MECHANISMS OF THE DOWNREGULATION OF PROLACTIN RECEPTOR AND THEIR ROLE IN CELL PROLIFERATION

Bentley Varghese

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Supervisor of Dissertation

Signature _____

Dr. Serge Y. Fuchs, M.D., Ph.D., Professor, Cell Biology

Graduate Group Chairperson

Signature_____

Dr. Daniel S. Kessler, Ph.D., Associate Professor, Cell and Developmental Biology

Dissertation Committee

Dr. J. Alan Diehl, Ph.D., Professor, Cancer Biology

Dr. Roger A. Greenberg, M.D., Ph.D., Assistant Professor, Cancer Biology

Dr. Anil K. Rustgi, M.D., T. Grier Miller Professor, Chief of Gastroenterology, Medicine

Dr. Andrei Thomas-Tikhonenko, Ph.D. Associate Professor, Pathology and Lab. Med.

ABSTRACT

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Bentley Varghese

Dr. Serge Y. Fuchs, M.D., PhD.

Cells react to diverse stimuli by expressing specific receptors that recognize these stimuli and initiate specific signaling pathways that enable a cell to change with the environment. Downregulation of these signaling receptors represents the most direct method for limiting the magnitude and duration of downstream signal transduction. For cell surface transmembrane receptors, ligand-stimulated endocytosis is a major mechanism by which the ability of a cell to react to a ligand is restricted. In order to investigate the downregulation of the prolactin receptor (PRLr), we investigated the mechanism and key determinants in the endocytosis and downregulation of PRLr. In **Chapter 2**, we show that the endocytosis of PRLr is a ligand-induced process which requires the catalytic activity of the constitutively bound Janus kinase Jak2. In **Chapter 3**, we show that PRLr is internalized by a clathrin-dependent mechanism which requires phosphorylation of the conserved phosphodegron motif ($DS^{349}GRGS$) at Ser 349 and an active $SCF^{\beta\text{-}TrCP}$ E3 ligase complex. Optimal PRLr endocytosis is shown to be achieved via K63-linked polyubiquitination of the receptor. In **Chapter 4**, we show that PRL signaling promotes

cell growth in 2-D and 3-D culture systems where PRLr levels are increased/stabilized. In **Chapter 5**, we identify pyruvate kinase M2 (PKM2), a glycolytic enzyme whose role in tumorigenesis has been described, to be a novel interactor of PRLr. We show that prolactin (PRL) signaling works to inhibit PKM2 activity by the propagation of tyrosine-phosphorylated proteins. This inhibition of PKM2 prevents progression through glycolysis and allows PKM2 to take a pro-tumorigenic role. We show that the interaction between PRL signaling and PKM2 is required for optimal prolactin-dependent cell growth. In this thesis (model shown in Model 1), we show that there is a defined mechanism of PRLr downregulation which works to limit PRL signaling. If this mode of receptor downregulation is not properly executed, it can result in aberrant signaling whereby prolactin-mediated inhibition of pyruvate kinase M2 mediates the pro-tumorigenic effect of prolactin.

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Chapter 1

Introduction

1.1 Abstract

Cells react to diverse stimuli by expressing specific receptors that recognize these stimuli and initiate specific signaling pathways that enable a cell to change with the environment. Downregulation of these signaling receptors represents the most direct method for limiting the magnitude and duration of downstream signal transduction. For cell surface transmembrane receptors, ligand-stimulated endocytosis is a major mechanism by which the ability of a cell to react to a ligand is restricted. In order to investigate the downregulation of the prolactin receptor (PRLr), we investigated the mechanism and key determinants in the endocytosis and downregulation of PRLr. In **Chapter 2**, we show that the endocytosis of PRLr is a ligand-induced process which requires the catalytic activity of the constitutively bound Janus kinase Jak2. In **Chapter 3**, we show that PRLr is internalized by a clathrin-dependent mechanism which requires phosphorylation of the conserved phosphodegron motif ($DS^{349}GRGS$) at Ser 349 and an active $SCF^{\beta\text{-}TrCP}$ E3 ligase complex. Optimal PRLr endocytosis is shown to be achieved via K63-linked polyubiquitination of the receptor. In **Chapter 4**, we show that PRL signaling promotes cell growth in 2-D and 3-D culture systems where PRLr levels are elevated/stabilized. In **Chapter 5**, we identify pyruvate kinase M2 (PKM2), a glycolytic enzyme whose role in tumorigenesis has been described, to be a novel interactor of PRLr. We show that prolactin (PRL) signaling works to inhibit PKM2 activity by the propagation of

tyrosine-phosphorylated proteins. This inhibition of PKM2 prevents progression through glycolysis and allows PKM2 to take a pro-tumorigenic role. We show that the interaction between PRL signaling and PKM2 is required for optimal prolactin-dependent cell growth. In this thesis (model shown in Model 1), we show that there is a defined mechanism of PRLr downregulation which works to limit PRL signaling. If this mode of receptor downregulation is not properly executed, it can result in aberrant signaling whereby prolactin-mediated inhibition of pyruvate kinase M2 mediates the pro-tumorigenic effect of prolactin.

1.2 Introduction

PRLr is a homodimerized receptor that elicits intracellular effects through engaging the peptide hormone prolactin (PRL). Upon ligand binding, PRLr undergoes a conformational change which leads to activation of several kinases including protein tyrosine kinases (JAK2 and Src), serine/threonine kinases (such as Erk and Nek3), and lipid phosphoinositol 3 kinase. Depending on the context, this signal transduction program can result in normal mammary gland development and lactation or promote uncontrolled proliferation and increased survival of breast epithelium that contributes to a malignant phenotype (Clevenger et al., 2003; Clevenger et al., 2009; Clevenger et al., 2008; Swaminathan et al., 2008a; Wagner & Rui, 2008). Examples of altered PRL-PRLr action in human breast cancers include elevated levels of PRL (Hankinson SE et al., 1999; Tworoger SS et al., 2004; Tworoger SS and Hankinson SE, 2008), accumulation of PRLr due to its impaired

proteolysis (Li et al., 2006; Plotnikov et al., 2008; Plotnikov et al., 2009; Swaminathan et al., 2008a), and mutations that yield a constitutively active PRLr (Bogorad RL et al., 2008; Canbay E et al., 2004).

Endocytosis of signaling receptors is a major mechanism used by cells to restrict the magnitude and duration of signal transduction induced by extracellular ligands. Often times, receptor-mediated signaling can result in a feedback loop which limits signaling when it becomes aberrant or it is advantageous for the cell to halt signaling via the given receptor. In some cases, such as with the nerve growth receptor TrkA, internalized receptors can still signal within the endosomes (Geetha T et al., 2005).

Various factors regulate the internalization of signaling receptors, including receptor ubiquitination. For several receptors (such as epidermal growth factor receptor, growth hormone receptor, interferon α receptor chain 1 (IFNAR1)), ubiquitination is stimulated by the ligand. This stimulation is mediated by the ligand-induced transduction of a signal initiated by a kinase activity that is either intrinsic to the receptor itself (as for epidermal growth factor receptor (Galcheva-Gargova Z et al., 1995)) or provided by a receptor-associated kinase (as for growth hormone receptor, IFNAR1, and PRLr (Marijanovic Z et al., 2006; Deng L et al., 2007; Rui H et al., 1994;)).

Posttranslational modifications, such as ubiquitination have emerged as an important determinant in the endocytosis and sorting of cell surface receptors. Ubiquitin is a 76-amino acid protein that forms an isopeptide bond between its terminal glycine and a lysine residue of a target substrate. Each ubiquitin moiety

harbors seven lysine residues (K6, K11, K27, K29, K33, K48, K63) allowing for the formation of ubiquitin chains linked through its internal lysine residues. Ubiquitin can form ubiquitin chains of uniform or mixed linkage through these lysine residues, primarily Lys 29, Lys 48, and Lys 63 (Pickart CM and Fushman D, 2004). Monoubiquitination and multi-monoubiquitination have both been shown to play a role in endocytosis. Monoubiquitination can also signal for membrane trafficking, DNA repair, and histone regulation (Haglund K et al., 2003a). Substrates that are K48-polyubiquitinated typically are targeted for degradation by the 26S proteasome. K29- and K63- polyubiquitination often target substrates for involvement in DNA repair or receptor-mediated endocytosis (Haglund K et al., 2003a).

Ubiquitination has been found to be important for mediating the internalization of many yeast receptors (Bonifacino JS and Weissman AM, 1998; Hicke L and Dunn R, 2003), the nerve growth factor receptor TrkA [K63-linked polyubiquitination] (Geetha T et al., 2005), MHC class II proteins [K63-linked polyubiquitination] (Duncan LM et al., 2006), EGF receptor (Waterman H et al., 2002; Bonifacino JS and Traub LM, 2003), and Growth Hormone receptor (van Kerkhof P et al., 2001).

A report by Lu JC et al. (Lu JC et al., 2005) used the Chinese hamster lung cell line ts20, which contains a thermolabile ubiquitin-activating enzyme E1, and stably expressed wild type human PRLr in these cells. These stable lines were cultured at the permissive temperature (30^{o}C; active E1 enzyme) or the non-permissive temperature (42^{o}C; inactive E1 enzyme) and treated with radiolabeled and unlabeled bovine placental lactogen. The ratio of radiolabeled ligand that

internalized was determined and based on their results, they determined that the internalization of human PRLr is not dependent on ubiquitination. However, it has been shown that receptor internalization can be very cell type-specific so the study of human PRLr internalization in Chinese hamster lung cells does not replicate what occurs in human cells. Furthermore, the measurement of internalized radiolabeled ligand (which in this case is further complicated by the use of bovine placental lactogen instead of human prolactin) is not an accurate way to measure the internalization of a given receptor. Radiolabeled ligand can internalized due to phagocytosis or non-specific interaction of the ligand with a cell surface protein different than the receptor of interest. Instead, the disappearance of PRLr from the cell surface should be directly measured to get an accurate estimation of the amount of PRLr that has internalized. Due to these valid caveats, the conclusion made by the authors stating that ubiquitination is not important for PRLr endocytosis must be discounted. Instead, other work, including that from our lab, has established that PRLr ubiquitination is necessary for the efficient internalization and degradation of PRLr.

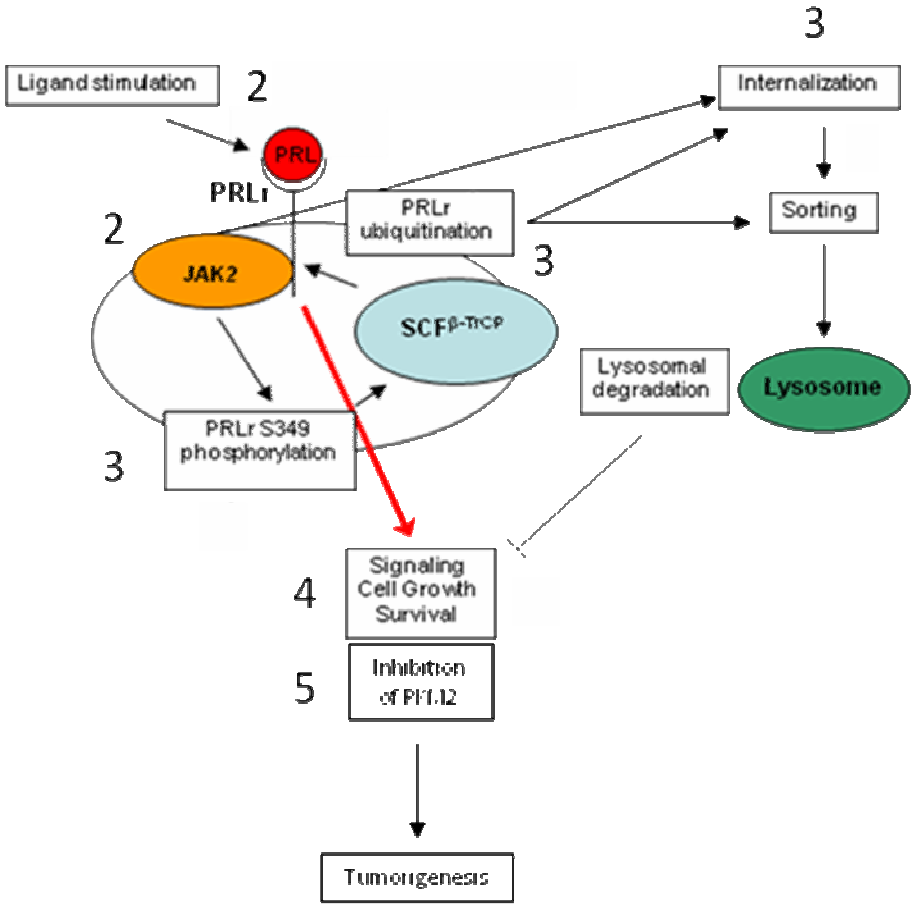
Pioneering studies in the early 1980s showed that prolactin was able to facilitate the lysosomal degradation of its receptor (Djiane J et al., 1979; Djiane J et al., 1981; Djiane J et al., 1982; Genty N et al., 1994; Hicke L, 1999). It was demonstrated that following stimulation of PRLr with its cognate ligand, PRL, the receptor gets phosphorylated on Ser349 within a well-conserved phosphodegron, $DS^{349}GRGS^{353}$ Activation of PRLr by ligand leads to Ser 349 and Ser 353

phosphorylation, likely following activation of Jak2, promoting interaction of the $SCF^{\beta\text{-}TrCP}$ E3 ligase complex with the well-conserved phosphodegron ($DS^{349}GRGS^{353}$) of PRLr. This interaction leads to receptor ubiquitination and subsequent lysosomal degradation (Li Y et al., 2004). The stabilization and accumulation of PRLr in breast cancer occurs as a consequence of impaired PRL-induced phosphorylation of the receptor and its subsequent escape from ubiquitin-mediated downregulation (Li Y et al., 2004; Li Y et al., 2006). This results in the continuous availability of receptor on the surface for ligand engagement and signal propagation. Recent work by Lu et al. demonstrated that inhibitors of both lysosomal pathway and of proteasomes impede the ligand-induced degradation of endogenous PRLr in PRL-deficient MCF7 breast cancer cells (Lu JC et al., 2005). While interpretation of these data is confounded by a known fact that many proteasome inhibitors suppress overall protein trafficking into the lysosomes indirectly by depleting the intracellular ubiquitin pool, it was proposed that, in this system, proteasomal function was required for limited cleavage of the receptor and generation of a receptor ECD-containing fragment, post internalization. It is currently unclear if this fragment represents an intermediate degradation product or a signaling unit, or whether this manner of receptor processing is cell-type specific (Lu JC et al., 2005).

The amount of PRLr on the cell surface controls both intensity and duration of PRL signals in cells and thereby cellular response to PRL. Alterations in PRLr levels can

therefore lead to aberrant downstream signaling in response to PRL resulting in disruption of cellular homeostasis. Early work examining the relative levels of PRLr in different breast cancer lines in comparison to a normal breast cell line indicated that the number of receptors per cell was high as 25,800 in T47D cells versus 1,700 in immortalized HBL-100 cells (Shiu RP, 1979). Subsequently, several studies have reported increased expression of PRLr mRNA in tumor tissue (corresponding to surrounding normal tissue) and in breast cancer cell lines (Laud K et al., 2000; Peirce SK and Chen WY, 2001; Touraine P et al., 1998). Similar results were obtained following analysis of PRLr protein levels by immunohistochemistry (Gill S et al., 2001; Reynolds C et al., 1997). Taken together, these observations and other experimental data obtained from cancer cell lines and primary tumor samples have postulated a positive link between increased receptor levels and breast cancer incidence, demonstrating the need to unravel the regulation of PRLr expression on the cell surface. The density of receptor on the surface is a cumulative consequence of events affecting de novo synthesis and subsequent fate of the receptor pre- and post- ligand binding.

Model 1



Model 1: This model shows the proposed relationship and key determinants which modulate the downregulation of PRLr and the consequences thereof. Each bold number indicates the numbered chapter in which each step is addressed.

Chapter 2

PRL and Jak2 promote internalization of PRLr

2.1 Abstract

Downregulation of signaling receptors represents the most direct method for limiting the magnitude and duration of downstream signal transduction. For cell surface transmembrane receptors, ligand-stimulated endocytosis is a major mechanism by which the ability of a cell to react to a ligand is restricted. PRLr has been shown to be degraded in an ubiquitination-dependent manner whereby phosphorylation of the phosphodegron motif ($DS^{349}GRGS$) on Ser 349 after ligand stimulation results in recruitment of the $SCF^{\beta\text{-}TrCP}$ E3 ligase complex. This E3 ligase complex ubiquitinates PRLr at target lysine residues that have yet to be characterized; this ubiquitination results in degradation of the receptor by the lysosome (Li Y et al., 2004). However, it was not known what determinants regulate the phosphorylation of Ser 349. Previous work from our lab established that PRLr is degraded in an ubiquitin-dependent manner but did not address the mechanism of internalization. As has been shown with other cell surface receptors, PRLr may be regulated in a feedback loop whereby the cell underwent a regulated program to restrict PRLr levels and thereby limit PRL signaling. In such a mode of downregulation, what role does ligand treatment and downstream effectors of PRL signaling play in limiting PRLr levels? In **Chapter 2**, we show that the endocytosis of PRLr is a ligand-induced process which requires the catalytic activity of the constitutively bound Janus kinase Jak2.

2.2 Introduction

The main signaling networks downstream of PRL/PRLr include the Jak-STAT, Ras-MAPK and PI3K-Akt pathways. These pathways impact crucial cellular processes like proliferation, survival, cytoskeletal effects and differentiation with well-established roles in the initiation and progression of cancer including mammary tumors. PRLr, analogous to other cytokine receptors, lacks intrinsic kinase activity and the receptor-Jak2 module acts to transmit signals downstream of ligand binding (Rui H, Lebrun JJ et al., 1994; Rui H, Kirken RA et al., 1994). PRL-mediated activation of the Jak-STAT signaling pathway results in transcriptional induction of milk protein genes and genes involved in cell proliferation like cyclin D1 (Chilton BS et al., 2005; Clevenger CV and Kline JB, 2001; Clevenger CV et al., 2003). PRL has been shown to activate the Ras-Raf-MAPK pathway in several mammary tumor cell lines, which promotes cell proliferation via multiple mechanisms. This is mediated in some cells by increased association of Shc with Jak2, as well as by the Grb2 and Sos complex (Das R and Vonderhaar BK, 1996). PRL has also been implicated in the activation of other MAPK such as JNK, which impact proliferation and apoptosis in cell systems like T47D, Nb2 and PC12 cells (Cheng Y et al., 2000; Olazabal I et al., 2000; Schwertfeger KL et al., 2000). Other kinases like c-Src, which is important in normal cellular physiology as well as mammary carcinoma, are activated in response to PRL and interface with PRLr- mediated signaling. Src functions upstream of PI3K or focal adhesion kinase (FAK)-Erk activation in PRL-

stimulated breast cancer cell lines (Acosta JJ et al., 2003). The activation of PI3K occurs by either the direct binding of the p85 subunit to PRLr or downstream of Src or Ras activation. The phosphoinositides generated by PI3K activate Akt, which transmits prosurvival and proliferatory signals by modulating cell-cycle regulators and also enable the recruitment of pleckstrin homology containing guanine nucleotide exchange factor (GEF), Vav, which activates the Rho family GTPases, leading to cytoskeletal rearrangements required for cell adhesion and migration. Moreover, PRLr signaling can transactivate other receptors involved in tumorigenesis. PRL treatment has been shown to induce tyrosine phosphorylation of human epidermal growth factor receptor (EGFR), leading to the activation of MAPK in breast cancer cell lines, suggesting that these pathways work in concert during the development of disease. This has implications for cancer therapeutics as it has been recently shown that a combination of anti-EGFR mAb, herceptin, and the PRL antagonist, PRL^{G129R} additively inhibited cell proliferation of T47D and BT474 cells as well as their growth in xenografts in athymic mice (Scotti ML et al., 2007). In addition to positive signal transduction, PRL/PRLr binding stimulates regulatory molecules capable of attenuating PRL generated signals. Included in this category are the SOCS family proteins, SOCS1 and 3, PIAS, CIS, and protein tyrosine phosphatases, PTP1B1 and TC-PTP which target the Jak-STAT pathway (Aoki N and Matsuda T, 2000; Aoki N and Matsuda T, 2002; Dif F et al., 2001; Tomic S et al., 1999; Tonko-Geymayer S et al., 2002).

2.3 Materials and Methods

Cell lines, constructs, and gene delivery

Human breast cancer T47D cells were kindly provided by Dr Z. Ronai (Burnham Institute, San Diego, CA, USA). Cells were cultured as previously described (Melck D et al., 2000). Negative control shRNA (Sigma, #SHC002) is a lentiviral pLKO.1-puro vector containing an irrelevant shRNA insert that does not target human and mouse genes. ShPRLr (Open Biosystems, #RHS3979-98492771) contains shRNA against human PRLr in the context of the same vector. Stable mass cultures of T47D containing these shRNAs were generated using viruses packaged in 293T cells co-transfected with indicated shRNA, VSV-G, and 8.2DeltaR plasmids. Mass cultures were selected in medium containing puromycin (2 μg/mL). CISH promoter-driven firefly luciferase reporter (Hankinson SE et al., 1999) was kindly provided by CV Clevenger (Northwestern University, Chicago, IL). Renilla luciferase expression vector was purchased (Promega). For luciferase assays, $2x10^5$ of T47D cells (shControl and shPRLr) were seeded into 24-well plates. Cells were transfected using LTX Lipofectamine reagent with pGL4-CISH reporter 9100 ng) and renilla luciferase reporter (1 ng). The transfected cells were starved for 24 h and treated or not with PRL (150 ng/mL) for an additional 24 hr. Luciferase reporter assay was performed using dual luciferase assay Promega kit according to manufacturer's instructions.

Human embryo kidney 293T cells, Jak2-null γ2A cells (Kohlhuber et al. 1997), and their derivatives were maintained and transfected as described elsewhere (Li et

al. 2004, Deng et al., 2007). Plasmids for expression of flag-tagged (Li et al. 2004) PRLr, as well as $Jak2^{WT}$ or $Jak2^{K882D}$ (Huang et al. 2001) were previously described. Plasmids for expression of hemagglutinin (HA)-tagged PRLr have been previously described (Swaminathan G et al., 2008b).

Reagents, antibodies, and immunotechniques

Antibodies against FLAG tag (M2, Sigma), HA tag (12CA5, Roche), beta-actin (Sigma), Jak2 (Upstate Biotech, Lake Placid, NY, USA), and PRLr (Zymed, San Francisco, CA, USA and Santa Cruz, Santa Cruz, CA, USA) were purchased. Human PRL was purchased from the National Hormone and Peptide program (AF Parlow). PRLr antagonist $PRL^{\Delta 1-9-G129R}$ was produced and purified as previously described (Bernichtein et al., 2003). AG490 and PP1 (Calbiochem, San Diego, CA, USA) were purchased. Immunoprecipitation and immunoblotting were performed as described previously (Li et al., 2004). Transfections were performed with Lipofectamine Plus or Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA) or FuGENE 6 (Roche) and analyzed after 48 h according to the manufacturer's recommendations.

Fluorescence-based internalization assay

This assay measures the loss of cell-surface immunoreactivity of epitope tagged or endogenous receptors using an ELISA assay as described previously (Barriere et al., 2006) with the following modifications. Briefly, 293T or γ2A cells transfected with HA-PRLr (or no receptor construct if studying endogenous PRLr) in addition to other constructs as indicated were serum starved and chilled on ice.

Internalization was initiated by incubation of cells with serum-free DMEM for indicated time periods at 37°C and terminated by placing the plate on ice. Levels of cell surface HA-PRLr or endogenous PRLr were analyzed by ELISA using anti-HA or anti-PRLr (Zymed, San Francisco, CA) primary antibodies respectively and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR, USA) followed by incubation with Amplex Red Ultra Reagent (Molecular Probes). Resulting fluorescence was measured (530 nm for excitation and 590 nm for emission) and expressed after subtracting the value obtained with mock transfected (endogenous receptor experiments used cells with a non-relevant control primary antibody) cells as percentage of fluorescence registered prior to internalization. Where indicated, pretreatment with monensin (100 μM in ethanol for 30 min prior to initiation of internalization) was used to prevent receptor recycling as described elsewhere for EGF receptor (Wang Y et al., 2002). In these cases, control cells received equal volumes of vehicle (ethanol).

2.4 Results

Elucidating the mechanism and identifying the determinants of PRLr downregulation is key to understanding how the cell limits PRL signaling and how these mechanisms can become aberrant and result in a disease state.

To determine the mode of regulation of PRL signaling, we first sought out to see if PRL signaling is affected by PRLr levels. For a given mode of receptor-based signaling in the face of decreased receptor levels, downstream signaling could be

maintained within the cell by increasing ligand amounts or by increasing the abundance of downstream effectors. Alternatively, decreasing the levels of the receptor may cause reduced signaling. In the case of PRLr, if a reduction in PRLr levels results in decreased PRL signaling, then the cell may look to degrade the receptor in cases where PRL signaling has become aberrant and must be controlled. If PRL signaling is dependent on receptor levels, then conditions whose pathogenesis is PRL signaling-dependent could be prevented by keeping PRLr levels in check. To address this question, we decided to use a breast cancer cell model where PRL signaling has been shown to be abundant. By altering PRLr levels in this 2-D culture system, we can study the effect of PRLr levels on PRL signaling. For this purpose, we chose the T47D breast cancer cell line due to its abundant PRL signaling. To this end, we created T47D stable lines that stably express a control shRNA or a PRLr-specific shRNA and asked whether shRNA-mediated knockdown of PRLr would result in decreased PRL signaling. Knockdown of PRLr in T47D stable cell lines halted the activation of a PRL-responsive CISH promoter (Figure 2.1), showing that the maintenance of receptor level is crucial for normal downstream signal transduction. Since PRL signaling is dependent upon PRLr levels, it is crucial to understand how the cell may limit the levels of PRLr. Deregulation of PRLr levels may explain disease states where PRL signaling has become aberrant.

In order to understand how the cell regulates receptor levels, we must investigate the mechanism of receptor endocytosis. Once the receptor is endocytosed, it can then enter the endosome and ultimately be degraded by the lysosome. However, the

question remains, what initiates the internalization of PRLr? In the case of a feedback loop, the upregulation or activation of downstream effectors or ligand-receptor binding works to initiate the attrition of signaling. To test this possibility, we sought to investigate the role of PRL and how it may affect receptor internalization. To study the mechanisms of receptor internalization, our lab has developed a novel method to directly study the disappearance of cell surface PRLr using a fluorescence-based internalization assay (described in Materials and Methods). By analyzing the rate of PRLr endocytosis using a highly sensitive fluorescence-based endocytosis assay that allows for the analysis of the internalization of receptor itself and not the internalization of radiolabeled ligand, we are able to more accurately study the endocytosis of PRLr. Other labs have commonly studied receptor internalization by observing the internalization of a radiolabeled ligand. However, this radiolabeled ligand could conceivably enter the cell independent of its interaction with internalizing receptors by general phagocytosis or by non-specific interactions with other cell surface molecules. By using our fluorescence-based method, we are accurately and directly studying the internalization of the PRLr.

To study the role of PRL in the internalization of PRLr, we expressed amino-terminally HA-tagged PRLr in 293T cells and observed the internalization kinetics (using our fluorescence-based internalization assay) in response to no treatment, the PRLr antagonist $PRL^{\Delta 1-9, G129R}$, PRL, or PRL in conjunction with the PRLr antagonist. In our initial studies, we found that PRL significantly increased the

initial internalization rate of its receptor in 293T cells. Treatment with the PRLr antagonist ($PRL^{\Delta 1-9,G129R}$), which allows for receptor dimerization but does not allow for downstream signal transduction, had no effect alone but reversed the stimulation of PRLr internalization by wild type PRL (Figure 2.4). These results indicate that PRL-induced signaling is a prime driving force behind the internalization of PRLr.

Since we established that PRL stimulation is crucial for the optimal internalization of PRLr, we wanted to investigate which signaling steps of the PRL signaling transduction pathway are necessary for PRLr endocytosis. One of the first events after PRL-PRLr binding is the phosphorylation and activation of the constitutively bound Janus kinase Jak2. Jak2 has a kinase activity which is required for PRL signaling. The importance of Jak2 activity is evidenced by the necessity of Jak2 for the phosphorylation of intracellular tyrosines of PRLr (Figure 2.2). These tyrosine phosphorylation events are necessary for effective PRL signaling. Furthermore, Jak2 activity is necessary for effective Ser 349 phosphorylation (Figure 2.2) and subsequent ubiquitination (Figure 2.3) of PRLr which leads to lysosomal degradation of the receptor (Swaminathan G et al., 2008b; Varghese B et al., 2008). PRLr is phosphorylated on Ser 349 which is part of a conserved phosphodegron motif ($DS^{349}GRGS$). Upon phosphorylation of the phosphodegron motif, the $SCF^{\beta\text{-}TrCP}$ E3 ligase complex is recruited to PRLr and ubiquitinates it at target lysines within the receptor.

Since Jak2 is important for the ubiquitination of PRLr which leads to receptor degradation, we sought to determine the role of Jak2 in PRLr internalization. To

determine the role of Jak2 kinase activity in PRLr endocytosis, we sought to pharmacologically inhibit Jak2 and see how this affected receptor internalization. Pharmacological inhibition of Jak2 is a preferred method since we can maintain the presence of the Jak2 protein while decreasing its catalytic activity, thus giving us an idea of the importance of Jak2 catalytic activity in PRLr endocytosis. For this purpose, 293T cells were pretreated with either Jak inhibitor AG490 or Src inhibitor PP1 and we measured the internalization of PRLr in the absence or presence of ligand using the fluorescence-based internalization assay. The usage of Src inhibitor PP1 was chosen to serve as a control since Src is a downstream effector of PRL signaling and we want to discover if any effect upon PRLr endocytosis is due to decreased Jak2 activity or because we are decreasing PRL signaling by decreasing the activity of a downstream effector. In these experiments, we found that while neither inhibitor affected basal PRLr endocytosis, the ligand-stimulated endocytosis of PRLr was impaired in cells treated with AG490 (Jak inhibitor) but not with the Src inhibitor PP1 (Figure 2.5). These data indicate that Jak2 activity is needed for efficient PRL-mediated PRLr internalization while decreased Src activity did not seem to significantly affect PRLr endocytosis. These data propose Jak2 as a key regulator of ligand-dependent PRLr internalization.

We further wanted to confirm the importance of Jak2 in regulating the internalization of PRLr. We sought to determine whether the addition of Jak2 could promote PRLr endocytosis. To this end, we decided to use γ2A cells which lack endogenous Jak2 and serve as a good system whereby we can add Jak2 and observe

the effects upon PRLr internalization by using our fluorescence-based internalization assay. Our studies of PRLr internalization in γ2A-derived cells (which lack endogenous Jak2) showed that expression of wild type Jak2 significantly increased the endocytic rate of the receptor, while this effect was neither observed in cells expressing inactive Jak2 nor in cells that lacked Jak2 protein (Figure 2.6). Given that inactive Jak2 has been implicated in the maturation and cell surface delivery of PRLr and EpoR (Huang et al. 2001), these data also suggest that, unlike for receptor maturation, Jak2's role in internalization does require its protein kinase activity. Taken together, these data suggest that the catalytic activity of Jak2 is necessary for ligand-facilitated acceleration of the initial rate of PRLr internalization.

2.5 Discussion

We established that PRL signaling is dependent upon receptor level, indicating that the study of PRLr downregulation and its key determinants is a valuable endeavor in that it will allow us to have insights into how PRL signaling becomes aberrant and subsequently can promote tumorigenesis. The relationship between enhanced PRL signaling and malignancies of the breast have been well-established but poorly understood. Our studies on the role of PRL signaling in the downregulation of PRLr have shown that treatment with PRL promotes Jak2 activity which works to promote the phosphorylation of the intracellular tyrosines of PRLr (crucial step for PRL signaling) and phosphorylation of Ser 349 of PRLr's phosphodegron motif (see Model 1). However, previous studies from our lab have

established that Jak2 is not the direct kinase which phosphorylates Ser 349 (data not shown). This phosphorylation event is necessary for PRLr degradation as phosphorylated Ser 349 of the conserved phosphodegron motif works to recruit the $SCF^{\beta\text{-}TrCP}$ E3 ligase complex which binds to PRLr and ubiquitinates the receptor on its target lysines. Additionally, we have shown that the ubiquitination of PRLr occurs in response to PRL in a manner dependent on Jak2 activity. Since Jak2 promotes the phosphorylation of the phosphodegron motif and the subsequent ubiquitination of the PRLr, both of which are necessary for effective ligand-mediated degradation of PRLr, we have therefore established Jak2 to play an important role in PRLr downregulation (see Model 1). These results may implicate Jak2 in the phosphorylation and activation of the yet to be identified kinase which phosphorylates PRLr on Ser 349 of the phosphodegron motif. This possible action of Jak2 would explain the importance of Jak2 in regulating the Ser 349 phosphorylation and ubiquitination of PRLr. Future studies aimed at identification of the PRLr Ser 349 kinase should focus on a Jak2-activated kinase.

Furthermore, in the investigation of PRLr downregulation, we have looked to see how the PRLr is internalized and what factors initiate its internalization by using a reliable and highly sensitive fluorescence-based internalization assay which directly studies the rate of receptor that becomes endocytosed as opposed to indirect studies using radiolabeled ligand. These elegant studies showed that PRLr internalization is largely a ligand-induced process. Ligand-induced internalization of PRLr could be abrogated by treatment with a PRLr antagonist which competes with PRL for the

ligand binding site of PRLr. However, treatment with the PRLr antagonist alone did not promote PRLr endocytosis, indicating that PRL-PRLr binding is not sufficient to promote receptor internalization. Furthermore, activation of PRL signaling is needed to activate the internalization of PRLr. Using this fluorescence-based internalization assay, we also identified Jak2 kinase activity, and not Src kinase activity, to be crucial for PRL-mediated internalization of the PRLr (see Model 1). Internalization studies using gamma2A cells, which lack endogenous Jak2, showed that optimal PRLr endocytosis was achieved when wild-type Jak2, and not a kinase inactive version, was introduced into the cell system.

Conclusions of Chapter 2:

- PRL signaling is dependent on levels of PRLr
- Active Jak2 promotes the tyrosine phosphorylation of PRLr in response to PRL
- Active Jak2 promotes the phosphorylation of Ser 349 of the phosphodegron motif of PRLr in response to PRL
- Active Jak2 is needed for the efficient PRL-induced ubiquitination of PRLr
- The internalization of PRLr is largely a PRL-driven process
- PRLr antagonist can prevent PRL-induced endocytosis of PRLr
- Jak2 activity is required for the efficient PRL-mediated internalization of PRLr

- Src activity is not required for the efficient PRL-mediated internalization of PRLr
- Active Jak2 protein promotes the PRL-mediated endocytosis of PRLr

Active Jak2 promotes and is necessary for the internalization of PRLr. The importance of Jak2 in the Ser 349 phosphorylation and ubiquitination of PRLr imply that the kinase which phosphorylates PRLr on Ser 349 is likely a Jak2-activated kinase. Identification of this kinase would be a useful next step in the elucidation of the mechanisms by which PRLr levels are regulated.

While Jak2 plays an important role in the downregulation of PRLr, it also is crucial for normal PRL signaling. In cancers driven by aberrant PRL signaling, pharmacological inhibition of Jak2 may not be a viable treatment as this would interfere with IL-3 and GM-CSF signaling, both of which utilize Jak2. This would be counterproductive and hinder the host's immunological response against the tumor.

The studies contained in **Chapter 2** are significant because they show that PRLr downregulation is a mainly a PRL-mediated process that is dependent on active Jak2. This is counter to the work by Piazza TM et al. that claimed PRLr endocytosis is a Src-dependent process (Piazza TM et al., 2009). However, these studies were performed using radiolabeled ligand and did not directly study the endocytosis of PRLr. Our studies, using the fluorescence-based internalization assay, accurately and reproducibly study the direct internalization of the receptor, allowing us to gain

accurate insights into the regulation of PRLr endocytosis. Our work showed that the PRL-mediated internalization of PRLr is Jak2-dependent and not Src-dependent. The presence of a minimal rate of PRLr endocytosis in the absence of ligand raises the possibility of an alternative yet minor ligand-independent internalization mechanism which may be Src-dependent. Such an alternative mechanism may allow for the slow turnover and subsequent renewal of cell surface PRLr even in the absence of active PRL signaling. In spite of any alternative internalization mechanisms, our studies show that ligand-mediated PRLr endocytosis is a Jak2 dependent process. These data propose Jak2 to not only be crucial for signaling downstream of the PRLr, as has been previously established, but also to be a key regulator of PRLr levels. We propose that upon hyperactive PRL signaling, the activation of Jak2 works to promote PRLr downregulation and thereby keep aberrant signaling in check. Jak2 is both needed for active PRL signaling and for the effective execution of a negative feedback loop.

PRL treatment and subsequent activation of Jak2 both work to promote receptor endocytosis and these events both promote Ser 349 phosphorylation, receptor ubiquitination, and receptor degradation. These facts lead us to believe that receptor ubiquitination may be the key linking factor which connects PRL and Jak2 to receptor internalization and subsequent degradation. While it has been previously shown that PRLr is ubiquitinated, the importance, nature, and specificity of PRLr ubiquitination and its importance for the internalization of PRLr have not been

established. These questions must be properly answered so that we may understand how the cell maintains and regulates PRLr levels.

Elucidating the mechanism by which PRLr levels are regulated will help us to understand how this regulation may go awry, resulting in aberrant PRL signaling and the induction of a disease state. Therefore, we will seek to investigate the nature of PRL-mediated PRLr endocytosis and the role of ubiquitination in this process in **Chapter 3**.

Figure 2.1

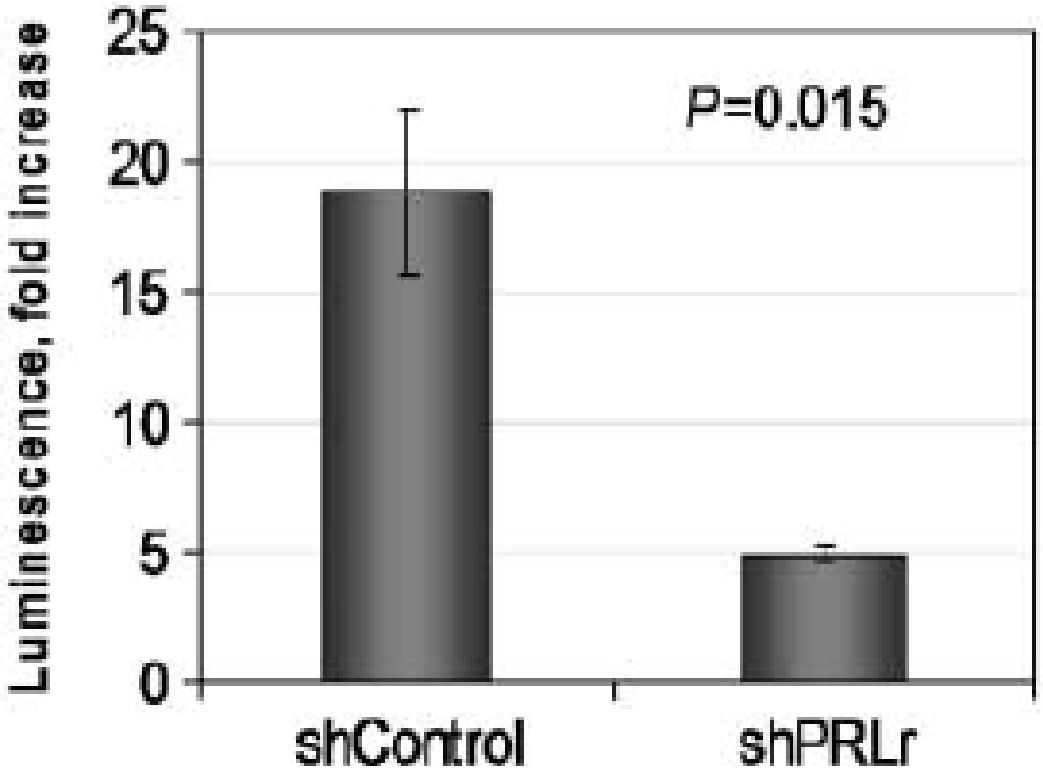


Figure 2.1: PRL-induced CISH promoter–driven luciferase activity in indicated T47D cell lines was performed as described in Materials and Methods.

Figure 2.2

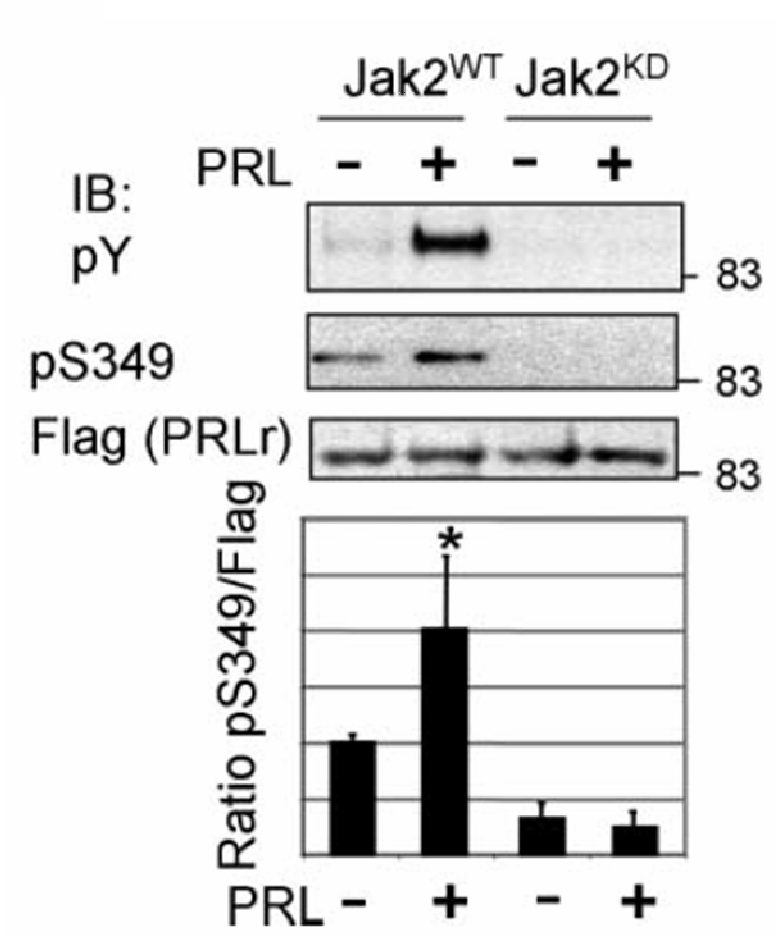


Figure 2.2: Phosphorylation of PRLr on Tyr residues (pY, upper panel) or on Ser349 (pS349, middle panel) in gamma2A cells expressing indicated Jak2 and treated as indicated with PRL (200 ng/ml for 30 min) was analyzed by immunoprecipitation of Flag-PRLr using anti-Flag antibody followed by immunoblotting using indicated phospho-specific antibodies. Ratios between pS349 and Flag signals corresponding to cell types and treatments are calculated as an average from four independent experiments (GS.E.M.) and depicted in the graph below. Asterisk denotes $P<0.05$ in the t-test relative to untreated $Jak2^{WT}$ cells. Experiment performed by Gayathri Swaminathan.

Figure 2.3

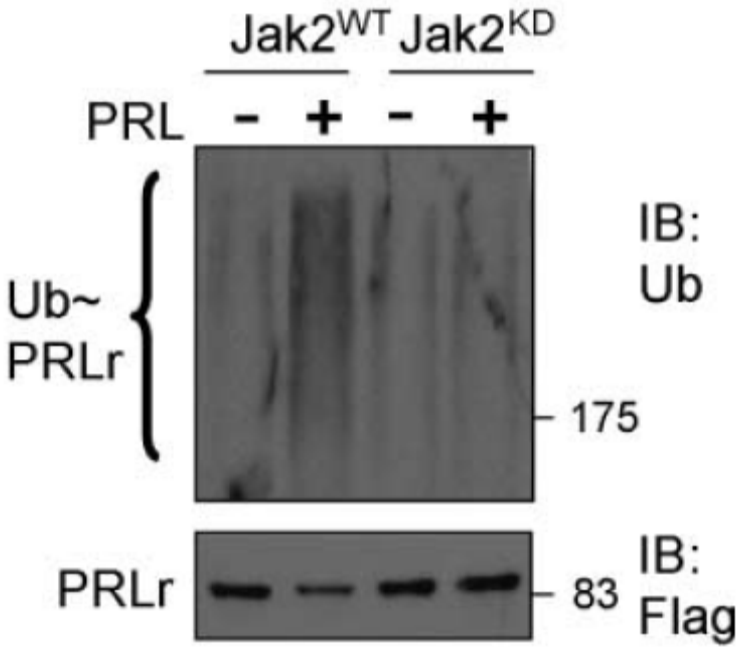


Figure 2.3: Flag-PRLr stringently immunopurified from denatured lysates of gamma2A cells expressing the indicated Jak2 and treated where indicated with PRL (200 ng/ml for 30 min) was analyzed by immunoblotting using either anti-ubiquitin (upper panel) or anti-Flag (lower panel) antibodies. Experiment performed by Gayathri Swaminathan.

Figure 2.4

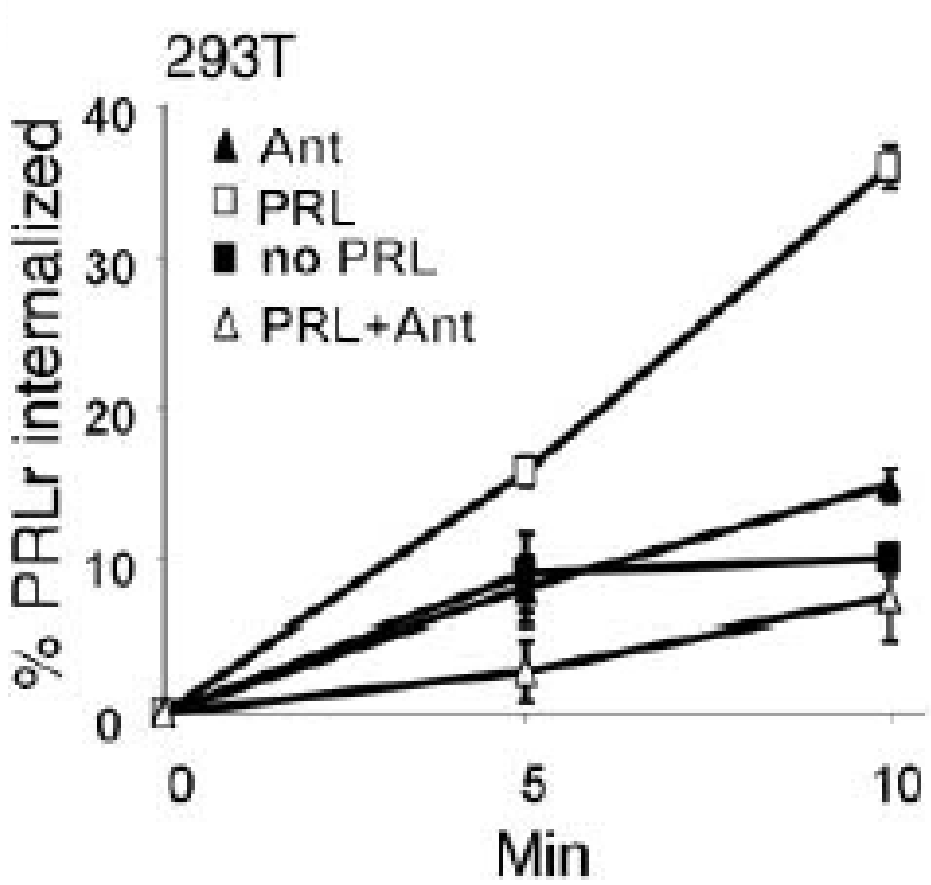


Figure 2.4: PRL signaling via Jak2 regulates initial internalization of PRLr. Effect of PRL (open squares and open triangles) or PRLr antagonist ($PRL^{\Delta 1\text{-}9,G129R}$, closed and open triangles) on the initial rate of internalization of amino-terminally HA-tagged PRLr expressed in 293T cells measured by a fluorescent assay as described in Materials and Methods.

Figure 2.5

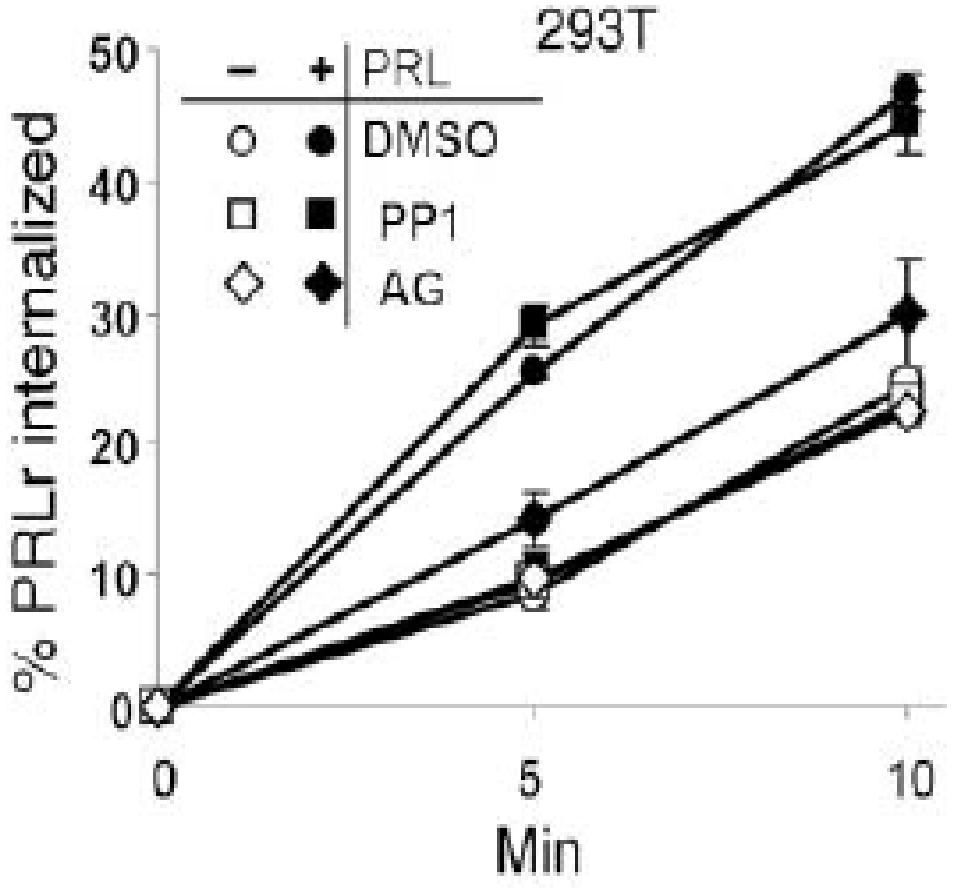


Figure 2.5: Effect of Jak inhibitor AG490 (50 mM, diamonds) or Src inhibitor PP1 (10 mM, squares) on the initial rate of internalization of PRLr in 293T cells in the presence (closed symbols) or absence (open symbols) of PRL (50 ng/ml) measured by a fluorescent assay. Inhibitors were added to cells 40 min before the internalization start. Controls are represented by treatment of cells with vehicle (DMSO, closed or open circles).

Figure 2.6

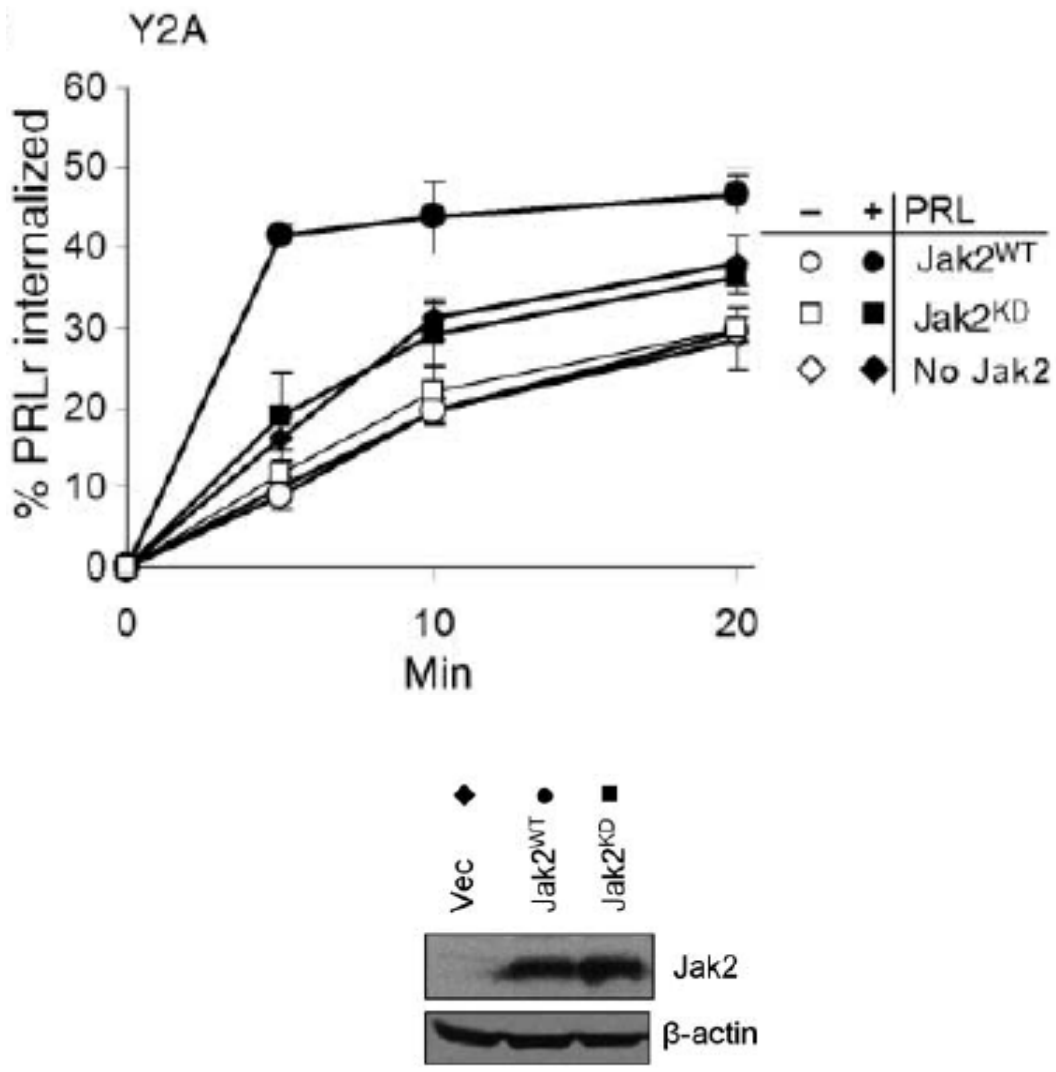


Figure 2.6: Effect of transient expression of $Jak2^{WT}$ (circles) or $Jak2^{KD}$ (squares) on the initial rate of internalization of PRLr in γ2A cells in the presence (closed symbols) or absence (open symbols) of PRL (50 ng/ml) measured by a fluorescent assay. Controls are represented by internalization of PRLr in cells that did not receive any Jak2 (closed or open diamonds circles). Bottom panel depicts immunoblotting analysis of Jak2 expression in these cells.

Chapter 3

Endocytosis of PRLr is dependent on K63-linked polyubiquitination of the receptor

3.1 Abstract

PRLr has been shown to be degraded in an ubiquitination-dependent manner whereby phosphorylation of the phosphodegron motif ($DS^{349}GRGS$) on Ser 349 after ligand stimulation results in recruitment of the $SCF^{\beta\text{-}TrCP}$ E3 ligase complex (Li Y et al., 2004). This E3 ligase complex ubiquitinates PRLr at target lysine residues that have yet to be characterized and results in degradation of the receptor by the lysosome (Li Y et al., 2004). Both PRL treatment and active Jak2 both work to promote receptor internalization and subsequent degradation. They also promote receptor ubiquitination. This leads us to believe that there is a connection between PRLr ubiquitination and the regulation of PRLr internalization. To characterize PRLr endocytosis, we sought to determine whether it was a caveolae- or clathrin-dependent process and whether ubiquitination conveyed specificity to the internalization of the receptor as has been shown for other cell surface receptors. In our studies to investigate these matters in **Chapter 3**, we determined that PRLr is internalized in a clathrin-dependent manner that requires phosphorylation of the phosphodegron motif (Model 2), intact β-TrCP (Model 2), and K63-linked receptor polyubiquitination (Model 2) for the optimal endocytosis of the PRLr.

3.2 Introduction

Cells react to diverse stimuli by expressing specific receptors that recognize these stimuli and initiate specific signaling pathways that enable a cell to change with the environment. Downregulation of these signaling receptors represents the most direct method for limiting the magnitude and duration of downstream signal transduction. For cell surface transmembrane receptors, ligand-stimulated endocytosis is a major mechanism by which the ability of a cell to react to a ligand is restricted. Furthermore, basal internalization independent of the ligand determines how responsive a naive cell would be to ligand stimulation. Mechanisms regulating the internalization of signaling receptors involve a dynamic exchange within the plasma membrane, which can result in bulk endocytosis and a cargo-specific clathrin-dependent endocytosis.

Endocytosis is the process by which molecules at the cell surface are trafficked into vesicular compartments within the cell. Once a molecule is endocytosed, it is trafficked to the early endosome and subsequently sorted. This sorting process can result in the endocytosed molecule to be recycled back to the cell surface or to progress through the endosome pathway and can ultimately be degraded by the lysosome. The vesicles of the endosome decrease in pH with vesicles of the early endosome being basic compared to the acidic environment of the lysosome. Degradative enzymes within the lysosome are activated upon this low pH and can work to degrade the internalized molecule/receptor. Alternatively, internalized receptors can be recycled back to the cell surface. Internalized receptors that get

recycled back to the cell surface first enter the early endosome and then bud off in a recycling endosome which fuses with the plasma membrane. In the case of receptor-mediated endocytosis, recycling can restore cell responsiveness to ligand and restore the signaling downstream of the receptor.

There are three basic mechanisms by which cargo is endocytosed: clathrin-mediated endocytosis, caveolae-mediated endocytosis, and dynamin- and clathrin-independent endocytosis (Dautry-Varsat A, 2001). Clathrin-mediated endocytosis requires clathrin-coated vesicles to internalize its cargo. Caveolae-mediated endocytosis involves small membrane invaginations that contain cholesterol, sphingolipids, and caveolin and is a method of receptor-mediated endocytosis in certain cell types. The dynamin- and clathrin- independent mode of endocytosis has yet to be clearly characterized (Dautry-Varsat A, 2001).

Ligand-induced internalization of cell surface receptors can occur through both clathrin-dependent or -independent pathways. In the clathrin-dependent pathway, receptors enter clathrin-coated vesicles, which are invaginations of the plasma membrane that concentrate endocytosed receptors. This process involves the interaction of the assembly polypeptide 2 (AP-2) clathrin adaptor complexes with specific endocytic signals located within the cytoplasmic domain of the receptors (Bonifacino JS and Traub LM, 2003). AP-2 complexes, involved in the assembly of clathrin triskelions at the plasma membrane, are composed of four components, including two adaptin subunits (α and β2) and two smaller subunits (μ2 and σ2). Each of these subunits has unique functions (Owen DJ et al., 2004)). Specific

endocytic motifs are essential for receptor clustering on the membrane and clathrin-dependent internalization of receptors. For example, both tyrosine- and leucine-based motifs can be recognized by the AP-2 complex via interaction with the μ2 subunit and with the β2 or α/σ2 hemicomplexes, respectively (Bonifacino JS and Traub LM, 2003; Chaudhuri R et al., 2007; Doray B et al., 2007).

In the case of many cell surface receptors, ligand-induced ubiquitination of the receptor promotes its interaction with clathrin and stimulates receptor endocytosis. Furthermore, ubiquitination plays an important role in post-internalization sorting of cargo receptors to the late endosomes and in subsequent lysosomal degradation (Höller D and Dikic I, 2004; Dikic I, 2003; Haglund K et al., 2003b).

PRLr is expressed in the majority of breast cancers (Ormandy CJ et al., 1997). Previous work from our lab has shown that the interaction between PRLr and β-TrCP, an F-box protein in the $SCF^{\beta\text{-}TrCP}$ E3 ligase complex that recognizes PRLr, is defective in human breast cancer cells compared to normal mammary epithelial cells (Li Y et al., 2006). This implies that aberrant PRL signaling plays an important role in carcinogenesis within the breast.

3.3 Materials and Methods

Cell lines, constructs, and gene delivery

Human embryo kidney 293T cells and their derivatives were maintained and transfected as described elsewhere (Li et al. 2004, Deng et al., 2007). Plasmid for

expression of flag-tagged (Li et al. 2004) PRLr was previously described. Plasmids for expression of hemagglutinin (HA)-tagged PRLr have been previously described (Swaminathan G et al., 2008b). The HA-tagged ubiquitin expression constructs were kindly provided by Yosef Yarden (Weizmann Institute, Israel). The knockdown of clathrin heavy chain was performed using a short hairpin obtained from Sigma (MISSION short hairpin RNA [shRNA] plasmid DNA; catalog no. SHDNACTRCN0000007982). The short hairpin constructs directed against β-TrCP (Li Y et al., 2004; Tang W et al., 2005) and the small interfering RNA against AP-2 (Barriere H et al., 2006) were previously characterized elsewhere. The knockdown of clathrin heavy chain was performed using a short hairpin obtained from Sigma (MISSION short hairpin RNA [shRNA] plasmid DNA; catalog no. SHDNACTRCN0000007982). The short hairpin constructs directed against β-TrCP (Li Y et al., 2004; Tang W et al., 2005) and the small interfering RNA against AP-2 (Barriere H et al., 2006) were previously characterized elsewhere.

Reagents, antibodies, and immunotechniques

Antibodies against FLAG tag (M2, Sigma), HA tag (12CA5, Roche), beta-actin (Sigma), and PRLr (Zymed, San Francisco, CA, USA and Santa Cruz, Santa Cruz, CA, USA) were purchased. Human PRL was purchased from the National Hormone and Peptide program (AF Parlow). Immunoprecipitation and immunoblotting were performed as described previously (Li et al., 2004). Monensin was purchased from Sigma. Transfections were performed with Lipofectamine Plus

or Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA) or FuGENE 6 (Roche) and analyzed after 48 h according to the manufacturer's recommendations.

Fluorescence-based internalization assay

This assay measures the loss of cell-surface immunoreactivity of epitope tagged or endogenous receptors using an ELISA assay as described previously (Barriere et al., 2006) with the following modifications. Briefly, 293T or γ2A cells transfected with HA-PRLr (or no receptor construct if studying endogenous PRLr) in addition to other constructs as indicated were serum starved and chilled on ice. Internalization was initiated by incubation of cells with serum-free DMEM for indicated time periods at 37°C and terminated by placing the plate on ice. Levels of cell surface HA-PRLr or endogenous PRLr were analyzed by ELISA using anti-HA or anti-PRLr (Zymed, San Francisco, CA) primary antibodies respectively and horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Molecular Probes, Eugene, OR, USA) followed by incubation with Amplex Red Ultra Reagent (Molecular Probes). Resulting fluorescence was measured (530 nm for excitation and 590 nm for emission) and expressed after subtracting the value obtained with mock transfected (endogenous receptor experiments used cells with a non-relevant control primary antibody) cells as percentage of fluorescence registered prior to internalization. Where indicated, pretreatment with monensin (100 μM in ethanol for 30 min prior to initiation of internalization) was used to prevent receptor recycling as described elsewhere for EGF receptor (Wang Y et al., 2002). In these cases, control cells received equal volumes of vehicle (ethanol).

3.4 Results

Various factors regulate the internalization of cytokine receptors, including receptor ubiquitination. Ubiquitinated receptors often internalize by interaction with proteins of the endocytic machinery that contain ubiquitin interacting domains. Such receptors (among them IFNAR1 [Kumar et al., 2007]) often are endocytosed via a clathrin-dependent pathway. We wanted to investigate the process of PRLr internalization and whether this is clathrin-dependent. To identify if PRLr is internalized via the clathrin-dependent pathway, we used a shRNA-mediated knockdown approach of clathrin to see how this would affect PRLr internalization. By knocking down clathrin heavy chain, we can impair clathrin-mediated internalization and observe whether the loss of the clathrin heavy chain has an effect upon PRLr internalization. If internalization of PRLr is impaired or decreased, then we can understand that clathrin plays a key role in the endocytosis of PRLr. To this end, we used our high throughput fluorescence based internalization assay using a tagged exogenous PRLr because it provides an accurate and precise way to look at the disappearance of the PRLr from the cell surface. Use of shRNA that efficiently knocked down the expression of clathrin heavy chain noticeably impaired the internalization of exogenous PRLr compared to the internalization of PRLr in which clathrin was intact (Figure 3.2). This result inferred that PRLr endocytosis is a clathrin-dependent process since efficient PRLr internalization could only be achieved in the presence of intact clathrin heavy chain protein.

However, this result was achieved using exogenous receptor. Since expression of an exogenous receptor can increase the load on the endocytic machinery, the internalization mechanism for PRLr may become altered in order to quickly deal with this now abundant protein. Thus, results obtained looking at the internalization kinetics of exogenous PRLr may not replicate that of the endogenous protein in normal settings. We sought to determine whether the internalization of the endogenous PRLr was a clathrin-dependent process and whether internalization studies using the exogenous receptor could recapitulate those performed using the endogenous receptor. To this end, we decided to use the fluorescence-based endocytosis assay to investigate the internalization kinetics of endogenous PRLr. In order to investigate whether the internalization of endogenous PRLr was a clathrin-dependent process, we decided to use a shRNA-mediated knockdown approach for the knockdown of clathrin heavy chain and observe whether internalization kinetics became altered. If the internalization kinetics of endogenous PRLr is deficient when clathrin heavy chain is knocked down, this would indicate that the endocytosis of PRLr is largely a clathrin-dependent process. Our studies indicate that the endocytosis of endogenous PRLr, like that of exogenous PRLr, is a clathrin-mediated process (Figure 3.1). This suggests that the fluorescence-based internalization assay can produce similar results using both exogenous receptor (which may be used for more complicated studies that require genetic manipulation) and for the endogenous receptor (which is more indicative of what occurs within the cell) in the case of clathrin heavy chain knockdown. These results taken together show that PRLr

internalization is clathrin-dependent and confirm our previous observations that the endocytosis of recombinant HA-tagged PRLr faithfully recapitulates the processes involved in the regulation of the endogenous receptor by use of this internalization assay (Swaminathan G et al., 2008b).

Previous work from our lab has shown that the $SCF^{\beta\text{-}TrCP}$ E3 ligase complex is needed for the effective ubiquitination and degradation of the PRLr (Li Y et al., 2004). Along these lines, we wanted to establish whether $SCF^{\beta\text{-}TrCP}$ plays a role in the endocytosis of PRLr. To this end, we decided to use the fluorescence-based internalization assay using exogenous PRLr to establish endocytic rates in the presence or absence of shRNA targeted for the knockdown of β-TrCP. By using shRNA to knockdown β-TrCP, we can determine whether the endocytic rates of PRLr are affected by β-TrCP. We decided to express HA-tagged PRLr in our model 293T cell system. These studies established that the rate of internalization of PRLr is highly deficient when β-TrCP is knocked down compared to PRLr in the presence of intact β-TrCP (Figure 3.4). This suggests that β-TrCP is necessary for efficient PRLr endocytosis.

To confirm the role of β-TrCP in the internalization of PRLr, we wanted to investigate the importance of the E3 ligase complex for the endocytosis of endogenous PRLr to see whether this would recapitulate the results obtained using exogenous PRLr. To this end, we performed the fluorescence-based internalization assay using 293T cells and studied the disappearance of endogenous PRLr from the cell surface either in the presence or absence of shRNA directed against β-TrCP.

These studies showed that the internalization of endogenous PRLr is dependent upon β-TrCP levels as knockdown of β-TrCP resulted in a deficient endocytic rate (Figure 3.3). This result indicates that β-TrCP is needed for the efficient endocytosis of endogenous PRLr. Taken together, these results show that PRLr is internalized in a manner dependent on the SCF β-TrCP E3 ligase complex.

Since β-TrCP is the major E3 ubiquitin ligase for PRLr, these data (Figure 3.3; Figure 3.4) suggest that the ubiquitination of PRLr is required for the efficient internalization of the receptor. However, knockdown of β-TrCP could affect PRLr internalization indirectly via the numerous other known β-TrCP targets (Fuchs SY et al., 2004; Yamaski L and Pagano M, 2004). To determine the role of PRLr ubiquitination in the internalization of PRLr in a more direct manner, we decided to compare the endocytic rates of wild type PRLr and of the ubiquitination-deficient $PRLr^{S349A}$ mutant, which does not efficiently recruit β-TrCP (Li Y et al., 2004). To this end, we expressed HA-tagged $PRLr^{WT}$ and HA-tagged $PRLr^{S349A}$ in 293T cells and used the fluorescence-based internalization assay to determine the endocytic rate of these receptors. Interestingly, the $PRLr^{S349A}$ mutant exhibited slower kinetics of endocytosis in 293T cells than $PRLr^{WT}$ (Figure 3.5). The facts that the $PRLr^{S349A}$ mutant is not effectively ubiquitinated and exhibits a deficient internalization rate compared to the wild type receptor are suggestive of PRLr ubiquitination playing a crucial role in promoting PRLr endocytosis.

Despite our data signifying the importance of PRLr ubiquitination in promoting the internalization of PRLr, these results could be explained by

differential rates of receptor recycling between the wild type PRLr and the S349A mutant. Our results (Figure 3.5) could be explained by the wild type and S349A mutant of PRLr both having similar endocytic rates but the S349A mutant having a high rate of internalized receptor that recycles back to the cell surface. In this case, despite the wild type and S349A mutant receptors having similar endocytic rates, the high number of mutant receptors that have recycled back to the cell surface will make it seem as though the S349A has a slower rate of internalization than the wild type receptor. In fact, the data obtained would be a result of the S349A mutant's ability to internalize and recycle at a quick rate and would complicate our interpretation of results looking at the role of ubiquitination in PRLr endocytosis. To understand the recycling rates of PRLr and determine whether our results obtained are an artifact of our system, we decided to investigate the endocytic rate of PRLr in the presence or absence of monensin, a well-established inhibitor of receptor recycling that has been used to differentiate between internalization and recycling phenotypes of various receptors, including EGF receptor (Wang Y et al., 2002). To this end, we expressed HA-tagged $PRLr^{WT}$ and HA-tagged $PRLr^{S349A}$ in 293T cells in the presence or absence of monensin and determined the endocytic rate of both receptors under both conditions using our fluorescence-based internalization assay. Results from these studies showed that pretreatment of the 293T cells with monensin neither altered the internalization rates of $PRLr^{WT}$ nor that of $PRLr^{S349A}$ (Figure 3.6). This indicates that PRLr cell surface recycling does not occur at an appreciable level during the initial time of PRLr internalization which is studied. Since monensin

pretreatment did not rescue the endocytic phenotype of $PRLr^{S349A}$ (Figure 3.6), this result confirms that PRLr internalization is dependent on the ubiquitination of PRLr and that interpretation of previous results (Figure 3.5) is not complicated by internalized PRLr that is being recycled back to the cell surface. These data taken together suggest that the ubiquitination of PRLr is required for its efficient endocytosis.

Clathrin-coated pits associate with internalized receptors through adaptor protein complexes (e.g., AP-2) which interact with the internalized receptors and membrane to form an invagination (Bonifacino JS and Traub LM, 2003). Previous findings from our lab concerning the interferon alpha receptor subunit 1 (IFNAR1) revealed that the ubiquitination of IFNAR1 stimulates its endocytosis by promoting the recruitment of the AP-2 components. Since we established that PRLr is internalized by a clathrin-dependent process, we wanted to investigate the importance of the AP-2 adaptor protein complex in regulating the endocytosis of PRLr. In order to determine the importance of the AP-2 complex for the internalization of PRLr, we decided to investigate the interaction between the alpha subunit of the AP-2 adaptor complex and PRLr. To this end, we expressed FLAG-tagged $PRLr^{WT}$ or FLAG-tagged $PRLr^{S349A}$ in 293T cells alone or in conjunction with a control shRNA construct or a shRNA construct directed for the knockdown of β-TrCP. Immunoprecipitation assays using the whole cell extracts from these cells were performed using a FLAG-specific antibody. These immunoprecipitates were assayed by SDS-PAGE and immunoblots were incubated with anti-FLAG and anti-

AP-2 antibodies to determine the interaction between PRLr and the alpha subunit of AP-2 under various conditions. These results showed that the wild type PRLr bound to the alpha subunit of AP-2 (Figure 3.7). However, this interaction was lost when the Ser 349 of PRLr was mutated to alanine ($PRLr^{S349A}$) or β-TrCP was knocked down using a specific shRNA construct (Figure 3.7). These findings show that effective PRLr internalization, as mediated in a clathrin-dependent manner, requires receptor ubiquitination as evidenced by the need for an intact phosphodegron motif ($DS^{349}GRGS$) and active E3 ligase complex ($SCF^{\beta\text{-}TrCP}$), both of which are needed for effective PRLr ubiquitination.

These results taken together establish that the ubiquitination of PRLr is important for the internalization of the receptor. However, the nature of PRLr ubiquitination and whether the ubiquitin linkage specificity matters for PRLr internalization have yet to be determined. To understand the linkage specificity of PRLr ubiquitination and subsequent receptor internalization, we sought to determine how the specific linkages of PRLr ubiquitination affect the interaction of the AP-2 adaptor protein complex with PRLr. To this end, we expressed FLAG-tagged PRLr in 293T cells along with various ubiquitin constructs. These ubiquitin constructs were expressed to high levels so as to overtake the endogenous ubiquitin system resulting in ubiquitination of substrates utilizing the exogenous ubiquitin that has been expressed. Lysates from these cells were immunoprecipitated using an anti-FLAG antibody, assessed by SDS-PAGE, and immunoblots were blotted with anti-FLAG and anti-AP-2 alpha chain antibodies. If the interaction between the PRLr

and AP-2 was deficient in the presence of a given ubiquitin construct, then we can determine that the type of ubiquitination linkage which is absent in these cells is necessary in regulating the efficient internalization of PRLr. Our data showed that the specificity of ubiquitination is important for the PRLr-alpha adaptin interaction as optimal PRLr-AP-2 interaction was seen in the presence of K63 polyubiquitination (Figure 3.8). Samples expressing the Ub K0 null mutant and Ub R48K reverse knock-in mutant exhibited a weak PRLr-AP-2 interaction that was significantly restored to that in the presence of Ub WT when the Ub R63K mutant was expressed. Additionally, mutation of Lys 63 of ubiquitin (Ub K63R) greatly decreased the PRLr-AP-2 interaction compared to sample expressing Ub WT. While mutation of Lys 48 of ubiquitin also decreased PRLr-AP-2 interaction, this decrease was not to the extent of the Ub K63R sample. Furthermore, expression of Ub R48K reverse knock-in mutant did not increase the PRLr-AP-2 interaction compared to the Ub K0 lysine null mutant. These data taken together suggest that while K48 linkages are involved in PRLr ubiquitination and interaction with AP-2 adaptor protein complex, they are not crucial for PRLr-AP-2 interaction like K63 linkages. These results show that K63-linked polyubiquitination is crucial for the effective interaction of PRLr and AP-2 adaptor protein complex and implies that K63-linked polyubiquitination of PRLr is necessary for the effective internalization of PRLr.

The AP-2 adaptor protein complex has been shown to interact with internalized receptors and clathrin proteins to mediate clathrin-dependent endocytosis of cell surface receptors. Our data has shown that PRLr does interact

with AP-2 in an ubiquitination-dependent manner, for the purposes of receptor endocytosis. However, we have not established that AP-2 adaptor protein complex is necessary for the internalization of PRLr. We next sought to determine if PRLr endocytosis is dependent upon AP-2. To this end, we expressed a control siRNA or siRNA directed against AP-2 alpha chain in 293T cells along with untransfected cells and assessed the internalization kinetics of endogenous PRLr using the fluorescence-based endocytosis assay. If siRNA-mediated knockdown of AP-2 resulted in a significant inhibition of PRLr internalization, then we can conclude that PRLr endocytosis is highly AP-2-dependent. Our results from these studies indicated that knockdown of AP-2 significantly decreased the rate of PRLr internalization while expression of the control siRNA showed nearly identical internalization kinetics to those seen in untransfected cells (Figure 3.9). While PRLr in the presence of siRNA directed against AP-2 alpha chain did show low levels of receptor internalization, this low rate of internalization is similar to that seen in the absence of the ligand (Figure 2.4). These data taken together indicate that PRLr internalization is largely an AP-2-dependent process. These results are consistent with the idea that ubiquitination may stimulate PRLr endocytosis by promoting the interaction of PRLr with AP-2, which is essential for mediating the efficient internalization of PRLr in a clathrin-dependent manner.

Our data has shown that AP-2 and clathrin are required for mediating PRLr internalization. We have also shown that the interaction of PRLr and the AP-2 adaptor protein complex is dependent on K63-linked polyubiquitination. However,

we have yet to confirm the nature of ubiquitination of PRLr that is necessary for the endocytosis of PRLr. To determine the nature of PRLr ubiquitination that is required for the effective internalization of PRLr, we decided to investigate whether polyubiquitination was needed for PRLr endocytosis or whether monoubiquitination alone was sufficient. To this end, we expressed HA-tagged PRLr in conjunction with wild type ubiquitin (WT) or lysine null ubiquitin mutant (K0), which can only propagate monoubiquitination on target substrates, in 293T cells and assessed the internalization kinetics of exogenous PRLr using the fluorescence-based internalization assay. These studies showed that expression of the Ub K0 mutant (which lacks internal branching lysines and can only be used to monoubiquitinate a substrate) noticeably delayed the initial internalization of PRLr compared to wild type ubiquitin (Figure 3.10). These data indicate that polyubiquitination is necessary for efficient PRLr endocytosis and that monoubiquitination alone is not sufficient to drive this process.

While we have established that polyubiquitination is needed for efficient PRLr endocytosis, we do not know the nature of this polyubiquitination, the nature of its internal branching linkages, and whether linkage specificity is important for the internalization of PRLr. To investigate this, we decided to observe the internalization kinetics of PRLr in the presence of single point Lysine mutants of ubiquitin compared to the wild type and lysine null ubiquitin proteins. To this end, we expressed HA-tagged PRLr in the presence of Ub WT, Ub K48R, Ub K63R, or Ub K0 in 293T cells and assessed the internalization kinetics of PRLr using the

fluorescence-based internalization assay. These studies showed that PRLr internalization in the presence of Ub K48R was similar to that in the presence of wild type ubiquitin while expression of Ub K63R resulted in similar endocytic rates of PRLr as those in the presence of Ub K0 (Figure 3.11). These results indicate that the loss of K48-linked polyubiquitination did not affect PRLr internalization while loss of K63-linked polyubiquitination resulted in an attenuated rate of PRLr endocytosis, suggesting that K63-linked polyubiquitination and not K48-linked polyubiquitination is necessary for efficient PRLr endocytosis.

We wanted to confirm the role of K48-linked and K63-linked polyubiquitination and decided to investigate the endocytic rates of PRLr in the presence of the reverse knock-in mutants of ubiquitin. To this end, we expressed HA-tagged PRLr in conjunction with Ub WT, Ub R48K (K0 mutant with residue 48 reverted back to lysine), Ub R63K (K0 mutant with residue 63 reverted back to lysine), or Ub K0 lysine null mutant in 293T cells and observed the internalization rates of PRLr by utilizing the fluorescence-based internalization assay. These studies showed that while expression of the Ub R48K reverse knock-in slightly increased PRLr endocytic rates above those seen in the presence of Ub K0, re-expression of Lys 63 in Ub K0 (Ub R63K) restored the internalization rate of PRLr to that seen in the presence of wild type ubiquitin (Figure 3.12). This indicates that K63-linked polyubiquitination alone is sufficient to drive PRLr endocytosis. Taken together, these results show that expression of ubiquitin mutants that lack the internal Lys 63 (Figure 3.11, Ub^{K63R}; Figure 3.12, Ub^{R48K}) robustly attenuated the endocytic

rate of PRLr, indicating that K63-linked (but not K48-linked) polyubiquitination is required for the efficient internalization of PRLr.

3.5 Discussion

In **Chapter 3**, we showed that PRLr internalization is a clathrin-dependent process which utilizes and requires the AP-2 adaptor protein complex. The interaction of PRLr and the AP-2 complex is dependent upon Ser 349 phosphorylation and recruitment of β-TrCP, suggesting that the ubiquitination of PRLr is necessary for receptor-AP-2 interaction. Our results showed that K63-linked polyubiquitination specifically was needed for optimal PRLr-AP-2 interaction. The internalization of PRLr was shown to be dependent upon Ser 349 phosphorylation, subsequent recruitment of β-TrCP, and the ubiquitination of PRLr. The decreased internalization kinetics of non-ubiquitinated PRLr are not due to enhanced recycling kinetics but are likely due to a lack of interaction between ubiquitinated receptor and proteins of the endocytic machinery which can recognize the ubiquitinated receptor and target it for internalization. Furthermore, we showed that K63-linked polyubiquitination is needed for the efficient internalization of PRLr.

Conclusions of Chapter 3:

- PRLr internalization is a clathrin-dependent process
- PRLr internalization is dependent on the AP-2 adaptor protein complex
- Interaction of PRLr and AP-2 is dependent on Ser 349 phosphorylation, recruitment of active β-TrCP, and K63-linked polyubiquitination of PRLr

- β-TrCP is required for efficient PRLr endocytosis
- Ser 349 phosphorylation and subsequent ubiquitination of PRLr is necessary for efficient PRLr internalization
- Recycling of the receptor back to the cell surface does not occur during the initial stage of PRLr endocytosis
- PRLr is internalized in a K63-linked polyubiquitination dependent manner

We have established a developing model for PRLr downregulation whereby PRL binds to the receptor, the receptor undergoes a conformational change, and the constitutively bound Jak2 proteins become activated and phosphorylate the intracellular tyrosines of PRLr. PRLr is subsequently phosphorylated on its phosphodegron motif at Ser 349 by a yet to be identified kinase in a Jak2-dependent manner. This phosphorylation results in the recruitment of β-TrCP, which ubiquitinates PRLr. While efficient PRLr internalization requires K63-linked polyubiquitination, other substrates of the $SCF^{\beta\text{-TrCP}}$ E3 ligase complex including IFNAR1 (internalized in a K48- and K63-polyubiquitination dependent manner), β-catenin (K48-polyubiquitinated), and IκB (K48-polyubiquitinated) are differentially ubiquitinated. How can substrates of the same E3 ligase complex be ubiquitinated and degraded in a manner depending on differing ubiquitin linkages? While β-TrCP is responsible for recognizing and binding the substrates, it is the associated E2 ubiquitin-conjugating enzyme which confers the specificity of ubiquitination. It may

be that the K63-linked ubiquitin specific E2 Ubc13 may preferentially form a complex with $SCF^{\beta\text{-}TrCP}$ in the case of PRLr as opposed to other E2 enzymes which may preferentially form polyubiquitin chains of other linkages. In the case of β-catenin and IκB, which are K48-linked polyubiquitinated, it may be that the K48-linkage specific E2 Cdc34 may form in complex with $SCF^{\beta\text{-}TrCP}$ while $SCF^{\beta\text{-}TrCP}$ can form complexes with Ubc13 or Cdc34 on IFNAR1. These E2-E3 complexes may form differentially on certain substrates based on steric hindrance. The orientation of the $SCF^{\beta\text{-}TrCP}$ complex may be such that the space between the phosphodegron motif (recognized and bound by β-TrCP) and the target lysines of the substrate may sterically prefer one E2 enzyme over another. This theory could explain how these substrates of $SCF^{\beta\text{-}TrCP}$ could be differentially ubiquitinated despite sharing the same E3 ligase. Under this idea, the formation of the E3 ligase complex with Skip1, Cullin1, β-TrCP, the RING finger protein, the substrate, and the E2 enzyme would be a very dynamic complex that is constantly forming and reforming at the target substrate.

Alternatively, the specificity of ubiquitination required for the degradation of certain β-TrCP substrates could be conveyed by the interaction of proteins of the endocytic machinery which have ubiquitin-binding domains (such as UBDs or UIMs) which may bind the ubiquitin chains of certain substrates. Due to the conformation of proteins subsequent to ubiquitination, certain proteins with ubiquitin-binding domains may be able to complex with certain substrates depending on their ubiquitination status. While K48-linked polyubiquitin chains tend to have a

zig-zag structure, K63-linked polyubiquitin chains tend to have a more linear structure. The shape of these chains may regulate which proteins of the endocytic machinery (containing ubiquitin-binding domains) can interact with a given ubiquitinated substrate. Such proteins of the endocytic machinery may also convey specificity for whether an internalized receptor, such as PRLr, are efficiently sorted for lysosomal degradation or recycled back to the cell surface. Studies investigating and testing these possibilities of how polyubiquitin chains of a certain linkage type are added could have great implications for the ubiquitination field as a whole.

Since our studies on PRLr internalization indicated that optimal endocytosis occurred in the presence of K63-linked polyubiquitination, it may be that ubiquitin chains of different linkages can be differentially recognized by the endocytic machinery. This would allow for certain receptors to be targeted for a unique signaling role or for degradation by the lysosome or proteasome. In the case of PRLr, effectively K63-polyubiquitinated PRLr that is internalized does not seem to signal (data not shown) within the endosome contrary to what has been shown for other proteins such as the nerve growth receptor TrkA (Geetha T et al., 2005). How the cell can recognize differential ubiquitin linkages is unknown and remains a mystery. However, the specific ubiquitination of PRLr that is necessary for receptor internalization indicates that there is a specified program for receptor downregulation and for controlling downstream signaling and keeping it in check.

These studies are significant in that they show that the endocytosis of PRLr is primarily a clathrin-dependent process, contrary to the conclusions established by

Piazza TM et al. that PRLr internalization is caveolin-dependent (Piazza TM et al., 2009). The work done by Piazza TM et al. studied PRLr endocytosis by looking at the internalization of radiolabeled ligand. Our work utilized the fluorescence-based internalization assay which directly studies the endocytosis of PRLr in an accurate and precise way. While our studies establish that PRL-mediated internalization of PRLr is primarily a clathrin-dependent process, it is possible that an alternative yet minor caveolin-dependent (and likely Src-dependent) mechanism for PRLr endocytosis exists.

This work also establishes that ubiquitination of the PRLr is necessary for the efficient endocytosis of the receptor. A previous report by Lu JC et al., that used a Chinese hamster lung cell line ts20 (which contains a thermolabile ubiquitin-activating enzyme E1) that stably expressed wild type human PRLr, concluded that PRLr internalization was independent of ubiquitination (Lu JC et al., 2005). These stable lines were cultured at the permissive temperature (30^{o}C; active E1 enzyme) or the non-permissive temperature (42^{o}C; inactive E1 enzyme) and treated with radiolabeled and unlabeled bovine placental lactogen. Based on the ratio of radiolabeled ligand that internalized, they concluded that the internalization of human PRLr is not dependent on ubiquitination. However, it has been shown that receptor internalization can be very cell type-specific so the study of human PRLr internalization in Chinese hamster lung cells does not replicate what occurs in human cells. Furthermore, the measurement of internalized radiolabeled ligand (which in this case is further complicated by the use of bovine placental lactogen instead of

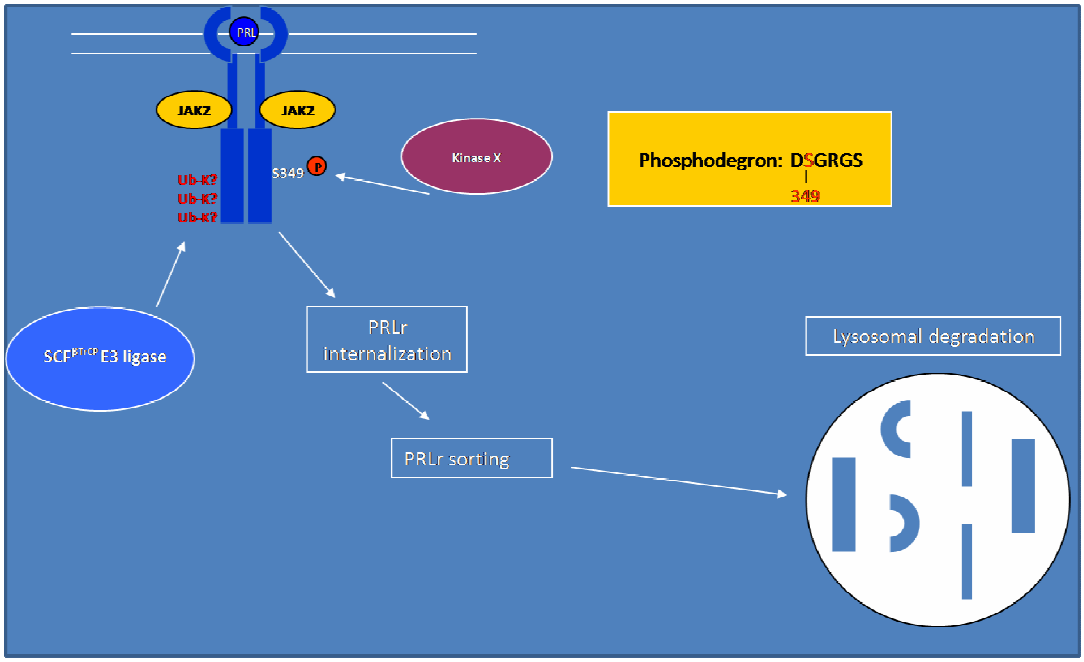
human prolactin) is not an accurate way to measure the internalization of a given receptor. Radiolabeled ligand can internalize due to phagocytosis or non-specific interaction of the ligand with a cell surface protein different than the receptor of interest. The disappearance of PRLr from the cell surface should be directly measured to get an accurate estimation of the amount of endocytosed PRLr. In light of these caveats, the conclusion made by the authors stating that ubiquitination is not important for PRLr endocytosis must be discounted. Our work is significant in that it shows that PRL-mediated endocytosis of PRLr is a process dependent on receptor polyubiquitination, specifically of the K63 linkage. These studies favor the view in the field of PRLr downregulation that receptor ubiquitination plays a key and important role. These studies establish for the first time that PRLr internalization is dependent on K63 polyubiquitination of the receptor. This is in agreement with previous studies showing the importance of Ser 349 phosphorylation, β-TrCP interaction, and receptor ubiquitination in the degradation of PRLr.

One viable way to control tumorigenic growth within the breast which is promoted by elevated PRL signaling would be to promote the downregulation of PRLr. It has been shown that primary breast cancer tissues and breast cancer cell lines exhibit elevated PRLr levels and a deficiency in Ser 349 phosphorylation of the PRLr (Li Y et al., 2006). Other studies from our lab have shown that the cannibinoid anandamide can inhibit the growth of breast cancer cell lines by promoting Ser 349 phosphorylation and subsequent PRLr degradation (Plotnikov A et al., 2009). The use of anandamide-like molecules may prove to be a viable treatment for breast

cancers that exhibit aberrant PRL signaling as it would promote Ser 349 phosphorylation without promoting active PRL signaling.

Having established that PRLr is internalized in a manner dependent on K63-linked polyubiquitination and then subsequently degraded, the following question remains to be answered: what are the consequences of the deregulation of PRLr levels? If this regulated program of PRLr internalization and downregulation does not occur, what effect do elevated PRLr levels have upon cell growth? These questions are addressed in further detail in **Chapter 4**.

Model 2



Model 2: Stimulation of PRLr with PRL leads to Jak2 activation, which promotes the phosphorylation of Ser 349 within the conserved phosphodegron motif of PRLr. The β-TrCP E3 ligase is then recruited and ubiquitinates PRLr at target lysine residues. This ubiquitination (specifically K63-linked polyubiquitination) promotes the internalization and subsequent degradation of the PRLr.

Figure 3.1

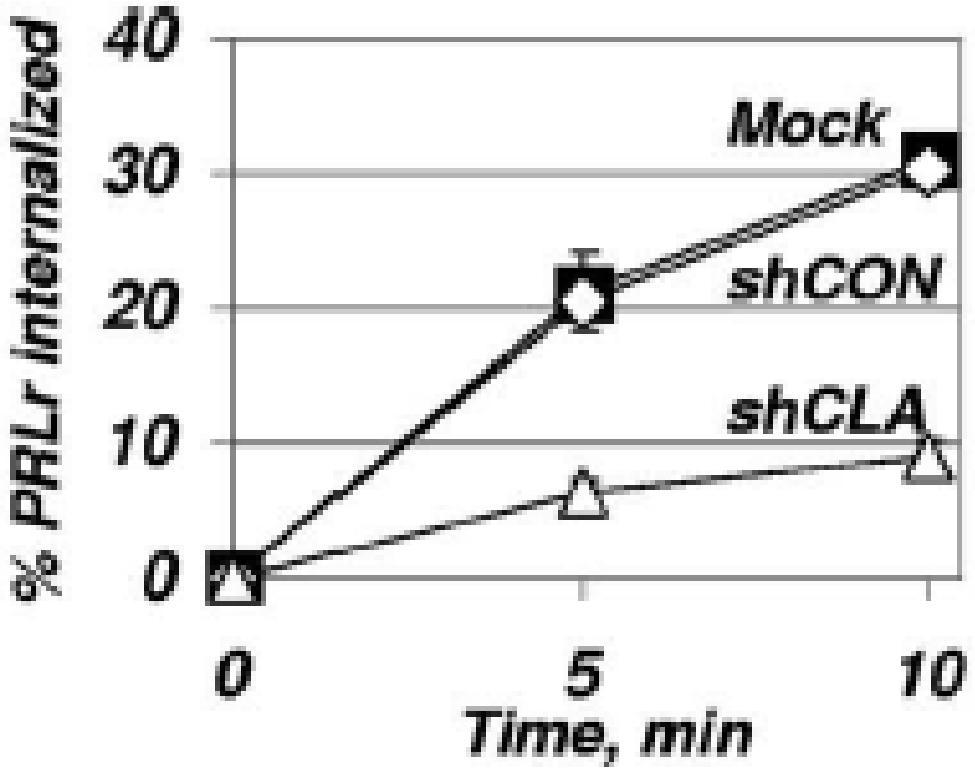


Figure 3.1: Efficiency of the clathrin-dependent internalization of PRLr is dependent on PRLr ubiquitination. Internalization of endogenous PRLr in 293T cells that were left untransfected (Mock [squares]) or were transfected with shRNA against clathrin heavy chain (shCLA) or green fluorescent protein (shCON [diamonds]) analyzed by the fluorescence-based assay using anti-PRLr antibody as outlined in Materials and Methods.

Figure 3.2

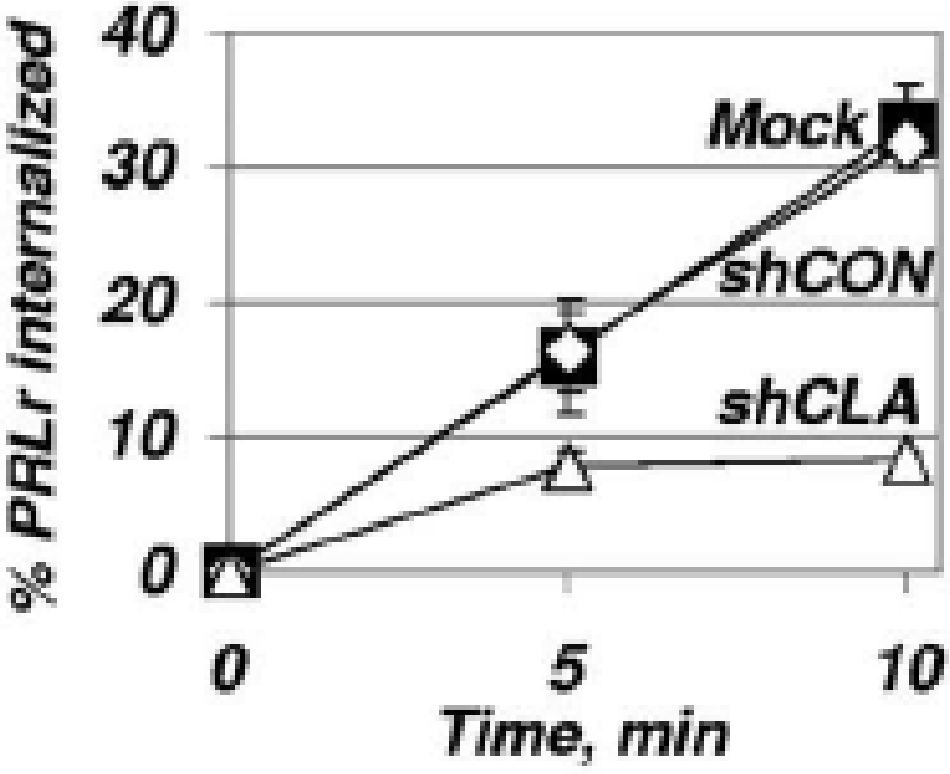


Figure 3.2: Internalization of HA-tagged $PRLr^{WT}$ exogenously expressed in 293T cells that were left untransfected (Mock [squares]) or were transfected with shRNA against heavy-chain clathrin (shCLA) or green fluorescent protein (shCON [diamonds]) analyzed by the fluorescence-based assay using anti-HA antibody as outlined in Materials and Methods.

Figure 3.3

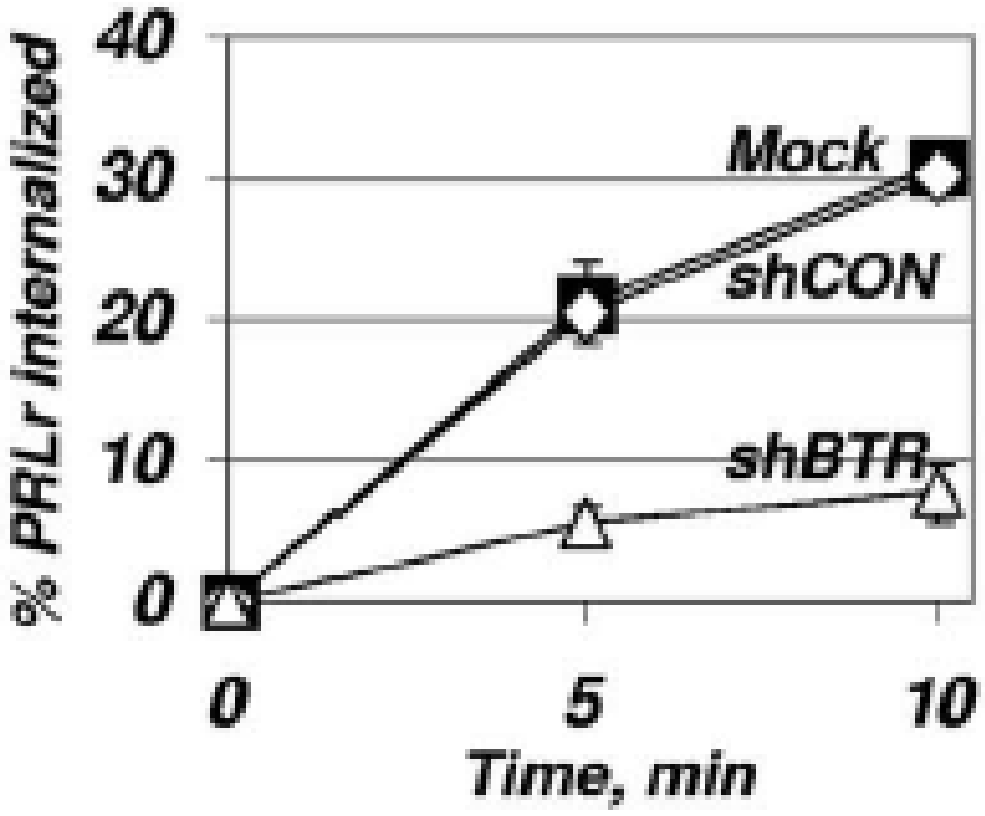


Figure 3.3: Internalization of endogenous PRLr in 293T cells that were left untransfected (Mock [squares]) or transfected with shRNA against β-TrCP2 (shBTR) or green fluorescent protein (shCON [diamonds]) analyzed as outlined for Figure 3.1.

Figure 3.4

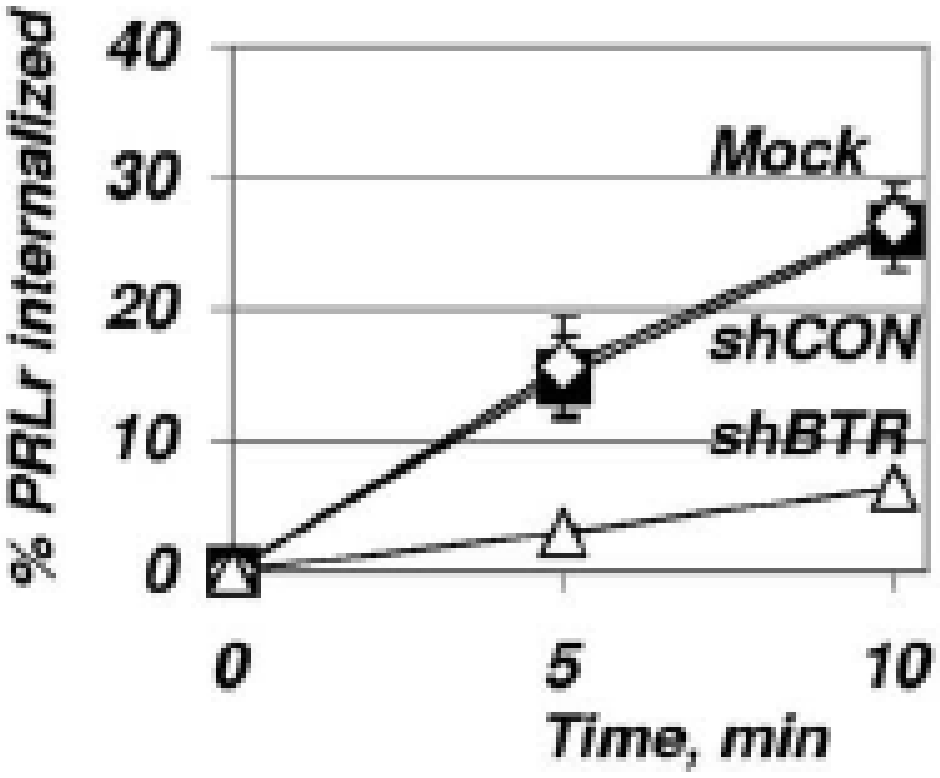


Figure 3.4: Internalization of HA-tagged PRLrWT exogenously expressed in 293T cells that were left untransfected (Mock [squares]) or transfected with shRNA against β-TrCP2 (shBTR) or green fluorescent protein (shCON [diamonds]) analyzed as outlined for Figure 3.2.

Figure 3.5

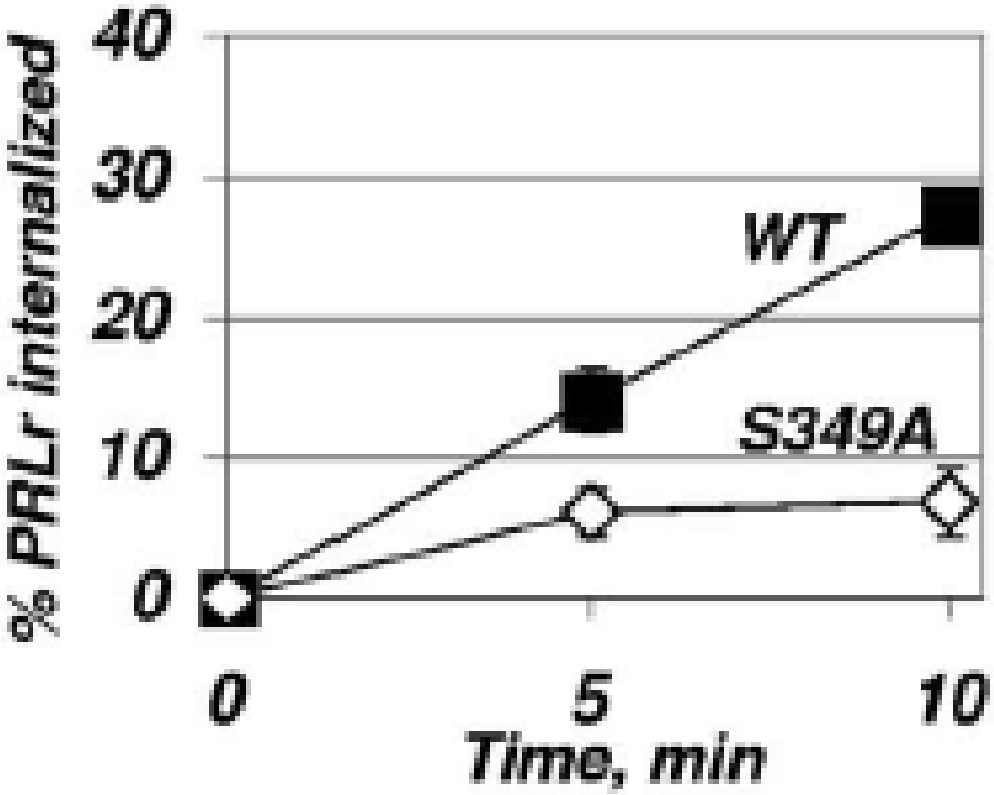


Figure 3.5: Internalization of a WT or ubiquitination-deficient S349A mutant of HA-tagged PRLr expressed in 293T cells was analyzed as described for Figure 3.2.

Figure 3.6

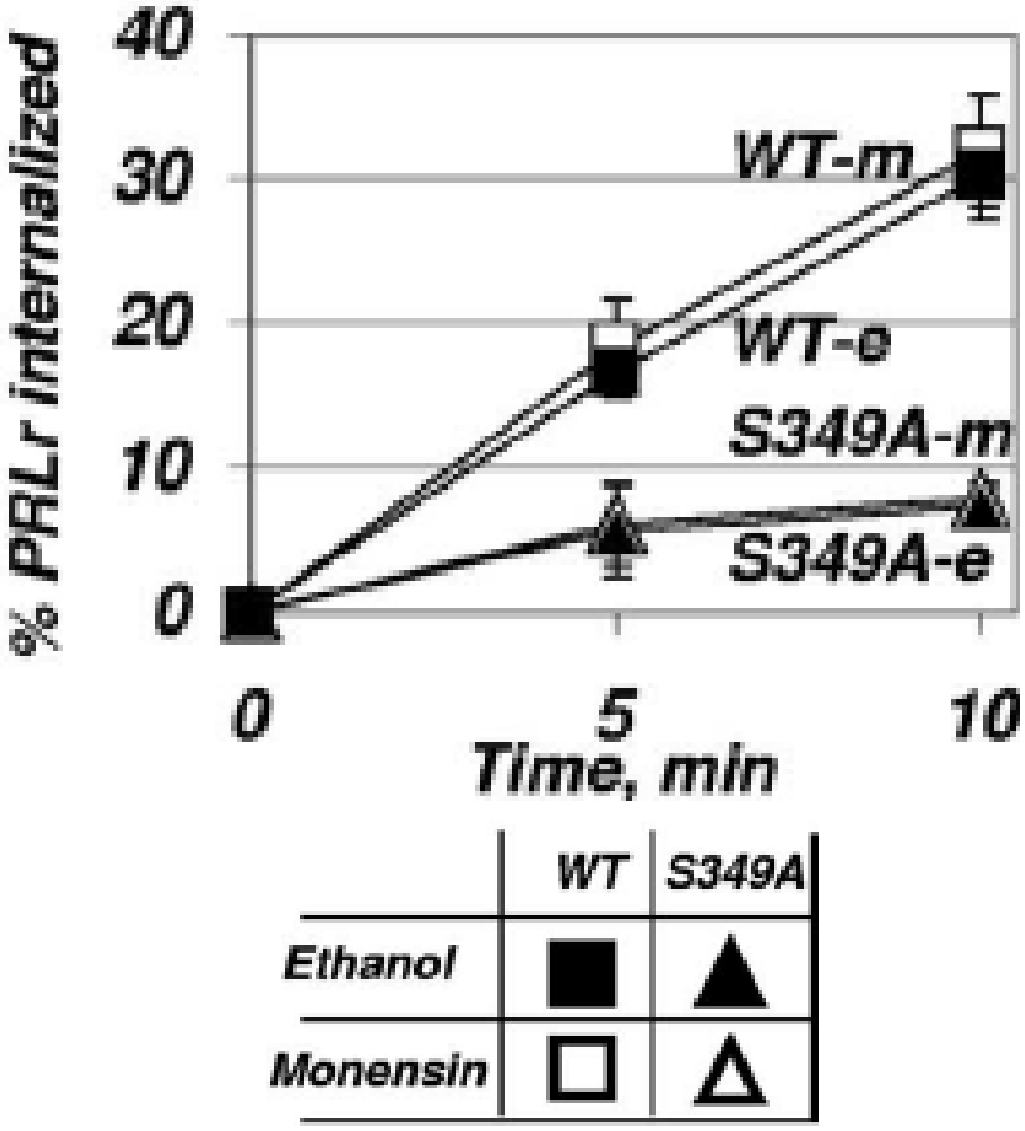


Figure 3.6: Rates of internalization of WT or ubiquitination-deficient S349A mutant HA-tagged PRLr expressed in 293T cells pretreated with 100 μM monensin (-m) or vehicle (ethanol) (-e) were analyzed as described for Figure 3.2.

Figure 3.7

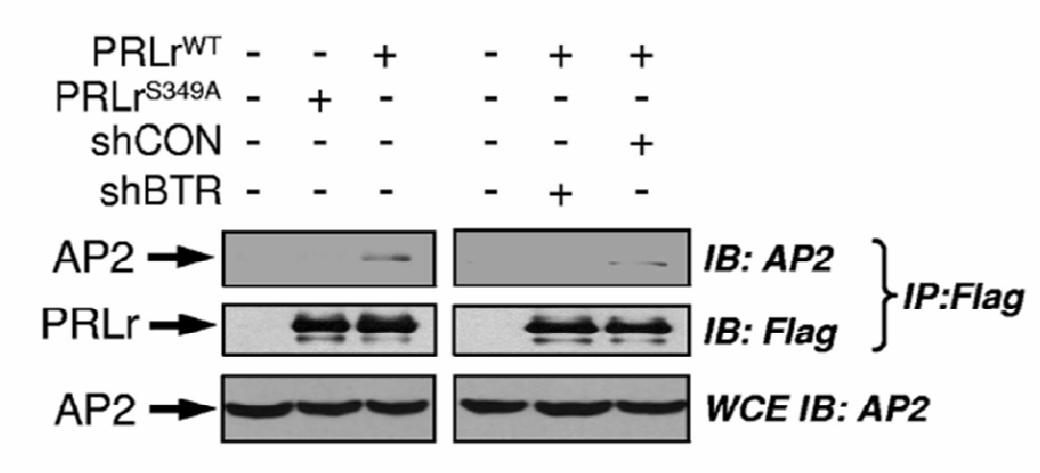


Figure 3.7: Ubiquitination of PRLr promotes its interaction with components of AP-2, which are required for PRLr internalization. Interactions between Flag-tagged PRLr (WT or S349A mutant) and the endogenous alpha subunit of the AP-2 complex in 293T cells (transfected with the indicated plasmids and treated with PRL [50 ng/ml for 5 min]) were analyzed by immunoprecipitation (IP) using M2 anti-Flag antibody followed by immunoblotting (IB) using the indicated antibodies. Material used for immunoprecipitation was normalized to yield comparable levels of PRLr in all lanes. Levels of the alpha subunit in whole-cell extracts (WCE) were also assessed. Experiment was performed by Christopher Carbone.

Figure 3.8

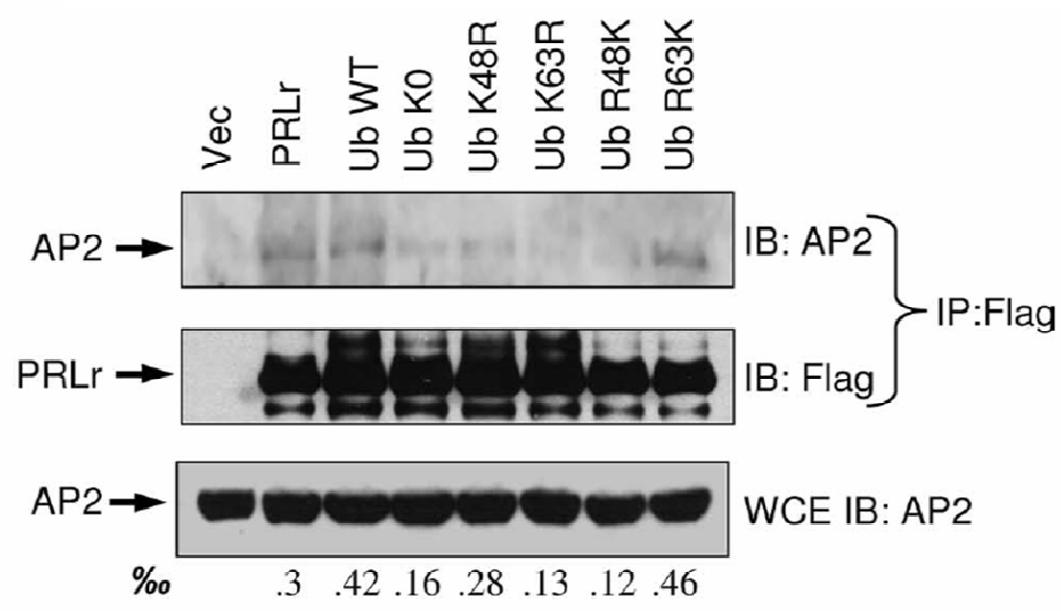


Figure 3.8: Interaction of Flag-tagged PRLr with an endogenous alpha-adaptin complex in 293T cells expressing the indicated ubiquitin constructs was analyzed by immunoprecipitation (IP) using M2 anti-Flag antibody followed by immunoblotting (IB) using the indicated antibodies. Material used for immunoprecipitation was normalized to yield comparable levels of PRLr in all lanes. Levels of the alpha subunit in whole-cell extracts (WCE) were also assessed. The results obtained with a fraction of alpha-adaptin bound to PRLr (per mille) are depicted in the bottom panel, Vec, vector; IB, immunoblotting; WCE, whole-cell extract.

Figure 3.9

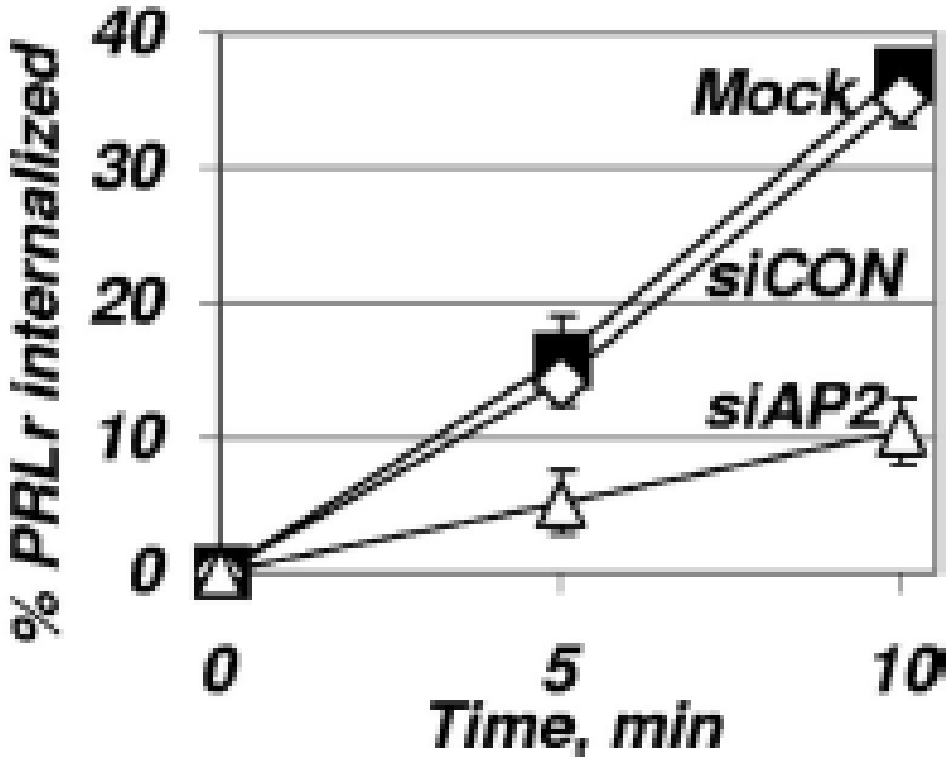


Figure 3.9: Internalization of endogenous PRLr in 293T cells that were left untransfected (Mock [squares]) or were transfected with small interfering RNA against the α-adaptin subunit of AP-2 (siAP2) or luciferase (siCON [diamonds]) was analyzed by the fluorescence-based assay using anti-PRLr antibody as outlined for Figure 3.1.

Figure 3.10

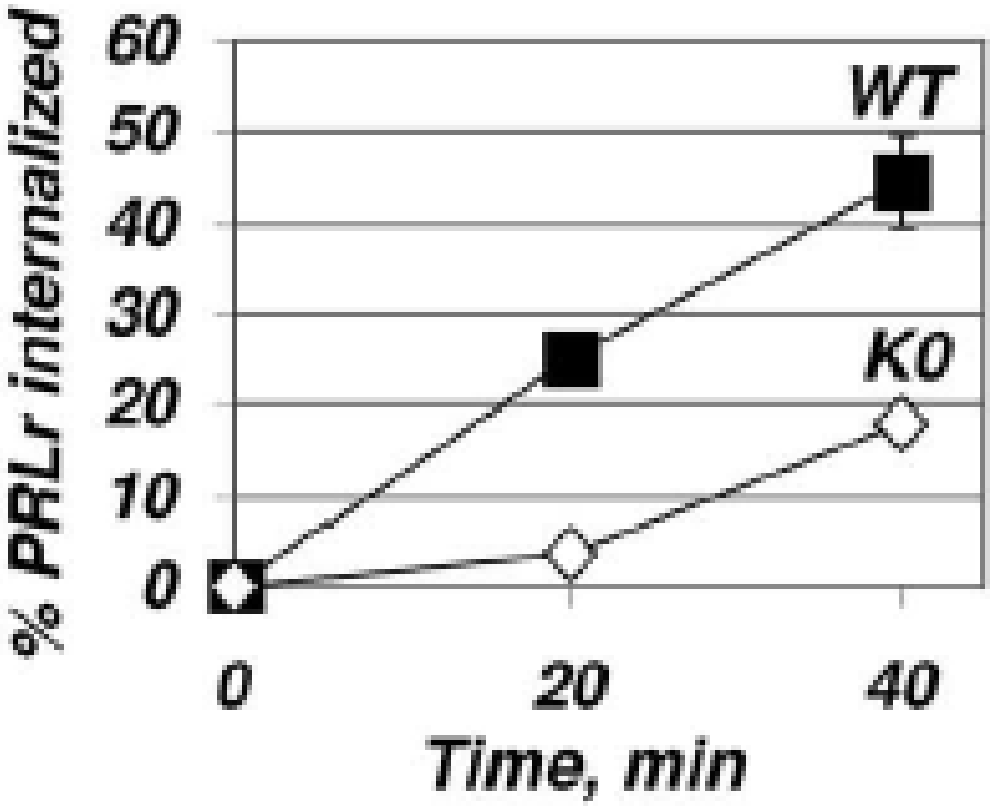


Figure 3.10: Internalization of HA-tagged PRLr co-expressed with either WT ubiquitin or K0 mutant in 293T cells was analyzed as described for Figure 3.2.

Figure 3.11

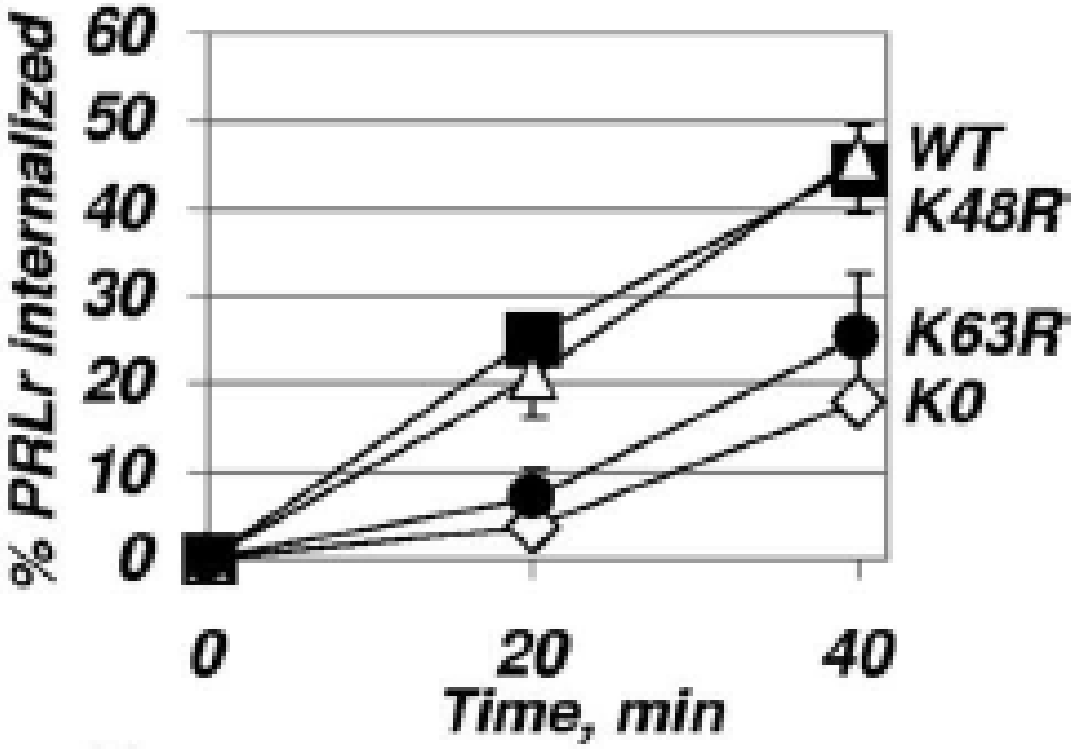


Figure 3.11: Internalization of HA-PRLr co-expressed with the indicated direct ubiquitin mutants was measured as described for Figure 3.2. WT ubiquitin, squares; K48R, open triangles; K63R, closed circles; K0, open diamonds.

Figure 3.12

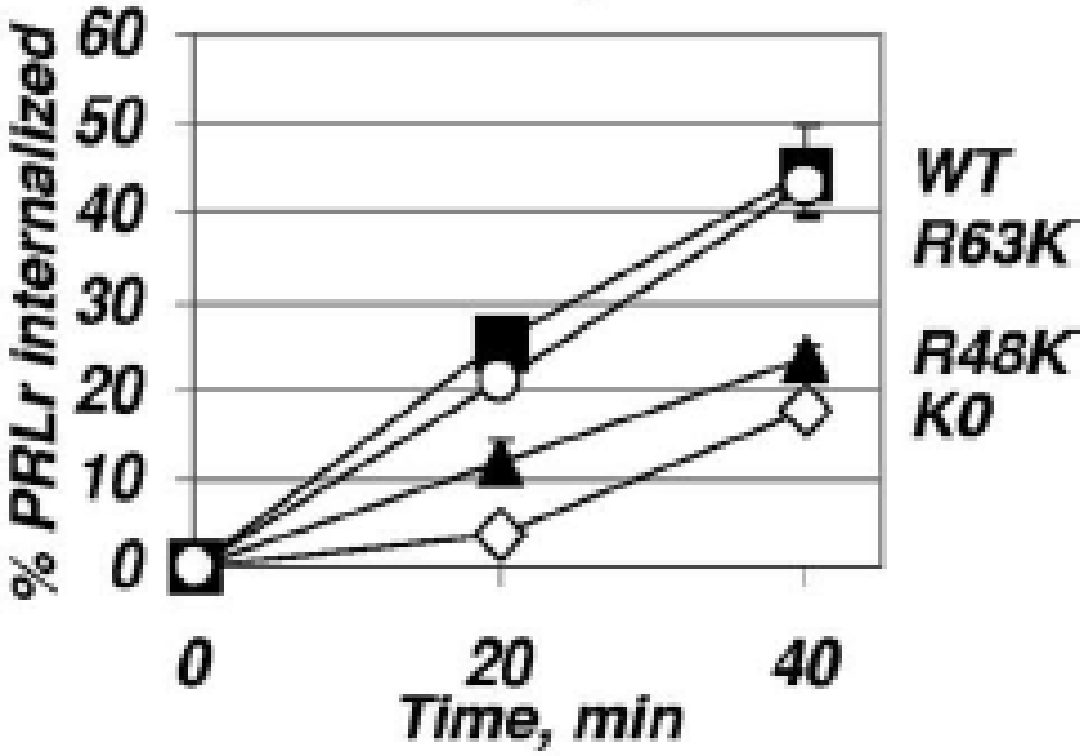


Figure 3.12: Internalization of HA-PRLr co-expressed with the indicated reverse ubiquitin mutants was measured as described for Figure 3.2. WT ubiquitin, squares; R63K, open circles; R48K, closed triangles; K0, open diamonds.

Chapter 4

Elevated PRLr levels promote cell growth

4.1 Abstract

Signaling by the polypeptide hormone prolactin (PRL) is mediated by its cognate receptor (PRLr). PRLr is commonly stabilized in human breast cancer due to decreased phosphorylation of residue Ser 349, which when phosphorylated recruits the β-TrCP E3 ubiquitin ligase and facilitates PRLr degradation. Impaired PRLr turnover results in an augmented PRL signaling and PRL-induced transcription. In **Chapter 4**, we show that human mammary epithelial cells harboring degradation-resistant PRLr display accelerated proliferation and increased invasive growth. Conversely, a decrease in PRLr levels achieved by genetic means in human breast cancer cells dramatically reduced transformation and tumorigenic properties of these cells. The consequences of the alteration of PRLr turnover for the homeostasis of mammary cells and development of breast cancers is evidenced by enhanced cell growth properties both *in vitro* and *in vivo*.

4.2 Introduction

Malignant transformation of cells and development of tumors result from several key events that include stimulation of cell proliferation and inhibition of cell death. The pituitary hormone prolactin (PRL), which is also secreted by mammary

epithelia, plays a central role in mammary gland development and function. In addition, several lines of evidence strongly implicate the role of PRL in breast tumorigenesis (Clevenger CV et al., 2003). First, PRL promotes growth of human breast cancer cells acting as a survival agent and as a mitogen (Das R and Vondehaar BK, 2007; Perks CM et al., 2004), and up to 95% of primary human breast cancers are positive for PRL and its receptor, PRLr (Reynolds C et al., 1997; Gill S et al., 2001; Bhatavdekar JM et al., 2000). Second, transgenic mice locally expressing PRL within mammary epithelia develop tumors (Wennbo H et al., 1997; Rose-Hellekant TA, 2003), whereas genetic ablation of PRLr severely delays the development of SV40 large T antigen–induced breast carcinomas (Oakes SR et al., 2007). Third, mutant PRLrs that are characterized by high levels of constitutive signaling have been recently identified in human breast tumors (Canbay E et al., 2004; Bogorad RL et al., 2008). Finally, epidemiologic studies link elevated levels of circulating PRL with increased risk of breast cancer (Hankinson SE et al., 1999; Tworoger SS et al., 2004) and its metastases (Mujagic Z and Mujagic H, 2004), as well as with decreased taxane therapeutic efficacy (Frontini L et al., 2004; Lissoni P et al., 2002; Lissoni P et al., 2001) that could be reversed by pharmacologic suppression of PRL levels (Lissoni P et al., 2002).

Because a high proportion of human breast cancer cells secrete their own PRL, the autocrine effects of PRL may account for the limited success of inhibitors of pituitary PRL synthesis/release against human breast cancers (Clevenger CV et al., 2003). Antagonists of PRLr kill human breast cancer cells in vitro and abrogate

the tumorigenesis in the xenograft models, showing that persistent signaling induced by locally secreted PRL is essential for growth and survival of these cells (Goffin V et al., 2005; Fuh G, Wells JA., 1995). However, PRL also induces proteolytic degradation of PRLr via receptor ubiquitination facilitated by the $SCF^{\beta\text{-}TrCP}$ E3 ubiquitin ligase that is recruited to the substrate in a manner that requires phosphorylation of Ser 349 within the phosphorylated degron (Li Y et al., 2004; Swaminathan G et al., 2008). Given that this ligand-induced PRLr down-regulation limits the extent of PRL signaling, it is not clear how PRL maintains the survival of breast cancer cells. Whereas levels of PRLr are decreased in the breast cancer intratumoral stromal compartment, the levels of PRLr in tumor cells are not decreased in comparison with benign mammary cells (Reynolds C et al., 1997; Clevenger CV et al., 1995), suggesting a possibility that down-regulation and degradation of PRLr in tumor cells might be impaired. Indeed, we have reported that phosphorylation of PRLr on Ser 349 within its phosphorylated degron is impaired in breast cancer cells and tissues that exhibit increased stability of PRLr and ensuing high levels of its expression (Li Y et al., 2006).

In this Chapter, we sought to investigate the outcomes of PRLr stabilization in breast cancer. Our studies reveal that abrogation of PRLr phosphorylation on Ser 349 in near-normal human mammary epithelial cells contributes to the development of a transformed phenotype. These studies suggest that decreasing the levels of PRLr in human breast cancer cells is detrimental for their growth, invasion, and tumorigenicity. Collectively, these findings suggest that an altered degradation (and

resulting accumulation) of PRLr might play a role in human breast cancers and could be targeted for anticancer therapies.

4.3 Materials and Methods

Cell lines, DNA constructs, and chemicals.

MCF10AΔp53 derivative cell line, in which p53 expression is knocked down (MCF10A) were a generous gift of Alan Eastman (Levesque AA et al., 2005). Generation of the MCF10AΔp53 cells stably expressing wild type or S349A mutant PRLr was previously described (Yamashita H et al., 2006). Human breast cancer MCF7 cells were a gift from Ze'ev Ronai (Burnham Institute, San Diego, CA). Human recombinant PRL was kindly provided for a fee by Dr. A.F. Parlow (National Hormone and Peptide Program, Bethesda, MD).

Analysis of cell growth, invasion, and tumorigenesis.

Growth in a two-dimensional culture was analyzed using the staining with trypan blue. The number of live cells in each well was counted. Results from three independent experiments are presented as average $\pm$ SE. For the analysis of cell growth in a three-dimensional culture, cells were mixed with Matrigel Basement Membrane Matrix (BD Biosciences) and cultured in complete medium for indicated number of days. Invasion assays were done in Boyden chambers supplied with polyethylene terephthalate filter inserts containing 0.8-um pores (BD Company). Filters were coated on ice with 50 AL of Matrigel Basement Membrane Matrix and incubated for 30 min (37 degrees C). MCF10AΔp53-derived or T47D-derived cells (5×10^4) were plated in 300 μL of Matrigel (diluted in 0.1% bovine serum albumin–

DMEM/F-12, 1:3) into the upper chamber. The lower chamber was filled with 700 µL of DMEM/F-12 medium supplied with 10% fetal bovine serum. Non-invaded cells in the inserts were removed with cotton swabs after 48 h of incubation. Invaded cells on the underside were fixed with absolute methanol for 2 min, stained with H&E solution (Sigma), and photographed using either 5x or 10x objectives. Tumorigenesis assays were carried out in NCRNU-M (Taconic) or in NSG mouse model (NOD-SCID, IL2Rgnull; The Jackson Laboratory) female mice, which also obtained pellets of 17beta-estradiol and PRL (purchased from Innovative Research of America). Cells were implanted s.c. or into abdominal mammary glands, and the growth of tumors was measured by caliper at indicated days after cell injection. Signal quantification and statistical analysis. Digital images were processed with Adobe Photoshop 7.0 software. For some experiments, band intensities and percentage of surface covered by cell growth were quantified by densitometry (ImageJ software). The statistical differences were analyzed using two-tailed Student's t test.

4.4 Results

In **Chapter 4**, we sought to determine the effect of stabilized PRLr on cell growth and whether elevated levels of PRLr can contribute to the transformation of human mammary epithelial cells. We first wanted to investigate elevated levels of PRLr had any consequence for cell growth. To this end, we studied the growth kinetics of MCF10AΔp53 mammary epithelial cell lines that stably expressed wild

type PRLr, the proteolytically stable S349A mutant of PRLr, or a puro-resistance vector as a control. In these studies, we observed that MCF10AΔp53 derivatives that express stabilized PRLr (S349A) grow faster in tissue culture than those expressing wild type PRLr, which showed greater growth kinetics than cells expressing the control vector (Figure 4.1). These results suggest that elevated levels of PRLr promote cell growth in a 2-D culture system.

We next wanted to investigate whether elevated levels of PRLr would promote 3-dimensional growth, suggestive of the ability of these cells to grow in 3 dimensions like a tumor would grow. To this end, the MCF10AΔp53 cell lines previously used (Figure 4.1) were grown in three-dimensional cultures in Matrigel and pictures of the cell structures that grew were used to study the morphology. Through these studies, the analysis of cell growth in three-dimensional cultures in Matrigel revealed significant differences in both the rate of growth and morphology between all examined cell types. Whereas vector-transduced puro cells grew slowly and formed well-defined spherical aggregates, WT cells formed numerous smaller spheroids. Remarkably, cells expressing the S349A PRLr mutant rapidly deviated from spherical growth to a pattern of irregular and poorly defined masses, forming a network of branches and meshes and, eventually, filling the entire culture space (Figure 4.2). Three other independent S349A individual clones displayed similarly fast tumor-like growth and morphology (data not shown), indicating that differences in cell growth were not clone specific but mediated by the S349A mutant of PRLr. In

summary, these data indicate that the increased stability of PRLr contributes to a transformed phenotype in human mammary epithelial cells.

Aggressive and irregular growth of S349A cells in Matrigel point to changes in their ability to grow invasively. We next sought to determine whether elevated PRLr levels enhanced the invasive properties of mammary epithelial cells. To this end, MCF10AΔp53-derived cell lines used previously (Figure 4.1; Figure 4.2) were allowed to migrate through Boyden chambers in order to provide an indication of their invasiveness. Indeed, these *in vitro* invasion assays revealed a superior ability of S349A cells (compared with WT cells which were more invasive than puro cells) to penetrate through Matrigel and insert pores in Boyden chamber assays (Figure 4.3). Cell motility and invasiveness is a complex process positively regulated among others by pathways that involve mitogen-activated protein kinase (MAPK), PI3K, and Rho family GTPases, all of which are activated by PRL (Clevenger CV et al., 2003). These data suggest that stabilization and increased levels of PRLr in breast cells contribute to a transformed *in vitro* phenotype, which is reflected by accelerated cell growth and increased motility and/or invasive abilities.

While having established the importance of PRLr levels in promoting 2-D growth, 3-D growth, and invasiveness of breast epithelial cells, we wondered whether this propagation towards a transformed phenotype was an merely an *in vitro* phenomenon or this transformation could be further demonstrated *in vivo*. To this end, we compared the tumorigenic growth of various MCF10AΔp53 derivatives (used previously in Figure 4.1) injected into the flanks of the NCRNU-M

immunocompromised mice that were implanted with pellets releasing estradiol and PRL. MCF7 breast cancer cells served as a positive control. These experiments showed that the MCF7 cells grew rapidly and continuously; the mice that were injected with these cells developed large tumors and had to be sacrificed by day 24. Although MCF10AΔp53 derivatives displayed a period of growth and formed distinct tumors, this growth was relatively short and was followed by tumor regression within 4 weeks after injection. Intriguingly, tumor regression proceeded significantly slower in S349A cells compared with either WT or puro cells ($P < 0.05$; Figure 4.4). Similar results were obtained when NSG immunodeficient mice were used as hosts on either intraflank or intramammary gland injection of human cells (data not shown). These data suggest that stabilization of PRLr promotes the growth of MCF10AΔp53 cells *in vivo* but is not sufficient for maintaining the tumorigenic phenotype.

4.5 Discussion

Whereas numerous epidemiologic and experimental data support important roles of PRL signaling in human breast cancers, the mechanisms that lead to constitutive activation of PRLr signaling that occur in primary human mammary tumors are poorly understood. Recent identification of gain-of-function mutations in PRLr in women with benign breast tumors (Bogorad RL et al., 2008) and the fact that PRLr levels are elevated in human breast carcinoma (Reynolds C et al., 1997) suggest that PRLr and PRL signaling are conducive to tumor cell growth and

survival in at least a subset of breast cancer cases. However, besides activating diverse signaling pathways, PRL also stimulates down-regulation of its own receptors. We previously found that PRLr is stabilized in some human breast cancers and tissues due to an impaired phosphorylation of PRLr on Ser 349, which is required for recruitment of β-TrCP ubiquitin ligase followed by PRLr ubiquitination and degradation (Li Y et al., 2004; Li Y et al., 2006).

Here, we investigated the consequences of PRLr stabilization and accumulation that was expected to contribute to elevated PRL signaling (Li Y et al., 2006). In this study we used the approach of investigating the stabilization of PRLr in nontumorigenic mammary epithelial cells by expressing the PRLr S349A mutant. Data from experiments using this approach clearly show that increased levels of PRLr in human mammary cells play a key role in developing and maintaining their transformed phenotype. These data indicate that human breast cancers gain growth and invasive advantages by stabilizing the PRLr and suggest that PRL signaling, in general, and regulation of PRLr, in particular, are important for mammary tumorigenesis. This hypothesis is consistent with the published data showing that knockout of PRLr in mice prevents mammary tumorigenesis induced by SV40 large T antigen (Oakes SR et al., 2007) and that either PRLr antagonists (Goffin V et al., 2005) decrease levels of PRLr and suppress growth of human breast cancer cells.

Conclusions of Chapter 4:

- Elevated levels of PRLr enhance 2-dimensional culture growth
- Elevated PRLr levels promote irregular 3-dimensional cell growth

- PRLr stabilization increases cell invasiveness
- Stabilized PRLr increases the tumorigenicity of mammary epithelial cells *in vivo*

In studies looking at the 2-dimensional culture growth of MCF10AΔp53 breast epithelial cell lines that stably expressed a control puro vector, wild type PRLr, or the stable S349A mutant of PRLr, we noticed that stable expression of the S349A mutant of PRLr resulted in a significant growth advantage by 24 hours in culture (Figure 4.1). These data indicate that elevated PRLr levels can promote 2-dimensional growth of these cells. However, the rate of growth of the WT and S349A cell lines from 24 to 48 hours is nearly identical despite the elevated levels of PRLr found in the S349A stable line compared to the WT stable line. It is likely that early on, elevated PRLr levels provide a significant growth advantage as evidenced by the growth of the S349A cell line within the first 24 hours. However, this increase in cell proliferation results in the quick utilization of nutrients which prevents an increase in the rate of growth of these cells from 24 to 48 hours. Additionally, this cell growth results in an increase in cellular waste products which result in acidification of culture media and prevent efficient cell growth. These factors prevent the exponential increase in growth rate of the S349A stable line. However, the WT stable line by 24 hours is able to seed and form necessary cell-cell attachments and from 24 to 48 hours is able to catch up and recapitulate the growth rate seen of the S349A cells. Despite the experimental limitations of these cell culture studies, experiments looking at the growth rate of these cell lines in 3-D

culture, *in vivo*, and their invasive properties confirm to us the pro-tumorigenic effects of elevated PRLr levels.

Whereas our current data clearly point to the importance of maintaining PRLr levels for breast cancer cell tumorigenicity, future studies should reveal additional genetic events that cooperate with stabilized PRLr during formation of tumors. Although stabilization of PRLr, along with knockdown of p53 tumor suppressor protein, temporarily allowed near-normal MCF10A to grow in nude mice, these genetic changes were clearly insufficient to sustain tumorigenesis (Figure 4.4). Given an aggressive phenotype of these cells *in vitro*, they seem to lack a systemic factor when implanted in mice. It is plausible that activation of other oncogenes (e.g., c-Myc) is required for angiogenesis in these tumors; under this scenario, stabilized PRLr is likely to promote survival of tumor cells deprived of nutrition and oxygen. On the other hand, the fact that expression of stabilized PRLr slowed down tumor regression may reflect prolonged PRL signaling, which might be insufficient in transplanted human cells given that mouse PRL poorly activates human PRLr. Generation of human PRL knock-in mice will enable testing of this possibility.

These studies are significant in that they establish the importance of elevated PRLr levels in promoting the growth, invasiveness, and tumorigenicity of mammary epithelial cells. A deregulation of PRLr levels leading to aberrant PRL signaling may be a key contributing factor to the development and progression of breast cancers. While elevated PRL signaling has been shown to occur in many breast cancers, these data confirm the importance of stabilized PRLr levels in promoting

this hyperactive signaling. However, the mechanism by which PRLr propagates these pro-tumorigenic phenotypes is not completely understood. While PRL signaling results in the upregulation of cyclin D1, the downregulation of p21, p27, Bad, and caspase 9, the tumorigenic effects of PRL signaling are likely mediated through other additional downstream effectors. In order to investigate the mechanism by which PRLr promotes tumorigenicity, we performed a proteomics-based screen to identify novel interactors of PRLr which may play a role. In this screen, we identified pyruvate kinase M2, or PKM2, and in **Chapter 5** we investigate how PKM2 works in conjunction with PRLr to promote a PRL-induced tumorigenic phenotype; this explores one such mechanism by which PRLr enhances cell growth and tumorigenicity.

Figure 4.1

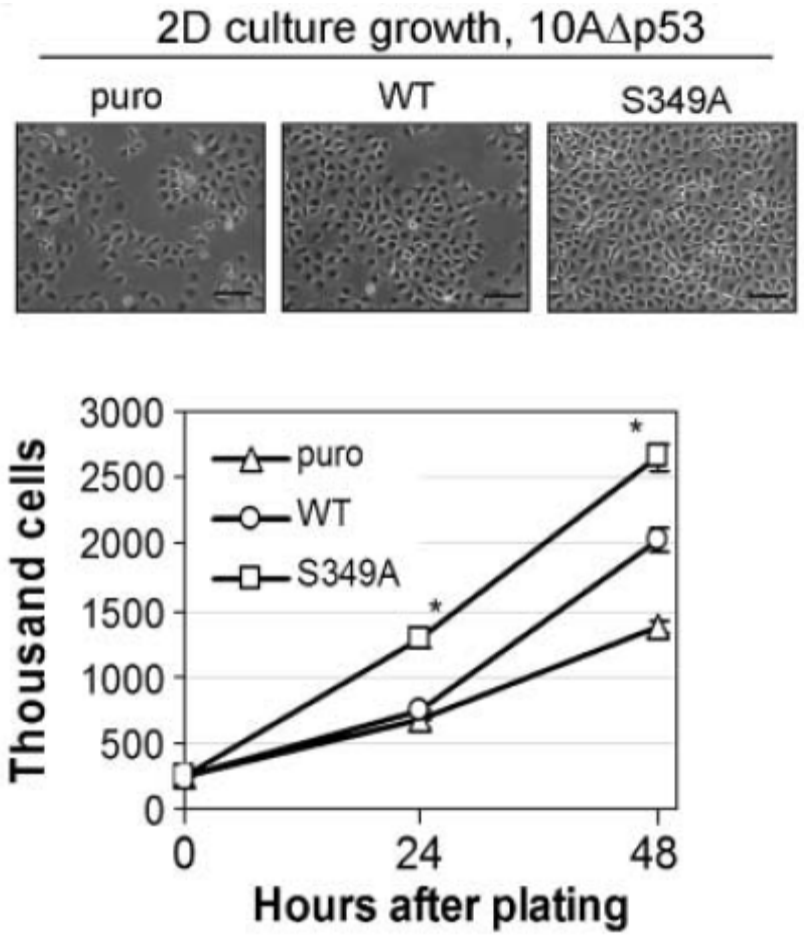


Figure 4.1: Expression of stabilized PRLr mutant augments growth of human mammary epithelial cells. Representative pictures of indicated MCF10AΔp53 cells at 24 h after seeding. Scale bar, 100 um. Points, numbers of live cells that were calculated using trypan blue at 24 and 48 h after seeding; bars, SE. The differences between number of S349A and WT cells were statistically significant ($P \leq 0.01$). Experiment was performed by Alexander Plotnikov.

Figure 4.2

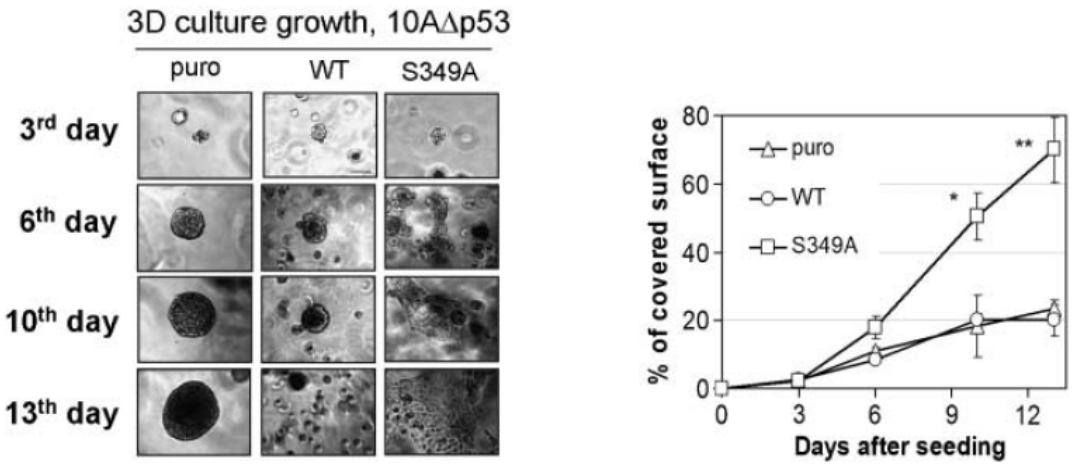


Figure 4.2: Representative morphology of indicated MCF10AΔp53 cell lines grown in three-dimensional cultures at the indicated day after plating. The experiment was repeated twice. Scale bar, 15 um. Points, percentage of surface covered by cell growth calculated from nine of 10 magnification pictures (triplicates); bars, SE. The differences between the surfaces covered by either S349A or WT cells were statistically significant at 10 d ($P \leq 0.05$) and 13 d ($P \leq 0.01$). Experiment performed by Alexander Plotnikov.

Figure 4.3

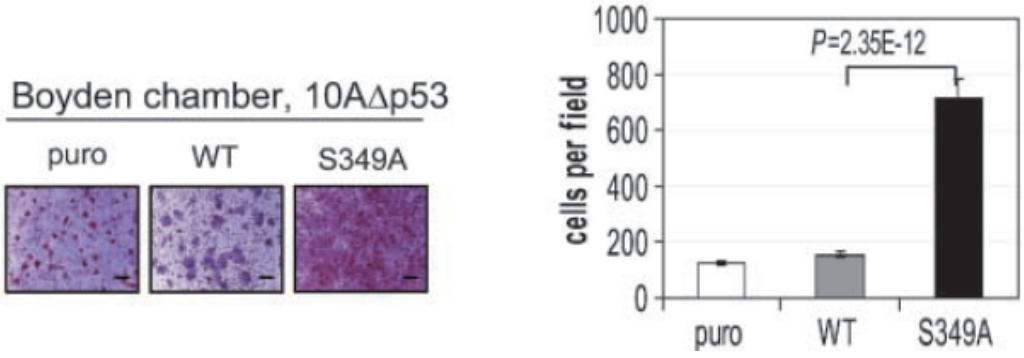


Figure 4.3: Analysis of invasiveness and tumorigenicity of MCF10AΔp53-derived cell lines. Migration of indicated cell lines through Boyden chambers was analyzed as described in Materials and Methods. The undersides of membrane inserts containing invaded cells fixed and stained with H&E were photographed 48 h after plating using a 5x objective. Scale bar, 100 um. Columns, number of invasive cells calculated from nine of x 10 magnification pictures (in triplicates); bars, SE. Experiment performed by Alexander Plotnikov.

Figure 4.4

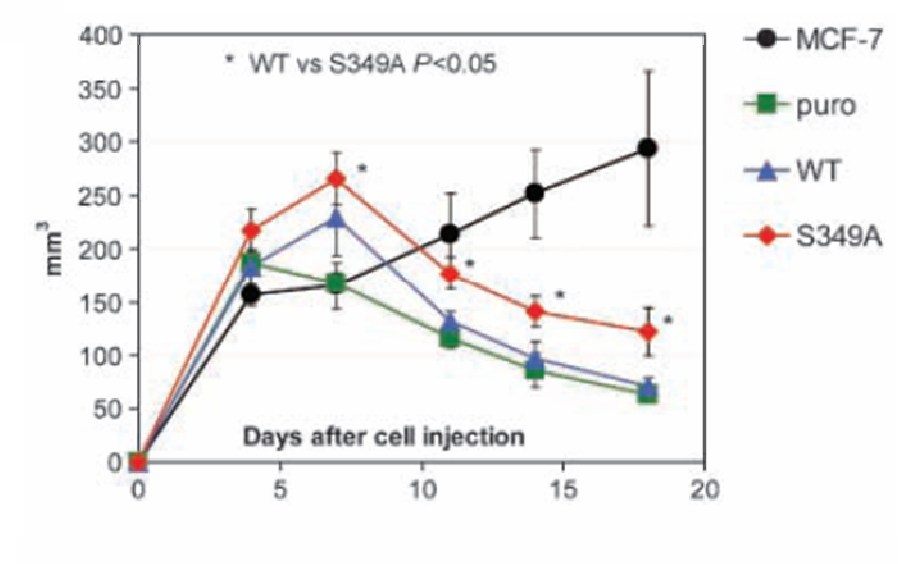


Figure 4.4: Volume of growth of indicated MCF10AΔp53 mammary epithelial cells and MCF7 breast cancer cells (positive control) injected into the flanks of the NCRNU-M mice was calculated as described in Materials and Methods. *, statistically significant ($P < 0.05$) growth difference between WT and S349A clones. Experiment performed by Alexander Plotnikov.

Chapter 5

Prolactin inhibits activity of pyruvate kinase M2 to stimulate cell proliferation

5.1 Abstract

Many studies have shown that PRL levels are associated with various breast cancer risk factors. A long-lasting reduction in PRL levels is associated with parity (Clevenger et al., 2003), which has been found to be protective against breast cancer (Russo J et al., 2005). Additionally, PRL levels positively correlate with mammographic density, a factor strongly associated with increased breast cancer risk (Clevenger et al., 2003). Elevated PRL levels are linked to long term use of oral contraceptives, a strongly correlated risk factor for mammary carcinoma (Clevenger et al., 2003). The correlation of PRL levels with these risk factors suggests that signaling downstream of PRLr, which mitigates the activities of PRL, plays a pertinent role in the etiology of breast cancer.

Prolactin receptor (PRLr) is a cytokine receptor whose signaling has been found to be crucial in the pathogenesis and maintenance of breast cancer and other human malignancies. In our efforts to study the regulation of prolactin receptor levels and its involvement in cancer, we used a Mass Spectrometry-based approach to identify novel interactors of the prolactin receptor. One such interactor we found in our screen was Pyruvate Kinase M2 (PKM2), a glycolytic enzyme known to be important in tumorigenesis. We studied the interaction of PRLr and PKM2 and in

Chapter 5 we show that PRL signaling inhibits PKM2 activity, thereby preventing progression through glycolysis and allowing PKM2 to promote the usage of glucometabolites to create lactate which is broken down as an alternative energy source. Furthermore, we show that this inhibition is dependent on PRLr level, PRLr tyrosine phosphorylation, Jak2 activity, and is necessary for effective PRL-dependent growth (Model 3).

5.2 Introduction

In 1924, Otto Heinrich Warburg postulated that the fundamental cause of cancer is a metabolic switch that occurs within a tumor cell; subsequent to this transformation, tumor cells obtain energy not through the oxidative phosphorylation of pyruvate, as in normal cells, but by alternative pathways of lactic acid fermentation and glutaminolysis. Warburg observed that though tumor cells take up more glucose than normal cells, they do not utilize this glucose for energy production via oxidative phosphorylation (Warburg O, 1956). Alternatively, tumor cells recruit the byproducts of glucose for the synthesis of building blocks (i.e. lipids, amino acids, nucleic acids) important for a growing tumor. Warburg discovered an increase in lactate levels even in the presence of oxygen. Although tumor cells do not normally progress through glycolysis and the citric acid cycle (TCA), lactate production can occur as a result of the breakdown of glutamine and serine, though the processes of glutaminolysis and serinolysis, respectively (Mazurek S et al., 2005).

A reemergence of Warburg's ideas on tumor metabolism has occurred in recent years. Pyruvate Kinase M2, an isoform of pyruvate kinase, has been found to be a key regulator of the tumor metabolome and a major player in understanding Warburg's view of cancer. PKM2 is an isoenzyme of pyruvate kinase which regulates the last step of glycolysis by catalyzing the conversion of phosphoenolpyruvate to pyruvate. Pyruvate kinase exists as various isoforms: M1 isoform (found in normal cells), M2 isoform (found in highly proliferating cells), and the L isoform (found in the liver).

This M2 isoform of pyruvate kinase has been shown to be expressed only in highly proliferating cells and in tumor cells. PKM2 is less active than its counterpart, found in normal cells, allowing for the metabolites of glycolysis to be utilized for the production of lipids, amino acids, and nucleic acids and the production of lactate (which is broken down for energy), as opposed to entering the Krebs cycle. Recent work concerning PKM2 has elucidated that the activity of PKM2 is inhibited upon binding to phosphotyrosine residues (Christofk HR et al., 2008a; Christofk HR et al., 2008b). Signaling pathways, such as Src signaling and HPV type 16 E7 oncoprotein signaling, that become hyperactive in cancer often result in the inhibition or inactiviation of PKM2 (Eigenbrodt E et al., 1992; Zwerschke W et al., 1999; Presek P et al., 1988). However, current literature suggests that prolactin signaling promotes pyruvate kinase activity and does not work to inhibit it (Costello LC and Franklin RB, 2002; Arunakaran J et al., 1993; Arunakaran J et al., 1992). Our work investigates the relationship between PRLr and

PKM2 and whether PRLr plays any regulatory role on the activity of PKM2 and its role in tumorigenesis.

By investigating the regulation of PRLr levels and elucidating the mechanism of internalization and subsequent lysosomal degradation, we can establish a new paradigm for understanding how cytokine signaling is regulated and how these processes or regulation may go awry in the case of human disease.

5.3 Materials and Methods

Cell lines, constructs, and gene delivery

MCF10AΔp53 derivative cell line, in which p53 expression is knocked down (MCF10A) were a generous gift of Alan Eastman (Levesque AA et al., 2005). Generation of the MCF10AΔp53 cells stably expressing wild type or S349A mutant PRLr was previously described (Yamashita H et al., 2006). Human breast cancer T47D cells were kindly provided by Dr Z. Ronai (Burnham Institute, San Diego, CA, USA). Cells were cultured as previously described (Melck D et al., 2000). Negative control shRNA (Sigma, #SHC002) is a lentiviral pLKO.1-puro vector containing an irrelevant shRNA insert that does not target human and mouse genes. ShPRLr (Open Biosystems, #RHS3979-98492771) contains shRNA against human PRLr in the context of the same vector. Stable mass cultures of T47D containing these shRNAs were generated using viruses packaged in 293T cells co-transfected with indicated shRNA, VSV-G, and 8.2DeltaR plasmids. Mass cultures were selected in medium containing puromycin (2 μg/mL). CISH promoter-driven firefly luciferase

reporter (Hankinson SE et al., 1999) was kindly provided by CV Clevenger (Northwestern University, Chicago, IL).

Human embryo kidney 293T cells and their derivatives were maintained and transfected as described elsewhere (Li et al. 2004, Deng et al., 2007). Plasmids for expression of flag-tagged (Li et al. 2004) or V5-tagged (Miller et al. 2005) PRLr, as well as JAK2WT or JAK2K882D (Huang et al. 2001) were previously described. Plasmids for expression of hemagglutinin (HA)-tagged PRLr have been previously described (Swaminathan G et al., 2008b). The HA-tagged ubiquitin expression constructs were kindly provided by Yosef Yarden (Weizmann Institute, Israel). Flag-PKM2^{WT} and FLAG-PKM2^{K433E} cloned into pLHCX retroviral vectors have been previously described (Christofk HR et al., 2008). The knockdown of clathrin heavy chain was performed using a short hairpin obtained from Sigma (MISSION short hairpin RNA [shRNA] plasmid DNA; catalog no. SHDNACTRCN0000007982). PRL-deficient MCF7 cells (Schroeder MD et al., 2002), rat lymphoma Nb2-11C cells (Gertler A et al., 1985), and control and PKM2-specific shRNA lentiviral vectors (Christofk HR et al., 2008a) have been previously described.

Reagents, antibodies, and immunotechniques

Antibodies against FLAG tag (M2, Sigma), HA tag (12CA5, Roche), beta-actin (Sigma), Jak2 (Upstate Biotech, Lake Placid, NY, USA), and PRLr (Zymed, San Francisco, CA, USA and Santa Cruz, Santa Cruz, CA, USA) were purchased. Human PRL was purchased from the National Hormone and Peptide program (AF

Parlow). PRLr antagonist $PRL^{\Delta 1-9-G129R}$ was produced and purified as previously described (Bernichtein et al., 2003). AG490 and PP1 (Calbiochem, San Diego, CA, USA) were purchased. Immunoprecipitation and immunoblotting were performed as described previously (Li et al., 2004). Transfections were performed with Lipofectamine Plus or Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA) or FuGENE 6 (Roche) and analyzed after 48 h according to the manufacturer's recommendations.

Pyruvate kinase activity assay and Lactate measurement assay

Pyruvate kinase activity was determined in 293T cells, MCF10A stable lines ($PRLr^{WT}$ or $PRLr^{S349A}$), or PRL-deficient MCF7 cells in the absence or presence of prolactin. The activity of pyruvate kinase activity was measured by a coupled enzymatic assay using lactate dehydrogenase (LDH). The change in absorbance at 340 nm due to the conversion of NADH to NAD+ was measured by a Varian CARY 1E UV-Vis Spectrophotometer. Enzymatic reactions contained cell lysate (50 µg), HEPES, pH 7.5 (10 mM), KCl (50 mM), MgCl2 (10 mM), ADP (2 mM), PEP (10 mM), NADH (0.5 mM), and LDH (25 units). Lactate levels were determined using a fluorescence-based lactate measurement kit as per the manufacturer's instructions (BioVision, Mountain View, CA).

Cell growth assay

The rat lymphoma Nb2-11C cell line was electroporated with a vector control (Vec), wild type murine Flag-PKM2 ($PKM2^{WT}$), or murine Flag-$PKM2^{K433E}$ ($PKM2^{KE}$). The PRL-deficient MCF7 cell line was transduced with a control shRNA

or shRNA targeting PKM2 (alone or in addition to an expression vector expressing a non-targetable murine PKM2 protein (wild type or K433E mutant)). $1x10^6$ cells were plated in triplicate for each sample and timepoint and cultured in the absence or presence of PRL (200 ng/ml). Trypan-blue negative live cells were counted at 24 and 48 hours after plating.

5.4 Results

A decrease in PRLr levels results in an attenuation of the signaling program, thereby halting the downstream phenotypes of proliferation, differentiation, and survival depending on the cellular context. In **Chapter 4**, we showed that elevated PRLr levels resulted in enhanced cell growth and promoted the transformation of mammary epithelial cells. However, the mechanism(s) by which PRLr promotes cell growth and transformation is yet to be clearly understood. Our lab sought out novel interactors of PRLr that may play a role in these processes. In an effort to investigate other proteins which may be important for PRLr-mediated cell growth and transformation, we performed a proteomics-based screen to identify novel interacting proteins of PRLr. To this end, FLAG-tagged PRLr was expressed in 293T cells and cell lysates were immunoprecipitated with anti-FLAG antibody, resolved by SDS-PAGE, and visualized by Colloidal Coomassie staining. Bands of interest were excised, trypsin-digested, and analyzed by LC-MS/MS. After obtaining results from this screen, the presence of peptides derived from PRLr itself and a phosphor-serine/threonine binding protein 14-3-3 that has been previously described as an

interactor of PRLr (Olayioye et al., 2003) validated our screen. In this effort, we identified the glycolytic enzyme pyruvate kinase M2 (PKM2) to be a novel interactor of PRLr (Figure 5.1; unpublished data).

While we identified PKM2 to be a novel interactor of PRLr, we wanted to confirm that this interaction occurred within the cell and was not an artifact of our proteomics-based screen. To this end, we expressed FLAG-tagged PRLr and HA-tagged PKM2 in 293T cells and assessed the interaction between PRLr and PKM2 by direct and reciprocal co-immmunoprecipitation (using anti-FLAG and anti-HA antibodies) followed by immunoblotting analysis. These data showed that PRLr and PKM2 co-immunoprecipitated with each other (Figure 5.2). These results indicate that PRLr and PKM2 are interacting proteins.

While we have established that PRLr and PKM2 interact, these studies were performed with exogenous proteins. We wanted to establish that the endogenous PRLr and PKM2 proteins in fact interact within the cell and that their interaction recapitulates the results seen using exogenous proteins. To this end, we immunoprecipitated endogenous PRLr from 293T cells treated with or without PRL using anti-PRLr antibody and resolved these immunoprecipitates by SDS-PAGE. Samples were immunoblotted with anti-PRLr and anti-PKM2 antibodies. Results from these studies showed that PKM2 co-immunoprecipitated with PRLr in lysates that were treated with or without PRL (Figure 5.3). This indicated that PRLr and PKM2 are bonafide interactors within the cell. Subsequent analysis of the effect of the ligand on the binding of PRLr to PKM2 did not yield conclusive results (data not

shown). These results taken together with the data from the proteomics screen suggest that PKM2 is indeed a protein that constitutively associates with PRLr.

Unlike pyruvate kinase M1 (PKM1), which is found in normal tissues, pyruvate kinase M2 has been shown to be expressed only in highly proliferating cells and in tumor cells (Mazurek S, 2007). Furthermore, in contrast to PKM1, PKM2 activity has been shown to be inhibited by the binding of PKM2 to phosphotyrosine residues in a manner that is dependent upon the integrity of a key amino acid within PKM2, lysine 433 (Christofk HR et al., 2008b). Expression of PKM2 (but not PKM1) and the integrity of K433 were shown to be important for cell proliferation and tumorigenicity (Christofk HR et al., 2008a). The current paradigm views the inhibition of PKM2 as a key event in preventing the progression of metabolites through the Krebs cycle and in promoting the utilization of metabolites for the production of lipids, amino acids, and nucleic acids. Therefore, PKM2 has been established as a key regulator of the Warburg effect: though tumor cells take up more glucose than normal cells, they do not utilize this glucose for energy production via oxidative phosphorylation (Warburg O, 1956). Alternatively, tumor cells channel the byproducts of glucose metabolism for the synthesis of building blocks (i.e. lipids, amino acids, nucleic acids) important for a growing tumor (Vander Heiden MG et al., 2009). In normal tissues, a stimulation of activity of PKM1 by PRL has been previously demonstrated (Arunakaran J et al., 1992; Arunakaran J et al., 1993; Costello LC and Franklin RB, 2002; Kumaran B et al., 1988). However, it is not

known how PRL that induces a cascade of tyrosine phosphorylation would affect the activity of PKM2 in rapidly proliferating cells.

Since we identified PKM2 to be a bonafide interactor of PRLr and PKM2 has been shown to be inhibited in a manner dependent on the binding of PKM2 to phosphotyrosine residues, we wanted to determine whether the PRLr-PKM2 interaction had any consequence for PKM2 activity. For this study, we decided to use the 293T human embryo kidney cell line since it expresses PKM2 but not PKM1 (Christofk HR et al., 2008a) and expresses low levels of endogenous PRLr (Li Y et al., 2004) so as to be amenable to experiments requiring the expression of exogenous forms of PRLr. To study the effect of PRL signaling upon PKM2 activity, we treated 293T cells with PRL for various timepoints and assessed the PKM2 activity of these lysates. The PKM2 enzymatic activity was assessed using a coupled enzymatic-based spectrophotometric assay that is well established in the study of pyruvate kinase enzymatic activity (Bergmeyer HU, 1963). Results from these experiments showed that exposure to PRL led to a noticeable inhibition of pyruvate kinase activity which became more robust over time (Figure 5.4). The extent of this inhibition was greater than that reported in cells treated with the insulin-like growth factor (Christofk HR et al., 2008b).

Once we established that PRL signaling works to inhibit PKM2 activity, we wanted to see if this effect was dependent upon PRLr levels. If the interaction between PRLr and PKM2 leads to this inhibition, we believe that increased PRLr levels would increase the amount of PKM2 within the cell that is PRLr-bound,

thereby further decreasing PKM2 activity within the cell. To study this hypothesis, we expressed wild type PRLr, PRLr S349A mutant, or an empty vector in 293T cells, treated them with or without PRL, and assessed the PKM2 kinase activity of the lysates using the coupled enzymatic-based spectrophotometric assay. These results showed that expression of exogenous wild type PRLr increased PRL-mediated inhibition of PKM2 to 25% while expression of the proteolytically stable PRLr S349A mutant increased PKM2 inhibition to 40% (Figure 5.5). These results indicate that increased levels of PRLr increase the inhibitory effect of PRL upon PKM2 activity.

We wanted to establish whether the effect of PRL treatment in the inhibition of PKM2 activity is cell-type specific and if PRL-mediated inhibition of PKM2 does occur in other cells, whether this effect is dependent on PRLr levels. For this investigation, we decided to use human mammary epithelial MCF10A cell lines that stably express wild type or S349A mutant versions of PRLr. Studies using these cells would tell us whether PRL-mediated inhibition of PKM2 is cell-type specific and whether it occurs in near normal breast epithelial cells. To this end, MCF10A-PRLr WT and MCF10A-PRLr S349A stable lines were treated with or without PRL. The PKM2 activity within these cell lysates was assessed using the coupled enzymatic-based spectrophotometric assay. Results from these experiments showed that a robust inhibition of PKM2 activity by PRL was observed in MCF10A cell lines that stably express wild type PRLr (40%); even stronger inhibition was seen in cells that harbor the proteolytically stable S349A mutant (60%) of PRLr (Figure 5.6).

These data show that PRL treatment results in PKM2 inhibition in MCF10A mammary epithelial cells and that this PKM2 inhibition is dependent on PRLr levels, indicating that these processes are not cell-type specific.

Having established that PRL treatment works to inhibit PKM2 activity and that this inhibition is dependent upon PRLr levels, we sought to determine the importance of PKM2 inhibition. PKM2 has previously been shown to regulate energy production within cancer cells. While normal cells obtain energy in the presence of oxygen by the breakdown of glucose via oxidative phosphorylation, cancer cells have been shown to undergo the conversion of glucose to lactate, or aerobic glycolysis, even in the presence of oxygen. Furthermore, cancer cells undergo the alternative energy production pathways of glutaminolysis and serinolysis, which produce energy by the breakdown of the amino acids glutamine and serine respectively, and result in the production of lactate. Since these alternative energy production pathways which occur in cancer cells result in elevated lactate levels, an increase in lactate production within the cell is indicative of a glycolytic switch. Tumor cells switch their dependence of energy production pathways from oxidative phosphorylation to aerobic glycolysis, glutaminolysis, serinolysis, and other such alternative pathways. In order to determine if the PRL-mediated inhibition of PKM2 resulted in such a change in energy dependence, the levels of lactate production were measured. To this end, MCF10A mammary epithelial cell lines stably expressing wild type PRLr or the S349A mutant of PRLr were treated with or without PRL. The lactate levels within the cell lysates was ascertained using

a fluorescence-based lactate measurement kit. These results showed that PRL treatment increased the levels of lactate within the cell and this effect was more robust in cells expressing the proteolytically stable S349A mutant of PRLr (Figure 5.7). The extent of lactate production (Figure 5.7) was inversely proportional to the pyruvate kinase inhibition (Figure 5.6) in these cells, indicating that PRL treatment promotes the inhibition of PKM2 and a switch in energy dependence within the cell.

We wanted to confirm that the PRL-mediated inhibition of PKM2 is dependent upon PRLr levels. By investigating the effect of PRL treatment on PKM2 activity in another cell line, we can confirm that the PRL-mediated inhibition of PKM2 is not a cell-type specific effect. To this end, we took T47D breast cancer cell lines (which express noticeable levels of endogenous PRLr) that stably express a control shRNA targeted against green fluorescent protein (GFP) or a shRNA construct targeted against PRLr (Plotnikov A et al., 2009), treated them with or without PRL, and assessed the PKM2 kinase activity in their cell lysates by using the coupled enzymatic-based spectrophotometric assay. These studies showed that while PRL treatment resulted in a 35% decrease in PKM2 activity in control cells (shCON), cells with stable shRNA-mediated knockdown of PRLr (shPRLr) did not exhibit any change in PKM2 activity after PRL treatment (Figure 5.8). These data indicate that PRLr levels are crucial for PRL-mediated inhibition of PKM2 as knockdown of PRLr abrogated this effect.

We further wanted to establish whether a switch in energy dependence occurs in cells that exhibit PRL-dependent inhibition of PKM2. To investigate this, we

sought to determine the lactate levels in the T47D stable cell lines which showed PRL-mediated inhibition of PKM2 when PRLr was intact (Figure 5.8). To this end, the shCON and shPRLr T47D stable lines were treated with or without PRL and the lacate levels within the cell lysates was determined using a fluorescence-based lactate measurement kit. These studies showed that in response to PRL treatment, lactate levels increased in control cells (shCON) but not in cells where PRLr was knocked down (shPRLr), indicating that increased lactate level production in response to PRL is dependent on PRLr levels (Figure 5.9). Similar to the results obtained using the MCF10A cell lines, PRL treatment of the T47D cell lines resulted in increased lactate levels which coincided with an inhibition of PKM2 activity. Together, these results indicate that the inhibition of PKM2 and accumulation of lactate in response to PRL are regulated by the abundance of PRLr, indicating that a switch in energy dependence occurs when PKM2 activity is inhibited and is dependent on PRL treatment and PRLr levels.

Our data establish that PRL treatment works to inhibit the activity of PKM2. However the mechanism by which this occurs or what determinants are key for this inhibition are unknown. We next sought to investigate how PRL inhibits PKM2 activity. Given that binding of phosphotyrosines was shown to elicit such inhibition (Christofk HR et al., 2008b) and that PRLr (which interacts with PKM2) undergoes tyrosine phosphorylation (Rui H et al., 1992; Rui H, Kirken RA, et al., 1994) which is required for the activation and tyrosine phosphorylation of downstream effectors, we next tested the role of the tyrosine phosphorylation of PRLr in PKM2 inhibition.

To this end, we expressed wild type PRLr, a PRLr mutant where all intracellular tyrosines are mutated to arginine (YF mutant of PRLr), or an empty vector as a control in 293T cells and assessed the PKM2 activity of the cell lysates by using the coupled enzymatic-based spectrophotometric assay. Results from these studies showed that expression of the PRLr mutant that lacks Tyr residues in its intracellular domain abrogated the PRL-induced inhibition of PKM2 in 293T cells (Figure 5.10). This suggests that tyrosine phosphorylation of PRLr is necessary for the PRL-mediated inhibition of PKM2.

Since our results show that tyrosine phosphorylation of PRLr is needed for the inhibition of PKM2 and tyrosine phosphorylation of PRLr is a required step for active PRL signaling, we sought to determine if the inhibition of PKM2 is dependent upon the active signaling downstream of PRLr. To this end, we expressed wild type PRLr, a gain-of-function I170L mutant of PRLr, or a control vector in 293T cells and measured the PKM2 activity within cell lysates by using the coupled enzymatic-based spectrophotometric assay. These data showed that expression of the gain-of-function I170L mutant of PRLr (which is found in some breast cancers and benign tumors and exhibits constitutive Tyr phosphorylation that can be further stimulated by ligand (Bogorad RL et al., 2008; Canbay E et al., 2004)) resulted in a robust inhibition of PKM2 even in the absence of ligand; these results were further augmented with PRL treatment (Figure 5.11). This suggests that PRL promotes the inhibition of PKM2 by propagating PRL signaling and not by a signaling-independent effect.

The importance of active PRL signaling for the inhibition of PKM2 led us to query whether active PRL signaling was needed for the PRL-mediated increase in lactate levels which are indicative of a switch in energy dependence. To this end, we expressed wild type PRLr, the I170L mutant of PRLr, or a control vector in 293T cells and assessed the lactate levels in the cell lysates by using the fluorescence-based lactate measurement kit. Results from these studies showed that expression of the gain-of-function I170L mutant of PRLr increased lactate levels even in the absence of ligand; PRL treatment further augmented this effect (Figure 5.12). This suggests that active PRL signaling promotes a switch in energy dependence as indicated by elevated lactate levels. These results taken together show that Tyr phosphorylation of PRLr is required for the inhibition of PKM2 by PRL treatment.

The PRL-mediated inhibition of PKM2 may either be directly regulated by PRLr or mediated by the recruitment of proteins containing phosphorylated Tyr residues to PRLr that would interact with PKM2 and decrease its activity. We wanted to investigate the role of two downstream kinases which have been found to be tyrosine phosphorylated and are activated in response to PRL treatment. Jak2 (the Janus kinase constitutively bound to PRLr) and Src are two major tyrosine kinases induced by PRL (Clevenger CV et al., 2003). We sought to investigate whether these downstream kinases were important for the PRL-mediated inhibition of PKM2. We decided to investigate the role of these kinases in PRL-mediated inhibition of PKM2 by pharmacologically inhibiting Jak and Src activity and observing whether the inhibition of PKM2 in response to PRL was altered. To this end, 293T cells were

treated with AG490 (Jak inhibitor), PP1 (Src inhibitor), or ethanol (vehicle control) and the PKM2 activity of cell lysates was assessed using the coupled enzymatic-based spectrophotometric assay. These results showed that pre-treatment of cells with the Jak inhibitor AG490 (but not with the Src inhibitor PP1) attenuated the PRL-mediated inhibition of PKM2 (Figure 5.13). This implies that Jak activity, but not Src activity, is necessary for the PRL-mediated inhibition of PKM2.

While we showed, by pharmacological inhibition, that Jak activity is needed for efficient PRL-mediated inhibition of PKM2, we have not established whether this dependence is upon Jak2 or the activities of all Jak kinases. To investigate if this effect is Jak2 specific, we decided to take a genetic approach and discover if the PRL-mediated inhibition of PKM2 is affected by shRNA-mediated knockdown of Jak2 or another Janus kinase. To this end, we expressed a shRNA targeted against Jak2, a shRNA targeted against Tyk2 (which is not bound to PRLr), or a control shRNA construct in 293T cells and we assessed the PKM2 activity in cell lysates by using the coupled enzymatic-based spectrophotometric assay. These experiments showed that knockdown of Jak2 (which is constitutively bound to PRLr) but not knockdown of another Janus kinase (Tyk2) prevented the PRL-mediated decrease in PKM2 activity (Figure 5.14). This shows that Jak2 is needed for the effective PRL-mediated inhibition of PKM2 and that this effect is not true of all Janus kinases.

Having established the importance of Jak2 in regulating the PRL-mediated inhibition of PKM2, we sought to determine whether Jak2 was needed for promoting a PRL-mediated switch in energy dependence. By using a genetic approach we can

establish what role, if at all, Jak2 plays in this switch. To this end, we expressed a shRNA targeted against Jak2, a shRNA targeted against Tyk2, or a control shRNA in 293T cells (similar to the approach taken with Figure 5.14) and assessed the lactate levels of cell lysates by using the fluorescence-based lactate measurement kit. These data showed that shRNA-mediated knockdown of Jak2, but not knockdown of Tyk2, resulted in a loss of lactate level increase in response to PRL treatment (Figure 5.15). This shows that Jak2 is crucial in regulating the switch of energy dependence in response to PRL.

While our data has suggested Jak2 to play an important role in the PRL-mediated inhibition of PKM2 and concomitant switch in energy dependence (as evidenced by elevated lactate levels), we wanted to confirm the role that Jak2 plays in this process. Would the enhancement of Jak2 activity result in an augmented inhibition of PKM2 activity? To investigate this, we decided to use constitutively active forms of Jak2 and observe how the inhibition of PKM2 was affected in response to PRL. To this end, we expressed wild type Jak2, constitutively active TEL-Jak2 fusion protein, constitutively active V617F mutant of Jak2, or a control vector in 293T cells and assessed the PKM2 activity within cell lysates by using the coupled enzymatic-based spectrophotometric assay. Our results showed that expression of constitutively active Jak2 proteins (TEL-Jak2 fusion protein and $Jak2^{V617F}$ mutant found in hematologic malignancies (Valentino L and Pierre J, 2006)) led to a robust suppression of PKM2 activity even in cells that did not receive

PRL (Figure 5.16). These results taken together suggest that PRL-induced Jak2 activity may be required and sufficient for the inhibition of PKM2.

Our data has shown that PRL treatment works to inhibit PKM2 activity and promote a switch in energy dependence. However, we do not know what the consequence of the PRLr-PKM2 interaction is for PRL mediated cell growth and transformation. While elevated PRLr levels result in enhance cell growth and transformation, we do not know how the mechanism by which PRL signaling enhances these phenotypes. To investigate whether the inhibition of PKM2 plays a role in PRL-mediated cell growth, we decided to overexpress PKM2 and observe whether this enhanced cell growth in response to PRL treatment. To this end, we expressed FLAG-tagged wild type PKM2, FLAG-tagged K433E mutant of PKM2 (which cannot bind phosphotyrosine residues and is not subsequently inhibited), or a control vector in the rat lymphoma Nb2-11C cell line (which has been shown to exhibit PRL-dependent growth) and assessed their growth kinetics in the absence or presence of PRL over a 48 hour time period. In rat lymphoma Nb2-11C cells that are highly sensitive to PRL-stimulated growth (Gertler A et al., 1985), expression of murine PKM2 further promoted cell proliferation in the presence but not in the absence of PRL (Figure 5.17). Remarkably, when the K433E mutant of PKM2 (which is insensitive to inhibition by binding to phosphotyrosine residues, (Christofk HR et al., 2008a; Christofk HR et al., 2008b)) was introduced into the cells, it did not further increase PRL-stimulated proliferation despite being expressed at levels

comparable with the wild type protein (Figure 5.17). These results suggest that the inhibition of PKM2 may contribute to PRL-induced cell proliferation.

Having implicated a role for PKM2 in promoting PRL-mediated growth, we wanted to determine whether the affect of PRL on PKM2 activity and the switch in energy dependence are reliant on the interaction between PRL signaling and PKM2 (and the subsequent inhibition of PKM2 activity) or whether these effects can be replicated by knockdown of the PKM2 protein. For these studies, we used a sub-clone derived from the MCF7 human breast cancer cell line; this sub-clone is deficient in producing its own PRL and, therefore, is highly sensitive to the effects of exogenously added PRL (Schroeder MD et al., 2002). We used shRNA constructs for the knockdown of PKM2 and re-expressed murine forms of the PKM2 protein which cannot be targeted by the shRNA construct in this MCF7 sub-clone (the cells were treated with or without PRL). PKM2 activity of the cell lysates was assessed by using the coupled enzymatic-based spectrophotometric assay. These studies showed that the knockdown of PKM2 abrogated the PRL-induced suppression of PKM2 activity (Figure 5.18). The PRL-mediated inhibition of PKM2 was restored in cells, where PKM2 was knocked down, by the expression of wild type PKM2 but not the K433E mutant of PKM2. These results suggest that PRL-induced suppression of PKM2 activity is dependent on the presence of a PKM2 protein which can bind phosphotyrosine residues and subsequently be inhibited.

Having established the need for an inhibitable PKM2 protein for the PRL-mediated suppression of PKM2 activity, we wanted to investigate whether an

inhibitable PKM2 protein is required for the PRL-induced increase in lactate levels which are indicative of a switch in energy dependence. To this end, we expressed a control shRNA or a shRNA directed against PKM2 alone or in conjunction with non-targetable murine wild type or K433E mutant PKM2 proteins in the MCF7 sub-clone, which is responsive to PRL, and treated them with or without PRL. The lactate levels within these cell lysates were determined by using the fluorescence-based lactate measurement kit. These results showed that knockdown of PKM2 abrogated the PRL-induced increase in lactate levels; the PRL-mediated increase in lactate levels were restored by expression of the non-targetable wild type PKM2 protein but not the K433E mutant of PKM2 (Figure 5.19). This suggests that the presence of an inhibitable PKM2 protein is needed for the PRL-induced augmentation of lactate levels, which are indicative of a switch in energy dependence.

Our data has shown that the presence of an inhibitable PKM2 protein is necessary for the PRL-mediated inhibition of PKM2 activity and increase in lactate levels. This leads us to ask the question of whether the presence of PKM2 is necessary for PRL-mediated cell growth. While previous results showed that the inhibitable PKM2 promotes PRL-dependent growth (Figure 5.17), we did not establish whether PKM2 is necessary for PRL-dependent cell growth. To this end, we used the MCF7 sub-clone (that is responsive to PRL) and expressed a control shRNA or shRNA directed against PKM2 and used a functional knock-in approach (Christofk HR et al., 2008a; Christofk HR et al., 2008b) and expressed murine

PKM2 (wild type or K433E mutant) that is insensitive to the specific shRNA directed against the human PKM2 enzyme. The growth kinetics of these cells in the presence or absence of PRL was assessed over a 48 hour time period. Knockdown of PKM2 abrogated PRL-stimulated cell proliferation (Figure 5.20). Whereas expression of non-targetable wild type murine PKM2 rescued PRL-stimulated cell proliferation in cells where PKM2 was knocked down, expression of the phosphotyrosine-insensitive K433E mutant of PKM2 failed to do so (Figures 5.20). These data suggest that the inhibition of PKM2 by PRL is necessary for PRL-induced cell proliferation.

5.5 Discussion

Collectively our data demonstrate that PKM2 constitutively binds to PRLr and undergoes a phosphotyrosine-mediated inhibition in response to PRL (Model 3). This inhibition is dependent on PRLr levels and its tyrosine phosphorylation and requires the catalytic activity of Jak2 (Model 3). Furthermore, PRL-mediated PKM2 inhibition plays an important role in PRL-induced cell proliferation. While there is an ever increasing appreciation for the significance of metabolic changes in general and inhibition of PKM2 in particular for cell proliferation and tumorigenesis induced by pro-mitogenic stimuli and diverse oncogenes (DeBerardinis RJ et al., 2008; Vander Heiden MG et al., 2009), our data suggest the importance of PKM2 inhibition in mediating the pro-tumorigenic effects of prolactin, whose receptor interacts with PKM2. Further studies are needed to investigate whether receptors for

other extracellular mitogens of polypeptide origin can also interact with PKM2 and to determine the role of such an interaction and its consequences for proliferation and tumorigenesis.

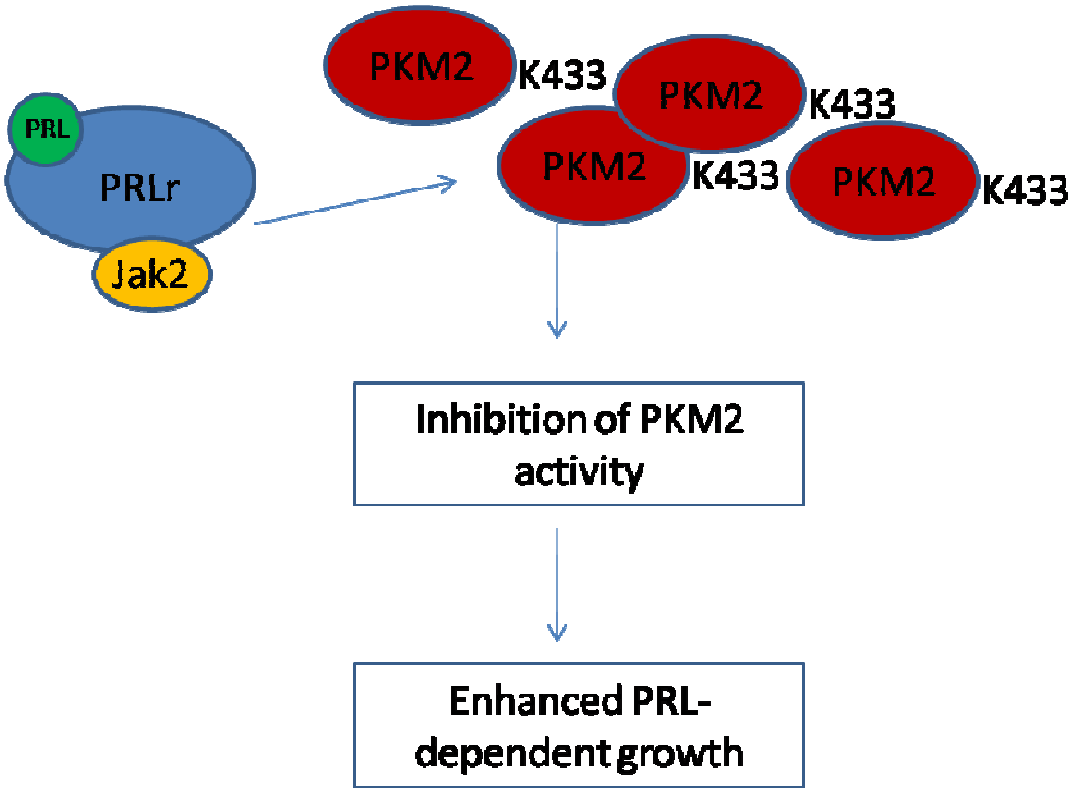
Conclusions of Chapter 5:

- PKM2 is a novel interactor of PRLr
- PRL treatment induces the inhibition of PKM2 kinase activity
- Elevated PRLr levels promote PRL-mediated inhibition of PKM2 and a switch in energy dependence (as evidenced by enhanced lactate levels)
- PRLr is required for the PRL-induced suppression of PKM2 activity and switch in energy dependence
- Phosphorylation of the intracellular tyrosines of PRLr are required for PRL-mediated inhibition of PKM2
- Constitutively active PRLr promotes PKM2 inhibition and a switch in energy dependence even in the absence of PRL
- Active Jak2 is enhances and is required for PRL-induced inhibiton of PKM2 and switch in energy dependence
- Expression of an inhibitable PKM2 protein enhances PRL-dependent growth
- Expression of an inhibitable PKM2 protein is required for PRL-dependent growth and switch in energy dependence
- The presence of an inhibitable PKM2 protein is required for PRL-dependent growth

While the inhibition of PKM2 by PRL results in increased PRL-dependent growth, shRNA-mediated knockdown of PKM2 only stunts PRL-mediated growth, contrary to logic. How can the necessity of the presence of a protein (PKM2) be reconciled to the fact that must be inhibited to promote a tumorigenic effect? While the PRL-mediated inhibition of PKM2 leads to a halt in progression through glycolysis and promotes alternative energy production pathways (i.e., lactate breakdown, glutaminolysis, serinolysis), the tumorigenic effect of PKM2 may not be strictly due to its role in glycolysis. Irrespective of its catalytic activity, PKM2 may act as an interactor which promotes the tumorigenic effects of other oncogenes. Under this proposed idea, PKM2 may serve to promote the activity of oncogenes or inhibit the activity of tumor suppressors in order to promote tumorigenic growth, thus leading to the necessity of the presence of PKM2 for PRL-mediated growth. However, if this PKM2 protein cannot be inhibited, then glycolysis is allowed to progress and the pro-tumorigenic effects of PKM2 are counterbalanced by the lack of a switch to alternative energy production pathways. Presumably if PKM2 can be inhibited, alternative energy production pathways can occur and PKM2 is free to act as a docking site for other proteins or as an activator of oncogenes or suppressor of tumor suppressors. This would reconcile the fact that optimal PRL-dependent growth can only occur in the presence of a PKM2 protein that becomes inhibited. Future studies looking at the mechanisms of PKM2-mediated tumorigenicity could provide valuable insights into these matters.

These studies in **Chapter 5** are significant in that they propose a novel mechanism by which PRL mediates cell proliferation in a manner dependent on PKM2 (Model 3). Previous work looking at the effect of PRL on glycolysis has shown that PRL signaling promotes progression through glycolysis and promotes PKM1 activity in normal cells. However, our studies paint a novel role for PRL signaling in the regulation of pyruvate kinase in cancer cells (PKM2). Furthermore PKM2, originally thought to play its role in the inhibition of glycolysis, seems to play the part of a key regulator of PRL-dependent cell growth and tumorigenesis.

Model 3



Model 3: Increased PRLr levels and enhanced PRLr and Jak2 activity promote the interaction of PRL and PKM2 which leads to the inhibition of PKM2 activity, by binding phosphotyrosine residues (in a PKM2 K433 dependent manner), and to a switch in energy dependence (as evidenced by increased lactate levels). This inhibition of PKM2 activity enhances cell growth in a PRL-dependent manner.

Figure 5.1

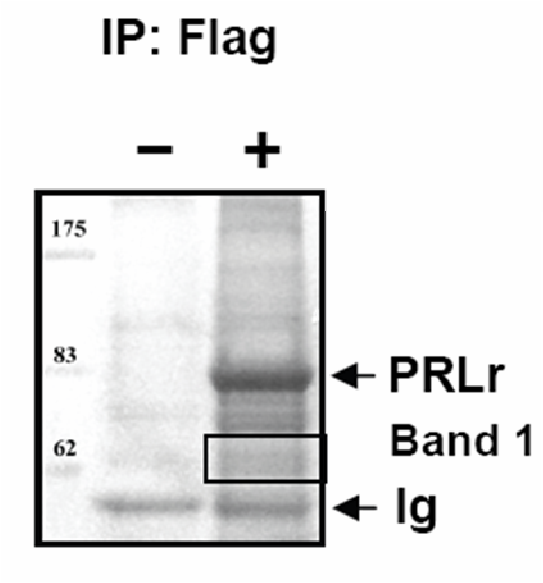


Figure 5.1: Whole cell lysates from human embryonic kidney 293T cells transfected to express Flag□tagged PRLr (Li et al., 2004) and the corresponding control vector were immunoprecipitated with Flag M2 agarose (Sigma) followed by stringent washes to minimize non□specific interactions. The proteins that co□purified with PRLr were resolved by SDS□PAGE and visualized by Colloidal Coomassie staining. Indicated proteins (Band 1) were excised, digested with trypsin and analyzed by LC□MS/MS. The results were searched against the NIH database using the SEQUEST software. Experiment performed by Gayathri Swaminathan.

Figure 5.2

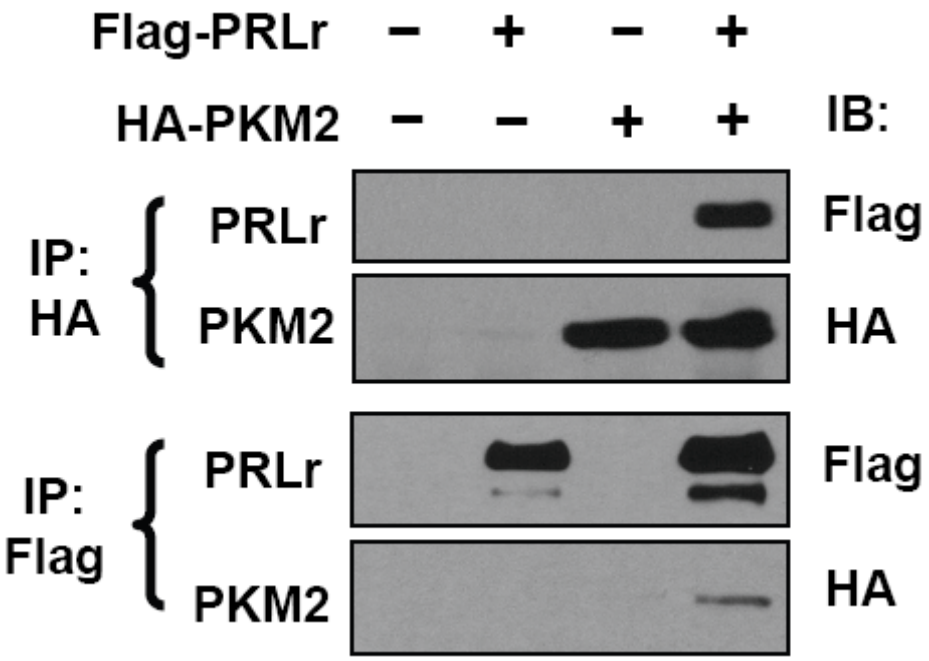


Figure 5.2: 293T cells transfected with Flag□tagged PRLr and HA□tagged PKM2 as indicated were lysed and immunoprecipitation□immunoblotting assays using anti□Flag (Sigma) and anti□HA antibody (12CA5, Roche) were carried out as depicted. Experiment performed by Gayathri Swaminathan.

Figure 5.3

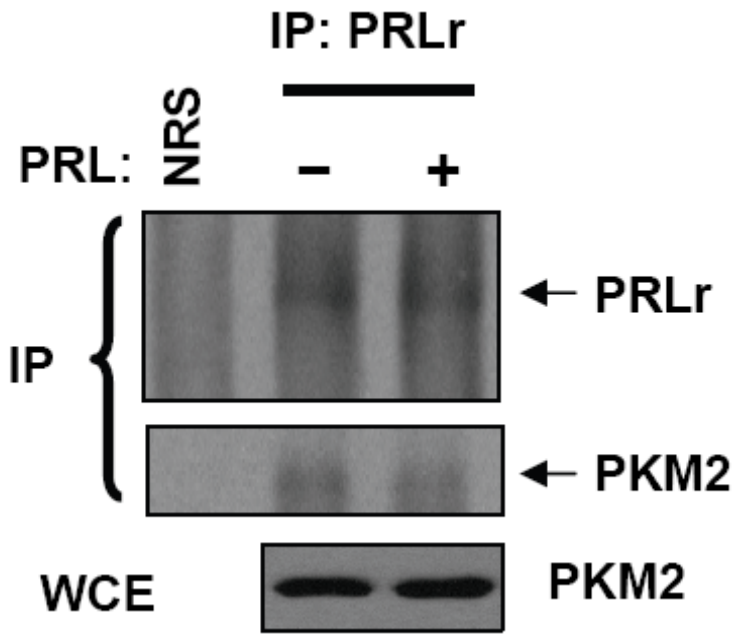


Figure 5.3: Immunoprecipitation of endogenous PRLr from lysates from 293T cells treated (or not) with human PRL (purchased from the National Hormone and Peptide program and used at 100ng/ml for 30 min) were carried out using anti□PRLr antibody (H□300, Santa Cruz) or naïve rabbit serum (NRS). Levels of PKM2 in whole cell extracts are also shown. Experiment performed by Alexander Plotnikov.

Figure 5.4

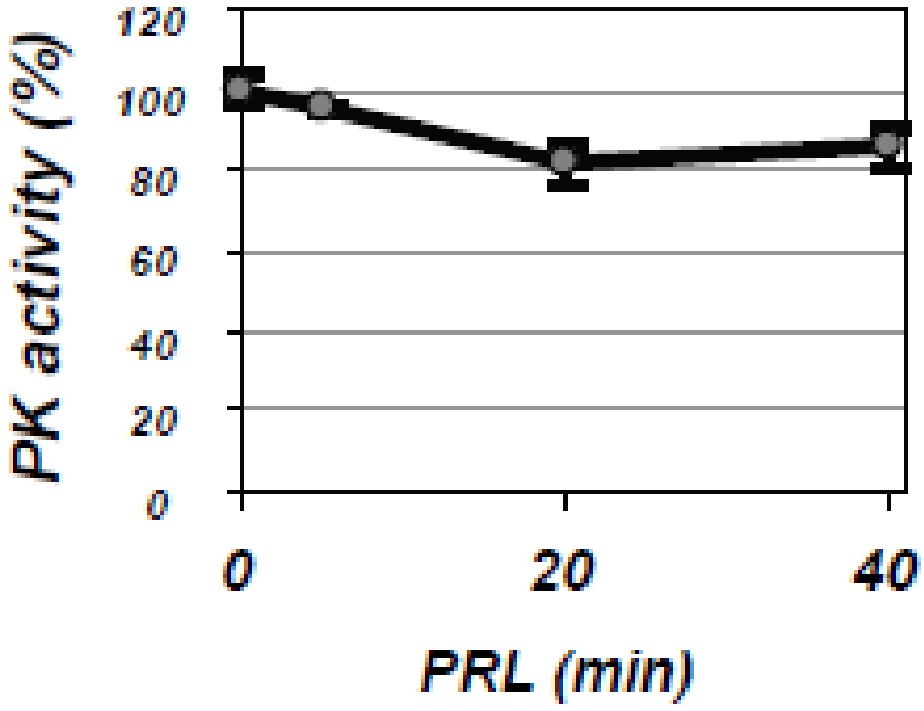


Figure 5.4: Pyruvate kinase activity was determined in lysates (50µg) from 293T cells treated with PRL (100 ng/ml for indicated timepoints) by a coupled enzymatic□based spectrophotometric assay that registers a decrease in absorbance at 340 nm using Varian CARY 1E UV□Vis Spectrophotometer as described in (Bergmeyer HU, 1963). The reagents for the assay including phosphoenolpyruvate, nicotinamide adenine dinucleotide, reduced form (NADH), adenosine diphosphate and lactic dehydrogenase were from Sigma. Average data from four independent experiments (each in triplicate) are presented as % of activity measured in cells that did not receive PRL (± S.D.).

Figure 5.5

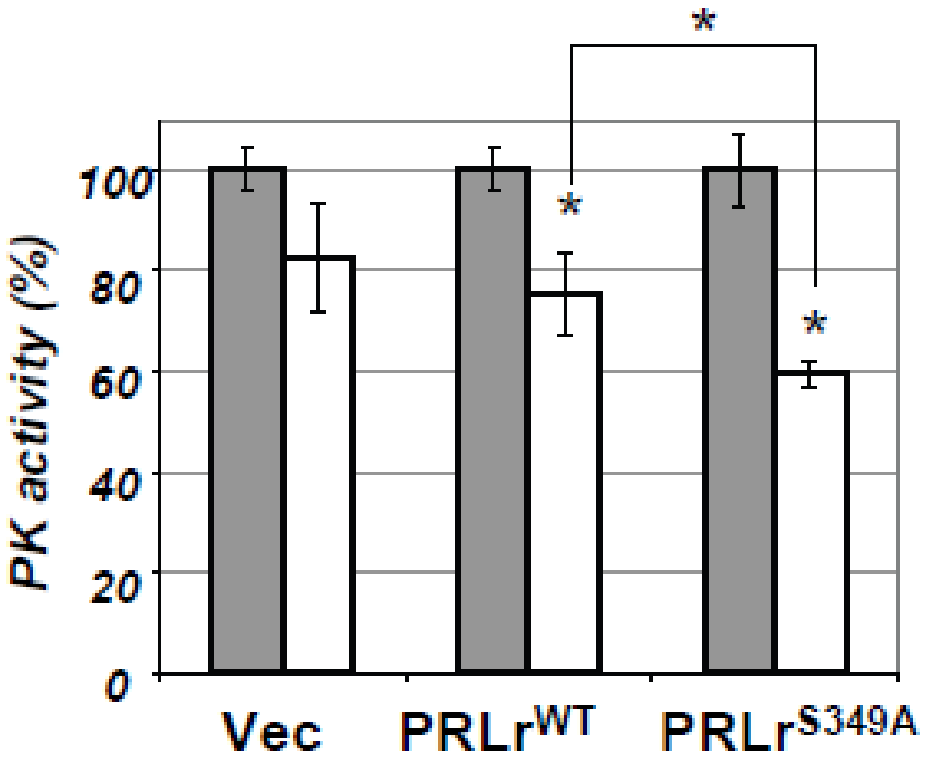


Figure 5.5: Activity of pyruvate kinase was determined (as in Figure 5.4) in lysates from 293T cells transfected with vector control (pcDNA3) or vectors for expression of PRLr (wild type or S349A mutant) and treated (100 ng/mL prolactin for 20 minutes, white bars) or not (grey bars) with PRL. Here and in similar subsequent figures, the average data from three independent experiments (each in triplicate) are presented as % of activity of non□treated control cells (± S.D.). Asterisks denote statistical significance of obtained differences ($p<0.05$ in the Student's t□test).

Figure 5.6

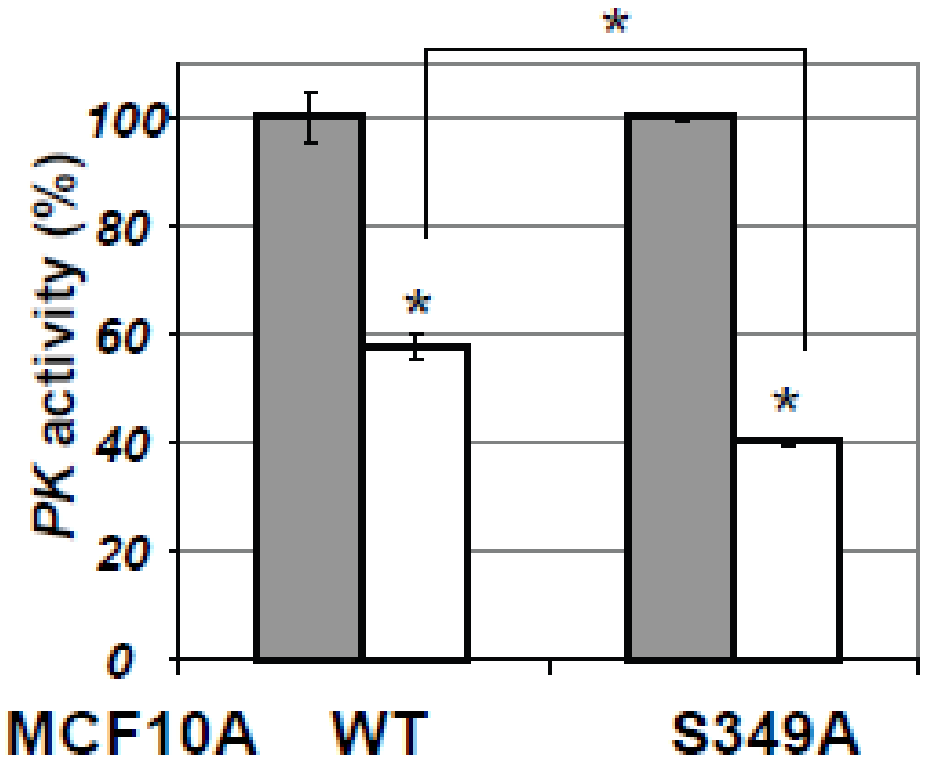


Figure 5.6: Pyruvate kinase activity was measured in the lysates from MCF10A□derived cells expressing wild type or S349A mutant of PRLr (described in details in (Plotnikov et al., 2009)) as in Figure 5.5.

Figure 5.7

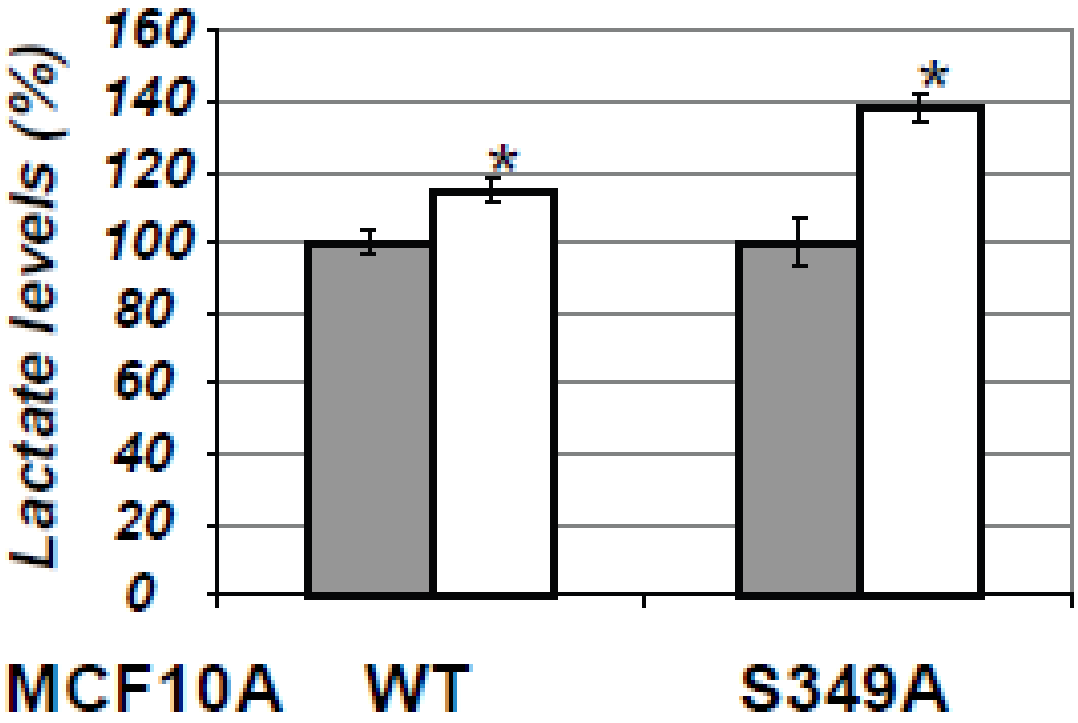


Figure 5.7: Lactate levels in the lysates from cells used in Figure 5.6 were determined using a fluorescence□based lactate measurement kit (BioVision). Asterisks signify that the difference in lactate levels between the treated and untreated samples is significant as determined by the Student's t□test ($p<0.05$).

Figure 5.8

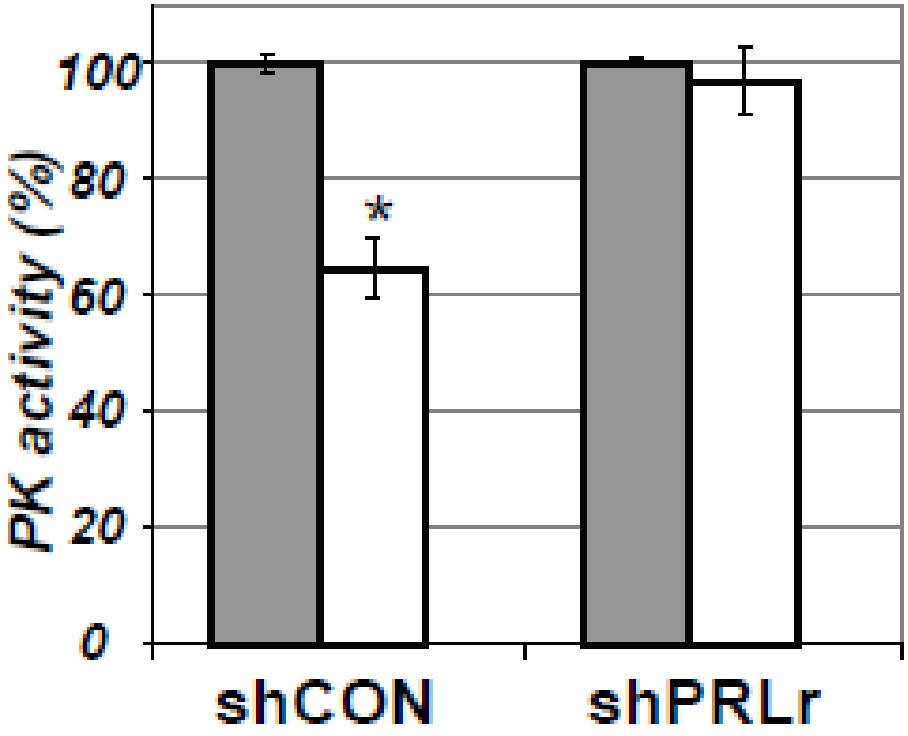


Figure 5.8: Pyruvate kinase activity was measured in the lysates from T47D□derived cells that harbor shRNA against GFP (shCON) or against PRLr (shPRLr). Cells were described in details in (Plotnikov et al., 2009)). Asterisks denote statistical significance of obtained differences ($p<0.05$ in the Student's t□test).

Figure 5.9

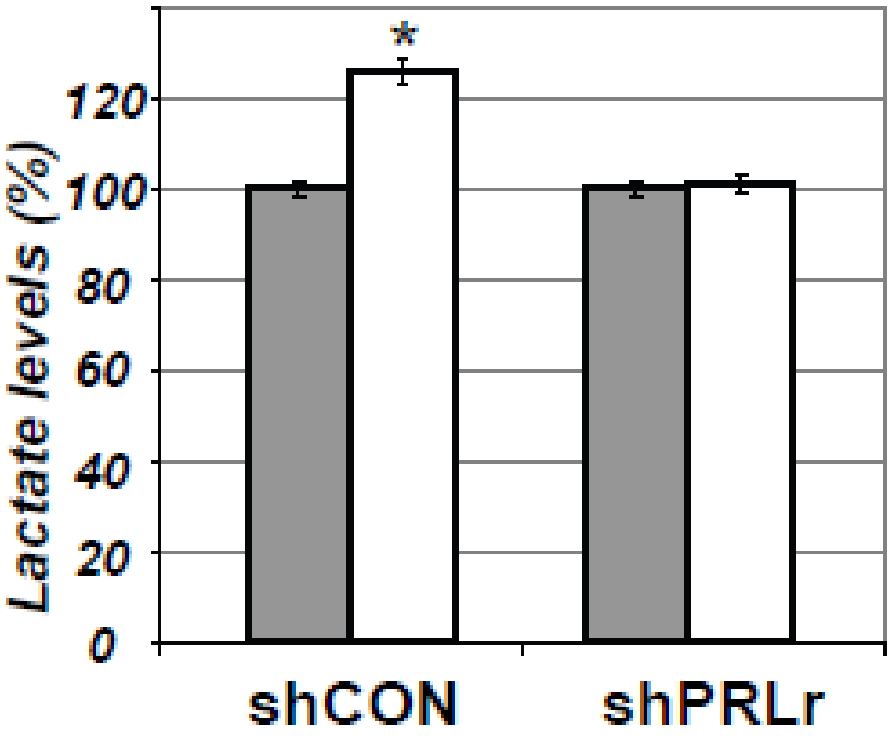


Figure 5.9: Lactate levels in the lysates from cells used in Figure 5.8 were determined as outlined in Figure 5.7.

Figure 5.10

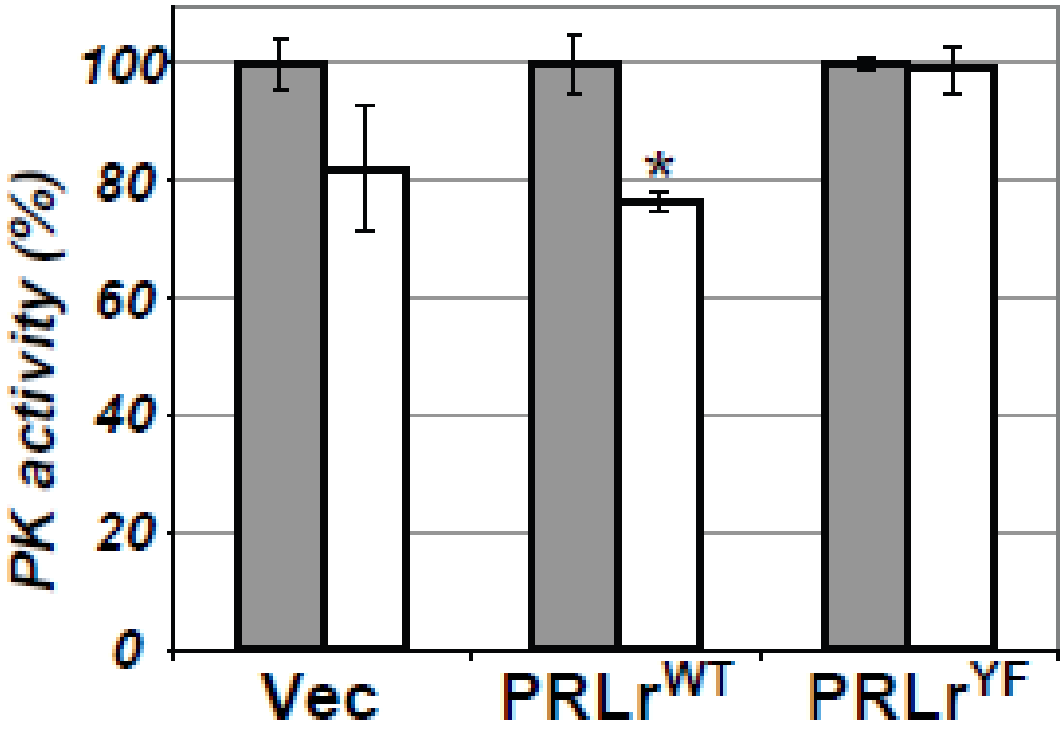


Figure 5.10: Activity of pyruvate kinase was determined (as in Figure 5.4) in lysates from 293T cells transfected with vector control (pcDNA3) or vectors for expression of PRLr (wild type or YF mutant (all intracellular tyrosines mutated to phenylalanine, described in (Swaminathan et al., 2008b)) and treated (100 ng/mL prolactin for 20 minutes, white bars) or not (grey bars) with PRL. Average data from four independent experiments (each in triplicate) are presented as % of activity measured in cells that did not receive PRL (± S.D.). Asterisks denote statistical significance of obtained differences ($p<0.05$ in the Student's t□test).

Figure 5.11

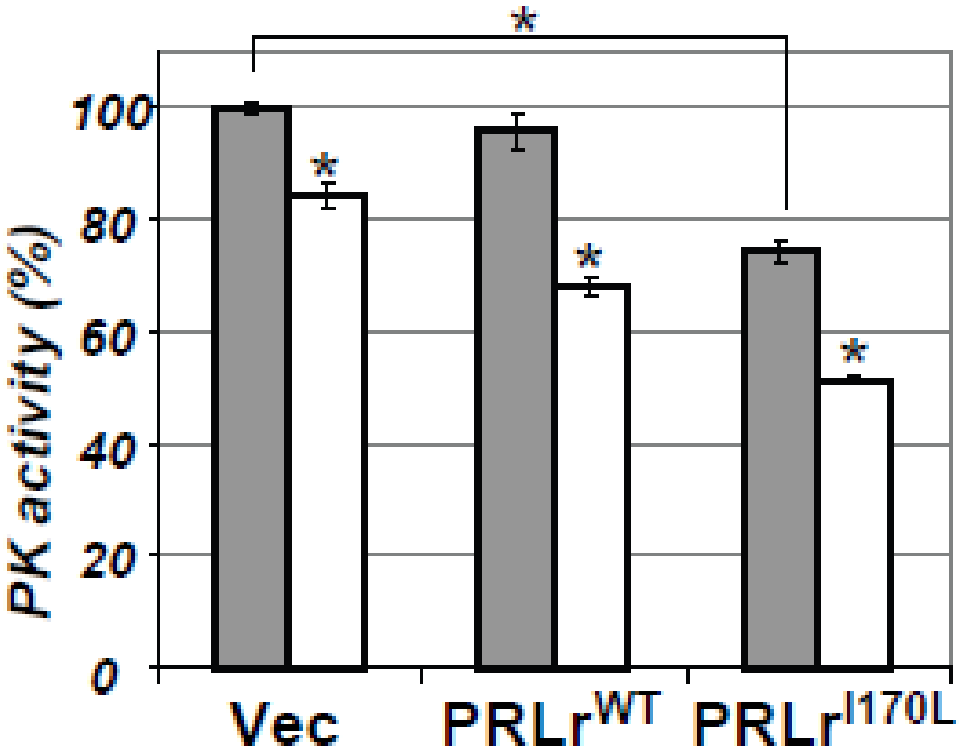


Figure 5.11: Pyruvate kinase activity was determined in lysates from 293T cells transfected with vector control (pcDNA3) or vectors for expression of PRLr (wild type or I170L mutant constructed by site directed mutagenesis in the backbone of pcDNA3□HA□PRLr, described in (Varghese et al., 2008)). Asterisks denote statistical significance of obtained differences ($p<0.05$ in the Student's t□test).

Figure 5.12

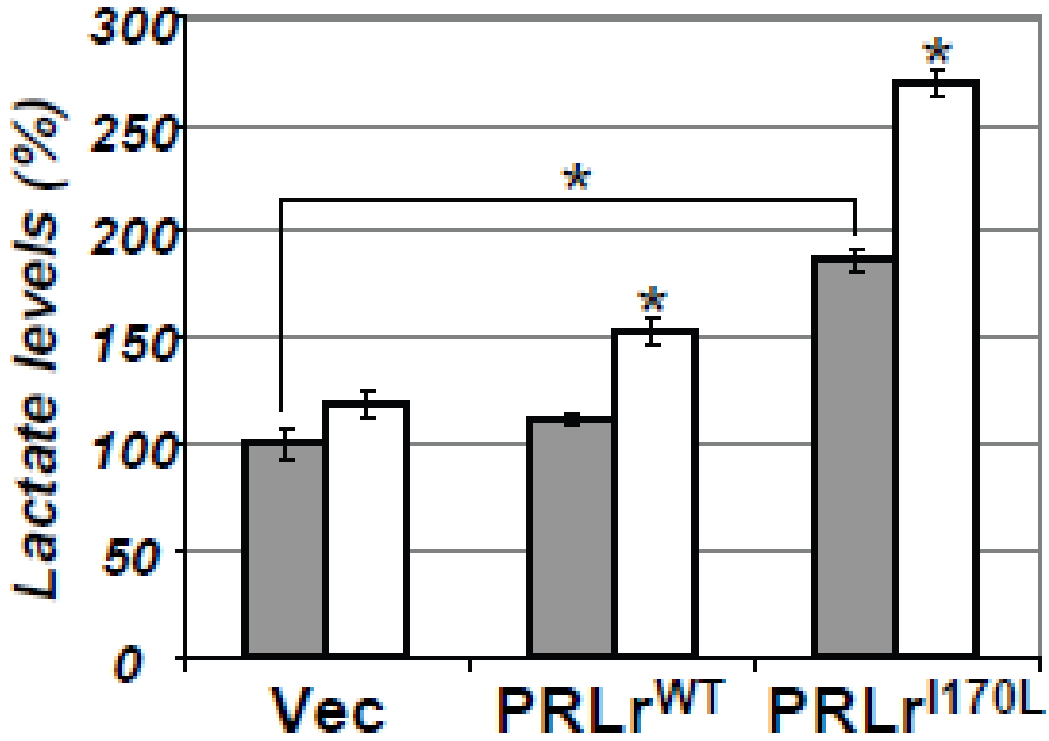


Figure 5.12: Lactate levels in the lysates from cells used in Figure 5.11 were determined as outlined in Figure 5.7.

Figure 5.13

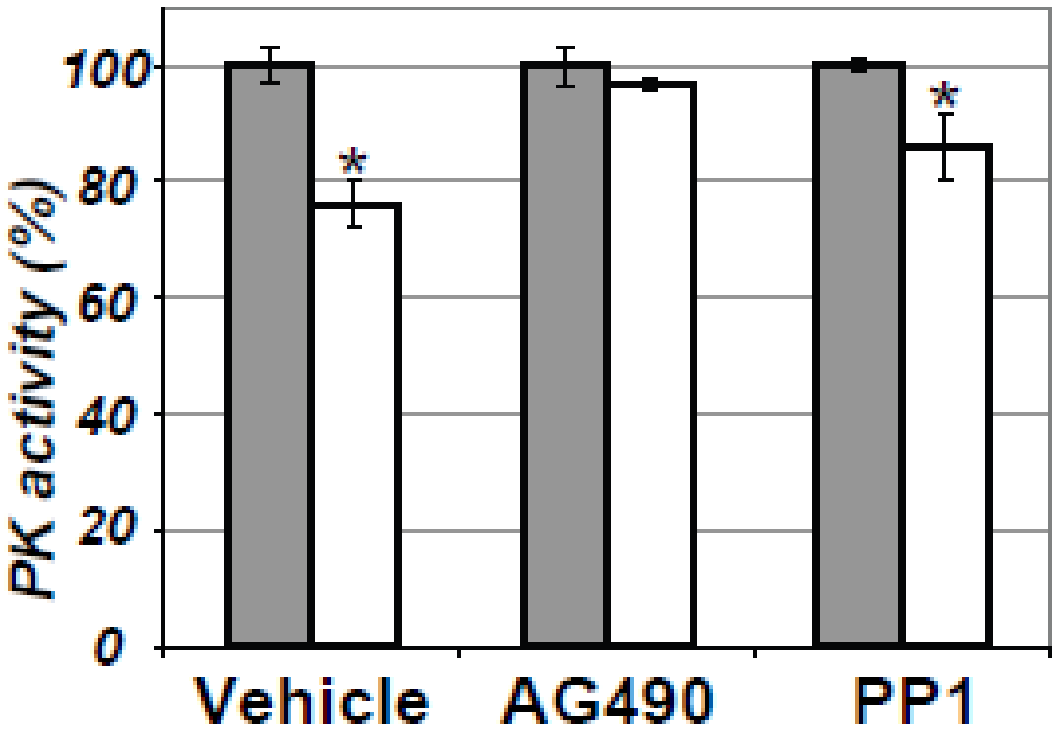


Figure 5.13: Pyruvate kinase activity was determined in lysates from 293T cells pretreated with ethanol (Vehicle), Jak inhibitor AG490 (AG490, 50 μM), or Src inhibitor PP1 (PP1, 10 μM) purchased from Calbiochem. Asterisks denote statistical significance of obtained differences ($p<0.05$ in the Student's t□test).

Figure 5.14

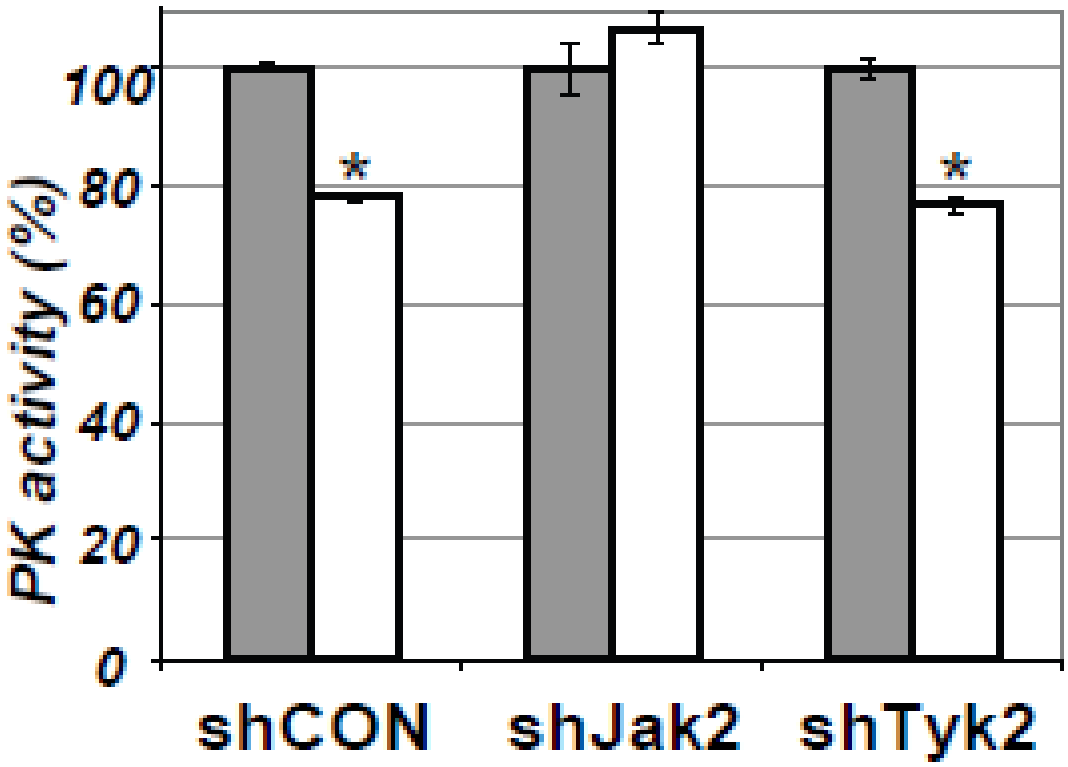


Figure 5.14: Pyruvate kinase activity was determined in lysates from 293T cells transfected with a control shRNA (shCON), shRNA targeting Jak2 (shJak2), or shRNA targeting Tyk2 (shTyk2, all constructs described in (Kumar et al., 2008)). Asterisks denote statistical significance of obtained differences ($p<0.05$ in the Student's t□test).

Figure 5.15

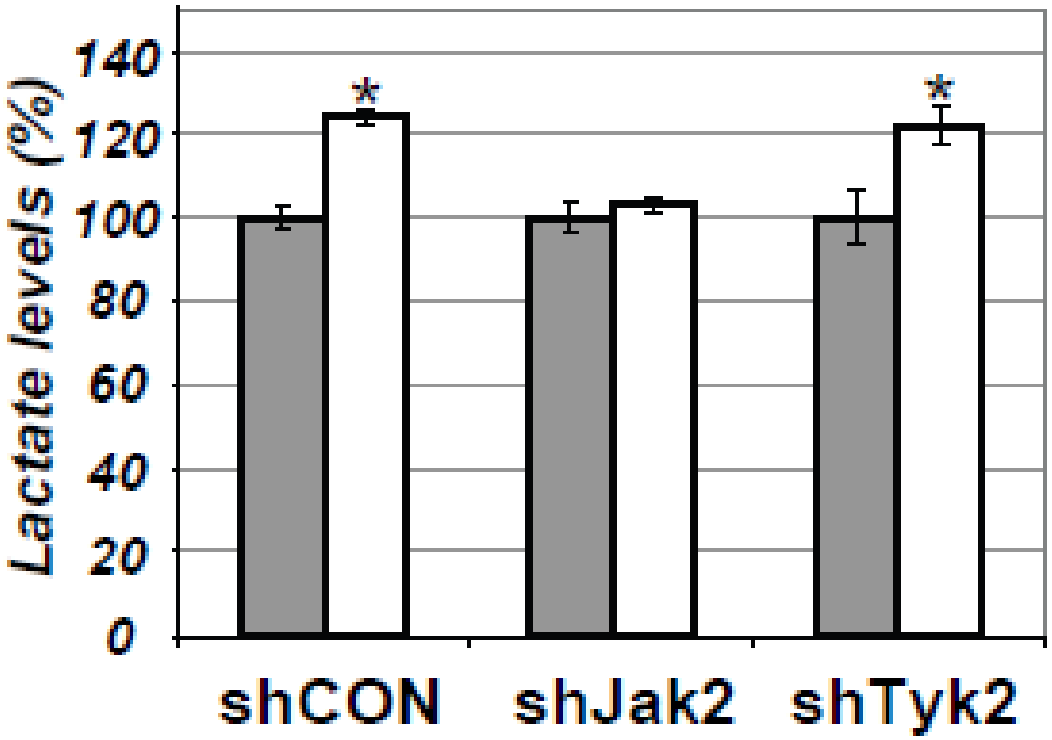


Figure 5.15: Lactate levels in the lysates from cells used in Figure 5.14 were determined.

Figure 5.16

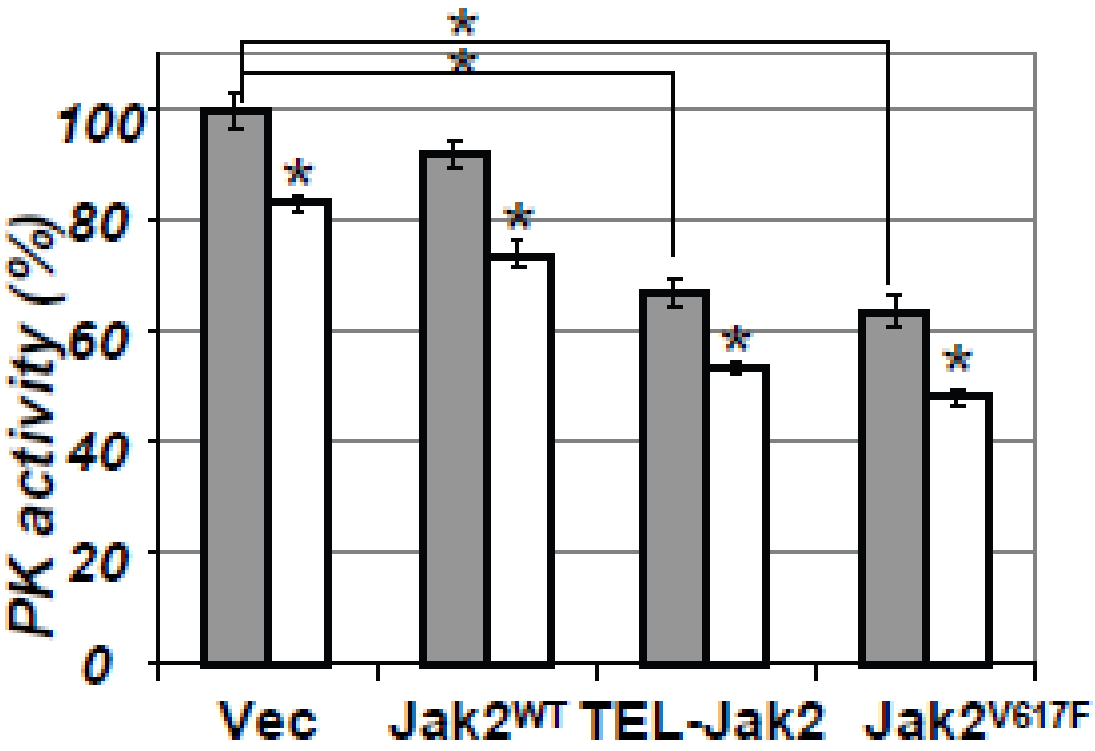


Figure 5.16: Pyruvate kinase activity was determined in lysates from 293T cells transfected with vector control (pcDNA3) or vectors for expression of Jak2 (wild type or TEL□Jak2 fusion or V617F mutant). Asterisks denote statistical significance of obtained differences ($p<0.05$ in the Student's t□test).

Figure 5.17

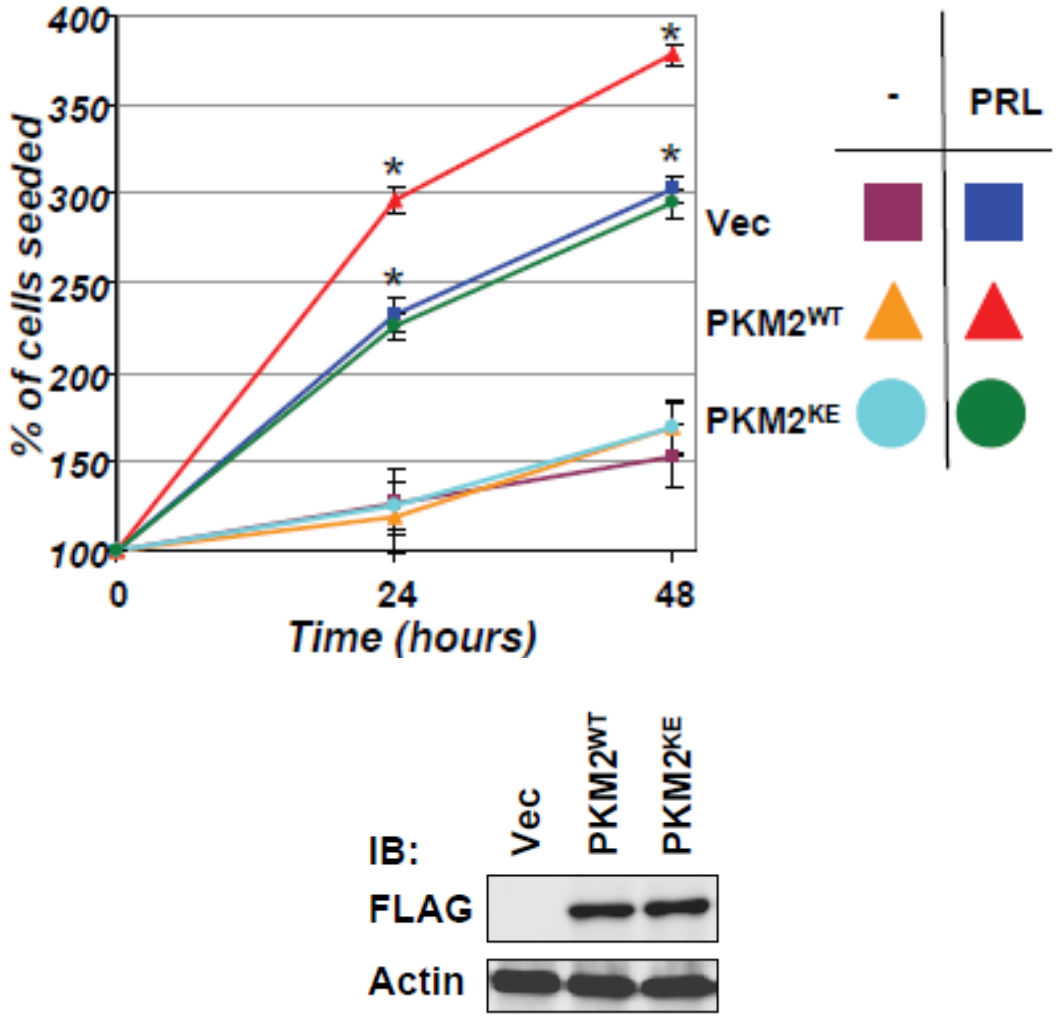


Figure 5.17: The rate of proliferation of rat lymphoma Nb2□11C cells electroporated with a vector control (Vec), wild type murine Flag□PKM2 ($PKM2^{WT}$), or murine Flag□$PKM2^{K433E}$ ($PKM2^{KE}$). $1x10^6$ cells were plated in triplicate for each sample and timepoint and cultured in the absence or presence of PRL (200ng/ml). Trypan□blue negative live cells were counted at 24 and 48 hours after plating. Flag□$PKM2^{WT}$ and FLAG□$PKM2^{KE}$ cloned into pLHCX retroviral vectors have been previously described (Christofk HR et al., 2008). Average data from three independent experiments (each in triplicate) are presented as % of number of initially seeded cells (± S.D.). Asterisks signify that the difference in growth rates between the treated and untreated samples is significant as determined by the student's t□test ($p<0.05$). Material from experiment shown in top panel was analyzed by immunoblotting using anti□Flag (Sigma) and anti□beta actin (Sigma) antibodies in bottom panel.

Figure 5.18

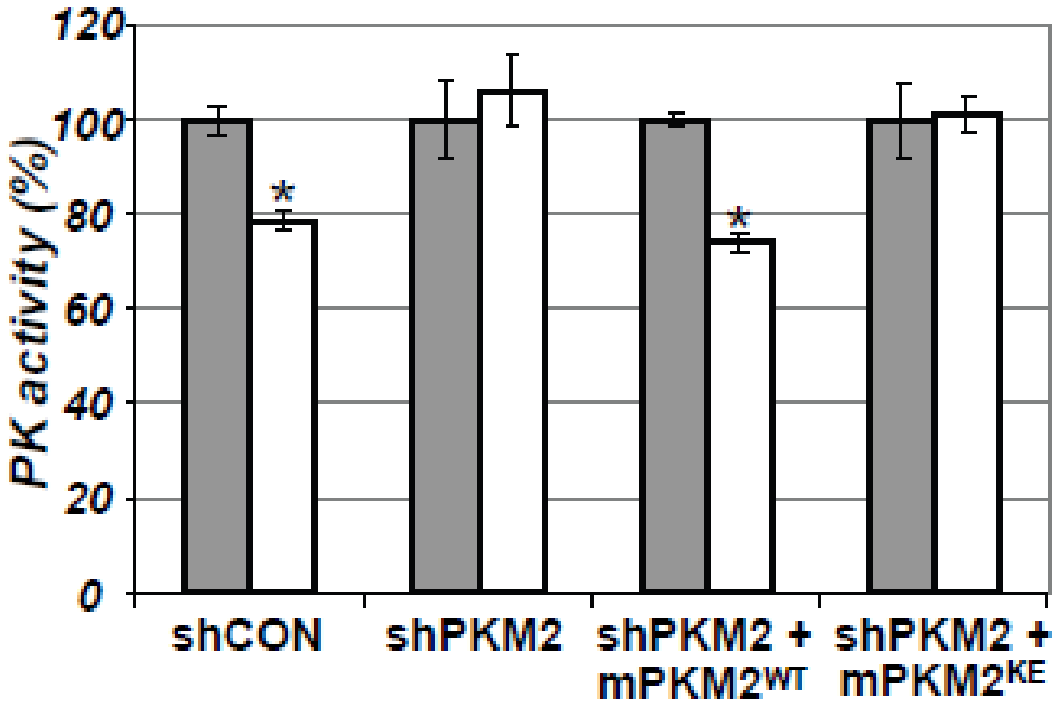


Figure 5.18: Pyruvate kinase activity was determined (as in Figure 5.4) in lysates from PRL□deficient MCF7 cells transduced with a control shRNA or shRNA targeting PKM2 (alone or in addition to an expression vector expressing a non□targetable murine PKM2 protein (wild type or K433E mutant)) and treated (200 ng/mL prolactin for 30 minutes, white bars) or not (grey bars) with PRL. Asterisks denote statistical significance of obtained differences ($p<0.05$ in the Student's t□test). PRL□deficient MCF7 cells (Schroeder et al., 2002) and control and PKM2□specific shRNA lentiviral vectors (Christofk et al., 2008a) have been previously described.

Figure 5.19

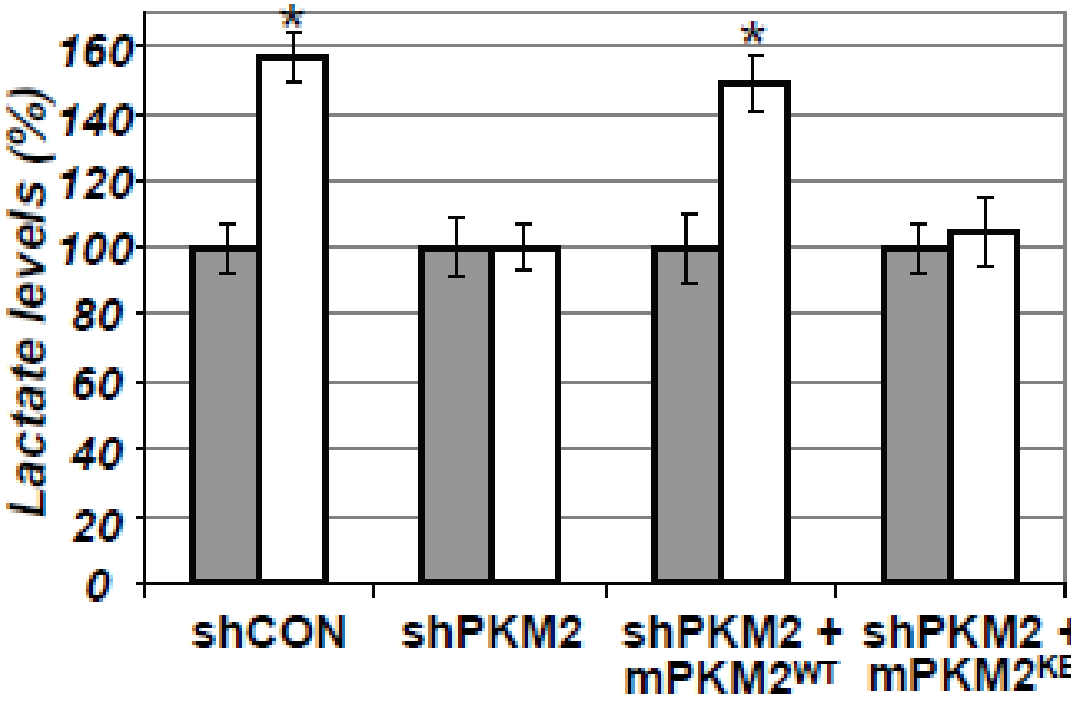


Figure 5.19: Lactate levels in the lysates from cells used in Figure 5.18 were determined.

Figure 5.20

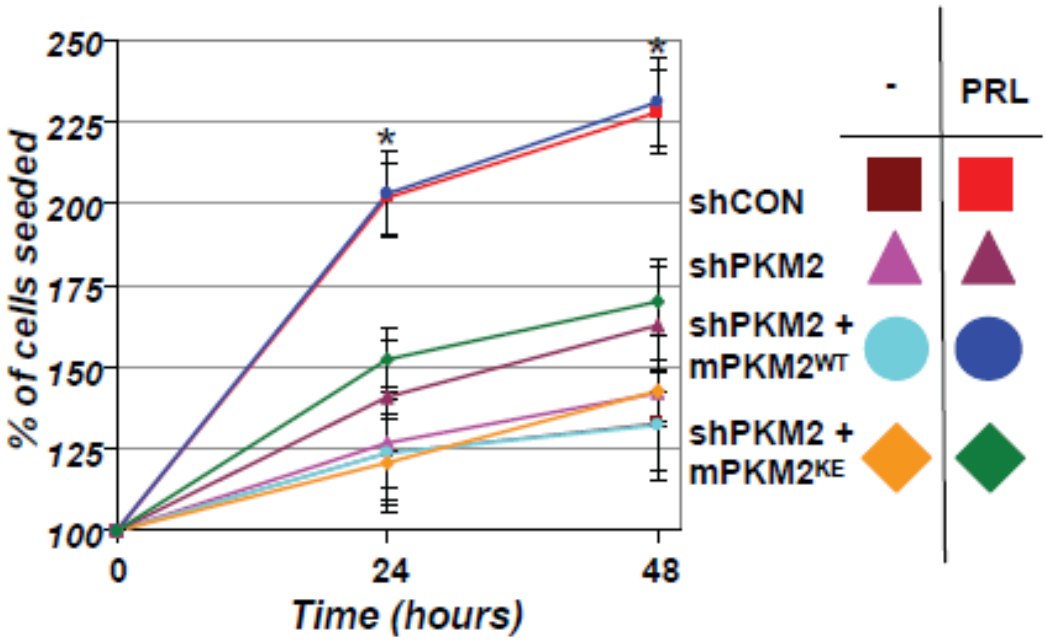


Figure 5.20: The rate of proliferation of MCF7□derived cells described in Figure 5.18 was determined as outlined in Figure 5.17. Average data from three independent experiments (each in triplicate) are presented as % of number of initially seeded cells (± S.D.). Asterisks denotes statistically significant differences ($p<0.05$ in the student's t□test).

Chapter 6

Discussion and Future Directions

In this thesis, we have investigated the downregulation of PRLr, its key determinants, and how elevated PRLr levels can result in a tumorigenic phenotype (Model 1). In **Chapter 2**, we showed that PRL signaling is dependent on the level of PRLr and that the internalization of PRLr is a ligand-induced process that depends upon Jak2 activity (Model 1). It is likely that upon ligand binding to PRLr, a conformational change occurs within the intracellular tail of each receptor subunit which brings the constitutively bound Jak2 proteins in close proximity. These Jak2 proteins then work to phosphorylate each other and phosphorylate key intracellular tyrosine residues on PRLr which need to be phosphorylated for effective PRL signaling. In addition to the role of Jak2 in PRL signaling, Jak2 also promotes the kinase activity (either directly or indirectly) of another yet to be identified kinase which phosphorylates PRLr on Ser 349, a key serine residue of the phosphodegron motif of PRLr. Upon phosphorylation of Ser 349, the $SCF^{\beta\text{-}TrCP}$ E3 ligase complex is recruited to PRLr and ubiquitinates the receptor at target lysine residues, leading to subsequent degradation of the PRLr. The kinase which phosphorylates Ser 349 of the phosphodegron leading to recruitment of β-TrCP E3 ligase, PRLr ubiquitination, and receptor degradation, has yet to be identified. Studies aimed at the identification of this putative kinase and the mechanisms of its activation might be of translational

value since this may be the key in regulating PRLr levels. The identification of small molecules that stimulate PRLr phosphorylation and turnover should benefit those patients whose malignancies depend on PRL signaling. However if the chosen target(s) promotes Ser 349 phosphorylation, there may be some augmentation of tyrosine phosphorylation of PRLr, similar to the effect of Jak2. If such viable targets act like Jak2 in promoting PRL signaling and driving receptor degradation, any treatment hitting these targets may have counterproductive effects.

In **Chapter 3**, we have shown that PRLr endocytosis is dependent on Ser 349 phosphorylation (part of the phosphodegron motif), β-TrCP activity, and is a clathrin-dependent process which utilizes the AP-2 adaptor protein complex to tether the PRLr into clathrin-coated vesicles. The internalization of PRLr was shown to be dependent on K63-linked polyubiquitination of the receptor as these ubiquitin linkages may be recognized by proteins of the endocytic machinery, thereby targeting PRLr for efficient internalization and subsequent degradation (Model 2). The way in which the cell can recognize the specific ubiquitin linkages and determine receptor fate is yet to be determined and remains an interesting avenue for further study. Our studies showed that the interaction of PRLr and the AP-2 adaptor protein complex (which acts as an adaptor to couple internalized receptors and clathrin into clathrin-coated vesicles) is dependent on the K63-linked polyubiquitination of PRLr. It is possible that AP-2 itself or associated proteins possess the ability to act as an 'ubiquitin reader', whereby the linkages found on the PRLr can be identified and it is then decided whether the endocytic machinery will

work to internalize PRLr. While some have proposed that the fates and signaling properties of differentially ubiquitinated substrates is due to the different shapes of the polyubiquitin chains being sterically read by other interacting proteins. However, the possibility remains that there is an 'ubiquitin reader' protein that can bind polyubiquitin chains and identify the linkages. Such a protein would be of a great deal of interest and would provide a breakthrough in the ubiquitin field. If such a protein exists, it is likely a protein of the endocytic machinery that contains an ubiquitin-interacting domain; this protein could bind to an ubiquitinated substrate, mark it for internalization, and target it for degradation (whether by the proteasome or lysosome) or altered signaling (such as the nerve growth receptor TrkA which is K63-polyubiquitinated and once internalized, signals in an altered fashion inside the vesicles).

While our studies suggested that K63-linked polyubiquitination targets PRLr for internalization and subsequent degradation, our data did show that PRLr is also K48-linked polyubiquitinated. The purpose of this K48-linked polyubiquitination and what role it may play in PRL signaling have yet to be determined. Furthermore, if PRLr is polyubiquitinated via K63 and K48 linkages, what is the fate of PRLr that is polyubiquitinated with a mix of both linkages? Is this receptor still targeted for lysosomal degradation? Is PRL signaling altered? These questions still remain to be investigated.

In **Chapter 4**, we show that enhanced PRLr levels can lead to increased growth (both 2-D and 3-D) and invasiveness and this can cause a non-tumorigenic

cell line to have enhanced growth properties both in culture and with in vivo mouse models. This proposed the importance of PRLr in tumorigenesis yet the possible mechanism by which PRLr enhances cell growth was unknown (Model 1). These data show that the study of PRLr downregulation is a worthy endeavor in that elevated PRLr levels can result in a pro-tumorigenic phenotype. While the exact relationship of PRL signaling and tumorigenesis, especially that in breast cancers, is yet to be clearly understood, the presence of aberrant PRL signaling in breast cancers has been established for many years. Our work in **Chapter 5** looking at the relationship of PRL signaling and PKM2, which plays a well-established role in cancer development and maintenance, provides us with a provocative mechanism by which the PRLr promotes tumorigenesis as seen in **Chapter 4**.

In **Chapter 5**, we found that PRLr interacts with PKM2 and that PRL signaling inhibits PKM2 in a manner dependent on PRLr Tyr phosphorylation, PRLr level, and Jak2 activity (Model 3). This PRL-mediated PKM2 inhibition was found to be a key factor in regulating PRL-mediated cell growth; this signaling crosstalk between PRLr and PKM2 likely plays a key role in PRL-mediated tumorigenesis. Is the interaction between PRLr and PKM2 responsible for the ability of PRL to inhibit PKM2 activity? Our data points to this fact but it still must be proven. We have yet to determine the effect of PRL on PRLr-PKM2 binding and what regions/motifs of PRLr interact with PKM2.

Future studies need to be focused on identifying the protein whose phosphotyrosine residues binds PKM2 and results in the inhibition of its enzymatic

activity in response to PRL. This protein could be PRLr itself, Jak2, or another effector of PRL signaling which we did not study. Identifying this protein may prove to have its own technical challenges. The protein which directly inhibits PKM2 (in response to PRL) is likely an important effector of PRL signaling. Any interference with the tyrosine phosphorylation of this protein may prevent inhibit PKM2 activity and PRL signaling at the same time, making it difficult to tease out the protein which directly inhibits PKM2. If this protein can be identified, inhibition of this protein using a small molecule inhibitor may provide a way to abrogate the pro-tumorigenic effect of PRLr while keeping normal PRL signaling relatively intact. Understanding the mechanism by which PRL signaling inhibits PKM2 activity may give choice insights into the role of PRL in breast cancer and other human malignancies. Since our data shows that constitutively active Jak2 resulted in PKM2 inhibition even in the absence of PRL, this may implicate other cytokine receptors, which signal through Jak2, to promote tumorigenesis by the inhibition of PKM2. The inhibition of PKM2 activity, by various cytokine signaling pathways or any signaling pathways that activate Jak2, may prove to be a widespread mechanism by which normal signaling pathways become aberrant and lead to tumorigenesis.

By investigating the mechanism of downregulation of PRLr, we have provided new insights into how levels of PRLr are maintained within the cell. We showed that elevated levels of PRLr are sufficient to promote cell proliferation, invasion, and tumorigenic growth in near normal breast epithelial cell lines. One novel mechanism by which PRLr exerts its pro-tumorigenic effect is by the

inhibition of PKM2, which acts as a key regulator of the energy production pathways within the cell, allowing for a tumor cell to hijack normal energy production pathways for metabolites that are used to create the building blocks of a growing tumor.

Future studies into the regulation of PRLr levels and the interplay between PRLr and PKM2 will provide us new insights into the role of PRL signaling and tumorigenesis within the breast, giving us hope of elucidating the enigmatic role of PRL signaling in breast cancer pathogenesis.

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