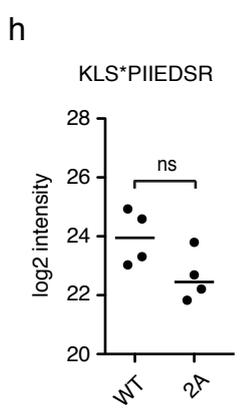
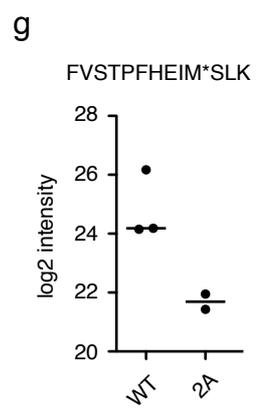
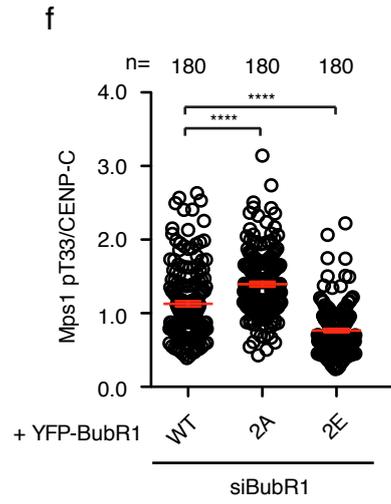
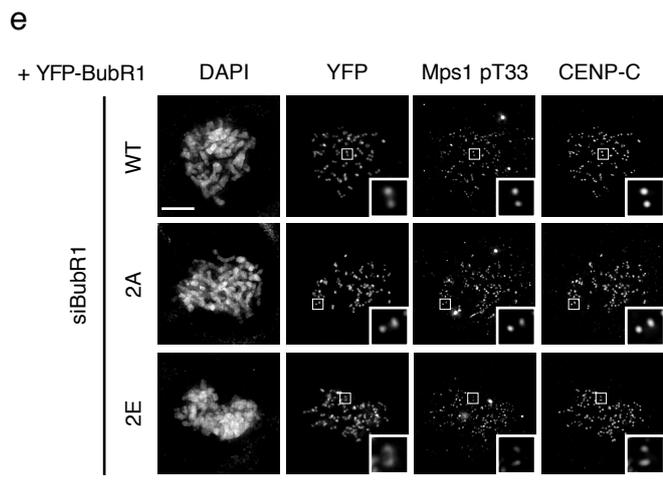
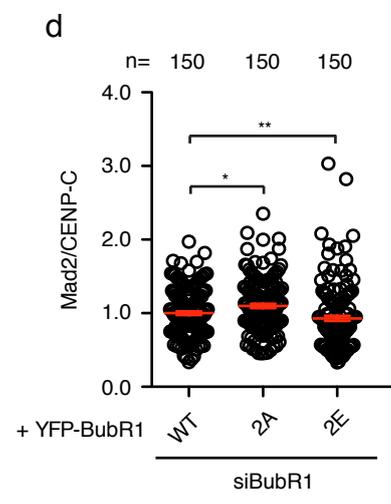
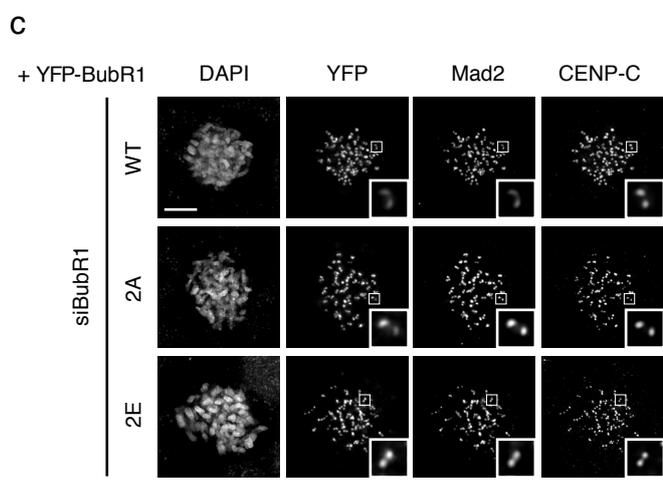
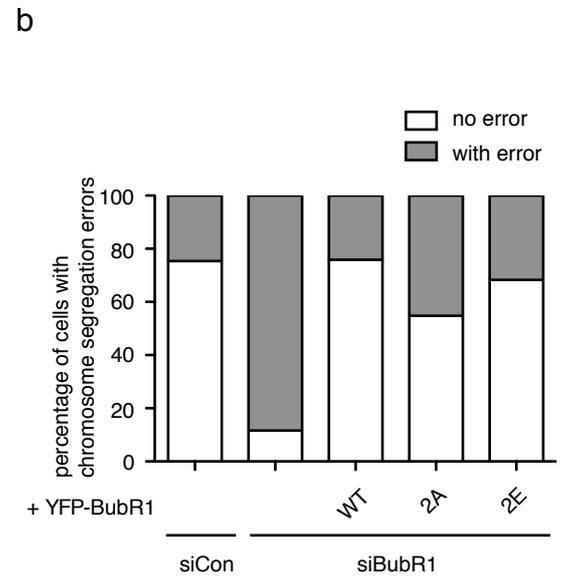
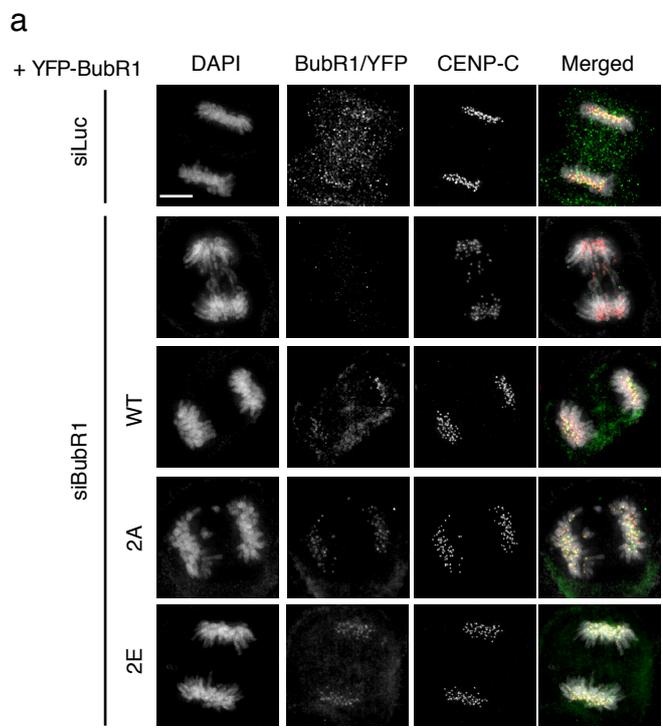


Supporting Information

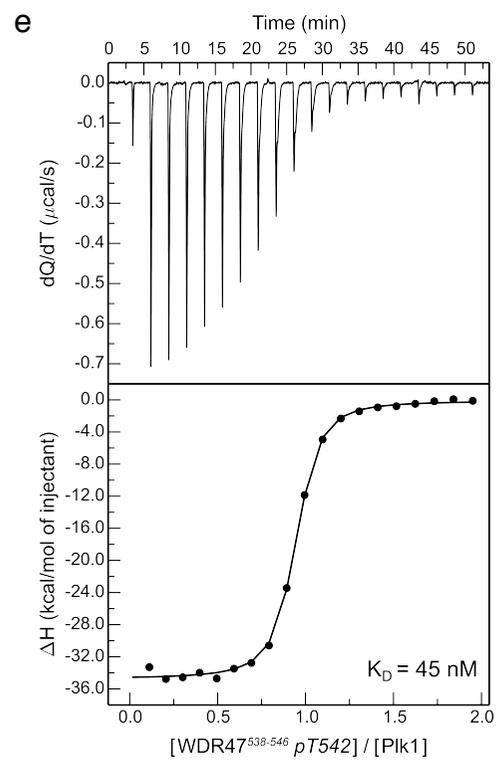
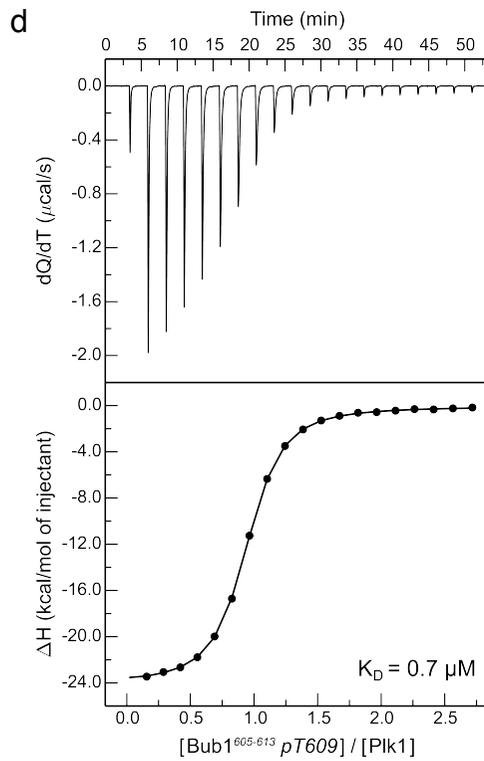
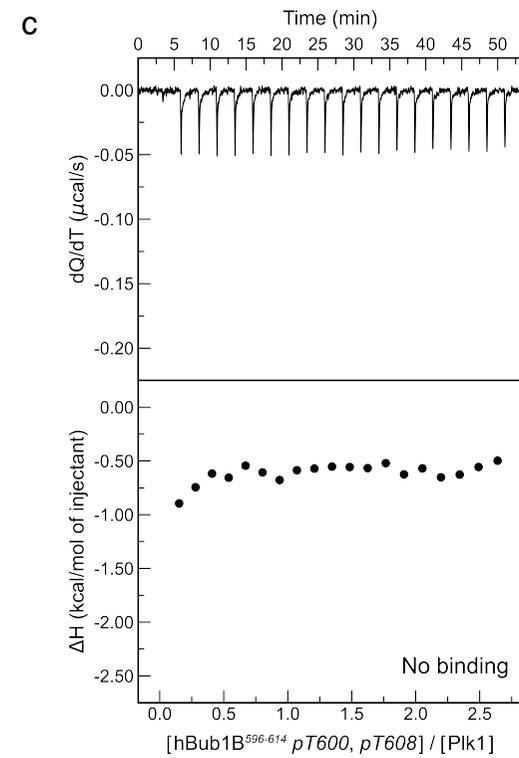
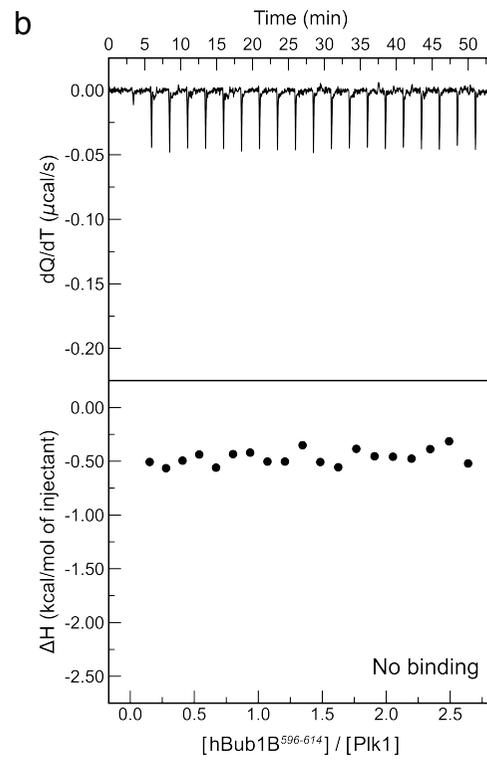
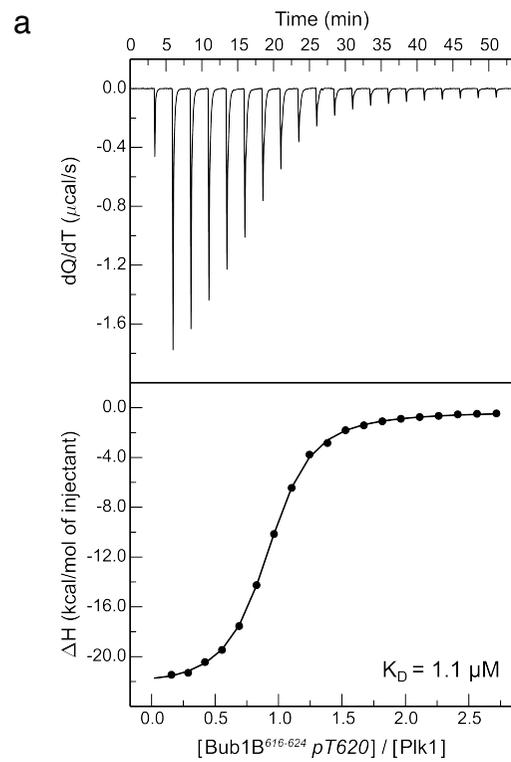
1. Supplementary Figures 1-5



Supplementary Figure 1

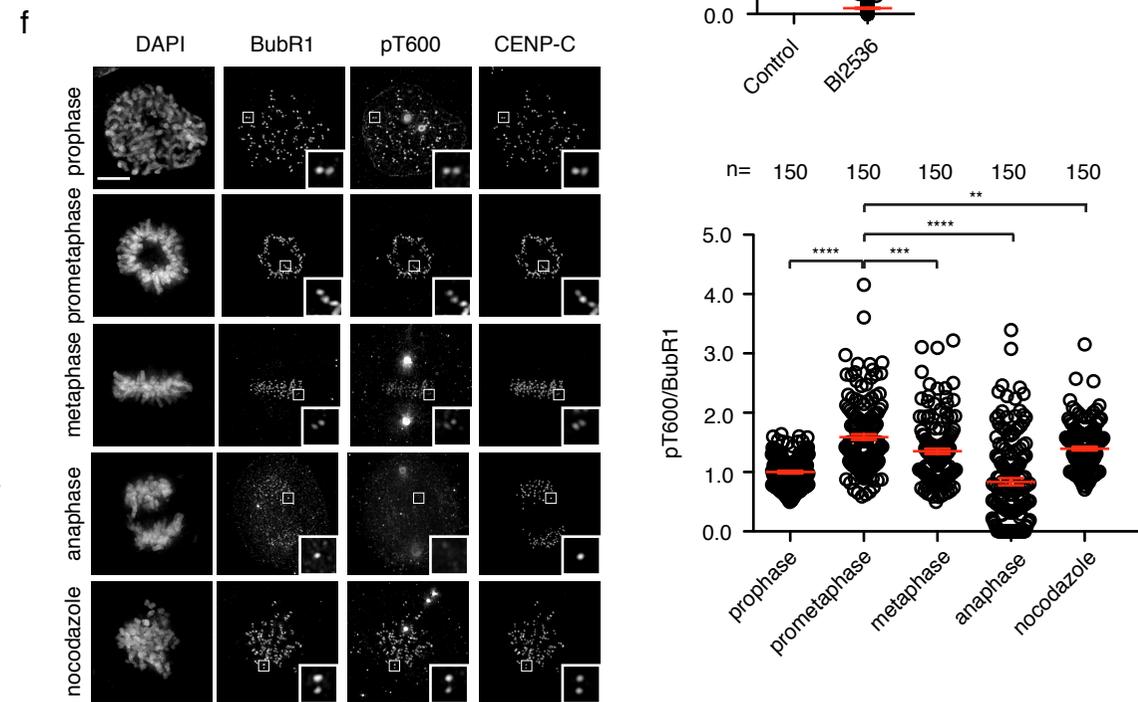
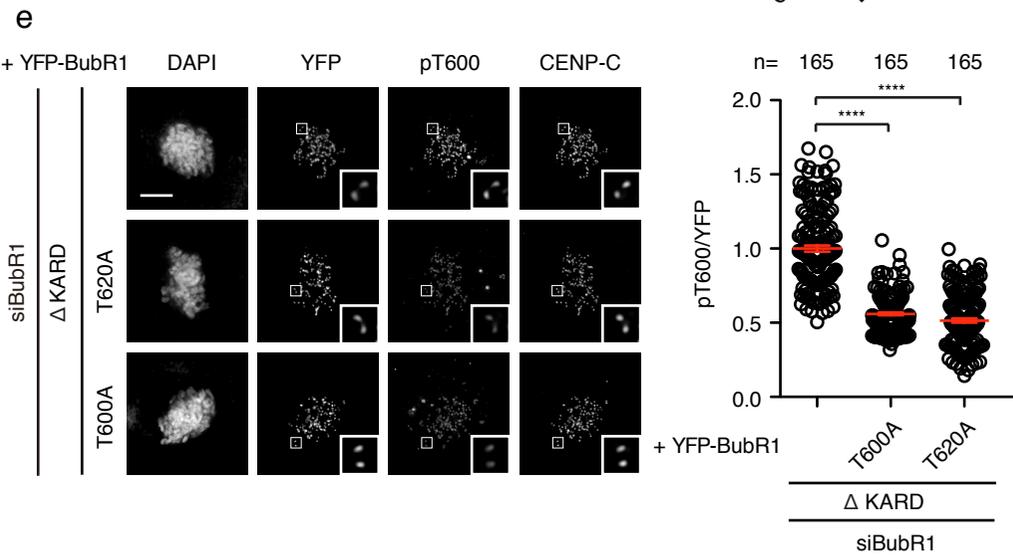
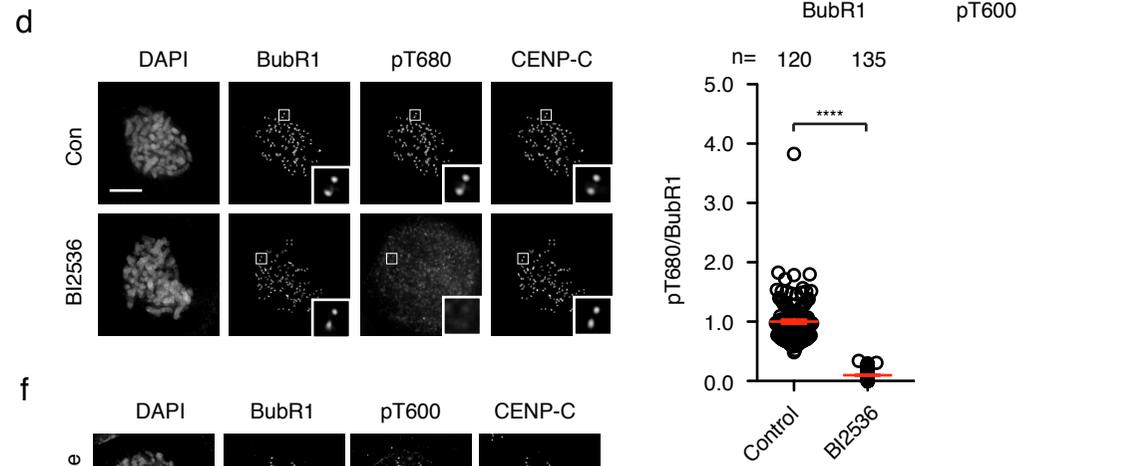
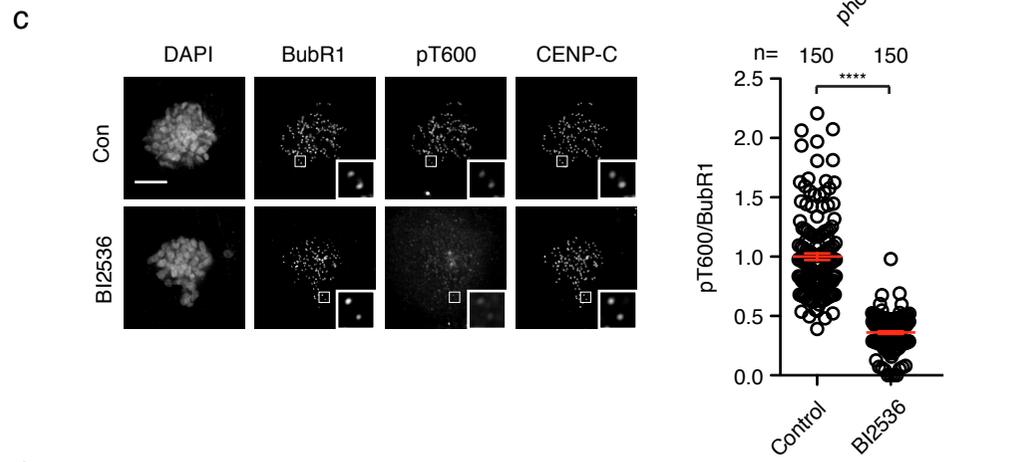
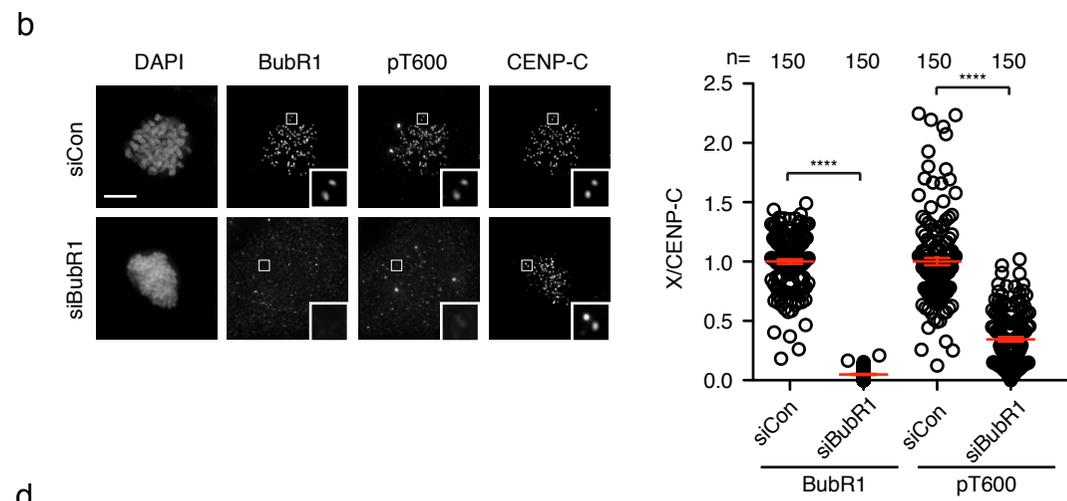
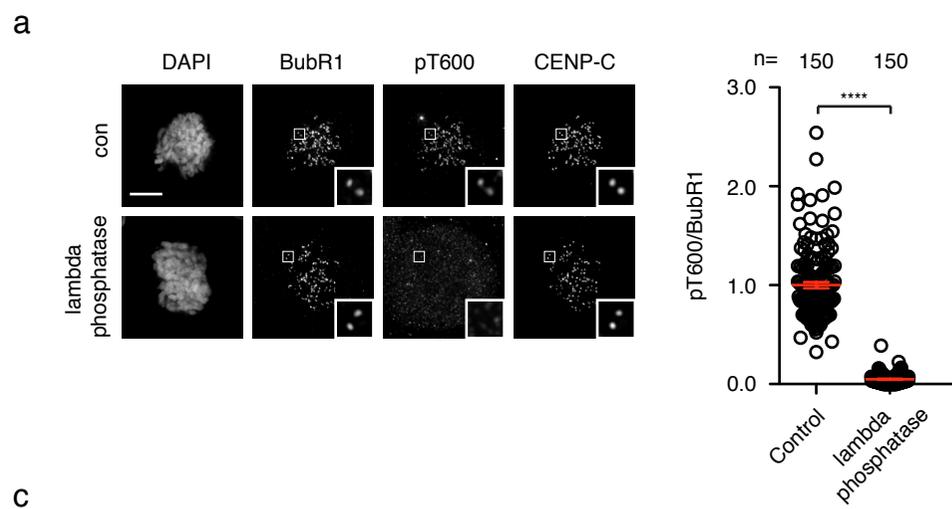
a Representative images of chromosome segregation in cells transfected with siRNA oligos against luciferase as control or siRNA oligos against BubR1 with RNAi-resistant constructs expressing YFP-BubR1. The cells were released from RO3306 into the medium for 60 min before fixation and staining with corresponding antibodies. Scale bar is 10 μ m. **b** Quantification of segregation errors including lagging chromosomes and chromosome bridge from **a**. More than 100 mitotic cells quantified in each condition. **c** Representative images of mitotic cells transfected with the same procedures as in **a**. The cells were released from RO3306 into the medium with nocodazole (200 ng/ml) for 45 min before fixation and staining with corresponding antibodies. Scale bar is 10 μ m. **d** Quantification of Mad2 on kinetochores against CENP-C from **c**. Mean values of 150 kinetochores from 10 cells for each condition were presented. The red line indicates the mean value which was set to 1 for wild type BubR1 (WT) and the rest was normalized to it. Bar is standard error of the mean. Mann-Whitney u-test was applied. * means $P < 0.1$; ** means $P < 0.01$. **e** Representative images of mitotic cells transfected with the same procedures in **a** and **c**. The cells were released from RO3306 into the medium with nocodazole (200 ng/ml) for 45 min before fixation and staining with corresponding antibodies. Bar is 10 μ m. **f** Quantification of kinetochore signals of Mps1 pT33 against CENP-C from **e**. Mean values of 180 kinetochores from 12 cells for each condition were presented. The red line indicates the mean value which was set to 1 for wild type BubR1 (WT) and the rest was normalized to it. Bar is standard error of the mean. Mann-Whitney u-test was applied. **** means $P < 0.0001$. **g,h** Peptide intensity measured by quantitative mass spectrometry from immunoprecipitate of YFP-BubR1 wild type or 2A mutant. **g** The peptide with T620; **h** The peptide with S670. The line

means median value. Mann-Whitney u-test was applied only to **h** as **g** does not have enough sample. ns means not significant.



Supplementary Figure 2

Recombinant Plk1 (367-603aa) was expressed and purified. The binding between recombinant Plk1 and the following peptides was measured by ITC: **a** peptide BubR1⁶¹⁶⁻⁶²⁴ with T620 phosphorylated; **b** peptide BubR1⁵⁹⁶⁻⁶¹⁴; **c** peptide BubR1⁵⁹⁶⁻⁶¹⁴ with T600/T608 phosphorylated; **d** peptide Bub1⁶⁰⁵⁻⁶¹³ with T609 phosphorylated; **e** peptide WDR47⁵³⁸⁻⁵⁴⁶ with T542 phosphorylated.



Supplementary Figure 3

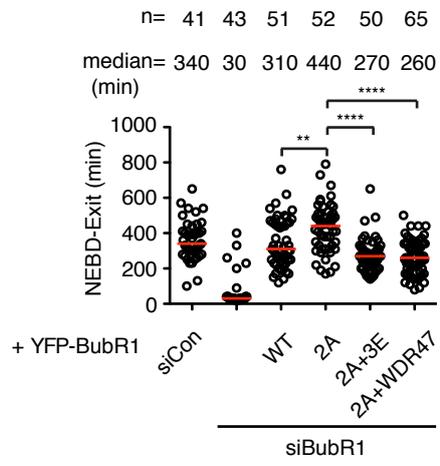
a Representative images of mitotic cells treated with lambda phosphatase. The cells were released from RO3306 into the medium with nocodazole (200 ng/ml) for 45 min before fixation, lambda phosphatase treatment and staining by corresponding antibodies. The quantification of kinetochore signals of BubR1 pT600 against BubR1 was shown on the right. **b** Representative images of mitotic cells transfected with siRNA oligos against luciferase or BubR1. The cells were treated similarly as in **a**. The quantification of kinetochore signals of BubR1 pT600 against BubR1 was shown on the right. **c-d** Representative images of mitotic cells treated with BI2536. The cells were treated similarly as in **a** except being released into the medium with both nocodazole and BI2536. The quantification of kinetochore signals of BubR1 pT600 (**c**) or pT680 (**d**) against BubR1 was shown on the right. **e** Representative images of mitotic cells transfected with siRNA oligos against BubR1 and RNAi-resistant constructs expressing YFP-BubR1. The cells were treated similarly as in **a**. The quantification of kinetochore signals of pT600 against YFP-BubR1 was shown on the right. **f** Representative images of cells at distinct mitotic phases. The cells were released from thymidine arrest for 8 hrs before fixation and staining with corresponding antibodies except the cell in the last panel was treated the same as in **a**. The quantification of kinetochore signals of pT600 against BubR1 was shown on the right. Mean values of 150 kinetochores from 10 cells for each condition were presented except for **d** (control: 120 kinetochores from 8 cells; BI2536: 135 kinetochores from 9 cells) and for **e** (165 kinetochores from 11 cells). The red line indicates the mean value which was set to 1 for control sample (**a-d**) or BubR1 Δ KARD (**e**) or prophase (**f**) and the rest was normalized to it. Bar is standard error of the mean. Mann-Whitney u-test was applied for all the quantification in **a-f**. ** means $P < 0.01$; *** means

P<0.001; **** means P<0.0001. The scale bar for all the images is 10 μ m.

a

wt 593 ITGFRNVTICPNPEDTCDFARAARFVSTPFHEIMSLKD(39)LSPIIEDSREAT 680
2A 593 ITGFRNV**A**ICPNPED**A**CDFARAARFVSTPFHEIMSLKD(39)LSPIIEDSREAT 680
2A+3E 593 ITGFRNV**A**ICPNPED**A**CDFARAARFVSTPFHEIMSLKD(39)LSPIIE**EEEE**EAT 680
2A+WDR47 593 ITGFRNV**A**ICPNPED**A**CDFARAA**IHTSTPRNP**IMSLKD(39)LSPIIEDSREAT 680

b

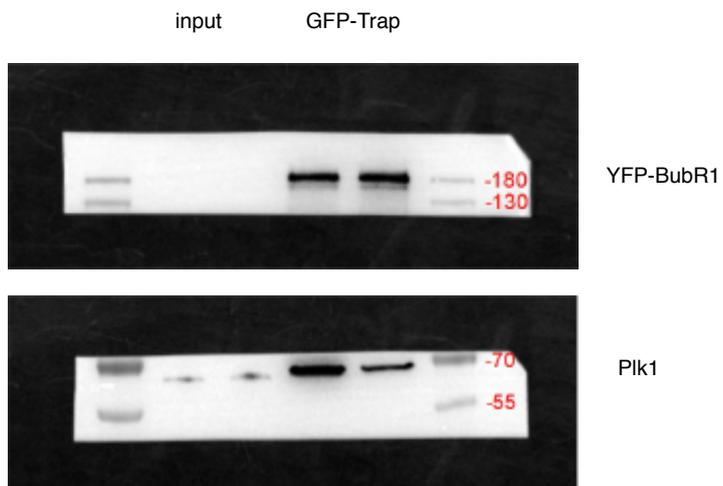


Supplementary Figure 4

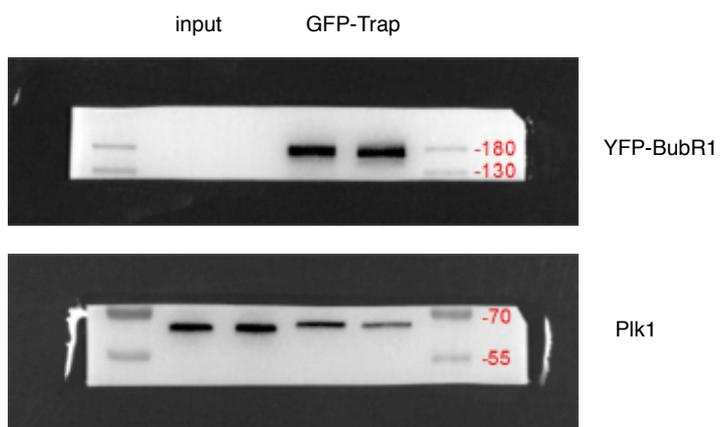
a Amino acid sequences of the engineered proteins. **b** Plot showing the time from NEBD to mitotic exit of the cells transfected with siRNA oligos against luciferase or siRNA oligos against BubR1 with RNAi-resistant constructs expressing YFP-BubR1. Low dose of nocodazole (30 ng/ml) was applied into the medium before live cell imaging conducted. Each circle represents the time spent in mitosis of a single cell. Red line indicates the median time. The number of cells analyzed per condition is indicated above (n = X). Mann-Whitney u-test was applied. ** means $P < 0.01$; **** means $P < 0.0001$.

Fig 2e

Replicate 1



Replicate 2



Supplementary Figure 5

The above are all uncropped blots for Figure 2e.