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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.

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-γH2AX (sc101696, Santa Cruz), anti-Mcm10 (A300-131A, Bethyl lab), anti-DNApolD1 (A304-005A, Bethyl lab), anti-H3 (sc8654, Santa Cruz), anti-H4 (ab10158, Abcam).

2.4. Flow cytofluorimetric 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 cytofluorimeter (Beckman Coulter).

2.5. Immunofluorescence

For immunofluorescence analysis, HCT116 cells were pulsed 48 h post-shRNA infection with 20 μM 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× 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×, 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× for 10 min. After blocking with 1% BSA in PBS 1× 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×), and stained with DAPI. Images were obtained using a confocal microscopy (Leica DM IRE2) with a 63× 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). Mander's 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 μM IdU (Sigma-Aldrich) for 15 min, 200 μM CldU for 15 min and 200 μM thymidine for 1 h. Genomic DNA was prepared in agarose plugs (0.25×10^5 cells per plug) and DNA