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The Role of Spy1 Protein Regulation In Breast Cancer

Mohammad Al Sorkhy
University of Windsor

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Mohammad Al Sorkhy

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Abstract

Cell growth and proliferation are tightly controlled by the cyclic regulation of the cyclin dependent kinases (Cdks). Cdks are positively regulated through interactions with regulatory Cyclin partners as well as being negatively regulated through interactions with families of Cdk inhibitors (CKIs). The Spy1/RINGO family of proteins have emerged as a unique class of 'Cyclin-like' proteins capable of directly binding both to the Cdks, as a positive regulatory partner, as well as to at least one member of the CKI's, p27^{Kip1}, as a negative regulator. Abnormally elevated levels of Spy1 promote cell proliferation, inhibit apoptosis and are implicated in aggressive tumorgenesis in all cell/tissue types studied to date. Understanding how Spy1 protein is regulated is essential in resolving how it contributes to normal and abnormal growth processes. Herein, we demonstrate that Spy1 is degraded in a cell-cycle-dependent manner via the ubiquitin-proteasome system. We have resolved the E3 ligase and essential phosphorylation sites mediating Spy1A degradation. Furthermore, we show that Spy1 protein is stabilized in subsets of human breast cancer samples. Using a stable mutant of Spy1 we demonstrate that this represents an oncogenic modification *in vitro* and accelerates tumor formation *in vivo*. We further show that these oncogenic properties are largely dependent upon the unique activation of Cdk1 and the subsequent inhibition of the anti-apoptotic regulator FOXO1. Utilizing Spy1 mutants unable to bind to the primary effectors p27 and Cdk2, we have found that Spy1-mediated effects in the breast rely on direct interactions with each of these effectors via separable functional mechanisms. This work reveals novel mechanisms regulating the progression, and potentially the etiology, of human breast cancers and may be of considerable therapeutic relevance.

DEDICATION

I dedicate this work to my family, my parents in particular, and to my other half; my wife, Tamara.

Acknowledgments

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List of Abbreviations

ATP ………………………………………………………………………………… *adenosine triphosphate*
CAK…………………………………………………………………………………... *Cyclin activating kinase*
CDK .... *Cyclin dependent kinase*
CKI .... *cyclin dependent kinase inhibitor*
DN……………………………………………………………………………..... *dominant negative*
E3 ………………………………………………………………..….…*ubiquitin protein isopeptide ligase*
EGF………………………………………………………………..…………... *Epidermal growth factor*
G0 .... *Gap0*
G1 .... *Gap1*
G2 ……………………………………………………………………………….…*Gap2*
GSK .... *Glycogen synthase kinase*
HA ....*hemagglutin*
Hect ………………………………………………..............*homologous to E6AP carboxyl terminal*
FOXO1……………………………………………..…………… *fork head box o class protein 1*
MOI .... *multiplicity of infection*
NEDD4 …………………………….....*neural precursor cell-expressed developmentally down regulated*
PAGE .... *polyacrylamide gel electrophoresis*
p21 .... ……………*CIP1*
p27 .... *KIP1*
PBS ……………………………………………………………..……*phosphate bufferedsaline*
PCR ……………………………………………………………………………*polymerase chain reaction*
PEI .... *polyethylenimine*
QRT-PCR…………………………………………………………………*quantitative real time PCR*
RO water……………………………………………………………….*Reverse Osomsis water*
RINGO .... *Rapid Inducer of G2/M progression in Oocytes*
SAGE ……………………………………………………………….*serial analysis of gene expression*
SCF …………………………………………………………………….….…......*Skp1-Cullin1-F-box*
SDM .... *site directed mutagenesis*
SE……………………………………………………………………………………….*slandered error*
siRNA ………………………………………………………………………………….*small interference RNA*
Spy1A .... *Spy1/RINGO A*
TBST ……………………………………………………………......*Triss buffered saline and Tween 20*
TMA………………………………………………………………………………………. *tissue micro array*
Ub …………………………………………………………………………….…………....*ubiquitin*
UPS …………………………………………………………………….………....*ubiquitin proteasome system*
WT …………………………………………………………………………………………………....*wild type*
X-Spy1 .... *Xenopus Spy1*

Chapter 1

General Introduction

Introduction

The cell division cycle is a tightly orchestrated series of events designed to coordinate DNA synthesis, cell growth and division into two daughter cells. Proper movement through this cycle is the critical component regulating the growth and development of all organisms. This seemingly straightforward cycle is cleverly designed to detect any errors in this program and respond by halting cell growth or sending cells into programmed cell death or apoptosis. Misregulation of the cycle in a manner that eludes detection by this surveillance system has potentially devastating consequences for the organism including the initiation of tumorigenesis, degenerative diseases or a host of developmental disorders. Hence, how this cycle is regulated has been the focus of many research groups for decades and elution of the key regulators represents some of the greatest scientific advancements of our time.

Central Cell Cycle Events

Eukaryotic cells of all orders of complexity share a very similar cell division cycle. The cycle is characterized by two major cellular events: the accurate duplication of the genome in DNA synthesis, or S phase, and the separation of complete sets of chromosomes into daughter cells in mitosis, or M phase. These pivotal events are separated by two gap phases, G1 and G2 [1].

Terminally differentiated adult cells spend the majority of their time in G1 phase where the cell increases in size through the coordination of a host of biosynthetic activities and monitor the conditions of both the internal and external environment of the cell to ensure that successful completion of both DNA synthesis and division are possible. Based on environmental and developmental signals, cells in G1 may

temporarily or permanently exit the cell cycle and enter quiescent or arrested phase known as G0. During G0, cells are metabolically active and capable of performing many functional roles, however they are unable to divide. If conditions are optimal, cells proceed through to recruit initiator of replication proteins at specific sites called replication origins forming the Replication Origin Complex (ORC). In eukaryotic cells, assembly of Cell Division Cycle 6 (cdc6) with the ORC forms a pre-replicative complex (pre-RC) which is required for loading of the Mini Chromosome Maintenance (MCM) proteins onto DNA. The MCM proteins have helicase activity, essential in the unwinding of the DNA to facilitate formation of the replication fork to allow primase and DNA polymerase to begin replicating the lagging and leading strand respectively.

Following successful synthesis of DNA, or S-phase, the second gap phase occurs. G2 is a much shorter phase of the cell cycle which plays an important role in ensuring that the cell is capable of successful and error-free division. Entry into G2 activates kinases which subsequently phosphorylate cdc6 to trigger disassembly of the ORC, ensuring that synthesis of DNA occurs only once per cycle. The final phase of the cell cycle, M phase, is composed of two major events: nuclear division (mitosis) and cell division (cytokinesis). Mitosis is the process of distributing the duplicated chromosomes into two nuclei. Following mitosis, the cell divides by cytokinesis to produce two identical cells that will reenter the cycle at G1or G0 depending on the their environment [1].

Regulation of the Cell Cycle

The correct course of successive cell cycle phases is ensured by the production and destruction of proteins known as Cyclins. These proteins must bind to their catalytic

subunit known as the Cyclin-dependent kinases (Cdks). Cdks are a group of serine/threonine protein kinases that play an essential role in the regulation of eukaryotic cell cycle division. The catalytic activity of Cdks is regulated by protein–protein interactions, post-translational modification of Cdks by phosphorylation or dephosphorylation, and the binding of Cdk inhibitors (CKI) [2]. To initiate Cdk activation, Cdks must be bound to their regulatory Cyclin partners that are synthesized and degraded in a cell-cycle-dependent manner [3]. Following activation, these protein complexes move cells through the stages of the cell cycle while constantly monitoring DNA integrity and the nutrient/energy levels of the cell. Tight regulation of the cell cycle is crucial for the health of cells and organisms and aberrant regulation of the Cyclin-Cdk complexes are implicated in many different growth disorders [4].

Cyclin Regulation

Cyclins form a family of structurally and functionally similar proteins, now encompassing 5 major families (A, B, C, D and E) [5]. Cyclins are characterized by the regulation of their protein levels, which fluctuate throughout the different stages of the cell cycle. All Cyclins share a conserved region of about 100 amino acids called the Cyclin box, which binds to and activates Cdks [6] Most The primary Cyclins known to regulate essential cell cycle movements ubiquitously in all mammalian somatic cells are categorized into two main groups: the G_1 Cyclins (Cyclins C, D1 and E), which control growth phase G_1 and the G_1/ S transition, and the so-called mitotic Cyclins (Cyclins A and B) which regulate the G_2/ M transition and mitosis.

Regulation of Cyclin-Dependent Kinases (Cdk)

Cyclin-dependent kinases (Cdks) are a group of serine/threonine protein kinases that play an essential role in the regulation of eukaryotic cell cycle division. Up to date there are eight Cdks have been characterized; however the most established regulators of the somatic cell cycle are Cdk1, Cdk2, Cdk4 and Cdk6 [7]. Cdks are constitutively expressed, but their catalytic activity is regulated by protein–protein interactions, and both inhibitory and activating phosphorylation. When Cdks are found in their inactive monomeric form, the conserved region of the kinase, known as the T loop, protects the catalytic cleft and prevents the binding of substrates [8]. Upon Cyclin binding, conformational changes in the Cdk cause the Cyclin Activating Kinase (CAK) to phosphorylate a threonine residue (Thr161 in Cdk1, Thr160 in Cdk2) on the T loop. This in turn exposes the catalytic cleft of the Cdk [8]. Cdk phosphorylation is not always associated with activation. In fact, phosphorylation of two residues, Thr14 and Tyr15, by kinases such as Wee1 and Myt1 inhibits Cdk activity. Thus, a Cdk must be dephosphorylated by the phosphatase Cdc25 on Thr14 and Ty 15 to become fully active [9].

Cyclin D1–Cdk4/6

In humans there are three closely related Cyclin D proteins Cyclin D1, Cyclin D2 and Cyclin D3. These proteins are expressed in an overlapping, semi-redundant fashion in most proliferating cells and collectively regulate the progression of cells through the cell cycle [10]. Transcriptional and translational regulation of CyclinD is controlled by a number of mitogenic factors during G1 phase. Cyclin D is relocalized out of the nucleus during S phase and the protein is degraded, through phosphorylation by GSK-3b [11].

The Cyclin D–Cdk4/6 complex can be activated by CAK-mediated phosphorylation [12]. The principal known substrates of Cdk4 and Cdk6 are the retinoblastoma protein, pRb, and other members of the pocket protein family. When these substrates are in their hypophosphorylated forms, the E2F-facilited transcription of genes essential for DNA synthesis is inhibited [4]. However, when phosphorylated by Cyclin D-Cdk4/6 this initiates the earliest events for preparing cells to enter into S-phase of the cycle. In addition, it is known that Cyclin-D-Cdk4/6 can bind and sequester the Cdk inhibitors, p21 and p27, thereby providing an additional level of control over Cdk activity [12]. Hence, Cyclin D-Cdk4/6 plays a major role in moving cells through early-mid G1 phase of the cell cycle.

Cyclin E–Cdk2

The next Cyclin to be expressed is Cyclin E in mid-G1 phase [13]. There are two members of this family; Cyclin E1 and Cyclin E2, they are closely related and often co-expressed and referred as Cyclin E [14]. Cyclin E preferably binds and activates Cdk2, which then phosphorylates a set of target proteins that facilitate the initiation of S phase [15]. The Cyclin E-Cdk2 complex has been shown to phosphorylate S phase-specific proteins such as NPAT (Nuclear-protein, atxia telagiectasia locus), which is involved in the activation of histone gene transcription. Cyclin E-Cdk2 phosphorylates Rb family proteins on different sites than Cyclin D-Cdk4-6. These phosphorylation sites are critical for full inactivation of the Rb proteins, thereby initiating movement of cells into S-phase of the cell cycle [16]. Another crucial task of the Cyclin E-Cdk2 complex is to phosphorylate the Cdk inhibitor p27 and Cyclin E itself, targeting both of these critical

proteins for degradation [17]. Cyclin E is regulated by ubiquitin-mediated degradation, through the SCF (Skp1/Cullin/F box) family of ubiquitin ligases [14].

Cyclin A-Cdk2

Cyclin A is expressed after Cyclin E at the G1/S border, and also complexes with Cdk2 [18]. There are two isoforms of the type-A Cyclins, A1 the embryonic form and A2 the somatic form [19]. The S phase transition and the regulation DNA replication are dependent on the activity of Cyclin A-Cdk2. Cyclin A-Cdk2 phosphorylates Cdc6 following the initiation of replication, to trigger relocalization of the critical pre-RC protein to prevent re-replication of DNA [20]. Cyclin A-Cdk2 has also been found to phosphorylate Skp2 and Cdc20, two components of proteolytic pathways involved in degrading proteins primarily in G1/S phase of the cell cycle progression, thereby playing a key role in ensuring unidirectional movement of the cycle [21].

Cyclin B-Cdk1

There are three known B-type Cyclin family members; including Cyclin B1, B2 and B3. Each partner with and activate the G1/M Cdk, Cdk1. Cyclin B1 has been found to be the main mitotic regulator in somatic cells of the three [22]. Although Cyclin B1 is continuously shuttled between the cytoplasm and nucleus throughout interphase, Cyclin B1 accumulates in the cytoplasm because the rate of its nuclear export exceeds its nuclear import [23]. In somatic cells Cyclin B1 is expressed in late S and G2 phases, binding with inactive Cdk1 in mid-G2. This complex is held in an inactive state through the stable inhibitory phosphorylation of the Cdk1 subunit on Thr14 and Tyr15 by the Wee1 and Myt1 kinases, which prevents premature initiation of mitosis [24]. At late G2, the Cdc25 phosphatase family remove the phosphates on Tyr15 and Thr14, prohibiting activation of

the complex [25]. Entry into mitosis requires this active complex to relocalize quickly to the nucleus. Neither Cyclin B1 nor Cdk1 has a strong Nuclear Localization Signal (NLS). While Cyclin B1-Cdk1 can bind to importin β, the rate at which is it localized to the nucleus does not describe the rapid accumulation at the onset of mitosis [26]. Pines and Hunter suggested that Cyclin B1-Cdk1 complex associates with another protein that has a functional NLS, such as Cdc25 or p21 [27]. The Donoghue group described a relationship between Cyclin B1 and an 'orphan' Cyclin, Cyclin F which may represent a binding relationship involved in the onset of mitosis [28].

Once in the nucleus, active Cyclin B-Cdk1 targets both structural proteins involved in the execution of mitotic events, and regulatory proteins that are necessary for the control and timing of these processes. A novel target of Cyclin B-Cdk1 is the transcription factor FOXO1 (Fork head box O class proteins). The FOXO family of proteins exhibit tumor suppressive functions through the transcriptional control of genes involved in cell proliferation, apoptosis and oxidative stress [29]. Huang and others showed that Cyclin B-Cdk1 phosphorylation of FOXO1 at Ser249 inhibits its transcriptional activity. Hence demonstrating an additional role for Cyclin B1-Cdk1 complex in the regulation of cell fate decisions.

Cdk Inhibitors

Cdk inhibitors are divided into two categories differing in structure and function. The INK4 (^{IN}hibitor of Cdk^4) family of inhibitors, which includes p16^{INK4a} (p16), p15^{INK4b} (p15), p18^{INK4c} (p18) and p19^{INK4d} (p19), specifically target Cyclin D-dependent kinases, at least in part, by preventing Cyclin-Cdk assembly. The CIP/KIP (*Cdk inhibiting protein*) family of inhibitors are composed of p21^{CIP1} (p21), p27^{KIP1} (p27) and p57^{KIP2}.

The latter are characterized by an N-terminal Cyclin–Cdk binding domain and a less well conserved C-terminal domain with structural motifs that are suggestive of a role in protein–protein interactions [30]. In contrast to the INK4 inhibitors which bind only the Cdk subunit, the CIP/KIP inhibitors bind the Cyclin-Cdk complex targeting both Cyclin D-Cdk4/6 complexes and Cyclin E/A–Cdk2 complexes. Structural studies of p27 binding to Cyclin A-Cdk2 have demonstrated that p27 binds to the Cyclin-Cdk interface, with part of the molecule contacting the ATP-binding lobe of the Cdk [12]. This in turn blocks ATP binding and disrupts the conformation of the catalytic cleft, causing Cdk2 activity inhibition. Concentrations of p27 that are sufficient for the inhibition of Cdk2 do not necessarily inhibit Cyclin D1–Cdk4 [31]. Futhermore, a positive role in Cdk regulation has also been suggested by data showing that p21 and p27 enhance the association of Cyclin D-Cdk4 complex by stabilization it, and may facilitate the complex nuclear localization [32]. Hence the CIP/KIP family members have differing activities when bound to Cyclin E/A-Cdk2 and Cyclin D-Cdk4/6 and much more remains to be discovered with regard to these specific activities and their physiological relevance.

p27

p27^{Kip1} plays a crucial role in regulating the response of cells to extracellular signals such as low nutrient levels and contact inhibition and p27 plays a major role in halting cell growth in conditions of cellular aging [33,34]. Although p27 mRNA levels are generally constant throughout the cell cycle, p27 protein levels are under tight translational control [35]. Proteolytic degradation is considered the most important mechanism for regulating p27 levels. In this process, the p27 protein is marked for degradation through a series of phosphorylation events [35]. One pathway leading to p27

proteolytic degradation is mediated via phosphorylation on a C-terminal residue Thr187 by Cyclin-E-Cdk2 [36]. This phosphorylation occurs in late G1-early S phase and facilitates the interaction of p27 with the Skp2-dependent E3 ligase complex, which subsequently results in p27 degradation [35]. Recent data demonstrated that phosphorylation of Tyr88 by oncogenic tyrosine kinases can facilitate Thr187 phosphorylation, leading to p27 degradation at the G1-S transition [37]. Upon cell cycle entry, p27 is captured and sequestered by the Cyclin D-Cdk complex. This will decrease the levels of active p27 to below threshold, thereby allowing the partial activation of Cyclin E-Cdk2 complexes [38]. Consequently, the Cyclin E-Cdk2 complex facilitates its own activation by phosphorylating Thr187 of p27, thereby initiating the degradation of its inhibitor [17]. Some residual molecules of p27 remain bound to Cyclin D-Cdk complexes throughout successive cycles and are only freed when growth factors are removed from the environment. This results in the inhibition of Cyclin E-Cdk and the arrest of the cell in G_1 phase. Thus p27 uptake by Cyclin D-Cdk4 complexes can promote the kinase activity of the Cyclin E-Cdk2 complex and help to establish the order of activation [39].

Like many cell cycle regulatory proteins p27 is also regulated by cellular localization. Phosphorylation of p27 on Ser10 by hKis kinase causes p27 cytoplasmic localization [40], while its phosphorylation at Thr157 by Akt kinase has been shown to impair its nuclear import; mutation of Thr157 to alanine resulted in an exclusively nuclear p27 mutant [41].

Role of p27 as a Tumor Suppressor

The function of p27 as a cell cycle regulator suggests that alterations in level and/or localization could abrogate normal cell cycle progression. In fact several lines of evidence suggest that p27 functions as a tumour suppressor; low p27 expression levels have been recorded in many human cancers, including breast and colon cancers and are correlated with a poor prognosis [42]. p27-null mice develop pituitary adenomas are more susceptible to tumorigenesis in response to radiation and oncogenic retroviruses [43,44]. Mutations in the gene encoding p27 are extremely rare and hence abnormal regulation primarily occurs at the protein regulation and localization level. This is supported by data in mouse models whereby mice lacking p27, or with a reduced p27 gene dosage, are more susceptible to tumorigenesis in response to radiation and oncogenic retroviruses[45]. Reduction of p27 gene dosage also accelerates tumorigenesis in mice lacking alleles of other tumor suppressors, including the retinoblastoma tumor suppressor gene Rb and the phosphatase PTEN (a negative regulator of mitogenic intracellular signaling through PI3K) [46]. p27 deficiency also accelerates lymphomagenesis in Myc-overexpressing lymphoma models [43] and mammary tumorigenesis in mice overexpressing ErbB2 [47]. Recent data has demonstrated a relationship between p27 and ErbB2, a receptor frequently elevated in human breast cancers, and p27 that may be important in the initiation or progression of breast cancer [47]. It was noted that reduced p27 expression is correlated with ErbB2 overexpression, at least in node-negative breast cancer and ErbB2 overexpression and activation reduce p27 stability [48]. Furthermore, antibody-mediated inhibition of ErbB2 or treatment with ErbB kinase inhibitors increases p27 levels and enhance its association with Cyclin E–

Cdk2, leading to cell cycle arrest [49]. Similarly, overexpression of p27 in ErbB2-overexpressing fibroblasts inhibits focus formation *in vitro* and tumor formation *in vivo* [50]. Since a reduction in p27 expression also demonstrates resistance *in vitro* to inhibitors targeted to ErbB receptors, breast cancers with low p27 levels may also negatively respond to these therapies [51]. These data support an important therapeutic relevance for monitoring p27 levels in breast cancer patients, as well as potentially pointing to novel therapeutic strategies for subtypes of the disease.

Cell Cycle Checkpoints

Cellular systems possess control circuits which act as surveillance mechanisms to monitor the completion of crucial cellular events and thereby regulate cell cycle progression. There are two categories of checkpoint mechanisms: an intrinsic surveillance system that operates throughout the cell cycle in order to ensure successive events; and extrinsic mechanisms triggered by the detection of an error in the DNA, or conditions of division, such as DNA damage, misregulation in DNA replication or DNA assembly. Loss of any of these mechanisms reduces the fidelity of cellular events and may lead to uncontrolled proliferation thereby contributing to tumorigenesis [52].

Of the checkpoint mechanisms known DNA damage checkpoints and spindle checkpoints are among the most well studied, in part because of the clear link to human disease. The DNA damage response is largely conserved in yeast and mammalian cells; with the exception that mammalian cells are able to undergo apoptosis since the goal is not the survival of a damaged cell but survival of the organism. The G1/S checkpoint constitutes a primary regulator of the DNA damage response. Three genes associated with this checkpoint are best described to mediate this response: ATM (*ataxia*

telangiectasia mutated) [53], p53 and p21 [54]. At the G1/S checkpoint, cell cycle arrest induced by DNA damage is p53-dependent. Usually, p53 levels are low, but DNA damage can lead to rapid increase of p53 activity [55]. p53 stimulates the transcription of different genes including p21 and Mdm2 [56]. The induction of p21, a CKI, results in Cdk inhibition and cell cycle arrest by inhibiting the replication of damaged DNA [57] . On the other hand, the ubiquitin ligase Mdm2 provides a crucial negative feedback loop by promoting p53 degradation [58].

Different surveillance proteins recognize DNA damage and signal to primary cascade of kinases to initiate the DNA damage signaling pathway; two of the initial kinases activated are ATM, and Ataxia Telangiectasia and Rad3 Related (ATR). In response to DNA damage, ATM/ATR phosphorylate p53 and the transducer kinase Chk2 which in turn further phosphorylates p53 at different sites to increase its transcriptional activity, resulting in p21 blocking the cell cycle, at least at the G1/S checkpoint (Fig. 4) [59]. G2 phase of the cell cycle is also capable of responding to the DNA damage signal; in this phase cells are able to initiate a cell cycle arrest in the presence or absence of p53. The entry into mitosis is prevented by maintaining Cdk1 in its inhibited form through inactivating phosphorylation or by sequestration of components of the Cdk1- Cyclin B complex [60]. This is achieved by the protein kinases Chk1 and Chk2, which are activated upon DNA damage in an ATM-dependent manner that phosphorylate Cdc25 [61]. Phosphorylation of Cdc25 inhibits its activity and promotes its binding to 14-3-3 proteins, sequestering it outside the nucleus and preventing it from activating Cdk1- Cyclin B and mitotic entry [62]. Figure 1 is a schematic diagram of some of the primary regulators of cell cycle arrest upon DNA damage. Genes coding for p53 and *ATM* are

frequently mutated in human cancers suggests that the function of checkpoints is important in cancer prevention [63] .
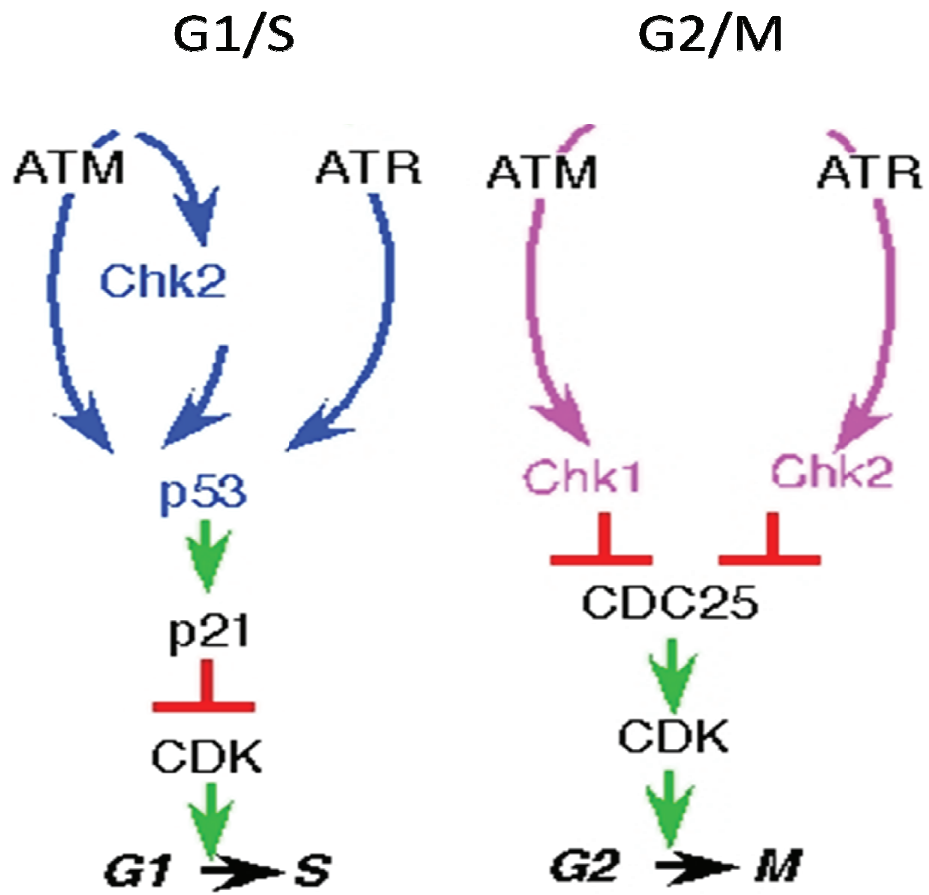
Figure 1. Schematic diagram showing the cellular response to DNA damage.

Posttranslational Modifications (Ubiquitination)

Protein ubiquitination is the main mechanism involved in the selective degradation or processing of intracellular proteins in eukaryotic cells. In this process, the addition of multiple ubiquitin molecules to a target protein is followed by its degradation in a large protease complex, the 26S proteasome. The broad biological role of the ubiquitin–proteasome proteolytic system (UPS) has been identified to encompass gene expression, cellular stress response, antigen presentation, DNA repair, programmed cell death, the cell cycle and tumorigenesis [64].

The covalent attachment of ubiquitin, a highly conserved 76-amino acid polypeptide, to lysine residues of a substrate protein, is required for proteasomal degradation. Protein ubiquitination involves a series of three classes of enzymes: the ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme Ubc or E2, and the ubiquitin-protein ligase E3 (Figure 2). E1 first activates one ubiquitin molecule via the formation of a high-energy thiol ester bond between the carboxyl-terminal glycine of ubiquitin and the thiol group of a cysteine residue of E1. The ubiquitin is then transferred to a cysteine residue on one of the E2 members. E2 enzymes may catalyze the attachment of the single ubiquitin to a substrate protein directly, or transfer the ubiquitin to an E3 protein. The E3 enzyme can catalyse the formation of an isopeptide bond between the carboxyl-terminal glycine of ubiquitin and the ε-amino group of lysine residues on a target protein. The attachment of additional ubiquitin molecules by an E3 enzyme eventually results in a multiply (poly) ubiquitinated substrate [65].
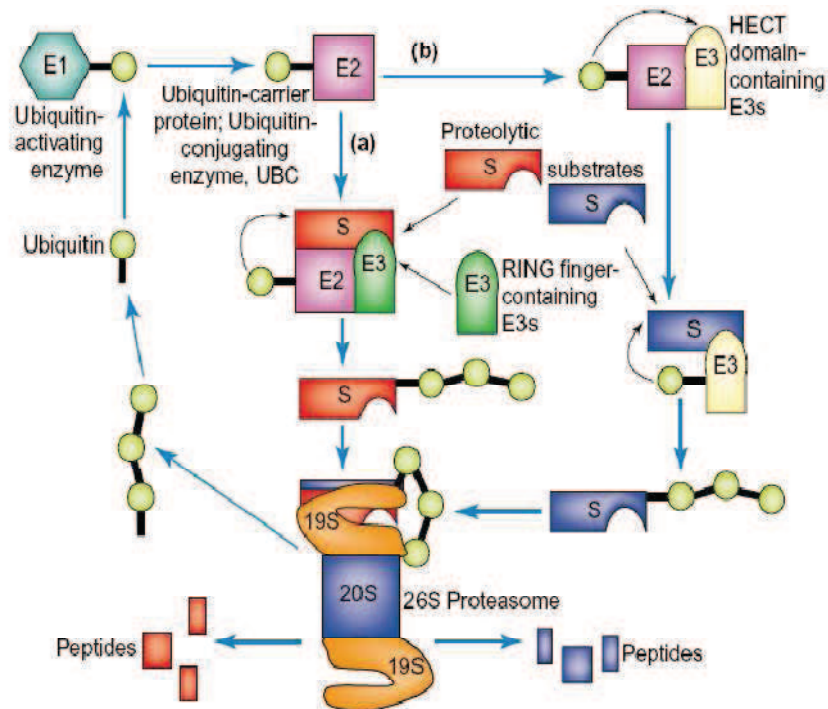
Figure 2. The ubiquitin–proteasome system (UPS). Ubiquitin is first activated to a high-energy intermediate by E1. It is then transferred to a member of the E2 family ofenzymes. From E2 it can be transferred directly to the substrate that is bound specifically to a member of the ubiquitin ligase family of proteins, E3 (a). This occurs when E3 belongs to the RING finger family of ligases. In the case of a HECT-domain-containing ligase (b), the activated ubiquitin is transferred first to the E3 before it is conjugated to the E3-bound substrate. Additional ubiquitin moieties are added successively to the previously conjugated moiety to generate a polyubiquitin chain. The polyubiquitinated substrate then binds to the 26S proteasome complex, where the substrate is degraded to produce short peptides, and free and reusable ubiquitin is released through the activity of de-ubiquitinating enzymes (DUBs). (Adapted from Ciechanover A., and Ben-Saadon, [66]).

Ubiquitin–Proteasome System in Cancer

A few studies have linked E2 to cancer, while E1 has never been characterized in any tumor [67]. On the other hand, numerous reports have indicated that E3 misregulation often promotes cancer development. This usually results from the stabilization of oncogenic products due to a malfunction of their E3 ligases, or the accelerated turnover of tumor suppressor gene products due to overexpression of their responsible E3 ligases [68]. Figure 3 represents a schematic of E3 oncogenic involvement.

An example of E3 enzymes implicated in cancer is the human E6-associated protein (E6AP). E6AP has been identified as a ubiquitin-protein ligase of the tumor suppressor protein p53 in the presence of E6 oncoprotein from human papillomavirus types 16 and 18 [69]. Polyubiquitinated p53 is then rapidly degraded by the 26S proteasome, which may lead to tumorigenesis. Putative E3 proteins from various organisms have been found to contain a conserved region, termed the Hect domain (homologous to E6AP carboxyl-terminus), of approximately 350 amino acids homologous to the carboxyl-terminus of E6AP [69].

Nedd4 ubiquitin-protein ligase is a member of the Hect-E3 protein ligase family. Nedd4 contains an N-terminal calcium-dependent lipid binding domain (C2), a specific protein–protein interaction domain (WW), and Hect domain in the C-terminal region [70]. Reports have implicated Nedd4 and its yeast homologues Rsp5/Npi1/Pub1 in multiple biological functions, including transcription and membrane transport [71]. Nedd4 has been found to be a proto-oncogene for PTEN, one example of the undeniable biological significance of this class of proteins [72]. Table 1 summarizes the tumor

associated E3 ligases and their substrates. Another example of the importance of the ubiquitin system in cancer biology is highlighted by the regulation of the Cdk inhibitor p27. As discussed previously, p27 is tightly regulated via ubiquitin mediated proteolysis. Elevated levels of p27 have been found in Skp2-knockout mouse tissues and embryonic fibroblast cells [45]. An increasing amount of evidence has shown that high expression levels of Skp2 and Cks1 proteins are found in different human cancers, such as non-small cell lung carcinomas [73]. Therefore, Skp2 and Cks1 are believed to play a pivotal role in the degradation of p27 in human cancers. High levels of another E3 responsible for p27 degradation, Pirh2, are also found to be inversely correlated with p27 levels in human head and neck cancers, as well as poor prognosis for these patients [74].
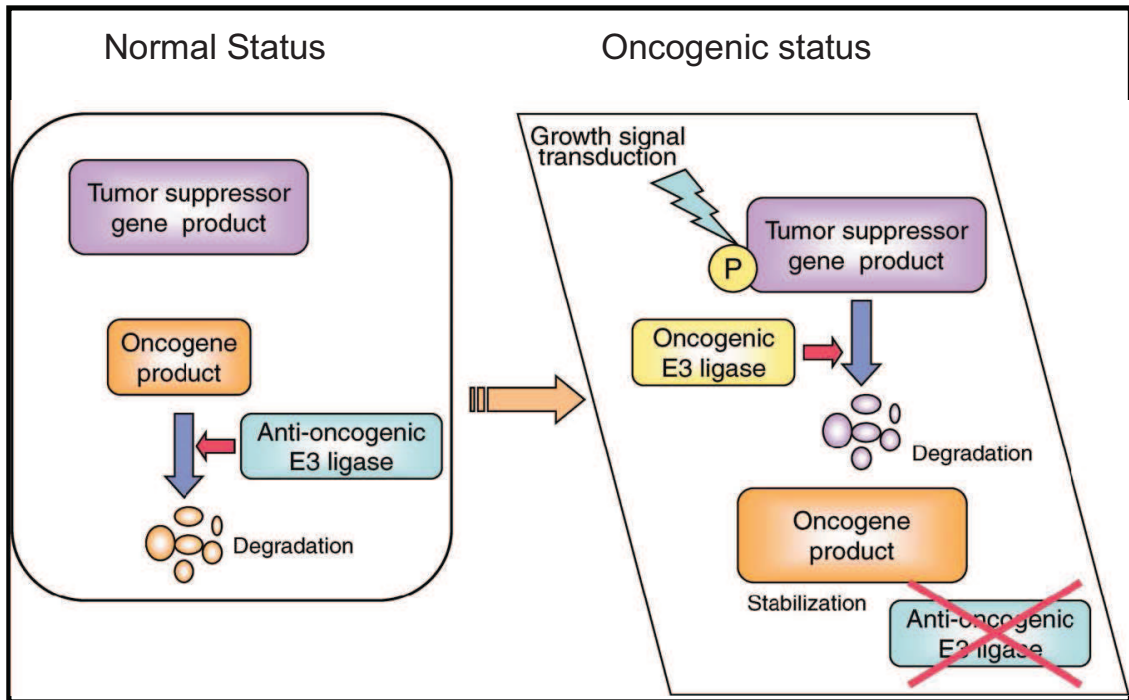
Figure 3. Accumulation of oncogenic products and enhanced degradation of tumor suppressor gene products in cancer cells. In normal cells, tumor suppressor gene products negatively regulate transformation and cancerous growth while oncogene products are quickly degraded by their E3 ligases. Thus E3 ligases which target oncogenes are known as anti-oncogenic E3 ligases. Defects in the anti-oncogenic E3 ligase and overexpression of the oncogenic E3 ligases, which target tumor suppressor gene products, often promote carcinogenesis and cancerous growth (adapted from Kitagawa et al [68]).

Type	Example	Tumor- Associated substrates
HECT	Nedd4-1/2	TβRI Smad2, 4, PTEN
	Smurf	TβRI Smad1, 2, 5, 7
	WWP1	P63, TβRI, Smad2, 4
	Itch	P63, p73, Smad2
	ARF-BP1(Huwe1)	P53
	E6AP-E6	P53
Single RING	Mdm2	P53, RB
	COP1	P53
	Pirh2	P53, p27
	c-Cbl	EGFR
	BRCA1	RPB8, RNAPII, ER
	Arkadia	Smad7
	TRIM32	Abi2
SCF	βTrCP	β-catenin, IκB, cdc25, Wee1, Period.
	Skp2	P27, p57, p130, Tob1,c-Myc
	Fbw7	c-Myc, c-Myb, CyclinE, c-Jun, Notch, mTOR
	FBX4-αCrystallin	Cyclin D1
Cul4	Cdt2/PCNA	Cdt1, p21andRad23B
	CSA	
	XPC	

Table 1. Tumor-associated E3 ubiquitin ligases and their substrates. These ligases are classified into five types: HECT-type, single RING-type, SCF-type, ECV-type, and Cul4-base. Specific E3 ligases and their reported substrates, which are associated with cancer, are also indicated. (Adapted from Kitagawa et al [68])

Speedy/RINGO Family Members

Xenopus Speedy (xSpy) was identified in a screen for genes that conveyed resistance to a *rad1* deficient strain of *Schizosaccharomyces pombe* when treated with UV or gamma irradiation [75]. Shortly after, another group identified a structurally identical protein that they called X-RINGO (Rapid INducer of G2/M progression in Oocytes) [76]. Ectopic expression of xSpy1 in G2 arrested oocytes resulted in meiotic maturation in the absence progesterone [75]. Down regulation of endogenous RINGO caused a delay in oocyte maturation following induction with progesterone [76].

The human homolog of xSpy1/RINGO, Spy1, possesses 40% homology to the *Xenopus* family member [77]. Six mammalian Spy1/RINGO family members have been characterized to date [78]; Table 2 shows a full list of Speedy/RINGO family members along with their tissue and species specificity. The originally characterized Spy1 is Spy1A1, but will be referred to as Spy1 throughout this work. All family members share a conserved core region known as the Speedy/RINGO box found to be crucial for Cdk binding [78].

Name	Alternate Name	Tissue Expression	Species	Length (AA)	Cdk Preference
X-RINGO	X-RINGO A, Is26	Oocyte	*Xenopus laevis*	299	Cdc2/Cdk2
X-Spy 1	X-RINGO B, Is27	Oocyte	*Xenopus laevis*	298	Cdc2/Cdk2
Speedy/ RINGO A1	Spy A 1, RINGO 3	Ubiquitous (high in testis)	*Homo sapiens/ Mus musculus*	286/ 283	Cdk2
Speedy/ RINGO A2	Spy A2	Ubiquitous (high in testis)	*Homo sapiens/ Mus musculus/ Sus scrofa*	313/ 310/ 311	Cdc2/Cdk2
Speedy/ RINGO B	RINGO 4	Testis only	*Mus musculus*	268	Cdc2
Speedy/ RINGO C	RINGO 2	Testis, liver, placenta, bone marrow, kidney, small intestine	*Homo sapiens*	293	Cdc2/Cdk2
Speedy/ RINGO D	RINGO 5	NA	*Mus musculus*	339	NA
Speedy/ RINGO E	RINGO 1	NA	*Homo sapiens*	336	Cdc2/Cdk2/Cdk5

Table 2. Members of the Speedy/RINGO family. Known Spy1/RINGO family members with their tissue and species specificity and preferred binding partners indicated. Modified from Gastwirt [79].

Spy1 Regulation of the Mammalian Cell Cycle

In mammalian cells, overexpression of Spy1 has been shown to increase cell proliferation and reduce the G1 phase population in comparison to control cells [77]. The acceleration of cell proliferation caused by Spy1 overexpression is dependent on Cdk2 activation, while knockdown of endogenous Spy1 using siRNA caused a significant reduction in Cdk2 kinase activity and subsequent increase in cell number [77]. These results support that Spy1 is an essential protein in cell proliferation. Interestingly, Spy1 was also found to bind to the cell cycle regulator p27 *in vivo* and *in vitro*, and to co-localize to the nucleus with p27 [80]. Although Spy1 functions like a Cyclin, it was found to bind to the Cdk binding site of p27 rather than the Cyclin binding site. Furthermore, Spy1 was found to enhance cell growth and proliferation in a p27-dependent manner [80]. Interestingly, Spy1-Cdk2 complex was shown to phosphorylate p27 at Thr187 leading to its proteasomal degradation and enhanced cell cycle progression [81]. To date, Spy1 binding and activation of Cdk2 and enhanced p27 degradation is the accepted model by which Spy1 promotes cell proliferation and overcomes p27-mediated cell cycle arrest.

The Role of Spy1 in the DNA Damage Response

In response to DNA damage, cells activate checkpoints to inhibit cell cycle progression and allow DNA damage repair, or program themselves to undergo cell death (apoptosis). As described in more detail above, different mechanisms are in place to ensure that Cdk activity is precisely controlled upon DNA damage, including Chk1/Chk2-mediated phosphorylation and degradation of Cdc25 phosphatases, and p53-mediated Cdk inhibition by p21 transcription [82].

Overexpression of Spy1 was demonstrated to prevent Cdk inhibition, cell cycle arrest and promote cell survival following treatment of genotoxic substances, such as cisplatin and camptothecin [83]. It was later demonstrated that these effects are due to the inhibition of apoptosis rather than an increase in cell proliferation [84]. To examine the role of Spy1 in ultraviolet-induced apoptosis, cells overexpressing Spy1 were exposed to different doses of UV radiation in comparison to mock cells. Cells overexpressing Spy1 displayed less sensitivity to DNA damage and expressed fewer apoptotic markers such as AnnexinV and caspase activation, these results were found to be p53 and Cdk2 dependent [85].

Mechanistically, inhibition of Cdk2 by small molecule inhibitors or by siRNA has been shown to activate checkpoint signaling leading to Chk1 feedback, and activating the specific checkpoint [86]. These results demonstrate the importance of Cdk2 inhibition in checkpoint activation upon DNA damage. Thus, full activation of Cdk2 may negatively affect checkpoint activation. Spy1 overexpression results in inhibition of Chk1 activating phosphorylations, and also prevents phosphorylation of both the single strand binding protein RPA and the Histone variant H2A.X [84]. Cells expressing a Cdk2 non-binding mutant of Spy1 fail to demonstrate these effects on checkpoint signaling, demonstrating the essentiality of Spy1-Cdk2 interaction in this phenomenon [84]. Figure 4 shows a proposed model of the effect of Spy1 activation of Cdk2 on the DNA damage response.
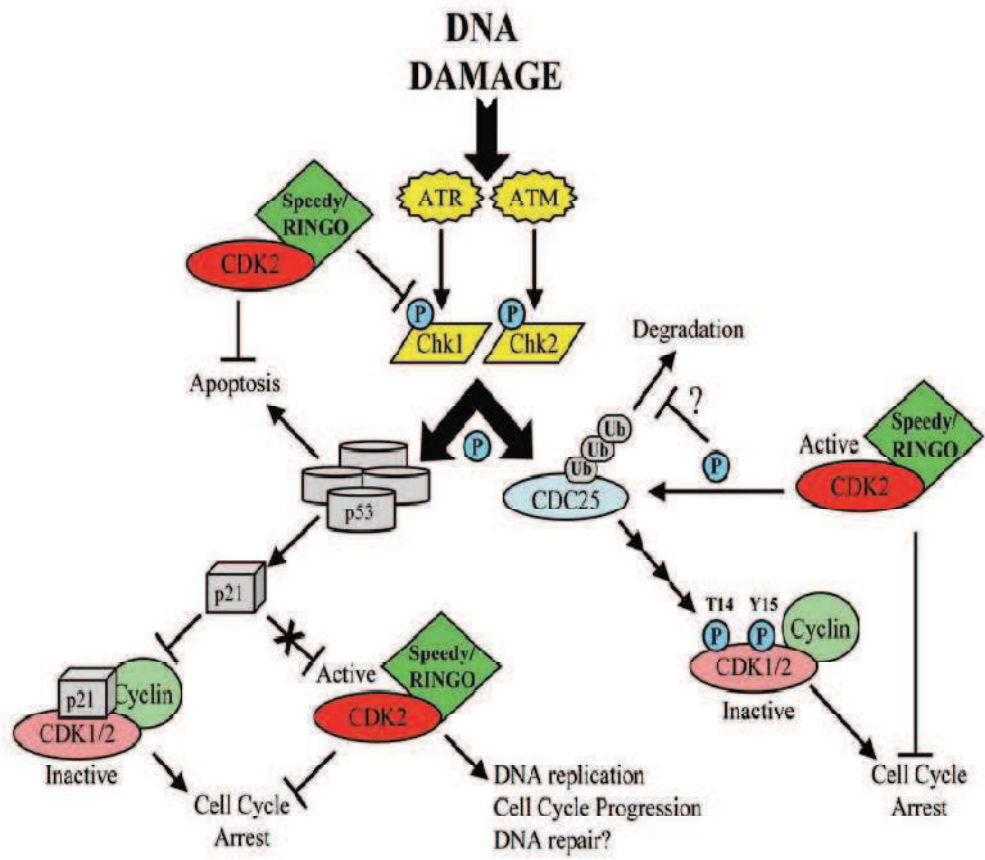
Figure 4. Proposed role of Spy1/RINGO in regulation of DNA damage responses. An overall perspective is presented on the role of Spy/RINGO in regulating CDKs in response to DNA damage and thereby affecting cell cycle progression and apoptosis. (adapted from Gastwirt et al. [79])

The Role of Human Spy1 in Tumorigenesis and Normal Mammary Gland Development.

The initial evidence linking Spy1 to breast cancer came from a SAGE analysis study by Zucchi and colleagues in 2004, where they found Spy1 is among 50 genes upregulated in breast ductal carcinoma [87]. Following this, Golipour et al performed a comprehensive study showing the role of Spy1 in mammary gland development and tumor formation [88]. Spy1 protein and RNA levels are tightly regulated during the development of the mammary gland [88]. Spy1 levels were elevated in the proliferating virgin gland and remained elevated in early stages of pregnancy. By late pregnancy, Spy1 levels dropped dramatically, coincident with terminal differentiation of the gland. Utilizing the BALB/c mouse cell line (HC11) Spy1 exhibited abrogated cellular morphology and failed to form the proper acini in cell culture. Moreover, Spy1-overexpressing HC11 cells were able to accelerate tumor formation when transplanted into the cleared fat pads of BALB/c mice. Glands transplanted with HC11-Spy1 developed visible tumors before 14 weeks post surgery while control glands were unable to form tumors in the same time interval. This study clearly indicates that Spy1 protein may have the ability to accelerate tumor formation and may play a potential role in cancer initiation [88] . Shortly after, Qing and colleagues found high levels of Spy1 in hepatocellular carcinoma (HCC) [89]. It was also found that Spy1 was overexpressed in HCC samples as compared with the adjacent normal tissue. They also were able to correlate high levels of Spy1 with a poor prognosis for hepatocellular carcinoma patients [89].

Collectively, these studies along with the known roles of the primary effectors of Spy1, Cdk2 and p27, in carcinogenesis clearly indicate that Spy1 plays a major role in cancer development and/or initiation, and paves the way for further investigation of the possible roles of Spy1 in tumorigenesis.

Hypothesis and Objectives

This research aims to elucidate how Spy1 protein is regulated and modified throughout the cell cycle as well as to investigate the possible role of Spy1 protein levels in breast cancer initiation and progression. Our working hypothesis is that: *misregulation of the Spy1 protein is a critical event in the initiation of breast cancer and thus the promotion of Spy1 degradation will represent a novel and specific therapeutic strategy in the treatment of breast cancer.*

This hypothesis will be tested through the following objectives:

- To elucidate the molecular mechanisms controlling Spy1 regulation throughout the cell cycle.

- To determine the functional significance of Spy1 degradation in normal and abnormal growth conditions.

- To elucidate the importance of the Spy1-p27 interaction for cell cycle progression.

The results from these questions will provide critical information with regard to the regulation of a novel cell cycle regulator. Future work may provide promise toward the development of novel drug targets and/or biomarkers in the treatment and detection of breast cancer.

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Chapter 2
The Cyclin-Dependent Kinase activator, Spy1A, is Targted for Degradation by the Ubiquitin Ligase NEDD4

Introduction

Members of the Speedy/RINGO family are unique cyclin-like regulators of the cell division cycle. There are now four members characterized in mammals exhibiting distinct tissue expression patterns and functional specificity [1]. The originally characterized family member Spy1A1, herein referred to as Spy1A, is expressed constitutively in most human tissues; it shortens the G1/S transition through activation of CDK2 and is essential for cell proliferation to occur [2]. Activation of CDKs by Spy1/RINGO proteins is thought to occur in an atypical fashion, independent of cyclin binding and in the absence of CDK phosphorylation within the T-loop [3]. Spy1A can also act in a unique fashion to prevent inhibition of CDK2 by $p27^{Kip1}$ (p27), this occurs through direct interactions with the p27 protein and results in enhanced degradation of p27 [4,5]. At a cellular level Spy1A also plays a role in the DNA damage response, functioning to enhance cell survival and promote cell proliferation in lieu of apoptosis [6,7]. Our lab and others have demonstrated that Spy1A is capable of promoting precocious development and tumorigenesis in the mammary gland and that Spy1A protein levels are implicated in invasive ductal carcinoma of the breast [8,9]. Hence, determining how Spy1A protein levels are regulated may reveal novel information regarding the dynamics of cell cycle control during normal and abnormal growth conditions.

In mammals, Spy1A mRNA is known to be up-regulated during G_1/S; however regulation at the protein level has not been studied [2]. The *Xenopus* homologue of Spy1A, X-Spy1, has been shown to undergo steps of proteasome dependent processing and degradation in a manner dependent on the initiation and progression of meiotic events [10]. Degradation of X-Spy1 occurs following meiosis I and is mediated by the ubiquitin ligase Siah-2, this depends on phosphorylation of X-Spy1 on a carboxy terminal

residue S243 [10]. Cyclin proteins in general are tightly regulated temporally and spatially through the cell cycle, controlled on a fundamental level by the ubiquitin-proteasome system (UPS). The UPS is the primary mechanism involved in the selective degradation of intracellular and membrane-bound proteins, and aberrations in this critically important system are correlated to many diseases including cancer [11,12]. Ubiquitination involves the conjugation of ubiquitin to a substrate protein via a concerted effort from three classes of enzymes: the ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme E2, and the ubiquitin-protein ligase E3 [13]. The E3 enzyme catalyzes the formation of a chain of ubiquitin molecules which then targets the substrate protein for degradation by the 26S proteasome [12,14,15]. Given the functional cyclin-like properties of Spy1A it is a valid hypothesis that Spy1A may also be subject to a cell cycle dependent ubiquitin-mediated proteolysis, however whether the mammalian somatic cell cycle regulates this critical protein in the same manner as that seen during oocyte maturation in *Xenopus* warrants investigation.

Herein we demonstrate that Spy1A is ubiquitinated and degraded during G_2/M phase of the cell cycle. We have determined 3 key amino acids within the N-terminal region of Spy1A which are essential to support regulated degradation of the protein and we have demonstrated that the C-terminal region, known to regulate X-Spy1 degradation, is dispensable for degradation of the mammalian homologue. We have resolved that the E3 ligase Neuronal-Precursor-Cell-Expressed Developmentally Downregulated-4 (Nedd4) is capable of binding to Spy1A, and that dominant negative forms and knockdown of Nedd4 reduce ubiquitination and subsequent degradation of Spy1A. Furthermore, we show that non-degradable forms of Spy1A do not trigger intrinsic cell

cycle checkpoints but rather promote cell proliferation; demonstration that this mechanism may contribute to tumorigenesis.

Experimental procedures

Cell Culture - Human mammary breast cancer cells, MCF7 (ATCC) and human embryonic kidney cells, HEK293 (293; ATCC), were maintained in DMEM medium (Sigma) containing 2mM L-glutamine (Sigma), penicillin and streptomycin (Invitrogen), and were cultured in a 5% CO_2 environment. MCF7 cells were supplemented with 10% (vol/vol) fetal calf serum (Sigma) and 293 cells were supplemented with 10% fetal bovine serum.

Plasmids and Mutagenesis - The Nedd4-PCEP plasmid (Nedd4), dominant negative Nedd4-PCEP plasmid (Nedd4[DN]) and empty vector control (PCEP) were provided by Dr. Dale S. Haines (Temple University School of Medicine). HA-Ubiquitin (HA-Ub) was provided by Dr. Sylvain Meloche (Université de Montréal). Creation of Myc-Spy1A-PCS3 vector was described previously (Porter et al 2002). QuikChange Multi-Site-Directed Mutagenesis (SDM; Stratagene) was used to incorporate new silent sites into the original Spy1-pJT0013 vector [2] in order to facilitate the cloning of deletion mutants A (DMA), B (DMB), C (DMC), G (DMG) and Z (DMZ). A BglII site was inserted by altering nucleotide 256 from T to C using the primers #A043 5'-GACGATTTAATTCAAGATCTCTTGTGGATGGACTGCTGC-3' and #A044 5'-GCAGCAGTCCATCCACAAGAGATCTTGAATTAAATCGTC-3' to construct the pRA01 vector. Using the pRA01 plasmid a Mlu site was also added by altering nucleotide 175 from C to G using #A004 5'-CAACAAATCTAAACGCGTCAAAGGACCTTGTCTGG-3' and #A005 5'-

CCAGACAAGGTCCTTTGACGCGTTTAGATTTGTTG-3' to make the vector pRA02. The pRS2 vector was constructed from Spy1-pJT0013 by creating an NdeI site just after the stop codon using the primers #A045 5'-GTCTTGTGTCCATATGTGTTTTGTGGTGACCC-3' and #A046 5'-GGGTCACCACAAAACACATATGGACACAAGAC-3'. The pRS1 vector was constructed by creating a MluI site in the Spy1-pJT0013 plasmid by altering nucleotide nucleotide 175 from C to G using primers #A004 5'-CAACAAATCTAAACGCGTCAAAGGACCTTGTCTGG-3' and #A005 5'-CCAGACAAGGTCCTTTGACGCGTTTAGATTTGTTG-3'. DMA was created by digesting wild-type Spy1A (in pRS1) with NdeI and MluI in order to remove the first 57 amino acids of the protein. DMB was created by digesting wild-type Spy1A (in pRA02) with MluI and BglII in order to remove 27 amino acids. DMC was created by digesting wild-type Spy1A (in pRA01) with BglII and NcoI in order to remove 61 amino acids. DMG was created by digesting wild-type Spy1A (in pJT0013) with NcoI and BbsI in order to remove 94 amino acids. Finally, DMZ was created by digesting wild-type Spy1A (in pRS1) with BbsI and NdeI in order to remove the last 47 amino acids. Gel electrophoresis of these digestions was run on a 1% agarose gel; the desired band was excised and gel-extracted (Bio Basics) for ligation using T4 DNA ligase (Fermentas). For all five deletion mutants, linkers containing a silent restriction site, PstI, and complementary sticky ends were designed, commercially synthesized (Sigma), annealed and utilized in the ligations. In each case, 20 µL ligation reactions were carried out at 22°C for 2-4 hrs. containing a 1:3 vector to linker ratio. Ligations were transformed into DH5α cells and selected for ampicillin resistance, mini-prepped, and digested with PstI

(Fermentas) to detect the correct ligation. The five Spy1A deletion mutants (depicted in Fig. 3A), spanning the length of the gene, were moved from the pJT0013 into pCS3 using EcoRI and XbaI sites flanking the gene.

SDM was also carried out using the PCS3 vector to generate the Spy1A-T15A, Spy1A-T33A, Spy1A-S22A and Spy1A-S247A mutants. Spy1A-T15A was designed using the primers #A151 5'-GAGACACCACCTACTGTCGCTGTTTATGTAAAATCAG-3' and #A152 5'-CTGATTTTACATAAACAGCGACAGTAGGTGGTGTCTC-3'; Spy1A-T33A was designed using the primers #A-153 5'-CAGCCTAAAAAGCCCATTGCACTGAAGCGTCCTATTTG-3' and #A154 5'-CAAATAGGACGCTTCAGTGCAATGGGCTTTTTAGGCTG-3'; Spy1A-S22A was designed using the primers #A139 5'-GTTTATGTAAAATCAGGGGCCAATAGATCACATCAGC-3' and #A140 5'-CTGATGTGATCTATTGGCCCCTGATTTTACATAAAC-3; Spy1A-S247A was designed using the primers #A143 5'-GGATTGTCTTCATCATCAGCGTTATCCAGTCATACTGCAGGGGTG-3' and #A144 5'- CACCCCTGCAGTATGACTGGATAACGCTGATGATGAAGACAATCC-3'. Successful cloning in all cases was determined by DNA sequencing (Robarts Sequencing Facility; Univ. of Western Ontario).

Inhibitors and Antibodies - The following antibodies were used: Spy1A (NB 100-2521; Novus), Nedd4 (ab14592; Abcam), Myc (9E10 and C19; Santa Cruz), HA (Y11 and F7; Santa Cruz), Actin (MAB1501R; Chemicon), Cyclin E (551157; BD Pharmingen), IgG (SC66186; Santa Cruz). The following inhibitors were used: N-Acetyl-L-leucyl-L-leucyl-

L-norleucinal N-Acetyl-Leu-Leu-Norleu-al (LLNL; Sigma A6060); MG132 (Sigma C2211); Cyclohexamide (Sigma C7698); nocodazole (Sigma M1404) , thymidine (Sigma T1895) and Lactacystin (Boston Biochem I-116).

Transfections - Calcium phosphate precipitation transfections were carried using 10 µg of DNA per 10 cm tissue culture plate. 250 µL $CaCl_2$ was incubated with the DNA for 30 sec., 250µL 2x BBS at pH 7.01 was added while vortexing and the solution was incubated for 10 min. The mixture was added slowly to the cells and then incubated in 3% CO_2 for 12-16 hrs. Media was then changed and plates were returned to 5% CO_2 for at least 12 hrs. prior to harvest.

Cell Synchronization and Flow Cytometry - 293 cells were synchronized using double thymidine block. Briefly, cells were cultured in media containing 2 mM thymidine for 16 hrs., followed by release into normal media for 8 hrs. and then a second thymidine block for 14 hrs., and then released into media containing 70 ng/ml nocodazole (with or without 10 µM MG132 as indicated). MCF7 cells were synchronized by being cultured in a serum-free media for 48 hrs., followed by release into media containing serum and 70 ng/ml nocodazole. 293 and MCF7 cells were trypsinized at specified times, washed twice in PBS, and then either used immediately or fixed and stored at -20°C. Fixation was carried out by resuspending cells at 2×10^6 cells in 1 mL of PBS, followed by slow addition of an equal amount of 100% ethanol. Within 1 week, fixed cells were pelleted, washed, and resuspended in 300 µL of PBS. Samples were then prepared for flow cytometry by treating with 1 µL of 10 mg/mL stock of DNase free RNase (Sigma) and 50 µL of 500 mg/mL propidium iodide stock solution. Data was collected using a Beckman

Coulter FC500 (Biology Dept.; U of Windsor) and cell cycle profiles were analyzed using CPX Beckman Coulter FC500 software.

Immunoblotting - Cells were lysed in 0.1% NP-40 lysis buffer (0.1% NP-40, 1M Tris pH 7.5, 0.5M EDTA, 5M NaCl) containing protease inhibitors (PMSF 100 µg/mL, aprotinin 5µg/mL, leupeptin 2µg/mL) for 30 min on ice. Bradford Reagent was used to determine the protein concentration following the manufacturer's instructions (Sigma). Aliquots of lysates containing 20-30 µg protein were subjected to electrophoresis on denaturing SDS-10% polyacrylamide gels and transferred to Polyvinylidene Fluoride-Plus transfer membranes (Osmonics Inc.) for 2 hrs. at 30V using a wet transfer method. Blots were blocked for 2 hrs. in TBST containing 3% non-fat dry milk (blocker) at room temperature. Primary antibodies were reconstituted in blocker and incubated over night at 4°C at a 1:1000 dilution for all antibodies, and secondary antibodies were used at a 1:10,000 dilution in blocker for 1 hr at room temperature. Blots were washed three times with TBST following incubation with both the primary and secondary antibodies. Washes were 6 min each following the primary antibody and 10 min each following the secondary antibody. Chemilumiminescent Peroxidase Substrate was used for visualization following the manufacturer's instructions (Pierce). Chemiluminescence was quantified on an Alpha Innotech HD2 (Fisher) using AlphaEase FC software.

Immunoprecipitation reactions were carried out using equal amounts of protein (~200µg/mL) incubated with 2 µg of primary antisera, as indicated, overnight at 4°C. This was followed by the addition of protein A–Sepharose (Sigma) and incubated at 4°C with gentle rotation for an additional 2 hrs. Complexes were washed extensively with 0.1% NP-40 lysis buffer and resolved by 10% SDS-PAGE.

In Vivo Labeling - 293 cells were treated with 10µM MG132 and 70 ng/ml nocadozol for 14 hrs. followed by incubation in phosphate-free media for 2 hrs. and cells were labeled with [^{32}P]orthophosphate (0.3 mCi/ml) (PerkinElmer) for 4 h at 37°C. Cells were lysed and immunoprecipitated with Myc antisera. Immunoprecipitations were washed rigorously with TBST and samples were analyzed by 10% SDS page gel. Gels transferred to PVDF membranes were visualized using a Cyclone phosphoimager and quantified using OptiQuant software (Perkin Elmer; Biology Dept.; U of Windsor).

In Vivo Ubiquitination Assays - 293 cells were plated and transfected appropriately in a 100-mm dish. 24 hrs. after transfection cells were treated with 10 µM MG132 for 14 hrs. Cells were then collected, pelleted by centrifugation, lysed in 200 µl of preboiled lysis buffer [50 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 1% SDS, and 1 mM DTT], and further boiled for an additional 10 min. Lysates were clarified by centrifugation at 13,000 rpm on a microcentrifuge for 10 min. Supernatant was diluted 10 times with 0.5% NP40 buffer and immunoprecipitated with anti-Myc antibody. Immunoprecipitates were washed 3 times and resolved by 10% SDS-PAGE, followed by immunoblotting with anti-HA antibody.

siRNA Knockdown Experiments – siRNA against Nedd4-1 was synthesized by inserting the oligo 5'GATGAAGCCACCATGTATA into the pSUPER-basic vector, as previously described [16]. As a control, LacZ siRNA (siCntl) was synthesized and inserted into pSUPER-basic vector as described (21). 293 cells were transfected using 12 µg of either Neddd4 siRNA or siCntl per 100 mm tissue culture plate and total protein was isolated from cell cultures and resolved using 12% SDS polyacrylamide gels as described above.

Results

Spy1A protein levels are regulated in a cell cycle dependent fashion.

MCF7 and 293 cells were blocked in G_1 using serum starvation and thymidine block procedures respectively. Cells were released into serum and nocodozole containing media and populations enriched in G1 or G_2/M phases of the cell cycle were collected as determined by flow cytometry analysis (Fig. 1A and C). Cell lysates from the respective populations were immunoblotted for endogenous Spy1A expression (Fig. 1B & D; upper panels). Cyclin E was utilized as a control for the cell cycle stage (Fig. 1B; middle panel), and Actin was used as the loading control (Fig. 1B and D; lower panels). These data demonstrate that Spy1A protein levels are greatly decreased during G_2/M phase of the cell cycle and support that, like many important cell cycle proteins, Spy1A is tightly regulated in a cell cycle dependent fashion.

FIGURE 1. *Spy1A protein levels are regulated in a cell cycle dependent fashion*. (A) Upper panel; Flow cytometry profiles for MCF7 cells either untreated (Cntl), collected immediately following release from serum starvation (G1) or collected 16 hrs. following release into nocodozole containing media. Lower panel; Percentage of cells in each phase of the cell cycle as determined by flow cytometry analysis software. (B) Cell lysates from each population described in A were immunoblotted with α-Spy1A, α-Cyclin E and α-Actin. (C) Upper panel; Flow cytometry profiles for 293 cells either untreated (Cntl), blocked by double thymidine block (G1) or blocked and then released into media containing serum and nocodazole (G2). Lower panel; Percentage of cells in each phase of the cell cycle as determined by flow cytometry analysis software. (D) Cell lysates from each population described in C were immunoblotted with α-Spy1A and α-Actin.

A

Cntl   G1   G2

G0G1
24.7%

S
4.3%

G2M
63.0%

G0G1
60.5%

G0G1
86.5%

G2M
22.6%

S
4.5%
4.3%

% Cells

100
90
80
70
60
50
40
30
20
10
0

Cntl   G1   G2

■ G1
■ S
■ G2/M

B

Cntl   G1   G2

Spy1A →

IB: Spy1A

Cyclin E →

IB: Cyclin E

Actin →

IB:Actin

C

Cntl   G1   G2

G0G1
54S:%

14 6%
G2M
26.8%

G0G1
96.3%

S
3.4%
G2M
0.9%

G0G1
8.8%

S
11.6%

77.2%

% Cells

120

100

80

60

40

20

0

Cntl   G1   G2

■ G1
■ S
■ G2/M

D

Cntl   G1   G2

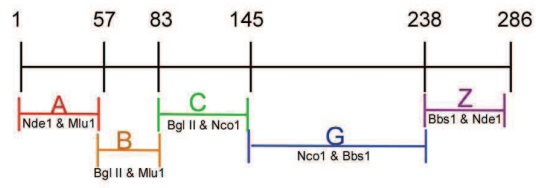Spy1A →

IB: Spy1A

Actin →

IB: Actin
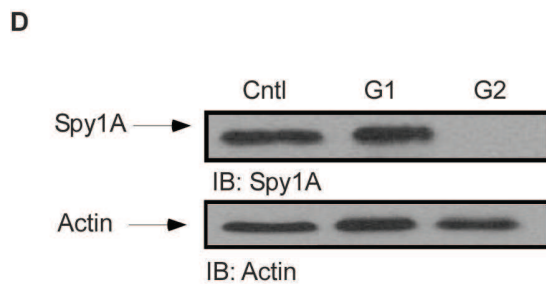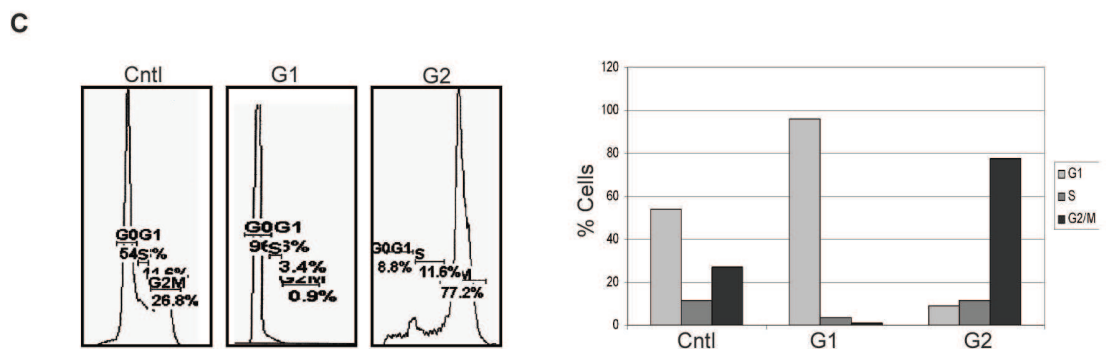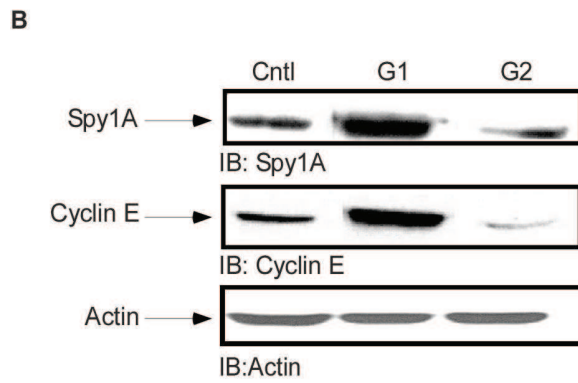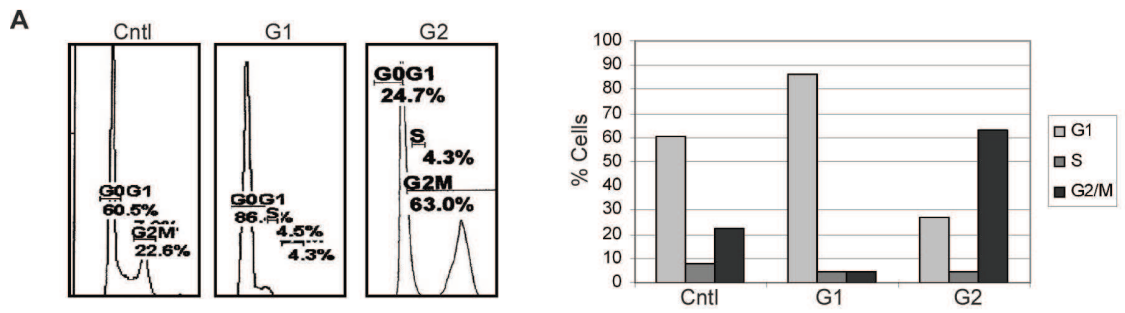
Spy1A degradation depends on phosphorylation within the N-terminal region.

Using a panel of Spy1A deletion mutants (Fig. 2A), we began to narrow down the region within the Spy1A protein that was necessary for degradation. We first determined whether deletion of any of the regions of Spy1A would result in stabilization of the protein. 293 cells were transfected with wild-type Spy1A or deleted versions of the Spy1A protein, DMA-DMZ. Cells were synchronized at G_2/M and levels of Spy1A were monitored by immunoblotting (Fig. 2B; upper left panel). All deletion mutants of Spy1A were degraded by G_2/M phase with the exception of the mutant lacking the first 57 amino acids (DMA). Asynchronous cells demonstrate that all deletion mutants were expressed (Fig. 2B; upper right panel). Collectively, these data demonstrate that the N-terminal region of Spy1A is essential to mediate degradation of the protein and that unlike the *Xenopus* homolog of Spy1 the C-terminal region is dispensable for degradation.

Phosphorylation is often the key event regulating recognition of the substrate protein by the E3 [11]. To determine whether deletion of the N-terminal region of Spy1A altered the phosphorylation status of the protein, orthophosphate labeling was performed on G2 populations of cells expressing either wild-type or Spy1A deleted of its N-terminal region (DMA) in the presence of MG132. Over 3 experiments a significant decrease in the incorporation of orthophosphate was repeatedly observed when the N-terminal region of Spy1A was deleted (Fig. 2C). From this information, we conclude that there is at least one phosphorylation site present within the N-terminal region of Spy1A that may play a significant role in regulating Spy1A stability.

FIGURE 2. *Spy1A degradation relies on the N-terminal region.* (A) A schematic diagram for the different Spy1A deletion mutants is depicted and restriction sites used for cleavage of the region are indicated. (B) 293 cells were transfected with Myc-Spy1A-PCS3 (wt) or the different deletion mutant DMA-DMZ (A-Z). Transfected cells were treated with nocodazole (left hand panels; synchronous) or no treatment (lower panel; asynchronous) for 16 hrs. post transfection. Lysates were immunoblotted with α-Myc or α-Actin. (C) 293 cells were transfected with Myc-Spy1A-PCS3 (wt) or deletion mutant A (DMA). 12 hrs post-transfection cells were treated with nocodazole and MG132 for 14 hrs. followed by orthophosphoric acid ^{32}P for an additional 4 hrs. Cells were lysed, immunoprecipitated with α-Myc and imaged on a Cyclone phosphorimager (upper blot). Immunoblot for α-Myc was used as a control (lower blot). Incorporation of ortho-phosphate was quantified using OptiQuant software; right panel represents results of one representative experiment of 3.

A

B

Synchronous Population

Deletion mutants

wt   A   B   C   G   Z

Myc-Spy1A →

IB:Myc

Actin →

IB:Actin

Asynchronous Population

Deletion mutants

wt   A   B   C   G   Z

IB: Myc

IB:Actin

C

MG132

wt   DMA
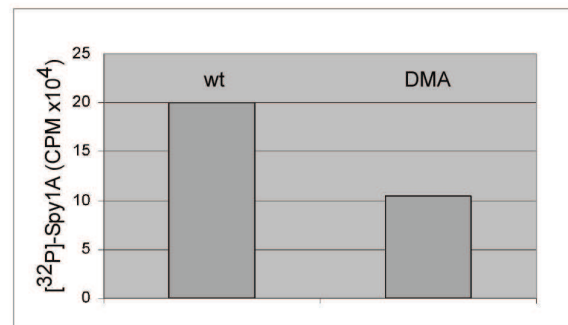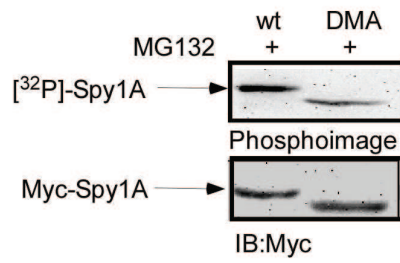 +   +

[^{32}P]-Spy1A →
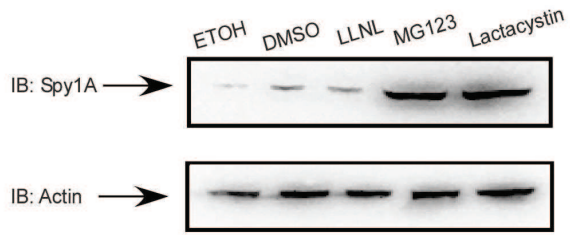
Phosphoimage
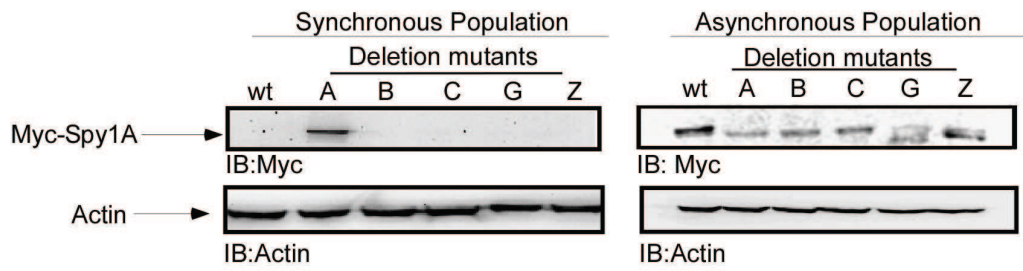
Myc-Spy1A →

IB:Myc

Spy1A steady state levels are proteasome dependent.

After determining the timing of Spy1A degradation during cell cycle progression, we set out to investigate the mechanism by which this occurs. To determine whether this mechanism was proteasome dependent we studied 293 cells in the presence or absence of the proteasome inhibitors MG132, Lactacystin and the calpain inhibitor LLNL. Spy1A protein levels were significantly elevated in the presence of MG132 as well as Lactacystin but not in the presence of the calpain inhibitor or the vehicle controls (Fig. 3A; upper panel; ETOH – vehicle for LLNL, DMSO – vehicle for MG132 and Lactacystin). This data implicates that Spy1A abundance is proteasome dependent. 293 cells were then utilized for an *in vivo* ubiquitination assay where cells were transiently transfected with HA-tagged ubiquitin (HA-Ub) and Myc-tagged Spy1A (Myc-Spy1A) in the presence of MG132 followed by immunoprecipitation with α-Myc (Fig. 3B; lower panel). Immunoblotting with α-HA revealed that Spy1A was labeled with HA-ubiquitin *in vivo* (Fig. 3B; upper panel). The experiment was then repeated using endogenous Spy1A and immunoprecipitation with α-HA. Immunoblotting with α-Spy1A revealed that Spy1A was indeed labeled with HA-ubiquitin *in vivo* (Fig. 3C; upper panel). Collectively these results demonstrate that Spy1 protein levels are regulated via the UPS. To determine whether the stability shown by DMA is due to lack of ubiquitination 293 cells were utilized in an *in vivo* ubiquitination assay. Cells were transiently transfected with HA-tagged Ubiquitin (HA-Ub) and either Myc-tagged Spy1A (Myc-Spy1A), DMA or DMB in the presence of MG132 followed by immunoprecipitation with α-Myc (Fig 3D; lower panel) Immunoblotting with α-HA revealed that Spy1A and TMB were

labeled with HA-ubiquitin *in vivo*, whereas TMA was not. This result demonstrates that lack of ubiquitination is responsible for the stability of the N-terminal deletion of Spy1A.

FIGURE 3. *Spy1A steady state levels are proteosome dependent*. (A) Cells were treated for 14 hrs with a calpain inhibitor (LLNL; 25µM) or proteasome inhibitors (MG132; 10µM and Lactacystin; 10µM) as well as the relevant vehicle control for LLNL (ETOH) and MG132 and lactacystin (DMSO). Lysates were analyzed by immunoblotting with α-Spy1A and α-Actin. (B) 293 cells were transfected with HA ubiquitin (HA-Ub), Myc-Spy1A-PCS3 (Myc-Spy1A) and PCS3 empty vector (Myc-Cntl), 12 hr. post-transfection 10µM MG132 was added for 14hr. α-Myc immunoprecipitations were immunoblotted with α-HA and α-Myc. (C). 293 cells were transfected with HA Ubiquitin (HA-Ub), or empty vector control (PMT123), 12 hr. post-transfection 10µM MG132 was added for 14hr. α-HA immunoprecipitations were immunoblotted with α-Spy1A and α-IgG-mouse. (D) 293 cells were transfected with HA ubiquitin (HA-Ub), Myc-Spy1A, PCS3 empty vector, PMT123 empty vector, Spy1A-deletion mutant A (DMA) and Spy1A deletion mutant B (DMB) 12 hr. post-transfection 10µM MG132 was added for 14hr. α-Myc immunoprecipitations were immunoblotted with α-HA and α-Myc

A

ETOH DMSO LLNL MG123 Lactacystin

IB: Spy1A →

IB: Actin →

B

Myc-Cntl   -   -   +
Myc-Spy1A +   +   -
HA-Ub   +   -   +
MG132   +   +   +

100Kd
70KD
HA-Ub-Spy1A
40KD

IP: Myc
IB: HA

Myc-Spy1A →

IP: Myc
IB: Myc

C

PMT123   -   +
Ha-Ub   +   -
MG123   +   +

100Kd
HA-Ub-Spy1A
70KD
40KD

IP: HA
IB: Spy1A

IgG →

IP: HA
IB: IgG

D

Myc-Spy1A +   -   +   -   -
Ha-Ub   +   +   -   +   +
DMA   -   +   -   -   -
DMB   -   -   -   +   -
PMT123   -   -   +   -   -
PCS3   -   -   -   -   +
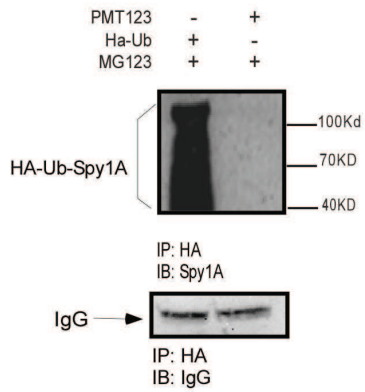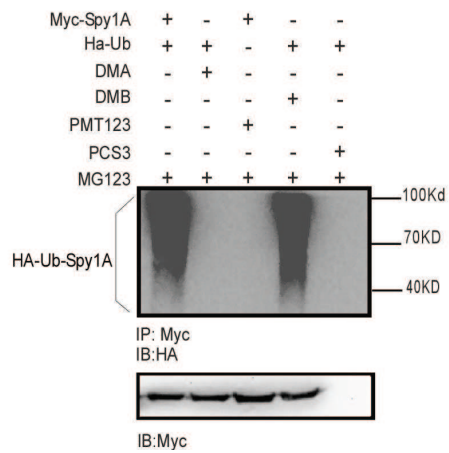MG123   +   +   +   +   +

100Kd
70KD
HA-Ub-Spy1A
40KD

IP: Myc
IB:HA

IB:Myc

53
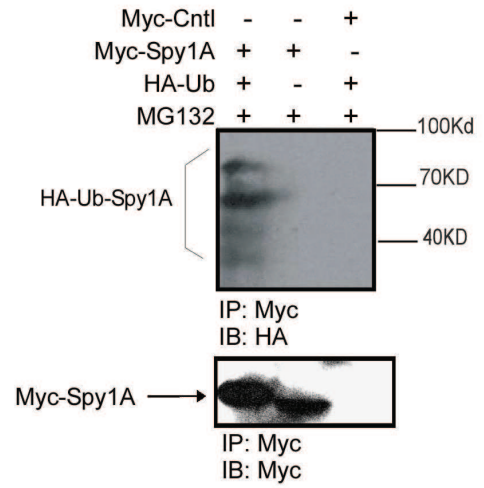
The E3 ligase Nedd4 regulates Spy1A degradation.

There are many different E3 ubiquitin ligase enzymes that are able to function in the ubiquitination pathway. To determine which E3 ligase functions in the degradation of Spy1A a protein blast for the N-terminal region of Spy1A revealed a weak potential interaction region for WW domain containing proteins, PPxxxxY spanning from P11-Y17 in the Spy1A sequence. Immunoprecipitation with endogenous Spy1A followed by coomassie staining revealed a predominant band enriched in G2 populations of cells at approximately 110-115 kD (data not shown). It is known that the 114 kD WW domain-containing ligase Nedd4 (product of <u>n</u>euronal precursor cell-<u>e</u>xpressed <u>d</u>evelopmentally <u>d</u>own-regulated gene 4), while preferring the canonical PPxY sequence, also binds to a variety of proline rich regions with phosphorylated threonine or serine residues to trigger ubiquitination and subsequent degradation [15,17,18]. Due to this we investigated the potential role of Nedd4 in Spy1A degradation. Interestingly, Nedd4 is a family of conserved E3 ubiquitin ligases found to function as both proto-oncogenes as well as tumor suppressors. Nedd4 is known to both mono-, di- and poly-ubiquitinate its target proteins, where polyubiquitinated proteins are selectively targeted for degradation by the proteosome (21). Clarifying the biology of the Nedd4 family and relevant substrates may provide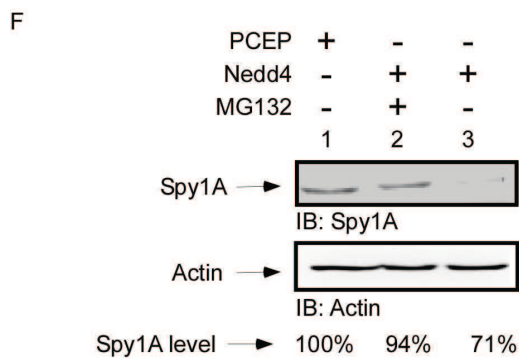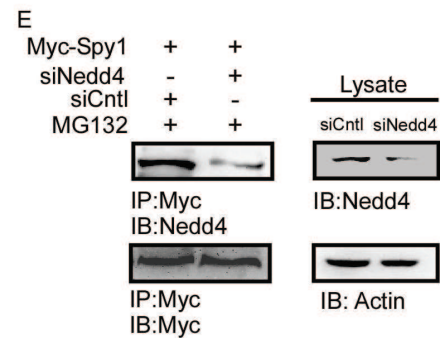 important information for tumorigenesis [19-23]. Co-immunoprecipitation of lysates overexpressing exogenous Nedd4 as well as Myc-Spy1A in the presence of MG132 demonstrates that Nedd4 interacts with Spy1A *in vivo* (Fig. 4A). These results were then confirmed by using endogenous binding in two different cell lines 293 and NIH3T3 (Fig. 4B). To further investigate whether Nedd4 is functioning as an ubiquitin ligase for Spy1A, we repeated the co-immunoprecipitation experiment using

overexpression of wild-type Nedd4 or dominant negative Nedd4 (Nedd4[DN]) in the presence of HA-Ub. Nedd4[DN] contains a single amino acid substitution which prevents the formation of a thioester bond with ubiquitin and hence renders Nedd4 inactive [12,14,15]. Immunoblotting for Spy1A, followed by quantification, revealed that HA-Ub incorporation was significantly decreased in the presence of Nedd4[DN] (Fig. 4C). To further establish whether Nedd4-1 was required for both binding and degradation of Spy1A, 293 cells were transfected with either siNedd4 or an siRNA control (siLacZ; siCntl) followed by immunoblotting with α-Nedd4 (Fig. 4D upper panel), α-Spy1A (Fig. 4D Middle Panel) and α-actin (Fig. 4D lower panel). Densitometry of detected bands demonstrates that Spy1A protein levels are accumulating when Nedd4 levels are reduced with siRNA. To examine the effect of knockdown of Nedd4-1 on the binding interaction between Spy1A and Nedd4, 293 lysates overexpressing Myc-Spy1A and either siNedd4 or siCntl were immunoprecipitated with α-Myc (Fig. 4E lower panel). Immunoblotting with α-Nedd4 shows that Nedd4-Spy1 binding was significantly reduced following knockdown of Nedd4-1 (Fig. 4E upper panel) To assess the effect of Nedd4 on endogenous Spy1A, Nedd4 was transfected into 293 cells in the presence and absence of MG132, and endogenous levels of Spy1A were measured. Overall Spy1A protein levels were consistently decreased in 2 separate experiments by at least 20% when Nedd4 was transiently transfected in the absence of MG132 as compared to when MG132 was present (Fig. 4F). To confirm the effect of Nedd4[DN] on Spy1 protein stability, Nedd4[DN], Nedd4 and Myc Spy1A were transfected into 293 cells. 12 hrs. post-transfection cells were released, at 16 hrs post-transfection 50 µg/ml cycloheximide was added to prevent *de novo* protein synthesis. Over 3 separate experiments immonoblotting for Spy1A,

followed by quantification, showed that Spy1A stability was decreased in the presence of Nedd4 but was in fact significantly more stable in the cells transfected with Nedd4[DN] (Fig. 4G). Collectively, this data demonstrates a novel relationship between two proteins previously implicated in tumorigenesis; Spy1A and Nedd4.

FIGURE 4. *The E3 ligase Nedd4 regulates degradation of Spy1A.* (A) 293 cells were transfected with empty vectors (PCS3 or PCEP), Nedd4-PCEP (Nedd4) or Myc-Spy1A-PCS3 (Myc-Spy1A) and treated with MG132 for 14 hrs prior to harvest. α-Myc immunoprecipitations were immunoblotted with α-Nedd4 (upper panel) and α-Myc (lower panel). Lysates from NIH3T3 cells served as positive control for Nedd4 expression (+; lane 5). (B) 293 and NIH3T3 cells were treated with 10μM MG132 for 14 hr., lysates were immunoprecipitated with α-Nedd4 or α-IgG and immunoblotted with α-Spy1A (upper panel) or α-IgG (lower panel). (C) 293 cells were transfected with empty vectors (PMT123 or PCEP), Nedd4-PCEP (Nedd4), HA Ub-PMT123 (HA-Ub) or Nedd4-PCEP Dominant Negative (Nedd4^{DN}) and treated with MG132 for 14 hrs. prior to harvest. α-HA immunoprecipitations were analyzed by immunoblotting for α-Spy1A (upper panel) and α-HA (middle panel). Lysates were used to demonstrate Nedd4/Nedd4^{DN} transfection (lower blot). Densitometry of Ha-Ub-Spy1A was performed and equalized using α-HA blot (right hand panel). This is one representative experiment of 3. (D) 293 cells were transfected with siRNA against Nedd4 (siNedd4) or siRNA against LacZ (siCntl). Lysates were immunoblotted with α-Nedd4 (upper blot), α-Spy1A (middle blot) and α-Actin (lower blot). Densitometry of Spy1 levels was performed and equalized to the Actin loading control (right hand panel). This is one representative experiment of 3. (E) 293 cells were cotransfected with Myc-Spy1A and either siNedd4 or siCntl, 12 hr. post-transfection 10μM MG132 was added for 14hr. α-Myc immunoprecipitations were immunoblotted with α- Nedd4 (upper panel) or for α-Myc (lower panel). (F) 293 cells were transfected with PCEP empty vector control (lane 1) and Nedd4-PCEP (Nedd4; lanes 2 & 3). Cells were either treated with MG132 (lane 2) or

DMSO (lanes 1 & 3). Lysates were immunoblotted with α-Spy1A and α-Actin. Densitometry for Spy1A levels was carried out and is depicted after equalization with Actin levels (right hand panel). One representative experiment of two. (G) 293 cells were transfected with Myc-Spy1A-PCS3 (Myc-Spy1A) along with either PCEP empty vector (Cntl), Nedd4-PCEP (Nedd4) and, Nedd4-PCEP Dominant Negative (Nedd4^{DN}). 16 hrs. after transfection cyclohexamide was added and cells were collected at 30, 75 and 120 min. time points. Cell lysates were analyzed by immunoblotting for α-myc antibody. Actin was used as a loading control (lower panel). Densitometry over 3 separate experiments is demonstrated in the lower panel. Error bars represent SEM ** = p<0.001. (H) 293 cells were transfected with Myc-Spy1A-PCS3 (Myc-Spy1A) along with either sicontrol (*siCntl*), Nedd4-PCEP (*Nedd4*) and siNedd4. 16 h after transfection cyclohexamide was added, and cells were collected at 30-, 75-, and 120-min time points. Cell lysates were analyzed by immunoblotting for α-Myc antibody. Actin was used as a loading control (*lower panel*). *Lower panels* (G and H*) represent densitometry where Spy1A expression is equalized with actin over three separate experiments. *Error bars* represent S.E. Statistics reflect Nedd4, Nedd4DN, or siNedd4 transfections as compared with control transfections at each time point. *, $p < 0.05$; **, $p < 0.01$.

A

PCS3	+	+	−	−	
PCEP	−	+	−	+	
Nedd4	+	−	+	−	
Myc-Spy1	−	−	+	+	
MG132	+	+	+	+	+
	1	2	3	4	+

Nedd4 →

IP: Myc
IB: Nedd4

Myc-Spy1A →

IP: Myc
IB: Myc

B

NIH3T3   293

IP: Nedd4   IgG   Nedd4   IgG

Spy1A →

IB: Spy1A

IgG →

IB: IgG

C

PCEP	−	−	+	+
MT123	−	−	−	+
HA-Ub	+	+	+	+
Nedd4	+	−	−	−
Nedd4DN	−	+	−	−
MG132	+	+	+	+

HA-Ub-Spy1A

100Kd
70KD
40KD

IP: HA
IB: Spy1A

Nedd4 →

IP: HA
IB: Nedd4

D

siCntl   siNedd4

Nedd4 →

Spy1A →

Actin →

E

Myc-Spy1	+	+
siNedd4	−	+
siCntl	+	−
MG132	+	+

Lysate

siCntl   siNedd4

IP:Myc
IB:Nedd4

IB:Nedd4

IP:Myc
IB:Myc

IB: Actin

F

PCEP	+	−	−
Nedd4	−	+	+
MG132	−	+	−
	1	2	3

Spy1A →

IB: Spy1A

Actin →

IB: Actin

Spy1A level →   100%   94%   71%

59

G

IB:Myc

IB:Actin

H

IB:Myc

IB:Actin

Residues T15, S22, and T33 are essential for Spy1A degradation.

Cell cycle regulatory proteins which are targeted to the UPS rely on signal transduction mechanisms to control the timing of this essential event. We have demonstrated that the N-terminal region of Spy1A is essential for mediating degradation. Hence, we focused on elucidating essential sites within this region that may target the protein for degradation. Utilizing the NetPhos 2.0 Server tool residues T15, S22, and T33 were isolated as potential phosphorylation sites [24]. Site-directed mutagenesis was performed to alter Spy1A residues T15, S22, and T33 to non-phosphorylatable alanines. Additionally we generated a similar mutation at S247 in the C-terminal region to serve as a control. 293 cells were transfected with the relevant constructs prior to synchronization at G_2/M. Surprisingly, mutation of all of T15, S22, and T33 to a non-phosphorylatable alanine prevented degradation and ubiquitination of Spy1A (Figs. 5A & B). Blotting asynchronous cell populations revealed that protein expression was not affected (Fig. 5A; right panel) and flow cytometry analysis demonstrated that effects at T15, S22 and T33 were not due to a failure of the mutant Spy1 expressing cells to properly arrest in G2 phase (Fig. 5E; upper panel). This suggests that phosphorylation, or maintenance of charge of all three of T15, S22, and T33 is essential in regulating the turnover of Spy1A. To further assess the effect of these mutations on Spy1A degradation, 293 cells were transfected and then treated with 50 µg/ml cyclohexamide 16hrs. post-transfection. Immunoblotting for Spy1A showed that cells transfected with the mutants have stabilized Spy1A levels (Fig. 5C). Quantifying 3 separate experiments demonstrate that indeed all 3 mutations significantly enhance the stability of Spy1A protein (Fig. 5C; right hand panel). To assess whether these sites are phosphorylated *in vivo* a triple mutant (Spy1A-

TST) was created where all 3 elucidated sites were mutated to a nonphosphorylatable alanine (T15A, S22A and T33A). Phosphorylation of Spy1A-TST at G2/M was compared to that of wt-Spy1A using an orthophosphate labeling experiment. A significant decrease in phosphorylation was observed with the triple mutant (Fig. 5D), demonstrating that Spy1A is phosphorylated at residues T15, S22 and T33 during G2 phase of the cell cycle. Importantly, these mutations provide us with a valuable tool to assess the essentiality of Spy1A degradation on cell cycle dynamics.

Aberrant Spy1A degradation enhances cell proliferation but does not trigger a cell cycle arrest. Cyclin protein levels serve as a monitoring mechanism for the cell to ensure that each phase of the cell cycle is complete before the next is initiated; such checkpoint mechanisms are essential in protecting the integrity of the cell. The Spy1/RINGO family members have been functionally characterized as novel cyclin-like proteins; hence we utilized the Spy1 degradation mutants in order to determine whether progression of the somatic cell cycle requires the timely degradation of Spy1A. Cells from figure 5A which were overexpressing Spy1-wt, Spy1-T15A, Spy1-T33A, Spy1-S22A or Spy1-S247A were analyzed via flow cytometry analysis (Fig. 5E). Wild-type and mutant Spy1A constructs revealed very similar cell cycle profiles (Fig. 5E asynchronous cells; lower panel), demonstrating that prevention of Spy1A degradation in this cell type does not trigger a cell cycle arrest.

To test the effects of ablating Spy1A degradation on cell proliferation live and dead cell populations were monitored by trypan blue analysis. Spy1A and mutant constructs significantly enhance cell proliferation as compared to mock with p values of 0.01 for mock:WT, 0.001 for mock:Spy1A-T15A, 0.0004 for mock:Spy1A-T33A, and

0.001 for mock:Spy1A-S22A (these stats are not reflected in Fig. 5F). There was no statistical change in the number of dead cells from one transfection to another (Fig. 5F; grey bars). Interestingly, Spy1 degradation mutants statistically enhanced proliferation over Spy1A alone by 20-60% (Fig. 5F; black columns). p-values for these comparisons were 0.009 for WT:Spy1A-T15A, 0.002 for WT:Spy1A-T33A and 0.03 for WT:Spy1A-S22A.

Collectively, these data demonstrate that residues T15, S22, and T33 within the N-terminal region of Spy1A are important phosphorylation sites for mediating the degradation of the protein. Furthermore, preventing degradation of Spy1A does not trigger cell cycle arrest but rather results in enhanced cell proliferation; hence representing a mechanism which may contribute to tumorigenesis.

FIGURE 5. *Phosphorylation on T15, T33 and S22 is needed for Spy1A degradation*. 293 cells were transfected with Spy1A wild-type (wt), Spy1A-T15A (T15A), Spy1A-T33A (T33A), Spy1A-S22A (S22A) or Spy1-S247A (S247A). (A) Cell lysates were treated with nocadazole (G2 population; left panels) or untreated (Asynchronous population; right panel). Half of the population was kept for flow cytometry analysis (Fig. 5D). The remainder were lysed and immunoblotted with α-Myc for Spy1A (upper panels) and α-Actin (lower panels). (B) All samples were co-transfected with HA-Ub followed by treatment with MG132 for 14 hrs. Lysates were immunoprecipitated with α-Myc and immunoblotted for α-HA (upper panel) or α-Myc (lower panel). (C) 16 hrs. after transfection cyclohexamide was added, cells were collected at 30, 75 and 120 min. time points. Cell lysates were immunoblotted with α-Myc. Actin was used as a loading control (lower panel). Spy1 bands were quantified using densitometry and values corrected for using Actin. Relative densitometry over 3 separate experiments. Error bars represent SEM ** = $p<0.001$ (D) 293 cells were transfected with Myc-Spy1A-PCS3 (wt) or the triple mutant Spy1A-TST. 16 hrs post-transfection cells were treated with nocodazole and MG132 for 14 hrs. followed by orthophosphoric acid ^{32}P for an additional 4 hrs. Cells were lysed, immunoprecipitated with α-Myc and imaged on a Cyclone phosphorimager (upper blot). Immunoblot for α-Myc was used as a control (lower blot). Incorporation of ortho-phosphate was quantified using OptiQuant software, right panel represents results of one representative experiment of 2. (E) Cells from (A) were analyzed by flow cytometry. CPX analysis was carried out to determine the % of cells in each population and are depicted above the schematic of the cell cycle profiles. (F) Alive and dead cells were counted at 36 hrs. post-transfection using trypan blue exclusion. Error bars reflect

standard deviation between 3 separate transfections and a standard T-test was performed assuming equal variance. Statistical data shown reflects comparisons between the wt transfected cells and mutant transfected cells * p≤ 0.05, ** p≤ 0.01.
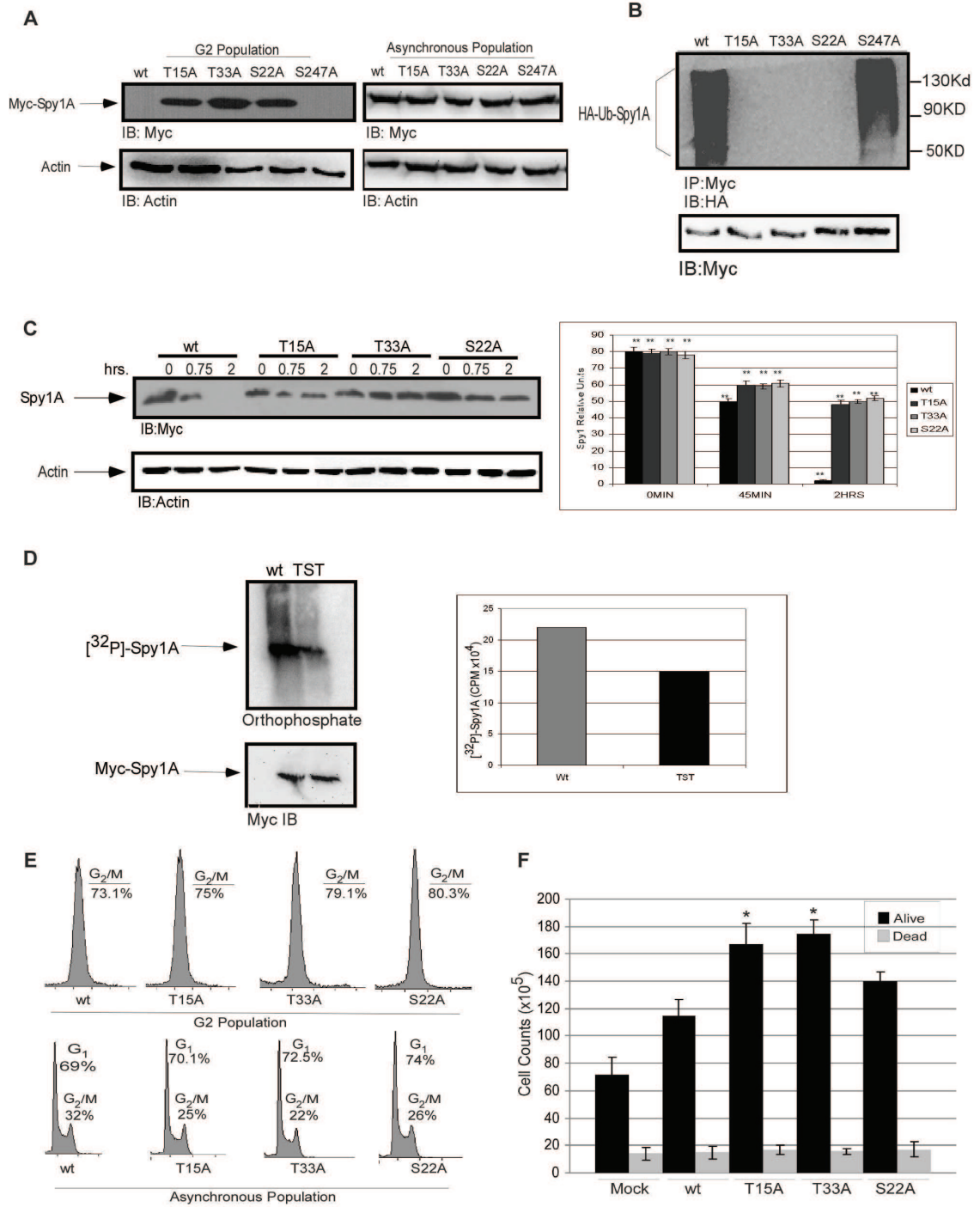
Discussion

Importance of Spy1A degradation in cell cycle regulation. Tight regulation over the protein levels of cyclins and activity of their respective kinase is known to be one mechanism by which the cell ensures the proper timing of cell cycle events [25]. More recently it has come to light that CDKs can also be activated by members of the Speedy/RINGO family. These proteins lack any sequence homology with cyclins however our data demonstrates that, like the cyclins, Spy1A is tightly regulated at the protein level through the cell cycle. The importance of Speedy/RINGO proteins in the cell cycle is irrefutable, expression of Spy1A is essential for cells to progress through DNA synthesis, overexpression enhances cell proliferation and deregulated levels lead to aberrant growth [5,8] [9]. In immortalized cells our results demonstrate that non-degradable mutants of Spy1A do not trigger a cell cycle arrest but rather promote significantly enhanced proliferation over Spy1 wild-type expression alone. Whether preventing degradation results in Spy1 activation of unique CDKs, and whether this contributes toward the proliferative phenotype of these mutants remains to be determined. Most importantly, these data support the possibility that altered degradation of the Spy1A protein is an unchecked cell cycle event which contributes toward proliferation and may play a mechanistic role in human tumorigenesis.

The Spy1A degradation mechanism. Herein, we demonstrate a novel interaction between Spy1A and the E3 ligase Nedd4 which mediates the degradation of Spy1A. This demonstrates that in addition to functional differences, the mammalian Spy1A isoform is subject to differential protein regulation as compared to its *Xenopus* counterpart [10]. The domain structure of Nedd4 family members are very similar and contain a series of

typically two to four WW domains which function as recognition sites for specific substrates or adaptor proteins [26,27]. The WW domains of Nedd4 preferentially recognize PPxY motifs in their substrates [28]. The N-terminal region of Spy1A lacks this consensus site; however is known that Nedd4 can also interact with phosphorylated threonine or serine residues to trigger ubiquitination and subsequent degradation [15] [18]. Notably all deletion mutant constructs for Spy1A, depicted in figure 2A, were found to interact with Nedd4 *in vivo* (data not shown). This suggests that the Nedd4-Spy1A interaction relies on at least 2 separate binding regions in the Spy1A protein; resolution of these required binding regions remain to be determined. Following mutagenesis of 3 potential phosphorylation sites within the N-terminal region of Spy1A, we have determined that preservation of amino acids 15-33 is generally important for Spy1A degradation. T15 is completely conserved among the mammalian Spy1A homologues, and is preceded by a highly conserved proline rich region (PPTV); whether these sites are involved in proteolysis of other Spy1/RINGO family members remains to be determined. Furthermore, the Nedd4 family consists of nine members, all containing WW domains. We know from overexpression assays using Nedd4-1 cDNA that this member of the Nedd4 family is capable of interacting and promoting the ubiquitination and degradation of Spy1A. Additionally, specific knockdown of Nedd4-1 prevented the degradation of Spy1 and interactions between Spy1-Nedd-4, collectively these data strongly support that Nedd4-1 is the specific isoform mediating Spy1 degradation. Whether other members of the Nedd4 family are also capable of regulating the degradation of Spy1A is currently not known. It is known that the Nedd4 family are capable of also mono- and di-ubiquitinating their substrate proteins. While we can not rule out that these modifications

also occur, our data demonstrate that Spy1A can be polyubiquitinated by Nedd4 and that this targets the protein for degradation.

Spy1A-Nedd4 interaction in cancer. From the current catalogue of known Nedd4 substrates it appears that Nedd4 can act as both a proto-oncogene, as well as a tumor suppressor under different circumstances. For example, Nedd4 has been shown to mediate the degradation of the vascular endothelial growth factor receptor 2 (VEGF-R2) [19]. VEGF-R2 is a positive regulator of cell proliferation, migration, and angiogenesis [29], and it is known to be up-regulated in colon [30], brain [31], and breast cancer [32]. In addition, Nedd4 has been shown to lead to the down-regulation of the insulin-like growth factor 1 receptor (IGF-1R) [20], which has been implicated in both the initiation and development of many human cancers types [33]. Our data provides further evidence that Nedd4 can function like a tumor suppressor to regulate the levels of proteins stimulating cell growth mechanisms. Conversely, Nedd4, or Nedd4 family members, have been shown to regulate the degradation and function of important tumor suppressor genes such as the phosphatase and tensin homolog 1 (PTEN), p53 and the p53 family member, p73 [21-23]. This novel interaction between the Spy1/RINGO family and Nedd4 strengthens the possibility that Nedd4 substrate specificity may contribute to oncogenesis, thereby allowing for the accumulation of proliferative proteins such as Spy1A. Further resolving how this mechanism is functioning in human cancers is an important direction that may provide a novel direction in the design of cancer therapeutics.

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Chapter 3

Misregulation of Spy1 Protein levels represent an oncogenic alterations

Introduction

The Speedy/RINGO family of proteins are novel regulators of the cell division cycle, capable of activating the cyclin-dependent kinases (Cdks) independent of cyclin binding and phosphorylation within the Cdk T-loop [1]. In *Xenopus* oocytes, the Speedy/RINGO family member X-Spy1 was shown to prematurely activate Cdk2, and to allow progression through the G_2/M checkpoint via activation of the MAPK pathway, thus promoting rapid oocyte maturation [2]. The human Speedy/RINGO homologue, herein referred to as Spy1, is constitutively expressed in most human tissues and is essential for somatic cell cycle progression [3]. Ectopic expression of Spy1 subsequently promotes rapid cell cycle progression through a shortened G_1/S phase that is attributed, at least in part, to the activation of Cdk2 [3]. Furthermore, Spy1 can prevent the inhibitory affects of the tumor suppressor $p27^{Kip1}$ on Cdk2 by directly promoting p27 degradation, suggesting yet another mechanism by which Spy1 can enhance both normal and aberrant cell growth [4-6]. Spy1 also plays a role in the DNA damage response, functioning to promote cell survival and override protective barriers in the presence of damaged DNA [7,8]. Collectively, these data support a role for Spy1 in promoting carcinogenesis. Indeed, SAGE analysis has shown that Spy1 is expressed at elevated levels in one case of invasive ductal carcinoma of the breast, Spy1 protein levels have also been implicated as a prognostic marker in hepatic carcinogenesis and ectopic overexpression of Spy1 can accelerate mammary tumorigenesis *in vivo* [9-11]. Hence, how Spy1 levels are regulated and how this contributes to the initiation and/or progression of tumorigenesis is of high priority for understanding both normal and abnormal cell growth programs.

Spy1 protein levels are tightly regulated during the cell cycle, being degraded via ubiquitin mediated proteolysis utilizing at least two separate mechanisms [12,13]. We have resolved three residues within the N-terminal region of the protein, T15, S22, and T33, essential for targeting Spy1 for Nedd4-mediated degradation in G2/M phase of the cell cycle [12]. Mutation of these residues generates a non-degradable form of Spy1 (Spy1-TST) which was found to significantly enhance cell proliferation over that of wild-type Spy1 [12]. Herein, we demonstrate that elevated levels of Spy1 protein are implicated in subsets of human breast cancer cell lines. Utilizing the nondegradable mutant of Spy1 we show that that altering Spy1 protein stability or expressing very high levels of Spy1-WT protein is a transforming event. Interestingly, preventing Spy1 protein degradation results in an enhanced activation of Cdk1 kinase activity during mitosis and subsequent inhibition of the pro-apoptotic regulator FOXO1. Collectively this data supports a unique relationship between the stabilization of Spy1 protein and mammary tumorigenesis.

Materials and Methods

Cell Culture

The mouse embryonic fibroblast cell line NIH 3T3 (NIH), human embryonic kidney cell line HEK 293 (293) and HTB 231 were purchased from ATCC and maintained in DMEM medium (Sigma) supplemented with 10% (vol/vol) fetal calf serum (Sigma) for NIH3T3 cells, and fetal bovine serum for 293 cells. The BALB/c mouse mammary epithelial cell line HC11 (kindly provided by Dr. C. Shermanko; University of Calgary) and MCF7 cells were maintained in RPMI 1640 medium (Hyclone) containing 10% (vol/vol) fetal calf serum and supplemented with 5 µg/ml insulin (Sigma), and 10 ng/ml

EGF (Calbiochem). MCF10A series cell line were maintained in DMEM-F12 media containing 0.5 ug/ml hydrocortisone, 10 ug/ml insulin, 20ng/ml human EGF and 5% (vol/vol) horse serum heat inactivated. The MMTV-Myc cell line was derived in the lab by E. Kirou. Briefly, mammary adenocarcinomas were freshly dissected from multiparous female mice, then tumor tissue was directly placed in fresh, collagenase buffer (10 mM Hepes, 2.5% FBS, RPMI 1640 supplemented with Lglutamine). Tissues were homogenized for 25 Min, and cells were subsequently isolated by multiple centrifugations. All cell lines were maintained in a media containing 2 mM L-glutamine (Sigma), penicillin (Invitrogen), and streptomycin (Invitrogen), and were cultured in a 5% CO_2 environment.

Plasmid and Mutagenesis Creation of the Myc-Spy1-PCS3 vector was described previously [14]. Myc-Spy1-TST-PCS3 was created using Quik Change PCR Multi Site-Directed Mutagenesis (SDM) (Stratagene) in 3 sequential steps. An alanine mutation was created at T15 in Spy1-PCS3 to form Spy1-T15A using primers #A151 5'-GAGACACCACCTACTGTCGCTGTTTATGTAAAATCAG-3' and #A152 5'-CTGATTTTACATAAACAGCGACAGTAGGTGGTGTCTC-3'; an alanine mutation was then introduced at S22 to produce Spy1-T15A-S22A using primers #A139 5'-GTTTATGTAAAATCAGGGGCCAA TAGATCACATCAGC-3' and #A140 5'-CTGATGTGATCTATTGGCCCCTGATTTTACATAAAC-3; and a third alanine mutation was introduced at T33 to form Myc-Spy1-TST-PCS3 using primers #A-153 5'-CAGCCTAAAAAGCCCATTGCACTGAAGCGTCCTATTTG-3' and #A154 5'-CAAATAGGACGCTTCAGTGCAATGGGCTTTTAGGCTG-3'. Successful cloning in all cases was determined by DNA sequencing (Robarts Sequencing Facility; Univ. of

Western Ontario). Plasmids for FLAG-FOXO1 (9036), HA-Cdk1 (1888), HA-Cdk1 dominant negative (Cdk1-DN) (1889) and the luciferase reporter construct, 3xIRS, which contains three copies of the FOXO response element in the promoter of the IGFBP1 gene (13511), were obtained from Addgene. The plasmid for flag-FOXO1 vector harboring an amino-acid substitution at S249 (FOXO1-A3/S249A) was kindly provided by Dr H Huang, University of Minnesota. Luciferase control plasmids were kindly provided by Dr. B. Vogelstein, John Hopkins university, Baltimore. Ras-V12 was kindly provided by Dr. S. Lowe, Cold Spring Harbor Laboratory, New York.

Antibodies

Primary antibodies were as follows: Myc (9E10 and C19; Santa Cruz), HA (Y11 and F7; Santa Cruz), andCdk1 (ab31687; Abcam). . HRP conjugated secondary mouse antibody (A9917) and rabbit antibody (A0545) were purchased from Sigma.

Transfection

Cells were transfected using polyethylenimine (PEI) branched reagent Sigma (408727), 10 µg of DNA (unless otherwise indicated) was mixed with 50 µL of 150 mM NaCl and 3 µL of 10 mg/ml PEI for 10 Min then added to a 10 cm tissue culture plate. Transfection media was changed after 8 h for NIH cells and remained overnight for the 293 cells.

Cell Synchronization and Flow Cytometry

Cells were synchronized using double thymidine block; in brief cells were cultured in a media containing 2 mM thymidine for 16 h released to normal media for 8 h, followed by a 14 h block in 2 mM thymidine and then released in 70 ng nocadozol. NIH cells were synchronized by being cultured in a 2% serum containing media for 24 h, followed by release in standard culture media.

Flow cytometry analysis; 293 cells were collected at indicated times, washed twice in PBS, and then either used immediately or frozen for future analysis. Frozen cells were resuspended at 2 x 10^6 cells in 1 ml of PBS, fixed by the drop wise addition of an equal amount of ethanol, and frozen at 80 ^{o}C. Within 1 week, fixed frozen cells were pelleted, washed, and resuspended in 300 µl of PBS. Samples of resuspended cells or fresh cells were treated with 1 µl of 10 mg/ml stock of DNase free RNase (Sigma) and 50 µl of 500 mg/ml propidium iodide stock solution. A minimum of 300,000 cells were analyzed per treatment using a Beckman Coulter FC500 (Biology Dept. U of Windsor).

Immunoblotting (IB) and Immunoprecipitation (IP)

Total protein was isolated from cell cultures by harvesting cells and lysing them in 0.1% NP-40 lysis buffer (5 ml 10% NP-40, 10 ml 1M Tris pH 7.5, 5 ml 0.5M EDTA, 10 ml 5M NaCl up to 500 ml RO water) containing protease inhibitors (10 µl/ml PMSF stock solution 10 mg/ml, 3 µl/ml aprotinin stock solution 20 mg/ml, 10 µl/ml leupeptin stock solution 1 mg/ml) for 30 Min on ice. For all cell lysates; Bradford Reagent was used to determine the concentration of proteins in lysates following the manufacturer's instruction (Sigma). Aliquots of lysates containing 20-30 µg protein were subjected to electrophoresis on denaturing SDS-10% polyacrylamide gels and transferred to PVDF-Plus 0.45 micron transfer membranes (Osmonics Inc.) for 2 h at 30V using a wet transfer method. Blots were blocked for 2 h in TBST containing 3% non-fat dry milk (blocker) at room temperature, primary antibodies were reconstituted in blocker and incubated over night at 4°C, secondary antibodies were used at a 1:10,000 dilution in blocker for 1 h at room temperature. Blots were washed three times with TBST following incubation with both the primary and secondary antibodies. Washes were 6 Min each following the primary antibody and 10 Mineach following the secondary antibody.

Chemilumiminescent Peroxidase Substrate was used for visualization following the manufacturer's instruction (Pierce). Chemiluminescence was quantified on an Alpha Innotech HD2 (Fisher) using AlphaEase FC software.

For immunoprecipitation, equal amounts of protein were incubated with primary antisera as indicated overnight at 4°C, followed by the addition of 10 ul protein A–Sepharose (Sigma) and incubated at 4°C with gentle rotation for an additional 2 h. These complexes were then washed 3 times with 0.1% NP-40 lysis buffer and resolved by 10% SDS-PAGE.

Quantiative/Real-Time Reverse Transcriptase PCR (Q-RT-PCR)

Total RNA was extracted using the RNAeasy Plus Mini Kit (Qiagen) and reverse transcribed using 200 U Superscript II (Invitrogen), 0.5 g Oligo dT's and 0.5 g random nanomers (Sigma) according to the manufacturer instructions. Q-PCR was carried out using SYBR green detection (Applied Biosystems) with 400 nM of each primer (Table 1; Suppl. Mat.) and PCR was performed using ABI Prism 7300 thermocycler. Data was analyzed using ABI 7300 software and represented $\log_{10}$ relative quantification (RQ) relative to control. Custom primers were designed using Primer Express software (Applied Biosystems).

Kinase Assays

Cells were transfected with the indicated plasmids, cultured in 10% FBS and lysed in 0.1% NP–40 lysis buffer. 16 h post-transfection IP was carried out as described above and precipitates washed four times prior to the addition of 50 μl of kinase buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 20 mM EGTA, 50 mM ATP, 10 mCi of [γ–^{32}P]ATP) and 74 μg/ml H1 histone (Boehringer Mannheim). Reactions were incubated for 10 min at 30°C, sample buffer was added to stop the reaction and 50 μl of each

sample were analyzed by 10% SDS–PAGE, followed by transferring to PVDF membrane. The amount of incorporated phosphorylation was visualized using a Cyclone storage phosphor system and quantified using OptiQuant software (Perkin Elmer, Biology Department UofW).

Luciferase Assay

Cells were transfected with the appropriate luciferase reporter construct in the presence or absence of a variety of constructs as indicated in the figures. Cells were harvested 24 h post-transfection and 50 ul of cell suspension was mixed with 50 ul of with Bright-glo reagent (E2620; Promega). Luminescence spectra of the samples were measured using a plate reader (Wallac Victor 1420; PerkinElmer 3TM-1420).

Soft Agar Assay

Two layers of agar/media mixture were plated into 60 mm culture dishes. Briefly 0.6 g of Noble agar (UBS, 9002-18-0) was suspended in 100 ml DI water to yield a 0.6% bottom agar concentration. This mixture was completely dissolved and then was poured into two 50 ml tubes, sealed with tape and placed at 40°C for 40 minutes. Simultaneously, DMEM media enriched with 2X fetal bovine serum was also incubated at 40°C. The bottom agar layer contained 1.5 ml of 6% agar solution and 1.5 ml of media solution and was poured and plated. When solidified a top agar layer was prepared containing a 3% agar solution. Cells ($\sim$2.5 x 10^{5}) were harvested, counted and added to the top agar + media mix and then promptly layered onto of the bottom agar layer. Plates are set for 1 h in the cell culture hood and then incubated at 37°C in 5% CO_2 for 12-14 days. Triplicate transfections for each experiment were observed using light microscopy and pictures were taken using an Apha Innotech HD2 camera colonies were counted manually under the light microscope.

Focus Assay

Low passage cells were grown in 10 cm plates and then transfected with the indicated constructs using the PEI method described above. Upon reaching confluency media with reduced serum (2% calf serum) was gently changed every other day for up to two weeks. The plates were stained with 0.5% crystal violet and colonies were taken using an Apha Innotech HD2 camera and counted manually.

Fat Pad Transplants

Fat pad transplant assays were conducted using BALB/c mice, which are syngenic for the HC11 cell line. Mice were maintained following the guidelines of the Canadian Council on Animal Care and the assay carried out as outlined in U. of Windsor AUPP #06-19 and previously described [11]. In brief; 5×10^5 cells were injected into the cleared fat pad of 4th inguinal mammary glands of 22 day old mice and allowed to grow for 1 to 8 weeks. Tumor presence was monitored weekly by palpitation of the gland. Animals were sacrificed humanely at the specified time points and glands dissected for analysis.

Results

Spy1 protein stability is implicated in human breast cancer cell lines

Spy1 protein levels are elevated in human hepatocarcinoma [10]. To determine whether Spy1 levels may be misregulated at the expression or protein level in subsets of human breast cancer we utilized a panel of well characterized breast cancer or breast normal cell lines (Fig. 1 A-B). Samples from non-tumorigenic human breast cell line (MCF10A) was ran along with cell lines established from tumors driven by MMTV-c-Myc (MMTV-Myc), or the MCF10A series human breast cell lines derived from the MCF10A normal lines above. In addition, the well characterized human breast cancer lines MCF7, MDA-MB 231, Sum 149 were all tested for Spy1 expression levels using Q-RT-PCR (Fig. 1A) and western blotting (Fig. 1B)of matched samples. Our data demonstrates Spy1 protein levels were significantly elevated in the MDA-MB 231 and MCF10CA1A lines over other breast cancer subtypes. Importantly, when comparing relative expression at the mRNA and protein levels is it clear that subsets of breast cancer lines regulate Spy1 protein levels differently than others. This data supports that aberrant regulation of Spy1 protein levels are implicated in subsets of human breast cancers.

Figure 1. *Spy1 protein levels are elevated in human breast cancer cell lines.* (A-B) A panel of breast cancer cell lines were analyzed for Spy1 protein and RNA. (A) QRT-PCR analysis for Spy1 RNA for the same breast cancer cell lines.(B). Western blot analysis for Spy1 protein levels for the indicated cell lines.
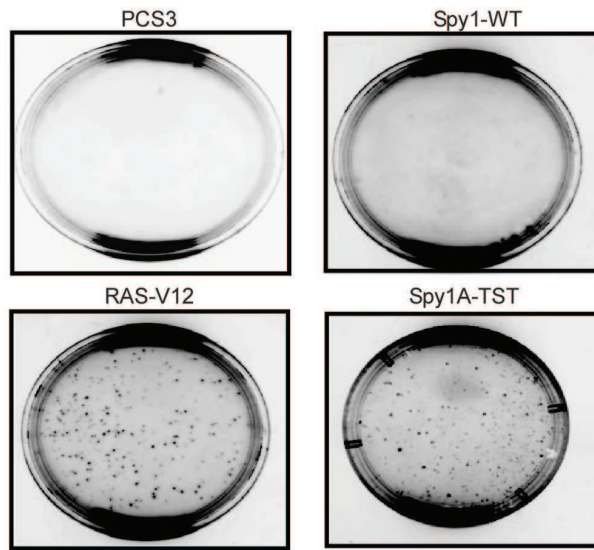
A

B
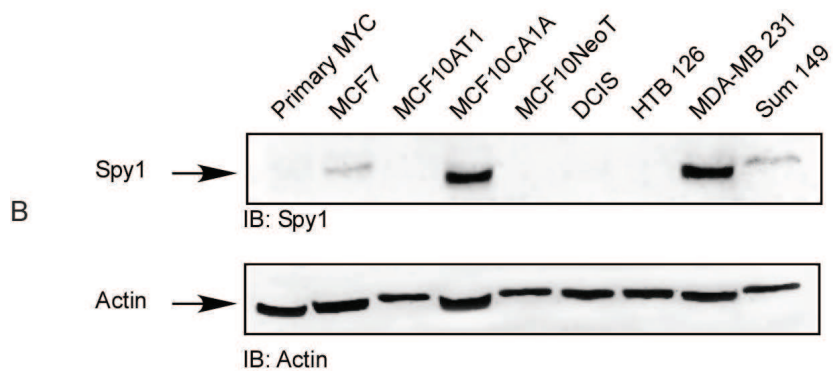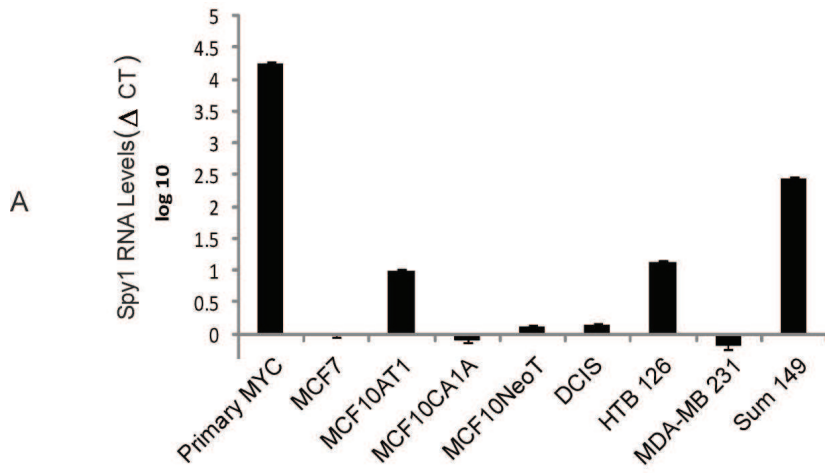
Stable Spy1 protein has oncogenic properties

To test the functional significance of stabilizing Spy1 protein we first performed a soft agar assay utilizing Spy1 wild-type (Spy1-WT), the non-degradable form of Spy1 (Spy1-TST) along with activated Ras (Ras-V12) as a positive control and empty PCS3 as a negative control (Fig. 2A). Colonies were formed in the presence of Ras-V12 as well as Spy1-TST but no colonies were present in the negative control or Spy1-WT. Quantification over three different transfections demonstrated that Spy1-TST yielded 4 times more colonies than the Spy1-WT counterpart (Fig. 2B). Densitometry analysis of protein levels of transfected Spy1 normalized using Actin levels shows more than a 2 fold increase in the overall Spy1 protein levels (Fig. 2C). This is not surprising given that we are utilizing asynchronous cells and there are multiple mechanisms for regulating Spy1 protein levels [12,13]. Functionally this suggests that Spy1-TST-mediated oncogenic properties are not explained merely by the accumulation of overall amounts of protein. To further test this result on the parameters of contact inhibition we performed a foci formation assay using NIH cell lines (Fig. 2D). Approximately 6-fold more foci were formed from the Spy1-TST and Ras-V12 expressing cells as compared to foci derived from the Spy1-WT counterparts, negligible foci were formed in the case of the negative control cells as well (Fig. 2E). Densitometry of relative protein levels, when normalized to Actin, demonstrate that Spy1-TST resulted in less than a 1.5 fold increase in overall protein, further supporting as above that Spy1-TST effects on cell growth properties are not adequately explained by increases in overall protein levels (Fig. 2F l). To further test the effects of overall protein concentration on colony formation, a soft agar assay was performed using increasing concentrations of both Spy1-WT and Spy1-TST (Fig. 2G).
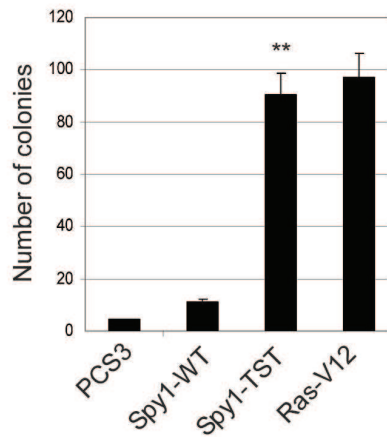
When transfected with 20-30 ug of DNA, Spy1-WT yielded significant colony growth, demonstrating that at very high levels the WT protein is capable of exhibiting transforming properties. However, even when relative protein levels are measured at a higher level for Spy1-WT (ie. comparing 20 ug of Spy1-WT with 7.5 ug of Spy1-TST; Fig. 2H; lower panel) Spy1-TST yielded at least 2 fold more colonies. Collectively, this data supports the hypothesis that threshold levels or stabilization of the Spy1 protein trigger an oncogenic mechanism that may contribute toward Spy1-mediated tumorigenesis. The significant increase in colonies seen with the non-degradable Spy1 over Spy1-WT supports that the G2/M degradation mechanism previously described may provide a protective barrier against this oncogenic pathway.

Figure2. *Spy1 stable protein induces colony formation.* HEK 293 cells (A-C) or NIH 3T3 cells (D-G) were transfected with empty myc-tagged vector PCS3 as mock, wild-type myc-Spy1 (Spy1-WT), activated Ras (Ras-V12) and nondegradable myc-Spy1 (Spy1-TST). (A) Soft agar assay was performed and foci were visualized after 14 days using light microscopy. Representative pictures from one of 3 separate experiments. Within each experiment assays were seeded in triplicate. (B) Total numbers of foci were counted over 3 separate plates for each experiment, three different transfections were performed. The graph is a representative of one of three experiments. Errors bars reflect SEM between triplicate experiments. *t* test was performed;** P≤ 0.01. (C) Western blot analysis was carried out to confirm protein expression for cells transfected in A-B. Protein levels were quantified using densitometry followed by normalization for Actin levels. (D) Colony formation assays were conducted and representative views of each plate recorded using light microscopy. (E) Quantification of the number of colonies over 3 separate plates from one representative experiment of 3. Errors bars reflect SEM between triplicate experiments. *t* test was performed;** P≤ 0.01 (F) Western blot analysis was carried out to confirm protein expression from D-E. Protein levels were quantified using densitometry followed by normalization for Actin levels. (G) 293 cells were transfected with different concentrations of both Spy1-WT and Spy1-TST. Soft agar assay was carried out and plates photographed and quantified on day 14. Foci were averaged over 3 separate plates for each experiment, three different transfections were performed. Errors bars reflect SE between triplicate experiments. *t* test was performed;** P≤ 0.01,.(H) Representattive western blot analysis of one experiment from cells used in seeding the soft agar assay described in G upper panel.

A

PCS3

Spy1-WT

RAS-V12

Spy1A-TST

B

Number of colonies

PCS3   Spy1-WT   Spy1-TST   Ras-V12

**

C

Myc-Spy1-WT	-	-	+	-
Myc-Spy1-TST	-	-	-	+
Ras-V12	-	+	-	-
PCS3	+	-	-	-

Spy1 →

IB: Myc

Actin →

IB: Actin

Spy1 relative units

Spy1-WT   Spy1-TST

D

| PCS3 | Spy1-WT |
| RAS V12 | Spy1A-TST |

E

F

G

H

Spy1 →   IB:Myc

Actin →   IB:Actin
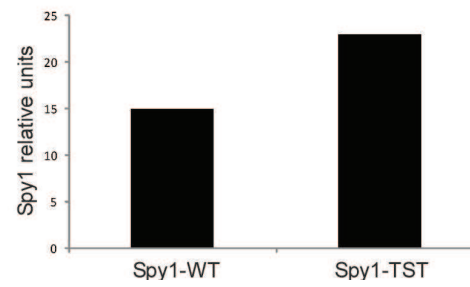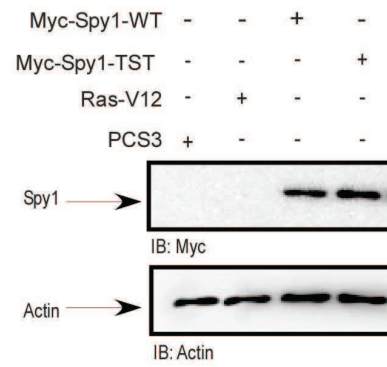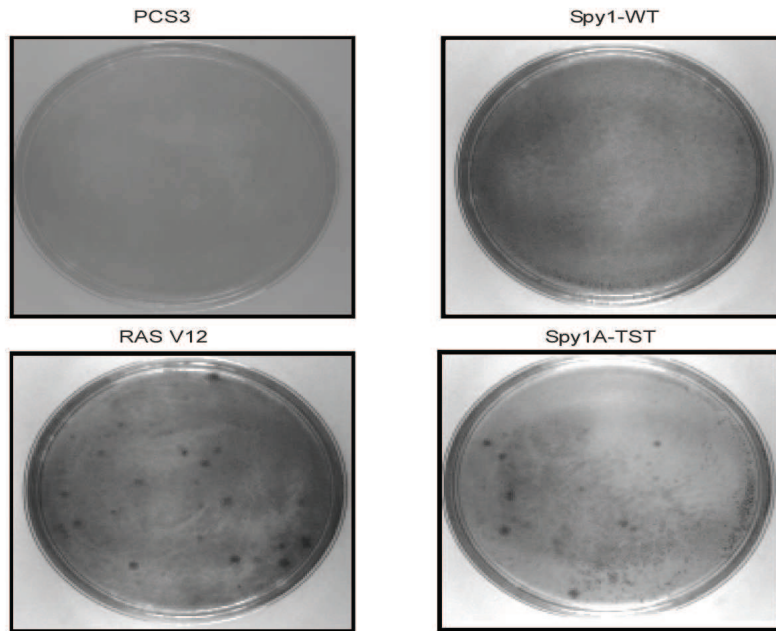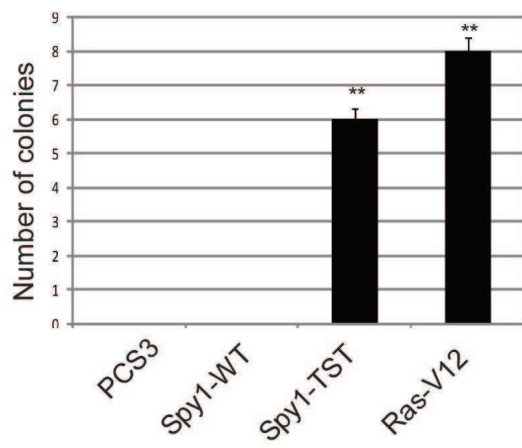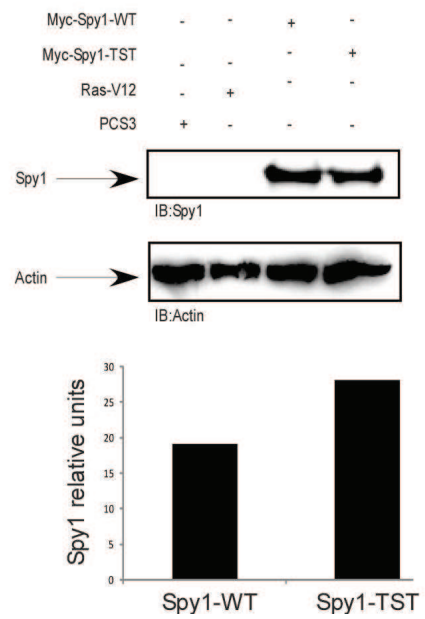
Stable Spy1 protein binds and activates Cdk1 uniquely during mitosis

We previously demonstrated that Spy1-TST remains stabilized at prometaphase of mitosis upon nocodazole treatment, a time point where Spy1-WT was completely degraded [12,13]. Hence, we sought to determine whether aberrant degradation of Spy1 will result in elevated Cdk1 activity during mitosis. Cdk1 has been shown to play an important role in different human cancers, aberrant Cdk1 activation has been described in number of primary tumors [15] providing a potential novel mechanism for Spy1-mediated oncogenic effects.

Reciprocal immunoprecipitation of exogenously expressed Spy1-TST (Fig. 3A; left panels) or Cdk1 (Fig.l 3A; right panels) demonstrate that these proteins interact in cells blocked at prometaphase of mitosis using nocodazole. We also examined the interaction between the endogenous Cdk1 and Spy1-TST (Fig. 3B). This figure demonstrates t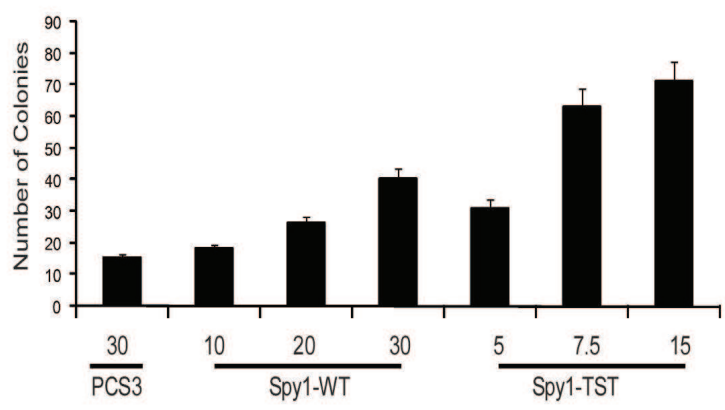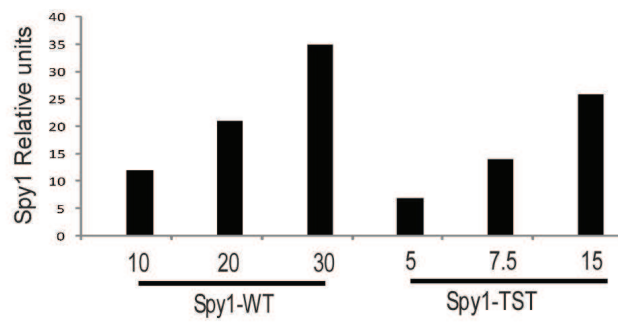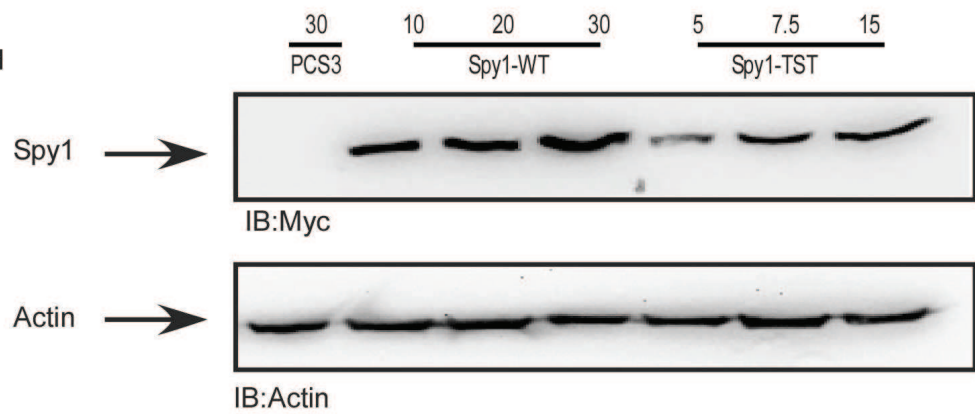hat endogenous Cdk1 interacts with Spy1-TST at prometaphase of mitosis,. To further investigate if Spy1-TST expression leads to unique activation of Cdk1, cells synchronized with nocodozole overexpressing Spy1-WT or Spy1-TST were immunoprecipitated with Cdk1 antibody and the lysates were subjected to an *in vitro* kinase assay using histone 1 (H1) as a substrate (Fig. 3C). Spy1-TST significantly increased histone phosphorylation (Fig. 3C; left panels) demonstrating approximately a 3-fold increase over control transfected cells and approximately a 2-fold increase over Spy1-WT transfected cells (Fig. 3C; right panels).

Figure 3. *Spy1 stable protein binds and activates Cdk1.* HEK293 cells were transfected with Myc-Spy-WT or Myc-Spy1-TST in the presence or absence of HA-Cdk1.(A) cells were treated with 70 ng Nocodazole for 16 h. Equal amounts of protein were immunoprecipitated with either Myc antibody (right lanes; left hand panel) or HA antibody (right lanes; right hand panel). In both cases immunoprecipitation with IgG was used as a negative control (left hand lanes). Precipitates were analyzed using 10% SDS PAGE followed by immunoblotting as indicated. Left 3 panels indicate IPs and right panels represent the lysate loading controls. One representative experiment of 2. (B). Equal amounts of protein were immunoprecipitated (left panels) with Cdk1 antibody, analyzed by 10% SDS PAGE followed by immunoblotting Myc antibody (Fig2B; right panels). Cells transfected as above were lysed and analyzed using 10% SDS PAGE followed by immunoblotting. (C). Cells were treated with 70 ng nocadazole for 16 h. Equal amount of protein was immunoprecipitated with Cdk1 antibody and subjected to H1 phosphorylation assay. Samples were analyzed using 10% SDS PAGE and then imaged on a Cyclone phosphorimager then the same membrane was probed using Cdk1 antibody and imaged on an Alpha Innotech HD2. Phosphate incorporation was quantified using OptiQuant software; right panel represents results of one representative experiment of 3. Errors bars reflect SEM. *t* test was performed *, $P<0.05$;**,$P<0.01$.

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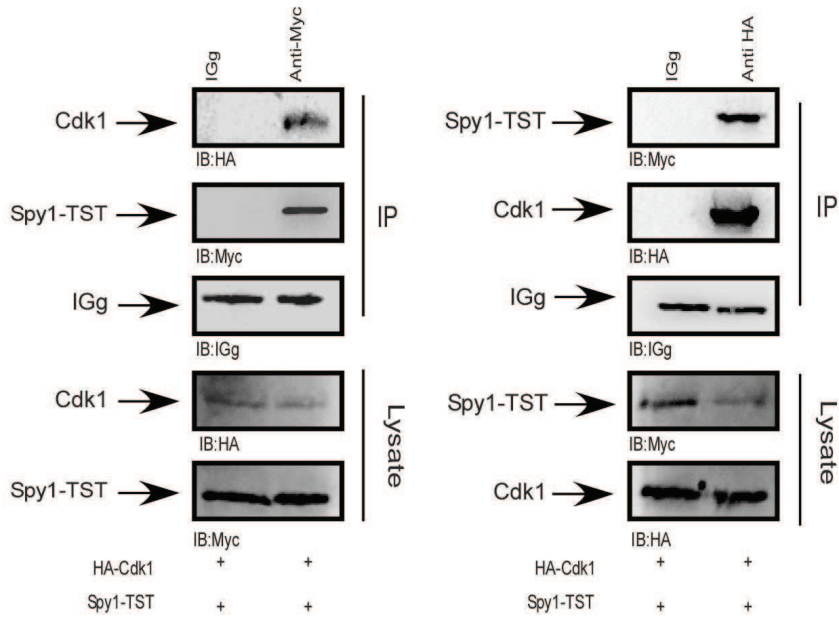
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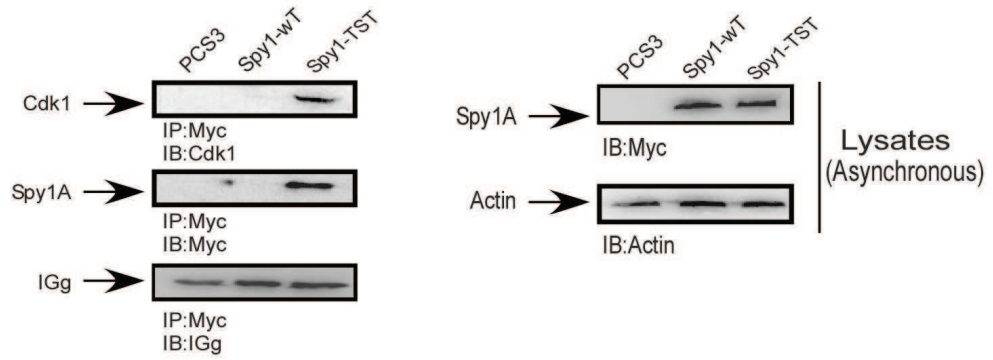
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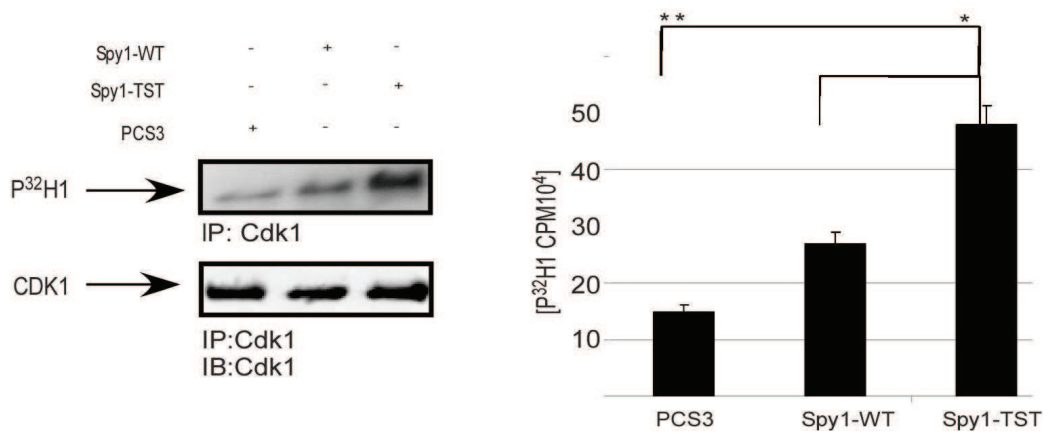
Spy1-TST oncogenic activity is dependent upon activation of Cdk1

Spy1 protein is known to regulate cell cycle progression at least in part through the direct binding to Cdks [3]. In somatic cells the primary partner for Spy1 appears to be the G1/S Cdk Cdk2, and Cdk2 activity is essential for overexpressed levels of Spy1to enhance proliferation [3]. Despite this, Spy1 is capable of also binding and activating Cdk1 [16]. To determine the role of the Spy1 Cdk effectors on Spy1- mediated transformation we utilized a mutant form of Spy1-TST where the aspartic acid residue at position 90 is mutated to a nonpolar alanine group (Spy1-TST/D90A), a modification previously demonstrated to significantly reduce Spy1-Cdk binding [16]. The Spy1-TST/D90A mutation produced significantly fewer colonies in a soft agar assay than Spy1-TST, being greater than 5-fold less transforming (Fig. 4A & B). To further investigate the relative contribution of each kinase individually to this effect, soft agar assays were performed using Spy1-TST in the presence of either Cdk1-DN or Cdk2-DN (Fig. 4C). Interestingly, Cdk1-DN reduced colony formation by ~60% with high statistical significance over 3 separate trials while Cdk2-DN demonstrated reduced colony numbers but this result was not statistically significant (Fig. 4C & D). This was not due to inefficient function of the dominant negative construct as the Cdk1-DN transfection reduced overall Cdk1 kinase activity as efficiently as the Cdk2-DN transfection (Fig. 4E). Collectively this data supports that the oncogenic function of Spy1-TST is dependent, at least in part, on the binding and activation of Cdk1.

Figure 4. *Spy1-TST mediated colony formation is Cdk1 dependent.* (A) Soft agar assays of 293 cells transfected with the indicated constructs were imaged 14 days post-transfection using light microscopy and pictures were taken using an Apha Innotech HD2 camera, this is one representative experiment of three. (B) Quantification of colony number over three separate trasfections. (C) Soft agar assays of 293 cells transfected with Spy1-TST in the presence or absence of Cdk dominant negative constructs (Cdk1-DN or Cdk2-DN) are depicted at 14 days post-transfection using light microscopy and pictures were taken using an Apha Innotech HD2 camera , this is one representative experiment of three. (D) Quantification of colony number over three separate transfections. Errors bars reflect SEM. *t* test was performed *, P<0.05;**,P<0.01.(E) 293 cells were transfected with Myc-tagged empty vector control (PCS3), Myc-Spy1-TST (Spy1-TST) and Cdk1/Cdk2 dominant negative (Cdk1/Cdk2-DN). Equal amount of protein was immunoprecipitated with Cdk1/Cdk2 antibody and subjected to H1 phosphorylation assay. Samples were analyzed using 10% SDS PAGE and then imaged on a Cyclone phosphorimager (upper panel) then the same membrane was probed using Cdk1/Cdk2 antibody and imaged on an Alpha Innotech HD2 (lower panel). Phosphate incorporation was quantified using OptiQuant software; lower graph represents results of one representative experiment of 2.

A
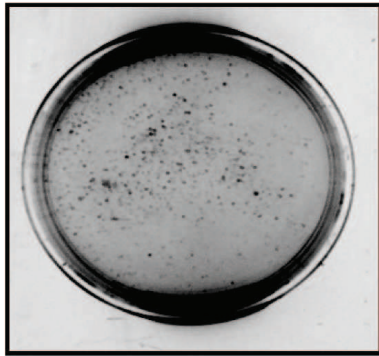
RAS-V12

Spy1-TST

Spy1-TST/D90A

B

C

Spy1-TST+ Cdk1 DN

Spy1-TST+ Cdk2 DN

Spy1A-TST

D

E

Cdk1-DN	-	+	-
Spy1 TST	-	+	+
PCS3	+	-	-

P^{32}H1 →

IP: Cdk1

Cdk1 →

IP:Cdk1
IB:Cdk1

Cdk2-DN	-	+	-
Spy1 TST	-	+	+
PCS3	+	-	-

P^{32}H1 →

IP: Cdk2

CDK2 →

IP:Cdk2
IB:Cdk2
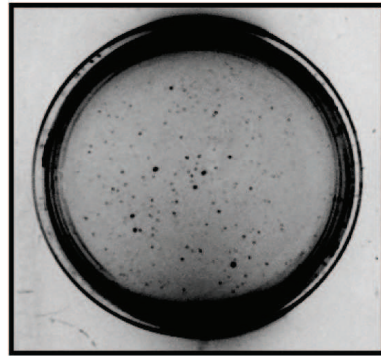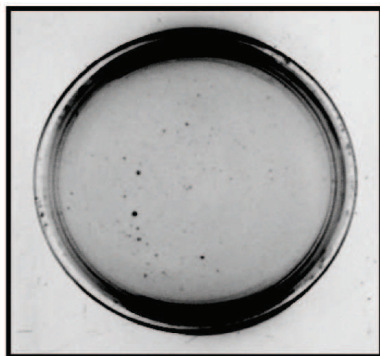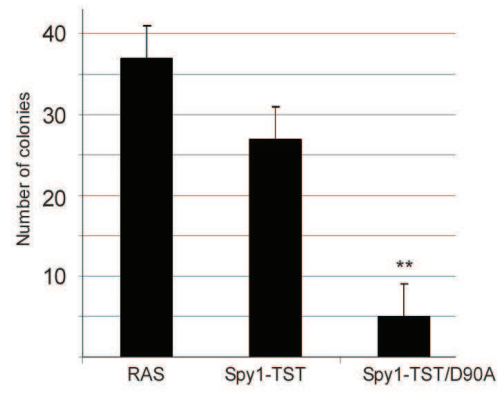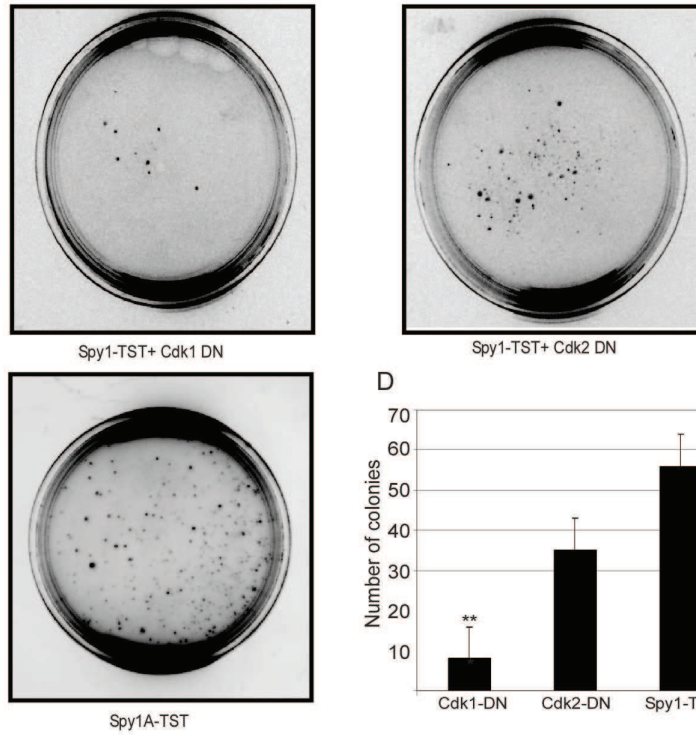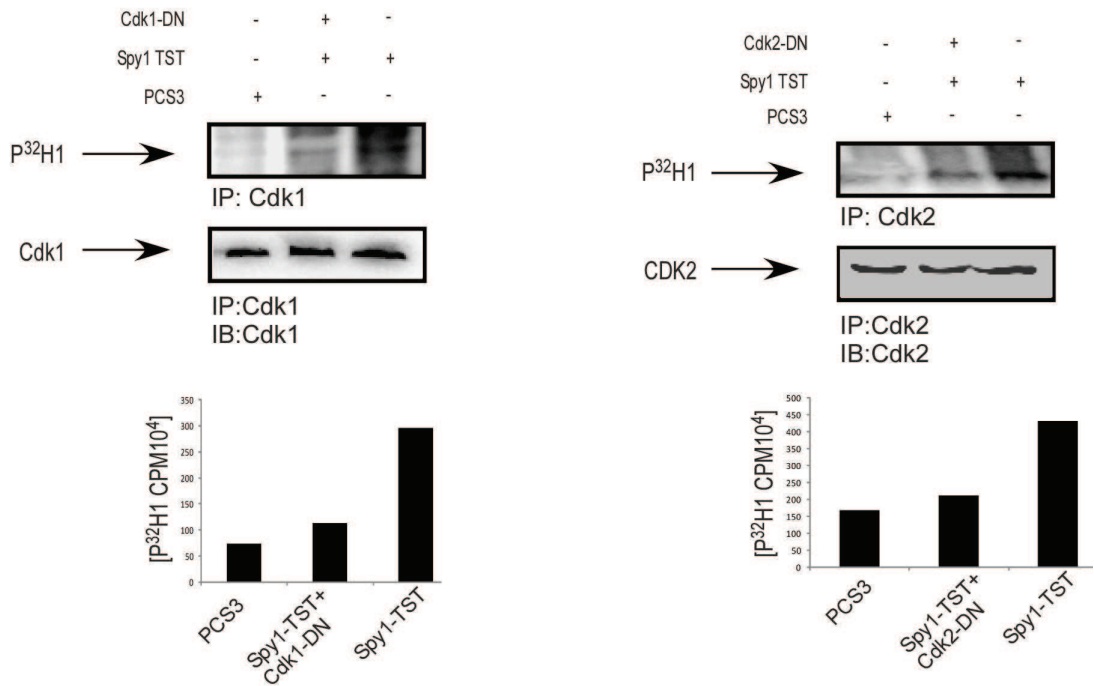
Cdk1 activation by Spy1-TST inhibits the apoptotic mediator FOXO1

Huang and colleagues reported that Cdk1 activation inhibits the transcriptional and apoptotic activities of the transcription factor FOXO1, thereby potentiating Ras-mediated oncogenesis [17]. To investigate the effect of Spy1-TST on FOXO1-induced apoptosis, 293 cells were transfected with FOXO1 and the effects of FOXO1 mediated apoptosis were studied in the presence or absence of Spy1-TST and/or variants of Cdk1 (Fig. 5A). As previously reported, FOXO1 induced robust apoptosis when expressed alone and this effect was diminished with ectopic expression of Cdk1 (Fig. 5A; right top panel compared to left middle panel). Double transfection with FOXO1 and Spy1-TST resulted in significant reduction of the FOXO1-induced apoptosis activity (Fig. 5A, left bottom panel). To test if this reduction was mediated through Cdk1 activation a Cdk2-DN was transfected along with Spy1-TST and FOXO1 (Fig. 5A; bottom panel). The effect of Spy1-TST was indeed reversed by the introduction of the Cdk1- DN, supporting that Spy1-TST inhibition of FOXO1 is due to Cdk1 activation. To further investigate the effect of Spy1-TST on FOXO1 transcriptional activity, a series of luciferase assays were performed (Fig. 5C-E). Ectopic expression of Spy1-TST reduced FOXO1 transcriptional activity as significant as the expression of Cdk1 (Fig. 5C). To check if this result is due to Cdk1 phosphorylation of FOXO1 we utilized a mutant of FOXO1 mutant unable to be phosphorylated by Cdk1, FOXO1-S249A [17,18]. Ectopic transfection of Spy1-TST with FOXO1-S249A prevented the inhibition of FOXO1 transcription by Cdk1 and by Spy1-TST (Fig. 5D). Furthermore, Spy1-TST was unable to suppress FOXO1-mediated transcription in the presence of the dominant negative form of Cdk1, Cdk1-DN (Fig. 5E). Collectively these data support that the transcription factor FOXO1 is a downstream

target of the Cdk1/Spy1 complex and represents a potential mechanism of Spy1-TST-mediated oncogenesis.

Figure 5. *Spy1 stable protein inhibits FOXO induced apoptosis through Cdk1 activation.* (A) Flow cytometry profiles of 293 cells transfected with the indicated constructs. Percentage of cells in each phase of the cell cycle as determined by CPX analysis are indicated on the histograms. Each profile represents no less than 100,000 cells and is one representative profile of 3 repeats. (B) Percent of cells from each treatment with DNA content less than G1 (sub-G1) are graphically presented. Errors bars reflect SE between different transfections. (C-E) Luciferase reporter activity for FOXO in cells expressing the indicated constructs. Errors bars reflect SEM. *t* test was performed *, P<0.05;**,P<0.01.

A

PCS3

SUB-G1
10%

FOXO1

SUB-G1
48%

FOXO+Cdk1

SUB-G1
18%

FOXO+Spy1-WT

SUB-G1
38%

FOXO+Spy1-TST

SUB-G1
22%

FOXO+Spy1-TST+DNCdk1

SUB-G1
39%
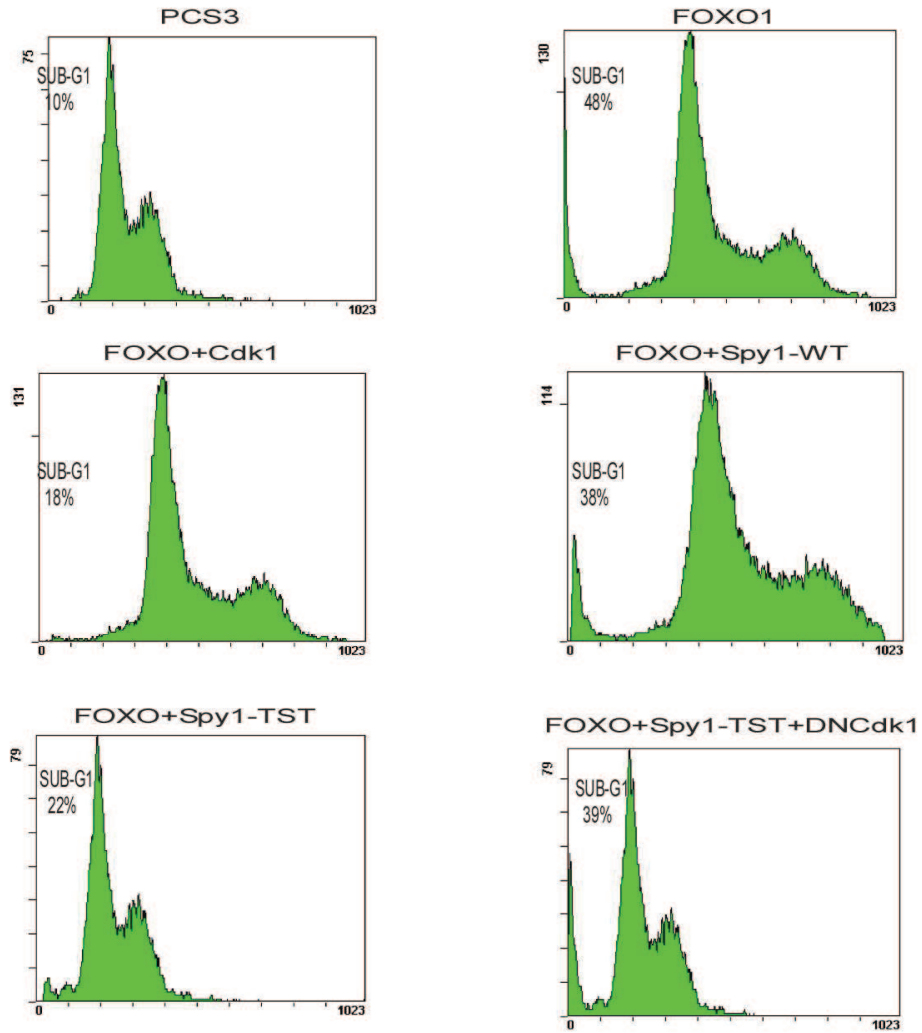
PCS3	+	-	-	-	-	-
Spy1-WT	-	-	-	+	-	-
Spy1-TST	-	-	-	-	+	+
FOXO1	-	+	+	+	+	+
Cdk1	-	-	+	-	-	-
Cdk1-DN	-	-	-	-	-	+

B
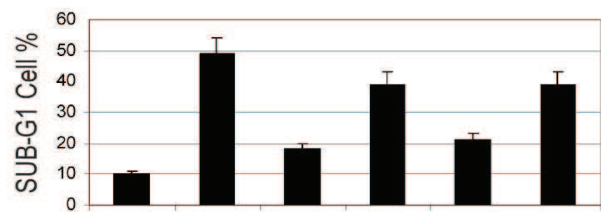
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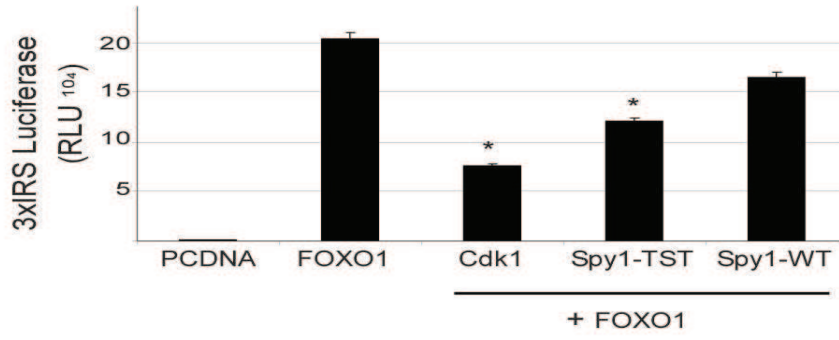
D
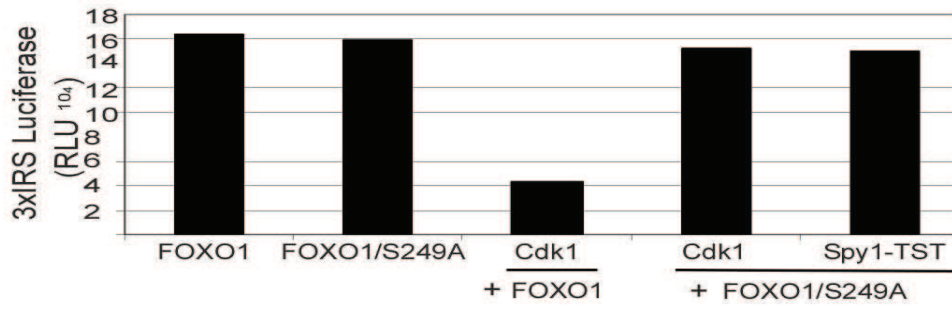
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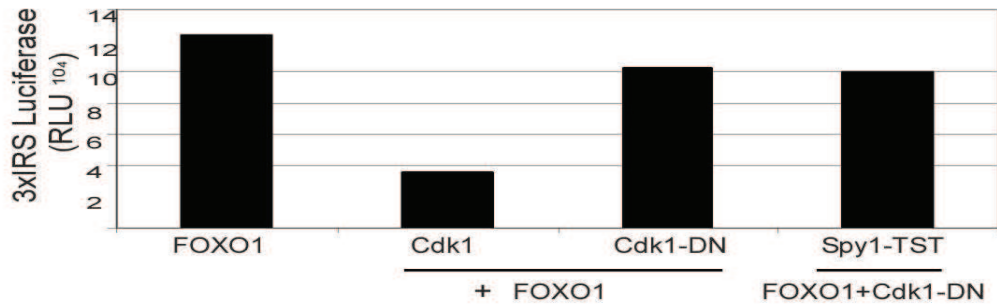
Spy1 stable protein accelerates tumor formation in vivo

Spy1 significantly accelerates tumor formation when HC11 cells overexpressing Spy1 were injected into cleared fat pad of BALB/c mice [11]. Mammary fat pad transplants were performed to determine the effect and timing of Spy1-TST *in vivo* as compared to the overexpression of Spy1-WT (Fig. 6A-C). The fourth inguinal mammary glands of 22 day old BALB/c were removed to prevent endogenous stem cells from re-colonizing the mammary fat pad. Left cleared fat pads were injected with control Spy1-WT-HC11, whereas right cleared fat pads were injected with Spy1-TST-HC11 cells. One week following transplantation, mice were palpated daily to determine the onset of tumor growth. Tumor onset occurred significantly more rapidly in the Spy1-TST injected glands, with 50% of the mice presenting with tumors by day 8 as compared to day 13 in the Spy1-WT mouse population (Fig. 6A). After 5 weeks post-transplantation, mice were sacrificed to determine the extensiveness of tumor growth. While 100% of the glands exhibited invasive tumors at this time the average total area of Spy1-TST tumors was found to be almost twice that of the Spy1-WT (Fig. 6B). These data support that stabilization of Spy1 protein promotes the onset and enhances tumor growth over that of Spy1-WT overexpressing glands.

Figure 6. *Spy1 stable protein accelerates tumorigenesis in vivo.* (A) Percent of mice presenting with palpable tumours from 0-19 days post-transplant. Each data point represents 4 mice transplanted with cells transfected with the indicated constructs; the whole experiment was repeated three times using three different transfections. Mann-Whitney Test was performed ($p<0.05$). (B) Total tumor area was calculated for both Spy1–HC11 (Spy1-WT) and Spy1-TST-HC11 (Spy1-TST) transplanted glands. Results were taken from 45 transplants using cells from 3 separate transfections. Errors bars reflect SEM between transplants from different transfections.

A
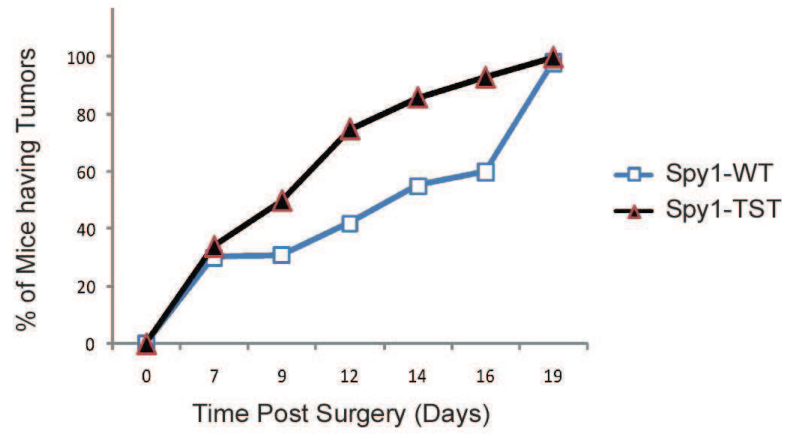
B
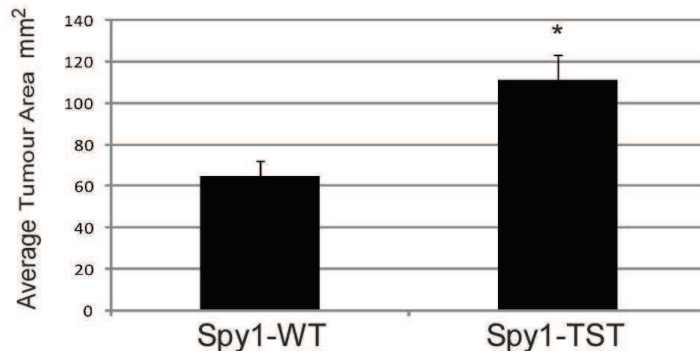
Discussion

Our lab and others have shown that Spy1 protein is regulated during the cell cycle; peaking during G1 and being reduced in G2/M phase of the cell cycle [12,13]. Elevated Spy1 protein levels have been implicated in invasive ductal carcinoma and other types of cancer and ectopic expression of the protein promotes mammary tumorigenesis [9-11]. We performed a number of *in vitro* and *in vivo* assays to investigate the role of Spy1 protein stability on tumorigenic properties of the cell. Colony and focus formation assays utilizing either wild-type Spy1 (Spy1-WT) or a non-degradable mutant of Spy1 (Spy1-TST) demonstrate that the stable form of Spy1 and exceptionally high levels of Spy1-WT protein, exhibit classical oncogenic properties. Our results support that this is not simply due to a generalized accumulation of the Spy1 protein in that equalizing relative protein levels between Spy1-WT and Spy1-TST still results in a several fold increased transformation response in the non-degradable form of Spy1. In fact, Spy1 accumulation was actually found to occur to a modest level in an asynchronous population of cells, which may be accounted for by alternative pathways mediating Spy1 degradation as recently demonstrated [13].

What then is mediating the oncogenic properties of the non-degradable form of Spy1? In mammalian somatic cells Spy1 is known to directly bind and activate Cdk2, functioning like an atypical cyclin [3]. While Spy1 prefers to bind to the G1/S Cdk in these cell types it has been demonstrated in that Spy1 can bind to more than one Cdk partner when overexpressed [19] while its *Xenopus* counterpart was found to bind and activate both Cdk1 and Cdk2 [1]. In this report we have demonstrated that when Spy1 protein is stabilized it is capable of binding and activating Cdk1 in cells arrested with

nocodazole, indicating a prometaphase arrest. This is a stage where Spy1-WT is usually absent. This activation of Cdk1 appears to play a role in the oncogenic properties of Spy1. Using colony formation assay and Spy1 mutant that does not bind to Cdk1 and Cdk1 dominant negative constructs, we have demonstrated that Cdk1 activation is an important factor in the oncogenic properties of the Spy1 stable mutant.

Recently, Huang and others demonstrated that Cdk1 specifically phosphorylates the transcriptional factor FOXO1 at serine 249, this phosphorylation has been shown to inhibit the transcriptional activity of FOXO1 and also was found to counteract FOXO1-induced apoptosis [17]. Several reports have shown that Cdk1 plays an important role in different human cancers. Aberrant Cdk1 profiles have been reported in multiple primary tumors [20,21]. Cdk1 upregulation was reported in human cancer tissues compared to normal tissues [20,22]. Moreover, Cdc25C, an upstream activator of Cdk1, was found to be upregulated exclusively in its active form in some human cancers [23]. Here we show that the abnormal activation of Cdk1 by Spy1 is mimicking the effect of overexpression Cdk1 that Huang had shown previously. Our results clearly show that Spy1 activation of Cdk1 inhibits FOXO1 transcriptional activity and reduces FOXO1-induced apoptosis.

Recent studies have found that overexpression of Spy1 will accelerate the rate of mammary tumorigenesis in the virgin mammary gland [11]. In this present study, we have demonstrated, using mammary fat pad experiments, that stabilized levels of Spy1 protein significantly increases the rate of of tumorigenesis *in vivo* as well as the overal tumour growth. Within two weeks post-transplantation a significant difference was detected via palpation for the Spy1-TST injected sides of the BALB/c mice. Differences in the size and total area of tumours were detected beginning at 5 weeks post-surgery.

Whereas it is known that Spy1 overexpression can lead to invasive and abnormal morphogenesis [11], we have seen that Spy1-HC11-TST cells significantly accelerate the width of tumour growth, leading to the possibility that Spy1 stable cell lines increases the invasiveness of tumorigenesis *in vivo*. This interestingly corresponds when we study the metastatic potential of the cell lines expressing Spy1 protein. Interestingly, Spy1 protein levels are expressed at considerable higher levels in cell lines known to possess metastatic potential *in vivo* while, mRNA levels of the same cell lines were not abrogated when analysed. This supports the hypotheses that Spy1 protein regulation plays a role in tumour formation and progression.

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Chapter 4

Direct interactions with p27 and Cdk2 regulate Spy1-Induced Mammry Tumorigineses.

Introduction

Cell cycle regulation is an intricately controlled process that plays a critical role in all cell fate decisions. The catalytic cyclin dependent kinases (Cdk)s are dependent on the production and destruction of their regulatory subunits, the Cyclin family of proteins. Additionally, the Cyclin-Cdk complexes are subsequently regulated by Cdk inhibitors (CKI). It is crucial that this regulation be tightly controlled to prevent the onset of tumorigenesis, for which uncontrolled cellular proliferation is a hallmark characteristic. An atypical 'cyclin-like' protein known to play a crucial role in regulating somatic cell proliferation is a member of the Speedy/RINGO class of proteins Spy1A1, herein referred to as Spy1 [1,2]. Spy1 is capable of binding to, and activating, both Cdk1 and Cdk2 leading to increased rates of cell proliferation and inhibition of apoptosis when overexpressed [1,3,4]. Unlike other Cyclin-like proteins, Spy1 has been shown to directly bind to the CKI p27 ultimately leading to its degradation [5]. The region regulating interactions with Cdks are well conserved among the Speedy/RINGO family, being widely referred to as the S/R Box [6]. In order for the Cyclin-Cdk complex to become fully activated it must be phosphorylated on a conserved Threonine residue, Thr 160 in the case of Cdk2, which is located near the T-loop [7]. Additionally, Cdks are inhibited by the phosphorylation of 2 residues present within the ATP binding region of the Cdk (Thr 14 and Thr 15 in higher eukaryotes) and activated by dephosporylation of these sites by the Cdc25 phosphatase family [8]. Interestingly, Spy1 is capable of activating Cdks in the absence of the activating phosphorylation and dephosphorylation of the corresponding residues [9]. Hence, offering the possibility that Spy1-bound Cdks can be activated in circumstances where Cyclin-Cdk complexes would otherwise be inactive.

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The mammary gland is an extensively characterized model system for studying normal development as well as the processes involved in the onset and progression of tumorigenesis, in part because the majority of development occurs postnatally and the gland is not essential for the viability of the organism [10]. The mammary gland undergoes multiple rounds of proliferation, differentiation and apoptosis corresponding to puberty/pregnancy, lactogenesis and involution respectively [10-13]. Entry and exit from each of these developmental stages is tightly controlled by a number of signaling pathways responding to alterations in circulating hormones; misregulation of these carefully orchestrated cues represent potential triggers for mammary tumorigenesis. We have demonstrated that Spy1 levels are high in epithelial cells during the proliferative phases of mammary development and are significantly reduced during differentiation [14]. This follows the same trend as that of the proto-oncogene c-Myc, a gene overexpressed in approximately 70% of all human breast cancer cases [15,16]. Interestingly, c-Myc is capable of activating Spy1 transcription independent of *de novo* protein synthesis and like, c-Myc, Spy1 overexpression in the mouse mammary gland induces rapid and invasive tumorigenesis [14].

Cell cycle regulators play critical roles in both normal mammary development as well as in the onset and progression of mammary tumorigenesis. p27 is biphasically regulated during normal mammary development where levels are high during differentiation and low during proliferative and apoptotic phases [17]. Additionally, p27 is differentially localized throughout the gland being high within epithelial tissues, especially in the terminally differentiated layers [17,18]. Low levels of p27 have been correlated with increased Cdk2-cyclin E activity when a cohort of the breast cancer

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population was studied [18,19]. Hence, p27 and subsequent Cdk2 activity are differentially regulated throughout mammary development. Importantly, it was found that elevated Cdk2 kinase activity and decreasing levels of p27 correlate positively with tumorigenic potential and negatively with patient prognosis [20,21]. Thus, targeting either p27 or Cdk2 may prove to be a valuable therapeutic approach in treating mammary tumorigenesis and indeed there are currently a number of Cdk drug inhibitors in various stages of clinical trials [22].

Herein, we will define the essentiality of the direct interaction between Spy1-Cdk2 and Spy1-p27, using mutants of Spy1 unable to interact selectively with either of theses down stream effectors. We test the essentiality of these interactions on mammary growth, proliferation and tumorigenesis *in vitro* and *in vivo*. Our data demonstrates that abrogation of direct binding between Spy1 and p27 significantly inhibits degradation of the CKI and subsequently reduces the proliferative capacity of Spy1 *in vitro*. In opposition, abrogated direct binding to Cdk2 demonstrated little effect on Spy1-mediated proliferation *in vitro* however data supports that the Spy1-Cdk2 interaction may be pivotal in how cells elicit checkpoint responses to stress. *In vivo* we have determined that preventing Spy1 interactions with both p27 and Cdk2 significantly delayed the onset of mammary tumorigenesis and reduced overall tumour burden with similar efficiency. Thus, the direct interaction between Spy1 and key effectors p27 and Cdk2 play a crucial role in mediating separable aspects effects of Spy1-mediated tumorgenesis *in vivo*.

Results

Spy1 protein levels are elevated in human breast cancer tissues

Spy1 has been shown to be highly expressed in an individual SAGE analysis study [23], protein levels have been implicated as a prognostic marker in hepatocarcinoma [24] and we have demonstrated regulation at both the expression and protein level in a variety of breast cancer cell lines (unpublished data). To study the levels of Spy1 protein in specific forms of human breast cancer, we utilized tissue microarrays (TMAs) consisting of 96 cores from breast cancer patients as well as cores taken from either pair-matched or normal breast samples. Combining values over 2 separate TMA's of four different breast cancer tissues; invasive ductal carcinoma, infiltrated ductal carcinoma, intraductal carcinoma and invasive lobular carcinoma, show that Spy1 is significantly elevated in all four types of breast cancer as compared to pair-matched adjacent tissue or a normal tissue samples (Fig. 1). Overall levels of Spy1 were highest in invasive lobular carcinoma and the most significant changes in levels were found in intraductal carcinoma. These data support that aberrant regulation of Spy1 protein levels are implicated in all subsets of human breast cancers studied.

Figure 1. *Spy1 protein levels are elevated in human breast cancer tissues.* TMAs containing cores from invasive ductal carcinoma (IvDC), infiltrated ductal carcinoma (IfDC), intraductal carcinoma (IDC) and invasive lobular carcinoma (ILC) as well as pair-matched normal (PM-N) or cancer-free patients (CF-N) were analyzed for Spy1 expression. The Spy1 signal intensity was normalized to nuclear stain (TOTO-3/PI) signal. Patient numbers are indicated below the sample (N). Data shown is mean ±s.d. Student's *t*-test was performed *, P<0.05;**,P<0.01.
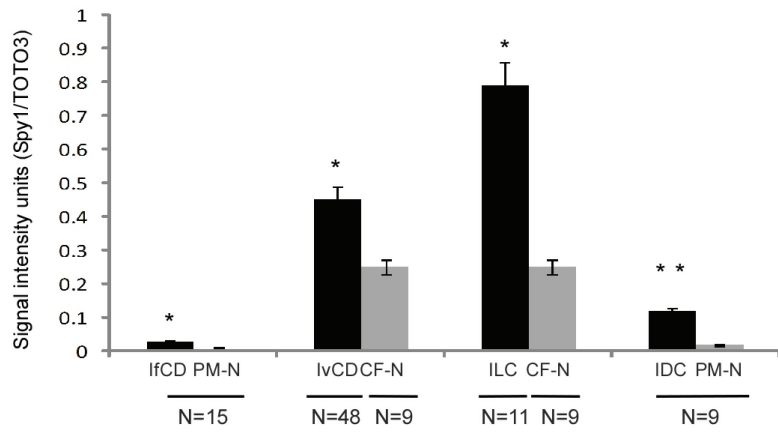
Determination of the p27 binding region within Spy1

Using a panel of Spy1 deletion mutants previously described [25] we began to narrow down the region within the Spy1 protein that was necessary for p27 binding. Previously, it was determined that truncation of Spy1 at aa215 was able to bind to p27 while truncation at aa160 was not able to bind to p27, demonstrating that the 55aa difference between these constructs represented a sequence essential for binding to p27 [5] (Fig. 2A; indicated beneath panel). We first determined whether any our panel of Spy1 deletion mutants would result in abrogation of binding to p27. 293 cells were transfected with wild-type Spy1 (WT) or versions of the Spy1 protein harboring the specified deletions (DMA-DMZ) (Fig. 2B). Cells were lysed and equal amounts of protein immunoprecipitated with Myc antibody and analyzed by western blot. We determined that deletion of aa145-239 (DMG) was essential for the binding between Spy1 and p27,confirming previous reported results [5]. While there is a considerable variety in the recognition motifs for p27 binding partners, it has been noted that p27 binding favors positively charged amino acids on the binding partner [26]. Alignment of the determined p27-binding region within Spy1 sequences from a number of different organisms noted a region of high similarity containing a string of 4 highly conserved positive amino acids (Fig. 2C). Hence, we generated a mutant of Spy1 containing alanine substitutions for arginines 170 and 174 (Spy1-R170) as well as a mutant containing alanine substitutions for arginines 179 and 180 (Spy1-R179) (Fig. 2D).

Figure 2. *Generation of Spy1 – p27 binding mutants.* (A) A schematic diagram of the Spy1 deletion mutants used for screening. Previously defined [5] truncation of Spy1 which retained p27 binding (aa215; depicted by dotted line below), or lost p27 binding (aa160; depicted by solid line below). (B) 293 cells were transfected with Myc-Spy1-PCS3 (WT) or the different deletion mutants DMA-DMZ depicted above in the presence of HA-tagged p27 (HA-p27). Transfected cells were treated with MG132 (10μM) for 14 hrs prior to harvest, lysates were immunoprecipitated with Myc antibody and immunoblotted with HA antibody (upper panel) and Myc antibody (lower panel). This is one representative experiment of 3. (C) Alignment of the amino acid sequence within a conserved region of DMG from several species. Conserved positively charged residues to be mutated are noted with a box. Amino acid #s are indicated after the species in brackets. (D) Region G of Spy1 depicting the Arginine. (R) residues which were mutated to Alanine (A) to create Spy1-R170 and Spy1-R179 mutations.

A


```
1        57   83      145              238    286
|        |    |       |                |      |
|--A--|       |--C--|                      |Z|
Nde1 & Mlu1   BglII & Nco1              Bbs1 & Nde1
   |--B--|          |-------G-------|
   BglII & Mlu1     Nco1 & Bbs1
```

```
- - - - - - - - - - - - - - ■
                            215
|--------------------■
                    160
```

B

Myc-Spy1   WT   DMA   DMB   DMC   DMG   DMZ

p27 →

IP:Myc
IB:HA

Spy1A →

IP:Myc
IB:Myc
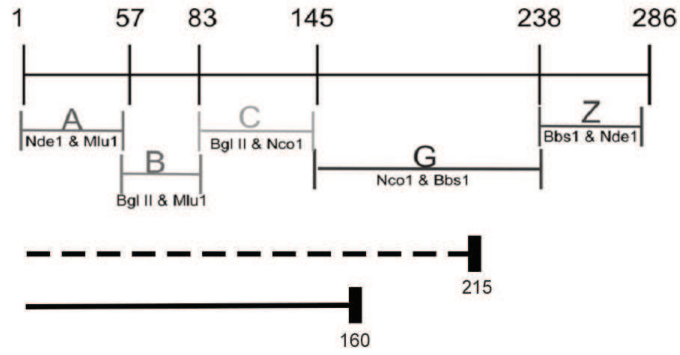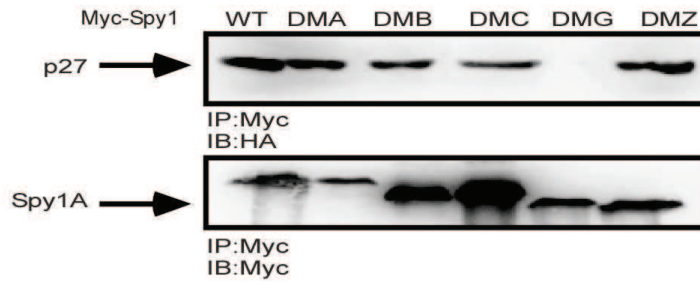
C

		Total % Homology
HumanA1 (160-184)	FLKLRDQLWDRIDYRAIVSRRCCEE	100
HumanA2 (160-184)	FLKLRDQLWDRIDYRAIVSRRCCEE	100
Pig (160-184)	FLKLRDQLWDRIDYRAIVSRRCCEE	90
Bovine (160-184)	FLKLRDQLWDRIDYRAIVSRRCCEE	89
Mouse (159-183)	FLKLRDQLWDRIDYRAIVSRRCCEE	85
Xenopus (150-174)	FLRLRDDFWAKMNYRAVVSRRCCEE	50
Zebrafish (163-177)	FLKQRDQLWARIEYRAAVSRRCCEE	65
Schistosoma mansoni (171-195)	FSRQKEALWARMGYRAVVSYRCCKE	45

D

R170   R179
 A   A   A A
LKLRDQLWDRIDYRAIVSRRCCEEVMAIAPTHYIWQRERSVHHSGAVR

Spy1-R170 and -179 do not bind to p27

To test the necessity of these conserved arginine residues for binding to p27, Myc-tagged Spy1-WT, Spy1-R170, or Spy1-R179 were transfected in combinations with HA-tagged p27 in the presence of MG132 (Fig. 3A). Immunoprecipitation/immunoblotting studies demonstrate that Spy1-R170 and Spy1-R179 mutants both reduce binding to p27 as compared to Spy1-WT binding (Fig. 3A). Reciprocally, immunoprecipitation using Myc antibody isolated p27 when Spy1-WT was overexpressed but not the mutant Spy1 constructs (Fig. 3B). Cdk2 continued to bind to Spy1 in the presence of both mutations (Fig. 3B). We also examined the interaction between the endogenous p27 and either Spy1-WT or Spy1-mutants. Following immunoprecipitation for the Myc-tagged Spy1 proteins, endogenous p27 protein was detected as part of a complex with Spy1-WT but not with either of the Spy1 non-binding mutants (Fig. 3C). Collectively, these results demonstrate that mutation of Spy1 at either arginines 170/174 or 179/180 abrogates binding interactions with the CKI p27. These will provide a valuable tool in assessing the specific role for Spy1-p27 interactions in functional experiments.

Figure 3. *R170 and R179 mutants of Spy1 abrogate binding to p27.* 293 cells were transfected with constructs indicated above the panels and treated with 10μM MG132 for 14 h. prior to lysis. Equal amounts of protein were immunoprecipitated followed by immunoblotting as indicated below each panel (upper panels). For each experiment cell lysates were also ran and immunoblotted to demonstrate transfection efficiencies (lower panels). (A) Overexpression of all constructs and immunoprecipitation for HA-tagged p27. One representative experiment of 3. (B) Overexpression of all constructs and immunoprecipitation for Myc-tagged Spy1 constructs. One representative experiment of 3. (C) Transfection of Spy1-WT or mutant constructs and analysis using endogenous p27. Cells were maintained in 2% serum containing media for 14 h. following transfection to elevate endogenous p27 levels. One representative experiment of 2.

A

p27	-	+	+	+	+
Spy1-WT	+	-	+	-	-
Spy1-R170	-	-	-	+	-
Spy1-R179	-	-	-	-	+

Spy1 →
IP:HA
IB:Myc

p27 →
IP:HA
IB:HA

p27 →
Lysate
IB:HA

Spy1 →
Lysate
IB:Myc

B

p27	-	+	+	+	+
Spy1-WT	+	-	+	-	-
Spy1-R170	-	-	-	+	-
Spy1-R179	-	-	-	-	+

p27 →
IP:Myc
IB HA

Spy1 →
IP:Myc
IB Myc

Cdk2 →
IP:Myc
IP:Cdk2

Spy1 →
IB Myc

p27 →
IB HA

Lysate

Actin →
IB:Actin

C

PCS3	+	-	-	-
Spy1-WT	-	+	-	-
Spy1-170	-	-	+	-
Spy1-R179	-	-	-	+

p27 →
IP:Myc
IB:p27

Spy1 →
IP:Myc
IB:Myc

Spy1 →
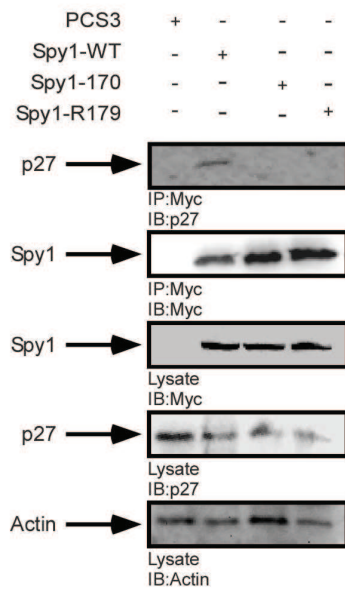Lysate
IB:Myc

p27 →
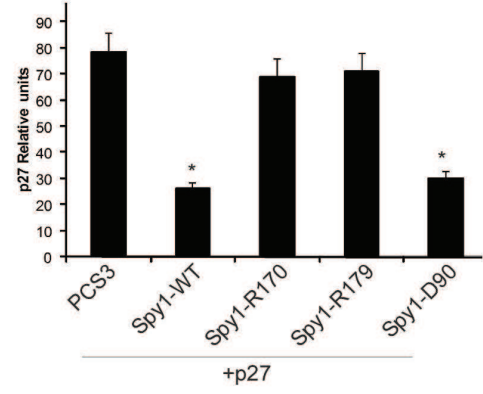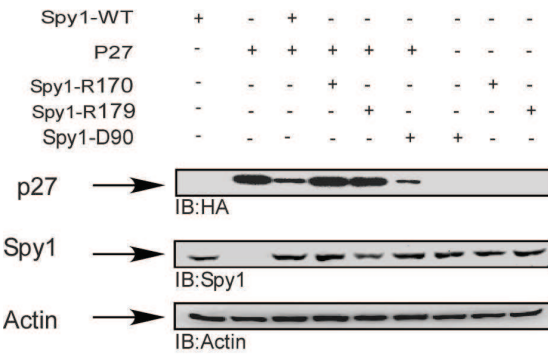Lysate
IB:p27

Actin →
Lysate
IB:Actin
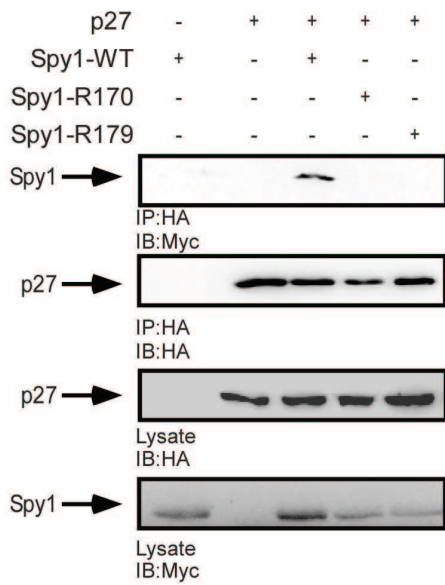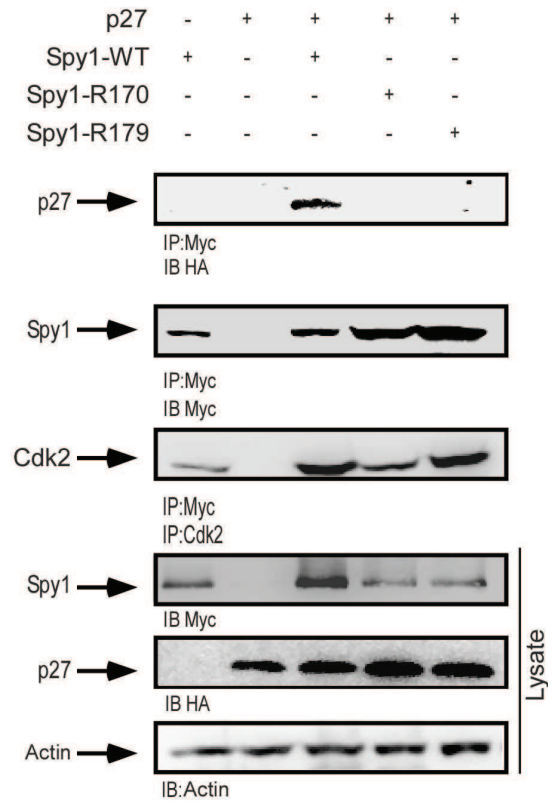
Effect of Spy1 mutants on p27 degradation

Direct binding of Spy1 protein to p27 enhances p27 degradation and subsequently activates Cdk2 kinase activity [2,5]. Importantly, Spy1 also directly binds to Cdk2 to activate kinase activity and it is known that Cdk2 phosphorylation of p27 on T87 promotes p27 degradation [27] Therefore, to determine whether the direct binding of Spy1 to p27 and/or Cdk2 is critical for Spy1-mediated degradation of p27 we utilized 293 cells transfected with either the Spy1-p27 non-binding mutants (Spy1-R170 and Spy1-R179) or the Spy1 mutant unable to interact with Cdk2 (Spy1-D90) and the lysates were monitored by western blot (Fig. 4A; left panel). Results from 3 separate experiments were quantified by densitometry and differences in the protein levels of p27 were analyzed for statistical significance (Fig. 4A; right panel). Cells transfected with Spy1-WT and Spy1-D90 resulted in a significant reduction in overall p27 protein levels, quantified as less than half that seen in cells overexpressing an empty vector control, or the Spy1-p27 non binding mutants (Fig. 4A). To further confirm these results we used radioactive sulfur (S^{35}) incorporation in a pulse chase assay, immunoprecipitated for p27, followed by SDS-page (Fig. 4B; left panel) and phosphor-image analysis to determine relative stability of p27 protein levels over 3 separate experiments (Fig. 4B; right panel). IgG was used as a control for the immunoprecipitation and lysates used for control over transfections (Fig. 4B; left bottom panels). Quantification of this data demonstrates that Spy1-WT and Spy1-D90 significantly reduced the stability of p27 protein levels over the empty vector (PCS3) control, however the Spy1-R170/R179 mutants were unable to significantly impact p27 turnover, hence demonstrating that the direct binding between Spy1 and p27 is essential for Spy1 effects on p27 protein turnover.

To examine the effect of Spy1 mutants on cell proliferation, cells were transfected with the indicated constructs and cell number after 24 hr. was assessed by trypan blue exclusion (Fig. 4C). Cells transfected with the Spy1 mutants unable to interact with p27 showed a significant decrease in the total cell number as compared to Spy1-WT, being comparable to that of the empty vector control (PCS3). Importantly, the Spy1 mutant unable to interact with Cdk2 (Spy1-D90) resulted in a significant increase in the total number of cells over the empty vector control, with numbers comparable to that of Spy1-WT. Collectively, these results demonstrate that abrogating the direct binding between Spy1 and p27 is detrimental for Spy1-mediated proliferation while abrogating the direct binding between Spy1 and Cdk2 is not.

Figure 4. *R170 and R179 mutants inhibit p27 down regulation.* 293 cells were transfected with HA-tagged p27 in the presence or absence of Myc-tagged empty–vector (PCS3), Spy1-WT, Spy1-R170, Spy1-R179 or Spy1-D90. (A) Lysates were immunoblotted with HA (upper panel), Spy1 (middle panel) and Actin (lower panel) antibodies. Left panel reveals one representative blot of 3. Right panel reflects densitometry of p27 levels equalized with actin over 3 separate experiments. Error bars represent SEM. *= $P<0.05$ (B) Pulse chase assay was conducted, followed by immunoprecipitation for HA-tagged p27 and S^{35} incorporation measured by phosphor image analysis (image; upper left panel). Immunoblot using IGg antibody was used as a control (lower left panel). Right panel represents quantification using OptiQuant software. This is one representative experiment of 3, error bars represent SEM, *= $P<0.05$ (C) 30 h post-transfection cells were collected and counted using trypan blue exclusion. Error bars represent SEM. *= $P<0.05$ This is one representative experiment of 3

A

Spy1-WT	+	-	+	-	-	-	-	-
P27	-	+	+	+	+	+	-	-
Spy1-R170	-	-	-	+	-	-	+	-
Spy1-R179	-	-	-	-	+	-	-	+
Spy1-D90	-	-	-	-	-	+	+	-

p27 → IB:HA

Spy1 → IB:Spy1

Actin → IB:Actin

B

Spy1-WT	-	+	-	-	-
P27	+	+	+	+	+
Spy1-R170	-	-	+	-	-
Spy1-R179	-	-	-	+	-
Spy1-D90	-	-	-	-	+

$p27^{S35}$ → IP:HA

IGg → IP:HA
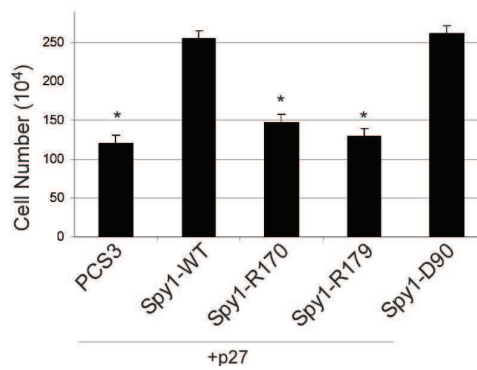IB:IGg

Spy1 → IB:Myc   ⎤ Lysate

p27 → IB:HA

C

127
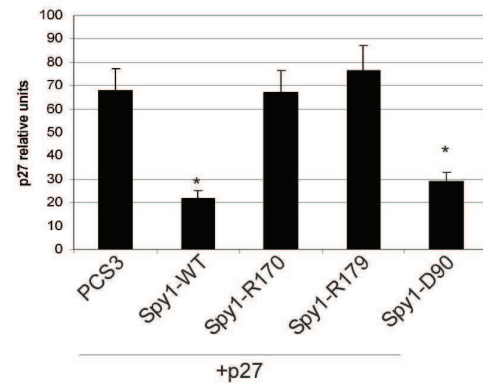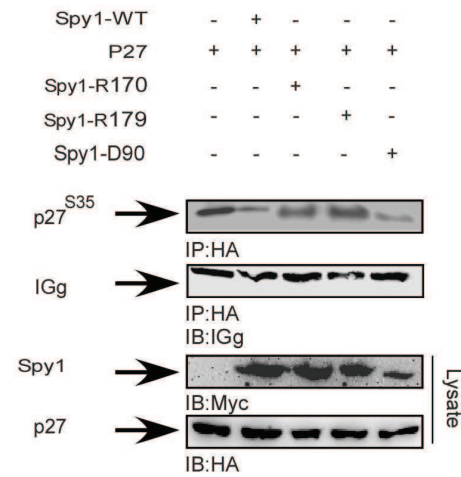
Spy1 direct binding to both p27 and Cdk2 are important for Spy1-mediated tumorigenesis in vivo

It has previously been shown that overexpression of Spy1 *in vivo* leads to accelerated rates of mammary tumorigenesis [14]. To determine if this is dependent on Spy1's interaction with either Cdk2 or p27, fat pad transplantation was performed on the cleared inguinal mammary gland of Balb/C mice using wild-type Spy1 (Spy1-WT) expression on one side of the mouse and Spy1 mutants defective for either Cdk2 binding (Spy1-D90) or p27 binding (Spy1-R170) on the opposite side. One week following surgery, approximately 70% of all Spy1-WT glands had visible tumors while only 15% of all Spy1-D90 glands and 10% of all Spy1-R170 glands had visible tumors (Fig. 5A). At day 21 post-transplant ~70% of the Spy1-D90 glands had detectible tumors, similar to that of Spy1-WT glands while the Spy1-R170 glands were still ~60% tumor-free. However, by day 28 post-transplant 100% of Spy1-WT glands had detectable tumours, only 10% remained tumor-free for Spy1-D90 and ~20% were tumor-free for the Spy1-R170 mutant. Mice were humanely sacrificed at day 28 post-surgery due to the size and invasiveness of the Spy1-WT glands and glands were removed and studied. There were dramatic differences in the overall tumor size observed with Spy1-WT glands being much larger and more invasive than both the Spy1-D90 and Spy1-R170 tumors (Fig. 5B). These data indicate that Spy1 direct interactions with both p27 and Cdk2 are important for Spy1-mediated tumorigenesis *in vivo*. It further suggests that inhibition of both of these functional pathways is required to significantly reduce tumorigenic effects over time.

Figure5. Spy1 tumorogenic activities are p27 and Cdk2 dependent.

Cleared mammary fat pads were transplanted with cells expressing Spy1-WT (WT) on the left and either Spy1-R170 (R170) or Spy1-D90 (D90) on the right. (A) Graphic depiction of the percent of mice remaining free from palpable tumours at time points following transplantation. Treatments occurred in groups of 3 using 3 separate colonies of infected cells.. Mann-Whitney Test was performed (p<0.05). (B) Mice were humanely sacrificed at the indicated time points and glands showing visible tumors were isolated and measurements of height, length and width of the tumors were recorded. Total average tumour area over 45 mice transplanted with either R170 or D90 (right gland) and Spy1-WT (left gland) from three separate infections are depicted.*,P<0.05.

A
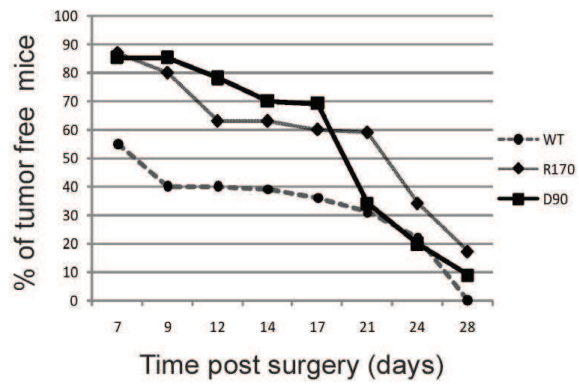
B
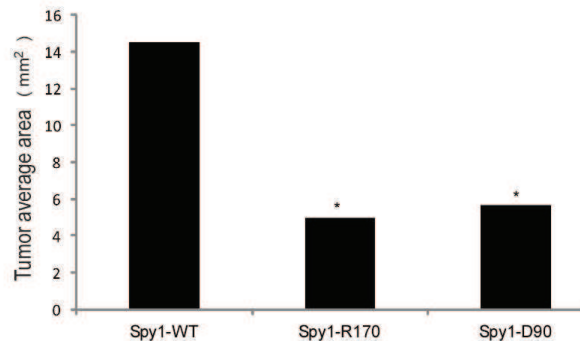
Materials and methods

Cell culture

Mouse embryonic fibroblast cell line, NIH3T3 (NIH) and human embryonic kidney cell line, HEK293 (293) were both purchased from ATCC and maintained in DMEM medium (Sigma) supplemented with 10% (vol/vol) fetal calf serum (Sigma; NIH) or fetal bovine serum (Hyclone). HC11, a non-immortalized BALB/c mouse mammary epithelial cell line (provided by Dr. C. Shermanko; University of Calgary) were maintained in RPMI 1640 medium (Hyclone) containing 10% (vol/vol) fetal calf serum (Sigma), supplemented with 5 µg/ml insulin (Sigma), and 10 ng/ml EGF (Calbiochem). All cell lines were maintained in a media containing 2mM L-glutamine (Sigma), penicillin (Invitrogen), and streptomycin (Invitrogen), and were cultured in a 5% CO_2 environment.

Plasmid and mutagenesis

Creation of Myc-Spy1 in PCS3 was described previously [1]. Flag-Spy1-PEIZ was generated by moving Flag-Spy1 from Spy1-PJT0013 previously described [2] through EcoR1 and Xba1 restriction sites into the lentivirus vector PEIZ (provided by Dr. B. Welm, University of Utah). Spy1-D90 and Spy1-R170 were created in Myc-Spy1-PCS3 using Quik Change PCR Multi Site-Directed Mutagenesis (SDM). Myc-Spy1-R170-PCS3 mutation was made using primers # A315 5'-GTTAAGGGACCAGCTCTGGGATGCAATTGACTATGCGGCTATTGTAAGCAGG- 3' and # A316 5'-CCTGCTTACAATAGCCGCATAGTCAATTGCATCCCAGAGCTGGTCCCTTAAC-3' ;Myc-Spy1-D90-PCS3 was made using primers #A123 5'-GATTTCTTGTGGATGGCATGCTGCTGTAAAATTGC-3' and #A124 5'-GTTAAGGGACCACTGGGATGSAAGACTATGCGGCTATTGTAAGCAGG 3',

Spy1-R170-PIEZ lentivirus plasmid was created by engineering an EcoR1 site upstream Spy1 in Myc-Spy1-PCS3 using primers #A532 5'-CTTGATTTAGGTGACACTATAGAATTCAAGCTTGTTCTTTTTG-3' and #A533 5'-CAAAAAGAACAAGTAGCTTGAATTCTATAGTGTCACCTAAATCAAG-3'. Myc-Spy1-R170 was moved to PIEZ lentivirus vector via the EcoR1 and XbaI sites. Successful cloning in all cases was determined by DNA sequencing (Robarts Sequencing Facility; Univ. of Western Ontario).

Fat pad transplants

BALB/c mice were maintained following the Canadian Council on Animal Care guidelines at the University of Windsor (protocol #06-19). HC11 cells infected and selected for the relevant lentiviral constructs were injected into the cleared fat pad of the fourth mammary glands in 22-day old mice (250,000 cells per gland) as previously described [14]. Tumor incidence was monitored every 2 to 4 days beginning one week after surgery by palpitation of the gland. Glands were allowed to grow for 2 to 4 weeks following surgery. Collected glands were either paraffin embedded for immunohistochemistry or flash frozen for protein, genomic or mRNA analysis.

Quantitative reverse transcriptase PCR (Q-RT-PCR)

Total RNA was extracted using the RNAeasy Plus Mini Kit (Qiagen) and reverse transcribed using 200 U Superscript II (Invitrogen), 0.5g Oligo dT's and 0.5 g random nanomers (Sigma) according to the manufacturer instructions. Q-PCR was carried out using SYBR green detection (Applied Biosystems) with 400 nM of each primer (Table 1; Suppl. Mat.) and PCR was performed using ABI Prism 7300 thermocycler. Data was analyzed using ABI 7300 software and represented $\log_{10}$ relative quantification (RQ)

relative to control. Custom primers were designed using Primer Express software (Applied Biosystems).

Immunoblotting

Cells were harvested and lysed in 0.1% NP-40 lysis buffer (5 ml 10% NP-40, 10 ml 1M Tris pH 7.5, 5 ml 0.5M EDTA, 10 ml 5M NaCl up to 500 ml RO water) containing protease inhibitors (10 μl/ml PMSF stock solution 10 mg/ml, 3 μl/ml aprotinin stock solution 20 mg/ml, 10 μl/ml leupeptin stock solution 1 mg/ml) for 30 Min on ice. Bradford Reagent was used to determine the protein concentration following the manufacturer's instruction (Sigma). Aliquots of lysates containing 20-30 μg protein were subjected to electrophoresis on denaturing SDS-10% polyacrylamide gels and transferred to PVDF-Plus 0.45 micron transfer membranes (Osmonics Inc.) for 2 h at 30V using a wet transfer method. Blots were blocked for 2 h in TBST containing 3% non-fat dry milk (blocker) at room temperature, primary antibodies were reconstituted in blocker and incubated over night at 4°C, secondary antibodies were used at a 1:10,000 dilution in blocker for 1 h at room temperature. Blots were washed three times with TBST following incubation with both the primary and secondary antibodies. Washes were 6 Min each following the primary antibody and 10 Min each following the secondary antibody. Chemilumiminescent Peroxidase Substrate was used for visualization following the manufacturer's instruction (Pierce). Chemiluminescence was quantified on an Alpha Innotech HD2 (Fisher) using AlphaEase FC software.

For immunoprecipitation, equal amounts of protein were incubated with primary antisera as indicated overnight at 4°C, followed by the addition of 10 ul protein A–Sepharose (Sigma) and incubated at 4°C with gentle rotation for an additional 2 h. These

complexes were then washed three times with 0.1% NP-40 lysis buffer and resolved by 10% SDS-PAGE.

Transfections and infections

Cells were transfected overnight using polyethylenimine (PEI) branched reagent Sigma (408727), 10 µg of DNA was mixed with 50 µL of 150 mM NaCl and 3 µL of 10 mg/ml PEI for 10 Min then added to a 10 cm tissue culture plate.

Lentiviral infections were carried out using a modified protocol from Welm et. al [28]. In brief; PEI transfection of Lenti-XTM 293 producer cell lines (Cat. No. 632180, Clontech, Mountain View, CA, USA) was conducted as described above, 16 h post-transfection, media containing viral particles was concentrated, tittered and stored at -80°C (detailed protocol; Appendix 1). Cells were screened for ZS-Green using flow cytometry. HC11 infections were optimized using $2x10^5$ cells/well; 6-well plate to determine the multiplicity of infection (MOI), an MOI of 10 was determined for HC11. Briefly, HC11 were seeded with media lacking antibiotics for 18 h the media removed and transfection media lacking antibiotics and serum is added. Virus particles were added in final concentrations of $8*10^5$ TU(transfection unit = MOI * cell number), and polybrene added to a final concentration of 10 ug/ml and incubated for 4 h. After 4 h media was changed to media lacking antibiotic only.

Antibodies

The generation of Spy1 antibody was previously described [1]. Myc antibodies both mouse (9E10) and Myc rabbit (C19), the HA probes (Y11 and F7) and p27 (F8) were purchased from Santa Cruz. Actin antibody (MAB150R) was purchased from Calbiochem. HRP conjugated secondary mouse antibody (A9917) and rabbit antibody (A0545) were purchased from Sigma.

Pulse chase

16 h post-transfection with indicated constructs cells, serum free media was replaced with Cys-Met free media (D0422; Sigma) supplemented with dialyzed FBS (F0392; Sigma) for 1 h. S^{35} was added to a final concentration of 500 µCi for 4 h. followed by 4 washes with 1X PBS and supplementation with growth media containing 2 mM Cys-Met. Cells were lysed at indicated time points, ran on an SDS-page gel. Incorporated sulfur was visualized using a Cyclone storage phosphor system and quantified using OptiQuant software (Perkin Elmer, Biology Department UofW).

Tissue microarray (TMA) analysis

Paraffin embedded TMA slides consisting of 165 tissue cores in total were purchased from U.S. Biomax (cat # BR721 and BR962). Slides were deparaffinized in xylene and decreasing percentages of ethanol according to the manufacturer's instructions. Antigen retrieval was performed at 95°C in citrate buffer (1mM EDTA, pH 8.0 or 0.01M sodium citrate buffer, pH 6.0). Slides were washed in 1x PBS and subjected to immunohistochemistry. Nuclei were counterstained with TOTO-3 (T-3600 Molecular Probes). The fluorescent signal was detected and quantified by ScanArray Express (Perkin Elmer Inc.). Spy1 signal intensity was normalized to nuclear stain signal.

Discussion

Spy1 is an atypical Cdk activator known to increase cell proliferation and inhibit apoptosis when overexpressed in mammalian cells [1]. In addition to directly binding and activating Cdks,. *in vitro* and *in vivo* experiments demonstrate that Spy1 directly binds and co-localizes with nuclear forms of with p27, functioning to override p27-mediated cell cycle inhibition [2]. Like Cyclin E-Cdk2, Spy1 overexpression is also associated with an increase in phosphorylation of p27 at T187 leading to its proteasomal degradation and enhanced cell cycle progression [5]. Whether the effects on p27 degradation are mediated through the direct binding of Spy1 to Cdk2 or p27 was not yey resolved. To address this question, we utilized a Cdk2 binding mutant (Spy1-D90) that has been previously described [6], as well as creating a p27 non-binding mutant (Spy1-R170) in assays to assess Sp1 effects on p27 turnover. In addition we determined the effects of these specific Spy1 binding mutants on cell proliferation. We determined that direct binding of Spy1 to p27 was required to promote p27 degradation, as well as Spy1-mediated proliferation. We also demonstrated that Spy1 direct binding to Cdk2 is dispensable for Spy1 mediated degradation of p27. Surprisingly, Spy1 direct binding to Cdk2 was also dispensable for Spy1-mediated proliferation. This could be attributed redundancy, it is known that Spy1 is capable of binding to Cdk1 when its overexpressed [29] and Cdk1-Cyclin E has the ability to regulate G1/S [30]. In addition to enhancing cell proliferation, Spy1 plays a role in increasing cell viability during cellular responses to DNA damage [3],It has been shown that these effects are drivin by the direct binding and activation of Cdk2 by Spy1 [4]. Collectively, these data highlight the significance of Spy1 binding to p27 and Cdk2 for Spy1 mediated cell proliferation and apoptosis,

suggesting that these cellular phenomena are regulated by Spy1 via two separable pathways.

Role of Spy1-p27 binding in tumorigenesis

Elevated expression of Spy1 has been implicated in one SAGE analysis study of invasive ductal carcinoma of the breast and elevated protein levels implicated in hepatocarcinoma [23,24]. Our lab has demonstrated that overexpression of Spy1 accelerates the rate of mammary tumorigenesis in the virgin mammary gland [14]. In this present study, we show that abrogating direct interactions between Spy1 and either p27 or Cdk2 significantly decreases the rate of tumorigenesis and overall tumour size *in vivo*. Low levels of p27 protein has been implicated in many human cancers including that of the breast [31]. Lung cancer is associated with one of the highest mortality rate of all forms of cancer, many studies have demonstrated that the loss of p27 is associated with increased tumor size, poor prognosis, high tumor grade and poor patient outcome [31]. In addition, constitutive activation of Cdk2 results in mammary gland hyperplasia, fibrosis, and mammary tumors in MMTV–cyclin D1–Cdk2 derived cell line [32]. Our results show the significance of Spy1 binding to both p27 and Cdk2 in driving the tumorogenic activities of Spy1.

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Chapter 5

General Discussion

In this work I endeavored to shed light on the role of a novel cell cycle regulator, Spy1, in the normal cell cycle as well as its role in tumor initiation and progression. Spy1 protein is a cyclin-like protein which exhibits no amino acid sequence homology to cyclins but has the ability to bind and activate Cdk1 and Cdk2 independent of cyclin binding [1].

It is well established that misregulation of cell cycle regulators such as Cdks, CkIs and cyclins are linked with oncogenesis. This abrogated regulation results in the inability to properly respond to cellular stress and DNA damage signals during specific checkpoints designed to halt the cell in such circumstances. Spy1 is not only found to bind and activate Cdks in normal cell cycle conditions, it is also known found to override DNA damage by decreasing cellular sensitivity to apoptotic signals [2]. As such, losing control of crucial regulator like Spy1 is a great danger for normal cell cycle integrity. On the other hand, cell cycle regulators, including Spy1, do not perform in isolation; instead, they are active via multiple interactions that form an intercalating surveillance network to ensure proper cell cycle progression. Therefore, understanding how Spy1 is synthesized and degraded is essential in resolving how it contributes to normal and abnormal cellular processes.

Breast and cervical cancers are the most common malignancies in women worldwide. Uncontrolled cell proliferation, which is associated with the loss of the proper cell cycle control, is a prominent feature in these cancers [3]. The sequential progression through the cell cycle depends on regulating the abundance of several proteins through ubiquitin-mediated proteolysis. Degradation is a precisely timed and specific event (see Chapter 1 for more details). Cyclin E deregulation impairs progression through S phase

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[4], while cyclin B1 overexpression correlates to aneuploidy and high proliferation in human mammary carcinomas [5]. To date two reports have discussed the mechanism regulating Spy1 protein levels in mammalian cells [6,7]. Chapter 2 of this work was the first to describe a mechanism regulating Spy1 turnover in a mammalian system, in addition to exploring the degradation mechanism of Spy1 and mapping important residues on the protein crucial for degradation, we were able to prove that Spy1 misregulation has an impact on the cell cycle, like other core cyclins. Our data revealed that stabilization of Spy1 protein levels enhances cell proliferation over the wild-type protein. The second report that came shortly after our work described the same cyclic profile of Spy1, as well as further describing an alternative degradation mechanism that involves the E3 ligase SCF^{Skp2} [6]. The fact that these two reports outlined two different degradation mechanisms of Spy1 stresses the importance of tight regulation of Spy1 protein. It is well known that most cell cycle regulators, especially those regulated by the ligase SCF^{Skp2}, have multiple degradation mechanisms involving different E3 ligases [8].

Based on our data we carried on to see if this stable form of Spy1 is capable of initiating tumors or has any oncogenic activities. Performing different colony formation assays we were able to show that the stable form of Spy1 has oncogenic activities and was able to accelerate tumor formation in Balb/c mouse model. These results, along with work from our lab demonstrating the role of wild type protein in tumor formation, clearly highlight the importance of Spy1 protein in cancer development and progression [9]. The onset of Spy1-induced tumor formation described in this work was considerably more rapid than that seen in Golipour et al. [9]. This difference could be attributed to the differences in gene delivery mechanisms used; Golipour et al. utilized a retroviral

infection of a cell population cultured over a long period of time, whereas this work utilized lentiviral infections to generate 3 separate populations of infected cells grown over limited number of divisions. The switch in theses systems was made because the retrovirus infections were not found to maintain stable overexpression, while it appears from this data that the lentiviral infections are much more efficient in this regard. Although this work focuses on one aspect of Spy1 regulation, we can not neglect a very important dimension of Spy1 regulation and its involvement in cancer development. Transcriptional regulation of Spy1 has been studied previously in our lab, Golipour et al., have found that Spy1 is a direct downstream target of the transcriptional factor c-Myc [9]. Moreover, Spy1 protein and mRNA levels are uniquely elevated in tumor tissues drivin by the c-Myc oncogene as compared to normal mammary gland tissues. The importance of these results comes from the fact that c-Myc is overexpressed in about 70% of breast cancer cases [10]. It is very clear that aberrant Spy1 protein levels are implicated in cancer development and progression, it is possible that transcriptional up regulation can be attributed to c-Myc-mediated tumorgenesis, our work also demonstrates that Spy1 protein stability needs to be studied in more depth to resolve how this protein is regulated an all forms of human cancer.

Cell cycle regulators do not work alone, Spy1 can directly bind and activate Cdk2 and Spy1 also directly interacts with p27, enhancing its degradation and overriding a p27-induced cell cycle arrest [11,12]. Considering the role of p27 in tumorgenesis (chapter 1 for review), elevated levels of Spy1 may result in the degradation of this crucial cell cycle inhibitor, thus accelerating the onset of tumorgenesis. While the proliferative effect of Spy1 depends on the presence of endogenous p27 in mouse embryonic fibroblasts, Spy1

is still capable of binding Cdk2 in p27 null cells, suggesting that Spy1 has independent roles represented by direct interactions with both Cdk2 and p27 [12]. To address the importance of each of these interactions individually on tumor formation and development, I have constructed a p27 binding mutant (R170A) and used a previously described Cdk2 mutant (D90A) [13]. Utilizing these mutants in a Balb/c mouse model we have demonstrated a significant reduction in tumorogenic properties, including tumor onset *in vivo* compared to wild-type Spy1. This delay in tumor formation highlights the importance of Spy1 binding to both Cdk2 and p27, the fact that tumors still formed in these mutations necessitate the need to develop a vector inhibiting interaction with both p27 and Cdk2. It is possible that there are yet other important Spy1 effectors which remain to be elucidated but represent important mediators in mammary tumorgenesis. Work from our lab implicates Spy1 in the Wnt/β-Catenin pathway. Despite its numerous functions, β-Catenin plays a role in cell adhesion and invasion is considered one of the most important regarding its contribution to tumorgenesis [14] Our data shows that Spy1 interacts with mediators of β-Catenin signaling and stability, Axin and LRP6. Axin is an essential scaffolding protein in the Wnt pathway which appears to play a role in both the degradation and stabilization/activation of β-Catenin [15,16]. LRP6 is a membrane bound receptor which forms a complex with a number of other proteins, including Axin, to recruit β-Catenin to the membrane and stimulate signaling [15,17]. These data may add another dimension to Spy1 role in tumorgenesis and support the idea that it is not only one or two interactions that derives the functionality of Spy1, it is rather an intercalated cycle of interactions that they have to be addressed independently and in combinations in primary cell lines and eventually in mouse models. In addition, it is essential to test this

data using primary cells since the HC11 cells are immortalized have a mutated form p53 and can form tumors by them selves after prolonged time [9]. Hence, whether Spy1 is acting to promote tumorgenesis only, or Spy1 may initiate tumorgenesis is not yet resolved.

Collectively our data supports that Spy1 interactions with p27 are essential in regulating Spy1 proliferative-functions in mammary tumorgenesis and that interactions with Cdk2 override normal protective cellular barriers, abrogating both interactions is an essential next step as a proof of principal for testing Spy1 as a putative therapeutic target. To address this conclusively, wild-type Spy1 and mutated forms os Spy1 need to be tested using primary cell lines and eventually genetic mouse models.

Perspectives and Future Directions

To clearly understand the development and progression of cancerous cells it is essential that we elucidate the mechanisms and interactions of normal regulators of the cell cycle. Spy1, a cell cycle protein elevated in different kinds of cancers including breast and brain, is tightly regulated during the cell cycle and that elevated levels of Spy1 was shown to promote tumor formation in mammary cells. In this body of work we were able to elucidate key molecular mechanism mediating Spy1 degradation during the cell cycle as well as to demonstrate that Spy1 protein regulation may be an important mediator of tumorgenesis. Further more, we addressed the roles of two downstream effectors of Spy1; Cdk2 andp27, Spy1-mediated tumor formation as well as mammary proliferation and differentiation. Although we have seen some delay in tumor formation, the need of Spy1-p27-Cdk2 double binding mutant is an important next step for this project. In addition, moving these experiments to include primary cell lines or genetic

models should be the destination for this follow up. The implications of transcriptional regulators such as c-Myc in Spy1 mediated tumorgenesis remain to be resolved. Determination of how Spy1 is differently regulated among tumor types is also an important future direction.

This work is an important first step in determining how Spy1 is regulated in normal cells, and how this is differs in specific forms of cancer. Importantly, these studies are the first to begin to resolve the involvement of key Spy1 effectors in Spy1-medaited tumorgenesis. This work may provide essential information for the development of future therapeutics for breast cancer and other forms of disease.

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Vita Auctoris

Mohammad Al Sorkhy was born in the year of 1975 in Zarqa, Jordan. He obtained an Honours B.Sc. in Applied Biological Sciences from Jordan University of Science and Technology in 1997. From there he went to graduate school at the same school where he obtained a Master degree in Microbiology in the year of 2000. Currently he is a candidate for the Doctor of Philosophy degree at the Department of Biology at the University of Windsor and he is hoping to graduate in the summer of 2010.