SUPPLEMENTAL FIGURES

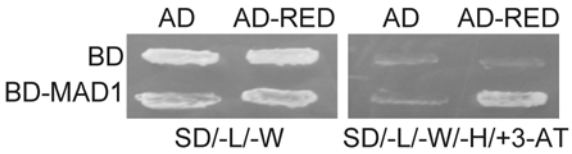

FIGURE S1. **Yeast two-hybrid analysis for the MAD1-RED interaction.** The full-length MAD1 was fused to the DNA-binding domain (BD) of yeast Gal4 transcription factor and RED to the activation domain (AD) of yeast Gal4. Synthetic dextrose minimal solid medium (SD) lacking leucine and tryptophan (SD/−L/−W) was used to cultivate yeast transformants, and the SD medium lacking leucine, tryptophan, and histidine and containing 3-aminotriazole (SD/−L/−W/−H/+3AT) was used to assay for expression of *HIS3* reporter gene.

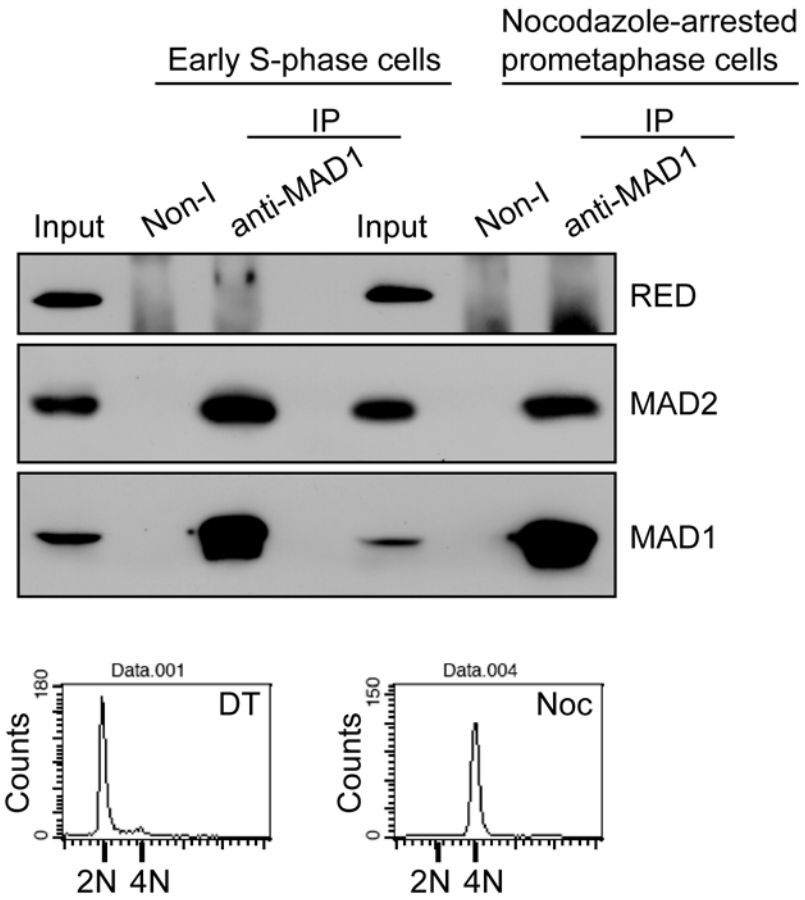

FIGURE S2. **Coimmunoprecipitation assay for the MAD1-RED interaction in the early S-phase and M-phase cells.** Cells were arrested in the early S-phase with a double-thymidine (DT) block and in prometaphase with nocodazole (Noc). Cells were collected for a co-immunoprecipitation assay using rabbit anti-MAD1 antibodies and DNA content analysis by flow cytometer as described in FIG. 2*C*. The purified rabbit non-immune (non-I) antibody was used as a control for the coimmunoprecipitation assay. The precipitated proteins were analyzed by immunoblot (IB) analysis using rabbit anti-RED, rabbit anti-MAD1, or mouse anti-MAD2 antibodies. Eighty micrograms of cell lysates were loaded as an input control.

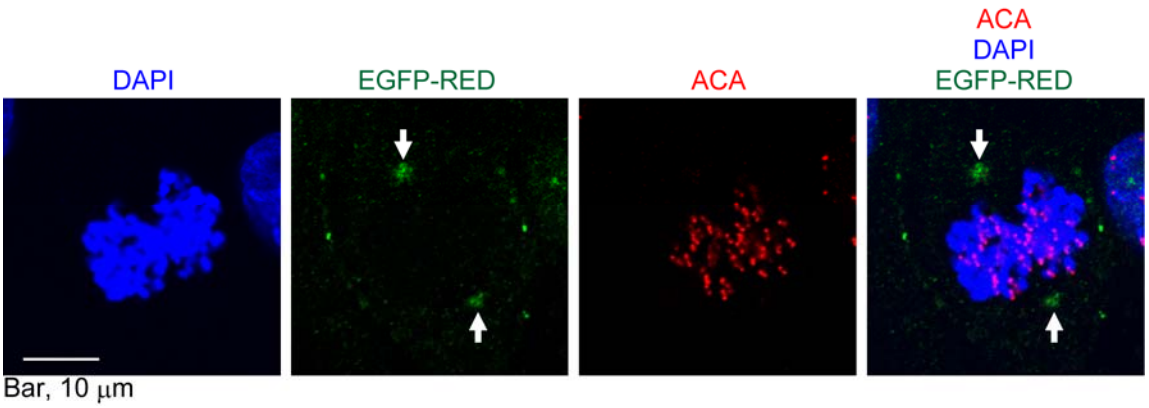

FIGURE S3. **Subcellular localization of EGFP-RED in prometaphase cells.** Cells were transfected with EGFP-RED for 24 hours. The transfected cells were stained for EGFP fusion proteins and kinetochores using anti-GFP and human ACA antibodies, respectively. DNA was stained with DAPI. The arrows indicate the location corresponding to the spindle poles. Scale bar, 10 μm.

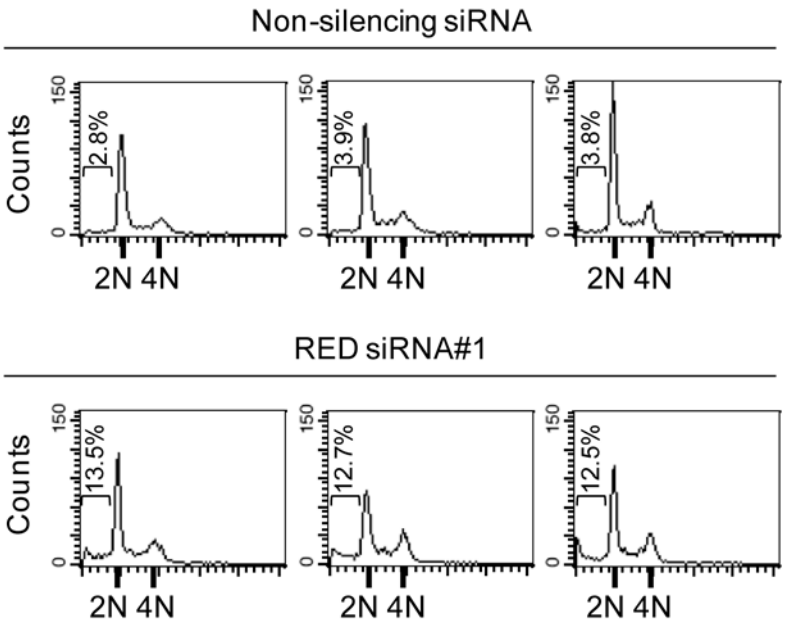

FIGURE S4. **Effect of RED RNAi on cell viability.** HeLa cells were treated with the non-silencing siRNA or RED siRNA#1 duplexes for 72 hours. Then, the DNA-content histograms for the non-silencing siRNA or RED siRNA#1-treated cells were analyzed by flow cytometer. ~12.9% ($p < 0.0001$) of the RED-depleted cells were in sub-G1, whereas ~3.5% of the non-silenced cells were in sub-G1, indicating that depletion of RED might cause apoptosis after a 3-day incubation.

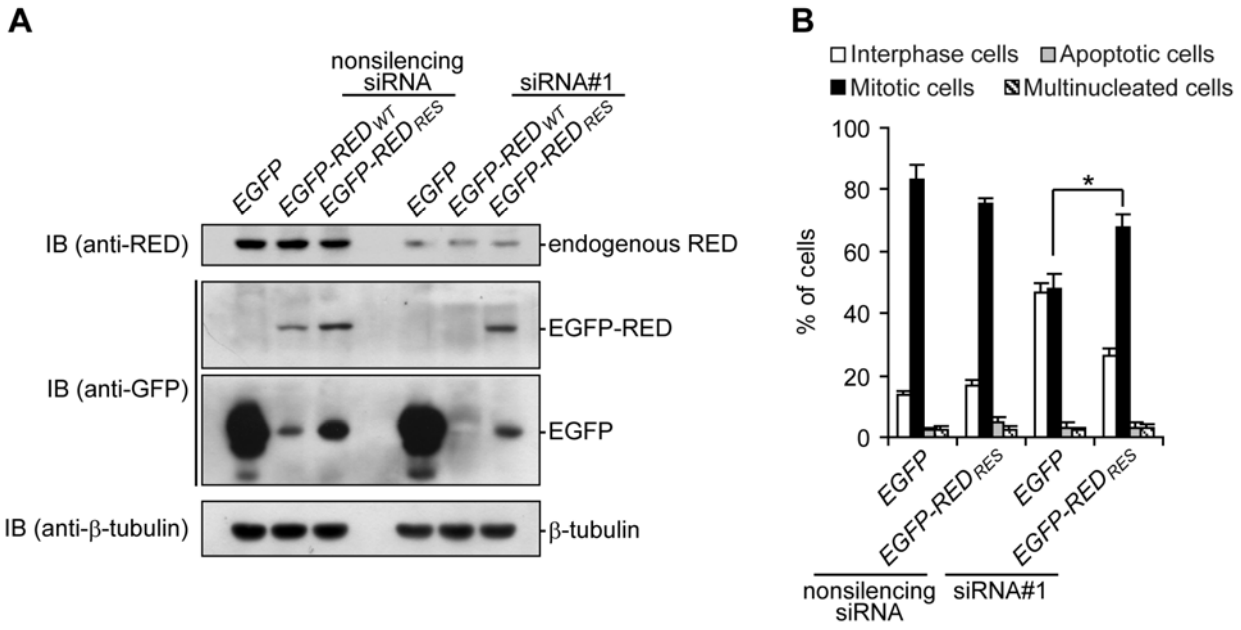

FIGURE S5. **Rescue of the SAC-defective phenotype caused by RED siRNA duplexes.** *A*, *EGFP-RED*$_{RES}$ is resistant to RED siRNA#1 duplex. Cells were cotransfected with 100 pmoles of non-silencing siRNA or RED siRNA#1 duplexes and 150 ng of *EGFP*, *EGFP-RED*$_{WT}$ or *EGFP-RED*$_{RES}$ plasmid DNAs. Cells were lysed and analysed by western blot with antibodies against RED, GFP and β-tubulin (loading control). *B*, *EGFP-RED*$_{RES}$ can rescue the defect in the SAC caused by RED siRNA-directed depletion of RED. Cells were cotransfected with non-silencing siRNA or RED siRNA#1 duplexes and *EGFP* or *EGFP-RED*$_{RES}$ constructs. The transfected cells were treated with nocodazole (50 ng/ml) for 18 hours and then collected for analysis. Two hundred GFP-positive cells were scored for each of four independent experiments. The data shown in graph are the means ± s.d. from four independent experiments. * $p < 0.001$ (ANOVA).

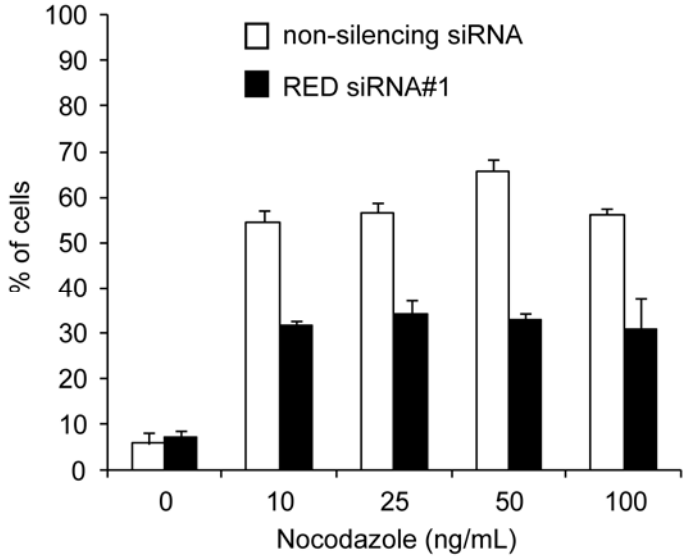

FIGURE S6. **The response of RED-depleted cells to different amounts of nocodazole.** After HeLa cells were transfected with the non-silencing siRNA or RED siRNA#1 duplexes for 24 hours, cells were treated with different amounts of nocodazole for 18 hours and then collected for analysis. To determine the percentage of cells in mitosis, two hundred cells were scored for each of three independent experiments. The data shown in the graph are the means ± s.d. from three independent experiments. The Y-axis label in the graph is the percentage of cells in mitosis.

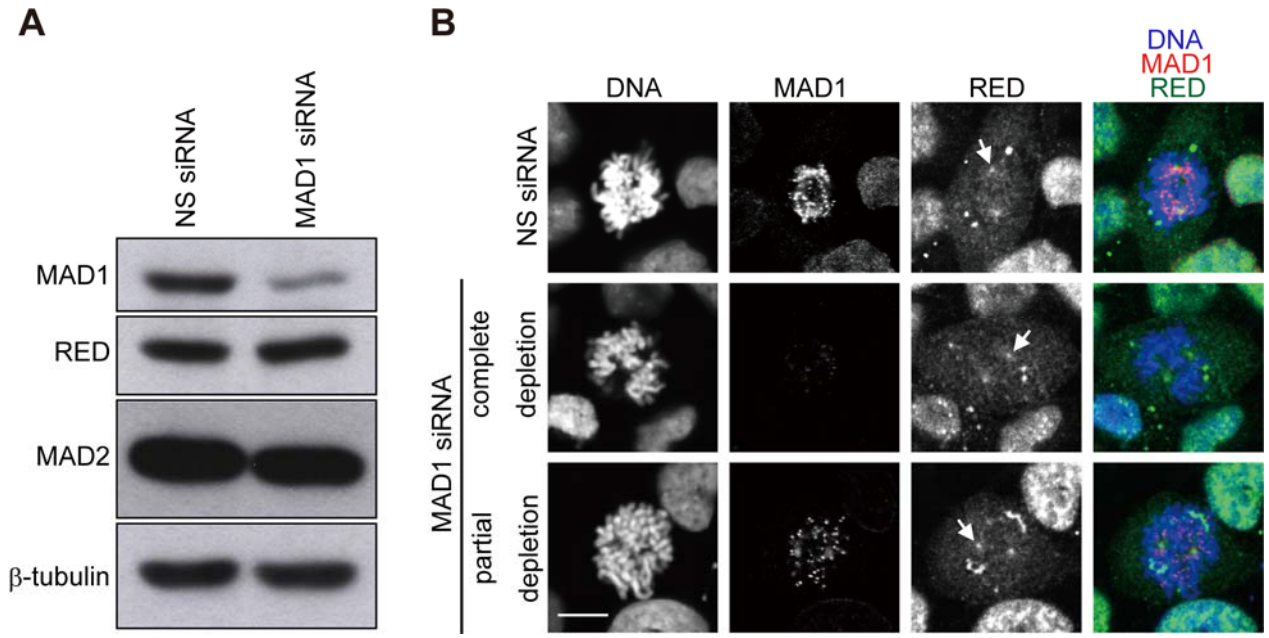

FIGURE S7. **Effect of MAD1 RNAi on localization of RED.** *A*, depletion of MAD1 protein. Cells were treated with the non-silencing (NS) or MAD1 siRNA duplexes for 48 hours. Cells were lysed and analysed by western blot with antibodies against MAD1, RED, MAD2, and β-tubulin (loading control). *B*, localization of RED in MAD1-depleted prometaphase cells. Non-silenced or MAD1-depleted cells were stained for DNA, MAD1 and RED as described in FIG. 4*A*. The arrows indicate the location of RED corresponding to the spindle poles. Scale bar, 10 μm.

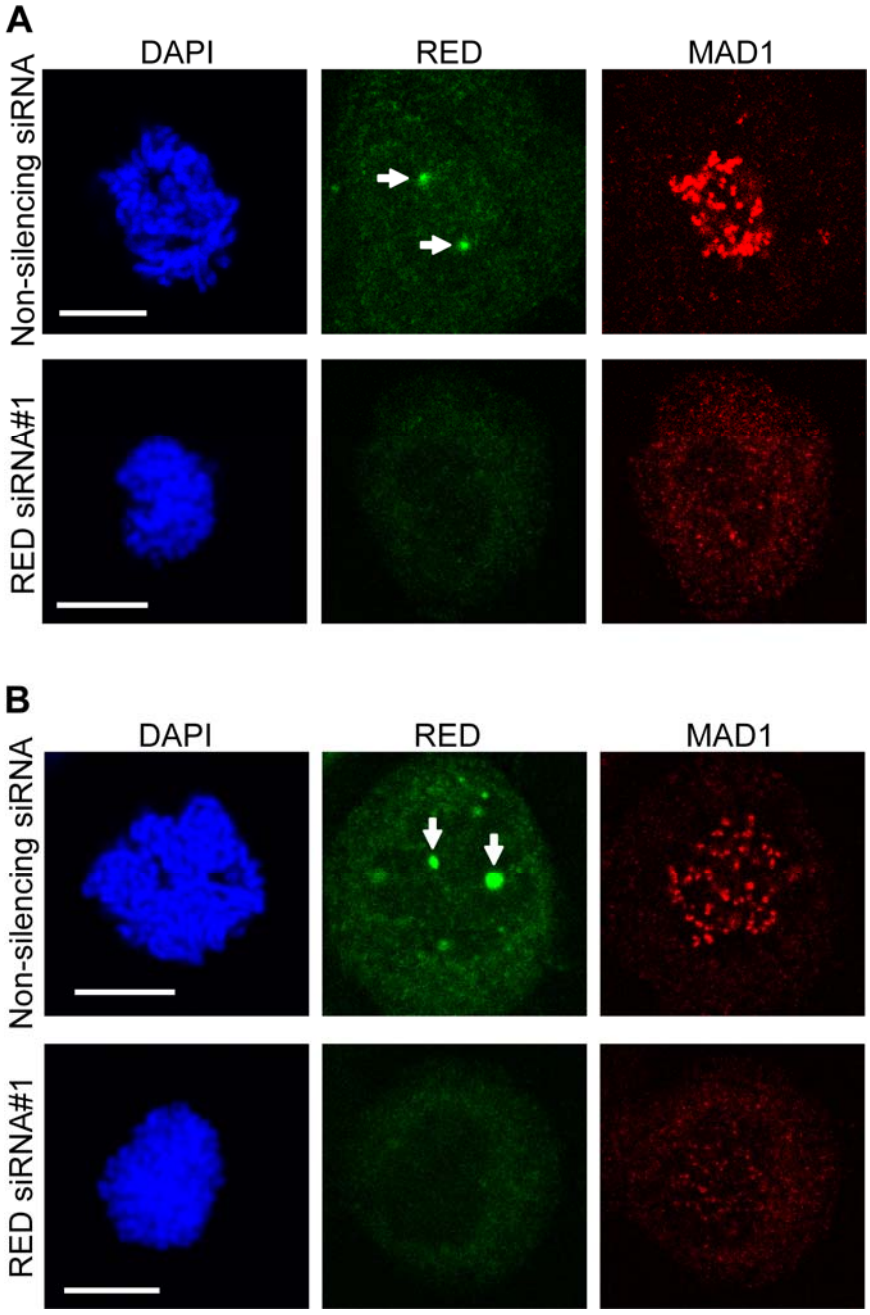

FIGURE S8. *A*, localization of MAD1 in prometaphase cells. After cells were transfected with the non-silencing siRNA or RED siRNA#1 duplexes for 42 hours, cells were fixed by paraformaldehyde for immunofluorescence staining. Staining for MAD1 and RED was performed as described in FIG. 4*A*. The chromosomal DNA was stained with DAPI. The stained cells were visualized using a confocal laser microscope. The arrows indicate the locations of the spindle poles. Scale bar, 10 μm. *B*, localization of MAD1 in the nocodazole-treated prometaphase cells. After cells were transfected

with the non-silencing siRNA or RED siRNA#1 duplexes for 42 hours, cells were treated with nocodazole (10 μM) for one hour and then fixed by paraformaldehyde for immunofluorescence staining. Staining for MAD1 and RED was performed as described in FIG. 4*A*. The chromosomal DNA was stained with DAPI. The stained cells were visualized using a confocal laser microscope. The arrows indicate the locations of the spindle poles. Scale bar, 10 μm.

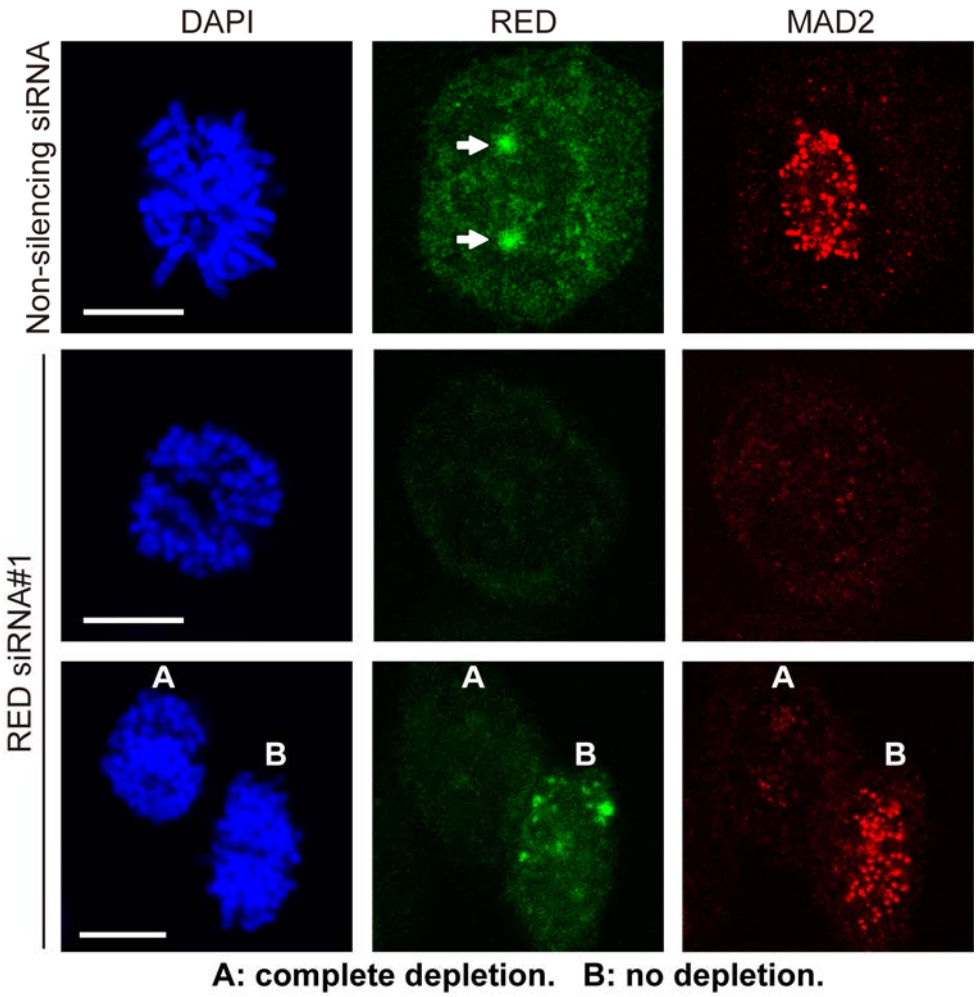

FIGURE S9. **Localization of MAD2 in the RED-depleted prometaphase cells.** After cells were transfected with the non-silencing siRNA or RED siRNA#1 duplexes for 42 hours, cells were fixed with paraformaldehyde and simultaneously stained with rat anti-MAD2 (made in this study) and rabbit anti-RED antibodies, followed by Cy3-conjugated anti-rat IgG and FITC-conjugated anti-rabbit IgG antibodies. The chromosomal DNA was stained with DAPI. The stained cells were visualized using a confocal laser microscope. The arrows indicate the locations of the spindle poles. Scale bar, 10 μm.

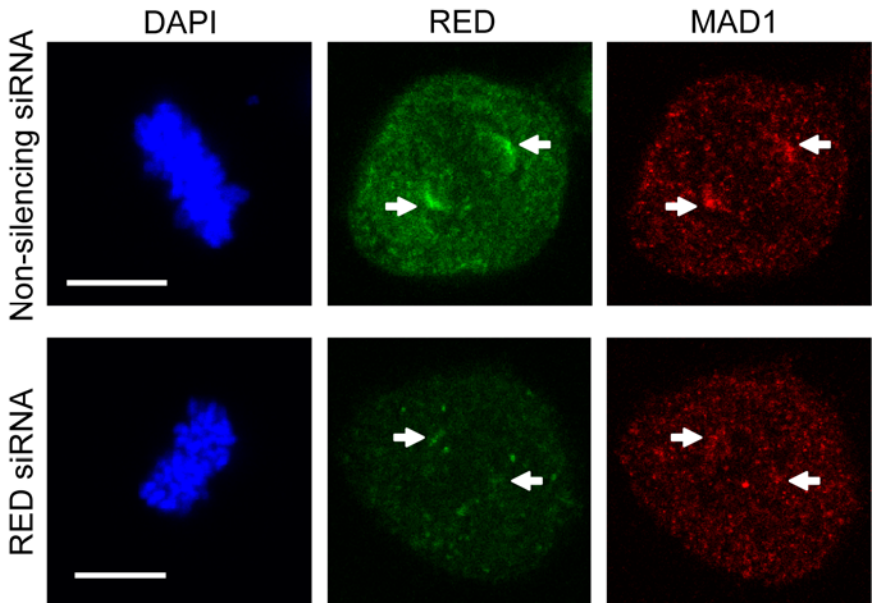

FIGURE S10. **Localization of MAD1 in the RED-depleted metaphase cells.** After cells were transfected with the non-silencing siRNA or RED siRNA#1 duplexes for 42 hours, cells were fixed by paraformaldehyde for immunofluorescence staining. Staining for MAD1 and RED was performed as described in FIG. 4*A*. The chromosomal DNA was stained with DAPI. The stained cells were visualized using a confocal laser microscope. The arrows indicate the locations of the spindle poles. Scale bar, 10 μm.

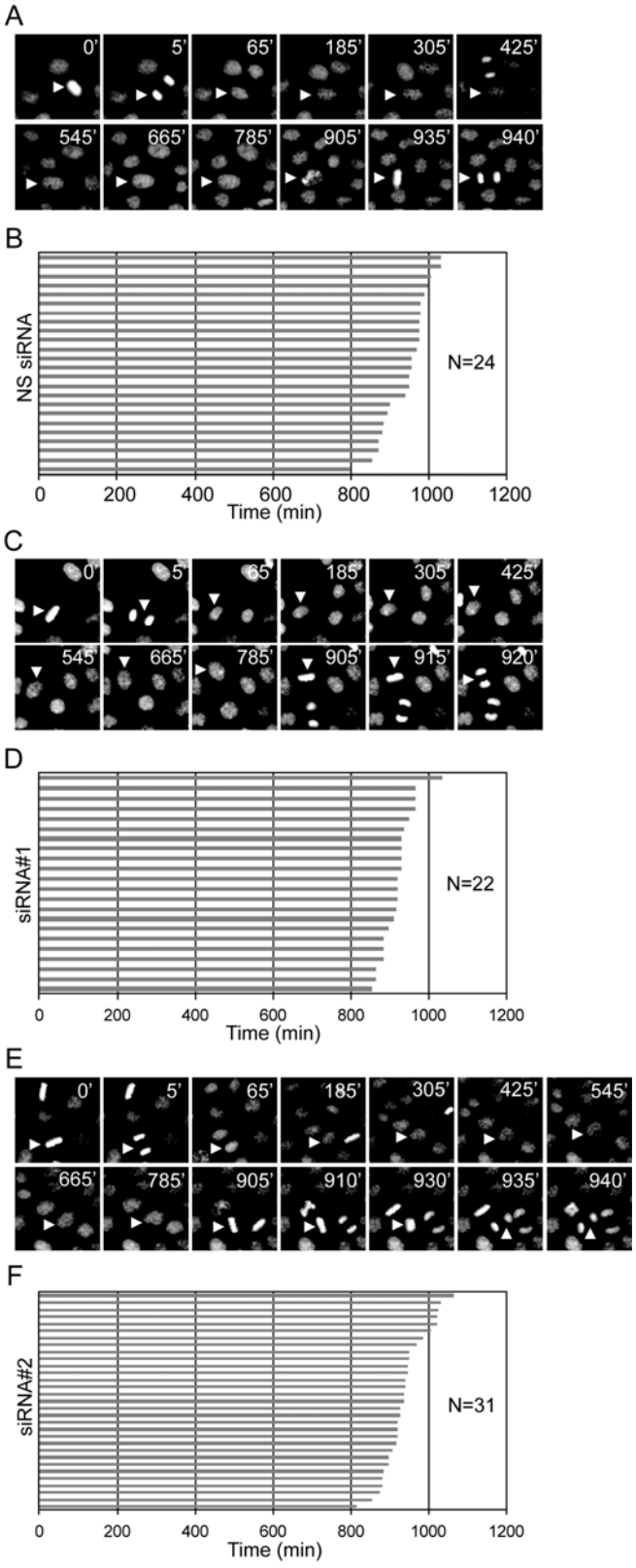
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FIGURE S11. **Effect of RED RNAi on cell doubling time.** H2B-GFP HeLa cells were transfected with the indicated siRNA duplexes and then arrested in the early S-phase by a thymidine block. Cells were released from early S-phase arrest for measurement of cell doubling time using live cell time-lapse fluorescence microscopy. The time point at the metaphase right before occurrence of anaphase was designated as T_0, and the timing from T_0 to the metaphase right before occurrence of anaphase of the next round of cell cycle was measured from live cell movies. *A*, *C* and *E*, a series of frames from live cell videos of H2B-GFP HeLa cells transfected with the indicated siRNA duplexes. The arrowheads indicate the cells that were used for measurements of cell doubling time. *B*, *D* and *F*, doubling time of H2B-GFP HeLa cells transfected with the indicated siRNA duplexes. The timing for each group of cells, selected randomly, is shown in the graphs. N, number of cells selected at random from two independent experiments.

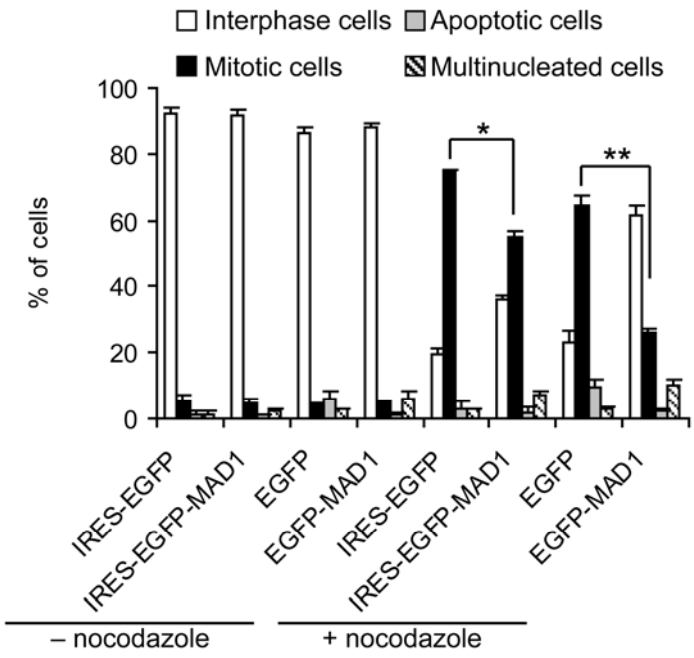

FIGURE S12. **The effect of ectopic expression of MAD1 on the SAC.** *EGFP* or *EGFP-MAD1* fusion gene was transcribed by the CMV promoter, and its translation was under the control of the disabled EMCV *IRES* sequence or not. The disabled *IRES* sequence allows moderate expression of EGFP and EGFP-MAD1 fusion proteins. Cells were transfected with *IRES-EGFP*, *IRES-EGFP-MAD1*, *EGFP* or *EGFP-MAD1* construct for 24 hours. The transfected cells were then treated with nocodazole (50 ng/ml) or mock-treated with DMSO for 18 hours and then collected for analysis. Two hundred GFP-positive cells were scored for each of three independent experiments. The data shown in graph are the means ± s.d. from three independent experiments. * $p < 0.001$, ** $p < 0.001$ (ANOVA). Compared to EGFP, moderate expression or overexpression of EGFP-MAD1 caused a failure of cells to properly arrest in mitosis after nocodazole treatment.

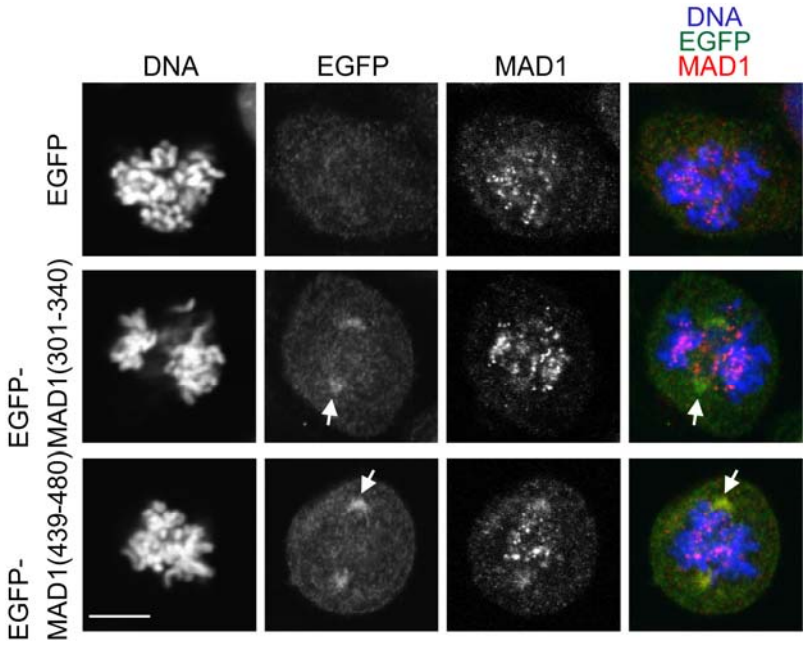

FIGURE S13. **Localization of MAD1 in the cells expressing EGFP-MAD1(301-340) or EGFP-MAD1(439-480).** Cells were transfected with *IRES-EGFP*, *IRES-EGFP-MAD1(301-340)* or *IRES-EGFP-MAD1(439-480)* for 24 hours. The transfected cells were fixed with paraformaldehyde and stained for EGFP fusion proteins and MAD1 using chicken anti-GFP and rat anti-MAD1 antibodies, respectively. DNA was stained with DAPI. The arrows indicate the location corresponding to the spindle poles, where EGFP-MAD1(301-340) and EGFP-MAD1(439-480) are localized. Compared to the cells expressing EGFP, MAD1 could localize normally to kinetochores in 100% (13/13) of the prometaphase cells expressing EGFP-MAD1(301-340) and 100% (22/22) of the prometaphase cells expressing EGFP-MAD1(439-480), indicating that expression of EGFP-MAD1(301-340) and EGFP-MAD1(439-480) did not interfere with the kinetochore localization of endogenous MAD1 in prometaphase cells. Scale bar, 10 μm.

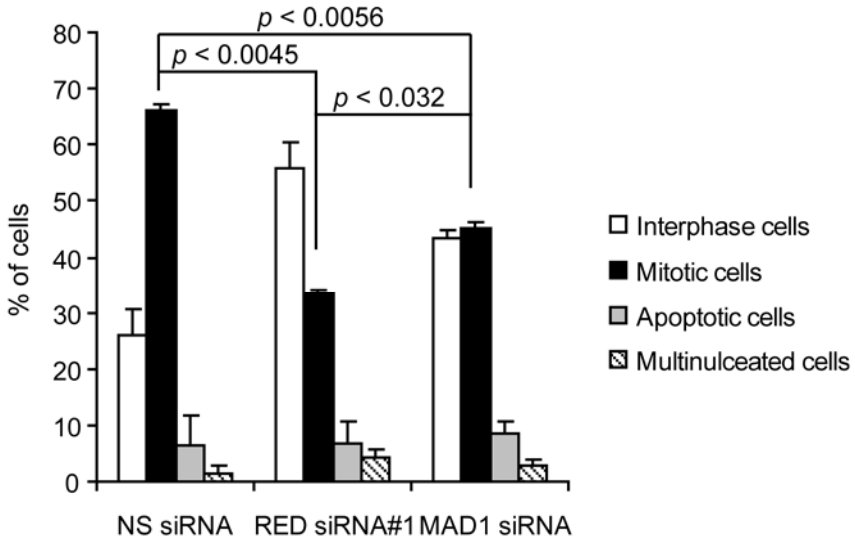

FIGURE S14. **A comparison of the severity of the SAC defect between MAD1- and RED-depleted cells.** Cells were transfected with the non-silencing siRNA, MAD1 siRNA, or RED siRNA#1 duplexes for 24 hours. The transfected cells were treated with nocodazole (50 ng/ml) for 18 hours and then collected for analysis. The analysis was performed as described in FIG. 5*B*. Two hundred cells were scored for each of three independent experiments. The data shown in graph are the means ± s.d. from three independent experiments. Each *p* value was obtained by the Student *t*-test.