HDAC2/3 binding and deacetylation of BubR1 initiates the spindle assembly checkpoint silencing

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Abbreviations

SAC, spindle assembly checkpoint; HDR, homology-directed repair; MCC, mitotic checkpoint complex; HDAC, Histone deacetylase; NEBD, nuclear envelope breakdown; NAD, nicotinamide adenine dinucleotide; KT-MT, kinetochore-microtubule; HDACi, HDAC inhibitors; MTT, 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide

Abstract

BubR1 acetylation is essential in spindle assembly checkpoint (SAC) signaling. Here we show that BubR1 deacetylation is a signal that initiates the mitotic exit. Sustained BubR1 acetylation arrests the cells in metaphase, although chromosome congression is achieved. BubR1 deacetylation was coordinated with dephosphorylation in mitotic exit, suggesting the presence of a coordinated acetylation-phosphorylation code in mitotic signaling. HDAC2 and 3 bound to acetylated BubR1 exclusively in mitosis and led to the polyubiquitination of BubR1. Subsequent degradation of BubR1 resulted in the disassembly of the mitotic checkpoint complex (MCC). Importantly, BRCA2 was required for HDAC2/3 association with acetylated BubR1 in nocodazole-arrested cells. Plk1, PP2A, PCAF, and BubR1 were found in mitotic BRCA2 complex, suggesting that BRCA2 acts as a signaling scaffold for BubR1 modification. Furthermore, we show that Plk1 is required for BRCA2 to localize at the prometaphase kinetochore. Inhibition of Plk1 resulted in the loss of BRCA2 from the kinetochore, and so did PCAF, consistent with the loss of BubR1 acetylation. Concordantly, BRCA2-dysfunctional cells exhibited resistance to Trichostatin A, which was restored when BRCA2 That loss of Brca2 conferred resistance to various HDAC inhibitors was was introduced.

corroborated by the experiments in mouse pancreatic organoids. These results suggest that BRCA2-BubR1 acetylation/deacetylation pathway is an important decision-making point for HDACi response. Taken together, BRCA2 is a signaling platform for BubR1, and BubR1 deacetylation is a cue to SAC silencing.

Introduction

Individuals with germ-line mutations of the breast cancer susceptibility gene *BRCA2* are predisposed to early-onset cancer. BRCA2-deficient cells exhibit genetic instability [1] and chromosome instability as well [2], manifested by the accumulation of DNA lesions, chromosome breaks, radial chromosomes, translocations, and aneuploidy [3, 4]. It is now revealed that BRCA2 is a multifaceted protein, functioning throughout the cell cycle: it is involved in homology-directed repair (HDR), stalled replication fork protection, telomere homeostasis, and mitotic fidelity [4].

BRCA2-deficient cells display chromosome missegregation, which can be explained by the role of BRCA2 in fine-tuning the spindle assembly checkpoint (SAC). It recruits PCAF acetyltransferase that acetylates BubR1 in mitosis at a single lysine (K250), which is adjacent to D-box and KEN motif degrons [5]. This activity is critical in modulating APC/C activity in mitosis [6]. Disruption of the interaction between endogenous BRCA2 and BubR1 in mice by the ectopic expression of the BubR1-binding domain of BRCA2 results in tumorigenesis without apparent HDR defect [5], suggesting that chromosome missegregation can be oncogenic.

Concordantly, mice heterozygous for the acetylation-deficient *BubR1* allele (substitution of the lysine 243 [*K*250 in human] to arginine) exhibit similar level and spectrum of tumorigenesis, when compared to transgenic mice disrupted of endogenous BRCA2-BubR1 interaction [5], without additional genotoxic insults [7]. The results from the two different mouse models that interfered with K250 acetylation of BubR1 confirmed that BubR1 acetylation represents one axis of BRCA2's tumor

suppressor mechanism. Moreover, further analysis of the mice heterozygous for BubR1 acetylation (K243R/+) taught us that BubR1 acetylation has dual roles: maintenance of the SAC and stabilization of chromosome-microtubule attachment [7].

Any chromosomes unattached with the spindle in prometaphase generates "wait anaphase signal", which activates the SAC that ultimately inhibits anaphase promoting complex/ cyclosome (APC/C) [8]. SAC signal is initiated by Mad1 and Mad2 localization to the unattached kinetochore. Mad2 undergoes a conformational change from open Mad2 to a closed Mad2, then forms a complex with BubR1, Bub3, and Cdc20, constituting the MCC [9, 10]. Sustained MCC binding to APC/C inhibits metaphase-anaphase transition, and thus maintains SAC until satisfied.

Silencing of SAC is the start of chromosome segregation, as APC/C becomes active, ubiquitinating cyclin B and securin for destruction. Compared to the mechanism of SAC signal generation and MCC formation, the signal that initiates SAC silencing is relatively less defined. As premature APC/C activation will lead to anaphase onset with unattached chromosomes, the mechanism that controls SAC silencing is essential for chromosome integrity. One important mechanism that is involved in exiting from mitosis is the role of TRIP13 [11-13] and p31(comet). TRIP13 and p31(comet) are involved in disassembling MCC by extracting Mad2 from MCC, and thus is essential for SAC silencing [14, 15].

In addition to the role of TRIP13 and p31-comet in extracting Mad2 for SAC inactivation, *in vitro* biochemical study showed that destruction of BubR1 silences SAC [16]. Importantly, MCC was not maintained in Mouse Embryonic Fibroblasts (MEFs) from K243R/+ mice because acetylation-deficient BubR1 was readily recognized by APC/C and destructed in mitosis, resulting in the premature onset of anaphase with unequal chromosome segregation [7]. This result suggested that deacetylation of BubR1, which leads to the destabilization of BubR1, may be a cue to mitotic exit.

Here, we asked if specific deacetylases were involved in deacetylation of BubR1 in mitosis and silencing of SAC. Then we asked whether BRCA2 was involved; what made BRCA2 function in mitosis. Finally, we asked whether the results have clinical implications in treating BRCA2-deficient cancers.

Results

Sustained acetylation of BubR1 results in metaphase arrest, although SAC is satisfied

We asked if deacetylation of BubR1 could be a cue for SAC silencing. For this, mitotic progression in HeLa cells expressing wild-type, acetylation-deficient (*K250R*), and acetylation mimetic form (*K250Q*) of *BubR1* were compared and analyzed. To assess the effect of the various forms of ectopically expressed BubR1, endogenous *BubR1* expression was silenced by siRNA that targets 3'UTR, and mCherry-tagged *BubR1* expression plasmids were co-transfected.

BubR1 acetylation deficiency results in premature degradation in mitosis through APC/C-Cdc20mediated ubiquitination [6]. When the mitotic timing was measured from nuclear envelope breakdown (NEBD) to the anaphase onset, we corroborated that K250R expression leads to shortened mitosis and K250Q expression results in prolonged mitosis, (Fig. 1A & D, see the time point marked with an arrow, Supplemental movie 4 & 5). Interestingly, when Mps1 inhibitor Reversine [17] was treated, delay in mitosis by the expression of K250Q was eliminated (Fig. 1A), indicating that BubR1 acetylation is not just a blockade of ubiquitination and degradation but is a cue that is coordinated with Mps1 kinase activity in SAC signaling (Fig. 1A, + Reversine).

Importantly, *K250Q* expressing cells stayed in metaphase with congressed chromosomes for 45 minutes longer than WT, and 55 minutes longer than *K250R*-expressing cells (Fig. 1B & D, see bold lines, movies 3-5). It should be noted that depending on the expression level of WT or K250Q, the timing in metaphase was affected in individual cells. Nevertheless, the statistical analysis of more

than 60 cells (Fig. 1B) confirmed that K250Q expression achieves and maintains chromosome congression, even when checkpoint was maintained [7]. The congression of chromosomes in K250Q-expressing cells was sustained while being delayed in metaphase (Fig. 1D, Movie 5). This phenomenon may be the consequence of stabilized kinetochore (KT)-microtubule (MT) attachment by the maintenance of acetylated BubR1 [7]. Consistent with this, expression of mutant BubR1 defective in phosphorylation at the KARD domain, which is phosphorylated by Plk1 and recruits PP2A-B56 α to counteract Aurora B activity in KT-MT attachment [18], resulted in delayed mitosis (Fig. 1C) with problems in chromosome congression (Fig. 1D, BubR1-KARD3A). This data is consistent with the previous finding [19].

Expression of K250Q leads to sustained chromosome congression, yet chromosomes do not segregate. To corroborate that the congression is achieved upon K250Q expression, the movement of CENP-B, the centromere-binding protein located beneath kinetochore [20], was monitored. mCherry-tagged *CENP-B* was co-transfected in HeLa cells stably expressing Venus-tagged *BubR1* (Movie 7), - *K250R* (Movie 8), -*K250Q* (Movie 9), respectively, and the kinetochore alignment was monitored. CENP-B alignment in metaphase was prolonged in *K250Q*-expressing cells (Fig. 1E, Movie 12), compared to *WT-BubR1*-expressers (Fig. 1E, Movie 10). In contrast, CENP-B in *K250R*-expressing cells hardly aligned (Fig. 1E, Movie 11). Interestingly, BubR1 at the kinetochore diminished before the completion of metaphase, as assessed by CENP-B segregation (Fig. 1E, Venus). The fluorescence intensity of acetylation-mimetic form (K250Q, Movie 9) was stronger and remained aligned at kinetochore longer than that of the WT. K250R was never aligned and disappeared from the kinetochore faster (Fig. 1E, Movie 8) than the WT (Fig. 1E, Movie 7). In this experimental setting, expression of K250Q eventually led to degradation of BubR1 from kinetochore and exited from mitosis (Fig. 1E, Movie 9).

The effect of K250Q expression is similar to the cells lacking p31-comet, which functions in SAC silencing by titrating out MCC component Mad2 [14, 15, 21], or APC15, which is required for rapid turn-over of APC coactivator Cdc20 [22]. Taken together with the information from mice heterozygous for the acetylation-defective *BubR1* allele (K243R/+) [7], BubR1 deacetylation may be another mechanism for SAC silencing that leads to MCC disassembly.

BubR1 deacetylation by HDAC2/3 leads to the ubiquitination of BubR1 and mitotic exit.

Treatment of mitotic cells with Hesperadin, the inhibitor for Aurora B [23], or Mps1 inhibitor Reversine [17] results in mitotic exit. To test the hypothesis that the deacetylation of BubR1 may be a cue for mitotic exit, we asked whether inhibitor for Aurora B or Mps1 treatment affected BubR1 acetylation. HeLa cells were synchronized in prometaphase by single thymidine block and release, followed by 10 μ M Taxol treatment. Mitotic cells were enriched by mechanical shake-off (Fig. 2A, 0h) and then released to progress into cell division by changing with fresh media. Cell lysates for Western blot (WB) were collected in 0, 1.5, 3 h, respectively. WB analysis with an antibody specific to acetylated BubR1 (anti-AcK250) [6] confirmed that BubR1 in cells arrested in mitosis were acetylated as expected (Tax, 0h, see the level of phospho-histone H3, ph3, compared to Att).

Aurora B inhibition (+Hesperadin) or Mps1 inhibition (+Reversine) after taxol treatment led to the increase of dephosphorylated BubR1 (Fig. 2A, 2nd row, BubR1). In these cases, BubR1 acetylation markedly disappeared (Fig. 2A, Hes & Rev, 3h). Cyclin B (Fig. 2A, cyclin B) and phospho-Histone H3 were markedly reduced (Fig. 2A, ph3) upon Hesperadin or Reversine treatment, confirming that cells are exiting from mitosis.

Treatment of 10 µM of MG132 resulted in the maintenance of BubR1 acetylation (Fig. 2A, MG132). Cyclin B level persisted upon MG132 treatment (Fig. 2A, cyclin B), indicating that the cells are arrested in metaphase. When Hesperadin or Reversine was added along with MG132, acetylation of BubR1 was prolonged up to 1.5 h, but markedly diminished in 3 hours from release. Meanwhile, WB This article is protected by copyright. All rights reserved. to anti-BubR1 showed that dephosphorylated BubR1 increased. Owing to MG132 treatment, the total level of BubR1, cyclin B, and Cdc20 are maintained [22, 24], indicating that the cells are still in mitosis, regardless of Hesperadin or Reversine treatment (Fig. 2A, BubR1, cyclin B, Cdc20). Inhibition of Aurora B or Mps1 kinase results in the shutdown of kinetochore signaling and exit from mitosis [22, 25]. Therefore, the result suggests that BubR1 deacetylation is linked with dephosphorylation events and may function as a cue to mitotic exit.

Previous in vitro transfection experiments showed that class I HDACs bind to BubR1 [6]. However, the mitosis-specific association of deacetylases was not tested until now. We asked if class I HDACs bound to acetylated BubR1 in a mitosis-specific manner. For this, HeLa cells were treated or untreated with nocodazole and subjected to IP and WB. The result showed that HDAC2 and/or HDAC3 were capable of binding to BubR1 in nocodazole-treated mitotic cells, but not in untreated interphase cells. Interestingly, when Reversine was added to MG132 treated mitotic cells, the interaction between BubR1 and HDAC2/3 decreased, suggesting that HDAC2/3 bind to the acetylated BubR1 in mitosis. Note that the acetylated BubR1 is also decreased (Fig. 2B). A similar test was done for SIRT2, as reports had suggested that SIRT2, NAD+ (nicotinamide adenine dinucleotide)dependent deacetylase, is implicated in control of cell division [26, 27]. Also, SIRT2 was suggested to bind to BubR1 [28, 29]. However, we did not observe a mitosis-specific binding between SIRT2 and BubR1 (data not shown). It is possible that SIRT2 binding to BubR1 is independent of mitosis: it may be involved in interphase, regulating aging [28, 30]. SIRT2 deacetylation of lysine 668 is involved in aging [30], therefore supports the notion that the outcome of acetylation and deacetylation of these two sites are quite distinct [31]. However, we do not rule out the possibility that SIRT2 might be involved, because a report showed that SIRT2 also deacetylates K250 [29]. HDAC1 may function similarly in different cell types, as class I HDACs were all found to interact with BubR1 in transfection experiment [6].

degradation.

Then we asked if HDAC2 or HDAC3 association with BubR1 resulted in the ubiquitination of BubR1. Deacetylation (or unacetylated form) of BubR1 results in APC/C-mediated ubiquitination and degradation [6, 7]. Increasing amounts of FLAG-tagged HDAC2 or -HDAC3 expression vectors were transfected, and the mitotic cell lysates were subjected to IP followed by WB. The result showed that the expression of HDAC2 or HDAC3 resulted in marked increase of polyubiquitination in a dose-dependent manner (Fig. 2C). In input control, the blot showed that the level of acetylated BubR1 decrease with the increasing amount of HDAC2/3 (Fig. 2C, Ack250). Interestingly, total BubR1 level increased in the same blot, indicating that the decrease of acetylated BubR1 is not merely due to protein degradation. Also, the result suggests that there may be a feedback mechanism to replenish BubR1 when BubR1 is continuously deacetylated and degraded to balance the checkpoint activation and silencing. When HDAC2 or HDAC3 is depleted in nocodazole-treated cells, acetylated BubR1 level (AcK250) persisted, compared to control (Fig. 2D), corroborating that HDAC2/3 function in mitosis to deacetylate BubR1. These results confirm that HDAC2 and HDAC3 (class I HDACs) bind to acetylated BubR1 and deacetylate it, leading to poly-ubiquitination and subsequent

BRCA2 serves as a mitotic platform for BubR1 signaling

BRCA2 fine-tunes the SAC by acting as a scaffold for BubR1 and acetyl-transferase PCAF association at the kinetochore [5]. Therefore, we asked if BRCA2 was required for the association of HDACs to BubR1 as well. BRCA2 depletion led to marked decrease of acetylated BubR1 (AcK250) in nocodazole treated mitosis cells (Fig. 3A). As BubR1 is acetylated at K250 exclusively in mitosis, AcK250 was barely detected, and hence the depletion of BRCA2 had little effect in asynchronously growing cells (Fig. 3A, left panel). In IP and WB, the association between BubR1 with HDAC2 or HDAC3 decreased in mitosis. BubR1 association to HDAC2/3 was barely observed in asynchronous cells, and thus there was no effect of BRCA2 in interphase (Fig. 3A, right panel). Immunoprecipitation with monoclonal antibody specific to AcK250 (mAcBubR1) and subsequent WB confirmed that BRCA2 depletion in nocodazole arrested cells interfered with HDAC2/3 association with acetylated BubR1 (Fig. 2B).

When we examined the BRCA2 immune-complex, we found that BRCA2 was associated with BubR1, Plk1, and PP2A only in mitotic cells (Fig. 3C, Noc). In comparison, PCAF association to BRCA2 was found both in mitotic and non-mitotic cells (Fig. 3C). The previous report showed that PCAF binds to BRCA2 in interphase as well [32], which is consistent with the data shown here. However, it should be noted that the outcome of PCAF binding to BubR1 in interphase is quite different from mitotic binding. Acetylated BubR1 recruits PP2A-B56 α and antagonizes Aurora B activity on Ndc80, which stabilizes kinetochore-microtubule attachments for anaphase onset [7]. The finding that PP2A catalytic subunit and Plk1 were found in BRCA2 immune-complex, together with BubR1, suggests that all of these proteins may be found in a complex on the platform of BRCA2. We do not rule out the possibility that the proteins bind to mitotic BRCA2 independently. In this regard, this hypothesis needs further investigation.

Plk1 is required for BRCA2 to act as a mitotic scaffold for BubR1 acetylation

Entry into mitosis is regulated by timely controlled phosphorylation events by M phase kinases. As Plk1 phosphorylates BRCA2 at multiple sites [32, 33], and because Plk1 also phosphorylates BubR1 and stabilizes kinetochore-microtubule (KT-MT) attachment [18, 34], we tested whether Plk1 activity is required for BRCA2 to function in mitosis.

When immunoprecipitated with anti-BRCA2 antibody, Plk1 was found in a complex with BRCA2, as is BubR1, in nocodazole arrested HeLa cells (Fig. 4A, 0h). At this stage, BubR1 was acetylated (Fig. 4A, WB with anti-AcK250). The complex began to disappear when cells were released into mitosis (Fig. 4A, 30 minutes onwards). When nocodazole arrested cells were depleted of Plk1 by siRNA, BubR1 disappeared from BRCA2 immune-complex (Fig. 4B, BubR1), as is Plk1 (Fig. 4B, Plk1). Note that the acetylated BubR1 decreases in total cell lysates when Plk1 is depleted (Fig. 4B, input, This article is protected by copyright. All rights reserved.

Similarly, treatment of cells with BI 2536, a small molecule inhibitor specific for Plk1 [35-37], also failed to pull down BubR1 and Plk1 from BRCA2 immunoprecipitation (Fig. 4C, BI). Accordingly, BubR1 acetylation was lost upon BI 2536 treatment (Fig. 4B & C, anti-AcK250 in input control). Note that the BRCA2 immune-complex from nocodazole treated cells consistently involves BubR1 and Plk1 (Fig. 4C, Noc). Plk1 and BubR1 binding in mitosis is well established [18, 38, 39], and BRCA2 and Plk1 association is known, albeit in interphase cells [4, 32, 33]. The data shown here indicate a mitosis-specific binding of Plk1 and BRCA2. Therefore, the results altogether suggest the possibility that Plk1-BRCA2-BubR1 may form a prometaphase-specific tri-molecular signaling cascade.

It could be argued that inhibition of Plk1 interferes with entry into mitosis, therefore the loss of complex formation between BRCA2 and BubR1. Also, it needed to be assessed whether Plk1 affected BRCA2-BubR1 binding at prometaphase kinetochore. Cells were first nocodazole arrested, then challenged with BI 2536 for 1h and 4h, respectively. In immunoprecipitation, followed by Western blot, inhibition of Plk1 kinase activity led to loss of both Plk1 and BubR1 from the BRCA2 complex (Fig. 4D). Simultaneously, BubR1 acetylation was markedly reduced (Fig. 4D, input, AcK250).

Next, the localization of BRCA2 to kinetochores was assessed. Immunofluorescence assay coupled with metaphase chromosome spread showed that BI 2536 treatment abolished BRCA2 from the kinetochore (Fig. 4E, BI 2536), whereas nocodazole treated control cells displayed colocalization of BRCA2 and BubR1 at the kinetochore (Fig. 4E, Noc), as reported [5]. Co-treatment of BI 2536 with nocodazole (Noc + BI 2536) also abolished BRCA2 from the kinetochore. These data suggest that Plk1 is required for BRCA2 to localize at the kinetochore. It also showed that Plk1 inhibition interfered with the BRCA2 association with BubR1 at outer kinetochores, whereas BubR1 localization was not affected (Fig. 4E & F). Taken together, we postulate that the mitotic function of BRCA2 at kinetochore requires Plk1.

The observation that BRCA2 serves as a platform for modulating SAC signaling, particularly on BubR1, led us to think that BRCA2 status might affect the sensitivity to histone deacetylase (HDAC) inhibitors. To test this idea, cancer cell lines that possessed intact BRCA2 or absent with BRCA2 (Fig. 5A) were subjected to viability test, based on MTT (3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay after treatment with pan-HDAC inhibitor Trichostatin A (TSA) for 2 or 3 days. Capan-1, which is truncated with BRCA2 [40], exhibited far less sensitivity to TSA, compared to *BRCA2*-positive cell lines, HeLa, GM847, U2OS, and WI-38 (Fig. 5B & C). Meanwhile, Capan-1 displayed similar level of sensitivity to Plk1 inhibitor BI 2536, when compared to BRCA2-positive cells (Fig. 5D). These results suggest that the absence of BRCA2 imposed the cells to reduced sensitivity towards HDAC inhibitor (HDACi).

To corroborate that BRCA2 status specifies the sensitivity to HDACi, *BRCA2* was depleted from HeLa or WI-38 cells by transfection with siRNA. The result showed that the absence of BRCA2 significantly reduced the sensitivity to TSA, both in HeLa and WI-38 cells (Fig. 5E). Concordantly, ectopic expression of *BRCA2* in Capan-1 cells restored the sensitivity to TSA, comparable to HeLa cells (Fig. 5F & G). Taken together, these results suggest that the status of *BRCA2*, intact or dysfunctional, is an important factor in determining the sensitivity to HDACi. We suggest that this is because BRCA2 serves as the scaffold for mitotic signaling on BubR1 acetylation. As BRCA2 works downstream of Plk1, BRCA2 deficiency did not affect the sensitivity towards Plk1 inhibitor.

The result from the cancer cell lines could be argued for the different genetic backgrounds, therefore we decided to utilize the mouse pancreatic organoids to confirm that the *Brca2* status is related to the response to HDACi. Organoids are self-assembled 3-dimensional epithelial structures that are allowed to expand long term [41]. As dysfunctional BRCA2 is associated with pancreas cancer development [42], we decided to culture and tested the drugs in pancreatic organoids. Pancreatic organoids are regarded as stem cell cultures of the ductal epithelial cells [43]. We generated mouse organoids from conditional *Brca2* knockout mice (*Brca2*^{*F11/F11}) [5, 44] that was bred to ER-Cre-</sup>*

expressing transgenic mice (CAGGC-Cre). In this mouse, *Brca2* exon 11 can be deleted in a tamoxifen-inducible way. Mouse pancreas organoids were cultured with or without the treatment of 4-hydroxytamoxifen (4-OHT). To expand our scope of applicability in targeting the BRCA2-BubR1 complex with HDAC inhibitors, we used different types of HDAC inhibitors (Fig 6A, B). The result showed that *Brca2* mutant mouse organoids were resistant to pan-HDAC inhibitors TSA and 17 μ M of SAHA [45]. Unexpectedly, *Brca2*-deleted organoids were resistant to LMK-235, the HDACi reported to have effects on class II HDACs [46]. Control organoids were resistant to the treatment of depsipeptide FK-228 (10 nM) that has epigenetic effect and class I HDAC inhibition [47], while Brca2-depleted organoids responded mildly (Fig. 6A). The efficiency of the depletion of Brca2 in organoids after 4-OHT treatment was assessed by genomic PCR (Fig. 6B). The reason why Brca2-deficient organoids are resistant to class II HDACi, and less to class I type, is not fully understood. It could be because HDACi usually has pleiotropic effects as they are also involved in epigenetic modulation. Nevertheless, Brca2-deficient cells and organoids are resistant to HDACi.

Discussion

We showed that HDAC2/3 binds to acetylated BubR1 and leads to ubiquitination and degradation of BubR1. Acetylation-deficient BubR1, K250R, prematurely disappears from kinetochore, accompanied by chromosome missegregation. Meanwhile, K250Q expression leads to sustained BubR1 at kinetochore with prolonged metaphase. Therefore, HDAC2/3 binding and deacetylation of BubR1 at K250 lead to the disassembly of MCC, which is critical in SAC silencing and mitotic exit (Fig. 6C). SIRT2 deacetylase was also shown to bind and deacetylate K250 of BubR1 in vitro [29], but failed to show that SIRT2 expression led to BubR1 ubiquitination. As SIRT2 also acts on the C-terminus of BubR1 and functions in controlling aging [30], it is possible that SIRT2 preferentially functions in interphase, and only partially in mitosis. Nevertheless, with compelling lines of evidence, we suggest that HDAC2/3 binding and deacetylation of BubR1 is a signal to SAC silencing and mitotic exit. For BubR1 acetylation and deacetylation, BRCA2 is required. Furthermore, Plk1 is This article is protected by copyright. All rights reserved.

required for BRCA2 to localize at prometaphase kinetochores and bind to BubR1. Based on the presented data, it is reasonable to think that *BRCA2*-deficient cells will be relatively resistant to HDACi, while response to Plk1 inhibitor will not be affected. Indeed, that was the case. We have shown this in cultured cell lines and syngenic mouse pancreas organoids (Fig. 5 & 6A).

BubR1 links kinetochore-microtubule (KT-MT) attachment to SAC activity [48], and Plk1 phosphorylation on the KARD domain at the C-terminus of BubR1 is critical for the stabilization of KT-MT attachment [18]. When KARD domain is phosphorylated by Plk1, BubR1 can recruit PP2A-B56α to the kinetochore, which now antagonizes Aurora B kinase activity. As Aurora B destabilizes KT-MT attachment by phosphorylating KMN (Knl1-Mis12-Ndc80) network, which forms the platform for microtubule attachment at the kinetochore, Aurora B's kinase activity on KMN is essential for error correction [49, 50]. Now when amphitelic attachment is achieved, Plk1 phosphorylates BubR1, recruits phosphatase activity and antagonize Aurora B activity, stabilizing microtubule attachment to kinetochore (Figure 6C, illustrated in regular line arrow).

Interestingly, mouse cells deficient in BubR1 acetylation (K243R/+) exhibited failure in the stabilization of KT-MT attachment, accompanied by elevated Ndc80 phosphorylation and failure in recruiting PP2A-B56 α [7]. The result had suggested that BubR1 acetylation at the N-terminus cross-talks with the C-terminus phosphorylation. This idea that BubR1 acetylation cross-talks with phosphorylation was tested and proven in this study. BubR1 is deacetylated when cells exit from mitosis (Fig. 2A). When Aurora B or Mps1 inhibitor is treated with MG132, BubR1 is deacetylated, although the cells are in mitosis with sustained cyclin B level (Fig. 2A). From these results, we suggest an interesting module in SAC signaling that there may be a BubR1 phosphorylation-acetylation code, including deacetylation and dephosphorylation, which is also illustrated in the model (Fig. 6C).

It is interesting to note that BRCA2 immune-complex includes Plk1, BubR1, PCAF, and PP2A (Fig. 3C). How the complex is formed is not tested yet: whether they are in a complex together or undergo a dynamic change. Nevertheless, BubR1-HDAC2/3 binding in mitosis was lost without BRCA2. Therefore, we postulate that BRCA2 may be a signaling scaffold that brings acetylase, deacetylase, and phosphatase to BubR1. We suggest that BRCA2's participation in mitosis reinforces BubR1's function in mitosis, the stabilization of KT-MT attachment and SAC maintenance (Fig. 6C, bold arrow). Furthermore, BubR1 deacetylation is a cue for MCC disassembly. In prometaphase, Plk1 may act in both ways: directly phosphorylating BubR1 (Fig. 6C, regular arrows in left) and enabling BRCA2 to function in mitosis that reinforces BubR1 activity (Fig. 6C, bold arrow). Thus, Plk1-BRCA2-BubR1 forms a triangular signaling, ultimately leading to proper chromosome segregation (Fig. 6C).

Cancer cells are characterized by unlimited cell growth, and therefore the control of mitosis was suggested to be effective for cancer treatment. However, clinical trials of mitotic kinase inhibitors, including Plk1 inhibitors are controversial. On the other hand, HDAC inhibitors (HDACi) control cell division and fate but because they control epigenetic gene expressions at the global level, HDACi has pleiotropic effects [51]. It should be noted that HDACi target non-histone proteins as well, as shown here with BubR1. Interestingly, HDACi treatment often leads to mitotic arrest [52], suggesting that BubR1 may have been an important target. Various HDACi were developed and tested, but due to its pleiotropic activities on tumor cells and invading immune cells, clinical application is complicated. In this study, we showed that *BRCA2*-deficient cells exhibit reduced sensitivity to HDACi. Taking this information into account, assessing the status of *BRCA2* will be beneficial in HDACi application. Collectively, the results shown here have many implications in precision medicine.

Statistical analysis

SPSS software was used for all statistical analysis. Student's t test was used. Mean \pm SEM is shown wherever required.

Cell culture and Synchronization

HeLa cells were cultured in high glucose DMEM (10% FBS, 1% p/s) at 37°C, 5% CO₂. HeLa cells stably expressing H2B–GFP was provided by Toru Hirota (Japanese Foundation for Cancer Research, Japan). HDAC3-, BubR1-, K250R-, K250Q-inducible HeLa cells were generated by cloning the genes into pcDNA5/FRT/TO construct (a gift from J. Pines, Gurdon Institute, Cambridge, UK), and transfecting into HeLa-FRT/TO cells (a gift from S. Taylor), followed by selecting the proper clone as described [6, 7]. 1 µg/ml of doxycycline (Sigma-Aldrich, St. Louis, MO, USA) was treated to induce the expression of ectopic genes. To arrest the cells in S phase, 2.5 mM thymidine (Sigma-Aldrich, St. Louis, MO, USA) was used. After 16 hr, cells were washed twice with PBS and released into fresh media. Nocodazole (M1404; Sigma-Aldrich, St. Louis, MO, USA) of 200 ng/ml, 10 µM taxol (T7402; Sigma-Aldrich, St. Louis, MO, USA), 0.5 µM Reversine (R3904; Sigma-Aldrich, St. Louis, 200 nM Hesperadin (S1529; Selleckchem, Houston, Texas, USA), respectively, MO, USA), or together with 10 µM MG132 (474790; Calbiochem, La Jolla, CA, USA) was treated to enrich the cells in mitosis. Transfection of DNA constructs into cells was done using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) or microporation using Neon microporator (Invitrogen, Carlsbad, CA, USA) at a single pulse at 1300 V, 20 ms.

Constructs, antibodies, and siRNA

Various BubR1 constructs were generated by PCR and sub cloned into pcDNA3.1-mcherry for expression in cultured cells. K250R, K250Q *BubR1* mutants were generated by site-directed mutagenesis using pcDNA3.1-mcherry BubR1 as the template.

The following antibodies were purchased: anti-mCherry (ab125096; Abcam, Cambridge, UK), anti-Flag (M2; F1804, Sigma-Aldrich, St. Louis, MO, USA), anti-Cyclin B (H-433; Santa Cruz Biotechnology, CA, USA), anti-Mad2 (C-19; Santa Cruz Biotechnology, CA, USA), anti-PCAF (H369 and E-8; Santa Cruz Biotechnology, CA, USA), anti-Cdc20 (H-175; Santa Cruz Biotechnology, CA, USA), anti-beta actin (AC-15; Sigma-Aldrich), anti-BubR1 (612503; BD Biosciences, San Jose, CA, USA), anti-AcK250-BubR1 (polyclonal) [6], anti-AcK250-BubR1 (acetylated at K250; cRVGGAL-(Ac)K-APSQNRG, monoclonal), anti–PP2A C (BD Biosciences, San Jose, CA, USA), anti-CREST (90C1058; Cortex Biochem, San Leandro, CA, USA), anti-HDAC2 (ab7029, Abcam, Cambridge, MA, UK), anti-HDAC3 (ab7030; Abcam, Cambridge, MA, UK), anti-Plk1 (36-298; Abcam, Cambridge, MA, UK), anti-phospho-histoneH3 (06-570; Millipore, Billerica, MA, USA), anti-BRCA2 (Ab-1; Calbiochem/ Merck, Nottingham, UK), anti-BRCA2 (B2-4 antigen; custom made sheep polyclonal) and Alexa Fluor-657, -568, -488-conjugated secondary antibodies (Invitrogen).

The following siRNAs were used: HDAC2: 5'-GGAUUACAUCAUGCUAAGAUU-3'; HDAC3 3'UTR, 5'-GAGGACTACATTGACTTCCTGCAGA-3'; BubR1 3'UTR, 5'-GUC UCA CAG AUU GCU GCC U-3'; BRCA2: 5'- GAA GAA CAA UAU CCU ACU ATT-3'; Plk1 3' UTR: UUC CUU GGC UUU AUG CAC AUU

Microscope image acquisition, live imaging, and processing

Microscopic images of fixed cells were acquired with DeltaVision (GE Healthcare), equipped with a $100 \times$ objective lens (Olympus). The images were obtained with 0.2 µm distanced optical sections in z-axis. Each section was deconvoluted and projected into one image using the softWoRx software (Applied Precision) as previously described [6, 7]. For live cell imaging, cells were monitored using the UPlanFLN 40×/NA 1.30 oil lens on a microscope (Delta Vision; GE Healthcare) equipped with a charge-coupled device camera (Photometrics) in a CO₂ chamber at 37°C (Applied Precision). The cells were seeded in a glass-bottom dish containing culture media and images were acquired 3 or 5

min intervals as 2 sections with 7- μ m z steps using 40X, 1.35NA 0.10mm WD objective lens and maximally projected.

Immunoprecipitation (IP) and western blot (WB)

Cells were lysed in NETN buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris, pH 8.0, and 0.5% NP-40) supplemented with protease inhibitors for IP and WB analysis. Cells were incubated on ice for 20 min and supernatants were collected after centrifugation at 4°C, 1200 rpm. Cell lysates were precleared for 1 hr at 4°C with protein A/G beads and subjected to immunoprecipitation with indicated antibodies at 4°C overnight. Immune-complexes were precipitated by incubating with Protein A/G beads for 2 hr. Before elution with 3x SDS sample buffer, immunoprecipitated beads were washed 3 times with the lysis buffer.

Immunofluorescence on metaphase chromosome spreads

For chromosome spreads coupled with immunofluorescence assays, cells were incubated with 200 ng/ml nocodazole for 4 hr. To harvest the cells, media was removed and cells were washed with PBS, then pelleted. Cells were swelled in 0.2% KCl and 0.2% trisodium carbonate containing water and cytospinned at 850 rpm for 5 min. The slides were subjected to immunofluorescence assay as described [6].

Cellular Viability

Cells were seeded into 96-well plate (3 x 10³ cells/well) and incubated overnight. The following day, cells were treated with increasing concentration of Trichostatin A (T5882; Sigma-Aldrich, St. Louis, MO, USA) or BI 2536 (S1109; Selleckchem, Houston, Texas, USA) and incubated for 2 or 3 days. Cell viability was evaluated by MTT assay, using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, M2128; Sigma-Aldrich, St. Louis, MO, USA). The amount of dye formed was quantified using an ELISA plate reader at 540 nm.

Mouse pancreas organoid culture and HDACi treatment

Mouse protocol and animal experiment were approved by the Institutional Animal Care and Use of Committee (IACUC) of Seoul National University (SNU-130219-4-9). Mice pancreas organoids were isolated and cultured from conditional *Brca2* knockout mice, *Brca2^{F1//11}* [5, 44], that was crossed to ER-Cre mice (CAGGCre-ER, The Jackson laboratory, Maine). Mice pancreas was lysed in digestion solution containing collagenase and DNaseI and ductal cells were collected as described previously [41]. Collected ductal cells were washed with cold PBS and seeded on to matrigel matrix (Corning, NY, USA). Cells were supplemented with culture media composed of stem cell growth factors as described previously [41]. When cells started to form organoids, they were treated with 400 μ M 4-Hydroxytamoxifen (Sigma-Aldrich, St. Louis, MO, USA) to knock-out BRCA2 gene. Mouse organoids were re-plated onto 24 well plate and were treated with varying doses of Trichostatin A (Sigma-Aldrich, St. Louis, MO, USA), LMK-235 (Selleckchem, Houston, Texas, USA), FK-228 (Selleckchem, Houston, Texas, USA) and Suberoylanilide hydroxamic acid (Sigma-Aldrich, St. Louis, MO, USA). Organoids were imaged with Zeiss Axio Observer (Carl Zeiss AG, Oberkochen, Germany) after 5 days of drug treatment.

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Authors declare no conflict of interest.

Author Contributions

HL conceived, designed and coordinated the study, and wrote the paper. IP and M-SK designed, performed and analyzed the major part of the experiments. SP, HK, H-OL, EC performed experiments. All authors reviewed the results and approved the final version of the manuscript.

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Supporting Information

Movie 1. Time-lapse video microscopy of cells expressing H2B-GFP for Figure 1D, 1st row.

Movie 2. Time-lapse video microscopy of cells expressing H2B-GFP for Figure 1D, 2nd row.

Movie 3. Time-lapse video microscopy of cells expressing mCherry-BubR1-WT and H2B-GFP for Figure 1D, 3rd row.

Movie 4. Time-lapse video microscopy of cells expressing mCherry-K250R and H2B-GFP for Figure 1D, 4th row.

Movie 5. Time-lapse video microscopy of cells expressing mCherry-K250Q and H2B-GFP for Figure 1D, 5th row.

Movie 6. Time-lapse video microscopy of cells expressing mCherry-KARD3A [phosphorylation deficient (3A) mutant] and H2B-GFP for Figure 1D, 6th row.

Movie 7. Time-lapse video microscopy of BubR1 (green) in HeLa-FRT-BubR1-Venus cell line, transfected with mCherry-CENPB.

Movie 8. Time-lapse video microscopy of K250R (green) in HeLa-FRT-K250R-Venus cell line, transfected with mCherry-CENPB.

Movie 9. Time-lapse video microscopy of K250Q (green)in HeLa-FRT-K250Q-Venus cell line, transfected with mCherry-CENPB.

Movie 10. Time-lapse video microscopy of CENP-B (Red) in HeLa-FRT-BubR1-Venus cell line, transfected with mCherry-CENPB.

Movie 11. Time-lapse video microscopy of CENP-B (Red) in HeLa-FRT-K250R-Venus cell line, transfected with mCherry-CENPB.

Movie 12. Time-lapse video microscopy of CENP-B (Red) in HeLa-FRT-K250Q-Venus cell line, transfected with mCherry-CENPB.



Fig. 1. Expression of acetylation mimetic form of BubR1 leads to arrest in metaphase with prolonged chromosome congression. (A) The statistical timing of NEBD to anaphase onset upon expression of various forms of BubR1. Endogenous BubR1 was depleted with siRNA (siBubR1) that targets 3'UTR and mCherry-tagged BubR1 plasmids were transfected into HeLa cells, stably expressing H2B-GFP [6]. Cells were synchronized by thymidine block and then released for 6 hours. Reversine $(0.5 \,\mu\text{M})$ treated cases are marked. Cells were filmed with time-lapse microscopy in 5 min intervals for total of 24 hrs (Supplemental movie 1, siControl; movie 2, siBubR1; movie 3, WT-BubR1; movie 4, K250R-; movie 5, K250Q-, respectively). Each dot represents a single cell event, and the bars mark the mean values (mean \pm SEM; n> 50 cells in all cases). SPSS software was used and p values were obtained with student's t test (A-C). Right, WB analysis of endogenous and mCherry-tagged BubR1. Anti-Mad2 and anti-actin WB analysis show that the siBubR1 has little offtarget effect. (B) The graph is representing the timing from metaphase (congressed chromosomes) to anaphase onset (mean \pm SEM; n> 50 cells in all cases). (C) The statistical timing of NEBD to anaphase onset upon expression of KARD3A mutant form of BubR1 (mean \pm s.e.m; WT, n=10; KARD3A, n=28; p < 0.0001). (D) Captured images of representative cells expressing mCherrytagged WT-BubR1 (Movie 3), K250R- (Movie 4), or K250Q- (Movie 5), KARD3A- (Movie 6), depleted of endogenous BubR1. Images are shown from NEBD (nuclear envelope breakdown) to anaphase onset. Each frame is in 5 min interval. The duration of metaphase (B) is marked with a bold line, and the anaphase onset is marked with a red arrow. (E) The effect of BubR1 acetylation status in chromosome congression is assessed by centromere alignment, marked with CENPB. mCherry-tagged CENP-B [53] was transfected into HeLa-FRT-BubR1 (Movie 7 & 10), -K250R (Movie 8 & 11), -*K250Q* (Movie 9 & 12) -inducible cells. Expression of BubR1, K250R, K250Q, respectively, was induced by doxycycline treatment. Cells were subjected to time-lapse video microscopy in 3-minute intervals. The beginning of chromosome separation is marked with red arrows. Time 0, NEBD. Red, mCherry-CENP-B; Green, BubR1. Scale bar, 10 µm.





Fig. 2. BubR1 deacetylation acts as the signaling cue for SAC silencing. (A) HeLa cells were synchronized by thymidine block and arrested in prometaphase with taxol treatment, followed by mitotic shake off. Then they were released for mitotic progression in the presence of indicated drugs. Lysates were collected in 0, 1.5, and 3 h from arrest. Treatment with drugs during release is indicated. Western blot (WB) was performed with the antibodies indicated. Attached cells (Att) were employed for control. (B) HeLa cells were treated with nocodazole (Noc), followed by treatment with MG132 and Reversine (MG132 + Rev). The cell lysates were then subjected to IP and WB with the antibodies indicated (Right). Total cell lysates (2%) were subjected to WB for input control (Left). Untreated asynchronous cells (Asn) are included for control. Asynchronous cells immunoprecipitated with rabbit immunoglobulin (IgG) were employed for negative control. (C) Increasing amounts of FLAG-HDAC2- or HDAC3-expressing plasmid was transfected into 293 FT cells with Myc or HAtagged *ubiquitin*-expressing construct. Then the cells were arrested in mitosis by nocodazole treatment, followed by MG132 treatment. Lysates were then subjected to IP with anti-BubR1, followed by WB with 9E10 (anti-Myc) or anti-HA antibody. Same blot was reprobed for WB with anti-BubR1 (BubR1). Input control shows protein levels of acetylated BubR1 (AcK250), BubR1, and HDAC2/3. Fastest migrating band of BubR1 is marked (*) to compare with the upper migrating ladders of BubR1. The intensities of BubR1 and AcK250 in input were measured using the densitometer (Multi Gauge). The ratio of AcK250 /total BubR1, relative to control untransfected, is marked in numbers at the bottom of each lane. Levels of HDAC2 or HDAC3, relative to untransfected, is indicated as well. (D) HeLa cells were transfected with siRNAs for control (siLuciferase), HDAC2 (siHDAC2), or HDAC3 (siHDAC3). They were treated with nocodazole, followed by treatment with/without MG132. Then the cell lysates were subjected to WB.





Fig. 3. Depletion of BubR1 results in the loss of BubR1-HDAC2/3 binding. (A-B) HeLa cells were transfected with siRNA for control (siControl) or *BRCA2* (siBRCA2). Twenty-four hours later, asynchronous cells (Asn) or mitotic cells with nocodazole were harvested. Lysates were then subjected to IP with anti-HDAC2, anti-HDAC3, followed by WB with antibodies indicated. (B) Nocodazole treated cells were subjected to IP with monoclonal antibody to Ack250 (mACK250), followed by WB with antibodies indicated (C) Mitotic HeLa cells were collected by nocodazole treatment and shake-off. Asynchronously growing HeLa cells (Asn) and nocodazole treated cells (Noc) were subjected to IP with anti-BRCA2 antibody, followed by WB with antibodies indicated. IP with rabbit IgG was included for negative control. Three percent of the total cell lysates were employed for input control.



Fig. 4. Plk1 activity is required for the recruitment of BRCA2 to kinetochore and BubR1

acetylation. (A) HeLa cells were synchronized at prometaphase with nocodazole, and released into the mitotic cell cycle. Cells were harvested at 30 min intervals and subjected to IP with anti-BRCA2 antibodies and WB with the indicated antibodies. (B) HeLa cells transfected with control (siGFP) or *Plk1* siRNA (siPlk1) were nocodazole arrested. Then the cell lysates were subjected to IP and WB as in *A*. (C) HeLa cells were treated with nocodazole or BI 2536, and subjected to IP with anti-BRCA2 antibodies and WB with the indicated antibodies. (D) HeLa cells were nocodazole arrested, followed by incubation with BI 2536 for 1 or 4 hours. Cells lysate were then subjected to IP and WB as indicated. (E & F) Chromosome spreads from HeLa cells treated with nocodazole, BI 2536, or Nocodazole + BI 2536, were obtained under non-denaturing condition. Chromosome spreads were co-immunostained with CREST, anti-BRCA2, anti-BubR1 antibodies, respectively, and the chromosomes were counterstained with DAPI. Enlarged images of the insets are shown at the right. Merged image of Chromosome, BubR1, BRCA2 are shown. Green, BubR1; Red, BRCA2. Photographs were taken at a 1,000X magnification under a DeltaVision RT. (F) Co-localization of BubR1 and BRCA2 were evaluated in each group and indicated in the bar graph (mean \pm s.e.m; n=400 kinetochores; p < 0.001). Scale bar, 5µm.



Fig. 5. BRCA2-deficient cells are resistant to HDACi. (A) Assessment of BRCA2 in HeLa, U2OS, GM847, WI38 VA13/2R (WI-38) and Capan-1 cells by WB using anti-BRCA2 antibody. Same blot was reprobed with anti-Lamin A/C for loading control. (B-C) MTT cell viability assays were performed after treatment of TSA in increasing concentrations. Cell viability is represented as percentage of viable cells, compared to that of the control (0 nM). (D) MTT cell viability assays against BI 2536. (E) HeLa and WI-38 cells were transfected with siRNA for *BRCA2* or control (siLuciferase) and subjected to MTT assay after treatment with TSA (Top). WB with anti-BRCA2 was performed to assess the level of BRCA2 after depletion (bottom). (F) *BRCA2*-expression plasmid (*pEGFP-N1-BRCA2*) or control (*pEGFP-N1*) were transfected in Capan-1 and the cells were subjected to MTT assay after treatment with TSA. (G) Bar graph representing the MTT assay in (F) (Left). WB analysis of expression of *BRCA2* in (F-G). Results (B, D, E, F) are from eight replicates (mean \pm s.e.m). P values (C, G) were obtained with student's t test.



Fig. 6. HDACi treatment in mouse pancreatic organoids corroborates that Brca2-deficient cells are resistant to HDAC inhibitors. (A) Response to various HDAC inhibitors in pancreatic organoids derived from transgenic mouse harboring BRCA2 conditional knockout mice (*BRCA2^{F11/}*^{F11}). The *BRCA2^{F11}* allele was conditionally deleted by treating 4-hydroxytamoxifen (4-OHT) to organoids. Growing mouse pancreatic organoids were treated with vehicle (DMSO, NT), 500 nM trichostatin A (TSA), 17 µM suberoylanilide hydroxamic acid (SAHA), 3 µM LMK-235 and 100 nM FK-228 for 5days. Pictures were taken 5 days post treatment. (B) Conditional depletion of BRCA2 allele (*BRCA2^{F11/F11}*) with 4-OHT treatment in pancreatic organoids were assessed with PCR using the genomic DNA. (C) Working model. For BRCA2 to function in mitosis, Plk1 is required. Phosphorylated BRCA2 can now localize to the kinetochore, bringing PCAF to BubR1 for acetylation. Acetylated BubR1 can maintain SAC and also stabilizes KT-MT attachment. When SAC is satisfied, HDAC2/3 binds to BubR1 and deacetylates it, leading to ubiquitination and degradation. BubR1 degradation leads to disassembly of MCC and SAC silencing.