THE ROLE AND REGULATION OF CYCLIN G2 IN HUMAN OVARIAN CANCER CELLS

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ABSTRACT

Epithelial Ovarian Cancer (EOC) is the leading cause of cancer related death associated with gynecological malignancies. Survival is greatly impeded by poor screening methods, non-specific symptoms, and limited knowledge of the cellular targets that contribute to disease. Cell division is under direct regulation of the cyclin family. Typical cyclins will accumulate periodically to activate cyclin-dependent kinases (CDKs) leading to the unidirectional flow of the cell cycle, whereas the unconventional G-type cyclins (cyclin G1, G2, and I) act to oppose cell cycle progression. Indeed, dysregulation of the cycle cell is an important molecular mechanism that is frequently altered in cancers, including EOC. Interestingly, recent evidence has suggested that the loss of cyclin G2 is associated with cancer progression and poor survival. In this study, we have investigated the role and regulation of cyclin G2 in EOC cells. We found that cyclin G2 overexpression decreases the overall tumor burden by reducing proliferation, migration, and invasion. Interestingly, these anti-tumorigenic effects are mediated, at least in part, by enhancement the epithelial phenotype via attenuation of β-catenin signaling. Cyclin G2 promotes the degradation of β-catenin and directs its sub-cellular localization to the membrane, possibly through up-regulation of E-cadherin. In addition, we have previously shown that cyclin G2 is highly unstable, and degraded very quickly by the ubiquitin-proteasome pathway. This study also suggests the calpain-mediated proteolysis is a major factor that contributes to the degradation of cyclin G2. This process is dependent on the presence of the cyclin G2 C-terminal PEST motif, as well as epidermal growth factor signalling. Together, these data suggest that the loss of cyclin G2 in human malignancies, possibly through growth factor signaling and multiple downstream degradation processes, may contribute to the increased tumorgenicity of ovarian cancer cells.

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LIST OF ABBREVIATIONS

ALK7	Activin receptor-like kinase 7
APC	Adenomatous polyposis coli
ATP	Adenosine triphosphate
APC/C	Anaphase promoting complex/cyclosome
AKAPs	AT Rich Interactive Domain 1A (SWI-Like)
Bub	Budding uninhibited by benomyl
CIP	Calf intestinal phosphotase
CANP	Calpain
CA-125	Cancer antigen-125
CK1	Casein kinase 1
CKII	Casein Kinase 2
Cip/Kip	CDK interacting protein/Kinase inhibitory protein
Cdc	Cell division cycle
CTD	C-terminal domain
CSMD3	CUB and Sushi multiple domains 3
cAMP	Cyclic adenosine monophosphate
CDK	Cyclin dependent kinase
CCNE	Cyclin E
CKI	Cyclin-dependent kinase inhibitor
CAK	Cyclin-dependent kinase-activating kinase
CCNG2	Cycllin G2
CHX	Cycloheximide
DAPI	4',6-Diamidino-2-Phenylindole, Dilactate
DMSO	Dimethyl sulfoxide
Dvl	Disheveled
DTT	Dithiothreitol
DDR	DNA damage response
E2F	E2 Transcription Factor
E-CAD	E-cadherin
δEF1	δ-E2-box factor 1
EGF	Epidermal Growth Factor
EGFR	Epidermal growth factor receptor
EGFRK	Epidermal growth factor receptor kinase
EOC	Epithelial ovarian cancer
EMT	Epithelial-to-mesenchymal transition
EPO-R	Erythropoietin receptor
ER	Estrogen Receptor
EGTA	Ethylene glycol tetraacetic acid
EDTA	Ethylenediaminetetraacetic acid

ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
FAP	Familial adenomatous polyposis
FSP1	Fibroblast-specific protein
FSH	Follicle-stimulating hormone
FOX	Forkhead box protein
FRE	FOXO Response Element
FBE	FoxO3a-binding element
G-phase	Gap phase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GSK3	Glycogen synthase kinase 3
GADD45	Growth arrest and DNA damage 45
HGSC	High grade serous ovarian carcinoma
HMGA2	high mobility group AT-hook 2
HRT	Hormone replacement therapy
HER2	Human epidermal growth factor receptor 2
HE4	Human epididymis protein 4
HIF1α	Hypoxia-inducible factor 1-α
ICAT	Inhibitor of β-catenin
IGF	Insulin-like growth factor
KRAS	Kirsten rat sarcoma viral onco gene
LRP	Low density lipoprotein Receptor
LGSC	Low grade serous ovarian carcinma
LMP	Low malignant potential
LH	Luteinizing hormone
Lef	lymphoid enhancer-binding factor
MEK	MAPK/ERK kinase
MMP	Matrix metalloproteinase
MECOM	MDS1 and EVI1 complex locus
MET	Mesenchymal-to-epithelial transition
MES	Mesothelin
mRNA	Messenger ribonucleic acid
miRNA/miR	microRNA
MAPK	Mitogen-activated protein kinase
Mad	Mitotic arrest deficient
M-phase	Mitotic phase
Mdm2	Mouse double minute 2
MYC	Myelocytomatosis
CDH2	N-cadherin
NF1	Neurofibromatosis type 1
NTD	N-terminal domain

OC	Oral contraceptives
OSE	Ovarian surface epithelium
PPARγ	Peroxisome proliferator-activated receptor γ
PTEN	Phosphatase and tensin homolog
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PI3K	Phosphoinositide 3-kinase
PDGF	Platelet-derived growth factor
PCNA	Proliferating cell nuclear antigen
PEST	proline (P), glutamic acid (E), serine (S), and threonine (T)
PML-NB	Promyelocytic leukemia nuclear body
PKA	Protein kinase A
PKC	Protein kinase C
PP2A	Protein phosphatase 2A
RAS	Rat sarcoma
rhEGF	recombinant human epidermal growth factor
Rb	Retinoblastoma
RB1	Retinoblastoma 1
Ser	Serine
STIC	Serous tubal intraepithelial carcinoma
SCF	Skp, Cullin, F-box containing complex
Sip1	Smad interacting protein
Smad	Small body size- mothers against decapentaplegic
α-SMA	α-Smooth muscle actin
SDS	Sodium dodecyl sulfate
Skp2	S-phase kinase-associated protein 2
SH domain	Src homology domain
S-phase	Synthesis phase
TCF	T-cell specific factor
Thr	Threonine
TF	Transcription factor
TGFβ	Transforming growth factor-β
Tyr	Tyrosine
Ub	Ubiquitin
UPP	Ubiquitin-proteasome pathway
Ets1	V-ets erythroblastosis virus E26 oncogene homolog 1
VIM	Vimentin
BRAF	v-Raf murine sarcoma viral oncogene
Wnt	Wingless/integrated
ZEB	Zinc finger homeodomain enhancer-binding protein

CHAPTER 1

LITERATURE REVIEW

I. OVARIAN CANCER

There are more than 15 different types of ovarian cancer, which are grouped into three categories: epithelial, germ cell and stromal cancers. Epithelial ovarian cancer (EOC) is the most common and lethal type of ovarian cancer and accounts for 90% of all reported cases [1]. The lack of effective early detection markers for ovarian cancer, coupled with the vague, non-specific symptoms of this malignancy, results in the late presentation of women to clinicians who are already at an advanced stage of the disease. These factors contribute to the lethality of ovarian cancer and makes EOC the most fatal of all gynecologic malignancies, and the 5^{th} leading cause of cancer death in women [2].

I. 1. CLASSIFICATION:

I. 1.1 Stage and Grade

The stage of EOC is determined by assessing the dissemination of the cancer in the body. The stage becomes important for prognostication and determination of treatment options [3]. Stage I tumors are limited to the ovaries and exist without the production of ascites. Stage II tumors involves one or both ovaries with metastasis to other pelvic tissues, such as tubes or the uterus. Ascites containing malignant cells are also a component of stage II tumors. Tumors that are still limited to the true pelvis but have extension into the small bowel or omentum are characteristic of stage III. Stage IV tumors involve one or both ovaries with distant metastasis[4]. The stage of the disease at diagnosis is one of the most important prognostic factors and has a direct relationship with five-year survival rates: Stage I (93%), Stage II (70%), Stage III (37%), and Stage IV (25%).

EOC has been classically graded according to the degree of the differentiation of malignant cells. Grade 1 tumors are well differentiated, whereas grade 3 tumors are poorly differentiated [4]. Recently, ovarian cancer grading has moved toward a two-tiered system of low- or high-grade (Type I or Type II, respectively). This distinction is made mainly based on the pattern of progression and the molecular genetic changes [5] (Figure 1). Low-grade tumors usually follow a step-wise progression model, are large but slow growing, and are contained to the ovary. In contrast, high-grade tumors are highly aggressive, evolve rapidly and are associated with early and high degree of metastasis. Precursor lesions of high-grade carcinomas are elusive due to the early transition of the occult lesion to the clinically diagnosed carcinoma. In addition, high-grade cancers exhibit greater chromosomal instability than the low-grade counterparts [6].

I. 1.2 Histopathological Subtypes:

Although most early research focused on EOC as a whole, ovarian cancer is a heterogeneous disease. Recent studies have conceptually advanced the way ovarian cancer is diagnosed and described: from one disease with many subtypes, to several distinct diseases. The more common histological subtypes are serous, endometriod, mucinous, and clear cell; these display distinct morphological and molecular profiles [7] (Table 1). Of all the subtypes, those of a serous histopathology are the most common, making up more than 70% of all reported cases. Of the remaining subtypes, endometriod and clear cell each account for ~10% and mucinous for 3-5% of EOC [8].The subsequent sections of this literature review will concentrate mainly on the serous subtype and the terms “high-grade serous ovarian cancer” and “ovarian cancer” will be used interchangeably.

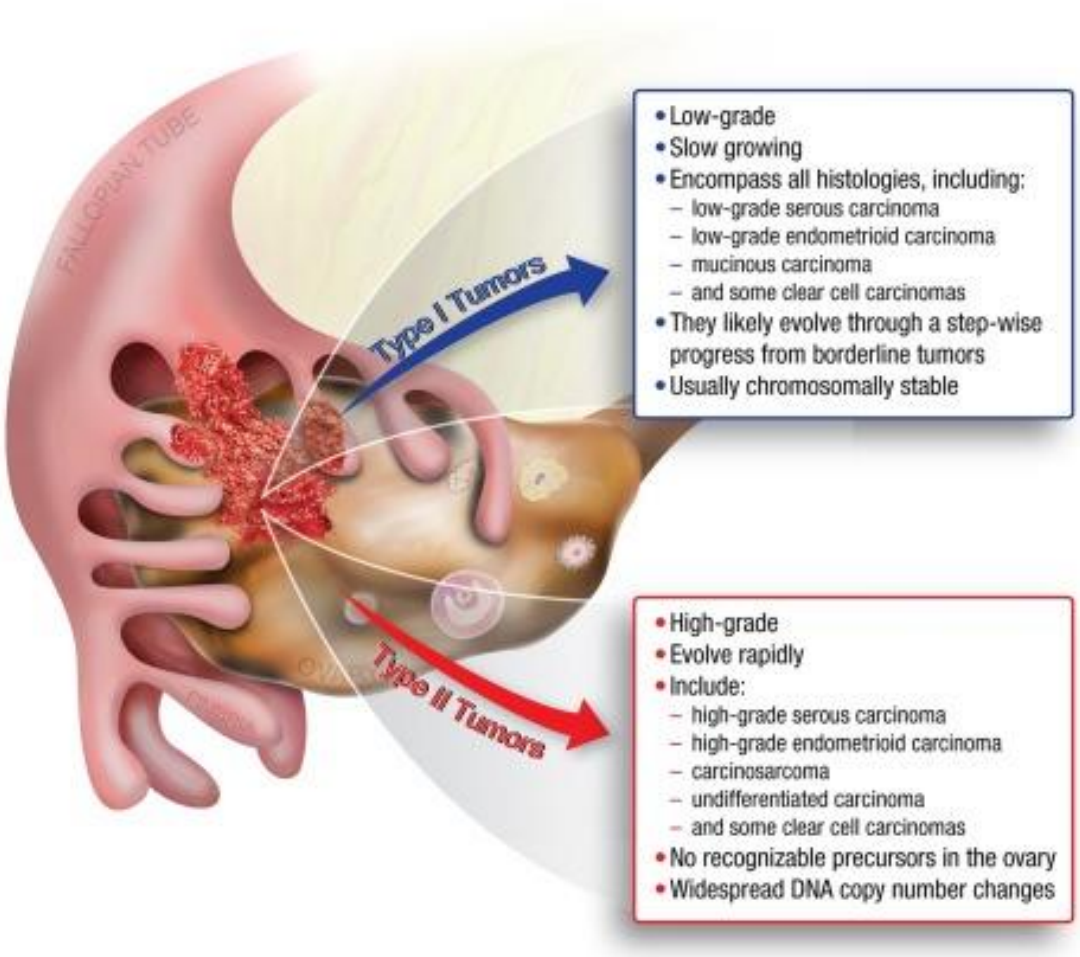

Figure 1: New grade classification of ovarian cancer subtypes. Type I tumors are low-grade, slow growing carcinomas that typically arise from well recognized precursors lesions (borderline tumors). In contrast, Type II tumors are high-grade and rapidly growing carcinomas. Typically, they have spread well beyond the ovary at the time of diagnosis. Figure obtained with permission from [9].

Table 1: Differentiation of ovarian Cancer Subtypes

	Serous High Grade	Serous Low Grade	Endometriod (Low/High)	Clear Cell (Low/High)	Mucinous (Low/High)
Precursor Lesion	STICs	Boarderline tumor	Endometriosis	Endometriosis	Boarderline tumor
Molecular Abnormalities	P53 BRCA1/2	BRAF KRAS	MMR deficiency ARIDA PI3KCA PTEN	ARIDA PI3KCA PTEN CTNNB1	KRAS HER2
Chromosomal Instability	High	Low	Low/High	Low/High	Low/High
Chemosensitivity	High	Intermediate	High	Low	Low

*adapted from [6]

High grade serous ovarian cancer

High grade serous ovarian cancers (HGSC) make up the majority of EOC cases diagnosed. HGSC are characterized as fast-growing and -disseminating aggressive tumors [10]. Overall, this subtype is by far the most lethal of all ovarian cancers. Most women will present at a late stage of diagnosis, when the 5-year survival rate has dropped to beneath 30% [11]. HGSC are characterized by high genomic instability, but only a few reoccurring mutations [12]. One such mutation found in over 90% of HGSC is in p53 [13]. The almost universal dysregulation of p53 in HGSC has helped to define the tissue of origin, which was once thought to arise predominantly from the ovarian surface epithelium. Examination of prophylactically removed fallopian tubes from women without ovarian cancer, but with a genetic risk of developing the disease, have shown a high degree of serous tubal intraepithelial carcinoma (STIC) and/or a p53 signature in the distal end of the fallopian tube identical to those seen in HGSC [14]. Therefore, it is now widely accepted that HGSCs begin as a precursor lesion in the fallopian tube that only secondarily seeds onto the surface of the ovary (Figure 2). Women with a heritable heterozygous mutation in BRCA1 or BRCA2 commonly develop HGSC [15], and these mutations are regarded as the greatest risk factor for the development of disease. Recently, HGSC have been classified into four subgroups relating to their gene content: proliferative, immunologic, mesenchymal, and differentiated [12]. These subtypes, however, have not been applied to clinical care.

Low-grade serous ovarian cancer

Low-grade serous ovarian cancers (LGSC) are much less common than HGSC, and represent a distinctively different disease. These cancers generally arise in younger women and have a slow pattern of progression [11]. When caught early, LGSC have a positive prognosis due

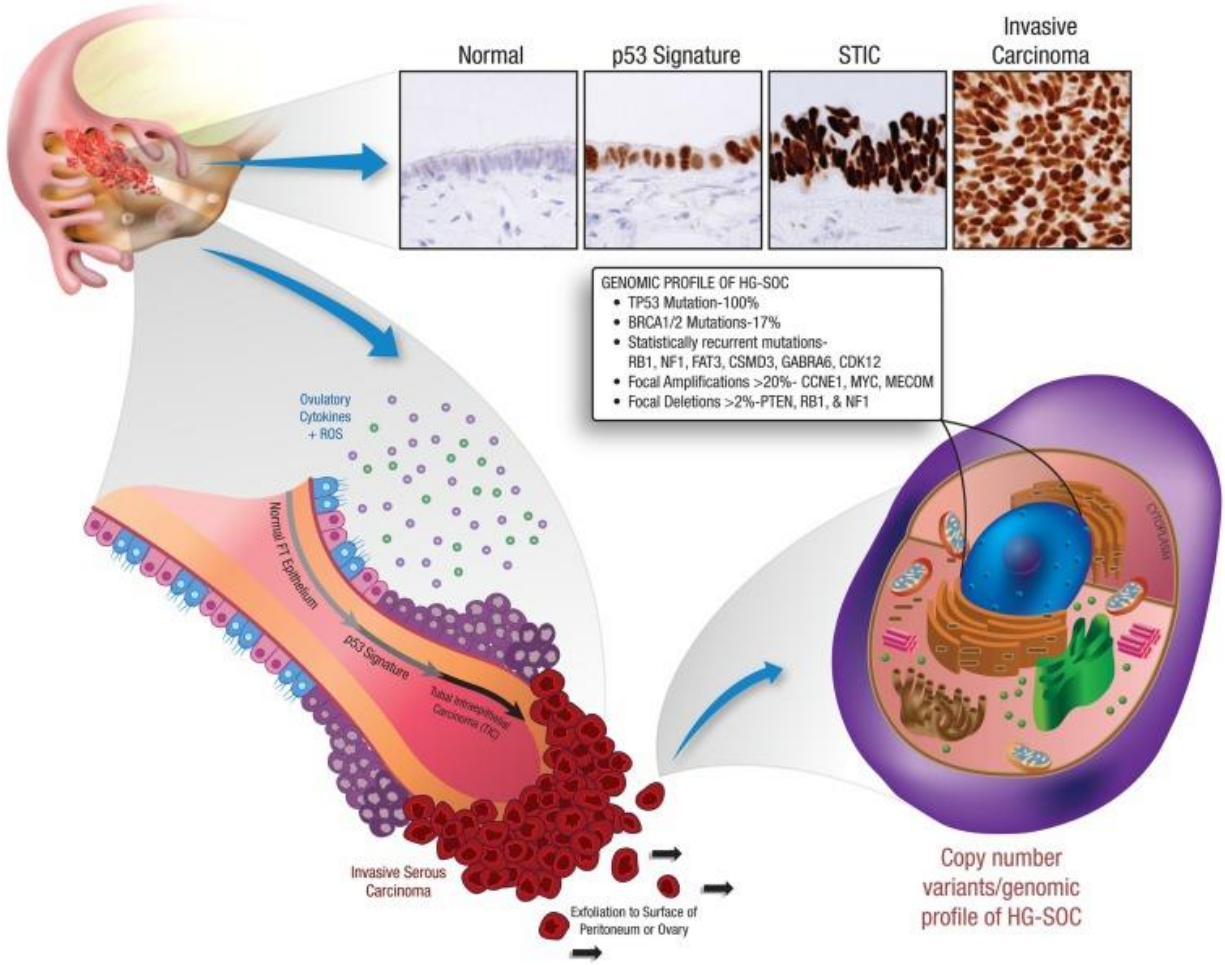

Figure 2: Development of HCSC from fallopian tube: precursor and genetic profile . HGSC originate from mutations in the TP53 tumor suppressor gene occurring in the secretory cells in the frimbrated end of the fallopian tube. These preneoplastic lesions are referred to as 'p53 signatures'. These cells progress to the development of serous tubal intraepithelial carcinoma (STIC), which is secondarily deposited on to the surface of the ovarian. HGSC are characterized by identical TP53 mutations and display a high degree of genomic instability and widespread copy number alterations throughout the genome. Figure obtained with permission from [9].

to surgical removal. However, as a result of the lack of symptoms and biomarkers available for diagnosis, LGSC are found at a late stage resulting in long-term survival similar to HGSC [16].

The origin of LGSC is less clear than HGSC. Commonly, LGSC are thought to arise from a step-wise malignant progression of epithelial entrapment in the ovarian stoma. Initially, epithelial inclusions transition into benign and then borderline tumors [17]. These borderline tumors begin as atypical proliferative serous tumors and evolve into invasive serous carcinomas [18]. The strongest support for this step-wise continuum is that identical mutations of either KRAS or BRAF have been observed in the epithelium of benign tumors adjacent to boarderline tumors, which suggests a common lineage [19]. However, since LGSC display a phenotype similar to that of the Müllerian epithelium, others suggest that a tubal origin of LGSC is more likely [20]. Those that favor the progression model, explain this phenomenon by Müllerian metaplasia of the ovarian surface epithelium.

LGSC are more genetically stable than their high-grade counterpart, and predominantly expresses mutations in KRAS and BRAF [17]. KRAS and BRAF are upstream of MAPK, and these mutations result in the constitutive activation of its signaling pathway leading to survival and an increased proliferative capacity. In contrast, mutations of p53 are highly uncommon in LGSC [6]. Generally, LGSC are also associated with low response rates to cytotoxic and hormonal agents [21], however a new MAPK inhibitor has shown positive treatment results in advanced ovarian cancers [22].

I. 2. ORIGIN OF EOC:

To date, the molecular events leading to the initiation and progression of HGSC remain poorly understood. For many years, it was widely believed that EOC originated from the coelomic epithelium that covers the ovarian surface. Hypotheses such as incessant ovulation and coelomic metaplasia put the ovarian surface epithelium (OSE) at the core of ovarian cancer development. However, these theories are strongly challenged by recent studies. There is strong evidence suggesting that the epithelial cells of the fallopian tube, particularly at the fimbriated end, is the origin of serous ovarian cancer [7]. The Müllerian hypothesis has been recently put forward. According to this hypothesis, most ovarian tumors arise from Müllerian-derived tissues, not the OSE [23] (Figure 3).

I. 2.1 The ovarian surface epithelium:

Fathalla proposed the incessant ovulation hypothesis in 1971 [24]. It is postulated that OSE cells undergo proliferation following ovulation to repair the damage caused by the release of egg. This increased proliferation may confer cells with an increased susceptibility to mutations, as well as entrapment within inclusion cysts, thus leading to malignant transformation. Dysplasia of the inclusion cysts was first described by Scully [25, 26] as early lesions of HGSC. The coelmic metaplasia hypothesis accounts for the appearance of Müllerian-type epithelium through prior metaplasia triggered by the microenvironment [23]. One major set-back in this hypothesis is the lack of *in situ* ovarian precursor cancer lesions. However, neoplastic transformation in the inclusion cyst could be immediate, via powerful stimulation by ovarian stromal factors, therefore significantly limiting the window in which early stage cancers can be detected [27].

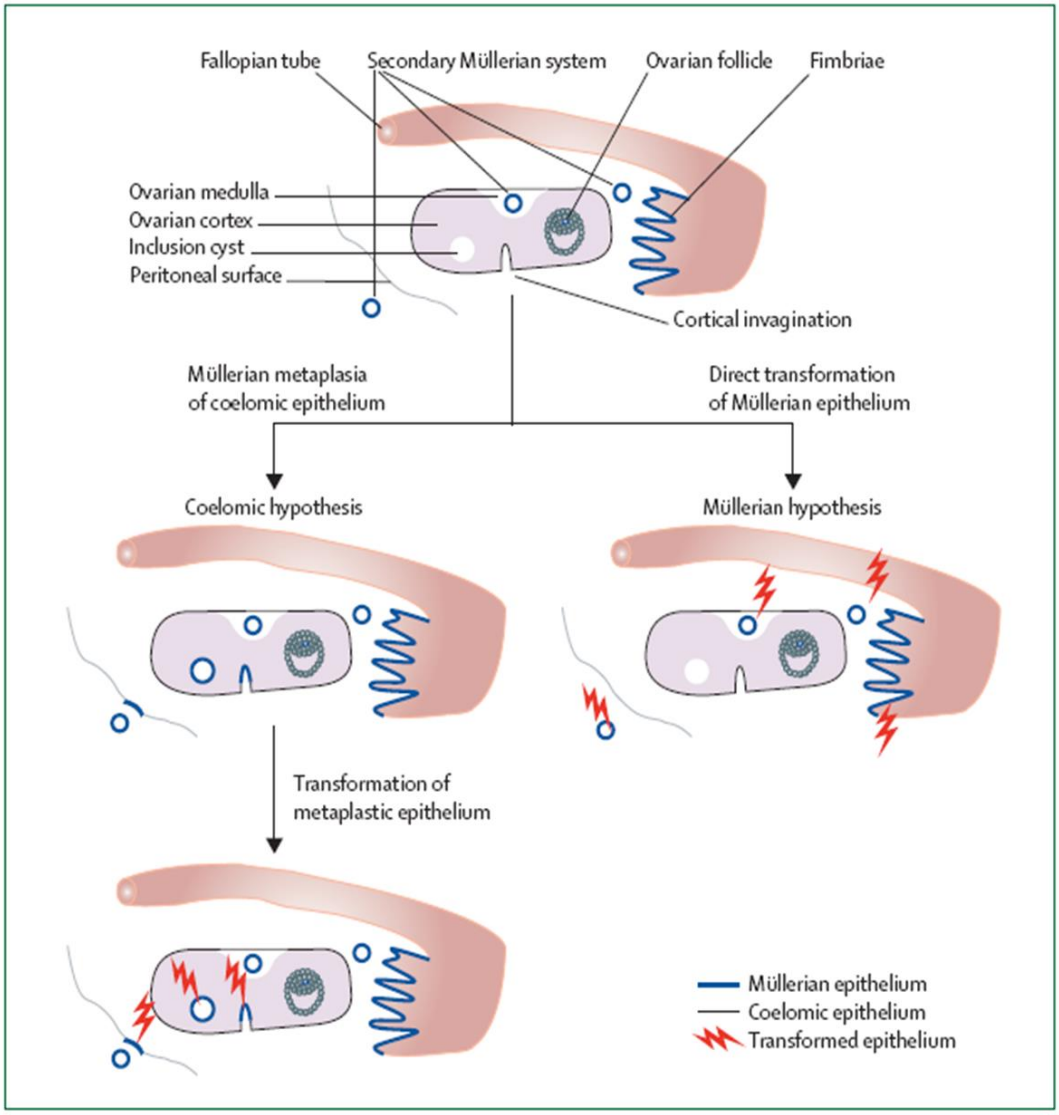

Figure 3: The surface epithelia versus Müllerian system hypotheses for the origin of ovarian carcinomas. According to the surface epithelia hypothesis, cortical invaginations and inclusion cysts, which are initially lined with coelomic epithelium (thin black line), undergo metaplasia and change to Müllerian-like epithelium (thick blue lines) before undergoing malignant transformation (lightening signs). The surface epithelium covering the ovary can give rise to primary tumors only after undergoing metaplasia to acquire characteristics of Müllerian epithelium. No intermediate metaplastic step is necessary with the Müllerian epithelium hypothesis, which stipulates that Müllerian-like tumors arise directly and exclusively from the Müllerian epithelium that is already present. Not illustrated: A new group of stem found in the region of hilum represents a transition region the OSE and tubal epithelium. These cells are activated following ovulation and possess increased malignant transformation potential following p53 mutation. Figure obtained with permission from [23].

I. 2.2 Fallopian tube fimbriae:

The Müllerian hypothesis stipulates that Müllerian-like ovarian cancers arise directly from Müllerian epithelium that covers the frimbrae, or components of the secondary Müllerian system [23]. This hypothesis attracted attention when Piek [28] demonstrated that 11 out of 12 fallopian tubes from prophylactic salpingo-oopherectomized women with BRCA mutations displayed dysplastic lesions on the fimbriae, whereas no abnormalities were found on the control. These lesions were characteristic of HGSC and it was thought that they could be deposited on to the ovary during the ovulatory sweep [27, 28]. More importantly, greater than 70% of women, irrespective of family history, had simultaneous STICs and HGSC, which display identical p53 mutations [29]. Furthermore, in areas of benign fimbriae, strong p53 staining was observed, known as the p53 signature, and was proposed to be the early cancerous lesion [30]. Presumably, exfoliation of STICs to the ovarian surface allows malignant cells to grow much faster due to the ovarian microenvironment, leading to the development of HGSC. Furthermore, mouse models of HGSC have explicitly shown the fallopian tube to be the primary site of cancer that only secondarily engulfs the ovary and spreads to the peritoneal cavity [31].

I. 2.3 Unifying Hypotheses:

Although no single origin theory has been universally accepted, a unifying hypothesis has suggested that the area between the fallopian tube and the ovary is a region of epithelial transition that is vulnerable to malignant transformations [27, 32]. The first experimental evidence has been put forth by Flesken-Nikitin [33] which supports the notion that susceptibility of transitional zones to malignant transformation may be explained by the presence of stem cell niches in those areas. The region of hilum, that represents a transition region between the OSE,

mesothelium and tubal epithelium, has been shown to harbor a high number of stem cell-like OSE cells [33]. These cells are activated following ovulation to repair the ovarian surface and have been shown to have increased malignant transformation potential following p53 mutation. Therefore, this small stem cell niche of the OSE may have important implications for understanding EOC initiation [33].

I. 3. RISK FACTORS:

There are many well-established prognostic factors that contribute to the stimulation or inhibition of ovarian tumorigenesis, and certain risk factors are consistently implicated in the lifetime risk of disease. Of these, a family history of breast or ovarian cancer remains the greatest risk factor in ovarian cancer development. In addition, reproductive and hormonal factors such as nulliparity, lack of oral contraceptive use, and hormone replacement therapy have all been implicated in increasing the risk of disease. Environmental and lifestyle factors are less convincing and have shown inconsistency with predicting risk. Some of these factors, such as obesity, may be associated with chronic inflammatory events, which could foster ovarian cancer development.

I. 3.1 Molecular and Genetic factors:

A family history of cancer caused by an inherited mutation in certain genes can increase the risk of ovarian cancer. Most of hereditary ovarian cancers are due to mutations in the BRCA1 and BRCA2 genes. The lifetime risk of developing ovarian cancer is estimated at 25% to 65% for BRCA1 carriers, and 15% to 20% for BRCA2 carriers [34]. Although it is rare to have a BRCA mutation in sporadic tumors [35, 36], evidence suggests alternative, non-mutational

mechanisms suppress BRCA in sporadic EOCs [37, 38]. Methylation at the BRCA-1 site correlates with a decrease in BRCA-1 mRNA and protein expression [39], which could potentially contribute to the progression of the disease [40]. Recently, a genomic analysis of HGSC has shed light on the molecular changes involved in development and progression of disease. In addition to p53, BRCA-1 and BRCA-2 mutations, four other frequently re-occurring mutations in RB1, NF1, CSMD3 and CDK12 were identified. Together, 30 somatic copy number alterations were identified (8 gains and 22 losses) in more than 50% of tumors. These analyses confirmed the high degree of genomic instability present in HGSC.

II. CYCLIN G2

The G type cyclins were first identified in screens for Src kinases [41] and later in screens for transcriptional targets of the tumor suppressor, p53 [42, 43]. Cyclin G1, cyclin G2, and cyclin I are the three members of the G type cyclins that form a distinct sub-group which reflect high sequence homology and some degree of functional similarity [44, 45]. Although cyclin G2 shares high sequence identity with the other G-types cyclins (71% homology to cyclin G1 and 41% homology to cyclin I) [46], their expression, promoter sequence, and regulation differ greatly, suggesting that these proteins have distinct and non-compensatory physiological functions [44, 47].

II. 1. STRUCTURAL FEATURES OF CYCLIN G2

II. 1.1 Protein domains:

The protein structure of cyclin G2 is characterized into three primary domains: the amino-terminal domain (NTD), a cyclin box of approximately 110 amino acids, and the carboxyl-terminal domain (CTD) [48]. The cyclin box of cyclin G2 resembles that of cyclin A, which is required for interaction with CDK-2, but the association with any cyclin dependent kinase partner has yet to be found [46, 49]. Therefore, although the association of cyclin G2 with other regulatory members of the cell cycle is possible, this interaction is likely not through the cyclin box [49]. Indeed, three identified cyclin G2 binding partners, cortactin, PP2A, and PPARγ, all associate with cyclin G2 via interactions outside of the cyclin box [50-52]. In addition, cyclin G2 contains a 46 amino acid CTD extension which includes a PEST (proline, glutamic acid, serine, and threonine) protein destabilization motif. The PEST motif is commonly

found in rapidly degraded proteins [53] and contains multiple potential phosphorylation sites that may subsequently influence its regulation [54, 55] (Figure 4).

II. 1.2 Phosphorylation and Protein Binding Domains:

Interrogation of the cyclin G2 sequence provided numerous potential phosphorylation sites and is indicative that phosphorylation may regulate much of cyclin G2 action. However, no phosphorylation site has been experimentally confirmed. Amino acids 272-294 are consistent with the epidermal growth factor autophosphorylation polypeptide sequences and are critical to complete a Shc phosphotyrosine binding site in the cyclin G2 CTD [42]. In addition, cyclin G2 has a putative SH2 domain-binding motif at the N terminus and a noncanonical SH3 domain-binding motif at the C-terminus, of which the SH3 is important for cortactin recruitment and cytoskeleton modulation [52]. Lastly, cyclin G2 has two putative Nuclear Receptor boxes and is an interesting target for interaction with nuclear receptors [50]. These sites implicate cyclin G2 as a downstream component of select signal transduction pathways or members of protein complexes that can regulate the function and/or stability of the protein [42, 56].

II. 2. CYCLIN G2 EXPRESSION AND DEGRADATION

II. 2.1 Growth factor regulation:

The expression of cyclin G2 mRNA level is regulated by a variety of growth inhibitory and mitogenic signals. For example, TGF-β, a potent growth inhibitor of normal cells, as well as related family members, Nodal and its receptor ALK, robustly up-regulate the expression of cyclin G2 [49, 54]. Likewise, when the Nodal-ALK7 downstream effectors, Smads-2 and -3, are

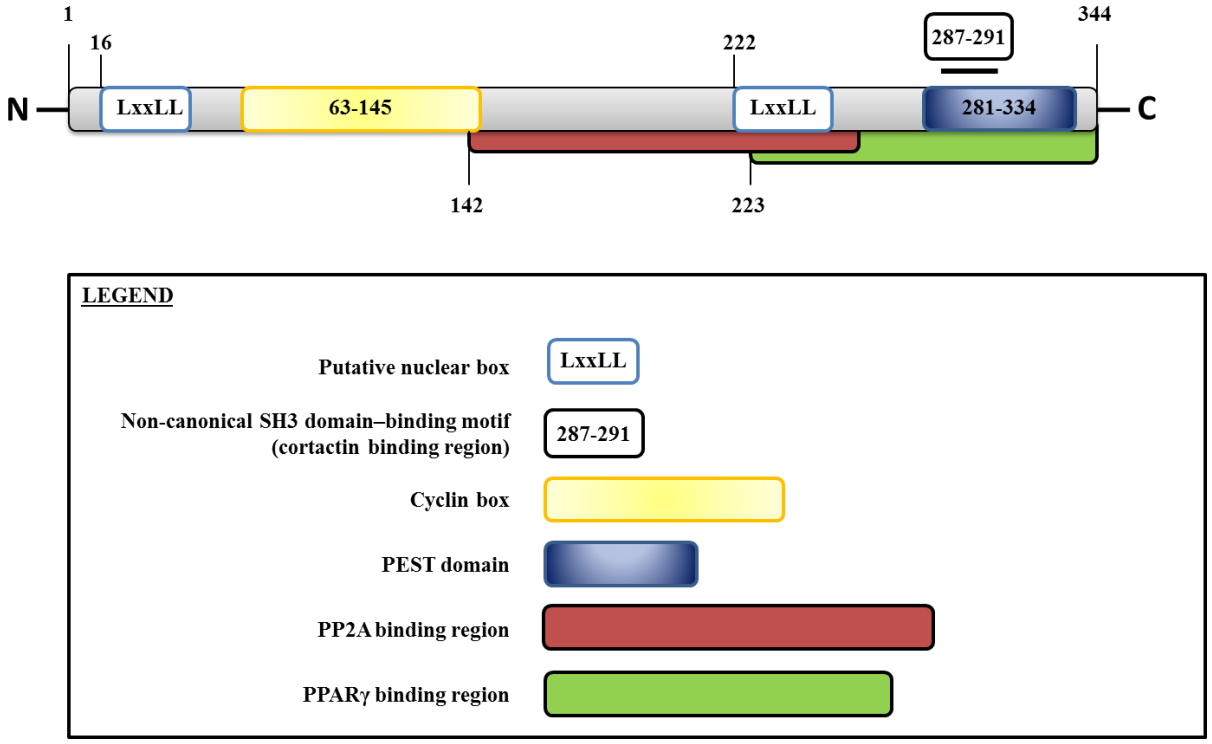

Figure 4: Domains of cyclin G2. Cyclin G2 is divided into three main regions: N-terminal, C-terminal, and the centrally located cyclin box. The C-terminal domain has been implicated in protein binding to PP2A, PPARγ, and cortaction (SH3 domain) and houses one of the two putative nuclear boxes. The C-terminal end is also tightly linked to cyclin G2 degradation via the presence of the destabilization PEST domain.

inactivated, cyclin G2 induction is abolished, suggesting that Nodal/ALK7 induces cyclin G2 through the canonical Smad2/3 pathway [54].

In addition, treatment with potent growth factors or hormone receptors, such as platelet derived growth factor (PDGF), insulin-like growth factor (IGF)1, human epidermal growth factor receptor (HER)-2, estrogen receptor (ER), and the erythropoietin receptor (EPO-R) decreased cyclin G2 mRNA levels. ER can inhibit cyclin G2 expression directly by forming a complex with the transcription factor, Sp1 on the cyclin G2 promoter [57], whereas PDGF, IGF, HER-2, and EPO depend, at least in part, on activated PI3K signaling. Indeed, most growth stimulating factors intersect with the PI3K pathway, and cells harboring PI3K mutations show dysregulation of cyclin G2 [45, 49, 58-61].

II. 2.2 The Phosphoinositide 3-kinase pathway and the Forkhead box protein (Fox)O subfamily of transcription factors:

The PI3K/Akt pathway is important in regulation of many cellular functions ranging from growth, proliferation, differentiation, and survival [58]. PI3K activation leads to downstream activation of Akt, also known as protein kinase B [58], while the known tumor suppressor, phosphatase and tensin homologue (PTEN), is the most notable inhibitor of PI3K/Akt signaling. Expression of the catalytic subunit of PI3K resulted in moderate down-regulation of cyclin G2 mRNA levels. On the other hand, upon PTEN stimulation, cyclin G2 mRNA showed significant up-regulation, similar to that of PI3K drug inhibition [62-64].

FoxO TFs are negatively regulated through direct phosphorylation by Akt [45], which leads to its nuclear exclusion and degradation [65]. Conversely, PTEN activates FoxO TFs by inhibition of Akt phosphorylation, promoting translocation of FoxO TFs to the nucleus [65]. Recently, various Forkhead response elements (FREs) have been identified on the cyclin G2 promoter [66], and FoxO3a has been shown to physically associate at some of these sites to stimulate cyclin G2 transcription. Inhibitory phosphorylation by Akt or use of dominant negative FoxO-mutants can abolish FoxO3a-induced expression of cyclin G2 [45, 67]. Alternatively, when FoxO is mutated so that it cannot be phosphorylated, Akt overexpression cannot facilitate the downregulation of cyclin G2 mRNA [67]. Therefore, FoxO action on the cyclin G2 promoter is highly dependent on the activity of Akt.

In addition, FoxO proteins are able to cooperate with other transcription factors to augment transcription [68, 69]. Nodal can induce cyclin G2 transcription by promoting synergistic interaction between FoxO3a and the Smads at the cyclin G2 promoter. Similarly, expression of the zinc finger-homeodomain transcription factor, δ-crystallin enhancer factor 1 (δEF1) can also lead to a synergistic induction of cyclin G2 mRNA [67].

II. 2.3 Regulation of cyclin G2 degradation:

In addition to transcriptional control, protein degradation is crucial in determining net protein expression. Recently, cyclin G2 has been found to be a highly unstable protein and is degraded quickly by the ubiquitin (Ub)-proteasome pathway (UPP), under the direction of the S-phase kinase-associated protein 2 (Skp2) at the PEST domain [54]. Interestingly, treatment with Nodal, a protein that is also involved in the regulation of cyclin G2 transcription, inhibited the

association of Skp2 with cyclin G2, resulting in protection from degradation [54]. This suggests that the effect of Nodal on the expression of cyclin G2 is twofold, in addition to regulating gene activity, it can also protect cyclin G2 from degradation.

II. 3. FUNCTIONS OF CYCLIN G2

II. 3.1 Cell cycle control:

The expression of cyclin G2 oscillates during the cell cycle and there is a consistent up-regulation of the protein in response to growth inhibitory signals and DNA damage [45, 46, 49, 70]. Cyclin G2 expression is commonly found to be highest when cells are in a quiescent state or at the G_o phase of the cell cycle and low in proliferating cells. Once cells are stimulated to progress through the G_o-G_1 transition, there is a rapid decline in the level of cyclin G2 expression, following re-accumulation of cyclin G2 expression in the late S/G_2 and G_2/M transition [45, 49]. The overall expression of cyclin G2 is atypical and does not mimic the cyclic appearance of the other cyclin family members [47], and high levels of cyclin G2 correlate with cell cycle arrest in many cells types [46, 47, 49, 70].

Role of PP2A and Microtubule Stability

One of the first identified binding proteins of cyclin G2 is protein phosphatase 2A (PP2A) [49]. PP2A is a highly conserved serine/threonine phosphatase that is essential for a plethora of signaling events, including cell cycle control. PP2A is made up of three subunits: the A- (scaffolding) subunit, the B- (regulatory) subunit, and the C- (catalytic) subunit. PP2A/B is responsible for target specificity and by extension, can direct the entire complex to distinct cellular locations [47, 71-73]. Cyclin G2 can directly associate with the B- and C- subunits of PP2A and co-localize in both the cytoplasm and nucleus. Furthermore, cyclin G2 may compete

with the PP2A/A subunit by acting as a scaffolding protein within the complex, and in this way, it is possible that cyclin G2 may alter the subset of PP2A targeted proteins and normal activity [49]. To facilitate this interaction, the carboxyl end, specifically the region between amino acids 142-344, is necessary for both the interaction with PP2A as well as cyclin G2-mediated cell cycle arrest; and therefore, the interaction between these two proteins may be relevant to the control of cyclin G2 function [49]

Both PP2A and cyclin G2 are able co-localize at the centrosome [49, 51], which implies their involvement in centrosome related functions. Indeed, overexpression of cyclin G2 results in stabilized microtubule bundles that were resistant to re-growth [51]. Evidence suggests that cyclin G2 may complex with PP2A to play a role in the maintenance of functional centrosomes that are needed for cytokinetic fidelity and progression through the cell cycle.

II. 3.2 Differentiation:

Cyclin G2 has also been associated with enhancing cellular differentiation: a process that is innately linked to cell cycle withdrawal. Importantly, cyclin G2 expression is often highest in terminally differentiated tissue [42]. Overexpression of cyclin G2 induces the differentiation of uterine stromal cells following implantation [74] and the formation of erythroblasts from erythroid-committed stem cells [75]. Additionally, cyclin G2 has been implicated in adipocyte differentiation [76, 77]. Peroxisome proliferator-activated receptor (PPAR)γ is a master regulator of adipocyte differentiation, and can be stimulated to induce transcription by forming a complex with cyclin G2 at the promoter of its target genes [50].

II. 3.3 DNA Damage repair:

Cyclin G2 induction has been suggested to play a role in the DNA damage response (DDR), and in conditions where cyclin G2 expression is depleted, cells show abnormal response to ionizing radiation and perturbations in DNA repair [44]. Following treatments with DNA damaging agents, cyclin G2 was found to be localized at centrosomes and resulted in cell cycle arrest [78], possibly through microtubule irregularities. More recently, cyclin G2 has been shown to co-localize with PP2A at promyelocytic leukemia (PML) nuclear bodies (PML-NBs) following double stranded breaks, and is necessary for their proper formation. In this context, the cyclin G2-PP2A complex is thought to dephosphorylate many factors necessary for a proper DDR [79].

II. 4. CYCLIN G2 DYSREGULATION IN CANCER AND OTHER DISEASES

Increasing evidence suggests that cyclin G2 exerts important anti-tumorigenic roles. First, cyclin G2 is upregulated by growth inhibitory signals, and many oncogenic signaling pathways inhibit cyclin G2 expression. In addition, multiple anticancer drugs can induce cyclin G2 expression [80-82]. Second, over-expression of cyclin G2 inhibits cell proliferation in several human cell lines [47, 56, 70], limits colony formation, and induces morphological changes [56, 83]. Similarly, most chemotherapeutic treatments correlated with an increase in cell cycle arrest, apoptosis and deregulated microtubule dynamics, which can all be attributed, at least in part, to an overexpression of cyclin G2. Finally, expression of cyclin G2 is negatively correlated with cancer progression and positively associated with patient survival. In both human oral cancer and papillary carcinoma of the thyroid, cyclin G2 is significantly lost as cells move through a step-wise cancer progression model. Normal tissue consistently expresses higher levels of cyclin G2

when compared to the precancerous lesions, and when cells undergo malignant transformation, cyclin G2 is almost completely lost [70]. Negative cyclin G2 status was also found to be correlated with more advanced stages of cancer in both acute leukemia and gastric cancers [84, 85]. In addition to its potential as an early mediator of cancer development, cyclin G2 was found to be a marker for prognosis and remission [84, 85]. Patients with a higher cyclin G2 level showed a better five-year survival rate [84, 86], with a reduced chance of relapse and greater potential for complete remission [85, 86]. A recent study reported that TGF-β and mutant p53 cooperate to oppose the activity of p63, and two key p63 target genes identified are Sharp1 and cyclin G2 [86]. Strikingly, interrogation of cDNA microarray data sets suggests that Sharp1 and cyclin G2 provide a minimal signature to predict metastasis-free survival [86]. Recently, Sharp1 has been described to suppress breast cancer metastasis by promoting the degradation of hypoxia-inducing factors [87], however the mechanism of cyclin G2 remains unknown.

The induction of cyclin G2 by the hypoxia induced factor (HIF)1α, and the specific role of cyclin G2 in hypoxic conditions contrasts the implicated role of cyclin G2 as an anti-tumorigenic protein. Indeed, in glioblastoma cells cyclin G2 enhanced hypoxia-induced invasion [52]. However, it is very important to note that the signaling dynamics may differ from carcinoma to gliomas, and Nodal/ALK7 and PP2A have also been shown to have tumor promoting roles in glioblastoma cell lines [88, 89].

II. 4.1 Genetic instability and epigenetic regulation:

Cyclin G2 is located at chromosome 4q21.1. Interestingly, the long arm of chromosome 4 at position 21 is characterized by genomic instability (Figure 5) [90, 91] and contains potential

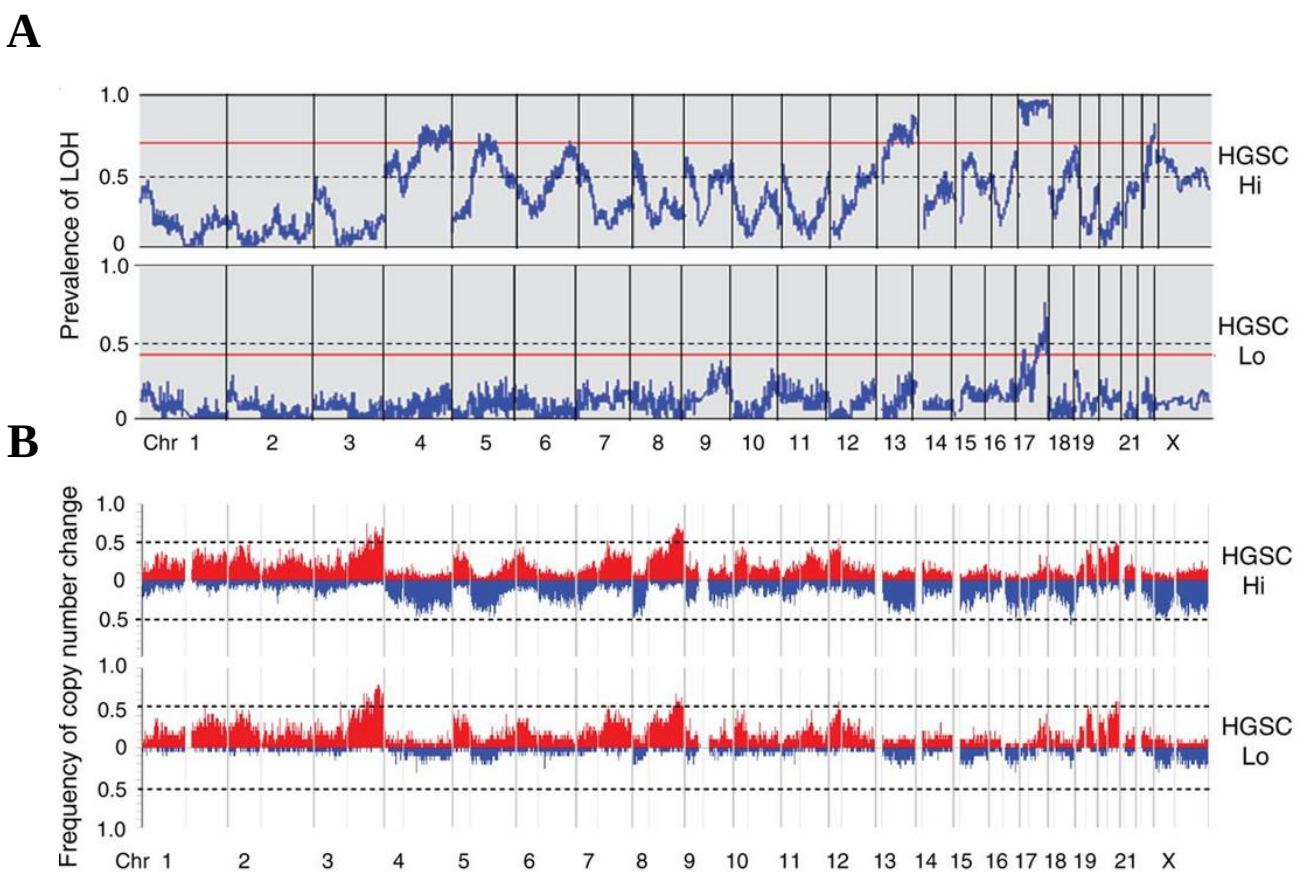

Figure 5: Loss of heterozygosity in HGSC. A) Prevalence of LOH in HGSC compared to LGSC. HGSC is characterized by chromosomal instability. The red horizontal line indicates a p-value of 0.05. Chromosome 4 shows high degree of allelic loss. **B)** Frequency of copy number changes in HGSC compared to LGSC. Gains are red, losses are blue. Chromosome 4 is characterized by apparent copy number losses. Figure obtained with permission from [91]

tumor suppressors [92-96], as well as cyclin G2 [97]. Furthermore, the cyclin G2 promoter region contains CpG islands that are susceptible to methylation, and in colorectal cancer cells cyclin G2 expression can be rescued following demethylation [98]. Therefore, in addition to regulation of protein expression and turnover by various signaling cascades, it is possible that cyclin G2 can be inactivated by a combination of frequent allelic loss of regions on chromosome 4 and promoter hypermethylation. It would be interesting to investigate if loss of heterozygousity at this critical position could be a potential marker for cancer development or a predicative marker for prognosis.

III. CALPAINS

The biological process known as protein turnover preserves the balance between *de novo* protein synthesis and protein degradation. This constant turnover helps to protect the integrity of proteins by reducing the amount of time exposed to the cellular environment and plays a key role in determining a protein's final concentration [99, 100]. Unlike several other post-translational events, proteolysis is irreversible, and is usually involved in the unidirectional control of cellular pathways [101]. For this reason, protein degradation is highly regulated and must be extremely selective for target substrates [100]. Several degradation specific mechanisms exist for the identification and destruction of cellular proteins. We have previously shown that cyclin G2 can be degraded by the ubiquitin proteasome pathway (UPP) [54], however it is possible that multiple mechanisms function in cohort to regulate cyclin G2 expression. Indeed, we have found that cyclin G2 can also be degraded in a calpain-dependent manner (Chapter 2). Here, protein cleavage by calpains will be discussed.

III. 1. GENERAL PROPERTIES OF CALPAINS:

Calpains were first discovered in the 1960s [102, 103], but the term 'calpain' was not adopted until about 20 years later [104] when it was determined that these proteins possess calcium-dependent activation and papain-like catalytic sites. There are two main isoforms of calpain that are ubiquitously expressed, calpain-1 and calpain-2. However, at least 15 additional ubiquitous and tissue specific forms have subsequently been identified [105-107] (Table 2). Calpain-1 and calpain-2 form heterodimers comprised of a common small subunit and similar, yet distinct, large subunit [108]. While calpain-1 and calpain-2 share various qualities, their major difference is the calcium concentration required for their activation *in vitro*. Micromolar

Table 2: Human Calpain Family

	Gene	Other names	Tissue Distribution	Protease activity	Association with small subunit
Calpain 1	CAPN1	M-calpain large subunit	Ubiquitous	Y	Y
Calpain 2	CAPN2	m-calpain large subunit	Ubiquitous	Y	Y
Calpain 3	CAPN3	p94, nCL-1	Skeletal muscles	Y	N
Calpain 5	CAPN5	hTRA-3, n-CL-3	Ubiquitous	Y	N
Calpain 6	CAPN6	CANPX	Placenta, embryonic muscles	N	N
Calpain 7	CAPN7	PalBH	Ubiquitous	Y	N
Calpain 8	CAPN8	nCL-2	Stomach	Y	N
Calpain 9	CAPN9	nCL-4	Digestive tract	Y	Y
Calpain 10	CAPN10	---	Ubiquitous	ND	ND
Calpain 11	CAPN11	---	Testies	ND	ND
Calpain 12	CAPN12	---	Hair follicle	ND	ND
Calpain 13	CAPN13	---	Ubiquitous	ND	ND
Calpain 14	CAPN14	---	ND	ND	ND
Calpain 15	SOLH	SOLH	Ubiquitous	ND	ND
CAPNS1	CAPNS1	Calpain small subunit, 30K, CAPN4	Ubiquitous	N	---
CAPNS2	CAPNS2	Calpain small subunit 2	Ubiquitous	N	---

*Y, yes; N, no; ND, not determined; ---, not applicable. Adapted from [109].

and milimolar calcium concentrations are required for the activation of calpain-1 and calpain-2, respectively [110]. In actuality, the calcium concentration required for a half-maximal calpain-2 activity is within the range of 400-800μM [111], or less when the large subunit has been N-terminally truncated [112].

III. 2. CALPAIN REGULATION AND FUNCTION

III. 2.1 Activation:

Although multiple avenues lead to the activation of calpain *in vivo*, the presence of calcium is fundamental for all calpain activation. However, it appears as though this basic requirement could never be physiologically achieved since the levels of calcium required for calpains greatly exceed normal intracellular calcium concentrations [111]. Subsequently, multiple theories have been presented and suggest that *in vivo* calpains actually require a lower level of calcium. In fact, interactions with activator proteins, post-translational modifications, and autolysis call all increase calpain activity and possibly reduce the calcium load [113-121]. It has been suggested that calpains may become active during calcium influx. In this way, calpains would be highly regulated in both a spatial and temporal fashion. On the other hand, phosphorylation may act as the switch that leads to the proper activation of calpain. For example, the Epidermal Growth Factor Receptor (EGFR) has been shown to play a role in the regulation of calpain-2 activity. ERK-dependent phosphorylation of calpain, downstream of EGFR signaling, results in a fully active protease at calcium concentrations below 1μM [114, 122, 123].

III. 2.2 Inhibition:

Calpain action is impeded by the endogenous inhibitor, calpastatin. In order to inhibit calpain, calpastatin tightly, yet reversibly, binds to multiple calpain domains, including the proteolytic core [124]. Interestingly, calpastatin action is also calcium dependent and targets only active calpains [125, 126]. Therefore, this provides a mechanism which prevents the *in vivo* hyper-activation of calpain. In addition, post-translational modifications such as phosphorylation have been found to regulate calpastatin activity with multiple phosphorylation sites having been identified for PKC, PKA, as well as cAMP-dependent kinase action [127]. Indeed, phosphorylation of calpastatin causes its inactivation and aggregation near the nucleus. Only through dephosphorylation following calcium influx does calpastatin become soluble, efficiently inhibiting calpain action [128-130].

III. 3. ACTION OF CALPAIN AND TARGET SPECIFICITY

III. 3.1 Target recognition

For substrate recognition, calpain does not target a single, typical amino acid consensus sequence. As a result, various amino acid residue stretches have been identified as potential sites vital to predicting calpain cleavage sites. For example, amino acids F/W/L/V, L/V, R/K, and R, K, L at position P3, P2, P1 and P1', respectively, are thought to be preferential for calpain recognition [131]. More recently, the calpain consensus site was suggested to be P, F, (F>L>P), (L>V), (L/F), (M>A>R), E, (R>K) at positions P5-P3' surrounding the cleavage site [132]. Furthermore, calpain targets are thought to harbor hydrophilic residues at position P5', P7', and P9', whereas P4' is likely to be unstructured [131]. Thus, in addition to the primary sequence, calpains likely depend on the 3D structure of the protein for target recognition [133, 134].

The PEST domain, found in many highly unstable proteins [53], has been suggested as an attractive target for calpain cleavage. Its amino acid composition results in the PEST domain acquiring an overall negative charge, which is postulated to sequester calcium ions that contribute to calpain activation. Furthermore, PEST motifs are found to make up a large proportion of unfolded or disordered regions of eukaryotic proteins, which are extremely sensitive to proteolysis [135]. In fact, 70% of calpain targets appear to be cleaved at irregularly structured areas, suggesting that calpains are able to detect protein disorder [136]. Although calpains target many of its substrates at the PEST domain [137-139], in some instances PEST was found to be dispensable to calpain action [140, 141].

IV. WNT/β-CATENIN SIGNALING

Signaling by the Wnt family is a fundamental pathway that directs cell proliferation, polarity, survival, and stem cell fate in embryonic and adult tissue [142]. The key regulator of the canonical Wnt pathway is β-catenin. β-catenin is a dynamic protein that is found in multiple subcellular compartments. Mutations in the Wnt/β-catenin pathway are often linked to a variety of pathological states, most notably cancer [143, 144].

IV. 1. THE β-CATENIN DESTRUCTION COMPLEX

IV. 1.1 Members:

The β–catenin destruction complex is the major cytoplasmic regulator that controls both the degradation, and prevents accumulation, of β-catenin. The main structural components of the complex are Axin and adenomatosis polyposis coli (APC). To this, effector proteins such as CK1, GSK3β, and PP2A are recruited.

Axin serves as the coordinating scaffold for the structural protein, APC, the kinases, GSK3β and CK1, the phosphatase PP2A, as well as for β-catenin [145]. It is also thought to be the rate limiting factor for the degradation of β-catenin [146]. Axin phosphorylation by GSK3β stabilizes the protein [145, 147] and subsequent CK1/ GSK3β phosphorylation increases its affinity for β-catenin [148-150]. APC is the largest structural core protein of the destruction complex that binds with both Axin and β-catenin. Phosphorylation of APC by CK1 (and possibly GSK3β), increases its affinity for β-catenin over Axin [151, 152]. GSK3β negatively regulates Wnt/β-catenin signaling through N-terminal phosphorylation of β-catenin within the destruction complex [153], which is believed to be the major event that induces β-catenin degradation.

GSK3β action generally requires a priming kinase acting 4-5 amino acids upstream of the GSK3β phosphorylation site. In regards to the destruction complex, CK1 acts as the priming kinase on β-catenin [154]. Furthermore, inhibition of GSK3β activity can occur via multiple pathways that include phosphorylation and interaction with protein binding partners that sequester GSK3β away from the destruction complex [155-157]. The role of PP2A in the β-catenin destruction complex is still unclear, and both positive and negative regulation has been described. PP2A has been shown to bind to both Axin and APC [158-160], and to dephosphorylate APC and β-catenin (in the absence of APC). Therefore, PP2A may dephosphorylate APC to progress β-catenin degradation (as discussed below), but may also stabilize β-catenin in the absence of proper complex formation. β-catenin is a highly conserved protein that is involved in a wide range of physiological systems [161]. The action of β-catenin is highly dependent on its structural composition, allowing for the formation of various protein complexes. The β-catenin protein possesses C-terminal, N-terminal and central domains. The C- and N-termini are structurally flexible, facilitating phosphorylation and transcription factor interaction, while the central region is the protein binding core and serves as an interaction platform for many proteins [162] (Figure 6). Overlapping binding sites disallows simultaneous binding of its protein partners, which is crucial for proper β-catenin activity and regulation [163-165].

IV. 1.2 The β–catenin destruction complex model:

Initially, Axin forms a complex with APC, GSK3β, CK1 and PP2A, and in the absence of Wnt signaling, new synthesized β-catenin is recruited to the complex and binds to Axin. The β-

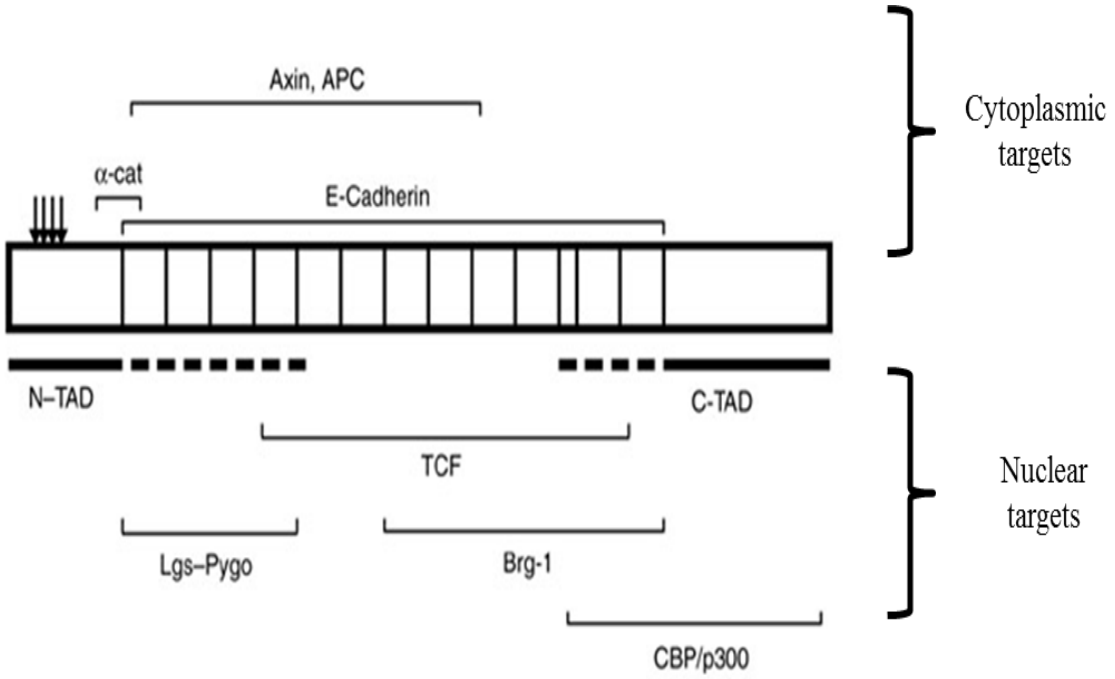

Figure 6: Domains of β-catenin. The primary structure of β-catenin, with its highly conserved Armadillo repeat domain (12 repeats boxed) and relatively unstructured N- and C- terminal domains. Its N-terminal GSK3β and CKI phosphorylation sites (arrows) are required for proteasome-mediated destruction, by β-TrCP. Two transcriptional activation domains (TAD) are indicated, which are important for nuclear signaling. The interaction domains with its binding partners are bracketed. Many of the β-catenin partners compete for the same binding site within the Armadillo repeat domain, which ensures mutual exclusivity. APC, adenomatous polyposis coli; α-cat, α-catenin; TCF; T-cell factor; Lgs-Pygo; Legless- Pygopus; CBP/p300, CREB-binding protein; Brg-1, also known as ATP-dependent helicase SMARCA4. Figure obtained with permission from [166].

catenin binding site is conveniently positioned between the GSK3β and CK1 binding sites, which allows for phosphorylation of the N-terminal domain of β-catenin at Ser45, Thr41, Ser37, and Ser33 [167]. At the same time, GSK3β and CK1 may also phosphorylate APC which coincides with a dramatic increase of APC binding affinity for β-catenin [168, 169]. APC is then able to outcompete Axin for the β-catenin binding domain, which frees Axin to accept an additional β-catenin. The SCF-E3 complex recognizes and binds to the phosphorylated β-catenin and ubiquinates the protein. However, the mechanism through which β-catenin exits the destruction complex is less clear. It is possible that APC/ β-catenin dissociate from the complex and the proteasome releases APC to re-join the destruction complex. Alternatively, since APC is a target of PP2A [158, 170, 171], dephosphorylation of APC could weaken its association with β-catenin, allowing it to leave the destruction complex. Repetition of this cycle leads to the decreased cytosolic, and thus nuclear accumulation of β-catenin (Figure 7).

IV. 2. THE FUNCTIONAL ROLES OF β-CATENIN

IV. 2.1 TCF/LEF:

Nuclear β-catenin associates with the TCF/Lef family of transcription factors to transcribe a diverse set of target genes [172]. In the absence of Wnt signaling, TCF/Lef possess limited transcriptional activity due to their interaction with Groucho transcriptional repressors [173, 174]. As β-catenin enters the nucleus, it displaces Groucho, forming a transcriptionally active complex with TCF and other co-activators [175]. TCF/ β-catenin transcription can be modified through a variety of signals. (i) TCF/LEF isoforms that lack the β-catenin binding domain antagonizes TCF/ β-catenin complex activity [176, 177]. (ii) Expression of nuclear

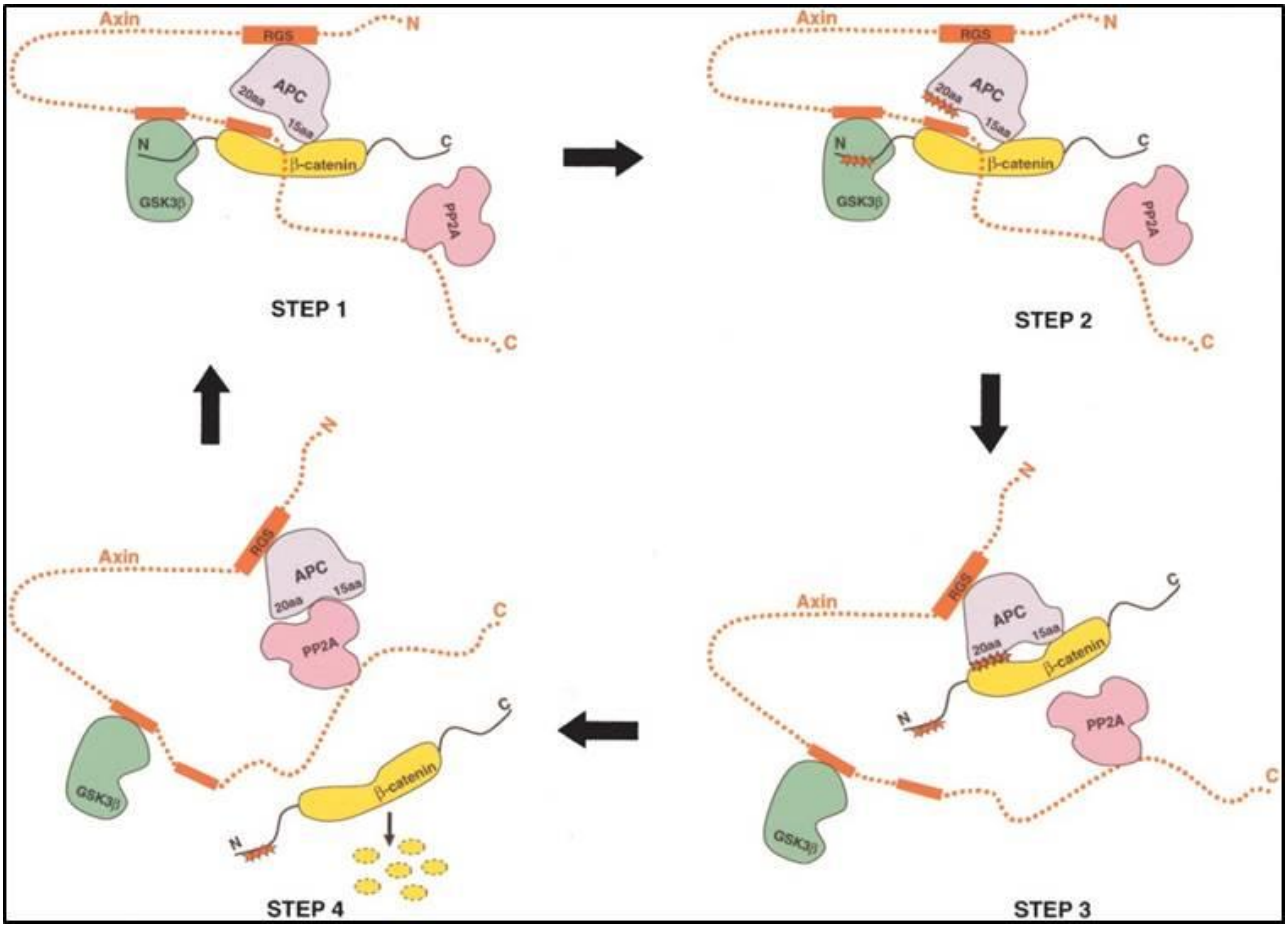

Figure 7: Working model of the β-catenin destruction complex. Step-wise progression of β-catenin destruction. β-catenin is recruited to the complex (step1) and phosphorylated by CK1 and GSK3β (step 2). In step 3, phosphorylation of APC increases its affinity for β-catenin, causing dissociation from Axin. Finally, APC is depohsporylated by PP2A, which releases phosphorylated β-catenin for degradation by the proteasome (step 4). Phosphorylation = red stars. Figure obtained with permission from [178].

antagonists, Chibby and ICAT, can bind to β-catenin to limit its interaction with transcription factors and co-activators, promoting its nuclear exclusion [179, 180]. Lastly, (iii) post-translational modifications such as phosphorylation, acetylation and sumoylation can lead to activation, repression, or degradation of TCF/LEF proteins [176, 177].

IV. 2.2 Adherens Junctions:

In addition, β-catenin binds to the cytoplasmic tail of E-cadherin to stabilize cellular adhesion junctions [181]. In turn, E-cadherin protects β-catenin from degradation and sequesters it from its nuclear targets [182]. When in complex, β-catenin connects E-cadherin to actin filaments, through its interaction with α-catenin [164, 183] to regulate cytoskeleton dynamics (Figure 8). Disassociation of the adherens junction by degradation, cleavage, or phosphorylation of E-cadherin can significantly increase the cytoplasmic pool of free β-catenin that once inside the nucleus can activate the promoter of its target gene [184-188]. Similarly, post-translational tyrosine phosphorylation of β-catenin reduces its affinity for E-cadherin and/or α-catenin leading to the dissociation of the E-cadherin/catenin complex and releasing β-catenin [184, 189-192]. In contrast, phosphorylation of the cytoplasmic tail of E-cadherin strengthens its association with β-catenin by a magnitude of several hundred-fold [164, 193, 194].

IV. 2.3 β-catenin and cancer:

Dysregulation of β-catenin, and the pathways it governs, is a common theme in malignancy [195-197]. Indeed, germline mutations of the APC gene are linked to a hereditary form of colorectal cancer, known as familiar adenomatous polyposis (FAP) [198, 199]. FAP

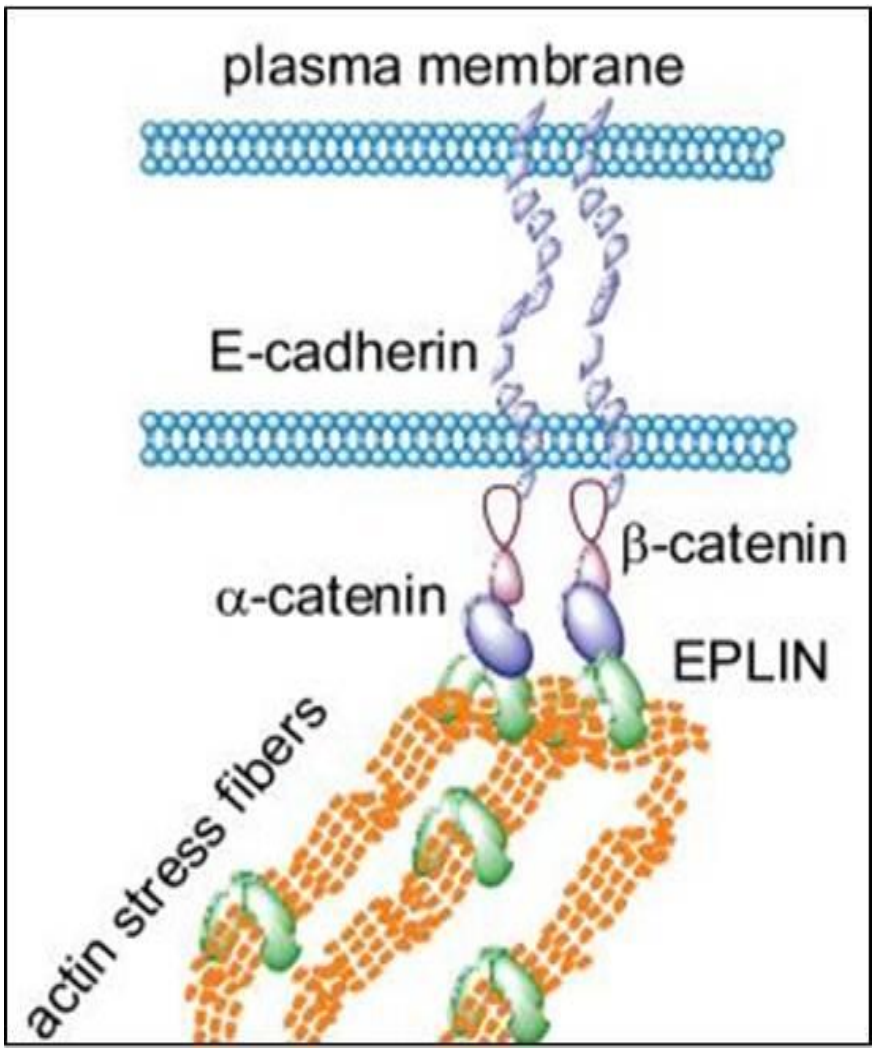

Figure 8: Epithelial adherens junction. β-catenin is part of a protein complex which forms the adherens junction. These junction mediate cell-to-cell signaling in epithelial cells and mediated extracellular interaction of E-cadherin. The intracellular tail of E-cadherin binds to β-catenin, which connects E-cadherin to the cytoskeleton through its interaction with α-catenin and actin filaments. Downregulation of E-cadherin results in the disintegration of adherens junctions, remodeling of the actin cytoskeleton and activation of β-catenin signaling. Figure obtained with permission from [200].

colorectal cancer results from the loss of the APC gene [201] and inappropriate stabilization of β-catenin [202]. Furthermore, activating mutations in β-catenin occur in a wide variety of tumors [203]. Nuclear accumulation of β-catenin can also act as a marker for distant metastasis in a variety of cancers [204-208], likely due to the activation of a transcriptional profile that leads to epithelial-to-mesenchymal transition (EMT) [209]. EMT is a process that is exploited in cancer systems and endows cells with higher invasive, metastatic and survival potential [209]. Since Wnt/β-catenin is implicated in the regulation of malignant programmes that stimulate the aggressive nature of cancer cells, β-catenin has become an attractive target for use in therapeutic treatments and clinical trials [210]. The role of EMT and cancer cells will be discussed in the next section.

V. EPITHELIAL-TO-MESENCHYMAL TRANSITION

Epithelial to mesenchymal transition (EMT) is a biological process that allows epithelial cells to assume mesenchymal characteristics, bestowing them with a greater migratory and invasive capacity, resistance to apoptosis, and increased secretion of extracellular matrix (ECM) components [211]. The process of EMT can also be reversed by mesenchymal-to-epithelial transition (MET), suggesting plasticity between these two pathways [212].

The importance of EMT is highlighted in development, where multiple cell types are derived from a single cell. In fact, cells can undergo multiple rounds of EMT and MET before terminal differentiation and the completion of development [209, 211]. However, it is now known that EMT aids wound healing in adult tissue and can be inappropriately exploited during carcinogenesis. Indeed, EMTs can be classified into three platforms that are based mainly on the biological context in which they occur [213]. Type I and II EMTs are not usually associated with disease or disorder, but are necessary for proper organ development and the regeneration of tissue. However, in cells that have undergone epigenetic changes that favor tumor growth, initiation of type III EMT can augment the oncogenic transformation to produce cells widely capable of invasion and metastatic disease. While the three classes of EMTs represent distinct physiological and pathological processes, a reproducible set of cellular changes are common to each platform and form the foundation of each pedigree [211, 213] (Figure 9).

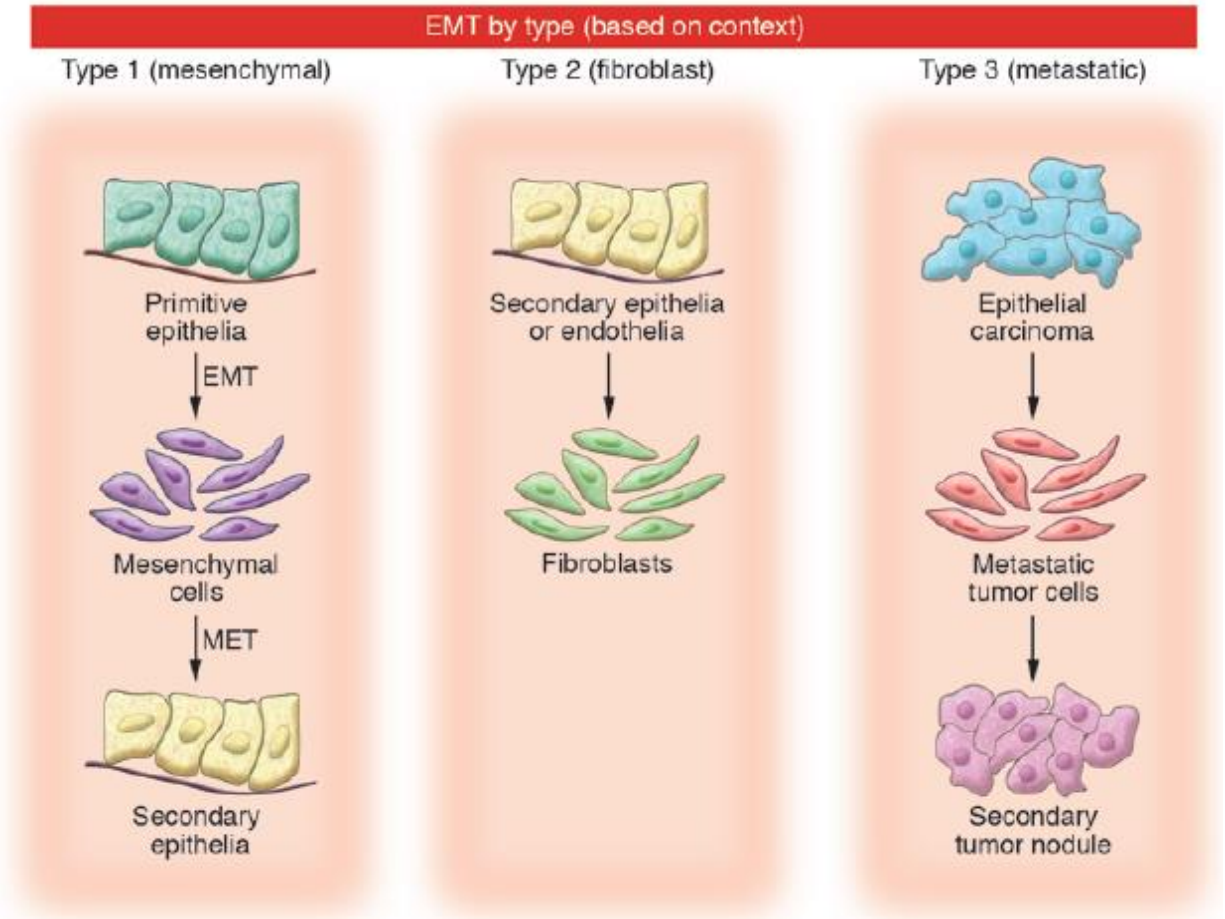

Figure 9: Types of EMT. Three types of EMT have been described. Type I occurs throughout embryonic development to form various epithelia throughout the body. Initially, primitive epithelium will transition into mesenchymal cells for migration, then revert back into new specialized epithelium. Type II EMT occurs as a result of inflammation during wound healing. Epithelial cells take on fibroblast-like features to infiltrate open spaces. Type III EMT is part of a metastatic process, where primary tumor cells transition to mesenchymal cells for metastasis to distant organs. Secondary lesions often undergo subsequent rounds of MET for efficient colonization. Figure obtained with permission from [213].

V. 1. BIOMARKERS OF EMT

V. 1.1 Cell surface markers:

E-cadherin

E-cadherin is the most classically recognized epithelial marker and participates in the proper arrangement of the adheren junctions. E-cadherin is a calcium-dependant cell-cell adhesion protein that consists of an E-cadherin binding extracellular domain, a single transmembrane domain, and as well as an intracellular domain capable of interacting with catenin proteins and actin filaments [214]. Downregulation of E-cadherin can occur through mutation, gene silencing via promoter hypermethylation or transcription factor action, and posttranslational modifications, including degradation [215]. Loss of E-cadherin, or a cadherin switch from E- to N-cadherin, has been implicated in the progression of EMT from both a developmental and cancerous standpoint [216, 217]. Disruption of adherens junction complexes can free cells from their restrictive cell-to-cell contacts, an event that allows for their dissemination outside of the epithelial barrier and leads to increased free β-catenin signaling in the nucleus [218].

V. 1.2 Cytoskeleton Markers:

Vimentin

Vimentin is often implicated in EMT of cancer cells, and is associated with increased invasiveness and metastasis [219-221]. During EMT, epithelial cells, which commonly only express the intermediate filament keratin, initiate the overexpression of vimentin, which contributes to EMT by remodelling cell shape and enhancing motility [219]. Although vimentin

provides cells with flexibility and resistance to overcome stress, its overall function in EMT is unknown [222].

β-catenin

EMT is highly dependent on Wnt/β-catenin signaling. As a biomarker, β-catenin is generally found either in the nucleus or cytoplasm of cells undergoing EMT as opposed to in a complex with E-cadherin at the membrane, which is characteristic of most epithelial cells [213].

V. 1.3 Transcription Factors:

Despite the variety of known EMT inducers, the overall response is reproducible. Indeed a number of transcription factors, including the Snail family, Twist, ZEB1, ZEB2, HMGA2, Ets1, Sip1, FOXC2 have all been suggested to regulate the transcriptional profile the dictates EMT.

Snail transcriptional factors (TFs) are the most widely characterized effectors of EMT, and include Snail 1 (Snail), Snail 2 (Slug), and Snail 3 (SMUC). All family members are part of the zinc-finger type of transcription factors and bind to the E-box (5'-CACCTG-3') in various promoters. However, compared to Slug, and SMUC, Snail binds to this sequence with a higher affinity and acts as the more potent regulator of E-cadherin [223]. Snail TFs have also been shown to form a complex with other transcriptional regulators in the nucleus, such as β-catenin and Smad2/3 [224, 225], to directly affect the activation or suppression of its target genes. Aside from E-cadherin, Snail TFs play a role in regulating the expression of various epithelial and mesenchymal markers. Expression of cytokeratins, occludins, and claudins are decreased, while fibronectin and vimentin are increased, by Snail/Slug [213, 223].

V. 2. INDUCERS OF EMT

V. 2.1 Growth Factors:

The induction of EMT is thought to depend on previous genetic insults of cancer cells that make them more susceptible to EMT-inducing signals [211]. Indeed, signaling from multiple growth-factors has been shown to increase the expression of EMT inducing TFs. Once activated, these TFs initiate the EMT process by forcing the expression of its targets genes and stimulating downstream signaling pathways. Specifically, TGF-β has been described as an important regulator of EMT mechanics [226] via the induction of Snail, ZEB, and Twist TFs [227]. TGF-β has also been reported to work in cohort with other signaling pathways such as Wnt/β-catenin [228, 229], and is dependent on a variety of transduction effectors [230-232] to elicit or augment its EMT-inducing potential.

V. 2.2 β-catenin:

Increased β–catenin in the nucleus or cytoplasm, resulting from the loss of E-cadherin at the membrane or β-catenin activating mutations, is linked to EMT [233, 234]. Wnt/β-catenin has been shown to downregulate the expression of the epithelial marker, E-cadherin, and upregulate the expression of mesenchymal markers, Fibronectin, MMP-7, Tcf/Lef, Twist, Zeb, Snail, and Slug [225, 235-241], as well as other genes that exacerbate the malignant phenotype, by both direct and indirect means [242-244]. In addition, Snail forms a transcriptionally active complex with β-catenin [225] and may participate in a positive feedback loop to enhance its own expression as well as the expression of other β-catenin target genes. Together, β-catenin signaling, possibly working in tandem with other signaling mechanisms, strongly regulates the acquisition of EMT phenotype and tumor biology.

V. 3. EMT AND CANCER PROGRESSION

Early in cancer progression malignant cells gain enhanced proliferative potential, which results in the growth of a locally confined tumor. Cancer cells undergoing EMT gain fibroblast-like cell markers, which confer a superior invasive and survival capacity. This alteration in cell type also makes cells receptive to mesenchymal signaling through interactions with surrounding stromal cells [211]. Cancer cells can then break down basement membrane barriers and invade through the endothelial lining to be systemically delivered through blood and lymph [227]. In the case of ovarian cancer, cells or cell spheroids are exfoliated from the primary site and enter the peritoneal cavity where they spread via malignant ascites to other peritoneal organs [245-247]. Ovarian spheroids maintain their mesenchymal characteristics, with reduced E-cadherin expression, and exhibit a more invasive and aggressive phenotype [248]. Indeed, the loss of E-cadherin allows for the transcriptional upregulation of the fibronectin receptor, integrin α5β1, which facilitates adhesion to the secondary site [249]. It is thought that cells undergo subsequent MET, due to either the presence of new microenvironmental cues or the lack of paracrine signals from the primary tumor, a state which favours metastasis formation and sustains rapid growth [250, 251]. Therefore, EMT/MET imparts a high level of regulation on various steps of cancer progression: EMT is important for the dissemination and aggressive behaviour of cancer cells leaving the primary tumor, whereas MET is vital for the efficiency of colonization at metastatic sites.

VI. RATIONALE, HYPOTHESIS, AND OBJECTIVE OF THE STUDY

Our lab has previously demonstrated that Nodal induces apoptosis and inhibits cell proliferation by activating its receptor, activin receptor kinase 7 (ALK7) in ovarian cancer cells [54, 252]. Using a Human Cell Cycle Gene Array, we found that one of the genes strongly induced by Nodal and ALK7 is cyclin G2. We selected cyclin G2 for further studies because its role in cell cycle progression is not clear and there are no reports regarding cyclin G2 in ovarian cancer.

Using overexpression and knockdown approaches, we found that cyclin G2 exerts an inhibitory effect on ovarian cancer cellular proliferation and partly mediates the antiproliferative effect of Nodal/ALK7 [54]. During this stage of the study, we also found that cyclin G2 is highly unstable and is degraded quickly by the ubiquitin(Ub)-proteasome pathway, and that Nodal/ALK7 can increase cyclin G2 stability by inhibiting its degradation [253]. Removal of the destabilization PEST domain in cyclin G2 greatly enhanced its stability, which suggests that it is a major target for cyclin G2. PEST sequences have been shown to serve as targets for the proteasome complex [254, 255] as well as calcium-dependant proteolysis by calpain [139, 256]. Future studies examining the mechanism of calpain activation and subsequent cyclin G2 proteolysis may describe a novel pathway that contributes to the unstable nature of cyclin G2. The possibility for cross-talk between calpains and the proteasome may account for the rapid degradation of cyclin G2. This instability is likely important for the normal progression of the cell cycle, however constitutive activation of one or more of these degradation pathways could easily contribute to the loss of cyclin G2 in pathological states, including cancer. Therefore, a

delicate balance between expression and degradation of cyclin G2 must be maintained in order to ensure homeostasis.

Increasing evidence has suggested that dysregulation of cyclin G2 may be an important step in cancer progression. However, the molecular function of cyclin G2 has yet to be well-defined. Diverse signaling pathways and genetic regulation tightly control the proper expression of cyclin G2 and dysregulation of any of these pathways would oppose normal homeostasis and may lead to pathology. Since recent evidence has consistently illustrated a role for cyclin G2 as an anti-tumor protein, one effect of decreased cyclin G2 expression may be directly linked to cancer progression. In fact, an inverse relationship is observed between cyclin G2 expression and malignancy, and cyclin G2 has been used as a marker to predict metastasis-free survival [70, 86, 257, 258]. Future studies will enhance our understanding of the function of the cyclin G2 protein and may reveal novel screening or therapeutic targets for this deadly disease. The significance of this work lies in improving our understanding of a relatively ambiguous protein, and its role in ovarian cancer development and progression.

Based on our findings and studies in other types of cancer, we hypothesize that 1) cyclin G2 exerts tumor-suppressive effects in ovarian cancer cells; 2) growth factors and their intracellular signaling pathways are involved in the regulation and action of cyclin G2 and; 3) cyclin G2 is degraded via multiple degradation pathways, including calpain-mediated proteolysis. Overall, we hypothesize that cyclin G2 suppresses ovarian cancer metastasis and its inhibition by calpain and growth factors is a critical event in ovarian cancer development. The objectives of my Ph.D. study were to further characterize the regulation of cyclin G2 and to

investigate the role of cyclin G2 in ovarian tumorigenesis. Specifically, I 1) investigated the role of cyclin G2 in ovarian tumorigenesis; 2) examined how cyclin G2 exerts its anti-proliferative and anti-invasive effects on EOC cells and; 3) determined the biochemical mechanisms that regulate cyclin G2 stability and degradation by calpain.

CHAPTER 2

CYCLIN G2 EXERTS ANTI-TUMOR EFFECTS BY ATTENUATION OF β-CATENIN SIGANLING

ABSTRACT:

Dysregulation of cyclin G2 (CCNG2) in a variety of human cancers has been reported; however, its role in tumorigenesis is not clear. The objective of this study was to investigate the function of cyclin G2 in ovarian cancer development. Overexpression of cyclin G2 inhibited cell proliferation, migration, invasion, and spheroid formation *in vitro* and tumor formation and invasion *in vivo*. Characterization of stable cells expressing cyclin G2 showed increased levels of E-cadherin, and decreased levels of vimentin, N-cadherin, Snail, and Slug, as well as a decrease in actin stress fibers, suggesting that cyclin G2 may exert its anti-tumor effects by inhibiting epithelial-to-mesenchymal transition in EOC cells. Cyclin G2 overexpression also resulted in a decrease in total β-catenin levels. On the other hand, phospho-β-catenin was induced by cyclin G2 overexpression. Furthermore, there was a decrease in nuclear β-catenin and an increase in membrane-associated β-catenin. Activation of β-catenin attenuated the effects of cyclin G2. Finally, quantitative PCR of human ovarian tumor samples revealed lower cyclin G2 levels in high-grade carcinoma compared to that of borderline or low malignant potential tumors. Taken together, these novel findings demonstrate that cyclin G2 has tumor-suppressing effects in EOCs by inhibiting EMT through attenuating β-catenin signaling.

INTRODUCTION:

Ovarian cancer is the most fatal gynecological malignancy and one of the leading causes of cancer related deaths in women [1, 2]. There are three types of ovarian cancer: epithelial (EOC), germ cell, and stromal cancers [3]. EOC is the most common type of ovarian cancer and contributes to 90% of all reported cases [1, 4-6]. Of these, high grade serous ovarian cancer (HGSC) is the most aggressive and lethal subtype of EOC and make up close to 70% of all diagnosed cases [7]. Due to the lack of effective screening markers, most cases are diagnosed at late stage, when patient prognosis is poor with a 5-year survival rate of less than 30% [8]. Recently, analysis of the genomic and epigenomic abnormalities of HGSC has shed some light on the molecular pathways altered in ovarian cancer progression [9]. HGSC are characterized by a high genomic instability that may lead to alternations in downstream signalling pathways.

Cyclin G2 belongs to a group of unconventional cyclins that include cyclin G1 and cyclin I, and unlike typical cyclins, they function to maintain the quiescent state of cells and cell cycle arrest. The expression of cyclin G2 is commonly found in terminally differentiated tissue [10] and recent evidence has suggested that cyclin G2 works in cohort with master differentiation factors, such as p63 and peroxisome proliferator-activated receptor γ (PPARγ) [11, 12]. Therefore, cyclin G2 may act as a differentiation factor in a variety of cellular contexts. Increasing evidence also suggests that cyclin G2 exerts important effects in cancer progression. It is upregulated in response to growth inhibitory signals, downregulated by oncogenic stimulators, and leads to the production of aberrant nuclear structures and deregulated microtubule networks [10, 12, 13]. The alteration of any of the transduction pathways in which cyclin G2 is involved in may affect the normal cellular physiological and increase the susceptibility for pathological

disorders. Cancer progression involves various genetic changes that cooperate to enhance the cell tumorigenic potential [12], and cyclin G2 is one of the many genes that have shown significant irregularities between normal and cancerous tissue [14-16].

Epithelial-to-mesenchymal transition (EMT) is a process by which epithelial cells acquire a motile and invasive phenotype, characteristic of mesenchymal-like cells [17, 18]. During EMT, cells gain mesenchymal markers, such as Snail, Slug, ZEB, and Twist, which are transcriptional repressors of the classical epithelial marker, E-cadherin [19-23]. Downregulation of E-cadherin results in the loss of adhesion junctions and activates β-catenin signaling, which can contribute to EMT [18]. EMT plays an important role in tumorigenesis, particularly during distant metastasis, of many cancer types, including ovarian [24]. The regulation of EMT depends on the signaling of various intracellular networks, such as the β-catenin pathway. Enhanced nuclear signaling of β-catenin has been reported to increase cell invasiveness and induce EMT in ovarian cancer cells [25, 26]. Nuclear accumulation of β-catenin is regulated in part by its localization at the cell membrane, through its association with E-cadherin, and by GSK3β-induced degradation via the β-catenin destruction complex [27, 28].

We have previously reported that cyclin G2 inhibited ovarian cancer cell proliferation [29]. To further understand the role of cyclin G2 in ovarian cancer development, we examined the function of cyclin G2 in EOC cells and investigated the mechanisms underlying the actions of cyclin G2. We demonstrate that the overexpression of cyclin G2 promotes the epithelial phenotype in ovarian cancer cells, which may account for the decreased aggressiveness of these cells, via the attenuation of β-catenin activity.

METHODS AND MATERIALS

Cell lines and cell culture

Four different epithelial ovarian cancer cell lines were used: ES-2, OVCAR-3 and SKOV3 were purchased from American Type Culture Collection (Manassas, VA, USA). SKOV3.ip1 were kindly provided by Dr. Mien-Chie Hung (University of Texas M.D. Anderson Cancer Center, Huston, Texas). SKOV3, SKOV3.ip1, and OVCAR-3 cells are most often referred to as high-grade serous ovarian cancer, while ES-2 cells were thought to originate from a clear cell tumor. However, in light of recent mutational analysis [30] it appears as SKOV3/SKOV3ip1 cells are more representative of clear cell tumors, while ES-2 and OVCAR-3 are possibly models of high-grade serous cancers. SKOV3/SKOV3ip.1 cells are p53-null and ES-2 and OVCAR-3 cells are p53-mutated ES-2, SKOV3, and SKOV3.ip1 were maintained in MyCoy's (Sigma-Aldrich) culture media, supplemented with 10% FBS (Gibco, Life Technologies), OVCAR-3 cells were maintained in RPMI-1640 (GE HyClone) culture media and supplemented with 10% FBS. To produce cells line with bioluminescence capability, firefly luciferase, pMir-luciferase (Life Technologies) was stably transfected into the SKOV3.ip1 cell line. To generate stable cell lines FLAG-cyclin G2 was cloned into the viral vector, pBabe-puro. In 293T, virus was produced by calcium phosphate co-transfection (18 hours) of 10μg of either pBabe-FLAG-cyclin G2 or pBabe-empty vector, as well as 3.5μg of the packaging plasmid for producing retroviral particles, pUMVC, and 6.5μg of the envelope plasmid. pCMV-VSVg. The media was changed and 24 hours later the virus was harvested by passing the media through a 0.45 μM filter. This media was subsequently used to infect each target cell line. Stable cell lines were maintained in puromyocin selection media and cyclin G2 expression was confirmed by

Western blot using an antibody against the FLAG tag or by qPCR using primers specific for cyclin G2.

Transient transfection

The FLAG-cyclin G2 plasmid was generated as described previously [29]. Transient transfection was carried out using Lipofectamine 2000 (Invitrogen, Life Technologies) according to the manufacturer's protocol. Small interfering RNAs (siRNA; 50 nM) were transfected into cells for 6 h using Lipofectamine 2000. To confirm gene-specific silencing effects, protein lysates were prepared at 24-48 h after transfection and subjected to western blot analysis. E-cadherin, GSK3β, and a scrambled control siRNA, which has no significant homology to any mammalian gene sequence, were purchased from GenePharma Company.

E-cadherin: 5-GAGUGAAUUUUGAAGAUUGTT-3

GSK3β: 5-CUCAAGAACUGUCAAGUAATT-3

Control siRNA: 5-UUCUCCGAACGUGUCACGUTT-3

Western blot and immunoprecipitation

Cell lysates were prepared and Western blot was performed as reported previously [31]. Briefly, cells were lysed in a buffer containing 150mM NaCl, 1% Nonidet P-40, 50mM Tris/HCl (pH 7.4) and supplemented with protease and phosphatase inhibitor cocktail just prior to use (Pierce, Thermo Scientific). Total protein was quantified by the Pierce BCA Protein Assay Kit (Thermo Scientific) and equal amounts of protein were separated by 12% SDS-PAGE and transferred onto Immuno-Blot PVDF membranes (BioRad). Primary antibodies used are listed in Table 1. Membranes were probed with secondary HRP-coupled antibodies for 1-2 hours at room temperature. Proteins were visualized by Luminata Classico Western HRP Substrate (Milipore).

Table 1: Antibody and Staining Reagents for Western blot and Immunofluorescence

Antibody	Company	Species	Dilution/Concentration
E-cadherin	BD Biosciences	Mouse	1:1000
Vimentin	Santa Cruz	Goat	1:500
Flag-M2	Sigma	Mouse	1:2000
β-catenin	Cell Signaling	Rabbit	1:2000
p-β-catenin (S33, 37, 41)	Cell signaling	Rabbit	1:1000
GSK3β	Cell signaling	Rabbit	1:1000
GAPDH	Santa Cruz	Mouse	1:5000
Lamin B	Santa Cruz	Goat	1:500
PP2A	BD BioScience	Mouse	1:1000
CCND1	Santa Cruz	Mouse	1:500
p473 Akt	SAB	Rabbit	1:1000
p308 Akt	SAB	Rabbit	1:1000
tAkt	SAB	Rabbit	1:1000
GSKα/β	Invitrogen	Mouse	1:1000
B-catenin-Alexa-488 (IF)	Cell Signaling	Mouse	1:100
F-actin (Phalloidian-FITC) (IF)	Sigma	---	50μg/mL
DAPI (IF)	Sigma	---	1:1000

Where indicated, cells were treated with 10μM SB-216763, to inhibit GSK3β activity, or 5 μM MG132, to inhibit the proteasome, prior to cell lysis.

For IP, cells were seeded in 10-cm dishes and cultured for 16 h. IP reactions were performed by incubating the same amount of cell lysate (300 μg) with anti-GSK3β antibodies (2μg, Cell Signalling), or its IgG control (Santa Cruz Biotechnology) at 4°C for 16 h. Following subsequent incubation with protein A/G agarose beads (GE Healthcare, Waukesha, WI) at 4°C for 3 h, the immune complexes were collected by centrifugation (10,000× *g*) at 4°C for 1 min, washed, and eluted into 50μl 2×SDS sample buffer. Samples were used for Western blot analysis.

Cellular Fractionation:

To separate the cytoplasmic and nuclear extracts from cultured cells, the Thermo Scientific NE-PER Extraction kit was used. Cells were cultured in 10 cm dishes and treated, as indicated. Cells were trypsinzed and pelleted by centrifugation at 500 x g for 5 minutes at 4°C. Cells were washed with PBS and cellular fractions were prepared according to manufacturer's protocol. Cytoplasmic and nuclear fractions were mixed with one-volume of SDS-sample buffer and run on SDS-PAGE gel.

Immunofluorescence imaging and microscopy

Cells were fixed with cold methanol for 5 minutes (1:1 volume), permeabilized with 0.2%Triton X-100 for 10 minutes, blocked with 1%BSA for 30 min, and incubated with anti-β-catenin-488 conjugate antibody (1:100) in PBS containing 1% BSA. Microscopy was performed using a Zeiss LSM 700 confocal microscope.

PCR and quantitative real-time PCR (qPCR)

Total RNA was extracted using Trizol reagent (Invitrogen, Life Technologies) and reversed transcribed to cDNA using SuperScriptIII (Invitrogen, Life Technologies) following the manufacturer's protocol. PCR was carried out in 20 µl volumes contained 1X reaction buffer, 0.5 units of Taq polymerase (New England BioLabs) and 10 µM of each forward and reverse primer. qPCR was carried out in 20µl volumes containing 1X EvaGreen qPCR master mix (Invitrogen), 300nM of forward and reverse primer on the Qiagen Rotorgene Q. Primers are listed in Table 2. Amplification was performed with an annealing temperature of 60° C and 30-35 cycles. Expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were also determined and used as an internal control. The relative expression levels of mRNA were quantified using comparative Ct (ΔΔCt) method.

Cell growth and clonogenic assays.

Cells stably transfected with cyclin G2 or an empty vector control were seeded at a density of 10,000 cells per well in a 6-well plate. Cells were maintained in media containing 10% FBS, trypsinized and counted using trypan blue on days 1, 3, and 5. In some experiments, cells were counted 2 days after transfection. Where indicated, cells were first transfected with either siRNA or plasmid DNA.

For clonogenic assay, cells were seeded onto 60 mm cultured dishes at the density of 1×10^3 cells/well. Cultures were maintained for 9-12 days in complete culture media until colonies appeared. The colonies were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The plates were then photographed and the numbers of visible colonies were counted.

Table 2: PCR Primer sequences

Primer	Sequence
CCNG2	F: GCTGAAAGCTTGCAACTGCCGAC R: GGTATCGTTGGCAGCTCAGGAAC
SNAIL	F: CCTCCCTGTCAGATGAGGAC R: CCAGGCTGAGGTATTCCTTG
SLUG	F: ATGAGGAATCTGGCTGCTGT R: CAGGAGAAAATGCCTTTGGA
HMGA-2	F: TGTAAAACGACGGCCAGTCACTACTCTGTCCTCTGCCTGT R: AACAGCTATGACCATGCTTGGAAAGGGAAGAGACTTGG
CDH2	F: ACAGTGGCCACCTACAAAGG R: CCGAGATGGGGTTGATAATG
VIMENTIN	F: GAGAACTTTGCCGTTGAAGC R: GCTTCCTGTAGGTGGCAATC
TCF1	F: CGGGACAGAGGACCATTACAACTAGATCAAGGAG R: CCACCTGCCTCGGCCTGCCAAAGT
LEF1	F: CGACGCCAAAGGAACACTGACATC R: GCACGCAGATATGGGGGGAGAAA
GAPDH	F:AAGGTCATCCCTGAGCTGAAC R: ACGCCTGCTTCACCACCTTCT
CDH1	F: TGCCCAGAAAATGAAAAAGG R: GTGTATGTGGCAATGCGTTC

Migration and invasion assay

Cells were grown to confluency in a 12-well plate and a wound was made using a 100-μL pipette tip, 24 hours after transfection or treatment with SB-216763 (Sigma-Aldrich). Ten points were marked down the wound, and pictures were taken at the same points 24 hours after the wound was made. The distance migrated by the cells was measured using Photoshop software. Additionally, migration assays were also carried out using 8 μm polycarbonate membrane transwell inserts (Costar, Corning Inc). Cells were collected using Accutase (Innovative Technologies Inc) and seeded on the top of the transwell insert at a density of 15,000 per filter. Cells were incubated with 1% FBS containing media while 10% FBS containing media was added outside the transwells to serve as a chemotactic agent. At 24 hours post plating, cells were fixed and stained with Harleco Hemacolor Staining Kit (EMD, Milipore). Non-migrated cells on the top of the membranes were wiped off using a cotton swab and membranes were liberated with a scalpel and mounted on slides for quantification. Migrated cells were visualized and photographed by a Nikon Eclipse TE2000-U fluorescence microscope (Nikon, Melville, NY, USA) at 100x total magnification. The number of cells migrated were counted using Image J.

Transwell invasion assays were performed in the same fashion as the transwell migration assay with the exception that transwell inserts were pre-coated with growth-factor-reduced Matrigel (BD Biosciences). After drying, the matrigel was reconstituted with 100μl serum free media for 1hour prior to cell seeding.

Where indicated, cells were transfected with siRNA or plasmid DNA 24 hours prior to migration/invasion assay.

Spheroid formation and 3D migration assay

Ovarian cancer spheres were formed by hanging drop. Briefly, cells were trypsinized and re-suspended in complete culture medium. 20µl droplets, each containing 20,000 cells, were plated into the inner surface of Petri dish cover. The covers were then inverted and placed on a dish containing 15ml of PBS. Formation of spheroids were examined and photographed on the fourth day after plating of the droplets.

For 3D migration assays, spheroids were embedded in rat-tail collagen I (BD Biosciences) in a 96 well plate. Collagen I (50 µL) was allowed to partially polymerize for 5 min, at which point spheres were transferred on to the base layer. An additional 75 µL of collagen I was distributed on the top of the spheres and allowed to polymerize completely at 37°C. Images were recorded immediately after polymerization, 3 and 6 days later. Cell migration was visualized by cellular penetration into the surrounding matrix.

Soft agar colony formation assay

To carry out colony formation assays, 1% agar and 2X culture media with 20%FBS was brought to 40°C using a water bath and then mixed at equal volumes to form the base agar. Using a 6-well plate, 1mL of base agar was added to each well and set aside for at least 5 minutes to solidify. Next, 0.7% agarose was heated to 40°C and adherent cells were trypsinzed for counting. The cell volume was adjusted to 100,000 cells per mL and 0.1mL of this suspension was added to a 10mL Falcon tube. Immediately prior to plating, 2mL of 2X media + 20% FBS and 2mL 0.7% agarose was added to cells and mixed gently. Plates were incubated at 37°C in a humidified incubator until visible colonies formed (~4 weeks). Cells were fed 2 times

a week with culture media + 10%FBS. Plates were fixed and stained with 0.5mL of 0.005% crystal violet for 1hour and colonies were counted using ImageJ.

Immunohistochemistry (IHC)

Tumors were freshly excised and fixed in 10% formalin overnight, followed by change to 70% ethanol and embedded in paraffin. Tissues were sectioned, de-paraffinized with xylene, passed through several changes of ethanol and then water, and stained with hematoxylin and eosin (H&E). For IHC, following deparaffinising and rehydrating, sections were boiled in a pressure cooker, washed with Tris-buffered-saline (TBS), and blocked with 10% goat serum. The blocking solution was replaced with primary antibody (anti-E-cadherin) and incubated at 4°C overnight. After washing, the sections were incubated with biotinylated secondary antibody at 25°C for 2 hours, followed by conjugated horseradish peroxidase provided by the Vectastain ABC kit (Vector, PK-4000). The slides were then stained with DAB, and Mayer's Hematoxylin for counter staining.

Tumor formation assay

Female 4-6 week old CD1 nude mice (Charles River Laboratories International, Inc.) were used for *in vivo* tumor studies. Prior to injection, SKOV3.ip1-luc or ES-2 stable cells were cultured in media containing 10% FBS in 12-175mL flasks to 60% confluency. Cells were washed two times with 1xPBS and trypsinzed with 0.2% trypsin. Cells were counted and resuspended in serum free media at $1x10^6$ for subcutaneous (SC) or $5x10^6$ for intraperitoneal (IP) injection in 150 µl media. Mice were injected either by SC or IP injection and weight, behaviour, and tumor size were recorded as indicated. Mice were sacrificed and photographed. Tumors were excised, weighed and measured for SC injection and total body weight was recorded for IP

injected mice. All animal protocols received institutional ethics approval for animal research and work was conducted in accordance to the guidelines of the Canadian Council on Animal Care.

Human ovarian cancer samples and database mining.

Human ovarian cancer samples were kindly provided by Dr. Barbara Vanderhyden, (University of Ottawa, Ottawa Hospital Research Institute, Center for Cancer Therapeutics, Ottawa, ON). Small samples of each tumor were placed in 1mL of Trizol and homogenized using Pro250 homogenizer on ice. Once homogenized, RNA extraction was completed according to manufacturer's protocol.

To evaluate the level of expression of cyclin G2 in human ovarian cancer tumor samples, the ovarian cancer dataset from the Gene Expression Omnibus (GEO: GSE26712) using the Affymetrix human U133A microarray platform was downloaded and analyzed for gene expression.

Statistical analyses

Results are expressed as mean ±SEM. One-way analysis of variance was used to determine the difference between multiple groups, followed by Student-Newman-Keul's test and comparison of two groups was performed by Student's t-test. GraphPad Prism software was used to conduct statistical testing and significance was defined as $p<0.05$.

RESULTS

Cyclin G2 supresses ovarian cancer cell proliferation, migration, invasion and spheroid formation

Previous reports have suggested that dysregulation of cyclin G2 may contribute to the tumorigenesity of cells [15, 16, 32]. Therefore, we investigated whether cyclin G2 overexpression in ovarian cancer cells could potentially exert anti-tumorigenic effects. Due to the highly unstable nature of cyclin G2 [29], we generated stable cells lines using retroviral vectors and confirmed the overexpression of cyclin G2 in various cells lines by detection of the FLAG tag using Western blot analysis (Figure S1). We found that overexpressing cyclin-G2 significantly reduced proliferation in multiple cell lines (Figure S2A), including the ovarian cancer cell lines SKOV3.ip1 and ES-2 (Figure 1A). Furthermore, overexpression of cyclin G2 strongly reduced the clonogenicity of ovarian cancer cell lines resulting in smaller and fewer colonies (Figure 1B and S2B).

Next, we investigated the role of cyclin G2 in migration and invasion. Using a scratch-wound assay, it was found that the overexpression of cyclin G2 strongly attenuated cell migration (Figure 1C and S3A). In transwell invasion assays, the SKOV3.ip1-cyclin G2 stable cells displayed decreased invasive capacity when compared to the empty vector control (Figure 1D). These results we comparable to various ovarian cancer cell lines tested (Figure S3B). In addition, using three-dimension cell culture as a model, we found that the SKOV3.ip1-cyclin G2 cells formed much looser spheroids, compared to the empty vector control cells, which formed well-defined and tight aggregates (Figure 1E, upper panel). When these spheroids were embedded into collagen I, cell migration and invasion were strongly inhibited as demonstrated

by reduced cellular penetration into the surrounding matrix (Figure 1E, lower panel). This experiment was also performed using several other EOC cells and similar results were obtained (Figure S4A and B).

Cyclin G2 inhibits *in vivo* tumor formation

To study the effect of cyclin G2 on *in vivo* progression of the tumor, SKOV3.ip1 cells stably overexpressing cyclin G2 or its empty vector control were injected subcutaneously into mice. Tumors started to form at 15 days after injection and their sizes were determined every 3 days until day 68. Cells overexpressing cyclin G2 formed small tumors that did not grow significantly over the course of the experiment. On the other hand, mice injected with control cells produced tumors that grew much larger (Figure 2A and B). Similarly, in ES-2 cells overexpressing cyclin G2, tumor formation was strongly inhibited (Figure S5). Immunohistochemistry revealed that unlike the tumors formed by control cells, tumors produced from cyclin G2-overexpressing cells failed to invade the surrounding muscle tissue layer (Figure 2C) and a clear muscle-tumor boundary could be distinguished (Figure 2C, white arrows). Furthermore, mice injected intraperitoneally with ES-2 cells stably transfected with cyclin G2 had decreased accumulation of ascites fluid, body weight, and belly distension when compared to the mice inoculated with control cells (Figure 2D and S5).

To determine if cyclin G2 is dysregulated during EOC development, we interrogated ovarian cancer microarray dataset on the Gene Expression Omnibus and found a lower cyclin G2 level in advanced grade epithelial ovarian cancer when compared to normal ovarian surface epithelium (Figure 2E). Consistent with these results, qPCR of human ovarian tumor samples

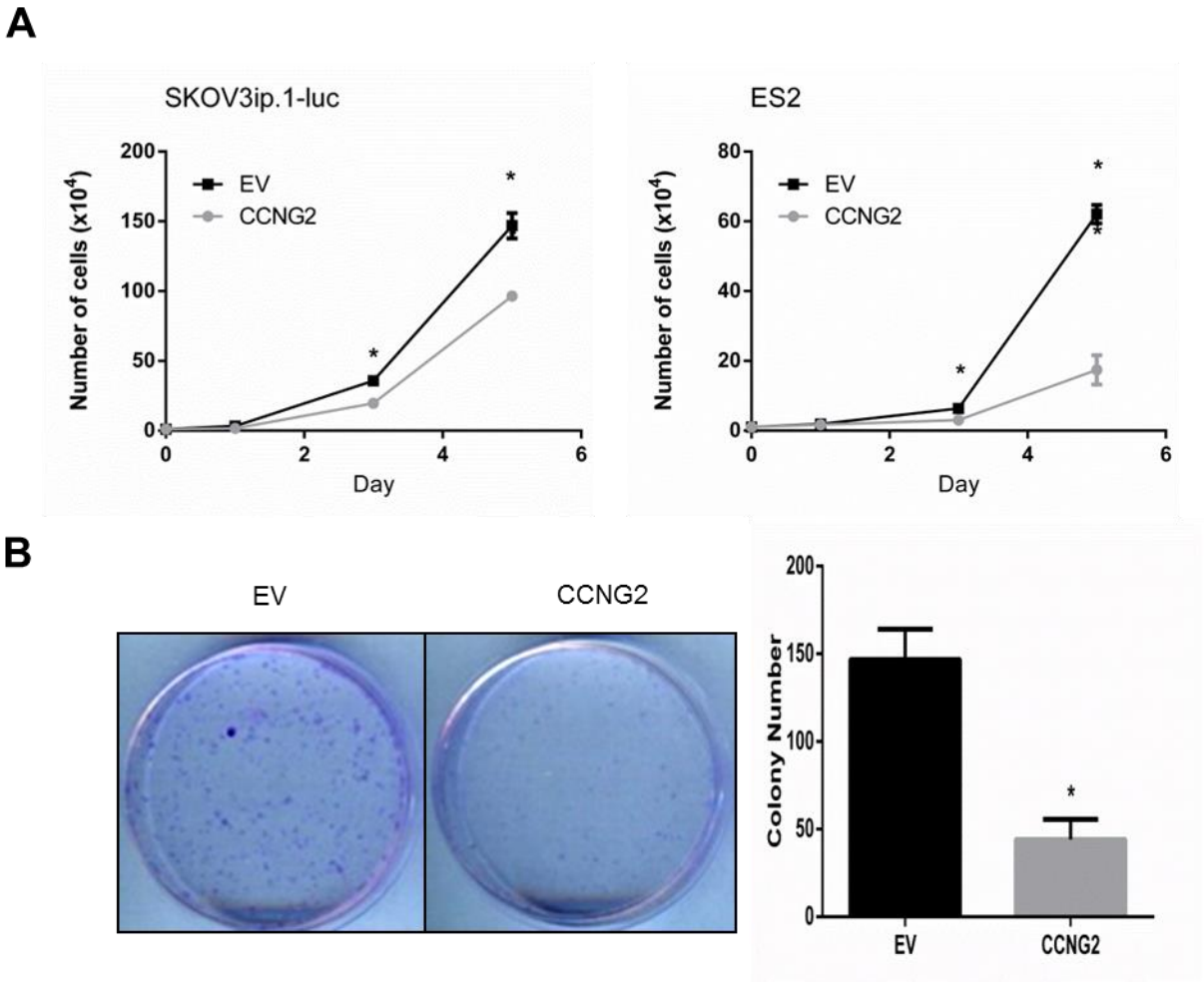

Figure 1: Cyclin G2 supresses ovarian cancer cell proliferation, migration and invasion. A) SKOV3.ip1 (upper panel) and ES-2 (lower panel) stable cells were plated at equal density and cultured for 1, 3, and 5 days. **B)** ES-2 stable cells were plated in very low density and observed until visible colonies formed. Representative pictures (upper panel) show crystal violet stained colonies. Colonies were counted using Image J and graphed (bottom panel).

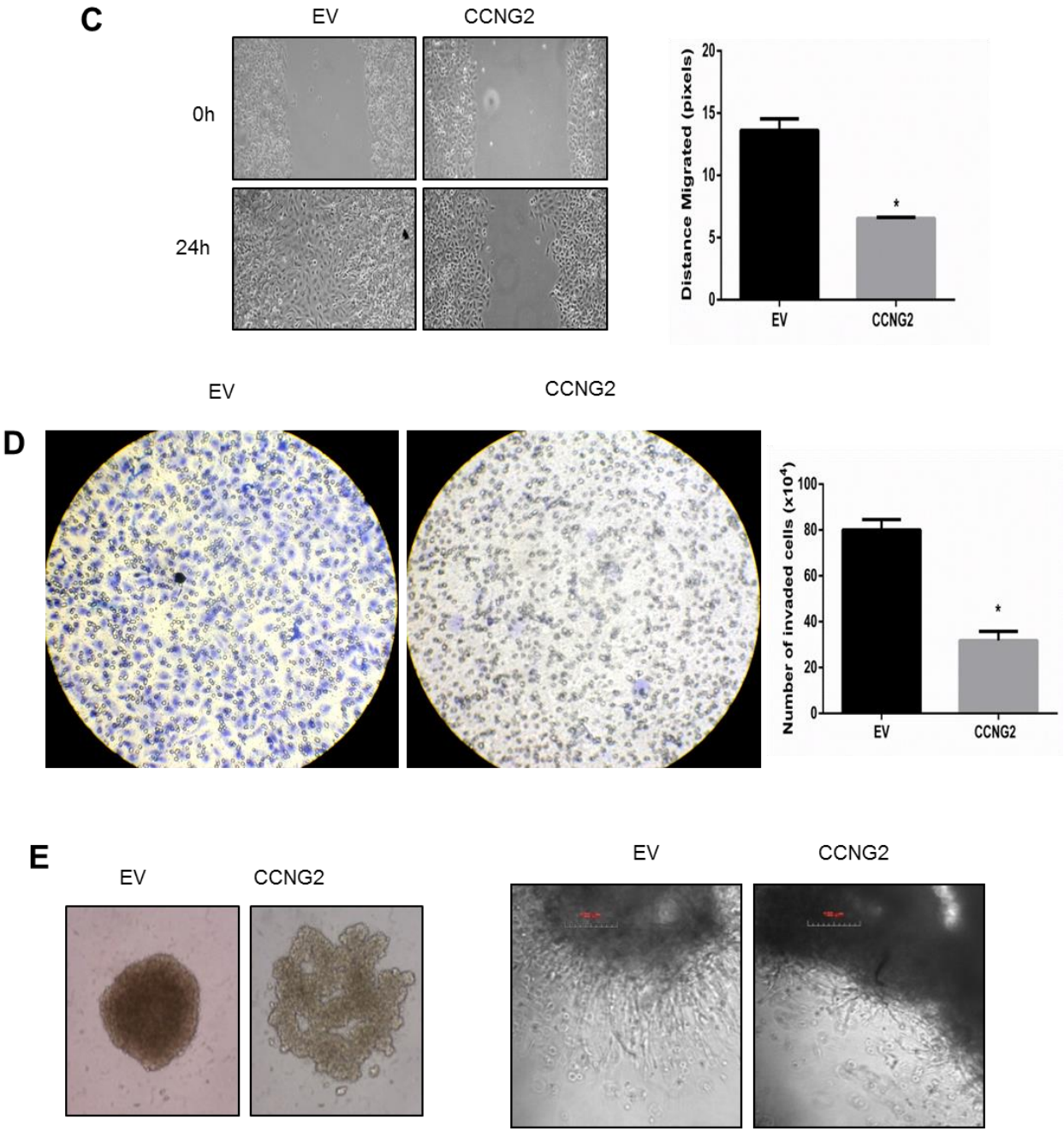

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Figure 1 (cont'd): Cyclin G2 supresses ovarian cancer cell proliferation, migration and invasion. C) Wound healing assay using SKOV3.ip1 stable cells. Pictures were taken right after the wound was created (0h) and 24h later. Overall wound closure was measured at consistent points down the wound and values were graphed. **D)** Matrigel-coated transwell-invasion assay of SKOV3.ip1 stable cells. Nuclei of cells that invaded through the transwell were stained with Harleco Hemacolor staining kit (blue) and visualized by light microscopy (pictures, left panel). Cells in 10 separate fields were manually counted and results were graphed (right panel). **E)** SKOV3.ip1-luc stable cell spheres were formed by the hanging drop culture method. Spheroids were observed and photographed 4 days after plating (left panel). CCNG2 spheres are much looser than the EV control. Resulting spheroids were embedded into collagen I and degree of migration was observed by cellular penetration into the surrounding matrix after 3 days (right panel). EV, empty vector; CCNG2, cyclin G2. *$p<0.05$ vs EV.

revealed lower cyclin G2 levels in high-grade carcinomas compared to borderline or low malignant potential tumors (Figure 2F). In all histotypes of ovarian cancer analyzed, including clear cell, mucinous, endometriod, and serous ovarian cancers, cyclin G2 mRNA was consistently higher in the borderline or low malignant potential tumors (Figure S6).

Cyclin G2 promotes an epithelial phenotype

Epithelial-to-mesenchymal transition (EMT) is a process critical for tumorigenesis and distant metastasis of many cancer types, including ovarian cancer [24] . Since we observed strong inhibitory effects of cyclin G2 on cell migration and invasion, we sought to determine if cyclin G2 was involved in the regulation EMT. We found that upregulation of cyclin G2 could potentiate the epithelial phenotype of ovarian cancer cells. Firstly, we observed that the cyclin G2 cells are more rounded and show greater cell-to-cell contacts, whereas control cells are more spindle-shaped and spread out evenly throughout the plate (Figure 3A). Next, we examined a panel of EMT markers in ovarian cancer cells and found that overexpression of cyclin G2 decreased the mRNA of the transcription factors, Snail and Slug (Figure 3B). Furthermore, we found that classical mesenchymal markers, vimentin and N-cadherin (CDH2), were decreased in cyclin G2 cells. On the other hand, the epithelial marker E-cadherin was up- regulated in cyclin G2 overexpressing cells (Figure 3C & D and S7). Similarly, in tumors formed from cyclin G2-overexpressing cells, E-cadherin levels were strongly enhanced, compared to control tumors (Figure 3D). In the same respect, the high-mobility group AT-hook 2 (HMGA2) protein, which has been shown to be a biomarker of ovarian cancer cells [33] and a marker of poorly differentiated cancers [34], was shown to be decreased in cyclin G2 overexpressing cells (Figure 3B).

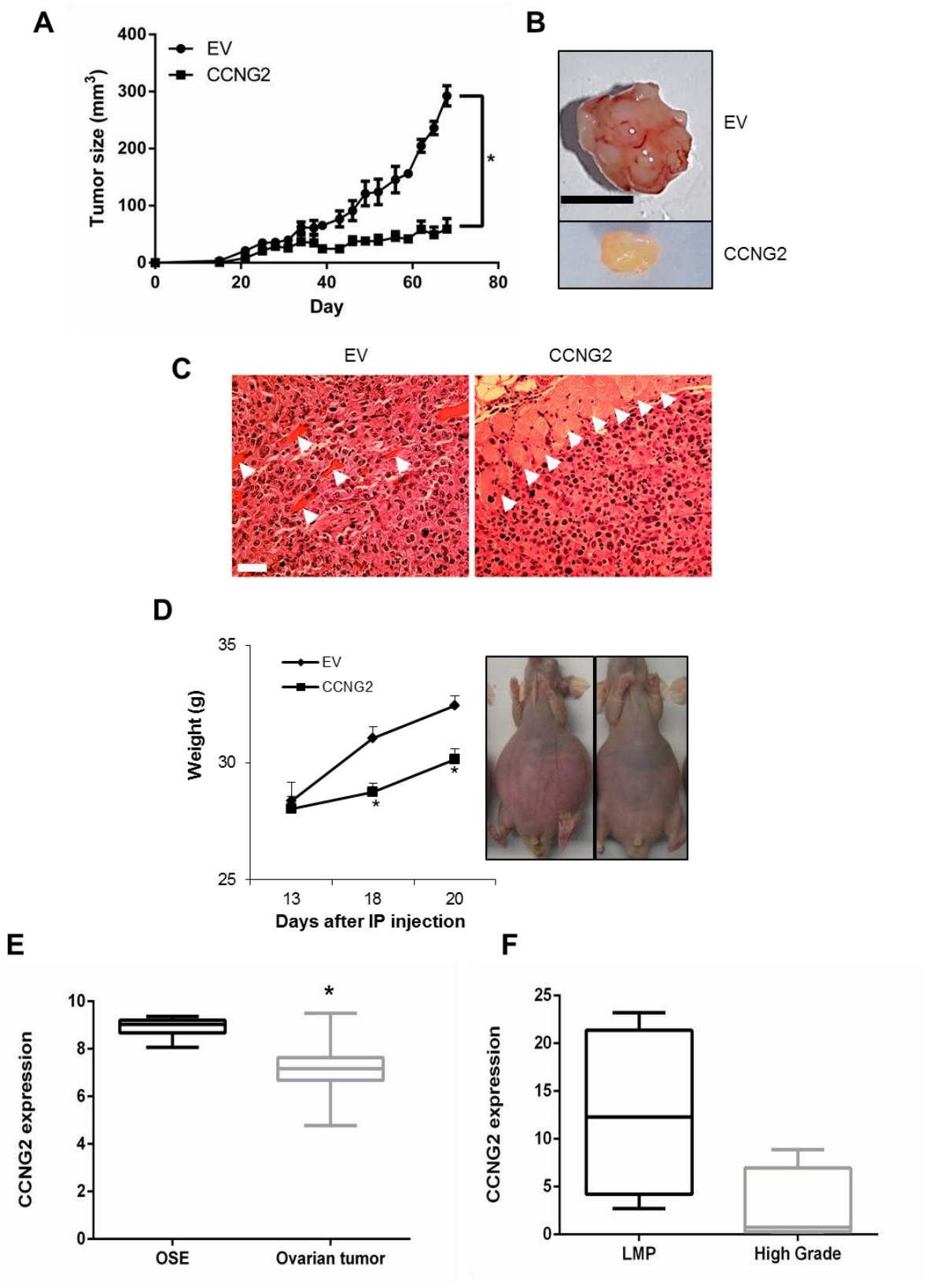

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Figure 2: Cyclin G2 inhibits *in vivo* tumor formation A) SKOV3.ip1 stable cells were injected subcutaneously (SC) into nude mice. Tumor sizes were measured once every 3 days after sizeable tumors began to form (day 15), and measured daily thereafter (mice were sacrificed on day 68). n=4 mice per group. **B)** Representative tumors obtained from SC injection. **C)** H&E staining of tumor sections showing invasion of tumor cells into surrounding smooth muscle (white arrows). Scale bar=50 mm. **D)** ES-2 stable cells were injected intraperitoneally into nude mice. Mice were weighed on days 12, 18 and before sacrifice on day 20. Representative pictures of injected mice showing ascites accumulation (left panel). n=3 mice per group**. E)** Interrogation of ovarian cancer microarray dataset from Bonome data set accessed via GEO (GEO accession: GSE26712). Lower cyclin G2 levels were found in late stage epithelial ovarian cancer when compared to normal ovarian surface epithelium (OSE). OSE, n=10. Tumor, n=185. **F)** qPCR analysis of cyclin G2 mRNA expression in human clinical samples. Ovarian cancer samples of low malignant potential (LMP) or high grade serous ovarian carcinoma (relative to the GAPDH internal control) (n=5 for each group). The box extends from the 25^{th} to 75^{th} percentile, while the mid-line is the median. The whiskers represent the minimum and maximum values. EV, empty vector; CCNG2, cyclin G2. *$p<0.05$ vs EV.

To further investigate the effect of cyclin G2 on EMT, we examined the organization of actin filaments and the formation of stress fibers in the control and cyclin G2 overexpressing cells. Using immunofluorescence, we observed a more bundled and circumferential arrangement of the F-actin in the cyclin G2 cells, (Figure 3E) whereas the empty-vector control group exhibited a more complex array of F-actin, which denotes morphological changes associated with mesenchymal-type cells.

E-cadherin mediates the anti-tumor effects of cyclin G2.

To determine if up-regulation of E-cadherin plays a major role in cyclin G2-regulated cell proliferation, migration, and invasion, we transfected control and cyclin G2-overexpressing SKOV3.ip1 cells with siRNA targeting E-cadherin or control siRNA. Transfection of E-cadherin siRNA strongly suppressed E-cadherin expression, as determined by Western blotting (Figure 4A). Knockdown of E-cadherin did not induce significant changes in cell proliferation in the control group; however, it significantly blocked the growth-inhibitory effects of cyclin G2 (Figure 4B). Furthermore, E-cadherin siRNA reversed the anti-migratory and -invasive effects of cyclin G2, in scratch-wound and transwell-invasion assays, respectively (Figure 4C and D). Finally, in the 3D hanging drop culture, knockdown of E-cadherin in the cyclin G2 cells rescued the well-defined and compact formation of spheroids (Figure 4E).

Cyclin G2 attenuates β-catenin nuclear translocation and signaling.

E-cadherin forms a complex with β-catenin at the adherens junction, sequestering it at the membrane, and inhibiting the activation of its target genes in the nucleus. Furthermore, β-catenin

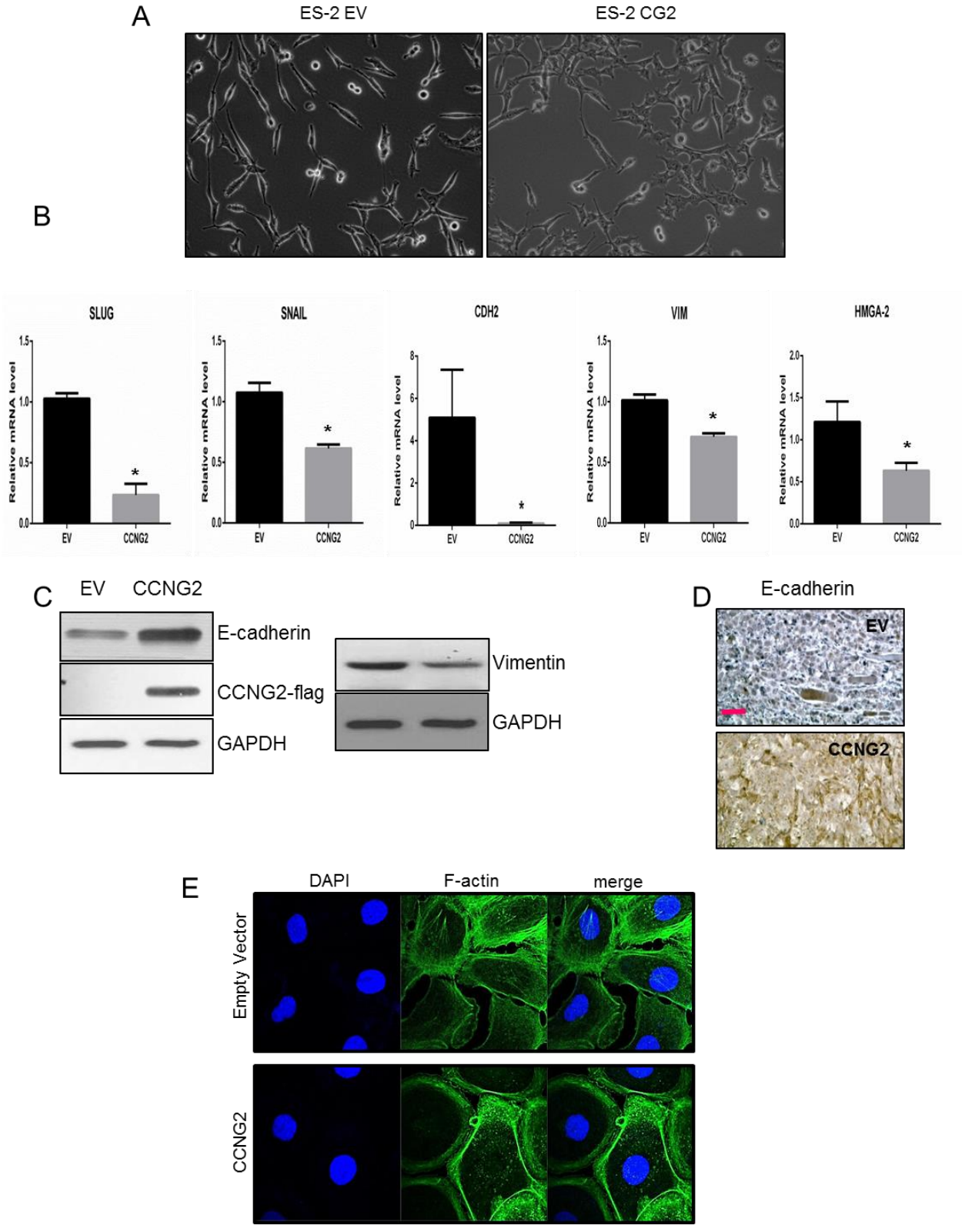

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Figure 3: Cyclin G2 inhibits EMT in EOC cells. A) Morphological changes of ES-2 cells overexpressing cyclin G2. Cyclin G2 overexpression induces greater cell-cell contacts, compared to the empty vector control cell line. **B)** mRNA levels of several EMT makers, snail, slug, high mobility group AT hook-2 (HMGA-2), N-cadherin (CDH2) and vimentin (VIM), in control and cyclin G2 overexpressing Skov3.ip1 cells. **C)** Protein expression of the EMT markers, E-cadherin (left panel) and Vimentin (right panel), by Western blot analysis from Skov3.ip1 stable cells. **D)** Immunohistochemistry of tumor sections obtained from mice subcutaneously injected with ES-2-empty vector or cyclin G2 stable cells, using an anti-E-cadherin antibody. Scale bar=50mm. **E)** Immunofluorescent staining of F-actin using phalloidin-FITC in SKOV3.ip1 stable cells. Images are of representative cells observed. EV, empty vector; CCNG2, cyclin G2. *$p<0.05$ vs EV.

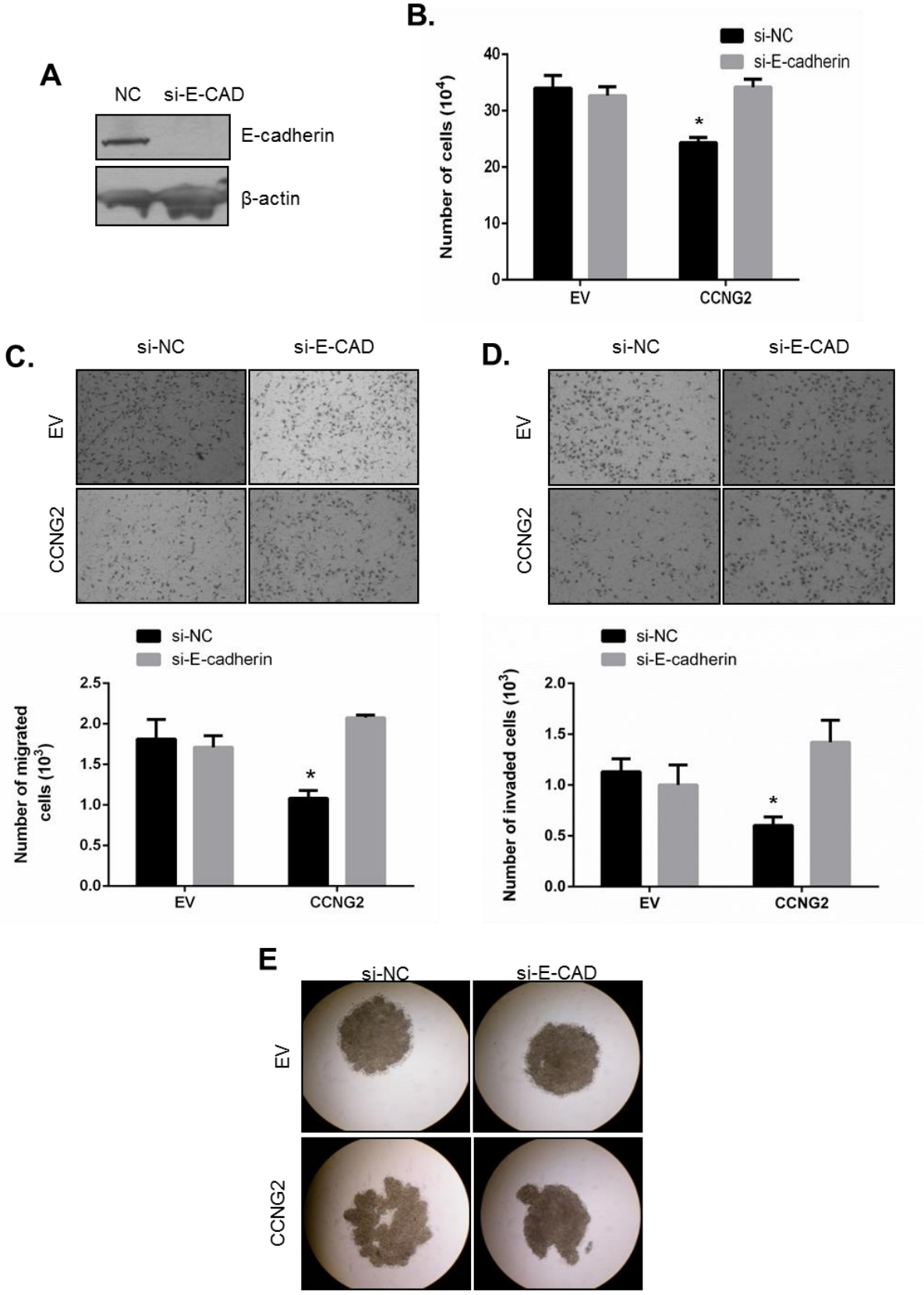

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Figure 4: E-cadherin mediates the anti-tumorigenic effect of Cyclin G2. (A) Knockdown of E-cadherin by negative control (NC) or E-cadherin (si-E-CAD) siRNA was confirmed by Western blotting. **(B)** SKOV3-ip.1 stable cells were transfected with NC or si-E-CAD. Cells were seeded at 10,000 cells per 6-well dish and counted two days post-transfection. Transwell migration **(C)** or invasion **(D)** assays for siRNA transfected SKOV3ip.1 stable cells. Knockdown of E-cadherin by siRNA increased migration and invasion, respectively, of the cyclin G2 cells similar to that of the control. Pictures and graphs are representative experiments. **(E)** SKOV3ip.1 stable cells expressing either cyclin G2 or its empty vector control were transfected with si-E-CAD or NC. Cells were trypsinized and re-suspended in culture medium containing 10% FBS for hanging drop culture and spheroid formation. Formation of spheroids were examined and photographed on the fourth day after plating of the droplets. At least 20 drops/group per experiment were analyzed. Representative spheres are shown. EV, empty vector; CCNG2, cyclin G2. *$p<0.05$ vs all other groups.

has been shown to downregulate E-cadherin through direct or indirect activation of its transcriptional repressors, such as Snail, Slug, Zeb, and Twist [35-38]. Therefore, we determined if cyclin G2 could also regulate β-catenin signaling in ovarian cancer cells. Immunofluorescence revealed that in cyclin G2-overexpressing cells, more β-catenin accumulated at the cell periphery in the cyclin G2 cells, whereas in the empty vector control cells, β-catenin was more diffusely organized with a greater proportion found in the nucleus (Figure 5A and S8A). Another major pathway that inhibits β-catenin signaling is via degradation by the β-catenin destruction complex, which is dependent on GSK3β phosphorylation at serine 33, 37, and 41 [39]. Interestingly, we also found that β-catenin levels were lower in cells that overexpress cyclin G2 than in control cells (Figure 5B). When comparing the nuclear and cytoplasmic fractions from cells that were treated with MG-132 to block protein degradation, cyclin G2 overexpression decreased the level β-catenin in both the cytoplasmic and the nuclear fraction, whereas phosphorylated β-catenin (S31, S37, S41) in the cytoplasm was strongly induced (Figure 5C)

Since β-catenin acts in the nucleus to stimulate gene transcriptional, we investigated the effect of cyclin G2 overexpression on various β-catenin target genes. A significant decrease in the mRNA of both Tcf-1 and Lef-1 was observed in the cyclin G2 overexpressing cells compared to the control cells (Figure 5D). Furthermore, protein levels of cyclin D1 and vimentin, both targets of the canonical β-catenin signaling pathway, were reduced as analyzed by Western blot (Figure S8B). To determine if inhibition of β-catenin is a critical for the observed anti-tumor effects of cyclin G2, we tested if constitutive activation of β-catenin (S33Y) could reverse the effect of cyclin G2. This plasmid is resistant to phosphorylation by GSK3β and will not be recognized by the proteasome for degradation. The β-catenin-S33Y plasmid is more stable

than the wild-type β-catenin and will accumulate in the cytoplasm and nucleus. In cyclin G2-overexpressing cells, transfection of a constitutively active β-catenin mutant (S33Y) reversed the effect of cyclin G2 on cell proliferation and migration (Figure 5E and F), suggesting that attenuation of β-catenin signaling is an important mechanism by which cyclin G2 exerts its anti-tumor effects.

Cyclin G2 destabilizes β-catenin via GSK3β

It is well documented that GSK3β phosphorylates β-catenin and is an integral part of the β–cateinin destruction complex, which includes APC, Axin, CKI and PP2A, for the efficient degradation of β-catenin by the proteasome [27]. To determine if GSK3β signaling could mediate the observed action of cyclin G2 on β-catenin stability, we treated cells with a GSK3β inhibitor, SB-216763, and analyzed the level of β-catenin in control and cyclin G2-overexpressing cells. Knockdown of GSK3β by siRNA or inhibition of GSK3β activity by treatment with SB-216763 prevented cyclin G2-mediated inhibition of β-catenin expression, and increased β-catenin protein level (Figure 6A and B). In addition, the inhibition of GSK3β blocked cyclin G2-induced E-cadherin expression (Figure S9).

To determine if GSK3β mediates the tumor-suppressive effects of cyclin G2, control and cyclin G2 cells were subjected to soft agar colony formation assay in the presence or absence of a GSK3β inhibitor, LiCl. Whereas the empty vector control cell colonies were larger regardless of LiCl treatment, the presence of LiCl in the culture media of the cyclin G2 cells was able to partially reverse the effect of cyclin G2, resulting in an increase in the size and number of colonies (Figure 6C). Lastly, to explain how cyclin G2 could regulate the GSK3/β-catenin axis,

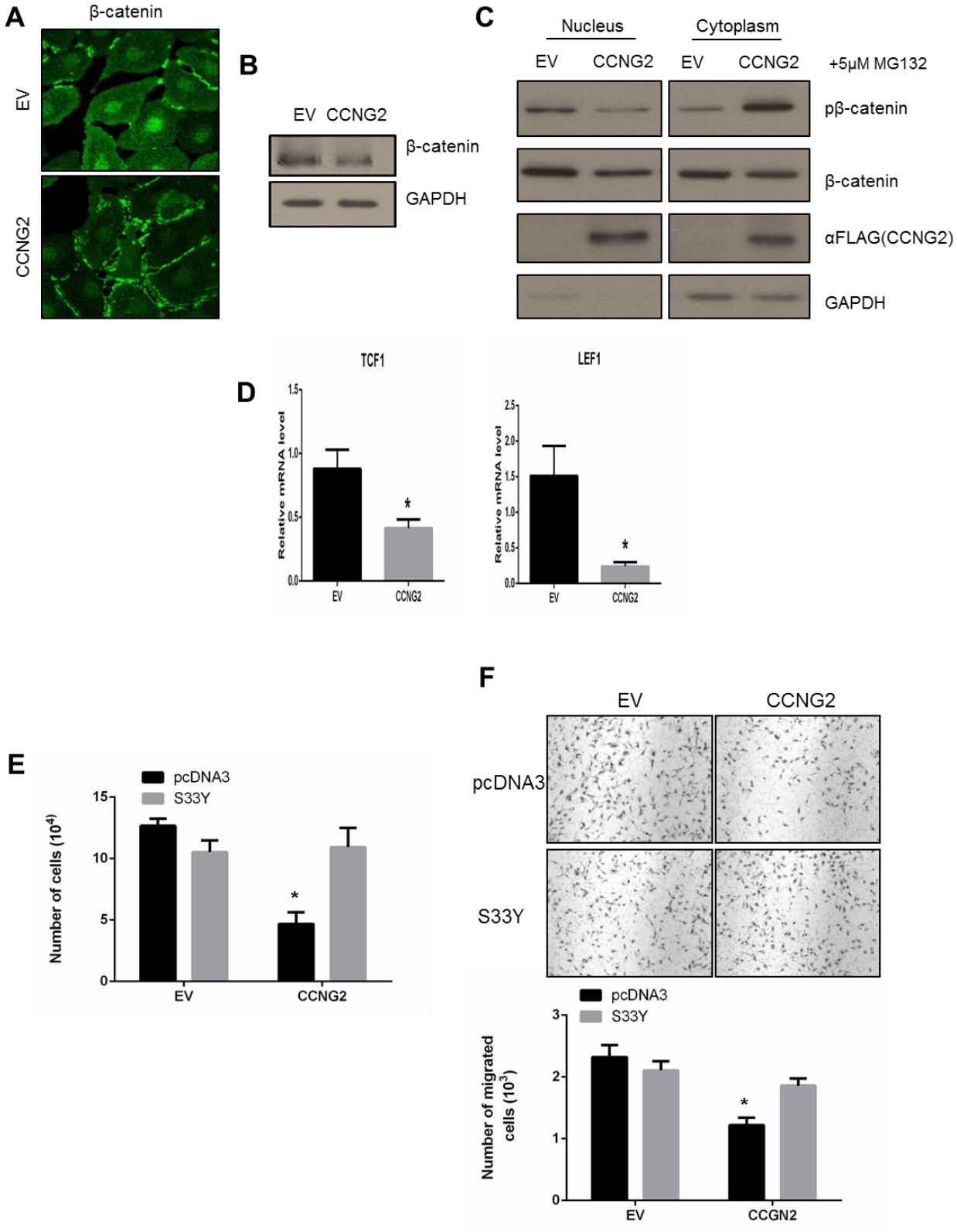

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Figure 5: Cyclin G2 attenuates β-catenin signaling. A) Immunoflourescnece staining of β-catenin in SKOV3.ip1 stable cells. Cyclin G2 overexpression directs β-catenin away from the nucleus to the membrane, while the empty vector cells show a higher nuclear stain. **B)** Western blot analysis of total level of β-catenin in SKOV3ip.1 stable cells. **C)** Cellular fractionation of SKOV3.ip1 stable cells. Cells were treated with 5μM MG132 and separated into cytosolic and nuclear fraction. Total and phosphorylated levels of β-catenin were analyzed by Western blot. GAPDH represents the cytosolic control. **D)** Relative transcript levels of β-catenin target genes, TCF1 and LEF1, as measured by qPCR. Representative experiments are shown. **E)** Proliferation assay of ES-2 stable cells transfected with β-catenin-S33Y or the empty vector control. Cells were counted two days post-transfection. **F)** Transwell migration assay of ES-2 stable cells transfected with β-catenin-S33Y or the empty vector control. EV, empty vector; CCNG2, cyclin G2. *$p<0.05$ vs all other groups.

we preformed co-immunoprecipiatation of GSK3β with known destruction complex members, such as β-catenin and PP2A (Figure 6D). Immunoprecipiatation revealed that cyclin G2 enhanced the association of destruction complex members GSK3β, β-catenin and PP2A, which could possibly augment β-catenin degradation and diminish its signaling.

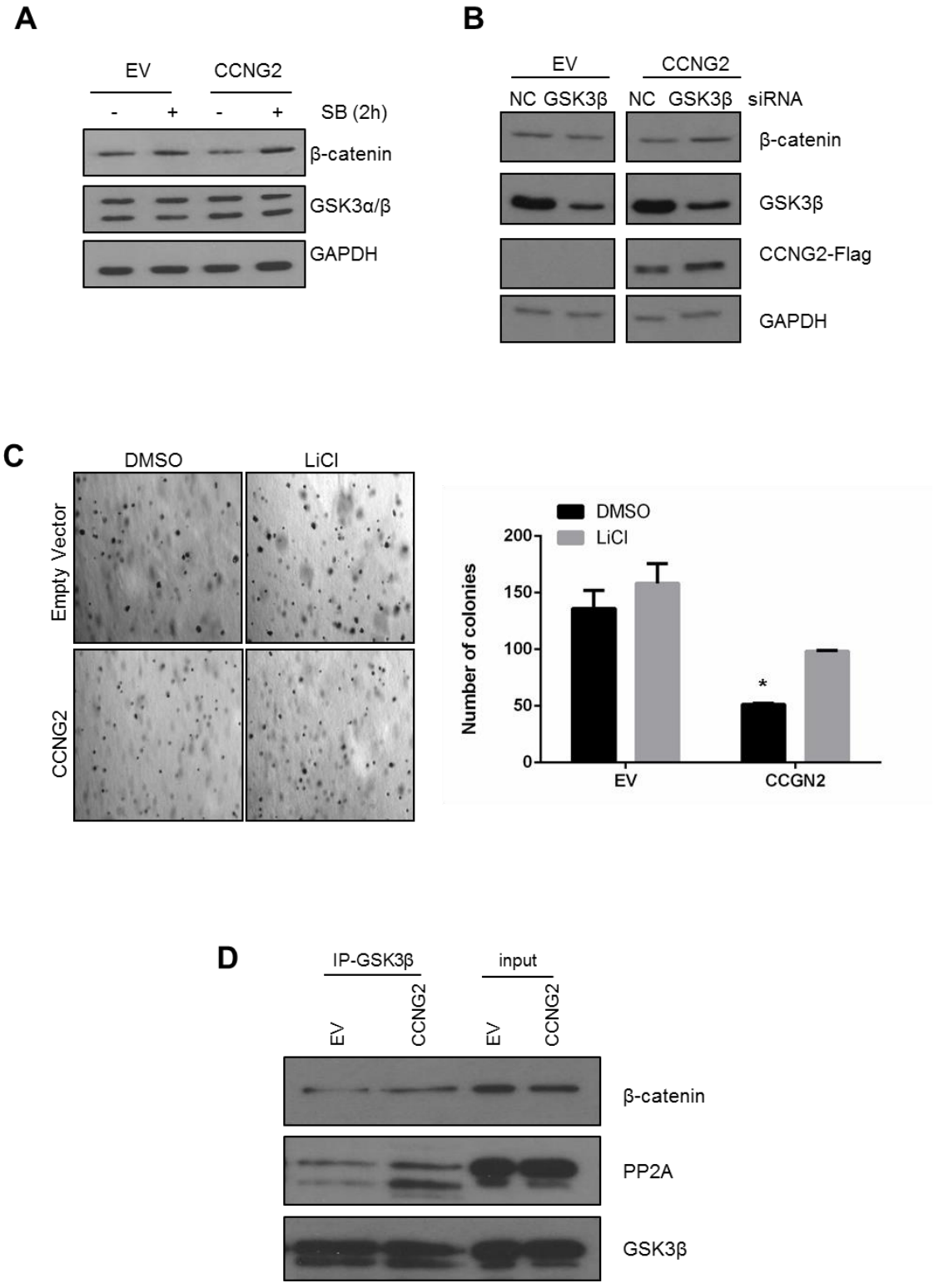

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Figure 6: Cyclin G2 destabilizes β-catenin via GSK3β. SKOV3.ip1 control or cyclin G2 stable cells were treated with a GSK3β inhibitor, 10μM SB216763 (SB) **(A)** or transfected with a GSK3β siRNA **(B).** β-catenin protein levels were determined by Western blot analysis. **C)** Colony formation assay. Cells were seeded on soft agar and treated with either 10 mM LiCl or DMSO bi-weekly for 4 weeks. Cyclin G2 cells formed smaller and fewer colonies compared to the empty vector or cyclin G2 cells treated with LiCl. **D)** ES-2 stable cells were immunoprecipitated using a GSK3β antibody and immunoblotted for β-catenin, PP2A, and GSK3β (as a control) to visualize β-catenin destruction complex members. EV, empty vector; CCNG2, cyclin G2. *$p<0.05$ vs all other groups.

DISCUSSION

The present study was carried out to test whether and how cyclin G2 is involved in ovarian cancer progression. Due to its highly unstable nature, we generated ovarian cancer cell lines that overexpress cyclin G2 and determined the effects of cyclin G2 overexpression on cell proliferation, migration, and invasion in vitro and tumor formation in vivo. In addition to the decreased proliferative potential of cyclin G2-expressing cells, we also observed strong anti-migratory and -invasive characteristic of these cells when compared to the control. *In vivo*, these results manifested to an overall decreased tumor volume and ascites accumulation in mice injected with ovarian cancer cells overexpressing cyclin G2. Clinical data reveals an inverse association between cyclin G2 expression and tumor grade or progression of human ovarian cancer patients, raising the possibility that cyclin G2 may be crucial to controlling the aggressiveness of ovarian cancer cells to a more malignant phenotype. These data are consistent with a previous report that suggested Sharp1 and cyclin G2 could provide a minimal signature to predict metastasis-free survival in breast cancer patients [12]. Therefore, it is possible that the cellular role of cyclin G2 extends beyond cell cycle control, and has far reaching effects in other molecular programs.

In addition, we have linked the expression of cyclin G2 to the regulation of multiple markers associated with EMT. Firstly, the transcription factors, Snail and Slug, which are highly involved in the initiation of EMT [21-23], were downregulated in the cyclin G2 overexpressing cells, which may account for the up- and down-regulation of E-cadherin and vimentin, respectively. Importantly, aside from the canonical markers of EMT, we found that HMGA2 is also downregulated by cyclin G2 overexpression. HMGA proteins have been identified as ideal

candidates for new prognostic and diagnostic biomarkers [40], and it has been demonstrated that HMGA2 is upregualated in ovarian cancers [41]. While not a classical marker of EMT, it has been reported that HMGA2 can serve as a better predictor of prognosis than E-cadherin, vimentin, or snail; and signifies an aggressive, de-differentiated cancer [34].

A major component in EMT is the reorganization of the actin network and the acquisition of stress fibers [42, 43]. During EMT, cells loose their cortical actin, which is associated with E-cadherin and α/β-catenin at epithelial adheren junctions, and acquire stress fibers, filopodia, and lamellopodia, allowing the cell to adopt a structure conducive for mobility [44]. In this study, we found that overexpression of cyclin G2 not only induced the expression of an epithelial marker, decreased the expression of mesenchymal markers, and suppressed cell migration and invasion, but also increased circumferential actin organization, augmenting the epithelial phenotype. One way by which cells adopt this new migratory-conducive morphology is via Rho-GTPase dependant signaling. While RhoA signaling can disrupt localization of E-cadherin at cell–cell adhesions and to promote a mesenchymal cell morphology [45], cadherin engagement can also inhibit RhoA activity [46, 47]. Therefore, the overexpression of E-cadherin, induced by cyclin G2, could inhibit RhoA signaling needed for actin cytoskeleton rearrangements. Together, these findings strongly support the notion that cyclin G2 is involved in the regulation of EMT and loss of cyclin G2 in advanced cancers could promote a mesenchymal phenotype.

The present study demonstrates that induction of E-cadherin by cyclin G2 was important to mediate the anti-tumor function of cyclin G2 in ovarian cancer cells. Overexpression of cyclin G2 not only correlated with increased E-cadherin expression, but knockdown of E-cadherin in

cyclin G2 overexpressing cells rescued the tumorigenic potential, similar to that observed in control cells. In ovarian cancer, loss of E-cadherin serves as prediction marker for poor prognosis [48]. Furthermore, ovarian cancer cells with low E-cadherin have been found to be more invasive [49]. Therefore, maintenance of the epithelial phenotype by cyclin G2, possibly via an induction of E-cadherin, is a likely functional mechanism through which cyclin G2 exerts its tumor suppressive effects.

β-catenin promotes EMT in ovarian cancer. Dissociation from the membrane, resulting from low expression of E-cadherin, allows β-catenin to transduce Wnt signaling by nuclear accumulation and stimulation of a transcription paradigm that promotes migration and invasion [26]. β-catenin regulates a diverse set of target genes, including direct and indirection activation of the transcriptional repressors of E-cadherin, including Snail, Slug, ZEB and Twist [13, 35-38]. Thus, increased β-catenin signaling leads to a decrease in the expression of E-cadherin [26], and can augment its own signalling. In addition, a major determinate of the overall level of β-catenin within the cells is via direct regulation of GSK3β-induced degradation [39]. Therefore, alternation of Wnt/β-catenin at any level of regulation has been shown to be important in ovarian cancer tumorigenesis [50].

Data presented within this study suggests that cyclin G2 can regulate β-catenin stability and localization to decrease β-catenin nuclear signaling. Firstly, upregulation of cyclin G2 caused β-catenin to associate strongly at the cell membrane. Since E-cadherin has been shown to recruit β-catenin to the cell membrane, it is possible that the increased expression of E-cadherin in cyclin G2 overexpressing cells could be, at least in part, responsible for the redistribution of β-

catenin. Furthermore, cyclin G2 overexpression cells had a decreased level of total and nuclear fractions of β-catenin, as well as an increased level of phospho- β-catenin, which implies that cyclin G2 may also regulate the intrinsic stability of β-catenin to inhibit its activity. This conclusion is further supported by findings that cyclin G2 decreased the expression of β-catenin downstream signaling partners and targets, such as Lef/Tcf transcription factors and vimentin, respectively. As expected, activation of β-catenin signaling by ectopic expression of a constitutively active mutant (β-catenin- S33Y), reversed the anti-proliferative and –migratory effects of cyclin G2. Therefore the inhibition of β-catenin signalling is an important mechanism underlying the anti-tumor effects of cyclin G2 in cancer cells.

One of the first identified binding partners of cyclin G2 is PP2A [13]. In addition, the association of PP2A in the GSK3β-dependent β-catenin destruction complex has been described, although the exact role of this protein is still unclear [51-53]. We found that inhibition of GSK3β activity via chemical inhibitors or through siRNA silencing blocked the effects of cyclin G2 on β-catenin and enhanced the tumorigenic potenital of the cells. Interestingly, we found that cyclin G2 overexpression enhanced the complex formation of GSK3β with other destruction complex members, such as β-catenin and PP2A. It is possible that cyclin G2 acts to correctly distribute one or more of these proteins to the destruction complex for efficient degradation of β-catenin. Therefore, the regulation of β–catenin by cyclin G2 in ovarian cancer cells is twofold: firstly by regulating is subcellular localization by recruitment to the membrane via E-cadherin, and secondly by decreasing its overall stability through promoting the formation of GSKβ dependant destruction complex . The mechanism by which cyclin G2 enhances this complex formation is an interesting avenue for future research.

Overall, our study aims to conceptually advance what is currently known regarding the cellular mechanisms of ovarian cancer, and highlights the importance of cyclin G2. Although many reports have linked decreased cyclin G2 expression with the tumorigenic phenotype, only few have attempted to explain the molecular function and/or mechanism of the protein. The present study presents evidence that cyclin G2 exerts potent anti-tumor effects on ovarian cancer cells and identifies the GSK3β/β-catenin/E-cadherin pathway as a key downstream mediator of cyclin G2 actions. Overexpression of cyclin G2 promotes epithelial phenotype by enhancing the formation of the β-catenin destruction complex to promote β-catenin degradation. Inhibition of β-catenin suppresses Tcf/Lef signaling, which directly or indirectly inhibits the transcription of β-catenin target genes, including the E-cadherin transcriptional repressor, Snail and Slug. Decreased Snail and Slug allows for increased E-cadherin levels, which in turn further sequesters β-catenin at the membrane. In addition, E-cadherin and β-catenin form a complex with F-actin, which modulates cytoskeleton dynamics and decreases the migratory capacity of cancer cells. On the other hand, when cyclin G2 is low, the formation of the β-catenin destruction complex is less efficient, leading to the accumulation of β-catenin in the nucleus. Transcription of Snail and Slug decreases expression of E-cadherin, resulting in the dissociation of the adherenes junction and circumferential actin arrangements, resulting in a more mesenchymal-like cell (Figure 7). Together, these changes may confer ovarian cancer cells with more aggressive characteristics. We suggest that the loss of cyclin G2 may define a critical turning point in oncogenesis that promotes EMT and cancer cell dissemination.

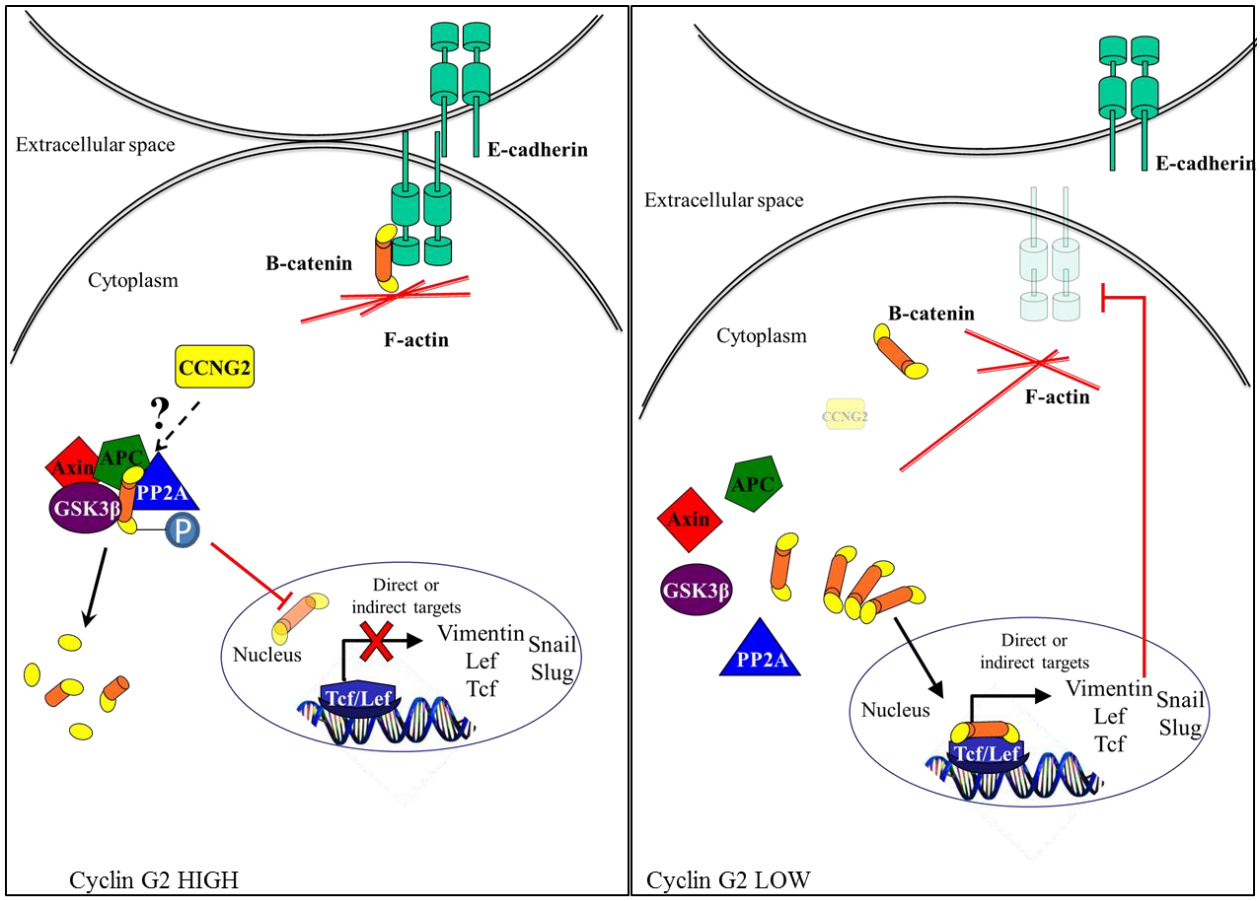

Figure 7: Proposed mechanism of cyclin G2 action in ovarian cancer cells. High expression of cyclin G2 promotes the formation of the β-catenin destruction, which leads to the degradation of β-catenin in the cytoplasm and limits its nuclear accumulation and signal transduction. Downregulation of the transcription factors Snail and Slug would allow for the re-expression of E-cadherin and may account for some of the anti-tumor effects of cyclin G2. On the other hand, when cyclin G2 expression is low, β-catenin degradation is supressed and can enter the nucleus to activate the transcription of its target genes. Downregulation of E-cadherin and upregulation of various mesenchymal markers may give epithelial ovarian cancer cells a more aggressive phenotype.

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Wenyi completed Figure 3B (CDH, VIM, HMGA2) and 5D

Christine completed Figure 2A

Weining contributed to Figure 2C and 3D.

Researchers at Sunnybrook Hospital completed Figure 2D and S5.

Yao-Yun Liang generated the SKOV3.ip1-luc cells

Dr. Barbara Vanderhyden provided ovarian tumor samples.

Supplementary Data

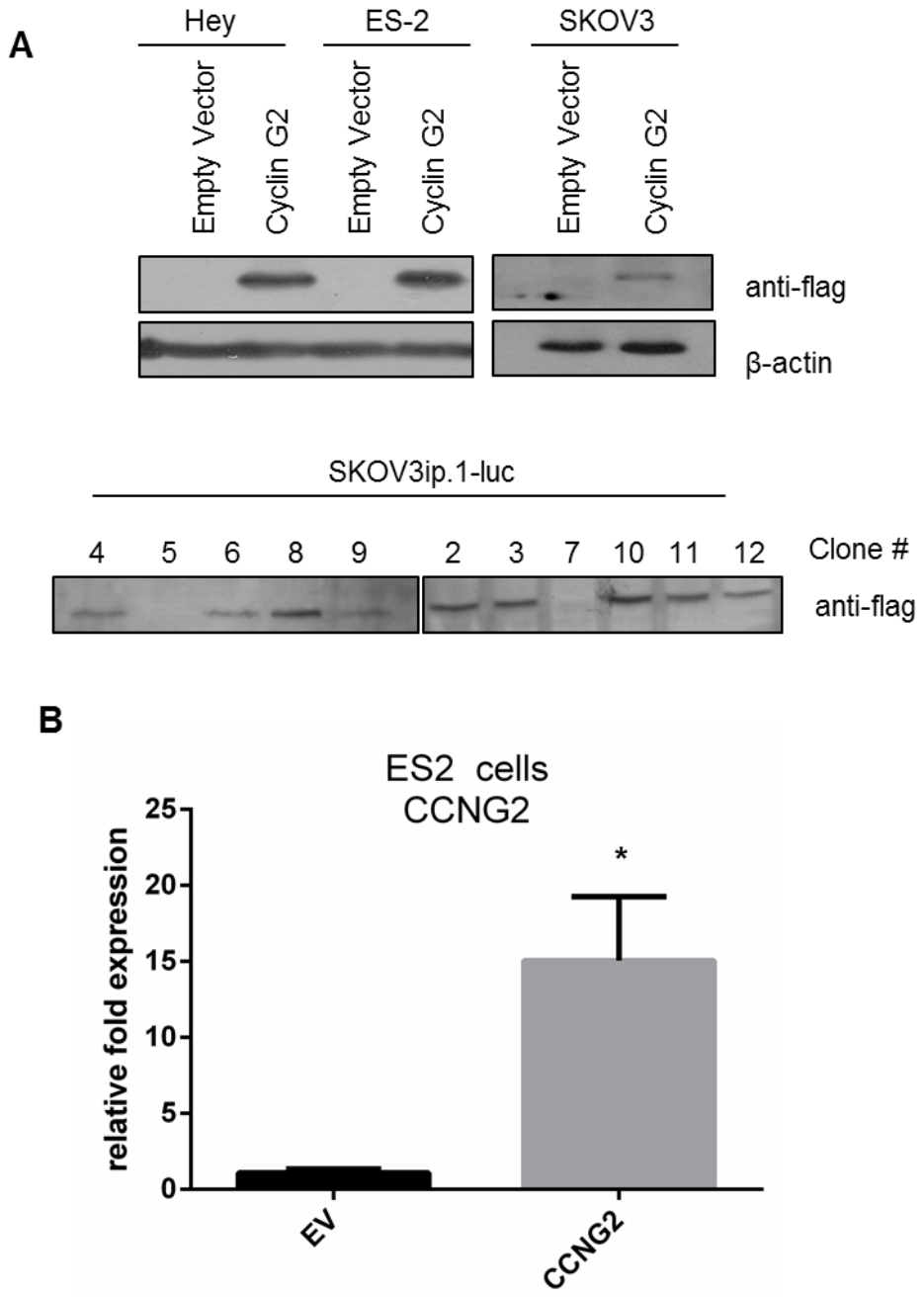

Figure S1: Confirmation of cyclin G2 overexpression in stable cell lines. **A)** Cells were infected with retrovirus carrying Flag-Cyclin G2 (CCNG2) or its empty vector (EV) pBabe as the control. Stable cell lines were maintained in puromyocin selection media and cyclin G2 expression was confirmed by Western blot using an antibody against the FLAG tag in each cell line tested. **(B)** RNA from ES-2 cells that stably express either CCNG2 or the EV control was extracted by trizol following manufacturer protocol and relative mRNA level for CCNG2 compared to the GAPDH internal control was quantified by qPCR. *$p<0.05$ vs EV.

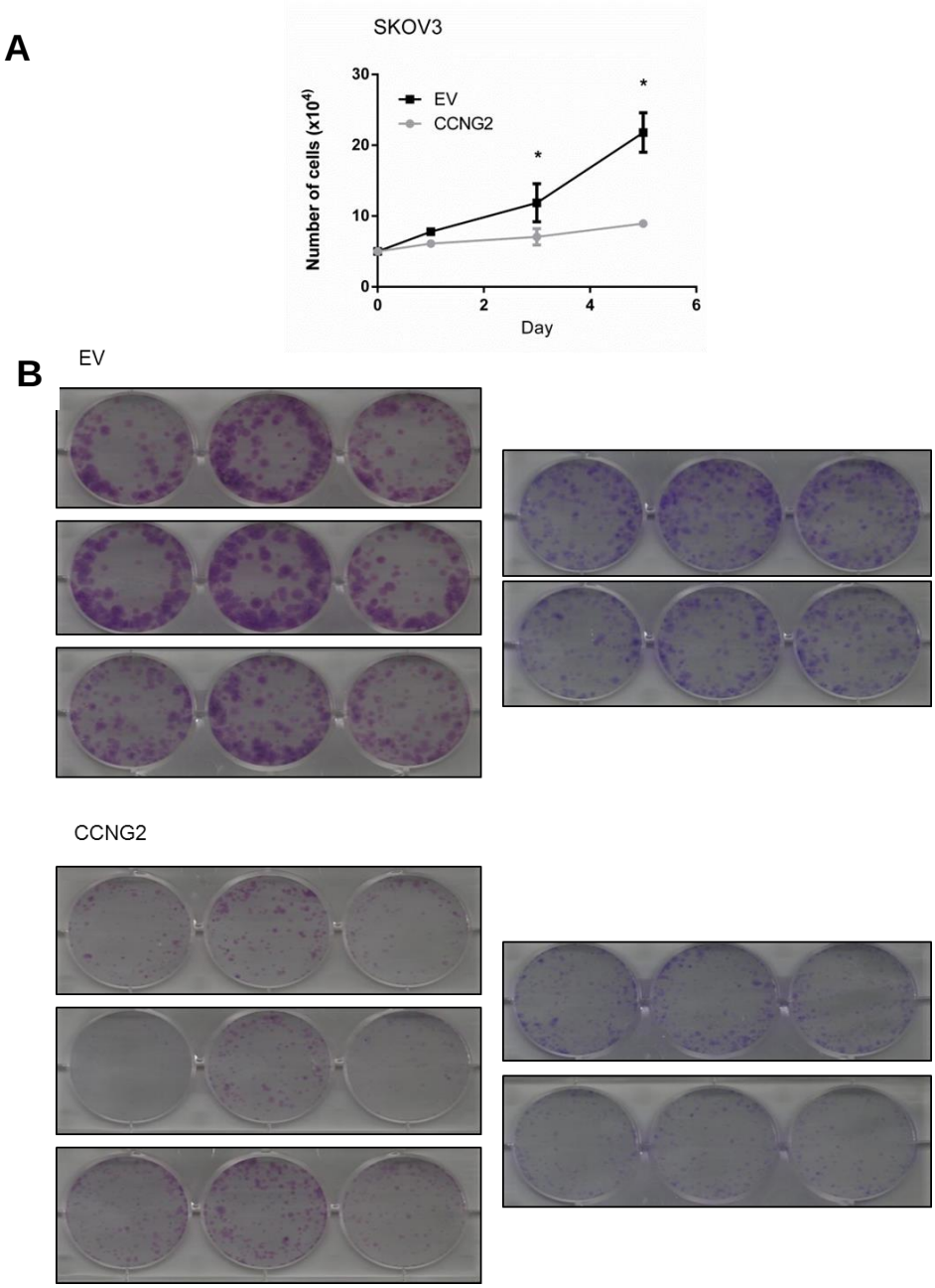

Figure S2: Cyclin G2 reduces proliferation and colony formation. **A)** Equal concentrations of either empty vector (EV) or cyclin G2 (CCNG2) stable cells for the SKOV3 cell line was seeded into 6-well plates in triplicate. Cells were cultured in media containing 10% FBS for 1, 3, and 5 days and cell numbers were determined by trypan blue exclusion assays. In all cell lines tested, the overexpression of cyclin G2 decreased the proliferative ability of the cells when compared to the empty vector control. **(B)** SKOV3.ip1 stable cells expressing either cyclin G2 or its empty vector control were seeded onto 60 mm cultured dishes at the density of 1×10^3 cells/well. Cultures were maintained for 9 days until the visible clones appeared. The colonies were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet, and photographed. *$p<0.05$ vs EV.

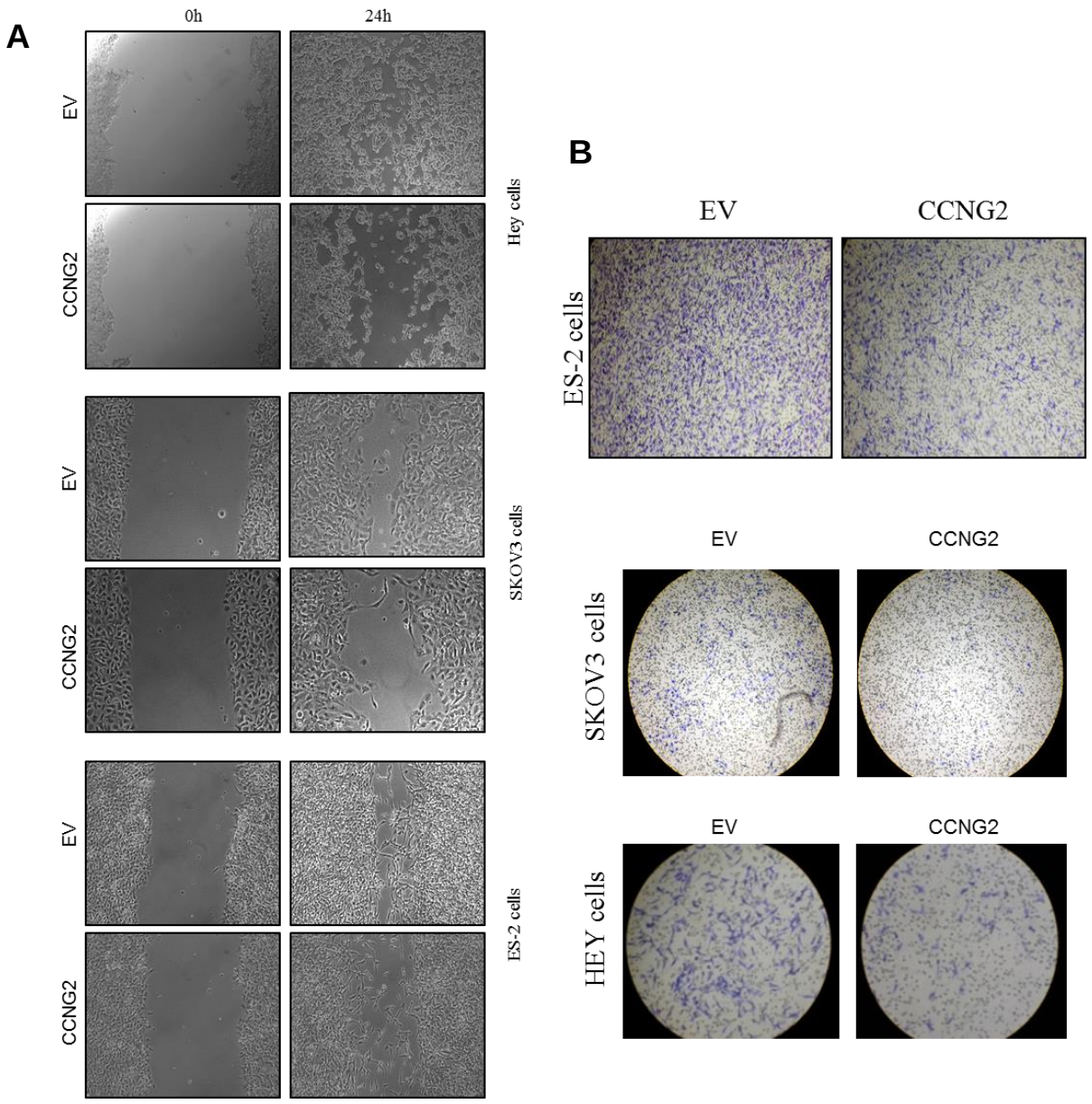

Figure S3: Cyclin G2 inhibits migration and invasion. A) HEY, SKOV3, and ES-2 cells expressing either empty vector (EV) or cyclin G2 (CCNG2) were cultured in 12-well plates. A wound was made by using 100 µl pipette tip when cells reach confluency. Cells were photographed and area of wound was quantified at 0 and 24 hours after wounding. A decrease in wound healing and a larger wound was observed in cyclin G2 stable cells as compared to control. **(B)** Various ovarian cancer cell lines that stably express cyclin G2 or its empty vector were seeded into matrigel coated transwells. Cells were allowed to invade through the matrigel for 24 h after which invaded cells were fixed and stained for their nuclei. Invaded cells were photographed and representative pictures are shown. A decrease in the number of invaded cells was noticed by the overexpression of cyclin G2.

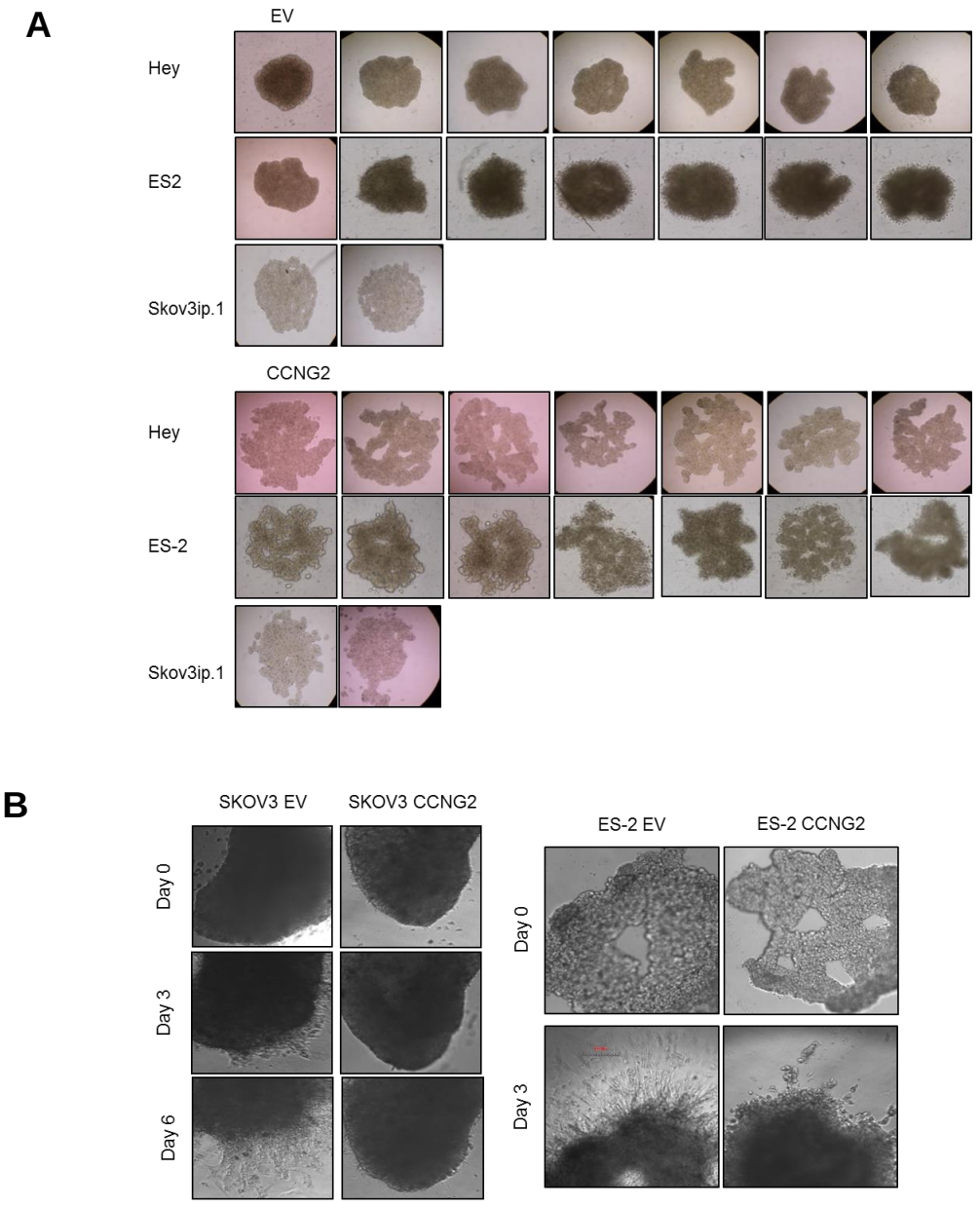

Figure S4: Cyclin G2 inhibits compact spheroid formation. A) Several EOC cell lines stably overexpressing cyclin G2 (CCNG2) or the empty vector (EV) were plated in 20µl droplets, each containing 20,000 cells, into the inner surface of petri dish cover. The covers were then inverted and placed on a dish containing 15ml of PBS. Formation of spheroids were examined and photographed after four days. The overexpression of cyclin G2 inhibited the formation of tight spheroids and covered a larger area when compared to the control. **B)** Spheroids formed from SKOV3 stable cells were encased in collagen I in a 96 well plate. Pictures were taken immediately following polymerization of the gel and after 3 and 6 days (left panel). Spheroid invasion of control and cyclin G2-ES-2 cells at 3 days after plating on collagen I (right panel). Cell invasion was visualized by penetration and movement through the matrix.

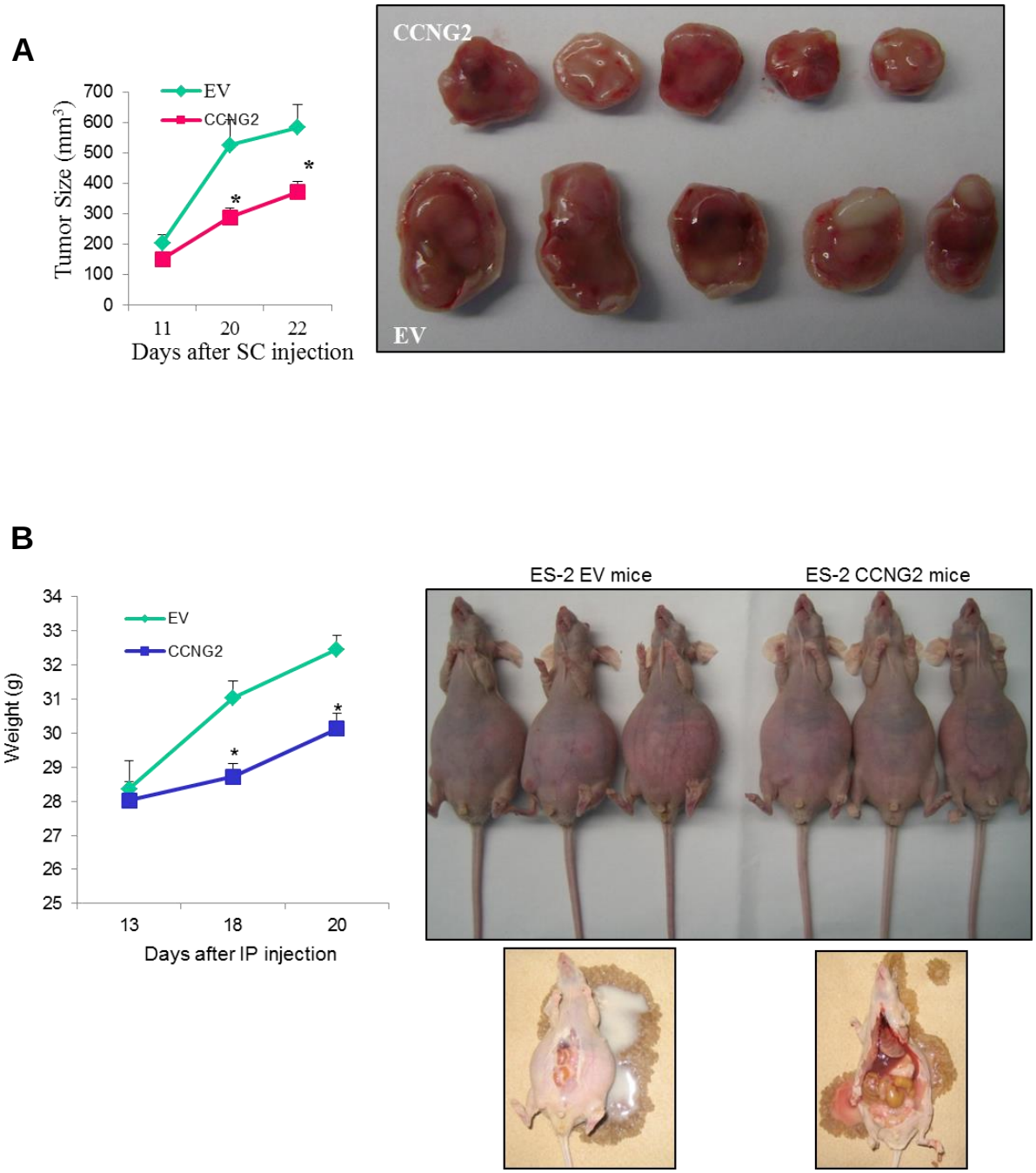

Figure S5: Effect of cyclin G2 on *in vivo* tumor formation and ascites fluid accumulation. **(A)** Control (EV) and cyclin G2 (CCNG2) ES-2 cells ($1X10^6$/mouse) were injected subcutaneously (SC) into nude mice. Tumor sizes were measured on day 11, 20 and 22 after injection (mice were scarified on day 22) (n=5 mice). Tumors obtained from cyclin G2 and control (right panel). **(B)** Cyclin G2 inhibits ascites fluid formation. Cyclin G2 and empty vector overexpressing ES-2 cells ($5X10^5$/mouse) were injected intraperitoneally into nude mice. Mice were sacrificed at day 20. Control mice are heavier (n=3) and had more ascites fluid. *$p<0.05$ vs EV.

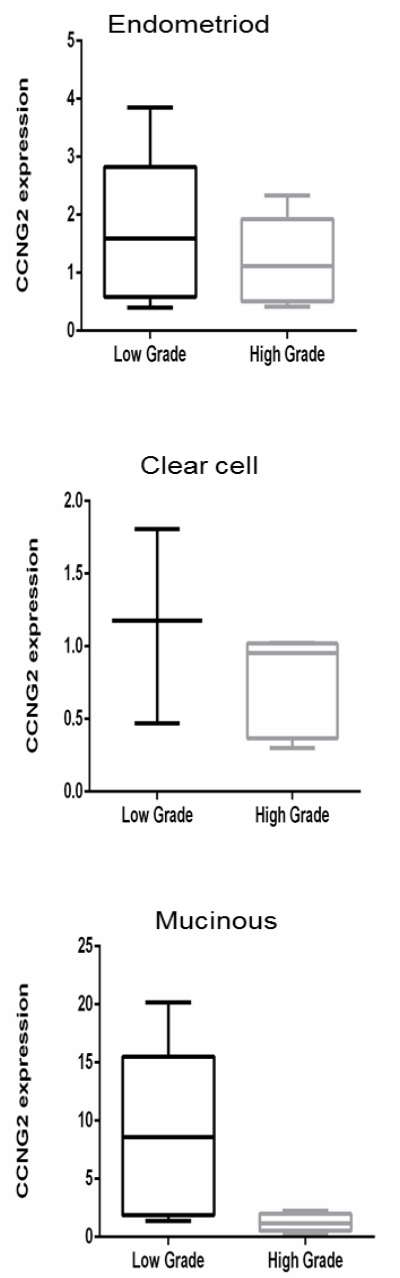

Figure S6: Cyclin G2 expression in ovarian cancer samples. qPCR analysis of cyclin G2 (CCNG2) mRNA expression in human clinical samples. Lower cyclin G2 mRNA levels were found in high grade cancer compared to low grade tumors in each histological subtype tested. The box extends from the 25th to 75th percentile, while the mid-line is the median. The whiskers represent the minimum and maximum values.Endometriod: low grade/high grade, n=5. Clear cell: low grade, n=3; high grade, n=5. Mucinous: low grade, n=5; high grade, n=4.

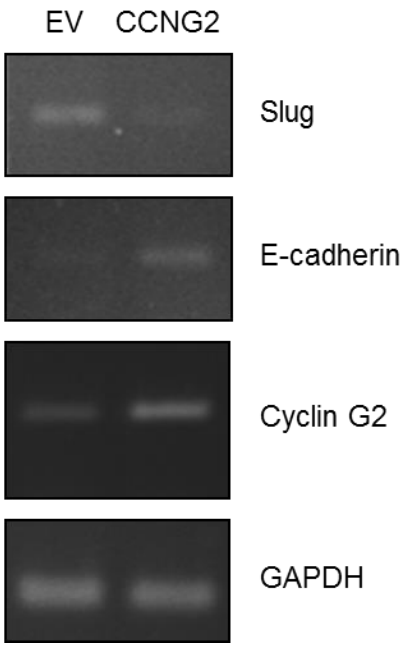

Figure S7: Cyclin G2 overexpression in Skov3.ip1 cells increase E-cadherin and decreases slug. SKOV3.ip1 stable cells that express either the empty vector or cyclin G2 were used to determine the mRNA level of slug, E-cadherin and cyclin G2 by PCR. Slug and E-cadherin show an inverse mRNA expression pattern with overexpression of cyclin G2 decreasing slug and increasing E-cadherin.

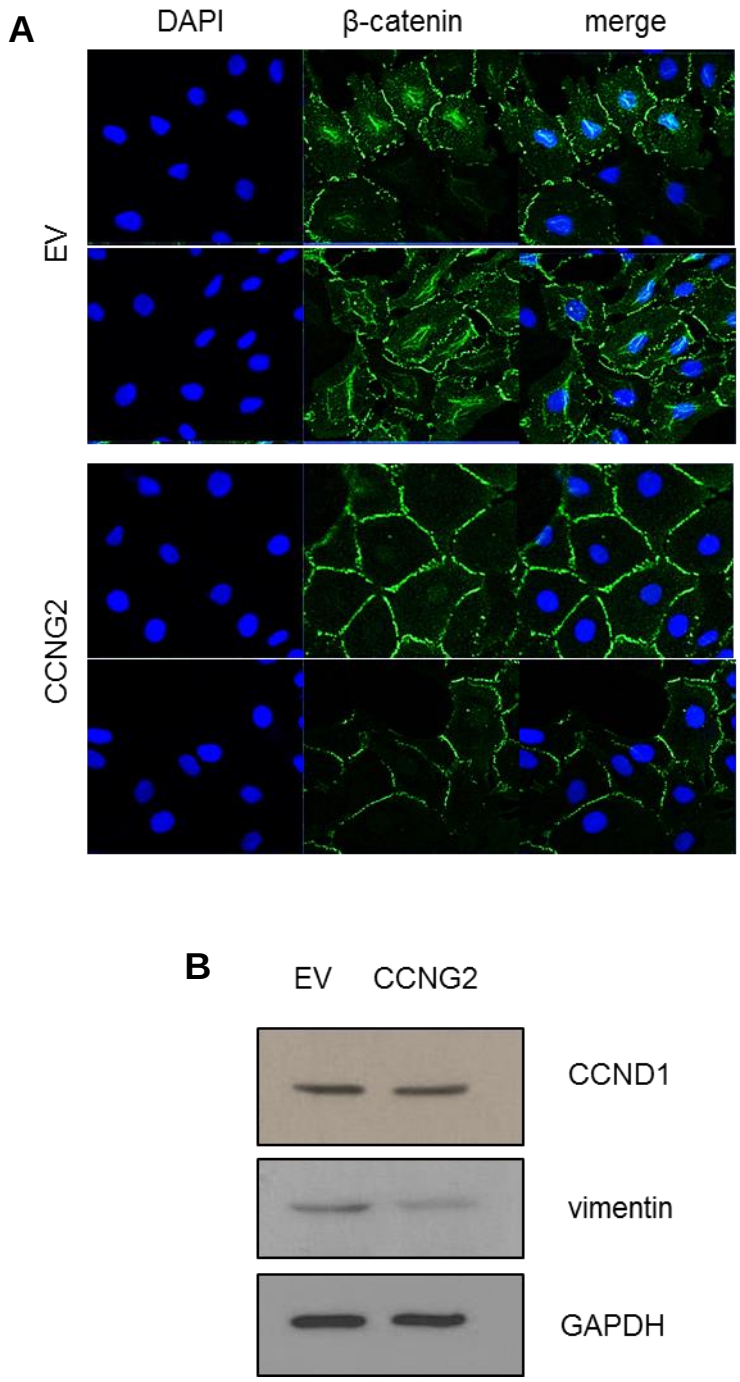

Figure S8: Cyclin G2 regulates β-catenin localization and target genes. A) Immunoflourescnece of β-catenin in SKOV3.ip1 empty vector (EV) or cyclin G2 (CCNG2) stable cells. Cyclin G2 overexpression directs β-catenin away from the nucleus to the membrane. **(B)** Western blot analysis of β-catenin target genes, cyclin D1 (CCND1) and vimentin.

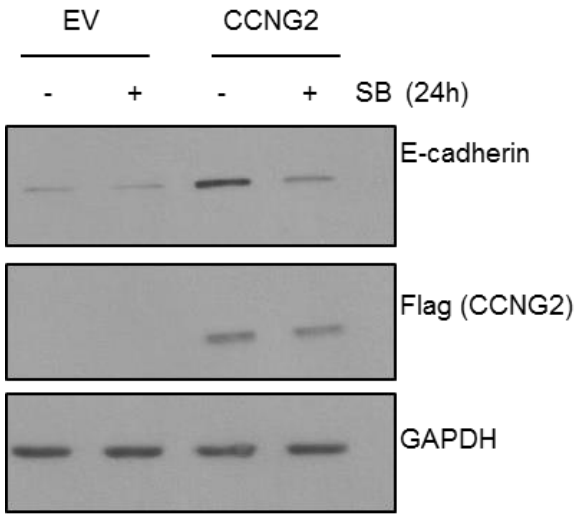

Figure S9: GSK3β is important for cyclin G2 action. Inhibition of GSK3β by SB-216763 decreased the cyclin G2-induced E-cadherin expression.

CHAPTER 3

CALPAIN-MEDIATED PROTEOLYSIS TARGETS CYCLIN G2 FOR DEGRADATION AND IS REGULATED BY EPIDERMAL-GROWTH FACTOR SIGNALING.

ABSTRACT

Cyclin G2 (CCNG2) is an atypical cyclin that can inhibit cell cycle progression and is often dysregulated in human cancers. We have previously shown that cyclin G2 is very unstable but its stability can be strongly enhanced by MG-132, an inhibitor of the proteasome and calpain-mediated degradation pathways. We further demonstrated that cyclin G2 can be degraded through the ubiquitin/proteasome pathway [1]. In this study, we determined if calpains, a family of calcium-dependent proteases, are also involved in cyclin G2 degradation. The addition of calpain inhibitors or silencing of calpain expression by siRNAs strongly enhanced cyclin G2 levels. On the other hand, incubation of cell lysates with purified calpains or increased calcium in the culture media resulted in a decrease in cyclin G2 levels. Interestingly, the effect of calpain was found to be dependent on the phosphorylation of cyclin G2. Using a kinase inhibitor library, we found that Epidermal Growth Factor (EGF) Receptor is involved in cyclin G2 stability and treatment with EGF induced cyclin G2 degradation. Cyclin G2 contains a PEST domain, which has been suggested to act as a signal for degradation. When the PEST domain was partially or completely removed, Calpain or EGF treatment failed to trigger degradation of cyclin G2. Taken together, these findings demonstrated that EGF-induced and calpain-mediated proteolysis contributes to the rapid destruction of cyclin G2.

INTRODUCTION

Cyclins encompass a group of closely related molecules [2]. Classical cyclins accumulate periodically to activate their associated cyclin-dependant kinases (Cdk) and stimulate the mitotic events that regulate the rate of cell division [3]. The concentration of cyclins oscillate during the cell cycle [4], which allows for the strong unidirectional flow of cellular division. Many cyclins are targeted for rapid degradation via the presence of a destruction box or a PEST domain. [5-7]. The Ubiquitin-Proteasome Pathway (UPP) is critical for the degradation of many short-lived proteins; and to date, all known cyclins are targeted by the UPP [4, 8, 9]. There are two main types of Ubiquitin ligases that mediate the cell cycle: the Skp-Cullin F-box (SCF) complex, which is active throughout the cell cycle, and the APC/C that is activated at the onset of anaphase and is crucial for cells to exit mitosis [4].

Cyclin G2 belongs to a group of unconventional cyclins that includes cyclin G1 and cyclin I [10, 11] and unlike typical cyclins, they function to maintain the quiescent state of cells and cell cycle arrest [11, 12]. The G-type cyclins are found in low levels in proliferating cells and are upregulated in response to diverse inhibitory signals [11, 13]. Despite having high amino acid sequence identity (53%), cyclin G1 and cyclin G2 have distinct expression patterns [10, 12]. While cyclin G1 and cyclin I maintain constant expression throughout the cell cycle, cyclin G2 is elevated in G_0 phase, is reduced as cells enter the cell cycle, and peaks again during the late S/G2 phase [10, 11]. Cyclin G2 contains distinct features that imply its temporal level and strict regulation. Firstly, a destabilizing domain, PEST, which controls the stability of many proteins, is located at the C-terminal end [10]; and secondly, a cyclin box, structurally similar to cyclin A, is centrally located within the protein [11]. However, little is known regarding cyclin G2

regulation. Interestingly, increasing evidence has suggested that cyclin G2 acts as a tumor suppressor. Indeed, overexpression of cyclin G2 inhibits proliferation in many cell lines [14, 15], and an inverse relationship is observed between cyclin G2 levels and cancer progression [10, 11, 16]. Furthermore, there exists a drastic difference in the level of cyclin G2 in normal versus malignant tissues [10, 16, 17].

Calpains are a family of intracellular, calcium-activated, cysteine proteases and can be divided into two main groups: calpain-1 (m-calpain) and calpain-2 (μ-calpain)[18]. They are ubiquitously distributed throughout all cells, and play important and diverse roles in basic physiology and pathology [18, 19]. Both isoforms have similar biochemical characteristics and differ mainly in the amount of calcium needed for their activation. Calpain-1 requires *in vitro* calcium levels in the millimolar range whereas calpain-2 is activated when calcium levels are in the micromolar range [19]. The end result of calpain activity is usually not simple destruction, but more often results in alteration of the target protein in a limited proteolytic manner. Under these conditions, the protein may become active, inactive or more susceptible to other digestive pathways [18, 19].

We have previously demonstrated that cyclin G2 is highly unstable and treatment with MG-132, an inhibitor of the proteasome and calpain, strongly enhanced cyclin G2 stability. We further showed that cyclin G2 can be degraded quickly by the ubiquitin(Ub)-proteasome pathway [20]. Herein, we report that cyclin G2 can be degraded by calpain, and that this process is dependent on prior phosphorylation and the presence of the PEST domain.

METHODS AND MATERIALS

Chemicals and reagents:

M2 anti-FLAG and anti-EGFR antibodies were purchased from Sigma-Aldrich and V5 antibody was from Invitrogen, Life Technologies. Purified calpains, calpeptin, N-Ac-Leu-Leu-norleucinal (ALLN), and A23187 were purchased from Calbiochem-Novabiochem Corp. Recombinant human EGF (rhEGF) was purchased from Invitrogen and calf intestinal phosphatase (CIP) was from New England Biolabs. Cyclohexamide (CHX) was obtained from Sigma-Aldrich.

Cell Culture and cell lines:

Two different gynecological cancer lines were used: SKOV3.ip1 were obtained from Dr. Mien-Chie Hung (University of Texas, M.D. Anderson Cancer Institue) and OV2008 cells were obtained from Dr. Benjamin Tsang (University of Ottawa, Ottawa Ontario). SKOV3.ip1 cells were maintained in MyCoy's culture media (Sigma-Aldrich), supplemented with 10%FBS (Gibco, Life Technologies), and OV2008 were cultured in RPMI-1640 (GE HyClone), supplemented with 10% FBS. To generate stable cell lines FLAG-cyclin G2 was cloned into a plasmid vector, pBabe-puro. In 293T, virus was produced by calcium phosphate transfection (18 hours) of 10μg of either pBabe-Flag-cyclin G2 or pBabe-empty vector, and 6.5μg VSVg and 3.5μg PUMVC for packaging. The next day the media was changed and 24 hours later the virus was harvested by passing the media through a 0.45 μM filter. This media was subsequently used to infect each target cell line. Stable cell lines were maintained in puromyocin selection media and cyclin G2 expression was confirmed by Western blot using an antibody against the FLAG tag.

Plasmids, RNA interference and transfection:

The FLAG-cyclin G2 and cyclin G2-V5 (WT, PEST24, and ΔPEST) plasmids were generated as described previously [1]. Transient transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Small interference RNA (50 nM) was transfected into cells for 6 h using Lipofectamine 2000. To confirm gene-specific silencing effects of calpain, protein lysates were prepared at 24-48 h after transfection and subjected to western blot analysis. Calpain-1, calpain-2, and a scrambled control siRNA, which has no significant homology to any mammalian gene sequence, were purchased from GenePharma Co:

Calpain-1 siRNA: 5-AAACUAGCUGGCAUCUUCTT-3

Calpain-2 siRNA: 5-GAAGUGGAAACUCACCAAATT-3

Control siRNA: 5-UUCUCCGAACGUGUCACGUTT-3

Treatment with kinase inhibitor library.

To determine which kinase is involved in cyclin G2 degradation, a kinase inhibitory library, which contains 80 known kinase inhibitors of well-defined activity, was purchased from Enzo Life Sciences. Cells were treated at indicated concentrations for 6 hours in the presence of CHX. Cells were lysed and analysed by Western blot for cyclin G2 expression. Some of the inhibitors used are listed in Table S1.

Table 1: Select kinase inhibitors used in Figure 4E

Kinase Inhibitor	Target
Tyrphostin 23	EGFRK
Tyrphostin 25	EGFRK
Tyrphostin 46	EGFRK, PDGFRK
Tyrphostin 47	EGFRK
Tyrphostin 51	EGFRK
Tyrphostin 1	Negative control for tyrosine kinase inhibitors
Tyrphostin AG 1288	Tyrosine kinases
Tyrphostin AG 1478	EGFRK
Tyrphostin AG 1295	Tyrosine kinases
Tyrphostin 9	PDGFRK

Western blot analysis. Cell lysates were prepared and Western blot was performed as reported previously [21]. Briefly, cells were lysed in a buffer containing 150mM NaCl, 1% Nonidet P-40, 50mM Tris/HCl (pH 7.4) and supplemented with protease and phosphatase inhibitor cocktail just prior to use (Pierce, Thermo Scientific). Total protein was quantified by the Pierce BCA Protein Assay Kit (Thermo Scientific) and equal amounts of protein were separated by 12% SDS-PAGE and transferred onto Immuno-Blot PVDF membranes (BioRad). Following primary antibody incubation (overnight at 4°C), membranes were probed with secondary HRP-coupled antibodies for 1-2 hours at room temperature. Proteins were visualized by Luminata Classico Western HRP Substrate (Milipore).

Calcium-induced degradation assay:

SKOV3.ip1 cells or OV2008 cells stably transfected with Flag-cyclin G2 were lysed using a degradation lysis buffer (10mM Tris/HCl, pH 7.4, 100mM NaCl, 1mM DTT, and 0.5% Triton-X) containing either 0, 0.5, or 5mM $CaCl_2$ for various 60 or 120 minutes. Reactions were

stopped with SDS sample buffer and analyzed by SDS/PAGE. Samples were immunoblotted with anti-Flag for cyclin G2 expression.

OV2008 cells were transfected with either full-length cyclin G2-V5 or PEST deletion mutatnts (PEST24 or ΔPEST). Following transfection cells were pretreated with or without 20μM ALLN for 30 minutes. Cells were then treated for an additional 2 hours with either DMSO as a control, or 1μM of the calcium ionophore, A23187. Cells were lysed and analyzed by Western blot. Cyclin G2 was detected using antibody against theV5-tag.

***In vitro* calpain degradation assay:**

Cells were lysed the degradation lysis buffer. Equal amounts of protein were separated into fresh 1.5mL tubes and supplemented with either vehicle control, $CaCl_2$, Calpain-1 or -2, calpeptin, or a combination of these, as indicated. Reactions took place for 1 hour at 30°C. Samples were immediately run on and SDS-gel, and immunobloted for cyclin G2 levels.

Where indicated, cells were treated with CIP and EGFR inhibitor (Tryphostin AG1478). For CIP treatment, 1 unit of CIP/μg of protein was added to the raw lysate for 30 mins at 37°C. For AG1478 treatment, 10μM of inhibitor was added to cell culture media for 30 mins prior to lysis. An additional 10μM of inhibitor was added to the raw lysate. The *in vitro* degradation assay proceeded as described above.

Casein zymography:

Zymography was used to determine the activity of calpain-1 and calpain-2. Cells were lysed with the degradation lysis buffer, mixed with one volume of SDS sample buffer (300mM

Tris, 40% glycerol, 0.02% bromophenol blue, and 100mM DTT, pH 6.8) and run immediately on a 12% acrylamide gel polymerized with casein.

Samples are run on the gel using a Tris-glycine buffer containing 1mM EGTA. Following electrophoresis, gels are incubated in 5mM $CaCl_2$ and 10mM DTT for 1 hour, buffer is refreshed and further incubated for 16 hours (overnight). Gels are then stained with Coomassie blue and calpain-1 and calpain-2 are visualized by clearing bands. Calpain-1 has high mobility in the gel and therefore can be easily distinguished from calpain-2. Degree of band clearing was proportional to calpain activity.

RESULTS

Calcium enhanced cyclin G2 degradation

OV2008 or SKOV3.ip1 cells that stably express cyclin G2 were seeded at equal densities and treated with CHX for 0-5 hours to block de novo protein synthesis (Figure 1A and B). Cyclin G2 degradation is evident within the first 1-2 hours following CHX addition, decreased thereafter and was almost completely abolished by hour 5. Calpain activation has been shown to be dependent on increased calcium concentrations [22]; therefore, to elucidate a role for the calcium-dependent activation of calpains in the degradation of cyclin G2, OV2008 cells were transiently transfected with Flag-cyclin G2 and whole cell lysates were incubated in buffers that contained different concentration of $CaCl_2$. Western blot analysis revealed that at each time point the expression of cyclin G2 decreased with increasing amounts of $CaCl_2$ (Figure 2B). Whole cell lysate incubated in high (5mM) concentrations of $CaCl_2$ showed a considerable increase in cyclin G2 degradation as compared to lower (0.5mM) concentrations.

To verify that cyclin G2 is degraded by calcium in living cells, the calcium ionophore, A23187, was added to the culture medium following transfection of full-length cyclin G2 or its PEST deletion mutants. A23187 has been shown to significantly enhance calpain activity, and that this activity could be inhibited by pretreatment with the calpain inhibitor, ALLN [23]. Compared to the control, treatment with 1μM A23187 drastically decreased the expression of full-length cyclin G2 with a much lesser effect on the levels of PEST deletion mutants (Figure 2C). Pre-incubation with ALLN abolished calcium-induced cyclin G2 degradation. Although ALLN strongly increased full cyclin G2 levels, this effect was reduced if the PEST domain was removed (Figure 2A and C).

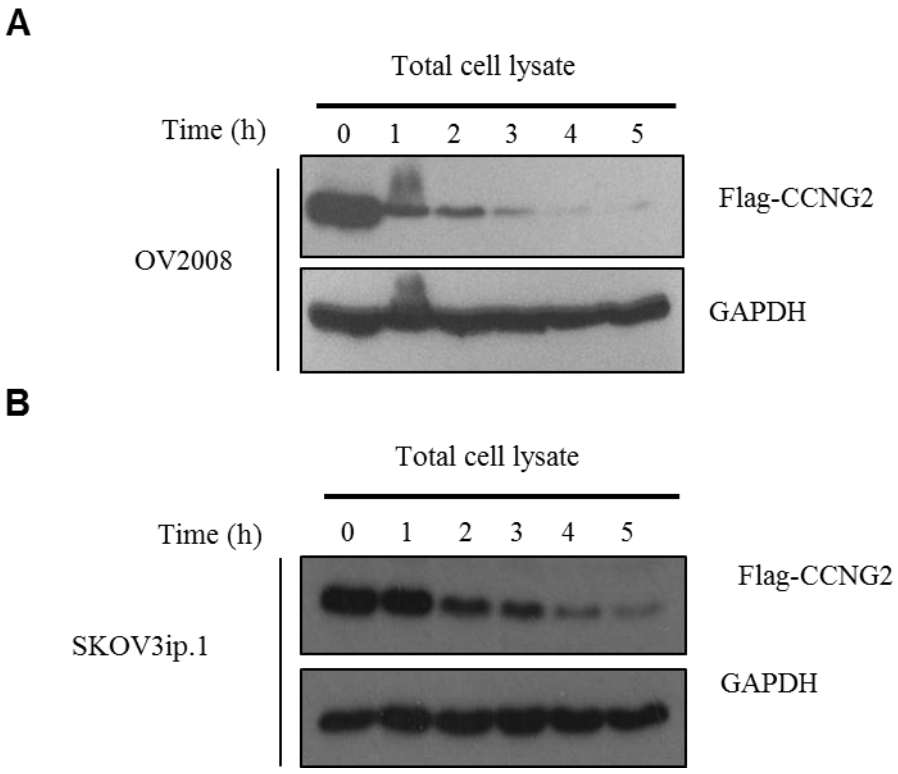

Figure 1: **Cyclin G2 is a highly unstable protein**. **A)** OV2008 or **B)** SKOV3.ip1 stable cells expressing cyclin G2 were seeded at equal densities and treated with 10μg/ml cychoheximide (CHX) to block *de novo* protein synthesis. Samples were lysed at 1 hour intervals up to 5 hours to monitor cyclin G2 stability. Western blot analysis showing expression level of Flag-cyclin G2 at each time point indicated. Cyclin G2 expression begins to decrease 1-2 hours post CHX treatment. CCNG2, cyclin G2

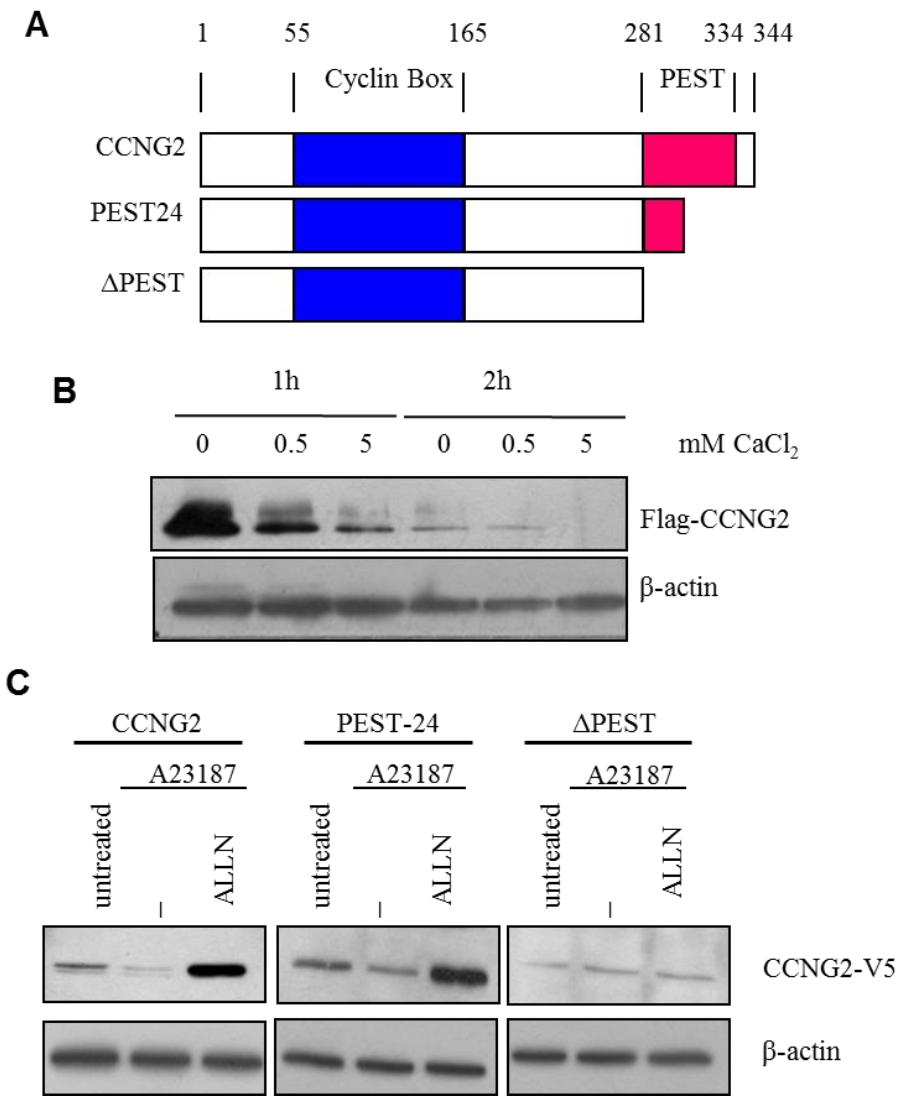

Figure 2: **Calcium acts at the PEST domain to decrease cyclin G2 stability**. **A)** Schematic structure of cyclin G2. Three constructs, full-length-cyclin G2, PEST24 (containing 24 amino acid of the PEST domain) and ΔPEST (complete removal of the PEST domain) have been generated. **B)** OV2008 cells were transfected with Flag-cyclin G2 and lysed in a buffer containing either 0 or 5mM $CaCl_2$ for 60 or 120 minutes. Reactions were stopped with SDS sample buffer, analyzed by Western blot for cyclin G2 (FLAG). Addition of $CaCl_2$ in the lysis buffer decreases the level of cyclin G2. High $CaCl_2$ concentration (5 mM) further decreased cyclin G2 expression, compared to the low $CaCl_2$ (0.5 mM) concentration. **C)** OV2008 cells were transfected with either full-length cyclin G2-V5, PEST24 or ΔPEST. Following transfection cells were treated with either DMSO as a control, 1μM A23187 or pre-incubated with 20μM ALLN for 30 minutes before treatment with 1μM A23187. Cells were lysed and analyzed by Western blot analysis. Cyclin G2 was detected using antibody against anti-V5. Increased intracellular Ca^2 decreased cyclin G2 expression, while ALLN treatment was able to protect cyclin G2 from calcium-induced degradation. CCNG2, cyclin G2.

Cyclin G2 was degraded by calpain-1 and calpain-2

To determine if calpain activation was indeed responsible for the degradation of the cyclin G2 protein, OV2008 cells, transiently transfected with Flag-cyclin G2 plasmid, were recovered in the presence or absence of a calpain inhibitor, calpeptin. As expected, inhibition of calpain resulted in an increase in cyclin G2 levels when compared to the DMSO control (Figure 3A). To further confirm the involvement of calpain in cyclin G2 degradation, siRNAs for both calpain-1 and calpain-2 were designed. Casein zymography confirmed that each siRNA knocked down its specific targets, with no cross-reactivity to each other (Figure 3B). Both siCAPN1 and siCAPN2 were able to protect cyclin G2 from degradation and resulted in a higher expression of the protein compared to the negative control siRNA (Figure 3C). Finally, to confirm that cyclin G2 is a substrate for calpain, and to determine to what degree the PEST domain is involved in this process, OV2008 cells were transfected with either full-length cyclin G2, PEST 24, or ΔPEST (Figure 2A) and cell lysates were incubated with purified calpains under various conditions (Figure 3D). Incubation with either calpain-1 or calpain-2 resulted in a decrease in the expression of the full-length cyclin G2. Calpain had no effect on cyclin G2 degradation in calcium free buffer or in the presence of a calpain inhibitor, calpeptin. Interestingly, the effect of calpain was found to be dependent on the presence of the complete PEST domain, since the purified calpain could still target cyclin G2 with the partial PEST deletion, but did not affect the level of the full PEST deletion mutant.

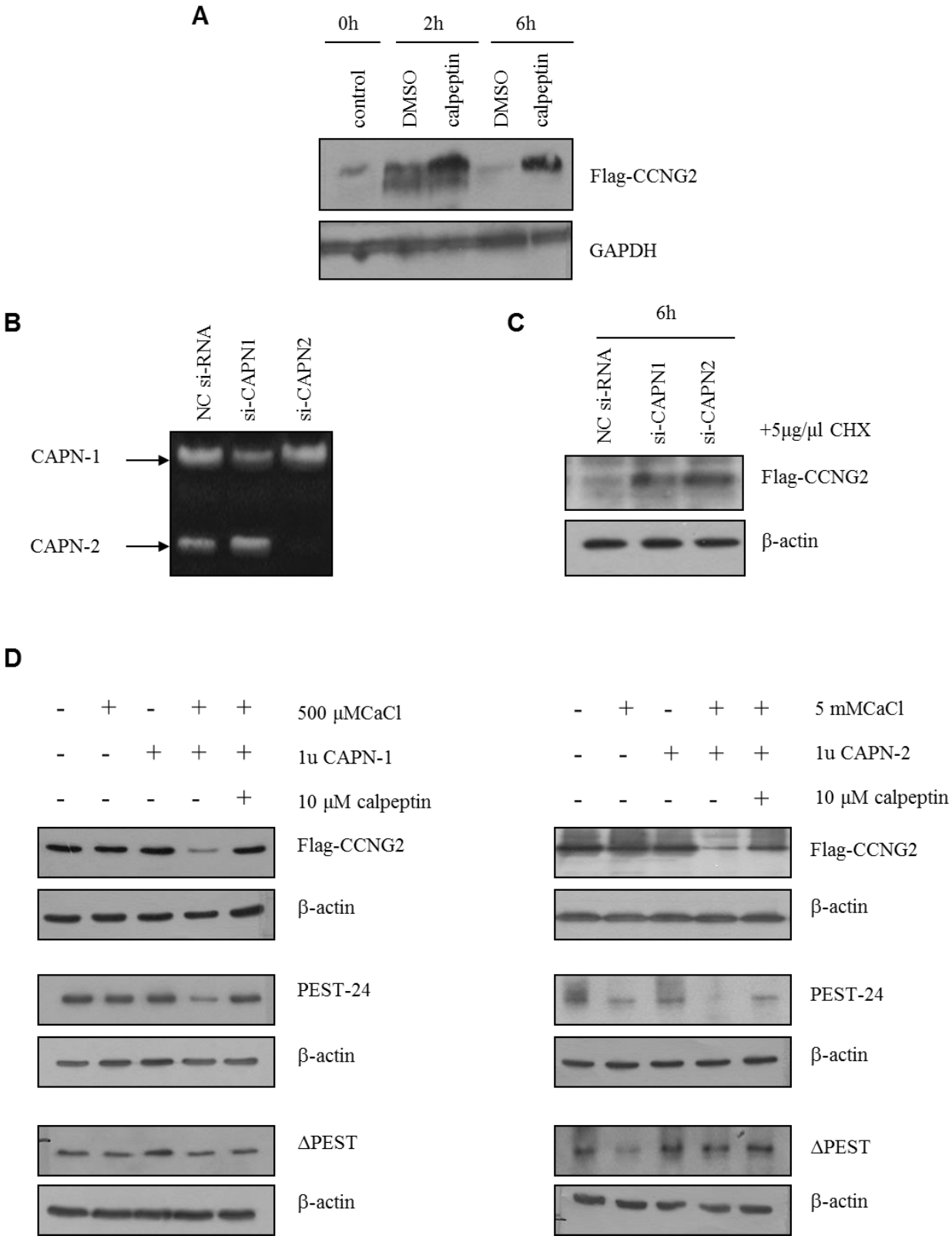

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Figure 3: Cyclin G2 is a target of calpain-mediated proteolysis. A) OV2008 cells were transiently transfected with Flag-cyclin G2 plasmid and recovered in the presence or absence of 50μM calpeptin for 2h and 6 hours. Cyclin G2 was detected using an antibody against Flag. In the presence of calpeptin, cyclin G2 degradation is decreased in comparison to the DMSO control at each time-point tested. **B)** siRNAs for calpain-1 (si-CAPN-1 and calpain-2 (si-CAPN-2) were generated. Casein zymography confirms that siRNA are specific for each calpain isoforms with no cross-reactivity to eachother. Calpain-2 is identified by higher mobility on the gel. **C)** Western blot of cyclin G2 expression following transfection of siRNAs for CAPN-1 or CAPN-2. OV2008 cells were transfected with siCAP1 or siCAPN2 for 6 hours prior to overnight (16 hour) transfection of Flag-cyclin G2. Cells were recovered for 6 hours in the presence of cycloheximide (CHX). Both calpain isoforms are able to increase cyclin G2, although si-CAPN2 resulted in a slightly higher retention of the cyclin G2 protein. **D)** OV2008 cells were transfected with either full-length cyclin G2 (CCNG2-V5), PEST 24 (mutant with a partial PEST deletion) or ΔPEST (mutant with a complete PEST deletion) and lysed in a buffer containing calcium, purified calpain, calpeptin, or a combination of the three. Cyclin G2 was detected by antibodies against V5. Incubation of protein lysate sample with calpain-1 or -2 lead to a decrease in cyclin G2 expression; however co-incubation with calpeptin was able to reverse this effect. Interestingly, the effect of calpain was found to be dependent on the presence of the complete PEST domain, since removal of this motif inhibited calpain-mediated degradation of cyciln G2. CCNG2, cyclin G2

Phosphorylation of cyclin G2 targets the protein for calpain-mediated proteolysis.

To further determine how calpains regulate the proteolysis of cyclin G2, we investigated the possibility of an upstream phosphorylation event that could label cyclin G2 as a target for activated calpains. Whole cell lysates from OV2008 cells that had been transfected with full length FLAG-cyclin G2 were pretreated with CIP, to dephosphorylate the protein, and subjected to purified calpain degradation. Similar to our previous results, both calpain-1 and -2 decreased cyclin G2 levels, however this effect was reversed by the CIP treatment, suggesting that phosphorylation of cyclin G2 is required for its proteolysis by calpain (Figure 4A). Examination of the cyclin G2 sequence using bioinformatics tools revealed multiple potential serine/threonine phosphorylation sites (Table 1), including CKII, PKC, and CDK5. However, treatment with inhibitors of these kinases did not protect cyclin G2 from degradation at the time point tested (Figure 4B-D). Therefore, we purchased a kinase inhibitory library to screen a variety of possible kinases. Using the kinase inhibitors supplied in the library, we found that treatment with the Epidermal Growth Factor Receptor Kinase (EGFRK) inhibitor, Tyrphostin AG1478, resulted in protection from degradation at the 2 hour time point and increased the expression of cyclin G2 to levels similar to that of the MG132-treated group (Figure 4E). To confirm that EGFR signaling participated in calpain-mediated degradation of cyclin G2, an *in vitro* degradation assay was performed in the absence or presence of the EGFRK-inhibitor. As shown in Figure 4F, treatment with calpain-2 strongly reduced cyclin G2 levels; however, the effect of calpain was reduced when cells were pre-treated with EGFRK-inhibitor.

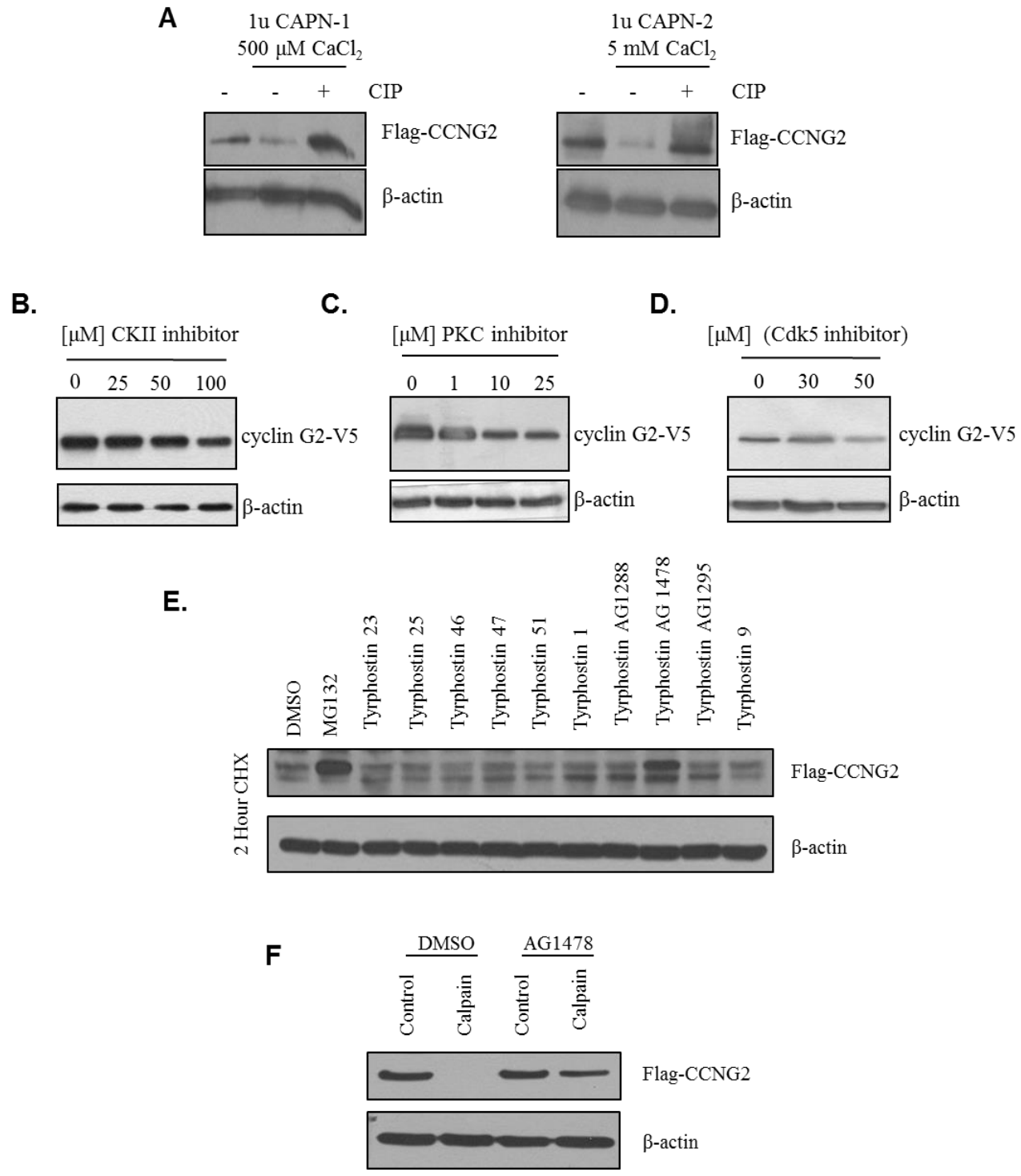

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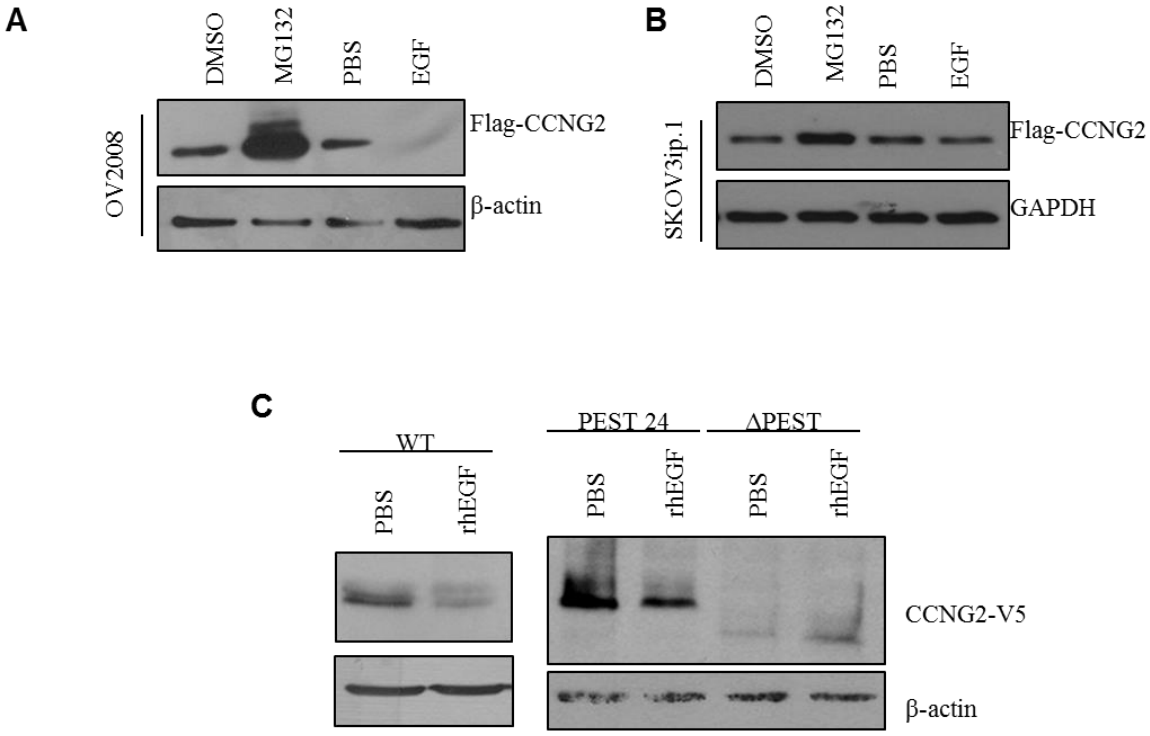
Figure 4: Phosphorylation is important for cyclin G2 stability. A) OV2008 cells transfected with Flag-cyclin G2 were pretreated with Calf Intestinal Phosphatase to dephosphorylate cyclin G2 and then incubated with purified calpain -1 (CAPN-1) or calpain-2 (CAPN-2) and calcium. Reactions were stopped with SDS-sample buffer and run on a SDS-PAGE gel. Degree of degradation was determined by Western blot analysis for Flag-cyclin G2. **B-D)** OV2008 cells were transfected with cyclin G2-V5 and recovered in the presence of various concentrations of either a CKII inhibitor, a general PKC inhibitor, or a Cdk5 inhibitor. CKII, PKC and CDK5 inhibitors had no protective effect on cyclin G2 as analyzed by Western blot for cyclin G2 expression. E**)** Effects of various kinase inhibitors on cyclin G2 degradation. MG132 (proteasome inhibitor) was used as a positive control. Treatment with 10μM Tyrphostin AG1478, which inhibits (EGFRK), protected cyclin G2 from degradation as analyzed by Western blot for Flag-cyclin G2. **F)** OV2008 stable cells that express cyclin G2 were treated with either DMSO control or Tyrphostin AG1478, for 30 minutes prior to lysing the cells. Whole cell lysates were further incubated with purified calpain and 5 mM calcium chloride for 1 hour. Treatment with Tyrphostin (AG1478) protected cyclin G2 from degradation when compared to the DMSO control as analyzed by Western blot analysis for Flag- cyclin G2. CCNG2, cyclin G2.

Table 2: Putative serine/threonine phosphorylation sites on cyclin G2

Site	Kinase Target
S-30	CKII
S-30	PKA
S-65	PKA
S-92	Cdc2
S-125	PKA
S-125	DNAPK
S-125	PKA
S-125	Cdc2
S-132	PKC
S-142	Cdc2
T-153	PKA
S-180	PKC
S-198	PKA
S-203	PKC
S-233	PKA
S-233	CKII
S-251	CKII
S-258	CKI
S-275	PKC
S-278	PKA
S-288	CKII
S-307	CKII
S-309	CKII
S-312	CKII
S-317	CKII
S-322	DNAPK
S-322	CKI
S-324	CKII
S-324	Cdc2
S-326	CDK5
S-326	GSK3
S-327	Cdc2
S-329	CKII
T-344	PKC

Activation of EGFR contributes to cyclin G2 degradation by calpain at the PEST domain.

To further investigate the effect of EGFR signaling on cyclin G2 degradation, we used cycloheximide to block *de novo* protein expression, and treated cells with rhEGF. As expected, in both OV2008 (Figure 5A) and SKOV3.ip1 (Figure 5B), treatment with MG-132 increased, whereas treatment with rhEGF decreased, the level of cyclin G2. Since we have previously indicated that the C-terminal PEST domain is critically involved in regulating cyclin G2 stability, the next step was to determine if the PEST domain was also important in regulating the action of EGFR on cyclin G2. Therefore, we transfected OV2008 cells with either the wild type, or partial (PEST24) or full PEST (ΔPEST) deletion mutants and then treated these cells with or without rhEGF for 30mins and chased with CHX for 2 hours. The rhEGF treatment decreased protein stability of both the wild-type and PEST24 cyclin G2 constructs, but did not affect the level of ΔPEST transfected cells. Interestingly, two putative tyrosines were found at residue 284 and 285 in the PEST domain, which may be phosphorylated by EGFR (Figure 5D). Lastly, we tested if EGF regulates calpain activity. OV2008 cells were treated with or without rhEGF and cell lysate was subjected to casein zymography. We found that rhEGF was able to stimulate calpain-2 activity, but had no effect on calpain-1 (Figure 6).



D

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H. sapiens              262 KPDLKKLVWIVSRRTAQNLHNSYYSVPELPTIPEGGCFDESESEDSCEDM 311
P. troglodyes           262 KPDLKKLVWIVSRRTAQNLHNSYYSVPELPTIPEGGCFDESESEDSCEDM 311
C. lupus                262 KPDHKKLVWIVSRRTAQSLHNSYYSVPELPTIPEGGCFDESESEDSCEDM 311
B. taurus               262 KPDLKKLVWIVSRRTAQNLHNSYYSVPELPTIPEGGCFDESE-------- 303
M. musculus             262 KPDLKKLVWIVSRRTAQNLHSSYYSVPELPTIPEGGCFDGSESEDSGEDM 311
R. norvegicus           262 KPDLKKLVWIVSRRTAQSLHNSYYSVPELPTIPEGGCFDGSESEDSGEDM 311
G. gallus               253 KPDHKKLVWIVSRRTAQNLQNSYYSVPELPTIPEGGCFNESESEDSCEDM 302
D. rerio                251 KPDHKKLVWIVSRRTAQNLHSSYCSIPELPTIPE-GVWDESESEDSSEDL 299
D. melanogaster         497 -PYKQRLVWKLSSRTLRVLRPINRFSSDLPTIEEGIP---NALDD----- 537
A. gambiae              213 -PYKQRLVWKLSSRTMRVLRPTDKLTSYLPTIAEHHHTHGHMSNS----- 256
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Figure 5: Legend on next page

Figure 5: EGFR signaling augments calpain-mediated degradation of cyclin G2. OV2008 **(A)** or SKOV3.ip1 **(B)** stable cells expressing cyclin G2 were treated with 10 μM MG132, to block protein degradation, or 20ng/mL recombinant human epidermal growth factor (rhEGF), and their vehicle control for 1 hour, and chased with cycloheximide (CHX) treatment to block translation for 2 hours. Treatment with MG-132 protected cyclin G2 from degradation, while treatment with rhEGF decreased cyclin G2 expression. **C)** Cells were transfected with either full-length cyclin G2-V5 or PEST deletion mutants (PEST24 or ΔPEST) and treated with rhEGF for 30 min before CHX addition for another 2 hours. rhEGF increased degradation of the wild-type and PEST24 transfected cells. Removal of the entire PEST domain resulted in the loss EGF activity as analyzed by Western blot for cyclin G2-V5. **D)** Predicated EGFR tyrosine kinase phosphorylation sites at residue 284 and 285 (red Y). This site is highly conserved throughout species, which may suggest important functional relevance. Generated with NCBI's MUSCLE (HomoloGene:3208). CCNG2, cyclin G2

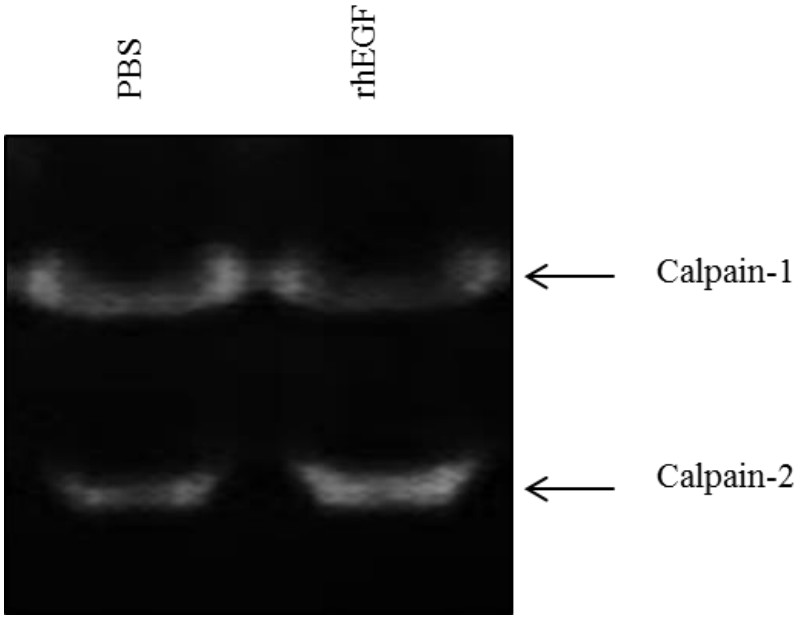

Figure 6: EGFR signaling activates calpain-2. OV2008 cells were treated with rhEGF for 24 hours. Cell lysates were subjected to caesin zymography for calpain actvity. Band clearing is proportional to calpain activity. EGF treatment increased the activity of calpain-2 but not calpain-1

DISCUSSION

We have previously demonstrated that cyclin G2 is a highly unstable protein and can be degraded via ubiquitin-proteosome pathway [1]. We also showed that the PEST domain is critically involved in the stability of cyclin G2. Since many proteins can be degraded through multiple mechanisms and several studies have reported that PEST sequence can be targeted by calpain for degradation [24, 25], we investigated if cyclin G2 can be degraded by the calpain pathway. In the present study, we provided the first evidence that cyclin G2 instability is mediated in part by proteolytic cleavage of calpain, and that the action of calpain is dependent on prior phosphorylation of cyclin G2 via the EGFR signaling pathway (Figure 7).

Several lines of evidence support the role of calpains in cyclin G2 degradation. Cyclin G2 stability was decreased in the presence of calcium. This increase in degradation is likely due to the activation of the calcium-activated protease, calpain, since pre-incubation with various calpain inhibitors, or knockdown of calpain-1 and calpain 2 by specific siRNAs resulted in a reduction of cyclin G2 degradation. Upon calcium binding, calpains undergo conformational rearrangements that allows for proper formation of the catalytic site [22]. These rearrangements are necessary for proper function of both calpain-1 and calpain-2, which differ mainly in their sensitivity to calcium. We observed that the high (5mM) concentration of $CaCl_2$, which activates calpain 2 [26], was more effective than the low (0.5 mM) concentration of $CaCl_2$, which activates calpain 1 [26], in the induction of cyclin G2 degradation. In addition, although incubation with either calpain-1 or calpain-2 decreased cyclin G2 protein level, this degradation was pronounced in the presence of calpain-2. Therefore, it is possible that calpain-2, is preferentially involved in the degradation of cyclin G2.

In this study, we observed that prior phosphorylation of cyclin G2 is necessary for calpain action and identified EGFRK as a potential upstream kinase responsible for the phosphorylation of cyclin G2. Pre-treatment of cyclin G2 with a phosphatase protected cyclin G2 from calpain-dependent cleavage. Similarly, previous studies have reported that calpain-mediated degradation can be induced by the phosphorylation of specific sites on the target protein, and that inhibition of these kinases, or mutation of the predicted phosphorylation site, is critical for calpain-mediated proteolysis [27, 28]. Therefore, it is possible that cyclin G2 cleavage by calpain takes place through a similar manner. Indeed, we have found that treatment with the EGFRK inhibition not only increased cyclin G2 stability, but also completely abrogated the action of calpain on cyclin G2. On the other hand, activation of EGFR by EGF induced the degradation of cyclin G2. Interestingly, previous reports have suggested that EGFR signaling may increase calpain (specifically calapin-2) activity [29-31], and we have observed the same effect. Therefore, it is possible that EGFR regulates cyclin G2 stability through two mechanisms. Firstly, to increase calpain activation and secondly, to phosphorylate cyclin G2, making it susceptible to degradation.

The PEST domain a polypeptide sequence enriched in proline, glutamic acid, serine and threonine and has been implicated in regulating the intrinsic stability of many proteins [32-34]. PEST domains have been regarded as recognition factors for many proteolytic proteins, including calpains, and protein kinases [33]. Phosphorylation at the PEST domain may also induce conformational changes elsewhere in the protein necessary for interaction with proteolytic components [33]. We have reported that the removal of the PEST domain greatly increased cyclin G2 stability [1]. In this study, we showed that both calpain and rhEGF treatment

induced the degradation of the wild-type and PEST24 cyclin G2 constructs, but had no effect on the stability of cyclin G2 when the PEST domain was completely removed. Therefore, the first 24 amino acids of the PEST domain appear critical for EGFR-induced degradation by calpain. Interrogation of potential phosphorylation sites by a Group-based prediction system (GPS) 2.1 provided two putative tyrosine residues, tyrosine 284 and tyrosine 285, located in the PEST domain, which may be phosphorylated by EGFR. Interestingly, both tyrosines are highly conserved across various species. This high degree of conservation may indicate an important regulatory role of this site, in terms of protein degradation. Future studies will determine if mutagenesis of these potential EGFR phosphorylation sites will abolish calpain-mediated cyclin G2 degradation. Alternatively, considering the diverse signalling cascades regulated by EGFR [35], it is possible that EGFR acts indirectly on cyclin G2 via its downstream kinase cascades.

In summary, data presented has outlined a novel mechanism that is responsible for degradation of cyclin G2 (Figure 7). Firstly, the activation of EGFR may lead to the phosphorylation of cyclin G2 at the PEST domain. This phosphorylation increases the susceptibility of cyclin G2 to degradation by calpains. Furthermore calpain-2 is activated downstream of EGFR [29], and may also contribute to the increased degradation of cyclin G2. This pathway, in combination with other proteolytic mechanisms, including the 26S proteasome [1], may be responsible for the fast turnover of cyclin G2 and mediates the highly unstable nature of this protein. EGFR activation is a common contributor to malignancy, and many reports have implicated increased EGF signaling as a driver of both ovarian and cervical cancer [36-39]. Since we have also observed that cyclin G2 can exert potent anti-tumorigenic effects (Chapter 2),

it is possible that inhibition of cyclin G2 via EGFR-induced activation of calpain is a significant event underlying the oncogenic actions of EGF and contributes to ovarian cancer development.

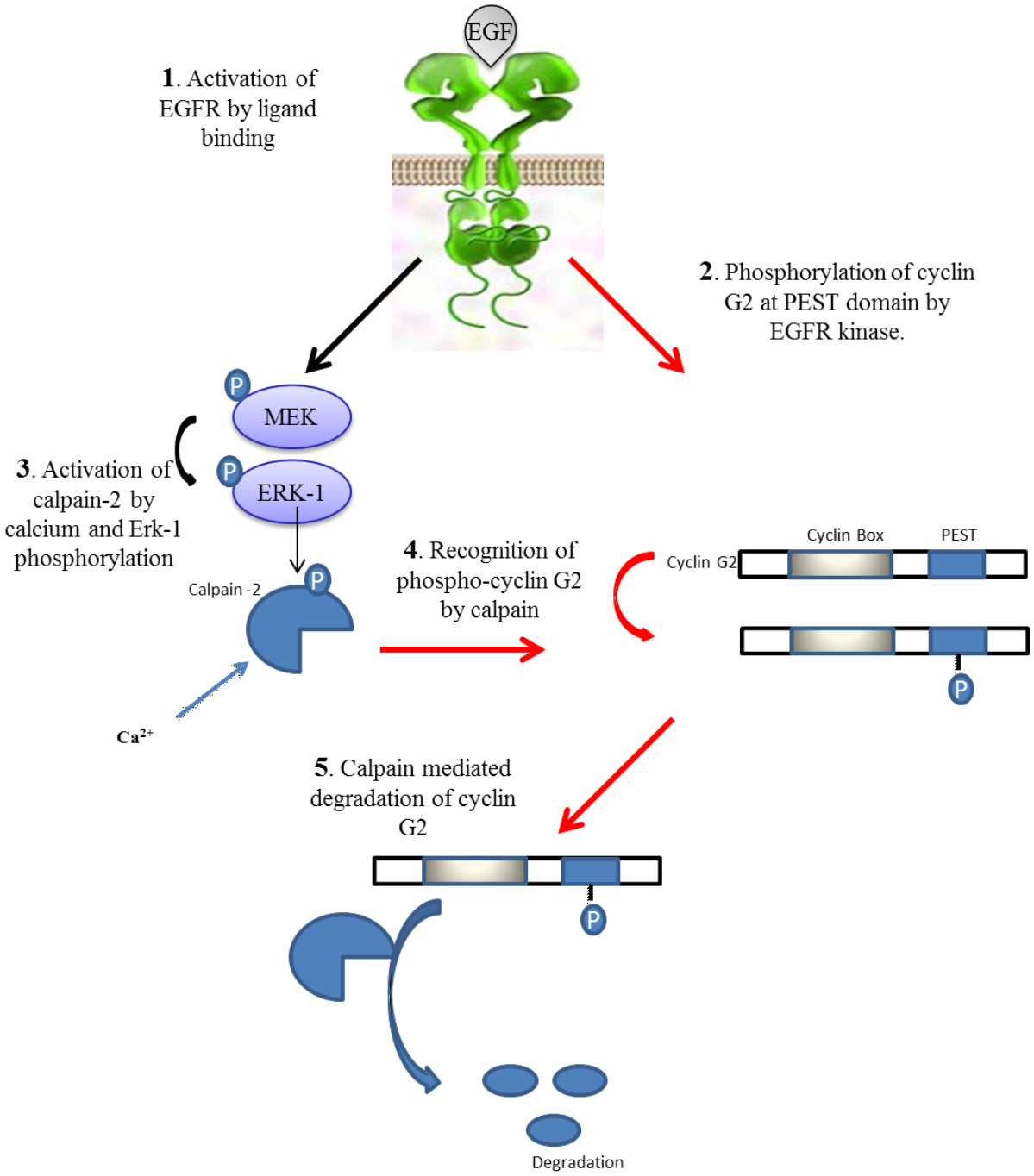

Figure 7: Proposed mechanism of cyclin G2 degradation by calpain. Epidermal Growth Factor (EGF) binds to its receptor, EGFR, for activation of its intracellular kinase action. Direction or indirect phosphorylation of cyclin G2 occurs downstream of EGFR activation and targets cyclin G2 for degradation by calpain. At the same time, EGFR can also activate calpain-2, which leads to efficient degradation of cyclin G2. Phosphorylated cyclin G2 is recognized by calpain-2 and results in proteolytic cleavage. Red arrows indicate areas of investigation of this study. Black arrows indicate previous reported actions.

ACKOWLEDGMENTS

We would like to thank Drs. Benjamin Tsang and Mien-Chie Huan for providing the OV2008 and SKOV3ip1 cells, respectively.

This work was supported by grants from the Canadian Institute for Health Research to Chun Peng and Ontario Graduate Scholarship, The Natonal Science and Engineering Research Council of Canada, and The Susan Mann Dissertation Scholarrship to Stefanie Bernaudo.

Shahin Khazai (Master's candidate) and Eilyad Honarpavar (Undergraduate thesis student) contributed to this work.

Shahin completed Figure 1B and 5B

Eilyad assisted me with Figure 4E and F

CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

SUMMARY

In this dissertation, I have investigated the role and regulation of cyclin G2 in epithelial ovarian cancer cells. The objectives of this study were to further characterize the function, mechanism of action and regulation of cyclin G2 in ovarian cancer cells. Specifically, we have: 1) investigated the role of cyclin G2 in ovarian cancer cells; 2) determined the mechanism by which cyclin G2 exerts it anti-tumorigenic effect, and 3) examined the mechanism of calpain-dependent proteolysis in cyclin G2 degradation;.

I. Cyclin G2 exert anti-tumor effects in ovarian cancer cells.

To further understand the role of cyclin G2 in ovarian tumor development, we have examined the function of cyclin G2 in ovarian cancer cells (Chapter 2). In EOC cell lines stably transfected with cyclin G2 cell proliferation, migration and invasion were significantly suppressed, as compared to cells transfected with a control vector. Furthermore, we assayed the ability of stable cells to form spheres by the hanging drop culture method. Previous reports have found a positive relationship between the extent of compact spheroid formation and invasive behavior [1]. Cyclin G2-overexpressing cells formed loose spheroids when compared to the control, and, more importantly, had limited ability to invade through a collagen network. Together, this data suggests that cyclin G2 exerts anti-tumorigenic effects in ovarian cancer cells.

In addition, we have provided *in vivo* data to support our hypothesis. When control and cyclin G2-overexpressing cells were injected subcutaneously into nude mice, the cyclin G2 cells formed significantly smaller tumors. Impressively, these cells failed to invade into the surround tissue layers, and formed a distinct boundary between the muscle and tumor tissues at the

primary site of injection. On the other hand the control cells easily infiltrated the neighboring sites. Similarly, mice receiving intraperitoneal injection of cyclin G2-overexpressing cells formed less ascites fluid compared to those injected with control cells. Investigation of human ovarian clinical samples revealed lower cyclin G2 levels in high-grade malignant carcinoma compared to that of normal, low-grade, or low malignant potential tumors. Overall, the decreased expression of cyclin G2 in ovarian tumors may indicate a more advanced cancer with aggressive characteristics.

In the future we would like to expand our understanding of how cyclin G2 may regulate the *in vivo* progression of EOC tumors. Since our data suggests that cyclin G2 inhibits migration and invasion and promotes an epithelial phenotype, it is likely that cyclin G2 may inhibit early dissemination of cancer cells. We have already performed subcutaneous and interperitoneal injection of control and cyclin G2 cells into nude mice and observed cyclin G2-induced tumor-suppressive effects. We would like to continue this study by using an orthotopic approach to better understand both primary and secondary tumor formation. An orthotopic mouse model for EOC has been described [2], in which ovarian cancer cells are injected under the ovarian bursa. This method takes advantage of the epithelial-stromal interactions that are critical to ovarian tumorigenesis, as well as EMT [3]. By this method, we can assay how cyclin G2 overexpression regulates the formation of primary lesions, as well as ascites accumulation and cell spreading throughout the peritoneal cavity. This model better replicates the human disease.

Further, our preliminary data suggests cyclin G2 expression is decreased in highly aggressive human clinical samples when compared to tissues from the normal ovarian surface or low grade ovarian cancer. In the future, it will be imperative to expand our clinical sample set to investigate if an inversely relationship exists between cyclin G2 expression and cancer progression, as seen in other cancer models [4]. One potential limitation is in choosing a proper control. While, it is now understood that most ovarian cancer do not actually arise from the ovarian, and that low- and high-grade cancer are also distinct diseases, extreme caution must be taken when choosing a control study. HGSC arises from the fimbriae of the fallopian tube [5], however primary lesions are elusive and samples are extremely rare. Therefore, for HGSC the tumor samples from stages I to IV may be a more suitable study. For LGSC, comparison of the borderline versus the invasive serous low-grade carcinoma, may provide cues in understanding the role of cyclin G2 in malignant transformation of these cells. Depending on availability, 10-20 samples of each group should be examined.

Lastly, we would like to investigate the role of cyclin G2 in the malignant transformation of a mouse model of ovarian cancer. m0505 and STOSE cells were obtained from Dr. Barbara Vanderhyden, Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada. The m0505 OSE cell line was established according to the protocol described in Gamwell et al.[6]. Upon long-term passage of the cells, the m0505 cell line spontaneously transformed, and was labeled Spontaneous Transformed Ovarian Surface Epithelial (STOSE) cells. Early passage M0505 cells grow slowly, having a doubling time of 48 h. The growth rate increases as m0505 cells reach >35 passages and cells begin to lose the epithelial “cobblestone” morphology. STOSE cells have a doubling time of 13 h, almost four times faster than their

untransformed M0505 counterpart [7]. Remarkably, the transformed show gene alterations that are consistent with the changes identifiedin human ovarian cancers by The Cancer Genome Atlas [6]. qPCR analysis of m0505 and STOSE cells revealed a lower cyclin G2 level in the sTOSE cells compared to m0505 (Figure 1A). This data suggests that the loss of cyclin G2 may contribute to early oncogenesis and the development of ovarian cancer.

Due to the highly unstable nature of cyclin G2 [8], we generated STOSE stable cells that overexpress either cyclin G2 or its empty vector control using retroviral vectors. We hypothesized that rescue of cyclin G2 expression in these cells could diminish their tumorigenic potential. Indeed, preliminary data shows that the overexpression of cyclin G2 in STOSE cells significantly decreased the proliferative capacity of these cells. In addition, we hope to generate a stable cell line for the m0505 cells that overexpresses cyclin G2-siRNA. We can use these cell lines to corroborate our hypothesis that cyclin G2 is involved in inhibiting oncogenesis. Briefly, expression of cyclin G2 in STOSE should inhibit processes such as proliferation, migration and invasion, while knockdown of cyclin G2 in m0505 cells should propagate the tumorigenic phenotype. These data can further define cyclin G2 as a protein with tumor suppressive effects and identify a role for cyclin in the malignant transformation of ovarian cancer cells. However, since the process of malignant transformation of mouse and human cells is substantially different, confirmation of these results in a human cell line is imperative.

II. Cyclin G2 exerts tumor-suppressive effects by promoting an epithelial phenotype and attenuating β-catenin signaling.

To explain the mechanism of cyclin G2 action we examined the molecular pathways altered in stable cells overexpressing cyclin G2 (Chapter 2). We suggest that cyclin G2 exerts its anti-tumor effects by promoting an epithelial phenotype in EOC cells. Specifically, cyclin G2 cells show increased levels of E-cadherin, a quintessential marker of epithelial cells, in addition to a decrease in the mesenchymal markers vimentin and N-cadherin. The transcription factors HMGA-2, Snail, and Slug were also decreased in the cyclin G2 cells, which may account the repression of the mesenchymal transcription profile. Lastly, cyclin G2 cells also displayed stronger circumferential f-actin and decreased actin stress fiber. Together these changes are characteristic of a more differentiated epithelial cell type. Knockdown of E-cadherin reversed the effect of cyclin G2 on proliferation, migration, invasion, and compact spheroid formation in the cyclin G2 overexpressing cells, suggesting that E-cadherin mediates the effects of cyclin G2.

In addition, overexpression of cyclin G2 resulted in differential subcellular localization and overall decrease in the β-catenin protein, when compared to the control. β –catenin is known to form a complex with E-cadherin at cellular junctions, and in cyclin G2 cells β-catenin expression was decreased in the nucleus, but strongly associated with the membrane. On the other hand, phosphor-β-catenin was induced by cyclin G2 overexpression. Since WNT/ β-catenin signaling has been shown to play an important role in epithelial-to-mesenchymal transition (EMT), cyclin G2 may inhibit EMT and tumor progression through attenuation of β-catenin signaling. Indeed, many β-catenin direct or indirect target genes are decreased in the cyclin G2 stable cells, including, TCF1 and LEF1, as well as the aforementioned, vimentin, HMGA-2,

Snail and Slug. Overexpression of a constituently active form of β-catenin rescued the tumorigenic phenotype in the cyclin G2 stable cells, leading to increased proliferation, migration and invasion. Lastly, GSK3β-induced degradation of β-catenin appears to be essential to cyclin G2-mediated inhibition of β-catenin signaling. Inhibition of GSK3β, increased β-catenin levels in the cyclin G2 cells, and could partly reverse the anti-tumorigenic effect of cyclin G2. Interestingly, the formation of the β-catenin destruction complex is enhanced in the cyclin G2 cells, as shown by the increased association of bona fide complex members, namely, GSK3β, β-catenin, and PP2A. Therefore, cyclin G2 regulation of β-catenin signaling is twofold: firstly by regulating is subcellular localization by recruitment to the membrane likely via binding to E-cadherin, and secondly by decreasing its overall stability through promoting the formation of GSKβ-dependent destruction complex. Overall, cyclin G2 may act to promote the epithelial differentiation of ovarian cancer cells and reduce tumor burden.

However, the exact mechanism behind cyclin G2-mediated regulation of the β-catenin destruction complex remains to be explained. It is possible that the association between cyclin G2 and the destruction complex is cell-cycle dependent and is differentially regulated based on the cellular milieu. In this case we can purchase cell cycle chemical inhibitors and preform immunoprecipitation to investigate whether the cell cycle stage can regulate complex formation. On the other hand, cyclin G2 may only transiently associate with one or more of the destruction complex members to enhance the complex formation of GSK3β and β-catenin. We can use a protein cross-linker prior to immunoprecipitation to stabilize the tentative association of cyclin G2 with β-catenin or GSK3β. Our recent data that shows PP2A is also enhanced within this complex. PP2A was previously identified binding partner of cyclin G2 [9]. Researchers showed

that cyclin G2 formed a complex with the regulatory (B) and catalytic (C) subunit of PP2A, which could alter PP2A activity and specificity [9]. How the possible interaction between cyclin G2 and PP2A subunits regulates the dynamics of the β-catennin destruction complex would be an interesting for future research.

Alternatively, cyclin G2 may be interacting with a completely novel partner to regulate the destruction complex. We can identify this partner by mass-spectrometry of pull-down samples from cyclin G2 stable cells. Knockdown of this newly identified cyclin-G2 partner should relieve the cyclin-G2 induced inhibition of β-levels and signaling.

III. Cyclin G2 is degraded by calpain-mediated proteolysis, which is dependent on the presence of the PEST domain and EGFR signaling.

Cyclin G2 contains a destabilization domain, PEST, at its C-terminal, which is suggested to be a target of multiple degradation pathways, including calpains [10]. Data presented in Chapter 3, describes a novel pathway of cyclin G2 degradation. Specifically, we present several lines of evidence that demonstrate that cyclin G2 can be targeted by calcium activated calpain-dependent proteolysis and that this cleavage event occurs at the PEST domain. Firstly, we have found that increasing either the intracellular or i*n vitro* calcium concentrations promoted the degradation of cyclin G2, while inhibition or knockdown of the calpain protease strongly inhibited degradation. In addition, removal of the PEST domain abolished the effect of both calcium and calpain, suggesting that it is critically involved in cyclin G2 stability. Furthermore, we showed that phorphorylation of cyclin G2 is required for its degradation by calpain. Finally, using a kinase inhibitory library, we found that treatment with the Epidermal Growth Factor

Receptor (EGFR) Kinase inhibitor resulted in protection of cyclin G2 from degradation, and that the first 24 amino acids of the PEST region appear to be critical for this regulation. These results, together with findings that cyclin G2 exerts potent anti-tumor effects on EOC (Chapter 2), suggest that rapid destruction of cyclin G2 may be one of the mechanisms by which EGF promotes cancer development.

Future study will investigate how EGF induces cyclin G2 degradation through the calpain pathway. It is possible that the EGFR, activated by EGF, can directly phosphorylate cyclin G2, making it a target for calpain. Indeed, interrogation of potential phosphorylation sites revealed two putative tyrosines within the first 24 amino acids of the PEST region that may be phosphorylated by EGFR. We will mutate these potential EGFR phosphorylation sites to determine if this will abolish calpain-mediated cyclin G2 degradation. This will help to solidify the idea that cyclin G2 and EGFR directly interact with each other prior to cyclin G2 degradation. In addition, immunoprecipitation can be performed to determine if EGFR and cyclin G2 are found in a complex within the cell. Since phosphorylation is a dynamic process, a protein cross linker may be needed to visualize this interaction. In addition, cyclin G2 phosphorylation can be determined *in vitro* by incorporation of [γ-^{32}P] ATP into cyclin G2. Cells expressing His-tagged cycliln G2 can be purified by Ni-NTA magnetic agarose beads and incubated in the presence of an activated form of EGFR (BIOMOL; BML-SE116-0010 Plymouth Meeting, PA). The mixture can be separated by SDS-PAGE and transferred to PVDF membrane for autoradiography.

Alternatively, if studies suggest that EGFR action on cyclin G2 is indirect, we can continue to examine what downstream kinases are activated by EGFR that can phosphorylate cyclin G2. To date attempts to identify an intermediate kinase that may be responsible for transduction of EGFR signals have yet to provide clues into cyclin G2 regulation. If the intermediate kinase is found, identification of the phosphorylation site would be critical to the understanding of cyclin G2 degradation. Computational prediction methods, mutational analysis and, determination of the *in vivo* phospho-site by mass spectrometry can help to confirm the importance of the implicated kinases.

Finally, since EGFR signaling increased calpain-2 activity, it is very likely that this also contributes to the decrease in cyclin G2 stability following EGF treatment. We will test if silencing of calpain 2 will partially reverse the effect of EGF on cyclin G2 degradation. Together, these studies will provide novel insights into how the proteolytic action of calpain and constitutive activation of EGFR signaling contribute to the decreased cellular concentration of cyclin G2.

CONCLUSION

Although cyclin G2 has been suggested to have anti-proliferative effects on cancer cells and is dysregulated during the progression of several types of cancer [4, 11-13], the role of cyclin G2 in cancer development is poorly understood. The present study provides evidence that cyclin G2 has anti-tumor effects and identifies the regulatory loop involving GSK3β/β-catenin/E-cadherin as a major downstream mediator of these actions. In addition, our study suggests that

increased oncogenic growth-factor signaling and protease activity during cancer development may significantly reduce the level of cyclin G2, which in turn, contributes to disease progression.

CHAPTER 5

ADDITIONAL PUBLICATIONS

I. Advances in Ovarian Cancer Management: Etiology and Overview

Ye G, Bernaudo S, and Peng C.
Department of Biology, York University, 4700 Keel Street, Toronto, ON M3J 1P3, Canada
Future Medicine 2012; Pages 6-46 (doi: 10.2217/ebo.11.356)

I contributed to this book chapter by assisting with the writing of the first draft. I was responsible for the addition of subsequent sections, such as "Biomarkers and Screening" and final editing of the chapter. I also helped to create the tables and information boxes used.

II. MicroRNA 376c enhances ovarian cancer cell survival by targeting activin receptor-like kinase 7: implications for chemoresistance.

Ye G[1], Fu G[1], Cui S[2], Zhao S[1,3], Bernaudo S[1], Bai Y[1], Ding Y[2], Zhang Y[4], Yang BB[5], and Peng C[1].
[1]Department of Biology, York University, 4700 Keel Street, Toronto, ON M3J 1P3, Canada
[2]Department of Histology and Embryology, Dalian Medical University, Dalian, 116044, China
[3]The Second Hospital of Hebei Medical University, Shijiazhuang, 050000, China
[4]Life Science Division, Tsinghua University, Shenzhen Campus, 518055, China
[5]Sunnybrook Research Institute, Sunnybrook Health Sciences Centre, and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON M4N 3M5, Canada

Journal of Cell Science 2011; 124(Pt 3):359-68.

I contributed to this paper by preforming all spheroid formation assays and collecting data. I also participated by writing a small section in Materials and Methods and provided editing for the final paper.

III. Regulation of membrane progestin receptors in the zebrafish ovary by gonadotropin, activin, TGF-beta and BMP-15.

Tan Q, Zagrodny A, Bernaudo S, Peng C.
Department of Biology, York University, 4700 Keele Street, Toronto, Ontario M3J 1P3, Canada.

Molecular and Cellular Endocrinology 2009; 312(1-2):72-9.

I contributed to this paper by preforming and optimizing the immunofluorescence assays on the whole oocyte. I also took the confocal microscopy pictures used in the publication. In addition to writing a small section in Materials and Methods, I also provided editing for the final draft of the paper.

IV. Cyclin G2 Is Degraded through the Ubiquitin-Proteasome Pathway and Mediates the Antiproliferative Effect of Activin Receptor-like Kinase 7

Xu G[1], Bernaudo S[1], Fu G[1], Lee DY[2], Yang BB[2], and Peng C[1]
[1]Department of Biology, York University, Toronto, ON, Canada M3J 1P3
[2]Sunnybrook and Women's College Health Science Centre and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada M4N 3M5

Molecular Biology of the Cell 2008: 19(11):4968-79.

I contributed to this paper by completing several of the revisions requested by reviewers. I preformed many of the proteasome inhibitor assays, pull down studies, skp2 overexpression and siRNA treatments on wild type and mutant constructs, immunoprecipitation and Western blot analyses of cyclin G2 in response to Skp2, ALK7 and Nodal and much of the fluorescence microscopy. I was also involved in writing and final preparation the manuscript.

APPENDIX

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II. CHAPTER 2

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III. **CHAPTER 3**

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IV. CHAPTER 4

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