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Human Speedy: a novel cell cycle regulator that enhances proliferation through activation of Cdk2

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The decision for a cell to self-replicate requires passage from G1 to S phase of the cell cycle and initiation of another round of DNA replication. This commitment is a critical one that is tightly regulated by many parallel pathways. Significantly, these pathways converge to result in activation of the cyclin-dependent kinase, cdk2. It is, therefore, important to understand all the mechanisms regulating cdk2 to determine the molecular basis of cell progression. Here we report the identification and characterization of a novel cell cycle gene, designated Speedy (Spy1). Spy1 is 40% homologous to the *Xenopus* cell cycle gene, X-Spy1. Similar to its *Xenopus* counterpart, human Speedy is able to induce oocyte maturation, suggesting similar biological characteristics. Spy1 mRNA is expressed in several human tissues and immortalized cell lines and is only expressed during the G1/S phase of the cell cycle. Overexpression of Spy1 protein demonstrates that Spy1 is nuclear and results in enhanced cell proliferation. In addition, flow cytometry profiles of these cells demonstrate a reduction in G1 population. Changes in cell cycle regulation can be attributed to the ability of Spy1 to bind to and prematurely activate cdk2 independent of cyclin binding. We demonstrate that Spy1-enhanced cell proliferation is dependent on cdk2 activation. Furthermore, abrogation of Spy1 expression, through the use of siRNA, demonstrates that Spy1 is an essential component of cell proliferation pathways. Hence, human Speedy is a novel cell cycle protein capable of promoting cell proliferation through the premature activation of cdk2 at the G1/S phase transition.

Introduction

Movement through the cell cycle is dependent on the activation and inactivation of catalytic proteins known as cyclin-dependent kinases (cdks).* To promote cell cycle progression, cdks must form an active complex with their regulatory cyclin partners (for reviews see Ohi and Gould, 1999; Yang and Kornbluth, 1999). Commitment of the cell to replicate the genome occurs during a highly regulated stage in late G1, known as the restriction point. Considerable effort has been spent attempting to understand the molecular mechanism by which a cell passes through this restriction point. Although most research focuses on the role of the cyclin D–cdk4/6–pRB–E2F cascade in mediating this switch, evidence shows that the retinoblastoma (RB) pathway alone cannot account for the G1/S control over this transition point (Santoni-Rugiu et al., 2000). Evidence is accumulating to implicate that a parallel pathway, operating through the proto-oncogene Myc, cooperates with the pRB–E2F pathway to control G1/S onset (Roussel et al., 1995; Leone et al., 1997; Beier et al., 2000). Importantly, all known pathways that play a role in moving cells through the restriction point converge on the control of the G1/S kinase, cdk2.

Because cdk2 is central in controlling cell proliferation, it is subject to many levels of regulation. Although cdk2 is expressed relatively ubiquitously throughout the cell cycle, it is highly regulated by a series of activating and inactivating phosphorylations (Ekholm and Reed, 2000). Additionally, cdk2 activity is limited by the availability of a cyclin binding partner. Cyclin proteins are regulated at the protein expression level, being synthesized and destroyed in an oscillating manner throughout the cell cycle, thereby assuring a narrow window of cdk activation (Ekholm and Reed, 2000). In

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*Abbreviations used in this paper: aa, amino acid(s); ATCC, American Type Culture Collection; cdk, cyclin-dependent kinase; GVBD, germinal vesicle breakdown; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; RB, retinoblastoma; RT, room temperature; RT-PCR, reverse transcriptase PCR; siRNA, small interference RNA

Key words: Speedy; siRNA; proliferation; restriction point; G1/S

mammalian cells, cdk2 forms active complexes with both cyclin E and cyclin A (Fang and Newport, 1991; for review see Ekholm and Reed, 2000). Crystallography studies demonstrate that cyclin binding allows for structural modifications within the catalytic cleft of the cdk molecule, enhancing cdk access to ATP and relative substrates (Jeffrey et al., 1995). Furthermore, both the formation and the activity of the cdk–cyclin complex are subject to inhibition by small inhibitory proteins (cdk inhibitors [CKIs]; Pines, 1994). Although much is known about the regulatory mechanisms governing cdk activation, questions still remain regarding the precise timing and manner by which all of these regulatory mechanisms function, particularly during times of cellular stress, DNA damage, and oncogenesis.

Previously, our laboratory reported the identification of a novel *Xenopus* cell cycle regulatory gene, X-Spy1 (Lenormand et al., 1999). Shortly after our publication of X-Spy1, Ferby et al. (1999) reported the identification of a *Xenopus* gene, p33^{ringo}, which is ~90% homologous to X-Spy1. Microinjection of either X-Spy1 or p33^{ringo} mRNA into *Xenopus* oocytes elicits rapid maturation in the absence of hormone. Additionally, X-Spy1 and p33^{ringo} are essential for progesterone-stimulated maturation of *Xenopus* oocytes (Ferby et al., 1999; Lenormand et al., 1999). Moreover, X-Spy1 and p33^{ringo} appear to be functionally redundant in the maturation process, as inhibiting only one of these proteins at a time with antisense oligonucleotides does not prevent maturation (Ferby et al., 1999). Interestingly, in *Xenopus* oocytes, X-Spy1 and p33^{ringo} were both found to bind and prematurely activate cdk2 in a p21-independent manner (Ferby et al., 1999; Lenormand et al., 1999). Recently, it has been reported that activation of cdk2 by p33^{ringo} does not rely on the phosphorylation of Thr161, suggesting a novel mechanism for cdk2 activation (Karaiskou et al., 2001). Thus, X-Spy1 and p33^{ringo} represent a new class of cell cycle regulatory proteins that directly interact with and activate cdk2, independent of classical regulatory mechanisms.

Here we report the identification and characterization of the human homologue of Speedy (Spy1). Similar to its *Xenopus* counterpart, human Speedy is able to induce maturation in *Xenopus* oocytes upon microinjection, suggesting a role in cell cycle progression. Human Speedy mRNA is present in a range of normal tissues and immortalized cell lines and is regulated in a cell cycle–dependent manner. We demonstrate that Spy1 interacts with human cdk2 independent of cyclin binding and stimulates its kinase activity in mammalian cells. Furthermore, Spy1 overexpression in various cell lines rapidly induces cell cycle progression, an action that is dependent on cdk2 activation. Importantly, we show that depleting endogenous Spy1 significantly decreases cell proliferation, demonstrating a physiological function for Spy1. Thus, human Speedy is a novel, essential inducer of cell cycle progression that functions through a unique pathway of cdk2 activation.

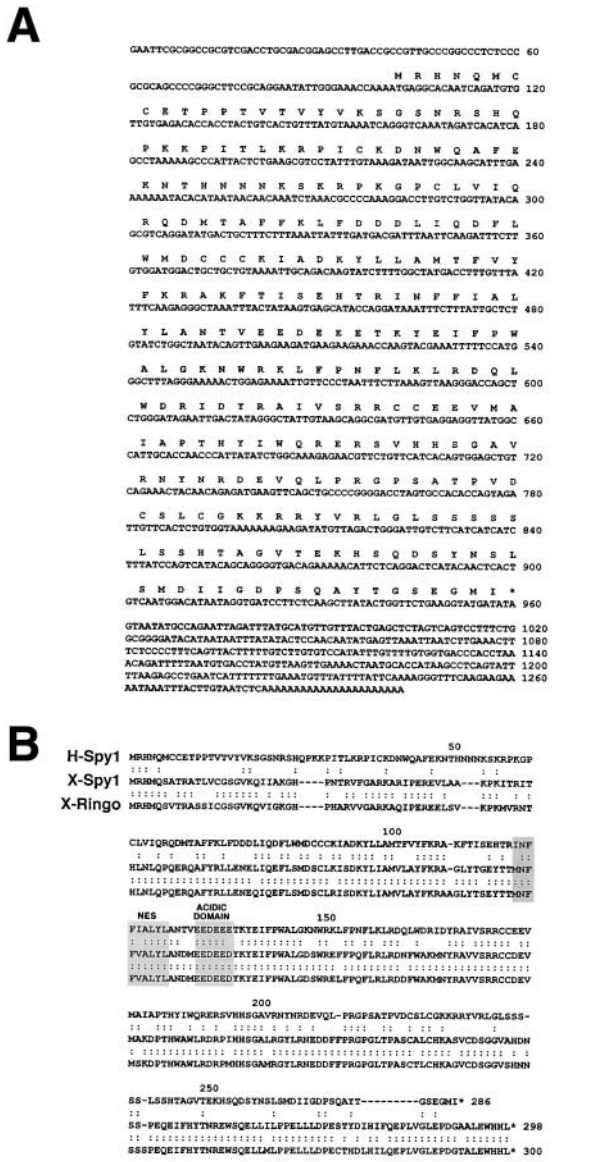

Figure 1. **Identification of human Spy1.** (A) Nucleotide sequence and predicted aa sequence of human Spy1. (B) The alignment of the amino acid sequence of *Xenopus* Speedy (X-Spy1) is shown in comparison with the human homologue (H-Spy1) and p33^{ringo} (X-Ringo). A potential nuclear export signal sequence and a conserved acidic domain are shaded. The GenBank/EMBL/DDBJ accession no. for human Spy1 is pending.

Results

Identification of human Speedy (Spy1)

A database search for proteins homologous to *Xenopus* Speedy yielded a partial match from an EST database. The corresponding human EST clone was from a testis cDNA library and contained 63% homology over a 170-bp region of X-Spy1. Primers corresponding to this human EST clone were designed and the corresponding full-length gene was isolated from a human testis cDNA library. A PCR-based serial dilution cloning method was used as described in the Materials and methods. PCR and dilution plating were repeated until a single clone was isolated. Sequencing of this 1,300-bp cDNA clone revealed an open reading frame encoding a predicted polypeptide of 286 residues (Fig. 1 A). This human clone shared an overall homology of ~40% with X-Spy1, including one stretch of 150 amino acids (aa) that shared ~70% homology (Fig. 1 B).

To confirm that the identified clone was the human ho-

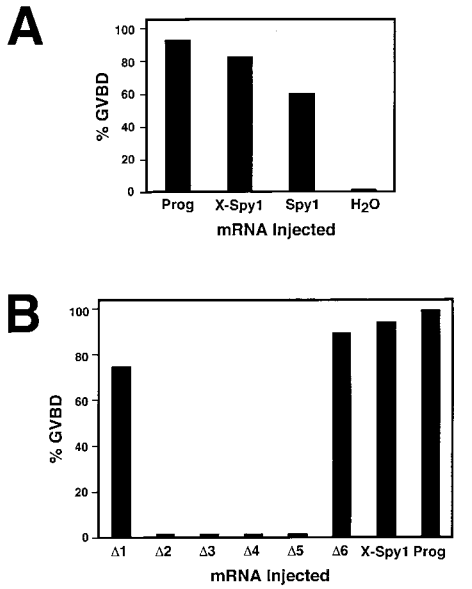

Figure 2. **Human Spy1 induces GVBD in *Xenopus* oocytes.** (A) Oocytes were injected with the indicated mRNA, incubated with progesterone (Prog) as a positive control, or injected with H_2O as a negative control, and scored for GVBD by the formation of a white spot on the animal pole. This graph is the composite of three independent experiments. (B) Deletion mutants of *Xenopus* Spy1 define a minimal activation domain of X-Spy1. Oocytes were injected with the indicated mRNA or incubated with progesterone and scored for GVBD. Deletion mutants are missing the following aa: Δ1 (5–53), Δ2 (54–94), Δ3 (98–137), Δ4 (137–170), Δ5 (173–218), and Δ6 (218–268).

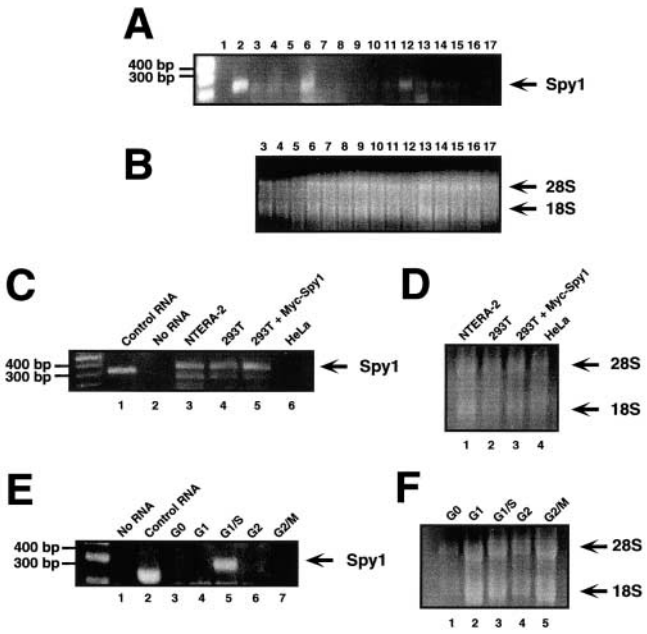

Figure 3. **Human Spy1 mRNA is present in a variety of human tissues and immortalized cell lines.** (A) RT-PCR of mRNA from 15 different human tissues. Lane 1 is a negative RT-PCR control containing no mRNA. Lane 2 is a positive RT-PCR control using control RNA with control primers. Human tissue samples are as follows: lane 3, thymus; 4, salivary gland; 5, liver; 6, fetal brain; 7, adrenal gland; 8, bone marrow; 9, fetal liver; 10, lung; 11, skeletal muscle; 12, thyroid; 13, brain/cerebellum; 14, heart; 15, placenta; 16, spleen; 17, trachea. (B) Control formaldehyde gel demonstrating the quality and quantity of the mRNA used in A. (C) RT-PCR of mRNA prepared from Ntera-2 cells (lane 3), 293T cells (lane 4), Spy1-transfected 293T cells (lane 5), and HeLa cells (lane 6). Lanes 1 and 2 are RT-PCR controls with a control mRNA and no mRNA, respectively. (D) Control formaldehyde gel demonstrating the quality and quantity of the mRNA used in C. Lanes 1–4 correspond to lanes 3–6 in C. (E) RT-PCR of mRNA prepared from synchronized 293T cells at various stages of the cell cycle. Lanes 1 and 2 are RT-PCR controls with no mRNA and control mRNA, respectively. (F) Control formaldehyde gel demonstrating the quantity and quality of the mRNA used in E. Lanes 1–5 correspond to lanes 3–7 in E.

mologue of Speedy, *Xenopus* oocytes were microinjected with mRNA encoding for either X-Spy1 or human Spy1. Oocytes were also treated with progesterone as a positive control or injected with water as a negative control. Oocytes were subsequently scored for germinal vesicle breakdown (GVBD) as indicated by the appearance of a white spot on the animal pole. As shown in Fig. 2 A, Spy1 induced oocyte maturation in the absence of hormone, although not as efficiently as X-Spy1 or progesterone. A subset of oocytes were fixed in 5% TCA and manually dissected to confirm GVBD (unpublished data). Spy1-induced maturation was notably slower than X-Spy1– or progesterone-induced maturation (unpublished data). Thus, we report the identification of human Spy1, a functional homologue of X-Spy1.

A series of X-Spy1 deletion mutants were assayed to ascertain which region is essential for activity. X-Spy1 was used for this analysis because X-Spy1 functions optimally in the *Xenopus* oocyte system. Six X-Spy1 deletion mutants were constructed by sequentially removing ~50 aa at a time. These mutants are designated Δ1–Δ6 and were analyzed for their ability to induce GVBD in *Xenopus* oocytes upon microinjection. Only two of these mutants retained the ability to induce oocyte maturation, Δ1 (corresponds to a deletion of aa 5–53) and Δ6 (lacks aa 218–268) (Fig. 2 B). These data imply that aa 54–218 are essential for the activity of X-Spy1, whereas the NH_2 and COOH termini are dispensable. Thus, the center region of the protein, which is also the most highly conserved between X-Spy1, $p33^{ringo}$, and Spy1 (Fig. 1 B), is required for X-Spy1 activity.

Speedy mRNA is expressed in a variety of human tissues and cell lines

To determine if Speedy mRNA is endogenously expressed in a variety of normal human tissues and immortalized cell lines, reverse transcriptase PCR (RT-PCR) was performed on mRNA isolated from 15 different human tissues as well as 3 different cell lines. Primers were used that are specific for the conserved region of human Speedy. As shown in Fig. 3 A, Spy1 mRNA is expressed at high levels in human fetal brain (lane 6) and the thyroid gland (lane 12). Significant amounts of Spy1 mRNA are also detected in human thymus (lane 3), salivary gland (lane 4), liver (lane 5), cerebellum (lane 13), heart (lane 14), and placenta (lane 15). As shown in Fig. 3 C, Spy1 mRNA is present in the human teratocarcinoma cell line Ntera-2 and the human embryonic kidney 293T cell line (lanes 3 and 4, respectively); yet Spy1 mRNA was not detected in the human epitheloid carcinoma cell line HeLa (lane 6). Notably, when mRNA levels for HeLa cells are increased 10-fold, Spy1 RNA can be detected (unpublished data). As a control, Fig. 3, B and D, demonstrates

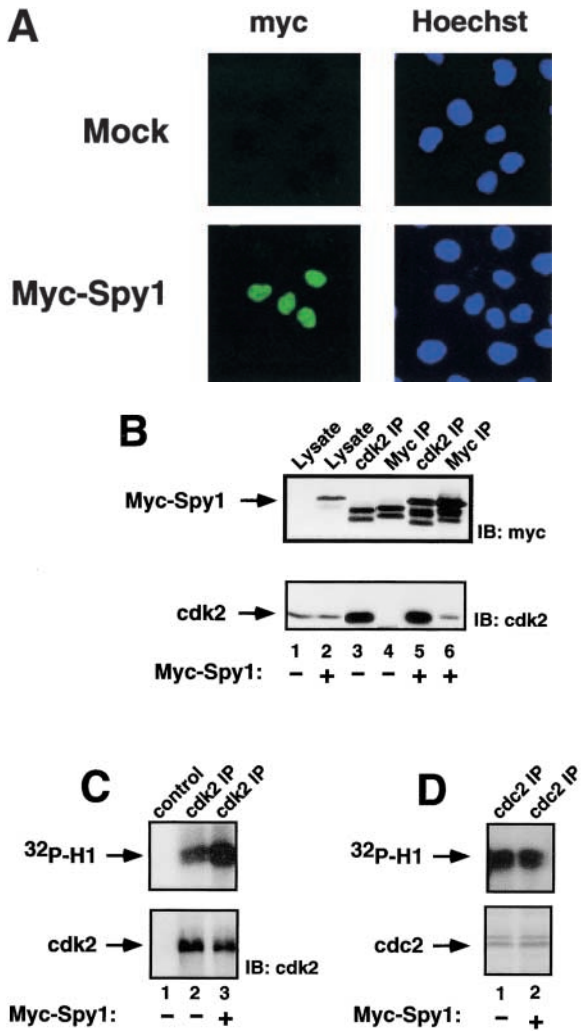

Figure 4. **Human Spy1 binds to and activates cdk2.** (A) Indirect immunofluorescence on COS-1 cells transiently transfected with human myc-tagged Spy1 (myc–Spy1; bottom left) or with empty vector (mock; top left) shows myc–Spy1 in the nucleus. The corresponding panels on the right are stained with Hoechst dye to illuminate the nuclei of all the cells present. (B) Coimmunoprecipitation of transiently transfected myc–Spy1 with endogenous cdk2 from 293T cells. Western blot analysis using antibodies against the myc epitope tag (top) or against cdk2 (bottom). Lane 1 is lysate from mock-transfected cells. Lane 2 is lysate from myc–Spy1-transfected cells. Lanes 3 and 4 are immunoprecipitations (IPs) from mock-transfected cells, and lanes 5 and 6 are immunoprecipitations from myc–Spy1-transfected cells using either anti-cdk2 (lanes 3 and 5) or anti-myc (lanes 4 and 6). (C) Top panel shows histone H1 phosphorylation assay. 293T cells were transiently transfected with empty vector (mock) or myc–Spy1. 24 h after transfection, cells were harvested and immunoprecipitated with antibodies against cdk2. Lane 1 is histone H1 alone (no IP). Lanes 2 (mock) and 3 (myc–Spy1) are cdk2 IPs. Bottom panel shows blot of cdk2 in IPs from mock and myc–Spy1 cells. (D) Top panel shows histone H1 phosphorylation assay. 293T cells were transiently transfected with empty vector (mock) (lane 1) or myc–Spy1 (lane 2). 24 h after transfection, cells were harvested and immunoprecipitated with antibodies against cdc2. Coomassie blue staining showing equal loading of cdc2 is presented in the bottom panel.

that the total mRNA used was of equal quality and quantity. PCR products were sequenced to confirm that the 360-bp band was indeed Speedy (unpublished data). Smaller bands

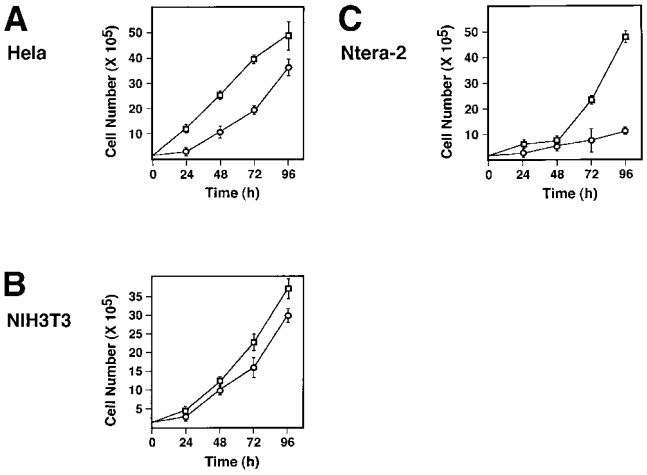

Figure 5. **Expression of Spy1 increases the rate of proliferation of mammalian cells.** Growth curves of (A) HeLa cells, (B) NIH3T3 cells, and (C) Ntera-2 cells. All cells were transiently transfected with myc–Spy1 (□) or empty vector (○). Cells were collected in triplicate at the indicated time point and counted via trypan blue exclusion. The graph is one representative experiment of at least three independent experiments. Error bars were calculated using SEM.

did not have informative sequences and are likely degradation products of Spy1. Thus, Spy1 mRNA is expressed in both a variety of immortalized cell lines as well as in several human tissue types.

Speedy mRNA is expressed in a cell cycle–dependent manner

To determine if Spy1 mRNA expression is cell cycle dependent, 293T cells were synchronized via double thymidine block and mRNA was prepared hourly after release of the cells. The cell cycle phase distribution was previously determined using flow cytometry analysis (unpublished data). At each time point, immunofluorescence was used to confirm morphological changes. Mitotic events occurred at ~8 h after release, which was consistent with earlier studies. The mRNA samples that correspond to each phase of the cell cycle (lane 3, G0; lane 4, G1; lane 5, G1/S; lane 6, G2; lane 7, G2/M) were subjected to RT-PCR using primers specific for human Speedy. As demonstrated in Fig. 3 E, Spy1 mRNA was only expressed in G1/S phase cells (lane 5). The quantity and quality of the total mRNA used in RT-PCR analysis is shown in Fig. 3 F.

Spy1 is localized in the nucleus and interacts with human cdk2

To determine the subcellular localization of Speedy, a myc-tagged human Speedy construct (myc–Spy1) was engineered. COS-1 cells were transiently transfected with myc–Spy1 or an empty vector as a control. Indirect immunofluorescence was then performed to examine the subcellular localization of Spy1. Treatment of cells with the c-myc (9E10) antibody showed that myc–Spy1 was expressed exclusively in the nucleus (Fig. 4 A, bottom left). Hoechst dye was used to stain the nuclei of all cells (Fig. 4 A, right).

Previously, our lab demonstrated that X-Spy1 was able to directly bind to cdk2 (Lenormand et al., 1999). As cdk2 and

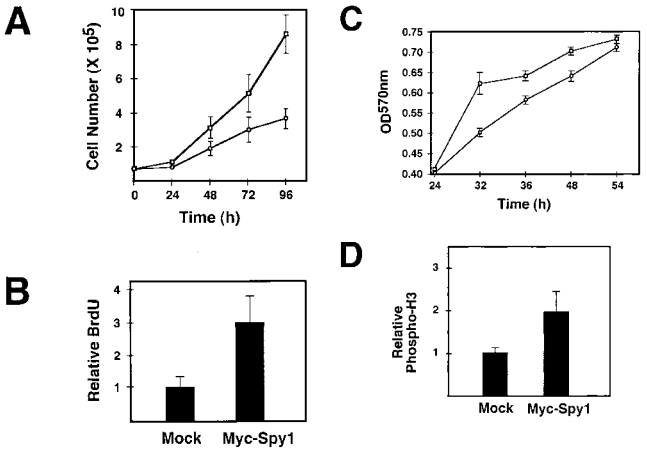

Figure 6. **Expression of Spy1 increases both the rate of cell replication and division.** (A) Growth curve of 293T cells transiently transfected with myc–Spy1 (□) or empty vector (○). Cells were collected at the indicated times, counted on a Coulter counter, and replated. The graph is the average of three independent experiments. Error bars were calculated using SEM. (B) Increase of BrdU incorporation in myc–Spy1-transfected 293T cells. Cells were harvested at day 2, stained for BrdU, and the positive cells were counted via fluorescence microscopy. Error bars represent the standard error of three experiments. (C) MTT analysis of 293T cells expressing myc–Spy1 (□) or empty vector (○). Cells were collected at the indicated times, treated, and then the absorbance was taken at 570 nm to determine the relative levels of MTT. Bars represent the standard error between four separate transfections within one representative experiment. (D) Histone H3 phosphorylation in myc–Spy1-transfected 293T cells. Cells were harvested at day 2, stained for phosphorylated histone H3, and the positive cells were counted via fluorescence microscopy. Bars represent the standard error of three separate experiments.

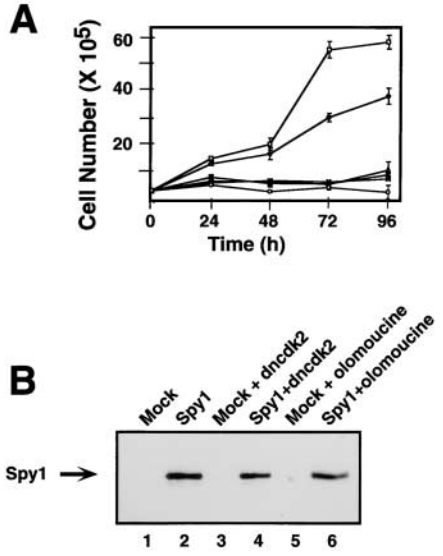

Figure 7. **Spy1-enhanced growth is dependent on cdk2 activation.** (A) Growth curve of 293T cells transiently transfected with Spy1 (□); mock (◇); Spy1 + dncdk2 (cdk2D145N) (Δ); mock + dncdk2 (X); Spy1 + 7 μM olomoucine (∇); mock + 7 μM olomoucine (○). Error bars represent the SEM between triplicate plates of one representative experiment. This experiment was repeated three times. (B) Western blot of lysates from each sample at 96 h. The blot was then probed for myc–Spy1 expression.

Spy1 are both nuclear, we investigated whether human Spy1 could directly interact with human cdk2. To accomplish this, 293T cells were transiently transfected with myc–Spy1 or an empty vector control. Cell lysates were subsequently coimmunoprecipitated with either cdk2 or c-myc antibodies. Immunoblot analysis showed that myc–Spy1 coimmunoprecipitates with endogenous cdk2 (Fig. 4 B). Specifically, immunoblotting for myc–Spy1 revealed that myc–Spy1 was present in a cdk2 immunoprecipitation from myc–Spy1-transfected cells (Fig. 4 B, lane 5), but not from mock-transfected cells (Fig. 4 B, lane 3). Similar results were obtained in the reverse direction. Immunoblotting for cdk2 showed that cdk2 was present in a myc–Spy1 coimmunoprecipitation in myc–Spy1-transfected cells (Fig. 4 B, lane 6), but not in mock-transfected cells (Fig. 4 B, lane 4). These results indicate that human Spy1, analogous to its *Xenopus* homologue, binds cdk2 in vivo.

We then wished to examine the kinase activity of cdk2. Lysates from mock- or myc–Spy1-transfected 293T cells were immunoprecipitated with antibodies against cdk2 and subjected to an in vitro histone H1 phosphorylation assay. Speedy-expressing cells had a significant increase in cdk2 kinase activity (Fig. 4 C, top). As a control, the protein levels of cdk2 were examined and found to be present in equivalent levels (Fig. 4 C, bottom). Although H1-associated kinase activity is widely used and highly indicative of the relative levels of kinase activity, the actual alteration in cdk2 activity on in vivo substrates may differ. These results indicate, consistent with prior observations in *Xenopus* oocytes, that human Spy1 binds to cdk2 and stimulates cdk2 kinase activity.

Interestingly, we have been unable to show consistent binding of Spy1 with any of the cyclins (unpublished data). Furthermore, when lysates from mock- or myc–Spy1-transfected cells were immunoprecipitated with antibodies against cdc2 and subjected to an in vitro histone H1 phosphorylation assay, we found no significant increase in cdc2 kinase activity in Spy1-expressing cells (Fig. 4 D, top) that have equal levels of cdc2 protein (Fig. 4 D, bottom).

Spy1 enhances cell proliferation in mammalian cells

To determine if Spy1 plays a role in regulating the mammalian cell cycle, growth curves were obtained comparing control-transfected cells against cells expressing myc–Spy1. Transiently transfected HeLa, NIH3T3, and Ntera-2 cells were counted every 24 h after transfection for 96 h. As shown in Fig. 5, A–C, cells overexpressing Spy1 grew significantly faster than mock cells. Trypan blue exclusion was used to distinguish between live and dead cells and no appreciable difference was noted in cell death (unpublished data). These data indicate that Spy1 expression enhances overall cell growth rate.

To determine which aspects of cell growth are affected by Speedy expression, several markers were examined in 293T cells transiently transfected with myc–Spy1 or an empty vector control. Fig. 6 A demonstrates that, like the other cell lines studied, 293T cells expressing myc–Spy1 proliferate at a considerably faster rate than cells with an empty vector

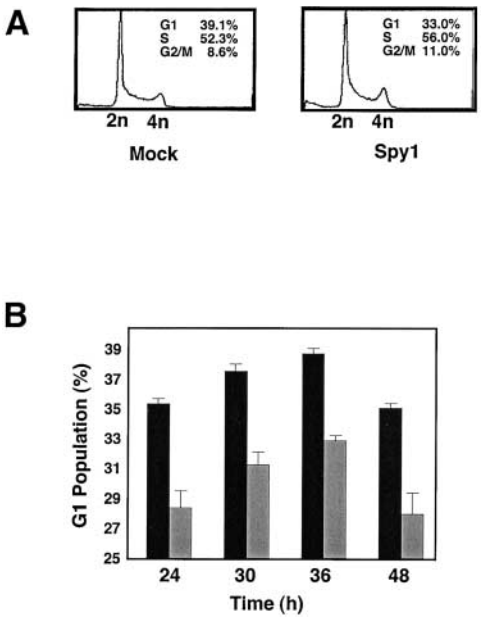

Figure 8. **Speedy increases cell division and decreases the G1 population.** 293T cells were transiently transfected with empty vector or myc–Spy1. Cells were collected 24, 30, 36, and 48 h after transfection and analyzed by flow cytometry. (A) A representative FACS® profile of cells overexpressing empty vector (mock) or myc–Spy1. (B) Graphic representation of the percentage of cells in G1 phase. Mock, black bars; myc–Spy1, gray bars. Error bars represent the SEM of four independent experiments.

control. To determine the percentage of cells synthesizing DNA at a given time, the incorporation of BrdU, a thymidine analogue, into DNA was studied in cell populations transfected with either mock or Spy1 (Wang et al., 1996). Using immunofluorescence microscopy, Spy1-expressing cells showed a threefold increase in BrdU incorporation (Fig. 6 B). Thus, cells expressing Speedy have an increase in DNA replication events, which is consistent with cells cycling more rapidly. Furthermore, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed on 293T cells transiently transfected with myc–Spy1 or empty vector to examine mitochondrial activity. This assay measures the activity of the mitochondrial enzyme succinic dehydrogenase (Twentyman and Luscombe, 1987). A significant increase in mitochondrial activity was seen in cells expressing Spy1 (Fig. 6 C). These data indicate that Speedy-expressing cells are undergoing cellular replication at a higher or more efficient rate than mock cells, consistent with the observation that Spy1 enhances cell proliferation.

To identify if Spy1-transfected cells were also successfully dividing more frequently than mock-transfected cells, we used a phospho-specific antibody to detect a form of histone H3 present only in mitotic nuclei (anti-PH3; Handzel et al., 1997). Using immunofluorescence, Spy1-expressing cells displayed a twofold increase in the phosphorylation of histone H3 (Fig. 6 D). This indicates that the presence of Spy1 induces cells to divide more rapidly. Taken together, Spy1-expressing cells replicate and divide at a faster rate than mock-transfected cells; therefore Spy1 functions in enhancing cell proliferation.

Spy1-enhanced cell proliferation is dependent on cdk2 kinase activity

To determine if Spy1 requires cdk2 kinase activity in order to enhance cell proliferation, we used a dominant-negative cdk2, cdk2D145N (van den Heuvel and Harlow, 1993). We additionally exploited a drug known to selectively inhibit cdk2 at low doses, olomoucine (Vesely et al., 1994). The dose of olomoucine used, 7 μM, is the IC50 value for cdk2 and cdc2 inhibition. This dose has no effect on any other kinase tested including both cdk4 and cdk6 (Vesely et al., 1994). Fig. 7 A demonstrates that Spy1 is unable to stimulate proliferation of cells where cdk2 kinase activity has been inhibited either through the use of the dominant-negative cdk2 or olomoucine. This is not due to an alteration in the expression of myc–Spy1 protein (Fig. 7 B).

Spy1 reduces the percentage of cells in G1 phase of the cell cycle

Flow cytometry was then employed to determine if Spy1 selectively alters one or more phases of the cell cycle. Fig. 8 A shows a representative flow cytometry profile of 293T cells transiently transfected with mock or Spy1. Notably, overexpression of Spy1 was not toxic to cells and did not initiate a cell cycle arrest. The graph shown in Fig. 8 B demonstrates that Spy1-expressing cells consistently exhibit a lower percentage of cells in G1 phase of the cell cycle. As G1 phase is the longest phase of the cell cycle, this may provide one explanation for Spy1-enhanced rate of cell proliferation. Of course, these results do not rule out the possibility that Spy1-expressing cells may also exhibit alterations in the timing of other phases of the cell cycle.

Spy1 expression is necessary for normal cell growth

To determine the physiological role of Spy1 in enhancing cell growth, small interference RNA (siRNA) techniques were employed. Transient transfection of 293T cells with siRNA constructs designed against Spy1 (siSpy) depletes the cells of Spy1 mRNA by 24 h (Fig. 9 B). Depletion of Spy1 mRNA persists over the time course of the experiment, from 24 through 96 h after transfection. Using a control siRNA directed against luc-GL2 (siCntl), a bacterial reporter, no effect was observed on Spy1 mRNA or overall RNA levels (Figs. 9, B and C). As demonstrated in Fig. 9 A, depleting Spy1 mRNA in 293T cells has a drastic effect on cell growth as indicated by trypan blue exclusion, which is especially evident by 96 h after transfection. Fig. 10 A demonstrates that the mitotic index of cells depleted of endogenous Spy1 for 72 h is significantly lower than that of siCntl cells, supporting that endogenous Spy1 is involved in promoting enhanced cell division. FACS® analysis of this same population of cells demonstrates that siSpy1-transfected cells have a higher percentage of cells in late G1/early S phase (68%) as compared with siCntl-transfected cells (54%) (Fig. 10 B). This alteration in cell cycle distribution has consistently been observed over a wide range of time points (unpublished data). Furthermore, under these experimental conditions, cdk2-associated H1 kinase activity in cells depleted of endogenous Spy1 decreases to ~35% of the siCntl-transfected cells (Fig. 10 C). Fig. 10 D demonstrates through Western

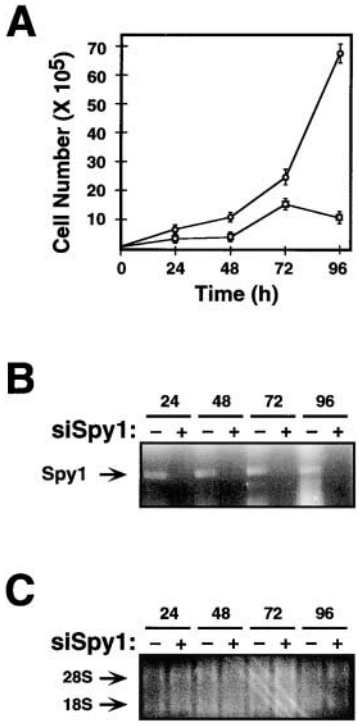

Figure 9. **Endogenous Spy1 is required for normal cell growth.** Spy1 RNA was depleted using RNA interference. (A) 293T cells were transiently transfected with siRNA directed against Spy1 (siSpy) (□) or siluc-GL2 control (siCntl) oligonucleotides (○). 24 h after transfection, triplicate plates were counted by trypan blue exclusion. The surviving cell number was graphed as an average of three plates (±SEM). (B) mRNA was isolated from remaining cells from each time point and was subjected to RT-PCR analysis. Lanes indicated with a plus have been treated with siSpy, and lanes indicated with a minus have been treated with siCntl. (C) Control 1.2% agarose formaldehyde gel demonstrating the quantity and quality of the mRNA used in B.

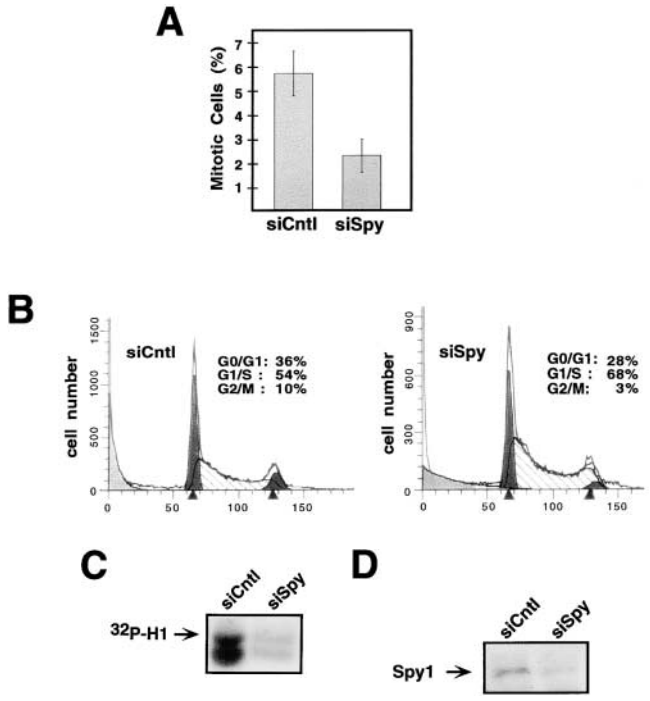

Figure 10. **Depletion of Spy1 protein decreases cdk2-associated kinase activity and slows movement of cells into M phase of the cell cycle.** (A) Mitotic index of cells expressing siSpy or siluc-GL2 (siCntl) 72 h after transfection. Error bars represent the average of three separate transfections. This is one representative experiment of three. (B) FACS® analysis of siSpy- and siCntl-expressing cells 72 h after transfection. Data were analyzed by M-cycle. One representative experiment of three is shown. (C) Cdk2-associated histone kinase activity was measured for cells transfected for 72 h with siSpy and siCntl. Data were quantitated using NIH Image 1.62. Similar alterations in kinase activity are observed over a range of time points. (D) Western blot control demonstrating that endogenous Spy1 protein is depleted 72 h after transfection of siSpy siRNA.

blot analysis that endogenous Spy1 protein is successfully depleted after transfection with Spy1 siRNA. Hence, these data demonstrate that depletion of Spy1 mRNA hinders cell proliferation, implicating endogenous Spy1 as an essential protein in normal cell growth.

Discussion

In this report, we characterize the novel human cell cycle gene, Spy1. Akin to its homologous *Xenopus* relative, Spy1 induces maturation of *Xenopus* oocytes upon microinjection. In mammalian cells, we demonstrate that Spy1 is a regulator of cell cycle progression. Overexpression of Spy1 increases proliferation in several mammalian cell lines, as indicated by cell growth curves. This may be explained by the ability of Spy1 to bind to and prematurely activate the G1/S kinase, cdk2. In support of this, flow cytometry studies using 293T cells demonstrate that Spy1 decreases the overall population of cells in G1 phase of the cell cycle. Furthermore, cells overexpressing Spy1 demonstrate an enhancement in the incorporation of BrdU and in MTT activity, respectively, indicating that both DNA synthesis and mitochondrial enzyme activity are enhanced. Taken together, Spy1 appears to be a potent regulator of cell cycle progression.

Previous studies demonstrated that the *Xenopus* Spy1 homologues, X-Spy1 and p33^{ringo}, bind to and activate cdk2 (Ferby et al., 1999; Lenormand et al., 1999). Moreover, p33^{ringo} was shown to bind to and activate cdc2 (Ferby et al., 1999). In these studies, X-Spy1 and p33^{ringo} are associated with rapid oocyte maturation, demonstrating the ability of X-Spy1 to progress cells through the G2/M transition of the cell cycle. Similar to these reports, we have found that human Speedy activates cdk2 in mammalian cells. Yet contrary to the *Xenopus* system, Spy1 in mammalian cells is associated with rapid progression of cells through G1/S. Furthermore, under our experimental conditions, we have been unable to demonstrate any direct binding of Spy1 with cdc2 or any enhanced activity of cdc2 in Spy1-expressing cells. We do, however, demonstrate that Spy1-expressing cells exhibit enhanced expression of phospho-histone H3, an indicator of cell division, and thus cannot rule out that Spy1 may exert some as yet undetermined effects in G2/M phase of the cell cycle. The apparent discrepancies between results obtained with *Xenopus* p33^{ringo} (Ferby et al., 1999; Karaiskou et al., 2001) and human Spy1 may possibly be explained by species differences in the regulatory properties of the Spy1 protein itself. It is evident from this report that Spy1 mRNA is

detectable primarily during G1/S phase of the cell cycle in mammalian cells. Additionally, our laboratory has preliminary data suggesting that Spy1 protein is degraded during G2 phase of the cell cycle (unpublished data). *Xenopus* studies, on the other hand, indicate that X-Spy1 and $p33^{ringo}$ protein exists at the G2/M border (Ferby et al., 1999; Lenormand et al., 1999). The fact that Spy1 is differently regulated between species is interesting and merits further investigation.

The possibility that Spy1 has biological function at various phases of the cell cycle depending on its production and destruction is itself interesting and reflects a characteristic common to the growing family of cyclin proteins. Furthermore, we demonstrate here that Spy1 mRNA is expressed in a cell cycle–dependent manner, similar to cyclin expression profiles. Yet, at the amino acid level, Spy1 is not significantly related to the archetypical cyclins. Future experiments determining the mechanism by which Spy1 activates cdk2 will reveal if Spy1 is indeed a potential cyclin-like protein. We cannot yet rule out that Spy1 is binding cdk2 via a complex containing other proteins. Perhaps Spy1 functions by altering the binding of cell cycle inhibitors to cdks.

Recently $p33^{ringo}$ was demonstrated to activate both cdc2 and cdk2 regardless of the phosphorylation status of Thr160 and Thr161, respectively, and in the absence of cyclin binding (Karaiskou et al., 2001). This implies that X-Spy1 and $p33^{ringo}$ activate their relevant cdk via a unique mechanism. Interestingly, we have been unable to observe any cyclin binding to Spy1 via coimmunoprecipitation (unpublished data). This suggests that Spy1, like $p33^{ringo}$, activates cdk2 via a unique pathway that is independent of cyclin binding. Consistent with this, depletion of Spy1 mRNA slows cell growth considerably, yet cells are still able to replicate and divide. Additionally, Spy1 mRNA is found at vastly different levels in select tissues, suggesting that Spy1-mediated cdk2 activation will have unique tissue-specific functions. It will be interesting to further determine how Spy1 binds and activates cdk2 and to determine the effects of Spy1 on cdk2–cyclin E/cyclin A complex formation. It is becoming increasingly evident that there are multiple pathways leading to cdk2 activation, ultimately promoting entry into S phase. The most well-established pathway leading to S phase is the RB pathway, comprising RB protein along with its upstream regulators cyclin D and cdk4/6 and pRB-regulated E2F transcription factors (Bartek et al., 1996). Accumulating evidence supports that the proto-oncogene Myc functions in parallel to RB in a pathway that is independent of E2F activation (Santoni-Rugiu et al., 2000). It will be interesting to determine if Spy1 is a critical component of one of these pathways regulating the onset of DNA replication. Moreover, it will be important to address if Spy1–cdk2 binding is altered during times of cellular stress or DNA damage, because Spy1 was initially isolated by its ability to rescue a Rad1-deficient strain of *Schizosaccharomyces pombe* from UV damage.

In summary, the results presented here characterize a novel human cell cycle gene that plays a role in the progression of mammalian cells through its ability to bind and activate cdk2. We have established that endogenous Spy1 is essential for the proliferation of mammalian cell lines and that Spy1 is expressed over a wide range of human tissues, opening up the possibility that this gene may be involved in human cell cycle disorders such as oncogenesis. There are numerous questions remaining regarding the regulation of Spy1; the answers of which may provide valuable insight into the mechanics of the mammalian cell cycle.

Materials and methods

Identification of human Speedy

A BLAST search of the EST database using X-Spy1 as the "bait" yielded one clone of significant homology (GenBank/EMBL/DDBJ accession no. AA424209). The EST clone was isolated from a human testis cDNA library. Thus, using primers directed against highly conserved regions of X-Spy1 and AA424209, we screened a human testis cDNA library (CLONTECH Laboratories Inc.) using a PCR-based dilution cloning method to isolate human Spy1. First, the library was plated on 10 plates at 100,000 plaque forming units/plate and allowed to grow for ~7 h and was then overlaid with SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin). PCR of the overlaid SM buffer (1 μl aliquot) was used to test for the presence of the desired clone on individual plates. The SM overlay containing a positive was then titered and plated out again on 10 plates at 10,000 pfu/plate. The PCR and titering were repeated with the positive plate replated onto 10 plates at 1,000 pfu/plate. After further PCR and titering, the positive plate at the 1,000 pfu/plate dilution was replated onto one plate. This plate was cut into 96 pieces and each was then placed in a 96-well plate followed by the addition of 100 μl SM buffer. At this dilution, each well should contain ~10–15 clones. Well no. C-12 tested positive and was replated at several dilutions to obtain a single clone containing the desired insert. Individual clones were picked and examined by PCR. One isolated plaque tested positive and the insert was shuttled to pSP64 and pcDNA3 vectors.

Plasmid construction and mRNA isolation

Human Speedy was isolated from a testis library in a λTriplEx vector (CLONTECH Laboratories Inc.). pTriplEx was excised from λTriplEx per the manufacturer's instructions. EcoRI and SacI sites were used to move human Spy1 from pTriplEx to pSB5, a modified version of pSP64(polyA) (Promega) containing EcoRI in the multicloning region with XhoI instead of EcoRI as the linearization site. To generate the myc–Spy1 clone, a NdeI site that incorporates the start codon of human Speedy was added via QuikChange site-directed mutagenesis (Stratagene). A pair of oligonucleotides (D1904/D1905) was inserted into the multicloning region of the pCS3 + MT vector at the BglII site, inserting a NdeI site in which the ATG was in frame with the myc tag coding region. Then Spy1 was ligated into pCS3 + MT via a three-part ligation using HindIII, NdeI, and XbaI sites to construct myc–Spy1. The X-Spy1 gene in the pSP64 vector was constructed as described previously (Lenormand et al., 1999). The deletion mutants of X-Spy1 were constructed by first inserting two silent restriction sites into X-Spy1 via QuikChange site-directed mutagenesis (Stratagene). A pair of oligonucleotides (D2423/D2424) inserted a NcoI silent site at aa 94 of X-Spy1 and a second pair of oligonucleotides (D2433/D2434) inserted a XhoI silent site at aa 137 of X-Spy1 in pSP64(poly A). Subsequently, X-Spy1 was cut with either NdeI/NcoI, NcoI/XhoI, or XhoI/BamHI, and in each case an ~100-aa piece of X-Spy1 was removed. Then ~50 aa were added back, via a pair of complementary oligonucleotides, in frame to create the deletion mutants lacking ~50 aa each. The pairs of oligonucleotides used were as follows: D2426/2427 for Δ1; D2428/D2429 for Δ2; D2435/D2436 for Δ3; D2437/D2438 for Δ4; D2439/D2440 for Δ5; and D2441/D2442 for Δ6. mRNAs were synthesized from pSP64 constructs using SP6 polymerase as described previously (Freeman et al., 1989).

The dominant-negative cdk2 vector (pCMV-cdk2D145N) was a gift from Ed Harlow and Sander van den Heuvel (Massachusetts General Hospital Cancer Center, Charlestown, MA), and has been previously described (van den Heuvel and Harlow, 1993).

Oocyte microinjections

Stage VI oocytes were dissected manually in modified Barth saline–Hepes (MBS-H) buffer. They were then either treated with progesterone (30 μM), microinjected with 50 nl of RNA at 1.0 mg/ml, or microinjected with 50 nl of diethylpyrocarbonate (DEPC)-treated H_2O and scored for GVBD as indicated by the appearance of a white spot on the animal pole, as described previously (Freeman et al., 1989).

Cell culture and synchronization

293T cells are a human embryonic kidney cell line (American Type Culture Collection [ATCC]), Ntera-2 cells are a human teratocarcinoma cell line

(ATCC), HeLa cells are a human epitheloid carcinoma cell line (ATCC), COS-1 cells are an SV40-transformed African green monkey kidney cell line (ATCC), and NIH3T3 cells are an embryonic, contact inhibited, NIH Swiss mouse cell line (ATCC). All of these cell lines were maintained in DME (GIBCO BRL) supplemented with 0.1% penicillin–streptomycin (Sigma-Aldrich) and 10% FBS. Ntera-2 cells were also supplemented with 4 mM L-glutamine (Sigma-Aldrich). Cells were incubated at 37°C in 10% (293T, COS-1, and Ntera-2 cells) or 5% CO_2 (HeLa and NIH3T3 cells).

Double thymidine block was used to synchronize the cell cycle. 293T cells were first treated with 100 mM thymidine in DME for 19 h at 37°C in 10% CO_2. Cells were then washed with Tris-saline and incubated in fresh media for 9 h. Thymidine/DME was then added for the second thymidine block, and cells were incubated for 17 h. The final Tris-saline wash and incubation in fresh media was followed by the beginning of time points, as cells exit simultaneously from G_o of the cell cycle.

Human Spy1 antibody isolation

The human Spy1 antibody was produced in collaboration with Pocono Rabbit Farm and Laboratory. Rabbits were immunized with KLH-conjugated peptides against aa 251–263 of human Spy1. ELISAs were performed using BSA-conjugated peptide, and further boosts were done every 2 wk with the KLH-conjugated peptide. Specificity of the antibody was determined using peptide-blocked antibody and preimmune serum derived from the same rabbit. The human Spy1 antibody specifically detects an endogenous protein of ~34 kD.

RT-PCR

Total RNA from 15 different human tissues was obtained from the Human Total RNA Master Panel II (CLONTECH Laboratories, Inc.). Total RNA was prepared from cells (3×10^6) using RNeasy (QIAGEN) per the manufacturer's instructions. The quantity and quality of mRNA were identified by running samples on a 1.2% agarose formaldehyde gel. 1 μg of RNA was reverse transcribed with 2.5 U of AMV reverse transcriptase following the supplier's suggestions (Promega). The reverse transcriptase reactant was mixed in a final volume of 20 μl together with 50 pmol of primer pairs and 25 μM of each deoxynucleoside triphosphate (dNTP) and amplified with 2.5 U of Tf1 DNA polymerase (Promega) for 30 cycles (denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 68°C for 1 min). This number of cycles was previously determined to be optimal for detecting the signal in a linear range. The primer sequences used were 5′-ATT GGG AAA CCA AAA TGA GGC-3′ (sense) and 5′-TCC TGG TAT GCT CAC TTA TAG-3′ (antisense). Positive control RNA and primers were included with the Access RT-PCR System (Promega) and used per the manufacturer's instructions. Amplification products were visualized and photographed using TAE/EtBr gel electrophoresis. Products were then gel purified using GeneClean (Bio101), following the manufacturer's instructions, and were cloned using the pGEM-T Easy Vector System. Each of the cloned products was then sequenced (UCSD Cancer Center DNA Sequencing Service).

Immunofluorescence microscopy

293T or COS-1 cells were plated onto glass coverslips (collagen coated for 293T cells) and cultured as described above. Cells were transiently transfected by calcium phosphate precipitation (Chen and Okayama, 1987) with 5 μg of myc–Spy1 in a 60-mm dish. 36–48 h after transfection, cells were fixed with 3% paraformaldehyde in PBS for 15 min and permeabilized with 0.1% Triton X-100 for 10 min. The 9E10 monoclonal myc antiserum (Santa Cruz Biotechnology, Inc.) was used to detect myc–Spy1 with FITC-conjugated goat anti–mouse secondary antibody (Boehringer).

For M phase marker assay (phospho-histone H3), 293T cells were incubated in blocking solution (0.1% Triton-X, 1.5% glycine, 2.5% FBS in PBS) for 30 min at room temperature (RT). An antisera that specifically recognizes the phosphorylated form of histone H3 (Upstate Biotechnology) was added at a 1:200 dilution in blocking solution for 1 h at RT. FITC-conjugated anti–mouse was then used at a 1:200 dilution.

For S phase marker assay (BrdU), 293T cells were treated as described above. In addition, 100 μl of 6 mg/ml BrdU labeling solution in PBS was added on the second day after transfection for 1 h at 37°C in 10% CO_2. Anti-BrdU (Becton Dickinson) was added to coverslips at a 1:50 dilution in blocking solution for 1 h at RT. FITC-conjugated anti–mouse was used at a dilution of 1:150 for visualization.

Immunoprecipitations and immunoblotting

Subconfluent 293T cells were transfected with 5 μg of DNA by calcium phosphate precipitation in a 10-cm dish. 24 h later, cells were harvested and lysed in 0.1% NP-40 lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 0.1% NP-40, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 10 μg/ml leupeptin).

For immunoprecipitation, cell lysates were clarified with protein A–Sepharose beads (Sigma-Aldrich) and subsequently incubated with primary antisera (myc–9E10, cdk2-D12, cdk2-M2, cdc2-p34 [17]; Santa Cruz Biotechnology, Inc.) as indicated overnight at 4°C, followed by the addition of protein A–Sepharose and incubation at 4°C with gentle rotation for an additional 1 h. These complexes were then washed extensively with 0.1% NP-40 lysis buffer and resolved by 10% SDS-PAGE. The membrane was immunoblotted with the indicated antisera followed by ECL (Amersham Pharmacia Biotech). The histone H1 kinase assays were performed as previously described (Kong et al., 2000). siRNA-treated cells were serum starved using media containing 0.2% FBS for 12 h before harvesting for histone kinase assays.

Cell proliferation assays

Cell growth curves. HeLa, NIH3T3, and Ntera-2 cells were seeded at a density of 10^5 cells/plate (10 cm) and were transfected in triplicate 24 h later with 5 μg of either empty vector or myc–Spy1. Every 24 h after transfection, three plates of both myc–Spy1 or empty vector control were trypsinized and counted by trypan blue exclusion. The average values of three plates are represented (±SEM). Coverslips were placed on each plate to assess transfection efficiency by indirect immunofluorescence. HeLa cells transfected with 40% efficiency, Ntera-2 cells with 45% efficiency, and NIH3T3 cells with 30% efficiency. Remaining cells for that time point were pooled, lysed, subjected to SDS-PAGE, and immunoblotted for myc–Spy1 expression. This experiment was repeated three times.

293T cells were seeded at a density of 5×10^4 cells/plate (60 mm) and were transfected 24 h later with 2.5 μg of either empty vector or myc–Spy1. 293T cells demonstrated a transfection efficiency of 50%, as assessed by immunofluorescence. Cells were trypsinized and counted every 24 h after transfection (day 1). The average values of three plates are represented (±SEM). Cell lysate samples from day 4 were analyzed for myc–Spy1 expression by immunoblotting.

Tetrazolium assay. 293T cells, transfected as described above, were split onto a 96-well plate. 20 μl of MTT (Sigma-Aldrich) stock (5 mg/ml in PBS) was added to each and incubated at 37°C in 10% CO_2 for 2 h. Then 100 μl of extraction buffer (20% SDS in DMF/H_2O, 2.5% of 80% acetic acid, 2.5% 1 M HCl, pH 4.7) was added to each well overnight with incubation at 37°C in 10% CO_2. Cells were then pulled at the indicated times and the absorbances were taken at 570 nm.

Inhibition of cdk2 growth curves. 293T cells were seeded at a density of 4×10^4 cells/plate (60 mm) and were transfected 24 h later with 5 μg of total DNA. Combinations of empty vector control and myc–Spy1 were used with or without cdk2D145N. Furthermore, myc–Spy1- or control-transfected cells were either treated with DMSO control or 7 μM olomoucine (Sigma-Aldrich). Cells were trypsinized and triplicate plates were counted every 24 h for each condition. The average values are represented (±SEM). Remaining cells at each time point were lysed, subjected to SDS-PAGE, and analyzed for myc–Spy1 expression by immunoblotting.

Flow cytometry

293T cells were transfected as described above with 10 μg of DNA. Cells were trypsinized at the indicated times, washed twice in PBS, resuspended at 2×10^6 cells in 1 ml of PBS, fixed by the addition of an equal amount of ethanol, and frozen. Within 1 wk, fixed cells were pelleted, washed, and resuspended in 300 μl of PBS. Samples were then treated with 1 μl of 10 mg/ml stock of DNase free RNase (Promega) and 50 μl of 500 mg/ml propidium iodide (Boehringer) stock solution. Data were collected on a flow cytometer within 6 h. The multicycle (M-cycle) program for cell cycle distribution histograms (Phoenix Flow Systems, Inc.) was used to analyze the data.

siRNA

RNA interference assays were conducted through the construction of siRNA for Spy1 (siSpy). The siSpy oligo (-GTACGAAATTTTTCCATGG-) was synthesized, purified, and duplexed by Dharmacon Research. As a negative control, a luciferase GL2 duplex siRNA was used (D-1000-Lu-5; Dharmacon Research).

siRNA transfection. Subconfluent 293T cells (10^5 cells/ml) were seeded in 10-cm dishes. Cells were transfected using calcium phosphate precipitation with 0.6 nM siSpy or siluc-GL2 duplex per plate. Cells were incubated at 3% CO_2 for 12 h and then returned to 10% CO_2 after changing the media.

siRNA growth curves. Every 24 h, plates were pulled in triplicate and both live and dead cells were counted by trypan blue exclusion. Surviving cells were averaged at each time point and their SEM was determined. Remaining cells were pooled for mRNA preparation as described above and sub-

jected to RT-PCR analysis for Spy1 mRNA production. The quantity and quality of mRNA was determined through formaldehyde gel electrophoresis.

Mitotic index. 293T cells were plated on collagen-coated coverslips and transfected with siSpy or siluc-GL2 control as described above. After the media change, cells were grown for 48 h, stained with Hoechst stain (0.7% NP-40, 4.7% formaldehyde, 11 μg/ml Hoechst stain in PBS), and analyzed by fluorescence microscopy.

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