SURVIVAL SIGNALS IN BREAST CANCER CELLS MEDIATED BY MYC STABILIZATION.

by

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2006

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ABSTRACT

SURVIVAL SIGNALS IN BREAST CANCER CELLS MEDIATED BY MYC STABILIZATION.

by

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Advisor: Professor David A. Foster

Estrogens, which have been strongly implicated in the development of breast cancer, enhance proliferation of mammary epithelial cells and particularly of estrogen receptor (ER) positive breast cancer cells. In the absence of serum growth factors, the ER positive MCF-7 breast cancer cell line undergoes apoptosis. However, estrogens, most commonly 17-β-estradiol (E2), can suppress apoptosis in MCF-7 cells deprived of serum. While E2 stimulates a short-term transient increase in Myc expression, E2 also stimulated a sustained increase in the expression of Myc that was detectable at 48 hr and pronounced at 5 days – the point where increased proliferation of MCF-7 cells in the absence of serum could be detected. The Myc-dependent survival signal generated by E2 at 5 days was in fact dependent upon basal levels of mammalian target of rapamycin (mTOR) and two upstream regulators of mTOR, phosphatidylinositol-3-kinase (PI3K) and phospholipase D (PLD). Phospholipase D (PLD), which is commonly elevated in ER negative breast cancer cells, also suppresses apoptosis in breast cancer cells. We thus hypothesized that during breast cancer progression when the ER positive breast cancer cells lose their estrogen receptor and become ER negative cells, elevated PLD activity promotes cell survival overcoming the loss of estrogen

dependence. Myc expression, which is elevated in a significant percentage of human cancers, is regulated at many different levels, including transcription, translation, as well as post-translationally. We report here that the sustained long-term elevation of Myc expression in response to both E2 and PLD is due to reduced turnover of Myc protein. The stabilization of Myc in response to E2 and PLD is due primarily to suppression of phosphorylation of Myc at Thr58 by glycogen synthase kinase-3β (GSK-3β). Consistent with previous reports demonstrating that phosphorylation of Myc at Thr58 targets Myc for ubiquitination and degradation by the proteasome, both E2 and PLD suppressed ubiquitination of Myc. The ability of both estrogen and PLD to suppress phosphorylation of Myc at Thr58, which promotes Myc stabilization, is likely a critical aspect of the survival signals generated by E2 and PLD in hormone-dependent and hormone-independent breast cancer and will be discussed in this work.

ACKNOWLEDGMENTS

First of all, I wish to express my deepest gratitude to my mentor Dr. Foster, for his support, and encouragement during all these years. He has provided me with guidance and good sense, and shared with me his invaluable experience.

My thanks to the other committee members: Dr. Patricia Rockwell, Dr. Derrick Brazill, Dr. Karen Hubbard and Dr. Neal Rosen for their time and advice.

I would also like to thank all my colleagues Noga Gadir, Avalon Garcia, Donggon Lyo, Ming Shi, Alfredo Toschi, and Yang Zheng.

I would like to express my gratitude to Derrick and Donggon for proofreading the manuscript.

Lastly, I thank my parents, my husband, and my son, for their love, support and patience. Without their encouragement I would not have been here today.

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CHAPTER I

INTRODUCTION

1.1 PHOSPHOLIPASE D

1.1.1 PLD FUNCTION

Phospholipase D (PLD) is a widely distributed enzyme found in bacteria, fungi, plants and animals. It has been implicated in several important cellular functions and, in mammals, is under the control of many hormones, neurotransmitters, growth factors and cytokines (Jenkins G.M. *et al.*, 2005). PLD hydrolyzes phosphatidylcholine (PC) to phosphatidic acid (PA) and choline (Figure 1.1.1). Also, PLD performs a transphosphatidylation reaction using primary alcohols such as ethanol or 1-butanol forming respectively phosphatidylethanol or phosphatidylbutanol (PBut). Ethanol and 1-butanol are preferentially used over water as the nucleophile by 1000-fold or more (Yang S.F. *et al.,* 1967, and Frohman M.A. *et al.*, 1999). This transphosphatidylation reaction occurs at the expense of the hydrolytic reaction, decreasing PA formation. PA can also be produced by diacylglycerol kinase and by acylation of glycerol 3-phosphate. In contrast, phosphatidylalcohols are uniquely formed by PLD. PA can be further metabolized to diacylglycerol (DAG) and lyso-PA (LPA) (Figure 1.1.1). Phosphatidylalcohols are also metabolically stable and accumulate in cells upon PLD activation. These properties have made phosphatidylalcohols useful markers of PLD activation in vitro and in vivo.

A PLD-type activity was first described by Hanahan and Chaikoff in carrot extracts in 1947 (Hanahan D.J. *et al.*, 1947, and Hanahan D.J. *et al.*, 1948). PLD activity was not demonstrated in a mammalian system until 1975, when Saito and Kanfer described a release of choline and ethanolamine from rat brain preparations (Saito M. *et al.*, 1975). The PA generated via PLD has been linked to many events of intracellular

signal transduction through the wide use of 1-butanol as an inhibitor of PA accumulation via PLD. In fact, it has been shown that PA is required for the activation of phosphatidylinositol 4-P 5-kinase (PI-4-P5-kinase) (Jenkins G.H. *et al.*, 1994), which generates PIP2, a critical co-factor for PLD. A new downstream target for PLD signaling is mammalian target of rapamycin (mTOR), which is also a target of the PI-3-kinase/Akt pathway (Sekulic A. *et al.*, 2000) (Figure 1.1.3). It was recently reported that PA is required for activation of mTOR. Also, PA was shown to interact with mTOR competitively with rapamycin (Fang Y. *et al.*, 2001, and Chen J. *et al.*, 2002). mTOR is a protein kinase that regulates both cell cycle progression and cell growth (Kuruvilla F.G. *et al.*, 1999). mTOR controls these processes by regulating translation, transcription, membrane traffic, and protein degradation (Schmelzie T. *et al.*, 2000, and Foster D.A. *et al.*, 2003).

Furthermore, PLD and its enzymatic product PA have been linked to vesicular traffic, secretion and endocytosis (Jones D. *et al.*, 1999) as well as exocytosis (Haslam R. J. *et al.*, 1993, Stutchfield J. *et al.*, 1993, and Xie M.S. *et al.*, 1991). Also, PLD has recently emerged as an enzymatic mediator of receptor internalization via endocytosis. Initially, epidermal growth factor receptor (EGFR) internalization was found to be blocked by treatment with 1-butanol, and EGF binding to the receptor has been found to activate PLD by increasing PA production (Kim Y.R. *et al.*, 2003). The internalization increased with overexpression of either PLD1 or PLD2 and decreased by overexpression of their catalytically inactive forms (Shen Y. *et al.*, 2001).

PLD has also been involved in different cellular processes including proliferation, cell survival, as well as apoptosis. Moreover, PLD seems to be a regulator of the cell cycle. However, all these functions have been mostly described in cancer cells; they will therefore be described in a later paragraph.

1.1.2 PLD STRUCTURE

PLD has recently been cloned from yeast, bacteria, plant, and mammalian sources (Morris A.J. *et al.,* 1996). Two mammalian PLD genes (PLD1 and PLD2), both with splice variants, have been reported (Hammond S.M. *et al.,* 1995, Hammond S.M. *et al.,* 1997, Colley W.C. *et al.,* 1997, Steed P.M. *et al.,* 1998). There is considerable homology between the two genes; however, there are significant differences in the regulation and subcellular distribution of PLD1 and PLD2 that will be described later. The defining feature of PLD enzymes is the catalytic motif designated HKD, denoting the HxxxxKxD sequence, where the amino acids are histidine (H), any amino acid (x), lysine (K) and aspartic acid (D) (Figure 1.1.2). Mammalian PLD1 and PLD2 both contain two HKD motifs, which are critical for enzymatic catalysis in vitro and in vivo (Sung T.C. *et al.,* 1997). Other highly conserved regions of the PLD genes are the phox consensus sequence (PX), the plekstrin homology (PH) domain and the PI4,5P2 binding site (Figure 1.1.2). The PH domain is thought to function in the localization of the protein (Sciorra V.A. *et al.,* 2002) but is not required for PLD enzymatic activity. The PX domain is thought to mediate protein-protein interactions or to bind phosphatidylinositol phosphates (PIP) (Xu Y. *et al.,* 2006). Finally, PLD1 has a conserved loop region that is not found in PLD2. The loop region has been proposed to function as a possible negative regulatory

element, as deletion of this region from PLD1 increased its basal activity threefold (Sung T.C. *et al.,* 1999).

Study of the subcellular localization of PLD1 and PLD2 has yielded varying descriptions. Reports on PLD1 in numerous cell lines have described perinuclear localization suggestive of a Golgi, endoplasmic reticulum or late endosomal distribution (Colley W.C. *et al.,* 1997, Sung T.C., Zhang Y. *et al.,* 1999, Freyberg Z. *et al.,* 2001, and Lucocq J. *et al.,* 2001) (Table 1.1.1). PLD1 has also been localized to the plasma membrane in unstimulated cells (Kim J.H. *et al.,* 1999). Several papers now report that upon stimulation, PLD1 translocates to the plasma membrane (Du G. *et al.,* 2003, Brown F.D *et al.,* 1998, and Huang P. *et al.,* 2005). However, PLD2 is most often reported to localize to the plasma membrane (Colley W.C. *et al.,* 1997, and Du G. *et al.,* 2004), but has also been localized to the cytosol (Honda A. *et al.,* 1999) and submembraneous vesicular compartments (Divecha N. *et al.,* 2000). PLD2 has been described as translocating to membrane ruffles in response to serum (Colley W.C. *et al.,* 1997), and epidermal growth factor (EGF) (Honda A. *et al.,* 1999). Also, both PLD1 and PLD2 have an absolute requirement for PI-4,5-bis-phosphate (PIP2) (Hammond S.M. *et al.,* 1995, and Colley W.C. *et al.,* 1997). PLD2 is constitutively active in vitro and this activity is unaffected by GTPases or PKCα (Colley W.C. *et al.,* 1997, Kim J.H. *et al.,* 1999, and Sarri E. *et al.,* 2003). However, PLD2 may be affected by PKCα in vivo (Slaaby R. *et al.,* 2000, and O'Luanaigh N. *et al.,* 2002) and its regulation will be described later. Also, both PLDs exhibit a Phosphatidic Choline (PC) specificity. The different characteristics of PLD1 and PLD2 are presented in Table 1.1.1.

CHARACTERISTICS	PLD1	PLD2
PKC/ARF/Rho Responsive	Yes	No
PIP2 Dependence	Yes	Yes
Molecular Weight kDa	~120	~106
Basal activity	Low	High
Substrate Specificity	PC	PC
Transphosphatidylation	Yes	Yes
Subcellular localization	PM,CEM,ES	PM,CEM

Table 1.1.1: Biochemical properties of Phospholipase D 1 and 2.

Presented are various characteristics of PLD 1 and 2, corresponding references are presented in the text. PM-plasma membrane; CEM-caveolae enriched membrane; ES-endosomes; PIP2-phosphatidylinositol-4, 5-bisphosphate.

1.1.3 PLD REGULATION

PLD has been shown to be responsive to several signaling proteins including, Protein Kinase C alpha (PKCα), the small GTPases RhoA, and ADP ribosylation Factor (ARF). In addition, Jiang H. and colleagues have recently shown that PLD activity is associated with the Ras-family GTPase RalA, which is required for the activation of PLD by both v-Src and v-Ras (Jiang H., Lu Z. *et al.,* 1995); the active complex including PLD1, RalA and Arf (Luo J.Q. *et al.,* 1997).

There is abundant evidence that PLD is regulated by PKC in most mammalian cells (Exton J.H., FEBS Lett., 2002). It was shown that different inhibitors of PKC reduced agonist activation of PLD activity. In addition, overexpression of phosphoinositide phospholipase C (PLC), which generates diacylglycerol and activates

PKC, resulted in increased PLD activity (Lee Y.H. *et al.,* 1994). Moreover, when the mammalian isozymes of PLD were cloned and expressed, PLD1 was shown to be stimulated by either PKC-α or β in vitro, whereas PLD2 was not affected (Hammond S.M. *et al.,* 1997, and Colley W.C. *et al.,* 1997). Deletional mutagenesis and binding studies have demonstrated the presence of PKC interaction sites in both the N- and C-terminal sequences of PLD1 (Sung T.C. *et al.,*1999, Park S.K. *et al.,*1998, and Min D.S. *et al.,*1998).

There is much evidence that RhoA, Rac1 and Cdc42Hs activate PLD1, but not PLD2 (Hammond S.M. *et al.,*1997, and Colley W.C. *et al.,*1997, Bae C.D. *et al.,*1998, and Sung T.C. *et al.,*1999). RhoA is the most efficacious, but Rac1 and Cdc42Hs also cause significant activation (Bae C.D. *et al.,* 1998). The interaction site for RhoA on PLD1 has been localized to sequences in the C-terminus by binding and activity measurements (Yamazaki M. *et al.,* 1999, and Cai S. *et al.,* 2001). In addition, studies of mutant forms of PLD1 in which the RhoA interaction sites have been mutated have indicated that the enzyme can be activated in vivo by direct binding of RhoA (Xie Z. *et al.,* 2002, and Du G. *et al.,* 2000). However, RhoA may also indirectly regulate the enzyme because of its effects on PI-4-P5-kinase, which is the enzyme that synthesizes PIP2 (Chong L.D. *et al.,* 1994, and Oude-Weernink P.A. *et al.,* 2000).

Moreover, it has been shown that PLD could be regulated by ADP ribosylation Factor (Arf). Arf was the first direct regulator of PLD1 to be recognized. All mammalian isoforms of Arf were shown to activate the enzyme in vitro (Brown H.A. *et al.,* 1995, and

Tsai S.C. *et al.,* 1998). However, PLD2 shows little or no response to Arf (Colley W.C. *et al.,* 1997, and Sung T.C. *et al.,* 1999). The interaction site on Arf for PLD has been localized to the N-terminus (Zhang G.F. *et al.,* 1995, and Jones D.H. *et al.,* 1999), but it is not known where Arf binds on PLD. In contrast to the many studies showing that PLD can be activated by Arf1 in vitro, there is less information on the role of this small G protein in the activation of the enzyme in vivo. On the other hand, Arf 6 is principally localized to the plasma membrane (Gaschet J. *et al.,* 1999, Radhakrishna H. *et al.,* 1997, and Caumont A.S. *et al.,* 1998) where it probably regulates PLD. As for RhoA, Arfs 1 and 6 have been reported to activate PI-4-P5-kinase and this could also control the activity of PLD through increases in PIP2 (Honda A. *et al.*, 1999, and Jones D.H. *et al.*, 2000).

Furthermore, PLD has been shown to be activated by the oncogenic tyrosine kinase v-Src. This activation has been found to be mediated by a GTPase cascade of Ras and RalA (Jiang H., Lu Z. *et al.,* 1995). Ras does not directly activate PLD, but there is evidence that Ras mediates the activation of PLD induced by v-Src in vivo. Further work has shown that RalA, a member of the Ras subfamily, is also required (Jiang H., Luo J.W. *et al.,* 1995). The precise interactions between Ras, RalA and PLD1 that lead to activation of the enzyme are not clear, but may involve Arf (Luo J.Q. *et al.*, 1998, and Kim J.H. *et al.*, 1998).

1.1.4 ROLE OF PLD IN BREAST CANCER

PLD has been implicated in cell proliferation and cell transformation; initially, PLD activity was found to be elevated in response to several growth factors including: platelet-derived growth factor (PDGF) (Plevin R. *et al.*, 1991), fibroblast growth factor (FGF) (Motoike T. *et al.*, 1993, and Sa G. *et al.*, 1999), epidermal growth factor (EGF) (Song J. *et al.*, 1994), insulin (Karnam P. *et al.*, 1997), insulin-like growth factor 1 (IGF-1) (Banno Y. *et al.*, 2003), and vascular endothelial growth factor (VEGF) (Seymour L.W. *et al.*, 1996). In addition, PLD activity was shown to be elevated in cells transformed by different oncogenes like v-Src (Song J. *et al.*, 1991), v-Ras (Carnero A. *et al.*, 1994, and Jiang H. *et al.*, 1995), v-Fps (Jiang Y.W. *et al.*, 1994), and v-Raf (Frankel P. *et al.*, 1999). Also, early studies demonstrated that PLD can cooperate with tyrosine kinases to induce transformation of cancer cells. In fact, elevated expression of either c-Src or the EGF receptor, in combination with elevated expression of either PLD1 or PLD2, was found to transform rat fibroblasts (Lu Z. *et al.*, 2000, Joseph T. *et al.*, 2001, and Ahn B.H. *et al.*, 2003). Both PLD1 and PLD2 have also been reported to induce anchorage-independent growth and enhance cell cycle progression of mouse fibroblasts (Min D.S. *et al.*, 2001). These studies provide a clear correlation between PLD activity and mitogenic or oncogenic signaling (Foster D.A., 2006).

Moreover, elevated PLD expression and activity have recently been reported in breast cancer tissues (Noh D.Y. *et al.*, 2000, and Uchida N. *et al.*, 1997). PLD activity has also been reported to be elevated in gastric (Uchida N. *et al.*, 1999) and renal cancers (Zhao Y. *et al.*, 2000) as well as in colorectal cancer (Yamada Y. *et al.*, 2003). Foster and

colleagues have also examined different breast cell lines where PLD activity was found to be similarly elevated (Zhong M. *et al.*, 2003, and Chen Y. *et al.*, 2003). Interestingly, there was a strong, although not complete, correlation between elevated PLD activity and a loss of the estrogen receptor. This observation suggested that elevated PLD activity in breast cancer could be providing a survival signal that is normally provided by estrogen in a developing tumor. Consistent with this hypothesis, inhibiting PLD activity in MDA-MB-231 cells, a breast cancer cell line with highly elevated PLD activity, resulted in apoptosis. However, this was not observed for MCF-7 breast cancer cells that are estrogen receptor positive with low PLD activity (Zhong M. *et al.*, 2003). This data indicate that not only PLD provides a survival signal to breast cancer cells but also is able to inhibit the apoptosis process.

Furthermore, elevated PLD activity has also been shown to prevent cell cycle arrest and apoptosis. High intensity Raf signaling induces cell senescence (Samuels M.L. *et al.*, 1994, and Kerkhoff E. *et al.*, 1998) or apoptosis if the cells are deprived of serum. It was demonstrated that elevated expression of either PLD1 or PLD2 can suppress the senescence and apoptosis induced by high intensity Raf signals (Joseph T. *et al.*, 2002). Similarly, rat fibroblasts overexpressing c-Src undergo apoptosis in response to growth factor deprivation, and both PLD1 and PLD2 were able to suppress the apoptosis induced by growth factor deprivation (Zhong M. *et al.*, 2003). These early studies in rodent fibroblasts indicated that in addition to enhancing cell proliferation, PLD could generate survival signals capable of suppressing default apoptotic programs. However, recent studies have shown that elevated PLD activity impacts upon tumor suppressor genes

implicated in cellular apoptotic programs. In fact, it was demonstrated that elevated PLD activity suppressed DNA damage-induced apoptosis by suppressing p53 (Hui L. *et al.*, 2004). Thus, the ability of PLD to inhibit p53 expression allows for progression through cell cycle checkpoints regulated by p53.

Classic studies from the Weinberg group have provided a framework for cooperating oncogenes in the transformation of primary human cells (Hahn W. *et al.*, 1999, and Land H. *et al.*, 1983). A model is provided in Figure 1.1.4, whereby growth factor signals such as the ones generated by an activated Ras or an overexpressed tyrosine kinase facilitate passage through a growth factor-dependent Restriction Point (Pardee A.B., 1974, and Zetterberg A. *et al.*, 1995). Oncogenes such as Myc and SV40 early region genes that cooperate with Ras in cell transformation facilitate gatekeeper override at a G1/S cell cycle checkpoint. Suppression of p53 is critical for the transformation of human cells using SV40 early region genes in combination with Ras, and telomerase (Hahn W.C. *et al.*, 1999, and Hahn W.C. *et al.*, 2002). The SV40 early region genes include Large T antigen, which neutralizes p53 and the retinoblastoma tumor suppressor gene Rb (Reich N.C. *et al.*, 1982, and Pipas J.M. *et al.*, 2001) and small t antigen (Hahn W. *et al.*, 2002), which is required to suppress protein phosphatase 2A (PP2A). In this regard, it is of interest that PLD also suppresses the activity of PP2A (Hui L. *et al.*, 2005) which is dependent upon both mTOR and MAP kinase. Thus, elevated PLD activity is able to achieve much of what the SV40 early region genes do – that being the suppression of the tumor suppressor genes p53 and PP2A (Foster D.A., 2006).

In an updated model (Figure 1.1.4), PLD, like SV40 early region genes, provides a gatekeeper override function that facilitates passage through cell cycle checkpoints. Consistent with this hypothesis and as discussed above, PLD suppresses p53, PP2A and Rb (Hui L. *et al.*, 2004, Hui L. *et al.*, 2005, and our unpublished work) as do the SV40 early region genes. Also of interest is that PLD increases Myc expression (Rodrik V. *et al.*, 2005), which cooperates with Ras in the transformation of primary rodent cells (Land H. *et al.*, 1983). In this context, elevated PLD activity leads to the transformation of cells with elevated expression of a tyrosine kinase (Lu Z. *et al.*, 2000, and Joseph T. *et al.*, 2001) by contributing to the override of gatekeeper function, which is important for the suppression of apoptosis. PLD ability to facilitate gatekeeper override certainly contributes to its ability to provide a survival signal in human breast cancer cells (Chen Y. *et al.*, 2003).

Finally, one important step in cancer development is metastasis and PLD has been implicated in this process. PLD activity has also been shown to affect cell motility (Aguirre Ghiso J.A. *et al.*, 1997) and more specifically, PLD2 was shown to stimulate cell protrusions in v-Src-transformed cells (Shen Y. *et al.*, 2002) which is also consistent with a role for PLD in cell migration. Rho family GTPases, which activate PLD1, have also been shown to regulate cell motility, and have been implicated in metastasis (Schmitz A.A. *et al.*, 2000). Moreover, PLD activity has been implicated in tumor invasion (Imamura F. *et al.*, 1993, and Pai J.K. *et al.*, 1994) and interestingly, MDA-MB-231 human breast cancer cells, which have very high levels of PLD activity, migrate and

invade matrigel in culture (Zhong M. *et al.*, 2003), whereas MCF-7 breast cancer cells, with relatively low PLD activity, do not (Sliva D. *et al.*, 2002).

Therefore, all PLD functions discussed above make PLD a good oncogene candidate and thus a good cancer therapeutic target.

1.1.5 __PLD AS A THERAPEUTIC TARGET__

At present, there are no good inhibitors of PLD other than alcohol, which prevents the production of PA by competing with water to form an inert phosphatidyl-alcohol (Exton J.H., 2002). Unfortunately, the level of alcohol required to effectively inhibit the production of PA is much too high to consider this strategy as a possibility (Foster D.A., 2004). However, the use of alcohol in cell culture studies has provided proof-of-principle that targeting PLD signals kills cancer cells where there is elevated PLD activity (Zhong M. *et al.*, 2003, and Chen Y. *et al.*, 2003). A major problem in targeting PLD directly is that PLD1 has also been implicated in vesicle budding and protein trafficking in the Golgi (Roth M.G. *et al.*, 1999). Therefore, drugs that suppress PLD1 directly will likely have side effects that will not be acceptable. Thus, PLD2 could be a better therapeutic target.

A more successful approach may be to target the signals that activate PLD activity. While several reports have shown elevated expression of PLD in human cancers (Zhong M. *et al.*, 2003, Zhao Y. *et al.*, 2000, Noh D.Y. *et al.*, 2000, and Uchida N. *et al.*, 1999), in general the increased level of PLD1 and/or PLD2 expression was small relative to the level of PLD activity. This implies that PLD activity in cancer cells is elevated in

response to oncogenic signals upstream from PLD. As discussed before, PLD activity is regulated by several monomeric G-proteins including Arf, Ral and Rho family GTPases (Joseph T. *et al.*, 2002, and Vieira A.V. *et al.*, 1996) and drugs that target these GTPases are being developed. Another possible target for suppressing PLD signaling are the enzymes that regulate the levels of PIP2. As described before, both PLD1 and -2 have a stringent requirement for PIP2 and PA activates PI-4-P-5-kinase, the enzyme that generates PIP2 in an apparent positive-feedback loop (Figure 1.1.3). Also, a potential target is the EGF receptor, which has been implicated in a wide variety of human cancers (Yarden Y. 2001). Importantly, several drugs that target the EGF and other tyrosine kinase receptors have been developed (Gschwind A. *et al.*, 2004) and are currently being evaluated (Foster D.A., 2006).

The most promising downstream target of PLD is mTOR. mTOR has been widely implicated as a critical target of survival signals generated by both PLD and PI3K (Foster D.A., 2004). All PLD survival effects that have been characterized have an mTOR requirement (Chen Y. *et al.*, 2003, Hui L. *et al.*, 2004, Hui L. *et al.*, 2005, and Rodrik V. *et al.*, 2005). mTOR has a PA requirement that is apparently competitive with rapamycin as discussed previously (Fang Y. *et al.*, 2001, and Chen Y. *et al.*, 2003). This makes mTOR an ideal target for suppression of PLD survival signals, not only because of its critical role in survival signaling, but also because there are already effective drugs that target mTOR with high specificity – rapamycin and rapamycin derivatives. Importantly however, elevated PLD activity confers resistance to rapamycin (Chen Y. *et al.*, 2003), presumably due to the competition between rapamycin and PA for mTOR. This

observation complicates the targeting of mTOR in cancer cells with elevated PLD activity. However, it was demonstrated that reducing PLD activity in human breast cancer cells increases sensitivity to rapamycin (Chen Y. *et al.*, 2003). Thus, a combination of drugs targeting the signals that activate PLD activity could make rapamycin, or rapamycin derivatives such as CCI-779 or RAD001, much more effective drugs in cancers where there is highly elevated PLD activity. Moreover, there is a limitation in targeting mTOR; in most studies reported to date, rapamycin was found to be cytostatic, not apoptotic, and blocked cell cycle progression in G1 (Neshat M.S. *et al.*, 2001, and Podsypanina K. *et al.*, 2001). It was recently reported that a combination of rapamycin and dexamethasone leads to apoptosis (Stromberg T. *et al.*, 2003) indicating that rapamycin is able to contribute to apoptosis under appropriate conditions. Consistent with this concept, it has been found that in the absence of serum, rapamycin induces apoptosis in some breast cancer cells *in vitro* (Chen Y. *et al.*, 2003), suggesting that serum provides a factor(s) that results in rapamycin inducing G1 arrest rather than apoptosis (Figure 1.1.5). In this regard, it is possible that depriving cancer cells of serum with anti-angiogenesis drugs to deprive cells of serum growth factors in combination with rapamycin could lead to apoptosis instead of G1 arrest (Foster D.A., 2006).

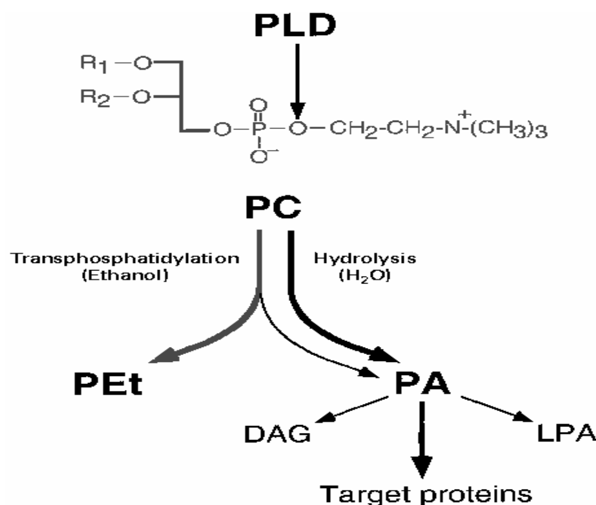

Figure 1.1.1: PLD specific transphosphatidyl reaction

PLD hydrolyses the distal phosphodiester bond in phospholipids such as PC (the structure shown above in grey). A phosphatidyl-enzyme intermediate is believed to form transiently which normally is hydrolysed by water, generating PA. Primary short-chain alcohols (e.g. ethanol) can substitute for water in a competing, transphosphatidylation, reaction. In the presence of ethanol the product of PLD-catalysed transphosphatidylation is PA ethyl ester or phosphatidylethanol (PEt). This reaction (thick grey arrow pointing to the left) occurs at the expense of the hydrolytic reaction (thin black arrow pointing to the right), decreasing PA formation as described in the text. PA can be further metabolized (thin black arrows) to diacylglycerol (DAG) and lyso-PA (LPA).

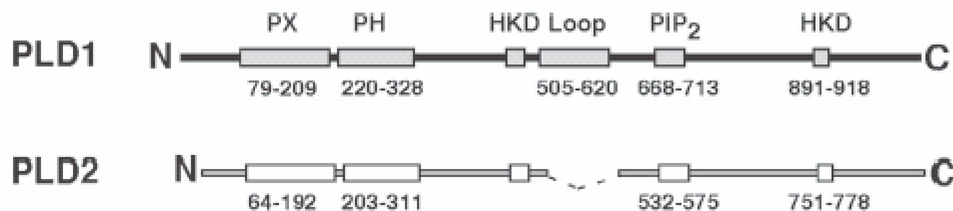

Figure 1.1.2: PLD genes
(Jenkins G.M. *et al.*, 2005)

Basic structure of PLD1 and PLD2. Linearized cartoon of structure showing amino (N) and carboxy (C) terminals with conserved domains as boxes. Domains shown are the catalytic HKD motif (HKD), phox consensus sequence (PX), plekstrin homology (PH), phosphatidylinositol bisphosphate (PIP2) and PLD1 loop region.

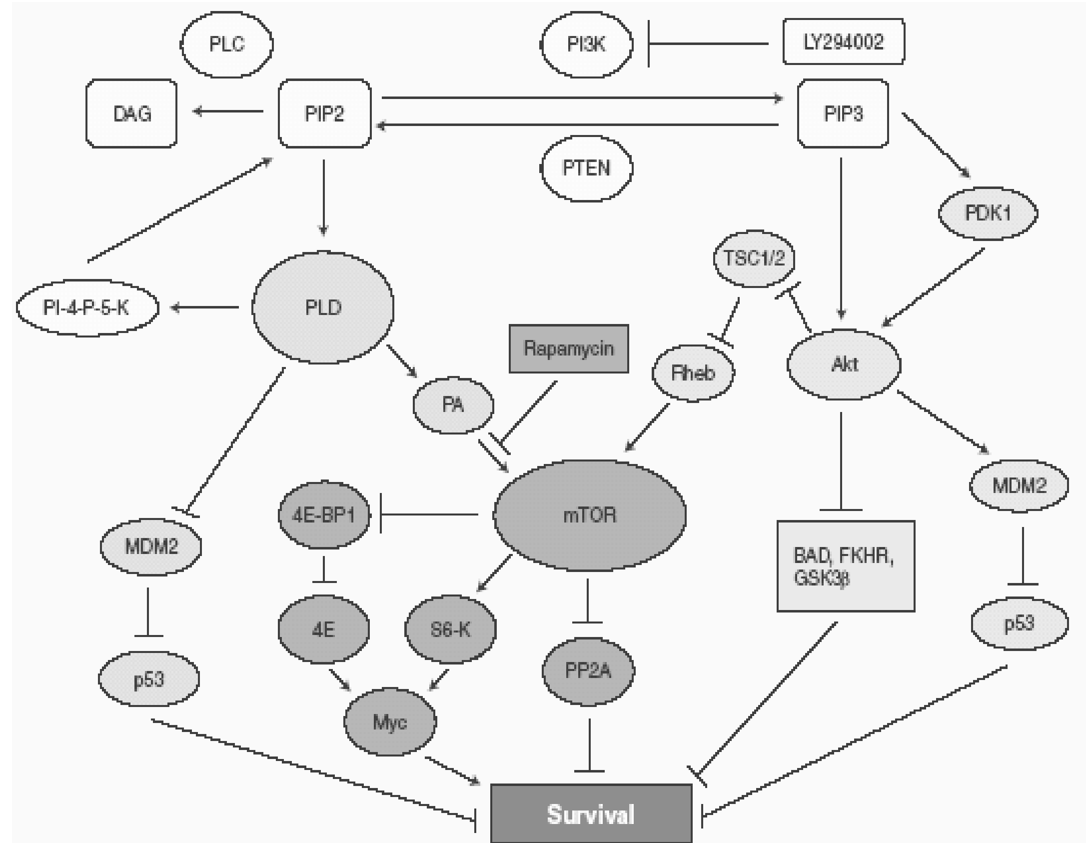

Figure 1.1.3: Survival signals targeting mTOR.
(Foster D.A., 2005)

mTOR is targeted by PI3K and PLD survival signals. Both PI3K and PLD are dependent upon PI metabolism. PLD is dependent upon PIP2 and PI3K converts PIP2 to PIP3. PLD-generated PA activates PI-4-P-5-kinase to generate more PIP2 and therefore provide a positive feedback loop. PIP3 recruits Akt and PDK1, the kinase that phosphorylates and activates Akt. Akt then phosphorylates several proteins that suppress cell cycle progression including the TSC1/2 complex, a GTPase-activating protein that suppresses the GTPase Rheb, which contributes to the activation of mTOR. PLD-generated PA competes with rapamycin to bind mTOR and is required for mTOR activation of S6-kinase and suppression of ribosomal subunit 4EBP1, leading to increased Myc expression. PLD also increases the expression of MDM2, which suppresses expression of p53. 4EBP1: 4E binding protein 1; GSK: Glycogen synthase kinase; PA: Phosphatidic acid; PDK: Phosphoinositide-dependent kinase; PI3K: Phosphatidylinositol-3- kinase; PIP: Phoshatidylinositol-4,5-bis-phosphate; PLC/D: Phospholipase C/D; PP2A: Protein phosphatase 2A; TSC: Tuberous sclerosis complex.

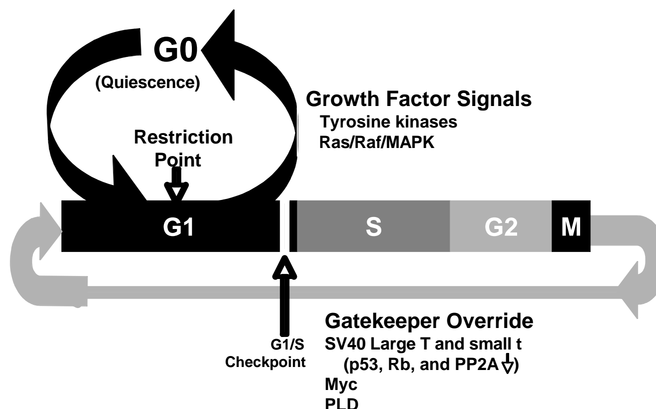

Figure 1.1.4: Oncogenic signaling and the cell cycle.
(Foster D.A., 2006)

Two complementation groups for cell transformation based on a model of Weinberg and colleagues are proposed to regulate progression through different stages of the cell cycle. As shown, there are growth factor-dependent signals prevent cells from exiting the cell cycle into a quiescent G0 state by facilitating passage through the Restriction Point. As decribed in the text, PLD cooperates with elevated tyrosine kinase expression to transform cells and also induces Myc expression and is therefore included in the gatekeeper override group. Although there is overlap in function between growth factor signals and gatekeeper override, the model does reflect the ability of growth factor signals to complement gatekeeper override to facilitate cell cycle progression and cell transformation.

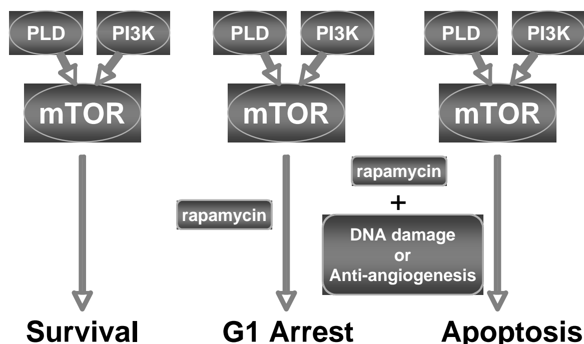

Figure 1.1.5: Strategies for targeting mTOR in anticancer therapies.
(Foster D.A., 2006)

The activation of mTOR by PLD, PI3K or both generates a survival signal (left panel). Rapamycin blocks the mTOR signals, but this only causes G1 arrest (middle panel). However, under the stressful conditions of DNA damage or serum deprivation rapamycin instead causes apoptosis.

1.2 <u>ESTROGEN AND ESTROGEN RECEPTORS</u>

1.2.1 ESTROGEN FUNCTION

The steroid hormone 17-β-estradiol (E2) is a key regulator of growth, differentiation, and exerts multiple biological effects on a wide array of target tissues, including the male and female reproductive tracts and the mammary gland (Hall J.M. *et al.*, 2001). In addition to its impact on the reproductive system, estrogen regulates bone structure (Termine J.D. *et al.*, 1998), cardiovascular function (Guzzo J.A., 2000), and the central nervous system (Hurn P.D. *et al.*, 2000). The predominant biological effects of E2 are mediated through two distinct intracellular receptors, Estrogen Receptor α (ER α) and Estrogen Receptor β (ER β), each encoded by unique genes (Giguère V. *et al.*, 1998). These ERs belong to the steroid/thyroid hormone superfamily of nuclear receptors (Olefsky J.M., 2000 and Beato M. *et al.*, 1995) and function as ligand-activated transcription factors (Evans R.M. *et al.*, 1988). All the steroids receptors, including ER, are phosphorylated after binding to their respective ligand (Weigel N.L., 1996). Upon binding of estrogen or similar ligand, ERs undergo conformational changes, an event that promotes ER homodimerization and subsequent phosphorylation (Weigel N.L., 1996) as well as high-affinity binding of the estrogen receptor to specific sites on DNA-estrogen response elements (EREs) promoting gene transcription (Figure 1.2.1). In the absence of estrogen, ERs are sequestered within nuclei of target cells and maintained in an inactive state by association with heat-shock proteins (Figure 1.2.1).

1.2.2 ***ESTROGEN RECEPTOR STRUCTURE***

The two receptors ER α and ER β share common structural and functional domains (Figure 1.2.2) bind to estrogen with high affinity, and bind estrogen response elements in a similar manner. They have six domains named A through F from N- to C-terminus. The three major functional domains of the ERs are: a variable N terminus (domains A and B) that modulates transcription in a gene and cell-specific manner through its N-terminal Activation Function-1 (AF-1); a central DNA-binding domain (DBD, consisting of the C domain), comprised of two functionally distinct zinc fingers through which the receptor interacts directly with the DNA helix; as well as the ligand-binding domain (LBD, domains E) that contains Activation-Function-2 (AF-2). The F domain plays a role in distinguishing estrogen agonists versus antagonists, perhaps through interaction with cell-specific factors (Montano M.M. *et al.*, 1995). Moreover, it was shown that ER β lacks a large portion of the carboxyl-terminal F domain. As suggested by the differences in structure, ER α and ER β are found to differ in many ways with respect to tissue distribution, transcriptional activities, and phenotypes in knockout models (Girdler F. *et al.*, 2000).

1.2.3 ***ESTROGEN RECEPTOR REGULATION***

The AF-1 domain is hormone-independent, whereas the AF-2 domain is hormone-dependent (Webster N.J. *et al.*, 1989). Both AF-1 and AF-2 are required for maximal ER transcriptional activity. However, with certain promoters, AF-1 and AF-2 can function independently (Tzukerman M.T. *et al.*, 1994). After hormone binding, dimerization and phosphorylation, ERs bind to DNA with high affinity through their

DNA-Binding Domain (C region, Figure 1.2.2) at specific sites, termed Estrogen Response Elements (ERE), as mentioned earlier. Also, ER α and ER β can form heterodimers that could also alter gene transactivation. Besides this classical mechanism of direct DNA binding, the two ER subtypes can also interact with DNA indirectly through tethering to other DNA-bound transcription factors, as it appears to be the case with the interactions between the ERs at AP-1 sites, where the receptor is tethered through the Fos/Jun complex (Webb P. *et al.*, 1999) (Figure 1.2.3). These transcription factors regulate genes involved in may cellular processes, including proliferation, differentiation and cell motility.

In contrast to the ligand-mediated transcriptional activity or genomic effects described above, the activity of the ER can also involve ligand-independent or non-genomic effects (Figure 1.2.1). This ligand-independent activity of the ER is a result of phosphorylation of the ER and creates cross-talk between the ER and other signaling pathways. Activation of ER α via phosphorylation at multiple sites by multiple kinases is important because of the interaction between growth factor signaling and the ER. Increased growth factor signaling may account for the loss of E2 dependence, thereby producing anti-estrogen resistant tumors. In addition, an association has been observed between elevated MAP kinase phosphorylation/activity and a poor response to endocrine therapy in breast cancer patients (Gee J.M. *et al.*, 2001).

1.2.4 ROLE OF ESTROGEN AND ESTROGEN RECEPTOR IN BREAST CANCER

Breast cancer is the second leading cause of cancer deaths in women today and the most common cancer (excluding nonmelanoma skin cancers) among women in North America (Greenlee R.T. *et al.*, 2000). There is substantial evidence to suggest that ovarian steroids play an important role in both the development and progression of breast cancer, with the risk of developing the malignancy related to the cumulative exposure of the breast to endogenous and exogenous estrogens (Henderson B.E. *et al.*, 2000). Breast cancer was first recognized to be an estrogen-dependent disease in 1896, when the British physician George Beatson demonstrated that oophorectomy induced regression of mammary tumors in a subset of premenopausal patients (Beatson G.T., 1896). Since then, a variety of clinical and epidemiological observations, with support from cell-culture studies have further substantiated the involvement of estrogens in the development and/or progression of the disease (Key T.J.A. *et al.*, 1988, Pike M.C. *et al.*, 1993, and Dorgan J.F. *et al.*, 1996). According to the standard paradigm, estrogens, through receptor-mediated processes, stimulate proliferation of normal and malignant cells through the induction of proteins involved in nucleic acid synthesis and the activation of growth-regulatory genes. The enhanced cell proliferation can increase the chance for errors in DNA repair, resulting in an accumulation of mutations. Over time, these mutations may contribute to progression from normal cell growth through hyperplasia to neoplasia. Evidence that estrogens can indeed initiate tumors is provided by their carcinogenicity in animal models, particularly the Syrian golden hamster, in which estrogen induced kidney tumors (Li J.J. *et al.*, 1987, and Liehr J.G. *et al.*, 1987). Also, recently, the National Toxicology Program listed, for the first time, steroidal estrogens as carcinogens.

The role of ER β in breast cancer growth and development is not as clear as the role of ER α (Palmieri C. *et al.*, 2002, and Gustafsson J.A. *et al.*, 2000). ER β might have a modulating effect in breast cancer because it is expressed in normal and malignant breast tissue, binds 17-β-estradiol and can heterodimerize with ER α (Ogawa S. *et al.*, 1998, Hall J.M. *et al.*, 1999, Cowley S.M. *et al.*, 1997, and Pettersson K. *et al.*, 1997). Overall, ER β appears to play a protective role against the development of breast, prostate and colon cancer. The majority of studies suggest that its presence is a good prognostic marker for breast cancer.

1.2.5 ESTROGEN RECEPTOR AS A THERAPEUTIC TARGET

As described above, the ER is an important target to develop drugs for the treatment and prevention of breast cancer (Jensen E.V. *et al.*, 2003). The interaction of estrogen with the ER can result in increased proliferation of target cells so the rationale for endocrine therapy is to block the interaction of estrogen with the ER. This goal can be accomplished by blocking the production of estrogen by ovariectomy, or inhibiting the conversion of steroidal precursors to estrogen using aromatase inhibitors. The ER can also be targeted directly using Selective Estrogen Receptor Modulators (SERMs) such as tamoxifen and raloxifene as competitive inhibitors of estrogen action, or by the removal and degradation of the ER by pure antiestrogens such as ICI 182,780 (fulvestrant) (Pearce S.T. *et al.*, 2004). Endocrine manipulations are among the least toxic and most effective therapies for the treatment of hormone responsive breast cancers.

SERMs act as estrogens in select target tissues but act as antiestrogens in other target tissues (Jordan V.C., 2001). The ideal SERM would be an estrogen agonist in bone, liver, the cardiovascular system and brain and an estrogen antagonist in the breast and uterus. The activity of tamoxifen is dependent on circulating levels of E2, which are high in premenopausal women and low in postmenopausal women. Tamoxifen is an antiestrogen in the breast, and decreases low density lipoprotein cholesterol levels in postmenopausal women. However, estrogenic activity of tamoxifen is observed in the uterus, which results in an increased incidence of endometrial cancer in postmenopausal women (Fisher B. *et al.*, 1998); thus the current tamoxifen treatment does not exceed 5 years. Also, current treatment strategies have shown that 5 years of adjuvant tamoxifen treatment is beneficial in pre- and postmenopausal women with ER-positive tumors (Early Breast Cancer Trialists' Collaborative Group, 1998). In addition, tamoxifen can be used for the prevention of breast cancer (Fisher B. *et al.*, 1998). Despite over 30 years of clinical experience with tamoxifen, for most patients, tumors that initially regress with tamoxifen will eventually recur and require alternate treatment. The mechanisms of cellular resistance to tamoxifen are under investigation (Clarke R., Skaar T.C. *et al.*, 2001 and Clarke R., Leonessa F. *et al.*, 2001, Ali S. *et al.*, 2002, Schiff R. *et al.*, 2003). However, tamoxifen resistance could also be explained by ER mutations, coregulator expression and recruitment, or interactions with other signaling pathways. Despite the possibility of drug resistance, there are potential treatments after the development of tamoxifen resistance. These include the use of aromatase inhibitors to block the production of estrogen (Baum M. *et al.*, 2002) as well as pure antiestrogens to degrade the ER (Howell A. *et al.*, 2002).

The aromatase inhibitors are particularly effective by inhibiting the conversion of androgens to estrogen in the tumor. Estrogen levels in the breast cancer tissue then fall to very low levels, much lower than with ovarian ablation in pre-menopausal women (Santen R.J. *et al.*, 1987). Today the aromatase inhibitors as a group are the most effective endocrine therapies for postmenopausal patients. Thus, depriving ER of its ligand is the most effective means of shutting off ER function. All three aromatase inhibitors anastrazole, letrozole, and the steroidal aromatase inhibitor exemestane, have been shown in randomized trials to be superior to tamoxifen as initial therapy (Dombernowsky P. *et al.*, 1998, Buzdar A.U. *et al.*, 1998, Rose C. *et al.*, 2003, and Nabholtz J.M. *et al.*, 2003). Indeed, aromatase inhibitors are effective therapy in tamoxifen-resistant patients, an observation that led to their use in sequence after tamoxifen in the adjuvant setting (Dombernowsky P. *et al.*, 1998, Buzdar A.U. *et al.*, 1998). However, the reverse sequence of aromatase inhibitors and then tamoxifen seems less effective. Only one completed adjuvant trial (ATAC) compared an aromatase inhibitor (anastrozole) directly with tamoxifen in patients with primary breast cancer after surgery (Baum M. *et al.*, 2002). Overall, the aromatase inhibitor for five years was statistically significantly superior to tamoxifen for five years in terms of disease-free survival and reduction in contralateral breast cancer. This study also examined the combination of anastrozole with tamoxifen, a combination that was inferior to anastrozole alone. Moreover, the three aromatase inhibitors have similar toxicity profiles compared with tamoxifen; they are associated with fewer side effects (Nordman I.C. *et al.*, 2005). While aromatase inhibitors are very effective therapy, de novo and acquired resistance to these treatments remain major problems (Osborne C.K. *et al.*, 2005).

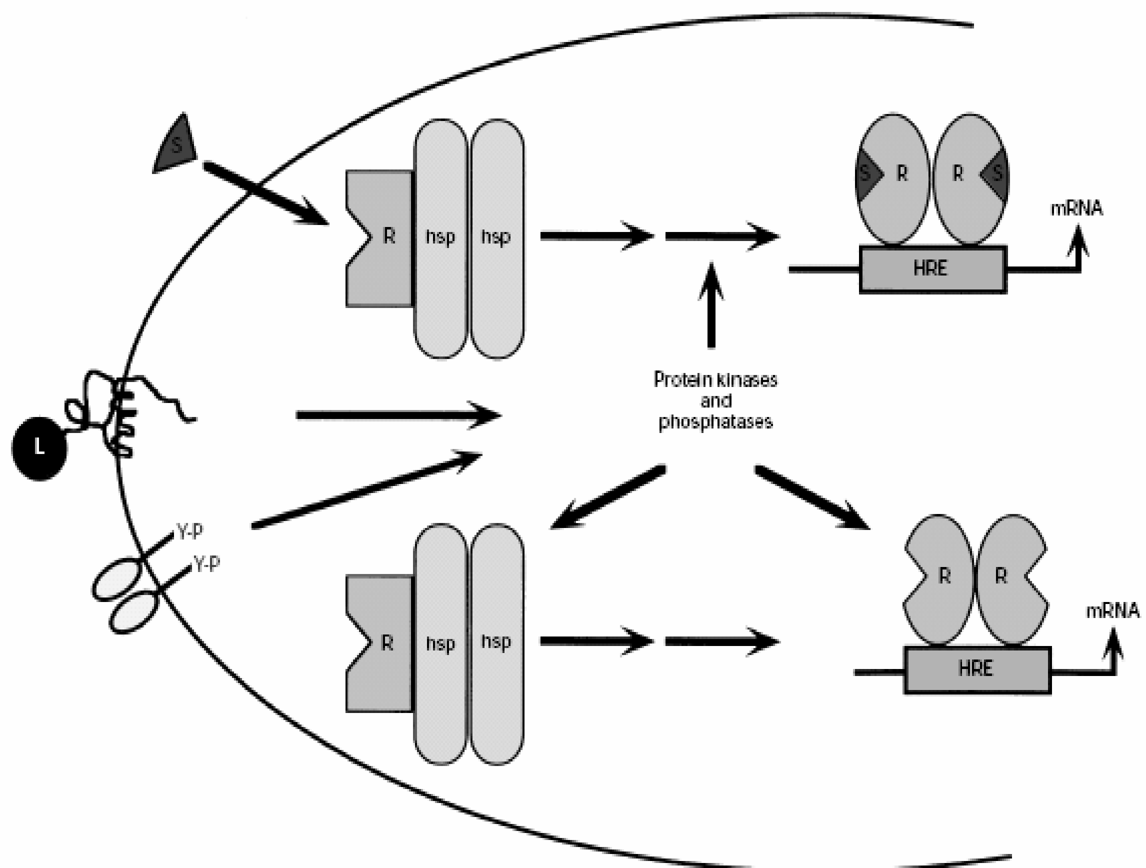

Figure 1.2.1: Steroid receptor activation.
(Weigel N.L. *et al.*, 1996)

The upper portion depicts the classical steroid (S)-dependent activation pathway. The lower portion depicts signals emanating from receptors such as the dopamine receptor or growth factor receptors that alter the kinase/phosphatase balance of the cell such that the receptors are activated in the absence of ligand. L represents a ligand binding to a membrane receptor that acts through a cAMP pathway. Y-P indicates the active form of a growth-factor receptor. R, receptor; hsp, heat-shock protein complex.

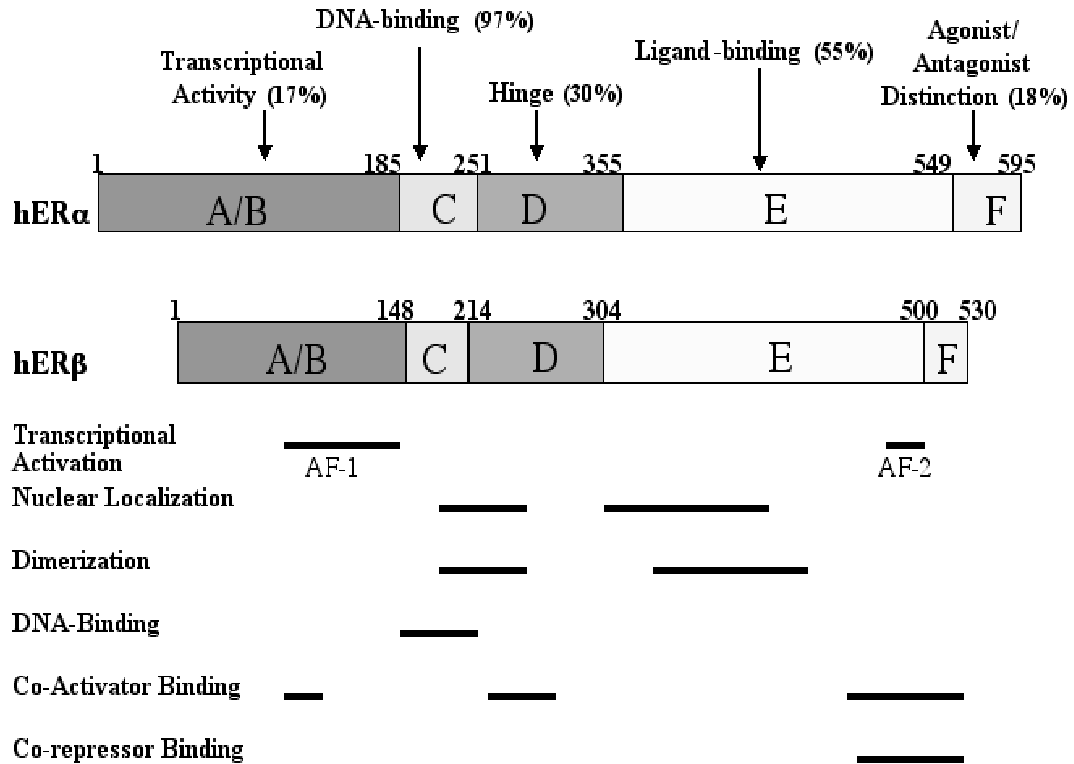

Figure 1.2.2: Functional Domains (% Homology between ER-α and ER-β). (Klinge C., 2000)

The domains of the receptors are indicated, as are the regions responsible for nuclear translocation, dimerization, DNA binding, hsp90 binding, and interaction with co-activators and co-repressors. The percentage of amino acid homology between regions A-F in ERα and ERβ is indicated by the numbers.

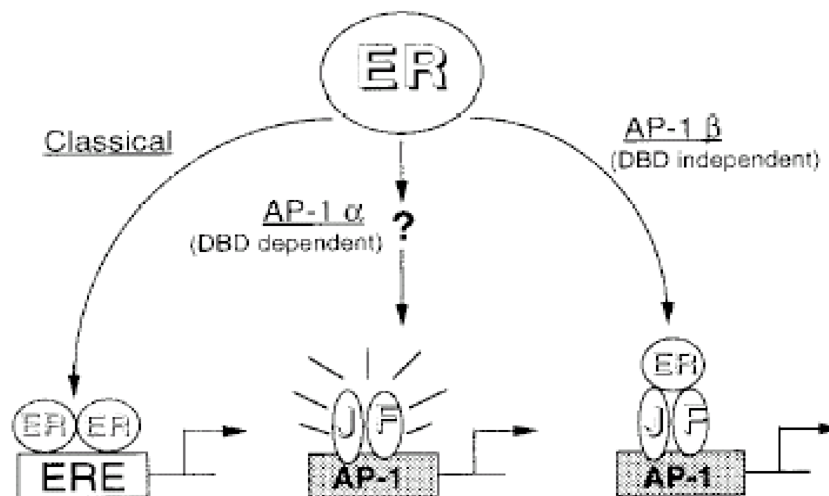

Figure 1.2.3: A Model of ER Action.
(Webb P. et al., 1995)

ER can stimulate promoter activity either by the classical pathway (left) in which the receptor binds to EREs in the promoter region of target genes or by either of two pathways leading to activation of genes regulated by AP-1 sites. In the AP-1 N pathway, which requires the receptor DBD and is activated by tamoxifen (middle), ER interacts with an unknown target protein activating a cascade that increases the transcriptional efficiency of Jun/Fos at their cognate AP-I site. In the AP-1 /3 pathway, which is Independent of the receptor DBD and is activated only by estrogen (right), ER is tethered to the AP-I site by interactions with Jun/Fos.

1.3 c-MYC

1.3.1 c-Myc FUNCTION

The c-myc gene was first discovered as the cellular homolog of the retroviral v-myc oncogene (Bishop J.M., 1982 and Bister K. *et al.*, 1986). The c-myc proto-oncogene was subsequently found to be activated in various animal and human tumors (Cole M.D., 1986 and Dalla-Favera R. *et al.*, 1982). It belongs to the family of myc genes that includes B-myc, L-myc, N-myc, and S-myc. The c-Myc protein is a transcription factor which is a potent regulator of cell growth, proliferation, differentiation, apoptosis and tumorigenesis (Cole M.D., 1986 and Lusher B. *et al.*, 1990). Myc is able to control all these biological activities by turning on and off the transcription of Myc target genes. Several hundred of Myc target genes have been identified (Figures 1.3.1, 1.3.2) (Dang C.V., 1999). However, it is important to distinguish between Myc functions in normal cells versus its functions in cancerous cells. Regulated c-myc gene expression is critical for controlled cell proliferation whereas deregulated, often constitutive, expression of c-myc characterizes oncogenic activation and is a frequent hallmark of tumor-derived cells (Dang C.V., 1999). Myc oncogenic functions will be described in a later paragraph.

Expression of c-Myc in the normal cell is tightly regulated by external signals, such as growth factors and extracellular matrix contacts, as well as by internal clocks, such as the cell cycle. The resting cell normally expresses little c-Myc, whereas cells stimulated by growth factors dramatically increase c-Myc expression as an immediate early response gene. c-Myc expression persists into the cell cycle, but then returns to its basal quiescent level in resultant resting daughter cells (Sears R.C., 2004). Abnormal or

ectopic overexpression of c-Myc in primary cells activates a protective pathway through the induction of p19/p14ARF and a p53-dependent cell death pathway. Hence, normal cells that overexpress c-Myc are eliminated from the host organism through apoptosis, thereby protecting the organism from lethal neoplastic changes. Normal embryonic development requires regulated expression of c-Myc as well as other myc family members. Mouse embryos in which both alleles of c-myc have been deleted by homologous recombination die in early development with a lack of primitive hematopoiesis (Dang C.V., 1999).

In physiological situations, the central role of c-Myc may be its promotion of cell replication in response to extracellular signals, by driving quiescent cells into the cell cycle. This function was originally thought to be elicited mainly via activation of transcription of the c-Myc target genes that are positive regulators of the cell cycle (Amati B. *et al.,* 1993), such as cyclins D1, D2, E and A, cdk4, e2f1, e2f 2, cdc25A and B, etc. (Barrett T.J. *et al.,* 1995, Amati B. *et al.,* 1993, Ben-Yosef T. *et al.,* 1998, and Dang C.V, 1999). In principle, promotion of cell cycle progression by c-Myc can also be achieved by suppression of transcription of growth inhibitory genes (Alexandrow M.G. *et al.,* 1998). Examples of these genes include gadd45 (Marhin W.W. *et al.,* 1997), cdk (cyclin-dependent kinase) inhibitors p21cip1 (Mitchell K.O. *et al.,* 1999, Coller H.A., *et al.* 2000), p19ARF (Dang C.V., 1999) and probably also p27kip1 (Amati B. *et al.,* 1993, Donjerkovic D. *et al*., 1999, Wu M. *et al.* 1999). There is evidence that under certain conditions the role of c-Myc in cell cycle progression may require only its activity of trans-suppression, not that of transactivation (Claassen G.F. *et al.,* 1999). In cell cycle

regulation, Myc is required for G1 to S phase progression of primary cells, and induction of c-Myc expression stimulates quiescent nontransformed cells to traverse G1 phase and enter S phase (Eilers M. *et al.*, 1989, Heikkila R. *et al.*, 1987 and Liao D.J. *et al.*, 2000).

Moreover, conflicting observations suggest that c-Myc is capable of both inducing and suppressing apoptosis. To induce a tumor, c-Myc may need not only to promote cell proliferation but also simultaneously to inhibit its tendency for cell death, so as to increase the cell number to form a tumor mass (Cory S. *et al.,* 1999, Lowe S.W. *et al.,* 2000). Therefore, the role of c-Myc in inhibiting apoptosis is easily connected to its tumorigenicity (Vakkala M. *et al.,* 1999). Also, there is only little evidence showing that c-Myc can induce apoptosis. The foundation of this concept of "c-Myc-induction of apoptosis" is built mainly on the systems where c-Myc expression is induced in a constant manner by approaches of transfection, viral-infection, or a specific promoter-driven c-myc transgene, while the expression level of the endogenous c-Myc is down-regulated (Prendergast G.C. *et al.,* 1999, Packham G. *et al.,* 1995). These artificial systems are different from the physiological situation in which c-Myc expression arises under its own promoter, specifically during G0/G1 transition of the cell cycle. Thus, a constantly high level of c-Myc may disrupt the cyclic pattern of expression of these genes; the cell may then be signaled to die (Liao D.J. *et al.,* 2000).

1.3.2 c-Myc STRUCTURE

The c-myc gene, located on human chromosome 8q24, is comprised of three exons (Battey J. *et al.*, 1983) (Figure 1.3.3). Its transcription may be initiated at one of the three promoters. Translation at the AUG start codon (exon2) produces a major 64kDa c-Myc protein, also called c-Myc2. In addition, a longer polypeptide of 67kDa results from translation initiated 15 codons upstream of the AUG at a CUG codon (exon1) yielding to c-Myc1 (Henriksson M. *et al.*, 1996, and Facchini L.M. *et al.*, 1998). An internal translationally initiated c-Myc 45kDa polypeptide was also recently recognized yielding to a shorter protein called c-MycS protein (Spotts G.D., 1997). The c-Myc protein is O-linked glycosylated and phosphorylated, and these modifications may alter the protein half-life. The N-Myc and L-Myc proteins share several regions of at least 80% amino acid sequence homology to c-Myc, including the Myc box I (MbI) and Myc box II (MbII) domains located in the N-terminus of c-Myc as well as a basic helix-loop-helix and leucine zipper region (BR-HLH-Zip), located in the C-terminus region of c-Myc (Figure 1.3.3). As these domains are also conserved among species, it is believed that they define important functional domains **(**Facchini L.M. *et al.*, 1998). This HLH-Zip domain is a dimerization motif; it mediates homotypic or heterotypic dimerization with other HLH-Zip proteins. The c-Myc dimerization domain was found to be necessary for cellular transformation, and the bHLHLZ protein Max was identified as a c-Myc obligate partner protein (Blackwood E. M. *et al.*, 1991).

The c-Myc RNA and protein have short half lives (30 min and 20 min, respectively) as compared to those of Max (3 hrs and >24 hrs, respectively), and in most

systems Myc appears to be the limiting, regulated component of the heterodimer (Facchini L.M. *et al.*, 1998). Dimerization of Myc and Max through the HLH LZ domain aligns the adjacent basic regions on each molecule to grip onto specific DNA hexanucleotide core sequences, termed E boxes (5'-CA[C/T]GTG-3'). Max can also bind other bHLH LZ Mad family proteins, which mediates transcriptional silencing. Mad levels, as opposed to Myc, increase during differentiation, and decreased expression of Mad2 (Mxi-1) protein has been implicated in the development of cancer in a mouse model (Zervos A.S. *et al.*, 1993). It is likely that both activation and repression functions of Myc are required for neoplastic transformation (Facchini L.M. *et al.*, 1998). The DNA bound Myc-Max heterodimer interacts through the Myc N-terminal region with a variety of proteins involved in transcription. The Mad-Max complexes, in contrast to Myc-Max complexes, recruit histone deacetylases that induce compact chromatin structures, which in turn limit access of transcription factors to DNA (Ayer D.E. *et al.*, 1993) (Figure 1.3.4).

1.3.3 c-Myc REGULATION

The expression of c-Myc was found to be regulated at every possible level: transcriptionally (initiation and elongation), post-transcriptionally (mRNA stability and translation) and post-translationally (protein stability) (Lusher B. *et al.*, 1990, Kelly K. *et al.*, 1983, and Sears R. *et al.*, 1999).

In general, c-Myc expression correlates tightly with the proliferative potential of the cell (Henriksson M. *et al.*, 1996). In quiescent cells, c-Myc expression is virtually undetectable. Upon mitogen or serum stimulation, there is a rapid and transient burst in

both c-myc mRNA and c-Myc protein expression as cells enter the G1 phase, followed by a gradual decline to low but detectable steady-state levels in proliferating cells (Campisi J. *et al.*, 1984). This induction of c-myc transcription occurs in the absence of de novo protein synthesis, indicating that c-myc is an immediate early gene and is directly downstream of mitogenic signaling cascades. A variety of overlapping control mechanisms cooperate to regulate c-Myc, allowing Myc expression and function to be rapidly and efficiently modulated in response to internal and external signals. c-myc RNA production is modulated transcriptionally at the level of initiation through mechanisms such as the homeostatic Myc negative autoregulation mechanism (Grignani F. *et al.*, 1990) as well as by transcriptional attenuation. c-Myc expression is also regulated posttranscriptionally at the levels of mRNA stability and protein translation. Indeed, and as mentioned earlier, c-myc mRNA and Myc protein are both short-lived molecules with half-lives of less than 30 min (Hann S.R. *et al.*, 1984). Therefore, to achieve functional Myc activity within the cell, active and continuous synthesis of both c-myc RNA and protein is required.

Regulation of c-Myc protein activity also occurs posttranslationally through direct and indirect mechanisms. These can potentiate or antagonize Myc activity, and include protein phosphorylation as well as interaction with other cellular proteins. Moreover, it has been found that c-Myc turnover is regulated in vivo by the ubiquitin proteasome pathway (Salghetti S.E. *et al.*, 1999). In many cases, proteins that are targeted for degradation by the ubiquitin proteasome pathway are first marked by phosphorylation of specific residues, allowing for their recognition by the ubiquitinylation machinery (Karin

M. *et al.*, 2000). There are two phosphorylation sites, Threonine 58 and Serine 62, in the N-terminus of c-Myc that are conserved across species and in c-Myc family members. These sites are particularly interesting as they are serum regulated and fall within a mutational hotspot found in c-myc alleles isolated from Burkitt's lymphomas. Moreover, Serine 62 is a target of the Extracellular Receptor Kinase (ERK) and Threonine 58 is targeted by Glycogen Synthase Kinase (GSK-3β) (Pulverer B.J. *et al.*, 1994). Thus, phosphorylation at both of these sites is regulated by Ras-activated signaling pathways: the Raf/MEK/ERK kinase cascade and the Phosphatidylinositol- 3-OH kinase (PI3K)/Akt pathway, which negatively regulates GSK-3β (Figure 1.3.5). Analysis of the effects of Serine 62 and Threonine 58 phosphorylation on c-Myc protein stability revealed that Serine 62 phosphorylation stabilizes c-Myc, while Threonine 58 phosphorylation destabilizes c-Myc. In addition, Threonine 58 phosphorylation requires prior phosphorylation of Serine 62 (Sears R. *et al.*, 2000). Thus, as depicted in Figure 1.3.5, mitogen stimulation of cell proliferation leads to new c-Myc protein synthesis and Ras activation. This promotes c-Myc protein stabilization through ERK-mediated phosphorylation of Serine 62. Ras activation also prevents the subsequent phosphorylation of c-Myc on Threonine 58 by Akt mediated inhibition of GSK-3β, further stabilizing c-Myc and increasing c-Myc protein levels. Later in G1 phase of the cell cycle, as Akt signaling drops, GSK-3β is reactivated and promotes c-Myc degradation by phosphorylating Threonine 58. This dual mechanism allows for precise regulation of c-Myc protein levels (Sears R., 2004) (Figure 1.3.5). Thus, phosphorylation events that alter c-Myc protein stability appear to play a very prominent role in the proper regulation of c-Myc expression levels during stimulation of cell proliferation. Since

Serine 62 phosphorylation must occur prior to Threonine 58 phosphorylation, this result suggested that a phosphatase could be involved in regulating c-Myc protein stability (Yeh E. *et al.*, 2004). Analysis with various inhibitors of known Serine/Threonine phosphatases suggested that protein phosphatase 2A (PP2A) might regulate c-Myc protein levels. Also, it has been shown that inhibition of PP2A using the chemical inhibitor, okadaic acid, the inhibitory siRNA directed against the PP2A catalytic subunit, or the simian virus 40 (SV40) small T antigen, which is a potent and specific inhibitor of PP2A, can increase c-Myc protein half-life (Yeh E. *et al.*, 2004). Moreover, Sears and colleagues have reported that PP2A can interact with the N-terminus of c-Myc and that Serine 62 is a direct target for dephosphorylation by PP2A. Therefore, a role for PP2A in destabilizing c-Myc is consistent with a number of studies showing that some PP2A holoenzymes exhibit tumor-suppressor activity and that inhibition of PP2A contributes to some human cancers (Sontag E. *et al.*, 1993, Kawabe T. *et al.*, 1997, and Li M. *et al.*, 1996).

One other player in the c-Myc degradation pathway is the ubiquitinylation machinery that poly-ubiquitinylates c-Myc, targeting it for proteolytic degradation. Ubiquitinylation of a target protein involves three enzymatic activities: the E1 ubiquitin activating enzyme, which is conserved, the E2 ubiquitin conjugating enzyme that is also common to multiple targets, and the E3 ubiquitin ligase, which contains the substrate-specific recognition subunit (Ciechanover A., 1994). Two recent papers have suggested that the F-box protein, Fbw7/hCdc4, is the substrate recognition subunit of a c-Myc E3 ubiquitin ligase (Yada M. *et al.*, 2004 and Welcker M. *et al.*, 2004). As ubiquitinylation

of c-Myc by Fbw7 was shown to be dependent on phosphorylation of Threonine 58, and Fbw7 is a known tumor suppressor, it is likely that Fbw7 plays an important role in c-Myc degradation (Sears R., 2004).

With a better understanding of how c-Myc protein levels are regulated, the therapies may be more effective and could be tailored to specific players in the c-Myc degradation pathway.

1.3.4 c-Myc ROLE IN CANCER DEVELOPMENT

c-Myc is expressed at elevated levels in most tumors (Spencer C.A. *et al.*, 1991). In addition, several tumors contain genetic alterations (i.e., translocations, gene amplifications and mutations in regulators of c-myc expression), which directly affect c-Myc expression (Nesbit C.E. *et al.*, 1999). As Weinberg and coworkers showed in the 1970s, rat embryo cells could be transformed when c-myc and ras were co-transfected. Since then, a variety of models have clearly indicated that c-myc is oncogenic. When immortalized rat fibroblasts are engineered to overexpress c-Myc, they acquire the ability to grow in soft agar, signifying loss of contact inhibition commonly found in tumor cells. Transgenic animals with tissue-targeted expression of c-Myc form tumors in the targeted tissues. Also, more recently it has been shown that the conditional induction of c-myc transgene in vivo in keratinocytes, mammary epithelial cells or hematopoietic cells can lead to reversible proliferation and clonal expansion, a hallmark of neoplasia (Dang C.V., 1999). Moreover, c-Myc has immortalizing properties as it extends the replicative life span of primary human cells, presumably by activating the expression of the htert gene

encoding the catalytic subunit of telomerase (Wang J. *et al.*, 1998). Also, the c-myc gene is rapidly induced by a variety of mitogens, and its level of expression is generally elevated in actively proliferating cells (Dang C.V., 1999). Although a link between c-Myc and cancer is well established both in vivo and in vitro, the molecular cellular mechanisms of c-Myc-mediated transformation are not fully known. It is important; however, to distinguish between c-Myc targets induced in normal cell homeostasis versus those induced in pathophysiological, neoplastic conditions in which c-Myc is overexpressed.

In a study of approximately 1000 breast cancer patients, amplification of c-myc was found in 17.1% (Berns E.M. *et al.*, 1992). For comparison, the oncogene HER2/neu was amplified in 18.7% of the cases analyzed (Berns E.M. *et al.*, 1992). Another report found that a group of patients with a rapid disease reoccurrence (mean disease free time: 1.4 years) or progression (mean disease free time: 1.4 years) had c-myc amplifications with a frequency of 56%, whereas patients with > 6.4 years of disease free survival had a frequency of 30% c-myc amplifications. Others have provided evidence that c-myc is in fact downstream of the receptor tyrosine kinase HER2/neu, which is commonly subject to oncogenic activation in breast cancer (Hynes N.E. *et al.*, 2001).

Moreover, transgenic mice have been generated to target the c-myc gene to the mammary glands (Amundadottir L.T., Merlino G. *et al.,* 1996, Nass S.J. *et al.,* 1997). In these c-myc transgenic mice, the transgene was expressed at high levels, specifically in the mammary and salivary glands of females (Stewart T.A. *et al.,* 1984). Spontaneous

carcinomas developped in mammary glands, at a frequency of roughly 50%, at about one year of age in the virgin females (Amundadottir L.T. *et al.,* 1995, 1996 and Rose-Hellekant T.A. *et al.,* 2000). Males though did not develop the tumors. Multiple pregnancies were found to significantly increase the incidence and shorten the tumor latency (Stewart T.A. *et al.,* 1984, Amundadottir L.T. *et al.,* 1995, 1996 and Nass S.J. *et al.,* 1997), indicating that certain physiological growth stimuli to the mammary gland, such as estrogen or progesterone, may serve as promoters of carcinogenesis.

As discussed previously, estrogens play complex roles in mammary gland development and carcinogenesis. The roles of estrogens in cell proliferation in their target organs are presumably exerted, in part, via a set of estrogen-responsive genes, including c-fos, c-jun, and c-myc (Schuchard M. *et al.,* 1993, Hyder S.M. *et al.,* 1994). Many in vitro studies and some in vivo experiments (mainly in uterine tissue) have shown that expression of c-myc mRNA is induced by treatment of estrogens (Schuchard M. *et al.,* 1993, Shiu R.P. *et al.* 1993, Hyder S.M. *et al.* 1994). However, it remains obscure if and how estrogen-ER signaling regulates c-Myc expression in human breast tumors (Miller T.L. *et al.,* 1993), as several reports show that overexpression and/or amplification of c-myc occurs preferentially in ER-negative tumors. Estrogen action on Myc expression will be described later in this work.

1.3.5 c-Myc AS A CANCER THERAPEUTIC TARGET

An advantage of c-Myc as a cancer therapeutic target may be the fact that it is downstream of multiple converging signaling pathways, which are affected by mutations in a number of different genes in diverse types of cancer. Recent experiments with mice

harboring conditional alleles of c-Myc suggest that even a short inactivation of c-Myc may be sufficient to stop tumor cell proliferation (Jain M. *et al.*, 2002). Different studies have suggested that a decrease in c-Myc activity could potentially inhibit tumor progression and may even lead to regression of existing tumors (Jain M. *et al.*, 2002 and Bazarov A.V. *et al.*, 2001). Unfortunately, transient side effects are expected after inhibition of c-Myc function by systemic treatment since c-Myc is widely expressed in regenerating compartments, like the hematopoietic system, epithelial linings of the digestive tract and the skin (Hermeking H., 2003). Also, as with other tumor-therapeutic approaches, inhibition of c-Myc function in tumors may be problematic because of the obstacles that tumors represent for drug-delivery. For example, vascularization is generally poor inside a tumor compared to surrounding tissues. A drug specific for the Myc protein or gene has to reach the nucleus of the target cell. This limits the approaches to small membrane permeable molecules as opposed to antibodies targeted against receptor tyrosine kinases, which reside at the cell surface (Hermeking H., 2003).

The currently conceivable strategies for therapeutically targeting Myc at the level of the gene, mRNA, or protein are depicted in Figures 1.3.6 and 1.3.7. One of these strategies is the use of Anti-sense DNA Oligonucleotides (aODNs) to target Myc. In fact, different studies suggest that the proliferation of different tumor cell lines could be inhibited by aODNs (Koster R. *et al.*, 1996, Leonetti C. *et al.*, 2001, Junghans M. *et al.,* 2000, Zhang Y.M. *et al.*, 2001, and Pastorino F. *et al.*, 2001). Also, Myc can be targeted by Anti-sense mRNA and two reports showed that the cells responded to this treatment by undergoing apoptosis (Chen J.P. *et al.*, 2001, and Ebinuma H. *et al.*, 2001). Moreover,

the use of triple-helix forming oligonucleotides (inhibition of gene transcription) (McGuffie E.M. *et al.*, 2002) or quadruplex-forming oligonucleotides (use of secondary DNA structures) have been shown to work effectively to inhibit c-Myc (Simonsson T. *et al.*, and Hurley L.H., 2001). Furthermore, Myc can be targeted by either ribozymes (Dolnikov A. *et al.*, 1996) or by using RNA interference (Lewis D.L. *et al.*, 2002), both strategies leading to apoptosis of the transfected tumor cells.

Also, it may be hard to identify drugs which specifically down-regulate c-myc, since a screen based on repression of c-myc would yield a large number of inhibitors of upstream mitogenic signaling cascades. Down-regulation of c-myc would only be a secondary effect in those cases. Moreover, transformation by c-Myc is dependent upon dimerization with the protein Max, since c-Myc/Max heterodimers facilitate binding of c-Myc to E-boxes in the vicinity of target genes, which mediate oncogenic effects of c-Myc (Amati B. *et al.*, 1993). Therefore, another strategy to inhibit Myc functions would be to interfere with the dimerization of c-Myc and Max (Figure 1.3.7) (Giorello L. *et al.*, 1998).

Gene product[a]	Regulation	Technique	Relevance to c-Myc
ARF or p19	Up	Guess	Apoptosis
CAD	Up	Promoter	Growth and metabolism
Cdc2	Up	Guess	Growth related
Cdc25A	Up	Guess	Growth related
Cyclin A	Up	Guess	Growth related
Cyclin D1	Up or down	Guess/diff	Growth related
Cyclin E	Up	Guess	Growth related
DHFR	Up	Promoter	Growth and metabolism
ECA39	Up	Diff	Amino acid transport
eIF-2α	Up	Guess	Growth-related metabolism
eIF4E	Up	Guess	Growth-related metabolism
ISGF3γ	Up	Guess	Stress response
LDH-A	Up	Diff	Growth and metabolism
MrDb	Up	Binding	Metabolism
ODC	Up	Diff/guess	Growth related
PAI-1	Up	Diff	?
α-Prothymosin	Up	Diff	Growth related
p53	Up	Diff/promoter	Growth related
RCC1	Up	Guess	Growth related
Rcl	Up	Diff	Growth related
Telomerase	Up	Guess	Immortality
TK	Up	Guess	DNA metabolism
Albumin	Down	Promoter	?
Collagens α1(I), α2(I), α3(VI), α1(III)	Down	Diff	Adhesion
C/EBPα	Down	Promoter	Differentiation
Gadd 45	Down	Diff	Growth
Ig lambda	Down	Promoter	?
LFA-1	Down	Guess	Adhesion
MHC class I	Down	Guess	Immune surveillance
Tdt	Down	Promoter	?
Thrombospondin	Down	Diff	Metastasis

Figure 1.3.1: Putative c-Myc target genes.
(Dang C.V., 1999)

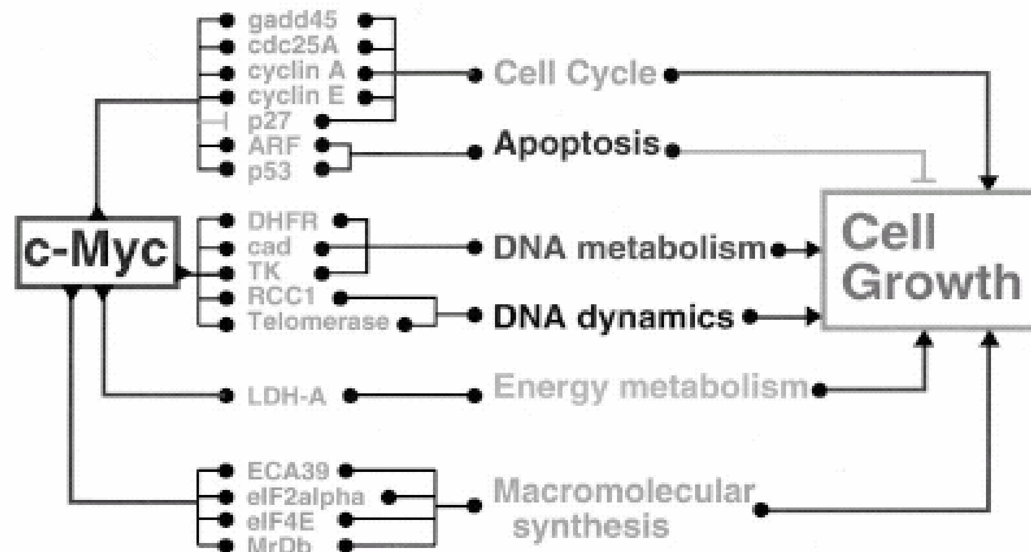

Figure 1.3.2: Links between c-Myc, selected putative target genes, cellular functions, and cell growth.
(Dang C.V., 1999)

This diagram illustrates the complexity of the connections between c-Myc and its putative target genes, which are shown clustered according to their functions. The various cellular functions cooperate to promote cell growth. It should be noted that this diagram does not reflect the controversies over the authentication of the various target genes.

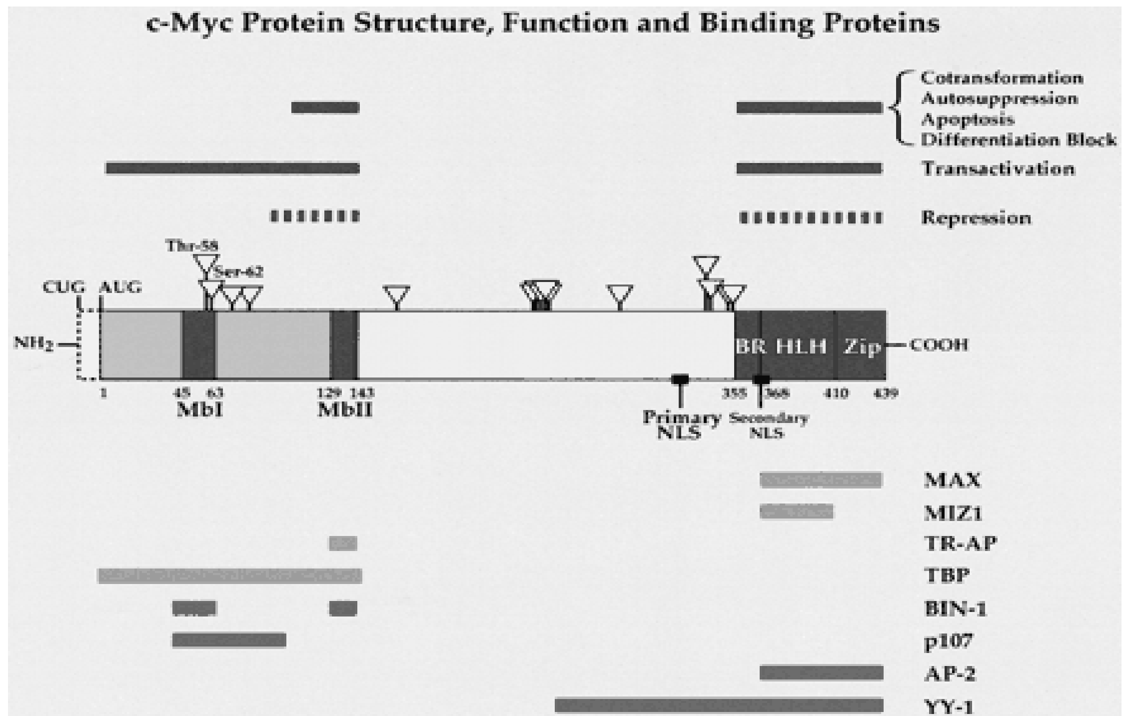

Figure 1.3.3: Structure of the human c-Myc protein including major functional domains and sites of phosphorylation (triangles).
(Facchini L. M., et al. 1998)

The aa region from 1–143 corresponds to the transactivation domain. Regions of the c-Myc protein required for Myc biological and transcription activity are indicated above the protein; regions involved in binding to cellular proteins are shown below. The broken line indicates that the domains required for Myc-mediated transcriptional repression are not yet well defined. Binding proteins that promote Myc function are shown in light grey and antagonists of Myc activity are shown in dark gray. BR, basic region (aa 355–367); HLH, helix-loop-helix (aa 368–410); Zip, leucine zipper (aa 411–439); MbI, Myc box I (aa 45–63); MbII, Myc box II (aa 129–141); NLS, nuclear localization signal.

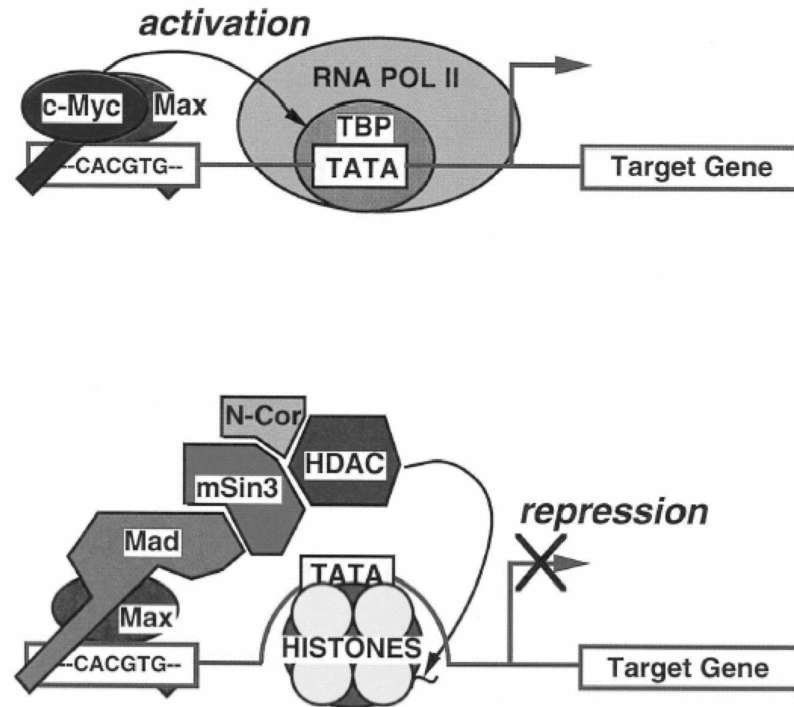

Figure 1.3.4: Models of c-Myc/Max and Mad/Max in transcriptional regulation. (Dang C.V., 1999)

The c-Myc/Max heterodimer is shown at the top tethered to the E box 59-CACGTG-39. c-Myc contacts TBP, although the molecular mechanisms involved in c-Myc transactivation are not known. The bottom diagram depicts the association of the Mad/Max heterodimer with the E box, as well as with mSin3, N-Cor, and histone deacetylase (HDAC). HDAC deacetylates histones, causing the locking of nucleosomal DNA and, consequently, inhibition of transcription. POL, polymerase.

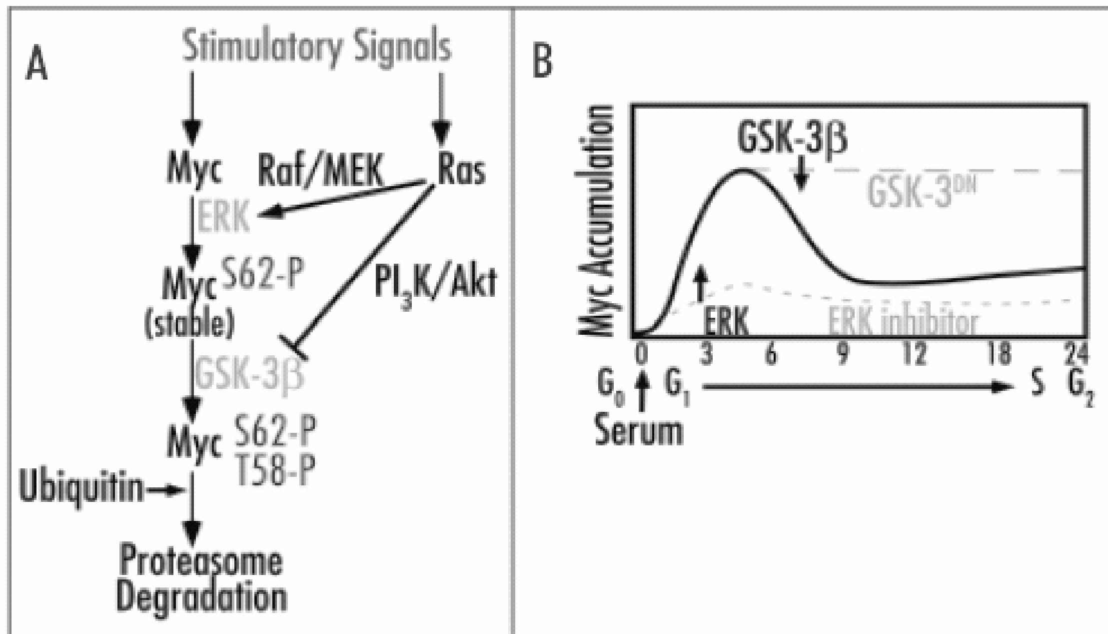

Figure 1.3.5: Phosphorylation events control c-Myc protein stability. (Sears R., 2004)

(A) Signaling pathways that regulate c-Myc phosphorylation and accumulation. (B) c-Myc protein accumulation profile in response to serum stimulation. ERK-mediated phosphorylation contributes to the rapid increase in c-Myc protein levels in early G1 while GSK-3β activation in late G1 leads to the downregulation of c-Myc. Effects of ERK inhibition and dominant negative GSK-3β (GSK3DN) on c-Myc accumulation are shown with dashed lines.

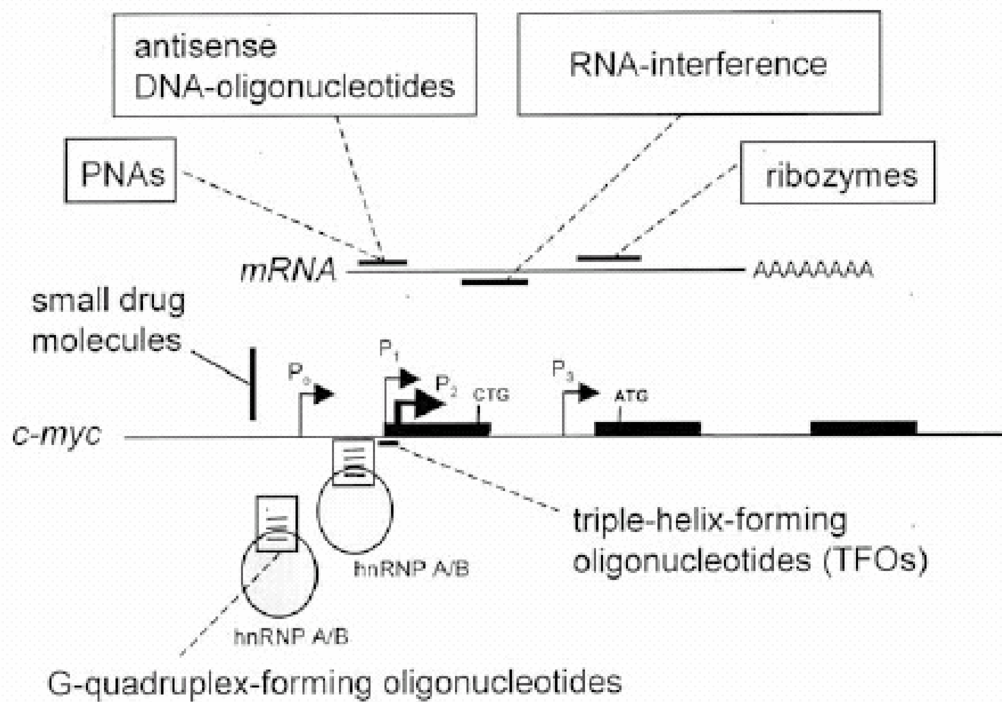

Figure 1.3.6: Strategies for inhibition of c-Myc expression.
(Hermeking H., 2003)

Map of the human c-*myc* gene depicting the pharmacological strategies for inhibition of c-*myc* expression described in the text. Transcription start sites are shown as arrows. Closed boxes represent exons. The 2 alternative start codons for the p67 and p64 c-MYC proteins are indicated by CTG and ATG, respectively. Similar strategies may apply to the N-*myc* gene.

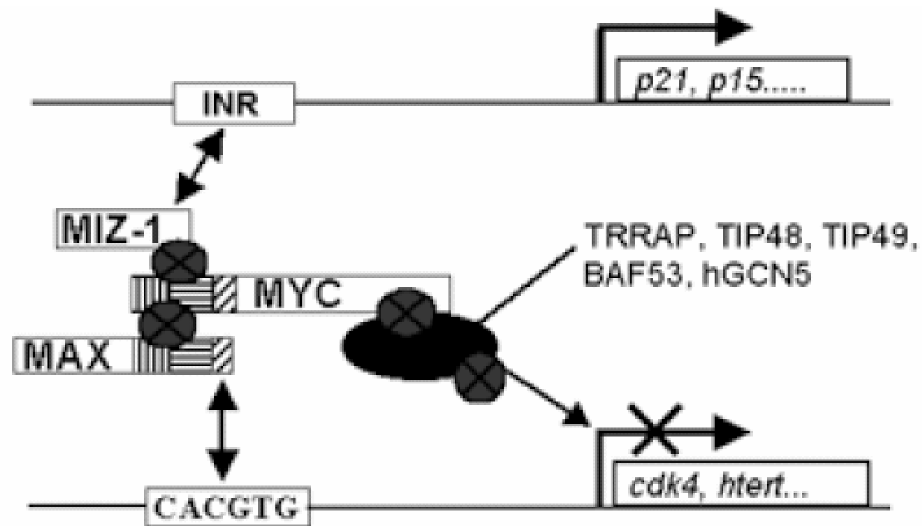

Figure 1.3.7: Pharmacological targeting of the c-Myc protein.
(Hermeking H., 2003)

Attractive points for pharmacological interference are depicted by crossed circles. Berg *et al.* identified a small molecule inhibitor of the MYC/MAX heterodimerization. The other points of interference proposed in this figure represent attractive pharmacological targets for future investigation. The *p21* and *p15* genes are examples of c-MYC-repressed genes, whereas *cdk4* and *htert* are c-MYC-induced genes. The arrows at the promoters indicate the outcome of the suggested treatments. The MYC, MAX and MIZ-1 proteins are shown as rectangles, with the basic region/helix-loop-helix/leucine zipper motif (BR-HLH-LZ) in different patterns. Transcriptional co-factors (TRRAP, TIP48/49, BAF53 etc.) or enzymatic activities (HAT (histone acetyltransferase), helicase) associated with the c-MYC N-terminus are shown as a black circle. A typical c-MYC DNA-binding site (E-box) is depicted as “CACGTG”. An initiator element (INR) is represented in the *p21* and *p15* genes. Similar strategies may apply to the N-MYC protein.

CHAPTER II

MATERIALS AND METHODS

2.1 CELLS, CELL CULTURE CONDITIONS AND TRANSFECTION

MCF-7 cells were obtained from the American Type Culture Collection and were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% bovine calf serum. Transfections for the siRNA were performed using a siRNA Kit (Cell Signaling) according to the vendor's instructions. Transfection efficiency (routinely in excess of 70%) was determined by transfection of the control siRNA (Cell Signaling), which expressed green fluorescent protein. Transient transfections of the PLD2 or mTOR plasmids were performed using Lipofectamine 2000 (Invitrogen) according to the vendor's instructions. Transfection efficiency was determined by transfection of pEGFP-C1 (Clonetech), which expresses green fluorescent protein (GFP). The percentage of green cells was determined microscopically and was in excess of 75%.

2.2 MATERIALS

Rapamycin was obtained from Sigma-Aldrich, wortmannin as well as cycloheximide, lactacystin and okadaic acid were obtained from Calbiochem. Polyclonal antibodies to poly-(ADP-ribose) polymerase (PARP) (human specific), Akt, phosphorylated Akt (Ser 473), ribosomal subunit S6-kinase (S6-kinase), phosphorylated S6-kinase (Thr 389), 4E-BP1 and phosphorylated 4E-BP1 (Thr 37/46) were obtained from Cell Signaling Technology as well as the Polyclonal antibodies to phosphorylated Myc (Thr58), GSK-3β and phosphorylated GSK-3β (Ser9). Antibodies to Myc and Actin were obtained from Santa Cruz Biotechnology. [^{3}H]-myristic acid was obtained from New England Nuclear. The antibody to phosphorylated Myc (Ser62) was obtained from Anaspec, and a polyclonal rabbit anti-ubiquitin antibody was obtained from Dako.

2.3 RNA ANALYSES

Total RNA was isolated using the RNAqueous commercial kit (Ambion) following the manufacturer's protocol. Three to five µg of total RNA/sample were run on a 0.8% agarose gel and transferred to nylon membrane for Northern blot analyses. A 2.1 Kbp NotI-SalI genomic fragment of human c-myc gene (ATCC) coding region was used as a probe for the mRNA product of the transgene. To normalize for variations in sample loading, blots were stripped and reprobed with a probe to 18 S rRNA (Ambion). All probes were labeled with [α-^{32}P] nucleotides using the random primers method (Invitrogen). Transgene mRNA signals were normalized to the loading control signal and quantified by PhosphorImager analysis (Molecular Dynamics).

2.4 PLASMIDS

The PcDNA3.1 control plasmid was obtained from Invitrogen. The plasmid expression vector for PLD2 (pCGN-PLD2) (Colley W.C. *et al.*, 1997) was a generous gift of Dr. Michael Frohman (SUNY-Stony Brook). pcDNA3.1-PLD2, containing a neomycin resistance gene, was constructed by excising the PLD2 sequence from pCGN-PLD2 with XbaI and SmaI and then inserting it into the XbaI and EcorV sites in pcDNA3.1. Cells were then selected with G418 sulfate over a period of 15 days. Pools of clones were then collected and used for experiments. pcDNA3.1-mTOR expression plasmids encoding WT, kinase-dead (KD; Asp2338Ala) rat mTOR (Brunn G.J. *et al.*, 1997) were kindly provided by Robert Abraham (Burnham Institute, San Diego, Calif.).

2.5 CELL VIABILITY AND APOPTOSIS ASSAYS

Cell viability was determined by trypan blue exclusion. After various treatments, cells were harvested, washed and treated with trypan blue at a concentration of 0.4% w/v. After 10 min, trypan blue uptake (dead cells) was determined by counting on a hemocytometer. Apoptosis was ascertained by cleavage of the caspases 3 and 7 substrates PARP as described previously (Zhong M. *et al.*, 2003, and Chen Y. *et al.*, 2003).

2.6 IMMUNOPRECIPITATION

The cells were washed twice with ice-cold phosphate-buffered saline and scraped into the modified radioimmune precipitation assay buffer: 50 mM Tris-HCl (pH 7.6), 1% Igepal CA-630, 0.25% sodium deoxycholate, 150mM NaCl, 10mM MgCl2, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, and 1X protease inhibitor mixture consisting of 0.5mM AEBSF (4-(2-aminoethyl)benzenesulfonyl fluoride), 1 μM leupeptin, 0.15 μM aprotinin, and 1 μM protease inhibitor E-64. The cells were then incubated at 4 °C for 25 min by gentle rocking, sonicated for 20 s on ice, and centrifuged at 12,000 X *g* at 4 °C for 10 min. The supernatant was precleared with protein G-Sepharose 4 Fast Flow beads (Amersham Biosciences), and 500 μg of the precleared proteins was adjusted to a volume of 500 μl in modified radioimmune precipitation assay buffer and then incubated with the antibody for overnight. The immunocomplex was captured by incubation with 50 μl of protein G-Sepharose 4 Fast Flow bead slurry and collected by centrifugation at 12,000 x *g* for 20 s at 4 °C. The beads were washed three times with modified radioimmune precipitation assay buffer and once with wash buffer (50 mM Tris, pH 7.6) and then subjected to Western blot analysis.

2.7 WESTERN BLOT ANALYSIS

Extraction of proteins from cultured cells was performed as previously described (Lu Z. *et al.*, 2000; Chen Y. *et al.*, 2003). Equal amounts of protein were subjected to SDS-PAGE using a 10% acrylamide separating gel, transferred to nitrocellulose membranes (Osmosis), membrane filters were then blocked for one hour at room temperature with 5% non-fat dry milk in isotonic phosphate-buffered saline (PBS: 136 mM NaCl, 2.6 mM KCl, 1.4 mM KH_2PO_4, 4.2 mM Na_2HPO_4) with 0.05% Tween 20 and then incubated with the appropriate antibodies diluted in 5% non-fat dry milk as described in the text. Depending upon the origin of the primary antibodies, either anti-mouse or anti-rabbit IgG was used for detection using the ECL system (Amersham).

2.7 PHOSPHOLIPASE D ASSAYS

PLD activity was measured by the ability of cells to convert the metabolically labeled [^{3}H]-PC into PBt in the presence of exogenously provided 1-BtOH. Cells were grown in 60 mm culture dishes to confluency and made quiescent as described above. These quiescent cells were labeled for 4-6 hr with [^{3}H]-myristate (40 Ci/mmol) at final concentration of 1 μCi/ml, and followed by 15 min of incubation with 1% (v/v) 1-BtOH. Afterwards, cells were placed on ice, washed twice with cold PBS and collected in 0.5 ml of methanol / 6 M HCl (50:1, v/v). Lipids were extracted by adding 0.5 ml of chloroform. Phase separation was achieved by the addition of 155 μl of 1 M NaCl and the organic phase was recovered after centrifugation. This was followed by reextraction through the addition of 350 μl H_2O, 115 μl of 1 M NaCl and 115 μl methanol. An aliquot of the obtained organic phase was counted in a liquid scintillation counter and the volume of each sample that had the same intensity of radioactivity was calculated according to the

readings. Lipids containing an equal amount of radioactivity were then dried under a stream of nitrogen and redissolved in 50 µl of chloroform / methanol (9:1, v/v). Samples were then spotted on a precoated silica (60A) plates and separated by thin layer chromatography (TLC) with a solvent system of ethylacetate/trimethylpentane/acetic acid/H_2O (9:5:2:10, v/v, upper layer). The transphosphatidylation product PBt was visualized by autoradiography of the TLC plates and the films were scanned in a densitometer for quantification.

CHAPTER III

SURVIVAL SIGNALS GENERATED BY ESTROGEN AND PHOSPHOLIPASE D IN MCF-7 BREAST CANCER CELLS ARE DEPENDENT ON MYC.

3.1 INTRODUCTION

A role for estrogens in the development of breast cancer has been indicated for over a century. It was demonstrated in 1896 that breast cancer remission could be induced by removal of the ovaries in a subset of breast cancer patients (Beatson G., 1896). Although not originally understood, the observed effects were the result of eliminating the primary source of estrogen in pre-menopausal woman. The effectiveness of anti-estrogen therapies such as tamoxifen and aromatase inhibitors in the prevention of breast cancer (Johnston S.R. *et al.*, 2003, and Jordan V.C. *et al*, 2004) also supports a critical role for estrogens in the promotion of breast cancer. The very low incidence of breast cancer in men is also consistent with a role for estrogens.

The role that estrogens, predominantly 17-β-estradiol (E2), play in the development of breast cancer is largely that of a tumor promoter - substances that do not directly lead to the mutations that convert a normal cell to a cancerous one. Tumor promoters typically stimulate the proliferation of cells that have acquired an activating mutation that facilitates the amplification of these “initiated” cells (Hennings H. *et al.*, 1993). One component of tumor promotion is the suppression of default apoptotic programs that are activated in cells with inappropriate growth signals or damaged genomes (Hanahan D. *et al.*, 2000). In this regard, it is of significance that E2 has been shown to suppress apoptosis in the estrogen receptor (ER) positive breast cancer cell line MCF-7 (Ahamed S. *et al.*, 2001, Fernando R.I. *et al.*, 2004, Huang Y., 1997, Teixeira C. *et al.*, 1995).

Breast epithelial cells typically express the ER in 15-25% of the cells of normal resting breast epithelia, whereas most (70%) breast cancers are ER positive (Ali S. *et al.*, 2002). These data indicate that in the development of breast cancer there is a selection for breast epithelial cells that express the ER. The apparent selection of ER positive breast epithelial cells in breast cancer further implicates estrogen in the development of breast cancer. Interestingly, most breast cancer cell lines are ER negative, indicating that the selection of breast cancer cell lines in culture involves the loss of estrogen responsiveness, a hallmark of poor prognosis in breast cancer (Sommer S. *et al.*, 2001). Thus, while breast cancers are still dependent upon estrogen, the tumors remain less aggressive. Gaining estrogen independence apparently provides a means for a tumor to become more aggressive.

The ability to gain independence from estrogen likely involves the activation of signals that lead to the suppression of apoptosis that was provided by estrogen. A candidate for replacing the estrogen-dependent suppression of apoptosis is phospholipase D (PLD), which is commonly elevated in breast cancer tissues (Noh D.Y. *et al.*, 2000, and Uchida N. *et al.*, 1997) and in breast cancer cell lines (Chen Y. *et al.*, 2003, and Zhong M. *et al.*, 2003). PLD was also shown to provide a survival signal in the ER negative breast cancer cell line MDA-MB-231 (Chen Y. *et al.*, 2005, and Zhong M. *et al.*, 2003). PLD cooperates with tyrosine kinases to transform rat fibroblasts (Hornia A. *et al.*, 1999, Joseph T. *et al.*, 2001, and Lu Z. *et al.*, 2000), suppresses apoptosis induced by high intensity Raf signaling (Joseph T. *et al.*, 2002), and prevents upregulation of the tumor suppressor p53 (Hui L. *et al.*, 2004). Thus, PLD is a good candidate oncogene in

cancers where elevated tyrosine kinase signaling is common – such as breast cancer. Elevated expression of tyrosine kinases such as the epidermal growth factor (EGF) receptor, Her2/Neu, and c-Src are commonly observed in breast cancer (Biscardi J.S. *et al.*, 1999).

These studies suggest that elevated PLD activity in breast cancer could provide survival signals that could allow an emerging breast cancer to progress to estrogen independence. Consistent with the hypothesis that PLD could generate survival signals, phosphatidic acid, the metabolite of PLD activity has been shown to target mTOR (Chen Y. *et al.*, 2003, Fang Y. *et al.*, 2001, and Fang Y. *et al.*, 2003), which has long been implicated in signals that suppress apoptosis and promote survival (Foster D.A., 2004, Schmelzie T. *et al.*, 2000, and Vivanco I. *et al.*, 2002).

In this section, we have investigated the ability of E2 to suppress apoptosis in the ER positive MCF-7 human breast cancer cell line. We provide evidence that an mTOR- and PLD-dependent activation of Myc is necessary for the suppression of apoptosis by E2 and that elevated PLD activity in these cells provides an estrogen independent survival signal.

3.2 RESULTS

3.2.1 E2 ENHANCES PROLIFERATION AND SURVIVAL OF MCF-7 CELLS IN THE ABSENCE OF SERUM

It was previously reported that MCF-7 cells, which are ER positive (Lacroix M. *et al.*, 2004), undergo apoptosis when deprived of serum; whereas, MDA-MB-231 breast cancer cells, which are ER negative (Lacroix M. *et al.*, 2004), survived under these conditions (Chen Y. *et al.*, 2005). After 1 day in the absence of serum, approximately 25% of MCF-7 cells lose viability and if maintained in the absence of serum, the percentage of non-viable cells goes to 68% at 5 days (Figure 3.2.1A). As shown in Figure 3.2.1B, there are corresponding increases in the cleavage of the caspase 7 substrate PARP indicating that the loss of cell viability was due to apoptotic cell death. Estrogens have been widely implicated in the proliferation of breast epithelial and human breast cancer cells (Foster J.S. *et al.*, 2001, and Foster J.S. *et al.*, 2003). Moreover, E2 has been reported to suppress apoptosis (Ahamed S. *et al.*, 2001, Fernando R.I. *et al.*, 2004, Huang Y., 1997, Perillo B. *et al.*, 2000, and Teixeira C. *et al.*, 1995). We therefore examined the effect of E2 on the proliferation and survival of MCF-7 cells deprived of serum. As shown in Figure 3.2.1C, there was a significant increase in the number of cells maintained in the presence of E2 relative to the untreated control cells. Significant differences in cell number between the E2 treated and untreated cells could be detected at the 5 day point where apoptosis was observed in Figures 3.2.1A and 3.2.1B.

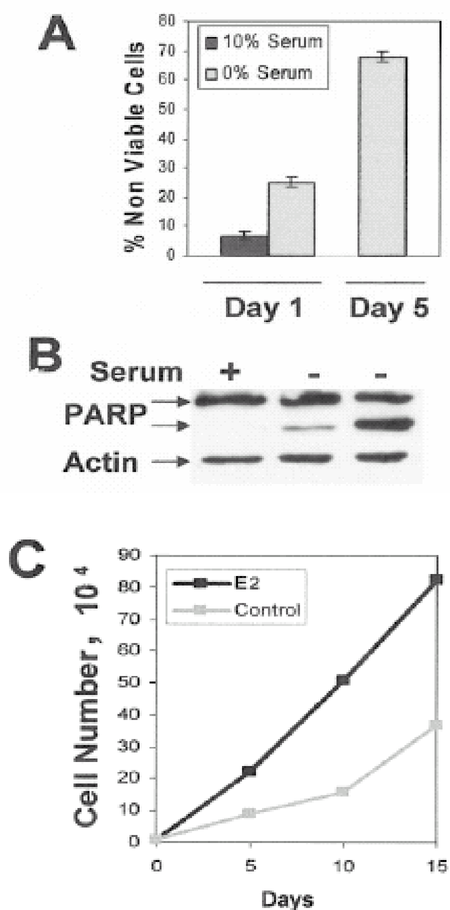

Figure 3.2.1: E2 enhances proliferation and survival of MCF-7 cells in the absence of serum. (A) MCF-7 cells were plated at $2x10^4$ cells/60-mm culture dish in DMEM with 10% bovine calf serum. After 24 h, the medium was changed to phenol red-free DMEM with either 0% or 10% charcoal dextran-treated serum as indicated. Both adherent and non-adherent cells were harvested at either 1 or 5 days of treatment, at which time the percentage of nonviable cells was determined by trypan blue exclusion. (B) MCF-7 cells were treated as in panel A, and lysates were prepared and analyzed for the level of PARP protein by Western blot analysis. Blots were stripped and reprobed with an antibody to actin as a loading control. The upper band represents uncleaved PARP, and the lower band represents cleaved PARP. (C) MCF-7 cells were plated as in panel A, and after 24 h (day 0) the medium was changed to phenol red-free DMEM depleted of serum. Ethanol (0.1%) as the control or E2 (2 nM) was then added, cells were harvested at the indicated time points, and cell number was determined. Medium with and without E2 was replenished every 48 h. Error bars in panels A represent the standard deviations for three independent experiments. Experiments in panels B, C are representative of three independent experiments.

We next examined the effect of E2 on cell viability and PARP cleavage in MCF-7 cells deprived of serum for 1 or 5 days. E2 increased cell viability (Figure 3.2.1D) and suppressed PARP cleavage (Figure 3.2.1E). The effect of E2 was observed at 5 days where the percentage of non-viable cells dropped from 68% to less than 25% in the presence of E2. These data indicate that E2 generates signals that allow MCF-7 cells to survive in the absence of serum.

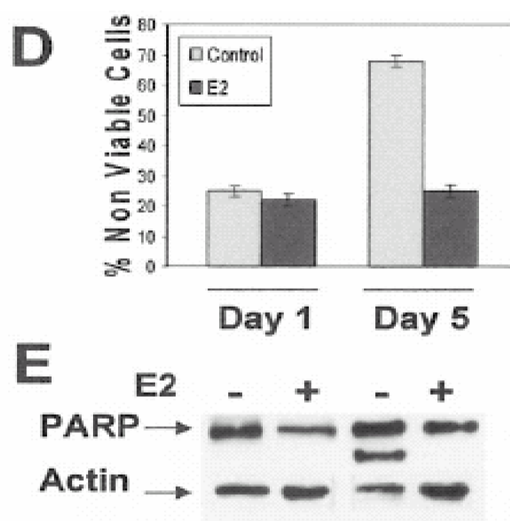

Figure 3.2.1. (Continue) (D) MCF-7 cells were plated and treated as in panel C. Cells were harvested and analyzed as in panel A. (E) Lysates from MCF-7 cells treated as in panel D were analyzed for PARP cleavage as in panel B. Error bars in panels D represent the standard deviations for three independent experiments. Experiments in panels E are representative of three independent experiments.

3.2.2 E2 STIMULATES A DELAYED SUSTAINED ELEVATION IN MYC EXPRESSION

Myc expression has been strongly correlated with survival signals (Pelengaris S. *et al.*, 2002). A transient induction of Myc by E2 has been reported to occur within 30 min after E2 treatment (Dubik D. *et al.*, 1992), and a second phase at 24 hr (Santos G.F.

et al., 1988). However, as indicated in Figure 3.2.1, the effects of E2 on the survival of MCF-7 cells are not apparent until 5 days after treatment. We therefore examined the expression of Myc in response to E2 over the 5-day time course where the survival effects of E2 on MCF-7 cells were observed. As shown in Figure 3.2.2A, there was a short-term transient increase in Myc expression in response to E2 as reported previously (Dubik D. *et al.*, 1992). There was also a corresponding increase in Myc RNA (Figure 3.2.2B). However, as shown in Figure 3.2.2C, there was a large stable increase in Myc expression that could be detected at 2 days and was increased at 5 days where the survival effects of E2 become apparent. Interestingly, there was no increase in myc mRNA levels during this time (Figure 3.2.2D).

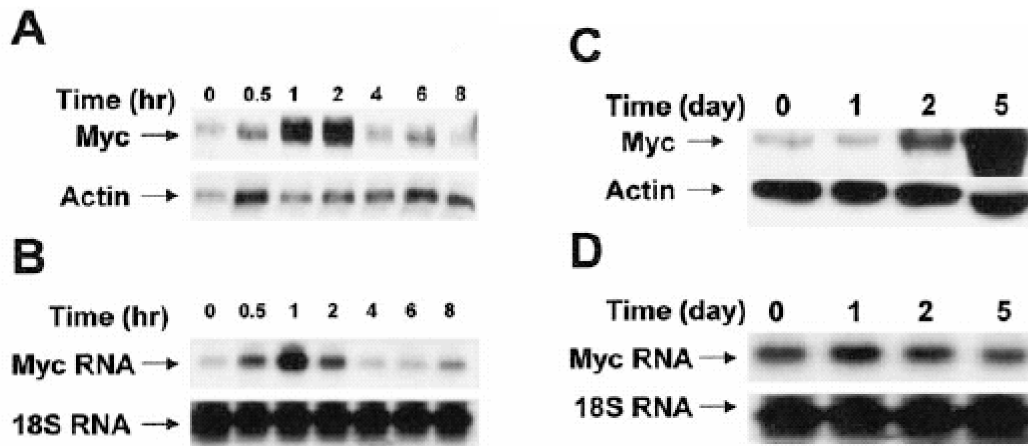

Figure 3.2.2: E2 stimulates elevated expression of Myc. MCF-7 cells were plated as for Figure 3.2.1 and placed in phenol red-free DMEM with 0% serum for 24 h. E2 (2 nM) was then added, and the cells were collected at the indicated times. Lysates were prepared and analyzed for the levels of Myc protein by Western blot analysis (A and C). Blots were reprobed with an antibody to actin to control for loading. (B and D) Northern blots analysis of the indicated samples were run using a c-*myc* probe. An 18S rRNA probe was used as a loading control. The experiment shown is representative of at least two independent experiments.

3.2.3. SUPPRESSION OF MYC EXPRESSION BLOCKS THE SURVIVAL EFFECTS OF E2

To determine if elevated Myc expression was required for the survival effects of E2, we treated the MCF-7 cells with Myc siRNA. MCF-7 cells were subjected to serum withdrawal in presence of E2 for 3 days at which time Myc siRNA and control siRNA were introduced by transient transfection. Cell viability and PARP cleavage were examined 48 hr later, which was at the 5-day time point of E2 treatment where the increased Myc expression was maximal and the survival effect was most apparent. As shown in Figure 3.3, Myc siRNA blocked E2 mediated cell survival (Figure 3.2.3A) and reversed the suppression of PARP cleavage (Figure 3.2.3B). The level of Myc protein was also examined and as shown in Figure 3.2.3C, Myc expression was very efficiently blocked by the Myc siRNA. These data indicate that Myc expression is required for the survival signals generated by E2.

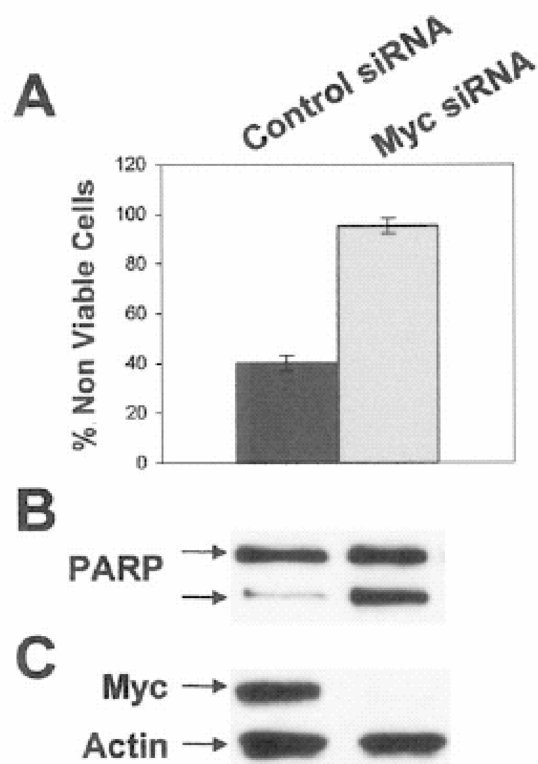

Figure 3.2.3: Suppression of Myc expression blocks the survival effects of estrogen. (A) MCF-7 cells were plated as in Figure 3.2.1, and 24 h later, the medium was changed to phenol red-free DMEM without serum containing E2 (2 nM). Seventy-two hours later, the cells were transiently transfected with Myc siRNA vector and the control siRNA vector. Forty-eight hours later, the cells were harvested, at which time the percentage of nonviable cells was determined by trypan blue exclusion. Error bars represent the standard deviations for three independent experiments. Transfection efficiency was determined by the percentage of green cells in the control siRNA culture, which expressed the green fluorescent protein, and was routinely in excess of 70%. MCF-7 cells from panel (A) were also analyzed for PARP cleavage (B) and Myc expression (C) as in Figure 3.2.1. Blots were reprobed with an actin antibody to control for loading. Experiments shown are representative of three independent experiments.

3.2.4. E2-INDUCED INCREASES IN MYC EXPRESSION ARE DEPENDENT ON BASAL LEVELS OF MTOR ACTIVITY IN MCF-7 CELLS

Although E2 has been reported to elevate Myc at the transcriptional level (Dubik D. *et al.*, 1992), Myc expression has also been reported to be dependent upon mTOR (the mammalian target of rapamycin), which regulates the translation of Myc RNA into Myc protein (West M.J. *et al.*, 1998). We therefore examined the effect of rapamycin on E2-induced cell survival and Myc expression. As shown in Figure 3.2.4, rapamycin reversed the E2-induced increase in cell survival (Figure 3.2.4A) and suppression of PARP cleavage (Figure 3.2.4B). Rapamycin also inhibited the E2-induced increase of Myc expression seen at 5 days (Figure 3.2.4C). We also examined the effect of E2 and rapamycin on the phosphorylation of the mTOR substrate S6-kinase. As expected, rapamycin suppressed phosphorylation of S6-kinase (Figure 3.2.4D). Somewhat surprisingly, E2 did not elevate S6-kinase phosphorylation, indicating that the E2-induced increase in Myc expression was not due to an effect upon mTOR. These data indicate that Myc expression induced by E2 is dependent upon basal levels of mTOR and further implicate Myc expression in E2-induced survival signals.

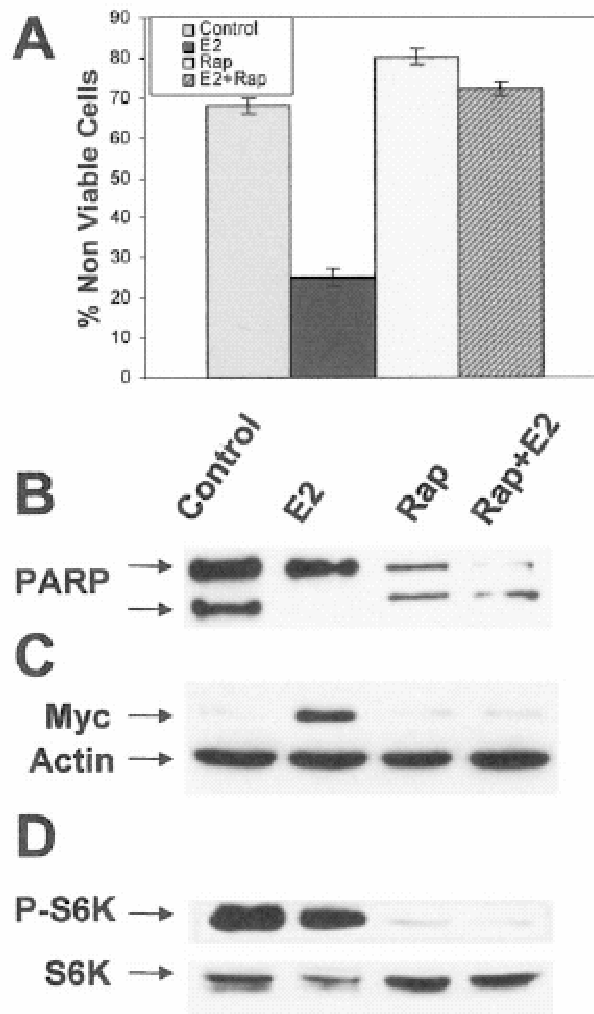

Figure 3.2.4: Survival signals generated by E2 are sensitive to rapamycin. (A) MCF-7 cells were plated as described for Figure 3.2.1 and placed in phenol red-free DMEM without serum after 24 h, along with either E2 (2 nM) or ethanol (0.1%) as the control. Ninety-six hours later (day 4) rapamycin (Rap) (20 nM) was added where indicated, cells were harvested 24 h later, and cell viability (A), PARP cleavage (B), and Myc levels (C) were determined as for Figure 3.2.3. Blots were reprobed with an actin antibody to control for loading. (D) Lysates were also analyzed for phosphorylated S6-kinase (P-S6K) and total S6-kinase (S6K) as a control for loading. Error bars in panel A represent the standard deviations for three independent experiments. Data in panels B, C, and D are representative of three independent experiments.

mTOR is activated in response to signals generated by phosphatidylinositol-3-kinase (PI3K) (Schmelzie T. *et al.*, 2000) and PLD (Chen Y. *et al.* , 2005). We therefore examined the requirement for PI3K and PLD in the E2 stimulated increase in cell survival, suppression of PARP cleavage and increased Myc expression. We used wortmannin to inhibit PI3K and primary alcohol (1-butanol) to block the production of phosphatidic acid by PLD. As a control for the primary alcohol, we used a tertiary alcohol (t-butanol), which does not interfere with PLD activity (Shen Y. *et al.*, 2001). The inhibitors were added either on day 4 (wortmannin) or on day 5 (alcohol) after the cells had been subjected to serum withdrawal. Cell viability, PARP cleavage, and Myc expression were examined 24 hr after the addition of wortmannin or 6 hr after addition of the alcohols.

As shown in Figure 3.2.5, the effect of E2 on cell viability (Figure 3.2.5A), PARP cleavage (Figure 3.2.5B), and Myc expression (Figure 3.2.5C) was reversed by both wortmannin and 1-butanol. t-butanol did not interfere with the effects of E2. We also examined the effect of 1-butanol and wortmannin on the phosphorylation state of S6-kinase, and as shown in Figure 3.2.5D, both 1-butanol and wortmannin suppressed the phosphorylation of S6-kinase in the E2-treated MCF-7 cells. To further establish that E2 induced cell survival and Myc expression was dependent upon PLD and mTOR, we employed catalytically inactive dominant negative mutants of PLD and mTOR. As shown in Figure 3.2.5E, the dominant negative PLD2 and mTOR reduced cell viability and increased PARP cleavage in E2-treated MCF-7 cells. These data further indicate a dependence on PLD and mTOR for E2-induced survival signals.

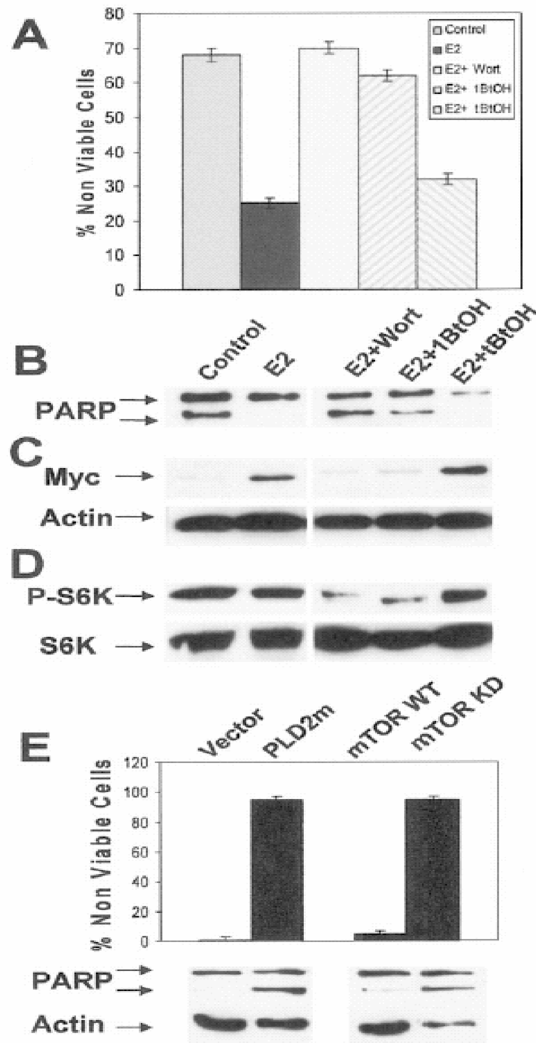

Figure 3.2.5: Survival signals generated by E2 are dependent on PI3K and PLD. MCF-7 cells were prepared as for Figure 3.2.4 and placed in serum-free, phenol red-free DMEM with or without E2 (2 nM) as indicated. Ninety-six hours later (day 4), wortmannin (Wort) (100 nM) was added as indicated. Twenty-four hours later the cells were treated with either the primary alcohol 1-butanol (1BtOH) or the tertiary alcohol *t*-butanol (3BtOH, tBtOH) (0.8%) as indicated for an additional 6 h. At this point, the cells were harvested and cell viability (A), PARP cleavage (B), Myc levels (C), and phosphorylated S6 kinase (PS6K) (D) were determined as for Figure 3.2.4. (E) MCF-7 cells were placed in serum-free, phenol red-free DMEM containing 2 nM E2. Three days later the cells were transiently transfected with either control empty vector or vector that expresses a catalytically inactive PLD2 (PLD2m), wild-type mTOR (mTOR WT), or a kinase-dead mTOR mutant (mTOR KD). Cell viability and PARP cleavage were determined 2 days later as for panel A. Error bars in panels A and E represent the standard deviations for three and two independent experiments, respectively. Experiments shown in panels B, C, and D are representative of three independent experiments.

The data in Figures 3.2.4 and 3.2.5 showed that while E2-induced survival was dependent upon mTOR signals, E2 apparently did not increase mTOR activity. To further establish this, we examined the effect of E2 on the mTOR activating signals of PI3K and PLD. To examine PI3K activity, we looked at the phosphorylation state of Akt, which gets phosphorylated when PI3K is activated (Vivanco I. *et al.*, 2002). As shown in Figure 3.2.6A, E2 did not increase phosphorylation of Akt at 5 days. PLD activity was measured directly using the transphosphatidylation reaction (Shen Y. *et al.*, 2001), and as shown in Figure 3.2.6B, E2 had no effect upon PLD activity. In contrast, EGF was able to increase both Akt phosphorylation and PLD activity in the MCF-7 cells (Figures 3.2.6A and 3.2.6B). These data, like the data in Figure 3.2.5D; indicate that E2 does not elevate the activity of either mTOR or its upstream regulators PI3K and PLD. The implication being that basal levels of mTOR activity are required for elevated Myc expression and survival signals generated by E2.

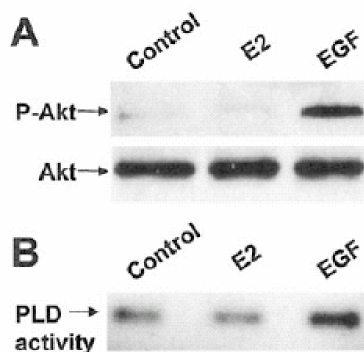

Figure 3.2.6: Effect of E2 on Akt phosphorylation and PLD activity. MCF-7 cells were prepared as for Figure 3.2.4 and placed in serum-free, phenol red-free DMEM with or without E2 (2 nM) as indicated. As a positive control, cells were also treated with EGF (100 ng/ml) where indicated. The cells were harvested 5 days later, and lysates were analyzed for the level of phosphorylated Akt and Akt protein by Western blot analysis (A). To determine the PLD activity, 0.8% 1-butanol was added at the 5-day time point, and the transphosphatidylation product phosphatidylbutanol was characterized 4 h later (B). Data shown are representative of three independent experiments. Error bars represent the standard deviations for three independent experiments.

3.2.5. ELEVATED EXPRESSION OF PLD2 IN MCF-7 CELLS INCREASES MYC EXPRESSION AND CONFERS ESTROGEN-INDEPENDENT SURVIVAL SIGNAL

Increasing PLD activity can lead to increased mTOR activity (Chen Y. *et al.*, 2003 and Fang Y. *et al.*, 2003), and elevated levels of PLD activity have been reported in several human cancers including breast (reviewed in Foster D.A. *et al.*, 2003). We reported previously that highly elevated PLD activity in the ER negative human breast cancer cell line MDM-MB-231 provides an mTOR-dependent survival signal (Chen Y. *et al.*, 2005). We therefore stably introduced the PLD2 gene, which is tolerated by cells much better than PLD1 (Joseph T. *et al* , 2001), into the MCF-7 cells (MCF-7 P2 cells) in order to determine whether elevating the level PLD activity in the MCF-7 cells could provide an estrogen-independent survival in these cells when deprived of serum. The levels of PLD activity and protein in the MCF-7 empty vector control and the MCF-7 P2 cells were evaluated, and as shown in Figure 3.2.7A, the MCF-7 P2 cells had more than 25 fold more PLD activity than the parental vector-transfected MCF-7 cells. We then examined the ability of these cells to survive in the absence of serum. As shown in Figure 3.2.7B, the MCF-7 P2 cells were more resistant than the MCF-7 cells to the apoptosis induced by serum withdrawal. However, E2 was still able to enhance survival of these cells indicating that elevated PLD activity alone may not provide the same degree of protection from apoptosis as that provided by E2. In Figure 3.2.7C, it is shown that the MCF-7 P2 cells have elevated Myc expression relative to the vector control MCF-7 cells. The elevated PLD activity did not have any effect on the level of myc mRNA (Figure 3.2.7D). The dependence of survival on Myc expression was examined using Myc siRNA to suppress Myc expression in the MCF-7 P2 cells. As shown in Figure 3.2.7E, the Myc

siRNA suppressed Myc expression and reversed the survival effects of PLD2 on the MCF-7 cells. This experiment was problematic in that the introduction of control siRNA into cells that were already under the stress of serum withdrawal increased the basal apoptosis to around 60%. However, in the cells that received the Myc siRNA, 100% of the cells underwent apoptosis (Figure 3.2.7E). If E2 was provided during the transfection, the results with the MCF-7 P2 cells were quite dramatic, in that virtually all of the cells receiving the control siRNA survived, whereas close to 100% of those that received the Myc siRNA underwent apoptosis (Figure 3.2.7E). These data indicate that elevated PLD activity provides a Myc-dependent survival signal in MCF-7 cells that, at least partially, overcomes E2 dependence.

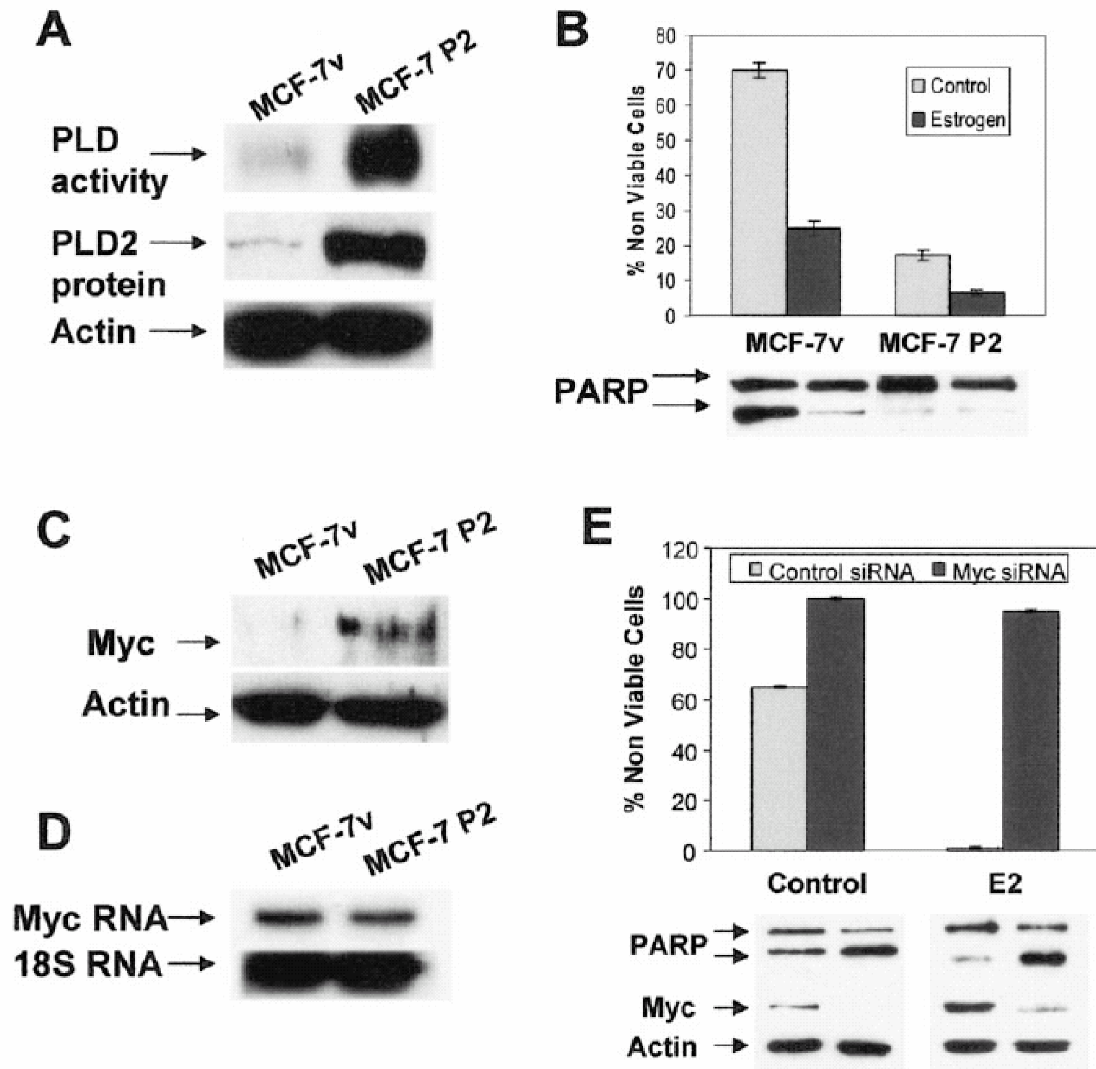

Figure 3.2.7: Elevated expression of PLD2 in MCF-7 cells increases Myc expression and confers an estrogen-independent survival signal. (A) MCF-7 P2 and control MCF-7v cells were generated as described in Materials and Methods. Cells were plated in DMEM with 10% serum and switched to DMEM with 0.5% serum after 24 h. PLD activity and protein levels were then determined by transphosphatidylation and Western blotting, respectively. (B) MCF-7v cells and MCF-7 P2 cells were prepared as for Figure 3.2.4 and placed in serum-free, phenol red-free DMEM with or without E2 (2 nM) as indicated. Cells were harvested after 5 days of treatment, at which time the percentage of nonviable cells and PARP cleavage were determined as for Figure 3.2.4. Myc protein (C) and RNA (D) levels in the MCF-7v cells and MCF-7 P2 cells were determined as for Figure 3.2.2. (E) The effects of Myc siRNA on cell viability, PARP cleavage, and Myc expression in the MCF-7 P2 cells were determined in the presence and absence of E2 as described for Figure 3.2.3. All experiments shown are representative of three independent experiments.

3.3 DISCUSSION

In this section, we have provided evidence that E2 provides a survival signal in the ER positive MCF-7 breast cancer cell line that is dependent upon a delayed increase in Myc expression. The E2 survival signal was also dependent upon basal levels of mTOR and its upstream regulators PI3K and PLD. Stable expression of PLD2 in MCF-7 cells provided an E2 independent survival signal that was also dependent upon elevated Myc expression. The key contributor to the survival of MCF-7 cells deprived of serum is apparently Myc expression. In this regard, it is of interest that E2 induced two phases of Myc expression, a short transient induction involving transcriptional activation as reported previously (Dubik D. *et al.*, 1992 and Santos G.F. *et al.*, 1988), and in addition, a large sustained induction that begins 2 to 3 days after treatment that did not involve an increase in myc RNA levels. Suppression of Myc expression reversed both E2 and PLD-dependent survival signals.

Activation of Myc has been reported to be dependent upon mTOR-dependent translation (West M.J. *et al.*, 1998). We demonstrated here that the E2-induced increase in Myc expression is dependent upon mTOR and two upstream activators of mTOR, PI3K and PLD. However, E2 did not increase mTOR, PI3K or PLD activity, indicating that only basal levels of mTOR activity were required for the E2 effects on Myc expression and cell survival. These data indicate that the delayed E2-induced increase in Myc expression is probably not dependent on increased translation. Since the increased Myc expression is apparently not dependent upon an increase in RNA levels or increased translation, it is most likely dependent upon increased stabilization of Myc protein.

Stabilization of Myc protein is regulated through the ubiquitin-proteasome pathway (Yeh E. *et al.*, 2004). The stabilization of Myc protein involves complex phosphorylation and dephosphorylation steps involving Erk, GSK-3β and protein phosphatase 2A (Yeh E. *et al.*, 2004). At this point it is not clear how E2 impacts on this pathway. E2 action on this pathway will further be decribed in the next chapter. The introduction of PLD2 into MCF-7 cells led to increased PLD activity and increased Myc expression which was also independent of an increase in Myc mRNA levels; however elevated PLD activity does increase S6-kinase phosphorylation (Chen Y. *et al.*, 2005, Hui L. *et al.*, 2004) and therefore could be enhancing mTOR-mediated translation of Myc. Whether PLD enhances Myc stabilization is not clear, but elevated PLD activity suppresses protein phosphatase 2A, which enhances Myc degradation (Hui L. *et al.*, 2005). This will also be discussed in the next chapter. Thus, PLD could conceivably enhance both Myc translation and stability. The survival signal generated by elevated PLD activity was dependent upon Myc. However, while the MCF-7 cells with elevated PLD activity could survive in the absence of serum, the combination of elevated PLD activity and E2 provided a stronger survival signal indicating that elevated PLD activity does not provide all that E2 accomplishes.

A model for survival signals generated by E2 and PLD in MCF-7 cells is shown in Figure 3.3.1. In this model, E2 provides a survival early in breast cancer progression. Elevated PLD or PI3K activity would enhance survival during the formation of a tumor mass prior to vascularization. In this simplified model, E2 increases Myc expression through enhanced stabilization Myc protein. The translation of Myc transcripts into Myc

protein is dependent upon basal levels of mTOR and elevated PLD activity in an emerging tumor would increase translation of basal levels of Myc transcript to Myc protein by activating mTOR. Progression to an ER negative phenotype likely involves additional genetic alterations that provide a metastatic potential that makes these tumor more aggressive and malignant.

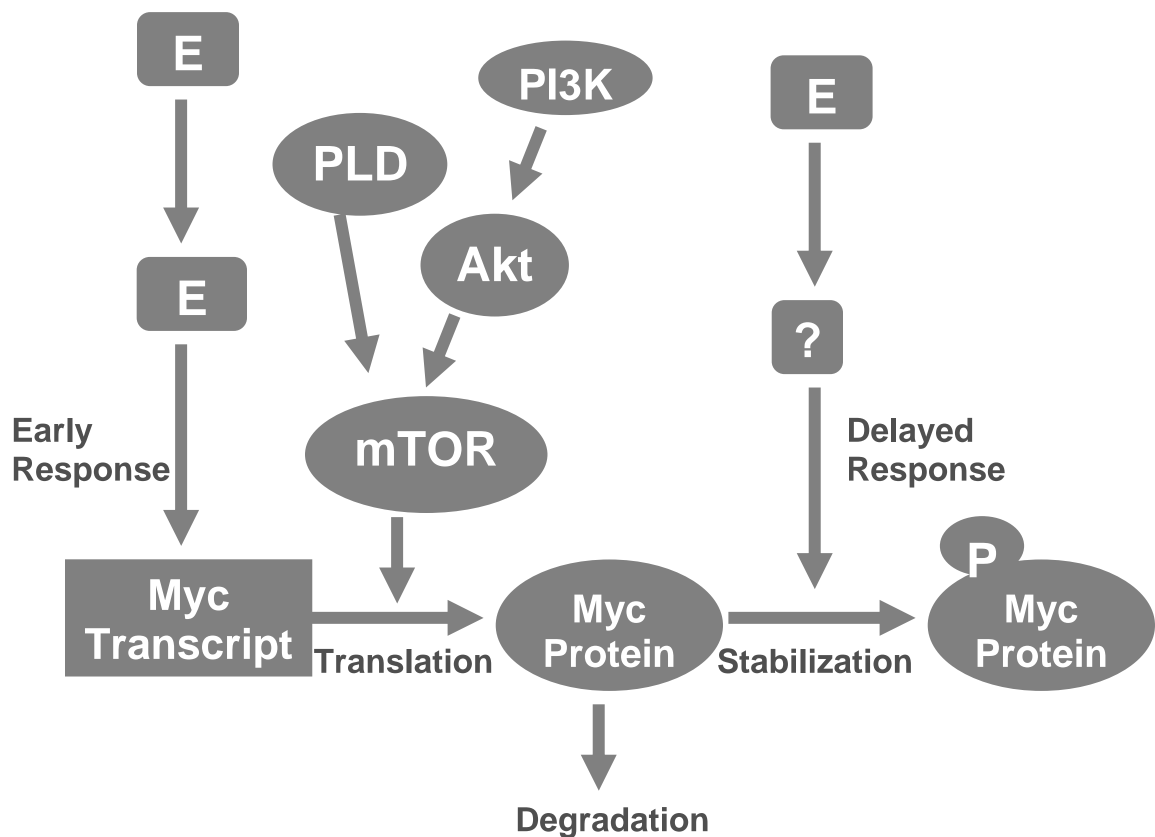

Figure 3.3.1: Model for E2-and PLD-driven Myc-dependent survival signals in MCF-7 cells. It is proposed that E2 induces an early transient increase in Myc expression at the level of transcription. Beginning on day 2 and peaking at day 5, there is a delayed increase in Myc expression that is likely due to stabilization of Myc protein through phosphorylation (P). Although there is no increase in mTOR activity induced by E2, the delayed increase in Myc expression is dependent upon basal levels of mTOR activity and the activity of the upstream regulators PLD and PI3K. In the absence of E2, it is proposed that elevated PLD activity can increase mTOR activity to translate basal levels of Myc transcript to increase Myc expression. It is not clear whether PLD would lead to increased Myc stabilization. Elevated PLD activity provides an E2-independent survival signal that could be critical for progression to an ER-negative status in breast cancer progression.

CHAPTER IV:

MYC STABILIZATION IN RESPONSE TO ESTROGEN AND PHOSPHOLIPASE D IN MCF-7 BREAST CANCER CELLS.

4.1 INTRODUCTION

One of the most common defects in human cancer cells is deregulated expression of the Myc oncogene. Myc is a transcription factor that stimulates the transcription of factors that facilitate progression through critical cell cycle checkpoints and suppresses transcription of factors that suppress cell cycle progression (Adhikary S. *et al.*, 2005). Elevated Myc expression has been reported in breast, colon, cervical, small cell lung carcinoma, osteosarcoma, glioblastoma, melanoma and myeloid leukemia (Pelengaris S. *et al.*, 2002), and Myc expression is believed to be elevated in a high percentage of human cancers (Nesbit C.E. *et al.*, 1999). Expression of Myc is regulated at many levels including: transcriptional, post-transcriptional (mRNA stability and translation), and post-translational (protein stability) (Lusher B. *et al.*, 1990, Kelly K. *et al.*, 1983 and Sears R. *et al.*, 1999, and West M.J. *et al.*, 1999). Thus, there are many ways in which Myc expression can be altered in human cancers. There are also mutations to the Myc gene that significantly enhance Myc stability and therefore expression (Gregory M.A. *et al.*, 2000, and Salghetti S.E. *et al.*, 1999). Most significantly, mutations at Thr58 lead to a stabilized Myc protein (Gregory M.A. *et al.*, 2000, and 2003). Phosphorylation of Myc at Thr58 has been shown to regulate Myc stability by targeting Myc for degradation by the proteasome (Yeh E. *et al.*, 2004). Thr58 is phosphorylated by glycogen synthase kinase-3β (GSK-3β), which phosphorylates many proteins fated for degradation by the protaseome (Doble B.W., 2003). As discussed in the previous chapter, estrogen treatment (E2) led to a bi-modal increase in Myc expression in the estrogen receptor positive MCF-7 human breast cancer cell line (Rodrik V. *et al.*, 2005). There was a short-term transient increase in Myc expression that peaked at about two hours. More striking

however was a large sustained stable increase in Myc expression that peaked at after five days of treatment. As shown in the previous chapter, this long-term increase in Myc expression was critical for E2 to suppress apoptosis in MCF-7 cells subjected to the stress of serum withdrawal. We also reported that elevated phospholipase D (PLD) activity could suppress apoptosis in MCF-7 cells deprived of serum, and that PLD similarly increased Myc expression (Chen Y. *et al.*, 2005, Rodrik V. *et al.*, 2005). PLD activity has been reported to be elevated in a substantial percentage of human breast cancers (Uchida N. *et al.*, 1997, and Noh D.Y. *et al.*, 2000) and has been proposed to provide a survival signal in breast and other cancers (Foster D.A. *et al.*, 2003, Foster D.A., 2004 and 2006). Importantly, PLD activity is elevated in several breast cancer cell lines that are E2 receptor negative (Zhong M. *et al.*, 2003, Chen Y. *et al.*, 2003, 2005, Zheng Y. *et al.*, 2006), suggesting the possibility that elevated PLD activity in breast cancer cells facilitates progression to hormone independence (Foster D.A., 2003). In this chapter, we provide evidence that the induction of Myc expression by both E2 and PLD is due to increased Myc stabilization mediated largely by the inhibition of GSK-3β as well as the suppression of Myc ubiquitination.

4.2 RESULTS

4.2.1 E2-INDUCED MYC EXPRESSION IS NOT DUE TO INCREASED TRANSCRIPTION OR TRANSLATION.

We previously discussed that E2 enhanced proliferation and survival of MCF-7 cells in the absence of serum that became apparent at 5 days (Rodrik V. *et al.*, 2005). The ability of E2 to promote proliferation and survival was dependent upon a sustained increase in Myc expression (Rodrik V. *et al.*, 2005). As shown in Figure 4.2.1A, Myc

protein level is elevated after 5 days of E2 treatment in MCF-7 cells without induction of myc mRNA. Since the level of Myc expression is also regulated at the level of translation (Dubik D. *et al.*, 1992), we examined whether E2-induced increases in Myc expression were dependent on protein synthesis. MCF-7 cells were treated with E2 for 5 days and cycloheximide (CHX), a protein synthesis inhibitor, was added to inhibit newly synthesized protein for the times indicated. Since E2 also induces a short-term transient increase in Myc expression-dependent on an increase in Myc translation (Dubik D. *et al.*, 1992), we also examined the effect of CHX on Myc expression in MCF-7 cells treated with E2 for 2 hours. As shown in Figure 4.2.1B, Myc protein levels induced by E2 for 5 days were very stable in the presence of the protein synthesis inhibitor, whereas the Myc levels induced by E2 at 2 hours dropped substantially in the presence of CHX. These data indicate that the high levels of Myc seen at 5 days of E2 treatment is not affected by the inhibition of protein synthesis and indicates that the increased Myc expression is not due to increased translation.

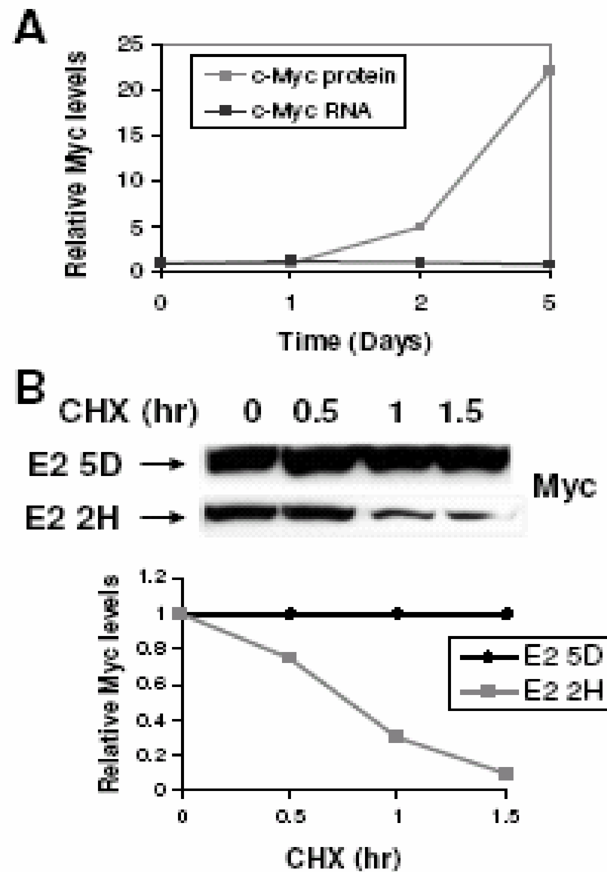

Figure 4.2.1: E2-induced Myc expression is not due to increased mRNA level or translation. (A) MCF-7 cells were plated at $2x10^4$ cells/60mm culture dish in DMEM with 10% bovine calf serum. After 24h, the medium was changed to phenol red free DMEM with 0% serum and E2 (2nM). Cells were collected at the indicated time points and lysates were analyzed for Myc protein and RNA levels as described in the previous chapter. Myc protein and RNA levels determined using densitometer quantification were normalized to levels observed in cells not treated with E2 (zero time point). (B) MCF-7 cells were plated as in (A). After 24h, the medium was changed to phenol red free DMEM with 0% serum. E2 (2nM) was then added for either 2 hours or 5 days as indicated. Cycloheximide (80µg/µL) (CHX) was then added for the indicated times. The cells were then collected and lysates were analyzed for the level of Myc protein using Western blot analysis. The relative Myc protein levels normalized to their respective levels in the cells not treated with CHX (zero time point) were determined by using densitometer quantification of the data shown in the upper panel. The data shown in (A) and (B) are representative of three independent experiments.

4.2.2 E2 INHIBITS MYC UBIQUITINATION

The data in Figure 4.2.1 indicate that the Myc protein generated at 5 days of E2 treatment is more stable than the Myc generated after 2 hr of treatment with E2. Importantly and as shown previously, it is at 5 days that the ability of E2 to suppress apoptosis becomes apparent (Rodrik V. *et al.*, 2005). Myc protein is degraded via the ubiquitin-proteasome pathway (Gregory M.A. *et al.*, 2000, and Salghetti S.E. *et al.*, 1999). We therefore examined whether long-term E2 treatment inhibited the ubiquitination of Myc. MCF-7 cells were treated with E2 for 5 days at which time Myc was recovered by immunoprecipitation. The Myc immunoprecipitates were then analyzed by Western blot analysis with an anti-ubiquitin antibody. As shown in Figure 4.2.2, E2 reduced the amount of ubiquitinated Myc protein in cell lysates in spite of the fact that there were highly elevated levels of Myc protein after E2 treatment. The proteasome inhibitor lactacystin was included to prevent degradation of ubiquitinated Myc.

These data provide evidence that E2 inhibits the ubiquitination of Myc protein, and consequently, degradation via the ubiquitin/proteasome pathway.

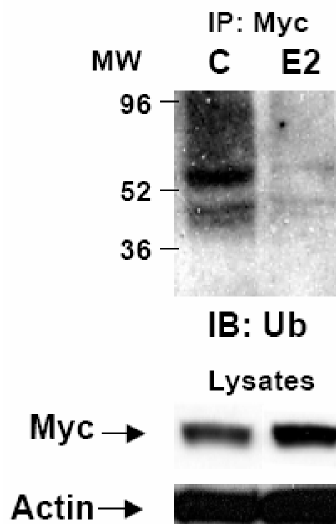

Figure 4.2.2: E2 inhibits ubiquitination of Myc. MCF-7 cells were plated as in Figure 4.2.1. 24 h later the medium was changed to phenol red-free DMEM without serum. E2 (2nM) was then added for 5 days where indicated. At day five, cells were treated with 10μM lactacystin for 6 hours, at which time cells were collected and lysates were immunoprecipitated with an anti-Myc antibody. Immunoprecipitates were then subjected to Western blot analysis using an anti-ubiquitin antibody (top panel). The lysates were also analyzed for the levels of total Myc by Western Blot analysis. This blot was reprobed with an antibody to actin to control for loading. The data shown are representative of three independent experiments.

4.2.3 <u>E2 INCREASES PHOSPHORYLATION OF MYC AT SER62 AND SUPPRESSES PHOSPHORYLATION AT THR58</u>.

The ubiquitination and stability of Myc is regulated by phosphorylation of Myc at two key sites – Ser62 and Thr58. Phosphorylation at Ser62 by MAP kinase suppresses ubiquitination and degradation; whereas the phosphorylation at of Myc at Thr58 by GSK-3β targets Myc for ubiquitination and degradation (Lutterbach B. *et al.*, 1994 and Sears R. *et al.*, 2000). Importantly, Ser62 must be phosphorylated before GSK-3β can phosphorylate Thr58, and phosphorylated Ser62 is dephosphorylated by phosphatase-2A (PP2A) (Yeh E. *et al.*, 2004). Dephosphorylation at Ser62 allows degradation to proceed by removing the stabilizing phosphate at Ser62. We therefore examined the effect of E2

on the phosphorylation status of Myc at Ser62 and Thr58. MCF-7 cells were treated with E2 for 5 days and lysates were then analyzed by Western blot with antibodies specific for phosphorylated Myc. As shown in Figure 4.2.3A, E2 increased phosphorylation Myc at Ser62 after 5 days of treatment compared to the control. However, total Myc protein was also elevated after E2 treatment. The increased phosphorylation of Ser62 was compared with increased Myc levels using densitometry scans from several experiments and as shown in Figure 4.2.3C, there were higher increases in phosphorylation at Ser62 than observed for total Myc protein, indicating that there was a net increase in Myc phosphorylated at Ser62. We next examined the phosphorylation state of Myc at Thr58, and as shown in Figure 4.2.3B, E2 strongly reduced the level of phosphorylated Myc at Thr58 compared to the control. The reduced levels of Myc phosphorylated at Thr58 was seen despite increased levels of Myc protein. Densitometric analysis of several experiments revealed that the suppression of phosphorylation at Thr58 in response to E2 was more than 20 fold (Figure 4.2.3C).

These data reveal that E2 suppresses phosphorylation at Thr58 – a site that when phosphorylated, targets Myc for ubiquitination and degradation. In addition, the data reveal that E2 also increases phosphorylation at Ser62 – a site that suppresses ubiquitination and degradation (Yeh E. *et al.*, 2004). While the effect of E2 on the phosphorylation at Ser62 was reproducible, the effect on phosphorylation at Thr58 was much more dramatic.

These data support the hypothesis that the elevated Myc expression in response to E2 is due to stabilization through elevated phosphorylation of Ser62 and reduced phosphorylation at Thr58.

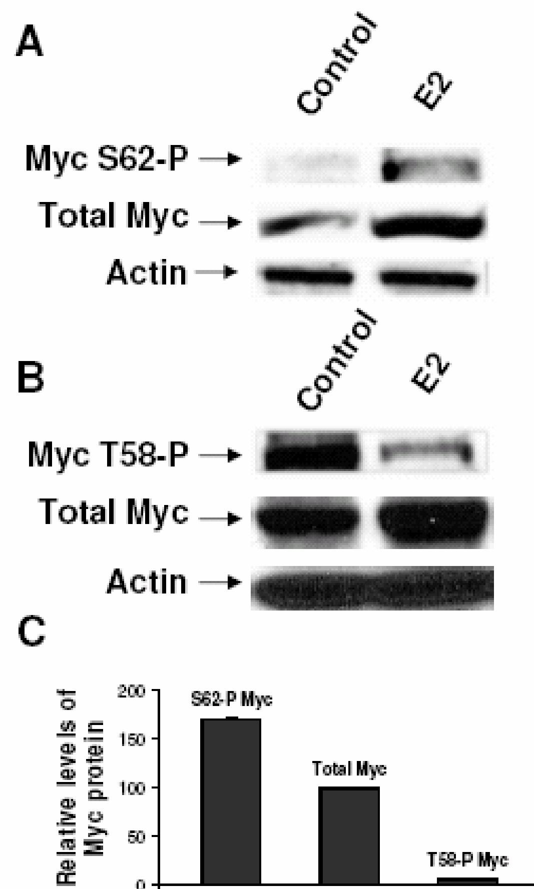

Figure 4.2.3: E2 increases phosphorylation of Myc at Ser62 and suppresses phosphorylation at Thr58. (A) MCF-7 cells were prepared and collected as described in Figure 4.2.2 and placed in serum-free, phenol red-free DMEM with or without E2 (2nM) as indicated. Lysates were prepared and analyzed for the levels of phosphorylated Myc protein at Ser62 by Western blot analysis. The blot was stripped and reprobed with an antibody to total Myc protein. The blot was also reprobed with an antibody to actin to control for loading. (B) MCF-7 cells were treated and collected as in (A) and the lysates were analyzed for the levels of phosphorylated Myc protein at Thr58. The blot was stripped and reprobed with antibodies to Myc and actin as in (A). (C) The levels of phosphorylated Myc in presence of E2 were normalized to their respective levels in the untreated control was determined using densitometer quantification. The relative changes in phosphorylated Myc for both Ser62 and Thr58 were compared to level of total Myc protein. Experiments shown in (A) and (B) are representative of three independent experiments. The data in (C) are the averages of three independent experiments normalized to the levels of Myc protein. Error bars represent the standard deviation.

4.2.4 <u>E2 INDUCES PHOSPHORYLATION OF GSK-3B AT THE NEGATIVE REGULATORY SITE SER9</u>.

Since the effect of E2 on phosphorylation at Thr58 was so dramatic, we examined the effect of E2 on GSK-3β. GSK3-β is a ubiquitous protein kinase that is inhibited by phosphorylation at Ser9 (Cross D.A. *et al.*, 1995, van Weeren P.C. *et al.*, 1998, and Srivastava A.K. *et al.*, 1998). Estrogen has been reported previously to inhibit GSK-3β in the hippocampus (Cardona-Gomez P. *et al.*, 2004). We therefore examined the effect of E2 on the phosphorylation state of GSK-3β at Ser9. MCF-7 cells were treated with E2 for five days and lysates were then analyzed by Western blot using an antibody that recognizes phosphorylated GSK-3β. As shown in Figure 4.2.4, the five-day E2 treatment led to a large increase in phosphorylation of GSK-3β at Ser9. The total GSK-3β protein level was not affected by E2 treatment.

These data indicate that the effect of E2 on Myc phosphorylation is largely due to the suppression of GSK-3β by inducing the phosphorylation of GSK-3β at Ser9.

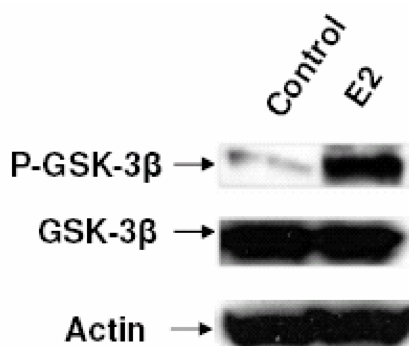

Figure 4.2.4: E2 suppresses GSK-3β activity. MCF-7 cells were prepared as described in Figure 4.2.2 and placed in serum-free phenol red-free DMEM in the absence or presence of E2 (2nM) for five days as indicated. Lysates were prepared and analyzed for the levels of phospho-GSK-3β (Ser9) by Western blot analysis. The blot was stripped and reprobed for the levels of total GSK-3β protein. The blot was reprobed with an antibody to actin to control for loading. The data shown are representative of three independent experiments.

4.2.5 <u>PLD-INDUCED MYC EXPRESSION IS NOT DUE TO INCREASED TRANSCRIPTION OR TRANSLATION.</u>

We previously demonstrated that elevated PLD activity in MCF-7 cells can provide a survival signal (Chen Y. *et al.*, 2003) that is dependent upon elevated expression of Myc (Rodrik V. *et al.*, 2005). It was proposed that elevated PLD activity could replace E2 dependence in breast cancer progression, since breast cancer cell lines with elevated PLD activity tend to be ER negative (Foster D.A. *et al.*, 2003, Chen Y. *et al.*, 2003, 2005, Rodrik V. *et al.*, 2005, and Zheng Y. *et al.*, 2006). We therefore extended our study on Myc expression in response to E2 to MCF-7 cells with elevated expression of PLD2 (Rodrik V. *et al.*, 2005). As shown in Figure 4.2.5A and as described previously (Rodrik V. *et al.*, 2005), Myc protein levels are elevated in MCF-7 cells with over-expressed PLD2 (MCF-7P2 cells) relative to MCF-7 vector control cells (MCF-7V cells) without a corresponding induction of myc mRNA levels. We next examined the effect of the protein synthesis inhibitor CHX on Myc protein levels in the MCF-7V and MCF-7P2 cells. CHX was added to the MCF-7V and MCF-7P2 cells and Myc protein levels were examined at 30 minutes intervals for 1.5 hr. As shown in Figure 4.2.5B, Myc protein levels in the MCF-7P2 were very stable over this period, whereas Myc levels in MCF-7V cells dropped substantially during the 1.5 hr incubation period. These data indicate that the elevated Myc expression in MCF-7P2 cells was not due to increased translation, whereas the Myc expression in MCF-7V cells was dependent on translation.

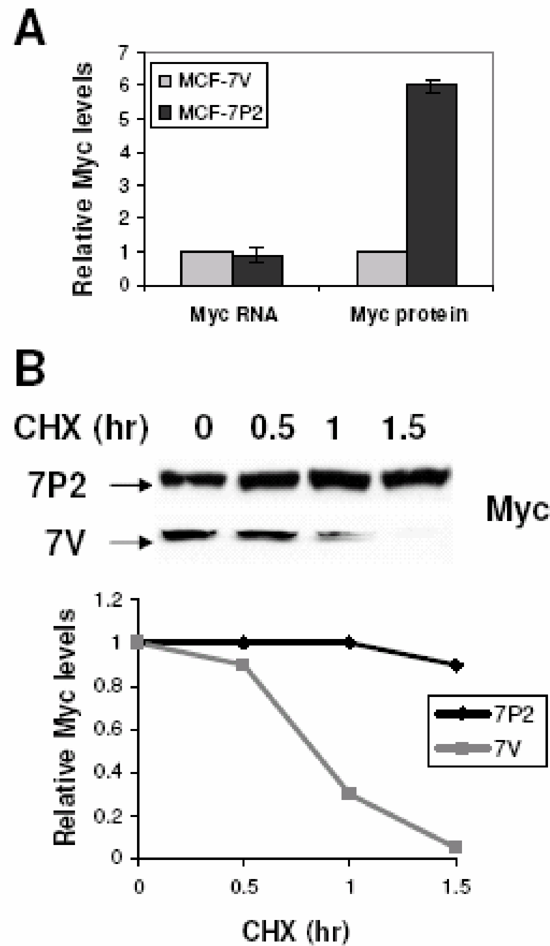

Figure 4.2.5: PLD-induced Myc expression is not due to increased mRNA level or translation. (A) MCF-7V control and MCF-7P2 cells were generated as described in the material and methods chapter. Cells were plated at $2x10^4$ cells/60 mm culture dish in DMEM with 10% bovine calf serum. After 24h, the medium was changed to phenol red free serum free DMEM for 5 days. Cells were then collected and lysates were analyzed for Myc protein and RNA levels as described in the previous chapter. Relative levels of Myc RNA and protein in MCF-7P2 cells normalized to levels in MCF-7V cells were determined using densitometer quantification. Error bars represent the standard deviations obtained from 3 independent experiments. (B) MCF-7V control and MCF-7P2 cells were plated as in (A). After 24h, the medium was changed to phenol red free DMEM with 0% serum for five days. Cycloheximide (80 µg/µL) (CHX) was then added for the indicated times. The cells were collected and lysates were analyzed for the level of Myc protein using Western blot analysis. Relative Myc levels normalized to the level in untreated cells (zero time point) were determined using densitometer quantification of the data shown in the upper panel. Experiments shown in (B) are representative of three independent experiments.

4.2.6 PLD SUPPRESSES MYC UBIQUITINATION IN MCF-7 CELLS.

The data in Figure 4.2.5 indicate that the Myc protein in MCF-7P2 cells is more stable than the Myc in the MCF-7V cells. We therefore examined whether ubiquitination of Myc was suppressed in the MCF-7P2 cells relative to the MCF-7V cells. MCF-7V and MCF-7P2 cells were immunoprecipitated with a Myc antibody and the immunoprecipitates were then analyzed by Western blot with an anti-ubiquitin antibody as in Figure 4.2.2. As shown in Figure 4.2.6, ubiquitinated Myc was substantially reduced in the MCF-7P2 cells relative to the MCF-7V cells.

These data provide evidence that overexpression of PLD2 in MCF-7 cells inhibits ubiquitination of Myc protein and thus its degradation via the proteasome pathway.

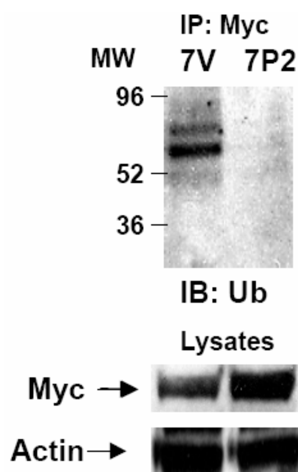

Figure 4.2.6: PLD inhibits ubiquitination of Myc. (A) MCF-7V and MCF-7P2 cells were plated as in Figure 4.2.5. 24 hr later the medium was changed to phenol red-free DMEM with 0.5% serum. At day five, cells were treated with 10μM lactacystin for 6 hours, at which time cells were collected and lysates were immunoprecipitated with an anti-Myc antibody. Cell lysates were immunoprecipitated with an anti-Myc antibody. The immunoprecipitates were then subjected to Western blot analysis using an anti-ubiquitin antibody as described in Figure 4.2.2. Lysates from MCF-7V and MCF-7P2 cells were treated and collected as above and analyzed for the levels of Myc by Western Blot analysis. The blot was reprobed with an antibody to actin to control for loading. The data shown are representative of three independent experiments.

4.2.7 <u>PLD INCREASES PHOSPHORYLATION OF MYC AT SER62 AND SUPPRESSES PHOSPHORYLATION AT THR58.</u>

As discussed above, ubiquitination of Myc involves the phosphorylation of Myc at Ser62 and Thr58. We therefore examined the effect of PLD2 expression on the phosphorylation of Myc at Ser62 and Thr58 in MCF-7 cells. MCF-7V and MCF-7P2 lysates were analyzed by Western blot analysis with phospho-specific Myc antibodies as described in Figure 4.2.3. As shown in Figure 4.2.7A, PLD2 over-expression resulted in increased phosphorylation of Myc at Ser62. As observed with E2 treatment of MCF-7 cells, total Myc was also elevated in the MCF-7P2 cells relative to the MCF-7V cells, and as observed in Figure 4.2.3C, there were higher increases in phosphorylation at Ser62 than observed for total Myc protein in the MCF-7P2 cells, indicating that there was a 50% increase in Myc with phosphorylated at Ser62 (Figure 4.2.7C). The phosphorylation state of Myc at Thr58 in MCF-7V and MCF-7P2 cells was then examined, and as shown in Figure 4.2.7B, the level of phosphorylated Myc at Thr58 in MCF-7P2 cells was dramatically reduced relative to the MCF-7V cells. Densitometric analysis from several experiments revealed that the suppression of phosphorylation at Thr58 in response to PLD over-expression was more than 50 fold (Figure 4.2.7C).

These data reveal that PLD, like E2, strongly suppresses phosphorylation at Thr58 and increases phosphorylation at Ser62. These data are consistent with the hypothesis that the stabilization of Myc in MCF-7 cells over-expressing PLD2 is due to elevated phosphorylation of Ser62 and reduced phosphorylation at Thr58.

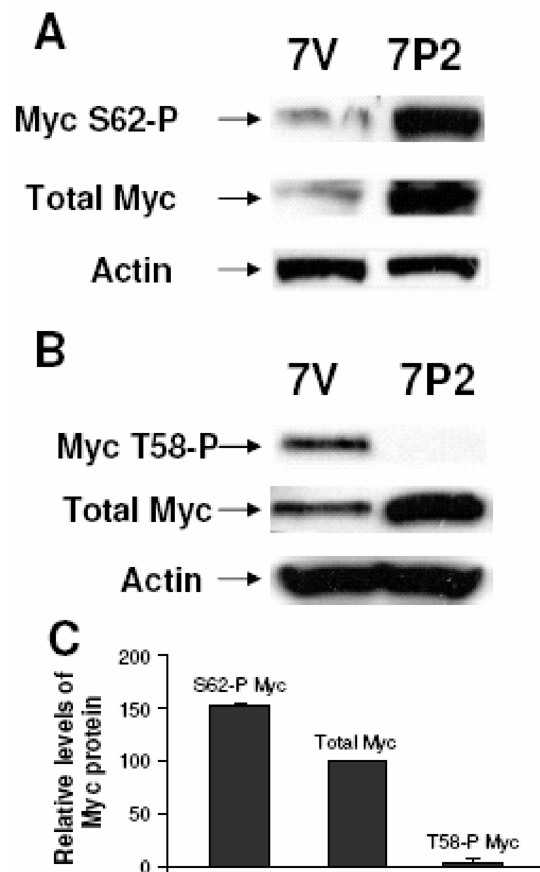

Figure 4.2.7: PLD increases phosphorylation of Myc at Ser62 and suppresses phosphorylation at Thr58. (A) MCF-7V and MCF-7P2 cells were prepared and collected as described in Figure 4.2.5 and placed in phenol red-free DMEM with 0.5% serum. Lysates were prepared five days later and analyzed by Western blot for the level of Myc phosphorylated at Ser62. The blot was stripped and reprobed with an antibody to total Myc protein. The blot was reprobed with an antibody to actin to control for loading. (B) MCF-7V and MCF-7P2 cells were treated and collected as in (A) and lysates were analyzed for the level of Myc phosphorylated at Thr58. The blot was stripped and reprobed with antibodies to Myc and actin as in (A). (C) The levels of phosphorylated Myc in the MCF-7P2 cells normalized to the level in the MCF-7V were determined using densitometer quantification. The relative changes in phosphorylated Myc for both Ser62 and Thr58 were compared to level of total Myc protein. Experiments shown in (A) and (B).

4.2.8 PLD INDUCES PHOSPHORYLATION OF GSK-3B AT THE NEGATIVE REGULATORY SITE SER9.

As shown in Figure 4.2.7, elevated PLD expression leads to a dramatic decrease in the phosphorylation of Myc at Thr58. We therefore examined whether PLD, like E2, induced an increase in the phosphorylation of the negative regulatory site Ser9. MCF-7V control or MCF-7P2 cells lysates were analyzed by Western blot analysis with an anti-Ser9 GSK-3β antibody as described in Figure 4.2.4. As shown in Figure 4.2.8, PLD2 over-expression in MCF-7P2 cells substantially increased phosphorylation of GSK-3β at Ser9 relative to the MCF-7V control cells. Total GSK-3β protein levels were not affected.

These data indicate that elevated PLD expression can inhibit GSK-3β by increasing phosphorylation on GSK-3β at Ser9.

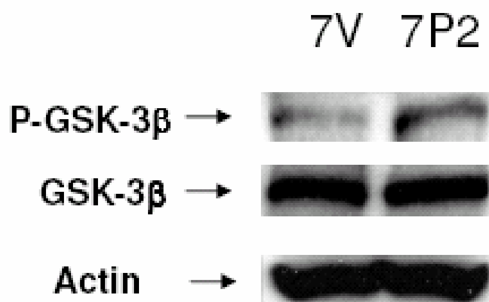

Figure 4.2.8: PLD induces phosphorylation of GSK-3β at the negative regulatory site Ser9. MCF-7 cells were prepared as described in Figure 4.2.5 and placed in placed in phenol red-free DMEM with 0.5% serum. Lysates were prepared five days later and analyzed for the levels of phospho-GSK-3β (Ser9) protein by Western blot analysis. The blot was stripped and reprobed for the level of total GSK-3β and actin as in Figure 4.2.4. The data shown are representative of three independent experiments.

4.3 DISCUSSION

Suppression of apoptosis is critical in tumor progression (Hanahan D.J. *et al.*, 2000). Myc has been implicated in the suppression of apoptosis and progression past cell cycle checkpoints where apoptosis is regulated (Pelengaris S. *et al.*, 2002). The regulation of Myc expression is complex and is controlled at the level of transcription, translation and stabilization (Sears R., 2004). As discussed in the previous chapter, prolonged exposure to E2 leads to very high levels of Myc expression that peaked at five days and that elevated Myc expression was required for the suppression of apoptosis in ER positive MCF-7 cells subjected to the stress of serum withdrawal (Rodrik V. *et al.*, 2005). E2 could be replaced with elevated PLD expression, which similarly increased Myc expression and suppressed apoptosis in MCF-7 cells subjected to serum withdrawal. In this work, we have characterized the mechanism for the sustained induction of Myc. We found that the increase in Myc expression by both E2 and PLD was due to stabilization. There was no significant effect on either myc mRNA levels or Myc translation. The effect was largely due to the suppression of phosphorylation of Myc at Thr58, which was dramatically reduced in response to both E2 and PLD. Myc is phosphorylated at Thr58 by GSK-3β (Lutterbach B. *et al.*, 1994 and Sears R. *et al.*, 2000). And consistent with the reduced phosphorylation of Myc at Thr58, both E2 and PLD stimulated a substantial increase in the phosphorylation of GSK-3β at Ser9, which suppresses GSK-3β activity (Cross D.A. *et al.*, 1995 and Srivastava A.K. *et al.*, 1998). A model for the E2 and PLD stabilization of Myc through suppression of GSK-3β is shown schematically in Figure 4.3.1. The phosphorylation of Myc at Ser62 was elevated in response to both E2 and PLD. The effect was not as dramatic as observed for Thr58, but the increased

phosphorylation at Ser62 is also consistent with increased stability of Myc. Myc must be first phosphorylated at Ser62 before it can be phosphorylated at Thr58 (Yeh Y. *et al.*, 2004), and then it must subsequently be dephosphorylated by PP2A before it can be ubiquitinated. We previously reported that PLD activity suppresses PP2A activity in MCF-7 cells (Hui L. *et al.*, 2005). Thus, the increased phosphorylation of Myc at Ser62 in response to PLD likely reflects the suppression of PP2A activity by PLD. We did not see a significant effect of E2 on PP2A activity in the MCF-7 cells. Thus, at this time we are not sure as to how E2 increases phosphorylation at Ser62. It is possible that the reduced phosphorylation of Myc at Thr58 in response to E2 reduced the ability of PP2A to recognize Myc. E2 was a stronger inducer of GSK-3β phosphorylation than was PLD and this could have led to reduced dephosphorylation of Myc at Ser62 by PP2A. While the impact of PP2A on the stability of Myc in response to E2 remains unclear, the effect of E2 on GSK-3β and phosphorylation at Thr58 is very apparent and is likely the major contribution to the stability of Myc in response to both E2 and PLD.

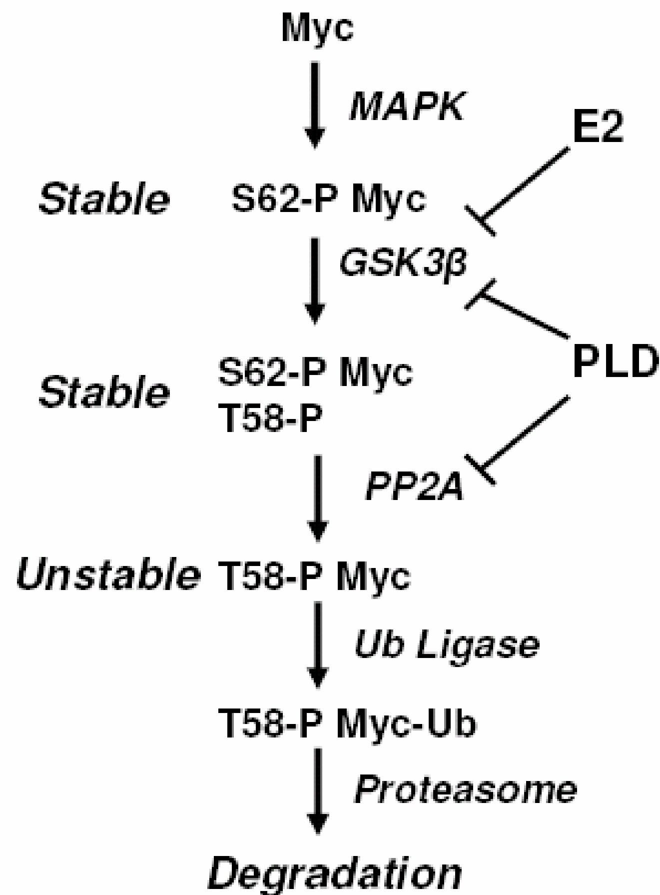

Figure 4.3.1: Model for Myc stabilization in response to E2 and PLD. MAP kinase (MAPK) phosphorylates Myc at Ser62 (S62-P), which stabilizes Myc. Myc, phosphorylated at Ser62, can then be phosphorylated at Thr58 (T58-P) by GSK-3β. The doubly phosphorylated Myc can then be dephosphorylated at Ser62 by PP2A. Myc phosphorylated only at Thr58 is recognized by a ubiquitin (Ub) ligase that leads to the ubiquitination of Myc and degradation by the proteasome (Sears R.C., 2004). Both E2 and PLD can suppress ubiquitination and degradation by suppressing the GSK-3β phosphorylation of Myc at Thr58. In addition, PLD can also suppress the PP2A (Hui L. *et al.*, 2005). The effect of E2 and PLD on Myc is the stabilization of Myc, which is required for the survival signals generated by both E2 and PLD (Sears R.C. *et al.*, 1999).

The data presented here clearly implicates GSK-3β in the stabilization of Myc in response to both E2 and PLD. Both E2 and PLD lead to increased phosphorylation of GSK-3β at the inhibitory Ser9 site. GSK-3β is phosphorylated at Ser9 by Akt (Cross D.A. *et al.*, 1995; Van Weeren P.C. *et al.*, 1998, and Srivastava A.K. *et al.*, 1998). We discussed in the previous chapter that E2 does not activate Akt as measured by phosphorylation of Akt at Ser 473 (Rodrik V. *et al.*, 2005). We have also reported that PLD does not increase phosphorylation of Akt (Hui L. *et al.*, 2004). Thus, we have a situation whereby both E2 and PLD lead to increased phosphorylation of GSK-3β at a site that is phosphorylated by Akt without increasing Akt phosphorylation. However, Akt-independent phosphorylation of GSK-3β at Ser9 has been reported (Desbois-Mouthon C. *et al.*, 2002), suggesting that there are alternative mechanisms for the phosphorylation of GSK-3β at Ser9 and suppressing GSK-3β activity. We discussed in the previous that the E2-induced increase in Myc and suppression of apoptosis was dependent upon mTOR, the mammalian target of rapamycin (Schmelzie T. *et al.*, 2000). PLD-induced Myc expression was also dependent upon mTOR (Chen Y. *et al.*, 2003). Preliminary studies indicate that rapamycin also suppresses the effect of both E2 and PLD on the phosphorylation of GSK-3β at Ser9, suggesting the involvement of mTOR in the suppression GSK-3β. In this regard it may be of significance that mTOR has a requirement for phosphatidic acid (Fang Y. *et al.*, 2001, and Foster D.A., 2004), the metabolic product of PLD. It is reasonable that if mTOR is involved in the suppression of PP2A, which would stabilize Myc, that mTOR might also be involved in the suppression of GSK-3β, which also contributes to the stabilization of Myc. How mTOR could lead to

the phosphorylation of GSK-3β at Ser9 in an apparent Akt-independent manner remains to be determined.

The importance of suppressing Myc phosphorylation at Thr58 is underscored by the properties of Myc mutants that cannot be phosphorylated at this site. Sears and colleagues reported recently that the stable Myc (T58A) mutant cannot be dephosphorylated by PP2A and replaces SV40 small T antigen in human cell transformation and tumorigenesis assays. Small-t antigen, which interacts with and inactivates PP2A (Pallas D.C. *et al.*, 1990, Yang S.I. *et al.*, 1991, and Chen W. *et al.*, 2003), may therefore exert its oncogenic potential by preventing dephosphorylation of Myc at Ser62, which prevents ubiquitination and results in Myc stabilization (Yeh E. *et al.*, 2004). The T58A Myc mutant was also defective in its ability to induce apoptosis (Hemann M.T. *et al.*, 2005). These studies further establish that the effects of both E2 and PLD are critical for the survival signals they generate.

CHAPTER V:

CONCLUSION

In this work, we have demonstrated that the E2-induced increase in Myc expression was dependent upon mTOR and two upstream activators of mTOR, PI3K and PLD. Moreover, we have shown that the increase in Myc expression by both E2 and PLD was not due to an increase in either mRNA levels or an increase in translation. However, this large increase in Myc was due to stabilization of the protein. We, in fact, demonstrated that E2- and PLD-induced Myc stabilization is dependent upon suppression of GSK-3β.

As discussed before, breast cancer progression involves the loss of the ER, thus rendering the tumor to become hormone-independent. The loss of ER expression in breast cancer correlates with poor prognosis in breast cancer (Sommer S. *et al.*, 2001). Also, as mentioned before, elevated PLD activity has been reported in a majority of breast cancer tissues that have been examined (Noh D.Y. *et al.*, 2000 and Uchida N. *et al.*, 1997). Moreover, we have shown in this work that elevated PLD activity in MCF-7 cells provides at least partial independence from E2 for survival. Thus, we have proposed that elevated PLD activity in human breast cancer provides a means for progression to hormone independence (Foster D.A., 2004). This was largely based on the observation that breast cancer cell lines with highly elevated PLD activity were all ER negative and most breast cancer cell lines with low PLD activity were ER negative (Zhong M. *et al.*, 2003; Chen Y. *et al.*, 2003; 2005, and Rodrik V. *et al.*, 2005). While these observations only provide correlative evidence for the involvement of PLD in progression to hormone independence in breast cancer, there is increasing evidence that PLD generates survival signals that would compensate for the loss of hormone responsiveness and dependence.

As we reported previously, and observed by others, E2 provides a survival signal in ER positive breast cancer cells (Perillo B. *et al.*, 2000, Foster J.S. *et al.*, 2001, Fernando R.I. *et al.*, 2004, and Rodrik V. *et al.*, 2005). PLD similarly provides a survival signal in breast cancer cells subjected to the stress of serum withdrawal (Zhong M. *et al.*, 2003, Chen Y. *et al.*, 2005), conditions that could occur in an emerging solid tumor prior to vascularization. The lack of serum would also restrict access to E2 and select for cells that could survive these conditions. Cells with elevated PLD activity would have the ability to survive these conditions. PLD has been reported to suppress p53 expression (Hui L. *et al.*, 1994) and to suppress PP2A activity (Hui L. *et al.*, 2005), both of which are critical for the transformation of human cells (Chen W. *et al.*, 2003). Since Myc has also been strongly implicated in the survival of breast cancer cells (Nesbit C.E. *et al.*, 1999 and Pelengaris S. *et al.*, 2002), the demonstration in this work that PLD, like E2, can promote the stabilization of Myc supports a critical role for the elevated PLD activity commonly seen in ER negative breast cancer cells. As discussed previously, targeting Myc in breast and other cancers has not yet proved to be a viable strategy for therapeutic intervention (Nesbit C.E. *et al.*, 1999). Therefore, targeting the signals that regulate Myc expression could be a viable strategy, and targeting PLD signals in hormone independent breast cancers could be especially effective for destabilizing Myc and inducing apoptosis.

CHAPTER VI:

REFERENCES

Adhikary S., and Eilers M. 2005. Transcriptional regulation and transformation by Myc proteins. Nat. Rev. Mol. Cell Biol. **6:**635-645.

Aguirre Ghiso J.A., Farias E.F., Alonso D.F., Arregui C., and Bal de Kier Joffe E. 1997. A phospholipase D and protein kinase C inhibitor blocks the spreading of murine mammary adenocarcinoma cells altering f-actin and ß1-integrin point contact distribution. Int. J. Cancer **71:**881-890.

Ahamed S., Foster J.S., Bukovsky A., and Wimalasena J. 2001. Signal transduction through the ras/Erk pathway is essential for the mycoE2 zearalenone-induced cell-cycle progression in MCF-7 cells. Mol. Carcinog. **30:**88-98.

Ahn B.H., Kim S.Y., Kim E.H., Choi K.S., Kwon T.K., Lee Y.H., Chang J.S., Kim M.S., Jo Y.H., and Min do S. 2003. Transmodulation between phospholipase D and c-Src enhances cell proliferation. Mol Cell Biol. **23:**3103-3115.

Alexandrow M.G., and Moses H.L. 1998. c-myc-enhanced S phase entry in keratinocytes is associated with positive and negative effects on cyclin-dependent kinases. Journal of Cell Biochemistry **70:**528-542.

Ali S., and Coombs C. 2002. Endocrine-responsive breast cancer and strategies for combating resistance. Nature Rev. Cancer **21:**101-112.

Amati B., **Brooks M.W.**, **Levy N.**, **Littlewood T.D.**, **Evan G.I.**, **and Land H.** 1993. Oncogenic activity of the c-Myc protein requires dimerization with Max. Cell **2:**233-245.

Amundadottir L.T., Johnson M.D., Merlino G., Smith G.H., and Dickson R.B. 1995. Synergistic interaction of transforming growth factor alpha and c-*myc* in mouse mammary and salivary gland tumorigenesis. Cell Growth and Differentiation **6:**737-748.

Amundadottir L.T., Merlino G., and Dickson R.B. 1996. Transgenic mouse models of breast cancer. Breast Cancer Research and Treatment **39:**119-135.

Amundadottir L.T., Nass S.J., Berchem G.J., Johnson M.D., and Dickson R.B. 1996. Cooperation of TGF alpha and c-Myc in mouse mammary tumorigenesis: coordinated stimulation of growth and suppression of apoptosis. Oncogene **13:**757-765.

Ayer D. E., Kretzner L., and Eisenman R. N. 1993 Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. Cell **72:**211-222.

Bae C.D., Min D.S., Fleming I.N., and Exton J.H. 1998. Determination of interaction sites on the small G protein RhoA for phospholipase D. J. Biol. Chem. **273:**11596-11604.

Banno Y., Takuwa Y., Yamada M., Takuwa N., Ohguchi K., Hara A., and Nozawa Y. 2003. Involvement of phospholipase D in insulin-like growth factor-I-induced

activation of extracellular signal-regulated kinase, but not phosphatidylinositol 3-kinase or Akt, in Chinese hamster ovary cells. Biochem. J. **369:**363-368.

Barrett J.F., Lewis B.C., Hoang A.T., Alvarez R.J.J., and Dang C.V. 1995. Cyclin A links c-Myc to adhesion-independent cell proliferation. Journal of Biological Chemistry **270:**15923-15925.

Battey J., Moulding C., Taub R., Murphy W., Stewart T., Potter H., Lenoir G., and Leder P. 1983. The human c-myc oncogene: structural consequences of translocation into the IgH locus in Burkitt lymphoma. Cell **34:**779-787.

Baum M., Budzar A.U., Cuzick J., Forbes J., Houghton J.H., Klijn J.G., and Sahmoud T., ATAC Trialists' Group. 2002. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer: first results of the ATAC randomised trial. Lancet **359:**2131-2139.

Bazarov A.V., Adachi S., Li S.F., Mateyak M.K., Wei S., and Sedivy J.M. 2001. A Modest Reduction in c-myc Expression has Minimal Effects on Cell Growth and Apoptosis but Dramatically Reduces Susceptibility to Ras and Raf Transformation. Cancer Res. **61:**1178-1186.

Beato M., Herrlich P., and Schutz G. 1995. Steroid hormone receptors: many actors in search of a plot. Cell **83:**851-857.

Beatson G. 1896. On the treatment of inoperable cases of the mamma: suggestions for a new method of treatment with illustrative cases. Lancet **2:**104-107, 162-165.

Ben-Yosef T., Yanuka O., Halle D., and Benvenisty N. 1998. Involvement of Myc targets in c-*myc* and N-myc induced human tumors. Oncogene **17:**165-171.

Berns E.M., Klijn J.G., Van Putten W.L., Van Staveren I.L., Portengen H., and Foekens J.A. 1992. c-*myc* Amplification is a Better Prognostic Factor than *HER2/neu* Amplification in Primary Breast Cancer. Cancer Res. **52:**1107-1113.

Biscardi J.S., Tice D.A., and Parsons S.J. 1999. c-Src, receptor tyrosine kinases, and human cancer. Adv. Cancer Res. **76:**61-119.

Bishop J.M. 1982. Retroviruses and cancer genes. Adv. Cancer Res. **37:**1-32.

Bister K., and Jansen H.W. 1986. Oncogenes in retroviruses and cells: biochemistry and molecular genetics. Adv. Cancer Res. **47:**99-188.

Blackwood E., and Eisenman R.N. 1991. Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. Science **251:**1211-1217.

Brown H.A., Gutowski S., Kahn R.A., and Sternweis P.C. 1995. J. Biol. Chem. **270:**14935-14943.

Brown F.D., Thompson N., Saqib K.M., Clark J.M., Powner D., Thompson N.T., Solari R., and Wakelam M.J. 1998. Phospholipase D1 localises to secretory granules and lysosomes and is plasma-membrane translocated on cellular stimulation. Curr. Biol. **8:**835-838.

Brunn G.J., Hudson C.C., Sekulic A., Williams J.M., Hosoi H., Houghton P.J., Lawrence Jr. J.C., and Abraham R.T. 1997. Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. Science **277:**99-101.

Buzdar A.U., Jonat W., Howell A., Jones S.E., Blomqvist C.P., Vogel C.L., Eiermann W., Wolter J.M., Steinberg M., Webster A., and Lee D. 1998. Anastrozole versus megestrol acetate in the treatment of postmenopausal women with advanced breast carcinoma: results of a survival update based on a combined analysis of data from two mature phase III trials. Arimidex Study Group. Cancer **83:**1142-1152.

Cai S., and Exton J.H. 2001. Determination of interaction sites of phospholipase D1 for RhoA. Biochem. J. **355:**779-785.

Campisi J., Gray H.E., Pardee A.B., Dean M., and Sonenshein G.E. 1984. Cell-cycle control of c-myc but not c-ras expression is lost following chemical transformation. Cell **36:**241-247.

Cardona-Gomez P., Perez M., Avila J., Garcia-Segura L.M., and Wandosell F. 2004. Estradiol inhibits GSK3 and regulates interaction of E2 receptors, GSK3, and beta-catenin in the hippocampus. Mol. Cell Neurosci. **25:**363-373.

Carnero A., Cuadrado A., del Peso L., and Lacal J.C. 1994. Activation of type D phospholipase by serum stimulation and ras-induced transformation in NIH3T3 cells. Oncogene **9:**1387-1395.

Caumont A.S., Galas, M.C., Vitale N., Aunis D. and Bader M.F. 1998. Regulated exocytosis in chromaffin cells. Translocation of ARF6 stimulates a plasma membrane-associated phospholipase D. J. Biol. Chem. **273:**1373-1379.

Chen J., and Fang Y. 2002. A novel pathway regulating the mammalian target of rapamycin (mTOR) signaling. Biochem. Pharmacol. **64:**1071-1077.

Chen J.P., Lin C., Xu C.P., Zhang X.Y., Fu M., Deng Y.P., Wei Y., and Wu M. 2001. Molecular therapy with recombinant antisense c-myc adenovirus for human gastric carcinoma cells in vitro and in vivo. J. Gastroenterol. Hepatol. **16:**22-28.

Chen W., and Hahn W.C. 2003. SV40 early region oncoproteins and human cell transformation. Histol. Histopathol. **18:**541-550.

Chen Y., Zheng Y., and Foster D.A. 2003. Phospholipase D confers rapamycin resistance in human breast cancer cells. Oncogene **22:**3937-3942.

Chen Y., Rodrik V., and Foster D.A. 2005. Alternative phospholipase D / mTOR survival signal in human breast cancer cells. Oncogene **24:**672-679.

Chong L.D., Traynor-Kaplan A., Bokoch G.M., and Schwartz M.A. 1994. The small GTP-binding protein Rho regulates a phosphatidylinositol 4-phosphate 5-kinase in mammalian cells. Cell **79:**507-513.

Ciechanover A. 1994. The ubiquitin-proteasome proteolytic pathway. Cell **79**:13-21.
Claassen G.F., and Hann S.R. 1999. Myc-mediated transformation: the repression connection. Oncogene **18:**2925-2933.

Clarke R., Skaar T.C., Bouker K.B., Davis N., Lee Y.R., Welch J.N., and Leonessa F. 2001. Molecular and pharmacological aspects of antiestrogen resistance. J Steroid Biochem. Mol. Biol. **76:**71-84.

Clarke R., Leonessa F., Welch J.N., and Skaar T.C. 2001. Cellular and molecular pharmacology of antiestrogen action and resistance. Pharmacol Rev. **53**:25-71.

Cole M.D. 1986. The myc oncogene: its role in transformation and differentiation. Annu. Rev. Genet. **20:**361-384.

Coller H.A., Grandori C., Tamayo P., Colbert T., Lander E.S., Eisenman R.N., and Golub T.R. 2000 Expression analysis with oligonucleotide microarrays reveals that MYC regulates genes involved in growth, cell cycle, signaling, and adhesion. PNAS **97:**3260-3265.

Colley W.C., Sung T.C., Roll R., Jenco J., Hammond S.M., Altshuller Y., Bar-Sagi D., Morris A.J., and Frohman M.A. 1997. Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. Curr. Biol. **7:**191-201.

Cory S., Vaux D.L., Strasser A., Harris A.W., and Adams J.M. 1999. Insights from Bcl-2 and Myc: malignancy involves abrogation of apoptosis as well as sustained proliferation. Cancer Research **59:**1685s-1692s.

Cowley S.M., Hoare S., Mosselman S., and Parker M.G. 1997 Estrogen receptors alpha and beta form heterodimers on DNA. J. Biol. Chem. **272:**19858-19862.

Cross D.A., Alessi D.R., Cohen P., Andjelkovich M., and Hemmings B.A. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature **378:**785-789.

Dalla-Favera R., Gelmann E.P., Martinotti S., Franchini G., Papas T. S., Gallo R.C., and Wong-Staal F. 1982. Cloning and characterization of different human sequences related to the onc gene (v-myc) of avian myelocytomatosis virus (MC29). PNAS **79:** 6497-6501.

Dang C.V. 1999. c-Myc target genes involved in cell growth, apoptosis, and metabolism. Mol. Cell Biol. **19:**1-11.

Desbois-Mouthon C., Blivet-Van Eggelpoel M.J., Beurel E., Boissan M., Delelo R., Cadoret A., and Capeau J. 2002. Dysregulation of glycogen synthase kinase-3β signaling in hepatocellular carcinoma cells. Hepatology **36:**1528-1536.

Divecha N., Roefs M., Halstead J.R., D'Andrea S., Fernandez-Borga M., Oomen L., Saqib K.M., Wakelam M.J., and D'Santos C. 2000. Interaction of the type Ialpha PIPkinase with phospholipase D: a role for the local generation of phosphatidylinositol 4,5-bisphosphate in the regulation of PLD2 activity. EMBO J. **19:**5440-5449.

Doble B.W., and Woodgett J.R. 2003. GSK-3: tricks of the trade for a multi-tasking kinase. J. Cell Sci. **116:**1175-1186.

Dolnikov A., King A., Luxford C., Symonds G., and Sun L.Q. 1996. Ribozyme-mediated suppression of v-myc expression abrogates apoptosis in transformed monocytes. Cancer Gene Ther. **3:**289-295.

Dombernowsky P., Smith I., Falkson G., Leonard R., Panasci L., Bellmunt J., Bezwoda W., Gardin G., Gudgeon A., Morgan M., Fornasiero A., Hoffmann W., Michel J., Hatschek T., Tjabbes T., Chaudri H.A., Hornberger U., and Trunet P.F. 1998. Letrozole, a new oral aromatase inhibitor for advanced breast cancer: double-blind randomized trial showing a dose effect and improved efficacy and tolerability compared with megestrol acetate. J. Clin. Oncol. **16:**453-461.

Donjerkovic D., Zhang L., and Scott D.W. 1999. Regulation of p27Kip1 accumulation in murine B-lymphoma cells: role of c-myc and calcium. Cell Growth and Differentiation **10:**695-704.

Dorgan J.F., Longcope C., Stephenson Jr. H.E., Falk R.T., Miller R., Franz C., Kahle L., Campbell W.S., Tangrea J.A., and Schatzkin A. 1996. Relation of prediagnostic serum estrogen and androgen levels to breast cancer risk, Cancer Epidemiol. Biomark Prev. **5:**533-539.

Du G., Altshuller Y.M., Kim Y., Han J.M., Ryu S.H., Morris A.J., and Frohman M.A. 2000. Dual requirement for rho and protein kinase C in direct activation of phospholipase D1 through G protein-coupled receptor signaling. Mol. Biol. Cell **11:**4359-4368.

Du G., Altshuller Y.M., Vitale N., Huang P., Chasserot-Golaz S., Morris A.J., Bader M.F., and Frohman M.A. 2003. Regulation of phospholipase D1 subcellular cycling through coordination of multiple membrane association motifs. J. Cell. Biol. **162:**305-315.

Du G., Huang P., Liang B. T. and Frohman M.A. 2004. Phospholipase D2 localizes to the plasma membrane and regulates angiotensin II receptor endocytosis. Mol. Biol. Cell **15:**1024-1030.

Dubik D., and Shiu R.P. 1992. Mechanism of E2 activation of c-myc oncogene expression. Oncogene **7:**1587-1594.

Ebinuma H., Saito H., Kosuga M., Wakabayashi K., Saito Y., Takagi T., Nakamoto N., Okuyama T., and Ishii H. 2001. Reduction of c-myc expression by an antisense approach under cre/loxP switching induces apoptosis in human liver cancer cells. J. Cell Physiol. **188:**56-66.

Eilers M., Picard D., Yamamoto K.R., and Bishop J.M. 1989. Chimaeras of myc oncoprotein and steroid receptors cause hormone-dependent transformation of cells. Nature **340:**66-68.

Early Breast Cancer Trialists' Collaborative Group. 1998. Tamoxifen for early breast cancer: an overview of the randomised trials. Lancet **351:**1451-1467.

Evans R.M., and Hollenberg S.M. 1988. Zinc fingers: Gilt by association. Cell **52:**1-3.

Exton J.H. 2002. Phospholipase D-structure, regulation and function. Reviews of Physiology, Biochemistry and Pharmacology (Amara S.G., Bamberg E., Blaustein M.P., Grunicke H., Jahn R., Lederer W.J., Miyajima A., Murer H., Pfanner N., Schultz G., Schweiger M. Eds.), Springer-Verlag, Berlin. **144:**1-94.

Exton J.H. 2002. Regulation of phospholipase D. FEBS Lett. **531:**58-61.

Facchini L.M., and Penn L.Z. 1998. The molecular role of Myc in growth and transformation: recent discoveries lead to new insights. FASEB J. **12:**633-651.

Fang Y., Vilella-Bach M., Bachmann R., Flanigan A., and Chen J. 2001. Phosphatidic acid-mediated mitogenic activation of mTOR signaling. Science **294:**1942-1945.

Fang Y., Park I.H., Wu A.L., Du G., Huang P., Frohman M.A., Walker S.J., Brown H.A., and Chen J. 2003. PLD1 regulates mTOR signaling and mediates Cdc42 activation of S6K1. Curr. Biol. **13:**2037-2044.

Fernando R.I., and Wimalasena J. 2004. Estradiol abrogates apoptosis in breast cancer cells through inactivation of BAD: Ras-dependent nongenomic pathways requiring signaling through ERK and Akt. Mol. Biol. Cell. **15:**3266-3284.

Fisher B., Costantino J.P., Wickerham D.L, Cecchini R.S., Cronin W.M., Robidoux A., Bevers T.B., Kavanah M.T., Atkins J.N., Margolese R.G., Runowicz C.D., James J.M., Ford L.G., and Wolmark N. 1998. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl. Cancer Inst. **90:**1371-1388.

Foster D.A., and Xu L. 2003. Phospholipase D in cell proliferation and cancer. Mol. Cancer Res. **1:**789-800.

Foster D.A. 2004. Targeting mTOR-mediated survival signals in anticancer therapeutic strategies. Exp. Rev. Anticancer Ther. **4:**691-701.

Foster D.A. 2006. Phospholipase D survival signals as a therapeutic target in cancer. Current Signal Transduction Ther. **1:** in press.

Foster J.S., Henley D.C., Ahamed S., and Wimalasena J. 2001. Estrogens and cell cycle regulation in breast cancer. Trends Endocrinol. Metab. **12:**320-327.

Foster J.S., Fernando R.I., Ishida N., Nakayama K.I., and Wimalasena J. 2003. Estrogens down-regulate p27Kip1 in breast cancer cells through Skp2 and through nuclear export mediated by the ERK pathway. J. Biol. Chem. **278:**41355-41366.

Frankel P., Ramos M., Flom J., Bychenok S., Joseph T., Kerkhoff E., Rapp U.R., Feig L.A., and Foster D.A. 1999. Ral and Rho dependent activation of phospholipase D in v-Raf transformed cells. Biochem. Biophys. Res. Comm. **255:**502-507.

Freyberg Z., Sweeney D., Siddhanta A., Bourgoin S., Frohman M., and Shields D. 2001. Intracellular localization of phospholipase D1 in mammalian cells. Mol. Biol. Cell **12:**943-955.

Frohman M.A., Sung T.C., and Morris A.J. 1999. Mammalian phospholipase D structure and regulation. Biochim. Biophys. Acta. **1439:**175-186.

Gaschet J., and Hsu V.W. 1999. Distribution of ARF6 between membrane and cytosol is regulated by its GTPase cycle. J. Biol. Chem. **274:**20040-20045.

Gee J.M., Robertson J.F., Ellis I.O., and Nicholson R.I. 2001. Phosphorylation of ERK1/2 mitogen-activated protein kinase is associated with poor response to anti-hormonal therapy and decreased patient survival in clinical breast cancer. Int. J. Cancer. **95:**247-54.

Giguère V., Tremblay A., and Tremblay G.B. 1998. Estrogen receptor β: reevaluation of E2 and antiE2 signaling. *Steroids* **63:**335-339.

Girdler F., and Brotherick I. 2000. The oestrogen receptors (ER alpha and ER beta) and their role in breast cancer: a review. Breast **4:**194-200.

Giorello L., Clerico L., Pescarolo M.P., Vikhanskaya F., Salmona M., Colella G., Bruno S., Mancuso T., Bagnasco L., Russo P., and Parodi S. 1998. Inhibition of cancer cell growth and c-Myc transcriptional activity by a c-Myc helix 1-type peptide fused to an internalization sequence. Cancer Res. **16:**3654-3659.

Greenlee R.T., Murray T., Bolden S., and Wingo P.A. 2000. Cancer statistics, CA Cancer J. Clin. **50:**7-33.

Gregory M.A., and Hann S.R. 2000. c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. Mol. Cell Biol. **20:**2423-2435.

Gregory M.A., Qi Y., and Hann S.R. 2003. Phosphorylation by glycogen synthase kinase-3 controls c-myc proteolysis and subnuclear localization. J. Biol. Chem. **278:**51606-51612.

Grignani F., Lombardi L., Inghirami G., Sternas L., Cechova K., and Dalla-Favera R. 1990. Negative autoregulation of c-myc gene expression is inactivated in transformed cells. EMBO J. **9:**3913-3922.

Gschwind A., Fischer O.M., and Ullrich A. 2004. The discovery of receptor tyrosine kinases: targets for cancer therapy. Nat. Rev. Cancer. **4**:361-370.

Gustafsson J.A., and Warner M. 2000. Estrogen receptor beta in the breast: role in estrogen responsiveness and development of breast cancer. J. Steroid Biochem. Mol. Biol. **74:**245-248.

Guzzo J.A. 2000. Selective E2 response modulators: a new age of E2 in cardiovascular disease? Clin. Cardiol. **23:**15-17.

Hahn W.C., Counter C.M., Lundberg A.S., Beijersbergen R.L., Brooks M.W., and Weinberg R.A. 1999. Creation of human tumour cells with defined genetic elements. Nature **400:**464-468.

Hahn W.C., Dessain S.K., Brooks M.W., King J.E., Elenbaas B., Sabatini D.M., DeCaprio J.A., and Weinberg R.A. 2002. Enumeration of the simian virus 40 early region elements necessary for human cell transformation. Mol. Cell Biol. **22:**2111-2123.

Hall J.M., and McDonnell D.P. 1999. The estrogen receptor beta-isoform (ERbeta) of the human estrogen receptor modulates ERalpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology **140:**5566-5578.

Hall J.M., Couse J.F., and Korach K.S. 2001. The Multifaceted Mechanisms of Estradiol and Estrogen Receptor Signaling. J. Biol. Chem. **276:**36869-36872.

Hammond S.M., Altshuller Y.M., Sung T.C., Rudge S.A., Rose K., Engebrecht J., Morris A.J., and Frohman M.A. 1995. Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family. J. Biol. Chem. **270:**29640-29643.

Hammond S.M., Jenco J.M., Nakashima S., Cadwallader K., Gu Q., Cook S., Nozawa Y., Prestwich G.D., Frohman M.A., and Morris A.J. 1997. Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5-bisphosphate, ADPribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C-a. J. Biol. Chem. **272:**3860-3868.

Hanahan D.J., and Chaikoff I.L. 1947. The phosphorus-containing lipides of the carrot. J. Biol. Chem. **168:**233-240.

Hanahan D.J., and Chaikoff I.L. 1948. On the nature of the phosphorus-containing lipides of cabbage leaves and their relation to a phospholipide-splitting enzyme contained in these leaves. J. Biol. Chem. **172:**191-198.

Hanahan D, and Weinberg R.A. 2000. The hallmarks of cancer. Cell **100:**57-70.

Hann S.R., and Eisenman R.N. 1984. Proteins encoded by the human c-myc oncogene: differential expression in neoplastic cells. Mol. Cell. Biol. **4:**2486-2497.

Haslam R.J., and Coorssen J.R. 1993. Evidence that activation of phospholipase D can mediate secretion from permeabilized platelets. Adv. Exp. Med. Biol. **344:**149-164.

Heikkila R., Schwab G., Wickstrom E., Loke S.L., Pluznik D.H., Watt R., and Neckers L.M. 1987. c-myc antisense oligodeoxynucleotide inhibits entry into S phase but not progress from G0 to G1. Nature **328:**445-449.

Hemann M.T., Bric A., Teruya-Feldstein J., Herbst A., Nilsson J.A., Cordon-Cardo C., Cleveland J.L., Tansey W.P., and Lowe S.W. 2005. Evasion of the p53 tumour surveillance network by tumour-derived MYC mutants. Nature **436:**807-811.

Henderson B.E., Ross R., and Bernstein L. 1988. Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation award lecture. Cancer Res. **48:**246-253.

Hennings H., Glick A.B., Greenhalgh D.A., Morgan D.L., Strickland J.E., Tennenbaum T., and Yuspa S.H. 1993. Critical aspects of initiation, promotion, and progression in multistage epidermal carcinogenesis. Proc. Soc. Exp. Biol. Med. **202:**1-8.

Henriksson M., and Luscher B. 1996. Proteins of the Myc network: essential regulators of cell growth and differentiation. Adv. Cancer Res. **68:**109-182.

Hermeking H. 2003. The MYC Oncogene as a Cancer Drug Target. Current Cancer Drug Target **3:**163-175.

Honda A., Nogami M., Yokozeki T., Yamazaki M., Nakamura H., Watanabe H., Kawamoto K., Nakayama K., Morris A.J., Frohman M.A., and Kanaho Y. 1999. Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell **99:**521-532.

Hornia A., Lu Z., Sukezane T., Zhong M., and Foster D.A. 1999. Antagonistic effects of protein kinase C α and δ on both transformation and phospholipase D activity mediated by the EGF receptor. Mol. Cell. Biol. **19:**7672-7680.

Howell A., Robertson J.F., Quaresma Albano J., Aschermannova A., Mauriac L., Kleeberg U.R., Vergote I., Erikstein B., Webster A., and Morris C. 2002. Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. J. Clin. Oncol. **20:**3396-3403.

Huang P., Altshuller Y.M., Chunqiu Hou J., Pessin J.E. and Frohman M.A. 2005. Insulin-stimulated plasma membrane fusion of Glut4 glucose transporter-containing vesicles is regulated by phospholipase D1. Mol. Biol. Cell **16:**2614-2623.

Huang Y. 1997. Estrogen increases intracellular p26Bcl-2 to p21Bax ratios and inhibits taxol-induced apoptosis of human breast cancer MCF-7 cells. Breast Cancer Res. Treat. **42:**73-81.

Hui L., Abbas T., Pielak R., Joseph T., Bargonetti J., and Foster D.A. 2004. Phospholipase D elevates the level of MDM2 and suppresses DNA damage-induced increases in p53. Mol. Cell. Biol. **24:**5677-5688.

Hui L., Rodrik V., Pielak R.M., Zheng Y., and Foster D.A. 2005. mTOR-dependent Suppression of Protein Phosphatase 2A is Critical for Phospholipase D Survival Signals in Human Breast Cancer Cells. J. Biol. Chem. **280:**35829-35835.

Hurley L.H. 2001. Secondary DNA Structures as Molecular Targets for Cancer Therapeutics. Biochem. Soc. Trans. **29:**692-696.

Hurn P.D., and Macrae I.M. 2000. Estrogen as a neuroprotectant in stroke. J. Cereb. Blood Flow Metab. **20:**631-652.

Hyder S.M., Stancel G.M. and Loose-Mitchell D.S. 1994. Steroid hormone-induced expression of oncogene encoded nuclear proteins. Critical Reviews in Eukaryotic Gene Expression **4:**55-116.

Hynes N.E., and Lane H.A. 2001. Myc and Mammary Cancer: Myc is a Downstream Effector of the ErbB2 Receptor Tyrosine Kinase. J. Mammary Gland Biol. Neoplasia **6:**141-150.

Imamura F., Horai T., Mukai M., Shinkai K., Sawada M., and Akedo H. 1993. Induction of *in vitro* tumor cell invasion of cellular monolayers by lysophosphatidic acid or phospholipase D. Biochem. Biophys. Res. Commun. **193:**497-503.

Jain M., Arvanitis C., Chu K., Dewey W., Leonhardt E., Trinh M., Sundberg C. D., Bishop J.M., and Felsher D.W. 2002. Sustained Loss of a Neoplastic Phenotype by Brief Inactivation of MYC. Science **297:**102-104.

Jenkins G.H., Fisette P.L., and Anderson R.A. 1994. Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. J. Biol. Chem. **269:**11547-11554.

Jenkins G.M. and Frohman M.A. 2005. Phospholipase D: a lipid centric review. Cell Mol. Life Sci. **62:**2305-2316.

Jensen E.V., and Jordan V.C. 2003. The estrogen receptor: a model for molecular medicine. The Dorothy P. Landon AACR Prize for Translational Research. Clin. Cancer Res. **6:**1980-1989.

Jiang H., Luo J.Q., Urano T., Lu Z., Foster D.A., and Feig L.A. 1995. Involvement of Ral GTPase in v-Src-induced Phospholipase D activation. Nature **378:**409-412.

Jiang H., Lu Z., Luo J.Q., Wolfman A., and Foster D.A. 1995. Ras mediates the activation of phospholipase D by v-Src. J. Biol Chem. **270:**6006-6009.

Jiang H., Luo J.Q., Urano T., Frankel P., Lu Z., Foster D.A., and Feig L.A. 1995. Involvement of Ral GTPase in v-Src-induced phospholipase D activation**.** Eur. J. Pharmacol. **302:**183-190.

Jiang Y.W., Song J., Zang Q., and Foster D.A. 1994. Phosphatidylcholine-specific phospholipase D activity is elevated in v-Fps-transformed cells. Biochem. Biophys. Res. Comm. **203:**1195-1203.

Johnston S.R., and Dowsett M. 2003. Aromatase inhibitors for breast cancer: lessons from the laboratory. Nat. Rev. Cancer **3:**821-831.

Jones D.H., Bax B., Fensome A., and Cockcroft S. 1999. ADP ribosylation factor 1 mutants identify a phospholipase D effector region and reveal that phospholipase D participates in lysosomal secretion but is not sufficient for recruitment of coatomer I. Biochem. J. **341:**185-192.

Jones D.H., Morris J.B., Morgan C.P., Kondo H., Irvine R.F., and Cockcroft S. 2000. Type I phosphatidylinositol 4-phosphate 5-kinase directly interacts with ADP-ribosylation factor 1 and is responsible for phosphatidylinositol 4,5-bisphosphate synthesis in the golgi compartment. J. Biol. Chem. **275:**13962-13966.

Jordan V.C. 2001.Selective estrogen receptor modulation: a personal perspective. Cancer Res. **61:**5683-5687.

Jordan V.C. 2004. Selective E2 receptor modulation: concept and consequences in cancer. Cancer Cell **5:**207-213.

Joseph T., Wooden R., Bryant A., Zhong M., Lu Z., and Foster D.A. 2001. Transformation of cells overexpressing a tyrosine kinase by phospholipase D1 and D2. Biochem. Biophys. Res. Comm. **289:**1019-1024.

Joseph T., Bryant A., Wooden R., Kerkhoff E., Rapp U.R., and Foster D.A. 2002. Phospholipase D overcomes cell cycle arrest induced by high-intensity Raf signaling. Oncogene **21:**3651-3658.

Junghans M., Kreuter J., and Zimmer A. 2000. Antisense delivery using protamine-oligonucleotide particles. Nucleic Acids Res. **28:**E45.

Karin M., and Ben-Neriah Y. 2000. Phosphorylation meets ubiquitination: The control of NF-kB activity. Annu. Rev. Immunol. **18:**621-63.

Karnam P., Standaert M.L., Galloway L., and Farese R.V. 1997. Activation and translocation of Rho and ADP ribosylation factor by insulin in rat adipocytes. Apparent involvement of phosphatidylinositol 3-kinase. J. Biol. Chem. **272:**6136-6140.

Kawabe T., Muslin A.J., and Korsmeyer S.J. 1997. HOX11 interacts with protein phosphatases PP2A and PP1 and disrupts a G2/M cell-cycle checkpoint. Nature **385:**454-458.

Kelly K., Cochran B.H., Stiles C.D., and Leder P. 1983. Cell-specific regulation of the *c-myc* gene by lymphocyte mitogens and platelet-derived growth factor. Cell **35**:603-610.
Kerkhoff E., and Rapp U.R. 1998. High-intensity Raf signals convert mitotic cell cycling into cellular growth. Cancer Res. **58:**1636-1640.

Key T.J.A., and Pike M.C. 1988. The role of oestrogens and progestagens in the epidemiology and prevention of breast cancer, Eur. J. Cancer Clin. Oncol. **24:**29-43.

Kim J.H., Lee S.D., Han J.M., Lee T.G., Kim Y., Park J.B., Lambeth J.D., Suh P.G., and Ryu S.H. 1998. Activation of phospholipase D1 by direct interaction with ADP-ribosylation factor 1 and RalA. FEBS Lett. **430:**231-235.

Kim J.H., Kim Y., Lee S.D., Lopez I., Arnold R.S., Lambeth J.D., Suh P.G., and Ryu S.H. 1999. Selective activation of phospholipase D2 by unsaturated fatty acid. FEBS Lett. **454:**42-46.

Kim Y.R., Cha H.Y., Lim K., Hwang B.D., Hoe K.L., Namgung U., and Park S.K. 2003. Activation of epidermal growth factor receptor is responsible for pervanadate-induced phospholipase D activation. Exp. Mol. Med. **35:**118-24.

Klinge C.M. 2000. Estrogen receptor interaction with co-activators and co-repressors. Steroids **65:**227-251.

Koster R., Blatt L.M., Streubert M., Zietz C., Hermeking H., Brysch W., and Sturzl M. 1996. Consensus-interferon and platelet-derived growth factor adversely regulate proliferation and migration of kaposi's sarcoma cells by control of c-myc Expression. Am. J.Pathol. **149:**1871-1885.

Kuruvilla F.G., and Schreiber S.L. 1999. The PIK-related kinases intercept conventional signaling pathways. Chem. Biol. **6:**R129-R136.

Lacroix M., and Leclercq G. 2004. Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res. Treat. **83:**249-289.

Land H., Parada L.F., and Weinberg R.A. 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature **304:**596-602.

Lee Y.H., Kim H.S., Pai J.K., Ryu S.H., and Suh P.G. 1994. Activation of phospholipase D induced by platelet-derived growth factor is dependent upon the level of phospholipase C-gamma 1. J. Biol. Chem. **269:**26842-26847.

Leonetti C., Biroccio A., Benassi B., Stringaro A., Stoppacciaro A., Semple S.C., and Zupi G. 2001. Encapsulation of c-myc antisense oligodeoxynucleotides in lipid particles improves antitumoral efficacy in vivo in a human melanoma line. Cancer Gene Ther. **8:**459-468.

Lewis D.L., Hagstrom J.E., Loomis A.G., Wolff J.A., and Herweijer H. 2002. Efficient Delivery of siRNA for Inhibition of Gene Expression in Postnatal Mice. Nat. Genet. **32:**107-108.

Lowe S.W., and Lin A.W. 2000. Apoptosis in cancer. Carcinogenesis **21:**485-495.
Li J.J., and Li S.A. 1987. Estrogen carcinogenesis in Syrian hamster tissues: role of metabolism. Fed. Proc. **46:**1858-1863.

Li M., Makkinje A., and Damuni Z. 1996. The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. J. Biol. Chem. **271:**11059-11062.

Liao D.J., and Dickson R.B. 2000. c-*Myc* in breast cancer Endocrine-Related Cancer **7:**143-164.

Liehr J.G., Hall E.R., Avitts T.A., Randerath E., and Randerath K. 1987. Localization of estrogen-induced DNA adducts and cytochrome P-450 activity at the site of renal carcinogenesis in the hamster kidney. Cancer Res. **47:**2156-2159.

Lu Z., Hornia A., Joseph T., Sukezane T., Frankel P., Zhong M., Bychenok S., Xu L., Feig L.A., and Foster D.A. 2000. Phospholipase D and RalA cooperate with the EGF receptor to transform 3Y1 rat fibroblasts. Mol. Cell. Biol. **20:**462-467.

Lucocq J., Manifava M., Bi K., Roth M.G., and Ktistakis N.T. 2001. Immunolocalisation of phospholipase D1 on tubular vesicular membranes of endocytic and secretory origin. Eur. J. Cell Biol. **80:**508-520.

Luo J.Q., Liu X., Frankel P., Rotunda T., Ramos M., Flom J., Jiang H., Feig L.A., Morris A.J., Kahn R.A., and Foster D.A. Functional association between Arf and RalA in active phospholipase D complex. 1998. Proc. Natl. Acad. Sci. USA **95:**3632-3637.

Luo J.Q., Liu X., Hammond S.M., Colley W.C., Feig L.A., Frohman M.A., Morris A.J., and Foster D.A. 1997. RalA interacts directly with the Arf-responsive, PIP2-dependent Phospholipase D1. Biochem. Biophys. Res. Commun. **235:**854-859.

Lusher B., and Eiseman R.N. 1990. New light on Myc and Myb. Part I. Genes Dev. **4:**2025-35.

Lutterbach B., and Hann S.R. 1994. Hierarchical phosphorylation at N-terminal transformation-sensitive sites in c-Myc protein is regulated by mitogens and in mitosis. Mol. Cell Biol. **14:**5510-5522.

Marhin W.W., Chen S., Facchini L.M., Fornace A.J.J., and Penn L.Z. 1997. Myc represses the growth arrest gene gadd45. Oncogene **14:**2825-2834.

McGuffie E.M., and Catapano C.V. 2002. Design of a novel triple helix-forming oligodeoxyribonucleotide directed to the major promoter of the c-myc gene. Nucleic Acids Res. **30:**2701-2709.

Miller T.L., Huzel N.J., Davie J.R., and Murphy L.C. 1993. c-myc gene chromatin of estrogen receptor-positive and -negative breast cancer cells. Molecular and Cellular Endocrinology **91:**83-89.

Min D.S., Kwon T.K., Park W.S., Chang J.S., Park S.K., Ahn B.H., Ryoo Z.Y., Lee Y.H., Lee Y.S., Rhie D.J., Yoon S.H., Hahn S.J., Kim M.S., and Jo Y.H. 2001. Neoplastic transformation and tumorigenesis associated with overexpression of phospholipase D isozymes in cultured murine fibroblasts. Carcinogenesis **22:**1641-167.

Mitchell K.O., and El-Deiry W.S. 1999. Overexpression of c-Myc inhibits p21WAF1/CIP1 expression and induces S-phase entry in 12-*O*-tetradecanoylphorbol–13-acetate (TPA)-sensitive human cancer cells. Cell Growth and Differentiation **10:**223-23.

Montano M.M., Muller V., Trogaugh A., and Katzenellenbogen B.S. 1995. The carboxy-terminal F domain of the human E2 receptor: role in the transcriptional activity of the receptor and the effectiveness of antiE2s as E2 antagonists. Mol. Endocrinol. **9:**814-25.

Morris A.J., Engebrecht J., and Frohman M.A. 1996. Structure and regulation of phospholipase D. Trends Pharmacol. Sci. **17:**182-185.

Motoike T., Bieger S., Wiegandt H., and Unsicker K. 1993. Induction of phosphatidic acid by fibroblast growth factor in cultured baby hamster kidney fibroblasts. FEBS Lett. **332**:164-168.

Nabholtz J.M., Bonneterre J., Buzdar A., Robertson J.F., and Thurlimann B. 2003. Anastrozole (Arimidex) versus tamoxifen as first-line therapy for advanced breast cancer in postmenopausal women: survival analysis and updated safety results. Eur. J. Cancer **39:** 1684-1689.

Nass S.J., and Dickson R. 1997. Defining a role for c-Myc in breast tumorigenesis. Breast Cancer Research and Treatment **44:**1-22.

Nesbit C.E., Tersak J.M., and Prochownik E.V. 1999. MYC oncogenes and human neoplastic disease. Oncogene **18:**3004-3016.

Neshat M.S., Mellinghoff I.K., Tran C., Stiles B., Thomas G., Petersen R., Frost P., Gibbons J.J., Wu H., and Sawyers C.L. 2001. Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. Proc. Natl. Acad. Sci. USA **98:**10314-10319.

Noh D.Y., Ahn S.J., Lee R.A., Park I.A., Kim J.H., Suh P.G., Ryu S.H., Lee K.H., and Han J.S. 2000. Overexpression of phospholipase D1 in human breast cancer tissues. Cancer Lett. **161:**207-214.

Nordman I.C., Spillane A.J., and Hamilton A.L. 2005. The aromatase inhibitors in early breast cancer: who, when, and why? Med. J. Aust. **183:**24-27.

O'Luanaigh N., Pardo R., Fensome A., Allen-Baume V., Jones D., Holt M.R., and Cockcroft S. 2002. Continual production of phosphatidic acid by phospholipase D is essential for antigen-stimulated membrane ruffling in cultured mast cells. Mol. Biol. Cell **13:**3730-3746.

Ogawa S., Inoue S., Watanabe T., Hiroi H., Orimo A., Hosoi T., Ouchi Y., and Muramatsu M. 1998. The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro. Biochem. Biophys. Res. Commun. **243:**122-126.

Olefsky J.M. 2001. Nuclear Receptor Minireview Series. J. Biol. Chem. **276:**36863-36864.

Osborne C.K., and Schiff R. 2005. Aromatase inhibitors: future directions. J. Steroid Biochem. Mol. Biol. **95:**183-187.

Oude-Weernink P.A., Schulte P., Guo Y., Wetzel J., Amano M., Kaibuchi K., Haverland S., Vob M., Schmidt M., Mayr G.W., and Jakobs K.H. 2000. Stimulation of phosphatidylinositol-4-phosphate 5-kinase by Rho-kinase. J. Biol. Chem. **275:**10168-10174.

Packham G., and Cleveland J.L. 1995. c-Myc and apoptosis. Biochimica et Biophysica Acta. **1242:**11-28.

Pai J.K., Frank E.A., Blood C., and Chu M. 1994. Novel ketoepoxides block phospholipase D activation and tumor cell invasion. Anticancer Drug Des. **9:**363-372.

Pallas D.C., Shahrik L.K., Martin B.L., Jaspers S., Miller T.B., Brautigan D.L., and Roberts T.M. 1990. Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. Cell **60:**167-176.

Palmieri C., Cheng G.J., Saji S., Zelada-Hedman M., Warri A., Weihua Z., Van Noorden S., Wahlstrom T., Coombes R.C., Warner M., and Gustafsson J.A. 2002. Estrogen receptor beta in breast cancer. Endocr. Relat. Cancer **9:**1-13.

Pardee A.B. 1974. A restriction point for control of normal animal cell proliferation. Proc Natl. Acad. Sci. USA. **71:**1286-1290.

Park S.K., Min D.S., and Exton J.H. 1998. Definition of the protein kinase C interaction site of phospholipase D. Biochem. Biophys. Res. Commun. **244:**364-367.

Pastorino F., Stuart D., Ponzoni M., and Allen T.M. 2001. Targeted delivery of antisense oligonucleotides in cancer. J. Control Release **74:**69-75.

Pearce S.T., and Jordan V.C. 2004. The biological role of estrogen receptors alpha and beta in cancer. Crit. Rev. Oncol. Hematol. **1:**3-22.

Pelengaris S., Khan M., and Evan G. 2002. c-MYC: more than just a matter of life and death. Nat. Rev. Cancer **2:**764-776.

Perillo B., Sasso A., Abbondanza C., and Palumbo G. 2000. 17β-estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two E2-responsive elements present in the coding sequence. Mol. Cell. Biol. **20:**2890-2901.

Pettersson K., Grandien K., Kuiper G.G., and Gustafsson J.A. 1997 Mouse estrogen receptor beta forms estrogen response element-binding heterodimers with estrogen receptor alpha. Mol. Endocrinol. **11:**1486-1496.

Pike M.C., Spicer D.V., Dahmoush L., and Press M.F. 1993. Estrogens, progestogens, normal breast cell proliferation, and breast cancer risk. Epidemiol. Rev. **15**:17-35.

Pipas J.M., and Levine A.J. 2001. Role of T antigen interactions with p53 in tumorigenesis. Semin. Cancer Biol. **11:**23-30.

Plevin R., Cook S.J., Palmer S., and Wakelam M.J. 1991. Multiple sources of sn-1,2-diacylglycerol in platelet-derived-growth factor-stimulated swiss 3T3 fibroblasts. Biochem J. **279:**559-65.

Podsypanina K., Lee R.T., Politis C., Hennessy I., Crane A., Puc J., Neshat M., Wang H., Yang L., Gibbons J., Frost P., Dreisbach V., Blenis J., Gaciong Z., Fisher P., Sawyers C., Hedrick-Ellenson L., and Parsons R. 2001. An inhibitor of mTOR reduces neoplasia and normalizes p70/S6 kinase activity in Pten+/- mice. Proc Natl Acad Sci USA **98:**10320-10325.

Prendergast G.C. 1999. Mechanisms of apoptosis by c-Myc. Oncogene **18:**2967-2987.

Pulverer B.J., Fisher C., Vousden K., Littlewood T., Evan G., and Woodgett J.R. 1994. Site-specific modulation of c-Myc cotransformation by residues phosphorylated in vivo. Oncogene **9:**59-70.

Radhakrishna H., and Donaldson J.G. 1997. ADP-ribosylation factor 6 regulates a novel plasma membrane recycling pathway. J. Cell Biol. **139:**49-61.

Reich N.C., and Levine A.J. 1982. Specific interaction of the SV40 T antigen-cellular p53 protein complex with SV40 DNA. Virology **117:**286-290.

Rodrik V., Zheng Y., Harrow F., Chen Y., and Foster D.A. 2005. Survival signals generated by E2 and phospholipase D in MCF-7 breast cancer cells are dependent on Myc. Mol. Cell Biol. **25:**7917-7925.

Rose C. 2003. A comparison of the efficacy of aromatase inhibitors in second-line treatment of metastatic breast cancer. Am. J. Clin. Oncol. **26:**S9-S16.

Rose-Hellekant T.A., and Sandgren E.P. 2000. Transforming growth factor alpha- and c-myc-induced mammary carcinogenesis in transgenic mice. Oncogene **19:**1092-1096.

Roth M.G., Bi K., Ktistakis N.T., and Yu S. 1999. Phospholipase D as an effector for ADP- Ribosylation factor in the regulation of vesicular traffic. Chem. Phys. Lipids **98:**141-152.

Sa G., and Das T. 1999. Basic fibroblast growth factor stimulates cytosolic phospholipase A2, phospholipase C-γ1 and phospholipase D through distinguishable signaling mechanisms. Mol. Cell Biochem. **198:**19-30.

Saito M., and Kanfer J. 1975. Phosphatidohydrolase activity in a solubilized preparation from rat brain particulate fraction. Arch. Biochem. Biophys. **169:**318-323.

Salghetti S.E., Kim S.Y., and Tansey W.P. 1999. Destruction of Myc by ubiquitin-mediated proteolysis:Cancer-associated and transforming mutations stabilize Myc. EMBO J. **18:**717-726.

Samuels M.L., and McMahon M. 1994. Inhibition of platelet-derived growth factor- and epidermal growth factor-mediated mitogenesis and signaling in 3T3 cells expressing ΔRaf-1:ER, an estradiol-regulated form of Raf-1. Mol. Cell Biol. **14:**7855-7866.

Santen R.J., Boucher A.E., Santner S.J., Henderson I.C., Harvey H., and Lipton A. 1987. Inhibition of aromatase as treatment of breast carcinoma in postmenopausal women. J. Lab.Clin.Med. **109:**278-289.

Santos G.F., Scott G.K., Lee W.M., Liu E., and Benz C. 1988. Estrogen-induced post-transcriptional modulation of c-myc proto-oncogene expression in human breast cancer cells. J. Biol. Chem. **263:**9565-9568.

Sarri E., Pardo R., Fensome-Green A., and Cockcroft S. 2003. Endogenous phospholipase D2 localizes to the plasma membrane of RBL-2H3 mast cells and can be distinguished from ADP ribosylation factor-stimulated phospholipase D1 activity by its specific sensitivity to oleic acid. Biochem. J. **369:**319-329.

Schiff R., Massarweh S., Shou J., and Osborne C.K. 2003. Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. Clin. Cancer Res. **9:**447S-54S.

Schmelzie T., and Hall M.N. 2000. TOR, a central controller of cell growth. Cell **103:**253-262.

Schmitz A.A., Govek E.E., Bottner B., and Van Aelst L. 2000. Rho GTPases: signaling, migration, and invasion. Exp. Cell Res. **261:**1-12.

Sciorra V.A., Rudge S.A., Wang J., McLaughlin S., Engebrecht J., and Morris A.J. 2002. Dual role for phosphoinositides in regulation of yeast and mammalian phospholipase D enzymes. J.Cell Biol. **159:**1039-1049.

Schuchard M., Landers J.P., Sandhu N.P., and Spelsberg T.C. 1993. Steroid hormone regulation of nuclear proto-oncogenes. Endocrine Reviews **14:**659-669.

Sears R.C. 2004. The life cycle of c-myc: from synthesis to degradation. Cell Cycle **3:**1133-1137.

Sears R.C., Leone G., DeGregori J., and Nevins J.R. 1999. Ras enhances Myc protein stability. Mol. Cell **3:**169-179.

Sears R., Nuckolls F., Haura E., Taya Y., Tamai K., and Nevins J.R. 2000. Multiple ras-dependent phosphorylation pathways regulate Myc protein stability. Genes Dev. **14:**2501-2514.

Sekulic A., Hudson C.C., Homme J.L., Yin P., Otterness D.M., Karnitz L.M., and Abraham R.T. 2000. A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. Cancer Res. **60:**3504-3513.

Seymour L.W., Shoaibi M.A., Martin A., Ahmed A., Elvin P., Kerr D.J., and Wakelam M.J. 1996. Vascular endothelial growth factor stimulates protein kinase C-dependent phospholipase D activity in endothelial cells. Lab. Invest. **75:**427-437.

Shen Y., Xu L., and Foster D.A. 2001. Role for phospholipase D in receptor-mediated endocytosis. Mol. Cell. Biol. **21:**595-602.

Shen Y., Zheng Y., and Foster D.A. 2002. Phospholipase D2 stimulates cell protrusion in v-Src-transformed cells. Biochem. Biophys. Res. Commun. **293:**201-206.

Shiu R.P., Watson P.H. and Dubik D. 1993. c-*myc* oncogene expression in estrogen-dependent and -independent breast cancer. Clinical Chemistry **39:**353-355.

Simonsson T., and Henriksson M. 2002. c-myc suppression in Burkitt's lymphoma cells. Biochem. Biophys. Res. Commun. **290:**11-15.

Slaaby R., Du G., Altshuller Y.M., Frohman M.A., and Seedorf K. 2000. Insulin-induced phospholipase D1 and phospholipase D2 activity in human embryonic kidney-293 cells mediated by the phospholipase C g and protein kinase C a signaling cascade. Biochem. J. **351:**613-619.

Sliva D., Rizzo M.T., and English D. 2002. Phosphatidylinositol 3-kinase and NF-κB regulate motility of invasive MDA-MB-231 human breast cancer cells by the secretion of urokinase-type plasminogen activator. J. Biol. Chem. **277:**3150-3157.

Sommer S., and Fuqua S.A. 2001. Estrogen receptor and breast cancer. Semin Cancer Biol. **11:**339-352.

Song J., Pfeffer L.M., and Foster D.A. 1991. v-Src increases diacylglycerol levels via a type D phospholipase-mediated hydrolysis of phosphatidylcholine. Mol. Cell Biol. **11:** 4903-4908.

Song J., Jiang Y.W., and Foster D.A. 1994. EGF induces the production of biologically distinguishable diglyceride species from phosphatidylinositol and phosphatidylcholine: evidence for the independent activation of type C and type D phospholipases. Cell Growth Differ. **5:**79-85.

Sontag E., Fedorov S., Kamibayashi C., Robbins D., Cobb M., and Mumby M. 1993. The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation. Cell **75:**887-897.

Spencer C.A., and Groudine M. 1991. Control of c-Myc Regulation in Normal and Neoplastic Cells. Adv. Cancer Res. **56:**1-48.

Spotts G.D., Patel S.V., Xiao Q., and Hann S.R. 1997. Identification of downstream-initiated c-Myc proteins which are dominant-negative inhibitors of transactivation by full-length c-Myc proteins. Mol. Cell. Biol. **17:**1459-1468.

Srivastava A.K., and Pandey S.K. 1998. Potential mechanism(s) involved in the regulation of glycogen synthesis by insulin. Mol. Cell. Biochem. **182:**135-141.

Steed P.M., Clark K.L., Boyar W.C., and Lasala D.J. 1998. Characterization of human PLD2 and the analysis of PLD isoform splice variants. FASEB J. **12:**1309-1317.

Stewart T.A., Pattengale P.K., and Leder P. 1984. Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. Cell **38:**627-637.

Stromberg T., Dimberg A., Hammarberg A., Carlson K., Osterborg A., Nilsson K., and Jernberg-Wiklund H. 2003. Rapamycin sensitizes multiple myeloma cells to apoptosis induced by dexamethasone. Blood **103:**3138-3147.

Stutchfield J., and Cockcroft S. 1993. Correlation between secretion and phospholipase D activation in differentiated HL60 cells. Biochem. J. **293:**649-655.

Sung T.C., Roper R.L., Zhang Y., Rudge S.A., Temel R., Hammond S.M., Morris A.J., Moss B., Engebrecht J., and Frohman M.A. 1997. Mutagenesis of phospholipase D defines a superfamily including a trans-Golgi viral protein required for poxvirus pathogenicity. EMBO J. **16:**4519-4530.

Sung T.C., Altshuller Y.M., Morris A.J., and Frohman M.A. 1999. Molecular analysis of mammalian phospholipase D2. J. Biol. Chem. **274:**494-502.

Sung T.C., Zhang Y., Morris A.J., and Frohman M.A. 1999. Structural analysis of human phospholipase D1. J. Biol. Chem. **274:**3659-3666.

Teixeira C., Reed J.C., and Pratt M.A. 1995. Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. Cancer Res. **55:**3902-3907.

Termine J.D., and Wong M. 1998. Post-menopausal women and osteoporosis: available choices for the maintenance of skeletal health. Maturitas **30:**241-245.

Tsai S.C., Adamik R., Hong J.X., Moss J., Vaughan M., Kanoh H., and Exton J.H. 1998. Effects of arfaptin 1 on guanine nucleotide-dependent activation of phospholipase D and cholera toxin by ADP-ribosylation factor. J. Biol. Chem. **273:**20697-20701.

Tzukerman M.T., Esty A., Santiso-Mere D., Danielian P., Parker M.G., Stein R.B., Pike J.W., and McDonnell D.P. 1994. Human E2 receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. Mol. Endocrinol. **8:**21-30.

Uchida N., Okamura S., and Nagamachi Y. 1997. Increased phospholipase D activity in human breast cancer. J. Cancer Res. Clin. Oncol. **123:**280-285.

Uchida N., Okamura S., and Kuwano H. 1999. Phospholipase D activity in human gastric carcinoma. Anticancer Res. **19:**671-675.

Vakkala M., Lahteenmaki K., Raunio H., Paakko P., and Soini Y. 1999. Apoptosis during breast carcinoma progression. Clinical Cancer Research **5:**319-324.

Van Weeren P.C., De Bruyn K.M., De Vries-Smits A.M., Van Lint J., and Burgering B.M. 1998. Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. J. Biol. Chem. **273:**13150-13156.

Vieira A.V., Lamaze C., and Schmid S.L. 1996. Control of EGF receptor signaling by clathrin-mediated endocytosis. Science **274:**2086-2089.

Vivanco I., and Sawyers C.L. 2002. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nature Rev. Cancer **2:**489-501.

Wang J., Xie L.Y., Allan S., Beach D., and Hannon G.J. 1998. Myc Activates Telomerase. Genes Dev. **12:**1769-1774.

Webb P., Lopez G.N., Uht R.M., and Kushner P.J. 1995. Tamoxifen activation of the E2 receptor/AP-1 pathway: potential origin for the cell-specific E2-like effects of antiE2s. Molecular Endocrinology **9:**443-456.

Webster N.J., Green S., Tasset D., Ponglikitmongkol M., and Chambon P. 1989. The transcriptional activation function located in the hormone-binding domain of the human oE2 receptor is not encoded in a single exon. EMBO J. **8:**1441-1446.

Weigel N.L. 1996. Steroid hormone receptors and their regulation by phosphorylation. Biochem. J. **319:**657-667.

Welcker M., Orian A., Jin J., Grim J.A., Harper J.W., Eisenman R.N., and Clurman B.E. 2004. The Fbw7 tumor suppressor regulates glycogen synthase kinase 3

phosphorylation-dependent c-Myc protein degradation. Proc. Natl. Acad. Sci. USA **101:**9085-9090.

West M.J., Stoneley M., and Willis A.E. 1998. Translational induction of the *c-myc* Oncogene via activation of the FRAP/TOR signaling pathway. Oncogene **17:**769-780.

Wu M., Bellas R.E., Shen J., Yang W., and Sonenshein G.E. 1999. Increased p27Kip1 cyclin-dependent kinase inhibitor gene expression following anti-IgM treatment promotes apoptosis of WEHI 231 B cells. Journal of Immunology **163:**6530-6535.

Xie M.S., Jacobs L.S., and Dubyak G.R. 1991. Regulation of phospholipase D and primary granule secretion by P2-purinergic- and chemotactic peptide-receptor agonists is induced during granulocytic differentiation of HL-60 cells. J. Clin. Invest. **88:**45-54.

Xie Z., Ho W.T., and Exton J.H. 2002. Mechanisms of regulation of phospholipase D1 and D2 by the heterotrimeric G proteins G13 and Gq. J. Biol. Chem. **277:**11979-11986.

Xu Y., Seet L.F., Hanson B., and Hong W. 2001. The Phox homology (PX) domain, a new player in phosphoinositide signaling. Biochem. J. **360:**513-530.

Yada M., Hatakeyama S., Kamura T., Nishiyama M., Tsunematsu R., Imaki H., Ishida N., Okumura F., Nakayama K., and Nakayama K.I. 2004. Phosphorylation-dependent degradation of c-Myc is mediated by the F-box protein Fbw7. EMBO J. **23:**2116-2125.

Yamada Y., Hamajima N., Kato T., Iwata H., Yamamura Y., Shinoda M., Suyama M., Mitsudomi, T., Tajima K., Kusakabe S., Yoshida H., Banno Y., Akao Y., Tanaka M., and Nozawa Y. 2003. Association of a polymorphism of the phospholipase D2 gene with the prevalence of colorectal cancer. J. Mol. Med. **81:**126-131.

Yamazaki M., Zhang Y., Watanabe H., Yokozeki T., Ohno S., Kaibuchi K., Shibata H., Muka, H., Ono Y., Frohman M.A., and Kanaho Y. 1999. Interaction of the small G protein RhoA with the C terminus of human phospholipase D1. J. Biol. Chem. **2774:** 6035-6038.

Yang S.I., Lickteig R.L., Estes R., Rundell K., Walter G., and Mumby M.C. 1991. Control of protein phosphatase 2A by simian virus 40 small-t antigen. Mol. Cell. Biol. **11:**1988-1995.

Yang S.F., Freer S., and Benson A.A. 1967. Transphosphatidylation by phospholipase D. J. Biol. Chem. **242:**477-484.

Yarden Y. 2001. The EGFR family and its ligands in human cancer. signaling mechanisms and therapeutic opportunities. Eur. J. Cancer **4:**S3-8.

Yeh E., Cunningham M., Arnold H., Chasse D., Monteith T., Ivaldi G., Hahn W.C., Stukenberg P.T., Shenolikar S., Uchida T., Counter C.M., Nevins J.R., Means A.R., and Sears R. 2004. A signaling pathway controlling c-Myc degradation that impacts oncogenic transformation of human cells. Nat. Cell Biol. **6:**308-318.

Zervos A. S., Gyuris J., and Brent R. 1993. Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell **72:**223-232.

Zetterberg A., Larsson O., and Wiman K.G. 1995. What is the restriction point? Curr. Opin. Cell Biol. **7:**835-842.

Zhang G.F., Patton W.A., Lee F.J.L., Liyanage M., Han J.S., Rhee S.G., Moss J., and Vaughan M. 1995. Different ARF domains are required for the activation of cholera toxin and phospholipase D. J. Biol. Chem. **270:**21-24.

Zhang Y.M., Rusckowski M., Liu N., Liu C., and Hnatowich D.J. 2001. Cationic liposomes enhance cellular/nuclear localization of 99mTc-antisense oligonucleotides in target tumor cells. Cancer Biother. Radiopharm. **16:**411-419.

Zhao Y., Ehara H., Akao Y., Shamoto M., Nakagawa Y., Banno Y., Deguchi T., Ohishi N., Yagi K., and Nozawa Y. 2000. Increased activity and intranuclear expression of phospholipase D2 in human renal cancer. Biochem. Biophys. Res. Commun. **278:**140-143.

Zheng Y., Rodrik V., Toschi A., Shi M., Hui L., Shen Y., and Foster D.A. 2006. Phospholipase D couples survival and migration signals in response to stress in human breast cancer cells. J. Biol. Chem. **281:** in press.

Zhong M., Shen Y., Zheng Y., Joseph T., Jackson D., Beychenok S., and Foster D.A. 2003. Phospholipase D prevents apoptosis in v-Src-transformed rat fibroblasts and MDA-MB-231 breast cancer cells. Biochem. Biophys. Res. Comm. **302:**615-619.