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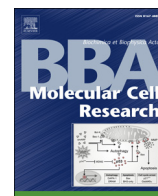
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Direct non transcriptional role of NF-Y in DNA replication



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ABSTRACT

NF-Y is a heterotrimeric transcription factor, which plays a pioneer role in the transcriptional control of promoters containing the CCAAT-box, among which genes involved in cell cycle regulation, apoptosis and DNA damage response. The knock-down of the sequence-specific subunit NF-YA triggers defects in S-phase progression, which lead to apoptotic cell death.

Here, we report that NF-Y has a critical function in DNA replication progression, independent from its transcriptional activity. NF-YA colocalizes with early DNA replication factories, its depletion affects the loading of replisome proteins to DNA, among which Cdc45, and delays the passage from early to middle-late S phase. Molecular combing experiments are consistent with a role for NF-Y in the control of fork progression. Finally, we unambiguously demonstrate a direct non-transcriptional role of NF-Y in the overall efficiency of DNA replication, specifically in the DNA elongation process, using a *Xenopus* cell-free system.

Our findings broaden the activity of NF-Y on a DNA metabolism other than transcription, supporting the existence of specific TFs required for proper and efficient DNA replication.

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1. Introduction

NF-Y is a transcription factor (TF) composed of the sequence-specific subunit NF-YA and the NF-YB/NF-YC histone-fold domain—HFD—dimer [1], which plays a pioneer role in the regulation of promoters containing the CCAAT box. NF-Y subunits are highly conserved in eukaryotes, from yeast to man, and dramatically expanded in plants. NF-Y binding affects the pattern of histone modifications, the binding of other TFs to neighboring sites, the recruitment of co-activators and, ultimately, the function of the RNA Pol II machinery [2–4]. It targets a multitude of genes involved in cell cycle regulation, apoptosis and DNA damage response [5–12].

NF-Y binding activity is regulated during the cell cycle due to the oscillation of NF-YA levels: while the expression of NF-YB and NF-YC subunits doesn't change during cell cycle progression, NF-YA levels,

and consequently NF-Y binding activity, are highest in S phase and lower in G2/M [9].

We, and others, recently reported that NF-YA knockdown in human and mouse cells triggers apoptosis, possibly because of DNA replication defects [13–15]. In particular, we found that NF-YA loss leads to the accumulation of BrdU negative cells with an S-phase DNA content, representing non-cycling S-phase cells that increase as apoptosis is inhibited. NF-YA inactivation delays the passage from early to late S-phase and DSBs occurred in S-phase cells. Altogether, these data raised the question whether NF-Y could have additional functions other than transcription and suggest that NF-Y could participate to S-phase progression and intra-S damage checkpoint [13].

A strong correlation between replication and transcription exists in metazoans, given the similarities between the two processes, both requiring the recruitment of multisubunit complexes to specific genomic sites and the association and movement of polymerases along the chromatin fiber. In addition, temporal changes in replication are coordinated with changes in gene transcription (for a review see Refs. [16, 17]). The interplay between transcription and replication involves both replication factors, such as ORCs, the MCM complex and Geminin [18–22], that can be involved in transcription regulation, and,

Abbreviations: TF, transcription factor; ORC, origin recognition complex; pre-RC, pre-replication complex; MCM, mini-chromosome maintenance; BrdU, 5-bromo-2'-deoxyuridine; PI, propidium iodide.

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reciprocally, sequence-specific TFs playing a role in replication origins activity, such as Myc, Spi-1/PU.1, and Hox proteins, whose depletion or overexpression affects DNA replication [23–25].

It is therefore possible that the organization of replication and transcription relies on common regulatory factors, and direct or indirect recruitment of replication proteins can be mediated by a combination of TFs. Studies of chromatin markers and DNaseI-hypersensitive regions annotated by ENCODE identified a correlation between replication timing and active chromatin modifications (H3K4me3 and H3ac) [26–29], suggesting that an open chromatin structure may favor the binding of proteins of the pre-replication complex. In general, early-replicating origins have been found in actively transcribed genes, while origins activated in late S-phase are associated with non-transcribed and heterochromatic regions [30–32].

In this study, we investigated the role of NF-YA in DNA replication in mammalian cells and *Xenopus* egg extracts. We showed that NF-YA colocalizes with early-replication factories and its inactivation severely affects S phase progression: the recruitment of replisome proteins on DNA is impaired and an increase in fork asymmetry and global forks density is observed in NF-YA knocked-down cells. Through the analysis of gene expression profiles, we determined that NF-YA loss doesn't cause significant transcriptional defects in DNA replication genes categories, suggesting its direct role in DNA replication.

Finally, the role of NF-Y in the advancement of replication forks has been demonstrated through a cell-free system derived from *Xenopus* eggs: the loss of NF-Y activity by immunodepletion or NF-Y-dominant negative expression triggers S phase-defects, associated to reduced DNA elongation and destabilization of the replisome.

2. Materials and methods

2.1. Cell lines and inactivation

Human colon carcinoma HCT116 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal calf serum (FCS). HCT116 cells were infected with shRNA lentiviral particles (MOI = 4) and harvested 48 h post-infection, as previously described [13]. Human lung adenocarcinoma epithelial A549 cells and spontaneously immortalized human keratinocyte HaCat cells were cultured in Dulbecco's Modified Medium (DMEM) supplemented with 10% FCS. The cells were infected with shRNA lentiviral particles (MOI = 6) and harvested 72 h post-infection, if not differently indicated.

Synchronization at the G1/S boundary was obtained by culturing HCT116 cells in IMDM without FCS for 24 h followed by the administration of 0.5 mM of Mimosine (Sigma Aldrich) for additional 16 h, or by culturing A549 and HaCat cells with 10% FCS-DMEM supplemented of 0.5 mM Mimosine for 20 h.

2.2. Cell proliferation analysis

Percentages of viable cells at the indicated time points upon NF-YA depletion versus SHC cells were determined by colorimetric cell viability assay (MTT).

2.3. Immunoblots

For Western blot analysis, NF-Y-inactivated and control cells were lysed in lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, 1 mM EDTA, protease and phosphatase inhibitors). Equivalent amount of chromatin enriched extracts, prepared as described by Mendez and Stillman [33], were resolved by SDS-PAGE, electrotransferred to PVDF membrane and immunoblotted with the following antibodies: anti-NF-YA (sc10779, Santa Cruz), anti-NF-YB (Pab001, GeneSpin), anti-Geminin (sc13015, Santa Cruz), anti-CDC45 (sc20685, Santa Cruz), anti-ORC2 (sc28742, Santa Cruz), anti-TopBP1 (sc22858, Santa Cruz), anti-PCNA (sc56, Santa Cruz), anti-MCM7

(sc9966, Santa Cruz), anti- γ H2AX (sc101696, Santa Cruz), anti-Mcm10 (A300-131A, Bethyl lab), anti-DNApo1D1 (A304-005A, Bethyl lab), anti-H3 (sc8654, Santa Cruz), anti-H4 (ab10158, Abcam).

2.4. Flow cytometric cell cycle analysis

Biparametric BrdU/PI cell cycle analysis was performed 48 h post-infection as previously described [13]. DNA distribution of propidium iodide (PI)-stained cells was analyzed upon 72 h from shCTR or shNF-YA transduction by an Epics cytometer (Beckman Coulter).

2.5. Immunofluorescence

For immunofluorescence analysis, HCT116 cells were pulsed 48 h post-shRNA infection with 20 μ M BrdU for 20 min, treated with 2 N HCl for 30 min at room temperature, followed by the addition of 0.1 M borate buffer, pH 8.5. Cells were washed and incubated with mouse anti-BrdU antibody (Beckton Dickinson) for 1 h at 4 °C [13]. After washing, cells were incubated with TRITC anti-mouse (#T5393, Sigma Aldrich) for 1 h at 4 °C. Finally, cells were stained with HOECHST (14533, Sigma Aldrich) and spotted onto microscope slides by cytospin centrifugation. Staining of BrdU/DAPI was analyzed by confocal microscopy (Leica DM IRE2).

For NF-YA/PCNA colocalization studies, HCT116 cells were seeded on coverslips and synchronized in Early-, Middle- and Late-S phase as explained above. Cells were washed twice in PBS 1 \times and soluble proteins were pre-extracted with Triton X-100 buffer (0.5% Triton X-100, 20 mM Hepes-KOH (pH 7.9), 50 mM NaCl, 3 mM MgCl₂, 300 mM Sucrose) for 2 min. Cells were rinsed gently in PBS 1 \times , fixed in cold methanol/acetone (1:1, v/v) for 5 min, washed again and re-permeabilized with 0.05% Triton-X 100 in PBS 1 \times for 10 min. After blocking with 1% BSA in PBS 1 \times for 15 min, samples were first incubated overnight at 4 °C with anti-NF-YA (sc-10779, Santa Cruz) and anti-PCNA (sc-56, Santa Cruz) antibodies (1:100 in 1% BSA in PBS1X), then for 1 h with the relative secondary antibodies, anti-rabbit FITC-conjugate (#F7512, Sigma Aldrich) and anti-mouse TRITC-conjugate (#T5393, Sigma Aldrich) diluted 1:200 in 1% (w/v) BSA in PBS 1 \times , and stained with DAPI. Images were obtained using a confocal microscopy (Leica DM IRE2) with a 63 \times objective. The assessment of colocalization of PCNA and NF-YA was performed using NIH ImageJ Just Another Colocalization plug-in (JACoP). JACoP provides the Pearson correlation coefficient (PCC), Manders' overlap M1 and M2 coefficients and Costes' randomization p-values for a pair of images in the red and green channels. Briefly, PCC measures the pattern similarities between two images, with values ranging from +1 (complete positive correlation) to -1 (negative correlation). Manders' coefficients indicate an actual overlap of the signals and represents the true degree of colocalization: M1 is an indicator of the proportion of the red signal (PCNA) coincident with a signal in the green channel (NF-YA) and M2 is the reverse of M1. M1 and M2 have a value ranging from 0 (no colocalization) to 1 (all pixels colocalize). Costes' randomization is a statistical significance algorithm based on the PCC: random images are created by shuffling pixels in the green channel and a new PCC is calculated for each randomized image. The PCC of the original image is then compared with the PCCs of the randomized images and the significance (p-value) is calculated. The p-value (expressed as percentage) is inversely correlated to the probability that the PCC of the original image is obtained by chance. For each early-S image analyzed, the Costes' p-value from Costes' randomized analysis was 100%, indicating high probability that the colocalization observed was not due to chance.

2.6. DNA combing analysis

Cells were labeled successively with 25 μ M IdU (Sigma-Aldrich) for 15 min, 200 μ M CldU for 15 min and 200 μ M thymidine for 1 h. Genomic DNA was prepared in agarose plugs (0.25 \times 10⁵ cells per plug) and DNA

combing on silanized coverslips was performed as described [34], except that agarose plugs were melted for 30 min at 65 °C before adding 1 U of β -agarase (New England Biolabs) for overnight digestion at 42 °C. IdU, CldU and single-stranded DNA were detected with mouse anti-BrdU (347580, 1:20, Becton Dickinson) and Alexa 546 Goat anti-mouse (A11030, 1:50, Molecular Probes), rat anti-BrdU (Abc-117-7513, 1:20, Seralab) and Alexa 488 goat anti-rat IgG (A11017, 1:50, Molecular Probes), mouse anti-ssDNA (MAB3034, 1:300, Millipore) and Alexa 647 goat anti-mouse IgG2a (A21241, 1:50, Molecular Probes), respectively. IODs, fork asymmetry, fork velocity and GIFD were measured as described [34]. 26 and 17 IODs, 32 and 29 fork asymmetries, 64 and 59 fork velocities, as well as 42 and 32 Forks, were measured for HCT116 shCTR and shNF-YA cells, respectively. Fork asymmetry has been calculated by the formula [(Long green track / short green track) – 1] \times 100 [35]. The non-parametric test of Mann–Whitney was applied to compare data sets from HCT116 shCTR and shNF-YA.

2.7. Chromatin immunoprecipitation (ChIP)

Chromatin was prepared 48 h post-shRNA infection and immunoprecipitation with anti-NF-YA, anti-NF-YB and anti-IgG (sc2027, Santa Cruz) antibodies performed as previously described [36,37]. As for ChIP shown in Fig. 3, 8 μ g of chromatin (fragment at most 500 bp) were incubated with the relevant antibody for 8 h and an extra 2 h with Protein A-coated magnetic beads. Beads were then washed in incubation buffer, incubation buffer 500 mM salt and LiCl washing buffer before elution in 1% SDS buffer. Reverse crosslink, protein digestion and DNA cleanup were performed under standard procedures (see Ref. [38]). PCRs were conducted in triplicate on ChIPed DNA and input DNA in a 96-well plate using SYBR green reagent on an Applied Biosystem *Real-time* PCR instrument. Data are presented as % Input value. ChIP experiments shown in Supplementary Fig. 3 were performed in triplicate with anti NF-YB antibody (Gene Spin), as previously described [13,37]. PCRs were conducted in triplicate in a 96-well plate using SYBR green reagent on a Roche Light Cycler 480 II *Real-time* PCR instrument.

2.8. *Xenopus* egg extract, replication assays and chromatin isolation

Metaphase-arrested *Xenopus laevis* egg extract and demembrated *X. laevis* sperm nuclei were prepared as described in Gillespie et al. [39]. Extracts were supplemented with 250 μ g/ml cycloheximide, 25 mM phosphocreatine and 15 μ g/ml creatine phosphokinase, and incubated with 0.3 mM CaCl₂ for 15 min to release from metaphase arrest. For DNA synthesis assays, sperm nuclei were incubated at 4–10 ng DNA/ μ l in activated extracts, supplemented with ³²P-dATP. When indicated, the following recombinant proteins were added to the reaction: 100 nM p27^{kip1} and 100 μ g/ml geminin, 500 nM NF-Y trimer, 500 nM NF-YA or 750 nM NF-YA dominant negative mutant (NF-YA DN) [40, 41]. DNA synthesis was assessed in extract supplemented with α -[³²P]dATP by TCA precipitation, as described [39]. Immunodepletions were obtained performing two rounds of incubation of the extract with Protein A-magnetic beads conjugated with antibodies anti-NF-YA (sc-10779, Santa Cruz), anti-NF-YB (Pab001, GeneSpin) or rabbit IgGs as a control, for 30 min at 4 °C. Chromatin isolation was performed by diluting 10 μ l of DNA synthesis reaction in ice-cold NIB buffer (50 mM KCl, 50 mM HEPES–KOH pH 7.6, 5 mM MgCl₂, 0.5 mM spermidine, 0.15 mM spermine, 2 mM DTT + phosphatase inhibitors), under-laid with NIB + 20% sucrose (w/v). After centrifugation at 2100 g for 5 min at 4 °C, the cushion was washed and chromatin was compacted by spinning at 13,000 g for 2 min. The resulting pellet was resuspended in 1 \times SDS sample buffer and proteins separated on 4–12% Bis–Tris gradient SDS–PAGE gels (Invitrogen), electrotransferred to PVDF membranes (GE Healthcare) and probed with antibodies against Mcm3, Mcm4, Mcm10, DNA polymerase α , Cdc45 (produced in the lab of J.J. Blow), NF-YA (H209, sc-10779 and G2, sc-17753, Santa Cruz), γ H2AX

(Upstate). Coomassie staining of histone proteins was used as loading control. Elongation assay was performed as previously described [42].

3. Results

3.1. NF-Y is associated to early DNA replication sites

To address the question whether NF-Y participates to the DNA replication process, we first analyzed the precise cellular localization of the DNA-binding subunit NF-YA in HCT116 human cells. Proliferating cell nuclear antigen (PCNA) has been chosen to mark replication factories and follow their changes during the cell cycle. Characteristic patterns of PCNA distribution can be distinguished by confocal microscopy, as shown in Fig. 1: numerous small foci scattered in the nucleus, associated with the replication of hyperacetylated transcribed chromatin, are evident in early S-phase cells [43]. During mid S-phase, discrete perinuclear foci are detected, while, in late S-phase, cells show few and larger intranuclear foci [44]. A partial overlap between NF-YA and early S-replication foci was observed, while no colocalization was detected in middle and late replication domains (Fig. 1, left panel). To statistically quantify the colocalization of PCNA and NF-YA signals, we used the Pearson's correlation coefficient (PCC) [45], which shows a significant correlation in early S-cells with respect to middle and late S-cells (Fig. 1, right panel). Also the Mander's coefficients, measuring what fraction of PCNA fluorescence is found in NF-YA positive area (M1) and *vice versa* (M2), suggested a positive relationship between NF-YA and early S-factories. These results are consistent with the idea that NF-Y chromatin binding partakes in the DNA replication process.

3.2. Depletion of NF-YA affects S-phase progression and the recruitment of replisome proteins

We previously showed that the depletion of NF-YA triggers an increase in BrdU-negative population with an S-phase DNA content, representing non cycling S cells [13]. We therefore examined the distribution pattern of replication foci within the nucleus, to study the effect of NF-Y depletion on the progression of the replication process. Confocal microscopy analysis of synchronized control and NF-Y-inactivated cells was performed, as illustrated in Fig. 2A (upper panel). Distinct focal BrdU distributions, monitored by immunofluorescence staining, correspond to the replication of different chromatin domains during S-phase progression, as described above for PCNA. At 4 h from the release, all BrdU incorporating cells showed an early S-phase pattern: the percentage of early S-phase cells was arbitrarily set at 100%, in both shCTR and shNF-Y cells (Fig. 2A, lower panels, and Supplementary Fig. 1). In control cells, early replicating population dropped to about 62% and 27% within 6 and 8 h from release, with middle S-phase cells at 38% and 54%, respectively. By 10 h, >65% of cells accumulated in late S-phase, and only 18.3% were still in early S-phase. On the contrary, NF-YA inactivation resulted in a delay in the passage from early to middle S-phase: within 12 h from release, 51% of cells still showed an early S-phase pattern and only about 13% had a late S-phase foci distribution. We further investigated the effects on replication progression upon NF-YB inactivation, which does not significantly affect S-phase progression [13]: indeed, the passage through the different S-phase stages was only marginally delayed with respect to control cells.

Western blot analysis of chromatin-enriched extracts was performed to investigate the effects of NF-YA or NF-YB depletion on the loading on DNA of key replication proteins, in asynchronous cells (Fig. 2B). Both NF-YA and NF-YB depletion triggered a decrease in the respective NF-Y partner, as a consequence of the failure to form the NF-Y heterotrimeric complex, which is required for the DNA binding of the transcription factor [46–48]. The chromatin binding of ORC2, one of the six subunits of the origin recognition complex, which enables the formation of the pre-replication (pre-RC) complex, was not affected by the inactivation of NF-Y subunits. A weak increase of PCNA and

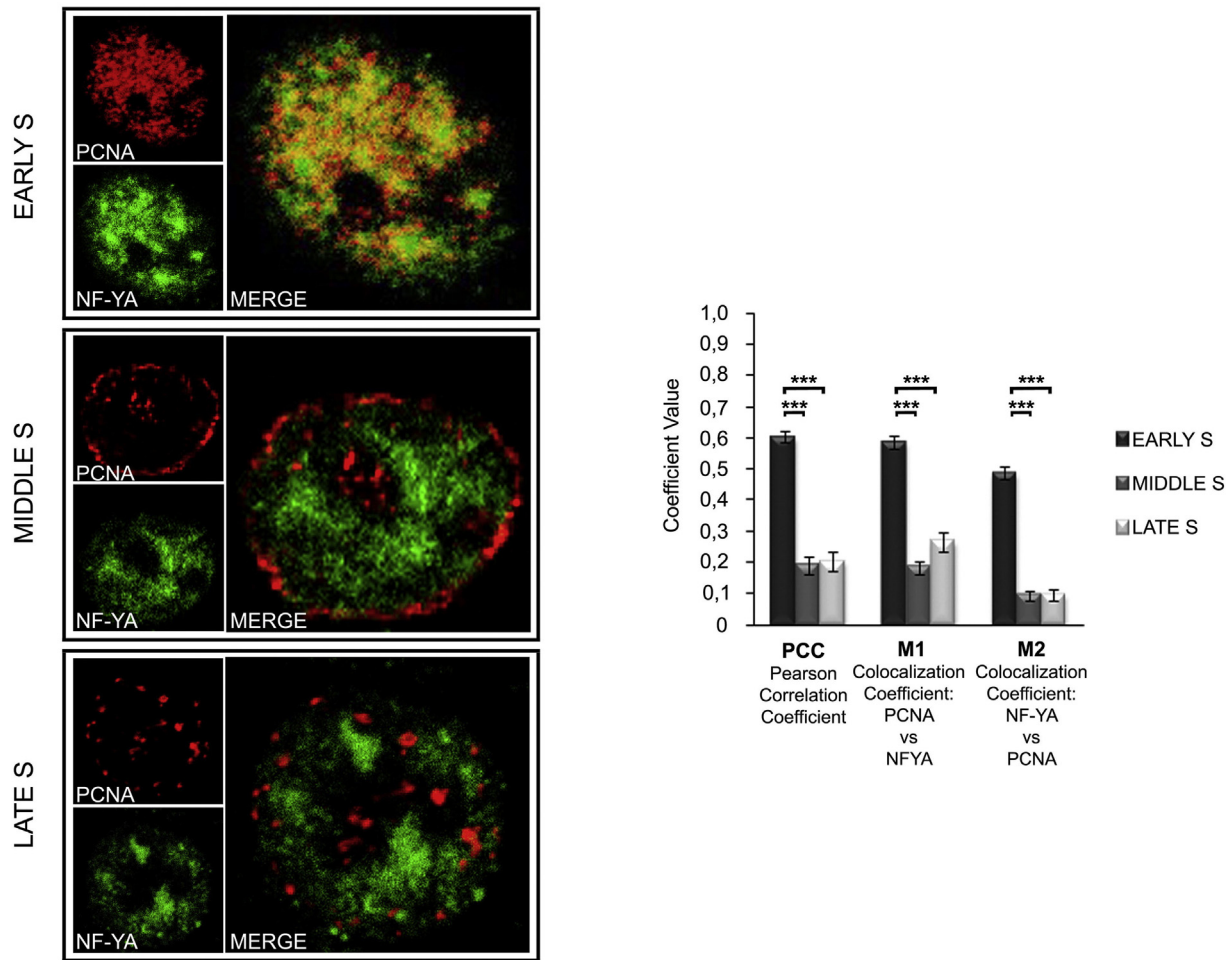


Fig. 1. NF-YA preferentially colocalizes with early-replication sites. Left panel: Representative confocal microscopy images of NF-YA (green) and PCNA (red) localization in early, middle and late-S HCT116 cells. The merge images show the superimposition of the green and red signals; the yellow color is indicative of colocalization of the two fluorescent probes. Right panel: The colocalization between NF-YA and PCNA, in early, middle and late-S cells, was calculated by means of PCC (Pearson Correlation Coefficient) and Mander's M1 and M2 coefficients (values shown as means \pm SEM of at least 30 cells for each S-phase; statistical significance analyzed with Student's t-test, *** p < 0.0001).

DNAPolD1 was detected in shNF-YB cells. Differently, the chromatin levels of the replication factors Mcm7, Cdc45, PCNA, DNAPolD1 and Geminin strongly increased upon NF-YA loss (Fig. 2B). The recruitment of Mcm10 and TopBP1, which promote the loading of Cdc45 and polymerases, didn't change either in shNF-YA or in shNF-YB cells. These data were in agreement with the existence of significant replication defects only following NF-YA loss (Fig. 2A) [13].

The chromatin levels of replication factors were then analyzed in cells synchronized at the beginning of the S phase following a time-course release from Mimosine-induced arrest (Fig. 2C). Compared to control cells, the levels of ORC2 and Mcm10 didn't change in shNF-YA cells, while Mcm7 and Geminin increased at early time points, consistently with previous observations. Conversely, the levels of Cdc45, PCNA and DNAPolD1, which rose in asynchronous extracts, presumably as a consequence of the accumulation of cells unable to progress through and exit the S-phase, were reduced in synchronized shNF-YA with respect to shCTR cells.

A similar alteration in the loading of replication factors was observed following NF-YA inactivation in human lung adenocarcinoma epithelial A549 cells. NF-YA loss clearly impaired cell viability and increased SubG1 events and cleaved-PARP1 protein levels (Supplementary Fig. 2A–C), as previously shown in HCT116 cells [13]. A decrease in the loading of replisome proteins was even observed in asynchronous shNF-YA compared to shCTR cells (Supplementary Fig. 2D). The impairment

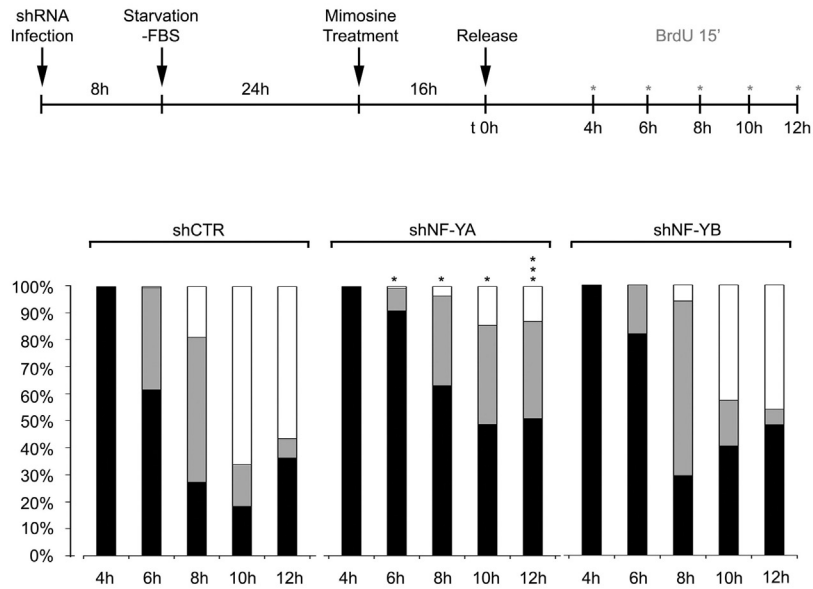
in chromatin recruitment of PCNA and Cdc45 was confirmed in Mimosine-released synchronous cells (Supplementary Fig. 2E). Finally, we checked whether the same defects were induced by NF-YA abrogation in a non-tumor spontaneously immortalized cell line (HaCaT). NF-YA-inactivated HaCaT cells showed increased apoptosis (Supplementary Fig. 3A), consistently to the expression of γ H2AX and cleaved-PARP1 (Supplementary Fig. 3C). NF-YA loss reduced BrdU-positive cell population (from 40% in shCTR to 8% in shNF-YA cells) and accumulated non-cycling S phase cells (BrdU-negative cells with an S-phase content DNA) (Supplementary Fig. 3B), as observed in HCT116 cells [13]. The levels of PCNA and Cdc45 decreased both in asynchronous and synchronous chromatin extracts (Supplementary Fig. 3D, E).

Therefore, we concluded that the loss of NF-YA affects the loading of replication proteins involved in fork progression.

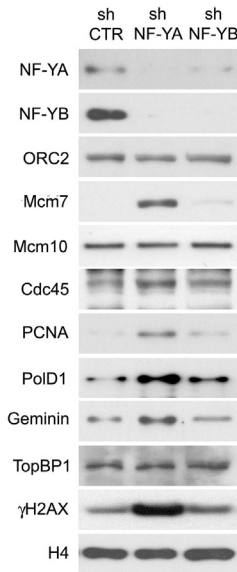
3.3. Relocation of Cdc45 around origins upon NF-YA inactivation

We next decided to investigate whether NF-Y could have a role in the recruitment of replication proteins at specific origins (ORIs) containing the NF-Y-motif. First of all, we verified by chromatin immunoprecipitation (ChIP) whether NF-Y was bound to four ORC1-associated (ORC1⁺) origins [28], containing CCAAT-elements, namely PCNA-P1, PCNA-P2, cMYC and UPR/MCM4: NF-Y enrichment over negative

A



B



C

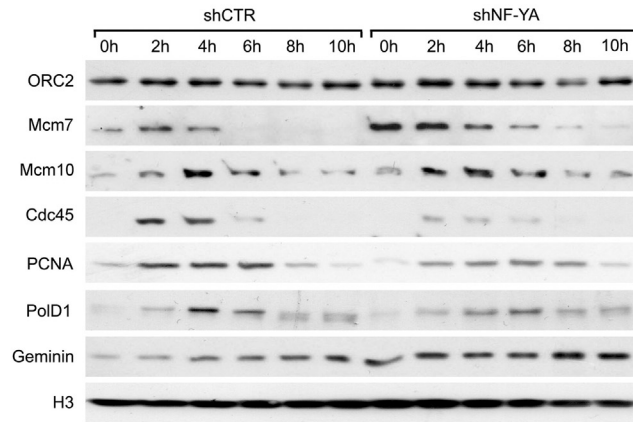


Fig. 2. NF-YA inactivation affects S-phase progression. (A) Upper panel: schematic representation of the experimental design for the analysis of BrdU-positive replication factories in HCT116 inactivated cells, released following Mimosine-induced G1/S arrest. Red asterisks indicate time points of BrdU pulse. Lower panel: distribution of the S phase patterns 4 h, 6 h, 8 h, 10 h and 12 h after the release from Mimosine-induced synchronization of shCTR, shNF-YA and shNF-YB cells. Black bars: Early S; gray bars: Middle S; white bars: Late S. Data are the means of three biological replicates \pm SD (Suppl. Fig. 1). To compare means across shCTR and shNF-YA or shNF-YB cell distributions, Mann–Whitney test was utilized; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$. (B) Expression analysis of the indicated proteins in chromatin enriched extracts of asynchronous HCT116 cells 48 h post-infection with shCTR, shNF-YA and shNF-YB. Histone H4 was used as loading control. (C) Western blot analysis of the indicated proteins in chromatin enriched extracts of shCTR and shNF-YA HCT116 cells released for the indicated time points from Mimosine-induced G1 arrest.

chromatin regions was observed and reduced following NF-YA inactivation (Supplementary Fig. 4A and B). Then, we analyzed the recruitment of the pre-RC proteins to ORC⁺/NF-Y⁺ regions, upon the depletion of NF-YA (Fig. 3A) and NF-YB subunits (Fig. 3B). The binding to origins and flanking regions (−1000/+1000 bp) of ORC1 and ORC2, as well as HBO1, a HAT chromatin remodeler contributing to

pre-RC recruitment [49], was not affected by NF-Y loss. Differently, the loading of Cdc45, which converts the pre-RC into pre-IC complex and is important for fork stabilization and progression [50], was reduced at origin sites and more diffuse in flanking regions following NF-YA loss. A similar pattern was observed in shNF-YB cells, although to a lesser degree.

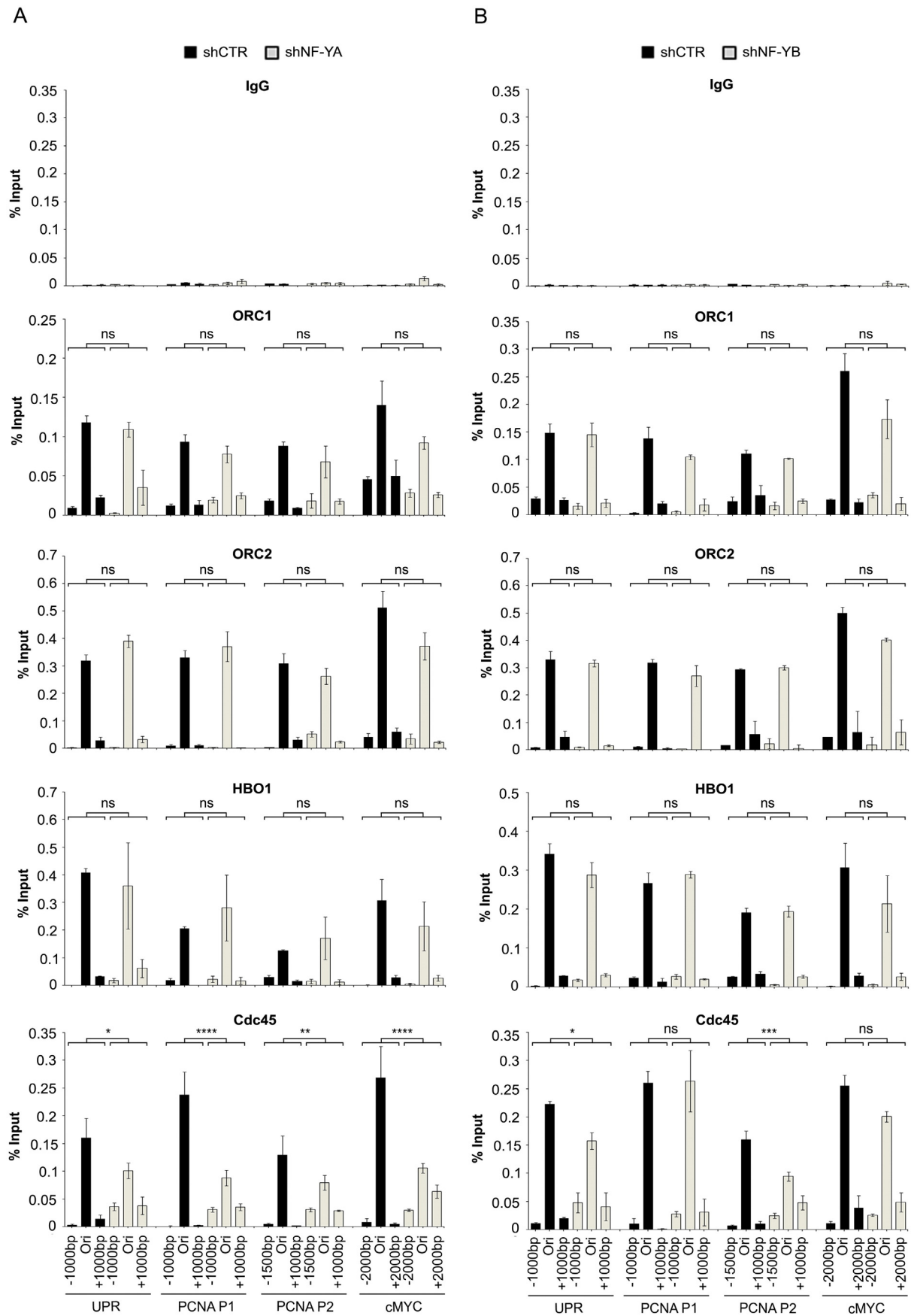


Fig. 3. Effects of NF-Y inactivation on chromatin binding of replication factors. qChIP analysis on chromatin from shNF-YA (A) and shNF-YB (B) HCT116 cells compared to shCTR cells. Antibodies used are indicated on the top of each panel and amplified regions are indicated at the bottom. The enrichment was calculated as percentage of IP recovery (% Input). p-Values were calculated for each origin as interaction between shRNA treatment and amplified region (–1000/Ori/+1000) with a two-way ANOVA; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

These data indicate that NF-Y binding is not necessary for recognition and proper recruitment of ORC proteins, but its loss affects the loading of Cdc45 around origins, consistently with possible defects in the normal progression of DNA replication forks.

3.4. Characterization of DNA replication defects

To characterize the defects in DNA replication progression induced by the lack of NF-YA, we performed DNA molecular combing experiments in control and NF-YA-inactivated cells, and determined the density and spacing of replication origins, as well as the rate and symmetry of fork progression, quantitatively and on single DNA molecules [34].

Asynchronous shCTR and shNF-YA cells were sequentially labeled by consecutive pulses of halogenated nucleotides (IdU and CldU), DNA was purified, combed on silanized glass coverslips and nucleotides incorporation on newly synthesized DNA was detected by fluorescence immunostaining (Fig. 4 and Supplementary Fig. 5). An increase in the overall velocity of replication forks was observed in the absence of NF-YA (mean velocity 2.27 kb/min in shNF-YA versus 1.71 kb/min in shCTR cells) (Fig. 4A). We determined replication fork asymmetry, indicative of DNA damage-induced fork pausing, by calculating the length ratio between two divergent forks departing from the same replication origin: a significant increase in fork asymmetry was detected in shNF-YA cells, suggesting the occurrence of fork stalling (108.5% asymmetry

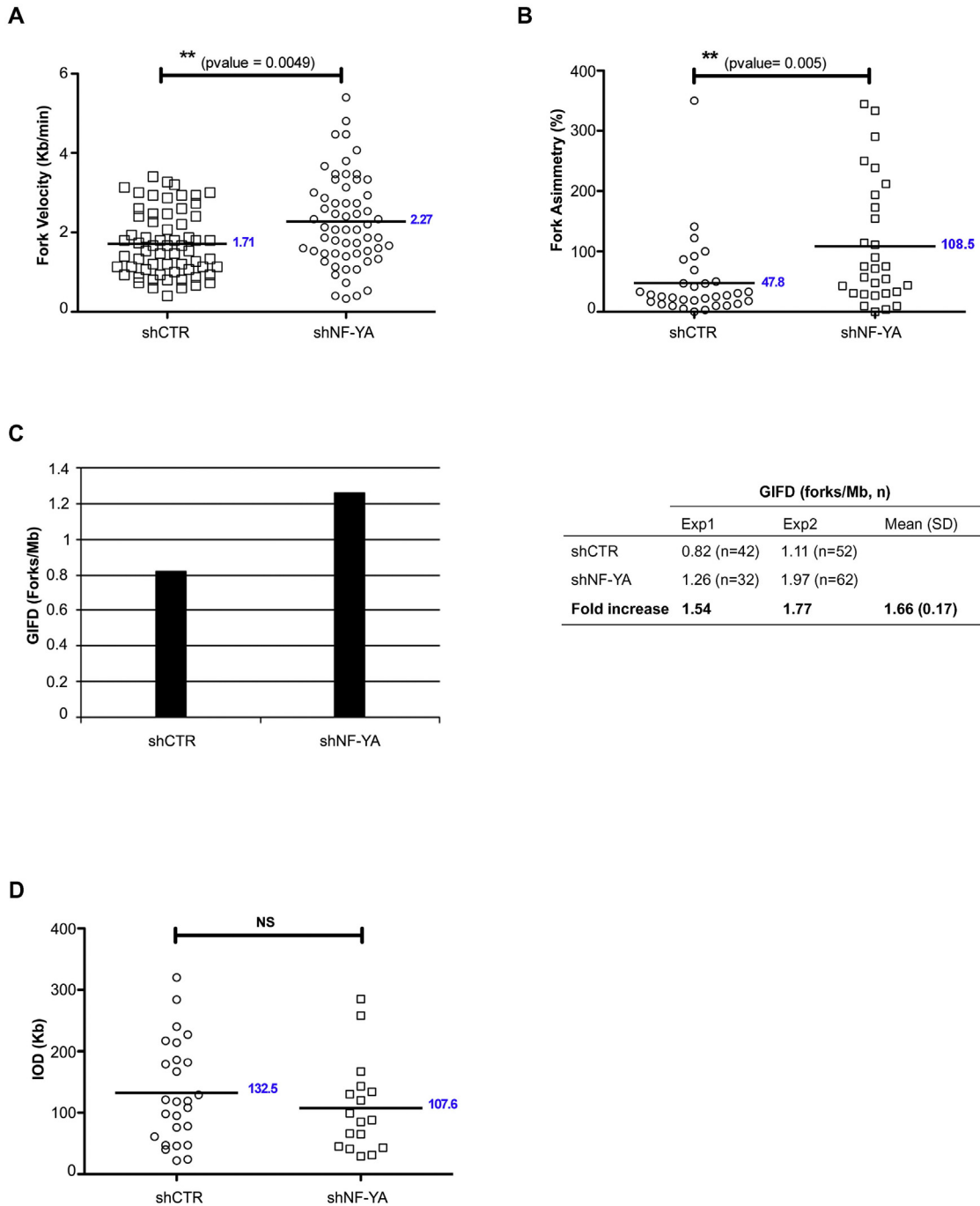


Fig. 4. DNA Combing analysis on shCTR and shNF-YA HCT116 cells. (A) Fork Velocity, (B) Fork Asymmetry: values were calculated as [(Long / Short tract) – 1] × 100, (C) Left panel: Global Instant Fork Density (GIFD). Right panel: Fold Increase (±SD) of the GIFD in shNF-YA versus shCTR in two biological replicates (shown in Fig.4C, left panel, and Suppl. Fig. 5C), (D) Inter-Origin Distances (IODs). p-Values were calculated with two-tailed Mann–Whitney test.

in shNF-YA versus 47.89% in control cells) (Fig. 4B). When replication forks fail, previously licensed origins, that otherwise remain dormant, can be activated to allow complete genome replication (reviewed in Refs. [51,52]). To test this possibility, we analyzed the Global Instant Fork Density (GIFD), calculated as the number of forks divided by total length of DNA examined, corrected for the fraction of cells in S-phase. As shown in Fig. 4C, GIFD raised from 0.82 forks/Mb in shCTR cells to 1.26 forks/Mb in shNF-YA cells: on average, an increase of 1.66-fold (± 0.17) was observed upon NF-YA depletion compared to control cells. This result can be the effect of the firing of more origin clusters, or more origins per cluster. A reduction of IOD (intra-cluster Inter-Origin Distance) after NF-YA loss was observed (Fig. 4D), consistently with the activation of more origins per cluster, to mitigate the damaging effects of fork collapse. Nevertheless, we could not rule out that more origin clusters fired, as a consequence of defects in the replication-timing program.

All together these data corroborate that NF-YA inactivation significantly affects DNA replication and the increase of GIFD and fork asymmetry are consistent with a role of NF-Y in forks progression.

3.5. NF-Y has a role in the DNA replication process beyond its transcriptional activity

NF-Y is a well known transcriptional regulator of cell cycle genes [13, 53,54]: the analysis of gene expression profiling of NF-Y-depleted HCT116 cells highlighted that several cell cycle genes were indeed down-regulated [6,13]. In particular, G2/M genes were found to be significantly affected following NF-YB knock down. Therefore, the defects in DNA replication following NF-Y depletion could be the consequence of transcriptional down-regulation of cell cycle genes, specifically those controlling the S phase. We analyzed the GO pathways affected by NF-YA and NF-YB inactivation with the Kobas software: specific DNA replication terms, among which *DNA replication*, *DNA strand elongation*, *G1/S transition*, *replication fork*, *activation of the pre-replicative complex*, were significantly down-regulated with p-values $\leq E-12$, but only in shNF-YB cells (Fig. 5A). None of the indicated categories, except the generic *DNA replication* term (p-value $3.05E-02$), were significantly affected by NF-YA depletion. Some of the genes belonging to DNA replication pathways were validated by qRT-PCR (Fig. 5B), confirming their robust down-regulation in NF-YB-inactivated cells. Despite these transcriptional effects, NF-YB depletion didn't delay the G1/S transition and the progression through the S phase was only marginally affected (Fig. 2). On the other hand, NF-YA inactivation does not significantly hit the expression of genes involved in DNA replication, indicating that the defects observed in S-phase progression cannot be ascribed uniquely to its transcriptional activity.

3.6. Direct role of NF-Y in DNA replication in *Xenopus* cell-free system

The *Xenopus* cell-free system is used to study the specific role of proteins in DNA replication, independently from interference of other DNA metabolisms, notably transcription [39]. Following fertilization, *Xenopus* eggs undergo 11 rounds of cell division without transcription, which takes place only after this stage [55]. Most of the proteins required for cell cycle progression are accumulated in the egg, supporting DNA replication under the same cell cycle controls as occur in vivo [56]. *Xenopus* NF-YA and NF-YB proteins (297 aa and 207 aa, respectively) are about 90% identical to the mouse and human proteins (Supplementary Fig. 6A) [57], and the antibodies raised against mouse/human NF-Y subunits recognize single bands corresponding to NF-YA and NF-YB proteins in *Xenopus* extracts (Supplementary Fig. 6B and C).

Using these antibodies, we examined whether and how NF-Y is recruited to chromatin during DNA synthesis in egg extract. Fig. 6A shows the timing of chromatin binding of known replication factors [58]: Mcm3, a component of the pre-RC, is rapidly loaded onto chromatin to allow origin licensing, followed by the replication initiator factor

Mcm10 [59], which is required for the chromatin binding of Cdc45 and DNA Pol α [60]. NF-YA is recruited to chromatin at late stages of DNA replication, 30 min after the addition of sperm chromatin, building up on chromatin even when DNA replication has been completed, indicating that NF-Y doesn't have a role in licensing or initiation of DNA replication. The association of NF-YA is prevented in egg extracts supplemented with p27^{Kip1}, an inhibitor of the cdk2-cyclin E complex, which allows pre-RC assembly but prevents the initiation of replication [61], or with Geminin, which targets the pre-RC component Cdt1, thus inhibiting DNA replication [62] (Fig. 6B and Supplementary Fig. 6D).

These data suggest that NF-Y binds to chromatin once replication is initiated: we therefore investigated whether NF-Y recruitment is necessary for efficient DNA replication, by immunodepleting NF-YA or NF-YB from *Xenopus* egg extracts (Supplementary Fig. 6E, F). We evaluated the kinetics of DNA replication in extracts depleted of NF-Y in comparison to control extracts depleted with non-immune antibodies (Fig. 6C), by measuring incorporation of radioactive dATP into newly synthesized DNA at different time points. The replication rate (between 120 and 180 min) of both NF-YA and NF-YB depleted extracts was reduced by about 40% compared to the control extract. However, the addition of recombinant NF-Y to depleted extract at 120 min was not able to restore the replication efficiency (Fig. 6D), possibly because one or more replication factors are co-depleted from the extract following NF-Y depletion. A second set of experiments was performed by adding to the egg extract a recombinant dominant negative NF-YA mutant (NF-YA-DN), which interacts with the NF-YB/YC dimer but is not able to bind to DNA [3,41]. The addition of NF-YA-DN to the extract affected DNA replication, similarly to NF-Y immunodepletion, but in this case efficient replication was restored by adding back wild type (wt) recombinant NF-YA (Fig. 6E and F). The comparison of chromatin binding of replication factors in control and NF-YA depleted extracts showed that the pre-RC proteins Mcm3 and Mcm4 were efficiently recruited to chromatin, while the binding of Cdc45, PCNA and DNA Pol α was clearly reduced, as a consequence of NF-Y depletion. Next, we investigated whether DNA damage occurred upon the loss of NF-Y activity, as observed in mammalian cells: Western blot analysis didn't detect any increase in the expression levels of γ H2AX both in NF-Y-depleted extracts and NF-Y-DN supplemented extracts (Supplementary Fig. 6G).

Finally, to address whether NF-Y was required for fork elongation, sperm nuclei were incubated with extracts in the presence of aphidicolin, to arrest DNA replication after initiation occurred [42]. Chromatin was then isolated and used as a template in fresh extracts containing p27^{Kip1} to prevent any further initiation event, and elongating DNA was labeled with ³²P ATP to monitor fork progression, either in the presence of wt NF-YA or NF-YA-DN (Fig. 6H). The results highlighted that forks elongating in the presence of additional wt NF-YA progressed at the same rate as control, but in the presence of NF-YA-DN, the forks elongated less efficiently. Consistently with the observations in mammalian cells, we concluded that NF-Y DNA binding is required for efficient DNA replication in *Xenopus* egg extract, participating to the elongation process after the initiation of replication.

4. Discussion

Transcription factors (TFs) play a major role in regulating gene expression through their sequence specific binding to regulatory regions. In addition to transcriptional regulation, TFs influence other DNA metabolic processes, such as DNA replication. The sequence specific TF NF-Y has been largely shown to regulate gene transcription in multiple organisms, from yeast to humans. Moreover, in human adenovirus and murine gammaherpesvirus, it was clearly established that the removal of CCAAT boxes affects DNA replication substantially and that NF-Y is directly implicated [63,64]. Our previous observations highlighted S-phase perturbation following NF-YA loss also in human cells [13], hinting at a possible function of NF-Y in DNA replication.

A

Term	ID	P-value	
		shNF-YA	shNF-YB
DNA replication	GO:0006260	3,05E-02	6,34E-22
DNA dependent DNA replication	GO:0006261	NS	7,24E-19
DNA strand elongation involved in DNA replication	GO:0006271	NS	6,00E-16
DNA repair	GO:0006281	NS	4,05E-15
DNA strand elongation	REACT_932	NS	2,64E-14
Cell cycle G1/S phase transition	GO:0044843	NS	1,10E-13
DNA replication initiation	GO:0006270	NS	1,57E-13
Activation of the pre-replicative complex	REACT_1095	NS	7,10E-13
Replication fork	GO:0005657	NS	2,80E-12
Cell cycle DNA replication	GO:0044786	NS	2,80E-12
Nuclear DNA replication	GO:0033260	NS	1,32E-11
Synthesis of DNA	REACT_2014	NS	1,59E-11
S phase	REACT_899	NS	3,03E-11
G1/S transition	REACT_1783	NS	1,88E-10
Regulation of DNA dependent DNA replication	GO:0090329	NS	1,92E-4
Regulation of DNA replication	GO:0006275	NS	7,9E-4

B

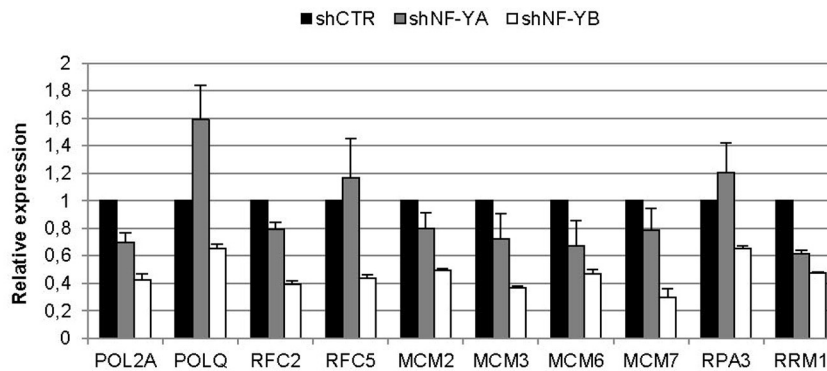


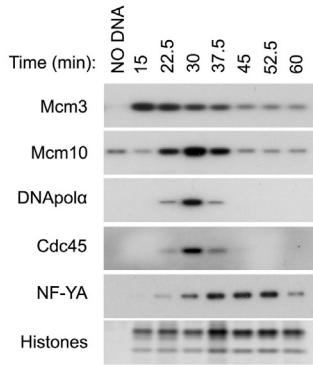
Fig. 5. Transcriptional analysis of genes associated to the DNA replication process in NF-YA- and NF-YB-inactivated cells. (A) DNA replication categories and relative p-values identified with Kobas analysis among down-regulated terms in shNF-YA and shNF-YB cells. (B) *Real-Time* PCR validation of the indicated genes, belonging to the DNA replication categories listed above (panel A), in shCTR, shNF-YA and shNF-YB cells.

Here we showed that NF-YA, the CCAAT-binding subunit of the complex, is associated to early-S replication factories (Fig. 1) and its depletion delays the progression from early to middle-late S (Fig 2), suggesting that NF-Y is not involved in replication initiation, rather it may take part to the elongation step. Indeed, NF-Y is not necessary for pre-RC assembly, as ORCs are properly recruited to origins and flanking regions also when NF-Y is inactivated (Fig. 3). On the contrary, the binding of Cdc45, which has a central role in the loading of DNA Pol α and interacts with the elongating DNA polymerases δ and ϵ to facilitate

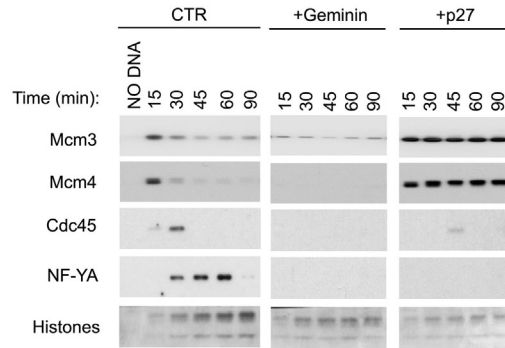
and increase the processive movement of the replication fork [65], is decreased at origins and increased in the surrounding regions upon NF-YA loss. These data suggest again that NF-Y depletion triggers replication forks stalling, with replication complexes not moving along the DNA and blocked as soon as replication starts, or improperly located within origins in the absence of NF-Y bookmarking.

The analysis of chromatin enriched extracts following NF-YA knock down highlights that the loading on DNA of PCNA, Cdc45 and DNA PolD1 decreases in synchronous cells, while it increases in the

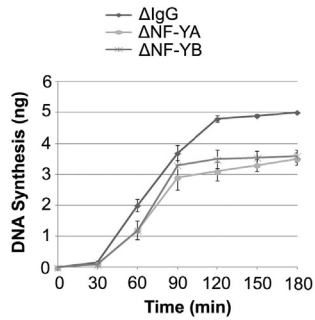
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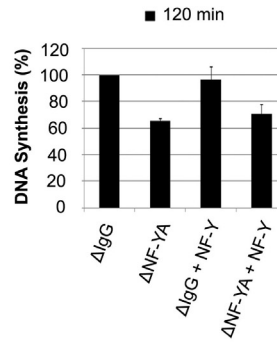
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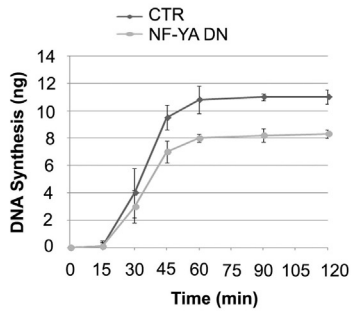
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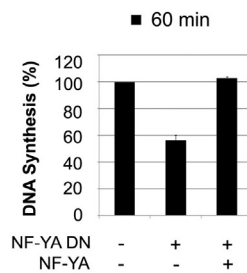
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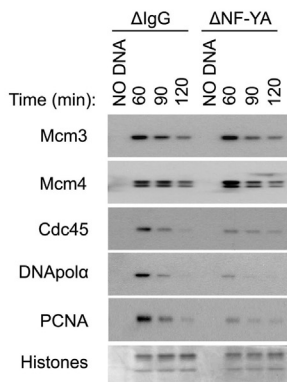
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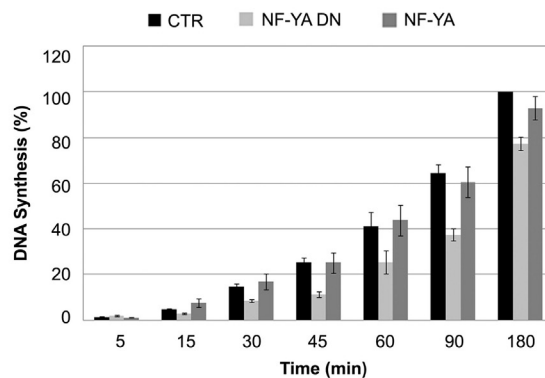
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asynchronous population (Fig. 2): these results are consistent with the existence of unproductive replication forks and with the accumulation of S-phase arrested cells, which are not able to progress through and exit the S-phase.

To prove that fork progression is impaired when NF-Y activity is abrogated, we performed DNA combing analysis on single cells: surprisingly, the velocity of ongoing replication forks increases, but the number of origins, measured as Global Instant Fork Density, is significantly higher in shNF-YA-cells, supporting the hypothesis of increased rate level of replication fork stalling. A doubling in the percentage of fork asymmetry is also consistent with defects in fork elongation or its control. Taken together, these data suggest that NF-YA loss triggers local fork stalling and the activation of the S phase checkpoint. This presumably induces global inhibition of late-firing origins and the activation of dormant origins to complete DNA replication, consistently with the observed increased GIFD. The inhibition of NF-Y activity could affect the stability of replication forks, thus leading to fork arrest but also uncontrolled progression of forks moving away at increased speed, thus resulting in faster fork velocity. Despite the local acceleration of fork progression and the increased number of active origins (GIFDs), this is not sufficient to compensate the overall replication length, which is significantly reduced in shNF-YA cells (Fig. 2). Changes in fork velocity of elongating forks may impact on replicative fidelity, thus triggering DNA damage and subsequent arrest of cells within S-phase, consistently with increased levels of γ H2AX and accumulation of non-cycling S-phase cells [13]. The role of NF-Y in the control of replication fork progression could be ascribed to the correct recruitment of the replication machinery, the control of the replication checkpoint, or the regulation of the chromatin structure. Literature data support these hypotheses: the microsequencing of NF-Y-containing fraction from CH27 cells identified a peptide that overlaps the coding sequence of RPA2 (Replication Protein A) 32 kDa, in addition to the expected peptides corresponding to NF-YA, NF-YB and NF-YC subunits [66]. Therefore, NF-Y could interact with the p32 subunit of the heterotrimeric complex (RPA), which plays an important function in DNA replication and metabolism, such as DNA repair, recombination and DNA damage checkpoints [67]. Another possible partner interacting with NF-Y is TopBP1 (Topoisomerase II β Binding Protein), a protein required for initiation of DNA replication, the maintenance of replication forks in case of stalled replication, DNA repair and DNA damage signaling [68]. The existence of a complex between NF-Y and TopBP1 has been already described; in particular, TopBP1 can directly interact with the NF-YA subunit. NF-Y/TopBP1 interaction has a key role in conferring new DNA binding properties to mutant gain-of-function p53, but the complex could have additional non-transcriptional functions, such as in DNA replication, that need to be explored.

As for the last hypothesis, that is NF-Y could control DNA replication through chromatin regulation, NF-Y binding to DNA is indeed important in the establishment of some histone post-translational modifications (PTMs) associated to active promoters [69], including H3K4me3 [26–28], and might be required for replication fork progression by setting up or preserving a permissive chromatin conformation.

Since NF-Y is an important transcriptional activator of cell cycle genes, we examined the possibility that transcriptional defects triggered by the loss of NF-Y activity are the cause of DNA replication impairment. The analysis of the expression profiles from NF-Y-inactivated cells showed that NF-YB, but not NF-YA depletion, severely affects various

genes that participate to DNA replication (Fig. 5) [13]. Despite this, a severe effect in the progression through the S phase is observed following NF-YA, but not NF-YB inactivation. Although we cannot formally rule out that NF-YA-inactivation hits one or a handful genes required for DNA replication, these observations are not in line with a simplistic view of effects entirely secondary to the transcriptional role of NF-Y.

The experiments performed in a cell-free system derived from *Xenopus* eggs, in which transcription is absent [39,58], unambiguously assessed a direct role of NF-Y in DNA replication. As observed in mammalian cells, NF-Y is necessary not for initiation, but for overall efficiency of DNA replication also in the *Xenopus* system (Fig. 6). The decrease of DNA synthesis by the addition of the DNA-binding NF-YA mutant indicates that DNA association is required. Moreover, the kinetics of replication observed in the DNA elongation assay with extracts added of NF-YA-DN indicates that NF-Y has a role in the elongation process, by mechanisms still unknown. It could be that NF-Y increases the efficiency of DNA replication through the interaction with other key proteins, taking into account the ineffective rescue of DNA synthesis following the administration of recombinant NF-Y in NF-Y-immunodepleted extracts.

While NF-Y depletion similarly delays DNA replication and affects the loading of Cdc45, PCNA and DNA Polymerases in human and *Xenopus* extracts, the activation of DNA damage is observed only in human cells. This could be the result of different degrees of replication stress induced in the two experimental systems, and therefore the activation of different checkpoints. We should also consider that shNF-YA cells are analyzed 48 h post-infection, time that allows the activation of rescue measures, including a transcriptional response, not possible in the *Xenopus* egg system. Finally, it could be that some factors are limiting in egg extracts preparations [70], with replication stress not triggering the same biological response observed in HCT116. Thus, the discrepancy observed can be due two quantitative and qualitative differences induced by NF-Y loss in mammalian and *Xenopus* systems.

The data presented here put a new light on past results. NF-YA is regarded as the limiting and regulated subunit of the trimer [69] and protein levels were shown to increase at the onset of S-phase in elutriation experiments [9,71]: the higher levels of NF-YA in S-phase might be specifically required for optimal CCAAT-origins function, as well as the loading of the trimer on the newly synthesized DNA. In addition, while the trimer is ubiquitous in cycling cells, NF-YA, but not NF-YB, is absent in some post-mitotic cells, such as myocytes and monocytes. The down-regulation of the subunit was suggested to favor terminal differentiation by switching-off cell cycle genes [72]: a block of DNA replication might well be another key consequence of the physiological removal of NF-YA in differentiating cells.

5. Conclusions

Taken together, the data presented here suggest a new non-transcriptional mechanism through which NF-Y could regulate cell proliferation, corroborating the already established connection between gene transcription and DNA replication. Based on our findings, NF-Y may act as a factor that facilitates DNA replication participating to the elongation process, presumably through functional interactions with other cellular proteins and/or establishing or maintaining a chromatin structure that allows the progression of the replication fork. Further studies will be required to provide mechanistic insight into how NF-Y contributes to DNA replication through non-transcriptional activity.

Fig. 6. NF-YA associates to replicating chromatin and is required for proper DNA replication in *Xenopus laevis*. (A, B) Chromatin was isolated from a replication reaction at the indicated time-points, separated by SDS-PAGE and immunoblotted with the indicated antibodies. Histones were stained with Coomassie as a loading control. When indicated, the replication reaction was supplemented with the CDK inhibitor p27^{KIP1} or the licensing inhibitor Geminin. (C) DNA synthesis was monitored in a time-course experiment based on the incorporation of α 32P-dATP, in mock (Δ IgG) or NF-Y depleted (Δ NF-YA and Δ NF-YB) extracts. (D) Quantification of global DNA replication after addition of recombinant NF-Y protein complex to Δ IgG or Δ NF-YA extracts. (E) DNA synthesis was monitored in a time-course experiment based on the incorporation of α 32P-dATP in extracts with or without addition of a Dominant Negative-NF-YA mutant (NF-YA-DN). (F) Quantification of global DNA replication (\pm SEM) after addition of recombinant NF-YA protein to control extracts or extracts containing NF-YA-DN. (G) Time-course of replication factors association to chromatin in Δ IgG and Δ NF-YA extracts. (H) Fork elongation analysis in extracts supplemented with recombinant wt NF-YA or NF-YA DN mutant, compared to control extract.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbamcr.2015.12.019>.

Transparency document

The Transparency document associated with this article can be found, in online version.

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