DAZ-interacting Protein 1 (Dzip1) Phosphorylation by Polo-like Kinase 1 (Plk1) Regulates the Centriolar Satellite Localization of the BBSome Protein during the Cell Cycle

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The function of the primary cilia, which is assembled in most vertebrate cells, is achieved by transport in and out of kinds of signaling receptors. The BBSome protein complex could recognize and target membrane proteins to the cilia, but how the BBSome itself is transported into the cilia is poorly understood. Here we demonstrate that the centrosome protein Dzip1 mediates the assembly of the BBSome-Dzip1-PCM1 complex in the centriolar satellites (CSs) at the G0 phase for ciliary translocation of the BBSome. Phosphorylation of Dzip1 at Ser-210 by Plk1 (polo-like kinase 1) during the G2 phase promotes disassembly of this complex, resulting in removal of Dzip1 and the BBSome from the CS. Inhibiting the kinase activity of Plk1 maintains the CS localization of the BBSome and Dzip1 at the G2 phase. Collectively, our findings reveal the cell cycle-dependent regulation of BBSome transport to the CS and highlight a potential mechanism that the BBSome-mediated signaling pathways are accordingly regulated during the cell cycle.

The primary cilium, an antenna-like organelle that extends from the basal body, is structurally dynamic during the cell cycle, with assembly after mitosis and disassembly before mitotic entry. Ciliary dysfunction often correlates with developmental disorders and abnormal cell proliferation (1). Assembling a functional cilium involves the precise arrangement of different proteins at the ciliary membrane, axoneme, basal body, and centriolar satellites (CSs).3 The BBSome, which was first identified in a patient with Bardet-Biedl syndrome, plays important roles in ciliary function (1). It consists of BBS1, -2, -4, -5, -7, -8, and -9 proteins (2) and is assembled by sequential joining of BBS1, BBS4, BBS5, and BBS8 to the BBS2-BBS7-BBS9 core complex (3). The BBSome can bind and target membrane vesicles to the ciliary membrane by reading sorting signals presented by the ciliary membrane-destined proteins on the vesicles (4). Through BBS4 and BBS8 subunits (2), the BBSome is recruited to the CS by pericentriolar material protein 1 (PCM1), a key component residing in the CS for recruiting proteins to the centrosome (5). In the CS the BBSome complexes with the intraflagellar transport (IFT) complex for the ciliary transportation of itself and its associated cargoes (6). The BBSome also recruits Rab8, the guanine nucleotide exchange factor for Rab8, to the basal body to activate Rab8 for ciliary assembly (2).

DAZ interacting protein 1 (Dzip1), a zinc finger-containing protein originally identified in zebrafish, is predominantly expressed in human embryonic stem cells and germ cells (7, 8). Fish with a Dzip1/iguana mutation develop an abnormal midline and body shape (9, 10) with defects in the Hedgehog (Hh) signaling pathway (8, 11). Dzip1 is also involved in Hh signaling in mice (12) by regulating the turnover of Gli proteins (12–14). In addition to its role in the regulation of Hh signaling, Dzip1 is also required for ciliogenesis in zebrafish Kupffer’s vesicle cells (15, 16) and in cultured mammalian cells (12, 17, 18). We previously showed that Dzip1 is phosphorylated at Ser-520 by GSK3β kinase after mitosis and that phosphorylation increases its GDP dissociation inhibitor displacement factor (GDF) ability to facilitate the production of Rab8GDP at the basal body during ciliogenesis (18). In this study we show that a portion of Dzip1 that localizes at the CS binds to PCM1, thereby mediating the recruitment of the BBSome to the CS. We also show that this process is negatively regulated by the activation of polo-like kinase 1 (Plk1), a serine/threonine kinase, during the G2 phase.

Results

Dzip1 Interacts with the BBSome and Regulates the Ciliary Translocation of the BBSome—Recently, we showed that Dzip1 regulates ciliary membrane assembly after mitosis (18). To understand whether Dzip1 regulates additional processes during ciliogenesis, we examined the dynamic localization of several proteins that localize to the cilia. Comparing that in the control cells, the ciliary localization of BBS1 and BBS5 were
both impaired in Dzip1 stable knockdown 1308-3 cells (Fig. 1, A and B), which show shortened cilia and a decreased ratio of ciliation (18). The loss of ciliary localization of BBS1 and BBS5 in 1308-3 cells could be recovered by introducing the RNA interference (RNAi)-resistant form of Dzip1 (Fig. 1, A and B), suggesting that the function of the BBSome is impaired by Dzip1 knockdown. We then tested the interaction between Dzip1 and the BBSome. The results showed that endogenous Dzip1 was immunoprecipitated from G0 phase NIH 3T3 cells and probed for the indicated BBSome subunits. E, Myc-Dzip1 binds GFP-tagged BBS1, BBS2, BBS4, BBS5, BBS8, and BBS9 proteins in HEK 293T cells arrested at the G0 phase. Immunoprecipitation was performed using an anti-GFP antibody, and Western blotting was conducted using an anti-Myc antibody. The relative binding affinity of Dzip1 with the BBSome subunits was each normalized to that in BBS1-expressing cells.

**FIGURE 1.** Dzip1 interacted with the BBSome and regulates its ciliary translocation. A and B, Dzip1 knockdown in cells impairs the ciliary localization of BBS1 and BBS5. The RNAi control cells, Dzip1-silenced (1308-3) cells, or 1308-3 cells expressing RNAi resistant Dzip1 were immunostained for the indicated proteins. The boxed areas in the main image are zoomed in the right panels and presented as separate channels. The values are the means ± S.D.; 50 cells per sample were counted in each of three independent experiments. ***, p < 0.001. DNA was stained by DAPI. C, Dzip1 and the BBSome subunits partially co-localized at the CS. NIH 3T3 cells arrested at the G0 phase were immunostained for the indicated proteins. Scale bars, 5 μm. D, Dzip1 interacts with the BBSome. Endogenous Dzip1 was immunoprecipitated from G0 phase NIH 3T3 cells and probed for the indicated BBSome subunits. E, Myc-Dzip1 binds GFP-tagged BBS1, BBS2, BBS4, BBS5, BBS8, and BBS9 proteins in HEK 293T cells arrested at the G0 phase. Immunoprecipitation was performed using an anti-GFP antibody, and Western blotting was conducted using an anti-Myc antibody. The relative binding affinity of Dzip1 with the BBSome subunits was each normalized to that in BBS1-expressing cells.
which forms a core scaffold for BBSome assembly (3, 21), resulted in the dissociation of BBS4, BBS5, Dzip1, and PCM1 from the BBSome (Fig. 2E), but PCM1 knockdown only mildly affected the association of Dzip1 with the BBSome (Fig. 2F), suggesting a sequential binding of BBSome subunits to CS. Cells transfected with Dzip1 or non-targeting control siRNAs were immunostained for BBS1, BBS4, BBS5, Cep290, and PCM1. Scale bars, 5 μm. E and F, the integrity of the BBSome is required for its binding to Dzip1. BBS1 or PCM1 was silenced in HEK 293T cells at the G0 phase expressing GFP-BBS8. The indicated proteins were analyzed on a sucrose density gradient. Note that BBS1 knockdown resulted in the redistribution of the BBSome subunits, PCM1 and Dzip1 in lower density fractions of the gradient (arrowheads).

FIGURE 2. Dzip1 linked the BBSome and PCM1 to form the BBSome-Dzip1-PCM1 complex. A and B, Dzip1 knockdown impairs the interaction between PCM1 and the BBSome. GFP-BBS8-expressing HEK 293T cells were arrested at the G0 phase and silenced for the RNAi control or Dzip1. Thereafter, the distribution of the indicated proteins was examined. Compared with the control, Dzip1 knockdown resulted in the distribution of PCM1 in lower density fractions (arrowheads). C, Dzip1 knockdown impairs the interaction between BBS4 and PCM1. GFP-BBS4 was immunoprecipitated from G0 phase cells, and the indicated proteins were analyzed. The relative RNAi efficiency of Dzip1 was normalized to that in GFP-expressing cells. Asterisks denote the IgG heavy chain. D, Dzip1 knockdown decreases the localization of BBSome subunits at the CS. Cells transfected with Dzip1 or non-targeting control siRNAs were immunostained for BBS1, BBS4, BBS5, Cep290, and PCM1. Scale bars, 5 μm. E and F, the integrity of the BBSome is required for its binding to Dzip1. BBS1 or PCM1 was silenced in HEK 293T cells at the G0 phase expressing GFP-BBS8. The indicated proteins were analyzed on a sucrose density gradient. Note that BBS1 knockdown resulted in the redistribution of the BBSome subunits, PCM1 and Dzip1 in lower density fractions of the gradient (arrowheads).

Dzip1 Phosphorylation by Plk1 Promotes Dissociation of Dzip1 from the CS at the G2 Phase—We previously reported that Dzip1 localizes to the centrosome and the CS from the G0/G1 phase until the early G2 phase (18). Herein, we studied how this is achieved and whether this regulates the
CS localization of the BBSome during the cell cycle. As Plk1 is recruited to the CS by PCM1 at the G2 phase (20), we speculated that Plk1 regulates Dzip1 removal from the CS. Monitoring the expression and localization changes of Cyclin B1, which only shows centriolar localization at the early G2 phase and cytoplasmic localization as well as centriolar localization at the middle G2 phase to finally accumulate at the centrosome and the nucleus at the late G2 phase (26), we found that Plk1 showed a clean centriolar localization at the early and middle G2 phases, and notably, it became concentrated at both the CS and the centrioles at the late G2 phase (Fig. 4A, upper panels). By super-resolution microscopy, we found that inhibition of Plk1 kinase activity by treating cells with the Plk1 inhibitor BI2536 maintained the localization of Dzip1 in the CS at the G2 phase (Fig. 4A and B). In accordance with this, Dzip1 was observed a clean centriolar localization at the early and middle G2 phases, and notably, it became concentrated at both the CS and the centrosome and the nucleus at the late G2 phase (26). We then investigated how Plk1 regulates the removal of Dzip1 from the CS. Immunoprecipitation and pulldown assays showed that Plk1 bound with Dzip1 through its polo-box domain (PBD) (Fig. 4C; supplemental S2, C and D). However, Dzip1 was hardly pulled down by using the Plk1 PBD-2A mutant (supplemental Fig. S2D), indicating that the interaction of Dzip1 with Plk1 is dependent on priming phosphorylation(s) of Dzip1. The in vitro kinase assay showed that Plk1 phosphorylated full-length GFP-Dzip1 immunoprecipitated from G2-phase cells (supplemental Fig. S2E). Phosphor-peptide mass spectrometry (MS) identification showed that Dzip1 was phosphorylated at Ser-210 in G2-phase cells (Fig. 4D). When the cells were treated with BI2536, the ratio of phosphor-Ser-210-containing peptide to its corresponding peptide that was captured sharply decreased from ≥90% to 5%, indicating that inhibition of Plk1 eliminates the phosphorylation of Dzip1 at Ser-210. To specifically test the ability of Plk1 to phosphorylate this site of Dzip1, we further performed in vitro kinase assay using GFP-tagged fragments of wild-type Dzip1149–250 and its
S210A mutant Dzip1149–250:S210A as Ser-210 was the only site predicted as potential sites for Plk1 phosphorylation within amino acids 149–250 of Dzip1. Consistent with that shown by MS identification, Plk1 could phosphorylate Dzip1149–250 but not Dzip1149–250:S210A (Fig. 4E). Moreover, treating cells with BI2536 or expressing the Dzip1 S210A mutant, both, significantly increased the binding of Dzip1 with PCM1 (Fig. 4F and G), but the Dzip1 S210D mutant, which mimicked the phosphorylated form of Dzip1 at Ser-210, showed much weaker PCM1 binding intensity compared with that of Dzip1 WT (Fig. 4H). Taken together, these results demonstrate that phosphorylation of Dzip1 at Ser-210 by Plk1 disrupted its binding to PCM1 and promotes its removal from the CS in G2 phase cells.

The BBSome-Dzip1-PCM1 Complex Disassembles upon Dzip1 Phosphorylation at Ser-210 by Plk1—Next, we investigated whether Plk1 kinase activity also affects the binding of the BBSome to PCM1. The sucrose density gradient centrifugation assay showed that the BBSome subunits in G2-phase cells still concentrated in the same higher density fractions, whereas PCM1 and Dzip1 shifted down and remained in different fractions with the BBSome subunits, indicating that the binding of the BBSome with PCM1 and Dzip1 largely decreased at the G2 phase (Fig. 5A). Notably, when G2-phase cells were treated with BI2536, PCM1 and Dzip1 shifted to the same higher density fractions with the BBSome, indicating that inhibiting Plk1 maintained formation of the BBSome-Dzip1-PCM1 complex in cells (Fig. 5B). Consistently, whereas Cep290 still localized in the CS, BBS4 and the other BBSome subunits disappeared from the CS in G2-phase cells, which could be recovered by BI2536 treatment (Fig. 5C and D). To directly examine the binding ability of the BBSome with PCM1 at the G2 phase, we also performed immunoprecipitation assays. The results showed
that whereas the binding of Dzip1 and BBS1 with PCM1 nearly totally disappeared, Plk1 inhibition by BI2536 rescued these interactions (Fig. 5E).

Finally, to understand whether this BI2536-induced localization was due to dephosphorylation of Dzip1 at Ser-210, G2-phase cells expressing GFP-tagged wild type, the S210A/D mutant of Dzip1 or GFP alone was respectively subjected to a sucrose density gradient centrifugation assay. In cells expressing GFP-Dzip1WT, GFP-Dzip1S210D, or GFP, PCM1 and GFP-Dzip1, but not the BBSome subunits, shifted to lower sucrose density gradient fractions (Fig. 6, A and B), indicating that PCM1 and Dzip1WT or Dzip1S210D failed to efficiently establish a complex with the BBSome. However, most PCM1 and GFP-Dzip1S210A, but not GFP-Dzip1WT and GFP-Dzip1S210D, redistributed in higher density gradient fractions with the BBSome (Fig. 6C), indicating that dephosphorylation of Dzip1 at Ser-210 maintains the association of the BBSome with PCM1. Consistently, the CS localization of the BBSome subunits and GFP-Dzip1 itself were both maintained in G2-phase cells by introducing the S210A mutant but not the WT and the S210D mutant (Fig. 6, E and F). We also confirmed that introducing exogenous Dzip1 did not significantly affect the expression levels of the examined proteins (supplemental Fig. S3B). Together, these results demonstrate that phosphorylation of Dzip1 at Ser-210 by Plk1 promotes dissociation of the BBSome from PCM1 and the removal of the BBSome and Dzip1 from the CS at the G2 phase.

**Discussion**

Assembly of a functional cilium requires trafficking of membrane vesicles from the cell body to the ciliary membrane and the establishment of the signaling receptor proteins localizing at specific sites within it. In this study, we demonstrate that Dzip1-mediated targeting of the BBSome to the CS is essential for the translocation of the BBSome into the primary cilium at
the G₀ phase. When Plk1 is recruited to the CS by PCM1 and its kinase activity elevates at the G₂ phase, the BBSome-Dzip1-PCM1 complex disassembles due to Plk1-dependent phosphorylation of Dzip1 at Ser-210, which disrupts the interaction between Dzip1 and PCM1 as well as the CS localization of the BBSome (Fig. 7). It is well known that the BBSome mediates kinds of receptor protein trafficking in and out of the cilia. Therefore, the formation of the BBSome-Dzip1-PCM1 complex and the Dzip1-mediated translocation of the BBSome with cargo-containing membrane vesicles to the CS must play important roles for regulation of these signaling pathways. Given the dynamic CS localization of the BBSome during the cell cycle, it is conceivable that these signaling pathways are accordingly turned on and off by cells. The precisely regulated turning on-and-off of these signaling pathways may be crucial for the transcriptional regulation of their downstream target genes that are essential for progression of the cell cycle, such as the Hh signaling downstream genes D-type and E-type cyclins (27, 28).

**FIGURE 6.** Dephosphorylation of Dzip1 at Ser-210 partially recovered the assembly of the BBSome-Dzip1-PCM1 complex and the CS localization of the BBSome at the G₂ phase. A–D, non-phosphorylation of Dzip1 at Ser-210 preserves the association of the BBSome and PCM1 in cells at the G₂ phase. GFP-BBS8-expressing HEK 293T cells at the G₂ phase were transfected with GFP (A), GFP-Dzip1WT (B), GFP-Dzip1S210A (C), or GFP-Dzip1S210D (D). Arrowheads indicate the redistribution of the indicated proteins. Note that the BBSome subunits, PCM1, and Dzip1 were found in the same high density fractions in cells expressing non-phosphorylated Dzip1 (C). E and F, non-phosphorylation of Dzip1 at Ser-210 preserves the CS localization of the BBSome subunits. NIH 3T3 cells expressing GFP-Dzip1WT, GFP-Dzip1S210A, or GFP-Dzip1S210D were synchronized to G₂ phase, and the indicated proteins were immunostained. Note that in addition to the centriolar localization like that of GFP-Dzip1WT, GFP-Dzip1S210A also showed the CS localization (E). Numbered boxes are zoomed in the right panels and presented as separate channels. The values are the means ± S.D.; 30 cells per sample were counted in each of three independent experiments (F). **, p < 0.01. Scale bars, 5 μm.

**FIGURE 7.** A model elucidating the regulation of BBSome CS localization by Dzip1 and Plk1 during the cell cycle. See the details in the “Discussion” section.
Regulation of the CS Localization of BBSome in Cell Cycle

Besides Dzip1, other CS-localized proteins also regulate the ciliary translocation of the BBSome. Among these proteins, PCM1 functions as a scaffold to host other CS proteins, and it is essential for the formation of the CS and assembly of the cilia. It interacts with Cep72 to recruit Cep290 to the CS. Both Cep72 and Cep290 are required for the CS localization of BBS4 and ciliary translocation of BBS4 and BBS8 during ciliogenesis (25). We found that depletion of PCM1 results in the disappearance of Dzip1 from the CS but that Dzip1 knockdown has no affect on the CS localization of Cep290 and PCM1, indicating that Dzip1 functions downstream of the PCM1-Cep290 axis for the assembly of the BBSome in the CS rather than regulating the integrity of the CS. Unlike Dzip1, Cep131, another CS protein that also interacts with the BBSome and is not involved in BBSome assembly, restricts ciliary recruitment of the BBSome (22). NPHP5, a protein that localizes at the transition zone, is required for the integrity of the BBSome, but its loss-of-function will mistakenly allow the malformed BBSome (lacking BBS2 and BBS5) to enter the cilium (29). Comparing the different roles of Dzip1, Cep131, and NPHP5 in regulating the ciliary transport of the BBSome, we propose that whereas all these proteins function as gatekeepers for proteins entering the cilium, they execute discriminated functions in the process.

Finally, as the BBSome could recruit Rabin8 to the basal body to activate Rab8GTP production for ciliary assembly (2), the dynamic CS localization of the BBSome provides a possibility that the production of active Rab8 at the basal body is dynamic during the cell cycle, with a relatively high level from the G0 to G1 phases that begins to progressively decrease at the G2 phase. On the other hand, removal of Dzip1 from the CS by Plk1 also helps to decrease the production of Rab8GDP at the G2 phase, as a portion of Dzip1 also functions as a GDF (GDP dissociation inhibitor displacement factor) at the centrosome (18). Therefore, we speculate that both of these two lines may cooperatively function to inhibit cilia assembly or to promote cilia disassembly during the G2 phase, which underlie alternative mechanisms that are different from the PCM1-Plk1-HDAC6 (20) and HEF1-Aurora A-HDAC6 (30) axes for ciliary dynamic control during the cell cycle.

Experimental Procedures

**Antibodies and Reagents**—BII536 (Axon Medchem, catalog no. 1129), rabbit anti-Dzip1 (Abgent, catalog no. AP8926c), anti-BBS4 (Proteitech, catalog no. 12766-1-AP), anti-BBS5 (Proteitech, catalog no. 14569-1-AP), anti-CEP290 (Proteitech, catalog no. 22490-1-AP), anti-PC1 (Santa Cruz Biotechnology, catalog no. sc67204), anti-Cyclin A2 (Santa Cruz Biotechnology, catalog no. sc751), anti-Cyclin B1 (Santa Cruz Biotechnology, catalog no. sc594), goat anti-BBS1 (Santa Cruz Biotechnology, catalog no. sc49790), mouse anti-Plk1 (Santa Cruz Biotechnology, catalog no. sc17783), anti-GAPDH (Proteitech, catalog no. 60004-1-lg), anti-GFP (MBL, catalog no. M048-3), anti-Myc (Sigma, catalog no. M4439), and acetylated α-tubulin (Sigma, T7451) were purchased from the indicated companies. For the immunoprecipitation assay, serum anti-Dzip1 was produced in rabbit immunized with a peptide of mouse Dzip1 (KPSSTSPSPQELRTN). All animal experiments were performed according to approved guidelines.

**Cell Culture, Synchronization, and Transfection**—NIH 3T3 and HEK 293T cells were from American Type Culture Collection (ATCC). Cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum under standard culture conditions and were confirmed without mycoplasma contamination. Cells arrested at the G0 phase were obtained by culturing for 36 h in medium supplemented with 0.5% serum. Cells blocked at the G1/S transition were treated with thymidine for 18 h followed by a brief interruption for 9 h and then treatment with thymidine for an additional 18 h. S- and G2/M-phase cells were obtained after the induction of G1/S release for 4 or 8 h, respectively. The efficiency of synchronization was confirmed by Western blotting using antibodies against the cyclin proteins. To manipulate the kinase activity of Plk1, BI2536 (100 nM) was added to the medium. Transient cDNA transfections were carried out on cells using MegaTran transfection reagent (Origene, catalog no. TT20002) according to the manufacturer’s instructions. The Dzip1 stable knockdown cell line 1308-3 was maintained as previously described (18).

**Molecular Cloning and RNAs**—Human BBS1, BBS2, BBS4, BBS5, BBS8, and BBS9 and mouse Dzip1 were cloned from the human B-cell and mouse embryo brain (E14) cDNA libraries, respectively. The full-length PCNTB, Plk1-PCNTB, and Plk1KD-PCNTB cDNAs (19) were kind gifts from Dr. Kunsoo Rhee (Seoul National University, Republic of Korea). The siRNA sequence for human BBS1 was 5’-GUUACAGGGCAUCAGCA-3’. The DNA fragments for expressing the shRNA targeting human Dzip1 (sense sequence: 5’-GATCCCCGAGATCAACCGTACCAGATATGTTGGACTTGATCTGCTTTAGATGTCCTTTTAT-3’) were generated by annealing the indicated pairs of oligonucleotides and ligating into the pSuper-RetroPuro vector (OligoEngine). The knockdown sequences of human and mouse PCM1 were identical to those previously described (20).

**Immunofluorescence Microscopy**—The cells were fixed by 4% paraformaldehyde in phosphate-buffered saline (PBS) and then extracted with 0.2% Triton X-100 in PBS or directly fixed by cold methanol followed by immunostaining with the indicated primary and secondary antibodies. DNA was stained with DAPI. To visualize the localization of the BBSome subunits in the CS, cells were fixed as previously described (2). Images were captured by the confocal immunofluorescence microscope (Carl Zeiss, LSM-710NLO and DuoScan) or by the three-dimensional structured super-resolution illumination microscope (Nikon, i-Stitch) as previously described (18).

**Immunoprecipitation and Immunoblotting**—NIH 3T3 or HEK 293T cells transfected with the indicated plasmids were lysed using IP buffer (50 mM HEPES (pH 7.0), 125 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, and 0.5 mM PMSF) on ice for 15 min. The lysates were centrifuged at 20,000  ×  g for 15 min, and the supernatants were incubated with the primary antibody-coated beads for 1.5 h at 4 °C on a rotator. After 6 washes with IP buffer, the beads were collected, and the bound proteins were analyzed by Western blotting. Each IP and Western blotting assay was repeated independently at least twice. The intensities of the indicated bands were quantified using ImageJ software (National Institutes of Health).

**Sucrose Density Gradient Ultracentrifugation**—BBSome assembly was assessed as previously described (21) with modi-
fications. Briefly, proteins were extracted from HEK 293T cells cultured under the indicated experimental conditions with IP buffer and concentrated to ~100 μl with Microcon centrifugal filter devices (50,000 molecular weight cutoff, Millipore). The proteins were then loaded onto a 1.4-ml 10–40% sucrose density gradient in PBS/Triton X-100 (138 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, and 0.04% Triton X-100 (pH 7.4)) and centrifuged at 166,000 × g for 20 h. Fractions (~100 μl each) were carefully collected from the top, combined with loading buffer, and analyzed by Western blotting.

Protein Expression and Purification and in Vitro Kinase Assay—Wild-type and 2A-mutant GST-PBD were expressed and purified from Escherichia coli BL21 cells as previously described (20). As the N terminus of Dzip1 was difficult to purify from prokaryotic cells, we purified full-length GFP-Dzip1 and the GFP-Dzip1 fragment from HEK 293T cells by immunoprecipitation. Beads coated with equal amounts of GFP-Dzip1 or its mutant were combined with Plk1 kinase (Life Technologies, catalog no. PV3501). The reaction was supplemented with 10 μCi [γ-³²P]ATP and incubated for 30 min at 30 °C. Loading buffer was added to stop the reaction. After electrophoresis of samples by SDS-PAGE, the gel was exposed to X-ray film for 6 h or overnight.

Phosphor-peptide Identification by Mass Spectrometry—Full-length GFP-tagged mouse Dzip1 was immunoprecipitated from HEK 293T cells that were synchronized at the G2 phase. Before harvest, the cells were incubated with or without BI2536 for 4 h. The samples were electrophoresed by SDS-PAGE and Coomassie Brilliant Blue-stained to visualize the protein bands. The GFP-Dzip1 bands were cut down and subjected to MS analysis. The phosphor-peptides of Dzip1 were identified as previously described (18). The spectrum showing “intensity versus m/z” in this manuscript is a snap from the software of MS results analysis. The b and y ion pairs are each labeled to show the molecular weight of the peptide fragments, and the peak of phosphorylation is marked in green.

Statistical Analysis—Statistical analysis was performed using Microsoft Office Excel 2007. We selected at least 150 samples in total in each of our experiment to achieve an adequate power of >0.8. p values were calculated by the paired t test from the mean values of the data. Significant differences were marked with asterisks (**, *p < 0.001; **, *p < 0.01).

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