# Investigating the Molecular Mechanism of ERp29-regulated Cell Cycle Arrest in Breast Cancer

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# Publications

**Gao D**, Bambang IF, Putti TC, Lee Y K, Richardson DR, Zhang D. **ERp29 induces Breast Cancer cell growth arrest and survival through modulation of activation of P38 and upregulation of ER stress protein P58 (IPK)** Laboratory Investigation *advance online publication 7 November 2011; doi: 10.1038/labinvest.2011.163*

## TABLE OF CONTENTS

# Summary

Endoplasmic reticulum protein 29 (ERp29) is a novel endoplasmic reticulum (ER) luminal protein and plays a critical role in protein unfolding and secretion. Recently, it was found that ERp29 is a novel tumor suppressor which drives the proliferative MDA-MB-231 breast cancer cells into a dormant state. However, the mechanism underlining this process is not fully understood. In this thesis, some aspects of the mechanism of how ERp29 induces tumor cell dormancy are studied. These studies provided evidence that overexpression of ERp29 induces breast cancer cell cycle arrest by modulating endoplasmic reticulum stress. Overexpression of ERp29 down-regulates the expression of eIF2α, a key ER transcription factor, and up-regulates the cyclin-dependent kinase, $p27^{kip1}$, a tumor suppressor. High expression of eIF2α was found in three proliferative breast cancer cell lines -- BT549, MDA-MB-231 and SKBR3, suggesting its potential as a marker of tumor aggressiveness. $P58^{ipk}$ was also markedly increased, and appeared to inhibit eIF2α phosphorylation. Silencing of eIF2α in ERp29-overexpressed MDA-MB-231 cells dramatically induces up-regulation of $p27^{kip1}$. Data showed that the downstream target of eIF2α, cyclinD1, translocated into the cytoplasm of the ERp29-overexpressed MDA-MB-231 cells, in contrast to the accumulation of cyclinD1 inside the cell nuclei, in ERp29-silenced MCF7 cells. Using immunofluorescence imaging, the translocation of cyclinD1 into the cytoplasm was shown to be phosphorylation-dependent, as phosphorylated cyclinD1 also translocated to the cytoplasm in the ERp29-overexpressed MDA-MB-231 while in the ERp29-silenced MCF7, phosphorylated cyclinD1 accumulated inside the nuclei, which facilitates tumor growth.

# List of Tables

# List of Figures

# List of Abbreviations

| | |
|---|---|
| APS | Ammonium persulfate |
| ATF4 | Activating transcription factor 4 |
| ATF6 | Activating transcription factor 6 |
| BSA | Bovine serum albumin |
| CDK | Cyclin-dependent kinase |
| CHOP | C/EBP homologous protein |
| DAPI | 4'-6-Diamidino-2-phenylindole |
| DTT | Dithiothreitol |
| EDTA | Ethylenediaminetetraacetic acid |
| eIF2α | eukaryotic translation initiation factor 2-α subunit |
| ER | Endoplasmic reticulum |
| ERp29 | Endoplasmic reticulum protein 29 |
| FBS | Fetal bovine serum |
| GRP78 | Glucose-regulated protein 78 |
| GRP94 | Glucose-regulated protein 94 |
| HRP | Horseradish peroxidase |
| IRE1 | Inositol-requiring enzyme 1 |
| Nrf2 | NF-E2 related factor 2 |
| PBS | Phosphate buffered saline |
| PBST | Phosphate buffered saline with Tween-20 |
| PDI | Protein disulfide isomerase |
| p-eIF2α | Phosphorylated-eIF2α |
| PERK | PKR-like endoplasmic reticulum kinase |
| PVDF | Hybond-P Polyvinylidene Fluoride |
| Rb | Retinoblastoma |
| RIPA | Radio-Immunoprecipitation Assay |
| rER | Rough endoplasmic reticulum |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophoresis |
| sER | Smooth endoplasmic reticulum |
| SiRNA | Small RNA |
| SR | Sarcoplasmic reticulum |
| SRP | Signal recognition particles |
| TEMED | N,N,N,N -tetramethyl-ethylenediamine |
| UPR | Unfolded Protein Response |
| XBP-1 | X-Box binding protein 1 |

# Chapter 1

# INTRODUCTION

## 1.1 Breast Cancer

### 1.1.1 Definition of Breast Cancer

Normal cells reproduce themselves in a healthy way because of proper regulatory functions of certain genes inside their nuclei. However, if mutation occurs, some of these genes will be turned on while others will be turned off; leading to cells that growing and dividing without regulatory control and thus forming a tumor. A tumor can be benign, that is not harmful to health, or it can be malignant, resulting in growth out of control and spread across the whole body. Breast cancer,-refers to the malignant cancer that originates from breast cells. Breast cancer mostly originates in the cells of lobules or ducts. Cancers originating from ducts are known as ductal carcinomas; those originating from lobules are known as lobular carcinomas. It can also originate at a lesser frequency, from stromal tissues, which include the fatty and fibrous connective tissues of the breast.

### 1.1.2 Incidence of Breast Cancer Worldwide

Breast cancer is the most common cancer among women world-wide (1).In the more-developped countries, the breast cancer incidences are the highest (2). In 2002, It was estimated that 636,000 new cases occurred in developed countries and 514,000 more occurred in developing countries (1). Breast cancer is also the most important cause of neoplastic deaths among women; the estimated number of deaths in 2002 was 410,000 world-wide (1). The incidence of breast cancer is low (less than 0.02%) in most countries

from sub-Saharan Africa, in China and in other countries of eastern Asia, except Japan. The highest rates (0.08%-0.09%) are recorded in North America, in regions of South America, including Brazil and Argentina, in northern and western Europe, and Australia. (Figure 1) In rural areas, the rate of breast cancer is lower than the unban areas (2).

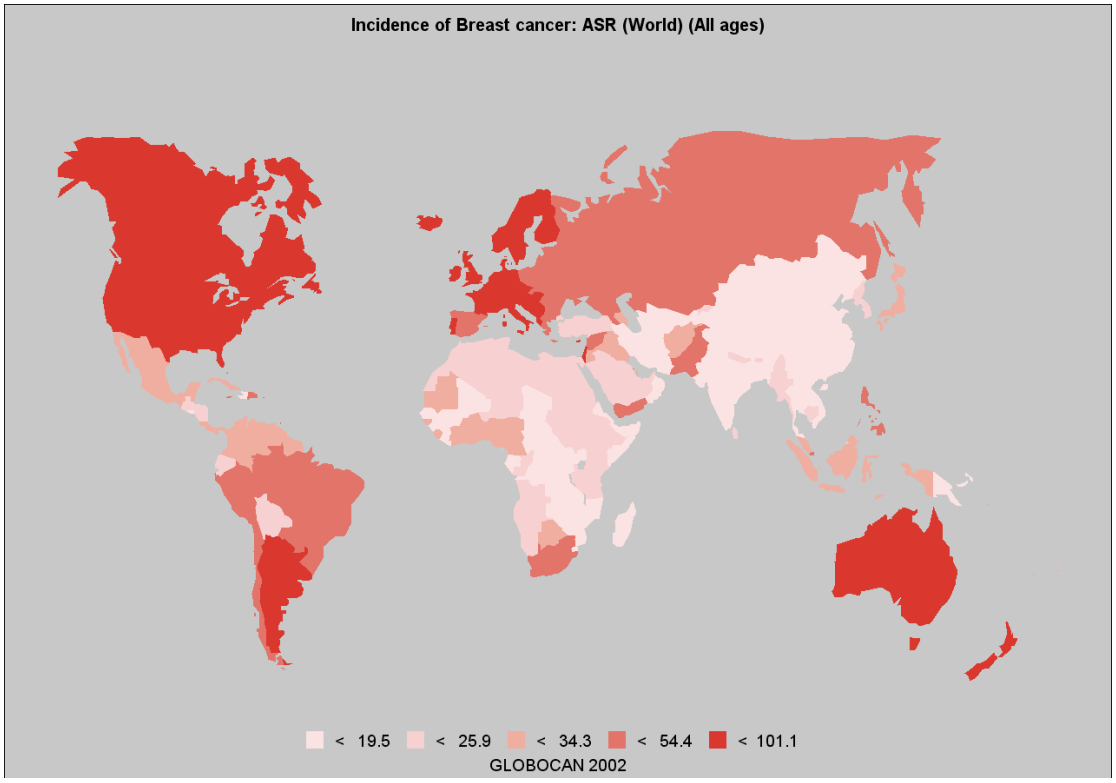



**Figure 1 Incidence of breast cancer world-wide.**
Data is sourced from World Cancer Report 2008, International Agency for Research on Cancer

### 1.1.3 Incidence of Breast Cancer in Singapore

Breast cancer is the most common cancer among Asian women (3) and among Singapore women (4). During 2005 to 2009, breast cancer was the top number 1 cancer with the highest incidence among Singapore women (Figure 2) (5). It was also the number 1 cancer resulting in death among females in Singapore.During the past four decades, since 1968, when Singapore experienced rapid economic growth and transited from a developing country to a developed industrial society, the breast cancer incidence grews steadily (6)

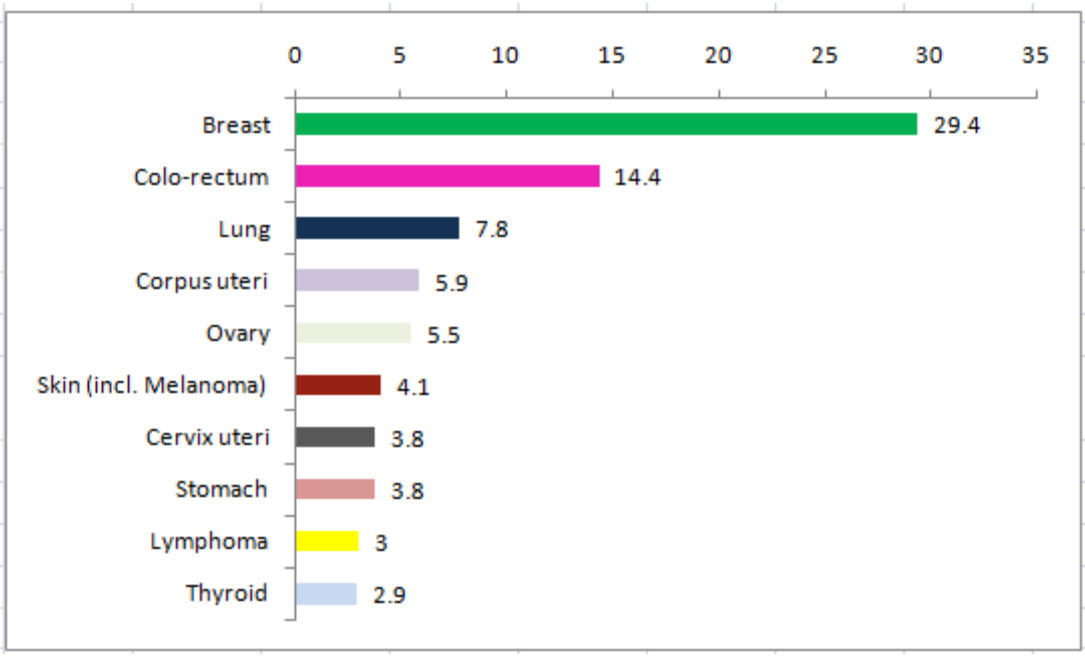



**Figure 2: Ten Most Frequent Cancers in Singapore Females (%), 2005 – 2009**
Data were obtained from the Singapore Cancer Registry Interim Annual Registry Report Trends in Cancer Incidence in Singapore 2005-2009 (5)

### 1.1.4 Risk factors of breast cancer

A wide range of genetic or life-style related factors may increase the risk of having breast cancer. Firstly, gender, age, and family history may play an important role. Most fundamentally, being a woman means that the chance of getting breast cancer is much higher as compared to being a man. Also, if a woman is older than 50 years of age or has a close relative with breast cancer, then her chance of getting breast cancer increases significantly (7). Exposure to the hormones such as estrogen and progesterone may also lead to breast cancer. Therefore, women with longer menstral periods (due to earlier onset of menstruation or later age of menopause) may suffer a higher risk of breast cancer. Similarly, combined hormone therapy involving both estrogen and progesterone exposes the subjects to greater risk of having breast cancer at a more advanced stage(8). Interestingly, women who never got pregnant or are pregnant at a later age (after 30 years old) are also at a higher risk of getting

breast cancer. On the contrary, multiple pregnancies at a younger age (below 30 years old) reduce breast cancer risk(9).

In order to lower the risk of having breast cancer, keeping a healthy life style is important. For example, consumption of alcoholic drinks increases the risk of having breast cancer (7). Having no more than one cup of alcoholic drink per day is thus recommended to avoid getting the disease. Watching one's weight is important as well, since obese women are at greater risk of getting breast cancer (7).

### 1.1.5 Stages of Breast Cancer

**Table 1 Staging of Breast Cancer.**
Adapted from http://www.cancer.gov/cancertopics/wyntk/breast/page7

| Stages | Definition |
|---|---|
| Stage 0 | Cell grows abnormally but not invasive. For example, Ductal Carcinoma In Situ or Lobular Carcinoma In Situ |
| Stage I | breast cancer. Cancer cells have invaded breast tissue beyond the original place of breast. The tumor is no more than 2 centimeters across. |
| Stage II | The tumor is no more than 2 centimeters across. The tumor cell has spread to the lymph nodes under the arm.<br>or<br>The tumor is between 2 and 5 centimeters. But has not spread to the lymph nodes under the arm.<br>or<br>The tumor size is between 2 and 5 centimeters. And has spread to the lymph nodes under the arm.<br>or<br>The tumor is larger than 5 centimeters. But has not spread to the lymph nodes under the arm. |
| Stage IIIA | The tumor is no more than 5 centimeters across. And has spread to underarm lymph nodes that are attached to each other or to other structures. Or the cancer may have spread to lymph nodes behind the breastbone<br>or<br>The tumor is more than 5 centimeters across. The cancer has spread to underarm lymph nodes that are either alone or attached to each other or to other structures. Or |

| | |
|---|---|
| | the cancer may have spread to lymph nodes behind the breastbone. |
| Stage IIIB | A tumor of any size that has grown into the chest wall or the skin of the breast. It may be associated with swelling of the breast or with nodules (lumps) in the breast skin:<br>The cancer may have spread to lymph nodes under the arm.<br>or<br>The cancer may have spread to underarm lymph nodes that are attached to each other or other structures. Or the cancer may have spread to lymph nodes behind the breastbone.<br>Or<br>Inflammatory breast cancer is a rare type of breast cancer. The breast looks red and swollen because cancer cells block the lymph vessels in the skin of the breast. When a doctor diagnoses inflammatory breast cancer, it is at least Stage IIIB, but it could be more advanced. |
| Stage IIIC | A tumor of any size. It has spread in one of the following ways:<br>The cancer has spread to the lymph nodes behind the breastbone and under the arm.<br>Or<br>The tumor cell has spread to the lymph nodes above or below the collarbone. |
| Stage IV | The cancer has spread to other organs, such as the bones or liver. |

### 1.1.6 Treatment of Breast Cancer

There are many treatment options that women with breast cancer can choose from. The most common one is surgery, which may include removing only cancerous tissue or the whole breast together with some lymph node. Surgery that removes only the cancerous tissue is a lumpectomy or a segmental mastectomy. Surgery that removes the whole breast is called mastectomy. Stage 0 breast cancer can be cured by lumpectomy while stage 1 or stage 2 may need a mastectomy. Besides surgery, there are other options including radiation therapy, hormone therapy, chemotherapy, and targeted therapy. Surgery is often combined with other treatment such as radiation therapy or chemotherapy. Surgery and radiation therapy are types of local therapy. They remove or destroy cancer cells within the breast. Hormone therapy, chemotherapy, and targeted therapy are types of systemic therapy. The drug enters the bloodstream and destroys or controls cancer throughout the body. For stage 4 metastatic

cancer, surgery, radiation therapy, chemotherapy, and targeted therapies are combined to manage the disease.

**Table 2 Treatment of breast cancer.**
Content was sourced from http://www.breastcancer.org/treatment/ on 28 Mar 2011

| Therapy | Descriptions | |
|---|---|---|
| **Surgery** | **Types of surgery** | **Description** |
| | **Lumpectomy (breast-conserving surgery)** | Removal of only the tumor and a small amount of surrounding tissue. |
| | **Mastectomy** | Removal of all of the breast tissue |
| | **Prophylactic mastectomy** | Preventive removal of the breast to lower the risk of breast cancer in high-risk people. |
| | **Prophylactic ovary removal** | A preventive surgery that lowers the amount of estrogen in the body |
| | **Cryotherapy, also called cryosurgery, uses extreme cold to freeze and kill cancer cells** | Uses extreme cold to freeze and kill cancer cells |
| **Chemotherapy** | A systemic therapy that uses medicine to go through the blood system to weaken and destroy breast cancer cells in the whole body | |
| **Radiation therapy** | A highly targeted, highly effective way to destroy cancer cells that may stick around after surgery. It can reduce the risk of breast cancer recurrence by about 70%. It is relatively easy to tolerate and its side effects are limited to the treated area. | |
| **Hormonal therapy** | Medicines treat hormone-receptor-positive breast cancers in two ways: by lowering the amount of the hormone estrogen in the body and by blocking the action of estrogen on breast cancer cells. It can also be used to help shrink or slow the growth of advanced-stage or metastatic hormone-receptor-positive breast cancers | |
| **Targeted Therapies** | **Types** | **Description** |
| | **Herceptin** | Works against HER2-positive breast cancers by blocking the ability of the cancer cells to receive growth signals |
| | **Tykerb** | Works against HER2-positive breast cancers by blocking certain proteins that can cause uncontrolled cell growth. |
| | **Avastin** | Works by blocking the growth of new blood vessels that cancer cells depend on to grow and function. |

## 1.2 Endoplasmic Reticulum Stress and Unfolded Protein Response

### 1.2.1. Structure and function of the endoplasmic reticulum

The endoplasmic reticulum (ER) is a membranous organelle in eukaryotic cells that is a single compartment (10). Structurally distinct domains of this organelle include the nuclear envelope (NE), the rough ER (rER) and the smooth ER (sER) (Figure 2) and the regions that contact other organelles (11). The morphology of ER may not be homogenous but may differ in different cell types or may have different functions. The two subregions of the ER, both rough and smooth, are visually distinct. This may be because they contain different membrane proteins (10). The rough ER, with ribosomes on its surface, is the place where translation of a secretoty protein or a membrane protein and the cotranslational translocation across the ER membrane occurs. It contains signal recognition particles (SRP) which recognize newly synthesised polypeptide from the membrane-bound ribosome. The ribosome-SRP complex together with the nascent polypeptide is targeted to the ER membrane by interaction with the heterotrimeric SRP receptor. As translocation proceeds, the nacent polypeptide is translocated across the ER membrane via the macromolecular machinery called a translocon. Because protein translocation is important for all the eukaryotic cells, they all have rER. In contrast, sER only exists in certain cell types, including steroid-synthesizing cells, liver cells, neurons, and muscle cells. The primary activities of the sER are very different in each of these cell types. For example, in liver cells, the sER is important for detoxification of hydrophobic substances. In steroid-producing cells, it is the site of many of the synthesis steps. In muscle cells, it is called sarcoplasmic reticulum (SR)

and is primarily involved in calcium release and uptake for muscle contraction and in neurons, although less well established; it is also probably required for calcium handling. Thus, the sER is also a cell type-specific suborganelle of the ER.

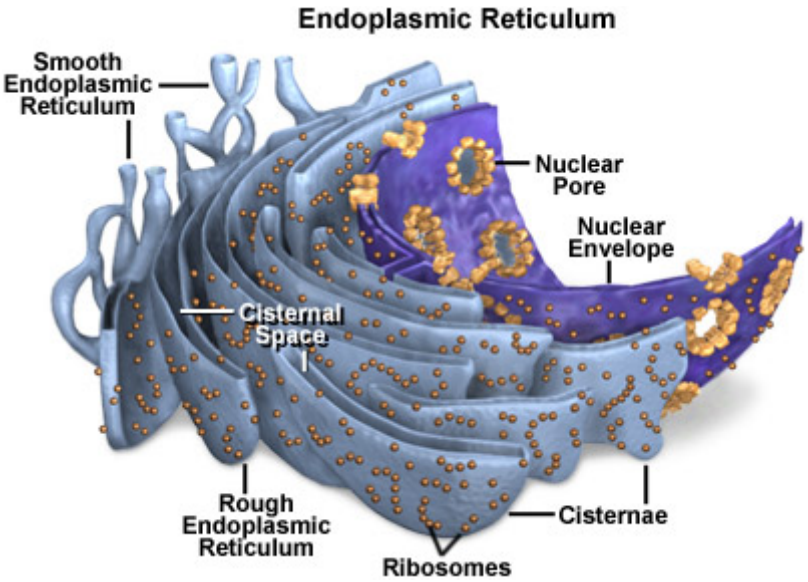



**Figure 3 Structure of Endoplasmic Reticulum**.
The picture is sourced from
http://micro.magnet.fsu.edu/cells/endoplasmicreticulum/endoplasmicreticulum.html on December 21, 2011

### 1.2.2. Definition of ER Stress

The ER is a primary place where secretory proteins or membrane proteins are synthesized (11).During this process, newly synthesized proteins are folded into proper conformation and undergo post translational modifications such as N-linked glycosylation and disulfide bond formation (12). For maintaining the diverse functions of the newly synthesized protein, it is very important that the nascent polypeptide is properly folded to become a mature protein. The ER provides stringent quality control systems to ensure that only correctly folded proteins exit the ER and unfolded or misfolded proteins are retained and ultimately degraded (13). If the influx of nascent, unfolded polypeptides exceeds the folding

and/or processing capacity of the ER, unfolded protein accumulate inside the ER lumen, and the normal physiological state of the ER is perturbed. This situation is termed ER stress.

### 1.2.3. Unfolded Protein Response (UPR)

When the ER suffers from ER stress, a signaling pathway called unfolded protein response (UPR) is activated to return the ER to its normal physiological conditions. This signal pathway down-regulates nascent poly-peptides entering the ER and up-regulates molecular chaperones to increase the folding ability of the ER (12). Also, transcription of genes encoding secretory proteins and translation of secretory proteins are brought down, and clearance of misfolded proteins are increased (14). There are mainly three transducers involved in the signal transduction of the UPR, namely IRE1，ATF6, and PERK (14). Firstly, the unfolded protein binds to the luminal domain of IRE1, triggers its autophosphorylation and oligomerization. It then endonucleolytically cleaves its substrate X-box binding protein-1(XBP-1) mRNA. The spliced mRNA is then ligated and encodes an activator of UPR target genes. Secondly, the activation of ATF6 leads to its transportation from the ER to the Golgi apparatus, and its cleavage by the Golgi-resident proteases S1P and S2P. After the cleavage, a cytosolic DNA-binding portion is released to enter the nucleus to activate gene expression. Thirdly, PERK also contains a protein kinase domain which undergoes autophosphorylation and oligomerization. Its activation phosphorylates its downstream target -the eukaryotic translation initiation factor 2-α subunit (eIF2α). This leads to the global translation shut down and thus prevents newly synthesized protein localization in the ER. Also, the phosphorylation of eIF2α activates a transcription factor ATF4 to activate more

UPR target genes (Figure 4).

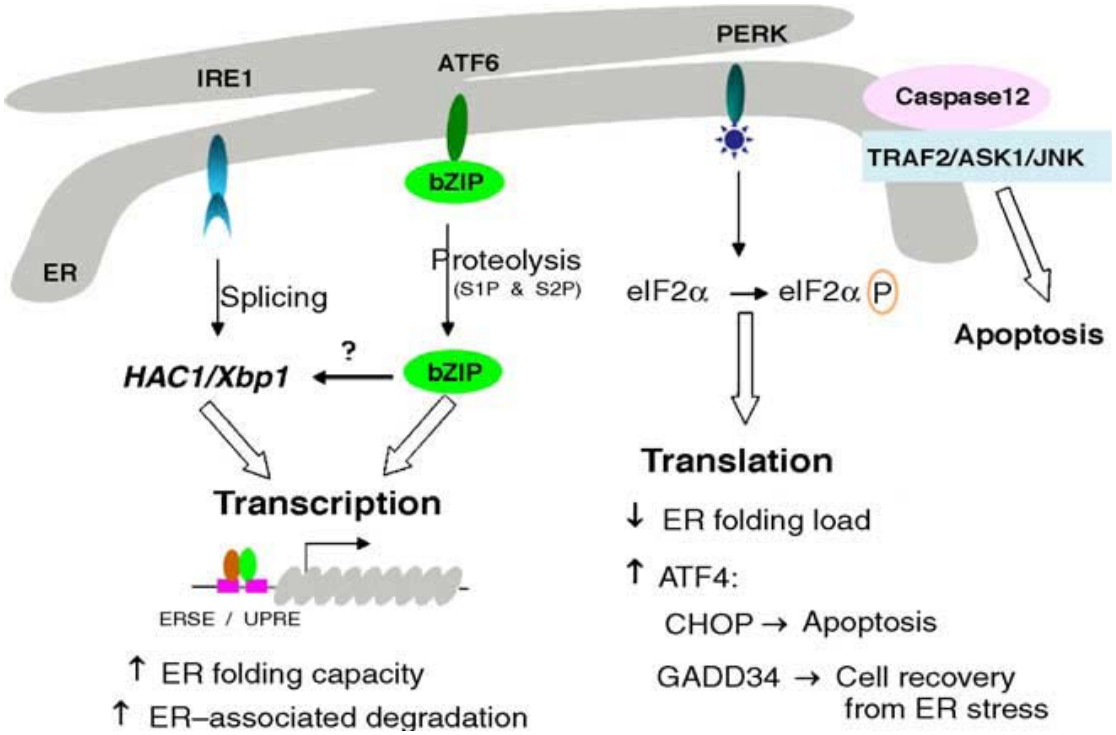



**Figure 4 Signal transduction of unfolded protein response.**
The picture is sourced from X Shen et al The unfolded protein response—a stress signaling pathway of the endoplasmic reticulum J Chem Neuroanat. 2004 Sep;28(1-2):79-92.

### 1.2.4 Unfolded Protein Response in Cancer

Solid tumors are continuously challenged by a restricted supply of nutrients and oxygen due to insufficient vascularisation. Therefore, the stress conditions such as hypoxia, nutrient deprivation and pH changes, activate the UPR pathway. The UPR is a cytoprotective pathway but prolonged activation of UPR can lead to apoptosis (15). Under the conditions related to cancer formation, the role of the UPR in tumor development is ambiguous (16). The recent researches focused on this are summarized in Table 3. Brifely, on the one hand, some components of UPR, such as PERK, GRP78, and ATF4 are activated during cancer genesis to

promote tumor survival (17) .Tumor cell survival is achieved by adapting the tumor cells to hypoxia and facilitating angiogenesis (18) or by increased expression of growth factors in tumor cells (19). One essential transcription factor in the UPR pathway, the XBP1, has been demonstrated to be necessary for cancer cell survival under hypoxia (20). The other component, GRP78, has also been proven to be critical for tumor cells to grow (21). Nevertheless, the expression level of GRP78 is shown to be significantly correlated with cancer reccurence and survival, with the high expression linked to higher reccurence and more death (22).

On the other hand, activation of these molecules- PERK, eIF2α, GRP78 are reported to induce cell cycle arrest and as such suppress cancer cell growth (23) (24) For GRP78 and PERK, the role in cancer development is ambiguous, and awaiting further clarification.

**Table 3 Unfolded Protein Response(UPR) in tumor development**

| Year | Author | Components of UPR | Study | Role |
|---|---|---|---|---|
| 2006 | D.R. Fels, et al. | PERK<br>eIF2α<br>ATF4 | The PERK/eIF2a/ATF4 axis adapts tumor cell to hypoxia stress | Pro-survival |
| 2006 | J.D. Blais, et al. | PERK | PERK-dependent translational regulation promotes tumor cell adaptation and angiogenesis in response to hypoxic stress | Pro-survival |
| 1999 | J.W. Brewer, et al. | eIF2α | Translational arrest induced via eIF2α phosphorylation causes cell cycle arrest | Tumor suppresive |
| 2004 | D.J. Perkins,et al. | eIF2α | Defects in translational regulation mediated by the eIF2α inhibit | Facilitaes malignant transformation |

| | | | | |
|---|---|---|---|---|
| | | | antiviral activity and facilitate the malignant transformation of human fibroblasts | |
| 2007 | B.Drogat,et al. | IRE1 | IRE1 signaling Is essential for ischemia-induced vascular endothelial growth factor-a expression and contributes to angiogenesis and tumor growth in vivo | Contributes to angiogenesis and tumor growth |
| 2004 | L. Romero-Ramirez, et al. | XBP-1 | XBP1 is essential for survival under hypoxic conditions and is required for tumor growth | Pro-survival |
| 2007 | A.S. Lee,et al. | GRP78 | GRP78 is highly expressed in tumors | Pro-survival |
| 1996 | C.Jamora,et al. | GRP78 | Knock-down of GRP78 inhibits tumor progression | Promotes tumor progression |
| 2006 | C. Denoyelle,et al. | GRP78 | ER stress upregulates UPR to inhibit tumor growth | Tumor suppression |

### 1.2.5 eIF2α

UPR activation can be mediated by three major signal transduction pathways, one of which includes activation of the eukaryotic initiation factor 2 α subunit(eIF2α). eIF2 is a multimeric protein which binds to GTP and initiator methionyl-tRNAi (Met-tRNAi), and mediates the association of Met-tRNAi to the 40s ribosomal subunit (25). It consists of three subunits α, β and γ. The α subunit, named eIF2α, has a phosphorylation unit at the Ser51 position and its phosphorylation by PERK shuts off general translation to protect cells from

ER stress (26). Meanwhile, EIF2α is a key translation initiation factor that regulates the rate of protein synthesis during cell proliferation. Overexpression of eIF2α is frequently found in tumors. For instance, expression of eIF2α was found to be positively correlated with classification of lymphoma behavior (27). A significantly increased expression of eIF2α in aggressive thyroid carcinoma exists compared to conventional papillary carcinoma (28). Expression of eIF2α was increased markedly in both benign and malignant neoplasms of melanocytes and colonic epithelium (29). Generally, eIF2α expression may have a strong linkage with tumor cell aggressiveness.

### 1.3 ERp29

#### 1.3.1 Structure and Function

ERp29 was first isolated and its cDNA cloned from rat enamel cells (30) and rat liver cells (31). Tissue expression of ERp29 was examined by immunoblotting (32) and northern blotting (31). Its expression was detected in all the tissues (32). A topology study identified ERp29 as an ER luminal protein known as reticuloplasmin. It was subsequently identified as a reticuloplasmin with an ER-retention motif, KEEL, present at the carboxyl-terminus (30). However, unlike other reticuloplasmins, it lacks the calcium-binding motifs and does not contain glycosylation sites. Moreover, it is highly homologous with members of the protein disulfide isomerase family, but lacks the thioredoxin-like (cys-X-X-cys) catalytic moieties that distinguish this class of reticuloplasmins (30). It exists mainly as a dimer and may also be involved in some higher-order homo- and/or heterocomplexes (33). Further research indicated that ERp29 is a constitutively expressed housekeeping gene which is conserved in all mammals (34).

Under ER stress, ERp29 is drastically induced like other reticuloplasmins such as GRP78 and GRP94. ERp29 was found to interact with the ER chaperone BiP/GRP78 (31). Two-fold higher levels of ERp29 were observed during the secretion of enamel proteins from the cells. After this period, ERp29 was down-regulated (32). These results corroborate that ERp29 may have an essential role in secretory-protein synthesis.

In order to further explore the function of ERp29, an ERp29-overexpressed FRTL-5 cell line was established. The overexpressed ERp29 was observed to be concentrated in the ER microsome. Moreover, overexpression of ERp29 resulted in enhancement of thyroglobulin (Tg) secretion.

On the contrary, ERp29 silencing attenuates Tg secretion (35). The overexpression of ERp29 can also induce the expression of ER chaperones such as GRP94, Calnexin, BiP, ERp72, PDI and PERK (36). The interaction of ERp29 with other ER chaperones (GRP94, Calnexin, BiP ERp72) and PERK was also observed.

Overall, these findings serve to highlight the important role of ERp29 in the secretion of proteins from the ER.

### 1.3.2 Role of ERp29 in carcinogenesis

As a novel ER chaperone, the role of ERp29 in carcinogenesis is currently ambiguous. Firstly, ERp29 is found to be intensively expressed in infiltrating basal-cell carcinoma of the skin (37). Secondly, in a recent study, endogenous ERp29 was up-regulated in xenografts of MCF7 cells compared to *in vitro* cultured MCF7 cells. In order to further the studies, MCF-7 cell line overexpressing wild-type or dominant-negative ERp29 were constructed, along with

the mock-transfect cell line as a control. These three cell lines grew at a similar rate *in vitro*. However, xenografts expressing a dominant-negative ERp29 grew significantly less than the tumors from the mock-transfected cell line or cells expressing wild-type ERp29. In addition, morphological examination showed that tumors from wild-type ERp29 overexpressing cells had a more aggressive pattern as compared to tumors derived from the mock-transfected or ERp29-dominant negatively expressing cells. In this study, the results seem to indicate that ERp29 may be involved in tumorigenesis (38).

In contrast, in another recent study, the expression of ERp29 was reduced with tumor progression. ERp29 overexpression led to cell cycle arrest in G0/G1 phase in the proliferative MDA-MB-231 breast cancer cell line. Moreover, it also led to a phenotype change and mesenchymal-epithelial transition. ERp29 overexpression decreased cell migration and reduced cell transformation.The genes involved in cell proliferation is highly reduced while those of some tumor suppressor are up-regulated. ERp29 is proven to negatively regulate cell growth in breast cancer cells (39), while silencing of ERp29 in MCF-7 cells enhanced cell aggressive behavior.

Overall, the role of ERp29 in carcinogenesis is controversial, and further research is needed to clarify whether it is an oncogene or a tumor suppressor.

### 1.4 Regulation of Cell Cycle

Cell cycle is defined as the ordered process that occurs during cell division. In eukaryotic cells, cell cycle includes four distinctive phases- G1, G2, S and M. During the G1 phase, a cell synthesizes materials for cell duplication and division, followed by the S phase, in which

DNA is synthesized. In the M phase, cell division occurs, leading to cell duplication. The cell cycle is a well regulated process in which cyclins and cyclin-dependent kinases(CDKs) play important roles. In the G1 phase, cyclin-D, cyclin-E, as well as cyclin-D- and cyclin-E-dependent kinases are critical mediators deciding whether the cell will progress smoothly through this phase.

Cyclin-D1 is a well-studied G1 cyclin that regulates cell cycle progression and cell growth. Past studies revealed that it is exported from nucleus to cytoplasm during the S phase (40). Another study demonstrated that its nuclear localization is related to malignant cell transformation (41). Indeed, in the current study, the cyclinD1 nuclear localization in breast cancer cells is shown to be regulated by the key molecule-ERp29. More will be discussed in relation to this phenomenon in the Results and Discussion section

### 1.5 Hypothesis

The preliminary results in our laboratory suggest that ERp29 induces tumor cell dormancy in breast cancer, although the molecular mechanism under this process is not fully elucidated. As overexpression of ERp29 induces ER stress and activates unfolded protein response, whether the ER stress signaling pathway is involved in ERp29-mediated cell cycle arrest is still a question. Here in my thesis, we hypothesized that ERp29 induces cell cycle arrest in breast cancer through the ER stress signaling pathway. The aim of this research is to clarify what signal molecules in the ER stress signal pathway are regulated by ERp29 and how cell cycle regulators are modified, leading to cancer cell dormancy.

# Chapter2

# MATERIALS AND METHODS

## 2.1 Materials

### 2.1.1 Antibodies

The following antibodies shown in Table 4 were used in western blotting and immunofluorescence:

**Table 4: Antibodies used in this study**

| Antibodies | Dilutioin factor used | Company, country of manufacturing | Used for |
|---|---|---|---|
| **Rabbit-anti-ERp29** | 1:2500 | Acris, Hiddenhayse,Germany | Western Blot |
| **Rabbit-anti-eIF2α** | 1:1000 | Cell Signaling Beverley, MD, USA | Western Blot |
| **Rabbit-anti-phospho-eIF2α** | 1:500 | Cell Signaling Beverley, MD, USA | Western Blot |
| **Rabbit-anti-α-tubulin** | 1:1000 | Cell Signaling Beverley, MD, USA | Western Blot |
| **Rabbit-anti- $p21^{Waf1/Cip1}$** | 1:100 | Cell Signaling Beverley, MD, USA | Western Blot |
| **Rabbit-anti-$p27^{Kip1}$** | 1:100 | Cell Signaling Beverley, MD, USA | Western Blot |
| **Mouse-anti-CDKN2B** | 1:400 | Sigma-Aldrich Steinheim, Germany | Western Blot |
| **Rabbit-anti-ATF4** | 1:500 | Santa Cruz CA, USA | Western Blot |
| **Rabbit-anti-Nrf2** | 1:500 | Santa Cruz CA, USA | Western Blot |
| **Mouse-anti-β-actin** | 1:10000 | Sigma-Aldrich Steinheim, Germany | Western Blot |
| **Mouse-anti-cyclinD1/2** | 1:400 | Upstate Biotechnology Inc. (Lake Placid, NY, USA) | Immunofluorescence |

### 2.1.2 Cell lines

The human breast cancer cell lines MDA-MB-231, SKBr3, BT549 and MCF-7 together with non-tumorigenic cell lines MCF10A and MCF12A were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). ERp29-transfected MDA-MB-231 and its vector-transfected control cells were maintained in a medium supplemented with 10% FBS and G418 (2 mg/ml). shRNA/ERp29-transfected MCF-7 cells and its vector-transfected control cells were maintained in a medium supplemented with 10% FBS and G418(1 mg/ml ). All cells were maintained at 37 °C with 5% CO2 in a humidified incubator.

## 2.2 Methods

### 2.2.1 Cell culture

MDA-MB-231 and MCF-7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). SKBr3 cells were cultured in McCoy5A medium with 10% FBS. BT549 cells were cultured in RPMI 1640 medium with 10% FBS. The human non-tumorigenic MCF10A and MCF12A mammary epithelial cell lines were grown in mammary epithelial cell complete medium (MEGM), supplemented with bovine pituitary extract (BPE).

To thaw frozen cells, the cells were removed from frozen storage and thawed quickly in a 37°C water bath by gently agitating the vial. As soon as the ice crystals melted, cells were pipeted gently into a culture flask containing 10 ml pre-warmed growth medium.

To subculture the cells, medium was discarded. Cells were washed with 1x ice-cold phosphate-buffered saline (PBS, pH7.4) to get rid of the excess medium. 1 ml of trypsin-EDTA was added to detach the cells. After detachment, 1 ml of FBS was added to neutralize the trypsin. Cells were moved into a new culture flask and 10 ml of culture medium was added and the culture flask put into the incubator under the conditions of 37°C, 5% $CO_2$.

### 2.2.2 ERp29 expression vector construction

ERp29 of human origin was amplified using its full length cDNA, the forward primer (5-ATATGAATTCATGGCTGCCGCTGTGC-3'with BamHI site) and the the reverse primer (5'-TCAGGATCCCTACAGCTCCTCCTCTTT-3'with EcoRI site). The product of this reaction was ligated with pcDNA3.1 (+) vector (Invitrogen, Oregon, USA) at the BamH1 and EcoR1 sites. DNA sequencing confirmed the validity of the ERp29 gene.

### 2.2.3 Production of ERp29-overexpressing single stable clone in MDA-MB-231 breast cancer cell

The ERp29-pcDNA3.1 vector, obtained as previously described, was used to transfect MDA-MB-231 breast cancer cells to generate ERp29-overexpressing clones. Briefly, cells were cultured in a 6-well plate until 60%-70% confluence. One microgram of plasmid vector was diluted in Opti-MEM® reduced serum medium (Invitrogen, Oregon, USA) and mixed with an appropriate amount of diluted lipofectamine and then transfection was done according to the manufacturer's protocol. After 48h of transfection, G418 was added to select positive transfectants. Serial dilutions were performed for single clone generation. The ERp29 expressions in these clones were confirmed by reverse-transcription PCR and immunoblot assay. Two ERp29-overexpressing clones (clones B and E) were used in the following experiments.

### 2.2.4 Buffer preparation

#### 2.2.4.1 1X SDS electrophoresis running buffer

| | Final Concentration | Amount |
|---|---|---|
| **Tris-base** | **25mM** | **3.03g** |
| **Glycine** | **192mM** | **14.40g** |
| **SDS** | **0.1% (w/v)** | **1.0g** |
| **Milli-Q Water** | | **To 1L** |

#### 2.2.4.2 1X western blot transfer buffer

| | Final Concentration | Amount |
|---|---|---|
| **Tris-base** | 25mM | 3.03g |
| **Glycine** | 192mM | 14.40g |
| **Methanol** | 20%(v/v) | 200ml |
| **Milli-Q Water** | | To 1L |

#### 2.2.4.3 RIPA(Radio-Immunoprecipitation Assay) buffer:

1% Igepal

1% sodium deoxycholate

0.15 M sodium chloride

0.01 M sodium phosphate, pH 7.2

2 mM EDTA

### 2.2.5 Casting of denaturing polyacrylamide gels

#### 2.2.5.1 Compositions for the 10%and 12% resolving gel

| | 10%(ml) | 12%(ml) |
|---|---|---|
| **30% acrylamide** | **3.33** | **3.96** |
| **1.5M** Tris-HCl, pH 8.8 | **2.5** | **2.5** |
| **Milli-Q water** | **4** | **3.39** |
| **10% SDS** | **0.1** | **0.1** |
| **10% APS** | **0.05** | **0.05** |
| **TEMED** | **0.007** | **0.005** |
| **Total** | **10** | **10** |

#### 2.2.5.2 Compositions for the 4% stacking gel

| | 4%(ml) |
|---|---|
| **30% acrylamide** | **0.66** |
| **1M Tris-HCl,pH7.0** | **1.26** |
| **Milli-Q water** | **3** |
| **10% SDS** | **0.05** |
| **10% APS** | **0.025** |
| **TEMED** | **0.005** |
| **Total** | **5** |

### 2.2.6 Western blotting

#### 2.2.6.1 Total cell lysates

When cells were grown to 80% confluence, the medium was discarded and then washed with PBS. After the remaining medium was washed off, cells were treated by trypsin-EDTA and then collected into a microfuge tube. The cell pellet was washed three times with ice-cold phosphate-buffered saline (PBS, pH 7.4). Cells were then resuspended in cold RIPA buffer pH7.4 supplemented with protease inhibitors and phosphatase cocktail inhibitors I and II and kept on ice for 1 hour. Cell lysates were then centrifuged at 4$^{o}$C at 12000 rpm, and the supernatants containing the total cell lysate proteins were collected.

#### 2.2.6.2 Protein concentration measurement

Protein concentrations were determined using the Coomassie Plus Bradford assay (Pierce,

Rockford, IL) In each cuvette, 50 µl of protein extracts were diluted by 450 µl of sterilized water, and then 1 ml of Coomassie Blue reagent was added into the cuvette. The sample was incubated for 10 min and its protein concentration was determined using a spectrophotometer (Beckman Coulter DU® 800,VWR).

**2.2.6.3 Running an SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) gel**

10-12% SDS-PAGE gels were prepared for protein electrophoresis (refer to Tables 2.2.3.1 and 2.2.3.2). 40 µg of the total protein with loading dye (Laemmli loading dye), A 3X stock comprises of: 1M Tris-HCl pH 6.8, 2.4 ml 20% SDS, 3 ml Glycerol (100%), 3 ml β-mercaptoethanol, 1.6 ml Bromophenol blue (0.006g) was loaded into each well of the SDS-PAGE gel and run using the Mini-PROTEAN 3 Electrophoresis Cells (Bio-Rad, Hercules, CA, USA) under 70 V for 30 min and 100 V for 1 hour until the dye front reached the edge of the gel.

**2.2.6.4 Transfer of proteins to PVDF membrane**

The proteins were then transferred onto a Hybond-P Polyvinylidene Fluoride (PVDF) membrane (GE Healthcare, Uppsala, Sweden) using the wet transfer apparatus (Bio-Rad, Hercules, CA) at 100 V for 1 h.

**2.2.6.5 Antibody hybridization**

After complete transfer was effected, the membrane was washed using Tris-buffered saline containing 0.1% Tween-20 (TBS-T) and blocked with 5% non-fat milk (Santa Cruz

Biotechnology, Inc., CA, USA) in TBS-T at room temperature for 1 h. The membrane was then incubated overnight with respective antibodies at 4ºC. TBS-T was used to wash off the unbound excess primary antibodies. Then, secondary antibodies – the HRP-conjugated goat anti-mouse IgG (Molecular Probes, Invitrogen, Oregon, USA) at 1:5000 dilutions in TBS-T or HRP-conjugated goat anti-rabbit IgG (ZYMED® Laboratories Inc. San Francisco, CA, USA) at 1: 10 000 dilutions in TBS-T were applied for 2 hours and TBS-T was used to wash off the unbound secondary antibodies.

#### 2.2.6.6 Signal detection

The chemiluminescent signals were detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA). Signals were then captured with the MULTI GENIUS BioImaging System (Syngene, Frederick, MD, USA) and the signal intensities were analyzed using the GeneTools software (Syngene, Frederick, MD, USA). The same membrane was then stripped and reprobed with anti-β-actin antibody which was the control to normalize for even protein loading.

### 2.2.7 Immunofluorescence and confocal microscopy

The cells were grown on glass coverslips using a 6-well plate. In each well, three glass coverslips were placed. After cells were grown for 24 h, they were washed with warm PBS at at ~37°C. The cells were then fixed with 4% paraformaldehyde (Sigma-Aldrich, Steinheim, Germany) in PBS for 30 min. After fixation, the cells were washed with PBS and then permeabilized with 0.1% Triton X-100 for 10 min. The cells were washed with 0.02% PBS-T

and blocked with 3% bovine serum albumin (BSA) in PBS-T for 1 h. After that, cells were incubated with anti-CyclinD1/2 primary antibody over-night at 4℃ with gentle shaking. The cells were then washed with 0.02% PBS-T and then incubated with Alexa Fluor® 488 (1:200 dilution, Invitrogen, Inc., Carlsbad, CA) for 2 h. Slides were mounted using the antifade mounting fluid containing DAPI and the images were visualized and captured using the Olympus Fluoview FV500 fluorescent microscope (Olympus, Japan). Raw images were analyzed using the Olympus FV10-ASW Viewer Software (Olympus, Japan).

**2.2.8 siRNA treatment**

siRNAs against p38 (sip38, SignalSilence® p38MAPK siRNA II, #6243) was purchased from Cell Signaling Technology® (Beverley, MD, USA). siRNA against eIF2α (eIF2α siRNA(h), sc-35272) and control siRNA (Control SiRNA-A:sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). MDA-MB-231 cells were plated in six well plates and grown to about 50% confluence before treatment with siRNA at a final concentration of 100pM with LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Cells were collected at 48 h post-transfection and the inhibition of p38 and eIF2α by siRNA was verified by Western blotting (see section 2.2.4).

**2.2.9 Statistical method**

Student’s T-test is used for analyzing data. The student T-test is done by an online calculator which is available at studentsttest.com

# Chapter 3

# RESULTS

## 3.1 ERp29 regulates transcription factor eIF2α and Nrf2 in ER stress signaling

eIF2α is an important translation initiation factor. Its phosphorylation regulates global protein synthesis (42). It is known that ER stress signaling of PERK/p-eIF2α translationally regulates protein synthesis and induces G1 arrest by phosphorylation of eIF2α (43). In order to explore how ERp29 modulates ER stress signaling, MDA-MB-231 cells stably overexpressing ERp29 were used. Total proteins were extracted from this cell line and the respective control cell line, and the level of ERp29 was detected by Western blotting. (Fig 5 left panel) In this (MDA-MB-231) clone, expression of ERp29 was nearly two-fold higher than the mock-transfected cell line. Western blots performed to examine the expression level of eIF2α, showed it was down-regulated in conjunction with ERp29 overexpression (Figure 5 left panel ) Meanwhile, knock-down of ERp29 in the MCF7 cell line slightly increased the expression of both eIF2α and its phosphorylated form (Figure 5 right panel ).

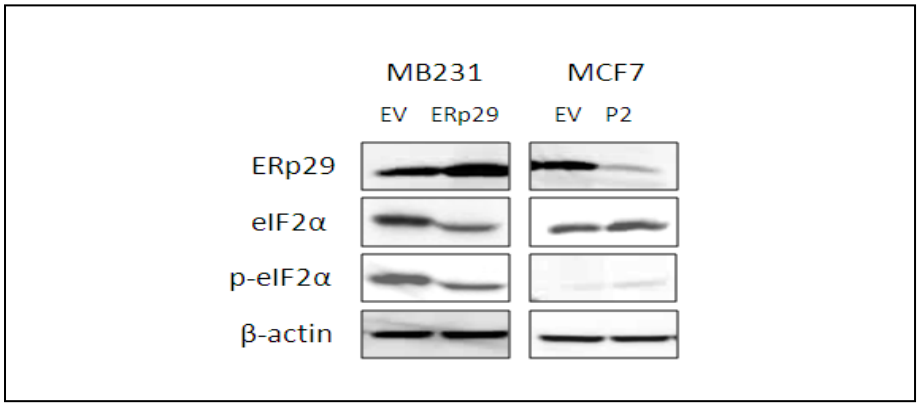


**Figure 5. ERp29 overexpression down-regulates translation initiation factor eIF2α**. Western blotting was performed using protein lysate from ERp29 overexpressing MB231 cells(left panel,ERp29) together with ERp29 silenced MCF7 cells(right panel,P2).40ug protein was loaded in each well and separated by SDS PAGE. The expression of basal eIF2α together with its phosphorylated form were examined using anti-eIF2α antibody (or anti-phosphoSer51-eIF2α antibody (Cell Signaling, USA) . β -actin is used as a loading control.

However, it is found that over-expression of ERp29 did not markedly enhance the relative phosphorylation of eIF2α (p-eIF2α/eIF2α) in ERp29-overexpressing MDA-MB-231 cells. Instead, the basal level of eIF2α was markedly reduced by ERp29. These data indicate that overexpression of ERp29 in MDA-MB-231 cells disturbs ER stress signaling by affecting the basal expression of eIF2α rather than by regulating its phosphorylation. Also, eIF2α is an important translation initiation factor which controls global protein synthesis. As such, the results so far appear to show that ERp29 may play a role in tumor dormancy by decreasing the level of eIF2α to suppress the cellular protein synthesis for energy conservation.

Besides eIF2α, another transcription factor which acts down-stream of PERK, the NF-E2 related factor 2 (Nrf2), which is ubiquitously expressed and responds to oxidative stress within cells, has also been studied. A role for Nrf2 activation during the UPR was established following the identification of Nrf2 as a PERK substrate (44). It was found that PERK-dependent activation of Nrf2 contributes to redox homeostasis and cell survival following Endoplasmic Reticulum Stress. Some preliminary results have shown that the level of ERp29 is highly reduced in highly proliferative cancer cells such as MDA-MB-231 when compared to MCF7 cells which on the contrary are low-proliferative cells (39). Moreover,

over-expression of ERp29 in MDA-MB-231 and SKBr3 strongly inhibited cell growth (39). On the other hand, knock-down of ERp29 in the MCF7 cell line promoted cell proliferation (39). Thus, it may be concluded that ERp29 suppresses cell growth in breast cancer cells to induce dormancy. However, the molecular mechanism underlying this phenomenon is not fully understood. Therefore, in the current dissertation, the author attempt to investigate how ERp29 may regulate another effector of PERK, Nrf2 in breast cancer cell lines. The levels of eIFα and Nrf2 in a panel of breast cancer cell lines including the non-tumorigenic MCF10A and MCF12A cells, low-proliferative MCF7 cells and high-proliferative MDA-MB-231, SKBr3 and BT549 cells were examined. As shown in Figure 6 and Figure 7, eIF2α and Nrf2 are highly increased in high-proliferative MDA-MB-231, SKBr3 and BT549 cells when compared with the low-proliferative MCF7 cells.

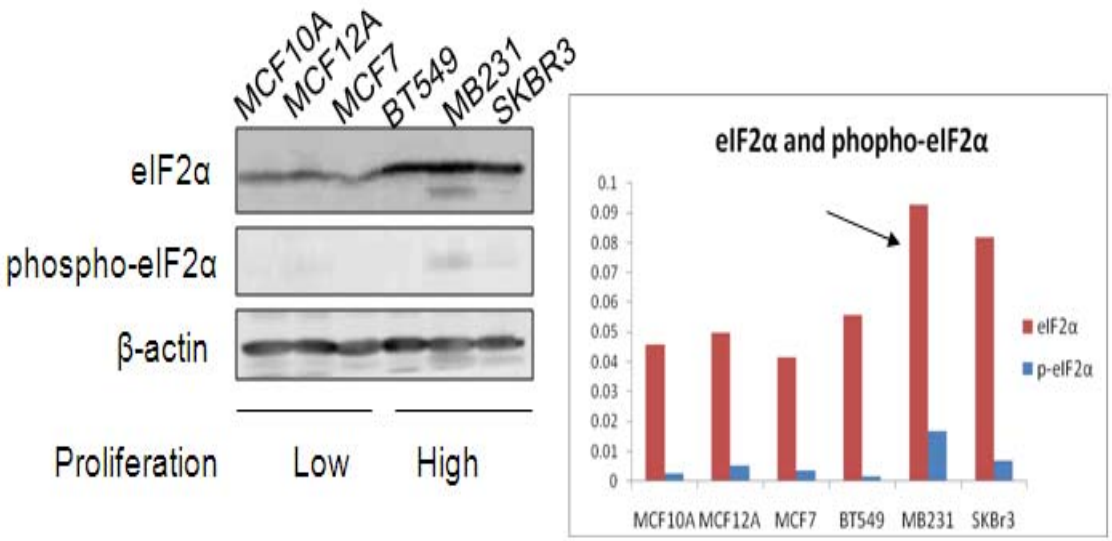
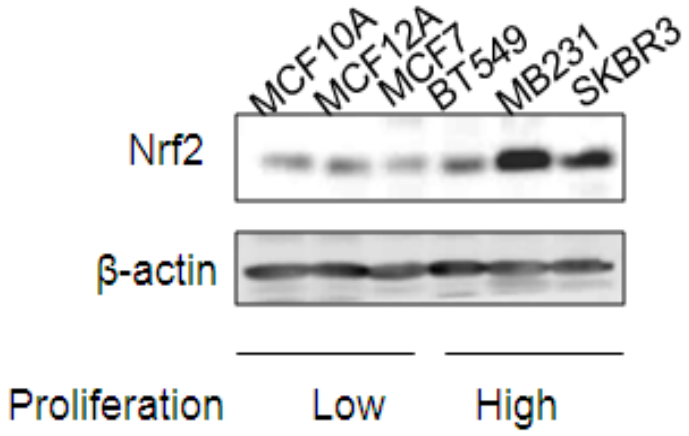



**Figure 6. Expression of eIF2α in breast cancer cell lines** Total protein was extracted from a subset of breast cancer cell lines. Western blot was performed using anti-eIF2α antibody. β-actin is used as a loading control. The arrow indicates highest expression of eIF2α in MB231.



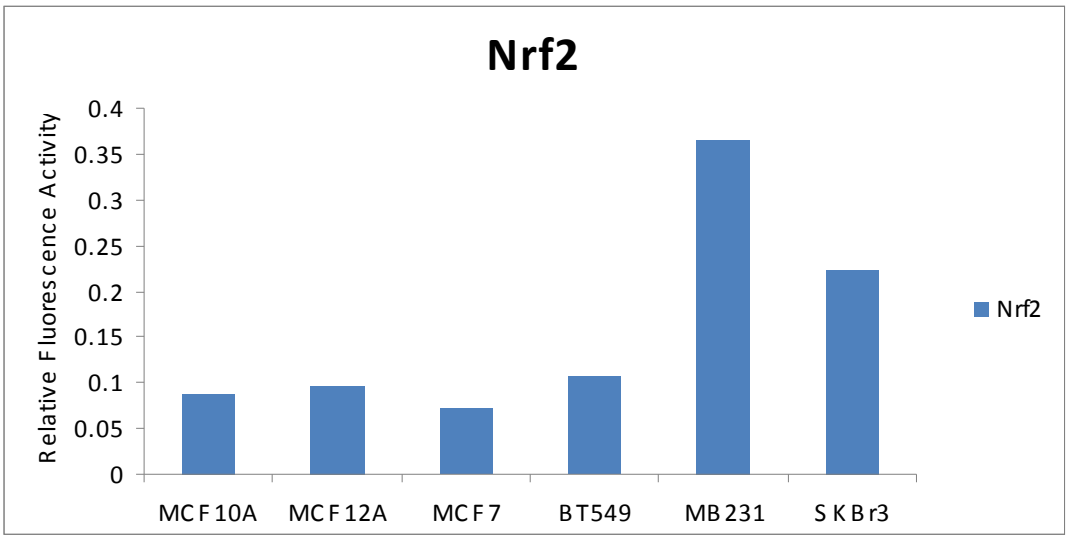




**Figure 7. Expression of Nrf2 in breast cancer cell lines** Total protein was extracted from a subset of breast cancer cell lines. Western blotting was performed using anti-Nrf2 antibody. β-actin is used as a loading control. Nrf2 expressed most high in MB231 cell line.

Since the ERp29 expression is low in MDA-MB-231, ERp29 was overexpressed in this cell line to determine whether the Nrf2 expression will be altered. As expected, Nrf2 is down-regulated when there is ERp29 overexpression (Figure 8). ERp29 was also knocked down in MCF7 which showed the highest ERp29 expression among the panel of cells

examined (39). However, the level of Nrf2 did not increase as predicted. This could be due to insufficient knock-down of ERp29 for this cell line, or due to the possibility that the mechanism of regulation for Nrf2 in MCF7 is different from that in other cell lines. To further investigate for these findings, ERp29 was also knocked down in MDA-MB-231 and the expected increase in expression of Nrf2 was observed (Figure 9).

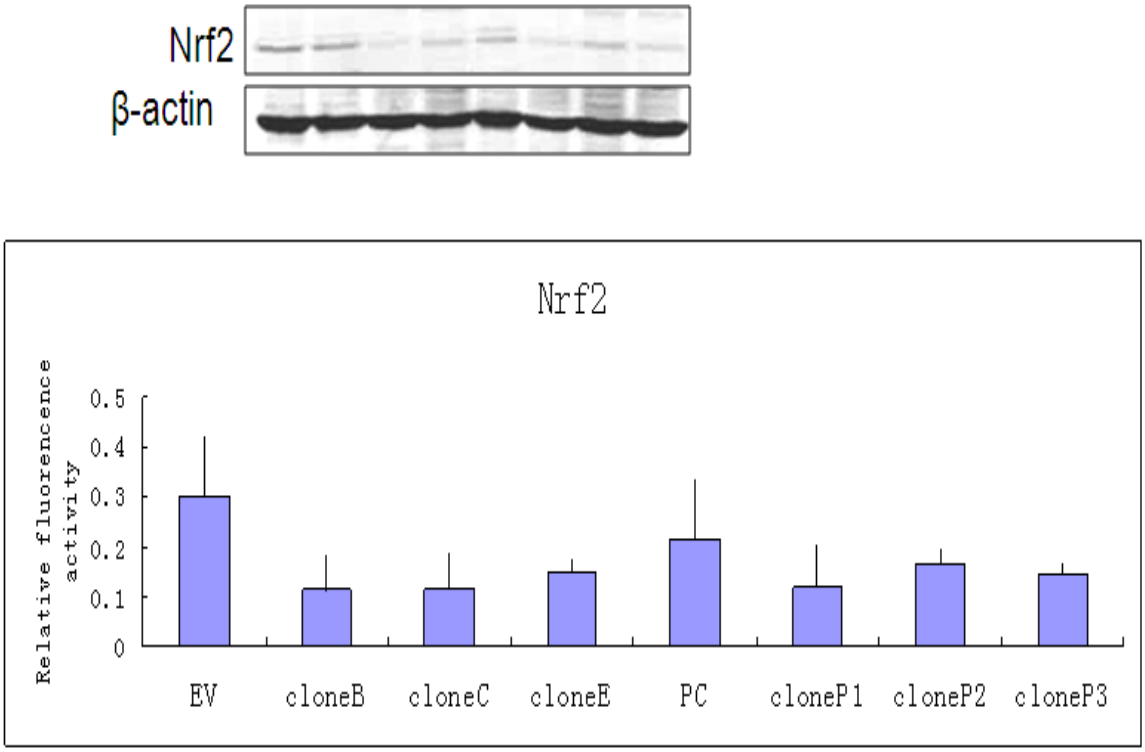



**Figure 8. Expression of Nrf2 in ERp29 overexpressing MB231 or ERp29 silenced MCF7.** Total protein lysates was extracted from ERp29 overexpressed MB231(clone B, clone C and clone E) or ERp29 silenced MCF7(clone P1, cloneP2 and cloneP3). Western blotting was performed using anti-Nrf2 antibody(Santa Cruz, USA) . Data shown represent the average from triplicate experiments.

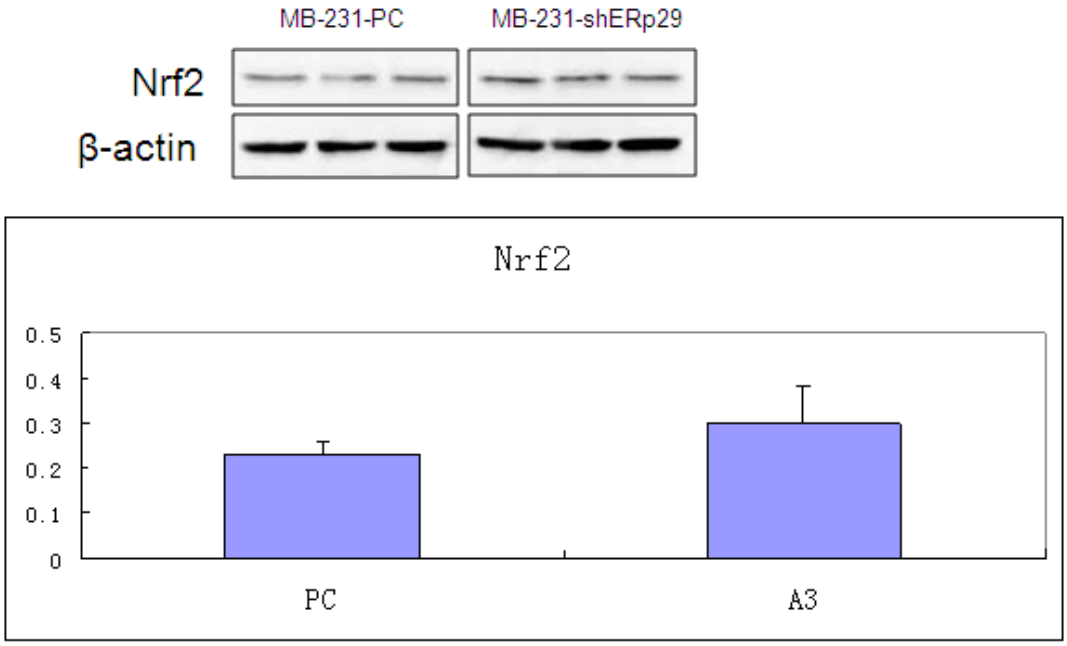


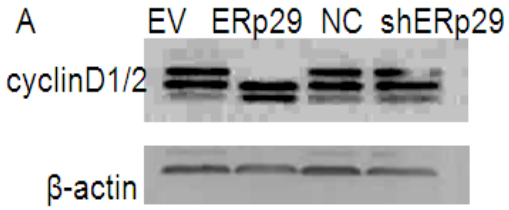

**Figure 9. Expression of Nrf2 in ERp29 silenced MB231(A3)** . Total protein lysate from mock-transfected control cell line(PC) or ShRNA transfected ERp29 silenced MCF7 cell line were used for Western blotting, data shown represent the average from triplicate experiments. β-actin was used as a loading control.

### 3.2 ERp29 overexpression regulates cell cycle mediators and inhibitors in breast cancer

Transitions between cell cycle phases are regulated by the activity of specific cyclin-dependent kinases (CDKs). Among them, CDK1/CDK2 regulates G2/M phase transition while CDK2/CDK4/CDK6 regulates G1/S phase transition. CDK protein expression levels stay constant throughout the cell cycle, while their binding partners (such as cyclins) and post-translational modifiers (including kinases and phosphatases) undergo periodic oscillations to regulate DNA synthesis and cell division. In breast cancer, cyclin D1 and E, as well as the CDK inhibitors p21 (Waf1/Cip1; hereafter referred to as p21) and p27 (Kip1; hereafter referred to as p27) are important in cell-cycle control and as potential

oncogenes / tumor suppressor genes. They are regulated in breast cancer cells following mitogenic stimuli including activation of receptor tyrosine kinases and steroid hormone receptors, and their deregulation frequently impacts on breast cancer outcome, including response to therapy. It will be interesting to examine how ERp29 overexpression regulates the key cyclins or cyclin-dependent kinases and impacts the cell cycle progression in breast cancer. Gene array was performed to measure relative changes in transcription of cell cycle regulatory proteins. As shown in Figure 10B, the expression of kinase inhibitor p15 is dramatically up-regulated by 719.3 fold, while on the other hand, the expression of cyclinD2 is significantly down-regulated by 162.4 fold. Western blot results showed that cyclins D1/2 were down-regulated and degraded with ERp29 overexpression (Figure 10A and Figure 12). Meanwhile, the expressions of cyclin-dependent kinase inhibitors p15/p21/p27 were up-regulated with ERp29 overexpression. (Figure 11,left panel).

A




B

| Gene Name | Relative level (ERp29/Ctrl) |
|---|---|
| CDKN2B (p15) | 719.3 |
| CyclinD1 | -1.71 |
| CyclinD2 | -162.4 |
| CyclinD3 | -2.35 |
| CDK6 | -8.2 |

**Figure 10 Expression level of important cell cycle regulators**. A. Total protein lysates extracted from ERp29 overexpressing and silenced MB231 and respective control cell lines were examined by immunoblotting. Anti-cyclinD1/2 antibody was used in immunoblotting. Beta-actin was used as a loading control. B.Gene array data showing key cyclins, cyclin-dependent kinase and cyclin-dependent kinase inhibitor which was regulated by ERp29 overexpression

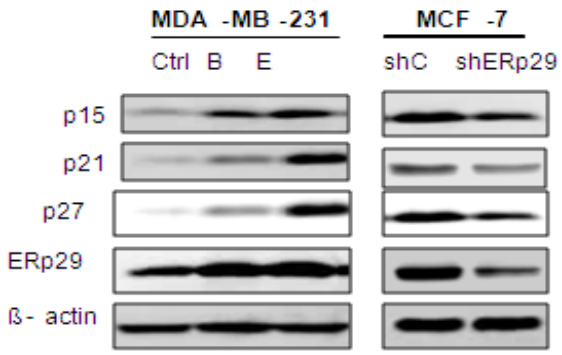



**Figure 11 ERp29 regulates CDK inhibitors**. Total cell lysates from ERp29 overexpressing MDA-MB-231(B and E)and ERp29 silenced MCF-7 and their respective control cell line was extracted. Western blot was performed using anti-p15, anti-p21,anti-p27 and anti-ERp29 antibodies. Beta- actin was used as a loading control.

Cyclin D1 and cyclin D2 are key mediators regulating G1/S transition through formation of complexes with Cyclin-Dependent Kinases (CDKs) to promote cell cycle progression. On the contrary, CDK inhibitors p15, p21 and p27 inhibit cell cycle progression from G1 phase. Therefore, ERp29 may down-regulate G1 cyclins (Figure 12) and up-regulate CDK inhibitors(Figure 11) to induce cell cycle arrest in G0/G1 phase for dormancy to commence.

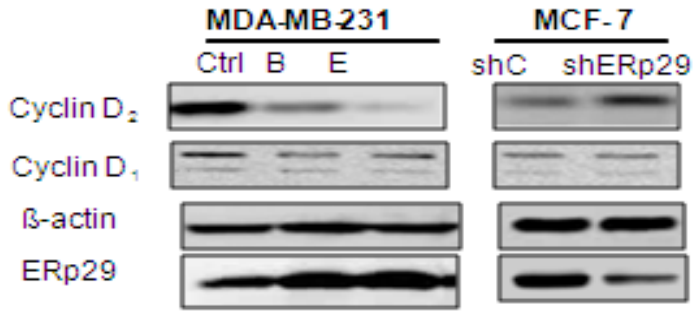
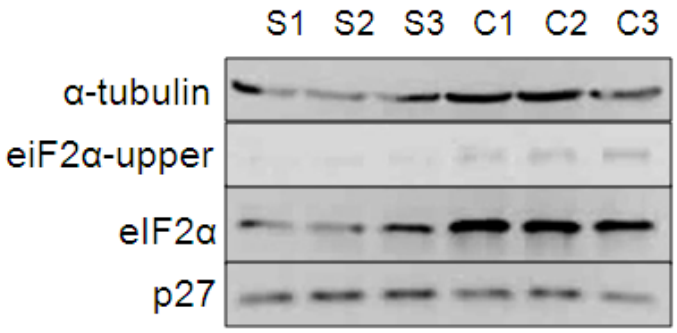



**Figure 12 ERp29 regulates G1 cyclins in MDA-MB-231 and MCF7.** Total cell lysates from ERp29 overexpressing MB231 (B and E) and ERp29 silenced MCF7 (shERp29) was examined by Western blotting. Anti-cyclinD1 and anti-cyclinD2 were used in Western blotting. Beta- actin was used as a loading control.

While it is shown that ERp29 up-regulates CDK inhibitors to induce cell cycle arrest, the pathway involved in this regulation is however still unknown. As it was previously found that the key translation initiation factor eIF2α is down-regulated with ERp29 overexpression, it is possible that this down-regulation may induce heightened expression of CDK inhibitors. To test this hypothesis, eIF2α was knocked down in MDA-MB-231 breast cancer cells and the expression of the CDK inhibitor p27 was examined. As shown in Figure 13, when eIF2α was silenced in MDA-MB-231, the expression of p27 increased. Therefore, the results indicate that ERp29 may up-regulate CDK inhibitor p27 through down-regulation of eIF2α.



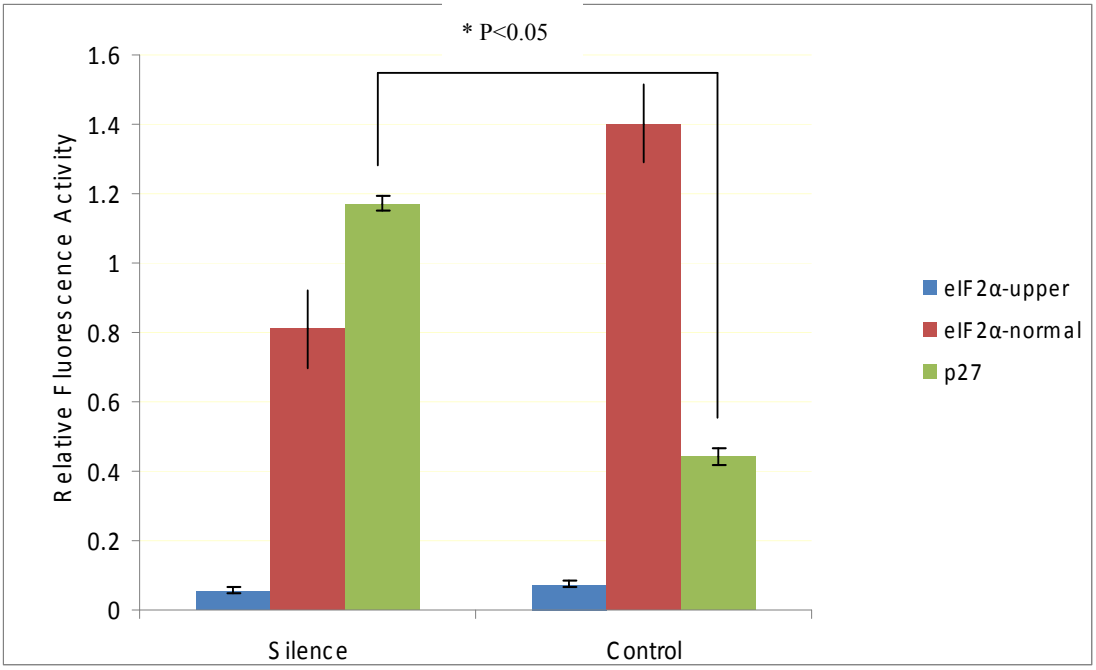




**Figure 13 Silencing of eIF2α up-regulates p27 expression**. Cells were transferred to grow in 6-well plate to 50% confluence and were transfected with eIF2α SiRNA, after 48 hours, cells were detached by trpsin and cell lysates were collected and subject to western blot. Both expression of eIF2α and p27 were examined using respective antibody. α-tubulin was used as a loading control.

### 3.3 ERp29 regulates cellular localization of the cell cycle regulator cyclinD1

Increased levels of cyclin D1 occur in a large segment of human cancers. Amplification of the cyclin D1 locus accounts for a low proportion of the total number of cancers that overexpresses cyclin D1; the mechanisms contributing to cyclin D1 overexpression in the

remainder have not been firmly established as yet. Notably, a large body of work strongly suggests that enforced overexpression of cyclin D1 is not likely to be the essential transforming property of cyclin D1. More recently, work has revealed that expression of a cyclin D1 mutant that is refractory to nuclear export and proteolytic degradation at the G1/S boundary is a highly transforming mutant and functions independent of additional oncogenes *in vitro*.

As discussed above, ERp29 down-regulates cyclinD1/2 expression in breast cancer cells, but the mechanism underlying this process is unknown. Also, it remains to be seen whether ERp29 regulates cyclinD1/2 subcellular localization. An immunofluorescence study was performed to determine the location of cyclinD1/2 with ERp29 overexpression or knock down. As shown in Figure 14, cyclinD1/2 is localized in both the nucleus and cytoplasm in the vector-transfected MDA-MB-231 control cells, while it is translocated from nucleus to cytoplasm to form aggregates in the ERp29 overexpressing cells (clone B and clone E).

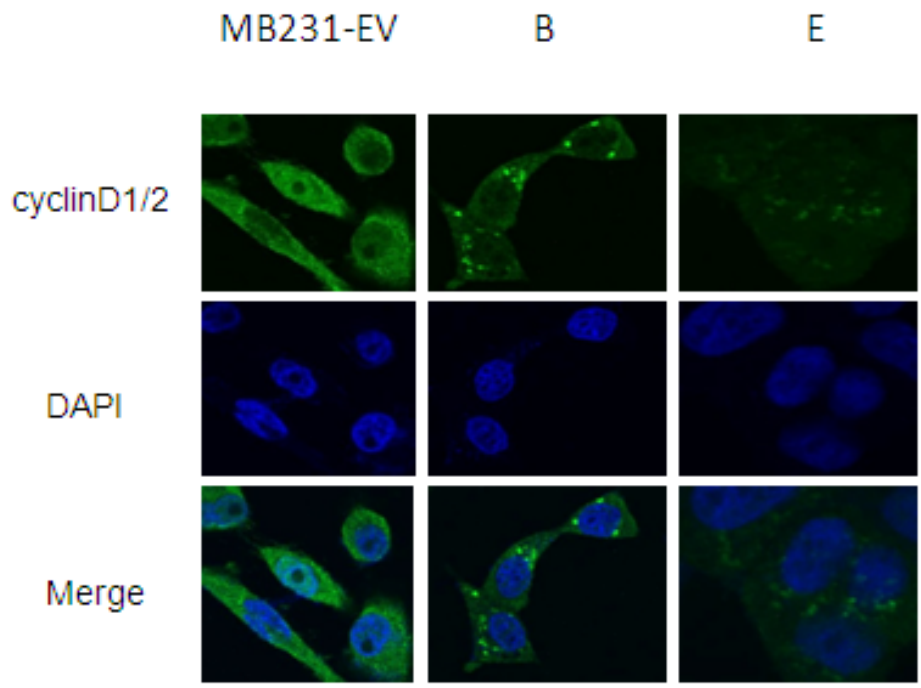



**Figure 14 ERp29 regulates cyclinD1/2 subcellular localization in MDA-MB-231 cells**. MDA–MB-231 cells(control-EV, B, E) were cultured in a 6-well plate with coverslips. After cells attaches onto the coverslip, discard medium and remove the coverslips from wells. Cells were fixed with 30μl of 4%paraformaldehyde for 30 min and then washed with PBS. Cells were then permeabilized using 30μl of 1% Triton-x-100 for 10min.After wash with 0.02% PBST, cells were then blocked with 3% BSA for 1 h in room temperature. After blockage, cells were incubated with anti-cyclinD1/2 antibodies at 4℃ overnight and then incubate with secondary antibodies for 1 hour. Cells were then incubated in dyes for 30min. Following which the coverslip was mounted and sealed.

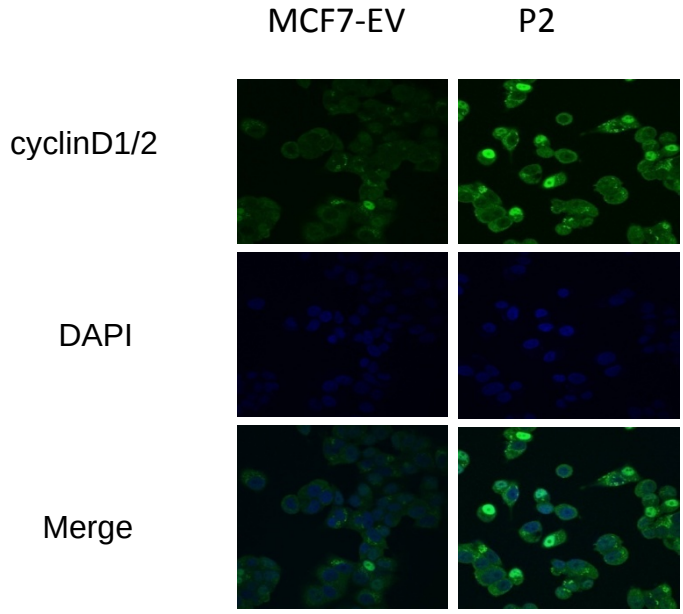



**Figure 15 ERp29 regulates cyclinD1/2 subcellular localization in MCF7 cells**. MCF7 cells (control-EV, P2) were cultured in a 6-well plate with coverslips. After the cells attached onto the coverslip, discard the medium and remove the coverslips from wells. Cells were fixed with 30µl of 4%paraformaldehyde for 30 min and then washed with PBS. Cells were then permeabilized using 30 µl of 1% Triton-x-100 for 10min.After washing with 0.02% PBST, cells were then blocked with 3% BSA for 1 h at room temperature. After blockage, the cells were incubated with anti-cyclin D1/2 antibodies at 4℃ overnight and then incubated with secondary antibodies for 1 hour. The cells were then incubated in dyes for 30min. Following which the coverslip was mounted and sealed.

As cyclinD1 nuclear export is phosphorylation dependent, phosphorylated cyclinD1 subcellular localization was examined. As shown in Figure 16, phosphorylated cyclinD1 remained inside the nucleus of the vector-transfected MDA-MB-231. During the onset of tumorigenesis, tumor suppression breaks down when the cell is no longer capable of shuttling phosphorylated cyclinD1 from the nucleus during the S-phase (41). With the phosphorylated cyclin D1 being retained in the nucleus, the former can lead to cell cycle progression and consequently uncontrolled cancerous cell growth. The mechanism underlying crippling of cyclin D1 nuclear export in MDA-MB-231 is currently unknown but may result from direct

mutations within cyclin D1 or mutations that target the upstream signaling pathway that regulate nuclear export of cyclin D1 complexes. In the current dissertation, it was found that overexpression of ERp29 leads to nuclear export of phosphorylated cyclinD1. As shown in the published results (39), ERp29 overexpression suppressed tumor cell growth and caused cell cycle arrest in the G0/G1 phase, and this is likely to be due to ERp29-induced cyclinD1 export. Therefore, these data suggest that ERp29 can suppress tumor growth for dormancy induction not only through down-regulation of cyclinD1 expression, but also by regulating its subcellular localization.

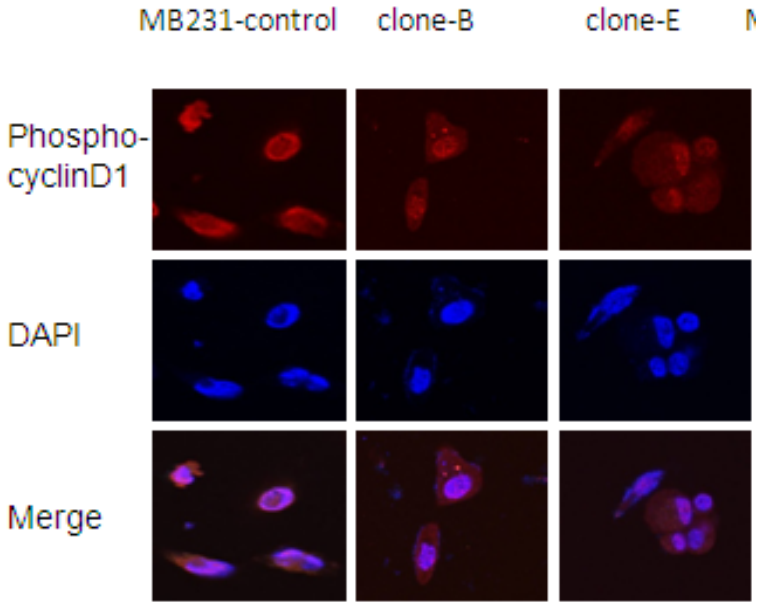



**Figure 16 ERp29 regulates cyclinD1 nuclear export in MDA-MB-231 cells**. MDA –MB-231 cells(control, B, E) were cultured in a 6-well plate with coverslips. After cells attached onto the coverslip, discard the medium and remove the coverslips from wells. Cells were fixed with 30μl of 4%paraformaldehyde for 30 min and then washed with PBS. Cells were then permeabilized using 30μl of 1% Triton-x-100 for 10min.After washing with 0.02% PBST, cells were then blocked with 3% BSA for 1 h at room temperature. After blockage, cells were incubated with anti-cyclinD1/2 antibodies at 4℃ overnight and then incubate with secondary antibodies for 1 hour. Cells were then incubated with dyes for 30min, following which the coverslip was mounted and sealed. The arrow indicates cytoplasm phospho-cyclinD1

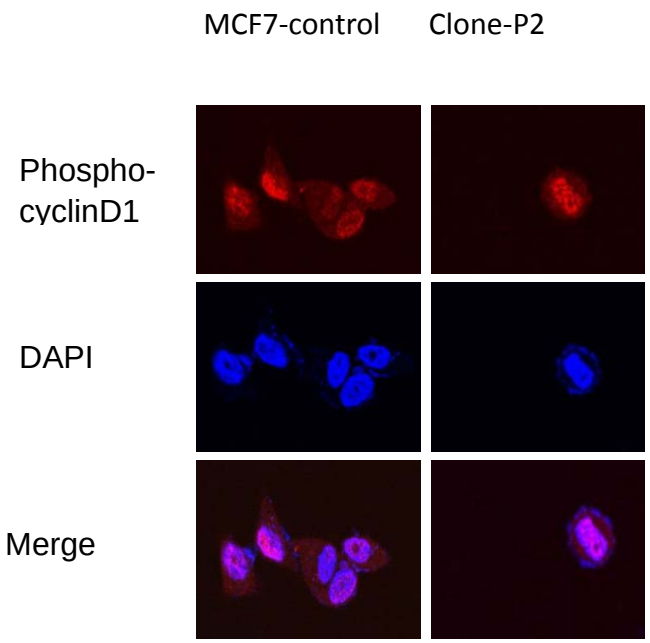



**Figure 17 ERp29 regulates cyclinD1 nuclear export in MCF7 cells**. MCF7 cells(control, P2) was cultured in a 6-well plate with coverslips. After cells had attached onto the coverslip, discard medium and remove the coverslips from wells. Cells were fixed with 30µl of 4%paraformaldehyde for 30 min and then washed with PBS. Cells were then permeabilized using 30µl of 1% Triton-x-100 for 10min. After washing with 0.02% PBST, cells were then blocked with 3% BSA for 1 h at room temperature. After blockage, cells were incubated with anti-cyclinD1/2 antibodies at 4℃ overnight and then incubated with secondary antibodies for 1 hour. Cells were then incubate with dyes for 30min. Following which the coverslip was mounted and sealed. The arrow indicates cytoplasm phospho-cyclinD1

### 3.4 ERp29 up-regulates ER stress induced molecular chaperone p58$^{IPK}$

p58$^{ipk}$ is an ER resident chaperone which is induced during the UPR. Previously it has been shown that over-expression of ERp29 in MDA-MB-231 led to activation of XBP-1, one of the ER proteins that regulate p58$^{ipk}$ (45). p58$^{ipk}$ may interact with PERK and attenuate PERK-mediated eIF2α phosphorylation under conditions of stress. Under the condition of cancer, it may be activated to cope with the ER stress. In the current study the aim is to determine if ERp29 overexpression can modulate ER stress signaling in breast cancer cells to induce tumor cell dormancy. As such, Western blot was performed to determine if p58ipk is

regulated by differential expression of ERp29. As shown in Figure 18, in ERp29 overexpressing MDA-MB-231, the expression of $p58^{ipk}$ is markedly enhanced. On the other hand, ERp29 knock-down in MCF7 cells down-regulated its expression. The up-regulated p58ipk functionally counteracts the upsteam kinase of eIF2α to inhibit its phosphorylation and attenuates activation of the p-PERK/p-eIF2α pathway.

PERK is a type-I ER transmembrane protein. It has a luminal domain bound by the ER chaperone BiP/GRP78 in non stress conditions. It also has a cytoplasmic domain containing kinase activity. Upon ER stress, BiP releases the luminal domain of PERK, allowing PERK to dimerize and autophosphorylate to become active (17). PERK activation then directly leads to eIF2a phosphorylation at Ser51 and translation inhibition (17).Some of the effects of PERK are mediated through the transcription factor ATF4, which is translationally upregulated by ER stress in an eIF2a phosphorylation-dependent manner (17).

Interestingly, in addition to $p58^{ipk}$, it has been observed that expression of basal PERK was highly increased whereas phosphorylation of PERK was weakly detectable in ERp29 overexpressing dormant cells as compared to the control cells (Figure.18B, left panel). It is reasonable that inactivation of PERK is mostly due to the increased level of $p58^{ipk}$ which binds to PERK and represses PERK phosphorylation. Over-expression of ERp29 increased the expression of BiP/GRP78 in the ERp29 overexpressing MDA-MB-231 cells. However, knockdown of ERp29 in MCF-7 cells also resulted in an increase of BiP/GRP78. Furthermore, expression of the eIF2α phosphorylation-dependent transcription factors ATF4 was not changed in the ERp29 overexpressing dormant cells. In contrast, knockdown of ERp29 in MCF-7 cells activated the expression of ATF4, consistent with the enhanced eIF2α

phosphorylation in the ERp29-knockdown MCF-7 cells. (Figure 5 right panel) These results indicate that the ER-dependent pro-apoptotic signaling is markedly suppressed by ERp29 in these dormant cells.

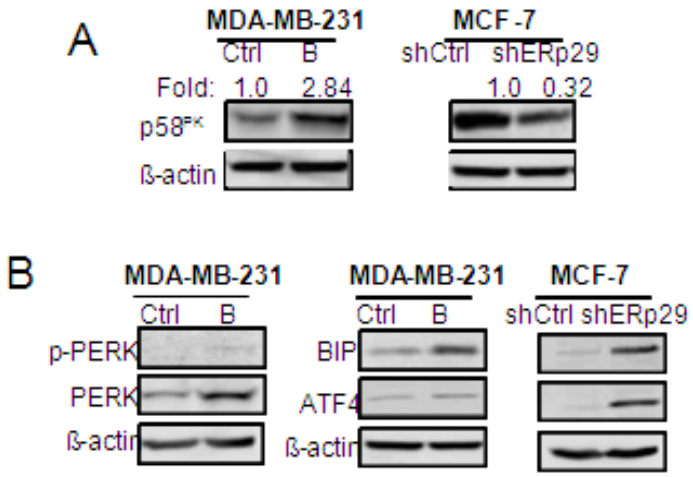



**Figure 18. ERp29 modulates ER stress signaling**. Total cell lysates were extracted from ERp29 overexpressing MDA-MB-231 cells (B) or ERp29 silenced MCF7 cells(shERp29). Western blot was performed using anti-p58IPK, anti-PERK, anti-p-PERK, anti-BIP and anti-ATF4 antibodies. β-actin was used as a loading control

# Chapter 4

# DISCUSSION

Organisms across different kingdoms have evolved ways to sense unfavorable environments and cope with stressful conditions (e.g., drought, cold, nutrient deprivation or heat shock) in order to survive. This in turn results in a suspension in development or growth and in the manifestation of stress-resistant phenotypes. These machineries provide organisms an adaptive advantage to resume development and/or growth after the stress is eased, as such they can generate offspring. This stage is defined as dormancy in plants, diapause in nematodes or hibernation in mammals.

Cancer recurrence is frequent - for example, 20-45% of patients with breast or prostate cancer will relapse years or decades later (46). Recurrence may, to some extent, be due to the presence of solitary dormant cancer cells. It is not common for disseminated cells that arrived in a secondary site to proliferate or even form large metastases of clinical importance. In fact, most cells that arrive at a secondary site will undergo apoptosis or remain in the tissue as solitary dormant cells (47). These cells are defined as quiescent cells which do not proliferate nor undergo apoptosis. In breast cancer, metastasis can occur many years after treatment. Though chemotherapy is used to prevent metastatic recurrence, it is not always successful. This is due to a large number of dormant cells which are disseminated *in vivo* and which are not sensitive to be killed by chemotherapy (48). The longest interval between primary treatment of breast cancer and tumor recurrence can be 20 to 25 years (46). A lack of insight into the mechanism of cancer cell dormancy has been a major stumbling block in the understanding of the intricacies of metastatic growth. Understanding of the signaling

pathways inducing tumorigenic quiescence may provide important information for preventing disease relapse in a large number of cancer patients.

Currently, very little is known about the mechanism of these dormant cells. Recently, a novel endoplasmic reticulum protein ERp29 was found that it was highly expressed in dormant breast cancer cell lines while down-regulated in highly-proliferative breast cancer cell lines. Moreover, overexpression of ERp29 in the MDA-MB-231 breast cancer cell line leads to G0/G1 cell cycle arrest and decreased apoptosis (39). However, the mechanism underlying these phenomena is not fully understood. In this thesis project, the aim is to explore the molecular mechanisms underlying the ERp29-mediated dormancy characteristics in breast cancer. In the ERp29-induced dormant cells, the basal level of the key transcription factor eIF2α is found to be down-regulated. The down-regulation of eIF2α in turn promotes cyclinD1 translocation to the cytoplasm for degradation. Moreover, down-regulation of eIF2α up-regulates CDK inhibitors p21, p27 and p15 to induce cell cycle arrest in the G0/G1 phase. On the other hand, the increased expression of ERp29 also down-regulates another transcription factor, Nrf2, which functions downstream of PERK and regulates ER and oxidative stress signaling. In cancer cells, activation of Nrf2 is reported to facilitate protein degradation (49) during the unfolded protein response(UPR) and therefore in ERp29-induced dormant cells, the down-regulation of Nrf2 may be an indication of decreased protein synthesis as part of the dormancy-induced energy conservation measure. Please refer to Figure 19 for schematics depicting the molecular pathways involved in ERp29-induced tumor dormancy.

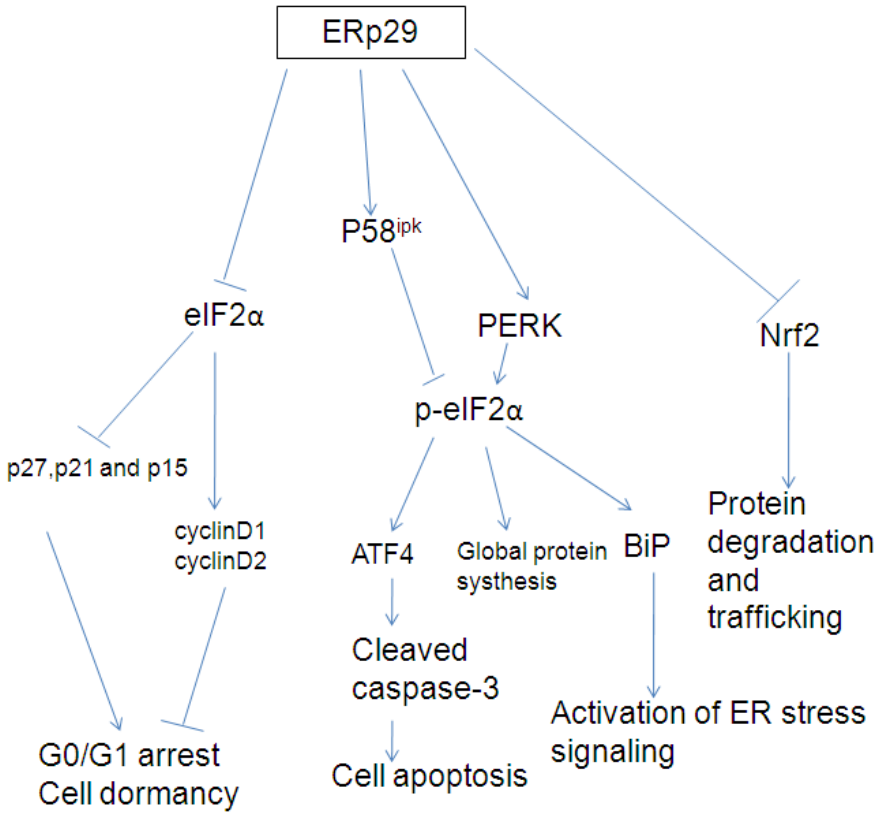



**Figure 19 Schematics showing the molecular players involved in ERp29-induced signaling for tumor dormancy**. Recently in our lab, we found that ERp29 is down-regulatled in breast carcinoma. It negatively regulates cell proliferation in breast cancer cells. In a dormant cell line, MCF7, ERp29 is extensively expressed. But the mechanism underlying these phenomena is not fully understood. Hereby in this thesis, we have explored the molecular mechanism of ERp29-mediated cell cycle arrest in breast cancer. In the ERp29-induced dormant cells, the basal level of key transcription factor eIF2α is down regulated. The down-regulation of eIF2α promotes cyclinD1's translocation to the cytoplasm for degradation. Moreover, down-regulation of eIF2α up-regulates CDK inhibitors p27,p21and p15 to induce cell cycle arrest in the G0/G1 phase. On the other hand, the increased expression also down-regulates another transcription factor, Nrf2, which functions downstream of PERK and regulates ER and oxidative stress signaling. In cancer cells, activation of Nrf2 is reported to facilitate protein degradation during the unfolded protein response and therefore In a ERp29-induced dormant cell, Nrf2 is down-regulated. ERp29 overexpression also up-regulates molecular chaperone p58ipk,which functions to inhibit PERK and the phosphorylation of eIF2α. Phosphorylation of eIF2α up-regulates transcription factor ATF4 and ER chaperone BiP.

It is well known that ER stress signaling is involved in the induction of tumor cell dormancy (50). In the current study, the role of ER stress signaling in ERp29-mediated cell cycle arrest was investigated, leading to breast cancer cell dormancy. eIF2α is a key molecule known to be involved in ER stress signaling (43). The PERK-eIF2α axis is activated during stressful conditions to facilitate tumor cell survival and dormancy. Activation of eIF2α by phosphorylation shuts down general protein translation to relieve ER stress and to promote cell survival (26).

Interestingly, the results show that ERp29 down-regulates basal eIF2α expression rather than regulating eIF2α phosphorylation. It is thus possible that down-regulation of eIF2α reduces global protein synthesis, thereby diminishing the ER stress in breast cancer cells and favoring cell survival and dormancy.

Cell growth arrest and cell survival are two main hallmarks of tumor cell dormancy (50). In the current study, it is observed that the cell cycle mediators cyclinD1 and cyclinD2, which act downsteam of eIF2α, are down-regulated to induce cell cycle arrest in the G0/G1 phase. Meanwhile, the cyclin-dependent kinase inhibitors, p15, p21 and p27 are up-regulated to suppress cell cycle progression.

Tumor cells entering a state of dormancy will require adequate survival signals in order to cope with hash conditions such as hypoxia, nutrient deprivation and acidosis. In the current investigation, it is found that survival signals in ER stress signaling were activated to maintain cell survival in dormant breast cancer cells. A putative ER chaperone, $p58^{ipk}$ was up-regulated in ERp29 -induced dormant cells and was down-regulated in ERp29-silenced

MCF7 cells. p58IPK was originally identified as a cellular inhibitor of the interferon-induced serine/threonine protein kinase (PKR) which is recruited by the influenza virus to inhibit PKR function in eIF2α phosphorylation through a direct interaction with PKR. The down-regulation of $p58^{ipk}$ in ERp29-silenced MCF7 cells induces increased expression of the transcription factor ATF4 and the ER chaperone BIP. Studies have revealed that BIP can serve as a survival signal and its upregulation mediates stress resistance to restore ER homeostasis. The up-regulation of BIP and PERK signaling may serve as survival signals that protect dormant tumor cells from hash microenvironment conditions and chemotherapeutic stress (24).

Resistance towards apoptosis or programmed cell death is a key factor for the survival of a malignant cell. Therefore, it is very important for dormant tumor cells to acquire such a resistance. In this research project, $p58^{ipk}$ was focused on it as a possible mediator for apoptotic resistance in ERp29-induced dormant cells. A recent study had demonstrated that $p58^{IPK}$ can interact with and inhibit PERK phosphorylation, leading to a decreased phosphorylation of eIF2α (51). In the ERp29-induced dormant cells, silencing of $p58^{IPK}$ stimulated eIF2α phosphorylation and activated the expression of ATF4/CHOP and cleavage of caspase-3, leading to an increased cell apoptosis. This was further supported by the early studies that eIF2α phosphorylation mediated stress-induced apoptosis (48). Therefore, up-regulation of $p58^{IPK}$ may facilitate cell survival under stress in the ERp29-induced dormant cells by repressing eIF2α phosphorylation. It is well known that GADD34, a downstream target of the activated p-eIF2α, also inhibits eIF2α phosphorylation by a feedback loop. These studies provide a clue that induction of $p58^{IPK}$ by ERp29 may help to

protect cells from ER stress by de-phosphorylating eIF2α. Activation of p-eIF2α/ATF4/CHOP pro-apoptotic pathway by silencing $p58^{IPK}$ correlated to the increased sensitivity of dormant cells to doxorubicin treatment (private communication, manuscript submitted). Collectively, all the data indicate that $p58^{IPK}$ is an important coordinator in modulating the crosstalk between the ERp29-mediated ER stress and ER-dependent pro-apoptotic signaling by inhibiting eIF2α phosphorylation in the ERp29-induced dormant cells. Thus far, we have demonstrated that ERp29 regulates cell cycle mediators

It has been demonstrated that ERp29 regulates cell cycle mediators expression to induce cell cycle arrest. In addition, it has been also shown that ERp29 up-regulates survival signals to protect dormant cancer cell from apoptosis. Taken together, these results indicate that ERp29 expression favors tumor cell dormancy. However, contradictory results also exist. In a study done by Mkrtchian *et al*., ERp29 expression was associated with larger and more malignant phenotype of xenografts *in vivo* using MCF7 breast cancer cells as a model (38). In their study, three different MCF7 stable transfectants were established: mock-transfected cells, cells overexpressing wild-type ERp29 and a dominant-negative mutant form of ERp29. These transfectants grew at similar rates *in vitro*. However, when injected with fibroblasts *in vivo*, the wild type ERp29-overexpressing cells grew significantly larger xenografts and formed more aggressive tumors than the mouse model injected with cells overexpressing the dominant-negative ERp29. As such, they hypothesized that ERp29 might be involved in cancer cell interaction with their microenvironment. This hypothesis was supported by the fact that endogenous ERp29 was upregulated in the xenografts. Thus they concluded that expression of ERp29 correlates with more aggressive phenotype and may have an oncogenic

function.

Results in the current study showed that ERp29 down-regulates Nrf2. Nrf2 is reported to be a direct PERK substrate and that the PERK-dependent activation of Nrf2 is critical for survival signaling (51). However, in ERp29-induced dormant cells, an up-regulation of Nrf2 as a survival response was not observed. On the contrary, Nrf2 was down-regulated in ERp29-induced dormant cells. This may be due to a different function of Nrf2 in these dormant cells. For example, it has been reported that Nrf2 may play a role in protein degradation (49). Therefore, in the dormant cells in which protein synthesis was shut down, Nrf2 may be down-regulated to reduce the energy cost for cell survival.

The study of cancer cell dormancy may lead to the discovery of therapeutic targets to prevent cancer relapse. For instance, a cell adherent molecule, β1-integrin(Intβ1), was shown to play an important role to induce metastatic growth and resistance to chemotherapy / radiation in the dormant cells (52). The inhibitor of this molecule is already under clinical trial to maintain the tumor cells in quiescence. Here in this dissertation, we have discussed the molecular mechanism of a novel endoplasmic protein, ERp29 in inducing cancer cell dormancy. It will be meaningful to research further, for example, how enhancing ERp29 expression in vivo will prevent cancer relapse.

**FUTURE WORKS**

The PERK/eIF2α/ATF4 module of ER stress signaling is demonstrated to confer survival advantage for tumor growth under hypoxia (17). However, whether this module controls the ERp29-mediated cell dormancy studied in this thesis is not yet fully worked out. It has been

shown that ERp29 up-regulates the expression of PERK and down-regulates the expression of eIF2α, but the activation of PERK through its phosphorylation was not studied in the current research. It would be worthwhile to examine whether PERK is activated and involved in phosphorylation of eIF2α.

The work thus far has shown that Nrf2 is down-regulated by ERp29 in ERp29-induced dormant cells. However, whether Nrf2 is activated or translocated to the nucleus is not clear. The phosphorylation of Nrf2 promotes Nrf2 dissociation from Keap1 as well as its nuclear translocation. Thus, even though the amount of Nrf2 is decreased, the extent of its activation should also be investigated in relation to the ERp29 level.

On the other hand, it is also necessary to examine the downstream effect of the cell cycle regulatory mechanism to further explore how ERp29 regulates cell cycle arrest at G0/G1 phase. Though several G1 cyclins, CDKs and CDK inhibitors were examined, it is meaningful to investigate other G0/G1 cell cycle arrest regulators. For example, it is unclear whether the phosphorylated form of Retinoblastoma (Rb) protein and transcriptional factor E2F are regulated by an increased level of ERp29 during cancer cell dormancy.

Previous results had shown that xenografts with ERp29 transfection forms less proliferative tumors *in vivo,* but as to how one could kill these dormant cells remains unsolved. In the future, it is important to attempt to remove these dormant cells by targeting the ERp29-regulated ER stress signal pathway. To fulfill this task, it is necessary to confirm which part of the ER stress signaling is activated to maintain ERp29–induced tumor cell dormancy. So far it has been shown that ERp29 down-regulates eIF2α, a key translation

initiation factor that controls global protein synthesis, and that ERp29 up-regulates the key ER chaperone BIP and the key transcription factor ATF4. To further explore how ER stress signaling is involved in ERp29-induced tumor cell dormancy, it is possible to knock down BIP and ATF4 to see if the dormancy is perturbed. Briefly, in ERp29-mediated dormant cells, BIP or ATF4 can be knocked down by siRNA. The BIP-knocked down or ATF4-knocked down cells can then be injected into mouse models, and subsequently their tumorigenic activity and sensitivity to chemotherapeutic drugs further investigated. This may enable the researchers to determine potential molecular targets for killing off dormant cancer cells and thus develop effective therapeutics to prevent cancer relapse.

## CONCLUSION

Cancer cell dormancy is one of the means for tumor cells to survive in harsh conditions and resist chemotherapeutic measures targeting their elimination. Studying the mechanism for cancer cell dormancy may help in preventing recurrent metastatic disease. Unfortunately, the mechanism of cancer cell dormancy is so far poorly understood. Previously, it has been found that a novel endoplasmic reticulum protein ERp29 induces cell dormancy in breast cancer. In this thesis project, the author tried to elucidate the molecular mechanisms underlying ERp29-mediated cancer cell dormancy in breast cancer.

The current results demonstrated that ERp29 modulate the ER stress signaling pathway in dormant cells. The key translation initiation factor eIF2α is down-regulated to shut down global protein synthesis. Meanwhile, one of the important ER chaperones, $p58^{ipk}$, is

up-regulated to facilitate cell survival under ER stress, where as the transcription factor Nrf2 is down-regulated to reduce excess energy cost for protein degradation. In summary, the author has provided proof-of-concept evidence that ERp29-mediated cell dormancy is induced through regulation of ER stress signaling.

With the further pursuit of knowledge of the molecular mechanism underlying ERp29-mediated cell dormancy, it would be possible to target one or more molecules in cancer therapy to block cell dormancy and enhance the effect of chemotherapeutic drugs.

Reference List


(1) International Agency for Research on Cancer. World Cancer Report 2008. 4 A.D. Aug.

(2) Porter PL. Global trends in breast cancer incidence and mortality. Salud Publica de Mexico 2009;51:s141-s146.

(3) Shin HR, Joubert C, Boniol M, Hery C, Ahn S, Won YJ, Nishino Y, Sobue T, Chen CJ, You SL, Mirasol-Lumague M, Law S, Mang O, Xiang YB, Chia KS, Rattanamongkolgul S, Chen JG, Curado M, Autier P. Recent trends and patterns in breast cancer incidence among Eastern and Southeastern Asian women. Cancer Causes and Control 2010 Nov 1;21(11):1777-85.

(4) Siew-Eng Lim, Michael Back, Esther Quek, Philip Iau, Thomas Putti, John E.L.Wong. Clinical Observations from a Breast Cancer Registry in Asian Women. World journal of Surgery 2011;31(7):1387-92.

(5) Singapore Cancer Registry Interim Annual Registry Report Trends in Cancer Incidence in Singapore 2005-2009. 2011 Jun 21.

(6) Trends in Cancer Incidence in Singapore. 2011.

(7) Kelsey JL, Bernstein L. Epidemiology and Prevention of Breast Cancer. Annual Review of Public Health 1996 Jan 1;17(1):47-67.

(8) Kelsey JL. Breast Cancer Epidemiology: Summary and Future Directions. Epidemiologic Reviews 1993 Jan 1;15(1):256-63.

(9) Kelsey JL, Berkowitz GS. Breast Cancer Epidemiology. Cancer Research 1988 Oct 15;48(20):5615-23.

(10) Voeltz GK, Rolls MM, Rapoport TA. Structural organization of the endoplasmic reticulum. EMBO J 2002;3:944-50.

(11) Baumann O, Walz B. Endoplasmic reticulum of animal cells and its organization into structural and functional domains. International Review of Cytology. Volume 205 ed. Academic Press; 2001. p. 149-214.

(12) Schroder M, Kaufman RJ. The mammalian unfolded protein response. Annual Review of Biochemistry 2005 Jul 1;74(1):739-89.

(13) Anelli T, Sitia R. Protein quality control in the early secretory pathway. EMBO J 2008 Jan 23;27(2):315-27.

(14) Shen X, Zhang K, Kaufman RJ. The unfolded protein response--a stress signaling pathway of the endoplasmic reticulum. Journal of Chemical Neuroanatomy 2004

Sep;28(1-2):79-92.

(15) Ma Y, Hendershot LM. The role of the unfolded protein response in tumour development: friend or foe? Nature Reviews Cancer 2004 Dec;4(12):966-77.

(16) Verfaillie T, Garg AD, Agostinis P. Targeting ER stress induced apoptosis and inflammation in cancer. Cancer LettersIn Press, Corrected Proof.

(17) Fels DR, Koumenis C. The PERK/eIF2α/ATF4 Module of the UPR in Hypoxia Resistance and Tumor Growth. Cancer Biology & Therapy 2006 Jul;5:7:723-8.

(18) Blais JD, Addison CL, Edge R, Falls T, Zhao H, Wary K, Koumenis C, Harding HP, Ron D, Holcik M, Bell JC. Perk-Dependent Translational Regulation Promotes Tumor Cell Adaptation and Angiogenesis in Response to Hypoxic Stress. Mol Cell Biol 2006 Dec 15;26(24):9517-32.

(19) Drogat B, Auguste P, Nguyen DT, Bouchecareilh M, Pineau R, Nalbantoglu J, Kaufman RJ, Chevet E, Bikfalvi Aa, Moenner M. IRE1 Signaling Is Essential for Ischemia-Induced Vascular Endothelial Growth Factor-A Expression and Contributes to Angiogenesis and Tumor Growth In vivo. Cancer Res 2007 Jul 15;67(14):6700-7.

(20) Romero-Ramirez L, Cao H, Nelson D, Hammond E, Lee AH, Yoshida H, Mori K, Glimcher LH, Denko NC, Giaccia AJ, Le QT, Koong AC. XBP1 Is Essential for Survival under Hypoxic Conditions and Is Required for Tumor Growth. Cancer Res 2004 Sep 1;64(17):5943-7.

(21) Jamora C, Dennert G, Lee AS. Inhibition of tumor progression by suppression of stress protein GRP78/BiP induction in fibrosarcoma B/C10ME. Proceedings of the National Academy of Sciences 1996 Jul 23;93(15):7690-4.

(22) Siamak Daneshmand, Marcus L.Quek, Ed Lin, Charlotte Lee, Richard J.Cote, Debra Hawes, Jie Cai, Susan Groshen, Gary Lieskovsky, Donald G.Skinner, Amy S.Lee, Jacek Pinski. Glucose-regulated protein GRP78 is up-regulated in prostate cancer and correlates with recurrence and survival. Human Pathology 2007;38(10):1547-52.

(23) Brewer JW, Hendershot LM, Sherr CJ, Diehl JA. Mammalian unfolded protein response inhibits cyclin D1 translation and cell-cycle progression. Proceedings of the National Academy of Sciences of the United States of America 1999 Jul 20;96(15):8505-10.

(24) Ranganathan AC, Zhang L, Adam AP, guirre-Ghiso JA. Functional Coupling of p38-Induced Up-regulation of BiP and Activation of RNA-Dependent Protein Kinase-Like Endoplasmic Reticulum Kinase to Drug Resistance of Dormant Carcinoma Cells. Cancer Res 2006 Feb 1;66(3):1702-11.

(25) Kimball SR. Eukaryotic initiation factor eIF2. The International Journal of Biochemistry & Cell Biology 1999 Jan;31(1):25-9.

(26) Wek RC, Jiang HY, Anthony TG. Coping with stress: eIF2 kinases and translational control. Biochem Soc Trans 2006 Feb 1;34(Pt 1):7-11.

(27) Wang S, Rosenwald IB, Hutzler MJ, Pihan GA, Savas L, Chen JJ, Woda BA. Expression of the Eukaryotic Translation Initiation Factors 4E and 2{alpha} in Non-Hodgkin's Lymphomas. Am J Pathol 1999 Jul 1;155(1):247-55.

(28) Wang S, Lloyd RV, Hutzler MJ, Rosenwald IB, Safran MS, Patwardhan NA, Khan A. Expression of Eukaryotic Translation Initiation Factors 4E and 2α Correlates with the Progression of Thyroid Carcinoma. Thyroid 2004 Jul 9;11(12):1101-7.

(29) Rosenwald IB, Wang S, Savas L, Woda B, Pullman J. Expression of translation initiation factor eIF-2 is increased in benign and malignant melanocytic and colonic epithelial neoplasms. Cancer 2003;98(5):1080-8.

(30) Demmer J, Zhou C, Hubbard MJ. Molecular cloning of ERp29, a novel and widely expressed resident of the endoplasmic reticulum. FEBS Letters 1997 Jan 27;402(2-3):145-50.

(31) Mkrtchian S, Fang C, Hellman U, Ingelman-Sundberg M. A stress-inducible rat liver endoplasmic reticulum protein, ERp29. European Journal of Biochemistry 1998;251(1-2):304-13.

(32) Hubbard MJ, McHugh NJ, Carne DL. Isolation of ERp29, a novel endoplasmic reticulum protein, from rat enamel cells. European Journal of Biochemistry 2000;267(7):1945-57.

(33) Mkrtchiana S, Baryshev M, Matvijenko O, Sharipo A, Sandalova T, Schneider G, Ingelman-Sundberg M. Oligomerization properties of ERp29, an endoplasmic reticulum stress protein. FEBS Letters 1998 Jul 24;431(3):322-6.

(34) Sargsyan E, Baryshev M, Backlund M, Sharipo A, Mkrtchian S. Genomic organization and promoter characterization of the gene encoding a putative endoplasmic reticulum chaperone, ERp29. Gene 2002 Feb 20;285(1-2):127-39.

(35) Baryshev M, Sargsyan E, Mkrtchian S. ERp29 is an essential endoplasmic reticulum factor regulating secretion of thyroglobulin. Biochemical and Biophysical Research Communications 2006 Feb 10;340(2):617-24.

(36) Park S, You KH, Shong M, Goo TW, Yun EY, Kang SW, Kwon OY. Overexpression of ERp29 in the thyrocytes of FRTL-5 cells. Molecular Biology Reports 2005 Mar 1;32(1):7-13.

(37) Cheretis CM, Dietrich FM, Chatzistamou IM, Politi KM, Angelidou EM, Kiaris HP,

Mkrtchian SM, Koutselini HM. Expression of ERp29, an Endoplasmic Reticulum Secretion Factor in Basal-Cell Carcinoma. American Journal of Dermatopathology 2006 Oct;28(5):410-2.

(38) Mkrtchian S, Baryshev M, Sargsyan E, Chatzistamou I, Volakaki AA, Chaviaras N, Pafiti A, Triantafyllou A, Kiaris H. ERp29, an endoplasmic reticulum secretion factor is involved in the growth of breast tumor xenografts. Mol Carcinog 2008;47(11):886-92.

(39) Bambang IF, Xu S, Zhou J, Salto-Tellez M, Sethi SK, Zhang D. Overexpression of endoplasmic reticulum protein 29 regulates mesenchymal-epithelial transition and suppresses xenograft tumor growth of invasive breast cancer cells. Lab Investigation 2009 Sep 21;89(11):1229-42.

(40) Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes & Development 1998 Nov 15;12(22):3499-511.

(41) Alt JR, Cleveland JL, Hannink M, Diehl JA. Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. Genes & Development 2000 Dec 15;14(24):3102-14.

(42) Wek RC, Cavener DR. Translational Control and the Unfolded Protein Response. Antioxidants & Redox Signaling 2007 Aug 30;9(12):2357-72.

(43) Hamanaka RB, Bennett BS, Cullinan SB, Diehl JA. PERK and GCN2 Contribute to eIF2 Phosphorylation and Cell Cycle Arrest after Activation of the Unfolded Protein Response Pathway. Mol Biol Cell 2005 Dec 1;16(12):5493-501.

(44) Cullinan SB, Zhang D, Hannink M, Arvisais E, Kaufman RJ, Diehl JA. Nrf2 Is a Direct PERK Substrate and Effector of PERK-Dependent Cell Survival. Mol Cell Biol 2003 Oct 15;23(20):7198-209.

(45) Zhang D, Putti TC. Over-expression of ERp29 attenuates doxorubicin-induced cell apoptosis through up-regulation of Hsp27 in breast cancer cells. Experimental Cell Research 2010 Dec 10;316(20):3522-31.

(46) Karrison TG, Ferguson DJ, Meier P. Dormancy of Mammary Carcinoma After Mastectomy. Journal of the National Cancer Institute 1999 Jan 6;91(1):80-5.

(47) Fehm T, Mueller V, Marches R, Klein G, Gueckel B, Neubauer H, Solomayer E, Becker S. Tumor cell dormancy: implications for the biology and treatment of breast cancer. APMIS 2008;116(7):742-53.

(48) Naumov GN, Townson JL, MacDonald IC, Wilson SM, Bramwell VHC, Groom AC, Chambers AF. Ineffectiveness of Doxorubicin Treatment on Solitary Dormant Mammary Carcinoma Cells or Late-developing Metastases. Breast Cancer Research

and Treatment 2003 Dec 1;82(3):199-206.

(49) Cullinan SB, Diehl JA. Coordination of ER and oxidative stress signaling: The PERK/Nrf2 signaling pathway. The International Journal of Biochemistry & Cell Biology 2006 Mar;38(3):317-32.

(50) Ranganathan AC, Adam AP, Zhang L, Aguirre-Ghiso JA. Tumor cell dormancy induced by p38SAPK and ER-stress signaling: an adaptive advantage for metastatic cells? Cancer Biology & Therapy 2006 Jul 1;729-35.

(51) Cullinan SB, Diehl JA. PERK-dependent Activation of Nrf2 Contributes to Redox Homeostasis and Cell Survival following Endoplasmic Reticulum Stress. Journal of Biological Chemistry 2004 May 7;279(19):20108-17.

(52) Barkan D, Chambers AF. beta1-Integrin: A Potential Therapeutic Target in the Battle against Cancer Recurrence. Clinical Cancer Research 2011 Sep 7.

# APPENDIX 1

**Reagents**

*1. Bio-Rad (USA)*

30% acrylamide

Ammonium persulfate (APS)

N', N', N', N'-Tetramethylethylenediamide (TEMED)

*2. Merck(USA)*

Ethanol(70%)for sterile use

Methanol(20%)for Western blot transfer buffer

*3. Sigma-Aldrich (Steinheim, Germany)*

Phosphatase cocktail inhibitors I and II

*4. Pierce (Rockford, IL,USA)*

Coomassie PlusTM Protein Assay Reagent

SuperSignal West Pico Chemiluminescent Substrate

*5. Promega (USA)*

Modified porcine trypsin (Sequencing grade)

*6. Roche(Indianapolis, IN,USA)*

Complete, EDTA-free protease inhibitor cocktail tablets

*7. USB Coporation (USA)*

Dithiotreitol (DTT)

Glycine

Soduim dodecyl sulfate (SDS)

Sodium chloride

Tris-base

*8. Invitrogen (Eugene, OR, USA).*

Geneticin sulphate (G418)

Fetal bovine serum

LipofectamineTM 2000 transfection reagents

# APPENDIX 2

## ENDOPLASMIC RETICULUM PROTEIN 29 (ERP29)-INDUCED BREAST CANCER CELL DORMANCY INVOLVES MODULATION OF ENDOPLASMIC RETICULUM STRESS VIA ACTIVATION OF P38 AND UP-REGULATION OF P58$^{IPK*}$

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Running title: ERp29 activates p38 and up-regulates p58$^{IPK}$ to induce cell dormancy

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**Endoplasmic reticulum protein 29 (ERp29) is a novel ER luminal protein and plays a critical role in protein unfolding and secretion. However, its function in cancer progression is not fully understood. Recently, we reported that ERp29 is a novel tumor suppressor which drives the proliferative MDA-MB-231 breast cancer cells into a dormant state. Here, we provide evidence that the ERp29-induced cancer cell dormancy was modulated by activation of p38 and up-regulation of p58$^{IPK}$. In this dormant cell model, over-expression of ERp29 significantly down-regulates the expression of urokinase receptor (uPAR), $\beta_1$-integrin and epidermal growth factor receptor (EGFR), and highly increases the phosphorylation of p38 and reduces MMP-9 secretion. Blockage of p38 phosphorylation by pharmacological inhibitor significantly down-regulates the expression of basal eukaryotic translation initiation factor 2α (eIF2α) and cyclin $D_2$ and up-regulates the expression of cyclin-dependent kinase inhibitors (p15 and p21). Further, we show that p58$^{IPK}$ was markedly increased to inhibit eIF2α phosphorylation. Reduction of p58$^{IPK}$ by RNA interference stimulated eIF2α phosphorylation and increased the expression of eIF2α phosphorylation-dependent activating transcription factor 4 (ATF4) and growth arrest and DNA damage-inducible protein (GADD153/CHOP) and the activation of caspase-3, leading to enhanced cell apoptosis and sensitivity to doxorubicin. Our studies indicate that activated p38 regulates the expression of checkpoint molecules to induce $G_1$ arrest while the up-regulated p58$^{IPK}$ inhibits eIF2α phosphorylation to relieve excessive ER stress, coordinately modulating the ERp29-induced cell dormancy and survival. Our findings suggest a novel regulatory mechanism of ERp29-induced cancer cell dormancy in breast cancer.**

Breast tumor cell dormancy is a significant clinical problem in cancer treatment. Most of the dormant cells do not express proliferation markers and are resistant to conventional therapies that target actively dividing cells (1). These dormant cells can survive for a long time in blood and/or bone marrow and recapitalize a proliferative characteristic to form a local and/or distant metastasis when cells are activated under certain condition. Although highly prevalent, little is known about the mechanisms responsible for the regulation of dormancy in these cells due to lack of suitable experimental models and limited clinical accessibility to the microscopic size of dormant tumors.

Currently there is no efficient therapeutic treatment that can keep tumor cells in dormant state for extended periods of time and prevent them from localization and proliferation. Therefore, understanding the mechanisms of inducing tumor cell dormancy is of clinical importance in suppressing tumor cell reactivation and cancer recurrence. This may lead to novel strategies to prolong the extent of dormant state to block tumor formation or to enhance signaling pathways that stimulate dormant cells into apoptosis or to improve their sensitivity to chemotherapy.

Tumor dormancy represents the inability of cells to produce tumors or the capability to form small nodules after prolonged latency. In general, dormant cells are characterized as cell cycle $G_0/G_1$ arrest or the similar proliferative and apoptotic rates leading to a little overall gain in cell mass (2). Several mechanisms have been proposed to explain the tumor dormancy. These include impaired angiogenesis by which tumor growth is blocked due to inability to form new and functional blood vessels; cellular dormancy by which tumor cells are in quiescent state due to cell cycle arrest, and immunosurveillance by which tumor mass growth is blocked by active immune response (3-5). Mechanistic studies in human squamous carcinoma cells have shown that urokinase receptor ($uPAR^2$), epidermal growth factor receptor (EGFR), extracellular signal-regulated kinase (ERK) and p38 activities play critical roles in regulating tumor cell dormancy (2, 6, 7). The uPAR is defined to be a central regulator of a balance between p38 and ERK activation as down-regulation of uPAR favors p38 activation over ERK to induce tumor cell dormancy (8, 9). In addition, activated ATF6α-Rheb-mTOR signaling, regulated in part by p38, was found to be essential in maintaining cell survival for quiescent but not for proliferative squamous carcinoma cells (10).

Tumor cell dormancy has also been mechanistically linked with endoplasmic reticulum (ER) stress signaling. Accumulation of misfolded/unfolded proteins in the ER lumen results in ER stress and subsequently initiates an integrated signal transduction pathway known as Unfolded Protein Response (UPR) to ameliorate the protein load on the ER. The UPR is mediated by at least three ER proximal sensors, PKR-related ER kinase (PERK), inositol requiring enzyme-1 (IRE-1) and activating transcription factor 6 (ATF6) (11, 12). PERK is a serine/threonine kinase that phosphorylates eukaryotic translation initiation factor 2α (eIF2α) at $Ser^{51}$ on stress and consequently attenuates global protein translation and induces $G_0/G_1$ arrest (13), a characteristic of dormancy of cells. Hence, cells may coordinate growth arrest and survival signals by activating ER stress, conferring dormant cells a resistance to stress-induced death. The role of p38 activation in inducing tumor cell dormancy by regulating PERK/p-eIF2α pathway has been established in human squamous carcinoma cells (14, 15). The mechanistic link between ER stress and tumor cell dormancy through p38 activation suggests an important role of p38-regulated network in modulating tumor cell quiescence, survival and apoptosis (14-16)

We have established a “dormancy-like” breast cancer cell model by exogenously over-expressing ER protein 29 (ERp29) in the high proliferative and invasive MDA-MB-231 cells (17). Over-expression of ERp29 in MDA-MB-231 cells results in $G_0/G_1$ arrest and significantly drives the proliferative cells into a quiescent state *in vitro*, thus leading to a marked delay in the onset of tumorigenesis and formation of small nodule of xenograft tumors *in vivo* (17). The ERp29-induced dormant cells are also characterized by loss of proliferative markers (e.g., fibronectin, uPAR, $\beta_1$-integrin, EGFR, Ki-67) and gain of molecules that are involved in cessation of cell growth and tumor suppression (e.g., cyclin-dependent kinase inhibitors p15 and p21, E-cadherin) (17). Significantly, the established dormant cell model phenotypically resembles that of epithelial MCF-7 cells (17) and demonstrates high resistance and increased survival to doxorubicin treatment (18). It

was reported that desensitizing doxorubicin is another important clinical characteristic of solitary dormant tumor cells (1). Based on the cellular behavior of the ERp29-overexpressed MDA-MB-231 cells *in vitro* and *in vivo*, we have implicated a pivotal role of ERp29 in inducing tumor cell dormancy, but the mechanisms remain elusive. Interestingly, we observed that, in addition to stimulating UPR by enhancing X-box binding protein 1 (XBP-1) slicing, ERp29 regulates PERK/p-eIF2α pathway by significantly reducing the expression of basal eIF2α, but not by increasing its phosphorylation (18). This may implicate a different regulatory mechanism of ERp29 in inducing tumor cell dormancy and survival.

In this report, we present evidence that uPAR/$\beta_1$-integrin/EGFR and its downstream focal adhesion kinase (FAK) /phosphorylated -ERK (p-ERK) are significantly decreased while p38 is highly phosphorylated in the ERp29-induced dormant MDA-MB-231 cells. Activation of p38 down-regulates cyclin $D_2$ and up-regulates p15 and p21, leading to $G_0/G_1$ arrest. Furthermore, we show that p38 activation by ERp29 negatively regulates the expression of basal eIF2α, while the up-regulated $p58^{IPK}$ inhibits the phosphorylation of eIF2α. Finally, we demonstrate that silencing of $p58^{IPK}$ facilitates eIF2α phosphorylation- dependent activation of ATF4/CHOP/caspase-3 pro-apoptotic signaling, leading to an increased sensitivity to chemotherapeutic treatment and cell death. Our results thus demonstrate that p38 activation and up-regulation of $p58^{IPK}$ are central regulators in sustaining ERp29-induced cell dormancy and survival.

## EXPERIMENTAL PROCEDURES

*Antibodies and reagents* – The following antibodies were used in this study: mouse anti-uPAR from R&D Systems (Minneapolis, MN), rabbit anti-EGFR (c-term) from Epitomics (Burlingame, CA), rabbit anti-ERp29 from Acris (Hiddenhayse, Germany), rabbit anti-PERK, rabbit anti-phospho-PERK (Thr980), rabbit anti-$p15^{INK4B}$, rabbit anti-$p21^{Wafi/Cip1}$, rabbit anti-$\beta_1$-integrin, rabbit anti-eIF2α, mouse anti-p-eIF2α ($Ser^{51}$), rabbit anti-$p58^{IPK}$, rabbit anti-FAK, rabbit anti-p38, mouse anti-GADD153/CHOP, rabbit anti-phospho-p38 (Thr180/Tyr182), and rabbit anti-cleaved caspase-3 from Cell Signaling Technology (Beverley, MD), rabbit anti-ATF4 and rabbit anti-Bip/GRP78 from Novus Biologicals (Littleton, CO), mouse anti-β-actin from Sigma-Aldrich (St. Louis, MO). SB203580 was purchased from Calbiochem (San Diego, CA). Complete, EDTA-free protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Indianapolis, IN) and phosphatase cocktail inhibitors I and II were from Sigma-Aldrich. Geneticin sulphate (G418) and lipofectamine$^{TM}$ 2000 transfection reagent were supplied from Invitrogen (Eugene, OR).

*Cell Culture* – MDA-MB-231 and MCF-7 breast cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen). MDA-MB-231 cells transfected with pcDNA/ERp29 or pcDNA, and MCF-7 cells transfected with shRNA/ERp29 or control shRNA were generated as previously described (17). All the transfected cells were maintained in DMEM supplemented with 10% FBS and G418 (Invitrogen, 2 mg/ml for MDA-MB-231 transfectants, 1 mg/ml for MCF-7 transfectants). Cells were cultured at $37^0$C with 5% $CO_2$ in a humidified incubator.

*Immunofluorescence and Confocal Microscopy* – Immunofluorescent analysis was performed as described earlier (17, 18). Briefly, cells plated on coverslips were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20min and permeabilized with 0.1% Triton X-100 for 10min. The cells were then

incubated overnight with primary antibodies against Ets-1 or cyclin $D_2$ at $4^0$C, followed by incubation with Alexa Fluor 488-conjugate streptavidin (1:1000, Invitrogen) for 1h at room temperature. After final washes with PBS, the coverslips were mounted using the antifade mounting fluid containing DAPI and the images were examined and captured using Olympus Fluoview FV500 confocal laser scanning microscope (Olympus, Japan). Raw images were analyzed using Olympus FV10-ASW 1.7 Viewer software (Olympus).

*Treatment with pharmacological inhibitor* – Cells were equally seeded in each well (~$5\times10^5$/per well) of 6-well plates and incubated at $37^0$C in $CO_2$ incubator. When cells reached ~90% confluence, these cells were treated with SB203580 (final conc. 40μM) or dimethyl sulfoxide (DMSO) as previously described (19). After 24h post-treatment, cells were collected and the total proteins were extracted for Western blot analysis.

*RNA Interference and Transfection* – Small interfering RNAs (siRNAs) against $p58^{IPK}$ (Cell Signaling Technology) and control siRNA (Santa Cruz Biotechnology) were used for gene knockdown according to the procedures as previously described (18). Cells at 60-80% confluence were transfected with siRNA at a final concentration of 100nM of the respective siRNA using Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Twenty four or 48 h after transfection, cells were harvested for Western blot analysis.

*Cell Viability assay* – Cell viability was analyzed with a 3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay kit (Promega Corporation, Madison, WI) according to the manufacturer's instruction. Briefly, cells ($5\times10^5$ cells/per well) were seeded in each well of 6-well plates. Cells at 60-80% confluence were treated with $p58^{IPK}$/siRNA or control siRNA for 24h and cell viability was assessed. Alternatively, abovementioned siRNA-treated cells were further treated with doxorubicin (final conc. 200μg/ml) or DMSO for 24h and cell viability was analyzed as followed. Cells were incubated with MTS solution for 4h and the absorbance of formazon at 492nm was measured with Infinite F200 microplate reader (TECAN Austria GmbH, Grodig, Austria). The absorbance of cells treated with scramble siRNA was defined as 100% survival (control), and the remaining data were converted to a percentage of the control.

*Western Blot Analysis* – Western blotting was performed by standard protocol as previously described (17). Briefly, cell lysates were extracted with RIPA Buffer (1% Igepal, 1% sodium deoxycholate, 0.15M sodium chloride, 0.01M sodium sulphate, pH 7.2 and 2mM EDTA), supplemented with protease inhibitors (Roche Diagnostics) and phosphatase cocktail inhibitors I and II (1:100, Sigma-Aldrich). Cell lysates were centrifuged at 13,000×g for 20min at $4^0$C and protein-containing supernatant was collected. The total proteins (30-50μg) were separated by 10% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked with 5% skim milk for 1h at room temperature and probed with the indicated primary antibodies. Goat-anti-mouse horseradish peroxidase (HRP) (Upstate Biotechnology, Lake Placid, NY) or goat-anti-rabbit HRP secondary antibody (ZyMED Laboratories Inc, San Francisco, CA) were used as secondary antibody. The chemiluminescent signal was developed with Supersignal® West Pico Chemiluminescent substrate (Pierce, Rockford, IL). Signal intensity was analyzed using the GeneTools software (Syngene, Frederick, MD). The level of β-actin was used as a loading control.

*Zymography* – Cells at 50-60% confluence were washed twice with sterilized PBS buffer (pH 7.4) and then incubated with serum-free DMEM for 48 h. The conditioned medium was collected and centrifuged at 3000×g for 15min at $4^0$C to remove cell debris. The supernatant was concentrated 80-100-fold using Biomax Ultrafree Centrifugal Filter Unit (Millipore, Bedford, MA) with a 10kDa

pore diameter cutoff. The total secreted proteins (30-50μg) were mixed with SDS sample buffer in the absence of reducing reagents and resolved in 7.5% polyacrylamide gels containing 0.1% gelatin under nonreducing conditions. MMP activity was assessed by gelatin zymography. Briefly, the gels were washed for 1h at room temperature with 50mM Tris-HCl buffer (pH 7.5) containing 2.5% Triton X-100 and 5mM $CaCl_2$ and then washed extensively with Milli-Q water. After removing SDS, the gels were further incubated overnight at 37°C in digestion buffer (50mM Tris, pH 7.5, 5mM $CaCl_2$, 0.1mM ZnCl, 150mM NaCl) to allow proteolysis of the gelatin substrate, followed by staining with 5% Coomassie Brillient Blue R-250 and de-staining in methanol/acetic acid/water (10:10:80). The gelatinolytic activities were detected as clear bands against blue background.

*Statistical Analyses* – Student's *t* test was used to analyze the significance of differences. Two-tailed $p<0.05$ was considered as significant. All cell culture experiments were performed in triplicate. Data are presented as mean ± standard deviation (SD).

## RESULTS

*Over-expression of ERp29 induces cell dormancy through down-regulation of uPAR/$\beta_1$-Integrin/EGFR* – We reported that over-expression of ERp29 in MDA-MB-231 cells resulted in cell growth arrest and drove proliferative cells into a dormant state (17). Mechanistic study revealed a transcriptional down-regulation of uPAR, $\beta_1$-integrin and EGFR in the ERp29-induced dormant MDA-MB-231 cells (17). Immunoblotting analysis further demonstrated a significant decrease of protein expression of uPAR/$\beta_1$-integrin/EGFR complex in these dormant cells (clone B and E) (Fig.1A, left panel). uPAR is an important membrane receptor in regulating both cell growth and invasion (20, 21). Its expression is highly regulated by v-ets erythroblastosis virus E26 oncogene homolog 1 (Ets-1) in invasive breast cancer (22). We showed that Ets-1 was down-regulated by ERp29 (Fig.1A, right panel), further supporting a mechanistic link of ERp29 in attenuating uPAR in these dormant cells. Immunofluorescent analysis indicated that Ets-1 was expressed in both nucleus and cytoplasm in the control cells and over-expression of ERp29 resulted in a significant reduction of Ets-1 in these dormant cells (Suppl. Fig. 1A). Given that activation or loss of this complex decides the cell destination between proliferation and dormancy, we further investigated whether ERp29-mediated cell growth arrest and dormancy in MDA-MB-231 cells was associated with the interruption of downstream signaling regulated by this complex.

*Over-expression of ERp29 activates p38 phosphorylation and decreases FAK expression and MMP-9 secretion* – Two important signaling pathways under this complex include FAK/p-ERK and cell division cycle 42 (Cdc42)/phosphorylated-p38 (p-p38) (3). Activation of FAK/p-ERK facilitates cell proliferation and tumorigenesis whereas activation of Cdc42/p-p38 promotes cell dormancy and $G_1$ arrest. In addition to inhibition of p-ERK in the ERp29-induced dormant MDA-MB-231 cells (17), here we showed that expression of FAK was highly reduced in the ERp29-induced dormant cells comparing to the proliferative MDA-MB-231 cells (Fig. 1B, left panel), while knockdown of ERp29 in MCF-7 cells enhanced the expression of FAK (Fig.1B, right panel). Inhibition of FAK was found to induce cell dormancy in human carcinoma cells (23). Accompanying with the inhibition of FAK in the dormant cells, the level of p-p38 was significantly enhanced, although the expression of basal p38 was highly decreased (Fig. 1B, left panel). The ERp29-enhanced phosphorylation of p38 was further verified in MCF-7 cells where knockdown of ERp29 resulted in a reduction of p-p38 (Fig. 1B, right panel). Clearly, these findings indicate that over-expression of ERp29 in MDA-MB-231 cells

markedly induces p38 phosphorylation and decreases FAK expression, leading to a signaling balance that favors p38 over ERK activity to induce a dormant state.

It has been reported that matrix metalloproteinases (MMPs) are the important downstream molecules regulated by uPAR/β1-integrin, p38 and ERK signaling and have critical role in matrix degradation and cell invasion (24, 25). To determine whether the reduced expression of uPAR/β1-integrin and activated p38 in the ERp29-induced dormant cells result in loss of MMP activity, both the vector-transfected control cells and the dormant cells (clone B as an example) were serum starved for 48h and the secretion of MMPs was assessed using gelatin zymography. As shown in Figure 1C, the dormant cells (clone B) showed a significantly decreased (~5.4 fold) secretion of MMP-9 based on the intensity of negatively stained band. However, the secretion of MMP-2 was not affected. These results further support a less invasive capacity of ERp29-induced dormant cells (17).

*p38 activation induces $G_1$ arrest through up-regulating cyclin-dependent kinase inhibitors p15 and p21 and down-regulating cyclin $D_2$* – To further establish the molecular changes in the ERp29-induced dormant cells, the expression of cyclin $D_1$/$D_2$ and cyclin-dependent kinase inhibitors p15 and p21 was examined. Consistent with our early report that transcription of cyclin $D_2$ was highly inhibited by ERp29 (17), we demonstrate that protein expression of cyclin $D_2$ was significantly decreased whereas cyclin $D_1$ was only slightly reduced in the ERp29-induced dormant cells compared to the proliferative mock-transfected control cells (Fig.2 A, left panel). Further, immunofluorescent analysis revealed that cyclin $D_2$ was highly reduced in the nucleus and aggregated to form particles in the cytoplasm, indicating a translocation and aggregation of cyclin $D_2$ in cytoplasm in the ERp29-induced dormant cells (Suppl. Fig. 1B). On the other hand, knockdown of ERp29 in MCF-7 cells resulted in an up-regulation of cyclin $D_2$, with no effect on cyclin $D_1$ (Fig. 2A, right panel). Subsequently we investigated the role of ERp29 in regulating the expression of p15 and p21 which were transcriptionally up-regulated by ERp29 (17). As indicated in Figure 2A, both p15 and p21 were highly expressed in these dormant cells, and knockdown of ERp29 in MCF-7 cells led to a great reduction of p15 and moderate decrease of p21. These results further substantiate the molecular mechanism underlying the ERp29-induced $G_0$/$G_1$ arrest in the dormant cells.

It was reported that p38 activation resulted in inhibition of cyclin $D_1$/$D_2$ in the dormant D-HEp3 cells (8). To further establish whether the enhanced p38 phosphorylation is involved in the ERp29-mediated up-regulation of p15 and down-regulation of cyclin $D_2$ in the ERp29-induced dormant cells, these cells (clone B cells were used as a model) were treated with 40μM of SB203580, a specific inhibitor of p38 phosphorylation (18, 19, 26). After 24h post-treatment, the cells were harvested and the level of protein expression was assessed by Western blotting. As shown in Figure 2B, concurrent with a significant reduction of p38 phosphorylation, the expression of p15 was highly decreased while that of cyclin $D_2$ was markedly increased in the inhibitor-treated dormant cells, compared to the cells treated with DMSO. Clearly, these results indicate that p38 phosphorylation is critical in modulating the expression of these checkpoint proteins in the ERp29-induced $G_1$ arrest cells. .

*Activation of p38 inhibits basal eIF2α expression and enhances eIF2α phosphorylation* – It has been demonstrated that activated p38 functionally links the ER stress signaling in modulating cell dormancy, survival and apoptosis (15). Moreover, activation of eIF2α by phosphorylation induces $G_1$ arrest and cell survival by blocking cyclin $D_1$/$D_2$ translation/stability (13). However, previous studies revealed that over-expression of ERp29 highly inhibited the expression of basal eIF2α, while the eIF2α phosphorylation was not markedly changed (18, Fig. 3A, left panel). This is further supported

by the notion that knockdown of ERp29 in MCF-7 cells increased the expression of basal eIF2 (Fig. 3A, right panel). These data demonstrate a significant role of ERp29 in negatively regulating the expression of basal eIF2α in our dormant cell models. Interestingly, reduction of ERp29 also increase the level of p-eIF2α and the relative phosphorylation of eIF2α was moderately enhanced (~1.76-fold increase) (Fig. 3A, right panel), implicating an activation of PERK/p-eIF2α when ERp29 was reduced in MCF-7 cells (Fig. 3A, right panel). In other words, ERp29 expression attenuates activation of PERK/ p-eIF2α pathway.

Since p38 phosphorylation suppresses cyclin $D_2$ and increases p15 in the ERp29-induced dormant cells (Fig. 2B), we propose that p38 phosphorylation might affect the basal eIF2α expression. To address this possibility, p38 phosphorylation was blocked with inhibitor SB203580 in the dormant cells (clone B). As shown in Figure 3B, when p38 activation was highly reduced in these cells, the expression of basal eIF2α was markedly increased (~3.4-fold), while the relative phosphorylation of eIF2α was significantly reduced when compared to the cells treated with DMSO (0.24 *vs* 1.0, 4.2-fold decrease) (Fig.3B, left panel). Similar scenarios were also observed in MCF-7 cells treated with this inhibitor: reduction of p38 phosphorylation led to 2-fold increase of basal eIF2α and approximately 6.5-fold reduction of eIF2α phosphorylation compared to the cells treated with DMSO (Fig. 3B, right panel). These data suggest that p38 phosphorylation negatively regulates the expression of basal eIF2α, but positively regulates eIF2α phosphorylation.

However an important observation is that highly activated p38 in the ERp29-induced dormant cells (Fig.1B, left panel) was unable to increase eIF2α phosphorylation (18, Fig.3 A, left panel). Furthermore, reduction of p-p38 (Fig.1B, right panel) in the ERp29-knockdown MCF-7 cells was unable to decrease the relative phosphorylation of eIF2α. Instead, relative phosphorylation of eIF2α was moderately increased (Fig. 3A, right panel). Considering these apparently contradictory notions, we propose that other factors that functionally counteract eIF2α phosphorylation by p-p38 might be concomitantly regulated by ERp29 and are responsible for the inhibition of eIF2α phosphorylation in the ERp29-induced dormant cells.

*ERp29-mediated up-regulation of p58$^{IPK}$ inhibits phosphorylation of eIF2α* – Our previous studies demonstrated that over-expression of ERp29 in MDA-MB-231 cells led to activation of XBP-1 (18). One of the ER proteins that are specifically regulated by XBP-1 is p58$^{IPK}$ (27). p58$^{IPK}$, an inhibitor of protein kinase, can directly bind to PERK to inhibit PERK phosphorylation, resulting in inhibition of eIF2α phosphorylation (28, 29). We then examined the expression of p58$^{IPK}$ in the dormant cells and investigated its role in regulating eIF2α phosphorylation. As shown in Figure 4A, p58$^{IPK}$ was markedly up-regulated (2.84-fold) in the ERp29-induced dormant cells (clone B), and knockdown of ERp29 in MCF-7 cells highly reduced its expression. Significantly, when the up-regulated p58$^{IPK}$ was silenced with siRNA in the dormant cells (clone B), phosphorylation of eIF2α was restored (3.2-fold increase) without highly affecting the expression of basal eIF2α (Fig. 4B, left panel). Similar results were also observed in MCF-7 cells in which silencing of p58$^{IPK}$ markedly stimulated eIF2α phosphorylation by 2.56-fold increase (Fig. 4B, right panel). These results suggest that the up-regulated p58$^{IPK}$ plays a critical role in inhibiting eIF2α phosphorylation in the ERp29-induced dormant cells.

In addition to p58$^{IPK}$, we also observed that expression of basal PERK was highly increased whereas phosphorylation of PERK was weakly detectable in these dormant cells (clone B) as compared to the control cells (Fig. 4C, left panel). It is reasonable that inactivation of PERK is mostly due to the increased level of p58$^{IPK}$ which binds to PERK and represses PERK phosphorylation (29).

Over-expression of ERp29 increased the expression of Bip/GRP78 in the dormant cells (Fig. 4C, middle panel). However, knockdown of ERp29 in MCF-7 cells also resulted in an increase of Bip/GRP78 (Fig. 4C, right panel). Clearly, there is no direct mechanistic link between these two molecules. Furthermore, expression of eIF2α phosphorylation-dependent transcription factors ATF4 was not changed in the ERp29-induced dormant cells (Fig. 4C, middle panel). In contrast, knockdown of ERp29 in MCF-7 cells activated the expression of ATF4 (Fig. 4C, right panel), consistent with the enhanced eIF2α phosphorylation in the ERp29-knockdown MCF-7 cells (Fig. 3A, right panel). These results indicate that the ER-dependent pro-apoptotic signaling is markedly suppressed by ERp29 in these dormant cells.

*Silencing of p58$^{IPK}$ activates p-eIF2α/ATF4/CHOP pathway in the ERp29-induced dormant cells* – The aforementioned data demonstrated that over-expression of ERp29 up-regulates p58$^{IPK}$, leading to inhibition of eIF2α phosphorylation. Given that silencing of p58$^{IPK}$ in the ERp29-induced dormant cells re-activates phosphorylation of eIF2α (Fig. 4B, left panel), reduction of p58$^{IPK}$ might be responsible for activation of eIF2α phosphorylation-dependent ATF4/CHOP pro-apoptotic pathway. As expected, silencing of p58$^{IPK}$ in dormant cells (clone B) led to a significant increase of both ATF4 and CHOP expression (Fig. 5A, left panel). Consistently, similar results were also achieved in MCF-7 cells in which silencing of p58$^{IPK}$ greatly increased the expression of ATF4 and CHOP (Fig. 5A, right panel). In consistent with previous studies that p58$^{IPK}$ over-expression decreased eIF2α phosphorylation-dependent expression of ATF4/CHOP (28), our data further demonstrate that p58$^{IPK}$ is a key regulator of the p-eIF2α/ATF4/CHOP pathway and its up-regulation is crucial in maintaining the ERp29-induced cell survival.

*Silencing p58$^{IPK}$ interrupts dormancy and induces cell apoptosis* – Previous studies have shown that over-expression of ERp29 in the ERp29-induced dormant cells markedly increased the resistance to doxorubicin while knock-down of ERp29 in MCF-7 cells sensitized to it (18). Furthermore, the above data demonstrate an important role of p58$^{IPK}$ in inhibiting eIF2α phosphorylation and pro-apoptotic signaling in these dormant cell models. We next examined if the up-regulated p58$^{IPK}$ plays a critical role in the ERp29-mediated cell survival and drug resistance.

To determine whether silencing p58$^{IPK}$ results in sensitivity to doxorubicin and causes cell death, the ERp29-induced dormant cells and MCF-7 cells were respectively treated with p58$^{IPK}$ siRNA or scramble control siRNA for 24h, followed by 24h treatment with doxorubicin (200ng/ml). The viability of these treated cells was examined. As indicated in Figure 5A, the dormant cells (clone B) and MCF-7 cells treated with p58$^{IPK}$ siRNA showed an enhanced expression of cleaved caspase-3, indicating an activation of cell apoptosis after p58$^{IPK}$ silencing. Further studies showed that these p58$^{IPK}$ siRNA-treated cells had a highly decreased cell viability compared to the cells treated with control siRNA (Fig. 5B, column 3 *vs* 1; column 7 *vs* 5). When these siRNA-treated cells including those treated with control siRNA were further exposed to doxorubicin, the dormant cells pre-treated with p58$^{IPK}$ siRNA showed a 3.0-fold reduction of cell viability over the dormant cells pre-treated with control siRNA (column 4 *vs* 2), suggesting that silencing of p58$^{IPK}$ significantly sensitized these dormant cells to this drug. A similar effect was also observed in MCF-7 cells where repression of p58$^{IPK}$ enhanced the cytotoxicity to doxorubicin, leading to approximately 2.5-fold decrease of viable cells compared to the cells pre-treated with control siRNA (Fig. 5B, column 8 *vs* 6). As a result, silencing of p58$^{IPK}$ in the ERp29-induced dormant cells disrupts the dormant state and increases cell death by activating ATF4/CHOP/Caspase-3 apoptotic signaling.

## DISCUSSION

Tumor cell dormancy is a critical step for tumor cells to escape from radiotherapeutic and chemotherapeutic treatment in cancer patients. It is therefore clinically important to understand the mechanisms underlying tumor cell dormancy to prevent cancer recurrence. In the present study, we demonstrated that ERp29 is one of the potential factors driving proliferative tumor cells into dormancy in breast cancer. In the ERp29-induced dormant cell model, we provide evidence that over-expression of ERp29 significantly reduced the expression of uPAR, $\beta_1$-integrin and EGFR, the essential players in the decision between tumor cell proliferation and dormancy (7). Reduction of this complex leads to attenuation of FAK/p-ERK and activation of p38 and consequently propels tumor cells into quiescence by down-regulating eIF2α expression, reducing cyclin $D_2$ and increasing p15 and p21. Importantly, we demonstrate that the up-regulated $p58^{IPK}$ in the dormant cells plays a critical role in attenuating eIF2α phosphorylation and inhibiting ATF4/CHOP/Caspase-3 pro-apoptotic pathway, leading to an enhanced cell survival.

The role of uPAR/$\beta_1$-integrin/EGFR complex and its downsteam molecules (e.g., FAK and p38) in modulating tumor cell dormancy has been well described in dormant squamous tumor cell models (2, 8, 9, 16, 23). The current studies in our established ERp29-induced dormant cells further support the significance of loss of the uPAR/$\beta_1$-integrin /EGFR complex in tumor cell dormancy by activating p38 phosphorylation and down-regulating FAK. Phosphorylation of p38 due to the loss of this complex has been demonstrated to be a central regulator in regulating cell dormancy, survival and apoptosis (3, 30). A significant link of p38 phosphorylation with cell cycle arrest at $G_0/G_1$ phase and cell survival *via* ER stress was mechanistically established by the fact that activation of p38 enhanced PERK/p-eIF2α signaling by stimulating phosphorylation of eIF2α (14), leading to induction of $G_0/G_1$ arrest by down-regulating cyclin $D_1$ (12, 13). Interestingly, in our established ERp29-induced dormant cell models, the expression of basal eIF2α was highly inhibited without highly affecting its phosphorylation (Fig. 3A, left panel). In addition, the expression of cyclin $D_2$, but not $D_1$, was highly decreased (Fig. 2A), and the cyclin-dependent kinase inhibitors p15 and p21 were markedly increased (Fig. 2A). These data may reveal a novel mechanism of ERp29 in driving proliferative cells into $G_1$ arrest and cell dormancy.

We demonstrate a critical role of p38 phosphorylation in the ERp29-mediated $G_1$ arrest. This is reflected by the fact that inhibition of p38 phosphorylation by pharmacological inhibitor in the ERp29-induced dormant cells reversed the expression of cyclin $D_2$ and inhibited the expression of p15 (Fig. 2B). Meanwhile, we also demonstrate that inhibition of p38 phosphorylation restored the level of basal eIF2α (Fig. 3B), indicating the importance of p38 phosphorylation in negatively regulating the basal eIF2α expression in the ERp29-induced dormant cells. C-JUN/AP-1 is one of the transcription factors regulating eIF2α expression (31). Transcription factor profile analysis revealed that c-JUN/AP-1 is one of the key transcription factors that are negatively regulated by p38 phosphorylation (16, 32). It was found to be reduced in dormant D-HEp3 cells (16) and similarly in the ERp29-induced dormant tumor cells (17). The ERp29-mediated down-regulation of basal eIF2α is mostly due to the inhibition of c-Jun via activation of p38. Collectively, the ERp29-mediated activation of p38 negatively regulates eIF2α expression.

ER stress signaling is an important determinant in deciding cell dormancy, survival and autophagy (11, 15, 33). Over-expression of ERp29 in the ERp29-induced dormant cells leads to activation of ER stress by stimulating a splicing of transcription factor XBP-1 (18). In contrast to an

enhanced phosphorylation of eIF2α, a mechanism leading to attenuation of general protein synthesis and activation of ATF4/CHOP under ER stress (34), over-expression of ERp29 is unable to increase eIF2α phosphorylation (18, Fig. 3A), although p38 is highly activated (Fig. 1B) and PERK is highly expressed (Fig. 4C) in the ERp29-induced dormant cells. PERK is one of the specific kinases that phosphorylate eIF2α (13). Activation of PERK induces cell survival in response to ER stress (35) and suppresses autophagy and lysosomal dysfunction (36). In the ERp29-induced dormant cells, expression of the key regulators including p-p38 and PERK are significantly increased, while eIF2α phosphorylation is not further improved. This may implicate that dephosphorylation of eIF2α that counteracts the phosphorylation exerted by its upstream kinases is concomitantly stimulated in the ERp29-induced dormant cells.

We reported that $p58^{IPK}$, a downstream molecule of XBP-1 activation (27), is significantly up-regulated in the ERp29-induced dormant cells. $p58^{IPK}$ was originally identified as a cellular inhibitor of the interferon-induced serine/threonine protein kinase (PKR) which is recruited by influenza virus to inhibit PKR function in eIF2α phosphorylation through a direct interaction with PKR (37, 38). Recent study further demonstrated that $p58^{IPK}$ can interact with and inhibit the PERK phosphorylation, leading to a decreased phosphorylation of eIF2α (29). In the ERp29-induced dormant cells, silencing of $p58^{IPK}$ stimulated eIF2α phosphorylation and activated the expression of ATF4/CHOP and cleavage of caspase-3 (Fig. 5A), leading to an increased cell apoptosis. This is further supported by the early studies that eIF2α phosphorylation mediated stress-induced apoptosis (39). Therefore, up-regulation of $p58^{IPK}$ may facilitate cell survival under stress in the ERp29-induced dormant cells by repressing eIF2α phosphorylation. It is well known that GADD34, a downstream target of the activated p-eIF2α, also inhibits eIF2α phosphorylation by feedback loop (40). Nevertheless, it should be noted that induction of $p58^{IPK}$ is directly regulated by ATF6 or IRE1/XBP-1-mediated arm of the UPR and is an upstream molecule that can directly bind to PERK to inactivate PERK/p-eIF2α (27, 41). Our studies provide a clue that induction of $p58^{IPK}$ by ERp29 may help to protect cells from the p-p38-activated excessive ER stress by de-phosphorylating eIF2α (42). As such, disturbing the signaling balance of p-p38-activated PERK/p-eIF2α and $p58^{IPK}$-repressed PERK/p-eIF2α at the steady state could impair the dormancy in the ERp29-induced dormant cells. As demonstrated, activation of p-eIF2α/ATF4/CHOP pro-apoptotic pathway by silencing $p58^{IPK}$ correlated to the increased sensitivity of dormant cells to doxorubicin treatment (Fig. 5B). Collectively, our data indicate that $p58^{IPK}$ is an important coordinator in modulating the crosstalk between the ERp29-mediated ER stress and ER-dependent pro-apoptotic signaling by inhibiting eIF2α phosphorylation in the ERp29-induced dormant cells.

In summary, we demonstrate the molecular mechanism underlying the ERp29-induced breast tumor cell dormancy. As illustrated in Figure 6, over-expression of ERp29 down-regulates the expression of uPAR/$\beta_1$-integrin/EGFR and subsequently activates p38 phosphorylation. Activation of p38 inhibits basal eIF2α expression, reduces cyclin $D_2$ and increases p15 and p21, leading to $G_1$ arrest. On the other hand, the up-regulated $p58^{IPK}$ in the ERp29-induced dormant cells suppresses the activation of p-p38/p-PERK/ p-eIF2α by repressing eIF2α phosphorylation, thus preventing cells from undergoing an ER-dependent apoptosis driven by extensive activation of ATF4/CHOP/caspase-3 pathway. Consequently, p38 phosphorylation and $p58^{IPK}$ up-regulation play key roles in modulating the ERp29-induced cell dormancy and survival.

## REFERENCES


1. Naumov, G. N., Townson, J. L., MacDonald, I. C., Wilson, S. M., Bramwell, V. H., Groom, A. C., and Chambers, A. F. (2003) *Breast Cancer Res. Treat* **82**, 199-206.
2. Yu, W., Kim, J., and Ossowski, L. (1997) *J. Cell Biol*.**137**, 767-77.
3. Aguirre-Ghiso, J. A. (2007) *Nat. Rev. Cancer* **7**, 834-46.
4. Almog, N. (2010) *Cancer Lett.* **294,** 139-46.
5. Udagawa, T. (2008) *APMIS* **116**, 615-28.
6. Ranganathan, A. C., Adam, A. P., and Aguirre-Ghiso, J. A. (2006) *Cell Cycle* **5**, 1799-807.
7. Allgayer, H., and Aguirre-Ghiso, J. A. (2008) *APMIS* **116**, 602-14.
8. Aguirre Ghiso, J. A., Kovalski, K., and Ossowski, L. (1999) *J. Cell Biol*. **147**, 89-104.
9. Aguirre-Ghiso, J. A., Liu, D., Mignatti, A., Kovalski, K., and Ossowski, L. (2001) *Mol. Biol. Cell* **12,** 863-79.
10. Schewe, D. M., and Aguirre-Ghiso, J. A. (2008) *Proc. Natl. Acad. Sci. USA* **105**, 10519-24.
11. Wang, G., Yang, Z. Q., and Zhang, K. (2010) *Am. J. Transl. Res*. **2**, 65-74.
12. Rutkowski, D. T., and Kaufman, R. J. (2004) *Trends Cell Biol.* **14**, 20-8.
13. Brewer, J. W., and Diehl, J. A. (2000) *Proc. Natl. Acad. Sci. USA*. **97**, 12625-30.
14. Ranganathan, A. C., Zhang, L., Adam, A. P., and Aguirre-Ghiso, J. A. (2006) *Cancer Res.* **66**, 1702-11
15. Ranganathan, A. C., Adam, A. P., Zhang, L., and Aguirre-Ghiso, J. A. (2006) *Cancer Biol. Ther*. **5**, 729-35.
16. Adam, A. P., George, A., Schewe, D., Bragado, P., Iglesias, B.V., Ranganathan, A. C., Kourtidis, A., Conklin, D. S., and Aguirre-Ghiso, J. A. (2009) *Cancer Res*. **69**, 5664-72.
17. Bambang, I F., Xu, S., Zhou, J., Salto-Tellez, M., Sethi, S. K., and Zhang, D. (2009) *Lab. Invest*. **89**, 1229-42.
18. Zhang, D., and Putti, T. C. (2010) *Exp. Cell Res*. **316**, 3522-31.
19. Wong, L. L., Chang, C. F., Koay, E. S., and Zhang, D. (2009) *Int. J. Oncol*. **34**, 1291-301.
20. Ahmad, A., Kong, D., Wang, Z., Sarkar, S. H., Banerjee, S., and Sarkar, F. H. (2009) *J. Cell Biochem*. 108, 916-25.
21. Kunigal, S., Lakka, S. S., Gondi, C. S., Estes, N., and Rao, J. S. (2007) *Int. J. Cancer* **121**, 2307-16.
22. Hsu, T., Trojanowska, M., and Watson, D. K. (2004) *J. Cell Biochem*. **91**, 896-90.
23. Aguirre Ghiso, J. A. (2002) *Oncogene* **21**, 2513-24.
24. Ahmed, N., Oliva, K., Wang, Y., Quinn, M., and Rice, G. (2003) *Br. J. Cancer* **89**, 374-84.
25. Loesch, M., Zhi, H. Y., Hou, S. W., Qi, X. M., Li, R. S., Basir, Z., Iftner, T., Cuenda, A., and Chen, G. (2010) *J. Biol. Chem*. **285**, 15149-58.
26. Kim, M. S., Lee, E. J., Kim, H. R., and Moon, A. (2003) *Cancer Res*. **63**, 5454-61.
27. Lee, A. H., Iwakoshi. N. N., and Glimcher, L. H. (2003) *Mol. Cell Biol.* **23**, 7448-59.
28. van Huizen, R., Martindale, J. L., Gorospe, M., and Holbrook, N. J. (2003). *J. Biol.*

*Chem*. **278**, 15558-64**.**
29. Yan, W., Frank, C. L., Korth, M. J., Sopher, B. L., Novoa, I., Ron, D., and Katze, M. G. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 15920-5.
30. Bulavin, D. V., and Fornace, A. J. Jr. (2004) *Adv. Cancer Res*. **92**, 95-118.
31. Ferreiro, I., Joaquin, M., Islam, A., Gomez-Lopez, G., Barragan, M., Lombardía, L., Domínguez, O., Pisano, D. G., Lopez-Bigas, N., Nebreda, A. R., and Posas, F. (2010) *BMC Genomics* **11**, 144.
32. Hümbelin, M., Safer, B., Chiorini, J. A., Hershey, J. W., and Cohen, R. B. (1989) *Gene* **81**, 315-24.
33. Verfaillie, T., Salazar, M., Velasco, G., and Agostinis, P. (2010) *Int. J. Cell Biol*. **2010**, 930509.
34. Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Lu, P. D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D. F., Bell, J. C., Hettmann, T., Leiden, J. M., and Ron, D. (2003) *Mol. Cell* **11**, 619-33.
35. Harding, H. P., Zhang, Y., Bertolotti, A., Zeng, H., and Ron, D. (2000) *Mol. Cell* **5**, 897-904
36. Park, M. A., Curiel, D. T., Koumenis, C., Graf, M., Chen, C. S., Fisher, P. B., Grant, S., and Dent, P. (2008) *Autophagy* **4**, 364-7
37. Lee, T. G., Tomita, J., Hovanessian, A. G., and Katze, M. G. (1992) *J. Biol. Chem*. **267**, 14238-43.
38. Polyak, S. J., Tang, N., Wambach, M., Barber, G. N., and Katze, M. G. (1996) *J. Biol. Chem*. **271**, 1702-7.
39. Srivastava, S. P., Kumar, K. U., and Kaufman, R. J. (1998) *J. Biol. Chem.* **273**, 2416-23.
40. Novoa, I., Zeng, H., Harding, H. P., and Ron, D. (2001) *J. Cell Biol*. **153**, 1011-22.
41. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001) *Cell* **107**, 881-91
42. Rutkowski, D. T., Kang, S. W., Goodman, A. G., Garrison, J. L., Taunton, J., Katze, M. G., Kaufman, R. J., and Hegde, R. S. (2007) *Mol. Biol. Cell* **18**, 3681-91.

# FOOTNOTES

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[2]The abbreviations used are: ERp29, endoplasmic reticulum protein 29; uPAR, urokinase receptor; EGFR, epidermal growth factor receptor; MMP-9, matrix metalloproteinase 9; UPR, unfolded protein response; eIF2α, eukaryotic translation initiation factor 2 alpha; p-eIF2α, phosphorylated eIF2α; ATF4, activating transcription factor 4; GADD153/CHOP, growth arrest and DNA damage-inducible protein; PERK, PKR-like endoplasmic reticulum kinase;

IRE-1, inositol requiring enzyme-1; XBP-1, X-box binding protein 1; FAK, focal adhesion kinase; ERK, extracellular signal-regulated kinase; p38, mitogen-activated protein kinase 14; SB, SB203580; Ets-1, v-ets erythroblastosis virus E26 oncogene homolog 1; DMSO, dimethyl sulfoxide; siRNA, small interfering RNA; c-JUN; **jun** proto-oncogene/ transcription factor AP-1; Bip/GRP78, glucose-regulated protein, 78kDa.

# FIGURE LEGENDS

FIGURE 1. Over-expression of ERp29 decreases the level of uPAR/β1-integrin/EGFR complex and activates p38 phosphorylation. A, The uPAR/β1-integrin/EGFR complex is highly reduced in the ERp29-induced dormant cells (clone B and E) (left panel). In the dormant cells, Ets-1, a transcription factor of uPAR, was markedly reduced by ERp29 (right panel), leading to a down-regulation of uPAR. B, Over-expression of ERp29 in MDA-MB-231 cells (left panel) attenuates the expression of FAK and basal p38, but significantly stimulates p38 phosphorylation. Knock-down of ERp29 in MCF-7 cells (right panel) increases expression of basal p38 while reduces its phosphorylation. Total proteins from the vector-transfected control cells and ERp29-over-expressed dormant cells (clone B and E) were extracted and the expression of the indicated proteins was examined by Western blotting as described in "Materials and Methods". The level of β-actin was used as a loading control. C, Zymography of MMPs. Cells were serum-starved for 24 h and the secreted MMPs were assayed by gelatin zymography as described in "Materials and Methods". Note that MMP-9 activity is significantly reduced in the dormant cells (clone B).

FIGURE 2. ERp29 results in cell cycle G1 arrest by affecting checkpoint protein expression. A, Over-expression of ERp29 significantly decreases cyclin D2 expression and increases p15 and p21 expression (left panel) while silencing of ERp29 in MCF-7 cells reverses the expression patterns of these molecules (right panel), as assessed by Western blotting. B, p38 phosphorylation involves the ERp29-mediated down-regulation of cyclin D2 and up-regulation of p15 in dormant cells. The ERp29-induced dormant cells (clone B) were treated with p38 phosphorylation-specific inhibitor SB203580 (40μM) or DMSO (control) for 24h and the expression of proteins was analyzed by Western blotting as described in "Materials and Methods". The cells treated with inhibitor show a decreased p38 phosphorylation, increased cyclin D2 and reduced p15 expression compared to the cells treated with DMSO. SB, SB203580.

FIGURE 3. p38 phosphorylation involves the ERp29-mediated down-regulation of eIF2α. A, Expression of basal eIF2α is highly decreased while the relative phosphorylation of eIF2α (p-eIF2α/eIF2α) is similar in the dormant cells (clone B). Knockdown of ERp29 in MCF-7 cells results in an up-regulation of basal eIF2α and a moderate increase of the phosphorylation of eIF2α. B, Inhibition of p38 phosphorylation by inhibitor SB203580 increases expression of basal eIF2α, but dramatically reduces its phosphorylation in both ERp29-induced dormant cells and MCF-7

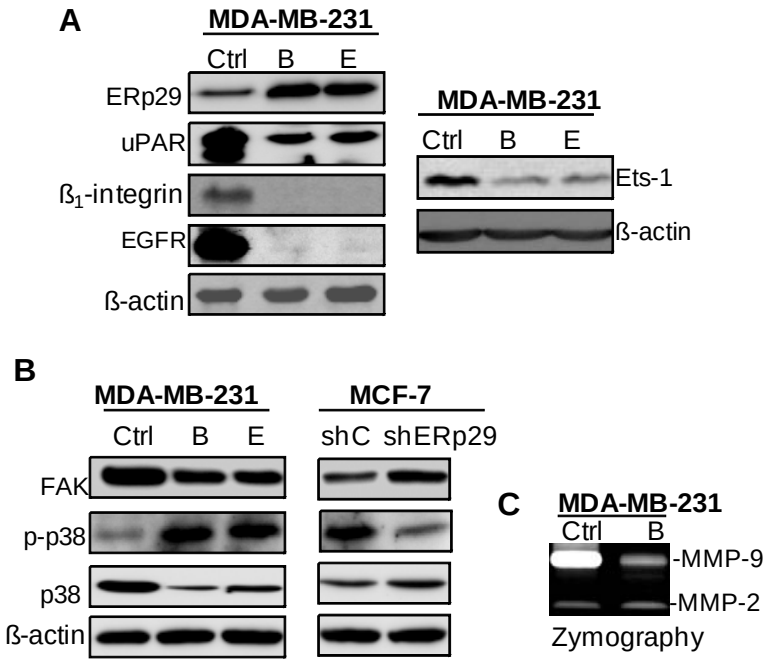
cells compared to the DMSO-treated control cells. Cells were respectively treated with DMSO and inhibitor following the same procedure as described in Figure 2 and the expression of proteins was assessed by Western blotting. Data represent an average of fold changes of relative phosphorylation of eIF2α normalized to basal eIF2α from three independent experiments. SB, SB203580.

FIGURE 4. ERp29 regulates the expression of p58IPK, PERK and ATF4. A, ERp29 highly up-regulates p58IPK. Data represent a fold change of p58IPK after normalized to β-actin, compared to the control cells. B, Effect of ERp29 on other molecules that response to UPR. Immunoblot analysis shows an up-regulation of PERK and BIP in the ERp29-induced dormant cells. However, BIP is also significantly expressed in the ERp29-knockdown MCF-7 cells (right panel). ATF4 expression is not highly increased in dormant cells, but instead, it is markedly increased in the ERp29-knockdown MCF-7 cells, implicating an activation of eIF2α downstream signal.

FIGURE 5. Silencing of p58IPK stimulates eIF2α phosphorylation, activates eIF2α phosphorylation-dependent ATF4/CHOP pro-apoptotic pathway and increases doxorubicin-induced cell apoptosis. A, Silencing of p58IPK enhances eIF2α phosphorylation. The ERp29-induced dormant cells (clone B) and MCF-7 cells were respectively treated with 100nM of p58IPK-specific siRNA (p58IPK/siRNA) or scramble siRNA for 24h and the expression of proteins was analyzed by Western blotting. As indicated, reduction of p58IPK by siRNA markedly increased the phosphorylation of eIF2α without highly affecting the level of basal eIF2α. Data represent a fold change of relative phosphorylation of eIF2α in the p58IPK/siRNA-treated cells normalized to the control cells. B, Silencing of p58IPK activates ATF4\CHOP pathway and increases expression of cleaved caspase-3. The ERp29-induced dormant cells and MCF-7 cells were treated as described above and the indicated protein expression was analyzed. The cleavage caspase-3 (17 and 12 kDa) was expressed in the p58IPK/siRNA treated cells. C, Silencing of p58IPK sensitizes both the ERp29-induced dormant cells and MCF-7 cells to DOX. Cells were treated with p58IPK /siRNA or scramble siRNA for 24h, followed by treatment with DOX (final conc. 200μg/ml) or DMSO for 24h. Cell viability was assessed using MTS as described in "Materials and Methods". As indicated, p58IPK silence and DOX synergistically increased cell death in both types of cells. Data represent a mean of three independent experiments. Bars: standard deviation (SD); DOX, doxorubicin. * $p< 0.01$; ** $p< 0.001$

FIGURE 6. **Novel molecular mechanism of ERp29-induced cancer cell dormancy.** In the ERp29-induced dormant cells, uPAR/β1-integrin/EGFR complex is highly decreased, leading to activation of p38. p38 phosphorylation inhibits the expression of basal eIF2α and cyclin $D_2$ and increases the expression of p15 and p21, thus causing $G_1$ arrest. On the other hand, over-expression of ERp29 up-regulates $p58^{IPK}$ by enhancing XBP-1 splicing. The up-regulated $p58^{IPK}$ functionally counteracts the up-stream kinases of eIF2α to inhibit eIF2α phosphorylation and attenuate activation of p-PERK/p-eIF2α pathway. Reduction of $p58^{IPK}$ by RNA interference (siRNA) leads to activation of eIF2α phosphorylation-dependent ATF4/CHOP/cleaved caspase-3

pathway and cell apoptosis. Activation of p38 and up-regulation of $p58^{IPK}$ are the critical molecular events in maintaining the ERp29-induced cell dormancy. XBP-1u: unspliced XBP-1; XBP-1s; spliced XBP-1. XBP-1 is activated by deleting 26 nucleotides by a protein kinase and endoribonuclease, inositol-requiring 1α (IRE1α).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Zymography

Fig 1

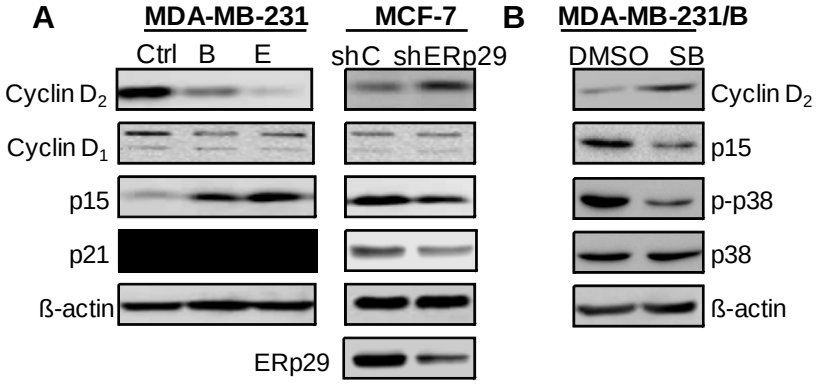
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Fig 2

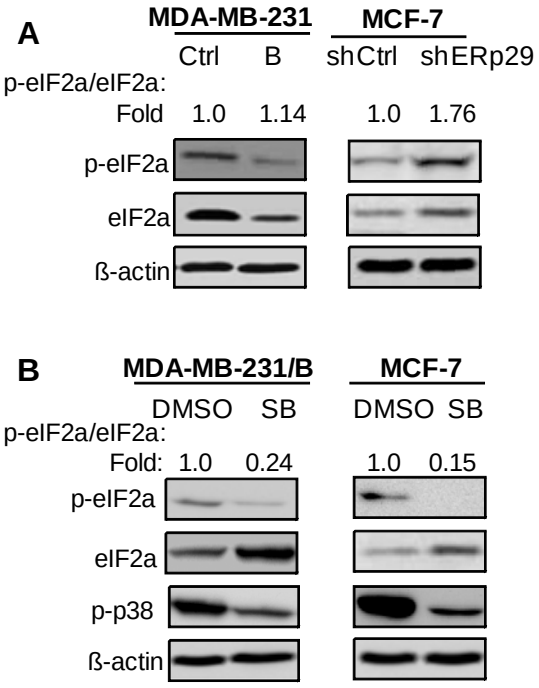
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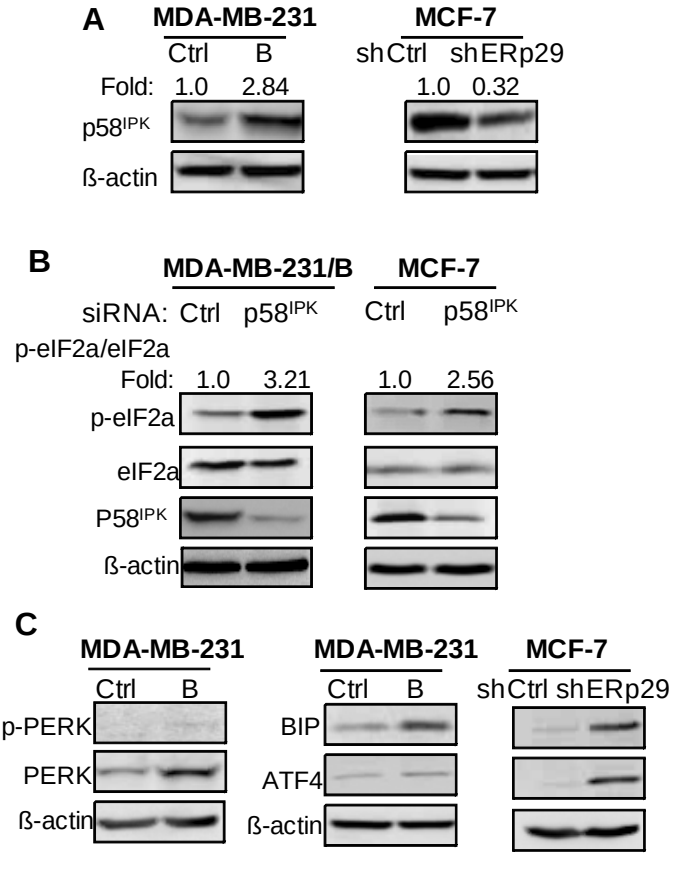
Fig 3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MCF-7
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p-eIF2a/eIF2a
Fold: 1.0 3.21
1.0 2.56
p-eIF2a
eIF2a
P58IPK
ß-actin
C
MDA-MB-231
MDA-MB-231
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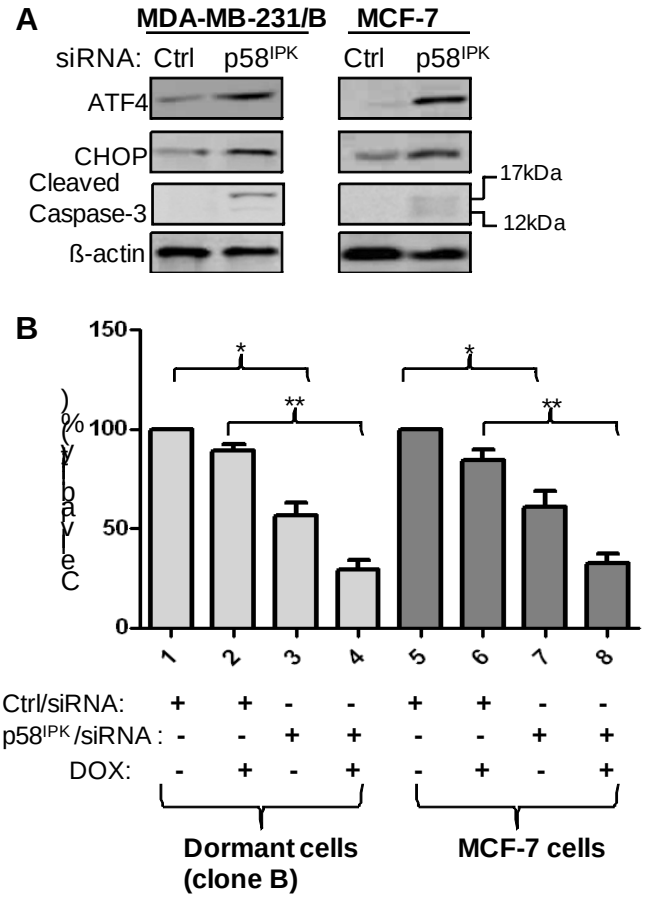
Fig 4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17kDa
12kDa
ß-actin
B
150
100
50
0
*
**
*
**
1 2 3 4 5 6 7 8
Ctrl/siRNA: + + - - + + - -
p58IPK/siRNA : - - + + - - + +
DOX: - + - + - + - +
Dormant cells (clone B)
MCF-7 cells

Fig 5

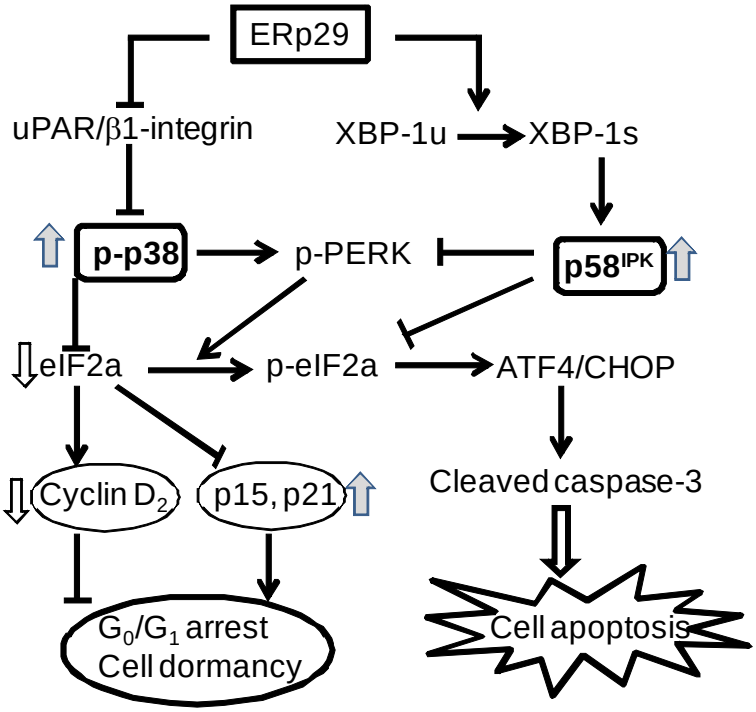
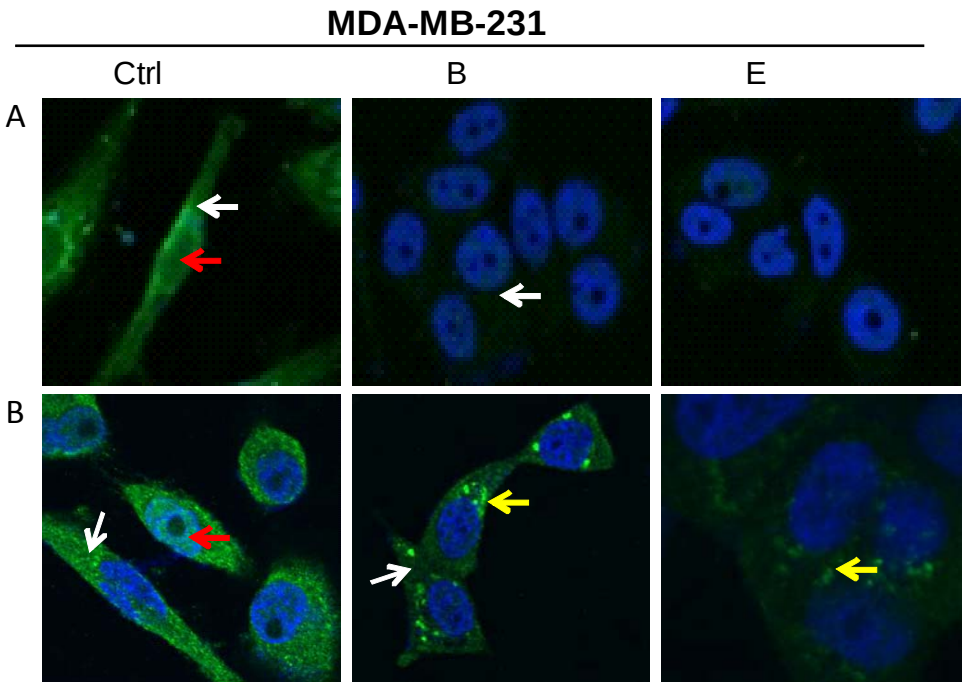

ERp29
uPAR/β1-integrin
XBP-1u
XBP-1s
p-p38
p-PERK
p58IPK
eIF2a
p-eIF2a
ATF4/CHOP
Cyclin D2
p15, p21
Cleaved caspase-3
G0/G1 arrest
Cell dormancy
Cell apoptosis

Fig. 6

Supplementary Figure 1




Supplementary Figure 1. **Immunofluorescent analysis of the expression of Ets-1 and cyclin $D_2$.** ***A***, Ets-1 (green) is expressed in both nucleus (red arrow) and cytoplasm (white arrow) in the vector-transfected MDA-MB-231 control cells. It is highly reduced in both nucleus and cytoplasm in the ERp29-induced dormant cells (clone B and E). ***B***, Cyclin $D_2$ (green) is expressed in both nucleus (red arrow) and cytoplasm (white arrow) in the vector-transfected MDA-MB-231 control cells and is translocated from nucleus to cytoplasm to form aggregation (yellow arrow) in the ERp29-induced dormant cells (clone B and E). Nucleus are stained with DAPI (blue).