Molecular Analysis of FER/Actin Complexes in Prostate Cancer

by

Daniel Thiruganaratnapathy

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Department of Medicine, Division of Experimental Medicine

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“The human body is a work of art woven together by God, the Artist.”
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Abbreviations

AR    androgen receptor
ATP   adenosine triphosphate
ABP   Actin binding proteins
BPH   Benign prostate hyperplasia
BSA   Bovine serum albumin
Cyt.D Cytochalasin D
DAPI  4'-6-Diamidino-2-phenylindole
DHT   5α-dihydrotestosterone
DMSO  Dimethyl sulfoxide
DPC-1 Dog prostate cancer-1
DTT   dithiothreitol
EFC   Extended FCH
F-BAR FCH-BAR
FBS   Fetal Bovine Serum
FCH   Fps/Fes/Fer/CIP4 homology
FER   FPS/FES Related
GF    Growth Factor
GST   Glutathione S-Transferase
HBSS  Hanks’ Balanced Salt Solution
IGF   Insulin-like growth factor
IgG   Immunoglobulin
IL-6  Interleukin-6
IPTG  isopropyl-b-D-thiogalactopyranoside
LC-ESI Liquid Chromatography-Electrospray Ionisation Tandem
MS    Mass Spectrometry
<table>
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<tr>
<th>Abbreviation</th>
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<tr>
<td>NES</td>
<td>Nuclear Exit Signal</td>
</tr>
<tr>
<td>Na$_3$VO$_4$</td>
<td>sodium orthovanadate</td>
</tr>
<tr>
<td>NH$_4$Cl</td>
<td>Ammonium Chloride</td>
</tr>
<tr>
<td>(NH$_4$)$_2$CO$_3$</td>
<td>Ammonium Bicarbonate</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear Localization Signal</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PSA</td>
<td>Prostate Specific Antigen</td>
</tr>
<tr>
<td>PCa</td>
<td>Prostate Cancer</td>
</tr>
<tr>
<td>pY</td>
<td>phosphotyrosine</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology 2</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcription 3</td>
</tr>
<tr>
<td>UGM</td>
<td>Urogenital Sinus Mesenchyme</td>
</tr>
<tr>
<td>WL</td>
<td>Whole lysate</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
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**Amino Acids**

<table>
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<tr>
<th>Letter</th>
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<tbody>
<tr>
<td>F</td>
<td>Phenylalanine</td>
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<td>Y</td>
<td>Tyrosine</td>
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**Purines and Pyrimidines**

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<tr>
<td>A</td>
<td>Adenine</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
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Abstract

Prostate cancer (PCa) is among most prevalent causes of cancer death in North American men. Hence mechanisms of progression are poorly understood. The host laboratory reported on the up-regulated FER tyrosine kinase in PCa, comparatively to the benign prostate, and on its nuclear accumulation in higher grade tumors. In vitro, FER also contributed to cell survival and growth. In searching for FER partners, the tyrosine phosphorylated (pY)-actin was discovered for the first time in mammalian cells. In this study, pY-actin levels were correlated to aggressive phenotypes of PCa cell lines. FER was shown to phosphorylate actin directly and to bind actin via its SH2 domain. Furthermore, levels of complexes increased in response to stress, once targeting cytoskeletal actin in a process accentuating levels of predominantly nuclear, globular and tyrosine phosphorylated actin. Considering data in amoeba, it is proposed that FER and pY-actin provide tumors with cell survival advantages to resist stress, thereby contributing to PCa progression.
Résumé

Le cancer de la prostate (CaP) est une cause majeure de décès en Amérique du Nord. Or, les mécanismes de progression demeurent obscurs. Le laboratoire d’accueil a rapporté la surexpression de la tyrosine kinase FER dans le CaP comparativement à la prostate bénigne, et son accumulation dans le noyau des cellules tumorales de haut grade. In vitro, FER contribue à la survie et la croissance des cellules du CaP. La recherche des partenaires de FER a conduit à la découverte de l’actine et de sa phosphorylation sur tyrosine (pY), une première chez les cellules de mammifères. Dans cette étude, nous démontrons que les niveaux de pY-actine corrélat avec le phénotype agressif des lignées cellulaires de CaP. FER phosphoryle l’actine directement et s’y lie via son domaine SH2. De plus, les niveaux de complexes augmentent en réponse à un stress qui cible l’actine du cytosquelette, en un processus qui accentue les niveaux nucléaires d’actine globulaire phosphorylée sur tyrosine. Considérant les données chez l’amibe, il est proposé que FER et la pY-actine confèrent aux tumeurs, des avantages de survie et de résistance au stress qui contribuent à la progression du CaP.
1- INTRODUCTION
1.1 THE PROSTATE

The prostate gland is a chestnut-shaped organ located below the neck of the bladder and surrounding the urethra. Exclusively part of the male urogenital system, its principle function is to provide the proteins and ions that compose approximately 30% of the seminal fluid that make up the ejaculate (Hayward and Cunha 2000).

1.1.1 PRE- AND POST- NATAL DEVELOPMENT OF THE PROSTATE

Development of the prostate is initiated at 10 weeks of fetal development with the growth of prostatic buds (Kellokumpulehtinen, Santti et al. 1980). The urogenital sinus mesenchyme (UGM), derived from the embryonic mesoderm of the fetus, contains androgen receptors (AR), which are stimulated by testicular androgens. This induces epithelial budding and the subsequent appearance of three interrelated cell subtypes, basal, luminal and neuroendocrine, which in turn directs and patterns the differentiation of prostatic smooth muscles (Cunha, Donjacour et al. 1987; Hayward and Cunha 2000). This development of prostatic tissue is primarily dependent on exposure to androgens, rather than sex determining genetic factors. Hence, the UGM of a female fetus can form a functional prostate if exposed to androgens, at the proper developmental stage (Jost 1968).

The biologically active androgen responsible for prostate development, via AR nuclear signaling, is a testosterone derivative called 5α-dihydrotestosterone (DHT). DHT is formed by the reduction of testosterone by an enzyme called 4,3-ketosteroid-5α-reductase, which is localized in the epithelial and stromal compartments of the prostate (Levine, Wang et al. 1996).
During childhood the prostate has a slow rate of growth and is fairly small, weighing about 2g (Coffey 1987). At puberty, it undergoes an exponential growth period, reaching a peak doubling time of 2.76 years, until reaching full maturity in adult (Coffey 1987). The average healthy adult prostate weighs about 20g, and returns to its slow rate of growth. Normal healthy prostate cells are rather quiescent as a result of slow rates of apoptosis and growth (Isaacs 1985).

1.1.2 THE MATURE PROSTATE

The fully developed prostate in an adult contributes to reproductive success in all species. The prostate gland is responsible for the secretion of the prostate fluid, which makes part of the ejaculate. This fluid contains a number of proteins involved in semen gelation, coagulation, liquefaction, as well as in the coating and uncoating of spermatozoa (Aumuller and Seitz 1990). Thereby, prostatic secretory products keep the sperm healthy and fit for natural ovum fertilization. However, as proven by in vitro fertilization technologies, it is clear that sperm fluidity and capacitation can be achieved without the requirement of prostatic secretions (Hirokawa 1909)

One of the principal secretory products of the prostate is Prostate Specific Antigen (PSA), a 34kDa glycoprotein, responsible for liquefying semen and permitting sperm motility (Balk, Ko et al. 2003). Indeed this glycoprotein is normally expressed in prostatic luminal epithelial cells, secreted in the ductal lumen and expelled in the semen during ejaculation. Under normal conditions, the protein does not cross the basement membrane surrounding glands or prostatic acini, and thus is generally not found in the bloodstream. However, under abnormal conditions such as benign prostatic hyperplasia (BPH) and PCa,
PSA leaks into the prostatic stroma and the bloodstream (Balk, Ko et al. 2003). Hence, BPH and PCa can to a certain extent be conveniently screened for by monitoring free and bound PSA levels in the bloodstream (Moore, Kuhrik et al. 1992). Above and beyond its activity on seminal proteins, PSA has been shown to cleave the insulin-like growth factor (IGF) binding protein-3, making IGF-I locally available for growth stimulation, while inhibiting programmed cell death (Cohen, Graves et al. 1992). PSA expression is usually depended on the availability of androgens. However, PSA expression has been found to be androgen-independent in PCa patients who suffer a relapse after androgen ablation therapy. It is now known that this androgen-independent expression of PSA is mediated by AR signaling under growth factor (GF) stimulation, which provides survival and growth advantages to prostatic tumor cells (Hakariya, Shida et al. 2006). PCa cells exhibit a wide array of such androgen-independent survival/growth pathways in the latter stages of the cancer. Deciphering the key regulators and players in such pathways can lead to new therapeutic avenues for delaying and eliminating the progression of PCa.

1.1.3 ANATOMY OF THE PROSTATE

The prostate can be divided into four respective zones; the peripheral, central, transition and anterior fibromuscular zone (Kirby 1996). The peripheral zone is composed of up to 70% of the prostate, and is found surrounding the distal urethra. More than 70% of prostatic cancers originate from this region of the prostate. The central zone, found surrounding the ejaculatory ducts, is composed of approximately 20-25% of the prostate, and accounts for about 25% of the cancers. The transition zone, surrounding the proximal urethra, represents merely 5% of the prostate, but continues to grow throughout a man’s
lifetime. This zone is rarely associated with carcinoma, but is responsible for BPH. Finally, the anterior fibromuscular zone is composed of muscles and fibrous tissues, and accounts for no more than 5% of the total prostate weight. The human prostate may also be divided into “virtual” (non-atomically defined) lobes; anterior (the transition zone), posterior (peripheral zone), lateral (all zones) and median (central zone) (Kirby 1996).

1.2 PROSTATE PROLIFERATIVE DISEASES

1.2.1 BENIGN PROSTATIC HYPERPLASIA

BPH is the most common prostate disease among aged men, affecting about 70-80% of men in their 70’s and 80’s. It is noncancerous, and caused by an increase in epithelial and stromal prostate cells (Padayatty, Marcelli et al. 1997). A benign prostate can weigh up to 150g, resulting from an increase in the peak doubling time of the prostate (4.5 years) (Padayatty, Marcelli et al. 1997). Increases in the proliferation rate, hypertrophy and distension of the stromal component of the prostate can be observed, while the rate of apoptosis remains as in normal tissue (Tanagho 1992; Colombel, Vacherot et al. 1998). BPH is highly common but may be asymptomatic in men and therefore often does not require treatment. When symptomatic, BPH patients experience lower tract urinary problems consequent to urethral compression (Colon and Payne 2008). Drugs to shrink the prostate gland, relax peri-urethral muscles in the prostate and lessen urinary symptoms are the most common and effective treatment options, together with surgery to remove excess tissues surrounding the prostatic urethra.

It is well established that BPH is not a precursor to PCa. Studies have revealed equal probabilities of developing PCa in men with or without BPH (Tanagho 1992). As
aforementioned, PCa arises in the peripheral zone of the prostate gland, as opposed to the transition zone in BPH (Deburuyne 2002).

1.2.2 PROSTATE CANCER

PCa is the leading cause of cancer amongst Canadian men, as well as one of the leading causes of cancer related mortalities (Canadian Cancer Society 2008). It was estimated that 24,700 new cases of PCa will have occurred in 2008, including 4,300 deaths resulting from the disease (Canadian Cancer Society 2008). In the last couple of decades, there has been a sharp rise in the diagnosis of PCa. This rise can be attributed to the discovery of circulating PSA (Stamey, Yang et al. 1987), which has become a marker. More public awareness in the aging male population has increased screening, which nowadays comprises the blood PSA test, along with digital rectal exam. These diagnostic techniques have greatly contributed to aid in the early detection of PCa. As a result of treatments in earlier stages of the disease, mortality rates caused by PCa have declined significantly between 1995 and 2004 (Canadian Cancer Society 2008). Nevertheless, PCa still remains amongst prevalent cancer threats to Canadian men, and much research into the prevention is required to unravel its etiology.

1.2.2.1 CLINICAL MANAGEMENT

Compared with other types of cancer, prostate cancer is relatively slow growing. The best chance for cure is when the disease is caught at its early stages, implying that tumor cells are localized within the prostate, and that efficient treatment options, such as surgery or radiation, exist. Unfortunately, a significant percent will experience a
recurrence, indicating that some subsets of tumor cells had already gained metastatic ability. One study of 1,439 men who underwent prostatectomy, revealed a 15% rate of recurrence after surgery (Cooperberg, Pasta et al. 2005). Moreover, while this is less frequent, a certain proportion of patients present with spread disease at diagnosis. In such circumstances endocrine therapies aimed at inhibiting the production of testicular androgens and/or blocking androgen binding to the AR. The response is initially favorable in a majority of patients, but only temporarily and followed by progression of the disease. At this point, therapeutic strategies involve chemotherapeutic drugs, which prolong life expectancy for a very short period of time, but unfortunately also harbor an environment that favors the evolution of the cancer into a highly aggressive, androgen-independent state for which there is presently no cure (Feldman and Feldman 2001).

1.2.2.2 RISK FACTORS

Studies have identified some key risk factors associated with PCa. Age is the most prevalent risk factor, as the risk of occurrence increases dramatically after the age of 50. Almost 2 out of 3 PCa patients are over the age of 65 (Canadian Cancer Society 2008). Genetic disposition and family history is the second most consistent risk factor of PCa (Steinberg, Carter et al. 1990). The risk of encountering PCa increases twice as high in men who have a first-degree relative with the disease, and four times as high for individuals who have more than one first-degree relative with PCa (Steinberg, Carter et al. 1990).

Geographic and ethnic differences are also amongst risk factors associated with PCa. Asian countries have significantly lower rates of PCa, with China having 4% of Canada’s incidence rate (Whittemore 1989). Genetic factors, along with cultural practices
(dietary habits) can be attributed to these differences (Whittemore 1989). In the United States, black men have a 50-60% higher incidence rate of PCa as opposed to white men (Whittemore 1989). Furthermore, a proposed model suggests that 0.6% of Caucasian men have an autosomal dominant inheritance of a rare high-risk allele that increases the risk factor by over 80% (Whittemore 1989; Carter, Beaty et al. 1992). The contribution of dietary habits can not be ignored when discussing risk factors of PCa, as observed amongst Japanese men who immigrated to North America (Whittemore 1989). Their rate of mortality from PCa, following migration, was significantly higher, likely resulting from changes in dietary consumption. Epidemiological studies clearly demonstrate a link between high fat intake and PCa, and furthermore an inverse association between the occurrence of PCa and the consumption of food rich in products such as lycopene, genistein and selenium (Mills, Beeson et al. 1989; Peterson and Barnes 1993; Gallagher and Fleshner 1998). Hence, consumption of certain dietary sources has been linked to the occurrence of PCa, while other nutritional sources to the prevention of the cancer.

1.2.2.3 GENOMIC AND EPIGENETIC CHANGES AND ONCOGENES

As is the case with various cancers, PCa has been proposed to result from the accumulation of genetic and molecular alterations in prostate cells that ultimately lead to malignant transformation and uncontrolled growth (Cathie Garnis 2004). In the case of prostate cancer, this linear hypothesis of carcinogenesis may not hold and was revisited recently on the basis of genome-wide profiling. Microarray comparative genomic hybridization of a series of PCa patient tumor DNA, has allowed a distinction of apparently favorable versus aggressive types, according to specific alterations (Lapointe, Li et al.
2007). Nonetheless, it appears that overall alterations favor tumor cell survival and growth by endorsing self-sufficiency in growth signals, insensitivity to growth-inhibitors, evasion from apoptosis, augmented replicative potential, promotion of angiogenesis and metastasis (Hanahan and Weinberg 2000). Mutations, deletions, chromosomal rearrangements or amplification of certain genes, can pave the way for inactivation of tumor suppressor genes, expression of oncogenic proteins and the activation of proto-oncogenes (Hanahan and Weinberg 2000).

The structure and function of oncogenes have been a major endeavor for half a century. Over 50 proto-oncogenes have been mapped in the human genome, and many cancer related mutations in these genes, then termed oncogenes, have been detected (Abelson, Simon et al. 1995). Such activated oncogenes lead to the expression of constitutively active oncoproteins, which favor the aforementioned survival/growth characteristics in tumor cells. A well researched example is the Ras proto-oncogene, which in normal cells expresses the RAS protein needed for GF-receptor signaling and culminating in DNA synthesis and growth (Goodsell 1999). However, a single mutation in the Ras gene results in an oncogene, which expresses a constitutively active RAS protein that promotes extensive cell division and growth (Paul and Mukhopadhyay 2004). This tumor gene has been strongly linked to various cancers, including bladder and prostate (Weber and Gioeli 2004). A number of different oncogenes and proto-concogenes such as BCL-2 (Segal, Cohen et al. 1994), Src (Chang, Bai et al. 2008), p53 (Scott, Earle et al. 2003) and Ras (Mukhopadhyay, Cinar et al. 2007) have been reported in PCa. Several of these genes encode Tyrosine Kinases (TKs), which are key in signaling pathways. It is
important to note that none of these oncogenes have been conclusively correlated with the initiation and progression of PCa (Peehl 1993).

1.3 TYROSINE KINASES

TKs are key enzymes playing a role in cancer, as several TKs are protein products of oncogenic translation. TKs are critically important for healthy cells, as they mediate signaling cascades and thus influence growth by controlling biological processes such as cell survival/death, proliferation, differentiation, metabolism and motility, in response to external and internal stimuli (Manash 2004). The fundamental enzymatic responsibility of a TK is to catalyze the phosphorylation of specific tyrosine (Y) residues of target proteins by conveniently transferring a phosphate group from adenosine triphosphate (ATP) to the Y residue. This post-translational modification is a pivotal component of normal cellular communication and maintenance of homeostasis (Schlessinger 2000). Though this TK activity is tightly regulated and controlled in normal cells by the antagonistic effects of phosphatases, in cancer cells these TKs acquire transforming functions due to mutations, overexpression, or autocrine-paracrine stimulation (Manash 2004). The result is a constitutive activation of what is normally a controlled signaling pathway, allowing for subsequent activation of other signaling proteins and second messengers that ultimately affect cell growth leading to malignancy (Manash 2004).

Deciphering the functional roles of oncogenic TKs in PCa, along with their mechanisms of expression and activation, may lead to the identification of novel meaningful markers in regards to prognosis and also, ultimately lead to new therapeutic targets to better manage progression. The host lab was one of the first to investigate TKs in
prostatic cells, given the theoretical possibility that dysfunctional TK expression/activity may contribute to the inherent survival/growth advantages found in aggressive forms of PCa. Our group had subsequently reported the up-regulation of the FER (FPS/FES Related) kinase in PCa, and furthermore, demonstrated the kinase’s influence on PCa growth (Allard, Zoubeidi et al. 2000; Zoubeidi, Rocha et al. 2009).

1.3.1 THE FER KINASE

The FER kinase is a member of the small FPS/FES family. The mouse Fer gene was originally identified through its homology with the V- abl oncogene. It encodes a non-receptor, 94kDa protein kinase, traditionally considered cytoplasmic (Pawson, Letwin et al. 1989). FER has a wide range of functions, including cell-cell adhesion (Rosato, Veltmaat et al. 1998), interaction (Kim and Wong 1995), motility (Craig, Senis et al. 2002), survival and proliferation (Allard, Zoubeidi et al. 2000). This was primarily demonstrated by its association to several signaling pathways through protein-protein interactions in diverse cell systems, including cytoskeleton regulating proteins such as cortactin, whereby interaction with FER mediates a growth factor dependent phosphorylation (Kim and Wong 1998). The SH2 domain of FER has also been implicated in EGFR and PDGF stimulated interactions and subsequent phosphorylation (Kim and Wong 1995). FER has also been implicated in cellular adhesion complexes, whereby the N-terminal of FER was shown to bind p120 catenin and regulate cadherin function by modulating cadherin-beta-catenin interaction (Lee 2005).

In theory the structure allows FER to interact and Y-phosphorylate other proteins. For instance as illustrated in Figure 1, FER has several unique domains, beginning with an
amino-terminal Fps/Fes/Fer/CIP4 homology (FCH) domain, followed by a coiled-coil region, a Src-homology-2 (SH2) domain, the carboxy-terminal kinase domain, which contains the ATP binding domain, the Nuclear Localization Signal (NLS) and the autophosphorylation site (Y714) (Hao, Heisterkamp et al. 1989).

The combined FCH domain and coiled-coil regions are now recognized as the F-BAR (FCH-BAR) or EFC (extended FCH) domain (Itoh, Erdmann et al. 2005). This particular domain was first described in PCH adaptor proteins involved in the regulation of cytokines and actin dynamics (Lippincott and Li 2000). It has now been identified in various proteins implicated in membrane trafficking, binding and tabulation, presumably driven by the oligomerization of F-BAR itself (Lippincott and Li 2000). Conventionally, the coiled-coil domain allows proteins to interact with the coiled-coil domains of other proteins, as well as with itself to form oligomers, and in the case of FER to form homotrimeric complexes (Craig, Zirngibl et al. 1999). Though this oligomerization of FER favors trans-autophosphorylation, it is not essential for the autophosphorylation of the kinase (Craig, Zirngibl et al. 1999). More importantly, the coil-coil domain could be involved in aiding to stabilize the interactions between FER and its substrates in order for FER to be in close proximity and subsequently allow or promote phosphorylation (Shibuya, Hanafusa et al. 1980).

SH2 domains, although chemically different in sequences (approximately 100-amino-acid region), can be found in various different proteins involved in signal transduction, particularly amongst kinases (Koch, Anderson et al. 1991). The primary function of such SH2 domains is to recognize and interact with Y-phosphorylated motifs pertaining to specific cellular proteins (Anderson, Koch et al. 1990), thereby encouraging
SH2 domain containing proteins to physically associate with Y-phosphorylated proteins, particularly via Y-sites of phosphorylation (Pawson and Scott 1997). Specificity in the recruitment of Y-phosphorylated proteins by SH2 domains is vital and achieved by the three to five residues located immediately C-terminal to the pY-site (Fantl, Escobedo et al. 1992). Irregular interactions may lead to the recruitment of inappropriate proteins, hence to undesirable or inappropriate activation of pathways. Since SH2 domains are involved in so many signaling pathways, much focus has been placed on this domain with therapeutic ambitions, as a target for drug design (Machida and Mayer 2005). Our group has reported evidence of the FER SH2 domain directly interacting with pY-STAT3 in PCa cells (Zoubeidi, Rocha et al. 2009), together with pY-AR (unpublished data).

The functional domain of FER is referred to as the catalytic or kinase domain, as it is responsible for transferring a phosphate group from an ATP to the tyrosine site of another substrate protein. The ATP binding sequence (Gly-X-Gly-X-X-Gly-X-Lys) in TKs is believed to be the consensus ATP binding site (Zeng, Aleshin et al. 1998) which corresponds to the 571-592 amino acid sequence of FER. Within the catalytic domain are also found the important NLS motif and the Y714 autophosphorylation site of Fer. The NLS domain, as the name implies, permits proteins access into the nucleus of a cell (Kalderon, Roberts et al. 1984). These sequences, composed of one or more positively charged lysine or arginine residues, are usually exposed at the surface of a folded protein, and are typically used to target proteins to the cell nucleus through the Nuclear Pore Complex or via its recognition by cytosolic nuclear transport receptors (Faustino, Nelson et al. 2007). The autophosphorylation, in the presence or absence of the coiled-coil domain, is necessary to attain optimal FER activity (Hao, Heisterkamp et al. 1989).
Figure 1. Structural domains of FER. A cartoon representation of the different domains of FER.
1.3.2 FER KINASE IN PROSTATE CANCER

Very little is known concerning FER in regards to cancer. The host lab reported on increased expression of the FER kinase associated with human prostate tissues that underwent malignant transformation (Allard, Zoubeidi et al. 2000). The FER protein was negligibly detected in the benign prostate, but significantly overexpressed in the cancerous prostate. Furthermore, the FER protein was found in the cytoplasm and nucleus of prostate tumors from patient specimens and human PCa cell lines (Allard, Zoubeidi et al. 2000; Zoubeidi, Rocha et al. 2009), with an apparently higher distribution in the nucleus of the more aggressive cell lines. FER was also detected at high levels in the highly aggressive dog prostate cancer cell line (DPC)-1 and observed in both subcellular compartments (Olivier 2003), while no FER protein was detected in the normal dog prostate or freshly dispersed prostate cells (Allard, Zoubeidi et al. 2000).

Further analysis in human PCa cell lines, notably in the aggressive androgen-independent PC-3 cell line using antisense Fer cDNA constructs and Fer siRNA, led to the conclusion that FER played a major role in the regulation of PCa cell survival and growth (Allard, Zoubeidi et al. 2000; Zoubeidi, Rocha et al. 2009). FER was found to be constitutively expressed in all PCa cell lines, including in the PC-3 model (Allard, Zoubeidi et al. 2000). As aforementioned, FER has a variety of established partners and functions, along with the ability to mediate signaling. However, attempts to reveal interactions between FER and identified partners, such as paxillin (adhesion), β-catenin, p120Cas (cell junction) and cortactin (cytoskeleton) in PCa cells failed to reveal any specific complex (Allard, Zoubeidi et al. 2000). Hence, it was hypothesized that the FER kinase, by its structure and competency, may likely bind and Y-phosphorylate alternative
signaling molecules as partners or substrates to promote survival and growth benefits in PCa cell lines.

1.3.3 FER KINASE PARTNERS IN PROSTATE CANCER

The host lab identified three specific pY-proteins that were found to interact with FER in the PC-3 cell line. Two of these proteins are well established players in PCa. One is the transcription factor STAT3 shown to promote metastatic progression of PCa (Abdulghani, Gu et al. 2008). Our group has shown evidence, demonstrating that the FER kinase in cooperation with interleukin (IL)-6, directly binds and phosphorylates STAT3 to promote progression of PCa (Zoubeidi, Rocha et al. 2009).

The second protein found to interact with FER is AR itself, the key transcription factor not only involved in the development of the prostate but also in the progression of PCa (Heinlein and Chang 2004). Of interest, AR interacts with pSTAT3, which contributes to the regulation of IL-6 mediated AR transcriptional activity on target genes in PCa cells (Aaronson and Iyengar 2001). Ongoing studies in the lab demonstrate that FER regulates the mechanism of AR activation by Y-phosphorylation and controls IL-6 mediated PCa cell growth, as well as the transcriptional regulation of the PSA gene (unpublished data). Hence, the interaction and influence of FER on STAT3 and AR appears to be significant in the context of PCa.

The other pY-protein found interacting with FER in PC-3 cells was actin. Such an interaction between FER and actin was reported in rat testicular extracts where several other cytoskeletal proteins formed complexes with FER (Chen, Lee et al. 2003). However
Y-phosphorylated actin (pY-actin) has never been detected in mammalian cells, let alone in human cancer cells.

1.4 ACTIN

Actin is a 42kDa glycoprotein found abundantly in cells (Poglazov 1983) and discovered from muscle extracts (Straub 1942). Traditionally, actin is regarded as a structural protein restricted to muscle cells, involved in the regulation of muscle contraction (Chabra 2008). There are four different actin isomers linked to muscle cells: two striated muscle (α-skeletal and α-cardiac), two smooth muscle (α - and β-) (Chabra 2008). It was later discovered in non-muscle cells as well, and in fact two distinct genes encode cytoplasmic β- and γ- actins (Hatano and Oosawa 1966). Non-muscular actin provides mechanical support for cell shape, by its dynamic capacity to assemble into filaments and disassemble back to monomers (Kabsch and Vandekerckhove 1992), on the basis of the monomeric protein having two conformations; globular (G-) and filamentous (F-) (Chabra 2008). It ensures control of cell motility and muscle contraction, notably by activating myosin ATPase thus allowing activated myosin to move along actin filaments and produce the required force (Kabsch and Vandekerckhove 1992). Intriguingly, actin is also found in the cell nucleus, though its function is unclear.

1.4.1 ACTIN STRUCTURE

Actin consists of 375 amino acid residues arranged as a single chain. The sequence is highly conserved between species and the aforementioned types of actin (Bray 1973). Based on the crystal structure of monomeric actin, the protein may be subdivided into domains; small (residues 1–144 and 338–375) and large (residues 145–337), as shown in
the right and left sections of Figure 2, respectively (Chabra 2008). The small domain is further divided into subdomain I (residues 1–32, 70–144 and 338–375) and subdomain II (residues 33–69). The large domain is divided into subdomain III (residues 145–180 and 270–337) and subdomain IV (residues 181–269). The N- and C-terminal ends of actin are both present in subdomain I. Interestingly, actin also has cation and nucleotide binding domains, both located in the interdomain cleft of actin (Chabra 2008). The actin monomeric conformation between G- and F- is influenced by ions bound at the cation binding domain. The nucleotide-binding site is usually occupied by ATP, also shown in Figure 2 (Engel, Fasold et al. 1977).

Subdomain II of G-actin has been well documented to form direct complexes with DNase I (Khaitlina and Strzelecka-Golaszewska 2002). More specifically, residues 38-52, exposed at the top of subdomain II form the DNase I-binding loop (Moraczewska, Gruszczynska-Biegala et al. 2004). Binding provokes the inhibition of DNase I activity. These residues are the most dynamic elements of the actin structure (Moraczewska, Gruszczynska-Biegala et al. 2004). Cleaving the DNase I-binding loop of actin (residues 38-52) was shown to inhibit the motility of actin filaments (Schwyter, Kron et al. 1990). Located next to this loop is Y53, whose phosphorylation in amoeba was also reported (Kishi, 1998). In vitro studies showed that Y53 phosphorylation in actin hampers DNase I binding (Graceffa and Dominguez 2003). The extent of these findings is poorly understood thus far.
Figure 2. Atomic Structure of Actin. A cartoon representation of actin and its subdomains I, II, III & IV (Otterbein and Graceffa 2001).
1.4.2 ACTIN POLYMERIZATION

Actin monomers have the capability of self-assembling to form polymers of filaments (Chabra 2008). In vitro studies revealed that polymerization is initiated only once the concentration of G-actin monomers surpasses a critical concentration ($C_C$). Hence, at low levels polymerization does not occur. This $C_C$ is influenced and affected by experimental conditions such as temperature, pH and salt concentration (Asakura, Kasai et al. 1960). Whether these in vitro findings reflect the physiological state of a cell is unknown.

The polymerization of actin is essentially a condensation reaction. It involves four steps as illustrated in Figure 3. The first step is called activation, whereby ions bind ($\text{Mg}^{2+}$, $\text{K}^+$, $\text{Ca}^{2+}$) at the cation binding site of actin. This induces the second step resulting from ion-induced conformational G to F change that promotes nucleation (Gaszner, Nyitrai et al. 1999), implying that at this stage, actin monomers are in a conformationally unique structure, referred to as F-actin monomers, allowing the formation of relatively stable oligomers (Rich and Estes 1976). The monomers are then added at both ends, forming filaments, in a process requiring ATP (Chabra 2008). The process is reversible and dissociation may occur simultaneously, leading to ATP hydrolysis whereby the conformation is returned to G-actin. Finally, annealing or assembly of filaments concludes the process by locking protomers into position (Chabra 2008).
Figure 3. Actin Polymerization. The four steps involved in conformational changes and polymerization of actin monomers to form filaments (Chabra 2008).
1.4.3 ACTIN BINDING PROTEINS

Actin binds a number of different proteins, referred to as actin binding proteins (ABP). Today, approximately 162 distinct proteins have been identified to directly interact with actin (Pollard and Cooper 1962). ABPs are categorized into seven distinctive groups, which include monomer-binding proteins, filament depolymerizing proteins, filament end-binding proteins, filament severing proteins, cross-linking proteins, stabilizing proteins and motor proteins. Monomer-binding proteins bind exclusively to G-actin and prevent its polymerization (e.g. DNase I). Filament-depolymerizing proteins bind to filaments and promote the conformational conversion to G-actin (e.g. cofilin). Filament end-binding proteins bind at the ends of F-actin, thus preventing the addition of monomers. Filament severing proteins bind in between filaments of actin at the sides, thereby cutting polymerized F-actin into two pieces (e.g. gelsolin). Cross-linking proteins contain more than one binding site for F-actin, and thus promote the formation of filament bundles and branching filaments (e.g., Arp2/3). Stabilizing proteins prevent depolymerization of actin filaments, by binding to the sides of F-actin and stabilizing the polymer (e.g., tropomyosin). Finally, motor proteins move along filaments of actin, and act as a track to promote cell motility (e.g., the myosin family of motors). Overall, cell locomotion depends on the rapid assembly of actin filaments (Pollard and Cooper 1962). Several ABPs, such as DNase-I, myosin and cofilin, bind at the same loci at the surface of actin (residues 38-52) and as a result can be expected to compete with each other (McGough, Pope et al. 1997; Dos Remedios, Chhabra et al. 2003; Moraczewska, Gruszczynska-Biegala et al. 2004).

Careful analysis of various ABPs reveals that some of these proteins are directly involved in oncogenic activities and pathways, notably EGF receptor (Mendelsohn and
Baselga 2000), ERK (Garber 2006), Abl (Muller, Oma et al. 2005), p53 (Cronauer, Schulz et al. 2004) and DNAse I (Shiokawa, Kobayashi et al. 2002). Best documented are the actin interactions with Abl and DNAse I. For instance, Abl activity is inhibited when bound to F-actin, and subsequently the nuclear translocation of Abl is prevented (Woodring, Hunter et al. 2003). For DNase I, an endonuclease implicated in DNA fragmentation during apoptosis and cell death, activity being inhibited by G-actin binding would promote survival, as shown in breast cancer cells (Eulitz and Mannherz 2007).

1.4.4 NUCLEAR ACTIN

The traditional dogma is of actin being merely a cytoplasmic protein involved in structural scaffolding. However, several recent reports have clearly identified actin in the nucleus of cells. Results on nuclear actin were initially regarded as artifacts, primarily due to the lack of a NLS within despite the presence of two nuclear exist signals (Wada, Fukuda et al. 1998), the abundance of cytoplasmic actin, difficulties encountered in cleanly isolating the nuclei and its size believed to be too large to diffuse through the nuclear pore. A final argument which persisted was the failure to detect nuclear actin when labeled with fluorescent phalloidin. However, it was discovered that phalloidin specifically binds actin at the interface between polymerized F-actin subunits (Vandekerckhove, Deboen et al. 1985), and eventually nuclear actin appeared to be predominantly consisting of G-actin monomers (Meijerman, Blom et al. 1997). Hence, phalloidin staining would not have recognized the globular actin found in the nucleus.

The first convincing evidence of nuclear actin was published in 1977, in Xenopus-Leavis oocytes, where it was possible to perform careful hand isolation of nuclei and avoid
contamination with cytoplasmic proteins (Clark and Merriam 1977). In this model, actin constituted 6% of nuclear proteins. Soon after, it was discovered that actin was also found present in mammalian rat neurinoma cells (Polyakova, Chudinovskaya et al. 1986), wherein exposure to dimethylsulfoxide (DMSO) was shown to induce an increase in levels of nuclear actin. Other stressors such as hydroxyurea, deferoxamine and hydrogen peroxide have also shown to induce nuclear accumulation of G-actin (Kwak, Kim et al. 2004). Recently, actin has been shown to be a component of the SWI/SNF-like BAF chromatin remodeling complexes in yeast, fly and human cells (Olave, Reck-Peterson et al. 2002). The implication of this finding is significant, as the BAF chromatin remodeling complex is a known human tumor suppressor complex (Olave, Reck-Peterson et al. 2002). A possible involvement of nuclear actin with transcriptional events concerning protein-coding genes was also observed in Rat2 fibroblasts and HeLa cells (Scheer, Hinssen et al. 1984).

Amongst proposed mechanisms, explaining actin translocation into the nucleus, are interactions with other proteins that have a NLS sequence. Putative co-transporters such as coflin, have been suggested to be involved, particularly owing to the presence of the SV-40 type nuclear translocation signal (Nishida, Iida et al. 1987). During times of cellular stress, actin has been shown to move into the nucleus, possibly by its interaction with coflin (Pendleton, Pope et al. 2003). The function of nuclear actin and its chaperone(s) is still very unclear.
1.4.5 TYROSINE PHOSPHORYLATION OF ACTIN

There exists no published evidence thus far of pY-actin present in mammalian cells, and of TKs phosphorylating actin on Y residues. However, the serine and threonine phosphorylation of actin has been well documented. Studies have demonstrated that epidermal growth factors promote the serine phosphorylation of actin, and subsequent polymerization of the protein in HER14 fibroblasts (Vandelft, Verkleij et al. 1995). Furthermore, stress and the actin-fragmin kinase have been shown to be involved in the threonine phosphorylation of actin, on threonine 202, 203 and 558 residues (Furuhashi, Hatano et al. 1992; Shirai, Sasaki et al. 2006). Interestingly, actin-fragmin interactions were shown to severe F-actin, and only when complexed to fragmin was actin phosphorylated. In the context of this thesis, in dealing with the FER Y-kinase, only the Y-phosphorylation of actin was specifically investigated. There are 15 Y-residues in the actin sequence, and thus far two have been shown to be phosphorylated, Y53 and Y219 in the Amoeba and in chicken B-cells, respectively.

In the Amoeba, actin was found to be Y-phosphorylated in cells that had recovered from stress, in a process proposed to withstand cell death (Kishi, Clements et al. 1998). Various stressors such as oxygen depletion, inhibitors, heat shock and nutritional starvation were all inducers. The Y53 found phosphorylated was identified by mass spectrometry (Liu, Shu et al. 2006) and is located in subdomain II (Figure 2). The Y53-phosphorylation of actin inhibited actin nucleation and elongation (Liu, Shu et al. 2006), thus preventing filament formation.
In chicken B-cells, the major site of actin tyrosine phosphorylation was the residue 219, whose dephosphorylation by the Src homology protein phosphatase, SHP-1, occurred upon antigen stimulation and implicates direct interaction with SH2 domain of SHP-1 (Baba, Fusaki et al. 2003). The relationship to actin dynamics has not been investigated in this study.

1.5 FER AND ACTIN

The observation of a relationship between FER and actin was initially obtained while seeking for FER partners among Y-phosphorylated proteins in parental PC-3 cells and its derived PRO4 cell variant. A 42kDa Y-phosphorylated protein was found in complex with FER, which was later identified as actin (Zoubeidi 2003). Based on the six gene classification mentioned above, prostate cells would primarily express non-muscle cytoplasmic form of β-actin. Further, subcellular fractionation studies revealed FER/actin complexes in the cytoplasm and nucleus together with detectable FER and pY-actin in the chromatin fraction. Moreover, levels of pY-actin depended on FER activity, as experiments carried out with catalytically inactive FER revealed a substantial decrease in pY-actin levels. It appeared that FER complexes with pY-actin occurred through binding with the FER SH2 domain. Lastly, preliminary experiments performed on cells exposed to Cytochalasin D (Cyt.D; 2µM for 2 hrs) revealed significant changes in the Y-phosphorylation of actin. Cyt.D is cell permeable mycotoxin, derived from a fungal metabolite, which specifically targets actin dynamics by inhibiting its polymerization and inducing the rapid depolymerization of actin filaments (Casella, Flanagan et al. 1981).
2- HYPOTHESIS AND OBJECTIVES
2.1 HYPOTHESIS

The above data coupled with the overexpression of FER in PCa and its established role in survival/growth, raises the intriguing possibility of FER being involved in the regulation of actin Y-phosphorylation in mechanisms linked to cell survival/growth in PCa.

2.2 OBJECTIVES AND SPECIFIC AIMS

The relationship between FER and actin, as well as the Y-phosphorylation of actin, are quite novel findings, considering that there is no published evidence of such phosphorylation in mammalian cells. The central goal of this study is to shed light on the mechanism(s) linking FER to pY-actin in prostate cancer, and to determine whether this could be linked to growth characteristics that accompany cancer cells. Deciphering such a role could be instrumental in understanding the progression of PCa.

The objectives of this study are to use PCa cell lines to: 1- Confirm and further analyze the interaction between FER and actin, including the cellular distribution of these complexes; 2- Validate the Y-phosphorylation of actin and investigate the significance of pY-actin in diverse cell lines 3-Ascertain relationship to stress by targeting actin dynamics; 4-determine and confirm site(s) of phosphorylation; 5- Observe the cellular localization of pY-actin; 6- Verify if actin is a direct substrate of FER; 7- Analyze consequence of targeting actin dynamics on cell fate.
3- MATERIALS AND METHODS
3.1 CELL LINES

The parental human prostate carcinoma cell lines, PC-3 (CRL-143) and LNCaP (CRL-1740), were obtained from the American Type Culture Collection (ATCC; Rockville, MD). The PC-3M variants, PRO4 and LN4, were provided by Dr. I. Fidler (Texas) and the LNCaP variant, C4-2, from Dr L. Chung (Virginia). The dog prostate carcinoma cell line, DPC-1, was developed by Anidjar et al. (Anidjar, Villette et al. 2001) and further characterized in the host lab. All PCa cell lines were cultured in RPMI 1640 growth medium (Invitrogen, Ontario, Canada) containing L-glutamine supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin/ streptomycin/neomycin antibiotics (Invitrogen).

3.2 DRUG TREATMENT

Cyt.D (Sigma, Oakville, Canada) was obtained and stored frozen at -20°C as a stock solution of 5mg/ml in DMSO. The drug was applied to the PCa cells at a final concentration of 1µg/ml (2µM), as reported (Schliwa 1982), for different time intervals ranging from 0-96hrs. Medium with serum containing or not Cyt.D was changed every 48hrs.

3.3 ISOLATION OF NORMAL DOG PROSTATE EPITHELIAL CELLS

Prostates were obtained at the euthanasia of adult mongrel dogs enrolled in other ongoing experimental protocols carried out by diverse investigators at the McGill University Health Center Research Institute (MUHC-RI). Prostates were weighed and then dissected to remove the capsule. The remaining tissue was uniformly minced (3mm³) and
a representative aliquot was frozen at -80°C for subsequent extraction. Tissue minces were subsequently re-suspended in a solution of 1% type I collagenase (Invitrogen) in Hanks’ Balanced Salt Solution (HBSS) containing D-glucose, dibasic sodium phosphate, monobasic potassium phosphate, potassium chloride and sodium chloride (Invitrogen) to dissociate cells, as reported (Chevalier, Bleau et al. 1981). Each digestion step took place at 37°C under rapid agitation for 15-20 minutes. Depending on the amount of prostate tissues to dissociate, the procedure was repeated three to four times. At each step, cells were recovered by centrifugation (10 minutes at 2000g.), placed on ice and pooled together. An aliquot was withdrawn to estimate the overall cell number by counting under a hemacytometer. Cells were next centrifuged at low speed and stored as pellets (~10⁷ cells per tube) at -80°C until protein extraction and analysis.

3.4 PREPARATION OF CELLULAR PROTEIN EXTRACTS

Cells in monolayers were washed with ice-cold PBS and pre-incubated for 10 minutes on a shaker at 4°C with PBS containing 1mM sodium orthovanadate (Na3VO4) (Sigma), a phosphotyrosine phosphatase inhibitor (Siegel, Meyer-Alber et al. 1999). The cells were lysed and extracted using a cold lysis buffer called RIPA (25 mM Tris•HCl pH 7.4, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing 1mM Na3VO4 and a Protease Inhibitor Cocktail (Roche, Montreal, Canada). Cell lysates were scraped on ice, collected and centrifuged at 13 000g for 10 minutes at 4°C, yielding the cellular crude extract in the supernatant.
3.5 IMMUNOFLUORESCENCE

Approximately $10^4$ PRO4 cells resuspended in complete RPMI medium (10% FBS and 1% antibiotic solution) were seeded on sterile coverslips in 6-well tissue culture plates and grown overnight at 37°C. Cells were then rinsed with cold phosphate buffered saline (PBS; 150mM NaCl and 50mM Na$_2$HPO$_4$, pH 7.4) containing Na$_3$VO$_4$, with all subsequent manipulations following fixation conducted on ice. Briefly, cells were fixed using 3.7% para-formaldehyde in PBS for 15 minutes. This was followed by triplicate washes, 5 minutes each, with PBS containing 50mM Ammonium Chloride (NH$_4$Cl). Cells were then permeabilized on coverslips with 0.1% Triton X-100 in PBS for 5 minutes, followed by another PBS wash for 5 minutes. Prior to primary antibody exposure, cells were incubated with a 0.5% bovine serum albumin (BSA) in PBS (blocking solution) for 1 hour to minimize non-specific binding of antibodies. Primary antibodies of interest were diluted 1:500 for anti-FER, 1:400 for anti-actin (Cell Signaling, monoclonal) and 1:200 for polyclonal pY53 actin antibody (ECM Biosciences, Kentucky, USA) in blocking solution and exposed to fixed cells for 1 hour. Following triplicate washes with PBS, cells were incubated with fluorescently-labeled secondary antibodies, coupled to either Alexa Fluor® (Alexa Fluor®488, Invitrogen) or rhodamine (Rhodamine CY3, Invitrogen), and diluted 1:1000 in blocking solution. The nucleus of the cells was visualized using 4’-6-Diamidino-2-phenylindole (DAPI), known to form fluorescent complexes with double-stranded DNA (Coleman and Goff 1985). Cells were washed one final time and slides were mounted and stored in the dark.

A fluorescent G-actin probe (DNase I Texas Red conjugate, Invitrogen) having a bright red fluorescence was used to selectively bind and detect unpolymerized G-actin in
fixed cells. The probe consists of a specific amino acid sequence that has a strong affinity for G-actin at the DNAse I binding loop (Cramer, Briggs et al. 2002). It was used as recommended by company, except for a 2-fold dilution since the intensity was too strong. It was diluted 1:3000.

Fluorescence was visualized using an inverted Olympus IX-81 microscope (Olympus, Center Valley, USA) equipped with a CoolSnapHQ digital camera (Tucson, AZ) and analyzed using the ImagePro+ software version 5.0.1 (Median Cybernetics, Silver Spring, USA). Confocal images were taken using a Leica DMLFSA confocal microscope (Leica Microsystems, Wetzlar, Germany), and images were analyzed using the aforementioned ImagePro+ software. All cell images were taken at 80x magnification.

3.6 PROTEIN QUANTIFICATION

The protein concentrations of cell extracts were determined through a colorimetric technique using a bicinchoninic acid (BCA) based protein assay (BSA Protein Assay Kit, Thermo Scientific, Rockford, USA).

3.7 FER ANTIBODIES

Rabbit polyclonal FER antibodies were prepared by the host lab using a construct of the FER SH2 domain generated from a 420bp EcorI-EcorI of the dog Fer cDNA (residues 451 to 564) fused to Glutathione S-Transferase (GST) and subcloned in the pGEX-3Z vector (pGEM®-3Z Vector, Promega, Wisconsin, USA). The 44kDa fusion protein was induced in Escherichia Coli at 30°C with 0.4mM isopropyl-b-D-thiogalactopyranoside (IPTG) for 4hrs. Proteins were extracted by sonication in PBS.
followed by solubilization in 0.1% Triton X-100 for 30 minutes at room temperature, according to Zoubeidi et al (2009). The fusion protein was purified on a Glutathione Sepharose™ 4B column (GE Healthcare), as recommended by the manufacturer. Purity was assessed by Coomassie Blue staining after electrophoresis (SDS-PAGE), as described below. The GST protein (29kDa) was similarly produced and used to assess specificity.

The immunization protocol (1mg FER fusion protein per injection) was as reported earlier (Allard, Zoubeidi et al. 2000), following the collection of a blood aliquot to prepare pre-immune serum and corresponding immunoglobulins (IgGs). FER antibodies were partially purified from the antiserum and pre-immune rabbit serum (control IgGs) on protein-G and A Sepharose beads (Invitrogen, Burlington, ON, Canada) prior further purification by an affinity column consisting of the FER SH2 domain covalently coupled to agarose beads.

3.8 IMMUNOPRECIPITATION

Protein extracts (500µg) were immunoprecipitated overnight at 4°C with appropriate antibodies (mouse or rabbit), at a dilution of 100µg:1µg. Primary antibodies included rabbit polyclonal anti-FER (as prepared above), actin (Cytoskeleton, Denver, USA), and pY53-actin together with mouse monoclonal pY- (Cell Signalling, Boston, USA) and FLAG (Sigma). 30µl of protein G agarose beads (Roche, Ontario, Canada) were next added for 2hrs at 4°C and incubated with gentle rocking. Immunocomplexes were recovered by centrifugation for 5 minutes at 10 000g and washed four times with lysis buffer. Proteins of interest were dissolved in a Laemmli protein loading buffer (0.313M
Tris-HCl, 10% SDS, 0.05% bromophenol blue and 50% glycerol) and then analyzed by Western blotting.

3.9 PULL DOWN ASSAYS WITH THE FER SH2 DOMAIN

The purified FER-SH2 GST fusion protein generated above (section on FER antibodies) was used to perform the pull down assays, according to Zoubeidi et al (2009). Briefly, protein extracts (750µg) from PRO4 cells were incubated with 7.5µg of the FER-SH2 (Zoubeidi, Rocha et al. 2009) domain fusion protein on a shaker overnight, at 4°C. Glutathione Sepharose beads were then introduced to interact with and pull down the FER-SH2 GST fusion protein complex along with any proteins that interact with the FER-SH2 domain. The mixture was centrifuged, washed three times with PBS, and analyzed via Western blotting.

3.10 WESTERN BLOTTING

Whole cellular protein extracts (50µg) or immunoprecipitated proteins (as outlined above) were resolved by electrophoresis in polyacrylamide gels (running gel: 10-12% acrylamide/ bisacrylamide, 1.5M Tris-HCl pH 8.8, 0.1% sodium dodecyl sulfate (SDS), 0.1% Temed and 0.03% ammonium persulfate) for 2-3 hrs at 100 volts. Subsequently proteins were electroblotted for 30 minutes/gel at 100 volts (4°C) onto nitrocellulose membranes (BioRad, California, USA). Membranes were blocked using either milk or BSA (3-5%) in TBS buffer (20mM Tris-HCl pH 7.4, 150mM NaCl). Primary antibodies were diluted 1:1000, unless otherwise specified, in blocking solution and used to incubate membranes overnight at 4°C on a shaker. Following washes in TBS, membranes were
incubated for 1 hour at room temperature in secondary goat anti-rabbit or anti-mouse antibodies coupled to horseradish peroxidase (Pierce, Rockford, USA). Following washes, proteins were immunodetected using a chemiluminescence horseradish peroxidase enzyme based antibody detection kit (HyGlo, Denville, New Jersey, USA), whereby antibodies were exposed to chemiluminescent reagent for 1 minute. Bands were scanned using a Hewlett Packard Scan Jet ADF densitometric scanner and analyzed by ImageQuant 350 (GE Healthcare, Buckinghamshire, England). Statistical analyses to measure correlation coefficient and standard deviations were performed on Excel (Microsoft, Washington, USA). The significance of differences were statistically verified according to the Student’s t-test, using a significance level of $\alpha=0.05$.

3.11 IN VITRO KINASE ASSAYS

A preparation of GST-tagged human recombinant FER kinase (catalytic domain) was obtained (Invitrogen) to phosphorylate commercial β-actin, from rabbit muscle cells (β-actin, Cytoskeleton), in vitro. The assay was carried out using 1µg of each respective protein in a phosphorylation buffer containing 25mM Tris-HCl, pH 7.2, 5mM β-glycerophosphate, 10mM MgCl$_2$, 0.5mM EDTA, 200µM ATP, 0.01% Triton X-100 and 0.5mM sodium vanadate. Prior to commencing the reaction 2.5mM of fresh dithiothreitol (DTT) was added. The kinase reaction was initiated upon addition of [γ-$^{32}$P]ATP (0.829mbQ/assay; specific activity; 111TBq/mmol; Perkin Elmer; Woodbridge, Canada) for 30 minutes at 30°C in a total volume 40µl/tube. Controls included either FER or actin alone. Reactions were terminated by cooling tubes on ice for 10 minutes and then adding the Laemmli protein loading buffer (5X). Samples were heated to 95°C for 5 minutes,
loaded as duplicate in two separate SDS-PAGE gels, and separated electrophoretically. Radiolabeled bands, loaded on one gel, were detected after 3 hrs of exposure using a Phosphoimager equipped with ImageQuant. The other gel was processed for Western blotting as a control using actin antibodies.

3.12 FER KNOCK DOWN

A validated siRNA with the sequence: CAGATAGATCCTAGTACAGAA (Hs_FER_5 HP Validated siRNA, Qiagen) was designed to target the human Fer gene, as reported Zoubeidi et al (2009). A non-mammalian fluorescent construct with the sequence: AATTCTCCGAACGTGTCACGT (Alexa Fluor 488 1022563, Qiagen, Ontario, Canada) was obtained to use as both a negative control and as a control to measure transfection efficiency. PRO4 cells were transfected using the Lipofectamine 2000 as a transfection reagent (Invitrogen), according to manufacturer’s instructions. The day before transfection, approximately 0.5X 10^6 cells were plated in a 100mm culture dish and allowed to attach overnight in complete growth medium without antibiotics.

Just prior transfection, constructs (600pmol) were diluted using 30µl Lipofectamine in 3ml of serum-free medium and allowed to complex for 10 minutes. Preparations were then transferred into the culture dishes, allowing for cell transfection to occur for 4hrs. The medium was renewed and cells were further cultured in the complete growth medium for 72hrs to attain significant knockdown of the Fer gene. Cyt.D (2µM) was introduced during the last 6hrs of incubation. Cells were then washed with PBS containing 1mM Na3VO4, lyzed to recover protein extracts for analysis as described above.
3.13 MASS SPECTROMETRIC ANALYSIS

The 42kDa band pertained to be pY-actin once resolved on a Coomassie blue G250 stained SDS-PAGE gel, was cut out and minced into cubes. The experimental protocol was performed in collaboration with Dr. Bernard Gibbs (Department of Medicine, McGill Univeristy). Briefly, the minced gels were destained in a 50% methanol/5% acetic acid in water solution. Once the Commassie Blue stain was removed, the gel pieces were dehydrated in 200µl of acetonitrile until turning opaque-white. A speedvac was used to dry samples between steps. After dehydration, proteins were reduced using 50µl of 10mM DTT for 30 minutes. Samples were dried and alkylated in 50µl of 50mM iodoacetamide for 30 minutes, and then washed with 100µL of 100mM ammonium bicarbonate for 10 minutes. Finally, they were dehydrated with acetonitrile and then rehydrated with ammonium bicarbonate ( (NH$_4$)$_2$CO$_3$ ). This process was repeated three times, after which gel pieces were dried. Trypsin (Promega) was prepared in 50mM of (NH$_4$)$_2$CO$_3$ at a final concentration of 20ng/µl, and used to digest proteins. Excess, unabsorbed trypsin was then removed, and the samples were incubated in 20µl of 50mM (NH$_4$)$_2$CO$_3$ overnight at 37°C. Proteins were finally extracted from the gel with 30µl of 100mM (NH$_4$)$_2$CO$_3$, and analyzed through Liquid Chromatography Electrospray Ionisation Tandem (LC-ESI) Mass Spectrometry (MS) on a QSRAR MS instrument. The data from the LC-ESI MS results were analyzed using an algorithm program offered by Matrix Science called MASCOT, searching specifically for actin fragments exceeding predicted mass by 80daltons, compatible with the addition of a phosphate group.
3.14 SITE-DIRECTED MUTAGENESIS

Human beta-actin plasmids (GC-T1780) were obtained from GeneCopoeia (Maryland, USA). Primers incorporating the appropriate mutation of Y53 to phenylalanine (F), were designed using the PrimeX Automated Sequencing software. The oligos were commercially synthesized and HPLC purified (Invitrogen), to aid plasmid mutation. The in vitro mutagenesis of actin was carried out using the QuikChange Site-Directed Mutagensis Kit (Stratagene). Successful mutation was verified by sequencing the mutant plasmids at the McGill University and Genome Quebec Innovation Center. The mutated plasmids were transformed into XL1-Blue supercompetent E.Coli cells and amplified. The amplified plasmids were then lysed and purified using the Plasmid Maxi Prep Kit (Qiagen).

The mutant Y53F actin plasmids were then transfected in PRO4 cells using SuperFect Transfection Reagent (Qiagen) for 48hrs in order to be expressed and analyzed. In some instances, cells were treated with Cyt.D, as detailed below in results.

3.15 CELL GROWTH

Cell growth was monitored by MTT assays (Sigma) according to Zellweger et al. (Zellweger, Miyake et al. 2001). PRO4 (2 x 10^3 cells) were plated on 96-well plates and cultured in serum-supplemented medium for 24hrs. Cells were then exposed to Cyt.D at a concentration of 2μM for increasing time intervals (from 0 to 96hrs). Medium was replaced twice a day. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) was dissolved in PBS at a concentration of 5mg/ml, filter-sterilized and stored at 4°C. For assays, 20μL of the stock solution was added to each well, and the cells were incubated for 4 hrs at 37°C. The media was then removed, and DMSO (200μL) was
added to solubilise the formazan. Number of surviving cells was assessed with a standard microplate reader to determine the absorbance at a wavelength of 540nm. Each experimental data point represented average values obtained from eight replicates, and each experiment was done at least twice. Values were recorded daily up to 96 hrs. MTT results were analyzed on EXCEL. The significance of differences were statistically verified according to the Student’s t-test, using a significance level of \( \alpha=0.05 \). Cell viability was also assessed by a trypan blue exclusion, whereby a cell suspension is exposed to 0.4% trypan blue, and then analyzed through a hemocytometer. The dead cells are observed in distinctive blue colour, since the diazo dye is absorbed only through the membrane of cells that are no longer viable.
4- RESULTS
4.1 ACTIN INTERACTS WITH FER

The host lab had gathered biochemical evidence of a specific interaction between FER and actin in the parental PC-3 cell line and its derived PRO4 variant. Moreover these two proteins appeared to be present in nuclear protein extracts from PRO4 cells. To get further insight, we sought to first pursue these studies by investigating if complexes were common in other PCa cell models and next determine the intracellular distribution of FER and actin by immunofluorescence.

4.1.1 BIOCHEMICAL EVIDENCE OF FER/ACTIN COMPLEXES

Confirmatory biochemical experiments were initially conducted, with a new generation of rabbit FER polyclonal antibodies, generated in the lab, using the FER-SH2 domain fused to GST as the antigen. IgGs had been purified from the antiserum and characterized. Figure 4A reproduces some of the FER antibodies specificity data, recognizing the FER-SH2 domain (44kDa) in Western blot analysis whereas no band was detected with pre-immune IgGs. FER antibodies also revealed a predominant 94kDa protein in PCa cell extracts, while no bands were detected with pre-immune IgGs. In addition, FER antibodies detected the myc-tagged recombinant FER protein once expressed after transient transfection of the dog fer cDNA in PCa cells (Fig. 4B). The transfected cells exhibited two bands, representing the endogenous and exogenous FER proteins. They also had the ability to immunoprecipitate the FER protein, as illustrated by a blot probed afterwards with the same FER antibodies.

The presence of complexes between FER and actin was next demonstrated by immunoprecipitation of FER in PCa cell lysates with FER antibodies, followed by Western
Figure 4. FER antibodies recognize the FER-SH2 domain and the FER protein in PCa cells

(A) The fusion protein FER-SH2-GST was induced (+) in E.Coli (left panel: Coomassie stained gel, lane 3). The fusion protein was next purified by ultrafiltration (30kDa cutoff membranes) and used to generate rabbit polyclonal FER antibodies, which were purified from the antiserum as described in Methods. The GST protein (lane 2) was similarly produced as a control. Arrows indicate the position of the FER-SH2 fusion and GST proteins. Molecular weight standard markers are on the left (Stds, lane 1). The Western blot of the FER-SH2 domain (lane 4; 50 µg protein) with FER antibodies and control IgGs (lane 5) are shown on the right.

(B) Right upper panel: Western blot with FER antibodies (top left) to detect endogenous FER (94kDa) in control LNCaP cells (-) and ectopic FER migrating slightly above 94kda after transient transfection of canine myc-his tagged fer cDNA in LNCaP (+), next probed with myc antibodies (top right). Lower panel: FER antibodies recognized immunoprecipitated FER (94kDa) and FER by direct blotting of proteins from PC-3 cell lysates (W.L.). A control immunoprecipitate with rabbit IgGs, next probed with FER antibodies, is included (bottom middle).
blot analysis with actin antibodies. This is depicted for PRO4 cells in Figure 5A. A direct Western blot of actin is also shown, in parallel with the FER/actin complex. As expected, no complex was detected when proteins were immunoprecipitated with pre-immune IgGs and probed with FER antibodies, thus demonstrating specificity of the immunoprecipitation.

The same approach was applied to protein extracts prepared from PC-3 and its variant, LN4, which along with PRO4 are reported to be more aggressive derivatives of PC-3 (Pettaway, Pathak et al. 1996). As shown in Figure 5B, complexes between FER and actin were present in these cell lines. They were detected in all PCa models tested, including androgen-sensitive LNCaP model (data not shown). Direct Western blots of actin and FER are also shown. Taken together, these findings indicate that FER and actin complexes are present in various PCa cell lines, irrespective of phenotype, suggesting a common function. Levels of complexes also appeared higher in PRO4 and LN4 than in PC-3., a finding further demonstrated below.

4.1.2 EVIDENCE OF FER/ACTIN COMPLEXES IN SITU IN PCA CELLS

Immunofluorescence analysis was used to study the localization of FER and actin in PRO4, along with their interactions using newly generated FER and commercial actin antibodies. This was achieved by confocal microscopy, a method which offers the advantage of analyzing proteins in more depth into cellular compartments. Figure 6 pertains to such confocal images, with sections at the level of the nucleus and cytoplasm presented in the upper (referred to as nuclear plane) and lower (cytoplasmic plane) panels, respectively. In the nuclear section FER antibodies (top left) strictly stained the nucleus red, whereas in the cytoplasmic section, the FER red staining (lower left) was spread
Figure 5. FER/actin complexes in PCa cell lines

(A) Proteins (500µg) from PRO4 cells were immunoprecipitated with control IgGs and FER antibodies and Western blotted with actin antibodies to detect complexes (42kDa). A direct blot of actin (42kDa) in whole cell lysates (W.L.: 50µg protein) is also shown. The position of molecular weight markers is indicated.

(B) Proteins (500µg) from PC-3, LN4 and PRO4 cell lysates were similarly immunoprecipitated with FER and probed with actin antibodies. Commercial actin (1µg) was included (right lane). Direct blots were carried out in parallel on cell lysates to detect actin (42kDa) and FER (94 kDa).
Figure 6. FER/actin complexes in situ

PRO4 cells were cultured on coverslips and processed for confocal immunofluorescence microscopy as described in Methods. Sections of PRO4 cells were taken through the nucleus (upper panels) and the cytoplasm (lower panels). Left: FER antibodies (red); Middle: Actin (green) antibodies; Right: Merge of FER and actin signals. Magnification: 80X (see Methods).
throughout. The actin signal is shown in green (Fig. 6). In the nuclear plane (top middle), actin antibodies did not show a specific nuclear localization in contrast to FER. The actin signal in the cytoplasm (lower middle) was more intense and distributed throughout the cell. Merged images (magnified in right panels) revealed co-localization within both planes in yellow, suggesting the presence of complexes between FER and actin. In addition, the yellow staining was more abundant in the cytoplasm in support of potentially more FER/actin complexes in this compartment. Nevertheless, the nuclear signal appeared significant. Taken together, these results not only confirm earlier biochemical findings of FER/actin complexes, but also illustrate their cytoplasmic and nuclear presence within the overall distribution pattern of actin and FER in these compartments of PRO4 cells.

4.2 ACTIN TYROSINE PHOSPHORYLATION

4.2.1 ACTIN IS TYROSINE PHOSPHORYLATED IN PRO4 CELLS

As aforementioned, the Y-phosphorylation of actin has been reported only in protozoan and chicken B cells. Biochemical evidence of specific Y-phosphorylation of actin was obtained in the host lab while searching for Y-phosphorylated FER partners in PCa cells. For this purpose, FER immunoprecipitates prepared from the parental PC-3 cell line and its derived PRO4 variant had first been probed with pY-antibodies. The predominant 42kDa pY-protein interacting with FER was shown to be actin, and revealed to be Y-phosphorylated. This is shown in Figure 7, by converse immunoprecipitations of actin and pY-proteins from PRO4 cell lysates, followed by SDS-PAGE and Western blotting with each of pY- and actin antibodies. Actin Y-phosphorylation was demonstrated
Figure 7. Evidence of pY-actin in PRO4 cells

Proteins (500µg) from cell lysates were incubated with antibodies to immunoprecipitate actin (left) and pY (right), then Western blotted to detect pY (left) and actin (right), respectively. As a negative control, samples were immunoprecipitated with IgG in both panels. The identity of the upper band in the actin blot of IP-pY proteins (position of immunoglobulins) is unknown. A direct blot of actin from whole cell lysates (W.L., 50µg proteins) is also shown in the right panel as a positive control (42kDa). The position of molecular weight markers is indicated alongside the gel.
in both instances, in agreement with earlier findings (Zoubeidi 2003; Zoubeidi, Rocha et al. 2009). The use of pre-immune IgGs as negative controls of immunoprecipitation ensured specificity. Further analyses were preferably performed by immunoprecipitating proteins with pY-antibodies and then by probing blots with actin antibodies.

4.2.2 LEVELS OF pY-ACTIN CORRELATE WITH AGGRESSIVE PHENOTYPES OF PROSTATE CANCER MODELS

To investigate if the extent of actin Y-phosphorylation was of relevance, various PCa cell models were analyzed, as mentioned above. Direct actin blots were performed in parallel to allow comparisons, by reporting phosphorylated levels on actin.

4.2.2.1 LEVELS OF pY-ACTIN IN THE PC-3 MODEL SYSTEM

Results on the androgen-independent/insensitive (AR negative) parental PC-3, and the PRO4 and LN4 derivatives are shown in Figure 8. Briefly, these cell lines were isolated from the metastastic PC-3 variant, which was orthotopically implanted in the prostate of nude mice and led to tumors in the prostate (PRO4) and metastasis in lymph nodes (LN4), respectively (Pettaway, Pathak et al. 1996). In vitro, LN4 exhibits the highest rate of proliferation and are followed in order by PRO4 and PC-3 (data not shown). The first panel exhibits pY-actin levels. The results indicate that the more aggressive cell lines have highest levels of pY-actin, in the following order LN4>PRO4>PC3. Of interest were levels assessed in parallel within this experiment in the androgen-sensitive (AR
Figure 8. Levels of pY-actin and FER in PC-3, its variants, and parental LNCaP

Protein extracts (500μg) from PC-3, PRO4, LN4 and LNCaP cell lines were immunoprecipitated with pY-antibodies and detected with actin antibodies (upper row in left panel). Actin and FER were directly blotted using whole lysates of each cell line (50 μg protein; lower series in left panel). ImageJ was used to quantify the intensity of the Y-phosphorylated and total actin bands respectively. Intensities were subsequently analyzed on Excel to calculate the ratios presented graphically in the right panel, which illustrates the percent of actin that is Y-phosphorylated in each cell line.
positive) parental LNCaP model, which appeared in the order of the parental PC-3. FER was equally expressed amongst these cell lines (Fig. 8, last panel).

In Figure 8, pY-actin levels were quantified, using Image J software, and normalized against their respective actin levels (relatively constant as shown in the middle panel) in each of these cell lines. The histogram represents the percent of total actin that is phosphorylated. The analysis revealed that over 15% of total actin was Y-phosphorylated in the more aggressive LN4 model. In comparison, 2-3% of actin was Y-phosphorylated in the parental PC-3 and LNCaP models. The experiment was repeated three times and similar results were obtained. Statistical analyses, using the Student T-test, confirmed significant differences between the pY-actin levels in PC-3, PRO4 and LN4 (P<0.006), whereas levels in LNCaP were not significantly different from PC-3.

4.2.2.2 LEVELS OF pY-ACTIN IN THE LNCAP MODEL SYSTEM

Based on the above, a similar approach was used to compare pY-actin levels in the parental androgen-sensitive LNCaP cell line to its C4:2 variant, which in nude mice was reported to be more aggressive and no longer responsive to androgens while still expressing the AR (El Etreby, Liang et al. 2000). The results in Figure 9 show higher levels of pY-actin in the C4:2 than in LNCaP cells (upper panel). Levels of actin (middle panel) were relatively equal in the two cell lines. FER was equally expressed amongst these cell lines (last panel). The graphical analysis in Figure 9 quantitatively substantiates that a higher percent of actin was phosphorylated in the C4:2 derivatives (P<0.005), with levels of 10% comparatively to 3% in the parental LNCaP. The experiment was repeated three times and similar results were obtained.
Figure 9. Levels of pY-actin and FER in parental LNCaP and derivative C4:2

Protein extracts (500µg) were immunoprecipitated with pY-antibodies and detected with actin antibodies (left panel) as in Fig. 8. Actin and FER were also directly blotted using whole lysates of each cell line (50µg protein; two lower series in left panel). Bands were analyzed using ImageJ to calculate the percent of actin that is Y-phosphorylated in LNCaP and C4:2 cell lines, as graphically illustrated in the right panel.
4.2.2.3 LEVELS OF pY-ACTIN IN CANINE PROSTATE CELLS AND THE DPC-1 CELL LINE

The host lab previously reported that FER is not expressed in the normal or hyperplastic dog prostate. This was shown by Western blotting of protein extracts from prostate tissue homogenates as well as from epithelial cells freshly isolated from the dog prostate (Allard, Zoubeidi et al. 2000). To expand the present studies on the relationship between FER and actin Y-phosphorylation, prostate cells, herein called D0, were isolated by collagenase digestion as described in Methods and previously reported (Chevalier, Bleau et al. 1981). Extracts were next evaluated for pY-actin against the highly aggressive DPC-1 (dog prostate cancer cell line) isolated from a dog with spontaneous prostate tumors (Anidjar, Villette et al. 2001), and known to express FER (Olivier 2003).

Figure 10 first confirmed with newly generated FER antibodies earlier results on FER expression in DPC-1 whereas no band was detected in D0 cells (lower panel). As expected, actin (middle panel) was present at significant levels in protein extracts from the two cell preparations. Results on pY-actin are shown in the upper panel of Figure 10.

Strong levels were observed in the DPC-1 cells, whereas none were detected in cells freshly isolated from the dog prostate. The quantitative analysis of pY-actin levels indicated that approximately 10% of actin was Y-phosphorylated in the DPC-1 cell line. The experiment was repeated and similar results were obtained with levels in DPC-1 being statistically different from Do cells (P<0.005). The presence of complexes between FER and actin had been shown by immunoprecipitations of FER and Western blotting of actin and also by immunofluorescence (Olivier 2003).
Figure 10. Levels of pY-actin and FER in normal dog prostate cells and the DPC-1 cell line

Epithelial cells were isolated from the canine prostate (D0) and processed along with cultured DPC-1 cells to obtain whole extracts as described in Methods. Proteins (500 µg) were immunoprecipitated with pY-antibodies and blotted with actin antibodies (left panel) as in Figs. 8 and 9. Actin and FER from each cell lysate were directly blotted (50 µg protein; lower portion of left panel). Bands were analyzed (as in Figs 8 and 9) to illustrate the percent of actin that is Y-phosphorylated in normal dog prostate cells and the DPC-1 cell line (right).
Taken together these findings of a correlation between the Y-phosphorylation of actin and the aggressive properties of human and dog PCa models together with the overexpression of FER in PCa suggest significance of complexes and pY-actin in this disease.

4.3 REGULATION OF Y-PHOSPHORYLATION OF ACTIN IN RESPONSE TO CYT.D, A DRUG THAT DIRECTLY TARGETS ACTIN DYNAMICS

The host lab had obtained preliminary data on actin Y-phosphorylation being increased in PRO4 and PC-3 cells upon 2 hrs exposure to Cyt.D (1µM), a drug known to directly affect actin dynamics by blocking the addition of G-actin monomer to the growing F-actin that forms the cytoskeleton (Flanagan and Lin 1980). This finding was of particular interest since actin was observed to undergo Y-phosphorylation in Amoeba in response to environmental and nutritional stress (Kishi, Clements et al. 1998). On the opposite, the triggering of the antigen receptor signaling pathway in chicken B-cells was accompanied by the dephosphorylation of pY-actin by the PTP-1B phosphatase (Baba, Fusaki et al. 2003). It was thus postulated that stressful rather than physiological conditions would increase actin Y-phosphorylation in PCa cells, and indeed when PC-3 or PRO4 cells were stimulated to grow in the presence of IL-6, no increase in pY-actin levels were detected (lab’s unpublished data). Accordingly, it was decided to pursue this line of investigation on FER in relation to the Y-phosphorylation of actin in PCa cells, using Cyt.D to modulate levels and subsequently observe its influence on actin dynamics.
4.3.1 PRO4 CELL RESPONSIVENESS TO CYT.D IS ACCOMPANIED BY ENHANCED pY-ACTIN LEVELS.

PRO4 and LN4 cell lines were selected to conduct kinetic experiments with Cyt.D treatment, as these cell lines exhibited high levels of Y-phosphorylated actin under basal conditions. Briefly, Cyt.D was prepared in growth medium and serum at a concentration of 1µM as used on DLD-1 and HT-29 colorectal carcinoma cell lines (Milsom and Rak 2005). PCa cells were exposed over a period of 72 hrs and periodically harvested to prepare protein extracts for pY-actin level determination. Representative blots of protein immunoprecipitation with pY-antibodies and detection of actin by Western Blot with actin antibodies are shown in Figure 11. The first series (lower panel; Fig. 11A) points out constant levels of actin in LN4 protein extracts together with the constitutive Y-phosphorylation of actin prior to and throughout Cyt.D treatment. The relative level was in the order of 11%, and following treatment for 72 hrs, the levels of actin Y-phosphorylation remained within the 11% range similar to the basal level (right panel). In contrast results in the PRO4 cells (lower panels, Fig. 11B) showed an increase in the Y-phosphorylation of actin by 24 hrs of treatment, reaching a maximal peak at 48 hrs, and followed by a decrease of phosphorylation at 72 hrs. Levels of actin were constant (shown in lower series). The percentage of total actin phosphorylation is also depicted graphically (right panel). With initial basal levels of about 3%, a 3-fold increase in the phosphorylation was observed in response to 48 hrs exposure to Cyt.D. The microscopic observation of cultured cells showed morphological differences in response to Cyt.D treatment. Visibly, LN4 cells looked healthy and remained attached over the time-period of exposure to the drug while PRO4 cells remained attached for up to 48 hrs, after which they began detaching. After 4 days of Cyt.D treatment, the PRO4 cells had completely detached, whereas LN4 cells were
Figure 11. Effects of Cyt.D on pY-actin levels in PCa cells LN4 (A) and PRO4 (B) cells were exposed to a Cyt.D stress for 0, 24, 48, 72 and 96 hours and lysed to immunoprecipitate proteins (500ug) with pY-antibodies and detect actin by Western blotting (left panels), including whole lysates (50ug proteins). As aforementioned, ImageJ was used to quantify levels of Y-phosphorylated actin (%) over total actin in response to stress, shown graphically for each respective cell line in right panels.

NB: Kinetics were repeatedly reproduced three times. The time-point 96hours (panel B) on detached (presumably dead) cells was incorporated in the last series and is presented as preliminary results.
still forming monolayers. A Trypan blue exclusion test on the detached cells revealed complete blue staining, indicating that all cells were no longer viable. Hence, it was presumed that detached cells represented those that died from the stress induced by Cyt.D. Preliminary results of detached cells from 96 hrs treated PRO4 cells were harvested to prepare protein extracts for analysis, and as indicated by results in lower panels of Figure 11, there was a complete loss of pY-actin, while actin was still detected.

Taken together, these results indicate that the Y-phosphorylation of actin is modulated by Cyt.D more rapidly in PRO4 as opposed to LN4 cells, which appeared more resistant to the drug. The fact that the basal pY-actin levels in LN4 cells were within the range of the peak levels, attained from stressed PRO4 cells at 48hrs, suggests that pY-actin levels may be saturated in LN4 cells. Based on the above, PRO4 cells were selected as a better model for further analysis of actin Y-phosphorylation in relation to FER.

4.3.2 FER/ACTIN COMPLEXES VARY IN PARALLEL WITH ACTIN TYROSINE PHOSPHORYLATION UPON CYT.D TREATMENT

The 48 hrs time frame of Cyt.D exposure was retained to determine if the interaction between FER and actin would be similarly modulated by the drug in parallel to pY-actin levels. For this purpose, protein extracts from PRO4 cells exposed to Cyt.D for 0, 24 and 48 hrs were processed to assess levels of FER/actin complexes by immunoprecipitating FER and detecting actin via Western blotting. Representative results in Figure 12A hint that treatment led to a progressive increase in complexes between the two proteins (top panel). Once quantified, a positive linear correlation with time was observed (r=0.9816)
Figure 12. Cyt.D regulates FER/actin complexes and pY-actin levels in PRO4 cells

Cells were exposed to Cyt.D for 0, 24 and 48 hrs. (A) Proteins were immunoprecipitated with FER (top left) and pY- antibodies, and next blotted for actin (42kDa). Direct actin blots are included. The rates of complex formation and actin Y-phosphorylation over time of Cyt.D treatment are shown graphically (right panels). (Values are the average of three experiments) (B) Graphical analysis of Y-phosphorylated actin relative to FER/actin complexes.
with up to a 5-fold change in levels of complexes by 48hrs. The increase was represented by a slope of 0.08% (complex formation/hour).

The progressive increase of pY-actin in cells treated over 48 hrs and constant levels of actin are also shown by immunoprecipitation and Western blotting (Fig. 12A), thereby reproducing earlier results. Once quantified, the extent of actin Y-phosphorylation (over actin level) also increased linearly with time (r=0.9999), and at a rate of 0.16% actin being Y-phosphorylated/hour. Therefore when actin was bound to FER upon Cyt.D treatment, more became phosphorylated. Given this parallel increase in levels of both complexes and pY-actin levels, a graph was constructed to determine the extent of the correlation. Figure 12B shows a linearity between the two processes with a high correlation coefficient (r=0.9844) and a slope of 2.1. Such a value suggests that the rate of actin phosphorylation is more rapid than the rate of complex formation. Further work is necessary to understand significance.

4.3.3 ACTIN DIRECTLY INTERACTS WITH THE SH2 DOMAIN OF FER

Protein-protein interactions in signaling complexes involving proteins containing pY-motifs often occur through the direct binding of the pY-motif in one partner with a SH2 domain present in the interacting protein (Pawson and Scott 1997). FER contains a SH2 domain (Figure 1), which the host lab had generated as a fusion construct with GST and was used to produce antibodies. Since this domain was shown to directly interact with pY-STAT3 in IL-6 stimulated PC-3 cells (Zoubeidi, Rocha et al. 2009), the FER-SH2 construct was used to perform pull down assays on protein extracts from PRO4 cells exposed to Cyt.D.
Figure 13. Actin binding to the FER-SH2 domain

(A) Proteins from PRO4 cells, control and Cyt.D treated (24hrs), were pulled-down with the GST tagged FER-SH2 fusion protein and GST control, as described in Methods to probe actin.

(B) Same as in (A) but over time of Cyt.D exposure i.e. pull down with the SH2 domain of FER and actin blot (top). Direct actin blots are included. (Results were reproduced twice). Bands were quantified and levels of actin interacting with the FER-SH2 domain over time of Cyt.D treatment were represented graphically (values from one experiment).

(C) Correlation between the actin bound to the SH2 domain vs complexed to the FER protein (values from one experiment).
Figure 13A illustrates Western blots of actin retained in pulled down assays from protein extracts of PRO4 cells, untreated and Cyt.D treated for 24hrs. The faint band detected at Time 0 reproduced earlier findings of the FER-SH2 domain being capable of complexing and interacting with actin. Of interest, more complex formation was observed in the Cyt.D treated PRO4 cells whereas no actin band was present when the GST control construct was used for pull down. Representative results of pull down assays illustrating the interaction between actin and the FER-SH2 domain over 48 hrs of Cyt.D treatment are shown in Figure 13B. The interaction with the SH2 domain of FER was vividly more apparent in the treated cells, and as shown graphically, increased linearly with time of Cyt.D treatment. The correlation was elevated \( (r=0.9985) \) and the level of actin bound to the FER-SH2 domain was 0.05\% per hour. This suggests a direct interaction between the SH2 domain of FER and actin, increasing in parallel when pY-actin levels are augmented and thus likely mediated by pY-motifs in actin. Since both the formation of FER/actin complex and actin interaction through the SH2 domain increase in parallel with time of Cyt.D treatment, their correlation was investigated graphically. Figure 13C demonstrates a linear relationship with a high correlation value \( (r=0.97) \). The slope was 0.60, suggesting that 60\% of actin in complex with FER, occurred through the FER-SH2 domain. These findings support a direct binding of pY-motifs in actin with the FER-SH2 domain, and raise the issue of the role of FER as a kinase.
4.4 IDENTIFICATION OF ACTIN Y-PHOSPHORYLATION SITE AND DETECTION

Of the three variants of the human actin family of proteins, the β-actin is most likely the primary form found in PCa cells. As mentioned in the Introduction, the actin structure comprises 15 Y-residues, and 2 were shown to be phosphorylated; on positions 53 in protozoan and 219 in chicken B cells (Kishi, Clements et al. 1998; Baba, Fusaki et al. 2003).

4.4.1 IDENTIFICATION OF pY-SITE(S) IN ACTIN FROM PRO4 CELLS

To identify the site(s) of Y-phosphorylation, LC-ESI-MS analysis was performed on the protein. Initially, actin was purified from PRO4 cells by immunoprecipitating the protein from cell lysates with actin antibodies. This step was followed by SDS-PAGE to recover actin from the gel for proteolysis and MS sequencing. Results from two attempts failed to detect actin fragments compatible with an increase of 80 daltons due to the addition of a phosphate group.

In order to optimize the sample preparation and favor maximal pY-actin levels in hopes of detecting phosphorylated fragments, we selected to pursue the approach of Cyt.D exposure, the premise being that the treatment would increase the likelihood of studying sites in the actin molecules that become phosphorylated. PRO4 cells were thus treated with Cyt.D for 48 hrs, and lyzed to extract proteins. To specifically isolate pY-actin, we took advantage of results obtained with the FER-SH2 domain and therefore pulled down proteins with the FER-SH2/GST construct. Proteins were resolved by SDS-PAGE and stained with Coomassie blue. This is shown in Figure 14A (lanes 2 and 3), where the
Figure 14. Identification of actin phosphorylation

(A) Coomassie Blue stained gel of actin, partially purified from PRO4 exposed to Cyt.D for 48 hrs via binding to the FER SH2 domain, as achieved by pull down. Lane 1: commercial actin (1µg; positive control); lanes 2 & 3: proteins pulled down from PRO4+Cyt.D. The 42kDa band from cells was cut and processed for digestion and mass spectrometry analysis as described in Methods.

(B) LC-ESI results analyzed through the MASCOT database identified an actin peptide of molecular mass compatible with one phosphorylated residue (+80 daltons). A loss of 30 daltons is also incurred on the Glutamine-43 residue owing to fragmentation that occurs during the ionization process. The low Expect (E-) value indicates a high significance that the sequence hit was not found by chance (E-values range from 0 to 1).
42kDa protein band, presumed to contain actin, aligned precisely with commercially available actin (positive control in lane 1). Actin was identified by Western blotting performed in parallel after transfer on membranes (data not shown).

The 42kDa band from FER-SH2 pull down was consequently cut out from the gel for extraction, trypsin-digestion into fragments and analysis by LC-ESI-MS. The computational analysis of results not only confirmed the successful isolation of actin, but also revealed a potentially phosphorylated actin fragment. Figure 14B illustrates the MS results obtained from the MASCOT analysis, whereby a characteristic increase of 80 daltons associated with a phosphorylation was detected within the fragment extending from residues 42-63. This fragment includes the Y53 residue identified in amoeba cells, but also contains two serine residues which could also be sites of phosphorylation.

4.4.2 DETECTION OF pY53 ACTIN AND EFFECTS OF CYT.D

A phosphospecific pY53-actin polyclonal antibody raised against a short peptide of the sequence correlating with the highly homologous actin sequence identified in amoeba cells, and containing a phosphate group on Y-residue 53 was commercially attained. The antibody was initially tested by performing Western Blot and immunoprecipitation experiments, respectively. Results shown in Figure 15, left panel, demonstrate that this antibody was not very efficient in detecting directly pY53-actin using lysates of control PRO4 cells. Moreover, no specific bands were detected at 42kDa on enriched preparations of proteins immunoprecipitated with both pY53-actin and pY-antibodies from these series of cell extracts, including after 48 hrs of Cyt.D exposure. The control consisting of
Figure 15. Characterization of pY53-actin antibodies

Protein extracts from PRO4 cells, control (-) and 24 hrs treated with Cyt.D (+) were immunoprecipitated (500µg) with pY53-actin antibodies (lanes 4, 5) and pY- antibodies (lanes 6, 7). Controls include commercial actin (lane 1; 1µg), proteins from whole cell lysate (W.L., 50µg in lane 2) and an IgG immunoprecipitate of 500µg proteins from control cells (lane 3). Membranes were probed with specific antibodies to detect pY53-actin (left) and actin (right), respectively.

Extra bands are high (~50kDa) and low (~32kDa) molecular weight immunoglobulins, revealed according to the use of polyclonal and monoclonal antibodies, respectively.
commercial actin was also negative. Hence, it appeared that the antibody was incapable of detecting pY53-actin from the aforementioned extracts on Western blots.

In contrast, when actin antibodies were used for Western blotting on PRO4 extracts immunoprecipitated with the pY53-actin antibody, a clear positive signal was detected at the 42kDa position (Figure 15, right panel). The signal was also seen when enriched pY-proteins were obtained through immunoprecipitation with pY-antibodies. The signal intensity was strong and appeared more elevated in the Cyt.D series. These findings indicate that the pY53-actin antibody is capable of immunoprecipitating pY53-actin from human PCa cells. It was thus assumed that the antibody recognizes the pY53-actin in its native form, but fails to detect this specific protein when denatured in gels. Consequently, subsequent studies of pY53-actin from cell lysates were carried out by immunoprecipitation followed by actin blots.

The successful application of pY53-actin antibody provided an opportunity to analyze further the modulation of the Y-phosphorylation of actin in relation to Cyt.D exposure and determine whether the increase in phosphorylation involves the Y53 residue of actin. For this purpose, the pY53-actin antibody was used for immunoprecipitation of extracts from treated PRO4 cells over time (0, 24 and 48 hrs) and actin was detected by Western blotting using actin antibody. Results illustrated in Figure 16 (top panel) are very similar to earlier findings on overall pY-actin. They revealed increasing levels of pY53-actin with time of exposure to Cyt.D. The quantification by scanning was reported to actin levels, as measured by direct blots of actin (lower panel), and is represented graphically in Figure 16 (right panel). A linear correlation was observed \((r=0.9937)\). The rate of actin Y53 phosphorylation was 0.10% per hour, a value corresponding to more than 60% of
Figure 16. Effect of Cyt.D exposure on pY53-actin levels

Proteins from untreated (T=0) and Cyt.D treated PRO4 cells, at different time intervals, were immunoprecipitated with pY53-actin antibodies and probed with actin antibodies (upper panel). Controls include immunoprecipitates of cell extracts with IgG, blotted for actin (negative; right lane in upper panel) and a direct actin blots of PRO4 cell lysate (W.L.; left lane in upper panel). Direct actin blots from respective cell extracts were also included (lower panel). A graphical analysis of the percent of actin that is Y53-phosphorylated, as a function of exposure (time) to Cyt.D is also provided (right panel).
overall rate of 0.16% for global pY-actin (Figure 11D). From these findings, it can be assumed that the increased levels of Y-phosphorylation of actin in response to Cyt.D may be explained, at least in part, by the Y53 residue. This suggests that Y53 contributes to a large extent to the overall Y-phosphorylation. However, the involvement of other tyrosine residues cannot be ruled out.

4.4.3 CONFIRMATION OF THE pY53 RESIDUE OF ACTIN

To ascertain that the identified pY53 residue of actin was specific, site directed mutagenesis experiments were conducted. Briefly, the human actin cDNA, tagged with a FLAG epitope (approximately 1kDa), was mutated at Y53 whereby the residue was replaced by phenylalanine (Y53F). The mutated plasmid was re-sequenced which confirmed the successful mutation of the appropriate residue, while the integrity of the rest of the plasmid was preserved. The mutated actin cDNA was next transfected in PRO4 cells along with wild-type (WT) actin cDNA, and expression of recombinant actins (WT and mutant) was confirmed with FLAG antibodies (data not shown). This enabled us to study the effects of Cyt.D in transfected cells. Due to the 48 hrs of transfection, a shorter exposure period (12 hrs) to Cyt.D was investigated.

Figure 17 shows the direct blots, using FER and FLAG antibodies, on non-transfected and transfected PRO4 cells as shown in the top two left panels. The results reveal the successful expression of the FLAG-tagged actin at 43kDa, while as expected no band was detected in the control non-transfected series. Constant levels of the FER protein in cell lysates confirmed equal loading.
Figure 17. Consequence of mutating Y 53 in actin on levels of Y-phosphorylated actin induced by Cyt.D in PRO4 cells

The mutated (F53Y) along with wild-type (WT) human actin cDNA were transfected in PRO4 cells, and exposed to Cyt.D for 12 hrs. In the top two left panels, proteins from whole lysates were probed with FER (1st) and FLAG- (2nd) antibodies. In rows 3-5, proteins (500µg) were immunoprecipitated with the following antibodies: pY53 actin (3rd), pY (4th) and FER (5th) and probed with FLAG-antibodies. The right panel illustrates graphically the percent loss in Y-phosphorylation of F53Y mutated actin (over exogenously expressed WT-actin).
A preliminary analysis of pY53-actin was next performed using the pY53-actin antibody for immunoprecipitation of recombinant WT-actin and mutated-actin, which were then blotted with the FLAG antibody. A positive FLAG signal at 43kDa was only detected in the series of PRO4 cells overexpressing WT-actin but not in the series with mutated actin (third left panel of Fig. 17). Thereby, confirming the specificity of the pY53-actin antibody and the successful mutation of the Y53 site. Moreover, when proteins were immunoprecipitated with pY-antibodies and probed with the FLAG antibody (fourth panel), the cells transfected with Y53 mutated actin cDNA demonstrated a decrease in signal by approximately 40%, as opposed to those transfected with WT-actin (Fig. 17, right panel). The importance of this pY53 site is currently being the matter of deeper investigations in the host lab.

This implies that other Y-sites (asides Y53) may contribute to the Y-phosphorylation of actin, induced by Cyt.D. The consequence of this mutation on levels of FER/actin complex was determined by immunoprecipitating FER and probing with FLAG antibody. Results in the last panel indicate no significant change in levels. Whether additional pY sites and/or FER binding domains are involved remain to be established. The results suggest that the Y53 residue is one amongst other Y-residues on actin, which is phosphorylated.

4.5 IN SITU ANALYSIS OF pY(53)-ACTIN IN PCA CELLS

Immunofluorescent microscopy had already revealed in control PRO4 cells (Fig. 6) that actin, as detected with both phalloidin and actin antibody was primarily in the cytoskeleton, whereas FER was distributed in both the cytoplasm and the nucleus.
Figure 18. Intracellular distribution of pY53-actin in PRO4 cells

(A) Cells were processed for immunofluorescence and stained with antibodies to pY53-actin (red; top left). Cytoskeletal actin was evidenced using a phalloidin probe (green; top right). In the lower panels are the DAPI staining (left) and merged image of the three signals (right). Below is a graphical line profile of pY53-actin and DAPI staining intensities across a PRO4 cell.
Figure 18. Intracellular distribution of pY53-actin in PRO4 cells

(B) Same as (A) but imaged through confocal microscopy and showing a nuclear plane across a PRO4 cell. Phalloidin probe in green (top left); pY53-actin (top right) in red; and merge signals (lower panel in the middle).
Complexes between FER and actin were also predominantly observed in the cytoplasm. Based on the successful use of this pY53-actin antibody in immunoprecipitation, immunofluorescence microscopy experiments were next conducted, to verify if the protein conformation would be sufficiently preserved for detection with the pY53-actin antibody. The results of staining in control PRO4 cells are illustrated in Figure 18A, with pY53-actin antibody (red), phalloidin (green) and DAPI (blue).

They reproduced earlier data (from Figure 6) on the detection with phalloidin of F-actin in the cytoskeleton. Intriguingly, a positive signal was obtained with the pY53-actin antibody, and was predominantly detected in the nuclear compartment. A faint signal was seen in the cytoplasm in close proximity of the nucleus. As mentioned in the introduction, various reports have shown the presence of actin in the nucleus of mammalian cells (Pederson and Aebi 2002), but to our knowledge the distribution of pY- and more specifically pY53-actin was unknown up to now. The pY53-actin antibody thus permits the identification of the pY53-actin distribution in PRO4 cells. In addition the merged image revealed a purple fluorescence resulting from blue DAPI (bound to DNA) and pY53-actin in red. This is graphically illustrated in Figure 18A by line profiles of the fluorescent intensity of pY53-actin and of DAPI across the cell. The fluorescent profiles of two are comparable and overlap within the nucleus, thereby suggesting the possibility of pY53-actin having an affinity for DNA.

Confocal microscopy was next performed in parallel using the pY53-actin antibody (red) and phalloidin (green) to confirm the above distribution of pY53-actin, as well as its relationship to F-actin. Representative images in the nuclear plane of a cell are shown in Figure 18B (DAPI staining was not included in the analysis). As expected, pY53-actin was
Figure 19. Effects of Cyt-D exposure on actin

PRO4 cells were exposed to Cyt.D for 48 hrs and fixed for immunofluorescence analysis using the phalloidin probe for cytoskeletal (F-) actin (green; 1st panel) and pY53-actin (red; 2nd panel) antibodies. Nuclei were counterstained with DAPI (blue; 3rd panel). The merged image is provided in the last panel.
again predominantly nuclear, while F-actin was distributed throughout the cell. Merged image failed to show co-distribution as no yellow staining was observed. This would argue that the Y53-phosphorylation of actin is unlikely attributed to F-actin.

To address the question of whether stress response to Cyt.D would modify the distribution, PRO4 cells were exposed to the drug over time, fixed, and similarly stained. Figure 19 shows the changes that incurred after 24 hrs. The F-actin staining (phalloidin in green) appeared substantially reduced and disassembled, being still in the cytoplasm but concentrated around the nucleus in comparison to cytoskeletal actin in control cells. The pY53-actin (red) distribution remained in the nucleus, and the merged image revealed again the purple color from the pY53-actin and DAPI staining, which was also evidenced in control cells.

Since it is established that Cyt.D affects actin dynamics, and we observed diminished levels of F-actin, while total actin levels were unchanged in Western blotting (Figure 16), we attempted to examine G-actin in comparison to F-actin using a G-actin probe known to specifically bind the globular conformation of actin, at the DNAse I binding loop, in addition to the phalloidin probe. For this purpose, control cells and cells exposed to Cyt.D for 24 and 48 hrs were compared. Phalloidin staining reproduced earlier findings on the F-actin cytoskeletal distribution and changes induced by Cyt.D, in the disassembly of the filaments as above in Figure 19 (at 24 hrs) and also shown in Figure 20 for up to 48 hrs. Interestingly, at 48 hrs the F-actin signal had drastically decreased.
Figure 20. Analysis of F- and G-actin

PRO4 cells exposed to Cyt.D for 0 (untreated control), 24, and 48 hrs were processed for immunofluorescence as in Fig. 19, to detect F-actin with the phalloidin probe (green) and globular actin with a G-actin probe (red). Merged images are shown.
The probe for G-actin revealed a nuclear red staining, resembling the pY53-actin distribution in control cells (Figure 20, untreated cells). In the treated series, the G-actin red signal intensity increased, and remained strictly nuclear. This is illustrated in the merged images of Figure 20, where staining with the F- and G-actin probes did not merge. Since the total actin levels did not change, we postulate that the decrease in F-actin parallels an increase in G-actin. A preliminary attempt to quantify the intensities of both respective probes, further suggested that this may indeed be the case, but further analysis is required.

The distribution of FER was analyzed in a similar fashion upon exposure to Cyt.D (48 hrs), as illustrated in Figure 21. Control PRO4 cells (left panels) illustrate a co-localization of FER with DAPI in the nucleus, along with a clear distribution throughout the cytoplasm. This is consistent with past findings, whereby FER is distributed throughout the cytoplasm and nucleus of PCa cells. However, upon exposure to Cyt.D, FER is predominantly co-localized with DAPI, while the cytoplasmic distribution is lost. A nuclear translocation of FER, in parallel to that of G-actin, is observed upon exposure to Cyt.D.

4.6 ACTIN, A SUBSTRATE OF FER

Our findings showed that actin interacts with the FER kinase, and that there exists a correlation between the phosphorylation of actin and its interaction with FER. However, it is unclear whether actin is a substrate of FER or not.
Figure 21. Effects of Cyt. D on FER intracellular distribution

PRO4 cells, untreated (left, control) and exposed to Cyt.D for 48hrs (right) were analyzed by immunofluorescence to detect FER (red; top panels). DAPI (blue) counterstaining is shown in middle panels. Merged images are in lower panels.
4.6.1 IN VITRO KINASE ASSAY

To answer the question of actin being a FER substrate, an in vitro kinase assay was performed using a 59kDa catalytic domain of the human FER kinase (Lys541-Thr822) as the enzyme, radio-labeled [γ\(^{32}\)P]-ATP as the phosphate donor, and non phosphorylated commercial actin as the substrate. Results of the kinase assay are shown in Figure 22. In the first lane where the kinase assay was performed with FER alone, a transfer of \(^{32}\)P onto FER was observed, indicating that in presence of ATP, FER was capable of phosphorylating itself. In the second lane, the assay was performed with only the substrate (actin), in which case no radioactive band was observed at 42kDa. This was intended to be a negative control. The need for a kinase to phosphorylate actin is shown the last lane, where the complete kinase assay was performed with FER, actin, and labeled ATP. Two radioactive bands were detected, one corresponding to the phosphorylation of actin at 42kDa, along with the autophosphorylation of FER at 59kDa observed above actin. The lower panel is a Western blot for actin, confirming the presence of actin in lanes 2 and 3. The catalytic domain of FER could not be detected with the FER antibody raised against the N-terminal SH2 domain (See Figure 1). These results demonstrate that the catalytic domain of FER is capable of directly transferring a phosphate group to actin. FER is also observed to add a phosphate group onto itself. Whether this autophosphorylation occurs on Y714 and is a prerequisite to actin Y-phosphorylation is yet to be determined. It was observed that the FER band appeared reduced in the presence of actin.
Figure 22. FER directly phosphorylates actin in vitro

The FER catalytic domain (59kDa) was used as the enzyme to phosphorylate commercial actin (42kDa) in the presence of [γ-\(^{32}\)P]ATP through an in vitro kinase assay, as described in Methods. The top panel illustrates the \(^{32}\)P-labeled bands detected in the gel with a Phosphoimager: FER (catalytic domain) alone (lane 1), commercial actin alone (lane 2) and in the complete assay, both FER and actin (lane 3). Proteins were transferred and membrane was blotted using actin antibody as a control (lower panel).
4.6.2 EFFECT OF FER KNOCKDOWN ON pY53 ACTIN LEVELS

To verify whether these results upheld in an in vivo environment within PCa cells, the role of FER in actin Y-phosphorylation was investigated using a siRNA approach to silence FER. Two siRNAs were recently used successfully by the host lab to modulate FER levels as confirmed by both real time-PCR, and Western blotting (Zoubeidi, Rocha et al. 2009). The expression of FER was thus downregulated within PRO4, using the more potent siRNA for 48 hrs, and cells were next exposed to Cyt.D for 6hrs to subsequently analyze effects on actin Y53-phosphorylation. Preliminary results in Figure 23 show that FER protein levels, as detected with FER antibodies, were indeed reduced by FER siRNA whereas actin levels were unchanged. It was measured from scanning the bands that FER was downregulated by 50% (Figure 21). The analysis of pY53-actin revealed a similar reduction (second left panel), which corresponded to 50% when scanned, as illustrated graphically in Figure 23. As expected, with the decrease in FER levels, a parallel decrease in levels of activated pY-FER by a little over 50% was observed and is shown in the third panel of Figure 23. Further work is underway to validate these results and identify other potential Y-sites implicated in this response.

4.7 EFFECTS OF CYT.D. ON GROWTH

Earlier reports from the lab established FER as a determinant factor for PCa cell survival and growth and response to IL-6 (Allard, Zoubeidi et al. 2000; Zoubeidi, Rocha et al. 2009). We demonstrated above that FER was critical for actin Y-phosphorylation and binding in control cells, and increased with Cyt.D treatment. This raised the question of
PRO4 cells were transfected with siRNAs, FER and control, further cultured for 48 hrs prior exposure to Cyt.D for 6hrs, and lyzed for subsequent analysis. Left: Direct blotting of FER (1\textsuperscript{st}) and actin (2\textsuperscript{nd}). Immunoprecipitations with pY53-actin antibody and actin blot (3\textsuperscript{nd}), and with pY-antibodies next blotted for FER (4\textsuperscript{th}). Right: Changes in levels of FER and pY53-actin, reported in control and FER downregulated cells, are quantified and graphically analyzed.
how PRO4 cell survival is affected by Cyt.D exposure. For this purpose, PRO4 cells were essentially grown in conditions used above to analyze FER/actin complexes and pY-actin, with the exception that they were plated on 96 well plates. The plated cells were continuously exposed to 1µM Cyt.D in serum supplemented culture medium and compared to control cells. Growth was measured daily through MTT assays. Representative results of two experiments presented in Figure 24 illustrate that cells were able to grow equally well in the control and Cyt.D treated series up until 48 hrs. At 72 hrs we began to observe cell loss. Death was evidenced by trypan blue exclusion in floating cells collected at 96 hrs, where complete cell detachment was observed. These findings indicate that the formation of maximal FER/actin complexes and Y-phosphorylation of actin occurred at a time when cells were still growing prior to the detrimental effects of Cyt.D leading to growth inhibition and cell death.

Collectively, the present findings, coupled with the role of FER in PCa cells and the literature on pY53-actin in the amoeba, support that this mechanism involving FER and pY-actin in the nucleus may be part of a stress response for cells to survive. This assumption is further substantiated by higher pY-actin levels in the more aggressive PCa cell lines. Indeed as tumors progress, more aggressive treatments are required. Nevertheless, further work at the cellular level is necessary to better understand how these complex formation and subsequent actin Y-phosphorylation affect cell fate.
Figure 24. Effects of Cyt. D exposure on cell growth

PRO4 cells were exposed to Cyt.D in serum supplemented medium for up to 96 hrs (as in Methods). MTT assays were performed over time on PRO4 cells exposed to Cyt.D (yellow bars) vs controls (purple bars), as described in Methods. Values represent means of 8 wells per point ±standard deviation.
5- DISCUSSION
Protein kinases play an important role in regulating a variety of different cellular processes, such as metabolism, DNA damage repair, survival/apoptosis, proliferation and cell motility in response to environment. Hence, it is no surprise that events such as the upregulation, expression, constitutive activation or mutation of kinases can result in uncontrolled cell growth leading to cancer, invasion and metastasis. Consequently research groups have invested many efforts in identifying oncogenic kinases, in hopes of defining more suitable markers of prognostic value and therapeutically targeting them as a form of treatment.

The host lab had published data revealing that the expression of FER was upregulated in prostate tumors and associated with survival/proliferation of PCa cell lines. Subsequently, in studying potential downstream partners of FER, three Y-phosphorylated proteins were identified. These proteins included STAT3, AR and actin. The AR and STAT3 have been well documented to play important roles in PCa. However, very little is known in regards to actin Y-phosphorylation, its relationship to FER and more importantly in PCa.

Though FER has been demonstrated to interact with ABPs such as cortactin (Kim and Wong 1998), there is no published evidence of direct interaction with actin, other than complexes observed in rat testes (Chen, Lee et al. 2003). To investigate this prospective relationship between FER and actin, it was first sought to validate the existence of such complexes in PCa cells through confocal immunofluorescence. Merged images of FER and actin substantiated this finding, and revealed complexes in the same cell compartments. Biochemical evidence provided further support, as immunoprecipitating one protein, the other was detected on Western blot. Interestingly, confocal results not only supported the
presence of complexes, but also revealed co-localization of FER and actin. This was observed primarily in the cytoplasm, with sparse complexes also observed in the nucleus. Thereby raising the question as to how actin may reach the nucleus.

Actin has long been considered a cytoplasmic protein, excluded from the nucleus, although various studies have depicted actin in isolated nuclei. Results of nuclear actin were initially dismissed, owing to abundance of actin and the strong possibility of cytoplasmic contamination. In 1977, convincing evidence was finally published of actin in a “dynamic equilibrium between the nucleus and cytoplasm” of *Xenopus* oocytes in the context of DMSO treatment (Clark and Merriam 1977). This opened the door to the idea that actin enters the nucleus via passive diffusion, since in theory the size, mass and shape was close to the cutoff (40kDa) for nuclear pores (Tzfira and Citovsky 2005). Nonetheless, despite the small molecular weight, many proteins with similar masses require facilitated nuclear import (Minakhina, Myers et al. 2005). The active nuclear transport of actin was considered, but it was soon discovered that actin lacked a NLS, while containing two functional, leucine-rich NES (Wada, Fukuda et al. 1998). Hence, it was presumed that actin potentially forms a complex with another protein chaperone that has a NLS, and subsequently undergoes active nuclear transport. The exact method of actin nuclear import is yet to be identified, but putative proteins with NLS sequences such as coflin and c-Abl have been proposed (Pendleton, Pope et al. 2003). FER is known to contain a NLS in the kinase domain that drives its nuclear translocation (Ben-Dor, Bern et al. 1999). Interestingly, the host lab and the present study have clearly demonstrated the presence of FER in the nucleus, and a higher proportion of nuclear FER in more aggressive PCa cells (Allard, Zoubeidi et al. 2000). Furthermore, FER controls the nuclear translocation of
STAT3 via the IL6 mediated pathway (Zoubeidi, Rocha et al. 2009). The immediate question arises whether FER may play a similar role with actin. Hence, an attractive possibility is that the nuclear import and export of the FER-actin complex could be regulated via the NLS and NES in the two respective proteins.

The discovery of the pY-actin as a FER partner in PCa cells is a novel finding, as there is no previous evidence of pY-actin in mammalian cells, but solely in Amoeba and chicken-B DT40B cells (Jungbluth, von Arnim et al. 1994). However, the active TK responsible for phosphorylating actin was not identified in either system. Owing to the novelty of pY-actin in human PCa cells and its relationship to FER, the Y-phosphorylation of actin became of great interest.

During the investigation, a combination of pY and actin antibodies was used in immunoprecipitation/Western blot experiments to compare pY-actin in diverse prostate systems. Levels varied according to cell phenotypes, being absent in the normal cells of the dog prostate, but elevated in all PCa cell lines tested, both human and dog. Within each cell model, the least aggressive parental cells exhibited lower levels than their more aggressive derivatives (ie. LN4>PRO4>PC-3, C4:2>LNCaP). In addition, levels were equivalent in the DPC-1 and LN4 model. Findings in the dog model are intriguing since other than humans and primates, dogs are the only large mammals that spontaneously develop PCa (LeRoy and Northrup 2009). Hence, dogs with naturally occurring PCa are relevant models of investigation for the disease in humans, but relatively rare in incidence and not available for research. This highlights the value of the DPC-1 cell line. Substantiating previous results, proteins extracted from the normal and non-dividing dog prostate cells revealed no FER, while expression was upregulated in normal dividing cells.
in vivo and in vitro (Allard, Zoubeidi et al. 2000) and as shown here was elevated in DPC-1. These findings suggest that there may exist a relationship or at the very least a correlation between FER and the levels of pY-actin in PCa cells of different phenotypes. Studies on pY-actin in normal proliferating cells are ongoing.

The role of actin Y-phosphorylation remains enigmatic. Evidence in the two available system, Amoeba and chicken B-cells, suggest a link with actin dynamics, whereby phosphorylation (under stress) promoted depolymerization (Liu, Shu et al. 2006), while dephosphorylation (during antigen receptor stimulation) favored polymerization (Baba, Fusaki et al. 2003). In the amoeba study, half of the actin molecules in spores were Y-phosphorylated as a defense mechanism to withstand stress, thus promoting survival (Kishi, Clements et al. 1998). To determine whether actin molecules in PCa cells responded in similar manners, PRO4 cells were either stimulated to grow with IL-6 or stressed using Cyt.D to specifically target the cytoskeletal actin, allowing for the dismantling and a diminishment of cytoskeletal F-actin. Unexpectedly, the G-actin probe revealed a parallel accumulation in and around the nucleus. The analysis of pY-actin showed increased levels to cells exposed to Cyt.D, but not IL-6 (unpublished data), strongly suggesting that the modulation of actin Y-phosphorylation is related to cell stress response rather than growth stimulation. Consequently, PRO4 cells were treated with the drug and analyzed in a kinetic fashion to observe the response in terms of actin Y-phosphorylation. Growth assays revealed that cells resist for the first 48 hrs of treatment, during which time maximal increase in pY-actin was observed. Growth inhibition and cell detachment was observed afterwards, after four days of treatment, death occurred in all cells released in the medium. A parallel decrease in the actin Y-phosphorylation was
observed, with complete loss at 96 hrs. As in the Amoeba study, these results would support that the Y-phosphorylation of actin may represent a resistance mechanism for cell survival. This may also explain why more aggressive and treatment resistant PCa cell lines contain highest levels of pY-actin and nuclear FER. This will be of critical importance to PCa progression since in later stages of the disease, cancer cells exhibit increased survival and resistance characteristics. If this holds true, a higher concentration of Cyt.D would be necessary to induce similar effects in more aggressive LN4 cells and other cancer cell types would likely express pY-actin (study in progress).

Determining sites of Y-phosphorylation in actin became of importance, as classically this would be a prerequisite for the functional analysis. This information would aid in the elucidation of any potential regulation mechanisms, help in the determination of upstream kinases and facilitate the development of phospho-specific antibodies. LC-ESI-MS analysis showed evidence that in Cyt.D stressed PRO4 cells, a peptide sequence of actin comprising Y53 and adjacent serines could potentially be phosphorylated. Given that Y53 was identified in Amoeba, site-directed mutagenesis of the Y53 site resulted in 40% loss of overall Y-phosphorylation. This indicates that additional Y-residues likely contribute to stress mediated actin phosphorylation, and also supported by the slower rate of Y53-phosphorylation as opposed to total Y-phosphorylation in response to Cyt.D stress. Future studies must be conducted to identify the other sites and determine their potential relevancy to stress response.

Having knowledge of one Y-phosphorylation site, a phospho-specific pY(53)-actin antibody recognizing the peptide sequence between amino acid residues 42-58 of actin from Amoeba, was commercially attained. Owing to the fact that this sequence is identical

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in all isoforms of actin and well conserved throughout most eukaryotic cells, the antibody was capable of recognizing native pY53-actin from human PCa cells. However, difficulty was confronted when actin was denatured, as the antibody failed to recognize pY53-actin on a direct Western blot. The dilemma was overcome by specifically immunoprecipitating pY53-actin, and subsequently Western blotting actin. Results substantiated the MS and mutagenesis results, as Y53 of actin was biochemically found to be Y-phosphorylated and enhanced in Cyt.D treated cells, in a similar kinetic fashion as overall pY-actin. These results support the hypothesis that actin phosphorylation on Y53, as in the Amoeba, contributes in part to survival/resistance mechanisms, in response to stress (Kishi, Clements et al. 1998). Further work is in progress to analyze cell response to other types of stressors.

The potency of Cyt.D to dismantle the cytoskeletal F-actin and cause a nuclear accumulation of G-actin occurred in a parallel fashion as the increase in pY-actin and pY53-actin. This prompted an immunofluorescence analysis of cells using the pY53-actin antibody. Astonishing results were obtained, as for the first time the cellular localization of pY53-actin was demonstrated and predominantly detected in the nucleus. This novel finding speaks in favour that the nuclear G-actin is Y53-phosphorylated as opposed to the cytoplasmic actin. This was evidenced in control cells, and remained nuclear and continued to accumulate in response to the stress induced by Cyt.D. Such an accumulation of nuclear actin was reported in cells upon exposure to stressors such as DMSO, urea and hydrogen peroxide (Polyakova, Chudinovskaya et al. 1986; Kwak, Kim et al. 2004). Thereby, suggesting that this could involve Y-phosphorylation. The present results are in agreement with both the inhibition of F-actin polymerization and the destabilization of the
F-actin conformation upon Y-phosphorylation (Kishi, Clements et al. 1998). In the *amoeba*, the phosphorylation of Y53 resulted in an increase in actin’s critical concentration, a reduction in the rate of actin polymerization and a substantial reduction in actin’s ability to inhibit DNAse I activity (Liu, Shu et al. 2006). A change in actin’s critical concentration as well rate of polymerization could substantially alter the ratio of G-to F-actin.

ABPs, such as c-Abl and DNAse I, are known preferentially bind one conformation over the other, specifically F- vs G- respectively, whereby changes in the ratio of G- vs F-actin have considerable downstream effects (Carter, Christopherson et al. 1997). For instance the binding of actin to c-Abl or DNAse I inhibit their enzymatic activity and prevent their nuclear translocation (Woodring, Hunter et al. 2001). Hence, altering the complex equilibrium between actin and its partners through actin Y-phosphorylation could significantly alter cellular behaviors. Further studies are necessary to better understand the effects of actin Y-phosphorylation on other downstream proteins, such as DNAse I and c-Abl.

A linear relationship was observed between the overall rate of actin Y-phosphorylation and FER/actin complex formation, upon exposure to Cyt.D over time. Recalling that the FER kinase has a SH2 domain that binds pY-motifs, it was hypothesized that FER may directly interact with pY-actin via the SH2 domain of FER. Indeed, SH2 domain pull down assays confirmed such actin binding to the FER SH2 domain. Thus, these data strongly suggest that pY-actin interact directly with FER, and more specifically through its SH2 domain. Interestingly, the rate of interaction through the SH2 domain accounted for about 60% of total FER/actin complex formation. Hence, another domain of
FER likely accounts responsibility for endorsing a direct interaction with actin, possibly non-phosphorylated. Hence, the coiled-coil domain of FER (Craig, Zirngibl et al. 1999) may promote such an interaction between actin and FER. An example was demonstrated between F-actin and the coiled-coil domain of HS1 (hematopoietic lineage cell-specific protein 1), another Y-phosphorylated protein (Hao, Zhu et al. 2005). Deletion of the coiled-coil domain on HS1 abolished interaction with F-actin. The domain in actin governing this interaction is unknown and thus far no coiled-coil domain has been identified. Computational analysis revealed a high probability that it may contain one. Hence, the assertion follows that the coiled-coil domain may instigate the initial interaction between FER and actin, and following phosphorylation, the SH2 domain of FER may further stabilize and protect the interaction and Y-phosphorylation, respectively. The coiled-coil domain of FER has been generated and will be used in pull-down assays to shed light on its involvement in actin binding.

Having established a direct interaction between FER and actin, the major question remained whether FER acts as a TK in phosphorylating actin. As mentioned, the kinase involved in the Y-phosphorylation of actin is yet to be discovered. None was identified for serine, whereas threonine phosphorylation in the plasmodium of Physarum polycephalum, was attributed to the actin fragmin kinase (AFK) (Furuhashi and Hatano 1992). The host lab has published data presenting evidence of STAT3 as a substrate of FER (Zoubeidi, Rocha et al. 2009) and obtained data (unpublished) showing that FER phosphorylates the AR. Moreover, the possibility that FER controls levels of pY-actin was raised by transfection of PCa cells with fer cDNA to express catalytically inactive vs active FER. In the present study we validated such a relationship between FER and actin, using an in vitro
kinase assay with the FER catalytic domain. FER alone was capable of phosphorylating itself, and proficient in phosphorylating actin in the presence of ATP. The main limitation of extrapolating these in vitro results to in vivo situations is that the catalytic domain of FER may not account for structural variations of endogenous FER and of additional factors that influence actin phosphorylation in the context of a cell. Hence, in vivo FER knockdown experiments using siRNA downregulated FER by approximately 50%, and similarly reduced the extent of actin Y-phosphorylation in Cyt.D treated cells. Therefore, it appears that FER is necessary for the Y-phosphorylation of actin, and does so directly, implying that actin is a substrate of FER.

Taken together, when combining the in vitro and in vivo results, along with results on co-localization, direct interactions, and nuclear translocation of both FER and actin in response to stress, it becomes evident that FER plays the role of a TK, and may represent a nuclear chaperone for actin, whereby the kinase’s NLS sequence can compensate for actin’s lack of NLS, and thus allow the complex entry into the nucleus via active transport. Further studies are required to demonstrate whether FER is responsible for the nuclear accumulation of pY-actin.

The precise role of nuclear pY-actin (and pY53-actin), as well as the potential significance of other Y phosphorylated residues, will be addressed in future studies as this may reveal pertinent information in regards to PCa progression. Despite accumulating evidence of nuclear actin and its involvement in transcription, chromatin remodeling, and ribonucleoprotein packaging (Olave, Reck-Peterson et al. 2002; Bettinger, Gilbert et al. 2004), there is no previous knowledge of the existence (or role) of pY-actin in the nucleus of cells. Our immunofluorescence and preliminary fractionation results suggest that pY53-
actin may reach the DNA. Since FER directly binds and phosphorylates STAT3 and AR in growing PCa cells, the question is raised as to whether actin may interact with such transcription factors, as well as the chromatin and DNA in a response to stress. The presence of pY-actin will be investigated in response to diverse therapies as well as in other forms of cancers given possible links with mechanisms promoting survival and resistance to stress.
6 CONCLUSION
This study demonstrates for the first time the Y-phosphorylation of actin in mammalian cells and more specifically on actin Y53 residue of human PCa cells. This phosphorylation appears to be catalyzed directly by the FER kinase, which has been linked to PCa cell survival and growth. Furthermore, the Y-phosphorylation levels of actin correlate with the aggressive phenotypes of PCa cells, and are increased in response to stress. This occurs in parallel with G-actin accumulation in PCa cell nucleus. Taking into consideration the predominantly nuclear localization of pY-actin, changes in actin dynamics and potential effects on ABPs, this relationship between FER and pY-actin may be highly relevant for PCa, given the increasing proportion of nuclear FER with progression and its involvement in PCa cell proliferation.
7 REFERENCES


