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The SUMO protease SENP1 is required for cohesion maintenance and mitotic arrest following spindle poison treatment

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ABSTRACT

SUMO conjugation is a reversible posttranslational modification that regulates protein function. SENP1 is one of the six SUMO-specific proteases present in vertebrate cells and its altered expression is observed in several carcinomas. To characterize SENP1 role in genome integrity, we generated *Senp1* knockout chicken DT40 cells. *SENP1*$^{-/-}$ cells show normal proliferation, but are sensitive to spindle poisons. This hypersensitivity correlates with increased sister chromatid separation, mitotic slippage, and apoptosis. To test whether the cohesion defect had a causal relationship with the observed mitotic events, we restored the cohesive status of sister chromatids by introducing the *TOP2α*$^{+/-}$ mutation, which leads to increased catenation, or by inhibiting Plk1 and Aurora B kinases that promote cohesin release from chromosomes during prolonged mitotic arrest. Although TOP2α is SUMOylated during mitosis, the *TOP2α*$^{+/-}$ mutation had no obvious effect. By contrast, inhibition of Plk1 or Aurora B rescued the hypersensitivity of *SENP1*$^{-/-}$ cells to colcemid. In conclusion, we identify SENP1 as a novel factor required for mitotic arrest and cohesion maintenance during prolonged mitotic arrest induced by spindle poisons.

1. Introduction

SUMOylation controls diverse cellular processes including DNA repair, chromosome organization and mitotic progression. Vertebrate and mammalian cells typically express three SUMO paralogues that are processed/matured by SUMO specific protease (Ulp/SENPs) family of enzymes [1]. SUMOylation is also reversed by SENPs. Thus, SENPs are required for maintaining the physiological level of SUMOylated and un-SUMOylated substrates. Mammals have six SENPs, SENP1, 2, 3, 5, 6 and 7. Several studies indicated a role for SENPs in cancer development. SENP1 is overexpressed in several carcinomas, including prostate, colon, breast, thyroid and bladder cancer [2–5], and it has been implicated in prostate tumorigenesis [3,6].

Previous work highlighted a critical role for SUMOylation in mitotic chromosome structure and segregation [7]. The metaphase to anaphase transition is a key step in mitosis. In metaphase, bipolar attachments of spindles to kinetochores ensure proper movement and alignment of the chromosomes to the metaphase plate. To allow sister chromatid separation, the cohesion holding the sister chromatids together has to be dissolved. Cohesion is mediated by a group of proteins called cohesins [8] and by catenation arising during DNA metabolism [9,10]. Chromatid separation during anaphase occurs via DNA decatenation, which is mediated by topoisomerase II (or TOP2α invertebrate/mammalian cells) [11,12], and via cohesin dissolution, which is mediated by a protease called separase [8]. During prolonged prometaphase/metaphase arrest induced by microtubule poisons, Plk1 and Aurora B mitotic kinases down-regulate cohesion by promoting cohesin release from chromosome arms [13–15].

Microtubule spindles are one of the most successful cancer chemotherapeutic targets. Microtubule active compounds, hereafter called spindle poisons, suppress microtubule dynamics [16]. This activates the spindle checkpoint, which in turn leads to transient mitotic arrest and inhibition of cell proliferation. Abnormal premature exit from mitotic arrest is known as "mitotic slippage" [17].

Here we established *SENP1*$^{-/-}$ cells in the avian cell line, DT40. *SENP1*$^{-/-}$ cells show normal proliferation but are hypersensitive to spindle poisons. Flow cytometric analysis revealed that *SENP1*$^{-/-}$ cells are deficient in sustaining a prolonged prometaphase/metaphase arrest and are prone to undergo mitotic slippage. The increased mitotic slippage events in *SENP1*$^{-/-}$ cells further correlate with higher frequency of endoreplication and apoptosis in these cells. The mitotic kinases Plk1- and Aurora B-mediated sister chromatid separation, but not TOP2α-mediated decatenation are involved in the colcemid-induced cytotoxicity. Together, our findings reveal SENP1 as a novel factor involved in the maintenance

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of cohesion during prolonged mitotic arrest induced by chemotherapeutic anti-microtubule drugs.

2. Materials and methods

Detailed information on constructs and methods is provided in the Supplementary Material accompanying the manuscript.

3. Results

3.1. Generation of SENP1$^{-/-}$ DT40 cells

Gene disruption of the chicken *Senp1* gene (NCBI; Gene ID: 426185) was achieved by substituting exons 5 and 6 of the genomic *SENP1* loci with the selection markers, puro and bsr, respectively. *Senp1* disruption was confirmed by Southern and Western blot analysis (Fig. 1B and C). *SENP1*$^{-/-}$ cells grew similarly with WT (Fig. 1D). Thus, SENP1 is not required for DT40 cell proliferation in unperturbed conditions.

3.2. SENP1$^{-/-}$ cells are sensitive to spindle poisons

We tested the sensitivity of *SENP1*$^{-/-}$ cells to various DNA damaging agents such as ionizing radiation, known to cause single and double strand breaks, the alkylating agent methyl methanesulfonate, and topoisomerase poisons, etoposide and camptothecin. We found *SENP1*$^{-/-}$ cells not to be hypersensitive towards any of these drugs or treatments (data not shown).

Because SUMOylation appears to play an important role in mitosis [7] and budding yeast mutants of the SUMO protease Ulp2 are sensitive to the spindle poison benomyl [18], we further tested the sensitivity of *SENP1*$^{-/-}$ cells to spindle poisons. To this end, we exposed cells to spindle poisons for 24 h, which corresponds to three doubling times in unperturbed conditions, and measured cellular viability. *SENP1*$^{-/-}$ cells were sensitive to all spindle poisons analyzed (Fig. 2A–D). We further reconstituted *SENP1*$^{-/-}$ cells with a chicken *SENP1* transgene. The *SENP1* transgene rescued the sensitivity of *SENP1*$^{-/-}$ cells to colcemid to WT levels (Fig. 2E), thus confirming a role for SENP1 in cell viability during prolonged mitotic arrest induced by spindle poisons. We further note that no obvious spindle or spindle pole body aberrations were observed in *SENP1*$^{-/-}$ cells in unperturbed conditions (Fig. 2F).

3.3. SENP1$^{-/-}$ cells are prone to undergo mitotic slippage when exposed to prolonged prometaphase/metaphase arrest

Previously it was reported that following spindle poison treatment, the spindle assembly checkpoint (SAC) becomes satisfied on abnormal spindles and cells proceed in mitosis via mitotic slippage [19]. We investigated the cell cycle profile following treatment with spindle poisons (Fig. 3A). WT and *SENP1*$^{-/-}$ cells displayed the same cell cycle profile and mitotic index (MI) prior to addition of colcemid or 5 h following colcemid addition (Fig. 3A and B). At 11 h, however, the two *SENP1*$^{-/-}$ clones, but not WT cells, were no longer able to maintain the prometaphase/metaphase arrest; more than half of *SENP1*$^{-/-}$ cells accumulated in sub-G1, and a fraction of cells showed a DNA content higher than 4N (Fig. 3A). At 18 h, while the majority of WT cells were still in prometaphase/metaphase, virtually no *SENP1*$^{-/-}$ cells were showing this arrest (Fig. 3A). Defective mitotic arrest in *SENP1*$^{-/-}$ cells was also observed following treatment of cells with vinorelbine and taxol (data not shown). Thus, *SENP1*$^{-/-}$ cells are predisposed to undergo mitotic slippage following prolonged prometaphase arrest induced by microtubule poisons.

We noticed that the mitotic index was slightly lower in *SENP1*$^{-/-}$ cells than WT cells 5 h after addition of colcemid treatment. Thus, we monitored the mitotic index every hour after the initial 5 h of colcemid treatment (Fig. 3B). The mitotic index of WT cells constantly increased and reached a peak at 7 h of colcemid treatment.

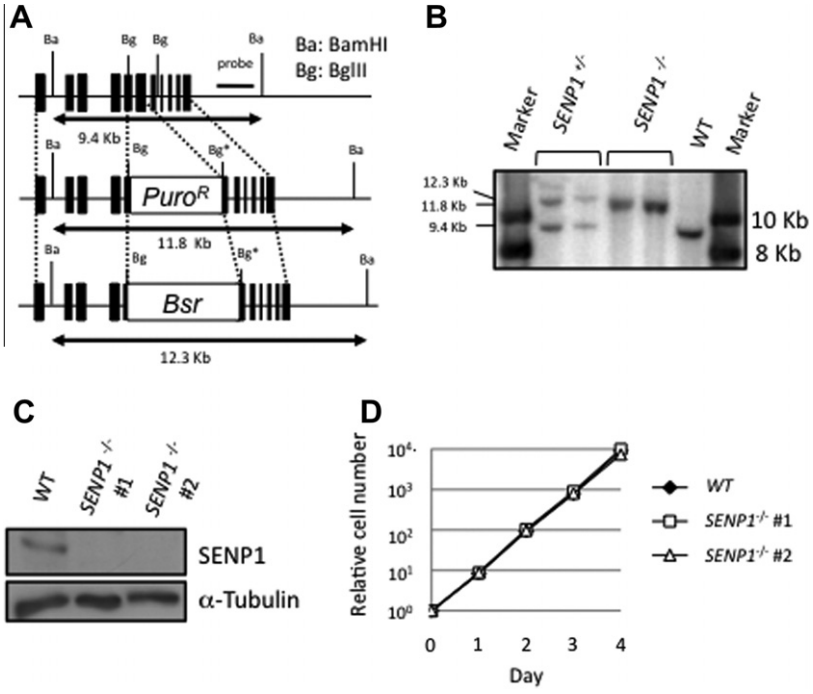

Fig. 1. Generation and characterization of *Senp1* knockout cells. (A) Schematic depiction showing a part of the *Senp1* locus and the targeting constructs. The filled boxes represent exons. The thick arrows show the BamHI fragment before and after targeting. The BglII site artificially made in exon 6 in targeting constructs is shown marked by an asterisk. (B) Southern blot analysis of BamHI-digested DNA using the probe indicated in (A). (C) Western blot analysis of indicated genotypes with anti-human SENP1 antibody. α-tubulin was used as a loading control. (D) Growth curve of WT and *SENP1*$^{-/-}$ cells.

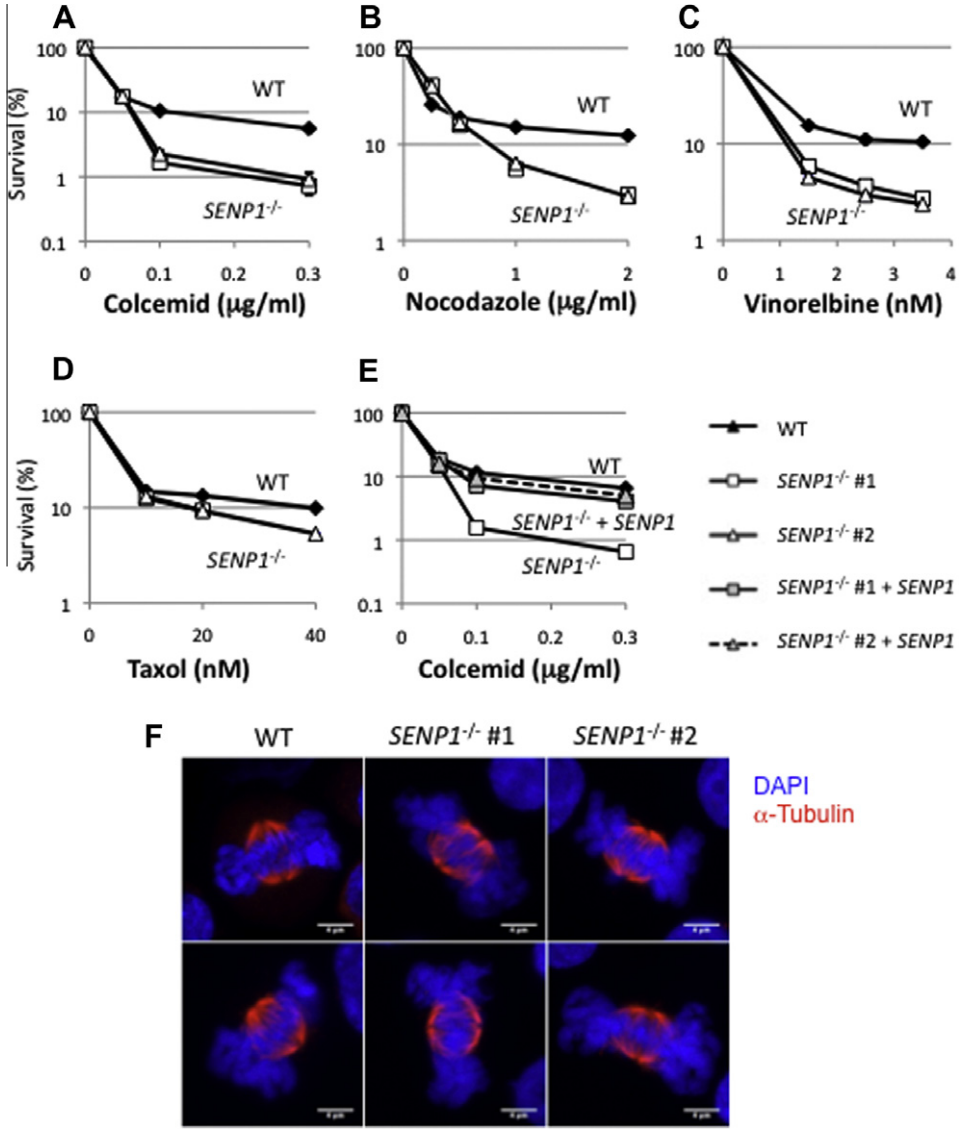

Fig. 2. Hypersensitivity of *SENP1*$^{-/-}$ cells to spindle poison drugs. (A–D) Cellular viability was measured at 24 h following exposure to various spindle poisons: colcemid (A), nocodazole (B), vinorelbine (C) and taxol (D) at the indicated concentrations. (E) Colcemid sensitivity of the *SENP1*$^{-/-}$ cells is complemented by a chicken *SENP1* transgene. (A–E) The concentration of spindle poisons is displayed on the *x*-axis on a linear scale, while the viability is displayed on the *y*-axis on a logarithmic scale. Error bars show the standard deviation for three independent experiments. Note that in most cases, error bars are smaller than the symbols for the data points. (F) Immunostaining of condensed chromosome and spindle dynamics with DAPI (blue) and α-tubulin (red) at metaphase in unperturbed conditions. Bar, 4 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

By contrast, such significant increase was not observed in *SENP1*$^{-/-}$ cells. Notably, at 5 and 7 h of incubation with colcemid, the viability of *SENP1*$^{-/-}$ cells was not affected, thus making it unlikely that the lower mitotic index observed was due to increased cell death. We therefore conclude that the increased sensitivity of *SENP1*$^{-/-}$ cells to the anti-microtubule poisons results from a defect in maintaining a prolonged metaphase arrest. In further support of this conclusion, we observed that the increased mitotic slippage phenotype of *SENP1*$^{-/-}$ cells correlated with an increase in the number of pre-apoptotic and apoptotic cells (Fig. 3C) [20].

Since we observed populations of both WT and *SENP1*$^{-/-}$ cells having DNA content higher than 4N following prolonged colcemid treatment, we hypothesized that this may be due to endoreplication. We analyzed the status of DNA replication by using BrdU-pulse-labeling flow cytometric analysis. As expected, a small population of WT cells is engaged in endoreplication (Fig. 3D), a typical phenotype of p53-deficient cells [21,22]. Consistent with a larger population of *SENP1*$^{-/-}$ cells undergoing mitotic slippage (Fig. 3A), we also observed an increased percentage of *SENP1*$^{-/-}$ cells undergoing endoreplication in comparison with WT cells. In addition, *SENP1*$^{-/-}$ cells showed an increase in sub-G1 and G1 populations likely due to increased apoptosis, in line with our previous results (Fig. 3A and C). In summary, loss of SENP1 caused a defect in maintaining prolonged metaphase arrest leading to re-initiation of DNA replication as well as apoptosis.

3.4. SENP1$^{-/-}$ *cells are cohesion defective and their sensitivity to colcemid is suppressed by inhibiting Plk1- and Aurora B-mediated sister chromatid separation*

We further addressed if similarly to budding yeast *ulp2* mutants [23,24], *SENP1*$^{-/-}$ cells show cohesion defects when arrested at metaphase. Because prolonged colcemid treatment induces mitotic slippage and reduces the population of metaphase *SENP1*$^{-/-}$ cells

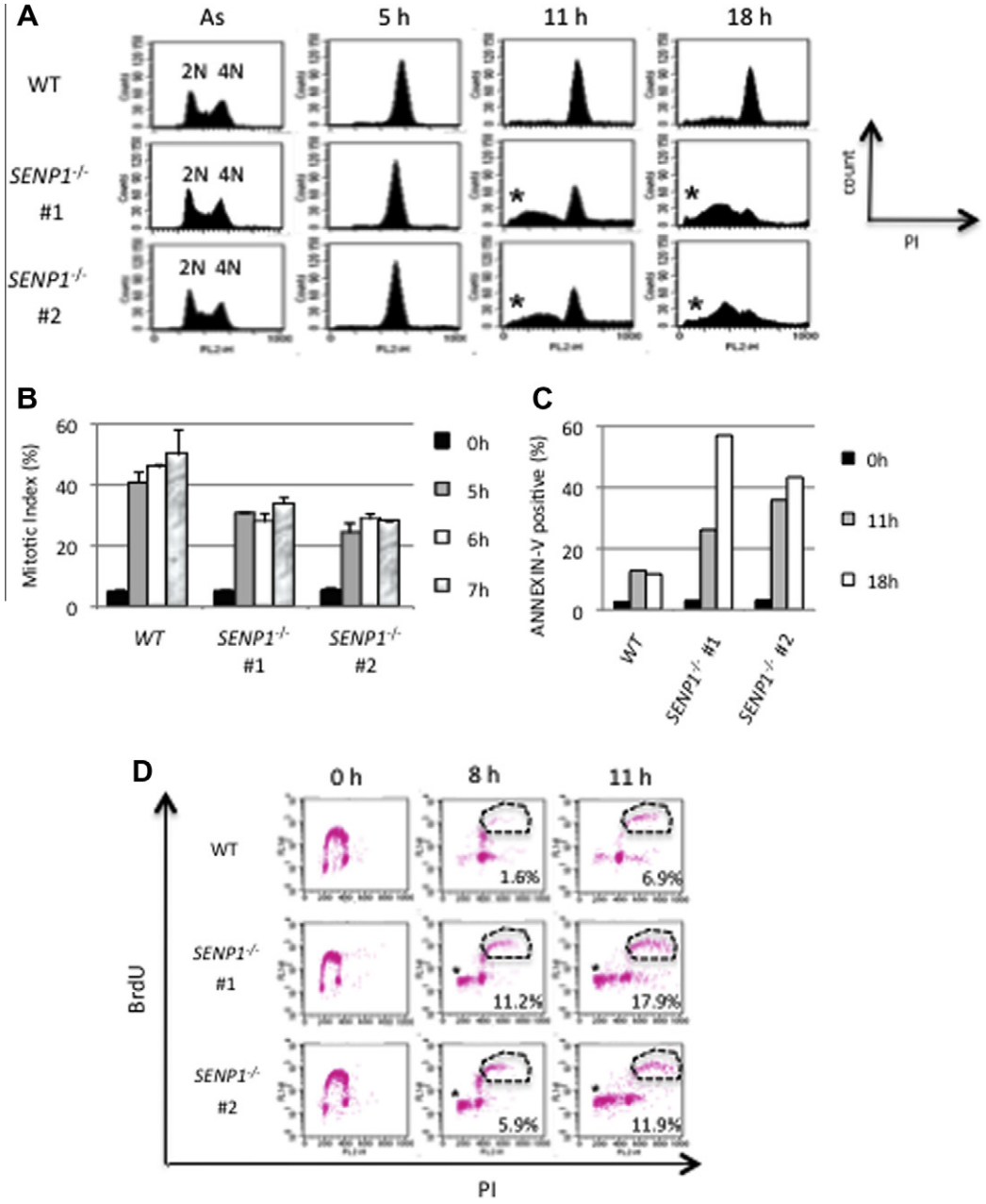

Fig. 3. Hypersensitivity of *SENP1*$^{-/-}$ cells to spindle poisons is due to mitotic slippage. (A) Asynchronous WT and the *SENP1*$^{-/-}$ cells were treated with colcemid. Samples were collected 0, 5, 11 and 18 h after addition of colcemid for cell cycle analysis by FACS. The asterisk indicates a sub-G1 population that is increased in *SENP1*$^{-/-}$ cells. (B) Mitotic Index was measured by immunofluorecence using an antibody against phosphorylated histone H3 serine 10. Samples were collected at 0 h and every hour from 5 to 7 h after addition of colcemid. H3-ser10 positive cell are plotted. At least 300 cells for each strain were analyzed. (C) Apoptotic cells following 0, 11 and 18 h of treatment with colcemid were detected by an anti-ANNEXIN V antibody. (D) Cell cycle profile with BrdU incorporation. To analyze the cell cycle and the DNA replication status, BrdU were incorporated for 10 min at 0, 8 and 11 h following colcemid treatment and cells were collected for analysis. The asterisk indicates a sub-G1 population that is increased in *SENP1*$^{-/-}$ cells. Cells undergoing endoreplication are shown in a gate and the calculated percentage is displayed.

(Fig. 3A), we performed chromosome analysis on metaphase spreads following 2 h of colcemid treatment, when no obvious effect of colcemid or of the *SENP1*$^{-/-}$ mutation on cell viability or on the frequency of mitotic slippage events are observed. In *SENP1*$^{-/-}$ cells an increased number of cells showed sister chromatid separation in comparison with WT (Fig. 4A). The aggravated sister chromatid separation defect observed in *SENP1*$^{-/-}$ cells compared to WT lay open the possibility that the defect might have a causal relationship with the sensitivity of the mutant cells to prolonged colcemid treatment.

SUMOylation promotes TOP2α-mediatedsister chromatid separation [25] and SENP1 deSUMOylates TOP2α, promoting its release from chromosomes following TOP2α-mediated decatenation [26]. Thus, it is possible that in *SENP1*$^{-/-}$ cells SUMOylated TOP2α promotes sister chromatid separation and induces colcemid sensitivity. We thus investigated if reduced TOP2α activity suppressed the colcemid sensitivity of *SENP1*$^{-/-}$ cells. Because *Top2α* is essential in chicken cells, we generated *TOP2α*$^{+/-}$ cells (Supplementary Fig. 1). Consistent with these cells having reduced amounts of TOP2α, they showed resistance to the topoisomerase II inhibitors,

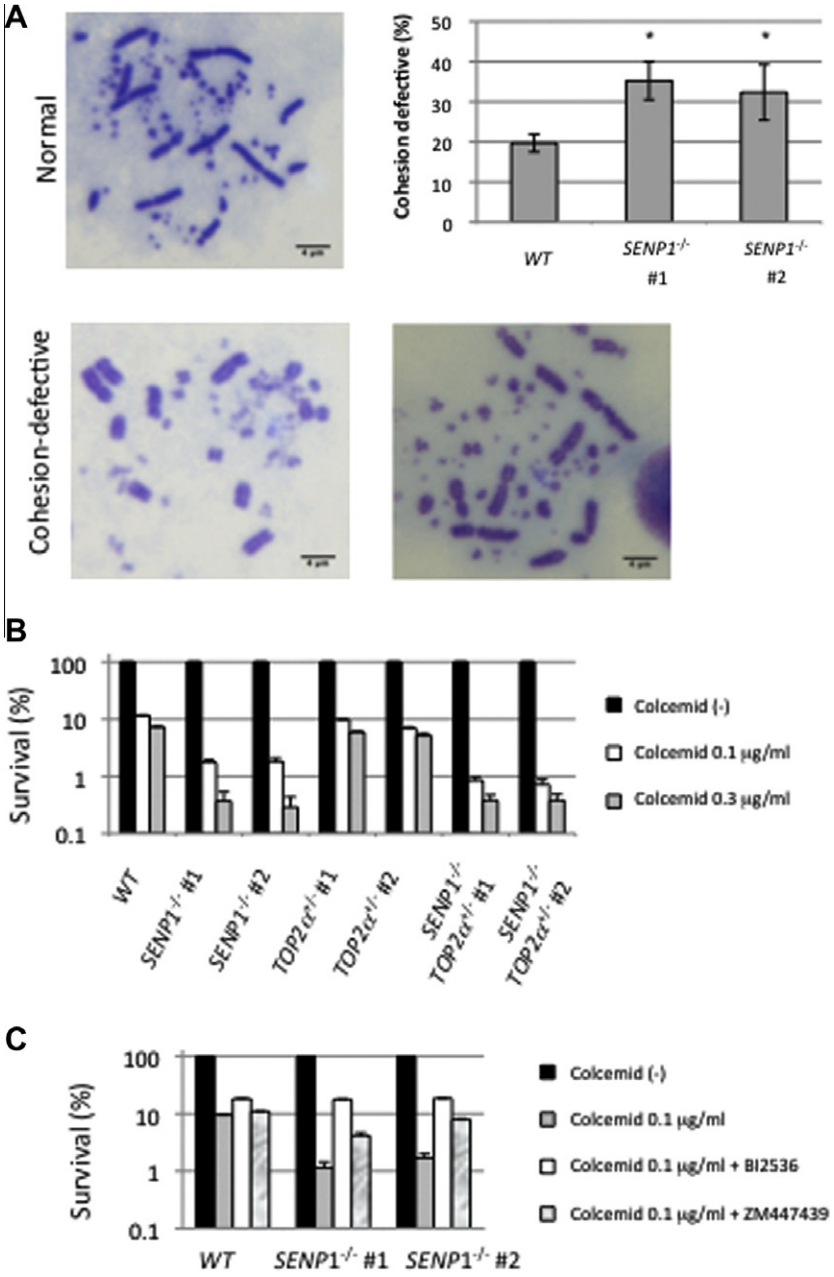

Fig. 4. Hypersensitivity of *SENP1*$^{-/-}$ cells to colcemid is suppressed by inhibiting Plk1 or Aurora B kinases, but not by reducing TOP2α-mediated decatenation. (A) Cohesion defects estimated from Giemsa-staining of mitotic chromosomes. Representative pictures of normal and cohesion-defective metaphase spreads are illustrated. Bar, 4 μm. The percentage of cohesion defective WT and *SENP1*$^{-/-}$ cells is indicated. The values represent average values of cohesion defective percentage and error bars as calculated from three independent experiments. The following number of metaphase spreads were analyzed in each experiment: WT: 123, 259, 96; *SENP1*$^{-/-}$ #1: 126, 129, 96; *SENP1*$^{-/-}$ #2: 123, 247, 96. The p value between WT and *SENP1*$^{-/-}$ #1 was 0.00196, the one between WT and *SENP1*$^{-/-}$ #2 was 0.01295. (B) The sensitivity of WT, *TOP2α*$^{+/-}$, *SENP1*$^{-/-}$ and *SENP1*$^{-/-}$ *TOP2α*$^{+/-}$ cells to 0.1 and 0.3 μg/ml colcemid after 24 h of treatment. The viability of colcemid-untreated cells was considered as 100%. (C) WT and *SENP1*$^{-/-}$ cells treated with 0.1 μg/ml colcemid for 2 h were further incubated with colcemid in the presence of 10 nM of BI2536 (Plk1 inhibitor, BI) or 2 μM of ZM447439 (Aurora B inhibitor, ZM) as shown on the top of the figure. Cellular viability was measured after 24 h of treatment and the viability of colcemid-untreated cells was considered as 100%.

ICRF-193 and Etoposide (Supplementary Fig. 1C and [27]). However, the *TOP2α*$^{+/-}$ mutation did not rescue the colcemid sensitivity of *SENP1*$^{-/-}$ cells (Fig. 4B), indicating that increased TOP2α SUMOylation and consequent TOP2α-mediated decatenation does not account for the hypersensitivity of *SENP1*$^{-/-}$ cells to colcemid.

In vertebrate cells, during prolonged prometaphase arrest induced by treatment of cells with spindle poisons, Plk1 and Aurora B mitotic kinases promote cohesin dissociation from chromosome arms [13–15]. If the colcemid sensitivity displayed by *SENP1*$^{-/-}$ cells (Figs. 2A–D) is due to failure in maintaining mitotic cohesion (Fig. 4A), then by inhibiting the Plk1- or Aurora B-mediated pathway of cohesin removal, the colcemid sensitivity of these cells should be rescued. Indeed, inhibition of either kinases using specific small molecule inhibitors, BI2536 and ZM447439 [28–30], significantly rescued the hypersensitivity of *SENP1*$^{-/-}$ cells to colcemid (Fig. 4C) and reduced/abolished the elevated levels of mitotic slippage observed in *SENP1*$^{-/-}$ cells (data not shown). These results confirm a link between sensitivity of cells to spindle poisons and chromatid separation induced by dissolution of cohesion during metaphase arrest.

4. Discussion

Profound changes in chromosome structure and nuclear architecture occur during mitosis. These changes are accompanied by extensive phosphorylation events and accumulation of specific SUMO conjugates [31–33]. The mechanisms underlying these events, the substrates involved and the interplay between SUMOylation enzymes and the activity of mitotic kinases remains largely unknown. In this study, we generated for the first time *Senp1* gene knockout cells and revealed SENP1 as a novel factor required for the maintenance of sister chromatid cohesion, cell viability and mitotic arrest following treatment of cells with spindle poisons. Thus, SENP1 is a new player that can significantly affect the resistance of malignant cells to anti-microtubule chemotherapeutic agents.

The decrease in *SENP1*$^{-/-}$ cell viability following treatment with spindle poisons appears to be due to elevated levels of mitotic slippage accompanied by apoptosis and endoreplication. Previous work established that spindle poisons hinder the correct attachment of kinetochores to the spindles and this in turn leads to activation of the SAC [34,35]. However, SAC is not able to arrest cells permanently [36,37] and studies in different organisms have shown that mitotic slippage (that is, escape from metaphase arrest in the presence of an unsatisfied SAC) involves inhibition of mitotic Cdk1 activity via different mechanisms such as inhibitory phosphorylation of Cdk1, activation of Cdk1 inhibitors or, most prominently in vertebrate cells, a continuous degradation of cyclin B via APC-mediated proteolysis [38–42]. The increased incidence of mitotic slippage events in *SENP1*$^{-/-}$ cells following spindle poison treatment is likely due to an anticipated exit from metaphase arrest. If this is due to poor activation of SAC, premature down-regulation of Cdk1, enhanced chromatid separation, or a combination of these effects is not yet clear.

In this study we obtained evidence for enhanced sister chromatid separation occurring in *SENP1*$^{-/-}$ cells. In support of the notion that the sister chromatid separation defect of *SENP1*$^{-/-}$ cells plays an important role in mediating the colcemid sensitivity and associated mitotic defects, we observed that by inhibiting Plk1- and Aurora B-mediated pathway controlling the dissolution of cohesion during prolonged prometaphase/metaphase arrest [13,15], the colcemid sensitivity and the increased mitotic slippage events of *SENP1*$^{-/-}$ cells were suppressed. However, given the diverse functions and numerous substrates for Plk1 and Aurora B kinases in mitosis [43–45], we cannot rule out that mechanisms besides cohesion dissolution are responsible or play a role in the observed suppression. In addition, while the enhanced chromatid separation mechanism alone can explain the colcemid sensitivity and anticipated anaphase entry of *SENP1*$^{-/-}$ cells, formally we cannot exclude an effect of increased SUMOylation due to defective SENP1 activity on mitotic kinases such as SAC or Cdk1.

In budding yeast, Ulp2 and the Polo-like kinase, Cdc5, were proposed to act in a mutually antagonistic manner to modulate the maintenance/dissolution of mitotic cohesion [24]. In vertebrate cells, the Polo-like kinase, Plk1, down-regulates cohesion maintenance mechanisms by promoting cohesin release [13–15]. Our findings indicate that SENP1 counteracts sister chromatid separation during spindle poison-induced mitotic arrest. While the targets of SENP1 in this process remain to be uncovered, budding yeast Ulp2 is thought to mediate cohesion maintenance via down-regulating SUMOylation of Pds5 [24,31]. The proposed mechanism envisages that SUMOylation of Pds5, which is increased in *ulp2* cells, helps cohesin release, possibly because SUMO moieties on Pds5 weaken cohesion or interfere with the ability of Pds5 to insulate cohesin from yet unknown cohesin-destabilizing factors, such as Wapl in vertebrate cells [13,46,47]. Whether a crosstalk mechanism between Plk1 and SENP1 exists in vertebrate cells and if SENP1 acts in parallel or opposing fashion with Plk1 in what regards the control of cohesion dissolution during prolonged metaphase arrest remain subjects for future work.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.08.066.

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