The hsSsu72 phosphatase is a cohesin-binding protein that regulates the resolution of sister chromatid arm cohesion

Hyun-Soo Kim^{1,2}, Kwan-Hyuck Baek¹,
Geun-Hyoung Ha^{1,2}, Jae-Chul Lee¹,
Yu-Na Kim³, Janet Lee¹, Hye-Young Park¹,
Noo Ri Lee¹, Ho Lee⁴, Yunje Cho⁵ and
Chang-Woo Lee^{1,2,*}

¹Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon, Korea, ²Center for Molecular Medicine, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, Korea, ³Department of Microbiology, College of Medicine, Gyeongsang National University, Jinju, Korea, ⁴Division of Cancer Biology, Research Institute, National Cancer Center, Goyang, Korea and ⁵Department of Life Science, Pohang University of Science and Technology, Pohang, Korea

Cohesin is a multiprotein complex that establishes sister chromatid cohesion from S phase until mitosis or meiosis. In vertebrates, sister chromatid cohesion is dissolved in a stepwise manner: most cohesins are removed from the chromosome arms via a process that requires polo-like kinase 1 (Plk1), aurora B and Wapl, whereas a minor amount of cohesin, found preferentially at the centromere, is cleaved by separase following its activation by the anaphase-promoting complex/cyclosome. Here, we report that our budding yeast two-hybrid assay identified hsSsu72 phosphatase as a Rad21-binding protein. Additional experiments revealed that Ssu72 directly interacts with Rad21 and SA2 *in vitro* and *in vivo*, and associates with sister chromatids in human cells. Interestingly, depletion or mutational inactivation of Ssu72 phosphatase activity caused the premature resolution of sister chromatid arm cohesion, whereas the overexpression of Ssu72 yielded high resistance to this resolution. Interestingly, it appears that Ssu72 regulates the cohesion of chromosome arms but not centromeres, and acts by counteracting the phosphorylation of SA2. Thus, our study provides important new evidence, suggesting that Ssu72 is a novel cohesin-binding protein capable of regulating cohesion between sister chromatid arms.

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Introduction

Proper chromosome alignment and segregation during mitosis rely on the cohesion between sister chromatids. This is mediated by a protein complex called cohesin, which includes heterodimeric ATPases made of Smc1 and Smc3 proteins in association with the regulatory subunit, Rad21 (a mammalian isoform of Scc1), which is in turn associated with the SA1 or SA2 variants of the Scc3 protein (Michaelis *et al*, 1997; Losada *et al*, 1998, 2000; Sumara *et al*, 2000; Haering *et al*, 2002). In higher eukaryotes, the removal of cohesin from sister chromatids occurs in a stepwise manner. During prophase, the bulk of cohesin is removed from chromosome arms via a process that requires Wapl and involves the polo-like kinase 1 (Plk1) and aurora B kinases, but does not result in the proteolytic cleavage of Rad21 (Losada *et al*, 1998, 2002; Waizenegger *et al*, 2000; Gimenez-Abian *et al*, 2004; Gandhi *et al*, 2006; Kueng *et al*, 2006). Recent studies have shown that protein phosphatase 2A (PP2A) and shugoshin (Sgo) together function to protect centromeric cohesin during early mitosis (Kitajima *et al*, 2006; Tang *et al*, 2006). More specifically, PP2A localizes at the centromere via Sgo, and prevents the phosphorylation of cohesin by Plk1 and potentially by aurora B (McGuinness *et al*, 2005; Tang *et al*, 2006). Therefore, PP2A counteracts the Plk1-mediated phosphorylation of the SA1/2 cohesin subunits, thereby preventing their dissociation from centromeric chromatin. After prophase, a minor amount of cohesin, found preferentially at the centromeres, is cleaved by anaphase-promoting complex/cyclosome-activated separase, which cleaves Rad21 at the metaphase-to-anaphase transition (Hauf *et al*, 2001; Nasmyth, 2002). This process removes all the remaining cohesin from the chromosomes and triggers the separation of sister chromatids. Despite the appeal of such a simple model, however, the related mechanisms are not yet fully understood.

The yeast protein, Ssu72, was initially identified as a transcription/RNA processing factor through its physical interaction with the TFIIB transcription factor (Pappas and Hampsey, 2000). Subsequent studies implicated Ssu72 in the regulation of cell viability in yeast, and showed that it is highly conserved among eukaryotic organisms (Pappas and Hampsey, 2000). Two groups independently reported that Ssu72 has phosphatase activity and its sequence contains the CX5R signature motif of PTPases; based on this, Ssu72 was thought to be a member of a new phosphatase subfamily in which the so-called aspartate loop is the phosphatase active site (Ganem *et al*, 2003; Meinhart *et al*, 2003). Recombinant yeast Ssu72 proteins were found to exhibit phosphatase activity against the synthetic substrate, *p*-nitrophenyl phosphate, and both phosphatase domain and aspartate mutants of Ssu72 showed decreased phosphatase activity (Ganem *et al*, 2003; Meinhart *et al*, 2003). Another study

*Corresponding author. Department of Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon 440-746, Korea. Tel.: +82 31 299 6121; Fax: +82 31 299 6109; E-mail: cwlee@med.skku.ac.kr

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found that the phosphatase activity of Ssu72 could dephosphorylate serine 5 at the C-terminal domain (CTD) of RNA polymerase II (Krishnamurthy *et al*, 2004). However, the exact functions and physiological substrates of Ssu72 remain unclear even in yeast.

In this study, we report that our yeast two-hybrid assay identified hsSsu72 as a Rad21-binding protein. Additional experiments revealed that Ssu72 directly interacts with Rad21 and SA2 *in vitro* and *in vivo*, and associates with mitotic sister chromatids. Interestingly, Ssu72 depletion or mutational inactivation of Ssu72 phosphatase activity causes premature sister chromatid arm separation, whereas the overexpression of Ssu72 yields high resistance to the resolution of arm cohesion. Further studies showed that Ssu72 counteracts the phosphorylation of SA2 but not that of other cohesin subunits, and it dephosphorylates SA2. Collectively, these results provide important new evidence, suggesting that Ssu72 is a novel cohesin-binding phosphatase capable of regulating the cohesion between sister chromatid arms.

Results

Ssu72 associates and co-localizes with Rad21 and SA2

To gain insight into the mechanisms underlying sister chromatid dissociation, we performed a conventional yeast two-hybrid assay using a human fetal kidney cDNA library, and identified Ssu72 as an hsRad21-binding protein (Figure 1A; Supplementary Figure S1). To confirm this finding, we first raised both rabbit polyclonal and mouse monoclonal antibodies against a peptide from hsSsu72 (LDRNKRIKPRPERFQNC). Experiments showed that the affinity-purified antibodies specifically and efficiently recognized both endogenous and exogenous Ssu72 proteins (Supplementary Figure S2). Specifically, the antibodies recognized a 28-kDa Ssu72 polypeptide in HeLa cell extracts, and this immunoreactivity could be depleted by transfection of HeLa cells with Ssu72-targeting shRNAs. Extracts from asynchronously growing HeLa cells were immunoprecipitated with the polyclonal anti-Ssu72 antibody or normal IgG (control), and immunoblotting was performed with antibodies against Rad21, SA2, Smc1, Smc3 and Mad2 (negative control) (Figure 1B, upper panel). Similarly, HeLa cell extracts were immunoprecipitated with an anti-Rad21 antibody or control IgG, and the resulting immunoprecipitates were immunoblotted as indicated (Figure 1B, lower panel). Notably, Ssu72 and Rad21 were present in the complexes *in vitro* and *in vivo*, and Ssu72 also showed interactions with the cohesin subunits, SA2, Smc1 and Smc3. Next, we prepared cellular extracts from HeLa cells stably expressing a Myc-tagged Rad21/Sccl (Myc-Rad21) fusion protein under the control of the inducible 'Tet-on' promoter (Figure 1C; Hauf *et al*, 2001). Subsequent immunoprecipitation and immunoblotting analyses revealed that the Myc-Rad21 fusion proteins formed a complex with endogenous Ssu72, as well as with Smc1 and Smc3 (positive controls) (Figure 1C). To further determine whether Ssu72 interacts directly with Rad21, we generated full-length His- and Glutathione S-transferase (GST)-tagged fusion proteins (His-Ssu72 and GST-Rad21, respectively) (Figure 1D, left panel). Pull-down assays revealed that His-Ssu72 bound to GST-Rad21. In addition, we generated full-length GST-SA2 and incubated it

with His-Ssu72 (Figure 1D, right panel). Unexpectedly, we found that Ssu72 also interacted directly with SA2.

To define the domains responsible for the Ssu72–Rad21 and Ssu72–SA2 interactions, we incubated a series of GST-fused Ssu72 deletion mutants with HeLa cell extracts. As shown in Figure 1E, the Ssu72 COOH-terminal domain (amino acids 121–194) formed a complex with Rad21, whereas both the central and COOH-terminal regions (amino acids 61–194) of Ssu72 were required for the interaction with SA2. To exclude the possibility that the interactions between Ssu72 and the cohesin subunits might be indirectly mediated through chromatin, we treated nuclear extracts from HeLa cells stably overexpressing HA-tagged Ssu72 (HeLa-HA-Ssu72) with DNase, and then subjected the extracts to immunoprecipitation with an anti-HA antibody or control IgG, followed by immunoblotting with the indicated antibodies (Figure 1F). Again, all of the tested cohesin subunits (Smc1, Smc3, SA2 and Rad21) were found in complexes with Ssu72, as shown by silver nitrate staining (data not shown) and immunoblotting (Figure 1F), indicating that Ssu72 forms a complex with the cohesin subunits.

As cohesins are loaded onto chromatin, most cohesin complexes are detected in chromatin fractions (Losada *et al*, 2002; Kueng *et al*, 2006). To test whether Ssu72 could also be found as a chromatin-bound protein, we removed the soluble proteins from HeLa cells by pre-extraction, and co-stained the remaining fraction with anti-Ssu72 and anti-Rad21 antibodies, and CREST antiserum (Figure 1G; Supplementary Figure S3). Both Ssu72 and Rad21 were detected near chromosomes during interphase (G2) and prophase (pro). However, consistent with the behaviour of Rad21, Ssu72 was dissociated from metaphase (meta) chromosomes. We further separated HeLa cell lysates into soluble cytoplasmic supernatants, insoluble pellets and chromatin-bound fractions for additional experiments. Consistent with previous findings (Losada *et al*, 2002; Kueng *et al*, 2006), we observed the presence of the cohesin subunits, Smc3, SA2 and Rad21, in the insoluble pellet and chromatin-bound fractions (Figure 1H). Under our experimental conditions, Ssu72 was also detected in the insoluble pellet and chromatin-bound fractions of asynchronously grown cells. In contrast (and consistent with our immunostaining results), the levels of Ssu72 were significantly reduced in the chromatin-bound fractions of mitotic cells (Figure 1H).

Aberrant expression of Ssu72 causes defects in the dissociation of cohesin from chromatin

To examine whether aberrant expression of Ssu72 affects the association or dissociation of chromatin cohesion in mitotic cells, we generated doxycycline-inducible HeLa cells expressing Rad21-RFP or SA2-RFP fusion proteins (Figure 2A). These HeLa-Rad21-RFP and HeLa-SA2-RFP cells were co-transfected with or without expression plasmids encoding Ssu72 and GFP-tagged H2B (H2B-GFP) or CFP-tagged H2B (H2B-CFP) and maltose-binding protein fused with GFP (MBP-GFP). The RFP fusion proteins were induced by doxycycline, and the RFP emissions (reflecting the intensity of Rad21 or SA2 protein expression) were digitally monitored by time-lapse microscopy (Figure 2B). Nuclear envelope breakdown (NEBD), which occurs as a cell enters mitosis, was determined by both the appearance of sister chromatid disorganization, as indicated by H2B-GFP or H2B-CFP, and

Figure 1 The hsSsu72 phosphatase forms a complex with cohesin subunits and associates with sister chromatids. (A) The amino-acid sequence of the hsSsu72 protein. The polo-box-binding motif (PBB, red), a conserved protein phosphatase domain (PPase, purple), the putative LxCxE motif (blue), and the putative destruction box (D-box, orange) are indicated. (B) Asynchronous HeLa cell extracts were immunoprecipitated with normal immunoglobulin IgG or a polyclonal anti-Ssu72 antibody, and immunoprecipitates were immunoblotted with anti-Rad21, anti-SA2, anti-Smc1, anti-Smc3 or anti-Mad2 (negative control) antibodies. Similarly, immunoprecipitation was performed with normal IgG or an anti-Rad21 antibody, followed by immunoblotting with anti-Ssu72, anti-SA2 or anti-Mad2 (a negative control) antibodies. Note that the generated anti-SA2 antibody specifically recognized endogenous SA1 and SA2 polypeptides in the HeLa cells. (C) HeLa cells expressing Myc-tagged Rad21/Sccl (HeLa-Myc-Rad21) were cultured in the absence (–) or presence (+) of 2 μ g/ml doxycycline (Doxy) and further incubated with 100 ng/ml nocodazole. Cell extracts were immunoprecipitated with an anti-Myc antibody, and subsequently immunoblotted with the indicated antibodies. (D) Purified His-Ssu72 was incubated with beads bound to either GST (G)-Rad21 (left) or GST (G)-SA2 (right). The beads were analysed by immunoblotting with anti-Ssu72, anti-Rad21 or anti-SA2 antibodies. (E) HeLa cell lysates were incubated with beads bound to GST (G) alone or to a series of GST (G)-fused Ssu72 deletion mutants. Bound proteins were resolved and immunoblotted with anti-SA2 and anti-Rad21 antibodies. (F) Nuclear extracts (NE) from HeLa-HA-Ssu72 cells (overexpressing HA-tagged Ssu72) were treated with DNase (0.5 U/ μ g) for 30 min, and then immunoprecipitated with an anti-HA antibody or control IgG. The immunoprecipitates were analysed by immunoblotting with anti-Smc1, anti-Smc3, anti-SA2, anti-Rad21 and anti-HA antibodies (right panels). Chromosomal DNA from nuclear extracts was analysed by ethidium bromide staining (left panels). (G) HeLa cells were extracted with 0.2% Triton X-100, and then fixed and qco-stained with an anti-Ssu72 antibody (green), an anti-Rad21 antibody (red) and CREST (purple). DNA was visualized by DAPI staining. ‘Pro’ and ‘Meta’ indicate prophase and metaphase, respectively. (H) Extracts from HeLa cells cultured in the absence or presence (Noco) of nocodazole were separated into soluble cytoplasmic supernatant (Supt), insoluble pellet (Pellet) and chromatin-bound (Chro) fractions. The fractions were analysed by immunoblotting using anti-Smc1, anti-Smc3, anti-SA2, anti-Rad21, anti-Ssu72 and anti-ERK1 (as a control for cytoplasmic supernatant fractions) or anti-lamin B (as a control for chromatin-bound fractions) antibodies.

the nuclear envelope permeability of MBP-GFP after cytoplasmic photobleaching (Figure 2B; Supplementary Figure S5). The Rad21-RFP signals on chromatin decreased within 4 min after NEBD in control cells, whereas this signal was markedly prolonged for up to 16 min post-NEBD in Ssu72-overexpressing cells (Figure 2C and D; Supplementary Figure S4A). The SA2-RFP signals on chromatin gradually decreased in control cells, becoming barely detectable after 3 min post-NEBD (Figure 2E and F; Supplementary Figure S4B). In Ssu72-overexpressing cells, however, the SA2-RFP signal was clearly visible at 4 min post-NEBD and was maintained even in metaphase chromosomes, indicating that Ssu72 overexpression appears to decrease the dissociation of cohesin from mitotic chromatin. We then investigated the effect of Ssu72 expression on cell cycle progression, and found that cells overexpressing Ssu72 showed a significant mitotic delay compared with control cells (Supplementary Figure S6A–D); this delay arose largely from the elongation of prometaphase and metaphase, while the timings of interphase and mitotic exit were unaffected (Supplementary Figure S6C–E).

HeLa-Rad21-RFP cells were transfected with CFP-tagged H2B (H2B-CFP) and GFP-tagged MBP (MBP-GFP) expression plasmids, along with shRNA specifically targeting Ssu72 (which significantly depleted Ssu72 expression (data not shown)) or luciferase (as a control), and cultured in the presence of doxycycline for 48 h (Figure 2G and H; Supplementary Figure S4B). In control cells, the RFP signal appeared on mitotic chromosomes, remained present until prophase, and then decreased beginning at 180 s post-NEBD. In Ssu72-depleted cells, however, the RFP signal sharply decreased at 120 s post-NEBD. To further investigate the effects of Ssu72 depletion on cell cycle progression, we analysed mitotic progression by flow cytometry. However, Ssu72-depleted cells did not show any meaningful change in the proportion of cells at interphase or mitotic cell cycle progression (Supplementary Figure S7).

Ssu72 regulates sister chromatid cohesion between chromosome arms

To determine the effect of Ssu72 expression on sister chromatid cohesion, we transfected HeLa cells stably expressing Myc-tagged versions of Rad21/Sccl (Myc-Rad21) (Hauf *et al*, 2001) or SA2 (Riedel *et al*, 2006) with either control luciferase shRNA or Ssu72 shRNA, and analysed Rad21 and SA2 staining by confocal microscopy. As shown in Figure 3A, both Myc-Rad21 and Myc-SA2 clearly appeared as chromatin-bound proteins in prophase/prometaphase control cells, whereas Ssu72-depleted cells showed significant displacement of these cohesin proteins from prophase/prometaphase chromatin. About 87% of the prophase/prometaphase control cells showed chromatin binding of Myc-Rad21, whereas only 40–50% of Ssu72-depleted prophase/prometaphase cells were Myc-Rad21 positive (Figure 3B), indicating that depletion of Ssu72 leads to the premature dissociation of cohesin from mitotic chromosomes. Next, we generated expression plasmids encoding Ssu72 WT, Ssu72 C12S (in which cysteine 12, located in the CX5RS signature motif of the protein phosphatase (PPase), was mutated to a serine) (Supplementary Figure S8), or the Ssu72 shRNA. HeLa cells transfected with these plasmids were cultured and treated with the microtubule inhibitor, colcemid, and sister chromatid cohesion was analysed by chromosome spreading and

Giemsa staining. Three types of mitotic chromosome patterns were observed: (1) ‘closed’ (cohesed chromosome arms), (2) ‘partial open’ (partial loss of arm cohesion) and (3) ‘open’ (complete loss of arm cohesion) (Figure 3C). Treatment of HeLa cells with a microtubule inhibitor normally abrogates cohesion between chromosome arms but not at the centromeres, forming the X-shaped ‘open’ arm pattern. However, asynchronous HeLa cells treated with a microtubule inhibitor usually show mixed-type mitotic chromosome spreads comprising about 45% open, 45% partial open and ~10% closed conformations (Figure 3D). Interestingly, cells overexpressing Ssu72 showed a significant increase in the percentage of closed arms, from 10% (in mock-transfected cells) to 50% in Ssu72-overexpressing cells, and a sharp reduction in the open type, from 45 to 15%, respectively (Figure 3D). In contrast, cells overexpressing the phosphatase-dead mutant of Ssu72 (Ssu72 C12S) showed a very similar pattern to control cells, with only a slight increase in the open phenotype. These data indicate that overexpression of WT but not phosphatase-dead mutant Ssu72 appears to prevent the dissociation of chromosome arm cohesion.

We next transfected HeLa cells with control luciferase shRNA or Ssu72 shRNA and treated the cells with colcemid. In contrast to the behaviour of the Ssu72-overexpressing cells described above, the chromosomes from these Ssu72-depleted cells showed a significant increase in the open phenotype (about 75%) compared with control cells (about 35%), and a marked decrease in the partial open (from 47% in controls to 15% in Ssu72-depleted cells) and closed (from 18 to 10%, respectively) phenotypes (Figure 3E), indicating that the depletion of Ssu72 causes premature dissociation of arm cohesion. Notably, we did not observe any changes in the dissociation of cohesion at the centromeres of Ssu72-overexpressing or -depleted cells.

To confirm that sister chromatid arm cohesion is maintained by Ssu72 expression, we generated shRNA-insensitive versions of Myc-tagged Ssu72 (shi Myc-Ssu72 WT and -Ssu72 C12S), and examined the abilities of these constructs to rescue the phenotypes of Ssu72-depleted cells (Figure 3F). As shown in Figure 3G, cells transfected with shSsu72 alone clearly showed endogenous Ssu72 knockdown, whereas cells co-transfected with shSsu72 and shi Myc-Ssu72 WT or -Ssu72 C12S MT expressed Ssu72 WT and C12S MT, respectively. Chromosome spreading assays revealed that overexpression of shi Myc-Ssu72 WT in Ssu72-depleted HeLa cells significantly recovered the resolution of sister chromatid arm cohesion (the open phenotype increased from 15 to 50%) (Figure 3G). In contrast, cells overexpressing the phosphatase-dead mutant of Ssu72 (Ssu72 C12S) showed a pattern similar to that of Ssu72-depleted cells. Collectively, our results suggest that Ssu72 regulates the maintenance and resolution of sister chromatid cohesion at the chromosome arms.

Depletion of Ssu72 causes the premature dissociation of cohesin

In vertebrate cells, cohesin complexes are removed from sister chromatids in a stepwise manner. During prophase/prometaphase, cohesin is first dissociated from the chromosome arms by phosphorylation of the cohesin subunits, which is mediated by phosphorylation of SA2 (Hauf *et al*, 2005). Therefore, we tested the effect of Ssu72 depletion on

the expression and phosphorylation of cohesin subunit proteins during the various stages of the cell cycle. HeLa cells were transfected with shLuciferase (shLuc) or shSsu72 and then synchronized by double-thymidine block (G1/S phases), doxorubicin treatment (G2), or nocodazole treatment (mitosis). Extracts from these synchronized HeLa cells were separated into chromatin and non-chromatin soluble fractions, and these fractions were immunoblotted with anti-

Ssu72, anti-SA2, anti-Rad21 and anti-phospho SA2 S1224 (which recognizes SA2 phosphorylated at serine 1224) antibodies (Figure 4A). Consistent with a previous report (Kueng *et al*, 2006), the hyperphosphorylated form of SA2 was detected in the non-chromatin fractions of mitotic cellular extracts. Interestingly, however, the levels of hyperphosphorylated SA2 were significantly increased in Ssu72-depleted cells (Figure 4A), indicating that Ssu72 depletion augments the

hyperphosphorylation of SA2. In addition, we observed a slower migrating band of SA2 polypeptides in mitotic non-chromatin fractions, which becomes faster migrating after λ -phosphatase treatment (Figure 4B). To further analyse the effect of Ssu72 depletion on cohesin subunit expression and phosphorylation levels in mitotic chromosomes, HeLa cells were transfected with shLuc or shSsu72, synchronized by double-thymidine block followed by a 6-h release, and then treated with nocodazole (as indicated) to enrich cells at the early phase of mitosis, prior to metaphase (Figure 4C). Interestingly, the relative level of hyperphosphorylated SA2 (Figure 4C, arrowhead) in the soluble non-chromatin supernatant was significantly increased in Ssu72-depleted cells, whereas that of hypophosphorylated SA2 in the chromatin fraction was sharply decreased in Ssu72-depleted cells after only 2 h of nocodazole treatment. In contrast, the control cells still retained hypophosphorylated SA2 in the chromatin fraction following 6 h of nocodazole treatment. Similarly, the amount of Rad21 detected in the chromatin fraction was also decreased in Ssu72-depleted cells subjected to only 2 h of nocodazole treatment. These results suggest that Ssu72 depletion leads to the premature dissociation of cohesin from chromosome arms.

To further validate that Ssu72 is involved in sister chromatid arm cohesion, we performed chromatin immunoprecipitation (ChIP) assays against two cohesin-associated regions on the chromosome arms: an arm region on chromosome 7 (116357745–116357949: primer 88) and the H19 imprinting control region (ICR) on chromosome 11 (1977613–1977821: primer 96). These regions reportedly include binding sites for cohesins and the zinc finger insulator protein, CTCF, which is required for the positioning of cohesin on DNA (Wendt *et al*, 2008). We used ChIP-qPCR to measure the relative amounts of cohesin (Rad21) at these cohesin-binding sites in Ssu72-depleted cells at G2 and early mitosis (Figure 4D and E). As a control, we analysed Scc2-depleted cells, in which the chromosomal loading of cohesin is sharply reduced. The abundance of bound cohesin sites was reduced in mitotic cells versus those in G2; this was as expected, because the arm cohesins at these sites are dissociated from the chromosome through the so-called ‘prophase removal’. In addition, and consistent with a previous report (Wendt *et al*, 2008), Scc2-depleted cells showed a remarkable reduction in the cohesin levels at these cohesin-binding sites. With regard to Ssu72, the cohesin signals were reduced at the cohesin-binding sites of Ssu72-depleted cells synchronized at both

G2 and mitosis. Thus, our results suggest that Ssu72 is involved in sister chromatid arm cohesion as early as the G2 phase of the cell cycle.

Ssu72 regulates cohesion at the chromosome arms in a Wapl-independent manner

Previous important studies have shown that depletion of Wapl prevents arm cohesins from dissociating from sister chromatids during mitosis, indicating that Wapl is essential for the resolution of chromosome arm cohesin and cell cycle progression (Gandhi *et al*, 2006; Kueng *et al*, 2006). To examine the potential interplay between Ssu72 and Wapl, we used shSsu72-encoding plasmids to generate HeLa cells in which Ssu72 was stably knocked down (hereafter called ‘Ssu72 KD cells’) (Figure 5A). Control HeLa and Ssu72 KD cells transfected with siLuc or siWapl were synchronized by double-thymidine block followed by a 6-h release, and further synchronized by nocodazole treatment for 4 h to enrich for cells at early mitosis. As shown in Figure 5B, the depletion of Wapl from control cells led to a significant increase of the cohesin protein (SA2 and Rad21) levels in the chromatin fractions; this is consistent with a previous report that Wapl is essential for the prophase removal of sister chromatid arm cohesins (Kueng *et al*, 2006). In Ssu72 KD cells, however, Wapl depletion failed to rescue the premature dissociation of arm cohesion caused by Ssu72 depletion, raising the possibility that Ssu72 regulates cohesion at the chromosome arms in a Wapl-independent manner. Next, we analysed the relative timing kinetics of cohesin dissociation from sister chromatids in Ssu72- and Wapl-depleted cells (Figure 5C and D). Quantitative live-cell imaging analysis revealed that after NEBD, the majority of the SA2-RFP proteins diffused from the nucleus; <5% of SA2-RFP was detected on the sister chromatids of shLuc- or shSsu72-transfected cells after NEBD (Figure 5D; $t = 5$ min). In Wapl-depleted cells, however, SA2-RFP largely remained on the sister chromatids, with only about 20% of the SA2-RFP signal diffused to the non-chromatin cell regions (Figure 5D; $t = 5$ min).

To further examine the links between Ssu72 and Wapl in regulating sister chromatid arm cohesion, we tested whether Ssu72 could antagonize the functional interaction of Wapl with sister chromatid cohesin. Inducible HeLa-SA2-RFP cells were co-transfected with shLuc, siWapl and shSsu72 singly or in combination, along with the H2B-GFP expression plasmid (Figure 5E–G). Our results revealed that Wapl depletion sharply inhibited the dissociation of SA2-RFP from sister

Figure 2 Aberrant expression of Ssu72 causes defects in the dissociation of cohesin from chromatin. (A–H) Inducible HeLa cells expressing Rad21-RFP (HeLa-Rad21-RFP) or SA2-RFP (HeLa-SA2-RFP) were co-transfected with expression plasmids encoding GFP-tagged H2B (H2B-GFP) and Ssu72 (pMyc-Ssu72 or pHA-Ssu72) or empty backbone vector (pMyc or pHA; controls). At 12 h post-transfection, cells were induced with 2 μ g/ml doxycycline (Doxy) and further cultured for 48 h. (A) Cell extracts were analysed by immunoblotting as indicated. ‘Endo’ designates the endogenous Rad21 or SA2 proteins. (B) Representative time-lapse microscopic images of chromosomes in the G1/S, G2 and nuclear envelope breakdown (NEBD) phases of the cell cycle (left panels). Representative time-lapse images of cells expressing MBP-GFP and H2B-CFP before and after photobleaching, and NEBD-phase cells. NEBD was defined by the appearance of sister chromatid disorganization (indicated by arrowheads) and the dispersal of MBP-GFP throughout the cell after photobleaching. (C, D) Time-lapse images taken at 2-min intervals show the false-coloured GFP (H2B-GFP) and RFP (Rad21-RFP) emissions. The time of NEBD is shown as t_0 . The relative amounts of Rad21-RFP on the chromatin were quantified in Ssu72-overexpressing ($n = 11$) and control ($n = 6$) cells (mean $\pm$ s.d.). (E, F) Time-lapse images taken at 1-min intervals show the false-coloured GFP (H2B-GFP) and RFP (SA2-RFP) emissions. The relative amounts of SA2-RFP on the chromatin were quantified in Ssu72-expressing ($n = 7$) and control ($n = 4$) cells (mean $\pm$ s.d.). (G, H) Inducible HeLa-Rad21-RFP cells were co-transfected with an expression plasmid encoding H2B-CFP (to visualize the chromosomes), MBP-GFP (as a marker for NEBD) and shRNAs against Ssu72 (shSsu72) or luciferase (shLuc, control). At 12 h post-transfection, cells were induced with doxycycline. Time-lapse images taken at 20-s intervals show the false-coloured CFP (H2B-CFP), GFP (MBP-GFP) and RFP (Rad21-RFP) emissions from control luciferase shRNA- or Ssu72 shRNA-transfected cells. The relative amounts of Rad21-RFP on the chromatin were quantified in Ssu72-depleted ($n = 15$) and control ($n = 11$) cells (mean $\pm$ s.d.).

Figure 3 Ssu72 regulates the maintenance and resolution of cohesion between sister chromatid arms. (A) HeLa cells stably expressing Myc-tagged Scc1/Rad21 (HeLa-Myc-Rad21) or Myc-tagged SA2 (HeLa-Myc-SA2) were transfected with control luciferase shRNA or Ssu72 shRNA and treated with 200 ng/ml nocodazole for 4 h, and the shaking-off method was used to collect a population enriched in cells at the early stage of mitosis. Mitotic cells (2×10^5 /ml) were incubated in a hypotonic buffer, spun onto coverslips using Cytospin, and stained with CREST serum (purple), and anti-Myc (green) and anti-Ssu72 (red) antibodies. DNA was stained with DAPI. (B) HeLa-Myc-Rad21 cells were transfected with luciferase shRNA, Ssu72 shRNA #1 or Ssu72 shRNA #2 (see Materials and methods), and stained as above. Prophase/prometaphase cells displaying chromatin-bound Myc-Rad21 fusion protein staining were scored, and the results are expressed as percentages out of more than 100 prophase/prometaphase cells. (C–E) HeLa cells were transfected with expression plasmids encoding Myc-tagged Ssu72 WT, Ssu72 C12S (a phosphatase-inactive mutant), or a control Myc vector (pMyc), and then cultured and treated with colcemid (0.04 mg/ml). Chromosomes were spread and visualized by Giemsa staining. The percentage of mitotic chromosome spreads was calculated from at least 600 cells per transfectant. HeLa cells were transfected with shRNA expression plasmids targeting either Ssu72 (shSsu72) or luciferase (shLuc, negative control), and then cultured and treated with colcemid, and chromosomes were analysed as described above. Extracts from HeLa cells transfected with expression plasmids encoding the Myc epitope, Myc-Ssu72 WT, Myc-Ssu72 C12S, shLuc or shSsu72 were immunoblotted with anti-Myc or anti-Ssu72 antibodies. (F, G) To generate an shRNA-insensitive Ssu72-encoding construct, five silent mutations were introduced into the Ssu72 gene sequence, which was then cloned into a Myc-tagged vector (F). Extracts from HeLa cells transfected with the indicated plasmids were immunoblotted with an anti-Ssu72 antibody (G). Chromosome spreads prepared from HeLa cells transfected with the indicated plasmids were visualized by Giemsa staining. The percentage of mitotic chromosome spreads was calculated from at least 300 cells per transfectant.

chromatids (Figure 5F, middle panels, and 5G). However, Ssu72 depletion clearly counteracted the association of SA2-RFP with sister chromatids in Wapl-depleted cells (Figure 5F, lower panels, and 5G), suggesting that the depletion of Ssu72 results in the premature dissociation of cohesin subunits from sister chromatids. We believe that this is likely to occur via the augmentation of SA2 hyperphosphorylation at the stage before Wapl regulates the dissociation of arm cohesion during

early mitosis. Taken together, these results further support our contention that Ssu72 selectively regulates sister chromatid arm cohesion.

Ssu72 phosphatase dephosphorylates SA2

As specific phosphorylations of the Rad21 and SA1/2 subunits are required for the prophase/prometaphase removal of arm cohesion (Losada *et al*, 2000; Hauf *et al*, 2005), we tested

Figure 4 Depletion of Ssu72 causes the premature dissociation of cohesion. (A, B) HeLa cells were transfected with shRNAs against Ssu72 (shSsu72) or luciferase (shLuc; control). At 12 h post-transfection, cells were synchronized at the G1-S boundary by double-thymidine block, at the G2 phase by doxorubicin treatment, or at mitosis by nocodazole treatment. (A) Synchronized cells were separated into chromatin and non-chromatin supernatant fractions, resolved by SDS-PAGE (10%), and immunoblotted with anti-SA2, anti-Rad21, anti-phospho SA2 serine 1224 (pSA2-S1224), anti-tubulin (as a non-chromatin-fraction marker), anti-histone H3 (as a chromatin-fraction marker) and anti-Ssu72 antibodies. (B) Synchronized cells were separated into chromatin and non-chromatin supernatant fractions, and cellular extracts were incubated with or without λ phosphatase (λ PP), resolved by SDS-PAGE (6%), and immunoblotted with anti-SA2, anti-Erk2 (as a non-chromatin-fraction marker) and anti-histone H3 antibodies. (C) HeLa cells were transfected as indicated, synchronized by a double-thymidine block followed by a 6-h release, and then treated with nocodazole (as indicated) to enrich for cells in the early phase of mitosis, prior to metaphase. The cells were then separated into soluble non-chromatin supernatant (Supt) and insoluble chromatin (Chro) fractions, resolved by SDS-PAGE (10%), and immunoblotted with anti-SA2, anti-Rad21, anti-Ssu72, anti-Erk2 and anti-histone H3 antibodies. (D, E) HeLa cells were transfected with siLuc, shSsu72 and siScc2 (a positive control), respectively, and synchronized at G2 and mitosis. Cellular extracts were immunoblotted with anti-Ssu72, anti-Scc2 and anti-actin antibodies. ChIP was performed with Rad21 or control IgG antibodies on cells synchronized at G2 or mitosis, and qPCR analyses were performed using primer pairs specific for the cohesin and CTCF-binding sites. The relative transcript levels were normalized with respect to that of the control siLuc in G2 phase (mean of $n = 3$; error bars, $\pm$ s.d.).

the phosphorylations of SA2 and/or Rad21 by Cdk1 and Plk1 (Figure 6A, data not shown), and then examined whether these phosphorylations could be counteracted by the phosphatase activity of Ssu72 (Figure 6B–D). We generated a His-tagged CTD of the SA2 cohesin subunit (residues 895–1232, His C-SA2) (Figure 6B); this fragment contained the mitosis-specific phosphorylation sites, which comprise a cluster of 12 serine and threonine residues (Hauf *et al*, 2005). Although full-length SA2 was efficiently phosphorylated by Plk1

in vivo (data not shown), the purified SA2, GST-SA2 and His C-SA2 proteins appeared to be more efficiently phosphorylated by Cdk1 than by Plk1 *in vitro* (Figure 6A). We then reacted His C-SA2 with recombinant Cdk1/cyclin B kinase in the presence of [γ - 32 P]ATP, and reacted the resulting *in vitro*-phosphorylated His C-SA2 with GST (negative control), GST-Ssu72 WT, the GST-Ssu72 C12S mutant or λ PPase (positive control) (Supplementary Figure S8E; Figure 6B). In agreement with the above results, the levels of phosphorylated

Figure 5 Ssu72 regulates chromosome arm cohesion in a Wapl-independent manner. (A) Extracts from control HeLa cells and two different Ssu72 KD cells (Ssu72 KD #1 and #2) were immunoblotted with anti-Ssu72 and anti-actin antibodies. (B) Control HeLa and Ssu72 KD cells were transfected with siRNAs against Wapl (siWapl) or luciferase (siLuc). At 12 h post-transfection, cells were synchronized by double-thymidine block followed by a 6-h release, and then treated with nocodazole for 4 h to enrich for cells at the early phase of mitosis. The synchronized cells were separated into insoluble chromatin and soluble non-chromatin fractions, and immunoblotted with anti-SA2, anti-Rad21, anti-phospho SA2 S1224 (ⓈSA2 (S1224)), anti-histone H3, anti-Erk2, anti-Wapl and anti-Ssu72 antibodies. (C–G) Inducible HeLa-SA2-RFP cells were co-transfected with the H2B-GFP expression plasmid and shLuc, siWapl and shSsu72 singly or in combination. (C) The SA2-RFP signals on both chromatin and non-chromatin regions were digitally monitored by time-lapse microscopy. (D) The relative ratio of non-chromatin-binding SA2-RFP fluorescence to the total SA2-RFP signal was determined by densitometric analysis (mean $\pm$ s.d.); shLuc-transfected cells ($n = 5$), shSsu72-transfected cells ($n = 12$) and siWapl-transfected cells ($n = 11$). (E) Extracts from cells transfected as indicated were immunoblotted with anti-RFP (SA2), anti-Ssu72, anti-Wapl and anti-actin antibodies. (F) The SA2-RFP signal was digitally monitored by time-lapse microscopy. (G) The relative amounts of SA2-RFP on the chromatin were quantified in Wapl-depleted ($n = 8$), Ssu72-depleted ($n = 11$) and shLuc-transfected control ($n = 4$) cells (mean $\pm$ s.d.).

SA2 were significantly reduced by incubation with GST-Ssu72 WT, but not with GST or the GST-Ssu72 C12S mutant. Similarly, we purified GST-Rad21, which is also known to be phosphorylated by Plk1 (Sumara *et al*, 2002; Hornig and Uhlmann, 2004), incubated it with Plk1 in the presence of; [γ - 32 P]ATP (Figure 6C), and then further reacted the *in vitro*-phosphorylated GST-Rad21 proteins with GST, GST-Ssu72 WT, the GST-Ssu72 C12S mutant or λ PPase. In contrast to our findings with SA2, the phosphorylation of Rad21 was not counteracted by Ssu72 WT, but the level of phosphorylated Rad21 was markedly reduced by control λ PPase treatment

(Figure 6C). To confirm these findings, we isolated the SA2-cohesin complexes from synchronized mitotic cell extracts, and then incubated the complexes with purified GST-Ssu72 WT, GST-Ssu72 C12S or λ PPase (positive control) (Supplementary Figure S8E; Figure 6D). Phosphorylation of SA2, which was clearly recognized by both the anti-phospho-serine and anti-phospho-threonine antibodies, was markedly counteracted by the addition of purified Ssu72 WT but not the phosphatase-inactive mutant Ssu72 (Ssu72 C12S).

To further examine the hypophosphorylation or dephosphorylation of SA2 in Ssu72-overexpressing cells, HeLa-Con

Figure 6 Ssu72 dephosphorylates SA2. (A) Purified GST-SA2 or GST-Rad21 was reacted with recombinant Cdk1/cyclin B1 or Plk1 in the presence of radio-unlabelled ATP. *In vitro*-phosphorylated GST-SA2 or GST-Rad21 was incubated with purified His-Ssu72. The eluted beads were immunoblotted with anti-phospho-threonine (ⓅThr), anti-phospho-serine (ⓅSer), anti-SA2 and anti-Ssu72 antibodies. (B) His-tagged C-terminal SA2 peptides (His C-S-A2) were incubated with recombinant Cdk1/cyclin B1 in the presence of [γ^{32} P]ATP, washed and then reacted with purified GST, GST-Ssu72 WT, GST-Ssu72 C12S or λ phosphatase (λ PPase). The graph shows the relative signal intensities of the radio-labelled His C-S-A2 peptides. (C) Purified GST-Rad21 proteins were incubated with Plk1 proteins in the presence of [γ^{32} P]ATP, washed and then reacted with purified GST, GST-Ssu72 WT, GST-Ssu72 C12S (2 or 4 μ g/ml) or λ PPase. (D) Dephosphorylation of SA2 by Ssu72. Nocodazole-treated (100 ng/ml, 12 h) HeLa cells were lysed and immunoprecipitated with an anti-SA2 antibody, and the resulting SA2-cohesin complexes were incubated with purified GST-Ssu72 WT, GST-Ssu72 C12S MT (1 or 2 μ g/ml) or λ PPase (control). The bound SA2 proteins were resolved by SDS-PAGE and detected using antibodies against phospho-serine (ⓅSer), phospho-threonine (ⓅThr), SA2 or Smc1. (E) HeLa-Con and HeLa-Ssu72 cells were cultured and treated with nocodazole for 4 h. Endogenous SA2 was immunoprecipitated from cell extracts using an anti-SA2 antibody or normal IgG (negative control), and the eluted SA2 complexes were immunoblotted with anti-SA2, anti-phospho-serine and anti-phospho-threonine antibodies. (F) SA2-cohesin complexes were isolated from HeLa-Con or HeLa-Ssu72 cell extracts using a polyclonal anti-SA2 antibody, and the SA2 immunocomplexes were analysed by two-dimensional SDS-PAGE followed by immunoblotting with a polyclonal anti-SA2 antibody. (G) Nocodazole-treated (100 ng/ml, 12 h) control and Ssu72-knockdown cells were lysed and immunoprecipitated with an anti-SA2 antibody, and the resulting SA2 complexes were immunoblotted with anti-SA2, anti-phospho-serine (ⓅSer) and anti-phospho-threonine (ⓅThr) antibodies.

(control HeLa cells) and HeLa-Ssu72 cells were treated with nocodazole, and endogenous SA2 complexes were immunoprecipitated from cellular extracts (Figure 6E). Interestingly, significantly less phosphorylated SA2 was recognized by the anti-phospho-threonine antibody (Figure 6E, asterisk) in Ssu72-overexpressing cells. In these cells, small amounts of SA2 were detected only in the faster electrophoretic migration range (indicative of dephosphorylated SA2), suggesting that SA2 phosphorylation may be counteracted by Ssu72 overexpression. In contrast, Rad21 phosphorylation was not affected by Ssu72 overexpression (data not shown). We then analysed the SA2 immunocomplexes by two-dimensional SDS-PAGE and subsequent immunoblotting with an

anti-SA2 antibody (Figure 6F). As expected, the higher molecular weight and higher isoelectric point (pI) polypeptides of SA2 were clearly reduced in cells overexpressing Ssu72, implying that Ssu72 may be involved in the dephosphorylation or hypophosphorylation of SA2. Similarly, we examined whether the depletion of Ssu72 affected the hyperphosphorylation of SA2 *in vivo*. Control HeLa and Ssu72 KD cells were treated with nocodazole and the endogenous SA2 complexes were immunoprecipitated from cellular extracts (Figure 6G). As expected, more highly phosphorylated SA2 was recognized by the anti-phospho-threonine antibody in Ssu72-depleted cells compared with control cells, but a little change by anti-phospho-serine antibody in consistent with Figure 6E.

Furthermore, we tested whether the expression of a non-phosphorylatable SA2 mutant (SA2 4A; Hauf *et al*, 2005) could prevent the dissociation of arm cohesins in Ssu72-depleted cells. We first generated plasmids encoding SA2 WT or SA2 4A, in which four putative phosphorylation sites were mutated, and transfected into HeLa cells together with shSsu72 (Supplementary Figure S9A and B). Interestingly, the expression of SA2 WT in Ssu72-depleted cells clearly rescued the resolution of sister chromatid arm cohesion induced by Ssu72 depletion (Supplementary Figure S9C). Moreover, the expression of SA2 4A significantly augmented sister chromatid cohesion compared with that in Ssu72-depleted cells expressing SA2 WT (the partial open phenotype was 40% in SA2 4A-expressing cells versus 25% in SA2 WT-expressing cells) (Supplementary Figure S9C), indicating that the non-phosphorylatable SA2 mutant rescued the premature dissociation of chromatid cohesin caused by Ssu72 depletion. Collectively, these findings indicate that the function of Ssu72 in the hypophosphorylation/dephosphorylation of SA2 depends on its phosphatase activity.

Discussion

It is vital for cells to shield chromosome arm cohesion prior to mitosis. The dissolution of chromosome arm cohesion is triggered by the mitotic kinase-mediated phosphorylation of cohesin subunits. Recent studies have shown that phosphorylation of SA2 by Plk1 is important for the dissociation of the cohesin complex from chromosome arms during prophase/prometaphase (Sumara *et al*, 2002; Hauf *et al*, 2005). The expression of a mutated, non-phosphorylatable SA2 was found to prevent the loss of cohesion between sister chromatid arms (Hauf *et al*, 2005), suggesting that protection of cohesin subunits from phosphorylation may be required to stabilize sister chromatid arm cohesion. Recent studies have shown that PP2A and Sgo collaborate to function as a 'protector' that prevents phosphorylation of cohesin by Plk1 and aurora B, thereby shielding centromeric cohesins (Kitajima *et al*, 2006; Riedel *et al*, 2006; Tang *et al*, 2006). In terms of arm cohesion, we herein propose a model in which the Ssu72 phosphatase appears to shield chromosome arm cohesion by interacting with Rad21 and SA2, and potentially by protecting SA2 from hyperphosphorylation by a yet-unknown mechanism. Notably, the amino-acid sequence of Ssu72 includes a polo-box-binding (PBB) motif, which is recognized by Plk1, as well as potential phosphorylation sites for aurora B kinase (HS Kim and CW Lee, unpublished data). This raises the possibility that the Plk1 and/or aurora B kinase could phosphorylate Ssu72, thereby contributing to its stability or activity. Future studies will be required to determine whether this model involves the phosphorylation of Ssu72 by Plk1 or one or more of the aurora kinases.

Cohesin is subject to complicated temporal and spatial regulation, thereby ensuring the proper establishment, maintenance and dissociation of cohesion. Cohesin becomes stably cohesive once it is formed. In particular, cohesin bound to chromatin during the G2 phase become cohesive when a cell suffers a double-strand break in one of its chromosomes (Ström *et al*, 2007; Unal *et al*, 2007). Recent studies have revealed that Eco1 is critical for generating cohesion through the acetylation of Smc3, and acts after chromatin binding to help cohesin become cohesive

(Ben-Shahar *et al*, 2008; Unal *et al*, 2008). However, the molecular mechanisms through which cohesin maintains sister chromatid cohesion prior to the onset of mitosis are not yet known. Our present results indicate that mutational inactivation or depletion of Ssu72 phosphatase decreases the cellular ability to maintain sister chromatid arm cohesion, leading to premature chromosome resolution. In addition, we found that the Ssu72 phosphatase is capable of dephosphorylating (or even hypophosphorylating) SA2. These findings significantly advance our understanding of the way in which cohesin maintains chromosome arm cohesion and protects against the premature removal of cohesion at prophase/prometaphase.

Given this, we examined some potential mechanisms through which the loss or inactivation of Ssu72 might induce the dissociation of chromosome arm cohesion. We tested whether depletion of Ssu72 could alter the formation of cohesin subunit-containing complexes, thereby decreasing arm cohesion. Immunoprecipitation experiments on Ssu72-depleted cell extracts showed that the depletion of Ssu72 did not affect the interactions of Rad21 with SA2 with the two structural subunits, Smc1 and Smc3 (data not shown). However, the prevention of cohesin dissociation caused by Wapl depletion was sharply antagonized in Ssu72-depleted cells (Figure 5B and E–G). This prompted us to ask: When does Ssu72 maintain the stability of chromosome arm cohesion? One possibility is that the phosphatase activity of Ssu72 promotes and sustains the premature dissociation of arm cohesins by inhibiting SA2 hyperphosphorylation until the onset of Wapl-mediated cohesin dissociation, which follows mitotic entry. This model might be attractive because Ssu72 appears to be involved in maintaining sister chromatid cohesion in the G2 phase. Furthermore, cohesion between sister chromatid arms in Ssu72-depleted cells was reduced in both G2-phase and mitotic cells, indicating that the premature dissociation of cohesin in Ssu72-depleted cells may begin during the G2 phase of the cell cycle. In addition, preliminary studies have indicated that the interaction between Rad21 and Wapl appears to be significantly increased in Ssu72-depleted cells (HS Kim and CW Lee, unpublished data). It is therefore possible that the recruitment of Ssu72 to the cohesin complex may antagonize the interaction of cohesin subunits with Wapl. We are currently working to corroborate this model.

Ssu72 was originally identified based on its physical interaction with the yeast transcription factor, TFIIB (Pappas and Hampsey, 2000). A subsequent study showed that Ssu72 was essential in yeast and known to act as a CTD phosphatase to dephosphorylate serine 5 of RNA pol II (Krishnamurthy *et al*, 2004). The phosphorylation of CTD regulates transcription and facilitates the recruitment of RNA processing factors during transcription (Orphanides and Reinberg, 2002). However, although we cannot exclude the possibility that Ssu72 functions to control the transcription of cohesin subunits, we found that Ssu72 overexpression did not significantly affect the mRNA levels of the genes encoding RAD21, SA2, SMC1 and SMC3 (Supplementary Figure S10). These findings are consistent with a previous report, suggesting that Ssu72 is not involved in the regulation of basal transcriptional activity (St-Pierre *et al*, 2005).

Most of the prior studies on Ssu72 have focused on the regulation of transcription and proliferation in yeast. Here,

we propose that human Ssu72 has an essential function in maintaining sister chromatid arm cohesion by directly and functionally interacting with Rad21 and SA2 during the G2 and mitotic phases. This model is supported by our observation that mutating the cysteine in the highly conserved CX5RS signature motif of Ssu72 not only abolished the phosphatase activity of Ssu72, it also dysregulated sister chromatin cohesion. Our structural analysis revealed that this mutation would logically perturb the conformation of the catalytic loop, potentially explaining the loss of phosphatase activity (Supplementary Figure S8).

In summary, we herein provide evidence that collectively identifies Ssu72 as a novel cohesin-binding protein essential for chromosome cohesion. These findings significantly advance our understanding of the mechanisms responsible for maintaining chromosome arm cohesion and protecting against premature cohesion removal at prophase/prometaphase.

Materials and methods

Generations of plasmids, shRNAs and siRNAs

The full-length cDNA sequences of the human Ssu72, Rad21 and SA2 genes were PCR amplified using oligo-dT primers. The Ssu72 C12S allele was generated by site-directed mutagenesis. cDNAs for Ssu72 WT, Ssu72 C12S and Ssu72 Δ 1–12 were subcloned into the Myc epitope- or HA epitope-encoding pcDNA3.1 vector to generate pMyc-Ssu72 WT, C12S and Δ 1–12 and pHA-Ssu72, respectively. MBP-GFP plasmid was provided by EUROSCARF (Lénárt and Ellenberg, 2006).

For shRNA synthesis, the following gene-specific sequences were generated using pSuper vector (Oligoengine): Ssu72 shRNA #1, 5'-A ACAGGGACTCACGTGAAGCT-3'; Ssu72 shRNA #2, 5'-AAGACCTGTT TGATCTGATCC-3'; Luciferase shRNA, 5'-CTACGCGGAATACTTCGA-3', and gene-specific sequences for siRNA synthesis were Luciferase shRNA, 5'-CUACGCGGAUACUUCGA-3' Wapl siRNA, 5'-CGGACU ACCCUUAGCACAA-3' and Scc2 siRNA, 5'-GCAUCGGUAUCAAGU CCCA-3'.

Constructions of inducible and stably transfected cell lines

To generate HeLa cells for inducible expression of Rad21-RFP or SA2-RFP fusion proteins, HeLa Tet-on cells were transfected with the pTRE2-hydro vector (BD Biosciences Clontech) containing the respective cDNAs and the RFP tag fused in-frame. Hygromycin-resistant clones were selected in culture media containing 200 μ g/ml hygromycin and induced with 2 μ g/ml doxycycline for 48 h, and expression of RFP-fused proteins was examined by immunoblotting analysis. HeLa-HA-Ssu72 and Ssu72-knockdown cell lines were generated by transfection of HeLa cells with the pIRES puro3 vector (BD Biosciences Clontech) containing the full-length Ssu72 cDNA sequence and with the pSuper puro vector (Oligoengine) containing the shRNA sequence against Ssu72 shRNA #2, respectively. Puromycin-resistant clones were selected by growth in medium containing 5 μ g/ml puromycin, and were tested in immunoblotting and immunofluorescence assays.

Cell culture, cellular fractionation and cell synchronization

HeLa cell lines were grown in DMEM containing 10% fetal bovine serum (FBS; Hyclone). For fractionation of cell extracts, soluble cytosolic supernatants were prepared using PA buffer (150 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM DTT and a mixture of protease inhibitors). Pellet fractions were collected by the dissolution of nuclei in XBE2 buffer (10 mM HEPES (pH 7.5), 300 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM PMSF, 1 mM DTT and a mixture of protease inhibitors). Chromatin fractions were subsequently prepared by sonication of the insoluble pellet fractions in XBE2 buffer. For synchronization at G1/S boundary, cells were grown in the presence of 1 mM thymidine (Sigma) for 14 h, washed with PBS, grown in fresh medium for 12 h and then treated with thymidine. After an additional 14 h, the cells were again washed in PBS and added with fresh medium. For

synchronization G2, the cells were grown in the presence of 50 nM doxorubicin. For synchronization at mitosis, the cells were grown in the presence of 200 ng/ml nocodazole and collected by shake-off. The cells were harvested at the indicated time points after release.

Antibodies

Rabbit polyclonal and mouse monoclonal antibodies to a KLH-conjugated peptide corresponding to residues 85–101 of human Ssu72 were generated and affinity purified (Supplementary Figure S2). We also prepared peptide antibodies against human SA2 (SSRGSTVRSKSKPSTGKRKVV) and human SA1/SA2 (DLPPSKNRRERTELKPDFFD) peptides. Other antibodies used in this study were obtained as follows: anti-Rad21 (Bethyl Laboratories and upstate), anti-Smc1 (Bethyl Laboratories), anti-Smc3 (Bethyl Laboratories), anti-Scc2 (Bethyl Laboratories), anti-Smc2 (Bethyl Laboratories), anti-Topoisomerase II alpha (Bethyl Laboratories), anti-Erk2 (Santa Cruz Biotechnology), anti-Lamin B1 (Abcam), anti-Tubulin (Ab frontier), Histone H3 (upstate), anti-CREST (Immunovision), anti-Plk1 (Santa Cruz Biotechnology), anti-actin (Sigma), anti-Mad2 (BD Biosciences Clontech), anti-Cyclin B (Santa Cruz Biotechnology), anti-Securin (Zymed), anti-Wapl (Bethyl Laboratories), anti-Myc (Roche), anti-HA (Roche), anti-phosphoserine (Sigma), anti-phosphothreonine (Cell signaling) and anti-phospho-SA2 Serine 1224 antibody was kindly provided by Dr Jan-Michael Peters.

Live-cell imaging and fluorescence photobleaching assay

To estimate the signals of RFP and GFP emissions, HeLa-Rad21-RFP and HeLa-SA2-RFP cells were transfected with an expression plasmid encoding H2B-GFP, induced by doxycycline treatment, and then imaged in Δ T 0.15-mm dishes in DMEM medium containing 10% FBS. The confocal pinhole was adjusted to an optical slice thickness larger than z-sampling rate and most time-lapse recordings were performed in parallel at multiple stage positions. Over the course of 24 h, 0.3-s exposures were taken every 20–60 s using an LSM500 META confocal microscope fitted with a $\times$ 20 NAO.75 objective lens (Carl Zeiss). Photobleaching assay was performed with a photobleaching program of the Carl Zeiss confocal software, according to the manufacturer's instructions. Briefly, HeLa-Rad21-RFP and HeLa-SA2-RFP cells were transfected with the expression plasmids encoding H2B-CFP and MBP-GFP, and then bleached using 488 nm laser beam at 80–100% intensity. We selected the cytoplasm (ROI) of whole cell for bleaching. The bleaching time ranged from 20 to 30 s depending on the size and localization of bleach ROIs.

Recombinant protein purification, GST-pull down and in vitro binding assays

GST or His₆-tagged fusion constructs for expression in *Escherichia coli* cells were generated by in-frame insertion of PCR fragments encoding Ssu72 WT, Rad21 and SA2 into the pGEX-KG or pVFT1S vectors (Pharmacia). Recombinant protein purification method was previously described (Kim *et al*, 2009). For the GST-pull-down assay, the fusion proteins were adsorbed onto glutathione-Sepharose bead (Amersham Biosciences) and incubated with whole cell extracts (2 mg) from asynchronous HeLa cells for 4 h at 4°C. The bound proteins were separated by SDS-PAGE and then analysed by immunoblotting with the appropriate antibodies. For the *in vitro* binding assay, purified His-Ssu72 and GST-Rad21 or SA2 were incubated and pulled down with GST-Rad21 or SA2-containing glutathione-Sepharose. The bound proteins were separated by SDS-PAGE and then analysed by immunoblotting with Ssu72, Rad21 and SA2 antibodies.

Immunoprecipitation, immunoblot and flow cytometer assay

For immunoprecipitation from total cell extracts, asynchronous or nocodazole-treated cells were resuspended in buffer A (100 mM Tris-HCl (pH 7.5), 20 mM EDTA, 1% NP40, 1 mM PMSF, 1 mM DTT and a protease inhibitor cocktail). The supernatants (soluble cytoplasmic fractions) were obtained and the cell pellets were resuspended in buffer B (100 mM Tris-HCl (pH 7.5), 20 mM EDTA, 300 mM NaCl, 1% NP40, 1 mM PMSF, 1 mM DTT and a protease inhibitor cocktail), centrifuged and then obtained the supernatants (soluble pellet fractions) of pellet. The mixed extracts (soluble cytoplasmic and pellet supernatants) were diluted with a salt-free buffer to reduce the salt concentration to 150 mM, and the samples were centrifuged and then analysed by immunoprecipitation. For

immunoblot assays, the cells were synchronized as described above or left asynchronized, harvested by scraping, washed twice in cold PBS, and then lysed in TNN buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP40, 1 mM PMSF, 1 mM DTT and a protease inhibitor cocktail). For flow cytometric analyses, cells were fixed and stained with propidium iodide for 5 min and then the DNA contents of 10 000 cells per sample were analysed on a Becton Dickinson FACScan cytometer using the CellQuest and WinMD12.8 software packages.

Immunostaining and chromosome spreading assays

For immunostaining, cells were cultured directly on glass coverslips, washed with PBS (in the case of pre-extraction immunostaining, cells were pre-extracted with 0.2% Triton X-100 in PBS for 10 min and then washed with PBS), fixed in 4% paraformaldehyde, and then incubated with the indicated primary and secondary antibodies. For chromosome spreading assays, cells were treated with 100 ng/ml colcemid or 200 ng/ml nocodazole for 4 h, and mitotic cells were collected by the shaking-off method. Mitotic cells (2×10^5 /ml) were incubated in a hypotonic buffer (50 mM Tris (pH 7.4) and 55 mM KCl), fixed with freshly made Carnoy's solution (75% methanol and 25% acetic acid), dropped onto glass slides, and dried at 80°C. Slides were stained with 5% Giemsa (Merck) or DAPI, washed with PBS, air-dried, mounted and processed for fluorescence microscopy.

ChIP and ChIP-qPCR

For ChIP assays, cells were fixed in culture medium with 1% formaldehyde for 15 min. The cells washed twice in PBS and collected by centrifugation at 3000 r.p.m. at 4°C. Cells were resuspended in ChIP lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1% NP40, 1 mM PMSF, 1 mM DTT and a protease inhibitor cocktail), incubated on ice for 10 min, and sonicated until chromatin DNA was sheared into 500–700 bp fragments. Immunoprecipitations were performed in the cell extracts using either chip grade anti-Rad21 (Abcam) or normal IgG in combination with Protein-A Sepharose. Precipitates were washed sequentially for 5 min each using TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl) and buffer III (0.25 M LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), respectively. Precipitates were then washed three times with 1 ml of TE buffer, and extracted in the solution containing 1% SDS and 0.1 M NaHCO₃. Elutes were pooled and heated at 65°C for 6 h to overnight to reverse the formaldehyde crosslinking. DNA fragments were purified with a QIAquick spin kit (Qiagen). qPCR reactions were performed with the SYBR Green PCR Master Mix in a MicroAmp optical 96-well reaction plate (Applied Biosystems) using the ABI PRISM 7000 SDS v1.1 instrument. The following specific primers were used: Primer 96 (H19 ICR): Forward 5'-TG TGGATAATGCCCGACCTGAAGATCTG-3' Reverse 5'-ACGGAATTGGT TGTAGTTGTGGAATCGGAAGT-3', and Primer 88: Forward 5'-AGATG TTATCATTTATGTCTCGC-3', Reverse 5'-GGCATCTACCTATACTGCG-3'.

Real-time RT-PCR

Total RNAs were extracted by RNeasy Mini Kit (Qiagen) and quantified by UV spectrometry. To prepare RNA for PCR analyses,

1 µg of RNAs were converted to cDNA using ImProm-II Reverse Transcription System (Promega). qPCR reactions were performed with the SYBR Green PCR Master Mix in a MicroAmp optical 96-well reaction plate (Applied Biosystems). Quantification of mRNA expression for the genes was performed by real-time quantitative PCR using the ABI PRISM 7000 SDS v1.1 instrument.

Two-dimensional SDS-PAGE

To test the dephosphorylation of SA2 in HeLa Con and HeLa HA-Ssu72 cells by two-dimensional SDS-PAGE, SA2 immunocomplexes were prepared from whole cell lysates and rehydrated in sample buffer supplemented with 50 mM DTT and the appropriate ampholytes. Isoelectric focusing was performed overnight using the Protean IEF Cell System (BioRad) with pH 3–11 NL immobililine DryStrip isoelectric focusing strips (Amersham PLC). The protein complexes were separated in the second dimension by SDS-PAGE, followed by transfer to a nitrocellulose membrane.

Purification of endogenous cohesin, *in vitro* kinase assay and cohesin dephosphorylation assay

For the *in vitro* kinase assay, bead-bound cohesin or the various purified GST-Ssu72 proteins were washed twice with kinase buffer (100 mM Tris-HCl (pH 7.5), 2 mM EDTA (pH 8), 20 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT and 1 mM PMSF) and reacted with 0.4 µg of recombinant Cdk1/Cyclin B (Invitrogen), Plk1 (Invitrogen) or aurora B (Sigma) proteins in the presence of radio-unlabelled ATP or [γ -³²P]ATP (10 µCi) at 30°C for 1 h. The reaction was stopped by the addition of SDS sample buffer, and resolved by SDS-PAGE and visualized by autoradiography. For the cohesin dephosphorylation assay, phosphorylated cohesin complexes were purified from nocodazole-arrested cell extracts by immunoprecipitation using anti-SA2 antibody. Bead-bound phosphorylated cohesin complex was washed twice with phosphatase buffer and reacted with purified GST-Ssu72 WT, GST-Ssu72 C12S or λ phosphatase (NEB) at 30°C.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

References

- Ben-Shahar TR, Heeger S, Lehane C, East P, Flynn H, Skehel M, Uhlmann F (2008) Eco1-dependent cohesin acetylation during establishment of sister chromatid cohesion. *Science* **321**: 563–566
- Gandhi R, Gillespie PJ, Hirano T (2006) Human Wapl is a cohesin-binding protein that promotes sister chromatid resolution in mitotic prophase. *Curr Biol* **16**: 2406–2417
- Ganem C, Devaux F, Torchet C, Jacq C, Quevillon-Cheruel S, Labesse G, Facca C, Faye G (2003) Ssu72 is a phosphatase essential for transcription termination of snoRNAs and specific mRNAs in yeast. *EMBO J* **22**: 1588–1598
- Gimenez-Abian JF, Sumara I, Hirota T, Hauf S, Gerlich D, de la Torre C, Ellenberg J, Peters JM (2004) Regulation of sister chromatid cohesion between chromosome arms. *Curr Biol* **14**: 1187–1193
- Haering CH, Lowe J, Hochwagen A, Nasmyth K (2002) Molecular architecture of SMC proteins and the yeast cohesin complex. *Mol Cell* **9**: 773–788
- Hauf S, Waizenegger IC, Peters JM (2001) Cohesin cleavage by separase required for anaphase and cytokinesis in human cells. *Science* **293**: 1320–1323
- Hauf S, Roitinger E, Koch B, Dittrich CM, Mechtler K, Peters JM (2005) Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2. *PLoS Biol* **3**: e69
- Horning NC, Uhlmann F (2004) Preferential cleavage of chromatin-bound cohesin after targeted phosphorylation by Polo-like kinase. *EMBO J* **23**: 3144–3153

- Kim HS, Jeon YK, Ha GH, Park HY, Kim YJ, Shin HJ, Lee CG, Chung DH, Lee CW (2009) Functional interaction between BubR1 and securin in an anaphase-promoting complex/cyclosomeCdc20-independent manner. *Cancer Res* **69**: 27–36
- Kitajima TS, Sakuno T, Ishiguro K, Iemura S, Natsume T, Kawashima SA, Watanabe Y (2006) Shugoshin collaborates with protein phosphatase 2A to protect cohesin. *Nature* **441**: 46–52
- Krishnamurthy S, He X, Reyes-Reyes M, Moore C, Hampsey M (2004) Ssu72 Is an RNA polymerase II CTD phosphatase. *Mol Cell* **14**: 387–394
- Kueng S, Hegemann B, Peters BH, Lipp JJ, Schleiffer A, Mechtler K, Peters JM (2006) Wapl controls the dynamic association of cohesin with chromatin. *Cell* **127**: 955–967
- Lénárt P, Ellenberg J (2006) Monitoring the permeability of the nuclear envelope during the cell cycle. *Methods* **38**: 17–24
- Losada A, Hirano M, Hirano T (1998) Identification of Xenopus SMC protein complexes required for sister chromatid cohesion. *Genes Dev* **12**: 1986–1997
- Losada A, Yokochi T, Kobayashi R, Hirano T (2000) Identification and characterization of SA/Scp3p subunits in the Xenopus and human cohesin complexes. *J Cell Biol* **150**: 405–416
- Losada A, Hirano M, Hirano T (2002) Cohesin release is required for sister chromatid resolution, but not for condensin-mediated compaction, at the onset of mitosis. *Genes Dev* **16**: 3004–3016
- McGuinness BE, Hirota T, Kudo NR, Peters JM, Nasmyth K (2005) Shugoshin prevents dissociation of cohesin from centromeres during mitosis in vertebrate cells. *PLoS Biol* **3**: e86
- Meinhart A, Silberzahn T, Cramer P (2003) The mRNA transcription/processing factor Ssu72 is a potential tyrosine phosphatase. *J Biol Chem* **278**: 15917–15921
- Michaelis C, Ciosk R, Nasmyth K (1997) Cohesins: chromosomal proteins that prevent premature separation of sister chromatids. *Cell* **91**: 35–45
- Nasmyth K (2002) Segregating sister genomes: the molecular biology of chromosome separation. *Science* **297**: 559–565
- Orphanides G, Reinberg D (2002) A unified theory of gene expression. *Cell* **108**: 439–451
- Pappas Jr DL, Hampsey M (2000) Functional interaction between Ssu72 and the Rpb2 subunit of RNA polymerase II in *Saccharomyces cerevisiae*. *Mol Cell Biol* **20**: 8343–8351
- Riedel CG, Katis VL, Katou Y, Mori S, Itoh T, Helmhart W, Gálová M, Petronczki M, Gregan J, Cetin B, Mudrak I, Ogris E, Mechtler K, Pelletier L, Buchholz F, Shirahige K, Nasmyth K (2006) Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I. *Nature* **44**: 53–61
- St-Pierre B, Liu X, Kha LC, Zhu X, Ryan O, Jiang Z, Zacksenhaus E (2005) Conserved and specific functions of mammalian ssu72. *Nucleic Acids Res* **33**: 464–477
- Ström L, Karlsson C, Lindroos HB, Wedahl S, Katou Y, Shirahige K, Sjögren C (2007) Postreplicative formation of cohesion is required for repair and induced by a single DNA break. *Science* **317**: 242–245
- Sumara I, Vorlauffer E, Gieffers C, Peters BH, Peters JM (2000) Characterization of vertebrate cohesin complexes and their regulation in prophase. *J Cell Biol* **151**: 749–762
- Sumara I, Vorlauffer E, Stukenberg PT, Kelm O, Redemann N, Nigg EA, Peters JM (2002) The dissociation of cohesin from chromosomes in prophase is regulated by Polo-like kinase. *Mol Cell* **9**: 515–525
- Tang Z, Shu H, Qi W, Mahmood NA, Mumby MC, Yu H (2006) PP2A is required for centromeric localization of Sgo1 and proper chromosome segregation. *Dev Cell* **10**: 575–585
- Unal E, Heidinger-Pauli JM, Koshland D (2007) DNA double-strand breaks trigger genome-wide sister-chromatid cohesion through Eco1 (Ctf7). *Science* **317**: 245–248
- Unal E, Heidinger-Pauli JM, Kim W, Guacci V, Onn I, Gygi SP, Koshland DE (2008) A molecular determinant for the establishment of sister chromatid cohesion. *Science* **321**: 566–569
- Waizenegger IC, Hauf S, Meinke A, Peters JM (2000) Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase. *Cell* **103**: 399–410
- Wendt KS, Yoshida K, Itoh T, Bando M, Koch B, Schirghuber E, Tsutsumi S, Nagae G, Ishihara K, Mishihiro T, Yahata K, Imamoto F, Aburatani H, Nakao M, Imamoto N, Maeshima K, Shirahige K, Peters JM (2008) Cohesin mediates transcriptional insulation by CCCTC-binding factor. *Nature* **451**: 796–801