Regulation of Pygopus (hPygo2) expression in prostate cancer by E74-Like Factor 1 (Elf-1) transcription factor

by

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ABSTRACT

Prostate cancer is a worldwide health concern. Pygopus2 (hPygo2) protein is required for growth in breast, ovarian, cervical and prostate cancer. hPygo2 expression is regulated by the Rb protein via the ETS factor Elf-1 in cervical and breast cancer. Additionally, the ETS family has confirmed roles in carcinogenesis and proliferation. The mechanism of hPygo2 expression has not been elucidated in prostate cancer. My hypothesis proposes that hPygo2 expression is regulated by Elf-1 bound to its promoter region. Prostate cancer cell lines were used to show protein levels of hPygo2, Elf-1 and ETS. ChIP assays confirmed varying binding capability of Elf-1 and ETS factors to the proximal promoter region between cell lines. Elf-1 knockdown experiments were performed, results show no change in hPygo2 protein levels but show reduction in 22Rv1 mRNA levels. These results suggest that Elf-1 might not be exclusively involved in the activation of Pygopus expression in prostate cancer.

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List of Symbols, Nomenclature or Abbreviations

AMACR - α-Methylacyl-CoA Racemase

ANOVA - Analysis of variance

APC - Adenomatous polyposis coli

AR - Androgen receptor

BCL9 - B-cell CLL lymphoma 9

BPE - Bovine pituitary extract

BPH - Benign prostatic hyperplasia

BRCA1 - Breast cancer 1 gene

BRCA2 - Breast cancer 2 gene

CBP - CREBB binding protein

cDNA - Complementary DNA

ChIP - Chromatin immunoprecipitation

CK1α - Casein kinase 1 alpha

Ct - Cycle threshold

dNTP - Deoxynucleotide

DRE - Digital rectal exam

DVL - Dishevelled

EBRT - External beam radiation therapy

ECL - Enhanced chemiluminescence

EDTA - Ethylenediaminetetraacetic acid

EGF - Epidermal growth factor

ELF-1 - E74-Like Factor 1

EN2 - Engrailed 2

EPCA-2 - Early prostate cancer antigen2

ERK-1 - Extracellular signal-regulated kinase

ETS - E26 transformation-specific

EZH2 - Enhancer of zeste homolog 2

GSK3β - Glycogen synthase kinase 3 beta

HOXB13 - Homeobox B13

HAT - Histone acetyltransferase

hPygo2 - Human pygopus 2

HPV - Human papillomavirus

HPV16 - Human papillomavirus 16

HPV18 - Human papillomavirus 18

IgG - Immunoglobulin G

KLK-4 - Kallikrein-related peptidase 4

LiCl - Lithium chloride

LHRH - Luteinizing hormone–releasing hormone

LRP - Lipoprotein receptor-related protein

ml - Milliliter

mM - Millimolar

min - Minute

ng - Nanogram

NaCl – Sodium Chloride

$NaHCO_3$ - Sodium bicarbonate

NHD - NH2-terminal homology domain

PBS - Phosphate-buffered saline

PCA3 - Prostate cancer antigen 3

PCR - Polymerase chain reaction

PIN - Prostatic intraepithelial neoplasia

PMSF - Phenylmethylsulfonyl fluoride

PNT - Pointed

pRb – Phosphorylated Rb

PSA - Prostate-specific antigen

PSCA - Prostate stem cell antigen

PVDF - Polyvinylidene fluoride

PYGO - Pygopus

Rb - Retinoblastoma

rDNA - Ribosomal DNA

RIPA - Radioimmunoprecipitation assay buffer

RT-PCR - Retro transcriptase PCR

RT-qPCR - Real time quantitative PCR

Sec - Seconds

SDS - Sodium dodecyl sulfate

siRNA - small interfering RNA

Src-3 - Steroid receptor coactivator-3

tPSA - Total PSA

TBST - Tris-Buffered Saline Tween 20

TMPRSS2 - Transmembrane protease, serine 2

µg - Microgram

µl - Microliter

uPA - Urokinase

uPAR - Urokinase receptor

‘UTR – Untranslated region

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Chapter I - Introduction

1.1.1 Incidence and Statistics

Prostate cancer is a growing health concern in many countries, especially in Western society. Worldwide, prostate cancer is the most diagnosed non-skin cancer in men and its mortality is second only to lung and bronchial cancer (Adams and Ferrington, 2014). In Canada, the incidence continues to rise causing it to be the most frequently diagnosed cancer amongst Canadian men. According to Canadian Cancer Statistics published for 2014, one in eight men is expected to be diagnosed with prostate cancer in their lifetime. Currently, 23 600 new prostate cancer cases are expected, representing 24% of all new male cancer cases (Cuzick et al., 2014; Canadian Cancer Society's, 2014). In the United States for 2014, prostate cancer represents the second leading cancer in estimated new cancer cases and deaths in males, representing 27% (233,000 cases) of all new cancer cases in men according to the American Cancer Society (Siegel et al., 2014).

Improved treatments and early detection of prostate cancer have reduced the number of prostate cancer related deaths but there is no effective cure for advanced disease (Shen and Abate-Shen, 2010; Sfanos and Marzo, 2012). Locally confined disease and early diagnosis results in 100% survival within 5-years, however, in case of advanced disease and metastasis, the 5-year survival is reduced to 33% (MacVicar and Hussain, 2013).

1.1.2 Anatomy and histology of the prostate gland

The normal prostate is a gland located under the bladder around the urethra. Its main function is to produce secretory components for the seminal fluid. Since the prostate gland lacks a globular structure, it is defined as having 3 zones according to the classic work of McNeal; the central zone, the transition zone and the peripheral zone which harbors the majority of prostate carcinomas (McNeal, 1969, 1980, 1981; Timms, 2008; Shen and Abate-Shen, 2010) *Figure 1.1*. This structure with an acinar morphology originates from the endodermal epithelial and meso-dermal stromal cells. Normal development of the prostate starts in the Wolffian and urogenital sinus (McNeal, 1981).

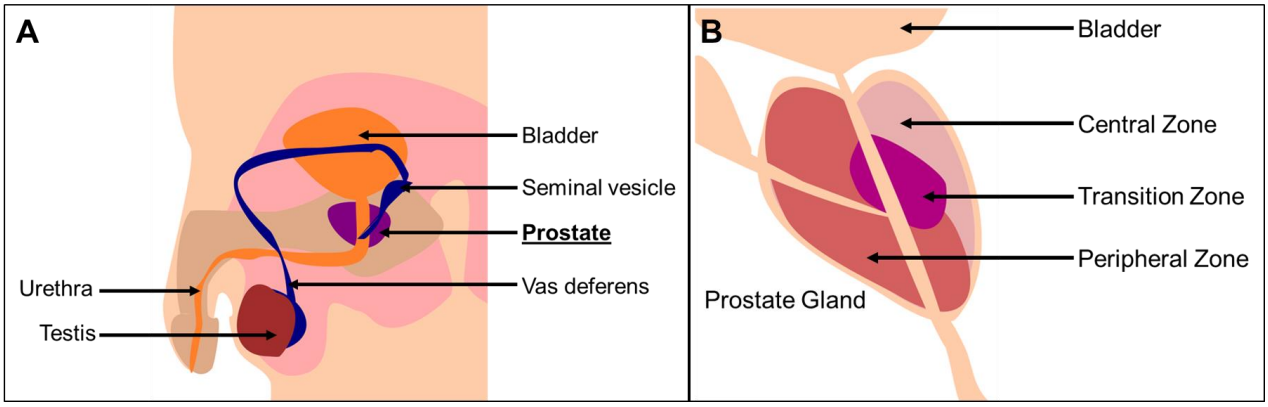

Figure 1. 1 A- Prostate gland location B- Zonal division of the prostate gland
Adapted from McNeal (1981) by permission

The normal prostate epithelium has three differentiated epithelial cell types, luminal, basal and neuroendocrine cells *Figure 1.2***.** Luminal cells are the predominant cells forming a continuous layer in the prostate epithelium producing secretory proteins. They are androgen receptor positive and express the markers cytokeratins 8 and 18, and CD57. The basal cells, also known as myoepithelial cells are located beneath the luminar epithelium and do not produce

secretory proteins. They present low androgen receptor levels and express p63, cytokeratins 5 and 14, and CD44 (Abate-Shen and Shen, 2000; DeMarzo *et al.*, 2003). The least populous epithelial prostate cells are the androgen independent neuroendocrine cells. While their function is still unclear, they express chromogranin A, serotonin and neuropeptides, and it is believed that they provide signals for growth of luminal cells (Sfanos and Marzo, 2012; Shen-Li *et al.*, 2000; Shen and Abate-Shen, 2010). Noticeably, an increase in the presence of neuroendocrine-like cells is typical of small cell carcinoma, a very aggressive form of prostate cancer (Shen and Abate-Shen, 2010).

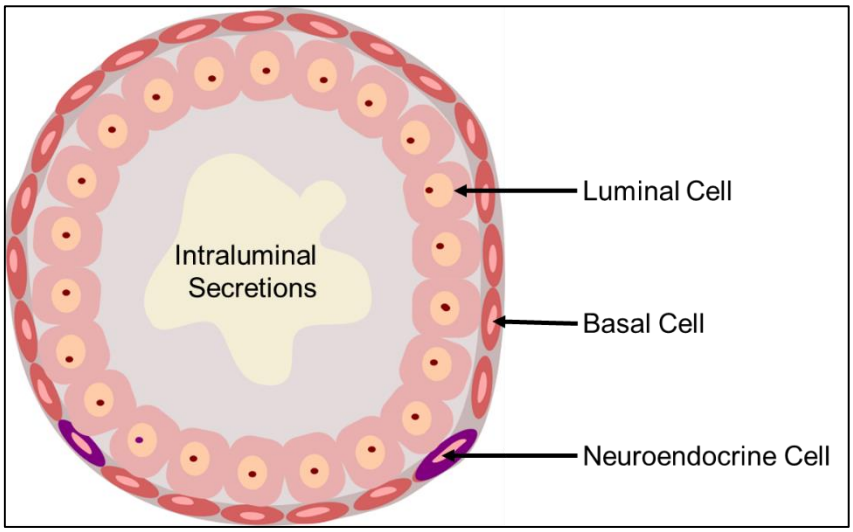

Figure 1. 2 - The three differentiated cell types within the prostate epithelium
Adapted from Abate-Shen and Shen (2000) by permission

1.1.3 Prostate cancer initiation and progression

Prostatic intraepithelial neoplasia (PIN) is widely considered a precursor of prostate cancer; findings suggest that its presence initiates when certain processes such as inflammation, oxidative stress and DNA damage affect the normal prostatic epithelium. PIN is characterized by an increase in luminal cells, reduction of basal cells and altered structure in the cells resulting in less adhesion and cytoskeleton changes (Shen and Abate-Shen, 2010; DeMarzo *et al.*, 2003). Progression of PIN into adenocarcinoma is due to the activation of oncogenes, inactivation of tumor suppressors and re-activation of pathways involved in development. Advanced forms of PIN show an acinus architecture lined by luminal-like cells that seem to be malignant (DeMarzo *et al.*, 2003). Histological evidence suggests that an abundant number of carcinomas have zones of high-grade PIN from which carcinoma glands seem to arise (DeMarzo *et al.*, 2003).

Adenocarcinoma is characterized by the complete absence of basal cells and the sustained proliferation of luminal-like cells. The previous thriving epithelial cells can lose their polarity and cell adhesion, gaining migratory properties that allow them to metastasize to other parts of the body, usually lymph nodes and bone (Shen and Abate-Shen, 2010). *Figure 1.3*

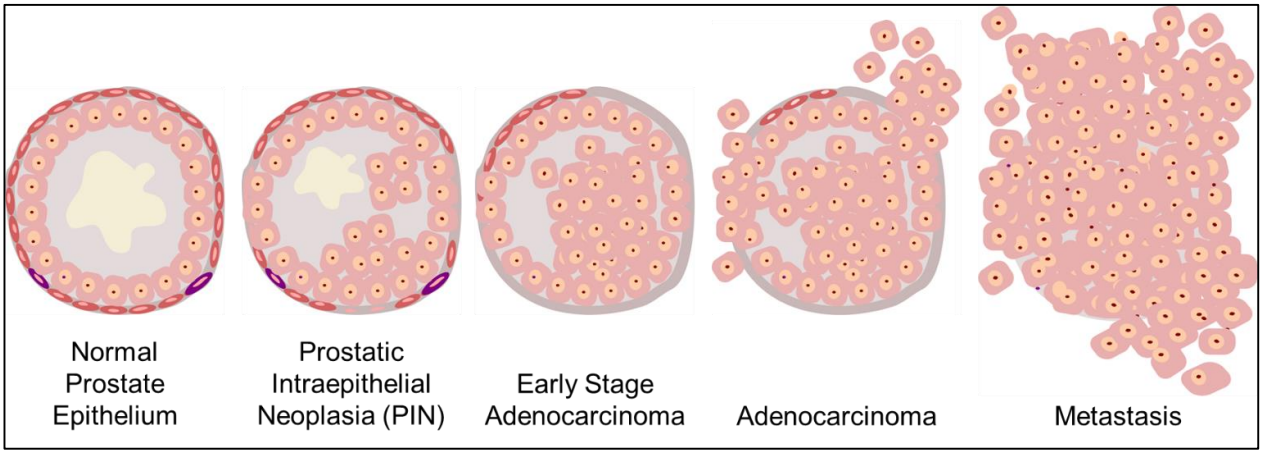

Figure 1. 3 - Prostate cancer progression
Adapted from Abate-Shen and Shen (2000) by permission

1.1.4 Risk factors

Understanding the different factors that determine the risk of developing prostate cancer is a very challenging and extensive task. There are, however, at least three well-known risk factors identified: age, race and heredity. The following section discusses these along with other risk factors mentioned in the literature.

1.1.4.1 Age

Age is the principal risk factor for prostate cancer. A rapid increase on the incidence of prostate cancer after the age of 50 is noticeable in a significant proportion of the population. In fact, prostate cancer incidence increases faster with age compared to other kind of cancers. Precursor lesions can be detected in men under the age of 40, however the majority of prostate cancer diagnoses occur in men after 65 years of age (Cuzick *et al.*, 2014; Fradet *et al.*, 2009). In Canada, around 100 men are diagnosed of 100 000 between 50-54 years, 500 per 100 000 men between 60-64 years and more than 700 per 100 000 men that are over the age of 80 (Fradet *et al.*, 2009; McDavid *et al.*, 2004). Although there is no sole cause for this manifestation, it is hypothesized that among other factors, the relationship between age and prostate cancer is significantly due to the increase of oxidative stress during aging. Oxidative stress has been linked to different kinds of cancer by increasing DNA mutations or DNA damage, genome instability and cell proliferation (Visconti and Grieco, 2009).

1.1.4.2 Race and geography

Prostate cancer incidence shows variation between different ethnic groups and countries. African-American men are the most affected group having 58% greater prostate cancer incidence than Caucasian males, 144% greater mortality and are also more likely to be diagnosed in an advanced stage while Hispanic men have 14% lower incidence and 17% lower mortality compared to Caucasian males in the United States (Fradet *et al.*, 2009; Cuzick *et al.*, 2014). The lowest incidence rates of prostate cancer are in Asia, the lowest annual incidence registered is 1.9 per 100,000 individuals per year in the city of Tianjin, China. The highest incidences are in North America, more specifically the United States having 197 per 100,000 individuals per year. These differences are caused by a combination of genetic factors, external factors like environmental exposure and even differences in health care (Grönberg, 2003). While African American ancestry seems to be a significant factor for incidence and outcome in individuals, there is also a geographic influence and increase of risk with individuals introduced to the western society lifestyle (Sfanos and Marzo, 2012). Migration studies have revealed an increase of prostate cancer risk after migration to North American countries by analyzing Japanese people moving to the USA. Their acquired risk is noticeable, however, it only represents 50% of the risk for Caucasian men and 25% of the risk of African-American men. These examples suggest that the differences between ethnic and geographical populations are real and not only an analysis influenced by lack of registered incidence on the health care system of different countries (Grönberg, 2003).

1.1.4.3 Genetic factors and heredity

Family history and genetics play an important role in prostate cancer incidence. Men who have a first-degree relative with prostate cancer have a higher risk than men without that relationship. Also, younger men (less 65 years old) who have a first-degree relative with prostate cancer have a higher risk,

getting diagnosed approximately 6 years earlier than men with no family history (Fradet *et al.*, 2009). Interestingly, men whose brothers are diagnosed have a greater risk of developing prostate cancer than those whose fathers had the disease, suggesting a link to chromosome X (Grönberg, 2003). More than two first-line relatives with the disease increases the incidence risk by 5-11 times (Bratt, 2002).

A portion of the family history risk is explained by mutation of known genes but only in rare cases. Some of these high-risk genes include BRCA1 and BRCA2 which increases 6 times the risk in men younger than 65, and HOXB13 increasing the risk 4 times. High risk studies have been focused mainly in androgen receptor, vitamin D-receptor and other genetic polymorphisms (Grönberg, 2003; Cuzick *et al.*, 2014). After all these findings, family history has been considered a risk factor but the majority of the prostate cancers appear not to be exclusively caused by hereditary factors. While genetic polymorphisms have been linked to cancer, only a handful of examples are clearly established.

1.1.4.4 External exposure and lifestyle

The relationship between prostate cancer and exposure to environmental factors has been studied extensively by epidemiological investigation. Exposure to radiation, chemicals and diet have been the principal studied risk factors. Ionising radiation and ultraviolet radiation have been both linked to prostate cancer. It is known that high exposure to cadmium correlates highly with incidence of prostate cancer but it is a rare case when talking about an entire population (Cuzick *et al.*, 2014). Exposure to chemicals such as pesticides might increase the risk of prostate cancer. As an example, methyl bromide has been linked to prostate cancer in farm workers (Fradet *et al.*, 2009). Exposure to cigarette smoke or being a smoker is associated with a moderate increase of risk of mortality once diagnosed with prostate cancer. Also, aggressive cancers have been associated with smokers suggesting that smoking might play part in metastatic disease. Other

evidence suggests smoking encourages tumor growth in prostate cancer patients, but there is no evidence of a direct correlation between smoking and prostate cancer initiation (Cuzick *et al.*, 2014; Fradet *et al.*, 2009).

Diet, weight and physical activity have been factors studied in relation to prostate cancer. A sedentary lifestyle has been linked to higher PSA concentrations in blood and while there is no clear link between specific diet regimes, it has been reported that there is a relationship between prostate cancer and dietary fat (Cuzick *et al.*, 2014). Noticeably, obesity has been associated with prostate cancer with an increased risk for aggressive disease and mortality. Studies suggestthat prostate cancer is associated with a diet that includes high consumption of dietary fats, red meat and dairy (Chan *et al.*, 1998). While these exogenous factors might be involved in the development of prostate cancer, there is no sufficient evidence generated from randomized trials to suggest any changes on related behaviors or lifestyle (Heidenreich *et al.*, 2014).

1.1.5 General guidelines for diagnosis and treatment

1.1.5.1 Diagnosis

The main tools for diagnosis of prostate cancer are: the digital rectal exam, PSA levels and transrectal ultrasound guided biopsies. Diagnosis is based on the combination of these results and histopathological results from biopsies (Smith *et al.*, 2007).

Prostate specific antigen (PSA) levels have been widely used as first screening for prostate cancer but there is controversy about its variation, accuracy and specificity for detection. The main concern is that a threshold level indicating the highest risk for diagnosis has not been defined (Heidenreich *et al.*, 2014, 2008) . Based solely on PSA measurement, the risk of

developing prostate cancer within 7-years of testing was 34% for men with PSA values of 3-6 ng/ml, 44% for values between 6-10 ng/ml and 71% for those with values greater than 10 ng/ml (Aus *et al.*, 2004; Prensner *et al.*, 2012). Further information about PSA as a biomarker is described in the next sections.

The standard method to obtain material in order to provide histopathological analysis is the transrectal ultrasound guided biopsy. Usual size for biopsies is 18G core and number of biopsies required for detection of prostate cancer is not clearly defined (Guichard *et al.*, 2007). Studies show higher detection of prostate cancer when using at least 10 biopsy cores but each procedure has to be adapted to the patient consideration and other values such as PSA level and prostate volume (Guichard *et al.*, 2007; Heidenreich *et al.*, 2008). Repeats of biopsies sets are recommended if biopsies are negative and PSA levels are high or other observations suggesting prostate cancer are detected (Klotz, 2005).

1.1.5.2 Treatment

The condition of the patient, tumor grade and progression of the disease within the prostate gland and metastasis to other tissues are all factors taken into account to select a treatment option. Active surveillance is the common start point for tumors that are small, low-grade and confined to the prostate tissue. Active surveillance includes regular PSA and DREs tests and examination of repeated biopsies during time in order to determine if the cancer has progressed and there is the need for therapy (Klotz, 2005).

Radical prostatectomy is the most used treatment with generally effective results for stage T1 and T2 tumors that have not spread outside the prostate gland. It consists of an operation to remove the prostate gland, seminal vesicles and depending on diagnosis some of the tissue

around it. Additionally, for men with high risk prostate cancer, a pelvic lymphadenectomy is performed which surgically removes groups of lymph nodes to try to prevent recurrent disease (Heidenreich *et al.*, 2014). There is still controversy in how to select patients that should have a lymphadenectomy from others. This surgery has been correlated to survival rates of 95%, 90% and 79% at 5, 10 and 15 years respectively (Ward *et al.*, 2005; Heidenreich *et al.*, 2014).

Radiation therapy, generally EBRT (external beam radiation therapy), can be used to treat prostate cancer at any stage but it is usually selected when the health and age of the patient is a concern. It consists of radiation beams that are targeted to the prostate gland. In patients with high-risk disease, this type of treatment has been shown to improve 5-year disease survival but does not insure against relapse or initiation of disease in surrounding areas (Bolla *et al.*, 2002). This therapy can be used in combination of other treatments, however, surgical removal of the prostate is not common and is very difficult after radiotherapy. To attempt to prevent relapse, radiation therapy with dose escalation is usually accompanied by androgen deprivation therapy (Pollack *et al.*, 2000). The second most common radiation therapy is perineal brachytherapy which consists of implanting radioisotope seeds directly to the tumor trying to avoid affecting surrounding tissues. Low-dose seed implants are used to treat low-grade cancers contained within the prostate gland, which release low level radiation steadily over several months. It is mainly used for localized cancer using iodine-125 or palladium-103 isotopes (Blank *et al.*, 2000). High-dose radiation is reserved for patients with high-grade tumors, the dose is administered through injections in the prostate, concentrating in the affected areas.

Hormonal therapy is widely used especially when facing aggressive, metastatic and/or recurrent disease usually after surgery or radiation therapy. Androgen deprivation therapy is performed using luteinizing hormone-releasing hormone agonists (LHRH) which provide a rapid

reduction in testosterone levels using different approaches: complete androgen deprivation, intermittent androgen deprivation, neoadjuvant hormone therapy and adjuvant hormone therapy (Crawford and Hou, 2009; McLeod *et al.*, 1997). Intermitting hormone therapy consists on stopping androgen deprivation once PSA detection has diminished and stabilized, but resumes if PSA increases again. Neoadjuvant hormone therapy consists on using androgen deprivation before local treatment with the goal of reducing the size of the tumor and potentially aiding in the effectiveness of the main treatment. Lastly, adjuvant hormone therapy is used directly after surgery or radiation therapy to attempt the eradication of cancer cells possibly remaining. Initially prostate cancer tumors respond to hormonal therapies but it is later when androgen-independent tumors emerge.

Chemotherapy is the treatment selected for patients with aggressively recurrent and/or metastatic disease that is resistant to hormonal therapies. Docetaxel is commonly the drug selected for treatment, which inhibits microtubule formation in mitosis and has been tested in combination with other therapies like radiation or surgery (Adams and Ferrington, 2014). According to randomized clinical phase III trials, Docetaxel in combination with prednisone is the regime of choice for men with castration-resistant disease resulting in increase of survival of 3 months, and improvement of pain and quality of life compared to Mitoxantrone, another common chemotherapeutic agent used as a second-line treatment for metastatic and hormone-refractory prostate cancer (Heidenreich *et al.*, 2008). Additionally, new agents such as Cabazitaxel have been generated and tested as the next generation of taxane chemotherapy designed to overcome resistance to Docetaxel treatment (Crawford *et al.*, 2015).

1.1.6 Biomarkers

Biomarkers are molecules identified as indicators of a particular state in living organisms. They are often used in diagnosis and treatment of prostate cancer. Research findings report unique changes in biological markers can be used to identify or suggest the initiation, progression or aggressiveness of disease. Currently, prostate cancer diagnosis is frequently based on the prostate-specific antigen (PSA), which has provided many advantages like enhanced detection at early stages of disease but comprises disadvantages due to low specificity potentially resulting in misdiagnosis (Miller, 2012). The following section discusses current biomarkers used in diagnosis, prognosis and treatment of prostate cancer under 2 categories: 1) Serum biomarkers, referring to biomarker molecules in blood plasma identified by serum assays; and 2) Tissue biomarkers, referring to proteins identified and expressed in prostate cancer tissue specimens.

1.1.6.1 PSA and serum biomarkers

Prostate specific antigen (PSA), also named kallikrein-3, is a serine protease very widely used and established as a serum biomarker for diagnosis and staging of prostate cancer (Partin *et al.*, 1993). Low levels of PSA are detectable in the normal prostate gland but higher levels have been associated with disease progression. PSA has the advantage of only being produced by prostate tissue but challenges arise due to PSA detection sensitivity and specificity. A large number of studies report a correlation between PSA and cancer progression however this relationship is not always consistent (Crawford and Abrahamsson, 2008). Total serum PSA concentration (tPSA) is regularly used, and screening is often recommended by clinicians every year along with digital rectal examination (DRE) for men older than 50 years old. Recent

controversy has arisen due to evidence that suggests that tPSA values can vary a lot in patients with no disease and patients with benign prostate hyperplasia (BPH), potentially resulting in misdiagnosis (Martin *et al.*, 2012; Prensner *et al.*, 2012). Due to these challenges additional prostate serum biomarkers have been studied.

Kallikrien-4 (KLK-4), found in serum and tissue, is an androgen-dependent serine protease that has been studied as a potential biomarker. Findings have shown correlation between elevated levels of KLK-4 in serum and prostate cancer progression, and have suggested that KLK-4 increases proliferation and motility of cancer cells (Martin *et al.*, 2012). Seiz *et al.*, in 2010, reported that KLK-4 is expressed in healthy prostate and it is upregulated in early-stage disease but not late-stage prostate cancer. The roles of KLK-4 have not been completely elucidated but could present an effective way to diagnose and stage patients in conjunction with PSA (Harada *et al.*, 2003).

Additional serum markers such as early prostate cancer antigen (EPCA-2) and prostate cancer gene 3 (PCA3) have been shown to be elevated in serum from prostate cancer patients but not present in healthy patients. Unfortunately, their specificity to prostate cancer and effective use as diagnostic and prognostic biomarkers needs further evaluation (Martin *et al.*, 2012; Velonas *et al.*, 2013).

1.1.6.2 Tissue biomarkers

Androgen receptor (AR) is a nuclear receptor that is activated by androgens such as testosterone or dihydrotestosterone (Balk and Knudsen, 2008). Once bound to these hormones it is translocated into the nucleus where it acts as a DNA-binding transcription factor that can regulate normal gene expression in the prostate gland, but it also is involved in the initiation and

progression of prostate cancer (Heinlein, 2004). Androgen depletion therapies have been common in prostate cancer treatment for over 40 years resulting in an initial disease regression but unfortunately for aggressive disease, later progressing to an androgen independent stage (Debes and Tindall, 2004; Feldman and Feldman, 2001).

The progression from primary prostate cancer into metastatic and castration-resistant disease has been associated with overexpression of androgen receptor (Linja *et al.*, 2001). Because AR is often upregulated in prostate cancer and therefore triggering its transcriptional activation of genes involved in growth, encouraging cancer progression even in absence of androgens; it has been considered a promising biomarker not only as a therapeutic target but also as a predictor of metastatic, aggressive and androgen-independent disease.

α-methyl-co-racemase (AMACR) is a biomarker often used in clinical diagnosis as high levels of the protein are associated with prostate cancer. AMACR is used primarily for immunohistochemistry of biopsy tissues for the detection of prostate carcinoma (DeMarzo *et al.*, 2003). It is used in conjunction with other biomarkers to confirm diagnosis of prostate cancer along with absence of basal cells. Unfortunately, AMACR is also detectable in high-grade prostatic intraepithelial neoplasia (PIN) and occasionally on benign glands, which challenges the diagnosis of low to medium grade prostate carcinoma (Esfahani *et al.*, 2015; Qu *et al.*, 2014; Martin *et al.*, 2012).

Urokinase-type plasminogen activator (uPA), a serine protease, interacts with its receptor called uPAR, bound to the cell membrane. Their interaction has been implicated in the proliferation and metastasis of different cancers mainly due to its ability to activate conversion of plasminogen to plasmin and activate different signalling events degrading matrix proteins (Sheng, 2001). In prostate cancer uPA and uPAR levels are elevated compared to normal

prostate tissues. Both activator and receptor are used as diagnostic markers due to their unique expression pattern during prostate cancer and efforts are still being made for them to be used as therapeutic targets (Li and Cozzi, 2007; Dong *et al.*, 2008; Sheng, 2001).

Steroid receptor coactivator-3 (Src-3), is a tyrosine kinase that has been identified as being recruited to the PSA promoter and able to interact with androgen. Findings have shown that this interaction correlates with tumorigenesis in breast, ovary and prostate cancer (Zhou *et al.*, 2005). High expression of Src-3 in serum correlates with cell proliferation and hormone-independence since patients that have higher expression of Src-3 are more likely to relapse (Zhou *et al.*, 2005; Fizazi, 2007). Src-3 has been identified to be a potential indicator of metastasis and progression, inhibitors of Src-3 are being currently evaluated to assess their effect in controlling cancer progression (Martin *et al.*, 2012).

Loss of E-cadherin expression, a protein that mediates calcium-dependent cell-cell adhesion and their bond to the extracellular matrix, has been related to invasion and metastatic ability of prostate cancer cells (Mao *et al.*, 2010). Lower levels of active E-cadherin have been found in metastatic prostate cancer cell lines and promotes the release of the cells from their extracellular matrix and tissue (Kuefer *et al.*, 2005). These findings suggest that this protein could potentially be used as an epithelial to mesenchymal transition biomarker (Zeisberg and Neilson, 2009).

Enhancer of Zeste homolog 2 (EZH2), is a protein associated with cancer metastasis in variety of cancers such as breast, renal and prostate cancer (Varambally *et al.*, 2002) . EZH2 has been correlated with low levels of E-cadherin expression found in tumors with poor prognosis. EZH2 expression is significantly higher in prostate cancer cells compared to ones with BPH or PIN (Dhanasekaran *et al.*, 2001; Martin *et al.*, 2012). In tumors, EZH2 expression is higher in

disease with a Gleason score of more than 7. EZH2 has been identified as a potential therapeutic target mainly due to its properties as a regulator of tumor metastasis at different stages (Varambally *et al.*, 2002; Shen and Abate-Shen, 2010; DeMarzo *et al.*, 2003).

Prostate stem cell antigen (PSCA) is a glycoprotein present in a large proportion of prostate cancers (DeMarzo *et al.*, 2003). Although it is also found in healthy prostate tissue, higher levels of PSCA correlate to later cancer stage, Gleason score and androgen independence. PSCA was expressed in 94% of primary tumors and 100% of metastatic samples according to Gu *et al.*, 2000 studies. For these reason, PSCA is considered a highly potential therapeutic marker in addition to being a diagnostic tool.

Engrailed-2 (EN2) is a member of the HOX gene family involved in embryonic development and shown to be re-activated in cancer progression. High levels of EN2 has been identified in PC-3, DU145 and LNCaP cell lines and elevated expression is correlated in tumor samples of genetically high risk men (Morgan *et al.*, 2011). It is a potential diagnostic tool because it can be detected in urine and according to Killick *et al.*, 2013, had a sensitivity of 66.7% and specificity of 89.3% to detect cancer using an ELISA based detection system. It has the potential advantage of being used non-invasively for early detection.

Genomic analysis is also widely used for studying biomarkers in the use of prostate cancer and trying to detect individuals who are more at risk. BRCA1/2 have the most potential for use as markers since there is an association of BRCA2 mutations with aggressive disease and poor survival (Velonas *et al.*, 2013). Another example is prostate cancer antigen 3 (PCA3), which is expressed in only prostate and is currently used mainly due to its detection in urine and prostatic fluid. PCA3 is overexpressed in 95% of prostate cancer patients compared to those with healthy or BPH prostate glands (Velonas *et al.*, 2013; Martin *et al.*, 2012).

To date, the most frequent and detectable gene fusion in prostate cancer is TMPRSS2:ERG (Tomlins *et al.*, 2005). TMPRSS2 is an androgen related serine protease that is secreted by prostate epithelial cells and is fused with ERG protein, a member of the ETS family, which will be discussed in the next sections. This fusion has a high predictive value but no diagnostic tests are available at the moment (Hessels *et al.*, 2007). Findings claim that the detection of this fusion along with the regular serum PSA testing would improve the effectiveness of diagnosis or prognosis but the value of TMPRSS:ERG detection is yet to be determined, mainly due to lack of correlation with Gleason scores and its presence in good prognosis patients (Demichelis *et al.*, 2007; Martin *et al.*, 2012).

Novel biomarkers present an opportunity to resolve the challenge of diagnosis, staging and treating prostate cancer. While many potential candidates have emerged there is a deficiency of strong well-sustained biomarkers for diagnosis and treatment able to exclusively detect cancer initiation, progression and/or metastasis.

1.1.7 Pygopus

Novel biomarkers present an opportunity to cover the necessity for effective molecular targets in their use in diagnosis, prediction and potential therapies. Our laboratory has concentrated its efforts on a novel biomarker called hPygo2 (Lake and Kao, 2003; Andrews *et al.*, 2007, 2008, 2009; Kennedy *et al.*, 2010; Popadiuk *et al.*, 2006; Tzenov *et al.*, 2013; Andrews *et al.*, 2013). Pygopus2 is one of the two isoforms of the Pygopus proteins originally discovered as a nuclear factor of canonical Wnt/β-catenin transcription complex of the Wnt signaling pathway in Drosophila (Kramps *et al.*, 2002; Belenkaya *et al.*, 2002). Soon after, Pygopus homologous proteins were identified with roles in organogenesis first in *Xenopus* and also in mammals (Lake and Kao, 2003; Li *et al.*, 2004).

As reviewed by Polakis, 2012, members of the Wnt signaling pathway are involved in countless processes in development, growth and differentiation, which make their abnormal activation and mutations play a critical role in cancer progression. Without exception, Pygopus is highly expressed in different malignancies such as breast, ovarian, glioblastomas and gliomas, cervical cancer, prostate and hepatic cancer (Andrews *et al.*, 2007; Popadiuk *et al.*, 2006; Tzenov *et al.*, 2013; Wang *et al.*, 2010; Chen *et al.*, 2011; Yang *et al.*, 2013; Zhang *et al.*, 2015). Findings in development and cancer research have shown that pygopus has Wnt-independent roles mainly due to its function as a chromatin modifier and recent discovered roles in ribosome biogenesis (Andrews *et al.*, 2009; Chen *et al.*, 2010; Andrews *et al.*, 2013). The following section discusses Pygopus2 function and its potential as a biomarker in prostate cancer.

1.1.7.1 Pygopus in the Wnt signaling pathway

Association of the Wnt/ β-catenin signaling pathway with cancer has been widely studied, mainly due to its requirement on growth and differentiation events (Clevers, 2002). Mutations of components of this pathway have been linked to a variety of human cancers like colorectal, breast, ovarian and cervical cancer (Andrews *et al.*, 2007). Activation of the canonical Wnt pathway causes an accumulation of β-catenin in the cytoplasm that is then translocated to the nucleus to act as a co-activator of TCF/LEF transcription factors binding to DNA and therefore initiating gene transcription (Thompson *et al.*, 2002; Polakis, 2012).

In the absence of Wnt stimulation, β-catenin is phosphorylated by a destruction complex that includes axin, glycogen synthase 3β (GSK3β), casein kinase 1α (CK1α) and the Adenomatous polyposis coli (APC) tumor suppressor. Degradation depletes nuclear β-catenin and therefore, inhibition of gene transcription since TCF/LEF transcription factors are inactive due to their binding to transcriptional corepressors such as Groucho *Figure 1.4*.

Pathway activation is initiated by the binding of a Wnt ligand to the Frizzled family receptor, which disrupts the destruction complex, resulting in cytoplasmic β-catenin accumulation and nuclear translocation. Together with several proteins, β-catenin binds and activates the TCF/LEF transcription factor, inducing gene transcription. Proteins forming the activation complex of TCF/LEF with β-catenin include B-cell CLL/lymphoma 9 protein (BCL9), CREB-binding protein (CBP) and Pygopus (Pygo) *Figure 1.5* (Thompson *et al.*, 2002; Kramps *et al.*, 2002; Lake and Kao, 2003).

Negative and positive acting components of the Wnt signaling pathway are affected in cancer cells. In cancer, a number of negative suppressing components are found to be mutated while positive components are activated (Thompson *et al.*, 2002). One factor commonly

identified in the disruption of the Wnt pathway is APC which is the most frequently mutated gene in human cancers. APC causes adenomatous polyposis which generated polyps in the large intestine at an early age which turn into colorectal cancer. This loss of function is required for cancer progression and it disrupts the ability to regulate β-catenin stability (Polakis, 2012).

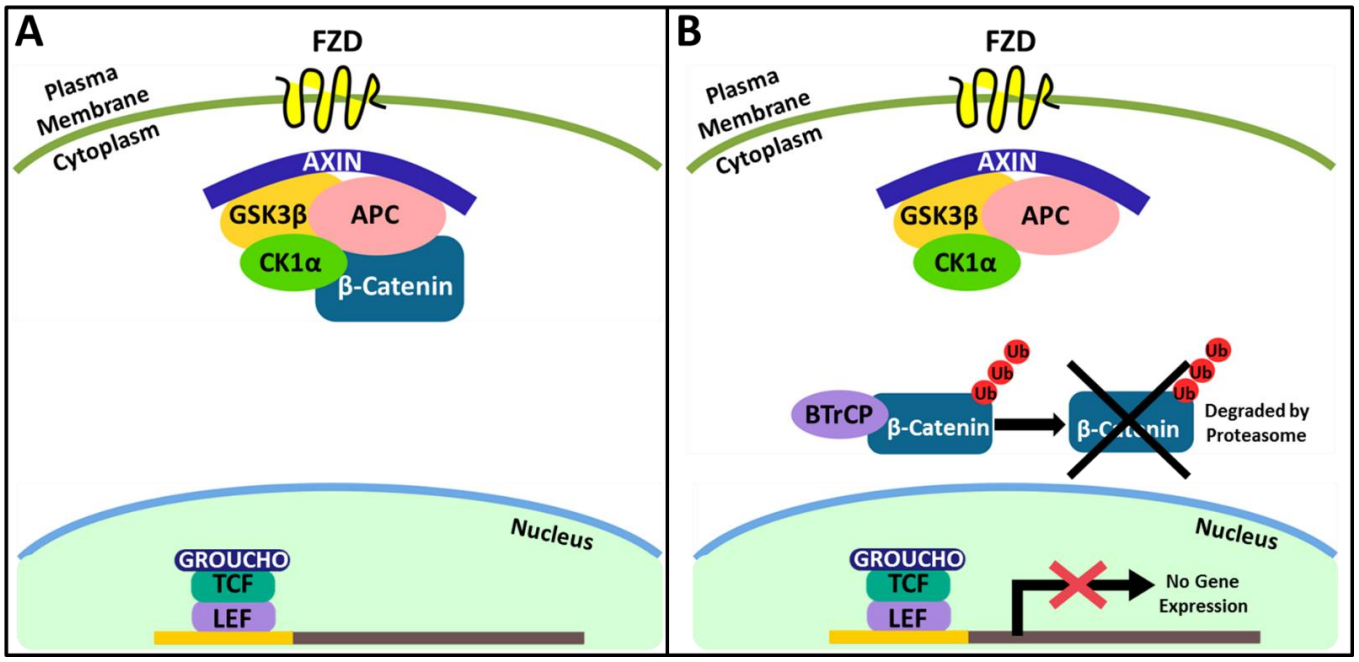

Figure 1. 4–Inactive Wnt Pathway. A-Destruction complex in the cytoplasm and repression of gene expression in the nucleus. Axin, glycogen synthase 3β (GSK3β), casein kinase 1α (CK1α) and the Adenomatous polyposis coli (APC) tumor suppressor conform the destruction complex in absence of Wnt stimulation. B- Degradation of β-catenin in the cytoplasm by the proteasome, TCF/LEF transcription factors are inactive due to their binding to transcriptional corepressors such as Groucho.

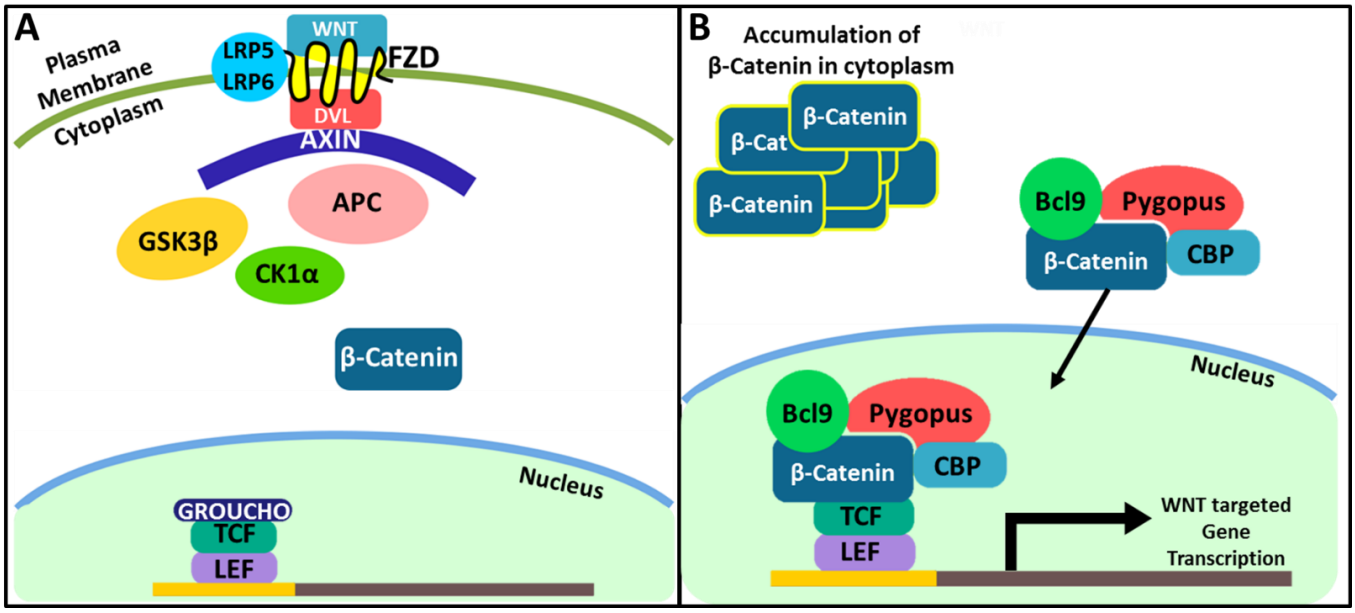

Figure 1. 5- Active Wnt Pathway. A- Wnt ligand binding to FZD (Frizzled) and LRP (Lipoprotein receptor) receptors, disrupting destruction complex. B- β-catenin accumulation and translocation to the nucleus where Pygopus among other factors form a complex to activate target gene transcription.

Pygopus proteins contain an evolutionary conserved PHD domain that controls chromatin access for transcription complexes and binding (Thompson *et al.*, 2002; Kramps *et al.*, 2002; Belenkaya *et al.*, 2002). Additionally, Pygopus proteins also contain an N-terminal homology domain (NHD), which is required to bind LEF/TCF and start Wnt-independent transcription (Jessen *et al.*, 2008). While much of the initial studies focused on the role of Pygopus in canonical Wnt-mediated gene transcription, Wnt-independent roles of Pygopus2 were revealed when knockdown experiments eliminated both β-catenin dependent and independent genes required for forebrain and retina development in *Xenopus* (Lake and Kao, 2003). Furthermore, Pygopus Wnt-independent functions have been studied in development and in a variety of cancer malignancies (Popadiuk *et al.*, 2006; Thompson *et al.*, 2002; Song *et al.*, 2007).

1.1.7.2 Pygopus relationship to cancer

High expression levels of hPygo2 have been reported in different malignancies such as breast, ovarian, glioblastomas and gliomas, cervical cancer, prostate and hepatic cancer (Andrews *et al.*, 2007; Popadiuk *et al.*, 2006; Tzenov *et al.*, 2013; Wang *et al.*, 2010; Chen *et al.*, 2011; Yang *et al.*, 2013; Zhang *et al.*, 2015). As reported by Popadiuk *et al.*, 2006, six different epithelial ovarian cancer cell lines, including Wnt-active and Wnt-inactive cells, overexpressed nuclear hPygo2 compared to normal ovarian epithelial cells. Additionally, 82% of patient tumors also overexpressed hPygo2 compared to benign disease, and when hPygo2 knockdowns were performed, both Wnt-active and Wnt-inactive cancer cell lines demonstrated growth arrest. These results suggest that even in the absence of Wnt signaling, hPygo2 is expressed and required for growth and therefore important for its potential as a diagnostic biomarker and therapeutic agent (Popadiuk *et al.*, 2006).

In breast cancer, hPygo2 levels were elevated in malignant tumors and knockdown experiments resulted in halted growth of MCF-7 and MDA-MB-468 cell lines. Along with anchorage-independent assays, these results demonstrate a requirement for hPygo2 in breast cancer growth (Andrews *et al.*, 2007). According to Wang *et al.*, 2010, tumor grade correlated with the overexpression of hPygo2 in glioma tissue samples. Additionally, when knocking down hPygo2 expression in the glioblastoma cell line U251, results showed reduction in proliferation and arrest of the cells suggesting that hPygo2 is required for growth (Wang *et al.*, 2010). The requirement for Pygopus2 in cervical cancer was also assessed showing high hPygo2 levels in higher-grade lesions than in normal epithelial cells using a cervical cancer progression microarray. Pygopus2 protein and mRNA levels were significantly greater in HPV-positive

cervical cancer cells compared to uninfected cells confirming a relationship between E7 protein, retinoblastoma protein, Elf-1 factor and expression of hPygo2 (Tzenov *et al.*, 2013).

In prostate cancer, Dr. Kao's lab found elevated expression of hPygo2 in androgen-dependent and independent cell lines compared to normal epithelial control and as expected, hPygo2 knockdown resulted in reduction in growth and proliferation in cell lines. Furthermore, hPygo2 was expressed in most radical prostatectomy tissue samples assessed by immunohistochemistry (unpublished). Recent work has reported the role of hPygo2 in ribosome biogenesis during cancer cell growth, showing hPygo2 binding to known ribosome production proteins like treacle and UBF (upstream binding factor) in nucleoli of cancer cell lines and potentially recruiting histone acetyl transferases (HAT)s to rDNA promoter to increase transcription for cell growth and proliferation (Andrews *et al.*, 2013). Pygopus2 knockdown experiments resulted in growth arrest in both p53 positive and negative cell lines, down-regulation in rRNA production, and triggering of ribosomal stress pathway resulting in cell arrest at G1 phase in p53-positive cells (Andrews *et al.*, 2013).

1.1.8 Pygopus relationship to the ETS factor family

The ETS transcription factor family of proteins, activate or repress the expression of genes involved in myriad biological processes. ETS factors' function in cellular proliferation, differentiation, transformation and apoptosis have increased the interest to research their molecular mechanisms and how they control transcription (Seth and Watson, 2005). Their capability to be involved in these processes could give insight into controlling and understanding cancer development.

In breast, ovarian and cervical cancer hPygo2 was found to be activated by Elf-1 (E74-like-1) factor, an ETS family transcription factor regulated by the retinoblastoma protein (Rb) (Andrews *et al.*, 2008; Tzenov *et al.*, 2013). The following section discusses the ETS transcription factor family, their roles in cancer and their potential activation mechanism of hPygo2 in prostate cancer.

1.1.8.1 ETS family

The ETS family in humans consist of 27 highly evolutionarily conserved transcription factors that have critical roles in biological processes such as cell proliferation, differentiation, transformation and apoptosis *Table 1.1* (Hsu *et al.*, 2004). The discovery of this large family of conserved genes started with the identification of the v-ets oncogene (E26) of avian leukemia virus (Oikawa and Yamada, 2003; Hsu *et al.*, 2004). ETS proteins share a conserved winged helix-turn-helix DNA binding domain called the ETS domain, which binds to DNA sequences containing GGAA/T exclusively. Additionally, some of the ETS proteins also have a pointed domain (PNT) used in protein-protein interactions *Figure 1.6*. More than 200 ETS target genes have been identified and the number of verified genes that are positively or negatively regulated

by ETS transcription binding sites are constantly increasing (Sementchenko and Watson, 2000). ETS transcriptional regulation is dependent on interaction with other factors and it is known that translational modifications can affect the activity of different ETS proteins involved in compartmentalization, transactivation or stability (Seth and Watson, 2005).

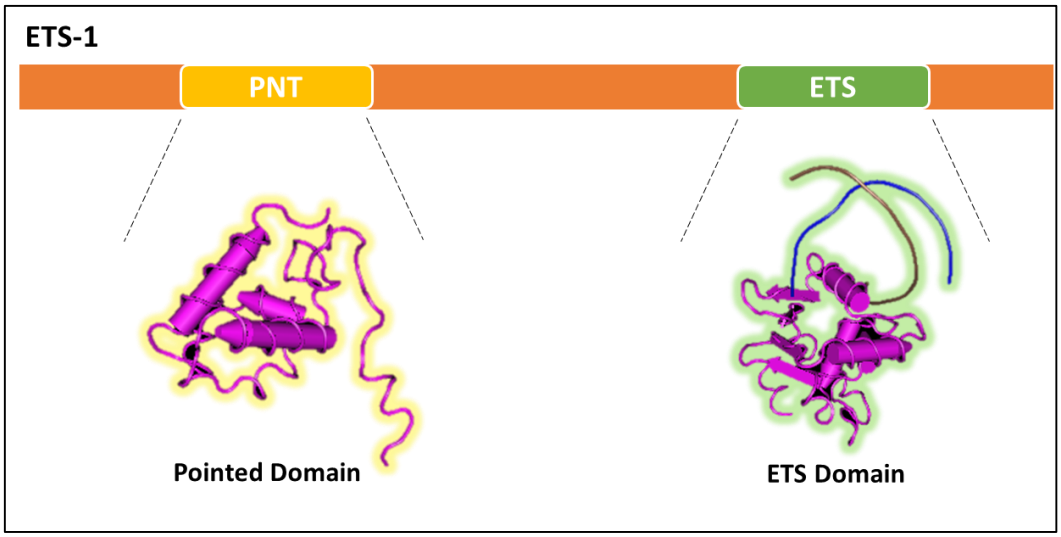

Figure 1. 4 – ETS-1 prototype member of ETS family, PNT and ETS Domains
Crystalized structures of domains obtained from MMDB public accessions (Mackereth *et al.*, 2004; Newman *et al.*, 2015)

Table 1. 1 - ETS subfamilies and members
Adapted from Gutierrez-Hartmann *et al.*, 2007

Subfamily	Mammalian family members
ELF	ELF1, ELF2, ELF4
ELG	GABPα
ERG	ERG, FLI1, FEV
ERF	ERF, ETV3
ESE	ELF3, ELF5, ESE3
ETS	ETS1, ETS2
PDEF	SPDEF
PEA3	ETV4, ETV5, ETV1
ER71	ETV2
SPI	SPI1, SPIB, SPIC
TCF	ELK1, ELK4, ELK3
TEL	ETV6, ETV7

1.1.8.2 ETS factors in carcinogenesis

ETS transcription factors have very important targets that include oncogenes, tumor suppressor genes, apoptosis and differentiation related genes, and not surprisingly, invasion and metastasis related genes. Consequently, aberrant expression of ETS factors can create a chain of changes that contributes to cancer initiation, progression and metastasis (Seth and Watson, 2005). The presence of the ETS sequence in the oncogenic virus E26 was the first time a connection was confirmed between ETS factors and cancer (Sementchenko and Watson, 2000; Seth and Watson, 2005). Different ETS factors are modified (deleted or mutated) and have abnormal expression patterns in leukemia, prostate cancer, breast cancer and sarcomas (Oikawa, 2004; Oikawa and Yamada, 2003). The following table summarizes examples of ETS family target genes involved in different processes of carcinogenesis *Table 1.2.*

Table 1. 2 - ETS family targets involved in carcinogenesis
Based on Hsu *et al.*, 2004; Seth and Watson, 2005; Oikawa and Yamada, 2003; Sementchenko and Watson, 2000

Carcinogenesis process	ETS family targets
Cell proliferation	CDK, Cyclin E1
Cell adhesion	VE-Cadherin, VE-Integrin, CAMs
Cell motility and migration	C-Met, Vimentin
Cell survival	Bcl-2
Cell invasion	uPA, uPAR, PAI, MMPs, TIMPs
Metastasis	Osteopontin, PTHrP, RANTES, CD44
Angiogenesis	Integrin b3, VEGF, Flt-1/KDR, Tie2

1.1.8.3 ETS factors in prostate cancer

Chromosomal rearrangements remain the principal studies involving ETS family genes in prostate cancer. Successful analyses of chromosomal rearrangements have elucidated important genes with aberrant expression able to have oncogenic properties in leukemia, lymphomas and sarcomas (Tomlins *et al.*, 2008; Shaikhibrahim and Wernert, 2012). These rearrangements occur when promoter or enhancer elements of one gene are juxtaposed to an oncogene causing an aberrant expression of the protein. In the case of ETS factors in prostate cancer, these rearrangements fuse two genes, resulting in a fusion protein that has a new or aberrant activity involved in cancer progression (Martin *et al.*, 2012; Turner, 2010) . As previously discussed, the main gene fusion involving ETS family in prostate cancer occurs with the prostate-specific serine protease TMPRSS2 gene and most commonly the ERG gene. Other family members have also been identified being fused to TMPRSS2 including ETV-1, ETV-4 and ETV-5 (Shaikhibrahim and Wernert, 2012). Since TMPRSS2 is an androgen-regulated protein, these rearrangements increase the expression of ETS members in response to androgens. As reviewed by Shaikhibrahim and Wernert, 2012; several studies suggest that ETS rearrangements are the

key for prostate cancer initiation but there is controversy indicating that they might aid cancer progression once initiation has already started. Overexpression of TMPRSS2-ERG in primary prostate cancer induced invasion but not proliferation while fusions with ETV-1 and ETV-4 increased invasion, growth and proliferation in prostate cancer cell lines (Turner, 2010; Oikawa and Yamada, 2003).

Several ETS genes have been studied for their properties in cancer progression and invasion. For example, the genes encoding ETS-1, FLI1, ERG, ELF-1 and PDEF are highly expressed in tumors while *ETV*-4 and *ELK*-1 are not expressed in prostate cancer tissues. Expression analysis of all the ETS family members in prostate cancer have been reported by quantitative RT-PCR using tissue from normal prostate glands and moderately differentiated tumor glands from radical prostatectomies, these analyses have reported that only 3 family members, *EHF*, *ELK-4* and *ETS-2* exhibit significant differences in expression between normal and tumor glands. In cell lines, qRT-PCR was performed to detect levels of expression of ETS family members in the androgen dependent cells VCaP and LNCaP, and the androgen independent cells PC3 and DU-145. Each cell line has a unique expression pattern of ETS family member without a clear correlation to their androgen status or aggressiveness (Shaikhibrahim *et al.*, 2011; Shaikhibrahim and Wernert, 2012). Investigation of the roles, expression and importance of ETS family members in prostate cancer is currently at an early stage since attention brought initially by the TMPRSS2-ERG fusion.

1.1.8.4 Pygopus and the Elf-1 factor

As mentioned in previous sections, high expression levels of hPygo2 have been reported in different cancer cell lines and tissues in breast, ovarian, glioblastomas and gliomas, cervical cancer, prostate and hepatic cancer (Andrews *et al.*, 2007; Popadiuk *et al.*, 2006; Tzenov *et al.*, 2013; Wang *et al.*, 2010; Chen *et al.*, 2011; Yang *et al.*, 2013; Zhang *et al.*, 2015). In prostate cancer, findings show a similar trend with elevated expression of hPygo2 in androgen-dependent and independent cell lines compared to normal epithelial control and reduction in growth and proliferation of cell lines when hPygo2 is eliminated by knockdown (Kao *et al.*, unpublished).

The mechanism of Pygopus2 regulation has not yet been elucidated in prostate cancer, however previous evidence in cervical and breast cancer indicated that hPygo2 gene expression is regulated by the Retinoblastoma protein (Rb) via Elf-1, an ETS factor involved in tumorigenesis in breast, ovarian and cervical carcinomas (Andrews *et al.*, 2008; Tzenov *et al.*, 2013). The retinoblastoma tumor suppressor mechanism regulates Elf-1 in a similar matter to E2F. When Rb protein is active, its pocket region interacts with the N-terminal LXCXE motif of Elf-1 factor when it is bound to the promoter blocking its transactivation activity but when Rb is phosphorylated by Cyclin D1-cdk4 complexes, the change in conformation de-represses Elf-1 at the promoter region being able to activate target gene expression, in this case of hPygo2 *Figure 1.7* (Wang *et al.*, 1993; Adnane *et al.*, 1995; Alt *et al.*, 2000; Andrews *et al.*, 2008). The previous mechanism is important due the fact that loss and aberrant expression of Rb is a key factor in prostate cancer progression which would allow Elf-1 to initiate hPygo2 transcription and therefore promote growth (Sharma *et al.*, 2010; Kao *et al.*, unpublished).

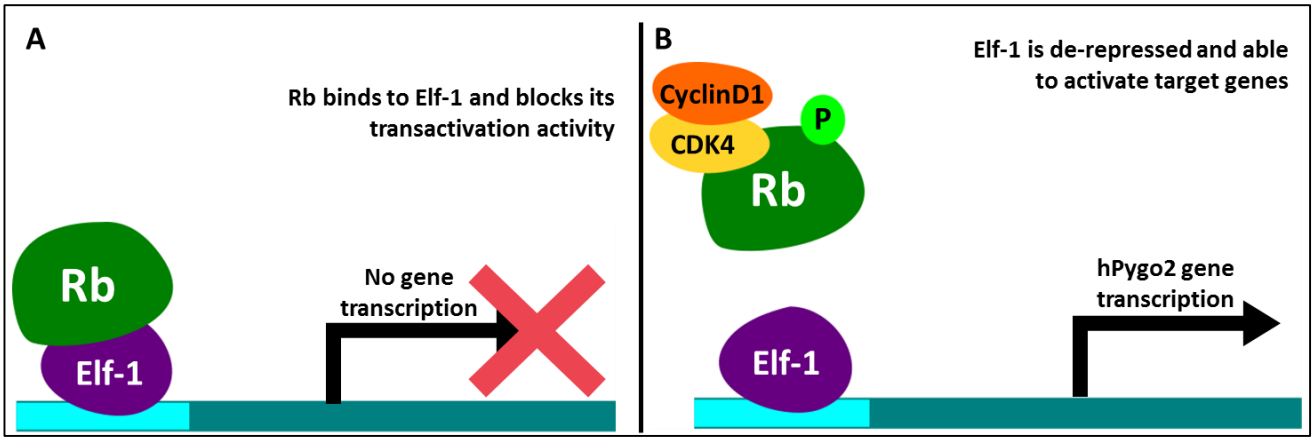

Figure 1. 5 - Pygopus expression is regulated by Elf-1 via Rb. A- Rb binds to Elf-1 blocking its activation of gene expression at the promoter region. B- CyclinD1 and CDK4 phosphorylate Rb, de-repressing Elf-1 at the promoter and starting gene transcription.

1.1.8.5 HPV infection and its relationship to pygopus and prostate cancer

Human papilloma virus (HPV) is a transmitted infection that has been associated with development of several epithelial cancers including cervical, anal, penile and vaginal cancers (Aghakhani *et al.*, 2011; Dillner *et al.*, 1998). In particular, HPV16 and HPV18 are known to cause around 70% of cervical cancer cases (Carozzi *et al.*, 2003). Since HPV-16 and 18 are transmitted sexually it has been hypothesized that HPV may also be a risk factor for prostate cancer. A large number of studies have tried to correlate this infection to the initiation and progression of prostate cancer, however, most of the results are subject to large controversy due to lack of clear correlations (Aghakhani *et al.*, 2011; Hisada *et al.*, 2000; Effert *et al.*, 1992; Al-Maghrabi, 2007).

In cervical cancer, Tzenov *et al.*, 2013 confirmed a mechanistic relationship between E7 protein, one of the primary oncoproteins of high risk HPV, and Elf-1 transactivation regulating hPygo2 protein. The findings reported that hPygo2 overexpression in cervical cancer is due to

E7 protein inducing the degradation of the Rb, consequently de-repressing Elf-1 and initiating hPygo2 gene transcription *Figure 1.8* (Tzenov *et al.*, 2013). While the presence of HPV 16 and 18 has been assessed before in different prostate cancer cell lines and tissues, the presence of E7 protein acting upon Rb protein and initiating Elf-1 activation of target genes has not been elucidated in prostate cancer (Tu *et al.*, 1994; Moyret- Lalle and Marcais, 1995; Dillner *et al.*, 1998; Adami *et al.*, 2003; Noda *et al.*, 1998).

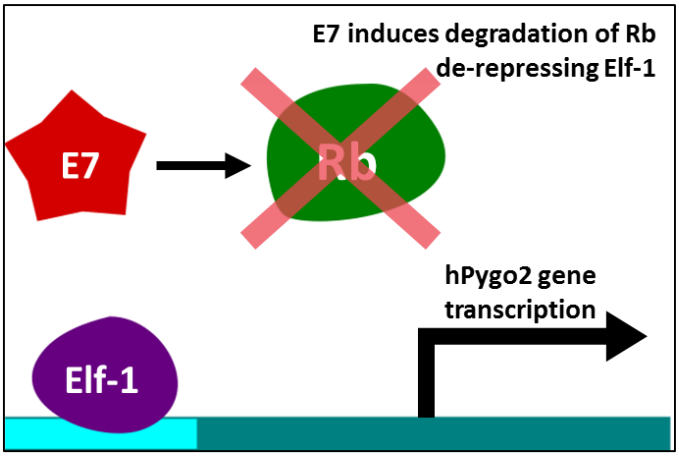

Figure 1. 6 - E7 induces degradation of Rb, de-repressing Elf-1 and activating hPygo2 gene transcription in cervical cancer.

1.1.9 Rationale

The mechanisms regulating hPygo2 gene activation are unknown in prostate cancer. Previous findings have suggested hPygo2 has an important role in prostate cancer and is required for cancer cell growth and proliferation (Andrews *et al.*, 2007; Popadiuk *et al.*, 2006; Tzenov *et al.*, 2013; Wang *et al.*, 2010; Chen *et al.*, 2011; Yang *et al.*, 2013; Zhang *et al.*, 2015). In breast and cervical carcinomas, hPygo2 regulation by the ETS factor Elf-1 depends on degradation of Rb protein and since loss and aberrant expression of Rb is a key factor in prostate cancer progression it could potentially have a similar mechanism (Shen and Abate-Shen, 2010; Andrews *et al.*, 2007; Tzenov *et al.*, 2013) . Moreover, numerous findings have reported increased expression of several ETS factors and their potential roles in cancer progression (Seth and Watson, 2005).

This thesis will address the mechanism of expression of hPygo2 in prostate cancer. It will evaluate the hypothesis: ***hPygo2 expression is regulated via Elf-1 ETS factor bound to its promoter region.***

1.1.10 Objectives

- Assess the presence of HPV 16 and HPV 18 E7 proteins in prostate cancer cell lines and possible role on hPygo2 regulation.
- Determine the levels of hPygo2, Elf-1 and additional ETS factors at a protein level in different prostate cancer cell lines.
- Assess the binding capability of Elf-1 and additional ETS factors to hPygo2 proximal promoter region.
- Evaluate the effect on hPygo2 expression by performing a knockdown of the Elf-1 factor in prostate cancer cell lines.

Chapter II – Materials and Methods

2.1 Cell culture

The following five characterized prostate cancer cell lines, 22Rv1 (Sramkoski *et al.*, 1999), LNCaP (Horoszewicz *et al.*, 1983), DU 145 (Stone *et al.*, 1978), MDA PCa 2b (Navone *et al.*, 1997) and PC-3 (Kaighn *et al.*, 1979) were obtained from the American Tissue Culture Collection (ATCC; CRL-2505, CRL-1740, HTB-81, CRL-2422 and CRL-1435 respectively, Virginia, USA). 22Rv1 and LNCaP were cultured in Roswell Park Memorial Institute 1640 Medium (RPMI 1640; Gibco Life Technologies; California, USA) while DU 145, MDA PCa 2b and PC-3 were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco Life Technologies; California, USA), 10% Fetal Bovine Serum (FBS; Gibco Life Technologies California, USA) was added to complement both media. Passage ratio was 1:3 and cells were passaged approximately every 3 days. Normal prostate epithelial cell line, PrEC (Sobel *et al.*, 2005) was obtained from Lonza (Lonza Group Ltd; CC-2555; Basel, Switzerland) and grown in their specific Prostate Epithelial Cell Growth Medium (Clonetics PrEGM; Lonza Group Ltd; Basel, Switzerland). Source, androgen status and PSA presence has been previously confirmed for all of the prostate cell lines *Table 2.1*.

Table 2. 1 - Prostate cell lines, their source, androgen sensitivity, androgen receptor and PSA status

Cell Line	Source	Androgen Sensitivity	Androgen Receptor	PSA	Reference
PrEC	Prostate Epithelial Cells	Androgen Sensitive	Positive	Positive	(Sobel *et al.*, 2005)
22Rv1	Prostate Cancer Cells from Xenograft line	Androgen Sensitive	Positive	Positive	(Sramkoski *et al.*, 1999)
LNCaP	Lymph Node Metastasis (Caucasian Male)	Androgen Sensitive	Positive - Mutated	Positive	(Horoszewicz *et al.*, 1983)
DU 145	Central Nervous System Metastasis (Caucasian Male)	Androgen Independent	Negative	Negative	(Stone *et al.*, 1978)
MDA Pca 2b	Bone metastasis (African-American Male)	Androgen Sensitive	Positive	Positive	(Navone *et al.*, 1997)
PC-3	Lumbar Metastasis (Caucasian Male)	Androgen Independent	Negative	Negative	(Kaighn *et al.*, 1979)

Additionally, normal human endocervical primary cell line HEN and the following immortalized cancer cell lines *in vitro* by HPV 16 and 18, HEN 16 and HEC 18 (Donations by Dr. Pater's Laboratory, Memorial University) (Yang *et al.*, 1996; Tsutsumi *et al.*, 1992) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco Life Technologies; California, USA). For HEN primary cells, 50 µg/ml bovine pituitary extract (BPE; Gibco Life Technologies; California, USA) and 5 ng/ml epidermal growth factor (EGF; Gibco Life Techonologies; California, USA) were added to complement the media. For HEN 16 and HEC 18 cell lines, 10% Fetal Bovine Serum (FBS; Gibco Life Technologies; California, USA) was added to DMEM media.

2.2 RNA extraction and cDNA generation

Total RNA extraction was performed using the RNeasy Mini Kit (Qiagen; C#74104; Hilden, Germany) on 6-well multiple well plates (Corning Inc.; New York, USA) with approximately 1 x 10^6 cells per well at the time of extraction following manufacturer's instructions. Synthesis of complementary DNA was implemented by following Invitrogen's M-MLV Reverse Transcriptase protocol with a total final volume of 20 µl per sample. Procedure started with incubation of 1 µg of extracted RNA, 0.2 µg random primers (oligo-dT; Invitrogen; California, USA), 0.5 mM dNTP and dH2O at 65 °C for 5 min followed by incubation of First Strand Buffer (FS Buffer, Invitrogen; California, USA), 10mM DTT and 10 units of Recombinant Ribonuclease Inhibitor (RNase Out, Invitrogen; California, USA) for 2 min at 37°C. Lastly, 50 units of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen; California, USA) were added to samples and incubated at 25°C for 10 min, 37°C for 50 min and 70°C for 15 min, using the GeneAmp PCR System 9700 (Applied Biosystems; California, USA) (Tzenov *et al.*, 2013; Chang *et al.*, 2010).

2.3 Reverse transcription PCR (RT-PCR) analysis and Real time Quantitative PCR

Each PCR reaction was prepared by adding 1X PCR buffer-MgCl, 5 mM MgCl, 200 µM dNTP, 1 unit of Platinum Taq Polymerase (Invitrogen; California, USA), dH2O, 2 µl of each primer (Final concentration of 0.8 M) and 1 µg of the respective cDNA for a final volume of 50 µl. Primer sequences are listed in *Table 2.2* (Dharmacon; Colorado, USA). Each PCR reaction with their specific primers was subjected to the following conditions as described in *Lake and Kao, 2003:* 94°C for 4 min followed by 35 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec finalizing with 7 min at 72°C, using a GeneAmp PCR System 9700 (Applied

Biosystems; California, USA). The PCR products were run in 2% agarose gel electrophoresis, stained with SYBR Safe Gel DNA Stain (SYBR Safe; Invitrogen; California, USA) and analyzed with Gel Doc EZ Imager (Biorad; California, USA). RT-PCR analysis was performed to detect presence of HPV 16 and 18 E7 protein in cervical cell lines and prostate cancer cell lines.

Real time quantitative PCR (RT-qPCR) was performed to measure levels of Elf-1 and hPygo2 in Elf-1 siRNA treated samples and non-targeting siRNA control samples using the Step One Plus Real time PCR system and software (Applied Biosystems; California, USA). cDNA samples were amplified using RT2 SYBR green master mix (Qiagen; Hilden, Germany) and 1.25 µM of the primers listed in *Table 2.3* using the standard 2-hour long run in the system (Andrews *et al.*, 2008). Data was analyzed using the relative quantitative comparative threshold cycle (ΔΔCt) method using β-actin as control and one-way repeated measures ANOVA followed by an Fisher's LSD statistical analysis for multiple comparisons ($p<0.05$ for significance) using Prism 6 software (Graphpad Prism, California, USA).

Table 2. 2 - Primer sequences used in RT-PCR analyses

	Forward Primer (5'-3')	Reverse Primer (5'-3')	Reference
E7 FOR HPV16	TCC AGC TGG ACA AGC AGA AC	CAC ACC CGA AGC GTA GAG TC	(Tzenov *et al.* , 2013)
E7 FOR HPV18	AAC ATT TAC CAG CCC GAC GA	TCG TCT GCT GAG CTT TCT AC	(Tzenov *et al.* , 2013)
ELF-1	CCA GTC ACC CAT GTG TCC GTC AC	CAC AGA TAT ATT TGG CGT AGT GGC TG	(Tzenov *et al.* , 2013)
β-ACTIN	ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG	ATG GCT GGG GTG TTG AAG GTC TC	Invitrogen Control (Andrews *et al.* , 2008)

Table 2. 3 - Primer sequences used in Real Time qPCR

	Forward Primer (5'-3')	Reverse Primer (5'-3')	Reference
ELF-1	CCA GTC ACC CAT GTG TCC GTC AC	CAC AGA TAT ATT TGG CGT AGT GGC TG	(Tzenov *et al.* , 2013)
HPYGO2	GTC CCC CAC TCC ATG GCC GCC TCG	GCT TCT TTT CTG GAC TCT TC	(Andrews et al., 2008)
β-ACTIN	ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG	ATG GCT GGG GTG TTG AAG GTC TC	Invitrogen Control (Andrews et al., 2008)

2.4 Protein extraction, SDS Page and immunoblotting

Protein was extracted from 80-85% confluent plates in radioimmunoprecipitation assay buffer (RIPA buffer, .01% SDS; 1.1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris-HCl pH 8.1; 167mM NaCl) adding 1 µM PMSF and protease inhibitors (Thermo Fisher Scientific; Massachusetts, USA). Samples were incubated at 4°C for 30 min, homogenized by resuspension, centrifuged for 10 min at 4°C, and then collected.

Total protein quantities were normalized by Bio-Rad protein assay (Bio-Rad Laboratories Inc.; California, USA) with a standard curve generated using BSA as reference. Equivalent protein sample of 25µg were loaded onto 10% SDS-denaturing polyacrylamide gels or 4-20% Mini-PROTEAN gels (Mini-PROTEAN TGX; Bio-Rad Laboratories Inc.; California, USA) for knockdown experiments, and subsequently transferred to polyvinylidene difluoride membrane (PVDF Trans-Blot® Turbo™ membrane, Bio-Rad Laboratories Inc.; California, USA) using the Western Blot Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories Inc.; California, USA). After transfer, membranes were blocked for 1 hour with 5% Skim Milk in Tris-Buffered Saline and Tween 20 solution (TBST), later to be incubated overnight at 4°C with their specific

antibodies and appropriate dilution *Table 2.4.* TBST washes were performed before incubating for 1 hour at room temperature with the respective secondary antibody, Donkey anti-rabbit IgG-HRP or goat anti-mouse IgG-HRP (Abcam; Cambridge, UK), and then washed again. Immunodetection was achieved using Clarity™ ECL Western Blotting Substrate (Bio-Rad Laboratories Inc.; California, USA) and CL-Xposure™ Film (Thermo Fisher Scientific; California, USA). Densitometry analysis was performed using scanned film at an 8-bit conversion using peak gel analysis tool of ImageJ software. Peak area and percentage of each band was divided by their respective loading control band in order to calculate a particular relative density. (Popadiuk *et al.*, 2006)

Table 2. 4 - Antibodies, source and dilution used in protein immunoblots.

Antibodies	Source	Dilution
hPygo2	Rabbit Polyclonal generated in Laboratory (Andrews et al., unpublished)	1:5000
AR	Rabbit Polyclonal (Santa Cruz Biotechnology, sc-816)	1:1000
Rb	Rabbit Polyclonal (Active Motif, 61585)	1:2500
pRb	Rabbit Monoclonal (Cell Signaling, D20B12)	1:5000
Elf-1	Rabbit Polyclonal (Santa Cruz Biotechnology, sc-631)	1:1000
Erg-1	Rabbit Polyclonal (Santa Cruz Biotechnology, sc-354)	1:5000
Ets-1	Rabbit Polyclonal (Santa Cruz Biotechnology, sc-111)	1:5000
Ets-1	Rabbit Polyclonal (Santa Cruz Biotechnology, sc-350)	1:1000
Elk-1	Rabbit Polyclonal (Santa Cruz Biotechnology, sc-355)	1:5000
Erk-1	Rabbit Polyconal (Santa Cruz Biotechnology, sc-94)	1:1000
B-actin	Mouse Monoclonal (Sigma-Aldrich, A5316)	1:5000

2.5 Chromatin immunoprecipitation

22Rv1, DU-145 and PC-3 cells were seeded in 100 mm dishes (Corning Inc.; New York, USA) and grown to 80-85% confluence. Cross-links between protein and DNA were achieved by incubating for 10 min with 0.75% formaldehyde, followed by 125mM glycine to quench the process. After, cells were washed with PBS, harvested and resuspended in lysis buffer (50 mM Tris-HCl (pH 8.0); 150 mM NaCl; 1 mM EDTA; 1% Triton X-100; 0.1% Na-deoxycholate; dH2O) containing protease inhibitors. Cross-linked cells were sonicated to produce 500 bp genomic DNA fragments using sonicator at 15% power repeating 7 times the following: 10 seconds pulse and 20 seconds pause; for a total of 1:10 min treatment.

Collected chromatin was pre-cleared with preblocked protein A beads (50% slurry, Millipore; Massachusetts, USA) at 4° C for 1 hour. Supernatants were incubated overnight at 4 °C with 2 µg of their respective antibodies followed by addition of 20 µl protein A agarose beads and incubated for 1 hour *Table 2.5*. The pellets extracted were then washed for 5 min twice with 1 ml of each of the following buffers, low salt immune complex wash buffer, high salt immune complex wash buffer, LiCl immune complex wash buffer, and TE buffer *Table 2.6.*

Protein-DNA complexes were eluted in 180 µl of elution buffer (1.0% SDS; 100 mM $NaHCO_{3;}$ dH2O) for 30 min and cross-links were reversed overnight at 65°C. DNA was purified using QIAquick PCR purification kit (Qiagen; Hilden, Germany) and eluted in 30 µl dH2O. All samples were amplified by real-time quantitative PCR using RT2 SYBR green master mix (Qiagen; Hilden, Germany) with hPygo2 proximal promoter region primers (hPygo2 -102u: 5'-CAG GCG TAG CGT CTC GTC CGG TC-3', hPygo2 +74d: 5'-CCG AGC TGC AGC AAC CAC AAA GTG-3'). Analysis was performed using the relative quantitative comparative threshold cycle (ΔΔCt) method against input chromatin levels and one way ANOVA analysis for

multiple comparisons against IgG followed by an uncorrected Fisher's LSD statistical analysis ($p<0.05$ for significance) using Prism 6 software (*Appendix A2)* (Graphpad Prism, California, USA) (Matthews *et al.*, 2006; Andrews *et al.*, 2008; Tzenov *et al.*, 2013).

Table 2. 5 - Antibodies used for chromatin immunoprecipitation assay

Antibodies	Source
IgG	Rabbit Polyclonal (Millipore, 12-370)
Elf-1	Rabbit Polyclonal (Santa Cruz Biotechnology, sc-631)
Erg-1	Rabbit Polyclonal (Santa Cruz Biotechnology, sc-354)
Ets-1	Rabbit Polyclonal (Santa Cruz Biotechnology, sc-111)
Elk-1	Rabbit Polyclonal (Santa Cruz Biotechnology, sc-355)

Table 2. 6 - Chromatin Immunoprecipitation buffers and formulations

ChIP Buffers	Formulations
Lysis Buffer	1% SDS; 10 mM EDTA; 50 mM Tris-HCl pH 8.1; dH2O
ChIP Dilution Buffer	.01% SDS; 1.1% Triton X-100; 1.2 mM EDTA; 16.7 mM Tris-HCl pH 8.1; 167 mM NaCl; dH2O
Low Salt Immune Complex Wash Buffer	0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl pH 8.1; 150 mM NaCl; dH2O
High Salt Immune Complex Wash Buffer	0.1% SDS; 1% Triton X-100; 2 mM EDTA; 20 mM Tris-HCl pH 8.1; 500 mM NaCl; dH2O
LiCl Immune Complex Wash Buffer	250 mM LiCl; 1.0% IGEPAL-CA630; 1% Deoxycholic acid; 1 mM EDTA; 10 mM Tris-HCl pH 8.1; dH2O
TE Buffer	10 mM Tris-HCl pH 8.1; 1 mM EDTA; dH2O

2.6 Antisense Knockdowns

Small interfering RNA's (SiRNA) were used to perform knockdown experiments for ELF-1 (ELF-1 A, Santa Cruz Biotechnology and ELF-1 B designed by Andrews *et al.*, 2008, target Sequence: 5' GAAAGAGAA-CACTGAGAAA, GE Dharmacon Healthcare Inc.; Colorado, USA) proteins, using siGENOME Non-Targeting siRNA Pool as a control (Dharmacon Healthcare Inc.; Colorado, USA). 22Rv1 and PC-3 cells were seeded in 6-well plates, seeding $1x10^5$ cells per well, 24 hours before transfection. Transfection was achieved

using Lipofectamine® RNAiMAX (Invitrogen; California, USA) using different concentration of duplexes (5nM, 10nM and 20nM) following manufacturer's instructions and collecting cells after 24 hours of transfection for RNA and 48 hours for protein extraction. Western blots were performed to confirm the protein knockdowns and changes to hPygo2, pRb and Rb proteins. RNA samples were used to generate cDNA as previously described and analyzed by qRT-PCR.

Chapter III – Results

3.1 Human papilloma virus E7 protein was not detectable in prostate cancer cell lines

The presence of E7 protein mRNA expressed by HPV16 and 18 in prostate cancer cell lines was assessed by RT-PCR using a normal endocervical primary cell line HEN as negative control, cancer cell lines harboring HPV 16 and 18, HEN 16 and HEC 18 as positive controls and the following prostate cancer cell lines: 22Rv1, PC-3 and DU-145. The presence of E7 mRNA was then assessed using E7 primers for HPV16 and E7 primers for HPV18 along with β-actin primers. In cervical cancer, E7 protein induces the degradation of the retinoblastoma tumor suppressor protein (Rb) releasing and allowing the transactivation of Elf-1 protein for hPygo2 transcription (Tzenov et al., 2013). The presence of E7 protein in prostate cancer cell lines could suggest a similar mechanism in the requirement of hPygo2 for growth in prostate cancer. Results indicate that E7 HPV16 or E7 HPV18 appears to not be expressed in prostate cancer cell lines compared to the respective cancer endocervical cell lines suggesting that HPV infection is not contributing to the proposed mechanism *Figure 3.1*.

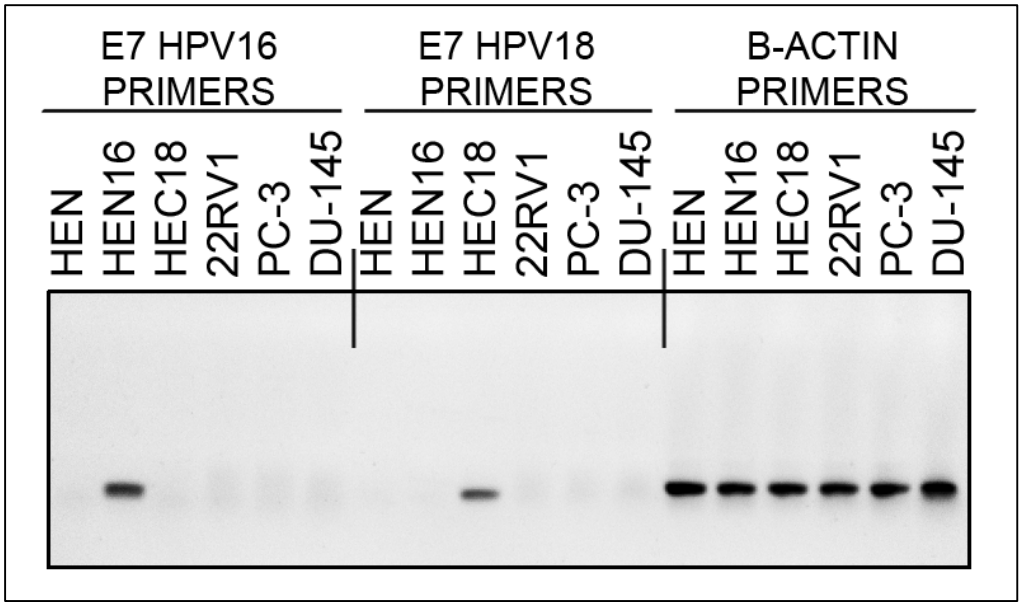

Figure 3. 1 - RT-PCR for the detection of E7 protein in prostate cancer cell lines

RT-PCR was performed on the normal endocervical cell line HEN, endocervical cancer cell lines HEN 16 and HEC 18 and prostate cancer cell lines 22Rv1, PC-3 and DU-145. β-actin was used as a loading control. Presence of E7 protein was only detectable in HEN 16 and HEC 18 for their respective primers. No amplification of E7 protein in prostate cancer cell lines was detectable.

3.2 Differential expression of Elf-1, hPygo2 and additional ETS factors in different prostate cancer cell lines

Previous findings in breast, ovarian and cervical cancer cell lines have supported the mechanistic relationship between Rb, which regulates the activation of hPygo2 gene expression via Elf-1 activity (Andrews *et al.*, 2009, 2008; Tzenov *et al.*, 2013). On that premise, the presence and protein levels of androgen receptor, phosphorylated Rb (pRb) (inactive), hPygo2 and, ETS factors Elf-1, Erg-1, Ets-1 and Elk-1 were assessed by immunoblot. Total Erk antibody was used as a loading control. Cell lines used were the normal prostate epithelial cell line PrEC, androgen receptor positive prostate cancer cell lines: 22Rv1, LNCaP and MDA PCa 2b, and

androgen receptor negative prostate cancer cell lines: DU-145 and PC-3. Androgen receptor status was concordant with the known status (*Table 2.1*) for each cell line.

hPygo2 protein levels varied between prostate cancer cell lines but show differences from the normal PrEC cell line, lowest expression in the prostate cancer cell lines is shown in LNCaP and MDA PCa 2b. pRb protein levels correlated with hPygo2 and Elf-1 expression only in 22Rv1 and PC-3 cell lines, while in the rest of the cell lines it was present at lower levels. Elf-1 antibody detected different transcript variants in the prostate cancer cell lines, there is evidence of five different Elf-1 variants with protein activity (Okada *et al.*, 2011; Gerloff *et al.*, 2011; Yang *et al.*, 2013). Presence of Rb levels were detected in all of the cell lines except for very low on DU-145, known to have a mutated Rb protein status (Ikediobi *et al.*, 2006). Interestingly, this last cell line has a high expression of hPygo2 protein levels. For the remaining ETS factors, Erg-1 was expressed in all of the cell lines while Elk-1 was highly expressed in all of the cell lines including normal epithelium. Interestingly, Ets-1, the prototype member of the ETS family, was expressed highly in 22Rv1 and DU-145, cell lines in which hPygo2 levels were also higher. These protein levels confirm the presence of hPygo2 in different prostate cancer cell lines regardless of their androgen status and the status of Elf-1 and additional ETS factors. *Figure 3.2*.

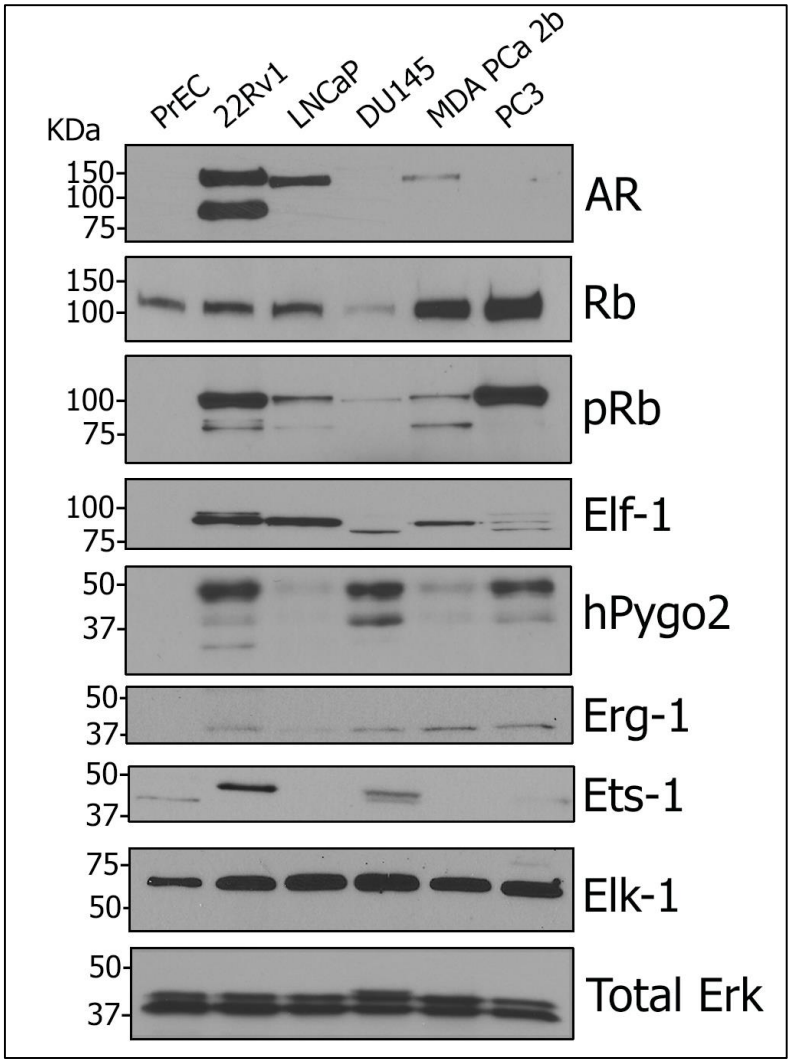

Figure 3. 2 - Protein expression levels of hPygo2, Elf-1 and ETS factors

Protein expression levels of hPygo2, pRb, AR, Elf-1, Erg-1, Ets-1, Elk-1 and Erk-1 as loading control were detected by immunoblot. Androgen receptor status is concordant with the known status for each cell line. hPygo2 expression is present at different levels in all of the prostate cancer cell lines and not on normal prostate epithelium cell line PrEC. Phosphorylation of the retinoblastoma protein is present at different levels in all of the prostate cancer cell lines but not on PrEC. Rb expression is present on all the cell lines except for DU-145 with a very low expression, concordant to its Rb mutated status. Erg-1 showed low protein levels in all of the cell lines while Elk-1 showed high expression in all of the cell lines. Ets-1 shows high expression in 22Rv1 and DU-145, cell lines that also show a high expression of hPygo2 levels.

3.3 Elf-1 and Ets-1 factors bind to the proximal promoter region of hPygo2 gene in 22Rv1 and PC-3 prostate cancer cell lines

Chromatin immunoprecipitation assays were performed in 22Rv1, Du-145 and PC-3 cell lines in order to assess the binding capability of ETS factors to the proximal region of hPygo2 promoter. The following cell lines were chosen because 22Rv1 shows high protein expression of hPygo2 and ETS factors, DU-145 shows high expression of hPygo2 but mutated Rb (which might give an insight of a different mechanism) and PC-3 has high protein expression of hPygo2. The ETS factors tested were Elf-1, Erg-1, Ets-1 and Elk-1. IgG was used as a negative control to compare against occupancy of ETS factors at the proximal promoter region (-102u to +74d). Immunoprecipitation, purification, real time q-PCR and analysis by relative quantitative comparative threshold cycle (ΔΔCT) was performed. Percentage of input values were normalized to IgG and statistical analysis was performed using a repeated measures ANOVA and Fisher's LSD statistical analysis for multiple comparisons to obtain significance against IgG binding capacity to the specific region ($p<0.05$). In 22Rv1 cells, higher levels of binding of Elf-1 and Ets-1 to the proximal promoter region of hPygo2 are significantly higher compared to IgG occupancy. In PC-3 cells, Elf-1 and Ets-1 also show higher levels of occupancy at the proximal promoter region but again only for Elf-1 this association is significant. In DU-145 cells, high levels of binding for all the ETS factors are seen but only Ets-1 and Elk-1 appear to be significant compared to IgG. As stated previously, the binding capability of Elf-1 could suggest activity at proximal regions of hPygo2 promoter and therefore possible induction of pygopus gene expression *Figure 3.3.*

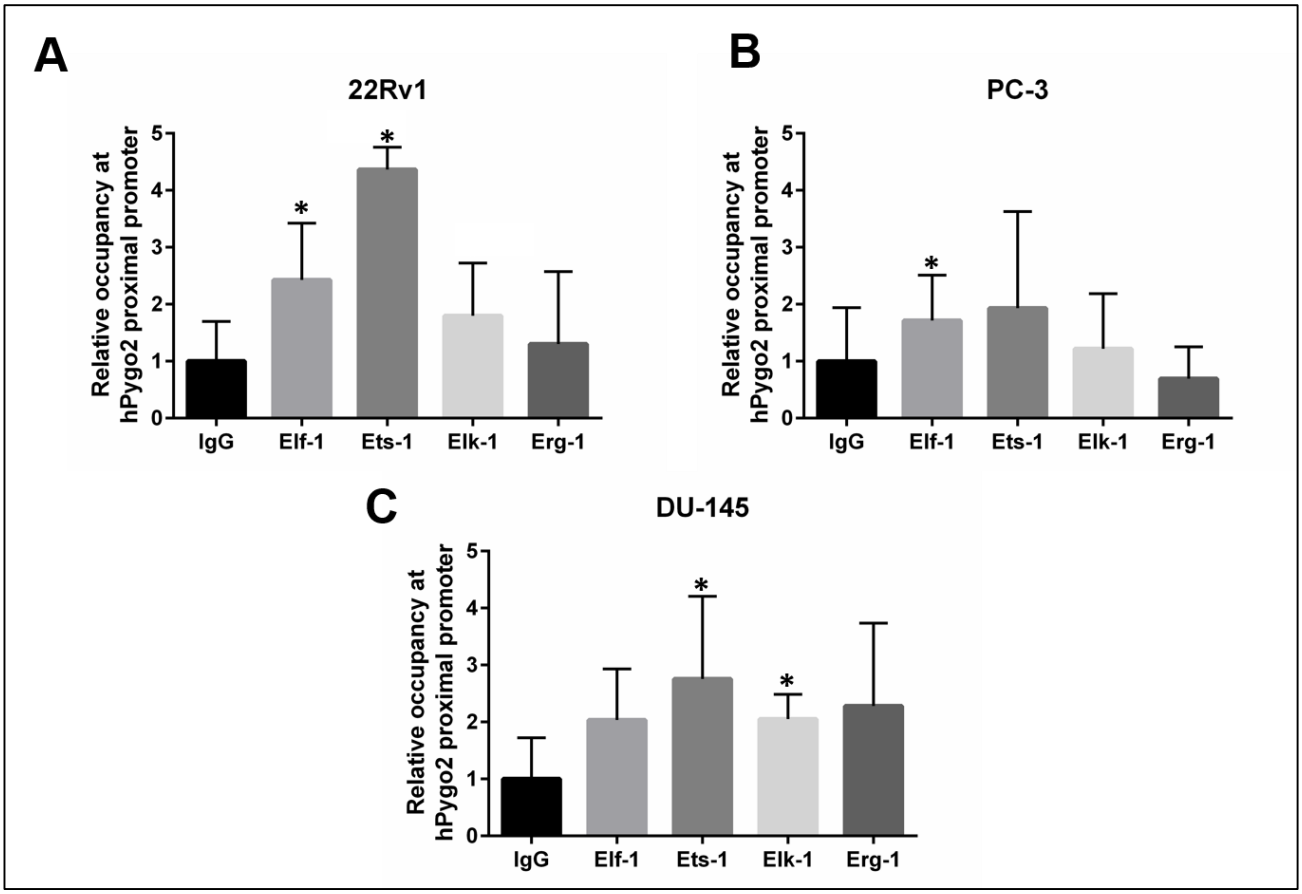

Figure 3. 3 - Detection of ETS factors binding to the proximal promoter region of hPygo2 by chromatin immunoprecipitation

Chromatin Immunoprecipitations amplifying for the proximal promoter region from -102u to +74d of hPygo2 were performed in 22Rv1, PC-3 and DU-145 cell lines in order to assess the binding capability of Elf-1, Ets-1, Elk-1 and Erg-1 factors against IgG. Analysis by relative quantitative comparative threshold cycle (ΔΔCT) was performed, results were normalized and compared to the binding capability of IgG using comparative measures ANOVA and Fisher's LSD statistical test ($p<0.05$). IgG normalization takes into account control variance and data input correlates each data set to their specific IgG mean.

3.4 Elf-1 knockdown reduces mRNA levels but not protein levels of hPygo2 protein

Elf-1 silencing experiments were performed in order to assess its effect on hPygo2 protein levels. Knockdown of Elf-1 was achieved using 2 different Elf-1 siRNA duplexes (Elf-1 siRNA A and B) in different concentrations and a non-targeting siRNA duplex used as control using the 22Rv1 cell line *Figure 3.4 – A*. Elf-1 knockdown was performed using the lowest functional concentration of 5nM of the siRNA-A duplex. Elf-1 expression, change in hPygo2 expression, and pRb were assessed by immunoblot where protein was collected at 24 hours, 48 hours and 72 hours after transfection. Using siRNA-A, there was no noticeable detectable change in hPygo2 protein expression after 24 hour and 48 hours after transfection. However, at 72 hours the non-targeting siRNA could possibly have an off target effect on pRb resulting in unexpected high expression of Elf-1 and hPygo2. Interestingly, at 72 hours using Elf-1 siRNA, hPygo2 expression is lower compared to the 72 hour non-targeting control *Figure 3.4 – B & C*.

Relative Elf-1 and hPygo2 mRNA expression in the 22Rv1 cell line for the Elf-1 siRNA-A and non-targeting siRNA treated samples were analyzed using real time qPCR. Results show reduction in levels of Elf-1 in knockdown samples compared to the samples treated with a non-targeting siRNA. Pygopus mRNA levels are significantly lower in Elf-1 siRNA-A treated samples ($p<0.05$) *Figure 3.5*.

Subsequently and due to possible non-targeting siRNA effects on the previous cell line, another Elf -1 knockdown was performed using the same two siRNA Elf-1 duplexes in PC-3 cell line. Results show Elf-1 silencing was successful but there is no significant change in hPygo2 protein expression *Figure 3.6 – A & B***.** These findings suggest that, unlike breast and cervical cancer, Elf-1 is not exclusively involved in the activation of hPygo2 expression in prostate cancer and the analysis of other transcription factors or mechanisms should be evaluated.

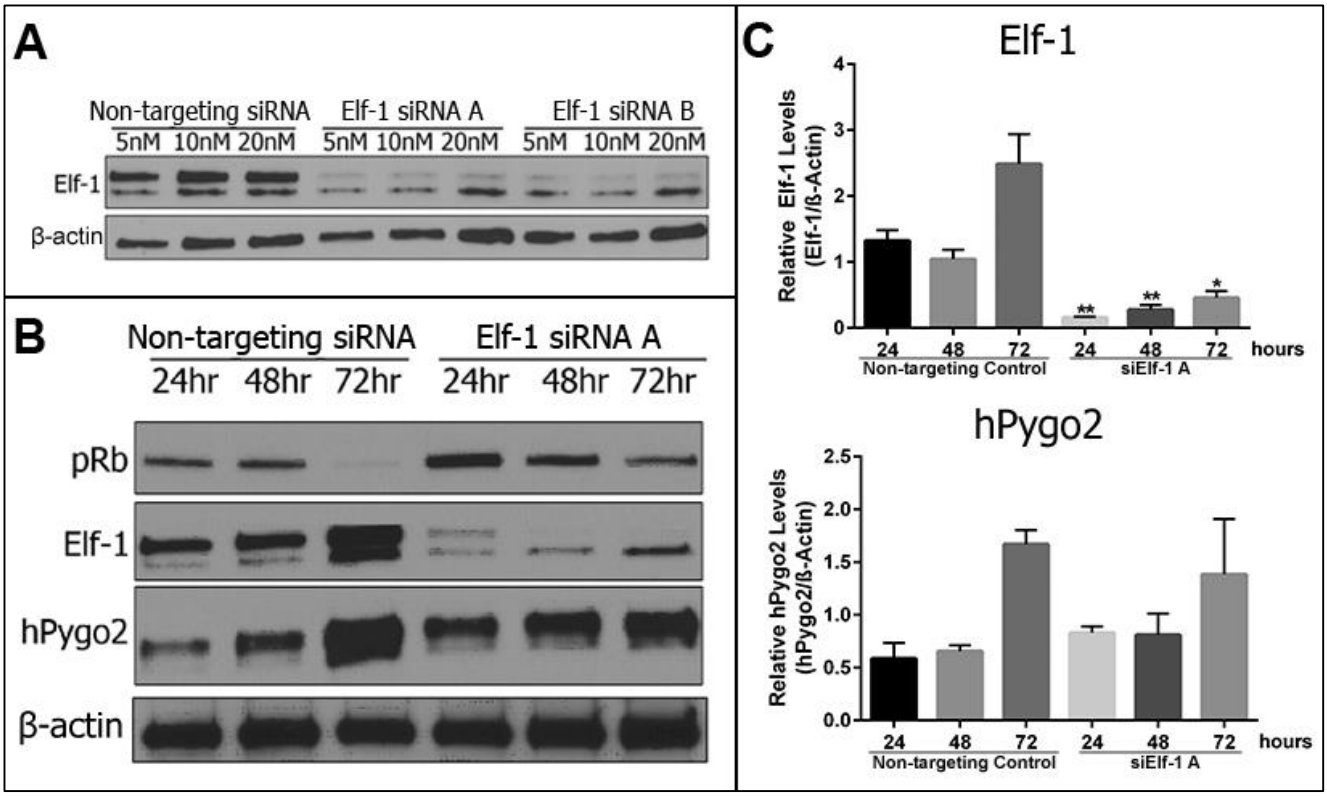

Figure 3. 4 - Effect of silencing Elf-1 in hPygo2 protein expression using 22Rv1 cell line

A- Elf-1 knockdown in a 22Rv1 cell line was performed using two different siRNA duplexes against a non-targeting siRNA duplex in different concentrations (5nM, 10nM, 20nM and 40nM) and protein levels were analyzed by immunoblot. Even a 5nM concentration shows considerably lower Elf-1 protein expression in both cases. B- Elf-1 knockdown was performed using 5nM concentration of the first Elf-1 siRNA duplex, protein was collected at 24, 48 and 72 hours. Levels of hPygo2 protein expression were not reduced by Elf-1 silencing. C- Densitometry showing Levels of Elf-1 and hPygo2. Elf-1 expression is lower on Elf-1 siRNA A treated samples. However, there is only an evident change on 72 hour hPygo2 expression of siRNA treated against non-targeting control possibly due to an off target effect of the non-targeting control siRNA. No significant change was obtained by comparative measures ANOVA and Fisher's LSD statistical test ($p<0.05$).

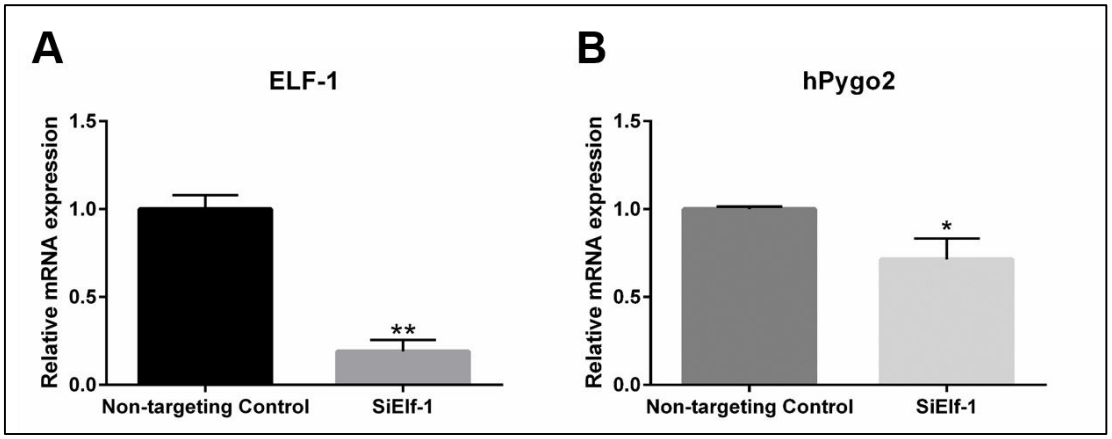

Figure 3. 5 - Pygopus mRNA expression in 22Rv1 cell line treated with Elf-1 siRNA A

RT-qPCR analysis shows a reduction in Elf-1 in Elf-1 siRNA A treated samples compared to non-targeting samples, significant reduction of hPygo2 in knockdown samples is shown. Ct values were normalized to β-actin as control. Significance was obtained by comparative measures ANOVA and Fisher's LSD statistical test ($p<0.05$).

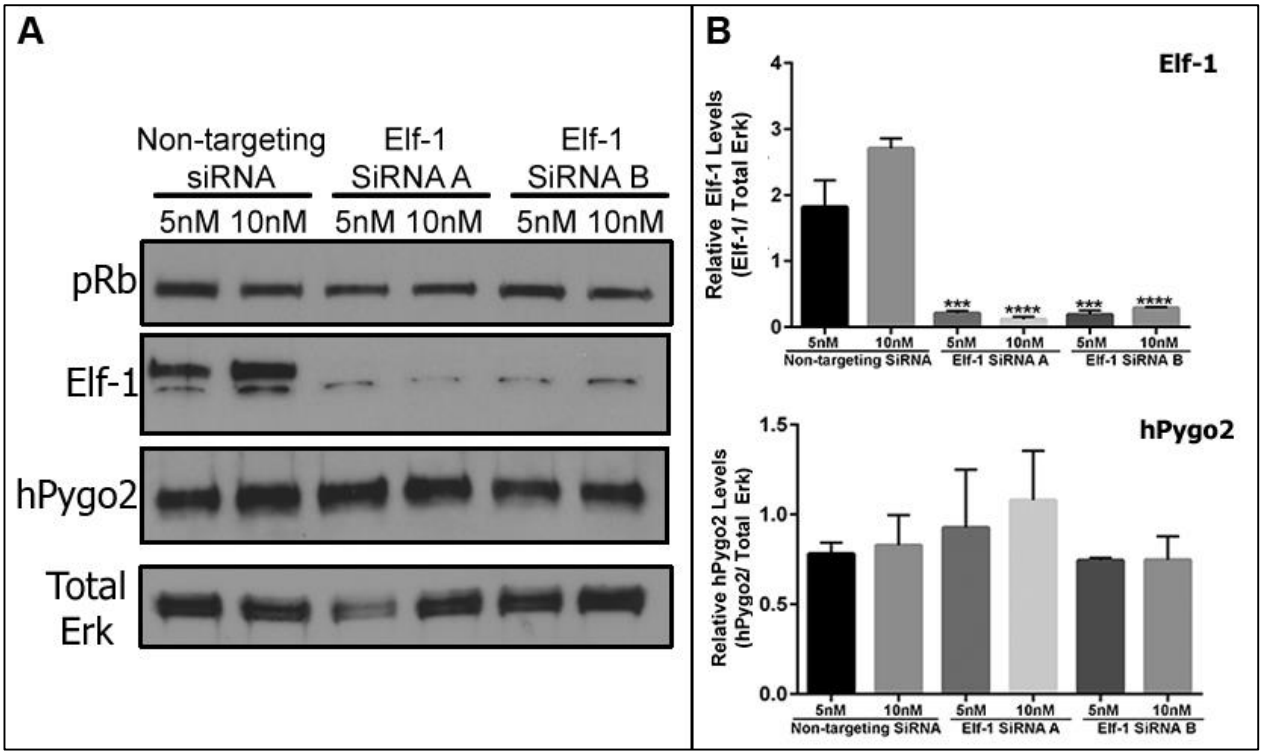

Figure 3. 6 - Effect of silencing Elf-1 in pygopus protein expression using PC-3 cell line

A - Elf-1 knockdown in PC-3 cell line was performed using two different siRNA duplexes against a non-targeting siRNA duplex at 5nM and 10nM concentrations, protein levels were analyzed by immunoblot. Both concentrations shows considerably lower Elf-1 protein expression yet no change on hPygo2 protein expression is noticeable. B – Densitometry showing Elf-1 and hPygo2 levels. Results show significant reduction of Elf-1 using both SiRNAs. However, no significant change in hPygo2 was detected against the respective non-targeting control. Significance was obtained by comparative measures ANOVA and Fisher's LSD statistical test ($p<0.05$).

Chapter IV – Discussion

4.1 The relationship between HPV and prostate cancer was not detectable

Current literature on the role of HPV infection in prostate carcinoma is controversial. The first involvement of HPV infection in carcinoma was identified in cervical cancer where HPV E6 and E7 protein target Rb for degradation, releasing E2F transcription factor and activating the aberrant expression of growth and proliferation genes (zur Hausen, 1977; Moody and Laimins, 2010). HPV infection in prostate carcinoma is of importance in relation to hPygo2 expression because it could explain its requirement for cell growth and proliferation. In cervical cancer, the presence of HPV infection generates E7 protein, which induces the degradation of Rb protein, and in a similar matter to E2F, Elf-1 is de-repressed initiating *hPygo2* gene transcription (Tzenov *et al.*, 2013). Numerous studies have examined the role of HPV infection in prostate carcinogenesis with inconsistent and contradictory results (Pascale *et al.*, 2013; Lin *et al.*, 2011; Moody and Laimins, 2010; Martinez-Fierro *et al.*, 2010; Rosenblatt *et al.*, 2003). I performed RT-PCR in order to assess the presence of E7 protein in prostate cancer cell lines and therefore the possibility of having a similar mechanism to that of cervical cancer. Results showed that HPV 16 and HPV18 E7 protein is expressed in their respective endocervical cancer cell lines but is not expressed in prostate cancer cell lines. This result suggests that there is no relationship between HPV infection and expression of hPygo2 in prostate cancer cell lines. Additional experiments with HPV 16 and 18 infected prostate cancer cell lines and tumors could be performed in order to analyze interaction of E7 protein with Rb and the expression levels of Elf-1 and hPygo2 proteins.

4.2 Differential expression of ETS factors in prostate cancer cell lines expressing pygopus protein

ETS proteins play an important role in the regulation of expression of genes involved in cellular proliferation, differentiation, transformation and apoptosis (Sementchenko and Watson, 2000). The relationship between hPygo2 expression and the ETS factor family relies in its mechanism of regulation. In breast and cervical cancer, hPygo2 was found to be activated by Elf-1 (E74-like-1), an ETS family transcription factor regulated by Rb (Andrews *et al.*, 2008; Tzenov *et al.*, 2013). As an initial step to assess the possibility of a similar mechanism in prostate cancer, protein levels of Rb, pRb, Elf-1, hPygo2, Erg-1, Ets-1 and Elk-1 were analyzed by immunoblot. Regardless of its androgen receptor status, all of the prostate cancer cell lines showed expression of Elf-1 and hPygo2 proteins. As expected, no expression of these two proteins was detected in the normal prostate epithelial cell line PrEC. Interestingly, hPygo2 expression only appeared to correlate with high expression of Elf-1 in 22Rv1 and PC-3 cell lines. When additional ETS factors were tested, Erg-1 protein was expressed at lower levels than other ETS factors, high levels of Ets-1 were present in 22Rv1 cells and Elk-1 was expressed in all of the cell lines but at a lower level in the normal PrEC. Unique expression of ETS family members between different prostate cancer cell lines have been reported before by Shaikhibrahim and Lindstrot (2011). Their results were similar to mine, reporting that Elf-1 (including variants) has higher levels in PC-3 cell, Elk-1 was higher in LNCaP cells, and Erg and Ets-1 were higher in in DU-145 and PC-3 cells (Shaikhibrahim and Lindstrot, 2011). The evident differences between ETS factor expression levels in different prostate cancer cell lines could be explained by the different tissue origins and metastatic properties of the cell lines making it a plausible reason of why hPygo2 expression varied as well.

4.3 Presence of ETS factors binding to the proximal pygopus promoter region in prostate cancer cell lines

Chromatin immunoprecipitation (ChIP) was performed in 22Rv1, Du-145 and PC-3 cell lines to assess the binding capability of Elf-1 and additional ETS factors to the proximal region of hPygo2 promoter. Results confirmed high and statistically significant occupancy of Elf-1 at the proximal promoter region in 22Rv1 and PC-3 cell lines. Moreover, Ets-1 had high occupancy at this region in all of the cell lines but it was only significant in 22Rv1 and DU-145. The presence of Elf-1 at the h*Pygo2* proximal promoter region was concordant with results in breast, ovarian and cervical cancer (Andrews *et al.*, 2008; Tzenov *et al.*, 2013).

Interestingly, Elf-1 may not be the only factor present at the proximal promoter region. Genome-wide analyses using a human T cell line have reported overlapping functions and redundant occupancy of different ETS factors at consensus gene regions, and while some of the ETS factors are present at specific binding sites, these sites are further away and distinct from transcription start sites (Hollenhorst *et al.*, 2007). The possibility of redundant occupancy of ETS factors in this region is likely due to their conserved domain and preference for consensus binding sites. This phenomenon, however, has never been studied in prostate carcinoma cell lines. While most of the ETS proteins bind DNA as monomers, cooperation among different ETS factors and other transcriptional factors have been demonstrated where protein-protein interactions help regulate DNA binding, localization and transcriptional regulation of target genes (Kodandapani and Pio, 1996; Li *et al.*, 2000).

Reports show that blocking Ets-1 activity with an inverse plasmid upregulates other ETS family members such as Elf-1, Elf-2, Elk-1, Etv-5 and Spi-1 but there is no evidence of direct interaction between the factors (Shaikhibrahim and Lindstrot, 2011; Shaikhibrahim and Wernert,

2012). These findings raise the possibility of protein interactions within the ETS factor family including Elf-1 which could suggesta different mechanism of hPygo2 expression in prostate cancer.

4.4 Elf-1 knockdown reduces mRNA levels but not protein levels of hPygo2 protein

Elf-1 knockdowns were performed in 22Rv1 and PC-3 cell lines in order to assess Elf-1 ability to regulate hPygo2, in both cases there was no evident change in protein levels of hPygo2 expression. However, hPygo2 mRNA levels were significantly reduced in 22Rv1. These results indicate that unlike breast and cervical cancer, Elf-1 is not exclusively involved in the activation of hPygo2 expression in prostate cancer and a redundant mechanism is likely in place to ensure activity of this important protein for growth. These experiments reject the hypothesis of Elf-1 exclusively regulating hPygo2 activation. The possibility of different ETS factors being involved in the expression mechanism of hPygo2 needs to be evaluated. Since Ets-1 and Elf-1 show binding to the same proximal promoter region, it is possible that expression is being regulated by both or their interaction. Unfortunately, Ets-1 siRNA knockdown in prostate cancer has not been reported and my preliminary knockdown experiments were unsuccessful (*Appendix A5).* The use of an inverse plasmid to block Ets-1 expression as shown by Shaikhibrahim and Lindstrot (2011) would be very useful to confirm Elf-1 upregulation in different prostate cancer cell lines and observe its effect on hPygo2 expression.

4.5 Conclusions

Pygopus expression is regulated by the Rb protein via the ETS factor Elf-1 in cervical and breast cancer (Andrews *et al.*, 2008; Tzenov *et al.*, 2013). The ETS family has roles in regulating target genes involved in proliferation, transformation and apoptosis. Previous evidence in cervical cancer cell lines report that HPV 16 and HPV18 E7 protein degrades Rb, de-repressing Elf-1 and initiating hPygo2 expression (Tzenov *et al.*, 2013) . To assess the possibility for a similar mechanism in prostate cancer, RT-PCR was performed for the detection of E7 protein in HPV infected endocervical cancer cell lines and prostate cancer cell lines. The presence of E7 was not detectable in the prostate cancer cell lines indicating that HPV infection is not a requirement for prostate cancer progression.

Variation in protein levels of ETS factors were identified between cell lines. However, Elf-1 and hPygo2 levels were higher in prostate cancer cells compared to the normal prostate PrEC cell line. To evaluate the binding capability of Elf-1 and ETS factors to the proximal promoter region of hPygo2, chromatin immunoprecipitation assays were performed resulting in high occupancy of Elf-1 in 22Rv1 and PC-3 cell lines. In 22Rv1, PC-3 and DU-145, Ets-1, another ETS factor, also showed high occupancy at the region suggesting Elf-1 might not exclusively regulate hPygo2.

To evaluate this, Elf-1 knockdowns were performed in 22Rv1 and PC-3 cell lines in order to assess the ability for Elf-1 to regulate h*Pygo2*; in both cases there was no change in hPygo2 protein levels. However, hPygo2 mRNA levels were significantly reduced in 22Rv1 cells. These results suggest that unlike breast and cervical cancer, Elf-1 might not be exclusively involved in the activation of pygopus expression in prostate cancer.

4.6 Future directions

The possibility of HPV E7 protein involvement in hPygo2 expression mechanism could be tested with known HPV 16 and 18 infected prostate cancer cell lines and tumor tissues to consequently analyze the expression levels of E7 protein, Rb, Elf-1, hPygo2 and additional ETS factors. Immunoprecipitations could be performed in order to confirm possible interactions between ETS family members, particularly Elf-1 and Ets-1. Overexpression vectors and blocking vectors of Ets-1 could be designed and transfected into different prostate cancer cell lines in order to evaluate the effect on Elf-1 and hPygo2 expression levels.

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Appendices

A1- ChIP Ct Values

Table A 1 - Ct values for 3 repetitions of chromatin immunoprecipitation assays for 22Rv1, DU-145 and PC-3 cell lines

	CT VALUES				CT VALUES				CT VALUES		
22RV1	REPEAT 1	REPEAT 2	REPEAT 3	**PC-3**	REPEAT 1	REPEAT 2	REPEAT 3	**DU-145**	REPEAT 1	REPEAT 2	REPEAT 3
Input	22.8328	19.309971	22.64655	Input	23.19832	24.12594	19.70994	Input	24.35308	24.30936	24.3238
Input	22.87971	19.408028	22.63814	Input	23.06955	24.08734	19.70141	Input	24.46077	24.30614	24.43694
Input	22.86482	18.963547	22.53842	Input	23.21966	24.15218	19.6674	Input	24.44057	24.31267	24.41135
IgG	34.10062	31.791277	35.65702	IgG	35.06413	33.74571	33.22698	IgG	34.06916	34.61302	37.03959
IgG	33.95923	30.025846	34.71248	IgG	34.49023	33.66729	31.41587	IgG	33.97977	35.06092	33.89942
IgG	35.33806	29.67452	34.52285	IgG	34.93179	33.8809	31.85049	IgG	34.06125	35.19434	36.10358
Elf-1	33.90597	30.969563	33.27305	Elf-1	34.2067	33.71796	31.2623	Elf-1	33.66601	33.59492	36.52449
Elf-1	34.05619	29.529421	33.32225	Elf-1	34.8522	34.28618	30.45097	Elf-1	32.94632	33.76887	35.46181
Elf-1	33.34143	29.681479	33.69221	Elf-1	35.59743	33.54503	30.4614	Elf-1	32.93803	34.69654	33.84104
Ets-1	33.22099	26.7719	33.28963	Ets-1	32.90247	34.12549	29.63543	Ets-1	33.40115	33.69521	35.14545
Ets-1	33.11826	27.695719	32.69748	Ets-1	34.12352	34.72675	31.39712	Ets-1	33.04694	34.1795	34.14508
Ets-1	33.0318	27.906841	32.77897	Ets-1	33.90061	34.07385	30.15009	Ets-1	32.58971	34.11958	33.7721
Elk-1	36.07978	29.290609	33.97073	Elk-1	35.44766	34.7078	30.66718	Elk-1	33.18248	34.12771	35.18562
Elk-1	34.94757	28.495888	34.12189	Elk-1	35.29787	34.39912	30.59116	Elk-1	33.23258	34.56599	36.04354
Elk-1	35.11702	28.516527	34.56851	Elk-1	35.72315	34.60854	31.81981	Elk-1	33.09251	35.90012	34.31923
Erg-1	33.87727	31.923014	34.94833	Erg-1	36.10727	34.96323	31.4219	Erg-1	32.26826	35.8955	34.57745
Erg-1	33.91021	37.118668	34.7306	Erg-1	36.62906	34.5088	31.46153	Erg-1	32.77705	35.88513	35.3998
Erg-1	33.7887	31.900423	34.2495	Erg-1	36.95311	35.40457	31.74523	Erg-1	34.58423	35.10019	34.17093

A2- ChIP Statistical Analysis - Data Entry

22Rv1

	% of Input Ct Values			Normalized to IgG =1 Each ETS factor value divided by their IgG value (Specific Repeat)		
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3
IgG	0.00176776	0.006151	0.002326	0.51766933	1.801312114	0.681018556
Elf-1	0.00580092	0.008232	0.006201	3.2815131	1.338296	2.666278
Ets-1	0.0079467	0.028749	0.009138	4.49535812	4.673704	3.929241
Elk-1	0.00218507	0.017637	0.003	1.23606913	2.867193	1.289918
Erg-1	0.00481067	0.001664	0.00213	2.72134293	0.27058	0.91574

PC-3

	% of Input Ct Values			Normalized to IgG =1 Each ETS factor value divided by their IgG value (Specific Repeat)		
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3
IgG	0.002959	0.011679	0.002208	0.526877	2.079872	0.393251
Elf-1	0.00391	0.013883	0.005805	1.321646	1.188717	2.628612
Ets-1	0.003109	0.010036	0.008578	1.050672	0.859298	3.884504
Elk-1	0.002179	0.006814	0.005149	0.736637	0.583471	2.331594
Erg-1	0.000815	0.005541	0.002932	0.275611	0.474414	1.327559

DU-145

	% of Input Ct Values			Normalized to IgG =1 Each ETS factor value divided by their IgG value (Specific Repeat)		
	Repeat 1	Repeat 2	Repeat 3	Repeat 1	Repeat 2	Repeat 3
IgG	0.012792	0.005282	0.003049	1.816766	0.750208	0.433026
Elf-1	0.027784	0.01507	0.003291	2.172081	2.852932	1.07955
Ets-1	0.022853	0.010897	0.013485	1.786587	2.063086	4.422889
Elk-1	0.023116	0.009505	0.007778	1.807154	1.799373	2.551158
Erg-1	0.037161	0.003261	0.01011	2.905107	0.617293	3.31587

Analysis was performed using the relative quantitative comparative threshold cycle (ΔΔCt) method against input chromatin levels and repeated measures ANOVA analysis for multiple comparisons against IgG followed by an uncorrected Fisher's LSD statistical analysis ($p<0.05$ for significance) using Prism 6 software (Graphpad Prism, California, USA)

A3- Elf-1 knockdown in 22Rv1 Ct values

Table A 2 - Ct values for Elf-1 Knockdown in 22Rv1

		CT VALUES	
1st REPEAT	B-actin	Elf-1	Pygopus
Non-targetting C	20.29896	27.774904	31.13721
Non-targetting C	20.61861	27.645544	31.78226
SiRNA Elf-1	20.35332	30.005264	31.89041
SiRNA Elf-1	20.59588	30.205086	31.82758

		CT VALUES	
2nd REPEAT	B-actin	Elf-1	Pygopus
Non-targetting C	19.28202	27.196129	30.55829
Non-targetting C	19.47549	27.036165	30.77224
SiRNA Elf-1	20.77568	30.50515	32.77407
SiRNA Elf-1	20.81149	30.746807	32.87446

		CT VALUES	
3rd REPEAT	B-actin	Elf-1	Pygopus
Non-targetting C	20.3917	27.105814	31.92888
Non-targetting C	20.6238	26.888554	31.68921
SiRNA Elf-1	20.37768	28.902636	31.62083
SiRNA Elf-1	20.89203	28.994549	32.59383

		CT VALUES	
3rd REPEAT	B-actin	Elf-1	Pygopus
Non-targetting C	20.0489	27.563349	31.02946
Non-targetting C	20.0208	27.501993	31.16687
SiRNA Elf-1	18.92608	28.345097	30.9332
SiRNA Elf-1	18.89705	30.896122	29.71433

A4- Expression of Ets-1 variants in prostate cancer cell lines

Expression of Ets-1 in different prostate cancer cell lines and the expression of the three main splicing variants that could be identifiable. The ETS-1 gene encodes three different variants, full length ETS-1 p51 protein, ETS-1 p42 lacking exon VII, and ETS-1 p27 missing exons III-VI (Koizumi *et al.*, 1990; Shaikhibrahim *et al.*, 2011). In order to assess expression of the different Ets-1 variants, immunoblot analysis was performed using the rabbit polyclonal C-20 anti-ETS-1 antibody directed against the DNA-binding domain in PrEC, 22Rv1, LNCaP, MDA PCa 2b, DU-145 and PC-3 cell lines. Results show high expression of the full length protein ETS-1 p-51 in normal prostate epithelial cell line PrEC, LNCaP, DU145, MDA PCa 2b, PC-3. The variant ETS-1 p42 is highly expressed in PC-3 and ETS-1 p27 variant appears to only be expressed in PrEC, DU-145 and MDA PCa 2b. These results provide evidence that ETS-1 variants protein levels are expressed differently depending on the prostate cell line.

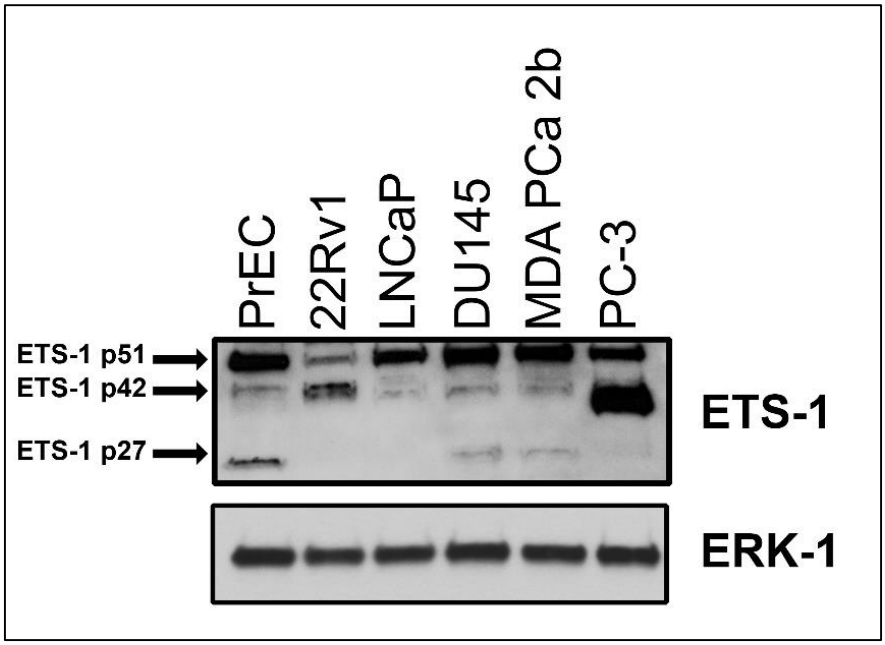

Figure A 1 - ETS-1 variants protein expression in prostate cell lines

Protein expression levels of the main three protein variants of ETS-1 were identified by immunoblot. Full length ETS-1 p51 protein appears to be expressed in all of the cell lines including the normal prostate epithelium however there is lower expression of it in 22Rv1 cell line. Spliced variant ETS-1 p42 is highly expressed in PC-3, followed by 22Rv1 compared to PrEC cell line. Lower expression of the second spliced variant ETS-1 p-27 is identifiable in PrEC, DU-145 and MDA PCa 2b.

A5- ETS-1 siRNA knockdown was unsuccessful at the protein level

ETS-1 knockdown experiments were performed in the 22Rv1 and PC-3 cell line using an ON-TARGETplus ETS-1 siRNA (*ON-TARGETplus siRNA with target sequences: 5'-GAUAAAUCCµGUCAGUCUU-3'; 5'-GGACCGµGCµGACCUCAAU-3'; 5'-GGAAUUACUCACµGAUAAA-3' and 5'-GCAUAGAG-AGCUACGAUAG-3*', GE Dharmacon Healthcare Inc; Colorado, USA) targeting 4 different variants of ETS-1 protein. Unexpectedly, Ets-1 protein levels were no different from that of the non-targeting control siRNA sµggesting that the knockdown was ineffective. Repeats were performed for each experiment using a range of concentration from 5nM to 40nM with the identical negative result. While siRNA transfection

protocol was performed identically as previously mentioned in Elf-1 knockdown experiments, a literature review confirmed the ON-TARGETplus ETS-1 siRNA has not been validated in prostate cell lines. For both cell lines, hPygo2 protein levels were analyzed by immunoblot to observe any potential effect induced by the siRNA transfection. Results show no change in hPygo2 levels and are not conclusive due to the lack of ETS-1 knockdown confirmation *Figure A2*. An *in silico* analysis using siRNA-Check tool from National Cancer Institute (In Silico Solutions, 2013) was performed obtaining each of the four siRNA sequences provided in the ON-targetplus ETS-1 siRNA from Dharmacon resulting in the identification of the target gene and mRNA genBank entries that confirmed they correspond to ETS-1 mRNA homologs and splice variants. Results also show that siRNA sequences target exons IV and X *Figure A3*. Additional experiments need to be implemented in order to confirm the effectiveness of the ON-TARGETplus ETS-1 siRNA provided.

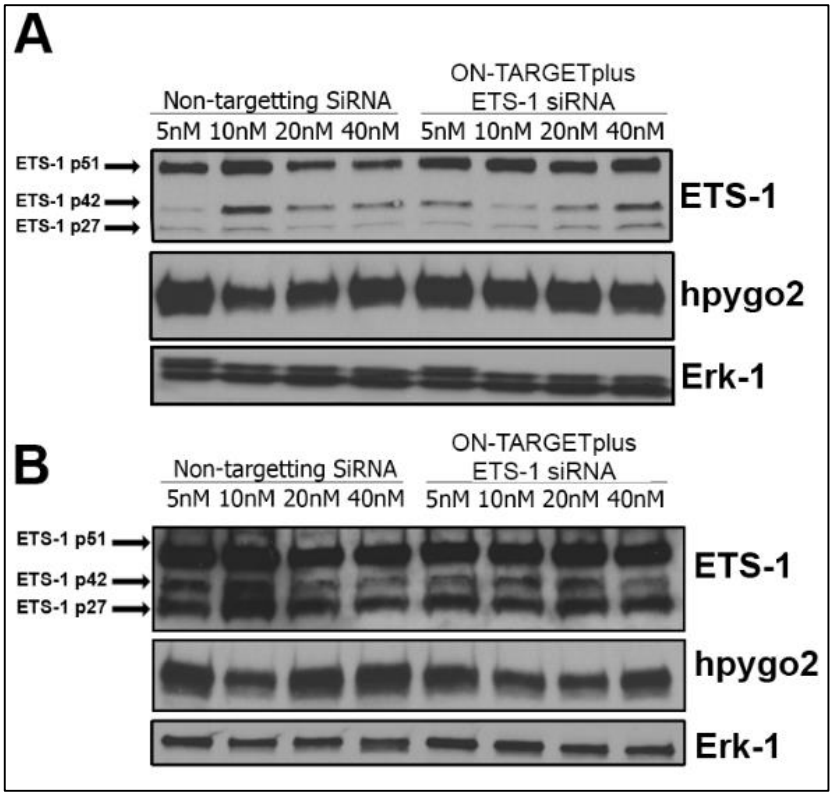

Figure A 2 - ETS-1 siRNA knockdown in 22Rv1 and PC-3

A- ETS-1 siRNA knockdown was performed on 22Rv1 cell line but the effectiveness could not be evaluated due to the lack of reduction on ETS-1 protein levels, hPygo2 levels did not show any change following knockdown. B- ETS-1 siRNA knockdown was performed on PC-3 cell line showing the same case, the lack of reduction on ETS-1 protein levels and hPygo2 levels did not show any change in protein levels compared to non-targeting control.

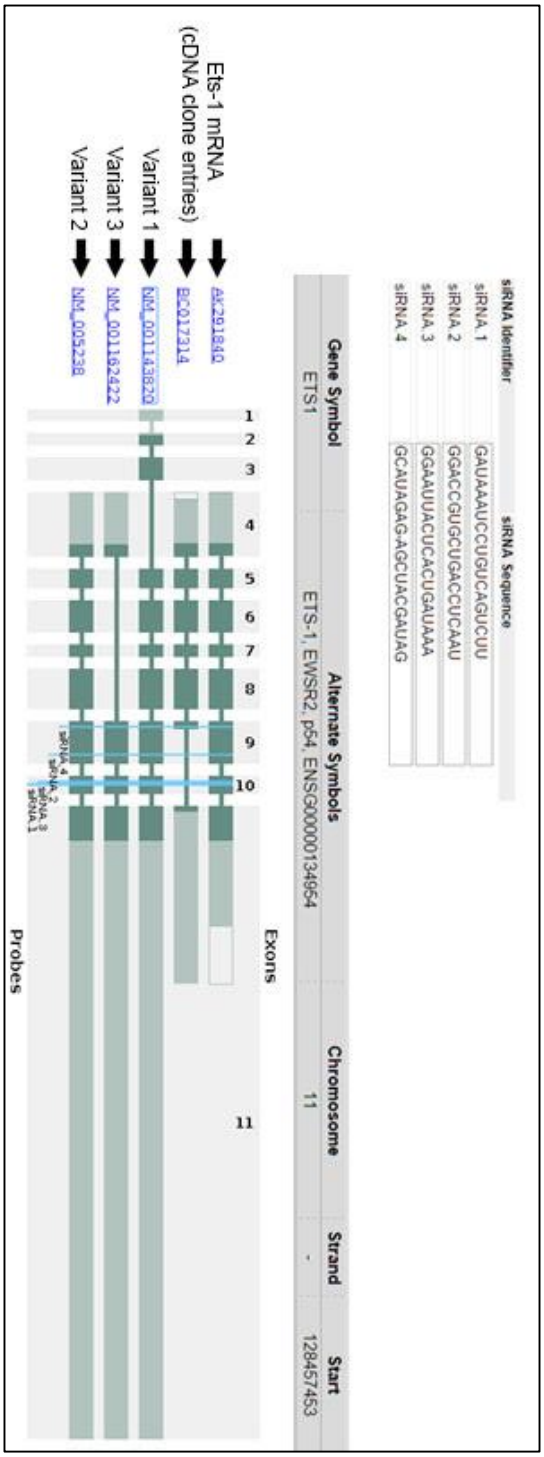

Figure A3- ETS-1 siRNA target in silico analysis

In silico analysis using siRNA-Check tool from National Cancer Institute was performed using the siRNA sequences provided in the ON-targetplus ETS-1 siRNa. Results confirm siRNA sequences target ETS-1 mRNA homologs and the three variants previously mentioned.