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Original article

Short-term effect of American summer treatment program for Japanese children with attention deficit hyperactivity disorder

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Abstract

We reported the results of the 3-week summer treatment program (STP) for children with attention deficit hyperactivity disorder (ADHD) in 2006. The STP was based on methods established by Professor Pelham in Buffalo, NY and has been used in a number of studies and at a number of sites in the U.S. This is the first STP outside North America. Thirty-six children age 6–12 years with ADHD participated. The collection of evidence-based behavioral modification techniques that comprises the STP's behavioral program (e.g., point system, daily report card, positive reinforcement, time out) was used. Most children showed positive behavioral changes in multiple domains of functioning, demonstrated by significant improvement in points earned daily, which reflect behavior frequencies. Only one child with ADHD co-morbid with pervasive developmental disorder required an individualized program for excessive time outs. The ADHD rating scale, symptoms of oppositional defiant disorder, and hyperactivity/inattention in Strength and Difficulties Questionnaires evaluated by parents significantly improved after STP. Although the 3-week STP was much shorter than most STPs run in the U.S., the program is more intensive than typical outpatient treatment, providing 105 h of intervenion in 3 weeks. The short-term effect of the STP was demonstrated for Japanese children with ADHD.

Keywords: Attention deficit hyperactivity disorder; Psychosocial treatment; Behavior modification; Summer treatment program

1. Introduction

Treatment of attention deficit hyperactivity disorder (ADHD) in children has become a national priority in Japan [1]. Particularly, the management of behavioral problems in children with ADHD is a significant issue for teachers and health professionals in Japan. It is clear from a great deal of research that there are three evidence-based short-term treatments for ADHD—medication with a CNS stimulant, behavior modification, and the combination of the two [2,3]. In the U.S. there is widespread agreement that multimodal treatment is best for many if not most ADHD children. However, providing comprehensive treatment for such children is difficult in the Japanese hospital setting. This is largely due to the shortage of specialists who are trained in the provision of evidence-based psychosocial treatments, and the lack of communication between the fields of medicine, psychology, and education. Miyamoto recently administered a questionnaire survey to 486

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parents who have children with ADHD [4]. Thirty-one percent of the parents who responded were not satisfied with medical service, because treatment was mainly medication and evidence-based psychosocial treatments were not commonly provided. This finding regarding parental preference for psychosocial or combined treatment is common among parents in the U.S. [5]. Clearly, we need to establish comprehensive approaches to intervention for ADHD in Japan.

A recent review of psychosocial treatments for ADHD has concluded that there is substantial support for three behavioral approaches to ADHD—parent training, school intervention, and child-focused interventions [3]. With respect to the latter, the only evidence-based, child-focused approach is intensive summer programs focused on peer relationships. The STP was developed to treat ADHD children's difficulties in peer relationships in an intensive summer experience that focuses on learning sports skills and social skills, improving follow-through with adult requests and commands, as well as improving academic achievement. The American STP usually last for 5–8 weeks [6].

The effectiveness of the STP has been documented in multiple studies in North America over the past decade [6–12]. These studies have documented substantial improvements in multiple domains of functioning, including peer relationships, compliance with adult requests, and classroom functioning, as assessed through direct observations and rating scales. Positive effects have been demonstrated in multiple geographic regions of the U.S. and Canada, and with multiple subject populations reflecting a variety of socioeconomic classes and ethnicities. However, there has never been an STP conducted with a primarily Asian sample.

In this paper, we report the results of the first Summer Treatment Program (STP) outside North America for children with ADHD. We established an STP in 2005 with the assistance of two STP specialists from Buffalo (EMG, ARG). This STP lasted 2 weeks and generated positive evaluations by staff and parents [13]. The staff members felt that even the 2-week STP dramatically improved the behavior of the 22 participants. However, consistent with results from U.S. studies, we concluded that 2 weeks was not long enough for several children. The purpose of this study was to evaluate whether a 3-week version of the American STP was effective with Japanese children with ADHD.

2. Patients and methods

Thirty-six Japanese children (Age 6–12 y, male:female 32:4) in and around Kurume City participated. Thirty children had ADHD, and six children had a diagnosis of ADHD co-morbid with high functioning pervasive developmental disorder (HFPDD). Among the 30 children with ADHD, 24 were combined type and 6 were predominantly inattentive type. The children were divided into three groups by age with 12 in each group; Group A (6–9 y, 6 had ADHD and 6 had ADHD co-morbid with HFPDD), Group B (8–10 y, all had ADHD), Group C (10–12 y, all had ADHD). Nineteen children had participated in the 2005 STP (5 in Group A, 7 in Group B, 7 in Group C). All parents were Japanese except for two children with American and British fathers, respectively. All the participants were diagnosed with ADHD according to DSM-IV criteria by pediatric neurologists either at Kurume University Hospital or St. Mary's Hospital and their IQs were all above 70. Thirteen children had co-morbid oppositional defiant disorder, but none had conduct disorder.

The staff consisted of 40 members: 9 clinical psychologists; 21 student counselors from the Department of Psychology, Kurume University; 1 pediatrician from the Department of Pediatrics & Child Health, Kurume University; 7 special education teachers in Kurume City; and 2 part-time consultants (EMG and ARG) from the State University of New York at Buffalo. The first author (YY) studied STP in Buffalo for 5 weeks in 2003 [14].

Training for STP staff has been well specified and documented [6]. After an introductory lecture by the first author, intensive training for student counselors was provided every weekend for 3 months before STP by the clinical psychologists (lead counselors), who had been trained by Buffalo staff and had participated in the first STP in 2005. The teachers had six training sessions by the special education teacher who had participated in the STP in 2005. The STP was run at Kanamaru municipal elementary school with the assistance of Board of Education in Kurume City. This study protocol was approved by the Ethical Committee of Kurume University and the written informed consent was obtained from the parents and the children.

2.1. Procedures and techniques used in the STP

The STP procedures include a variety of behavioral components that have been extensively described and documented elsewhere [6,15]. A few minor modifications from the original program manual due to the cultural difference were made in consultation with Buffalo STP specialists. Children attended the STP from 9:30 AM to 4:35 PM on weekdays for 3 weeks. Children were placed in three age-matched groups of 12 that stayed together throughout the day. The children with symptoms with HFPDD were all in the youngest group. Each group spent 1 h daily in classroom sessions conducted by special education teachers, during which individualized paper and pencil and computer-assisted learning was provided. The remainder of each group's day consisted of recreationally based group activities, supervised by a team of 6–8 counselors. The intervention was implemented across classroom and recreational settings while children were

Table 1
Summer treatment program: a typical day.

Time	Activity
9:30–10:00	Arrivals
10:00–10:15	Morning discussion
10:15–10:25	Transition/bathroom
10:25–11:25	Sports skills training (Soccer)
11:25–11:35	Transition/bathroom
11:35–12:35	Academic learning center
12:35–12:45	Transition/bathroom
12:45–13:05	Lunch
13:05–13:20	Lunchtime recess
13:20–13:30	Transition/bathroom
13:30–14:30	Designated game (Soccer)
14:30–14:40	Transition/bathroom
14:40–15:40	Swimming
15:40–15:50	Transition/bathroom
15:50–16:05	End of day recess
16:05–16:30	Departure

engaged in classroom tasks and group-based recreation. The typical STP schedule is shown in Table 1. Sixteen children remained on their currently-prescribed medication (short-acting methylphenidate) during the STP. Compliance with medication dosing in the morning and at lunch was strictly checked by the medical staff.

2.1.1. Intervention Components

2.1.1.1. Point system. In a systemic reward/response–cost program, children earned points for appropriate behavior (e.g., helping, compliance, good sportsmanship) and lost points for inappropriate behavior (e.g., teasing, noncompliance, rule violations) throughout the day. The points children earned were exchanged for privileges (e.g., weekly field trips on Friday) and social honors. Counselors recorded points taken from and awarded to each child throughout the day. Staff members received extensive training in the point system, and the reliability of the point system and frequency counts has been well documented [6,11]. Before the program began, children received a booklet that described the point system and each mother was asked to explain the point system to her child. On the first day of the STP, lead counselors explained the point system and activity rules to the children. Staff members reviewed activity rules prior to each activity, and during the activities gave specific behavioral feedback and instructions to the children in addition to informing them of points earned and lost whenever they exhibited behaviors included in the point system. Children were disciplined for prohibited behaviors (i.e., intentional aggression, intentional destruction of property, repeated noncompliance), with discipline taking the form of loss of privileges or time out from the ongoing activities.

2.1.1.2. Daily Report Card. Daily report cards (DRCs) were implemented for the children in STP. DRCs included individualized target behaviors from the classrooms and from the recreational activities. We provided standardized DRC in the first week, and the three target behaviors and criteria for meeting daily goals were individually revised in an ongoing manner in the second and third week. Parents provided positive reinforcements at home to reward children for reaching daily goals.

2.1.1.3. Sports skills and social skills training. Children received intensive coaching and practice in sports participation and skills. One hour daily was devoted to small-group skills training either soccer or kick baseball and an additional hour was devoted to playing the same sports game. One hour was spent in swimming instruction. The emphasis was on teaching skills and sports rules because many children with ADHD are not fully aware of sports rules and do not have well developed sports skills and coordination. The instruction was reinforced through the point system. When children violated sports rules, counselors informed them of point losses in addition to providing corrective feedback and instruction. In addition, children's attention to the game and knowledge of game were assessed by asking each child 'attention questions' during each game period. Children earned points when they answered questions correctly. Social skills training was provided in brief, daily group sessions that included instruction, modeling, role-playing, and practice in key social concepts such as "good communication".

2.1.1.4. Learning Center. Children spent 1 h daily in Learning Center: 30 min in an academic class and 30 min in a computer-assisted-instructional classroom. Behavior in the classrooms was managed with a simplified point system that included both reward (earning points for work completion and accuracy) and response–cost (losing points for violating classroom rules) components. Children received academic assignments that were individualized according to each child's needs, usually including reading, spelling Kanji (Chinese characters), and arithmetic problems.

2.1.1.5. Parent training. Parents participated in two sessions before the beginning of the STP in which they learned general behavioral principles such as reinforcement systems, appropriate commands, and time out, and were taught to implement home-based rewards for children's performance during STP day as measured by the DRC. A weekly parent meeting with lead counselors and developmental specialists was provided at night to discuss children's individual problems during the STP.

2.1.2. Dependent Measures

Behavioral data were based on frequency counts of the behaviors described in the point system in the recreational and learning center settings. These included individual points taken from or awarded to children, number of time outs, and rule violations. Behaviors were

recorded by counselors and teachers, collected by the data manager, and input into a computer spreadsheet at the end of the day. Thus, daily behavioral data were available to lead counselors and developmental specialists to monitor children's progress and revise treatment programs as necessary. A composite dependent measure was constructed across settings (recreation and classroom), valence of behavior (negative and positive behaviors), and specific categories (peer relations, compliance, academic work completion) by computing the net total of points (points earned minus points lost) for each child for each day.

Parents completed a questionnaire on ADHD, oppositional defiant disorder (ODD), and conduct disorder rating scale based on DSM-IV criteria, and the Strengths and Difficulties Questionnaire (SDQ) [16] 2 weeks before the STP and 1 week after the STP. Parent satisfaction questionnaires [6,11] were mailed to parents 1 week and 4 months after the STP. These ratings ranged from 1 (very much unsatisfied) to 5 (very much satisfied).

Analysis of variance (ANOVA) was used for the analysis of each point system-related efficacy measure. Paired *t*-tests were performed for the comparison of rating scales before and after STP. All statistical tests were performed at $p = 0.05$ significance level.

3. Results

3.1. Group results

None of the 36 children dropped out during the 3-week STP. Most of the children showed positive behavioral changes in multiple domains of functioning, as shown by a significant improvement in the average net points earned by groups ($p < 0.01$) (Fig. 1). The eldest group (Group C) earned significantly more points than the youngest group (Group A) (1820.5 ± 268.6 vs 876.8 ± 268.6, $p = 0.039$). Both children with and without co-morbid HFPDD significantly improved at the end of the STP ($p < 0.01$); however, daily average points were significantly higher in the ADHD group compared to the ADHD + HFPDD group ($p < 0.01$). The average number of time outs per day per child was significantly lower in Group B (0.8 ± 0.9) and Group C (0.6 ± 0.7) compared to Group A (2.3 ± 2.9) ($p = 0.039$). The daily average numbers of rule violations by groups are shown in Fig. 2. The frequency of rule violations was significantly higher in Group A compared to Groups B and C ($p = 0.028$); however, rule violations significantly decreased by the end of the STP in all the groups.

Parent ratings showed significant improvement in ADHD scores, ODD scores (Fig. 3), and hyperactivity/inattention and conduct problems ($p < 0.05$). Parent-rated emotional problems, peer relationship problems, and pro-social behavior did not significantly improve, however.

Ninety-seven percent of the parents 1 week after the STP, and 93% after 4 months, were satisfied with the STP. The average ratings were 4.6, and 4.4, respectively, on the 5-point scale, reflecting very high levels of parent satisfaction.

3.2. Case examples

One 7-year-old boy with ADHD co-morbid with HFPDD in the youngest group lost many points and served an excessive number of time outs. Because he disliked being hot during sports skills and game periods, and would become upset when he lost points, he often ran away from the activity area. Consequently, he lost many points (minus 9000 points) due to repeated non-compliance and running away. He required an individualized program from the second week, in which he could get his favorite Mushi-King^{R} sticker on his card every time he was not in time out for 5 minutes, a DRO schedule of reinforcement. He could exchange each sticker

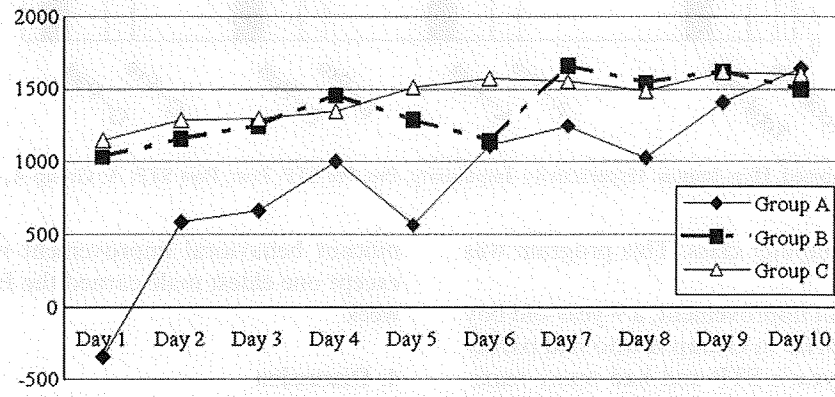

Fig. 1. Average daily points each group earned every day significantly improved day by day. The STP in 2006 started from Tuesday and the point system did not start on the first day. The days on which the point system worked were Wednesday and Thursday in the first week (Day 1–2), Monday through Thursday in the second and third week (Day 3–6, Day 7–10). In total, data on 10 days were evaluated.

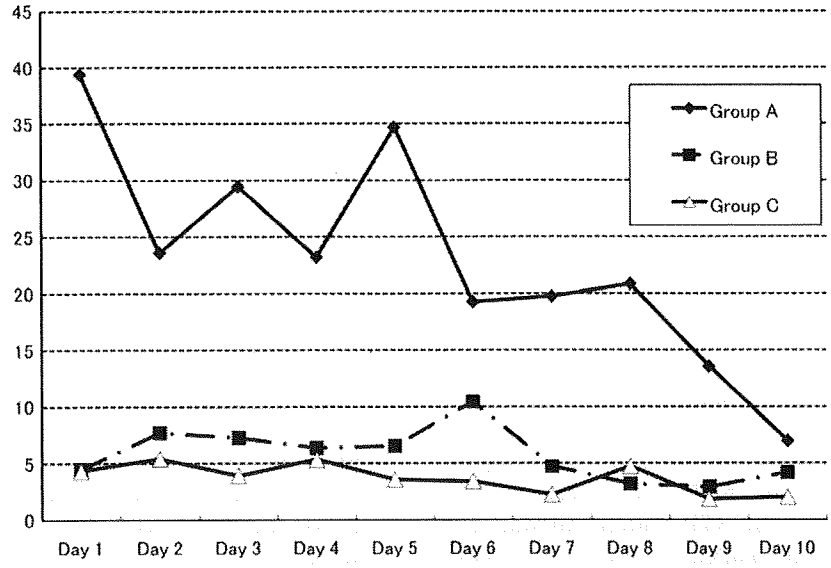

Fig. 2. Daily frequencies of rule violations in Group A were significantly higher compared to Groups B and C, however this rate decreased to the same level at the end of STP.

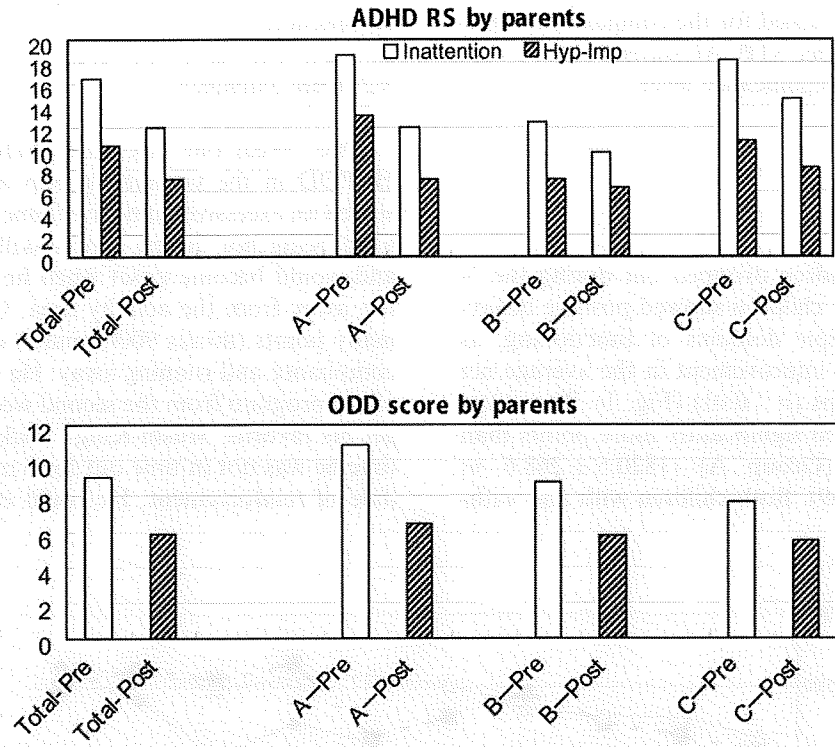

Fig. 3. Total; all groups combined, Hyp–Impuls: Hyperactivity–Impulsivity, Pre; Pre STP, Post; Post STP, A; Group A, B; Group B, C; Group C.

for 2 min activity in computer class. This program was very effective.

As a final indication of improvement, a 9-year-old boy with combined type ADHD in Group B who had participated in the previous year's STP and had many maximum-length time outs behaved extremely well in this STP. Another 8-year-old boy with HFPDD+ADHD who had excessive time outs in STP 2005 also showed significant behavioral improvement in 2006. All children except one eldest male earned the Friday field trips each week.

4. Discussion

This study provides preliminary evidence in Japanese children of the effectiveness of an intervention for

ADHD—the STP—that has a solid evidence base in North America. We document that a version of the STP that has been modified to fit with Japanese culture and school schedules is effective. We discuss these results below.

First, it has become increasingly evident that ADHD should be regarded as a chronic disorder with a poor long-term course and that models of treatment should be those pertinent for a chronic and refractory disease [17]. The most common treatment of ADHD is medication, mainly CNS stimulants. Medications have a large evidence base with short-term efficacy but have a number of limitations, including off-label use of short-acting methylphenidate for children and adults with ADHD in Japan. Furthermore, long-term studies of medication fail to document long-term benefits. It is thought that medication may not have a sufficient impact on peer relationships, parenting skills, and academic functioning, all of which are widely thought to be the mediators of long-term outcome in children with disruptive behavior disorders [3].

The second most common treatment for ADHD is behavior modification in the form of behavioral parent training, behavioral school interventions, and child-focused behavioral interventions such as the STP. Like medication, behavior modification has large evidence with short-term efficacy but has also limitations. The lack of specialists providing behavior modification and the need to work in the home, school, and peer settings makes it difficult to implement. Unlike medication, however, behavioral interventions can teach skills that overcome some of the key functional impairments associated with ADHD [6]. Indeed, the goals of STP are to improve the children's peer relationships, interactions with adults, academic performance, and self-efficacy, while training their parents in behavior management. These are the components which predict the prognosis of children with ADHD and which are not sufficiently improved by medication alone.

We started the Japanese version of the STP in 2005. The American STP usually last for 7–8weeks, and there are preliminary data showing that a 5-week, partial day time period is effective [6]. However there have been no systematic parametric studies looking at the efficacy of shorter STPs. In Japan, the duration of summer vacation is less than 6 weeks, necessitating a shorter STP duration. Therefore, the purpose of this study was to evaluate whether an STP 3 weeks in length was effective for Japanese children with ADHD.

It is notable that none of the 36 children dropped out during the STP. This low dropout rate was extraordinary because some children who participated in our STP hated to play outside in summertime in their original schools, but they actively engaged in the outdoor sports activities during the STP. We believe that this was made possible by the intensive coaching by counselors and the positive reinforcement of participation through the point system. Pelham and Hoza reported that during a six-year period (1987–92), the drop-out rate from the STP was less than 3%, which was remarkably low compared to other intervention programs [11]. Thus, the dropout rate for Japanese children was comparable to that for the American program.

A few modifications were made to the program between 2005 and 2006 to maximize the efficacy of the 3-week program. For example. in 2005, it took a week for younger children to fully understand the point system. Thus, we provided a booklet that described the point system to families before this STP began and asked mothers to explain the point system to their children. This might have made it easier for children to understand the point system more quickly.

The significant improvement in net points earned, and decrease in time outs and rule violations during the STP, was observed at both the group and individual level. The only exception was the 7-year-old boy with ADHD co-morbid with HFPDD who required an individualized program but who subsequently improved during the final week of the program. These experiences suggest that STP might be effective not only in children with ADHD but also in some children with ADHD co-morbid with HFPDD because the STP is well structured with clearly defined rules and consequences that make it much easier to understand and function compared to the ordinary classroom. Additional studies will be necessary to determine whether a longer-duration program would be more beneficial for these ADHD + HFPDD children.

Parent rating scales showed significant improvement in ADHD scores, ODD scores, and hyperactivity/inattention and conduct problems ($p < 0.05$). Emotional problems, peer relationship problems, and pro-social behavior did not significantly improve, however. We are not sure why peer relationship problems and pro-social behavior did not improve. One possible explanation is that the evaluations were made by parents and that improvement in peer relationships was hard to evaluate at home. Most measures of peer relations for ADHD are gathered in peer or school settings rather than home settings [18]. Alternatively, it might be because 3 weeks was not long enough to improve social behavior. Finally, it may be that the parent-training program that we employed was insufficient to improve behavior at home across multiple domains. In the American STPs, behavioral parent training is always a component and consists of 8, weekly, 2-h group sessions. This level of parent training is the standard minimal amount in North America [3], and we may need to increase our parent training in Japan to approximate this intensity.

It is estimated that 30–50% of children with ADHD have co-morbid ODD or CD. In our STP study, 13 chil-

dren had ODD, but none had CD. No evidence exists for differential response to treatments—either pharmacological or behavioral—for children with ADHD, ODD, or CD [3,19]. Furthermore, studies that had participants with ADHD alone and co-morbid with other disruptive behavior disorders reported behavioral or pharmacological treatment to be effective irrespective of co-morbid diagnosis. This is specifically true for the STP, where many studies have shown effectiveness that is equal in children with and without co-morbid ODD and CD [6].

A limitation of this study is that we only looked at the short-term efficacy of the STP. Longer-term follow-up will be necessary to evaluate whether the improvements in behavior seen in the STP will maintain. A promising development is that we encouraged teachers from the children's original classrooms to observe the STP, and more than 20 teachers visited. We also encouraged teachers to continue the DRC; however, the DRC was not always continued between home and school. In Buffalo, follow-up parent training and school interventions as a part of follow-up treatment are established by program therapists and/or paraprofessionals who work directly with teachers to ensure generalization to the children's school environment. Such follow-up was also given in the MTA study, in which the STP was the child-focused treatment and after which parent training and school intervention were delivered [20]. The improvement of the follow-up program in Kurume STP is an issue for further development. We are planning to establish a follow-up program in which school counselors at each elementary school work as behavior consultants to continue the DRC and other advice to school teachers. All the lead counselors participated in STP are working as school counselors at elementary school in Kurume City.

An important long-term benefit of the STP in Japan is that student counselors and teachers have the opportunity to learn fundamental skills for behavioral management that they can then carry forward in their work with children. The STP was useful to promote collaboration among many professionals from different disciplines, which had been lacking in Japan. More than 1000 undergraduate students have been trained through working in STPs in North America, and they have gone on to work in the educational, medical, or psychological fields [6]. We hope that the Japanese STP will fulfill a similar training role.

In addition to treatment and training, the STP will be useful to facilitate clinical research in Japan, because objective evaluation was possible by analyzing the daily records from the STP (e.g., daily report card, rule violations). The STP is also an ideal setting in which to evaluate the efficacy of medication, behavior modification, and combined treatments in a natural setting. Dozens of such studies have been conducted in the STP [12]. For example, Fabiano and colleagues have recently demonstrated that combined treatment of behavioral and pharmacological treatment could dramatically reduce the dose of medication necessary for normalizing functioning in ADHD children using an STP with different intensities of behavior modification and multiple doses of methylphenidate [9].

In summary, we have shown that the STP can be successfully implemented with Japanese children and that the program showed significant benefit for the children. The STP should be promoted in other sites in Japan as a component of comprehensive treatment for children and families with ADHD.

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Report

SIRT2 downregulation confers resistance to microtubule inhibitors by prolonging chronic mitotic arrest

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Abbreviations: APC/C, anaphase promoting complex/cyclosome; hiMSC, human immortalized mesenchymal stem cells; MTIs, microtubule inhibitors; TAP, two tandem affinity tag

Key words: SIRT2, spindle checkpoint, microtubule inhibitors, chronic mitotic arrest, cell death, tetraploidization, centrosome

We previously identified SIRT2, a deacetylase for tubulin and histone H4, as a protein downregulated in gliomas, and reported that exogenously-expressed SIRT2 arrests the cell cycle prior to entry into mitosis to prevent chromosomal instability in response to microtubule inhibitors (MTIs) such as nocodazole, characteristics previously reported for the CHFR protein. We herein investigated the effects of SIRT2 downregulation on sensitivity to MTIs using HCT116 cells, a mitotic checkpoint-proficient near-diploid cancer cell line used for studying checkpoints. We found that SIRT2 downregulation confers resistance to MTIs as well as that of BubR1, a well-characterized mitotic checkpoint protein, though by a different mechanism. While BubR1 suppression abolished spindle checkpoint functions, which is a requirement for cell death after release from the spindle checkpoint, SIRT2 downregulation prolonged chronic mitotic arrest from sustained activation of the mitotic checkpoint and consequently prevented a shift to secondary outcomes, including cell death, after release from chronic mitotic arrest. Consistent with this notion, BubR1 downregulation was dominant over SIRT2 knockdown in regard to mitotic regulation in the presence of nocodazole. These results suggest that SIRT2 functions to release chronic mitotic arrest in cells treated with MTIs, leading to other outcomes. We also found that SIRT2 downregulation caused centrosome fragmentation in response to nocodazole prior to the alteration in spindle checkpoint function, implying not only a novel function of SIRT2 for centrosome maintenance upon exposure to mitotic stress caused by MTIs, but also the existence of a centrosome-mediated signaling pathway to sustain the spindle checkpoint. Therefore, this study highlights a novel pathway leading to resistance to MTIs, in which SIRT2 downregulation participates.

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Introduction

Sir2, an NAD^+-dependent protein deacetylase, extends the lifespan and is involved in both aging, diseases of aging and the cell cycle.[1-4] The spindle checkpoint monitors the proper attachment of microtubules to the kinetochores of sister chromatids, and the integrity of bipolar mitotic spindles ensures faithful transmission of chromosomes during cell division.[5] Either unattached or untensioned kinetochores provoke the spindle checkpoint, which leads to cell cycle arrest at metaphase, and thus prevents the missegregation of chromosomes by preventing the onset of anaphase through a signaling cascade that results in the suppression of the anaphase promoting complex/cyclosome (APC/C).[6] It has been established that several evolutionally conserved proteins, including BubR1, Bub1, Bub3, Mad1, Mad2, Cenp-E and Mps1, are required for spindle checkpoint function.[7]

The spindle checkpoint is also activated in response to various microtubule inhibitors (MTIs), such as nocodazole, that prevent the attachment of microtubules to the kinetochores by depolymerizing microtubules. Taxanes (e.g., paclitaxel/taxol) that are used as chemotherapeutic drugs for cancer inhibit the dynamic instability of the spindle and allow microtubule attachment to the kinetochores, but prevent the generation of tension across kinetochores, and thus also provoke the spindle checkpoint.[8]

The mitotic arrest observed upon treatment with MTIs is dependent on the spindle checkpoint, and long-term treatment with MTIs can have several outcomes. The first is chronic mitotic arrest, which by definition is not committed to other outcomes. Chronic mitotic arrest is sometimes reversible (i.e., cells can undergo normal division) upon withdrawal of MTIs. The state of chronic mitotic arrest is not permanent and shifts to secondary outcomes. One secondary outcome is execution of the cell death pathway directly from mitosis. Another is mitotic slippage or adaptation, in which cells exit long-term mitotic arrest while still

in the presence of MTIs, fail cytokinesis, enter pseudo-G_1, and become tetraploid. These cells could then execute cell death, become senescent or continue dividing.

Cells showing sensitivity to MTIs exhibit cell death directly from mitosis or from the tetraploid state, or senescence from the tetraploid state. As a corollary, cells showing resistance to MTIs exhibit chronic mitotic arrest in the presence of MTIs and resume normal division upon withdrawal of MTIs, or continued cycling in the tetraploid state. Intriguingly, the activation of the spindle checkpoint is responsible for cell death in response to MTIs.[9,10] Consistent with this notion, checkpoint defects are frequent in human cancers and can confer resistance toward chemotherapy[11,12] and a robust spindle checkpoint is required for sensitivity to MTIs in vitro.[13] For example, suppression of BubR1 or Mad2 by RNA interference in paclitaxel-treated cancer cells abolishes checkpoint functions, resulting in paclitaxel resistance through an escape from cell death during mitosis with the loss of the mitotic index and Cdk1 activity.[14] The identification of molecular characteristics predictive of MTI sensitivity or resistance thus could aid in selecting patients to receive chemotherapy.

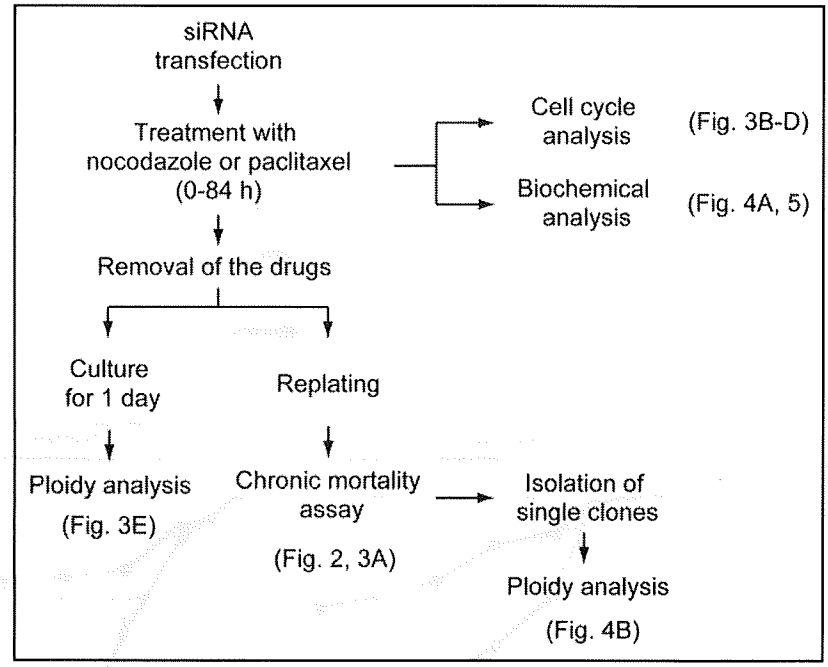

Figure 1. Schematic representation of the present study. siRNA-mediated knockdown of SIRT2 was performed to know the effect of SIRT2 downregulation on mitotic progression in the presence of MTIs, as well as that of BubR1, a well-characterized spindle checkpoint protein spindle checkpoint proteins. The simultaneous knockdown of SIRT2 and BubR1 was also performed.

We previously identified SIRT2, an NAD-dependent deacetylase that targets tubulin and histone H4, as a protein downregulated in gliomas and glioma cell lines, which are characterized by aneuploidy.[15] We observed that exogenously expressed SIRT2 functions in the prophase to block the entry to chromosome condensation and subsequent hyperploid cell formation in glioma cell lines in response to mitotic stress.[16] SIRT2 is thus a novel mitotic checkpoint protein that functions in the prophase to prevent chromosomal instability, characteristics previously reported for the CHFR protein.[4] In this study, we investigated the involvement of SIRT2 downregulation in spindle checkpoint regulation and sensitivity to MTIs in the HCT116 cell line, a checkpoint-proficient near-diploid cell line, which is frequently used in studies of checkpoint functions in response to MTIs.[10,11,17-26] We found that SIRT2 downregulation confers resistance to MTIs as has been reported for the downregulation of canonical spindle checkpoint proteins such as BubR1, Mad1 and Mad2.[11,14] However, the mechanism conferring resistance to MTIs by SIRT2 downregulation is different than that of the downregulation of canonical spindle checkpoint proteins. SIRT2 downregulation sustains mitotic arrest at the spindle checkpoint provoked by MTIs and consequently prevents cell death after the release from chronic mitotic arrest, while BubR1 suppression abolishes spindle checkpoint functions, which is a requirement for subsequent cell death. These results suggest a novel pathway for conferring resistance to MTIs and that SIRT2 could function to promote the release of chronic mitotic arrest provoked by MTIs, leading to other outcomes.

Furthermore, we observed that SIRT2 downregulation leads to centrosome fragmentation in the presence of nocodazole at an early stage. This implies not only a novel function of SIRT2 for centrosome maintenance, but also the existence of a centrosome-mediated signaling pathway linking to the spindle checkpoint. A possible relationship between this phenomenon and resistance to MTIs is discussed.

Results

Induction of resistance to MTIs upon SIRT2 downregulation. To examine whether SIRT2 is involved in resistance to MTIs and spindle checkpoint functions, cells were transfected with siRNA to SIRT2 or negative control siRNA, treated with nocodazole and paclitaxel, which elicit mitotic stress by inducing microtubule depolymerization and polymerization, respectively, and then analyzed for various effects related to the spindle checkpoint. The experimental design in this study is illustrated in Figure 1. We used the colorectal cancer cell line HCT116, a mitotic checkpoint proficient near-diploid cell line, which is frequently used for studying the relationship between the spindle checkpoint and resistance to MTIs. The level of SIRT2 expression in HCT116 was comparable to that of a human normal fibroblast cell line (TIG-1) (data not shown).

We first examined whether SIRT2 downregulation modulates the sensitivity to MTIs in HCT116 cells. Transfection of siRNA against SIRT2 into HCT116 cells resulted in a dramatic and reproducible reduction in SIRT2 protein, in contrast to cells transfected with negative control siRNA (Fig. 2A). More than 70% of the protein was depleted in SIRT2 siRNA-transfected cells, as judged by western blotting. SIRT2 suppression was also obtained using a plurality of different siRNAs to SIRT2 and a similar phenomenon

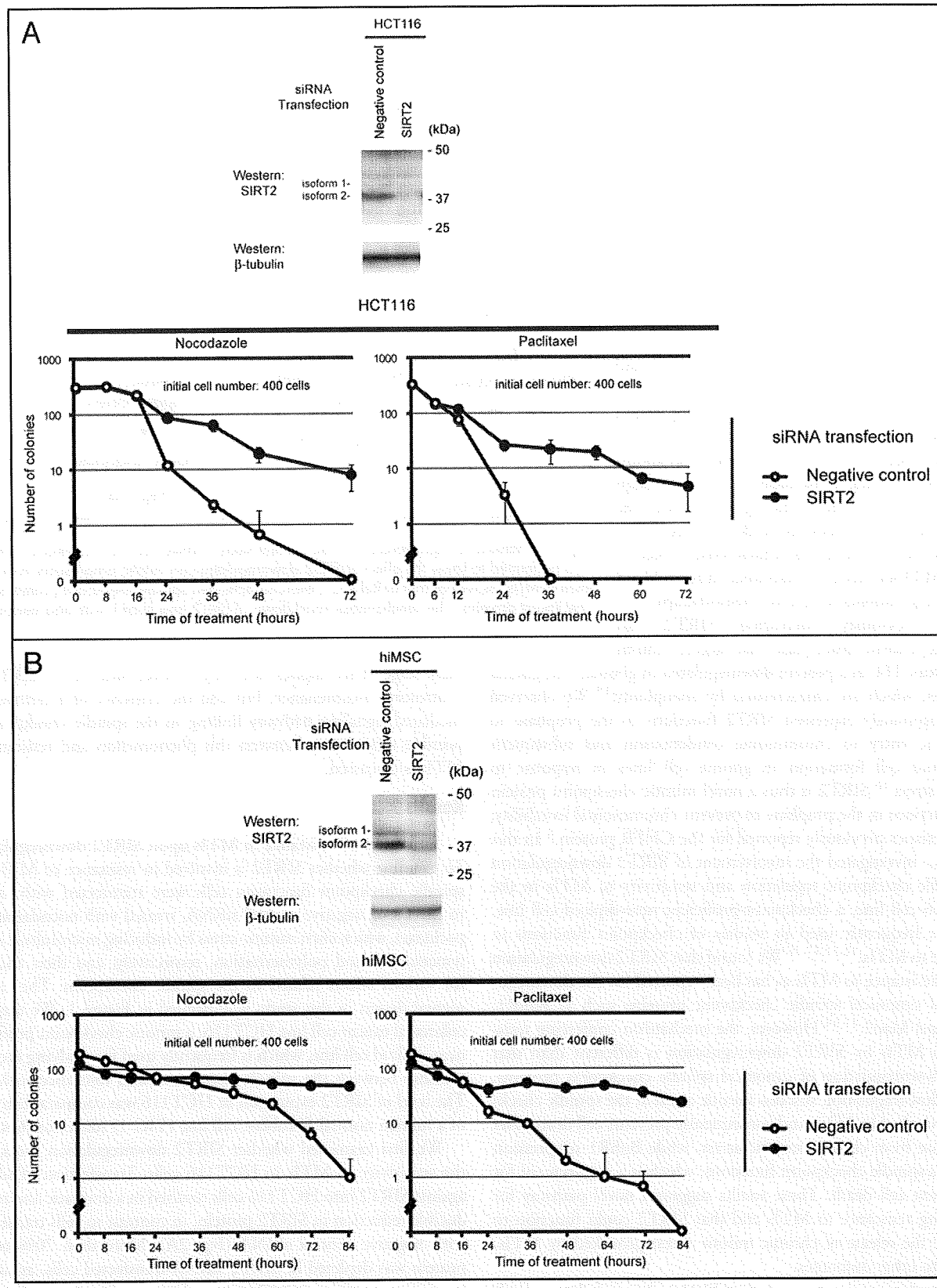

Figure 2. For figure legend, see page 1281.

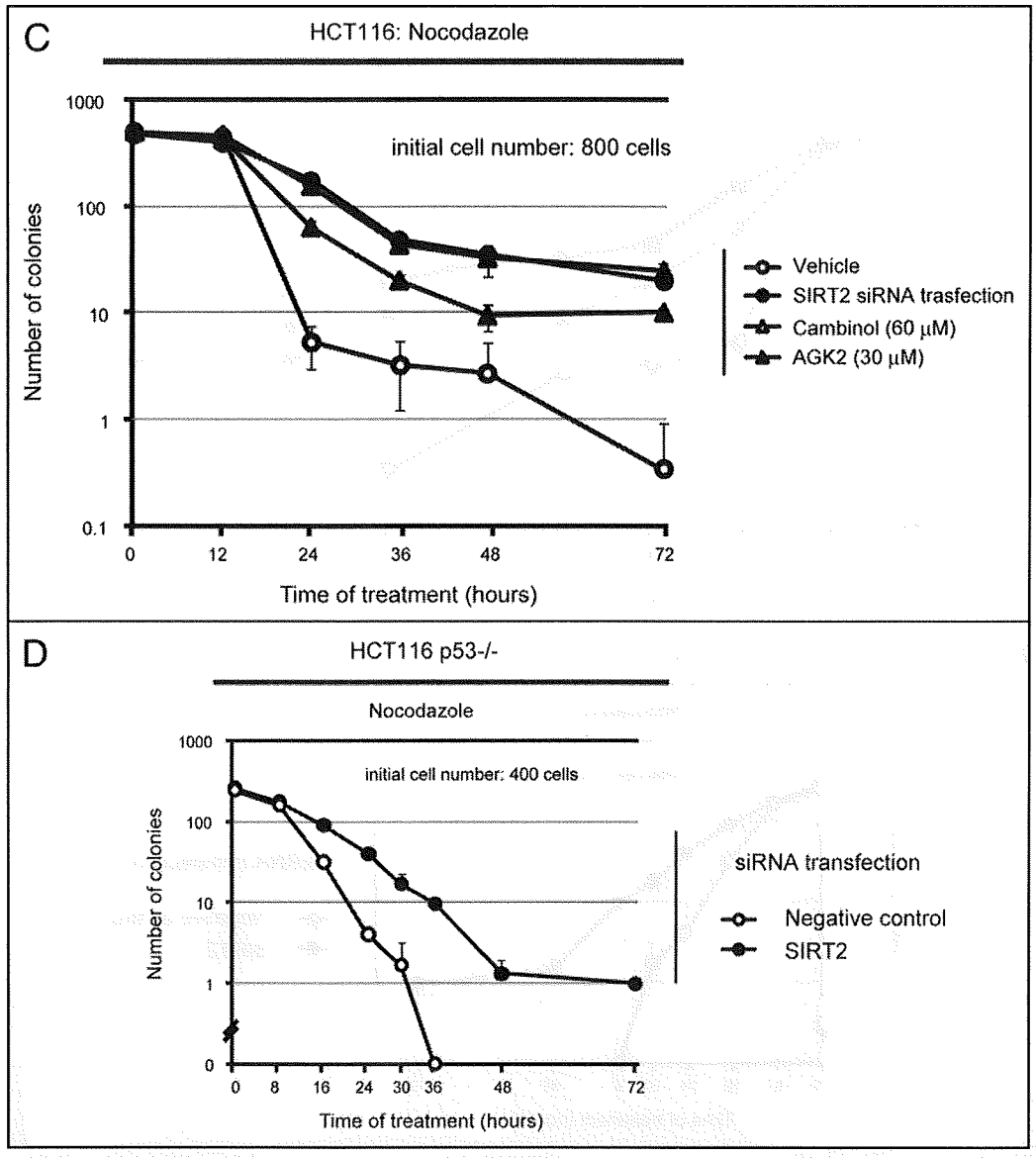

Figure 2. SIRT2 suppression confers resistance to MTIs. (A) Chronic mortality assays for HCT116 cells with siRNA-mediated SIRT2 suppression followed by exposure to MTIs. Cells were incubated in the presence of MTIs for the indicated times, incubated in drug-free medium for 10 days, and then colony numbers were scored. Assays were performed in triplicate and error bars denote standard deviation. Knockdown by siRNA was confirmed by western blotting. (B) Chronic mortality assays for hiMSC cells with siRNA-mediated SIRT2 downregulation followed by exposure to MTIs. Knockdown by siRNA was confirmed by western blotting. (C) Chronic mortality assays for HCT116 cells with cambinol (an inhibitor of SIRT2 and SIRT1) and AGK2 (a potent preferential inhibitor of SIRT2). (D) Chronic mortality assays for HCT116 $p53^{-/-}$ cells with siRNA-mediated SIRT2 downregulation followed by exposure to nocodazole.

was obtained in other cell types (data not shown). SIRT2 downregulation did not affect the population doubling time or the cell cycle distribution in the absence of MTIs in HCT116 cells (data not shown, see also Fig. 3D), as is consistent with a previous study using SIRT2 deficient chicken DT40 cells.[27]

We examined the effect of SIRT2 suppression on the survival of cells following MTI treatment by chronic mortality assays. Cells were treated with nocodazole or paclitaxel for the various periods indicated in Figure 2A, followed by the removal of the drugs, and the colony number was scored after 10 days. As shown in Figure 2A, SIRT2 suppression significantly increased resistance to both nocodazole and paclitaxel for treatment times in excess of 24 h. Similar results were obtained by using a plurality of different siRNAs to SIRT2 (data not shown). HCT116 cell clones stably expressing exogenous SIRT2 protein exhibited lower resistance to nocodazole as compared to the parental HCT116 upon transfection of siRNA against SIRT2 (Fig. S1). Thus, this resistance was attributable to SIRT2 suppression but not to off-target effects of siRNA to SIRT2. We observed that SIRT2 suppression increased resistance to both nocodazole and paclitaxel also in a human

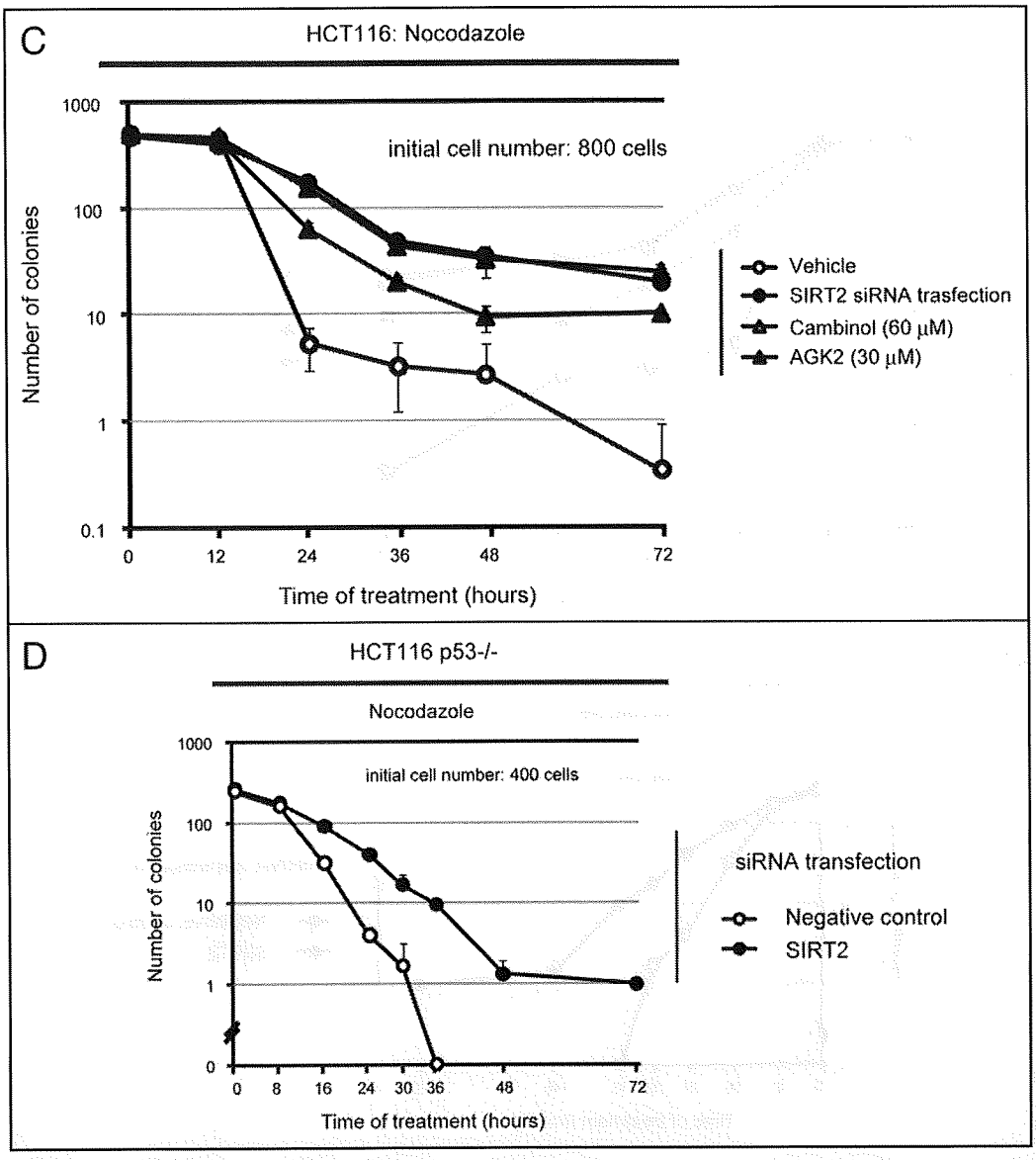

Figure 2C and D. SIRT2 suppression confers resistance to MTIs. (C) Chronic mortality assays for HCT116 cells with cambinol (an inhibitor of SIRT2 and SIRT1) and AGK2 (a potent preferential inhibitor of SIRT2). (D) Chronic mortality assays for HCT116 $p53^{-/-}$ cells with siRNA-mediated SIRT2 downregulation followed by exposure to nocodazole.

immortalized mesenchymal stem cell (hiMSC) line[28] as shown in Figure 2B, suggesting that the resistance to MTIs upon SIRT2 suppression is not limited to HCT116 cells. Cambinol, an inhibitor of SIRT1 and SIRT2, and AGK2, a potent preferential inhibitor of SIRT2, conferred resistance to nocodazole in HCT116 cells (Fig. 2C). Although we cannot rule out the possibility that inhibition of SIRT1 may also contribute to resistance to MTIs, this result implies that resistance to MTIs in SIRT2-suppressed HCT116 cells depends on the deacetylase activity of SIRT2. We observed significant resistance to nocodazole upon suppression of SIRT2 also in HCT116 $p53^{-/-}$ cells, where the p53 gene was disrupted by homologous recombination from HCT116 ($p53^{+/+}$)[29] (Fig. 2D). This suggests that the presence of functional p53 does not affect nocodazole resistance to MTIs between cells with SIRT2 suppression and control cells.

Suppression of SIRT2 prolongs chronic mitotic arrest in the presence of nocodazole, in contrast to suppression of BubR1. It is known that suppression of BubR1 and Mad2 abolishes spindle checkpoint functions in cancer cells, resulting in resistance to MTIs.[14,21] We confirmed that transfection of siRNA against BubR1 into HCT116 cells resulted in a significant reduction in BubR1 protein (Fig. 3A) and conferred resistance to nocodazole, although the degree of resistance seems to be less than that with SIRT2 suppression. We examined the possibility that SIRT2 suppression leads to resistance to MTIs through the inactivation of the spindle checkpoint, as is the case with BubR1 suppression. For

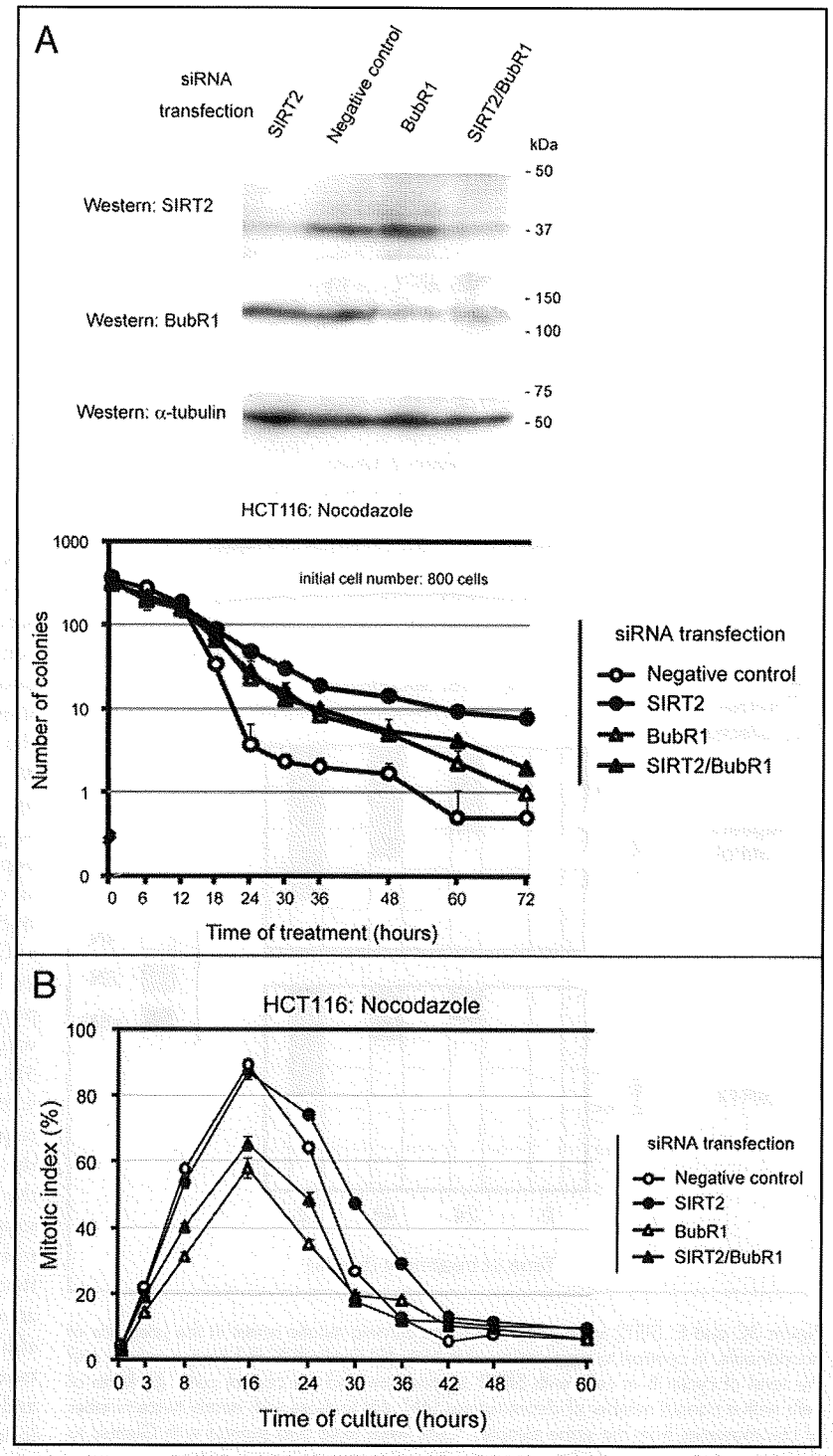

Figure 3A and B. SIRT2 suppression prolongs chronic mitotic arrest in the presence of nocodazole, in contrast to suppression of BubR1. (A) Chronic mortality assays for HCT116 cells with suppression of SIRT2, BubR1 or both. Knockdown by siRNA was confirmed by western blotting. (B) Mitotic indexes of the same samples. Mitotic index was determined by FACS analyses using anti-MPM-2 antibodies. N = 3.

comparison, we used cells with BubR1 suppression as well as cells with SIRT2 suppression in the subsequent analyses.

It is known that suppression of BubR1 and Mad2 abolishes spindle checkpoint functions in cancer cells, resulting in resistance to MTIs that correlate with suppression of Cdk1 activity and suppression of mitotic index. To know whether this is the case for suppression of SIRT2, we determined the mitotic index in nocodazole-treated HCT116 cells. We performed siRNA-mediated knockdown of SIRT2 alone, BubR1 alone or for both SIRT2 and BubR1. We cultured the cells in the presence of nocodazole, and then analyzed the mitotic index, Cdk1 activity and cell cycle progression during exposure to nocodazole.

FACS analyses using anti-MPM-2 (biochemical marker for prophase, prometaphase and metaphase; lost after anaphase onset) showed that the mitotic index reached a peak at 16 h of treatment in all samples and then decreased (Fig. 3B). The mitotic indexes in cell with SIRT2 suppression were nearly the same as those of the control until 16 h of treatment, but strikingly, the mitotic index at 24 to 36 h of treatment was significantly higher in cells with SIRT2 suppression than in the control, although it decreased with time. In contrast, the mitotic indexes of cells with BubR1 suppression were lower as compared to the control at all time points, and the kinetics of the mitotic index were comparable among the three samples. The suppression of the mitotic index in cells with BubR1 suppression is consistent with a previous report.[21] The same phenomenon has been reported for the downregulation of other mitotic checkpoint proteins, notably Mad2, which functions in a single pathway to BubR1,[30] and Mad1, which forms a complex with Mad2.[11,21] These results suggest that SIRT2 suppression and BubR1 suppression sustain and abolish the spindle checkpoint functions provoked by nocodazole, respectively. In addition, this suggests that SIRT2 suppression prolongs chronic mitotic arrest in the presence of MTIs and consequently prevents other outcomes following chronic mitotic arrest, including cell death, while BubR1 suppression abolishes spindle checkpoint functions, which is a requirement for subsequent cell death. By immunostaining BubR1 in cells with SIRT2 suppression in the presence of nocodazole, we observed BubR1-positive foci on chromosomes as well as in the control cells (Fig. S2), showing that the spindle checkpoint function is retained in cells with SIRT2 suppression.

When both SIRT2 and BubR1 are simultaneously suppressed, the persistence of mitotic index observed with suppression of SIRT2 alone was cancelled and the result closely resembled that

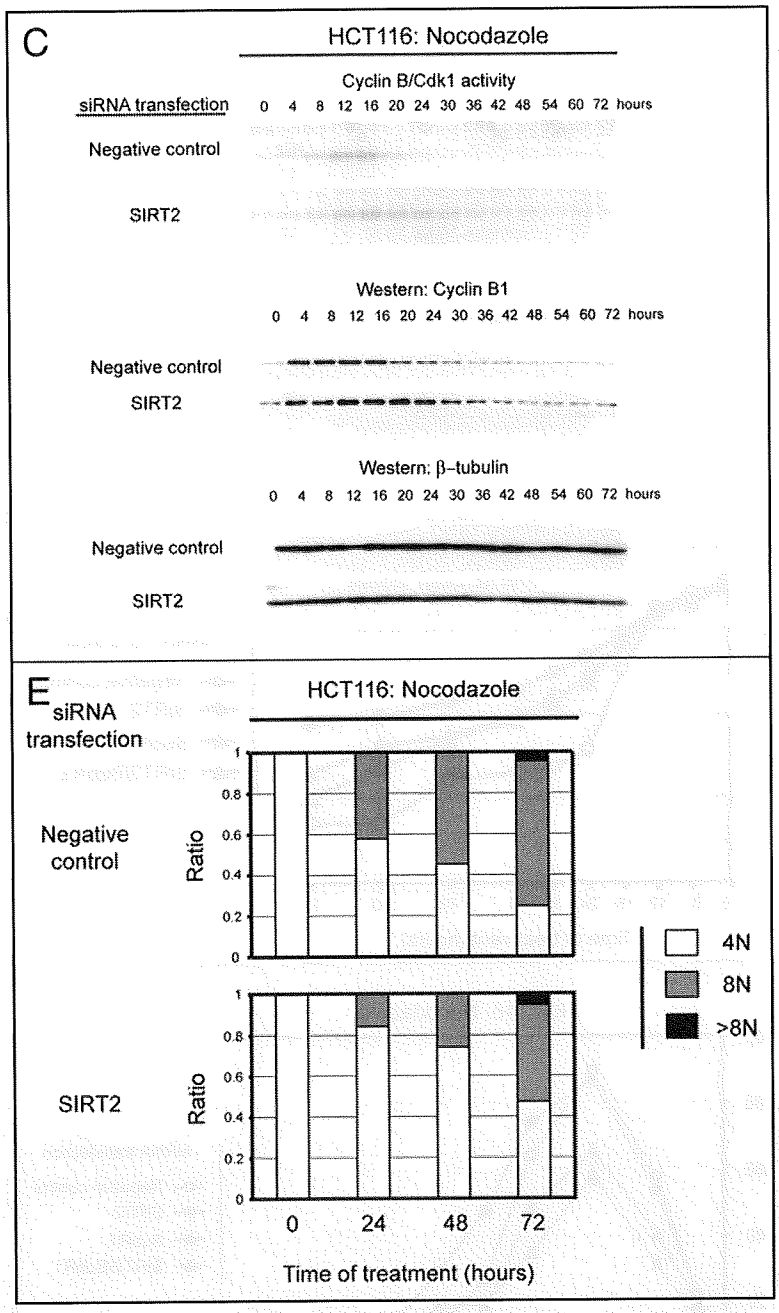

Figure 3C and E. SIRT2 suppression prolongs chronic mitotic arrest in the presence of nocodazole, in contrast to suppression of BubR1. (C) Cyclin B/Cdk1 kinase activity and the level of cyclin B in cells with SIRT2 suppression and the control cells. (E) Ratio of cells with a normal number of chromosomes (4N; 46) to those with nearly twice number of chromosomes from the same samples. Cells were fixed and stained with Giemsa to determine chromosome number. At least 100 metaphase cells were scored.

with suppression of BubR1 alone (Fig. 3B). These results suggest that BubR1 suppression was dominant over SIRT2 suppression in regard mitotic regulation in the presence of nocodazole, and are consistent with the notion that SIRT2 suppression sustains mitotic checkpoint functions that depend on BubR1. It is noteworthy that in chronic mortality assays, nocodazole resistance in cells with simultaneous suppression of SIRT2 and BubR1 closely resembled those with suppression of BubR1 alone (Fig. 3A), as is the case for the mitotic index analysis (Fig. 3B).

We measured cyclin B/Cdk1 activity, whose inactivation is required for the onset of anaphase, in cells with SIRT2 downregulation in the presence of nocodazole. As shown in Figure 3C, cyclin B/Cdk1 activity reached a peak after 12–16 h of treatment in cells with SIRT2 downregulation as well as in the control. While the activity dropped after 20 h of treatment in the control, it was still detectable until 30–36 h of treatment. This result is consistent with the data obtained in Figure 3B, supporting the hypothesis that SIRT2 suppression prolongs chronic mitotic arrest provoked by MTIs.

We performed cell cycle analyses in cells with suppression of SIRT2 alone, BubR1 alone, and both SIRT2 and BubR1 during nocodazole treatment. Figure 3D shows that the patterns of cell cycle progression and accumulation from the 2C to the 4C population are comparable in all samples up to 16 h treatment with nocodazole, except that cells with BubR1 suppression showed a slight accumulation of the 2C population in the absence of nocodazole. We observed that cells with 8C DNA content appeared after 36 h and increased up to 72 h in all the samples. It is striking that upon suppression of SIRT2 alone, cells exhibited a smaller population with >4C DNA content at all time points as compared to the control. In contrast, cells with BubR1 suppression exhibited a greater population of >4C DNA content as compared to the control after 24 h of treatment. Ploidy analysis showed that an 8N population appeared by 24 h treatment both in cells with SIRT2 downregulation and the control cells (Fig. 3E). The ratio of 8N by 24 h treatment was 16% and 42% in cells with SIRT2 suppression and control cells, respectively (Fig. 3E). The ratio of 8N increased as the period of treatment increased in both groups, and the ratio was significantly lower in cells with SIRT2 suppression than in control cells at any period of treatment. Considering the as a whole, we conclude that SIRT2 suppression prolongs chronic mitotic arrest provoked by MTIs, while BubR1 suppression abolishes it and that the mechanism conferring resistance to MTIs differs between suppression of SIRT2 and BubR1, in that SIRT2 prevents other outcomes following chronic mitotic arrest, including cell death, while BubR1 suppression abolishes spindle checkpoint functions, which are required for subsequent cell death. Based on this insight, it is logical to conclude that BubR1 suppression is dominant over SIRT2 suppression, and that SIRT2 suppression conferred nocodazole resistance also in HCT116 $p53^{-/-}$ cells (Fig. 2D), since the spindle checkpoint functions do not necessary require functional p53.[31,32]

Suppression of outcomes after chronic mitotic arrest contributes to resistance to nocodazole in cells with SIRT2 suppression. Cells showing sensitivity to MTIs exhibit cell death directly from mitosis or from the tetraploid state, or senescence from the tetraploid state. All the data shown in Figures 2 and 3 strongly suggest that SIRT2 suppression confers resistance to MTIs by suppression of cell death during mitosis and/or suppression of mitotic slippage leading to tetraploidy, which has been reported to be eliminated by apoptosis in HCT116 and HCT116 $p53^{-/-}$ cells.[24] Thus, we next sought to know whether these outcomes are suppressed in cells with SIRT2 suppression.

To date, it has been reported that the p38 MAP kinase cascade is essential for paclitaxel-induced apoptosis.[33,34] Also it has been reported that the activation of caspase-3 is observed in cells that undergo cell death both during mitosis and cell death from the tetraploid state after mitotic slippage.[35] We performed western blotting to detect phospho-p38 MAP kinase (Thr180/Tyr182), an activated form of p38 MAP kinase, and cleaved caspase-3 (Asp175), an activated caspase-3 resulting from cleavage adjacent to Asp175 of caspase-3, in cells with suppression of SIRT2, BubR1 or both, followed by nocodazole treatment.

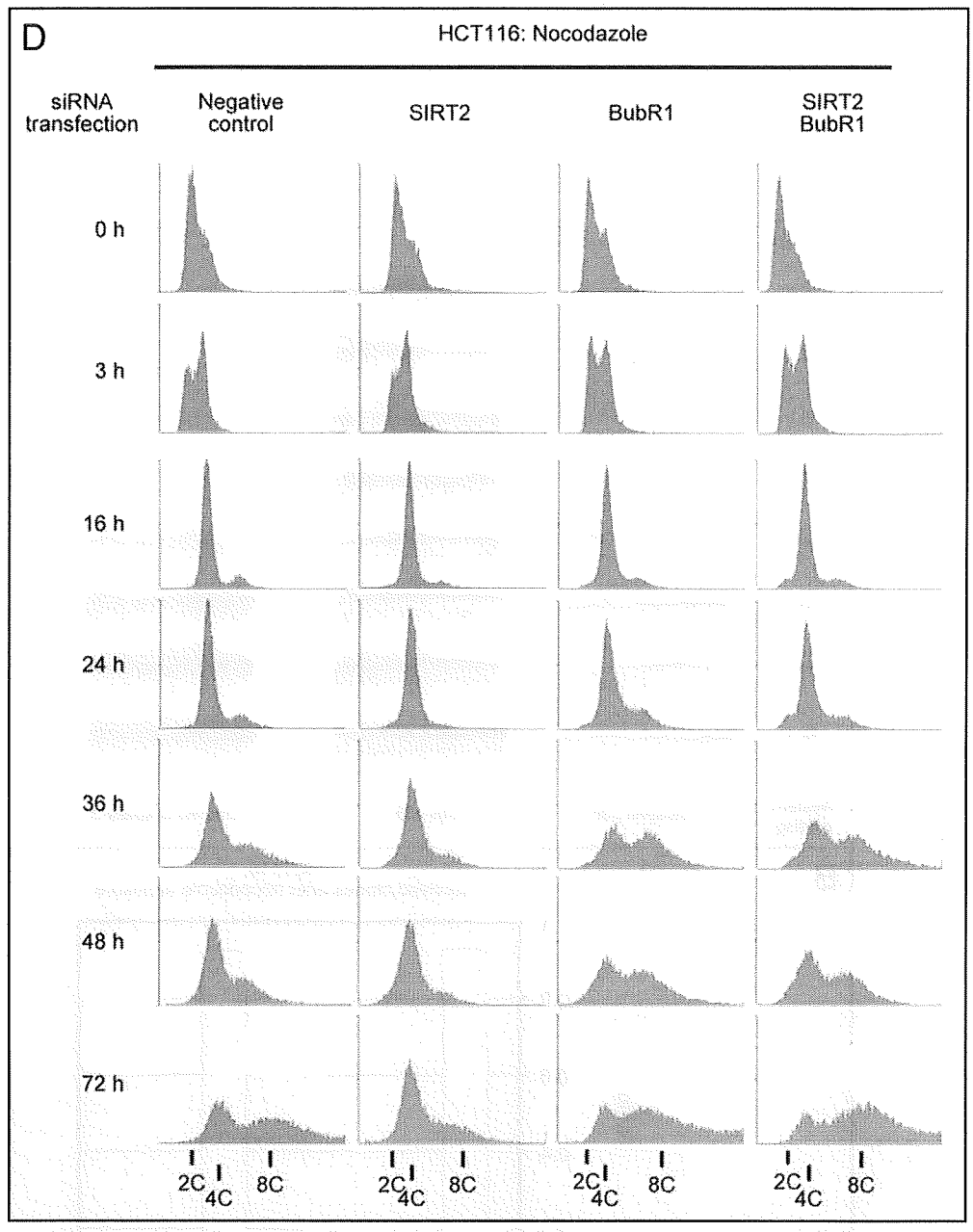

Figure 3D. SIRT2 suppression prolongs chronic mitotic arrest in the presence of nocodazole, in contrast to suppression of BubR1. (D) Cell cycle analysis for cells with suppression of SIRT2, BubR1 or both after prolonged exposure to nocodazole.

We observed that p38 MAP kinase was activated after 24 h of treatment and that activated caspase-3 was detected around the same time as activated p38 MAP kinase in the control cells, but not in cells with SIRT2 suppression (Fig. 4A). By contrast, in cells with SIRT2 suppression, both the activation of p38 MAP kinase and caspase-3 were not detected until after 30 h of treatment with a 6 h delay vs. the control (Fig. 4A). The delay of activation of p38 and caspase-3 observed in cells with SIRT2 suppression is consistent with prolonged chronic mitotic arrest observed in those cells (Fig. 3B–D). Thus, resistance to MTIs in cells with SIRT2 suppression contributes at least partially to the suppression of cell death during mitosis.

It has been reported that nocodazole-induced p53-dependent c-Jun N-terminal kinase (JNK) activation reduces apoptosis in HCT116 cells.[20] We observed rapid activation of SAPK/JNK in nocodazole-treated HCT116; however, no difference was observed between cells with SIRT2 suppression and the control cells (Fig. 4A), suggesting that this pathway is not involved in nocodazole

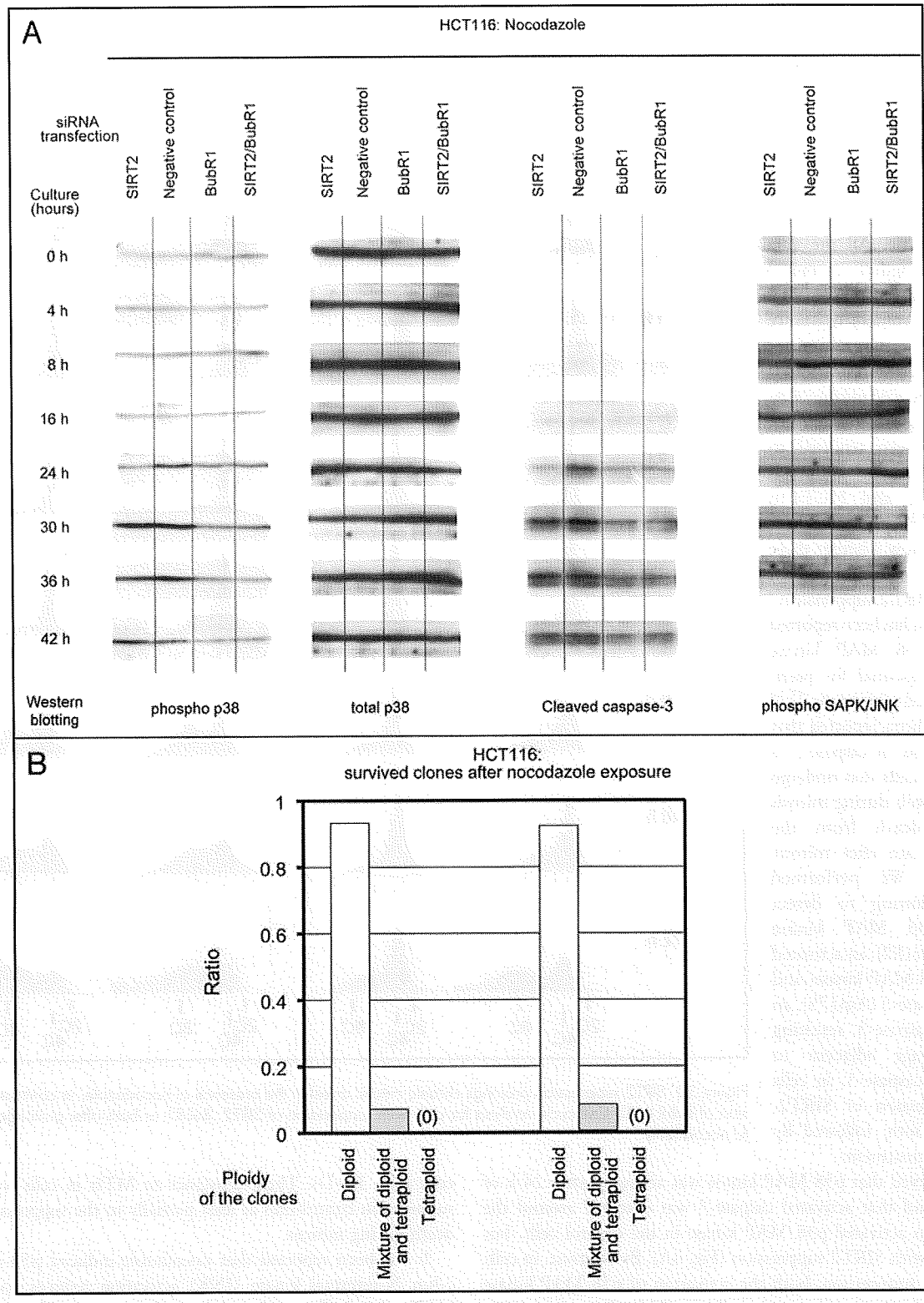

Figure 4. Suppression of outcomes after chronic mitotic arrest is attributable to resistance to nocodazole in cells with SIRT2 suppression. (A) Activation of p38 and caspase-3 derived from cell death during mitosis is delayed in cells with SIRT2 suppression. Cells with suppression of SIRT2, BubR1 or both after prolonged exposure to nocodazole were harvested and subjected to western blotting using the indicated antibodies. (B) Modal chromosomal number analysis of cell clones obtained after 36 h nocodazole treatment following siRNA transfection. At least 30 clones were isolated and analyzed.

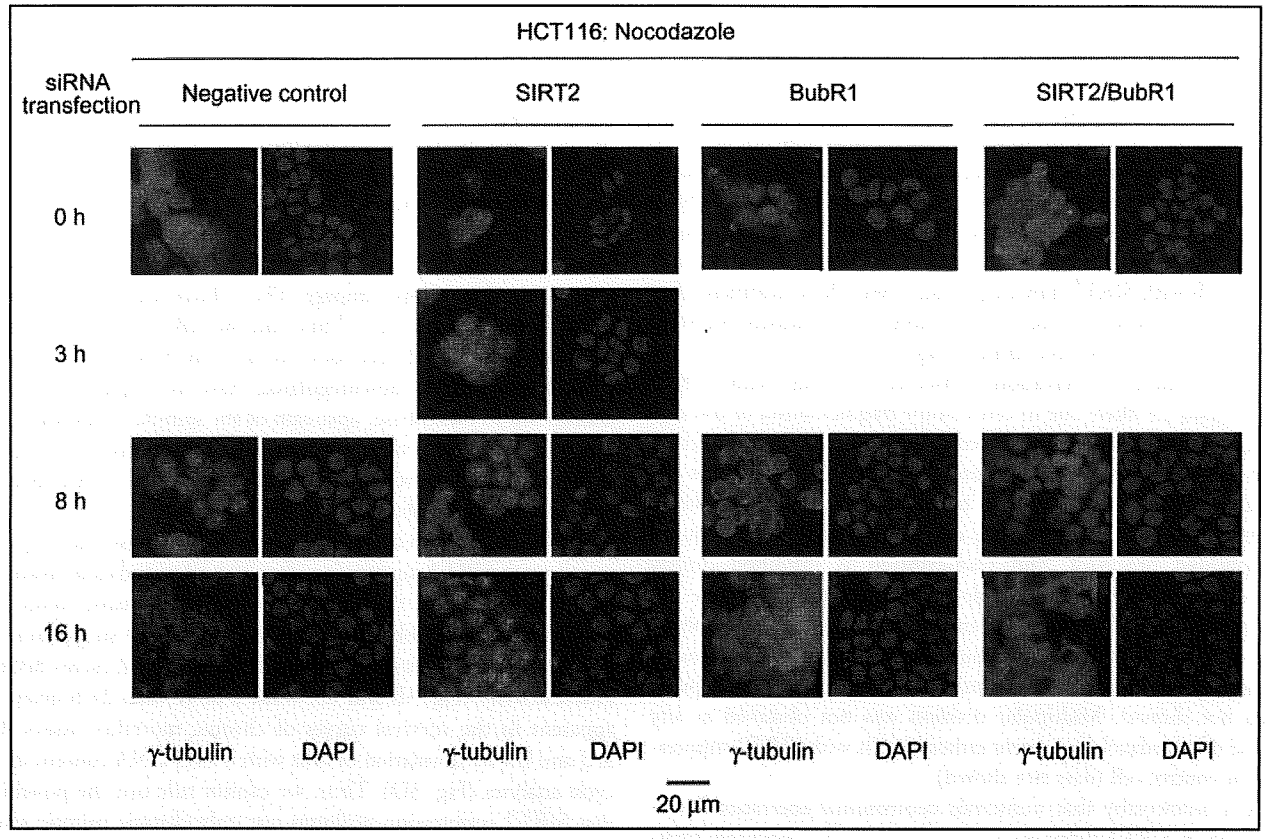

Figure 5. SIRT2 downregulation causes centrosome fragmentation in a nocodazole-dependent manner. Cells with suppression of SIRT2, BubR1 or both after prolonged exposure to nocodazole were subjected to immunostaining using anti-γ-tubulin antibody. Cells were counterstained with DAPI.

resistance. This is consistent with the fact that the effects of SIRT2 suppression on resistance to MTIs are not dependent on the p53 status in HCT116 cells (Fig. 2D).

In contrast, in cells with BubR1 suppression, significant activation of p38 was not detected, implying that activation of p38 depends on spindle checkpoint activation which requires BubR1 to function. Moderate activation of caspase-3 was observed in cells with BubR1 downregulation. As is the case for Figures 2 and 3, the results of western blotting using cells with simultaneous suppression of SIRT2 and BubR1 resembled that of BubR1 alone (Fig. 4A). Again, these results support the notion that SIRT2 suppression and BubR1 suppression (i.e., the inactivation of the spindle checkpoint) confer resistance to MTIs with different mechanisms, in which the latter is dominant.

As shown in Figure 3E, mitotic slippage was suppressed in cells with SIRT2 suppression. We next sought to provide supportive evidence that the suppression of mitotic slippage leading to tetraploidization also contributes to resistance to MTIs in cells with SIRT2 suppression. Since it has been reported that tetraploid cells are eliminated by apoptosis in HCT116 and HCT116 $p53^{-/-}$ cells,[24] it was expected that tetraploidies formed after mitotic slippage would be eliminated during chronic mortality assays, and that the difference in the tetraploid cell population observed between cells with SIRT2 suppression and control cells would contribute to the difference of resistance to MTIs observed between them. To confirm this, we performed ploidy analysis for cell clones obtained in chronic mortality assays after 36 h nocodazole treatment following siRNA transfection. We found that more than 90% of clones consisted of diploid cells exclusively, and the remaining were a mixture consisting of mostly diploid cells with a few tetraploid cells, both for samples with SIRT2 downregulation and the control (Fig. 4B). We did not observe clones consisting of tetraploid cells exclusively in either sample (Fig. 4B). This result fits the hypothesis that the suppression of tetraploid formation by mitotic slippage also at least partially contributes to resistance to MTIs in cells with SIRT2 suppression, along with the suppression of cell death during mitosis. Since it is most likely that both suppression of cell death during mitosis and mitotic slippage are consequences of prolonged chronic mitotic arrest, we propose that prolonged chronic mitotic arrest is the primary cause of resistance to MTIs in cells with SIRT2 suppression.

SIRT2 suppression leads to the formation of supernumerary centrosomes in a nocodazole-dependent manner. Recently, it has been reported that SIRT2 is associated with the centrosome during prophase,[36] while SIRT2 maintains a largely cytoplasmic localization during interphase. Taken together with the insight obtained in the present study, this prompted us to examine the possible link between prolonged chronic mitotic arrest in cells with SIRT2 suppression and any alteration of centrosome function.

Immunostaining was performed using anti-γ-tubulin (a centrosome-specific marker) on cells with suppression of SIRT2, BubR1 or both, followed by nocodazole treatment. As shown in Figure 5, in the absence of nocodazole, one or two spots were observed and structural or numerical aberrations were not observed in any of the samples. Upon nocodazole treatment for 8 h and 16 h, the same trend was observed in cells with suppression of BubR1, both SIRT2 and BubR1, and the control cells. Surprisingly, more than 3 and sometimes close to 10 spots were observed in about half of the cells with SIRT2 downregulation after 8 h of treatment (Fig. 5). This trend was observed more notably after treatment for 16 h, although the spots became diffuse (Fig. 5).

The numerous aberrations observed in cells with SIRT2 suppression are likely due to centrosome fragmentation or disorganization, which has been reported to be observed in conjunction with DNA damage during mitosis.[37-39] We cannot rule out the possibility that this is caused by centrosome overduplication, although this is unlikely because centrosome duplication requires complicated processes, and transcription is inhibited during mitosis. After 24 h of nocodazole treatment, clear spots were not observed in any of the samples, most likely due to disassembly of the centrosome components themselves by prolonged inhibition of tubulin dynamics or prolonged mitotic inhibition of transcription (data not shown). Multipolar division was not observed at any period of nocodazole treatment either in cells with SIRT2 suppression or control cell (data not shown).

It is noteworthy that numerous centrosomal aberrations were observed in SIRT2 suppressed cells after 3 h of nocodazole treatment, while differences in nocodazole resistance, mitotic index, cell cycle distribution and cyclin B/Cdk1 activity between cells with SIRT2 downregulation and control cells were observed only after 24 h of treatment. This raised the possibility that the centrosomal aberrations were responsible for the subsequent differences, although we cannot determine whether these two phenomena are linked or independent. One thing that is certain is that SIRT2 is involved in maintaining centrosome structure upon exposure to mitotic stress caused by MTIs.

Discussion

The present study using siRNA-mediated SIRT2 downregulation demonstrates that SIRT2 suppression confers resistance to MTIs by prolonging chronic mitotic arrest and consequently preventing other secondary outcomes, including cell death, after release from chronic mitotic arrest. In other words, an important function of SIRT2 could be to promote the release of chronic mitotic arrest provoked by MTIs, leading to secondary outcomes.

In a previous study, we found that SIRT2 can block the entry to chromosome condensation and consequently suppresses mitotic index in the presence of MTIs.[16] SIRT2 is thus a novel mitotic checkpoint protein that functions in early metaphase, prior to the spindle checkpoint, to prevent further progression of mitosis, characteristics previously reported for the CHFR protein.[4] It has been reported that CHFR suppression by siRNA sensitizes oral squamous cancer cells to MTIs by reducing the ability to arrest at G_2/M phase.[40] We thus expected prior to the present study that SIRT2 suppression would sensitize HCT116 cells to MTIs; however, all the data obtained in the present study demonstrate that SIRT2 suppression confers resistance to MTIs. Insights obtained by these two studies are compatible, assuming that SIRT2 acts to block chromosome condensation at early metaphase in response to mitotic stress, while at the later stage, it acts to release the spindle checkpoint at an appropriate time in order to lead to other outcomes, including cell death during mitosis and cell death of tetraploids after mitotic slippage. The cellular response to exposure to MTIs depends on the cell line and the cell context, and is regulated by complicated processes. In fact, HCT116 cells, in which CHFR expression is downregulated, have an impairment of the checkpoint to metaphase, upstream of the spindle checkpoint,[41] so it is conceivable that the impact of SIRT2 suppression on mitotic regulation was observed at the spindle checkpoint rather than the checkpoint to metaphase in HCT116 cells.

We observed both activation of p38 MAP kinase and caspase-3 in cells with SIRT2 suppression after 30 h of nocodazole treatment with a 6 h delay vs. the control (Fig. 4A). The same trend (i.e., around a 6 h delay observed in cells with SIRT2 suppression vs. the control) was observed for the mitotic index and cyclin B/Cdk1 kinase activity (Fig. 3B and C), while a more than 24 h delay was apparent in the survival curves of chronic mortality assays (Fig. 2A) and the accumulation of cells with a >4C DNA content in cell cycle analyses (Fig. 3D). Thus, we cannot rule out the possibility that SIRT2 suppression prolongs not only chronic mitotic arrest, but also any process following it such as mitotic slippage, both of which contribute to resistance to MTIs.

The present study demonstrates for the first time that SIRT2 is involved in maintaining centrosome structure upon exposure to mitotic stress caused by MTIs, but not in normal cultures. Intriguingly, centrosomal aberration is the earliest event we know of so far that distinguishes cells with suppression of SIRT2 alone. It has been reported that centrosomes seem also to be affected by stresses such as heat, mitotic stress, radiation or viral infections, and centrosome fragmentation may normally work as a backup checkpoint mechanism.[42] This illustrates that a mitotic centrosome is capable of responding to a wide variety of stimuli and is thus influenced by environmental factors, and therefore its reaction to stress is part of signal transduction pathways and cell cycle checkpoints. In this context, there is a possibility that compromising SIRT2 function mediates as yet unknown signals to halt mitotic progression by prolonging chronic mitotic arrest in the presence of mitotic stress through centrosome fragmentation. We do not know whether centrosomal aberration by SIRT2 suppression followed by exposure to nocodazole or other events observed at a later stage, such as persistent activation of cyclin B/Cdk1 and suppression of mitotic slippage, are cause-and-effect sequences of events or independent. It is not clear whether substrates of SIRT2 identified so far as well as SIRT2 interacting proteins can account for this potential function. Interestingly, centrosomal aberration was suppressed by simultaneous suppression of SIRT2 and BubR1. Although the precise mechanism is unknown, it is tempting to

speculate that BubR1 may perform its functions on the spindle checkpoint provoked in part via centrosome regulation in an opposite manner to SIRT2.

A possible link between the integrity of the mitotic checkpoint and sensitivity to MTIs is presumed; however, some cancer cells with normal spindle checkpoints exhibit resistance to MTIs.[13] The present study raises the possibility that SIRT2 is a new diagnostic marker candidate with high accuracy for predicting the sensitivity to MTIs, particularly for spindle checkpoint-proficient cancer cells, in order to avoid unnecessary treatment. We observed that siRNA-mediated SIRT2 suppression did not confer resistance to MTIs in some colorectal cancer cell lines, although the levels of SIRT2 expression and the degree of siRNA-mediated suppression of SIRT2 are comparable to those of HCT116 cells. Thus, it seems that whether SIRT2 downregulation leads to resistance to MTIs depends on the cellular context and the status of other molecules. Further study to reveal what determines the difference in outcome for SIRT2 downregulation is required to determine whether SIRT2 can be used as a molecular marker of tumors that will respond to MTIs.

Materials and Methods

Cell culture and siRNA transfection. All cells were cultured in the appropriate medium containing 10% fetal bovine serum (Invitrogen). HCT116 and HCT116 $p53^{-/-}$ cells were kindly provided by Dr. Bert Vogelstein (Baltimore). hiMSC cells[28] were kindly provided by Dr. Junya Toguchida (Kyoto University). Nocodazole and paclitaxel were obtained from Sigma and kept in DMSO. Nocodazole was used at a final concentration of 200 nM and 400 nM in HCT116 cells and hiMSC cells, respectively, and paclitaxel was used at a final concentration of 20 nM in both cell lines. Cambinol and AGK2 were obtained from Calbiochem and kept in DMSO.

TAP-SIRT2, a construct encoding a fusion of SIRT2 with two tandem affinity peptide (TAP) tags (a streptavidin binding peptide and a calmodulin binding peptide) was generated from the pNTAP plasmid (Stratagene). HCT116 cell clones stably expressing TAP-SIRT2 were obtained by transfection using Lipofectamine 2000 (Invitrogen) followed by selection for 12–14 days in the presence of 800 μg/ml G418 (Invitrogen).

siRNAs corresponding SIRT2 and BubR1 mRNAs were obtained from Qiagen and Ambion, respectively. Both siRNAs have been validated for knockdown by quantitative RT-PCR. Negative control siRNA was obtained from Qiagen. Cells at 50% confluence were transfected with 40 nM siRNA by using Lipofectamine 2000, according to the manufacturer's instructions.

Chronic mortality assays. Cells transfected with siRNA were seeded in 60-mm dishes in triplicate and treated with nocodazole or paclitaxel for the indicated durations. The cells were washed with PBS once and incubated in drug-free medium for 10 days. The resultant colonies were stained with 1% crystal violet/70% ethanol and then counted using NIH Scion Image software (freeware).

Western blotting and immunocytochemistry. Antibodies to SIRT2 were raised as we previously reported.[16] For western blotting analysis, equal amounts of protein samples or protein samples derived from an equal number of cells were separated on 10%, 12.5% or 15% polyacrylamide gels and transferred to a nitrocellulose membrane (Amersham Pharmacia). Western blotting was carried out using polyclonal anti-SIRT2 antibody (1:2,000), monoclonal anti-cyclin B antibody (1:600, Santa Cruz), monoclonal anti-BubR1 antibody (1:600, Rockland), polyclonal anti-p38 MAP kinase (1:1,000, Cell Signaling Technology), polyclonal anti-phospho p38 MAP kinase (Thr180/Tyr182) (1:1,000, Cell Signaling Technology), polyclonal anti-phospho SAPK/JNK (Thr183/Tyr185) (1:1,000, Cell Signaling Technology), monoclonal anti-cleaved-caspase-3 (Asp175) (1:1,000, Cell Signaling Technology), and anti-β-tubulin (1:2,000, Amersham Pharmacia) as a primary antibody. A 1:4,000 dilution of peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin (Amersham Pharmacia) was used as the secondary antibody. Can Get Signal Immunoreaction Enhancer Solution™ (Toyobo) was used as an antibody diluent. Signals were visualized using the ECL detection system (Amersham Pharmacia). For immunostaining, the cells were fixed with 4% paraformaldehyde/PBS for 20 min at room temperature and permeabilized with 0.2% Triton X-100/PBS for 20 min at room temperature. Immunostaining was carried out using monoclonal anti-γ-tubulin antibody (1:500, Sigma) and anti-BubR1 antibody (1:500) as a primary antibody with 5% skim milk/TBS as an antibody diluent. FITC-conjugated anti-mouse immunoglobulin (1:2,000, Chemicon) was used as a secondary antibody. The slides were counterstained with DAPI and viewed under a Leica confocal microscope (TCS SP2). Leica confocal software was used to produce images.

Microscopic analysis of chromosomes. For metaphase preparation, cells were treated with a hypotonic solution of 75 mM KCl for 15 min at room temperature. Next, the cells were fixed with methanol/acetic acid (3:1) and dropped onto glass microscope slides. The slides were dried and stained with 5% Giemsa for 30 min.

Flow cytometry analysis. For DNA content evaluation, cells were collected by trypsinization, fixed in 4% paraformaldehyde/PBS at room temperature and then in cold 70% ethanol/30% PBS, and stored at -20°C until flow cytometry analysis. For mitotic index evaluation, cells prepared as described above were incubated with anti-phospho-Ser/Thr-Pro, and MPM2 antibody (Upstate) and FITC-conjugated anti-mouse immunoglobulin (Upstate) as primary and secondary antibodies, respectively, according to manufacturers' instructions. Flow cytometric analyses were performed using an EPICS ELITE 4.0 (Coulter). For each analysis, 10,000 gated events were collected to permit analysis of cell populations. Data analysis and figure generation were performed using the WinMDI 2.9 software program (freeware).

Cyclin B/Cdk1 kinase assays. Cyclin B/Cdk1 kinase assays were performed as previously reported.[43] Briefly, whole cell extracts were prepared in 50 mM Tris (pH 7.9), 120 mM NaCl, 0.5% NP-40, 10 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM EGTA, 1.5 mM $MgCl_2$, 1 mM DTT, and a cocktail of protease inhibitors (Roche). Whole cell extracts were incubated with 1.5 μg histone H1 (Calbiochem) as