

Brief Report

Proteasome Activity is Required for Centromere Separation Independently of Securin Degradation in Human Cells

Juan F. Giménez-Abián^{1,2,‡}

Laura A. Díaz-Martínez^{1,‡}

Karin G. Wirth³

Consuelo De la Torre²

Duncan J. Clarke^{1,*}

¹Department of Genetics, Cell Biology & Development; University of Minnesota Medical School; Minneapolis, Minnesota USA

²Proliferación Celular; Centro de Investigaciones Biológicas CSIC; Madrid, Spain

³Klinik und Poliklinik für Innere Medizin II; Klinikum der FSU Jena; Jena, Germany

[‡]These authors contributed equally to this work.

*Correspondence to: Duncan J. Clarke; Department of Genetics, Cell Biology & Development; University of Minnesota Medical School; 420 Washington Ave SE; Minneapolis, Minnesota 55455 USA; Tel.: 612.624.3442; Fax: 612.626.6140; Email: Duncan.J.Clarke-2@umn.edu

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KEY WORDS

anaphase promoting complex, cyclosome, Pds1, securin, separase, proteasome, centromere cohesion, VELCADE®

ABBREVIATIONS

APC anaphase promoting complex
RNAi RNA interference

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ABSTRACT

Loss of centromere cohesion during anaphase in human cells is regulated by the spindle assembly checkpoint and is thought to depend on a ubiquitin ligase, the Anaphase Promoting Complex/Cyclosome (APC). APC-Cdc20 adds ubiquitin chains to securin inducing its destruction by the proteasome and these events correlate with the loss of sister chromatid cohesion and the onset of anaphase. But whether securin destruction is necessary and sufficient for anaphase initiation is not clear. Therefore, we asked if proteasome activity is needed for anaphase onset in human cells that lack securin. We find that even in the absence of securin, a metaphase block with cohered sister centromeres can be enforced in the absence of proteasome activity. Therefore, other targets of the proteasome must be degraded to allow anaphase onset.

The anaphase promoting complex (APC) is thought to initiate sister chromatid separation by ubiquitin-targeted degradation of the anaphase inhibitor Pds1/securin. Prior to anaphase, securin binds to and inhibits separase, a protease that can cleave a member of the cohesin complex that holds sister DNA molecules together. However, securin minus human and yeast cells are viable,¹⁻³ and can regulate anaphase onset,⁴⁻⁶ indicating that this protein cannot be the sole factor controlling sister separation. Still, a number of studies have used proteasome inhibitors to achieve a G₂/M arrest in mammalian cells, making the assumption that proteasome inhibition blocks securin degradation and thus prevents sister separation.^{7,8} Most of these studies used FACScan analysis of DNA content as their tool to measure cell cycle arrest and clearly this analysis cannot distinguish G₂ cells from cells arrested in mitosis.

Apart from securin, several other proteasome substrates are ubiquitinated by the APC and degraded around the time of anaphase onset. These include cyclin B and Sgo1, the latter being implicated in the control of sister cohesion.⁹⁻¹⁴ Indeed, recent reports have provided evidence that cyclin B might play a redundant role with securin in blocking anaphase progression. Mutant forms of cyclin B1 or overexpression of cyclin B1 was shown to block anaphase onset.¹⁵⁻¹⁷

To test directly whether a factor other than securin must be degraded by the proteasome for anaphase to initiate, we asked if mammalian cells lacking securin can be arrested in metaphase in the presence of proteasome inhibitors. Our rationale therefore was to test in the absence of securin if other proteasome targets might inhibit anaphase onset. We first accumulated securin *-/-* or securin *+/+* cells (see ref. 1) in *c*-mitosis with nocodazole to avoid the effects of proteasome inhibition at other cell cycle stages (Fig. 1B and I). (This procedure was used because we found that the proteasome inhibitor induced arrest at several cell cycle points including G₂ phase; data not shown.) Then we released the cells from nocodazole arrest in the presence or absence of the proteasome small molecule inhibitor VELCADE® taking samples for biochemical and chromosome analysis.

Upon release, both cell lines formed metaphase plates (Fig. 1C–E and data not shown) and executed anaphase in a timely manner without VELCADE® (Fig. 1F–H, P and R); cyclin B1 and Sgo1 (and securin in the securin *+/+* cells) were degraded, histone H3 was dephosphorylated (Fig. 1A) and most of the formerly arrested cells exited mitosis within 2 hrs (Fig. 1A, P and R). At low frequency, anaphase bridges or laggards were observed in securin *-/-* cells (Fig. 1G and H), consistent with published data.¹ In contrast, VELCADE® blocked most cells in metaphase (Fig. 1A, M and N) with stabilized cyclin B1 and Sgo1, and some decrease in phospho-H3 (Fig. 1A, Q and S), even though metaphase plates formed normally (Fig. 1J–L). Chromosomes in these blocked metaphase plates showed no centromere opening, indicating that centromere cohesion can resist the pulling forces of the spindle even in cells in which arm cohesion had been lost (judged by

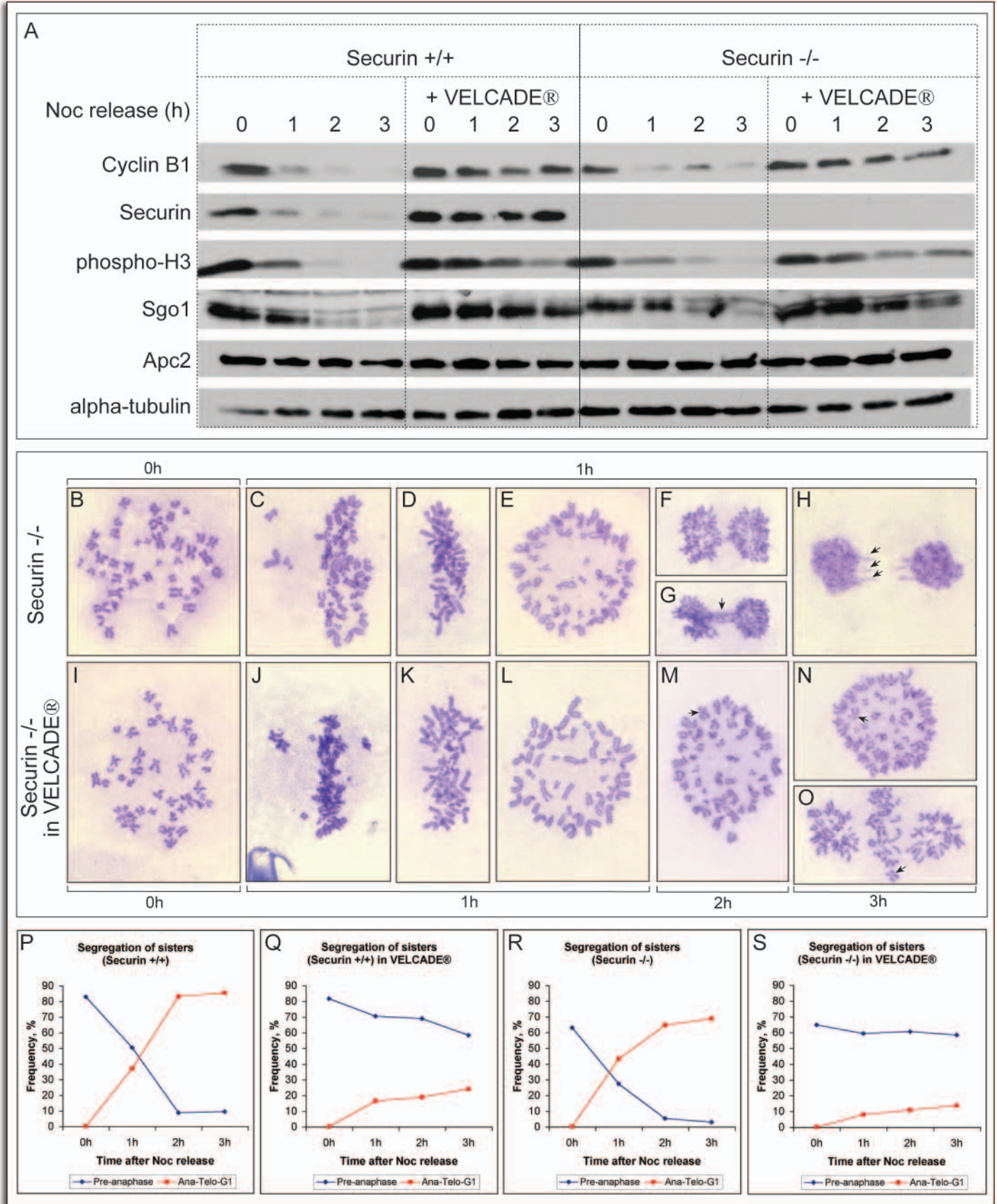


Figure 1 (previous page). Proteasome inhibitor enforced block in securin $-/-$ cells. Hct116 (parental) or securin $-/-$ cells were accumulated in c-mitosis with nocodazole then shaken-off and washed in medium $+/-$ VELCADE® (1 μ M) and incubated with or without VELCADE® for 0-3 hrs. (A) Western blots of the indicated proteins versus Apc2 and alpha-tubulin loading controls (0-3 hrs after nocodazole release). (B-O) Chromosome preparations (methanol-acetic acid fixed material stained with Giemsa) showing metaphase plate formation and anaphase onset in securin $-/-$ cells with or without VELCADE®: (B,I) c-mitosis: chromosomes are scattered in the cytoplasm due to the absence of a mitotic spindle; (C,J) Prometaphase: side views - several chromosomes lie away from the spindle equator; (D,E,K,L) Metaphase plate formation: D and K are side views, E and L are polar views; (M,N) Prolonged metaphase arrest with VELCADE® - polar views - well defined centromere regions, arrows; (F) Normal anaphase; (G,H) Chromosome bridges (arrows), sometimes observed in anaphase securin $-/-$ cells, as previously reported. I In the occasional anaphases that occurred in the presence of VELCADE® (O), some chromosomes segregated to the poles indicating spindle function, while others lagged at the plate, some having apparently retained centromere attachment (arrow) but with open arms. (P-S) Kinetics of sister segregation after nocodazole release $+/-$ VELCADE®.

arm opening; Fig. 1O, arrow) during the prolonged nocodazole arrest. Some cells did leak into anaphase with VELCADE® (Fig. 1O), but this correlated with a decrease in phospho-H3 positivity and a slight drop in cyclin B1 and Sgo1 levels (Fig. 1A).

We conclude that metaphase arrest can be enforced, at least transiently, in the absence of securin but in a proteasome-dependent manner, and that the arrest correlates with the presence of endogenous cyclin B1 and Sgo1. Since metaphase arrest occurred in the presence of VELCADE® in securin $-/-$ cells, this arrest can be enforced without prior inhibition of separase by securin. These data agree with similar studies that indicated that at least a transient metaphase arrest can be induced by cyclin B in mammalian cells. Whether securin and cyclin B are the only factors that must be degraded in order for anaphase to proceed efficiently and with fidelity is not known. We speculate, however, that Sgo1 degradation is not required for anaphase onset, since the decay of Sgo1 protein was delayed relative to securin and cyclin B upon release from nocodazole (Fig. 1A) and therefore probably occurs subsequent to sister chromatid separation. It should be noted that we have not formally excluded the possibility that the metaphase block in the presence of VELCADE® might be due to activation of the spindle assembly checkpoint, rather than being a direct consequence of stabilizing an anaphase inhibitor. Metaphase plates did appear to form normally in the presence of VELCADE®, but we would not have detected subtle defects using this methodology. It is also possible that a signaling component of the spindle checkpoint must be degraded by the proteasome in order for the checkpoint to be turned off once all of the chromosomes have congressed to the equatorial plate.

In summary, here we present evidence that securin degradation is not sufficient for centromere separation or anaphase onset in human cells. These data may explain why securin null mice are viable and why cells from these mice do not have spindle checkpoint defects.^{18,19} Conversely, several studies have indicated that securin degradation is not necessary for centromere separation and anaphase onset in human cells, but that a lack of securin degradation does impede chromosome arm separation, compromising the fidelity of chromosome segregation.^{14,20} Together these studies might explain why human securin is typically over-expressed in tumor cells rather than being inactivated. Furthermore, these data are in agreement with separase RNAi studies that suggest separase is more critical for chromosome arm separation than for centromere separation in humans.²¹

EXPERIMENTAL PROCEDURES

Hct116 mammalian cells were grown under standard conditions in DMEM high glucose, with L-glutamine, 110 mg/L sodium pyruvate and pyridosine hydrochloride, supplemented with 10% FBS (Gemini), 100 U/mL Penicillin and 100 μ g/mL Streptomycin. After a double thymidine block, nocodazole was added (using a final concentration of 0.3-0.5 μ M) 6 hrs after the second thymidine release. Thymidine arrest/release was performed as described.¹⁴ Cells were collected by mitotic shake off 14 hrs after the second thymidine release (8 hr nocodazole arrest) and released (after three washes) into nocodazole-free medium. For VELCADE® treated cells: VELCADE® (1 μ M; Millennium Pharmaceuticals) was added to nocodazole arrested cells before shake off and also added to the nocodazole-free medium and it was also present during the washes. Chromosome preparations and biochemistry were performed as described in the accompanying article.¹⁴ Photomicrographs were obtained using a Zeiss Axioplan II microscope and an alpha Plan Fluor 100x/1.45 n.a. objective, captured with an AxioCam MRC5 camera (Axiovision software).

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