Polo-like kinase-1 triggers histone phosphorylation by Haspin in mitosis

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Abstract

Histone modifications coordinate the chromatin localization of key regulatory factors in mitosis. For example, mitotic phosphorylation of Histone H3 threonine-3 (H3T3ph) by Haspin creates a binding site for the chromosomal passenger complex (CPC). However, how these histone modifications are spatiotemporally controlled during the cell cycle is unclear. Here we show that Plk1 binds to Haspin in a Cdk1-phosphorylation-dependent manner. Reducing Plk1 activity decreases the phosphorylation of Haspin and inhibits H3T3ph, particularly in prophase, suggesting that Plk1 is required for initial activation of Haspin in early mitosis. These studies demonstrate that Plk1 can positively regulate CPC recruitment in mitosis.

Keywords cyclin-dependent kinase; haspin; histone modification; mitosis; polo-like kinase-1

Subject Categories Cell cycle; Chromatin, Epigenetics, Genomics & Functional Genomics

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Introduction

Histone modifications coordinate a variety of chromatin functions, including gene transcription and DNA damage responses, and mitosis appears to be no exception [1]. For example, phosphorylation of Histone H3 at threonine-3 (H3T3ph) by Haspin [2] provides a binding site for Survivin, a subunit of the Chromosomal Passenger Complex (CPC), and therefore modulates Aurora B function, particularly at centromeres [3–8]. H3T3ph also may be involved in the displacement from mitotic chromatin of H3K4-binding complexes such as TFIID [1,9]. Phosphorylation of histone H3 at serine-10 (H3S10ph, generated by Aurora B) and Histone H2A at threonine-120 (H2AT120ph, generated by Bub1) are additional histone modifications that may facilitate displacement of HP1 and recruitment of

Shugoshin to mitotic chromatin, respectively [8,10–12]. However, the key question of how the location and timing of such histone modifications in mitosis are controlled remains incompletely answered.

We previously found that Aurora B plays a significant role in regulating Haspin and H3T3ph. If Aurora B or other components of the CPC are inhibited or depleted, H3T3ph declines significantly, though it is not abolished [13]. Furthermore, because the product of Aurora B-stimulated Haspin activity, H3T3ph, is also a binding site for the CPC, a positive feedback loop is formed that can drive H3T3ph generation and CPC localization on chromatin [13]. This process is further enhanced by the ability of Aurora B to inhibit the chromatin localization of the Repo-Man-PP1 complex that dephosphorylates H3T3ph [14–16]. The Bub1-H2AT120ph pathway plays an additional role in CPC recruitment [8,12] that might boost Aurora B and H3T3ph specifically at centromeres [13]. However, Haspin produces H3T3ph mainly on chromosome arms early in mitosis, before concentrating at centromeres [2,17,18], and the mechanism by which Haspin activity is initially triggered is unknown.

We previously found that Haspin is phosphorylated at consensus motifs of Cyclin-dependent kinase (Cdk) and Polo-like kinase-1 (Plk1) in mitosis [13]. Here, we report that regulation of Haspin by Plk1 is particularly important for the deposition of H3T3ph early in mitosis, and may serve as a trigger for the Aurora B-H3T3ph feedback loops that drive CPC localization.

Results and Discussion

Plk1 is required for normal phosphorylation of Haspin and H3T3 in mitosis

We first examined the effect of Plk1 inhibitors on Haspin in mitotic cells. HeLa Tet-On cells expressing low levels of myc-Haspin in the absence of doxycycline induction [2] were arrested in mitosis with nocodazole, and then treated with kinase inhibitors for 1 h (in the presence of MG132 to prevent mitotic exit). Two distinct Plk1 inhibitors, BI 2536 [19] and GSK461364A [20] caused a similar increase in the mobility of Haspin in gels, consistent with reduced phosphorylation in mitosis (Fig 1A). Furthermore, inhibition of Plk1 caused a

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Figure 1. Plk1 activity is required for normal phosphorylation of Haspin and H3T3 in mitosis.

- A** Uninduced HeLa Tet-On/myc-Haspin cells were treated with 0.6 μ M nocodazole for 18 h. Mitotic cells collected by shake-off were replated in the presence of nocodazole with MG132 and indicated inhibitors for 1 h. Immunoblots of cell lysates are shown.
- B** HeLa cells were transfected with control, Plk1, Aurora B or Haspin siRNA and, after 48 h, subjected to immunofluorescence.
- C** RPE1 cells were released from double thymidine block and, after 7 h, kinase inhibitors were added for 2 h, followed by immunofluorescence microscopy. Scale bar, 5 μ m.
- D** Quantification of H3T3ph and H3S10ph staining from the experiment shown in (C). See supplementary Methods.

partial but substantial reduction in the phosphorylation of H3T3, indicating that Plk1 contributes to Haspin activity in prometaphase-like cells. In these conditions, Plk1 inhibition did not influence H3S10ph, a product of Aurora B activity (Fig 1A). Interestingly, although the reduction in the apparent molecular weight of Haspin was greater than that seen upon inhibition of Aurora B (with either ZM447439 or Hesperadin [21,22]), Plk1 inhibition had a lesser effect on H3T3ph than did inhibition of Aurora B in nocodazole-arrested prometaphase cells (Fig 1A).

We then determined the effect of reduced Plk1 activity in cells entering mitosis. Chemical inhibition of Plk1 during mitotic entry strongly reduced H3T3ph in HeLa cells (supplementary Figs S1A, S5) and depletion of Plk1 by RNA interference (RNAi) also decreased H3T3ph levels and the apparent molecular weight of Haspin in mitotic HeLa Tet-On/myc-Haspin cells (supplementary Fig S1B). Immunofluorescence microscopy following Plk1 RNAi revealed that this reduction in H3T3ph was most apparent in prophase (Fig 1B), when H3T3ph is predominantly found on chromosome arms [2]. We confirmed this finding using kinase inhibitors in a non-tumor cell line, RPE1. In control cells, H3T3ph and H3S10ph both rose rapidly

in prophase. Plk1 inhibitors strongly delayed the prophase increase in H3T3ph, and partially decreased H3T3ph in prometaphase cells (Fig 1C, D). Plk1 inhibitors also led to a modest reduction in H3S10ph (also seen in HeLa cells; supplementary Fig S1A, B). However, this is unlikely to be an H3T3ph-mediated effect on Aurora B because direct inhibition of Haspin did not cause the same result (Fig 1C,D), and it could be due to phosphorylation of the CPC by Plk1 [23,24] or another Haspin-independent mechanism. As expected, Aurora B inhibitors strongly but incompletely decreased H3T3ph [13] and almost eliminated H3S10ph [21,22]. Notably, H3T3ph in prophase cells was more resistant to Aurora B inhibitors than Plk1 inhibitors, but this pattern was reversed in prometaphase cells (Fig 1D, supplementary Fig S1C). We conclude that Plk1 is required for normal phosphorylation of Haspin and for full phosphorylation of H3T3, particularly in prophase.

Cdk phosphorylates Haspin and promotes binding to Plk1

Plk1 binds to many substrates through its Polo box domain (PBD), a phospho-specific recognition module that binds to specific

phosphorylated sites often generated by Cyclin B-Cdk1 [25,26]. Previous mass spectrometry of Haspin from mitotic cells revealed eight phosphorylated S/T-P motifs that are potential targets of Cdk [13]. We confirmed that recombinant kinase-dead MBP-Haspin was phosphorylated in a Cdk1-dependent manner in mitotic extracts (supplementary Fig S2A), and that Cyclin B1-Cdk1 phosphorylated Haspin *in vitro* (Fig 2A). Furthermore, Haspin immunoprecipitated from mitotic but not interphase cells carried the mitotic (phospho-) protein monoclonal-2 (MPM-2) epitope (Fig 2B) whose consensus motif resembles both Cdk target sites and optimal PBD-binding sequences [25]. Cdk inhibition abolished recognition by MPM-2

(supplementary Fig S2B). Therefore, Haspin is phosphorylated by Cdk in mitosis.

To determine if the PBD of Plk1 can bind phosphorylated Haspin, we carried out Far-Western experiments. GST-PBD bound to Haspin immunoprecipitated from mitotic but not interphase cells, and this interaction was abolished by mutation of key residues in the binding pocket of the PBD (GST-PBDmut containing mutations H538A/K540M; Fig 2B, supplementary Fig S2C). In addition, wild-type (WT) GST-PBD, but not GST-PBDmut, bound to recombinant MBP-Haspin more strongly when Haspin was phosphorylated *in vitro* by Cyclin B1-Cdk1 (Fig 2C). Finally,

Figure 2. Phosphorylation by Cyclin B-Cdk1 allows Haspin to bind Plk1-PBD.

- A Kinase-deficient (KD) MBP-Haspin or MBP-CREB was used as a substrate in an *in vitro* kinase assay for GST-Cyclin B1/GST-Cdk1 (or control GST-Wee1) activity in the presence of $\gamma^{32}\text{P}$ -ATP.
- B Myc-Haspin was immunoprecipitated from uninduced HeLa Tet-On/myc-Haspin cell extracts, and subjected to immunoblotting or Far-Western analysis.
- C MBP-Haspin KD was phosphorylated *in vitro* with GST-Cyclin B1/GST-Cdk1 and then subjected to Far-Western analysis.

GST-PBD was able to more efficiently “pull-down” endogenous Haspin from mitotic cell extracts than from asynchronous cell extracts, and this interaction was abolished if the extract was treated with λ phosphatase (supplementary Fig S2D), or if the cells were previously treated with the Cdk inhibitor Roscovitine (supplementary Fig S2E). Therefore, Haspin displays a Cyclin B-Cdk1-dependent interaction with the phospho-specific PBD of Plk1 in mitosis.

Haspin contains a single S-pT-P docking site for Plk1

The optimal sequence for PBD binding is S-pS/pT-P [25]. Human Haspin contains such a single motif, S-T₁₂₈-P, in the N-terminal region. Although the surrounding sequence is poorly conserved [27], the motif itself is widely retained in vertebrates (Fig 3A). The predicted Cyclin B-Cdk1 target within this motif, T128, has been confirmed as a phosphorylation site in cells in numerous mass spectrometry studies [28]. Indeed, we found that Haspin immunoprecipitated from mitotic but not interphase cells was recognized by antibodies to the S-pT-P motif, and that the mutation T128A abolished this recognition (Fig 3B). Therefore, Haspin is phosphorylated at T128 in mitotic cells.

To determine if the S-pT₁₂₈-P motif serves as a docking site for the PBD in mitosis, we conducted Far-Western analysis using myc-Haspin and mutants immunoprecipitated from mitotic cells. While GST-PBD bound to myc-Haspin WT, binding to myc-Haspin T128A was essentially eliminated (Fig 3C). In contrast, mutation of 11 Aurora B target sites in Haspin (Haspin 11A; [13]) had no effect on PBD binding (Fig 3C). In addition, GST-PBD, but not GST-PBDmut, was able to “pull-down” myc-Haspin WT, but not myc-Haspin T128A, from mitotic cell extracts (Fig 3D). Finally, we conducted co-immunoprecipitation experiments from mitotic extracts of HeLa cells co-expressing full-length HA-Plk1 and myc-Haspin (Fig 3E). Immunoprecipitates of myc-Haspin WT contained HA-Plk1 WT, but not HA-Plk1 PBDmut, while myc-Haspin T128A immunoprecipitates did not contain HA-Plk1 WT. Conversely, HA-Plk1 WT immunoprecipitates contained myc-Haspin WT but not myc-Haspin T128A, and HA-Plk1 PBDmut did not bring down myc-Haspin WT. Therefore, the S-T₁₂₈-P motif of Haspin is the docking site for Plk1 in mitosis.

Plk1-dependent phosphorylation of Haspin requires T128

We found that the apparent molecular weight of the Haspin T128A mutant was considerably lower than Haspin WT (Fig 3B-E), suggesting that preventing Plk1 binding to Haspin reduced phosphorylation at a number of sites in addition to T128 itself, presumably mainly sites that are direct targets of Plk1. In accordance with this, myc-Haspin T128A migrated in SDS-PAGE at a position very similar to that of Haspin WT from cells treated with the Plk1 inhibitors BI 2536 or GSK461364A, and Plk1 inhibition showed little effect on the migration of myc-Haspin T128A (Fig 3F, supplementary Fig S3A). Therefore, PBD-dependent binding of Plk1 to Haspin through the S-pT₁₂₈-P motif leads to increased phosphorylation of Haspin at Plk1 target sites. Indeed, we previously detected mitotic phosphorylation sites on Haspin [13] that match the known consensus for Plk1 (D/E/N-x-S/T- Φ ; [29–31]), consistent with the idea that Haspin is a direct target of Plk1.

Phosphorylation of Haspin by Plk1 promotes H3T3ph in mitosis

To determine if the direct binding of Plk1 to Haspin contributes to the activity of Haspin towards H3T3ph in cells, we compared the ability of Haspin WT and Haspin T128A mutant to restore mitotic H3T3ph in cells depleted of endogenous Haspin. Because overexpression of Haspin causes anomalous H3T3ph throughout the cell cycle [2], we used HeLa Tet-On cells and plasmids containing a doxycycline-responsive promoter to drive siRNA-resistant Haspin expression at low levels. As expected, Haspin RNAi strongly reduced H3T3ph in nocodazole-arrested cells. After transfection and doxycycline treatment, myc-Haspin WT was able to substantially restore H3T3ph, but the myc-Haspin T128A mutant was less effective (Fig 4A, supplementary Fig S3B). In a parallel experiment in the absence of doxycycline, when myc-Haspin was expressed below detectable levels, the defect in the ability of myc-Haspin T128A to restore H3T3ph was clear (Fig 4A). The partial loss of function of Haspin T128A in nocodazole-arrested prometaphase cells is consistent with partial loss of H3T3ph in prometaphase cells observed upon Plk1 RNAi or inhibition (Fig 1, supplementary Fig S1). Thus, binding to Plk1 contributes to H3T3 phosphorylation by Haspin.

Plk1 inhibition had little effect on the residual H3T3ph produced by myc-Haspin T128A (supplementary Fig S3C), consistent with the idea that T128 phosphorylation promotes binding and phosphorylation of Haspin by Plk1. To determine if direct phosphorylation of Haspin by Plk1 regulates Haspin action, we mutated seven sites in Haspin that match the Plk1 consensus motif: four residues known to be phosphorylated in mitosis (S151, S211, S317, S349) [13], and additional three in regions not covered by our previous mass spectrometric analysis (S47, S53, T334). The myc-Haspin 7A mutant retained the S-pT-P epitope and binding to GST-PBD (supplementary Fig S3D). However, like myc-Haspin T128A, it was partially defective in its ability to support H3T3ph in Haspin RNAi-rescue experiments (Fig 4B). Therefore, we conclude that phosphorylation of Haspin by Plk1 is required for full generation of H3T3ph in mitosis.

Role of Plk1 and Aurora B pathways of Haspin regulation

We were interested in the relationship between previously defined Aurora B-dependent mechanisms that regulate H3T3ph [13–16], and the Plk1-dependent pathway defined here. Co-treatment with inhibitors of Aurora B and Plk1 essentially abolished detectable H3T3ph in nocodazole-arrested prometaphase-like cells (supplementary Fig S4; also Fig 1A), suggesting that these are two major pathways responsible for Haspin activity toward H3T3. It appears that Aurora B and Plk1-mediated Haspin phosphorylation can act at least partly independently: co-inhibition of Aurora B and Plk1 causes a greater decrease in the apparent molecular weight of Haspin in mitosis than inhibition of either kinase alone (Fig 1A), and the Haspin T128A mutant remains moderately sensitive to Aurora B inhibition despite its near-complete loss of Plk1-dependent phosphorylation (Fig 3F, supplementary Fig S3A). Furthermore, weak but detectable H3T3ph is present in early prophase cells even when Aurora B is inhibited, while H3T3ph is delayed but partly recovers by late prometaphase when Plk1 is inhibited (Fig 1C, D, supplementary Figs S1C, S4).

Nevertheless, generation of H3T3ph is strongly influenced by Aurora B activity, even in prophase cells, and H3T3ph is partly

Figure 3. Plk1 binding to Haspin requires the PBD of Plk1 and T128 of Haspin.

A Alignment of vertebrate Haspin sequences surrounding the conserved STP motif. See supplementary Methods.

B Myc-Haspin was immunoprecipitated from extracts of asynchronous or nocodazole-arrested mitotic HeLa cells expressing myc-Haspin (WT or T128A), and subjected to immunoblotting with anti-myc or anti-S-pT-P motif antibodies.

C Myc-Haspin was immunoprecipitated from extracts of nocodazole-arrested mitotic HeLa cells expressing myc-Haspin (WT, T128A or 11A), and subjected to immunoblotting or Far-Western analysis.

D Lysates of nocodazole-arrested mitotic HeLa cells expressing myc-Haspin (WT or T128A) were subjected to pulldowns using GST-PBD and controls, followed by immunoblotting with anti-myc antibodies.

E Lysates of nocodazole-arrested mitotic HeLa cells cotransfected with myc-Haspin (WT or T128A) and HA-Plk1 (WT or PBDmut) plasmids were subjected to immunoprecipitation with anti-myc or HA antibodies, followed by immunoblotting. For ease of interpretation, lanes were spliced as shown to correct loading order. Asterisk indicates a non-specific band.

F HeLa cells were transfected with myc-Haspin WT or T128A plasmids, arrested in mitosis, and treated with inhibitors and MG132 for 1.5 h as described in Fig 1A. Lysates were subjected to immunoblotting.

Figure 4. Phosphorylation of Haspin at T128 and Plk1 target sites is required for full H3T3ph generation and normal Aurora B localization in mitosis.

A HeLa Tet-On cells were transfected with indicated siRNAs, followed the next day by transfection with doxycycline-inducible myc-Haspin WT or T128A plasmids. At 24 h after DNA transfection, cells were blocked in mitosis by nocodazole treatment for 18 h, in the presence or absence of doxycycline, and lysates of cells collected by shake-off were analyzed by immunoblotting.

B HeLa Tet-On cells were transfected with inducible myc-Haspin plasmids (WT, 7A or T128A) in the presence of doxycycline and analyzed as in (A).

C Model for Haspin regulation in mitosis. See text for details.

D HeLa cells were released from thymidine block and, after 7 h, 0.3 μM nocodazole was added with kinase inhibitors for 3 h before fixation. The ratio of Aurora B at centromeres versus chromosome arms was determined by immunofluorescence microscopy. Means ± s.d. are shown, $n = 10$, ** $P < 0.001$ by Bonferroni multiple comparison test. Example images are shown in supplementary Fig S5A.

reduced even at centromeres in Plk1-inhibited prometaphase cells (Fig 1C, D). These patterns of inhibition suggest that the Cyclin B-Cdk1-primed phosphorylation of Haspin by Plk1 could enhance subsequent triggering of Aurora B-H3T3ph feedback loops. Indeed, instigation of the Plk1 pathway by Cdk activity early in mitosis may serve to activate a population of Haspin, generating low H3T3ph on chromatin. Recruitment of the CPC by H3T3ph would then bring Aurora B into proximity with Haspin (and Repo-Man) to trigger the feedback loops that drive full H3T3ph and CPC localization in mitosis (Fig 4C) [13,14]. Consistent with this, Plk1 inhibition or T128A mutation decreases the accumulation of Aurora B at centromeres in prometaphase (Fig 4D, supplementary Figs S5, S6), and Plk1 inhibition delays activation of Aurora B in inhibitor washout

experiments [4], as measured by phosphorylation of the Aurora B substrate CENP-A S7 (supplementary Fig S7).

Role of multiple contributions to Haspin regulation

It is likely that multiple inputs allow H3T3ph and CPC recruitment to be controlled in time, space and extent during the cell cycle. Phosphorylation of Haspin T128 by the master mitotic regulator Cyclin B-Cdk1 restricts H3T3ph to mitosis. The additional need for recognition of the S-pT₁₂₈-P motif by Plk1 acts as a coincidence detector or “AND gate” [26], ensuring that Haspin generates H3T3ph only when both active Cdk1 and Plk1 are present. The involvement of Plk1 may also help target Haspin on chromosome

arms, a function consistent with the role of Plk1 in regulating sister chromatid cohesion and condensin II on arms early in mitosis [32–34]. Cyclin B-Cdk1 activity is low early in mitosis, and gathers strength throughout prophase and into prometaphase [35,36]. The ability of the Plk1-PBD to transform a single Cdk-dependent phosphorylation event at T128 of Haspin into multiple Plk1-dependent phosphorylation events may amplify the Cdk1 signal, ensuring that H3T3ph is generated early in prophase.

Why, in the absence of Plk1 activity, is Aurora B-mediated activation of Haspin (and inactivation of Repo-Man-PP1) [13,14] insufficient to generate H3T3ph in prophase? One possibility is that Aurora B cannot be fully activated upon Plk1 inhibition. However, when Plk1 is inhibited, Aurora B largely retains activity on chromosome arms in prophase as measured by H3S10 phosphorylation (Fig 1). Another possibility is that Haspin is simply a less abundant and kinetically favorable substrate for Aurora B than H3S10. Indeed, Haspin is an inferior substrate for Aurora B compared to H3 *in vitro* [13]. Furthermore, it appears more difficult to eliminate H3S10ph by depleting the CPC than to prevent other Aurora B functions [37]: Aurora B phosphorylates H3S10 before its alternative target H3S28 *in vitro* [38]; H3S28ph and H3T3ph are dephosphorylated more efficiently than H3S10ph [15]; and Haspin-mediated CPC localization is not required for H3S10ph, even though it influences other Aurora B substrates [4,7]. Therefore, Haspin may only become a “good” substrate for Aurora B once the feedback loop is underway and an Aurora B-independent means of Haspin activation, such as that provided by Cdk1 and Plk1, may be needed in prophase. It is also possible that activation of Plk1 by Aurora B [39] provides an additional Plk1-dependent means by which Aurora B activates Haspin.

Implications for Plk1 function

The mechanism we propose suggests a positive role for Plk1 in regulating Aurora B, consistent with work showing that inhibition of Plk1 compromises Aurora B functions [23,24,40,41]. However, Plk1 can also play an antagonistic role toward Aurora B: phosphorylation of BubR1 by Plk1 recruits PP2A to kinetochores to dephosphorylate Aurora B substrates [42–45]. We think it likely that this dichotomy reflects different mechanisms. Regulation of Haspin and H3T3ph by Plk1 early in mitosis may help determine the extent and location of CPC recruitment to chromatin, including inner centromeres. In contrast, kinetochore-bound Plk1 may play a distinct role in counteracting Aurora B through PP2A, particularly later in prometaphase. These two pathways may act together to ensure dynamic regulation of kinetochore-microtubule attachments.

Conclusions

We propose that the Cdk1-Plk1 pathway serves as a timing input to trigger Haspin activity early in mitosis (Fig 4 Ci); Aurora B-H3T3ph feedback loops then act as amplifiers of this signal [13,14] (Fig 4 Cii); and the Bub1-H2AT120ph pathway supplies a spatial controller to train the amplification loop on the centromere [8,12,13], leading to the accumulation of H3T3ph and the CPC at centromeres by prometaphase (Fig 4 Ciii). While this paper was under revision, Ghenoiu *et al* [46] also reported that Plk1 regulates Haspin activity, and that this may be due to the release of an autoinhibitory interaction between

the N-terminal and kinase domains of Haspin. This new function helps explain previous reports implying a positive role for Plk1 in regulation of CPC activity in mitosis, and these findings should provide a framework for understanding the regulation of other proteins that are substrates of both Plk1 and Aurora B in mitosis.

Materials and Methods

Cell culture, cell synchronization, transfection, immunoprecipitation, immunoblotting and immunofluorescence staining were conducted essentially as described [2–4,13]. Point mutations were introduced into a siRNA-resistant Haspin cDNA [13] with the QuikChange II XL site-directed mutagenesis kit (Stratagene). Nocodazole was used at 0.3–3 μ M and mitotic cells were collected by “shake-off”; MG132 was used at 20 μ M; ZM447439 at 2 μ M; Hesperadin at 12.5–25 nM; GSK461364A at 250 or 400 nM; and BI 2536 at 100 nM. Chromosome condensation, measured as the increase in variance of DNA staining intensity as chromosomes condense, was used to track progression through early mitosis. Fluorescence microscopy was carried out using Nikon TE2000-U or ECLIPSE microscopes equipped with CCD systems. *In vitro* kinase assays, using recombinant proteins produced in *E. coli* (or, for GST-Cdk1/GST-Cyclin B1, in an insect baculovirus system) were carried out as described [2]. Further details can be found in the supplementary Methods.

Supplementary information for this article is available online: <http://embor.embopress.org>

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Author contributions

LZ, XT, CZ, FW and JMGH carried out the experiments. FW and JMGH conceived and directed the project and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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