IDENTIFICATION OF PYGOPUS 2 AS A COMPONENT OF THE RIBOSOMAL RNA TRANSCRIPTION COMPLEX IN CANCER

ZHUJAN HE

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By

©Zhijian He

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Abstract

Background: Pygopus was initially validated as a co-activator for the canonical Wnt signaling pathway, a critical intracellular cascade implicated both in development and cancer. Further studies indicated that Pygopus appears to have both Wnt-dependent and Wnt-independent functions. Our lab has demonstrated that human Pygopus 2 (hPygo2) was overexpressed and required for Epithelial Ovarian Cancer cell growth in the absence of Wnt signaling. **Purpose:** The purpose of this thesis was to investigate the mechanism of Wnt-independent requirement of hPygo2 for Epithelial Ovarian Cancer cell growth.

Hypothesis and Objectives: I hypothesized that hPygo2 achieves Wnt-independent functions in Epithelial Ovarian Cancer via interacting with protein factors not related to Wnt signaling. Therefore, three objectives were proposed in this thesis: 1) to determine minimal domain(s) of hPygo2 required for Wnt-independent Epithelial Ovarian Cancer cell growth; 2) to identify potential protein factors that interact with this critical domain; and 3) to characterize the identified interactions.

Results: *Objective 1).* The transfection of hPygo2-specific antisense oligonucleotides in Wnt-inactive Epithelial Ovarian Cancer cell line, SKOV-3 led to cell growth arrest, as shown previously. To assess the sequence requirements of hPygo2 for SKOV-3 cell growth, plasmids encoding various mutant hPygo2 with site-specific alterations within the transcriptional activation domain (N-terminal

Homology Domain, NHD) or Wnt-mediating domain (Plant Homeodomain, PHD) were transfected in the hPygo-2-specific antisense treated SKOV-3 cells. Like wild-type hPygo2, mutant hPygo2 proteins with alterations in the PHD that prevented their Wnt-mediating ability restored the growth levels of antisense-treated SKOV-3 cells. On the other hand, hPygo2 protein with mutations in the transcriptional activation NHD domain, failed to restore cell growth. *Objective 2).* Using a proteomics approach, I identified several possible proteins including p68, RNA helicases II/Gu, Nop56, and Treacle protein from SKOV-3 nuclear extracts that bound to the NHD domain. *Objective 3).* The treacle protein was identified to have the highest probability of interaction with hPygo2. This interaction was confirmed *in vitro and in vivo*, in cancer cells. Immunofluorescence assays indicated that Treacle colocalized with hPygo2 in the nucleoli of SKOV-3 and HeLa cancer cells. Actinomycin D treatment in HeLa cells suggested that hPygo2 is a fibrillar component of the nucleolus and may be involved in transcription or early modification of premature ribosomal RNAs.

Conclusions: The NHD, but not the PHD domain, of hPygo2 protein is required for Wnt-independent SKOV-3 cell proliferation. The hPygo2 interacts with Treacle through its NHD domain and co-localizes with Treacle to the fibrillar compartment of nucleolus in cancer cells. Thus, these results suggest one of the Wnt-independent functions of hPygo2 is involved in ribosomal biogenesis.

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Lastly, I am greatly indebted to my wife Lin Zheng and my family. I indeed thank their tremendous love and support throughout the program.

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Abbreviations

℃	degrees Celsius
β-TrCP	β-transducin repeat-containing protein
µg	microgram
µl	microlitre
AMD	actinomycin D
ASO	antisense oligonucleotide
APC	adenomatous polyposis coli
A	adenosine
T	thymine
C	cytosine
G	guanine
BCL-9	B-cell lymphoma-9
CBP	CREB binding protein
Co-IP	co-immunoprecipitation
CK-I	Casein Kinase I
CRC	colorectal cancer
CREB	cAMP responsive element binding protein
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
Dsh,Dvl	dishevelled

EOC	epithelial ovarian cancer
FAP	familial adenomatous polyposis
FBS	fetal bovine serum
Frz	frizzled
GSK-3β	glycogen synthase kinase-3beta
GST	glutathione S transferase
ICC	immunocytochemistry
LRP	low density lipoprotein receptor-related protein
ml	millilitre
mM	milliMolar
mRNA	messenger ribonucleic acid
NHD	N-terminal homology domain
NLS	nuclear localization sequence
nM	nanoMolar
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pygo	pygopus
PHD	plant homeodomain
RNA	ribonucleic acid
TCS	Treacher Collins Franceschetti Syndrome

SDS-PAGE	sodium docecyl sulfate-polyacrylamide gel electrophoresis
shRNAs	short hairpin RNAs
siRNAs	small interfering RNAs
TCF	T-cell factor
TCOF1	Treacher Collins-Franceschetti syndrome 1
TPBS	Triton X-100 in PBS
Wnt	wingless/Int

CHAPTER 1
INTRODUCTION

1.1 Pygopus, a Crucial Component Originally Identified in Wnt Signaling

Pygopus was initially identified as a co-activator for the canonical Wnt signaling pathway, a critical intracellular cascade implicated both in development and cancer. Given its central role in the Wnt signaling pathway, I would like to first discuss the Wnt signaling pathway and its biological implications.

1.1.1 Wnt Signal Transduction Pathways

The mouse proto-oncogene *int-1* was originally identified as a mammalian homolog of the segment polarity gene *Wingless in Drosophila* (Nusslein-Volhard and Wieschaus, 1980; Cabrera et al., 1987; Rijsewijk et al., 1987). The name "Wnt", derived from terms "Wingless" and "int-1", was then used to describe a cluster of metazoan genes which encode protein ligands that can trigger a number of intracellular cascade events. In the past two decades, the Wnt gene family has been expanded to 19 members in the human genome including *Wnt1, Wnt3a, Wnt8* (Katoh, 2002). The ligands encoded by these Wnt genes are evolutionarily conserved glycoproteins that have similar amino acid sequences normally with 22-24 cysteine residues (Katoh, 2002). They function via directly

binding to a 7-trans-membrane Frizzled (Fzl) receptor family (Bhanot et al., 1996). Given that at least 10 Frizzled protein isoforms have been found, the combination of Wnt ligands binding to Fzl receptors is intricate and not well characterized (reviewed by van Amerongen et al., 2008). The other family of co-receptors that Wnt ligands bind to is the low-density lipoprotein receptor-related proteins (LRP) corresponding to Arrow in *Drosophila* and LRP5 and 6 in vertebrates (He et al., 2004a; Wehrli et al., 2000).

The different combinations of Wnt/Fzl/LRP binding complexes can activate various intracellular pathways (reviewed by van Amerongen et al., 2008). The most well characterized one involves a key transducer β-catenin, referred to as the canonical Wnt signaling pathway or the Wnt/β-catenin signaling pathway. Pygopus has been implicated only in the canonical Wnt/β-catenin pathway. The other pathways which are activated without the participation of β-catenin, defined as non-canonical Wnt signaling pathways, are not discussed here. Unless otherwise specified, therefore, the phrase "Wnt signaling" will imply "canonical Wnt signaling pathway" in the following sections.

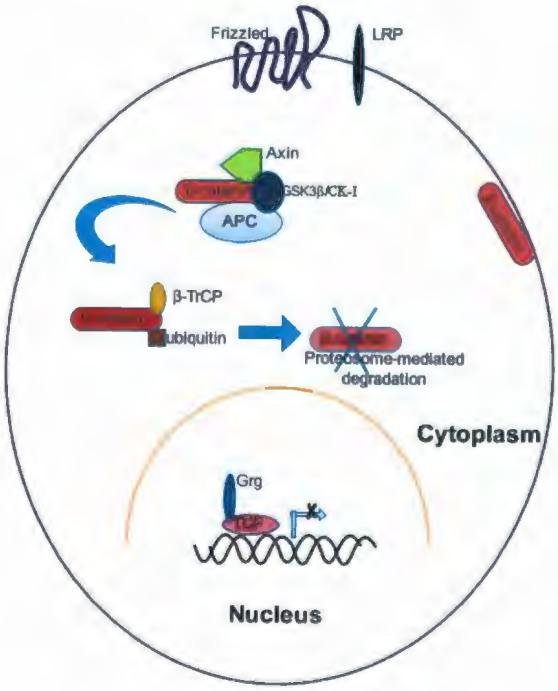
β-catenin has a role in epithelia as a component of cell adherens junctions. It is stably bound to the cell membrane proteins of the cadherin family in the absence of Wnt ligand, thus participating in cell adhesion (Kemler, 1993; Hulsken et el., 1994; Huber et al., 1997; Wheelock and Johnson, 2003). In the

absence of ligand binding, the "off" stage of Wnt signaling (Figure 1.1A), the surplus newly synthesized cytoplasmic β-catenin not associated with cadherin molecules, is targeted by a destruction complex that includes two scaffolding proteins, Axin and Adenomatous Polyposis Coli (APC). These two proteins facilitate two kinases, Glycogen Synthase Kinase-3 beta (GSK-3β), and Casein Kinase I (CK-I) to phosphorylate the N-terminal region of cytoplasmic β-catenin at residues Ser-45, Ser-33, Ser-37, and Thr-41 sequentially (Amit et al., 2002; Van Noort et al., 2002; Wu and He, 2006). Phosphorylated cytoplasmic β-catenin forms a complex with β-transducing repeat-containing protein (β-TrCP) and ubiquitin proteins for proteosome-mediated degradation (Amit et al., 2002; Kishida et al., 1998; Patel and Woodgett, 2008; Peifer et al., 2000; Piao et al., 2008; Willert et al., 1999). As a result of this phosphorylation/degradation mechanism, cytoplasmic β-catenin is maintained at a low level in the absence of Wnt ligand.

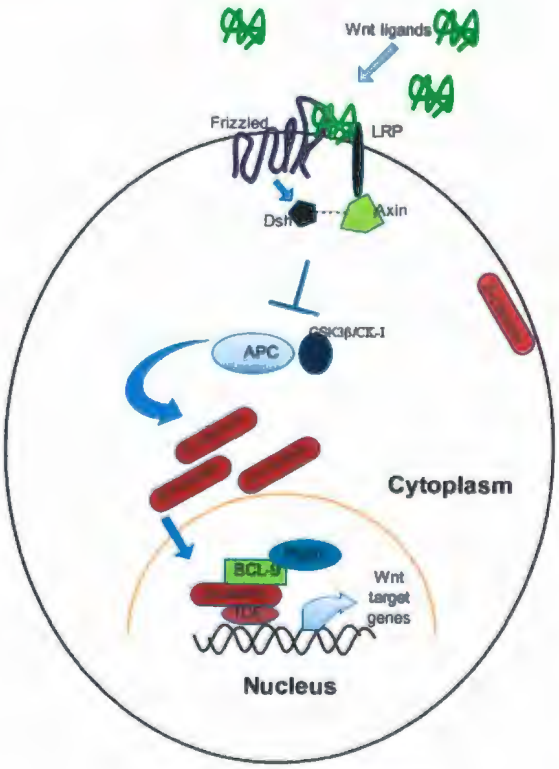
The binding of Wnt ligands to Frz/LRP receptors activates a Frz-interaction protein known as Dishevelled (Dsh) (Figure 1.1B). Furthermore, Wnt stimulation leads to phosphorylation of the LRP6 receptor at Pro-Pro-Pro-(SerTrp)Pro (PPP(S/T)P) amino acid residues where it may anchor the Axin protein to the plasma membrane (Tamai et al., 2004; Tolwinski et al., 2003) and additional evidence suggests direct contact between Dsh and Axin proteins

Figure 1.1 Canonical Wnt signal transduction pathway. A. "off" Stage of Wnt Signaling: β-catenin is either asscociated with cell membrane or degraded upon proteosome-mediated means following the phosphorylation by Axin/APC/GSK-3β/CK-I scaffolding complex and the addition of β-TrCP and ubiquitin. **B. "on" Stage of Wnt Signaling:** The binding of available Wnt ligands to receptors (Fzl and Lrp) leads to the activation of Dsh and then the inhibition of the degradation complex, and ultimatedly results in the accumulation and translocation of β-catenin into the nucleus to activate Wnt target genes by recruiting BCL-9 and Pygo to form the TCF/ β-catenin transcriptional complex.

Wnt signaling pathway



A. "Off" stage of Wnt signal pathway

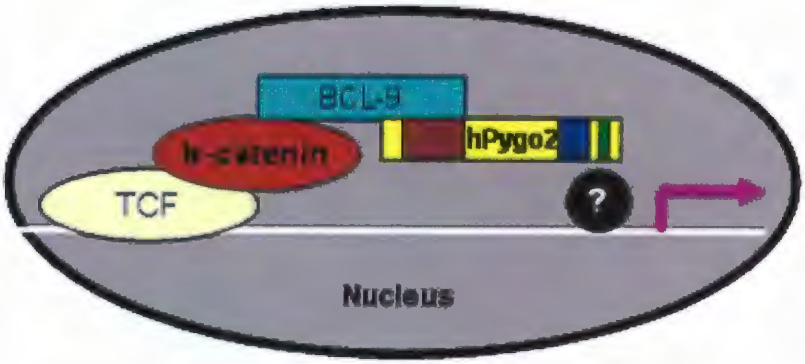


B. "On" stage of Wnt signal pathway

following Wnt ligand/receptor binding (Piao et al., 2008; Zeng et al., 2008). Regardless, the activation of Dsh and its association with Axin are events that inhibit the assembly of the disruption complex (Axin, APC, and GSK-3β), which relieves cytosolic β-catenin from phosphorylation/degradation (Schwarz-Romond et al., 2007; Zeng et al., 2008). Unphosphorylated β-catenin can consequently accumulate in the cytoplasm and enter into the nucleus by a mechanism that is not fully understood. In the nucleus, stabilized β-catenin activates Wnt target genes through interaction with T-cell factor (TCF) DNA binding proteins. In the absence of Wnt stimulation, TCF forms a complex with Groucho as a repressor of target genes since the functional interaction between Groucho and the histone deacetylase (HDAC) protein Rpd3, may impede access of the basal transcriptional activation complex to DNA (Cavallo et al., 1998; Chen et al., 1999). During Wnt signaling, β-catenin enters into the nucleus, binds to TCF, and switches the TCF/Groucho complex in a transcriptional repression mode to an activation mode by recruiting multiple cofactors. Of these cofactors, the Histone acetyltransferase CBP/p300 and a chromatin remodeling component Brg-1 are proposed to interact with TCF/β-catenin comlex and facilitate Wnt target gene transcription by inducing chromatin remodeling (Barker et al., 2001; Hecht et al., 2000). The recruitment of Pygopus as a co-activator to the β-catenin/TCF transcriptional complex under the mediation of BCL-9 protei n (Figure 1.2) is required for the

Figure 1.2 β-catenin/TCF Transcriptional Complex in the Canonical Wnt Signaling pathway. The complex composes of TCF, β-catenin, BCL-9, and hPygo2. The PHD domain (in brown color) of hPygo2 interacts with HD-1 domain of BCL-9 and associates with the complex for Wnt signaling. NHD domain (blue color) of hPygo2 may contain strong transcriptional activity by recruiting unknown factors.

β-catenin/TCF Transcriptional Complex



— Nuclear Localization Sequence (NLS)

— N-terminal Homology Domain (NHD)

— Plant Homeodomain (PHD)

activation of canonical Wnt signaling pathway (Hoffmans et al, 2005; Städeli and Basler, 2005).

To evaluate the exact consequences associated with active Wnt signaling, Wnt target genes have been intensely researched in the last 20 years. These target genes include those that encode vascular endothelial growth factor (*VEGF*) (Zhang et al., 2001) and fibroblast growth factors (*FGFs*) (Chamorro et al., 2005); cyclin D1 (Tetsu and McCormick, 1999) and *MYC* (He et al., 1998), both of which can regulate cell growth and proliferation; siamois (Carnac et al., 1996; Lemaire et al., 1995) and brachyury (Arnold et al., 2000; Smith et al., 1991), which regulate cell differentiation during embryonic development. Some Wnt signaling pathway components such as Axin, Fzl, LRPs, and TCFs can also be target genes for autoregulating feedback (reviewed by Klaus and Birchmeier, 2008; http://www.stanford.edu/~rnusse/wntwindow.html). In *Drosophila*, the naked cutical (*nkd*) gene containing Wingless responsive elements (WREs) was well characterized not only as a target gene activated by Wnt signaling but also as a feedback antagonist to regulate Wnt signaling (Chang et al., 2008; Zeng et al., 2000).

1.1.2 Wnt Signaling in Development

The Wnt signal transduction pathway plays evolutionarily conserved and critical roles in animal development by controlling biological

programs of embryonic development and adult tissue homeostasis (Reviewed in Klaus and Birchmeier, 2008; Grigoryan et al., 2008).

The implications of Wnt signaling in embryonic development were mostly determined by the analysis of different animal embryos that contain gain- or loss-of-function mutant Wnt proteins or pathway components. For instance, the *Wingless* (*Wg*, *Wnt1* homolog in *Drosophila*) mutant fly embryos displayed abnormal patterning in the overlying ventral cuticle, similar to that observed in embryos with mutations on Wnt signaling components such as Dsh and Amadillo (the Drosophila homolog of β-catenin), suggesting that Wnt signaling is require for normal segmentation and polarity in Drosophila (Nüsslein-Volhard and Wieschaus, 1980). Xenopus embryos are also widely used as models to study the effects of Wnt signaling in vertebrate development. The injection of mRNAs that encode signaling positive regulators including Wnt1, Dsh, β-catenin or dominant-negative versions of GSK-3β into the 4 celled embryo of Xenopus resulted in duplicated Axis formation (McMahon and Moon, 1989; Dominguez et al., 1995; Guger and Gumbiner, 1995; Behrens et al., 1996) whereas injections of pathway inhibitors such as Axin led to the loss of primary axis formation (Zeng et al., 1997; Behrens et al.,1998). These studies implied that dysregulation of Wnt signaling affected normal development of the body axis.

Wnt3a, one of the ligands that activate Wnt signaling, was

elucidated as a regulator for the posterior development of mouse embryos. It regulates axis patterning and presomitic mesoderm (PSM) formation, and directs the segmentation of presomitic mesoderm into somites by targeting the segment boundary determination genes Mesp2 and Ripply2 in the PSM (Dunty et al., 2008; Nakaya et al., 2005).

The roles of Wnt signaling in the specification and maintenance of stem cells in various adult organs were revealed in transgenic mice with conditional loss- or gain-of-function mutations of β-catenin. The affected tissues and organs included skin appendages (hair follicles and teeth), central nervous system, heart, bone, neural crest, eye, gastrointestinal tract, kidney, mammary gland, hematopoietic system and other organs (Reviewed in Grigoryan et al., 2008). For instance, transgenic mice containing loss-of-function mutant β-catenin displayed the formation of cysts instead of hair follicles by inhibiting the production of hair progenitor cells (Huelsken et al., 2001). More further analysis of gain-of–function mutation of β-catenin revealed increased hair cell progenitors and demonstrated that the Wnt signaling regulates the bulge stem cell of the hair follicle to specify the hair cell fate (Ito et al., 2007; Malanchi et al., 2008). In another example, mice with depleted *TCF-4* completely lost the stem cell compartment of the small intestine (Korinek et al., 1998). Later research identified that Wnt signaling controls intestinal stem cell renewal, in cooperation with Bone

Morphogenetic Protein (BMP) signaling (He et al., 2004b).

Recent evidence implicated Wnt signaling in heart stem cell renewal and differentiation. For instance, the defective regeneration of zebrafish heart was observed following the inhibition of Wnt signaling by the inhibitor DKK (Stoick-Cooper et al., 2007). Another research group identified that the multipotent isl1(+) cardiovascular progenitors can differentiate into the three major heart cells including cardiac, smooth muscle, and endothelial cells (Moretti et al., 2006). More recent studies from the same lab demonstrated that Wnt signaling regulates the regeneration and differentiation of isl1(+) cardiovascular progenitor cells during cardiogenesis (Qyang et al., 2007). The activation of Wnt signaling by introducing Wnt-3a ligands into isl1(+) progenitor cells of the secondary heart field could inhibit the progenitor cell differentiation and thus result in the accumulation of isl1(+) progenitors. This inhibition effect on heart stem cell differentiation gave rise to a defective morphology of outflow tract (OFT) in the heart.

1.1.3 Wnt Signaling and Cancer

The previous section discussed the important roles of Wnt signaling in normal animal development. However, the desregulation of Wnt signaling is associated with a number of malignancies, such as colon cancer (de

Lau et al., 2007; Hadjihannas et al., 2006), ovarian cancer (Boerboom et al., 2005), breast cancer (Benhaj et al., 2006), melanoma (Larue and Delmas, 2006; Murakami et al., 2001), and prostate cancer (Chen et al., 2004; Lu et al., 2009; Verras and Sun, 2006). Familial Adenomatous Polyposis (FAP), for example, is a hereditary disease with inactivating mutations of the human *APC* gene. Patients presenting with multiple pre-malignant lesions such as aberrant crypt foci and small polyps in the intestine (Gaspar et al., 2008; Powell et al., 1992; Seno et al., 2002) may develop into multiple colonic adenomas (Groden et al, 1991; Hinoi et al., 2007; Zauber et al., 2003). Polyps may also occur in the upper gastrointestinal tract and carcinomas may also develop in the brain and thyroid gland in patients.

Compared to FAP, colorectal cancer (CRC) is a more frequently occurring disease, and up to 85% of all sporadic CRCs contain loss-of-function mutations in the APC gene (Giles et al., 2003; Joyce and Pintzas, 2007; Segditsas and Tomlinson, 2006). These alterations, including insertions, deletions, and nonsense mutations in the *APC* gene give rise to the retention of β-catenin and deregulation of Wnt target genes. In addition, the gain-of-function mutations in β-catenin itself and the inactivating mutations of axin have been identified in approximately 10% of the remaining CRCs and a few CRC cell lines (Webster et al., 2000), providing support of the hypothesis that mutations causing stabilized nuclear β-catenin may be sufficient for the development of colonic neoplasms.

Activating mutations of the Wnt pathway are not restricted to CRCs. Upper gastrointestinal tumors, for instance, contain a high number of β-catenin mutations (Lynch et al., 2005; Ogasawara et al., 2006). Also, mutations occurring in the key mediator β-catenin itself are associated with neoplastic transformation in one subtype of epithelial ovarian cancer (Zhai et al., 2002), which will be discussed in the next section.

The identification of *Wnt1*, originally called *int-1*, as an oncogene in mouse breast tumors attracted researchers' interest to investigate the association of Wnt signaling with breast cancer. Different from other cancers, evidence is lacking to conclude that the deregulation of Wnt signaling in human breast cancers is entirely due to mutant APC, Axin or β-catenin (Howe and Brown, 2004; Ueda et al., 2001). A recent study on human breast cancer cell lines found accumulated β-catenin in nuclei and up-regulated expression of the Wnt target gene cyclin D1. The overexpression of multiple Wnt ligands and receptors observed in this study may account for the activation of Wnt signaling in human breast cancer cells (Benhaj et al., 2006).

1.1.4 Wnt Signaling and Epithelial Ovarian Cancer

Carcinomas are referred to as cancerous epithelial tumors. Approximately 85%-90% of ovarian cancers are Epithelial Ovarian Carcinomas

(EOC) (Auersperg et al., 2001; Landen et al., 2008), a deadly gynecologic malignancy that is derived from transformation of ovarian surface epithelial cells. Patients diagnosed with EOC are mostly at an advanced stage since early stage disease is usually asymptomatic, limiting the median survival time to less than 2 years (Jemal et al., 2007). EOC tumors become refractory to most available chemotherapy drugs and the recurrence rate is close to 100% (Christie and Oehler, 2006; Jemal et al., 2007). In addition, EOC is histologically categorized into at least 5 distinct subtypes: serous, mucinous, endometrioid, clear cell and undifferentiated. The undifferentiated epithelial ovarian carcinomas tend to proliferate and spread most quickly (Christie and Oehler, 2006).

Understanding the mechanism of this disease is important to improve treatment options. Although the Wnt signaling pathway plays an important role in many malignancies, such as colorectal cancer (Verma et al., 2003), breast cancer (Benhaj et al., 2006) and hepatocellular carcinoma (Kim et al., 2008), in EOC, only one cell line of endometrioid origin has been shown to display high levels of constitutive Wnt signaling (Kildal et al., 2005; Zhai et al., 2002). Cell lines derived from the other four EOC subtypes do not. These observations suggest that Wnt signaling is important only in one subtype of EOC tumors.

1.2 Human Pygopus2 (hPygo2) in Epithelial Ovarian Cancer

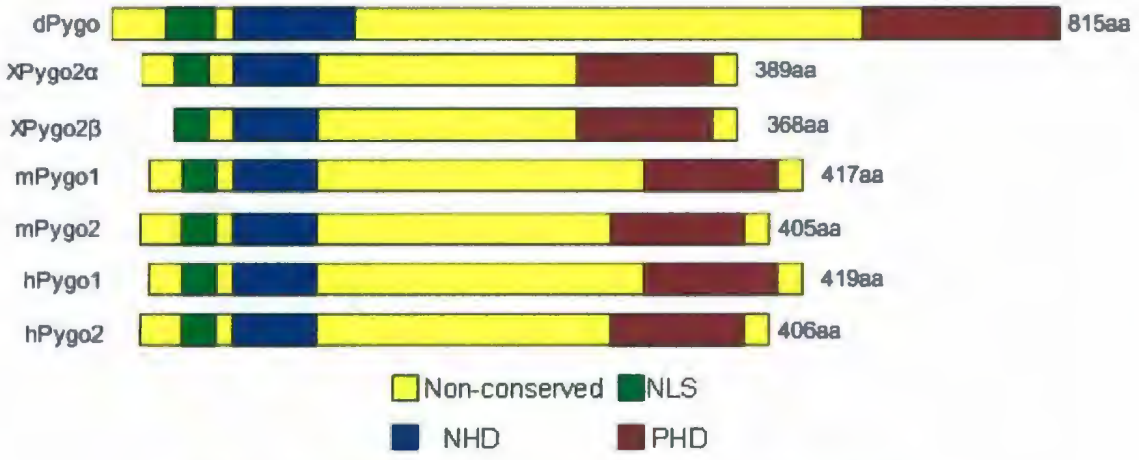
1.2.1 Pygopus proteins and their functional domains

Pygopus is a component of the β-catenin/TCF transcriptional complex essential for activation of Wnt signaling pathway. The extreme similarities in phenotypes between Pygo mutants and other Wg signaling mutants resulted in the identification of the prototype Pygopus protein in *Drosophila* (dPygo) (Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002). A yeast-two-hybrid assay initially showed that the legless protein (*Drosophila* homolog of human BCL-9) interacted with Pygo (Kramps et al., 2002) and thus may link Pygo to β-catenin. Subsequent studies revealed that Pygo may function as an anchor for the nuclear retention of β-catenin which is mediated by Legless (Krieghoff et al., 2006; Townsley et al., 2004; Tolwinski and Wieschaus, 2004). Alternatively, a "Pygo->legless->β-catenin" chain protein complex was proposed by which Pygo and Legless conferred their co-activator function to β-catenin for activating Wnt gene transcription (Hoffman et al., 2005; Stadeli and Basler, 2005).

dPygo contains several conserved regions corresponding to its vertebrate counterparts. In *Xenopus*, two Pygopus isoforms, α- and β- were found to be required for the development of the normal body axis (Belenkaya et al., 2002) and brain patterning (Lake and Kao, 2003). Human pygopus proteins have two versions, -1 and -2, abbreviated as hPygo1 and hPygo2 hereinafter (Figure 1.3). hPygo1 and hPygo2

Figure 1.3 Conserved domains of Pygopus proteins in different species. Top Panel: Pygopus proteins contain several conserved domains including the Nuclear Localization Sequence (NLS, green color), NHD domain (blue color), and PHD domain (brown red color) in different species such as *Drosophila, Xenopus, Mouse, and Human*. **Bottom Panel:** Conserved amino acid sequences among different species (Cited from Li et al., 2004. Cloning and developmental expression of mouse pygopus 2, a putative Wnt signaling component. *Genomics*. **84:** 398-405).

Conserved Domains of Pygopus Proteins in different species



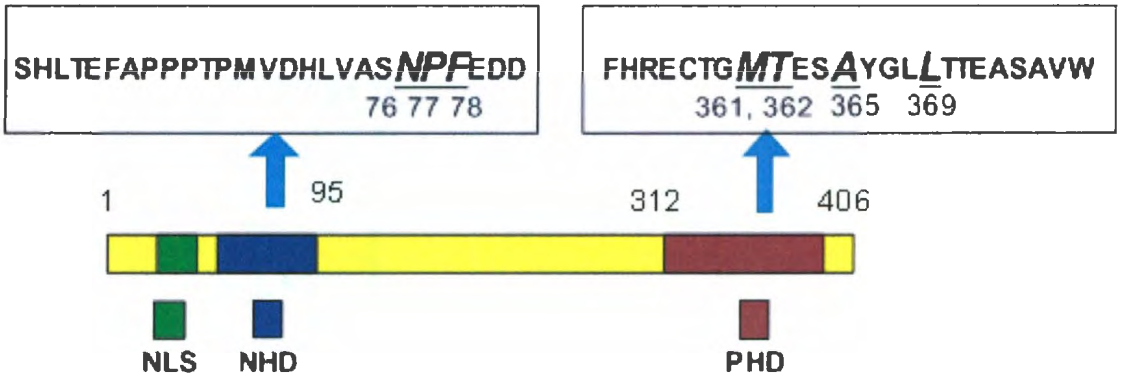
Conserved Sequences of Pygopus Proteins

proteins, like dPygo, share several conserved domains. For example, at the C-terminus, they contain a highly conserved zinc-binding domain known as the Plant Homeodomain (PHD) finger. In Wnt signaling, the PHD of Pygopus directly interacts with HD1 domain of Legless/BCL-9 (Kramps et al., 2002). Four specific residues (corresponding to hPygo2: Met361, Thr362, Ala365, Leu369) within the PHD domain of Pygopus (Figure 1.4) are required for its association with BCL-9 to form the β-catenin/TCF transcription complex and thus functions as a co-activator in transmission of Wnt signals (Townsley et al., 2004).

The PHD finger motif was found in proteins that participate in chromatin remodeling (Aasland et al., 1995). Chromatin remodeling requires loosening and reorganizing the nucleosome structure, namely the histones and condensed DNA complex (Ausio et al., 2003). This may be achieved by modifications on Histone proteins such as the addition of methyl groups (methylation) or acetyl groups (acetylation) by the appropriate methytransferases, acetyltransferases and other chromatin remodeling factors to specific residues on exposed core histone tails (Sterner et al., 2000). For example, the SET1-type methyltransferase complex can promote the trimethylation of the histone H3 tail at lysine 4 (H3K4me3) (Sierra et al., 2006). The PHD fingers from a variety of chromatin remodeling proteins such as BPTF (Li et al., 2006), ING2/Yng1 (Pena et al., 2006), and RAG2 proteins (Ramon-Maiques et al., 2007) can specifically

Figure 1.4 Structural domains of human Pygopus 2 Protein (hPygo2). hPygo2 protein contains conserved domains including the NLS (green color), NHD domain (N-terminal, blue color), and PHD domain (C-terminal, brown red color). A *NPF* (Asn76, Pro77, and Phe78) motif in the NHD domain was found to have strong transcriptional activity. In the PHD domain of hPygo2, four amino acids in *Drosophila* corresponding to the residues (Met361, Thr362, Ala365, and Leu369) have been shown to be critical for its interaction with BCL-9 (Legless in *Drosophila*) protein and required for Wnt gene transcription and embryonic development.

Human Pygopus 2 Protein



and selectively bind to the lysine 4 trimethylated histone H3 tail. Interestingly, a complex consisting of the PHD of Pygo and HD1 of BCL9 can specifically bind to H3K4me3, suggesting that this interaction may link chromatin remodeling with Wnt target gene transcription (Fiedler et al., 2008).

Other conserved domains of Pygopus are the N-terminal homology domain (NHD), adjacent to an N-terminal Nuclear Localization Sequence (NLS) (Li et al., 2004; Stadeli and Basler, 2005). The NHD and NLS domains are linked to the C-terminal PHD finger region by a long proline-rich non-conserved domain. Wnt target gene transcription not only depends on the interaction of Pygo PHD with legless/BCL-9 to form the Pygo>BCL9>β-catenin>TCF transcriptional complex, but also requires the regulation of a functional NHD domain. The NHD domain exhibits strong transcriptional activity dependent on a conserved NPF tri-peptide (Asn76, Pro77, and Phe78) (Figure 1.4) and site-mutations within this NPF motif abolishes its transcriptional activity *in vitro* as well as dramatically decreases Wnt signaling *in vivo* (Stadeli and Basler, 2005). The mechanism by which the NPF motif confers transcriptional activity to the NHD, however, is lacking.

Several possibilities were discussed recently for the activity of the NHD. Firstly, it was reported by de la Roche and Bienz (2007) that Pygo constitutively associated with dTCF in *Drosophila* tissue cultured cells and salivary glands which depended on the NHD of Pygo but not on legless. These

researchers also modified the former accepted model of TCF transcriptional machinery and proposed that Pygo associated with dTCF at the "off" stage of Wnt signaling and indirectly brought β-catenin in a legless-dependent manner to dTCF following Wnt stimulation.

A subsequent study identified that the Med12 and Med13 components, encoded by *skd* and *kto* genes in *Drosophila,* are required for Wnt target gene transcription. These two components are subunits of the mediator complex crucial for the RNA polymerase II-dependent gene transcription. *skd* and *kto* mutant flies displayed absent expression of Wnt target genes even when the constitutively active form of Armadillo (Drosophila homolog of β-catenin) was expressed, suggesting that these two genes functioned as further downstream components of β-catenin in Wnt signaling. This study also demonstrated that Med12 and Med13 were required for the transcriptional activity of Pygo NHD. The indirect interaction of Med12, 13 with the Pygo NHD determined by GST-pulldown and coimmunoprecipitation assays suggested that there may be some intermediate factors connecting the Med 12&13 to the Pygo NHD (Carrera et al., 2008).

Direct interaction between the Pygo NHD and the ETO domain of TAF4 was demonstrated in early 2009. TAF4 is a subunit within the TFIID complex of the basal transcription machinery. The TFIID complex, composed of

TATA-binding protein (TBP) which has promoter recognition activity and TBP-associated factors (TAFs) with co-activator functions, can bind to the promoter and enhancer regions of DNA and assist the initiation of RNA polymerase II-dependent transcription. The well established Wnt target gene *nkd* was used to reflect the activation of Wnt signaling and deletion mutation analysis of TAF4 identified the ETO domain at the N-terminal region of TAF4 was required for *nkd* expression under Wnt stimulation by an active form of Armadillo (Wright and Tjian, 2009).

1.2.2 hPygo2 is required for EOC growth in the Absense of Wnt Signaling

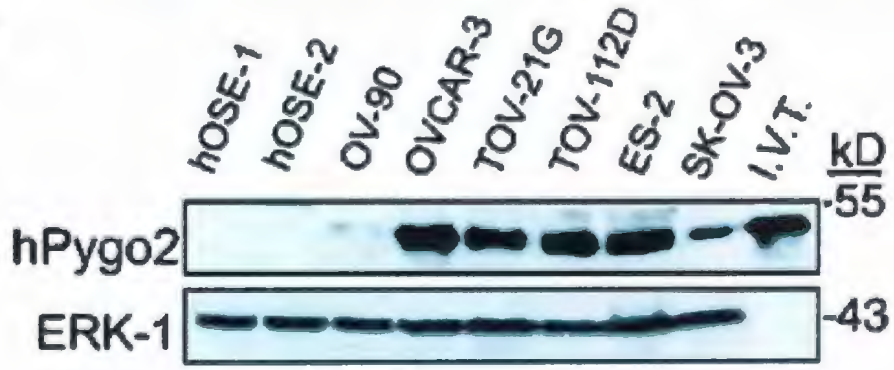
As mentioned in section 1.1.4, EOC can be divided into 5 histological subtypes. While Wnt signaling is restricted mainly to the endometrioid subtype (Kildal et al., 2005; Zhai et al., 2002), hPygo2 is unexpectedly overexpressed in all subtypes of EOC (Popadiuk et al., 2006) (Figure 1.5). hPygo2 is overexpressed in six malignant EOC cell lines and in the nuclei of 82% of 125 archived patient EOC tumors as compared to immortalized normal ovarian surface epithelial cells or benign disease tissues. These EOC cell lines and tumor tissues were derived from all 5 histological subtype origins including not only the Wnt-active (endometrioid) subtype but Wnt-inactive (other subtypes) origins. Furthermore, the depletion of hPygo2 protein in the EOC cell lines halted cell growth,

Figure 1.5 A. EOC subtypes and EOC cell lines. EOC has 5 major histological subtypes: endometrioid, clear cell, serous, mucinous, and undifferentiated. Cell lines including TOV-112D, TOV-21G, SKOV-3, ES-2, and OV-90 were derived from and represent each of these subtypes, respectively. Constitutive activation of Wnt signaling is only restricted to one subtype (endometrioid). All the other subtypes exhibit either very low level or inactive Wnt signaling properties. **B. The protein expression of hPygo2 in EOC cell lines (Immunoblot).** hPygo2 protein is overexpressed in all Wnt-active and Wnt-inactive EOC cell lines comparing to non-malignant human ovarian surface epithelial cells. (Popadiuk et al., 2006)

A

EOC Subtypes	Endometrioid	Clear Cell	Serous	Mucinous	Undifferentiated
Representing Cell lines	TOV-112D	TOV-21G	SKOV-3 OVCAR-3	ES-2	OV-90
Wnt Signaling	Constutively Active	Inactive or Low level			

B



suggesting its requirement for cancer growth regardless of Wnt signaling status (Popadiuk et al, 2006). An hypothesis was then proposed in which hPygo2 might play essential roles in EOC, independent of Wnt signaling.

The notion that Pygopus has roles in the absence of, or independently of Wnt signaling was supported by experiments in embryonic development. Wnt-independent roles of Pygo2 in lens development were revealed using a conditional Pygo2 knockout mouse model (Song et al., 2007). It was observed that deprivation of Pygo2 caused a microophthalmia phenotype by reducing expression of the Pax6 gene required for lens development in the $Pygo2^{-/-}$ null mice. Since the conditional deletion of β-catenin in mice did not produce similar lens defects to $Pygo2^{-/-}$ null mice, the authors proposed that Pygo2 has crucial functions in lens development in a Wnt-independent manner (Song et al., 2007). These observations were consistent with previously published work in our lab in which the NHD of Pygo2 alone was able to restore eye and head patterning gene expression in Pygo2 deficient *Xenopus* embryos (Lake and Kao, 2003).

Thus, evidence from both cancer and embryonic development strongly suggest that Pygopus has functional roles which are independent of Wnt signaling. How these requirements are fulfilled mechanistically is not completely known.

1.3 Purpose of the Thesis

The purpose of my thesis was to investigate the role of hPygo2 protein in EOC cancer cells independent of Wnt signaling. The following three major objectives were proposed in my thesis to address the hypothesis that hPygo2 fulfils Wnt-independent functions in EOC via interacting with factors not related to Wnt signaling.

Objective 1). Determine the function of each domain of hPygo2 protein in EOC cell proliferation in the absence of Wnt signaling.

Objective 2). Identification of hPygo2 interacting proteins in Wnt-inactive cell lines.

Objective 3). Characterization of the interactions between potential proteins and hPygo2

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell Culture

The epithelial ovarian cancer (EOC) cell line SKOV-3 and cervical cancer cell line HeLa were purchased from American Type Culture Collection (Manassas, VA). All the cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented by 10% fetal bovine serum (FBS) (Gibco), 100 units/ml penicillin, and 100 μg/ml streptomycin; and maintained in a humidified atmosphere with 5% CO_2 at a temperature of 37℃.

2.2 Western Blotting

Total proteins from tissue cultured cells were extracted in cell culture lysis reagent (CCLR) (Promega). Protein concentration was measured using the Bio-Rad assay reagent (Anon, 1991). Approximately 50μg of total cell lysate was loaded per sample onto a 10% SDS-denaturing polyacrylamide gel. Separated proteins were then transferred (blotted) to nitrocellulose membranes (Hybond-ECL™, Amersham). Blots were probed using hPygo2 rabbit antisera (Hausenfeffer-3I4B) (Andrews et al., 2007) or anti-treacle IgY (Abcam) antibodies, and then visualized by enhanced chemiluminescence (Amersham). Blots were reprobed with either monoclonal anti-β-actin (Sigma)

or polyclonal anti-ERK1 to further confirm the equal loading of total protein as described (Popadiuk et al., 2006).

2.3 Cell Rescue Asssay

In brief, SKOV-3 cells were seeded in triplicate at a density of $4x10^4$/well in 12-well plates 24 hours prior to transfection of plasmid constructs (cloned in pCS^{2+} vector) expressing either wildtype hPygo2 or hPygo2 mutants with mutations within the NHD or PHD domain, respectively (Figure 2.1A.). The next day, Phosphorothioate Antisense oligos hPy-ON8 (Invitrogen) (5'-C*C*T*CTGGCCAGAAAC*T*T*T-3'; * represents a phosphorothioate bond) specifically targeting the 3' UTR of hPygo2 mRNA (Figure 2.1B) was transfected to deplete endogenous hPygo2 protein (Popadiuk et al., 2006). It contains phosphorothioate modifications on 3 mers of each end marked in bold dark font. The mismatched (5'-G*C*C*TGAGCTAATCATT *G*T*T-3') (Invitrogen) phosphorothioated oligos (MM oligos) which presumably are unable to bind hPygo2 mRNA and have no knockdown activity, were used as a negative control (Popadiuk et al., 2006). Cell growth was assayed by cell counting using a hemacytometer 24 and 48 hours after transfection of the Antisense or MM oligos. Cell numbers are the average from 3 independent experiments. Western blots were performed to determine the endogenous and exogenous hPygo2 protein levels. Rescuing

Figure 2.1 A. Rescuing plasmids. The DNA sequences of hPygo2 wild-type coding region were amplified and inserted into pCS^{2+} plasmid vector. Site-mutations were made in the PHD or NHD domain of hPygo2 using this pCS^{2+}/hPygo2 wildtype coding region plasmid as a template. All wildtype and mutant plasmids were used for the rescue assay. **B. Antisense Oligos for depleting hPygo2 protein.** Antisense Oligos hPy-ON8 specifically targeting 3'-UTR of hPygo2 mRNA were used to knockdown the endogenous hPygo2 expression in the rescue assay.

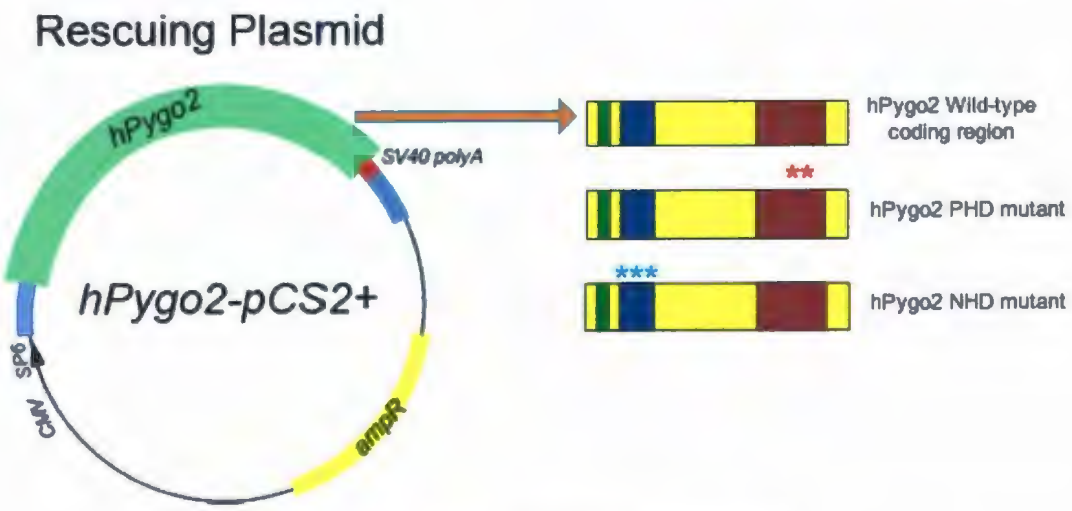


Figure 2.1A. Plasmid constructs for cell rescue assay

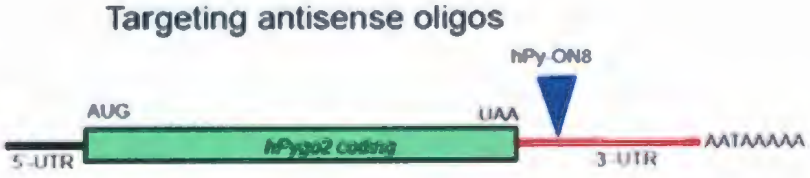


Figure 2.1B. ASOs for depleting hPygo2 protein in cell rescue assay

plasmids, Antisense and MM Oligos used in the cell rescue assay are shown in Figure 2.1A and B and described in detail below.

2.3.1 Plasmid constructs

All the plasmid constructs (Figure 2.1) were transfected at 150ng/well via Effectene® Transfection Reagent (Qiagen) as per the manufacturer's protocol.

1) pCS^{2+} empty vector (as a negative control): generously provided by Dr. Dave Turner, the Department of Biological Chemistry, University of Michigan.

2) pCS^{2+}/hPygo2 coding region (as a positive control): Engineered by Phillip Andrews.

3) pCS^{2+}/hPygo2 PHD mutant: Using pCS^{2+}/hPygo2 as a template, two PHD mutants were generated by Quik-Change® XL Site-Directed Mutagenesis Kit (Stratagene). The first one contained a leucine to alanine alteration at position 369 within the PHD domain termed "hPygo2 L369A". The other one, named "hPygo2 T362A, A365V", had combinational two sites mutations in which the threonine residue at position 362 was altered to an alanine and the alanine residue at position 365 was changed to valine. The dual-site mutant was more efficient for abolishing the interaction with BCL-9 (see results section 3.1) and was therefore used for the rescue assay.

4) pCS^{2+}/hPygo2 NHD mutant: The N-terminal homology domain (NHD) of Pygopus was previously shown to exhibit strong transcriptional potential a

dependent on a conserved NPF tri-peptide (corresponding to the amino acids Asparagine, Proline and Phenylalanine at position 76-78 of hPygo2) (Stadeli and Basler, 2005). Here also using pCS2+/hPygo2 wildtype as a template, these three residues at position 76-78 were all mutated into Alanines by QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene).

2.3.2 Antisense Oligonucleotides

Either phosphorothioated Antisense oligos hPy-ON8 or MM control oligos were transfected at a final concentration of 250nM via Effectene® Tranfection Reagent (Qiagen). Media were changed 24 hours after transfection.

2.3.3 Experimental groups

All the experimental groups are indicated in Table 3.1. Group 1 and 2 are co-transfections of pCS^{2+} empty vector with either Antisense oligos hPy-ON8 or MM Oligos. Group 3 and 4, 5 and 6, and 7 and 8 are co-transfections of pCS^{2+} hPygo2 wildtype coding region, pCS^{2+} hPygo2 PHD mutant and pCS^{2+} hPygo2 NHD mutant respectively with either Antisense oligos hPy-ON8 or MM Oligos.

Table 2.1 Experimental groups of co-transfections

Co-Transfections / Group Names	Rescuing plasmids	Oligonucleotides
1. EV+AS	pCS $^{2+}$ Empty Vector (EV)	Antisense oligos hPy-ON8
2. EV+MM	pCS^{2+} Empty Vector (EV)	MM oligos
3. WT+AS	pCS^{2+}/hPygo2 Wildtype Coding Region (WT)	Antisense oligos hPy-ON8
4. WT+MM	pCS^{2+}/hPygo2 Wildtype Coding Region (WT)	MM oligos
5.PHD Mut 2+AS	pCS^{2+}/ hPygo2 PHD mutant 2 (T362A, A365V)	Antisense oligos hPy-ON8
6.PHD Mut 2+MM	pCS^{2+}/ hPygo2 PHD mutant 2 (T362A, A365V)	MM oligos
7.NHD Mut+AS	pCS^{2+}/ hPygo2 NHD mutant (NPF76-78AAA)	Antisense oligos hPy-ON8
8.NHD Mut+MM	pCS^{2+}/ hPygo2 NHD mutant (NPF76-78AAA)	MM oligos

2.4 Mass-spectrometry

2.4.1 Production of GST-fusion Proteins

DNA sequences encoding only the hPygo2 NHD or PHD domain were amplified using the following PCR primers: NHD---forward, 5'-GCGAATTCGTCCCCCACTCCATGGCCGCCTCG-3'; reverse, 5'-GCCTCGAGCCAAGGAATGGAGGGGCTGCAAC-3'; PHD--- forward, 5'-CGGAATTCATGGCTCCAAAGAAGAAGCGTAAGGTAC-3'; reverse, 5'-GCCTCGAGTCACCCATCGTTAGCAGCC-3'; and were inserted into EcoRI/XhoI restriction sites of the pGEX 4T-1 vector containing the Glutathione S Transferase (GST) tag. The resulting products were then mini-prepped using Wizard Plus SV Miniprep Kit (Promega) and sequenced using an Applied Biosystems 3730 DNA Sequencer (Sequencing performed by Dr. Elizabeth Perry in the Genomics and Proteomics (GaP) Facility, Memorial University). Plasmids pGex-4T-1-NHD and pGex-4T-1-PHD were transformed into BL-21 Codon Plus RP competent *E.coli* cells (Stratagene) and grown in 250 ml Luria-Bertani (LB) media supplemented with 50ug/ml ampicillin at 37 ℃ for nearly 3 hours. 0.1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and the cells were allowed to grow an additional 3 hours for the production of GST fused proteins. The GST-fusion proteins were lysed from the BL-21 cells and collected in 1×PBS solution supplemented with 1×Protease Inhibitor (PI) and 0.1% Triton X-100.

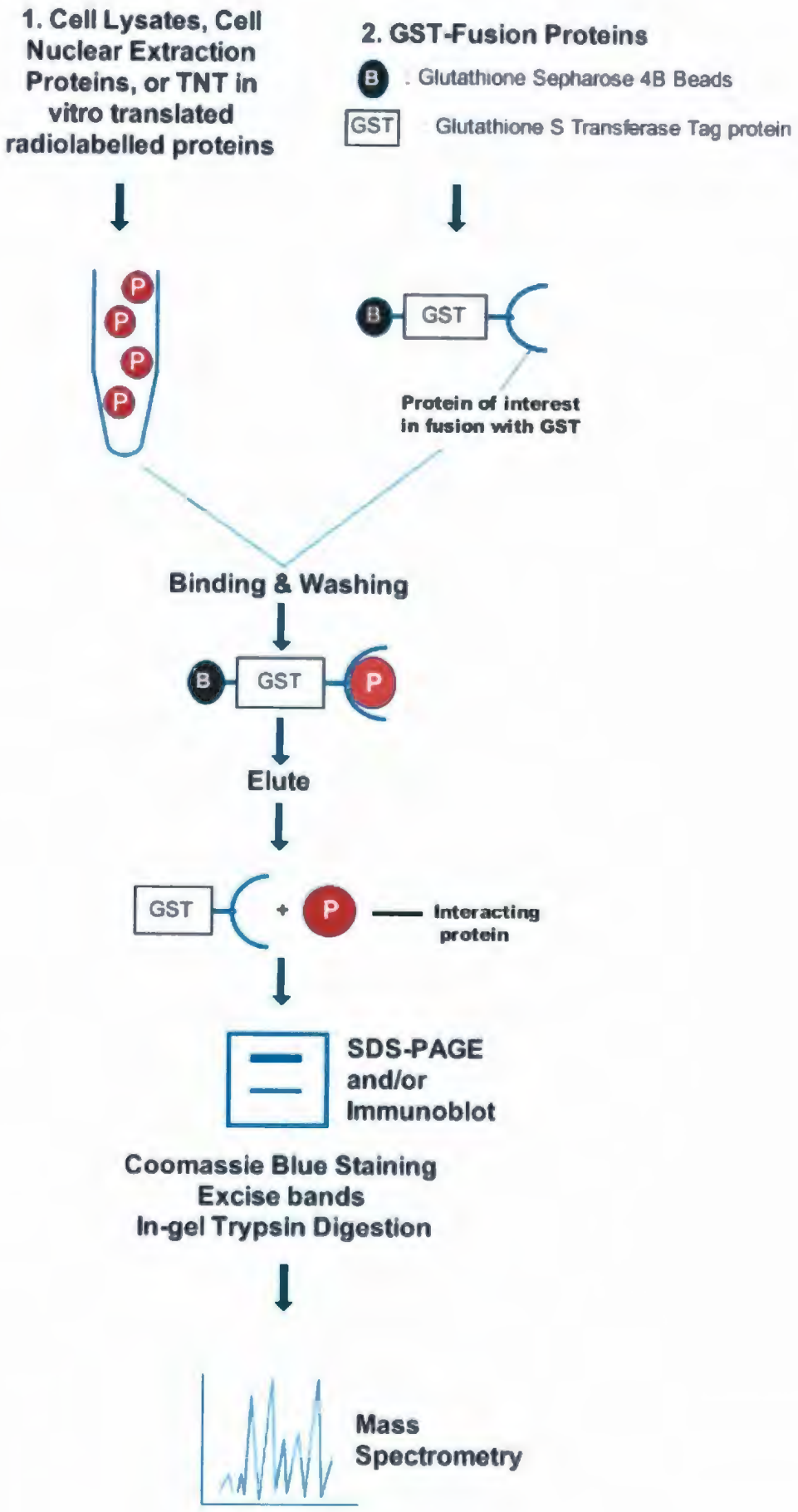
2.4.2 GST-pulldown assay

The rationale of the GST-pulldown assay is illustrated in Figure 2.2. The NHD or PHD domain of hPygo2 was fused into GST proteins respectively. The GST-Fusion-NHD or GST-Fusion-PHD protein was subsequently incubated with Glutathione Sepharose® 4B beads (Amersham) at 4℃ for 1 hour according to the manufacturer's protocol. The beads were washed at least five times with GST pulldown buffer to avoid non-specific binding. Nuclear proteins were extracted from EOC SKOV-3 cells using the NE-PER™ Nuclear and Cytoplasmic Extraction kit (Pierce) and incubated with previous GST protein-containing beads for 3 hours. After washing the beads 10 times with buffer, proteins bound to the beads were resolved and separated by 10% SDS-PAGE. Gels were then stained with Coomassie Brilliant Blue (Sigma) followed by destaining in destain-buffer and visualized with a digital camera to analyze the protein bands.

2.4.3 In-gel digestion and Mass-spectrometry

The basic principal for protein identification by mass-spectrometry is that: 1) Each sample protein is digested and cleaved into smaller peptides; 2) Peptides are then ionized and selected peptides are fragmented to produce mass spectra while some of these peptides do not ionize. Other peptides are fragmented multiple times to generate multiple mass spectra; 3) Acquired

Figure 2.2. Diagram of GST pulldown assay. GST-Fusion Proteins were immoblized onto Glutathione Sepharose 4B beads and incubated with cell lysates, nuclear extraction proteins, or in vitro translated radiolabelled proteins to allow the interacting-proteins to bind. After washing the beads, bound proteins were eluted and loaded on SDS-PAGE and subsequently identified by Immunoblot or Mass Spectrometry.


1. Cell Lysates, Cell Nuclear Extraction Proteins, or TNT in vitro translated radiolabelled proteins
2. GST-Fusion Proteins
B Glutathione Sepharose 4B Beads
GST Glutathione S Transferase Tag protein
P
B
GST
Protein of interest in fusion with GST
Binding & Washing
Elute
Interacting protein
SDS-PAGE and/or Immunoblot
Coomassie Blue Staining
Excise bands
In-gel Trypsin Digestion
Mass Spectrometry

mass-spectra are searched in a peptide sequence database; 4) The list of matched peptides is used to identify corresponding proteins. (Reviewed in Topaloglou, 2006)

Differential bands representing differential proteins pulled down by GST-NHD or GST-PHD fusion proteins were compared and bands of interest were excised into silicon tubes. Proteins in each band were digested via the Trypsin Profile IGD kit (Sigma). The digested peptides were extracted in 0.1% trifluoroacetic acid and 50% acetonitrile, dried by Speed-Vacuum Method and identified by a Dionex Ultimate 3000 HPLC coupled with an Applied Biosystem QSTAR XL MALDI-TOF mass-spectrometry (Performed by Ms. Lidan Tao at the Genomics and Proteomics (GaP) Facility, Memorial University). Mass-Spectrometry results were analyzed by Analyst (Applied Biosystem) and MASCOT software and the best matching candidate proteins were profiled from Pubmed protein database.

2.5 Characterizing protein-protein interactions

2.5.1 Immunfluorescence(IF) microscopy

IF was performed as described (Popadiuk et al., 2006). Briefly, cells grown on glass chamber slides were fixed in 4% paraformaldehyde (30 minutes) followed by 2×washes with PBS and 3×washes with 0.2% Triton-X 100/PBS (0.2% TPBS). Fixed cells were blocked in 5% normal goat serum (NGS) for 30 minutes and incubated with primary Antisera (diluted in 1.5% normal goat serum/PBS) overnight. Cells were washed for 30 minutes in 0.2% TPBS and then incubated with secondary antibodies. 4',6-diamidino-2-phenylindole (DAPI) was used for nuclear staining. For biotinylated secondary antibody, samples were incubated with fluorescein isothiocyanate (FITC)-Streptavidine for 30 minutes at room tempreture. Lastly, stained cells were washed 3 times in 0.1% TPBS with 10 minutes each time, mounted in 10% glycerol/PBS and viewed under fluorescence microscopy.

2.5.2 Co-Immunoprecipitation (Co-IP) assay

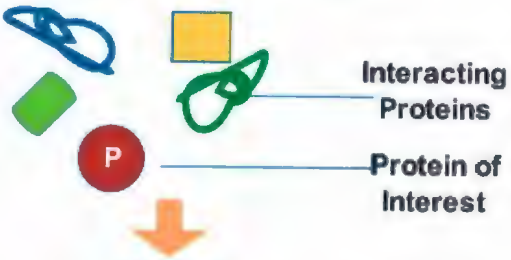
The Co-IP assay is a common method utilized for verifying protein-protein interactions. Typically, complexes encompassing protein of interest and its interacting proteins in cell lysates are captured by specific antibodies against the protein of interest, which are then immobilized on Protein A or G affinity beads. The interacting proteins are subsequently

elucidated by immuno-blot or Mass-spectrometry.

For this experiment (Figure 2.3), SKOV-3 cell monolayers at 90% confluence were washed with PBS and lysed in 1×Triton Media (1×TM) with 1×PI/PMSF. They were then extracted in cell lysates and incubated with antibodies overnight at 4℃. The next day, Protein A or G Sepharose™ CL-4B (GE Healthcare) beads were added and incubated for one hour at 4℃ followed by 10 times washes with 1×TM. Beads were spun down and bound proteins were subsequently resolved in 2×SSB and loaded on 8% SDS-PAGE. Specific interacting proteins were probed using primary antibodies and detected by enhanced chemiluminescence (Amersham).

Figure 2.3 Diagram of Co-immunoprecipitation Assay. 1. Incubate the antibody against protein of interest with cell lysates; 2. Immobilize the antibody, protein of interest, and interacting protein complexes onto protein A/G beads; 3. Wash the beads to eliminate non-specific binding proteins; 4. Elute binding proteins and immunoblot the interacting protein.

Step 1
Extract Proteins in cell lysates

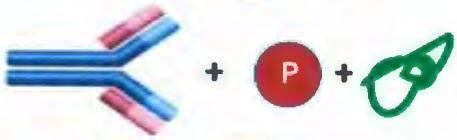


Step 2
Capture proteins by antibody against protein of interest

Step 3
Immobilization of antibody, protein complexes onto Protein A/G Beads

Binding and Washing beads

Elute



Immunoblot using antibodies against interacting proteins

2.5.3 GST-pulldown assay with in vitro translated radiolabeled proteins

The rationale of the GST-pulldown assay was discussed in section 2.3.2, Figure 2.2. For this experiment, successive deletion fragments of hPygo2 were PCR amplified (except GST-NHD32-47aa) and inserted into the EcoRI and XhoI sites of pGex4T-1(Figure 2.4). PCR primers used in the construction of hPygo2 deletion fragments are listed in Table 2.1. For the GST-NHD32-47aa, two oligonucleotides were directly annealed and formed double-stranded DNA fragments containing a 5' EcoRI site and a 3' XhoI overhang which can be inserted into pGex4T-1 vector. The above plasmid constructs were then used to produce corresponding GST-fusion proteins.

Complementary full-length protein or fragments of specific domains which were identified to interact with hPygo2, were synthesized and radiolabeled using in vitro transcribed/translated TnT SP6 System kit (Promega) and incubated with previous GST-Fusion proteins. Bound proteins were isolated by SDS-PAGE and in vitro translated proteins were detected using autoradiography instead of immunoblots.

Figure 2.4 GST fused successive deletion fragments of hPygo2 protein. Successive deletion fragments of hPygo2 were PCR amplified and inserted into pGex 4T-1 vector. 1: empty vector that only produces GST tag protein; 2: GST-fused PHD domain; 3: GST-Central (non-conserved) domain; 4 to 7: GST-NHD, GST-NHD1-47aa, GST-NHD32-47aa, and GST-NHD48-95aa fusion proteins, respectively.

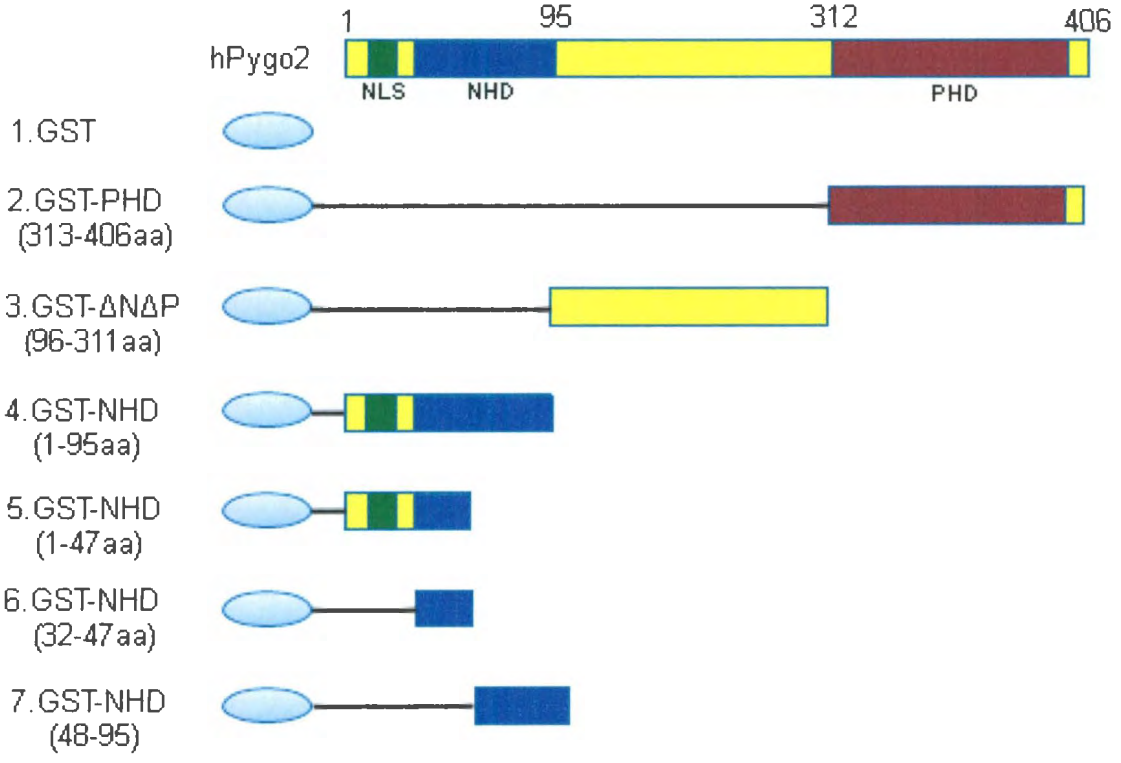

1
95
312
406
hPygo2
NLS
NHD
PHD
1.GST
2.GST-PHD
(313-406aa)
3.GST-ΔNΔP
(96-311aa)
4.GST-NHD
(1-95aa)
5.GST-NHD
(1-47aa)
6.GST-NHD
(32-47aa)
7.GST-NHD
(48-95)

Table 2.2 Primers used for amplifying successive deletion fragments of hPygo2

Construct	Forward Primer	Reverse Primer
2. PHD	5'-CGGAATTCATGGCTCCAAAGAAGAAGCGTAAGGTAC	5'-GCCTCGAGTCACCCATCGTTAGCAGCC
3. ΔNΔP	5'-CGGAATTCGCATCCAACCCTTTTGAAGATGAC	5'-CGGTCGAGTCAGCCAGGGGGTGCCAAGCTGTTG
4. NHD	5'-GCGAATTCGTCCCCCACTCCATGGCCGCCTCG	5'-GCCTCGAGCCAAGGAATGGAGGGGCTGCAAC
5. NHD1-47aa	5'-GCGAATTCGTCCCCCACTCCATGGCCGCCTCG	5'-GCCTCGAGCTTCCTTCGCTTCTTTTCTGGACTC
6. NHD32-47aa	5'-AATTCAAGCAGGGCAAGGCCGGTCTGCAAAT-	5'-TCGAGCTTCCTTCGCTTCTTTTCTGGACTCTTC-
	GAAGAGTCCAGAAAAGAAGCGAAGGAAGC	ATTTGCAGACCGGCCTTGCCCTGCTTG
7. NHD48-95aa	5'-GCGAATTCTCAAATACTCAGGGCCCTGCATAC	5'-GCCTCGAGCCAAGGAATGGAGGGGCTGCAAC

2.5.4 Actinomycin D (AMD) treatment

HeLa cells were seeded in chamber slides and grown overnight in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented by 10% fetal bovine serum (FBS) (Gibco), 100 units/ml penicillin, and 100 μg/ml streptomycin; and maintained in a humidified atmosphere with 5% CO2 at a temperature of 37℃. Next day, cells were treated with medium containing 50ng/ml AMD for 2 hours at 37℃ and analyzed by indirect immunofluorescence microscopy. Cells treated with medium containing Ethanol (vehicle for AMD) were used as controls.

CHAPTER 3
RESULTS

The hPygo2 protein was originally identified as an essential co-mediator in the canonical Wnt signaling pathway. It was later found overexpressed in and required for not only Wnt-active but also Wnt-inactive EOC cell growth (Popadiuk et al., 2006). The purpose of my thesis is to determine what is(are) the role(s) of hPygo2 in EOC cells in the absence of Wnt signaling. To this end, three objectives were proposed in my thesis: 1) determine which specific domain(s) of hPygo2 is(are) required for Wnt-inactive EOC cell growth using the cell rescue assay; 2) identify potential proteins interacting with this domain via proteomics technology; 3) characterize the interactions between potential proteins and hPygo2 which are critical for EOC growth in the absence of Wnt signaling.

3.1 Cell Rescue Assay

Previous studies used cell rescue assays to demonstrate the specificity of the requirement of hPygo2 for EOC cell growth (Popadiuk et al, 2006). Cell growth, as determined by EOC cell number, was significantly reduced when the level of hPygo2 was reduced by the transfection of phosphorothioated antisense oligonucleotides (oligos), which specifically targeted the non-coding sequences of hPygo2 mRNA. Cell growth arrest was prevented by the co-transfection of plasmids expressing wild-type hPygo2 protein.

In an attempt to isolate which domain(s) of hPygo2 is (are) required for Wnt-inactive EOC cell growth, I performed similar rescue experiments but co-transfected mutant hPygo2 plasmids in which critical residues within the NHD or PHD domains were altered. From these experiments, the requirement of the domain(s) of hPygo2 for EOC cell proliferation could then be determined depending on whether or not mutations introduced in this domain disrupt the ability of hPygo2 to restore growth from hPygo2-depleted SKOV-3 (Wnt-inactive) cells. In addition, the proline-rich domain linking the NHD to PHD of hPygo2 is not conserved among different species and has not exhibited any significant function in development and cancer. Therefore no mutant plasmids originating from this domain were involved in the cell rescue assays.

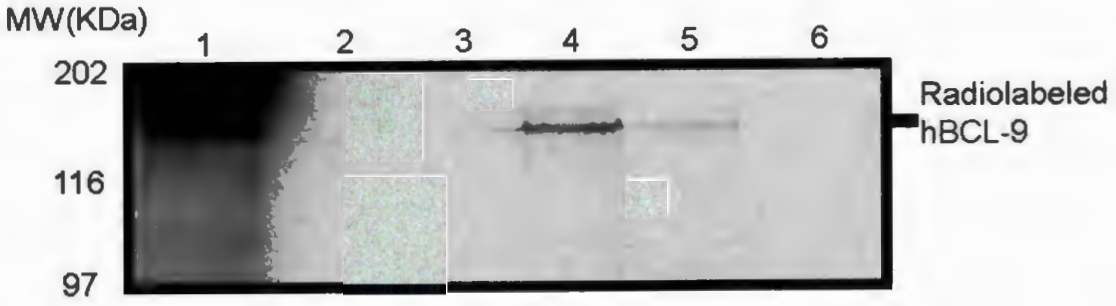
3.1.1 hPygo2 PHD mutant can not bind human BCL-9 protein

The Plant Homeodomain (PHD) of Pygopus directly interacts with the HD1 domain of Legless/BCL-9 (Hoffmans and Basler, 2004; Townsley et al., 2004), and functions as a canonical Wnt signaling-dependent co-activator for the β-catenin/TCF transcription machinery. Recent evidence also indicated that a complex comprised of the PHD of Pygo and HD1 of BCL-9 can specifically bind to methylated lysine 4 residues on histone H3 tails suggesting its involvement in the initiation step of Wnt target gene transcription (Fiedler et al., 2008).

Specifically, four residues (corresponding to hPygo2: Met361, Thr362, Ala365, Leu369) in the PHD domain were essential for the interaction of the PHD with BCL-9 in the β-catenin/TCF transcriptional activation complex (Townsley et al., 2004). Other than this association, very limited functions were demonstrated for the PHD.

Because of their importance for the function of the PHD, the four critical residues within the PHD were selectively altered to generate hPygo2 PHD mutant proteins that were unable to bind BCL-9. A single-point mutant in which Leucine at position 369 was converted to Alanine (hPygo2-L369A) and a double-site mutant in which Threonine at position 362 and Alanine at 365 were altered to Alanine and Valine respectively (hPygo2-T361A, A364V), were made as described in section 2.4.1. To validate whether these mutants abolished the association with BCL-9, GST-pull down assays were performed using GST-fused above mutant proteins to test interactions with *in vitro* translated radiolabeled human BCL-9 proteins (hBCL-9). As shown in Figure 3.1, the GST-fused wildtype hPygo2 protein (lane 4) interacted with radiolabeled hBCL-9. This level of interaction was substantially diminished to approximately 50% after introducing the L369A mutation (lane5). In contrast, the Pygo2-T362A, A365V mutant had completely lost the potential to bind BCL-9

Figure 3.1 GST Pulldown Assay: GST-PHD double point mutant abolished the interaction with human BCL-9(hBCL9). Lane 1: 10% input of in vitro translated radiolabeled hBCL-9 protein (MW: approximately 170KDa); **Lane 2 and 3:** GST alone and GST-NHD fragment proteins incubated with radiolabeled hBCL-9; **Lane 4, 5 and 6:** GST-hPygo2 wildtype, GST-hPygo2 L369A single point mutant and T362A, A365V dual-site mutant incubated with radiolabeled hBCL-9 respectively.


MW(KDa)
1
2
3
4
5
6
202
116
97
Radiolabeled
hBCL-9

(lane 6). Therefore, DNA sequences encoding the latter dual-site PHD mutant protein were subcloned into the pCS^{2+} expression vector and co-tranfected with hPy8-ON Antisense oligos to determine if it could rescue growth arrest in SKOV-3 cells (see sections 2.4.1 and 2.4.3 for the experimental design and section 3.1.3 for the cell rescue assay results).

3.1.2 Amino acid substitution of NPF to AAA abolished the transcriptional activity of hPygo2 NHD domain

The N-terminal homology domain (NHD) of Pygopus exhibits strong transcriptional potential (Stadeli and Basler, 2005). A conserved NPF tri-peptide within the NHD (corresponding to the amino acids Asn76, Pro77, and Phe78 of hPygo2) was shown to be an essential motif for this activity, since site-mutations within this motif resulted in substantial abolishment of transcriptional potential *in vitro* as well as a dramatic decline of Wnt signaling *in vivo*. Apart from this transcription potential very little activity of the NHD region was reported.

In order to investigate whether the NHD was required for EOC growth, I mutated the NPF triplet motif of the NHD into three Alanine residues (NPF->AAA). As expected from previous studies (Stadeli and Basler, 2005), DNA tethered, Gal4 dependent transcription assays (performed in collaboration with P. Andrews, Appendix 1) showed that this NPF->AAA mutant was deficient in

transactivation activity. In these assays, the NPF->AAA NHD mutant fragment was fused to the GAL4 DNA binding domain (DBD), and its ability to stimulate transcription when co-transfected with the reporter plasmid, pFR-Luc was quantified in relative luciferase unit (RLU) values. It displayed approximately equal luciferase activity as compared to background obtained from Gal4DBD alone while the RLU values were increased over 2 fold when Gal4DBD-fused to wild-type NHD was expressed. These assays confirmed that the NPF motif is required for the transcriptional activity of the NHD of hPygo2.

3.1.3 A functional NHD domain is required for rescuing cell number decrease observed with Antisense treatment

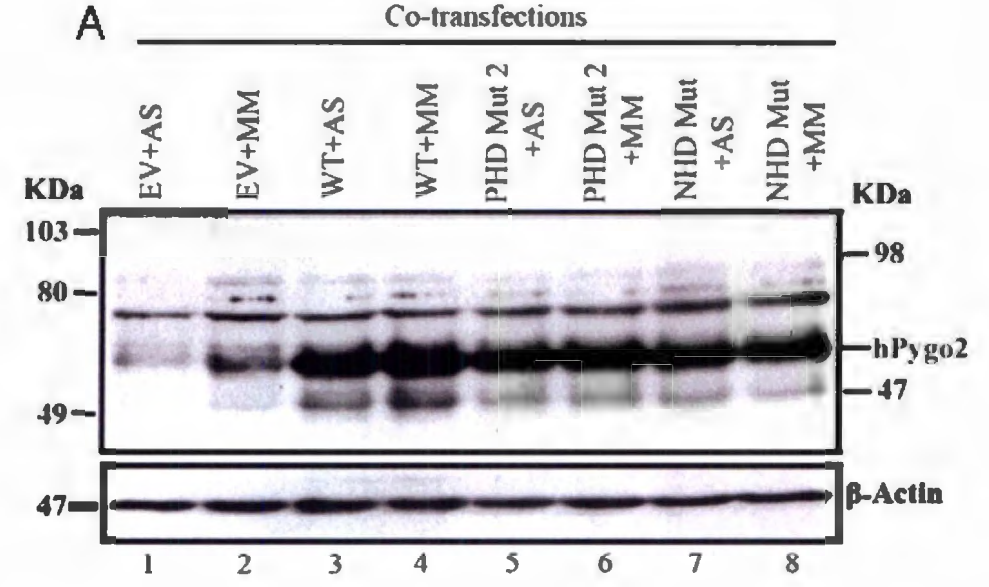
Previous studies demonstrated the specific requirement of hPygo2 for EOC growth from the observation that wildtype hPygo2 had rescued cell growth (Popadiuk et al., 2006). In the present rescue experiments, co-transfecting the wildtype hPygo2 with the hPy8-ON Antisense Oligos was therefore included as a positive control. On the other hand, co-transfection of empty vector with the hPy8-ON Antisense Oligos was used as a negative control. The co-transfection of plasmids that encode either the hPygo2 PHD mutant or the NHD mutant proteins with Antisense oligos were individually compared (controlled) by co-transfecting them with Mismatched (MM) Oligos as the MM Oligos had no effect on hPygo2

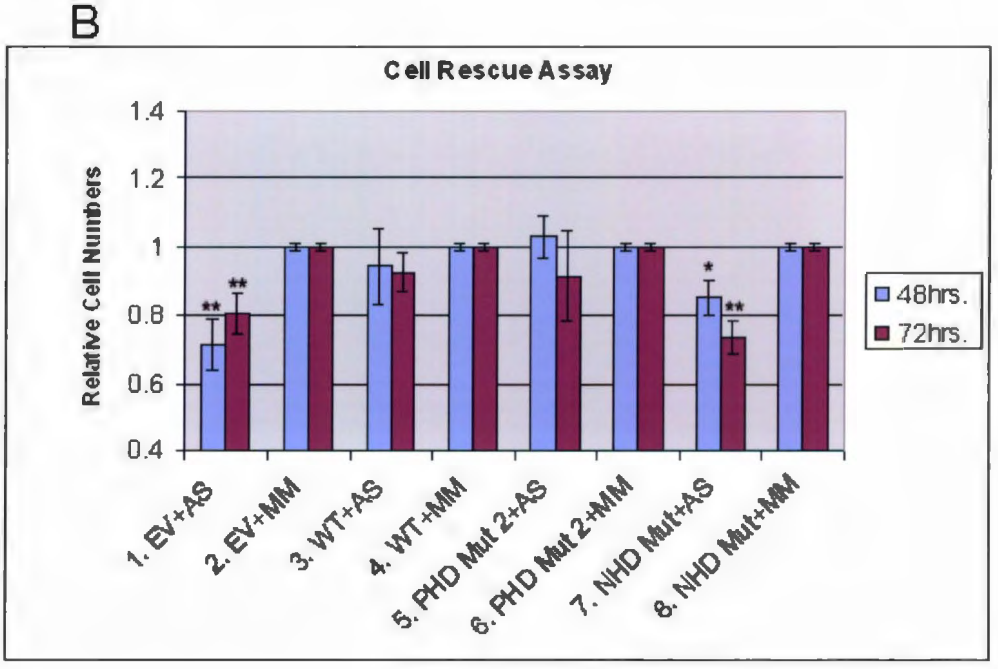
protein levels and cancer cell growth (Popadiuk et al, 2006).

Prior to determining the effects of these rescuing plasmids on cell growth arrest, western blots were performed not only to confirm that hPy8-ON Antisense oligos were able to deplete endogenous hPygo2 protein, but also to verify that the hPygo2 wildtype or mutant proteins were sufficiently expressed. As shown in Figure 3.2A, the levels of endogenous hPygo2 protein were significantly decreased by the co-transfection of pCS^{2+} empty vector (EV) with the hPy-ON8 Antisense Oligos (lane 1) as compared to the pCS^{2+} EV plus mismatched (MM) control oligos (lane 2). On the other hand, the loss of endogenous hPygo2 protein following Antisense treatment was compensated by the largely exogenous expression of hPygo2 wildtype (lane 3), PHD mutant (lane 5) or NHD mutant proteins (lane 7). Moreover, the hPygo2 wildtype (lane 4), PHD mutant (lane 6) or NHD mutant proteins (lane 8) were also significantly expressed when co-transfected with MM oligos.

The cell rescue assay results are shown in Figure 3.2B. Consistent with previous studies, the co-transfection of pCS^{2+} EV with hPy8-ON displayed nearly 30% reduction ($P<0.01$) in the number of SKOV-3 cells 48 hours after transfection and about 20% reduction ($P<0.01$) after 72 hours (lane 1) as compared with the control treatment (pCS^{2+} EV plus MM oligos, lane 2). This implied that pCS^{2+} EV, as the negative control, was unable to recover cell growth from hPygo2

Figure 3.2A. Western blot for rescue assay. Cell lysates were extracted 48hours after co-transfection. hPygo2 protein was detected with hPygo2 polyclonal rabbit antisera (about 50KDa). β-actin (47KDa) was re-probed to confirm the equal loading of total proteins. **B. Cell Rescue Assay.** Cell numbers counted in all the Antisense experimental groups were normalized by corresponding MM control groups. Colomns are the averages of three experiments repeated in triplicate; Bars, standard deviation. Unpaired t test were performed to determine statistical significance. A single star ($P<0.05$) indicates significant difference and a double star ($P<0.01$) indicates very significant difference between the Antisense experimental groups and corresponding MM control groups.


A
Co-transfections
EV+AS
EV+MM
WT+AS
WT+MM
PHD Mut 2 +AS
PHD Mut 2 +MM
NHD Mut +AS
NHD Mut +MM
KDa
103
80
49
47
KDa
98
hPygo2
47
β-Actin
1
2
3
4
5
6
7
8


B
Cell Rescue Assay
Relative Cell Numbers
1.4
1.2
1
0.8
0.6
0.4
48hrs.
72hrs.
1. EV+AS
2. EV+MM
3. WT+AS
4. WT+MM
5. PHD Mut 2+AS
6. PHD Mut 2+MM
7. NHD Mut+AS
8. NHD Mut+MM

knockdown.

The co-transfection of pCS^{2+}/hPygo2 wildtype plasmids with hPy-ON8 Antisense Oligos resulted in no significant difference (approximately 5% at 48 hours, P=0.20; and 8% at 72 hours, P=0.14) in SKOV-3 cell numbers (lane 3) in contrast to that of corresponding controls (pCS^{2+}/hPygo2 wildtype plus MM oligos, lane 4).

Similar results were observed when hPygo2 PHD mutant protein (lane 5) was expressed after antisense treatment. There appeared to be either slightly (nearly 3%, P=0.82) more cells at 48 hours or slightly fewer cells (not statistically significant, P=0.43) at 72 hours in relation to control treatment (pCS^{2+}/ hPygo2 PHD mutant plus MM oligos, lane 6),indicating that mutations on the critical residues within the PHD did not affect its ability to reverse the decrease in EOC cell numbers following antisense treatment. Thus, while the PHD domain is required for the interaction of Pygo with BCL-9, it is unlikely required for EOC cell growth, at least in the Wnt-inactive SKOV-3 cells.

When hPygo2 NHD mutant proteins were co-transfected with antisense oligos into SKOV-3 cells, there were significantly fewer cells remaining (18% at 48 hours, P<0.05; 29% at 72hours, P<0.01; Figure 3.2B, lane 7) as compared to those cells transfected with control reagents (pCS^{2+}/hPygo2 NHD mutant plus MM oligos, lane 8). These findings indicate that mutation of the

NHD, but not the PHD disabled the ability of exogenous hPygo2 to restore SKOV-3 cell numbers reduced by depletion of endogenous hPygo2.

3.2 Proteomics

The cell rescue assays described previously (Section 3.1) demonstrated the requirement of the NHD of hPygo2 protein for SKOV-3 cancer cell growth. It is possible that mutation of the NPF motif within the NHD prevents interaction of Pygo with other proteins, and that these interactions are essential for cell growth. To begin testing this hypothesis, proteomics experiments were performed to identify candidate NHD-interacting proteins. These analyses were performed in collaboration with Phillip Andrews and Keelia Farrell.

Our approach was to couple glutathione-S-transferase (GST)-pulldown assays with Mass-spectrometry. In brief, GST alone, GST fused to the NHD (GST-NHD), GST fused to the NHD mutant (GST-NHD NPF->AAA), or GST fused to the PHD (GST-PHD) were immobilized into Glutathione Sepharose beads individually and then used to probe nuclear fraction proteins of SKOV-3 cells. The nuclear proteins that specifically bound to GST-NHD, but not GST alone or GST-PHD beads, after stringent washes, were separated by SDS-PAGE and visualized by Coomassie Blue staining. The differential proteins pulled down by GST-NHD, but not GST-NHD NPF->AAA, indicate possible proteins that specifically associate with the NPF residues. These possible binding proteins were then digested, "in-gel", into peptides using the Trypsin Profile IGD digestion kit (Sechi and Chait, 1998) and analyzed by MALDI-TOF mass-spectrometry.

MASCOT software was then used to identify peptide sequences and search matched candidate proteins from the protein database at www.pubmed.com.

Mass-spectrum analysis was repeated two times and no differential protein bands were visualized when comparing GST-NHD pulldown and GST-NHD NPF->AAA pulldown. However, comparing the proteins that interact with GST-NHD, but not GST alone or GST-PHD, identified a number of candidate proteins including RNA binding protein p68, RNA helicase II/Gu, Nop56, and Treacle, which are briefly described below. These proteins that potentially interact with the NHD domain were identified in both repeated experiments.

p68 is a member of the DEXD-box (Asp-Glu-Ala-Asp) family of RNA helicases involved in RNA metabolism such as RNA splicing and splicing site selection. It may also regulate gene transcription by binding to estrogen receptor α, p53, RNA polymerase II, MyoD and CREB binding protein (CBP) respectively and thus stimulate their functions (Caretti et al, 2006; Bates et al, 2005; Guil et al, 2003; Liu 2002; Rossler et al, 2001; Rossow et al, 2003). Recent research has shown that p68 is overexpressed in colon cancer and can be a potential target for colon cancer treatment (Shin et al., 2007). The other study revealed that downregulating the p68 led to reduced splicing products of 32S pre-rRNA which demonstrated the involvement of p68 in ribosomal biogenesis (Jalal et al, 2007).

RNA Helicase II/Gu is also a member of the DEAD-box family of proteins related to p68 and p72 RNA helicases. In addition to its involvement in RNA unwinding and RNA folding, it was shown to interact with c-Jun and stimulate c-Jun-mediated gene activation (Valdez et al., 2002). It was later found to participate in rRNA processing since siRNA mediated knockdown of RNA Helicase II/Gu caused significant inhibition of 28S and 18S rRNA production. This participation was also evident through functional interaction with ribosomal protein L4 (Yang et al, 2005).

Nop56 is one of the four core small nucleolar ribonucleoproteins (snoRNPs) besides fibrillarin, Nop58 and 15.5 KDa proteins. They belong to the box C/D class of snoRNPs which direct the most common modification of ribosomal RNAs (rRNAs) known as 2'-O-Methylation (Gautier et al, 1997; Tran et al, 2003).

As a nucleolar phosphoroprotein, Treacle is encoded by *TCOF1* gene, which is mutated in individuals with hereditary Treacher-Collins Franceschetti syndrome (TCS), an autosomal dominant disorder of craniofacial development (reviewed in Dixon et al, 2007). It was found localized in the nucleolar compartment in HeLa cells, interacting with upstream binding factor (UBF, a critical transcription factor for RNA polymerase I) and required for ribosomal RNA synthesis (Valdez et al, 2004). A recent study also demonstrated its

participation in pre-RNA methylation during ribosome biogenesis (Gonzales et al, 2005a).

In summary, our proteomic analyses suggested that the NHD of hPygo2 may interact with a variety of nucleolar proteins encompassing p68, RNA helicase II/Gu, Nop56, and Treacle. Characterization of these interactions may contribute to reveal critical roles of hPygo2 in cancer cell proliferation.

3.3 Characterizing the Interaction between hPygo2 and Treacle

Nucleoli are subnuclear compartments where ribosomal RNAs (rRNAs), signal recognition particles as well as proteins involved in gene expression and cell proliferation are synthesized. It is the center of ribosomal biogenesis which involves the process of rRNA synthesis, maturation, and assembly of rRNA and ribosomal proteins into the large and small ribosome subunits (Scheer and Hock, 1999; Carmo-Fonseca et al., 2000). Many of the identified nucleolar proteins are associated with human diseases (Pederson, 1998; Visintin and Amon, 2000; Gonzales et al., 2005a, b).

In section 3.2, multiple nucleolar proteins were identified as highly probable candidates found in SKOV-3 nuclei that interact with the NHD of hPygo2. Among these candidates, Treacle protein was of particular interest due to its highest predicted possibility of interaction. This section will describe a series of analyses confirming the interaction of hPygo2 with Treacle and will begin to explore the possibility of hPygo2 participating directly in ribosome biogenesis.

The Treacle protein is encoded by the *TCOF1* gene. It is a nucleolar phosphoprotein, required not only for rRNA transcription by interacting with upstream binding factor (UBF) but also for early modification (methylation) of pre-rRNA (Gonsales et al, 2005a; Valdez et al, 2004). In humans, mutation of the *TCOF1* gene is associated with Treacher-Collins

Franceschetti syndrome (TCS) (Dixon et al, 2006), whereby affected individuals display an array of craniofacial defects (Dixon et al., 1997; Dixon et al., 2007; Isaac et al., 2000). The cellular origin of TCS is complex, but studies in mice indicate that *TCOF1* is required for rapid proliferation of neural fold cells during a critical time of brain growth. This growth requirement is considered to be linked to the role of TCOF1 protein (Treacle) in ribosomal biogenesis (Dixon et al., 2000).

The observations in TCS and in *TCOF1*-deficient mouse embryos are consistent with studies using *pygo2*$^{-/-}$ mice (Li et al., 2007; Schwab et al., 2007; Song et al., 2007), and *Xenopus* embryos in which Pygo2 was depleted using antisense morpholino technology (Lake and Kao, 2003). These Pygo-defective embryos displayed head defects analogous to both humans with TCS and *TCOF1*-defective transgenic mouse models. Taken together with our proteomics data indicating that hPygo2 interacts with Treacle, these studies supported my hypothesis that hPygo2 may be involved in ribosomal biogenesis *in addition* to, or *in lieu* of its canonical role in TCF/β-catenin-mediated transcriptional activation.

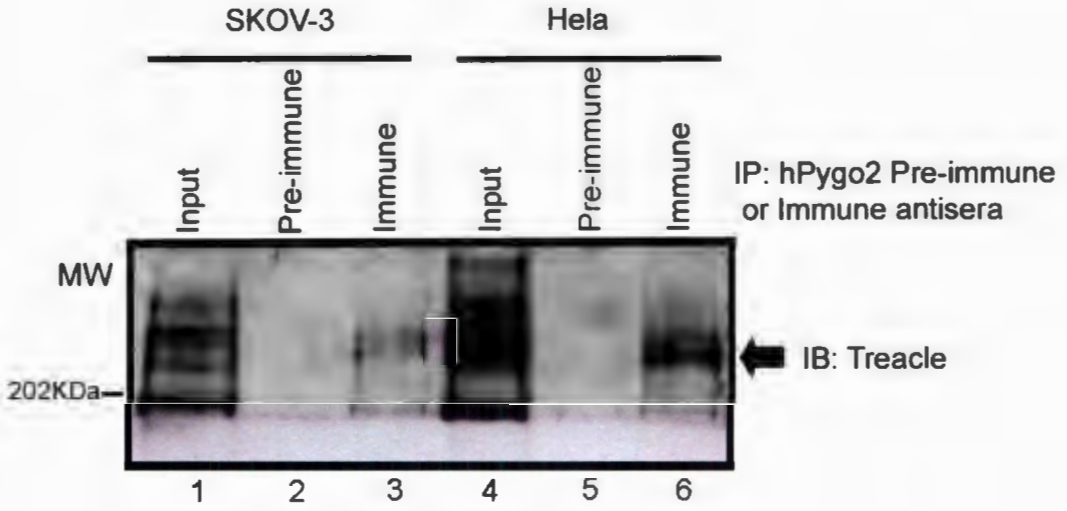
The following sections are focused on characterizing the interaction of Treacle with hPygo2, followed by further investigation of the involvement of hPygo2 in ribosomal biogenesis. This involvement may generally exist in highly proliferative cells, such as EOC (Popadiuk et al., 2006)

and breast cancer cells (Andrews et al, 2007) in which hPygo2 was shown to be required for their malignant growth. Therefore, the following studies included both (EOC) SKOV-3 cells and the well-established cancer cell line-HeLa cells derived from cervical carcinoma.

3.3.1 Treacle interacts with hPygo2 *in vivo* in SKOV-3 and HeLa cells

The physical interaction of Treacle and hPygo2 was further examined by co-immunoprecipitation (Co-IP) in SKOV-3 and Hela cells. Total SKOV-3 or Hela cell lysates were prepared and immunoprecipitated with hPygo2 polyclonal rabbit antisera (hPygo2 immune). The co-immunoprecipitated proteins were separated by SDS-PAGE and analysed for the presence of Treacle by immunoblotting with anti-treacle chicken antibody. As shown in Figure 3.3, hPygo2 immunoprecipitated endogenous Treacle proteins in SKOV-3 cells (lane 3) and Hela cells (lane 6). SKOV-3 or Hela cell lysates were directly loaded as inputs to show the presence of endogenous Treacle protein (lane 1 and 4). In contrast, pre-immune rabbit serum used at the same concentration as the immune antisera did not precipitate any Treacle protein from the SKOV-3 (lane 2) and Hela cell lysates (lane 5). The above results clearly demonstrated that Treacle and hPygo2 interact, *in vivo,* in SKOV-3 and HeLa cancer cells.

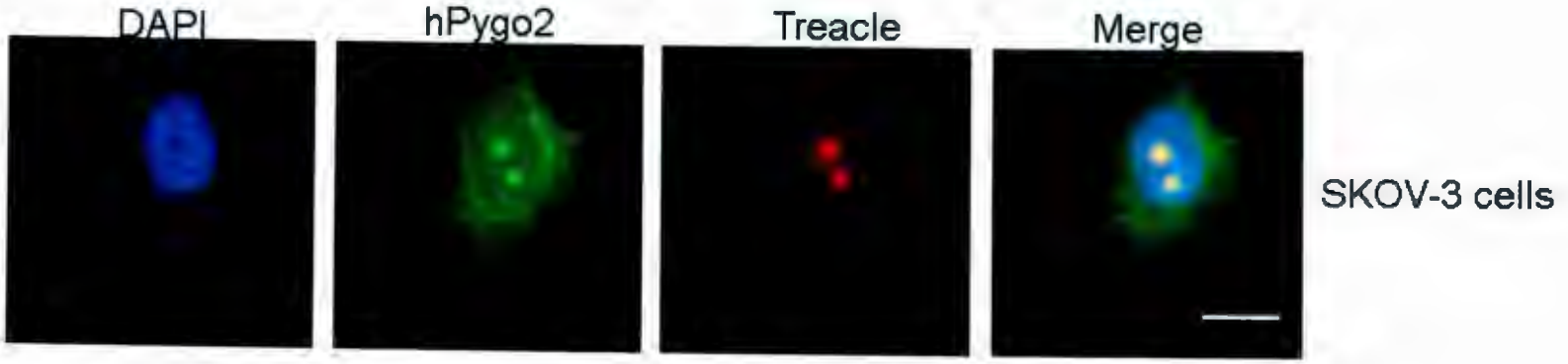
Figure 3.3 Co-immunoprecipitation (Co-IP) assay for hPygo2 and Treacle in cancer cells. SKOV-3, and HeLa cell lysates were incubated with either pre-immune rabbit serum or hPygo2 polyclonal rabbit antisera (hPygo2 immune). Co-immunoprecipitated proteins were blotted with chicken anti-Treacle IgY. **SKOV-3 cells**: lane 1, cell lysate input; lane 2, IP with pre-immune; lane 3, IP with hPygo2 immune. **Hela cells**: lane 4, cell lysate input; lane 5, IP with pre-immune; lane 6, IP with hPygo2 immune.


SKOV-3
Hela
Input
Pre-immune
Immune
Input
Pre-immune
Immune
IP: hPygo2 Pre-immune
or Immune antisera
MW
202KDa
IB: Treacle
1
2
3
4
5
6

3.3.2 Treacle colocalizes with hPygo2 in the nucleoli of SKOV-3 cells

Treacle was identified by mass-spectrometry as a candidate that interacts with the NHD domain of hPygo2 protein. To verify this possibility, the subcellular localization of both proteins was analyzed by immunofluorescence microscopy. SKOV-3 cells were fixed and incubated with hPygo2 polyclonal rabbit antisera (hPygo2 immune) and anti-Treacle chicken (IgY) antibody. The secondary antibody for the hPygo2 antiserum was a biotinylated goat-anti-rabbit immunoglobulin G (IgG), detected using fluorescein isothiocyanate (FITC)-linked Streptovidin. Cy3-conjugated to goat anti-chicken IgG was used to detect Treacle protein. Treacle was localized to nucleoli of SKOV-3 cells (Figure 3.4, red fluorescence) as expected (Valdez et al, 2004). hPygo2 (Figure 3.4, green fluorescence) was found colocalized in the nucleoli with Treacle. hPygo2 was also found in the perinuclear region of SKOV-3.

Figure 3.4. Treacle and hPygo2 colocalized in the nucleoli of SKOV-3 cells. SKOV-3 cells were indirectly stained with antibodies against Treacle (chicken anti-Treacle IgY, red) and hPygo2 (hPygo2 polyclonal rabbit antisera, Green). 4',6-diamidino-2-phenylindole (DAPI) was used to indicate nuclear staining (blue). Scale bar, 10μm.


DAPI
hPygo2
Treacle
Merge
SKOV-3 cells

3.3.3 Mapping protein interaction domains

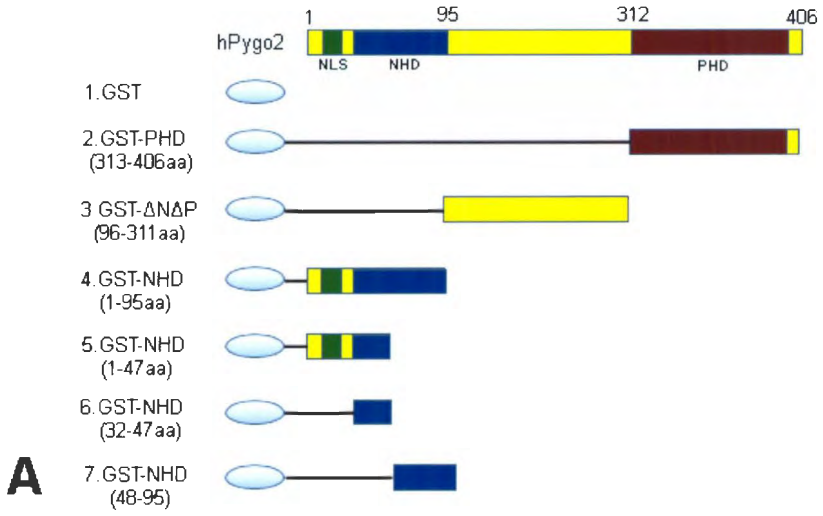
The previous sections provided evidence that hPygo2 interacted with Treacle in cancer cells: immunofluorescence revealed that both proteins colocalized to nucleoli and Treacle co-immunoprecipitated in cell lysates using antisera directed specifically against hPygo2. In this section, I described analyses where I used GST pulldown assays coupled with *in vitro* translated S^{35} radiolabeled proteins to map the domains and/or regions required for the interaction between the two proteins. Mapping data is useful to determine the specific residues or protein sequence domains that are required for the function of the protein.

Deletion mapping was performed to determine the sites within the NHD that are required for its interaction with Treacle (Figure 3.5). A series of truncated DNA sequences encoding fragments of the NHD (within residues 1-95) were subcloned into pGex 4T-1 plasmid vectors. DNA sequences encoding the hPygo2 PHD (residues 312-406) or intermediate non-conserved domains (ΔNHDΔPHD) (residues 96-311) were also subcloned into the pGex 4T-1 vector to be used as negative controls. Corresponding GST-fusion proteins were produced as described as in section 2.5.3 (Figure 2.4 and Figure 3.5A).

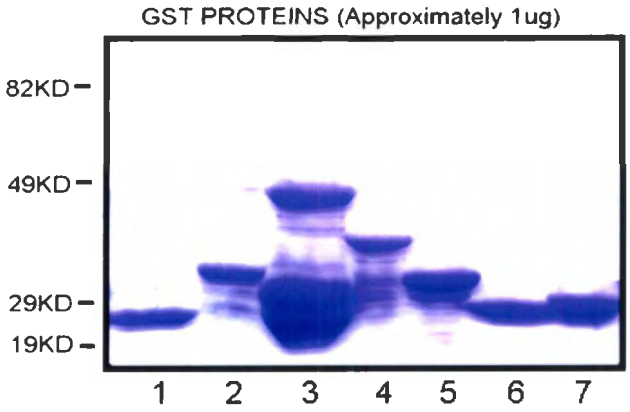
A cDNA clone containing the coding sequence of human *TCOF1*, 5' and 3' untranslated regions (cDNA in pCMV-SPORT6 vector, Clone ID: 3921754) was purchased from Open Biosystems, Thermo Fisher Scientific,

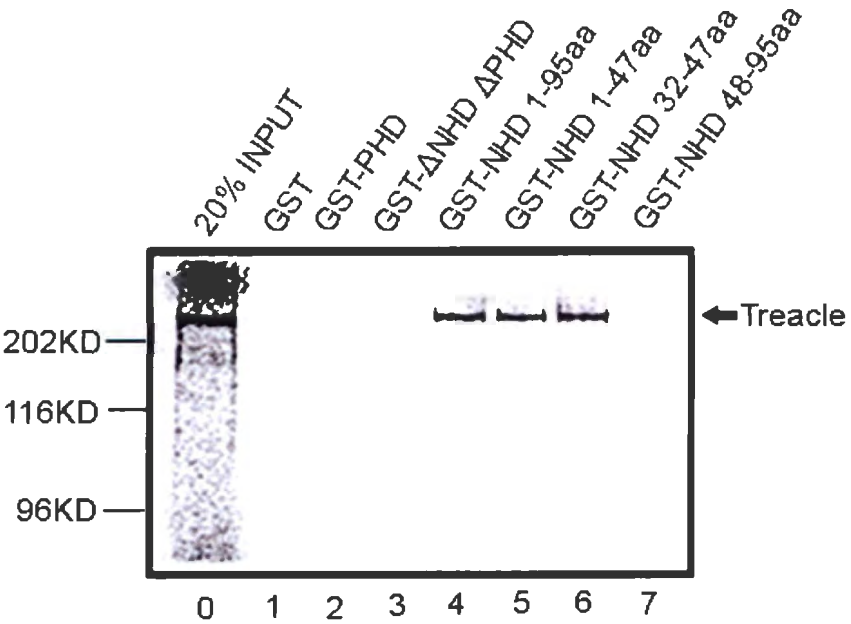
Huntsville, AL. Radiolabeled full length Treacle was translated, *in vitro,* from this clone and then used to test all the GST-fusion proteins via autoradiography. It was loaded without incubation with any GST-fusion proteins as an input shown in lane 0 of Figure 3.5B. The result clearly showed that GST-NHD (Figure 3.5B, lane 4), but not GST alone, GST-PHD and GST-ΔNHDΔPHD (lane 1, 2, 3 respectively), could pull down *in vitro* translated Treacle protein. In addition, GST fused to residues 1 to 47 (GST-NHD$^{aa\ 1-47}$, lane 5) and residues 32 to 47 (GST-NHD$^{aa\ 32-47}$, lane 6) of the NHD, but not GST-NHD$^{aa\ 48-95}$ (lane 7), were able to pull down Treacle protein. The minimal region within the NHD required for interaction with Treacle, therefore, was narrowed down from the whole NHD domain to the region encompassing amino acid residues 32-47.

Figure 3.5 Deletion mapping-GST Pulldown Assay. A. Schematic of the successive deletions of hPygo2 fused into GST. Approximately 1μg of each GST-fusion product was examined on the gel. **B.** The GST fusion products and in vitro translated Treacle protein were used for Pulldown Assay. ^{35}S labeled Treacle proteins which were pulled down can be presented in corresponded position by autoradiography. Lane 1: Treacle 20% input; Lane 2: GST; Lane 3: GST-PHD; Lane 4: GST-ΔNHDΔPHD; Lane 5: GST-NHD; Lane 6: GST-NHD 1-47aa; Lane 7: GST-NHD 32-47aa; Lane 8: GST-NHD 48-95aa.


1
95
312
406
hPygo2
NLS
NHD
PHD
1.GST
2.GST-PHD
(313-406aa)
3 GST-ΔNΔP
(96-311aa)
4.GST-NHD
(1-95aa)
5.GST-NHD
(1-47aa)
6.GST-NHD
(32-47aa)
7.GST-NHD
(48-95)

A


GST PROTEINS (Approximately 1ug)
82KD
49KD
29KD
19KD
1
2
3
4
5
6
7

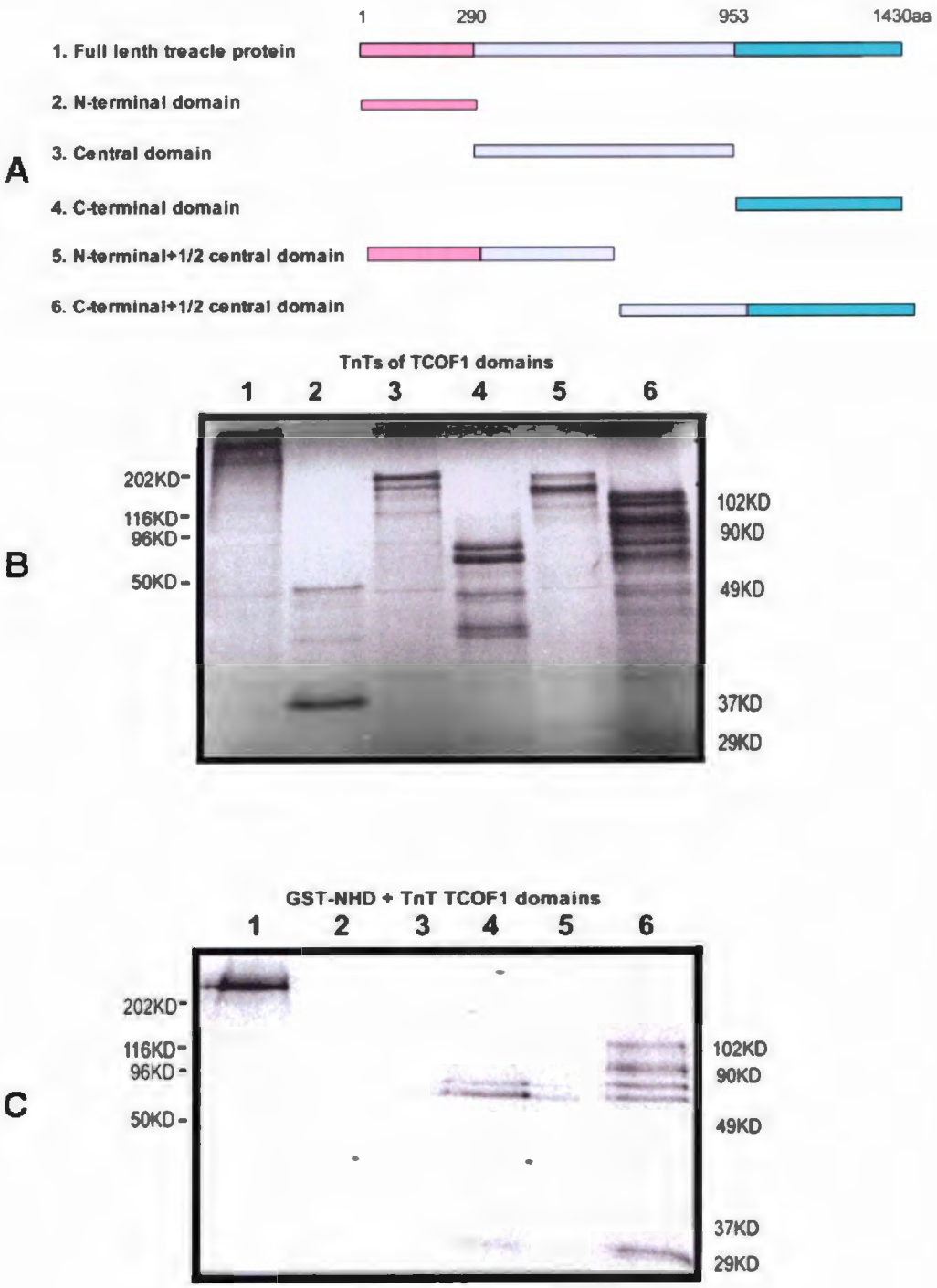

20% INPUT
GST
GST-PHD
GST-ΔNHD ΔPHD
GST-NHD 1-95aa
GST-NHD 1-47aa
GST-NHD 32-47aa
GST-NHD 48-95aa
Treacle
202KD
116KD
96KD
0
1
2
3
4
5
6
7

B

The above in vitro GST-pulldown assays suggested that Treacle can interact with a region consisting of 15 residues (residues 32-47) within the NHD domain of hPygo2. I next asked which region of Treacle is required for its association with hPygo2. To answer this question, DNA sequences encoding separate domains of Treacle were amplified using previous pCMV-SPORT6/*TCOF1* construct as a template, cloned into the pCS^{2+} plasmid vector and translated *in vitro* into radiolabeled protein fragments. As shown in Figure 3.6B, multiple bands were detected for each *in vitro* translated protein fragment. One possible cause for the lower molecular mass proteins is due to partial protein degradation. Although multiple bands were detected, each uppermost (highest molecular weight) band is well matched to the expected size for each protein fragment.

The GST-NHD fusion proteins were immobilized into Glutathione Sepharose beads and incubated with the radiolabeled Treacle fragments in a GST-pulldown assay. As shown in Figure 3.6C, *in vitro* translated and radiolabeled full length Treacle (1-1411aa, lane 1), C-terminal (954-1411, lane 4) and C-terminal+ last partial central domains (667-1411aa, lane 6) are able to bind the GST-NHD. On the contrary, neither the N-terminal (1-290aa, lane 2) nor the central (291-953, lane 3) domains of Treacle as well as N-terminal+the first partial central region (1-666, lane5) can associate with GST-NHD. These results indicated that the C-terminal domain (954-1411aa) of

Figure 3.6A.B. In vitro translated Treacle fragments. Different fragments of Treacle domains (A) were in vitro translated, loaded on SDS-PAGE gel and visualized by autoradiography (B). This B panel represents 20% input for the GST pulldown shown in C. **C. Interaction of the hPygo2 NHD domain and Treacle fragments- GST Pulldown Assay.** The GST-NHD fusion protein was used to test all in vitro translated radiolabeled full length Treacle or Treacle fragments as below: lane1, Full-length Treacle; lane2, N-terminal domain; lane3, Central domain; lane4, C-terminal domain; lane5, N-terminal+first half Central domains; lane6, C-terminal+last 1/3 Central domains of Treacle.


A
1
290
953
1430aa
1. Full lenth treacle protein
2. N-terminal domain
3. Central domain
4. C-terminal domain
5. N-terminal+1/2 central domain
6. C-terminal+1/2 central domain
B
TnTs of TCOF1 domains
1 2 3 4 5 6
202KD
116KD
96KD
50KD
102KD
90KD
49KD
37KD
29KD
C
GST-NHD + TnT TCOF1 domains
1 2 3 4 5 6
202KD
116KD
96KD
50KD
102KD
90KD
49KD
37KD
29KD

Treacle protein is required for its interaction with the hPygo2 NHD.

3.3.4 hPygo2, Treacle and UBF co-segregated in Hela cells treated with Actinomycin D (AMD)

A great deal of detailed information on the structure and function of the nucleolus as the machinery for ribosomal biogenesis has accumulated for many decades (Olson, 2004). The nucleolar compartment contains three ultrastructural subregions: the core region termed the fibrillar centre (FC), the dense fibrillar component (DFC) surrounding the FCs, and the most outer layer termed granular component (G). The transcription of ribosomal DNA (rDNA) by RNA polymerase I mostly occurs either in the FC or at the border of FC and DFC (Boisvert et al, 2007; Huang, 2002). The synthesized ribosomal RNA (rRNA) precursors including the 47S and 45S pre-rRNA transcripts undergo modifications, which typically include methylation and pseudouridylation. The modified precursors are then cleaved into 28S, 5.8S or 18S rRNA products mainly within the DFC region by recruiting early processing factors known as snoRNPs. The late processing events take place at the outer most layers, the GC region where cleaved rRNAs are assembled with ribosomal proteins into ribosome subunits. Assembled ribosome subunits are subsequently exported into nucleoplasm and cytoplasm for generating ribosomes (reviewed in Boisvert et al, 2007).

The ultrastructural organization of the nucleolus is related to the transcriptional activity of RNA polymerase I which was demonstrated by Actinomycin D experiments (Shav-Tal et al., 2005; Unuma et al, 1973). Actinomycin D (AMD) is a chemotherapy drug that directly binds to rDNA and inhibits the transcriptional activity of RNA polymerase I. It is well established that AMD treatment causes nucleolar components within the fibrillar (FC and DFC) subregions to re-orient into nucleolar "caps", which segregate from the granular(GC) components (Shav-Tal et al., 2005). The change in the localization of a protein upon AMD treatment depends on the functional step with which it is involved during ribosomal biogenesis (Smetana et al, 2001; Unuma et al, 1973).

Upstream binding factor (UBF) combined with other basic RNA polymerase I factors form the transcriptional initiation complex with RNA polymerase I. Previous research used UBF as a contrast for investigating the localization of Treacle protein in HeLa cells with AMD treatment via immunofluorescence (Valdez et al, 2004). It has been shown that Treacle only co-segregated with upstream binding factor (UBF), but not with the rRNA processing factor RNA helicase II/Guα (one of the GC components), into the same subregion of nucleoli in HeLa cells treated with 50ng/ml AMD for 2 hours. This study therefore suggested that Treacle was more likely to act as a fibrillar component that facilitates rRNA transcription or early pre-RNA

processing (modification) than functioning in rRNA late processing events such as splicing, cleavage or ribosome subunit assembly and export (Valdez et al, 2004). Later studies verified that Treacle was required for rDNA transcription and involved in pre-rRNA methylation (Gonzales et al., 2005a).

In this study, four nucleolar components including UBF, Treacle, Fibrillarin and B23 were used as markers to identify the subnucleolar localization of hPygo2. While UBF and Treacle represent transcription and pre-rRNA modification (methylation) factors (Valdez et al, 2004; Gonzales et al, 2005a), Fibrillarin is mostly involved in early splicing of pre-rRNA (Amin et al., 2007; Chen and Huang, 2001; Tollervey et al, 1991), which is localized in the DFC region of the nucleolus. In contrast, B23 protein as a GC component is only recruited in late processing events and ribosome subunit assembly (Smetana et al, 2001).

After AMD treatment (50 ng/ml AMD for 2 h) in HeLa cells, the above four markers were co-immunostained with hPygo2 individually and then viewed under confocal scanning fluorescence microscopy. As shown in Figure 3.7, hPygo2 colocalized with UBF, Treacle and Fibrillarin, but not B23 in the nucleoli of untreated HeLa cells. In AMD treated HeLa cells, hPygo2 co-segregated with Treacle and UBF to the same region at the nucleolar caps, which was distinct from Fibrillarin. B23 also re-localized to the nucleoplasm but was organized circumferentially around the nucleoli. It did not

Figure 3.7. hPygo2 co-segregated with Treacle and UBF in the Hela cells treated with AMD. HeLa cells were treated with 50ng/ml actinomycin D for 2h, stained by indirect immunofluorescence and observed by confocal microscopy. Nucleoli (arrows) were shown in nuclei by Differential interference contrast (DIC) images. (**A**) Treacle (red) localization in untreated and treated cells is compared with hPygo2 (green); (**B**) UBF (red) localization in untreated and treated cells is compared with hPygo2 (green); (**C**) Fibrillarin (red) localization in untreated and treated cells is compared with hPygo2 (green); (**D**) B23 (red) localization in untreated and treated cells is compared with hPygo2 (green). Scale bar, 10μm.

co-segregate with any of the markers, including hPygo2. These results suggested that hPygo2 is localized to the fibrillar region (FC and DFC), but not the GC subcompartment of nucleoli.

Taken together with my results demonstrating strong interaction of hPygo2 with Treacle, these observations suggested that hPygo2 may be involved in ribosomal biogenesis by participating in either rDNA transcription or pre-rRNA modification.

CHAPTER 4
DISCUSSION

hPygo2 protein is overexpressed and required for the growth of epithelial ovarian malignancies in the absence of Wnt signaling. Here in my thesis, I further identified that the N-terminal homology domain (NHD) of hPygo2, but not the Wnt-mediating PHD domain, is essential for the Wnt-independent EOC cell proliferation. In addition, I verified the strong interaction between the hPygo2 NHD region and the Treacle protein. These results suggest that one of the Wnt-independent functions of hPygo2 is involved in ribosomal biogenesis.

4.1 Cell Rescue Assay

To investigate the potential roles of hPygo2 protein in the absence of Wnt signaling, I have conducted a cell rescue assay to determine which domain of hPygo2 is required for Wnt-inactive cell growth. In this assay plasmids expressing either hPygo2 or hPygo2 mutants were co-transfected with Antisense Oligos to observe whether they can recover the growth of hPygo2-depleted cells.

The SKOV-3 cell line represents a Wnt-inactive EOC cell line, and was used as a model for rescue assays and proteomics assays to investigate the Wnt-independent mechanism of hPygo2 protein for cancer growth. One of the plasmids that rescued growth of Antisense Oligo-transfected cells was a version of hPygo2

that contains alterations on the critical residues required by hPygo2 to interact with lgs/BCL-9 within the PHD domain (Townsley et al., 2004). One possible caveat for using this plasmid in a rescue assay is that the critical residues may also be used by hPygo2 for its interactions with proteins besides BCL-9. Therefore, in the event that the PHD mutants could not rescue cell growth, I would not be able to distinguish whether it was due to the abolishment of the interaction with BCL-9 or with other, unknown proteins. However, this issue was largely abrogated since the rescue assay results indicated that the PHD mutant did efficiently rescue cell growth, similar to results obtained with the positive control-hPygo2 wildtype plasmid, suggesting that the interaction of hPygo2 with BCL-9 and its involvement in Wnt signaling are not necessary for EOC cell growth.

The same issue may also be applied to the NHD mutant rescuing plasmid. The rescue assay results, however, showed that the NHD domain containing alterations in NPF motif, which previously was shown to be required for the transcriptional activity of hPygo2 (Stadeli and Basler, 2005), substantially lack the ability to rescue SKOV-3 cell growth, suggesting that it is required for growth.

Another consideration with the rescue assay results is that the knockdown effects by Antisense Oligos hPy-ON-8 were not ideal (approximately 20%-30%) even they are statistically significant. It might because those AS Oligos

were targeted to 3'-untranslated region (UTR), and not the coding region, and may not be able to efficiently suppress hPygo2 mRNA. Alternatively, small interference RNAs (siRNA) or small hairpin RNAs (shRNAs) targeting hPygo2 mRNA could also be used to downregulate hPygo2 expression through a RNA interference pathway. (Chen et al., 2009; Hamilton and Baulcombe, 1999; Simpson et al., 2008)

The western blotting assay was used to determine hPygo2 protein levels. In these analyses, it was not possible to distinguish the endogenous from exogenous hPygo2 protein because they were expressed at the same size in the experimental groups 3-8 (Figure 3.3A, lane 3-8). Alternate approach would be to use an epitope-tag-containing vector (Chavand et al., 2001) instead of the pCS^{2+} vector to construct rescuing plasmids and then the tagged-wildtype hPygo2 or tagged-mutant hPygo2 could be discerned from the endogenous protein by size or by an antibody specifically against the tag. However, the tag itself may affect the function of hPygo2 protein in cancer cells and therefore may significantly influence its rescuing ability. More tests using different tag-hPygo2 rescuing plasmids should be conducted.

4.2 hPygo2 Interacting proteins

The current understanding of the function of hPygo2 is that it is not

only a co-activator in the canonical Wnt signaling pathway but also a transcriptional regulator through Wnt-independent mechanisms (Hoffmans and Basler, 2004; Townsley et al., 2004; Popadiuk et al., 2006; de la Roche and Bienz, 2007; Song et al., 2007). The possibility of hPygo2 involvement in a Wnt-independent mechanism may due to transcriptional activity of its NHD. My cell rescue assay results suggested the importance of the NHD, especially the NPF motif, for EOC cell growth.

I hypothesized that there are factors that specifically bind to the NPF residues required for the function of hPygo2. The GST fusion proteins including GST-NHD or GST-NHD NPF-AAA mutant were used to probe nuclear extracts of SKOV-3 cells, and I attempted to determine the proteins that bound to the NPF. However, perhaps due to the limited separation rate of one dimensional small-sized SDS-PAGE gels and the low resolution of visualization by Coomassie blue staining, it was not possible to distinguish any protein bands that associated with the GST-NHD exclusive of the GST-NHD NPF-AAA mutant. Future experiments may involve the use of 2-dimension gel electrophoresis and/or staining approaches with higher resolution such as the silver staining method (Mortz et al., 2001).

Although I was not able to find NPF specific binding factors up to present, in collaboration with my colleagues, we nonetheless identified several candidate proteins using proteomics technology including p68, RNA helicase

II/Gu, Nop56, and Treacle that interacted with the NHD domain of hPygo2. p68 and RNA helicase II/Gu both belong to the DEAD box (Asp-Glu-Ala-Asp) family of RNA helicases (Cordin et al., 2006). p68 is increasingly expressed during benign to malignant transformation in the colon which is very similar to the up-regulation of hPygo2 protein in EOC cells as compared to normal ovarian epithelial cells. p68 interacts with β-catenin directly and enhanced the ability of β-catenin to activate Wnt target gene transcription, suggesting that p68 is very important for the activation of Wnt signaling in colon cancer (Shin et al., 2007). However, whether it interacts directly with hPygo2 in EOC has not been characterized. Furthermore, whether it plays the same crucial roles in the Wnt-inactive EOC cells as in the colon cancer, or whether its interaction with hPygo2 can promote oncogene transcription for EOC progression, needs to be resolved.

RNA helicase II/Gu is one of the nucleolar proteins whose function mainly involves RNA unwinding. It also has been identified as a cofactor for c-jun-activated transcription (Valdez et al., 2002). It was reported to be involved in ribosomal RNA processing because its suppression by siRNA led to an 80% reduction in both 18S and 28S rRNA production. The underlying mechanism for this effect has not been identified (Yang et al., 2005). However, the function of RNA helicases Gu in ribosomal RNA processing may relate to another hPygo2 interacting protein-Treacle which was characterized in this thesis and it is possible

that this Gu protein forms a complex with Treacle and hPygo2 to affect ribosomal biogenesis in cancer cells. Therefore, whether it is colocalized with hPygo2 and whether it interacts with hPygo2 to cooperatively regulate c-jun gene transcription and/or ribosomal RNA synthesis for cancer proliferation need to be further identified.

In addition, Nop56 is one of the core small nucleolar ribonucleoproteins (snoRNPs) belonging to the box C/D class of snoRNPs which direct the most common modification of ribosomal RNAs (rRNAs) known as 2'-O-Methylation (Gautier et al, 1997; Tran et al, 2003). Proteomic analyses from other studies identified the interaction of Treacle with Nop56 associated pre-ribosomal complexes (Hayano et al., 2003). Therefore, verification of the association of hPygo2 with Nop56 will suggest its possible involvement in pre-rRNA modification (methylation).

Nucleoli are very important subcellular compartments for producing ribosomal RNAs, signal recognition particles and regulatory proteins involved in cell proliferation. Many nucleolar proteins can regulate mitosis, cell-cycle progression and proliferation, relating the nucleoli to human disease. Therefore, the colocalization of Treacle and hPygo2 in the nucleoli and their *in vivo* association as determined by Co-IP assays in SKOV-3 and HeLa cells raises the intriguing possibility that their interaction plays critical roles for cancer

progression. I will discuss the significance of the interaction between hPygo2 and Treacle in the next section.

4.3 The Significance of Interaction between hPygo2 and Treacle

TCOF1 encodes a nucleolar phosphoprotein called Treacle, which is mutated in patients with hereditary Treacher-Collins Franceschetti syndrome (TCS), an autosomal dominant, congenital craniofacial disorder. Most *TCOF1* mutations identified in TCS patients likely result in truncated treacle products because of the occurrence of premature stop codons. TCS is suggested to be caused by haploinsufficiency or dominant-negative effects, which result in loss-of-function and rapid digestion of truncated Treacle protein products (Dixon et al., 2007; Isaac et al., 2000).

The full length Treacle protein is spatiotemporally expressed in the neuroepithelium throughout the E8 stage and more strongly in the frontonasal and branchial arch mesenchyme at E9.5 in the mouse embryo (Dixon et al., 2006). It is required for the transcription of ribosomal RNA and the methylation of pre-rRNA in the growth of the developing anterior nervous system (Valdez et al., 2004; Gonzales et al., 2005a).

Similarly, mouse Pygopus2 (mPygo2) is expressed starting at day E7 and peaks at day E17 in the mouse embryo. It was observed within multiple

regions in the developing brain with the strongest expression at the mesencephalon (Li et al., 2004). Moreover, mPygo2-/- mice have severe brain defects or exencephaly phenotypes (Schwab et al., 2007) which resemble those of TCOF-/+ embryos (Dixon et al., 2006). The interaction of Treacle and hPygo2 characterized in this thesis is consistent with interdependent roles in embryonic development. The requirement of treacle for ribosomal RNA transcription in development and cancer cell lines (Valdez et al., 2004) suggests the intriguing possibility that a Wnt-independent role of hPygo2 in cancer may relate to a role in Treacle-dependent rRNA transcription in development of the mammalian nervous system..

Further studies on the requirement of the interaction between Treacle and hPgyo2 proteins for EOC cell proliferation as well as head development are warranted.

4.4 Future Directions

Studies in my thesis indicated that the NHD domain of hPygo2 was required for Wnt-independent cancer cell growth and Treacle was identified as an NHD-interacting protein. Therefore, in future studies, an initial experiment to perform would be one designed to determine the cell growth requirements of the hPygo2-Treacle interaction. The minimal residues in the NHD of hPygo2 required for its interaction with Treacle could be identified. Similarly, identification of the

minimal residues within the C-terminal domain of Treacle protein required for interacting with hPygo2 will be necessary. Mutations of either interaction sites will be used in cell rescue assays as previously described in section 2.1. The results will be able to indicate if the interaction between hPygo2 and Treacle is required for cancer cell proliferation.

Interestingly, the preliminary studies revealed that hPygo2 interacts with the C-terminal domain of Treacle from which most mutations in humans causing truncated proteins occurred. In addition, this domain is thought to be responsible for the nucleolar localization of Treacle protein (Winokur et al., 1998). Given the putative role for Pygopus as a nuclear "anchor" for transcriptional complexes (Townsley et al., 2004), it is possible that truncated treacle proteins resulting from C-terminal deletions fail to interact with Pygopus and therefore lose the ability to localize to nucleoli. Therefore, the requirement of the Pygo-Treacle interaction for retention of Treacle in the nucleoli should be evaluated. For instance, experiments could be designed to determine if the knockdown of hPygo2 protein by Antisense Oligos or siRNAs prevents the normal localization of Treacle to nucleoli in cancer cells. Similarly, we could observe the localization of Treacle protein in $Pygo^{-/+}$ transgenic mice.

Lastly, my immunofluorescence, co-IP and actinomycin D experiments showed that hPygo2 interacted with Treacle in cancer cells and co-

segregated with Treacle and UBF, but not fibrillarin or B23 following AMD treatment, suggesting that hPygo2 may be involved in rRNA transcription or early processing rather than late processing events. In order to determine if hPygo2 is involved in rRNA transcription, Antisense Oligos or siRNAs to knockdown hPygo2 can be transfected into SKOV-3 and HeLa cells. Production of rRNA could be determined by nuclear run-on-assays of the 45S pre-rRNA (Glibetic et al., 1995), pulse-chase metabolic labeling of pre-rRNA (Hennning et al., 2003), or by RNase protection assay (Yang et al., 1998). Moreover, to investigate possible roles of hPygo2 in pre-rRNA methylation, pre-rRNA methylation assays can be carried out as described (Gonzales et al., 2005a) following hPygo2 knockdown. In these assays, the reduction in pre-rRNA production or pre-rRNA methylation levels will be expected when cancer cells are transfected with hPygo2 antisense oligos or siRNA.

CHAPTER 5
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Appendix

Transcriptional Reporter Assays

To determine transcription activity, Luciferase Assasys were performed as described (Andrews et al., 2008). The reporter plasmid, pFR-Luc (Stratagene), consists of the luciferase gene driven by Gal4-binding sites and a basic transcriptional promoter (TATATA). The genes of interest were cloned into pM-Gal4 (Clontech) plasmid vectors expressing Gal4 DNA binding domain (Gal4DBD). When pFR-Luc is transfected into cancer cells, little or no luciferase is expressed as the background expression level for the cell line used. When it was co-transfected with previously constructed pM-Gal4 plasmids, luciferase gene expression was measured, resulting from the association of Gal4-binding sites with the Gal4DBD in fusion with protein products encoded by the genes of interest. Therefore, the transcriptional activity of genes of interest can be quantified by relative luciferase activity.

For this thesis, MCF-7 cells were seeded in triplicate 24 hours before transfection at a density of $1.5x10^5$ per well in 12-well plates. pM-Gal4 plasmids containing the genes of interest were co-transfected with pFR-Luc reporter plasmids into cells using Lipofectamine and Plus Reagent (Invitrogen). pRSV-βgal plasmids (Promega) expressing β-galactosidase to determine transfection efficiency were also co-transfected. Luciferase activities were measured using a

luciferase assay reagent (Promega) and a Monolight 2010 Luminometer 48 hours after transfection according to the manufacturer's directions. Relative luciferase unit (RLU) values were normalized to β-galactosidase activity in each sample.

Appendix Figure: NHD NPF-AAA mutant lost transcription activity determined by a transcriptional reporter assay. Gal4DBD alone (Column 1), Gal4DBD in fusion with NHD wildtype (Column2), and Gal4DBD in fusion with NHD NPF-AAA mutant protein (Column 3) were co-transfected with pFR-Luc reporter plasmids into MCF-7 cells. RLU were measured and normalized to β-galactosidase activity in each sample. (Data from Mr. Phillip Andrews)

Appendix Figure