Glycogen Synthase Kinase-3β (GSK3β) Negatively Regulates PTTG1/Human Securin Protein Stability, and GSK3β Inactivation Correlates with Securin Accumulation in Breast Tumors*[S]

Mar Mora-Santos[‡1], M. Cristina Limón-Mortés[‡], Servando Giráldez[‡2], Joaquín Herrero-Ruiz[‡], Carmen Sáez[§3], Miguel Á. Japón[§], Maria Tortolero[‡], and Francisco Romero[‡4]

From the [‡]Departamento de Microbiología, Facultad de Biología, Universidad de Sevilla, Apartado de Correos 1095, 41080 Sevilla and the [§]Instituto de Biomedicina de Sevilla (IBIS), Hospital Universitario Virgen del Rocío/Consejo Superior de Investigaciones Científicas (CSIC)/Universidad de Sevilla, and Departamento de Anatomía Patológica, Hospital Universitario Virgen del Rocío, 41013 Sevilla, Spain

PTTG1, also known as securin, is an inactivating partner of separase, the major effector for chromosome segregation during mitosis. At the metaphase-to-anaphase transition, securin is targeted for proteasomal destruction by the anaphase-promoting complex or cyclosome, allowing activation of separase. In addition, securin is overexpressed in metastatic or genomically instable tumors, suggesting a relevant role for securin in tumor progression. Stability of securin is regulated by phosphorylation; some phosphorylated forms are degraded out of mitosis, by the action of the SKP1-CUL1-F-box protein (SCF) complex. The kinases targeting securin for proteolysis have not been identified, and mechanistic insight into the cause of securin accumulation in human cancers is lacking. Here, we demonstrate that glycogen synthase kinase-3β (GSK3β) phosphorylates securin to promote its proteolysis via SCF^βTrCP E3 ubiquitin ligase. Importantly, a strong correlation between securin accumulation and GSK3β inactivation was observed in breast cancer tissues, indicating that GSK3β inactivation may account for securin accumulation in breast cancers.

Mitosis is a process that results in the segregation of sister chromatids into two newly made cells. During anaphase, sister chromatids synchronously lose cohesion, allowing the replicated chromosomes to segregate to opposite ends of the cell. Separase is the key regulator for this segregation. This cysteine protease cleaves the cohesin subunit SCC1/RAD21, which opens the cohesin ring and causes it to dissociate from chromosomes. In vertebrates, separase activation is regulated by both securin (PTTG1[5] or pituitary tumor-transforming gene 1) and phosphorylation-dependent cyclin B1 binding (1, 2). Securin is a chaperone protein that, following chromosome alignment, is ubiquitylated and degraded, leaving separase unbound and active (3, 4). Securin is degraded by the 26 S proteasome at the metaphase-to-anaphase transition via the anaphase-promoting complex/cyclosome (APC/C). APC/C is an E3 ubiquitin ligase that requires the additional function of CDC20 or CDH1, two adaptor proteins (5, 6). Moreover, not only is securin degraded via APC/C, but some phosphorylated forms of securin are also degraded by the action of SKP1-CUL1-F-box protein (SCF) complex (7). The SCF complex is the other major ubiquitin ligase responsible for the specific ubiquitylation of many regulators of the cell cycle (8–10).

There is much evidence that securin is important for cellular responses to genotoxic stresses (11–13). We previously described the role of human securin in cell cycle arrest after UV irradiation, when it probably prevents cell proliferation during DNA damage repair (14). Irradiation causes rapid proteasome-dependent securin degradation that is mediated by SCF^βTrCP E3 ubiquitin ligase and prevented by inhibitors of GSK3 (15). A role for securin in cancer pathogenesis is supported by the observation of increased expression of securin in different tumors (16–19) and in samples under a putative metastasis program (20).

Glycogen synthase kinase-3, GSK3, is a serine/threonine protein kinase that has been found to phosphorylate many proteins and play important roles in a variety of cellular processes such as cell proliferation, differentiation, microtubule dynamics, cell cycle, and apoptosis (21, 22). In fact, a consensus motif and context-based computational analysis of identified *in vivo* protein phosphorylation sites indicates that GSK3 is one of the kinases with the most substrates in the cell (23).

Identification of GSK3β as a Regulator of Human Securin

GSK3β has been known to play an inhibitory role in cell cycle progression and cell proliferation, at least partly through its regulation of cyclin E, cyclin D1, CDC25A, and c-Myc stability. GSK3β phosphorylation mediates rapid degradation of both cyclin D1 and cyclin E. Ras signal inactivates GSK3β through the PI3K/AKT pathway and results in accumulation of stabilized cyclins, triggering cell cycle progression (24, 25). Inactivation of GSK3β leads to accumulation of CDC25A phosphatase, another GSK3-regulated protein degradation substrate, in early cell cycle phases, accelerating the S phase entry (26). At the same time, mitogen signaling also inhibits the GSK3-mediated degradation of c-Myc, resulting in the activation of its target genes, including cyclin D1, cyclin E, and other cell cycle mediators (27, 28). GSK3 thus has both direct and indirect roles in regulation of cell cycle progression. This study reports that GSK3β phosphorylates human securin to promote its proteolysis via SCF$^{\beta TrCP}$ E3 ubiquitin ligase in normal cell cycle and that accumulation of securin strongly correlates with GSK3β inactivation in breast tumors.

EXPERIMENTAL PROCEDURES

Plasmids, Point Mutations, and Sequencing—pCDNA3- 2HA-hSec, pRSET-A hSecΔC, pRSET-A hSecΔN, pGEX4T2, pGEX4T2 hSec, pGEX4T2 hSec Nter, pEGFP-N1, pCS2HA-βTrCPΔF, pCDNA3-HA GSK3β, pCDNA3-HA GSK3β K85A, and empty vectors were previously described (13–15, 29–32). hSec S183A/S184G was constructed using the Transformer site-directed mutagenesis kit from BD Biosciences. Sequencing of point mutations was performed on both strands with an automatic sequencer.

Cell Culture, Cell Synchronization, Drugs, FACS Analysis, Transient and Stable Transfection, and Lysis—Routinely, HeLa, HCT116 and Cos-7 cells were grown in Dulbecco's modified Eagle's medium (Lonza) as described (14). HeLa cells enriched in the G_1, S, G_2, or M phase were obtained as described previously (33). HeLa G_1 cells were obtained by incubating cells for 16 h in 6 mM butyrate. HeLa G_1/S cells were obtained by performing a double-thymidine block (*i.e.* two 16-h incubations in 2.5 mM thymidine, with an 8-h release in between). Cells enriched in S phase were harvested 4 h after release from the second block. Cells harvested 8 h after release were further enriched for a G_2 population by rinsing extensively to remove mitotic cells. Mitotic arrested cells were obtained by incubation for 16 h in medium containing 5 μM nocodazole. Purity of the phases was confirmed by flow cytometry. When indicated, cells were pretreated with LiCl (10–100 mM), 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8, 50 μM), CT99021 (10 μM), *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal (100 μM), or cycloheximide (50 μg/ml) and harvested at various times. FACS analysis was used to determine the drug effects on the cell cycle.

DNA constructs were transiently transfected by electroporation into Cos-7 cells or using the Lipofectamine method into HeLa cells. 18 or 48 h after transfection, respectively, cells were harvested and lysed. HCT116$^{sec-/-}$ cells (34) were stably transfected with pCDNA3-2HA-hSec to obtain HCT116$^{sec-/-}$2HA-hSec. Cell lysis was performed at 4 °C in Nonidet P-40 buffer. When necessary, extracts were treated with λ-protein phos-phatase (λ-PP (7)). Protein concentration was determined using the Bradford assay.

Small Interfering RNA (siRNA) Assays—Cells were interfered with GSK3β siRNA (35) using the Oligofectamine method to suppress the expression of the endogenous gene. EGFP siRNA was used as a nonspecific control. Cells were harvested 48 h after transfection, and reduction of protein levels was confirmed by Western blotting. In some experiments, cells were both transfected and interfered. In these cases, we used the Lipofectamine method.

Electrophoresis, Western Blot Analysis, and Antibodies—Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and gels were electroblotted onto nitrocellulose membranes and probed with the following antibodies: anti-HA-peroxidase monoclonal antibody (Roche Applied Science), anti-PTTG and anti-GSK3α/β monoclonal antibodies (Santa Cruz Biotechnology), anti-Cdc25A monoclonal antibody (NeoMarkers), anti-GFP polyclonal antibody (Immunology Consultants Laboratory), anti-α-tubulin monoclonal antibody (Sigma), and anti-GSK3β monoclonal antibody (BD Biosciences). Peroxidase-coupled donkey anti-rabbit IgG and sheep anti-mouse IgG were obtained from GE Healthcare. Immunoreactive bands were visualized using the enhanced chemiluminescence Western blotting system (ECL, GE Healthcare).

Affinity Chromatography and in Vitro Kinase Assays—The expression of the GST or His$_6$ fusion proteins was induced in *Escherichia coli* BL21 (DE3) cells by incubation with 1 mM isopropyl-β-D-thiogalactoside for 3 h at 37 °C. Fusion proteins were purified from bacterial lysates via their affinity to glutathione-Sepharose (GE Healthcare) or nickel-nitrilotriacetic acid-agarose (Qiagen), respectively. For affinity chromatography assays, cellular lysates (200–500 μg) were incubated for 2 h with GST fusion proteins (100–500 ng) bound to the Sepharose beads. Beads were washed six times in lysis buffer, and bound proteins were eluted by the addition of SDS-sample buffer heated at 95 °C for 5 min. Finally, the samples were subjected to SDS-PAGE. For *in vitro* kinase assays, purified GSK3β (Invitrogen) was incubated with the GST or His$_6$ fusion proteins, [γ-^{32}P]ATP, and GSK3β kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 0.1 mM Na$_3$VO$_4$, 2 mM DTT, and 100 μM unlabeled ATP) for 15 min at 30 °C. His$_6$-Tau was used as a positive control. Reactions were terminated by adding 4× SDS-sample buffer, and proteins were analyzed by SDS-PAGE and autoradiography.

Coimmunoprecipitation Experiments—Cellular lysates (1–2 mg) were incubated with normal rabbit serum for 30 min and subsequently with protein A-Sepharose beads (GE Healthcare) for 1 h at 4 °C. After centrifugation, beads were discarded, and supernatants were incubated for 2 h with polyclonal anti-GSK3β (Santa Cruz Biotechnology), anti-hPTTG (29) antibodies, or normal serum followed by protein A-Sepharose beads for 1 h. Beads were washed, and bound proteins were solubilized by the addition of SDS-sample buffer heated at 95 °C for 5 min.

Tissue Microarray and Immunohistochemistry—1-mm-diameter tissue cores from 95 paraffin-embedded invasive breast adenocarcinomas and three normal breast controls were arrayed in a recipient paraffin tissue block. Specimens were
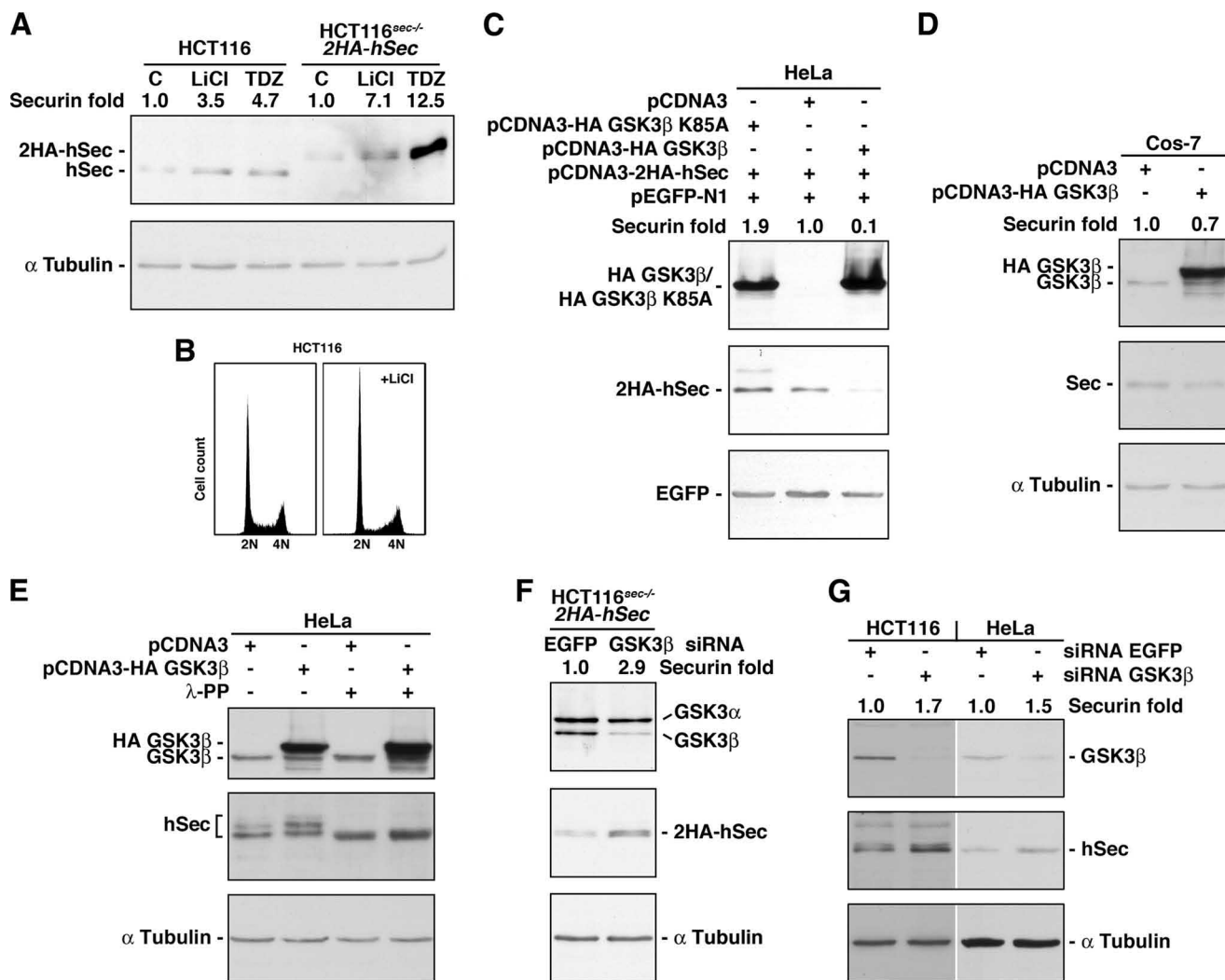
FIGURE 1. **Securin protein levels are negatively regulated by GSK3β.** *A*, asynchronously growing HCT116 and HCT116$^{sec-/-}$2HA-hSec cells were untreated (*C*) or incubated with 10 mM LiCl or 50 μM TDZD-8 (*TDZ*) for 24 h (*n* = 2). Western blots of extracts were analyzed for securin and α-tubulin levels. *B*, FACS analysis of HCT116 cells treated or not treated with 30 mM LiCl for 18 h. *C*, HeLa cells were transiently transfected with the indicated plasmids (*n* = 2) and, after 48 h, lysates were analyzed by Western blotting. *D*, Cos-7 cells were transiently transfected with pCDNA3-HA GSK3β or empty vector (*n* = 3), and extracts were treated with λ-PP and analyzed by Western blotting. *E*, HeLa cells were transiently transfected with the indicated plasmids (*n* = 2) and, 4 h before harvesting, cells were treated with the proteasomal inhibitor *N*-acetyl-L-leucyl-L-leucyl-L-norleucinal. Where indicated, extracts were treated with λ-PP. Membranes were probed with the indicated antibodies. *F*, HCT116$^{sec-/-}$2HA-hSec cells were interfered with the indicated siRNA(s) and, after 48 h, extracts were analyzed by Western blotting. *G*, similarly, HeLa and HCT116 cells were interfered with the indicated siRNA(s), and extracts were analyzed by Western blotting.

retrieved from the archives of the Department of Pathology, Hospital Universitario Virgen del Rocío, with the approval of local ethical committees. 4-μm-thick paraffin sections were mounted on silanized microscopy slides for immunohisto-chemistry. Sections were deparaffinized in xylene and rehy-drated in graded alcohols according to standard methods and then immersed in 3% hydrogen peroxide in water to exhaust endogenous peroxidase activity. Epitope unmasking was per-formed in all cases in 1 M Tris, 0.01 M EDTA buffer (pH 9.0) using a microwave oven. Sections were covered with 10% nor-mal swine serum in PBS and then incubated with primary anti-body overnight at 4 °C in a humid chamber. Primary antibodies and dilutions used were rabbit polyclonal anti-GSK3β (Santa Cruz Biotechnology) at 1:200, rabbit monoclonal anti-phos-pho-GSK3β (Cell Signaling) at 1:50, mouse monoclonal anti-PTTG (Santa Cruz Biotechnology) at 1:100, and mouse monoclonal anti-Ki67 antigen (Dako) at 1:100. Secondary per-

oxidase-dextran-conjugated antibodies (EnVision, Dako) were applied 1 h at room temperature, and 3-3′-diaminobenzidine was used as the peroxidase chromogenic substrate. Sections were slightly counterstained with hematoxylin solution (Gill II, Merck), dehydrated, cleared in xylene, and mounted in Pertex (Medite). Immunostains of phospho-GSK3β and securin were assessed using digital images and classified by two observers as positive in cases with more than 10% moderate-to-intensely positive-staining cells or as negative in any other cases. Associ-ation between phospho-GSK3β and securin (or Ki67 antigen proliferation index or tumor grade) was evaluated using Fish-er's exact tests with the aid of the GraphPad Prism software.

RESULTS

Identification of GSK3β as a Potential Regulator of Securin— We have previously described that degradation of securin in response to ultraviolet radiation was mediated by SCF^{βTrCP}
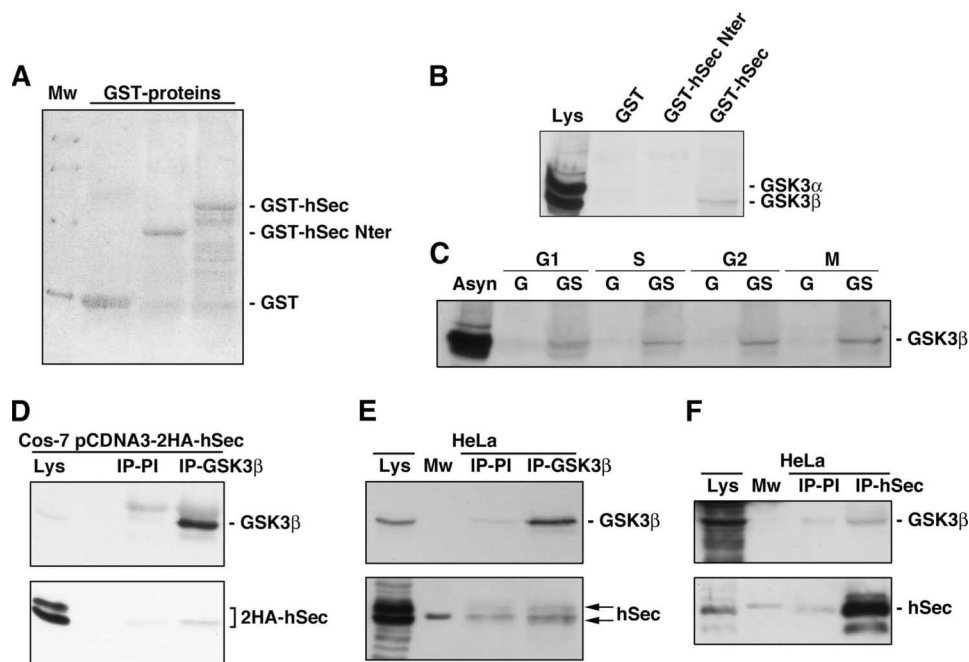
FIGURE 2. **GSK3β interacts with securin.** *A*, Ponceau S staining of GST fusion proteins expressed in bacteria and purified on glutathione-Sepharose columns. *Mw*, molecular mass markers (95, 66, 45, and 31 kDa). *B*, GST proteins were incubated with Nonidet P-40 extracts from HeLa cells, washed, and immunoblotted with an anti-GSK3α/β antibody. *Lys*, lysate from HeLa cells. *C*, GST (*G*) and GST-hSec (*GS*) were incubated with extracts from HeLa cells arrested in the different phases of the cell cycle and, after washing, blotted with anti-GSK3β antibody. *Asyn*, extracts from asynchronously growing HeLa cells. *D*, Cos-7 cells were transiently transfected with pCDNA3-2HA-hSec and extracts immunoprecipitated (*IP*) with rabbit anti-GSK3β or normal rabbit serum (*PI*). Immunoprecipitated materials were analyzed by Western blotting. *Lys*, extracts from Cos-7 transfected cells. *E*, Nonidet P-40 extracts from HeLa cells were used to immunoprecipitate GSK3β, and complexes were analyzed by immunoblotting. *IP-PI*, immunoprecipitation with normal rabbit serum. *Lys*, lysate from HeLa cells. *Mw*, molecular mass markers. *F*, the complementary experiment was also performed using securin immunoprecipitation from λ-PP-treated HeLa cells extracts. Membrane was blotted with anti-GSK3β.

ubiquitin ligase and that GSK3 inhibitors prevented this degradation (15), so it was decided to explore the role of this kinase in the regulation of securin in non-irradiated cells. Several experiments were performed to determine whether GSK3 negatively regulates securin. It is known that transcriptional activation of securin begins in G_1 but that the maximum expression is reached later, in the G_2 phase (36). To focus on the role of GSK3 on protein stability, it was first decided to obtain a cell line expressing securin from a constitutive promoter to avoid possible indirect effects on transcriptional regulation. Human HCT116 colorectal cells knocked out for securin (HCT116$^{sec-/-}$) were stably transfected with hemagglutinin-tagged securin (HCT116$^{sec-/-}$2HA-hSec, see "Experimental Procedures"). Next, HCT116 and HCT116$^{sec-/-}$2HA-hSec cells were treated with LiCl or TDZD-8, two potent GSK3 inhibitors (37, 38). As seen in Fig. 1*A*, both endogenous securin in HCT116 cells and 2HA-securin in stably transfected HCT116$^{sec-/-}$2HA-hSec cells accumulated in cells treated with either LiCl or TDZD-8. The securin level rises as cells advance through the cell division cycle (36). FACS analysis was performed to ensure that GSK3 inhibition did not cause a significant fraction of the cell to move into and remain in the late cell cycle phases. If this were the case, the observed stabilization of securin could be due to an indirect effect of cell cycle position rather than a direct effect of GSK3 inhibition. Under the time course of the experiment, 18 h, inhibition of GSK3 in HCT116 cells did not significantly alter the cell cycle profile (Fig. 1*B*). In fact, LiCl treatment provoked a slight increase of cells in the G_1 phase, demonstrating that securin stabilization was not an indi-

rect effect of the progress of the cell cycle to late phases. Next, the 2HA-securin ectopically produced level was monitored in HeLa cells overproducing kinase inactive (HA GSK3β K85A) or wild type (HA GSK3β). Fig. 1*C* shows that the 2HA-securin protein level was reduced in cells expressing wild type GSK3β and augmented in cells expressing kinase inactive GSK3β. Moreover, endogenous securin protein level was also reduced in Cos-7 or HeLa cells overexpressing wild type GSK3β and, when proteasome was inhibited in these conditions, securin degradation was avoided, and several retarded bands of the protein were observed in SDS-PAGE gels (Fig. 1, *D* and *E*). These bands were reduced to a single band after λ-PP treatment, indicating that GSK3β provokes securin phosphorylation *in vivo*. Finally, experiments were also carried out using GSK3β-specific siRNA. As seen in Fig. 1*F*, knockdown of GSK3β stabilized 2HA-securin in HCT116$^{sec-/-}$2HA-hSec cells. Knockdown of GSK3β did not affect levels of GSK3α, demonstrating that this stabilization of 2HA-securin was due to loss of GSK3β rather than to loss of both GSK3β and GSK3α. Furthermore, GSK3β down-regulation also increased endogenous securin levels of both HeLa and HCT116 cells (Fig. 1*G*). Taken together, these results show that GSK3β negatively regulates securin *in vivo*.

GSK3β Interacts with Securin—In vitro binding experiments demonstrated that GSK3β associates with securin. Lysates from HeLa cells were incubated with full-length GST-securin, GST-hSec, or truncated GST-securin (residues 1–123), GST-hSec Nter, bound to glutathione-Sepharose. After washing the resin, proteins were Western blotted for detection of GSK3α/β. As seen in Fig. 2, *A* and *B*, GSK3β from cellular extracts associ-
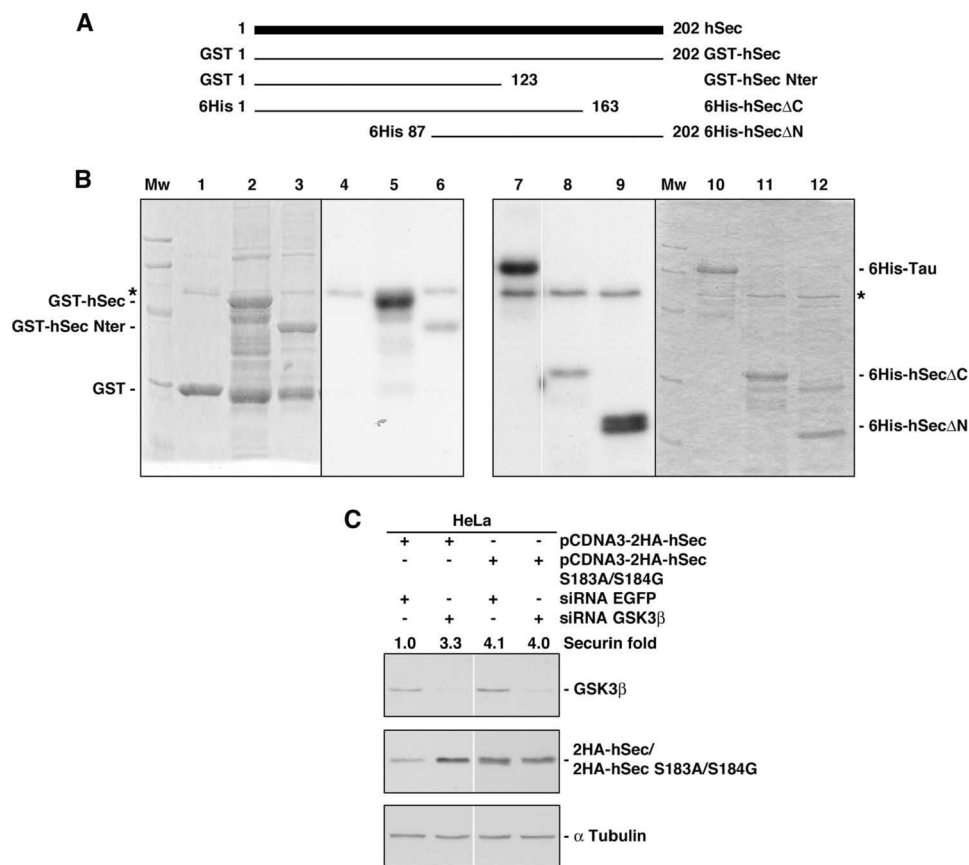
FIGURE 3. **GSK3β phosphorylates securin.** *A*, schematic representation of securin and fusion proteins. *B*, purified GSK3β (marked with an *asterisk*) was incubated with GST- or His-securin (or derivatives) fusion proteins, [γ-^{32}P]ATP, and GSK3β kinase buffer. Reactions were analyzed by SDS-PAGE, stained with Coomassie Blue (*lanes 1–3* and *10–12*), and autoradiographed (*lanes 4–6* and *7–9*). His$_6$-Tau was used as a positive control of GSK3β kinase activity. *Mw*, molecular mass markers (95, 66, 45, 31, and 21 kDa). *C*, HeLa cells were transiently transfected with the indicated plasmids, along with siRNA oligonucleotides against GSK3β or EGFP, and collected after 48 h. Extracts were treated with λ-PP and Western blotted for GSK3β, HA, and α-tubulin detection.

ated with full-length securin but not with securin deleted for residues 124–202. Interestingly, GSK3α from cellular extracts did not associate with securin, confirming the results shown in Fig. 1*F* that stabilization of securin was due to loss of GSK3β. Furthermore, to know whether this binding changes during the cell cycle, we performed similar experiments using extracts from HeLa cells arrested in G$_1$, S, G$_2$, and M. However, no difference was observed (Fig. 2*C*). On the other hand, interaction between securin and GSK3β was also detected *in vivo*. To this aim, lysates from Cos-7 cells transiently transfected with pCDNA3-2HA-hSec were incubated with anti-GSK3β polyclonal antibody or with normal serum. The immunoprecipitated material was detected for the presence of 2HA-securin. As seen in Fig. 2*D*, 2HA-securin was present in GSK3β immunoprecipitates but not in normal rabbit IgG precipitates. In the same way, complexes between endogenous securin and GSK3β could also be detected in HeLa cells after coimmunoprecipitation experiments in both directions (Fig. 2, *E* and *F*). Therefore, although protein interaction between kinases and their substrates tend to be transient and are notoriously difficult to detect by pulldown or coimmunoprecipitation experiments, we were able to observe a small but consistent *in vivo* association between securin and GSK3β and observe that the C-terminal part of securin is involved in this interaction.

Securin Is a Substrate for GSK3β—To determine whether GSK3β directly phosphorylates securin, an *in vitro* kinase assay

with purified GSK3β was performed using GST- or His-securin fusion proteins as substrates (Fig. 3*A*). The results show strong phosphorylation of GST-hSec (Fig. 3*B*, *lane 5*) and His$_6$-hSecΔN (Fig. 3*B*, *lane 9*) when compared with phosphorylation of GST-hSec Nter (Fig. 3*B*, *lane 6*) or His$_6$-hSecΔC (Fig. 3*B*, *lane 8*). These results suggested that the residues phosphorylated by GSK3β kinase were localized mainly in the carboxyl-terminal fragment of the protein (residues 164–202). The classic GSK3β consensus motif is (S/T)*XXX*(S/T)*, where *X* is any amino acid and potential GSK3β phosphorylation sites are underlined (39, 40). GSK3β often requires that its substrates first be phosphorylated at a serine or threonine residue in the +4 position relative to the GSK3β phosphorylation site. This residue is referred to as the priming site (indicated by an asterisk). An examination of the amino acid sequence of securin revealed two putative phosphorylation sites in positions ^{183}SS*XX*S*T*^{188}, so it was decided to mutate residues Ser^{183}-Ser^{184} and study the *in vitro* GSK3β phosphorylation of this mutant protein and analyze the effect of GSK3β on its *in vivo* stability. Securin S183A/S184G showed some phosphorylation *in vitro*, probably due to other phosphorylation sites (not shown), but nevertheless, the amount of securin S183A/S184G was higher than that of the wild type securin, suggesting that the mutant protein is more stable (Fig. 3*C*, *lane 3 versus lane 1*). More importantly, knockdown of GSK3β did not affect the level of securin S183A/S184G in contrast with the accumula-
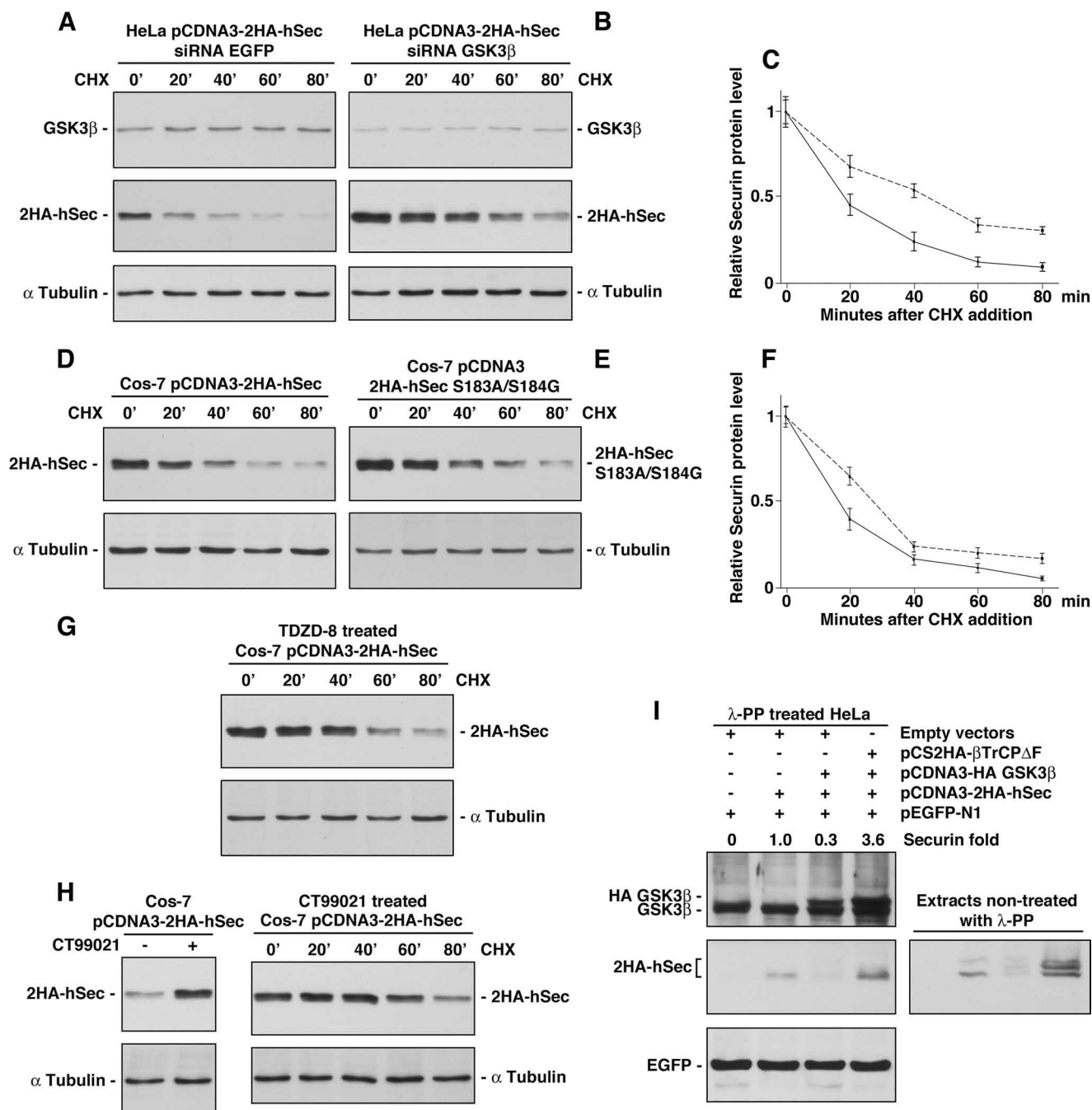
FIGURE 4. **SCF^{βTrCP} mediates the degradation of securin induced by GSK3β.** *A* and *B*, HeLa cells were transfected with pCDNA3-2HA-hSec and interfered with EGFP or GSK3β siRNA and, after 48 h, cycloheximide (*CHX*) was added to the medium, and cells were collected at the indicated times (*n* = 2). Extracts were treated with λ-PP and analyzed by Western blot. *C*, quantification of securin protein levels presented in *A* and *B* using the ImageJ software: HeLa 2HA-hSec siRNA EGFP (*continuous line*) and HeLa 2HA-hSec siRNA GSK3β (*dashed line*). *Error bars* represent the S.D. *D* and *E*, Cos-7 cells were transiently transfected with the indicated plasmids, and expression was induced for 18 h. Later, cycloheximide was added to the medium, and cells were collected at the indicated times (*n* = 3). Extracts were treated with λ-PP and analyzed by Western blot. *F*, quantification of securin protein levels presented in *D* and *E* using the ImageJ software: 2HA-hSec (*continuous line*) and 2HA-hSec S183A/S184G (*dashed line*). *Error bars* represent the S.D. *G* and *H*, Cos-7 cells were transiently transfected with pCDNA3-HA-hSec, and expression was induced for 18 h. Where indicated, TDZD-8 or CT99021 was added 2 h and 30 min later. Cycloheximide was added to the medium, and cells were collected at the indicated times (*n* = 3). Extracts were treated with λ-PP and analyzed by Western blot. The *left panel* of *H* shows the increase of HA-hSec produced by CT99021. *I*, HeLa cells were transiently transfected with the indicated plasmids, and extracts were treated or not treated with λ-PP and analyzed by Western blotting.

tion observed in the case of wild type securin, demonstrating that these residues are a target *in vivo* for this kinase.

Phosphorylation of Securin by GSK3β Promotes SCF^{βTrCP} Ubiquitin-mediated Proteolysis during an Unperturbed Cell Cycle—Some phosphorylated forms of securin are known to be unstable and are degraded by the action of the SCF complex (7).

The question then arose whether GSK3β has a role in the turnover of securin. If this is the case, the GSK3β knockdown is expected to prolong the half-life of securin. As seen in Fig. 4, *B* and *C*, securin was stabilized in cells interfered with GSK3β siRNA. The half-life of securin was ~18 min in cells with active GSK3β (interfered with EGFP siRNA, Fig. 4, *A* and *C*). In con-
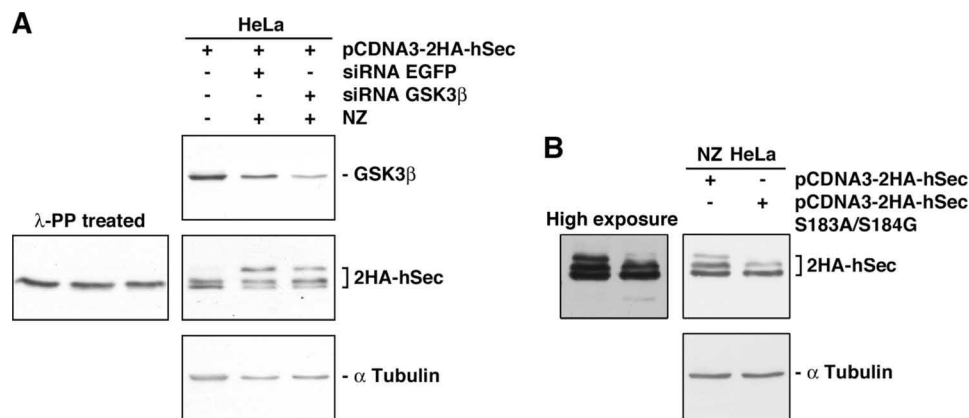
FIGURE 5. **GSK3β contributes to the state of phosphorylation of securin in mitosis.** *A*, HeLa cells were transiently transfected with the indicated plasmid and siRNA, and 24 h later, they were treated with nocodazole (*NZ*) for 24 h (*n* = 2). Where indicated, extracts were treated with λ-PP. Membranes were analyzed by Western blot. *B*, HeLa cells were transiently transfected with the indicated plasmid and treated with nocodazole as above. Extracts were Western blotted for HA and α-tubulin detection.

trast, the half-life of securin was prolonged to 45 min in cells where GSK3β was interfered. Similar results were obtained in cells where GSK3β was inhibited pharmacologically (TDZD-8 or the specific GSK3β inhibitor CT99021 (41) (Fig. 4, *G* and *H*)). Moreover, given that GSK3β knockdown did not alter the amount of securin mutated in residues Ser[183]-Ser[184] (Fig. 3*C*), it was predicted that it would have a longer half-life than wild type securin. This was indeed the case, as shown in Fig. 4, *E* and *F*. The half-life of the S183A/S184G mutant was longer than that of wild type securin (Fig. 4, *D* and *F*).

We previously reported that ultraviolet radiation-induced degradation of securin is mediated by SCF[βTrCP] (15). To know whether this E3 ubiquitin ligase is also responsible for securin degradation promoted by GSK3β phosphorylation in non-irradiated cells, a dominant-negative variant of βTrCP lacking the F-box domain was used (32). Given that the securin level was reduced in cells overexpressing wild type GSK3β (Fig. 1, *C* and *D*), we expected that if βTrCP is involved in this down-regulation, the dominant-negative version would revert the GSK3β effect. As shown in Fig. 4*I*, expression of βTrCPΔF completely avoids the securin fall promoted by GSK3β. These results show that degradation of securin induced by GSK3β phosphorylation along a normal cell cycle is mediated by SCF[βTrCP] ubiquitin ligase.

Regulation of Securin by GSK3β in Mitosis—It is known that the main phosphorylated form of securin appears in cells arrested in metaphase after treatment with nocodazole and is Cdc2-dependent (36). Mitotically phosphorylated securin is observed as a band whose migration is retarded in SDS-PAGE gels. To explore whether GSK3β could also play a role in securin phosphorylation in mitosis, we analyzed the effect of GSK3β knockdown on the pattern of securin phosphorylated bands in cells treated with nocodazole. HeLa cells were transfected with 2HA-hSec and interfered with EGFP or GSK3β siRNA, nocodazole was added after 24 h, and mitotic cells were harvested 24 h later by gentle shake-off. As seen in Fig. 5*A*, knockdown of GSK3β reduced the mitotic securin phosphorylated band level (*third lane*). Confirmation that these bands are phosphorylated forms of securin was obtained by the λ-PP treatment of extracts, in which all the retarded bands were packed in a single band. In fact, as expected, the securin S183A/S184G mutant, a

bad substrate for GSK3β (Fig. 3*C*), did not produce the retarded phosphorylated bands in nocodazole-treated cells (Fig. 5*B*). Together, these results suggest a role for GSK3β in securin phosphorylation in mitosis.

Correlation between GSK3β Inactivation, Securin Accumulation, and Grade of Breast Tumor Malignancy—Securin is overproduced in a wide variety of human cancers, and its accumulation correlates with poor clinical outcome (16–19). Mechanistic insight into the cause of securin accumulation in these cancers is lacking. Given that GSK3β is frequently inactivated in a variety of tumors (39, 40, 42), we tested whether there was a correlation between securin overproduction and GSK3β inactivation using human tumor tissue arrays derived from invasive breast cancers of different histological grades (Fig. 6 and supplemental Table S1). Immunohistochemistry was performed with anti-securin and anti-GSK3β antibodies and an antibody that recognizes GSK3β when it is phosphorylated on serine 9 (phospho-GSK3β), a modification that inactivates GSK3β (39, 40, 42). As control, the specificity of antibodies was tested using securin and GSK3β knockdown experiments in HeLa and HCT116 cells (supplemental Fig. S1). As expected, siRNA hSec down-regulated securin in both immunohistochemistry and Western blot and also in both HeLa and HCT116 cells. Similarly, siRNA GSK3β reduced GSK3β expression. Moreover, siRNA GSK3β also increased the amount of securin in immunohistochemistry. These results confirm the specificity of the antibodies used in Fig. 6.

Fig. 6*A* shows that 35 out of 86 informative tumor tissues stained positive for phospho-GSK3β (40.7%) and that securin was overproduced in 26 out of the 35 tumors containing inactive phospho-GSK3β (*p* < 0.001, using Fisher's exact tests). In contrast, no significant correlation between total GSK3β and securin expression was observed (*p* = 0.7) because most tumors showed strong immunoreactivity for total GSK3β, with only 11 tumors classified as weakly immunostained. Moreover, most inactive phospho-GSK3β tumors had a high proliferative index (21 out of 35, *p* < 0.02) and were G2 or G3 grade (32 out of 35, *p* < 0.02). These findings provide a strong correlation between GSK3β inactivation, securin accumulation, and grade of malignancy in breast cancers.

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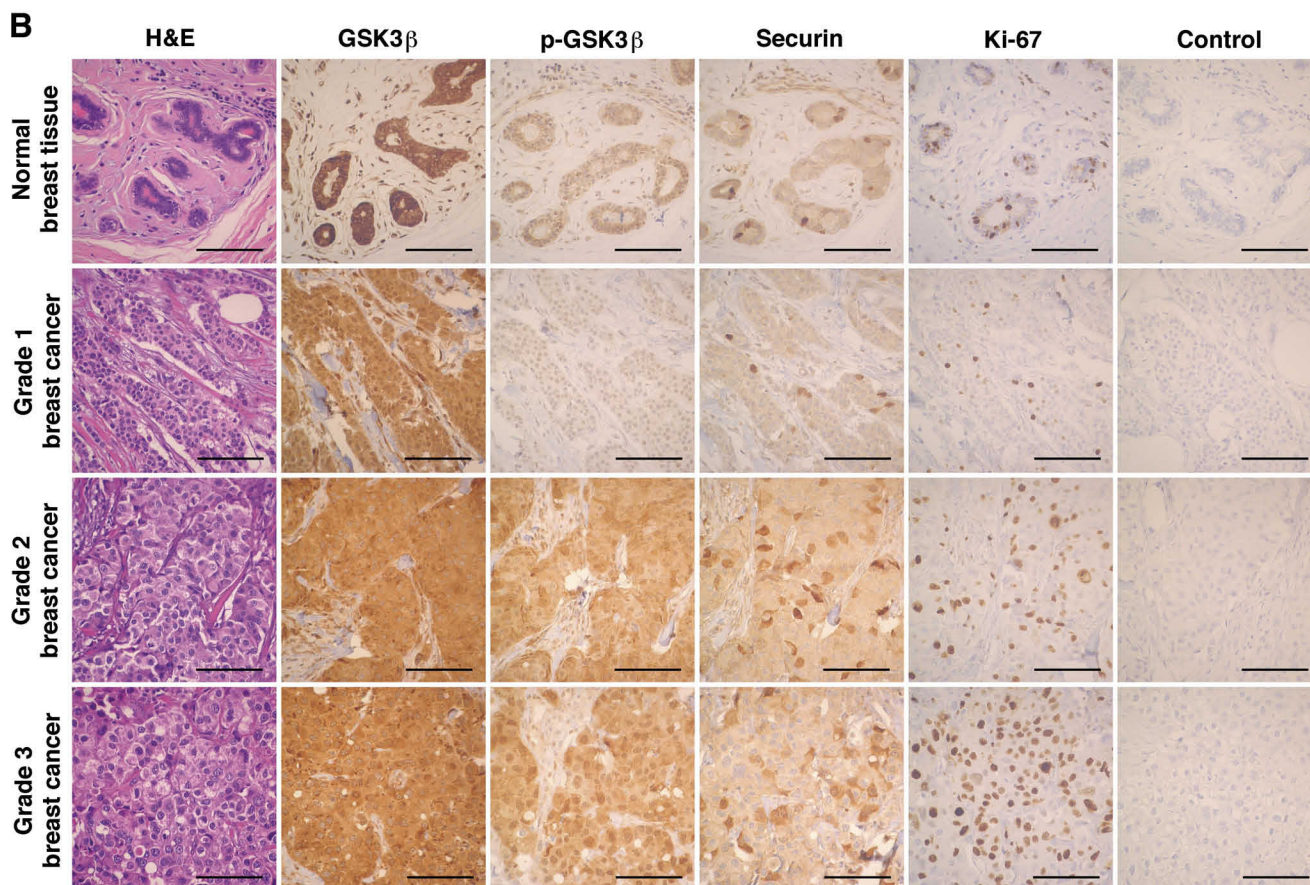|		p-GSK3β		
		Negative	Positive	p-value
Securin	Negative	38	9	< 0.001
	Positive	13	26	
Proliferative index[a]	Low	34	14	< 0.02
	High	17	21	
Tumour grade	G1	16	3	< 0.02
	G2/G3	35	32	

B

FIGURE 6. **Inactivation of GSK3β correlates with accumulation of securin, proliferation index, and grade of malignancy of breast tumors.** *A*, summary of the results obtained from the immunohistochemical staining for phospho-GSK3β (*p-GSK3β*), securin, and Ki67 using a multiple human breast cancer tissue microarray. The proliferative index cut-off value was 20%. *B*, representative examples of GSK3β, phospho-GSK3β, securin, and Ki67 immunostains in normal breast and tumors of different histological grades. *H&E*, hematoxylin and eosin stain. *Control*, absence of primary antibody. *Scale bar*, 100 μm.

DISCUSSION

In this study, we identify GSK3β as a key kinase that regulates the stability of securin. GSK3β phosphorylates securin, promoting its proteolysis via ubiquitylation by the E3 ubiquitin ligase SCF^βTrCP. Importantly, we report a strong correlation between GSK3β inactivation and securin accumulation in breast cancers, suggesting that GSK3β inactivation may account for the prevalence of securin accumulation in human cancers.

The periodic fluctuations observed in securin levels throughout the cell cycle have been attributed to the action of the APC/C ubiquitin ligase. Securin is degraded in metaphase coincident with cyclin B (4, 43), and its proteolysis is controlled by the spindle checkpoint (4). Securin degradation is catalyzed by

both CDC20 and CDH1, is mediated by an R*XX*L destruction box (D-box) and a KEN-box, and is inhibited only when both sequences are mutated (43). Analysis of securin degradation in living cells reveals that it is degraded in metaphase by APC/ C^CDC20, although a securin mutant with a defective D-box but an intact KEN-box is degraded later, in anaphase (4). Furthermore, out of mitosis, the stability of securin depends on its phosphorylation state, such that hyperphosphorylated forms are rapidly destroyed via the SCF E3 ubiquitin ligase (7). The nature of the phosphorylation events that trigger securin degradation is not known.

Securin stability is also markedly reduced after UV radiation (14). We have reported that UV radiation induced the destruc-

tion of securin that is mediated by SCF$^{\beta TrCP}$ ubiquitin ligase and prevented by GSK3 inhibitors, suggesting that this kinase plays a role in regulating securin levels following exposure of cells to DNA damage (15).

In this study, we identify for the first time a kinase that regulates the abundance of securin in non-stressing conditions. Two observations strongly implicate GSK3β in the regulation of securin stability throughout a normal cell cycle. First, decreasing cellular GSK3β activity, as a result of using RNA interference, chemical inhibitors, and a dominant-negative mutant of GSK3β, promotes the accumulation of securin. Second, increasing the cellular levels of GSK3β leads to a reduction in securin levels. One possibility was that the reduction of GSK3β decreased the level of a transcriptional activator of securin. It has been reported that securin is a target of the β-catenin/T cell factor signaling pathway (44, 45), and it is well known that β-catenin is a substrate of GSK3β, promoting its ubiquitylation and proteasome degradation (46, 47). Nevertheless, our results provide evidence that the effect of GSK3β on securin abundance was not a consequence of gene transcriptional activation because it was also observed in stable or transiently transfected cells expressing securin from a constitutive promoter. Moreover, the effect of GSK3β on destabilization of securin was not a consequence of the influence of GSK3β on cell cycle progression. FACS analysis showed that in our experimental conditions, inhibition of GSK3β did not significantly alter the cell cycle profile.

Several observations support the conclusion that GSK3β directly regulates securin stability. First, securin and GSK3β interact both *in vitro* and *in vivo*, and the C-terminal part of securin is involved in this interaction. Second, GSK3β directly phosphorylates securin. Recombinant securin was phosphorylated by GSK3β in an *in vitro* kinase assay, GSK3β overexpression provoked *in vivo* securin phosphorylation in cells in which proteasomal degradation was inhibited, and knockdown of GSK3β altered the phosphorylation pattern of securin in cells arrested in mitosis. Finally, a mutated form of securin that was insensitive to knockdown of GSK3β kinase in asynchronous cells had a phosphorylation pattern altered in mitosis and a longer half-life. Bioinformatics analysis revealed the presence of several putative GSK3β phosphorylation sites in the securin protein, two of them localized in the carboxyl-terminal moiety of the protein, serines 183 and 184. As the experiments of phosphorylation *in vitro* demonstrated that this fragment was preferentially phosphorylated by GSK3β, we decided to mutate Ser^{183} and Ser^{184} to alanine and glycine, respectively. The S183A/S184G securin mutant did not accumulate in cells interfered for GSK3β. The pattern of *in vivo* phosphorylation of this mutant was altered in cells arrested in mitosis and, consistent with this, it was similar to the phosphorylation pattern of wild type securin in cells interfered for GSK3β. GSK3β promotes the turnover of securin *in vivo* and, as expected for a mutant in a residue target for this kinase, the half-life of securin S183A/S183G was longer than that of the wild type securin. Taken together, these results indicate that in an unperturbed cell cycle, securin stability is controlled by GSK3β phosphorylation in serines 183 and/or 184. However, we cannot exclude that GSK3β phosphorylates other additional residues of securin. In fact, the half-life of wild type securin in cells where GSK3β was chemically inhibited or interfered by siRNA was even longer than that of the S183A/S184G mutant.

The physiological significance of GSK3β securin regulation after UV irradiation and during a normal cell cycle remains to be elucidated. There is accumulative knowledge about the rigorous control of securin level in the cell, regulated not only by the APC/C during metaphase-to-anaphase transition but by the SCF at other cell cycle stages. Unsurprisingly, we speculated about the possibility that securin has additional roles to that of its function as an inhibitor of separase. In line with this, it is interesting to mention results obtained in mouse oocytes revealing a role for securin at the G$_2$ to M transition suggesting a more general mechanism whereby physiological levels of co-competing APC/C substrates function in modulating the timing of cell cycle transitions (48).

Until now, pathways leading to securin accumulation in human cancers have not been identified. Importantly, GSK3β inactivation is a common occurrence in human cancers, and we observed significant correlation between GSK3β inactivation and securin accumulation in breast cancers. Indeed, 40.7% of breast cancer tissues examined contained inactive GSK3β. Interestingly, 74.9% of tissues containing inactive GSK3β also contained high levels of securin, supporting the conclusion that derailment of GSK3β signaling correlates with the accumulation of securin in a subset of breast cancers.

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