Securin-independent regulation of separase by checkpoint-induced shugoshin-Mad2 complex

Susanne Hellmuth¹, Laura Gómez-H², Alberto M. Pendás², and Olaf Stemmann¹

¹ Chair of Genetics, University of Bayreuth, 95440 Bayreuth, Germany

² Molecular Mechanisms Program, Centro de Investigación del Cáncer and Instituto de Biología Molecular y Celular del Cáncer (CSIC-Universidad de Salamanca), 37007 Salamanca, Spain

Separation of eukaryotic sister chromatids is timed by the spindle assembly checkpoint (SAC) and ultimately triggered when separase cleaves cohesion-mediating cohesin¹⁻³. Silencing of the SAC in metaphase activates the ubiquitin ligase APC/C resulting in the proteasomal destruction of the separase inhibitor securin¹. In the absence of securin mammalian chromosomes still segregate on schedule but how separase is regulated under these conditions remains enigmatic^{4,5}. Here, we show that human shugoshin 2 (Sgo2), an essential protector of meiotic cohesin with unknown function in the soma^{6,7}, is turned into a separase inhibitor upon association with SAC-activated Mad2. Sgo2-Mad2 can functionally replace securin and sequesters most separase in *SECURIN*^{-/-} cells. Acute loss of securin and Sgo2, but not the individual depletions, result in separase deregulation associated with premature cohesin cleavage and cytotoxicity. Like securin^{8,9}, Sgo2 is a competitive inhibitor that uses a pseudo-substrate sequence to block separase's active site. APC/C-dependent ubiquitylation and action of the AAA-ATPase Trip13 in conjunction with the Mad2-specific adaptor p31^{comet} liberate separase from Sgo2-Mad2 *in vitro*. The latter mechanism facilitates a considerable degree of sister chromatid separation in *SECURIN*^{-/-} cells that lack APC/C activity. Thus, our study unravels an unexpected Sgo2 function in mitotically dividing cells and a separase regulatory mechanism that is independent of securin while retaining supervision by the SAC.

1 All eukaryotic anaphases are triggered when chromosomal cohesin is cleaved by the
2 essential Cys-endopeptidase separase^{3,10}. To prevent premature loss of sister chromatid
3 cohesion, separase needs tight control. It is competitively inhibited by association with
4 securin for most of the cell cycle. Only in metaphase the E3 anaphase-promoting complex
5 or cyclosome (APC/C) mediates the degradation of securin via the ubiquitin-proteasome-
6 system, thereby activating separase¹¹. Securin destruction is timed by the spindle assembly
7 checkpoint (SAC), which keeps the APC/C co-activator Cdc20 inactive until all kinetochores
8 are properly attached to spindle microtubules¹.

9 Unexpectedly, securin is dispensable in human cells and mice^{4,5}. This can partially be
10 explained by Cdk1-cyclin B1-dependent regulation of separase¹²⁻¹⁷. Murine *CDC20*^{-/-}
11 embryos arrest in metaphase with cohered chromosomes because they can neither
12 degrade cyclin B1 nor securin¹⁸. Interestingly, double knock-out of *CDC20* and *SECURIN*
13 resulted in an arrest with separated sisters and this defect was rescued by constitutive SAC
14 activation¹⁸, which argues for existence of a SAC-dependent but securin-independent
15 control of separase. Rather than being SAC-stimulated, the binding of Cdk1-cyclin B1 to
16 separase is dampened by early mitotic cyclin B1 phosphorylations¹⁹. However, we had
17 previously identified a link between the SAC and the cohesin protector shugoshin (Sgo) in
18 that human Sgo2 - much like Cdc20 - is bound by the essential SAC component Mad2 (7).
19 Interestingly, murine Sgo2 and separase interacted upon their co-expression in Hek293
20 cells (Extended Data Fig. 1a; see, however, Extended Data Fig. 1b). Together, this led to the
21 idea that SAC-activated Mad2 could enable Sgo2 to bind and inhibit separase. Indeed, when
22 endogenous human separase was isolated by immunoprecipitation (IP) from untransfected,
23 prometaphase arrested Hek293T or untransformed RPE-1 cells, Sgo2 (but not the related
24 Sgo1) and Mad2 co-purified - along with the known interactors securin and cyclin B1 (Fig.
25 1a and Extended Data Fig. 1c). In contrast to separase, Sgo2 and Mad2 were undetectable
26 in a securin-IP and - *vice versa* - securin and cyclin B1 were absent from a Sgo2-IP (Fig. 1a).
27 Together with previous results²⁰, this argues for co-existence of three mutually exclusive
28 complexes in human mitotic cells: Separase-securin, separase-Cdk1-cyclin B1 and separase-
29 Sgo2-Mad2. Following immunodepletions of the three inhibitors from taxol-arrested
30 Hek293T, HCT116 and HeLa-K cells, the relative amounts of associated separase were
31 quantified. This revealed that on average 59, 35, and 6% of total separase is sequestered by

32 securin, Sgo2, and cyclin B1, respectively (Extended Data Fig. 1d). Notably, in mitotic
33 *SECURIN*^{-/-} cells most separase (85%) was in complex with Sgo2 (Fig. 1b).

34 To test whether Sgo2 binding to separase required Mad2, endogenous Sgo2 was depleted
35 by RNAi and replaced by siRNA-resistant, transgene-encoded variants. Subsequent IP of co-
36 expressed Myc-separase showed that the Mad2-binding deficient Sgo2-R153D was unable
37 to interact with separase, while Sgo2-N58I, which can no longer interact with protein
38 phosphatase 2A (PP2A)⁷, retained Mad2- and separase-binding (Fig. 1c). The SAC-shugoshin
39 link is conserved in *Xenopus* with the curious difference that here Sgo1 rather than Sgo2
40 binds Mad2 and separase (Extended Data Fig. 2)⁷. Thus, the separase-shugoshin interaction
41 depends on active Mad2 and is conserved in vertebrates.

42 The separase-Sgo2-Mad2 complex was present in G1-, early S-, G2-, and prometaphase
43 arrested cells (Fig. 1d) but disappeared when taxol-treated HeLa-K cells were driven into an
44 anaphase-like state by SAC abrogation with the aurora B kinase inhibitor ZM-447439 (Fig.
45 1e). This cell cycle distribution mirrored that of Cdc20-Mad2, whose formation in
46 interphase requires Mad1-dependent Mad2 activation at nuclear pore complexes (NPC)²¹.
47 Consistently, separase-Sgo2-Mad2, like Cdc20-Mad2, became (almost) undetectable in
48 unsynchronized HeLa-K cells depleted of Mad1 and/or the NPC component TPR (Fig. 1f).
49 Thus, conformationally activated Mad2 enables Sgo2 to sequester separase through all of
50 the cell cycle but a short period of SAC inactivity during late mitosis.

51 Overexpression of a Cdk1-cyclin B1-resistant and stabilized separase-S1126A variant causes
52 premature sister chromatid separation (PCS) in Hek293T cells^{12,22}. This PCS phenotype was
53 aggravated by siRNA-mediated depletion of endogenous Sgo2 and alleviated by
54 simultaneous, slight over-expression of Sgo2-WT from an siRNA-resistant transgene (Fig.
55 1c). Sgo2-R153D failed to rescue PCS in this cellular assay, while transgenic Sgo2-N53I
56 remained functional. Together, this suggested that Sgo2 might indeed have an inhibitory
57 effect on separase and that this requires Mad2- but not PP2A-binding to Sgo2.

58 By recruiting PP2A to (peri)centromeres Sgo2 exerts essential cohesin protective functions
59 throughout meiosis I but - similar to securin - it is dispensable in somatic cells^{6,7}. What if
60 securin and Sgo2-Mad2 could functionally replace each other as crucial negative regulators
61 of separase? Then, co-depletion of securin and Sgo2 should result in detrimental

deregulation of separase. First, overall effects on cell viability/proliferation were assessed by clonogenic assays. As expected, the knock-down of securin or Sgo2 alone had no or only a small inhibitory effect on HeLa-K colony formation, respectively (Fig. 2a and Extended Data Fig. 3a). In sharp contrast, hardly any clones grew when both separase interactors were depleted at the same time. Similarly, Sgo2 depletion virtually extinguished colony formation in *SECURIN*^{-/-} cells, whereas it only halved colony numbers in the parental HCT116 (Extended Data Fig. 3b). The same tendencies were found for Hek293T cells although the effects were less pronounced here (Extended Data Fig. 3c). Live imaging of histone H2B-eGFP expressing HeLa-K cells revealed that the individual knock-downs did not affect mitosis but when securin and Sgo2 were both missing, the ability to form proper metaphase plates was profoundly compromised (Extended Data Fig. 4a). Securin- and Sgo2-less HeLa-K cells were also hallmarked by the otherwise uncommon absence of cohesin from early mitotic chromatin (Extended Data Fig. 4b). Consistently, the individual knock-down of securin or Sgo2 had no effect on cohesion, while the simultaneous removal of both resulted in PCS as judged by chromosome spreads from prometaphase-arrested Hek293T cells (Extended Data Fig. 5a, b). The PCS phenotype was further fortified by chemical inhibition (with EGCG) of Pin1, a peptidyl-prolyl-isomerase required for Cdk1-cyclin B1 dependent separase inhibition (Fig. 2b and Extended Data Fig. 5a, b)¹⁹. In keeping with the separase-Sgo2-interaction being dependent on Mad2, partially removing Mad2, while without effect on its own, inhibited colony formation and exacerbated PCS in conjunction with RNAi of securin (Fig. 2b, Extended Data Fig. 3 and 5a, b). All these phenotypes could be explained by precocious separase-dependent cleavage of cohesin. Indeed and consistent with a previous study in yeast²³, the characteristic fragments of cohesin's Rad21 subunit were detectable in prometaphase-arrested cells if - and only if - securin and Sgo2 had both been depleted (Fig. 2c). Likewise, a transiently expressed separase activity sensor was maximally cleaved in securin- and Sgo2-depleted HeLa-K cells already in a taxol arrest, whereas in mock-depleted cells it was only cleaved upon addition of ZM-447439 (Extended Data Fig. 5c). Screening of separase mutants identified two variants (V4 and V5) unable to interact with Sgo2-Mad2 (Extended Data Fig. 6). When endogenous separase was replaced by these variants, depletion of securin was sufficient to induce PCS (Fig. 2d). This strongly argues that it is a direct separase inhibitory function of Sgo2 that works redundantly with securin to prevent PCS.

94 In interphase, separase is excluded from the nucleus but has an established function in
95 centriole disengagement²⁴. Therefore, this licensing step of centrosome duplication rather
96 than cleavage of chromosomal cohesin was assessed upon depletion of securin and Sgo2.
97 Premature centriole disengagement in G2-phase was 3-fold increased in securin/Sgo2
98 doubly depleted relative to singly- or mock-depleted Hek293T cells (45% versus 10-15%,
99 respectively) (Extended Data Fig. 7). Thus, Securin- and Sgo2-less cells contain active
100 separase already in interphase and, hence, are expected to lose sister chromatid cohesion
101 immediately upon nuclear envelope breakdown.

102 Shugoshins are hallmarked not only by an N-terminal coiled-coil but also by a C-terminal
103 SGO-box that binds to Bub1-phosphorylated histone H2A (25). Consistently,
104 phosphorylated but not unphosphorylated H2A peptide bound immobilized Sgo2 (Extended
105 data Fig. 8a-c). Importantly, pre-incubation with phospho-H2A but not unphosphorylated
106 H2A or a chemically similar but irrelevant phospho-H3 peptide suppressed Sgo2's ability to
107 bind Mad2 (Extended data Fig. 8a-d). *Vice versa*, pre-incubation of immobilized Sgo2 with
108 Mad2 prevented subsequent phospho-H2A-Sgo2 interaction (Extended data Fig. 8e, f).
109 Phospho-H2A did bind to Sgo2, however, when previous Sgo2-Mad2 complex formation
110 was not possible due to use of Sgo2-R153D or Mad2 Δ C instead of the corresponding wild
111 type proteins. Thus, Mad2 and phospho-H2A bind Sgo2 in a mutually exclusive manner. We
112 propose that different pools of Sgo2 either associate with chromatin or bind Mad2 and
113 inhibit separase.

114 Incubation of human separase-securin complex in securin-degrading, anaphase-like
115 *Xenopus* egg extracts followed by affinity purification of separase generates active protease
116 that specifically cleaves ³⁵S-labeled Rad21 (17). Pre-incubation of this separase with *in vitro*-
117 expressed Sgo2 and *E. coli*-expressed, purified Mad2 blocked Rad21 cleavage (Fig. 3a).
118 Inhibition also occurred with Sgo2-N53I instead of wild type Sgo2 but failed when Sgo2 or
119 Mad2 were omitted or replaced by Sgo2-R153D or Mad2 Δ C, respectively. Thus, the PP2A-
120 independent but Mad2-dependent inhibition of human separase by Sgo2 can be
121 recapitulated *in vitro*.

122 How does shugoshin inhibit separase? Could it act like securin, i.e. by occupying the
123 catalytic site with a non-cleavable pseudo-substrate sequence^{8,9}? Using interaction blocking
124 antibodies, protein fragments and point mutations, we mapped sites within X.l. Sgo1 that

125 are important for separase-interaction (Extended Data Fig. 9). Nearby putative pseudo-
126 substrate sites (ϕ ExxX with ϕ being a hydrophobic residue) were then changed into
127 consensus sites (ϕ ExxR) and the resulting variants screened for cleavage by active *Xenopus*
128 separase. Notably, *X.l.* Sgo1-S135R (but not -F288R or wild type) was cleaved by separase
129 and this cleavage was greatly pronounced in presence of wild type Mad2 as compared to
130 Mad2 Δ C (Extended Data Fig. 10).

131 To independently confirm this finding, we switched back to the human system and asked
132 whether a specific ϕ ExxX-to- ϕ ExxR mutation could also turn *H.s.* Sgo2 into a separase
133 substrate. Indeed, ³⁵S-labeled Sgo2-M114R (but not -F95R or -S126R) was fragmented in
134 presence of active separase and wild type Mad2 (Fig. 3b). Sgo2-M114R cleavage - like that
135 of Rad21 (26) - was further enhanced upon phosphorylation by Polo-like kinase 1. To test *in*
136 *vivo*-cleavage, HeLa-K cells were transfected to express Flag-tagged Sgo2- or securin
137 variants. Subsequent IPs from prometaphase lysates demonstrated that securin-F118R
138 (positive control) and Sgo2-M114R were both fragmented, whereas the wild type proteins
139 remained unprocessed (Fig. 3c). Cleavage of Sgo2-M114R was separase-specific because it
140 was suppressed by introduction of a separase interaction-compromising, second mutation
141 (amino acids 239-42 to Ala) into Sgo2 (Fig. 3c and Extended Data Fig. 11a). Thus, shugoshin
142 resembles securin in acting as a competitive inhibitor of separase but differs in that it
143 requires Mad2 binding to do so.

144 *X.l.* Sgo1 and *H.s.* Sgo2 are very different in sequence (21% similarity) and length (663
145 versus 1265 residues). Yet, the relative order and distance of functional elements is the
146 same in both proteins (Extended Data Fig. 11b). This suggests that Mad2 binding and
147 separase inhibition evolved prior to duplication of a primordial *SGO* gene and were later
148 lost randomly from one *SGO* gene but retained in the other due to selective pressure.

149 In contrast to securin, *H.s.* Sgo2 is not (or only slowly and incompletely) degraded in late
150 mitosis. This begs the question of how separase is liberated from Sgo2-Mad2 when cells are
151 ready to undergo anaphase. The Cdc20- and Mad2-containing mitotic checkpoint complex
152 (MCC) is disassembled by the combined action of the AAA-ATPase Trip13 and its Mad2-
153 specific adaptor p31^{comet} (27). We tested whether this molecular machine could also
154 dismantle separase-Sgo2-Mad2. The complex was immunoprecipitated with separase

antibody from securin-depleted, taxol-arrested HeLa-K cells and incubated with different combinations of recombinant Trip13- and p31^{comet} variants. Beads were then washed to remove detached proteins, incubated with ³⁵S-Rad21 to assay for activity of the immobilized separase and finally analysed for retained proteins. Remarkably, wild type Trip13- and p31^{comet} quantitatively displaced Sgo2 and Mad2 from separase, thereby leaving it proteolytically active (Fig. 4a). While Trip13 alone partially disassembled separase-Sgo2-Mad2, Walker-A or -B mutant variants were inactive even in presence of p31^{comet}. Separase activation by Trip13 was also spoiled by presence of p31^{comet}-variants defective in Mad2- or Trip13 interaction. To assess the role of Trip13 and p31^{comet} *in vivo*, securin-depleted HeLa-K cells were additionally transfected with siRNAs against Trip13 and p31^{comet}, synchronized in prometaphase with taxol and then released by ZM-447439. To our surprise, late mitotic events, like the de-phosphorylation of Cdc27 and histone H3 and the degradation of cyclin B1, were only slightly delayed or occurred largely on schedule in the Trip13- and p31^{comet}-less cells relative to control-treated cells (Fig. 4b, upper half). In contrast, the separase-Sgo2-Mad2 complex was profoundly stabilized in the absence of Trip13 and p31^{comet} as revealed by separase-IP and immunblotting (lower half). Thus, separase-Sgo2-Mad2 is actively dismantled by Trip13 and p31^{comet} and might depend on this molecular machine for its disassembly in late mitosis even more than the MCC.

The above results suggest that sister chromatid separation in the absence of APC/C^{Cdc20} activity could be possible when separase is chiefly controlled by Sgo2-Mad2 instead of securin. To test this prediction, taxol-arrested *SECURIN*^{-/-} and parental HCT116 cells were supplemented with the two APC/C inhibitors proTame and Apcin (or carrier solvent), released by ZM-447439 addition and analysed by time-resolved IP-immunoblotting and chromosome spreading. Indeed, 35 minutes after Aurora B inhibition up to 60% of chromosomes were separated in *SECURIN*^{-/-} cells but only 20% on average in parental HCT116 cells despite persistence of Cdc27-phosphorylation and cyclin B1 in both (Fig. 4c, d). While separase-associated securin levels also stayed constant in APC/C-inhibited HCT116 cells, Sgo2 and Mad2 (which appeared overexpressed in *SECURIN*^{-/-} cells) disappeared from separase irrespective of securin status (Fig. 4d). Consistent with Sgo2-Mad2 being the primary separase inhibitor, this went along with strongly increased separase auto-cleavage in *SECURIN*^{-/-} cells. Thus, activation of Sgo2-Mad2-inhibited separase occurs at least partially independent of APC/C. We noted, however, that sister chromatid separation was

187 more effective in the absence of proTame and Apcin even in *SECURIN*^{-/-} cells (Fig. 4c).
188 Given that APC/C-associated MCC is disassembled also upon ubiquitylation of Cdc20 (28)
189 and given the recent identification of separase and Sgo2 as APC/C interactors/substrates²⁹,
190 we tested whether separase-Sgo2-Mad2 could also be dismantled by APC/C-dependent
191 ubiquitylation *in vitro*. Indeed, incubation of the immobilized complex with E1, E2s (Ube2c
192 and Ube2s) and active APC/C^{Cdc20} in the presence of ubiquitin and ATP displaced Sgo2 and
193 Mad2 and rendered separase proteolytically active (Extended Data Fig. 12). Dissociation
194 was accompanied by ubiquitylation of separase. The complex stayed intact, however, when
195 Ube2c was replaced by a dominant-negative variant or when APC/C^{Cdc20} was omitted. These
196 results suggest that APC/C-dependent ubiquitylation represents a second mode of
197 separase-Sgo2-Mad2 disassembly.

198 We propose a model of bifurcated regulation of separase downstream of the SAC (Fig. 4e).
199 Next to the APC/C^{Cdc20}-securin axis, a second major branch is represented by mammalian
200 Sgo2 (Sgo1 in amphibians) which is turned into a direct, competitive inhibitor of separase
201 by SAC-activated Mad2. Both branches employ Trip13-p31^{comet} (and APC/C-dependent
202 ubiquitylation) for disassembly of their respective Mad2-containing complexes. However,
203 while this already liberates separase from shugoshin, the canonical branch additionally
204 requires proteasomal destruction of securin. At least in the cell lines tested herein, securin
205 and Sgo2 can compensate for loss of the other. The reason for this seeming redundancy
206 remains to be clarified but the different requirements for protein degradation might make
207 it beneficial at times to rely on one or the other mode of separase regulation.

References

- 209 1 Musacchio, A. The Molecular Biology of Spindle Assembly Checkpoint Signaling Dynamics. *Curr Biol* **25**,
210 R1002-1018, doi:10.1016/j.cub.2015.08.051 (2015).
- 211 2 Nasmyth, K. & Haering, C. H. Cohesin: its roles and mechanisms. *Annu Rev Genet* **43**, 525-558,
212 doi:10.1146/annurev-genet-102108-134233 (2009).
- 213 3 Uhlmann, F., Wernic, D., Poupart, M. A., Koonin, E. V. & Nasmyth, K. Cleavage of cohesin by the CD clan
214 protease separin triggers anaphase in yeast. *Cell* **103**, 375-386, doi:S0092-8674(00)00130-6 [pii] (2000).
- 215 4 Mei, J., Huang, X. & Zhang, P. Securin is not required for cellular viability, but is required for normal
216 growth of mouse embryonic fibroblasts. *Curr Biol* **11**, 1197-1201, doi:S0960-9822(01)00325-6 [pii] (2001).
- 217 5 Pfliegerhaa, K., Heubes, S., Cox, J., Stemmann, O. & Speicher, M. R. Securin is not required for chromosomal
218 stability in human cells. *PLoS biology* **3**, e416, doi:05-PLBI-RA-0748R2 [pii] 10.1371/journal.pbio.0030416
219 [doi] (2005).
- 220 6 Llano, E. *et al.* Shugoshin-2 is essential for the completion of meiosis but not for mitotic cell division in
221 mice. *Genes & development* **22**, 2400-2413, doi:10.1101/gad.475308 (2008).
- 222 7 Orth, M. *et al.* Shugoshin is a Mad1/Cdc20-like interactor of Mad2. *The EMBO journal* **30**, 2868-2880,
223 doi:10.1038/emboj.2011.187 (2011).
- 224 8 Boland, A. *et al.* Cryo-EM structure of a metazoan separase-securin complex at near-atomic resolution.
225 *Nature structural & molecular biology* **24**, 414-418, doi:10.1038/nsmb.3386 (2017).
- 226 9 Lin, Z., Luo, X. & Yu, H. Structural basis of cohesin cleavage by separase. *Nature* **532**, 131-134,
227 doi:10.1038/nature17402 (2016).
- 228 10 Wirth, K. G. *et al.* Separase: a universal trigger for sister chromatid disjunction but not chromosome cycle
229 progression. *J Cell Biol* **172**, 847-860, doi:jcb.200506119 [pii] 10.1083/jcb.200506119 (2006).
- 230 11 Zou, H., McGarry, T. J., Bernal, T. & Kirschner, M. W. Identification of a vertebrate sister-chromatid
231 separation inhibitor involved in transformation and tumorigenesis. *Science* **285**, 418-422, doi:7655 [pii]
232 (1999).
- 233 12 Boos, D., Kuffer, C., Lenobel, R., Korner, R. & Stemmann, O. Phosphorylation-dependent binding of cyclin
234 B1 to a Cdc6-like domain of human separase. *The Journal of biological chemistry* **283**, 816-823,
235 doi:M706748200 [pii] 10.1074/jbc.M706748200 [doi] (2008).
- 236 13 Hellmuth, S. *et al.* Positive and negative regulation of vertebrate separase by Cdk1-cyclin B1 may explain
237 why securin is dispensable. *The Journal of biological chemistry* **290**, 8002-8010,
238 doi:10.1074/jbc.M114.615310 (2015).
- 239 14 Huang, X. *et al.* Preimplantation mouse embryos depend on inhibitory phosphorylation of separase to
240 prevent chromosome missegregation. *Molecular and cellular biology* **29**, 1498-1505, doi:MCB.01778-08
241 [pii] 10.1128/MCB.01778-08 (2009).
- 242 15 Huang, X. *et al.* Inhibitory phosphorylation of separase is essential for genome stability and viability of
243 murine embryonic germ cells. *PLoS biology* **6**, e15, doi:07-PLBI-RA-1294 [pii]
244 10.1371/journal.pbio.0060015 (2008).
- 245 16 Huang, X., Hatcher, R., York, J. P. & Zhang, P. Securin and separase phosphorylation act redundantly to
246 maintain sister chromatid cohesion in mammalian cells. *Mol Biol Cell* **16**, 4725-4732, doi:E05-03-0190 [pii]
247 10.1091/mbc.E05-03-0190 (2005).
- 248 17 Stemmann, O., Zou, H., Gerber, S. A., Gygi, S. P. & Kirschner, M. W. Dual inhibition of sister chromatid
249 separation at metaphase. *Cell* **107**, 715-726, doi:S0092-8674(01)00603-1 [pii] (2001).
- 250 18 Li, M., York, J. P. & Zhang, P. Loss of Cdc20 causes a securin-dependent metaphase arrest in two-cell
251 mouse embryos. *Molecular and cellular biology* **27**, 3481-3488, doi:10.1128/MCB.02088-06 (2007).
- 252 19 Hellmuth, S. *et al.* Human chromosome segregation involves multi-layered regulation of separase by the
253 peptidyl-prolyl-isomerase Pin1. *Molecular cell* **58**, 495-506, doi:10.1016/j.molcel.2015.03.025 (2015).

- 254 20 Gorr, I. H., Boos, D. & Stemmann, O. Mutual inhibition of separase and Cdk1 by two-step complex
255 formation. *Molecular cell* **19**, 135-141, doi:S1097-2765(05)01346-8 [pii] 10.1016/j.molcel.2005.05.022
256 [doi] (2005).
- 257 21 Rodriguez-Bravo, V. *et al.* Nuclear pores protect genome integrity by assembling a premitotic and Mad1-
258 dependent anaphase inhibitor. *Cell* **156**, 1017-1031, doi:10.1016/j.cell.2014.01.010 (2014).
- 259 22 Holland, A. J. & Taylor, S. S. Cyclin-B1-mediated inhibition of excess separase is required for timely
260 chromosome disjunction. *Journal of cell science* **119**, 3325-3336, doi:jcs.03083 [pii] 10.1242/jcs.03083
261 (2006).
- 262 23 Clift, D., Bizzari, F. & Marston, A. L. Shugoshin prevents cohesin cleavage by PP2A(Cdc55)-dependent
263 inhibition of separase. *Genes Dev* **23**, 766-780, doi:10.1101/gad.507509 (2009).
- 264 24 Tsou, M. F. & Stearns, T. Mechanism limiting centrosome duplication to once per cell cycle. *Nature* **442**,
265 947-951, doi:nature04985 [pii] 10.1038/nature04985 (2006).
- 266 25 Kawashima, S. A., Yamagishi, Y., Honda, T., Ishiguro, K. & Watanabe, Y. Phosphorylation of H2A by Bub1
267 prevents chromosomal instability through localizing shugoshin. *Science* **327**, 172-177,
268 doi:10.1126/science.1180189 (2010).
- 269 26 Hauf, S. *et al.* Dissociation of cohesin from chromosome arms and loss of arm cohesion during early
270 mitosis depends on phosphorylation of SA2. *PLoS biology* **3**, e69, doi:04-PLBI-RA-0677R1 [pii]
271 10.1371/journal.pbio.0030069 (2005).
- 272 27 Eytan, E. *et al.* Disassembly of mitotic checkpoint complexes by the joint action of the AAA-ATPase TRIP13
273 and p31(comet). *Proceedings of the National Academy of Sciences of the United States of America* **111**,
274 12019-12024, doi:10.1073/pnas.1412901111 (2014).
- 275 28 Reddy, S. K., Rape, M., Margansky, W. A. & Kirschner, M. W. Ubiquitination by the anaphase-promoting
276 complex drives spindle checkpoint inactivation. *Nature* **446**, 921-925, doi:10.1038/nature05734 (2007).
- 277 29 Bakos, G. *et al.* An E2-ubiquitin thioester-driven approach to identify substrates modified with ubiquitin
278 and ubiquitin-like molecules. *Nature communications* **9**, 4776, doi:10.1038/s41467-018-07251-5 (2018).

279 **Acknowledgements**

280 We thank Stefan Heidmann and Peter Wolf for critical reading of the manuscript and Jutta
281 Hübner and Markus Hermann for technical assistance. This work was supported by a grant
282 (STE997/4-2) from the Deutsche Forschungsgemeinschaft (DFG) to O.S. and by MINECO
283 (BFU2017-89408-R) and Junta de Castilla y Leon (CSI239P18). CIC-IBMCC is supported by
284 the Programa de Apoyo a Planes Estratégicos de Investigación de Estructuras de
285 Investigación de Excelencia cofunded by the Castilla–León autonomous government and
286 the European Regional Development Fund (CLC–2017–01).

287 **Author contributions**

288 L.G.-H. and A.M.P. first discovered the interaction between separase and Sgo2. S.H. carried
289 out all experiments except for the one shown in Extended Data Fig. 1a, which was
290 conducted by L.G.-H.. S.H. and O.S. co-designed the research and wrote the paper.

291 **Author information**

292 Reprints and permissions information is available at www.nature.com/reprints.

293 The authors declare that they have no competing interest.

294 Correspondence and requests for materials should be addressed to [olaf.stemmann@uni-](mailto:olaf.stemmann@uni-bayreuth.de)
295 bayreuth.de.

Figure 1: Mad2-dependent binding of human Sgo2 to separase.

a, Endogenous proteins were immunoprecipitated from taxol-arrested Hek293T cells and analyzed by immunoblotting. Mock = unspecific IgG. **b**, Lysates from taxol-arrested *SECURIN*^{-/-} cells were subjected to consecutive immunodepletions and analyzed by immunoblotting. **c**, Transgenic Hek293 cells depleted of Sgo2 or control-treated (*CTRL.*) were transfected to express siRNA resistant Sgo2 variants as indicated, induced with doxycycline to express Cdk1-cyclin B1 resistant, stabilized Myc-separase-Ser1126Ala, arrested in prometaphase, and then analyzed by IP-immunoblotting and chromosome spreading. Shown are averages (bars) of three independent experiments (dots). WT = wild type. **d**, HeLa-K cells synchronized in the indicated cell cycle phases were subjected to IP-immunoblotting. **e**, Taxol-arrested HeLa-K cells were released with ZM-447439 and subjected to time-resolved IP-immunoblotting. **f**, Control-, Mad1-, or TPR-depleted, thymidine-arrested HeLa-K cells were analyzed by IP-immunoblotting.

Figure 2: Co-depletion of Securin and Sgo2 is cytotoxic and results in premature sister chromatid separation and cohesin cleavage.

a, Exemplary photographs of clonogenic assay with HeLa-K cells transfected with the indicated siRNAs. Scale bar = 1 cm. **b**, siRNA-transfected Hek293T cells were supplemented with taxol and EGCG and analyzed by chromosome spreading. Shown are averages (bars) of three independent experiments (dots). **c**, siRNA-transfected HeLa-K cells were taxol-treated and analyzed by IP-immunoblotting. **d**, Securin- and separase-depleted Hek293T cells expressing siRNA-resistant, transgenic separase-WT, -ΔEEEL (V4), or -MxxIxEE to AxxAxAA (V5) were taxol- and EGCG-treated and analyzed by chromosome spreading and IP-immunoblotting. Shown are averages (bars) of three independent experiments (dots). Arrow head marks auto-cleaved separase.

Figure 3: Sgo2 is a Mad2-dependent, competitive inhibitor of separase.

a, Protease-dead (PD) or active separase was incubated with variants of recombinant Sgo2 and Mad2 and assayed for its ability to cleave ³⁵S-labeled Rad21. Relevant upper and lower parts of the same gel are shown. **b**, ³⁵S-labeled Sgo2 variants were incubated with Mad2 and, where indicated, Polo-like kinase 1 (Plk1) before being assayed for *in vitro*-cleavage by active (ac) separase. Relevant upper and lower parts of the same gel are shown. **c**, Sgo2- and securin-depleted HeLa-K cells were transfected to express the indicated siRNA-resistant, Flag-tagged Sgo2- or securin variants, taxol-arrested and analyzed by IP-immunoblotting.

Figure 4: Trip13-p31^{comet}-dependent disassembly liberates separase from Sgo2-Mad2.

333 **a**, Experimental scheme, corresponding immunoblots and autoradiograph (relevant upper
334 and lower parts of the same gel are shown). A = G184A; B = E253Q; QF = Q83A, F191A; PK =
335 P228A, K229A. **b**, siRNA-transfected, taxol-arrested HeLa-K cells were released with ZM-
336 447439 (ZM) and subjected to time-resolved IP-immunoblotting. **c**, Taxol-arrested
337 *SECURIN*^{-/-} and parental HCT116 cells were supplemented with ZM and APC/C inhibitors or
338 carrier solvent (mock) and analyzed by time-resolved IP-immunoblotting and chromosome
339 spreading. Shown are averages (bars) of four independent experiments (dots). **d**, IP-
340 immunoblotting analysis of one experiment (red diamonds) from (c). **e**, Model of bifurcated
341 regulation of separase. Activation of Mad2 by SAC signaling inhibits APC/C^{Cdc20}, thereby
342 stabilizing securin, and enables shugoshin to directly inhibit separase. Liberation of
343 separase from securin requires Trip13-p31^{comet}-dependent dissociation of Mad2 from
344 Cdc20 followed by APC/C-dependent degradation of securin. In contrast, Trip13-p31^{comet}-
345 dependent dissociation of Mad2 from shugoshin leads to direct activation of associated
346 separase. Note that the alternative dissociation of Mad2 from its targets by APC/C^{Cdc20}-
347 dependent ubiquitylation and the Cdk1-cyclin B1-dependent inhibition of separase were
348 omitted for clarity.
349

350 **Methods**

351 **Antibodies**

352 Antibodies generated within this study are from Charles River Laboratories. Antigenic
353 peptides (Bachem) were coupled via terminal Cys to Maleimide activated KLH
354 (ThermoFisher) prior to immunization. Antibodies were affinity-purified against
355 immobilised antigens - proteins coupled to NHS-activated sepharose (GE Healthcare) or
356 peptides coupled to Sulfo-link (ThermoFisher). The following antibodies were used for
357 immunoblotting according to standard protocols. Antibodies directed against human
358 proteins: Rabbit or guinea pig anti-Sgo2 (1,5 µg/ml; 'ab 1'; anti-DVPPRESHSHSDQSSKC),
359 rabbit anti-Sgo2 (1 µg/ml; 'ab 2'; anti-KSEDLSSERTSRRRRC), guinea pig anti-TRIP13 (raised
360 against full length Trip13), rabbit anti-p31^{comet} (raised against isoform 2 of full length
361 p31^{comet}), rabbit anti-separase¹⁷, mouse anti-securin (1:1,000; MBL, DCS-280), mouse anti-
362 Flag (1:2,000; Sigma-Aldrich, M2), rabbit anti-Sgo2 (1:1,000; Bethyl, A301-262A), rabbit
363 anti-Sgo1 (1:500, Abcam ab21633), mouse anti-Mad2 (1:800; Santa Cruz Biotechnology,
364 17D10), rabbit anti-Mad2 (1:1,000; Bethyl, A300-300A), mouse anti-Mad1 (1:1,000; Sigma-
365 Aldrich, 9B10), mouse anti-APC7 (1:800; ThermoFisher, PA5-20948), rabbit anti-
366 *phospho*Ser10-histone H3 ('H3-pS10'; 1:1,000; Millipore, 06-570), mouse anti-PP2A-C
367 (1:1,000; Millipore, 1D6), mouse anti-cyclin B1 (1:1,000; Millipore, 05-373), goat anti-Cdc27
368 (³⁰), mouse anti-topoisomerase IIα (1:1,000; Enzo Life Sciences, 1C5), mouse anti-cyclin A2
369 (1:200; Santa Cruz Biotechnology, 46B11), mouse anti-Rad21 (1:800; Santa Cruz
370 Biotechnology, B-2), rabbit anti-Rad21 (1:1,000; Bethyl, A300-080A). Antibodies directed
371 against *Xenopus* proteins: Rabbit anti-Mad2 (raised against full-length protein), four
372 different rabbit anti-Sgo1 (raised against amino acids 200-300, 300-400, 400-500, and 500-
373 600 of *X.l. Sgo1*)⁷, rabbit anti-separase³¹. Other antibodies: Mouse anti-Myc (hybridoma
374 supernatant 1:50; DSHB, 9E10), rat anti-HA (1:2,000; Roche, 3F10), rabbit anti-ovalbumin
375 (1:1,000; ThermoFisher, PA1-196), mouse anti-ubiquitinated proteins (1:1,000; Millipore,
376 FK2), mouse anti-GFP³², and mouse anti-α-tubulin (hybridoma supernatant 1:200; DSHB,
377 12G10). For immunoprecipitation experiments, the following affinity matrices and
378 antibodies were used: Mouse anti-Myc agarose (Sigma-Aldrich, 4A6), mouse anti-Flag M2-
379 Agarose (Sigma-Aldrich), mouse anti-Rad21 coupled to protein G sepharose (GE
380 Healthcare). Rabbit anti-separase (human), rabbit anti-securin³⁰, rabbit anti-Sgo2 and rabbit
381 anti-Sgo1 (human) were coupled to protein A sepharose (GE healthcare). To precipitate

382 *Xenopus laevis* (X.l.) separase from CSF-extract corresponding separase antibody or
383 unspecific rabbit IgG were coupled to magnetic protein A Dynabeads® (Invitrogen). For
384 non-covalent coupling of antibodies to beads, 10 µl of the respective matrix were rotated
385 with 2-5 µg antibody for 90 min at room temperature and then washed three times with
386 LP2 lysis buffer. For immunofluorescence microscopy (IFM), rabbit anti-Cap-E (anti-
387 CAKSKAKPPKGAHVEV) and mouse anti-Rad21 (1:500; Millipore, 05-908) were used. Isolated
388 centrosomes were stained with rabbit anti-centrin-2, guinea-pig anti-C-Nap1 and mouse
389 anti-γ-Tubulin (Sigma-Aldrich, GTU-88) as previously described³³. Secondary antibodies (all
390 1:500): Cy3 donkey anti-guinea pig IgG and Cy3 goat anti-rabbit IgG (Invitrogen), Marina-
391 Blue goat anti-mouse IgG (ThermoFisher), Alexa Fluor 488 goat anti-rabbit IgG, and Alexa
392 Fluor 488 goat anti-mouse IgG (both Invitrogen).

393 **Cell lines**

394 hTERT RPE-1 cells were purchased from ATCC (CRL-4000). Hek293 Flp-In TRex cells were
395 purchased from Invitrogen (R78007). All other cell lines were gifts: Hek293T from Marc W.
396 Kirschner (Harvard Medical School), HeLa-K from Daniel Gerlich (IMBA), SECURIN knock-out
397 and parental HCT116 from Christoph Lengauer (Johns Hopkins). Validation procedures for
398 purchased cell lines are described by the corresponding manufacturers. All other cell lines
399 were authenticated via visual inspection of typical morphology, by immunoblotting
400 analyses (e.g. absence of securin), cell synchronization behavior, efficiencies of different
401 transfection reagents and resistance to certain antibiotics. Cell lines were not tested for
402 mycoplasma contamination but microscopic inspections of their fluorescently labeled DNA
403 contents were inconspicuous.

404 All human cells were cultured in DMEM (Biowest) supplemented with 10% FCS (Sigma-
405 Aldrich) at 37°C and 5% CO₂. Generation of a stably transgenic Hek293-FlpIn-TRex line
406 (Invitrogen) expressing Myc₆-separase-S1126A upon induction with doxycycline has been
407 described¹³. For transient expression of FLAG₃-Tev₂-Sgo2 variants (WT, R153D, N53I,
408 M114R, RKK124-126A, LSE127-129A, HSDQ239-42A) Hek293T or HeLa-K cells were
409 transfected with the corresponding pCS2-based plasmids using a calcium phosphate-based
410 method or Lipofectamine 2000 (Invitrogen). For time-lapse experiments HeLa cells stably
411 expressing histone H2B-eGFP were used. Additionally, H2B-mCherry-Scc1₁₀₇₋₂₆₈-eGFP¹⁹ was
412 transiently transfected to visualise premature separase activation.

Cell treatments

For synchronization in early S-phase, cells were treated with 2 mM thymidine (Sigma-Aldrich) for 20 h. Synchronization of cells in prometaphase was done by addition of taxol (LC Laboratories) to 0.2 µg/ml 6 h after release from a single thymidine block. G2 arrest was achieved by addition of 10 µM RO-3306 (Santa-Cruz Biotechnology) 4 h after G1/S release. To analyse cells in G1-phase, cells were harvested 15 h after release from a single thymidine block. For the 'taxol-ZM override' experiments, taxol-arrested mitotic HeLa-K cells were harvested by shake-off and released for the indicated times by reseeding into medium supplemented with ZM-447439 (5 µM, Tocris Biosciences), taxol (0.2 µg/ml), cycloheximide (30 µg/ml, Sigma-Aldrich) and, where indicated, with proTame (6 µM, Boston Biochemicals) and Apcin (20 µM, Tocris Bioscience). To further enrich endogenous separase-Sgo2-Mad2 complex for later isolation, securin was depleted by RNAi.

Immunoprecipitation

1×10⁷ cells were lysed with a dounce homogenizer in 1 ml LP2 lysis buffer (20 mM Tris-HCl pH 7.7, 100 mM NaCl, 10 mM NaF, 20 mM β-glycerophosphate, 5 mM MgCl₂, 0.1% Triton X-100, 5% glycerol), combined with benzonase (ad 30 U/l; Santa-Cruz Biotechnology), and incubated on ice for 1 h. To preserve phosphorylations, lysis buffer was additionally supplemented with 50 nM calyculin A (LC-Laboratories) and 1µM microcystin LR (Alexis Biochemicals). Corresponding lysates were centrifuged at 2,500 g for 10 min followed by incubation of 1 ml cleared lysate with 10 µl of antibody loaded beads for 4 h or overnight at 4°C and washed 5x with LP2. In some cases, immobilized hSgo2 variants (purified from G1 cells) or endogenous Separase-Sgo2-Mad2 complex (purified from mitotic cells) were starting material for further experiments before bound proteins were eluted by boiling in SDS-sample buffer. For consecutive immunoprecipitation from *SECURIN*^{-/-} cells, lysate used for the first purification was kept and served as origin for the second precipitation. For immunoprecipitation of *X.l.* separase, CSF-arrested *Xenopus* egg extract was prepared as previously described³⁴ and combined with cycloheximide (100 µg/ml), recombinant human Δ90-cyclin B1 (23 ng/µl ≈ 500 nM)¹⁹ and sperm nuclei (2.000 µl⁻¹). After 15 min at room temperature, the egg extract was released into anaphase II by addition of CaCl₂ (0.6 mM). Previously prepared mock- or *X.l.* separase antibody-coupled magnetic beads were equilibrated in CSF-XB followed by addition of anaphase extract (minimal volume of 500 µl)

and consecutive incubation for 45 min at 18°C. After re-isolation, beads were washed 5 times in CSF-XB supplemented with 300 mM NaCl and 0.01 % Triton X-100.

RNA interference

For efficient knockdown, cells were calcium phosphate or RNAiMax (Invitrogen) transfected with 70-100 nM siRNA duplex of

SECURIN: 5'-UCUUAGUGCUUCAGAGUUUGUGUGUAU-3',

SGO2: 5'-GAACACAUUUCUUCGCCUATT-3',

MAD2: 5'-GAGUCGGGACCACAGUUUAUU-3',

MAD1: 5'-AACCAGCGGCUCAAGGAGGUU-3',

P31^{comet}: 5'-GGCUGCUGUCAGUUUACUUTT-3',

TRIP13: 5'-CUGAUGAAGUGUCAGAUCA-3'

TRP: 5'-GGGUGAAGAUAGUAAUGAAUUCTT-3'.

Transfected cells were grown for 12-24 h before synchronization procedures were applied.

LUCIFERASE siRNA (GL2) was used as negative control (*CTRL.*).

Immunofluorescence staining and microscopy

Hela-K transfected with the indicated siRNAs were grown on poly-lysine coated glass coverslips and processed 8 h after thymidine release in presence of BI-2536 (10 nM) to slow down prophase in early mitotic cells. To get rid of soluble proteins, cells were pre-extracted (PBS, 0.3% Triton X-100) for 5 min washed once with PBS and fixed with fixation-solution (PBS, 3.7% formaldehyde, 0.3% Triton X-100) for 10 min at room temperature.

Subsequently coverslips were washed twice with quenching-solution (PBS, 100 mM glycine), incubated with permeabilization-solution (PBS, 0.5% Triton X-100) for 5 min, washed once with PBS and then incubated in blocking-solution (PBS, 1% (w/v) BSA) for 1 h at room temperature. Coverslips were transferred into a wet chamber and incubated with primary antibodies for 1 h followed by 4 washes with PBS-Tx (PBS, 0.1% Triton X-100). After incubation with fluorescently labelled secondary antibodies for 40 min, samples were washed once, stained for 10 min with 1 µg/ml Hoechst 33342 in PBS-Tx and washed again 4 times. Finally, coverslips were mounted in 20 mM Tris-HCl pH 8.0, 2,33% (w/v) 1,4-diazabicyclo(2.2.2)octane, 78% glycerol on a glass slide. IFM of fixed cells was performed on a DMI 6000 inverted microscope (Leica) using a HCX PL APO 100x/1.40-0.70 oil objective.

To identify early mitotic nuclei DNA morphology (commencing condensation) and

condensin staining intensity were examined. For representative images Z-stacks series over 4 μm in 0.2 μm increments were collected, deconvoluted and projected into one plane using the LAS-AF software. Chromosome spreads were prepared using Canoy's solution as described³⁵. Spreads were observed with the Zeiss Axioplan 2 Imaging microscope using a Plan-APOCHROMAT 100x/1.40 Oil objective. A cell was counted as suffering from PCS when exhibiting loss of cohesion of > 50% of its chromosomes. At least 100 spreads were counted per condition. To assess centriole engagement status, centrosomes were isolated from RO-3306 arrested HeLa-K cells (4×10^6) 36 h after transfection of indicated siRNAs and stained as previously described³³.

Clonogenic assays

12 h after siRNA transfection, HeLa-K, Hek293T, HCT116 parental or *SECURIN*^{-/-} cells were seeded in 10-cm dishes (100 cells per plate and condition). Cells were grown for 10 days and then fixed in ice-cold methanol for 10 min. Staining was performed as described³⁶ except for the use of ethanol instead of glutaraldehyde. The number of colonies per plate with a minimal area of 20 (circularity 0.00-1.00) was determined with ImageJ particle analysis software.

***In vitro* disassembly of separase-Sgo2-Mad2 complex**

10 μl of immobilized separase-Sgo2-Mad2 complex was incubated with 2 mg/ml recombinant variants of Trip13 (WT, A: Walker A mutant Gly184Ala, B: Walker B mutant Glu253Gln) and RGS-His₆-p31^{comet} (WT, QF: Q83A/F191A Mad2-binding deficient, PK: P228A/K229A Trip13-binding deficient) in EDTA/EGTA-free lysis buffer supplemented with ATP (1 mM) in a final volume of 30 μl for 30 min at 18°C. Subsequently, 15 μl of the corresponding supernatant was removed for later analysis. Beads were washed three times with LP2 and then equilibrated in cleavage buffer (10 mM Hepes-KOH pH 7.7, 50 mM NaCl, 25 mM NaF, 1 mM EGTA, 20% glycerol). To monitor separase activity, 2 μl of *in vitro* translated ³⁵S-Rad21-GFP were added to a volume of 30 μl . Following incubation for 30 min at 30°C, reactions were stopped by boiling in SDS-sample buffer. Assaying disassembly by *in vitro* ubiquitylation (Extended Data Figure 12) was performed essentially as described³⁰ with the exception that instead of securin 10 μl immobilized separase-Sgo2-Mad2 complex were added as substrate.

Bacterially expressed proteins

pET28-vector encoded, His₆-SUMO₁-tagged³⁷ variants of Trip13 (NP_004228) and p31^{comet} (NP_055443) were expressed individually in *E. coli*. Rosetta 2 DE3 (Novagen). Bacteria were lysed in LP1 (PBS, 5 mM imidazole, 0.5 mM DTT and additional 400 mM NaCl) and purified over Ni²⁺-NTA-Agarose (Qiagen) according to standard procedure. Following elution with PBS supplemented with 250 mM imidazole, 0.5 mM DTT and additional 400 mM NaCl (pH adjusted to 7.5 with HCl), proteins were dialyzed at 4°C against LP1 in presence of His₆-SenP2 (10 ng per 100 µg of protein) and then rotated for 3 h over 9/10th the amount of fresh Ni²⁺-NTA-Agarose. Supernatants containing pure Trip13 and p31^{comet} were dialyzed against 50 mM Hepes-KOH pH 7.7, 10 % glycerol, 100 mM NaCl, 5 mM MgCl₂, 1 mM EDTA and 1mM DTT. Human Mad2 and X.l. Mad2 were purified as described previously⁷.

H2A-peptide binding assay

Thr-9-phosphorylated or unmodified histone H2A-peptide (QAVLLPKKTESHKAKGK) were from Bachem; Ser-10-phosphorylated or unmodified histone H3-peptides were purchased from Eurogentec (AS-61702 and AS-64611). 1 mg each of the H2A peptides were conjugated to 10 mg/ml maleimide activated ovalbumin (ThermoFisher) according to manufacture's instructions and dialyzed against CSF-XB³⁴ containing 0.5 mM DTT. Reactions (final volume of 30 µl) containing 10 µl immobilized FLAG₃-Tev₂-Sgo2 and 1 µg ovalbumin-coupled-H2A peptide, 5 µg free H2A- or H3 peptide, or 4 µg of recombinant Mad2 were assembled and incubated for 30 min at 18°C. Unbound peptide/protein was removed by four washes with CSF-XB and the second reaction was assembled with the corresponding counterpart and again incubated for 30 min at 18°C. Samples were again washed (four times with CSF-XB containing 0.01 % Triton X-100) before beads were eluted by boiling in SDS-sample buffer.

Mapping experiments in X.l. Sgo1

N-terminally tagged Myc₆- or FLAG₃-tagged X.l. Sgo1 variants were *in vitro* translated in rabbit reticulocyte lysate (TNT Quick, Promega) according to manufactures protocol. Mapping by the help of interaction blocking antibodies, 16 µl Sgo1 were combined with 2.5 µg anti-X.l. Sgo1 and 5 µg recombinant X.l. Mad2 and incubated for 30 min at 18 °C. Then, 10 µl of X.l. Separase loaded magnetic beads (isolated from anaphase egg extract) were added and reactions were incubated for 30 min at 18 °C. Beads were washed four times

536 with CSF-XB, 0.01 % Triton X-100 and twice with CSF-XB, 0.01 % Triton X-100, 300 mM NaCl
537 and finally eluted by boiling in SDS-sample buffer. In all other cases 12.5 µl Sgo1 and 5 µg
538 Mad2 were pre-incubated in 300 µl CSF-XB, 0.01 % Triton X-100 followed by addition of 10
539 µl immobilized Separase.

540 **Separase-inhibition and -cleavage assays**

541 For separase inhibition 10 µl of FLAG₃-Tev₂-Sgo2 bound to anti-FLAG beads and 4 µg
542 recombinant Mad2 were incubated in cleavage buffer (total volume of 28 µl) for 10 min at
543 18°C followed by the addition of 2.5 µl active (ac, S1126A variant) or protease dead (PD,
544 S1126A + C2029S) separase¹⁷. After a 10 min incubation at room temperature, 2 µl *in vitro*
545 translated, ³⁵S-labeled Rad21-GFP were added. Reactions were stopped after 30 min by
546 boiling in SDS-sample buffer. Samples were separated by SDS-PAGE and blotted onto PVDF
547 membrane (SERVA), which was cut and analysed by immunoblotting prior to reassembly
548 and autoradiography. For Fig. 3b, 2 µl of each *in vitro* translated, ³⁵S-labeled Sgo2 variant
549 was combined with 1 µg human Mad2 and, where indicated, 0.1 µg Plk1 (ProKinase; No.
550 0183-0000-1) in modified cleavage buffer (10 mM Hepes-KOH pH 7.7, 50 mM NaCl, 25 mM
551 NaF, 20% glycerol, 1 mM ATP, 10 mM MgCl₂) and a total volume of 15 µl. After 15 min at
552 30°C, 2.5 µl separase (ac or PD) were added and reactions were incubated for 30 min at
553 room temperature. For Extended Data Fig. 10, 2 µl of each *in vitro* translated, ³⁵S-labeled
554 X.l. Sgo1 variant were combined with 1 µg X.l. Mad2-WT or -ΔC10 and 10 µl of immobilized
555 X.l. separase. Samples were further processed and analysed as described above.

556 **Live-cell imaging**

557 Cells in phenol red-free medium were seeded into µ-slide 8-well chambered coverslips
558 (Ibidi) and kept in an atmosphere of 37°C and 5% humidified CO₂ during microscopy on a
559 DMI 6000 inverted microscope (Leica). For imaging of unperturbed mitosis, GFP and DIC
560 images were captured 6 h after release from thymidine arrest in 10 min intervals over a
561 period of 15 h, through a HCX PL APO 40x/0.85 CORR objective. Changes in focus plane due
562 to mitotic rounding of the cells were compensated by collecting Z-stacks at each time point.
563 Captured images from each experiment were analysed using the corresponding LAS-AF
564 software (Leica).

565 **Statistics and Reproducibility**

566 Experiments analysed by immunoblotting were repeated 2-4 times with similar results (2-4

567 biological replicates). For quantitative analyses of chromosome spreads, clonogenic assays,
568 and IFM specimen the investigators were blinded to sample allocation.

569 **Data availability**

570 The authors declare that all data supporting the findings of this study are available within
571 the paper and its supplementary information files. If there is reasonable request additional
572 source data can also be provided from the corresponding author.

Supplementary References

- 573 30 Hellmuth, S., Bottger, F., Pan, C., Mann, M. & Stemmann, O. PP2A delays APC/C-dependent degradation of
574 separase-associated but not free securin. *The EMBO journal* **33**, 1134-1147, doi:10.1002/embj.201488098
575 (2014).
- 576 31 Gorr, I. H. *et al.* Essential CDK1-inhibitory role for separase during meiosis I in vertebrate oocytes. *Nature*
577 *cell biology* **8**, 1035-1037, doi:ncb1467 [pii] 10.1038/ncb1467 [doi] (2006).
- 578 32 Hellmuth, S., Gutierrez-Caballero, C., Llano, E., Pendas, A. M. & Stemmann, O. Local activation of
579 mammalian separase in interphase promotes double-strand break repair and prevents oncogenic
580 transformation. *The EMBO journal* **37**, doi:10.15252/embj.201899184 (2018).
- 581 33 Schockel, L., Mockel, M., Mayer, B., Boos, D. & Stemmann, O. Cleavage of cohesin rings coordinates the
582 separation of centrioles and chromatids. *Nat Cell Biol* **13**, 966-972, doi:10.1038/ncb2280 (2011).
- 583 34 Murray, A. W. Cell cycle extracts. *Methods Cell Biol* **36**, 581-605 (1991).
- 584 35 McGuinness, B. E., Hirota, T., Kudo, N. R., Peters, J. M. & Nasmyth, K. Shugoshin prevents dissociation of
585 cohesin from centromeres during mitosis in vertebrate cells. *PLoS biology* **3**, e86, doi:04-PLBI-RA-0678R1
586 [pii] 10.1371/journal.pbio.0030086 (2005).
- 587 36 Franken, N. A., Rodermond, H. M., Stap, J., Haveman, J. & van Bree, C. Clonogenic assay of cells in vitro.
588 *Nature protocols* **1**, 2315-2319, doi:10.1038/nprot.2006.339 (2006).
- 589 37 Butt, T. R., Edavettal, S. C., Hall, J. P. & Mattern, M. R. SUMO fusion technology for difficult-to-express
590 proteins. *Protein Expr Purif* **43**, 1-9, doi:10.1016/j.pep.2005.03.016 (2005).
- 591 38 Holland, A. J., Bottger, F., Stemmann, O. & Taylor, S. S. Protein phosphatase 2A and separase form a
592 complex regulated by separase autocleavage. *The Journal of biological chemistry* **282**, 24623-24632,
593 doi:M702545200 [pii] 10.1074/jbc.M702545200 [doi] (2007).
- 594 39 Schagger, H. Tricine-SDS-PAGE. *Nature protocols* **1**, 16-22, doi:10.1038/nprot.2006.4 (2006).

Extended Data Figure Legends

Extended Data Figure 1: Mammalian Sgo2 interacts with separase.

a, Hek293T cells were co-transfected with expression vectors for the following *M.m.* proteins: GFP-separase, Flag-SGO2 and securin. Immunoprecipitation was carried out from transfected cells with either an anti-Flag and anti-GFP antibody and analysed by immunoblotting with the indicated antibodies. **b**, Sgo2 and Mad2 specifically interact with separase also in untransformed cells. Taxol-arrested RPE1 cells were subjected to IP-immunoblotting analyses as indicated. **c**, Even in securin expressing cells, a considerable fraction of separase is sequestered by Sgo2. Taxol-arrested Hek293T, HCT116, and HeLa-K cells were subjected to IP-immunoblotting analyses using the indicated antibodies. ab 1 = anti-DVPPRESHSHSDQSSKC (corresponding to amino acids 230-245 of human Sgo2); ab 2 = anti-KSEDLSSERTSRRRRC (corresponding to amino acids 1234-1249 of human Sgo2); rb = rabbit; mo = mouse. Given below are the relative intensities (in %) of the separase signals (sum of full-length and N-terminal auto-cleavage fragment). Note the considerable co-depletion of separase upon Sgo2-IP from HeLa-K (right panels). **d**, Murine separase does not interact with human Sgo2 and, therefore, cannot be used to study separase regulation in human cells. Securin-depleted Hek293T cells expressing GFP-tagged human separase-Ser1126Ala or murine separase-Ser1121Ala were taxol arrested and then subjected to IP-immunoblotting analyses using the indicated antibodies. WCE = whole cell extracts.

Extended Data Figure 2: Sgo1 rather than -2 interacts with Mad2 and separase in *Xenopus*.

The indicated variants of *in vitro*-expressed *Xenopus laevis* (*X.l.*) shugoshins and an excess of *E. coli* expressed Mad2 (to mimic SAC signaling) were incubated in anaphase egg extract (left). Following IP with anti-*X.l.* separase or mock-IgG from these mixtures, isolated proteins were detected by immunoblotting (middle and right). R170A = Mad2-binding deficient Sgo1; Δ C = Sgo1-binding deficient, C-terminally truncated Mad2.

Extended Data Figure 3: Lack of securin and Sgo2 or Mad2 have synergistic cytotoxic effects.

a-c, Clonogenic assays with HeLa-K (**a**), *SECURIN*^{-/-} and parental HCT116 (**b**), and Hek293T cells (**c**) transfected with the indicated siRNAs. Shown are percentages of colony numbers relative to the control (bars) of three independent experiments (dots).

Extended Data Figure 4: Depletion of securin and Sgo2 but not the individual knock-downs result in impaired chromosome alignment and premature loss of chromosomal cohesin.

a, HeLa-K cells transfected with the indicated siRNAs and a histone H2B-eGFP expression plasmid were observed by video fluorescence microscopy to assess metaphase plate formation. Shown are averages (bars) of three independent experiments (dots) counting at least 50 mitotic cells each. Scale bar = 5 μ m. **b**, Eight hours after release from a thymidine arrest, HeLa-K cells transfected with the indicated siRNAs were pre-extracted, fixed, and examined by fluorescence microscopy for cohesin-negative early mitotic chromatin. Shown are representative images (left) and averages (bars) of three independent experiments (dots) counting prophase nuclei that were still round but already stained positive for condensin (100 each). Scale bar = 5 μ m.

Extended Data Figure 5: Depletion of securin and Sgo2 but not the individual knock-downs result in premature loss of cohesion and unscheduled separase activity.

a, Hek293T cells transfected with the indicated siRNAs were supplemented with taxol and analyzed by chromosome spreading. Shown are averages (bars) of three independent experiments (dots). **b**, Exemplary immunoblots of cells analysed in (a) and Fig. 2b. Star denotes unspecific band. **c**, siRNA-transfected HeLa-K cells expressing separase activity sensor (H2B-mCherry-Rad21^{aa107-268}-eGFP) were released from a taxol arrest by ZM-447439 addition at time zero and analysed by time-resolved immunoblotting.

Extended Data Figure 6: Identification of Sgo2-binding deficient separase variants.

Taxol-arrested Hek293T cells expressing transgenic Myc-tagged wild type separase (WT) or one of the indicated variants (V1-5) were analysed by IP-immunoblotting using the indicated antibodies. The investigation of PP2A-binding deficient variants was motivated by the notion that Sgo2-Mad2, like PP2A, preferentially interacts with full-length rather than auto-cleaved separase (see, for example, Fig. 3c)³⁸. Note that V3 cannot bind PP2A but Sgo2-Mad2 indicating overlapping but not identical binding sites.

Extended Data Figure 7: Depletion of securin and Sgo2 but not the individual knock-downs result in premature disengagement of centrioles.

HeLa-K cells transfected with the indicated siRNAs were released from a thymidine block and arrested in G2 phase with the Cdk1 inhibitor RO-3306. Corresponding lysates were used for immunoblotting and centrosome isolation followed by immunofluorescence microscopy to assess the degree of centriole disengagement as revealed by two C-Nap1 foci. Shown are representative images and averages (bars) of three independent experiments (dots) counting 100 centrosomes each. Scale bar = 1 μ m.

Extended Data Figure 8: Mutually exclusive binding of Sgo2 to Mad2 or phosphorylated histone H2A.

a-d, Pre-charging of Sgo2 with phosphorylated histone H2A blocks subsequent Mad2 binding. **a**, Experimental scheme for experiments shown in (b-d). Beads loaded with the indicated Flag-Sgo2 variants were consecutively incubated first with phosphorylated or un-phosphorylated H2A or H3 peptide (input 1) and then with wild type or, where indicated, C-terminally truncated (Δ C) Mad2 (input2). Following washing (supernatant 2), bound proteins were visualized by immunoblotting. **b**, Usage of free H2A peptides. **c**, Usage of ovalbumin-coupled H2A peptides to facilitate their detection by standard glycine-SDS-PAGE/immunoblotting. **d**, Phosphorylated histone H3 peptide does not bind to Sgo2. Free H2A and H3 peptides, used to interrogate immobilized Sgo2, were separated by Tricine-SDS-PAGE³⁹ and analysed by Coomassie-staining and immunoblotting. **e**, Pre-charging of Sgo2 with Mad2 blocks subsequent phospho-H2A binding. Experimental scheme (top) and corresponding immunoblotting analysis. Note that H2A peptides were coupled to ovalbumin for ease of detection. **f**, Cartoon illustrating that Sgo2 can bind to H2A phosphorylated on Thr-121 or Mad2 but not both at the same time.

Extended Data Figure 9: Mapping separase-binding sites on *X.l.* Sgo1.

a, Covering amino acids 200-300 of *X.l.* Sgo1 with polyclonal antibodies impairs its binding to separase. Region-specific polyclonal *X.l.* Sgo1 antibodies A-D were characterized (left) and added to *in vitro*-expressed *X.l.* Sgo1 and *E. coli*-expressed Mad2 (prey) as indicated. Immobilized *X.l.* separase isolated by IP from anaphase egg extracts was then added as bait

to these mixtures. Separase beads were washed and finally probed for associated proteins by immunoblotting. Mock = unspecific IgG; * = unspecific band. **b-c**, Identification of two sites within *X.l.* Sgo1 that are relevant for separase binding. Different *in vitro* translated (IVT) *X.l.* Sgo1 fragments and variants thereof were combined with Mad2-WT or - Δ C as indicated (prey). Separase beads as in (a) were combined with these mixtures, washed and analyzed for associated proteins by immunoblotting. **d-f**, *X.l.* Sgo1-143-145A and -254-256A (shown in red) are compromised in separase binding but retain Mad2 binding. **d**, The indicated full-length *X.l.* Sgo1 variants were assessed for Mad2-dependent separase binding as in (b, c). **e**, The indicated *X.l.* Sgo1 fragments and variants thereof were combined with Mad2-WT or - Δ C, immunoprecipitated via their Myc-tag and assessed for Mad2 binding by immunoblotting. **f**, Summary of the mapping experiments. MIM = Mad2-interaction motif; n.d. = not determined.

Extended Data Figure 10: Point mutation turns *X.l.* Sgo1 into a Mad2-dependent separase substrate.

³⁵S-labeled *X.l.* Sgo1 variants were incubated with Mad2-WT or - Δ C before being assayed for *in vitro*-cleavage by *X.l.* separase, which had been immunoprecipitated from anaphase egg extracts. Note that gels were blotted onto membranes, which were cut and subjected to immunoblotting (upper panels) prior to reassembly and autoradiography (lower panel).

Extended Data Figure 11: *X.l.* Sgo1 and *H.s.* Sgo2 share the same order and spacing of separase- and Mad2 binding sites.

a, *H.s.* Sgo2-127-129A and -239-242A are compromised in separase binding but retain Mad2 binding. The indicated full-length Sgo2 variants were immunoprecipitated via their Flag-tag from transfected, taxol-arrested Hek293T cells and assessed by immunoblotting for Mad2- and separase binding. See also illustration on right. **b**, Cartoon comparing the arrangement of functional domains and alignment of pseudo-substrate and Mad2 binding site of *X.l.* Sgo1 and *H.s.* Sgo2. Note that the separase interaction site around position 144 of *X.l.* Sgo1 and around position 128 of *H.s.* Sgo2 was omitted for clarity.

Extended Data Figure 12: *In vitro*-disassembly of separase-Sgo2-Mad2 by APC/C^{Cdc20}-dependent ubiquitylation.

Immobilized separase-Sgo2-Mad2 complex isolated as in Fig. 4a was incubated with ATP, ubiquitin, E1, Ube2s, wild type or dominant-negative (DN) Ube2c and APC/C^{Cdc20} (or reference buffer). After removal of the supernatant and washing, the beads were incubated with ³⁵S-Rad21 before being analysed by autoradiography and immunoblotting. Note that the autoradiograph shows the relevant upper and lower parts of the same gel. Ubiquitylation of purified securin served as positive control.

Figure 1

Figure 2

Figure 3

Figure 4