FLASH is required for histone transcription and S-phase progression

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Cajal bodies are nuclear subdomains that are involved in maturation of small ribonucleoproteins and frequently associate with small nuclear RNA and histone gene clusters in interphase cells. We have recently identified FADD-like IL-1β-converting enzyme (FLICE) associated huge protein (FLASH) as an essential component of Cajal bodies. Here we show that FLASH associates with nuclear protein, ataxia-telangiectasia, a component of the cell-cycle-dependent histone gene transcription machinery. Reduction of FLASH expression by RNA interference results in disruption of the normal Cajal body architecture and relocalization of nuclear protein, ataxia-telangiectasia. Furthermore, FLASH down-regulation results in a clear reduction of histone transcription and a dramatic S-phase arrest of the cell cycle. Chromatin immunoprecipitation reveals that FLASH interacts with histone gene promoter sequences. These results identify FLASH as an important component of the machinery required for histone precursor mRNA expression and cell-cycle progression.

Cajal bodies | coiled bodies | cell cycle

Cajal bodies (CBs) are multifunctional nuclear organelles present in a wide variety of animal and plant cell nuclei (reviewed in refs.1 and 2). CBs contain high concentrations of small ribonucleoproteins and are thought to play roles in their assembly and maturation (reviewed in ref. 3). In particular, the U7 small nuclear ribonucleoproteins and other factors involved in histone precursor mRNA processing are known to accumulate within CBs (1, 4). Notably, CBs also associate with the major histone gene clusters in a variety of organisms, including mammals, amphibians, and dipterans (5, 6). In addition to participating in various RNA-processing activities, CBs have also been implicated in transcriptional regulation of the cell-cycle-dependent histone genes. Phosphorylation of a CB component p220/nuclear protein, ataxia-telangiectasia (NPAT) by cyclin E/Cdk2 is required for activation of histone transcription, exit from G_1, and progression through S phase (7–12). Taken together, these observations suggest that CBs are intimately involved in histone gene expression.

In this study, we identify FADD-like IL-1β-converting enzyme (FLICE) associated huge protein (FLASH) (13) as a component of the histone gene expression machinery. Although FLASH was originally identified as a component of the apoptotic signaling complex known as the death-inducing signaling complex (DISC) that is assembled in response to Fas ligand binding (13, 14), we have recently shown that FLASH is an essential component of CBs and is required for maintenance of their structure (15). We show that FLASH colocalizes with the histone transcriptional activator, NPAT, in CBs and is required for efficient expression of histone genes.

Results

FLASH Down-Regulation Results in S-Phase Block. One of the hallmarks of proteins that are involved in expression of the cell-cycle-dependent histone genes is that perturbation of their function results in an accumulation of cells in S phase. Accordingly, we found that treatment of cells with short hairpin RNAs (shRNAs) targeting FLASH (shFLASH) resulted in a dramatic block of cells within S-phase of the cell cycle (Fig. 1*a*). Such a block was observed in all cell lines tested (HEK293, HeLa, MCF-7, SAOS2, 3T3 and MEFs) reaching up to 70% after 72 h (see Fig. 5, which is published as supporting information on the PNAS web site). These findings were confirmed through use of a colony-forming assay, revealing that down-regulation of FLASH resulted in a remarkable reduction in growth of the shFLASH-treated cells (Fig. 1*b*). Western blot in Fig. 1*c* confirms FLASH protein levels down-regulation after shRNA treatment.

Another hallmark of genes involved in histone gene expression is that their protein levels are up-regulated during S phase. Endogenous FLASH expression showed a clear cell-cycle-dependence, peaking during S-phase, when cells were synchronized by thymidine block and deoxycytidine release (Fig. 1*d*). Consistent with these observations, we found that the number of FLASH-positive bodies was correlated with the cell cycle. Primary (IMR90) cells were used for this analysis, as they are diploid. As shown in Fig. 1*e*, the number of FLASH bodies in BrdU-positive (S-phase cells) was typically four, whereas in BrdU-negative cells, the number was typically two.

FLASH Interacts with NPAT and Is Bound to Histone Gene Promoters. FLASH foci in primary cells such as IMR90 were typically of two distinct sizes. Strikingly similar findings were reported for NPAT, wherein the larger foci corresponded to the large cluster of histone genes on human chromosome 6p21 and the smaller signals corresponded to the small cluster located at 1q21 (7). We found that NPAT and FLASH foci were 100% concordant in every cell line tested (Fig. 2*a* and Fig. 6, which is published as supporting information on the PNAS web site), suggesting that FLASH localizes to the histone gene clusters. To test this idea more stringently, we performed chromatin immunoprecipitation (ChIP). By using primers specific for four different histone gene promoters, we showed that HA-tagged FLASH, but not HA-p21 (negative control) coprecipitated with histone gene chromatin

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Abbreviations: CB, Cajal bodies; FLASH, FADD-like IL-1β-converting enzyme (FLICE)-associated huge protein; NPAT, nuclear protein, ataxia-telangiectasia; shRNA, short hairpin RNA.

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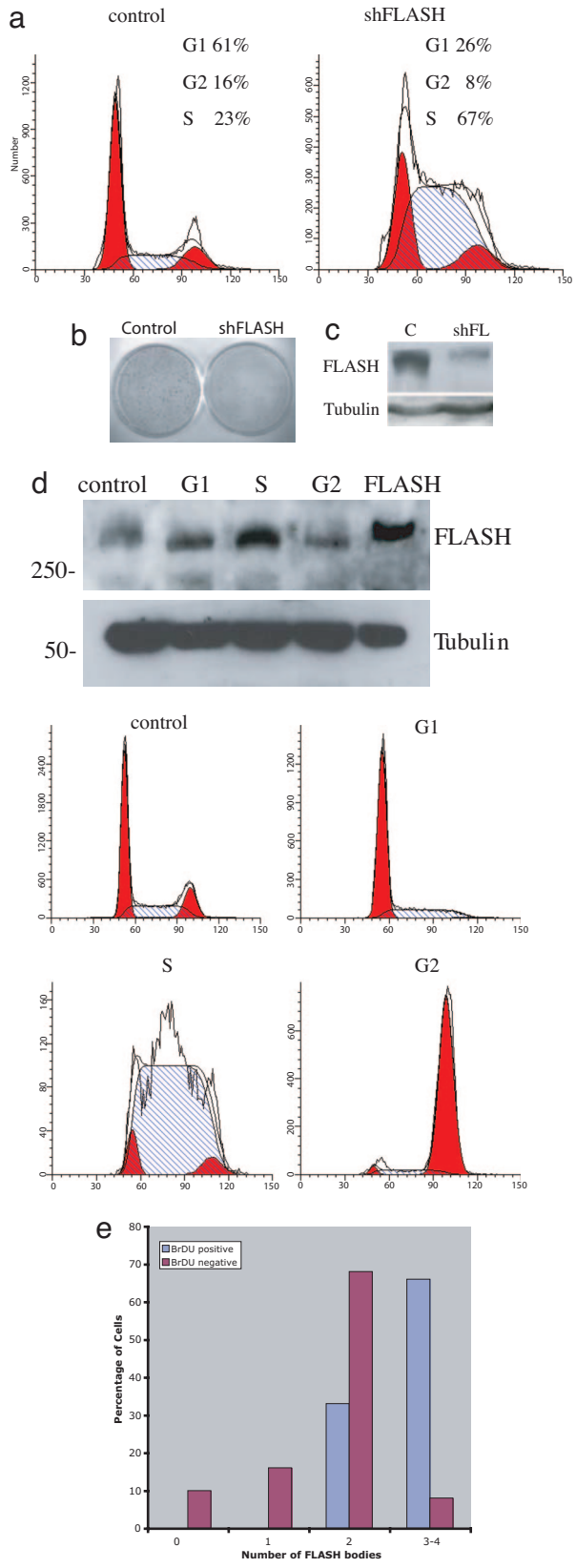

Fig. 1. Down-regulation of FLASH results in S-phase block. (*a*) Cell-cycle distribution of HeLa cells transfected with GFP-spectrin and either pSUPER-scrambled (control) or pSUPER-FLASH-1 (shFLASH). Cells were stained with PI, and GFP-positive cells were analyzed by flow cytometry for DNA content. Percentages of cells in G_1, S, or G_2/M calculated by using ModFit program are indicated. Identical results were obtained by using pSUPER-FLASH-2 (data not shown). (*b*) Colony-forming assay of SAOS-2 cells transfected with pBabe-puro and either pSUPER-scrambled (control) or pSUPER-FLASH-1 (shFLASH), selected for 2 weeks with 1 μg/ml puromycin. Cells transfected with pSUPER-FLASH-1 show a large reduction in colony numbers. (*c*) Western blot showing that transfection of MCF-7 cells with pSUPER-FLASH-1 (shFL), but not with a scrambled vector (control), results in reduction of FLASH protein levels. (*d Upper*) Western blot showing FLASH expression in MCF-7 cells untreated (control) or treated with either 2 mM thymidine for 16 h (G_1), followed by 4-h release in 24 μM deoxycytidine (S), or treated with 50 ng/ml nocodazole for 16 h (G_2). As a positive control, cells transfected with GFP-FLASH (FLASH) were loaded. The Western blot was reprobed with tubulin to show equal loading. (*d Lower*) Cell-cycle distribution of an aliquot of cells used for the Western blot. (*e*) Percentage of IMR90 cells containing 0, 1, 2, or 3–4 FLASH bodies positive (blue) or negative (red) for BrdU incorporation. The number of FLASH bodies per cell increases in S phase cells as reported for CBs.

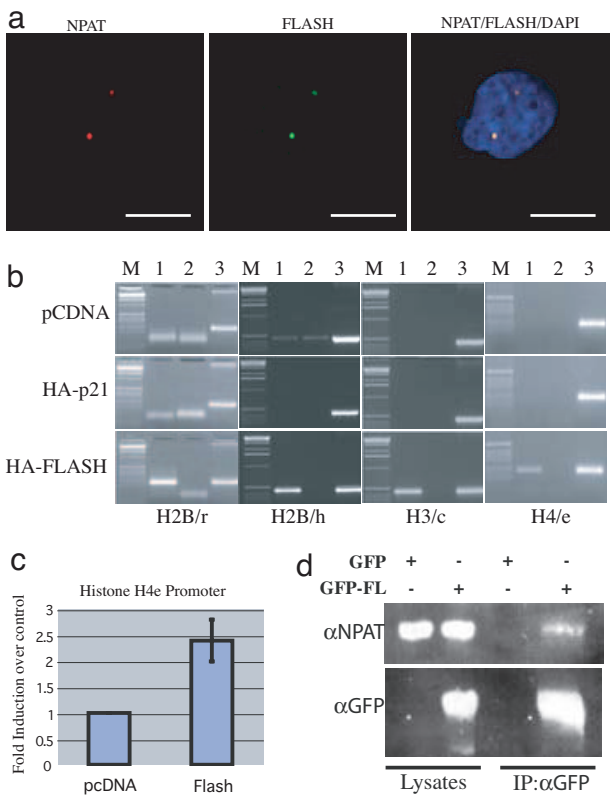

Fig. 2. FLASH interacts with p220/NPAT and binds to histone gene promoters. (*a*) Coimmunostaining by using anti-FLASH (green) and anti-NPAT (red) antibodies in SAOS-2 cells shows that the endogenous proteins colocalize (Scale bar = 5 μm). (*b*) Chromatin IP showing that FLASH binds to promoter sequences for histones: H2B/r, H2B/h, H3/c, and H4/e. Cells were transfected with either an empty vector (pCDNA3), with HA-FLASH (HA-FLASH), or with a vector coding for an unrelated HA-tagged protein (HA-p21). PCR was performed on DNA extracted from lysates subjected to IP with anti-HA antibody (lane 1) or with an unrelated anti-tubulin antibody (lane 2). As a positive control, PCR was performed on DNA extracted from untreated cells (lane 3). A positive PCR band appears only in HA-FLASH-transfected cells immunoprecipitated with anti-HA antibody but not in control-transfected or control-immunoprecipitated samples. (*c*) Luciferase assay on H1299 cells transfected with a luciferase reporter gene under the control of histone H4/e promoter sequence together with an empty vector (pCDNA) or HA-FLASH (FLASH). (*d*) HEK-293 cells were transfected with pEGFP or GFP-FLASH, and nuclear extracts were subjected to IP with anti-GFP antibody. Western blot against NPAT shows that endogenous NPAT coimmunoprecipitates with GFP-FLASH.

(Fig. 2*b*). Furthermore, we found that overexpression of FLASH up-regulated the activity of a histone H4 promoter in a luciferase assay (Fig. 2*c*), suggesting a direct role of FLASH in histone gene

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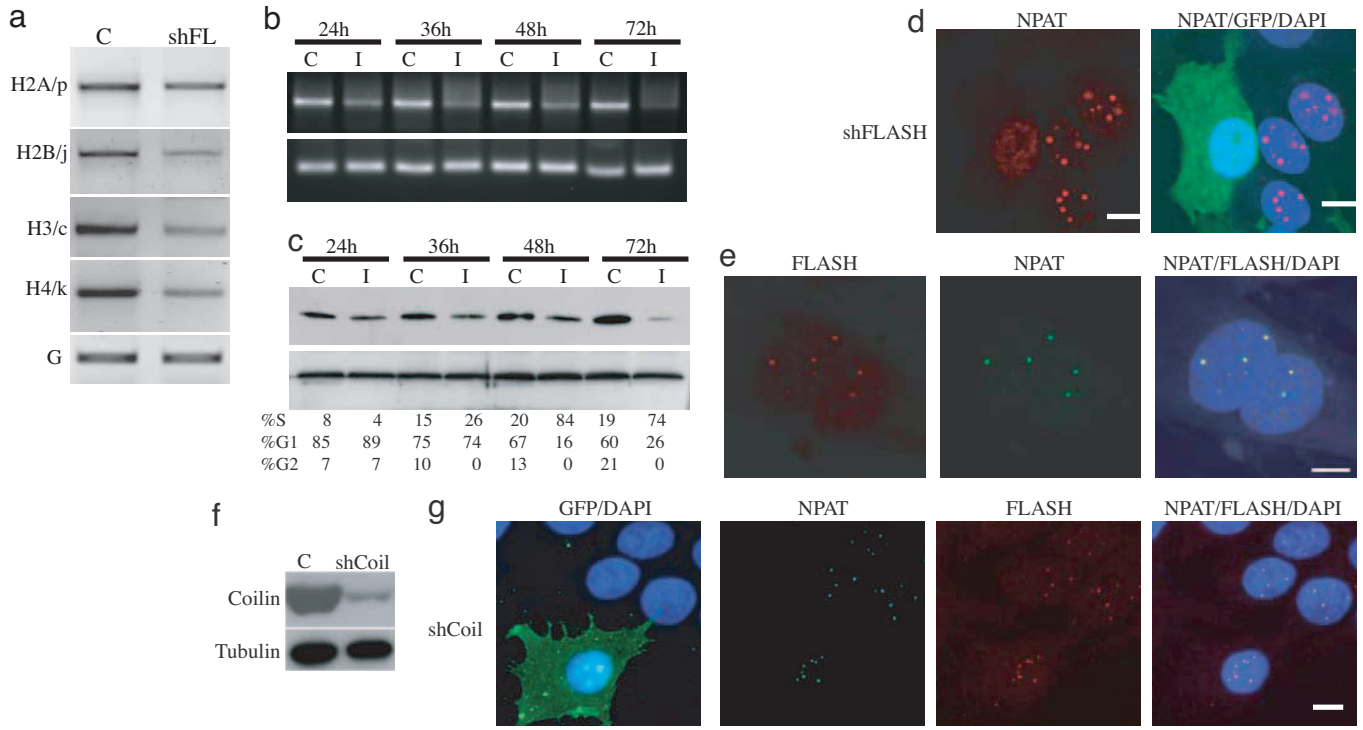

Fig. 3. FLASH down-regulation affects histone gene mRNA and protein levels. (*a*) Representative RT-PCR showing mRNA steady state levels for histones H2A/p, H2B/j, H3/c, and H4/k in HeLa cells transfected with pGFP-spectrin and either pSUPER-scrambled (control) or pSUPER-FLASH-1 (shFLASH) (1:5 ratio). GFP-positive cells were sorted by flow cytometry 48 h after transfection, and total RNA was extracted. Twenty-four cycles of amplification were performed after checking that this reaction was in the linear amplification range. GAPDH amplification (G) was used to demonstrate equal loading. Down-regulation of FLASH results in a significant reduction of mRNA levels for all of the histones tested. HeLa cells were transfected with pGFP-spectrin and either pSUPER-scrambled (C) or pSUPER-FLASH-1 (I) (1:5 ratio), and harvested for protein and RNA extraction 24, 36, 48, and 72 h after transfection. Representative RT-PCR (*b*) and Western blot (*c*) showing mRNA and protein steady state levels for histone H4 (*Upper*). For the PCR, 24 cycles of amplification were performed after checking that this reaction is still in the linear range. GAPDH amplification was used to demonstrate equal loading, and tubulin was used to confirm equal loading in the Western blot (*Lower*). An aliquot of cells from the same experiment was used to analyze cell-cycle distribution of the cells, and the results are reported below the Western blot. (*d*) Immunofluorescence of MCF-7 cells transfected with pSUPER FLASH (shFLASH) together with pGFP-spectrin (5:1 ratio) and stained with anti-NPAT antibodies. (*e*) Immunofluorescence of coilin −/− MEFs by using antibodies against FLASH (red) p220/NPAT (green) shows that the protein still colocalize. (*f*) Western blot of MCF-7 cells transfected with pSUPER-coilin (shCoil) or with a control vector (C) and collected 48 h after transfection, using an anti-coilin antibody. A strong reduction of coilin protein levels is observed. (*g*) Immunofluorescence using antibodies against p220/NPAT (green) and FLASH (red) showing that in MCF-7 cells in which coilin has been down-regulated by transfection with pSUPER-coilin (shCoil) together with pGFP-spectrin for 48 h (first panel; green), and FLASH and NPAT still colocalize. Please note that, to use four colors, NPAT was stained with a secondary antibody emitting in far red and falsely colored in green to show the overlap with FLASH in red.

transcriptional regulation. Although we were unable to immunoprecipitate sufficient quantities of endogenous FLASH with the available antibodies, transient transfection of GFP-tagged FLASH, followed by coimmunoprecipitation analysis revealed that FLASH and NPAT interact *in vivo* (Fig. 2*d*). These findings demonstrate that, like NPAT, FLASH is localized to histone gene promoters.

FLASH Is Required for Efficient Histone Gene Expression. CB components such as NPAT and CDK2/cyclin E have been shown to play a major role in S phase regulation and histone precursor mRNA transcription (7, 8, 12). Disruption of their function results either in an S phase block or a complete failure to enter S from G_1 (8, 10, 11). We therefore investigated whether the S phase block we observed after FLASH down-regulation (Fig. 1*a*), was accompanied by changes in histone mRNA levels. Fig. 3 shows that depletion of FLASH resulted in a pronounced reduction of both histone mRNA (as measured by RT-PCR for four distinct histone messages; Fig. 3 *a* and *b*) and protein (as measured by Western blotting for histone H4; Fig. 3*c*) levels. Moreover, this reduction was paralleled by an S phase block (Fig. 3*c*). To verify whether FLASH down-regulation affected also general transcription, we measured BrU incorporation before and after FLASH down regulation by shRNA. As shown in Fig. 7, which is published as supporting information on the PNAS web site, FLASH down-regulation does not affect general transcription.

As shown in Fig. 3*d*, down-regulation of FLASH resulted in redistribution of NPAT to a diffuse nuclear staining instead of the normal focal pattern. This phenomenon seems to be an early event after FLASH down-regulation, because ≈98% of the cells showed NPAT delocalization 24 h posttransfection. Complete delocalization was observed in all cells within 36 h. The FLASH interaction with NPAT is coilin independent, because down-regulation of coilin by shRNA in human cells did not affect colocalization of these two proteins (Fig. 3 *e* and *f*); FLASH and NPAT also colocalized in coilin knockout mouse cells (Fig. 3*g*). 48 h after FLASH shRNA transfection, NPAT levels remained comparable with controls, although at longer time points the protein began to be degraded (Fig. 4*a*). Again, coilin depletion had no effect on NPAT protein levels (Fig. 4*a*). Similarly down-regulation of FLASH resulted in reduction of coilin protein levels (already visible 48 h after transfection; data not shown). Although more detailed studies are required to investigate the degradation pathways of these proteins, our data suggest that after delocalization from CBs, coilin and NPAT are

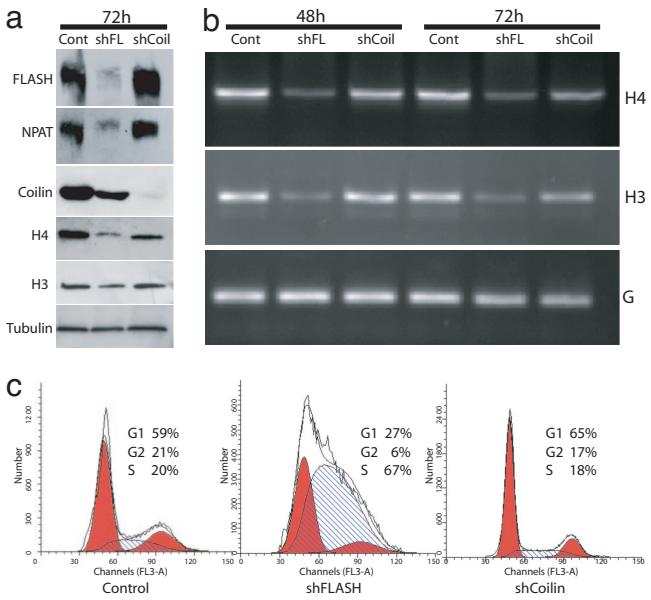

Fig. 4. Down-regulation of coilin does not affect cell-cycle and histone transcription. HeLa cells were transfected with either a pSUPER-FLASH-1 (shFL) or pSUPER-coilin (shCoil) or pSUPER-scrambled sequence (Cont) together with pGFP-spectrin. An aliquot of each sample was used for cell-cycle detection, mRNA, and protein extraction. (*a*) Western blot for the indicated proteins was performed on HeLa cells treated as described earlier for 72 h. Whereas FLASH down-regulation results in the decrease of histones H4 and H3 and NPAT levels, coilin down-regulation produces only a very modest effect on histone H4 protein levels. (*b*) RT-PCR for histones H4/k and H3/c using mRNA of HeLa cells treated as described earlier for 48 and 72 h. After 72 h, down-regulation of coilin produces a modest effect on histone genes mRNA levels. (*c*) Cell-cycle analysis of cells transfected as described earlier for 72 h. Coilin down-regulation does not affect cell-cycle regulation.

targeted for degradation. Taken together, our findings demonstrate that, like NPAT, FLASH plays an important role in the expression of the cell-cycle-dependent histone genes. Moreover, this function of CBs seems to be coilin-independent because coilin down-regulation by shRNA has very little effect and at much longer time points on histone transcription and on cell-cycle regulation (Fig. 4 *b* and *c*).

Discussion

FLASH was originally identified as a protein involved in one of the main apoptotic signaling pathways, as part of the multiprotein complex known as the death-inducing signaling complex (DISC) (13). Such a role for FLASH has been seriously questioned (16) and the biological function of the protein has remained elusive for several years. Here we identify a physiological role for FLASH in histone gene expression. Our data clearly show that FLASH colocalizes with NPAT to the major histone gene clusters. FLASH forms a complex with NPAT and is recruited to histone gene promoters. FLASH expression peaks during S phase of the cell cycle and down-regulation of the protein by shRNA results in the inability of cells to progress through S phase. Like NPAT, FLASH is required for Cajal body homeostasis, and down-regulation of FLASH results in reduced expression of histone precursor mRNA and protein. We conclude that FLASH is an important component of the machinery required for expression of the cell-cycle-dependent histone genes.

As detailed earlier, FLASH and NPAT share a number of common features: both proteins localize to histone gene clusters, are important for proper S-phase expression of histone genes, and are required for maintenance of Cajal body structure. Despite these similarities, a number of differences are also apparent. FLASH and NPAT do not share significant regions of sequence similarity. On transcription or translation block, FLASH is rapidly degraded, whereas NPAT persists for longer (see ref. 15). Most importantly, depletion of NPAT results in a failure to enter S-phase (9), whereas down-regulation of FLASH blocks progression through S-phase (Figs. 1*a* and 5). Thus NPAT seems to be required for the CDK2/cyclin E-mediated transition from G_1 to S, resulting in accumulation of cells in G_1. This consideration suggests that, although the two genes are each involved in the regulation of histone gene transcription, NPAT plays a role in regulating S-phase entry that is independent of FLASH.

Precisely how Cajal bodies are involved in FLASH and NPAT function is unclear. Previous work on NPAT suggested that phosphorylation of NPAT by CB-bound CDK2/cyclin E (7–10, 12) leads to the assembly of histone gene transcription complexes. Because the U7 small nuclear ribonucleoproteins accumulates in CBs and is required for processing of the cell-cycle-regulated histone precursor mRNAs, the prevailing view holds that components involved in histone gene expression are preassembled in CBs and then translocated to the adjacent sites of histone gene transcription. Our data are consistent with this hypothesis. Future work will be required to determine whether FLASH is involved in the other CB functions, such as the maturation of small ribonucleoproteins. In conclusion, we have established that FLASH is essential for the normal homeostasis of CBs and have demonstrated an important functional role for this protein in histone transcription and cell-cycle progression.

Materials and Methods

Cell Cultures and Transfections. Human primary dermal fibroblasts, H1299, SAOS-2, and IMR90 cells were grown in RPMI medium 1640 (Invitrogen, Carlsbad, CA), HeLa and MCF-7 cell lines and MEFs were grown in DMEM (Invitrogen) at 37°C in a humidified atmosphere of 5% (vol/vol) CO_2 in air. All of the media were supplemented with 10% (vol/vol) FBS (Invitrogen). Transient transfections were performed with the Calcium Phosphate Transfection kit (Invitrogen) according to the manufacturer's protocol, except for MEFs and MCF-7, which were transfected with FUGENE 6 (Roche, Basel, Switzerland) according to the manufacturer's protocol, and H1299, which were transfected with lipofectamine 2000 (Invitrogen).

Cell-cycle synchronization with thymidine block and deoxycytidine release or nocodazole block was performed as described in ref. 17 for the indicated time.

Plasmids. FLASH cDNA was amplified by RT-PCR. Forward primer, 5′-atggcagcagatgat-3′; reverse primer, 5′-cagttttacgtctatt-3′ (GenBank no. 16306505) from Normal Epidermal Human Keratinocytes (NEHK) (American Type Culture Collection, Manassas, VA) RNA, and then cloned in-frame with the HA tag into pcDNA by using the NheI and XhoI unique restriction sites. The insert was then subcloned in-frame with an amino-terminal GFP tag in pEGFP-C3 vector (BD Clontech, Mountain View, CA).

The pSUPER-FLASH-1, pSUPER-FLASH-2, pSUPER-Coilin, and pSUPER-scrambled vectors were generated by insertion in pSUPER vector (OligoEngine, Seattle, WA) of oligos targeting the following sequences: FLASH-1, 5′-gattgtctgagtttccaca-3′ (this sequence is 100% identical both in human and mouse FLASH); FLASH-2, 5′-aagggagaagtccttgataat-3′; Coilin 5′-agttgctgagaattctg-3′; scrambled, 5′-aattctccgaacgtgtcacgt-3′. pGFP-spectrin was kindly provided by R. F. Kalejta (Institute for Molecular Virology, University of Wisconsin, Madison, WI) (18).

Immunofluorescence and BrdU Incorporation. Immunofluorescence was performed as described in ref. 17. The following antibodies were used for immunofluorescence: anti-coilin antibody, ab11822 (Abcam, Cambridge, U.K.); anti-NPAT antibody,

611344 (BD Transduction Laboratories, San Jose, CA); anti-BRCA1 (D-9), sc-6954 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-TRF2, IMG-124 (Imgenex, San Diego, CA); and anti-MRE11, MS-MRE11-PX1 (Genetex, San Antonio, TX).

For FLASH, we used anti-FLASH antibody M300, sc-9088, lot no. B040 (Santa Cruz Biotechnology) for all of the immunofluorescence shown. In addition, similar staining (data not shown) was obtained by using the rabbit anti-FLASH antibody purified clone 522 generated by T.G.H. and the rabbit anti-FLASH antibody SL1133 and SL1134 generated in our laboratory.

For BrdU incorporation, IMR90 cells were cultured in RPMI medium 1640 containing 10 μg/ml BrdU (Sigma, St. Louis, MO) for 60 minutes, washed two times in PBS, fixed in 4% paraformaldehyde and stained with anti-FLASH antibody. To detect BrdU, the cells were fixed once more with 4% paraformaldehyde, treated with 3 M HCl for 15 min, washed three times in PBS and incubated with anti-BrdU antibody no. 1170376 (Roche).

Cell-Cycle Detection. Cell Cycle was analyzed by flow cytometric evaluation of DNA content according to the Nicoletti method (19). Cells were transfected with a 5:1 ratio of pSUPER-FLASH-1, pSUPER-Coilin, or pSUPER-scrambled together with a plasmid expressing pGFP-spectrin. Cells were gated for GFP expression to allow analysis only of transfected cells, and 20,000 events were evaluated by using the Cell Quest Program (BD, Franklin Lakes, NJ) and ModFit LT software (Verity Software; BD).

Colony-Forming Assays. Colony-forming assay was performed on SAOS2 cells transfected with pSUPER-FLASH-1 or pSUPER-scrambled together with a pBabe-puro vector for selection as described (17).

Histone mRNA Evaluation. RT-PCR for replication-dependent histones was performed by using the following primers: histone H2A/p (NM_021064), forward 5′-atgtctggacgtggccaagca-3′, reverse 5′-agcttgttgagctcctcgtg-3′; histone H2B/j (AF531291), forward 5′-ccgaagaagggctccaagaa-3′, reverse 5′-ttatttggagctggtgtacttg-3′; histone H3/c (AF531276), 5′-agctcgcaagtctaccggcg-3′, reverse 5′-cgtttagcgtgaatagcgca-3′; histone H4/k (NM_003546), forward 5′-caaagttctgcgcgacaaca-3′, reverse 5′-gccgccaaagccatacaggg-3′.

Western Blotting. Western blots were performed as described (17) by using the following antibodies: anti-FLASH, M300, sc-9088, lot no. B040 (Santa Cruz Biotechnology), anti-FLASH antibody rabbit SL1133 and SL1134 generated in our laboratory; anti-Histone H4, #2592 (Cell Signaling, Beverly, MA); anti-Histone H3 no. 9715 (Cell Signaling); anti-GFP monoclonal antibody, 632375 (BD Clontech), anti-NPAT antibody, 611344 (BD Transduction Laboratories); anti-coilin, ab11822 (Abcam).

Immunoprecipitation. Hek-293 cells were transiently transfected with 20 μg of GFP-FLASH or pEGFP control vector and harvested 48 h after transfection (20). Nuclear extracts were prepared by using Cell Lytic Nuclear Extraction kit (Sigma). Immunoprecipitation was performed as described, incubating 3 mg of nuclear extracts with anti-GFP polyclonal antibody, 632460 (BD Clontech) (17). Membranes were probed with the anti-NPAT antibody, 611344 (BD Transduction Laboratories).

ChIP. SAOS2 cells (2×10^6) were transfected with either pcDNA, HA-FLASH, or an unrelated gene HA-p21. ChIP was performed as described (17) by using mouse anti HA antibody (BABCO, Denver, PA) or an unrelated, anti-β-tubulin antibody for the immunoprecipitation step.

DNA samples were then analyzed with 35 cycles of PCR to amplify the indicated histone promoter sequences. The primers used were as follows: for histone H2B/r (7) 5′-ggatttgcgaatcctgattgggca-3′, 5′-agcactgtgtagctataaagcgcc-3′; for histone H3/c, 5′-gagtctgaacgtttctggtg-3′, 5′-ccgccggtagacttgcgagct-3; for histone H2B/h, 5′-actagaaacataatatccgt-3′, 5′-cttcttggagcccttctcgg-3′; for histone H4/e (7), 5′-gcgggacttcccgccgacttcttc-3′, 5′-gcagtactttacggtggcgcttagc-3′ (please note that these primers also amplify the promoter region of histone H4/d giving an amplification product that differs only for 4 bp; therefore, either gene or both can be pulled down in this assay).

Luciferase Assay. H1299 cells were transfected in 96-well plates with 80 ng per well of HA-FLASH or pCDNA control vector together with 60 ng per well of a construct containing the luciferase gene under the control of histone H4/e promoter (H4e-Luc) and a Renilla luciferase reporter (1.2 ng per well). Thirty-six hours later, the luciferase activity was quantified by using a commercially available kit (Dual-Glo-Luciferase Reporter Assay System; Promega, Madison, WI) with a Perkin–Elmer Victor2 luminometer. Histone H4/e-Luc was obtained by cloning a fragment of histone H4/e gene from nucleotides -120 to $+4$ obtained by PCR amplification with the following primers: 5′-ggctagccctatttcggtttggccctt-3′ and 5′-cctcgagttgtggcctctgacctgaggc-3′ containing Nhe-I and Xho-I sites. This fragment corresponds to a promoter fragment previously described (7, 21).

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