Spy1A is a cyclin-like protein required for progression through the G1/S phase of the cell cycle. Elevated Spy1A protein levels have been implicated in tumorigenesis and are attributed to overriding the DNA damage response and enhancing cell proliferation. Understanding how Spy1A is produced and degraded is essential in resolving how it contributes to normal and abnormal growth processes. Herein, we demonstrate that Spy1A is degraded in a cell cycle-dependent manner during mitosis via the ubiquitin-proteasome system. We have resolved the E3 ligase and essential phosphorylation sites mediating Spy1A degradation. Furthermore, we have determined that non-degradable forms of Spy1A do not trigger cell cycle arrest but, rather, contribute to uncontrolled cell growth. Further investigation into the regulation of Spy1A may reveal novel strategies for understanding the etiology and progression of specific growth disorders.

Members of the Speedy/RINGO family are unique cyclin-like regulators of the cell division cycle. There are now five 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 cyclin-dependent kinase 2 (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 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 laboratory 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 G1/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 after meiosis I and is mediated by the ubiquitin ligase Siah-2; this depends on phosphorylation of X-Spy1 on a C-terminal residue Ser-243 (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. The ubiquitin-proteasome system 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 26 S 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 G1/M phase of the cell cycle. We have determined three 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 down-regulated-4 (Nedd4) is capable of bind-
ing to Spy1A and that dominant negative forms and knock-down of Ned4 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 Dulbecco’s modified Eagle’s medium (Sigma) containing 2 mM l-glutamine (Sigma), penicillin, and streptomycin (Invitrogen) and were cultured in a 5% CO₂ environment. MCF7 cells were supplemented with 10% fetal bovine serum.

**Plasmids and Mutagenesis**—The Ned4-PCEP plasmid (Ned4d), dominant negative Ned4-PCEP plasmid (Ned4ΔDN), and empty vector control (PCEP) were provided by Dr. Dale S. Haines (Temple University School of Medicine). HA-tagged ubiquitin (HA-UB) was provided by Dr. Sylvain Meloche (Université de Montréal). siRNA against Ned4-1 was synthesized by inserting the oligo 5’-GATGAAAGCACCACATATACTTCTGTGCCTGTGGATGGACTGCTGC-3’ into the pSUPER-basic vector, as previously described (16). As a control, LacZ siRNA (scntI) was synthesized and inserted into pSUPER-basic vector as described (19). Creation of Myc-Spy1A-PCSM3 vector was described previously (2). QuikChange multi-site-directed mutagenesis (Stratagene) was used to incorporate new silent sites into the original Spy1a-pJT0013 vector (2) to facilitate the cloning of deletion mutants A (DNA), B (DBM), C (DMC), D (DMG), and Z (DMZ). A BglII site was inserted by altering nucleotide 256 from T to C using the primers A143 (5’-CACCCCTGCAGTATGACTGGATAACGCTGA-3’); Spy1A-S247A was designed using the primers A-153 (5’-CAGCCTAAAAGCCCTTGCACTGAAGCGTCCTATTTG-3’); Spy1A-T33A was designed using the primers A-151 (5’-GACGATTTATCCAAGATCTCTTGTGGATGGTTTTATGTAAAATCAG-3’). DMA was created by digesting wild-type Spy1A (in pRS1) with BbsI and Ndel 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 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 h 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.

Multi-site-directed mutagenesis 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 A152 (5’-CACCCCTGCAGTATGACTGGATAACGCTGA-3’); Spy1A-T33A was designed using the primers A153 (5’-CAGCCTAAAAGCCCTTGCACTGAAGCGTCCTATTTG-3’); Spy1A-T15A was designed using the primers A150 (5’-CACCGACAG-TCCATCCACAGAAGATTTGTTAATATCCGTC-3’) to construct the pRA01 vector. Using the pRA01 plasmid, an Mlu site was also added by altering nucleotide 175 from C to G using A004 (5’-GACGATTTATCCAAGATCTCTTGTGGATGGTTTTATGTAAAATCAG-3’). DMA was created by digesting wild-type Spy1A (in pRS1) with BbsI and Ndel 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 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 h 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.

Multi-site-directed mutagenesis 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’-GAGCACACCACTAC-TGTCCGTGTATTGTAAATACGAG-3’); and A152 (5’-CTGATTATTACATACACCGACAGTAGGTGGTCTTC-3’); Spy1A-T33A was designed using the primers A-153 (5’-CAGCCTAAAAGCCCTTGCACTGAAGCGTCCTATTTG-3’); Spy1A-T15A was designed using the primers A150 (5’-GAGCCTAATGAGCCTGCACTGATAACGCTGA-3’); and A144 (5’-CACCCCTGCACTGATAACGCTGA-3’). Successful cloning in all cases was determined by DNA sequencing (Robarts Sequencing Facility; University of Western Ontario).

**Inhibitors and Antibodies**—The following antibodies were used: Spy1A (NB 100-2521; Novus), Ned4 (ab14592; Abcam), Myc (9E10 and C19; Santa Cruz), actin (MAB1501R; Chemicon), cyclin E (551157; BD Pharmin-gen), IgG (SC66186; Santa Cruz). The following inhibitors were used: N-acetyl-l-leucyl-l-leucyl-l-norleucinal (LLNL; Sigma A6060); MG132 (Sigma C2211); cyclohexamide (Sigma C7698); nocodazole (Sigma M1404); 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 10–12 μg of DNA per 10-cm tissue culture plate. 250 μl of CaCl₂ was incubated with the DNA for 30 s, 250 μl of 2× BBS (50 mM BES, 280 mM NaCl, 1.5 mM NaHPO₄) 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% CO₂ for 12–16 h. The medium was then changed, and plates were returned to 5% CO₂ for at least 12 h before harvest.

**Cell Synchronization and Flow Cytometry**—293 cells were synchronized using a double thymidine block. Briefly, cells were cultured in medium containing 2 mM thymidine for 16 h followed by release into normal medium for 8 h and then a second thymidine block for 14 h and then released into medium containing 70 ng/ml
nocodazole (with or without 10 μM MG132 as indicated). MCF7 cells were synchronized by being cultured in a serum-free medium for 48 h followed by release into media containing serum and 70 μg/ml nocodazole. 293 and MCF7 cells were trypsinized at specified times, washed twice in phosphate-buffered saline, 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 phosphate-buffered saline 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 phosphate-buffered saline.

FIGURE 1. Spy1A protein levels are regulated in a cell cycle-dependent fashion. A, left panel, flow cytometry profiles for MCF7 cells either untreated (Cntl), collected immediately after release from serum starvation (G1), or collected 16 h after release into nocodazole-containing media. Right 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 (IB) with α-Spy1A, α-cyclin E, and α-actin. C, left 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). Right 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.
Samples were then prepared for flow cytometry by treating with 1/1000 of 10 mg/ml stock of DNase free RNase (Sigma) and 50/1000 of 50 mg/ml propidium iodide stock solution. Data were collected using a Beckman Coulter FC500 (Biology Department, University of Windsor), and cell cycle profiles were analyzed using CXP Beckman Coulter FC500 software.

**Immunoblotting**—Cells were lysed in 0.1% Nonidet P-40 lysis buffer (0.1% Nonidet P-40, 1 M Tris, pH 7.5, 0.5 M EDTA, 5 M NaCl) containing protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin) 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 of protein were subjected to electrophoresis on denaturing 10–12% SDS-polyacrylamide gels and transferred to Polyvinylidene Fluoride-Plus transfer membranes (Osmonics Inc.) for 2 h at 30 V using a wet transfer method. Blots were blocked for 2 h in Tris-buffered Tween containing 3% nonfat dry milk (blocker) at room temperature. Primary antibodies were reconstituted in blocker and incubated overnight at 4 °C at a 1:10,000 dilution for all antibodies, and secondary antibodies were used at a 1:10,000 dilution in blocker for 1 h at room temperature. Blots were washed 3 times with Tris-buffered Tween after incubation with both the primary and secondary antibodies. Washes were 6 min each after the primary antibody and 10 min each after the secondary antibody. Chemiluminescent 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 (0.1% Nonidet P-40 lysis buffer 0.1% Nonidet P-40, 1 M Tris, pH 7.5, 0.5 M EDTA, 5 M NaCl) containing protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 2 μg/ml leupeptin) 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 of protein were subjected to electrophoresis on denaturing 10–12% SDS-polyacrylamide gels and transferred to Polyvinylidene Fluoride-Plus transfer membranes (Osmonics Inc.) for 2 h at 30 V using a wet transfer method. Blots were blocked for 2 h in Tris-buffered Tween containing 3% nonfat dry milk (blocker) at room temperature. Primary antibodies were reconstituted in blocker and incubated overnight 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 h at room temperature. Blots were washed 3 times with Tris-buffered Tween after incubation with both the primary and secondary antibodies. Washes were 6 min each after the primary antibody and 10 min each after the secondary antibody. Chemiluminescent 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.

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**In Vivo Labeling**—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 (right-hand panel; asynchronous) for 16 h post-transfection. Lysates were immunoblotted (IB) with α-Myc or α-actin. C, 293 cells were transfected with Myc-Spy1A-PCS3 (wt) or deletion mutant A (DMA). 12 h post-transfection cells were treated with nocodazole and MG132 for 14 h followed by [32P]orthophosphoric acid for an additional 4 h. Cells were lysed, immuno- precipitated with α-Myc, and imaged by Cyclone phosphorimaging (upper blot; left panel). The immunoblot for α-Myc was used as a control (lower blot). Incorporation of orthophosphate was quantified using OptiQuant software; the right panel represents results over three separate experiments. Error bars reflect S.E. t test was performed; **, p ≤ 0.01.
for 4 h at 37 °C. Cells were lysed and immunoprecipitated with Myc antisera. Immunoprecipitations were washed rigorously with Tris-buffered Tween, and samples were analyzed by 10% SDS page gel. Gels transferred to polyvinylidene difluoride membranes were visualized using Cyclone storage phosphor system and quantified using OptiQuant software (PerkinElmer and Biology Department, University of Windsor).

In Vivo Ubiquitination Assays—293 cells were plated and transfected appropriately in a 100-mm dish. 24 h after transfection cells were treated with 10 μM MG132 for 14 h. 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 dithiothreitol) 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% Nonidet P-40 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.

RESULTS

Spy1A Protein Levels Are Regulated in a Cell Cycle-dependent Fashion—MCF7 and 293 cells were blocked in G1 using serum starvation and thymidine block procedures, respectively. Cells were released into serum, and nocodazole-containing media and populations enriched in G1 or G2/M phases of the cell cycle were collected as determined by flow cytometry analysis (Fig. 1, A and C). Cell lysates from the respective populations were immunoblotted for endogenous Spy1A expression (Fig. 1, B and 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. 1, B and D, lower panels). These data demonstrate that Spy1A protein levels are greatly decreased during G2/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.

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 G2/M, and levels of Spy1A were monitored by immunoblotting (Fig. 2B; upper left panel). All deletion mutants of Spy1A were degraded by G2/M phase with the exception of the mutant lacking the first 57 amino acids (DMA). Asynchronous
Nedd4 Regulates Spy1A Turnover

A

| PCS3 | PCEP | Nedd4 | Myc-Spy1 | MG132 |
|------|------|-------|----------|-------|
|     + |     + |     + |       + |       + |

IP: Myc
IB: Nedd4

Myc-Spy1A

IP: Myc
IB: Nedd4

B

NIH3T3
IP: Nedd4 IgG

293
IP: Nedd4 IgG

Spy1A

IB: Spy1A

IgG

IgG

C

PCEP

PM123

HA-Ub

Nedd4

Nedd4

MG132

HA-Ub-Spy1A

IP: HA
IB: Spy1A

100Kd
70Kd
40Kd

D

siCntl

siNedd4

Nedd4

Spy1A

Actin

E

Myc-Spy1

siNedd4

siCntl

MG132

Lysate

IP: Myc
IB: Nedd4

IP: Myc
IB: Nedd4

IB: Actin

F

PCEP

Nedd4

MG132

1 2 3

Spy1A

Actin

Spy1A level

100% 94% 71%
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 three 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.

**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 proteasome-dependent we studied 293 cells in the presence or absence of the proteasome inhibitors MG132, lactacystin, and the calpain inhibitor LLLNL. 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 LLLNL; DMSO, vehicle for MG132 and lactacystin). These data imply that Spy1A abundance is proteasome-dependent. 293 cells were then utilized for an *in vivo* ubiquitination assay where cells were transiently transfected with 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 ubiquitin-proteasome system. 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-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.

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 ligase Nedd4 (product of gueronal precursor cell-expressed developmentally down-regulated gene 4), although 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). Because of 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 protooncogenes as well as tumor suppressors. Nedd4 is known to mono-, di-, and polyubiquitinate its target proteins, where polyubiquitinated proteins are selectively targeted for degradation by the proteasome (19). 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 func-
tions as a ubiquitin ligase for Spy1A, we repeated the co-immunoprecipitation experiment using overexpression of wild-type Nedd4 or dominant negative Nedd4 (Nedd4DN) in the presence of HA-Ub and MG132. Nedd4DN contains a single amino acid substitution that 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 Nedd4DN (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 over three separate experiments demonstrate that Spy1A protein levels accumulate when Nedd4 levels are reduced with siRNA. To examine the effect of knockdown of Nedd4-1 on Spy1A-Nedd4 binding, 293 lysates overexpressing Myc-Spy1A and either siNedd4 or siCntl were immunoprecipitated with α-Myc and either α-Nedd4 or α-actin (Fig. 4D, left panels). Immunoblotting with α-Nedd4 shows that Nedd4-Spy1 binding was significantly reduced after 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 with when MG132 was present (Fig. 4F). To confirm the effect of Nedd4DN on Spy1 protein stability, Nedd4DN or siNedd4wt- Nedd4 and Myc-Spy1A were transfected into 293 cells. 12 h post-transfection cells were released, and at 16 h post-transfection 50 μg/ml cycloheximide was added to prevent de novo protein synthesis. Over three separate experiments immunoblotting for Spy1A followed by quantification showed that wt-Spy1 had a half-life of ~1 h and that stability was decreased significantly in the presence of Nedd4 but was significantly more stable in the cells transfected with Nedd4DN and siNedd4 (Fig. 4, G and H). Collectively, these data demonstrate a novel relationship between two proteins previously implicated in tumorigenesis, Spy1A and Nedd4.

**Residues Thr-15, Ser-22, and Thr-33 Are Essential for Spy1A Degradation**—Cell cycle regulatory proteins, which are targeted to the ubiquitin-proteasome system, 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 sites within this region that may target the protein for degradation. Utilizing the NetPhos 2.0 Server tool residues Thr-15, Ser-22, and Thr-33 were isolated as potential phosphorylation sites (24). Site-directed mutagenesis was performed to alter Spy1A residues Thr-15, Ser-22, and Thr-33 to non-phosphorylatable alanines. Additionally we generated a similar mutation at Ser-247 in the C-terminal region to serve as a control. 293 cells were transfected with the relevant constructs before synchronization at G2/M. Surprisingly, mutation of all of Thr-15, Ser-22, and Thr-33 to a non-phosphorylatable alanine prevented degradation and ubiquitination of Spy1A (Figs. 5, A and B). Blotting asynchronous cell populations revealed that protein expression was not affected (Fig. 5C, right panel) and flow cytometry analysis demonstrated that effects at Thr-15, Ser-22, and Thr-33 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 Thr-15, Ser-22, and Thr-33 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 16 h post-transfection. Immunoblotting for Spy1A showed that cells transfected with the mutants have stabilized Spy1A levels (Fig. 5C). Quantifying three separate experiments demonstrate that indeed all three 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 three elicited sites were mutated to a nonphosphorylatable alanine (T15A, S22A, and T33A). Phosphorylation of Spy1A-TST at G2/M was compared with 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 Thr-15, Ser-22, and Thr-33 during the 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 to determine whether progression of the somatic cell cycle requires the timely degradation of Spy1A. Cells from Fig. 5A, which overexpressed 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 with 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, gray 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 Thr-15, Ser-22, and Thr-33 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 that may contribute to tumorigenesis.

**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 demonstrate 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 that 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 with 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, it 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 Fig. 2A, were found to interact with Nedd4

**Nedd4 Regulates Spy1A Turnover**
FIGURE 5. Phosphorylation on Thr-15, Thr-33, and Ser-22 is needed for Spy1A degradation. 293 cells were transfected with Spy1A wild-type (wt), Spy1A-T15A (T15A), Spy1A-T33A (T33A), Spy1A-S22A (S22A), or Spy1A-S247A (S247A). A, cell lysates were treated with nocodazole (G2 population; left panels) or untreated (Asynchronous population; right panel). Half of the population was kept for flow cytometry analysis (D). The remainder were lysed and immunoblotted (IB) with α-Myc for Spy1A (upper panels) and α-actin (lower panels). B, all samples were cotransfected with HA-Ub followed by treatment with MG132 for 14 h. Lysates were immunoprecipitated (IP) with α-Myc and immunoblotted for α-HA (upper panel) or α-Myc (lower panel). C, 16 h after transfection cyclohexamide was added, and 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 were corrected for using actin. Relative densitometry is shown over three separate experiments (right-hand panel). Error bars represent S.E. Statistical data reflects comparisons between the wt-transfected and mutant-transfected cells at each time point. **, p < 0.01. D, 293 cells were transfected with Myc-Spy1A-PCS3 (wt) or the triple mutant Spy1A-TST. 16 h post-transfection cells were treated with nocodazole and MG132 for 14 h followed by [32P]orthophosphoric acid for an additional 4 h. Cells were lysed, immunoprecipitated with α-Myc, and imaged by Cyclone phosphorimaging (upper blot). An immunoblot for α-Myc was used as a control (lower blot). Incorporation of orthophosphate was quantified using OptiQuant software; the right panel represents results of one representative experiment of two. E, cells from A were analyzed by flow cytometry. CXP 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 h post-transfection using trypan blue exclusion. Error bars reflect S.D. between three separate transfections, and a standard t test was performed assuming equal variance. Statistical data shown reflect comparisons between the wt-transfected cells and mutant-transfected cells. *, p ≤ 0.05; **, p ≤ 0.01.
in vivo (data not shown). This suggests that the Nedd4-Spy1A interaction relies on at least two separate binding regions in the Spy1A protein; resolution of these required binding regions remain to be determined. After mutagenesis of three 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. Thr-15 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 and 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 diubiquitinating their substrate proteins. Although we cannot 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 (20). vascular endothelial growth factor receptor 2 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 (21), 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 (19, 22, 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 functions in human cancers is an important direction that may provide a novel direction in the design of cancer therapeutics.

Acknowledgments—We thank Drs. D. S. Haines and D. Rotin for providing plasmids for this study. We are grateful to Drs. P. Vacratsis, M. Crawford, and S. Anavoranich for valuable advice and to J. Ritchie and D. Myers for help on this manuscript.

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