# REGULATION OF CYCLIN G2 DEGRADATION IN HUMAN OVARIAN CANCER CELLS

**Shahin Khazai**

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# Abstract

Epithelial ovarian cancer (EOC) has the highest mortality rate among gynecological malignancies due to its non-specific symptoms, poor screening methods, lack of reliable biomarkers, and our limited knowledge of the cellular and molecular mechanisms underlying the disease. As in all cancers, uncontrolled cell growth resulting from a deregulated cell cycle lies at the core of the cellular aspects of EOC. Normal cell cycle regulation is maintained through temporal expression of canonical cyclin molecules, which then direct progression through the cycle by their direct binding to and activating cyclin-dependent kinases (CDKs). Cyclin G1, G2, and I, constitute a group of unconventional cyclins whose expression results in cell cycle arrest. Cyclin G2 expression has been shown to negatively regulate cell cycle and its levels are lowered in various types of cancer including ovarian, breast, oral, gastric, and kidney. Our lab has previously reported that cyclin G2 is an unstable protein, which can be degraded through the ubiquitin proteasome pathway. The aim of this study was to further examine how the degradation of cyclin G2 is regulated. Through a series of *in vitro* assays, we showed that cyclin G2 is a target of calpain's proteolytic activity in a number of human ovarian cancer cells. Furthermore, through the use of inhibitors of various protein kinases we found that inhibition of epidermal growth factors receptor (EGFR) in those cells led to protection of cyclin G2 from degradation. Finally, we demonstrated that stimulation of cells with epidermal growth factor (EGF) also resulted in the degradation of cyclin G2. Considered together, these findings suggest that activation of EGFR and calpain promote cyclin G2 degradation.

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# List of Abbreviations

| | |
|---|---|
| ALK7 | Activin receptor-like kinase 7 |
| ATP | Adenosine triphosphate |
| APC/C | Anaphase promoting complex/cyclosome |
| CIP | Calf intestinal phosphatase |
| CK1 | Casein kinase 1 |
| CK2 | Casein kinase 2 |
| Cip/Kip | CDK interacting protein/Kinase inhibitory protein |
| Cdc | Cell division cycle |
| cDNA | Complementary DNA |
| cAMP | Cyclic adenosine monophosphate |
| CCNG2 | Cyclin G2 |
| CDK | Cyclin-dependent kinase |
| CKI | Cyclin-dependent kinase inhibitor |
| CHX | Cycloheximide |
| DMSO | Dimethyl sulfoxide |
| Dvl | Disheveled |
| DTT | Dithiothreitol |
| DDR | DNA damage response |
| E2F | E2 transcription factor |
| EV | Empty Vector |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| EOC | Epithelial ovarian cancer |
| EMT | Epithelial-to-mesenchymal transition |
| ER | Estrogen receptor |
| ERK | Extracellular signal-regulated kinase |
| FBS | Fetal Bovine Serum |
| FOX | Forkhead box protein |
| FRE | FOXO response element |
| FBE | FOXO3a-binding element |
| GSK3 | Glycogen synthase kinase 3 |
| GAPDH | Glyderaldehyde-3-phosphate dehydrogenase |
| HRP | Horse radish peroxidase |
| HER2 | Human epidermal growth factor receptor 2 |
| HIF1α | Hypoxia-induced factor 1 alpha |
| IGF-1 | Insulin-like growth factor 1 |
| KRAS | Kirsten rat sarcoma viral oncogene |
| MEK | MAPK/ERK kinase |

| | |
|---|---|
| MET | Mesenchymal-to-epithelial transction |
| mRNA | Messenger RNA |
| miRNA/miR | microRNA |
| MAPK | Mitogen-activated protein kinase |
| Mdm2 | Mouse double minute 2 |
| MYC | Myelocytomatosis |
| PPARγ | Peroxisome proliferator-activated receptor γ |
| PTEN | Phosphatase and tensin homolog |
| PBS | Phosphate-buffered saline |
| PI3K | Phosphoinositide 3-kinase |
| PDGF | Platelet-derived growth factor |
| PAGE | Polyacrylamide gel electrophoresis |
| PVDF | Polyvinylidene fluoride |
| PEST | Proline (P), glutamic acid (E), serine (S), threonine (T) |
| PKA | Protein kinase A |
| PKB | Protein kinase B |
| PKC | Protein kinase C |
| PP2A | Protein phosphatase 2A |
| RIPA | Radioimmunoprecipitation assay |
| RAS | Rat sarcoma |
| rhEGF | Recombinant human epidermal growth factor |
| Rb | Retinoblastoma |
| Skp2 | S-phase kinase-associated protein 2 |
| SCF | Skp, Cullin, F-box containing complex |
| Sip1 | Smad interacting protein |
| Smad | Small body size- mothers against decapentaplegic |
| SDS | Sodium dodecyl sulphate |
| SH domain | Src homology domain |
| SEM | Standard error of the mean |
| TBS-T | TBS + 0.5% Tween-20® |
| TF | Transcription factor |
| TGFβ | Transforming growth factor beta |
| TBS | Tris-buffered saline |
| Ub | Ubiquitin |
| UPP | Ubiquitin-proteasome pathway |
| UTR | Untranslated region |
| BRAF | v-Raf murine sarcoma viral oncogene |
| Wnt | Wingless/integrated |
| ZEB | Zinc finger homeodomain enhancer-binding protein |

# Chapter 1: Introduction

## 1.1: The Lifecycle of Eukaryotic Cells

### 1.1.1: Overview of Regulatory Networks of Cellular Life

Appropriate and steady regulation is the key to maintain the complexity and organization of any system that dares to go against the grain of the universal increase of entropy. The eukaryotic cells of the human body, each containing about $10^9$ interacting protein molecules at any given time[1], are a perfect embodiment of regulated complexity, which is absolutely needed for the normal functioning of these cells. Controlled growth and proliferation at cellular level results in normal development and functioning of the body. On the other hand, uncontrolled growth of cells can lead to many diseases, including cancer[2]. At the molecular level, cell growth is induced by the activation of cyclin-dependent kinases (CDKs), which follows their binding to their positive regulators, cyclins[3,4]. The proliferation of all eukaryotic cells is governed by the cell cycle, where temporally controlled biochemical events promote or halt progression through it, depending on the intracellular as well as extracellular conditions[5]. Upon completion of mitosis and cytokinesis, the two daughter cells can be viewed as entering a new cycle, where the environment as well as the biological clues will decide their fate.

### 1.1.2: Cyclins and CDKs

Cyclins are a class of closely related proteins[6] that contain a conserved stretch of ~110 amino acids known as the cyclin box[7]. The grouping of cyclins is based on their structural similarities as well as their temporal expression during the cell cycle[8,9]. The cyclin box facilitates the formation of CDK-cyclin complexes[10] and this binding switches on the serine/threonine kinase

activity of CDKs, which then promotes cell cycle progression through phosphorylation of their substrates[3,11]. Even though cyclins and CDKs are the canonical drivers of the cell cycle, it is striking that of the 13 CDK and 25 cyclin genes identified in the human genome so far, only a handful are directly and exclusively involved in cell cycle regulatory pathways[2]. For instance, in addition to cell cycle regulation in different cell types, CDK7-cyclin H as well as CDK8, CDK9, CDK10, and CDK11 also regulate transcription through phosphorylating different subunits of RNA polymerase II[12-14]. While intracellular levels of CDKs remain constant throughout the cell cycle, the expression and amount of most cyclins oscillate with the cell cycle[15], and their levels are regulated by rapid degradation via ubiquitin-proteasome pathway (UPP)[16,17] . Cyclins destined for destruction are ubiquitinated on their particular lysine residues by specific E3 ligases, which marks them for degradation in the proteasome machinery. This degradation process and rapid turnover is dependent on the presence of a destruction box motif in the case of cyclins A and B, or a PEST sequence when it comes to degradation of cyclins D and E[18,19].

### 1.1.3: Cell Cycle

As mentioned before, after completing mitosis and cytokinesis, the two daughter cells enter a new cycle, starting with the $G_1$ (Gap 1) phase of the cell cycle. During this phase, cells grow in size and duplicate their organelles while synthesizing the necessary proteins for DNA replication[20]. Terminally differentiated cells or actively proliferating ones that experience stress factors such as lack of nutrients or hypoxic conditions might exit the cell cycle and enter a quiescent state, commonly known as $G_0$ phase, where cell metabolism continues, but cell cycle progression and subsequent cell division are blocked[21]. During $G_0$, while cells continue to metabolize, they will not actively progress through the cell cycle. Another point of crucial decision making in $G_1$ phase is

the restriction point (R), also known as the point of no return. The name stems from the fact that once a cell passes through this point in its cycle, it becomes committed to continue advancing through the cell cycle, even in the absence of growth stimulatory signals[22]. Consistent with the pattern of cancer cells' uncontrolled growth, the R point is always bypassed in these cells to maintain a continuous progression through the cell cycle[23]. Direct association of CDK4 and CDK6 with cyclin D1, cyclin D2, and cyclin D3 are the driving forces behind $G_1$ phase[24]. To transition to the next phase of the cell cycle, S (synthesis), the formation of CDK2-cyclin E complex is required[25]. During this phase, cell's nuclear genome is replicated and errors in replication are corrected[26]. Progression through S phase requires the presence of CDK2-cyclin A complex[27,28]. $G_2$ phase separates S phase from mitosis (M) and it is the most variable phase of the cell cycle among different species. Cell cycle is halted at the G2/M checkpoint in the presence of DNA-damaging stress[29]. Finally, CDK1-cyclin A signals entry into M and a complex of CDK1 and cyclin B maintains progression through mitosis[30]. The various combinations of CDK-cyclin complexes during the cell cycle are summarized in Figure 1.

In contrast to cyclins, CDK inhibitors (CKIs) such as $p15^{INK4}$, $p16^{INK4}$, $p21^{WAF1/CIP1}$, and $p27^{KIP1}$ negatively regulate CDK activity by binding to CDK-cyclin complexes to halt the cell cycle when needed[11,31]. In addition to this internal regulatory network, cell cycle is also subject to extracellular stimuli, both positive and negative. Inhibitory signals can cause the cell cycle exit and entry into $G_0$ as mentioned before. Growth stimulatory signals such as epidermal growth factor (EGF) and subsequent activation of kinase activity of its receptor (EGFR) can force cells to exit $G_0$ and enter the cell cycle through upregulation of cyclin D1 for instance[32].

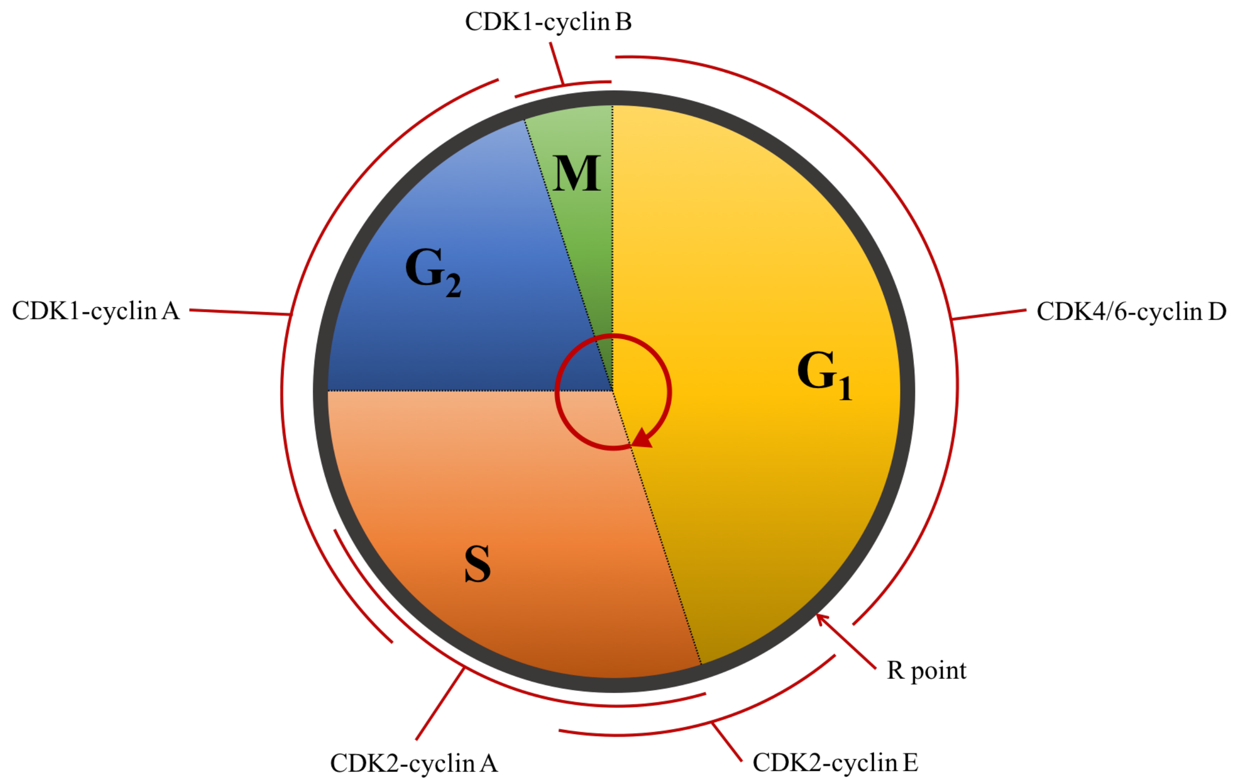



**Figure 1: The formation of CDK-cyclin complexes throughout the cell cycle.** To initiate and progress through each phase, formation of specific CDK-cyclin complexes is needed. Similarly, in order for the cell to move on to the next phases, previously present cyclin proteins must undergo degradation, which then leads to deactivation of their CDK partners. The length of each phase of the cell cycle is highly variable amongst cells and are ultimately determined by the cell type and the environment in which the cells find themselves. The circular arrow at the centre shows the direction of the cycle.

# 1.2: Ovarian Cancer

## 1.2.1: An Overview of Ovarian Cancer

At the core, cancer is a disease of the genes; the highly celebrated and tightly regulated orchestra of the collective gene expression that maintains the balance between life and death of the cell is now left with a mad conductor with total disregard for the latter. As we saw, a rigorous regulatory network ensures the proper growth of healthy cells through the actions of CDK-cyclin complexes as well as the complimentary effects of CKIs and extracellular stimuli. The deregulation of this network in cancer stems from a broad range of genes that undergo mutations, translocations, as well as epigenetic modifications[33,34], the net result of which is the loss or inactivity of tumour suppressor genes and unchecked activation of oncogenes, notably the ones that stimulate growth.

Ovarian cancer (OVCA) is the collective term given to many different types of cancerous lesions that arise and cause disease in the ovary. More than 30 types of ovarian malignancies have been identified so far, making ovarian cancer a highly heterogeneous disease. These malignancies can be grouped into three main categories: epithelial, germ, and stromal cell carcinomas[35]. Of the three, epithelial ovarian cancer (EOC) accounts for 90% of all cases of OVCA, and it also has the highest mortality rate[36]. Poor screening methods and lack of specific EOC biomarkers often lead to late diagnoses of the disease, which in turn contribute to its high mortality rate. In addition, the symptoms caused by EOC are non-specific and often similar to those of other gastrointestinal, genitourinary, and gynaecological conditions[37]. Finally, the high resistance of EOC cells to most available chemotherapeutic drugs makes the situation even more dire for the patients[38]. Overall ten-year survival rate of up to 90% when treatment starts at stage I, compared to <6% when the

disease is being treated at stage IV highlights the absolute importance and necessity for early detection of EOC[37]. Although only 1.3% of women will develop EOC in their lifetime, the aforementioned factors make this disease the most lethal of all gynaecological malignancies, and the fifth leading cause of cancer-related deaths in women[39,40].

The choice of treatment for EOC patients depends on the stage at which the cancer is diagnosed, and the correct choice is essential, considering the poor prognosis and high relapse rate of EOC[35]. The assignment of EOC stages is based on the extent to which cancer cells have spread through the body and away from the ovaries. The description of EOC stages and their corresponding ten-year survival rates, when diagnosed and treated timely, according to latest guidelines of International Federation of Gynecology and Obstetrics (FIGO) are summarized in Table 1[41].

**Table 1: Characteristics of tumours and the overall ten-year survival rates for different stages of EOC when detected and treated**

| Stage # | Tumour characteristics | Ten-year survival rate (%) |
|---|---|---|
| **I** | Tumour limited to ovaries or fallopian tube(s) | 73-90 |
| **II** | Tumour in one or both ovaries or fallopian tubes with extension into pelvic region (below pelvic brim) or primary peritoneal cancer | 45-55 |
| **III** | Tumour in one or both ovaries or fallopian tubes, or primary peritoneal cancer spread to outside of pelvic region and/or metastasis to retroperitoneal lymph nodes | 21 |
| **IV** | Distant metastasis beyond pelvis | <6 |

### 1.2.2: Tumourigenesis and Genetic Aberrations in Ovarian Cancer

With respect to ovarian cancer, a universal model for its pathogenesis and progression is not available, but attempts have been made to classify the resulting tumours based on clinical and molecular genetics studies[133]. The current model describes two main types of ovarian cancer based on the aforementioned classification criteria. Type I tumours consist mainly of cancerous lesions that find their way to the site of the ovaries and/or fallopian tubes and cause further transformation of cells at the site, as well as malignancies that arise from endometrioid, fallopian, and germ cells themselves. Most type I ovarian cancer tumours are considered low grade tumours. On the other hand, type II tumours are almost always the result of cancerous lesions in the fallopian tube(s) and histologically speaking, most of them fall in the category of high grade tumours[134].

As mentioned earlier, cancer is always associated with genetic aberrations, and the combinatorial effects of these aberrations can confer different characteristics to different tumours arising from the same tissue. In this regard, ovarian cancer can be calssified into two groups of low- and high-grade, or Type I and Type II, respectively. Low-grade serous ovarian cancer (LGSOC) is characterized by slow-growing tumours, and the process of tumourigenesis seems to follow a step-wise fashion. This class of tumour often shows less genomic instability, is less aggressive, and more responsive to treatment. However, due to lack of reliable biomarkers it often goes undetected and only shows clear symptoms at later stages. Cases of LGSOC showcase expression of amphiregulin (growth factor deregulation), loss of heterozygosity (LOH) on chromosome $X_q$, microsatellite instability, and mutations in *PIK3CA*, *BRAF*, and *KRAS*, and *ERBB2*, resulting in uncontrolled growth[37]. On the other hand, tumours of high-grade serous ovarian cancer (HGSOC) are more aggressive and grow fast, and respond poorly to available

treatments. Increased genomic instability is a feature of this type of cancer, which probably accounts for its more aggressive nature. HGSOC genetic aberrations are often characterized by mutations in *TP53*, *BRCA1* and *BRCA2*, and LOH on chromosomes $7_q$ and $9_p$, and an abundance of mitotic markers associated with rapid growth[42-44].

An important step in tumourigenesis of EOC and most other forms of carcinoma is epithelial-to-mesenchymal transition (EMT), which involves epithelial cells acquiring mesenchymal multipotent stem cells characteristics[45]. A direct consequence of this transition is that these cells no longer require cell-cell adhesion and their ability to migrate and invade other tissues are greatly enhanced[46]. It is important to note that during development[47] as well as wound healing in adults[48], EMT is frequently employed by the body, but the process is highly regulated. In development, neural crest cells give rise to a slew of cell types such as endocrine, neurons and glial, as well as melanocytes to name a few[49], and in wound healing, after the first round of EMT which carries the cells to the site of injury, they undergo mesenchymal-to-epithelial transition (MET) to then cover the wound as fresh and healthy epithelial cells. Healthy epithelial cells covering ovarian surface regularly undergo EMT for post-ovulatory wound healing as well[50]. However, the loss of EMT regulation can give rise to epithelial cells that can then migrate and invade other tissues, resulting in tumour growth[46]. In summary, cancer cells that have undergone EMT rely less on cell-cell adhesion and acquire more migratory and invasive characteristics like mesenchymal cells. In case of ovarian carcinoma, the cancerous cells disseminate into the peritoneal cavity and can spread to other sites through ascites. Once at a secondary site suitable for growth, the process of MET enables them to adhere to the cells at the site and to start proliferating again, eventually invading the new site and establishing colonies[128]. Multiple cellular

and molecular markers associated with EMT in EOC cells have been identified. A common feature in majority of carcinomas is the loss of cell-cell adhesion and an increase in their migration and invasion capabilities[34]. This is in part due to the loss of E-cadherin, which maintains epithelial cell structure by controlling their polarity[51,52]. Downregulation of E-cadherin at the genetic level is attributed to mutations, epigenetic modifications, and altered transcription factor activity, and post-translationally, E-cadherin is subject to increased degradation in cells undergoing EMT[46,53]. Transcription factors (TFs) that negatively regulate *CDH1* (E-cadherin) expression and are upregulated in EOC include Snail, Slug, and SIP1[45]. In addition, an increase in the levels of many growth factors has been shown to be upregulated, which also contribute to EMT. Transforming growth factor-β (TGF-β), epidermal growth factor (EGF), hepatocyte growth factor (HGF), endothelin-1 (ET-1), and bone morphogenetic protein 4 (BMP4) have all been found to drive EMT, although their contribution is not equal and depending on the type of genetic and transduction pathway alterations in each EOC case they show differential effects[46].

## 1.3: Cyclin G2

### 1.3.1: Discovery of G Type Cyclins

As with most of our subjective and imperfect attempts to classify nature in order to simplify its study for ourselves, and as more mammalian cyclins were discovered, we learned that not only all cyclins do not promote cell cycle progression, but there are those that have the exact opposite effect. Enter G type cyclins. The serendipitous discovery of G type cyclins in 1993 came about as Tamura and his colleagues set to screen a rat fibroblast cDNA library with a mixture of *c-src* kinase domains as probe[54]. A year later, it was shown that this new cyclin's mRNA was upregulated in response to gamma irradiation of cells as a transcriptional target of p53[55]. Finally, in 1996 two

distinct G type cyclins in murine and human cells were cloned: cyclin G1 and its homologue cyclin G2, encoded by *CCNG1* and *CCNG2*, respectively[6]. These two cyclins share 53% primary sequence and cyclin G2 and cyclin A, the closest relative of G type cyclins, are 26% similar in their amino acid sequence[6].

It soon became clear that cyclin G1 mRNA was constitutively active throughout the cell cycle, but that of cyclin G2 showed oscillating behaviour with peak expression at late S phase[6]. Of more interest is the negative effect of cyclin G2 on cell cycle progression, which will be discussed later. Together with cyclin I, they now constitute the unconventional G type cyclin group. All three are highly expressed in terminally differentiated tissues; cyclin G1 mRNA is abundant in cardiac and skeletal muscle cells and cyclin G2 and I are present at high levels in cerebellum[56]. Unlike cyclin G1 which is a transcriptional target of p53[55,57], cyclin G2 expression is p53-independent[6,10,15,58], but its negative effect on cell cycle progression is at least partially mediated through the activity of p53[59]. Despite the homologous nature of cyclin G1 and cyclin G2, their distinct transcriptional response to p53, differential regulation during development, and mRNA expression patterns during the cell cycle, it is safe to assume that these two proteins play different and non-overlapping roles at the cellular as well as organismal levels. An additional structural difference between cyclin G1 and cyclin G2 is the lack of a distinct destruction box or PEST sequence in cyclin G1, whereas cyclin G2 harbours a PEST motif at its C-terminal, making it 46 amino acids longer than cyclin G1[6]. The function and significance of PEST sequence are discussed later. Overexpression of cyclin G2 has been shown to exert a robust cell cycle halt in a myriad of cell types (Table 2), and consistent with this, cyclin G2 mRNA is significantly upregulated in

quiescent cells and its levels rapidly drop upon re-entry into the cell cycle[60], as well as apoptotic cells such as in negatively selected self-reactive T cells[15].

**Table 2: Cell lines in which overexpression of cyclin G2 halts the cell cycle**

| Cell line | Reference number |
| --- | --- |
| CHO | 56, 59 |
| GES-1 | 61 |
| HCT116 | 62 |
| HEK293 | 56 |
| HeLa | 63 |
| MCF7 | 64, 65 |
| Murine B cell lines | 15 |
| Murine splenic B cells | 66 |
| OV2008 | 67 |
| SCC15 | 68 |
| SGC-7901 | 69 |
| SKBr3 | 64 |
| U2OS | 59 |

### 1.3.2: Structural Features of Cyclin G2

Positioned at 4q21.1 in the human genome, *CCNG2* encodes a 344-amino acid long protein weighing ~39 kDa. Presented in Figure 2 are the structural features of cyclin G2 protein. The N-terminal half of cyclin G2 harbours its cyclin box stretching from residues 55-165 (Figure 2). Although formation of CDK-cyclin complexes relies on the presence of cyclin box[10], as of today no evidence for a cyclin G2-binding CDK is available, despite its overall similarity to cyclin A, which can form complexes with both CDK1 and CDK2. Even more interestingly, the few bona fide binding partners of cyclin G2, namely PP2A[56], PPARγ[70], and cortactin[71] bind this protein almost completely independent of the cyclin box residues, indicating the existence of novel functions of this domain that are yet to be identified.

In its C-terminal half, cyclin G2 contains a PEST domain, stretching from residues 281-334. The PEST hypothesis was introduced three decades ago[72], stating that most unstable proteins whose intracellular half-lives are ≤ 2 hours, harbour a stretch of amino acids rich in proline (P), glutamic acid (E), serine (S), and threonine (T), and that harbouring these residues in a small cluster is involved in rapid degradation and therefore short half-lives of those proteins. Consistent with this hypothesis, published data from our lab has demonstrated that cyclin G2 PEST domain does indeed contribute to the overall instability of cyclin G2 protein and its partial or full deletion, significantly protects cyclin G2 from degradation by the proteasome machinery[67]. PEST domain has also been proposed to be involved in calpain-mediated protein degradation as in the cases of IκBα[73] and ABCA1[74]. It is worth mentioning that no conserved sequence or motif has been proposed to indicate a bona fide PEST domain, but this is rather determined through calculating the PEST score for any stretch of amino acids[19,72].

In addition, cyclin G2 has two LxxLL motifs: 17-LLGLL-21 and 224-LEILL-228[70]. LxxLL is a short motif that facilitates many protein-protein interactions and is especially involved in modulating transcription through its presence in several transcription cofactors[75]. Finally, following the initial cloning of Cyclin G2 and interrogation of its primary sequence, it was shown to contain an 282-NxxY-285 motif similar to that of EGFR's auto-phosphorylation site capable of binding to Shc phosphotyrosine-binding domain[6]. However, as of today no evidence of direct phosphorylation of cyclin G2 on its Y285 has been presented.

### 1.3.3: Function and Binding Partners of Cyclin G2

Protein phosphatase 2A (PP2A) was the first bona fide binding partner of cyclin G2 to be identified[56]. PP2A is a versatile serine/threonine phosphatase[76], involved in a wide range of seemingly unrelated cellular functions such as cell cycle regulation, signal transduction, translational control, and endosome trafficking[77-81]. Active PP2A is a trimeric complex comprised of scaffolding, regulatory, and catalytic subunits designated as A, B, and C subunits, respectively[56]. The scaffolding and catalytic subunits each have two isoforms, and the regulatory subunit which controls PP2A substrate recognition and subcellular localization comes in more than sixteen different types, giving PP2A its broad involvement in cellular processes[56]. The minimal requirement of residues of cyclin G2 for direct association with PP2A are 142-241, and this complex formation seems to take place through replacing the scaffolding subunit of active PP2A since cyclin G2-PP2A complexes have been shown to contain both regulatory and catalytic subunits, but never the scaffolding[56]. In this respect, it is tempting to think of cyclin G2 not only as an alternative scaffolding subunit for the regulatory and catalytic ones to bind to, but possibly as having novel regulatory effects on the function(s) of PP2A. Overexpression of cyclin G2 and

its direct association with PP2A results in rapid accumulation of cyclin G2 at the centrosomes[59] as well as halting of the cell cycle, with a significant increase in the population of cells in G1, unable to proceed to S phase[56]. Ectopic expression of cyclin G2 also results in the formation of aberrant nuclei and an increased resistance of microtubules to degradation, which are indicative of of defective mitotic and/or cytokinetic processes[56,59]. Strikingly, overexpression of only the last 100 residues of cyclin G2's C-terminal is sufficient to halt the cell cycle[59], suggesting the presence of mechanisms other than direct binding to PP2A for halting of the cell cycle.

Cyclin G2 is also involved in adipogenesis through direct interaction with peroxisome proliferator-activated receptor γ (PPARγ)[70]. Cyclin G2-PPARγ complex drives the expression of adipose-specific genes such as *aP2*, *FATP-1*, and *LPL*[82,83]. Residues 223-344 of cyclin G2 are involved in this binding and of interest is that this binding happens in the same region where PPARγ also binds its ligand[70]. Even though in this scenario cyclin G2 can be thought of as a transcription cofactor, none of its LxxLL motifs are involved in binding to PPARγ and mutational analysis of those sites (LLGLL→LFAAL and LEILL→LFAAL) revealed no discernable effect on the downstream activation of PPARγ target genes[70]. A more detailed map of the residues involved and possibly new protein-protein interaction motifs remain to be elucidated.

A cohort of *in vivo* as well as *in vitro* studies recently performed in our lab have broaden our understanding of the anti-tumour effects of cyclin G2 in different EOC-derived tumour cells, shedding more light on the role(s) of cyclin G2 in tumourigenesis. The broad range of studies ranging from the use crude cell lysates to using animal models support the view that cyclin G2 is a negative regulator of cancer cells' growth and proliferation[15]. To list the highlights of these recent

findings, overexpression of cyclin G2 in these cells reduced their proliferation, migration, invasion, and spheroid formation, all of which are directly related to the aggressiveness of EOC tumours[84]. In animal models injected with EOC-derived cancer cells the tumour sizes were inversely proportional to the presence of ectopically expressed cyclin G2. Of more directly applicable results to therapeutic solutions was that it was shown cyclin G2 inhibits EMT in these cells and this was shown to be through significant reduction of Wnt/β-catenin signal transduction pathway at the cellular and molecular levels. As it was mentioned before, EMT is a crucial step towards cancer cells becoming more migratory and aggressive and losing their anchorage dependence and can freely migrate and invade near or far tissues in the body, a characteristic of stage III and IV of metastatic EOC cases[41]. Finally, in glioblastoma cells cyclin G2 directly interacts with cortactin and this association leads to phosphorylation of cortactin. Interestingly, cyclin G2 is a positive factor in expansion of glioblastomas, especially under hypoxic conditions, where cyclin G2 levels are increased[71].

Figure 2 demonstrates the regions of cyclin G2 that are involved in biding to its partners. It is worth noting that all known binding partners of cyclin G2 associate with the protein through the regions present in the C-terminal half of the protein, and the roles of cyclin G2 N-terminal half remains open for further investigation.

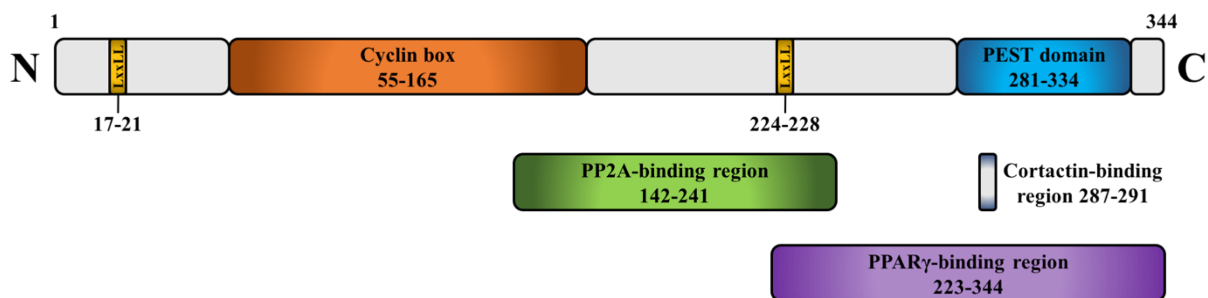



**Figure 2: Schematic representation of cyclin G2 protein, its verified domains, and binding regions to its partners.** The C-terminal half of cyclin G2 corresponds to the binding regions of its various partners, through which it exerts its various effects in the cell. The C-terminal PEST domain (blue) of cyclin G2 has been shown to be involved in its degradation via proteasome machinery as well as proteolytic activity of calpains.

### 1.3.4: Regulation of Cyclin G2

The big picture of cyclin G2 regulation at transcriptional as well as translational levels is painted by downregulation in response to growth stimulatory factors and an upregulation when growth and proliferation are impeded, consistent with high levels of cyclin G2 mRNA in quiescent cells and its power to halt cell cycle progression (Table 1). *CCNG2* promoter has binding sites for FOXO3a, a member of the forkhead box (FOX) transcription factors (TFs), which positively regulates the expression of cyclin G2[60,66,85]. Phosphoinositide 3-kinase-Akt (PI3K-Akt) pathway can phosphorylate FOXO3a, leading to its nuclear exclusion and inactivity as a TF, and therefore PI3K-Akt activity is at least partially involved in downregulation of cyclin G2 expression[60,64]. In fact, inhibition of PI3K results in dephosphorylation of FOXO3a, which is then able to translocate to the nucleus to drive the expression of cyclin G2[85]. Within the same line of reasoning, findings from our lab showed that binding of Nodal, a member of the TGF-β superfamily, to its receptor (ALK7) slows the proliferation of ovarian cancer cells[86] and further investigation provided evidence that *CCNG2* was amongst the genes that were upregulated as a consequence of this interaction[85]; activation of ALK7 results in direct interaction between Smad proteins and FOXO3a and this complex induces transcription of *CCNG2* by binding to its promoter. Activation of canonical Smad pathway downstream of ALK7 activation[87], and their ability to inhibit PKB activity fits nicely into the big picture of cyclin G2 regulation in the context of PI3K-Akt-FOXO3a regulatory network.

Following the trend of cyclin G2 downregulation in response to growth stimulatory signals, overexpression of the receptor HER2 in breast cancer cells resulted in a significant downregulation of *CCNG2* mRNA and antibody-mediated blocking of HER2 led to a rapid accumulation of cyclin

G2 in the nucleus[64]. Genotoxic stresses which lead to DNA breaks lead to rapid elevation of cyclin G2 levels followed by a G1/S checkpoint activation. Under these conditions, cyclin G2 is recruited to the centrosomes where it degrades CDK2 in a p53-dependent manner. DNA damage and cyclin G2 upregulation also lead to phosphorylation and activation of Chk2, which then halts the cell cycle[62].

Cyclin G2 is also negatively regulated in response to a variety of growth stimulatory factors. For instance, estrogen-occupied estrogen receptor (ER) can form a complex with Sp1, which can then directly bind to cyclin G2 promoter and prevent its transcription by removing RNA polymerase II[88]. Similarly, insulin and its analogue, X10, as well as insulin-like growth factor 1 (IGF-1) also downregulate cyclin G2's both mRNA and protein levels[89]. In our lab we have demonstrated that stimulating cells with EGF leads to rapid degradation of cyclin G2 protein, and therefore have provided evidence for regulatory effects of EGFR signalling on cyclin G2. The details of this process remain to be identified and provide novel and exciting research opportunities. As mentioned before, cyclin G2 is also subject to degradation by the proteasome machinery and the presence of its full length C-terminal PEST domain is inversely proportional to the stability of the protein, indicating that cyclin G2 must be under tight regulation and go through rapid turnover in cells[67]. Our lab has also shown that cyclin G2 is a target of calpain-mediated proteolysis in different cell lines, offering new insight into cyclin G2 regulation at protein level.

**1.3.5: Cyclin G2 and Cancer**

Considering the bigger picture of cyclin G2 regulation and the type of cellular functions it is involved in, it should not come as a surprise that *CCNG2* is significantly downregulated in a

variety of malignancies including ovarian[85], breast[90,91], oral[68], gastric[92], thyroid[93], esophageal[94], prostate[95], kidney[96] and colorectal[97] cancers. To add more context to this, the levels of cyclin G2 are inversely proportional to the stage of the primary tumours and poorly differentiated carcinomas show lower levels of cyclin G2 compared to well-differentiated samples[98]. Published work from our lab has provided evidence for significantly lower levels of *CCNG2* expression in EOC cells compared to normal ovarian surface epithelium[84]. In addition, cyclin G2 expression is upregulated in response to anti-tumour agents, which cause cell cycle arrest, consistent with the previous findings on cyclin G2 effect on cell cycle, growth, and proliferation (Table 2). Similarly, cyclin G2 has recently been shown to inhibit EMT processes in ovarian cancer-derived cell lines by attenuating Wnt/β-catenin signalling[84]. Taken together, it is not surprising that two recent studies have suggested that levels of *CCNG2* can be used as a prognostic tool in breast[129] and pancreatic cancer[98]. It seems quite feasible that more data from primary tumour samples will enable us to use *CCNG2* as a more useful prognostic tool in various types of malignancies.

## 1.4: Calpains

Protein turnover is the collective term given to the processes that ensure the balance between the levels of protein synthesis and degradation which cells employ to maintain homeostasis. In contrast to the instructions for protein synthesis, which are universally carried out by the ribosomal machinery, evolution has equipped cells with different mechanisms to degrade proteins, such as ubiquitin-protease pathway (UPP) as well as a very large family of protein-degrading enzymes known as proteases or peptidases[99].

The superfamily of calpains (calcium-dependent papain-like enzymes) consists of cytoplasmic cysteine proteases whose activation and function are dependent on the presence of and binding to calcium ions[100,101]. In humans, there are at least 15 genes encoding different calpains[102], almost half of which are classified as ubiquitous (calpains 1, 2, 5, 7, 10, 13, and 15), and the rest are expressed in a more tissue-specific manner (Calpain 3, 6, 8, 9, 11, and 12)[101]. Although specific physiological functions of calpains are not yet very well-defined, the variety of diseases where calpain deregulation is documented is a clear indication of the diverse roles calpains play in maintaining human health. Alzheimer's and other neurodegenerative diseases[103], cataract[101], limb-girdle muscular dystrophy type 2A[104], type 2 diabetes[105], and metastasis in cancer[106] are among those conditions where calpain malfunction contributes to the disease state. Similar to their wide-yet-not-fully-understood physiological roles, at the cellular level too, calpains are involved in a myriad of processes such as signal transduction, cell cycle progression and proliferation, apoptosis, differentiation, membrane fusion, platelet activation, and of course, calcium-regulated processes[102,107-110]. Interestingly, while most proteases recognize and cut consensus primary sequences within their target proteins, calpain substrate recognition is more

structurally dependent[101,111], even though the primary sequence of their targets are still important[111]. In this way, calpains have been shown to also recognize and cut the bonds between domains[101] . A consequence of this particular mechanism of proteolysis is that the calpain targets are not always fully digested, but instead the resulting fragment(s) can still be biologically active. One well-studied case of this type of proteolysis is the digestion of protein kinase C by calpain, which results in an active kinase domain whose function is no longer dependent on effector molecules or calcium[101,112]. In this regard, calpains are called bio-modulators because they can exert this kind of regulatory effect on their substrates[101].

The two conventional and ubiquitous calpain 1 and calpain 2, also known as μ-calpain and m-calpain, respectively are the better-studied members of the calpain superfamily. The 'm' and 'μ' refer to the [$Ca^{2+}$] requirements of the two calpains *in vitro*: micromolar and macromolar, respectively[113]. Both these calpains are heterodimers comprised of a large ~80 kDa catalytic subunit and a regulatory ~30 kDa subunit[101]. The catalytic subunit consists of domains I, IIa and IIb, III, and IV, and the regulatory subunit contains V and VI domains. Domains IV and VI facilitate the interaction between the two subunits. In the absence of $Ca^{2+}$ binding, calpain is inactive because of the steric hindrances between IIa and IIb domains. Following conformational changes upon binding to calcium ions, the two domains interact and form the active catalytic site of calpain[101]. Figure 3 shows the ways through which intracellular calcium concentration increases, which in turn lead to activation of calpains. While different genes encode the catalytic subunits of calpain 1 and calpain 2 (*CAPN1* and *CAPN2*, respectively), the regulatory subunit is encoded by the same gene (*CAPN4*) and is shared in joining μ-calpain and m-calpain catalytic subunits[114]. ERK, one of the downstream effectors of EGFR signal transduction pathway has also

been shown to activate m-calpain through phosphorylation[115]. Endogenously, calpain inhibition is carried out by the cytosolic calpain inhibitor, calpastatin[116]. There is also evidence for inhibition of m-calpain activity upon becoming phosphorylated by protein kinase A[117]. Various commercial short peptide as well as nonpeptidic calpain inhibitors are available, but their specificity is not very high[101,107,118].

Preliminary data from our lab suggest that cyclin G2 is a target for calpain 1 and calpain 2 *in vitro* in different human ovarian cancer cells. In addition, we also have provided evidence that the PEST domain of cyclin G2 is not only involved in its degradation via the ubiquitin-proteasome pathways (UPP), but that the same trend is observed with respect to degradation by calpain as well; truncated or fully deleted PEST domain increases stability of cyclin G2 compared to when the full-length PEST domain is present. Overexpression of HER2 in breast cancer cells leads to degradation of IκBα in a calpain-dependent manner[73]. IκBα is similar to cyclin G2 in size and it too harbours a C-terminal PEST domain, and so interesting parallels can be drawn from the studies of IκBα degradation.

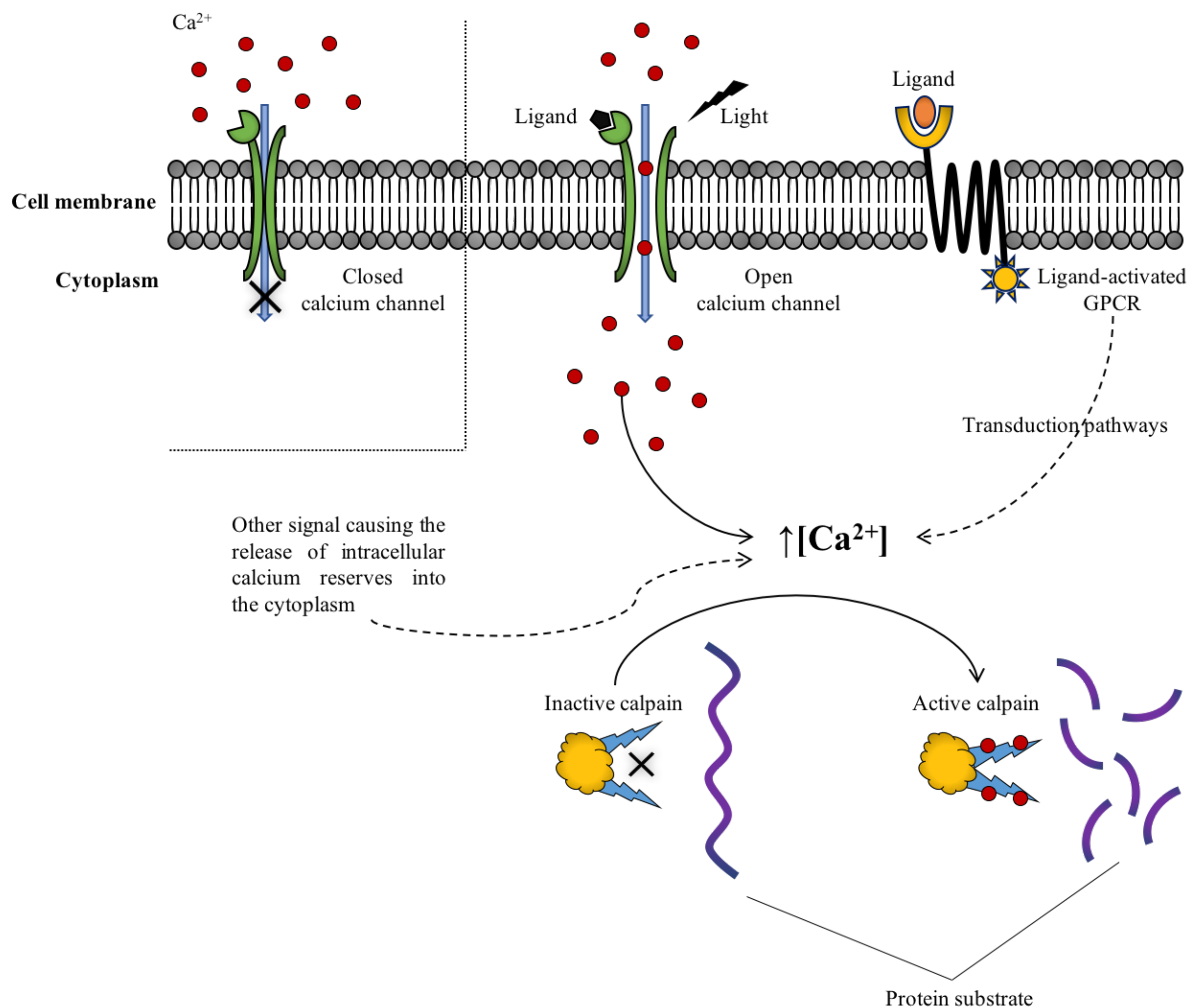



**Figure 3: Activation of calpain as a consequence of increasing intracellular calcium concentration.** Intracellular [$Ca^{2+}$] increases in response to ligand- or light-activated calcium channels (green). Alternatively, G-protein coupled receptor (GPCR) activation as well as other signals that promote the release of intracellular reserves of calcium also cause an increase in intracellular [$Ca^{2+}$]. Upon binding to calcium ions, calpain goes through conformational changes, exposing its catalytic domain for recognition and proteolysis of its protein substrates. Structural features of this activation process are discussed in the text.

## 1.5: Epidermal Growth Factor Signalling

Epidermal growth factor (EGF) is a short protein which upon binding to its receptor, epidermal growth factor receptor (EGFR/ErbB1) induces one of the most diverse signal transduction pathways ever discovered and studied[119]. The formation of ligand-receptor complex induces conformational changes in EGFR and brings two inactive subunits together to form dimers followed by trans-autophosphorylation of the two subunits on their specific tyrosine residues, forming docking sites and activating signals for a myriad of downstream effectors[120]. EGFR can also be activated through binding to its other ligand, transforming growth factor-α (TGFα)[121]. The diversity of the downstream pathways stemming from EGFR activation is also the result of the fact that aside from forming homodimers, EGFR can heterodimerize with its three homologues, ErbB2 (HER2), ErbB3 (HER3), and ErbB4 (HER4) and the type of ligand bound to these receptors determines the resulting heterodimers and the downstream pathways they activate[120]. ErbB2 has no known ligand, but its association with other members of the ErbB family and its subsequent kinase activity has been well established[122]. EGF is a mitogen whose signal is translated to an leveling up of the processes such as proliferation, differentiation, and survival as well as an increase in protein synthesis[123,124]. Overall, EGFR signalling is associated with cell survival and enhanced proliferation. Given the nature of these signals and the different ways ErbB family members can form active dimers, it comes as no surprise that dysregulated ErbB signalling is prevalent in many types of cancer[120]. The enhanced activity of ErbB family can be the result of gain-of-function mutations as well as receptor and/or ligand overexpression in a myriad of cell and cancer types[124,130].

In our lab we have shown that stimulating ovarian cancer cells with EGF leads to rapid degradation of cyclin G2 protein, consistent with our overall knowledge of the negative effects of cyclin G2 on cell cycle and tumour formation. We have additionally provided evidence that inhibition of EGFR by Tyrphostin AG 1478 protects cyclin G2 from degradation in the presence or absence of EGF. Taking into consideration that EGFR signalling can activate both calpain activity as well as PI3K-Akt signalling pathway, we show new connections between EGF signalling and cyclin G2 regulation both at transcriptional and translational levels.

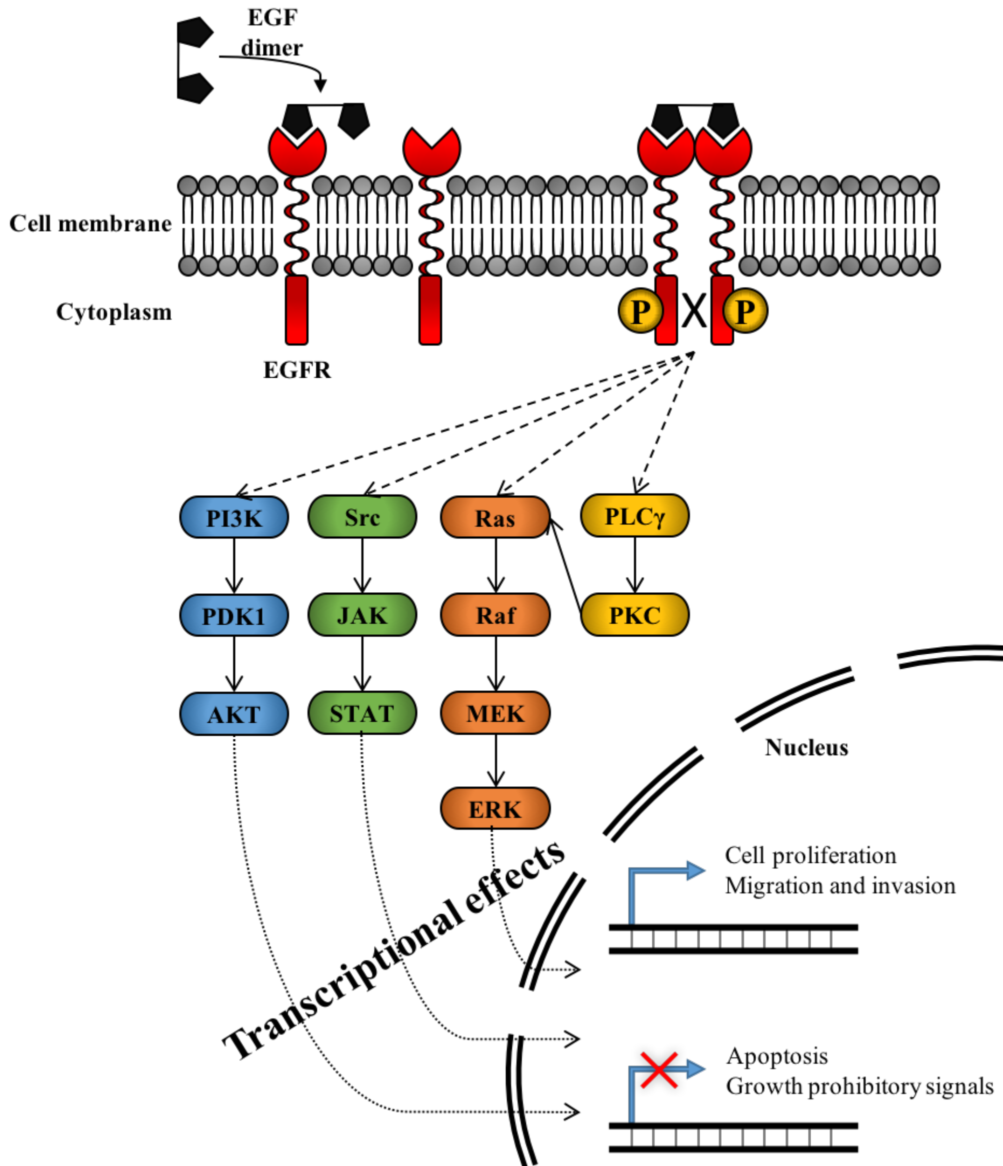



**Figure 4: Simplified EGFR signalling pathways.** EGFR (red) undergoes conformational changes upon binding to EGF (black), which facilitate the formation of EGFR homodimers. The cytoplasmic domains of each monomer transphosphorylates the other on a number of tyrosine residues. The phosphorylated tyrosine residues serve as docking sites for various proteins, which then relay the signal through four main pathways: PI3K/AKT (blue), STAT (green), MAPK/ERK (orange), and PLCγ/PKC (yellow). The genes activated downstream of EGFR promote cellular proliferation as well as migration and invasion capabilities of the cell while suppressing apoptosis and other growth inhibitory signals. For simplicity, only the better-known proteins from each pathway have been selected to be shown.

## 1.6: Rationale, Hypothesis, and Objectives of Present Study

Our lab has provided evidence that the interaction of Nodal with its receptor, ALK7, results in the halting of human ovarian cancer cell proliferation[86]. Further studies identified *CCNG2* (cyclin G2) was amongst the upregulated genes as the result of Nodal-ALK7 interaction and it was also partially responsible for exerting the aformentioned antiproliferative effect on those cells[85]. Unlike canonical cyclins which promote cell cycle progression and cellular proliferation[15], ectopic expression of cyclin G2 can result in halting of the cell cycle (references listed in Table 2). More recently, our lab showed that cyclin G2 has anti-tumour effects *in vivo* and that its expression levels are significantly lower in human epithelial ovarian canrcinoma[84]. Other lines of evidence have shown significant downregulation of cyclin G2 in a variety of other cancers as well[98]. Taken together, it is suggestive that downregulation or loss of cyclin G2 might be one of the contributing factors to the development of EOC. Given the severity of EOC and poor prognosis of patients, and the recent discoveries demonstrating the role of *CCNG2* in the development of ovarian cancer, it is important to investigate the mechanisms which govern degradation of cyclin G2 in these cells.

Preliminary data from our lab showed that in human ovarian cancer cells, cyclin G2 is quickly degraded, and this rapid turnover is due to the presence of a PEST sequence in the C-terminal end of cyclin G2. PEST sequences serve as regulatory motifs in proteins destined for degradation by both the proteasome pathway[131] as well as calpain-mediated proteolysis[73]. Our lab has demonstrated that cyclin G2 is indeed degraded through the UPP. Additional data found in our lab suggested that degradation of cyclin G2 was connected to intracellular calcium levels as well as the presence of calcium in crude cell lysate. Taken together, this suggested a role for calpain in meditating the degradation of cyclin G2. Additional data showed a role for phosphorylation as a

necessary step in cyclin G2 degradation and results from other work in our lab pointed us to the involvement of EGFR signalling in destruction of cyclin G2 protein.

Considering our findings about cyclin G2 in human ovarian cancer cells, we hypothesize that i) cyclin G2 can be degraded by calpain in these cells, and that ii) EGFR signalling is at least partially involved in the degradation of cyclin G2. The focus and purpose of the present study were to unravel more details that govern the rapid turnover and degradation of cyclin G2 in human ovarian cancer cells. The overall objective of this project was to show the involvement of calpain in the degradation of cyclin G2 and the role of growth stimulatory pathways on the fate of the protein. More specifically, we investigated i) degradation of cyclin G2 *in vitro* by calpain and ii) the involvement of EGFR signalling in cyclin G2 turnover, a novel aspect of cyclin G2 regulation.

# Chapter 2: Materials and Methods

## 2.1: Cell Lines and Cell Culture

All original and modified cell lines were incubated at 37°C in humidified atmosphere containing 5% $CO_2$. Unless otherwise stated, all media were supplemented with 10% heat-inactivated fetal bovine serum (incubated at 56°C water bath for 30 minutes) and 1% Penicillin-Streptomycin (P/S) cocktail for cell passage or initial seeding for downstream treatments. Cells were passaged when reached 80-90% confluency and were seeded at $3x10^5$ cells/well of 6-well plates or $1x10^6$ cell/10-cm plates. For stably transfected cells with pBABE-puro empty vector or the vector containing *CCNG2* cDNA, 10 µg/ml puromycin was added to the media to select for cells retaining the exogenous DNA constructs. Table 3 lists the cell lines and their tissue origins as well as the media type and supplements used for this project. Cells stably expressing the empty vector (EV) or FLAG-*CCNG2* construct were generated by Dr. Stephanie Bernaudo in our lab[84].

**Table 3: Original and modified cell lines, tissue origins, and respective media and supplements**

| Cell Line | Tissue Origin | Media + Supplements |
|---|---|---|
| **OV2008** | Human cervical carcinoma (ATCC)[a] | RPMI-1640[b] + 10% FBS[c] + 1% P/S[d] |
| **OV2008-EV** | OV2008 cells stably transfected with empty pBABE-Puro vector | RPMI-1640 + 10% FBS + 1% P/S + 10 µg/ml puromycin[e] |
| **OV2008-CG2** | OV2008 cells stably transfected with pBABE-Puro harbouring 3XFLAG-*CCNG2* cDNA | RPMI-1640 + 10% FBS + 1% P/S + 10 µg/ml puromycin |
| **SKOV3** | Human ovarian carcinoma (ATCC) | DMEM[f] + 10% FBS + 1% P/S |
| **SKOV3.ip1[g]** | SKOV3 cells intraperitoneally injected into mice and recovered afterwards | DMEM + 10% FBS + 1% P/S |
| **SKOV3.ip1-EV** | SKOV3.ip1 cells stably transfected with empty pBABE-Puro vector | DMEM/McCoy[h] + 10% FBS + 1% P/S + 10 µg/ml puromycin |
| **SKOV3.ip1-CG2** | SKOV3.ip1 cells stably transfected with pBABE-Puro harbouring 3XFLAG-*CCNG2* cDNA | DMEM/McCoy + 10% FBS + 1% P/S + 10 µg/ml puromycin |
| **ES2** | Human ovarian carcinoma (ATCC) | McCoy's 5A + 10% FBS + 1% P/S |
| **ES2-EV** | ES2 cells stably transfected with empty pBABE-Puro vector | McCoy's 5A + 10% FBS + 1% P/S + 10 µg/ml puromycin |
| **ES2-CG2** | ES2 cells stably transfected with pBABE-Puro harbouring 3XFLAG-*CCNG2* cDNA | McCoy's 5A + 10% FBS + 1% P/S + 10 µg/ml puromycin |
| **HEY[i]** | Human ovarian carcinoma | DMEM + 10% FBS + 1% P/S |

[a] American Type Culture Collection (Manassas, VA, USA), [b]RPMI-1640: Roswell Park Memorial Institute medium-1640 (GE HyClone, Logan, UT, USA, SH30027.02), [c]FBS: fetal bovine serum (Life Technologies, Burlington, ON, Canda, 12483-020), [d]P/S: Penicillin-Streptomycin (Multicell, 450-201-EL), [e]Puromycin (InvivoGen, ant-pr), [f]DMEM: Dulbecco's Modified Eagle Medium (GE HyClone, Logan, UT, USA, SH30243.01), [g]SKOV3.ip1 cells were obtained from Dr. Mien-Chie Hung (MD Anderson Cancer Centre, Houston, TX, USA), [h]McCoy's 5A (Sigma-Aldrich, Oakville, ON, Canada, M4892), [i]HEY cells were obtained from Dr. Ted Brown (Mount Sinai Hospital, Toronto, ON, Canada).

## 2.2: Transient Transfection

Plasmid DNA for transient transfections was purified from 100-150 ml overnight cultures of *E. coli* DH5α grown in LB media with 100 µg/ml ampicillin, using PerfectPrep Endofree Maxi Kit (5 Prime, 2300120). The concentration and purity of each preparation was measured using a NanoDrop2000c instrument (Thermo Scientific). Only samples with A260/A280 ratio of ≥1.8 were used for subsequent transient transfections. Cells of 70-90% confluency were transiently transfected overnight (16-18 h) with 3µg of DNA per well of 6-well plates and 7 µg of DNA per 10 cm plates, using Lipofectamine 2000 (Invitrogen, 11668019) following the manufacturer's instructions with the following deviation: 1/5 of the total volume of the media added to cells consisted of the mixture of plasmid DNA and Lipofectamine 2000 in OMEM (Opti-MEM®, Invitrogen, 31985070) and the rest was comprised of media in which the cells were originally cultured in, supplemented with 1% P/S and no serum. After overnight transfection, the transfection mixture was first removed, followed by the appropriate treatment. Per case treatments are described in the Results section.

## 2.3: Cell Lysate Preparation and Western Blotting

Cells were washed with room temperature 1X PBS pH 7.4, and collected and lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS) supplemented with protease inhibitor cocktail (Thermo Scientific, 88266). Where applicable, total protein concentration of cell lysate was measured using Pierce® BCA Protein Assay (Thermo Scientific, 88266). The cell lysate was then mixed with 5X SDS sample buffer (0.25 M Tris-HCl pH 6.8, 0.25% bromophenol blue, 0.5 M DTT, 50% glycerol, 10% SDS) and boiled for 5-10 minutes. Protein ladder and equal amounts of

protein samples were then loaded onto the wells of 12% SDS-polyacrylamide gels (recipe in Table 4) placed in 1X Tris-Glycine running buffer (0.3% Tris, 1.44% glycine, 1% SDS) and proteins were separated by electrophoresis at room temperature (at 100V until samples reached the separating gel, followed by 150V until desired separation was achieved, judging by the ladder markers). The contents of each gel were electrotransferred at 100V for 60 min on ice onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, 162-0177) in transfer buffer (10% Tris-glycine running buffer, 20% methanol). The membranes were then blocked using 5% skim milk (no name®, 20052038_EA) dissolved in TBS while gently rocking at room temperature for 1 hour. Each membrane was then incubated overnight at 4°C with the appropriate primary antibody prepared in either 5% skim milk or BSA (BioShop, ALB001.100). The following day, each membrane was washed 3 time for 10 minutes with TBS-T, followed by incubation with HRP-conjugated anti-mouse or anti-rabbit IgG at room temperature. The membranes were washed again 3 times for 10 minutes with TBS-T and then incubated with HRP substrate (Millipore, EBLUC0500) for 3-4 minutes. Antigen-antibody complexes were visualized using an ECL detection system and photo-sensitive film developed in a Konica Minolta SRX-101A system (Konica Minolta Medical Imaging) or using a MicroChemi scanner and GelCapture Chemi analysis software (DNR Bio-Imaging Systems Ltd.). Where necessary, the band signals were quantified using Image Studio Lite 3.1 (LI-COR Inc.).

**Table 4: SDS-polyacrylamide stacking and resolving gels recipes**

| Gel layer | $dH_20$ (ml) | 30% Acrylamide (ml) | 1.5 M Tris, pH 8.6 (ml) | 1.0 M Tris, pH 6.8 (µl) | 10% SDS (µl) | 10% APS (µl) | TEMED (µl) |
|---|---|---|---|---|---|---|---|
| **Resolving** | 3.3 | 4 | 2.5 | – | 100 | 50 | 10 |
| **Stacking** | 1.9 | 0.320 | – | 315 | 25 | 10 | 3 |

**Table 5: Antibodies used for western blot analysis**

| Antibody | Company | Species | Dilution |
|---|---|---|---|
| **FLAG** | Sigma | Mouse | 1:1000 |
| **GAPDH** | Santa Cruz | Mouse | 1:10000 |

### 2.4: *In Vitro* Calcium-Induced Degradation Assay

SKOV3.ip1 and ES2 cells stably transfected with FLAG-*CCNG2* construct were washed with room temperature PBS and lysed under non-reducing and non-denaturing conditions in RIPA buffer containing 0, 0.5, or 5 mM $CaCl_2$ and incubated for 60 and 120 minutes at room temperature (~22°C) or at 37°C. Aliquots of each reaction were mixed with 5X SDS sample buffer and immunoblotted with anti-FLAG antibody to detect cyclin G2 levels.

### 2.5: *In Vitro* Calpain-Induced Degradation Assay

*Studying the effects of calpain on cyclin G2 degradation*: SKOV3.ip1, ES2, and HEY cells were transiently transfected with FLAG-*CCNG2* construct overnight as described before. The next day cells were washed with room temperature PBS and lysed under non-reducing and non-denaturing conditions in calpain degradation buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM DTT, 0.5% Triton-X). Cell lysate total protein concentration was measured using Pierce® BCA Protein Assay (Thermo Scientific, 88266). From each cell lysate, five aliquots each containing 480 μg total protein were taken and incubated with 0 or 500 μM $CaCl_2$, and in the presence or absence of 12 units of calpain-1 (porcine erythrocytes, Calbiochem, 208712) as well as a calpain inhibitor, calpeptin (Calbiochem, 03-34-0051) and incubated in a 30°C water bath for 1 hour. Reactions were stopped by adding 5X SDS sample buffer and subsequently subjected to western blot analysis with anti-FLAG antibody to monitor the levels of cyclin G2.

*Investigating the effects of EGFR inhibition on calpain-mediated degradation of cyclin G2*: SKOV3.ip1, ES2, and HEY cells were transiently transfected with FLAG-*CCNG2* construct overnight as described before. The following day transfection mixture was removed and cells were

treated with 10 μM of an EGFR inhibotr, Tyrphostin AG 1478 (Sigma-Aldrich, T4182) or DMSO vehicle in serum-free media for 30 min. Cells were washed with room temperature PBS and lysed under non-reducing and non-denaturing conditions in calpain degradation buffer. Total protein concentration of each cell lysate was determined using Pierce® BCA Protein Assay. Aliquots from cell lysates each containing 480 μg total protein were mixed with 1 μM Tyrphostin AG 1478 or 1:1000 DMSO (Sigma, D4540), in the presence or absence of calpain-1 and incubated in a 30°C water bath for 1 hour. Reactions were stopped by addition of 5X SDS sample buffer and western blot analysis was carried out using anti-FLAG antibody to observe the levels of cyclin G2.

### 2.6: Kinase Inhibitor Library Assay

SCREEN-WELL® kinase inhibitor library (Enzo Life sciences, BML-2832-0500) and EGFR kinase inhibitor, Tyrphostin AG 1478 (Sigma, T4182) stocks were prepared in DMSO at 10 mM. The stocks were diluted to working concentrations in the appropriate cell media containing antibiotics in the absence of serum. Cells were incubated with the appropriate kinase inhibitor or DMSO control plus 10 μg/ml cycloheximide (Sigma) to block protein *de novo* synthesis for 30-120 minutes. 10 μM MG-132 (Selleckchem, S2619), a proteasome inhibitor was included as positive control when cyclin G2 was protected from degradation. Table 4 lists the kinase inhibitors used in these assays.

**Table 6: Kinase inhibitors, their targets, and working concentrations (all stocks 10 mM)**

| Inhibitor Name/ID | Target | S/T/Y* Kinase | Concentration Used (µM) |
|---|---|---|---|
| **Tyrphostin AG 1478** | EGFR | Y | 10 |
| **Wortmannin** | PI3K | – | 1 |
| **PP2** | Src | Y | 10 |
| **BML-257** | Akt | S/T | 10 |
| **Rapamycin** | mTOR | S/T | 0.01 |
| **GW5074** | cRAF | S/T | 1 |
| **Y-27632** | ROCK | S/T | 10 |
| **ZM449829** | JAK3 | Y | 1 |
| **5-Iodotubercidin** | CK1 | S/T | 10 |
| **Apigenin** | CK2 | S/T | 10 |
| **5-Iodotubercidin** | ERK | S/T | 10 |
| **Hypericin** | PKC | S/T | 10 |
| **Piceatannol** | Syk | Y | 10 |
| **SU-4312** | Flk1 | Y | 10 |
| **SP-600125** | JNK | S/T | 50 |
| **PD-98059** | MEK | S/T | 50 |
| **SB-203580** | p38 | S/T | 10 |

*S: serine, T: threonine, Y: tyrosine

### 2.7: Epidermal Growth Factor Treatments

Lyophilized recombinant human EGF (Invitrogen, PHG0311) was reconstituted in sterile PBS pH 7.4 to a final concentration of 10 mg/ml as the stock. Unless indicated, cells were serum-starved for ~18 hours overnight, followed by a 30-minute treatment with 10 µg/ml cycloheximide (CHX) and subsequent addition of media with antibiotics in the absence of serum containing 20 ng/ml EGF to each well or plate for 1-2 hours. Treatment with 10 µM MG-132 was included as positive control for cyclin G2 protection.

### 2.8: Statistical Analyses

Results are expressed as mean ±SEM. One-way analysis of variance (ANOVA) was used to determine the difference between multiple groups in each experiment and Tukey's post-hoc analysis was utilized to decide the statistical difference between the specific groups. GraphPad Prism 6.0 software was used to conduct statistical testing and significance was defined as $p<0.05$.

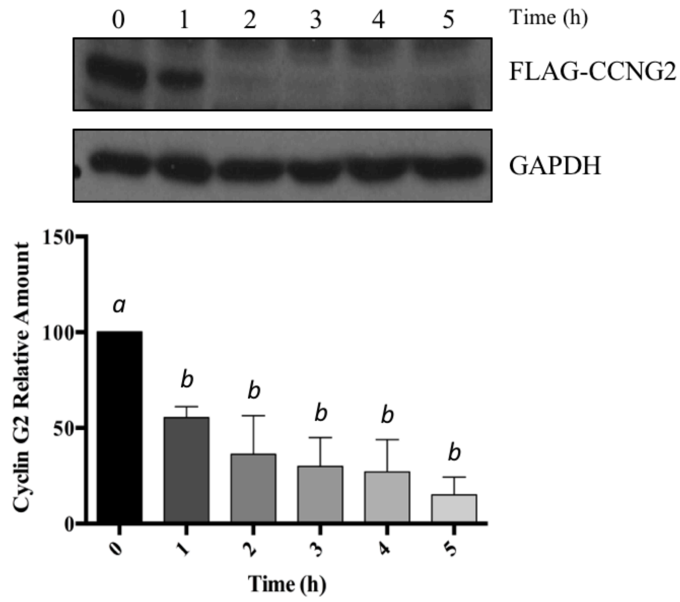
# Chapter 3: Results

### 3.1: Cyclin G2 is an Unstable Protein with a Short Half-Life

A few years ago, our lab demonstrated the unstable nature of cyclin G2 protein in OV2008 cells[67]. However, through profiling a number of cell lines, a more recent study showed that OV2008 are actually of cervical cancer origin as opposed to EOC[132]. Therefore, we started to use other EOC cell lines to study the stability of cyclin G2 in those cells. Where possible, OV2008 cells were used as a positive control.

In one series of experiments, OV2008, SKOV3.ip1, and ES2 cells stably transfected with a FLAG-*CCNG2* construct (stable cells) were used to monitor the stability of cyclin G2 protein. After overnight serum starvation and when *de novo* protein synthesis was blocked by addition of cycloheximide (CHX), a significant reduction in the levels of protein were observed at around 1 hour compared to the start of the experiment (Figure 5A, 5B, 5C). We also tested the stability of cyclin G2 in ES2 and HEY cells through transient transfection. To do this, ES2 and HEY cells were transiently transfected with a FLAG-*CCNG2* construct overnight, with a subsequent treatment with 10 μg/ml CHX in serum-free media. Sample collection and analysis were performed exactly the same as in the stable cells (Figure 5D, 5E). In both stable and transiently transfected cells Western blot analysis and subsequent quantification of the bands demonstrated a rapid decline in the levels of cyclin G2 as soon as 1 hour, with the most and least degradation observed in ES2 and SKOV3.ip1 cells, respectively.

**A**

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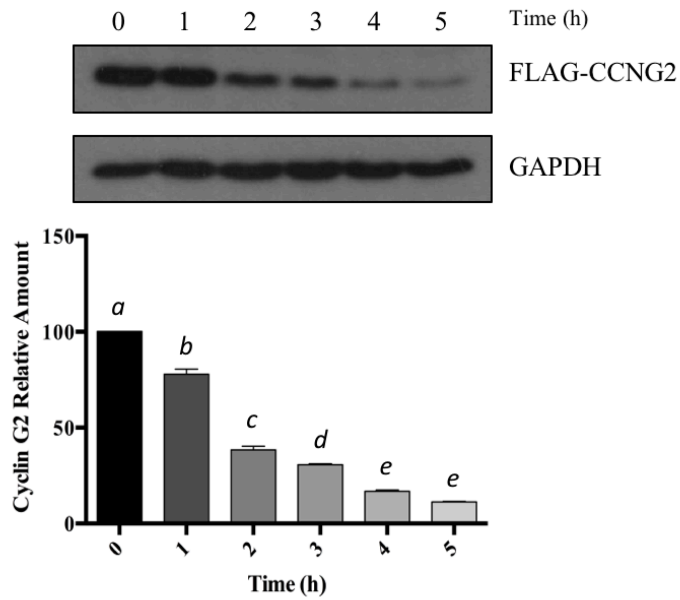


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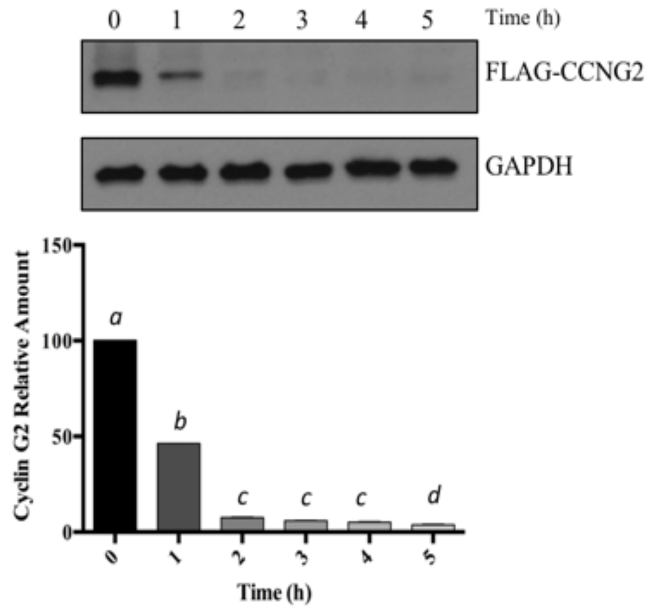


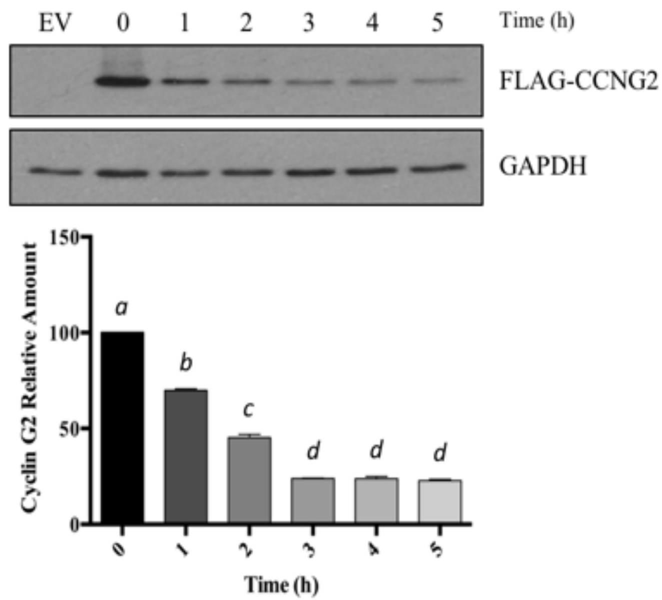


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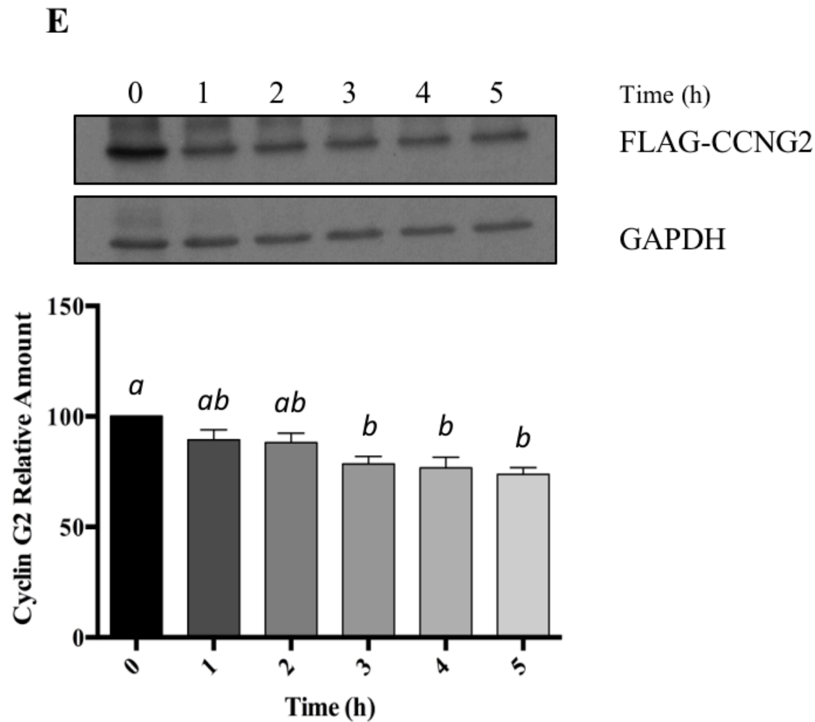



**Figure 5: Cyclin G2 is an unstable protein and is degraded rapidly in human ovarian cancer cell lines.** (A) OV2008, (B) SKOV3.ip1, and (C) ES2 cells stably expressing FLAG-*CCNG2* were treated with CHX and samples were taken at the intervals indicated. Transiently transfected (D) ES2 and (E) HEY cells were analyzed in the same way. Levels of cyclin G2 at each time interval were monitored using western blotting and graphs were generated by quantifying bands as previously described. Data represent mean±SEM of three experiments. Differences were considered significant at values of $p<0.05$.

## 3.2: Addition of Calcium Induces Cyclin G2 Degradation in a Concentration-Dependent Manner *In Vitro*

As mentioned earlier, cyclin G2 harbours a destabilizing PEST domain in its C-terminal, which has been shown to enhance cyclin G2 degradation via the proteasome machinery in human ovarian cancer cells[67]. To investigate whether or not cyclin G2 is susceptible to degradation by calpain as well, we set up a series of experiments to explore these questions. Since an increase in the intracellular concentration of calcium enhances the activity of calpains[101], we investigated the effect of supplementing crude cell lysate with additional source of calcium to see if this in turn led to degradation of cyclin G2. Equal numbers of SKOV3.ip1 and ES2 cells stably expressing FLAG-*CCNG2* were seeded and upon attachment to the plate substrate were collected and lysed. Equal amounts of each cell lysate were incubated with lysis buffer containing increasing concentrations of $CaCl_2$ and incubated for 1 or 2 hours at 37°C for SKOV3.ip1 cells and 1 hour at 37°C as well as room temperature for ES2 cells. Blotting for FLAG to detect levels of cyclin G2 revealed that addition of calcium did indeed result in increased degradation of cyclin G2 (Figure 6). Additionally, higher concentration (5 mM) of calcium resulted in more degradation of cyclin G2 compared to when 0.5 mM calcium was present.

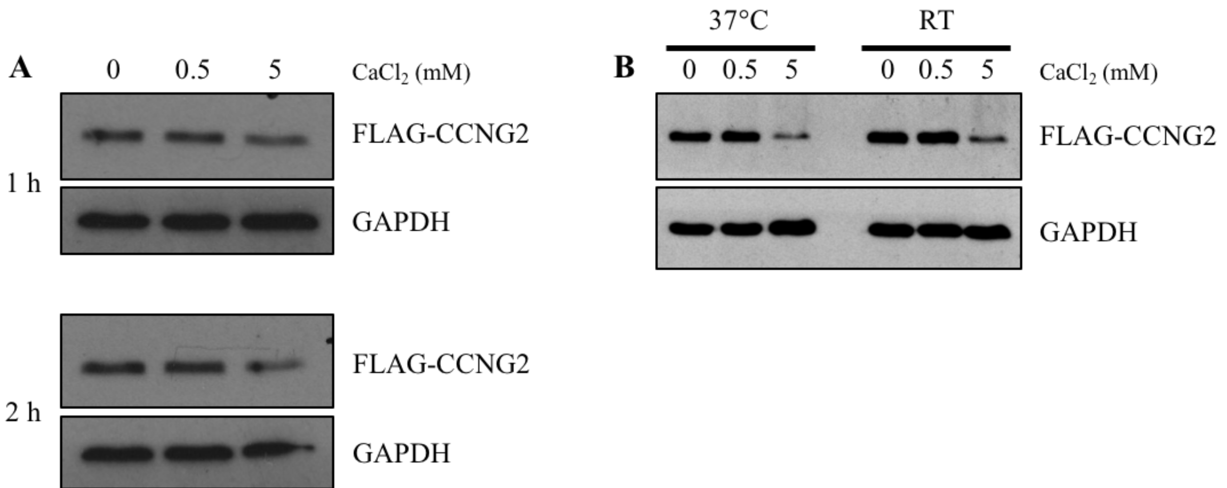
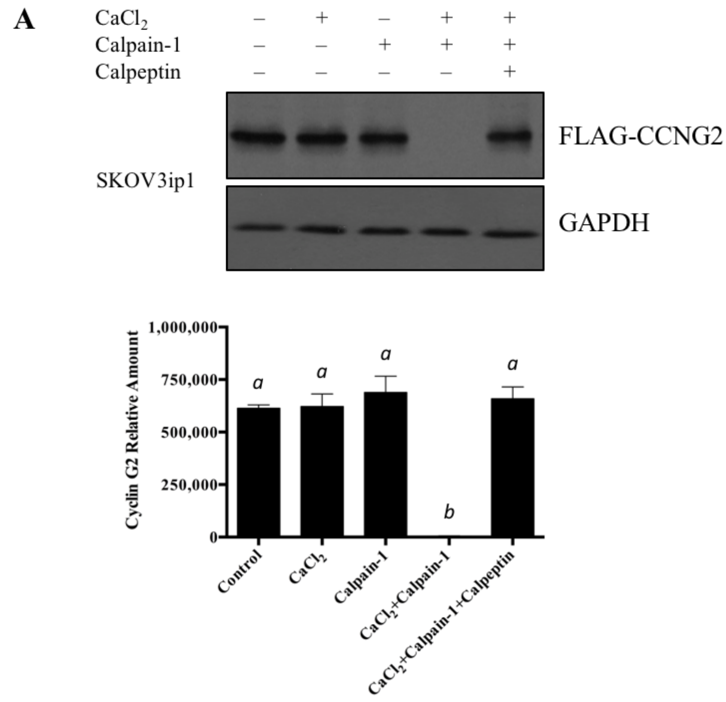
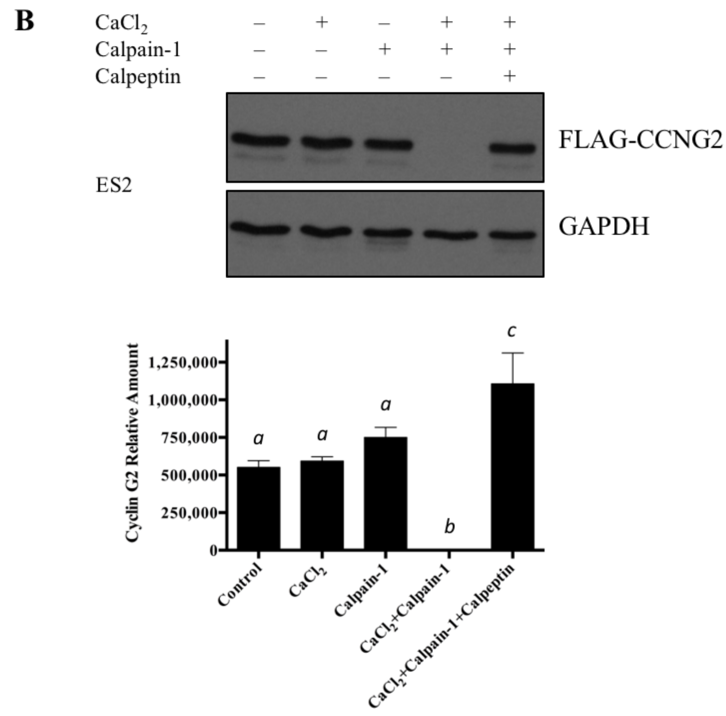



**Figure 6: Addition of calcium causes degradation of cyclin G2 *in vitro*.** (A) SKOV3.ip1 cells stably expressing FLAG-*CCNG2* were lysed and incubated with increasing amounts of calcium at 37°C. (B) Stable ES2 cells were lysed and incubated with increasing amount of calcium for 1 hour at temperatures indicated. 5 mM $CaCl_2$ caused the most degradation of cyclin G2 in both cell lines.

### 3.3: Cyclin G2 is Degraded by Calpain-1 *In Vitro*

Degradation of cyclin G2 in response to addition of calcium in cell lysates prompted us to verify if the activity of calpain is in fact at least one driving force behind this phenomenon under the experimental conditions. To do this, equal densities of SKOV3.ip1, ES2, and HEY cells were transiently transfected overnight with a FLAG-*CCNG2* construct and subsequently lysed in calpain degradation buffer with or without 0.5 mM $CaCl_2$, active calpain-1, and calpeptin, a calpain inhibitor. Western blot analyses (Figure 7) confirmed that in the presence of added calcium, active calpain-1 caused a reduction in the levels of cyclin G2 protein. Addition of $CaCl_2$ or calpain alone had no obvious effect on cyclin G2 levels as compared to the negative control. Addition of calpeptin to cell lysate protected cyclin G2 from degradation by inhibiting the activity of calpain-1. However, these results should be considered with caution since calpain-1 failed to cause degradation of cyclin G2 significantly as shown in Figure 9.

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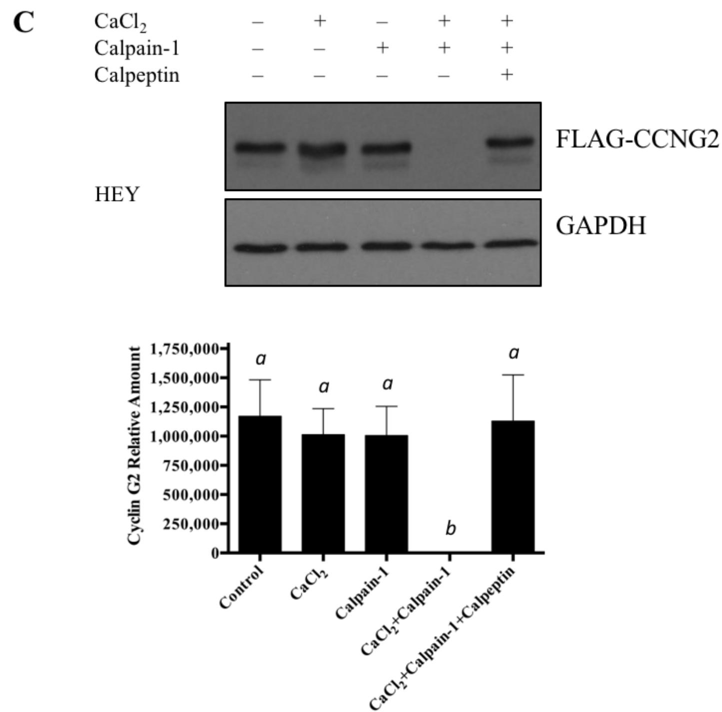



**Figure 7: Calpain-1 degrades cyclin G2 *in vitro*.** Various human ovarian cancer cells, (A) SKOV3.ip1, (B) ES2, and (C) HEY, were transiently transfected and aliquots of cell lysate were incubated with the ingredients as indicated in each panel. When supplemented with extra calcium, calpain-1 activity leads to dramatic degradation of cyclin G2 and this process is significantly blocked when calpeptin, a calpain inhibitor, is added to the cell lysate. Labels on the left of the blots denote the cell line used. Graphs represent mean±SEM (n=3), and the differences were considered significant at values of $p<0.05$.

### 3.4: Inhibition of EGFR Kinase Activity Protects Cyclin G2 from Degradation

Unpublished data from our lab have demonstrated a role for phosphorylation in the process of calpain-mediated proteolysis of cyclin G2 in OV2008 cells. This discovery prompted us to use a kinase inhibitor library to find out if cyclin G2 could be protected from degradation when various protein kinases were inhibited. Serum-starved OV2008 cells stably expressing a FLAG-*CCNG2* construct were pretreated with a variety of kinase inhibitors for 1 hour, followed by treatment with epidermal growth factor (EGF) and CHX for an additional hour or shorter. EGF is able to bind to and subsequently induce rapid activation of EGFR and its downstream signalling pathways. Western blot analyses showed that cyclin G2 was protected from degradation when EGFR was inhibited (Figure 8A). In a similar fashion, but in the absence of EGF, ES2 cells were transiently transfected and levels of cyclin G2 were monitored when the cells were treated with different kinase inhibitors (Figure 8B). The results suggest that EGFR kinase activity is involved in regulation of cyclin G2 degradation, and inhibition of its kinase activity, regardless of the presence or absence of EGF stimulation of the cells.

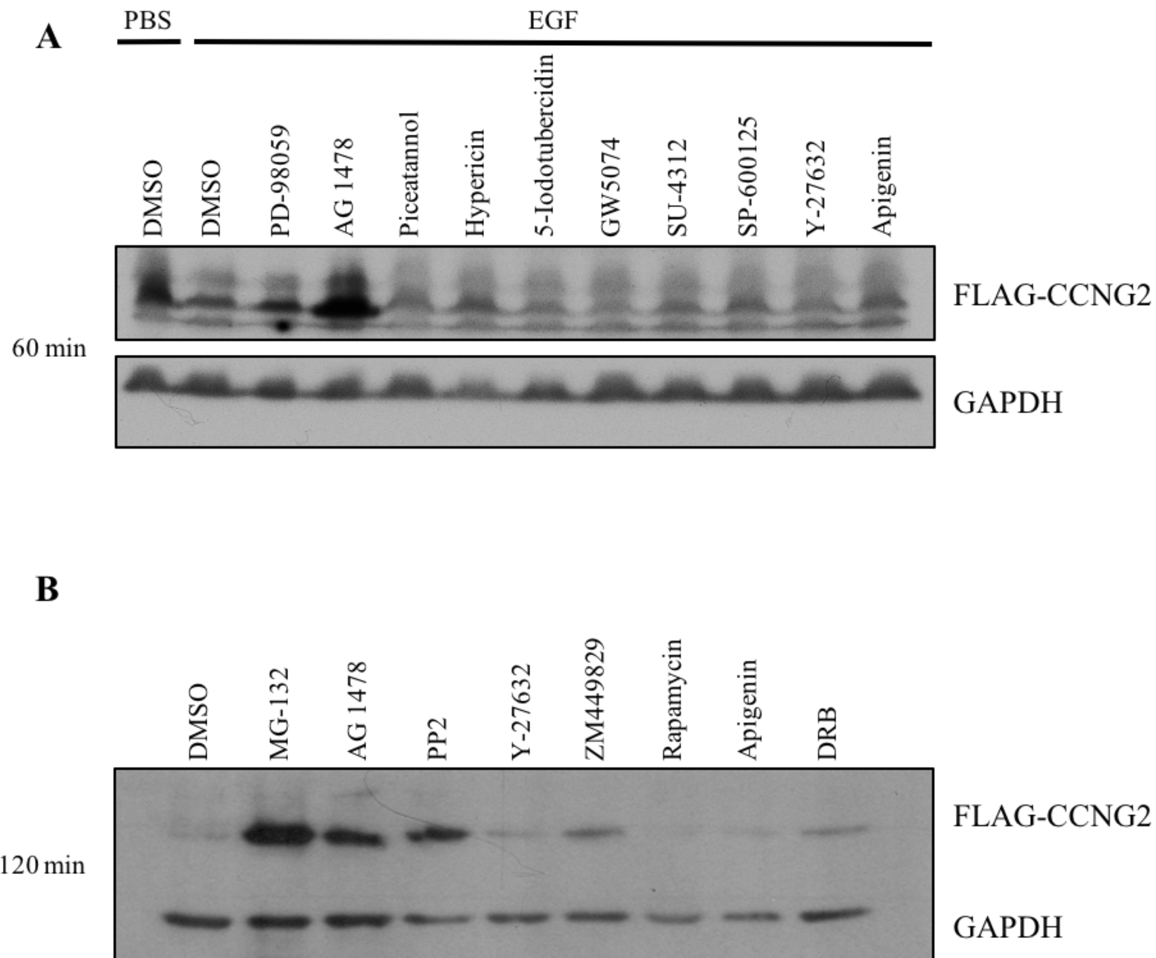
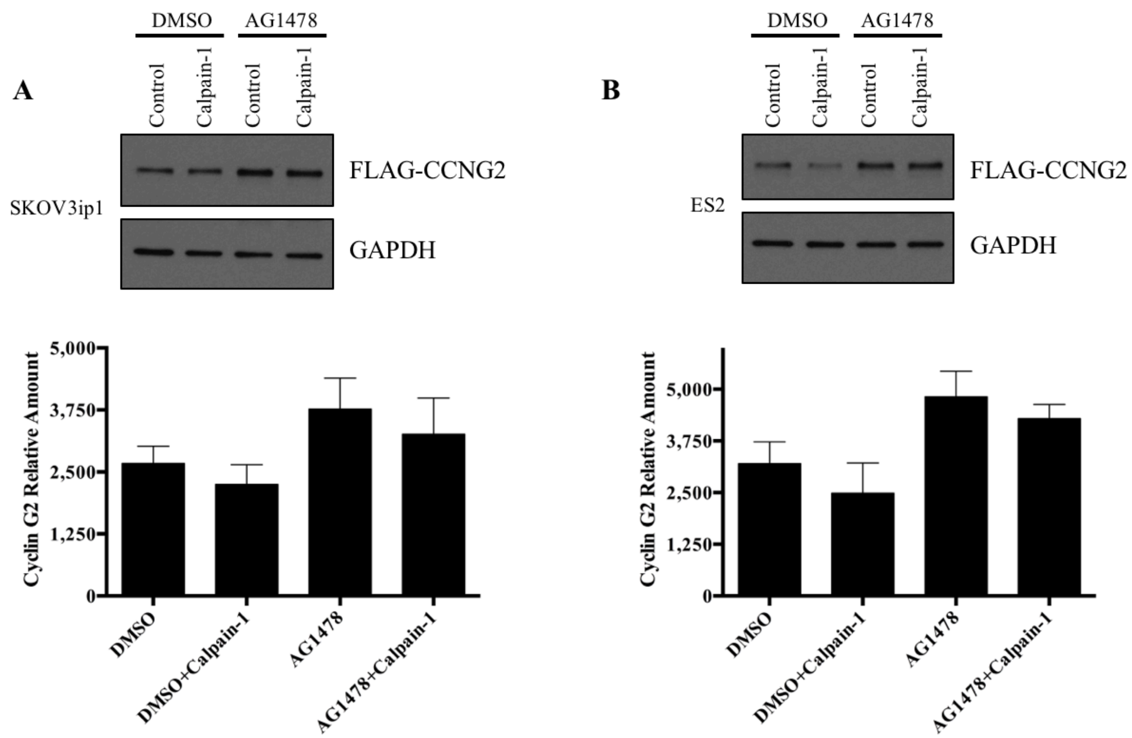



**Figure 8: Inhibition of EGFR blocks cyclin G2 degradation.** (A) OV2008 cells stably expressing a FLAG-*CCNG2* construct were treated with various kinase inhibitors followed by EGF treatment. In the presence of Tyrphostin AG 1478, an inhibitor of EGFR, cyclin G2 was not degraded. (B) ES2 cells were transiently transfected with a FLAG-*CCNG2* construct, followed by treatment with various kinase inhibitors. Inhibition of EGFR protected cyclin G2 protein from degradation. Inhibition of Src by PP2 also showed some protective effect. The experiment presented in B panel was conducted and the figure was created by Ms. Alina Kopteva. MG-132, an inhibitor of proteasome, was included as the control since it protects cyclin G2 from degradation.

### 3.5: Effect of EGFR Inhibitor on Calpain-Mediated Degradation of Cyclin G2

To further investigate the connection between EGFR activity and calpain-mediated degradation of cyclin G2, SKOV3.ip1, ES2, and HEY cells were transiently transfected as described before, followed by a 30-minute treatment with Tyrphostin AG 1478, a potent inhibitor of EGFR, or vehicle DMSO. Cells were then lysed in calpain degradation buffer and the lysate was incubated with Tyrphostin AG 1478 or DMSO in the presence of added calcium to ensure the continued inhibition of EGFR. As depicted in Figure 9, inhibition of EGFR resulted in some degree of protection against cyclin G2 degradation when cell lysates were incubated with active calpain-1. However, due to large variation among the experiments, no stastistical significance among different treatment groups was observed. Fine-tuning of the experimental conditions can shed more light on the connection between EGFR inhibition and calpain-mediated degradation of cyclin G2.



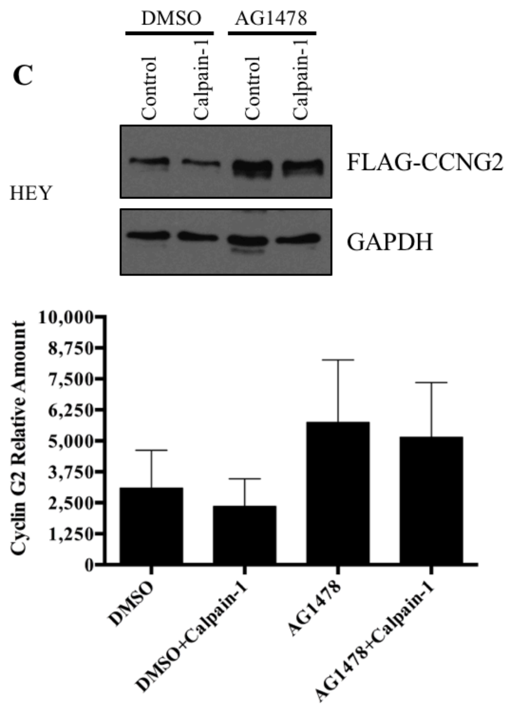




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**Figure 9: Effects of EGFR inhibition on cyclin G2 degradation by calpain-1.** Human ovarian cancer cells, (A) SKOV3.ip1, (B) ES2, and (C) HEY, were transiently transfected with FLAG-*CCNG2* construct overnight and treated with an inhibitor of EGFR or DMSO. Lysed cells were incubated with active calpain-1 in the presence or absence of EGFR inhibitor. Graphs represent mean±SEM (n=3).

### 3.6: EGF Stimulation Leads to Degradation of Cyclin G2

According to the results presented here, EGFR seems to play a positive role in degradation of cyclin G2 in the human ovarian cancer cells we have used. As we saw earlier, one of the ligands that activates EGFR kinase activity if epidermal growth factor (EGF). To study the effects of EGFR activation alone on the fate of cyclin G2 in human ovarian cancer cells, cells under investigation were serum-starved (except in one case), followed by a treatment of EGF for various time lengths as indicated in each figure.

To test the effect of EGF on cyclin G2 degradation, OV2008 and SKOV3.ip1 cells stably expressing a FLAG-*CCNG2* construct were serum-starved and subsequently treated with increasing amounts of EGF for an hour in the presence of CHX. We saw a decline in the levels of cyclin G2 after an hour when cells were treated with 20 ng/ml EGF (Figure 10A). To study whether the presence of serum has any effect on EGF treatment, SKOV3.ip1 stable cells were treated with increasing amounts of EGF in the presence or absence of serum in the media. Western blot analysis revealed that the presence of serum had no discernable effect on levels of cyclin G2 (Figure 10B). To assay the effect of 20 ng/ml EGF treatment on the levels of cyclin G2 as a function of time, ES2 and SKOV3.ip1 stable cell lines were incubated with EGF or MG132, an inhibitor of the proteasome machinery, along with their vehicles, plus 10 μg/ml CHX. MG132 was used as positive control for protection against degradation as it has been shown that cyclin G2 is a target for degradation by the proteasome[2]. It became obvious that after 2 hours of treatment, levels of cyclin G2 had decreased in both cell lines. This decline was much more obvious in ES2 cells, where after the first hour, all traces of cyclin G2 were gone, except where cells were treated with MG132. The more enhanced degradation of cyclin G2 in these two cell lines corroborates with the results of

cyclin G2 stability in various human ovarian cancer cell lines as before. Figure 11 summarizes these findings.

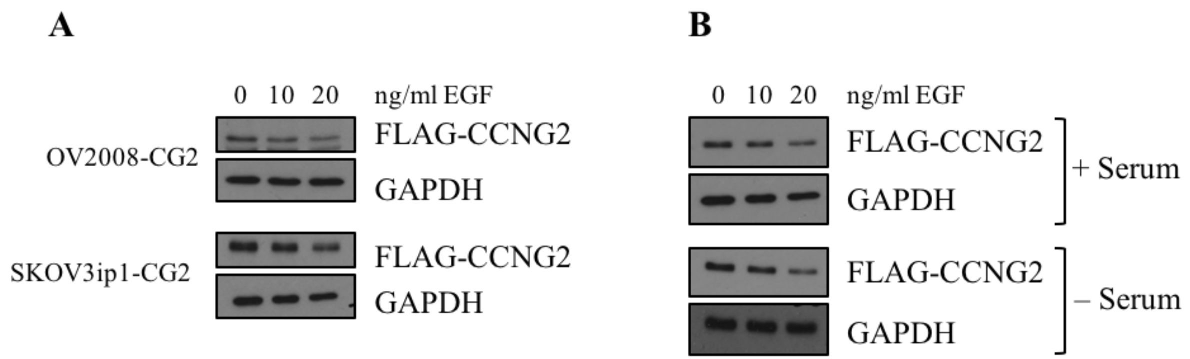


**Figure 10: EGF stimulation causes degradation of cyclin G2.** (A) Cyclin G2 was degraded in OV2008 and SKOV3.ip1 cells upon EGF stimulation. (B) Presence or absence of serum had no effect on cyclin G2 degradation in response to EGF treatment of SKOV3.ip1 cells overexpressing cyclin G2.

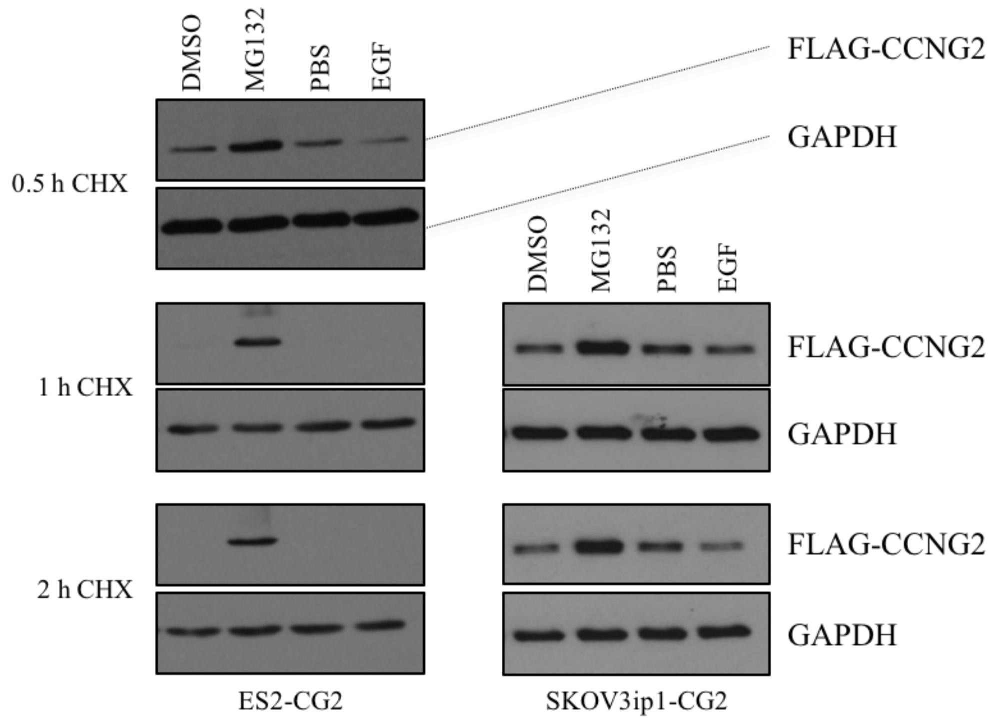


**Figure 11: Inhibition of proteasome protects cyclin G2 from degradation.** In both ES2 (left panel) and SKOV3.ip1 cells stably expressing a FLAG-*CCNG2* construct EGF caused cyclin G2 degradation. MG132, a proteasome inhibitor, which also has inhibitory effects on calpain activity, protected cyclin G2 in both cell lines and under different experimental conditions.

# Chapter 4: Discussion

Increasing evidence points towards potent negative effects of cyclin G2 on cell cycle progression and proliferation. More specifically, and focusing on enhanced capability of cancer cells to migrate and invade, our lab has demonstrated that cyclin G2 overexpressing ovarian cancer cells had a reduced ability to migrate and invade when compared to the control group. Consistent with the effects of cyclin G2 at the cellular level, our lab has also shown that the tumour size in mice models was overall smaller when the animals were injected with cells that were overexpressing cyclin G2 when compared to the control cells that only expressed the empty vector. Looking at the bigger picture, numerous experiments ranging from cultured cells to primary clinical samples of tumours show an inverse relationship between the levels of cyclin G2 and the stage of cancer. Taking into consideration that cyclin G2 not only can halt the cell cycle, but also limit cells' ability to migrate and invade we can conclude that this protein is involved in not only control of cell cycle, but also in cell mobility and anchorage dependence.

As part of the present study, we assayed the stability of cyclin G2 protein in a number of human ovarian cancer cells. Degradation of cyclin G2 in cells overexpressing it is visible as early as 1 hour, and the levels are barely detectable after 5 hours when *de novo* protein synthesis was blocked by using cycloheximide (CHX). The results are in agreement with the previous findings of our lab that cyclin G2 is an unstable protein[67]. These findings are also in harmony with the context of the growth of cancer cells; to bypass the growth inhibitory signals, cancer cells often eliminate the anti-proliferative signals both at genetic and protein levels. Knowing the negative effects of cyclin G2 on cell cycle progression and its anti-tumour effects it makes perfect sense that cyclin G2 is degraded quickly in cancer cells.

Since the original proposal of PEST hypothesis[72], increasing evidence has shown that proteins harbouring PEST domains are indeed short-lived and that their degradation is at least in part carried out by the catalytic activity of calpains, a class of proteases whose activation relies on the presence of calcium[101]. When bound to calpain, calcium ion causes a conformational change in the protease, leading to its activation. In this study, we demonstrated that calpains play a role in regulating cyclin G2 degradation. First, *in vitro* studies using crude cell lysates of human ovarian cancer cells overexpressing cyclin G2 showed that addition of calcium to the cell lysate did in fact cause a decrease in the levels of cyclin G2 when compared to the control groups. Second, addition of calpain to the cell lysate in the presence of $CaCl_2$ caused a dramatic decrease in the levels of cyclin G2. Third, addition of calpeptin, an inhibitor of calpain, reversed the effect of calpain in the presence of $CaCl_2$, and protected cyclin G2 from degradation.

Adding to our understanding of this process, unpublished data from our lab also shows that phosphorylation is a required step in degradation of cyclin G2 by calpain; treatment of cell lysates with calf intestinal phosphatase (CIP) rescued cyclin G2 from degradation. To gain a deeper understanding and trying to find the protein kinase that phosphorylates cyclin G2, the protein's primary sequence was interrogated using Group-Based Prediction System (GPS) software and epidermal growth factor receptor (EGFR) was obtained as one of the candidates. This finding is particularly interesting because EGFR activation has been shown to also upregulate calpain activity[113,115]. To look for potential kinases involved in cyclin G2 degradation, a kinase inhibitor library was used to selectively block the activity of EGFR as well as other protein kinases downstream of it to see the effects on the levels of cyclin G2 in human ovarian cancer cells. Our results showed that inhibition of EGFR protected cyclin G2 from degradation in these cells,

whether the cells were stimulated with EGF or not. Inhibition of EGFR also protected cyclin G2 from degradation in cell lysates with added calpain. Therefore, activity of EGFR is involved in degradation of cyclin G2. Whether there is a direct interaction between EGFR and cyclin G2 where the latter becomes phosphorylated or if a downstream effector of EGFR is responsible for phosphorylating cyclin G2 remains to be explored. However, it is possible that EGFR is the kinase we are seeking, because even though signal transduction pathways are not at work once the cell is lysed, presence of Tyrphostin AG 1478, although not significantly, showed some protective effect against cyclin G2 degradation. Additionally, there is evidence that EGFR can be internalized and travel all the way to the nucleus where it drives the expression of genes such as cyclin D1[125]. The possibility of direct phosphorylation of cyclin G2 by EGFR remains to be investigated.

EGF binding and subsequent activation of EGFR and downstream transduction pathways are important events in cancer development. EGFR-induced signals and gene expression patterns lead to an increase in cell proliferation, migration and invasion, adhesion, and angiogenesis while suppressing apoptotic pathways or those that negatively regulate cell cycle progression[119,120,130]. Another set of evidence which shows involvement of EGFR activity in cyclin G2 degradation comes from the results where cells were stimulated with EGF. We showed that treating a number of human ovarian cancer cells with EGF resulted in a decrease in the levels of cyclin G2. Using an inhibitor of the proteasome machinery which also inhibits calpain activity, MG-132, resulted in the attenuation of the effect of EGF on cyclin G2 stability. In this context, it is important to make room for the fact that mutations of EGFR in ovarian cancer[126,127], which lead to an increased activity of the receptor and its downstream effectors and gene targets might mask the effect of added EGF; the receptors could already be fully active and additional EGF might not cause any

significant effect. It should be mentioned however that no attempts to characterize possible EGFR mutations have been made in our lab. It would be of interest to study the effects of EGF on cyclin G2 stability in healthy ovarian cells in addition to the investigations done in cancerous cells.

One surprising finding was the apparent lack of activity of calpain-1 in degrading cyclin G2 as shown in Figure 9. While addition of calcium and calpain-1 showed a significant decrease in levels of cyclin G2 in Figure 7, such effect was absent in similar conditions. Although not confirmed yet, maybe the presence of DMSO, both as control as well as the vehicle for Tyrphostin AG 1478, might have contributed to attenuation of calpain-1 proteolytic activity. Alternatively, calpain-1 stock might have lost its activity. Revision of the protocols and studying the effect of DMSO on calpain-1 will shed more light on this curious result.

At this point, it is worth mentioning a few overshadowing limitations that were experienced throughout the work presented here. First, we had no access to a reliable anti-cyclin G2 antibody to monitor the fate of endogenous cyclin G2. One consequence of this is that we were forced to work with tagged, exogenous cyclin G2 in order to detect and monitor its levels. In this regard, we have used both stable cells that constitutively express cyclin G2, which can pose a problem as mentioned earlier in Introduction (Table 2) with respect to the negative effects of cyclin G2 on cell cycle progression. Cell cycle which has deviated from its normal course will have other consequences in terms of the whole cellular molecular pathways, which can further us from a more physiologically-relevant conditions that cancer cells experience. A less intrusive way to study cyclin G2 was therefore to transiently express the FLAG-*CCNG2* construct to reduce the myriad of effects which overexpression of this protein entails. For example, in the case of ES2 cells stably

expressing cyclin G2, after a few passages they stopped growing, and an approach centring transient transfection was chosen over using stable cells. Considering levels of cyclin G2 have been reported to be significantly lowered in various types of cancer, its overexpression is a logical method to study its function in these cells. However, any experiment that leads to introducing higher than normal amounts of cyclin G2 in the cells, should also make room for the inhibitory effects of abundant cyclin G2 on the cell cycle progression. But as we know, proteins are not lone wolves in the cell, and any deviation from the normal levels of the proteins puts the cells in a very different situation. The other restriction, which has been explained in more detail under Future Directions was the lack of signal from expressing *CCNG2* constructs with truncated or deleted PEST domain. Considering the pivotal role of PEST domain in regulating the degradation of cyclin G2, exciting discoveries are waiting to be made in the field to further uncover details of not only the role of PEST domain in degradation of cyclin G2, but to identify the important amino acid residues that are involved in this process.

The fact that signal transduction pathways instigated by EGFR are a cascade of phosphorylation events, together with the findings in our lab indicating that treatment of cell lysates with CIP prevented cyclin G2 degradation, plus knowing that EGFR activation can also activate calpain, and that calpain-mediated protein degradation seems to depend on phosphorylation of the target protein, it is possible to propose a model for the mechanism of cyclin G2 degradation downstream of EGFR signalling cascades. Two major mechanisms are likely to be in play when it comes to degradation of cyclin G2 downstream of EGFR. **FIRST**, EGFR activation leads to phosphorylation of cyclin G2. Whether EGFR directly phosphorylates cyclin G2 or not as well as the spatial details of this process are excellent areas of investigation for the

future. In the same area, the residues phosphorylated on cyclin G2 are also waiting to be discovered. The phosphorylation event(s) then guide cyclin G2 degradation via two mechanisms, which leads us to the **SECOND** step. Cyclin G2 phosphorylation can act as a signal for ubiquitination and degradation by the UPP. As well, phosphorylation of cyclin G2 can result in interaction between cyclin G2 and calpain, which also leads to degradation of cyclin G2. Another major component of this model is the activation of calpain in response to EGFR activation. Figure 12 represents a view of this proposed model.

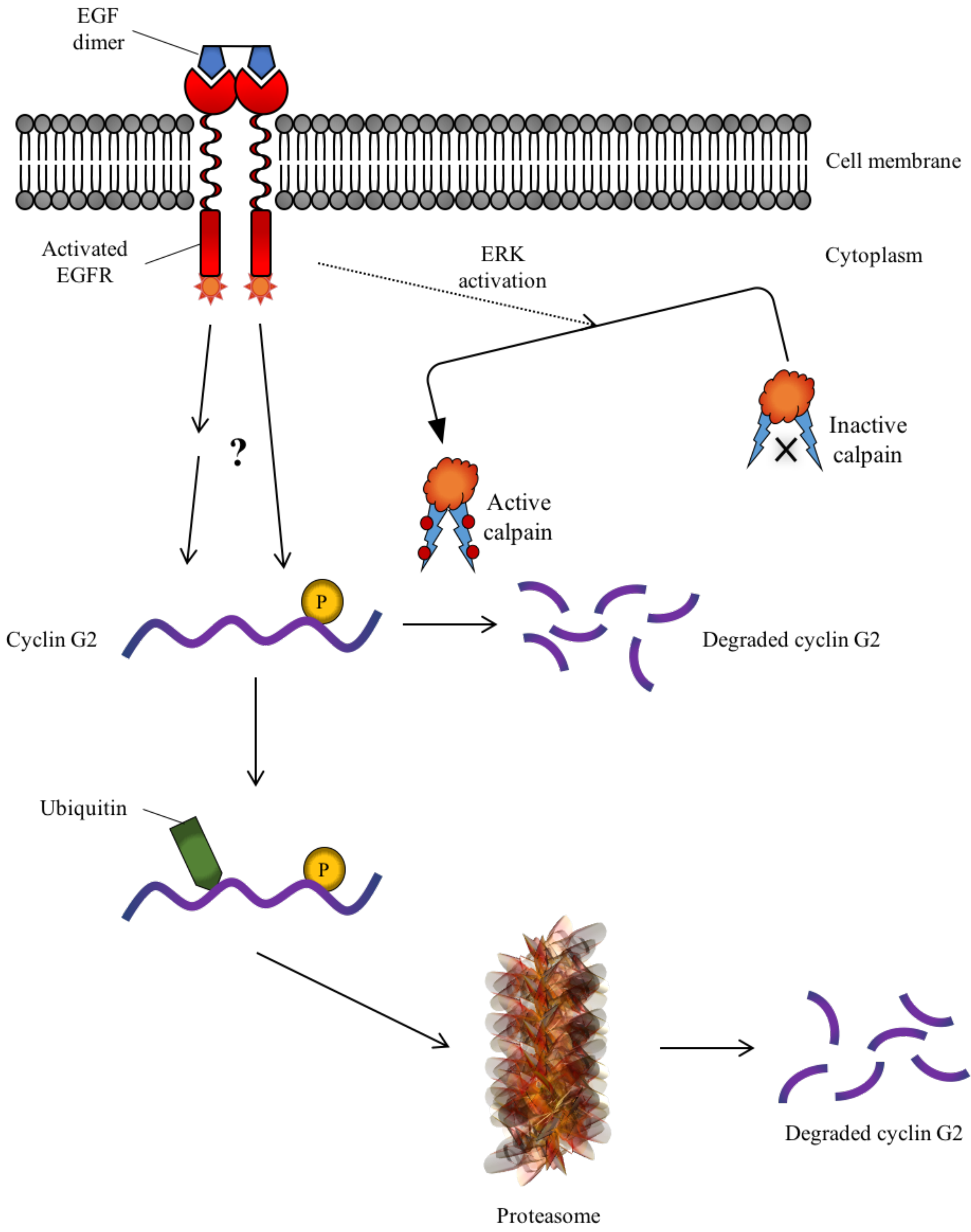


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**Figure 12: A proposed model for EGFR and calpain-mediated degradation of cyclin G2.** Activated EGFR directly or indirectly causes phosphorylation of cyclin G2. Phosphorylated cyclin G2 can be degraded by calpain protease activity, which can also be positively regulated through the action of ERK as a consequence of EGFR activation. Phosphorylated cyclin G2 is then ubiquitinated and sent to proteasome for degradation.

# Chapter 5: Summary and Future Directions

## 5.1: Summary

To summarize the findings, we have shown that cyclin G2 is an unstable protein, and is rapidly degraded in a number of human ovarian cancer cells. In addition to the previous findings by our lab that cyclin G2 is targeted by the proteasome for degradation, we have shown that cyclin G2 is also subject to degradation by calpain proteolytic activity. While investigating cyclin G2 degradation in the context of signal transduction pathways we have revealed that EGFR signalling decreases cyclin G2 stability. In accordance with published data that EGFR signalling also positively regulates calpain activity, we studied the effect of EGFR inhibition on calpain-mediated degradation of cyclin G2, but did not find a significant effect of EGFR inhibition on the fate of cyclin G2 in the presence of calpain-1. Finally, we have provided evidence that stimulation of cells with EGF causes cyclin G2 degradation in human ovarian cancer cells, and that blocking the proteasome machinery attenuates the effect of EGF on cyclin G2 degradation.

## 5.2: Future Directions

The crucial involvement of cyclin G2 PEST domain in its degradation has been demonstrated[67], but unfortunately we did not succeed in getting expression from the constructs with various PEST domain deletions to collect information on how these deletions would affect cyclin G2 stability in these cells. Although these constructs were isolated using the same method as the ones that expressed well, multiple attempts at sequencing them failed, and so it was not even possible to determine if the lack of expression could be due to a nonsense mutation or not. Additionally, this problem could have been caused by mislabeled or old bacterial stock that were not maintained well. Very recent unpublished results from our lab have reported successful

expression of these constructs and have narrowed down the problem to the inefficiency of anti-V5 antibody which was available at the time. Future studies that aim at investigating the stability of wild-type or modified versions of cyclin G2, should also monitor the stability of a number of other proteins, in order to provide a context in which stability of cyclin G2 can be better compared and contrasted to other proteins as well as in different cell lines, as stability of proteins can vary from one cell type to the next.

To study more aspects of cyclin G2 degradation and processes involved, fresh constructs with various modifications to the wild-type gene can be generated and used. More specifically, *CCNG2* can be mutated on the residues known to facilitate direct interactions between cyclin G2 and it known binding partners. Other mutations should focus on potential phosphorylation sites, which most likely serve as a starting signal for cyclin G2 degradation. Speaking of the involvement of kinases in degradation of cyclin G2, studying its phosphorylation state as a requirement for degradation can also be further studied through the use of constructs which harbour mutations of potential phosphorylation sites.

While chemical kinase inhibitors provide a relatively easy method to study the effects which inhibition of those kinases might have, some of them bear the disadvantage of low specificity for their target kinase; they could also inhibit other protein kinases. To have a tighter control over this process, siRNA could be used instead, to specifically silence only the protein kinase(s) under study. We are far from having full control over the processes employed by the cells to survive, and so the use of siRNA might prove to be an invaluable alternative or compliment to the use of chemical protein kinase inhibitors.

Finally, performing experiments in healthy ovarian cell lines as well as in cell lines from different tissue origins, will provide us with a much broader view of the processes involved in cyclin G2 function and regulation, and will hopefully make comparisons and the lessons learned from those comparisons a more fruitful endeavour. These findings will be further enriched when we incorporate and consider the microenvironments and microbiota in which cancer cells form in and are surrounded by, respectively.

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