

SHORT REPORT**TRAIP is a regulator of the spindle assembly checkpoint****Christophe Chopard¹, Patrick Meraldi², Tobias Gleich¹, Daniel Bachmann¹, Daniel Hohl¹ and Marcel Huber^{1,*}****ABSTRACT**

Accurate chromosome segregation during mitosis is temporally and spatially coordinated by fidelity-monitoring checkpoint systems. Deficiencies in these checkpoint systems can lead to chromosome segregation errors and aneuploidy, and promote tumorigenesis. Here, we report that the TRAF-interacting protein (TRAIP), a ubiquitously expressed nucleolar E3 ubiquitin ligase important for cellular proliferation, is localized close to mitotic chromosomes. Its knockdown in HeLa cells by RNA interference (RNAi) decreased the time of early mitosis progression from nuclear envelope breakdown (NEB) to anaphase onset and increased the percentages of chromosome alignment defects in metaphase and lagging chromosomes in anaphase compared with those of control cells. The decrease in progression time was corrected by the expression of wild-type but not a ubiquitin-ligase-deficient form of TRAIP. TRAIP-depleted cells bypassed taxol-induced mitotic arrest and displayed significantly reduced kinetochore levels of MAD2 (also known as MAD2L1) but not of other spindle checkpoint proteins in the presence of nocodazole. These results imply that TRAIP regulates the spindle assembly checkpoint, MAD2 abundance at kinetochores and the accurate cellular distribution of chromosomes. The TRAIP ubiquitin ligase activity is functionally required for the spindle assembly checkpoint control.

KEY WORDS: TRAF-interacting protein, TRAIP, Mitosis, Chromosome mis-segregation, Spindle assembly checkpoint

INTRODUCTION

The equal distribution of chromosomal DNA during mitosis is ensured by a highly complex process in which the control of APC/C (anaphase-promoting complex/cyclosome) activity is crucial (Foley and Kapoor, 2013; Kops et al., 2005; Pines, 2011). APC/C activity is regulated by the spindle assembly checkpoint (SAC) proteins MAD1, MAD2 (also known as MAD1L1, MAD2L1, respectively), BUBR1 (also known as BUB1B), BUB1, BUB3 and MPS1 (also known as TTK), which sense whether or not chromosomes are connected in a bi-oriented manner to opposite spindle poles through microtubules (Foley and Kapoor, 2013; Khodjakov and Pines, 2010; Santaguida and Musacchio, 2009). The strength of the SAC crucially depends on the MAD2 abundance at kinetochores (Collin et al., 2013; Heinrich et al., 2013). Defects in chromosome segregation control cause chromosomal instability and aneuploidy, common

characteristics of human solid tumors (Holland and Cleveland, 2012).

Ubiquitylation is a highly conserved biochemical process during which ubiquitin monomers are attached to proteins (Dikic et al., 2009; Welchman et al., 2005). The TNF-receptor-associated factor (TRAF)-interacting protein (TRAIP, also known as TRIP or RNF206) is a RING-type E3 ubiquitin ligase (Besse et al., 2007), but hitherto no *in vivo* substrate has been identified. Ectopically expressed TRAIP interacts with TRAF proteins and represses NF- κ B signaling (Besse et al., 2007; Lee et al., 1997), although this might not be physiologically relevant (Chopard et al., 2012). TRAIP is expressed ubiquitously at a low level (Lee et al., 1997; Su et al., 2004) in the nucleoli (Zhou and Geahlen, 2009) of interphase cells but is overexpressed in breast cancer cell lines (Zhou and Geahlen, 2009) and in basal cell carcinomas (Almeida et al., 2011).

Both TRAIP-knockout mice (Park et al., 2007) and mutants of NOPO (Merkle et al., 2009), the *Drosophila* homolog of mammalian TRAIP, die early in development owing to aberrant proliferation and mitosis. TRAIP transcription is strongly downregulated upon induction of cell cycle exit, and its knockdown decreases proliferation and causes a G1/S phase block in keratinocytes (Almeida et al., 2011). TRAIP interacts with CYLD (Regamey et al., 2003) and SYK (Zhou and Geahlen, 2009), two tumor suppressors (Bailet et al., 2009; Bignell et al., 2000; Coopman et al., 2000; Lee et al., 2005; Moroni et al., 2004), and with DNA polymerase η , which facilitates translesional synthesis after DNA damage (Wallace et al., 2014).

We report here that the TRAIP protein preferentially localizes during mitosis around condensed chromosomes in prometaphase and metaphase, and on chromosomal DNA in anaphase. Its functional inactivation accelerates the progression through early phases of mitosis, reduces MAD2 recruitment to kinetochores and leads to increased mitotic defects. Our findings strongly support the existence of a role for TRAIP in SAC regulation.

RESULTS AND DISCUSSION**TRAIP preferentially localizes at the periphery of chromosomes in early mitosis**

Analysis of endogenous TRAIP expression by immunofluorescence detection in mitotic cells showed localization around prometaphase chromosomes and colocalization with anaphase chromosomes (Fig. 1A). To determine the specificity of the antibody, immune detection of endogenous TRAIP in HeLa cells was analyzed 24 h after transfection with two small interfering (si)RNAs targeting TRAIP (siTRAIP1 and siTRAIP2) or a scrambled negative control siRNA (siCTRL). TRAIP mRNA and protein levels were reduced to ~30% in TRAIP-depleted cells compared with levels in control cells (supplementary material Fig. S1A). The immunofluorescent signal of endogenous TRAIP was observed in the nucleoplasm of both siCTRL- and siTRAIP-treated cells during interphase (Fig. 1B). After treatment of cells with a short detergent extraction before fixation, to remove soluble proteins (Nalepa

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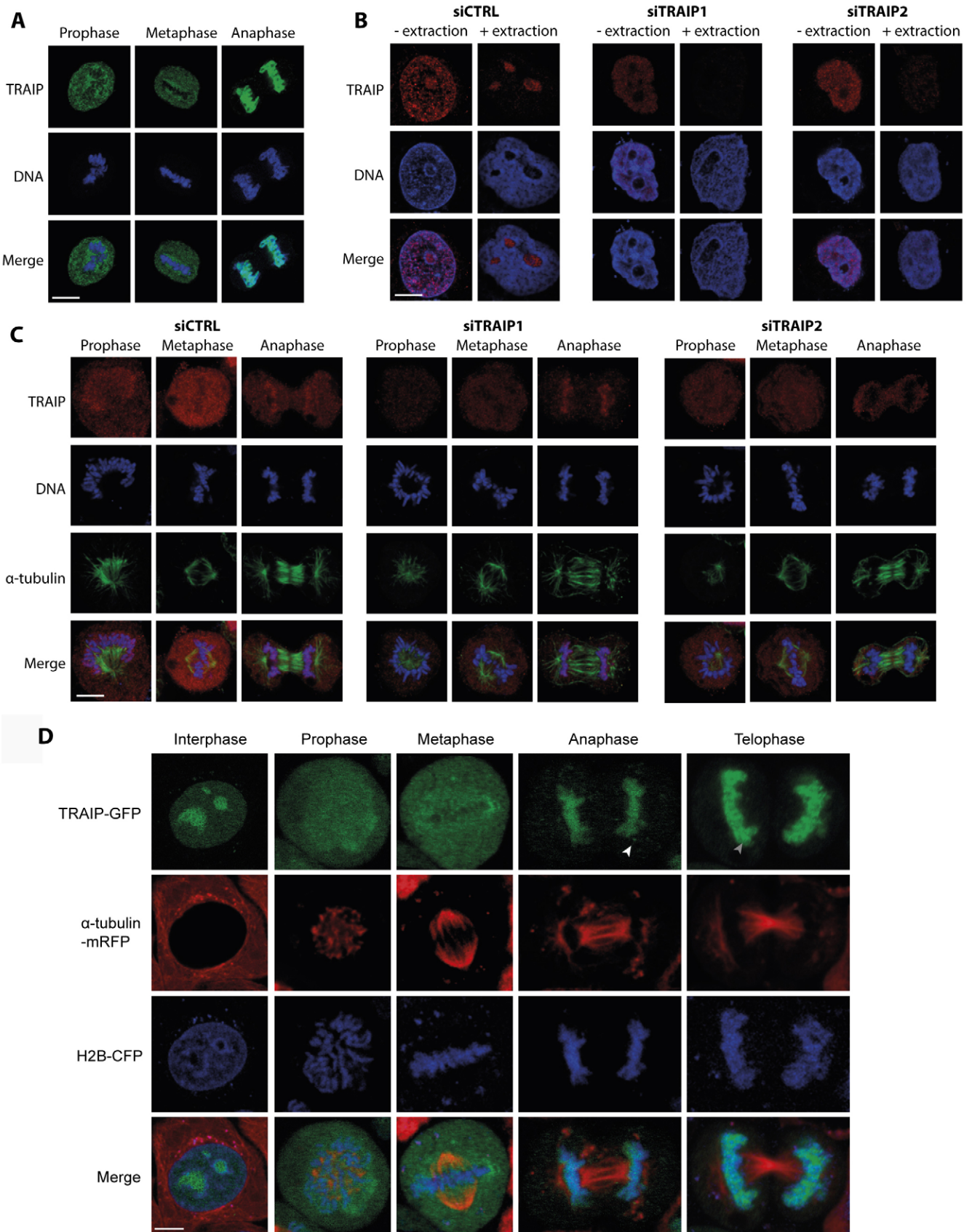


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Fig. 1. Endogenous and ectopic TRAIIP is a chromosomal peripheral protein during mitosis. (A) Confocal images of mitotic HeLa cells immunostained with anti-TRAIIP antibody (green). (B,C) Confocal images of HeLa cells in interphase (B) or mitosis (C) immunostained for endogenous TRAIIP (red) and α -tubulin (green), 24 h after treatment with the indicated siRNAs. Extraction was carried out with 0.1% Triton X-100 for 2 min before fixation (B). The settings for confocal imaging were identical within experiments. DNA was stained with DAPI (blue). (D) Confocal images of living HeLa cells stably expressing α -tubulin-mRFP (red) and H2B-CFP (blue) together with TRAIIP-GFP (green). PNB and NDF are marked by gray and white arrowheads, respectively. Scale bars: 5 μ m.

et al., 2013), the signal became nucleolar in controls and disappeared from TRAIIP-depleted cells. The signal in mitotic cells was localized close to congressing chromosomes in prometaphase and was associated with condensed chromosomes in anaphase in controls but was strongly reduced in siTRAIIP-treated cells (Fig. 1C).

To further validate TRAIIP localization, a lentiviral vector driving expression of a functional (supplementary material Fig. S2; Besse et al., 2007; Zhou and Geahlen, 2009) TRAIIP-GFP was used to infect HeLa cells stably expressing α -tubulin-mRFP and H2B-CFP (Beronja et al., 2010). In early mitotic phases, TRAIIP-GFP was dispersed throughout the cytoplasm and converged at the chromosome periphery, whereas in later phases it colocalized with chromosomes (Fig. 1D). During telophase, a small fraction accumulated in cytoplasmic particles, probably corresponding to nucleolus-derived foci (NDF) and pre-nucleolar bodies (PNB) (Dundr et al., 1997; Ma et al., 2007; Van Hooser et al., 2005).

The perichromosomal layer of TRAIIP appeared in prometaphase and disappeared in telophase or cytokinesis (Hernandez-Verdun and Gautier, 1994; Ma et al., 2007; Van Hooser et al., 2005). Nucleolar proteins and RNAs are known to bind to the surface of chromosomes at the beginning of mitosis and are incorporated again into newly formed nucleoli during telophase (Dundr et al., 1997; Van Hooser et al., 2005). Different biological functions have been assigned to chromosomal peripheral proteins (CPP) forming the perichromosomal layer (Hernandez-Verdun, 2011; Hernandez-Verdun and Gautier, 1994; Ma et al., 2007; Matsunaga and Fukui, 2010). Our analysis identified the nucleolar protein TRAIIP as a CPP and prompted us to investigate TRAIIP function in mitosis.

TRAIIP regulates progression through early phases of mitosis

We examined the consequences of silencing TRAIIP expression on the duration of mitosis using siRNAs and fluorescence time-lapse imaging of HeLa cells expressing H2B-EGFP and α -tubulin-mRFP (Toso et al., 2009), taking nuclear envelope breakdown (NEB) as starting time, $t=0$ min (Meraldi et al., 2004). In siCTRL-transfected cells, the median duration of mitosis was 116.7 min [95% confidence interval (CI)=111.7–123.3 min; range, 75 to 275 min]. The high variability of duration of mitosis was mainly caused by the time between NEB to anaphase onset (Lim et al., 2013; Mchedlishvili et al., 2012; Rieder et al., 1994; Toso et al., 2009), which varied from 20 to 200 min (median, 53.3 min; 95% CI=46.7–61.7 min). The time from anaphase to cytokinesis fluctuated only moderately from 45 to 85 min (median, 63.3 min; 95% CI=60–66.7 min) (Fig. 2A–D).

The average time taken to complete mitosis by siTRAIIP1- or siTRAIIP2-treated cells was reduced to 96.7 min (95% CI=95–101 min) or 90 min (95% CI=87.7–91.7 min), respectively

(Fig. 2A). Although the time from anaphase to cytokinesis with siTRAIIP1 (range, 45 to 95 min; median, 66.7 min; 95% CI=63.3–70 min) and siTRAIIP2 (range, 45 to 80 min; median, 58.3 min; 95% CI=56.7–63.3 min) was very similar to that of control cells, the time from NEB to anaphase onset was significantly reduced to 30 min (95% CI=28.3–33.3 min; range, 20 to 75 min) with siTRAIIP1 and 26.7 min (95% CI=26.7–28.3 min; range, 20 to 85 min) with siTRAIIP2 (Fig. 2B,C). In total, 90% of TRAIIP-depleted cells accomplished the onset of anaphase within 45 min after NEB compared to only 50% of control cells (Fig. 2E). In the skew-normal frequency distribution of anaphase onset times, a first population (peak of the distribution) with rapid and uniform chromosome congression and a second with longer anaphase onset times (active spindle checkpoint) could be distinguished (Meraldi et al., 2004; Rieder et al., 1994). In control siRNA-transfected HeLa cells, the skew-normal distribution of anaphase onset times had a peak (mode) at 31.8 ± 1.2 min, whereas in TRAIIP-depleted cells a shift to 28.4 ± 0.4 min (siTRAIIP1; mean \pm s.e.m.) or 24.2 ± 0.4 min (siTRAIIP2) was observed (Fig. 2F). The NEB to anaphase onset time in cells transduced with siRNA-resistant TRAIIP before siRNA transfection was indistinguishable from that of the control, whereas a catalytically inactive TRAIIP mutant (C25A) failed to rescue the siRNA-treated cells (Fig. 2G). Although MAD2 is a frequent siRNA off-target (Hübner et al., 2010; Sigoillot et al., 2012; Westhorpe et al., 2010), the expression of MAD2, MAD1, BUB1 and BUB3 remained unchanged after TRAIIP KD (supplementary material Fig. S1B,C). Taken together with the rescue data it is therefore unlikely that TRAIIP-knockdown phenotypes are caused by off-target effects. These findings suggest that the ubiquitin ligase TRAIIP is involved in the regulation of NEB to anaphase onset time.

TRAIIP loss increases the occurrence of chromosome alignment defects and lagging chromosomes

Because a reduced NEB to anaphase onset time could induce aberrant chromosome segregation (Meraldi and Sorger, 2005; Michel et al., 2001; Perera et al., 2007), we examined whether TRAIIP depletion affected chromosome behavior. Following TRAIIP depletion, the number of cells dividing with a bipolar spindle and giving rise to two living daughter cells was not significantly different from that of control cells. Mitotic indexes in siRNA-treated cells were examined by immunostaining with anti-phospho-histone H3(Ser28) and anti- α -tubulin antibodies. Although mitotic indexes were comparable to those of controls (Fig. 3A), the percentage of cells in prophase was reduced by 10–20% in TRAIIP-depleted compared with control cells (Fig. 3B), consistent with shorter NEB to anaphase onset times. In TRAIIP-depleted mitotic cells synchronized by a double-thymidine block, the percentage of non-aligned chromosomes at the metaphase plate was increased compared with that of the control (siRNA1, $38.5 \pm 4.9\%$; siRNA2, $37.1 \pm 7.6\%$; control, $19.6 \pm 7.5\%$; mean \pm s.d.) (Fig. 3C,E). More chromosome segregation errors were detected in mitotic cells treated with either siTRAIIP1 or 2 compared with the number observed in control cells ($15.7 \pm 4.5\%$ or $19.5 \pm 6.7\%$ versus $9.7 \pm 2.9\%$; mean \pm s.d.) (Fig. 3D,F). As expected, the number of chromosome alignment and segregation errors was increased in MAD2-depleted cells, supporting the reliability of our data (Fig. 3E,F). In summary, TRAIIP downregulation in HeLa cells leads to chromosome misalignment and segregation defects.

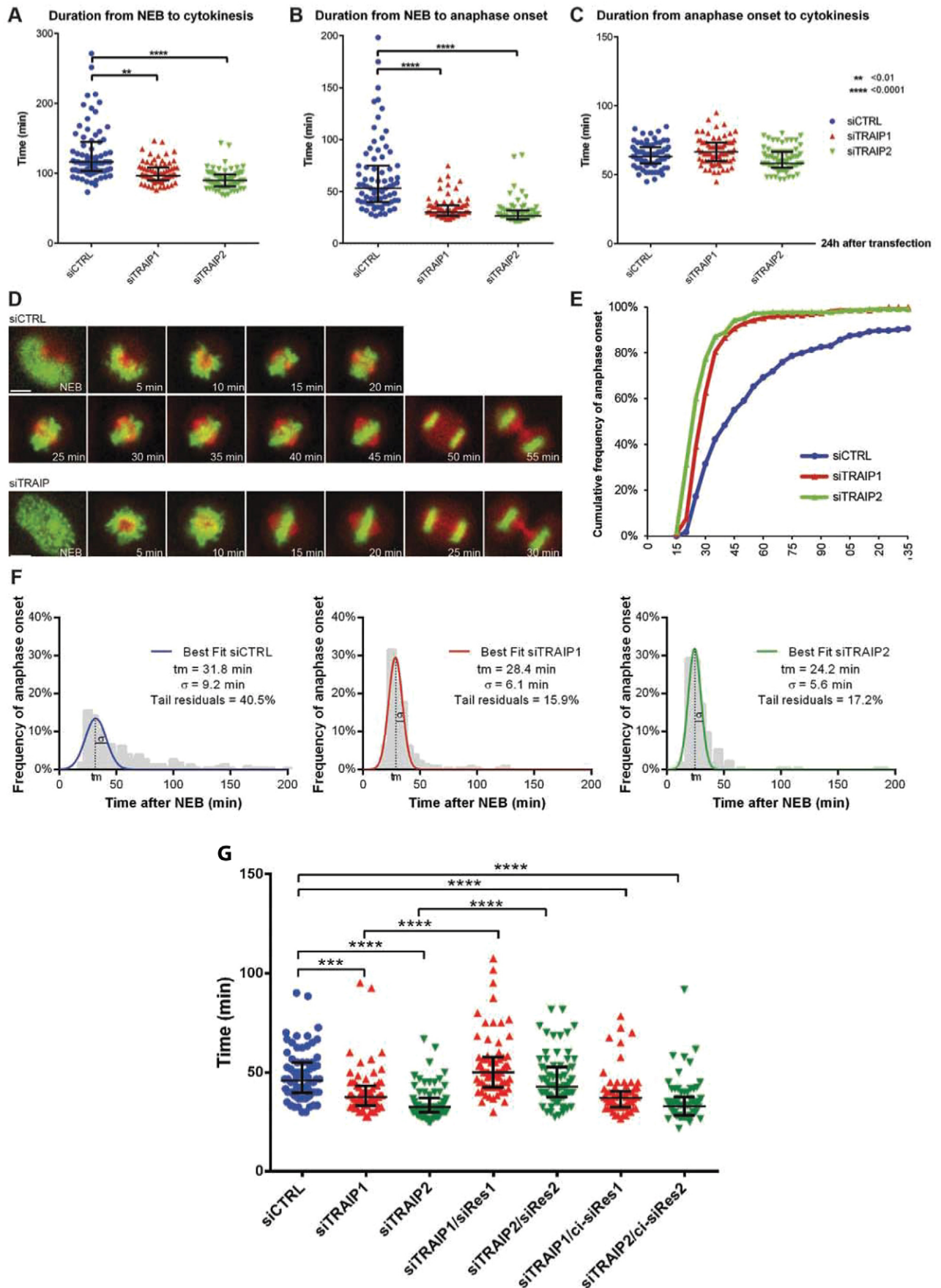


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Fig. 2. TRAIP depletion decreases the NEB to anaphase time.

(A–C) Scatter plots (indicating the median and interquartile range) of times taken to complete mitosis (A), times from NEB to anaphase onset (B) and from anaphase onset to cytokinesis (C) in siRNA-treated HeLa cells. $N=3$ independent experiments, $n=225$ cells. (D) Successive frames from live-cell movies. Red, α -tubulin; green, histone 2B. Scale bar: 5 μm . (E) Cumulative frequency plots of anaphase onset times (NEB=0 min). $N=3$ independent experiments, $n=225$ cells. (F) Frequency distribution of anaphase onset times. $N=3$ independent experiments, $n=225$ cells. (G) The decrease in NEB to anaphase onset time was rescued by the expression of siRNA-resistant wild-type but not by catalytically inactive (ci, C25A) TRAIP. The median and interquartile range are indicated; $N=3$ independent experiments, $n=180$ cells. ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$.

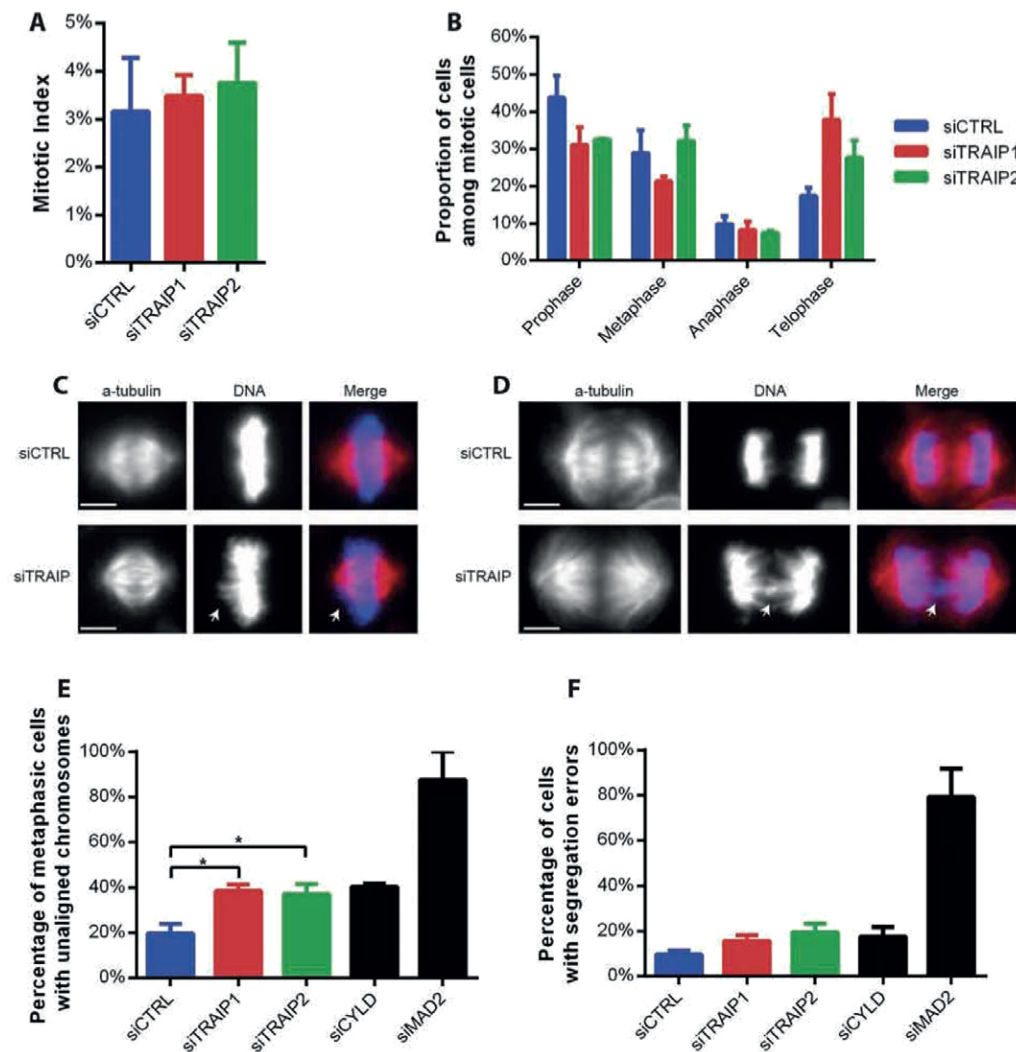
TRAIP loss decreases SAC function

Our results demonstrated that early mitosis and chromosome behavior are affected by TRAIP knockdown, suggesting a role of TRAIP in the SAC. To investigate whether TRAIP knockdown enabled HeLa cells to bypass the SAC, a taxol-induced mitotic arrest assay (Stegmeier et al., 2007a) was undertaken to assess mitotic index and nuclear morphology. CYLD- and MAD2-specific siRNAs were used as controls for mitotic entry delay and SAC bypass (Draviam et al., 2007; Meraldi et al., 2004; Stegmeier et al., 2007a; Stegmeier et al., 2007b). Only $13\pm 8\%$ (mean \pm s.d.) of MAD2-depleted or $51\pm 15\%$ of CYLD-depleted cells were

mitotic (Fig. 4A,B) (Stegmeier et al., 2007b), whereas the value was higher for control cells ($74\pm 7\%$). In TRAIP-depleted cells, a reduced mitotic index (siRNA1, $52\pm 16\%$; siRNA2, $53\pm 8\%$) (Fig. 4B) and multilobed nuclear morphology (Stegmeier et al., 2007a; Stegmeier et al., 2007b), similar to that of cells treated with MAD2-specific siRNA (Fig. 4A,C), was observed, demonstrating that loss of TRAIP led to a checkpoint-bypass phenotype. However, TRAIP depletion led to a smaller effect than MAD2 knockdown. Whether this reflects a phenotypic difference or is due to differences in the degree of mRNA knockdown remains to be seen. Our data indicated that TRAIP depletion was less effective compared with MAD2 knockdown (supplementary material Fig. S1A). To summarize, loss of TRAIP reduced mitotic arrest in response to the spindle poison taxol, hence leading to the bypass of the SAC.

TRAIP depletion decreases MAD2 levels at unattached kinetochores

The expression and/or localization of SAC proteins are finely modulated to regulate APC/C activity (Musacchio and Salmon, 2007) in a dose-dependent manner (Collin et al., 2013; Heinrich et al., 2013). The robustness of the SAC is, in part, determined by the amount of MAD2 recruited to kinetochores. To elucidate whether TRAIP deficiency is affecting the kinetochore localization

**Fig. 3. TRAIP depletion leads to chromosome alignment defects and lagging chromosomes.**

(A) Mitotic index of siRNA-treated HeLa cells. $N=3$ independent experiments, $n=7000$ cells. (B) Distribution of siRNA-treated HeLa cells in the different phases of mitosis. $N=3$ independent experiments, $n=250$ cells. (C,D) Representative images of siRNA-transfected HeLa cells in metaphase (C) and anaphase (D). Arrows show unaligned (C) or lagging chromosomes (D). Scale bars: 5 μm . (E) Fraction of cells with unaligned chromosomes at the metaphase plate. $N=3$ independent experiments, $n>100$ (siCTRL, siTRAIP1 and siTRAIP2); $N=2$ independent experiments, $n=14$ cells (siMAD2) and 60 cells (siCYLD). (F) The fraction of cells with segregation errors in anaphase. $N=3$ independent experiments, $n>150$ cells (siCTRL, siTRAIP1 and siTRAIP2); $N=2$ independent experiments, $n=69$ cells (siMAD2) and 45 cells (siCYLD). Results in A,B,E,F are shown as the mean \pm s.e.m.

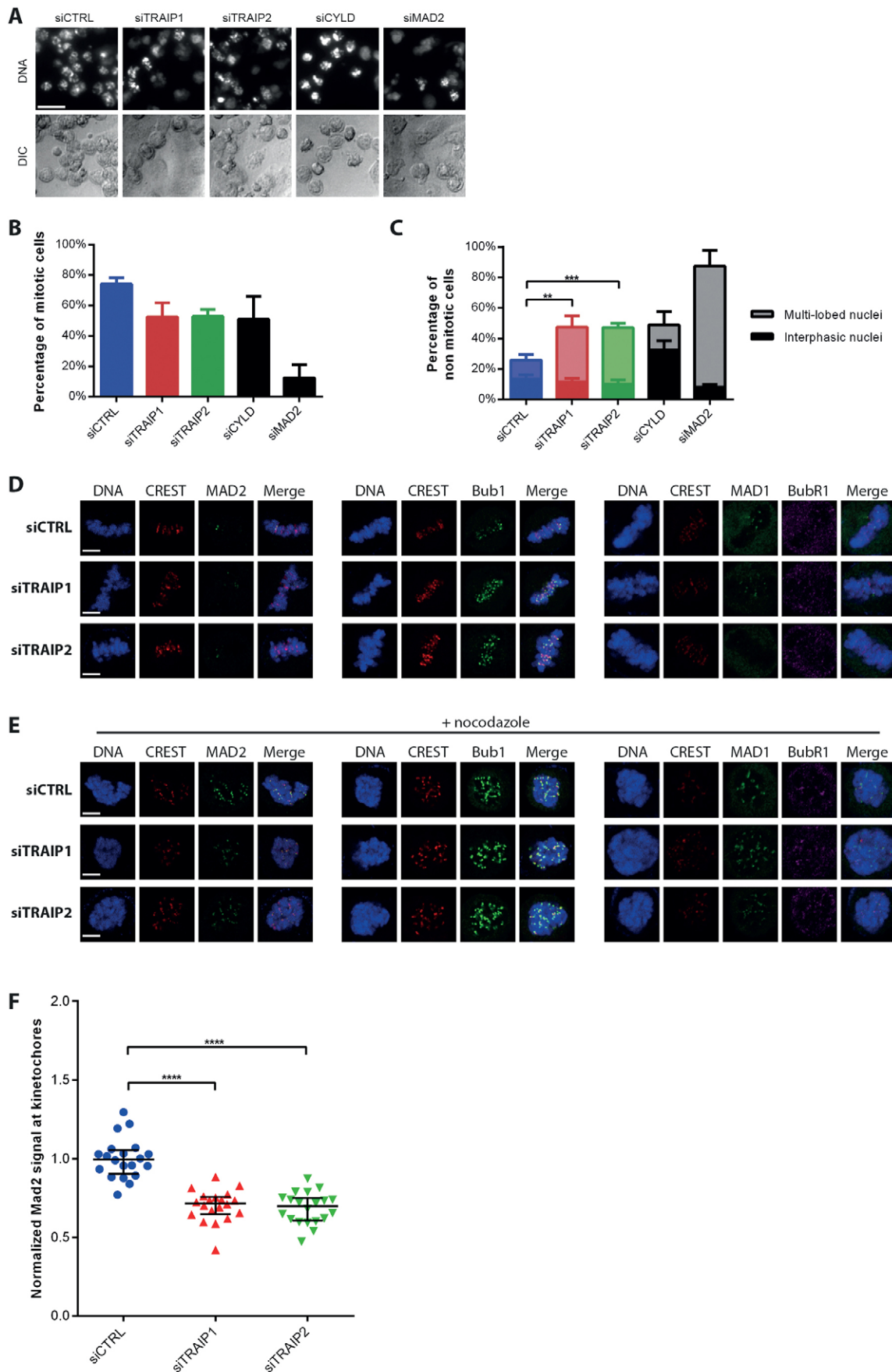


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Fig. 4. TRAIIP knockdown affects the spindle checkpoint by reducing the amount of MAD2 at unattached kinetochores. (A) Representative images of siRNA-transfected HeLa cells treated with 100 nM taxol for 24 h. Scale bar: 30 μ m. (B,C) The percentage of mitotic cells (B) and cells with either multilobed or interphase nuclei (C) was determined by cell rounding in DIC microscopy and DAPI staining. The results are represented as the mean \pm s.e.m. $N=4$ independent experiments, $n>2700$ cells (siCTRL, siTRAIP1 and siTRAIP2); $N=2$ independent experiments, $n=400$ cells (siMAD2 and siCYLD). (D,E) Confocal images of siRNA-treated HeLa cells immunostained for CREST (red) and either MAD1, MAD2, BUB1 (green) or BUBR1 (purple). Cells in E were treated with nocodazole (1 μ g/ml) for 1 h prior to analysis. Scale bars: 5 μ m. (F) Scatter plot of MAD2 intensities at single kinetochores in nocodazole-treated cells shown in E. Quantification of MAD2 levels relative to the CREST signal is shown, along with the median and interquartile range. The total mean signal for MAD2 in control cells was normalized to 1. $**P<0.01$, $***P<0.001$, $****P<0.0001$.

of SAC proteins, we performed immunofluorescence detection of MAD1, MAD2, BUB1 and BUBR1 proteins in TRAIIP-siRNA-treated cells (Fig. 4D,E). SAC proteins were correctly positioned at unattached kinetochores marked by an anti-CREST antibody in prometaphase cells. However, the amount of MAD2 recruited to the kinetochores was significantly reduced in TRAIIP-depleted cells compared with that of control cells (siRNA1, $70.2\% \pm 10.1\%$; siRNA2, $68.5\% \pm 9.9\%$; control, $100\% \pm 12.8\%$; mean \pm s.d.) (Fig. 4E,F). This might be physiologically relevant, because a reduction of 20% in MAD2 levels is sufficient to impair SAC activity in yeast (Heinrich et al., 2013).

In summary, we have demonstrated that TRAIIP changes its predominantly nucleolar localization in interphase cells to the perichromosomal layer in mitosis. The decrease in progression time from NEB to anaphase onset and in the binding of MAD2 to kinetochores in TRAIIP-depleted cells, leading to chromosome misalignment and segregation errors, implicates TRAIIP as a novel regulator of SAC activity that functions in a ubiquitin-ligase-dependent manner. Our findings most likely explain the cell death in TRAIIP-knockout mice and NOPO mutants in *Drosophila* embryos, because inaccurate chromosome segregation negatively impinges on the life of normal cells. The elucidation of the mechanism by which TRAIIP functions at the molecular level to regulate the SAC awaits identification of its substrates and interaction partners.

MATERIALS AND METHODS

Cell culture

Cells were cultured in DMEM containing 10% fetal bovine serum and appropriate antibiotics (200 μ g/ml G418, 0.25 μ g/ml puromycin). Cells were synchronized by a double-thymidine (2 mM) block. Transfection of siRNAs was performed after the first thymidine treatment.

Plasmids and lentivirus

TRAIIP cDNA was amplified from a human cDNA library and cloned into pEGFP-N3 (Clontech) or the lentiviral vector pCDH-CMV-MCS-EF1-Hygro (BioCat). TRAIIP cDNAs with the C25A mutation or resistance to siRNAs were constructed by PCR amplification. PCR products were verified by sequencing. LV-CFP expressing H2B-CFP was purchased (Addgene #25998). Lentivirus was produced as described previously (Wiznerowicz and Trono, 2003). Cells were infected (multiplicity of infection of 10) with lentivirus (Almeida et al., 2011) and selected with 800 μ g/ml hygromycin.

Protein extractions and TRAIIP autoubiquitylation

Proteins were extracted by cell lysis in 1% SDS in phosphate-buffered saline. For immunoblots, the following antibodies were used: anti-GFP (Clontech), anti-actin (Sigma), rabbit anti-TRAIIP (Abcam), goat anti-TRAIIP (Imgenex), anti-MAD2 (Covance) and anti-HA (Santa Cruz

Biotechnology). TRAIIP autoubiquitylation was carried out as described previously (Almeida et al., 2008). Signals from immunoblots were captured by using LAS4000 (GE Healthcare) and analyzed by using ImageJ.

RNA interference

siRNA targeting human TRAIIP mRNA (NM_005879.2), CYLD (Draviam et al., 2007; Sun et al., 2010) and MAD2 (Uzunova et al., 2012), and control siRNA (Origene), were transfected into cells at a final concentration of 30 nM using INTERFERin (Polyplus).

Live-cell imaging and immunofluorescence analysis

Experiments were performed at 37°C under 5% CO₂ in cell-culture chambers (MatTek) in Phenol-Red-free medium. For time-lapse experiments, 24 h after siRNA transfection, images were recorded every 5 min for 16 h using a 20 \times Plan-Neofluar NA 0.5 objective on a Axio Observer Z1 microscope (Zeiss). Confocal live-cell imaging experiments were performed using a 63 \times /1.4 oil objective on a LSM700 confocal laser-scanning microscope (Zeiss). Antibodies against the following proteins were used for immunofluorescence: GFP (Clontech), TRAIIP (Abcam), phospho-histone H3(Ser28) (Cell Signaling Technology), tubulin (Sigma), nucleolin C23 (Santa Cruz Biotechnology), MAD1 (Meraldi et al., 2004), MAD2 (Bethyl Laboratories), BUB1 (Klebig et al., 2009), BUBR1 (Klebig et al., 2009) and CREST (Klebig et al., 2009). Images were analyzed using ImageJ. Kinetochores levels of MAD2 were quantified as described previously (McClelland et al., 2007).

Taxol assay

The taxol assay in HeLa cells was carried out as described previously (Stegmeier et al., 2007a), except for DNA staining with DAPI. MosaiX images were acquired using a 20 \times objective on an Axio Imager microscope (Zeiss) equipped with differential interference contrast (DIC) and DAPI filters. Cells were scored using ImageJ.

RNA isolation and qRT-PCR analysis

RNA was purified using the RNeasy Kit (Qiagen) and cDNA was synthesized using Primescript-RT Kit (TakaRa). Quantitative PCR analysis was performed (Almeida et al., 2011) using primers for RPL13A (endogenous reference), TRAIIP, CYLD, MAD1, BUB1B, BUB3 (Qiagen) or MAD2 (Universal Probe Library, Roche).

Statistical analyses

Statistical significance ($*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$) was calculated using one-way ANOVA followed by Tukey's multiple comparisons test or two-way ANOVA followed by Tukey–Kramer's post hoc test (GraphPad Prism6).

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Competing interests

The authors declare no competing interests.

Author contributions

M.H. designed and supervised the present study. C.C., T.G. and D.B. performed experiments. C.C., P.M., D.H. and M.H. wrote and discussed the paper.

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Supplementary material

Supplementary material available online at <http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.152579/-DC1>

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