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Deacetylation of the mitotic checkpoint protein BubR1 at lysine 250 by SIRT2 and subsequent effects on BubR1 degradation during the prometaphase/anaphase transition

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ABSTRACT

Mitotic catastrophe, a form of cell death that occurs during mitosis and after mitotic slippage to a tetraploid state, plays an important role in the efficacy of cancer cell killing by microtubule inhibitors. Prolonged mitotic arrest at the spindle assembly checkpoint (SAC) is a well-known requirement for mitotic catastrophe and, thus, for conferring sensitivity to microtubule inhibitors. We previously reported that downregulation of SIRT2, a member of the sirtuin family of NAD+-dependent deacetylases, confers resistance to microtubule inhibitors by abnormally prolonging mitotic arrest and thus compromising the cell death pathway after mitotic slippage. Thus, turning off SAC activation after a defined period is an additional requirement for efficient post-slippage death. Here, we investigated whether SIRT2 deacetylates BubR1, which is a core component of the SAC; acetylation of BubR1 at lysine 250 (K250) during prometaphase inhibits its APC/C-dependent proteolysis and thus regulates timing in anaphase entry. We showed that SIRT2 deacetylates BubR1 K250 both in vitro and in vivo. We also found that SIRT2 knockdown leads to increased levels of BubR1 acetylation at prometaphase; however, this increase is not substantial to elevate the levels of total BubR1 or delay the transition from prometaphase to anaphase. The present study shows that SIRT2 is a deacetylase for BubR1 K250, although the abnormally prolonged SAC activation observed in SIRT2 knockdown cells is not accompanied by a change in BubR1 levels or by delayed progression from prometaphase to anaphase.

1. Introduction

The spindle assembly checkpoint (SAC) monitors the proper attachment of microtubules to the kinetochores of sister chromatids, and the integrity of bipolar mitotic spindles ensures the faithful transmission of chromosomes during cell division [1]. Either unattached or untensioned kinetochores provoke the SAC, which leads to cell cycle arrest at metaphase, and this mitotic arrest allows time for the correction of chromosome connections to the spindle; when these corrections are completed, the SAC is silenced. The SAC prevents the onset of anaphase through a signaling cascade that results in the suppression of the anaphase promoting complex/cyclosome (APC/C) [2]. It has been established that several evolutionally conserved proteins, including BubR1, Bub1, Bub3, Mad1, Mad2, Cenp-E, and Mps1, are required for SAC function [1].

The SAC is activated not only by naturally occurring mitotic errors but also in response to various microtubule inhibitors such as the microtubule depolymerizer nocodazole. However, the SAC cannot be satisfied in the presence of microtubule inhibitors. In that case, cells can have several outcomes that are collectively referred to as mitotic catastrophe [3]. The first outcome of mitotic catastrophe is mitotic death, in which mitotic derangement results in the activation of the cell death machinery with elevated Cyclin B1 levels before cells exit mitosis. However, SAC activation is not

Abbreviations: APC/C, anaphase promoting complex/cyclosome; SAC, spindle assembly checkpoint.

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permanent and is turned off after a defined period, although the mechanism by which it is deactivated is not clear. When mitotic death does not occur before SAC termination, another outcome of mitotic catastrophe is mitotic slippage, in which cells exit mitotic arrest, enter pseudo-G1 without cytokinesis, and then become tetraploid. These cells then either undergo cell death (either quickly or in a delayed fashion hereinafter referred to as post-slippage death), or become senescent, or continue dividing, Mitotic catastrophe accompanied by cell death and growth arrest is important for the cancer cell-killing efficacy of microtubule inhibitors [4].

Prolonged mitotic arrest by SAC is a well-known requirement for mitotic catastrophe and, thus, for conferring sensitivity to microtubule inhibitors. For example, knockdown of BubR1 or Mad2 abolishes SAC function and confers resistance to microtubule inhibitors [5]. On the other hand, in a previous study we showed that turning off SAC activation after a defined period of time is another requirement for efficient post-slippage death following the tetraploid state and identified SIRT2, a member of the sirtuin family of NAD+-dependent deacetylases, as a regulator of this process [6]. When SIRT2 knockdown cells were exposed to nocodazole, SAC activation occurred normally, with the same kinetics and at the same levels as for the control cells. However, SIRT2 knockdown leads to abnormally prolonged SAC activation and confers resistance to microtubule inhibitors by inhibiting post-slippage death [6]. The resistance to nocodazole observed in SIRT2 knockdown cells was reversed by shortening the duration of SAC activation to normal levels by simultaneous knockdown of autophagy-related genes [7]. Thus, an important function of SIRT2 could be to terminate SAC at the right time to induce post-slippage death.

Although SIRT2 has been reported to deacetylate histone H3, histone H4, FoxO1, FoxO3, and p300 [8], the molecular functions of these genes do not explain the role of SIRT2 in regulating SAC. However, acetylation of BubR1, one of the SAC core components mentioned above, at lysine 250 (K250) by PCAF acetyltransferase is required for checkpoint function through the inhibition of ubiquitin-dependent BubR1 degradation [9]. When the checkpoint is satisfied by the proper attachment of microtubules to the kinetochores, BubR1 is deacetylated and becomes a substrate of APC/C-Cdc20-dependent proteolysis. Thus, it has been proposed that BubR1 acetylation inhibits its degradation and regulates the timing of anaphase onset and thus that BubR1 acetylation/deacetylation serves as a molecular switch for the conversion of BubR1 from an inhibitor of the APC/C complex to its substrate [9]. These insights prompted us to examine the possibility that SIRT2 knockdown abnormally prolongs SAC activation in the presence of microtubule inhibitors and delays the onset of anaphase after prometaphase arrest (thus causing resistance to mitotic catastrophe) by increasing the stability of BubR1 via the elevated levels of K250 acetylation. Our previous observation that BubR1 knockdown was dominant over SIRT2 knockdown in regard to SAC regulation supports this hypothesis [6]. Thus, in this study, we investigated whether SIRT2 deacetylates BubR1, and whether, in that case, deacetylation of BubR1 by SIRT2 is required for the regulation of SAC by SIRT2 in the presence of microtubule inhibitors.

2. Materials and methods

2.1. Cell culture and siRNA transfection

Cell culture and siRNA transfection were performed as previously reported [6,7]. When we used plural siRNAs to knockdown SIRT2, we obtained the same phenomenon as previously reported regarding sensitivity to microtubule inhibitors [6]. We used the most potent of these siRNAs (SI02655471) (QIAGEN, Hilden, Germany) for this study.

2.2. Plasmids, immunoprecipitation, and immunoblotting

We obtained a mammalian expression plasmid containing human SIRT2 with a FLAG-tag cloned in pcDNA3.1+ [10] and a plasmid containing PCAF with a FLAG-tag cloned in pCI [11] from Addgene (Cambridge, MA, USA). A plasmid containing human BubR1 cloned in pME18S was purchased from Biological Resource Center, NITE (Tokyo, Japan). FLAG/CBP-tagged SIRT2 (wild type) driven by an EF-1α promoter was generated as described in our previous report [12]. SIRT2 (ΔNDAC), a mutant version of SIRT2 that lacks NAD-dependent deacetylase activity, was described in our previous report [6]. BubR1 (K250R), a mutant version of BubR1 in which lysine 250 is substituted with arginine, was generated using the KOD Plus Mutagenesis Kit (Toyobo, Osaka, Japan).

Transfection of 293T cells using the calcium phosphate DNA precipitation method, preparation of whole cell extracts, immunoprecipitation, and immunoblotting were performed as previously reported [6,7,13,14]. A monoclonal anti-FLAG M2 antibody (1:1000) was purchased from Sigma (St. Louis, MO, USA). Other antibodies used in this study are listed in our previous reports [6,7].

2.3. In vitro deacetylation assays

In vitro deacetylation assays were performed as previously reported [15]. We purified SIRT2 from 293T cells transfected with FLAG-tagged SIRT2 using the FLAG Tagged Protein Immunoprecipitation Kit (Sigma). We isolated acetylated BubR1 from 293T cells cotransfected with PCAF, and then performed immunoprecipitation using an anti-BubR1 antibody. We performed the deacetylation assays using immunoprecipitates generated with anti-BubR1 and eluted SIRT2 at 37 °C in a reaction buffer containing 50 mM Tris (pH 8.0), 4 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/mL bovine serum albumin, and with or without 0.5 mM NAD+.

2.4. Cyclin B1/Cdk1 kinase assays

Cyclin B1/Cdk1 kinase assays were performed as previously reported [6,14].

2.5. Flow cytometry analyses

Flow cytometry analyses were performed as previously reported [7].

3. Results

3.1. SIRT2 interacts in a complex with BubR1 and deacetylates lysine 250 (K250) in BubR1

We first used coimmunoprecipitation to examine whether SIRT2 can interact with BubR1. Initially, we used a colorectal cancer cell line HCT116, which is a mitotic checkpoint-proficient, near-diploid cell line that we used in our previous studies on the regulation of SAC by SIRT2 [6,7]. However, we could not observe any interactions between endogenous SIRT2 and BubR1 by coimmunoprecipitation, possibly due to the low efficiency of the precipitation and low levels of expression of endogenous SIRT2 and BubR1 (data not shown).

We next transiently transfected 293T cells with FLAG/CBP-tagged SIRT2 and a BubR1 expression vector. When extracts from these cells were used for the coimmunoprecipitation, SIRT2 was recovered in immune precipitates generated using an anti-FLAG antibody, as shown in Fig. 1A. BubR1 was also detected in these immunoprecipitates; in contrast, no BubR1 was detected in

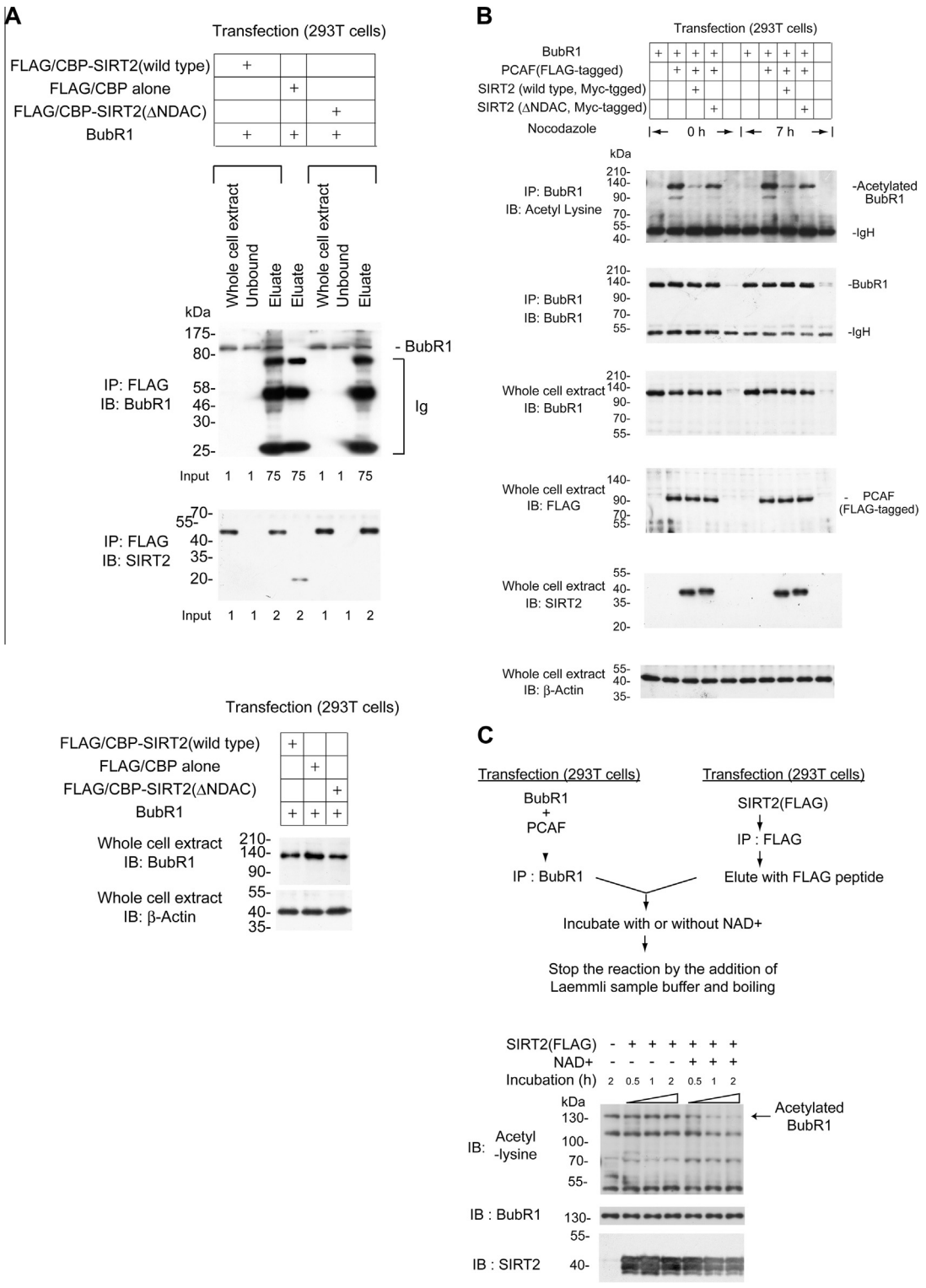

Fig. 1. SIRT2 deacetylates BubR1 K250 in vitro and in vivo. (A) SIRT2 and BubR1 physically interact. 293T cells were transfected with a combination of expression plasmids as indicated. Whole cell lysates were immunoprecipitated using an anti-FLAG antibody and then subjected to immunoblotting analysis using an anti-BubR1 antibody and anti-SIRT2 antibody. The representation (loading) of samples is indicated as a fold-cell-equivalent of the whole cell lysate. The expression of SIRT2 and BubR1 was also examined by immunoblotting. (B) Reversible acetylation/deacetylation of BubR1 lysine 250 mediated by PCAF and SIRT2. 293T cells were transfected with a combination of expression plasmids as indicated. Whole cell lysates were immunoprecipitated using an anti-BubR1 antibody and then subjected to immunoblotting analysis using an anti-acetyl-lysine antibody. The levels of total BubR1 protein in the immunoprecipitates and the expression of PCAF, SIRT2 (wild type), and SIRT2 (ΔNDAC) were also examined by immunoblotting. (C) SIRT2 deacetylates acetylated BubR1 K250 mediated by PCAF in vitro. SIRT2 and acetylated BubR1 were purified from transfected 293T cells and then mixed and incubated for the indicated time with or without NAD+. The levels of deacetylation were monitored by immunoblotting analysis using an anti-acetyl-lysine antibody. IB, immunoblotting; IP, immunoprecipitation.

immunoprecipitates from cells transfected with the BubR1 expression vector alone (Fig. 1A). This result suggests that SIRT2 interacts in a complex with BubR1 and thus supports the hypothesis that BubR1 is a target of SIRT2-dependent deacetylation.

To further examine whether BubR1 is a target of SIRT2-dependent deacetylation, we examined the acetylation levels of BubR1. Exogenously expressed BubR1 in 293T cells was recovered in immunoprecipitates generated with an anti-BubR1 antibody and then analyzed by immunoblotting using anti-pan-acetyl-lysine antibody. We could not detect acetylated BubR1, possibly because there was not enough endogenous BubR1 acetylase to acetylate the amount of exogenous BubR1 present (Fig. 1B). It has been reported that BubR1 K250 is acetylated by PCAF specifically at prometaphase or when SAC is activated by nocodazole [9]. Thus, we next cotransfected 293T cells with BubR1 and PCAF and analyzed the acetylation levels of BubR1. As shown in Fig. 1B, we detected acetylated BubR1 at similar levels in the presence or absence of nocodazole. In parallel, the same sample was analyzed with mass spectrometry. However, we could not detect the presence of acetylated BubR1 K250, since the ionization efficiency was not sufficient to detect the peptide containing K250 (data not shown). Therefore, we examined BubR1 (K250R), a mutant version of BubR1 in which K250 is replaced with arginine, and found that the mutant was barely acetylated by PCAF (Supplementary Fig. 1). This result is consistent with the findings of Choi et al. that K250 is the main target lysine in BubR1 for PCAF-dependent acetylation.

Importantly, cotransfection of SIRT2 but not SIRT2 (ΔNDAC), a mutant version of SIRT2 lacking an NAD+-dependent deacetylase activity, decreased the acetylation levels of BubR1 (Fig. 1B). This result suggests that BubR1 K250 is a target of SIRT2-dependent deacetylation.

To obtain further evidence for the SIRT2-dependent deacetylation of BubR1, we performed in vitro deacetylation assays with purified FLAG-tagged SIRT2 and acetylated BubR1. The data demonstrate that SIRT2 deacetylates BubR1 in an NAD+-dependent manner (Fig. 1C). Taken together, these data indicate that SIRT2 deacetylates BubR1 K250.

3.2. SIRT2 knockdown leads to elevated acetylation levels of endogenous BubR1 in cells arrested at prometaphase by nocodazole

We next sought to determine whether SIRT2 deacetylates BubR1 in vivo, particularly during SAC activation. We transfected HCT116 cells with either siRNA against SIRT2 or a negative control siRNA and then collected the transfected cells at prometaphase by treating them with nocodazole. The optimal length of nocodazole treatment was determined to be 16 h because SAC activation, as monitored by Cyclin B1/Cdk1 activity and MPM-2 positivity (which indicates mitotic cells), peaked in both SIRT2 knockdown cells and control cells after 16 h of treatment [6,7]. We found that BubR1 isolated from cells treated with nocodazole gave a retarded mobility shift due to phosphorylation, as previously reported [16], and that, consistent with a previous report, the total levels of BubR1 as well as the acetylation levels of BubR1 in nocodazole-treated cells were higher than those in cycling cells [9] (Supplementary Fig. 2). Notably, we observed that the level of BubR1 acetylation after 16 h of nocodazole treatment was higher in SIRT2 knockdown cells than in control cells (Fig. 2). This result supports the hypothesis that SIRT2 regulates levels of BubR1 acetylation.

On the other hand, total levels of BubR1 protein were comparable between the two samples (Fig. 2). This suggests that the elevated level of BubR1 acetylation observed in SIRT2 knockdown cells does not affect the stability of endogenous BubR1 in cells arrested at prometaphase by nocodazole. Given that the SAC prevents APC/C-dependent proteolysis, which is responsible for BubR1

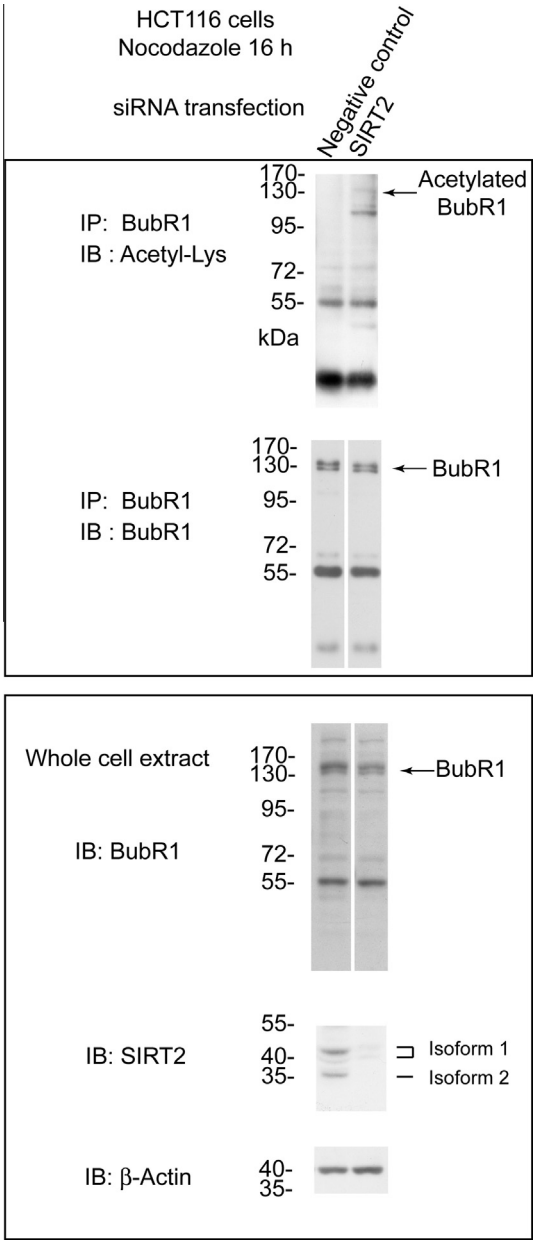

Fig. 2. SIRT2 knockdown leads to elevated levels of BubR1 acetylation. HCT116 cells were transfected with SIRT2 siRNA or the negative control siRNA and then cultured for 16 h in the presence of nocodazole (200 nM). Whole cell lysates derived from approximately 20 10 cm dishes (at 70% confluence) were immunoprecipitated using anti-BubR1 antibody and then subjected to immunoblotting using an anti-acetyl-lysine antibody. The levels of total BubR1 and SIRT2 in the immunoprecipitates and whole cell extracts were also examined by immunoblotting. IB, immunoblotting; IP, immunoprecipitation.

degradation, it is not surprising that elevated levels of BubR1 acetylation does not lead to an increase of the total BubR1 at prometaphase.

3.3. Elevated levels of BubR1 acetylation caused by SIRT2 knockdown do not affect BubR1 stability or the timing of anaphase entry

The data shown in Figs. 1 and 2 support the hypothesis that the abnormally prolonged SAC activation observed in SIRT2 knockdown cells may be mediated by BubR1 acetylation and the change in BubR1 protein levels. Thus, we examined whether the elevation of BubR1 acetylation levels by SIRT2 knockdown contributes to increased stability in BubR1 particularly during

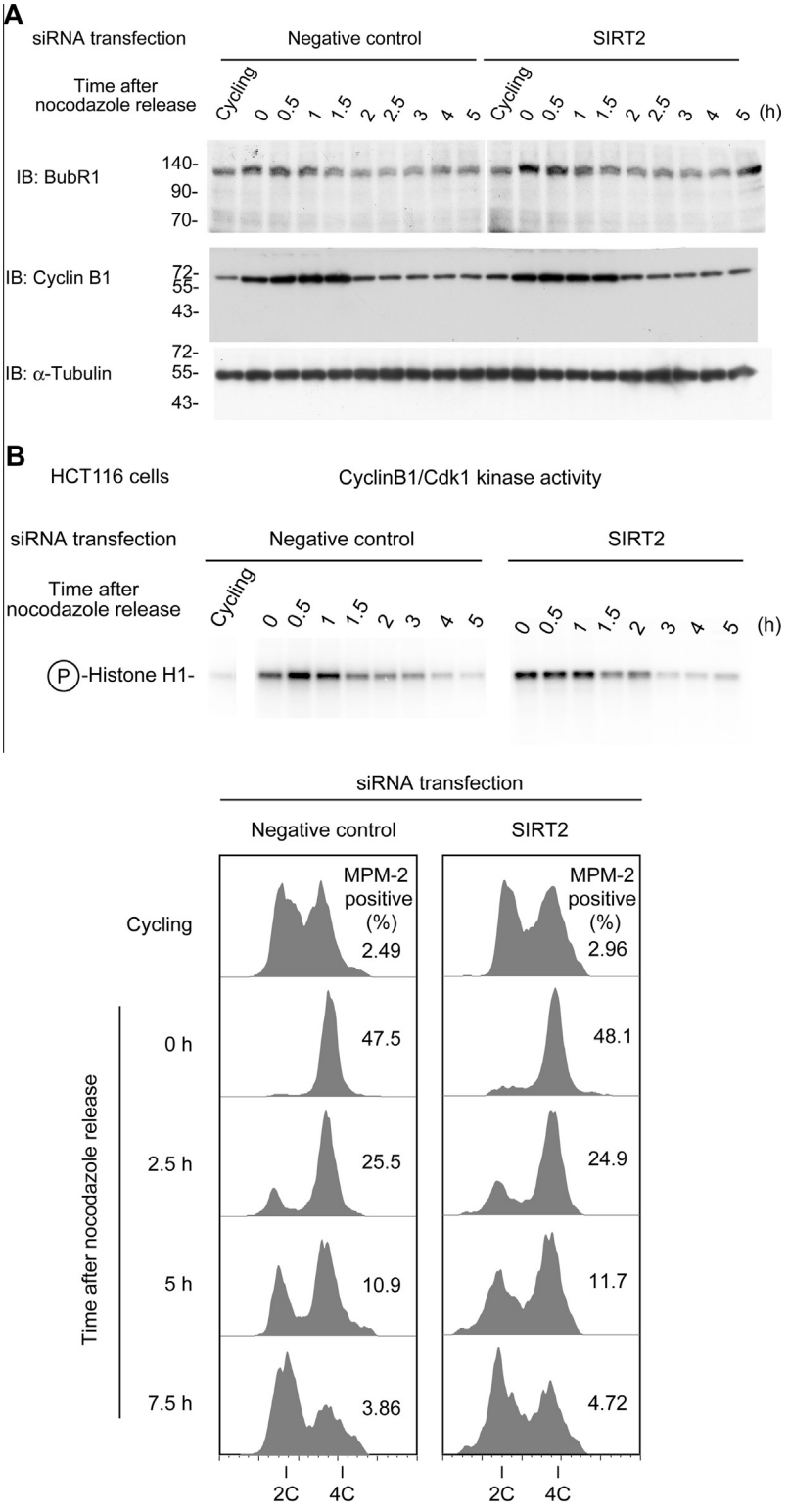

Fig. 3. Elevated levels of BubR1 acetylation observed in SIRT2 knockdown cells do not affect BubR1 stability or the timing of anaphase entry. (A) HCT116 cells were transfected with SIRT2 siRNA or the negative control siRNA and then cultured for 16 h in the presence of nocodazole. Prometaphase-arrested cells were collected by mechanical shake-off, released from the arrest by washing out the nocodazole, and harvested at the indicated times. Whole cell lysates were subjected to immunoblotting using an anti-BubR1 antibody and an anti-Cyclin B1 antibody. (B) Cyclin B1/Cdk1 kinase activity and cell cycle stage after nocodazole release. Cyclin B1-associated kinase activity was measured in Cyclin B1 immunoprecipitates from cell extracts prepared at the indicated times. Histone H1 was used as a substrate. Cell cycle progression was assessed by propidium iodide staining. The positivity of MPM-2 is also shown. IB, immunoblotting; IP, immunoprecipitation.

the prometaphase to anaphase transition, when APC/C-mediated degradation of BubR1 normally occurs, and whether, in that case, the transition from prometaphase to anaphase is reciprocally delayed.

To address these questions, we transfected HCT116 cells with SIRT2 siRNA or negative control siRNA. Next, we obtained nocodazole-arrested mitotic cells by mechanical shake-off, and then released the cells from mitotic arrest by washing out nocodazole.

The cells were then harvested at the indicated time points and analyzed for BubR1 levels (Fig. 3A). Cell cycle stages were determined according to Cyclin B1 levels, Cyclin B1/Cdk1 kinase activity (a biochemical marker for SAC activation), and MPM-2 positivity (a biochemical marker for prophase, prometaphase, and metaphase; lost after anaphase onset; Fig. 3B). BubR1 levels decreased during the transition from metaphase arrest in the control cells, as reported previously [9]. BubR1 level observed in nocodazole-treated SIRT2 knockdown cells was comparable to that observed in nocodazole-treated control cells; this result is consistent with those shown in Fig. 2. Notably, similar declines in total BubR1 levels observed over time after nocodazole release were observed in both SIRT2 knockdown cells and control cells. In addition, the prometaphase to anaphase transition, as monitored by Cyclin B1 levels, Cyclin B1/Cdk1 activity, and MPM-2-positivity, showed that the kinetics of SAC termination in SIRT2 knockdown cells and control cells were comparable (Fig. 3A and B), suggesting that timing of anaphase entry is comparable between the two samples.

Thus, the elevated levels of BubR1 acetylation observed in SIRT2 knockdown cells does not affect the level of total BubR1 during the transition from prometaphase to anaphase, as well as at prometaphase arrest. In other words, changes in levels of total BubR1 do not seem to be involved in the regulation of SAC by SIRT2. Although SIRT2 deacetylases K250 in BubR1 both in vitro and in vivo, deacetylation of BubR1 does not attenuate BubR1 stability during SAC activation or during prometaphase arrest.

We previously reported that SIRT2 knockdown causes mitotic arrest with abnormally prolonged SAC activation in the presence of nocodazole [6]. The results of the present study suggest that the abnormally prolonged mitotic arrest observed in SIRT2 knockdown cells in the presence of nocodazole is due to a delay in triggering SAC termination, but not due to a delay in progression after exiting from prometaphase arrest.

4. Discussion

We previously identified SIRT2, an NAD+-dependent deacetylase, as a non-canonical SAC regulator. When cells were exposed to nocodazole, SAC activation normally occurs in SIRT2 knockdown cells, with the same kinetics and levels as in control cells. However, SIRT2 knockdown leads to an abnormally prolonged SAC activation and confers resistance to microtubule inhibitors by inhibiting post-slippage death [6]. In the present study, we examined the possibility that SIRT2 serves this function via deacetylating and decreasing the stability of BubR1, a central SAC protein, for which acetylation at K250 inhibits ubiquitination-dependent proteolysis that controls the timed degradation of BubR1 and the correct timing for anaphase entry [9]. Our present study clearly showed that SIRT2 deacetylates BubR1 at K250 both in vitro and in vivo. However, although we observed that the levels of BubR1 acetylation increased in cells with SIRT2 knockdown, the elevated levels of BubR1 acetylation do not affect the levels of total BubR1 protein present at prometaphase or the kinetics of the decrease in total BubR1 levels observed during the transition from prometaphase to anaphase. Consistent with this result, the kinetics of the prometaphase to anaphase transition as assessed by Cyclin B1/Cdk1 activity and MPM-2 positivity were comparable between SIRT2 knockdown cells and control cells. Therefore, changes in the total level of BubR1 protein and in the regulation of the transition from prometaphase to anaphase are not involved in the regulation of SAC by SIRT2.

These observations do not contradict the findings of Choi et al. that the acetylation status of BubR1 regulates its degradation and the timing of anaphase entry [9]. The most plausible explanation for our result is that SIRT2 knockdown does not achieve BubR1 acetylation levels high enough to contribute to BubR1 stabilization. Choi et al. have reported that treatment of cells with trichostatin A, an inhibitor of the class I and II mammalian histone deacetylases (HDACs) but not class III HDACs such as SIRT1-7, resulted in the stabilization of BubR1 levels. Although inhibitors of the class III HDACs were not examined in their study, their results suggest that as-yet unidentified deacetylases belonging to the class I and II HDACs are more potent regulators of the deacetylation and destabilization of BubR1 than SIRT2 is.

We cannot explain the biological significance of the deacetylation of BubR1 K250 by SIRT2. Recently, it has been reported that BubR1 lysine 668 (K668) is acetylated by the acetyltransferase CBP and deacetylated by SIRT2 [17]. Unlike the acetylation of BubR1 K250, acetylation at K668 in BubR1 promotes the ubiquitination and degradation of BubR1. Taken together with the results of our present study, this finding raises the possibility that total BubR1 levels are regulated in a complex manner by PCAF, CBP, and SIRT2 via the acetylation status of K250 and K668. If both K250 and K668 of BubR1 are targets of acetylation/deacetylation in SAC regulation, elevated levels of BubR1 acetylation in response to SIRT2 knockdown may not necessarily lead to an increase in the total levels of BubR1. Although quantitation of the degrees of acetylation in both K250 and K668 of BubR1 is necessary, this may be an alternative model to explain why the elevated levels of BubR1 acetylation observed in SIRT2 knockdown cells do not modulate levels of total BubR1. Although North et al. have reported that SIRT2 knockdown and overexpression leads to an increase and decrease, respectively, of BubR1 in HeLa cells [17], we did not observe a significant change in the level of total BubR1 in response to SIRT2 knockdown and overexpression in HCT116 cells (Fig. 3 and unpublished observations). Thus, BubR1 lysine residues targeted for acetylation/deacetylation may differ among cell types.

Incidentally, we observed that the kinetics of SAC inactivation from nocodazole release, as monitored by Cyclin B1/Cdk1 activity and MPM-2-positivity, were comparable between SIRT2 knockdown cells and control cells. Thus, it is logical to conclude that the sustained SAC activation observed in SIRT2 knockdown cells in the presence of nocodazole is mediated by a delayed triggering of SAC termination rather than a delayed progression from prometaphase to anaphase.

A question remains to be answered: how does SAC termination in the presence of nocodazole delayed in SIRT2 knockdown cells? One possibility is that any functional changes accompanied by changes in BubR1 acetylation status may participate in a delayed triggering of SAC termination. For example, Park et al. using knock-in technology, generated an acetylation-defective BubR1 allele in mice via substitution of the K243 residue, which corresponds to K250 in human BubR1, with an arginine. The study reported that the acetylation of this site is important for BubR1 to counteract excessive Aurora B kinase activity at the KMT (KNL1/Mis12/Ndc80) network at the kinetochore, a process that is important for the stable maintenance of the kinetochore–microtubule interaction. Thus, it should be clarified whether this process is also present in SIRT2 knockdown cells and whether it would lead to a delay in the triggering of SAC termination.

Moreover, our recent study demonstrated that SIRT2 regulates the duration of SAC in the presence of nocodazole by suppressing basal autophagy levels [7]. It has been proposed that SIRT2 suppresses autophagy via the SIRT2–FoxO1–ATG7 axis [18]. We are currently investigating pathways and molecules controlled by autophagy that participate in SAC termination in the presence of nocodazole.

Competing interests

The authors declare that they have no competing interests.

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.2014.09.128.

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