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# Securin and Separase Modulate Membrane Traffic by Affecting Endosomal Acidification

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Securin and separase play a key role in sister chromatid separation during anaphase. However, a growing body of evidence suggests that in addition to regulating chromosome segregation, securin and separase display functions implicated in membrane traffic in Caenorhabditis elegans and Drosophila. Here we show that in mammalian cells both securin and separase associate with membranes and that depletion of either protein causes robust swelling of the trans-Golgi network (TGN) along with the appearance of large endocytic vesicles in the perinuclear region. These changes are accompanied by diminished constitutive protein secretion as well as impaired receptor recycling and degradation. Unexpectedly, cells depleted of securin or separase display defective acidification of early endosomes and increased membrane recruitment of vacuolar (V-) ATPase complexes, mimicking the effect of the specific V-ATPase inhibitor Bafilomycin A1. Taken together, our findings identify a new functional role of securin and separase in the modulation of membrane traffic and protein secretion that implicates regulation of V-ATPase assembly and function.

Key words: endosome, Golgi, membrane, pH, secretion, securin, separase, tumor, vesicle, V-ATPase

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Traffic

Separase is an evolutionally conserved proteolytic enzyme that ensures chromosome segregation during anaphase by cleaving the cohesin complex (1). Its activity is controlled by securin (pituitary tumor transforming gene 1, PTTG1), a member of a divergent class of anaphase inhibitors whose proteosomal degradation by the anaphase promoting complex (APC) is required to release separase and allow its activation (2,3). Numerous observations, however, suggest that each molecule is functionally implicated in other cellular events. Thus, securin is thought to participate in transcriptional regulation (4), DNA repair (5) and tumor promotion (6), whereas separase is proposed to orchestrate spindle dynamics during anaphase as well as cytokinesis (7). Emerging evidence indicates that both molecules may also play a potentially important role in membrane traffic and secretion (6,8–10).

Mammalian securin was discovered in pituitary tumors where hormonal secretion is aberrant (11), and has itself been shown to be secreted in human pituitary adenomas (10). Moreover, pituitary glands of transgenic mice engineered to overexpress securin display focal hyperplasia with a prominent Golgi apparatus and an increased number of secretory vesicles (12). Consistent with these observations, securin overexpression in cell lines is reported to enhance secretion of angiogenic factors including vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 (MMP-2) (13,14). Together, these observations support a role for securin in the regulation of protein secretion but the underlying mechanisms have yet to be defined.

Similar to the potential involvement of securin in secretion, several lines of evidence implicate separase in the control of membrane traffic in *Caenorhabditis elegans* and Drosophila. In C. elegans, separase has been found to regulate cortical granule exocytosis (8). It was also shown to control trafficking of Rab11-positive vesicles during cytokinesis and their incorporation into the plasma membrane at the cleavage furrow and midbody (9). In Drosophila, a functional genomic screen identified separase as a candidate gene required for constitutive protein secretion and Golgi organization (15). Thus, in addition to its role in cleavage of cohesin and chromosome segregation, separase appears to have an evolutionarily conserved function in membrane traffic and protein secretion that may be important for both normal cell physiology and oncogenesis. In light of these observations, we addressed the molecular mechanisms whereby the securin/separase complex might affect membrane trafficking in mammalian cells.

#### Results

### Securin and separase associate with membranes in mammalian cells

We have observed securin and separase to be abundantly expressed within the cytoplasm of a broad range of human tumor cell lines, including MDA-MB-231 breast carcinoma cells (Figure S1A,B, and data not

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shown). Their cytoplasmic localization outlines the biosynthetic/secretory pathway, as shown by colocalization of both molecules with markers specific for the endoplasmic reticulum (ER) (Calnexin), the cis-Golgi (GM130, giantin) and trans-Golgi network (TGN) (TGN46), as well as early endosomes (EEA1), (Figure S2A,B). Strong anti-securin and anti-separase antibody reactivity was also detected beneath the plasma membrane as early as 30 min following cell attachment to culture plates and was maintained in subconfluent cell cultures but was lost from cells that reached confluence (Figure S1C, and data not shown). Specificity of anti-securin and anti-separase antibody staining was validated by the observation that siRNA-mediated depletion of either protein, as assessed by western blot analysis, resulted in the loss of immunofluorescent staining of cells by the corresponding antibody (Figure S1D,E). Cell fractionation experiments confirmed securin and separase presence in the nucleus, but also demonstrated for the first time association of both molecules with mammalian cell membranes (Figure S1F). The subcellular distribution of securin and separase is thus consistent with as yet undiscovered membrane-associated functions.

## Securin and separase depletion causes TGN and endosome swelling independent of cell cycle

Based on these observations we addressed the possible implication of securin and separase in the regulation of membrane traffic in mammalian cells. Greater than 90% reduction of securin expression was achieved using any one of three different securin-specific siRNA oligonucleotide sequences as early as 24 h following transfection and remained stable for more than 72 h (Figure 1A,B). Comparably effective separase depletion was obtained using three different separase-specific siRNA sequences (Figure 1B). All subsequent morphological and functional studies were performed 72 h following siRNA oligonucleotide transfection, as depletion of both molecules was found to be maximal at this time-point (Figure 1A,B). Transient securin and separase depletion profoundly affected the morphology of early and late endosomes, the medial-Golgi and the TGN (Figure 1C). Immunofluorescence analvsis of EEA1- and Rab5-positive endosomes 72 h following either securin or separase siRNA transfection uncovered aberrantly enlarged early endosomes that clustered in the perinuclear region in 40-50% of cells (Figure 1C). Quantification of the observed endosomal changes revealed a significant increase in early-endosome size paralleled by a comparably significant decrease in their number (Figure 1D), possibly reflecting aberrant endosome fusion. The medial-Golgi and TGN, identified by anti-Mannosidase II (data not shown) and anti-TGN46 antibody staining, respectively, along with M6PR-positive late endosomes also underwent robust swelling (Figure 1C). By contrast, the cis-Golgi compartment and the ER remained unaltered, as assessed by anti-GM130 and anti-giantin antibody staining of the cis-Golgi, and anti-Calnexin, anti-Bip and anti-KDEL antibody staining of the ER (Figure 1C, and data not shown).

The observation that depletion of either securin or separase results in TGN and endosome swelling raises the possibility that the two molecules may function together, similar to the manner in which they control chromosome segregation. Consistent with the observations by others (16), we found that depletion of securin results in decreased separase expression at the protein level (Figure S3A). By contrast, separase depletion did not affect securin expression (Figure S3B), supporting the notion that the effector function whose loss leads to the observed phenotype may be primarily provided by separase.

Because of their implication in the control of cell division and the recent suggestion that separase mediates regulation of membrane traffic during cytokinesis (8,9), we addressed the possibility that the observed effects of securin and separase depletion are cell cycle dependent. However, double-immunofluorescence staining using the anti-phospho-Histone H3 Ser10 antibody (a marker of mitosis) in combination with antibodies against TGN46, M6PR and the early-endosome markers Rab5 and EEA1 failed to reveal any dependence of the observed TGN swelling and giant endosome formation on mitosis (Figure S3C,D). Moreover, swollen TGN and giant endosomes appeared irrespective of the cell cycle phase, and were also observed in securin- and separase-depleted, serumstarved, G<sub>0</sub>-synchronized cells (data not shown). Thus, in mammalian cells, securin and separase regulate TGN and endosome morphology independent of cell cycle.

# Securin and separase depletion impairs constitutive protein secretion and endocytic degradative and recycling pathways

We next addressed the functional implication of securin and separase depletion-induced TGN and endosome swelling by assessing the efficacy of constitutive protein secretion and receptor-mediated endocytosis, respectively, in cells depleted of either protein. To assess protein secretion, MDA-MB-231, Hela, HEK293T and HepG2 cells were transfected with a cDNA encoding horse-radish peroxidase (HRP) engineered to include an N-terminal basolateral sorting signal (ss-HRP) (15), and HRP activity released into the culture medium and retained in cells was measured by chemiluminescence. Securin and separase depletion led to a significant reduction of HRP secretion paralleled by a corresponding increase in intracellular levels in all four cell lines (Figure 2A, and data not shown). Thus, swelling of the TGN caused by securin or separase depletion is associated with decreased constitutive protein secretion.

Endosome-mediated receptor degradation and recycling were also significantly impaired by securin and separase depletion (Figure 2B,C). Receptor internalization was comparable in control and securin- and separasedepleted cells, as demonstrated by Alexa594-labeled EGF colocalization with EEA1-positive early endosomes in all samples after 20 min of incubation (Figure 2B). However, whereas delivery of EGF-Alexa594 from early to









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late endosomes/lysosomes was clearly visible at 1.5 h in control cells (Figure 2B), and almost completely cleared by 5 h (not shown), securin- or separase-depleted cells displayed delayed early to late endosome/lysosome delivery and impaired clearance of EGF-Alexa594. Remarkably, while EGF-Alexa594 fluorescence was no longer detectable in early (EEA1) and late (LAMP1) endosomes in vsv-g-siRNA-treated control cells 12 h following uptake, it was retained in both compartments in securinand separase-depleted cells (Figure 2B, 12 h, EEA1 and LAMP1). Similarly, internalization of Alexa488-labeled transferrin that is sorted for recycling was comparable at early time-points in control and securin/separasedepleted cells (Figure 2C). However, receptor recycling from early endosomes to the plasma membrane was significantly delayed in securin- and separase-depleted cells as shown by retention of Alexa488-transferrin fluorescence in early endosomes for more than 12 h following uptake (Figure 2C). Thus, tracking of receptors destined for degradation or recycling revealed that whereas early endocytic events were unperturbed by securin and separase depletion, later stages of cargo sorting for degradation and recycling were significantly delayed.

#### Rab5 activity and autophagy are not the underlying mechanisms of securin and separase depletion-induced formation of giant endosomes

Vesicle morphology and function are regulated by the activity of small GTPases of the Rab family (17). Among Rab family members, Rab5 plays a central role in regulating transport between the plasma membrane and sorting endosomes and promoting homotypic endosome fusion (18). The constitutively active, GTP-associated, Rab5 mutant (Rab5 Q79L) induces early-endosome fusion resulting in giant endosome formation and inhibition of transferrin recycling (19), whereas its constitutively inactive counterpart (Rab5 S34N) prevents fusion (19). Based on the similarity of the endosome phenotype observed upon securin and separase depletion to that associated with constitutively active Rab5, we assessed the amount of active, GTP-loaded Rab5 in cells depleted of securin and separase but found no increase compared to control cells (data not shown). Furthermore, comparison of

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early-endosome size in control and securin- or separasedepleted MDA-MB-231 cells stably expressing Rab5 Q79L, Rab5 S34N or empty vector revealed that securin or separase depletion caused giant endosome formation not only in cells expressing empty vector and Rab5 Q79L (as might be expected), but also in those expressing Rab5 S34N (Figure S3E). These observations strongly suggest that mechanisms independent of Rab5-mediated endosome fusion underlie securin- and separase-depletioninduced endosome enlargement. Because the observed giant endosomes may resemble autophagosomes that arise in a variety of pathological conditions, we addressed autophagy as a possible explanation for the phenotype induced by securin and separase depletion. However, TGN swelling and giant endosome formation occurred in securin and separase-depleted MDA-MB-231 cells stably silenced for Atg5 and Atg7, two key proteins in autophagosome formation (20) (Figure S3F,G). We also monitored cellular distribution of the autophagy marker LC3 (21) between the cytoplasm and giant endosome membranes, but could not detect a significant difference between control and securin- or separase-depleted cells (data not shown). These observations rule out autophagy as an explanation of the vesicular phenotype observed in response to securin and separase depletion.

## Securin and separase depletion causes defective endosome acidification

Having excluded the implication of Rab5 and autophagy, we addressed changes in vesicular pH as a candidate explanation for the observed endosomal phenotype, given that efficient acidification is required for the maintenance of endosome morphology and function (22,23). We first assessed early-endosome pH in control and securin and separase-depleted cells by direct measurement using the pH-sensitive fluorescein isothiocyanate (FITC) probe conjugated to EGF (see *Materials and Methods*). Similar to receptor degradation assays, EGF-FITC was first bound to its receptor (EGFR) on the plasma membrane at 4°C and the receptor–ligand complex was then allowed to internalize by incubating the cells at 37°C. Live-cell imaging was performed within 20 min following ligand–receptor complex internalization to allow pH assessment of the

**Figure 2:** Securin or separase depletion impairs (A) constitutive protein secretion, (B) receptor degradation and (C) recycling pathways. A) HRP activity determined in cell supernatants of MDA-MB-231, Hela, HepG2 and HEK 293T cells 72 h post-transfection of siRNA oligonucleotides targeting vsv-g (control), securin and separase. Bars indicate the mean  $\pm$  SD of HRP activity secreted in the culture medium normalized to total cellular protein content of experiments performed at least in triplicate. Significance of the statistical analysis, as performed by the Student's *t*-test, is denoted as follows: \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. B) Representative images of endosome degradative pathway monitored by internalization of EGF-Alexa594 complexed to EGFR in control and securin-and separase-depleted MDA-MB-231 cells 72 h post-siRNA transfection. Securin or separase depletion does not affect surface binding of EGF-Alexa594 or early steps of endocytosis as shown by plasma membrane staining (T0, red) and colocalization (yellow) of EGF-Alexa594 (red) with EEA1 (green) at 20 min following uptake. Securin and separase depletion delays delivery of EGF-Alexa594 from early EEA1-positive to late LAMP1-positive late endosomes as well as clearing of cargo from the latter in EEA1- and LAMP1-positive endosomes (green) in securin- and separase-depleted cells (inset). C) Similar to EGF, tracking of Transferrin-Alexa488 that is sorted for recycling displayed significant delay of receptor recycling from EEA1-positive early endosomes to plasma membrane in securin- and separase-depleted cells compared to controls. Note the retention of Transferrin-Alexa488 (green) in EEA1-labeled early endosomes (red) 12 h following uptake in securin- and separase-depleted cells compared to controls. Sole bar = 6  $\mu$ m.



Figure 3: Legend on next page.

early-endosomal compartment. A series of images were taken at 488 and 440 nm that correspond to pH-sensitive and pH-insensitive wavelengths of the FITC probe, respectively, and absolute pH values were obtained by calibration of the 488/440 nm ratio curve using the nigericin K<sup>+</sup> method, as described in *Materials and Methods*. pH measurement of early endosomes using this approach revealed an increase from 6.1 to 6.4 in both securinand separase-depleted cells (Figure 3A,B), supporting the notion that the observed impaired receptor recycling and degradation are due, at least in part, to defective endosomal acidification.

## *Perturbation of endosomal pH recapitulates the phenotype induced by securin and separase depletion*

To determine whether a change in pH may be responsible for the TGN and endosome swelling observed upon securin and separase depletion, cells were subjected to treatment by the macrolide antibiotic Bafilomycin A1 or NH<sub>4</sub>Cl. At nanomolar concentrations, Bafilomycin causes highly specific inhibition of vacuolar (H<sup>+</sup>)-ATPases (V-ATPases) that translocate protons across endosomal membranes and ensure endosomal acidification (23). Administration of NH<sub>4</sub>Cl, on the other hand, directly leads to endosome alkalinization. Similar to securin or separase depletion, inhibition of V-ATPase using Bafilomycin A1 and endosome alkalinization by NH<sub>4</sub>Cl resulted in the formation of giant EEA1- or Rab5-positive early endosomes that clustered in the perinuclear region, together with swelling of the TGN (TGN46) and M6PR-positive late endosomes (Figure 4A). A robust increase in early-endosomal pH, that surpassed 7.3 and could therefore not be precisely assessed because of the flattening of the sigmoidal curve beyond this value (24), (data not shown), paralleled by impaired constitutive protein secretion (Figure 4B) were also observed. Taken together, these data strongly suggest that the increase in endosomal pH associated with

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securin and separase depletion may underlie the observed changes in vesicle morphology and membrane trafficking.

## Securin and separase regulate membrane association of vacuolar ATPase domains

Having established that defective endosome acidification constitutes a common denominator of impaired endosomal morphology and function resulting from securin or separase depletion and V-ATPse inhibition, we addressed the possibility that securin and separase might participate in the regulation of V-ATPase activity. V-ATPases are large multisubunit complexes organized into a peripheral domain (V1), located on the cytoplasmic side of the membrane and responsible for ATP hydrolysis, and a membrane-embedded domain  $(V_0)$  composed of proteolipid and protein subunits that carries out proton transport across the membranes to the lumen or extracellular space. Regulation of V-ATPase activity is complex, and only partially understood (23), but there is substantial evidence to suggest that dissociation or inappropriate association of V<sub>1</sub> and V<sub>0</sub> domains can cause or accompany abrogation of V-ATPase function. Thus, glucose deprivation leads to release of the V1 domain into the cytosol (23) and loss of V-ATPase activity. By contrast, V-ATPase inhibition induced by alterations in endosomal membrane cholesterol content (25) or by the selective V-ATPase blocker salicylhalamide (26) is associated with redistribution of the cytosolic V1 domain from the soluble to the membranebound form. We, therefore, addressed possible changes in V-ATPase subunit distribution in response to securin and separase depletion. Post-nuclear supernatants (PNSs) of control and securin- or separase-depleted cells were subjected to high-speed ultracentrifugation to separate the membranes (MB) from the cytosol and assessed for the MB/cytosol distribution of  $V_1A_1$  and  $V_0d_1$  subunits. The  $V_1A_1$  subunit is representative of the  $V_1$  domain, whereas the V<sub>0</sub>d<sub>1</sub> subunit, although being part of the V<sub>0</sub> domain, is not a transmembrane protein (23) and may therefore

Figure 3: Depletion of securin and separase causes a decrease in endosome acidification and alters V-ATPase subunit association with membranes. A) Quantification of early-endosome pH determined by pH measurement in control and securin- and separase-depleted cells. EGF-FITC was bound to EGFR at 4°C and the complex internalized for 10 min at 37°C. Cells were imaged during the following 10 min to allow assessment of early-endosome pH. Absolute pH values were obtained by extrapolation of the calibration curve (Methods). The significance of differences between samples was established using the Student's t-test for unpaired samples (\*\*\*p < 0.001). B) Histograms showing the distribution of the pH values in (n = 108) control (n = 83), siSecurin and (n = 83) siSeparase early endosomes, respectively. Note the increase of the median pH from 6.1 (controls) to 6.4 in securin- and separase-depleted cells. Experiments were repeated three times. C) Assessment of membrane association of vacuolar ATPase domains in MDA-MB-231 cells 72 h after securin and separase depletion. MDA-MB-231 cells were cooled on ice, scraped and mechanically disrupted (*Methods*). PNSs were subjected to high-speed ultracentrifugation for the preparation of membrane (MB) and cytosol. Equal amounts of protein (30 µg) were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and blotted using antibodies recognizing the V1A1 and V0d1 V-ATPase subunits. Quantification of bands was performed by densitometry using IMAGEJ and results expressed as an MB to cytosol ratio (for the V<sub>1</sub>A<sub>1</sub> subunit) and an MB to PNS ratio (for the V<sub>0</sub>d<sub>1</sub> subunit), left panel. Error bars denote standard deviation of three independent experiments. Right panels: representative western blots displaying distribution of V1A1 and V0d1 V-ATPase subunits in si-vsv-g-treated cells (control) and securin- and separase-depleted cells used for assessment of V-ATPase assembly with IMAGEJ. Note the decrease of V1A1 and V0d1 V-ATPase subunits in PNS following securin and separase depletion. D) Assessment of membrane association of vacuolar ATPase domains 72 h after securin and separase depletion in serum- and glucose-deprived MDA-MB-231 cells. To induce V-ATPase disassembly, cells were serum- and glucose-deprived for 2 h before mechanical disruption and cell fractionation performed as in (C). Right panels: representative western blots displaying distribution of V1A1 and V0d1 V-ATPase subunits used for assessment of V-ATPase assembly.



**Figure 4: Endosome swelling in response to Bafilomycin A1 and NH4CI treatment.** Effect of Bafilomycin A1 and NH<sub>4</sub>CI treatment on (A) early endosome (EEA1), TGN (TGN46), late endosomes/lysosomes (M6PR) morphology, and (B) constitutive protein secretion in MDA-MB-231 cells treated with 100 nm Bafilomycin A1 for 48 h or 10 mm NH<sub>4</sub>CI for 72 h. Note the formation of giant early and late endosomes, swollen TGN and impaired protein secretion (ss-HRP assay). Scale bar = 8  $\mu$ m.

dissociate from the membrane-bound domain, similar to V<sub>1</sub>. In control cells the V<sub>1</sub>A<sub>1</sub> subunit was predominantly found in the cytosol with an MB/cytosol ratio of 0.6. Interestingly, this distribution was reversed upon securin and separase depletion, resulting in a 2- and 1.5-fold increase in the MB/cytosol ratio, respectively (Figure 3C). In addition to the reversal of the membrane to cytosol ratio of the V1A1 subunit, its total protein level in PNS was decreased (Figure 3C). Contrary to V1A1, the V0d1 subunit was entirely membrane-associated but similar to the V1A1 subunit, its total protein level decreased in PNS, whereas it remained constant in membranes, shifting the membrane to PNS ratio from 0.7 in controls to 2.3 and 1.7 in securinand separase-depleted cells, respectively (Figure 3C). The decrease in PNS levels of V-ATPase subunits was found to be because of a decrease in transcription of the corresponding genes (data not shown). Interestingly, the same decrease in V-ATPase subunit was observed upon treatment of cells with bafilomycin and NH<sub>4</sub>Cl (data not shown). Although the mechanism underlying the decrease in V-ATPase subunits is not currently known, it is conceivable that alkalinization of vesicles generates signals that are directly or indirectly responsible for the corresponding gene silencing. To determine the robustness of securin and separase depletion-dependent V-ATPase domain redistribution to membranes, we assessed the effect of separase and securin depletion in the context of forced V-ATPase dissociation by glucose deprivation. Control and securin- or separase-depleted cells were therefore starved of glucose and the V<sub>1</sub>A<sub>1</sub> and V<sub>0</sub>d membrane to cytosol or PNS ratio were determined. As expected, glucose depletion caused dissociation of V-ATPase with detachment of both V<sub>1</sub>A<sub>1</sub> and V<sub>0</sub>d subunits from the membrane (Figure 3D). However, whereas V<sub>0</sub>d detachment was observed in control cells subjected to glucose starvation (Figure 3D), its detachment in glucose-starved cells depleted of securin or separase was negligible (Figure 3D), raising its MB/PNS ratio to nearly 6 (Figure 3D). Similar observations were made for V<sub>1</sub>A<sub>1</sub>, with the MB/cytosol ratio between 4 and 5 (Figure 3D).

The reason for the observed decrease of  $V_1A_1$  and  $V_0d_1$ subunits in PNS following securin and separase depletion is unclear. However, the same observation was made upon Bafilomycin and NH<sub>4</sub>Cl treatment (data not shown) and it is remarkable that despite the decrease in their total protein level in PNS the membrane-associated fraction of both subunits remained constant. It is possible that securin and separase depletion-induced inhibition of V-ATPase dissociation activates a feedback loop to decrease expression of its subunits. Consistent with this possibility, a decrease in V<sub>1</sub>A<sub>1</sub> and V<sub>0</sub>d<sub>1</sub> transcripts was detected as early as 16 h following siRNA-mediated separase depletion (data not shown). Whatever the mechanism that underlies the observed reduction in  $V_1A_1$  and  $V_0d_1$ transcripts may be, the increase in the MB/cytosol and MB/PNS ratio of the two subunits in both resting conditions and upon forced V-ATPase dissociation by glucose starvation indicates that securin and separase depletion alters V-ATPase subunit distribution.

#### Discussion

Our observations provide the first evidence that securin and separase, best known for their role in the control of chromosome segregation in anaphase, are associated with mammalian cell membranes. Importantly, their depletion causes not only marked changes in TGN and endosomal morphology, but decreases constitutive protein secretion as well as endosomal receptor recycling and degradation. The implication of securin and separase in membrane trafficking is shown not to be cell cycledependent, suggesting a general role in the regulation of constitutive secretion and receptor recycling. Such a role may be important in both normal and malignant cells, where securin expression has shown to correlate with tumor progression (27). Efficient protein transport, secretion and growth factor receptor recycling are required for malignant cell growth and survival, and it will be of interest to determine to what degree securin- and separase-mediated control of membrane trafficking contributes to tumor cell growth and success in subverting their microenvironment.

We have identified a candidate mechanism whereby separase and securin participate in membrane trafficking as the regulation of vesicular acidification based on the finding that depletion of either molecule results in increased vesicular pH. The observed increase in pH of 0.3 units appears to be sufficient for biologically relevant changes as illustrated by observations that an increase in pH ranging between 0.3 and 0.9 units suffices to delay transferrin cycling (28-30). Vesicle alkalinization, either by NH<sub>4</sub>Cl treatment or by Bafilomycin-mediated V-ATPase inhibition, induces morphological and functional TGN and endosome changes that closely mimic those resulting from securin and separase depletion, strongly supporting this notion. Because V-ATPase provides the key physiological mechanism of endosome acidification, it would seem reasonable to assume that the defective acidification observed in the absence of securin and separase is related to altered V-ATPase function. In support of this view, we found that in the absence of either securin or separase, V-ATPase subunits undergo redistribution to the membranes, a change that has been associated with alteration or loss of proton pump function (23). Exactly how securin and separase may regulate a molecule as complex as V-ATPase remains to be determined and will require substantial additional work. Nevertheless, our observations have uncovered a function of securin and separase that will be worthwhile elucidating and that may help understand their implication in membrane trafficking in mammalian cells.

Interestingly, recent work in *C. elegans* has shown that separase localizes to the ingressing furrow and midbody

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during cytokinesis and that its depletion causes failure of cytokinesis that is not because of eggshell defects or chromosome nondisjunction. Moreover, depletion of separase caused the accumulation of Rab11-positive vesicles at the cleavage furrow and midbody that was not a consequence of chromosome missegregation, but that was mimicked by depletion of the vesicle fusion machinery (9). Earlier work had shown separase to localize to cortical granules during anaphase of the first meiotic division and to play a direct role in regulating cortical granule exocytosis in response to fertilization (8). Together, these observations suggest that in C. elegans separase may participate in regulating cytokinesis by ensuring appropriate vesicle delivery and fusion to the cell membrane that is required for cytokinesis to proceed normally (31). In mammalian cells, depletion of separase is not reported to cause defective cytokinesis and we did not observe giant vesicle accumulation at the cleavage furrow of dividing cells depleted of either separase or securin. It would therefore seem that in mammalian cells securin/separase-mediated control of chromosome segregation and membrane trafficking may proceed as parallel but not necessarily interdependent events.

Taken together, our observations provide evidence of an unsuspected implication of securin and separase in membrane traffic in mammalian cells. The present identification of a candidate mechanism whereby they participate in membrane traffic control sheds new light onto securin and separase and opens new avenues to explore the full spectrum of their functions.

#### **Materials and Methods**

#### Cells, reagents and antibodies

MDA-MB-231 and Hela cells were maintained in DMEM (Gibco #31966), supplemented with 10% fetal calf serum (FCS) at 37°C in a 5% CO2 incubator. Transferrin-Alexa Fluor 488 (T13342) and biotin-EGF (E-3477) were from Molecular Probes (Invitrogen), Bafilomycin A1 from Sigma (B1793). Rabbit anti-securin antibody (34-1500) was from Zymed Laboratories (Invitrogen), mouse anti-securin (ab3305), mouse anti-separase (ab16170), rabbit anti-separase (ab3762 and ab52158), mouse anti-Mannose 6 phosphate receptor cation independent (M6PR) (ab2733), rabbit anti-giantin (ab24586), mouse-anti-KDEL (ab2223), and rabbit anti-ATP6V $_0d_1$  (ab56441) antibodies were from Abcam, rabbit anti-TGN46 (NB110-40769) from Novus Biologicals, mouse anti-GM130 (611434) and mouse anti-EEA1 (610456) from BD Transduction laboratories (BD Biosciences), mouse anti-phospho-Histone H3 (Ser10) (#9706) and Stress antibody sampler kit (#9956) were from Cell Signaling Technology Inc., rabbit-anti ATP6V1A from Novus Biologicals (NBP1-33021) and mouse anti-v5 from Invitrogen (# 46-0705). Alexa Fluor 488- and 594-conjugated secondary antibodies were from Molecular Probes and Streptavidin-FITC from Dako.

#### Immunofluorescence microscopy

Cells grown on coverslips were fixed 24–72 h post-transfection with paraformaldehyde (PFA) 4% for 20 min at room temperature, washed, incubated with 50 mm NH<sub>4</sub>Cl for 10 min and permeabilized with 0.01% Triton X-100 in blocking buffer (PBS–FCS 10%) for 5 min. Fixed cells were incubated with primary antibodies diluted in blocking buffer for 45 min at room temperature, washed, incubated with diluted secondary antibodies for 45 min, washed, and mounted on glass support slides

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using Immu-Mount (Thermo Shandon, #9990402). DAPI (4',6-diamidin-2-phenylindol dihydrochloride, Roche) was used to visualize the nuclei. Confocal images were acquired on a Leica SP5 AOBS Laser-Scanning Confocal Microscope with a 100 × oil-immersion objective. Data were acquired using Leica Application Suite (LAS) software. ADDRE PHOTOSHOP 6.0 (Adobe Systems) was used to process all images. IMARIS 6.3.1 (Bitplane) was used to determine protein colocalization using Pearson's correlation. IMAGE J software (Image processing and analysis in Java) was used to calculate vesicle area as described in http://rsb.info.nih.gov/ij/docs/menus/analyze.html. At least 100 cells were analyzed for each sample.

#### siRNA oligonucleotide transfection

siRNA oligonucleotides (Qiagen) were transfected using Oligofectamine (Invitrogen). Transient securin and separase downregulation was achieved using three different oligonucleotides targeting the coding region (cds), the 5' and/or 3' untranslated gene regions (UTR). siRNA oligonucleotides targeting securin (PTTG1) were GCATTCTGTCGACCCTGGA (cds), AACGTTC GATGCCCACCA (cds) and GACCTGCAATAATCCAGAA (5' UTR). For separase, GCTTGTGATGCCATCCTGA (cds), GTCCTGTACCTAGGAAAGA (5' UTR), GCCTCATAACTGTTCTACC (3' UTR). siRNA oligonucleotides targeting the vsv-g (AAAGGAAACTGGAAAATG) and AllStars Negative Control siRNA (cat. num. 1027281) were used as control. Cells were transfected with annealed siRNA oligonucleotides (100 nm final concentration) and the knockdown efficiency determined by immunoblotting 24–72 h post-transfection.

#### **Cell fractionation**

Seventy-two hours after siRNA transfection, MDA-MB-231 cells were cooled on ice for 5 min, rinsed twice with cold PBS, scraped, centrifuged for 5 min at 300g and resuspended in homogenization buffer (HB, 250 mm sucrose, 3 mm imidazole pH 7.4). PNS was obtained by mechanical disruption of cells with a 22-G needle and centrifugation at 600g for 10 min. MB and cytosol were obtained from PNS subjected to high-speed ultracentrifugation (100 000 × g, 45 min) in a TLA120.2 rotor (Beckmann Coulter Ultracentrifuge). All steps were performed at 4°C. Equal amounts of proteins (30 µg) for all fractions were resolved on SDS-PAGE gradient gels and transferred onto a nitrocellulose membrane. Nitrocellulose membranes were blotted using anti-ATP6V0d1 and anti-ATP6V1A1 antibodies and the intensity of the bands was quantified by densitometry using IMAGE J program. Results are expressed as the ratio of the band intensities between MB/Cytosol and MB/PNS for the ATP6V1A1 and ATP6V0d1, respectively, in control and securin- and separase-depleted cells.

#### Western blot

Twenty-four to seventy-two hours after siRNA transfection, cells were placed on ice for 5 min, washed using ice-cold PBS, and lysed using 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Triton X-100, supplemented with 1 mM EDTA, 1 mM β-glycerolphosphate, 1 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and complete protease inhibitors cocktail (Roche) for 20 min on ice. Cell lysates were centrifuged for 5 min at 1200g to pellet the nuclei and cell debris, and protein concentration was measured using Bradford (Biorad). Equal amounts of proteins (50  $\mu$ g) were separated by SDS–PAGE using gradient acrylamide gels and transferred onto a nitrocellulose membrane. Western blots were revealed with SuperSignal Chemiluminescence (Pierce) and quantified by densitometry.

#### ss-HRP secretion assay

Briefly, 24 h after siRNA transfection, cells were transfected with plasmid carrying ss-HRP using Fugene (Roche) according to the manufacturer's instructions. Secreted and intracellular HRP activity was measured from supernatants and cell lysates, respectively, recovered 48 h after transfection. ss-HRP was measured using enhanced chemiluminescence (ECL, Pierce, #34095) as described (15). The secreted and intracellular HRP activity was normalized to the total protein content.

#### Transferrin internalization and recycling assay

Seventy-two hours after siRNA transfection, MDA-MB-231 cells were washed with 2× PBS and starved for 2 h in internalization medium (IM) (DMEM 0.01% BSA) to deplete endogenous transferrin. Cells were then cooled on ice for 5 min to block endocytosis, rinsed using ice-cold PBS, and incubated with ice-cold IM containing human transferrin conjugated to Alexa Fluor 488 (50  $\mu$ g/mL) for 1 h at 4°C to allow cell-surface binding of transferrin. Cells were then washed on ice using cold PBS–BSA 0.5% to remove unbound transferrin, and the probe was chased at 37°C for indicated time-periods in the presence of 100× excess unlabeled iron-saturated human transferrin (Calbiochem) to prevent fluorescent transferrin reinternalization. At the end of each time-point, cells grown on coverslips were fixed on ice using 4% PFA. Representative images of experiments performed three times are shown. Analogous results were obtained when transferrin-Alexa Fluor 488 was pulsed for 20 min at 37°C and then chased for indicated time-points at 37°C.

#### EGF internalization and degradation assay

The EGF assay was analogous to the transferrin assay with few modifications. Briefly, EGF-biotin conjugated to Alexa Fluor 594 was prepared by incubating EGF-biotin with streptavidin-Alexa Fluor 594 (5:1) for 30 min at 4°C. Labeled EGF was then diluted in IM (100 ng/mL) and allowed to bind to cell surface of serum-starved MDA-MB-231 cells for 1 h at 4°C. The internalization steps and immunofluorescence were performed as described for transferrin recycling.

#### pH measurement

Seventy-two hours after siRNA transfection or 48 h after Bafilomycin A1 100 nm treatment, MDA-MB-231 cells grown on glass coverslips were washed with  $2 \times$  PBS and starved for 2 h in IM. Cells were cooled on ice for 5 min, rinsed twice using ice-cold PBS and incubated with ice-cold IM containing human EGF-FITC (100 ng/mL) for 1 h at 4°C. Cells were then washed on ice using cold PBS-BSA 0.5% and the probe was chased for 10 min at 37°C to allow internalization of the ligand/receptor complex into early endosomes. Glass coverslips were then inserted into a perfusion chamber (Havard Apparatus) at 37°C in 1 mL of IM medium and imaged for 10 min with a video/CCD camera controlled by METAMORPH/METAFLUOR imaging software (Universal Imaging). Early-endosomal pH was measured by ratio fluorescence imaging as described (32,33) with the use of a Nipkow dual spinning disk confocal laser imaging system with a QLC module (Visitron systems GmbH). Images were acquired for 600 milliseconds at two different wavelengths, using the two lasers of 488 and 440 nm, with a 63×, 1.4 NA (numerical aperture) oil-immersion objective (Carl Zeiss AG). Image processing was performed as described previously (32). The significance of differences between samples was established using the Student's t-test for unpaired samples (\*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001). For calibration of endosomal pH measurements nigericin high K<sup>+</sup> method was used. Five different solutions containing nigericin (5 µg/mL) and monensin (5  $\mu\text{M})$  in mM, 125 KCl, 20 NaCl, 0.5  $\text{MgCl}_2,$  0.2 EGTA and 20 HEPES for pH 7.0 and 7.5, or 20 MES for pH 6.5, 6.0 and 5.5, were perfused on MDA-MB-231 cells previously loaded with EGF-FITC. A fivepoint exponential calibration curve was fitted and final pH values were extrapolated.

#### Generation of stable cell lines

Stable silencing of ATG5 and ATG7 genes was obtained by retrovirus infection of MDA-MB-231 cells with plasmids containing ATG5 and ATG7 shRNA sequences obtained from Dr Masashi Narita Cancer Research UK, Cambridge (UK). Rab5 constructs encoding the wild type and mutant (Q79L and S34N) protein were obtained from Dr Herald Stenmark (Centre for Cancer Biomedicine, Faculty of Medicine, University of Oslo, Norway) and subcloned in a modified version of pLVTHS transfer vector (Addegene) under the control of the Ef1alpha promoter (sequence available upon request). Retroviral and lentiviral production and infection of mammalian cells were performed according to standard procedures.

#### Real-time quantitative PCR

cDNA was obtained using a Superscript reverse transcriptase (Invitrogen) using 500 ng of total RNA as template. Real-time PCR amplification was done using a Taqman Universal PCR mastermix in an ABI Prism 7700 instrument (Applied Biosystems) according to the manufacturer's instructions. Relative quantification of target, normalized to endogenous control (Cyclophilin, Taqman Probes, Applied Biosystems), was done using a comparative method according to the manufacturer's instructions. Primers amplifying ATG5 and ATG7 transcripts were designed using Primer Design program (Applied Biosystems), sequence: ATG5 Fw 5'-caacttgttcacqctatacage3', Rev 5'-cacttgtcaqtgtcactqct-3'.

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#### **Supporting Information**

Additional Supporting Information may be found in the online version of this article:

Figure S1: Representative images of securin and separase localization in MDA-MB-231 cells. A) Securin and separase display abundant localization in the nucleus but also in the cytoplasm and beneath the plasma membrane. B) Examples of securin and separase colocalization in the cytoplasm of MDA-MB-231 cells obtained using IMARIS 6.3.1 software on confocal image scans. Note the output of colocalization results (vellow) showing abundant securin and separase colocalization in the perinuclear region and beneath the plasma membrane (inset). The focal plane of the colocalization image was selected to highlight cytoplasmic and plasma membrane colocalization. C) Examples of securin localization in subconfluent cell cultures (24 h) as compared to confluent cells (72 h). Note the prominent securin localization beneath the plasma membrane in cells attached to cell culture dishes for 24 h. D) Specificity of anti-securin and anti-separase (ab16170) antibodies assessed 72 h post-siRNA oligonucleotide transfection. Note the total disappearance of the anti-securin staining in securin siRNA-treated cells (upper panel) and the presence of residual background staining in separase siRNA-treated ones (lower panel). Scale bar =  $6 \mu m$ . E) Western blot analysis of MDA-MB-231 cell lysates in control (sivsv), securin (siSecurin), and separase (siSeparase) siRNA-treated cells 72 h post-treatment. Note the disappearance of securin (26 kDa) and full-length separase (220 kDa) and the presence of two aspecific bands (asterisk) unaffected by separase siRNA treatment. Two additional commercially available anti-separase antibodies (ab3762 and ab52158) were tested in the present study but both presented higher residual background staining in immunofluorescence and additional non-specific bands in western blots upon separase siRNA treatment. The ab16170 was therefore chosen for the present study. F) Western blot analysis of securin and separase distribution in purified cellular fractions. Membranes: crude membrane preparation; nucleus and cytosol (see Materials and Methods). Note the presence of full-length separase (220 kDa) in PNS, membranes and nucleus and the expression of cleaved separase forms in the nucleus. Asterisks indicate non-specific bands recognized by the anti-separase antibody. Similar to separase, securin was distributed among all fractions and displayed prominent localization in the cytoplasm. The transferrin receptor denotes purity of the MB fractions. Histone H3 of the nuclear fraction and tubulin of the cytosolic fraction.

Figure S2: Colocalization of securin and separase with ER, Golgi en edosomal markers. Representative images of colocalization of (A) securin with calnexin (ER marker), GM130 (*cis*-Golgi), TGN46 (TGN),

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and EEA1 (early-endosome marker), and (B) separase with TGN46 and giantin (*cis*-Golgi). The first column shows anti-securin (A) or anti-separase (B) staining, the second column shows anti-organelle-specific marker antibody staining. Merged images are shown in the third column and colocalization results obtained using IMARIS 6.3.1 software are illustrated in the fourth column. Scale bar = 8  $\mu$ m in all panels, except for the separase/giantin panel B = 14  $\mu$ m.

Figure S3: Western blot analysis of securin and separase expression in MDA-MB-231 cells 72 h post-siRNA oligonucleotide transfection. Securin depletion (A) achieved using three different siRNA oligonucleotides affects separase stability. Separase depletion (B) obtained using two different siRNA oligonucleotides does not affect securin expression. C) Quantification of phospho Histone H3 (Ser 10)-positive mitotic cells in control (sivsv) and securin- and separase-depleted cells 72 h post-siRNA oligonucleotide transfection. Results are expressed as the percentage of pH3 (Ser10)positive cells identified within the total number of counted cells (at least 500) for each sample. Error bars indicate the standard deviation of counts performed in three independent experiments. D) Distribution of cells displaying normal (N) or giant (G) EEA1-positive early endosomes within phospho Histone H3 (Ser 10)-positive (pH3+) and phospho Histone H3 (Ser 10)-negative (pH3-) cell populations in securin- and separase-depleted MDA-MB-231 cells. Seventy-two hours post-siRNA oligonucleotide transfection, cells were fixed and double-labelled for phospho Histone H3 and EEA1 marker. Images were acquired and early-endosome size was scored in pH3+ and pH3- cells using IMAGEJ as before (Figure 1D). Endosomes were classified as giant when presenting at least the double of the size of the average size value of normal endosomes. Note the equal distribution of cells having giant endosomes between pH3+ and pH3- cell populations. E) Representative images of early endosomes observed in MDA-MB-231 cells expressing empty vector, constitutively active (Rab5\_Q79L) or inactive (Rab5\_S34N) form of Rab5. Cells were treated with siRNA oligonucleotides targeting vsv-g (first row) or securin (second row) or separase (not shown) for 72 h and then fixed and processed for immunofluorescence. In agreement with the previous studies, expression of Rab5 Q79L expression induces giant early-endosomes' formation in vsv-g-treated control cells, whereas Rab5\_S34N expression results in the formation of early endosomes having significantly smaller size. Early endosomes were identified using anti-EEA1 antibody (green). Securin and separase depletion synergized with Rab5\_Q79L expression resulting in even larger earlyendosome structures. Remarkably, early-endosome fusion still occurred upon securin and separase depletion in Rab5 S34N-expressing MDA-MB-231 cells. Compare endosome size in vsv-g-treated Rab5\_S34N control cells versus securin-depleted Rab5\_S34N cells. F) qPCR analysis of ATG5 and ATG7 transcripts denoting 85% and 50% depletion of the corresponding genes in MDA-MB-231 stably silenced with ATG5 and ATG7 shRNA constructs. G) MDA-MB-231 stably silenced for ATG5 (shown) and ATG7 (not shown) were transfected with vsv-g (control) and securin (shown) or separase (not shown) siRNA oligonucleotides for 72 h. Early endosomes were identified using anti-EEA1 antibody (green). Note the formation of giant EEA1-positive endosomes in securin siRNA-treated MDA-MB-231 cells stably silenced for ATG5. Scale bar =  $8 \,\mu$ m.

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