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NIPA Defines an SCF-Type Mammalian E3 Ligase that Regulates Mitotic Entry

Florian Bassermann,[1] Christine von Klitzing,[1] Silvia Münch,[1] Ren-Yuan Bai,[1] Hiroyuki Kawaguchi,[2,3] Stephan W. Morris,[2] Christian Peschel,[1] and Justus Duyster[1,*]

[1]Department of Internal Medicine III
Technical University of Munich
81675 Munich
Germany

[2]Department of Pathology and Hematology-Oncology
St. Jude Children's Research Hospital
Memphis, Tennessee 38105

Summary

The regulated oscillation of protein expression is an essential mechanism of cell cycle control. The SCF class of E3 ubiquitin ligases is involved in this process by targeting cell cycle regulatory proteins for degradation by the proteasome, with the F-box subunit of the SCF specifically recruiting a given substrate to the SCF core. Here we identify NIPA (nuclear interaction partner of ALK) as a human F-box-containing protein that defines an SCF-type E3 ligase (SCF^{NIPA}) controlling mitotic entry. Assembly of this SCF complex is regulated by cell-cycle-dependent phosphorylation of NIPA, which restricts substrate ubiquitination activity to interphase. We show nuclear cyclin B1 to be a substrate of SCF^{NIPA}. Inactivation of NIPA by RNAi results in nuclear accumulation of cyclin B1 in interphase, activation of cyclin B1-Cdk1 kinase activity, and premature mitotic entry. Thus, SCF^{NIPA}-based ubiquitination may regulate S-phase completion and mitotic entry in the mammalian cell cycle.

Introduction

Cell cycle-specific proteolysis involves the addition of ubiquitin molecules along a cascade of E1, E2, and E3 enzymes to target proteins, thus marking them for degradation by the proteasome (Hochstrasser, 1996). The E3 ubiquitin-protein ligases are critical in these ubiquitination reactions, as they allow the transfer of activated ubiquitin from E2 enzymes to the target protein and mediate the specificity of substrate recognition (Weissman, 2001). Two types of E3 ligase complexes are prominent in controlling the abundance of cell cycle regulatory proteins: the APC and the SCF. The APC (anaphase-promoting complex) is a multisubunit complex that targets substrates for degradation only during mitosis and G1 and is responsible for targeting mitotic cyclins and proteins involved in sister chromatid cohesion and spindle function (Zachariae and Nasmyth, 1999). More recently, the SCF class of E3 ubiquitin ligases has been identified (Jackson and Eldridge, 2002). These complexes consist of four subunits: Skp1, Cul1, Roc1, and an interchangeable F-box protein (Fbp), the latter of which determines substrate specificity (Bai et al., 1996; Skowyra et al., 1997). Members of the F-box protein family have a bipartite structure: a shared N-terminal F-box motif that links F-box proteins to Skp1 and the SCF core complex and a C-terminal divergent protein-protein interaction motif, which selectively binds the cognate substrate(s) (Cardozo and Pagano, 2004). The core components of the SCF (Skp1, Cul1, Roc1) are abundant throughout the cell cycle, and ubiquitin ligase activity is thought to be regulated at the level of substrate availability and substrate phosphorylation (Jackson and Eldridge, 2002). Thus far, it has remained mostly elusive whether the F-box component contributes to the timing process of cell-cycle-dependent ubiquitination, although some data suggest that regulated abundance of Fbps may be involved (Galan and Peter, 1999; Zhou and Howley, 1998). Only recently, evidence of crosstalk between the APC and SCF has emerged that demonstrates a link between the two E3 ligases in terms of their interdependent regulation of enzymatic activity and cooperation in substrate ubiquitination (Ayad et al., 2003; Bashir et al., 2004; Wei et al., 2004). In this regard, a dual mode of degradation has been described for the Cdc25A phosphatase (Busino et al., 2003). While most cell-cycle-related functions of SCF complexes have been related to the G_1/S transition, other cell cycle transitions also require SCF-mediated protein degradation (Carrano et al., 1999; Nakayama et al., 2004; Strohmaier et al., 2001). Genetic experiments in yeast have shown that cells harboring certain mutants of Skp1 arrest as large, budded single-nucleus-containing cells with a G_2 DNA content, and several studies suggest a role for SCF-dependent proteolysis in *Xenopus* and mammalian cells in G_2 phase and mitosis (Ayad et al., 2003; Bai et al., 1996; Guardavaccaro et al., 2003; Michael and Newport, 1998).

Entry into mitosis is triggered by the activated cyclin B1/Cdk1 complex (also known as M-phase-promoting factor or MPF), the activity of which must be exquisitely controlled during the cell cycle (Minshull et al., 1989). In the course of mitosis, cyclin B1 is targeted for ubiquitination by the APC in anaphase to allow for subsequent mitotic exit (King et al., 1995; Zachariae and Nasmyth, 1999). Tight control of MPF activity is particularly important during interphase, since untimely activation can trigger premature mitotic entry, thereby compromising the fidelity of genome replication. Nuclear abundance of cyclin B1 is a major determinant of MPF activity, thus necessitating regulatory means for timing of the subcellular localization of this cyclin. The predominant cytoplasmic localization of cyclin B1, which is reversed only upon phosphorylation of the CRS (cytoplasmic retention signal) region, and the switchlike upregulation of Cdk1-cyclin B1 activity in response to an increase in cyclin B1 concentration have emerged as potentially relevant restraints (Pines and Hunter, 1991; Yang et al., 1998). In addition, the impact of nuclear

*Correspondence: justus.duyster@lrz.tum.de
[3]Present address: Department of Pediatrics, Tokyo Dental College, Ichikawa General Hospital, Chiba, Japan.

cyclin B1 has recently broadened with the identification of its S-phase-promoting potential (Moore et al., 2003). Therefore, identification of the mechanisms that control cyclin B1 expression and function spatially and temporally are crucial for understanding DNA replication and the timing of mitotic entry.

Recently, we identified NIPA (nuclear interaction partner of ALK) as a protein of widespread expression and exclusive nuclear subcellular location in a screen to identify phosphotyrosine-dependent interaction partners of the activated anaplastic lymphoma kinase (ALK) receptor tyrosine kinase. Constitutively active oncogenic fusion forms of ALK contribute to the development of certain human lymphomas and sarcomas (Duyster et al., 2001; Ouyang et al., 2003). Here we characterize NIPA as a human F-box protein that defines an SCF-type ubiquitin E3 ligase, the formation of which is regulated by cell-cycle-dependent phosphorylation of NIPA. This phosphorylation event commences in G_2 phase, results in dissociation of NIPA from the SCF core, and proves to be critical for proper G_2/M transition. We identify cyclin B1 as a substrate of the SCF^{NIPA}, which is accordingly targeted for ubiquitination during interphase only. In addition to the well-known APC-mediated degradation of cyclin B1 in mitosis, our data suggest an SCF-based ubiquitination process involving cyclin B1 in interphase, thereby providing a mechanism to inhibit premature nuclear accumulation of cyclin B1 during the mammalian cell cycle.

Results

Cell-Cycle-Dependent Expression and Phosphorylation of NIPA

We initially isolated NIPA as a human nuclear protein in a screen for activated ALK binding proteins (Ouyang et al., 2003). In an effort to determine whether NIPA plays a role in cell cycle control, we assayed for modifications of the protein throughout the mammalian cell cycle. NIPA expression was minimal in growth-arrested cells ($G_{0/1}$) and became abundant in S and G_2/M phases (Figure 1A). Moreover, the apparent molecular mass of NIPA was increased in G_2/M cells (Figure 1A). This mobility shift was sensitive to phosphatase treatment and thus most likely due to phosphorylation (Figure 1B). To assess NIPA levels and phosphorylation in the ongoing cell cycle, we released synchronized cells from either S phase or the G_2/M boundary and assayed for expression and phosphorylation of the protein. As depicted in Figure 1C, NIPA phosphorylation increased as cells approached G_2/M. Analysis of the cell cycle phase transition times of the NIH 3T3 cells used suggested an onset of NIPA phosphorylation in G2 phase, with a peak at the G_2/M boundary. After the G_2/M transition, NIPA phosphorylation and expression levels declined precipitously upon reentry into G_1 (Figure 1C, right panel). Using tryptic phosphopeptide analysis combined with the engineering of mutations of candidate phosphorylation sites, we determined that serine 354 is a major phosphorylation site during the course of cell-cycle-dependent phosphorylation of NIPA (Figure 1D).

We next generated polyclonal murine NIPA antibodies to demonstrate that these cell cycle effects are valid for the endogenous NIPA protein. As shown in Figure 1E, the NIPA antibody detects a protein of the expected molecular mass (66 kDa) in HeLa cells. The antibody was only reactive toward the human NIPA protein and did not detect the murine homolog in NIH 3T3 cells. When applied to indirect immunofluorescence in HeLa cells, the NIPA antiserum detected NIPA exclusively in the nucleus (Figure 1E). As observed with overexpressed NIPA (Figure 1A), endogenous NIPA became abundant in S phase and was phosphorylated at G_2/M (Figure 1F).

NIPA Is a Human F-Box Protein that Associates with Skp1

We next performed a yeast two-hybrid screen using the full-length NIPA protein as bait to screen a human testis cDNA library for additional NIPA binding proteins. Surprisingly, all specific clones identified from this screen encoded human Skp1 (data not shown). To confirm this association, we tested the ability of a recombinant GST-Skp1 fusion protein to interact with NIPA wild-type (wt) stably expressed in NIH 3T3 cells (Figure 2A).

Since many proteins known to associate with Skp1 are F-box proteins, we searched for an F-box motif in the NIPA sequence. Close examination of this sequence revealed an F-box motif in the N terminus of the NIPA protein (amino acids 170–210) (Figure 2B). Mutation of the core LP residues (L170 and P171) of the NIPA F-box abolished binding to Skp1, thus identifying this motif as the relevant binding region and suggesting that NIPA is a human F-box-containing protein (Figure 2A). Additionally, we identified binding determinants for NIPA in both the N and C termini of Skp1, which is thought to be typical for the Skp1-F-box interaction (data not shown) (Ng et al., 1998).

NIPA Is Part of a Functionally Active SCF Complex that Assembles in a Cell-Cycle-Regulated Manner

Since we confirmed that NIPA interacts with Skp1 and that this interaction requires a functional F-box motif, we examined whether NIPA assembles with the other known mammalian SCF subunits (Cul1, Roc1) to form an SCF complex. To test for in vivo complex formation, we precipitated Flag-NIPA from 293T cell lysates transfected with the constructs indicated (Figure 3A). As depicted in this figure, NIPA copurified with Skp1, Cul1, and Roc1 to form a complete SCF complex. Complex formation was also found to occur in vitro using a cell-free system, thus suggesting direct interaction (Figure 3B).

Having shown that NIPA can form an SCF complex (SCF^{NIPA}), we next determined whether SCF^{NIPA} possesses associated ubiquitination-promoting activity. As shown in Figure 3C, increased ubiquitination-promoting activity was observed after preincubation of GST-NIPA with extracts derived from cells transfected with the SCF core components Skp1, Cul1, and Roc1 (+SCF) (Figure 3C, compare lanes 3 and 4). When preincubated with untransfected extracts (–SCF) (lane 7) or not preincubated (lane 2), GST-NIPA exhibited only background activity, similar to GST alone (lane 1). These findings suggest that SCF^{NIPA} contains ubiquitin ligase activity and interacts with the ubiquitination machinery.

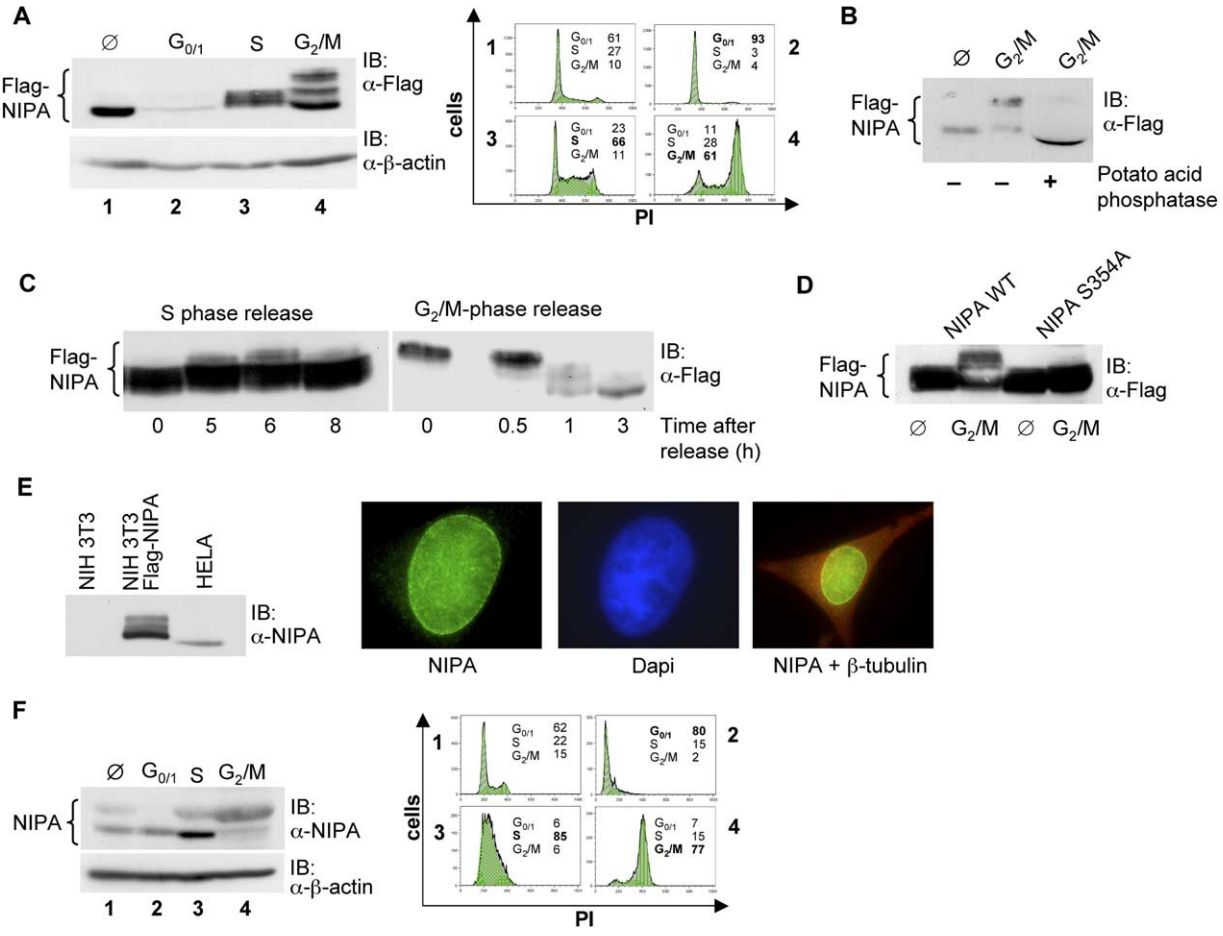

Figure 1. Cell-Cycle-Dependent NIPA Expression and Phosphorylation

(A) Analysis of NIPA at different stages of the cell cycle. (Left) Cycling cultures of an NIH 3T3 cell line expressing Flag-NIPA wt were arrested in $G_{0/1}$, S, or G_2/M or left untreated. Thereafter, cell lysates were prepared and processed for Western blotting with the antibodies indicated (IB, immunoblot). (Right) Cell cycle distribution of the cells shown in the left panel.

(B) Cells, unsynchronized (Ø) or synchronized at G_2/M, were lysed and the lysates treated with potato acid phosphatase for 10 min or left untreated as indicated.

(C) NIH 3T3 cells described in (A) were released from blocks in S phase (left panel) or at G_2/M (right panel), sampled at the given intervals, and processed for Western blotting.

(D) COS1 cells were transiently transfected with Flag-NIPA wt or Flag-NIPA S354A, enriched at G_2/M, or left unsynchronized, then collected for Western blotting.

(E) Characterization of a NIPA-specific antiserum. NIH 3T3 lysate (lane 1), NIH 3T3 lysate from cells transfected with human NIPA (lane 2), and HeLa lysate (lane 3) were resolved by SDS-PAGE and immunoblotted with an affinity-purified polyclonal murine NIPA antiserum. (Right) HeLa cells were subjected to indirect immunofluorescence using NIPA antiserum. DAPI and β-tubulin stains visualize the nucleus and cytoplasm, respectively.

(F) Analysis of endogenous NIPA at different stages of the cell cycle. HeLa cells were arrested in $G_{0/1}$, S, or G_2/M or left untreated, lysed, and processed for Western blotting with the NIPA antiserum described in (E).

We reasoned that the cell-cycle-dependent phosphorylation of NIPA from G_2 phase through mitosis (see Figure 1) might regulate its interaction with Skp1 and thus to the SCF core. To address this issue, we compared the binding of GST-Skp1 to NIPA expressed in either unsynchronized or G_2/M cells. Strong retention of NIPA with GST-Skp1 was observed only in unsynchronized cells, with no binding detected in G_2/M cells (Figure 3D). To verify these data in vivo, we precipitated endogenous NIPA from either interphase or G_2/M HeLa cells and assayed for binding to endogenous Skp1. Again, binding of NIPA to Skp1 was only observed in interphase cells and was not detectable in G_2/M cells (Figure 3E). Thus, cell-cycle-dependent phosphorylation of NIPA can regulate its binding to Skp1, and this posttranslational modification likely functions as a timing switch for the disassembly of the SCF^{NIPA} complex. Accordingly, a functional SCF^{NIPA} complex would only occur in the cell cycle segment from G_1 to G_2 phase.

Identification of Nuclear Cyclin B1 as an Ubiquitination Target of the SCF^{NIPA} Complex

Cyclin B1 levels oscillate during the cell cycle, with increasing levels as S and G_2 phases progress and a

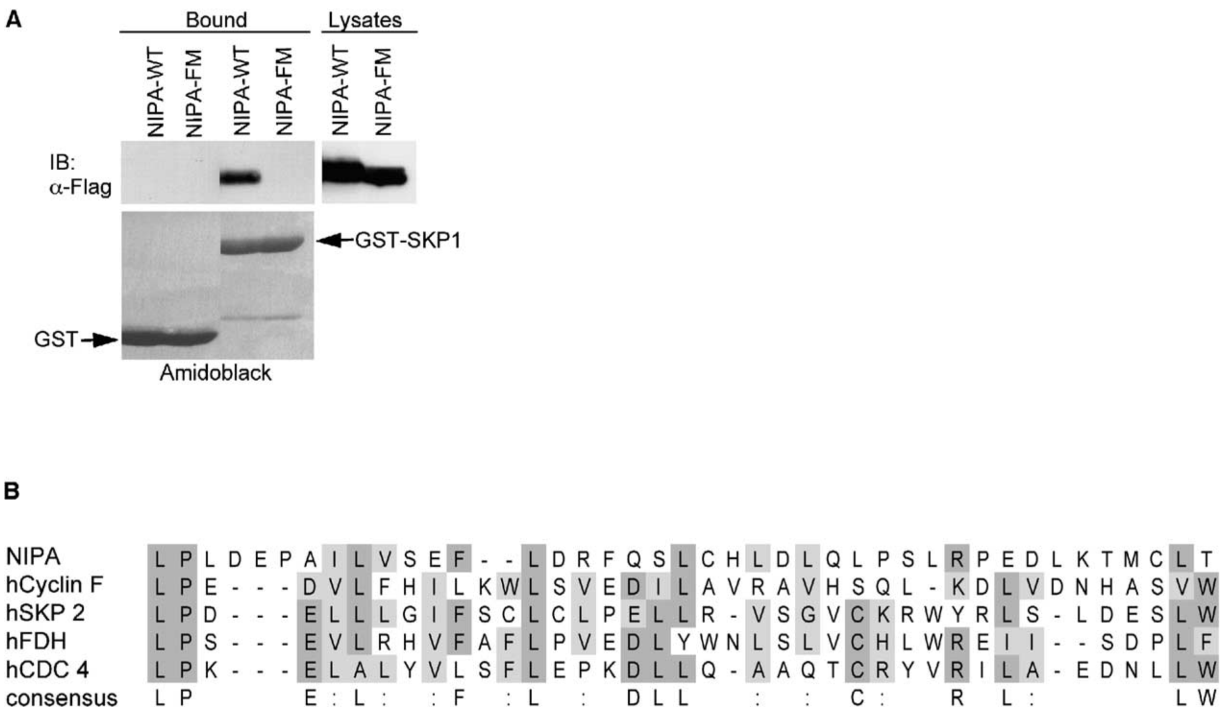

Figure 2. NIPA Is a Human F-Box Protein that Associates with Skp1

(A) NIPA interacts with Skp1 via the F box. Flag-NIPA wt and Flag-NIPA FM (F-box mutant) were expressed in 293T cells. Lysates were incubated with GST or GST-Skp1 protein, and the bound proteins were detected by Western blotting as indicated.

(B) CLUSTAL W alignments of F-box motifs comparing NIPA (aa 170–210) with known human F-box proteins. Identical residues (dark gray), similar residues (light gray), conservative residue differences (:).

peak at G_2/M. Cyclin B1 is largely cytoplasmic during interphase, and nuclear accumulation of phosphorylated cyclin B1 is mainly restricted to G_2/M (Hagting et al., 1999). As illustrated in Figure 4A, NIPA is phosphorylated and hence dissociated from the SCF core in nocodazole-arrested prometaphase cells, in which cyclin B1 strongly accumulates. Furthermore, we observed nuclear cyclin B1 levels in interphase cells to increase upon inhibition of the proteasome (Figure 4B). Thus, we investigated whether NIPA can interact with cyclin B1. We precipitated cyclin B1 from either interphase cells or nocodazole-arrested G_2/M cells and assayed for coprecipitated NIPA. Figure 4C demonstrates the binding of cyclin B1 to NIPA only in interphase cells, while no binding was seen in G_2/M cells. Thus, binding of NIPA to cyclin B1 only occurs when the SCF^{NIPA} complex is assembled, and disassembly of the SCF^{NIPA} complex via phosphorylation of NIPA abrogates the ability of NIPA to bind cyclin B1.

Given these observations, we sought to determine whether the SCF^{NIPA} complex can ubiquitinate cyclin B1. For this purpose, we established a reconstituted SCF system using proteins from baculovirus-infected Sf21 cells. Purified SCF^{NIPA}, SCF^{Cdc4} as a control SCF complex (Strohmaier et al., 2001), or SCF core complex ($SCF^{(ØF\text{-}box)}$ = Skp1, Cul1, Roc1) were assembled and incubated with GST-cyclin B1 as substrate. Beforehand, GST-cyclin B1 was preincubated with HeLa cell lysates. In this system, only the SCF^{NIPA} complex was able to efficiently ubiquitinate cyclin B1 (Figure 4D, upper panel, lane 2). Neither the SCF core complex alone nor the SCF^{Cdc4} complex showed significant ubiquitination activity toward cyclin B1 (lanes 3 and 4). Anti-ubiquitin Western blotting demonstrated efficient ubiquitination mediated by the SCF^{NIPA} complex (Figure 4D, lower panel).

We next carried out an in vitro ubiquitination assay using HeLa extracts that were either NIPA inactivated or reconstituted with purified protein to further investigate the requirement of NIPA for cyclin B1 ubiquitination. NIPA inactivation was performed using an siRNA approach (Figure 4E) described in detail below (Figure 5A). As shown in Figure 4F, NIPA inactivation caused a marked reduction of cyclin B1 ubiquitin ligation activity (lane 3). In contrast, upon reconstitution with purified Skp1-NIPA complex, ubiquitination of cyclin B1 was reinforced (Figure 4F, lane 5). Addition of purified Skp1-Skp2 complex was not able to restore cyclin B1 ubiquitination (lane 6). Of note, cyclin B1 was upshifted when preincubated; this alteration most likely represents phosphorylation of the protein and presumably conforms to the modification required for SCF^{NIPA}-mediated ubiquitination (Figure 4D, upper panel; Figure 4F). Importantly, the addition of purified Skp1-NIPA not only stimulated ubiquitination of cyclin B1 but markedly induced cyclin B1 degradation as measured by a degradation assay (Figure 4G).

We then tested for in vivo ubiquitination of nuclear cyclin B1 by SCF^{NIPA}. Because phosphorylation of NIPA at G_2/M should inactivate the SCF^{NIPA} complex, we

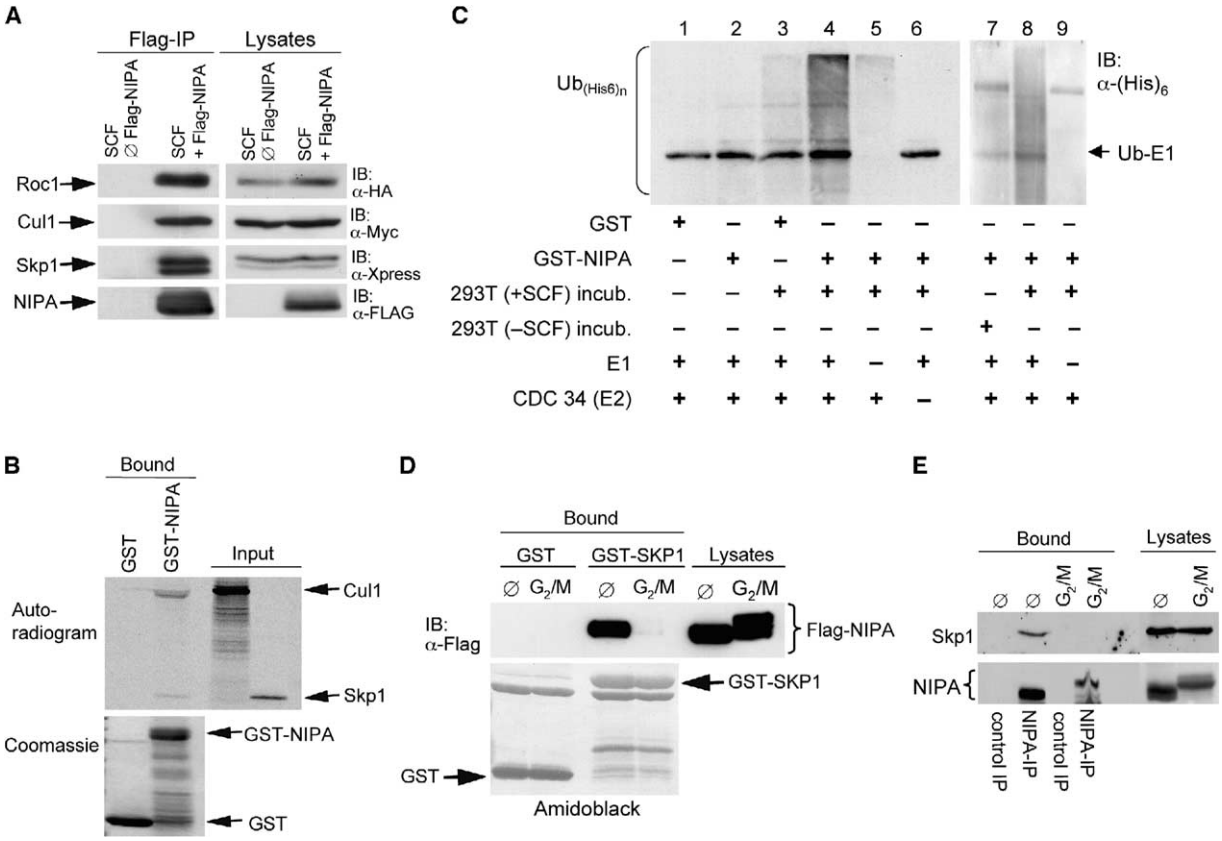

Figure 3. NIPA Defines a Functionally Active SCF Complex that Assembles in a Cell-Cycle-Regulated Manner

(A) NIPA interacts with SCF components in vivo. Xpress-Skp1, Myc-Cul1, and HA-Roc1 were cotransfected with or without Flag-NIPA in 293T cells and interactions determined by anti-Flag immunoprecipitation.

(B) NIPA interacts with SCF components in vitro. A glutathione-agarose precipitation assay using GST-NIPA wt or GST as control was performed to assess binding to in vitro-translated and ^{35}S-labeled Skp1 and Cul1.

(C) The SCF^{NIPA} possesses ubiquitination-promoting activity in vitro. Glutathione-agarose beads preloaded with GST or GST-NIPA and either preincubated in lysates of 293T cells transfected with Skp1, Cul1, and Roc1 (+SCF) (lanes 3–6) or in lysates of untransfected 293T (–SCF) (lane 7), or not preincubated (lanes 1 and 2), were processed for their ability to promote an in vitro ubiquitination reaction.

(D) NIPA associates with Skp1 in interphase only. GST-Skp1 or GST was incubated with lysates collected from unsynchronized or G_2/M-synchronized NIH 3T3 cells expressing Flag-NIPA wt, and binding was determined.

(E) In vivo binding of NIPA to Skp1 is restricted to interphase. Extracts of unsynchronized or G_2/M-synchronized HeLa cells were immunoprecipitated with purified NIPA antibody or with control IgG fractions. Immunoprecipitates and lysates were immunoblotted with either Skp1 or NIPA antibodies.

compared interphase cells with nocodazole-arrested G_2/M cells. The phosphorylation-deficient NIPA S354A mutant, which should assemble a constitutively active $SCF^{NIPA\text{-}S354A}$ complex, was included in the assay. Cyclin B1 was immunoprecipitated from nuclear cell extracts expressing the plasmids indicated in Figure 4H. In interphase cells, expression of both NIPA wt and NIPA S354A stimulated ubiquitination of cyclin B1 (Figure 4H, lanes 2 and 3). However, when cyclin B1 precipitated from G_2/M cells was examined, ubiquitination of the cyclin protein was observed only in the NIPA S354A sample (lanes 6 and 7). Notably, essentially no ubiquitination of cyclin B1 was observed when the cyclin B1 protein precipitated from the corresponding cytoplasmic cell extracts was examined (data not shown). Thus, nuclear cyclin B1 is targeted for ubiquitination by the SCF^{NIPA} complex in interphase only, whereas the $SCF^{NIPA\text{-}S354A}$ complex remains constitutively active and targets nuclear cyclin B1 throughout G_2/M.

NIPA Silencing Stabilizes Nuclear Cyclin B1 and Induces Premature Mitotic Entry

We used an siRNA knockdown approach to further assess the role of SCF^{NIPA} in controlling the nuclear abundance of cyclin B1. On both the RNA and protein levels, a significant reduction of NIPA expression was observed upon NIPA siRNA treatment (Figure 5A). Silencing of NIPA caused a considerable accumulation of cyclin B1 protein in nuclear extracts as compared to cells transfected with control siRNA (Figure 5A, right panel), whereas, on the RNA level, cyclin B1 was not affected (Figure 5A, left panel). We then tested the effect of silencing NIPA expression in HeLa cells arrested in S phase. Cells were synchronized by thymidine treatment, and pulse-chase analysis was performed. Nuclear cyclin B1 was degraded in cells transfected with control siRNA, whereas the levels of nuclear cyclin B1 remained constant in cells treated with NIPA siRNA (Figure 5B).

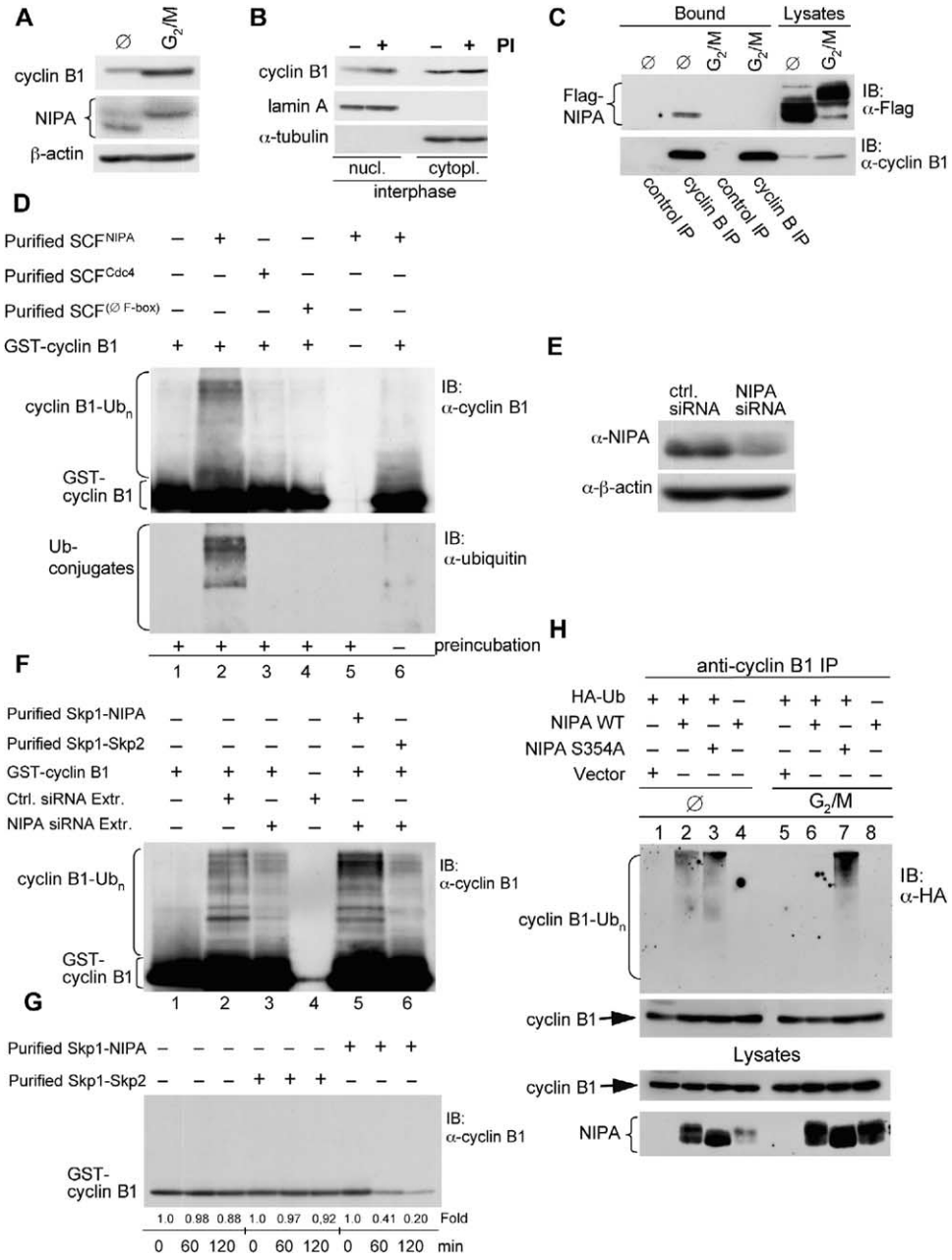

Figure 4. Identification of Nuclear Cyclin B1 as a Ubiquitination Target of the SCF^{NIPA} Complex

(A) Phosphorylation of NIPA correlates with cyclin B1 accumulation. HeLa cells were arrested at prometaphase using nocodazole or left untreated, then processed for Western blotting with anti-NIPA and anti-cyclin B1 antibodies.

(B) Proteasome-dependent degradation of nuclear cyclin B1 in interphase. HeLa cells were either left untreated or treated with the proteasome inhibitors (PI) LLnL and MG132 for 3 hr before nuclear extract preparation and Western blotting using a cyclin B1 antibody. The purity of the cell fractions was assessed using α-tubulin and lamin A as cytoplasmic or nuclear markers, respectively.

(C) Cell-cycle-dependent interaction of NIPA and cyclin B1 in vivo. NIH 3T3 cells expressing NIPA were either left untreated or synchronized at G_2/M. Normalized amounts of lysate were processed for cyclin B1 immunoprecipitation. A nonspecific rabbit antiserum was used as a control.

(D) In vitro ubiquitination of cyclin B1 using a purified SCF system. SCF^{NIPA}, SCF^{Cdc4}, or SCF core complex ($SCF^{ØF\text{-}box}$ = Skp1, Cul1, Roc1) were expressed with baculoviruses and purified from Sf21 cells. The insect cell-derived GST-cyclin B1 substrate was preincubated in HeLa lysate prior to the reactions. Samples were supplemented with ubiquitin, E1, E2 (Cdc34), and an ATP regeneration system.

(E) Western blot analysis of cells used in (F) and (G) with polyclonal NIPA antiserum.

(F) In vitro ubiquitination of cyclin B1 using reconstituted HeLa extracts. GST-cyclin B1 was incubated with extracts derived from HeLa cells treated with either control or NIPA siRNAs as described below. Recombinant insect cell-derived Skp1-NIPA or Skp1-Skp2 complexes were added as indicated.

(G) NIPA promotes destruction of cyclin B1 in vitro. Purified GST-cyclin B1 was incubated with extracts derived from HeLa cells treated with NIPA siRNA, and baculovirus-derived Skp1-NIPA or Skp1-Skp2 complexes were added, as indicated. The degradation reaction was performed under conditions that conserve proteasomal activity.

(H) SCF^{NIPA} ubiquitinates nuclear cyclin B1 in a cell-cycle-regulated manner in vivo. Expression plasmids encoding NIPA wt or NIPA S354A, or empty vector were each cotransfected with pCMV-HA-ubiquitin in COS1 cells. The cells were then either synchronized at G_2/M (lanes 5–8) or left unsynchronized (lanes 1–4). Following treatment with LLnL, nuclear extracts were prepared and subjected to cyclin B1 immunoprecipitation.

We reasoned that nuclear accumulation of cyclin B1 evoked by NIPA inactivation might affect the timing of mitotic entry. First, we determined the effect of NIPA inactivation on the cyclin B1/Cdk1 kinase activity using a histone H1 kinase assay. As depicted in Figure 5C, cyclin B1-associated kinase activity in cells released from G_1/S peaked significantly earlier in NIPA knockdown cells (5 hr) as compared to control cells (10 hr). Furthermore, kinase activity in NIPA knockdown cells remained at high levels throughout the period under observation. Next, we examined the kinetics of mitotic entry in cells released from G_1/S using phosphorylation of histone H3 as a mitotic marker. Premature mitotic entry was clearly observed in NIPA knockdown cells, while control cells displayed a physiological course of mitotic entry (Figure 5D). Of note, NIPA knockdown cells remained in mitosis longer than the corresponding control cells (Figure 5D). Western blot analysis of cells used in Figures 5C and 5D demonstrated efficient reduction of NIPA (Figure 5E).

We proceeded next to examine the cell cycle distribution of NIPA-inactivated cells. As shown in the 2D cell cycle analysis in Figure 5F, left panel, silencing of NIPA caused an increase of cells with a 4N DNA content. This finding likely reflects the prolonged mitosis observed in Figure 5D. We evaluated the specific mitotic forms of the 4N cells by indirect immunofluorescence. As shown in Figure 5F, middle and right panels, the majority of mitotic cells treated with NIPA siRNA were found to be arrested in prometaphase (characterized by spindle morphology and phospho-histone H3 pattern). While premature mitotic entry in NIPA knockdown cells is likely to be a consequence of elevated nuclear cyclin B1 levels, the mitotic block at prometaphase is presumably not cyclin B1-related. The observed prometaphase arrest may represent a compound phenotype resulting from the accumulation of multiple NIPA substrates. Alternatively, this mitotic arrest might occur due to activation of a mitotic checkpoint caused by the premature mitotic entry.

A Phosphorylation-Deficient NIPA Mutant, NIPA S354A, Retains Activity toward Cyclin B1 at G_2/M and Delays Mitotic Entry

If the SCF^{NIPA} complex normally dissociates as the cell cycle progresses toward G_2/M to allow for expression of cyclin B1, then expression of the phosphorylation-deficient mutant NIPA S354A, which forms a constitutively active $SCF^{NIPA\text{-}S354A}$ complex, would be predicted to block cyclin B1 accumulation and delay mitotic entry. We performed pulse-chase analysis of NIH 3T3 cells expressing either NIPA wt or NIPA S354A to evaluate nuclear cyclin B1 turnover at G_2/M. As shown in Figure 6A, turnover of cyclin B1 in nocodazole-arrested cells was only observed in cells expressing the NIPA S354A mutant. Thus, the $NIPA^{S354A}$ complex retains activity toward cyclin B1 at G_2/M.

To investigate the impact of the NIPA S354A mutant on mitotic entry, we generated cell lines with inducible expression of either NIPA wt or NIPA S354A (Figure 6B, left panel). Cells were arrested at G_1/S 24 hr postinduction, and the block was subsequently removed to permit synchronized cell cycle progression. Using 2D cell cycle analysis, an evident delay of mitotic entry was observed in cells expressing the phosphorylation-deficient NIPA S354A mutant as compared to NIPA wt- or vector-containing cells (Figure 6C). To rule out that this observation was the result of a proapoptotic effect of NIPA S354A, we applied TUNEL analysis to the inducible cell lines used and found no induction of apoptosis in the respective cells (Figure 6B, right panel). Immunofluorescence microscopy revealed that the apparent delay of NIPA S354A cells occurred at an early stage of mitotic entry (prophase), given that nuclear envelope breakdown had not yet occurred (Figure 6D) and histone H3 phosphorylation was not yet detectable (data not shown).

To correlate the extent of delay at mitotic entry to the expression level of NIPA S354A in terms of a dose-effect relationship, we generated single cell-derived NIH 3T3 clonal cell lines that stably express NIPA wt or NIPA S354A at various levels (Figure 6E, upper panel). As demonstrated by the 2D cell cycle analysis in Figure 6E, lower panel, expression of NIPA S354A resulted in a concentration-dependent accumulation at G_2/M. This dose-effect relationship, which correlates with the cell cycle effects observed in the inducible cell system (Figures 5C and 5D), argues against the possibility of clonal variations among the respective cell lines. Thus, the phosphorylation process involving NIPA at G_2/M, which times the dissociation of the SCF^{NIPA} complex, appears to be important for normal mitotic entry.

Discussion

Regulated protein ubiquitination and proteolysis are prerequisites for the integrity of the cell cycle, and the regulated activity of E3 ubiquitin ligases is central to this process. The most prominent groups of these proteins are the APC and SCF families of ubiquitin ligases. While the APC ligases are mainly active after metaphase and in G_1, SCF ligases target substrates for degradation at the G_1/S, S and G_2 phases of the cell cycle. In this study, we have identified NIPA as a human F-box protein that defines an SCF-type E3 ligase (SCF^{NIPA}). One of the prominent features of this ubiquitin ligase is that its activity oscillates throughout the cell cycle, and cell-cycle-regulated phosphorylation of NIPA constitutes the responsible timing circuit. We identify nuclear cyclin B1 to be a critical substrate of the SCF^{NIPA} complex in interphase. According to the oscillating activity of SCF^{NIPA}, this targeting process is terminated at G_2/M to allow for nuclear cyclin B1 accumulation, which is necessary for mitotic entry. Consistent with these data, evasion of this oscillation using the constitutively active $SCF^{NIPA\text{-}S354A}$ complex delays mitotic entry. In contrast, inactivation of NIPA by siRNAs results in nuclear accumulation of cyclin B1 during interphase, with the consequence of premature mitotic entry. Given the well-characterized mitotic ubiquitination of cyclin B1 by the APC, the identification of an SCF complex that targets cyclin B1 during interphase further defines an E3 ligase network that is operative in mitotic control.

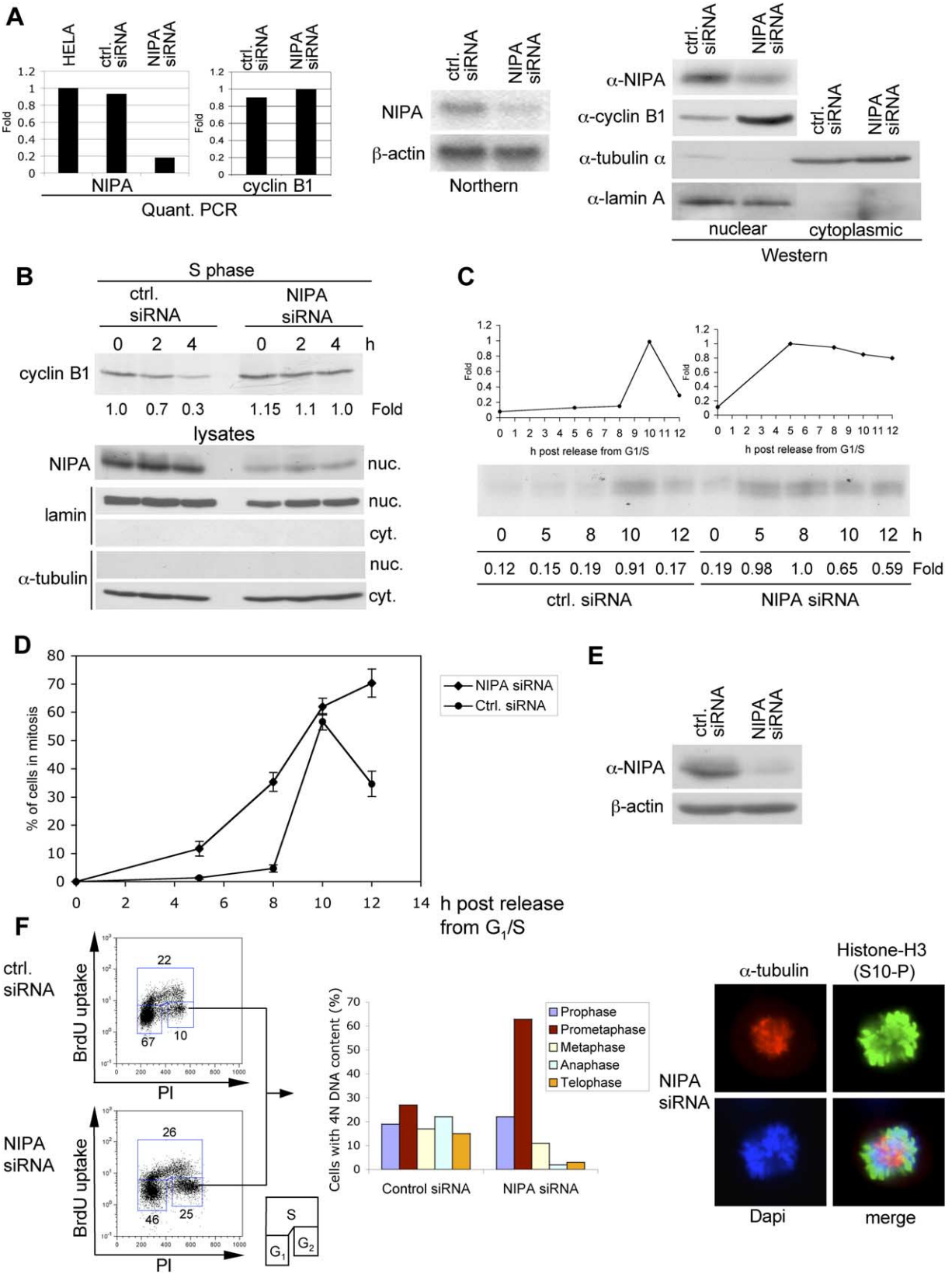

Figure 5. NIPA Silencing Stabilizes Nuclear Cyclin B1 and Induces Premature Mitotic Entry

(A) Silencing of NIPA results in nuclear accumulation of cyclin B1. HeLa cells were transfected with either control siRNA or NIPA siRNA oligonucleotides. Expression of NIPA and cyclin B1 RNAs was analyzed by quantitative PCR (left panel) or Northern blot analysis (middle

SCF^{NIPA} Functions as a Phosphorylation-Dependent Cell Cycle Oscillator

Protein degradation acts as a key trigger at various points of the cell cycle to control cell cycle transitions. Thus, mechanisms that precisely regulate the timing of this process must exist. SCF-mediated proteolysis is largely determined by the F-box component, which defines the substrate binding specificity of a respective SCF complex. The equilibrium between different F-box proteins and the limited amount of SCF core complexes is poorly understood but thought at least in part to be a matter of timely proteolysis of the F-box subunits by a mechanism involving autoubiquitination (Galan and Peter, 1999; Zhou and Howley, 1998). Furthermore, APC-dependent degradation has been reported for the F-box proteins Tome-1 and Skp2 (Ayad et al., 2003; Bashir et al., 2004; Wei et al., 2004).

In this report, we show that cell-cycle-dependent phosphorylation of an F-box protein, NIPA, acts as a timing circuit for the activity of its corresponding SCF complex. This mechanism may represent a more general principle to regulate SCF activity. Subsequent to the NIPA phosphorylation event at G_2/M, we observed expression levels of NIPA to decline as cells exited mitosis and entered G1. Therefore, phosphorylated NIPA may be a substrate for proteosomal degradation itself.

While we have not identified the serine/threonine kinase(s) responsible for NIPA phosphorylation as yet, it is intriguing to speculate that such a kinase could be stimulated apart from its physiologic cell-cycle-dependent activation by oncogenic tyrosine kinases as well. As previously reported, we cloned NIPA in complex with the oncogenic tyrosine kinase NPM-ALK and determined coexpression of activated ALK fusions to be associated with NIPA serine phosphorylation (Ouyang et al., 2003). In addition, we have observed NIPA serine phosphorylation when overexpressing in cells an activated form of the Abl tyrosine kinase v-Abl (F.B. and J.D., unpublished data). Such a mechanism could unravel a process by which these oncoproteins and perhaps other tumor-associated kinases interfere with cell-cycle-dependent proteolysis and faciliate inappropriate mitotic entry.

Cyclin B1 Is a Substrate of the SCF^{NIPA} Complex during Interphase

Cyclin B1/Cdk1 (MPF) controls mitotic entry, and the regulation of this complex occurs at both the level of enzymatic activity and subcellular distribution. Spatial control of cyclin B1 is a critical determinant of MPF activity because cyclin B1 can exert its ability to promote mitotic entry only when located within the nucleus. In addition to its role in promoting M phase, a recent report further revealed a function for cyclin B1 in supporting DNA replication when trapped to the nucleus (Moore et al., 2003). Thus, regulatory means for timing the nuclear translocation of cyclin B1 are vital to ensure the fidelity of S phase progression and mitotic entry. To date, the predominantly cytoplasmic localization of cyclin B1 in interphase, which is reversed only upon phosphorylation of the CRS region at G_2/M, and the switchlike upregulation of Cdk1-cyclin B1 activity to increases in cyclin B1 concentration have emerged as potential regulatory restraints (Pines and Hunter, 1991; Yang et al., 1998). These data identify a further mechanism explaining how the cell is protected from premature nuclear accumulation of cyclin B1 and, thus, premature mitotic entry: proteolytic degradation during interphase based on SCF-mediated ubiquitination. Previously, ubiquitination as a means of cyclin B1 regulation had been demonstrated only in mitosis, mediated by the APC ubiquitin ligase (Zachariae and Nasmyth, 1999). Our data suggest a dual mode of degradation for cyclin B1 involving the SCF^{NIPA} complex during interphase in addition to the known APC activity in mitosis. Cooperation and interdependent activity of the APC and SCF E3 ubiquitin ligases may be a more general oscillation mechanism in the control of cell-cycle-dependent protein abundance. In this regard, a recent report has described a similar mechanism involving the APC and SCF for ubiquitin-mediated degradation of human Cdc25a (Busino et al., 2003). In fission yeast, a dual mechanism of cell-cycle-regulated destruction involving both the APC/C and the $SCF^{Pop1/Pop2}$ complexes has been described for Cig2, a B-type cyclin active in S phase of the cell cycle (Yamano et al., 2000). Together, these data support our model of a dual mode of degradation for cyclin B1 in mammalian cells. The formal possibility exists that ubiquitination of cyclin B1 in interphase is mediated also in part by the APC. However, this mechanism seems rather unlikely, given a recent finding that restricts the interaction of the APC and cyclin B1 to mitosis (Yamano et al., 2004).

Since most known SCF substrates are phosphorylated prior to interaction with their respective F-box proteins, it is likely that cyclin B1 must be phosphorylated in order to interact with NIPA. In our experimental

panel). Protein expression in nuclear extracts was demonstrated using NIPA-antiserum and anti-cyclin B1 antibody. Immunoblots detecting lamin A and α-tubulin were used to assess the quality of the extract fractionations (right panel).

(B) SCF^{NIPA} degrades nuclear cyclin B1 in S phase. HeLa cells were treated with NIPA or control siRNAs and arrested in S phase. The extent of cyclin B1 degradation was assayed using pulse-chase analysis.

(C) NIPA inactivation results in premature activation of cyclinB1/Cdk1 kinase activity. HeLa cells were transfected with either NIPA or control siRNAs, synchronized at G_1/S, and subsequently released. Thereafter, cells were harvested at the indicated time points and processed for anti-cyclin B1 immunoprecipitation. Precipitates were subsequently applied to an in vitro histone H1 kinase assay.

(D) NIPA inactivation promotes premature mitotic entry. HeLa cells treated as in (C) were released from G_1/S and fixed at the indicated time points. Thereafter, cells were subjected to indirect immunofluorescence using phospho-histone H3 and α-tubulin antibodies, and DAPI staining as markers to distinguish mitotic cells. The graph represents the results from four independent experiments.

(E) Western blot analysis of cells used in (C) and (D) with polyclonal NIPA antiserum.

(F) NIPA inactivation causes a mitotic arrest in prometaphase. (Left) The cells described in (A) were processed for 2D cell cycle analysis. (Middle and right) Cells described in (A) were stained with anti-α-tubulin- (red), anti-histone-H3(S10-P)- (green), and DAPI (blue), then visualized by fluorescence microscopy. The fraction of cells with 4N DNA content was quantified with regard to the different stages of mitosis.

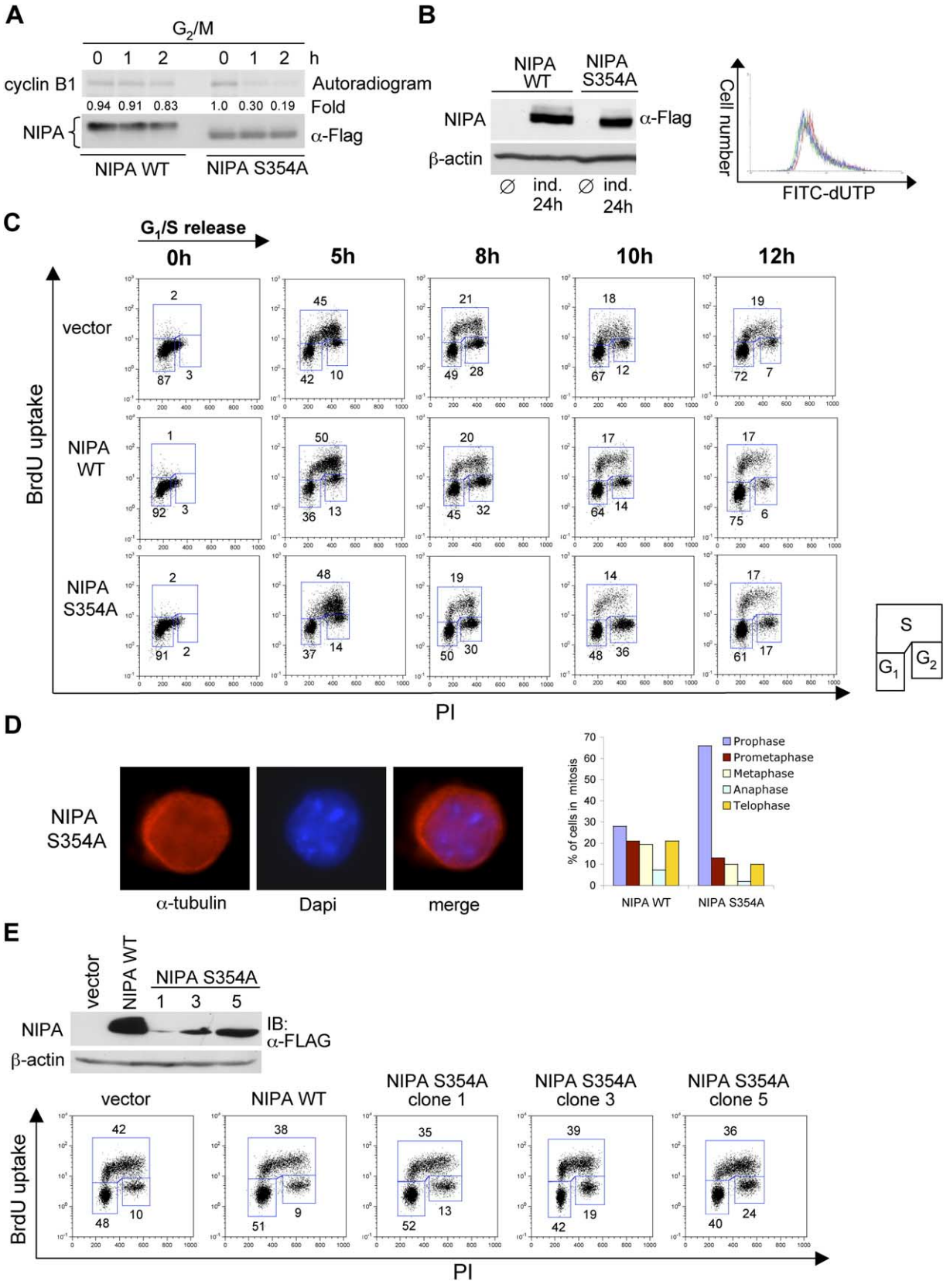

Figure 6. A Phosphorylation-Deficient NIPA Mutant, NIPA S354A, Retains Activity toward Cyclin B1 at G_2/M and Delays Mitotic Entry

(A) NIPA S354A degrades nuclear cyclin B1 at G_2/M. NIH 3T3 cells transfected with either NIPA wt or NIPA S354A were synchronized at G_2/M and subsequently pulsed with ^{35}S-methionine and cysteine. The extent of cyclin B1 degradation is shown in an autoradiogram of immunoprecipitated endogenous cyclin B1.

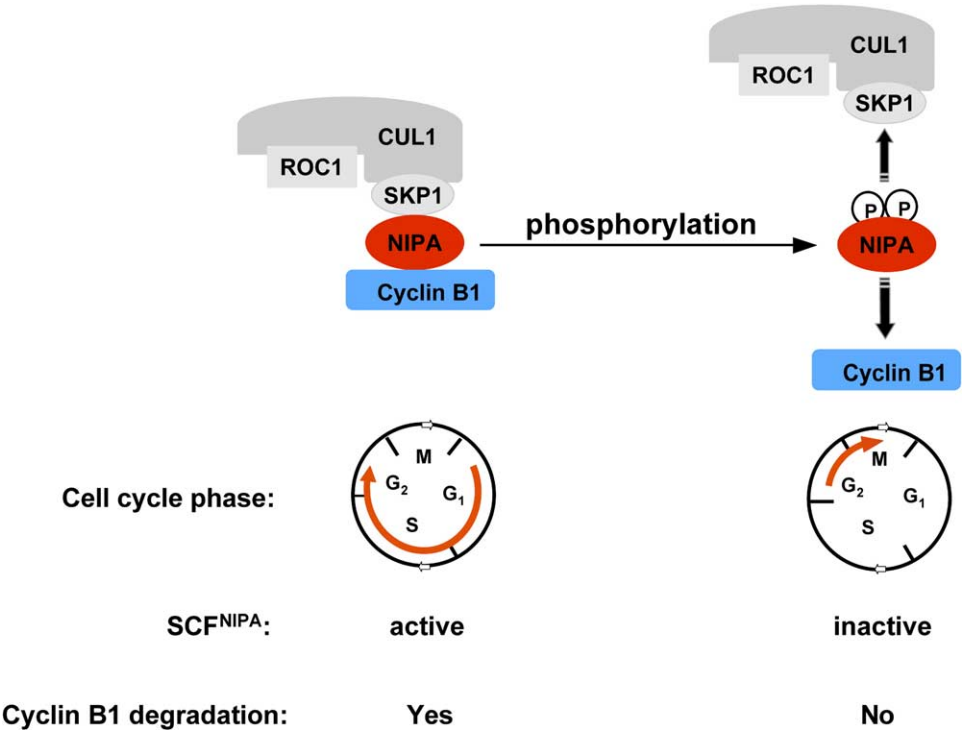

Figure 7. Model of SCF^{NIPA} Activity during the Cell Cycle

Phosphorylation of NIPA governs the ubiquitination activity of the SCF^{NIPA} complex, thereby allowing nuclear cyclin B1 levels to increase as cells progress toward G_2/M.

setting, in vitro ubiquitination of cyclin B1 using a reconstituted SCF system was only observed with purified cyclin B1 preincubated with cell lysates beforehand (Figure 4D). It is probably the case that this preincubation process involves phosphorylation of cyclin B1, which may represent a prerequisite for cyclin B1 degradation by the SCF^{NIPA}. We demonstrate that inactivation of NIPA using siRNA causes nuclear cyclin B1 to accumulate in interphase. This untimely accumulation associates with premature cyclin B1/Cdk1 activity and early mitotic entry. Recent studies demonstrate that nuclear localization is a prerequisite for cyclin B1 to be active, and premature localization of cyclin B1 to the nucleus induces DNA replication and evokes mitotic entry (Li et al., 1997; Moore et al., 2003). Thus, NIPA knockdown cells exhibit a phenotype with regard to their kinetics of mitotic entry that seems concordant with the ubiquitination activity of NIPA toward nuclear cyclin B1 during interphase. In contrast, the arrest in prometaphase we observed in NIPA knockdown cells is not likely to be a cyclin-B1-specific effect. Rather, this observation may represent a compound phenotype resulting from the accumulation of multiple yet-to-be-identified NIPA substrates. A further conceivable cause could be a mitotic checkpoint activated by premature mitotic entry.

In summary, our results suggest a role for the SCF^{NIPA} complex in timing mitotic entry. The underlying mechanism is based on the oscillating activity of the SCF^{NIPA}, which promotes degradation of nuclear cyclin B1 during interphase but allows for the accumulation of cyclin B1 through S and G_2 phases to trigger the G_2-to-M transition (Figure 7). Our data thus define a cellular mechanism that times the nuclear accumulation of cyclin B1 with mitotic entry.

Experimental Procedures

Plasmids and Antibodies

Details of the construction of plasmids pcDNA3.1-Flag-NIPA wt, -NIPA S354A, and -NIPA FM (F-box mutant) are available from the authors upon request. Point mutations of the NIPA cDNA were engineered using the QuickChange Site-Directed Mutagenesis Kit

(B) Generation of inducible cell lines expressing NIPA wt and NIPA S354A. (Left) Immunoblot showing expression in either uninduced or induced cells. (Right) TUNEL analysis of NIPA wt- (green), NIPA S354A- (blue), or empty vector-containing cells (green).
(C) Induced expression of the phosphorylation-deficient mutant NIPA S354A delays mitotic entry. Cell lines described in (B) were synchronized at G_1/S 24 hr postinduction. Cells were released from the block and their cell cycle distribution was measured at the indicated time points.
(D) NIPA S354A delays mitotic entry at prophase. Cells treated as in (C) were stained with anti-α-tubulin antibody (red) and DAPI (blue) and visualized by fluorescence microscopy at the 10 hr time point.
(E) NIPA S354A influences mitotic entry in a dose-effect-related manner. Stably transfected NIH 3T3 clones expressing NIPA wt, NIPA S354A, or empty vector were analyzed by 2D cell cycle analysis.

(Stratagene), while deletion mutants were prepared by PCR using standard cloning procedures. Mouse monoclonal antibodies were purchased from Sigma (anti-Flag [M2], anti-Myc [clone 9E10], anti-β-actin), Qiagen (anti-$[His]_6$), Invitrogen (anti-Xpress), and Santa Cruz (anti-α-tubulin, anti-lamin A). Polyclonal rabbit antibodies were from Santa Cruz (anti-HA tag [Y11], anti-cyclin A, anti-cyclin B1, anti-ubiquitin), Zymed (anti-Skp1), and Upstate (anti-phospho-H3).

Antibody Preparation

Bacterially produced GST-NIPA (BL-21 cells) was used to raise polyclonal antibodies in mice (Davids Biotechnology, Regensburg, Germany). The resulting serum was affinity purified.

Yeast Two-Hybrid System

The full-length NIPA cDNA was cloned into the yeast expression vector pBTM116 for use as the bait to screen a human testis library (Clontech) (9×10^6 clones) in the *Saccharomyces cerevisiae* L40 strain, as previously described (Bai et al., 2002).

Cell Culture, Cell Cycle Synchronization, and Transfection

NIH 3T3, HeLa, COS1, and 293T cells were cultivated in DMEM containing 10% FCS. Transient transfections of COS1 and 293T cells were performed using the DOTAP (Roche) and Lipofectamine 2000 (Invitrogen) transfection reagents. Stable transfections of NIH 3T3 cells have been described before (Bassermann et al., 2002). For inducible expression, the GeneSwitch expression system (Invitrogen) was used. Cells were treated with 2 mM thymidine or 70 μM quercetin for 16 hr at 37°C to arrest them in S phase or were arrested at G_2/M by sequential culture with 2 mM thymidine and 500 ng/ml nocodazole. For synchronization in $G_{0/1}$, cells were serum starved for 48 hr (0.5% FCS). Synchronization at G_1/S was performed using a double thymidine block.

Cell Cycle Analysis

For BrdU incorporation and DNA content analysis, cells were pulsed with 10 μM BrdU (BD Biosciences) for 40 min and subsequently stained according to a standard protocol. One- and two-dimensional flow cytometry was performed to detect either PI or both fluorescein and PI. To quantify cell cycle distribution, the FlowJo software (Tree Star Inc., Stamford, Connecticut) was utilized.

siRNA

siRNAs were purchased from Proligo and transfected into subconfluent HeLa cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The target sequence of NIPA siRNA was 5′-GTCCACGTCACTGCCTGTA-3′. A firefly luciferase siRNA served as the control.

Immunoprecipitation, Western Blotting, and Phosphatase Assays

Immunoprecipitations were performed as described (Duyster et al., 1995). Phosphoprotein digestion was carried out in a volume of 50 μl in phosphatase buffer (40 mM PIPES [pH 6.0], 1 mM DTT, 1 mM $MnCl_2$, 100 μg/ml BSA, 50 μM leupeptin) using potato acid phosphatase, type III (Sigma) at 100 μg/ml.

Northern Blotting and Quantitative PCR

Total RNA was extracted from HeLa cells using Trizol reagent (Invitrogen). Hybridization was performed with a ^{32}P-labeled human NIPA probe obtained by Hind III digestion of the full-length NIPA cDNA. Quantitative PCR was performed with GAPDH-, NIPA-, and cyclin B1-specific primers using standard procedures.

GST Fusion Proteins and Pull-Down Assays

NIPA wt, NIPA FM, and Skp1 were expressed in *E. coli* using pGEX vectors (Amersham). GST pull-down assays were performed as previously described (Bassermann et al., 2002).

Insect Cell-Derived Recombinant Proteins

Complementary DNAs encoding human NIPA and Cdc4 were inserted into the pBacPak-9 vector (Clontech). Baculoviruses expressing human GST-Skp1, HA-Cul1, Roc1, and Skp2 were kindly supplied by M. Pagano. All recombinant proteins were produced in Sf21 insect cells. The different complexes were formed by coexpression of the respective baculoviruses and purification performed using the GST and HA tags of Skp1 and Cul1, respectively.

Ubiquitination of Cyclin B1 Using a Reconstituted SCF System

To assay in vitro ubiquitination of cyclin B1 using a reconstituted system, purified GST-cyclin B1 (Upstate) was collected on glutathione-agarose beads and preincubated with HeLa lysate. GST-cyclin B1 was subsequently precipitated and washed three times in lysis buffer and ubiquitination buffer (40 mM Tris-HCl [pH 7.6], 5 mM $MgCl_2$, 1 mM DTT, 10% glycerol). Thereafter, GST-cyclin B1 was transferred to the ubiquitination reactions, which contained approximately 500 ng each of purified Skp1-NIPA or Skp1-Cdc4 together with Cul1-Roc1 complexes, 0.5 μg/μl ubiquitin, 1 μg E1 (Affinity, Inc.), 1.3 μg CDC34 (A.G. Scientific, Inc.), 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase, 0.5 mM ATP, and 1 μM ubiquitin aldehyde.

Protein Extraction for In Vitro Ubiquitination Assays

To generate cell extracts used for the experiments illustrated in Figure 4E, logarithmically growing HeLa cells were transferred to a nitrogen disruption bomb (Parr Instrument Company) and pressurized at 70 bar for 30 min. Following decrompession, the material was centrifuged at 10,000 × g for 10 min to obtain the S10 supernatant.

In Vitro Ubiquitination and Degradation Assay

The ubiquitination reaction mix contained 0.5 μg/μl ubiquitin, 1 μM ubiquitin aldehyde, 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase, 0.5 mM ATP, 20 μg HeLa extract, and 100 ng purified GST-cyclin B1 in ubiquitination buffer. Baculovirus-produced Skp1-NIPA or Skp1-Skp2 was added in similar amounts, as indicated.

The cyclin B1 degradation assay was performed in a similar manner, except that proteasome inhibitors, ubiquitin, and ubiquitin aldehyde were omitted and extracts were prepared as described in Carrano et al. [1999].

Histone H1 Kinase Assay

Cyclin B1 was immunoprecipitated from cell lysates as indicated above using anti-cyclin B1 (Santa Cruz). Precipitates were washed and transferred to the kinase reaction, which contained 80 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP, 1 μCi [γ-^{32}P] ATP (Amersham), 1 mM DTT, and 1 μg histone H1.

Immunofluorescence Microscopy

Staining procedures were performed essentially as described (Bai et al., 2002). Primary antibodies (anti-NIPA, anti-α-tubulin, anti-histone H3[S10-P]) were used at dilutions of 1:1000, 1:400, and 1:200, respectively.

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Accession Numbers

The GenBank accession numbers for the human and murine NIPA mRNAs are AJ537494 and AJ537495, respectively.