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# Nucleolar protein Spindlin1 recognizes H3K4 methylation and stimulates the expression of rRNA genes

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**The tandem Tudor-like domain-containing protein Spindlin1 has been reported to be a meiotic spindle-associated protein. Here we report that Spindlin1 is not associated with the spindle in mouse embryonic fibroblast cells during mitotic divisions. In interphase cells, Spindlin1 specifically localizes to the nucleoli. Moreover, Spindlin1 is a histone methylation effector protein that specifically recognizes H3K4 methylation. Finally, Spindlin1 localizes to the active ribosomal DNA (rDNA) repeats, and Spindlin1 facilitates the expression of rRNA genes.**

Keywords: Spindlin1; histone methylation; nucleolus; rRNA; transcription

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## INTRODUCTION

Histone lysine methylation is a pivotal regulator of chromatin structure and function (Shilatifard, 2006; Allis *et al*, 2007; Bannister & Kouzarides, 2011; Zhu & Reinberg, 2011). In most cases, histone lysine methylations are specifically recognized by effector proteins through which they exert their functions (Yun *et al*, 2011). Similarly to RNA polymerase II-mediated messenger RNA (mRNA) transcription, RNA polymerase I-mediated rRNA gene transcription is regulated by histone lysine methylations (McStay & Grummt, 2008). Active rDNA repeats are enriched for H3K4 and H3K36 methylations, whereas repressed rDNA repeats are enriched for H3K9 methylation (McStay & Grummt, 2008). Many chromatin modifiers have been reported to regulate rRNA gene transcription. The histone demethylases KDM2A and KDM2B are nucleolar proteins that repress rRNA gene transcription (Frescas *et al*, 2007; Tanaka *et al*, 2010); interestingly, two other closely related histone demethylases, PHF2 and PHF8, also localize to nucleoli and activate rRNA gene transcription by demethylating H3K9me1/2 (Feng *et al*, 2010; Wen *et al*, 2010; Zhu *et al*, 2010). Although enzymes that modify histone methylation are known to influence rRNA gene transcription, little is known about the roles of effector proteins in mediating rRNA transcriptional regulation.

Spindlin1 was initially identified as a meiotic spindle-binding protein in mice (Oh *et al*, 1997). The human homologue of Spindlin1 has been reported to be a nuclear protein that transforms NIH3T3 cells on overexpression (Gao *et al*, 2005). The crystal structure of the human Spindlin1 protein shows that it has a fold resembling tandem Tudor-like domains (Zhao *et al*, 2007). Several Tudor-like domain-containing proteins have been characterized as histone lysine methylation effector proteins (Botuyan *et al*, 2006; Du *et al*, 2006; Huang *et al*, 2006; Kim *et al*, 2006; Lee *et al*, 2008), which raised the possibility that Spindlin1 might also be a histone methylation effector protein.

Here we report that human Spindlin1 specifically recognizes H3K4 methylation *in vitro*. Moreover, Spindlin1 is a nucleolar protein that localizes to active rDNA loci and facilitates the expression of rRNA genes.

## RESULTS AND DISCUSSION

### Spindlin1 is a nucleolar protein

Spindlin1 has been reported to be a tandem Tudor-like domain-containing protein (Zhao *et al*, 2007) associated with the meiotic spindle (Oh *et al*, 1997). Because of our research interest in histone modification-mediated mitotic epigenetic inheritance (Xu & Zhu, 2010; Xu *et al*, 2010; Chen *et al*, 2011; Wu & Zhu, 2011), we decided to investigate whether Spindlin1 might be a histone lysine methylation-binding protein that specifies the kinetochore. To our surprise, indirect immunofluorescence

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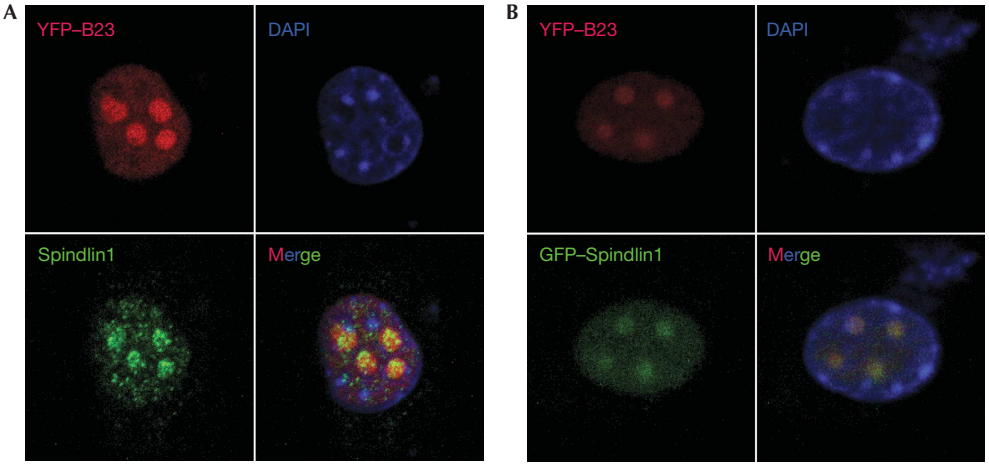


**Fig 1** | Spindlin1 is a nucleolar protein. (**A**) Endogenous Spindlin1 protein colocalizes with the nucleolar marker B23 in mouse fibroblast cells. (**B**) Green fluorescent protein (GFP)-tagged, ectopically expressed Spindlin1 fusion protein colocalizes with the nucleolar marker B23 in mouse fibroblast cells. DAPI, 4,6-diamidino-2-phenylindole; YFP, yellow fluorescent protein.

staining with antibodies specific for Spindlin1 showed that little Spindlin1 was localized to the mitotic spindle or centromeres in prometaphase, anaphase and telophase mouse embryonic fibroblast cells (supplementary Fig S1A–C online). In contrast, Spindlin1 appeared to be enriched at nuclear compartments that showed little 4,6-diamidino-2-phenylindole (DAPI) staining in interphase cells (supplementary Fig S1D online), which indicates that Spindlin1 might be a nucleolar protein.

Staining for Spindlin1 in mouse embryonic fibroblasts transfected with a nucleolar marker, yellow fluorescent protein-fused B23 (Zhu *et al*, 2010), showed Spindlin1 to be highly enriched in the nucleoli (Fig 1A). To exclude the possibility that Spindlin1 staining in the nucleolus was the result of nonspecific antibody crossreactivity, green fluorescent protein-fused Spindlin1 was co-transfected with yellow fluorescent protein-fused B23, and the colocalization of the fluorescent proteins clearly showed that Spindlin1 was a nucleolar protein (Fig 1B).

## Spindlin1 recognizes H3K4 methylation

The crystal structure of Spindlin1 showed structural features similar to the Tudor-like domain (Zhao *et al*, 2007), indicating that Spindlin1 might be a methylated histone-binding protein. To characterize the role of Spindlin1 as an effector protein for histone modifications, pull-down experiments were performed using mononucleosomes (supplementary Fig S2A online) assembled using biotin-labelled 601 nucleosome positioning sequence (Lowary & Widom, 1998) and core histones containing various methylated lysine analogues (MLAs; supplementary Fig S2B online; Simon *et al*, 2007; Jia *et al*, 2009). Recombinant Spindlin1 was selectively pulled down using mononucleosomes containing methylated H3Kc4 (Fig 2A), but not methylated H3Kc9 or H3Kc27 (supplementary Fig S3A online).

The specific interaction between H3K4me3 and Spindlin1 was confirmed by isothermal titration calorimetry (ITC) experiments, which showed an interaction between recombinant Spindlin1 and H3K4me3 peptide, with a $K_d$ of about $0.8 \times 10^{-6}$ mol/l (Fig 2B). Spindlin1 showed a reduced affinity towards H3K4me2 peptide with a $K_d$ of about $6 \times 10^{-6}$ mol/l and a further reduced affinity towards H3K4me1 peptide with a $K_d$ of about $11 \times 10^{-6}$ mol/l (Fig 2B) and no apparent interaction with H3K4me0 peptide (supplementary Fig S3B online). In addition, H3K9me3 and H3K27me3 peptides did not show significant interactions with recombinant Spindlin1 protein (supplementary Fig S3C online).

These results show a direct interaction between H3K4 methylation and Spindlin1, which is in agreement with a recent report that showed significant enrichment of Spindlin1 using affinity purification with nucleosomes containing H3K4me3 (Bartke *et al*, 2010).

Using the crystal structures of Spindlin1 (Zhao *et al*, 2007) and H3K4me3–JMJD2A Tudor domain complex (Huang *et al*, 2006), we modelled a structure of the Spindlin1 and H3K4me3 complex using docking methods (Chen *et al*, 2003). The docking results showed that the trimethylated K4 inserts into a pocket composed of residues Y170, F141 and Y177 (Fig 2C). The predicted binding mode indicates that residues Y177, Y170 and F141 on Spindlin1 interact with the trimethylated K4 by cation-$\pi$ interactions (Fig 2C).

Recombinant Spindlin1 proteins with Y170 or F141 mutated to alanine were then expressed and analysed for their binding abilities towards H3K4me3. As predicted by the structural data, Spindlin1 Y170A and F141A mutants showed greatly impaired interactions with H3K4me3, in both nucleosome pull-down (Fig 2D) and ITC (Fig 2E) experiments.

## Spindlin1 localizes to rDNA loci

Spindlin1 is a nucleolar protein (Fig 1) that specifically recognizes H3K4me3 (Fig 2), which indicates that Spindlin1 might be localized to rDNA loci and that Spindlin1 might potentially regulate the transcription of rRNA genes. To validate the presence of Spindlin1 at rDNA loci (illustrated in Fig 3A), chromatin immunoprecipitation (ChIP) experiments were performed using antibodies specific for Spindlin1. These experiments confirmed that Spindlin1 was enriched at rDNA loci, but not at two inactive loci, PRM3 (specifically expressed during spermiogenesis; Grzmil *et al*, 2008) and MyoD (specifically expressed during myogenesis; Tapscott *et al*, 1988; Fig 3B). In addition, control ChIP experiments were also performed with antibodies specific for H3K4me3 (Fig 3C) and a Pol I subunit RPA194 (Fig 3D).

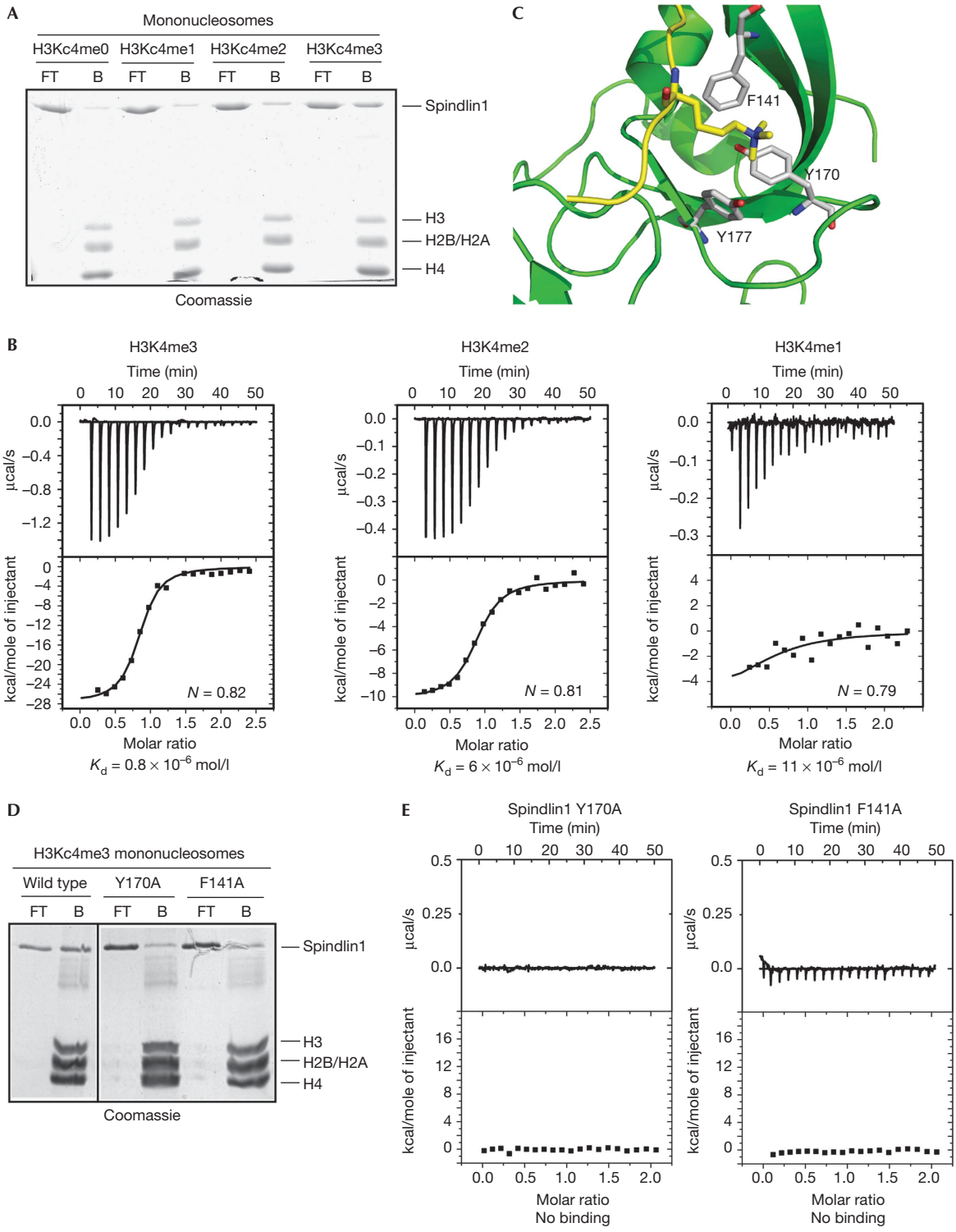


**Fig 2** | Spindlin1 is an H3K4me3-binding protein. (**A**) Nucleosome pulldown experiments showed that recombinant Spindlin1 recognized nucleosomes containing H3Kc4me2 and H3Kc4me3. (**B**) Isothermal titration calorimetry analysis showed binding between Spindlin1 and H3K4me3 peptide with a $K_d$ of about $0.8 \times 10^{-6}$ mol/l, binding between Spindlin1 and H3K4me2 peptide with a $K_d$ of about $6 \times 10^{-6}$ mol/l, and binding between Spindlin1 and H3K4me1 peptide with a $K_d$ of about $11 \times 10^{-6}$ mol/l. (**C**) A modelled structure of Spindlin1 and H3K4me3 complex, showing key residues caging the trimethylated H3K4. (**D**) Spindlin1 Y170A and F141A mutants showed reduced binding to mononucleosomes containing H3Kc4me3. (**E**) Spindlin1 Y170A and F141A mutants showed impaired interaction with H3K4me3 peptide.

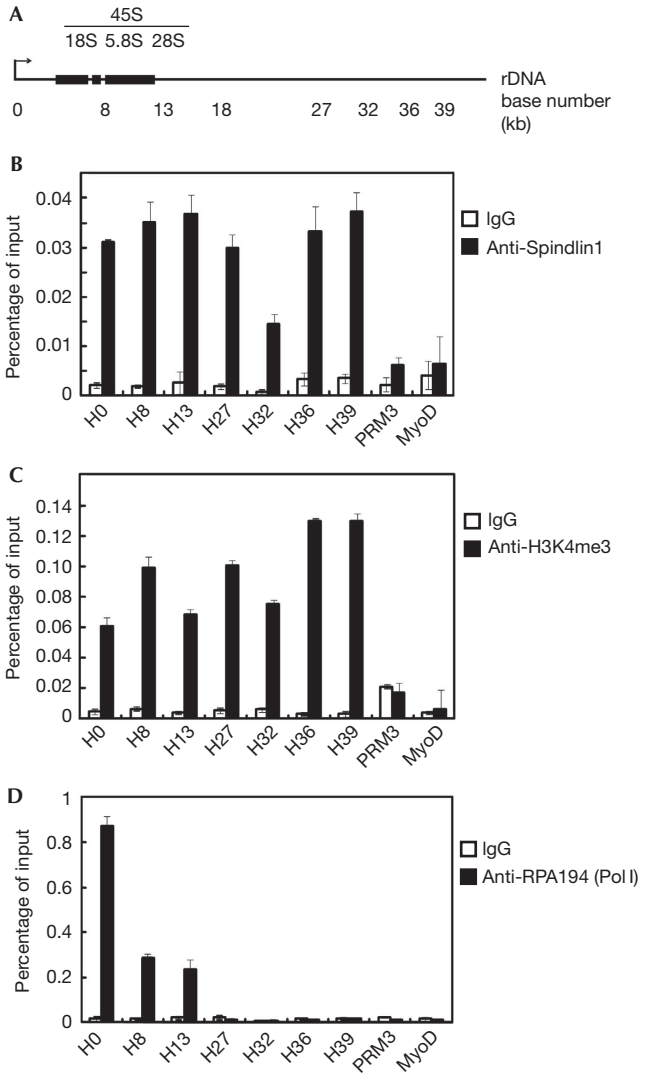


**Fig 3** | Spindlin1 occupies ribosomal DNA (rDNA) loci. (**A**) Schematic illustration of a single human rDNA repeat. (**B**) Chromatin immunoprecipitation (ChIP) experiments using HeLa cells showed Spindlin1 occupancy at the rDNA loci. (**C**) The H3K4me3 profile at rDNA loci as determined using ChIP. (**D**) The distribution profile of Pol I at rDNA loci as determined using ChIP against RPA194, a Pol I subunit. Error bars represent the standard deviation calculated from three independent experimental repeats. IgG, immunoglobulin G.

## Spindlin1 localizes to active rDNA repeats

Ribosomal DNA contains both active repeats that are enriched for H3K4 methylation and inactive repeats that are enriched for H3K9 methylation (McStay & Grummt, 2008). To investigate whether Spindlin1 is localized at the active rDNA repeats, we performed co-staining using antibodies against Spindlin1 and RPA194. The immunostaining results showed that Spindlin1 and RPA194 were largely colocalized, which indicates that Spindlin1 was localized at Pol I-engaged, active rDNA repeats (Fig 4A).

To further confirm that Spindlin1 localizes to active rDNA repeats, we decided to perform sequential ChIP experiments with a stable cell line expressing a Flag-tagged Spindlin1 using antibodies against Flag and RPA194 or H3K4me3. Attempts to establish a stable cell line that constitutively overexpresses Spindlin1 failed to yield a healthy cell line because continued overexpression of Spindlin1 led to apoptotic cell death, as previously reported (Yuan *et al*, 2008). Therefore, a stable HeLa cell line was established, in which an N-terminal Flag-tagged Spindlin1 was expressed under the control of a tetracycline-inducible promoter. In this cell line, Spindlin1 protein and mRNA levels were robustly induced by the addition of tetracycline (Fig 4B). Cells were collected for sequential ChIP after 72 h of tetracycline induction; RPA194 and H3K4me3 were robustly enriched at Flag–Spindlin-occupied chromatin (Fig 4C).

The above data collectively indicate that Spindlin1 was localized to active rDNA repeats, which is consistent with its role in recognizing H3K4 methylation.

## Spindlin1 facilitates rRNA gene expression

H3K4 methylation recognition proteins, such as NURF (Wysocka *et al*, 2006), TFIID (Vermeulen *et al*, 2007), CHD1 (Flanagan *et al*, 2005; Sims *et al*, 2005) and Sgf29 (Vermeulen *et al*, 2010; Bian *et al*, 2011), are often involved in regulating transcription. Another nucleolar H3K4me3-binding protein, PHF8, has been reported to regulate the transcription of rRNA genes (Feng *et al*, 2010; Zhu *et al*, 2010). To investigate whether Spindlin1 also has a role in regulating rRNA gene transcription, the expression level of Spindlin1 was modulated to assess its effect on pre-rRNA production.

On induction of the Flag–Spindlin1 in the above-mentioned stable cells, pre-rRNA level was significantly elevated in a dose-dependent manner (Fig 5A). In contrast, pre-rRNA level did not change in cells stably transfected with the empty vector (Fig 5B). In addition, the Spindlin1 Y170A and F141A mutants significantly lost their abilities to stimulate the expression of pre-rRNA (Fig 5B). ChIP experiments showed that the rDNA occupancies of these mutants were slightly reduced (supplementary Fig S4A online). In this context, we point out that impaired histone modification binding activity does not necessarily fully abolish the chromatin localization of these mutant effector proteins. First of all, the binding affinity between effector proteins and histone modifications are typically at the μM range, which indicates that the role of such interactions is to stabilize the complex, rather than to serve as the initial recruitment. In addition, other properties of Spindlin1, such as its interaction with DNA (Zhao *et al*, 2007), its interaction with the endogenous Spindlin1 due to its dimeric structure (Zhao *et al*, 2007) and other potential protein partners, might also contribute to the partial chromatin localization of these mutants.

To further establish the role of Spindlin1 in rRNA gene transcription, Spindlin1 was knocked down in HeLa cells using three different pairs of small interfering RNA (siRNA). Spindlin1 protein and mRNA levels were significantly reduced, especially in cells treated with siRNA pair 2 and pair 3 (Fig 5C). Pre-rRNA level was significantly reduced in cells that showed efficient Spindlin1 knockdown (Fig 5D). Finally, control experiments showed that Spindlin1, but not other Spindlin family members such as Spindlin2A or Spindlin2B, was specifically targeted by the siRNA pair 3 (supplementary Fig S4B online).

These data collectively support a role for Spindlin1 in facilitating the expression of rRNA genes, which is consistent

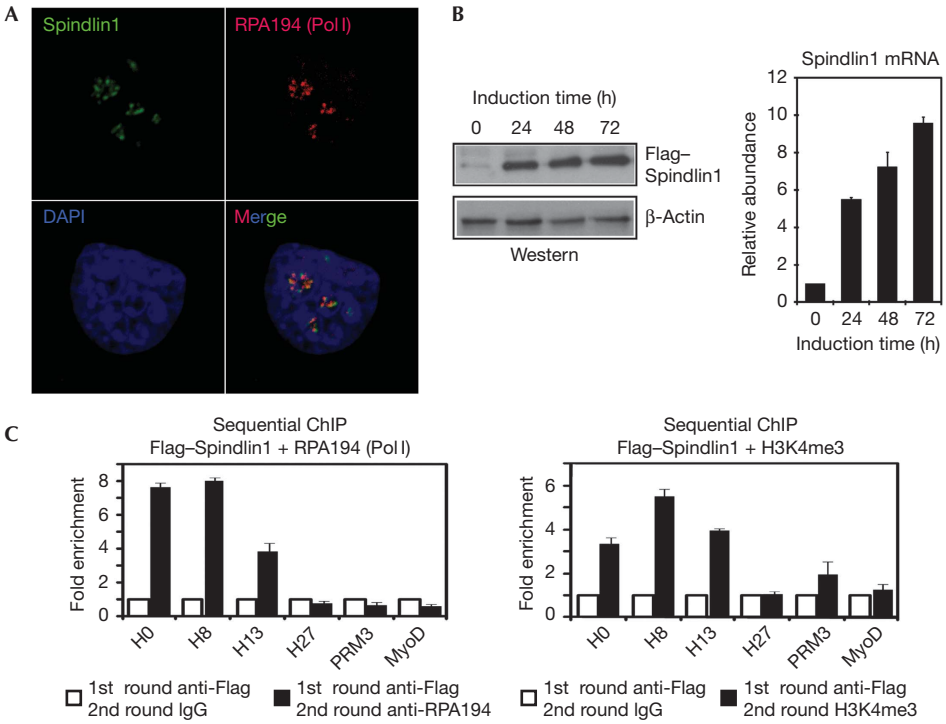


**Fig 4** | Spindlin1 occupies active ribosomal DNA (rDNA) repeats. (**A**) Endogenous Spindlin1 colocalizes with Pol I subunit RPA194. (**B**) Western blot analyses (left panel) and reverse transcription–polymerase chain reaction (right panel) verified the induction of ectopic Flag–Spindlin1 expression in HeLa cells. (**C**) Sequential chromatin immunoprecipitation (ChIP) showing co-occupancy of Flag–Spindlin1 with RPA194 (left panel) and H3K4me3 (right panel). Error bars represent the standard deviation calculated from three independent experimental repeats. DAPI, 4,6-diamidino-2-phenylindole; IgG, immunoglobulin G; mRNA, messenger RNA.

with its biochemical function in recognizing active chromatin modifications, H3K4me2 and H3K4me3 (Fig 2).

Spindlin1 belongs to the Spindlin family, which comprises Spindlin1, Spindlin2A, Spindlin2B and Spindlin3 (supplementary Fig S5 online). All these proteins are poorly studied at present; it would be interesting to investigate whether the other Spindlin family members are also chromatin modification effector proteins and/or whether they might also regulate the expression of rDNA.

## Spindlin1 is not a subunit of a stable complex

Because Spindlin1 is a relatively small protein lacking apparent functional domains other than its H3K4me3-binding domain, it is possible that other proteins might associate with Spindlin1 to facilitate its role in transcriptional regulation. Therefore, we attempted to isolate a stable protein complex associated with Spindlin1. Under stringent washing conditions (0.5 M KCl), two protein bands appeared to be enriched in affinity purification eluates from cells expressing Flag-tagged Spindlin1 (supplementary Fig S6 online). Unfortunately, these proteins were identified using mass spectrometry as HSP70 and HSPA8, contaminants common to Flag-tag affinity purifications (supplementary Fig S6 online). This result indicates that Spindlin1-associated protein(s), if any, might be only weakly or transiently associated with Spindlin1. Further investigation to identify such factor(s) will help to deepen our understanding of how Spindlin1 facilitates rRNA gene expression.

## METHODS

**Antibodies.** Antibodies against Spindlin1 were purchased from ProteinTech Group (12105-1-AP); antibodies against H3K4me3 were purchased from Millipore (04-745); antibodies against H3K4me2 were purchased from Millipore (07-030); antibodies against PRA194 (Pol I subunit) was purchased from Santa Cruz (SC-48385); and antibodies against Flag were purchased from Sigma (F1804).

**ChIP and RT–PCR experiments.** Primers for rDNA loci used in ChIP experiments were synthesized according to previously reported sequences (O'Sullivan *et al*, 2002). The following ChIP primers were used for PRM3: forward primer, GAAGTTATCCTG ACTCACAC, and reverse primer, CCAGAGCCCAGGCCACAG CC. The following ChIP primers were used for MyoD: forward primer, GAGAAGCTAGGGGTGAGGAA, and reverse primer, AGTCTGAATGCCCACCCACT.

The reverse transcription–polymerase chain reaction (RT–PCR) primers used for pre-rRNA and glyceraldehyde-3-phosphate dehydrogenase were described previously (Feng *et al*, 2010; Zhu *et al*, 2010). The following RT–PCR primers were used for Spindlin1: forward primer, ATGAAGACCCCATTCGGAAA, and reverse primer, TGTGGGATGTCCTCTTCTTC. Spindlin2A and 2B differ at only two bases in the coding region (supplementary Fig S5 online); the following RT–PCR primers were used for Spindlin2A/2B: forward primer, CAACGCACAGGAAGCCGAAG, and reverse primer, TGATGGGCTCATCTCCTTCC.

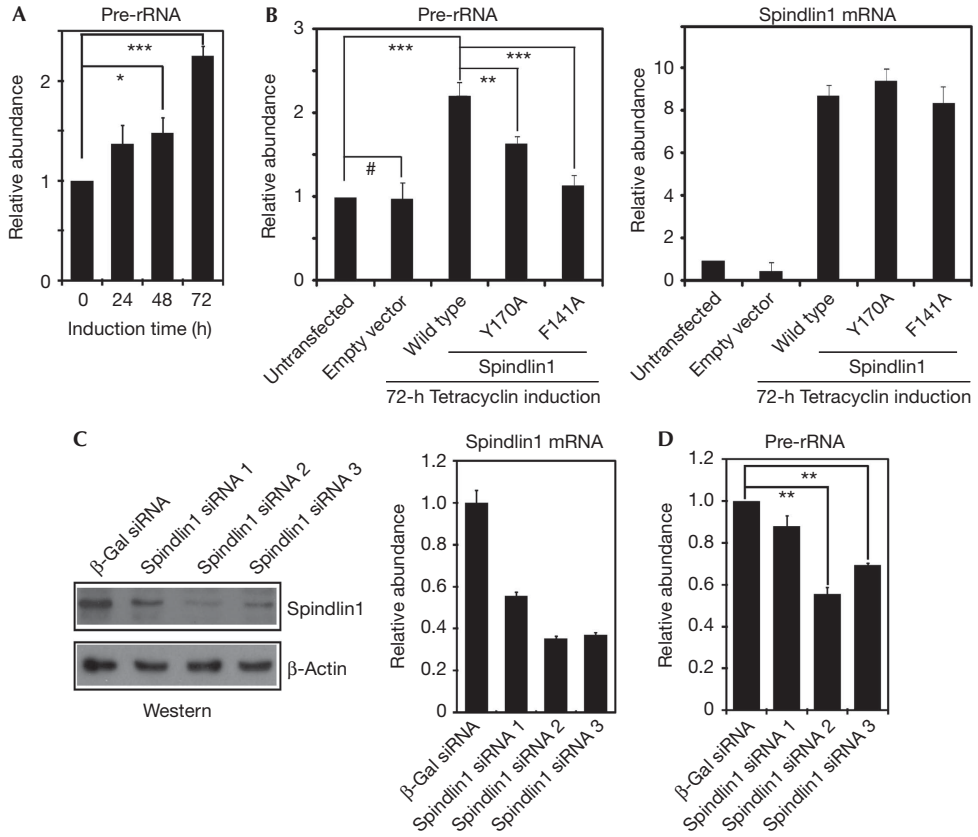


**Fig 5** | Spindlin1 facilitates the expression of ribosomal RNA (rRNA) genes. (**A**) Reverse transcription–polymerase chain reaction (RT–PCR) results revealed elevated pre-rRNA levels upon Flag–Spindlin1 induction. (**B**) RT–PCR results showing impaired stimulation of pre-rRNA expression in cells expressing the Spindlin1 mutants (left panel); the expression levels of the Flag–Spindlin1 were also determined by RT–PCR (right panel). (**C**) Western blot analyses (left panel) and RT–PCR (right panel) verified the efficiency of Spindlin1 knockdown in HeLa cells. (**D**) RT–PCR results showed significantly reduced pre-rRNA levels on Spindlin1 knockdown. Error bars represent the standard deviation calculated from three independent experiment repeats. $^{***}P<0.002$; $^{**}P<0.01$; $^{*}P<0.05$; and $^{\#}P>0.1$. mRNA, messenger RNA; siRNA, small interfering RNA; β-Gal, β-galactosidase.

ChIP–PCR and RT–PCR experiments were performed in triplicate to calculate the standard deviation. A *t*-test was performed for all the RT–PCR experiments measuring the levels of pre-rRNA to calculate corresponding *P* values.

**Stable cell line.** Complementary DNA coding for a Flag-tagged human Spindlin1 was inserted into pcDNA4/TO (Invitrogen) and transfected into HeLa cells. Stable clones were selected using Zeocin (100 μg/ml, Invitrogen).

**Methylated lysine analogue reactions and mononucleosome assembly.** MLA reactions were performed as previously described (Simon *et al*, 2007). Core histones were assembled into mononucleosomes using biotin-labelled 601 nucleosome positioning sequence (Lowary & Widom, 1998).

**Nucleosome pull-down assay.** Recombinant Spindlin1 protein (10 μg) was incubated with 5 μg of biotin-labelled mononucleosomes captured using Streptavidin agarose (Promega) in buffer containing 20 mM Tris–HCl (pH 8.0), 150 mM NaCl and 0.1% NP40 at 4 °C for 4 h. Unbound proteins were washed away with excess binding buffer, and proteins associated with the mononucleosomes were eluted using SDS–PAGE gel loading buffer.

**Isothermal titration calorimetry.** ITC experiments were performed using a MicroCal ITC titration calorimeter (ITC200, USA). Samples were prepared in titration buffer containing 50 mM HEPES (pH 8.0) and 150 mM NaCl. The reaction cell contained a degassed solution of 30 μM recombinant Spindlin1 and 20 2-μl aliquots of 300 μM H3K4me3, H3K9me3 or H3K27me3 peptides. Experimental data were analysed using the ORIGIN analysis software (MicroCal Software, USA).

**Structural modelling.** Using docking methodology (Chen *et al*, 2003), we constructed the complex of Spindlin1 with H3K4me3. The structure of H3K4me3 was extracted from the structure of JMJD2A Tudor domain–H3K4me3 complex (PDB code: 2GFA; Huang *et al*, 2006), and then was docked into the pocket of Spindlin1 (PDB code: 2NS2; Zhao *et al*, 2007) with ZDOCK program encoded in Discovery Studio (http://www.accelrys.com). The docking modes with the highest score were selected as candidates. After comparing with the binding mode of H3K4me3 in 2GFA, we selected one binding conformation of H3K4me3 in complex with Spindlin1.

**RNA interference.** siRNA pairs were transfected into HeLa cells using Lipofectamine 2000 (Invitrogen), and cells were collected

for analysis 72 h after transfection. The following siRNA sequences were used:

Spindlin1 siRNA pair 1: sense, GGAUUCAGCAUGGGUG GAAdTdT and antisense, UUCCACCCAUGCUGAAUCCdTdT;- Spindlin1 siRNA pair 2: sense, GCAAAGCAGUGGAACAUA UdTdT and antisense, AUAUGUUCCACUGCUUUGCdTdT; Spindlin1 siRNA pair 3: sense, GCAUUAUGCCUGAUUCCAAdTdT and antisense, UUGGAAUCAGGCAUAAUGCdTdT; and β-galactosidase siRNA pair: sense, AAGGCCAGACGCGAAUUAUdTdT and antisense, AUAAUUCGCGUCUGGCCUUdTdT.

**Affinity purification.** Affinity purification of Flag-tagged Spindlin1 was performed as previously described (Yuan *et al*, 2009). In brief, M2 anti-Flag agarose (Sigma) was equilibrated with buffer containing 20 mM Tris–HCl (pH 7.9), 1.5 mM $MgCl_2$, 0.42 M NaCl and 0.2 mM phenylmethylsulphonyl fluoride and incubated overnight at 4 °C with solubilized nuclear pellets derived from stably transfected cells. The resin was washed with buffer containing 20 mM Tris–HCl (pH 7.9), 1.5 mM $MgCl_2$, 0.2 mM phenylmethylsulphonyl fluoride and 0.5 M KCl, and proteins were eluted with the same buffer supplemented with 0.1 mg/ml Flag peptide.

**Supplementary information** is available at EMBO *reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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