The human Cranio Facial Development Protein 1 (Cfdp1) gene encodes a protein required for the maintenance of higher-order chromatin organization

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The human Cranio Facial Development Protein 1 (Cfdp1) gene maps to chromosome 16q22.2-q22.3 and encodes the CFDP1 protein, which belongs to the evolutionarily conserved Bucentaur (BCNT) family. Craniofacial malformations are developmental disorders of particular biomedical and clinical interest, because they represent the main cause of infant mortality and disability in humans, thus it is important to understand the cellular functions and mechanism of action of the CFDP1 protein. We have carried out a multi-disciplinary study, combining cell biology, reverse genetics and biochemistry, to provide the first in vivo characterization of CFDP1 protein functions in human cells. We show that CFDP1 binds to chromatin and interacts with subunits of the SRCAP chromatin remodeling complex. An RNAi-mediated depletion of CFDP1 in HeLa cells affects chromosome organization, SMC2 condensin recruitment and cell cycle progression. Our findings provide new insight into the chromatin functions and mechanisms of the CFDP1 protein and contribute to our understanding of the link between epigenetic regulation and the onset of human complex developmental disorders.

Chromatin organization is highly dynamic and subject to many epigenetic changes, mediated by histone modifying enzymes and ATP-dependent chromatin remodeling complexes1. These complexes are multi-protein molecular devices able to slide or displace nucleosomes, thus making DNA more accessible to specific binding proteins that control essential cellular processes, such as transcription, replication and DNA repair.

Over the last decade, growing evidence has shown that mutations in genes which encode the epigenetic regulators controlling chromatin configuration can promote cancer and human developmental disorders2–9. An emblematic case of these “chromatin diseases” is the developmental genetic syndrome called CHARGE10, which is caused by mutations in the gene encoding a member of the CHD family of ATP-dependent chromatin remodeling enzymes4,11. The identification of new candidate genes and proteins will challenge us to expand our understanding of how epigenetic alterations of chromatin structure can perturb development and trigger the onset of human diseases, and will have a significant impact on applied research.

One possible candidate in human developmental diseases is the Cranio Facial Development Protein 1 gene (Cfdp1). Cfdp1 is 139,815 bp long with 7 exons and 6 introns and maps to chromosome 16 in the band 16q22.2-q22.3, where it is proximally and distally flanked by Bcar1 and Tmem170A genes, respectively12–15. Cfdp1 expression has been detected in a wide range of human tissues, including cancer tissues. It encodes a protein of 299 amino acids, called CFDP1, belonging to the evolutionarily conserved family of Bucentaur (BCNT) proteins12–14. The CFDP1 protein is characterized by an 82-amino acid region located at the C-terminus, called the BCNT-C domain, which is highly conserved among almost all eukaryotes, while the N-terminal region is more divergent12–14.

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The functions of Cfdp1 orthologs have been investigated in different species\(^1\)\(^2\)\(^3\)\(^4\). In particular, the observation that mouse Cfdp1 is expressed during tooth development suggested an involvement of this gene in craniofacial development\(^5\)\(^6\)\(^7\). Further evidence linked the CFD1 protein to craniofacial development and osteogenesis in vertebrates\(^8\)\(^9\)\(^10\), although specific syndromes caused by mutations of Cfdp1 have not yet been identified.

An integrative global proteomic study provided evidence suggesting that the CFD1 protein interacts with members of the SNF2-related CBP activator protein (SRCAP) chromatin remodeling complex\(^21\) which catalyzes an ATP-dependent exchange of canonical histone H2A for variant H2A.Z in humans\(^22\). Intriguingly, truncating mutations of the Scap gene cause the rare Floating Harbor syndrome that, among other defects, includes craniofacial abnormalities\(^9\).

Drosophila YETI and yeast SWC5, two orthologs of CFD1, were found to be subunits of the d-Tip60 and Swr1 chromatin remodeling complexes, respectively\(^23\)\(^24\). Both d-Tip60 and Swr1 complexes share a dozen subunits with the SRCAP complex and are functionally and evolutionarily related to it, in that they also govern variant H2A loading onto chromatin\(^1\)\(^2\)\(^3\)\(^4\)\(^5\)\(^6\)\(^7\)\(^8\).

More recently, it has been shown that the CFD1 protein expressed in wild-type Drosophila melanogaster is able to bind salivary gland polytene chromosomes, strongly affecting chromatin organization and H2Av deposition in a dominant-negative fashion\(^25\). In addition to its possible role in chromatin remodeling, CFD1 may also have autonomous functions in transcriptional regulation, as suggested by its interactions with SMAD3 and Ewings Sarcoma (EWS) proteins, which are involved in the modulation of transcription\(^26\).

Thus far, studies on the in vivo function(s) of CFD1 in human cells are missing.

In the present work, by combining cell biology with reverse genetics and biochemical approaches, we performed a functional analysis of the role played by the CFD1 protein in human cells. We used Western blotting to detect two endogenous CFD1 isoforms of 50 and 35 kDa in HeLa, U2OS and MRC5 cell lines. Immunofluorescence microscopy (IFM) and chromatin fractionation assays, together with the expression of intact or truncated Flag-CFD1 proteins, suggest that the 50 kDa isoform is a chromatin-binding protein that interacts with the SRCAP chromatin remodeling complex. In addition, the depletion of endogenous CFD1 in HeLa cells drastically affects higher-order chromatin organization and cell cycle progression.

Results

Nuclear localization and chromatin association of CFD1 in human cell lines. We initially performed Western blotting assays on total protein extracts from HeLa, U2OS and MRC5 cell lines, using a mouse monoclonal antibody to CFD1 (see Materials and Methods). The results showed the presence of two sharp bands of about 50 kDa and 35 kDa in all three cell lines (Fig. 1A). The Cfdp1 gene is predicted to undergo alternative splicing, giving rise to two mRNAs which differ for the presence or absence of the last exon (http://www.uniprot.org/blast/?about=Q9UEE9-2). It is conceivable that the 50 kDa and 35 kDa bands represent the CFD1 isoforms 1 and 2, respectively, the shorter isoform lacking the last 82 amino acids which correspond to the evolutionary conserved BCNT domain.

Next, we used immunofluorescence microscopy (IFM) with the same monoclonal antibody to visualize the cellular localization of the endogenous CFD1. As shown in Fig. 1B, CFD1 is present in the nucleus of HeLa cells in interphase but is not seen on mitotic chromosomes. The staining of interphase nuclei was confirmed by additional IFM experiments performed in HeLa, U2OS and MRC5 cell lines. Immunofluorescence microscopy (IFM) and chromatin fractionation assays, together with the expression of intact or truncated Flag-CFD1 proteins, suggest that the 50 kDa isoform is a chromatin-binding protein that interacts with the SRCAP chromatin remodeling complex. In addition, the depletion of endogenous CFD1 in HeLa cells drastically affects higher-order chromatin organization and cell cycle progression.

Chromatin association of CFD1 requires both N- and C-terminal regions. To further validate the nuclear localization and chromatin binding ability of CFD1, as is the case for the Drosophila melanogaster ortholog YETI\(^1\), we expressed flag-tagged CFD1 from a full length cDNA and two truncated cDNAs that carried either the N-terminal or the C-terminal portion of the gene, in HeLa cells (see Materials and Methods). Worth noting, the Flag-CFD1-Nt variant exactly matches the putative isoform 2 of CFD1, in that it carries the first 217 amino acids and lacks the C-terminal BCNT domain (Fig. 2B).

IFM showed that Flag-CFD1, Flag-CFD1-Nt and Flag-CFD1-Ct are all able to enter the cell nuclei (Fig. 3C,D,E). A fractionation assay, however, showed all the three forms present in the soluble fractions, while only Flag-CFD1 is detectable in the chromatin-bound fractions (Fig. 2F). It appears that both Flag-CFD1-Nt and Flag-CFD1-Ct truncated forms are defective for the chromatin binding activity. We must conclude that two different regions of CFD1, one included in Flag-CFD1-Nt and the other in Flag-CFD1-Ct, are present simultaneously for chromatin binding. In addition, these results, together with the results obtained in synchronized HeLa cells (Fig. 2A), strongly suggest that the chromatin-binding activity is specific to the 50 kDa isoform of CFD1, while the 35 kDa isoform may have different functions.

Depletion of CFD1 in HeLa cells affects chromosome organization and condensin recruitment in mitosis. To investigate the function of CFD1 protein in human cells, we performed a cytological analysis
Figure 1. Expression and localization of CFDP1 protein in human cells (A) Western blotting of protein extracts from HeLa, U2OS and MRC5 cells with a mouse monoclonal antibody against human CFDP1 (bottom) and ISWI (hSNF2) as loading control (top). The intensity of the 35 kDa band differs between human cell lines tested, being lower in U2OS and MRC5 cells compared to HeLa; this observation suggests that the synthesis of the 35 kDa band (CFDP1 isoform 2) is subject to regulatory controls. Both 50 kDa and 35 kDa bands are also recognized by a rabbit polyclonal antibody to CFDP1 (see G). (B) Fixed HeLa cells were stained with the mouse monoclonal antibody to CFDP1 (red) and counterstained with DAPI (blue). The CFDP1 staining is clearly present in the interphase nuclei, while no significant staining was detected on mitotic chromosomes (pointed by arrows). (C,D,E) Human cell lines were also incubated with a rabbit polyclonal antibodies to human CFDP1 and with commercial antibodies to α-tubulin. From left to right panels: DAPI (blue), α-tubulin (green), CFDP1 (red) and merge in: (C) HeLa, (D) U2OS and (E) MRC5 cells. A significant fluorescent staining of interphase nuclei was observed in all the three lines. (F) and (G) Western blotting with mouse monoclonal and rabbit polyclonal antibodies to CFDP1, respectively; it appears that in RNAi-treated cells (RNAi lane) the intensity of both CFDP1 bands is strongly reduced compared to the mock-treated control cells (mock 100% lane); ISWI (hSNF2) was used as control (top). (H) The CFDP1 nuclear staining with monoclonal antibodies shows about 70% decreased in RNAi-treated HeLa cells compared to the mock.
of fixed HeLa cell preparations after RNAi-mediated knock-down of Cfdp1 (see Materials and Methods). The efficiency of CFDP1 protein depletion was monitored by Western blotting, as previously shown in Fig. 1F,G. Metaphase chromosome spreads from CFDP1-depleted cells displayed aberrant morphology and condensation defects when compared to mock-treated cells (Fig. 3A–C). In a total of 115 scored metaphase chromosome spreads, 45% of abnormally condensed figures were seen in Cfdp1-siRNA treated cells, while only 3% of such metaphases were found in the mock-treated controls. Thus, it appears that CFDP1 activity is required for the maintenance of proper higher-order chromatin organization in human cells. This behavior strongly resembles that of the Drosophila YETI protein24. In addition, the count of mitotic figures showed that the mitotic index in CFDP1 depleted cells is decreased about 75% compared to controls (Fig. 3P), indicating that the loss of CFDP1 can also affect cell cycle progression.

Given that the condensin complexes play a key role in chromosome condensation, we also studied the localization of SMC2, a subunit of the condensin I and II complexes32 in CFDP1-depleted HeLa cells. The results of this analysis are shown in Fig. 3D,E,H,I. In about 80% of metaphase cells (634 in total) a significant loss of chromosomal SMC2 was detected (Fig. 3Q). We also observed a high proportion (30%) of telophases with chromatin bridges (Fig. 3F,G,L,M,N,O,R). This abnormality may be the consequence of chromosome stickiness due to condensation defects. Together, these results strongly support a role for CFDP1 in chromatin organization.
Expression of Flag-YETI in HeLa cells mimics CFDP1 depletion. Our previous results suggest that when CFDP1 is expressed in wild-type fruit flies, it can physically interact with YETI to form inactive heterodimers; this would result in an overall depletion of functional YETI with a consequent disruption of chromatin organization and individual viability. To test whether YETI overexpression may affect cell behavior in human cells, we transiently transfected HeLa cells with Flag-Yeti and V5-Cfdp1 cDNA constructs. The IF staining showed that V5-CFDP1 and Flag-YETI are able to enter the nuclei (Fig. 4A–C). Moreover, in a fractionation assay both Flag-YETI and V5-CFDP1
were found in the chromatin-bound fraction, as well as in the soluble fraction (Fig. 4D). As shown in Fig. 4E, HeLa cells overexpressing Flag-YETI exhibit a significant decrease in mitotic index (about 50%), although the effect is not as strong as in CFDP1 depleted cells. By contrast, the overexpression of the V5-CFDP1 fusion protein did not affect mitotic index. In addition, in HeLa cells overexpressing V5-CFDP1 and Flag-YETI constructs, the V5-CFDP1 fusion protein is not able to rescue the mitotic index decrease (Fig. 4E).

**Co-IP assays in HeLa cells.** As discussed in the introduction, a global proteomic study provided evidence that the CFDP1 protein interacts with members of the SNF2-related CBP activator protein (SRCAP) chromatin remodeling complex. However, thus far these data have not been validated in vivo. To test whether CFDP1 interacts in vivo with members of the SRCAP complex, we performed a series of co-IP assays. HeLa cells were transfected with 3 different expression vectors containing different tagged proteins: CFDP1 tagged with V5 (V5-CFDP1), H2A.Z tagged with HA (HA-H2A.Z) and Arp6 tagged with Myc (Myc-Arp6). In parallel, HeLa cells were also transfected with two expression vectors containing CFDP1 tagged with V5 (V5-CFDP1) and SRCAP tagged with HA (HA-SRCAP). Cell extracts were immunoprecipitated with anti-V5 antibodies and then analyzed by Western blotting using antibodies against anti-Myc, HA and other known components of the SRCAP complex. As a negative control, we used HeLa cell extracts immunoprecipitated with Ms IgG.

As shown in Fig. 5, Myc-Arp6, P18\textsuperscript{Hamlet} and H2A were detected in V5-CFDP1 immunoprecipitates, but were absent in the Ms IgG control. By contrast, HA-H2A.Z and HA-SRCAP were not detected in the IP. These results suggest that CFDP1 interacts with Arp6 and P18\textsuperscript{Hamlet} which are known members of the SRCAP complex.

Independent experimental evidence has shown that YETI interacts with HP1\textgreek{a} in *Drosophila melanogaster*\textsuperscript{24,33}. Given that, we also analyzed V5-CFDP1 immunoprecipitates using antibodies against HP1\textgreek{a}. A reproducible HP1\textgreek{a} band, although of weak intensity, was apparent in the IP sample and absent in the control (Fig. 5), suggesting that the interaction between BCNT proteins and HP1\textgreek{a} is maintained during evolution.
result that corroborates our previous cycle progression. This could be due to the to chromatin (Fig. 4D) and produces a strong decrease in the mitotic index (Fig. 4E), with an impairment of cell symptoms of epigenetic diseases 6 and most known candidate genes are transcription factors and chromatin humans. Together with cognitive defects and growth abnormalities, craniofacial malformations are common in humans, which most likely result from alternative splicing. Both bands are the result of $\text{Cfdp1}$ expression, since their intensity strongly decreased following RNAi-mediated depletion of this gene (Fig. 1E).

Using IFM and chromatim fractionation we determined that CFDP1 has a nuclear localization and is found in the chromatin fraction in both interphase and metaphase (Figs 1 and 2). We also found that the chromatim-binding function of CFDP1 requires both its N- and C-terminal regions (Fig. 2). The N-terminal fragment used in our experiments lacks the last 82 amino acids as does the 35 kDa isoform. This region corresponds to the evolutionarily conserved BCNT-domain that was found to be crucial for the chromatim-binding activity of YETI in D. melanogaster 24. We speculate that the 50 kDa isoform of CFDP1 is the one that participates in chromatin organization, while the 35 kDa isoform may have a different and still undetected function.

The results of co-IP assays (Fig. 5) indicated that in HeLa cells CFDP1 interacts with members of the SRCAP chromatin remodeling complex that exchanges H2A and H2A.Z histones (22). We also found an interaction with endogenous H2A, but not with HA-H2A.Z (Fig. 5). This finding suggests that CFDP1, by interacting with H2A, may play a role in an H2A-H2B eviction reaction, rather than in H2A.Z-H2B deposition.

In the light of these results, it is conceivable that CFDP1 bounds the chromatin through interaction with members of the SRCAP complex (Fig. 5) and that the N- and C- terminally truncated variants are unable to enter the complex. However, it cannot be completely ruled out that CFDP1 may also have an autonomous chromatim-binding activity independent of its interaction with the SRCAP complex.

RNAi-mediated depletion of CFDP1 led to mitotic chromosomes with aberrant morphology and condensation defects as well as chromosome segregation defects such as chromatim bridges (Fig. 3). These defects recall those exhibited by Yeti null alleles in Drosophila melanogaster 24,34. Interestingly, the amount of SMC2 condensin, a major player in chromosome condensation that localizes to the axes of metaphase chromosomes, was strongly reduced in a high percentage of CFDP1-depleted cells (Fig. 3D,E). This could be a secondary consequence of drastic chromatim perturbations triggered by CFDP1 depletion. Alternatively, CFDP1 may play a direct role in the recruitment of condensin.

A recent study in fission yeast suggests that nucleosome eviction promotes condensin loading in mitosis 35. This is interesting in view of the interaction we detected between CFDP1 and members of the SRCAP complex. In fact, both H2A and H2A.Z histones have also been proposed to be involved in condensin binding to chromatim 36,37.

Moreover, the co-IP assays indicate that CFDP1 interacts with HP1$\alpha$, in the same way that Drosophila YETI interacts with HP1$\alpha$ 24,34. This finding suggests that BCNT proteins may be evolutionarily conserved mediators involved in the targeting of HP1 to chromatim remodeling regions.

We also found that Drosophila Flag-YETI expressed in HeLa cells enters the cell nucleus (Fig. 4A–C), binds to chromatim (Fig. 4D) and produces a strong decrease in the mitotic index (Fig. 4E), with an impairment of cell cycle progression. This could be due to the in vivo formation of inactive YETI-CFDP1 heterodimers (Fig. 4F), a result that corroborates our previous in vitro findings 29.

**Discussion**

**CFDP1 is required for chromatim organization in human cells.** We have carried out the first in vivo functional characterization of the Cfdp1 gene and its encoded protein in human cells. Two different isoforms of the protein can be detected in HeLa, U2OS and MRC5 cell lines (Fig. 1A), which most likely result from alternative splicing. Both bands are the result of Cfdp1 expression, since their intensity strongly decreased following RNAi-mediated depletion of this gene (Fig. 1E).

Using IFM and chromatim fractionation we determined that CFDP1 has a nuclear localization and is found in the chromatin fraction in both interphase and metaphase (Figs 1 and 2). We also found that the chromatim-binding function of CFDP1 requires both its N- and C-terminal regions (Fig. 2). The N-terminal fragment used in our experiments lacks the last 82 amino acids as does the 35 kDa isoform. This region corresponds to the evolutionarily conserved BCNT-domain that was found to be crucial for the chromatim-binding activity of YETI in D. melanogaster 24. We speculate that the 50 kDa isoform of CFDP1 is the one that participates in chromatin organization, while the 35 kDa isoform may have a different and still undetected function.

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**CFDP1 and craniofacial development.** Craniofacial malformations are developmental disorders of crucial biomedical and clinical interest, since they represent the main cause of infant mortality and disability in humans. Together with cognitive defects and growth abnormalities, craniofacial malformations are common symptoms of epigenetic diseases 8 and most known candidate genes are transcription factors and chromatim regulators 38–40.
Among craniofacial diseases, autosomal recessive primary microcephaly (MCPH) is a rare disorder characterized by a reduction in brain size and head circumference at birth and mild to severe mental retardation. Thus far, mutations in 12 genes have been found in patients with MCPH; these genes affect cell cycle regulation and DNA repair. Intriguingly, the MCPH1 gene encoding microcephalin shows some aspects in common with Cfdp1. Microcephalin was found to regulate one of the two condensin complexes present in the cell, condensin II. Similar to cells lacking CFDP1, MCPH1-depleted cells display aberrant chromosome condensation. It is unclear whether this is the major pathological mechanism in patients with MCPH1 mutations. Additional functions of MCPH1 during chromosome shaping and dynamics may be influencing. Similarly, depletion of CFDP1 may affect other processes in addition to mitosis. Given its interaction with the SRCAP chromatin remodeler, the function of CFDP1 may affect both higher-order chromatin organization throughout the cell cycle and gene regulation. In any case, in view of the similarities with MCPH1, it would be interesting to include Cfdp1 gene in the sequencing panels for microcephaly and related human disorders.

In conclusion, our findings provide new insight into the functions and mechanisms of the CFDP1 protein and contribute to our understanding of the link between epigenetic regulation and the onset of human craniofacial disorders.

Methods

Cytology and immunostaining. To analyze chromosome condensation, HeLa cells were treated at 37 °C for 2 h with 0.1 μg/mL colcemid, harvested by trypsinization and treated with hypotonic buffer (0.075 M KCl) for 10 min at room temperature. Cells were fixed with freshly made Fix solution (3:1 methyl alcohol/glacial acetic acid) at −20 °C. Then, chromosome preparations were made by air-drying method. For IF staining, HeLa cells were seeded on glass coverslips and 24 h later they were fixed for 15 min at room temperature (RT) in 2% formaldehyde in PBS. Cells were treated with hypotonic solution (75 mM KCl) for 30' at RT before fixation. After permeabilization in 0.2% Triton X-100 solution and washing in PBS, the cells were incubated in 3% bovine serum albumin (BSA) for 1 h and, subsequently, with primary antibodies for 2 h at RT. After several washes in PBS, the cells were incubated with secondary antibodies for 1 h at RT. After washing in PBS, the coverslips were mounted onto slides with anti-FADE solution containing the anti-bleaching agent DABCO (Sigma-Aldrich). Preparations were analyzed using a computer-controlled Nikon Eclipse 50i epifluorescence microscope equipped with a CCD camera. CFDP1 fluorescence intensity was assessed using the ImageJ software (http://rsbweb.nih.gov/ij/). About 400 nuclei were scored in three independent experiments. The primary antibodies were: mouse monoclonal anti-CFDP1 (1:100; Sigma-Aldrich); rabbit polyclonal anti-CFDP1 (1:200; Thermo Scientific); rabbit polyclonal anti-hSMC2; and rabbit anti-beta Tubulin (1:4000, Loading Control, Abcam). The secondary antibodies were: Donkey anti-rabbit IgG-CFL 555 (Santa Cruz Biotechnology; 1:200); and goat anti-mouse IgG-CFL 488 (Santa Cruz Biotechnology; 1:200).

CFDP1 constructs. To make Flag-CFDP1 constructs, sequences corresponding to full length (Flag-CFDP1), N-terminal (Flag-CFDP1-Nt) and C-terminal/BCNT (Flag-CFDP1-CT) of CFDP1 cDNA were cloned into the pENTR/D-TOPO (Invitrogen) and subcloned into pHRespuro3-MycFlag/GATCassette Gateway destination vector. V5-CFDP1 and Flag-YETI plasmid are described in our previous papers24,29. The constructs pCS2-5xMyc-Arp6, pcDNA3-HA-H2A.Z and pcDNA3-HA-SRCAP were described in ref. 47. For a description of the CFDP1 constructs, see Fig. S1.

Cell Cultures and RNAi treatments. HeLa cells, purchased from ATTC company, were cultured in 6-well plates in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS and a Penicillin/Streptomycin solution (Gibco, 15140122). Transfection was performed with 1 μg of CFDP1 esiRNA using Dharmafect (Thermo Scientific) according to the manufacturer’s protocol. Endoribonuclease-prepared siRNA (esiRNA) that targets CFDP1 was purchased from Sigma-Aldrich. Two days after transfection cells were harvested for cytological and immunoblotting analysis.

Cell cycle Synchronization. For cell cycle-dependent chromatin binding experiments, HeLa cells were synchronized in interphase (G1/S boundary) or metaphase using thymidine or thymidine/nocodazole blocks, respectively. Briefly, for G1/S boundary synchronization, cells were treated with 2 μM thymidine (Sigma, T9250) for 19 h, washed with PBS, harvested by centrifugation and frozen in liquid nitrogen. For metaphase synchronization, after thymidine treatment, HeLa cells were released from G1/S block in fresh media for 5 h, incubated with 40 nM nocodazole (Sigma, M14043) for 16 h and then harvested by mitotic shake-off. Mitotic cells were washed three times with PBS and released in fresh medium for 30’ before harvesting and freezing in liquid nitrogen. Interphase and metaphase cell samples were prepared by resuspending cells in Buffer A for subsequent chromatin fractionation assay.

Chromatin Fractionation Assay. HeLa cells (2 × 10^7) were resuspended in 1 mL of Buffer A (10 mM HEPES at pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol [DTT], 1 mM NaVO4, 5 mM 3-glycerophosphate, 0.1 mM phenyl methane sulphonyl fluoride [PMSE], 0.5 mM NaF, protease inhibitor cocktail [Roche]). Triton X-100 was added at 0.1% and cells were incubated for 5’ on ice. After centrifugation (4000 rpm for 5’ at 4°C), the nuclei enriched pellet was resuspended in 1 mL of Buffer B (3 mM EDTA, 2 mM EGTA, 1 mM DTT, 0.1 mM PMSE, protease inhibitors) and incubated for 5’ on ice. All the fractioned samples were load in a polyacrylamide gel and transferred onto PVDF membrane for Western blot analysis.

Immunoprecipitations. HeLa cells were transfected with 2 μg of V5-Cfdp1 and Myc-Arp6 or HA-H2A.Z expression vectors by using Lipofectamine 3000 reagent according to the manufacturer’s protocol, and 48 h later, they were lysated in IP buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EGTA, 5 mM EDTA, 20 mM NaF) supplemented with protease and phosphatase inhibitors cocktail (from Roche) and 1 mM PMSE.
Cleared lysates were immunoprecipitated O/N at 4 °C with 2 μg of mouse anti-V5 antibodies (Invitrogen) followed by 1 h of incubation with 30 μl of agarose-conjugated protein A/G (Santa Cruz Biotechnology). The beads were then washed three times in IP buffer and analyzed by immunoblotting.

Western blotting. HeLa cells were lysed in sample buffer at 10000 cells/μl. All the samples were load in a polyacrilamide gel, transferred onto PVDF membrane and probed with different antibodies: mouse monoclonal anti-CFDP1 (1:1000; Sigma-Aldrich); rabbit polyclonal anti-CFDP1 (1:1000, Thermo Scientific); mouse monoclonal anti-FLAG (1:1000; Sigma-Aldrich); mouse monoclonal anti-α-HA (1:1000, Cell Signaling); mouse monoclonal anti-c-Myc (1:1000, Clontech); mouse monoclonal anti-V5 (1:1000, Thermo Scientific); rabbit polyclonal anti-HI3 (1:1000, Cell Signaling); rabbit polyclonal anti-histone H2A (1:1000, Millipore); rabbit polyclonal anti-pH3(FAM121) (1:200)46; rabbit polyclonal anti-MEK2 (1:1000, Santa Cruz Biotechnology); rabbit polyclonal anti-histone H3 (1:15000, Abcam); rabbit polyclonal anti-H3p (1:5000)49; rabbit polyclonal anti-ISWI (1:5000)50. The bands were immunodetected using the Enhanced chemiluminescence (ECL) kit from Thermo Scientific.

Statistical analysis. Data analyses were performed using the GraphPad Prism softwares (GraphPad Software, Inc., La Jolla, CA, USA). All results are expressed as mean ± SD values from three independent replicate experiments. *P value of less than 0.05 (*P < 0.05, compared with the control group) are considered to be statistically significant by using two-tailed Fisher’s exact test.

Ethical approval and informed consent. All the methods were carried out in accordance with the approved guidelines.

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Conceived and designed the experiments: G.M., M.T.A., L.P., A.L., P.D.; Performed the experiments: G.M., M.T.A., F.A., E.P., L.P.; Analyzed the data: G.M., M.T.A., Y.P., L.P., A.L., P.D.; Wrote the paper: P.D.

Additional Information

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