apoptosis. BH3 domain only, pro-apoptotic member, BAD has been intensively investigated for its contribution in regulation of apoptosis at the mitochondrial level. Post-translational phosphorylation modulates BAD's function and, most survival factors impose inhibitory phosphorylations on BAD via active Akt and MAPK pathways.

$E_2$ rapidly induces Akt and ERK signaling pathways resulting in phosphorylation of BAD on two serine residues, and abolishes apoptosis in response to a variety of stimuli in MCF-7 cells. Therefore, we hypothesized that $E_2$ targets BAD for its anti-apoptotic effect. Data presented clearly show that $E_2$ effects are at least partly mediated via Ras originated ERK/$p90^{RSK}$ and PI-3 kinase/Akt pathways that add inhibitory phosphate groups onto corresponding serine 75 and 99 residues of BAD there by inactivating its pro-apoptotic tendencies. We demonstrate the significance of BAD and its functional serine residues in $E_2$ mediated anti-apoptosis with studies conducted with anti-sense mRNA and phosphorylation site mutants of BAD. Over-expression of wild type or phosphorylation site mutant of BAD did not enhance the basal cell death, which was remarkably lessened by the inhibition of endogenous BAD. $E_2$ could rescue cells from TNF$\alpha$ induced death in control cultures and in wild type BAD transfected cells but not in phosphorylation site mutant BAD expressing cells. This observation agrees with previous studies that demonstrate the importance of BAD's phosphorylation sites in growth factor mediated cell protection. This study shows for the first time that $E_2$ is an anti-apoptotic agent in breast cancer cells whose response is mediated by non-genomic cytoplasmic signal networks that converge on BAD in turn regulating the mitochondrial membrane potential change.

When transiently over-expressed, wild type or dual phosphorylation site mutant of BAD did not induce apoptosis, as observed in the first part of this study, but the rate of growth of these cells was remarkably low compared to the untransfected or control vector transfected cells. This suggested that the presence of extra amount of BAD could reduce the growth of MCF-7 cells. Similar phenomenon have been reported with the overexpression of BCL2 in malignant as well as normal epithelial cells. When over expressed, BCL2 arrests cell cycle in G0/G1 phase and this effect appears to depend on the amino terminal BH4 domain. Interestingly in a variety of epithelial cell types, endogenous BCL2 was shown to localize to the chromosomes of mitotic nuclei. This pattern of BCL2 expression is considered to be indicative of its special role in cell proliferation.

We hypothesized that BAD also exhibits a cell cycle related function in breast cancer epithelial cells, which was tested in the second part of this study. We evaluated the distribution pattern of nuclear BAD in several breast cancer cell lines. BAD overexpression lessened cyclin D1protein levels that agreeably correlate with the G1-S transition block observed. Cyclin D1 regulation by BAD was assayed at the transcriptional level using a luciferase reporter under the control of the cyclin D1 promoter. In asynchronous cells and in synchronous and $E_2$ treated cells, BAD over-expression significantly reduced the cyclin D1 reporter activity. Overexpression of a dual phosphorylation site mutant of BAD did not alter the cyclin D1 expression pattern or the cell cycle progression suggesting an involvement of these serine residues in BAD's nuclear functions. Furthermore, the BH3 domain deletion mutant and the S91A mutant, both of which carry intact S75 and S99 residues, exerted inhibitory effects similar to that of wild type BAD.

Wild type BAD overexpression as well as BAD depletion from nuclear extracts diminished the labeled AP-1/TRE DNA binding with the nuclear factors. This demonstrates that BAD is capable of binding and sequestering some components from the nuclear extracts which bind with AP-1/TRE element. Collectively our data suggest that in addition to pro-apoptotic functions, BAD regulates the cell cycle at least partly via AP-1/TRE element. These functions appear to depend on its S75 and S99 phosphorylation status that are also the targets of pro-survival mechanisms. Further, these results also suggest that the AP-1 transcription regulation by BAD is kept at its minimal level in normal cells as a result of the fine balance of its phosphorylation state.

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# Chapter 1

# Signal Pathways that Mediate 17-β Estradiol Induced Anti-apoptosis in Breast Cancer Cells

## 1.1 Introduction

### 1.1.1 Cancer

Cancer cells are characterized by two heritable properties; they (i) reproduce in defiance of normal restraints, and (ii) invade and colonize surrounding tissue and distant sites constituting the phenomenon, metastasis. Fundamental properties of malignancy are encoded in genes of the cancer cell, as evident by the inheritance of malignant traits by its progeny. Studies of genetic markers in cancers indicate a given human tumor is derived from a single cell (e.g. expression of unique immunoglobulin molecules in lymphomas and myelomas, translocation of chromosomes 9 and 22 (Philadelphia chromosome) in chronic myelogenous leukemia). However, further genetic changes as well as other environmental factors could result in a heterogeneous population of cancer cells in a given tumor and this heterogeneity may affect many aspects of cancer development i.e. invasiveness, resistance to tumor therapy and metastatic properties, hence the tumor progression.

Much of current cancer research is directed to the identification and characterization of genes whose products are involved in the transformation of a normal to a malignant cell. These studies led to the identification of oncogenes, where a mutation causes a change in a normal cellular protein that could lead to malignant change in the cell. These genes are dominantly acting since only one copy of the gene has to undergo the mutation. Many of these gene products are important in the normal transmission of growth-promoting signals from cell surface to the nucleus (e.g. *src, ras*), transcription regulation (e.g. *myc, c-jun*), or regulation of cell death (e.g. *bcl2*). The other class of genes, tumor suppressors, acts to suppress the malignant transformation in normal cells (e.g. *Rb*, *p53, BRCA1* and *2*). Both copies of the tumor suppressor gene must be lost, mutated or inactivated for malignant transformation. In normal tissue, there is a balance between cell proliferation and cell death. In tumors, this balance is disturbed

perhaps because of genetic defects that lead to abnormal cell proliferation and death. Aberrant cell proliferation and death rarely coincide as does the occurrence of cancer. Increased proliferation consequently triggers cell death and the reduced cell death is not significance in the absence of enhanced proliferation (103). This natural interdependency of the two key mechanisms keeps the tissue balanced. Accumulation of genetic damages in cells hinders their ability to restrain or counteract abnormal cellular processes. Acquisition of independency from mitogenic stimuli and noncompliance to growth inhibitory signals could be the result of such genetic mutations. Telomere erosion, which sets control for endless cell division is the next obstacle that sustained cell proliferation has to overcome. Telomeres are the long non-transcribed regions at the ends of chromosomes. As a result of repeated DNA replication these structures shortens and this phenomenon is termed as telomere erosion. The enzyme that repairs the eroded telomeres, telomerase is expressed in limited fashion in somatic cells; therefore the somatic cells are allowed only a limited number of cell divisions (105). Telomerase up-regulation has been observed in cancer (18).

Characterization of cell proliferation and death in cancer has provided the information that contrary to general belief, tumor cells usually proliferate more slowly than cells in normal tissues, such as the intestinal epithelium and bone matrix. A relatively small proportion of tumor cells may retain the capacity for unlimited proliferation and these stem cells are the important targets of cancer therapy. Furthermore, tumors have a high rate of cell death perhaps as a result of limited supply of nutrients, through the process of programmed cell death and /or necrosis (72). Also, the over-expression of oncogenes has been shown to induce apoptosis in tumors (75,122,151).

It has been suggested that immune surveillance may eradicate some tumors before their clinical detection. Expression of ‘non-self’ molecules such as the products of mutated oncogenes, which differ from those in normal cells, could promote an immune rejection response. Therefore, the tumors that either do not express these ‘non-self’ molecules or have mechanisms allowing them to evade the immune rejection response

may continue to grow (1). This type of information has led to the efforts to devise therapies that might induce tumor rejection by the host.

In solid tumors there are interactions between the constituent cells and the extra cellular matrix (ECM). These interactions may be modulated by growth factors and hormones that interact with specific receptors and propagate signal and gene expression, and adhesion molecules that interact with ECM components and initiate intracellular signaling (46,181,215). The ECM provides the surface for the cell attachment and a barrier for cell movement. Therefore, breakdown of extra cellular components through secretion of proteolytic enzymes (e.g. metalloproteinases, Cathepsins), which is thought to be an important mechanism in metastasis (229), may also assist in invasion, growth and the angiogenesis of a tumor. The inhibition of these proteases has been recognized as potential strategy to suppress tumor growth (88). Further, proper attachment of transformed as well as untransformed mammary epithelial cells to the ECM was shown to enhance their anti-apoptotic capacity. This acquired resistance to apoptosis depends, at least in part on NFκB mediated signaling, which was activated in this system by the interaction of epithelial integrin and the ECM (283).

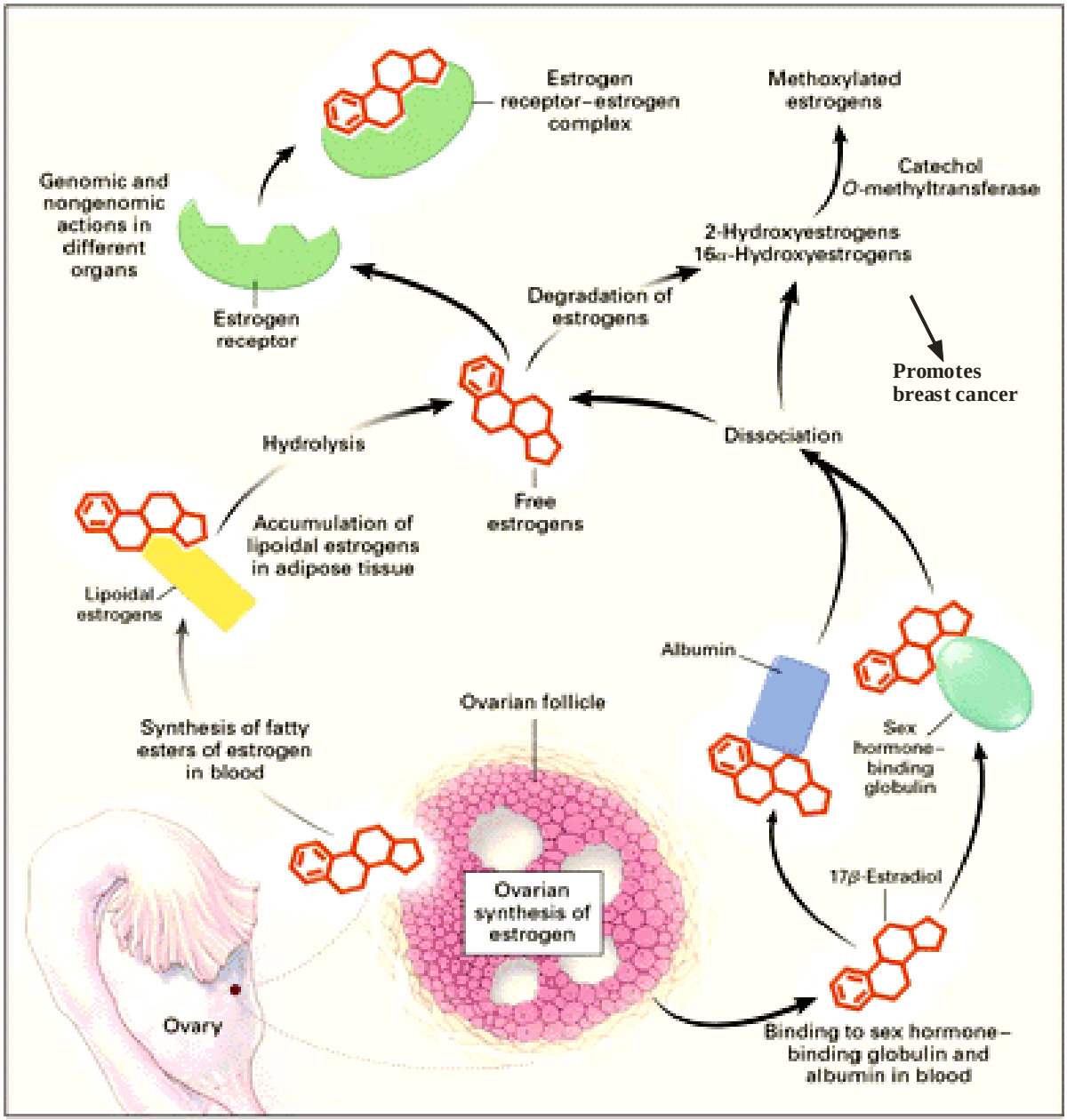
In order to sustain the growth, a solid tumor has to elicit the proliferation of blood vessels that provide nutrition to the cancer cells and remove catabolic products. This process of angiogenesis is stimulated by the release of several growth factors from tumor cells as well as the surrounding normal cells. Vascular endothelial growth factor (VEGF) has been shown to vastly contribute to this process. The synthesis and release of VEGF is stimulated by the hypoxic environment (223). Upregulation of VEGF and angiogenesis has also been observed in response to constitutive activation of other oncogenic proteins (e.g. EGFR, Raf, MEK, PI3K) acting at various levels on the Ras signaling pathway (219). Interestingly, a stronger expression of VEGF was observed in the surrounding stroma but not in the tumor in an animal model (97). This finding that the VEGF promoter of non-transformed cells is strongly activated by the tumor microenvironment may point to a need to analyze and understand the stromal cell collaboration in tumor angiogenesis.

While clinical observation has divided tumor development into a number of discrete categories of severity (benign, malignant, metastatic), the underlying biology can be described as a process of many changes. The genetic changes can affect the cell's growth potential, and the cells with such changes are selected for (or against) by the conditions they are exposed to. Increasing knowledge of signal transduction pathways in cells has demonstrated that many aspects of cell function are controlled by a balance of positive and negative signals received from inside and the outside of the cell. Thus the lack of ability or increased ability to respond to a specific signal may allow the cell to proliferate in the face of other signals that would normally prevent such proliferation. Cancer treatment can be considered as adding negative signals to the cellular environment. The process of genetic change may give rise to a subpopulation of cells that have evolved varied mechanisms of drug resistance, and these cells could survive and re-grow the tumor following chemotherapy. The genetic evolution of cancer cells and the signal that the cells receive from their extra cellular environment therefore could play critical roles in the growth and treatment of cancers (1).

### 1.1.2 Estrogen

#### Synthesis, Metabolism and Physiological Importance

Estrogens, an important class of steroid hormones, influence growth, differentiation and functions of many target tissues. All naturally occurring estrogens (estrone, estradiol and estriol) are $C_{18}$ steroids secreted by the theca interna and granulosa cells of the developing ovarian follicle, corpus luteum as well as the placenta. Estrogens are also produced by aromatization of androgens in adipose tissue, liver, skin and in breast epithelial cells. Both estrogens and precursor androgens are derived from cholesterol. The most potent naturally occurring estrogen, 17-β estradiol ($E_2$) is found in both free and protein bound forms in the circulation (Figure 1.1.2.1). After synthesis, $E_2$ is secreted into the blood stream where it binds with sex-hormone-binding globulin and albumin. Free estrogens diffuse into target tissues to exert their specific genomic or non-genomic effects. Enzymatic catabolism of estrogens yields the hydroxyl-estrogens and methoxy-estrogens (107). In the liver, estrogens are conjugated to sulfate or gluconate




***Modified from NEJM 346: 340-352***

**Figure 1.1.2.1**

**Synthesis, metabolism and physiological importance of estrogen**
See text for details

and made water-soluble. Then they are excreted by billiary secretion and a major part of it is reabsorbed prior to urinary passage (2).

Besides stimulating development and maturation of the female reproductive structures and secondary sexual characteristics, estrogens are necessary for cyclic endometrial changes that are important for reproduction, development of breast tissue, health of the skin and bone homeostasis. Estrogenic effects on blood coagulation, endothelial cells, intestinal motility, cholesterol metabolism and sodium and water conservation by renal tissue have also been appreciated giving estrogen a remarkable importance in the normal physiology (123,228). Importance of estrogen in male fertility, specifically to sperm count was reported recently. This study focused on the regulatory role of estrogen - induced fluid absorption during the transfer of sperm in fluid from the testis through the efferent ductules to the epididymis (116). Some pesticides such as DDT and many industrial chemicals have estrogenic or anti-estrogenic properties and may have deleterious effects on male and female health. Declining sperm counts all around the world is regarded as a reflection of the adverse effects of estrogenic compounds on male fertility (236).

**Estrogen Receptors, Classical *vs*. Non-Classical Functions of Estrogen**

Many of the diverse biological effects of sex steroids are hypothesized to occur as follows: the hormone enters the cytoplasm by diffusion, translocates to the nucleus, interacts with nuclear receptor, and binds DNA resulting in transcriptional activation of steroid responsive genes. The estrogen receptor (ER) when unattached to the ligand is loosely bound in the cytoplasm by a host of chaperone proteins. These molecules function to stabilize the receptor in the inactivated state, prevent the receptor from binding to the DNA elements, and contribute to cross-talk among different signal pathways (249,260,270). The exact location of the ER-chaperone complex is still uncertain. The possibility is that there is an equilibrium distribution between the cytoplasm and the nucleus that could be shifted upon the stimulation with the estrogenic ligand. When free estrogen diffuses into the cell, it binds with the ligand-binding domain of the ER. The chaperones dissociate and estrogen-ER complex then migrates to the

nucleus and bind to specific sequences of DNA called estrogen response elements (ERE, 5'GGTCAnnTGACC3' where n=any nucleotide). This DNA binding requires ER to be dimerized (287). A host of ERE carrying genes has been defined (Table 1.1.2.1). The estrogen-receptor complex not only binds to the EREs, but to co-activators or repressors thereby adding levels of regulation to the transcriptional activities of estrogen. Estrogens also regulate the transcription of genes that lack functional EREs by interacting with other nuclear factors. As an example, ER interacts with activating protein-1 (AP-1) subunits and modulates AP-1 dependent gene transcription (274).

Two types of nuclear ER, α and β which carry out estrogen-induced gene activation upon ligand binding have been demonstrated, depicting the classical steroidal hormone-receptor function. However the effects of nuclear cofactors on ERα and β ligand binding and the correlation of ligand binding and gene transactivation have not been completely resolved yet. Both isoforms are reported to originate from a single transcript and have near-identical affinities for $E_2$ (225) though their tissue distribution and functional mechanisms seems to differ considerably. ERα predominates in breast and uterine tissues (154) whereas ERβ is expressed in the central nervous system, endothelial cells and urogenital systems (Figure 1.1.2.2 (B)) (287). Though both receptors share many structural similarities (Figure 1.1.2.2 (A)) their functional differences may add to the existing complexity of estrogenic effects. The main difference between them lies within the AF-1 transactivation domain, which is inactive or even repressive in ERβ (109,154,296) in contrast to ERα whose AF-1 is active by itself. Interestingly both isoforms have virtually identical DNA binding domains suggesting that ERα and β interact with similar response elements. Outcome and level of maintenance of activated signal cascades by both receptors differ in the presence of estrogen agonists and antagonists. Several lines of human breast cancer cells and about 70% of breast cancers at initial diagnosis express estrogen receptors. About 50% of these tumors are dependent on $E_2$ for growth as evidenced by studies with anti estrogens (32,254).

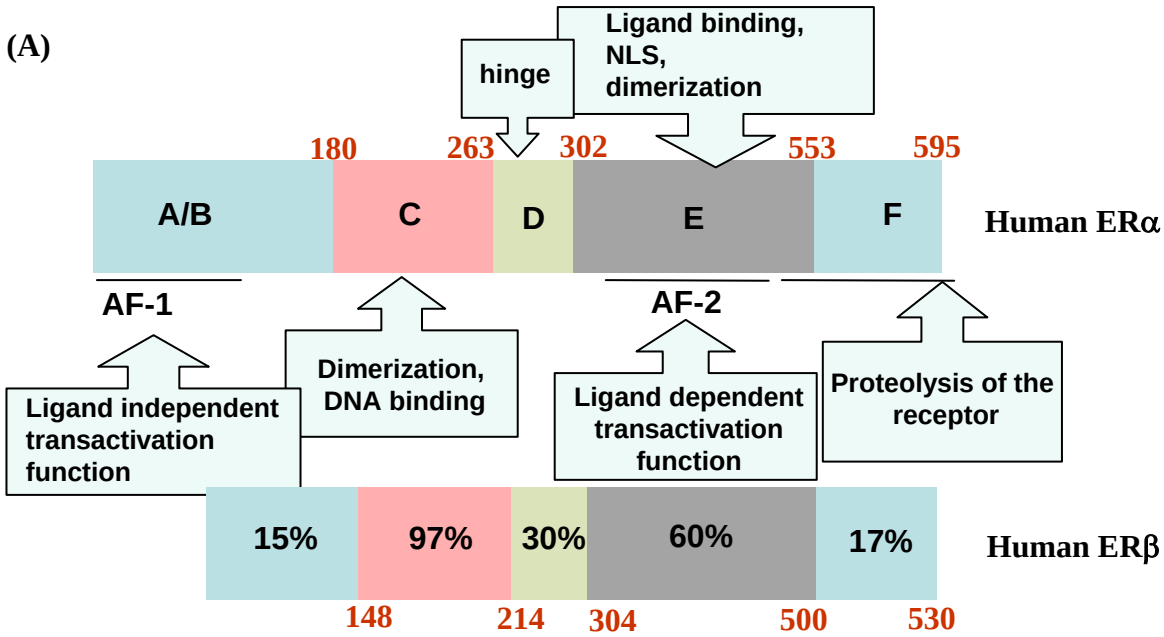
Estrogen and ER duet could yield to a magnitude of alternative pathways that may contribute to non-classical, non-genomic functions, some of which have been already described. As for many other nuclear receptors, ligand independent activation of ER

## Table 1.1.2.1

**List of Putative Estrogen /ER Target Genes**

*Modified from Genome* ***Biology 5:****R66*

| Gene name | Function |
|---|---|
| thrombospondin 1 | Cell adhesion [0007155] |
| Link guanine nucleotide exchange factor II | Neurogenesis [0007399] |
| Olfactomedin 1 | Signal transduction [0007165] |
| DnaJ (Hsp40) homolog, subfamily C, member 1 | Protein folding [0006457] |
| Insulin-like growth factor binding protein 4 | Signal transduction [0007165] |
| ATP-binding cassette, sub-family A (ABC1), member 3 | ATP-binding cassette (ABC) transporter [0004009] |
| Stanniocalcin 2 | Cell-cell signaling [0007267] |
| Hypothetical protein DKFZp761P0423 | Biological_process unknown [0000004] |
| Solute carrier family 38, member 1 | Amino acid transport [0006865] |
| Hypothetical protein FLJ20986 | Cation transport [0006812] |
| mRNA; cDNA DKFZp434E082 | Biological_process unknown [0000004] |
| Protein kinase H11 | Translational regulation, initiation [0006446] |
| GREB1 protein | High-affinity zinc ion transport [0006830] |
| Retinoblastoma binding protein 8 | DNA repair [0006281] |
| Seven in absentia homolog 2 (*Drosophila*) | Ubiquitin-dependent protein degradation [0006511] |
| Chemokine (C-X-C motif) ligand 12 (stromal cell derived factor 1) | Immune response [0006955] |
| Frizzled homolog 8 (*Drosophila*) | Biological_process unknown [0000004] |
| J domain containing protein 1 | Physiological processes [0007582] |
| NIF3 NGG1 interacting factor 3-like 1 (S. pombe | DNA methylation [0006306] |
| Hypothetical protein FLJ13710 | Developmental processes [0007275] |
| Hypothetical protein FLJ14299 | Transcription regulation [0006355] |
| cDNA: FLJ22159 fis, clone HRC00251, mRNA sequence | Biological_process unknown [0000004] |
| Sodium channel, voltage-gated, type I, beta polypeptide | Sodium transport [0006814] |
| Tumor protein D52-like 1 | Signal transducer [0004871] |
| Diacylglycerol kinase, zeta 104 kDa | Signal transduction [0007165] |
| Immunoglobulin superfamily, member 4 | Virulence [0009406] |
| Cyclic AMP-regulated phosphoprotein, 21 kD | Biological_process unknown [0000004] |
| *Helicobacter pylori* responsive 1 | Biological_process unknown [0000004] |
| cDNA FLJ13137 fis, clone NT2RP3003150, mRNA sequence | Biological_process unknown [0000004] |
| Hypothetical protein FLJ21918 | RNA processing [0006396] |
| Cytochrome c, somatic | Electron transport [0006118] |
| Progesterone receptor | Signal transduction [0007165] |
| S-adenosylmethionine decarboxylase 1 | Polyamine biosynthesis [0006596] |
| Interleukin 6 signal transducer (gp130, oncostatir M receptor) | Signal transduction [0007165] |
| Nuclear receptor interacting protein 1 | Transcription regulation [0006355] |
| Prostaglandin E synthase | Prostaglandin metabolism [0006693] |
| Chromosome 14 open reading frame 3 | Protein folding [0006457] |
| Ornithine decarboxylase antizyme inhibitor | Polyamine biosynthesis [0006596] |
| Protein phosphatase 1, regulatory (inhibitor) subunit 15B | Biological_process unknown [0000004] |
| Cathepsin D (lysosomal aspartyl protease) | Proteolysis and peptidolysis [0006508] |



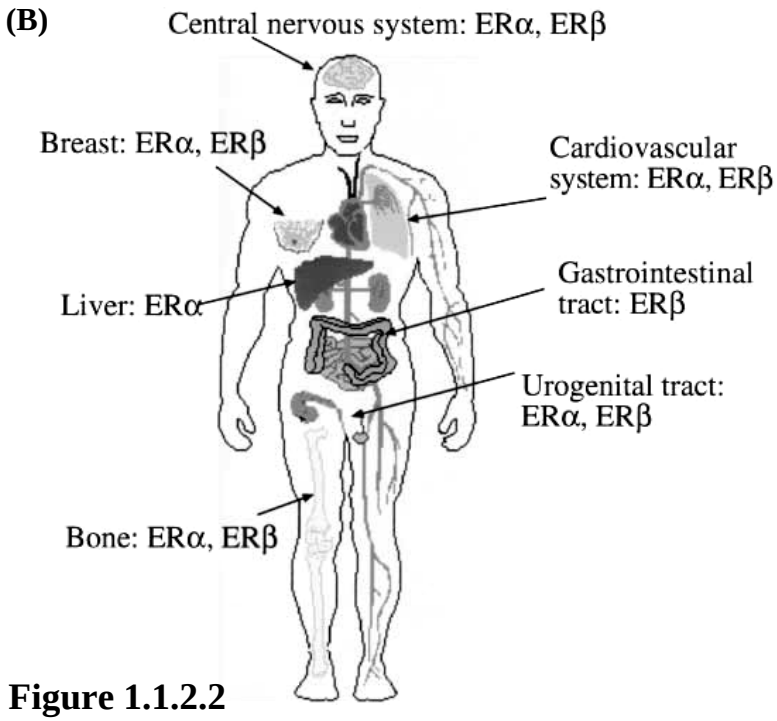




**Figure 1.1.2.2**

**Structure and tissue distribution of estrogen receptors (α and β)**

**(A)** Structure of human estrogen receptors. Function of various regions of the receptor are indicated in boxes. Numbers in red indicate position of amino acid residues. % indicates the percent homology between ERβ to ERα

**(B)** General distribution of both receptors in human

(mostly ERα) has been reported. ERs are members of the steroid hormone receptor family that are regulated by phosphorylation of tyrosine and serine residues. Changes in the phosphorylation could invariably modulate their function. Growth factors and other agents may possibly activate/inactivate these receptors through the activity of their downstream protein kinases. Phosphorylated sites vary depending on the identity of the activated kinase/s, thereby adding more intricacies to the receptor function. For an instance, PI-3 Kinase/Akt pathway phosphorylates ERα at consensus serine 167 activating the receptor. This activity confers protection against anti-estrogen therapy in breast cancer cells (43) possibly through blocking ER and anti-estrogen interaction. Similarly, resistance to tamoxifen was shown to be mediated by PKA through its target serine 305. Upon phosphorylation of this site, ERα was unable to acquire the inhibitory conformation when bound to tamoxifen, and this led to growth stimulation in the presence of this anti-estrogen (191). Additionally, serine 104, 106, 118 and 126 as well as tyrosine 357 have been identified as potential targets of various kinases and their phosphorylations appear to activate the receptor function.

Although in vitro studies suggest the two species of ER may play redundant roles, a dissimilar tissue distribution indicates otherwise. Therefore the generation of mice lacking individual or both ER forms was a very effective tool. Summary of reported phenotypes of ER null mice are shown in Table 1.1.2.2 (62). ERα appears to be critical for the reproductive function in both male and female mice, where as ERβ holds an intermediary effect. It is logical to argue that known genomic effects of estrogen are mostly mediated by the major isoform, ERα. ERβ appears to show some redundancy in its function in certain tissues. ERβ has been shown to posses opposing effects in response to estrogenic ligands (162), and may play a crucial role in maintaining a balanced state of estrogen effect on a particular tissue. This information should be born in mind when interpreting data of knock out studies. A single knock out may not necessarily provide the critical information regarding the particular ER; rather the effect may be the combination of that as well as the unchecked action of the other ER.

The genomic mechanism of estrogen described above is unlikely to explain the rapid effects observed in target tissues such as bone, vasculature, neural, uterus and breast

## Table 1.1.2.2

**Phenotypes of ER Deficient Mice** *(modified from Endocrine Reviews 20: 358-417)*

| ER knock out model | Phenotype |
|---|---|
| **Lethality of mutation** | |
| ERα KO | No |
| ERβ KO | No |
| **Fertility** | |
| ERα KO | Both sexes are infertile |
| ERβ KO | Female is subfertile (reduced litter size); males fertile |
| **General** | |
| ERα KO | Exhibit normal expression of the ERb gene |
| ERβ KO | Exhibit normal expression of the ERa gene |
| **Female reproductive tract** | |
| ERα KO | Tract undergoes normal pre- and neonatal development but is insensitive to E2<br>Responsive to mitogenic actions of androgens<br>Ovaries undergo normal pre- and neonatal development, but are anovulatory during adulthood, exhibit multiple hemorrhagic cysts, and no corpora lutea<br>30–40% incidence of ovarian tumors by 18 months of age |
| ERβ KO | Tract undergoes normal pre- and neonatal development and appears sensitive to ovarian estrogen cycling during adulthood |
| **Mammary gland** | |
| ERα KO | Undergoes normal prenatal development but is insensitive to estrogen-induced development during puberty and adulthood<br>Responsive to exogenous progesterone and prolactin |
| ERβ KO | Undergoes normal prenatal and pubertal development, virgin gland is grossly indistinguishable from that of age-matched wild-type<br>Undergoes normal differentiation and lactation during pregnancy and motherhood |

## Table 1.1.2.2 continued

| ER knock out model | Phenotype |
|---|---|
| **Male reproductive tract**<br>ERα KO | Tract undergoes normal pre- and neonatal development<br>Age-related phenotype of attenuated fluid resorption in efferent ducts leads to dilation of Rete testis, atrophy of the seminiferous epithelium, and decreasing sperm counts<br>Disrupted sperm function -inability to fertilize<br>Age-related decreases in testis weight<br>Age-related increases in seminal vesicle weight |
| ERβ KO | Undergoes normal pre- and neonatal development with no apparent defects in spermatogenesis that impede fertility |
| **Cardiovascular system**<br>ERα KO | reduced estradiol-induced angiogenesis and reduced basal levels of vascular nitric Oxide |
| **Behavior – Females**<br>ERα KO | Exhibit a lack of estradiol and progesterone-induced sexual behavior, increased aggression, |
| ERβ KO | Exhibit no defects in sexual behavior that impede fertility |
| **Behavior – Males**<br>ERα KO | Exhibit normal mounting and attraction toward wild-type females but a complete lack of intromission and ejaculation; display reduced aggression |
| ERβ KO | Exhibit no defects in sexual behavior that impede fertility |

tissue (214). These effects do not fit into the time frame of classical genomic activity and suggest the existence of rapid, non-genomic activity. Recent reports clearly show that $E_2$ is capable of activating kinase-regulated signal transduction pathways in MCF-7 and other cell lines (7,117,192,245). Most of these studies further elaborate cell/tissue context specific estrogenic activities. Further investigation into the estrogen signaling mechanisms has led to the hypothesis of cell surface estrogen receptors that could be coupled to cytosolic signal transduction pathways. Membrane impermeable estrogens ($E_2$-BSA) have been used to demonstrate possible membrane associated functions for decades. Even though many signal cascades have been defined as response to these compounds, it is important to appreciate the inherent difficulties of the approach such as leakage of minute, undetectable levels of $E_2$ that could signal the cellular responses. Expression of ER in cells lacking endogenous ER has been useful and it has tremendously helped to delineate signal networks associated with ER, yet it is not without setbacks. Artificial expression of the molecule doesn't always guarantee its optimum function. Lack of factors that are indispensable for ER action (and present in naturally ER positive cells) could retard the receptor function in the new environment. On the other hand, exogenously expressed ER may interact with host cell specific molecules producing unrelated effects. Therefore information from this type of studies should be interpreted with caution.

Evidence for exclusively membrane bound ER in many cell types is slowly emerging. Studies where overexpression of membrane anchored ERα was used to activate cytoplasmic signal transduction pathways (167) have attempted to explore the significance of membrane bound ER. Consequently, the structural determinant that is necessary for the localization and function of ERα at the plasma membrane was determined (224). Mutation of a specific serine phosphorylation residue (S522) to alanine significantly reduced the membrane localization of mouse ERα and the expression of this mutant in ER expressing cells abolished the binding of native ERα to the membrane. Interestingly, the nuclear function of this mutant was comparable to that of wild type ER in response to $E_2$. Determination of the exact amounts of exclusively membrane bound and nuclear ER levels may pose difficulties since ER constantly shuttle

between the nucleus and the cytoplasm, despite its predominantly nuclear localization (195). Based on microscopic evidence it is worth to speculate that the membrane pool of ERα represents a small proportion of the total immunoreacting receptors (189,195,224). Interpretation of data from ligand (estrogen) binding studies should also be done with caution since other estrogen binding entities that are not related to ER have been reported (275).

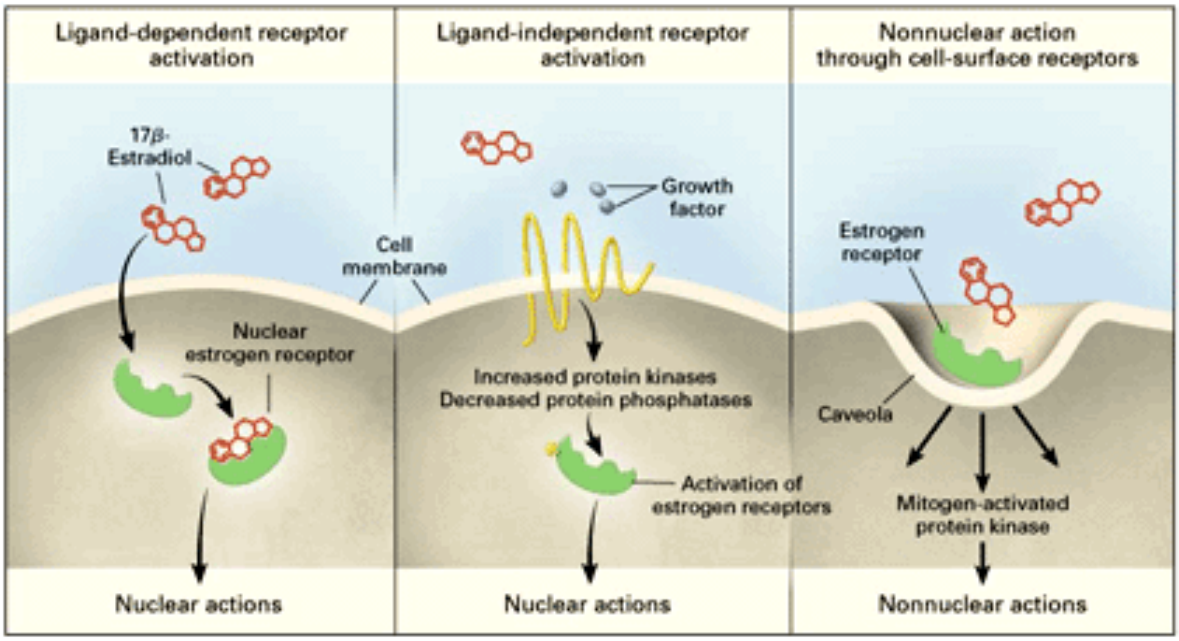
In physiological condition the signaling mechanisms described above may operate as individual entities or may co-exist and interact. The latter will add up to the existing complexity of our understanding in estrogenic effects. Genomic, non-genomic, classical and non-classical activities of $E_2$ and ER are demonstrated in the Figure 1.1.2.3.

**Estrogen and Breast Cancer**

**Estrogen and the Development of Breast Tissue**

The fundamental feature of breast cancer is that it occurs far more frequently in women than in men, although men have breast tissue and sometimes get breast cancer. Genetics, hormonal milieu and environment, all participate in the pathogenesis of breast cancer for a greater or lesser extent. Presence of higher levels estrogen appears to act on the breast to make it susceptible for tumor formation in females. In males, the plasma concentration and the production rate of estrogen are significantly lower than that in females (Figure 1.1.2.4). The inverse relationship is true for the level of testosterone in males and females. This fundamental difference of hormone levels is the key to the distinct male and female physiologies. Such differences could also make one gender more susceptible to specific disorders than the other.

Development of the breast tissue in female is a characteristically regulated by the action of estrogen (Figure 1.1.2.4). Unlike the tissues that are fully formed at birth, breast tissue in newborns consists only of a small duct, which grows rapidly at puberty and completes full growth only after the first pregnancy (2). The internal breast structures begin to expand rapidly under the influence of estrogen. Typically a girl's first period will begin about a year or two after her breasts begin to grow. With every menstrual cycle a new phase of growth occurs which includes extensive branching of the




***Modified from NEJM 346: 340-352***

## Figure 1.1.2.3

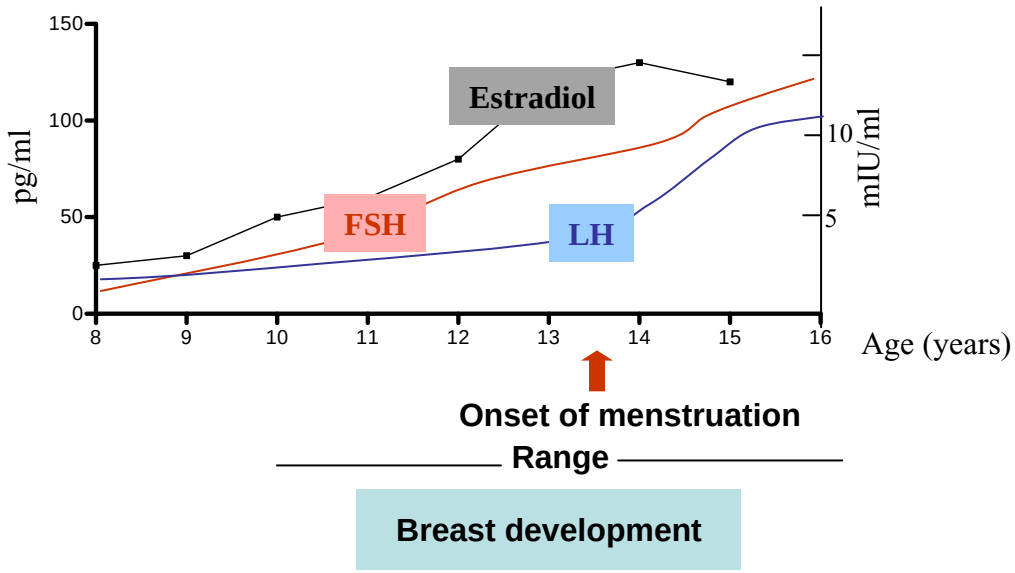
**Functional diversity of $E_2$-ER**

ER can be activated by 17-β-estradiol (E2) or independently of estrogen. Growth factors indirectly activate ER by increasing the activity of protein kinases which phosphorylate sites on ER. In the case of non-nuclear estrogen signaling pathway, cell membrane bound ER that may be found in caveolae, link their activity to mitogen activated protein kinase pathways. This results in rapid nonnuclear effect.

**(A)**

| Steroid | Plasma concentration (ng/dl) | |
|---|---|---|
| | Female | Male |
| Estradiol | Early follicular 6<br>Late follicular **50**<br>Mid follicular 20 | **3** |
| Progesterone | Follicular 100<br>Luteal 1000 | |
| Testosterone | 40 | 650 |

**(B)**




## Figure 1.1.2.4

**(A)** Plasma concentrations of female and male gonadal steroids (modified from *Physiology* 2nd edition chap55 pgs 998, 1000)
**(B)** Average chronological sequence of hormonal and biological events in normal female puberty (modified from *Physiology* chap 55 pg1015)

ductal system and organization of the internal structures. The immature breast cells are inefficient in repairing gene mutations and more likely to bind carcinogens. It is important to reduce the exposure of young women and girls to carcinogens that might damage DNA during the rapid phase of breast development. After first full term pregnancy, a high proportion of breast cells are transformed to mature, differentiated cells that are less sensitive to DNA damaging agents. Therefore susceptibility to mutations and resulting carcinogenesis may decline in the breast cells of women who have had an early full-term pregnancy (179). Accordingly, increased risk of breast cancer in nulliparous women can be explained. Similar level of risk has been observed in females who did not complete a full term pregnancy before the age of 30-35yrs. This could be the direct result of the undifferentiated breast cells being exposed to carcinogens and DNA damaging agebts for an extended period of time. If the cancer initiation process has already begun due to this reason, the full term pregnancy may not have a significant effect.

**Mechanisms of Estrogen Mediated Breast Cancer**

Diverse cellular and molecular mechanisms have been shown to mediate estrogen induced breast cancer progression. Clinical and epidemiological data have led to the conclusion that endogenous $E_2$ has a critical role in sporadic breast cancer in women. Two enzyme systems compete for an opportunity to alter the structure of $E_2$ molecules, but they do so at two different locations, the 2- and 16-carbon positions (Figure 1.1.2.1). The end products of such reactions are quite different in their biologic roles. Insertion of a hydroxyl radical at the 2-carbon site produces a neutral molecule while that at the 16-carbon location produces a genotoxic molecule, which increases the chances of spontaneous mutation (269).

Endogenous and exogenous estrogens may initiate and promote breast cancer via two key mechanisms in susceptible individuals. First, estrogen may also contribute to mammary carcinogenesis by stimulating the proliferation of a clone of precancerous cells. There is evidence that estrogen decreases cell cycle transit time, so that DNA damages and mutations could be passed to the daughter cells before repair (51) perhaps

due to defective DNA damage checkpoint functions. Cell cycle effects of estrogen have been studied extensively. These studies show that mitogenic abilities of estrogen are mostly mediated by genomic functions of estrogen. Estrogen-ER complex induces DNA synthesis, cell division and production of biologically active proteins such as pS2, transforming growth factor (TGF), and epidermal growth factor (EGF) that induce cell growth and differentiation (4). In breast cancer xenograft models using ER positive cell types, $E_2$ produced pre-malignant and malignant lesions at high frequency compared to untreated samples (239).

Second, estrogen prevents cell death, especially apoptosis through ER (226), mitogen activated protein kinase (MAPK) (251), phosphoinositide 3-kinase (PI-3K) (261) mediated pathways in cell cultures. Data in this dissertation demonstrate the significance of these types of cell signal pathways in estrogen-induced anti-apoptosis, in response to a variety of stimuli. Estrogen acts in synergism with anti-apoptotic proteins such as BCL2 (57,210) while abolishing the effects of pro-apoptotic molecules (129 and data in dissertation). The mitogenic and survival promoting effects of estrogen are further confirmed by observations that the anti-estrogens or estrogen withdrawal can either block breast cancer cell proliferation or induce apoptosis (129 and data in the dissertation). In clinical setting, traditional hormonal therapy for women with breast cancer included hormone ablation by surgical procedures (oophorectomy, adrenalectomy). Later on, anti-estrogens and aromatase inhibitors superseded this strategy (218). Paradoxically, hormonal therapy with high estrogen doses caused regression of ER positive breast cancer in post-menopausal women (142). This response was shown to directly correlate with the duration of the post-menopausal period. This clinical data may suggest that long-term exposure to very low levels of estrogen might alter the responsiveness of breast cancer to estrogen therapy. Similar phenomenon was later shown in cell culture systems using long-term estrogen deprived (LTED) MCF-7 cell variety. Further estrogen induced apoptosis in these cells when administered in higher doses (252). These observations clearly provide a clue that the adaptation of cancer cells to lower blood levels of estrogen might sensitize them to pro-apoptotic effects of estrogen. To extend the breast cancer treatment in pre- and post-menopausal women, a new regimen of cyclic treatment

strategies would need to be introduced. Here selective estroigen recptor modulators (SERMs) or aromatase inhibitors can be used for period of time to sensitize the cancer cells to apoptotic effects of estrogen (253)

**Environmental Estrogenic Compounds and Breast Cancer**

In biological regulatory pathways, molecules compete for their receptors. Such competition among molecules is based upon their structural similarities. However, many synthetic chemicals not belonging to the family of human hormones actively compete for receptors for that family. This natural phenomenon is well illustrated by the example of competition for receptors among estrogens and estrogen mimics such as, pesticides (DDT and heptachlor), plastic (polycarbonates) breakdown products, PAHs (polycyclic aromatic hydrocarbons), petroleum byproducts, polystyrene, plant estrogens such as coumestrol, equol and zearalenone. It has been suggested that these xenoestrogen molecules may have 3D structures that can fit in the ligand binding site in the steroid hormone receptor. Therefore, the total burden of estrogen in an individual is constituted of endogenous and exogenous estrogens. Exposure of previously unexposed women to xenoestrogens in addition to dietary changes may be partly responsible for higher rates of breast cancer in oriental females who migrate to the United States. In susceptible individuals, it is possible that environmental carcinogens and estrogenic compounds might synergistically enhance the development of breast cancer (78,128,233).

**Estrogen Action on Tumor Vascularization**

In both tumor growth and metastasis, angiogenesis plays an essential role. Growth factors, cytokines and extra cellular matrix have been shown to regulate endothelial function in vivo and in vitro (239). Estrogen action in endothelial cell function also has been appreciated pertaining to its cardiovascular activities (13). The evidence shows that estrogen guards against cardiovascular diseases in reproductive years and the protection wanes as women reach menopause (21,40). Mechanism/s of estrogen function in vascular component of breast cancer has been proposed, thus estrogen was shown to increase the expression of vascular endothelial growth factor (VEGF) and other

angiogenic factors in breast cancer cells (156,290). Paradoxically, $E_2$ inhibited the growth of breast cancer xenografts (of KPL-1 cells) in nude female mice by abolishing angiogenesis as well as the level of VEGF production, and the anti-estrogen ICI 182, 780 had opposing effects (156). Whether these cells are ER positive or negative was not defined. On the other hand, high levels of ER have been shown to inhibit proliferative, invasive and metastatic potential of a subset of tumor cells in vitro (11). Subsequent to ERα overexpression, in vivo down-regulation of VEGF and inhibition of vascularization was observed in tumor xenografts.

**Selective Estrogen Receptor Modulators (SERMs)**

Receptor mediated estrogenic functions are further modulated by a host of co-regulator proteins, and the complex nature of these interactions may impart distinctive effects in different target tissues. Estrogen has multiple desirable effects such as improved bone density, lipid profile, and benefits for the cardiovascular and central nervous systems. Unfortunately there are undesired effects including increased breast cancer risk, stimulation of endometrial hyperplasia and increased risk of venous thrombo-embolism (VTE). Estrogenic compounds that block or antagonize the effects of estrogen in specific tissues while retaining some of the beneficial effects in other tissues have shown great benefits in post-menopausal women as well as women who undergo ovariectomy early in the life. These pharmacological agents, known as selective estrogen receptor modulators (SERM) are mainstays in treatment of metastatic breast cancer and have shown promise as chemo-preventive agents for breast cancer and osteoporosis. SERMs function by competing for the ER binding to estrogen. Subsequent to ER-SERM interaction, conformational changes occur in ER thereby blocking ER mediated effects (190). SERMs posses varying degree of estrogenicity (complete or partial, agonists or antagonists) depending on the target tissue. This tissue selectivity is well demonstrated with Tamoxifen, an anti-estrogen used in breast cancer therapy. The rationale for its use is based on the fact that $E_2$ is a mitogen in ER-expressing breast cancer cells and, this action can be inhibited by an ER antagonist. Clinical data demonstrated that Tamoxifen abolished ER dependent breast cancer and promoted increase in bone mineral density

mimicking the estrogen effect in this tissue (136) as well as in the uterus (124). Receptor sub-type difference in ER could also influence the pharmacological effects of SERM. It has been shown that almost all SERMs have antagonistic effect on ERβ where as more differential effects are seen on ERα (190). Therefore it is possible that differential expression of ERs could at least partly contribute to the tissue selective effects of SERM. Structures of some SERMs and their mode of action are shown in Figure 1.1.2.5.

**Aromatase Inhibitors**

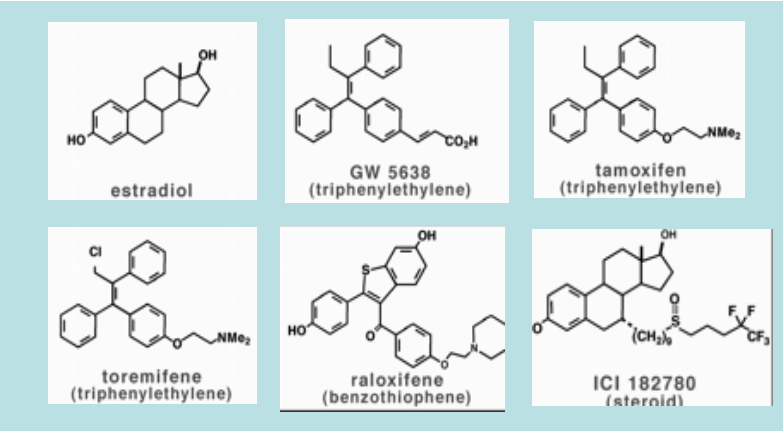
Another approach to anti-estrogen therapy is to lower the amount of estrogen being produced by the body. In post-menopausal women, estrogen is no longer produced by the ovaries, but is synthesized from testosterone in the peripheral tissues by a reaction catalyzed by the enzyme aromatase (40). Aromatase inhibitors block testosterone from being converted to estrogen thereby limiting the available estrogen level. Recent studies using third generation aromatase inhibitors (anastrozole, letrozole and exemestane) have shown better outcome and improved therapeutic ratio over hormonal approaches (progestins) and over Tamoxifen. These results have led recently to testing of aromatase inhibitors in the adjuvant setting for postmenopausal patients (218,268).

### 1.1.3 Apoptosis

**Different Ways to Die**

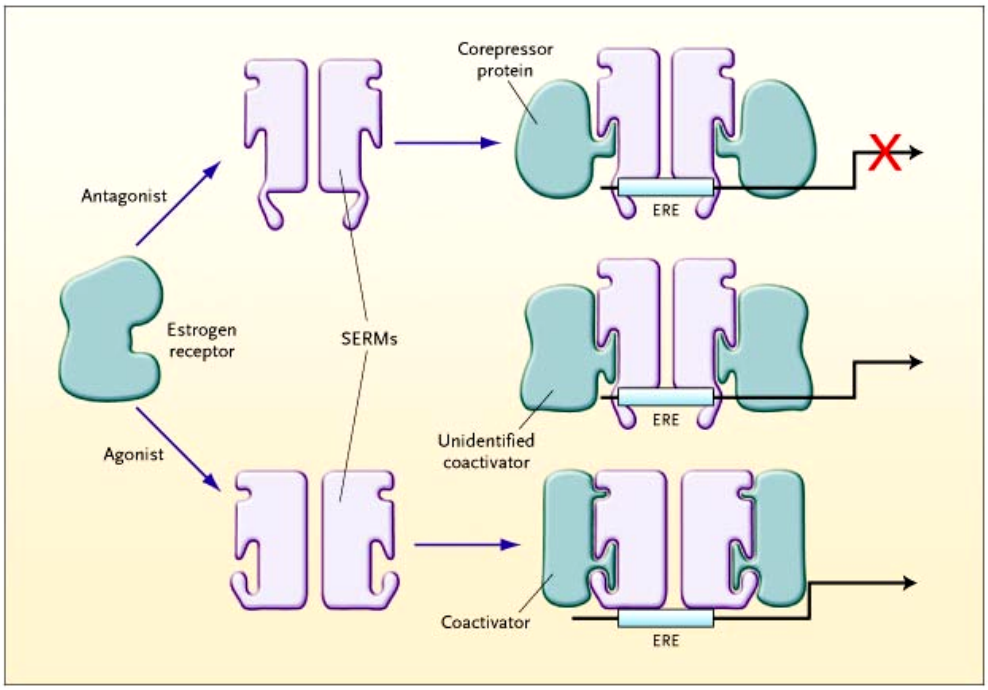
Cell proliferation and growth are among the most intensely investigated areas in the field of cancer research. The notion that programmed cell death (PCD) plays a major role in the life of multi-cellular organism has gained attention recently and convincing evidence is accumulating at a fast rate. When a cell of a multi-cellular organism dies by a mechanism exerted by proteins encoded by the host's genome, the process can be defined as physiological cell death. The purpose of this process is to eliminate unwanted host cells and is important in development and homeostasis, as a defense mechanism and as a final stage of the aging process. Most genetic or non-genetic changes that deregulate these cell death programs may underlie the etiology of cancer, autoimmune and developmental disorders.

**(A)**




***Modified from NEJM 346: 340-352***

**(B)**




*Modified from Science 29:2380-2381*

## Figure 1.1.2.5

**(A)** Structures of SERMs. The structure of estradiol is also shown for comparison
**(B)** Estrogen binding promotes ER comformational change that permits its spontaneous dimerization and facilitates the subsequent interaction of the dimer with EREs located within target genes. Estrogen also facilitates the interaction of the ER with coactivators. An antagonist-activated ER, on the other hand, interacts preferentially with a corepressor protein. Evidence suggests that the structure of some SERM–ER complexes favors corepressor recruitment and that of others favors affinity for known coactivators. The implication of this model is that SERM activity will be influenced by the relative levels of expression of the cofactors (corepressors and coactivators) in target cells.

The term ‘apoptosis’ has been used interchangeably with PCD and has immensely confused the understanding of regulated cell death. Activation of caspases (aspartate directed cystein proteases) is the final commitment step to apoptosis in a normal cell (39). Function of caspases is conserved throughout evolution from nematodes to mammals. In mammals the process is considerably more complex involving multiple forms of caspases and their targets. Resulting cell death is characterized by unique morphological features (described below). Other forms of cell death, which are morphologically and mechanistically distinct to apoptosis could exist and some of them have been described (178,255). It may now be the time to note that caspases are not the sole or unique effectors of cell death programs. In fact, the inhibition of caspases does not prevent the total cell loss due to apoptotic stimuli (14) or in severely compromised cells by growth factor withdrawal, metabolic toxins or physiological killers like glucocorticoids (178). Interestingly, organisms that do not express caspases undergo PCD that does not closely resemble classical apoptosis (17,91,161,177). Together it is now evident that there are multiple pathways leading to cell death. Some cells may have the required components for some pathways but not for others. Also cells may contain inhibitory mechanisms that rule out a particular program. Moreover, different pathways can co-exist in the same cell and are switched on by specific stimuli.

**Apoptosis**

For a tumor biologist defective apoptosis is one of the seven pillars upon which cancer develops and progresses. Defective cell cycle regulation, growth factor autonomy, defective senescence, angiogenesis, metastasis and cell invasion are the other six pillars (73). Apoptosis is a genetically encoded and highly regulated cellular response for a pro-death signal. This results in unique characteristic features such as cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation, intranucleosomal DNA fragmentation, phosphatidylserine exposure due to changes in the plasma membrane, and finally fragmentation into membrane enclosed apoptotic bodies sequestered by macrophages or other engulfing cells or neighbors (73,143,291).

**Mechanism of Apoptosis**

When discussing the central components of apoptosis, it is most appropriate to describe *C. elegans's* genes whose functions led to the discovery of higher eukaryotic homologues. The core machinery of apoptosis was first discovered in *C. elegans* and include Egl-1, Ced-3, Ced-4 and Ced-9 whose mammalian homologues are BH3-only proteins, caspases, Apaf-1 and BCL2-like proteins respectively (39). The process in the higher mammals is more complicated than in the worm though the basic makeup of the mechanism is comparable. Activation of caspases is responsible for the cleavage of vital components of the cell and it is during this process the characteristic morphological features of apoptosis (described above) are produced (39).

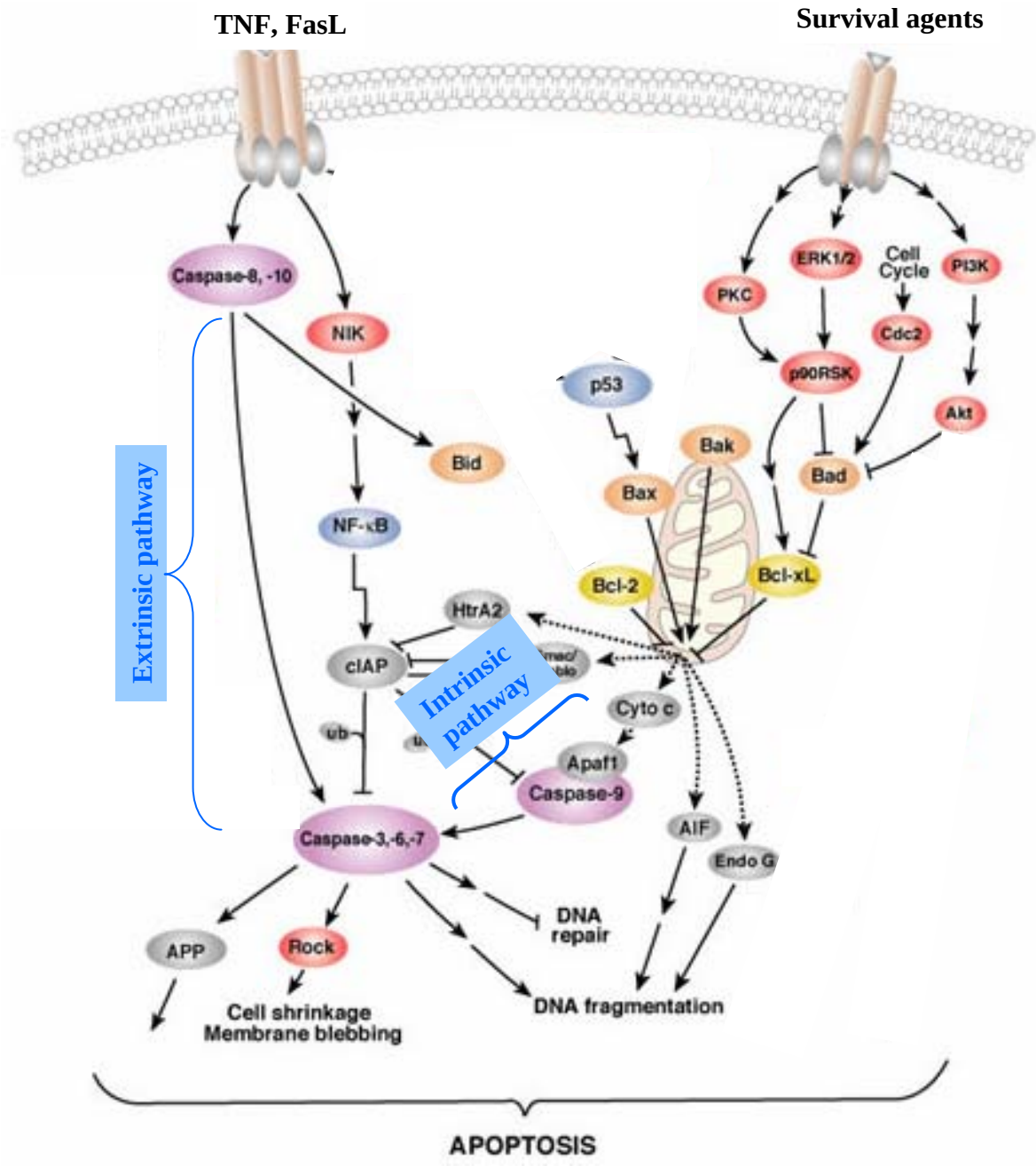
Major form of apoptosis in vertebrates is mediated by the mitochondria associated mechanism (102). Caspases are commonly activated by mitochondrial membrane permeabilization (MMP), or more specifically mitochondrial outer membrane permeabilization (MOMP) (103), which marks a point of no return from the death process. MMP leads to the release of proteins normally found in the space between the inner and outer mitochondrial membranes, such as cytochorme C and apoptosis inducing factor (AIF) to the cytosol. Though the exact mechanism of MMP is still being debated, it is clear that local effects on inner and outer mitochondrial membranes could lead to differences in the membrane potentials. Defective inner transmembrane potential ($\Delta\Psi m$) has been clearly observed before, during and after MMP (104). The mitochondrial respiratory chain produces energy which is stored as an electrochemical gradient or the $\Delta\Psi m$, negative inside of about 180-200 mV. This energy source pumps protons ($H^+$) that drive ATP synthesis. Loss of $\Delta\Psi m$ is a result of the ion equilibration across the inner membrane. This could lead to swelling of the matrix as water enters, which can result in sufficient swelling to break the outer membrane to produce MMP. MMP contributes to cell death in several different ways. In apoptosis by releasing cytochrome-C, in caspase independent cell death by releasing factors like AIF (262) and Htra2/Omi (114) and, by culminating mitochondrial functions that are essential for cell survival such as electron transport chain (101). Once in the cytosol, cytochrome C contributes to the formation of the apoptosome (Apaf-1/caspase 9/cytochrome-C) that activates the executer caspases

(39). A pathophysiological role for MMP is emerging and manifestations of deranged ΔΨm and release of intermembrane proteins are frequently observed in diseases with increased cell death (104).

In addition to the mitochondrial-mediated pathway, ligand binding and activation of the cell surface death receptors has also been implicated in activating caspases. Activated receptors initiate the recruitment of pro-caspases (eg. pro-caspase 8) and their adaptor molecules to form the caspase processing unit. This aggregation results in the transactivation of pro-caspases and consequent cleavage and activation of the down stream capases and other targets. A cascade of biochemical reactions follows, ensuring the final fate of the cell. The cell surface receptor cascade also contributes to the mitochondrial cytochrome – C release through the activation of caspase 8 and its down stream target BID (39). Finally both mechanisms converge into cleavage and activation of Caspases 3, 6 and 7 which carry out the proteolysis of death substrates (Figure 1.1.3.1). These pathways are tightly regulated by pro and anti- apoptotic mechanisms and the balance between them may govern the orderly demise of the cell.

**Caspases**

Caspases are a well-characterized family of cysteine proteases that are responsible for the execution of apoptosis. They are also important in other cleavage dependent functions such as cytokine maturation (10). As the name implies ('c' is for cysteine and 'aspase' is for aspartate) these enzymes are able to cleave the substrate after an aspartic acid residue. Each caspase is initially synthesized as a pro-caspase and requires a cleavage at specific site (which also is an aspartate) by another caspase to generate active enzyme suggesting a cascade activity of this family of proteases. Functional apoptotic program in mammals requires multiple caspases and the contribution of different caspases to different phases of apoptosis is becoming clearer (39). Currently, 11 human caspases have been identified: caspase 1-10 and caspase 14. The protein initially named caspase 13 was found to be the bovine homologue of caspase 4. Caspase 11 and 12 are murine homologues of human caspase 4 and 5 (73). Caspases are classified in several ways. Most important of those is the function-based classification where all caspases are fit into three subfamilies i.e.




*Modified from Cell Signaling Technology*

**Figure 1.1.3.1**
**General overview of apoptosis**
See text for details

initiators of apoptosis and inflammation (caspase 1, 5, 11), initiators of apoptosis (caspase 2, 8, 9, 10, 12) and effectors /executors of apoptosis (caspase 3, 6, 7) (73).

Caspases posses very strict substrate specificity. Target substrates can be subdivided based on their cellular functions i.e. (a) proteins directly involved in regulation of apoptosis, (b) protein kinases that mediate apoptosis, (c) structural proteins, (d) proteins required for cellular repair, (e) cell cycle regulatory proteins and (f) proteins involved in diseases. These are summarized in Table 1.1.3.1 (73). Mouse knock out studies have contributed enormously to the current knowledge on specific roles of individual caspases. Phenotypic details of these mice are shown in Table 1.1.3.2..

**Regulation of Caspase Activity and Apoptosis**

Regulation of caspase activity is implicated in apoptosis and to a lesser extent in cell proliferation and cellular differentiation. In the latter, limited activation of caspase has been demonstrated to be essential i.e. proliferation of lymphocytes (8), differentiation of red blood cells (35) and epidermal cells (87). A family of proteins termed 'inhibitors of apoptosis' (IAP) has been described to play a major role in caspase regulation hence apoptosis regulation. These proteins function as intrinsic regulators of the caspase cascade. There are other cellular proteins that modify specific caspases, but IAPs are the only proteins that can regulate initiator as well as executor caspases.

Several IAPs have been described to date e.g. the first mammalian homologue neuronal IAP (NIAP), X-chromosome linked IAP (XIAP), Survivin and Livin. They are characterized by the presence of one or more of baculoviral IAP repeats (BIR). This domain is important in binding and inactivation of caspases (173). In addition to apoptosis regulation these proteins have been shown to participate in cell cycle regulation and receptor mediated cell signaling. Both endogenous (mitochondria mediated) and exogenous (surface receptor mediated) apoptosis pathways are regulated by IAPs.

Regulatory proteins that bind and suppress the function of IAPs have been described. Mitochondrial protein Smac, which is released by a similar mechanism as cyctochrome-C, has been shown to bind to all the IAPs. In addition to Smac, other mitochondrial and cytoplamic IAP inhibitors have been shown to regulate IAP function.

## Table 1.1.3.1

**List of some caspase substrates**

*Modified from Oncogene 22: 8543*

| | |
|---|---|
| **Apoptotic and inflammatory regulators** | BID, Caspases, BCL2, BCLxl, XIAP, IkB, |
| **Protein kinases and other signal molecules** | Raf, AKT, PP2A, MEKK1, PKC, FAK |
| **Structural proteins** | Actin, Lamin A-C, β-catenin, |
| **Repair proteins** | PARP, ATM, DNA-PK, Rad51, Rad9 |
| **Cell cycle regulators** | Rb, p27, p21, Cdc27 |
| **Disease related proteins** | Huntingtin, ataxin-3, androgen receptor, atropin |

## Table 1.1.3.2
**Summary of phenotypic characteristics of caspase knockout mice**

*Modified from Oncogene 22: 8543*

| Caspase | Embryonic lethality | Developmental defects | Apoptotic defects |
|---|---|---|---|
| 1 | No | No | Defects in inflammatory response<br>Resistance to septic shock |
| 11 | No | No | Defects in lymphocyte apoptosis<br>Resistance to septic shock<br>Ischemic brain injury |
| 2 | No | Excessive oocyte generation | Defects in apoptosis induction in oocytes and lymphocytes<br>Compensatory activation of other caspases were reported |
| 3 | Yes | Neuronal hyperplasia | Defects in DNA fragmentation<br>Compensatory activation of other caspases were reported |
| 8 | Yes | Impaired cardiac muscle formation<br>Abdominal bleeding | Defects in activation of death receptor pathway |
| 9 | Yes | Neuronal hyperplasia | Defects in apoptosis induction by genotoxic stress |
| 12 | No | No | Defects in endoplasmic reticulum stress apoptosis |

IAPs can also be substrate targets of caspases, showing another level of apoptosis regulation. In some instances such cleavage yields a pro-apoptotic molecule that accelerates the death process where as in other instances it simply represents the final outcome of apoptosis (76,173).

**Endoplasmic Reticulum and Apoptosis**

Critical contribution of endoplasmic reticulum (End.Ret.) to apoptosis has been evaluated recently. The End.Ret. induces stress signaling in response to defective protein folding through the unfold protein response (UPR) that ultimately drives cell to apoptosis (15). This phenomenon if termed as ER-stress induced apoptosis. The mechanism by which ER-stress is coupled to caspase activation was revealed by the characterization of caspase 12 (198). Caspase 12 is specific in its function for ER-stress as has been demonstrated by null mouse studies (Table 1.1.3.2). In addition to the direct activation of caspase 12, End.Ret. acts in concert with mitochondria to propagate apoptotic signals. This has been shown to take place through several pathways. Increased cellular concentration of $Ca^{2+}$ is frequently observed with End.Ret. stress. This can potentially activate $Ca^{2+}$ dependent enzymes such as calcineurin whose down stream effector is the BCL2 family protein BAD. BAD is dephoshorylated (activated) by this enzyme thereby promoting the mitochondrial action of BAD (282). Further, increased $Ca2^{+}$ was shown to directly activate mitochondrial mediated cytochrome-C release (15). Multi-domain BCL2 family proteins (pro- as well as anti-apoptotic) have been shown to localize to the ER membrane (148,153). As in mitochondria, pro-apoptotic BAX like proteins inflict membrane damage that can be prevented by the expression of BCL2 group. Changes in End.Ret. membrane cause changes in $Ca^{2+}$ mobilization that may contribute to the apoptotic program as described above. Correct protein folding is essential to maintain the homeostasis of the cell. the End.Ret. acts as the sensor for this mechanism and stops the metabolic processes until the defect is corrected. It is when the protein folding capacity of ER is compromised due to a variety of reasons the UPR mediated signal directs the apoptotic program. It is likely that various apoptotic agents described thus far may also

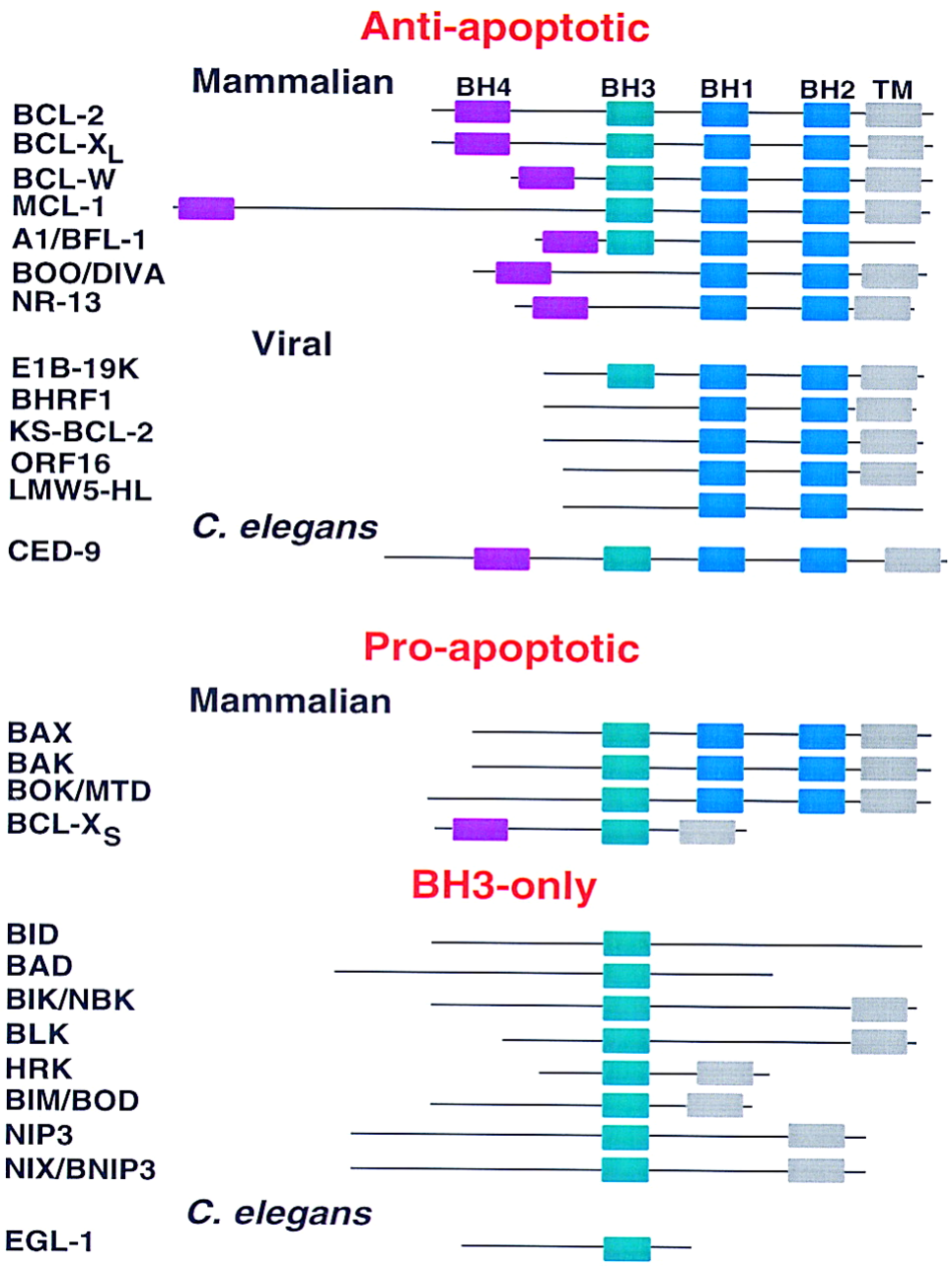
have effects on ER. The outcome could then be due to all these programs that work simultaneously.

**BCL2 Family Members and Apoptosis**

Apoptosis is highly conserved in multicellular organisms and information gathered from studying less complex species have been easily translated to more complex counterparts. Identification of remarkable similarity between the *C. elegans* ced-9 gene members and mammalian BCL2 family is the best case in point (5). Several proteins have been identified to be members of the family subsequent to the discovery of the founder member, BCL2. BCL2 was described in 1988 as a protein, which is involved in B-cell lymphomas (277). BCL2 transfected B cells were shown to be resistant towards apoptosis normally induced in B cells by IL-3 withdrawal: for the first time it was shown that the pathway toward tumorigenesis depends not only on the ability to escape growth control but also on the ability to prevent apoptosis. Interestingly, expression of human BCL2 in *C. elegans* reduced the amount of programmed cell death suggesting that the mechanism controlled by BCL2 in human is similar to that in nematodes (278).

**Structure and Function of BCL2 Proteins**

A hallmark of BCL2 family members is to form hetero or homo dimers, which may either, be a competition for neutralizing or, result in a synergetic effect. Possession of one or more of conserved BCL2 homology (BH, 1-4) domains determines the structural and functional capacities of each member. BCL2 and BAX subfamilies that contain BH 1-3 (Figure 1.1.3.2) show opposing anti-apoptotic and pro-apoptotic effects respectively. BH3 only proteins compose the third family, which is strictly pro-apoptotic. They may directly counteract the activities of anti- apoptotic members or induce death via alternative mechanisms (61,106). However it is clear that their apoptotic function is solely dependent on the presence of BAX subfamily members (301). At the C-terminus of most BCL2 and BAX and in certain BH3 only proteins is a hydrophobic region, which features as a trans-membrane domain important for their membrane targeting (61). Despite the structural similarities, BCL2 proteins differ in their subcellular localization.




*Modified from Genes and Dev 13: 1899-1911*

**Figure 1.1.3.2**

**BCL-2 family members**

BCL-2 homology regions (BH1-4) are denoted as is the carboxy-terminal hydrophobic trans-membrane (TM) domain

Among the anti-apoptotic members, BCL2 and BCLxl are found extensively membrane bound in mitochondrial and ER fractions while BCLw is mainly cytosolic. In the pro-apoptotic group, BAK is mostly membrane bound and BAX is cytosolic (61). Recent structural studies have provided insights regarding these differences. In BAX and BCLw, the C-terminal regions were shown to interact with the groove made up of BH1/2/3 and therefore, unavailable to interact with membranes (118). The hydrophobic groove is the target for the BH3 domain of the binding partner and, then there is a need for a competitive displacement of the C-terminal from the groove. Since BCL2 and BCLxl are mostly bound to the membrane through the C-terminal transmembrane domain, it is presumed that their hydrophobic groove is available to bind with interacting BH3 domains.

Pro-apoptotic BAX family members are critical for apoptosis in many cell types. Double knockouts of these genes have deleterious effects and results in prenatal death, where as single knockouts show little consequence (170) probably due to functional redundancy. Upon apoptotic signal, BAX and BAK undergo conformational changes and form homo-oligomers (193,200). This may require the rearrangement of their BH domains so that one molecule may act as the donor of the BH3 domain and the other as the acceptor (the hydrophobic groove). Once activated, BAX and BAK cause membrane damage, particularly the outer membrane of the mitochondria (influence MMP, described above). BCL2 and BCLxl over-expression has been shown to neutralize the effects of BAX and BAK (113,256).

Individual BH3 only proteins appear to have direct and indirect effects on pro-apoptotic BAX family proteins, contributing to apoptosis induction. BID, upon cleavage in response to cytotoxicity binds and directly activate BAX (165,169). BAD and BIK cannot directly activate BAX or BAK but instead binds anti-apoptotic BCL2 preventing their functions (5,22,165). This information supports the view of two modes of action for BH3 domains i.e. (1) activating BAX, BAK and (2) sensitizing by occupying the pocket of anti-apoptotic members. Table 1.1.3.3 summarizes the known functions of some BCL2 family members.

## Table 1.1.3.3
**Summary of functions BCL2 family members**

*Modified from Science 305: 626-629, Cell 75: 229, Nat.Gen 16: 358)*

| Family member | Functions |
|---|---|
| **Anti-apoptotic** | |
| BCL2 | Deficient mice have defects in renal development, loss of lymphocytes, melanocytes and hair pigmentation. Effects are reversed by BIM deficiency |
| BCLxl | Knock-out is embryonic lethal. Massive increase in apoptosis in erythroid and neuronal cells |
| BCLw | Deficient mice are viable. Males are impotent |
| Mcl | Knock-out is embryonic lethal |
| **Pro-apoptotic** (multidomain) | |
| BAX | Deficient mice are viable. Lymphocyte number is Increased |
| BAK | Develop normally |
| | BAX/BAK double knockouts die perinatally due to various developmental defects. Absence of MMP |
| **BH3 only** | |
| BAD | Knockout mice develop normally, with time display diffuse large B cell lymphoma. |
| BID | Knockout mice develop myeloproliferative disorder. Hepatocytes are resistance to Fas mediated apoptosis. |
| Noxa | Induced by p53. MEFs are resistant to DNA damage induced apoptosis when Noxa is knocked out. |
| BIM and BOD | Knockout mice show increased lymphoid cells. |

BCL2 family proteins have been shown to participate in other cellular functions in addition to apoptosis. Cell cycle transit from quiescence to proliferation was blocked when BCL2 proteins were over-expressed (64) but high levels of BCL2 did not affect the proliferating rate of continuously growing cultures (61). Most importantly the anti-apoptotic and cell cycle effects of BCL2 appear to be genetically separable i.e. cell cycle effect depends on the amino terminal BH4 domain (121,127). BAD was also shown to exert a cell cycle effect. BAD expression resulted in failure to block cell cycle progression in fibroblasts following release from growth arrest conditions. Using mutation analysis it was shown that BAD requires co-segregation with BCL-xl to exert this effect (50). Contrary to this, data in this dissertation show that BAD over-expression blocks cell cycle progression in breast cancer epithelial cells in growth arrest as well as continuously growing conditions. Further we demonstrate that endogenous BAD has a regulatory role in cell cycle progression at the level of cyclin D1 transcription.

The BCL2 family proteins are frequent targets of post-translational modification downstream of both survival and death signal transduction cascades and such modifications may often dictate their active versus inactive conformation, sub-cellular localization, and partner proteins (5). The ratio between pro and anti-apoptotic members helps to determine at least in part the susceptibility to a death signal (140,301).

**BAD**

BAD (**B**CL-/**B**CLxl **A**ssociated **D**eath promoter) is an exclusively BH3 domain only member sharing sequence homology only within the BH3 amphipathic α-helical domain (Figure 1.1.3.2**)** in the BCL2 family. It was originally cloned from mouse cDNA by its ability to bind with BCL2, both in yeast two-hybrid and direct biochemical interactions (294). Subsequently the human homologue, which is 37 amino acids shorter, was identified. Both mouse and human counterparts are expressed ubiquitously. BAD promotes cell death; and it is widely accepted as a pro-apoptotic protein. As for any other pro-apoptotic BCL2 member (i.e. BID, BIK, BAK and BAX) intact BH3 domain is important for BAD's function. It competes for the binding with BCLxl and BCL2 to a lesser extent, via the BH3 domain thereby affecting the level of heterodimerization of

these proteins with BAX or BAK (119,140,204). Possible interactions among BCL2 family members are shown in Figure 1.1.3.3.

**Inactivation of BAD**

Phosphorylation of BAD on several serine sites found in and out of the BH3 domain change the effectiveness of BAD as a death-promoting molecule. In the presence of most survival factors, cells phosphorylate BAD on serine 75 (murine S112) by Ras mediated ERK/RSK1 pathway (90). Subsequent phosphorylation on serine 99 (murine S136) by protein kinase B/AKT (70) promotes heterodimerization with 14-3-3 proteins. Such interaction then facilitates the phosphorylation at serine 118 (murine S155) a site within the BH3 domain, by PKA (175). Phosphorylation in the BH3 domain weakens the interaction with BCLxl thereby leading to the cell survival (22,82,119).

Recently another inactivating phosphorylation site, serine 133 (murine S170) outside the BH3 domain was shown to be important in cytokine induced cell survival (83), although it remains uncertain which kinase/s is responsible for the phosphorylation. Deletion of the BH3 domain was shown to completely abolish the pro-apoptotic function of BAD (140) whereas, point mutations within this domain (leucine 114, aspartate 119; murine L151 and D156 respectively) have not established any significant corelation with BAD's functions (personal communication Dr. A. Kelekar), (3).

**Activation of BAD**

BAD can also be activated by phosphorylation in response to death signals. To this end serine 91 (murine S128) has been shown as critical target for phosphorylation by stress activated JNK signaling pathway. Interestingly the activity of growth factor mediated signaling failed to inhibit apoptosis induced by BAD S91 phosphorylation, depicting opposing signaling mechanisms that converge on BAD (80).

Reversible phosphorylation is a key regulatory mechanism in cell survival and death, and has been shown to regulate the function of BAD. Members of major eukaryotic serine/threonine phosphatase families have been discovered to de-phosphorylate BAD, even though conflicting data for their specificities are obvious.

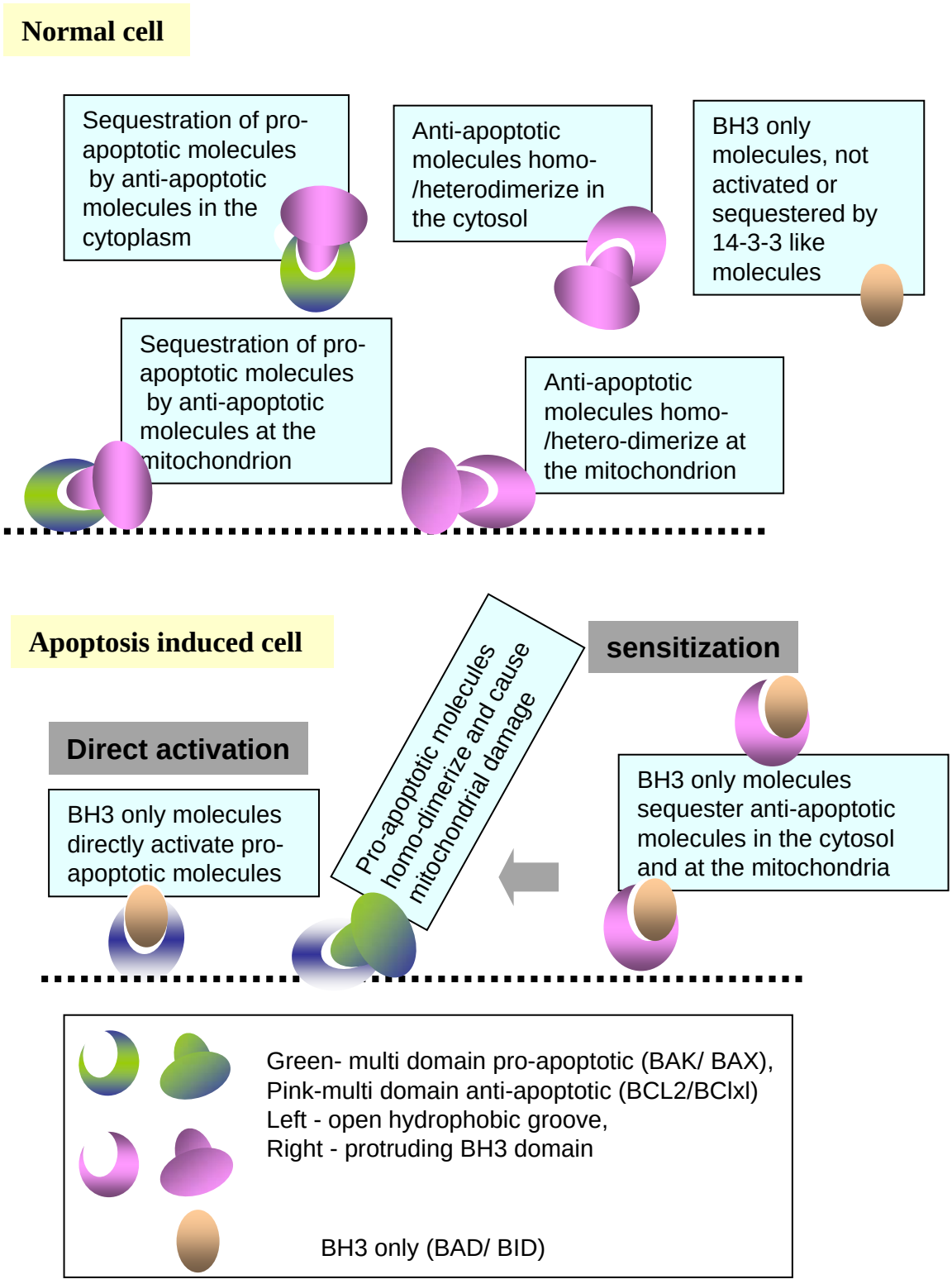



**Figure 1.1.3.3**
**Interactions among BCL2 family proteins**

BAD is a target for Ras activated phoshatase (PP1α) as well as calcineurin (PP2B) in Ca2+ induced apoptosis (19,247). 14-3-3 was shown to actively protect BAD against de-phosphorylation by PP2A in vitro implicating that 14-3-3 holds a regulatory role in BAD de-phosphorylation (55). On the other hand, BCL2 targets BAD for de-phosphorylation by PP1α. BCL2 possesses the consensus sequence (RIVAF) important for PP1α interaction and contributes to the formation of a trimolecular complex thereby facilitating the dephosphoryaltion of BAD by PP1α (20). These mechanisms depict the existence of regulatory processes that ensure latent status of BAD, which is constitutively expressed in normal cells.

BAD has been shown to undergo cleavage by caspase 3 resulting in a more potent pro-apoptotic molecule. This phenomenon was observed in apoptosis caused by interleukine (IL3) deprivation in murine myeloid precursor cells (59) and in response to Raloxifene (mixed estrogen agonist/antagonist) in human bladder cell line (147). Whether BAD is cleaved by proteases other than caspase 3 is not yet clear. In cells that do not express caspase 3 (e.g. MCF-7 (131)) BAD might not be effectively cleaved. Notably, in the studies with MCF-7 we have not observed cleaved form/s of BAD in response to the host of apoptotic stimuli that were used. Several other BCL2 members are also targets for proteases thus showing that cleavage is another post-translational modification of this family of proteins. Pro-apoptotic BID undergoes cleavage by caspase 8 and by granzyme B and promotes mitochondrial release of cytochrome C (169). In some circumstances anti-apoptotic BCL2 and BCLxl were shown to undergo cleavage thus forming potent pro-apoptotic molecules (54,58).

The cellular location of BAD is considered to be cytoplasmic since it is devoid of the membrane anchoring hydrophobic region, which is present in most other BCL2 family members (Figure 1.1.3.2). Upon its interaction with BCLxl or BCL2 that are anchored onto the mitochondria BAD may be found in the mitochondria. Active survival mechanisms inflict inhibitory phosphorylation of BAD and promote sequestration of BAD away from mitochondria. Whether BAD, either phosphorylated or not, has cellular locations other than the cytoplasm and mitochondria is currently unknown. The important phosphorylatable residues and domains of BAD are shown in Figure 1.1.3.4.

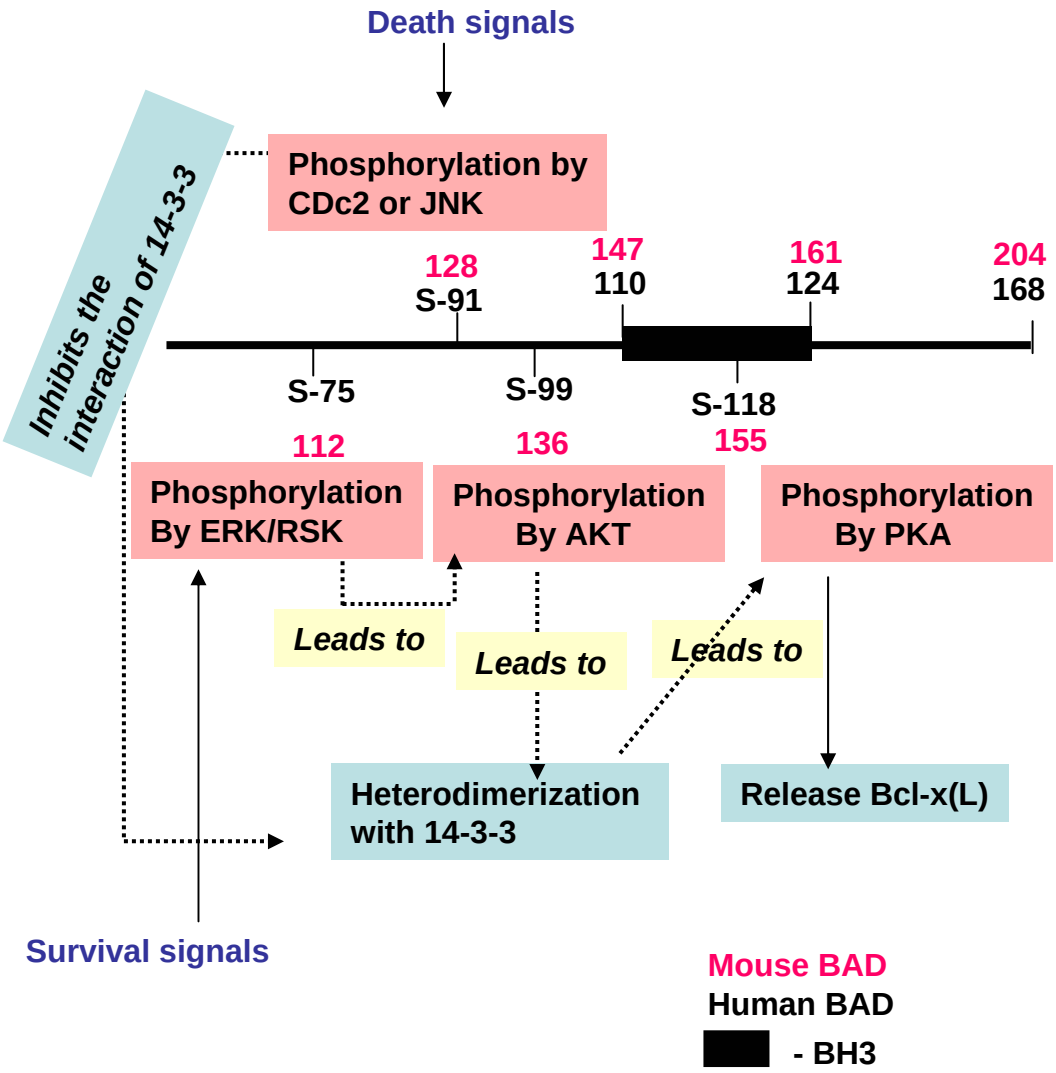



**Figure 1.1.3.4**
**Multi site phosphorylation of BAD**
See text for details

Information on phosphorylation of BAD may not be sufficient to explain its role in cell survival/death process. The large number of signaling pathways that target BAD is perhaps surprising, but there are indications that more may be discovered soon.

Recently transgenic mouse studies showed that survival factors are less protective against insults when BAD gene is knocked out. Moreover, the knocked-in unphosphorylatable BAD did not increase cell death, yet increased the animal' s susceptibility for carcinogens, highlighting the impact of BAD phosphorylation on three serine residues (75, 99 and 118 (murine 112, 136 and 155 respectively) (71,222)). These studies concluded that BAD is a sensitizing intermediate through which survival factors set the threshold for apoptosis.

**Apoptosis and Beast Cancer**

In an adult woman, the cell death in the form of apoptosis and cell proliferation are the key determinants of the mammary gland homeostasis (155). During the menstrual cycle apoptosis occurs at varying rates in response to changes in hormone levels in the breast tissue (220) as well as in the endometrium (66). Similarly, apoptosis plays a key role in the post-lactational involution subsequent to pregnancy (134). Apoptosis is also regulated by non-hormonal signals. Changes in apoptotic regulatory mechanisms due to genetic abnormalities may results in increased cell number, promoting tumor development and progression. This could promote metastasis by enabling the tumor cells to survive in the circulation and grow in distant places. Upon detachment from the basement membrane, epithelial cells undergo apoptosis, which is defined by the term anoikis (96). In order to metastasize, breast carcinoma cells should overcome anoikis. Proliferation and apoptosis also determine the growth dynamics of breast carcinomas in response to a variety of therapeutic measures. Evaluation of treatment-induced changes in these two parameters contributes to the understanding of the tumor's response (or resistance) to the therapy (172). Chemotherapy, radiation therapy as well as immunotherapy heavily rely on apoptosis to kill breast cancer cells (244) and the changes that decrease the ability of cells to activate the apoptotic response may play a key role in resistance to these therapies.

Studies suggest that once arisen, cancers have a very low threshold for apoptosis, which may be a consequence to enhanced proliferation. In contrast, a low threshold for apoptosis may not be a consequence of high rate of proliferation in normal cells e.g. intestinal epithelium. This very fact is exploited by the cancer therapies that target the rapidly dividing – more sensitive cancer cell DNA by genotoxic agents. Unfortunately cancer cells develop resistance for such therapies resulting in a deadly outcome.

Many death and survival genes, which are regulated by extracellular factors, are involved in apoptosis. There appears to be a critical set of apoptotic mediators regardless of the initiating agent, which are regulated by various survival mechanisms. These mechanisms are interconnected so that defects or manipulations in one can cause changes in the other (discussed in detail in the first part of this chapter). Studies suggest that anticancer drugs kill susceptible breast cancer cells by inducing the expression of death receptor ligands such as Fas (FasL), which then bind and activate Fas and death receptor pathway via caspase 8 (250). Interestingly most breast tumors express FasL as well as members of TNFα receptor family (29,139). There seem to be controversies in data regarding such receptor and ligand functions, mostly due to discrepancies in tissue culture conditions and drug concentrations. Additional studies are needed in breast cancer cell lines to determine the precise role of this membrane linked, extrinsic apoptosis pathway. However, the intrinsic (mitochondrial pathway) has been shown to play an important role in most anticancer drug therapies.

Much of the work on apoptosis and breast cancer has focused on BCL2 and its family members. BCL2 is important in pathogenesis of B-cell malignancies in human as the name implies (B-cell Lymphoma/Leukemia 2) but also plays a major role in other types of cancers. It has been recognized as a proto-oncogene, activation of which leads to malignancies. BCL2 family proteins play crucial roles in mitochondria-regulated cell death pathway (Figure 1.1.3.1). Expression of anti-apoptotic BCL2 is increased by $E_2$ in estrogen responsive MCF-7 (42) and breast cancer tissues (186). Environmental estrogens are thought to mimic this effect as these agents suppress TNFα induced apoptosis in ER positive but not in ER negative cells (149). Chemotherapeutic agents exert their effect also through BCL2 family members. For an instance, paclitaxtel,

doxorubicin and thiotepa upregulate pro-apoptotic and down regulate anti-apoptotic members of the BCL2 family (37,186,206). Some of these have been shown to sensitize MCF-7 cells to apoptosis by phosphorylating and inactivating BCL2 (256) related to activation of Cdc2, JNK and other kinases. Introduction of anti-BCL2 antibody gene via a plasmid, enhanced the chemotherapy induced apoptosis in MCF-7 cells and may represent a novel approach to cancer therapy (213). Microinjection of synthetic BCL2 peptide was shown to reduce the apoptosis induced in renal tubular epithelial calls where as the BAK and BAX peptides induced apoptosis in otherwise untreated cells (209). Clinically, increased levels of BAX are correlated with a good response to chemo and radiotherapy, whereas increased levels of BCL2 and BCLxl are correlated with poor response (44,171,299).

Protein kinase B (Akt) is particularly important in breast cancer as a signal intermediate for survival factors such as IGF-1. Dysregulation of Akt signaling as well as amplification of the Akt gene is a common occurrence in tumors (16,158,257). Akt phosphorylates and regulates proteins involved in various cellular pathways including apoptosis. Pro-apoptotic BAD and NFκB are examples for such Akt targets. NFκB is activated, while BAD is inactivated by Akt (231,295), and both these actions promote cell survival. The Ras/ERK pathway (one of several Ras oncogene pathways implicated in cancer) and Akt act in cooperation to induce NFκB activity (231). In MCF-7 cells, Akt suppressed TNFα induced apoptosis, which also correlated with increased NFκB activation (26,30). Further, a recent study shows a new model for anti-estrogen resistance that requires ERα activation by PI-3K and Akt in the absence of estrogen (43). The caspases have been studied in *in vitro* cancer models as potential cancer therapeutic targets owing to their central role in apoptosis (Figure 1.1.3.1). MCF-7 cells, which lack caspase 3 due to deletion in the *caspase 3* gene (131), showed increased sensitivity to chemotherapeutic agents upon caspase 3 reconstitution (293). Though caspase 3 is redundant in some settings, it is obviously a key factor towards an efficient apoptotic program. Therefore further studies may show whether promoting crosstalks between extrinsic and intrinsic apoptotic pathways (that eventually activate caspase 3 and other

executor caspases), and upregulation of these caspases are indeed useful strategies of cancer therapy.

## 1.2 Hypothesis and Rationale

Estrogens are growth inducers in normal and cancer breast tissue and, are capable of inhibiting apoptosis *in vitro* and in animal models. They appear to oppose apoptosis induced by intracellular (default death signals) as well as extra cellular factors such as chemotherapeutic agents. The goal of this study is to understand the underlying mechanisms and dissect specific signal transduction pathways by which $E_2$ exerts anti-apoptotic effects in breast cancer cells. Since growth of a malignant tumor is the sum of cell proliferation and cell death, understanding the factors that regulate both processes is crucial in understanding the biology of this cancer. It is likely that $E_2$ effects on MCF-7 (and other ER positive breast cancer cells) are more pronounced than in normal breast epithelial cells. This may be a result of distinct biological changes that occur in cancer cells during oncogenic transformation.

In response to intracellular and extracellular distress signals, cells enter a programmed death process called apoptosis. This is executed by death proteases (caspases) that are tightly regulated in a normal healthy cell. Among the mechanisms regulating caspase activity, BCL2 family of cytoplasmic proteins takes a centre stage. Pro- and anti-apoptotic members of this family interact with each other and integrate a diverse array of upstream survival and death signals and determine the fate of the cell. Disrupted regulation of apoptosis may be manifested as a variety of disorders.

In the presence of survival factors, BCL2 family member BAD gets phosphorylated on two serine residues (75 and 99), which are consensus targets for Akt and ERK induced $p90^{RSK}$. Phosphorylated BAD is sequestrated in the cytosol by 14-3-3 molecules. Non-phosphorylated BAD binds with anti-apoptotic members of the family such as BCLxl and BCL2 and abolishes their activity towards cell survival.

PI-3 kinase and its down stream targets mediate survival signals activated by a variety of agents in MCF-7 and other cell types (221). In addition to its well known down stream effector Akt, PI-3K also has been shown to have effects on all MAPK pathways (286).

Activation of Akt and MAPK (specifically ERK) are known to support cell survival and growth, and their effects in MCF-7 breast cancer cell line are very well documented.

In this study we hypothesize that $E_2$ induces inhibitory phosphorylation of pro-apoptotic protein BAD at two serine residues through the activation of ERK and PI-3 Kinase pathways, ensuring the survival of ER positive MCF-7 cells, in the presence of a host of apoptotic inducers.

## 1.3 Results and Discussion

**Figure 1.3.1**

**$E_2$ effects on $H_2O_2$, TNFα and serum withdrawal induced apoptosis are mediated by PI-3 kinase**

Previous work in our laboratory has shown that estrogens decreased cell death and apoptosis induced in MCF-7 cells by mitogen withdrawal (7). Recent work indicates that induction of cell growth by $E_2$ in MCF-7 cells may require activity of the PI-3K pathway (176) and the Ras/ERK pathway (48). Given this background we sought to determine if PI-3K plays an important role in $E_2$ action with respect to cell growth and apoptosis. In preliminary experiments we verified that $E_2$ promoted cell survival/growth under low serum conditions and further established that the PI-3K inhibitor wortmannin inhibited this effect of $E_2$.

**(A)** To confirm the requirement of PI-3K activity for the cell survival induced by $E_2$, MCF-7 cells were treated with $E_2$ in the presence or absence of LY294002, a specific PI-3K inhibitor (234) in serum free medium. In the absence of $E_2$, there was significant cell loss reflected in MTT uptake over a five-day period ($n= 4$, $p<0.05$). $E_2$ completely blocked this decrease and maintained MTT uptake at or above day 1 level. Treatment with LY294002 abolished the ability of $E_2$ to increase cell numbers as indicated by MTT uptake (day 2) or maintain cell survival (day 4, 5) and also reduced viability in the control cultures at day 4 and 5. These results suggest that survival of MCF-7 cells under conditions of serum withdrawal requires PI-3K activity and that the ability of $E_2$ to protect cells from death as well as to induce growth is blocked by abrogation of PI-3K

## Figure 1.3.1

**$E_2$ effects on H2O2, TNFα and serum withdrawal induced apoptosis are mediated by PI-3 kinase**

**(A)** MCF-7 cells were treated as indicated and MTT assay was performed to measure amount of live cells

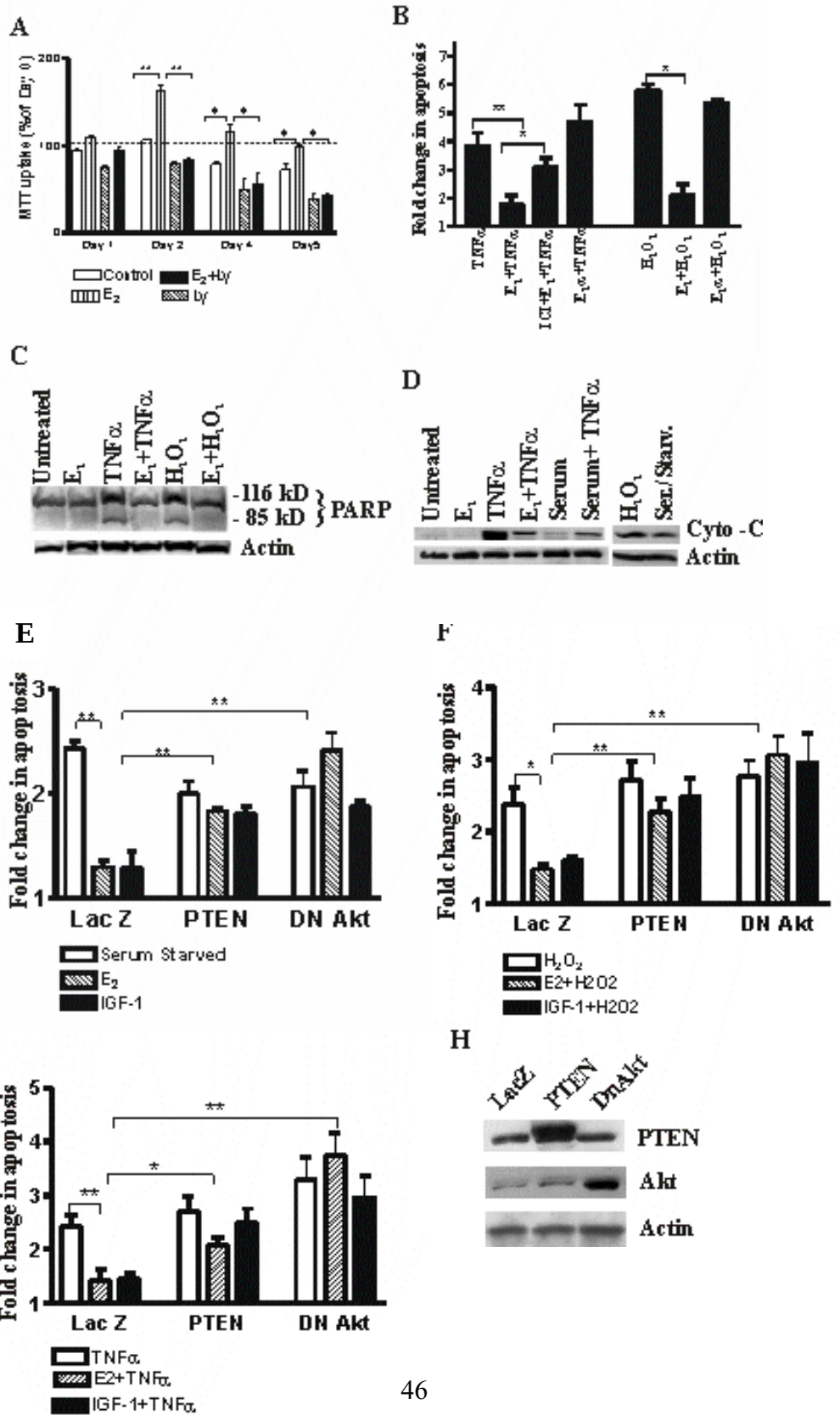
**(B)** Apoptosis was induced as indicated and DNA fragmentation was assayed

**(C-D)** Western blot demonstrate the PARP cleavage and cytochrome-C release in response to indicated agents.

**(E-G)** MCF-7 cells expressing indicated constructs were treated with various apoptotic agents and DNA fragmentation was assayed.

**(H)** Western blot demonstrates the exogenous expression of indicated molecules

(please see the text for detailed experimental conditions)


A
MTT uptake (%of Day 0)
Day 1
Day 2
Day 4
Day5
Control
E2+by
E2
by
B
Fold change in apoptosis
TNFα
E1+TNFα
ICI+E1+TNFα
E1α+TNFα
H1O1
E1+H1O1
E1α+H1O1
C
Untreated
E1
TNFα
E1+TNFα
H1O1
E1+H1O1
-116 kD
- 85 kD
PARP
Actin
D
Untreated
E1
TNFα
E1+TNFα
Serum
Serum+ TNFα
H1O1
Ser./Starv.
Cyto -C
Actin
E
Fold change in apoptosis
Lac Z
PTEN
DN Akt
Serum Starved
E2
IGF-1
F
Fold change in apoptosis
Lac Z
PTEN
DN Akt
H2O2
E2+H2O2
IGF-1+H2O2
G
Fold change in apoptosis
Lac Z
PTEN
DN Akt
TNFα
E2+TNFα
IGF-1+TNFα
H
LacZ
PTEN
DnAkt
PTEN
Akt
Actin

activity. MTT results simply demonstrate the amount of live cells present under given conditions and, in the case of $E_2$, this may reflect the sum of anti-apoptotic and mitogenic effects.

**(B)** The above results and prior observations suggest that $E_2$ may decrease apoptosis in MCF-7 cells. Since TNFα induces apoptosis through activation of caspase8 and, or JNK activation (39) in a variety of cell types including MCF-7 cells (41,108,267). We treated MCF-7 cells with TNFα in the presence of $E_2$, 17-α estradiol (an estrogen of low potency), $E_2$ plus an excess of the specific $E_2$ antagonist, ICI 182,780. TNFα increased apoptosis as measured by DNA fragmentation by 4-5 fold and $E_2$ was able to reduce this effect significantly, whereas 17α-estradiol had no protective effect. The presence of an excess of the $E_2$ antagonist blocked the protective effect of $E_2$ in TNFα treated cells.

**(C)** Poly (ADP-ribose) polymerase (PARP) cleavage, a well-known late event in the apoptosis process (86) was induced by TNFα and this cleavage was significantly blocked by $E_2$ pretreatment.

The reactive oxygen species, $H_2O_2$, is known to induce apoptosis in a variety of cell types including MCF-7 cells (146) and this apoptotic effect is decreased by pretreatment with $E_2$ or overexpression of BCL2 in a synergistic fashion (210). Furthermore, $E_2$ and overexpression of BCL2 exhibit additive inhibitory effects on cleavage of PARP (86) induced by $H_2O_2$. In confirmation of this data, we found that $E_2$ pretreatment can block DNA fragmentation as well as PARP cleavage induced by 2 mM $H_2O_2$ (Figure 1.3.1 B and C). Pretreatment with 17-α estradiol had no significant protective effect against $H_2O_2$ suggesting that $E_2$ effects are unlikely to be due to its potential free radical scavenger function (27).

**(D)** Apoptotic effects of certain stimuli including those activating the extrinsic pathway are manifested at least in part via their effects on mitochondrial membrane integrity (82) (106). Release of cytochrome-C from dysfunctional mitochondria contributes to the formation of the apoptosome, which ultimately activates the downstream caspase

pathways (39). Since the results presented in A-C would predict that $E_2$ may preserve mitochondrial integrity in the presence of apoptotic stimuli, we sought to determine whether $E_2$ would preserve mitochondrial integrity by assessing cytoplasmic cytochrome-C protein level. In untreated or $E_2$ only treated cells, cytoplasmic cytochrome-C was undetectable. As expected $H_2O_2$, TNFα and serum withdrawal increased cytochrome-C in the cytoplasm. Co-treatment with $E_2$ decreased cytochrome-C release induced by TNFα to a similar extent as that observed in cells co-treated with serum and TNFα. Taken together these results suggest that $E_2$ protects cells against apoptosis induced by TNFα, $H_2O_2$ and serum withdrawal (not shown) possibly by maintaining mitochondrial integrity.

**(E, F, G)** To establish the role of the PI-3K pathway in $E_2$-induced anti-apoptosis, we used adenoviral vectors to interfere with operation of this pathway. Cells were transduced with adenoviral wild type PTEN/MMAC that effectively decreases activity of the PI-3K pathway by dephosphorylation of PI-3K products (183,199). Over-expression of PTEN completely blocked the ability of $E_2$ or IGF-1 to prevent apoptosis induced by serum withdrawal (E), $H_2O_2$ (F) and TNFα (G) when compared to adenoviral LacZ (control) infected cultures. Akt/PKB is a downstream target of PI-3K pathway that promotes cell survival in a number of cell types (69,81). Similarly the overexpression of dominant negative Akt (DNAkt) enhanced apoptosis induced by the agents, and completely reversed the effects of either IGF-1 or $E_2$ on apoptosis prevention. These data together with data derived using chemical inhibitors strongly suggests that the PI-3K/Akt pathway is essential for the anti-apoptotic effects of $E_2$ or IGF-1 in MCF-7 cells. We repeatedly observed that DNA fragmentation (measured by the Cell Death ELISA) in viral transduced cells was below the levels observed in parental cells (1.8-2.5 *vs.* 4-5 fold, compare Figure 1.3.1B and E-G), which lead to an apparent decrease in the magnitude of apoptosis as measured by the cell death ELISA in the latter.

**(H)** A Western blot analysis showing the expression of the indicated constructs in MCF-7 cells.

### 1.3.2 $E_2$ induced rapid signal pathways impose phosphorylation on BAD

**Figure 1.3.2.1**

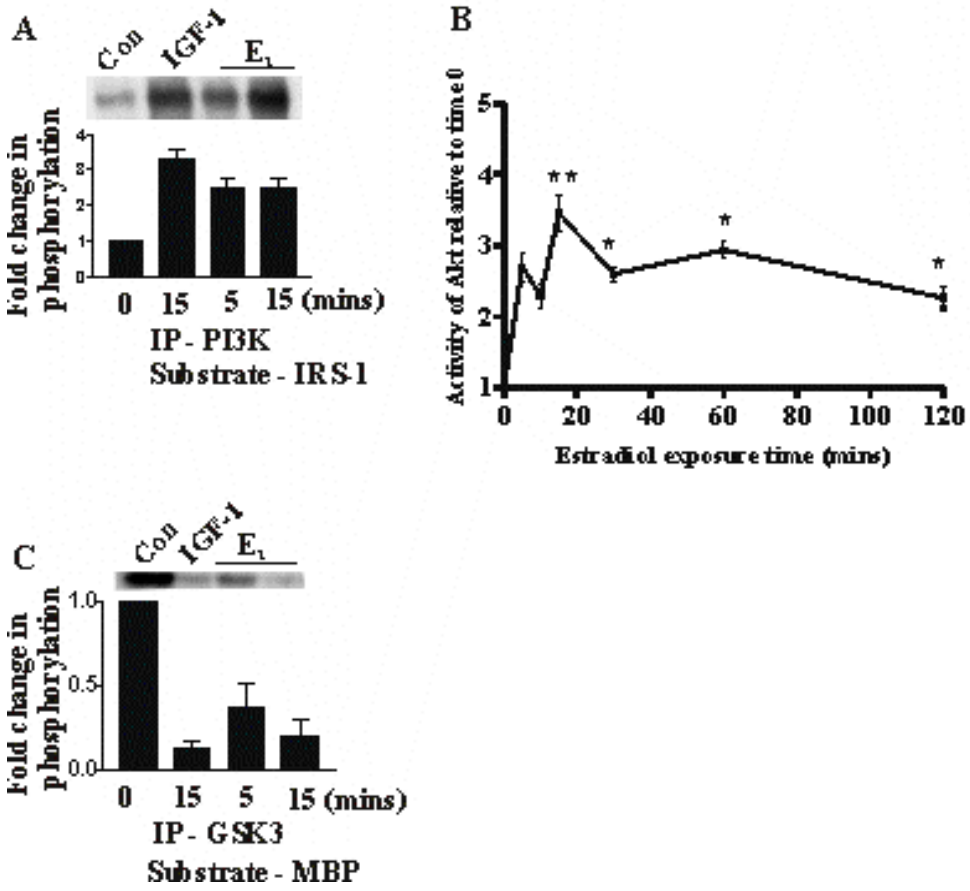
**$E_2$ activates the PI-3K/Akt pathway leading to phosphorylation of down stream targets**

The above data strongly suggests that the ability of $E_2$ to decrease apoptosis in MCF-7 cells is at least partly mediated by the PI-3K/Akt pathway. Transient activation (measured by S473 phosphorylation) of Akt by $E_2$ in MCF-7 cells has been reported (48,227). Our data suggest that the PI-3K/Akt pathway may need to be activated for a prolonged period to maintain BAD in an inactivated (phosphorylated) state.

**(A)** Kinetic behavior of the PI-3K response to $E_2$ was measured. PI-3K activation was determined by phosphorylation of IRS-1 on S612 by *in vitro* kinase assay using immunoprecipitates of the p85 subunit of PI-3K. $E_2$ was able to rapidly activate PI-3K approximately 3 fold, in growth arrested cells. Similar in magnitude to the activation elicited by IGF-1.

**(B)** A detailed time course of Akt catalytic activity in growth arrested MCF-7 cells, was measured by *in vitro* kinase assays (n=3) using Akt immunoprecipitates and BAD as the substrate.

**(C)** GSK3β is a downstream target of Akt (65). Active GSK3β effectively triggers rapid cyclin D1 turnover and affects the cell cycle regulation (77). GSK3β activity was determined by *in vitro* kinase assay using immunoprecipitates of GSK3β. $E_2$ rapidly and strongly inhibited GSK3β activity. As expected, another Akt activator IGF-1 also completely inhibited GSK3β activity. The effect of $E_2$ was sustained for 60 min (our unpublished data) and such a time course is compatible with the increase in cyclin D1 protein observed 3-4 hrs after $E_2$ addition to quiescent cells (94,95)**.**




## Figure 1.3.2.1

**$E_2$ activates the PI-3K/Akt pathway leading to phosphorylation of down stream targets**

**(A)** In vitro kinase assay with PI-3kinase immunoprecipitataes and IRS-1 as the substrate. Graph represents densitometry values from three independent experiments

**(B)** Time course for Akt activation by $E_2$ growth arrested cells. Graph was obtained from values of three independent experiments

**(C)** In vitro kinase with GSK3β immunoprecipitataes and MBP as the substrate

**Figure 1.3.2.2**

**$E_2$ activates ERKs and down stream target $p90^{RSK}$**

A number of studies reported that $E_2$ rapidly activates ERK1 and 2 in MCF-7 cells (130) and refs there in) and in a number of other cell types (152,246). However, others failed to observe $E_2$-mediated ERK activation in MCF-7 cells (47,85,176). $E_2$ induced ERK activity could be a result of activation of the Ras/Raf signal transduction pathway or may follow rapid increase in intracellular $Ca^{2+}$ (130). Previously we had demonstrated that increased cyclin D1 synthesis in response to estrogens is partly blocked by the MEK1 inhibitor PD98059 and that dominant negative ERK2 partially inhibited the induction of cell cycle transit by estrogens (7).

**(A)** Therefore we measured a detailed time course of ERK activation by $E_2$.
$E_2$ rapidly activated ERK with maximum activation of five fold observed within 15 mins and significant ERK activation was sustained for at least 120 mins (n=4).

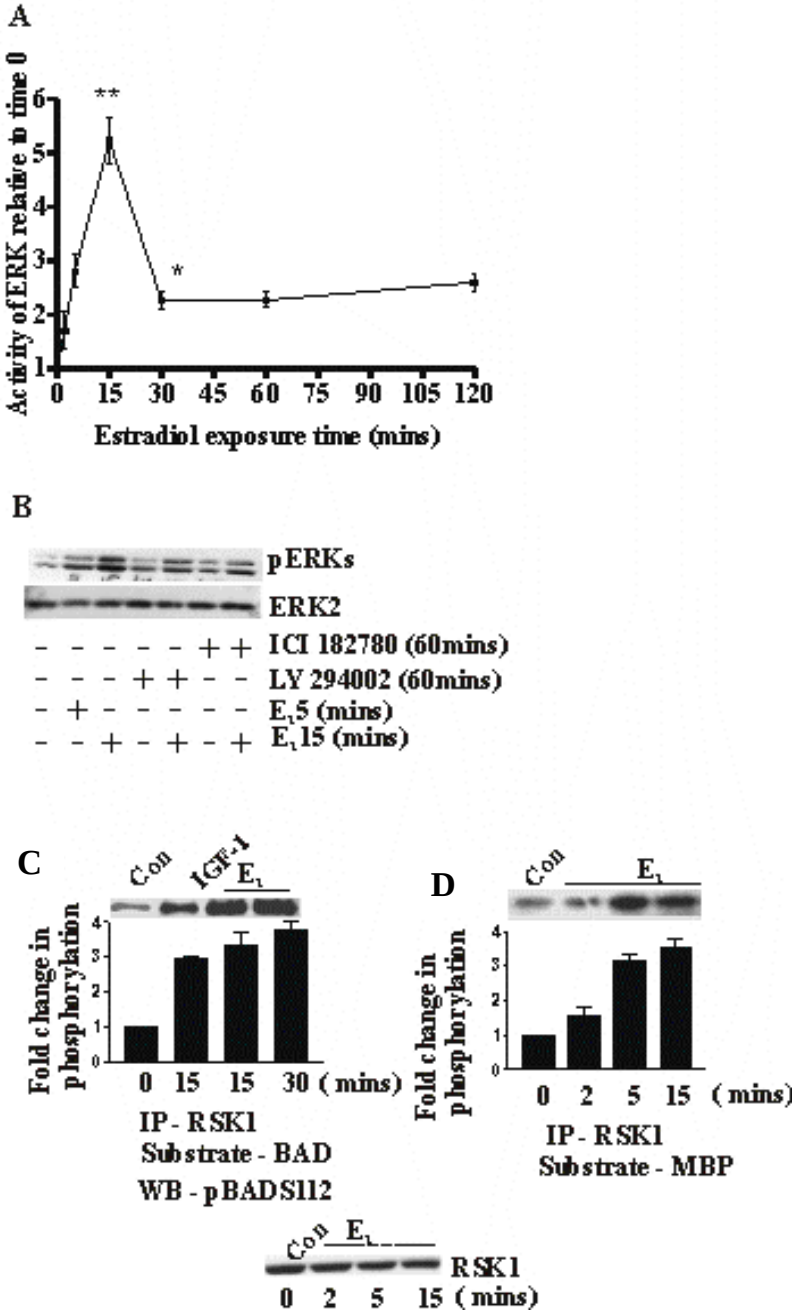
**(B)** Enhancement of ERK activity by $E_2$ was partly blocked by the specific estrogen antagonist ICI182, 780 and the PI-3K inhibitor LY294002.

**(C -D)** It is known that $p90^{RSK1}$ is phosphorylated at S364 and T574 residues by ERKs leading to its activation (67) and our results on BAD phosphorylation (C) imply that $E_2$ activates $p90^{RSK1}$. Therefore we directly determined that $E_2$ rapidly activates $p90^{RSK1}$ by *in vitro* kinase assay using immunoprecipitates of $p90^{RSK1}$ (D) which shows that $p90^{RSK1}$ is activated within 5min by $E_2$.

**Figure 1.3.2.3**

**Interactions of $E_2$ induced signal pathways**

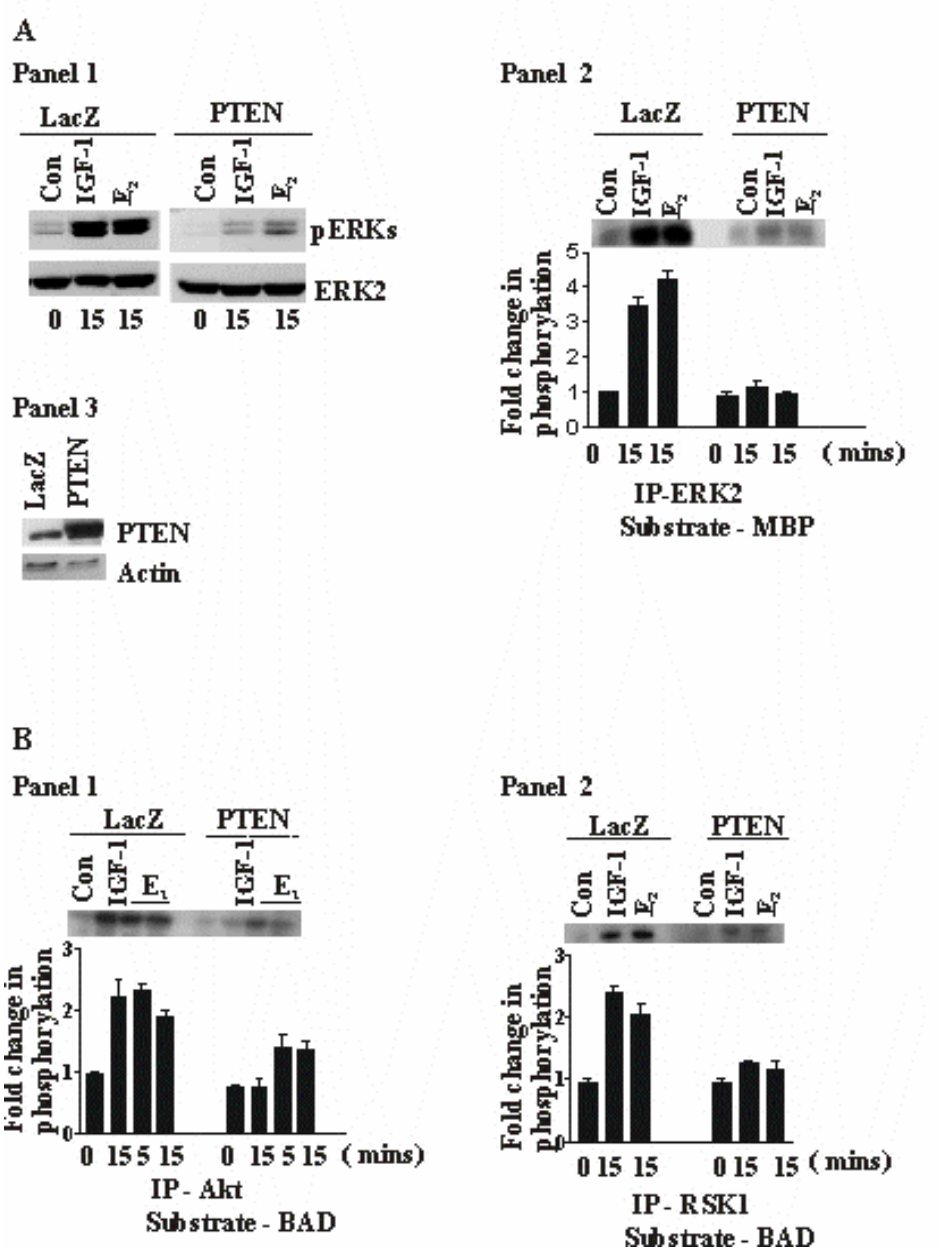
**(A panels 1-3)** Above results and other recent work (112,286) indicate that there is a crosstalk between the PI-3K and ERK pathways. We explored this interaction further by over-expression of adeno viral PTEN. $E_2$ or IGF-1 activated ERKs in control Ad virus infected cells, PTEN over-expression (approximately 3X over the basal level [panel 3])




## Figure 1.3.2.2

**$E_2$ activates ERKs and down stream target p90$^{RSK}$**

**(A)** Time course of $E_2$ activated ERK measured by in vitro kinase assays (n=4) **(B)** Western blot demonstrating phosphorylation of ERKs in response to indicated agents. **(C)** In vitro kinase assay (Non radioactive) using immunoprecipitates of p90RSK and BAD as the substrate. Western blot of the reaction demonstrates phospho BAD. **(D)** In vitro kinase assay using with immunoprecipitates of p90RSK and MBP as the substrate. Expression of p90RSK is shown in a Western blot




## Figure 1.3.2.3

**Interactions of $E_2$ induced signal pathways**

**(A)** MCF-7 cells expressing indicated viral vectors were treated with IGF or $E_2$. Western blot demonstrates the activation of ERK by phoshorylation (panel 1) In vitro kinase assay with immunoprecipitates of ERK and MBP as the substrate (panel 2). Level of expression of PTEN is shown in panel 3

**(B)** Panels 1-2 in vitro kinase assay with immunoprecipitates or Akt and p90RSK Using BAD as the substrate.

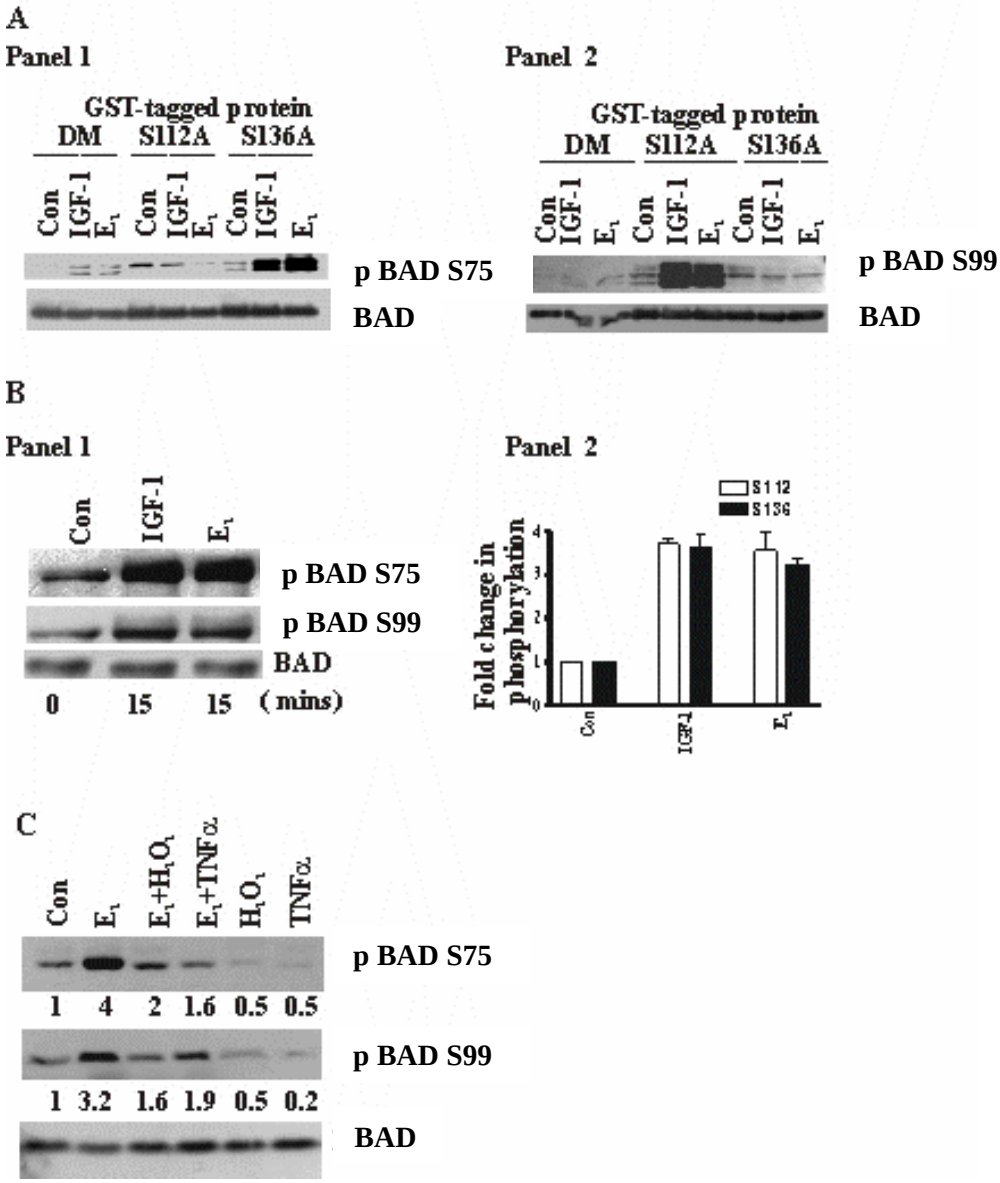
partly blocked ERK activation observed at 15mins of treatment with these two agonists. In confirmation of the data using phosphospecific ERK antibodies, PTEN also partly inhibited ERK2 activity measured by MBP phosphorylation in an *in vitro* kinase assay (n=3) (panel 2).

**(B panels 1-2)** In extracts from similarly treated cells, BAD phosphorylation by Akt and $p90^{RSK1}$ were measured *in vitro.* PTEN over-expression abolished the Akt and $p90^{RSK1}$ activation. These results suggest that the PI-3K pathway may regulate the ERK response in addition to the Akt response in MCF-7 cells and provide evidence for cross talk between these signal pathways in response to $E_2$ or IGF-1, under our experimental conditions.

**Figure 1.3.2.4**

**$E_2$ induces BAD phosphorylation**

**(A panels 1-2)** BAD is a pro-apoptotic BH3 domain-containing protein (5,106), which forms heterodimers with BCL2 or BCLxl (59) resulting in cytochrome-C release from mitochondria. Anti-apoptotic agents like IGF-1 induce BAD phosphorylation at specific serine residues, and phosphorylated BAD is sequestered away from its site of action in the mitochondria by binding to cytosolic 14-3-3 proteins (70). Since BAD is phosphorylated on serine 99 (S99) by Akt (70) and S75 is a target site for $p90^{RSK1}$ (82), we ascertained the ability of $E_2$ to induce phosphorylation of BAD at specific residues by using BAD phosphorylation site mutants as in vitro substrates [note: human S75 and S99 are equivalent to S112 and S136 in mouse]. Clearly $E_2$ or IGF-1 treated cell extracts were unable to induce phosphorylation of the serine to alanine mutant (S75A) while S99 phosphorylation was enhanced 3-4 fold. Similar specificity was observed when the S99A BAD mutant was used as the substrate (panel 1). When the double phosphorylation site mutant (DM) was used as a substrate, only a small fraction of the phosphorylation that was observed with single mutants was detectable. Multiple immunoreactive bands have been previously observed when recombinant BAD was used as a substrate (70).




## Figure 1.3.2.4

**$E_2$ induces BAD phosphorylation**

**(A)** Panel 1-2, GST-tagged BAD mutants were used as in vitro substrates with $E_2$ or IGF-1 induced cell lysates. Reactions were Western blotted for phospho- and total BAD

**(B-C)** Western blot demonstrating endogenous BAD phosphorylation induced by indicated agents.

**(B panels 1-2)** In vivo BAD phosphorylation was also measured by Western blot analysis. Following treatment with $E_2$ or IGF-1 for 15mins both S75 and S99 phosphorylations were enhanced (>3 fold) and total BAD levels were unchanged in this time frame. Panel 2 shows a graph obtained from three independent experiments.

**(C)** Since our results demonstrated that $E_2$ can induce BAD phosphorylation in vitro and in vivo and can prevent cell death in response to several stimuli, we studied the possible changes in phosphorylation status of BAD during apoptosis induction. $E_2$ increased endogenous BAD phosphorylation at S75 and S99 above the basal level several fold whereas TNFα and $H_2O_2$ decreased it below control level. Co-treatment with $E_2$ and $H_2O_2$ or TNFα restored the BAD phosphorylation to above control levels. Taken together these data strongly suggest that $E_2$ as well as IGF-1 exert anti-apoptotic actions partly through phosphorylation and inactivation of the pro-apoptotic BAD protein, and that phosphorylation of BAD is a consequence of activation of Akt and ERK/$p90^{RSK1}$

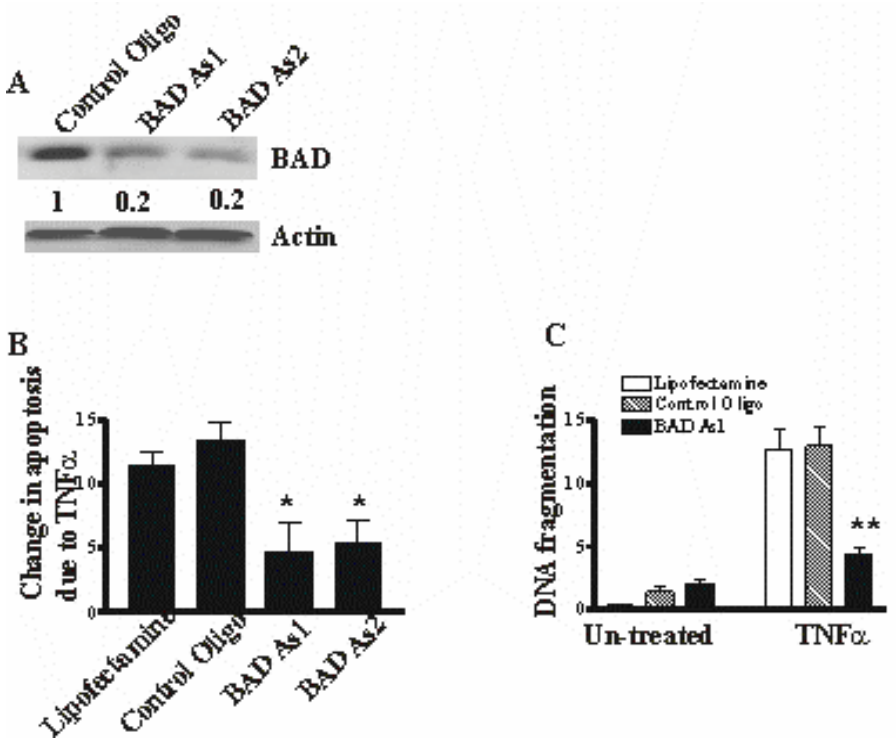
### 1.3.3 BAD is a key mediator of $E_2$ induced anti-apoptosis in MCF-7 cells

Since the effects of apoptotic agents on decreasing BAD phosphorylation (inactivation) were largely reversed by $E_2$, BAD inactivation could be a potential non-genomic effect of $E_2$, which results in cell survival. To better understand the involvement of BAD in cell death and survival in MCF-7 cells, experimental techniques were used to knock down the endogenous BAD and over-express wtBAD as well as phosphorylation site mutants of BAD.

**Figure 1.3.3.1**

**Inhibition of BAD is a critical event in $E_2$ induced anti-apoptosis in MCF-7 cells**

**(A)** MCF-7 cells transduced with antisense oligonucleotides to prevent the translation of BAD mRNA. The antisense sequences, BAD antisense 1 (BAD As 1) containing the complementary sequence to the translation initiation codon and BAD antisense 2 (BAD As 2) containing the complementary sequence to the translation termination codon were




## Figure 1.3.3.1

**Inhibition of BAD is a critical event in $E_2$ induced anti-apoptosis in MCF7 cells**

**(A)** Endogenous BAD was knocked down with anti sense mRNA to BAD. Western blot demonstrates the efficiency of the reduction of BAD

**(B)** Quantification of apoptosis by flow cytometry in anti sense mRNA treated cells in response to TNFα. Change in the apoptotic population due to TNFα is graphed against each cell population

**(C)** DNA fragmentation assay to measure apoptosis in response to TNFα in indicated cell populations

equally effective in downregulating BAD protein level whereas a scrambled oligonucleotide corresponding to BAD As1 (control oligo) was not.

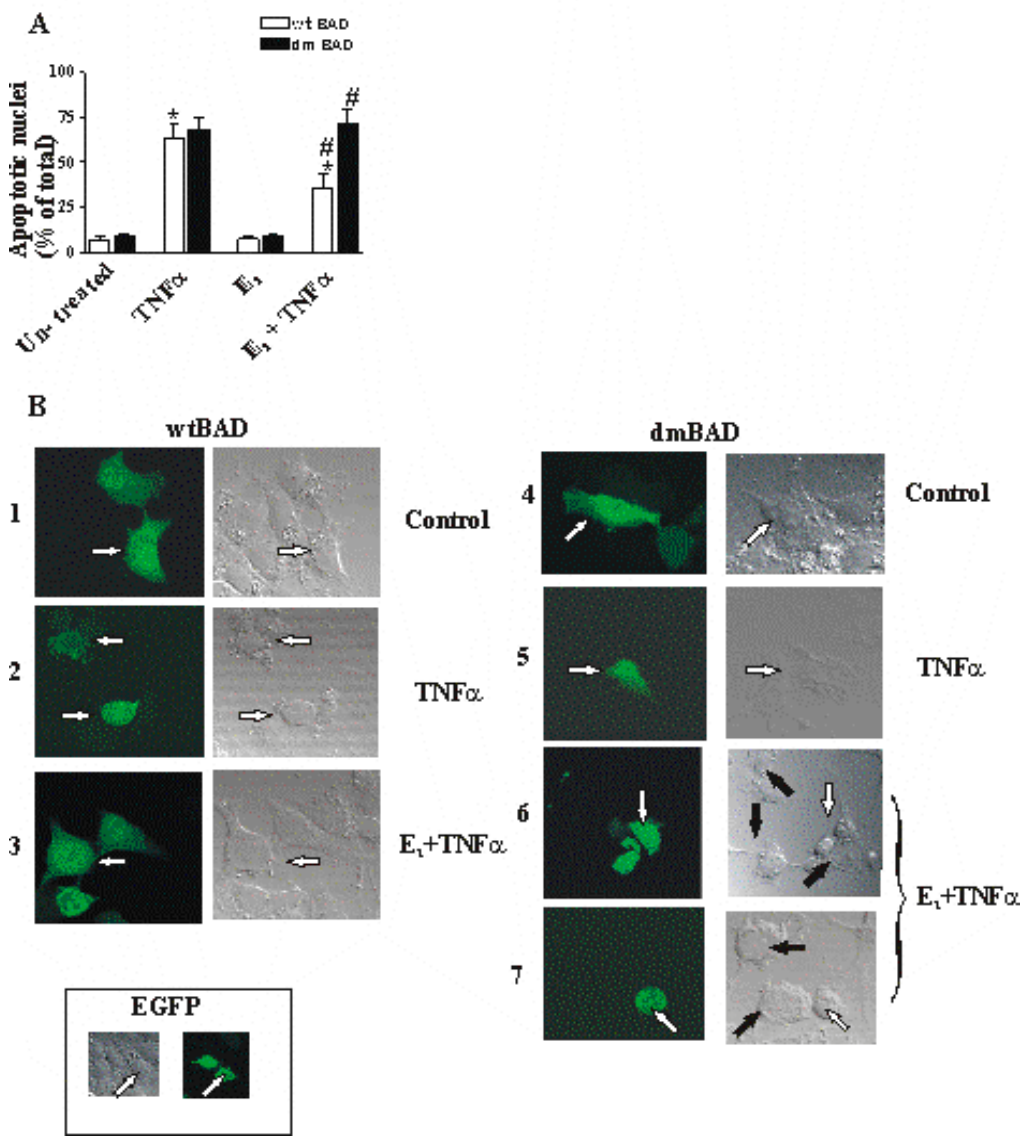
**(B)** Quantification of DNA by flow-cytometry revealed a large reduction of the sub $G_1$ fraction - apoptotic cells (203) in TNFα treated as well as serum-starved (not shown) cells in BAD As1 and BAD As2 treated, but not in control oligonucleotide treated cells. $E_2$ did not have an additional effect alone or together with TNFα in anti-sense treated cells compared to control oligonucleotide treated cells (not shown). Change in apoptosis due to TNFα was calculated by subtracting sub G1 fraction of untreated from sub G1 fraction of TNFα treated cells for each oligonucleotide treated population. Graph shows data from three independent experiments. Introduction of BAD As1 or BAD As2 significantly reduced the magnitude of apoptosis in response to TNFα (* = $p<0.05$), $H_2O_2$ and complete serum starvation (not shown).

**(C)** We next measured DNA fragmentation in oligonucleotide treated cells in the presence or absence of TNFα. TNFα induced 4-5 fold DNA fragmentation above the basal level (comparable to Figure 1.3.1) in control oligonucleotide or Lipofectamine treated cells. BAD As1 drastically reduced this effect (** = $p<0.005$) and maintained the DNA fragmentation near the basal level. Similar results were observed using BAD As2 (not shown). This data clearly suggest a critical involvement of BAD in cell survival and death in response to several agents in MCF-7 breast cancer epithelial cells and agrees with the reduction in basal BAD phosphorylation induced by TNFα and $H_2O_2$ in the absence of $E_2$ (Figure 1.3.2.4).

**Figure 1.3.3.2**

**Functional serine phosphorylation sites are important for apoptotic function of BAD**

**(A)** To assess the effects on BAD in $E_2$ induced cell survival and consequent changes in cellular morphology we transiently expressed wild type BAD (wtBAD) and dual phosphorylation site mutant of BAD (dmBAD) where S75 and S99 are mutated to unphosphorylatable alanine, together with an EGFP expression vector. Following the




# Figure 1.3.3.2

**Functional serine phosphorylation sites are important for apoptotic function of BAD**

**(A)** Cover slips cultures of MCF-7 cells transfected with wild type or double phosphorylation site mutant together with EGFP, were treated as indicated. Nuclei were stained with Hoechst 33342. Number of apoptotic nuclei in response to treatment is graphed for each cell group.

**(B)** Similarly treated cells were analyzed with confocal microscope to visualize cellular changes.

**(C)** Panel 1-3 Dose response for individual BAD vector in MCF-7 cells. In response to indicated agents, amount of apoptosis was assayed using DNA fragmentation, in cells expressing varying amounts of vectors. Panel 4, apoptotic response in pcDNAtransfected cells.

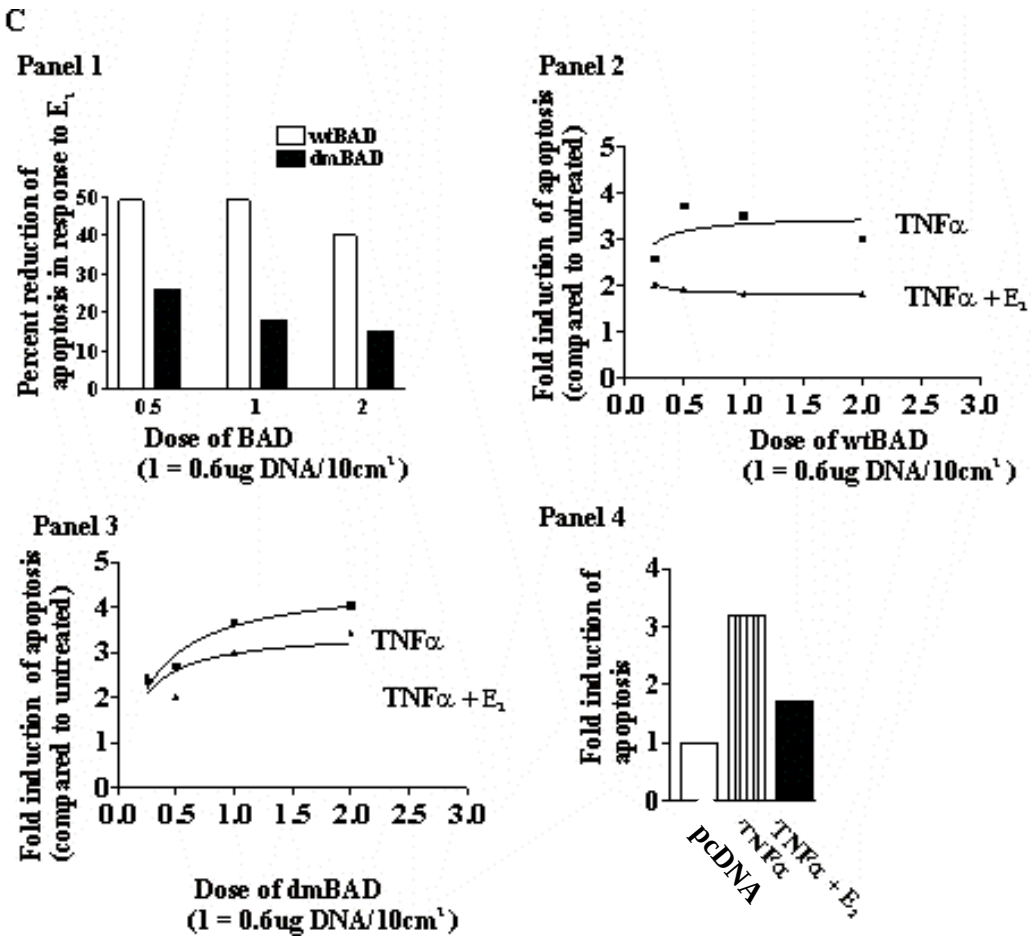



**Figure 1.3.3.2 continued**

desired treatments, cover-slip cultures were stained with Hoechst 33342 and mounted on to glass slides. $E_2$ reduced the TNFα induced apoptosis in wtBAD expressing cells by >50% and importantly had no protective effect in dmBAD expressing cells. Pre-treatment of $E_2$ had the same protective effect against TNFα in cells expressing the control vector pcDNA3 or EGFP alone (not shown). It must be emphasized that the overexpression of dmBAD did not alter the number of apoptotic nuclei compared to those in pcDNA3 or EGFP transfected cultures. There is an absolute requirement for TNFα to induce apoptosis in dmBAD as well as wtBAD expressing cells. The magnitude of apoptosis observed with cell counting (~ 8-9 fold) is somewhat higher than that observed with DNA fragmentation ELISA assay (4-5 fold in Figure 1.3.1) in response to TNFα. Possible explanation for such differences in magnitude may be the fact that only a fraction of all the cells (i.e. the transfected population) is counted in (A) whereas in Figure 1.3.1A, DNA fragmentation of the whole population is measured.

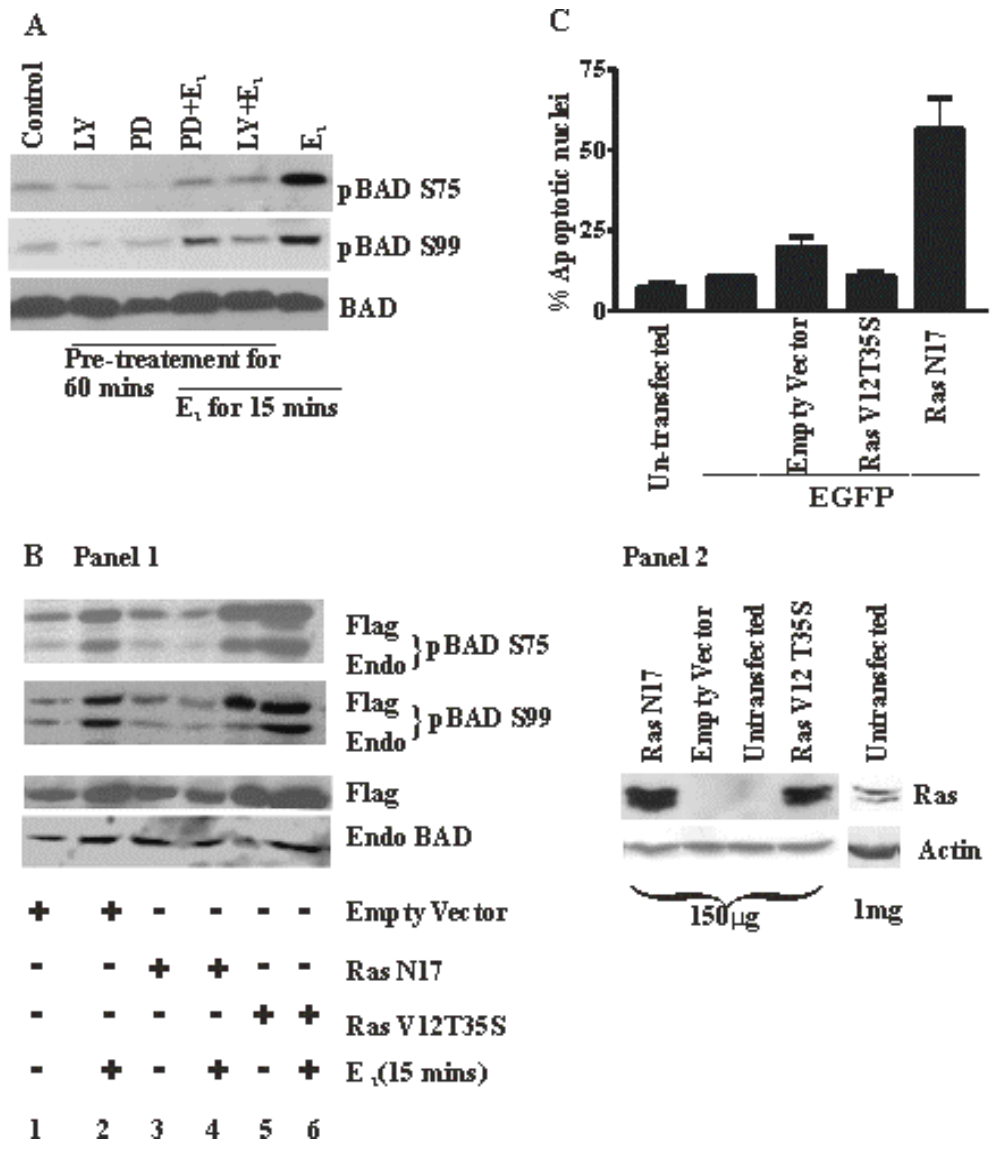
**(B)** Similarly transfected and treated cells were examined by confocal microscopy to further analyze the cellular morphological changes. TNFα induced cellular shrinkage and atypical structure of nuclei in MCF-7 cells as has been previously described for fibroblast (182) and neuronal cells (45). It was difficult to observe the chromatin clumping (typical for apoptosis) in TNFα treated cells due to shrinkage of the nuclei as opposed to this feature being readily apparent in paclitaxel treated cells (not shown). Under these conditions expression of exogenous wtBAD or most importantly, dmBAD (white arrows in fluorescence images and respective differential interference contrast (DIC) images) did not enhance basal apoptosis (panel 1 and 4). Treatment with TNFα in wt and dmBAD transfected cells clearly induced apoptotic changes (panel 2 and 5). While $E_2$ was able to impede morphological effects of TNFα in wtBAD expressing cells (panel 3) it was unable to counteract the effects of TNFα in dmBAD expressing cells (panel 6 and 7). Black arrows in panel 6 and 7 point to the untransfected cells that preserve the normal morphology. Under these conditions expression of EGFP alone did not induce morphological changes (white arrows in the insert). These observations further supports the proposed crucial role of BAD in MCF-7 cells and the critical importance of the two

specific phosphorylation sites in BAD for the anti-apoptotic effects of $E_2$. It is quite clear that dmBAD by itself does not induce apoptosis in the absence of TNFα. This suggests that in these mammary carcinoma cells, there are mechanisms to possibly sequester BAD away from mitochondria independent of S75 and S99 phosphorylation and binding to 14-3-3 proteins, thus making dmBAD ineffective as an apoptosis inducer by itself.

**(C Panels 1-4)** Anti-apoptotic effect of $E_2$ was further evaluated in cells expressing increasing levels of BAD. MCF-7 cells were transfected with 1/4x, 1/2x, 1x and 2x (x = regular dose = 0.6ug DNA for 10cm$^2$) of each construct and were treated with TNFα with or without $E_2$. DNA fragmentation was assayed as described above. $E_2$ reduced apoptosis by 45-60 percent in the wtBAD expressing cells irrespective of the dose. $E_2$ mediated protection was dampened by the overexpression of dmBAD by 50% or more (panel 1). A moderate dose dependency in response to dmBAD was observed in TNFα as well as the $E_2$+TNFα treated cells (panel 3). The values of apoptosis as measured by ELISA in pcDNA3, wtBAD and dmBAD were unrelated to the dose of DNA. Increasing doses of wtBAD did not influence the basal apoptosis, TNFα induced apoptosis or the protection offered by $E_2$ (panel 2). Panel 4 demonstrates the fold induction of apoptosis in pcDNA3 expressing cells at regular dose of DNA. In wtBAD transfected cells (panels 1 & 2) $E_2$ is able to decrease the apoptosis due to TNFα to the same extent as in untransfected or pcDNA3 transfected cells (panel 4) by 50-60% when measured by this method. Taken together this data strongly suggest that BAD is at least partly involved in $E_2$ induced anti-apoptosis, and dmBAD is not an apoptosis inducer by itself in MCF-7 cells. This reinforces our notion that in these cells, TNFα (and perhaps other apoptotic stimuli) is able to translocate cytoplasmic BAD to its mitochondrial sites of action irrespective of phosphorylation at S75 and S99 residues.

**Figure 1.3.4**

**$E_2$ induced BAD phosphorylation requires Ras activated signaling pathways and Ras function is critical for cell survival of MCF-7 cells**




## Figure 1.3.4

**$E_2$ induced BAD phosphorylation requires Ras activated signaling pathways, and Ras function is critical for cell survival of MCF7 cells**

**(A)** Phosphorylation of endogenous BAD in response to indicated agents demonstrated by Western blot.

**(B)** Endogenous and exogenous BAD phosphorylation in Ras transfected cells in the presence and absence of $E_2$. Western blot demonstrates the expression of endogenous and exogenous Ras in MCF-7 cells

**(C)** Quantification of apoptosis by counting apoptotic nuclei in indicated cell populations.

**(A)** Previous studies in our laboratory had demonstrated that $E_2$ induced $p27^{kip1}$ degradation and entry of quiescent MCF-7 cells into the cell cycle requires Ras activity (6,7,94). Other investigators have demonstrated that $E_2$ rapidly activated Ras in MCF-7 cells (144) and recent work in our laboratory (93) demonstrated that Ras activity is required for nuclear export and degradation of $p27^{kip1}$ in response to $E_2$. The phosphorylation of BAD at S75 and S99 has been proposed to be mediated by ERK/$p90^{RSK1}$ and PI-3K/Akt pathways, respectively, in addition to other mechanisms (82). Given the role of Ras discussed above and the evidence that the PI-3K and ERK pathways are activated by $E_2$ (as discussed above) we used chemical inhibitors LY294002 and PD98059 to inhibit these pathways respectively and determined the effects of these treatments on BAD phosphorylation. $E_2$ induced BAD phosphorylation on S75 as well as S99 was completely abrogated by LY294002 supporting the idea of cross talk between the MAPK/$p90^{RSK1}$ and PI-3K/Akt pathways. Similarly, PD98059 effectively reduced the phosphorylation of BAD at S75 as determined by Western blots, however the MEK inhibitor had considerably less effect on BAD S99 phosphorylation (compare $E_2$ *vs*. PD+$E_2$ on BAD phosphorylation on S99). None of the treatments changed total BAD levels in this time frame.

**(B)** Since Ras is a well-known upstream regulator of the PI-3K/Akt and MEK/ERK/$p90^{RSK1}$ pathways, we analyzed the effects of inhibition of endogenous Ras by using dominant negative Ras, RasN17 (DNRas) or selectively activating the MEK/ERK pathway with RasV12T35S (110) in transient transfection experiments. Flag tagged BAD was co-transfected with control empty vector, RasN17 or RasV12T35S into MCF-7 cells. Twenty-four hours after transfection, cells were serum withdrawn for another 24 hrs and stimulated with $E_2$ for 15mins. BAD phosphorylation was assessed by Western blotting. Clearly the ability of $E_2$ to increase both S75 and S99 phosphorylation (panel 1, lane2) of exogenous as well as endogenous BAD was inhibited by RasN17 (lane 4) which by itself did not regulate BAD phosphorylation (lane3). The MEK/ERK selective activator, RasV12T35S, by itself significantly activated BAD S75 phosphorylation and moderately activated exogenous BAD S99 phosphorylation (lane 5)

and $E_2$ was able to enhance this effect (lane 6). Taken together the results in (A) and (B) strongly indicate that the ability of $E_2$ to stimulate BAD phosphorylation and thereby prevent apoptosis is mediated through Ras activated MEK/ERK and PI-3K/Akt pathways. Expression of endogenous and exogenous Ras is shown in panel 2.

**(C)** To directly analyze the apoptotic effects of RasN17 in these cells, we stained nuclei of Ras transfected cells and studied them by confocal microscopy. Nuclei corresponding to EGFP expressing cells (co-transfected with Ras or control vector) were counted, and the graph shows the number of fragmented nuclei as a percentage of total number of cells. Transient expression of RasN17 induced 7-8 fold apoptosis over 48hr period where as the expression of RasV12T35S or empty vector had minimum effects on apoptosis observed in cells co-expressing EGFP. These results demonstrate that Ras function is required to protect MCF-7 cells from dying even in the presence of growth medium and that this effect may be mediated at least partly by phosphorylating and inactivating pro-apoptotic BAD. The observation that $E_2$ induced BAD phosphorylation was abolished by inactivation of endogenous Ras (B) further highlights the involvement of Ras mediated pathways in $E_2$ signaling and demonstrates that Ras plays a critical role in the anti-apoptosis mediated by $E_2$ in MCF-7 breast cancer cells.

# Chapter 2

# BH3 Domain only Protein BAD Regulates Cell Cycle Progression by Modulating Transcriptional Activities of AP-1

## 2.1 Introduction

### 2.1.1 Functional Multiplicity of BAD

BAD is a point where survival signaling pathways interact with the death machinery. Survival mechanisms inhibit BAD by adding inhibitory phospho groups on to it in order to prevent its participation in the apoptotic program. Recently a BAD knockout mouse study (222) showed that survival factors are less protective against apoptotic insults when the BAD gene is knocked out. Moreover, when the un-phosphorylatable BAD was "knocked-in" it did not increase cell death, yet increased the animal' s susceptibility for carcinogens, highlighting the impact of BAD phosphorylation on three serine residues (75, 99 and 118 (murine 112, 136 and 155 respectively). Absence of BAD in these animals also increased the incidence of some tumors like lymphoma. This study concluded that BAD is a sensitizing intermediate through which survival factors set the threshold for apoptosis. In the first part of our study (Chapter 1) we studied in detail, the importance of BAD phosphorylation on two of these critical serine sites, serine 75 and 99 in $E_2$ action as an anti-apoptotic agent. Over-expressed wild type (wt) BAD or dual phosphorylation site mutant (dm) of BAD did not increase the basal death level in MCF-7 cells. When challenged with apoptotic stimuli, cells expressing wtBAD but not the dmBAD were remarkably protected against apoptosis by $E_2$. Our study and the BAD knockout mouse study emphasize the significance of functional phosphorylation sites of BAD for its role as an apoptotic mediator.

Interestingly BAD was revealed to participate in nutrient metabolism, more precisely glucose, in mice (68). Here BAD plays an essential role as an assembly factor for the glucose-metabolizing-enzyme complex at the mitochondrion. BAD deficient mice displayed an abnormal glucose metabolism result from the inability to form the enzyme complex. The most exciting finding was that the mice carrying un-

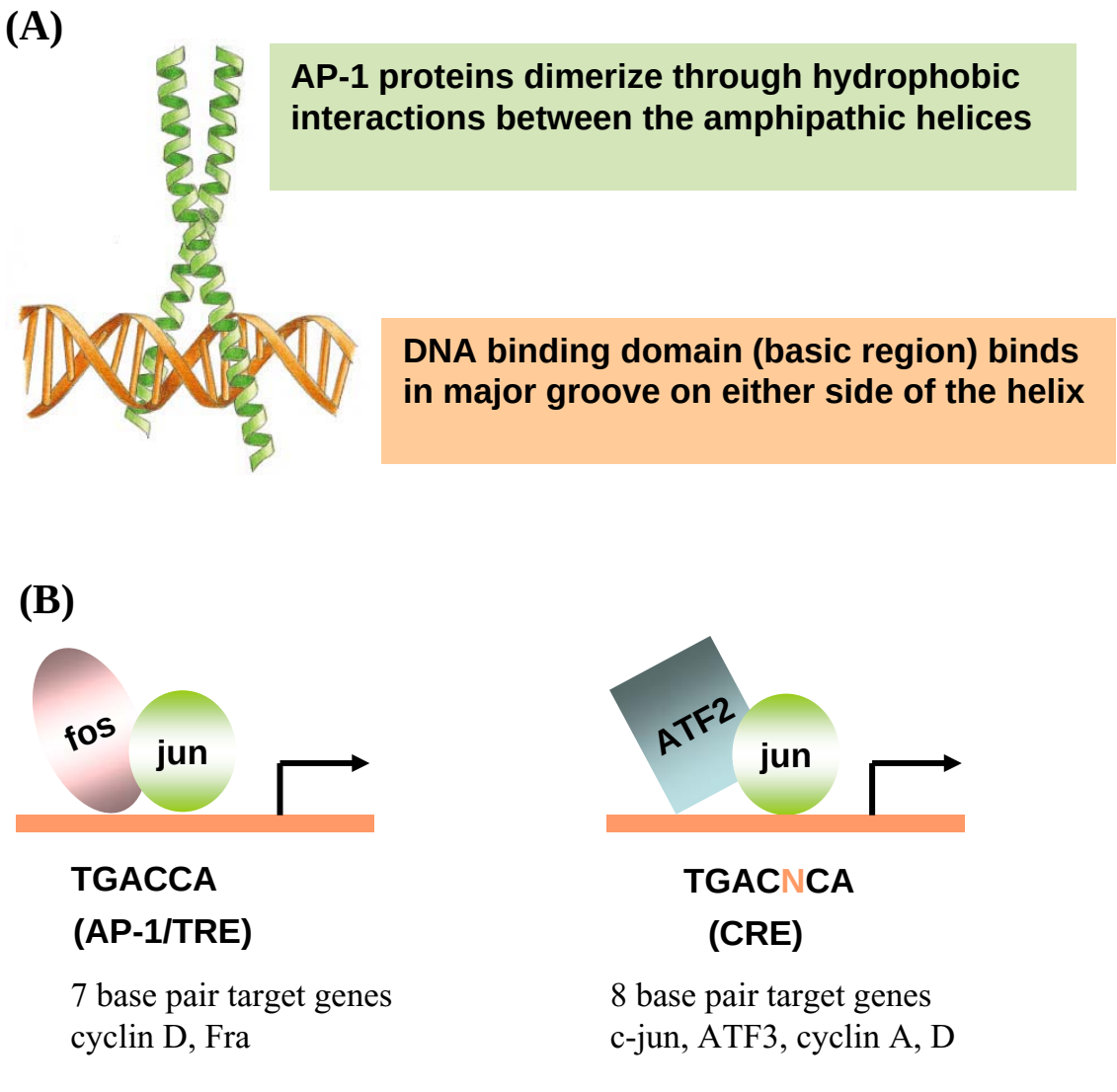
phosphorylatable form of BAD showed a defective glucose metabolism though the enzyme complex was assembled normally. This finding again suggests that phosphorylation and/or events secondary to phosphorylation are important for the "metabolic function" of BAD. Although BAD is believed to be inactive after the phosphorylation on these serine residues, this "apoptotically inactive" molecule appears to posses the capacity to perform other tasks.

Growth promoting effects of BAD has also been shown. Chicken embryo fibroblasts overexpressing wtBAD but not the control vector, increased their proliferation in the presence of growth factors. This growth promoting effect of BAD was determined to be dependent on S99 phosphorylation and subsequent 14-3-3 binding (188). All above findings suggest that BAD requires fully operative phosphorylation sites for its 'normal function'. It is important to note that the normal function of BAD extends beyond the apoptosis regulation contributing to the balanced state of cell physiology.

In our preliminary studies a wide spread expression of exogenous BAD was found in MCF-7 cells. Similarly unphosphorylated and phosphorylated forms of endogenous BAD were found in the nuclei of proliferating breast cancer cells. These observations warranted investigation of currently unknown nuclear functions of BAD in breast cancer cells and the second part of this study focuses on transcriptional effects of BAD on the cell cycle regulator protein cyclin D1.

### 2.1.2 Activating Protein (AP-1) Transcription Factor Family

AP-1 is a fundamental or critical class of transcription regulators that sense environmental information and translates it into expression of specific genes. This family consists of several basic region leucine zipper (bZIP) domain proteins that homo- or hetero- dimerize in order to bind with the DNA targets. The basic region harbors the actual DNA contact region and the leucine zipper enables the formation of the dimer with the partner molecule (Figure 2.1.2.1). The characteristic feature of AP-1 complexes in the cell is the heterogeneity in dimer formation. This results from the multiple AP-1 subunits being expressed at the same time. Originally AP-1 was divided into 4 main groups and sub-groups within them (276) .




## Figure 2.1.2.1

(A) AP-1 proteins homo-or hetero dimerize through leucine zipper domain in order to bind with the DNA targets. The basic region harbors the DNA contact region.
(B) Jun/fos heterodimers preferentially bind to 7 base pair AP-1/TRE where as Jun/ATF-2 binds to 8 base pair CRE

The first group, AP-1 is composed of jun, fos and fra gene products. Jun proteins form homo or hetero dimers with other members of the family and bind with high affinity to 7 base pair TRE (TGA G TCA), which is a phorbol ester 12-O- tetradecanoyl-phorbal-13-asetate (TPA) response element (280). Second group, ATF/CREB family proteins is further divided into two sub families (Table 2.1.2.1) depending on their binding partnerships. First sub class (ATF1) either homo- or hetero-dimerizes with CREB and bind with cyclic AMP response element (CRE) which is 1 base pair longer than TRE (276). Members of the second subclass (ATF2) complex with themselves as well as members of the first group (AP-1). In addition to the intra-group partnerships, AP-1 family transcription factors tend to interact with other non bZIP proteins (56,258). This variation in dimer formation could direct the AP-1 factors to promoter elements that posses very minor resemblance to their consensus sites. This in turn provides AP-1 factors a high level of flexibility in gene regulation potential (276).

**AP-1 as a Mediator of Cell Proliferation, Survival and Death**

A plethora of physiological and pathological stimuli induce and activate AP-1 proteins. Studies using cells and mice deficient in individual AP-1 proteins have immensely contributed to the understanding of their physiological functions. These involve the control of cell proliferation, growth, neoplastic transformation and apoptosis.

Among growth regulators AP-1 proteins are unusual because they are up regulated in response to growth as well as stresses that cause cell cycle arrest and/or apoptosis. This variable behavior might depend on the composition of AP-1 transcription complex, post-translational modification of individual member as well as other interacting factors. The clearest data on AP-1 induced apoptosis come from studies on the nervous system. Continuous c-fos activation was shown to precede neuronal cell death in response to neurotoxic agents (248). Inhibition of c-jun activity by dominant negative form of c-jun protected neuronal cells from apoptosis induced by neuronal growth factor (NGF) withdrawal (205). The activation of c-jun N-terminal Kinase (JNK) has been linked to induction of cell death in response to a variety of stimuli (25,52). Expression of c-jun mutant (S63/73A) that cannot be phosphorylated by JNK was employed to effectively

## Table 2.1.2.1

**Classification of AP-1 Family Proteins and Their Preferred DNA Elements**

| Family | Sub Family | Dimmers | Target element/s |
|---|---|---|---|
| AP-1 | Jun, Fos, Fra | Homo and hetero | 7 base pair TRE |
| ATF/CREB | CREB and ATF1 | Homo and hetero | 8 base pair CRE (cyclic AMP response element) |
| | ATF2-4, ATF6, ATFa | Homo and hetero dimers with themselves and Jun & Fos | 8 base pair CRE (cyclic AMP response element) |
| C/EBP | | Hetero dimers with both above families | Data not available |
| Maf | | Hetero dimers with both above families | Data not available |

block apoptosis (as effectively as JNK inhibitors) thereby showing the direct relationship between JNK and c-jun (92,238). Interestingly, there are other reports on physiological and pathological conditions in nervous system where JNK activation precedes c-jun phosphorylation without inducing apoptosis (238). Genotoxic stress induced apoptosis in response to UV radiation depends heavily on c-jun activation as demonstrated using c-jun and JNK null mouse fibroblast that are resistant to UV induced apoptosis (273). However in vivo observations show that AP-1 proteins in fact have protective roles in normal development. C-jun deficient embryos show massive liver apoptosis, which might result in lethality (28). Additionally JNK or c-jun independent apoptosis has been described in response to TNFα (174).

Different mechanisms of AP-1 actions described above may not be mutually exclusive. Various stimuli could induce subsets of AP-1 regulated genes that support cell proliferation or survival, or in extreme conditions cell death. AP-1 proteins, mostly members of Jun group, control cell growth through their ability to regulate the expression and function of cell cycle regulators such as cyclin D1, p53, p21(cip1/waf1), p19(ARF) and p16. For instance, c-Jun is able to positively regulate cell proliferation through the repression of tumor suppressor gene expression and function, and induction of cyclin D1 transcription (237,238,288). JunB, which upregulates cell cycle inhibitory proteins and represses cyclin D1 (207), antagonizes these actions. Table 2.1.2.2 summarizes the effects of Jun family proteins on cell cycle regulators.

**Cyclin D1 Regulation by AP-1**

Cyclin D1 protein regulates cell cycle progression at the G1 to S transition. It interacts with the respective kinase partner, cyclin dependent kinase 4 (CDK4) to enhance its action. This eventually results in the phosphorylation and inactivation of the retinoblastoma protein (Rb), which is inhibitory for the cell division (60). The human cyclin D1 gene promoter sequence contains an AP-1 element at position -954 (9). Accordingly, several AP-1 proteins have been shown to bind to this site. C-jun was shown to induce cyclin D1 transcription in transient expression studies (9) and phosphomimicking mutations of c-jun (mutation of serine 63 and serine 73 of c-jun to

## Table 2.1.2.2

**Effects of Jun Proteins on AP-1 Target Genes Involved in Cell Proliferation**
Modified from *Shaulian and Karin Oncogene 20: 2390-2400*

| Gene | C-jun | JunB | JunD |
|---|---|---|---|
| Cyclin D | Up | Down | - |
| p16 | Down | Up | - |
| p19 | - | - | Down |
| p53 and p21 | Down | Down | - |

negatively charged residues) further increased the extent of cyclin D transcription. Fos group members cooperate with c-jun to induce cyclin D levels (36). Whether cyclin D is the major mediator of AP-1 induced cell cycle progression was addressed by transducing cyclin D into c-jun -/- cells. Only 30% of the DNA synthesis was restored by cyclin D introduction (288). This demonstrates that AP-1 may require other effectors in addition to cyclin D to mediate its cell cycle effects (288).

**p53 Regulation by AP-1**

Another important c-jun target is p53, a negative regulator of cell proliferation. C-jun negatively regulates p53 expression and its down stream functions. In c-jun-/- cells a high basal p53 level was observed and this was reversed by stable expression of c-jun (235). A direct relationship between these two players was established when an AP-1 response element was found in the p53 promoter. Interestingly, while c-jun is a potent activator when bound to its target sites, on p53 it appears to repress the transcription of the gene. Most importantly deletion of p53 abrogated all cell cycle defects in c-jun -/- cells (235). DNA damage by UV exposure often results in c-jun phosphorylation by JNK (discussed above). On the other hand DNA damage triggers the up regulation of p53, which acts via p21 (cell cycle inhibitor) to suppress cell cycle progression. It is well established that c-jun activation in this situation leads to the down regulation of p53 and relief the growth arrest. Eventually cells may resume growth or may undergo apoptosis depending on the extent of DNA damage. In some settings cells require c-jun to undergo apoptosis in response to UV damage (discussed above) and in another setting, cells increased sensitivity to UV induced apoptosis in the absence of c-jun (235). These may appear contradictory yet may very well represent the true complexity of endogenous system that is governed by complex mechanisms mediated by multifaceted AP-1 proteins.

Another AP-1 induced and apoptosis related molecule is FasL. DNA damaging agents, which also activate JNK, such as UV, induce FasL. AP-1 directly induced FasL transcription through the AP-1 binding element found in FasL promoter (138).

**Regulation of c-jun by Phosphorylation**

C-jun is an immediate early gene whose expression level increases within an hour of the stimulation. However, its transcriptional functions depend on post-translational modifications, namely phosphorylation. About 8 phosphorylation sites of c-jun have been described to date. Two N-terminal serine sites, 63 and 73 are largely accepted as the most critical residues pertaining to the transactivation function. These are the well-known targets of JNK (164) as well as ERK1 and 2 (196,197). Upon phosphorylation of these residues conformational changes occur in c-jun to facilitate DNA binding (196 and refs therein). Subsequently, several more residues were identified (threonine 231, 239, serine 243 and 249), that are proximal to the DNA binding domain. Phosphorylation of these residues abolished c-jun and DNA binding (34). In vivo and in vitro studies identified kinases such as GSK3, CK2, ERK1 and ERK2 as responsible (196 and refs therein). This information suggested that c-jun activation requires phosphorylation of N-terminal S63 and S73 and dephosphorylation of C-terminal residues. Recently two more threonine residues (91 and 93) were identified whose phosphorylation was essential for the dephosphorylation of C-terminal residues. It is the conformational change that results from T91 and 93 phosphorylation that facilitates the accesses of phosphatases to the C-terminal sites (196). Currently the exact kinase/s that are responsible for the phosphorylation of these residues are not known. Evidence suggests that kinase other than JNK can be involved since in JNK null mice these sites were found to be phosphorylated (196). Collectively, it is conceivable that full activation of c-jun may require N-terminal serine phosphorylation by JNK (also by ERK), and C-terminal dephosphorylation which depends on the phosphorylation of two N-terminal threonine residues whose in vivo upstream kinase/s are remained to be identified.

**Estrogen Action on Cyclin D1 Promoter Depends on AP-1**

Estrogens are responsible for the proliferation of normal mammary epithelial and development and progression of breast cancer (Chapter 1.1 Introduction). They act in early G1 phase of the cell cycle, and both steroidal and non-steroidal anti-estrogens arrest estrogen-dependent cell lines in the G1 phase (12,33,272). Previous studies suggest that

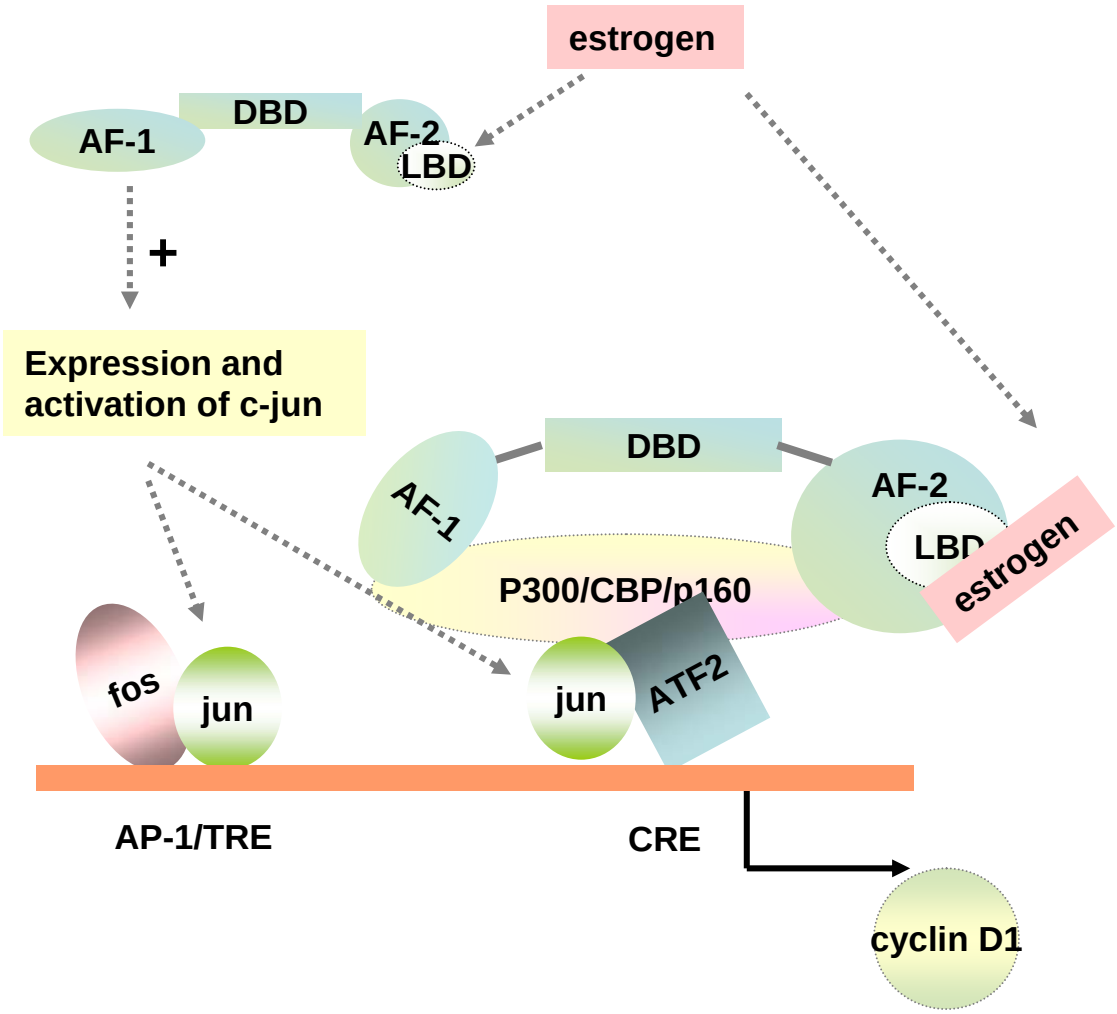
cyclin D1 may be involved in mediating the estrogen induced cell proliferation in normal and malignant breast epithelial cells (24,94,264). Cyclin D1 deficient mouse are defective in estrogen responsive proliferation of breast epithelium during pregnancy (242). Estrogens induce MCF-7 cell cycle in conditions where mitogen activated protein kinases are continuously inhibited. This correlated with the expression of cyclin D1 as well as the phosphorylation of retinoblastoma protein (12). Interestingly the increased expression of cyclin D1 upon over-expression of estrogen receptor (ER) has been noted (38). However, no ERE-related sequence has been found in the proximal cyclin D1 promoter (115).

In addition to the classical ERE dependent mechanism, an alternative pathway of ER action has been described. Here ER induces certain promoters that express AP-1 protein binding sites (49,99,159). The fundamental facts of ER action on AP-1/TRE elements have been demonstrated by several groups. First, DNA binding of Jun and Fos is needed for ER action, and ER appears to increase the intrinsic transcriptional activity of these factors when bound to DNA (160,212,274). Next, SERMs are able to active AP-1 target genes through binding to ERβ (187,284). Genetic dissection of ERα has provided important clues on the mechanism of ER action on AP-1. Activation functions (AF-1/AF-2) dependent and independent pathways of ERα have been shown to be important in estrogen induced AP-1 activities. In the AF dependent pathway, estrogen-ER actions points to the function of co-activator molecules. ER was shown to interact specifically with Jun proteins, but not with the Fos family proteins (271). Contrary to this, isolated ligand binding domain (LBD) of ER was shown unable to directly bind with AP-1 proteins, though it was able to induce AP-1 action (285). Hence the most conceivable mechanism could be that, ER may bind with the Jun/Fos recruited co-activator complex, which includes p300, CBP as well as p160 (100,271). ER could join the already recruited activator complex at the AP-1 binding sites and trigger the co-activator function to a higher state (160). This triggering model may explain how estrogen-ER could have promoter specific effects i.e. the promoters that carry more elements in addition to ERE may be more responsive to estrogen.

In the AF independent pathway, which has been observed in response to SERMs, ER appears to activate AP-1 mediated transcription without directly participating in the AP-1 complex. The finding that ER interacts with transcriptional co-repressors such as N-CoR only in the presence of SERM (284) suggests that ER may sequester N-coR and associated repressors namely histone deacytalases (HDACs) away from the AP-1 binding sites. This may allow the histone acetylases (HAT) in the co-activator complex (recruited by Jun/Fos) to act without inhibition (98). It is interesting to note that neither of these models requires ER and AP-1 protein direct interactions, or the DNA Binding domain (DBD) of ER.

The mechanism by which estrogen induces cyclin D1 in ERα positive cells has been linked to AP-1 responsive sites in the promoter. Among the known cyclin D1 promoter elements are AP-1/TRE and CRE (115) both of which can be activated by AP-1 protein binding (Figure 2.1.2.1B). The minimal cis-acting element in the cyclin D1 promoter responsive to estrogen was mapped to position –96 to –26, which contains a putative CRE (232). Mutations in this region caused complete loss of estrogen responsiveness suggesting an essential role of this element in mediating estrogen effect. Agreeing with the previous observations (276), c-jun and ATF-2 heterodimers were shown to be the most effective transcription factor in CRE transcription, and estrogen enhanced that basal effect. Remarkably, isolated CRE sequence of the cyclin D1 promoter was unable to induce transcription when removed from its natural environment (232). This shows the importance of adjacent DNA for CRE function. They may play a role in recruitment of co-regulators to the CRE site. This study also shows that the DNA binding domain and the activation function of ER are important for cyclin D1 induction by estrogen.

Estrogen induces c-jun expression as well as phosphorylation in an ER dependent manner (217,232), but does not affect expression of ATF-2 (232). Therefore, it is likely that ER plays a dual role in cyclin D1 expression (i) through activating c-jun and (ii) acting as a co-activator for c-jun/ATF-2 dimer thereby increasing CRE transcriptional activity (Figure 2.1.2.2).




## Figure 2.1.2.2

**Possible estrogen/ER and AP-1 actions at the cyclin D1 promoter**

Estrogen induces c-jun expression as well as its phosphorylation in an ER dependent manner. ER plays a dual role in cyclin D1 expression (1) through activating c-jun and (2) acting as a co-activator for c-jun/ATF-2 dimer thereby increasing CRE transcriptional activity

## 2.2 Hypothesis and Rationale

Preliminary observations that over-expressed wild type BAD inhibits cell cycle transit in MCF-7 cells led us to investigate likely cell cycle regulatory effects of this molecule. Additionally, BAD abolished the activating protein response element (AP-1/TRE luciferase) activation in MCF-7 and T47D cells. $E_2$ induced cell cycle progression depends on cyclin D1 and associated kinases as has been previously shown. Since cyclin D1 carries an active AP-1/TRE element at position –953 of its promoter, we hypothesized that BAD induced cell cycle block may result at least partly from an inhibitory action on this DNA element.

Presence of endogenous as well as exogenous BAD in the nucleus raised our curiosity as how this translocation could occur since BAD does not contain a typical nuclear localization signal (NLS), and there is no information about another molecule that may bind to BAD and facilitate its nuclear import. BAD co-immunoprecipitated with c-jun, which is a critical regulator of AP-1 functions, in the nuclear extract. This observation led to the hypothesis that BAD binds with c-jun as a normal regulatory mechanism and when present in extra amount further sequesters c-jun away from its site of action. Binding with c-jun and/or a subsequent step may facilitate BAD's translocation to the nucleus.

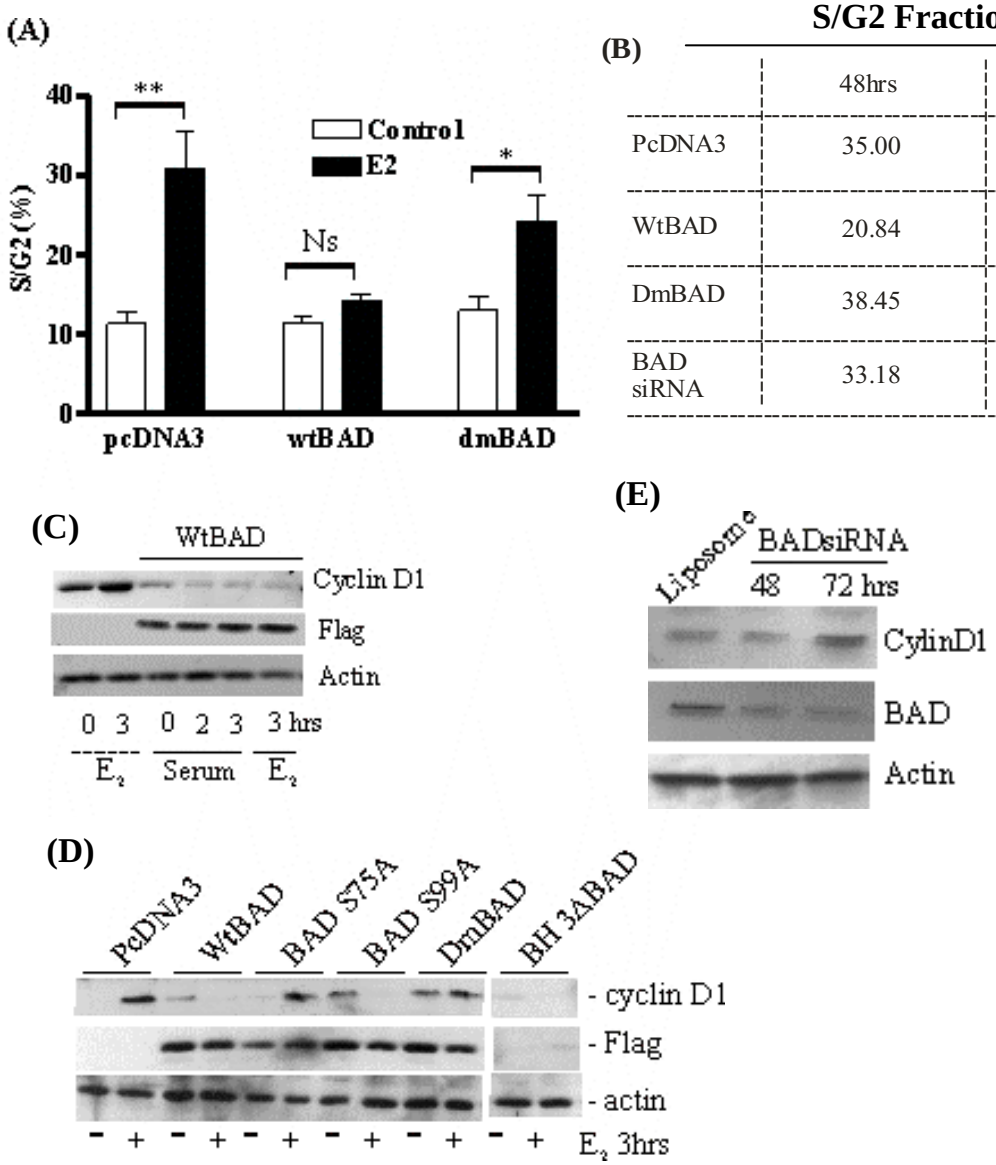
Further we hypothesize that the functional phosphorylation sites, S75 and S99 are important for this transcriptional regulatory function of BAD since the dual phosphorylation site mutant was incapable of inducing cell cycle block and the AP-1 transactivation.

## 2.3 Results and Discussion

**Figure 2.3.1**

**Over expression of BH3 only BAD impedes the breast cancer cell growth in culture**

Two plasmid constructs (Flag-BAD and GFP-BAD) were used to over-express BAD in MCF-7 as well as T47D, breast cancer cells. Presence of high amount of BAD did not kill these cells as we had observed previously (in contrast to the observations made by other authors). However BAD appeared to slow down the rate of their proliferation as




## Figure 2.3.1

**Over expression of BAD impedes the breast cancer cell growth in culture**

**(A)** MCF-7 cells were transfected with the indicated constructs, growth arrested and induced with $E_2$. DNA profile was assayed. Percent of S/G2 fraction is shown in the graph. **(B)** Cells expressing the indicated BAD constructs and cells where endogenous BAD was knockdown (siRNA) were kept in complete growth medium for indicated times and S/G2 fraction was analyzed (n=2). **(C -D)** Cells transfected and treated as in (A) were lysed and whole cell lysate was used for Western blot analysis. **(E)** asynchronous cells were transfected as indicated and Western blot analysis was done as in (C).

judged by visual observation of transfected cells. This observation prompted us to investigate whether in addition to its pro-apoptotic functions BAD plays any role in cell cycle progression.

**(A)** MCF-7 cells were growth arrested at G1/G0 using anti-estrogen ICI182, 780 in low serum conditions for 48 hours and induced with 5-10 nM $E_2$ for 17 hours. As measure of cell cycle progression, the DNA profile was analyzed by flow cytometry. Change in S/G2 fraction is graphed for various treatment conditions (n=4). BAD over-expression remarkably blocked $E_2$ induced G1-S progression compared to control vector (pcDNA3.1) transfected or untrasfected cells ($p<0.005$). Similar inhibitory effect was observed in BAD transfected cells in response to 5% FBS (not shown). Since known BAD's functions are mostly tied to its phosphorylation status, we questioned whether a relationship would exist between cell cycle regulatory function of BAD and its phosphorylation level. Dual phosphorylation site mutant (dmBAD) was expressed, growth arrested and treated as described above. Expression of dmBAD did not block the effect of $E_2$ on cell cycle progression as cells entered S phase in the presence of $E_2$ as much as observed in pcDNA3.1 transfected cells.

**(B)** Cells expressing indicated constructs or cells in which endogenous BAD was inhibited by siRNA, were maintained in complete growth medium. At indicated times cells were harvested and DNA profile was analyzed. Mean values S/G2 fraction from two independent experiments with duplicates for each treatment are shown. Marked reduction of S/G2 fraction by wild type BAD was observed at 48 hr post-transfection. Increased S/G2 fraction is apparent with BAD siRNA at 72 hr post-transfection, which clearly correlated with the expression levels of cyclin D1 (E).

**(C)** $E_2$ induced MCF-7 cell cycle progression through the regulation of cyclin D1 protein (12,95,263). Since BAD abolished $E_2$ induced cell cycle progression, a logical question is whether BAD influences the cyclin D1 level in these cells. Cells were transfected and growth arrested as described in (A) and treated with $E_2$ or 5% serum for indicated

periods. Whole cell extracts were obtained and Western blot analyses were performed using the indicated antibodies. $E_2$ up-regulated cyclin D1 in control cells, but not in wtBAD over-expressing cells. Further, wtBAD over-expression completely prevented serum from inducing cyclin D. These results indicate that cell cycle regulation by BAD requires its phosphorylation at S75 and S99.

**(D)** Various BAD mutant constructs were used to further elaborate involvement of BAD phosphorylation sites and other domains. Single phosphorylation site mutants of BAD had variable effects, where as the dmBAD was completely incapable of inhibiting cyclin D response in $E_2$ induced cells. This data suggests the involvement of functional phosphorylation sites in BAD's action. Further strengthening this idea, a BH3 domain mutant of BAD, which cannot interact with other BCL2 family members, but carries intact S75 and S99 phosphorylation sites, had an inhibitory effect similar to the wild type protein.

**(E)** Asynchronous MCF-7 cells were transfected with siRNA for endogenous BAD. At indicated times, cells were harvested and total protein was used in Western blot analysis. siRNA treatment at 72 hrs inhibited endogenous BAD >75%. Marked increase in cyclin D1 protein expression was observed that correlated with the reduction in BAD expression. Data presented is a representative of two independent experiments with comparable results.

In **(C-D)** exogenous BAD expression is assayed by Western blot analysis using anti-Flag antibody, and as a protein loading control, levels of actin are shown. Western blots shown are representatives of at least three experiments performed, with comparable results.

**Figure 2.3.2**

**Cyclin D1 is a Transcriptional Target for BAD**

Reduced cyclin D1 protein levels in response to an excess amount of BAD could reflect the destabilization of the protein and/or negative regulation of its transcription and/or translation. Therefore, effects of BAD on cyclin D1 transcription and translation were assayed.

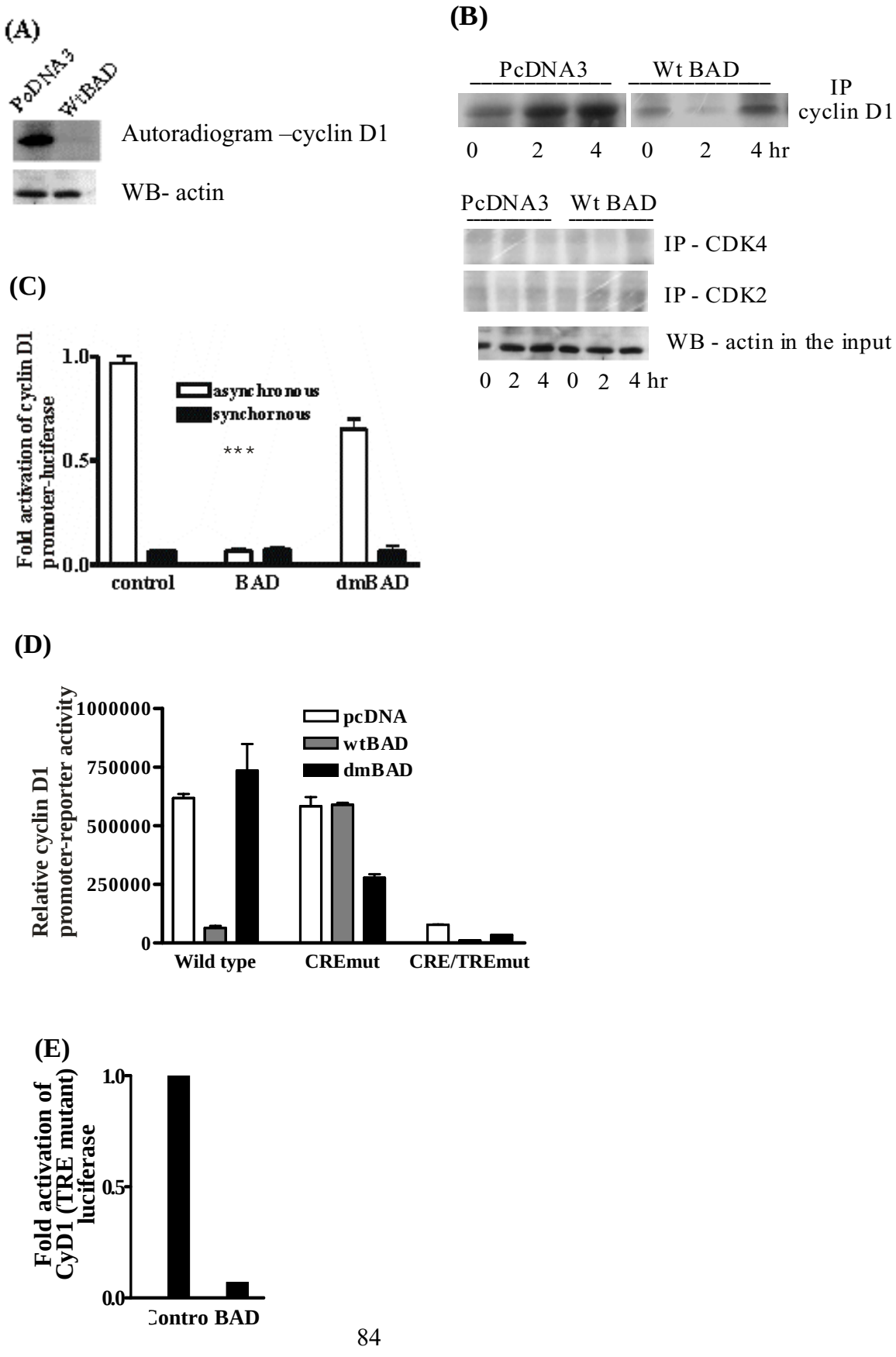
**(A -B)** Metabolic labeling of cultures with $^{35}$(S) labeled methionine was used to detect efficiency of cyclin D1 synthesis in asynchronous **(A)** as well as synchronous (G1 arrested) MCF-7 cultures in response to $E_2$ **(B)**. Cells were transfected and treated as indicated. Cyclin D1 immunoprecipitates of each sample were resolved in SDS-PAGE. Gels were dried and autoradiograms were obtained. Over-expression of wtBAD significantly reduced the basal cyclin D1 synthesis in asynchronous cells. $E_2$ enhanced the synthesis of cyclin D1 in growth-arrested cells in a time dependent manner. BAD completely abolished this effect at 2 hrs and partially at 4 hrs, demonstrating the ability to negatively regulate cyclin D1 production in response to a specific mitogenic stimuli as well as in complete growth medium. Neither BAD nor control vector expression influenced the newly synthesized CDK4 and CDK2 as observed with the immunoprecipitates of these proteins (B bottom panels). Amount of actin in the input is shown as a protein control for the immunoprecipitions. Autoradiograms shown are the representatives of at least three independent experiments with comparable results.

**(C)** A cyclin D1 promoter construct was used to demonstrate the involvement of BAD in cyclin D1 transcription control. Over-expression of BAD significantly reduced the promoter activity measured by the activity of the luciferase gene product (* $p<0.0001$); whereas expression of dmBAD had no negative effect on the cyclin D1 promoter. BAD induced inactivation of the cyclin D1 promoter is comparable to that was observed with synchrony of cells at G1 phase. Data presented is from three independent experiments with duplicates for each sample, with comparable results.

## Figure 2.3.2

**Cyclin D1 is a transcriptional target of BAD**

**(A)** Proliferating cultures of MCF-7 cells transfected with the indicated constructs were labeled with $^{35}$(S) methionine for 2 hrs. Total cyclin D1 was immnoprecipitated and newly synthesized cyclin D1 is shown in the radiogram. Bottom panel shows a Western blot of actin as the loading control. **(B)** MCF-7 cells expressing indicated constructs were growth arrested and induced with $E_2$ (10nM). $^{35}$(S) methionine was added simultaneously with $E_2$. Newly synthesized cyclin D1, CDK2 and CDK4 were detected by immunoprecipitation as described in (A). **(C)** Cyclin D1 promoter luciferase was co-expressed in MCF-7 cells with indicated constructs (1/3 of the total DNA content). Luciferase activity was assayed 36 hr post-transfection. **(D)** Cyclin D1 promoter mutants (CRE single and CRE/TRE double mutant) were used as in (C) (n=2). **(E)** TRE mutant of cyclin D1 promoter was used with or without BAD as in (C) (n=2).


(A)
PcDNA3
WtBAD
Autoradiogram –cyclin D1
WB- actin
(B)
PcDNA3
Wt BAD
IP
cyclin D1
0 2 4 0 2 4 hr
PcDNA3
Wt BAD
IP - CDK4
IP - CDK2
WB - actin in the input
0 2 4 0 2 4 hr
(C)
Fold activation of cyclin D1 promoter-luciferase
1.0
0.5
0.0
asynchronous
synchornous
***
control
BAD
dmBAD
(D)
Relative cyclin D1 promoter-reporter activity
1000000
750000
500000
250000
0
pcDNA
wtBAD
dmBAD
Wild type
CREmut
CRE/TREmut
(E)
Fold activation of CyD1 (TRE mutant) luciferase
1.0
0.5
0.0
Contro
BAD

**(D)** Mutant constructs of cyclin D1, CRE single mutant (CRE mut) and CRE and AP-1/TRE double mutant (CRE/TRE mut) preceding luciferase gene were expressed in MCF-7 cells with indicated BAD constructs or control plasmid. Relative luciferase activity 36 hr post trasnfection is shown in the graph. wtBAD failed to inactivate the cyclin D1 promoter when CRE is mutated, showing that BAD's influence is essentially mediated through the CRE rather than AP-1/TRE in this natural promoter. This observation agrees with the suggested critical role for CRE in cyclin D1 transcription in breast cancer cell.

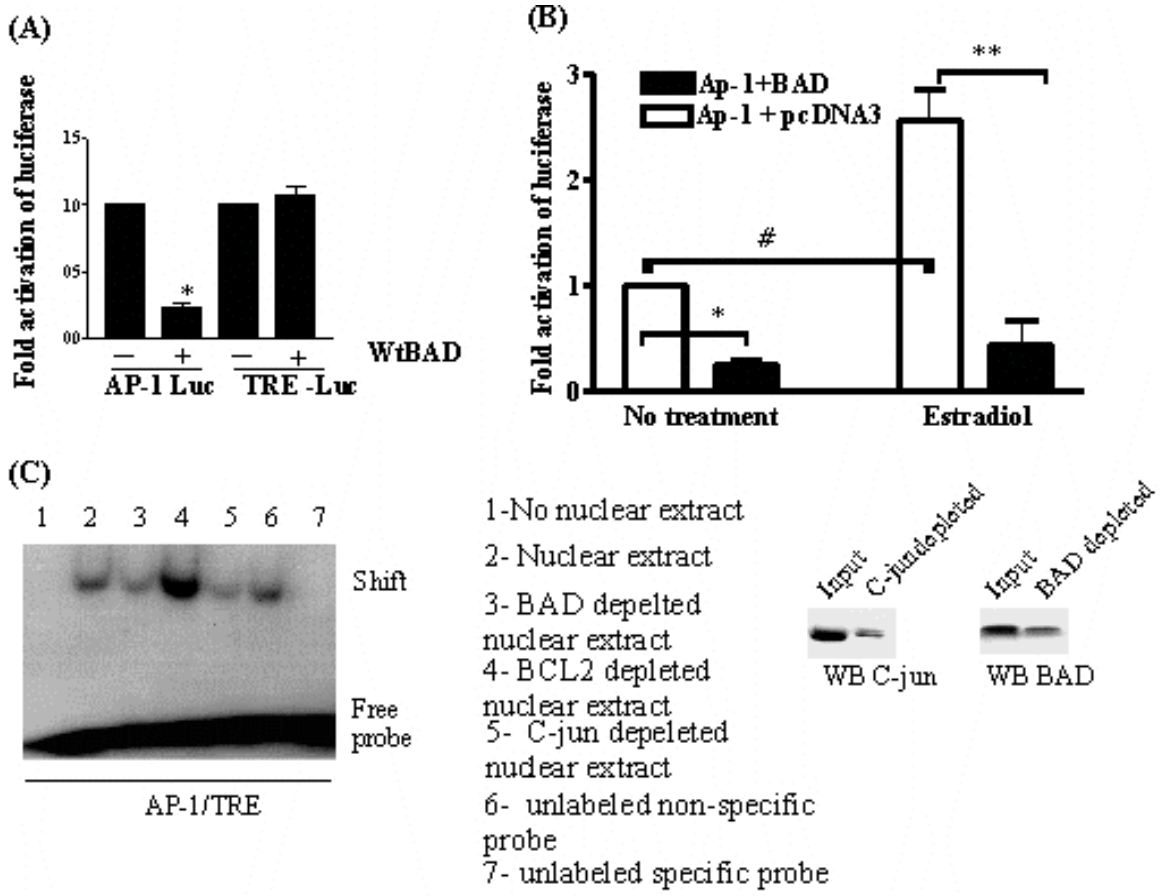
**(E)** A TRE mutant of cyclin D1 promoter (intact CRE) was used to further demonstrate the specificity of BAD's effect. wtBAD significantly inhibited the reporter activity showing supporting the data in (D). Data from two independent experiments with duplicated samples are shown in the graph.

**Figure 2.3.3**

**BAD targets AP-1/TRE Element through Association with AP-1 Proteins**

The evidence in Figure 2.3.1 argues that BAD can decrease synthesis of cyclin D1 as well as the activity of its promoter. The promoter of cyclin D is highly complex and is composed of various regulatory elements such as AP-1/TRE, SP-1 and CRE. Importance of AP-1/TRE and CRE for the cyclin D promoter activity in response to various agents has been shown previously (115). Data in Figure 2.3.2D-E shows the importance of these elements in the context of BAD regulatory effects. Influence of BAD on the activation of these elements was further determined.

**(A-B)** MCF-7 cells were transfected with AP-1/TRE luciferase with wtBAD or empty vector pcDNA3. In asynchronous **(A)** and synchronous (G1) **(B)** cells the basal and $E_2$ induced AP-1/TRE activation was measured. Over expression of BAD significantly (*$p < 0.05$) reduced the basal AP-1/TRE transactivation in asynchronous as well as synchronous cells. $E_2$ induced reporter activation (# $p < 0.005$) was significantly inhibited by the expression of BAD, but not the empty vector. Comparable data was obtained

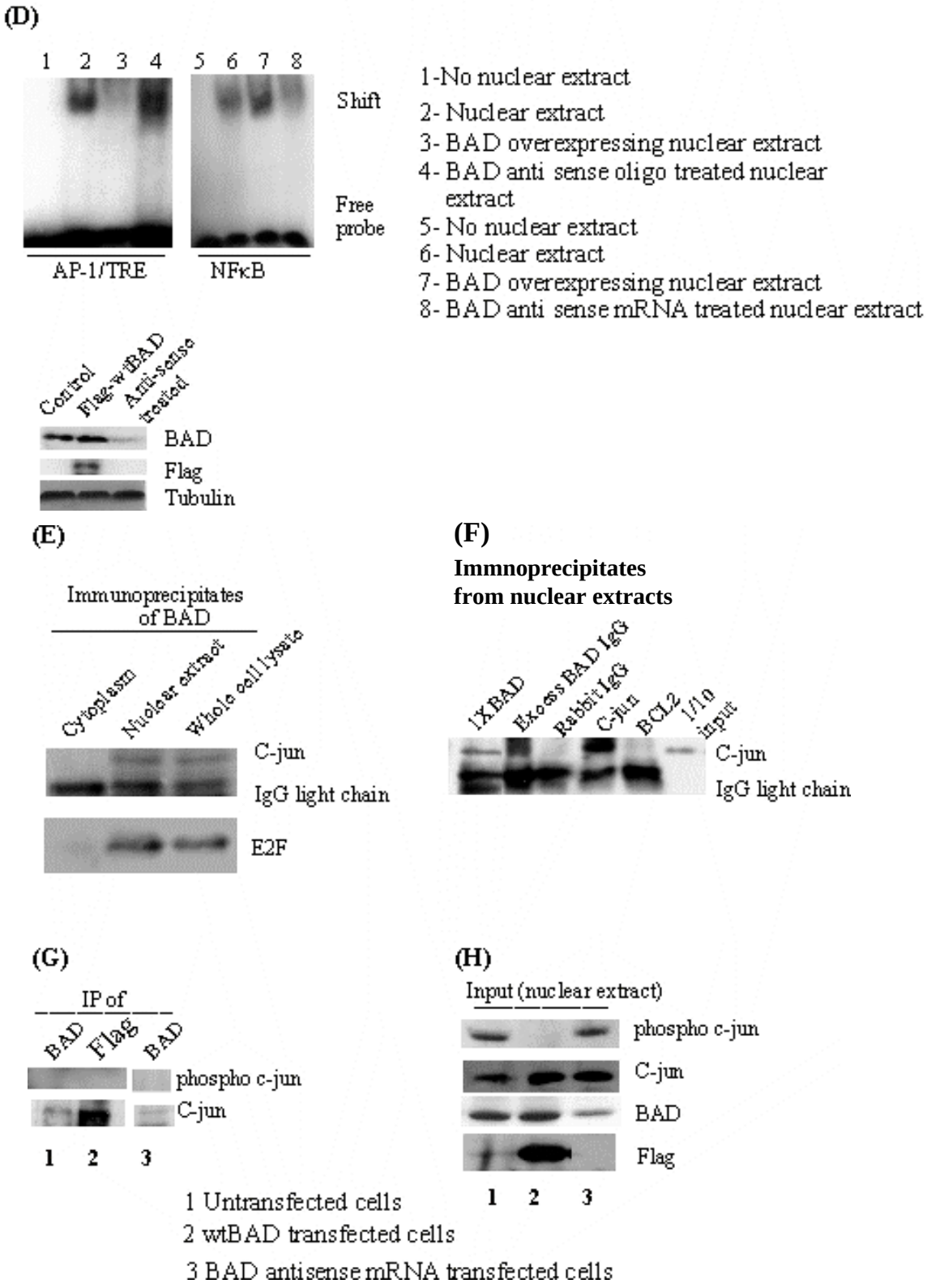



## Figure 2.3.3

**BAD targets AP-1/TRE element through association of the AP-1 proteins**

**(A-B)** MCF-7 cells were transfected with AP-1/TRE (control vector tetracycline response element) luciferase with wtBAD or empty vector pcDNA3. In asynchronous (A) and synchronous (B) cells the AP-1/TRE activation was measured. **(C)** EMSA assay with indicated nuclear extracts. Panel on the right shows the efficiency of c-jun and BAD depletions from the nuclear extract.

**(D)** EMSA assay with indicated nuclear extracts. Labeled NFκB sequence was used as a control probe. Bottom panel shows the expression of BAD in indicated nuclear extracts. **(E)** Western blot shows c-jun in immunoprecipitates of BAD from indicated MCF-7 extracts and E2F levles were determined by WB. **(F)** Western blot shows c-jun in immunoprecipitates of indicated proteins from MCF-7 nuclear extracts. **(G)** Immunoprecipitates of BAD or Flag from the indicated nuclear extracts were Western blotted for phosphorylated and unphosphorylated c-jun. **(H)** Western blot demonstrating expression of the indicated proteins in the nuclear extract input for (G)


(D)
1 2 3 4 5 6 7 8
Shift
Free probe
AP-1/TRE
NFκB
1-No nuclear extract
2- Nuclear extract
3- BAD overexpressing nuclear extract
4- BAD anti sense oligo treated nuclear extract
5- No nuclear extract
6- Nuclear extract
7- BAD overexpressing nuclear extract
8- BAD anti sense mRNA treated nuclear extract
Control
Flag-wtBAD
Anti-sense treated
BAD
Flag
Tubulin
(E)
Immunoprecipitates of BAD
Cytoplasm
Nuclear extract
Whole cell lysate
C-jun
IgG light chain
E2F
(F)
Immnoprecipitates from nuclear extracts
1XBAD
Excess BAD IgG
Rabbit IgG
C-jun
BCL2
1/10 input
C-jun
IgG light chain
(G)
IP of
BAD
Flag
BAD
phospho c-jun
C-jun
1 2 3
(H)
Input (nuclear extract)
phospho c-jun
C-jun
BAD
Flag
1 2 3
1 Untransfected cells
2 wtBAD transfected cells
3 BAD antisense mRNA transfected cells

using T47D breast cancer cells (data not shown). As a control, tetracycline response element luciferase (TRE) was used. BAD or pcDNA3 co-expression did not influence the activity of this reporter **(A)**, which does not carry an AP-1 element.

**(C)** To further assess the BAD influence on AP-1/TRE element, radio labeled element sequence (TGACTCA) was used as a probe in an electro mobility shift (EMSA) assay. Indicated nuclear extracts were incubated with the probe and change in nuclear factor binding was evaluated. Depletion of BAD from the nuclear extract (lane 3) reduced the nuclear extract and labeled probe binding to the same extent as did by c-jun depletion (lane 5). BCL2 depletion on the other hand, did not reduce the interaction between probe and nuclear factors. Western blot shows the efficiency of depletion of the indicated molecules from the nuclear extract.

**(D)** In the presence of exogenous BAD (lane 3) the nuclear factor and probe binding was significantly lower than that of control extract (lane 2). This effect was reversed when endogenous BAD was inhibited by BAD anti-sense mRNA (lane 4). Labeled sequence of NFκB was used as a control. Over-expression or diminution of BAD did not influence nuclear factors and NFκB binding. Western blot shows the expression of endogenous and exogenous BAD in the nuclear extracts.

Data from EMSA studies show that depletion of BAD from the nuclear extract or expression of exogenous BAD had inhibitory effects on AP-1/TRE element - nuclear factor binding. This could suggest that BAD interacts with AP-1/TRE element DNA binding factors in the nuclear extract and that when BAD is depleted from the latter, those that are bound to BAD are also removed. When in excess, BAD might compete with DNA binding by these factors and therefore decrease binding of nuclear factors to DNA. This is conceivable since a reduction of probe binding instead a supershift (which would indicate BAD/nuclear factor binding to DNA), which is observed in response to BAD over-expression (lane 3)

**(E-F)** Given that c-jun is one of the nuclear proteins that bind to the AP-1/TRE element we sought to determine whether BAD interacts with c-jun. Furthermore, c-jun has also been shown to participate in AP-1 dependent cyclin D transcription (9). Endogenous BAD complexed with c-jun in the nucleus but not in the cytoplasm of proliferating MCF-7 cells **(E)**. BCL2 did not complex with c-jun under this condition demonstrating the specificity of BAD and c-jun interaction **(F)**. E2F1 was measured as a nuclear marker.

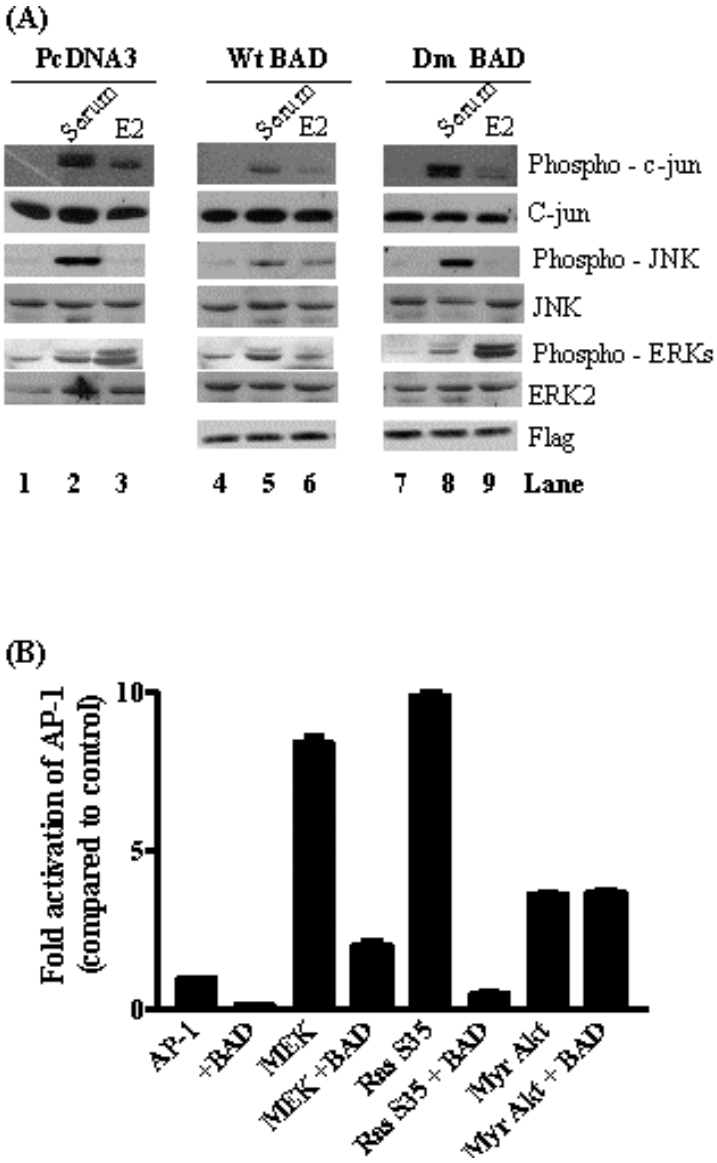
**(G)** Gene transactivation functions of c-jun are regulated by its phosphorylation status. We determined whether BAD and c-jun interaction depends on the phosphorylation of c-jun by measuring active c-jun (N-terminal phosphorylation at S63 and S73) levels in BAD immunoprecipitates. Unphosphorylated but not the phosphorylated form of c-jun was immunoprecipitated by endogenous as well as exogenous BAD in the nuclear extracts.

**(H)** Nuclear extract used in **(G)** was assayed for the expression of various proteins by Western blot. In BAD overexpressing cells, amount of c-jun phosphorylation is markedly low compared to control as well as BAD antisense treated cells. Of note, expression of total c-jun was not changed either by increasing or reducing the level of BAD in these cells.

**Figure 2.3.4**

**BAD Inhibits c-jun Phosphorylation via Signal Transduction Mechanisms**

**(A)** The data above suggests that BAD could prevent AP-1/TRE activation via its effects on c-jun. In normal conditions BAD appears to physically interact with un-phosphorylated c-jun in the nucleus (Figure 2.3.3.G). Since the over-expression of BAD completely abolished c-jun phosphorylation, we questioned, in addition to physical interaction, which is evident under normal conditions with endogenous BAD, whether exogenous BAD could prevent c-jun being phosphorylated by upstream kinases, thereby inhibiting its DNA binding. Activities of c-jun kinases were assayed by their




## Figure 2.3.4

**BAD inhibits c-jun phosphorylation via signal transduction mechanisms**
**(A)** MCF-7 transfected with indicated constructs were growth arrested and treated with $E_2$ or serum for 3 hrs. Western blot demonstrates the expression of indicated proteins in the whole cell extract.
**(B)** AP-1/TRE luciferase (1/4 of total DNA) was expressed in MCF-7 with indicated constructs. BAD or empty vector pcDNA3 was introduced at 1:1 ratio with the signaling intermediates.

phosphorylation status. In serum treated cells, active JNK induced c-jun phosphorylation, and this was inhibited by BAD over-expression (lanes 2 and 5).

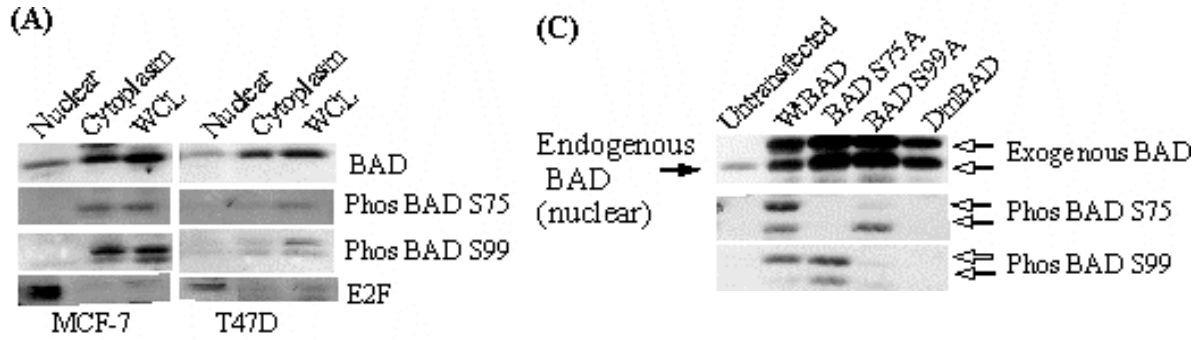
$E_2$ increased c-jun phosphorylation considerably through a mechanism, which appears to be JNK-independent. This correlated with the phosphorylation of ERK1 and 2, which was also inhibited by BAD overexpression (lanes 3 and 6). This suggests that c-jun activation in response to serum and $E_2$ are mediated by different mechanisms. BAD when overexpressed at least partly inhibit these mechanisms.
Expression of dmBAD did not have any effect on the signal molecules despite its comparable expression to wtBAD (lanes 7-9) suggesting an important role for phosphorylation in the signal transducing effects of BAD. The Western blot shown is a representative of more than three independent experiments with comparable results.

**(B)** To further clarify the effects of BAD on extra cellular signal activated protein kinases, activated constructs of signal intermediates were used. Expression of activated form of MEK1 and RasV12S35T mutant, both if which specifically activate ERK1 and 2 increased the AP-1 luciferase activity 8-10 fold above basal level. This effect is significantly reduced by the co-expression of BAD (at 1:1 ratio). The activity elicited by MyrAKT (constitutively activated) was not interfered by BAD, indicating a specific effect of BAD on the Ras/ERK signal pathway. Data from two independent experiments with two replicates per each treatment was used in the graph. Since our data shows that BAD can inhibit MEK1 mediated AP-1 activation, BAD might regulate ERK activation or downstream events which result in c-jun phosphorylation.

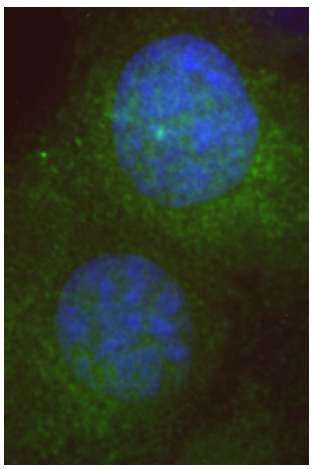
**Figure 2.3.5**

**Inhibitory Effects of Over-expressed BAD on AP-1/TRE Functions Depend on its Phosphorylation**

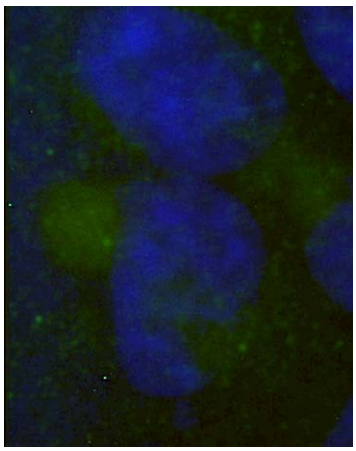
Cellular functions of BAD are mostly regulated by its level of phosphorylation i.e. hypo-phosphorylated BAD is apoptotically more active than the hyper-phosphorylated form. We wondered whether the nuclear effects of BAD would also depend on its phosphorylation, if so, to what extent. Phosphorylation site mutant BAD (dmBAD),



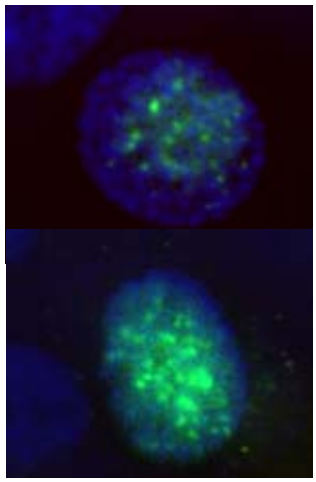

**(B)**



Endo.BAD -green
Nuclei – blue



Endo.phospho BAD
S112– green
Nuclei – blue



Endo.phospho BAD
S136 – green
Nuclei – blue

## Figure 2.3.5

**Inhibitory effects of over-expressed BAD on AP-1/TRE functions are dependent on its phosphorylation status**

**(A)** Extracts of proliferating MCF-7 and T47D cells were analyzed by Western blots to detect phosphorylated and total BAD.

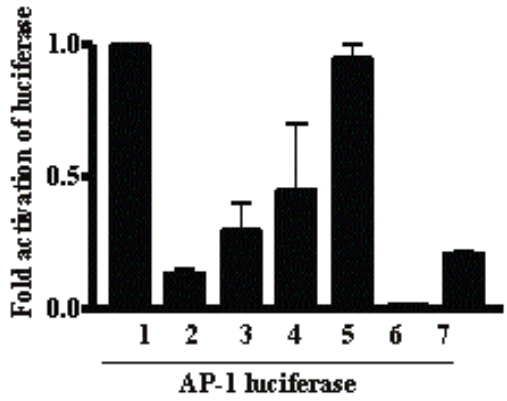
**(B)** Fluorescent images of immuno histochemically stained MCF-7 cells. Endogenous BAD (phosorylated and unphoshorylated) is shown in green and nuclei in blue. Images were merged to demonstrate BAD localization in the nucleus.

**(C)** Nuclear extracts of MCF-7 cells expressing various BAD mutants were analyzed by Western blot. Phosphorylation of BAD is detected using anti phospho BAD S75 and S99 antibodies.

**(D)** Indicated BAD mutants were co-expressed with AP-1/TRE luciferase reporter (1/4 of total DNA) in MCF-7 cells. Luciferase activity was assayed 36hr post transfection.

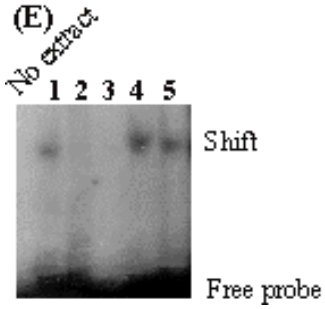
**(E)** EMSA with indicated nuclear extracts and labeled AP-1/TRE element.

(D)




1-pcDNA3.1
2-wtBAD
3-BAD S112A (serine 112 is mutated to alanine)
4-BAD S136A (serine 136 is mutated to alanine)
5-dmBAD (serine 112 and 136 are mutated to alanine)
6-BAD S128A (serine 128 is mutated to alanine)
7-BH3 domain deleted BAD

(E)




1-Nuclear extract from untransfected cells
2-BAD over expressing nuclear extract
3-BAD S112A over-expressing nuclear extract
4-BAD S136A over-expressing nuclear extract
5-dmBADover expressing nuclear extract

when expressed to the same level could not negatively regulate the cell cycle or the cyclin D1 protein level, as did the wtBAD (Figure 2.3.1A, C).

**(A)** Expression pattern of phosphorylated form of BAD was assayed in various cell compartments. In proliferating breast cancer cells (MCF-7 and T47D), endogenous BAD is present in nucleus and cytoplasm as shown by Western blots. However the level of phosphorylated forms in the nucleus is very low compared to cytoplasm. This suggests that the endogenous AP-1/TRE regulatory effects of BAD may not depend on its phosphorylation status. Similarly only the unphosphorylated endogenous BAD could complex with c-jun in the nucleus (Figure 2.3.3 F and data not shown). Data shown is a representative of three independent experiments with similar results.

**(B)** Immuno histochemistry technique was used to detect endogenous BAD in MCF-7 cells. Cells were incubated with BAD antibodies (total, phos BAD S75 and phos BAD S99) followed by fluorescent conjugated secondary antibodies (green). Nuclei were stained with Hoechst 33342 (blue). Images were merged to demonstrate localization of BAD in the nucleus. Images shown are the representatives of three or more experiments with comparable data.

Considering our observation that excess amount of wt BAD but not dmBAD plays a negative role on AP-1/TRE mediated cell cycle progression, it is reasonable to speculate that the inhibitory effect of BAD is phosphorylation dependent. If so, over-expressed BAD should be found in the nucleus.

**(C)** Various BAD mutants were expressed and nuclear extracts were assayed. Almost all the mutants expressed were present in the nucleus and they are phosphorylated on relevant residues in contrast to endogenous BAD. Note: Flag-BAD vectors give rise to two immunoreactive bands in Western blots. One at 23-25kD and the other around 30-35kD. The discrepancy between nuclear distributions of phosphorylated endogenous and exogenous BAD may relate to nuclear accumulation and dephosphorylation of these molecules in addition to other factors.

**(D-E)** Whether the dormant role of dmBAD in cell cycle regulation is a reflection of its effect on AP-1/TRE element was the next question we addressed. AP-1/TRE luciferase reporter assay (D) as well as the EMSA (E) clearly shows the neutral effects of dmBAD on this element. Graph in (D) was obtained from at least three independent experiments. Autoradiogram in (E) is a representative of two experiments with comparable results.

Collectively the data in this study show that (1) BAD has a regulatory role on AP-1/TRE mediated expression of cyclin D1. This effect is mediated by physical interaction with c-jun and possible sequestration from its site of action or from its activating kinases. It is very likely that, this effect of BAD at least partly contributes to the equilibrium state of AP-1/TRE functions, which are diverse and complex. (2) When present in excess, BAD appears to intervene with the c-jun phosphorylation (Figure 2.3.4A), in addition to physical interaction (Figure 2.3.3G). Both of these effects could contribute to the observed inhibitory effect. Interestingly the effects of exogenous BAD appear to depend on the functional phosphorylation sites.

# Chapter 3

# General Discussion

The study presented in this dissertation was designed to (1) dissect $E_2$ mediated signal mechanisms that are important for cell survival and, (2) analyze the significance of pro-apoptotic, BH3 domain only protein BAD in breast cancer cell survival and proliferation, in an *in vitro* breast cancer model.

## 3.1 Importance of BAD in $E_2$ Mediated Anti-Apoptosis

$E_2$ is known to support cell survival though paradoxical induction of apoptosis has also been described. $E_2$ induced apoptosis in rat embryo fibroblasts stably transfected with ERα (163) and MCF-7 cells transfected with Raf-1. In the latter the mechanisms were postulated to be mediated by ERβ and AP-1 transcription factors (89). Estrogen activated $p38^{MAPK}$ leading to apoptosis of ERα -stably transfected Hela cells (298). In addition, the response of neurons to estrogen depends on the ER subtype of the cells; ERβ undergo apoptosis, whereas cells with ERα are protected from apoptosis. Although the mechanisms underlying the effect of estrogen are not clear, apoptosis mediated by ERβ requires FasL (201). Decreased proliferation and increased apoptosis are associated with Jurkat T lymphocytes exposed to estrogen and decreased BCL2 proteins as well as mRNA levels were observed during this estrogen effect (133). Similarly, the thymic atrophy due to thymocyte apoptosis has been described with estrogen treatment (202). In bone tissue, estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts in an ER dependent manner (137). Furthermore, induction of apoptotic gene E9 was observed in estrogen-treated E8CASS (a MCF7 variant) cells and the apoptotic action of estrogen was suggested to be mediated by this molecule (266). MCF-7 cells that were long-term estrogen deprived underwent apoptosis in contrast to their survival response to estrogen under normal conditions (252) (discussed in 1.1.2 Introduction). Analogous findings have been reported in the prostate cancer cell line that is normally growth stimulated by androgenic compounds (135), and may constitute the common biologic response in hormone-responsive tissues. Cell death induced by

estrogen is not a common phenomenon and occurs in a few cell types in contrast to the survival benefits that are observed with many cell types.

Most of $E_2$'s actions on cell survival or death have been attributed to gene regulation. Emerging evidence for non-genomic actions of $E_2$ prompted us to examine the signaling mechanisms of $E_2$ with respect to regulation of cell survival in MCF-7 breast cancer cells. Our study shows that $E_2$ significantly reduces apoptosis induced by TNF$\alpha$, $H_2O_2$ and serum withdrawal utilizing signal transduction pathways that lead to phosphorylation and inactivation of pro-apoptotic protein BAD.

$E_2$ effects on BCL2 family members have been described previously: in cells where survival is promoted, $E_2$ increased mRNA (210) and protein levels of anti-apoptotic BCL2 (57,79) or down regulated pro-apoptotic BAK protein (166). In contrast to regulation of BCL2 and BAK at the transcriptional level, we find that $E_2$ rapidly induces phosphorylation of BAD at two serine residues (75 and 99) without altering BAD protein levels suggestive of activation of non-genomic signal pathways. Given the fact that pro-apoptotic effects of BAD are decreased by phosphorylation and consequent sequestration by 14-3-3 proteins (82), we determined whether $E_2$ induced phosphorylation of BAD is important for the anti-apoptotic functions of $E_2$.

Apoptotic stimuli used in this study increased cytochrome-C level in the cytoplasm and co-treatment with $E_2$ reduced this effect concomitant with improved cell survival. This is suggestive of the restoration of mitochondrial integrity at least to some degree in $E_2$-treated cells and may account for anti-apoptotic effects of $E_2$. In cells treated with TNF$\alpha$ and $H_2O_2$ the extent of BAD phosphorylation was lower than that observed in control cultures implicating the activation of BAD by these apoptotic stimuli. Accelerated dephosphorylation of BAD as well as the reduced phosphorylation as a result of deactivated survival signals can be presumed under these conditions. Estrogen treatment in this context restored phosphorylation to control levels. These experiments along with those demonstrating that $E_2$ prevents apoptosis in cells over-expressing wild type but not the double phosphorylation site mutant (S75A/S99A) of BAD suggest that BAD phosphorylation has a central role in apoptosis of MCF-7 cells. Remarkably the expression of BAD (S75A/S99A) did not cause apoptosis by itself, suggesting that in

addition to phospho-serine mediated cytosolic 14-3-3 binding there may be other mechanisms to sequester BAD away from mitochondria. These mechanisms effectively inactivating BAD are counteracted by apoptotic stimuli like TNFα.

Importance of BAD in MCF-7 cells was confirmed by using antisense oligonucleotides to suppress BAD protein translation and analyzing the effects of selected apoptotic agents on MCF-7 cell survival. Diminution of BAD in MCF-7 cells significantly reduced the extent of apoptosis induced by TNFα suggesting that BAD phosphorylation/inactivation has a central role in apoptosis of MCF-7 cells in response to stimuli like TNFα. Agents such as TNFα and $H_2O_2$ have been shown to exert mixed effects on BAD protein levels and phosphorylation. TNFα increased phosphorylation of BAD through PI3-K pathway in neutrophils (63), and in HeLa cells (208) where cell survival rather than the cell death was promoted by this agent. TNFα also induced the production of more potent truncated form of BAD in a caspase 3 dependent manner leading to apoptosis in Jurkat cells (59). Similarly $H_2O_2$ but not superoxide anion ($O_2^-$) has been shown to increase BAD protein levels in cardiomyocytes leading to apoptosis (281). Serum starvation may modulate activity of BAD and other apoptosis regulators and such effects may be reversed by addition of growth factors or by manipulating signal transduction pathways (23,295). Recent evidence from BAD knockout mice suggests that BAD may have an important pro-apoptotic role in cell types such as the mammary tissue. Importance of BAD was especially underscored in apoptosis that occurs in the absence of growth factors (222) much like the importance of BAD inactivation in the response to $E_2$ which is a well known growth factor to breast cancer cells. However it is also evident that BAD concentration or phosphorylation status could not be used to define survival or apoptosis in other cells such as PC12 cells (184).

**Estradiol Signals Through Two Interacting Pathways in its Anti-apoptotic Action**

In addition to genomic actions, $E_2$ has been known to exert rapid, likely nongenomic actions both in the whole animal and in cultured cells (126,226,265). Previously, we had observed that estrogens require the function of the Ras, mitogen-activated protein kinase kinase (MEK)1 and extracellular signal-regulated kinase (ERK) pathway to induce G1/S

phase transition (7) and elicit down-regulation of the Cdk2 inhibitor p27kip1 (93). Given that $E_2$ is now known to activate kinase-regulated signal transduction pathways in MCF7 and other cells (192) we have examined potential nongenomic pathways involved in its anti-apoptotic actions, in particular the PI-3K/Akt and ERK/RSK pathways, which mediate BAD inactivation and favors cell survival. The ability of dominant negative Akt or PTEN to reverse the protective effects of $E_2$ suggests an essential role for signal transduction through PI-3K/Akt in $E_2$ action in protecting cells from apoptosis. Akt is a known oncogene and is overexpressed in breast cancer cell lines when compared to untransformed breast epithelial cells, as well as in breast cancer specimens where the majority of cells are ER positive (261). Given the importance of PI-3K/Akt pathway to anti-apoptotic action of $E_2$ (194,211) we examined aspects of this pathway, which might influence cell survival. Our results clearly indicate that PI-3K/Akt has a critical role in $E_2$ action as a survival agent and a rapid and sustained activation of Akt in response to $E_2$ was found. Prolonged activity of Akt may be required to maintain BAD phosphorylation at S99 as well as to maintain GSK3β in the inactive state. Active GSK3β is required to stabilize cyclin D1 (77) and increased cyclin D1 protein is a characteristic response to $E_2$ in MCF-7 and other estrogen responsive cell (94 and refs there in). $E_2$ signaling has also been shown to closely associate with the PI-3K/Akt pathway in vascular endothelial cells (120) and ERα is phosphorylated by activation of the PI-3K/Akt pathway in MCF-7 cells in the absence of $E_2$ (43,259). These studies provide insights to growth factor induced signal mechanisms that couple and converge the genomic and non-genomic effects of ER.

In addition to Akt-mediated signaling, our data demonstrate that $E_2$ may also signal through Ras/ERK/$p90^{RSK}$ pathway since endogenous and exogenous BAD were effectively phosphorylated at S75, a well-known ERK/$p90^{RSK}$ phosphorylation site (82) in $E_2$-treated cells. Our studies with activating and inactivating Ras mutants provides strong evidence for this line of argument and show that BAD S75 is a target of Ras-mediated MEK/ERK activity. We could directly demonstrate that $E_2$ is able to activate $p90^{RSK1}$ a downstream target of ERK1 and 2 (31,300). Although the ability of $E_2$ to activate ERK remains controversial (47,176,251), ERK activation seems to be important

in the apoptotic response (90,241) perhaps through the regulation of BAD phosphorylation via $p90^{RSK}$. The prolonged activation of ERK in contrast to transient activation reported by others (192) may be important to maintain the phosphorylation and inactivation of BAD, acting in concert with Akt. It is also possible that BAD can be phosphorylated by kinases other than Akt and $p90^{RSK}$ and that such phosphorylations may enhance or diminish the pro-apoptotic capacity of BAD (80,111,150,175). The two signaling pathways that are described here may function as separate entities or interact in order to mediate $E_2$ effects. Previous studies have shown that Ras/ERK and PI-3K/Akt exhibit regulatory cross-talk (240,286,297). Regulation of ERK by $E_2$ through the PI-3K/Akt pathway has not been previously reported to our knowledge. Using both biological and chemical inhibitors we found that PI-3K may be required not only for $E_2$, but also, for IGF-1 induced ERK activation as well as BAD phosphorylation at S75, a likely target of the ERK/$p90^{RSK}$ pathway. Therefore it is likely that $E_2$ regulates ERK activation, in part, through PI-3K pathway and our data suggest that $E_2$ regulates a step upstream of PI-3K, possibly Ras function (230).

Expression of dominant negative Ras (RasN17) on the other hand interfered with $E_2$-induced BAD phosphorylation on both serine residues without affecting the basal BAD phosphorylation or protein levels supporting the notion that $E_2$ regulates PI-3K through Ras (145). However we cannot exclude the possibility that $E_2$ may regulate another kinase, which can phosphorylate BAD on S99. The ability of RasN17 to induce apoptosis in proliferating cells suggests that Ras plays a critical role in preventing apoptosis in MCF-7 cells as observed in other cells (289). Since 3'4'5' or 3'4'PI phosphates regulate a variety of signal pathways such as Akt and PDK that could lead to modulation of Ras/Raf/ERK pathway (221), non genomic signaling by $E_2$ may be quite complex (168). Furthermore, activation of PI-3K by $E_2$ through a direct interaction of the p85 subunit with ERα was reported in human vascular endothelial cells (243) and MCF-7 cells (261). Clearly further studies are needed to delineate signaling mechanisms and their interactions in the perspective of estradiol's anti-apoptotic and cell cycle effects.

The non-genomic signaling of $E_2$ is postulated to be mediated by membrane associated ER (141) and this hypothesis is further supported by the recent demonstrations

of membrane located ERα in MCF-7 and other cell types (185,216). According to these authors the fraction of plasma membrane bound ERα ranged from 3-20% of the total in MCF-7 cells. Agreeing with this notion our data shows that $E_2$ induced rapid ERK phosphorylation and the protective effects against apoptotic agents was substantially blocked by the anti-estrogen ICI 182,780.

## 3.2 BAD Has a Role in Cell Cycle Regulation

Above findings clearly define a role for BAD in $E_2$ mediated anti-apoptosis in breast cancer cells. To extend our knowledge on BAD regulated cell survival and function to another level, we assayed the transcriptional activities of BAD in the context of cell cycle progression as our data indicated the presence of BAD in the nucleus.

Cell proliferation and apoptosis are two major pathways that are important for the growth and development of a multi-cellular organism. The natural interdependency of these two key mechanisms keeps the tissue mass to a balance i.e. increased proliferation consequently triggers cell death through apoptosis and other programmed cell death process, where as reduced cell death is of no significance in the absence of enhanced proliferation. Just as cell death shapes tissue remodeling or development, cell death also maintains the course and nature of the immune response. Lymphocytes mediated, immune-directed programmed cell death provides a mechanism that is the basis for self-versus non-self discrimination and potentially the immnuorecognition and removal of damaged or cancerous cells (discussed in 1.1.1 Introduction).

Multitasking proteins such as Ras, Myc and c-Jun have been shown to participate in both cell proliferation and cell death and, our data strongly indicate a novel role for BAD in cell cycle control.

**The concentration of BAD modulates cell cycle progression**

Under normal conditions, BAD is phosphorylated on S75 and S99 and bound by the 14-3-3 like molecules a measure to reduce the sensitivity to apoptotic signals (71). However, there is a substantial amount of unphosphorylated BAD in a normal cell and the cytoplasm appears to be the major location for both forms. Known pro-apoptotic

functions of BAD mostly occur in the cytosol or the mitochondria. Localization of endogenous BAD in breast cancer cell nuclei strongly suggests an existence of a currently unknown reserve of this molecule. When present in an excess amount, BAD prevented cell cycle progression in response to growth factors and $E_2$, where as, BAD knockdown enhanced the cell cycle entry in a time dependent manner. A negative regulation on cell cycle by BAD is plausible in an unstimulated cell, since the reduction of BAD improves the progression from G1 to S. The observation that was made with BAD over-expression could then be an exaggerated or magnified effect of this normal endogenous effect of the molecule.

Similar phenomenon has been observed with BCL2 and BCLxl in malignant as well as normal epithelial cells. When overexpressed, BCL2 arrests cells in G0/G1 phase and this effect depends on the amino terminal BH4 domain (121). Interestingly in a variety of epithelial cell types, endogenous BCL2 was shown to localize to the chromosomes of mitotic nuclei (180). This pattern of BCL2 expression can be considered as an indicative of its special role in cell proliferation. Mutation of the conserved tyrosine residue within the BH4 domain of BCL2 and the equivalent residue Y22 in BCLxl eliminated their ability to delay S phase entry with out affecting the ability to inhibit apoptosis (125), leading to the conclusion that anti-apoptosis functions of these proteins can be genetically separated from their cell cycle function. The cell cycle delay effect of BCL2 and BCLxl has been measured as lengthened time to reach S phase from quiescence, which results from enhanced G0 arrest or delayed transit through G0-G1 (132). The reduction in S/G2 fraction concomitant with increased G1/G0 fraction in BAD overexpresing, asynchronously growing cells suggests the retardation of the passage from the latter. MCF-7 cells that are synchronized in G1 by anti-estrogen and low serum readily progress into S phase upon mitogenic stimuli (94) and this response is also reduced due to excess amounts of BAD. Studies are underway to clearly identify the exact location of the cell cycle arrest due to BAD under these conditions. Extending our analysis to normal mammary epithelial cells, we should be able to evaluate the physiological relevance of BAD's effect on the cell cycle.

**Cyclin D1 regulation by BAD through AP-1 proteins and their binding elements**

Regulation of cell cycle in ER+ breast cancer cell line MCF-7 has been extensively studied and the information on mitogenic effects of $E_2$ and other agents on these and other breast cancer cells are abundant (7,94,95). Cyclin D1 plays an important role at the G1 to S transition upon a mitogenic signal (94) and has been implicated as a diagnostic/prognostic marker of breast cancer. Transcription of the cyclin D1 gene and the stability of the gene product are tightly regulated hence the function of the molecule. Members of the AP-1 transcription factor family, particularly c-Jun has been shown to be involved in the transcription control of cyclin D1 (discussed in Chapter 2.1). c-Jun induces mRNA and protein levels of cyclin D1 in response to proliferative signals, which correlates with the progression of cell cycle. However, cell cycle defects in c-Jun-/- cells were only partially (30%) relieved by the overexpression of cyclin D1 suggesting that cyclin D1 is not the only target of c-Jun in this context.

An increased expression of cyclin D1 protein was observed in the BAD knockdown conditions, which correlates with the increased G1 to S transition. An inhibitory effect on the cell cycle by BAD or its down stream target/s could have been revoked by the decrease in BAD, resulting in this proliferative effect. This hypothesis is further supported by the overexpression studies where an increased amount of BAD abolished the cyclin D1 level in response to mitogens. De novo synthesis of cyclin D1 was markedly reduced under excess BAD expression, which is likely to contribute to the above observation. However, BAD's influence on the stability of the cyclin D1 protein cannot be ruled out.

The signal axis of c-Jun activation to cyclin D1 expression in MCF-7 cells appears to be obliterated by BAD as phosphorylation dependent activation of c-Jun and the upstream kinases were significantly reduced by its overexpression. BAD's influence on signaling pathways have not been described to date and require further studies. The activity change without a change in the expression levels strongly suggests a non-genomic action on these signal molecules by the excessive amount of BAD, presumably accelerated dephosphorylation. As per our hypothesis that the effects of exogenous BAD

are the exaggerated endogenous effects, the site of this action of BAD needs to be identified.

BAD's influence on c-Jun appears to be two fold since in addition to inhibiting its phosphorylation, BAD binds to the unphosphorylated or transcriptionally inactive c-jun in the nucleus. Additional to the known regulatory mechanisms that govern the level of phospho c-jun available for gene transactivation, BAD may function as a sequestering entity for c-jun thereby preventing it being phosphorylated by activating kinases, under normal conditions. The significance of this interaction can be postulated by the extent of BAD bound c-jun in the nucleus, which is approximately 10-15%.

Regulation of cyclin D1 in response to $E_2$ is mediated through AP-1 binding DNA elements such as TRE and CRE (Chapter 2.1 Introduction). Our studies exploring the transcriptional and translational control of cyclin D1 in BAD over-expressing cells show that BAD indeed negatively regulates the de novo synthesis of cyclin D1and the cyclin D1 promoter activation. Assay of cyclin D1 promoter –reporter constructs where AP-1 protein binding sites have been mutated (structure of cyclin D1 promoter, Figure 2.1.2.1B) shows that BAD's influence is essentially mediated through the CRE rather than AP-1/TRE in this natural promoter, an observation that agrees with the suggested critical role for CRE in cyclin D1 transcription in breast cancer cell (115).

Using minimal AP-1 binding DNA sequences in electro mobility shift (EMSA) and reporter assays, we further evaluated the inhibitory effects of BAD on AP-1 protein and DNA interaction. Level of BAD in the cell appears to influence the efficiency of this interaction in MCF-7 cells. When present in an excessive amounts BAD significantly reduces this interaction to an extent that is comparable to the dominant negative c-Jun. An increased protein-DNA interaction was observed under BAD knockdown condition in vivo and in vitro. Further, BAD over-expression markedly reduced the TRE-luciferase activity, which is dependent on the direct interaction of AP-1 nuclear factors and TRE sequence, in asynchronous and $E_2$ induced cells. Availability of active AP-1 transcription factors is critical to ensure proper DNA binding in above conditions and inhibition or unavailability of these factors can be presumed, to result in decreased DNA biding when BAD is present. This data clearly suggest a regulatory role for BAD, upstream to AP-1

protein and DNA interaction, an effect that is physiologically manifested by the cell cycle blockade.

**BAD Effects on Cell Cycle regulation depend on its phosphorylation**

It is well established that, when phosphorylated on two serine residues (S75 and S99) BAD is inactive in respective to its pro-apoptotic function (Chapter 1.1.3 Introduction). Agreeably, our data demonstrate the dephosphorylation-mediated activation of BAD by apoptotic agents and the restoration of BAD phosphorylation during the $E_2$ induced anti-apoptosis. Further, BAD dephosphorylation (achieved by knocking in the unphosphorylatable mutant of BAD) sensitized mice and mouse embryonic fibroblasts to apoptosis induced by death receptor signaling and DNA-damaging agents (71). Together, all above information suggest that BAD phosphorylation and dephosphorylation specifically regulate the response to apoptotic stimuli that involve mitochondrial dysfunction and, phosphorylated BAD is a critical mediator of survival signals in cells. Our data also suggest the critical involvement of BAD phosphorylation in its cell cycle regulatory function. Analogous to this, mono-site or multi-site phosphorylation of BCL2 in the flexible loop region enhanced its anti-apoptotic effect and retarded the cell cycle progression (74). This observation refutes the previous notion that the anti-apoptotic and cell cycle effects of BCL2 are functionally separable (121), and links these two functions through phosphorylation. Similarly, in the case of BAD, pro-apoptotic and cell cycle regulatory effects appear to be linked by phosphorylation.

In the context of nuclear/transcriptional effect, the phosphorylated or 'apoptotically inactive BAD' is notably important. The cell cycle block subsequent to the regulatory effects on AP-1 functions, observed with wild type BAD were not readily visible with the dual phosphorylation site mutant of BAD in which S75 and S99 are mutated to unphosphorylatable alanine despite the satisfactory expression level of this molecule. In contrast, BH3 domain mutant, which retains the intact S75 and S99 was as effective as the wild type BAD. A step subsequent to BAD phosphorylation is likely to mediate another AP-1 regulatory effect in addition to BAD mediated binding and sequestration of c-Jun in the nucleus. Inactivation of c-Jun activating kinases by the

overexpressed, phosphorylatable wild type BAD but not the phosphorylation site mutant supports the supposition that BAD regulates these signal transduction pathways in a phosphorylation dependent manner.

At least 50% of the cells that overexpressed wild type BAD were able to counter apoptotic stimuli when $E_2$ was present and these $E_2$ induced survival effects are partly mediated by Ras/ ERK signaling pathway. On the other hand, accumulation of BAD (through transient overexpression) in the cells over a period of time (> 72 hrs) was important to render cells unresponsive to mitogenic signals of $E_2$, that are also partly mediated by ERK mediated signaling mechanisms.

## 3.3 Summary

This study demonstrates that $E_2$ inhibits apoptosis in MCF-7 cells by enhanced phosphorylation of BAD, and indicates that BAD inactivation results from activation of Ras/ERK/$p90^{RSK}$ and Ras/PI-3K/Akt pathways by $E_2$. Data also indicate that BAD plays an essential role in apoptosis induced by $H_2O_2$, TNF$\alpha$ and serum withdrawal. Interestingly, data also indicate that even the double phosphorylation site mutant of BAD (S75/99A) is inactive as an inducer of apoptosis in the absence of an apoptotic stimulus (Figure 3.1), an observation that agrees with the BAD knockout study (71) where the knocked in BAD S75/99A mutant did not induce death in mouse or MEFs rather sensitized them to apoptotic agents like TNF$\alpha$ and growth factor withdrawal.

BAD also plays a decisive role when breast cancer cells have to respond to mitogenic signals. Its negative influence on AP-1 mediated cyclin D1 expression may play an important role to maintain the correct level of cell cycle progression under normal conditions. For a successful mitogenic response to occur, BAD's negative regulation needs to be decreased, possibly by dissociating BAD/c-Jun interactions that may be found proximal to chromatin (Figure 3.2). When mitogen fails to counteract the enhanced negative regulation of cell cycle by elevated levels of BAD, cells may submit to reduce proliferation and subsequently programmed cell death. The interplay of above discussed factors can be summarized by the scheme shown in Figure 3.3.

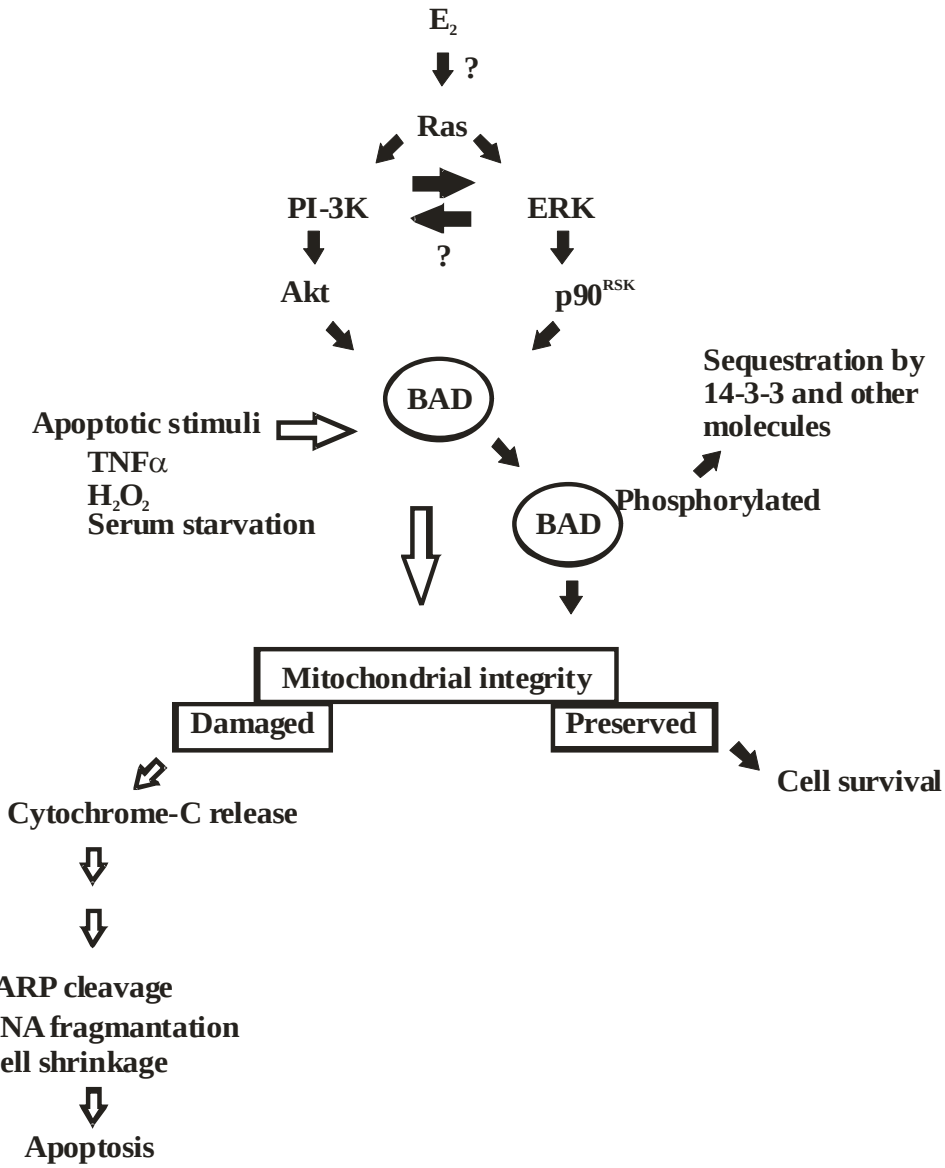



**Figure 3.1**

**Mechanisms that mediate $E_2$ induced anti-apoptosis**

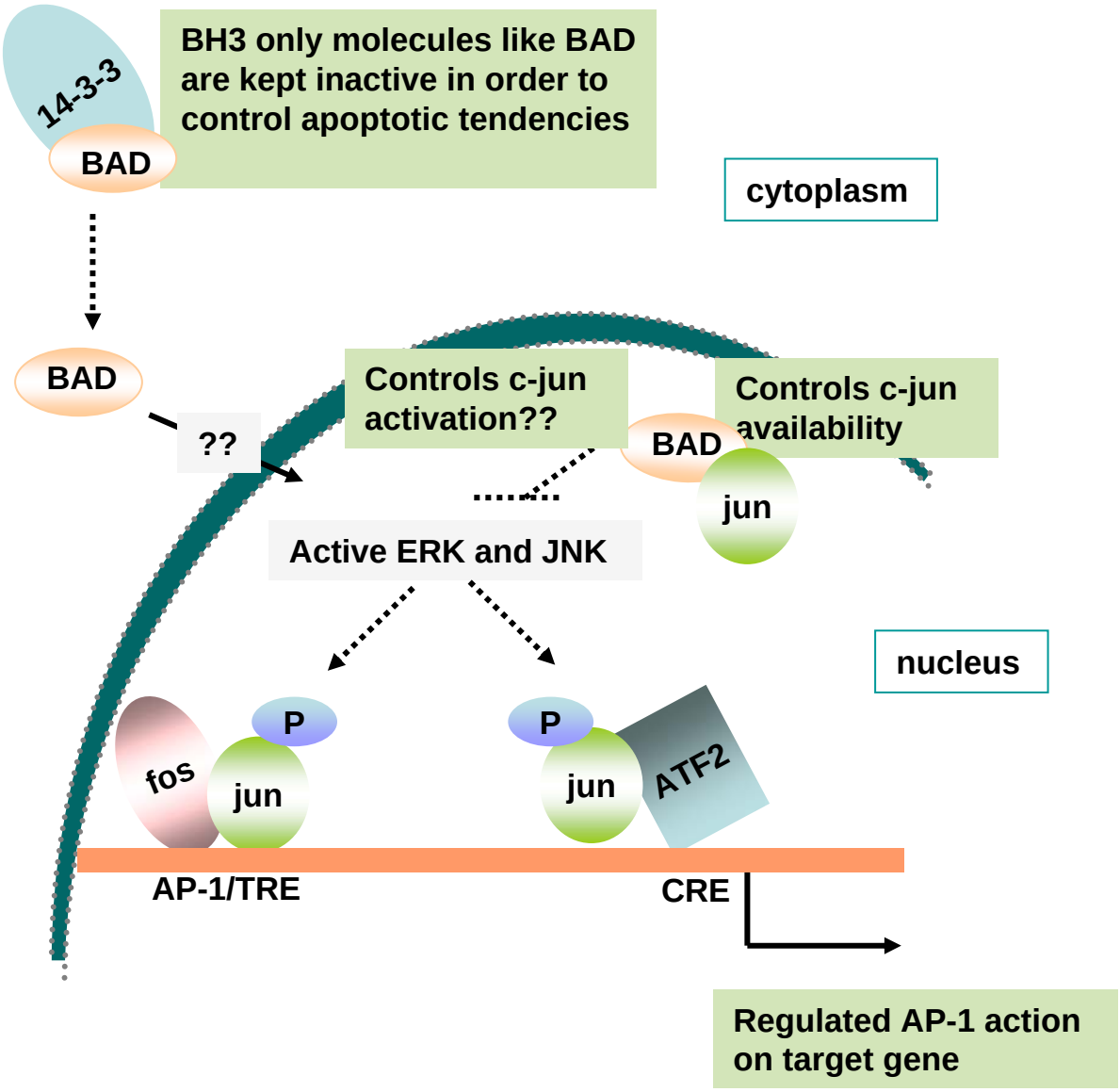



**Figure 3.2**
**Proposed regulatory role for BAD in AP-1 transcriptional functions**

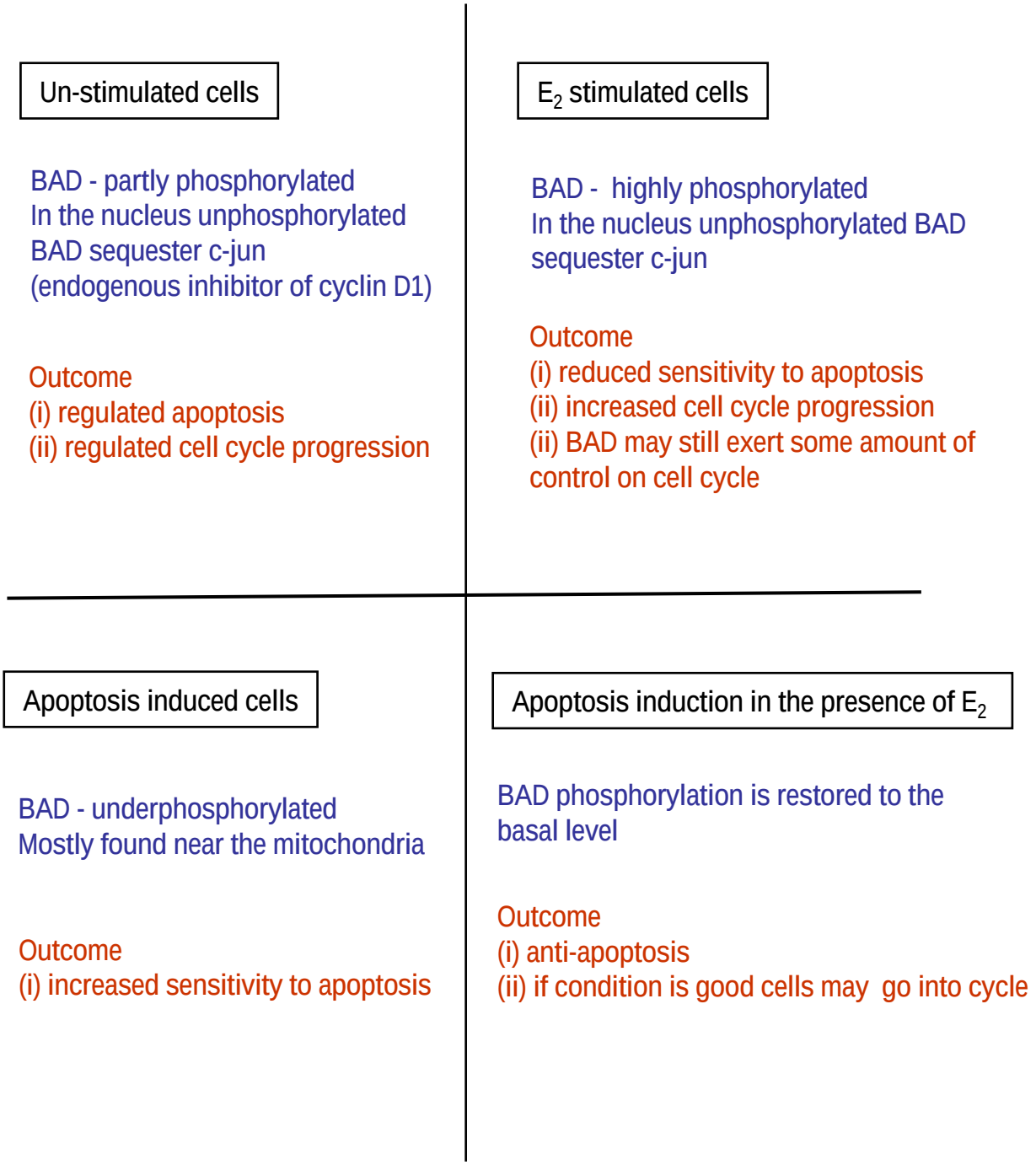



**Figure 3.3**

**Proposed outcomes due to $E_2$ and BAD interaction in MCF-7 cells**

## 3.4 Prospects

### Identification of genomic targets for BAD

Effects of exogenous BAD on AP-1 binding elements in cyclin D1 natural promoter warrant further investigation of genomic targets of BAD. This can be approached in several different ways.

(1) DNA sequences that bind with BAD (directly or indirectly) can be isolated by the Chromatin immuno precipitation (ChIP) coupled with BAD specific antibody. In this method protein/protein and protein/DNA are reversibly cross-linked and immunoprecipitated with BAD specific antibody. The resulting complex can be used to detect interacting proteins as well as nucleic acid sequences.

(2) Gene targets that are up or down regulated due to BAD overexpression can be analyzed by Gene array technique. The arrays tailored for specific pathways are commercially available.

### Significance of BAD phosphorylation in cell cycle regulation

Failure of the unphosphorylatable BAD mutant to suppress cell cycle progression calls for further verification of the role of these phosphorylation sites. Phospho-mimicking (serine sites mutated to glutamate or aspartate) mutation to BAD can be used to address this issue. If the cell cycle suppression and the up stream effects on c-Jun and cyclin D1 can be restored by these mutations, a precise role of BAD phosphorylation can be defined.

### Significance of BAD and c-Jun interaction in cyclin D1 expression

BAD and c-Jun interaction at the nuclear level was demonstrated by immunoprecipitation assays. Similarly, BAD depletion from nuclear extract reduced the AP-1/TRE and nuclear factor interaction to an extent that is comparable to that of dominant negative c-Jun. Sequestration of c-Jun by BAD can be deduced from these observations and can be further verified by mammalian two-hybrid technique, where BAD and c-Jun can be cloned into bait and target vectors respectively. In addition to c-Jun, other presumed AP-1 molecules can be used as targets in this technique.

Further, mutant forms of either molecule can be used to define the critical domains or residues that are necessary the interactions.

**BAD influence on signal mechanisms**

Overexpression of wild type BAD abolished ERK and JNK signaling in response to 2-3 hr treatment of $E_2$ and serum respectively. We also found that the ERK response for $E_2$ is bi-phasic (transient/rapid and sustained) and the rapid phase signal is presumed to inactivate BAD's apoptotic effects whereas the sustained signal may be required for the mitogenic effect. Our data suggest a role for BAD as a regulator for the second phase of $E_2$ induced ERK signaling, that is reflected by its effects on cell cycle progression. Detailed kinetics of $E_2$ induced ERK signaling under the conditions of BAD over-expression and knockdown is desired.

Accelerated dephosphorylation of these molecules by BAD is likely and, the activities of phosphatases need to be assayed. Within the cells, the interacting signal molecules are mostly found in clusters (enzyme and substrate docking via special domains) and BAD's role in modulating these interactions can be analyzed.

**Generation of cell lines that express BAD (wild type and mutants) in a regulated manner**

Cells that stably express the desired molecule are more advantageous than transient expression. When a stable expression is under a regulated promoter (e.g. tetracycline responsive) the molecule can be expressed at desired levels and the effects can be analyzed. Cell lines that express BAD (wild type and mutants) in a regulated manner can be very useful to carry out various experiments that have been suggested above.

# Chapter 4

# Materials and Methods

## 4.1 Cell Culture

MCF-7 cells (a gift from R. P. Shiu (84)) were cultured in Dulbecco's modified Eagles's medium (Sigma St. Louis, MO) supplemented with 5% fetal bovine serum (FBS), penicillin G and streptomycin at 37° C in 5% $CO_2$.

T47D (ATTC) were grown in DMEM/F12 (1:1) supplemented with 5% fetal serum, penicillin G and streptomycin at 37° C in 5% $CO_2$.

### Growth arrest

**Complete serum/growth factor withdrawal**

Cells (MCF-7) were kept in 1% FBS for 24 hrs in DMEM (phenol red [PR] free) medium and completely serum withdrawn for additional 36-48 hrs before the experiments.

**Using anti estrogen and low serum**

Cells (MCF-7 and T47D) were kept in 10nM ICI182,780 and 0.2% FBS for 48 hrs. Both these methods arrested cells at G1 (at least 80% of the population was found to be in G1 as measured by DNA profiling using flow cytometry).

### Treating growth arrested cells

Prior to treatments, cells were washed once with warm PBS and treated in medium free of serum and PR.

LY294002 (5μM in MTT assay, 20μM in other assays), PD98059 (50μM) [Sigma] and ICI182, 780 (1μM) [gift from Zeneca Pharmaceuticals], were used alone or added 1 hr prior to other growth factors and $E_2$.

$E_2$ was used at 1nM in MTT assay and 10nM in other assays.

IGF-1 was used at 10ng/ml. Cells were treated with DMSO or EtOH as vehicle controls.

## 4.2 Transient Expression of Desired Molecules with Adenoviral and Plasmid Vectors

### Adeno viruses

PTEN and DnAkt [kind gifts from Drs. R Bookstein, (53) and K Walsh (157) respectively] and control vector LacZ [Q.Biogene - Carlsbad, CA] were transduced at 60, 30 and 30 MOI respectively. Optimum MOIs were determined by analyzing DNA profiles (by flow cytometry) in response to virus dosage. Cells at 20-30% confluence were infected in complete growth medium for 2 hrs. After the infection, cells were recovered for 3-6 hrs in growth medium; growth arrested and treated as desired. To study DNA fragmentation with TNF$\alpha$ or $H_2O_2$, cells were maintained in DMEM (PR free) with 2.5% FBS for 48 hrs after infection followed by induction of apoptosis.

### Plasmids

Cells were transfected with various plasmid vectors using Lipofectamine Plus$^{TM}$ reagent according to the manufacture's [Invitrogen, Carlsbad, CA] protocol. Fifty to 80% transfection efficiency was obtained as determined by the expression levels of EGFP (microscopic and flow cytometry analysis).

Following is a list of plasmids used in this study.

Flag tagged human BAD wild type (wt) and human BAD serine75 and serine99 (equivalent to mouse serine112 and serine136 respectively) mutated to alanine were kindly donated by Dr. HG Wang (119)

GFP tagged human wtBAD

RasN17, RasV12T35S (kindly donated by Drs. G L Johnson and C M Counter (110) respectively)

AP-1 luciferase (kindly donated by Dr. Ben Dibling

Tetracycline response element (TRE) luciferase (ClonTech)

Human c-jun (kindly donated by Dr. Anders Strom)

Myristylated AKT (kindly donated by Dr. Antonio Cuadrado)

Cyclin D1 promoter luciferase constructs (kind gift from Dr. Pestell)

v-jun (Kindly donated by Dr. Vogt)

## 4.3 Endogenous BAD Knockdown

### Using BAD anti-sense oligonucleotides

BAD antisense oligonucleotides with phosphorothioate linkages were synthesized and purified by Sigma-Genosys, Woodlands, TX. Cells were transfected twice (48 hrs apart) with Lipofectamine Plus $^{TM}$ or RNAifect reagent according to the manufacture's [Invitrogen, Carlsbad, CA] protocol. Transfections were carried out in 6 well plates using 1–3 μg of oligonulceotide per well. Antisense oligonucleotides used in this study encoded complementary sequences to the coding regions including translation initiation (BAD antisense 1) and termination codon (BAD antisense 2) sites for the human BAD gene. Oligonucleotide sequences used were BAD antisense 1; 5'-TGGGATCTGTAACATGCT-3', BAD antisense1 (scrambled/control); 5'-GGTTAGGTCAATTACTCG-3', BAD antisense 2; 5'- CGAAGGTCACTGGGA-3'.

### Using BAD siRNA

For the RNA interference siRNA for human BAD was purchased from Cell Signal Technology (Cat# 6471). Transfection was done at the final concentration of 33nM of siRNA with RNAifect according to the manufacturer's protocol.

## 4.4 Cell Survival Assay using MTT

3-4,5-dimethylthiozol-2, 5-diphenyl-tetrazolium bromide (MTT) was used to measure the amount of functional mitochondria hence the amount of live cells.

At 20-30% confluence, cells were treated in triplicates in 24 well plates. At 24 hr intervals, MTT assay was performed according to the manufacture's [Sigma] protocol. Briefly MTT (5mg/ml) was added to equal one-tenth of the culture volume, and followed by incubation for 3 hrs. Medium was removed and the converted dye was solubilized in ice-cold isopropanol. Absorbency of dye was measured at 560nm with background subtraction at 630nm on a micro plate reader [EL 340 Bio Kinetics Reader- BIO-TEK® instruments].

## 4.5 Induction and Assessment of Apoptosis

(1) Apoptosis was induced with $H_2O_2$ (2mM) for 1.5 hrs, TNFα (10ng/ml) for 8 hrs or by complete serum withdrawal for 72 hrs, or with indicated constructs (where appropriate). Cells were pretreated with $E_2$ or IGF-1 for 1 hr prior to apoptotic stimuli.

ELISA assay kit [Cell death ELISA, Roche Diagnostics, Indianapolis IN] was used with slight modification to the manufacture's protocol to quantify DNA fragmentation. Briefly, 50,000 cells (of each treatment) were lysed in 100μl of lysis buffer and a fraction of the supernatant was subjected to reaction for 2 hrs with the immunocomplex of anti-DNA-conjugated with peroxidase which binds to nucleosomal DNA, and anti-histone-biotin which interacts with streptavidin-coated wells in a micro titer plate. At the end of the incubation, substrate was added and color development was quantified at 405nm wavelength.

(2) DNA content was analyzed by flow cytometry and the sub $G_1$ population was considered as apoptotic (203).

## 4.6 Microscopic Analysis of Fluorescent-labeled Cells (fluorescence and confocal microscopy)

Cells grown on cover slips were, transiently transfected with (i) indicated vectors and green fluorescent protein (EGFP, 15% of total DNA) for co-expression studies (we have previously validated the co-expression of plasmid constructs in MCF-7 cells (93)), (ii) Flag tagged constructs, or left untrasfected. All trasfections were carried out using Lipofectamine Plus$^{TM}$ reagent.

For anti-body staining: cover slips were fixed in 3% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 (in PBS) and blocked with (100% goat serum and 1% BSA) for 30 min at room temperature. Cover clips were incubated with primary antibody solutions (made in blocking buffer according to the manufacturer's instructions) overnight at $4^0$C. Cover slips were washed 5 times with PBS incubated with fluorescent conjugated secondary antibodies for 2 hrs at room temperature. After washes with PBS nuclei were stained with 3 μg/ml Hoechst 33342 [Sigma St. Louis, MO]. Cover slips

were mounted on to glass slides and analyzed using a fluorescence microscope (Leitz DMRB) or the confocal microscope.

For EGFP expressing cells: cover slips were fixed and nuclei were stained as above.

## 4.7 Western blot Analysis

Cells were washed once with ice-cold PBS and lysed in buffer containing 50mM Tris-HCl, 1% NP-40, 0.25% sodium deoxycholate, 150mM NaCl, 1nM EGTA, 1mM PMSF, 1μg/ml leupetin, 1mM $NaVO_3$, 1mM NaF.

For PARP cleavage detection, cells were lysed in ice-cold buffer containing 50mM Tris-HCL, 6.5M Urea, 5% mercaptoethanol, 2% SDS and protease inhibitors; sonicated for 20 seconds and briefly centrifugated to remove cell debris (292).

For cytochrome-C detection, cytosolic fraction was prepared as described under 'cell fractionation'.

Aliquots of cell extracts containing 50-100 μg of total proteins or immunoprecipitates of the indicated proteins were resolved on SDS-PAGE, transferred to nitrocellulose membranes and probed with specific antibodies to phospho ERKs (pTEpY) [Promega-Madison, WI], phosphor BAD (S112 and S136) [Upstate Biotechnology-Lake Placid, NY], ERK2, PTEN, BAD, PARP, cytochrome-C, Ras, phospho c-jun, cyclin D1, Akt [Santa Cruz Biotechnology-Santa Cruz, CA] and c-jun [Neomarkers], followed by the appropriate secondary antibody conjugated with HRP [Santa Cruz Biotechnology]. Proteins were detected by ECL™ [Amersham Life Science Ltd-Piscataway, NJ] and examined by X-ray films. The band intensities/ fold activities were measured by Sigma Gel™ software [Jandel Scientific-Winooski, VT].

## 4.8 Cell Fractionation

### Nuclear and cytosolic fractions

Cytosolic and nuclear fraction were prepared as described in (119) with some modifications. Cells (~10 million cells) were suspended in 150μl of hypotonic buffer containing 3.3mM Hepes, pH7.5, 1.7mM KCl, 0.25mM $MgCl_2$, 0.2mM EGTA, 0.2mM EDTA, 5μg/ml leupeptin, 0.3mM PMSF, 16.7mM NaF, 66.7μM $Na_3VO_4$ and 8.3mM

sodium β-glycerophosphate. After incubation on ice for 30 min, cells were homogenized with a Dounce homogenizer and centrifuged at 1000g for 5 min at 4°C to collect the nuclei. The resulting supernatant was centrifuged at 10000g for 10min at 4°C to obtain the heavy-membrane fraction (pellet) and the cytosol (supernatant).
The nuclear pellet was resuspended in 50μl of buffer containing 20mM Hepes pH7.5, 1.5mM $MgCl_2$, 420nM NaCl, 0.2mM EDTA, 25% v/v Glycerol, 0.3mM PMSF and 5μg/ml leupeptin, and incubated on ice with vigorous agitation (using small magnetic bar) for 30 min. Nuclear extract (supernatant) was recovered after centrifugation at 5000g for 10 min at 4°C.

## 4.9 Immunocomplex Kinase Activity Assays

### Radioactive method

Antibodies against ERK2, Akt, GSK3β, p85 (PI-3K) and protein A/G plus beads were purchased from Santa Cruz Biotechnology. Anti $p90^{RSK1}$antibody, soluble BAD and IRS-1 were purchased from Upstate Biotechnology-Lake Placid, NY. Myelin Basic Protein (MBP) was from GIBCOBRL-Carlsbad, CA. 100-200 μg total protein was immunoprecipitated with antibody in excess and protein A/G plus beads at 4°C overnight. Precipitates were washed three times with TG buffer (250mM NaCl, 20mM Tris, 0.5% NP 40) and once with kinase buffer (50mM Hepes pH 7.4, 15mM $MgCl_2$). Kinase reactions were performed by incubating immunoprecipitates and the specific substrates (0.2-1μg per sample) in kinase mixture (20μM ATP, 5μCi ($\gamma$ $^{32}P$) ATP, 1mM dithiothreitol and 0.1 mM $Na_3VO_4$ in kinase buffer) for 30 min at room temperature. Reactions were stopped with 4X Laemmli's sample buffer and proteins were resolved on SDS-PAGE. Band intensities of phosphorylated substrates in autoradiograms were measured by densitometry.

### Phosphorylation assay (non-radioactive) with BAD GST fusion proteins

p-GEX-$BAD^{wt/mutant}$ (kindly donated by Drs. P Cohen, (175) and AM Tolkovsky(279)) were grown in *E.coli* and proteins were isolated using glutathione-agarose beads (Sigma)

followed by elution in the presence of reduced glutathione. Ten μg of BAD protein was incubated with 75 μg of whole cell lysate in 50 μl of kinase buffer in the presence of 25μM ATP for 30 min in room teperature. At the end of the reaction 15 μl of agarose conjugated glutathione was added and shaken for 1 hr at 4°C. Beads were collected, washed three times with TG buffer and resuspended in 25 μl of 2X Laemmli's sample buffer.

## 4.10 Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts for EMSA was prepared as described above (3.8). Gel Shift assay system (Cat# E3300) was purchased from Promega (Madison, WI). The AP-1 and control elements were labeled with ($\gamma$-$^{32}$) ATP and non denaturing 4% acrylamide gels were prepared according to the manufacture's protocol. DNA binding reactions were carried out using 50μg of nuclear extract, radio labeled probe and gel shift binding buffer with relevant volume of nuclease free water as described by the manufacturer. At the end of the incubation period, reactions were resolved on non-denaturing acrylamide gel. Gels were dried and auto-radio grams were obtained.

## 4.11 Reporter Assay

AP-1/TRE and cyclin D1 luciferase constructs were expressed in MCF-7 and T47D cells as described in results and figure legends. After the desired periods of expression, cells were rinsed with PBS and lysed with minimal volume of 1X reporter lysis buffer (Promega Cat#E3971) [100 μl of lysis buffer for 1 well of a 6 well plate] on ice for 10 min. Supernatant was collected after brief centrifugation (12,000g for 2 min at $4^0$C). 50 μl of cell lysate and 100 μl of Luciferase Assay Substrate (Promega Cat#E4030) were mixed and emission of light was measured using the scintillation counter. For consistency in measuring luciferase activity, each sample reaction was initiated immediately before the measurement and reading. To measure the level of luciferase vector expression, EGFP was co-expressed and EGFP levels were analyzed by the Western blotting.

## 4.12 DNA Profile by Flow Cytometry Analysis

Single cell suspension of harvested cells was fixed in 70% cold ethanol, pelleted and washed with PBS. Cells were then incubated in propidium iodide staining solution (1μg/ml) in the presence of RNAse in PBS for 30 mins to 1 hr at room temperature, in dark.

## 4.13 Metabolic labeling with $^{35}$(S) methionine

MCF-7 cells were transiently transfected with desired constructs. Twenty four -48 hours after the transfection, cells were cyctine/methionine deprived for 1 hr (methionine free Dulbecco's MEM/F12) and labeled with Trans $^{35}$S (75μci/ml) in the same medium for desired period of times. Labeling medium was removed and the cells were washed twice with cold PBS. Cells were lysed and indicated proteins were immnuoprecipitated from whole cell extracts. Immunoprecipitates were resolved using 12% SDS-PAGE. Gels were dried and exposed to X-ray film. Intensity of the band denotes the amount of $^{35}$S methionine incorporated within the labeling period.

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# Vita

Romaine Ingrid Fernando was born in Wennappuwa, Sri Lanka on the 12th of April, 1968 and was raised in Wennappuwa. She completed her ordinary and advanced level education in Holy Family Balika Maha Vidyala in the same city. She earned her BVSc (DVM) from the Faculty of Veterinary Medicine in University of Peradeniya in 1995. After graduation, she was adsorbed as a probationary lecturer to the Department of Veterinary Clinical Studies. In 1999, she joined the Graduate School of Medicine, University of Tennessee, Knoxville to pursue her doctorate in Comparative and Experimental Medicine.