# Aurora B SUMOylation is Restricted to Centromeres in Early Mitosis and Requires RANBP2

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**Keywords:** SUMOylation, Aurora B, in situ proximity ligation assay (isPLA), RANBP2, mitosis

**SUPPLEMENTARY TABLE S1. ANTIBODIES USED IN THIS WORK**

**Immunofluorescence and PLA**

| PRIMARY | HOST | SOURCE |
|---|---|---|
| alpha-tubulin | mouse | T5178, Sigma-Aldrich |
| alpha-tubulin | rat | ab6161, Abcam |
| alpha-tubulin | chicken | ab89984, Abcam |
| Aurora B | rabbit | ab2254, Abcam |
| Aurora B | mouse | 611082, Becton Dickinson |
| Aurora BphThr232 | rabbit | 636102, BioLegend |
| Aurora BphThr232 | mouse | 600-401-677S, Rockland |
| Borealin | rabbit | ab74473, Abcam |
| Borealin | mouse | ab67126, Abcam |
| CREST | human | 15-234-0001 Antibodies Inc |
| CENP F | rabbit | NB 500-101, Novus Biologicals |
| CENPA | mouse | ab13939, Abcam |
| CENP A phThr3 | rabbit | NL41, Millipore |
| gamma-tubulin | mouse | T6557, Sigma-Aldrich |
| H3 phSer10 | mouse | 3H10, Millipore |
| INCENP | mouse | ab23956, Abcam |
| INCENP | mouse | B-4, SC-376514, Santa Cruz Biotechnology Inc. |
| RANBP2 | mouse | D-4 sc-74518, Santa Cruz Biotechnology Inc. |
| SUMO2/3 | mouse | M114-3, MBL Life Science |
| TPX2 | rabbiy | NB 500-179, Novus Biologicals |

| SECONDARY | CONJUGATION | SOURCE |
|---|---|---|
| anti-mouse | Texas Red | Vector |
| anti-mouse IgG | AlexaFluor 647 | Jackson Imm. |
| anti-mouse IgG | AMCA | Jackson Imm. |
| anti-mouse IgG | Rhodamine | Jackson Imm |
| anti-rabbit IgG | CY3 | Jackson Imm. |
| anti-rabbit IgG | FITC | Santa Cruz |
| anti-chicken IgG | Alexa 647 | Jackson Imm. |
| anti-human IgG di | AMCA | Jackson Imm. |
| anti-mouse/anti-rabbit | CY 3.5 | www.olink.com/products/duolink/how-use-duolink |
| anti-mouse/anti-rabbit | CY3 | www.olink.com/products/duolink/how-use-duolink |

**Western immunoblotting**

| PRIMARY | HOST | SOURCE |
|---|---|---|
| actin | goat | I-19 sc1862, Santa Cruz Biotechnology Inc. |
| alpha-tubulin | mouse | B512, Sigma- Aldrich |
| Aurora B | rabbit | N-ter 1-100, ab2254 Abcam |
| GFP | rabbit | ab6556, Abcam |
| GFP | mouse | 11814460001, Roche |
| NUP153 | mouse | QE5 AB81463, Abcam |
| RANBP2 | rabit | N-20, sc-15442 Santa Cruz Biotechnology Inc. |
| RANGAP1 | mouse | N-19, sc-1862 Santa Cruz Biotechnology Inc |

| SECONDARY | CONJUGATION | SOURCE |
|---|---|---|
| anti-rabbit IgG | HRP | sc-2004, Santa Cruz Biotechnology Inc |
| anti-mouse IgG | HRP | sc-2005, Santa Cruz Biotechnology Inc |
| anti-goat IgG | HRP | sc-2354, Santa Cruz Biotechnology Inc |

## SUPPLEMENTARY FIGURE LEGENDS

**Figure S1. Specificity controls for intramolecular PLA assays between Aurora B and SUMO2/3 peptides. A.** Positive controls for PLA reactions of Aurora B with established interacting partners, Borealin and INCENP. **B.** As a negative control, PLA amplification yielded no signal for the Aurora B and gamma-tubulin pair. **C.** RNA interference-mediated Borealin silencing in HeLa cells. After quantitative IF, the extent of Borealin depletion, relative to control cultures interfered with neutral siRNA (GL2), was quantified in the box plots (**** $p<0.0001$, extremely significant difference; Mann-Whitney test, 60 counted cells / group, 2 experiments).

**Figure S2.** Generation of stable cell lines expressing RNAi-resistant Aurora $B^{WT}$-GFP and Aurora $B^{K202R}$-GFP in a dox-inducible manner. **A.** Map of wild-type and K202R mutant Aurora B: both are rendered RNAi-resistant by introducing three close synonymous substitutions in the coding sequence (double arrowhead line) and can therefore be expressed in an Aurora B-silenced background. The coding sequences also carry in-frame Myc and GFP tags cloned downstream of a dox-inducible promoter. **B.** Western blot analysis (40 μg of protein extract per lane) of cells expressing either Aurora $B^{WT}$-GFP or mutant Aurora $B^{K202R}$-GFP RNAi-resistant sequences (78 KDa) probed with GFP antibody. – and + indicate the addition of Aurora-B specific siRNAs. A twin filter was probed with Aurora B antibody to control silencing of the endogenous Aurora B and selective expression of the constructs after dox induction in cells treated with Aurora B-specific siRNAs. Alpha-tubulin was used as a loading control. Only the regions where proteins of interest migrate are shown. **C.** Still images from video recorded cultures induced to express either wild-type or K202R Aurora B, monitored by the GFP signal appearance. **D.** Release from STLC-dependent inhibition of the kinesin kif11 activates Aurora B. IF to $AurB^{phThr232}$ shows that Aurora B activity is stimulated by STLC treatment (2 hours) and even further after STLC release (30 min) cells. The box-and-whiskers plot represent the distribution of $AurB^{phThr232}$ signals in individual cells from control (CTR) and STLC-released cultures (n, 80 cells, 2 experiments). Aurora $B^{phThr232}$ increased significantly in STLC release compared to CTR (**, $p<0.01$).

**Figure S3.** $AuroraB^{K202R}$ mutant interacts with CPC partners. **A.** INCENP PLA products were assessed in cells silenced for the endogenous Aurora B and expressing Aurora $B^{K202R}$: both Aurora B forms retain the ability to interact with INCENP. **B.** INCENP concentrates at centromeres in Aurora $B^{WT}$-GFP-expressing metaphase cells, while being largely diffuse over DAPI-stained chromosomes in metaphase cells expressing $AuroraB^{K202R}$-GFP. In anaphase and telophase both the Aurora $B^{WT}$/INCENP and the $AuroraB^{K202R}$/INCENP pairs localise at the midbody in an undistinguishable manner. **C.** Borealin signals concentrate at metaphase centromeres in cells interfered for the endogenous Aurora B and expressing Aurora $B^{WT}$-GFP; in contrast, cells reconstituted with Aurora $B^{K202R}$-GFP show impaired Borealin localisation at centromeres. All assays in the dox-inducible cell lines were repeated in 3 or 4 independent experiments.

**Figure S4.** RANBP2 silencing in HeLa cells. **A.** Protocol for RANBP2 silencing in HeLa cultures synchronised by thymidine arrest and release. Control cultures were treated with neutral siRNA, indicated as GL2(i). **B.** Western immunoblotting of protein extract (40 μg per lane) shows that RANBP2-specific siRNAs depleted the cells of RANBP2 as well as SUMO-conjugated RANGAP1 (signals are quantified in **C**); the nucleoporin NUP153 used for control dos not vary. Only the regions where proteins of interest migrate are shown. **D.** The panels show the enrichment in mitotic cells in synchronised cultures. Mitotic stages are identified from both their chromosome configuration (DAPI staining) and the spindle organisation (TPX2 staining). RANBP2 was homogeneously depleted in the interfered culture. 40x objective. **E.** High-resolution images showing a control metaphase, treated with neutral GL2 siRNA, with RANBP2 (green) at the spindle (TPX2, red) and at microtubule-attached kinetochores. Chromosomes are stained with DAPI. Below, TPX2 is unaffected in the RANBP2-interfered metaphase. 100 x objective.

**Figure S5.** RANBP2 silencing impairs Aurora B concentration at centromeres. **A.** In control cells interfered with neural siRNAs [GL2(i)], Aurora B IF signals (red) are centromere-restricted; in RANBP2-interfered cells, a chromosome-associated Aurora B fraction is evident in prometaphase and metaphase cells: the mask profile of DAPI (chromosomes) and Aurora B IF shows an almost total overlap in RANBP2-interfered panels. **B.** The histograms represent the percentage of cells displaying either centromere-restricted (CEN), or centromere/chromosome (CEN/CHROM) diffuse localisation of Aurora B in control and RANBP2-interfered samples. Differences are highly significant in prometaphase and metaphase cells (****, $p<0,0001$). Aurora B localisation at the midbody in anaphase and telophase does not vary, with or without RANBP2 (ns, non significant difference). At least 80 cells/samples were counted in 3 experiments and the frequency of patterns were compared using the $\chi 2$ test (two-sided). **C**. RANBP2 interference does not prevent the association of Aurora B with INCENP in PLA reactions.

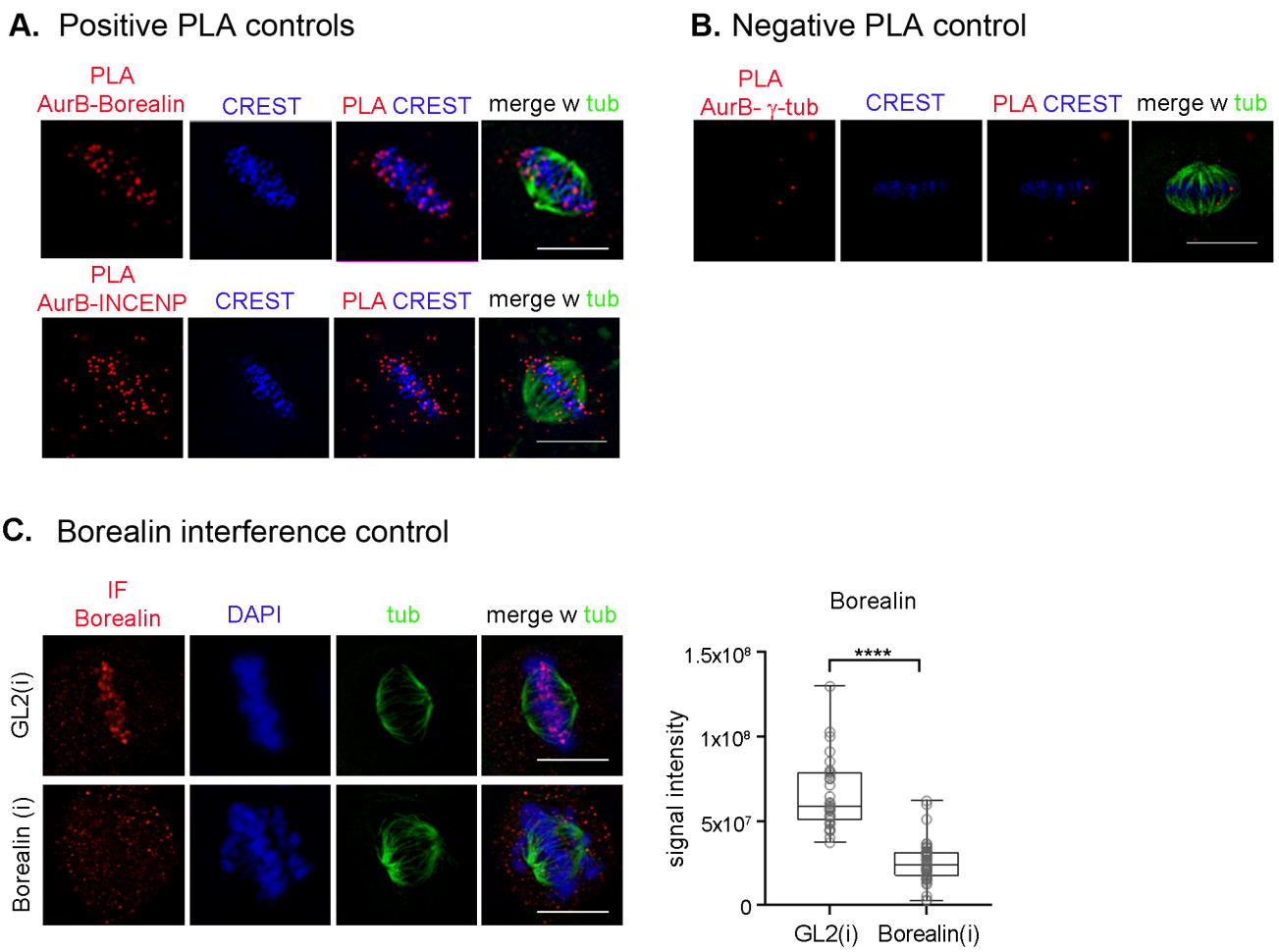
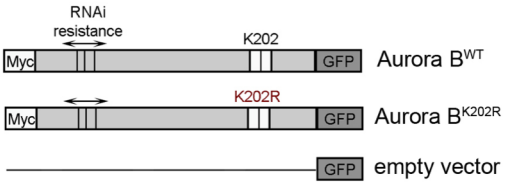
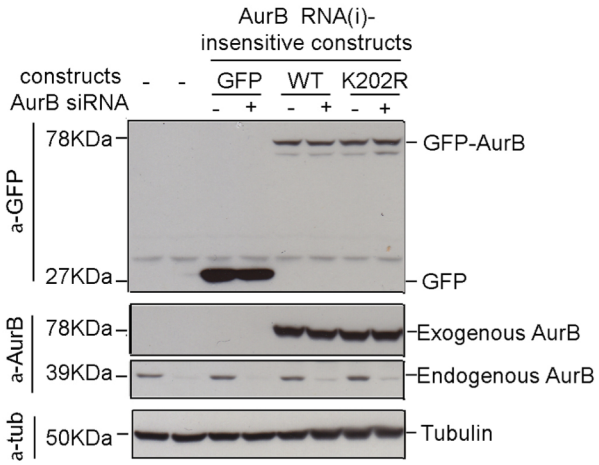
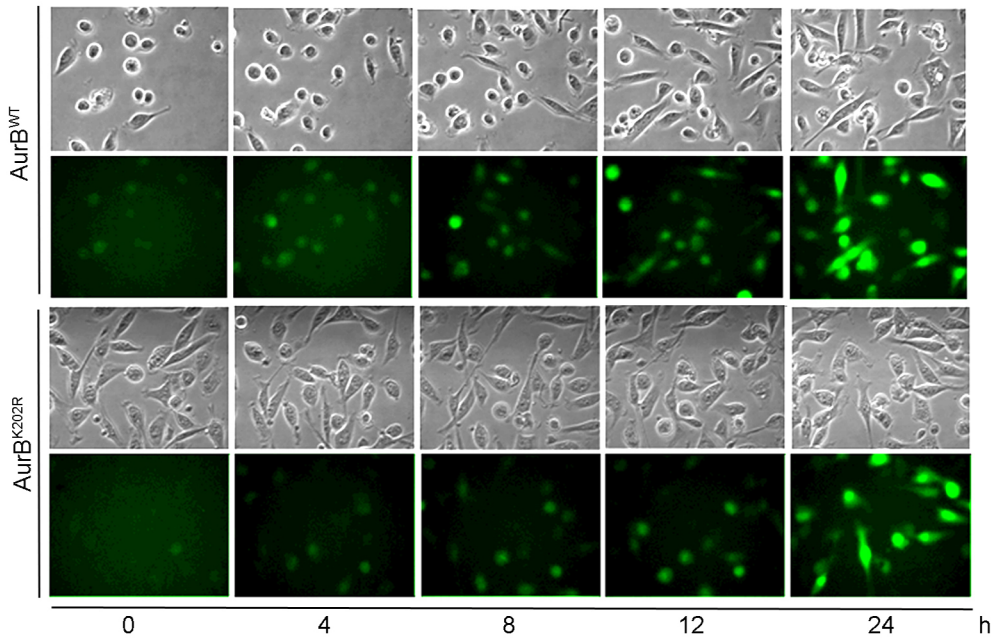

A. Positive PLA controls
PLA
AurB-Borealin
CREST
PLA CREST
merge w tub
PLA
AurB-INCENP
CREST
PLA CREST
merge w tub
B. Negative PLA control
PLA
AurB- γ-tub
CREST
PLA CREST
merge w tub
C. Borealin interference control
IF
Borealin
DAPI
tub
merge w tub
GL2(i)
Borealin (i)
Borealin
1.5x10⁸
1x10⁸
5x10⁷
0
signal intensity
****
GL2(i)
Borealin(i)

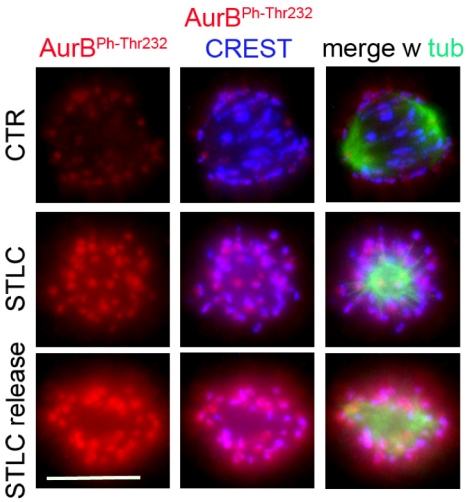
Figure S1

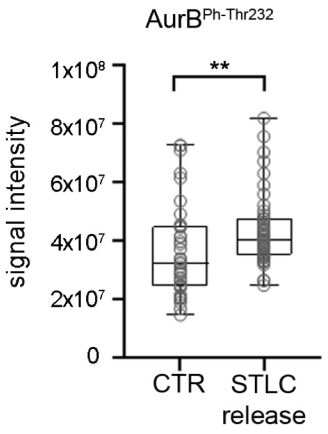
Figure S2


RNAi resistance
K202
Myc
GFP
Aurora B^WT
K202R
Aurora B^K202R
empty vector


AurB RNA(i)-insensitive constructs
constructs
AurB siRNA
GFP
WT
K202R
78KDa
27KDa
a-GFP
GFP-AurB
GFP
a-AurB
Exogenous AurB
39KDa
Endogenous AurB
a-tub
50KDa
Tubulin


AurB^WT
AurB^K202R
0
4
8
12
24
h


AurB^Ph-Thr232
AurB^Ph-Thr232
CREST
merge w tub
CTR
STLC
STLC release


AurB^Ph-Thr232
**
signal intensity
1x10^8
8x10^7
6x10^7
4x10^7
2x10^7
0
CTR
STLC release

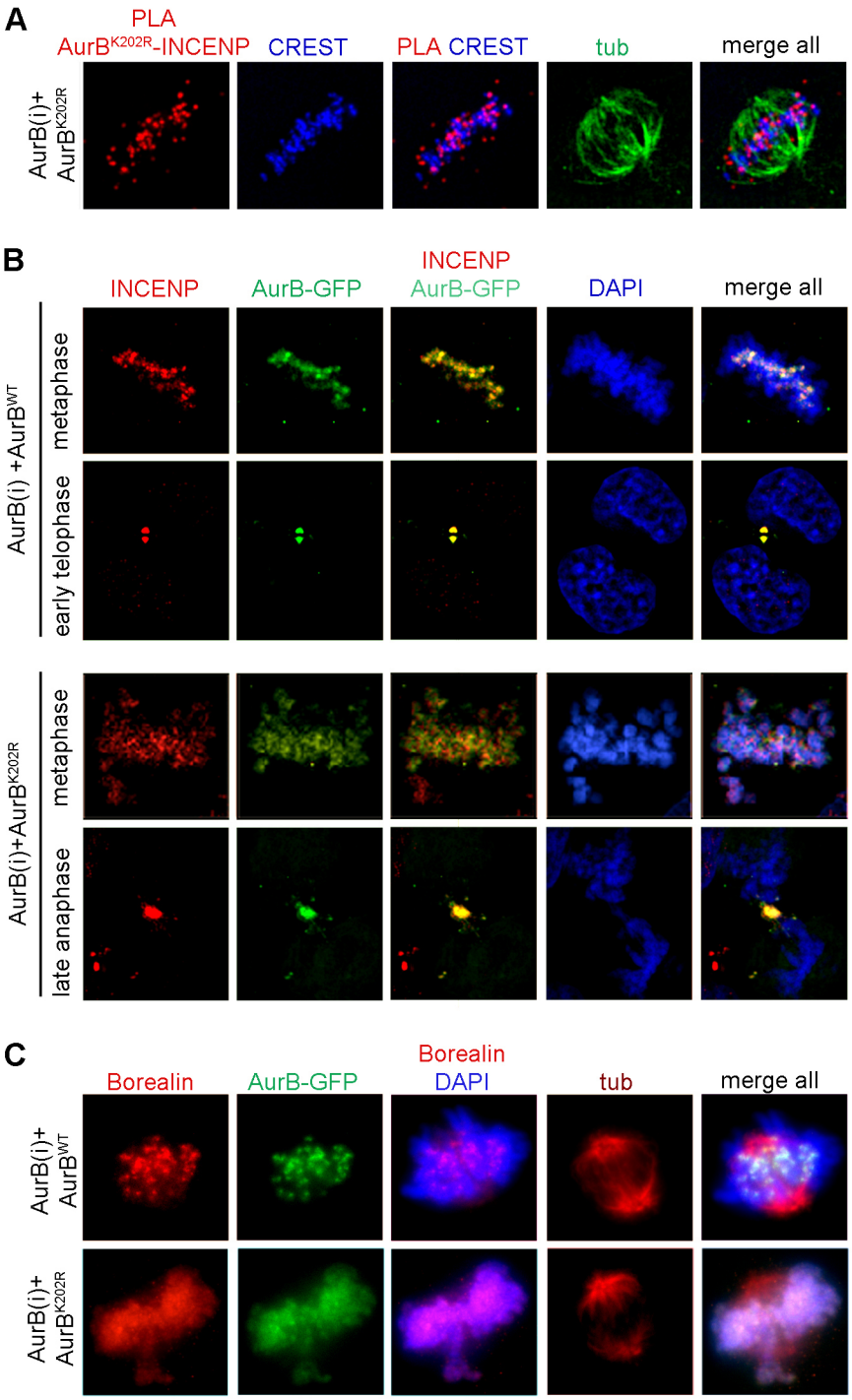
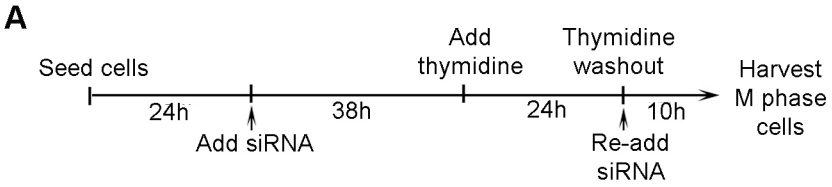
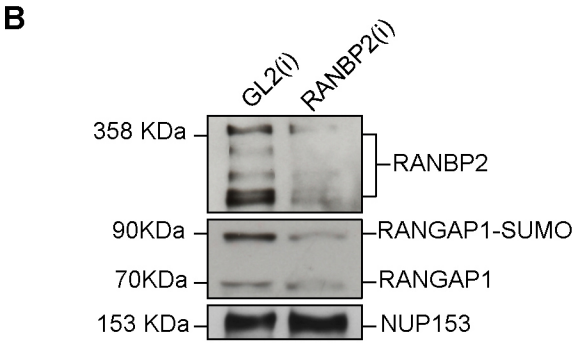
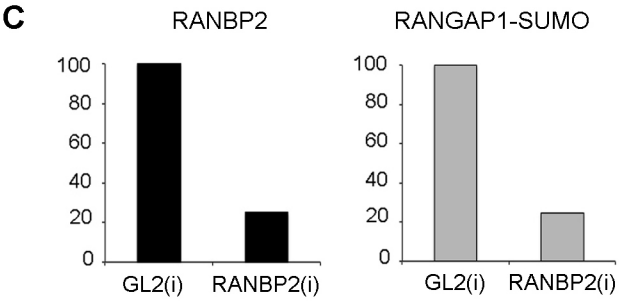
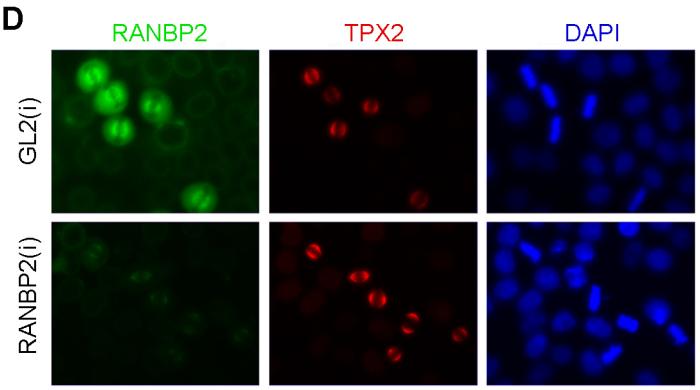

A
PLA
AurB$^{K202R}$-INCENP
CREST
PLA CREST
tub
merge all
AurB(i)+ AurB$^{K202R}$
B
INCENP
AurB-GFP
INCENP
AurB-GFP
DAPI
merge all
AurB(i) +AurB$^{WT}$
metaphase
early telophase
AurB(i)+AurB$^{K202R}$
metaphase
late anaphase
C
Borealin
AurB-GFP
Borealin
DAPI
tub
merge all
AurB(i)+ AurB$^{WT}$
AurB(i)+ AurB$^{K202R}$

Figure S3


A
Seed cells
Add thymidine
Thymidine washout
Harvest M phase cells
24h
38h
24h
10h
Add siRNA
Re-add siRNA


B
GL2(i)
RANBP2(i)
358 KDa
RANBP2
90KDa
RANGAP1-SUMO
70KDa
RANGAP1
153 KDa
NUP153


C
RANBP2
RANGAP1-SUMO
100
80
60
40
20
0
GL2(i)
RANBP2(i)


D
RANBP2
TPX2
DAPI
GL2(i)
RANBP2(i)

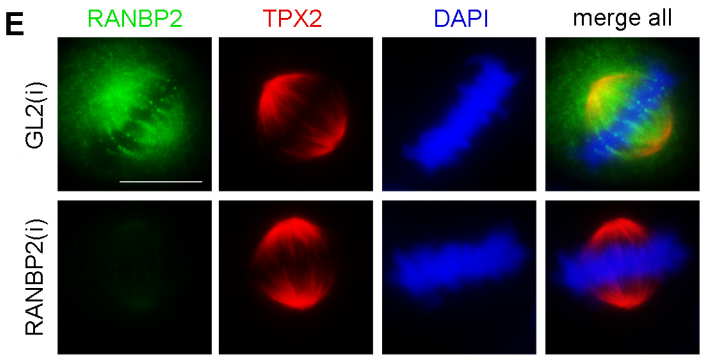
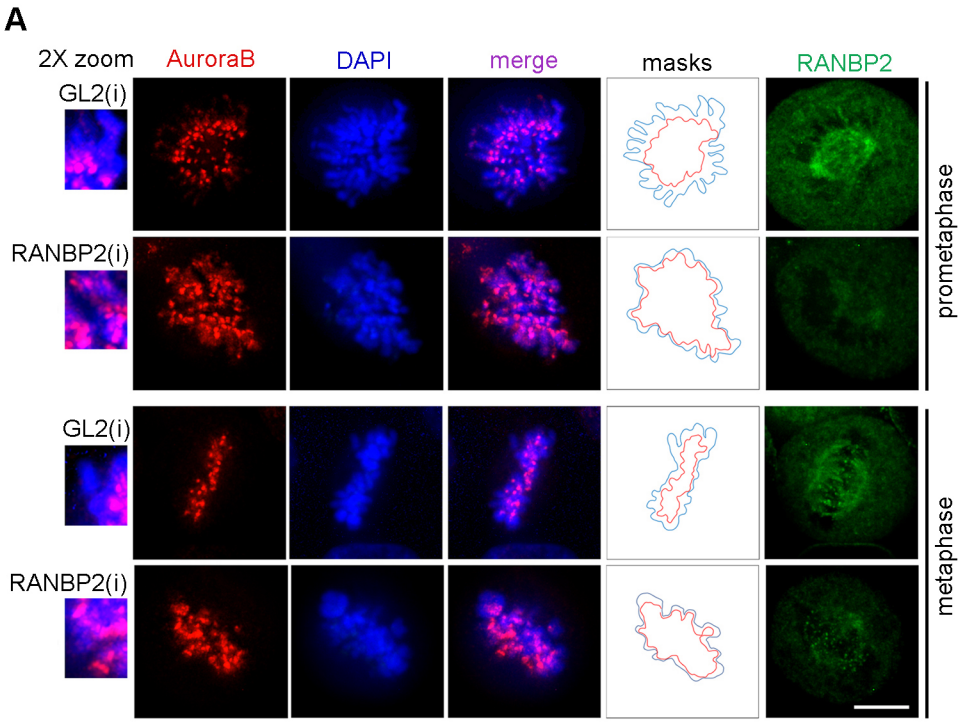
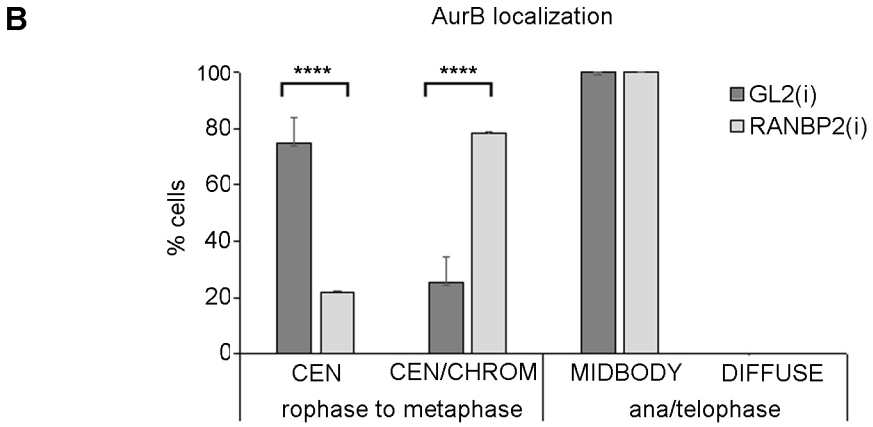

E
RANBP2
TPX2
DAPI
merge all
GL2(i)
RANBP2(i)

Figure S4


A
2X zoom
AuroraB
DAPI
merge
masks
RANBP2
GL2(i)
RANBP2(i)
prometaphase
GL2(i)
RANBP2(i)
metaphase


B
AurB localization
100
80
60
40
20
0
% cells
****
****
GL2(i)
RANBP2(i)
CEN
CEN/CHROM
MIDBODY
DIFFUSE
rophase to metaphase
ana/telophase

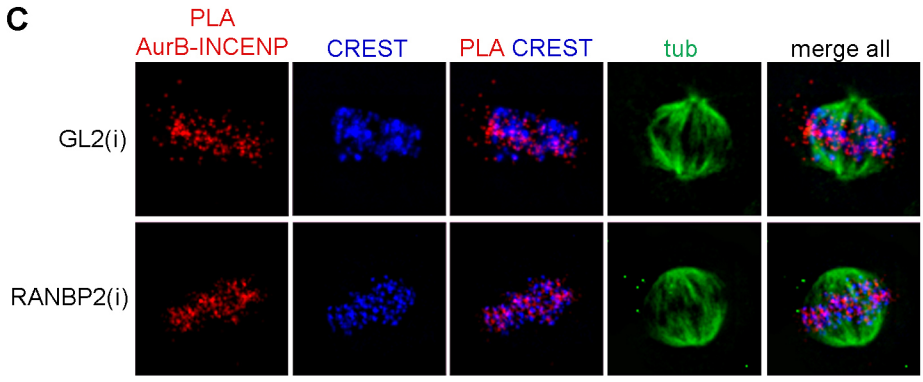

C
PLA
AurB-INCENP
CREST
PLA CREST
tub
merge all
GL2(i)
RANBP2(i)

Figure S5